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Multiple pharmacophores for the selective activation of nicotinic $\alpha 7$ -type acetylcholine receptors

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Abbreviations: nAChR, nicotinic acetylcholine receptors; GTS-21, 3-2,4,dimethoxy-benzylidene anabaseine; TMA, tetramethyl-ammonium; ETMA, ethyltetramethyl-ammonium; dEdMA, diethyldimethylammonium; QN, quinuclidine, MQN, 1-methyl-1-azoniabicyclo[2.2.2]octane iodide; QN-O, quinuclidinone; QN-OH quinuclidinol; EQN, 1-ethyl-1-azoniabicyclo[2.2.2]octane iodide; BQNE, E-3-benzylidene-1-azoniabicyclo[2.2.2]octane chloride; BQNZ, Z-3-benzylidene-1-azoniabicyclo[2.2.2]octane chloride; MBQNE, E-3-(4-methoxybenzylidene)-1-azoniabicyclo[2.2.2]octane chloride; MBQNZ, Z-3-(4-methoxybenzylidene)-1-azoniabicyclo[2.2.2]octane chloride; DME, 1,2-dimethoxy ethane; ABT-418, ((S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole; ACME, (cis-1-methyl-2,3,3a,4,5,9b,-hexahydro-1H-pyrrolo[2,3-f]quinoline)

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Abstract

The activation of heteromeric and homomeric nicotinic acetylcholine receptors (nAChR) was studied in *Xenopus* oocytes in order to identify key structures of putative agonist molecules associated with the selective activation of homomeric $\alpha 7$ receptors. We observed that selectivity between $\alpha 7$ and $\alpha 4\beta 2$ was more readily obtained than selectivity between $\alpha 7$ and $\alpha 3\beta 4$. Based on structural comparisons of previously characterized selective and nonselective agonists, we hypothesize that there existed at least three chemical motifs which, when present in molecules containing an appropriate cationic center, could be associated with the selective activation of $\alpha 7$ receptors. We identify the three distinct structural motifs, based on prototypical drugs as: the choline motif, the tropane motif, and the benzylidene motif. The choline motif involves the location of an oxygen-containing polar group such as a hydroxyl or carbonyl separated by two carbons from the charged nitrogen. The tropane motif provides $\alpha 7$ -selectivity based on the addition of multiple small hydrophobic groups positioned away from the cationic center in specific orientations. We show that this motif can convert the nonselective agonists quinuclidine and ethyltrimethyl-ammonium to the $\alpha 7$ -selective analogs methyl-quinuclidine and diethyldimethyl-ammonium, respectively. We have previously shown that the benzylidene group of GTS-21 converts anabaseine into an $\alpha 7$ -selective agonist. The benzylidene motif was also applied to quinuclidine to generate another distinct family of $\alpha 7$ -selective agonists. Our results provide insight for the further development of nicotinic therapeutics and will be useful to direct future experiments with protein structure-based modeling and site-directed mutagenesis.

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Introduction

The nicotinic acetylcholine receptors of the brain can be broadly divided into two classes, heteromeric beta-subunit containing receptors and homomeric $\alpha 7$ -type receptors. Homomeric $\alpha 7$ -type receptors have emerged as an exciting potential therapeutic target for several indications, and this has encouraged the development of $\alpha 7$ -selective agonists. This path of drug development relies on consideration of both features which distinguish the heteromeric receptors from the homomeric receptors and features which distinguish selective agonists from nonselective agonists.

Both heteromeric, beta subunit-containing, receptors and homomeric $\alpha 7$ -type receptors are pentameric. The heteromeric neuronal nAChR contain at least one or more alpha subunits ($\alpha 2$ – $\alpha 6$) and additional beta subunits ($\beta 2$ – $\beta 4$), with two agonist binding sites, located at the interface between alpha and beta subunits (Dani, 2001). Neuronal nicotinic receptor alpha subunits are classified as such based on sequence homology to the alpha subunits of muscle-type receptors (Heinemann et al., 1990), and essential conserved aspects of muscle and neuronal alpha subunits provide specialized subdomains that contribute to the primary face of an asymmetrical binding site for acetylcholine and other agonists. In contrast, there is structural homology between muscle-type gamma, delta, and epsilon subunits and neuronal $\beta 2$ and $\beta 4$ subunits that provide in these subunits the specialized subdomains for the complimentary face of the agonist binding site (Le Novere et al., 2002b). While the majority of heteromeric receptors in the mammalian brain are believed to contain just $\alpha 4$ and $\beta 2$ subunits (Flores et al., 1992), minor populations may contain additional subunits in various configurations (Turner and Kellar, 2005). The specializations for forming an agonist binding site appear to be lacking in the muscle $\beta 1$, as

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well as the neuronal $\alpha 5$ and $\beta 3$ subunits, so these have been identified as "structural subunits" (Gotti et al., 2006).

At least two emergent properties are likely to have come from the specialization of the non-alpha subunits in the agonist binding sites. These two properties are 1) the failure of heteromeric receptors to be activated efficiently by the ACh precursor choline (Papke et al., 1996), and 2) the conversion of the receptors to desensitized states with high affinity for agonist, in parallel to, or following, activation (Buisson and Bertrand, 2001; Higgins and Berg, 1988). These features are common to all heteromeric nAChR, including muscle-type receptors. In contrast to the heteromeric receptors, for the homomeric $\alpha 7$ receptors of the brain, choline is a fully efficacious agonist, and $\alpha 7$ receptors do not convert to high affinity desensitized states.

The homomer-forming subunit, $\alpha 7$ has been identified as phylogenetically ancestral to the more specialized subunits of the heteromeric receptors, and as such it contains subdomains able to contribute to either the primary or complimentary faces of up to five agonist binding sites per receptor (Le Novere et al., 2002a). While some unique biophysical properties may emerge from the presence of so many potential agonist binding sites (Papke et al., 2000), it is presumably the lack of certain specializations in the binding site that has made it relatively easy to identify agonists which will activate $\alpha 7$ receptors but not heteromeric receptors like those containing $\alpha 4$ and $\beta 2$ or $\alpha 3$ and $\beta 4$.

Our conceptual approach has been to classify "core agonist" structures as they represent different elaborations of the simplest cationic center of tetramethyl-ammonium (TMA). We have arranged into nine structurally-related families of agonists, compounds which have been functionally characterized in the published literature by ourselves or others, or in unpublished studies conducted in our laboratory (Figure 1). Note that agonists which selectively activate $\alpha 7$

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nAChR have been identified in most of the structural classes. Table 1 provides summaries of the function studies that have been conducted on these various compounds.

In addition to choline, a relatively simple structure associated with selective activation of $\alpha 7$ receptors is that of tropane (Papke et al., 2005a). We have recently published that unsubstituted quinuclidine will activate $\alpha 7$ receptors (Papke, 2006), and, as shown in Figure 1, a quinuclidine provides the core agonist cationic center for many structurally complex $\alpha 7$ -selective agonists. In the present study we evaluate in greater detail the activity of choline and the simple amines TMA and ethyltetramethyl-ammonium (ETMA) and extend a preliminary report (Leonik et al., 2007) on quinuclidine and related compounds. While quinuclidine itself is not $\alpha 7$ -selective, we report that hydrophilic side groups, as well as both minimal and large hydrophobic substitutions affect the activity profile of quinuclidines and can promote selectivity for the activation of $\alpha 7$.

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Methods and materials

Synthetic chemistry

Solvents and reagents were purchased from Aldrich and Acros. All solvents were dried overnight over CaH_2 and freshly distilled before use. ^1H and ^{13}C NMR spectra were obtained using VXR 300, Gemini 300 and 500, or Mercury 300 (300 MHz) spectrometers in CDCl_3 , CD_3OD , or D_2O solvents. EI Mass spectra were obtained on a Finnigan MAT 95Q spectrometer, or ESI spectra were obtained on a Bruker APEX II FTICR mass spectrometer. NMR spectral data for the new compounds synthesized are included in the supplemental material.

1-methyl-1-azoniabicyclo[2.2.2]octane iodide (MQN)(Chen and Benoiton, 1976; Kaminski et al., 1978)

A mixture of methyl iodide (0.54 ml), KHCO_3 (0.34g) and quinuclidine hydrochloride (50 mg) was stirred in methanol (6.8 ml) at room temperature for 12 h. The solvent was evaporated and CHCl_3 was added to the residue, and the mixture was stirred overnight. The mixture was filtered and the solvent evaporated under vacuum to afford methyl quinuclidine iodide as a white solid (85 mg) in 99% yield. MP 230 °C (dec). ^1H NMR (D_2O) δ (ppm) 2.01 (m, 6H), 2.20 (m, 1H), 2.93 (s, 3H), 3.43 (t, 6H), ^{13}C NMR (D_2O) δ (ppm) 57.4, 52.3, 23.9, 19.1. EI Calcd for $\text{C}_8\text{H}_{16}\text{N}$ (M^+): 126.1277, found: 126.1286.

1-ethyl-1-azoniabicyclo[2.2.2]octane iodide (EQN)

This compound was synthesized by the same procedure described above using 0.55 ml of ethyl iodide. The reaction afforded N-ethyl quinuclidine iodide as a white solid (81 mg) in 90% yield. MP 210 °C (dec). ^1H NMR (D_2O) δ (ppm) 1.31 (t, 3H), 2.01 (m, 6H), 2.22 (m, 1H), 3.22 (q, 2H), 3.40 (t,

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6H), ^{13}C NMR (D_2O) δ (ppm) 60.0, 54.5, 23.8, 19.5, 7.6. ESI-FT-ICR Calcd for $\text{C}_9\text{H}_{18}\text{IN}$ ($2\text{M}+\text{I}^+$): 407.1918, found: 407.1921.

E- and Z- 3-benzylidene-1-azoniabicyclo[2.2.2]octane chloride (BQNE, BQNZ)

The free base quinuclidinone was obtained by treating quinuclidinone hydrochloride with a 2M aqueous solution of $2\text{M K}_2\text{CO}_3$ (20 ml). Then, this solution was extracted three times with ether (30 ml) and the solvent was evaporated under reduced pressure. To a suspension of NaH (60% in mineral oil, 0.23 g, 5.84 mmol) in 1,2-dimethoxy ethane (DME) (6.4 ml) was added dropwise a solution of diethyl benzyl phosphonate (1.33 g, 5.84 mmol) in DME (2 ml) at room temperature, under argon. After this addition, a solution of quinuclidinone (0.4 g, 2.54 mmol) in DME (1.78 ml) was added dropwise. The reaction mixture was refluxed for 1.5 h. Then, the mixture was quenched carefully with water (30 ml) and the bulk of the DME was evaporated under reduced pressure. The aqueous phase was extracted with CH_2Cl_2 (3 x 30 ml) and the organic layer dried over MgSO_4 and evaporated under vacuum. The crude oil was purified by flash chromatography on silica gel using CH_2Cl_2 : MeOH: Et_3N 20:1:0.1 as eluent to give the Z isomer (153 mg) in a 30% yield and E isomer in a (82 mg) 16% yield. The hydrochloride salts of these isomers were obtained by adding ether-HCl to a solution of the free bases in a mixture of CH_2Cl_2 /ether. For the Z isomer: MP 200 °C (dec). ^1H NMR (CDCl_3) δ (ppm) 2.07 (td, 4H), 2.78 (m, 1H), 3.26 (m, 4H), 4.15 (s, 2H), 6.45 (t, 1H), 7.15-7.39 (m, 5H), ^{13}C NMR (CDCl_3) δ (ppm) 144.5, 137.7, 128.9, 128.7, 126.7, 122.0, 56.0, 48.0, 34.1, 28.1. ESI-FT-ICR Calcd for $\text{C}_{14}\text{H}_{18}\text{N}$ (M^+): 200.1439, found: 200.1423. For the E isomer: MP 200 °C (dec). ^1H NMR (CDCl_3) δ (ppm) 2.01 (t, 4H), 3.4 (m, 5H), 4.06 (s, 2H), 6.49 (s, 1H), 7.17-7.39 (m, 5H), ^{13}C NMR (CDCl_3) δ (ppm) 135.1, 132.2, 128.6, 128.3, 127.5, 125.7, 54.2, 46.7, 24.4, 23.7. ESI-FT-ICR Calcd for $\text{C}_{14}\text{H}_{18}\text{N}$ (M^+): 200.1439, found: 200.1423.

E- and Z- 3-(4-methoxybenzylidene)-1-azoniabicyclo[2.2.2]octane chloride (MBQNE/MBQNZ)

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These compounds were synthesized using the same procedure described above. A suspension of NaH (60 % in mineral oil, 0.868 g, 22.4 mmol) in DME (24 ml) was stirred at room temperature under argon. A solution of diethyl 4-methoxy benzylphosphonate (3.55 ml, 20.6 mmol) in DME (7 ml) was added dropwise to that solution. After this, a solution of quinuclidinone as a free base (1.12g, 8.96 mmol) in DME (6 ml) was added dropwise. The reaction mixture was refluxed for 1.5 h and quenched with water. The DME was evaporated under vacuum and the residue was dissolved in dichloromethane and washed with water. The organic layer was washed with brine and dried over MgSO₄. The mixture was purified by flash chromatography (silica gel, CH₂Cl₂:MeOH 35 :1). The two isomers, obtained as oils, were isolated as free bases giving the Z isomer (400 mg) in 20 % yield and the E isomer (59 mg) in 3% yield. For the Z isomer: ¹H NMR (CDCl₃) δ(ppm) 2.10 (td, 4H), 2.80 (t, 1H), 3.29 (dt, 2H), 3.39 (dt, 2H), 3.83 (s, 3H), 4.19 (s, 2H), 6.42 (t, 1H), 6.89 (d, 2H), 7.09 (d, 2H), ¹³C NMR (CDCl₃) δ (ppm) 159.2, 131.1, 130.0, 128.2, 125.2, 114.6, 55.7, 54.4, 47.1, 32.2, 25.3. EI Calcd for C₁₅H₁₉NO (M⁺): 229.1467, found: 229.1475. For the E isomer: ¹H NMR (CDCl₃) δ(ppm) 2.04 (m, 4H), 3.36 (m, 5H), 3.82 (s, 3H), 3.99 (s, 2H), 6.40 (s, 1H), 6.90 (d, 2H), 7.12 (d, 2H), ¹³C NMR (CDCl₃) δ (ppm) 159.3, 131.1, 129.9, 127.9, 125.5, 114.4, 55.7, 54.7, 47.0, 24.7, 24.2. EI Calcd for C₁₅H₁₉NO (M⁺): 229.1467, found: 229.1475.

