Supporting Information

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SI Methods

Degenerate PCR Cloning. The two cytoplasmic domains comprising the catalytic domains of adenylyl cyclase (AC) are well-conserved across all species (1). The sequences corresponding to GDCYYC and WQ(Y/F) DVW from the first conserved cytoplasmic domain, C1a and KIKTIGS, and WG (N/K) TVN from the conserved cytoplasmic domain, C2a, were used to design degenerate primers. To minimize the degree of degeneracy in the primers, the neutral base inosine was used when codons for conserved amino acids were fully degenerate in the third codon position. (All primers are listed at the end of *SI Methods*.)

RACE. Primers for 5′ and 3′ RACE for AC-*Apl*A, AC-*Apl*B, and AC-*Apl*C were designed based on clones isolated from degenerate PCR cloning. Initially, a small segment of the AC-*Apl*D C2a domain was identified by BLAST analysis of the *Aplysia* EST database (2) [National Center for Biotechnology Information (NCBI)]; a portion of ESTs EB257189.1 and EB335326 are similar to the C2a domains of transmembrane ACs. RACE primers are listed at the end of *SI Methods*. First-strand cDNA synthesis was completed using the BD Smart RACE cDNA Amplification Kit (Clontech). Following the manufacturer's directions, touchdown PCR was used to clone the remainder of the four *Aplysia* ACs (5 cycles: 94 °C for 30 s and 72 °C for 3 min; followed by 5 cycles: 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; followed by 27 cycles: 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min). The RACE PCR products were subcloned into the pCR 2.1 TOPO TA vector (Invitrogen). Constructs were sequenced by the Biopolymer/Genomics Core Facility, University of Maryland, Baltimore.

Domain Expression and Purification. Sequences for individual domains of each AC obtained by PCR were initially inserted into the pCR 2.1 vector (Invitrogen) and then inserted into expression vector pET32A (Novagen), which contains a His_6 tag. Constructs were transformed into Rosetta-gami cells (Novagen) for protein expression. Expression was induced with 1 mM IPTG. All buffers for domain purification contained 50 μg/mL PMSF, 1 mM benzamidine, and 3 mM TCEP [Tris(2-carboxyethyl) phosphine hydrochloride (for inhibition of proteases)]. Cell pellets were resuspended in buffer A (10 mM Na-Hepes, pH 7.6, 100 mM Na-phosphate) with addition of 5 mM imidazole, 100 mM NaCl, and 8 M urea. Pellets were sonicated three times for 10 s at power level 5 (550 Sonic Dismembrator; Fisher Scientific). The preparations were centrifuged at 10,000 × *g* for 5 min and the supernatants were applied to an Ni-NTA Superflow (Qiagen) column equilibrated with buffer A with 5 mM imidazole and 100 mM NaCl. To eliminate nonspecific binding, the column was washed with buffer A with 20 mM imidizaole and 300 mM NaCl. The protein was eluted with buffer A with 250 mM imidazole.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). Sequences of the primers are as follows:

Mutant 1. 5′-ACAAACTGTCTTCCAAGGGCGTGACCAACTGCGACATCCGCCT-3′
Mutant 2. 5′-AACTGCGACATCCGCCTGATGCAGTCCGACAAGTTCAACGCG-3′
Mutant 3. 5′-ATCCGCCTGATGCAGTCCGACAAGTTCAACGCGG-3′.

Calmodulin Overlay Assays. For calmodulin (CaM) overlay experiments, purified His_6-tagged cytoplasmic domains were separated by SDS/PAGE and transferred to nitrocellulose membranes (see *SI Methods* for details). Membranes were incubated overnight at 4 °C with binding buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM $CaCl_2$, 50 mM $MgCl_2$, and 1% BSA), and then probed with 100 ng/mL biotinylated CaM (Calbiochem) for 2 h, following manufacturer's instructions. For controls, 10 mM EGTA was added to all solutions to block CaM binding. CaM binding was visualized using streptavidin-conjugated alkaline phosphatase and chromogenic substrate BCIP/NBT (KPL).

Expression of Recombinant *Aplysia* ACs Using Baculovirus. Full-length ACs were cloned by PCR from *Aplysia* cDNA and inserted into a transfer vector, either BacPAK vector (Clontech) or pFastbac (Invitrogen). For the Clontech kit, vector DNA containing an AC gene was transfected into Sf21 cells along with BacPAK6 viral DNA provided in the kit. For the Invitrogen kit, the pFastbac vector containing an AC gene was transformed into DH10Bac cells containing a baculovirus shuttle vector (bacmid) with a mini*att*Tn7 target site and a helper plasmid. Recombinant viral clones were isolated twice to ensure purity. BTI-TN-5B1-4 insect cells (High Five cells; Invitrogen) were cultured in suspension at 27 °C in Ex-cell 405 medium (JRH Bioscience) supplemented with 50 μg/mL gentamicin and 0.1% pluronic F-68 (Invitrogen). High Five cells at a density of 1×10^6/mL were infected with baculovirus encoding an *Aplysia* AC gene or β-gal (as a control) at 1 plaque-forming unit per cell, and harvested after 48 h.

In the case of AC-*Apl*B, despite several independent attempts at expression, we obtained variable and very modest increases in AC activity as compared with membranes from High Five cells infected with baculovirus encoding β-gal. This may reflect an important difference in processing of the AC-*Apl*B protein between the High Five cells and *Aplysia* neurons, or possibly an error in the sequence that we identified (which was verified three times from independent groups of animals).

Membrane Preparation. High Five cells expressing recombinant *Aplysia* ACs were centrifuged at 300 × *g* for 5 min and resuspended at a density of 4×10^6 cells/mL in lysis buffer (20 mM Na-Hepes, pH 7.6, 49 mM NaCl, 16 mM KCl, 7.6 mM MgCl, 1 mM EGTA, 1 mM EDTA, and 1 mM DTT) with protease inhibitor mixture (10 μg/mL leupeptin, 10 μg/mL aprotinin, 25 μM *p*-nitrophenyl-*p*′-guanidinobenzoate hydrochloride, and 1 mM benzamidine). Cells were disrupted by nitrogen cavitation, equilibrating them at 750 psi on ice with shaking for 10 min. The cell lysate was centrifuged at 1,000 × *g* for 2 min to remove nuclei and large membrane fragments. The supernatant was centrifuged at 30,000 × *g* for 20 min. The pellet was rehomogenized in membrane resuspend buffer (50 mM K-Hepes, pH 7.6, 75 mM KCl, 1 mM DTT, and protease inhibitor mixture) and recentrifuged. The final pellet was suspended in resuspend buffer at 9×10^6 cells/mL. Membrane aliquots were stored in liquid nitrogen.

