A potentially novel nicotinic receptor in *Aplysia* neuroendocrine cells

by:

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Abstract

Nicotinic receptors form a diverse group of ligand-gated ionotropic receptors with roles in both synaptic transmission and the control of excitability. In the bag cell neurons of *Aplysia*, acetylcholine activates an ionotropic receptor, which passes inward current to produce a long-lasting afterdischarge and hormone release, leading to reproduction. While testing the agonist profile of the cholinergic response, we observed a second current that appeared to be gated only by nicotine and not acetylcholine. The peak nicotine-evoked current was markedly smaller in magnitude than the acetylcholine-induced current, cooperative (Hill value of 2.7), had an EC₅₀ near 500 μM, readily recovered from desensitization, showed Ca²⁺ permeability, and was blocked by mecamylamine, dihydro-β-erythroidine or strychnine, but not by α-conotoxin ImI, methyllycaconitine or hexamethonium. *Aplysia* transcriptome analysis followed by PCR yielded 20 full-length potential nicotinic receptor subunits. Sixteen of these were predicted to be cation-selective, and real-time PCR suggested that 15 of the 16 subunits were expressed to varying degrees in the bag cell neurons. The acetylcholine-induced current, but not the nicotine current, was reduced by double-strand RNA treatment targeted to both subunits ApAchR-C and E. Conversely, the nicotine-evoked current, but not the acetylcholine current, was lessened by targeting both subunits ApAchR-H and P. To the best of our knowledge, this is the first report suggesting that a nicotinic receptor is not gated by acetylcholine. Separate receptors may serve as a means to differentially trigger plasticity or safeguard propagation by assuring that only acetylcholine, the endogenous agonist, initiates large enough responses to trigger reproduction.
Introduction

The pentameric ligand-gated ion channel superfamily is a large group of receptors with a common structure. Vertebrate receptors of this type possess an extracellular disulfide-bonded cys-loop, and are gated by acetylcholine, glycine, gamma-aminobutyric acid (GABA), serotonin or zinc (Albuquerque et al., 2009; Thompson et al., 2010). Invertebrate receptors also have a cys-loop, and bind not only acetylcholine and GABA, but also glutamate and histamine (Norekian, 1999; Jones and Sattelle, 2007; Kehoe et al., 2009). Some prokaryotic versions are gated by H\(^+\) and GABA but lack the cys-loop (Corringer et al., 2010). The best studied of these channels are the nicotinic receptors, which despite acetylcholine being the endogenous ligand, are categorized by nicotine binding. That stated, there are examples of cholinergic ionotropic receptors being both nicotine-insensitive and acetylcholine-sensitive, such as in the nematode, *Caenorhabditis elegans* (Richmond and Jorgensen, 1999), and the mollusc, *Aplysia californica* (Kehoe and McIntosh, 1998), as well as chicken α3β2 (Hussy et al., 1994) and mammalian α9 (Elgoyhen et al., 1994; Rothlin et al., 1999) receptors in expression systems. However, there are no prior reports of nicotinic ionotropic receptors failing to respond to acetylcholine. In the present study, we provide evidence for ionotropic receptors in *Aplysia* neuroendocrine cells: one activated by nicotine alone and the other by acetylcholine alone.

The bag cell neurons are neuroendocrine cells that control reproduction in the marine snail, *Aplysia californica*. They are found in two clusters just rostral to the abdominal ganglion, and in response to acetylcholine undergo a ~30-min afterdischarge of depolarization and spiking (Kupfermann and Kandel, 1970; Kauer and Kaczmarek, 1985; Ferguson et al., 1989; White and Magoski, 2012). During the afterdischarge, hormones are released into the blood to initiate egg-laying behaviour (Arch, 1972; Chiu et al., 1979; Sigvardt et al., 1986). Aside from an example of a metabotropically-gated K\(^+\) channel (Kehoe, 1994), the vast majority of responses to acetylcholine in *Aplysia* neurons are ionotropic and inhibitory. Although in some neurons, like the small unpigmented cells of the pleural ganglion, the RB cluster of the abdominal ganglion and the bag cell neurons themselves, acetylcholine generates
While characterizing the bag cell neuron cholinergic ionotropic receptor, we found that nicotine failed to reproduce the acetylcholine response (White and Magoski, 2012). Rather, nicotine induced a second cationic response not mimicked by acetylcholine. Here we propose that these two currents are mediated by disparate receptors - based on magnitude, desensitization, antagonist profile, $\text{Ca}^{2+}$ permeability, and RNA inhibition. This may represent a unique example of two cholinergic ionotropic receptors on the same neuron, responding to different ligands. Such receptor diversity might allow for discrete activation of signaling pathways, or is an adaptation that ensures reproductive success in the face of marine venoms and toxins that act as nicotinic agonists and antagonists (Kem 1997; Dwoskin and Crooks, 2001; Schwarz et al., 2003; Bourne et al., 2010).
Materials and Methods

Animals and cell culture

Adult *Aplysia californica* (a hermaphrodite) weighing 150-500 g were obtained from Marinus Inc (Long Beach, CA, USA) and housed in an ~300-l aquarium containing continuously circulating, aerated artificial sea water (Instant Ocean; Aquarium Systems, Mentor, OH, USA) at 15°C on a 12/12 hr light/dark cycle and fed Romaine lettuce 5 times a week. For primary cultures of isolated bag cell neurons, animals were anaesthetized by an injection of isotonic MgCl₂ (~50% of body weight), the abdominal ganglion removed and treated with neutral protease (13.33 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN, USA) dissolved in tissue culture artificial sea water (tcASW) (composition in mM: 460 NaCl, 10.4 KCl, 11 CaCl₂, 55 MgCl₂, 15 N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid (HEPES), 1 mg/ml glucose, 100 U/ml penicillin and 0.1 mg/ml streptomycin, pH 7.8 with NaOH) for 18 hr at 20-22°C. The ganglion was then rinsed in tcASW for 1 hr, after which the bag cell neuron clusters were dissected from their connective tissue. Using a fire-polished Pasteur pipette and gentle trituration, neurons were dispersed in tcASW onto 35 x 10 mm polystyrene tissue culture dishes (353001; Falcon Becton-Dickinson, Franklin Lakes, NJ, USA). Cultures were maintained in a 14°C incubator and used within 1-3 d. Salts were obtained from Fisher Scientific (Ottawa, ON, Canada), ICN (Aurora, OH, USA), or Sigma-Aldrich (St. Louis, MO, USA).

Sharp-electrode, current-clamp and whole-cell, voltage-clamp recording

Current-clamp recordings were made from cultured bag cell neurons in normal artificial seawater (nASW; composition as per tcASW but lacking the glucose and antibiotics) using an AxoClamp 2B amplifier (Axon Instruments/Molecular Devices; Sunnyside, CA, USA) and the sharp-electrode, bridge-balanced method. Microelectrodes were pulled from 1.2 mm external, 0.9 mm internal diameter borosilicate glass capillaries (TW120F-4; World Precision Instruments; Sarasota, FL, USA) and had a resistance of 5-20 MΩ when filled 2 M K-acetate plus 10 mM HEPES and 100 mM KCl (pH 7.3 with KOH). Current was delivered with either Clampex software (version 8 or 10; Molecular Devices) or a
Grass S88 stimulator (Astro-Med; Longueuil, QC, Canada). Voltage was filtered at 3 KHz, using the Axoclamp Bessel filter and sampled at 2 KHz using a Digidata 1322A analogue-to-digital converter (Molecular Devices), Clampex and an IBM-compatible personal computer.

Voltage-clamp recordings were made from cultured bag cell neurons using an EPC-8 amplifier (HEKA Electronics; Mahone Bay, NS, Canada) and the tight-seal, whole-cell method. Microelectrodes were pulled from 1.5 mm external, 1.2 mm internal diameter borosilicate glass capillaries (TW150F-4; World Precision Instruments) and had a resistance of 1-2 MΩ when fire-polished and filled with regular intracellular saline (see below). Before seal formation, pipette junction potentials were nulled. After seal formation, the pipette capacitive current was cancelled and, following break through, the whole-cell capacitive current was also cancelled, while the series resistance (3-5 MΩ) was compensated to 80% and monitored throughout the experiment. Current was filtered at 1 KHz with the EPC-8 Bessel filter and sampled at 2 KHz as per current clamp. Data was gathered at room temperature (20-22°C).

Most voltage-clamp recordings were made in nASW, although in some cases Ca²⁺ was replaced with Mg²⁺ and 0.5 mM EGTA added to achieve Ca²⁺-free ASW. The recording pipette was filled with standard intracellular saline (composition in mM: 500 K-aspartate, 70 KCl, 1.25 MgCl₂, 10 HEPES, 11 glucose, 10 glutathione, 5 (EGTA), 5 adenosine 5′-triphosphate disodium salt hydrate (A3377; Sigma-Aldrich) and 0.1 guanosine 5′-triphosphate sodium salt hydrate (GTP) (G8877; Sigma-Aldrich); pH 7.3 with KOH). In some instances, the K⁺ was replaced with Cs⁺, or the GTP replaced with 10 mM guanosine 5′-[β-thio]diphosphate trilithium salt (GDP-[β]S) (G7637; Sigma-Aldrich). The free intracellular Ca²⁺ concentration was set at 300 nM by adding the appropriate amount of CaCl₂, as calculated by WebMaxC (http://www.stanford.edu/~cpatton/webmaxcS.htm). A junction potential of 15 mV was calculated for intracellular saline vs. nASW and compensated for by subtraction off-line.

