

Supporting Information

1: Material and Methods

1.1. Cloning and sequencing. *Aplysia californica* (60-300 g) were obtained from Marinus (Long Beach, CA) or the NIH *Aplysia* Resource Facility (Miami, FL) and kept in tanks with natural sea water at 15-18°C on a 12:12-hour light: dark cycle for up to 3 weeks. Before dissection, animals were anesthetized by injection of 60% (volume/body weight) isotonic MgCl₂ (337 mM). The molecular methods were similar to those we have described previously (1-3). Briefly, the CNS, peripheral organs or identified neurons were isolated and RNA/DNA was extracted and cloning and/or sequencing libraries were prepared as described (4, 5).

Initially, primers (5'-CGGAGTCTACGTCAGAGGATACC-3'; 5'-GACGATGCTGGGGTTCTTGCCGA-3') were designed to the HCN-like cDNA sequence from our *A. californica* expressed sequence tag (EST) database (1). The obtained fragment was extended by multiple 5'- and 3'-RACE (rapid amplification of cDNA ends) reactions (6). The complete coding region of acHCN was obtained from the CNS library by a polymerase chain reaction (PCR) using the following primers: 5'-ATGGGGCAGGAATGCGTGGCTGGA-3' and 5'-TTAAGGATCTGGTCCTTGAGATAGCCGGT-3'. The cDNA was purified using a Gel Extraction kit (Qiagen, Valencia, CA), ligated into pCR4-TOPO vector (Invitrogen, Carlsbad, CA), and transformed into One-Shot competent *E. coli* cells (Invitrogen). The clones were isolated, purified using a MiniPrep kit (Qiagen) and Sanger sequenced.

Additional validation of HCN sequences were performed using shotgun information in NCBI trace archives of the *Aplysia* genome project and following our RNA-seq data obtained from the CNS and several peripheral tissues as well as from identified neurons according to the protocols described (4).

Initial alignment of HCN proteins from different organisms was done using ClustalX (<ftp://ftp-igbmc.ustrasbg.fr/pub/ClustalX/>) with default parameters and the graphical output was produced by GeneDoc (<http://www.nrbsc.org/gfx/genedoc/gddl.htm>). The pore region, CNBD and transmembrane domains were predicted using SMART (<http://smart.embl-heidelberg.de/index2.cgi>) and TMPred (www.ch.embnet.org/software/TMPRED_form.html) respectively, and putative phosphorylation and glycosylation sites were obtained using PROSITE (www.expasy.org/cgi-bin/scanprosite).

1.2. In situ hybridization and expression analysis. *In situ* hybridization protocols were as reported for *Aplysia* preparations (1, 7, 8). pCR4-TOPO plasmid containing cDNA fragments of interest was linearized with appropriate restriction enzymes (Not I and Pme I in the case of acHCN). Transcription with T3- and T7 polymerases (Roche Diagnostics, Basel, Switzerland) was done to obtain antisense and sense (control) RNA probes respectively, following the Roche protocol for probe preparation with a DIG RNA labeling kit. RNA probes to acHCN were made to the first 1.5 kb of the channel's cDNA. This method is relatively sensitive as it allows detection of low abundance transcripts, such as *A. californica* two-pore potassium channel AcK2p1 (9, 10). Briefly, after dissection, the CNS was treated at 34°C for 30-50 min (depending on the size of an animal) with 10 µg/ml protease IX, fixed for 3-6 hours with 4 % paraformaldehyde in phosphate buffer solution (PBS, 0.1 M, pH=7.4) and desheathed. Desheathed ganglia were dehydrated by subsequent 10 min incubations in 30 %, 50 %, and 70 % methanol in PTW (0.1 % Tween 20 in PBS), then in 100 % methanol for 5 min. After re-hydration, the ganglia were treated with 10 µg/ml proteinase K (Roche) in PTW at room temperature for 1 hr, followed by post-fixation for 20 min at +4° C in 4 % paraformaldehyde and subsequent washes in 2 mg/ml glycine in PTW (2 times) and in PTW alone (2 times). Preparations were washed two times in 0.1 M triethanolamine hydrochloride, pH 8.0 (TEA HCl) and incubated in 2.5 µl/ml solution of acetic anhydride in TEA HCl for 5 min; then 2.5 µl/ml acetic anhydride was added, followed by 5 min incubation with agitation. After several washes in PTW, the ganglia were incubated in hybridization buffer (50 % formamide, 5 mM