Modeling

Molecular models of agonists were constructed with the Chem3D Ultra program (Cambridge Scientific). The structures obtained were optimized with the molecular mechanics force field within Chem3D; in some cases semi-empirical calculations with AM1 parameters were used. Conformational searching was employed to ensure that high energy local minima were not used for the overlay calculations. Estimates of common molecular features between

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groups of agonists were made by using the overlay routine resident within the program suite. The positively charged nitrogen atom found in all agonists was always used as one of the pairs of points used to generate the overlaid structures.

Clustal analysis

Clustal is an on-line utility to generate DNA or protein sequence alignments and comparisons. Binding loop protein sequences of $\alpha 4\beta 2$, $\alpha 3\beta 4$, and $\alpha 7$ were submitted to ClustalW2 online at The European Bioinformatics Institute (EMBL-EBI) website (<http://www.ebi.ac.uk/Tools/clustalw2>). Specifically, sequence of loops A, B, and C of the alpha was attached to the sequence of loops D, E, and F of the beta or $\alpha 7$. Sequences selected for $\alpha 4\beta 2$ and $\alpha 3\beta 4$, were homologous to the loop sequence indicated for $\alpha 7$ (Stokes et al., 2004) and the AChBP (Brejc et al., 2001).

ACh receptor clones

The human nAChR receptor clones were obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia PA).

Preparation of RNA

Subsequent to linearization and purification of cloned cDNAs, RNA transcripts were prepared in vitro using the appropriate mMessage mMachine kit from Ambion Inc. (Austin TX).

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Expression in Xenopus laevis oocytes

Mature (>9 cm) female *Xenopus laevis* African frogs (Nasco, Ft. Atkinson WI) were used as a source of oocytes. Before surgery the frogs were anesthetized by placing them in a 1.5 g/l solution of MS222 for 30 min. Oocytes were removed from an incision made in the abdomen.

Harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemical Corporation, Freehold NJ) for two hours at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO₃, 0.82 mM MgSO₄, 15 mM HEPES (pH 7.6), 12 mg/l tetracycline) in order to remove the follicular layer. Stage-5 oocytes were isolated and injected with 50 nl (5-20 ng) of each subunit cRNA. Recordings were normally conducted 2-5 days post-injection.

Electrophysiology

Experiments were conducted using OpusXpress6000A (Axon Instruments, Union City, CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the voltage and current electrodes were filled with 3M KCl. The oocytes were clamped at a holding potential of -60 mV.

Data were collected at 50 Hz and filtered at 20 Hz. The oocytes were bath-perfused with Ringer's solution. Agonist solutions were delivered from a 96-well plate using disposable tips. Flow rates were set at 2 ml /min for $\alpha 7$ and 4 ml/min for $\alpha 4\beta 2$ and $\alpha 3\beta 4$. Drug applications alternated between ACh controls and test solutions of ACh or other experimental agonists at varying concentrations.

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Experimental protocols and data analysis

Responses of $\alpha 7$ receptors are calculated as net charge (Papke and Papke, 2002), and responses of wild-type beta subunit-containing receptors are reported as peak currents. Each oocyte received initial control applications of ACh, then experimental drug applications, and follow-up control applications of ACh. For $\alpha 7$ receptors the control ACh concentration was 300 μ M, a concentration which is sufficient to evoke a maximal net charge response (Papke and Papke, 2002). For $\alpha 4\beta 2$ and $\alpha 3\beta 4$ receptors the ACh control was 100 μ M. Responses to experimental drug applications were calculated relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. Mean values and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration. For concentration-response relations, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated from the Hill equation.

Equation 1:

$$\text{Response} = \frac{I_{\text{Max}}[\text{agonist}]^n}{[\text{agonist}]^n + (\text{EC}_{50})^n}$$

where I_{max} denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. I_{max} , n , and the EC_{50} were all unconstrained for the fitting procedures, except in the case of the ACh response curves. Since ACh is our reference full agonist, for the ACh concentration-response curves the data were normalized to the observed ACh maximum and the I_{max} of the curve fits were constrained to equal one.

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Results

Choline and quinuclidine activation of $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChR

The only known $\alpha 7$ -selective agonist naturally occurring in the vertebrate nervous system is choline, and, as previously published, choline is a full agonist with potency 10-fold lower than that of ACh. As shown in Figure 2A, although choline is highly selective for $\alpha 7$, it does produce small but detectable responses from both $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChR. When normalized to the maximal ACh-evoked responses in these cell types, the $\alpha 3\beta 4$ were significantly more sensitive to choline than were the $\alpha 4\beta 2$ receptors ($p < .01$), with responses to 3 mM choline equal to $4.5 \pm 0.6\%$ ($n = 3$) and $2.3 \pm 0.3\%$ ($n = 5$) of the ACh maximum for $\alpha 3\beta 4$ and $\alpha 4\beta 2$, respectively.

Quinuclidine (QN) is a fully efficacious agonist of $\alpha 7$ receptors (Papke, 2006) and is a core element of many large $\alpha 7$ -selective agonists (Figure 1). We tested the agonist activity of QN on $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChR, and, as shown in Figure 2B, 300 μ M QN was capable of producing maximal activation of $\alpha 7$. At this concentration QN evoked currents from $\alpha 4\beta 2$ receptors that were only $3.2 \pm 0.7\%$ of the ACh maximal responses ($n = 5$), while for $\alpha 3\beta 4$ receptors the QN-evoked responses were $46 \pm 2\%$ of the ACh maximum.

Our observations therefore suggest that in order to identify the key elements for an $\alpha 7$ -selective agonist, comparisons between $\alpha 7$ and $\alpha 3\beta 4$ will be of more utility than comparisons between $\alpha 7$ and $\alpha 4\beta 2$. A similar observation has been published previously (Efang et al., 2001) regarding piperidyl- and pyrrolidyl- chromans which activated $\alpha 7$ and $\alpha 3$ -containing nAChR much more efficaciously than $\alpha 4$ -containing receptors. A Clustal analysis (Higgins, 1994) of $\alpha 7$, $\alpha 3\beta 4$, and $\alpha 4\beta 2$ sequences in the putative ACh binding loops (comparing $\alpha 7$ to $\alpha 4$

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and $\alpha 3$ in loops A, B, and C and to $\beta 2$ and $\beta 4$ in loops D, E, and F (Brejc et al., 2001)) indicated slightly greater sequence similarity between $\alpha 7$ and $\alpha 3\beta 4$ than between $\alpha 7$ and $\alpha 4\beta 2$. For these reasons we focused our studies on $\alpha 7$ and $\alpha 3\beta 4$ receptors. A good selectivity profile between these two subtypes will additionally have the therapeutic value of providing drugs with few potential side effects caused by the activation of ganglionic nAChR.

Activity profiles for TMA, ETMA, choline and ACh on $\alpha 7$ and $\alpha 3\beta 4$

It has been previously reported that tetramethylammonium (TMA) and ethyltrimethylammonium (ETMA) activate multiple subtypes of neuronal nAChR (Papke et al., 1996) when applied at high (1 mM) concentrations. Figure 3 shows full concentration-response studies for these compounds as well as for ACh and choline on $\alpha 7$ and $\alpha 3\beta 4$ receptors (curve fit values provided in Table 2). All four of these quaternary amines were full agonists for $\alpha 7$, with only choline showing a significant decrease in potency compared to ACh. In contrast, both TMA and ETMA were only partial agonists of $\alpha 3\beta 4$ receptors and also showed reduced potency compared to ACh (Table 2), while the activity of choline for $\alpha 3\beta 4$ was too low to be characterized in regard to potency.

Activity of small quinuclidine-based molecules on $\alpha 7$ and $\alpha 3\beta 4$ nAChR

Quinuclidine (QN) is an effective activator of $\alpha 7$ (Figure 2B) and a partial agonist of $\alpha 3\beta 4$ (Figure 5). As shown in Figure 4a, QN is comparable in size to the $\alpha 7$ -selective agonists tropane and DMP (Papke et al., 2005a). However, while overlays of DMP and tropane show reasonably good alignment of the charged nitrogen and hydrophobic elements, the overlay of tropane and QN highlights the absence of a small hydrophobic element common to tropane and

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DMP, a single methyl group. We therefore sought to test the hypotheses that the addition of a methyl group to QN in a similar position (MQN, Figure 4B) would produce a loss of $\alpha 3\beta 4$ activity. Additional QN-related structures are also provided in Figure 4B.

As shown in Figure 5, QN was a potent and fully efficacious agonist for $\alpha 7$ and a partial agonist for $\alpha 3\beta 4$, with an efficacy of 64% that of ACh. MQN was also fully efficacious for activating $\alpha 7$, though less potent than QN. With the addition of a methyl group to the QN core, essentially all activity was lost for $\alpha 3\beta 4$ receptors, consistent with the pharmacophore hypothesis discussed above. Interestingly, the additional oxygen of quinuclidinone (QN-O) or the hydroxyl group of quinuclidinol (QN-OH) also caused a loss of $\alpha 3\beta 4$ activation, and the spacing of these polar groups from the amine center of QN corresponds to the distance of the hydroxyl from the charged nitrogen in choline. QN-O and QN-OH were also reduced in efficacy for $\alpha 7$ compared to QN, and both showed decreases in potency compared to QN that were equal to (in the case of QN-O) or greater than (in the case of QN-OH) the potency difference between ETMA and choline for $\alpha 7$.

Ethyl-quinuclidine (EQN, Figure 4) was also synthesized and tested (Figure 6) to determine if a somewhat larger hydrophobic extension in that domain would improve $\alpha 7$ agonist activity. EQN evoked receptor-specific current in $\alpha 7$ -injected cells. However, it also evoked receptor-independent currents which could be recorded in both injected and uninjected cells. The nAChR-mediated current could be identified because it was eliminated by the co-application of 100 μ M mecamylamine. In $\alpha 7$ -injected cells, the mecamylamine-sensitive current was $58 \pm 3\%$ of the total current evoked by the application of 1 mM EQN ($n = 4$), and the net charge of the mecamylamine-sensitive current was only $23 \pm 2\%$ that of the current evoked by the application of 300 μ M ACh to the same cells. EQN also evoked currents in cells injected with $\alpha 3\beta 4$, but

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these currents were comparable to the current evoked in uninjected oocytes and were not blocked by mecamlamine (data not shown). Therefore, although our data suggest that EQN is a weak partial agonist for $\alpha 7$ nAChR, the ability of EQN to activate receptor-independent currents limited our ability to study this agent in detail using the *Xenopus* oocyte expression system. While it is clearly outside the scope of these experiments to investigate the nAChR-independent currents evoked by EQN in detail, we did wish to determine whether mediators of the response might be muscarinic AChRs, sometimes endogenously expressed by oocytes. Therefore, we compared the responses of uninjected oocytes to 1 mM EQN in Ringer's with or without our normal 1 μ M atropine, and determined that the responses of uninjected oocytes to EQN were not significantly different in the presence or absence of the mAChR antagonist atropine (data not shown).

Selective activation of $\alpha 7$ nAChR by diethyl, dimethyl ammonium

Since the addition of a single methyl group was sufficient to convert QN into an $\alpha 7$ -selective agonist, we hypothesized that a similar modification to the structure of ETMA would produce decreased activity on $\alpha 3\beta 4$ receptors without a large decrease in $\alpha 7$ activation. As shown in Figure 7, 300 μ M diethyldimethylammonium (dEdMA) produced only about a 2% activation of $\alpha 3\beta 4$ receptors, and even at a concentration of 3 mM, $\alpha 3\beta 4$ responses were only about 6% the ACh maximum. In contrast to the greater than 10-fold difference in efficacy for $\alpha 3\beta 4$, for $\alpha 7$ dEdMA was 90% as efficacious as ACh and only 2.5-fold less potent compared to ETMA.

Activity of benzylidene quinuclidines on $\alpha 7$ and $\alpha 3\beta 4$ nAChR

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While the addition of only a methyl group was sufficient to increase the $\alpha 7$ -selectivity for a quinuclidine-based agonist, larger hydrophobic groups are also often tolerated in $\alpha 7$ -selective agonists, often improving potency (Papke et al., 2005a), or, depending on additional side groups, affecting both potency and efficacy (Papke et al., 2004a). The conjugation of benzylidene groups to the non-selective agonist anabaseine account for the $\alpha 7$ -selectivity of GTS-21 (also known as DMXBA) and related compounds (Papke et al., 2004a). Therefore we synthesized a series of benzylidene quinuclidines (Figure 4B) to determine how effectively benzylidene groups would modulate the activity of quinuclidine-based agonists. Note that the relative orientation of the phenyl and quinuclidine groups about the double bond can lead to alternative E and Z structures (Figure 4B). Both of these isomers were made with either an unsubstituted benzene group (BQNE and BQNZ) or with a methoxy group at the 4 position (MBQNE and MBQNZ). As shown in Figure 8, the benzylidene quinuclidines in the E configuration were relatively potent $\alpha 7$ partial agonists, though less efficacious than QN or MQN (Table 1). The efficacy for $\alpha 7$ was further reduced for the benzylidene quinuclidines in the Z configuration. While the activity of BQNE and MBQNE was $\alpha 7$ -selective, BQNZ also produced significant activation of $\alpha 3\beta 4$ receptors (Figure 8) that was comparable to the activation of $\alpha 7$ -expressing cells.

