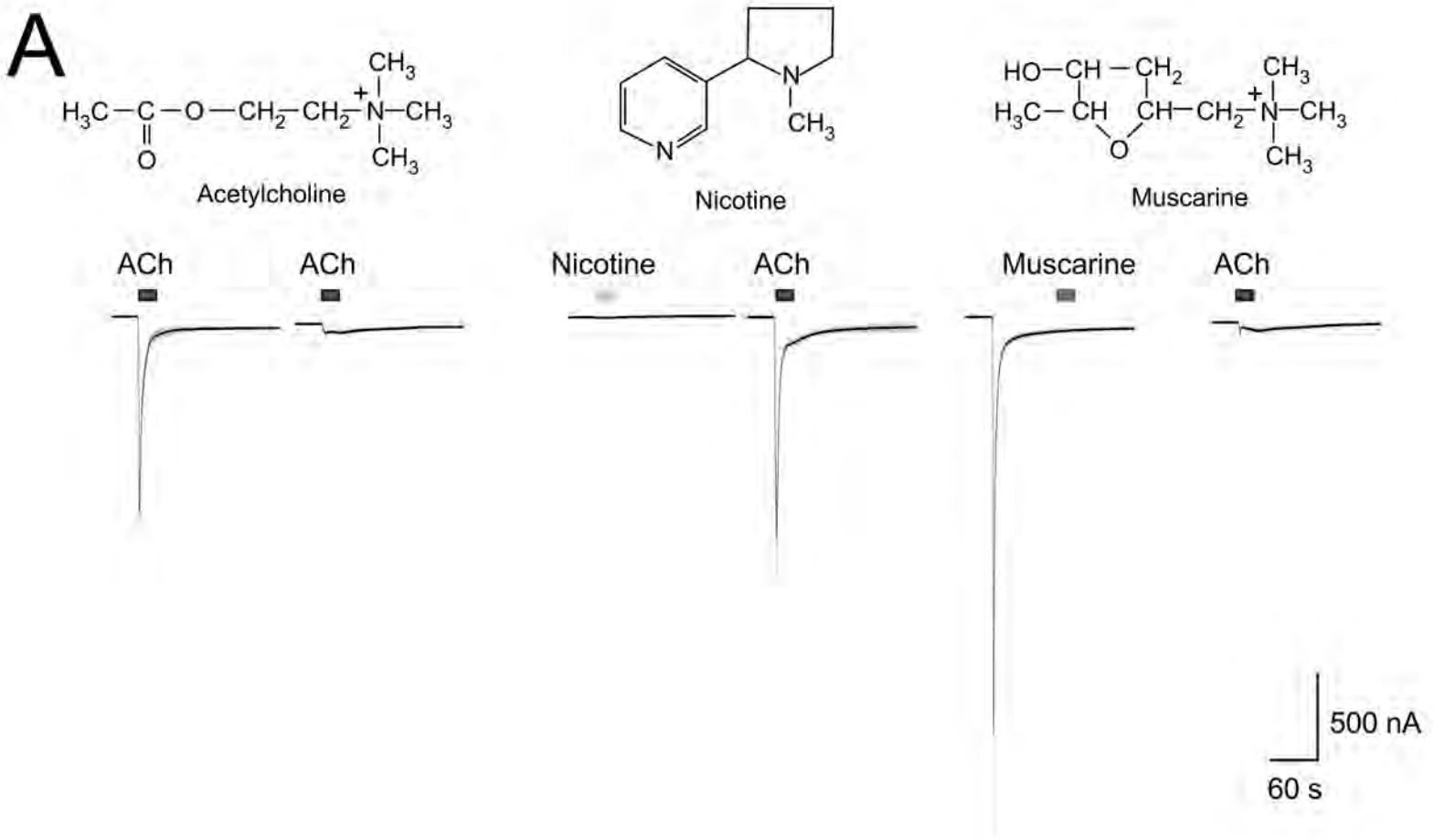

A
Acetylcholine
Nicotine
Muscarine
ACh
ACh
Nicotine
ACh
Muscarine
ACh
500 nA
60 s

