

NEURONAL NICOTINIC RECEPTORS



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Susan Wonnacott is Professor of Neuroscience and Christopher Sharples is a post-doctoral research officer within the Department of Biology and Biochemistry at the University of Bath. Their research focuses on understanding the molecular and cellular events underlying the effects of acute and chronic nicotinic receptor stimulation. This is with the goal of elucidating the structure, function and regulation of neuronal nicotinic receptors.

The nicotinic acetylcholine receptor (nAChR) arguably has the longest history of experimental study of any receptor, and is the prototype ligand-gated ion channel (LGIC). nAChRs in muscle and, to a lesser extent, in autonomic neurones have been characterised in detail. In contrast, nAChRs in the CNS have only lately become the focus of intense research efforts. The family of neuronal nAChRs in the brain and spinal cord is increasingly recognised to have therapeutic potential in a wide range of conditions, yet the physiological functions of these nAChRs remain enigmatic and their subunit composition, assembly, trafficking and regulation, and the significance of their heterogeneity, are still to be fully elucidated. Progress in these endeavours has been hampered by a dearth of subtype-selective tools. Historically, Nature has provided the majority of nAChR-selective compounds, but the nicotinic pharmacopoeia is now expanding with a number of novel synthetic ligands that have been developed in the last few years.

Historical perspective¹

The use of nicotine as an experimental tool was pivotal in Langley's formulation of the concept of a 'receptive substance'.² In these studies he showed that 'nicotine causes tonic contraction of certain muscles of fowl, frog and toad, and that this contraction is prevented... by curare'. Subsequently, Loewi and Dale developed the theory of neurohumoural transmission, and recognised acetylcholine (ACh) as an endogenous transmitter.³ Dale distinguished the actions of muscarine and nicotine, leading to the recognition of two pharmacologically distinct (and now known to be structurally and functionally unrelated) families of receptors for ACh, that take their names from these natural products.⁴ Neuromuscular and ganglionic

preparations lend themselves to physiological and pharmacological investigations, and there followed a period of intense study of the properties of nAChR-mediated transmission at these sites. nAChRs at the muscle endplate and in sympathetic ganglia could be distinguished by their respective preferences for C10 and C6 polymethylene bistrimethylammonium compounds, notably decamethonium and hexamethonium,⁵ providing the first hint of diversity among nAChRs.

Biochemical approaches to elucidate the structure and function of the nAChR protein in the 1970's were facilitated by the abundance of nicotinic synapses akin to the muscle endplate, in electric organs of the electric ray, *Torpedo*, and eel, *Electrophorus*. High affinity snake α -toxins, principally α -bungarotoxin (α -Bgt), enabled the nAChR protein to be purified, and subsequently resolved into 4 different subunits designated α , β , γ and δ .⁶ An additional subunit, ϵ , was subsequently identified in adult muscle. In the early 1980's, these subunits were cloned and sequenced, and the era of the molecular analysis of the nAChR commenced. The muscle endplate nAChR has the subunit combination and stoichiometry $(\alpha 1)_2\beta 1\epsilon\delta$, whereas the extrajunctional nAChR $(\alpha 1)_2\beta 1\gamma\delta$ predominates in foetal or denervated muscle.

Neuronal nAChRs

The biochemical and molecular approaches to studying the muscle nAChR eclipsed interest in neuronal nAChRs, as no model systems existed for these receptors. The credentials for ganglionic nAChRs were firmly established from electrophysiological and pharmacological approaches but the notion of nAChRs in the brain was viewed with scepticism. While α -Bgt, the defining ligand of muscle nAChRs, specifically bound to brain tissue, it did not antagonise central cholinergic functions.⁷ However, the tobacco smoking habit was increasingly accepted to be an addiction to nicotine, implying that nicotine exerts a powerful central effect. In 1980, [³H]-nicotine was reported to specifically label sites in the rat brain that have a unique nicotinic pharmacology.⁸ [³H]-Nicotine binding was not blocked by α -Bgt, and the anatomical distributions of binding sites for these two nicotinic ligands were quite distinct, raising the (then) novel and controversial prospect of nAChR heterogeneity in the brain. The first publication of a cloned neuronal nAChR subunit ($\alpha 3$) appeared in 1986.^{9,10} The nAChR credentials of the α -Bgt binding site in neurones were conclusively provided by cloning of the $\alpha 7$ nAChR subunit gene in 1990.¹¹ The current tally of neuronal nAChR subunits in mammals is eleven ($\alpha 2$ - $\alpha 7$, $\alpha 9$, $\alpha 10$, $\beta 2$ - $\beta 4$), with an additional subunit, $\alpha 8$, identified in avian species.^{12,13}

A distinct but related gene family of α and β subunits has been revealed in invertebrates.¹⁴ The *C. elegans*

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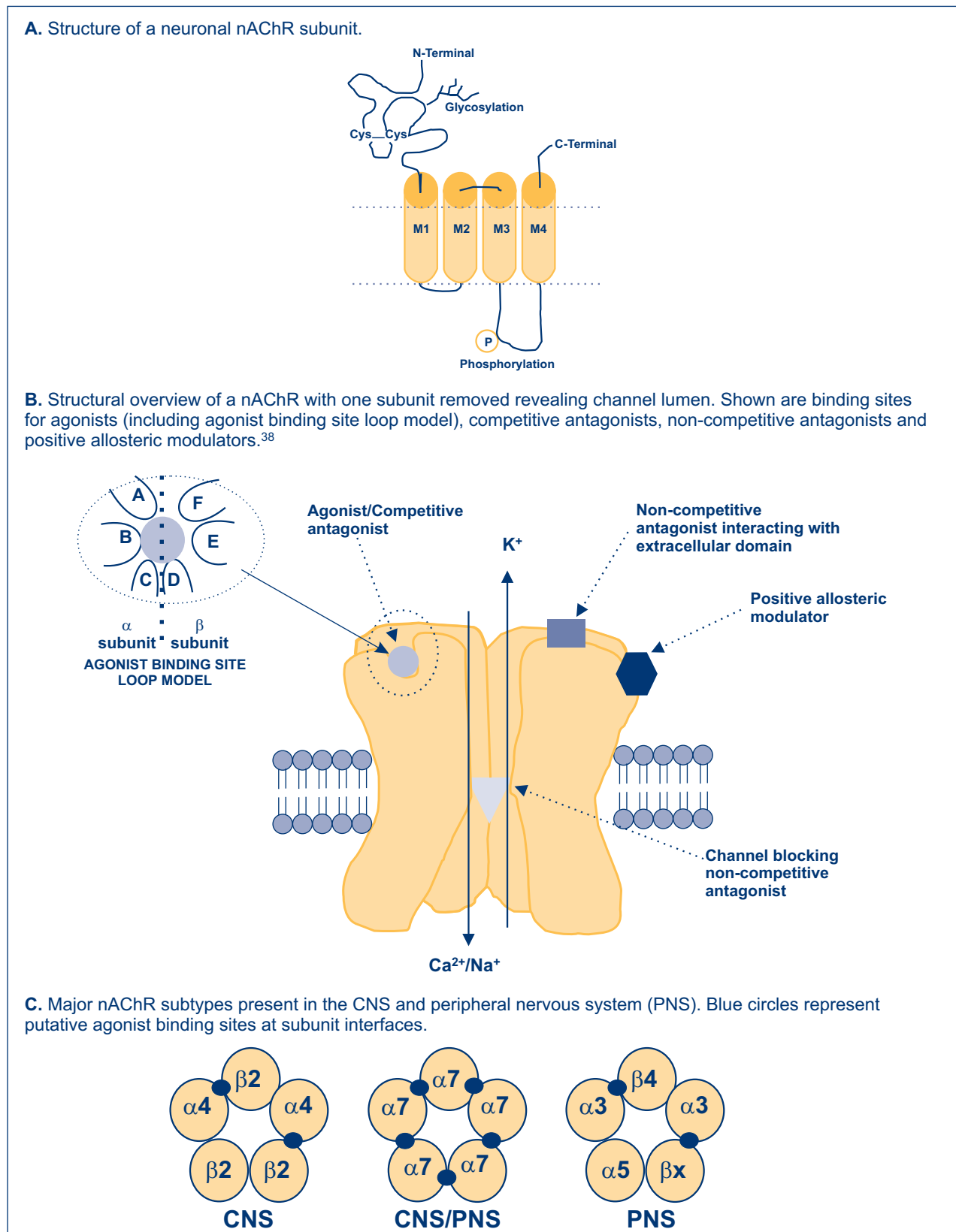
genome sequence incorporates 17 candidate nAChR subunits; nAChRs in nematodes are the target for the anthelmintic levamisole. In insects, nAChRs are the principal excitatory receptors in the CNS, and constitute a major pesticide target (e.g. for imidacloprid). Here we will focus on vertebrate nAChRs; the reader is referred to a recent review¹⁴ for a comprehensive account of insect nAChRs.