EDTA (Invitrogen), 5X SSC, 1X Denhardt solution (0.02 % ficoll, 0.02 % polyvinylpyrrolidone, 0.02 % BSA), 0.1 % Tween 20, 0.5 mg/ml yeast tRNA (Invitrogen)) at 50°C for 6-8 hrs. 0.5-2 µl of probe (0.5-0.8 µg/µl) was then added and hybridization proceeded at +50°C for 12 hr, followed by subsequent washes in stringent conditions (50 % formamide/ 5X SSC/ 1 % SDS (USB Corp., Cleveland, OH), then 50 % formamide/2X SSC/1 % SDS, and then 0.2X SSC, twice, for 30 min at +60°C each). Immunological detection was performed using components of the DIG Nucleic Acid Detection Kit (Roche). Development proceeded until background staining started to appear (10-20 min in the case of serotonin transporter, 1.5-3 hrs for HCN and 2-4 hrs for CNG transcripts). Ganglia were then fixed in 4 % paraformaldehyde in methanol for 1 hr and washed in 100% ethanol two times for 10 min.

Single-cell RNA-seq methods adapted to *Aplysia* were also described in detail (4) and transcript quantification was presented as Reads Per Kilobase per Million mapped Reads or RPKM values.

1.3. Expression of HCN channels in *Xenopus laevis* oocytes. For acHCN RNA synthesis two constructs were made from the coding region of a single acHCN transcript. Construct 1 started at a potential alternative start codon (CTG) 201 nucleotides upstream from the first ATG codon of acHCN and ended at the stop codon at the 3'-end of the transcript. It was obtained from *A. californica* CNS library by a PCR using the following primers:

5'-CTGGGACCAACTAGTGGCGCCGGGA-3' and

5'-TTAAGGATCTGGTCCTTGAGATAGCCGGT-3'. Construct 2 started at the first ATG codon of acHCN and ended at the stop codon at the 3'-end of the transcript. It was obtained from the same CNS library by a PCR using the following primers:

5'-ATGGGGCAGGAATGCGTGGCTGGA-3' and

5'-TTAAGGATCTGGTCCTTGAGATAGCCGGT-3'. These constructs were cloned into LingT plasmid – a modified BlueScript plasmid engineered to include 5'- and 3'-UTRs of the *Xenopus laevis* β-globin to facilitate expression in frog oocytes, presumably by increasing RNA stability (11), see **Figure S1**. The constructs were transformed into One-Shot competent *E. coli* cells (Invitrogen). The clones were isolated, purified using a MiniPrep kit (Qiagen) and sequenced. cRNA for oocyte injections was obtained by *in vitro* transcription of PmeI-linearized LingT plasmid using mMessage mMachinE T7 Ultra (Ambion), a high yield, capped RNA transcription kit. The integrity and quantity of the acHCN cRNA were determined by a 2100 Bioanalyzer (Agilent).

Mature (>9 cm) female African clawed frogs *X. laevis* (Xenopus Express, Plant City, FL) were used as a source of oocytes. Prior to surgery, frogs were anesthetized by placing the animal in a 2 g/L solution of MS222 (3-aminobenzoic acid ethyl ester). Oocytes were removed from an incision made in the abdomen. To remove the follicular cell layer, harvested oocytes were treated

with collagenase for 2 hours at room temperature in calcium-free ND96 solution (96 mM NaCl, 2 mM KCl, 1mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, adjusted to pH 7.5 with 10N NaOH). Subsequently, stage 5-6 oocytes were isolated and injected with ~25 ng of cRNA in 50 nl volume using a Micro4 injector (WPI, Berlin, Germany). Oocytes were kept at 17°C in sterile ND96 oocyte medium that was supplemented with 2.5 mM sodium pyruvate, 100 units per ml/1 penicillin, 100 mg per ml/1 streptomycin, and 5 % horse serum. Recordings were made three to seven days after injection. Oocytes injected with RNA produced from Construct 1 did not express a functional acHCN channel, i.e., no currents were generated following hyperpolarization of oocytes injected with Construct 1 RNA. Therefore, all the experiments described in the paper were performed on the oocytes injected with Construct 2 RNA.