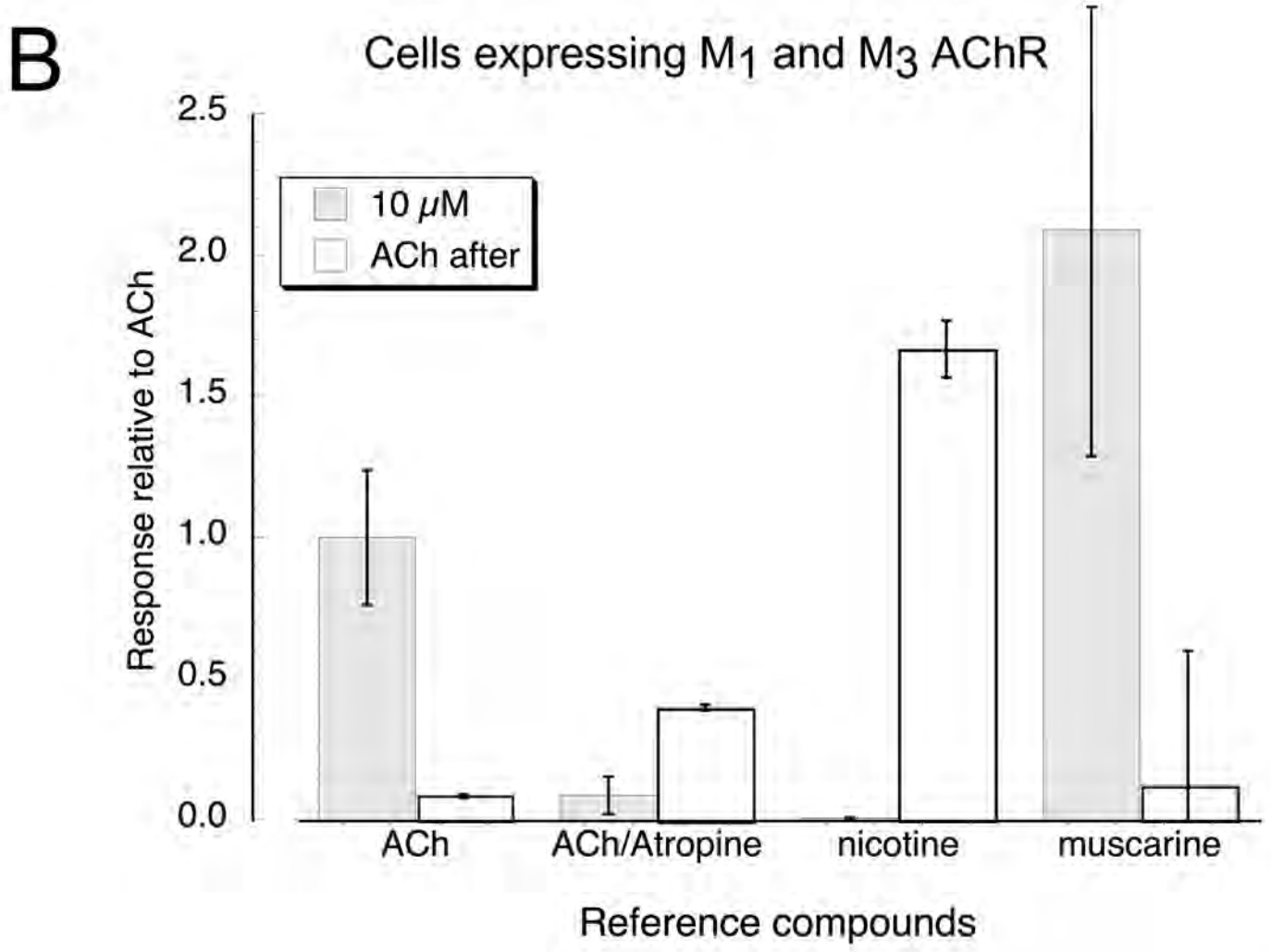
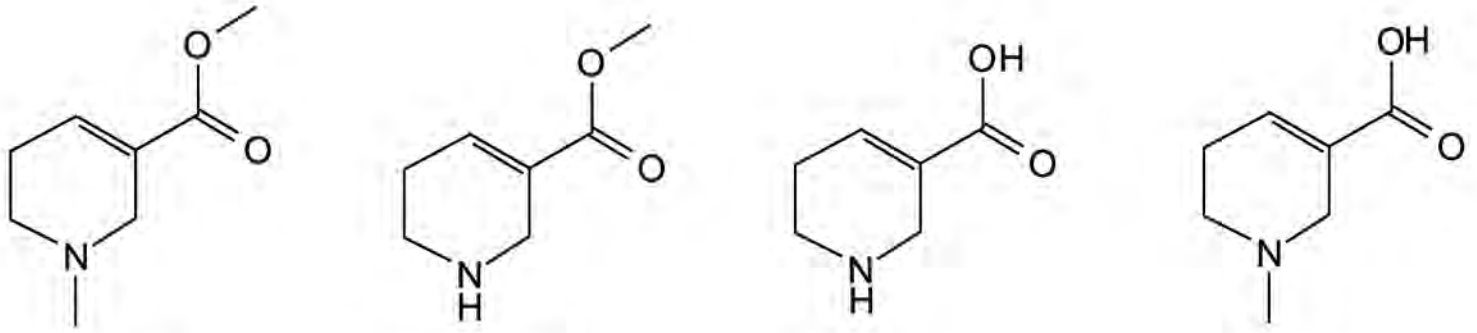

B
Cells expressing M1 and M3 AChR
10 μM
ACh after
Response relative to ACh
2.5
2.0
1.5
1.0
0.5
0.0
ACh
ACh/Atropine
nicotine
muscarine
Reference compounds