Heterogeneity of neuronal nAChR subunits and subtypes

nAChRs are pentameric LGICs and members of the superfamily of neurotransmitter receptors that includes the GABA_A, glycine and 5-HT₃ receptors.¹⁵

nAChRs are present (albeit in relatively low abundance) throughout the central and peripheral nervous systems. In contrast to their pivotal role in autonomic neurotransmission and in the initiation of muscle contraction, nAChRs in the CNS are considered to exert a more modulatory influence.^{16,17} Neuronal nAChRs are gaining credence as significant players in the nervous system from their relationship with a number of disease states in which they are perceived as novel drug targets.¹⁸ These include Alzheimer's and Parkinson's diseases, schizophrenia, Tourette's Syndrome and attention deficit disorder. In addition, neuronal nAChRs are also targets for analgesia, anxiolysis, neuroprotection and smoking cessation.

Figure 1. nAChR structure and heterogeneity



Neuronal nAChRs are composed of pentameric combinations of α and β subunits, giving the potential for a great variety of native neuronal nAChRs.¹² Heterologous expression of nAChRs in *Xenopus* oocytes and mammalian cell lines has established certain rules of assembly that limit the subtypes of native nAChRs that may exist. Pairwise combinations of $\alpha 2$, $\alpha 3$ or $\alpha 4$ subunits with $\beta 2$ and $\beta 4$ subunits can form functional nAChRs, whereas the $\alpha 5$ and $\beta 3$ subunits are generally unable to participate successfully in pairwise combinations, but they have been expressed in heteromers with at least two other types of subunits.^{12,19,20,21,22} The $\alpha 6$ subunit has been shown to form a functional nAChR in combination with $\beta 4$, but higher levels of expression were observed when $\alpha 6$ was co-expressed with at least two other types of subunit, including $\beta 3$.^{23,24} In contrast, the $\alpha 7$, $\alpha 8$ and $\alpha 9$ subunits are distinguished by their ability to form robust homomeric receptors in expression systems, although $\alpha 7\alpha 8$ heteromers also occur in avian tissues.^{11,25,26,27} The related $\alpha 10$ subunit is only incorporated into a functional nAChR when co-expressed with $\alpha 9$.¹³

In native systems, knowledge of the subunit composition of nAChRs is generally lacking and only a few major subtypes have been identified. These include the $\alpha 4\beta 2^*$ nAChR, which is relatively abundant in the CNS and constitutes over 90% of high affinity [³H]-cytisine binding in the rat brain (see below).²⁸ Based on the expression of $\alpha 4\beta 2$ nAChRs in *Xenopus* oocytes, the stoichiometry $(\alpha 4)_2(\beta 2)_3$ has been proposed.^{29,30} However, more complex subunit combinations may exist *in vivo* and an asterisk is used to denote the possible presence of additional types of subunit.¹² Indeed in chick a small proportion of $\alpha 4\beta 2^*$ nAChRs may contain $\alpha 5$ subunits and in rat striatum an nAChR composed of $\alpha 4, \beta 2, \alpha 5$ and $\alpha 6$ subunits has been proposed.^{31,32}

The other major subtype of neuronal nAChR is comprised of $\alpha 7$ subunits which are generally thought to form homomeric nAChRs in the CNS and peripheral nervous system.³³ In the chick brain and retina, the $\alpha 7$ subunit also forms heteromeric nAChRs with $\alpha 8$, and may combine with other subunits such as $\alpha 5$ in chick sympathetic neurones.^{25,27,34} In the peripheral nervous system, a variety of heteromeric $\alpha 3^*$ nAChRs may exist. The predominant subtype contains $\alpha 3$ and $\beta 4$ subunits, which may assemble together with $\alpha 5$ and/or $\beta 2$.^{35,36} This brief overview does not preclude the possibility that nAChRs comprised of different or more complex subunit combinations may also exist in the vertebrate nervous system. $\alpha 9$ and $\alpha 10$ are both expressed in mechanosensory hair cells while neither has been detected in the adult brain.¹³

nAChR structure: sites for ligand interaction

Individual nAChR subunits consist of a number of distinct functional domains (Figure 1A).³⁷ The large extracellular N-terminal domain contains putative glycosylation sites, a disulphide-linked cysteine loop between residues homologous to 128 and 142 of the $\alpha 1$ subunit, and the interface for agonist binding (loops A, B and C in α subunits and loops D, E and F in all subunits, Figure 1B).³⁷ In addition, α subunits are defined by the presence of a cysteine pair at positions homologous to $\alpha 1$ 192-193 in loop C. The loops that comprise the agonist binding site contain conserved residues, many of which possess aromatic side chains (tryptophan and tyrosine) which are proposed to make cationic- π interactions with agonists.³⁸

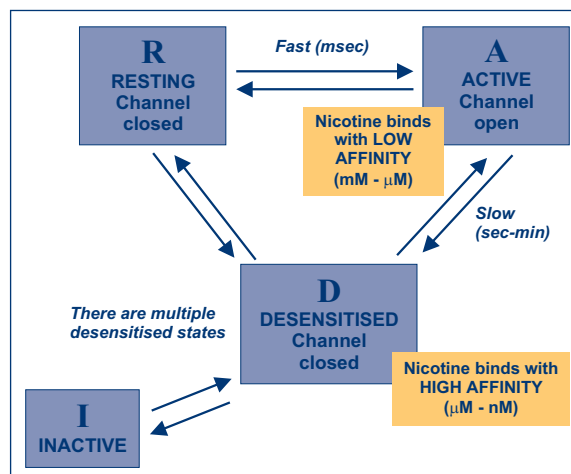
The polypeptide chain of nAChR subunits contains four hydrophobic, putative transmembrane segments

(M1-M4) that transverse the plasma membrane (Figure 1A); evidence indicates that M2 is α -helical and lines the cation pore.^{1,37} M3 and M4 are separated by a large, variable, intracellular loop which contains putative phosphorylation sites for Ser/Thr kinases.

Five subunits assemble to form a pentameric nAChR. Cryo-electron microscopy at 4.6Å resolution of *Torpedo* nAChRs in a resting, non-activated state has revealed a membrane spanning protein in which the bulk of the structure is extracellular.³⁹ It accommodates a cation conducting pore which narrows to a putative gate within the membrane bilayer. Two agonist binding sites are located at the interfaces of the *Torpedo* α/γ and α/δ subunits (analogous to α/β subunit interfaces in neuronal nAChRs, Figure 1C), approximately 20-40 Å from the ion channel.^{37,40} Most features deduced from this moderately resolved *Torpedo* nAChR structure have been confirmed with the determination of a high resolution (2.7Å) crystal structure of an ACh binding protein from the snail *Lymnaea stagnalis*.⁴¹ This protein is a structural and functional analogue of the extracellular domain of an nAChR pentamer, with closest similarity to the neuronal $\alpha 7$ nAChR.

Upon agonist binding, nAChRs undergo an allosteric transition from the closed, resting conformation to an open state which conducts the cations sodium, potassium and calcium. In the active (open) conformation, the nAChR binds agonists with low affinity (Figure 2). The continued presence of agonist

Figure 2. Relationship between major states that a nAChR may occupy



leads to ion channel closure and receptor desensitisation. In this condition, the nAChR is refractory to activation although it displays higher affinity for agonist binding. A multiplicity of desensitised states is proposed to exist.¹⁵ The rates of desensitisation and recovery differ between nAChR subtypes: for example, the $\alpha 7$ nAChR displays very rapid desensitisation.^{11,19} Prolonged agonist exposure may produce an inactivated state, from which recovery is very slow; the $\alpha 4\beta 2$ neuronal nAChR is prone to inactivation on chronic nicotine treatment.⁴² Transitions between resting, open and desensitised states are reversible and different ligands may stabilise different receptor states: agonists initially stabilise the activated (open) state whereas competitive antagonists preferentially stabilise the nAChR in a closed state, either the resting or desensitised configuration (Figure 2).

The nAChR presents a number of sites which may be targeted by ligands (Figure 1B). Agonists and competitive antagonists compete for the agonist binding site in the extracellular domain. As this site exists at the interface between adjoining subunits, it

offers the prospect of nAChR subtype-selective interactions. Non-competitive antagonists (NCAs) and allosteric modulators act at sites distinct from the agonist binding site.

Agonists (Table 1)

Nicotinic agonists interact with the nAChR agonist binding site to initiate an allosteric change within the pentameric complex which leads to opening of the cation conducting pore. The agonist interaction with the activated state occurs with low affinity. Hence the EC₅₀ values for agonist activation of nAChRs are distinct from the higher affinity interaction with the desensitised state which is measured in binding assays, as a consequence of the prolonged incubation conditions necessary to reach equilibrium (Figure 2, Table 1). nAChRs are the molecular targets for the offensive or defensive mechanisms of a wide variety of plants and animals, hence Nature has provided an array of potent ligands. These include the agonists (-)-nicotine, (-)-cytisine, (+)-anatoxin A, (+)-epibatidine, anabasine and anabasine. Synthetic agonists range from the classical “ganglionic agonist” dimethylphenylpiperazine (DMPP) developed in the 1960’s, to novel agonists created more recently in order to provide greater subtype selectivity and therapeutic efficacy. Typically, agonists bind with highest affinity at the $\alpha 4\beta 2$ nAChR and with 2-3 orders of magnitude lower affinity at $\alpha 7$ nAChRs (Table 1). They bind to $\alpha 3^*$ nAChRs with intermediate affinity. In terms of functional potency, a similar relationship is observed, except that EC₅₀ values at $\alpha 4\beta 2$ and $\alpha 3^*$ nAChRs are more similar. Binding affinities (K_i values) are typically 2-3 orders of magnitude higher (i.e. lower concentration) than EC₅₀ values for nAChR activation (Table 1).⁴³ Few agonists have sufficient nAChR subtype selectivity for the exclusive activation of a particular subtype.

