Acetylcholine-evoked afterdischarge
in Aplysia bag cell neurons

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Abstract

A brief synaptic input to the bag cell neurons of *Aplysia* evokes a lengthy afterdischarge and the secretion of peptide hormones that trigger ovulation. The input transmitter is unknown, although prior work shows that afterdischarges are prevented by strychnine. Because molluscan excitatory cholinergic synapses are blocked by strychnine, we tested the hypothesis that acetylcholine acts on an ionotropic receptor to initiate the afterdischarge. In cultured bag cell neurons, acetylcholine induced a short burst of action potentials followed by either return to near baseline or, like a true afterdischarge, transition to continuous firing. The current underlying the acetylcholine-induced depolarization was dose-dependent, associated with increased membrane conductance, and sensitive to the nicotinic antagonists, hexamethonium, mecamylamine and α-conotoxin ImI. While neither nicotine, choline, carbachol nor glycine mimicked acetylcholine, tetramethylammonium did produce a similar current. Consistent with an ionotropic receptor, the response was not altered by intracellular dialysis with the G-protein blocker, GDP-β-S. Recording from the intact bag cell neuron cluster showed acetylcholine to evoke prominent depolarization, which often lead to extended bursting, but only in the presence of the acetylcholinesterase inhibitor, neostigmine. Extracellular recording confirmed that exogenous acetylcholine caused genuine afterdischarges which, as per those generated synaptically, rendered the cluster refractory to further stimulation. Finally, treatment with a combination of mecamylamine and α-conotoxin ImI blocked synaptically-induced afterdischarges in the intact bag cell neuron cluster.

Acetylcholine appears elicit to the afterdischarge through an ionotopic receptor. This represents an expedient means for transient stimulation to elicit prolonged firing in the absence of ongoing synaptic input.
**Introduction**

Neuronal communication typically involves presynaptic neurons evoking postsynaptic action potentials through temporal and spatial summation. However, some neurons display the remarkable property of responding with prolonged firing, long after cessation of the initial stimulus. For these cells, synaptic input results in ionotropic receptor- and/or voltage-gated Ca\(^{2+}\) channel-mediated Ca\(^{2+}\) influx, which acts on nearby channels or causes biochemical change to produce plateau potentials and prolonged depolarization (Andrew and Dudek 1983; Egorov *et al.* 2002; Burgoyne 2007). Acetylcholine is particularly potent at initiating lengthy bursts through ionotopic receptor-induced depolarization (Elliott *et al.* 1992; Yamashita and Isa 2003) and lipid metabolism (Tieman *et al.* 2001), as well as metabotropic channel regulation by second messengers (Haj-Dahmane and Andrade 1996; Yan *et al.* 2009; Zhang *et al.* 2011) or tyrosine kinases (Swayne *et al.* 2009). The present study examines a prolonged response in the bag cell neurons of *Aplysia* following cholinergic ionotropic receptor activation.

The bag cell neurons form two distinct clusters at the base of the pleuroabdominal connectives just rostral to the abdominal ganglion (Coggeshall 1967; Smock and Arch 1977). They are normally silent, but upon brief extracellular stimulation of either the connective or the cerebral ganglion they depolarize and undergo a ~30 min afterdischarge (Kupfermann and Kandel 1970; Ferguson *et al.* 1989b); subsequently, the neurons become refractory to stimulation for ~18 hr (Kaczmarek *et al.* 1982; Magoski *et al.* 2000; Zhang *et al.* 2002; Magoski and Kaczmarek 2005). During the afterdischarge, egg-laying hormone (ELH) and other peptides are released into the blood to initiate egg-laying behavior (Arch 1972; Chiu *et al.* 1979; Sigvardt *et al.* 1986).

The afterdischarge is a well-studied and profound change in activity and excitability; surprisingly, the input transmitter is unknown and the only drug found to consistently prevent the afterdischarge is strychnine (Kaczmarek *et al.* 1978). While normally considered a glycineric antagonist (Bradley *et al.* 1953; Curtis *et al.* 1967), strychnine also blocks cholinergic responses and
synapses in *Aplysia* (Kehoe 1972b; Faber and Klee 1974). It is thought that select bag cell neurons receive afferent input from either the pleural or cerebral ganglia (Mayeri *et al.* 1979b; Haskins *et al.* 1981). Dye-conjugated microspheres injected into the cluster retrogradely label a small number of neurons in the pleural and cerebral ganglia; conversely, injection into a cerebral ganglion anterogradely stains neurons in either cluster, whereas injection into a pleural ganglion less reliably labels a few ipsilateral cells (Shope *et al.* 1991). This suggests that a cerebral presynaptic source is more likely.

We now show that acetylcholine is the only candidate neurotransmitter capable of sufficiently depolarizing bag cell neurons to generate afterdischarge-like spiking; moreover, both this response and afterdischarges evoked by cerebral stimulation are blocked by nicotinic antagonists. Our results suggest that acetylcholine in a key input transmitter to the bag cell neurons and demonstrate that transient ionotropic receptor activation can initiate a long-term change in activity fundamental to reproductive behavior.
Materials and Methods

Animals and cell culture

Adult *Aplysia californica* weighing 150-500 g were obtained from Marinus Inc. (Long Beach, CA, USA) and housed in a ~300 l aquarium containing continuously circulating, aerated sea water (Instant Ocean; Aquarium Systems, Mentor, OH, USA or Sea Chem; Madison, GA, 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 transferred to fresh tcASW for 1 hr, after which the bag cell neuron clusters were dissected from their surrounding 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 (430165; Corning, Corning, NY, USA or 353001; Falcon Becton-Dickinson, Franklin Lakes, NJ, USA). Cultures were maintained in tcASW in a 14°C incubator and used for experimentation within 1-3 d. Salts were obtained from Fisher Scientific (Ottawa, ON, Canada), ICN (Aurora, OH, USA), or Sigma-Aldrich (St. Louis, MO, USA).

Whole-cell, voltage-clamp and sharp-electrode, current-clamp recording from cultured bag cell neurons

Voltage-clamp recordings from cultured bag cell neurons were performed at room temperature (20-22°C) 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; Sarasota, FL, USA) and had a resistance of 1-2 MΩ when filled with regular intracellular saline (see below). Pipette junction potentials...
were nulled immediately before seal formation. 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 using an IBM-compatible
personal computer, a Digidata 1322A analogue-to-digital converter (Molecular Devices; Sunnyvale, CA,
USA), and the Clampex acquisition program of pCLAMP (version 8.2; Molecular Devices).

Recordings were made in normal ASW (nASW; composition as per tcASW but lacking glucose
and antibiotics) with regular intracellular saline in the pipette (composition in mM: 500 K-aspartate, 70
KCl, 1.25 MgCl₂, 10 HEPES, 11 glucose, 10 glutathione, 5 ethylene glycol-bis-(β-aminoethyl ether)-
N,N,N',N'-tetraacetic acid (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). The free Ca²⁺ concentration was set at 300 nM by adding an 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.