Activation and/or inhibition of 5HT₃ receptors and other nAChR

In order to more fully characterize the selectivity profile of the compounds used for this study, they were also tested at a concentration of 100 μ M for their ability to either activate (when applied alone) or inhibit (when co-applied with agonist) 5HT₃ receptors, as well as muscle type ($\alpha 1\beta 1\epsilon\delta$) and $\alpha 4\beta 2$ nAChR expressed in oocytes. While most serotonin receptors are G-protein coupled, 5HT₃ receptors are members of the cys-loop superfamily of ligand-gated ion channels,

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and, like $\alpha 7$ nAChR, 5HT₃ receptor subunits form functional homomeric assemblies. Some previously characterized $\alpha 7$ -selective agonists, such as tropisetron (Macor et al., 2001; Papke et al., 2005a), have been shown to be antagonists of 5HT₃ receptors (Table 1). Likewise, benzyldene anabaseines have been shown to interact at these receptors, either as antagonists or partial agonists (Machu et al., 2001).

None of the quinuclidines or linear amines used for the $\alpha 7$ experiments produced detectable activation of 5HT₃ receptors in oocytes. However, the benzyldene quinuclidines, like some benzyldene anabaseines, were effective antagonists, suggesting that the benzyldene group may allow these molecules to bind as competitive antagonists to the serotonin receptors.

The only compounds which produced detectable activation of muscle-type nAChR were the non- $\alpha 7$ -selective compounds, QN, TMA, and Z-forms of the benzyldene quinuclidines. These compounds, and to a lesser degree choline, also produced detectable responses in $\alpha 4\beta 2$ -expressing oocytes. These results support the classification of dEdMA, MQN, QN-O, QN-OH, and the E forms of the BQN compounds as $\alpha 7$ -selective agonists. Several of the compounds which did not activate the muscle-type or $\alpha 4\beta 2$ nAChR were inhibitory in co-application experiments, suggesting that they may bind to, but fail to activate these receptors, or alternatively may have channel blocking properties. Benzyldene anabaseines have been reported to both displace cytosine binding from $\alpha 4\beta 2$ -type receptors in brain membranes (Meyer et al., 1997) and produce use-dependent inhibition of $\alpha 4\beta 2$ receptors expressed in *Xenopus* oocytes (Meyer et al., 1998a), so inhibition of these nAChR subtypes by the BQN compounds might be produced by either or both of these mechanisms.

Modeling of multiple pharmacophores for selective activation of $\alpha 7$ nAChR

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Figure 9a highlights how the non-selective agonist QN can lead to three unique $\alpha 7$ -selective agonists. Figure 9b presents a model for three corresponding structural motifs to achieve selective activation of $\alpha 7$, and a typical (Beers and Reich, 1970) two-point pharmacophore model is represented to illustrate the geometric context of the selectivity model. This model was generated by overlaying a series of $\alpha 7$ agonists and identifying common disposition of like atom types. Specifically, based on prototypical drugs using these motifs we can identify them as: the choline motif, the tropane motif, and the benzyldiene motif.

The choline motif involves the location of an oxygen-containing polar group such as a hydroxyl or carbonyl separated by two carbons from the charged nitrogen. Note that the distance between the nitrogen and oxygen is ~ 3.4 angstroms, which, for example, in the case of 3-quinuclidinol, is likely too short to correspond to the hydrogen bond acceptor group described in existing nAChR pharmacophore models (Beers and Reich, 1970; Sheridan et al., 1986; Tonder et al., 2001). We therefore consider this selectivity motif to be distinct from the aforementioned pharmacophoric element.

The tropane motif relies on recognition of small hydrophobic groups in a geometric relationship quite distinct from the benzyldiene motif (discussed below). This motif is centered around the ammonium pharmacophore as illustrated in Figure 9B. The improvement in $\alpha 7$ -selectivity obtained with the addition of a methyl group to quinuclidine and ethyltrimethylammonium supports the hypothesis that tropane and other small selective agonists such as 1,1-dimethylpiperidine utilize a common (tropane) motif. This point is supported by the molecular overlay of QN and tropane shown in Figure 4.

Like the tropane motif, the benzyldiene motif is based on recognition of a hydrophobic group. However, it is spatially distinct from the tropane site. We consider that the $\alpha 7$

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recognition site for this motif is extended in length, on the order of 7-10 Å from the charged nitrogen common to all agonists. This estimate is based on inspection of models of benzyldene anabaseine, cinnamylidene anabaseine, and BQNE, in which the distance from the charged nitrogen to the end of the aromatic ring was measured. Further, since substituents on this aromatic ring can modulate activity of the agonist, the receptor pocket recognizing the benzyldene motif is likely to include residues that provide discrimination additional to simple recognition of the aromatic ring system. Interestingly, the bridged nicotine analog ACME (Figure 1) may provide an indication that the benzyldene selectivity filter could “start” as close as 3.7 Å from the charged nitrogen. In ACME, which is $\alpha 7$ selective, the ethyl chain which constrains the pyridine ring from free rotation is approximately in the same location as the benzylic carbon in compounds like BA and EBQN (Papke et al., 2005b).

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Discussion

While certain features have been proposed to be common to most $\alpha 7$ -selective agonists, the diversity of ligands identified to be $\alpha 7$ -selective is impressive (Mazurov et al., 2006). In general, drugs in commercial development have a cationic center, a hydrogen bond acceptor group, and one or more hydrophobic elements. The first two of these features were postulated in the earliest models for the pharmacophore for the muscle-type nAChR (Beers and Reich, 1970) and hence are not associated with $\alpha 7$ selectivity. However, the effective activation of both heteromeric and homomeric neuronal nAChRs by the simple quaternary amine TMA means that, while a hydrogen bond acceptor group may improve the activity for a specific type of ligand, it is not part of the minimal pharmacophore of a nonselective $\alpha 7$ agonist. The key element for $\alpha 7$ selectivity would then seem to be, in most cases, specific hydrophobic element(s). Most of the $\alpha 7$ -selective agonists shown in Figure 1 have very large hydrophobic groups, and many may be grouped into common classes based on the size and position of the groups. However, we have previously reported that the size of the hydrophobic element(s) need not be large (Papke et al., 2004b; Papke et al., 2005a; Papke et al., 2005b). The data of the present study support the hypothesis that a hydrophobic element as small as a single methyl group can provide selective activation of $\alpha 7$ nAChR. Furthermore, this small hydrophobic recognition element is distinct from the one recognizing large hydrophobic residues. Our results therefore suggest that there are at least three independent and distinct structural motifs which can be associated with $\alpha 7$ -selective agonists, which we have identified as: the choline motif, the tropane motif, and the benzylidene motif.

While agonists with the choline motif such as choline, QN-O, and QN-OH can selectively activate $\alpha 7$ receptors, the potency of these drugs is typically at least 10-fold lower

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than related agonists that lack the oxygen group (e.g. ETMA and QN). In the case of the QN compounds, there was also decreased efficacy in the $\alpha 7$ -selective compounds using the choline motif. The choline motif is also present in four other inactive compounds closely related to $\alpha 7$ agonists (gray in Figure 1), N-methyl-3-piperidinol, N,N-dimethyl-3-piperidinol 1-(2-hydroxyethyl)piperidine, and 1-(2-hydroxyethyl)pyrrolidine (data not shown). In the case of N-methyl-3-piperidinol and N,N-dimethyl-3-piperidinol, the hydroxyl group resides in a piperidine ring with a chair-like conformation. Though structurally similar to QN-OH, this latter compound places the six-membered piperidine ring in a boat-like conformation because of the bridgehead. This alters and locks the orientation of the hydroxyl group, which could reasonably be critical for activity. In the case of the hydroxyethyl compounds, the choline motif is entirely exocyclic, and thus the attached 5 or 6 membered ring structure may clash with the receptor in seeking to bind favorably.

Since, the $\alpha 7$ -selective compounds using the choline motif all seem to be less potent and/or efficacious for $\alpha 7$ than their most closely related non-selective analogs, the choline motif for $\alpha 7$ -selectivity seems to apply some cost to functionality. One hypothesis is that perhaps the polar groups of choline and of more complex non-functional choline analogs such as tropine make a spurious hydrogen bond with the receptor that impedes the proper orientation of the ammonium analog in the binding site, reducing binding affinity and/or potency for activation. Comparison of AR-R17779 and 3-hydroxy quinuclidine by overlay show nearly perfect overlap (Figure 10A). Because AR-R17779 cannot function as a hydrogen bond donor, it would avoid the spurious H-bond problem, and it is noteworthy that if AR-R17779 is modified so its oxygen atom becomes an H-bond donor (NH group) or a CH₂ group, $\alpha 7$ binding affinity is abolished (Mullen, 2000). Alternatively, functional groups of the receptor-bound drugs may interfere with

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the conformational changes required for channel gating, and although this would not necessarily affect binding affinity for the resting state of the receptor, it might affect potency for activation. In summary, features of the choline motif that may strongly disfavor binding and/or activation of heteromeric receptors, may only do so mildly for $\alpha 7$'s.

The tropane motif provides $\alpha 7$ selectivity via recognition of small hydrophobic groups clustered about the ammonium nitrogen. Given that methylpiperidine is not $\alpha 7$ selective, but addition of the second N-methyl group in the axial position above the piperidine ring plane renders it $\alpha 7$ selective, we suggest that the selectivity of MQN may not arise from the methyl group directly, but rather, the methyl group may position the agonist so that the bridgehead CH_2CH_2 unit fits into the tropane motif of the receptor. In tropane itself, a similar effect may be operative. Further, this tropane motif might be hypothesized to be the basis for other $\alpha 7$ -selective agonists such as 3-acetoxy-1,1 dimethyl-piperidine. However, it is also present in DMPP, which is not $\alpha 7$ selective. Although DMPP has two nitrogens, the phenyl substituted one is not basic, so an alternate binding mode with this position bearing the positive charge seems unlikely. It is possible that in binding to a non- $\alpha 7$ receptor, the phenyl group of DMPP provides a pathway for receptor activation that is unavailable to a simple piperidine. Whether or not this is the case for DMPP, it is quite clear that the position of the phenyl group with respect to the ammonium pharmacophore is quite critical since, as we discuss in the next section, $\alpha 7$ selectivity can be conferred to a member of the quinuclidine family by addition of a benzyldene group.

The third selectivity motif we identify is the benzyldene motif, in part because we have directly shown that addition of a benzyldene group to quinuclidine in the BQN series can confer selectivity. As significant is the historic precedent of the first large family of synthetic $\alpha 7$ -

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selective agonists, the benzylidene anabaseines such as GTS-21 (Papke et al., 2004a). Presumably, homologs of this motif account for the $\alpha 7$ -selectivity of agonists such as PNU-282987, SSR180711, and others in Figure 1 that have correspondingly large hydrophobic elements. Indeed, the molecular overlay of BQNE and SSR180711 shows an excellent coincidence of the aromatic moieties, using the charged nitrogen as an anchor point. (Figure 10B).

The configuration of the double bond in the benzylidene motif is important for selective activation of $\alpha 7$, and also significantly affects both the potency and efficacy of BQNs, since the E- isomers appear to be not only more selective but also more potent and efficacious (Table 1). However, this is only a modest difference, leading to the possibility that the recognition site in $\alpha 7$ for the benzylidene motif may be able to accommodate either geometric isomer. Another possibility is that the benzylidene motif does not apply to the Z-isomers. Instead, in the QNZ compounds the phenyl ring may locate in the same region of the receptor occupied when agonists like nicotine or anabaseine are bound, utilizing the “aryl-centroid” pharmacophoric site (Sheridan et al., 1986). BQNZ and the non-selective agonist anabaseine (AB) were compared by overlaying the two structures, and we found that the aryl ring of BQNZ but not the aryl ring of BQNE, resided close to the pyridine ring of anabaseine.(Figure 10C, D). This and all of the preceding comparative analyses are predicated on the simplifying idea that a common single productive binding mode leading to activation exists. If this is not the case, comparative analysis would become exceptionally complex. The latter possibility is supported by mutational studies of the conserved Y188 residue in $\alpha 7$ and $\alpha 4\beta 2$ (Horenstein et al., 2006). This residue has been hypothesized to play a key role in the sequence of intramolecular events coupling agonist binding to channel gating (Mukhtasimova et al., 2005). However, although the mutation of

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tyrosine 188 to phenylalanine had a large effect on ACh potency in $\alpha 7$, it had no effect on the potency of the benzylidene anabaseine 4OH-GTS-21 efficacy or potency in $\alpha 7$ (Horenstein et al., 2006). Further support for the hypothesis that gating can be initiated through multiple mechanisms came from the observation that the corresponding tyrosine to phenylalanine mutation in $\alpha 4\beta 2$ had little apparent effect on activation by ACh but produced a 200-fold increase in the efficacy of 4OH-GTS-21.