The neurotransmitter ACh is the endogenous agonist for nAChRs. ACh binds with a high affinity at $\alpha 4\beta 2$ nAChRs ($K_i \sim 10$ nM) but its utility as a nicotinic ligand is limited by its lack of selectivity for nAChRs versus muscarinic AChRs (mAChRs) and its susceptibility to hydrolysis. Therefore a mAChR antagonist (typically atropine) and an acetylcholinesterase (AChE) inhibitor must be added with ACh in biological preparations; some of these agents may also interact with nAChRs (see below).

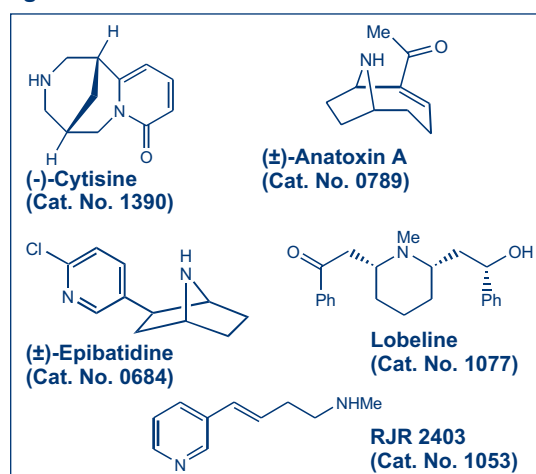
Modification of ACh by conversion of its ester group to a carbamate yields the hydrolysis-resistant analogue carbamylcholine (carbachol). This has reduced affinity at $\alpha 4\beta 2$ and $\alpha 7$ nAChRs and is more potent as a muscarinic agonist.^{44,45} The *N*-methyl derivative of carbachol, *N*-methylcarbamylcholine (MCC) recovers a high affinity for binding to $\alpha 4\beta 2$ nAChRs ($K_i \sim 2$ nM), comparable to ACh.^{46,47} *N*-methylation also confers substantial selectivity for nAChRs over mAChRs, in binding and functional assays.^{46,48} MCC has a permanently charged quaternary nitrogen atom which renders the molecule membrane impermeant and hence useful for the study of cell surface nAChRs.⁴⁷

The prototypic agonist for nAChRs is the tobacco alkaloid, (-)-nicotine. Nicotine binds to the $\alpha 4\beta 2$ nAChR with high affinity ($K_i = 1-11$ nM) whereas the $\alpha 7$ nAChR is 1000-fold less sensitive (although $\alpha 8^*$ nAChRs are more sensitive than $\alpha 7$ nAChRs to nicotine and other agonists).⁴⁹ Nicotine activates $\alpha 4\beta 2$ nAChRs with an EC₅₀ of 1 μ M, compared with 500 μ M for $\alpha 7$ nAChRs and 200 μ M for $\alpha 7/\alpha 8$ nAChRs.²⁷ $\alpha 9$ and $\alpha 9/\alpha 10$ nAChRs are unique in that nicotine is an antagonist at these subtypes (IC₅₀ = 31 μ M and 4 μ M respectively).^{13,26} Nicotine is widely used in behavioural studies: it crosses the blood brain barrier readily and its pharmacokinetics and

metabolism are well documented.⁵⁰ The principal metabolite of nicotine, cotinine, is devoid of significant nicotinic binding activity.⁴⁶ A minor tobacco alkaloid is anabasine, which is structurally related to nicotine: the pyrimidine ring of nicotine is replaced by a larger, unmethylated piperidine ring. Anabasine binds with mid-nanomolar affinities to $\alpha 4\beta 2$ and $\alpha 7$ nAChRs.⁵²

Trans-meta nicotine (RJR2403) is generated by opening the pyrrolidine ring of nicotine, and occurs naturally as a minor tobacco alkaloid. It is claimed to show functional selectivity for $\alpha 4\beta 2$ nAChRs compared with $\alpha 3^*$ nAChRs, which is an important consideration in developing drugs targeted at CNS dysfunctions.⁵¹ Thus it was found to be as potent as nicotine in activating $\alpha 4\beta 2$ nAChRs in rat thalamic synaptosomes but did not significantly activate $\alpha 3^*$ and $\alpha 7$ nAChRs expressed in the PC12 cell line. It was ten-fold less potent and considerably less efficacious than nicotine in inducing guinea pig ileum contractions, an effect probably mediated by $\alpha 3^*$ nAChRs.

Figure 3. Chemical structures of some nAChR agonists



(Bold text denotes compounds available from Tocris)

(-)-Cytisine occurs in a number of plants of the *leguminosae* family including laburnum. Its rigid structure has provided a template for modelling nicotinic ligands.⁵³ It is comparable to nicotine with respect to its high affinity binding at $\alpha 4\beta 2$ nAChRs ($K_i \sim 1$ nM) but has been used to distinguish sites with low affinity for cytisine amongst the population labelled by [³H]/[¹²⁵I]-epibatidine.^{47,54,55} However, in contrast to many nAChR agonists, including nicotine, the functional efficacy of cytisine is dependent on the identity of the β subunit present in the nAChR. (-)-Cytisine has full efficacy at nAChRs containing the $\beta 4$ subunit expressed in *Xenopus* oocytes, while at $\beta 2$ -containing nAChRs greatly reduced efficacy is observed.⁵⁶ Thus (-)-cytisine is a partial agonist at $\alpha 4\beta 2$ and $\alpha 3\beta 2$ nAChRs: this emphasises the importance of the β subunit in determining agonist interactions with neuronal nAChRs.

(+)-Anatoxin A is an azabicyclonone alkaloid originally isolated from freshwater blue green algae, *Anabaena flos aqua*.⁵⁷ Anatoxin A is a potent, semi-rigid, stereoselective agonist:⁵⁸ activity resides in the (+)-enantiomer which activates $\alpha 4\beta 2$ nAChRs at sub-micromolar concentrations (EC₅₀ = 48-93 nM).^{43,59} Anatoxin A binds to this nAChR subtype with higher affinity than nicotine. It has been exploited to characterise nicotinic currents in brain neurones and nAChR-mediated dopamine release from striatal preparations.^{60,61}

Epibatidine, originally obtained from skin extracts of the Amazonian frog, *Epibates Tricolor* is the

Table 1. Binding affinities (K_i)^a and functional potencies^b for nicotinic agonists at native or recombinant $\alpha 4\beta 2$ and $\alpha 7$ nAChRs and recombinant $\alpha 3\beta 4$ nAChRs. References are quoted in brackets, beneath the figures.