For voltage-clamp recording of the whole-oocyte responses oocytes were conditioned in the ND96 serum-free medium for 1 hr prior to recording and then placed in a small volume of bathing medium in a plastic chamber which was continuously perfused at a rate of 2 ml/min. The chamber solution was connected to a virtual ground of a current monitor head stage (VG-2A-x100, Molecular Devices, Sunnyvale, CA) through two 2 mM KCl 2% agar-bridged, Ag/AgCl reference electrodes. Microelectrodes (1-3 MΩ) were prepared with a P-2000 puller (Sutter Instrument Co., Novato, CA) from 1.2 mm borosilicate glass capillaries (WPI) and filled with a solution used for patch clamp recording of the mouse HCN channel (mBCNG-1) expressed in *X. laevis*

oocytes (12): 5 mM NaCl, 107 mM KCl, and 10 mM HEPES, pH 7.5. The concentrations of Na⁺ and K⁺ in this solution are in the biological range naturally occurring in *X. laevis* oocytes (13).

Currents were measured with a two-electrode voltage clamp (OC 725A, Warner, New Haven, Conn., USA) using a DigiData1200 acquisition system (Molecular Devices). Recordings were made at room temperature (~25°C). All control experiments were done in ND96 solution. Oocytes were clamped at a holding potential of -30 mV. This potential was chosen because it was the mean resting potential of the oocytes injected with acHCN RNA. Currents were allowed to stabilize for 10 min following electrode penetration. Oocytes were then hyperpolarized for 5 s from the -30 mV holding potential to more negative potentials ranging from -50 mV to -110 mV in steps of -10 mV. After each hyperpolarizing voltage step the currents were allowed to return to their base values for 5 s. To determine time constants, oocytes were hyperpolarized for 20 s from the -30 mV holding potential to more negative potentials ranging from -50 mV to -110 mV in steps of -10 mV.

Current amplitudes were calculated by subtracting the current values at ~120 ms after the beginning of hyperpolarization (following relaxation of capacitive currents), from the current values at 160 ms from the end of the hyperpolarizing voltage step, where the currents were stable. Tail currents were generated by hyperpolarizing oocytes from a holding potential of -30 mV to -110 mV for 2 s to activate the HCN channels and then stepped to -100 mV and in steps of 10 mV to -10 mV. Further depolarization was not done, because at the potentials positive to 0 mV native outward currents (present in the control oocytes) developed, which changed the shapes of tail currents in the acHCN mRNA-injected oocytes. The amplitudes of tail currents were determined at 140 ms after the beginning of the depolarizing step following relaxation of capacitive currents.

Modulation of the acHCN channel by cAMP and cGMP was determined using membrane permeable analogs: 8-Br-cAMP and 8-Br-cGMP (sodium salts), because the CNBD of HCN channels is intracellular. After measuring control currents, oocytes were perfused with a solution of a cyclic nucleotide in ND96 for 10 min before recording. This time was chosen because acHCN-mediated currents did not significantly change following longer perfusions with either 8-Br-cAMP or 8-Br-cGMP.

To test the effect of inhibitors on acHCN, oocytes were perfused with a solution of either Cs⁺ or ZD7288 (Tocris, Ellisville, MO) in ND96 for 10 min and then washed for either 10 min or 30 min following perfusion by Cs⁺ and ZD7288 respectively. In both cases, these were the minimal times required for the acHCN-mediated currents to return to their control values. Percent inhibition by Cs⁺ or ZD7288 was calculated at the potentials of their maximal inhibition of I_h, -110 mV and -90 mV respectively.

Experiments were performed on several oocytes from at least two different frogs under each condition. Data were analyzed using ClampFit (Molecular Devices) and SigmaPlot 9.0 software (SYSTAT Software Inc., Point Richmond, CA, USA). Values depicted in graphs represent the mean ± s.e.m. from at least four independent experiments involving at least four different oocytes.