Current-clamp recordings were made from cultured bag cell neurons in nASW using an
AxoClamp 2B (Axon Instruments/Molecular Devices) amplifier 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) and had a resistance of 5-20 MΩ
when filled with 2 M K-acetate plus 10 mM HEPES and 100 mM KCl (pH 7.3 with KOH). Current was
delivered with either Clampex or a S88 stimulator (Grass; Astro-Med; Longueuil, QC, Canada). Voltage
was filtered at 3 KHz, using the Axoclamp Bessel filter and sampled at 2 KHz as per voltage-clamp.

**Ensemble, extracellular and single neuron sharp-electrode, current-clamp recording from the intact
bag cell neuron cluster**

For extracellular recording, the abdominal ganglion with or without the attached central ring
ganglia was maintained in a 14°C nASW-filled chamber. A wide-bore, fire-polished glass suction
recording electrode (containing nASW) was placed over one of the two bag cell neuron clusters, while a
similar stimulating electrode was placed either at the rostral end of the pleuroabdominal connective corresponding to the recorded cluster, or over the rostro-medial area of the right cerebral ganglion. In most instances when the central ring was included, it was isolated from the abdominal ganglion by means of a custom-made chamber with a thin plastic barrier. The pleuroabdominal connectives passed through two, small slits in the barrier, and were covered by Vaseline for complete isolation (see Results for details). Stimulating current pulses were delivered with a Grass S88 stimulator and isolation unit while voltage was monitored using a Warner DP-301 differential amplifier (Warner Instruments; Hamden, CT, USA). Voltage was high pass filtered at 0.1 Hz and low pass filtered at 1 kHz using the DP-301 filters and acquired at a sampling rate of 2 kHz using Axoscope (version 9.0, Molecular Devices).

For intracellular recording from single bag cell neurons in the intact cluster, the sharp-electrode, bridge-balance method was again employed, but in this case using a Neuroprobe 1600 amplifier (A-M Systems; Sequim, WA, USA). To facilitate sharp-electrode impalement, ganglia were treated with 0.5 mg/ml elastase (E1250; Sigma-Aldrich) and 1 mg/ml collagenase/dispsase (10269638001; Roche) for 2 hr (Fisher et al., 1994; Kehoe, 1972c), then one of the clusters (usually the left) was de-sheathed using fine forceps. Voltage was filtered at 1 kHz using the Neuroprobe filter and acquired with Axoscope at 2 kHz. Extra- and intracellular recordings were sometimes carried out simultaneously by recording extracellularly from the cluster that was ipsilateral to the stimulated right cerebral ganglion, while recording intracellularly from a neuron in the contralateral cluster.

Drug application and reagents

For cultured bag cell neurons, the culture dish served as the bath, with transmitters and drugs being applied directly using a single-cell microperfusion system at ~1 ml/min. The perfusion system consisted of a micromanipulator-controlled square-barrelled 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 of control extracellular saline over the neuron, which was switched to agonist-containing saline for a specific amount of time by activating the appropriate
stopcock. Additional experiments, particularly those involving acetylcholine antagonists, saw the blocker introduced directly into the bath by pipetting a small volume of concentrated stock solution prior to pressure ejection of acetylcholine from an unpolished patch pipette (1-2 µm bore) for 2 sec at 75-150 KPa, using a PMI-100 pressure micro-injector (Dagan; Minneapolis, MN, USA). As was previously undertaken with bag cell neurons by Fisher et al. (1993), no perfusion was employed during the pressure-application protocol; however, the pipette was removed from the bath immediately after each ejection to minimize leakage and possible desensitization.

All drugs were made up as stock solutions in water and frozen at -20°C, then diluted down to a working concentration daily as needed: α-conotoxin ImI (3119; Tocris Bioscience, UK); acetylcholine chloride (A6625; Sigma-Aldrich); carbachol (212385; calbiochem); choline chloride (C1879; Sigma-Aldrich); guanosine 5′-[β-thio]diphosphate trilithium salt (GDP-β-S) (G7637; Sigma-Aldrich); glycine (G7126; Sigma-Aldrich); hexamethonium chloride (H2138; Sigma-Aldrich); mecamylamine hydrochloride (M9020; Sigma-Aldrich); methyllycaconitine citrate salt hydrate (M168; Sigma-Aldrich); neostigmine bromide (N2001; Sigma-Aldrich); nicotine (N0257; Sigma-Aldrich); oxotremorine sesquifurmarate (O9126; Sigma-Aldrich); Phe-Met-Arg-Phe amide (FMRFamide) (P4898; Sigma-Aldrich); serotonin (H9523; Sigma-Aldrich); strychnine (S0532; Sigma-Aldrich); tetramethylammonium chloride (TMA) (T19526; Sigma-Aldrich).

**Analysis**

The Clampfit analysis program of pCLAMP was used to determine the amplitude and time course of changes to membrane potential or holding current evoked by candidate 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 of the response (see Results for details). 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 the whole-cell capacitance (as determined by the EPC-8 slow capacitance compensation
For display, most current and voltage traces were filtered off-line between 20 and 80 Hz using Clampfit. The slow nature of the responses ensured that this second filtering brought about no change in amplitude or kinetics. Conductance was derived using Ohm's law \( G=I/V \) from the current during a 200 ms step from -60 to -70 mV. In cases where acetylcholine was applied twice, the subsequent application occurred after a ~10-min interval, with the peak current of the second response expressed as a percentage of the first response.

Afterdischarge duration was quantitated as the time from the initial extracellular spike after cessation of the stimulus. In some cases, the response went beyond 30 min and was truncated at that point. For display, extracellular voltage was filtered off-line to 80 Hz using Clampfit.

Data are presented as mean ± standard error of the mean. Statistical analysis was performed using Instat (version 3; GraphPad Software; San Diego, CA, USA). The Kolmogorov-Smirnov method was used to test data sets for normality. To test whether the mean differed between two groups, either Student's unpaired t-test (for normally distributed data) with the Welch correction as necessary (for unequal standard deviations) or the Mann-Whitney U-test (for not normally distributed data) was used. For three or more means, multiple comparisons involved an analysis of variance (ANOVA) followed by Dunn’s multiple comparison post hoc test. Fisher’s exact test, which examines the association between two variables, was used to test differences in frequency. The level of significance of the one- or two-tailed p-value was set at <0.05.
Prior work shows that most *Aplysia* neurons tested are acetylcholine-responsive, with the vast majority in the abdominal ganglion presenting hyperpolarization, and a minority showing either depolarization or a combined depolarizing-hyperpolarizing response (Tauc and Gerschenfeld 1961; Frazier *et al.* 1967). As such, at least four cholinergic receptor types are believed to exist: two distinctive Cl--conductances, one rapidly desensitizing and the other slowly desensitizing, mediate the hyperpolarizing responses (Kehoe 1972c; Gardner and Kandel 1977; Kehoe and McIntosh 1998); the depolarizing response is due to a non-selective cation conductance (Marty *et al.* 1976; Ascher *et al.* 1978a); and a fourth acetylcholine-induced response involves a G-protein dependent K+-conductance (Kehoe 1994). Some *Aplysia* neurons contain only one receptor type, yet others, such as those in the medial pleural ganglion, present both depolarizing and hyperpolarizing responses (Kehoe 1972b). The response of bag cell neurons to acetylcholine is not well characterized, with only one previous study noting modest depolarization (Bodmer and Levitan 1984).