Immunoblotting. For immunoblots, desheathed pleural ganglia or sensory neuron (SN) clusters were homogenized in 8 M urea; in some experiments, ganglia were homogenized in buffer with protease inhibitor mixture. This protease inhibitor mixture inhibits proteolysis, whereas urea blocks it completely; therefore, the shorter form of AC-*Apl*A observed in immunoblots (Fig. 3*A*) is unlikely to be an artifact of degradation during processing. Membranes from whole *Apl*ysia CNS ganglia and High Five cells expressing either

AC-*Apl*A, AC-*Apl*B, or AC-*Apl*C were prepared as described above. Equal amounts of protein were resolved on 8% (wt/vol) SDS/PAGE gel at 4 °C and transferred to a nitrocellulose membrane. To prevent nonspecific binding, nitrocellulose membranes were incubated with immunoblot buffer (0.1% Tween 20 in PBS, pH 7.6) with 5% milk powder overnight at 4 °C. The membranes were incubated with primary antibody against one of the three AC C2b domains (AC-*Apl*A-C2, 1:2,000; AC-*Apl*B-C2, 1:500; or AC-*Apl*C-C2, 1:1,000) in immunoblot buffer at room temperature for 2 h. As a control, primary antibody was preincubated with the corresponding antigen peptide (4 μg/mL). The membranes were washed with immunoblot buffer six times and incubated in HRP-coupled goat anti-rabbit secondary antibody (KPL), diluted 1:10,000 in immunoblot buffer for 1 h, and washed with immunoblot buffer six times. Membranes were developed using Super-Signal West Femto Maximum Sensitivity Substrate (Pierce) and visualized using a Molecular Imager (Bio-Rad).

Ca^{2+}/Mg^{2+} Buffers. Ca^{2+} buffers were designed with an iterative computer program using affinity constants from Blinks et al. (3), following the approach of Yovell and Abrams (4). Ca^{2+} buffers were prepared at three times their final concentration to produce a final total EGTA concentration in the AC assay solutions of 1 mM. Buffers were calibrated with a Ca^{2+} absorbance indicator, either 10 μM BAPTA (for low-Ca^{2+} buffers) or 25 μM 5,5′-dibromo-BAPTA (for high-Ca^{2+} buffers), with absorbance measured at 254 or 264 nm, respectively. Free Ca^{2+} was calculated using an apparent K_d of 167 nM for Ca^{2+}-BAPTA and 1.44 μM for Ca^{2+}-dibromo-BAPTA at an ionic strength of 100 mM (5).

Assays of AC Activity. Assays of AC activity (6) in High Five cell-membrane preparations were carried out in 60 μL. To reduce Ca^{2+} contamination, the assays contained no ATP-regenerating system, and therefore assay times were shortened to 4 min. Assay temperature was 30 °C. Assays were conducted in Ca^{2+}/Mg^{2+}/EGTA buffers ± 1 μM CaM (6). AC assay solution included 10 μM [α^{32}P] ATP (80 μCi/mL), 50 μM [^{3}H]cAMP (~3×10^5 cpm/mL), 0.5 mM IBMX, 3 mM $MgCl_2$, 75 mM KCl, 1 mM EGTA, 50 mM K-Hepes (pH 7.6), 1 mM DTT, and protease inhibitor mixture (described above). Assays were terminated by addition of unlabeled ATP and cAMP, plus sodium lauryl sulfate (7). cAMP was separated from precursor ATP as described by Salomon (7). The [^{3}H]cAMP enabled normalization for recovery following chromatography.

Quantitative Real-Time PCR. For qRT-PCR, total RNA from SN clusters or CNS ganglia was purified using a NucleoSpin RNA XS Kit (Macherey Nagel) or RNAqueous columns (Ambion), respectively. Full-length *Aplysia* AC genes were cloned into PCR 2.1 vectors as templates for standard curves. The concentrations of full-length cDNAs were calculated based on UV absorbance. Serial dilutions of full-length AC templates (ranging from 8×10^4 to 5 copies per reaction) for each AC isoform were used for the standard curve. The thermocycler protocol was 95 °C for 10 min and 40 cycles of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 30 s, and 1 cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. Quantitative RT-PCR was performed using Brilliant SYBR Green QPCR Master Mix (Stratagene) on an MX3000P thermocycler (Stratagene). Melting-curve assays were then performed to detect the formation of primer-derived dimers.

Immunocytochemistry. Dissociated SNs from the ventro-caudal (VC) cluster in the pleural ganglion were cultured either alone or together with L7 motor neurons (MNs) (8). Cultures were fixed either 24 h or 3 d after plating for SNs cultured alone or for SN-MN cocultures, respectively. Cells were fixed with 4% paraformaldehyde in 30% sucrose in PBS (pH 7.6) for 1 h at room temperature (9). The preparations were washed twice with 30% sucrose and 0.4% Triton X-100 in PBS, and twice with 0.4% Triton X-100 in PBS. Free aldehyde was quenched with 50 mM NH_4Cl in 0.4% Triton X-100/PBS and the preparations were washed three times with 0.4% Triton X-100 in PBS. To block nonspecific binding sites, the preparations were treated with 10% goat serum in PBS at 4 °C overnight before incubating with primary antibody. The cultured SNs were incubated with primary antibodies (AC-*Apl*A-C2, 1:100; AC-*Apl*B-C2, 1:50; or AC-*Apl*C-C2, 1:40) in 200 μL of 10% goat serum in PBS at 4 °C overnight. The preparations were washed six times with 0.4% Triton X-100 in PBS. For controls, the antibodies were preincubated with the antigen peptides for 30 min before the final dilution. The preparations were washed extensively with 0.4% Triton X-100 in PBS, and incubated with Alexa 568-conjugated goat anti-rabbit antibody (2 μg/mL) (Invitrogen) overnight at 4 °C. Immunofluorescence was visualized using a Zeiss 410 confocal laser scanning microscope.