1.4. Behavioral methods and electrophysiology. The behavioral methods were similar to those we have described previously (14-18). Briefly, the siphon, tail and central nervous system of *Aplysia californica* (100-150 gms) were dissected and pinned to the floor of a recording chamber filled with circulating, aerated artificial seawater at room temperature (**Fig. 4A**, main text). The siphon was partially split, and one-half was left unpinned. A controlled force stimulator was used to deliver taps of approximately 20 gm/mm², 500 msec duration to the pinned half, and withdrawal of the other half was recorded with a low mass isotonic movement transducer attached to the siphon with a silk suture. The peak amplitude of withdrawal was measured using a laboratory interface to a microcomputer and commercially available software, which also controlled the stimulation. A fixed capillary electrode was used to deliver AC electric shocks of 25 ma, 1 sec duration to the tail.

The preparation was rested for at least 1 hr before the beginning of training (**Fig. 4B**). In sensitization experiments, the reflex was tested once every 15 min, and a train of 4 shocks (with a 2 sec interval between shocks) was delivered to the tail 2.5 min before the fourth test (PostTest). In conditioning experiments, there were three blocks of four training trials each, with a 5 min interval between trials in a block and a 20 min rest between blocks. The response to the siphon tap CS was measured in a pretest 5 min before the first block (Pre), in test trials 15 min after each block (T1-T3), and in a final post-test 45 min after the last block (Post). During

paired training, the CS began 0.5 sec before the tail shock US on each trial. During unpaired training, the interstimulus interval was 2.5 min. Animals were randomly assigned to the training conditions. Experiments were continued only if the siphon withdrawal was between 1 and 5 mm on the first test, and greater than 3 mm in response to the shock.

The abdominal ganglion was surrounded by a circular well with the nerves led through a Vaseline seal, so that the ganglion could be bathed in a different solution than the rest of the preparation. ZD7288, APV, or both (Tocris, prepared as stock solutions in distilled water and diluted in ASW immediately before use) was applied for 30 min before and during the experiments. The data were analyzed with two-way (for sensitization) or three-way (for conditioning) ANOVAs with one repeated measure (test), followed by planned comparisons of the difference between the training groups (and for conditioning the reduction of that difference by each drug, i.e. the ZD7288 x training and APV x training 2-way interactions, as well as the APV x ZD7288 x training 3-way interaction) overall, and then at each test to define the time courses of those effects.

The electrophysiological methods were also similar to those we have described previously. The abdominal ganglion was partially desheathed and an LE siphon sensory neuron and/or LFS siphon motor neuron was impaled with a double-barreled microelectrode (7-15 Mohm filled with 2.5 M KCl). LE and LFS neurons were identified by their location, size, and electrophysiological characteristics (19) as well as their response to siphon stimulation (LE) or production of siphon movement (LFS). To examine the effect of ZD7288 on baseline evoked spiking of LFS and LE neurons (**Fig. 4E**) the experimental design was similar to that described above for behavioral sensitization, except that instead shocking the tail before the fourth test the abdominal ganglion was perfused with ZD7288 or ASW control, and instead of measuring siphon withdrawal the number of spikes in the first 1 s after the start of each siphon tap was measured.

To examine HCN current in LFS siphon motor neurons (**Fig. 5A**), a 6 sec step of hyperpolarizing current was passed through one barrel of the electrode and the “sag” or depolarization from the beginning to the end of the step was measured. This was repeated for 4 different step amplitudes ranging from 40 to 110 mV below resting potential (which was around -50 mV) before and after perfusion of the ganglion with ZD7288. The rate of spontaneous firing, resting membrane potential, and input resistance of the LFS neuron were also measured before and after perfusion with ZD7288 in some of these experiments. A similar design was also used to examine the effect of NO on the HCN current produced during hyperpolarizing steps in the range from 90 to 160 mV. DEA-NO (Enzo Life Sciences, Farmingdale, NY) was prepared as a stock solution in 10mM NaOH and diluted in ASW immediately before use.

To examine the effect of ZD7288 on the monosynaptic LE-LFS EPSP (**Fig. 5B**) the experimental design was similar to that described above for evoked spiking of LE and LFS neurons, except that brief intracellular current injection was used to produce a single action potential in an LE neuron once every 15-20 min and the peak of the EPSP in the LFS neuron was measured. To examine the effect of ZD7288 on the NMDA-like current in the LFS neuron, the late (50-75 msec after peak) component of the LE-LFS EPSP was measured. In some experiments the ganglion was first perfused with either the AMPA receptor antagonist CNQX (Tocris) or CNQX followed by CNQX plus the NMDA antagonist APV (Tocris) before ZD7288 was added.