**Acetylcholine depolarizes cultured bag cell neurons**

To satisfy the role of an input transmitter triggering the afterdischarge, acetylcholine should depolarize and evoke action potentials in bag cell neurons. Microperfusion of 1 mM acetylcholine dissolved in nASW near the soma of a cultured bag cell neuron (see Materials and Methods for details) induced a substantial depolarization under sharp-electrode current-clamp at -60 mV (n=20). With continued exposure of agonist, the peak depolarization lasted a few sec before returning to baseline within 3-5 min. In some cases, the response did not return to baseline but reached a new, depolarized steady-state. Acetylcholine delivered in this manner depolarized three of the 20 bag cell neurons sufficiently to induce spiking (not shown). In order to speed the rate of the depolarization, 1 mM acetylcholine was pressure applied for 1-2 sec near the soma (n=13) (see Materials and Methods for details), and produced a more robust and rapid response that consistently resulted in a brief burst of action potentials in 10 of the 13 neurons tested (Fig. 1A). Although the same concentration of

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acetylcholine was used for the initial microperfusion and pressure pressure application experiments, it is likely that the latter delivered a more rapid change in agonist concentration, allowing the neuron to reach threshold more consistently before desensitization occurred (see below). When the data sets for the acetylcholine-induced depolarization evoked by either microperfusion or pressure ejection were merged, the average was significantly different from zero at 36.8 ± 2.73 mV (n=33) (Fig. 1B). Occasionally, this depolarization led to afterdischarge-like spiking that continued long after the stimulus (n=5) (Fig. 1C).

In these cases, the depolarization followed a similar initial time course; however, instead of returning to -60 mV, the neuron reached a depolarized steady-state of ~40 mV and proceeded to fire action potentials. This is similar to potentials recorded intracellularly from neurons in the cluster during an afterdischarge caused by presynaptic stimulation (Mayeri et al. 1979b; Kauer and Kaczmarek 1985; Fisher et al. 1993), as well as from cultured neurons responding to a brief train of action potentials (Hung and Magoski 2007) or application of cAMP (Kaczmarek and Strumwasser 1981).

**A concentration-dependent, acetylcholine-induced inward current**

To further explore the acetylc holine-induced response, we characterized the properties of the underlying current in cultured bag cell neurons. Initial testing showed that application of acetylcholine by microperfusion, for as short as 10 sec, caused marked desensitization to subsequent applications, even following a 20-min wash. This may explain the difficulty we encountered in evoking spiking with this method (see above). Therefore, acetylcholine was applied only once per neuron by microperfusion over the soma under whole-cell voltage-clamp at -60 mV using a K+-based intracellular saline. Figure 2A shows example traces from four concentrations (10, 100, 300, 1000 µM) in separate neurons, revealing a relatively slow onset current at lower concentrations, becoming faster at higher doses. Return to baseline typically took 3-5 min in the continued presence of agonist. Delivering concentrations ranging from 1 µM to 10 mM, generated a dose-response curve with a Hill coefficient of 0.7, indicating either a lack of cooperativity or potential negative cooperativity, and an EC$_{50}$ of 267 µM (Fig. 2B).

Ascher et al. (1978a) showed that the depolarizing response to acetylcholine in right pleural ganglion neurons involved an increased cation conductance. To test if the bag cell neuron current was
due to channel opening, conductance was calculated using a 200-msec step from -60 to -70 mV (Fig. 2C, 
bottom). Three of these -10 mV steps were delivered to each neuron: two prior to the acetylcholine 
perfusion, separated by 1-2 min, and one at the peak of the acetylcholine-induced response. To account 
for leak and achieve a baseline conductance change over time, the first control current evoked by the 
step was subtracted from the second current (Fig. 2C, top). The second control current was then 
subtracted from the current produced by the step during the acetylcholine response (Fig. 2C, middle). 
The increase in conductance change between the peak acetylcholine response and the prior control 
period was clear and readily met the level of significance (n=9) (Fig. 2D).

Because nicotinic vs. muscarinic receptor classification is not clearly delineated in molluscs, it 
was necessary to see if oxotremorine, a muscarinic agonist shown to be effective in *Aplysia* (Dembrow 
et al., 2004), would mimic the acetylcholine-induced current. However, bath application of 20 µM 
oxotremorine failed to produce an appreciable current at -60 mV (n=4) (Fig. 2E, F). Furthermore, since 
strychnine, the only agent known to block the afterdischarge (Kaczmarek *et al*., 1978), is typically 
thought of as a glycnergic blocker (Bradley *et al*., 1953; Curtis *et al*., 1967), we also tested the effect of 
glycine. As shown in figure 2E and F, no detectible current was produced by glycine (n=8).

Interestingly, application of up to 3 mM nicotine (n=9) failed to replicate the acetylcholine-
induced current, and produced a significantly smaller current density of $-0.12 \pm 0.01 \text{ pA/pF}$, compared 
to $-5.8 \pm 0.7 \text{ pA/pF}$ for 1 mM acetylcholine (Fig. 2F). Tetramethylammonium (TMA), which mimics the 
excitatory action of acetylcholine without inducing metabotropic affects in both other *Aplysia* neurons 
(Ascher *et al*., 1978a) and the homologous caudodorsal cells of *Lymnaea* (Ter Maat and Lodder, 1980), 
was the only compound to evoke a current of similar density to acetylcholine (n=20) (Fig. 2F). The 
general cholinergic agonist, carbachol (n=6) (Schwartz *et al*., 1982), as well as specific $\alpha$-7 nicotinic 
receptor agonist, choline (n=9) (Kehoe 1972c; Papke *et al*., 1996; Alkondon *et al*., 1997), also failed to 
induce a response (Fig. 2F).

Even though serotonin (5-HT) is able to block the afterdischarge, previous attempts at observing 
5-HT-induced currents in cultured bag cell neurons have not been successful (Jennings *et al*., 1981). In
our cells, 5-HT never induced an inward current, but occasionally produced an outward current, and only through pressure application. As such, the average outward current density of $0.21 \pm 0.13$ pA/pF (n=14), which largely includes neurons where 5-HT provoked no current (Fig. 2F).