Morpholino Selection. Morpholino oligonucleotides, 25 bp in length, targeted to the initial coding sequence of each AC, were designed for each AC based on the manufacturer's recommendation (Gene Tools). For AC-*Apl*A, the morpholino sequence corresponded to −13 to 12 nucleotides. For AC-*Apl*B, the morpholino sequence corresponded to −37 to −12 nucleotides. For AC-*Apl*C, the morpholino sequence corresponded to −1 to 24 nucleotides. The sequences are as follows:

AC-*Apl*A 5′-$^{-13}$CTGCACCCGCATCAGGCCAACAGAT^{+12}-3′
AC-*Apl*B 5′-$^{-37}$GCTACTGGTATTTTTATTTGCAGAC^{-12}-3′
AC-*Apl*C 5′-$^{-1}$GTCTGAGTGGTGGTTGTTTTCCATT^{+24}-3′.

dsRNA Synthesis. The hypervariable TM2 domains of individual ACs, 650–850 nucleotides in length, were used to generate dsRNA. The T7 promoter was added to both the 5′ and 3′ ends of the TM2 domain-coding sequence by PCR, and RNA was transcribed in vitro using T7 polymerase (Ambion) following the manufacturer's protocol, except that the RNA was eluted with water. The sense strand and its complementary strand were hybridized at 75 °C for 5 min to generate dsRNA.

dsRNA and Morpholino Oligo Injections and Electrophysiology. In pilot experiments in which we injected siRNA for AC-*Apl*A into the hemocoel of intact animals and measured AC activity, we observed highly variable but significant knockdown of CaM-stimulated AC activity after 4 d, but not after 2 d. Therefore, all electrophysiological studies involving knockdown of ACs were done 4 d after intracellular injection of dsRNA or morpholinos.

Aplysia californica (*Aplysia* Resource Facility), weighing 60–80 g, were anesthetized by injection of isotonic $MgCl_2$, and their pleural-pedal ganglia were removed. Ganglia were secured with minuten pins in a chamber with a 1:1 mixture of $MgCl_2$ and artificial sea water. Pleural ganglia were desheathed to expose the ventro-caudal (VC) SN cluster; the clusters were separated and transferred to a new Petri dish with normal culture medium containing 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM Na-Hepes (pH 7.6) supplemented with nutrients [7 mM glucose, MEM essential and nonessential amino acids (0.2 × normal concentration; Invitrogen), and MEM vitamin solution (0.7× normal concentration; Invitrogen)], which was used both for microinjection and recording. For pressure injection, SNs in VC clusters were penetrated with beveled glass microelectrodes (7–10 MΩ) containing either morpholino oligos (600 μM) or dsRNA (500 ng/μL) in intracellular injection solution (400 mM KCl and 20 mM K-Hepes, pH 7.3). Alexa dextran 594 (10,000 MW, 7.5 mg/mL) was coinjected for detection of successfully injected cells. Some SNs were injected with Alexa dextran 594 alone or nonsense fluorescent morpholino as negative controls. After injection, SN clusters were transferred to L15 culture medium with 50% hemolymph and maintained in an incubator at

15 °C. Four days later, the cell clusters were brought out to room temperature for spike-broadening experiments conducted in normal culture medium with tetraethylammonium (TEA) and nifedipine. Neurons that were fluorescent were selected for recording with microelectrodes (12–20 MΩ) filled with 2 M KCl and 400 mM K-acetate. SN action potentials were elicited by injection of 2-ms depolarizing current pulses at a 15-s interval in the presence of 100 mM TEA and 20 μM nifedipine. TEA at this concentration blocks $I_{Kv,early}$ and nifedipine blocks dihydropyridine-sensitive Ca^{2+} channels, both of which are modulated by PKC (10, 11); under these conditions, 5-HT-induced spike broadening is entirely mediated by cAMP (12). Spike duration, measured from the peak of the action potential to the time at which it had decayed to 33% of the maximum amplitude, was recorded before and after the application of 10 μM 5-HT. Data were acquired digitally with a Modular Instruments interface and analyzed using Spike software (Hilal Associates).

Statistical Analysis. Statistical analyses were performed using SPSS software. Analysis of effects of Ca^{2+} on AC activity or of effects of morpholinos or dsRNA was done with ANOVA. Posthoc pairwise comparisons of treatments were done using the Sidak adjustment for multiple comparisons. Data are expressed as means ± SEM for means of multiple independent experiments. Data for individual representative experiments are expressed as means ± SD of results of replicate trials.

Primers.

Degenerate primers.

C1a domain	F: ATCAAGCTCGAGGG(I)GA(C/G)TG(C/T)TA(C/T)TA(C/T)TG (GDCYYC)
	R: CACGTCCTCGAGCCA(I)AC(A/G)TC(A/G)(C/G)A(C/T)TGCCA (WQ(Y/F)DVW)
C2a domain	F: CGGCAGCTCGAGAA(A/G)AT(A/C/T)AA(A/G)AC(I)(A/G)T(I)GG (KIKT(I/V)G)
	R: CCGGGACTCGAGAC(A/G)TT(I)AC(I)GT(I)TT(I)CCCCA (WG(N/K)TVN)
C1a to C2a domains	F: ATCAAGCTCGAGGG(I)GA(C/G)TG(C/T)TA(C/T)TA(C/T)TG (GDCYYC)
	R: CCGGGACTCGAGAC(A/G)TT(I)AC(I)GT(I)TT(I)CCCCA (WG(N/K)TVN)

RACE Primers.

AC-AplA.

5′ Outer primer	AGCTTCACGCCTGTCTGGTCGCACACGC
5′ Inner primer	ATGCGGCGTGGTTGGCGCGTGCCTC
3′ Outer primer	TTCCCACCCATGTGCTCGGCATCCTGGC
3′ Inner primer	ACAGGGAGCAGGAGTGGACGCTGAGACTGG
3′ Outer primer	TGGCATCAATGTGGGTCCTGTGGTGGCTGG
3′ Inner primer	TGTGATCGGGGCAAGGAAGCCTCAGTACG

AC-AplB.