The model presented in Figure 9 incorporates the disposition of selectivity motifs in a relative sense, but ultimately we seek to map this model into the receptor via mutagenesis studies, SCAM analyses, homology modeling and docking studies, in lieu of direct crystallographic data for $\alpha 7$ and $\alpha 3\beta 4$ receptors. Preliminary inspection of homology models for $\alpha 7$ based on the crystal structure of snail ACh binding protein suggest that the large benzylidene motif will require extended interactions at the subunit interface. Ongoing work will address why heteromeric receptors cannot accommodate the aforementioned selectivity motifs in the activated state of the receptor, and what the specific interactions are with $\alpha 7$. The results reported in this study demonstrate that $\alpha 7$ -selective agonists may be obtained by swapping a subset of molecular features of one agonist class with another, for example, conferring selectivity to a quinuclidine by addition of a benzylidene group. This portability can be exploited for the design and synthesis of new $\alpha 7$ agonists.

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Footnotes

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Figure Legends

Figure 1 Structural diversity of selective activators of $\alpha 7$ nAChR. Multiple structural classes of nicotinic agonists are represented. Circled compounds are $\alpha 7$ -selective. Compounds in filled gray circles had no significant agonist activity when tested at 300 μ M on $\alpha 7$, $\alpha 4\beta 2$, or $\alpha 3\beta 4$ receptors (data not shown).

Figure 2 A) Responses to ACh and choline obtained from single cells expressing either $\alpha 7$, $\alpha 4\beta 2$, or $\alpha 3\beta 4$ nAChR. Note that for $\alpha 7$, the 300 μ M control is a fully efficacious ACh concentration for the net charge response. Choline is approximately 10-fold less potent than ACh, and therefore 3 mM choline evokes nearly identical responses as 300 μ M ACh. For $\alpha 4\beta 2$ the 30 μ M ACh control is approximately the EC60 for peak current, and 3 mM choline evokes responses that are only 2% the ACh maximum. For $\alpha 3\beta 4$ the 100 μ M ACh control is approximately the EC45 for peak current, and 3 mM choline evokes peak currents that are approximately 5% the ACh maximum. B) Responses to ACh and QN obtained from single cells expressing either $\alpha 7$, $\alpha 4\beta 2$, or $\alpha 3\beta 4$ nAChR. Note that for $\alpha 7$, the 300 μ M control is a fully efficacious ACh concentration for the net charge response, but not for peak current. QN is more potent than ACh and therefore evokes a larger peak current but the same net charge. For $\alpha 4\beta 2$ the 30 μ M ACh control is approximately the EC60 for peak current, and for $\alpha 3\beta 4$ the 100 μ M ACh control is approximately the EC45 for peak current.

Figure 3 The effect of ACh, TMA, ETMA, and choline on $\alpha 7$ and $\alpha 3\beta 4$ nAChR subtypes. Concentration-response curves for $\alpha 7$ -expressing oocytes were calculated based on net charge and those for $\alpha 3\beta 4$ responses on peak current amplitudes. Responses were calculated relative to ACh control responses obtained from the same cells (see Methods) and subsequently normalized to the ACh maximum response determined in separate experiments (not shown). Values represent the averages (\pm SEM) of at least four oocytes.

Figure 4 Structural comparisons of nicotinic agonists. **4A.** We have previously reported that the relatively simple tropane and DMPP molecules shown in the top row are selective activators of $\alpha 7$ -receptors, and the overlay of these structures (second row, left) shows a similar disposition of the charge center and hydrophobic substituents. Quinuclidine (QN), which is not $\alpha 7$ -selective, is compared to tropane (second row, right). This comparison suggests the addition of a methyl group to QN to produce methylquinuclidine (MQN, Figure 4B) would make a better match to the

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common features of tropane and DMP. **4B** also presents the structures of EQN, BQN compounds, and other quinuclidines as discussed in the text.

Figure 5 The effect of quinuclidine compounds on $\alpha 7$ and $\alpha 3\beta 4$ nAChR subtypes. Concentration-response curves for $\alpha 7$ -expressing oocytes were calculated based on net charge and those for $\alpha 3\beta 4$ responses on peak current amplitudes. Responses were calculated relative to ACh control responses obtained from the same cells (see Methods) and subsequently normalized to the ACh maximum response determined in separate experiments (not shown). Values represent the averages (\pm SEM) of at least four oocytes.

Figure 6 Effects of EQN on cells expressing $\alpha 7$ and uninjected cells. Only $\alpha 7$ -injected cells showed response to the application of 300 μ M ACh. In contrast, inward currents were evoked by 1 mM EQN in both $\alpha 7$ -injected and uninjected cells. However, in $\alpha 7$ -injected cells some portion of the current was mecamylamine-sensitive. The rightmost panel shows the EQN-evoked currents in the absence and presence of 100 μ M mecamylamine.

Figure 7 The effects of diethyldimethylammonium (dEdMA) on cells expressing $\alpha 7$ and $\alpha 3\beta 4$. Representative traces are shown in the top panel compared to control ACh responses obtained from the same cells. For $\alpha 7$ the 300 μ M control is a fully efficacious ACh concentration for the net charge response. For $\alpha 3\beta 4$ the 100 μ M ACh control is approximately the EC45 for peak current. Concentration-response relationships are shown below. Values represent the averages (\pm SEM) of at least four oocytes.

Figure 8 The effect of benzylidene-quinuclidine compounds on $\alpha 7$ and $\alpha 3\beta 4$ nAChR subtypes. Concentration-response curves for $\alpha 7$ -expressing oocytes were calculated based on net charge and those for $\alpha 3\beta 4$ responses on peak current amplitudes. Responses were calculated relative to ACh control responses obtained from the same cells (see Methods) and subsequently normalized to the ACh maximum response determined in separate experiments (not shown). Values represent the averages (\pm SEM) of at least four oocytes.

Figure 9 A. Three unique modifications of a nonselective agonist lead to selectivity. Quinuclidine is a non-selective $\alpha 7$ agonist, but its 3-hydroxy, 3-benzylidene, and N-methyl analogs are $\alpha 7$ selective. **B.** Spatial model for $\alpha 7$ -selectivity filters. Three key recognition motifs are superimposed on an arbitrary Cartesian frame of reference to illustrate the spatial relationships between the motifs. Red signifies the universal ammonium (cationic) recognition motif for all nAChRs and the H bond acceptor of the classic nAChR pharmacophore. The dark

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blue region represents the small tropane motif which recognizes small aliphatic groups. The small light blue region represents the choline motif, a putative hydrogen bond acceptor. Green represents the benzylidene motif, an extended region that accommodates planar aromatic moieties. **C.** Alpha-selective agonists superimposed on the spatial model. The top row (tropane motif) compares MQN, DMP, and tropane, illustrating that a common element is a small hydrophobic group resting above the x-y plane. The N-methyl group may serve to position the agonist to take advantage of this binding pocket. The bottom row (benzylidene motif) compares EBQN with BA. The common element for these two $\alpha 7$ -selective agonists is an aromatic group in the third quadrant of the x-y plane.

Figure 10. Comparison of synthesized compounds with known $\alpha 7$ -selective and nonselective agonists. The comparison illustrates how known quinuclidine selective agonists can be classified in terms of whether they employ a selectivity pharmacophore. The relative orientation of molecules coincides with the framework shown in Figure 9. Panel **A** compares AR-R17779 (blue) with QNOH (red), which share the choline motif. Panel **B** compares SSR180711 (blue) with BQNE (red), both utilizing the benzylidene motif. Panel **C** illustrates how the Z isomer of BQN places the phenyl ring close to the region occupied by the pyridine ring of non-selective anabaseine. Panel **D** shows overlay of BQNE and anabaseine. There was failure to match the benzylidene motif with the pyridine ring of anabaseine with any reasonable overlay scheme. In **C** and **D**, the red molecules are the BQN isomers, the blue molecule is anabaseine (AB).

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Table 1: Characterization of putative $\alpha 7$ -selective agonists

(see Figure 1 for structures)

Compound	Summary*	Reference
A-844606	<u>Expression system:</u> <i>Xenopus</i> oocytes transfected cells and IMR-32 cells <u>nAChR subtypes studied:</u> human $\alpha 7$, $\alpha 4\beta 2$, rat $\alpha 7$, putative $\alpha 3\beta 4$ (IMR-32) <u>Effects on 5HT3 receptors:</u> Not studied <u>Summary:</u> Partial agonist of $\alpha 7$ with relatively little activation of other nAChR tested. Demonstrated to have antagonist activity for $\alpha 4\beta 2$ and more potently for putative $\alpha 3\beta 4$. Mechanism of antagonism not investigated.	(Briggs et al., 2008)
ABBF	<u>Expression system:</u> <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 7$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, Muscle (subunit composition not specified) <u>Effects on 5HT3 receptors:</u> Antagonist <u>Summary:</u> Putative full agonist for human $\alpha 7$ ($EC_{50} \approx 3 \mu M$) and an antagonist for other nAChR tested (IC_{50} s 1-8 μM). Mechanism of antagonism not investigated. Bound to 5HT3 receptors with high affinity (60 nM), comparable to rat brain membrane $\alpha 7$ sites (62 nM).	(Boess et al., 2007)
ACME & ACME-B	<u>Expression system:</u> <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 7$, $\alpha 3\beta 4$, $\alpha 4\beta 2$ <u>Effects on 5HT3 receptors:</u> Not studied <u>Summary:</u> The rigid nicotine analogs ACME and ACME-B were partial agonists for rat $\alpha 7$ nAChR (I_{max} , 10 and 20%, that of ACh, respectively). ACME-B was 2-fold more potent ($EC_{50} \approx 26 \mu M$) than the boron free form ACME. Potential antagonist activity of non- $\alpha 7$ nAChR not investigated.	(Papke et al., 2005b)
AR-R17779	<u>Expression system:</u> <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 7$, $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 3\beta 2$, $\alpha 3\beta 3\alpha 5$ <u>Effects on 5HT3 receptors:</u> None <u>Summary:</u> Initial characterization by Mullen et al. examined function at just $\alpha 7$ and no other nAChR. Claims for $\alpha 7$ -selectivity was based solely on binding studies with rat brain membranes. Subsequent study by Papke et al. at other nAChR subtypes and the 5HT3 receptor. It was confirmed to be a full agonist for $\alpha 7$ with negligible agonist or antagonist activity for other receptors tested.	(Mullen et al., 2000; Papke et al., 2004b)
benzylidene anabaseines	<u>Expression system:</u> <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 7$, $\alpha 2\beta 2$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 2\beta 4$, $\alpha 3\beta 4$, $\alpha 4\beta 4$ <u>Effects on 5HT3 receptors:</u> Partial agonist and antagonist activity related to side groups on benzylidene ring. <u>Summary:</u> Numerous benzylidene anabaseines have been studied. They all appear to be $\alpha 7$ -selective partial agonists, but vary widely in both potency and efficacy. They are effective antagonists of other nAChR subtypes and in some cases the inhibition has been shown to be use dependent.	(Kem et al., 2004; Machu et al., 2001; Meyer et al., 1998a; Meyer et al., 1997; Papke et al., 2004a)
choline	<u>Expression system:</u> <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 1\beta 1\gamma\delta$, $\alpha 3\beta 4$, $\alpha 3\beta 2$, $\alpha 4\beta 2$, $\alpha 7$, $\alpha 9$ <u>Effects on 5HT3 receptors:</u> Not studied <u>Summary:</u> Choline was initially identified as full agonist for $\alpha 7$ nAChR with little or no activity at heteromeric nAChR. The potency of choline for $\alpha 7$ is ten-fold lower than that of ACh. Antagonist activity was not studied in detail although channel block is likely to occur at high (> 1 mM) concentrations. Choline was later shown to also be an agonist for $\alpha 9$ nAChR (Verbitsky et al.).	(Papke et al., 1996; Verbitsky et al., 2000)
cinnamylidene anabaseines	<u>Expression system:</u> <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 1\beta 1\gamma\delta$, $\alpha 2\beta 2$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 4$, $\alpha 7$ <u>Effects on 5HT3 receptors:</u> Not studied <u>Summary:</u> Numerous cinnamylidene anabaseines have been studied. They all appear to be $\alpha 7$ -selective agonists or partial, but vary widely in both potency and efficacy. They are effective antagonists of other nAChR subtypes and in some cases the inhibition has been shown to be use dependent.	(de Fiebre et al., 1995; Meyer et al., 1998b)