Figure S1


Arecoline
Guvacoline
Guvacine
Arecaidine

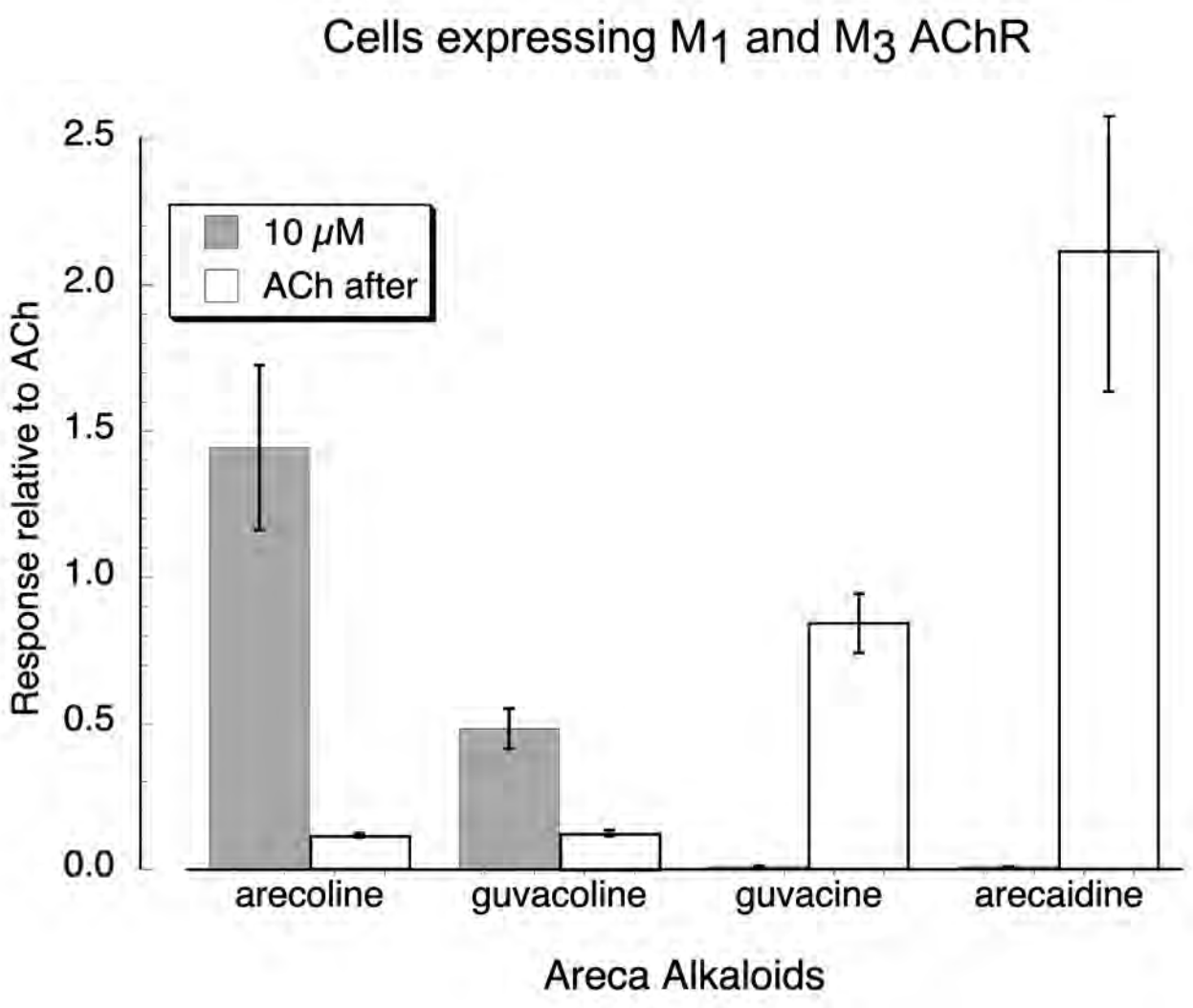
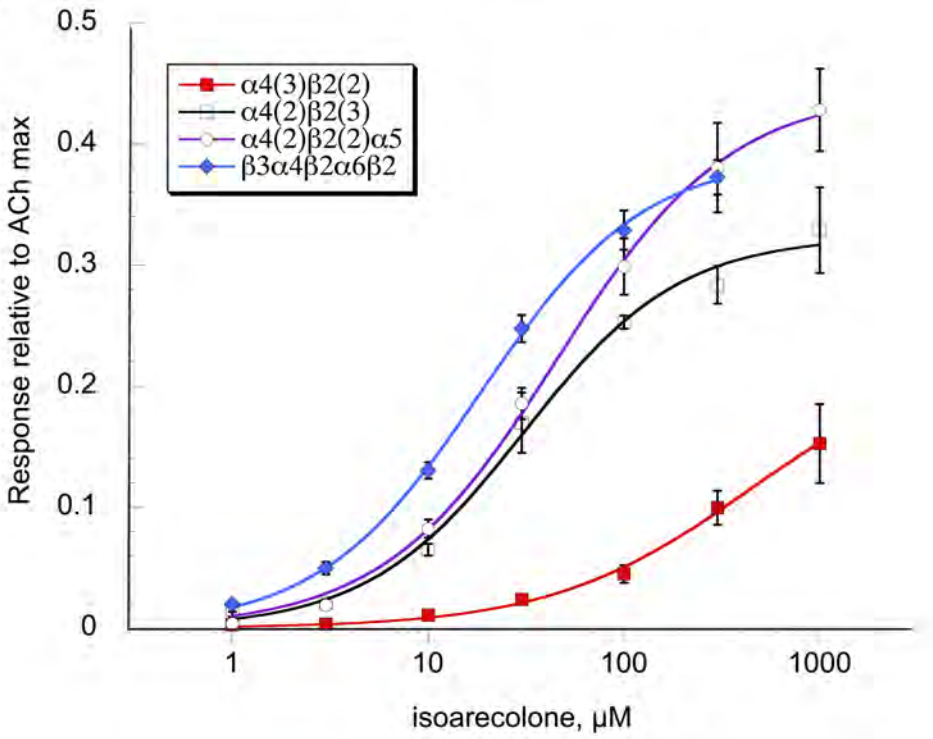

Cells expressing M1 and M3 AChR
10 μM
ACh after
Response relative to ACh
2.5
2.0
1.5
1.0
0.5
0.0
arecoline
guvacoline
guvacine
arecaidine
Areca Alkaloids

Figure S2

A


Isoarecolone Responses
relative to ACh maximum
α4(3)β2(2)
α4(2)β2(3)
α4(2)β2(2)α5
β3α4β2α6β2
Response relative to ACh max
0.5
0.4
0.3
0.2
0.1
0
1
10
100
1000
isoarecolone, μM

