Nicotinic ACh Receptors

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Introduction

The nicotinic acetylcholine receptor (nAChR) is the prototype of the cys-loop family of ligand-gated ion channels that also includes $GABA_A$, $GABA_C$, glycine and $5\text{-}HT_3$ receptors, as well as invertebrate glutamate-, histamine-, ACh-, and 5-HT-gated chloride channels.[1–3] More recently, homologous ligand-gated ion channels (albeit lacking the hitherto definitive 'cys-loop') have been identified in prokaryotes and are likely to represent the ancestral form from which current day 'cys-loop' receptors descended.[4,5] In vertebrates, nAChRs constitute a family of proteins serving many physiological functions:

- at the neuromuscular junction postsynaptic nAChRs mediate skeletal muscle contraction;
- in the autonomic nervous system, ganglionic nAChRs are responsible for fast excitatory transmission; they are also found in presynaptic nerve endings of sympathetic, parasympathetic and sensory neurons;
- 'neuronal' nAChRs are present throughout the brain and spinal cord, where they exert a largely modulatory influence;
- 'neuronal' nAChRs also occur on non-neuronal cells, including glial, immune and endothelial cells, where they are presumed to respond to paracrine ACh.

The structural and functional diversity within this receptor family[6] has kindled interest in nAChRs as potential therapeutic targets for a wide variety of medical conditions, and has spurred drug discovery programs. This has resulted in the development of subtype-selective ligands that complement the generous armamentarium of natural products directed at nAChRs, to provide an increasing portfolio of tools for nAChR research.[7,8]

nAChR Structure

nAChRs in vertebrate skeletal muscle, and their counterparts in the electrogenic organs of *Torpedo* and *Electrophorus*, were the first receptors to be studied and have been characterized in exquisite functional and structural detail. This was possible because the neuromuscular junction enabled detailed electrophysiological measurements of nAChR function to be made, in early studies by Langley and Dale, followed by the pioneering work of Katz and Miledi, and development of single channel recording by Neher and Sakmann.[1,8] *Torpedo* and *Electrophorus* electric organs provided a high density of

nAChRs that facilitated high resolution structural studies[9,10] and biochemical characterization.[1]

High affinity snake α-toxins, notably α-bungarotoxin (α-Bgt), enabled the nAChR protein to be purified from *Torpedo* electroplax and resolved into 4 different subunits, designated α,β,γ and δ.[11] An additional subunit, ε, was subsequently identified in nAChRs purified from adult skeletal muscle. In the early 1980s, these subunits were cloned and the era of the molecular analysis of nAChR commenced.[1] The muscle endplate nAChR has the subunit combination and stoichiometry $(\alpha1)_2\beta1\varepsilon\delta$, whereas extrajunctional $(\alpha1)_2\beta1\gamma\delta$ nAChRs predominate in fetal or denervated muscle, and electric organs.[12] Each of the five subunits comprising the nAChR spans the lipid bilayer to create a water-filled pore (Figure 1). Each subunit consists of 4 transmembrane segments; the second transmembrane segment (M2) lines the ion channel. The extracellular N-terminal domain of every subunit contains a 'cys-loop' that is the signature sequence of this ligand-gated ion channel family: two cysteine residues (Cys 128, 142, *Torpedo* α1 subunit numbering), separated by 13 amino acids, form a disulfide bond to create a loop that has been implicated in the transduction of agonist binding into channel opening.[13,14]

The principal agonist binding site resides in the N-terminal domain of α subunits, close to the pair of adjacent ('vicinal') cysteine residues (Cys 192, 193, *Torpedo* numbering) that define an α subunit. Mutagenesis and photoaffinity labeling experiments have highlighted the importance of 4 aromatic residues (Tyr 93, Trp 149, Tyr 190, Tyr 198, *Torpedo* numbering), consistent with 3 polypeptide loops of the α subunit (loops A-C) contributing to the principal agonist binding site.[14,15] These aromatic residues stabilize bound ligands through π-cation interactions.

The muscle nAChR subunits are arranged in clockwise order – α1, β1,δ,α1,γ/ε – with the two agonist binding sites occurring at the α-δ and α-γ/ε interfaces[12] (see Figure 3). The adjacent subunit (δ or γ/ε) also contributes to the agonist binding site (complementary site: polypeptide loops D-F). One consequence of this is that the α-δ and α-γ/ε binding sites are not identical, and this is reflected in differences in ligand affinity.[12,22] However, occupancy of both binding sites is required for effective opening of the channel.

Knowledge of ligand binding to nAChRs has been greatly augmented by the crystal structure of a soluble ACh binding protein (AChBP), first identified in the snail *Lymaea stagnalis* and subsequently also cloned from *Aplysia californica* and *Bulinus truncatus*.[16–18] Each subunit of this pentameric secreted protein is homologous to the N-terminal domain of a nAChR subunit (20-24% sequence identity), with conservation of all the residues implicated in ACh binding to the muscle nAChR. The ability to crystallize these proteins for X-ray diffraction studies has provided a high resolution view of the extracellular portion of the receptor, notably of the binding site at the interface between adjacent subunits, and the interaction of ligands with these sites.[14,17,19] Co-crystallization of the AChBP with different ligands, and molecular modeling approaches in which the crystal structure of the AChBP is modified to accommodate sequence differences found in the extracellular domains of various nAChR subunits, can be exploited to identify subtype-selective nAChR ligands.[20,21]

Whereas the function of the AChBP is to sequester ACh, binding of ACh to nAChRs increases the probability of channel opening, thus promoting cation flux. Upon agonist binding, nAChRs undergo an allosteric transition from the predominantly closed, resting conformation to an open state that allows an influx of Na^+, and – to some extent (depending on nAChR subtype, see Figure 3) – Ca^{2+}, accompanied by an efflux of K^+ under normal physiological conditions. In the closed state the ion channel is occluded by a 'hydrophobic girdle' that constitutes a barrier to ion permeation.[9] Agonist binding in the extracellular domain promotes a conformational change that results in a rotational movement of the M2 helices lining the pore, twisting the girdle to widen the pore by ~3 Å, sufficient for ion permeation.[9,23] At the muscle endplate, the ensuing depolarization elicits muscle contraction.

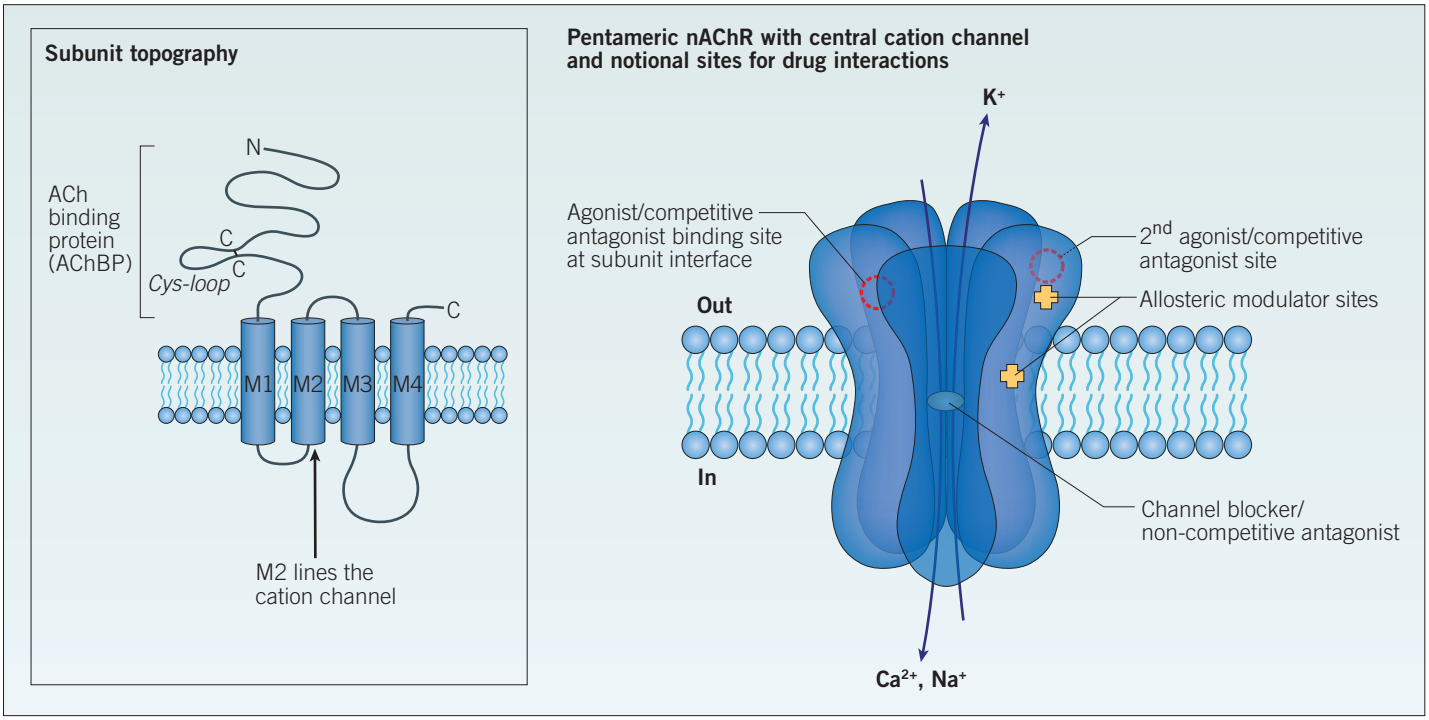
Despite the presence of agonist, the nAChR channel closes within seconds to minutes, in order to enter a desensitized state (Figure 2). In this condition, the nAChR is refractory to activation. Multiple desensitized states have been proposed to exist.[24] In the active (open) conformation, the nAChR binds agonists with low affinity (e.g. K_d of muscle nAChR for ACh ~50 μM). The desensitized states display higher affinity for agonist binding (K_d of muscle nAChR for ACh ~1-5 μM); thus the desensitized nAChR retains bound agonist despite its non-conducting state. The transitions between resting, open and desensitized states can be influenced by allosteric modulators (see below).

Sites on the nAChR for Ligand Interactions (Figure 1)

Agonists bind at subunit interfaces in the extracellular domain, as described above.[14] Agonists stabilize the mobile loop-C of the principal binding subunit in close apposition to the adjacent (complementary) subunit. Competitive antagonists also bind at or close to the agonist binding sites, preventing access to agonists. In contrast to agonists, competitive antagonists stabilize loop-C in an extended conformation that precludes channel opening.[17,19,25] Thus the channel remains closed and access to the agonist binding site is prevented.

In contrast to full agonists, exemplified by the endogenous ligand ACh, partial agonists activate the nAChR with low efficacy, despite interacting with the same agonist binding site.[26] Such ligands also exhibit competitive inhibition through competition with other, more efficacious agonists for occupancy of the binding site. Thus they combine weak agonism with a degree of antagonism.[8] The efficacy displayed by a partial

Figure 1 | General structure of a nAChR



Left: The topography of a single nAChR subunit. The two cysteine residues forming the signature cys-loop are indicated. The extracellular N-terminal domain corresponds to the AChBP.
Right: Schematic of a generic nAChR showing 5 subunits spanning the membrane to form a central ion channel. Notional sites of action of interacting drugs are shown: two agonist binding sites are indicated at extracellular subunit interfaces, consistent with a heteromeric nAChR. Competitive antagonist sites overlap the agonist binding sites. Extracellular and intramembrane sites have been proposed for different allosteric modulators. Non-competitive antagonists may occlude or block the ion channel, as indicated, or may act at other sites. See text for further details.

agonist is not an intrinsic property of the ligand but a consequence of the interaction with a particular nAChR subtype.[139] For example, the efficacies of synthetic agonists TC 2559 and sazetidine A at α4β2 nAChRs are determined by the subunit stoichiometry.[27] The complementary subunit has been implicated as the major determinant of efficacy, based on a comparison of cytisine and a novel agonist NS 3861.[37]

Non-competitive antagonists bind at sites distinct from agonist binding sites to prevent receptor activation or block channel function. Channel blocking compounds may interact specifically with residues in the mouth or lumen of the pore to occlude the ion channel. In addition, any small, positively charged species may be predicted to channel block, and many agonists, including ACh, do this at high concentrations.[28] The efficiency of channel blockade is 'state dependent' as access to the channel requires the channel to be open. Hence the speed of block will be influenced by the state of the receptor: resting, open or desensitized (Figure 2).

Allosteric modulators are non-competitive ligands that act at a variety of distinct sites to positively or negatively influence agonist interactions and/or nAChR function.[29] This diverse class includes inorganic cations, steroids, anesthetics and amines. Subtype-selective positive allosteric modulators (PAMs), in particular, are attracting attention as potential therapeutic drugs.[8] Their sites of interaction on the nAChR are diverse, ranging from non-canonical binding sites in the extracellular N-terminus to locations within the membrane spanning domains (Figure 1; see section on PAMs, p22).[30–32]

Diversity of nAChR Subtypes (Figure 3)

The classical studies of Paton and Zaimis[33] demonstrated that nicotinic responses in autonomic neurons are pharmacologically distinct from their counterparts in skeletal muscle, providing the first indication of nAChR heterogeneity. The quest for nAChRs in the brain, necessary to explain the psychoactive actions of nicotine, identified binding sites for ^{125}I-α-Bgt and [^{3}H]-nicotine.[34,35] Their distinct pharmacological profiles and anatomical distributions in rodent brain raised the (then) novel and controversial prospect of nAChR heterogeneity. Following the first publication[36] of a cloned neuronal nAChR subunit (α3) in 1986, eleven novel 'neuronal' nAChR subunits have been identified in mammals (α2-α7, α9, α10, β2-β4), with an additional subunit, α8, cloned from avian species.[6,8]

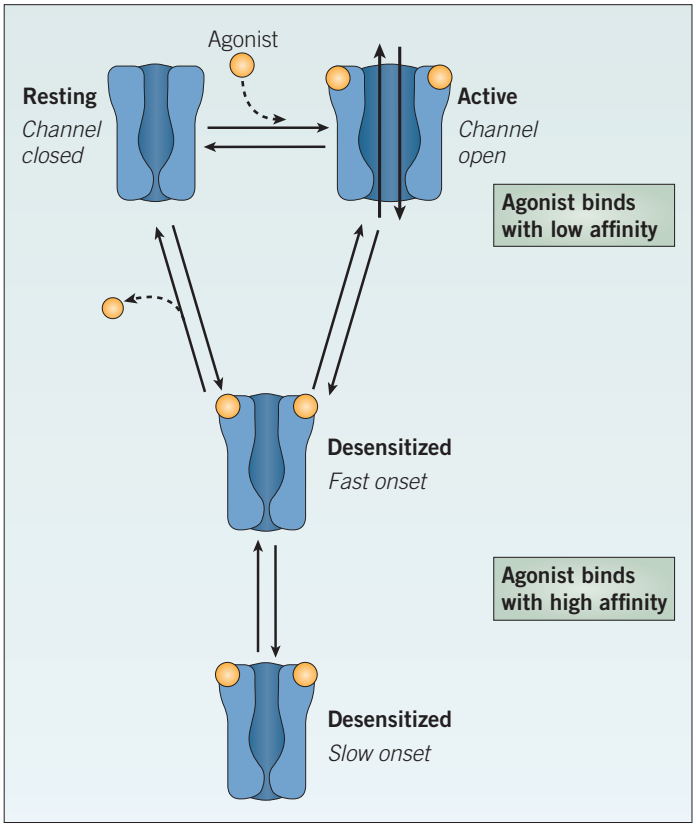
α2-α6 subunits form heteromeric nAChRs with β subunits. α subunits are defined by the presence of a pair of vicinal cysteines equivalent to those that characterize the muscle α1 subunit. This led to the supposition that all α subunits could constitute a principal agonist binding site in neuronal nAChRs.