**The acetylcholine-induced current is not dependent on G-proteins**

The evidence thus far suggests that the bag cell neuron acetylcholine current is ionotropic; however, if acetylcholine acts in a metabotropic manner, it would be G-protein coupled and blocked by GDP-β-S (a non-hydrolyzable form of GDP) (Eckstein *et al.* 1979). Dialysis of cultured bag cell neurons for 30 min with normal internal solution plus 10 mM GDP-β-S (replacing the 0.1 mM GTP) did not alter the current to pressure-applied acetylcholine (1 mM) at -60 mV (n=5) (Fig. 3A right), when compared to parallel controls dialyzed with internal solution containing GTP (n=7) (Fig. 3A left). Summary data indicate a slight, but not significant increase in the acetylcholine-induced current with GDP-β-S in the pipette (Fig. 3B). As a positive control, the outward current at -40 mV produced by pressure-applied FMRFamide (500 µM), a known metabotropic agonist in molluscs (Piomelli *et al.* 1987a,b; Brezina 1988; Fisher *et al.* 1993), was blocked by GDP-β-S (Fig. 3C, D). The summary data of FMRFamide following GDP-β-S dialysis in figure 3D includes data from neurons never exposed to acetylcholine (n=4/8), as well 10 min after application of acetylcholine (n=4/8), which were not significantly different from each other (data not shown, 2-tailed Mann-Whitney) and thus pooled.

**The acetylcholine response is sensitive to specific cholinergic blockers**

Most cholinergic currents of *Aplysia* neurons are blocked by traditional nicotinic antagonists; in particular, the inward current is blocked in a voltage-dependent manner by hexamethonium or d-tubocurarine (Kehoe 1972b; Ascher *et al.* 1978b), and in a voltage-independent manner by α-conotoxin ImI (Kehoe and McIntosh 1998). Because d-tubocurarine also blocks dopamine receptors in molluscan neurons (Ascher 1972; Berry and Cottrell 1975), we chose to examine the effects of the general antagonists hexamethonium (Paton and Zaimis 1948; Tauc and Gerschenfeld 1961), mecamylamine (Stone *et al.* 1956; Ueki *et al.* 1961; Ascher *et al.* 1979), methyllycaconitine (MLA), which at low doses preferentially blocks α7 nicotinic receptors (Ward *et al.* 1990; Alkondon *et al.* 1992; Mogg *et al.* 2002),
and α-conotoxin ImI (McIntosh et al. 1994; Johnson et al. 1995). Choline, which can preferentially activate α7 nicotinic receptors (Papke et al. 1996; Alkondon et al. 1997), was also tested for agonist-induced desensitization.

A pair of 2-sec acetylcholine (1 mM) pressure applications, separated by a minimum of 10 min, were used and expressed as a percentage of the second current vs. the first. Antagonists were introduced into the bath after the first acetylcholine application, and the relative effectiveness determined by calculating the percent remaining current evoked by the second application. Under control conditions, without the addition of any antagonist to the bath, the second application of acetylcholine produced a peak current of 54.7 ± 3.5% of the first (n=26) (Fig. 4A, G). Bath application of 500 µM hexamethonium (n=6) (Fig. 4B, G), 100 µM mecamylamine (n=12) (Fig. 4C, G), or 1 µM α-conotoxin ImI (n=6) (Fig. 4D, G) after the initial current had returned to baseline, blocked much of the second acetylcholine-induced current. The post-mecamylamine current was rapid while the post-hexamethonium or α-conotoxin ImI currents were relatively slow. Delivering both mecamylamine and α-conotoxin ImI together virtually eliminated the current evoked by the second acetylcholine application (n=5) (Fig. 4E, G). For either 1 µM MLA or 10 mM choline, only 1 out of the 7 neurons showed a measurable block; therefore, the average of total remaining current in both cases was not significantly different from control (n=7 and 7) (Fig. 4G). Considering that strychnine can block the afterdischarge (Kaczmarek et al. 1978), as well as cholinergic synaptic transmission in Aplysia central ring neurons (Kehoe 1972a; Faber and Klee 1974), we also tested its effects on cultured bag cell neurons. Compared to control, 500 µM strychnine significantly blocked the second acetylcholine-induced current (n=12), although not to the same extent as mecamylamine or α-conotoxin ImI (Fig. 4F, G).

The block of the acetylcholine response shows differential voltage-dependence

During the afterdischarge, the bag cell neurons are typically depolarized from a resting potential of -60 mV to spiking potential of between -40 and -30 mV (Mayeri et al. 1979a,b; Kaczmarek et al. 1982). Due to the voltage-dependent block by many of the nicotinic antagonists used here (Ascher et al. 1978b; Kehoe and McIntosh 1998), we examined if hexamethonium, mecamylamine and α-conotoxin...
ImI were still able to inhibit the cholinergic current at depolarized potentials. While holding at -30 mV, a second pressure application of 1 mM acetylcholine induced a current 68.0 ± 4.9% of the first (n=8) (Fig. 5A), which failed to reach the level of significance when compared to the run-down at -60 mV (Fig. 5F). At -30 mV, 500 μM hexamethonium was essentially the same as control, with the current at 56.7 ± 5.5% of the initial value (n=6) (Fig. 5B), and significantly different from the 16.6 ± 5.2% remaining current at -60 mV (Fig. 5F). Similarly, 100 μM mecamylamine resulted in a 22.5 ± 8.8% decrease of the initial current at -30 mV (n=6) (Fig. 5C), which again was significantly less than the 7.7 ± 3.3% remaining current at -60 mV (Fig. 5F). Clearly, hexamethonium, and to a lesser extent mecamylamine, are not effective antagonists of the cholinergic current at depolarized potentials. Alpha-conotoxin ImI (1 μM), on the other hand, was equally potent at blocking the current, with 14.0 ± 3.1% and 17.1 ± 8.5% remaining current at -60 mV and -30 mV (n=8), respectively (Fig. 5D, F). Moreover, as was the case at -60 mV, a combination of mecamylamine and α-conotoxin ImI completely blocked the second current at -30 mV (n=6) (Fig. 5E, F).

**Acetylcholine depolarizes bag cell neurons in the intact cluster**

Having established that bag cell neurons respond to acetylcholine with an ionotropic receptor, we next explored the response of neurons in the intact bag cell neuron cluster to acetylcholine. The bag cell neuron cluster contains a high concentration of acetylcholinesterase, similar to levels observed in neuropil of the abdominal ganglion (Giller and Schwartz 1971). Therefore, most experiments employed the acetylcholinesterase inhibitor, neostigmine, which has been previously shown to counteract the enzyme while not directly affecting cholinergic currents in *Aplysia* neurons (Bhattacharya and Feldberg 1958; Carpenter *et al.* 1976; Ascher *et al.* 1978b; Slater *et al.* 1986). In addition, nASW was used in favour of high-divalent sea water, given that the latter greatly reduces the likelihood of afterdischarge generation (Kaczmarek *et al.* 1982).