**Ca²⁺ imaging**

Ca²⁺ imaging was performed under whole-cell voltage-clamp using the standard intracellular saline, but supplemented with 1 mM of the Ca²⁺-sensitive dye, fura-PE3 (Vorndran et al., 1995), and the EGTA and Ca²⁺ removed. Following break-through, neurons were dye-filled by dialyzing for at least 10
Imaging was performed using a Nikon TS100-F inverted microscope (Nikon; Mississauga, ON, Canada) equipped with Nikon Plan Fluor 20X (numerical aperture = 0.5) objective. The light source was a 75 W Xenon arc lamp and a multi-wavelength DeltaRAM V monochromatic illuminator (Photon Technology International; London, ON, Canada) coupled to the microscope with a UV-grade liquid-light guide. Excitation wavelengths were 340 and 380 nm. Between acquisition episodes, the excitation illumination was blocked by a shutter, which along with the excitation wavelength, was controlled by an IBM-compatible computer, a Photon Technology International computer interface and EasyRatioPro software (version 1.10; Photon Technology International). The emitted light passed through a 400 nm dichroic mirror and a 510/40nm emission barrier filter prior to being detected by a CoolSNAP HQ\textsuperscript{2} ICX285 charge coupled device camera (Photometrics; Tuscon, AZ, USA). The high threshold value was left at maximum and, to reduce background, the low threshold value set to 400 arbitrary units of fluorescence. From a focal plane near the middle of the neuron, fluorescence intensities were sampled using a region of interest defined over the soma at 2-sec intervals and averaged 8 frames/acquisition. The ratio of the emission following 340 and 380 nm excitation (340/380) was taken to reflect free intracellular Ca\textsuperscript{2+} and saved for subsequent analysis. Threshold level, image acquisition, frame averaging, region of interest sampling and ratio calculations were carried out using EasyRatioPro.

**Drug application and reagents**

The culture dish served as the bath, with transmitters and drugs applied using either single-cell microperfusion or pressure ejection. The perfusion system consisted of a micromanipulator-controlled square-barreled glass pipette (~500 µm bore) positioned 300-500 µm from the soma and connected by a stopcock manifold to a series of gravity-driven reservoirs. This provided a constant flow (~0.5-1 ml/min) of control extracellular saline over the neuron, which was switched to agonist-containing saline by activating the appropriate stopcock. Additional experiments involved pressure ejection of an agonist from an unpolished patch pipette (1-2 µm bore; positioned ~10 µm from the soma) for 2 sec at 75-150 KPa, using a PMI-100 pressure micro-injector (Dagan; Minneapolis, MN, USA). For antagonists, the blocker was introduced directly into the bath by pipetting a small volume of concentrated stock solution.
prior to pressure application. As previously undertaken with bag cell neurons by Fisher et al. (1993) and our laboratory (White and Magoski, 2012), perfusion was, with one exception, not employed during pressure application; however, the pipette was removed from the bath immediately after each ejection to minimize leakage and possible desensitization.

Stock solutions of drugs were made in water and frozen at -20°C, then diluted down to a working concentration in the appropriate extracellular saline as needed: acetylcholine chloride (A6625; Sigma-Aldrich), α-conotoxin ImI (3119; Tocris Bioscience, UK), dihydro-β-erythroidine hydrobromide (d-β-E) (D149; Sigma-Aldrich), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (D5891; Sigma-Aldrich), hexamethonium bromide (H2138; Sigma-Aldrich), mecamylamine hydrochloride (M9020; Sigma-Aldrich), methyllycaconitine citrate salt hydrate (M168; Sigma-Aldrich), nicotine (N0257; Sigma-Aldrich), strychnine (S0532; Sigma-Aldrich), tetramethylammonium chloride (T19526; Sigma-Aldrich).

**In silico identification of cholinergic ionotropic receptor subunits**

We initially searched for putative *Aplysia* receptor subunits in the University of California Santa Cruz Sea Hare Genome Browser (http://genome.ucsc.edu/, September 2008 Broad 2.0/aplCal1 assembly) by employing the BLAST-like alignment tool (BLAT) with published acetylcholine receptor (AchR) subunit sequences from another mollusc, *Lymnaea stagnalis* (van Nierop et al., 2006). The BLAT queries used the following published *Lymnaea* receptor subunits: LnAchR-A (GenBank™ accession number DQ167344), LnAchR-B (DQ167345), LnAchR-C (DQ167346), LnAchR-D (DQ167347), LnAchR-E (DQ167348), LnAchR-F (DQ16734), LnAchR-G (DQ167350), LnAchR-H (DQ167351), LnAchR-I (DQ167352), LnAchR-J (DQ167354), LnAchR-K (DQ167353) and LnAchR L (DQ167355). This yielded 12 predicted partial sequences of equivalent *Aplysia* receptors (ApAchR-A to L). We lengthened these ApAchR sequences with Prot2Gene (courtesy Dr. P Liang, Brock University; http://genomics.brocku.ca/Prot2gene/), which allowed for precise exon prediction and mapping with large stretches of the *Aplysia* genome and full or nearly-full LnAchR protein sequences as input. Using the deep RNA sequencing project and large transcriptome shotgun assembly of the *Lymnaea* CNS (Sadamoto et al, 2012), we also found previously unidentified putative LnAchR subunits,
namely, LnAchR-J2 (DNA Database of Japan accession number FX184869), LnAchR-J3 (FX182518), LnAchR-K2 (FX183719), LnAchR-M (FX180550), LnAchR-O (FX183843), LnAchR-P (FX222775-partial), LnAchR-Q (FX182529) and LnAchR-R (FX183247) (contigs from the *Lymnaea* transcriptome shotgun assembly are available at [http://www.ddbj.nig.ac.jp](http://www.ddbj.nig.ac.jp)). Homologues of these additional AchRs were then found in *Aplysia* by BLAST of an *Aplysia* RNA sequence assembly from the Institute for Genome Sciences at the University of Maryland ([http://aplysiagenetools.org](http://aplysiagenetools.org)). In total, we identified 15 new putative *Aplysia* full-length cation-selective subunits (ApAchR-A, C, D, E, G, H, J2, J3, L, M, N, O, P, Q and R) and 5 anionic-selective subunits (ApAchR-B, F, I, K1 and K2). A sixteenth putative cation-selective subunit, which we have designated ApAchR-J1, was previously published in GenBank™ as accession number NP_001191486 (Moroz et al., 2006).

**PCR of full-length sequences**

Abdominal ganglia were dissected from *Aplysia* and the bag cell neuron clusters removed. Ganglia were snap-frozen in liquid N₂ and homogenized in lysis solution from a Norgen Total RNA isolation kit (17200; Norgen Biotek Corp, Thorold, ON, Canada). Total RNA was then isolated and purified from these ganglia using the Norgen kit. cDNA was synthesized by reverse transcription with an iScript™ cDNA synthesis kit (170-8890; Bio-Rad Laboratories, Mississauga, ON, Canada) using a mixture of poly-A and random hexamer primers. PCR amplification of ApAchR subunits was performed with a Techne Touchgene Gradient Thermocycler (Fisher Scientific) using 1 μL of cDNA as template, 40 pmol of forward and reverse primer sets against the 16 different cation-selective ApAchRs (Table 1), iTaq™ DNA Polymerase (170-8870; Bio-Rad Laboratories) and the following program: 3 min of denaturation at 95°C, 38 cycles at 95°C for 30 sec, annealing at 68°C for 30 sec and elongation at 72°C for 90 sec. Analysis of products was carried out on 1% agarose gels in TAE buffer stained with ethidium bromide. Fragments of interest were excised from the gel, purified with an UltraClean® GelSpin® DNA extraction kit (12400; MO BIO Laboratories Inc, Carlsbad, CA, USA) and sequenced by GénomeQuébec (Montréal, QC, Canada) using an Applied Biosystems 3730xl DNA Analyzer. GenBank™ database accession numbers for the sequences are as follows: ApAchR-A (KC417388),
ApAchR-Q (KC411665) and ApAchR-R (KC411666).