5′ Outer primer	CCACGTCATACTCGCCGTCCAGGCAGTCC
5′ Inner primer	GACATCGCGGATGGCGTCCAGCATGCGC
3′ Outer primer	CACTGGACTGCACCGAGGACAAGCCGG
3′ Inner primer	ATCAGGACGACGAGGCAGGGGAGAGAACG

AC-AplC.

5′ Outer primer	CCGTCTCCCGGCTCCAGGTCGTAGTCTCCG
5′ Inner primer	GCCCACGCGCATGTTCACGTTGGTGCCAG
3′ Outer primer	GTGTGCATCCGGGCTGACCAGCCGCACC
3′ Inner primer	GGTCCGGTGGTTGCTGGAGTGATTGGGGCC

AC-AplD.

5′ Outer primer	CGGGGCATCTTCGCCACCATCGTCAACTTCAACGAGTTCTATG
5′ Inner primer	AGACCAACCGCAGCTACTCCCGCAACCACGCGG
3′ Outer primer	CGGGGCATCTTCGCCACCATCGTCAACTTCAACGAGTTCTATG
3′ Inner primer	CATAGAACTCGTTGAAGTTGACGATGGTGGCGAAGATGCCCCG

Real-Time PCR Primers.

AC-*Apl*A	F: ACAGACTCCAAGCACAAG
	R: AAGAATAAGAGGAAGACCAATC
AC-*Apl*B	F: ACCTCCTCTCCTTGACCTC
	R: GCTATCACTGCCTCTACTGG
AC-*Apl*C	F: GAAGCACAGCAACGAGAGG
	R: GTGAAGCCGCAGATGTCC
AC-*Apl*D	F: GAACTAGAGAACGCCTAC
	R: AGATGATGTTGCTGATGG

dsRNA primers.

AC-*Apl*A	F: TAATACGACTCACTATAGGCAGTTCTCCCCGCAGGACTTGG
	R: TAATACGACTCACTATAGGCACCTCCTGCTCCCTGTTCTGTAGC
AC-*Apl*B	F:TAATACGACTCACTATAGGCAGTTCTACGTCATGATCACCTTCGTC
	R: TAATACGACTCACTATAGGCAGGAAATGCATGGTGATCAGC
AC-*Apl*C	F: TAATACGACTCACTATAGGCCCATATAACAGTTCTCATGGTGGC
	R: TAATACGACTCACTATAGGGCTTCCCATCTCAGAGGTATCC

1. Premont RT (1994) Identification of adenylyl cyclases by amplification using degenerate primers. *Methods Enzymol* 238:116–127.
2. Moroz LL, et al. (2006) Neuronal transcriptome of *Aplysia*: Neuronal compartments and circuitry. *Cell* 127:1453–1467.
3. Blinks JR, Wier WG, Hess P, Prendergast FG (1982) Measurement of Ca^{2+} concentrations in living cells. *Prog Biophys Mol Biol* 40:1–114.
4. Yovell Y, Abrams TW (1992) Temporal asymmetry in activation of *Aplysia* adenylyl cyclase by calcium and transmitter may explain temporal requirements of conditioning. *Proc Natl Acad Sci USA* 89:6526–6530.
5. Harrison SM, Bers DM (1987) The effect of temperature and ionic strength on the apparent Ca-affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. *Biochim Biophys Acta* 925:133–143.
6. Yovell Y, Kandel ER, Dudai Y, Abrams TW (1992) A quantitative study of the Ca^{2+}/calmodulin sensitivity of adenylyl cyclase in *Aplysia*, *Drosophila*, and rat. *J Neurochem* 59: 1736–1744.
7. Salomon Y (1979) Adenylate cyclase assay. *Adv Cyclic Nucleotide Res* 10:35–55.
8. Rayport SG, Schacher S (1986) Synaptic plasticity in vitro: Cell culture of identified *Aplysia* neurons mediating short-term habituation and sensitization. *J Neurosci* 6: 759–763.
9. Martin KC, et al. (1997) MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in *Aplysia*. *Neuron* 18:899–912.
10. Braha O, Edmonds B, Sacktor T, Kandel ER, Klein M (1993) The contributions of protein kinase A and protein kinase C to the actions of 5-HT on the L-type Ca^{2+} current of the sensory neurons in *Aplysia*. *J Neurosci* 13:1839–1851.
11. Sugita S, Baxter DA, Byrne JH (1994) Activators of protein kinase C mimic serotonin-induced modulation of a voltage-dependent potassium current in pleural sensory neurons of *Aplysia*. *J Neurophysiol* 72:1240–1249.
12. Dumitriu B, Cohen JE, Wan Q, Negroiu AM, Abrams TW (2006) Serotonin receptor antagonists discriminate between PKA- and PKC-mediated plasticity in *Aplysia* sensory neurons. *J Neurophysiol* 95:2713–2720.

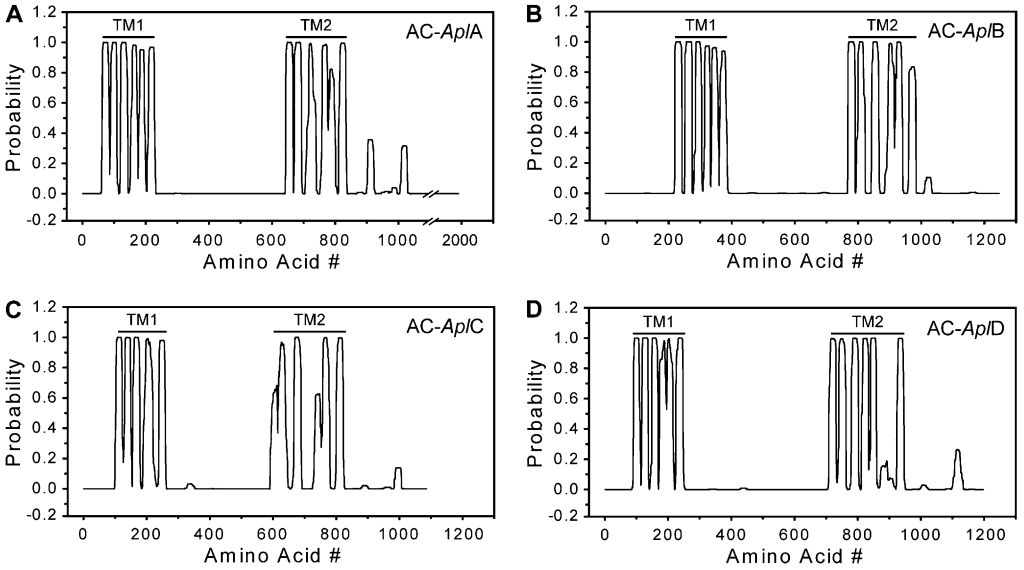

Fig. S1. Predicted transmembrane domain of four putative *Aplysia* ACs. Membrane-spanning α-helices were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Note each AC isoform has two predicted transmembrane domains, each of which contains a series of membrane-spanning α-helices. Other algorithms gave qualitatively similar results, with the number of predicted α-helices within each transmembrane domain ranging between 5 and 7.