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cocaine methiodide	<p><u>Expression system:</u> <i>Xenopus</i> oocytes and transfected cells</p> <p><u>nAChR subtypes studied:</u> $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 1\beta 1\gamma\delta$, $\alpha 7$</p> <p><u>Effects on 5HT3 receptors:</u> Not studied</p> <p><u>Summary:</u> While cocaine was an antagonist at multiple nAChR subtypes the methiodide derivative was a selective activator of rat, human and chick $\alpha 7$ receptors, expressed in oocytes and was also able to activate human $\alpha 7$ receptors transiently expressed in PC12 cells. Cocaine methiodide was a weak antagonist at other nAChR subtypes studied.</p>	(Francis et al., 2001)
compound-15b	<p><u>Expression system:</u> PC12 cells</p> <p><u>nAChR subtypes studied:</u> $\alpha 7$ and possibly others</p> <p><u>Effects on 5HT3 receptors:</u> Not studied</p> <p><u>Summary:</u> No data provided to support selectivity. Relatively affinity for $\alpha 7$ supported by α-btX binding experiments but contrasting data for $\alpha 4\beta 2^*$ receptors was not provided. Functional data was obtained from PC12 cells which may express $\alpha 7$ as well as other nAChR subtypes. Selective antagonists were not used to confirm that evoked responses were $\alpha 7$-mediated. The (+) isomer of 15b had 5-fold greater agonist activity but both isomers had similar affinity in binding assays.</p>	(Tatsumi et al., 2004)
compound-23	<p><u>Expression system:</u> cultured hippocampal neurons</p> <p><u>nAChR subtypes studied:</u> $\alpha 7$ (putative $\alpha 4\beta 2^*$ receptors in binding assays)</p> <p><u>Effects on 5HT3 receptors:</u> Affinity for 5HT3 receptors in binding experiments ($K_i \approx 10$ nM), nearly as high as for putative $\alpha 7$ receptors ($K_i \approx 3$ nM).</p> <p><u>Summary:</u> Only binding data is provided to support selectivity. There was high affinity for putative $\alpha 7$ and low affinity for $\alpha 4\beta 2^*$ receptors. However, the data also indicated high affinity for 5HT3 receptors, but no functional tests of 5HT3 receptors were conducted. Functional data were obtained from cultured neurons which predominantly express $\alpha 7$ nAChR and selective antagonists were used to support the conclusion that $\alpha 7$ receptors were activated. Efficacy was only about 40% and potency in the functional assay was 1000-fold lower than affinity obtained in binding assays.</p>	(Tatsumi et al., 2006)
compound-25	<p><u>Expression system:</u> cultured hippocampal neurons</p> <p><u>nAChR subtypes studied:</u> $\alpha 7$ (putative $\alpha 4\beta 2^*$ and muscle-type receptors in binding assays)</p> <p><u>Effects on 5HT3 receptors:</u> Not studied.</p> <p><u>Summary:</u> Only binding data is provided to support selectivity. There was high affinity for putative $\alpha 7$ and low affinity for $\alpha 4\beta 2^*$ and muscle type receptors. Functional data were obtained from cultured neurons which predominantly express $\alpha 7$ nAChR and selective antagonists were used to support the conclusion that $\alpha 7$ receptors were activated. Efficacy was only about 40% and potency in the functional assay was potency was 1000-fold lower than affinity obtained in binding assays.</p>	(Tatsumi et al., 2005)[
dimethyl piperidinium	<p><u>Expression system:</u> <i>Xenopus</i> oocytes</p> <p><u>nAChR subtypes studied:</u> $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 7$</p> <p><u>Effects on 5HT3 receptors:</u> Antagonist activity reported</p> <p><u>Summary:</u> A partial agonist (≥ 60 % efficacy compared to ACh), selective for $\alpha 7$ among the limited number of receptor subtypes tested. Antagonist activity at $\alpha 3\beta 4$ and 5HT3 receptors, but not for $\alpha 4\beta 2$.</p>	(Papke et al., 2005a)
JN403	<p><u>Expression system:</u> Transfected cells and <i>Xenopus</i> oocytes</p> <p><u>nAChR subtypes studied:</u> $\alpha 1\beta 1\gamma\delta$, $\alpha 3\beta 4$, $\alpha 3\beta 2$, $\alpha 4\beta 2$, $\alpha 7$</p> <p><u>Effects on 5HT3 receptors:</u> Relatively low potency antagonist ($IC_{50} \approx 20$ μM)</p> <p><u>Summary:</u> In the <i>Xenopus</i> oocyte experiments, JN403 was a relatively potent partial agonist, with an efficacy of about 50% that of ACh. 5HT3 receptors and other nAChR were evaluated with calcium flux measurements in transfected cells and JN403 was antagonist in these experiments.</p>	(Feuerbach et al., 2007)
PHA-543,613	<p><u>Expression system:</u> Transfected cells and cell lines</p> <p><u>nAChR subtypes studied:</u> putative $\alpha 3\beta 4$, $\alpha 1\beta 1\gamma\delta$, putative $\alpha 4\beta 2^*$ in rat brain membranes.</p> <p><u>Effects on 5HT3 receptors:</u> Inhibitory activity at ≈ 600 nM</p> <p><u>Summary:</u> Functional data for $\alpha 3\beta 4$ and $\alpha 1\beta 1\gamma\delta$ were obtained from calcium flux measurements and the compound was a weak antagonist in these assays, though a much more potent antagonist at 5HT3 receptors. Interactions with putative $\alpha 4\beta 2$ nAChR were only evaluated with very cursory</p>	(Wishka et al., 2006)

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	binding experiments. Functional data were obtained from cultured neurons which predominately express $\alpha 7$ nAChR and selective antagonists were used to support the conclusion that $\alpha 7$ receptors were activated.	
PHA-709829	<u>Expression system:</u> Transfected cells and cultured hippocampal neurons <u>nAChR subtypes studied:</u> putative $\alpha 3\beta 4$, $\alpha 1\beta 1\gamma\delta$, putative $\alpha 4\beta 2^*$ in rat brain membranes. <u>Effects on 5HT3 receptors:</u> Inhibitory activity at ≈ 300 nM <u>Summary:</u> Functional data for $\alpha 3\beta 4$ and $\alpha 1\beta 1\gamma\delta$ were obtained from calcium flux measurements and the compound was a weak antagonist in these assays, though a much more potent antagonist at 5HT3 receptors. Interactions with putative $\alpha 4\beta 2$ nAChR were only evaluated with very cursory binding experiments. No functional data were presented for $\alpha 7$ nAChR. Affinity for $\alpha 7$ nAChR supported only by binding data.	(Acker et al., 2008)
PNU-282987	<u>Expression system:</u> cultured hippocampal neurons, and other unspecified <u>nAChR subtypes studied:</u> putative $\alpha 1\beta 1\gamma\delta$, $\alpha 3\beta 4$, $\alpha 7$ <u>Effects on 5HT3 receptors:</u> Functional antagonist of the $IC_{50} \approx 5$ μ M. <u>Summary:</u> Reportedly there was negligible agonist or antagonist activity at either $\alpha 1\beta 1\gamma\delta$ or $\alpha 3\beta 4$, but no data are shown and methods for these determinations are not described. The claim for $\alpha 7$ -selectivity is based on the limited data that 1 μ M PNU-282987 displaced only 14% cytosine binding from rat brain membranes but displaced MLA binding from rat brain homogenates with a K_i of 27 nM. It was shown to evoke currents in rat hippocampal neurons in a concentration-dependent manner, but the data were insufficient to measure potency or efficacy.	(Bodnar et al., 2005)
PSAB-OFP	<u>Expression system:</u> Transfected cells and <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 2\beta 4$, $\alpha 3\beta 4$, $\alpha 4\beta 4$, $\alpha 3\beta 2$, $\alpha 4\beta 2$, $\alpha 7$ <u>Effects on 5HT3 receptors:</u> A 66% partial agonist, 10-fold more potent than 5HT <u>Summary:</u> PSAB-OFP is a potent and efficacious agonist of $\alpha 7$ nAChR with little activity for other nAChR. However, it is also a potent agonist for 5HT3 receptors.	(Broad et al., 2002)
S 24795	<u>Expression system:</u> <i>Xenopus</i> oocytes and hippocampal interneurons in brain slices <u>nAChR subtypes studied:</u> $\alpha 7$, putative muscle type nAChR (<i>Torpedo</i>), ganglionic (IMR32 cells), and $\alpha 4\beta 2^*$ and $\alpha 7^*$ in brain membranes <u>Effects on 5HT3 receptors:</u> Not studied <u>Summary:</u> Selectivity for $\alpha 7$ supported by binding studies not described in detail. Functional data from oocytes and hippocampal interneurons in brain sliced support the conclusion that S 24795 is a partial agonist for $\alpha 7$ nAChR.	(Lopez-Hernandez et al., 2007)
SSR-180711	<u>Expression system:</u> <i>Xenopus</i> oocytes cultured hippocampal neurons and transfected cells <u>nAChR subtypes studied:</u> $\alpha 4\beta 2$, $\alpha 7$, $\alpha 3\beta 2$, putative $\alpha 3\beta 4$ (IMR32 cells) and $\alpha 1\beta 1\gamma\delta$ (TE671 cells) <u>Effects on 5HT3 receptors:</u> Not studied <u>Summary:</u> A relatively potent partial agonist with selectivity for $\alpha 7$ compared to other nAChR subtypes tested established by binding and voltage clamp experiments.	(Biton et al., 2007)
TC-1698	<u>Expression system:</u> <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 4\beta 2^*$ $\alpha 3\beta 2$, $\alpha 3\beta 4$ $\alpha 1\beta 1\epsilon\delta$ $\alpha 7$ <u>Effects on 5HT3 receptors:</u> Not studied <u>Summary:</u> A potent full agonist for $\alpha 7$ nAChR with negligible activation of the heteromeric neuronal nAChR tested, but with low potency partial agonist activity for muscle nAChR. Effective competitive antagonist at $\alpha 4\beta 2$ nAChR.	(Marrero et al., 2004)
tilorone	<u>Expression system:</u> <i>Xenopus</i> oocytes transfected cells and IMR-32 cells <u>nAChR subtypes studied:</u> human $\alpha 7$, $\alpha 4\beta 2$, rat $\alpha 7$, putative $\alpha 3\beta 4$ (IMR-32) <u>Effects on 5HT3 receptors:</u> Not studied <u>Summary:</u> Partial agonist of $\alpha 7$ with relatively little activation of other nAChR tested. Demonstrated to have antagonist activity for $\alpha 4\beta 2$ and more potently for putative $\alpha 3\beta 4$. Mechanism of antagonism not investigated.	(Briggs et al., 2008)
tropane	<u>Expression system:</u> <i>Xenopus</i> oocytes <u>nAChR subtypes studied:</u> $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 7$ <u>Effects on 5HT3 receptors:</u> Neither agonist nor antagonist activity detected <u>Summary:</u> A partial agonist (25% efficacy compared to ACh), selective for $\alpha 7$	(Papke et al., 2005a)

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	among the limited number of receptor subtypes tested. An antagonist for other nAChR tested but not for 5HT3 receptors.	
tropinone	<p><u>Expression system:</u> <i>Xenopus</i> oocytes</p> <p><u>nAChR subtypes studied:</u> $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 7$</p> <p><u>Effects on 5HT3 receptors:</u> Neither agonist nor antagonist activity detected</p> <p><u>Summary:</u> A partial agonist (64% efficacy compared to ACh), selective for $\alpha 7$ among the limited number of receptor subtypes tested. Antagonist activity at $\alpha 3\beta 4$ but not $\alpha 4\beta 2$ or 5HT3 receptors.</p>	(Papke et al., 2005a)
tropisetron	<p><u>Expression system:</u> <i>Xenopus</i> oocytes</p> <p><u>nAChR subtypes studied:</u> $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 1\beta 1\gamma\delta$ for binding only</p> <p><u>Effects on 5HT3 receptors:</u> Competitive antagonist</p> <p><u>Summary:</u> A partial agonist (30% efficacy compared to ACh), selective for $\alpha 7$ among the limited number of receptor subtypes tested. An antagonist for other nAChR tested as well as for 5HT3 receptors.</p>	(Macor et al., 2001; Papke et al., 2005a)

*Compounds circled in Figure 1 have been introduced into the literature as $\alpha 7$ -selective agonists or partial agonists. However, the functional studies which provided the basis for this attribution have varied greatly in both the methods used and the number of receptor subtypes which were studied. Provided are brief synopses of the salient features of the studies cited and their conclusions. Note that in some cases arguments for selectivity were based largely on binding data, which would not discriminate between agonists and antagonists, rather than on functional studies. Also, some functional assays relied on cell lines (e. g. PC12 cells), which vary in their nAChR expression from lab to lab or over time in a single lab.

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Table 2: Activation of $\alpha 7$ and $\alpha 3\beta 4$ nAChR

agonist	$\alpha 7$		$\alpha 3\beta 4$	
	I_{\max}	$EC_{50} \mu M$	I_{\max}	$EC_{50} \mu M$
ACh	$1.0 \pm$	27 ± 3	$1.0 \pm$	110 ± 9
TMA	1.1 ± 0.1	30 ± 3	0.55 ± 0.03	350 ± 190
ETMA	1.0 ± 0.1	19 ± 2	0.78 ± 0.04	660 ± 90
Choline	0.90 ± 0.1	243 ± 8	0.04 ± 0.01	N.A.
dEdMA	0.95 ± 0.06	197 ± 37	0.06 ± 0.01	N.A.
QN	1.03 ± 0.02	7.2 ± 0.5	0.64 ± 0.01	180 ± 10
MQN	1.0 ± 0.1	40 ± 8	0.025 ± 0.006	N.A.
QN-O	0.73 ± 0.04	78 ± 11	$\geq 0.07 \pm$	$> 1,000$
QN-OH	0.60 ± 0.03	214 ± 15	$0.01 \pm$	N.A.
BQNE	0.28 ± 0.01	1.5 ± 0.2	<0.01	N.A.
BQNZ	0.11 ± 0.01	7.3 ± 0.7	0.10 ± 0.01	N.A.
MeOBQNE	0.41 ± 0.03	1.3 ± 0.2	$<.01$	N.A.
MeOBQNZ	$0.09 \pm .01$	1.1 ± 0.8	<0.01	N.A.