For intracellular recording, single bag cell neurons within the cluster were sharp-electrode current-clamped to -60 mV and acetylcholine bath-applied (see Materials and Methods for details). In four neurons tested without neostigmine pretreatment, acetylcholine (1 mM) produced an average...
depolarization of 6.1 ± 2.2 mV (Fig. 6A, D). A 20-min pretreatment with 2 µM neostigmine increased
the cholinergic depolarization to 24.8 ± 2.6 mV (n=17 neurons from separate clusters in different
abdominal ganglia) (Fig. 6B, D). In seven of the 17 bag cell neurons that depolarized, acetylcholine
induced continuous spiking similar to that of an afterdischarge (Fig. 6C). However, it was not possible
to accurately measure the duration of acetylcholine-induced afterdischarges using intracellular recording
because of difficulty in maintaining recordings of consistent duration. Acetylcholine likely activated
muscles within the pleuroabdominal connective (Coggeshall 1967) or neurons within the abdominal
ganglia that project to intrinsic muscles (Alevizos et al. 1991), which served to dislodge the recording
electrode. Thus, we also employed a dual recording of the contralateral cluster using an extracellular
suction electrode (see Materials and Methods for details). In four extracellularly recorded clusters,
acetylcholine induced an afterdischarge of 6.9 ± 1.2 min in duration (Fig. 6E, F).

Prior acetylcholine exposure changes the response of the bag cell neuron cluster

If acetylcholine is an input transmitter for the afterdischarge, it would be expected that clusters
stimulated by acetylcholine would more likely be refractory. Typically, bag cell neurons can be induced
to fire an afterdischarge through extracellular stimulation of the pleuroabdominal connective with a 5
Hz, 10-30 sec stimulus protocol (Kaczmarek et al. 1978). However, some bag cell neuron processes can
travel up the connective to reach the pleural ganglion (Haskins et al. 1981); thus, to avoid as much as
possible the antidromic stimulation of bag cell neuron processes, we chose to stimulate the right cerebral
ganglion near the region of the F cluster as per Ferguson et al. (1989b). The stimulation protocol
involved increasing the extracellular voltage from 10 V to up 25 V until the initiation of extracellular
spikes. Stimulation was terminated 5 sec after the first appearance of extracellular spikes or at 30 sec. If
no afterdischarge was initiated, a second 30-sec protocol was delivered after a 10-min rest period.
Stimulation of 25 separate bag cell neuron clusters consistently generated an afterdischarge with
an average duration of 14.6 ± 3.0 min (Fig. 7A left). However, in 11 clusters previously exposed to 1
mM acetylcholine in 2 µM neostigmine, stimulation failed to evoke an afterdischarge in nine cases (Fig.
7B), while the remaining two clusters presented durations of 7 and 17 min, respectively. This decreased
frequency of afterdischarge generation following prior acetylcholine exposure was significant (Fig. 7A right). Conversely, it is expected that clusters previously stimulated to the point of refractoriness would also fail to respond to acetylcholine. Figure 7C (left) shows an example of an intracellular recording from an intact bag cell neuron, in the presence of neostigmine, and the afterdischarge response to synaptic input. In four separate clusters, such previous stimulation rendered 1 mM acetylcholine ineffective at generating an afterdischarge (Fig. 7C right). Two clusters did not respond at all to acetylcholine, whereas, one cluster hyperpolarized and another depolarized slightly but not sufficiently to induce action potentials.

_Mecamylamine and α-conotoxin ImI block the afterdischarge_

Since the addition of mecamylamine and α-conotoxin ImI could block the cholinergic current in cultured bag cell neurons at both resting and depolarized membrane potentials, it was important to see if these antagonists also blocked the afterdischarge evoked by presynaptic stimulation. One cluster was recorded per animal. In control conditions, without the addition of antagonists, cerebral stimulation induced an afterdischarge of 21.0 ± 3.9 min in duration (n=8) (Fig. 8A, C left), which became refractory to subsequent stimulation. Pretreatment with both 100 µM mecamylamine and 1 µM α-conotoxin ImI completely blocked any afterdischarge due to stimulation in five of seven clusters (Fig. 8B left, C left). The other two clusters responded with normal afterdischarges of 18 and 30 min, respectively. The latter indicates the possibility that multiple factors may initiate an afterdischarge (see Discussion). For the five clusters blocked by the antagonists, subsequent stimulation after a 30-min wash restored the ability to generate an afterdischarge in three cases, with an average duration of 6.7 ± 0.5 min (Fig. 8B right, C left). The presence of mecamylamine and α-conotoxin ImI produced a significant decrease in the frequency of afterdischarge generation induced by cerebral stimulation compared to control (Fig. 8C right).
Discussion

We present evidence for acetylcholine being an input transmitter to the bag cell neurons: First, acetylcholine is the only tested transmitter capable of sufficiently depolarizing bag cell neurons, both in culture and within the intact cluster, to action potential threshold. Some cultured neurons display regenerative firing, long after cessation of agonist application. In the intact cluster, the addition of the acetylcholinesterase inhibitor, neostigmine, enables acetylcholine to induce an afterdischarge. Second, a combination of the nicotinic antagonists, mecamylamine and α-conotoxin ImI, block both the acetylcholine-induced current in cultured neurons as well as afterdischarges in the intact cluster evoked by cerebral ganglion stimulation. Third, refractory clusters are much less likely to respond to acetylcholine, whereas resting clusters exposed to acetylcholine become refractory to synaptic stimulation. Thus, along with a number of other factors (see below), acetylcholine has the potential to initiate the afterdischarge in vivo.

Strictly depolarizing response of acetylcholine in cultured bag cell neurons

Based on an increase in conductance at peak current and a lack of attenuation following GDP-β-S dialysis, the bag cell neuron acetylcholine response is consistent with an inward current through opening of an ionotropic receptor. GDP-β-S is well-established as preventing metabotropic receptor coupling in molluscan neurons (Lemos and Levitan 1984; Brezina 1988; Kehoe 1994; Magoski et al. 1995; Tam et al. 2011). Furthermore, the metabotropic agonist, oxotremorine, did not elicit a current, and in no instance did acetylcholine induce hyperpolarization or outward current in cultured bag cell neurons. Within the intact ganglia, acetylcholine occasionally caused hyperpolarization; however, since it was bath applied in nASW, extra-synaptic events may have contributed. The acetylcholine current we observe mimics the current mediated by a non-selective cation channel-type ionotropic receptor in Aplysia right pleural ganglion neurons (Marty et al. 1976; Ascher et al. 1978a,b; Kehoe and McIntosh 1998). Both responses show voltage-dependent block by hexamethonium or mecamylamine, voltage-independent block by strychnine or α-conotoxin ImI, and are recapitulated by TMA but not nicotine. As
per Ascher et al. (1978b) and Kehoe and McIntosh (1998), we find hexamethonium block to be strongly voltage-dependent (essentially not effective at -30 mV) and mecamylamine block to somewhat less influenced by depolarization. However, unlike other reports, the bag cell neuron current presents long-lasting desensitization and is insensitive to carbachol. The latter was surprising, but not unprecedented, since carbachol cannot reproduce the excitatory effect of acetylcholine on Aplysia gill (Weiss et al. 1984) or parapodial (Laurienti and Blankenship 1999) muscle.