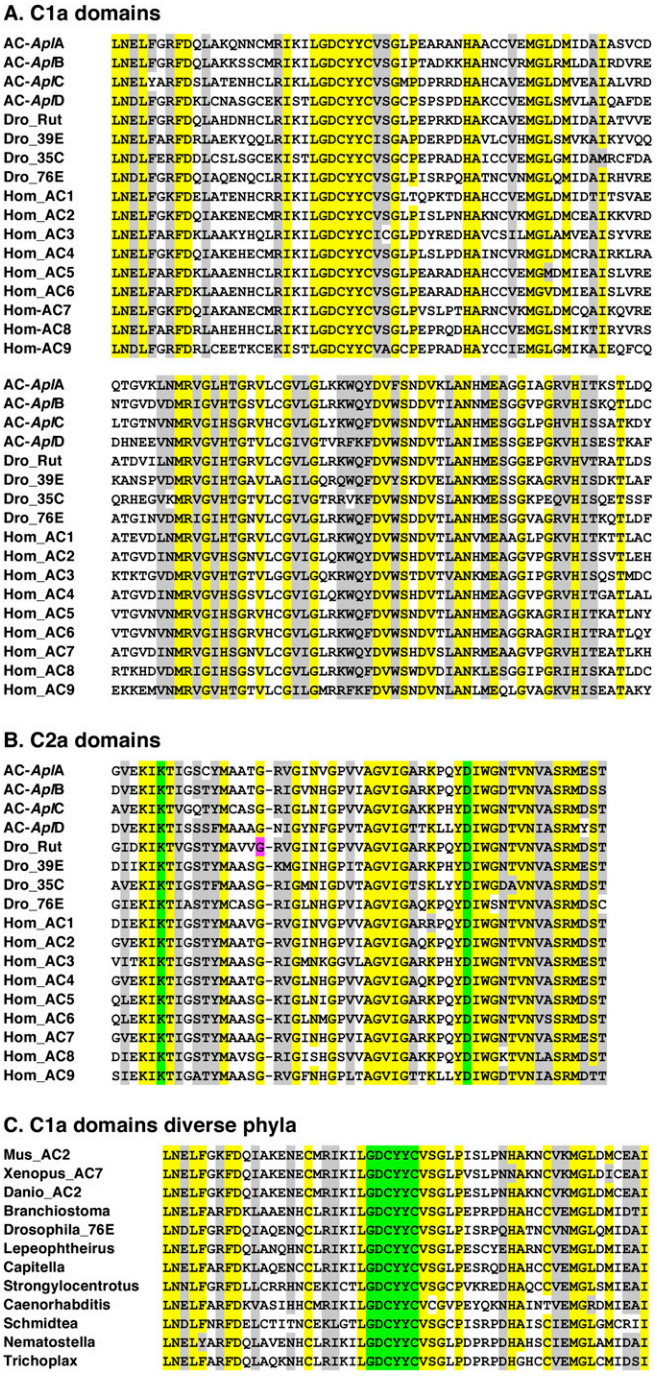

Fig. S2. Clustal analysis of highly conserved sequences within the ATP-binding regions of the C1 and C2 cytoplasmic domains of transmembrane ACs from *Aplysia*, *Drosophila*, humans, and diverse phyla. (*A*) Clustal analysis of a highly conserved sequence within the C1a domain of ACs from *Aplysia*, *Drosophila*, and humans. (*B*) Clustal analysis of two highly conserved sequences within the C2a domain of ACs from *Aplysia*, *Drosophila*, and humans; a gap between these two sequences is indicated by a dash. Yellow highlighting: identical amino acids conserved in 90% or more of the AC isoforms. Gray highlighting: conserved substitutions. In *B*, lysine and aspartate residues highlighted in green distinguish adenylyl cyclases from guanylyl cyclases (1). In the *Drosophila rutabaga* learning mutant, Gly1026, highlighted in purple, is altered to Arg, resulting in complete loss of rutabaga AC catalytic activity (2). (*C*) Clustal analysis of C1a domains from eight phyla, illustrating conservation of GDCYYC sequence (green highlighting). All sequences are from the NCBI, except for sequences from the Joint Genome Institute (JGI), which are indicated (http://genome.jgi-psf.org). For *C*, the species, (common name), gene or protein ID, and phylum are as follows:

Mus musculus (mouse) AC2 (Chordata)

Xenopus tropicalis (frog) AC7 (Chordata)

Danio rerio (zebrafish) AC2 (Chordata)

Branchiostoma floridae (lancelet) XM_002586782.1 (Cephalochordata, Chordata)

Drosophila melanogaster (fruit fly) 76E (Arthropoda)

Lepeophtheirus salmonis (copepod, crustacean) ADND01120628.1 (Arthropoda)

Capitella teleta (polychaete), JGI Protein ID 155468 (Annelida)

Strongylocentrotus purpuratus (sea urchin) AAGJ02145453.1 (Echinodermata)

Caenorhabditis japonica (nematode) ABLE02010385.1 (Nematoda)

Schmidtea mediterranea (planaria) AAWT01029706.1 (Platyhelminthes)

Nematostella vectensis (sea anemone) JGI Protein ID 234384 (Cnidaria)

Trichoplax adhaerens JGI Protein ID 21914 (Placozoa).

1. Baker DA, Kelly JM (2004) Structure, function and evolution of microbial adenylyl and guanylyl cyclases. *Mol Microbiol* 52:1229–1242.
2. Levin LR, et al. (1992) The *Drosophila* learning and memory gene rutabaga encodes a Ca^{2+}/calmodulin-responsive adenylyl cyclase. *Cell* 68:479–489.