| nAChR subtype/ compound | Binding affinity K_i (nM) | | | Functional potency EC_{50} (μ M) | | |
|----------------------------|---|---|----------------------|---|--|---|
| | $\alpha 4\beta 2^*$ | $\alpha 7$ | $\alpha 3\beta 4$ | $\alpha 4\beta 2^*$ | $\alpha 7$ | $\alpha 3\beta 4$ |
| Acetylcholine | 6.8-57 (47, 78, 170, 171) | 4000-10830 (45, 78, 172, 197) | 560-881 (65, 171) | 0.48-3 (20, 43, 59, 64, 90, 173, 174, 175) | 79-316 (59, 64, 78, 89, 90, 175, 176, 177) | 53-210 (64, 85, 90, 93, 178, 179) |
| Carbachol | 207-582 (44, 170, 180, 181) | 18000-580000 (45, 172, 197) | 3839 (65) | 2.5-29 (43, 44) | 296 (173) | n.d. |
| MCC | 3.8 (47) | 10580 (94) | n.d. | 3.2 (43) | n.d. | n.d. |
| (-)-Nicotine | 1-11 (44, 45, 47, 51, 52, 78, 170, 171, 180, 181, 182) | 400-8900 (45, 51, 52, 78, 94, 166, 172, 176, 182, 197) | 300-475 (65, 171) | 0.3-15 (43, 44, 52, 59, 64, 67, 68, 90, 173, 174, 175, 180, 183) | 18-91 (52, 59, 64, 89, 90, 175, 176, 177, 183, 184) | 5-410 (64, 85, 90) |
| Anabasine | 76-260 (52, 171) | 58 (52) | 2100 (171) | n.d. | 16.8 (52) | n.d. |
| (-)-Cytisine | 0.14-2.7 (47, 78, 170, 171, 173, 180, 181) | 1400-3883 (45, 78, 172, 197) | 56-195 (65, 171) | 0.019-71.4 (43, 44, 90, 174, 176, 180) | n.d. | 72-134 (85, 90, 178) |
| (+)-Anatoxin A | 3.5 (185) | 63.3-380 (185, 197) | 52.9 (65) | 0.048-0.093 (43, 59) | 0.58 (59) | n.d. |
| (-)-Anatoxin A | 390 (186) | 21000 (186) | n.d. | n.d. | n.d. | n.d. |
| (±)-Anatoxin A | 1.3-4.2 (47, 67) | 707-1840 (67, 94) | n.d. | n.d. | n.d. | n.d. |
| (+)-Epibatidine | 0.019-0.041 (63, 64) | 9.8-590 (64) | n.d. | 0.005-0.01 (43, 64) | 1.2-2 (64) | 0.02-0.07 (64) |
| (-)-Epibatidine | 0.01-0.06 (63, 64) | 3.1-350 (64) | n.d. | 0.004-0.02 (43, 64) | 1.1-2.2 (64) | 0.009-0.02 (64) |
| (±)-Epibatidine | 0.01-0.05 (47, 67, 170, 182) | 20.6-233 (67, 94, 182, 197) | 0.38 (65) | n.d. | 1.3-3.5 (177, 184) | n.d. |
| (±)-UB-165 | 0.27-0.44 (67) | 2790 (67) | n.d. | partial agonist (67) | 6.9 (67) | 0.27 (67) |
| RJR 2429 | 1 (68) | n.d. | n.d. | >100 (68) | n.d. | n.d. |
| ABT-418 | 3-44 (47, 69, 121) | >100000 | n.d. | 0.5-14 (69, 174) | 264 (184) | n.d. |
| RJR 2403 | 26 (51) | 36000 (51) | n.d. | 0.73-16 (51, 187) | 240 (187) | n.d. |
| SIB-1508Y | 4.6 (72) | n.d. | n.d. | 2.6 (72) | n.d. | 41 (72) |
| ABT-594 | 0.04 (75) | 1560-13800 (75) | n.d. | n.d. | n.d. | n.d. |
| A-85380 | 0.05 (188) | 148 (188) | 73.6 (65) | 0.7 (188) | 8.9 (188) | n.d. |
| GTS-21 (DMXB) | 85 (78) | 212-652 (78, 197) | n.d. | No activation (174) | 26-109 (78, 171, 184) | n.d. |
| Anabaseine | 32-75 (52, 78) | 58-759 (52, 78, 197) | n.d. | 4.2 (52) | 6.7 (52) | n.d. |
| (±)-AR-R17779 | 16000 (79) | 190 (79) | n.d. | n.d. | n.d. | n.d. |
| (-)-AR-R17779 | 16000 (79) | 92 (79) | n.d. | n.d. | 21 (79) | n.d. |
| Choline | 112000 (81) | 2380000 (94) | n.d. | n.d. | 1600 (82) | n.d. |
| DMPP | 9.4-400 (44, 170, 171, 186) | 160-2300 (172, 186) | 1300 (171) | 0.07-18 (43, 44, 90) | 19-75 (89, 90, 176, 177) | 10-92 (85, 90, 178) |
| Lobeline | 4-50 (171, 180) | 11000-13100 (153, 197) | 480 (171) | n.d. | No activation (89) | n.d. |

(Bold text denotes compounds available from Tocris)

^aBinding affinities reported for competition binding assays of [³H]-agonist binding (except [³H]-epibatidine) to brain membranes ($\alpha 4\beta 2^*$ nAChRs) and [³H]-agonist binding to heterologously expressed $\alpha 4\beta 2$ nAChRs, [¹²⁵I]-Bgt or [³H]-MLA binding to brain membranes or heterologously expressed $\alpha 7$ nAChR, [³H]-epibatidine binding to heterologously expressed $\alpha 3\beta 4$ nAChRs.

^bFunctional potencies reported at brain thalamic synaptosomes ($\alpha 4\beta 2^*$ nAChRs) or heterologously expressed nAChRs.

Values are the range from the references cited below each entry.

most potent natural agonist at nAChRs yet known.⁶² Its potent non-opioid analgesic activity awakened interest in neuronal nAChRs as targets for analgesic drugs.⁶³ Like anatoxin A, epibatidine also has a bicyclic moiety that confers some rigidity to its structure but, in this case, it is a smaller azabicycloheptane ring, coupled to a chloropyridyl moiety. Epibatidine has exceptionally high binding affinity at $\alpha 4\beta 2$ nAChRs ($K_i = 19$ pM) and about 10-fold lower affinity at $\alpha 3\beta 2$ nAChRs ($K_i = 230$ pM) and $\alpha 3\beta 4$ nAChRs ($K_i = 380$ pM).^{64,65} Binding affinity at $\alpha 7$ nAChRs is about 10,000-fold lower than at $\alpha 4\beta 2$ nAChRs ($K_i \sim 200$ nM). In contrast to anatoxin A, epibatidine is not particularly stereoselective and its enantiomers show equivalent biological activities.^{63,64} The functional potency of epibatidine is also exceptionally high, with sub-micromolar EC_{50} values for $\alpha 4\beta 2$, $\alpha 3\beta 2$, $\alpha 3\beta 4$ and $\alpha 8$ neuronal nAChR subtypes.⁶⁴ Only $\alpha 7$ and muscle nAChRs exhibited EC_{50} values in the low micromolar range.

UB 165 is a novel hybrid molecule, comprising the azabicyclononene bicycle of anatoxin A and the chloropyridyl moiety of epibatidine.⁶⁶ UB 165 exhibits stereoselectivity comparable to anatoxin A. It binds with high affinity to $\alpha 4\beta 2$ nAChRs but is a partial agonist at this nAChR type, in contrast to its full efficacy in activating other neuronal nAChR subtypes expressed in *Xenopus* oocytes.⁶⁷ This property was exploited to infer the participation of presynaptic $\alpha 4\beta 2^*$ nAChRs in the modulation of dopamine release in rat striatal preparations.

RJR 2429 resembles UB 165 both structurally and functionally. It is a quinuclidine derivative of nicotine, and displays high binding affinity at $\alpha 4\beta 2$ nAChRs but is unable to activate this nAChR subtype in an assay of rubidium efflux from rat thalamic synaptosomes.⁶⁸ However, in contrast to UB 165, RJR 2429 does not show selectivity for neuronal nAChRs over muscle nAChRs: it is a potent activator of human muscle nAChRs ($EC_{50} = 59$ nM) but is markedly less potent in activating $\alpha 3^*$ nAChRs in the rat PC12 cell line ($EC_{50} = 1.1$ μ M).⁶⁸

In addition to UB 165 and RJR 2429, several other synthetic nicotinic agonists have been described that show improved nAChR subtype selectivity, compared with nicotine. These include ABT-418 and SIB-1508Y. ABT-418, an isoxazole isostere of nicotine, has high affinity for $\alpha 4\beta 2$ nAChRs while displaying reduced functional potencies at other nAChR subtypes which could mediate peripheral side effects.⁶⁹ This molecule has cognition enhancing and anxiolytic properties, with reduced side effects compared to nicotine.^{70,71} SIB-1508Y, a 5-substituted ethynyl analogue of nicotine, retains potency at $\alpha 4\beta 2$ nAChRs (binding with low nanomolar affinity, comparable to nicotine) but has slightly reduced functional potency at $\alpha 3\beta 4$ and $\alpha 4\beta 4$ nAChRs stably expressed in HEK-293 cell lines.⁷² It exhibits similar potency but increased efficacy, compared with nicotine, in stimulating dopamine release from rat striatal slices, which led to its development as a potential therapy for Parkinson's disease.⁷³

The 3-pyridyl ethers comprise a distinct group of molecules that are structurally more distant from nicotine, incorporating the ether linkage found in ACh. These molecules exhibit very high affinity for $\alpha 4\beta 2^*$ nAChRs (comparable to epibatidine) and also display high selectivity, in terms of binding affinity, for $\alpha 4\beta 2$ nAChRs over $\alpha 3^*$ and $\alpha 7$ nAChRs. Two recently described examples of this class of compound are A-85380 and ABT-594, the latter being a chloropyridyl derivative of A-85380.^{74,75} The 5-iodo derivative of A-85380 is proposed to be an $\alpha 4\beta 2$ selective ligand versus $\alpha 7$ and $\alpha 3^*$ nAChRs.⁷⁶ ABT-594 has also

shown potent analgesic properties that, together with its weaker interaction with peripheral nAChRs, make it a promising therapeutic lead for the treatment of pain.⁷⁷

A further, structurally disparate group of compounds exhibit some degree of preference for the $\alpha 7$ nAChR. GTS-21 (also known as DMXB) is a synthetic, functionally selective agonist for $\alpha 7$ nAChRs ($K_i = 212$ nM; $EC_{50} = 26.21$ -109 μ M). Despite binding to $\alpha 4\beta 2^*$ nAChRs with a reasonable affinity ($K_i = 85$ nM), GTS-21 displays negligible agonist activity at this subtype.⁷⁸ GTS-21 is a 3-substituted derivative of anabaseine, an alkaloid that occurs naturally in nemertines (a marine worm). Anabaseine differs from the tobacco alkaloid anabasine only in bond order, but this difference seems to confer a functional selectivity for $\alpha 7$ nAChRs that is also preserved in GTS-21. Anabaseine has a moderate binding affinity at $\alpha 4\beta 2^*$ nAChRs ($K_i = 32$ nM) and a relatively high affinity at $\alpha 7$ nAChRs ($K_i = 58$ nM).⁵²