**Similarities to molluscan and mammalian nicotinic receptors**

The one cloned molluscan cation-selective acetylcholine receptor is from *Lymnaea* (LnAchRA), and while it is blocked by mecamylamine and α-conotoxin Iml, but not MLA, it differs from the bag cell neuron current by showing nicotine and choline sensitivity, as well as faster kinetics and little desensitization to repeated applications (van Nierop et al. 2005). The bag cell neuron cholinergic current is probably not mediated by an α7 nicotinic receptor, because the *Aplysia* α7-like conductance is Cl-selective, reproduced by nicotine and blocked by MLA (Kehoe and McIntosh 1998). The most striking difference between the bag cell neuron current and vertebrate nicotinic receptors is a lack of mimicry by nicotine. While nicotine can elicit an inward current, we find it differs from the acetylcholine-gated channel in reversal potential, cooperativity, Ca-sensitivity and antagonist profile (White and Magoski in preparation).

**Presynaptic role for acetylcholine in afterdischarge generation**

Either the afterdischarge is initiated by a cholinergic afferent input (likely from the cerebral ganglia) or the bag cells themselves are cholinergic and secrete acetylcholine to maintain the afterdischarge. Previous evidence indicates synaptic input is more likely, *i.e.*, the bag cell neurons are not labelled by anti-acetylcholine immunohistochemistry (Soinila and Mpitsos 1991). Also, the levels of acetylcholinesterase, which would breakdown acetylcholine released from any input, are comparable between the bag cell neuron cluster and the synaptic neuropil of the abdominal ganglion (where cholinergic transmission is expected to be common) (Giller and Schwartz 1971). Moreover, if the bag cell neurons were cholinergic, it would require a significant amount of choline acetyltransferase to
synthesize acetylcholine, yet the cluster contains only a low level of this enzyme (Giller and Schwartz 1968; McCaman and Dewhurst 1970). It is likely that processes travelling through or innervating the cluster give rise to this small signal. Further evidence for potential cholinergic input is the presence of small, clear vesicles near select bag cell neuron axon bundles; these are in contrast to most bag cell neuron neurites, which contain moderately dense core granules and innervate the connective tissue sheath (Chiu and Strumwasser 1981; Haskins et al. 1981).

Strychnine can block EPSPs from a presumed cholinergic input to anterior pleural cells (Kehoe 1972c), as well as the afterdischarge in bag cell neurons (Kaczmarek et al. 1978). Tubocurarine or hexamethonium were unable to block the afterdischarge in intact clusters (Kaczmarek et al. 1978). However, tubocurarine is known to inhibit receptors for other transmitters (Ascher 1972; Berry and Cottrell 1975), and such lack of specificity may explain why it was unable to prevent the afterdischarge. In addition, both ourselves and others (Kehoe and McIntosh 1998) find hexamethonium to be a poor blocker at -30 mV. Because the afterdischarge is associated with depolarization to -40 or even -30 mV (Mayeri et al. 1979a,b; Kaczmarek et al. 1982), hexamethonium could have been rendered ineffective. We that show the afterdischarge is blocked by a combination of the specific nicotinic antagonists, mecamylamine and α-conotoxin ImI. This cocktail also eliminates the acetycholine-induced current in cultured bag cell neurons at both resting and depolarized membrane potentials. That stated, we cannot rule out the possibility that the anatagonists may also act on the receptors for some unknown chemical input.

Possible cholinergic synaptic input to the bag cell neurons

Surprisingly little is known about the synaptic input to bag cell neurons; however, the homologous caudodorsal cells in Lymnaea receive cholinergic afferents (Ter Maat et al. 1983). Most bag cell neuron axons project to the vascularized sheath and rarely course more than 1 cm rostrally along the pleuroabdominal connective (Haskins et al. 1981). Nevertheless, some axons run through the connective core to the pleural or cerebral ganglia, and retrograde labeling of bag cell inputs reveals neurons in the pleural and cerebral ganglia (Shope et al. 1991). Furthermore, cobalt backfilling of the
connective stains a number of neurons in the cerebral ganglion at a site where focal extracellular stimulation induces afterdischarges (Ferguson et al. 1989b).

The cerebral F-cluster neurons have been implicated in bag cell neuron function (Ferguson et al. 1989b; Rubakhin et al. 1999). Neurons that stain for acetylcholine are observed near the F-cluster (Soinila and Mpitsos 1991), but their actual location has not been accurately described, so it is possible that we directly stimulated these cells in addition to the F-cluster neurons. The F-cluster is bordered by the C-cluster, which contains ectopic bag cell neurons - small populations of neurons in both the cerebral and pleural ganglia which produce ELH and other bag cell neuron peptides (Chiu and Strumwasser 1981, 1984). Even if focal stimulation spread to ectopic bag cell neurons in the cerebral C-cluster, the latter send their processes into the neuropil and not down the connective, and are ineffective at initiating afterdischarges on their own (Chiu and Strumwasser 1984; Painter et al. 1989).

**Acetylcholine-induced long-term change in activity**

Acetylcholine generates plateau potentials in cortical and hippocampal neurons by coupling muscarinic receptors to non-selective cation channels (Egorov et al. 2002; Tahvildari et al. 2008; Tai et al. 2010; Zhang and Seguela 2010; Zhang et al. 2011). Nevertheless, nicotinic receptors in substantia nigra neurons produce moderate bursting in Ca^{2+}-dependent manner (Yamashita and Isa 2003). Acetylcholine evokes plateau potentials and bursting in *Lymnaea* N1 and N2 feeding neurons through a presumed ionotropic receptor (Elliott and Kemenes 1992; Brierley et al. 1997; Straub et al. 2002). Also, cerebral cholinergic neurons modulate the *Aplysia* feeding central pattern generator (Susswein et al. 1996; Hurwitz et al. 2003; Dembrow et al. 2003, 2004). *Aplysia* interneuron L10 is cholinergic and makes excitatory connections (Koester and Alevizos 1989); for example, recruiting neurons R25/L25 to burst and trigger respiratory pumping (Byrne 1983; Koester 1989), as well as LUQ cells projecting to the kidney (Koester and Alevizos 1989).

Our results suggest cholinergic ionotropic receptors are capable of producing extended bursting in bag cell neurons. Although we find no evidence of metabotropic cholinergic receptors, second messenger production associated with maintenance of the afterdischarge may arise because of Ca^{2+}
influx or the autocrine effect of certain bag cell neuron peptides (Kaczmarek et al. 1978; Redman and Berry 1993; Wayne et al. 1999). Ca\(^{2+}\) influx may occur directly through the cholinergic ionotropic receptor or via voltage-gated Ca\(^{2+}\) channels recruited during the acetylcholine-induced depolarization. Elevated intracellular Ca\(^{2+}\) has the potential to activate some of the various non-selective cation channels that drive the afterdischarge (Wilson et al. 1996; Magoski 2004; Magoski and Kaczmarek 2005; Lupinsky and Magoski 2006; Gardam and Magoski 2009; Geiger et al. 2009). Finally, the afterdischarge can be sustained by the acetylcholine-evoked depolarization opening bag cell neuron persistent Ca\(^{2+}\) current (Tam et al. 2009).