C1a

A	*B*	*C*	*D*	Rut	39E	35C	76E	1	2	3	4	5	6	7	8	9	
	53	63	39	72	48	50	55	66	57	51	52	68	68	53	55	42	AC-*AplA*
		52	38	56	47	38	59	56	57	53	57	53	52	54	56	39	AC-*AplB*
			41	66	53	41	52	64	57	58	51	73	72	53	62	42	AC-*AplC*
				42	40	53	39	43	36	39	36	40	40	36	38	53	AC-*AplD*
					53	44	59	69	59	54	54	68	68	56	62	45	Dro-Rut
						43	49	49	46	61	46	50	50	44	53	41	Dro-39E
							40	40	36	41	38	40	39	35	42	51	Dro-35C
								54	56	49	52	58	57	53	52	39	Dro-76E
									57	54	54	69	68	56	61	42	Hom-AC1
										48	72	58	57	72	50	36	Hom-AC2
											48	56	56	47	57	42	Hom-AC3
												54	53	68	50	36	Hom-AC4
													93	57	64	42	Hom-AC5
														58	62	43	Hom-AC6
															50	38	Hom-AC7
																42	Hom-AC8
																	Hom-AC9

C2a

A	*B*	*C*	*D*	Rut	39E	35C	76E	1	2	3	4	5	6	7	8	9	
	49	56	43	60	52	45	50	62	55	53	55	60	60	53	57	46	AC-*AplA*
		49	40	46	46	43	55	48	56	48	56	50	50	55	49	44	AC-*AplB*
			43	55	49	45	51	54	54	53	51	67	65	52	56	43	AC-*AplC*
				43	42	54	39	43	43	40	42	44	44	41	46	53	AC-*AplD*
					48	42	51	60	54	50	54	59	59	53	58	42	Dro-Rut
						40	49	52	51	61	53	49	48	53	51	45	Dro-39E
							43	42	45	42	42	46	45	41	42	56	Dro-35C
								52	64	51	61	54	54	63	52	42	Dro-76E
									56	54	57	60	58	56	56	46	Hom-AC1
										51	77	58	58	78	56	49	Hom-AC2
											52	51	50	52	56	43	Hom-AC3
												57	57	75	54	47	Hom-AC4
													93	54	59	43	Hom-AC5
														54	58	43	Hom-AC6
															55	43	Hom-AC7
																45	Hom-AC8
																	Hom-AC9

Fig. S3. Sequence similarity among ACs from *Aplysia*, *Drosophila*, and humans. The values are percent similarity of the C1a and C2a domains determined using Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Abbreviated AC names on top are in the same sequence as AC names on the right. Human AC isoforms with the highest similarity to each *Aplysia* or *Drosophila* isoforms are indicated in red (highlighted isoforms differed by 2% or less; equally similar *Aplysia* or *Drosophila* isoforms are also highlighted). Shaded values signify high similarity in both C1a and C2a domains.