2: Results

Residues that mediate cyclic nucleotide gating. There are at least seven key residues in the CNBD that mediate gating of HCN channels by cyclic nucleotides: R591, T592, and E582 in the β -roll; R632, R635, I636, and K638 in the C-linker helix (amino acid numbers refer to the *Mus musculus HCN2 (mHCN2)*). *AcHCN* shares the conserved R591 (*AcHCN* Arg⁴⁷⁸) and E582 (*AcHCN* Arg⁴⁶⁹) residues of the β -roll, but has a Val residue (*AcHCN* Val⁴⁵⁸) substitution in place of T592. This threonine is predicted to form three hydrogen bonds with cGMP (as opposed to two with cAMP) and contribute to cGMP selectivity in the CNBD. Despite this substitution, *AcHCN* is indeed modulated by cGMP. The four C-helix residues are well-conserved between *mHCN2* and *AcHCN*; R632 as *AcHCN* Arg⁵¹⁹, I636 as *AcHCN* Ile⁵²³, K638 as *AcHCN* Lys⁵²⁵, while R635 occurs as the similarly charged Lys⁵²² in *AcHCN*. Previous experiments have demonstrated that if I636 is mutated into an aspartate residue, *mHCN2* becomes far more selective for cGMP compared to cAMP (20). Interestingly, *AcHCN* lacks the conserved T592 residue and retains the I636 residue, which suggests the channel

may be modulated more strongly by cAMP than cGMP. We compare these seven key CNBD residues among vertebrates and *Aplysia* in **Table S1**.

References for the Supporting Information

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Table S1. Amino acids that confer cyclic nucleotide sensitivity in HCN channels

Mouse HCN2	Human HCN2	<i>Aplysia</i> HCN
Arg ⁵⁹¹	Arg ⁶¹⁸	Arg ⁴⁷⁸
Thr ⁵⁹²	Thr ⁶¹⁹	Val ⁴⁷⁹
Glu ⁵⁸²	Glu ⁶⁰⁹	Glu ⁴⁶⁹
Arg ⁶³²	Arg ⁶⁵⁹	Arg ⁵¹⁹
Arg ⁶³⁵	Arg ⁶⁶²	Lys ⁵²²
Ile ⁶³⁶	Ile ⁶⁶³	Ile ⁵²³
Lys ⁶³⁸	Lys ⁶⁶⁵	Lys ⁵²⁵

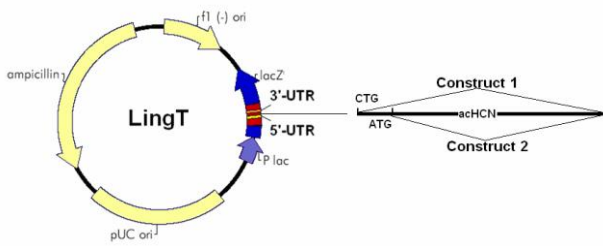


Figure S1. Schematic diagram of the constructs used to produce acHCN RNA. LingT plasmid is a modified BlueScript plasmid with added 5'- and 3'-UTRs of *X. laevis* β -globin and T-overhangs. Two constructs made from a single transcript were cloned into the plasmid: Construct 1 starting from the alternative start codon CTG and Construct 2 starting from the first ATG codon.

AcHCN : -----MGQE--CVACEAGGAASKASSTT----- : 21
 HsHCN1 : -----MEGGKPNSSNSRDDGNSVFPKASATGAPAAAEKR-IGTTPGGGGA-CA----- : 50
 HsHCN2 : MD-----ARGGGGRP---GESFPGAS---PTTTPPPPPPPPPKQPPPPPPAPPAPP---GPAPPQ : 53
 HsHCN3 : -----MEAEQR--PAAGASEGATPGLAEVPPVAPPATAASGP----- : 36
 HsHCN4 : MDKLPSPMRKRLYSLPQQVGAKAWIMDEEEDAE--EGAGGRQDPSRRSIRLRPLPS-SPSAAAGTESRSSALGADSE : 78