Real-time PCR

RNA was isolated from either Aplysia bag cell neuron clusters or abdominal ganglia (sans the
bag cell neurons) using the Norgen Total RNA isolation kit. RNA purity was analyzed by
spectrophotometry (NanoVue; GE Healthcare Bio-Sciences, Baie d’Urfe, QC, Canada), and cDNA
synthesized by reverse transcription using the iScript™ cDNA synthesis kit and 500 ng of total RNA
with a mixture of poly-A and random hexamer primers. Each forward and reverse primer (Table 2) was
designed by Primer3 (http://frodo.wi.mit.edu) to generate 100-150 bp amplicons. Prior testing ensured
that each primer pair had a 95-99% amplification efficiency using a 10-fold dilution series. The 20 μl
final reaction mixture contained 1 μL of cDNA, 10 μL of iQ™ SYBR® Green Supermix (170-8880;
Bio-Rad Laboratories) and 0.4 μmol of each primer. For both bag cell neuron and abdominal ganglion
cDNA, the relative expression of each cation-selective ApAchR was estimated in triplicate. Similar to
Van Nierope et al. (2005, 2006) and Lymnaea receptors, ApAchR expression was calculated relative to
Aplysia glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GenBank™ accession number
KC417387) expression. However, the relative expression of multiple genes could be influenced by
differences in primer pair efficiency, and thus must be considered an estimate. Real-time PCR was
performed in low-profile 96-well clear PCR multiplates (MLL-9601; Bio-Rad Laboratories) sealed with
Microseal “B” film (MSB-1001; Bio-Rad Laboratories) using a Bio-Rad Laboratories CFX96 real-time
PCR detection system with the following conditions: 95.0°C for 3 min, then 40 cycles at 95.0°C for 15
sec and annealing/extension at 60.0°C for 40 sec. Subsequently, PCR products were heated to 95.0°C
for 15 sec and a melt curve generated by measuring fluorescence during a temperature increase from
65.0°C to 95.0°C in 0.5°C/10 sec increments. Bio-Rad CFX Manager software (version 3.0) was used
to generate the cycle threshold (Ct) values of the transcripts.
Double-stranded RNA treatment

To examine the impact of reducing the expression of individual ApAchR subunits on acetylcholine- or nicotine-induced currents, bag cell neurons were incubated in long double-stranded ribonucleic acid (dsRNA) (Fire et al., 1998; Bhargava et al., 2004). cDNA fragments encoding ApAchR-C (543 bp), ApAchR-E (496 bp), ApAchR-H (507 bp) and ApAchR-P (526 bp) were separately PCR-amplified using iTaq™ DNA polymerase and gene-specific primers (AchR-C: forward, 5’- ACAACGCACCGAGACTTTACAGAT-3’; reverse, 5’- AAAACACAGTGAGGGCGACGAGGAT -3’; AchR-E: 5’-TGAGACCAAGTGAGATGTCGCTGGGTGAT-3`; reverse, 5’-ACTGTGTGTGGCAGGTGATCTGCACT-3`; AchR-H: forward, 5’- CACCGCCTGTGACAACTCCGACTAC-3`; reverse, 5’-TAGTAGCTGTCGCTCCCAGGCAACAC-3`; AchR-P: forward, 5’- AGCAGCCTTTACACCCCTCATCACC-3`; reverse, 5’- TCCACTTCTGTAGGTGCGTCGCTGTG-3’) extended on their 5’ ends with a T7 RNA promoter sequence (TAATACGACTCACTATAGGGAGA). Using 500 ng of bag cell neuron cluster cDNA (obtained as per Materials and Methods, PCR of full-length sequences), 5 cycles of PCR were performed with melting at 95°C for 30 sec, annealing at 68°C for 30 sec and elongation at 72°C for 50 sec, followed by 30 cycles of PCR with melting at 95°C for 30 sec, annealing at 72°C for 30 sec and elongation at 72°C for 50 sec. The PCR product was agarose gel purified with an UltraClean® GelSpin® DNA extraction kit and used to synthesize sense and antisense cRNA in the same reaction mix with T7 RNA polymerase (4 h at 37°C) from a MEGAscript® RNAi kit (AM1626; Life Technologies, Burlington, ON, Canada). Reactions were treated with DNasel and RNase (both from the MEGAscript® kit) for 1 h at 37°C and column purified according to the MEGAscript® kit protocol. As a negative control, a 450 bp dsRNA was prepared directed against the 5' untranslated region of the newt (Notophthalmus viridescens) retinoic acid receptor (GenBank™ accession number AY847515) using gene-specific primers (forward, 5’- AGCATGGACGGGATGCTGCTG-3`; reverse, 5’- GTTGGGTTCCTGACTGAGGA -3`) with T7 ends. Bag cell neurons were first cultured overnight at 14°C and then bath-incubated at 14°C in 600 ng/ml dsRNA for an additional 3-4 d. This method of long
dsRNA treatment has been successfully employed to knock down gene and protein expression in *Aplysia* bag cell (Hickey et al., 2013) and sensory neurons (Lee et al., 2009), as well as *Lymnaea* motor neurons (van Kesteren et al., 2006).

**Amino acid sequence analysis and phylogenetic tree generation**

Amino acid sequences were translated from nucleotide sequences and aligned using multiple sequence comparison by log-expectation (MUSCLE) (Edgar, 2004) with the default settings in Jalview 2.8 (Waterhouse et al., 2009). Proposed signal peptide sequences were identified with SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and transmembrane sites were predicted from ApAchR-A using SMART analysis (http://smart.embl-heidelberg.de/). The alignment was used to create a tree with the neighbor joining method using Clustalx 2.1 (http://www.clustal.org/) (Saitou and Nei, 1987). Positions with gaps were excluded by bootstrap resampling up to 1000 trials and a random number generator seed of 111. Bootstrap labels were placed on nodes and saved as a Philip tree for observation using Treeview 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The tree was rooted with the *Aplysia* GABA-A receptor α subunit (GenBank™ accession number AF322878) as an out-group and compared the ApAchRs to the following human nicotinic receptor subunits: α1 (NM_001039523), α2 (NM_000742), α3 (NM_000743), α4 (NM_000744), α5 (NM_000745), α6 (NM_004198), α7 (NM_000747), α9 (NM_000748), α10 (NM_020402), β1 (NM_000747), β2 (NM_000748), β3 (NM_000749), β4 (NM_000750), δ (NM_000751), γ (NM_005199), ε (NM_000080). Identity values between human and *Aplysia* subunits, as well as the *Aplysia* acetylcholine binding protein (accession number NM_001204559) were calculated using the pairwise alignment program from Jalview.

**Analysis**

The Clampfit analysis program of pCLAMP was used to determine the amplitude of changes to membrane potential or holding current evoked by neurotransmitters and drugs under current- or voltage-clamp. After at least 1 min of baseline, two cursors were placed immediately prior to the current or voltage change, while an additional two cursors were similarly positioned at the peak response. Clampfit then calculated the average current or voltage between the paired cursors. The maximal
amplitude of the response was taken as the difference between these average baseline and peak values. Current was normalized to cell size by dividing by whole-cell capacitance (as provided by the EPC-8 slow capacitance compensation circuitry). For display only, most current and voltage traces were filtered off-line to between 20 and 80 Hz using the Clampfit Gaussian filter. The slow nature of the responses ensured this second filtering brought about no change in amplitude or kinetics. Conductance was derived using Ohm's law \((G=I/V)\) and the current change during a 200-msec step from -60 mV to -70 mV. In cases where nicotine was applied twice, the second application occurred ~10 min after the first. The peak current of the second response was then expressed as a percentage of the first response.

For intracellular Ca\(^{2+}\), EasyRatioPro files were exported as .txt files and plotted as line graphs using Prism (version 3; GraphPad Software Inc; La Jolla, CA, USA). Analysis compared the steady-state value of the baseline 340/380 ratio with the ratio from a peak or new steady-state during agonist application. Averages of the baseline and new regions were determined by eye, if an obvious peak was present, or with adjacent-averaging. Change was the difference between the new and the baseline ratio.

Relative expression levels of ApAchR subunits were taken from real-time PCR data by normalizing to the expression of ApGAPDH, as done by Van Nierope et al. (2005, 2006) for LnAchR subunits. Delta Ct was calculated as the Ct of a given ApAchR minus the Ct of ApGAPDH. This was transformed by raising the base of 2 to the negative delta Ct of each ApAchR and plotted in Prism.

Data are presented as mean ± standard error of the mean. Statistical analysis was performed using Prism. The Kolmogorov-Smirnov method was used to test data sets for normality. To test whether the mean differed between two groups, either Student's paired or unpaired \(t\)-test (for normally distributed or the Mann-Whitney \(U\)-test (for not normally distributed data) was used. For three or more means, normally distributed data were compared using a standard one-way analysis of variance (ANOVA) followed by Dunnett or the Tukey-Kramer multiple comparisons test, while not normally distributed data were compared using the Kruskal-Wallis ANOVA (KW-ANOVA) and Dunn's multiple comparisons test. Means were considered significantly different if the 2-tailed p-value was <0.05.
Results

Nicotinic agonists depolarize cultured bag cell neurons

In cultured bag cell neurons under sharp-electrode current-clamp, a 2-sec pressure application of 1 mM acetylcholine induced a response that either depolarized the cell, which then recovered towards resting potential (Fig. 1A), or activated a burst of action potentials (Fig. 1B). In 16 neurons, the average depolarization from -60 mV was ~35 mV (Fig. 1F), with six neurons firing action potentials.