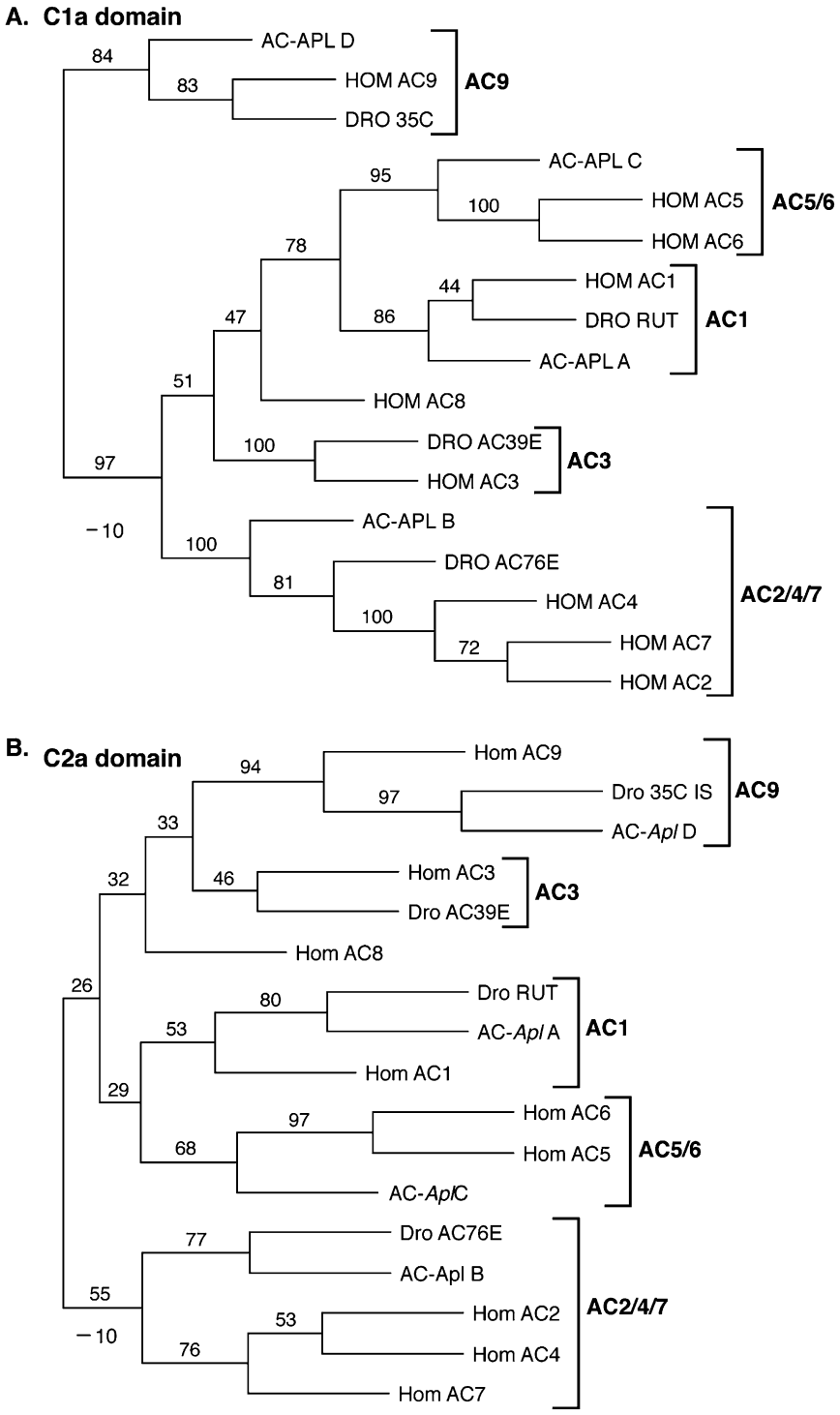

Fig. S4. Results of a PHYLIP analysis of the C1a and C2a domains of AC from humans (HOM), *Drosophila* (DRO), and *Aplysia* (APL). Brackets indicate groups of ACs based on mammalian isoforms. The numbers represent the percentage of trees generated by the PHYLIP program Neighbor (http://evolution.gs.washington.edu/phylip.html) that contained the grouping shown here (of a total of 1,000 replicate trees generated); scale bars represent 10% of trees. *C. elegans* AC NP 508018 (NCBI) was used as the outgroup for the C1a analysis and *Dictyostelium* ACA served as the outgroup for the C2a analysis.

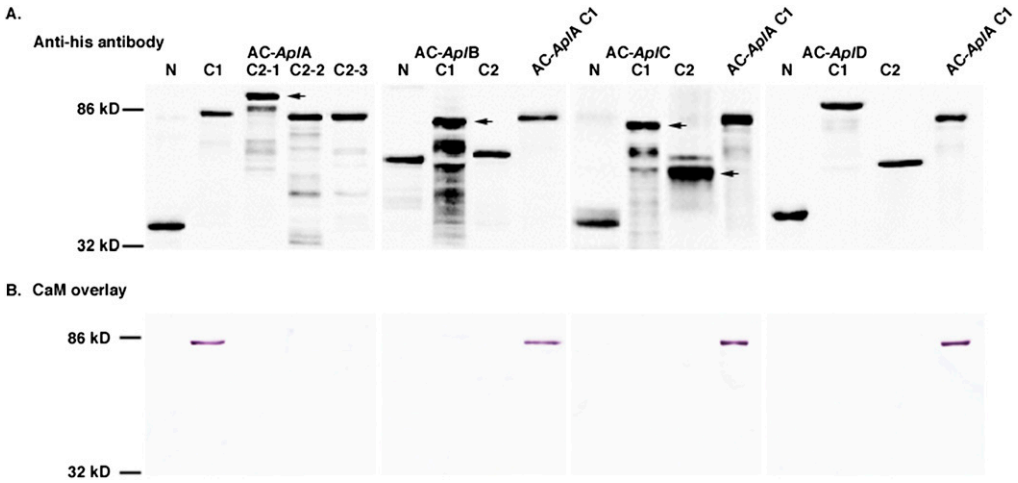

Fig. S5. CaM overlay assays identify CaM-binding domain in AC-*Apl*A. Purified His-tagged cytoplasmic domains of *Aplysia* ACs were separated by SDS/PAGE and transferred to nitrocellulose membranes. Due to its large size, the C2 domain of AC-*Apl*A was expressed as three overlapping fragments, C2-1, C2-2, and C2-3. (*A*) Nitrocellulose membranes were probed with an anti-His_6 antibody. Preparations of some domains yielded multiple bands after purification (presumably due to degradation); in these cases, the arrow indicates the band of interest. (*B*) CaM overlay assays in the presence of 1 mM Ca^{2+}. Note that only the AC-*Apl*A C1 domain binds CaM. As a positive control, the AC-*Apl*A C1 domain was included in all overlay assays together with the domains of each of the other ACs.

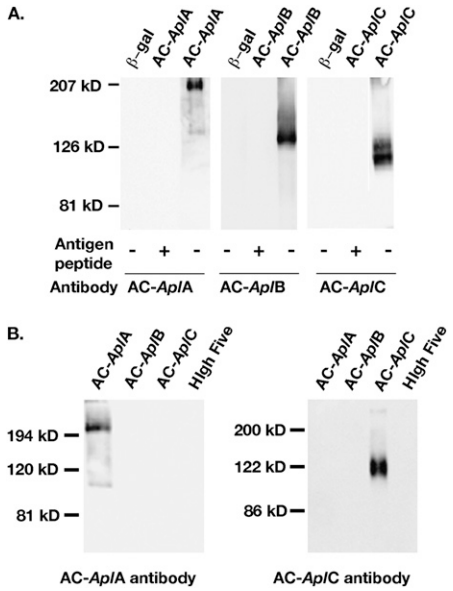

Fig. S6. Immunoblots of AC-*Apl*A, AC-*Apl*B, and AC-*Apl*C expressed in High Five insect cells. High Five cells were infected with baculovirus encoding each *Aplysia* AC isoform. (*A*) Membrane proteins from High Five cells expressing a single AC isoform were probed with an affinity-purified antibody against the corresponding AC C2b domain peptide. Control membranes were from cells infected with baculovirus encoding β-gal. As an additional control, antibodies were preincubated with the corresponding antigen peptide. (*B*) Test of antibody specificity. Antibodies raised against AC-*Apl*A or AC-*Apl*C peptides were tested with membranes from High Five cells expressing either AC-*Apl*A, AC-*Apl*B, or AC-*Apl*C. Note that each antibody was specific for the corresponding AC isoform.