AcHCN : ----- : -
 HsHCN1 : -----KEHGNVCFKVDGGGGGGGGGG----- : 74
 HsHCN2 : HPPRAEALPPEAADEGGPRGRLRSRDSSCGRPCTPGAASST-----AKGSPNGECGRGEPQCSP---AGPEGPARG : 120
 HsHCN3 : ----- : -
 HsHCN4 : GPARGAGKSSSTNGDCRRFRGSLASLGRSGGSGGTGSGSSHGHLHDSAEERRLIAEGDASPGEDRTPPGLAAEPPERPGAS : 158

AcHCN : -----SYLKDILNFFQPSDNKLMKLFQNK : 48
 HsHCN1 : -----EEFAGGFDAEGPRROY-----GFMCRQFTSMIQGVNKFSLRMFGSK : 118
 HsHCN2 : PKVSFSCRGAASGPAPGPGPAEEA-----GSEEAG---PAGEPRGSQASFMCRQFGALLIQGVNKFSLRMFGSK : 187
 HsHCN3 : -----IPKSGPEPK-----RRHLGTLLOFTVKNFSLRVFGSHK : 69
 HsHCN4 : AQPAAASPPPQQPPQASASCEQPSVDTAIVKVEGAAAGDQILPEAEVRLGQAGFMCRQFGALLIQGVNKFSLRMFGSK : 238

AcHCN : ALIKFKMRHRVGVNVIHEGNSFRFYWDLMLVLLIANLIIIPVAISFFNDLSTHWIVFNCLSDTVFELDIVINFRTG : 128
 HsHCN1 : AVEKEQERVKTAGFWIHPVSDFRFYWDLMLIMVGNLVIIPVGITFFTECTTTPWIIFNVASDTVFELDLIMNFRTG : 198
 HsHCN2 : AVEREQERVKAGAWIHPVSDFRFYWDLMLIMVGNLIIIPVGITFFKDETTAPWIVFNVSDFEFLMDLVLNFRGT : 267
 HsHCN3 : AVEKEQERVKAGAWIHPVSDFRFYWDLMLIMVGNLIVLPVGITFFKEENSPEWIVFNVSDFEFLDLVLNFRGT : 149
 HsHCN4 : AVEREQERVKAGFWIHPVSDFRFYWDLMLIMVGNLIIIPVGITFFKDETTAPWIVFNVSDFEFLIDLVLNFRGT : 318

S1 S2

AcHCN : IILNFADIEIILDPKLIKAKQYMKTWELDLSSVPMDYIFLMWDAEADEN-QLFHAGRALRMLRLAKLLSLLRLLRSLRM : 207
 HsHCN1 : VNEE-SSEIILDPKVIKMYLKSWEVVDLSSIPVDYIFLIV--EKGIQSEVYKTARALRIVRFTKILSLLRLLRSLRI : 275
 HsHCN2 : VTEI-NTEIILDPKIKKKYLRTWFEVVDVSSIPVDYIFLIV--EKGIQSEVYKTARALRIVRFTKILSLLRLLRSLRI : 344
 HsHCN3 : VVEE-GAEIILAPRATRTRYLRTWELVDLSSIPVDYIFLVVLEPRLEAEVYKTARALRIVRFTKILSLLRLLRSLRI : 228
 HsHCN4 : VVEI-NTEIILDPKIKKKYLKSWEVVDLSSIPVDYIFLIV--ETRIDSEVYKTARALRIVRFTKILSLLRLLRSLRI : 395

S3 S4

AcHCN : RYVQWEEFL----ATAGKEMRIENLIGMLLGHWDGCLQFLVPLIQDFPKDCWVSTEGEQEAHWAEQYTWALFKALSH : 283
 HsHCN1 : RYIHWEEIFHMTYDLASAVRIENLIGMMLLGHWDGCLQFLVPLLQDFPDCWVSLNEMVNDGHWGKOYSYALFKAMSH : 355
 HsHCN2 : RYIHWEEIFHMTYDLASAVRIENLISMMMLLGHWDGCLQFLVPLQDFPRNCWVSIINGMVNHSWSELYSEALFKAMSH : 424
 HsHCN3 : RYIHWEEIFHMTYDLASAVRIENLIGMMLLGHWDGCLQFLVPLQDFPDCWVSIINHMVNSHWGROYSHALFKAMSH : 308
 HsHCN4 : RYIHWEEIFHMTYDLASAVRIENLIGMMLLGHWDGCLQFLVPLQDFPDCWVSIINMVNNSHWGKOYSYALFKAMSH : 475