However, the α5 subunit is not capable of fulfilling this role as it lacks the critical tyrosine from loop C (Tyr 190, *Torpedo* α1 labeling).[38] β subunits lack the N-terminal vicinal cysteines but β2 and β4 subunits contain the tryptophan residue characteristic of loop D; hence these subunits can act like γ and δ muscle nAChR subunits to provide the complementary agonist binding site at an αβ interface. The absence of this key tryptophan residue in the β3 subunit makes it the true homolog of the muscle β1 subunit that does not contribute to an agonist binding site. Indeed, the sequence similarity between α5 and β3 subunits[4,6,8] (see Figure 3) is consistent with both having this role, although other subunits can also occupy the position equivalent to β1.[39,40] Issues of nAChR subunit nomenclature are reviewed by Hurst *et al.*[8]

nAChR Stoichiometry

Heteromeric neuronal nAChRs comprised of α and β subunits include two pairwise combinations of α2, α3, α4 or α6 with β2 or β4 subunits, to provide two agonist binding sites in an arrangement analogous to that of the muscle nAChR (Figure 3). The fifth position, corresponding to that occupied by the β1 subunit in muscle nAChR, can accommodate any subunit (although rules of assembly constrain the subunit combinations in a given nAChR, Figure 3). The subunit occupying this position (sometimes referred to as the 'accessory' subunit)[40] can influence assembly, trafficking and functional properties of the resultant nAChR subtype, including agonist potency, conductance, Ca^{2+} permeability and desensitization kinetics.[27,40] For example, agonists and competitive antagonists typically have higher potency at $(\alpha4)_2(\beta2)_3$ nAChRs, compared with $(\alpha4)_3(\beta2)_2$ nAChRs.[27] Nicotine upregulates α4β2 nAChRs by acting as a chaperone to stabilize the $(\alpha4)_2(\beta2)_3$ stoichiometry.[41,42] α3β4 nAChRs also exhibit stoichiometry-dependent differences in agonist and antagonist sensitivities and nicotine-induced upregulation, in heterologous expression systems.[37,43,44]

Figure 2 | Relationship between the major conformational states of a nAChR



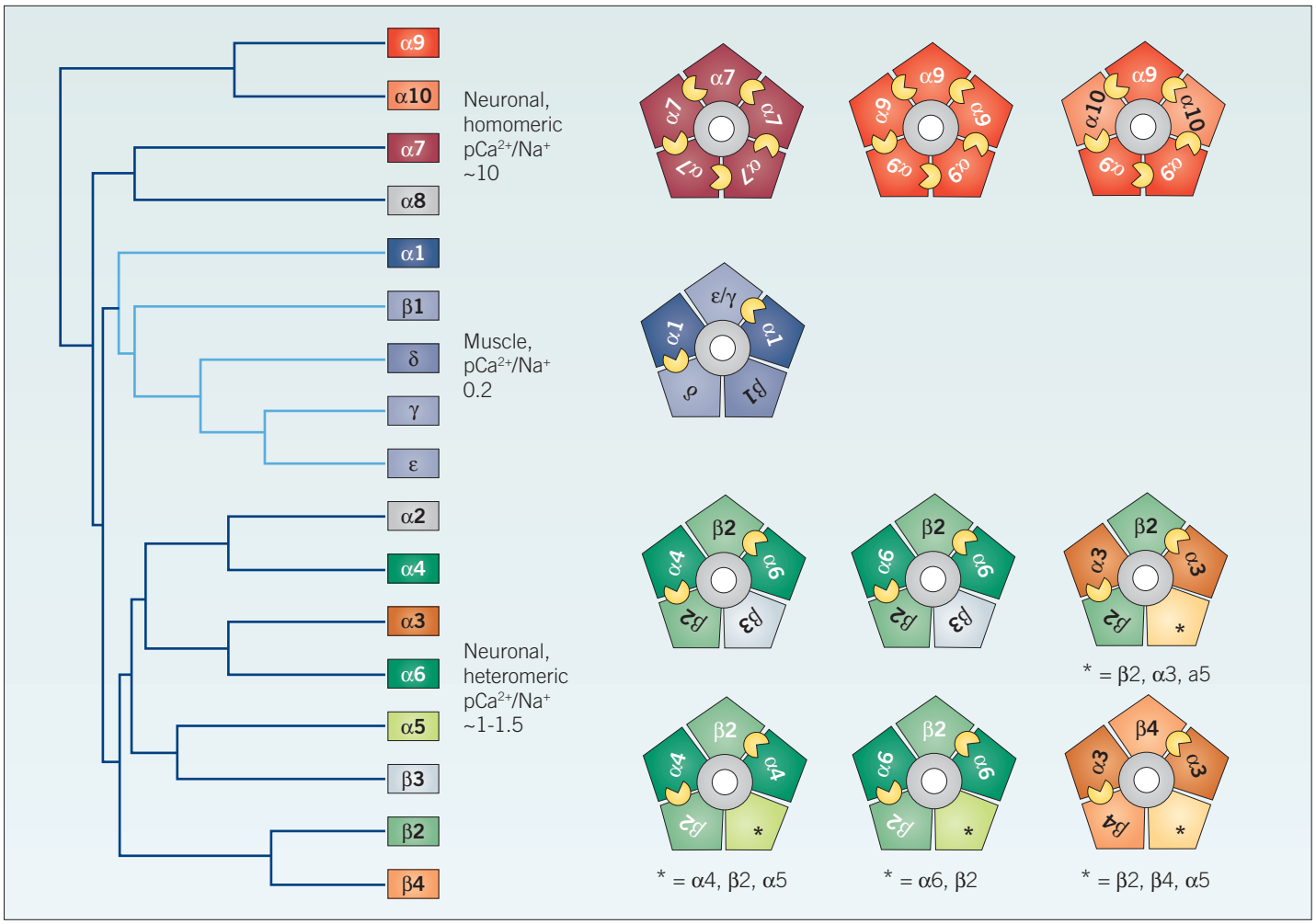
The fifth subunit contributes to a putative allosteric site analogous to the benzodiazepine binding site in $GABA_A$ receptors.[14] There is evidence that some allosteric modulators bind to neuronal nAChRs at this location, depending on which subunit occupies the fifth position. For example, the anthelmintic morantel acts at this site in mammalian α3β2 nAChRs to enhance channel gating.[30] Site-directed mutagenesis, combined with cysteine-substitution methods, indicated that β2 occupies the fifth position to create a non-canonical binding site (β2 – principal face; α3 – complementary face).[30] Conversely, the PAM NS 9283 is proposed to interact selectively with an α4β2 heteromer with stoichiometry $(\alpha4)_3(\beta2)_2$, which places α4 in the fifth position.[46] However, the precise subunit stoichiometry of native nAChRs remains poorly established.

In contrast, the α7, α8 and α9 subunits are distinguished by their ability to form robust homomeric receptors, sensitive to α-Bgt, when expressed in *Xenopus* oocytes. Evidence indicates that native α-Bgt-sensitive nAChRs in mammalian brain are predominantly homomeric, being formed of α7 subunits.[6] However, compared with heteromeric nAChRs, heterologous expression of α7 nAChRs is much less efficient in non-neuronal mammalian cultured cells. This paradox was resolved by the discovery that the endoplasmic reticulum-resident chaperone RIC-3 can promote the formation of functional α7 nAChRs in non-permissive cells.[47] This highlights the importance of ancillary proteins in regulating the assembly, trafficking, stabilization and turnover of nAChR subtypes.[48]

In a homopentamer, the same type of subunit provides both principal and complementary faces of the agonist binding site,[66] resulting in five putative binding sites per receptor homomer (Figure 3). An interesting question concerns the number of sites that must be occupied by an agonist for efficient channel activation. Elegant studies to determine the single channel activity of receptors composed of different proportions of wildtype and mutated (low conductance) subunits indicated that occupancy of 3 non-consecutive sites within a pentamer is necessary for full channel activity.[49]

Despite its ability to form homomeric nAChRs, in physiological systems the α9 subunit co-assembles with α10 to form heteromeric nAChRs, with the stoichiometry $(\alpha9)_2(\alpha10)_3$.[50,51] On its own, α10 appears to be incapable of forming a homomeric receptor.[52] In avian tissues α7α8 heteromers exist and nAChRs comprised of α7 and β2 subunits have been proposed to occur in heterologous expression systems and in mammalian brain.[53–55]

Figure 3 | Heterogeneity of vertebrate nAChRs



Left: Cladogram of all vertebrate nAChR subunits cloned to date, adapted from Novère *et al*[468]
Right: Cartoons illustrate viable pentameric mammalian subunit combinations, with putative agonist binding sites indicated by small yellow crescents between adjacent subunits. See text for further details.
*Indicates the possible inclusion of additional unspecified subunits

Invertebrate nAChRs

A distinct but related gene family of α and β nAChR subunits has been uncovered in invertebrates. The *C. elegans* genome sequence incorporates the largest number of such subunits, with at least 32 reported to date, 22 of which have been classified as α subunits.[56] This contrasts with a lower number of subunits in parasitic nematodes, where they represent clinical and veterinary targets.[56] Insects, including the fruit fly (*Drosophila melanogaster*), malaria mosquito (*Anopheles gambiae*), and honey bee (*Apis mellifera*) have 10-12 nAChR subunits that comprise the major excitatory receptor class in the insect central nervous system.[57,58] This distinct gene family of nAChR subunits has allowed the development of selective agonists – the neonicotinoids that target insect or helminth nAChRs, for pesticide or veterinary applications.[59] Mutagenesis, electrophysiology and computer modeling suggest that the subunit providing the complementary face of the binding site confers the selectivity for neonicotinoids of invertebrate over mammalian nAChRs. Concerns about the impact of neonicotinoids on 'friendly' insects, such as bees, highlights the challenge to generate more discriminating ligands that can distinguish between insect species.[60]

Distribution and Physiological Significance of nAChR Subtypes

Autonomic neurons (including sympathetic ganglia, parasympathetic innervation, sensory ganglia, chromaffin, neuroblastoma and PC12 cells) typically express α3, α5, α7, β2 and β4 subunits,[61,62] with the likely assembly of α3β4, α3β4α5, α3β4β2 and α7 nAChRs. Additional subunits (including α4, α6, α9 and α10) have been reported in dorsal root ganglia;[63–65] nAChRs in these sensory neurons are of interest as therapeutic targets for modulating nociceptive signals.

In the mammalian brain there is a heterogeneous distribution of α2-α7 and β2-β4 subunits, with distinct and often extensive and overlapping expression patterns.[8,66] α4, β2 and α7 are the most wide-spread subunits, with α4β2* (where * indicates the possible inclusion of additional unspecified subunits),[67] and α7 nAChRs having a somewhat complementary distribution.

In contrast to the well-established role of nAChRs in synaptic signaling at the neuromuscular junction and in sympathetic ganglia, there are rather few reports of neuronal nAChRs mediating cholinergic synaptic transmission in the CNS. However, there is abundant evidence in the brain for presynaptic nAChRs that modulate the release of many different neurotransmitters[68] and this has led to the supposition that the majority of nAChRs are located presynaptically. In addition, nAChRs also exist on somatodendritic regions,[69] in perisynaptic and extrasynaptic locations.[70,355] The current perspective is that presynaptic and extrasynaptic nAChRs modulate short and longer term neuronal activity in response to non-synaptic ('paracrine') levels of ACh (or choline, in the case of α7 nAChRs).[71,72] Nevertheless, a few examples of synaptic nAChRs mediating synaptic transmission at cholinergic synapses in the brain have been documented.[73,74]

The α7 nAChR is particularly prominent in hippocampus and cortex (including the prefrontal cortex (PFC)),[75] where it is commonly associated with GABAergic interneurons and glutamatergic synapses.[72,77] There are numerous accounts of the highly Ca^{2+} permeable α7 nAChRs contributing to synaptic plasticity and long term potentiation, notably in the hippocampus,[71,72,76,78] and working memory in the PFC.[355] Genetic linkage studies and pharmacological evidence have implicated α7 nAChRs in schizophrenia.[79] A substantial body of evidence has encouraged the aim of targeting α7 nAChRs to treat cognitive and attentional deficits in a variety of conditions including schizophrenia, ADHD and Alzheimer's disease.[8] α7 and α4β2 nAChRs also collaborate in modulating synaptic activity.[69,80]

α4β2 nAChRs with stoichiometry $(\alpha4)_2(\beta2)_3$ have high affinity for nicotine (and account for >90% of [^{3}H]-nicotine binding to brain tissues[81]). Transgenic knockout of either of these subunits eliminates nicotine self-administration, whereas virally targeted re-expression of the β2 subunit in mesolimbic areas of β2 knock-out mice recovers this behavior, implicating a role for α4β2 nAChRs in nicotine addiction.[82,83] A similar strategy has uncovered a role for β2* nAChRs in the prelimbic area of the PFC in attention.[84] α4β2 nAChRs are highly expressed in the thalamus.[85] Their putative role in thalamo-cortical circuitry provides a rationale for the ability of gain of function mutations in the channel-forming M2 domain, or adjacent M3 domain, of either the α4 or β2 subunit to give rise to autosomal dominant nocturnal frontal lobe epilepsy.[86]

The α3, α5 and β4 nAChR subunit genes form an evolutionarily conserved gene cluster, found on human chromosome 15, and they are often, but not always, co-expressed.[8] α3 and β4 nAChR subunits have a more restricted distribution in the CNS, compared with α4 and β2, whereas the α5 subunit is expressed more widely, at low to moderate levels.[87–89] Polymorphisms in these 3 genes, notably D398N within the coding region of the α5 subunit gene, are associated with increased vulnerability to tobacco addiction and/or risk of lung cancer.[90] This association inspired studies in mice that implicate α5-containing nAChRs in the medial habenular – interpeduncular nucleus (IPN) pathway in the negative regulation of nicotine consumption, making such receptors a potential target for smoking cessation.[91,92]

In cortex, thalamus, hippocampus and striatum, α5 is associated with α4 and β2 subunits in 15-40% of α4β2* nAChRs.[89] Somatodendritic or terminal α4β2α5 nAChRs have been identified in dopamine neurons in the ventral tegmental area (VTA) and substantia nigra and in corticothalamic projection neurons.[93–95] α5 confers increased Ca^{2+} permeability and enhanced rates of desensitization to heteromeric nAChRs,[40] can alter sensitivity to ACh, and renders α4β2 nAChRs resistant to nicotine-induced upregulation.[89, 96–98]

α6 and β3 subunits are commonly expressed together and exhibit a limited distribution, largely restricted to catecholaminergic neurons and neurons of the visual pathway.[6,99,100] These subunits contribute to nAChRs of complex subunit composition on dopaminergic terminals, including α6β2β3 and α4α6β2β3 nAChRs[93] (Figure 3). The latter subtype is inferred to have the highest sensitivity to nicotine (EC_{50} = 0.23 μM) and sazetidine A.[101,102] The β3 subunit is suggested to influence the assembly, stability and/or targeting of α6-containing nAChRs[103–105] and a dominant negative role has been inferred from studies of recombinant nAChR subunits.[106,107]

The α2 nAChR subunit has a limited expression pattern in the rodent CNS, being largely restricted to the IPN.[108] Its distribution in the primate brain appears to be more extensive.[109] Transgenic mice with a targeted deletion of the α2 subunit gene exhibit little change in phenotype, although some nicotine-modulated behaviors, including nicotine self-administration and withdrawal, are potentiated.[110] This is consistent with the role of the IPN in restraining addictive behavior, alluded to above.[91,92] α2β4 nAChRs are also inferred to participate in the motorneuron-Renshaw cell synapse of the mouse spinal cord, a rare model of nicotinic synaptic transmission in the CNS.[111]

Mechanosensory hair cells express α9 and α10 nAChR subunits that co-assemble to generate predominantly heteromeric nAChRs. α9α10 nAChRs exhibit an unusual mixed nicotinic/muscarinic pharmacology: although they are activated by ACh, nicotine and other agonists act as competitive antagonists at α9α10 nAChRs.[52,124] α9α10 nAChRs are highly Ca^{2+} permeable; they mediate the effects of the efferent olivocochlear system on auditory processing.[51,112] These subunits are not expressed in the brain but have been reported, together or separately, in a variety of non-neuronal cells and tissues, as well as in sensory neurons where they are putative antinociceptive targets.[113]

Expression of numerous 'neuronal' nAChR subunits has also been detected in diverse non-neuronal cells. These include astrocytes, macrophages, keratinocytes, endothelial cells of the vascular system, muscle cells, lymphocytes, intestinal epithelial cells and various cell-types of the lungs.[114] mRNAs encoding most nAChR subunits have been detected in such cells but the

identity and functional significance of assembled nAChRs in non-neuronal cells is less clear. α7 nAChRs in non-neuronal cells have excited interest because of their high Ca^{2+} permeability and ability to engage Ca^{2+}-dependent signaling cascades, but there are arguments for both adverse and beneficial influences.[115,116]

Nicotinic Ligands for Neuronal nAChR

Due to the critical roles of muscle and ganglionic nAChRs in normal vertebrate physiology, Nature has elaborated a diverse array of plant and animal toxins that target these receptors, and their counterparts in the CNS. The nicotinic pharmacopoeia continues to be augmented by the generation of synthetic ligands in response to the perceived validity of neuronal nAChRs as therapeutic targets. As a consequence, the listing that follows is primarily directed at neuronal nAChRs. The α7 nAChR, in particular, has attracted a substantial number of selective agonists, antagonists and allosteric modulators (see Tables 2, 3 and 7, respectively). There remains a scarcity of subtype-selective tools for heteromeric nAChRs, especially ligands that can discriminate between nAChR subtypes with subtle differences in subunit composition and stoichiometry. Most notable is the dearth of synthetic subtype-selective antagonists (see Table 3), perhaps reflecting the lack of therapeutic potential associated with nAChR blockade. Some compensation for this deficit is afforded by the α-conotoxins (Table 4). A striking development has been the generation of subtype-selective PAMs (Table 7). Representatives of these various classes of ligand that are currently available from Tocris are briefly discussed below. For more comprehensive accounts of some of the families of synthetic nicotinic ligands see References 7, 8 and 117.