AR-R17779, a novel, conformationally restricted analogue of ACh, is also reported to be a selective ligand for $\alpha 7$ over $\alpha 4\beta 2$ nAChRs, with respective K_i values of 190 nM and 16000 nM for the racemate.⁷⁹ The (-) enantiomer was shown to be a full and potent agonist at rat $\alpha 7$ homomers expressed in *Xenopus* oocytes.⁷⁹ It has anxiolytic properties and improves learning and memory performance in rats.⁸⁰

Choline, the product of ACh hydrolysis, is a selective agonist of $\alpha 7$ nAChRs ($EC_{50} = 1.6$ mM).^{81,82} Other nAChR subtypes are not activated by choline, and it has been employed to selectively stimulate $\alpha 7$ nAChRs.⁸² Whereas concentrations above 200 μ M activate $\alpha 7$ nAChRs, low concentrations (35 μ M) desensitise the $\alpha 7$ nAChR: as the local concentrations of choline in the brain are not precisely known, the physiological implications of this potential endogenous ligand are a matter of debate.⁸²

The synthetic compounds dimethylphenylpiperazine (DMPP) and methylphenylpiperazine (MPP) are both agonists at nAChRs. DMPP has a long history as a ganglionic agonist but shows little selectivity between neuronal nAChR subtypes, with respect to binding affinity and potency of activation. However, DMPP is a partial agonist at chicken $\alpha 7$ nAChRs expressed in *Xenopus* oocytes but is fully efficacious in eliciting Type IA currents (attributed to $\alpha 7$ nAChRs) in rat hippocampal neurones.^{60,83,84} DMPP was also shown to be a partial agonist at rat $\alpha 3\beta 4$ nAChRs stably expressed in HEK-293 cells.⁸⁵ It is membrane impermeant, due to the quaternary nitrogen atom, and has been used to selectively block cell surface nAChRs.⁸⁶

Lobeline, an alkaloid produced by the Indian tobacco *Lobelia inflata* deserves a mention because of its anomalous behaviour. Lobeline binds with high affinity to $\alpha 4\beta 2^*$ nAChRs. It was originally considered to be a nicotinic agonist, but it is now proposed to be an antagonist.⁸⁷ Although it was found to stimulate dopamine release from rat striatal slices at concentrations above 1 μ M, this effect is not nAChR-mediated as it cannot be blocked by the nAChR antagonist, mecamylamine. Indeed lobeline, at concentrations below 1 μ M, inhibits nicotine-stimulated dopamine release.⁸⁸ In addition, it has also been shown to inhibit nicotine-stimulated rubidium efflux from rat thalamic synaptosomes, an effect ascribed to $\alpha 4\beta 2^*$ nAChRs, and fails to activate human $\alpha 7$ nAChRs expressed in *Xenopus* oocytes.^{88,89}

Competitive antagonists (Table 2)

Competitive antagonists interact reversibly with the nAChR at, or close to, the agonist binding site, stabilising the receptor in a conformation with the channel closed and preventing access for agonists. Inhibition by competitive antagonists is surmountable with increasing agonist concentration. Like agonists, most competitive antagonists originate from a wide variety of natural sources. Unfortunately, there is a very limited range of subtype-selective agents and not all are commercially available. Binding affinities for competitive antagonists are given in Table 2 and these approximate more closely to functional potency, unlike agonists. IC₅₀ concentrations for functional blockade are not listed as these values will depend on the agonist concentration used. Typical concentrations for nAChR blockade are indicated in the text.

A classic antagonist is d-tubocurarine (d-TC), which is produced by the South American shrub *Chondodendron tomentosum*. It is well known as the arrow tip poison used by South American Indians and has had a long history as a nicotinic antagonist.² d-TC does not discriminate appreciably between nAChR subtypes and it is able to fully antagonise functional responses mediated by a wide variety of neuronal nAChRs at a concentration of approximately 10 μM.⁹⁰ It should be noted that the mechanism of inhibition by d-TC can be complex, also involving non-competitive interactions.^{83,90} In addition, d-TC is also a 5-HT₃ receptor antagonist: it inhibits 5-HT₃ receptor-mediated currents in hippocampal interneurons (full blockade at 1 μM) and in HEK-293 cells stably transfected with the 5-HT₃ receptor (IC₅₀ = 2.8 μM).^{91,92}

A purely competitive antagonist for neuronal nAChRs is dihydro-beta-erythroidine (DHβE), an alkaloid originating from *Erythrina* seeds. Sub-micromolar concentrations of DHβE block human and rat α4β2

and α3β2 nAChRs but it is 10-50-fold less potent at α3β4 and α7 nAChRs expressed in *Xenopus* oocytes.^{90,93} In hippocampal neurones, 100 nM DHβE blocked Type II currents, attributed to α4β2* nAChRs, whereas Type I currents mediated by α7 nAChRs were insensitive to DHβE concentrations below 10 μM.⁶⁰

The most well-established subtype-selective nicotinic antagonist is α-Bgt, an 8kDa peptide isolated from the venom of the Taiwanese banded krait, *Bungarus Multicinctus*. It was instrumental in the isolation and purification of muscle and *Torpedo* nAChRs but is also a highly potent and selective antagonist at α7 nAChRs, to which it binds with an affinity (K_i) of approximately 1 nM.⁹⁴ However, its association binding kinetics are very slow and typically a pre-incubation of one hour with the toxin would be necessary to achieve a complete blockade, using a low nanomolar concentration of α-Bgt (10 nM). This is commonly circumvented by increasing the concentration and decreasing the preincubation time. This strategy is possible because, even at micromolar concentrations, α-Bgt does not appear to interact with α/β heteromeric nAChRs. α-Bgt also exhibits very slow dissociation kinetics, such that functional blockade is not reversed by washout within the timescale of a typical experiment (e.g. 1 hour). The avian α8* nAChRs bind, and are blocked by, α-Bgt (K_i ~ 2 nM), and α9* nAChRs of cochlear hair cells are reversibly inhibited by nanomolar concentrations of α-Bgt.^{13,27}

The venom of *Bungarus Multicinctus* also contains a number of minor components with pharmacological activity. One such fraction exhibits specificity for inhibiting at least some α3* subtypes of neuronal nAChRs, in addition to α7 nAChRs.⁹⁵ This toxin has been given a variety of names but is now referred to as neuronal bungarotoxin (n-Bgt). α3β2 nAChRs are

Table 2. Binding affinities (K_i)^a for nicotinic antagonists in competition binding assays at native or recombinant α4β2* and α7 nAChRs and recombinant α3β4 nAChRs.

| nAChR subtype/ compound | K _i (nM) | | |
|---------------------------------|---|---|-------------------|
| | α4β2* | α7 | α3β4 |
| DHβE | 13.9-1900 (46, 47, 156, 170, 186, 195) | 25000-57900 (186, 197) | 218622 (65) |
| d-TC | 1000-25000 (44, 47, 170, 181) | 3400-7700 (45, 172, 181) | 22929 (65) |
| Methyllycaconitine (MLA) | 3700-6100 (47, 186) | 0.69-10.3 (94, 166, 172, 186, 197) | 3700 ^b |
| α-Bgt | > 1000 (186) | 0.35-3.5 (45, 94, 166, 172, 176, 186, 197) | n.d. |
| α-Conotoxin Iml | n.d. | 2400 (194) | n.d. |
| Hexamethonium | 28000-> 1000000 (47, 181, 182, 186) | 800000-880000 (45, 186) | > 1000000 (65) |
| Decamethonium | 460-120000 (47, 182, 186) | 124000-200000 (45, 186) | n.d. |
| Mecamylamine | 822000-> 1000000 (47, 181, 182, 186) | > 1000000 (186) | > 1000000 (65) |
| Chlorisondamine | 686000 (182) | n.d. | n.d. |

(Bold text denotes compounds available from Tocris)

^aBinding affinities reported for competition binding assays of [³H]-agonist binding (except [³H]-epibatidine) to brain membranes (α4β2* nAChRs) and [³H]-agonist binding to heterologously expressed α4β2 nAChRs, [¹²⁵I]-αBgt or [³H]-MLA binding to brain membranes or heterologously expressed α7 nAChRs, [³H]-epibatidine binding to heterologously expressed α3β4 nAChRs.

^bSharples, unpublished observation.

Values are the range from the references cited below each entry.

subject to pseudo-irreversible blockade by 100 nM n-Bgt whereas $\alpha 3\beta 4$ nAChRs are insensitive to this concentration: analysis of chimeric subunits has shown that the kinetics and affinity of n-Bgt blockade resides in the N-terminal domain of the β subunit.^{96,97} This toxin is not generally available, and has been superseded to some extent by the more stringent specificities of the *Conus* toxins.