B

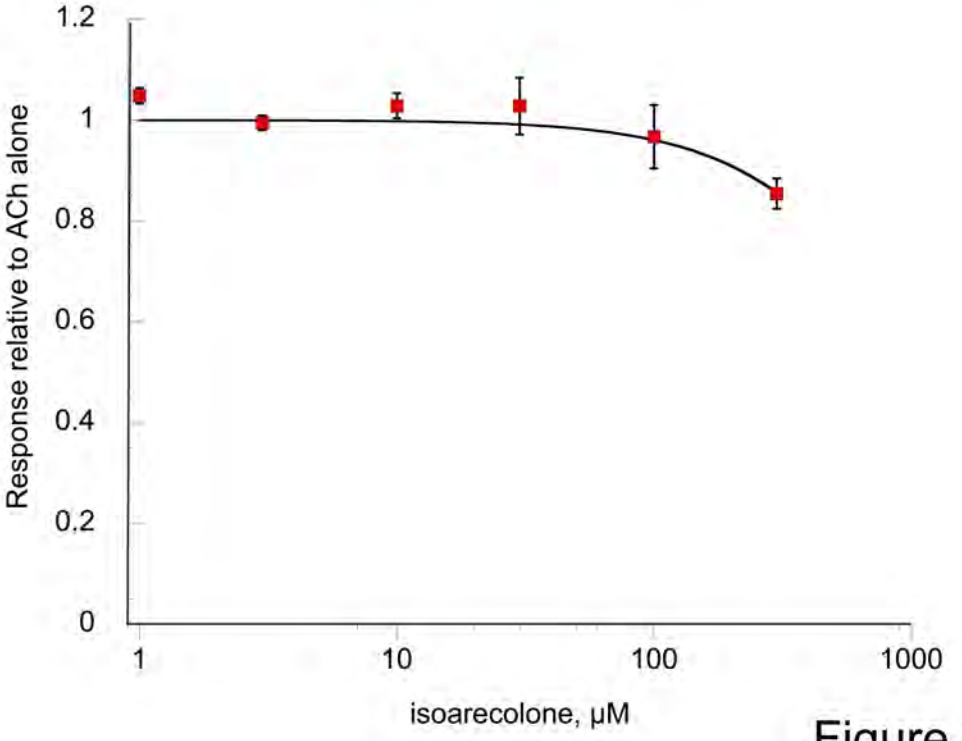

Inhibition of α7 responses
Response relative to ACh alone
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
isoarecolone, μM