Agonists

nAChR agonists are structurally diverse, comprising natural products from a variety of plant and animal species and synthetic molecules derived from structure activity relationship programs or screening of drug libraries.[8,117] In equilibrium binding studies, non-selective agonists like nicotine typically bind with highest affinity (K_i) to the $(\alpha4)_2(\beta2)_3$ nAChR, with 2-3 orders of magnitude lower affinity at α7 nAChRs and with intermediate affinity at α3* nAChRs. Binding reflects the high affinity desensitized state of the nAChR (Figure 2) that is stabilized during the prolonged incubation period of such assays. Agonists are ~10-fold more potent at α9α10 nAChRs compared with their affinities for α7 nAChRs.[118] With respect to functional potency, EC_{50} values are typically 1-3 orders of magnitude higher than K_i values. This difference is greatest for α4β2 nAChRs, so that EC_{50} values between subtypes may be more similar than suggested by their binding affinities. This has spurred the quest for subtype-selective nAChR agonists to exclusively activate a particular subtype in a mixed population.

Functional potency of novel ligands is typically assessed by electrophysiological recording or Ca^{2+} flux assays of recombinant nAChR subunits expressed in *Xenopus* oocytes or mammalian cells.[119,120,121,272] Evaluation in native systems is complicated by the likely presence of a multiplicity of nAChRs of complex and often unknown subunit composition. A practical aspect arises from the method of analysis of rapidly desensitizing currents, notably those recorded from cells expressing α7 nAChRs. Measurements of the peak height (amplitude) of electrophysiological responses to construct concentration response relationships, as is conventionally done, underestimate the potency of agonists activating α7 nAChRs; concentration response relationships based on net change analysis (area under the curve) provide more accurate (and more potent) EC_{50} values.[122] For more slowly desensitizing nAChRs, like α4β2 and α3β4, these two methods are in good agreement with respect to EC_{50} values and Hill coefficients.[123]

Classical agonists (Table 1)

These well-established agonists are non-selective and can activate all nAChR subtypes (with the possible exception of α9* nAChRs),[124] with varying degrees of potency and efficacy. The general rank order of potency is:

Epibatidine > Anatoxin A > Nicotine = Cytisine ≥ ACh > Carbamoylcholine

Acetylcholine chloride (ACh)

The endogenous agonist that activates all nAChRs

ACh is the endogenous agonist for all nAChR subtypes. It is a full agonist and a popular choice for activating nAChRs in electrophysiological experiments (commonly used at 100 µM). Its utility is compromised by (i) its lack of selectivity for nAChRs versus muscarinic receptors and (ii) its susceptibility to hydrolysis. A muscarinic antagonist (typically atropine, ~1 µM) and an acetylcholinesterase inhibitor may be necessary adjuncts; some of these agents may also interact directly with nAChRs. Choline, a substrate and hydrolysis product of ACh, is a selective, full agonist of α7 nAChRs (EC_{50} = 1.6 mM; about an order of magnitude less potent than ACh).[125,126] Choline has weak partial agonist activity at α3β4* nAChRs,[126] and also antagonizes nAChRs.[127]

(±)-Anatoxin A fumarate

A potent non-selective agonist with properties similar to those of ACh

Anatoxin A is a potent, semi-rigid, stereoselective agonist originally isolated from freshwater blue green algae, *Anabaena flos aqua*.[128] Activity resides in the natural (+)-enantiomer. At muscle nAChRs, anatoxin A is about 8 times more potent than ACh[129] but it is 20-100 times more potent than ACh in activating neuronal nAChR subtypes (EC_{50} values in the high nM – low µM range).[130,131] As a secondary amine, anatoxin A crosses the blood brain barrier readily, and the (+)-enantiomer is behaviorally effective when administered systemically at doses of 100-200 µg/kg in rats.[132] Despite similarities, its responses were qualitatively different from those of nicotine, a conclusion supported by subsequent studies.[133,134]

Carbamoylcholine chloride (carbachol)

A weak, non-selective agonist that is more potent at muscarinic receptors

Carbamoylcholine is the carbamate analog of ACh; it is hydrolysis-resistant but retains potency at muscarinic receptors and is commonly used as a non-selective muscarinic agonist. It has been frequently employed to study muscle nAChRs[135] but has lower affinity at α4β2 and α7 nAChRs.[136] N-Methylation of the carbamate nitrogen to yield N-methylcarbamoylcholine recovers high (nanomolar) binding affinity at α4β2 nAChRs, comparable to ACh.[136] N-Methylation also confers substantial selectivity for nAChRs over muscarinic receptors.

(-)-Cytisine

A relatively potent agonist with variable efficacy dependent on nAChR subtype and subunit stoichiometry

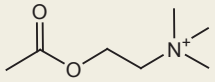
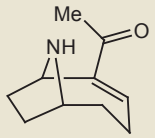
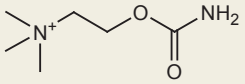
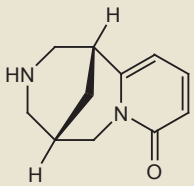
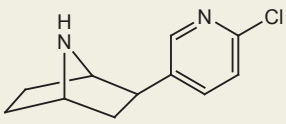
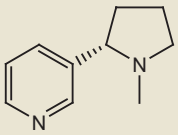
(-)-Cytisine is a rigid tricyclic quinolizidine alkaloid found in plants of the *Leguminosae* family. It is comparable to nicotine with respect to its high affinity binding to α4β2 nAChRs (K_i ~1 nM). However, its differential interactions with other nAChR subtypes has enabled subpopulations of nicotinic binding sites labeled by [^{3}H]-epibatidine to be distinguished by their high or low affinity for cytisine.[137] Its efficacy varies with subunit composition;[37] it has very low or negligible efficacy at $(\alpha4)_2(\beta2)_3$ nAChRs, but displays ~50% efficacy at $(\alpha4)_3(\beta2)_2$ nAChRs.[138,139] These properties have made cytisine a lead compound for drug discovery programs,[140] most notably for smoking cessation[141,142] and depression.[143] Cytisine has been shown to be effective in behavioral experiments at doses of 1-3 mg/kg in rats[144,145] or 1-5.6 mg/kg in mice.[146,147] It is less potent than nicotine *in vivo*, a fact ascribed to its lower lipophilicity.[144] Cytisine shows only partial generalization to nicotine in a drug discrimination test, attributed to its partial agonist profile at α4β2 nAChRs.[148,149] Like nicotine, it offers some protection against 6-hydroxydopamine lesions *in vivo*.[145]

(±)-Epibatidine

A very potent and efficacious non-selective agonist

(+)-Epibatidine was originally obtained from skin extracts of the Amazonian frog, *Epidobates tricolor*.[150] This bicyclic alkaloid (comprising an azabicycloheptane structure coupled to a chloropyridyl moiety) is one of the most potent nicotinic agonists, with both enantiomers showing similar activity. It binds to multiple heteromeric nAChRs with subnanomolar affinities.[151] The functional potency of epibatidine is also exceptionally high with sub-micromolar EC_{50} values for heteromeric and α8 neuronal nAChR subtypes, whereas α7 and muscle nAChRs exhibited EC_{50} values in the low micromolar range.[117,152,153] *In vivo*, epibatidine displays potent non-opioid analgesic activity[150] but its therapeutic window is very narrow, attributed to side effects arising from its lack of nAChR subtype selectivity. Both spinal and central loci for

Table 1 | Classical nAChR agonists

Agonist[a]	Structure	Comment/Selectivity[a]
Acetylcholine		Endogenous nAChR agonist; also activates muscarinic receptors. Readily hydrolyzed
(±)-Anatoxin A		Potent ACh-like, nAChR-specific, hydrolysis-resistant agonist
Carbamoylcholine (carbachol)		Non-hydrolyzable analog of ACh; also activates muscarinic receptors. α1β1γ/εδ > neuronal nAChRs
(-)-Cytisine		Potent partial agonist at α4β2 nAChRs; greater efficacy at other subtypes
(±)-Epibatidine		Very potent agonist. Heteromeric neuronal nAChRs > α7, α1β1γ/εδ nAChRs
(-)-Nicotine		Potent agonist. Heteromeric neuronal nAChRs > α7, α1β1γ/εδ nAChRs

(Bold text denotes compounds available from Tocris at time of publication)

[a] See text for details and references

epibatidine's antinociceptive actions have been proposed.[154–156] Effective doses reported for *in vivo* administration are: 0.25-10 µg/kg[156] or 0.1-1.0 µg[155] intrathecal (rat), 1-10 µg/kg s.c.,[154,157] 0.01-0.3 µg local infusion.[156]

(-)-Nicotine ditartrate

*The eponymous nicotinic agonist activates all nAChR subtypes except α9**

The tobacco alkaloid (-)-nicotine is the prototypic nAChR agonist that has been used historically to classify nAChRs. All nAChR subtypes are activated by nicotine (with the exception of α9 and α9α10 nAChRs, which are blocked by nicotine).[124] It binds preferentially and with high affinity to α4β2 nAChRs (K_i ~1 nM),[158] and activates neuronal nAChRs with EC_{50} values in the micromolar range.[8] Nicotine crosses the blood brain barrier readily and its pharmacokinetics and metabolism are well documented.[159] Recommended doses for *in vivo* research have been compiled.[160] Behavioral responses often show a bell-shaped dose-response profile with maximum responses in rats elicited by doses of 0.4 mg/kg s.c. or less. Doses are normally reported as the free base concentration of nicotine, and correspond to 3-times higher concentrations of the tartrate salt. Since the latter forms acidic solutions, it should be pH-neutralized for *in vivo* administration.

The primary metabolite of nicotine is (-)-cotinine.[159] It is credited with having weak agonist activity at certain nAChR subtypes[161,162] (EC_{50} = 340 µM for rat striatal dopamine release).[163] *In vivo*, its high concentration, relative to nicotine, and long half-life may promote nAChR desensitization, in both human smokers and animal subjects.[159,163]

Other synthetic agonists (Table 2)

A 582941

An α7 nAChR-selective partial agonist

A 582941 (Octahydro-2-methyl-5-(6-phenyl-3-pyridazinyl)-pyrrolo[3,4-c]pyrrole) is an α7 nAChR-selective agonist that binds with high affinity (K_i = 10 nM, rat) to these receptors.[164] It behaves as a partial agonist at α7 nAChRs (EC_{50} = 2.4 µM; 60% efficacy) but is devoid of appreciable activity at other nAChR subtypes tested. It was effective in a number of behavioral tests when given *in vivo* at doses of 0.01-1 µmol/kg (administered i.p. in rodents or i.m. in monkeys),[164] 1-10 mg/kg in rats[165,166] or 0.04-4 mg/kg in mice.[167]

A 844606

An α7 nAChR-selective partial agonist

A 844606 (2-(Hexahydro-5-methylpyrrolo[3,4-c]pyrrol-2(1*H*)-yl)-9*H*-xanthen-9-one) is an α7 nAChR-selective partial agonist derived from a well-known interferon inducer tilerone.[168] Tilerone itself possesses potent affinity for α7 nAChRs and has inspired structure-activity relationship studies.[169] A 844606 was found to bind with high affinity (IC_{50} = 11 nM, rat) and potently activated α7 nAChRs in *Xenopus* oocytes (EC_{50} ~ 2 µM).[168] A carbon-11 labeled version has been investigated as a potential PET ligand.[170]

1-Acetyl-4-methylpiperazine hydrochloride (AMP HCl)

A weak non-selective agonist

The hydrochloride (HCl) salt of 1-acetyl-4-methylpiperazine (AMP) is a brain accessible version of the nAChR agonist AMP methiodide.[171] It exhibits lower potency than the methiodide and differs from nicotine in its pharmacodynamic actions. AMP has been employed along with other agonists to demonstrate a nicotinic component within the visual responses of superior colliculus neurons in the rat.[172]

4-Acetyl-1,1-dimethylpiperazinium iodide

A weak non-selective agonist

4-Acetyl-1,1-dimethylpiperazinium iodide is a less potent analog of isoarecolone.[173] This class of compound is of interest because of its structural rigidity and similarity to the classical ganglionic agonist DMPP.[174]

(+)-Anabasine hydrochloride

A weak non-selective agonist

(+)-Anabasine is a tobacco alkaloid.[175] Unusually, anabasine shows similar binding affinity at α4β2 and α7 nAChRs (K_i ~ 0.5 µM).[176] Functional selectivity for α7 nAChRs is enhanced in the naturally occurring analog anabaseine (see below). Anabasine is effective behaviorally at 1-10 mg/kg s.c.[177,178] Intravenous LD_{50} in mice is 1.3 mg/kg, and high doses produced muscle co-ordination deficits (presumably by interacting with muscle nAChRs).[179]

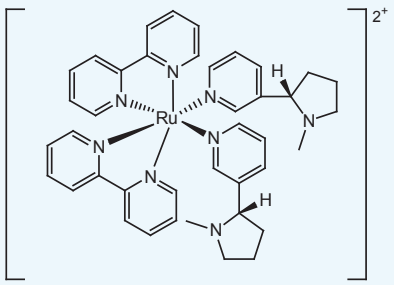
AR-R 17779 hydrochloride

An α7 nAChR-selective agonist

AR-R 17779 ((3*S*)-Spiro[1-azabicyclo[2.2.2]octane-3,5′-oxazolidine]-2′-one hydrochloride) was one of the first α7-selective agonists to be reported.[180] It is a structurally rigid spirooxazolidone with 100-fold greater affinity for binding to α7 nAChRs than α4β2 nAChRs. It activated α7 nAChR currents

Caged Nicotine

RuBi-Nicotine *Cat. No. 3855*



RuBi-Nicotine is a ruthenium-bisbipyridine-caged nicotine $[Ru(bpy)_2(Nic)_2]^{2+}$ that can be excited by irradiation with light in the visible spectrum.[469] The compound undergoes rapid photolysis to release nicotine (time constant 17.3 ns), and exhibits a high quantum yield. RuBi-Nicotine induces action potential propagation in Retzius neurons of leech ganglia, with no detectable toxicity at a concentration of 1 mM.[469]

Table 2 | Other nAChR agonists, including novel synthetic structures

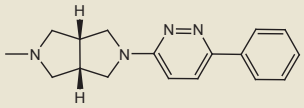
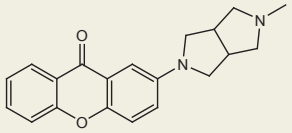
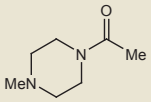
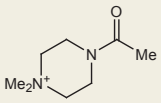
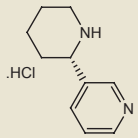
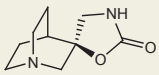
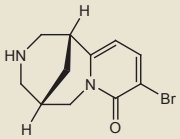
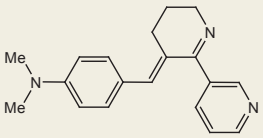
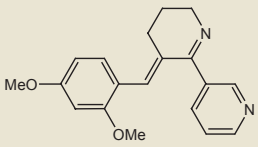
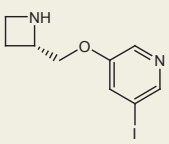
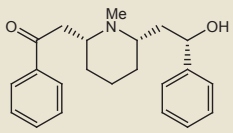
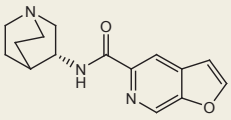
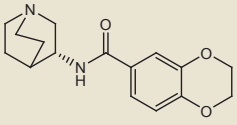
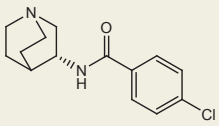
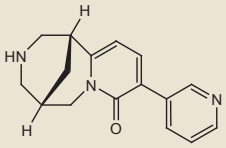
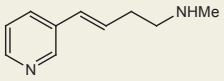
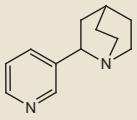
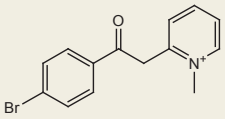
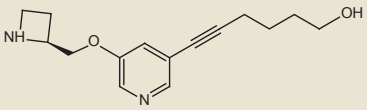
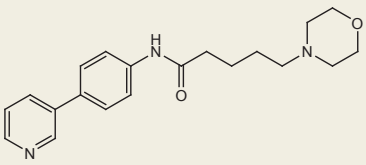
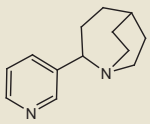
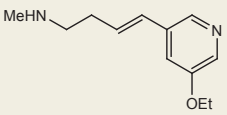
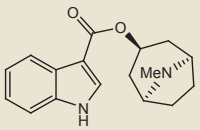
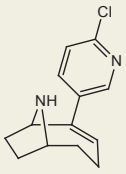
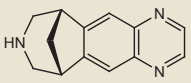
Agonist[a]	Structure	Comment/Selectivity[a]	Effective Concentration Range[b]	
			In vitro	*In vivo*
A 582941		An α7 nAChR-selective partial agonist	5-10 µM	0.1-10 mg/kg
A 844606		An α7 nAChR-selective partial agonist	5-10 µM	
1-Acetyl-4-methylpiperazine		A weak non-selective nAChR agonist, structurally related to isoarecolone and DMPP		
4-Acetyl-1,1-dimethyl-piperazinium		A weak non-selective nAChR agonist, structurally related to isoarecolone and DMPP		
(+)-Anabasine		A weak non-selective nAChR agonist, with relatively high affinity for α7 nAChRs		1-10 mg/kg
AR-R17779		An α7 nAChR-selective agonist	10-100 µM	1-2 mg/kg
3-Bromocytisine		A potent non-selective nAChR agonist	1-100 nM	0.1-0.2 mg/kg
DMAB-anabaseine		A weak nAChR agonist with some preference (but lower efficacy) for α7 nAChRs		2 mg/kg
GTS 21		A partial agonist at α7 nAChRs and a weak antagonist at α4β2 and other heteromeric nAChRs	5-100 µM	0.1-10 mg/kg
5-Iodo-A-85380		A potent β2*-selective nAChR agonist	10-100 nM	0.1-1 mg/kg
(-)-Lobeline		An atypical nicotinic partial agonist that also interacts with several non-nicotinic targets	0.1-10 µM	0.3-4 mg/kg
PHA 543613		An α7 nAChR-selective agonist, weak activity at 5-HT_3 receptors	0.3-30 µM	0.3-1.0 mg/kg
PHA 568487		An α7 nAChR-selective agonist		