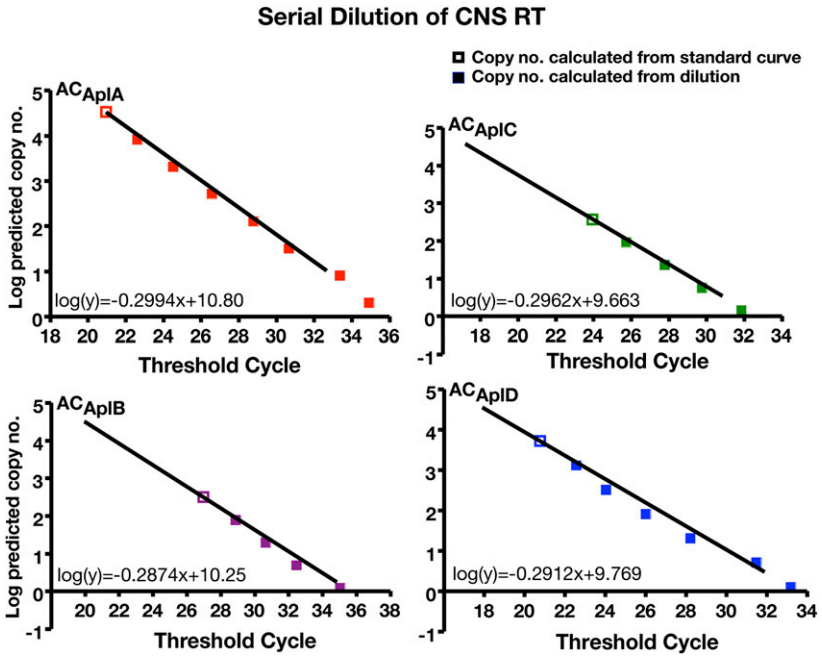

Fig. S7. Quantitative real-time PCR using cDNA from *Aplysia* CNS. Standard curves were generated using serial dilutions of plasmid DNA containing genes for each AC isoform. Equations and corresponding lines are fit to the plasmid standard curves (individual data points for standard curves are not shown). To test the linearity of the qRT-PCR technique, serial dilutions of cDNA from CNS were used as templates in the qRT-PCR. The highest copy number of cDNA from *Aplysia* CNS (open squares) was calculated from the standard curve based on the Ct value for the sample (e.g., a Ct of 21.0 for AC-*Apl*A corresponded to a gene copy number of 3.32×10^4). The copy-number values for the lower concentrations of cDNA were obtained by dividing the copy number for the highest concentration by the dilution factor; these calculated copy numbers are plotted against empirically obtained Ct values (filled squares). (See *SI Methods* for detailed methods.)

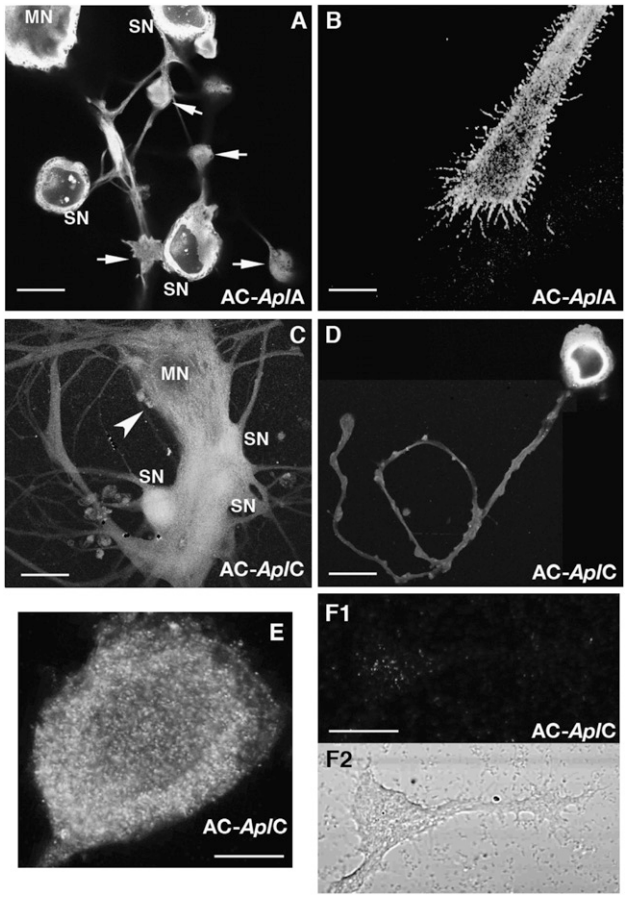

Fig. S8. Immunocytochemistry of dissociated VC cluster SNs, cultured either alone or with an L7 MN. Immunostaining with antibody against AC-*Apl*A (*A* and *B*) or AC-*Apl*C (*C*–*F*). (*A*) Both pre- and postsynaptic neurons express AC-*Apl*A in somata and processes, including regions of pre- and postsynaptic apposition, and also in growth cones (arrows). Staining within the outer circumference of somata may reflect the deep membrane invaginations (trophospongium) found in the cell bodies of these large neurons [similar patterns of staining have been observed for the receptors (1, 2)]. (*B*) Immunostaining of SN growth cone with anti-AC-*Apl*A antibody. Note the high level of AC-*Apl*A expression in growth cone. (*C*) Both pre- and postsynaptic neurons express AC-*Apl*C. (MN soma is indicated by an arrowhead.) (*D*–*F*) SN cultured alone. In *D*, the image is a montage; the SN soma is at a higher plane than the process to facilitate comparison of regions of maximal AC-*Apl*C expression. Note in *C* and *D* that AC-*Apl*C immunofluorescence is less intense in processes than in somata. (*E* and *F*) AC-*Apl*C is expressed at low levels in growth cone (*F*1) as compared with soma of the same neuron (*E*). (*F*2) Same field as *F*1 to show location of growth cone. [Scale bars, 50 μm (*A*, *C*, and *D*) and 10 μm (*B*, *E*, and *F*1).]

1. Jonas EA, Knox RJ, Kaczmarek LK, Schwartz JH, Solomon DH (1996) Insulin receptor in *Aplysia* neurons: Characterization, molecular cloning, and modulation of ion currents. *J Neurosci* 16:1645–1658.
2. Lee YS, et al. (2009) Identification of a serotonin receptor coupled to adenylyl cyclase involved in learning-related heterosynaptic facilitation in *Aplysia*. *Proc Natl Acad Sci USA* 106: 14634–14639.

Table S1. Predicted lengths of cytoplasmic domains of *Aplysia* ACs and CaM-sensitive ACs from humans (AC1 and AC8) and *Drosophila* (rutabaga)

	Total length (aa)	N	C1	C2
AC-*Apl*A	1,927	69	421	1,092
AC-*Apl*B	1,246	220	390	263
AC-*Apl*C	1,085	101	340	267
AC-*Apl*D	1,198	86	449	256
AC1	1,119	39	382	316
AC8	1,251	181	378	334
Rut	2,248	61	505	1,351

Predicted lengths of entire ACs are also included. Note that AC-*Apl*A and rutabaga AC both have unusually long C2 domains.