Figure S3

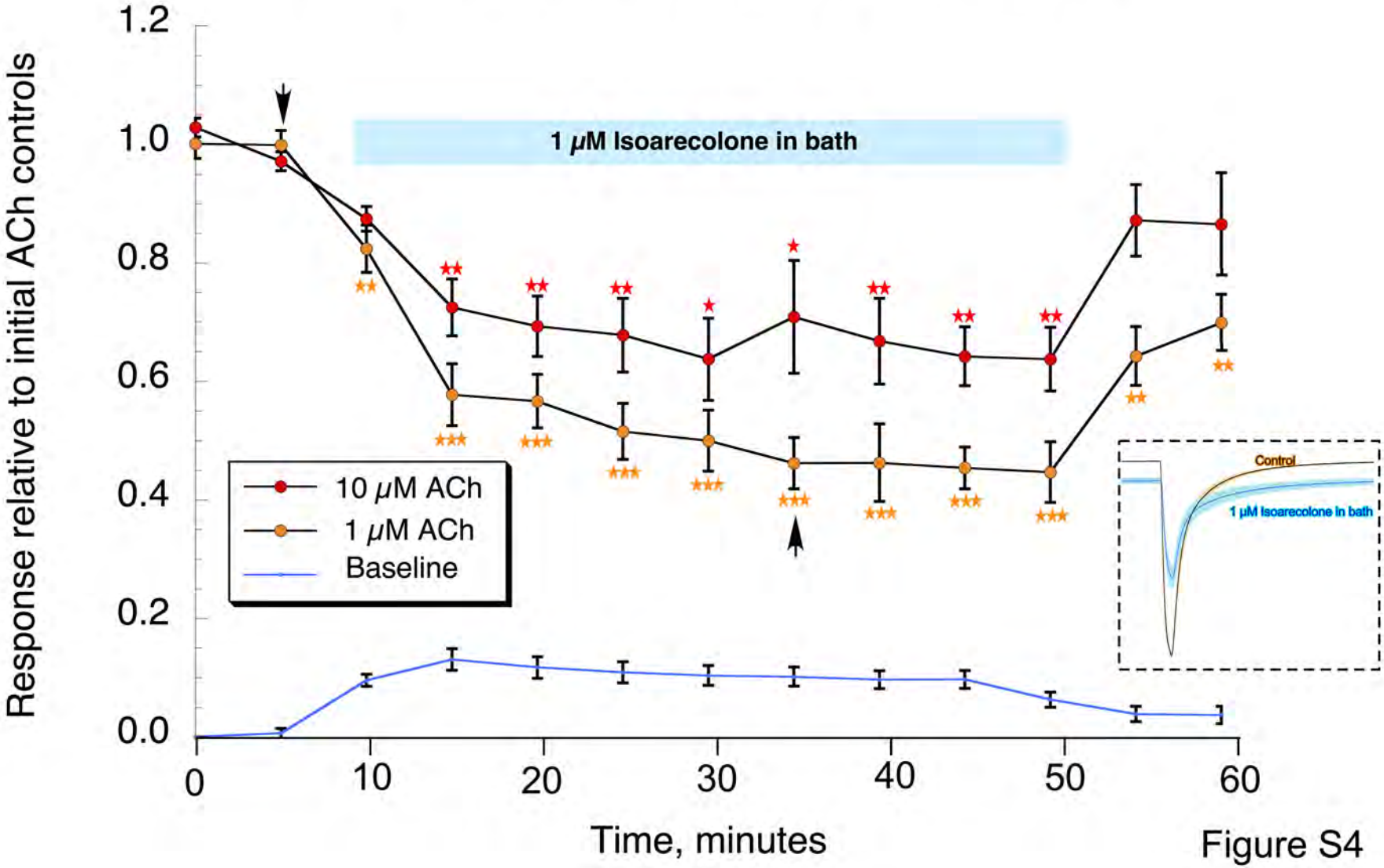
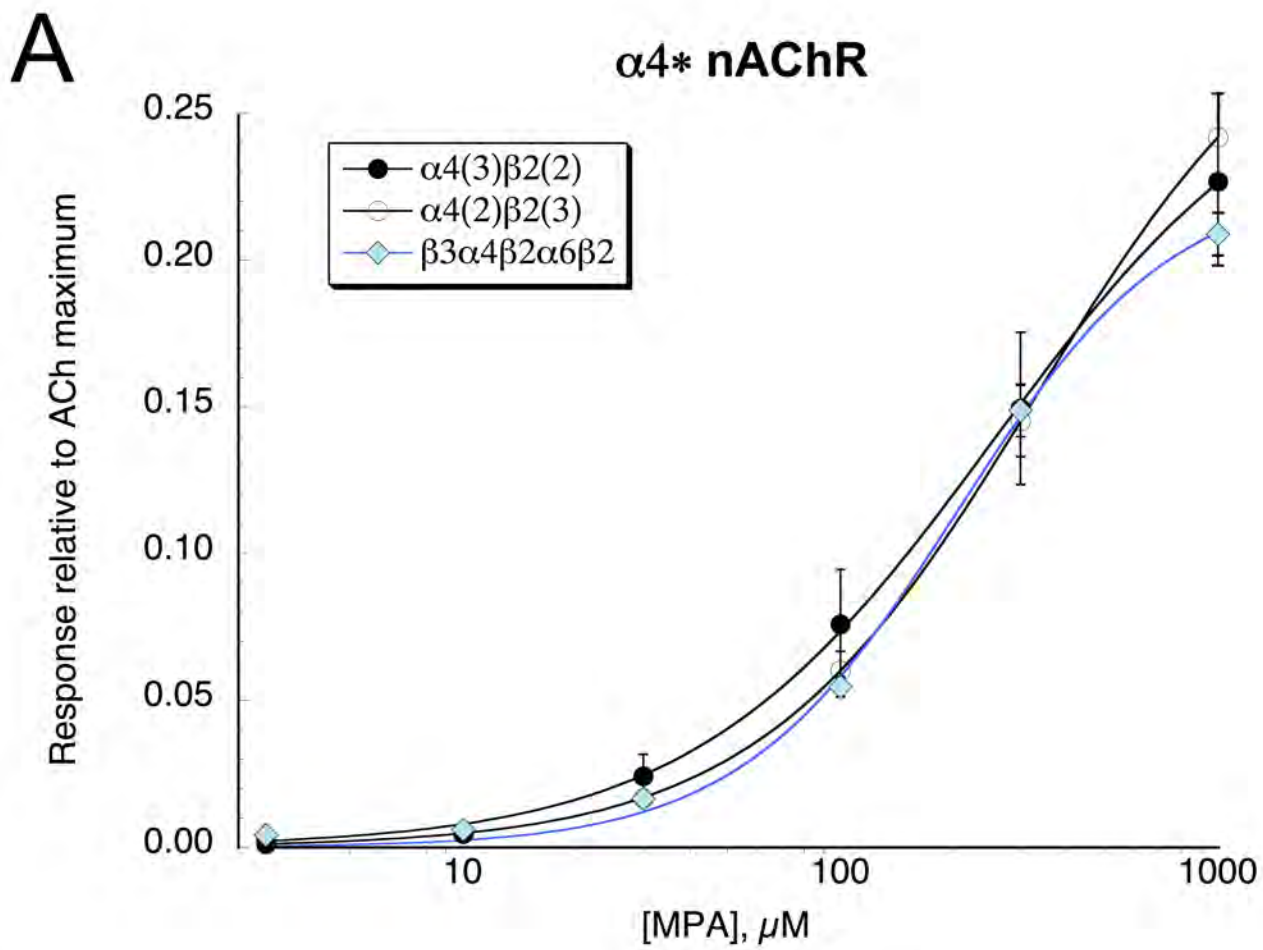

Effect of 1 μM Isoarecolone on ACh-evoked responses and steady-state current of α4β2 nAChR
Response relative to initial ACh controls
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 μM Isoarecolone in bath
10 μM ACh
1 μM ACh
Baseline
Control
1 μM Isoarecolone in bath
0
10
20
30
40
50
60
Time, minutes

Figure S4


A
α4* nAChR
α4(3)β2(2)
α4(2)β2(3)
β3α4β2α6β2
Response relative to ACh maximum
0.25
0.20
0.15
0.10
0.05
0.00
10
100
1000
[MPA], μM