Table 2 | (continued)

Agonist[a]	Structure	Comment/Selectivity[a]	Effective Concentration Range[b]	
			In vitro	*In vivo*
PNU 282987		An α7 nAChR-selective agonist	1-10 µM	1-30 mg/kg
3-pyr-Cytisine		A selective partial agonist at α4β2 nAChRs	10-100 µM	0.3-0.9 mg/kg
RJR 2403		An α4β2 nAChR-selective agonist	0.1-10 µM	10-200 mg/kg
RJR 2429		A broad spectrum agonist: α4β2> $\alpha_1\beta_1\gamma\delta$> α3β4* nAChRs		
S 24795		A weak α7 nAChR-selective agonist	10-100 µM	0.3-1.0 mg/kg
Sazetidine A		A potent stoichiometry-dependent agonist at α4β2 nAChRs	10-100 nM	0.01-3 mg /kg
SEN 12333		An α7 nAChR-selective full agonist	10-100 µM	1-10 mg/kg
TC 1698		An α7 nAChR-selective full agonist; partial agonist at $\alpha_1\beta_1\varepsilon\delta$ nAChRs; competitive antagonist at α4β2 nAChRs	10 µM	
TC 2559		An α4β2 nAChR-selective agonist	0.1-10 µM	0.3-10 mg/kg
Tropisetron		A 5-HT_3 receptor antagonist and α7 nAChR partial agonist; inhibits α3β4 nAChRs	1-10 µM	1-3 mg/kg
UB 165		A potent nAChR agonist, especially at α4β2 nAChRs	0.1-1 µM	
Varenicline		A partial agonist at α6β2β3*>α4β2>>α3β4 nAChRs; full agonist at α7 nAChRs	0.1-10 µM	0.1-3 mg/kg

(Bold text denotes compounds available from Tocris at time of publication)

[a] See text for details and references

[b] Range of concentrations typically used to achieve substantial activation of main target nAChR subtype in rodents (unless otherwise stated)

* Indicates the possible inclusion of additional unspecified subunits

in *Xenopus* oocytes (EC_{50} = 10 μM) whereas no activation of α4β2, α3β4, α3β2 or 5-HT_3 receptors was observed, at concentrations up to 1 mM.[181] Central effects were observed with doses of AR-R 17779 of 1-2 mg/kg s.c.[182,183] Its lack of effect at 20 mg/kg in attentional tasks has been interpreted as evidence that α7 nAChRs are not involved.[184]

3-Bromocytisine

A potent non-selective agonist with high affinity for α4β2 nAChRs

3-Bromocytisine is the most potent of a series of cytisine derivatives halogenated at different positions of the pyridine ring.[185,186] It is an order of magnitude more potent than cytisine but exhibits the same agonist profile as the parent molecule.[186,187] It binds to α4β2 nAChRs with picomolar affinity (K_i ~80 pM) and displays a biphasic activation curve with α4β2 nAChRs expressed in *Xenopus* oocytes (EC_{50} = 5 pM and 9 nM), consistent with a differential interaction with the two stoichiometries of this recombinant receptor.[187] It is effective *in vivo* at 0.1-0.2 mg/kg (rat).[188]

DMAB-anabaseine dihydrochloride

A weak agonist with some preference for α7 nAChRs

DMAB-anabaseine dihydrochloride (4-[(5,6-Dihydro[2,3´-bipyridin]-3(4*H*)-ylidene)methyl]-*N,N*-dimethylbenzenamine dihydrochloride) is a derivative of anabaseine (a naturally occurring toxin with a distinctive nicotinic profile favouring α7 nAChRs)[189] and has a nicotinic profile that has been superceded by the more efficacious derivative DMXB (GTS 21).[190] DMAB-anabaseine (2 mg/kg) produced some benefits in combating learning and auditory deficits.[190,191]

GTS 21

A partial agonist at α7 nAChRs and an antagonist at α4β2 nAChRs

GTS 21 (3-[(2,4-Dimethoxyphenyl)methylene]-3,4,5,6-tetrahydro-2,3'-bipyridine dihydrochloride; also known as DMXB), initially reported to be a nicotinic ligand with cognitive-enhancing properties,[190,484] was subsequently shown to be a partial agonist at α7 nAChRs and a weak antagonist at α4β2 and other heteromeric nAChRs.[485] Co-crystallization of the AChBP with GTS 21 indicates that the benzylidene substituent prevents full closure of binding site loop-C, which may explain its partial agonist / antagonist profile.[486] There are species differences in potency and efficacy of GTS 21 at rat (EC_{50} = 5 μM; 32% efficacy) and human (EC_{50} = 11 μM; 9% efficacy) α7 nAChRs, attributed to amino acid differences in the binding site loops.[487] Pharmacokinetic studies in rats indicate that it rapidly crosses the blood brain barrier and its elimination half-life from plasma is 1.7 h.[488] GTS 21 (0.1-10 mg/kg i.p.) is effective in rodent models of sensory gating and cognitive deficits[485,489] and GTS 21 improved attention and working memory in patients with schizophrenia.[79] Its principal hydroxy metabolites exhibit similar activity profiles to the parent compound.[8,490]

5-Iodo-A-85380 dihydrochloride

A potent β2-selective agonist

Iodination of A-85380 to generate an iodinated radioligand produced improved functional selectivity for β2-containing nAChRs over other nAChR subtypes.[192,193] This is useful for discriminating β2- from β4-containing nAChRs.[194] 5-Iodo-A-85380 is a potent agonist at β2* nAChRs (EC_{50} = 13 nM),[193] and is effective *in vivo*; for example, it improved auditory gating in mice when given at 0.1-1 mg/kg i.p.[195] and partially reduced L-DOPA induced motor dysfunction when given twice daily at ~0.2 μmol/kg.[196] 5-Iodo-A-85380 incorporating the short-lived radioisotope ^{123}I is in use in humans as a SPECT neuroimaging ligand for evaluating α4β2 nAChRs.[197]

(-)-Lobeline hydrochloride

A nicotinic partial agonist with multiple non-nicotinic targets

(-)-Lobeline, an alkaloid from the Indian tobacco *Lobelia inflata,* has been known as a ganglionic drug for over half a century but is an atypical nicotinic ligand. Lobeline binds with high affinity to α4β2* nAChRs (K_i ~10 nM), and displays agonist, antagonist (desensitizer) and potentiator actions.[198–200] Its partial agonist profile has prompted investigation of its utility in combating drug dependence.[201,202] However, lobeline is promiscuous and inhibits vesicular monoamine transporters (IC_{50} ~1 μM) and plasma membrane dopamine transporters (IC_{50} = 40-100 μM),[203] μ opioid receptors (IC_{50} = 1 μM)[204] and potassium channels (IC_{50} = 15 μM, K_V1.5 channel).[205] Lobeline (0.3-4 mg/kg) shows some nicotine-like effects *in vivo*,[206] although blockade of other targets may also contribute, for example, to its antidepressant-like effects.[207]

PHA 543613 dihydrochloride

An α7 nAChR-selective agonist with activity at 5-HT_3 receptors

PHA 543613 (*N*-[(3*R*)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide) is a novel α7-selective agonist (K_i = 8.8 nM; EC_{50} = 65 nM) with negligible activity at other nAChRs and weaker activity at 5-HT_3 receptors (K_i = 500 nM).[208] PHA 543613 displayed rapid brain penetration and was effective in behavioral tasks (0.3-1.0 mg/kg, rat)[208] and provoked neurochemical changes *in vivo* (0.3-1.0 mg/kg, mice).[209]

PHA 568487

An α7 nAChR-selective agonist

PHA 568487 is a quinuclidine α7-selective agonist, designed to overcome limitations in *in vivo* tolerability of previous structures.[210]

PNU 282987

A potent α7 nAChR-selective agonist

PNU 282987 (*N*-[(3*R*)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide) is a potent and selective α7 nAChR agonist (K_i = 27 nM; EC_{50} = 150 nM), with weak activity at 5-HT_3 receptors (K_i = 0.9 μM).[211,212] It has been widely used to target α7 nAChRs. It is reported to be effective *in vivo* at 1-30 mg/kg i.p.,[213,214] 1 mg/kg i.v.[212] or 10-40 nM injected locally into the brain.[215]

3-pyr-Cytisine

A weak α4β2 nAChR-selective partial agonist

3-pyr-Cytisine (1*R*,5S)-1,2,3,4,5,6-Hexahydro-9-(3-pyridinyl)-1,5-methano-8*H*-pyrido[1,2-*a*][1,5]diazocin-8-one) is the pyridine homolog of 3-bromocytisine (see above).[143] It is a relatively weak partial agonist at α4β2 nAChRs (EC_{50} = 30 μM) with little effect at α3β4 or α7 nAChRs. 3-pyr-Cytisine produced antidepressant-like effects in mice, when administered at 0.3-0.9 mg/kg i.p.[143] 3-pyr-Cytisine (0.3 mg/kg) did not affect intracranial self-stimulation thresholds in rats, in contrast to nicotine and varenicline.[216] However, it was as potent as nicotine as a secretagog when applied to PC12 cells (1-100 μM).[217]

RJR 2403 oxalate

An α4β2 nAChR-selective agonist

RJR 2403 (also known as *trans*-metanicotine or TC-2403) was first generated by opening the pyrrolidine ring of nicotine; it also occurs naturally as a minor tobacco alkaloid. It shows some functional selectivity for α4β2 nAChRs (K_i = 26 nM; EC_{50} = 730 nM) compared with other nAChR subtypes[218,219] and has been used to support the presence of presynaptic α4β2 nAChRs on projections to the dorsal raphe nucleus.[220] RJR 2403 is effective *in vivo*, with a profile that recapitulates some, but not all, of nicotine's effects, consistent with its greater nAChR subtype selectivity.[221] RJR 2403 also produced antinociception in mice when administered at 10-30 mg/kg[222] or 10-200 mg/kg;[223] it was at least 10-fold less potent than nicotine in both studies.

RJR 2429 dihydrochloride

An α4β2 nAChR-selective agonist

RJR 2429 ((±)-2-(3-pyridinyl)-1-azabicyclo[2.2.2]octane) has an unusual profile.[224] It conforms to the pattern expected of a partial agonist at α4β2 nAChRs, binding with high affinity (K_i = 1 nM) while acting as a more effective antagonist than agonist. However, it was more efficacious in eliciting striatal dopamine release (EC_{50} = 2 nM), suggesting an enhanced agonist action at more complex β2* nAChRs. It was also an exceptionally potent agonist at α1β1γδ nAChRs (EC_{50} = 60 nM), but was relatively weak at activating ganglionic (α3β4*) nAChRs.

S 24795

A weak α7 nAChR-selective partial agonist

S 24795 (2-[2-(4-bromophenyl)-2-oxoethyl]-1-methylpyridinium iodide) is a weak partial agonist at α7 nAChRs (EC_{50} = 34 μM; efficacy = 14% of ACh responses).[225] It showed a memory-enhancing effect in mice at 0.3-1.0 mg/kg,[226] and facilitated release of Aβ peptide from α7 nAChRs *in vitro* (10-100 μM) and *in vivo* (0.3-1.0 mg/kg).[227,228]

Sazetidine A dihydrochloride

A potent α4β2 nAChR-selective agonist with stoichiometry-dependent efficacy

Sazetidine A, an analog of A-85380 with an acetylene substituent in the 5 position (analogous to 5-iodo-A-85380), was first reported to be a potent desensitizing agent at α4β2 nAChRs.[229] Subsequent studies indicated an agonist action that is highly dependent on subunit stoichiometry, with very limited activation of $(\alpha4)_3(\beta2)_2$ nAChRs (6%) but full agonist activity at $(\alpha4)_2(\beta2)_3$ nAChRs (EC_{50} = 6 nM).[230] Sazetidine A also potently activated α4α6β2β3 nAChRs (EC_{50} = 19-44 nM), and was 5-10 times less potent, and less efficacious, at activating α6β2β3 nAChRs (EC_{50} = 0.15 μM).[102] It has much weaker affinity for α3β4 and α7 nAChRs.[231] Sazetidine A (3 mg/kg s.c.) reduced ethanol and nicotine self-administration in rats,[232] and ameliorated nicotine withdrawal-induced behavior when given systemically (0.01-0.1 mg/kg i.p.) or by local infusion into the hippocampus (10 pM - 1 nM),[233] raising interest in it as a lead compound for combating drug addiction.

SEN 12333

An α7 nAChR-selective agonist

SEN 12333 (*N*-[4-(3-pyridinyl)phenyl]-4-morpholinepentanamide), also known as WAY 317538, is an α7 nAChR-selective full agonist (K_i = 260 nM; EC_{50} = 1.6 μM).[234] While devoid of functional potency at other targets, inhibition of heteromeric nAChRs and other receptors at low micromolar concentrations was reported. SEN 12333 (1-10 mg/kg) was effective *in vivo* in a number of tests within a narrow therapeutic window, interpreted as reflecting an inverted 'U' dose-response relationship.[234,235]

TC 1698 dihydrochloride

An α7 nAChR-selective agonist

TC 1698 (2-(3-Pyridinyl)-1-azabicyclo[3.2.2]nonane dihydrochloride) is a potent and efficacious agonist at α7 nAChRs (EC_{50} = 440 nM), a partial agonist at muscle nAChRs (EC_{50} = 20 μM), and a competitive antagonist at α4β2 nAChRs (IC_{50} = 0.3 μM versus 30 μM ACh).[236] TC 1698 (10 μM) produced α-Bgt-sensitive changes in the JAK2 survival pathway *in vitro*.

TC 2559 difumarate

An α4β2 nAChR-selective agonist

TC 2559 ((*E*)-*N*-Methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine), the 5-ethoxy derivative of RJR 2403, is comparable with nicotine in eliciting striatal dopamine release (EC_{50} ~0.2 μM) but lacks activity at muscle or ganglionic nAChRs,[237] suggesting a selectivity for α4β2* nAChRs. This preference was confirmed using recombinant nAChRs expressed in cell lines.[238] In contrast to most other agonists, TC 2559 displayed high efficacy at low sensitivity $(\alpha4)_3(\beta2)_2$ nAChRs.[138] TC 2559 (0.3-10 mg kg) generalized to the nicotine discriminative stimulus[148] and when administered at 0.6-10 μmol/kg it showed similar efficacy to nicotine in amelioration of scopolamine-impaired memory, but differed from nicotine in other *in vivo* measures.[237]

Tropisetron

A potent α7 nAChR-selective agonist and 5-HT_3 receptor antagonist

Tropisetron, a 5-HT_3 receptor antagonist,[239] is a potent partial agonist at α7 nAChRs (EC_{50} = 1 μM).[181] It is a potent antagonist of α9α10 nAChRs (IC_{50} = 70 nM),[240] with higher concentrations inhibiting α3β4 nAChRs.[181] MLA-sensitive central or behavioral effects indicate that tropisetron (1-3 mg/kg) can activate α7 nAChRs *in vivo*.[241,242]

UB 165 difumarate

A potent partial agonist at α4β2 nAChRs

UB 165 comprises the azabicyclononene bicycle of anatoxin A and the chloropyridyl moiety of epibatidine and exhibits intermediate potency, with stereoselectivity comparable to that of anatoxin A. Therefore UB 165 is a potent agonist at α4β2* nAChRs (K_i ~0.3 nM; EC_{50} = 50 nM) but in contrast to the parent molecules, it is a partial agonist at this subtype.[243,244]

Varenicline tartrate

A potent β2 nAChR-selective partial agonist*

Varenicline (Chantix™ (USA); Champix™ (EU)) is a cytisine congener developed to exploit the α4β2* nAChR selectivity and partial agonism of the parent compound, as an aid to smoking cessation.[141] It is a partial agonist at α4β2 nAChRs (EC_{50} ~2 μM; 13% efficacy relative to ACh)[245,246] and α6β2β3* nAChRs (EC_{50} = 0.1-0.2 μM; 6-27%).[40,247] It also activates α3β4 nAChRs with lower potency and greater efficacy (EC_{50} ~50 μM; 75%) and is a relatively potent (possibly dependent on species), full agonist at α7 nAChRs (EC_{50} = 0.8-18 μM).[245,246] Genetic mouse models have been exploited to show that α4* nAChRs are necessary and sufficient for varenicline-induced reduction of alcohol consumption.[248] Varenicline is effective *in vivo* at doses of 0.01-6.0 mg/kg (given s.c. or p.o.)[249,250] and has a half-life of 4 and 17 hours in rats and humans respectively, with little metabolism.[251]

Antagonists

As already mentioned, there are rather few nAChR antagonists. In addition to their use as tools for defining nAChR responses and mechanisms, *in vitro* and *in vivo*, antagonists have also been employed to generate animal models of impaired nicotinic function, reviewed by Roegge *et al.*[252] Most have a long pedigree, many emanating from natural products, while some newer synthetic molecules are starting to arise from SAR (structure-activity relationship) programs. Increasing awareness of the subunit complexity of native nAChRs emphasizes the need for nAChR subtype-selective antagonists. However, that very complexity, including differences in stoichiometry and subtle species differences, makes the identification and rigorous characterization of subtype-selective ligands challenging.