Considering that a strong depolarization will provoke voltage-gated Ca^{2+} influx and potentially activate non-selective cation channels (Lupinsky and Magoski, 2006; Hung and Magoski, 2007; Gardam and Magoski, 2009; Tam et al., 2009), it was not surprising to find that three of the neurons reached a new steady-state of -53.6 ± 1.5 mV. On the other hand, a 2-sec pressure application of 3 mM nicotine stimulated the neurons much less, with an average depolarization of ~18 mV (n=15), that was significantly different from acetylcholine (Fig. 1F), and induced action potentials in only three of 15 neurons (Fig. 1D). We also assessed tetramethylammonium (TMA), which activates nicotinic receptors via the same quaternary ammonium ion motif as acetylcholine (Ascher et al., 1978; Schmitt et al., 1999).

At a level between nicotine and acetylcholine, the TMA-induced depolarization was ~24 mV (n=9) (Fig. 1E), which was significantly different from acetylcholine, but not nicotine (Fig. 1F), with spiking observed in just one of the nine neurons.

Nicotine produces a concentration-dependent inward current by opening an ionotropic receptor

We sought to obtain evidence for nicotine activating a receptor different from that of acetylcholine. Initially, cultured bag cell neurons were whole-cell voltage clamped at -60 mV in nASW using standard K^{+}-aspartate-containing intracellular solution and given repeated, 10-sec microperfusion applications of 3 mM nicotine every 10 min. Unlike the acetylcholine response, which we previously showed desensitizes with repeated doses (White and Magoski, 2012), nicotine failed to desensitize at the concentration and application-times involved. To generate a dose-response, multiple concentrations of nicotine (30 μM-10 mM) were delivered. Although 10 mM was always applied, due to a finite time of...
being able to hold a cell, not all doses were given to every neuron. Examples of responses to 100 μM, 300 μM, 1 mM and 3 mM nicotine are shown in figure 2A. When normalized to the initial maximal current at 10 mM, the resulting dose-response curve had a cooperative Hill value of 2.4, and an EC₅₀ of 543 μM (Fig. 2B). Our prior work found acetylcholine to have a non-cooperative Hill value of 0.7 and a smaller EC₅₀ of 267 μM (White and Magoski, 2012).

The current evoked by nicotine involved channel opening. Bag cell neurons were voltage clamped at -60 mV and given several 200-msec steps to -70 mV (Fig. 2C, bottom). Initially, two of these steps were delivered as control, separated by 1-2 min, followed by a third step 2 min later at the peak of the response to a 10-sec perfusion of 3 mM nicotine (concentration from the top of the curve). Using Ohm’s law, conductance was calculated from the magnitude of the current produced by the step. The change in conductance during baseline conditions was determined by comparing the first and second control step-currents. The conductance change due to nicotine came from comparing the second control step-current and the acetylcholine step-current. For display, leak was removed from both the second control step current and the acetylcholine step-current by subtracting the appropriate prior step-current. Compared to control (Fig. 2C, upper, black), there was an increase in conductance with nicotine (Fig. 2C, upper, grey) (n=11). The control change in conductance, taken 2 min prior to nicotine, revealed only a slight increase of ~3%, which was significantly different from the ~100% change in nicotine (Fig. 2E, left).

The nicotine response did not appear to involve metabotropic receptors. Cultured bag cell neurons were dialyzed under whole-cell voltage-clamp at -60 mV for 30 min with standard intracellular solution, where the normal 0.1 mM GTP was replaced with 10 mM GDP-βS, a non-hydrolysable form of GDP (Eckstein et al., 1979). Compared to parallel controls dialyzed with GTP (n=6), introduction of GDP-βS did not alter the peak current to pressure-applied 3 mM nicotine (n=5) (Fig. 2D, grey vs. black), and the average data failed to show a significant difference (Fig. 2E, right). Ourselves and others have employed GDP-βS to block G-protein-coupled receptor-dependent responses in both Aplysia and
Pharmacology of the nicotinic response

Given both a Hill value of 2.4 for nicotine vs. 0.7 for acetylcholine, and the lack of desensitization to repeated nicotine applications, it appears that nicotine may gate a different receptor than acetylcholine. To explore this further, classic nicotinic antagonists were tested on the nicotine current in cultured bag cell neurons whole-cell voltage-clamped at -60 mV. Nicotine (3 mM) was pressure-applied twice, for 2 sec, with a minimum of 10 min between deliveries. Antagonists were introduced into the bath after the first nicotine application, and the relative effectiveness of the blocker determined by the percent-remaining peak current evoked during the second nicotine application.

Under control conditions, the nicotine response did not desensitize, i.e., when no antagonist was delivered, the second application of nicotine elicited a peak current that was essentially equal (~100%) to the first current (n=34) (Fig. 3A, D). We previously established that acetylcholine readily desensitizes, and evoked a second current of just 55% of the first (White and Magoski, 2012). Several antagonist proved effective on the nicotine-induced response. The non-competitive cholinergic blocker, mecamylamine (100 μM) (Stone et al., 1956), reduced the second current to ~60% of the first (n=16) (Fig. 3B, D). This was a less robust block compared to acetylcholine, where our prior observation was that mecamylamine nearly eliminated the acetylcholine current (White and Magoski, 2012). The general competitive antagonist, dihydro-β-erythroidine (500 μM) (Folkers and Major, 1937), inhibited the second nicotine current to a level ~40% of the first (n=6) (Fig. 3B, D). However, dihydro-β-erythroidine did not block the acetylcholine response (control percent-remaining peak current = 54.7 ± 3.5%, n=26 vs. dihydro-β-erythroidine percent-remaining current = 49.7 ± 5.3%, n =7; p>0.05, unpaired Student’s t-test). Considering that strychnine blocks both the bag cell neuron afterdischarge and the acetylcholine current (Kaczmarek et al., 1978; White and Magoski, 2012), as well as other Aplysia cholinergic synapses (Kehoe, 1972), we examined its effect on the nicotine response, and observed the second current to be ~60% of the first (n=9) (Fig. 3C, D). Finally, the nicotine current was not altered...
by either the competitive antagonist, \(\alpha\)-conotoxin ImI (1 \(\mu\)M) \((n=11)\) (Paton and Zaimis, 1948) or the
non-competitive antagonist, hexamethonium (100 \(\mu\)M) \((n=6)\) (McIntosh et al., 1994), as well as
methyllycaconitine (1 \(\mu\)M) \((n=8)\), a potential selective \(\alpha_7\) receptor blocker (Ward et al., 1990; Alkondon
et al., 1992) (Fig. 3D). Our past work showed both \(\alpha\)-conotoxin ImI and hexamethonium strongly
reduce the acetylcholine-elicited current in bag cell neurons (White and Magoski, 2012).

The unpigmented small neurons from *Aplysia* right pleural ganglion present inward current in
response to both acetylcholine and the quaternary ammonium ion motif-containing agonists,
tetramethylammonium (TMA) and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Ascher et al.,
1978; Kehoe and McIntosh, 1998). When the latter two were tested on bag cell neurons, they evoked
responses that were distinct in magnitude and comparable to acetylcholine and nicotine, respectively.

Under whole-cell voltage-clamp at -60 mV, application of 10 mM TMA \((n=4)\), the most simple
quaternary agonist (Burn and Dale, 1915), produced a large current that at peak was similar in density to
that elicited by 1 mM acetylcholine \((n=13)\) (Fig. 4A, B, E). However, 10 mM DMPP \((n=9)\), a
quaternary piperazine (Chen et al., 1951), and 3 mM nicotine \((n=8)\), both of which contain an aromatic
ring, each provoked a 10-fold smaller response compared to acetylcholine or TMA (Fig. 4C, D, E).

**Ca\(^{2+}\)-dependence of the acetylcholine and nicotine responses**

Another factor that could distinguish between potentially disparate acetylcholine and nicotine
receptors is Ca\(^{2+}\) permeability. Ionotropic acetylcholine receptors vary widely in the ability to pass
Ca\(^{2+}\); for example, \(\alpha_7\) receptors conduct Ca\(^{2+}\) much more readily than other nicotinic receptors (Castro
and Albuquerque, 1995). We replaced extracellular Ca\(^{2+}\) with Mg\(^{2+}\), and observed changes in the
reversal potential of the bag cell neuron acetylcholine and nicotine responses. For these experiments,
Cs\(^+\) was substituted for K\(^+\) in the recording pipette to remove any potential confounding influence from
voltage-gated K\(^+\) currents. In initial tests, intracellular Cs\(^+\) did not alter the reversal potential of the
current brought about by pressure- or perfusion-applied 1 mM acetylcholine \((-15.9 \pm 1.4 \text{ mV for } \text{Cs}^+,
n=13 \text{ vs. } -16.1 \pm 1.9 \text{ mV for } \text{K}^+; n=10; p>0.05, \text{ unpaired Student’s } t\text{-test})\). To observe the reversal
potential of acetylcholine- or nicotine-induced currents, a 6-sec ramp from -60 to 0 mV was delivered.
during the peak of the response to a 10-sec perfusion of agonist and, to eliminate leak currents, subtracted from a prior control ramp. Figure 5A shows currents comparing the reversal potential of the acetylcholine-evoked current in nASW and Ca\textsuperscript{2+}-free conditions. For acetylcholine, the reversal potential in Ca\textsuperscript{2+}-free ASW (n=9) was not significantly different from nASW (n=13), with both being ~18 mV (Fig. 5C). However, for the nicotine-induced current there was a significant, 6 mV left-shift in the current-voltage curve from ~24 mV in nASW (n=16) to ~30 mV in the absence of Ca\textsuperscript{2+} (n=9) (Fig. 5B, C). Also, a comparison of reversal potentials in nASW also showed a significant difference between the more depolarized reversal for acetylcholine and the more hyperpolarized reversal for nicotine (Fig. 5C).