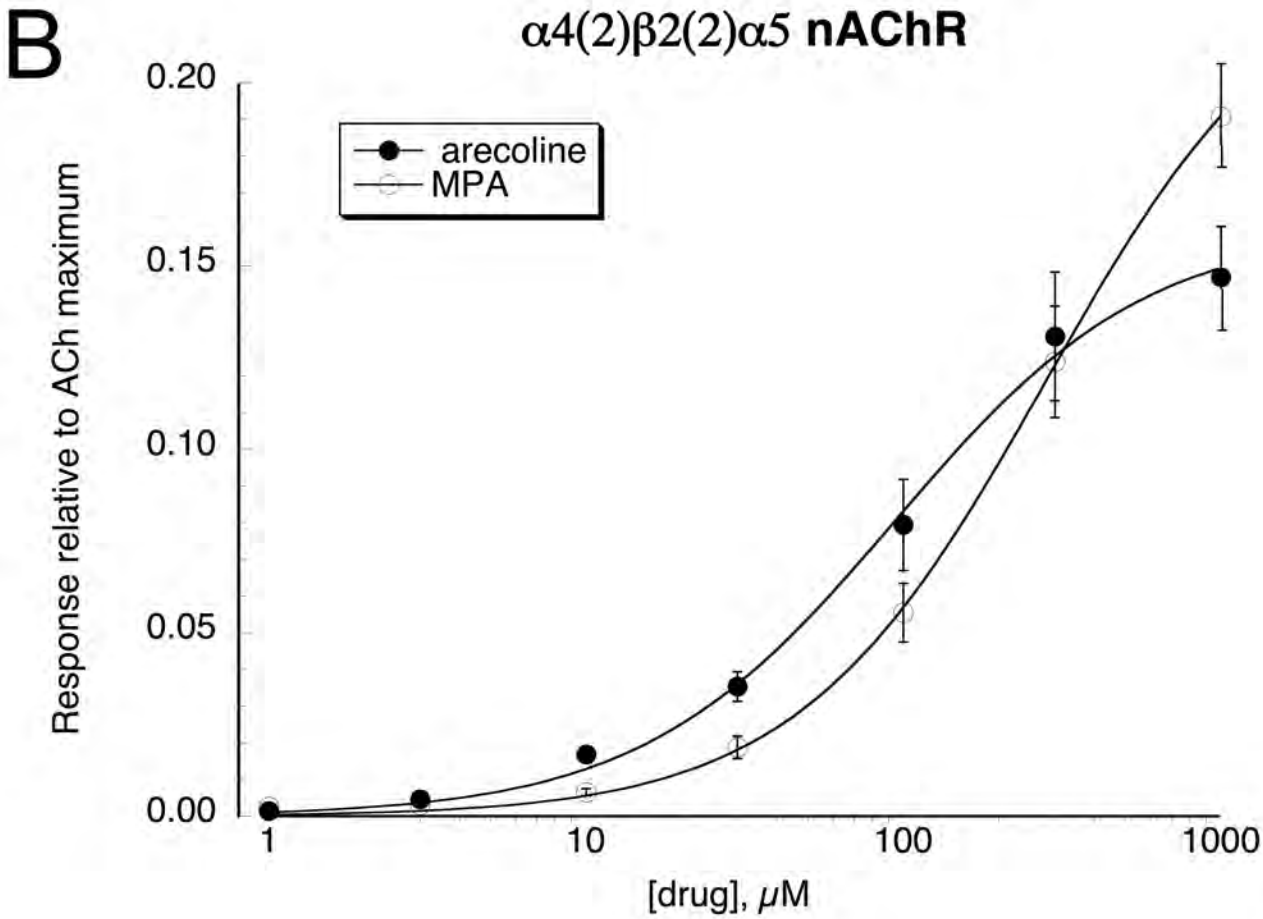

B
α4(2)β2(2)α5 nAChR
arecoline
MPA
Response relative to ACh maximum
0.20
0.15
0.10
0.05
0.00
1
10
100
1000
[drug], μM

Figure S5

Table 1

Partial agonist I_{max} values relative to ACh maximum

Receptor	arecoline*	isoarecolone	MPA
α4(3)β2(2)	0.036 ± 0.003	0.23 ± 0.03	0.28 ± 0.01
α4(2)β2(3)	0.054 ± 0.004	0.32 ± 0.01	0.31 ± 0.01
α4(2)β2(2)α5	0.16 ± 0.01	0.44 ± 0.01	0.23 ± 0.01
α6β2β3α4β2	0.056 ± 0.003	0.45 ± 0.05	0.23 ± 0.01

EC_{50} values (μM)

Receptor	arecoline*	isoarecolone	MPA
α4(3)β2(2)	75 ± 7	440 ± 170	253 ± 24
α4(2)β2(3)	14 ± 3	31 ± 5	327 ± 7
α4(2)β2(2)α5	94 ± 12	45 ± 4	280 ± 20
α6β2β3α4β2	21 ± 4	27 ± 1	204 ± 18

*Data on subtypes other than α4(2)β2(2)α5 are taken from [Papke, 2015 #5369]

Table 2

Responses of nAChR subtypes to 100 μM DMPA relative to ACh maximum response

α4(3)β2(2)	0.47 ± 0.12
α4(2)β2(3)	0.46 ± 0.05
α4(2)β2(2)α5	0.39 ± 0.02
α6β2β3α4β2	0.46 ± 0.01
α3β4	0.01 ± 0.00
α1β1εδ	0.40 ± 0.03
α7	0.41 ± 0.02
α7*	20.83 ± 5.29

* Net charge response to 100 μM DMPA plus 10 μM PNU-120596 relative to ACh alone

Legends

Figure S1: Characterization of reference compounds for muscarinic receptors. M_1 and M_3 AChR were expressed in *Xenopus* oocytes. These subtypes are coupled to G_q and activate calcium-dependent chloride currents. **A)** The structures of acetylcholine and the prototypical agonists used to discriminate nicotinic and muscarinic AChR are shown at the top. A two-application protocol was used in order to characterize these ligands. ACh or the test compounds were applied at 10 μM, and then after a three-minute wash period an additional 10 μM ACh application was made to determine whether the initial application was able to desensitize the receptor/channel system and decrease or eliminate further responses. Shown are the averaged raw data (see Methods) for the cells stimulated with either 10 μM ACh (n =5), 10 μM nicotine (n = 6), or 10 μM muscarine (n = 7) prior to the application of 10 μM ACh. **B)** Summary data for the experiments shown in A. For the first set of bars, 10 μM ACh was used for both applications, and the average of first ACh response was used to normalize the data for all the other experiments. Additionally, the second set of bars show the effect of 100 μM atropine co-application with the first 10 μM ACh application. Data are the averaged peak current response (± SEM) of at least five oocytes.