Competitive antagonists (Table 3)

By interacting with the nAChR at, or close to, the agonist binding site, the inhibition achieved by competitive antagonists can, by definition, be overcome by increasing the agonist concentration. Hence competitive antagonism is referred to as 'surmountable', shifting the concentration response relationship for the agonist to the right (e.g. (+)-tubocurarine block of ACh-evoked currents in *Xenopus* oocytes;[253] dihydro-β-erythroidine (DHβE) versus nicotine-evoked [^{3}H]-dopamine release).[186] Consequently the degree of functional blockade achieved by a given concentration of competitive antagonist will be influenced by the experimental conditions, notably the agonist concentration.

Benzoquinonium dibromide

A classical neuromuscular blocking agent

Benzoquinonium was developed as a muscle relaxant for surgical anesthesia in the 1950s.[254] It was employed in early studies of muscle nAChRs.[255] Benzoquinonium is not selective, inhibiting muscle and ganglionic nAChRs with comparable potency (IC_{50} = 6 μM)[256] and it is also used as an effective nicotinic antagonist in invertebrate preparations.[257,258] Benzoquinonium is also a weak allosteric potentiator of muscle and neuronal nAChR subtypes at 0.1-10 μM, via a site distinct from the agonist binding site that is shared by galanthamine and other novel ligands.[259]

bPiDDB

Centrally active compound with a profile consistent with selective and potent blockade of α6β2 nAChRs; weaker action at putative α3β4* nAChRs*

bPiDDB (1,1′-(1,12-Dodecanediyl)bis[3-methylpyridinium] dibromide) is a synthetic *N-n*-alkylnicotinium compound that potently blocked around 60% of nicotine-induced dopamine release from striatal slices (IC_{50} = 2 nM) in a competitive manner, without competing for radioligand binding to α4β2 or α7 nAChRs, which was interpreted as evidence of specificity for α6β2* nAChRs.[260,261] bPiDDB was effective in attenuating nicotine-evoked dopamine release *in vivo* in the rat following systemic (1-3 mg/kg s.c) or local administration (0.1-10 μM by reverse dialysis).[262] It also blocked nicotine-evoked noradrenaline release from rat hippocampal slices (putative α3β4* nAChRs)[194] in a non-competitive manner (IC_{50} = 430 nM).[446] This may reflect the sensitivity of β4* nAChRs to bPiDDB, reported in a study of recombinant nAChR subtypes expressed in *Xenopus* oocytes, which revealed a non-competitive mode of action.[263] bPiDDB did not block nicotine-evoked conditioned responding.[278] Originally envisaged as a smoking cessation agent, toxicity of bPiDDB has lead to the development of new analogs.[264]

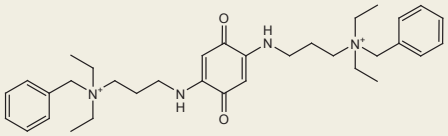
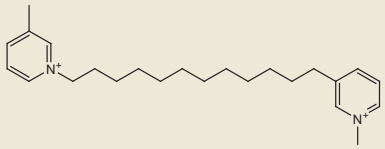
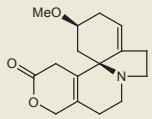
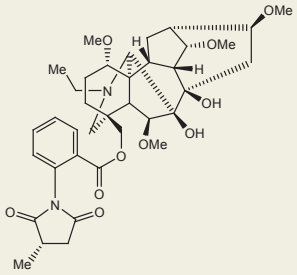
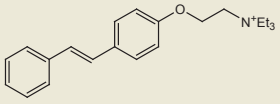
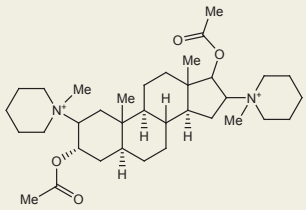
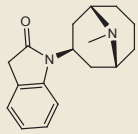
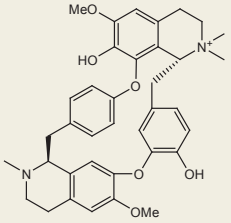
α-Bungarotoxin (α-Bgt)

A very potent, pseudo-irreversible antagonist of α1β1γ/εδ, α7-α9 and some invertebrate nAChRs*

A polypeptide snake toxin isolated from the venom of the Taiwanese banded krait, *Bungarus multicinctus,* α-Bgt was instrumental in the characterization and purification of muscle nAChRs.[1] This 8 kDa 'three finger' peptide is the most potent of the 'long' α-neurotoxins at muscle and *Torpedo* nAChRs. It binds to α1β1γ/εδ, α7-α9* and some invertebrate nAChRs with high affinity (K_i ~1 nM).[124,176,280] Nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography and modeling studies identified loop-C of the agonist binding site as the major locus of interaction, with fingers I and II of α-Bgt enveloping loop-C, which adopts an open or extended conformation. α-Bgt forms cation-π interactions with essential Tyr 190 within loop-C of muscle/*Torpedo* α1 subunit[265,266] and the equivalent Tyr 184 of the α7 nAChR.[267]

The high affinity binding shown by α-Bgt is a consequence of very slow association and dissociation kinetics, with

Table 3 | Competitive nAChR antagonists

Antagonist[a]	Structure	Comment/Selectivity[a]	Effective Concentration Range[b]	
			In vitro	*In vivo*
Benzoquinonium		A classical neuromuscular blocking agent; broad specificity	1-5 µg/ml (10-50 µM)	15-75 µM (limpet)
bPiDDB		α6β2* > α3β4*?	10-100 nM	1-3 mg/kg s.c.
α-Bungarotoxin		Pseudo-irreversible antagonist of muscle, α7, α8, α9* and some invertebrate nAChRs; requires lengthy preincubation	1-100 nM	
Dihydro-β-erythroidine		β2* >> β4*, α7, α8, α9*	1-10 µM	2-4 mg/kg s.c.
Methyllycaconitine		A potent competitive antagonist with selectivity for homomeric nAChRs. α7, α8, α9* > α6* > α3* > α4*, muscle	10-100 nM	4-10 mg/kg s.c.
MG 624		Putative α7-selectivity for chicken recombinant nAChRs; specificity for mammalian nAChRs uncertain	0.1-20 µM	n.d.
Pancuronium		Muscle >>> neuronal nAChRs	1-10 nM	
SR 16584		α3β4 >> α4β2, α7 (recombinant nAChRs)	0.5-10 µM	
(+)-Tubocurarine		Potent blocker of muscle nAChRs at nM concentrations; antagonist of neuronal nAChRs at µM concentrations; also blocks 5-HT_3 > $GABA_A$ receptors	10-30 µM (neuronal nAChRs)	

(Bold text denotes compounds available from Tocris at time of publication)

[a] See text for details and references

[b] Range of concentrations typically used to achieve substantial functional blockade of main target nAChR subtype in rodents (unless otherwise stated); *in vivo* data not given for neuromuscular blocking agents due to peripheral toxicity

* indicates the possible inclusion of additional unspecified subunits

n.d. = Not determined

s.c. = Subcutaneous delivery

implications for its practical application. Typically a pre-incubation of up to one hour with a low nanomolar concentration (10 nM) of toxin is necessary to achieve a complete blockade. This is commonly circumvented by increasing the concentration 10 or 100-fold and decreasing the preincubation time. This strategy is possible because, even at micromolar concentrations, α-Bgt does not appear to interact with other nAChR subtypes (α/β heteromeric neuronal nAChRs). The very slow dissociation kinetics (especially at muscle nAChRs) mean that functional blockade is not readily reversed by washout,[268] which may be advantageous or problematic. It does not access the brain if given peripherally *in vivo* but has been effective if administered by the intracerebroventricular route (i.c.v.; 1.25 nM).[269]

Dihydro-β-erythroidine (DHβE)

A non-α7 nAChR antagonist with a preference for β2-containing subtypes

An alkaloid originating from *Erythrina* seeds, DHβE is a purely competitive antagonist of neuronal nAChRs. The crystal structure of DHβE bound to the AChBP suggests a novel mode of interaction with loop-C of the agonist binding site.[270] Sub-micromolar concentrations of DHβE block recombinant α4β2 nAChRs but it is 10-50 fold less potent at other subtypes, including α3β4, α7 and α9 nAChRs.[124,263,271] A similar difference in sensitivity is shown by native β2* and β4* nAChRs.[186] α6-containing nAChRs have been difficult to express in heterologous systems but using chimeric constructs, α6β2* nAChRs appear to be about 10-fold less sensitive to DHβE than α4β2 nAChRs,[263,272] a result compatible with inferences from native systems.[273] DHβE is typically employed at a concentration of 1-10 μM to selectively block β2* nAChRs *in vitro*. DHβE is also effective *in vivo*, typically administered at concentrations of 2-4 mg/kg s.c in both rats[274,275] and mice.[276,277] Both higher and lower doses have also been reported, e.g. doses up to 10 mg/kg were employed to block nicotine's conditioned stimulus effects in rats,[278] while DHβE was used at 1 mg/kg to explore the roles of β2* nAChRs in motor function, in mice bearing hypersensitive Leu9′Ala mutations in the α4 subunit.[273]

Methyllycaconitine citrate (MLA)

A reversible α7 nAChR antagonist

MLA is a norditerpenoid alkaloid produced by *Delphinium* sp. It is a potent competitive antagonist, selective for α7 nAChRs.[279,470] MLA binds to α7 nAChRs with a K_i of approximately 1 nM, and inhibition of α7 nAChRs by MLA is rapid and reversible, making it a useful alternative to α-Bgt. Like α-Bgt, MLA also potently blocks α9 and α9α10 nAChRs and some invertebrate nAChRs with low nanomolar affinity,[124,280] and potently binds to the AChBP.[17] Crystallization of MLA bound to the AChBP shows that it binds at the subunit interface forming the agonist binding site without closing loop-C.[17] Unlike α-Bgt, MLA discriminates between α7 and muscle nAChRs, and is 3 orders of magnitude less potent at blocking the latter.[279] It is a weak antagonist of α4β2 nAChRs (IC_{50} ~0.2 μM), and may also bind non-competitively at the non-canonical α4-α4 interface of $(α4)_3(β2)_2$ nAChRs.[470] Of more practical concern for brain studies is MLA's ability to inhibit α6β2* nAChRs with only ~30-fold lower affinity (K_i ~30 nM) than at α7 nAChRs.[272,281] Therefore, this antagonist is selective, rather than specific, for α7 over other nAChR subtypes. This can be problematic for *in vivo* studies where the local concentration of MLA is not known, especially in areas of catecholaminergic cell bodies or innervation which exhibit high levels of α6 expression. MLA accesses the brain following systemic injection (4-10 mg/kg s.c. in rodents)[277,282] but brain uptake may be diminished after chronic nicotine treatment.[283] MLA is effective by i.c.v. administration (10 μg, rat).[284]

MG 624

A putative α7 nAChR antagonist but has other actions, especially in mammalian systems

The 4-oxystilbene derivative MG 624 (*N,N,N*-triethyl-2-[4-(2-phenylethenyl)phenoxy]ethanaminium iodide) is a selective and potent antagonist of recombinant chicken α7 nAChRs (IC_{50} = 100 nM). It is ~30 fold less potent at chicken muscle-type or α4β2 nAChRs.[285,286] MG 624 (100 nM) effectively blocked nicotine-induced airway contractions in murine trachea[287] and when applied at 20 μM, inhibited nicotine-induced angiogenesis in various *in vitro* preparations from different species.[288] The latter effect has been ascribed to specific blockade of α7 nAChRs. However, the nAChR subtype specificity of MG 624 in mammalian species is less well established. MG 624 potently attenuated nicotine-evoked [^{3}H]-dopamine and [^{3}H]-noradrenaline release in rat hippocampal slices (IC_{50} ~0.5 μM), in contrast to the α7 nAChR-selective antagonists α-conotoxin ImI, α-Bgt and MLA.[289] Moreover, although MG 624 rather weakly inhibited the binding of a putative α7 nAChR-selective radioligand, [^{3}H]-CHIBA-1001, to rat brain membranes (K_i = 1.6 μM), nicotine, α-Bgt and MLA failed to displace [^{3}H]-CHIBA-1001 binding.[290] The specificity of [^{3}H]-CHIBA-1001 is highly questionable and the ability of MG 624 to interact with this site confounds, rather than confirms, the α7 nAChR-credentials of this antagonist.

Pancuronium bromide

A potent, selective, non-depolarizing antagonist of muscle nAChRs

Pancuronium is a steroidal neuromuscular blocking agent[291] used clinically for reversible neuromuscular blockade in anesthesia and intensive care. Pancuronium is about 10-fold more potent than (+)-tubocurarine at muscle nAChRs.[292] IC_{50} values for inhibition of recombinant immature/extrajunctional muscle $(α1)_2β1γδ$ nAChRs and adult postsynaptic muscle $(α1)_2β1εδ$ nAChRs are in the low nanomolar range,[293,294] with $(α1)_2β1γδ$ nAChRs showing slightly greater sensitivity to pancuronium than their mature counterparts.[295] In contrast to (+)-tubocurarine, pancuronium has a preference for binding to the αδ interface over the αε site.[296] Sensitivity of neuronal nAChRs to pancuronium is 3 orders of magnitude lower than that of muscle nAChRs, with IC_{50} values in the micromolar range.[123]

SR 16584

A novel ligand showing selective antagonism at recombinant α3β4 nAChRs

SR 16584 (1,3-Dihydro-1-(3-exo)-9-methyl-9-azabicyclo[3.3.1] non-3-yl]-2*H*-indol-2-one) was identified through a SAR screen.[297] In ligand binding assays, SR 16584 displaces [^{3}H]-epibatidine binding to rat α3β4 nAChRs expressed in HEK cells with a K_i of 500 nM, but does not displace radioligand binding to α4β2 or α7 nAChRs, indicative of at least 200-fold selectivity for α3β4 nAChRs. This selectivity for α3β4 over α4β2 nAChRs is attributed to the larger size of the bicyclic azabicyclononane ring, compared with that of epibatidine, together with the introduction of the hydroindolinone ring. These features also confer an antagonist profile, demonstrated by the inhibition of epibatidine-induced Ca^{2+} influx in HEK cells transfected with α3β4 nAChRs (IC_{50} = 10 μM), with no evidence of α3β4 nAChR activation by SR 16584 alone.[297] Although the inhibition of [^{3}H]-epibatidine binding implies a competitive interaction with the agonist binding site, saturation binding experiments suggested that SR 16584 provokes a change in K_d as well as B_{max} interpreted as evidence of a degree of non-competitive binding.[297] It is not known if the inhibition of Ca^{2+} influx was surmountable at increasing epibatidine concentrations. This promising compound merits more studies (including its activity at α3β2 nAChRs) to provide a better defined pharmacological profile.