**Multiple mechanisms for initiation of the afterdischarge**

Brown et al. (1989) show that, in addition to pleuroabdominal connective stimulation, the afterdischarge can be initiated by activating pleural ectopic bag cell neurons (as opposed to cerebral ectopic bag cell neurons). This appears to be chemically mediated, as electrical coupling is not evident between pleural ectopic neurons and abdominal cluster neurons. Interestingly, these authors provide the only report of an afterdischarge being generated by intracellular stimulation of a single bag cell neuron in the cluster. Additionally, application of atrial gland extract to the cerebral ganglia can induce afterdischarges; however, this is likely poly-synaptic, since it only manifests when applied to the cerebral ganglia with an intact pleuroabdominal connective, and atrial gland extract has no effect on cultured bag cell neurons (Strumwasser et al. 1980). These alternative means may account for those rarer occasions where we failed to observe acetylcholine-induce refractoriness or pharmacological block of the afterdischarge. Furthermore, different external stimuli, such as mating or chemical signals from an egg mass, can cause afterdischarges (Begnoche et al. 1996). Given the importance to species propagation, a diversity of mechanisms for afterdischarge initiation and egg-laying is not surprising. It is testament to the survival strategies involved in reproduction.

In summary, acetylcholine evokes an afterdischarge in the intact bag cell cluster, likely due to presynaptic release from neurons in the cerebral ganglion, and opening of an ionotropic receptor.
Figure Legends

Figure 1. Depolarization of bag cell neurons by acetylcholine.

A, Under sharp-electrode current clamp, a 2-sec pressure application of acetylcholine (Ach) depolarizes a cultured bag cell neuron sufficiently for action potential generation in nASW from -60 mV. Inset shows three action potentials under an expanded time scale. B, Summary graph indicating the average depolarization of 36.8 ± 2.73 mV induced by acetylcholine is significantly different from zero (p<0.0001, 2-tailed one-sample t-test). C, Example of an acetylcholine application resulting in persistent spiking. The neuron continued to fire for 30 min, but the trace is truncated at 8 min for display.

Figure 2. Current responses in cultured bag cell neurons to the application of acetylcholine and related agonists.

A, Whole-cell voltage-clamp recordings in nASW with K+-based internal solution. Current induced by continuous microperfusion of 10 µM, 100 µM, 300 µM or 1 mM acetylcholine (Ach), at a holding potential of -60 mV. B, The dose-response curve for acetylcholine reveals an EC50 of 267 µM with little cooperativity, based on a Hill coefficient of 0.70 (n=2 for 1 µM, 5 for 3 µM, 3 for 10 µM, 6 for 30 µM, 16 for 100 µM, 6 for 300 µM, 9 for 1 mM, 8 for 3 mM, 7 for 10 mM). C, Subtracted current traces taken 1 min apart under voltage-clamp during a 10 mV hyperpolarizing step (lower). Prior to the addition of acetylcholine, essentially no conductance change occurs (upper); however, subtraction currents taken at the peak of the acetylcholine response demonstrate a large increase in conductance (middle). D, The percent change in conductance, from 19.4 ± 7.9% between consecutive steps in control conditions to 2935 ± 829% between steps in control and acetylcholine, is significantly different (p<0.01, 2-tailed paired Student’s t-test). E, Example current traces show that microperfusion of oxotremorine (oxo), a muscarinic agonist, and glycine, a member of the cys-loop ligand-gated ion channel family, of which the nicotinic receptor is also a member, do not induce a current. F, Summary graph of different agonists used. Only tetramethylammonium (TMA) produces a similar current (-4.5 ± 0.8 pA/pF) as acetylcholine.
The other agonists including, nicotine (0.12 ± 0.01 pA/pF), were all significantly
different (p<0.01, one way ANOVA, Dunnett’s multiple comparisons test). Null currents were included
in the summary of 5-HT-induced responses for a mean of 0.21 ± 0.13 pA/pF.

Figure 3. The acetylcholine-induced current is not dependent on G-protein activation.

A, Left, whole-cell current induced by a 2-sec pressure-application of 1 mM acetylcholine (Ach) in a
cultured bag cell neuron held at -60 mV, after a 30-min dialysis with a standard K⁺-based internal
solution containing 0.1 mM GTP. Right, in a separate neuron dialyzed for 30 min with the same internal
solution, except for 10 mM GDP-β-S replacing GTP, the acetylcholine induced current is similar in
magnitude. B, Summary data indicating no significant difference between the peak current density
induced by acetylcholine as a result of replacement of GTP (-3.9 ± 1.3 pA/pF) with GDP-β-S (-6.0 ± 2.2
pA/pF) (p>0.05, 2-tailed unpaired Student’s t-test). C, Acting as a positive control, pressure application
of 500 µM FMRFamide induces an outward current at a holding potential of -40 mV (left); this is
eliminated in a separate neuron dialyzed with GDP-β-S (right). D, The FMRF peak current density with
a GDP-β-S containing internal solution (0.01 ± 0.01 pA/pF) is significantly reduced compared to control
(0.24 ± 0.06 pA/pF) (p<0.01, 2-tailed Mann-Whitney U-test).

Figure 4. Nicotinic receptor antagonists block the acetylcholine-induced current.

A, Following an initial 1-2 sec pressure-application of acetylcholine (Ach) (1 mM) at a holding potential
of -60 mV, the current evoked by a second application 10 min later, is reduced to 54.7 ± 3.5% of its
initial value. B-D, Bath application of the nicotinic receptor blockers, hexamethonium (500 µM),
mecamylamine (100 µM), or α-conotoxin ImI (1 µM), potently blocks the second acetylcholine-induced
current, reducing it to 16.6 ± 5.2%, 7.7 ± 3.3%, or 14 ± 3.1% of the initial value, respectively. E, Adding
both mecamylamine and α-conotoxin ImI simultaneously almost completely eliminates the second
response to acetylcholine (1.0 ± 0.4% of the initial current). F, In a separate neuron, bath application of
strychnine (500 µM) after the first acetylcholine response further reduces the current evoked by the
second acetylcholine application to 23.9 ± 5.2% of the initial value. \( G \), Summary data showing the percent of remaining current between the second and first acetylcholine applications. Compared to control, there is significantly less residual current in the presence of hexamethonium (hex), mecamylamine (mec), \( \alpha \)-conotoxin ImI (ImI), mecamylamine and \( \alpha \)-conotoxin ImI together, or strychnine, but not with methyllycaconitine (MLA), an \( \alpha_7 \) nicotinic receptor-selective antagonist, or choline, an \( \alpha_7 \) nicotinic receptor desensitizing agonist (p<0.001, one way ANOVA, Dunn’s multiple comparisons test).