The venoms of *Conus* snails are a valuable source of subtype-selective antagonists for a variety of pharmacological targets. α -Conotoxins are small peptide toxins (14-17 amino acids) with a highly conserved disulphide bonding pattern, with specificity for nAChR subtypes.⁹⁸ It is not yet established if they all act in a truly competitive manner. Three well characterised α -conotoxins specific for neuronal nAChRs are α -conotoxins MII, Iml and AulB. α -Conotoxin MII was originally shown to be a potent and selective antagonist of $\alpha 3\beta 2$ nAChRs expressed in *Xenopus* oocytes; $\alpha 3\beta 2$ nAChRs are fully blocked by 100 nM toxin.^{99,100,101} At other heterologously expressed nAChRs, the potency of α -conotoxin MII is 2-4 orders of magnitude lower. The partial inhibition of nicotine- or anatoxin A-evoked dopamine release from rat striatal synaptosomes by α -conotoxin MII led to the proposition that a nAChR containing at least one $\alpha 3\beta 2$ interface contributed to this response.^{101,102} This interpretation may require modification in light of the high sequence similarity between the $\alpha 3$ and $\alpha 6$ subunits, and the observation that $\alpha 6\beta 2$ nAChRs expressed in *Xenopus* oocytes are susceptible to inhibition by α -conotoxin MII.¹⁰³ The absence of [¹²⁵I]- α -conotoxin MII binding (see below) in transgenic mice lacking the $\beta 3$ nAChR subunit suggests that this rare subunit (which shares a similar anatomical distribution with $\alpha 6$) may also participate in α -conotoxin MII binding sites.¹⁰⁴

α -Conotoxin AulB partially inhibits nicotine-evoked [³H]-noradrenaline and [³H]-ACh release from rat brain preparations and is a selective antagonist of $\alpha 3\beta 4$ nAChRs.^{105,106} It is less potent than α -conotoxin MII and 10 μ M α -conotoxin AulB is required to fully inhibit $\alpha 3\beta 4$ nAChRs. Another well characterised α -conotoxin is α -conotoxin Iml, which is a selective antagonist of $\alpha 7$ nAChRs, fully blocking responses at 1 μ M.^{107,108} α -Conotoxin Iml also blocks $\alpha 9$ nAChRs with an affinity 8-fold less than at $\alpha 7$ nAChRs.¹⁰⁷ One caveat with regard to α -conotoxins is that their exquisite specificity may limit extrapolation between species: it is reported that although α -conotoxin Iml inhibits rat $\alpha 7$ nAChRs, it blocks a non- $\alpha 7$ nAChR in bovine chromaffin cells.¹⁰⁹

Methyllycaconitine (MLA) is a norditerpenoid alkaloid produced by *Delphinium* sp. It is a competitive antagonist, selective for $\alpha 7$ nAChRs and, unlike α -Bgt, discriminates between $\alpha 7$ and muscle nAChRs.^{53,110} MLA binds to $\alpha 7$ nAChRs with a K_i of approximately 1 nM, and picomolar concentrations are reported to block $\alpha 7$ nAChR-mediated currents recorded from hippocampal neurones or *Xenopus* oocytes.^{111,112} Inhibition of $\alpha 7$ nAChRs by MLA is rapid and reversible, making it a useful complement to α -Bgt in the $\alpha 7$ pharmacological armoury. While muscle and $\alpha 4\beta 2$ nAChRs require micromolar concentrations for inhibition, $\alpha 3\beta 2^*$ nAChRs are about 10-fold more sensitive to MLA.¹¹³ Recent evidence suggests that MLA at concentrations of 1-50 nM may block a minor, non- $\alpha 7$ nAChR in rat striatum.^{32,114} In addition, non- $\alpha 7$ interactions of MLA have been indicated in avian preparations, where functional responses sensitive to MLA, but not α -Bgt, have been observed.^{34,115} MLA is also able to cross the blood-brain barrier and has been given intracerebrally in

behavioural studies.^{116,117} However, as the local concentration of MLA achieved is not known in these studies, results must be interpreted with caution as this antagonist is selective, rather than specific, for $\alpha 7$ nAChRs over other nAChR subtypes.

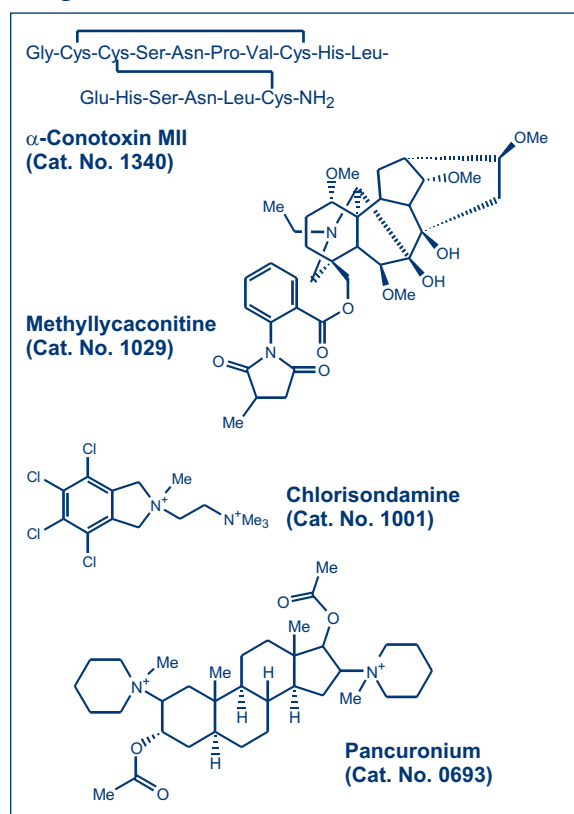
Strychnine, generally regarded as a competitive antagonist of glycine-gated LGIC, has been shown to competitively inhibit currents mediated by $\alpha 7$ nAChRs on rat hippocampal neurones ($IC_{50} = 1.2 \mu$ M),¹¹⁸ $\alpha 9$ and $\alpha 9/\alpha 10$ nAChRs are exquisitely sensitive to strychnine ($IC_{50} = 20$ nM).¹³ In contrast, strychnine was concluded to block currents mediated by $\alpha 4\beta 2^*$ nAChRs in a voltage-dependent, non-competitive manner.¹¹⁸

Non-competitive antagonists

Non-competitive antagonists (NCA) exert their effects by interacting with sites distinct from the agonist binding site, and therefore do not compete with agonists for binding. Commonly a NCA inhibits nAChR activity by blocking the intrinsic channel pore, by binding to sites within or near the entrance to the channel. As the action of a NCA is not surmountable by agonist, it is easier to predict effective concentrations required to achieve full blockade under conditions of varying agonist concentrations. However, if the NCA is a channel blocker, the nAChR must first be activated to allow the drug access to the channel. Such NCAs typically show voltage-dependent blockade of nAChRs.

The synthetic compound mecamylamine is the archetypal NCA for neuronal nAChRs. Mecamylamine blocks most neuronal nAChRs with IC_{50} values in the low micromolar range. $\alpha 7$ nAChRs are somewhat less sensitive than α/β heteromers, requiring 10 μ M for full blockade.⁹⁰ In addition, *N*-methyl-D-aspartate (NMDA) receptors are also transiently inhibited by mecamylamine at high micromolar concentrations.¹¹⁹ Mecamylamine crosses the blood brain barrier freely and is typically administered at a concentration of around 1 mg/kg in rats to block CNS nAChRs in behavioural studies.

Figure 4. Chemical structures of some nAChR antagonists



(Bold text denotes compounds available from Tocris)

Like mecamylamine, hexamethonium was also first recognised as a ganglionic nAChR blocking agent but the hydrophilic nature of this polymethylene bistrimethylammonium compound prevents it from crossing the blood-brain barrier.⁵ Comparative studies of the effects of mecamylamine and hexamethonium *in vivo* have been undertaken to establish if a particular behaviour is centrally or peripherally mediated, as peripherally administered hexamethonium is unable to block CNS nAChRs *in vivo*.

Chlorisondamine, a bisquaternary nicotinic antagonist, was also originally used as a ganglionic blocker. It is an irreversible open channel blocker for neuronal nAChRs and is unique in exerting a persistent blockade over a period of weeks when given *in vivo*. For example, it effectively blocked dopamine release from rat striatal synaptosomes *in vitro*, even when administered *in vivo* (10 mg/kg/day) several weeks before the sacrifice of the animal.¹²⁰ It is hypothesised that this long lasting inhibition may arise from an intracellular accumulation of the drug.¹²¹

Ibogaine, an alkaloid isolated from the African shrub *Tabernaemontana iboga*, is reported to have anti-addictive properties. It attenuates self-administration of cocaine and morphine in rats and is an NCA for neuronal nAChRs.¹²² It is able to block functional responses mediated by ganglionic $\alpha 3^*$ nAChRs in the PC12 and human neuroblastoma SH-SY5Y cell lines.^{123,124}

Many NCAs for neuronal nAChRs have alternative primary targets and cannot be considered to be specific nicotinic drugs. In compiling the list below, compounds are included that would not be deliberately chosen to antagonise nicotinic responses but awareness of such an interaction may be important when interpreting the effects of the drug. This list is not comprehensive; any positively charged compound small enough to approach the nAChR channel may indeed block the lumen and inhibit ion conductance: this also applies to nicotinic agonists, that become channel blockers in the high micromolar concentration range.^{125,126}

MK 801 (dizocilpine) is an anticonvulsant agent developed as a channel blocker of the NMDA receptor. It is able to perform the same function on neuronal nAChRs, where it is reported to be an open channel blocker at the $\alpha 4\beta 2$ ($IC_{50} = 15 \mu M$) and $\alpha 7$ nAChRs ($IC_{50} = 15 \mu M$).^{127,128} MK 801 (10-25 μM) has also been shown to partially inhibit nicotine-evoked [³H]-GABA release from rat hippocampal synaptosomes.¹²⁹ Studies on *Torpedo* nAChRs indicate that MK 801 may also interact with a non-luminal site, in addition to the channel.¹³⁰

Bupropion (ZybanTM) was originally developed as an antidepressant but is now marketed as an aid to smoking cessation. At low micromolar concentrations it non-competitively inhibits rat $\alpha 3\beta 2$, $\alpha 4\beta 2$ and $\alpha 7$ nAChRs expressed in *Xenopus* oocytes and nAChR-mediated rubidium efflux in the human SH-SY5Y cell line.^{122,131} In the former study, inhibition by bupropion was voltage-independent suggesting that it may not act within the lumen of the channel.