(+)-Tubocurarine chloride (TC)

Classical non-selective nAChR antagonist

A product of the South American shrub *Chondodendron tomentosum,* TC has an intriguing history since its early use as an arrow poison by South American natives.[298] It contributed to classical studies of muscle nAChRs, including the realization that the two non-identical agonist binding sites can be distinguished pharmacologically: TC binds with higher affinity to the α-ε interface than to the α-δ interface of adult mouse nAChRs.[12,299] Low nanomolar concentrations of TC block muscle nAChRs (IC_{50} = 18 nM) whereas it inhibits neuronal nAChRs at micromolar concentrations, with IC_{50} values between 1 and 20 μM.[123] While blockade of α4β2 nAChRs is clearly competitive,[123,253] a non-competitive mode of action has been inferred at α7 or α3β4 nAChRs.[123,300] TC also binds to the AChBP with high affinity.[301] Despite its rigid structure, TC adopted at least 3 different binding orientations at the intersubunit binding site. This versatility may account for the ability of TC to interact with other cys-loop receptors. TC is a potent antagonist of 5-HT_3 receptors, binding with a K_i of approximately 0.1 μM, with a concentration of 1-10 μM inhibiting 5-HT_3 receptor-mediated currents in hippocampal interneurons and transfected HEK-293 cells.[302–304] Higher concentrations of TC also block $GABA_A$ receptor function.[305,306]

α-Conotoxins (Table 4)

The α-conotoxins are highly selective, often very potent, typically competitive, peptide antagonists of nAChRs that are

Table 4 | α-Conotoxins

α-Conotoxin[a]	Structure	Selectivity[a]
ACV 1 (Vc1.1)	Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Asn-Tyr- Asp-His-Pro-Glu-Ile-Cys-NH_2	α9α10 nAChRs, plus actions at other nAChRs and other targets
AulB	Gly-Cys-Cys-Ser-Tyr-Pro-Pro-Cys-Phe-Ala- Thr-Asn-Pro-Asp-Cys-NH_2	α3β4 > α7 nAChRs
EI	Arg-Asp-Hyp-Cys-Cys-Tyr-His-Pro-Thr-Cys- Asn-Met-Ser-Asn-Pro-Gln-Ile-Cys-NH_2	α1β1δγ, α3β4 > α4β2 nAChRs
ImI	Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp- Arg-Cys-NH_2	Moderate potency at α7, α9 nAChRs (rat); species differences
MII	Gly-Cys-Cys-Ser-Asn-Pro-Val-Cys-His-Leu- Glu-His-Ser-Asn-Leu-Cys-NH_2	α3β2, α6-containing nAChRs
PIA	Arg-Asp-Pro-Cys-Cys-Ser-Asn-Pro-Val-Cys- Thr-Val-His-Asn-Pro-Gln-Ile-Cys-NH_2	α6β2* > α6β4 > α3β2 > α3β4 nAChRs
PnIA	Gly-Cys-Cys-Ser-Leu-Pro-Pro-Cys-Ala-Ala- Asn-Asn-Pro-Asp-Tyr-Cys-NH_2	α3β2 > α7 nAChRs

(Bold text denotes compounds available from Tocris at time of publication)

[a] See text for details and references

* Indicates the possible inclusion of additional unspecified subunits

produced by marine cone snails.[307] The venom from such organisms contains a cocktail of toxins to immobilize and kill their prey and predators. Careful analysis of the individual peptides has identified some discriminating research tools. The α-conotoxins are 12-19 amino acids in length and all share a conserved disulfide bonding pattern imposed by 4 conserved cysteine residues. Some α-conotoxins display a degree of selectivity for particular nAChR subtypes that has not been realized by other antagonists, making them therapeutic drug leads.[308] In addition to their deployment as selective antagonists they are also useful molecular probes for interrogating nAChR structure and function.

There are a few caveats associated with their experimental use as antagonists. Their exquisite selectivity can make extrapolation between species problematic, α-conotoxin ImI being a prime example (see below). Synthetic peptides can differ from naturally produced peptides that may undergo post-translational modification, which could modify their characteristics (e.g. α-conotoxin PnIA is sulfated).[307] As peptides, conotoxins are prone to stick to plastic surfaces etc., and BSA (0.1-1 mg/ml) is commonly added to counter this problem.[263,309] Their peptidergic nature precludes the usual routes of systemic administration *in vivo*.

ACV 1 (α-conotoxin Vc1.1) (Table 5)

A potent antagonist of α9α10 nAChRs, with actions at other targets

ACV 1 was identified in venom duct mRNA from *Conus victoriae*.[310] It was initially shown to be a competitive inhibitor of nicotine-stimulated catecholamine release and nicotine-evoked currents in chromaffin cells (IC_{50} = 1-3 μM), indicative of an interaction with α3β4* nAChRs.[310,311] It also blocked α6-containing nAChRs at sub-μM concentrations. It was effective in models of neuropathic pain,[309,312] attributed to its potent antagonism of α9α10 nAChRs[313] (Table 5a). It is regarded as a useful lead compound for this clinical condition.[313] However α9α10 nAChRs have been challenged as the therapeutic target; instead, an agonist action at $GABA_B$ receptors modulating Ca^{2+} channels has been proposed[314–316] and questioned.[113] The jury is still out.[313] ACV 1 has been administered *in vivo* by intrathecal (0.2 – 2 nM, rat)[317] and intramuscular routes (0.36 – 36 μg, rat).[313,318]

α-Conotoxin AuIB

A modest antagonist of α3β4 nAChRs, with weaker activity at α7 nAChRs

α-Conotoxin AuIB was initially purified from the venom of *Conus aulicus*.[346] It was found to be a selective antagonist of α3β4 nAChRs (IC_{50} = 0.75 μM) with little effect on other heteromeric nAChRs tested, as it does not block α9α10 nAChRs[318] but does display some activity at α7 nAChRs (34% block at 3 μM).[346] Its specificity for α3β4* nAChRs has been exploited in the characterization of nAChR subtypes modulating transmitter release and ionic responses *in vitro* (typically used at 5-10 μM).[347–349] α-conotoxin AuIB has also been administered into the brains of rats *in vivo*, by local microinjection (2.5-25 pM);[350,351] the i.c.v. route of administration was employed in mice (3.5-14 pM).[277] Intramuscular (0.36-36 μg) or intrathecal injections (0.02-2 nM) were reported to be effective in reversing signs of neuropathic pain,[317,318] although a partial inhibition of voltage operated Ca^{2+} channels (as reported for ACV 1) was also observed.[318]

α-Conotoxin EI

A structurally interesting α-conotoxin that preferentially blocks α1β1δγ and α3β4 nAChRs

α-Conotoxin EI was purified from the venom of *Conus ermineus*, an Atlantic fish-hunting *Conus*.[319] α-conotoxin EI competitively antagonizes muscle-type nAChRs. It binds preferentially to the α1/δ subunit interface of *Torpedo* nAChRs but is much less discriminating between the two agonist binding sites of mammalian muscle nAChRs. Structurally, α-conotoxin EI resembles α-conotoxins that target neuronal, rather than muscle, nAChRs.[320] Subsequently α-conotoxin EI has been found to block α3β4 nAChRs more effectively than α4β2 nAChRs when applied at 10 μM.[321] Potentiating effects following brief application of nanomolar concentrations of α-conotoxin EI were also noted.

Table 5 | Relative selectivity and potencies of select conotoxins

a) Selectivity of ACV 1[309,311]

nAChR Subtype	α9α10	α6/α3β2β2[a]	α6/α3β4[a]	α3β4	α3α5β2	α3β2	α3α5β4, α4β2, α7, α1β1δγ
IC_{50} (nM)	19	140	980	4200	7200	7300	>30,000

b) Potency of α-Conotoxin ImI at rat[323] and human[325] nAChRs

Species	Rat				Human			
nAChR Subtype	α7	α9	α1β1δγ	α3β4, α4β2	α3β2	α7	α3β4	α4β2,α1β1δε
IC_{50} (μM)	0.22	1.8	51	No effect	0.04	0.6	3.4	>10

c) Selectivity of α-Conotoxin PIA[339]

nAChR Subtype	α6/α3β2[a]	α6/α3β2β3[a]	α3β2	α6/α3β4[a]	α3β4	α4β2, α1β1δε
IC_{50} (nM)	0.69	0.95	74	30	518	>10,000

[a]Chimeric α3/α6 subunits comprised of the N-terminal extracellular domain of α6 and transmembrane domains of α3 subunits, to facilitate heterologous nAChR formation and expression[433]

α-Conotoxin ImI (Table 5)

A selective antagonist at rodent α7 and α9 nAChRs but potently blocks human α3β2 nAChRs

α-Conotoxin ImI is a 12 amino acid peptide originally isolated from *Conus imperialis*.[322] It was first described as a selective antagonist of rat α7 and α9 nAChRs, with muscle nAChRs requiring more than 2 orders of magnitude higher concentrations for blockade.[323] However, α-conotoxin ImI appears to show considerable species differences in its selectivity. It is more efficacious at producing neuromuscular blockade in frog preparations,[322] and its propensity to block nicotine-evoked catecholamine secretion in bovine chromaffin cells (IC_{50} = 2.5 μM) has been attributed to inhibition of α3β4* nAChRs in this species.[324] When tested on recombinant human nAChRs it proved to be most potent at blocking α3β2 nAChRs[325] (Table 5b).

α-Conotoxin ImI has been exploited to probe binding interactions with α7 nAChRs,[307,326] and with an AChBP,[17] as well as serving as a template for developing novel α7 nAChR-selective antagonists.[308] It has been used to define rodent α7 nAChR responses *in vitro*.[327,328] *In vivo*, i.c.v. injections (5-10 nM) in rodents produced complex seizures (likely due to blockade of α7 nAChRs in the hippocampus) with a high incidence of death, while i.p injections were without effect.[322]

α-Conotoxin MII

A potent and selective antagonist of α3β2 and α6-containing nAChRs

α-conotoxin MII, from *Conus magus*, was initially hailed as being a selective antagonist of α3β2 nAChRs (IC_{50} = 0.5 nM)[329] but was subsequently revealed to recognize α6-containing nAChRs,[330] which are at least as sensitive to inhibition by α-conotoxin MII (IC_{50} = 0.4 nM).[331] This specificity appears to extend to primate nAChRs.[332] α-conotoxin MII has been widely used *in vitro* to investigate the roles of α6β2* nAChRs in modulating nigrostriatal dopamine release in normal and Parkinsonian models (typical concentrations for selective blockade are 10-100 nM).[101,333,334] Intrathecal administration of α-conotoxin MII (0.02 – 2 nM) was effective in a neuropathic pain model.[317] It has also been administered locally by injection (0.25-25 pM),[335,336] reverse dialysis (1-10 μM),[337] or delivered i.c.v. (30 nM)[338] to achieve central blockade.

α-Conotoxin PIA (Table 5)

A potent and selective antagonist of α6-containing nAChRs

α-Conotoxin PIA was cloned from *Conus purpurascens*.[339] It blocked α6β2* nAChRs at low nanomolar concentrations and was two orders of magnitude less potent at α3β2 nAChRs. This was the first antagonist reported to distinguish between α6- and α3-containing nAChRs. It also preferentially blocks β2- over β4-containing nAChRs; the order of potency is α6β2* > α6β4 > α3β2 > α3β4 (Table 5c). Recovery from blockade by α-conotoxin PIA was quicker at α3-containing nAChRs. Recombinant rat and human subtypes were similarly sensitive to α-conotoxin PIA.[339] It is also effective on native nAChRs, partially blocking nicotine-stimulated dopamine release from striatal synaptosomes (IC_{50} = 1.5 nM),[340] consistent with the involvement of α6β2* nAChRs. α-Conotoxin PIA (1 nM) was used to define the presence of α6-containing nAChRs on GABAergic terminals.[341] I*n vivo* perfusion of α-conotoxin PIA into the rat ventral tegmental area by reverse dialysis (10 μM) attenuated nicotine-evoked increases in dopamine overflow and locomotion.[337] In this study, α-conotoxin PIA was an order of magnitude less potent than α-conotoxin MII, but its greater selectivity for native α6β2* nAChRs over α3β2* nAChRs was affirmed.

α-Conotoxin PnIA

A potent antagonist of α3β2 nAChRs, with weaker activity at α7 nAChRs

α-Conotoxin PnIA was purified from the venom of *Conus pennaceus*.[342] It preferentially inhibits α3β2 nAChRs (IC_{50} = 9.5 nM) while displaying weaker antagonism of α7 nAChRs (IC_{50} = 252 nM).[343] It has attracted most interest because the related α-conotoxin PnIB shows the reverse selectivity, prompting structure activity studies.[307,344,345] α-Conotoxin PnIA was less effective at α3β4, α4β2 and α1β1δγ nAChRs.[343] Its effect on α6-containing nAChRs does not appear to have been reported.

Non-competitive antagonists (Table 6)

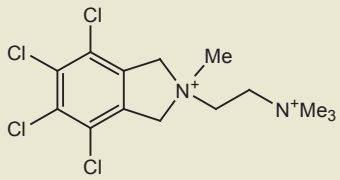
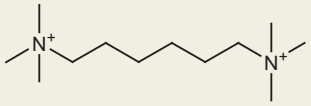
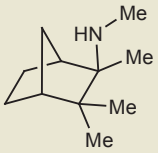
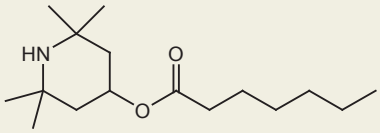
Non-competitive antagonists, by definition, do not compete for binding to the agonist binding sites. Hence they do not displace the binding of conventional agonist or competitive antagonist radioligands, and their inhibition is not surmountable with increasing agonist concentration (e.g. comparison of blockade of ACh-evoked currents by catestatin (non-competitive) and DHβE (competitive) at α3β4 nAChRs).[352] Insurmountable blockade has practical advantages for reliable nAChR inhibition. Non-competitive antagonists interact with distinct sites on the nAChR to inhibit receptor function; typically they block the nAChR channel, but a variety of sites and modes of antagonism are encountered.[353]

Catestatin

A peptide open channel blocker of α3β4 and other neuronal nAChRs

Catestatin, a naturally occurring, 21 amino acid fragment of chromogrannin A that is secreted from chromaffin cells, is a potent inhibitor of nicotinic-cholinergic-stimulated catecholamine secretion (IC_{50} ~0.8 μM).[352,354] The peptide confers an antihypertensive protection, while naturally occurring polymorphisms and knockout of catestatin result in varying degrees of hypertension. Catestatin inhibits recombinant rat α3β4 nAChRs expressed in *Xenopus* oocytes in a reversible, non-competitive, voltage-, and use-dependent manner.[352] This profile is consistent with open-channel blockade. Docking of the catestatin structure onto a homology model of the α3β4 nAChR indicated major interactions with the extracellular domain of the α3 subunit, at sites distinct from the agonist binding site, such that the peptide occludes the ion channel from the receptor vestibule, consistent with its non-competitive mode of action.[356] α7, α3β2 and α4β2 nAChRs were similarly sensitive to 0.1-10 μM catestatin.[352]

Table 6 | Non-competitive nAChR antagonists

Antagonist[a]	Structure	Comment/Selectivity[a]	Effective Concentration Range[b]	
			In vitro	*In vivo*
Catestatin	Arg-Ser-Met-Arg-Leu-Ser-Phe-Arg-Ala-Arg-Gly-Tyr-Gly-Phe-Arg-Gly-Pro-Gly-Leu-Gln-Leu	A peptide with specificity for α3β4 and other neuronal nAChRs	0.1-10 µM	
Chlorisondamine		Long-lasting inhibition of CNS nAChRs		10 mg/kg 5 µg, i.c.v.
Hexamethonium		Ganglionic blocker that does not cross blood brain barrier	1-100 µM	2-10 mg/kg; 12-18 ng, i.c.v.
Mecamylamine		Centrally active ganglionic blocker/ heteromeric nAChRs > α7	1-10 µM	1-3 mg/kg
TMPH		Slowly reversible inhibitor of heteromeric neuronal nAChRs	1-10 µM	

(Bold text denotes compounds available from Tocris at time of publication)
[a] See text for details and references
[b] Range of concentrations typically used to achieve substantial functional blockade of main target nAChR subtype in rodents (unless otherwise stated)

Chlorisondamine diiodide

A ganglionic blocker which produces long-lasting inhibition of central nAChRs

This bisquaternary nicotinic antagonist was originally used as a ganglionic blocker, inhibiting nAChRs that mediate synaptic transmission in sympathetic and parasympathetic ganglia.[357] When administered *in vivo* it is unique in producing a persistent blockade of nAChRs within the CNS. Inhibition lasts for weeks or even months, in contrast to a transient ganglionic blockade. For example, nicotine-evoked [^{3}H]-noradrenaline release was abolished from rat hippocampal synaptosomes prepared 3 weeks after administration of chlorisondamine *in vivo* (10 mg/kg s.c).[358] It is hypothesized that this long lasting inhibition may arise from an intracellular accumulation of the drug, based on the retention of tritium following administration of radiolabeled chlorisondamine.[358] Its persistent action is particularly useful for antagonizing brain nAChRs during lengthy behavioral protocols or chronic studies involving repeated or continuous nicotine administration.[359] However, it should be noted that systemic administration of centrally effective doses of chlorisondamine (10 mg/kg s.c) will elicit some transient ganglionic effects due to peripheral blockade. The drug can also be delivered locally into the brain (5 µg, i.c.v.) to interrogate the roles of discrete brain regions in nicotinic responses.[188] The long lasting inhibition allows for recovery from stereotaxic injection (3 days[188]) before behavioral testing. The nAChR selectivity of chlorisondamine is unclear.