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Table 3: Activation and/or inhibition of 5HT3 receptors and other nAChR

Compound	5HT3		$\alpha 1\beta 1\epsilon\delta$		$\alpha 4\beta 2$	
	Act.	Inhib.	Act.	Inhib.	Act.	Inhib.
QN	N	NS	3%	NS	$\approx 2\%$	$74 \pm 5\%$
TMA	N	NS	$<2\%$	NS	$19 \pm 6\%$	NS
ETMA	N	NS	N	NS	$\leq 2\%$	$40 \pm 9\%$
dEdMA	N	NS	N	NS	N	$52 \pm 5\%$
Choline	N	NS	N	NS	$\approx 1\%$	NS
MQN	N	NS	N	NS	N	$72 \pm 4\%$
QN-O	N	NS	N	NS	N	NS
QN-OH	N	NS	N	NS	N	NS
BQNE	N	$94 \pm 5\%$	N	$70 \pm 7\%$	N	$77 \pm 2\%$
BQNZ	N	$95 \pm 4\%$	$\approx 7\%$	NS	$\approx 1\%$	$74 \pm 6\%$
MeOBQNE	N	$94 \pm 5\%$	N	$90 \pm 2\%$	N	$81 \pm 2\%$
MeOBQNZ	N	$80 \pm 16\%$	$\leq 1\%$	$76 \pm 6\%$	N	$56 \pm 4\%$

Agonist activity (Act.) was evaluated by applying the compounds at a concentration of 100 μM and comparing those responses to appropriate control responses obtained from the same oocytes. Controls were 10 μM serotonin, 3 μM ACh, and 30 μM ACh for the 5HT3, $\alpha 1\beta 1\epsilon\delta$ and $\alpha 4\beta 2$ receptors, respectively. Those responses were then normalized to maximal agonist-evoked responses for each receptor subtype, as determined in other experiments. "N" indicates that application of the drug at a concentration of 100 μM did not produce responses above our level of detection (i.e. distinct from application artifacts of 3-5 nA). For 5HT3 receptors the detection threshold was 5% of the maximal serotonin-evoked responses. ACh-evoked responses for muscle type receptors are very large, so the detection threshold for responses evoked by the experimental compounds was 0.5% ACh maximum and for $\alpha 4\beta 2$ receptors the detection threshold was 1% of the ACh maximum. Antagonist activity (Inhib.) was determined by comparing the activation produced by control applications of agonist to those produced by co-application of agonists at the control concentrations plus the 100 μM of the experimental compounds. "NS" indicates that there was no significant inhibition of the co-application responses compared to control applications of agonist alone.

Structural Diversity of $\alpha 7$ -selective agonists

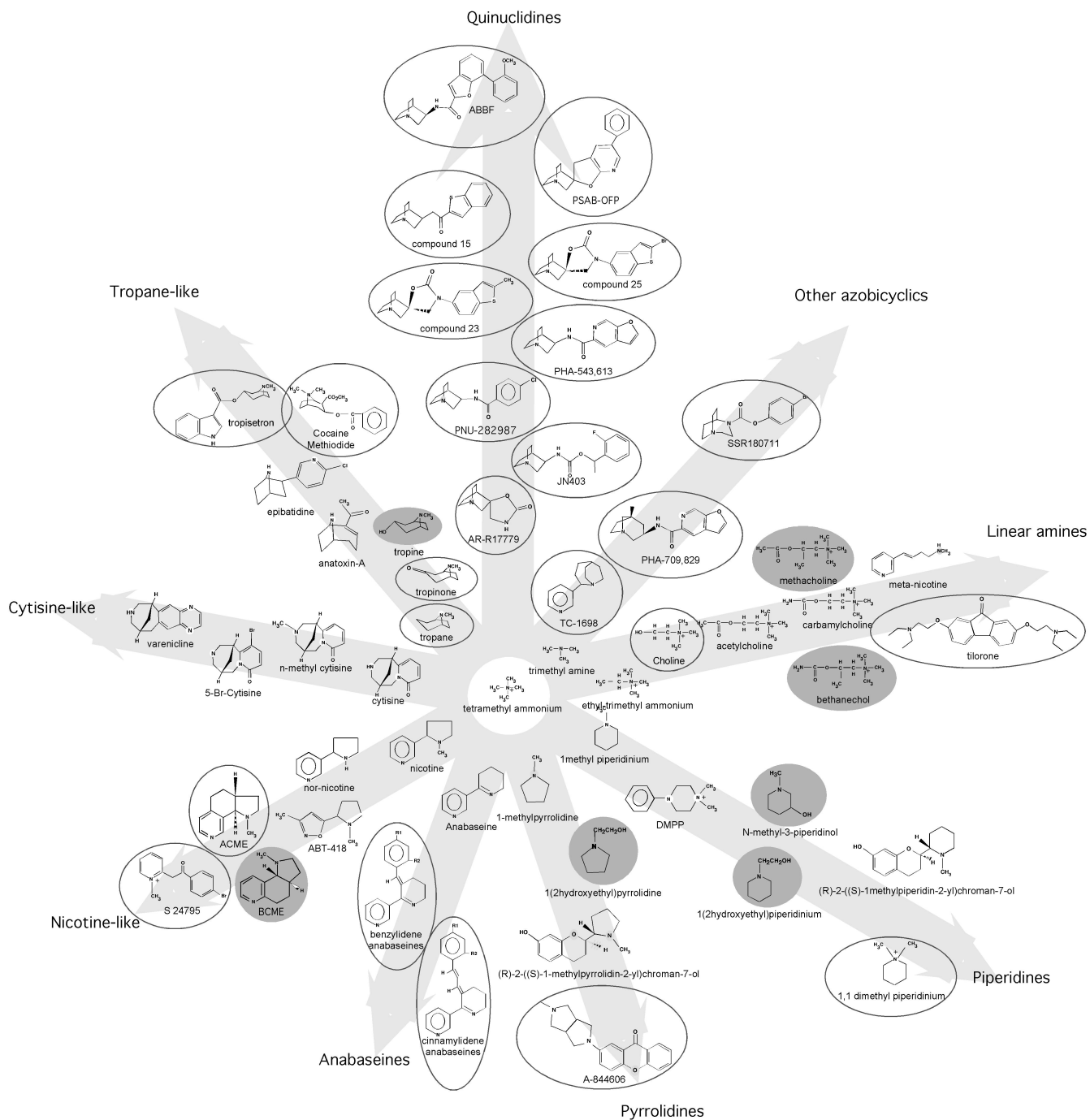
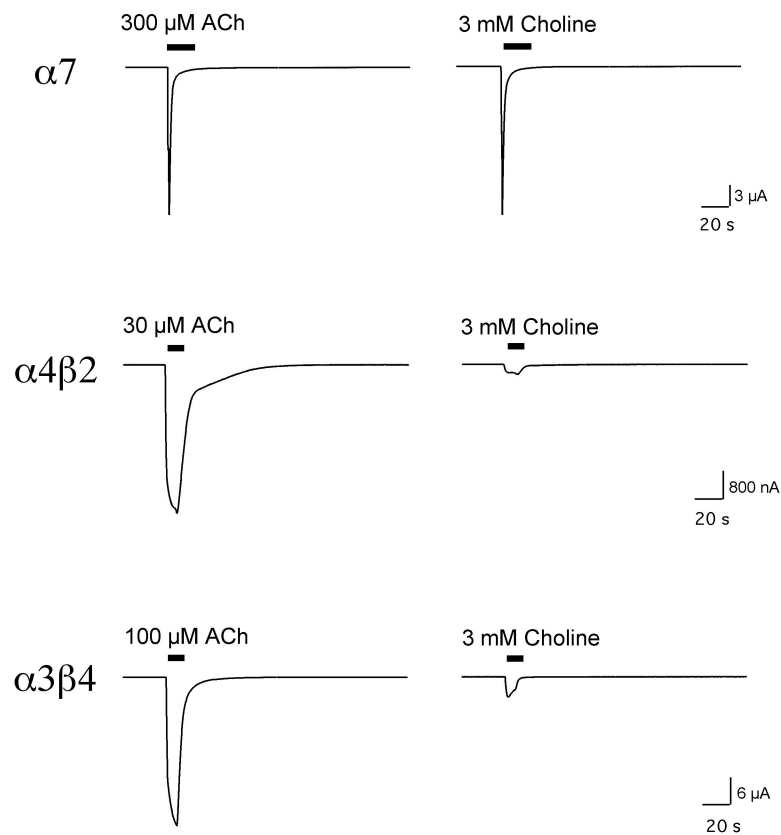
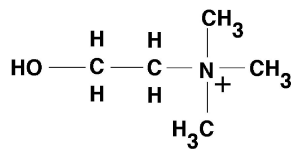
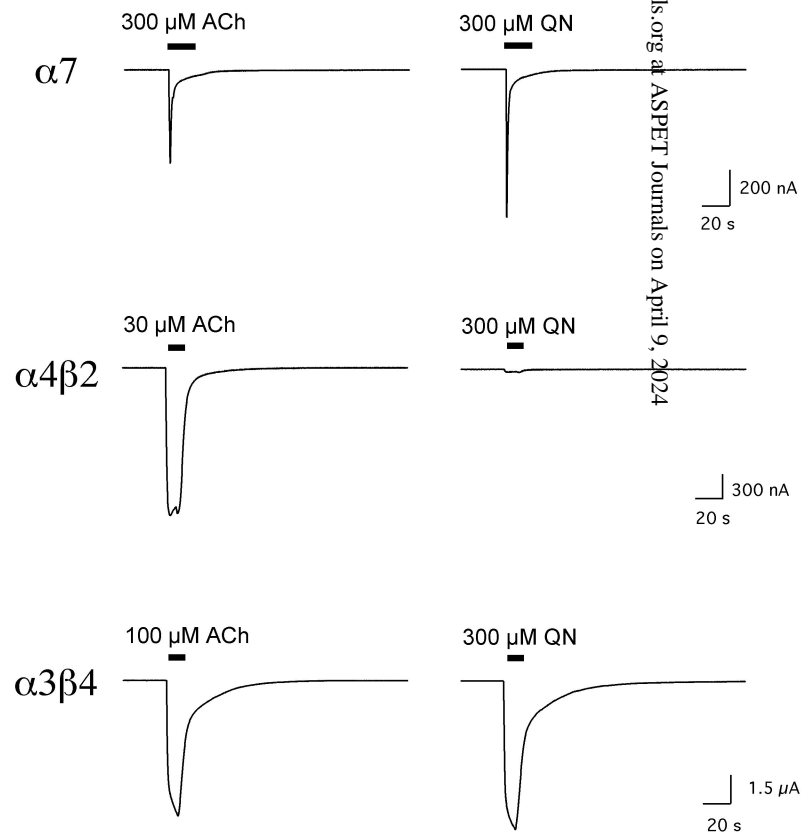
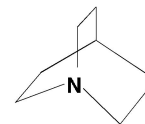
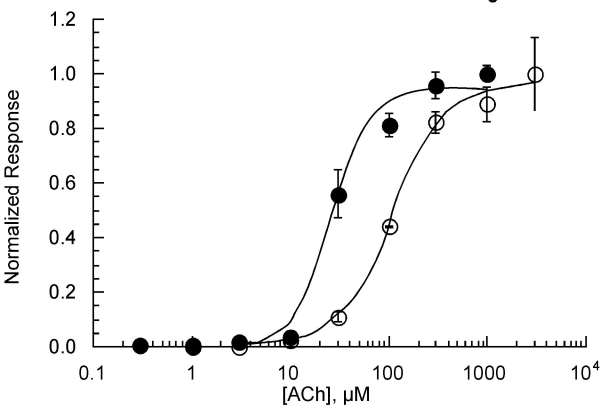
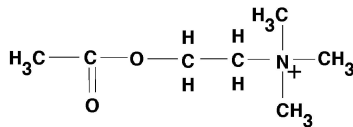
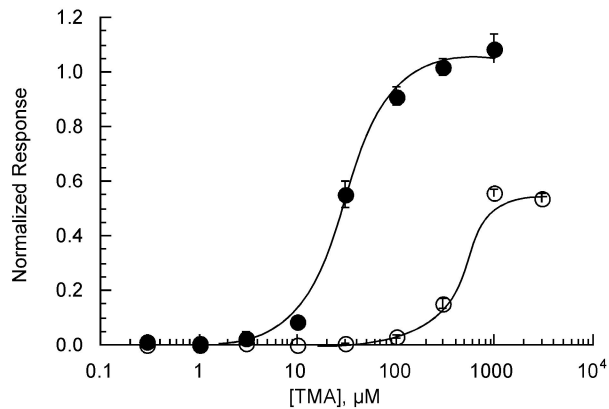
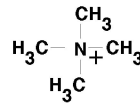


Figure 1

A**Choline****B****Quinuclidine**

ACh**TMA**

● $\alpha 7$
○ $\alpha 3\beta 4$

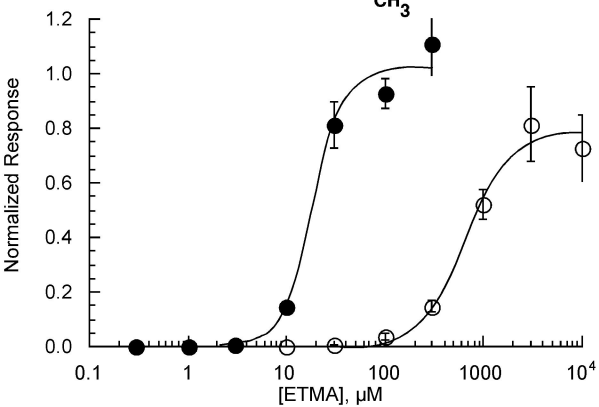
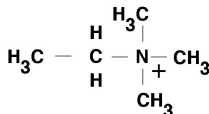
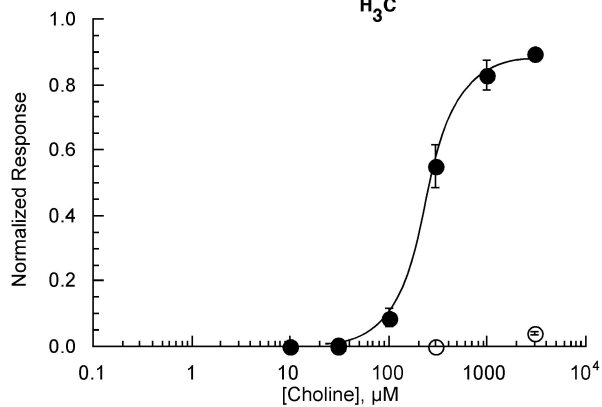
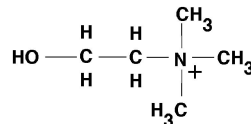
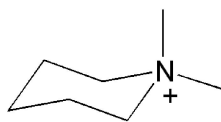
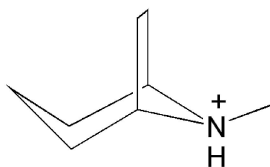
ETMA**Choline**