Our earlier work (White and Magoski, 2012) demonstrated that block of the acetylcholine response by α-conotoxin ImI left behind a small current at -60 mV in nASW – also shown here in Fig. 5D. To confirm that this remaining current was not mediated by the α-conotoxin ImI-insensitive nicotine response, we examined the reversal potential at the peak of the response to 1 mM acetylcholine in the presence of 1 μM α-conotoxin ImI (n=6). This remaining current presented an average reversal potential of ~17 mV, and was not significantly different from that for acetylcholine alone in nASW (Fig. 5C, E). There is also the prospect that, under control conditions, our 10-sec perfusion or 2-sec pressure applications of acetylcholine did in fact activate the nicotine receptor, but this portion of the response desensitized too quickly to be resolved. This was tested by pressure-applying 1 mM acetylcholine for 100 ms very close to bag cell neurons voltage clamped at different potentials while perfusing with a fast-flowing stream of nASW (>1 ml/min) (n=5). This resulted in a rapid-onset current that reversed at ~15 mV (Fig. 5F, G), which was is very similar to that resolved using the voltage ramp and 10 sec of acetylcholine perfusion.

The Ca\textsuperscript{2+}-sensitivity of the nicotine current reversal potential suggests that the channel(s) responsible pass Ca\textsuperscript{2+}. To bolster this, we added 1 mM of the Ca\textsuperscript{2+}-imaging dye, fura-PE3 (Vorndran et al., 1995; Kachoei et al., 2006; Geiger et al., 2009), to the intracellular solution, while excluding both EGTA and Ca\textsuperscript{2+}. After fura-loading the neurons with 10 min of whole-cell dialysis under voltage-clamp
at -60 mV, a 2-sec pressure application of 1 mM acetylcholine (n=13) induced a rapid, large inward current (Fig. 6A, lower), but no measureable increase in the 340/380 ratio (Fig. 6A, upper). In contrast, pressure application of 3 mM nicotine (n=11) evoked a much smaller current (Fig. 6B, lower), yet elevated intracellular Ca\(^{2+}\) (Fig. 6B, upper). The average density of the peak acetylcholine current (~2.5 pA/pF) was significantly different from that of nicotine (~0.1 pA/pF) (Fig. 6C); nonetheless, nicotine caused a significantly larger Ca\(^{2+}\) rise, based on an increase in the 340/380 ratio (Fig. 6D).

**Identification of potential nicotinic-type receptor subunits in Aplysia**

If nicotine and acetylcholine gate distinct receptors, one may expect *Aplysia* to have multiple ionotropic acetylcholine receptors genes. However, prior to the present study, only two nicotinic receptors have been published in GenBank\(^{TM}\): an *Aplysia* α1 subunit (accession number AF467898) and an *Aplysia* non-α subunit (AAL37250). Yet, van Nierop et al. (2006) reported 12 nicotinic receptors in the related pond snail, *Lymnaea* (LnAchR-A through L). Hence, we searched the University of California, Santa Cruz *Aplysia* genome using BLAT and Prot2Gene with LnAchR protein sequences as input. This produced predicted sequences for 12 *Aplysia* AchR subunits, which we named ApAchR-A through L, based on their similarity to the *Lymnaea* receptors (van Nierop et al., 2006). Subsequently, nine more receptors were found, and to continue the convention, designated ApAchR-J2 and J3 (similar to ApAchR-J1), K2 (similar to ApAchR-K1), as well as M, N, O, P, Q and R. With the exception of ApAchR-N, these additional subunits were first identified in a *Lymnaea* CNS transcriptome shotgun assembly as LnAchRs (Sadamoto *et al*., 2012), and then found via BLAST searches of an *Aplysia* RNA-sequence assembly transcriptome from the Institute of Genome Sciences, University of Maryland.

After achieving *in silico* sequence for all 21 subunits, 16 were identified as putative cation-selective ApAchRs based on an absence of the conserved Pro and Ala that line the pore adjacent to the second transmembrane domain of anion-selective cys-loop receptors (Galzi *et al.*, 1992; Jensen *et al.*, 2005). PCR was used to achieve overlapping full-length open reading frames for the putative cation-selective receptors: ApAchR-A, C, D, E, G, H, J1, J2, J3, L, M, N, O, P, Q and R. See Table 1 for primers against non-coding 5' and 3' untranslated regions as well as internal coding regions. ApAchR-E
is nearly identical to the published *Aplysia α1* (only a 4 amino acid difference), while ApAchR-J1 is identical to the *Aplysia* non-α receptor. Those five ApAchRs predicted to be anion-selective, i.e., ApAchR-B, F, I, K1 and K2, were not pursued further.

Comparing the *Aplysia* protein sequences with the archetypical muscle nicotinic receptor α1 subunit from *Torpedo californica* (GenBank™ accession number P02710) indicated that all putative cation-selective ApAchRs possess amino acid similarities to nicotinic receptors in the cys-loop ligand-gated channel superfamily, specifically, an extracellular N-terminal binding domain with two highly conserved Cys residues (corresponding to Cys128 and Cys142 in *Torpedo*), six agonist binding loops (loops A-F), a membrane spanning region containing the ion conduction pore, four conserved transmembrane domains (M1-4), and a variable intracellular loop between M3 and M4 (Unwin, 1998). Based on the presence of vicinal Cys192 and Cys193 (*Torpedo* numbering) (Kao et al., 1984; Sine, 2002), potential α receptors are ApAchR-A, C, D, E, G, H, L, M, N, O, P and R (aligned in Fig. 7). Conversely, a lack of vicinal Cys suggested ApAchR-J1, J2, J3 and Q are candidate β receptors. Among the α receptors, ApAchR-A, C, E, G and N contain all four highly-conserved aromatic amino acids (Tyr93, Trp149, Tyr190 Tyr198 in *Torpedo*) in loops A, B and C of the principal component of the agonist binding pocket (Arias, 2000). The binding pockets of ApAchR-D, M, P and R have a Phe substituted for Tyr at *Torpedo* equivalent Y198. Akk et al. (1999) reported that this swapping of aromatic residues in the *Torpedo* receptor does not affect binding and moderately impacts gating. However, ApAchR-H has Tyr substituted for Trp at corresponding W149 in *Torpedo*, which may well alter binding, given that this residue interacts with acetylcholine (Zhong et al., 1998; Arias, 2000). ApAchR-L is unlikely to possess full agonist-binding potential, due to a charged His substituted for Tyr in loop A at *Torpedo* equivalent Y93; similarly, loop C of ApAchR-O has a nonpolar Ser and a His at *Torpedo* equivalent Y190 and Y198, respectively, suggesting it may be an accessory subunit.

**Phylogeny and relative expression of Aplysia nicotinic receptors**

A radial tree was created using the putative cation-selective ApACHRs and the equivalent LnAchRs, with the exceptions of LnAchR-N, which we were unable to find, and LnAchR-P, the
sequence of which was too short. For this comparison, the amino termini nor the highly-variable regions between M3 and M4 were not included. The resulting hypothesis of relationship suggested that the ApAchRs appeared to have diverged from a common ancestor in three separate clades, with ApAchR-A and N arising on their own, ApAchR-C, E and J1-3 as one group, and ApAchR-D, G, H, L, M, O, P, Q and R as another group (Fig. 8A). Of course, such analysis does not provide the complete history, and can only imply what may have happened during evolution.

We next used the Aplysia GABA-A receptor as an out-group to root a second tree comparing Aplysia and human receptor subunits (Fig. 8B). This suggested many ApAchRs could have diverged from a common ancestor prior to the evolution of the human homomeric $\alpha_7$, 9 and 10, with ApAchR-H and P possibly being the oldest. The Aplysia subunits closest to human nicotinic receptors are ApAchR-E, sharing 51% and 47% sequence identity with $\alpha_2$ and $\alpha_3$, respectively, and ApAchR-C, being 42% and 43% identical to $\alpha_2$ and $\alpha_3$ (Table 3). Despite an overall highest similarity with $\alpha_2$, ApAchR-J1, J2, and J3 possess homologous amino acids in loops D and E of the complementary binding domain (Arias, 2000; Sine, 2002), and not surprisingly their next closest homologues are $\beta_2$ and $\beta_4$.