Hexamethonium bromide

A ganglionic blocker that does not access the brain

Hexamethonium was also first recognized as a ganglionic nAChR blocking agent, and used to treat hypertension, among other conditions.[360] It is a voltage-dependent, open channel blocker of ganglionic nAChRs.[361] The hydrophilic nature of this polymethylene bistrimethylammonium compound limits its ability to cross the blood-brain barrier and access the brain. Hence it is used in comparative *in vivo* studies with a centrally acting antagonist (typically mecamylamine) to establish if a particular behavior is centrally or peripherally mediated.[362,363] Typical doses for systemic administration are in the range 2-10 mg/kg.[364,365] Hexamethonium is relatively non-selective, blocking muscle and diverse neuronal nAChR subunit combinations with an IC_{50} value of ~10-20 µM,[246] and is used at 1-100 µM to block neuronal nAChRs *in vitro*.[366,367] It is an effective antagonist of central nicotinic effects if delivered directly into the brain.[365]

Mecamylamine hydrochloride

Widely used antagonist for in vitro and in vivo studies

Mecamylamine was also developed as a ganglionic blocker for the treatment of hypertension.[368] Unlike hexamethonium, this secondary amine readily crosses the blood brain barrier to exert both central and peripheral effects. More recently mecamylamine has been considered as a lead compound for the treatment of neuropsychiatric conditions.[8,369] It has become the

archetypal non-competitive antagonist for neuronal nAChRs. Molecular docking studies suggest that as a result of its predominantly protonated form, mecamylamine is attracted to the channel mouth and interacts with a luminal site, consistent with it being an open channel blocker.[370] Mecamylamine inhibits most neuronal nAChRs with IC_{50} values typically in the range 0.1-5 μM, with α3β4 appearing slightly more sensitive than other nAChRs.[246,271] Ten μM mecamylamine is typically used to achieve a complete block *in vitro*. Mecamylamine's antagonism of α7 nAChRs is readily reversible,[371] with higher concentrations needed for effective blockade of this subtype. Mecamylamine (1-10 μM) also antagonizes muscle nAChRs in a non-competitive manner and high concentrations of mecamylamine (100 μM) can transiently inhibit NMDA receptors.[135,371] Mecamylamine crosses the blood brain barrier freely and has been widely used as a general nicotinic antagonist in behavioral experiments. It is typically administered at 1-3 mg/kg in rodents to block CNS nAChRs *in vivo*, although doses as high as 10 mg/kg have been reported.[252] It has also been effective after i.c.v. administration.[372]

2,2,6,6-Tetramethylpiperidin-4-yl heptanoate hydrochloride (TMPH)

A potent and slowly reversible inhibitor of heteromeric neuronal nAChRs

TMPH is a synthetic derivative of the parent bis-tetramethylpiperidine compound BTMPS. The latter produces a nearly irreversible non-competitive block of neuronal nAChRs (IC_{50} ~200 nM) by interacting with the channel-forming domain of β subunits, whereas its inhibition of muscle nAChRs is readily reversible.[373] Similarly, low micromolar concentrations of TMPH produced a long-lasting inhibition of heteromeric nAChRs comprised of α3 or α4 with β2 or β4 subunits (IC_{50} ~100-400 nM), whereas blockade of α7 and muscle nAChRs was readily reversible, allowing the different classes of nAChRs to be distinguished.[374] However, incorporation of an additional subunit (either α5, α6 or β3) into heteromeric nAChRs resulted in less potent inhibition by TMPH. It was also effective *in vivo* (1-5 mg/kg),[375,376] inhibiting nicotine-induced analgesia, nicotine discrimination and levamisole-induced seizures. A selective action was implied by the insensitivity of nicotine-induced hypothermia and locomotor effects to TMPH (up to 20 mg/kg).[375]

Other compounds producing non-competitive inhibition of neuronal nAChRs

Many molecules that have other primary targets also act as non-competitive antagonists of nAChRs. These agents cannot be considered to be specific for nAChRs, but the interactions can be of pharmacological or physiological relevance or they may raise practical concerns. The examples mentioned here are representative of some of the more well-known or topical classes. Compounds in bold typeface are currently available from Tocris.

Antidepressants and antipsychotics

Buproprion was originally developed as an antidepressant but is now marketed as an aid to smoking cessation (Zyban™). Its principal pharmacological target is inhibition of dopamine and noradrenaline transporters, but at low micromolar concentrations it also acts as a non-competitive inhibitor of various neuronal nAChR subtypes.[377–379] The possibility that this interaction contributes to the efficacy of bupropion as a smoking cessation agent is debated.[380] The arguments are complicated by the efficacy of bupropion metabolites and species differences.[381] A photoreactive derivative of bupropion has been developed to interrogate its sites of interaction, reported to be within the M2 pore-forming domain of the nAChR.[382] Low micromolar concentrations of other antidepressants including **fluoxetine**, **sertraline** and **paroxetine** also inhibit nAChRs (IC_{50} = 1-12 μM),[377,383,384] although it is argued that this interaction is unlikely to contribute to their antidepressant actions.[385] Both typical (chlorpromazine, **haloperidol**) and atypical antipsychotics (**clozapine**, **quetiapine**)[386] and the anti-epileptic drug **lamotrigine**[387] also interact with nAChR channels.

Ca^{2+} channel blockers

Inhibitors of L-type voltage-operated Ca^{2+} channels inhibit nAChRs in chromaffin cells or neuroblastoma cell lines, with IC_{50} values in the low micromolar range. These drugs include **verapamil** and **diltiazem**, and the dihydropyridines **nimodipine**, **nifedipine** and **nitrendipine**.[388–390] The N/P/Q-type calcium channel blocker **ω-conotoxin MVIIC** and the N-type blocker **ω-conotoxin GVIA** have also been reported to block nAChRs expressed in *Xenopus* oocytes, with rat α3β4 nAChRs being more susceptible than α7 nAChRs.[389] The block of α3β4 nAChRs by ω-conotoxins was shown to be reversible, whereas these inhibitors exert a longer lasting inhibition of voltage-operated calcium channels. This difference has been exploited to discriminate between these two targets.[347]

NMDA receptor antagonists

(+)-MK 801 maleate ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) is an open channel blocker of the NMDA receptor (IC_{50} = 0.4 μM), with no effect on AMPA or kainate receptors.[391] It is also an open channel blocker of α4β2 (IC_{50} = 15 μM) and α7 nAChRs (IC_{50} = 15 μM);[392,393] being about 40 times less potent at nAChRs compared with NMDA receptors.[394] Other NMDA receptor blockers that also interact with nAChRs, typically in the low micromolar range, include the dissociative anesthetics **phencyclidine** and **ketamine,**[377,395] and the Alzheimer's drug **memantine**.[396] The endogenous tryptophan metabolite **kynurenic acid**, a competitive antagonist of the glycine site of NMDA receptors, has been reported to be a non-competitive antagonist of α7 nAChRs (IC_{50} = 7 μM),[398] although this is controversial.[399,400] In a recent report, kynurenic acid was advocated as a negative modulator of α7 nAChR-mediated enhancement of marijuana's rewarding effects.[482]

Steroids

Steroids, including **corticosterone**, **progesterone**, **estradiol** and **hydrocortisone**, inhibit native and recombinant neuronal nAChRs with IC_{50} values ranging from 0.1-10 μM.[401–403] Fluorescence spectroscopy has identified the membrane lipid – nAChR interface as the site of action of steroids (and also of free fatty acids).[404] Progesterone modulates α5 nAChR subunit expression, although this probably represents an indirect mechanism.[405]

Strychnine hydrochloride

Strychnine, from the plant *Strychnos nux-vomica*, is a potent competitive antagonist of glycine receptors. It also interacts with other members of the cys-loop family. Strychnine non-competitively blocked muscle α1β1γδ nAChRs (IC_{50} = 7 μM)[406] and heteromeric neuronal nAChRs ('type II' putative α4β2* nAChRs; IC_{50} = 38 μM).[407] In contrast it was a more potent, competitive antagonist of α9α10 nAChRs (IC_{50} ~20 nM)[118,124] and α7 nAChRs (IC_{50} = 1 μM).[407] Like (+)-tubocurarine, it has been shown to interact with multiple sites on the AChBP.[301]

Amyloid β-peptide 1-42 ($A\beta_{1-42}$)

There are numerous reports that $A\beta_{1-42}$, the endogenous agent that accumulates in Alzheimer's disease, interacts with nAChRs, principally the α7 subtype. However, the reported effects of $A\beta_{1-42}$ on nAChR function are very varied and not easily reconciled.[408] Non-competitive, voltage-independent block of α7 nAChRs in rat hippocampal cultures or *Xenopus* oocytes has been described (IC_{50} = 7.5 – 90 nM);[409,410] heteromeric α7β2 nAChRs in basal forebrain cholinergic neurons are purported to show high sensitivity to blockade by oligomeric $A\beta_{1-42}$.[54] However, others have failed to observe any antagonism or have reported activation of α7 nAChRs by very low (picomolar) concentrations of $A\beta_{1-42}$.[411] Inconsistencies in the literature may be due to variables such as the membrane environment of the nAChR and the physical state of $A\beta_{1-42}$.[408] There is an emerging view that α7 nAChRs (having a presynaptic, postsynaptic or glial location) may mediate some physiological and/or pathological effects of $A\beta_{1-42}$ at glutamatergic synapses.[412–415]

Positive Allosteric Modulators (Table 7)

Positive allosteric modulators (PAMs)[29] interact with sites separate from the agonist binding site, and alter the energy barrier between resting, open and/or desensitized states (Figure 2). They lower the energy barrier from resting to activated (open) nAChR, or raise the barrier from open to desensitized nAChR, resulting in an increased probability of receptor activation and hence a potentiation of agonist action.[8] PAMs have no discernible agonist activity[416] (although some potentiators, notably galanthamine and physostigmine, can themselves increase the frequency of single channel events).[417] They embrace a wide variety of structures, including the endogenous molecule SLURP-1 that is a PAM at peripheral α7 nAChRs.[418,419] The modulators are therapeutically attractive as they offer a means of enhancing the nicotinic effects of endogenous agonist (ACh), preserving the spatial and temporal specificity imposed by cholinergic innervation. The emergence of subtype-selective PAMs, notably α7-selective PAMs, has prompted extensive searches for new molecules with PAM activity, using conventional drug discovery approaches or novel *in silico* methods.[420–422]

As a result of comparing the properties of several PAMs, two classes of α7-selective PAMs have been distinguished, termed type I and type II.[29,416] Type I PAMs lower the energy barrier between resting and open states to enhance agonist responses (sensitivity / efficacy) without altering the response time-course (dictated by the open to desensitized transition of the receptor). NS 1738 is a commonly used representative.[423,424] Type II PAMs raise the barrier for desensitization, enhancing the agonist response and its duration. PNU 120596 was the first compound in this class to be described.[8] Type II PAMs are useful research tools for unveiling functional α7 nAChRs that are otherwise undetectable with methods that lack sufficient temporal and functional sensitivity to capture the normally very brief responses.[425,426]

Interrogation of the sites with which PAMs interact on the nAChR has identified a number of candidates. Type I and II PAMs have been proposed to bind at a common, mutually exclusive site located in an intrasubunit transmembrane cavity.[31,421,423,427] This interaction is analogous to that proposed for neurosteroids and volatile anesthetics binding to the $GABA_A$ receptor, and might correspond to a conserved modulatory site.[428] However, alternative, extracellular sites have been proposed for type I PAMs, notably the M2-M3 loop for genistein and NS 1738.[416,429] Homology modeling and mutagenesis has defined an extracellular site on α4β2 and α7 nAChRs for non-selective PAMs represented by galanthamine,[430,449] while photoaffinity labeling has identified multiple sites.[431] Moreover, at least 3 small molecule PAM binding sites were described in the extracellular domain of an α7 nAChR model, using an automatic pocket finding program.[422] Some modulators (e.g. morantel and zinc) are purported to bind at a non-canonical subunit interface[30,432] and galanthamine's greater potentiation of α5-containing nAChRs has invoked comparison with the benzodiazepine site of $GABA_A$ receptors.[40]

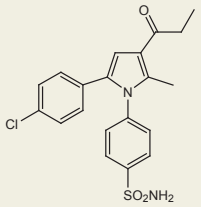
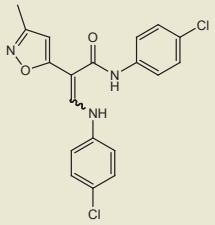
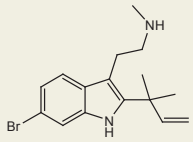
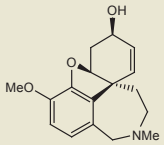
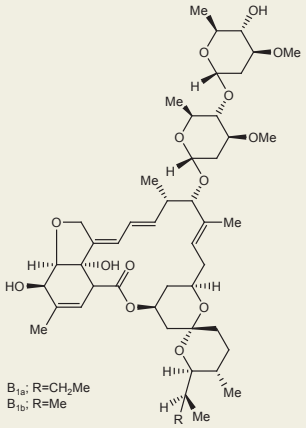
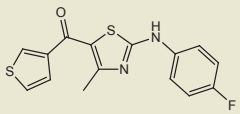
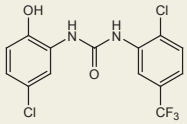
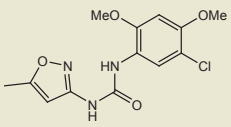
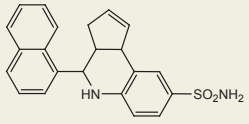
Both type I and type II PAMs have been reported to show *in vivo* efficacy.[457] This has raised concern that chronic administration of a type II PAM (modeling a long term therapeutic application) might have neurotoxic consequences due to prolonged Ca^{2+} influx[269] but this has not been supported by subsequent experimental observations.[426] However, despite enthusiasm for the PAM concept as a therapeutic approach, no positive allosteric modulators have progressed to clinical development thus far.[8]

A 867744

An α7-selective type II PAM with inhibitory actions at heteromeric nAChRs

A 867744 (4-[5-(4-chlorophenyl)-2-methyl-3-propionyl-1*H*-pyrrol-1-yl]benzenesulfonamide), an optimized pyrrolesulfonamide,[434] potentiated ACh-evoked responses in *Xenopus*

Table 7 | Allosteric potentiators

Positive Allosteric Modulator (PAM)	Structure	Comment/Selectivity[a]	Effective Concentration Range[b]	
			In vitro	*In vivo*
A 867744		α7-selective type II PAM; may inhibit heteromeric nAChRs	5-10 μM	n.d.
CCMI		α7-selective type I PAM; weak $GABA_A$ receptor potentiator	1-3 μM	0.3 mg/kg
Desformylflustrabromine		α4β2- and α2β2-selective PAM with type II characteristics; inhibitory at higher concentrations (≥10 μM)	1-5 μM	3-6 mg/kg
Galanthamine		Non-selective weak potentiator; inhibitory at higher concentrations (≥10 μM)	1-3 μM	1-3 mg/kg
Ivermectin		α7-selective type I PAM; activates several other Cys-loop receptors	30 μM	
LY 2087101		Weak type I PAM at α7 nAChRs; more potent potentiator of α4β2 nAChRs	1-10 μM (α4β2) 10-30 μM (α7)	
NS 1738		α7-selective type I PAM; inhibits α4β2 and α3β4 nAChRs at ≥10 μM	10 μM	10-30 mg/kg
PNU 120596		α7-selective type II PAM	2-10 μM	1-30 mg/kg
TQS		α7-selective type II PAM; inhibits other nAChRs at ≥10 μM	10 μM	

(Bold text denotes compounds available from Tocris at time of publication)

[a] See text for details and references

[b] Range of concentrations typically used to achieve substantial functional potentiation of main target nAChR subtype in rodents

oocytes expressing α7 nAChRs up to 7.3 fold (EC_{50} ~1 μM), without displaying any intrinsic agonist activity itself.[435] A 867744 shifted the concentration-response curve for ACh to the left, prevented desensitization in the continued presence of ACh and reactivated desensitized α7 nAChRs. Similar effects were observed on native α7 nAChRs in hippocampal slice preparations. Although A 867744 did not compete for [^{3}H]-MLA binding sites it did differ from other type II PAMs in partially displacing the binding of [^{3}H]-A 585539,[435] a novel α7-selective radioligand.[436] It is not clear if the locus of this interaction is responsible for the PAM activity of A 867744. A 867744 does not potentiate responses from α4β2 or α3β4 nAChRs or 5-HT_3 receptors, but low micromolar concentrations inhibited α4β2 and α3β4 nAChRs.[435] No cytotoxicity was observed following exposure of cultured cells to A 867744 for up to 3 days.[426]

CCMI

An α7-selective type I PAM

First described as Compound 6, CCMI ([*N*-(4-chlorophenyl)]-alpha-[(4-chlorophenyl)-aminomethylene]-3-methyl-5-isoxazole-acetamide) was identified as an α7-selective PAM in a screen of $GABA_A$ receptor PAMs.[269] It potentiated agonist-evoked currents (from 5% to ~50% of I_{max}) in *Xenopus* oocytes expressing α7 nAChRs (EC_{50} ~0.5 μM). Potentiation of $GABA_A$ receptors was weaker and there were no effects on α4β2, α3β4 or α1β1γδ nAChRs or 5-HT_3 receptors. The concentration-response curve for ACh was shifted to the left but the rapid desensitization kinetics were unaffected. These characteristics are consistent with a type I PAM. In contrast to type II PAMs, CCMI (1 nM – 3 μM) produced only modest increases in intracellular Ca^{2+} and no change in ERK phosphorylation, in cells also exposed to an α7 nAChR agonist.[426] CCMI was behaviorally effective in rodents at a dose (0.3 mg/kg) that was confirmed to penetrate the brain following i.v. or oral administration in mice.[269]

Desformylflustrabromine hydrochloride

A putative α4β2-selective PAM that also potentiates α2β2 nAChRs; inhibitory at higher concentrations