Figure 3

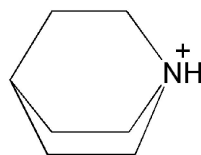
A



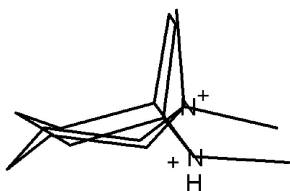
DMP



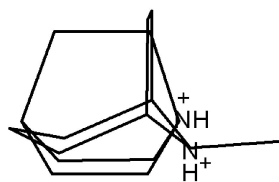
Tropane



QN

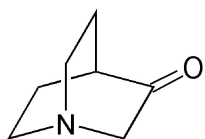


DMP vs Tropane

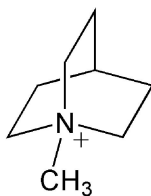


QN vs. Tropane

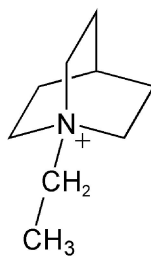
B



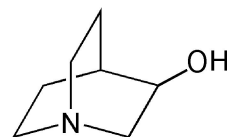
QN-O



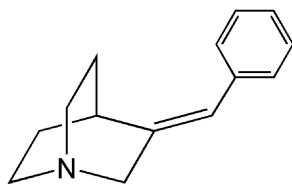
MQN



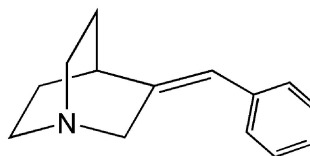
EQN



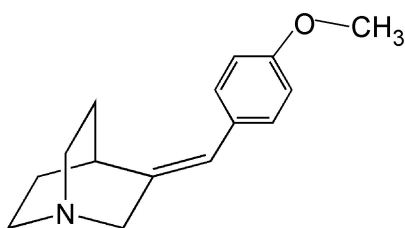
QN-OH



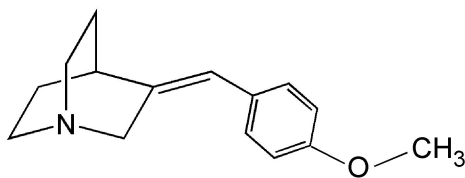
BQNE



BQNZ



MeOBQNE



MeOBQNZ

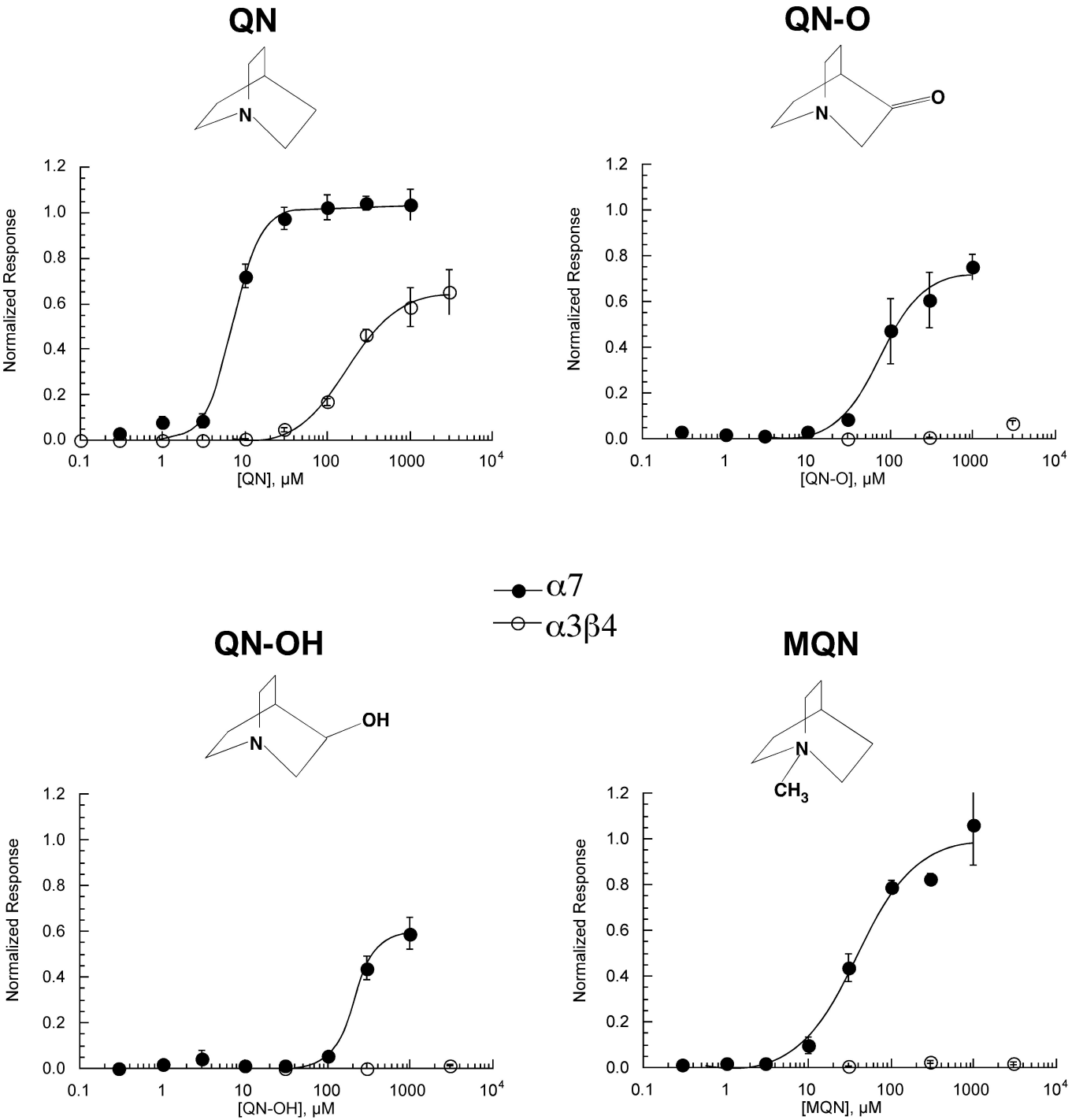


Figure 5

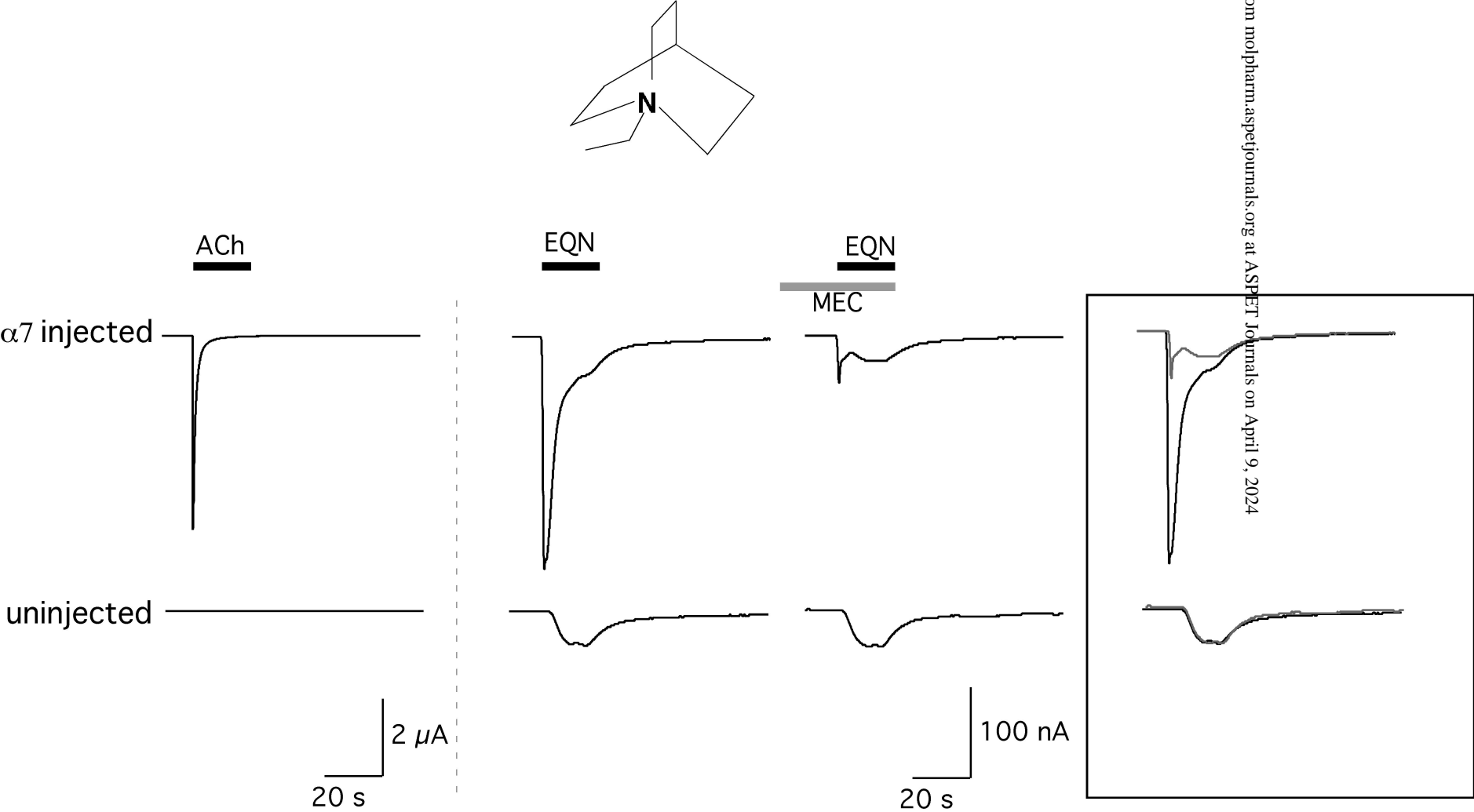
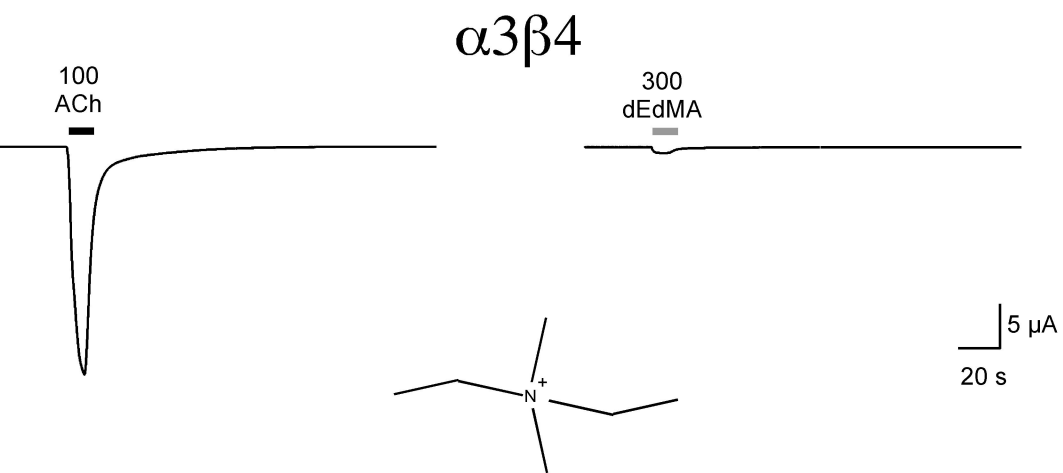
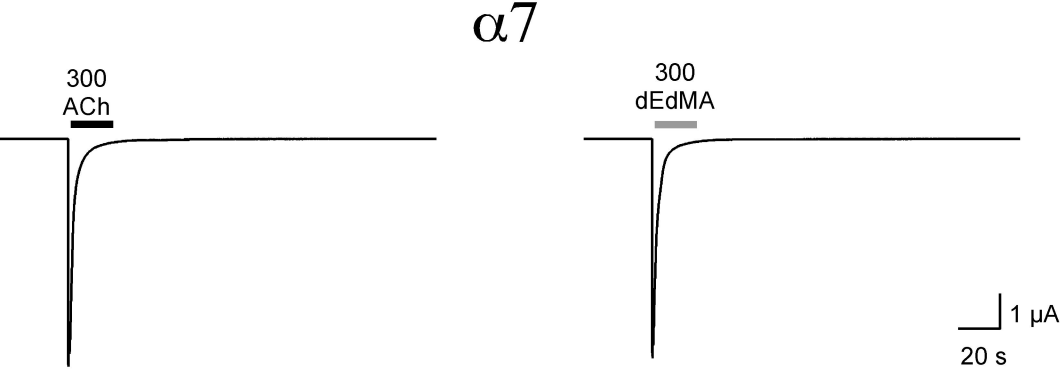