To gain an indication of which ApAchR subunits are potentially expressed in tissues of interest, real-time PCR was performed using cDNA from either bag cell neuron clusters or abdominal ganglion (sans the bag cell neuron clusters) and normalized to the Aplysia housekeeping gene, GAPDH. A similar undertaking was made by van Nierop et al. (2005, 2006) for LnAchR in different regions of the Lymnaea CNS. Interestingly, ApAchR-Q had the highest relative expression in the bag cell neurons, despite the sequence data suggesting it would not contribute to agonist binding. The next highest relative expression was the $\alpha$-like ApAchR-C, followed by ApAchR-A, D, E, G, L and M to a roughly equal degree, along with the $\beta$-like ApAchR-J1 (Fig. 8C, upper). There was limited relative expression of ApAchR-H, J2, J3, O, P and R, as well as a very small amount of ApAchR-N. For the abdominal ganglion, the relative expression of ApAchR-Q was again the greatest, with ApAch-A, C, D, E, G, H, J1, J2, L and M all being present to a lesser extent, while ApAch-J3, P and R were the least, and ApAch-N was, like the bag cell neurons, almost absent (Fig. 8C, lower). Note that during the PCR of full-length
sequences using the primers in Table 1, the band intensities of the various subunit products provided qualitatively similar results, with ApAch-N being consistently amplified in very low amounts. This stated, the apparent levels of relative expression of multiple genes could be influenced by differences in primer pair efficiency, and ultimately must be considered an estimate.

**dsRNA targeting of select AplR subunits reduces the acetylcholine or nicotine current**

We attempted to knock-down subunits contributing to the acetylcholine- or nicotine-induced currents. ApAchR-C and E were selected as candidates for the acetylcholine receptor, given that they should possess full binding potential for acetylcholine, are present in relatively high amounts in bag cell neurons, and appear most closely related to the prototypical \( \alpha 3 \) group (see Fig. 8B, C). Conversely, ApAchR-H and P were chosen as candidates for the nicotine receptor because they appear to be the oldest, are some of the least abundant subunits and lack a Trp at *Torpedo* equivalent W55 (see asterisk under loop D label in figure 7). Loss of this Trp more severely impairs gating by acetylcholine than by nicotine (Xie and Cohen, 2001; Corringer et al., 1998).

Initially, we incubated cultured bag cell neurons for 3-4 d in 600 ng/ml dsRNA directed against ApAchR-C or E. As a control, neurons were treated with 600 ng/ml dsRNA corresponding to the untranslated region of the newt retinoic receptor (which has no significant sequence similarity to any known *Aplysia* transcripts). Compared to control (n=5), exposure to ApAchR-C or E dsRNA (n=6 and 6) produced no change in the peak current density evoked by a 2-sec pressure application of 1 mM acetylcholine under whole-cell voltage clamp at -60 mV (Fig. 9C). However, including dsRNA for both ApAchR-C and E (300 ng/ml each) (n=14) did reduce the acetylcholine-elicited current by ~50% vs. control (n=14) (Fig. 9A, D). Yet, the combination of dsRNA for ApAchR-C and E did not change the current in response to pressure-applied 3 mM nicotine (n=11) relative to control (n=5) (Fig. 9E).

Conversely, treating neurons with both ApAchR-H and P dsRNA (300 ng/ml each) (n=10) attenuated the nicotine-evoked current, again by more than 50% in contrast with control (n=7) (Fig. 9B, F). While the response to acetylcholine was the same in neurons subjected to the ApAchR-H and P dsRNA cocktail (n=6) and control cells (n=10) (Fig. 9G).
Discussion

Ionotropic cholinergic receptors typically bind nicotine with high affinity and acetylcholine with low affinity, the latter thought to be necessary for high frequency input (Hurst et al., 2012). However, we find that in bag cell neurons, nicotine appears to trigger a separate receptor from acetylcholine. While there are ionotrophic receptors that are acetylcholine-sensitive and nicotine-insensitive, the reverse has not been previously reported, and we believe our data is novel in suggesting separate acetylcholine- and nicotine-activated receptors. First, the current gated by nicotine, but not acetylcholine, is blocked by the competitive antagonist, dihydro-β-erythroidine. Conversely, a different competitive antagonist, α-conotoxin ImI, as well as the non-competitive antagonist, hexamethonium, exclusively attenuate the acetylcholine-evoked current. Second, DMPP imitates the current elicited by nicotine, while the acetylcholine response is mimicked by TMA, which contains an ammonium ion motif, like acetylcholine, and may bind in a similar manner (Lape et al., 2009). Third, Ca\(^{2+}\) removal left-shifts the nicotine current reversal potential and nicotine elevates intracellular Ca\(^{2+}\), suggesting the nicotine receptor is Ca\(^{2+}\) permeable. Meanwhile, the acetylcholine current reversal potential is not altered by Ca\(^{2+}\)-free saline and acetylcholine does not change intracellular Ca\(^{2+}\), consistent with this receptor not passing Ca\(^{2+}\). Fourth, the two responses present differential sensitivity to dsRNA exposure, with the targeting of ApAchR-H and P reducing the nicotine-evoked current and ApAchR-C and E lessening the current produced by acetylcholine.

In addition to presenting different pharmacology and Ca\(^{2+}\) permeability, the nicotine current is also smaller in magnitude, more cooperative, and desensitizes less vs. acetylcholine. Thus, nicotine may activate a different receptor subtype with a different binding pocket. The highly-conserved aromatic amino acids in the principal component of the ligand-binding site are responsible for affinity, whereas the more variable complementary component mediates selectivity (Albuquerque et al., 2009). Differences in certain residues on the principle subunits could reduce affinity or agonist-induced channel activation such that the receptor is selective for one agonist over the other (Arias 2000). Alternatively, if
there are changes to key residues of the complementary subunits, it may result in the loss of selectivity for a particular agonist. A cholinergic ionotropic receptor failing to respond to nicotine is not unprecedented; for example, nicotine does not gate either the α9 or α9/10 nicotinic receptor (Elgoyhen et al., 1994, 2001). In the bag cell neurons, it is possible that nicotine evokes a smaller response because of a partial agonist effect on the acetylcholine receptor, as seen with the chicken α3β2 receptor (Hussy et al., 1994). This may occur through lower-affinity nicotine binding, failure to trigger full opening, or nicotine acting as a pore blocker (Kuryatov et al., 2000; Rush et al., 2002; Paradiso and Steinbach, 2003). For NMDA receptors, low-affinity agonists produce currents which decay more quickly and recover from desensitization faster (Lester and Jahr, 1992). However, these alternative mechanisms would likely not give rise to the differential block of the bag cell neuron nicotine and acetylcholine currents by dihydro-β-erythroidine and α-conotoxin ImI/hexamethonium, respectively. The cation-selective, but not the chloride-selective, acetylcholine receptor from Aplysia buccal and pleural neurons is also exclusively sensitive to conotoxin ImI/hexamethonium (Kehoe and McIntosh, 1998).

Differences between the magnitude and desensitization of the acetylcholine- and nicotine-gated currents do not necessarily distinguish between two receptors. For example, the single-channel conductance of human alpha 7 receptors is different when opened by acetylcholine compared to an allosteric agonist (Pałczyńska et al., 2012). Yet, conductance is not a function of the agonist for cholinergic currents from unpigmented right pleural Aplysia neurons (Ascher et al., 1978), a mouse muscle-like cell line (Papke et al., 1988), and rat alpha 4/beta 2 receptors (Akk and Auerbach, 1999). In bag cell neurons, if both acetylcholine and nicotine were acting on the same channel, one would not expect nicotine to exclusively increase intracellular Ca\(^{2+}\), or the nicotine-induced current to have a more negative reversal potential and be sensitive to Ca\(^{2+}\) removal. Similarly, it is probably not the case that the larger acetylcholine response masks a nicotine component, given that the acetylcholine-elicited current remaining after α-conotoxin ImI presented the same reversal potential as acetylcholine alone.

We also failed to uncover evidence that the nicotine receptor opens but then quickly desensitizes during acetylcholine delivery. Brief pressure application of acetylcholine under fast-flowing perfusion results
in a rapid-onset current which reverses at a voltage similar to the other acetylcholine responses, but not at a voltage like the nicotine response or a combination of the two responses. Thus, the possibility of two, distinct receptors appears plausible.

The large number of ApAchR subunits present in bag cell neurons is enigmatic, since there are only two distinct currents. The *Aplysia* transcriptome contains at least 20 acetylcholine receptors, 16 of which could be excitatory, based on the sequence of the conserved M1-M2 linker involved in ion permeability (Sine and Engel, 2006). Each subunit may have a specialized role in transmission, depending on agonist sensitivity, gating kinetics, permeability and desensitization. The seemingly high expression of ApAchR-Q is equally puzzling, considering that the lack of conserved amino acids in the binding pocket implies it likely does not bind acetylcholine. Its function could be structural, as is the case for the α5, β1 and β3 receptor subunits, which are thought to occupy the non-binding fifth position in certain cholinergic receptors (Arias, 2000). Alternatively, ApAchR-Q could have a non-synaptic role, such as receptor trafficking and assembly, like the vertebrate α5 subunit (Ramirez-Latorre et al., 1996), or represent a chemotaxic sensor, similar to channels found in *Caenorhabditis* chemosensory neurons (Yassin et al., 2001) and certain prokaryotes (Tasneem et al., 2005).