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**Figure 5. Differential voltage-dependence of nicotinic receptor antagonist block of the acetylcholine-induced current.**

- **A**, Following the same protocol as per figure 4, but at a holding potential of -30 mV, the second acetylcholine (Ach) (1 mM) current is 68.0 ± 4.9% of the first in control conditions. **B**, A 10-min application of hexamethonium fails to alter the acetylcholine-induce response at -30 mV (56.7 ± 5.5% remaining current at -30 mV vs. 16.6 ± 5.2% at -60 mV). Note that the percent current remaining at -30 mV in hexamethonium is similar to that of the control experiments at the same voltage. **C**, Mecamylamine inhibits the acetylcholine-induce response at -30 mV; however, by a reduced factor of three (22.5 ± 8.8% remaining current at -30 mV vs. 7.7 ± 3.3% at -60 mV). **D**, Alpha-conotoxin ImI blocks the acetylcholine-induced current equally well (14.0 ± 3.1% and 17.1 ± 8.5% current remaining at -60 mV and -30 mV, respectively). **E**, As with the case at -60 mV, the co-application of mecamylamine and \( \alpha \)-conotoxin ImI eliminated the current at -30 mV. **F**, Summary data comparing each antagonist at both resting (-60 mV; replotted from Fig. 4) and depolarized (-30 mV) potentials, reaches the level of significance for hexamethonium (hex) (p<0.0005, 1-tailed unpaired Student’s t-test) and mecamylamine (mec) (p<0.05, 1-tailed Mann-Whitney U-test), but not in control or \( \alpha \)-conotoxin ImI (ImI).
Figure 6. Intracellular and extracellular responses of bag cell neurons to acetylcholine within the intact cluster from the abdominal ganglion.

A, Under sharp-electrode current-clamp, an individual bag cell neuron is depolarized to a small extent by bath-applied acetylcholine (Ach) (1 mM), but not sufficient to induce action potentials. B, With a 30-min pretreatment in 2 µM of the acetylcholinesterase inhibitor, neostigmine, the acetylcholine-induced response is increased dramatically. C, In seven of the 17 neurons within individual bag cell neuron clusters from different animals, the acetylcholine-induced depolarization was sufficient to cause maintained action potential firing. D, The addition of neostigmine to the bath significantly increased the acetylcholine response of individual bag cell neurons within the intact cluster four-fold (from 6.1 ± 2.2 mV to 24.8 ± 2.6 mV) (p<0.01, 2-tailed Mann-Whitney U-test). E, Approximately 1 min after bath application of acetylcholine, the bag cell neuron cluster undergoes an afterdischarge characterized by the presence of extracellular spikes. F, Summary data showing an average acetylcholine-induced afterdischarge of 6.9 ± 1.2 min.

Figure 7. Pre- and post- acetylcholine responses to cerebral ganglion stimulation.

A, Left, Summary data indicating an afterdischarge duration of 14.6 ± 3.0 min in a control group of 25 clusters following cerebral ganglion stimulation. Right, For preparations previously exposed to bath-applied 1 mM acetylcholine in 2 µM neostigmine (post Ach), only two of 11 clusters show an afterdischarge. Compared to the control group, which consistently presented an afterdischarge, the frequency of afterdischarge generation subsequent to acetylcholine was significantly less (p<0.0001, 2-tailed Fisher’s exact test). B, Simultaneous intra and extracellular recording show that subsequent cerebral ganglion stimulation in nine of 11 intact clusters, previously exposed to acetylcholine in 2 µM neostigmine, failed to excite either the single cell or intact cluster past the period of stimulation. Small amplitude spikelets appear half way through the stimulation in the intracellular record. C, Left, An example of an intracellular recording (from a bag cell neuron in the cluster) during cerebral ganglion stimulation leading to an afterdischarge (in neostigmine). Right, Following a stimulation-induced
afterdischarge, acetylcholine hyperpolarized the single bag cell neuron within the cluster. In three other
cases, the cell either failed to respond to acetylcholine, or depolarized slightly without inducing action
potentials.

Figure 8. Response of the bag cell neuron cluster to cerebral ganglion stimulation.

A, Left, Extracellular recording from an intact bag cell cluster within the abdominal ganglion shows an
afterdischarge following stimulation of the right cerebral ganglion (recording truncated at ~4 min; actual
burst lasted >30 min). Right, Ten min after cessation of the first afterdischarge, the bag cell neuron
cluster is refractory to a second stimulation. B, Left, Pretreatment with 100 µM mecamylamine and 1µM
α-conotoxin ImI prevents an afterdischarge to a 30-sec stimulus at the cerebral ganglion. Right, Wash of
the ganglion with nASW for 30 min, followed by the same cerebral stimulus elicited an afterdischarge,
albeit shorter in duration. C, Left, Summary data reveals that the afterdischarge duration in control is
21.0 ± 3.9 min, where as pretreatment with nicotinic antagonists (mec/ImI) elimated the burst in five of
seven clusters. In three of the five bag cell neuron clusters blocked by mecamylamine/α-conotoxin ImI
pretreatment, subsequent washout allows for an average 6.7 ± 0.5 min afterdischarge upon cerebral
stimulation; duration expressed as the average of these three. Right, Compared to control, the frequency
of afterdischarge generation was rendered significantly less by addition of mecamylamine and α-
conotoxin ImI (p<0.007, 2-tailed Fisher’s exact test).
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Figure 2

A. HP = -60 mV

10 μM Ach
100 μM
300 μM
1 mM

200 pA
20 s

B. Current density (pA/pF)

[H] Log M

[Ac] 267 μM
Hill: 0.7

C. Control

Peak Ach (1 mM)

200 pA
60 ms

-60 mV
-70 mV

D. % ΔG

(n=9)

Control
Ach

E. HP = -60 mV

OxO

Glycine

40 pA
10 s

F. pApF

Ach
TMA
Nicotine
OxO
Glycine
Carbachol
Choline
5-HT

Bars represent means ± SEM.
Figure 3

**A** control GDP-β-S

![Graph showing current density (pA/pF) with Ach](image)

**B** Ach

![Bar graph showing current density (pA/pF) with control and GDP-β-S](image)

**C** control GDP-β-S

![Graph showing current density (pA/pF) with FMRFamide](image)

**D** FMRFamide

![Bar graph showing current density (pA/pF) with control and GDP-β-S](image)
Figure 5

A  
HP -30 mV
2nd Ach application
1st Ach application
500 pA
10 s

B  
Ach
hexamethonium (500 µM)
100 pA
20 s

C  
Ach
mecamylamine (100 µM)
1 nA
10 s

D  
Ach
α-conotoxin lml (1 µM)
100 pA
20 s

E  
Ach
mecamylamine/α-conotoxin lml
200 pA
10 s

F  
remaining Ach current (%)

control  hex  mec  lml  meclml
-60 mV  26  8  5  6  5
-30 mV  6  6  8  5  6

* Significant difference

Note: The figure shows the effects of Ach and various compounds on the remaining Ach current at different membrane potentials.
Figure 6

Ach (1 mM)

Ach (in neostigmine)

Ach (in neostigmine)

Ach-induced $\Delta V_m$ (mV)

Ach-induced duration (min)