Figure S2: Characterization of *Areca* alkaloids on M_1 and M_3 AChR expressed in *Xenopus* oocytes. The structures of alkaloids known to be present in *Areca* nuts are shown at the top. A two-application protocol as described for Figure 1 was used to characterize these ligands. Test compounds were applied at 10 μM, and then after a three-minute wash period 10 μM ACh was applied to determine whether the initial application was able to desensitize the receptor/channel system and decrease or eliminate further responses. All experiments were conducted on the same day and with the same set of injected oocytes. Data shown are the averaged response (± SEM) of at least five oocytes, normalized to the average of the first ACh response for the control cells shown in Figure S1.

Figure S3: Nicotinic AChR concentration-response studies with isoarecolone. **A)** Receptors with defined subunit composition were formed by the expression of the α4β2α6β2β3 concatamer [36] or the α4β2 dimer co-expressed with monomeric α4, β2, or α5 [35] to yield the subtypes indicated. After obtaining initial ACh control responses, sets of oocytes were stimulated with alternating applications of isoarecolone at increasing concentrations and repeats of the ACh control to confirm the stability of the responses. Shown are the average peak-current

responses of five or more cells (± SEM) normalized to the preceding ACh control responses and subsequently adjusted for the ratio of the ACh controls and the ACh maximum responses determined in other experiments [25, 43]. B) Inhibition of α7 ACh-evoked responses by co-applications of isoarecolone. After obtaining initial control responses to the application of 60 μM ACh, sets of oocytes were stimulated with alternating co-applications of 60 μM ACh and isoarecolone at increasing concentrations and repeats of the ACh control to confirm the stability of the responses. Responses were calculated as the net charge [37] of the co-application response divided by the net charge of the previous ACh control. A small but significant inhibition ($p < 0.01$) was detected only after 300 μM isoarecolone, the highest concentration tested.

Figure S4: Effect of isoarecolone bath activation on the phasic and tonic activation α4β2 receptors. RNA coding for α4 and β2 nAChR subunits was injected into *Xenopus* oocytes at a 1:1 ratio. Initial control responses to either 10 or 1 μM ACh were obtained (measured as peak current) and then the bath solution was switched to one containing 1 μM isoarecolone. Further ACh applications were made in solutions that also contained 1 μM isoarecolone. From the second ACh application on, the bath application of isoarecolone significantly reduced the initial control ACh-evoked responses obtained from the same cells as determined by pairwise t-tests (single stars represent $p < 0.05$, two stars represent $p < 0.01$ and three stars represent $p < 0.001$). Additionally there was a shift in the baseline holding current in the presence of 1 μM isoarecolone, indicative of steady-state receptor activation. Shown are the baseline data for the 1 μM experiment; similar data were obtained in the 10 μM experiment. All points are the averages of six oocytes (± SEM). The insert shows the averaged raw data for the two points indicated by the arrows.

Chemicals and reagents

Areca alkaloids were purchased from Toronto Research Chemicals, (Toronto, Canada). PNU-120596 was synthesized as previously described [31, 32]. Isoarecolone was provided by the NIDA drug supply program. The arecoline analogs, 1-(4-methylpiperazin-1-yl) ethanone (MPA), 4-acetyl-1,1-dimethylpiperazin-1-ium (DMPA), and 1-(4-ethylpiperazin-1-yl) ethanone (EPA) were synthesized as described in the supplemental materials. Other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Concentrated stock solutions of other test drugs were prepared in dimethyl sulfoxide (DMSO) and stored at -20 ºC. Working solutions were prepared freshly at the desired concentration from the stored stock.

*Synthetic methods and compound syntheses***.**

Reagents for chemical synthesis were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO). Melting points (uncorrected) were obtained on an MFB-595010M Gallenkamp apparatus equipped with a digital thermometer. NMR spectra (^{1}H and ^{13}C) were recorded on a Varian Mercury-300 (300 and 75.0 MHz, respectively) instrument using $CDCl_3$, CD_3OD, $(CD_3)_2SO$ or D_2O as solvent. Chemical shifts (δ scale) are reported in parts per million (ppm) relative to the peak of the internal standard TMS (δ = 0.00 ppm) for $CDCl_3$ or relative to the central peak of the solvent (δ = 3.31 ppm for CD_3OD, 4.79 for D_2O) in ^{1}H NMR and relative to the central peak of the solvent (δ = 77.16 ppm for $CDCl_3$ and 39.52 for (CD3)2SO (DMSO-d_6)) in ^{13}C NMR. Processing of the spectra was performed with MestReNova 6.0.2. Column chromatography was performed with silica gel (Sigma-Aldrich, 230-400 mesh) or neutral alumina. Reactions were monitored by TLC using 0.25 mm silica gel F-254 glass plates (EMD Millipore or neutral alumina TLC plates). All reagents were of reagent quality or were purified before use. Organic solvents were of analytical grade or were purified by standard procedures. Compound purity was more than 95% as determined by ^{1}H NMR analyses.