The major cholinergic receptor in vertebrate autonomic ganglion is the heteromeric, α-conotoxin ImI-sensitive α3β2/β4 (Listerud et al., 1991; Ramirez-Latorre et al., 1996; Conroy and Berg, 1995). Nicotine itself activates vertebrate α7 (Anand et al., 1993), the LnAchR-A homologue in *Lymnaea* (van Nierop et al., 2005), and the anionic-selective α7-like receptor in *Aplysia* (Kehoe and McIntosh, 1998). Block of the bag cell neuron acetylcholine-induced current by α-conotoxin ImI suggests either an α7- or α3β2-type receptor (Johnson et al., 1995; Ellison et al., 2004). However, the lack of activation by nicotine and the failure of MLA to reduce the current rules against α7. The possibility of a α3β2-type heteromeric receptor is strengthened by the homology of α2/α3 to the higher-expressing ApAchR-C and E, as well as the knockdown of the acetylcholine current by combined ApAchR-C and E dsRNA treatment. Assuming the nicotine response is mediated by a distinct receptor, the dsRNA experiments would suggest ApAchR-C or E are not involved, but rather ApAchR-H and P may contribute to the
channel gated by nicotine. Consistent with this, the absence of a key Trp in complementary loop D 
(Torpedo equivalent W55) of both ApAchR-H and P would likely impair acetylcholine binding (Xie and 
Cohen, 2001; Corringer et al., 1998). The remaining subunits either are more likely to be acetylcholine-
sensitive (ApAchR-A and G), possess an altered binding pocket (ApAchR-D, L, M, O and R), or are 
probably not present in significant enough abundance (ApAchR-N). These speculations are based on 
pharmacology and a limited application of dsRNA technology. Definitive proof would require 
heterologous expression of multiple combinations of ApAchR subunits and subsequent physiological 
characterization. Unfortunately, attempts at expressing invertebrate receptors in oocytes or cell lines has 
seen limited success. In fact, the only Lymnaea cation-selective receptor that has been expressed is 
LnAchR-A, and it is both acetylcholine- and nicotine-activated (van Nierop et al., 2005). 

If there are two, separate receptors in bag cell neurons, it begs the question: what is the 
endogenous agonist for the nicotine receptor? Like Aplysia, many other cloned invertebrate cys-loop 
receptors possess differences in binding pocket amino acids, and may respond to other transmitters 
(Tricoire-Leignel and Thany, 2010; Dent, 2006; Barbara et al., 2008). With the exception of 
acetylcholine, all classical neurotransmitters either inhibit or do not change the bag cell neuron 
membrane potential (Kaczmarek et al., 1978; Whim and Kaczmarek, 1998; White and Magoski, 2012). 
Thus, if there is an endogenous agonist for the nicotinic receptor, it may be a peptide, perhaps related to 
certain bungarotoxin-like peptides (Tsetlin, 1999), or a metabolite (Yassin et al., 2001; Grando, 2008). 
Because the putative nicotine receptor is Ca$^{2+}$-permeable, it would allow for Ca$^{2+}$ influx at voltages 
nearer to the resting membrane potential then that permitted by voltage-gated Ca$^{2+}$ channels (Tam et al., 
2009). For example, α7 receptors produce Ca$^{2+}$ transients in dendritic spines, while α3-containing 
receptors augment global Ca$^{2+}$ increases through Ca$^{2+}$-induced Ca$^{2+}$-release (Shoop et al., 2001). In 
addition, different states of receptor desensitization could confer a role in bag cell neuron plasticity, i.e., 
long after the acetylcholine response has desensitized, which presumably occurs during the 
afterdischarge, nicotine-gated channels could still be recruited to directly permit Ca$^{2+}$ entry.
An alternative and intriguing prospect is that the separate acetylcholine and nicotine receptors are adaptive. Bourne et al. (2010) suggest the role of acetylcholine binding protein in *Aplysia* may be to buffer dinoflagellate toxins, such as red tide. By extension, the overwhelming prominence of acetylcholine-sensitive receptors in the bag cell neuron cholinergic response may serve as a defence against protists or predatory worms which have co-evolved nicotine-mimetic toxins (Kem, 1997; Schwarz et al., 2003). The smaller-magnitude nicotinic response in bag cell neurons would be insufficient to permit toxin-mediated activation of reproduction. Because egg-laying behaviour lowers the defences of *Aplysia* (Mackey and Carew, 1983; Goldsmith and Byrne, 1993), this could protect the animal from predation.
Figure Legends

Figure 1  Depolarization of cultured bag cell neurons by nicotinic agonists.

A: under sharp-electrode current clamp, a 2-sec pressure application of 1 mM acetylcholine (Ach) depolarizes a cultured bag cell neuron in nASW from a resting potential of -60 mV.  B: in 6 of 16 neurons, the depolarization was sufficient to reach action potential threshold.  C: nicotine (Nic) at 3 mM also depolarizes bag cell neurons, although to a lesser extent than acetylcholine.  D: nicotine delivery resulted in action potentials in only 3 of 15 neurons.  E: the quaternary ammonium salt, tetramethylammonium chloride (TMA), also depolarizes bag cell neurons to a degree between that of acetylcholine and nicotine.  F: summary graph indicating the average depolarization for acetylcholine is significantly different from either TMA (p<0.01) or nicotine (p<0.001) using an ANOVA with Tukey-Kramer multiple comparisons test.

Figure 2  Current responses to nicotine application in cultured bag cell neurons.

A: under whole-cell voltage-clamp at -60 mV, 10-sec applications of 0.1, 0.3, 1 and 3 mM nicotine perfused close to a bag cell neuron elicits inward currents of increasing magnitude.  B: when the current is normalized to 10 mM, the fit of the dose-response curve provides a Hill value of 2.4 and an EC50 of 543 μM.  Each neuron saw the largest dose first, with some or all of the subsequent doses delivered at an interval of ~10 min.  C: leak-subtracted currents taken 1 min apart under voltage-clamp during a 10 mV hyperpolarizing step (lower).  Prior to the addition of nicotine, essentially no conductance change occurs (black); however, a subtraction current taken at the peak of the 3 mM nicotine response presents an increase in conductance (grey).  D: while voltage-clamped at -60 mV, pressure application of 3 mM nicotine induces similar inward currents in cultured bag cell neurons dialyzed for 30 min with standard intracellular solution containing either 1 mM GTP (black) or 10 mM GDP-βS (grey).  The apparent difference in the decay kinetics of the nicotine-evoked current between GTP and GDP-βS simply reflects variance in the data and is not significant (GTP τ = 105.1 ± 35.2 sec vs GDP-βS τ = 102.9 ± 71.7 sec; p>0.05 Mann-Whitney U-test).  E: left, summary data showing the percent change in conductance
between control and nicotine is significantly different (p<0.05, paired Student’s t-test). Right, summary data indicate no significant difference between the peak current density induced by nicotine as a result of replacing GTP with GDP-βS (p>0.05, Mann-Whitney U-test).

**Figure 3 Antagonist profile for the nicotine-induced current.**

*Figure 4 Quaternary ammonium-based agonists exhibit differential potency.*

Examples of currents from cultured bag cell neurons induced by 2-sec pressure applications of different nicotinic agonists at a held at -60 mV under whole-cell voltage-clamp. *A*: 1 mM acetylcholine, *B*: 10 mM TMA, *C*: 3 mM nicotine and *D*: 10 mM DMPP. *E*: summary data indicating that the average peak current density induced by TMA is similar to that provoked by acetylcholine. Conversely, DMPP results in a far smaller current density which is near to the response elicited by nicotine.