Other known NCAs for neuronal nAChRs include the neuroleptic, chlorpromazine and a number of psychoactive drugs.¹³² The dissociative anaesthetics phencyclidine (PCP) and ketamine are in this class.¹³³ In addition there are several classical NCAs which have been used to study muscle nAChRs but whose activity at neuronal nAChRs has not been well characterised. This includes pancuronium, histrionicotoxin and quinacrine.

A range of steroids, including corticosterone, progesterone, oestradiol, hydrocortisone and aldosterone, have been shown to inhibit neuronal

nAChRs expressed in the SH-SY5Y cell line, with IC_{50} values in the high nanomolar to micromolar range.¹³⁴ In addition, a similar range of steroids inhibited currents mediated by rat $\alpha 4\beta 2$ nAChRs stably expressed in the HEK-293 cell line, with IC_{50} values in the low micromolar range, and progesterone inhibited $\alpha 4\beta 2$ nAChRs expressed in *Xenopus* oocytes with an IC_{50} of 9 μM .^{135,136}

Numerous diverse blockers of voltage-operated calcium channels have been reported to inhibit rat $\alpha 3\beta 4$ nAChRs expressed in *Xenopus* oocytes, whereas $\alpha 7$ nAChRs were virtually unaffected.¹³⁷ These drugs include the N/P/Q-type calcium channel blocker, ω -conotoxin MVIIC, the N-type blocker, ω -conotoxin GVIA and the L-type blockers flunarizine, verapamil and diltiazem. All inhibited nicotinic currents with IC_{50} values in the low micromolar range. The ω -conotoxins were found to block the $\alpha 3\beta 4$ nAChR reversibly, whereas they exert a longer lasting inhibition of calcium channels. This difference can be used to discriminate between these two targets.¹³⁸ Dihydropyridine calcium channel blockers (nimodipine, nifedipine, nitrendipine and flunarizine) have also been observed to abolish nAChR-mediated ⁴⁵Ca²⁺ uptake in bovine chromaffin cells, an activity largely attributable to $\alpha 3\beta 4^*$ nAChRs.¹³⁹

The muscarinic antagonist atropine, the GABA_A receptor antagonist bicuculline, and the 5HT₃ receptor antagonist ICS-205,930 inhibit $\alpha 9^*$ nAChRs with IC_{50} values of 1 μM or less.¹³ Other nAChR subtypes are much less sensitive to these agents: atropine blocks $\alpha 7$ nAChRs with an IC_{50} of 120 μM .⁴⁵

Particularly intriguing are recent reports from several groups that β -amyloid peptide inhibits $\alpha 7$ nAChRs. It binds competitively with picomolar affinity to $\alpha 7$ nAChRs.¹⁴⁰ Recently β -amyloid₁₋₄₂ was shown to reversibly block $\alpha 7$ nAChRs on rat hippocampal neurones ($IC_{50} = 7.5$ nM) in a non-competitive manner, by an interaction with the N-terminal domain of the receptor.¹⁴¹ Moreover, β -amyloid₁₋₄₂ inhibited whole cell and single channel nicotinic currents in rat hippocampal interneurons at a concentration of 100 nM.¹⁴² β -Amyloid₁₋₄₂ has also been shown to stimulate the mitogen-activated protein kinase (MAPK) cascade in hippocampal slices via $\alpha 7$ nAChRs.¹⁴³ This encourages speculation about the role of nAChRs in the pathogenesis of Alzheimer's Disease.

Positive allosteric modulators

Positive allosteric modulators potentiate responses to nicotinic agonists by acting at a site other than the agonist binding site or channel area. Such effects are well established for other receptor classes (e.g. benzodiazepines and GABA_A receptors; glycine and NMDA receptors), but have only recently been recognised for neuronal nAChRs.

Acetylcholinesterase inhibitors, including galanthamine and physostigmine, have been shown to potentiate the functional effects of sub-maximal concentrations of nicotinic agonists on neuronal nAChRs, without themselves causing significant nAChR activation when applied alone.¹⁴⁵ This dual action (acetylcholinesterase inhibition and nAChR potentiation) has led to the development of these agents for the treatment of Alzheimer's Disease. Galanthamine (ReminylTM) (1-10 μM) potentiated ACh-evoked currents in HEK-293 cells stably expressing the human $\alpha 4\beta 2$ nAChR.¹⁴⁴ At higher concentrations (> 10 μM) galanthamine inhibited responses, possibly by acting as a channel blocker; physostigmine behaves in a similar manner. The site of action for their positive allosteric modulation is thought to be at, or close to, the binding site for the

monoclonal antibody FK-1, near lys 125 in the extracellular N-terminal domain of the neuronal nAChR α subunit.¹⁴⁵ Other allosteric potentiators include the opiate codeine, the anthelmintic drug, ivermectin and the neurotransmitter 5-HT. Codeine acts at the FK-1 antibody binding site to potentiate currents activated by ACh in PC12 cells, while ivermectin was shown to enhance currents through chick or human $\alpha 7$ nAChRs expressed in *Xenopus* oocytes.^{146,147} Sub-micromolar concentrations of 5-HT could mimic the potentiating effects of galanthamine in PC12 cells.¹⁴⁵ Recently, the steroid, β -estradiol, was reported to potentiate human, but not rat, $\alpha 4\beta 2$ nAChRs expressed in *Xenopus* oocytes distinct from the inhibitory effects of steroids.^{134,135,136,148} This is an example of positive allosteric modulation being species specific.

Radioligands (Table 3)

Nicotinic radioligands have provided invaluable tools for the identification, pharmacological characterisation and localisation of neuronal nAChRs. Table 3 summarises the binding affinities at relevant neuronal nAChR subtypes of the major nicotinic radioligands published.

[³H]-Nicotine binding to brain tissue was first described in detail by Romano & Goldstein.⁷ [³H]-Nicotine identifies a single population of sites in the CNS, in most reports, with a K_d of 1-10 nM and is considered to label primarily $\alpha 4\beta 2^*$ nAChRs, as [³H]-nicotine binding is almost totally absent from the brains of transgenic mice lacking the $\alpha 4$ or $\beta 2$ subunits.¹⁴⁹ [³H]-Nicotine binds to glass fibre filters, and this is remedied by pre-soaking the filters in poly-L-lysine or polyethyleneimine; non-specific binding to tissue preparations can be diminished by including a high concentration of Tris in the buffer.^{7,150,151} [³H]-Nicotine does not store well and addition of mercaptoacetic acid is advised.⁷

Specific nicotinic labelling by [³H]-ACh (in the presence of atropine and an acetylcholinesterase inhibitor) is comparable to that of [³H]-nicotine in anatomical distribution and pharmacological characteristics.^{9,152,153} [³H]-Cytisine also labels specific sites in the brain with an affinity ($K_d = 0.96$ nM) and distribution comparable to [³H]-nicotine;¹⁵⁴ immunoprecipitation analyses suggest that it also labels $\alpha 4\beta 2^*$ nAChRs.²⁸ In addition, one or more other, minor nAChR subtypes may be labelled by [³H]-cytisine.¹⁵⁵ [³H]-ABT-418 was also shown to bind to rat brain with a low nanomolar affinity ($K_d = 2.85$ nM) and the distribution of [³H]-ABT-418 binding sites in the brain, determined by autoradiography, is comparable to that of [³H]-cytisine.¹⁵⁶ Similarly, [³H]-MCC has been used to label sites in brain with high affinity ($K_d = 1$ nM) and a pharmacological profile resembling that of [³H]-nicotine and [³H]-cytisine binding sites.^{46,157}

Although each of these agonists can interact with a full range of neuronal nAChR subtypes (Table 1), as radioligands they predominantly label only $\alpha 4\beta 2^*$ nAChRs. This is because, in a binding assay, the prolonged incubation with agonist converts the nAChR to a high affinity desensitised state (Figure 2). Of the various nAChR subtypes, the desensitised $\alpha 4\beta 2$ nAChR has the highest affinity (~ 1 nM) for these particular agonists; lower affinity binding (> 10 nM) would not result in specific labelling under the conditions used.