Figure 6



diEthyl, diMethyl ammonium

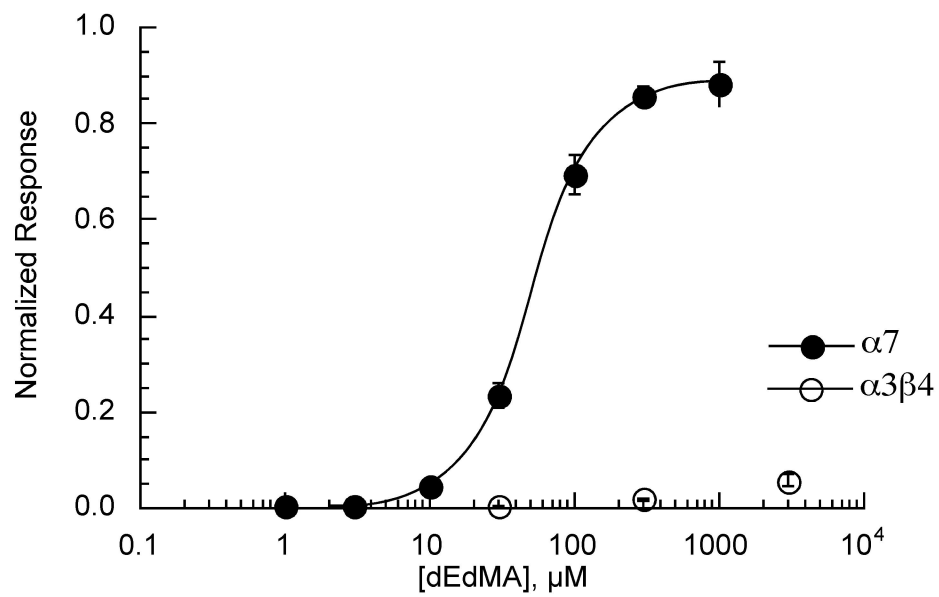
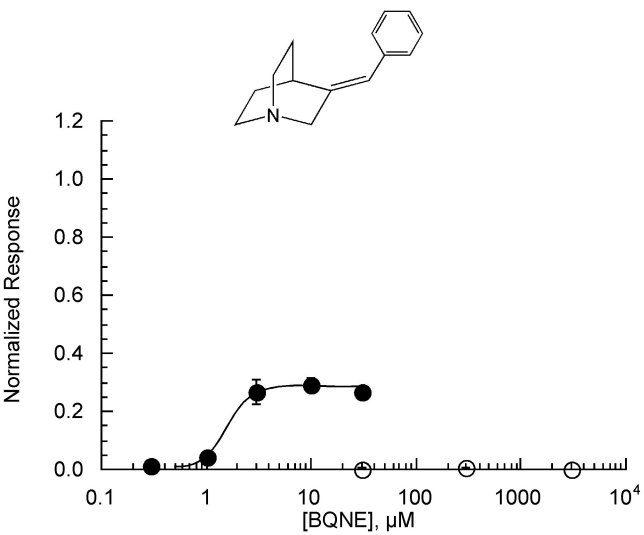
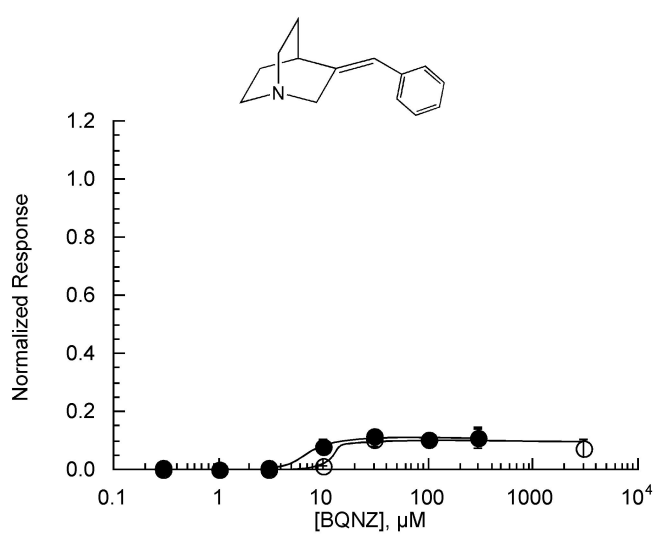


Figure 7

BQNE**BQNZ**

● $\alpha 7$
○ $\alpha 3\beta 4$

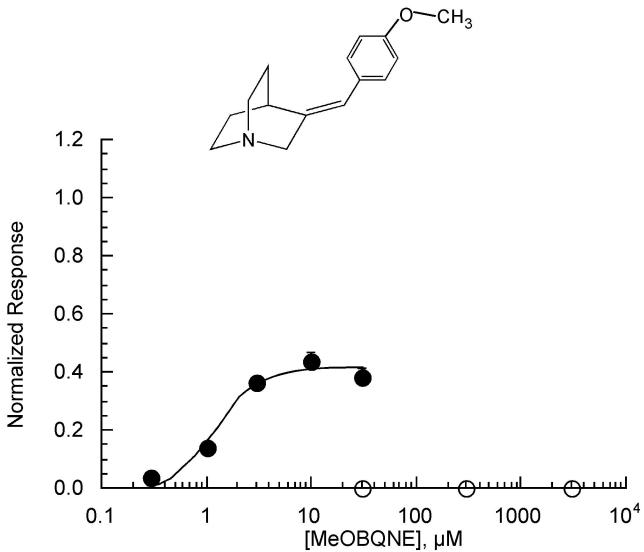
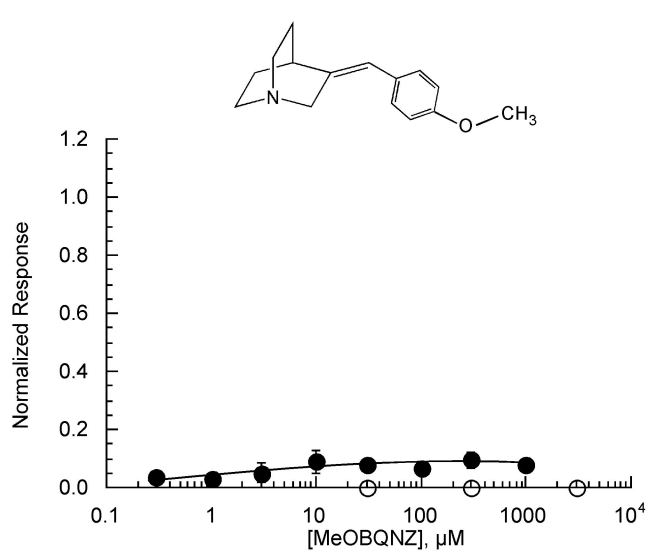
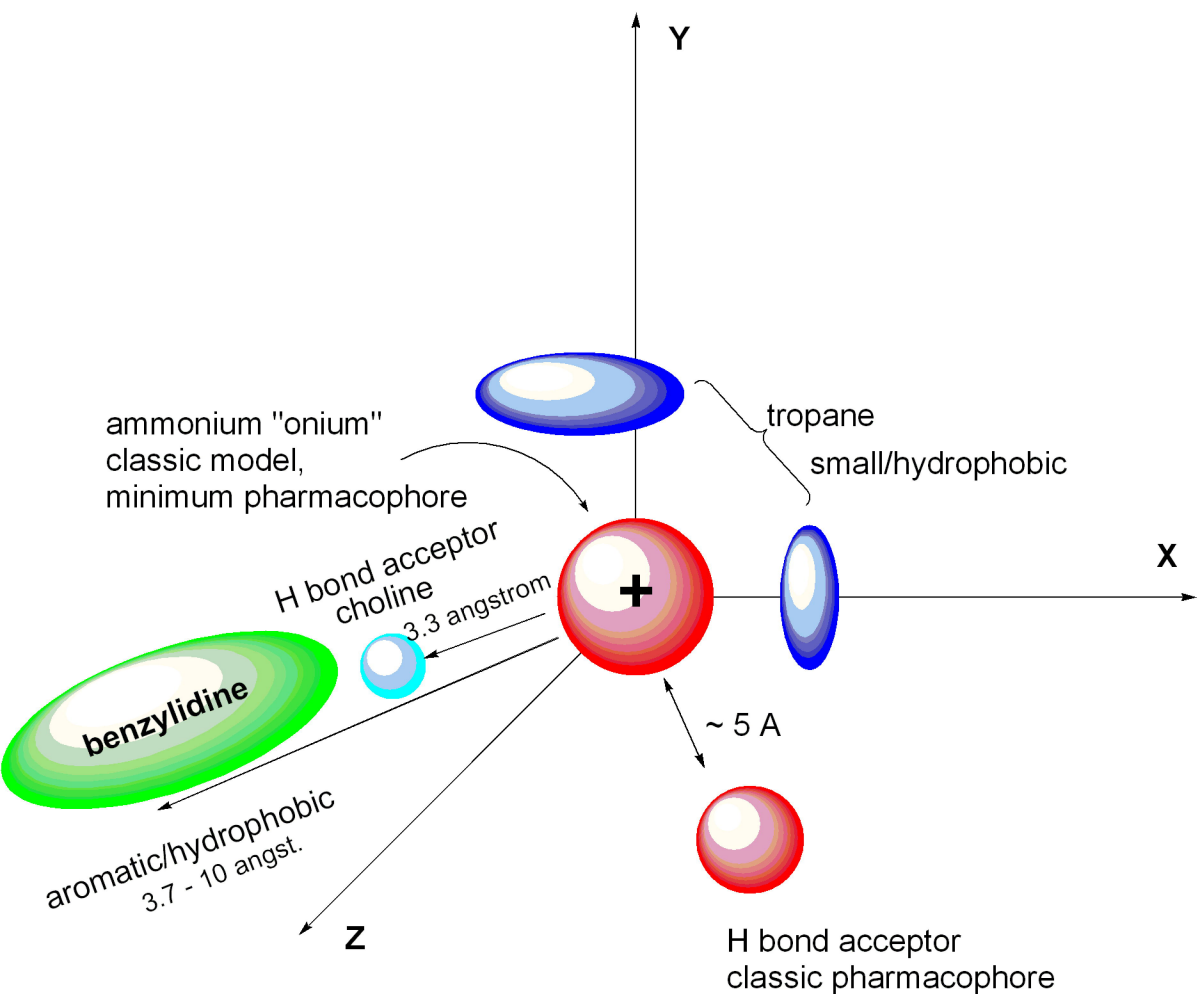
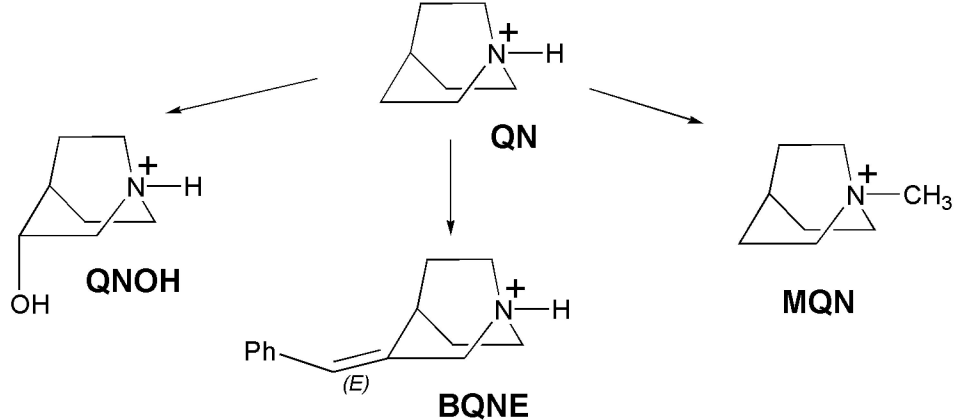
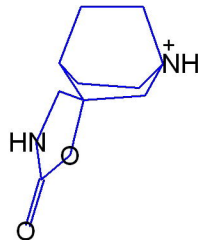
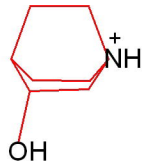
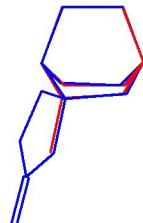
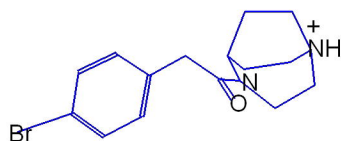
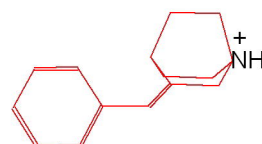
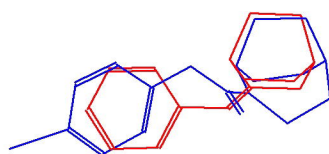
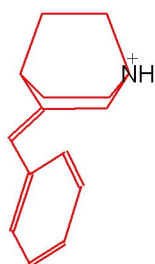
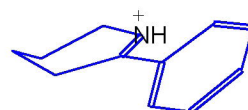
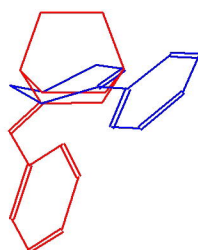
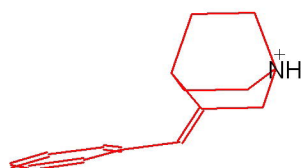
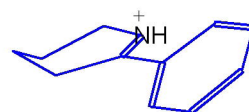
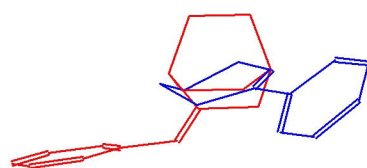
MeOBQNE**MeOBQNZ**

Figure 8



A**AR-R17779****QN-OH****B****SSR180711****BQNE****C****BQNZ****AB****D****BQNE****AB**