First identified as a natural product in bryozoan *Flustra foliacea* (a marine organism that is a common bryozoan in the North Sea),[437] desformylflustrabromine was found to potentiate α4β2 nAChRs by increasing the open probability of the receptor channel.[438] The synthetic, water soluble HCl salt has been studied in more detail and displays a complex profile. It potentiates ACh-evoked responses from recombinant α4β2 nAChRs up to 3-fold (EC_{50} = 120 nM), shifting the concentration response curve for ACh to the left,[439] and has a greater effect on responses to partial agonists.[440] There is some evidence that it can re-activate desensitized α4β2 nAChRs in the continued presence of agonist,[440] and desformylflustrabromine HCl (1 μM) can prevent the inhibition of α4β2 nAChRs by β-amyloid (1 μM).[213] However, at higher concentrations (>10 μM) desformylflustrabromine HCl inhibits α4β2 nAChRs, and this is likely due to channel blockade. Thus the concentration response curve for desformylflustrabromine HCl is bi-phasic.[439,441]

The specificity profile of desformylflustrabromine relies on a single study of recombinant nAChRs in *Xenopus* oocytes. The natural compound was reported to be devoid of potentiating activity at α3β2, α3β4, α4β4 and α7 nAChRs when tested at 10 μM with 100 μM ACh.[438] More recently desformylflustrabromine HCl was shown to potentiate and inhibit α2β2 nAChRs in a similar manner to its interactions with α4β2 nAChRs (EC_{50} = 446 nM; IC_{50} = 11 μM).[213] The lack of potentiation of α7 nAChRs has been confirmed, with the finding that low micromolar concentrations of desformylflustrabromine HCl inhibit α7 nAChRs (IC_{50} = 2-44 μM).[439,441]

In vivo, desformylflustrabromine HCl (3, 6 mg/kg s.c.) decreased nicotine self-administration (an α4β2* nAChR-mediated response)[82] without substituting for nicotine itself.[442] It is not clear if the potentiating or inhibitory activity (or both) contributed to this action. Brain penetration of desformylflustrabromine HCl was suggested by the presence of the compound in CSF for at least 90 minutes after systemic administration; the half-life for desformylflustrabromine HCl in blood was estimated to be 8.6 hours.[442]

Galanthamine

A weak non-selective potentiator, with greater efficacy at α5-containing nAChRs

Galanthamine is an acetylcholinesterase inhibitor that was among the first nAChR potentiators to be reported.[259] It did not discriminate between major nAChR subtypes and weakly potentiated (by ~30%) nAChR responses evoked by sub-maximal concentrations of nicotinic agonists, shifting the concentration-response curve to the left.[443,444] Alone, galanthamine can activate single-channel currents in muscle and neuronal cells but the probability of channel opening is too low to generate macroscopic (whole cell) currents.[417] Its locus of action was established as distinct from the agonist binding site.[445] nAChRs incorporating the α5 subunit show enhanced potentiation (200%) by galanthamine[40] and this has been exploited to distinguish native heteromeric nAChRs.[77] Higher concentrations of galanthamine (>1 μM) inhibit nAChR responses by acting as an open channel blocker.[40,443]

A number of other compounds also act as weak non-selective potentiators, in a similar manner to galanthamine, including the acetylcholinesterase inhibitor **physostigmine**, the opiate codeine, the neuromuscular blocking agent **benzoquinonium** (Table 3) and the neurotransmitter 5-HT.[259,417,447,448] Site-directed mutagenesis, electrophysiology and molecular docking has identified Thr 197 as important for the interaction of these diverse ligands with neuronal nAChRs: this residue lies close to both the agonist binding site and the cys-loop, leading to a rationale for its potentiating effect.[430,449] However, using the intrinsic photoreactivities of galanthamine and physostigmine to label sites of interaction within muscle-type nAChRs, multiple binding sites were identified.[431] The sensitivity to galanthamine conferred by the α5 subunit[40] raises further questions about its site and mechanism of potentiation.

Ivermectin

An α7-selective type I PAM with actions at other cys-loop receptors

Ivermectin is a macrocyclic lactone and an anthelminthic drug. At nanomolar concentrations it activates glutamate-gated chloride channels (cys-loop receptors exclusive to invertebrate species[3]). At micromolar concentrations ivermectin activates $GABA_A$ and glycine receptors, potentiates α7 nAChRs[32,450] and has no effect on $5\text{-}HT_3$ receptors. Ivermectin conforms to the criteria of a type I PAM at α7 nAChRs and potentiates ACh-evoked currents with an EC_{50} value of 6.8 μM.[451] Its site of action has been localized to an intrasubunit transmembrane cavity.[451] Another anthelmintic, levamisole, targets nAChRs in parasitic nematodes and is a common contaminant of cocaine.[452] It reputedly shows dual potentiation and inhibition of ACh-evoked responses recorded from *Xenopus* oocytes expressing human α3* nAChRs.[453] This dual behavior is reminiscent of the modulatory effects of galanthamine and physostigmine described above.

LY 2087101

Relatively non-selective PAM that potentiates α4β2 nAChRs and is a weak type I PAM at α7 nAChRs

LY 2087101, a (2-amino-5-keto)thiazole compound, has the characteristics of a weak type I PAM in its potentiation of peak currents evoked from α7 nAChRs, at concentrations of 3-30 μM.[454] It is a more potent potentiator of α4β2 nAChRs (EC_{50} = 1 μM) but without effect on α1β1δγ, α3β2 or α3β4 heterologously expressed nAChRs. Mutagenesis and computer docking simulations predict that LY 2087101 may bind to the α7 nAChR within a transmembrane intrasubunit cavity that is also a putative site for type II PAMs.[31]

NS 1738

A type I PAM selective for α7 nAChRs; inhibits α4β2 and α3β4 nAChRs

NS 1738 (*N*-(5-Chloro-2-hydroxyphenyl)-*N*′-[2-chloro-5-(trifluoromethyl)phenyl]urea) is a type I PAM at rat α7 nAChRs expressed in *Xenopus* oocytes (EC_{50} = 3.9 μM) or human α7 nAChRs expressed in GH4C1 cells (EC_{50} = 1.6 μM).[455] Maximal responses to ACh were increased 2-6 fold, with little effect on the potency of ACh or the desensitization kinetics of α7 nAChRs,[455] and it does not reactivate the desensitized receptor.[423] NS 1738 is a selective type I PAM for α7 nAChRs, giving no potentiation of α4β2, α3β4 or α1β1γδ nAChRs, or $5\text{-}HT_3$ receptors; inhibition of the heteromeric neuronal nAChRs was observed at concentrations of 10 μM and above.[455] In rats, NS 1738 shows modest brain penetration and it improved cognitive function *in vivo* in scopolamine-impaired rats (Morris water maze task), when administered at 10 and 30 mg/kg i.p.[455] NS 1738 was inferred to interact with the extracellular domain of α7 nAChRs, including the M2-M3 segment,[429] although other studies have proposed that it competes for the same (or overlapping) intrasubunit transmembrane site that binds PNU 120596.[423] Perhaps consistent with their binding to a common site, NS 1738 (30 mg/kg i.p.) partially blocked the antinociceptive effects of PNU 120596 in mice, without having any antinociceptive action itself.[424]

PNU 120596

Prototypical type II PAM for α7 nAChRs

First published in 2005,[456] PNU 120596 (*N*-(5-Chloro-2,4-dimethoxyphenyl)-*N*′-(5-methyl-3-isoxazolyl)-urea) has become the most widely used type II PAM, with over 60 citations to date. PNU 120596 selectively potentiates α7 nAChRs (EC_{50} = 200 nM).[456] It increases the magnitude of agonist-evoked responses several fold and extends the timecourse of responses by preventing desensitization; these properties led to the classification and definition of type II PAMs.[416] It has been widely used in a variety of *in vitro* studies to magnify α7 nAChR responses[425, 458-460] and is predicted to bind at an intrasubunit transmembrane site on the α7 nAChR.[31,427] Five PNU binding sites per α7 homomer are predicted and potentiation is highly co-operative, with occupancy of all 5 sites required for maximum potentiation.[461]

PNU 120596 accesses the brain after i.p. administration.[424] It is effective *in vivo* in rats against auditory gating deficits (1 mg/kg i.v.),[456] cognitive dysfunction (2 mg/kg i.p.),[462] cerebral ischemia (30 mg/kg s.c., 1 mg/kg i.v.)[463] and inflammatory hyperalgesia (30 mg/kg i.p.),[464] and provoked the overflow of dopamine (1 mg/kg i.p.).[465] In mice PNU 120596 is reported to be antinociceptive (4 and 8 mg/kg i.p).[424] Its sub-chronic administration[424] was without adverse effects, and no cytotoxicity was observed *in vitro*.[426] Daily injections (30 mg/kg; 7 days) did not alter brain ^{125}I-αBgt binding sites in rats, in contrast to the upregulation produced by repeated agonist administration.[466] PNU 120596 has some solubility issues. For *in vivo* administration it is typically prepared as a stock solution in 5% DMSO + 5% solutol dissolved in 0.9% sterile saline.[213]

TQS

An α7 nAChR-selective type II PAM

TQS (3a,4,5,9b-Tetrahydro-4-(1-naphthalenyl)-3*H*-cyclopentan[*c*]quinoline-8-sulfonamide) selectively potentiated α7 nAChR responses (up to 4-fold increase in peak current; EC_{50} = 3 μM) and decreased current decay times, consistent with the characteristics of a type II PAM.[416] It is devoid of agonist activity but, in contrast to PNU 120596, TQS inhibits α4β2, α3β4 and α1β1γδ nAChRs.[416,467] Mutagenesis and computer-docking simulations identify a transmembrane binding site close to that proposed for PNU 120596, using the same methodology.[428] A related compound, 4-BP-TQS, in which the naphthyl group is replaced by a 4-bromophenyl entity, is a potent, atypical, non-desensitizing agonist at α7 nAChRs, in addition to displaying type II PAM activity.[428,467] 4-BP-TQS does not compete for the orthosteric agonist binding site but interacts with the transmembrane site shared by TQS and PNU 120596.

Future Perspectives for nAChR Research

In the seven years since the previous version of this review there have been substantial developments in our understanding of the molecular structure of nAChRs, including insights into their subunit stoichiometry, assembly and trafficking, as

well as an increasing knowledge of their cellular functions and roles in physiological and pathological processes. However, like the proverbial onion, each layer of knowledge reveals further sets of questions. To help tackle these questions, this period has also seen a growth in the number of new nicotinic ligands published, with a welcome increase in the number of nAChR subtype-selective agonists and PAMs.[8]

These synthetic successes are likely to pave the way for more novel nicotinic probes. RuBi-Nicotine[469] (page 9) is an example: this caged nicotine will have applications for the rapid, localized activation of nAChRs. There is also the prospect of monitoring nAChR behavior via detection mechanisms coupled to subunit proteins, such as Förster resonance energy transfer (FRET)[471] or optochemical technology.[472] While the use of radioligands for quantifying nAChRs has declined in recent years, there has been a growth in the development of novel radioligands, based on subtype-selective nicotinic ligands, for *in vivo* analysis of nAChRs by positron emission tomography (PET) studies.[473,474] Antibodies remain problematic for identifying nAChRs, in brain tissue at least,[475] but peptide toxins offer alternative approaches. Fluorescently-labeled α-Bgt has been a valuable marker for labeling muscle and α7 nAChRs in cells and tissues,[476,477] and fluorescent labeling is being extended to the α-conotoxins with some success.[478] Biotinylated α-Bgt offers additional approaches, for example detection by fluorophore-labeled anti-biotin antibodies (for signal amplification)[479] or single particle tracking of nAChRs in live cells using streptavidin quantum dots.[70,479,480] Indeed, α-Bgt binding motifs have been incorporated into α3 subunits[483] and other membrane proteins[481] in order to take advantage of α-Bgt-linked detection agents.

With such technological advances and new chemical entities we can look forward to resolving some of the mysteries that still surround nAChRs and their roles in health and disease.

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Nicotinic Compounds Available from Tocris

Cat. No.	Product Name	Primary Action
Agonists		
4341	A 582941	Partial agonist at α7 nAChR
4477	A 844606	Selective α7 nAChR partial agonist
0351	1-Acetyl-4-methylpiperazine hydrochloride	nAChR agonist
0352	4-Acetyl-1,1-dimethylpiperazinium iodide	nAChR agonist
2809	Acetylcholine chloride	Endogenous neurotransmitter; agonist for all nAChR subtypes
1971	(+)-Anabasine hydrochloride	nAChR agonist
0789	(±)-Anatoxin A fumarate	nAChR agonist
3964	AR-R 17779 hydrochloride	α7-selective nAChR agonist
3549	3-Bromocytisine	Potent agonist of α4β4, α4β2 and α7 nAChR
2810	Carbamoylcholine chloride	Cholinergic receptor agonist; carbamate analog of acetylcholine
3110	(-)-Cotinine	Primary metabolite of nicotine
1390	(-)-Cytisine	Potent, selective neuronal nAChR agonist
2241	DMAB-anabaseine dihydrochloride	nAChR agonist
4125	3-pyr-Cytisine	Potent, selective neuronal nAChR agonist
0684	(±)-Epibatidine	Very potent nAChR agonist
1518	5-Iodo-A-85380 dihydrochloride	High affinity α4β2* and α6β2* subtype-selective nAChR agonist
1527	5-Iodo-A-85380, 5-trimethylstannyl N-BOC derivative	Precursor to 5-Iodo-A-85380 (Cat. No. 1518)
1077	(-)-Lobeline hydrochloride	nAChR agonist; interacts with other targets
3546	(-)-Nicotine ditartrate	Prototypical nAChR agonist
3092	PHA 543613 dihydrochloride	Potent and selective α7 nAChR agonist
3134	PHA 568487	α7-selective nAChR agonist
2303	PNU 282987	α7-selective nAChR agonist
1053	RJR 2403 oxalate	α4β2-selective nAChR agonist
1271	RJR 2429 dihydrochloride	β2-selective nAChR agonist
3855	RuBi-Nicotine	Caged nicotine; rapidly excitable by visible light
3518	S 24795	Partial agonist at α7 nAChR
2736	Sazetidine A dihydrochloride	α4β2-selective nAChR agonist
4441	SEN 12333	α7-selective nAChR agonist
4764	SIB 1553A hydrochloride	Subunit selective nAChR agonist
2518	TC 1698 dihydrochloride	α7-selective nAChR agonist
2737	TC 2559 difumarate	Selective agonist at α4β2 nAChR
2459	Tropisetron hydrochloride	Partial agonist at α7 nAChRs; potent 5-HT_3 antagonist
1348	UB 165 fumarate	nAChR agonist
3754	Varenicline tartrate	Subtype-selective α4β2 nAChR partial agonist
Antagonists		
3205	ACV 1	α9α10-selective nAChR antagonist; α-conotoxin
0424	Benzoquinonium dibromide	nAChR antagonist
2133	α-Bungarotoxin	Antagonist of muscle, α7, α8, α9, α10 and some invertebrate nAChRs
3221	bPiDDB	Orthosteric nAChR antagonist
2722	Catestatin	Non-competitive nAChR antagonist
1001	Chlorisondamine diiodide	nAChR antagonist; long lasting
3124	α-Conotoxin EI	α1β1δγ selective nAChR antagonist
3119	α-Conotoxin IMI	Rodent α7 and α9 antagonist; human α3β2 blocker
1340	α-Conotoxin MII	Potent α3β2- and α6β2-selective nAChR antagonist
3121	α-Conotoxin PIA	Selective antagonist of α6-containing nAChRs
3123	α-Conotoxin PnIA	Selective α3β2 nAChR antagonist
3120	α-Conotoxin AuIB	Selective α3β4 nAChR antagonist

Cat. No.	Product Name	Primary Action
2349	Dihydro-β-erythroidine hydrobromide	Antagonist for neuronal α4-containing nAChRs; exhibits a preference for β2-containing subtypes
2241	DMAB-anabaseine dihydrochloride	Antagonist at α4β2 and other nicotinic receptors
4111	Hexamethonium bromide	Non-competitive nAChR antagonist
2843	Mecamylamine hydrochloride	Non-competitive nAChR antagonist
1029	Methyllycaconitine citrate	Competitive α7-selective nAChR antagonist
1356	MG 624	Antagonist of chicken α7 nAChRs and rodent heteromeric nAChRs
0693	Pancuronium bromide	Nicotinic antagonist selective for muscle nAChRs
4424	SR 16584	Selective α3β4 nAChR antagonist
2438	TMPH hydrochloride	Neuronal nAChR antagonist
2820	(+)-Tubocurarine chloride	nAChR antagonist
Positive Allosteric Modulators		
4571	A 867744	Positive allosteric modulator of α7 nAChR
3837	CCMI	Positive allosteric modulator of α7 nAChR
3328	Desformylflustrabromine hydrochloride	Positive allosteric modulator of α4β2 nAChR
0686	Galanthamine hydrobromide	Cholinesterase inhibitor and nAChR potentiator
1260	Ivermectin	Positive allosteric modulator of α7 nAChR
4141	LY 2087101	Potentiator of α4β2 and α7 nAChRs
2995	NS 1738	Positive allosteric modulator of α7 nAChR
2498	PNU 120596	Positive allosteric modulator of α7 nAChR
4233	TQS	Positive allosteric modulator of α7 nAChR

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