In 1995, the binding of [³H]-epibatidine was characterised, and it was found to label 2 sites in the rat brain with sub-nanomolar affinities and very low non-specific binding.¹⁵⁸ The highest affinity binding site ($K_d \sim 10$ pM) accords with $\alpha 4\beta 2$ nAChRs.⁴⁷ [³H]-Epibatidine has proved to be a very useful radioligand for monitoring a variety of defined nAChR subtypes in heterologous expression systems. [³H]-Epibatidine labelled $\alpha 3\beta 4$ nAChRs expressed in HEK-293 cells and in *Xenopus* oocytes with K_d values of 0.3 nM and

Table 3. Binding affinities (K_d)^a of nicotinic radioligands.

| nAChR subtype/ radioligand | K_d (nM) | | | | | |
|--|---|--|-------------------------|------------------------------|---------------------------|---------------------------|
| | $\alpha 4\beta 2^*$ | $\alpha 7$ | $\alpha 3\beta 4$ | $\alpha 3\beta 2^*$ | $\alpha 3\alpha 5\beta 4$ | $\alpha 3\alpha 5\beta 2$ |
| [³ H]-Nicotine | 0.89-9 (44, 46, 181, 186, 189, 190) | – | – | – | – | – |
| [³ H]-Acetylcholine | 10 (191) | – | – | – | – | – |
| [³ H]-Cytisine | 0.15-0.96 (46, 154) | – | – | – | – | – |
| [³ H]-ABT-418 | 2.85 (156) | – | – | – | – | – |
| [³ H]-MCC | 1.07 (46) | – | – | – | – | – |
| [³ H]-Epibatidine | 0.011-0.086 (47, 170, 171, 192) | – | 0.306-4.9 (159, 171) | 0.013-0.14 (64, 159, 171) | 2.8 (159) | 0.25 (159) |
| 5-[¹²⁵ I]-A-85380 | 0.010 (76) | – | – | – | – | – |
| [¹²⁵ I]- α -Conotoxin MII | – | – | – | 0.93-1.9 (161, 196) | – | – |
| [¹²⁵ I]- α -Bgt | – | 0.4-1.66 (45, 166, 172, 176, 186, 193) | – | – | – | – |
| [³ H]-MLA | – | 1.86-2.2 (94, 163) | – | – | – | – |
| [¹²⁵ I]-MLA | – | 1.8 (167) | – | – | – | – |

(Bold text denotes compounds available from Tocris)

^aBinding affinities derived from saturation binding experiments carried out on brain membranes/primary cultured neurones (for $\alpha 4\beta 2^*$ or $\alpha 7$ nAChRs) or heterologously expressed nAChRs. Binding affinities of [¹²⁵I]- α -Conotoxin MII for $\alpha 3\beta 2^*$ were derived from quantitative autoradiography on brain slices.

Values are the range from the references cited below each entry.

4.9 nM respectively, while affinity for $\alpha 3\beta 2$ nAChRs expressed in *Xenopus* oocytes was 0.12 nM.^{65,159} $\alpha 4\beta 2$ nAChRs in transfected cell lines are labelled with higher affinity than $\alpha 3^*$ nAChRs ($K_d = 12$ pM).⁴⁷ In a comparative analysis of nicotinic radioligand autoradiography in brains of transgenic mice lacking the $\beta 2$ subunit, Zoli and colleagues identified one high affinity [³H]-epibatidine binding site that persisted in these animals, and tentatively assigned the subunit composition $\alpha 3\beta 4^*$.¹⁵⁵ One caution in using such a high affinity radioligand is the possibility of ligand depletion during an assay, such that the free radioligand concentration is diminished and quantitative analysis becomes unreliable; this can be avoided by decreasing tissue concentration and/or increasing assay volumes.^{47,158}

[¹²⁵I]-Epibatidine (in which the chlorine atom of epibatidine is replaced by [¹²⁵I] with little change in pharmacological properties) has recently become available and provides a higher specific activity ligand for labelling minor populations of binding sites.^{55,160} These sites were differentiated by their sensitivities to unlabelled cytosine and A-85380.⁵⁵ Indeed 5-[¹²⁵I]-A-85380, labels brain $\alpha 4\beta 2$ nAChRs with a low picomolar affinity ($K_d = 10$ pM);⁷⁶ it labels a single population of sites and thus offers improved binding selectivity for $\alpha 4\beta 2$ over $\alpha 3^*$ nAChRs compared to radiolabelled epibatidine (unlabelled 5-iodo-A-85380 displaced [³H]-epibatidine binding to $\alpha 3^*$ nAChRs in rat adrenal gland membranes with an affinity 1000-fold less than epibatidine).⁷⁶

A minor population of nAChR binding sites comprising $\alpha 3/\alpha 6\beta 2^*$ subunits has been identified directly, in rodent and monkey brain, using an iodinated version of α -conotoxin MII.^{161,196} Conventional "grind and bind" assays with this conotoxin are confounded by high non-specific binding. However, the labelled peptide works effectively in quantitative autoradiography: this technique was used to demonstrate monophasic [¹²⁵I]- α -conotoxin MII labelling with an affinity (K_d) of 0.9 nM and 1.9 nM for monkey and rodent brain respectively.^{161,196}

[¹²⁵I]- α -Bgt has been widely used as a definitive ligand for a distinct nAChR, now equated with the $\alpha 7$ subtype, in both ligand binding and autoradiography

studies.^{9,94,153,162,163} Longer incubation times are necessary to achieve equilibrium, but the very slow dissociation kinetics of this ligand minimise loss of binding during washing steps. In rodent brain it labels a single population of sites with $K_d \sim 1$ nM.¹⁵³ Both high (2000 Ci/mmol) and low (150 Ci/mmol) specific activity versions of [¹²⁵I]- α -Bgt are available commercially, depending on the sensitivity required, or it can be readily iodinated with intermediate specific activity (~ 700 Ci/mmol) by the chloramine T method.^{164,165,166} [³H]- α -Bgt is also available but has generally not been used to characterise neuronal nAChRs.

[³H]-MLA has been developed as an alternative to [¹²⁵I]- α -Bgt for labelling $\alpha 7$ nAChRs.^{94,163} This radioligand labels a single population of sites in rodent brain with low nanomolar affinity ($K_d \sim 1-2$ nM) and the distribution of binding sites closely parallels that of [¹²⁵I]- α -Bgt. An α -Bgt-insensitive fraction of [³H]-MLA binding was observed but may be explained by steric factors preventing access of the larger α -Bgt (but not MLA) to five binding sites on a homomeric $\alpha 7$ nAChR.⁹⁴ An iodinated version of MLA, generated using the chloramine T method, has also been described.¹⁶⁷

There is also interest in developing isotopically labelled nicotinic ligands for non-invasive *in vivo* imaging of brain nAChRs.^{168,169}

Future directions with nicotinic ligands

Despite the growing nicotinic pharmacopoeia, there is still a lack of subtype-selective agonists and antagonists, especially directed towards the less abundant nAChR subtypes. Presently only the α -conotoxins provide this degree of specificity. The design of more subtype-selective ligands will facilitate elucidation of the physiological roles and therapeutic potential of individual neuronal nAChR subtypes. This family of LGICs is increasingly perceived to have an important influence on central, as well as peripheral, nervous system activities: the pharmacological dissection of these roles and the development of nAChR subtype-selective pharmacophores are goals that will occupy researchers in this field for a good while longer yet!

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Nicotinic Receptor Ligands available from Tocris

Agonists

| | | |
|------|-----------------------------------|--|
| 0352 | 4-Acetyl-1,1-dimethylpiperazinium | Nicotinic agonist |
| 0351 | 1-Acetyl-4-methylpiperazine | Nicotinic agonist |
| 0789 | (±)-Anatoxin A | Nicotinic agonist |
| 1390 | (-)-Cytisine | Potent, selective neuronal nicotinic agonist |
| 0684 | (±)-Epibatidine | Very potent nicotinic agonist |
| 1077 | Lobeline | Nicotinic agonist |
| 1053 | RJR 2403 | CNS selective nicotinic agonist |

Antagonists

| | | |
|-------|--------------------------------------|--|
| 0424 | Benzoquinonium | Nicotinic antagonist |
| 1001 | Chlorisondamine | Nicotinic antagonist; slow offset |
| 1340* | α-Conotoxin MII | Potent, α3β2 and β3 subunit selective antagonist |
| 1029 | Methyllycaconitine | Neuronal nicotinic receptor antagonist |
| R1029 | [³ H]-Methyllycaconitine | Radiolabelled form of (1029) |
| 0693 | Pancuronium | Nicotinic (neuromuscular) antagonist |

Modulators of Acetylcholine Receptor Function

| | | |
|------|--------------------------------|--|
| 0388 | Ambenonium | Cholinesterase inhibitor |
| 0422 | Bromodechloroambenonium | Cholinesterase inhibitor |
| 0686 | Galanthamine | Cholinesterase inhibitor |
| 1260 | Ivermectin | Allosteric modulator of α7 nicotinic receptor |
| 0537 | MR 16728 | Stimulates ACh release |
| 0657 | α-NETA | Potent, selective, fluorescent choline acetyltransferase inhibitor |
| 0592 | β-NETA | Selective, fluorescent choline acetyltransferase inhibitor |
| 0750 | PG-9 maleate | Presynaptic cholinergic modulator |
| 0622 | Physostigmine | Cholinesterase inhibitor |
| 0751 | SM-21 | Presynaptic cholinergic modulator |
| 0965 | Tacrine | Cholinesterase inhibitor |
| 0739 | Tropanyl 2-phenylthiobutanoate | Presynaptic cholinergic modulator |
| 0653 | (±)-Vesamicol | Inhibits ACh storage |

*Local regulations may restrict the sales of this product in certain territories. Please consult your local Tocris Cookson office or distributor for further details.

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