**Figure 5 The reversal potential of the nicotine- but not the acetylcholine-induced current is sensitive to extracellular Ca$$^{2+}$$.*

*A*: leak-subtracted currents from separate cultured bag cell neurons under whole-cell voltage clamp with Cs$$^{+}$$-based intracellular saline. There is no difference in the reversal potential in nASW (*black line*) vs. Ca$$^{2+}$$-free medium (*grey*) of currents elicited by a 6-sec ramp from -60 to 0 mV at the peak of the
response to microperfused 1 mM acetylcholine. B: when the same ramp is applied at the peak of the 3 mM nicotine response, a left-shift of the reversal potential is observed in Ca^{2+}-free external compared to nASW. C: summary graph indicating no significant difference between the reversal potential of the acetylcholine-induced current in nASW alone vs. Ca^{2+}-free seawater vs. the current remaining in nASW after block by 1 μM α-conotoxin ImI (ImI) (p>0.05, KW-ANOVA, Dunn’s multiple comparisons test). However, there is a significant change in the reversal potential of the nicotine current in Ca^{2+}-free seawater vs. nASW (p<0.05, Mann Whitney U test). The reversal potentials for nicotine and acetylcholine in nASW are also significantly different (p<0.01, unpaired Student’s t-test). D: whole-cell voltage-clamp recordings in nASW from the same neuron held at -60 mV show that the current produced by a 2-sec pressure application of 1 mM acetylcholine is diminished with the subsequent introduction of 1 μM α-conotoxin ImI. E: a subtraction current evoked in nASW by a ramp from -60 to 0 mV at the peak of the residual response to 1 mM acetylcholine in the presence of 1 μM α-conotoxin ImI. F: pressure application of 1 mM acetylcholine for 100 ms in close proximity to the soma of a cultured bag cell neuron in a stream of nASW. A fast, inward current is evoked by the acetylcholine, which decreases in magnitude as the steady-state holding potential is changed from -60 mV through to -10 mV. G: the mean peak current density of the fast acetylcholine current at different voltages provides a reversal potential of ~-15 mV.

**Figure 6** Nicotine, but not acetylcholine, induces an increase in intracellular Ca^{2+}.

Simultaneous measurement of free intracellular Ca^{2+}, using fura-PE3 fluorescence, and agonist-induced current, under whole-cell voltage-clamp at -60 mV, in cultured bag cell neurons. A: following a 2-sec pressure application of 1 mM acetylcholine, that induces a large rapid current (bottom), no observable change is apparent in the intensity of the 340/380 fluorescent ratio (top). B: conversely, application of 3 mM nicotine elicited a relatively small inward current and a simultaneous rise in intracellular Ca^{2+}. C: summary data indicating a significantly smaller current density produced by nicotine compared to acetylcholine (p<0.0001, Mann-Whitney U-test). D: summary data showing an average increase in the
340/380 fluorescence ratio due to the nicotine-evoked current that is significantly different from acetylcholine (p<0.05, Mann-Whitney U-test).

**Figure 7** Protein sequence alignment for putative cation selective nAchR subunits of *Aplysia*.

MUSCLE-derived multiple sequence alignment of putative cation-selective *Aplysia* acetylcholine receptor subunits. Amino acids conserved in all ApAchRs are highlighted in dark purple, those moderately conserved in light purple, and residues in the minority are in grey. The N-terminal signal peptide for each subunit is shown in lower case. Based on analysis of ApAchR-A, the cys-loop, loops A-C of the principle binding component, loops D and E of the complementary binding component and the four transmembrane domains (M1-4) are labeled. The aromatic amino acids involved with ligand binding are numbered as per the sequence of the α1 nicotinic receptor from *Torpedo* and surrounded by boxes for clarity, including the conserved region required for cation permeability (CAT). Residues that contribute to loops D and E of the complementary binding domain are indicated by an asterisk.

**Figure 8** Phylogenetic tree and real-time PCR of *Aplysia* nicotinic receptor subunits.

A: radial tree of putative cation-selective acetylcholine receptors from *Aplysia* and *Lymnaea* using the neighbour-joining clustering algorithm, excluding the amino termini and the regions between M3 and M4. Bootstrap numbers placed on nodes are based on 1000 replicates (asterisk = 1000 iterations), with the scale bar representing substitutions per site. From a common node of origin (black dot in centre), the hypothesis of relationship implies that the receptors split into one (ApAchR-C, E, J1-3), two (ApAchR-D, G, H, L, M, O, P, Q, R) and three (ApAchR-A and N) distinct clades. B: tree of the ApAchRs and human nicotinic receptors rooted with the *Aplysia* GABA-A receptor as an out-group and bootstrap values. The majority of ApAchRs appear to have emerged before α7, 9 and 10. While ApAchR-J1, J2 and J3 could have a shared origin with most of the β receptors and other accessory subunits. Finally, there may be a mutual origin for ApAchR-C and E with the remaining α receptors and *Torpedo* α1. C: relative expression levels of ApAchRs in bag cell neuron cluster (upper) and abdominal ganglion (lower). Summary data for real-time PCR of ApAchR expression levels relative to the housekeeping
gene, ApGAPDH, with cDNA template derived from three separate pairs of bag cell neuron clusters or
three separate abdominal ganglia (sans the bag cell neurons). ApAchR-Q appears to have the highest
expression in both the bag cell neurons and the abdominal ganglion, with ApAchR-A, C, D, E, G, J1, L
and M likely at similar levels. The bag cell neurons seem to have less expression of ApAchR-H, J2, J3,
O, P and R, which is also the case for the abdominal ganglion, with the exception of slightly greater

Figure 9 dsRNA targeting of the acetylcholine and nicotine currents.

A: left, following incubation in 600 ng/ml dsRNA corresponding to the 5' untranslated region of the
newt retinoic acid receptor (acting as a control), a 2-sec pressure application of 1 mM acetylcholine
generates a large inward current in a cultured bag cell neuron whole-cell voltage-clamped to -60 mV.

Right, the magnitude of the acetylcholine-induced current is reduced by half after culturing in 300 ng/ml
dsRNA ApAchR-C and 300 ng/ml dsRNA ApAchR-E. B: left, subsequent to control dsRNA treatment,
delivery of 3 mM nicotine elicits a typical inward current at -60 mV. Right,
culturing in 300 ng/ml dsRNA of both ApAchR-H and ApAchR-P, attenuates the nicotine-evoked
current. C-G: summary data of the peak current density for the acetylcholine or nicotine responses after
treatment with different dsRNAs. The ordinate label applies to all graphs. C: there is no significant
difference in the current brought about by acetylcholine between control and neurons incubated in either
ApAchR-C or E dsRNA alone (p>0.05, ANOVA, Dunnett multiple comparison test). D: however, the
acetylcholine induced current is significantly different after culturing in a cocktail of ApAchR-C and E
dsRNA (p<0.05, unpaired Student’s t-test). E: the same ApAchR-C plus E dsRNA treatment does not
significantly alter the response to nicotine (p<0.05, unpaired Student’s t-test). F: instead, compared to
control, the current elicited by nicotine is rendered significantly smaller following incubation in 300
ng/ml each of ApAchR-H and P dsRNA (p<0.05, Mann-Whitney U-test). G: This combination of
ApAchR-H with P dsRNA does not result in a significant difference for the acetylcholine-elicited
current compared to control (unpaired Student’s t-test).
References


Kehoe J, McIntosh JM. Two distinct nicotinic receptors, one pharmacologically similar to the vertebrate alpha7-containing receptor, mediate Cl\(^{-}\) currents in *Aplysia* neurons. *J Neurosci* 18: 8198-8213, 1998.


Kem WR. Alzheimer's drug design based upon an invertebrate toxin (anabaseine) which is a potent nicotinic receptor agonist. *Invert Neurosci* 3: 251-259, 1997.


Figure 2

A HP -60 mV, K+ internal
100 µM Nic
300 µM
1 mM
3 mM 20 pA 20 s

C control
peak Nic (3 mM)
-60 mV -70 mV

D Nic (3 mM)
GDP-βS
GTP

B
normalized peak current

EC50: 543 µM
Hill: 2.4

E (n=11)

% ΔG

contrast Nic

peak current density (pA/pF)
contrast GDP-βS

6
5

-0.4
0.0
Figure 3

A  HP -60 mV
Nic (3 mM)

B  mecamylamine (100 μM)

C  strychnine (500 μM)

D  remaining peak Nic current (%)

control  34  16  6  9  11  6  8
nec  Dβ-E  strychnine  ImI  hex  MLA

* denotes significant difference from control.
Figure 5

A. Ach (1 mM, Cs\(^+\) internal)

B. Nic (3 mM, Cs\(^+\) internal)

C. Reversal potential (mV)

D. HP -60 mV

E. Ach (1 mM) in nASW + Iml (1 µM)
Figure 6

Ach (1 mM)  

HP -60 mV  

Nic (3 mM)  

0.05 (340/380)  30 sec  

0.4 nA  30 sec  

C

D

peak current density (pA/pF)

peak 340/380

13  11  

*  

13  11  

*
Figure 7
### Table 1: PCR primers for individual of *Aplysia* nicotinic receptor subunits

For each ApAchR, the first primer pair amplifies from the 5' untranslated region through to the internal coding region of the gene. The last primer pair amplifies from the internal coding region through to the 3' untranslated region. For those ApAchR where a middle primer pair is listed, it amplifies a strictly internal coding region. In all cases, the resulting PCR products overlap to provide full-length open reading frames.

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Table 2: Real-time PCR primers for individual of *Aplysia* nicotinic receptor subunits and GAPDH

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Table 3: Pairwise amino acid identity of *Aplysia* nicotinic receptor subunits with human nicotinic receptor subunits.

Percent sequence identity of aligned ApAchR and human AchR subunits, as well as the Aplysia acetylcholine binding protein (AchBP). Bold numbers reflect the highest scores.

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