Synthesis of MPA, 1-(4-methylpiperazin-1-yl)ethan-1-one

Acetic anhydride (3.75 mL, 39.67 mmol, 1.1 equiv) was slowly added to a solution of 1-methylpiperazine (4 mL, 36.06 mmol, 1 equiv) in CH_2Cl_2 (100 mL) at 0 °C. (Tsarev, 2015) The reaction mixture was stirred at room temperature for 5 minutes. 12N HCl (15 mL, 180.3 mmol, 5 equiv) was then added dropwise at 0 °C. After stirring at room temperature for 30 minutes, solvents were evaporated under reduced pressure to give MPA as a white solid (6.31 g, 35.32 mmol, yield 98 %). mp. 210-212 °C (lit. mp. 213.2-215 °C) ^{1}H NMR (300 MHz, CD_3OD) δ 4.65 (d, J = 11.4 Hz, 1H), 4.15 (d, J = 14.5 Hz, 1H), 3.65 – 3.46 (m, 3H), 3.24 – 2.99 (m, 3H), 2.93 (s, 3H), 2.15 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.7, 52.1, 51.8, 42.5, 41.9, 37.7, 21.0.

Synthesis of DMPA, 1-(4,4-dimethyl-4λ^4-piperazin-1-yl)ethan-1-one iodide

Acetic anhydride (1.87 mL, 19.83 mmol, 1.1 equiv) was slowly added to a solution of 1-methylpiperazine (2 mL, 18.03 mmol, 1 equiv) in CH_2Cl_2 (50 mL) at 0 °C. (Tsarev, 2015) The reaction mixture was stirred at room temperature for 5 minutes and then solvent was removed under reduced pressure to afford MPA (2.56 g, 18.02 mmol, yield 100 %). The crude compound was dissolved in acetone (40 mL), followed by addition of methyl iodide (5.61 mL, 90.1 mmol, 5 equiv) and the resulting mixture was stirred for 12 hours at room temperature. (Scates, 2008) The white precipitate was recovered and re-crystalized from MeOH to give DMPA iodide as bright yellowish crystals (2.10 g, 7.39 mmol, yield 41 %). mp. 274-275 °C (lit. mp. 268-272 – Scates, 2008; or 274.5-275.5 °C-Spivak, 1986). ^{1}H- NMR (300 MHz, D_2O) δ 4.03 – 3.89 (m, 4H), 3.64 – 3.56 (m, 2H), 3.56 – 3.48 (m, 2H), 3.28 (s, 6H), 2.20 (s, 3H). ^{13}C NMR (75 MHz, D_2O) δ 172.7, 60.8, 51.6, 40.5, 35.8, 20.6.

Synthesis of EPA (1-(4-ethylpiperazin-1-yl)ethan-1-one) (Romanens, 2015)

Acetic anhydride (1.64 mL, 17.33 mmol, 1.1 equiv) was slowly added to a solution of 1-ethylpiperazine (2 mL, 15.75 mmol, 1 equiv) in CH_2Cl_2 (50 mL) at 0 °C. The reaction mixture was stirred at room temperature for 5 minutes. Subsequently, 12N HCl (7 mL, 78.75 mmol, 5 equiv) was added dropwise at 0 °C. After stirring at room temperature for 30 minutes, solvents were evaporated under reduced pressure to give EPA as a white solid (3.00 g, 15.59 mmol, yield 99%) which was purified on neutral alumina, eluting with a gradient of EtOAc/MeOH (95:5 to 4:1) to provide the free base of EPA as an orange oil (1.92 g, 12.30 mmol, yield 78%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.72 – 3.62 (m, 2H), 3.57 – 3.47 (m, 2H), 2.57 – 2.38 (m, 6H), 2.09 (s, 3H),

1.12 (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.6, 52.5, 52.0, 52.0, 45.8, 40.9, 21.1, 11.5. HMRS $[M+H]^+$ calcd for $C_8H_{17}N_2O^+$ 157.13, found 157.1329.

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Heterologous expression of AChRs in Xenopus laevis oocytes

The human nAChR clones and concatamers were obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia PA). Mouse muscle clones were obtained from Jim Boulter (UCLA, Los Angeles CA) and Paul Gardener (University of Massachusetts Wooster MA). The human resistance-to-cholinesterase 3 (RIC-3) clone, obtained from Dr. Millet Treinin (Hebrew University, Israel) was co-injected with α7 to improve the level and speed of α7 receptor expression without affecting the pharmacological properties of the receptors [33]. The human mAChR clones were provided by Dr. Tony Morielli (University of Vermont, Burlington VT). Subsequent to linearization and purification of the plasmid cDNAs, cRNAs were prepared using the mMessage mMachine in vitro RNA transcription kit (Ambion, Austin, TX).

Oocytes were surgically removed from mature *Xenopus laevis* frogs (Nasco, Ft. Atkinson WI) and injected with appropriate nAChR subunit cRNAs as described previously [34]. Frogs were maintained in the Animal Care Service facility of the University of Florida, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee. In brief, the frog was first anesthetized for 15-20 min in 1.5 l frog tank water containing 1 g of 3-aminobenzoate methanesulfonate buffered with sodium bicarbonate. The harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemicals, Freehold, NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 12 mg/l tetracycline, pH 7.6) to remove the follicular layer. Stage V oocytes were subsequently isolated and injected with 50 nl of 5-20 ng nAChR subunit cRNA. [34] Oocytes were maintained in Barth's solution with calcium and recordings were carried out 2-17 days after injection.