S5 Pore

AcHCN : MLCIGYGRFFQNMSTWLTILSMLSGATCYALFIAHTTLLIQSEDTSRRLYNEKFKQVEEYMYRKLERSLRQRIIDYY : 363
 HsHCN1 : MLCIGYGAQAFVMSDLWI TMLSMIVGATCYAMFVGHATLIIQSLDSSRRQYQEKYKQVEQYMSFHKLPAEMRQKIHYY : 435
 HsHCN2 : MLCIGYGRQAFESMIDTWLTMLSMIVGATCYAMFVGHATLIIQSLDSSRRQYQEKYKQVEQYMSFHKLPAEMRQKIHYY : 504
 HsHCN3 : MLCIGYQQAFVGMEDVWL TMLSMIVGATCYAMFVGHATLIIQSLDSSRRQYQEKYKQVEQYMSFHKLPAEMRQRIHYY : 388
 HsHCN4 : MLCIGYGRQAFVGMEDVWL TMLSMIVGATCYAMFVGHATLIIQSLDSSRRQYQEKYKQVEQYMSFHKLPAEMRQRIHYY : 555

S6

AcHCN : EHRYQGMFDEETILSELNCKLKEVNVNHCNCRSLVASVEEFTNADAFVSEVSVSKLKFEVYQPGDYIIREGTMCTKMF : 443
 HsHCN1 : EHRYQGKIFDEENILNELNDLREIIVNFCRKLVAIMPILFANADNFVNTAMLTKLRFVFPQGDYIIREGAVGKMYFI : 515
 HsHCN2 : EHRYQGMFDEESILGELNGFLREIIVNFCRKLVAIMPILFANADNFVNTAMLTKLRFVFPQGDYIIREGIGKMYFI : 584
 HsHCN3 : EHRYQGMFDEESILGELSEFLREIIVNFCRGLVAIMPILFADNPFVNTAVLTKLRFVFPQGLLVVREGSVGKMYFI : 468
 HsHCN4 : EHRYQGMFDEESILGELSEFLREIIVNFCRKLVAIMPILFANADNFVNTSMLTKLRFVFPQGDYIIREGIGKMYFI : 635

AcHCN : QEGVIVITSDGEVATSLSDGSYFGEICLLTNARRMVASRAETVACLYSDAVEEFTAVLEERYVPMRRMTESVAERLTKI : 523
 HsHCN1 : QHGVAGVIT-KSSKEMKLDGYSYFGEICLLTKRRRTASVRADTYCRLYLSVINFNEVLEEYPMRRRAFETVALDRLERI : 594
 HsHCN2 : QHGVSVLIT-KGNKEMKLDGYSYFGEICLLTRGRRRTASVRADTYCRLYLSVINFNEVLEEYPMRRRAFETVALDRLERI : 663
 HsHCN3 : QHGLSVLIT-RGARDRLDGSYFGEICLLTRGRRRTASVRADTYCRLYLSVINFNEVLEEYPMRRRAFETVALDRLERI : 547
 HsHCN4 : QHGVSVLIT-KGNKEMKLDGYSYFGEICLLTRGRRRTASVRADTYCRLYLSVINFNEVLEEYPMRRRAFETVALDRLERI : 714

CNBD

AcHCN : GKNSIVSSRAD-----LEELQKMNVEI-----VMESTPIPTSAEDEDSDSDESSDGSKQKKT : 579
 HsHCN1 : GKNSILLQKQFQKDLNTGVFNQENELIKQIVKIDREMVAQAIAPINYPQMTLLNSTSSSTTPT--SRVRTQSPVYTT : 672
 HsHCN2 : GKNSILLHRVQHDNLNSGVFNQENAIIQELIVKIDREMVAQAEALGQRVGLFPPEPP-----PQVTSIAIATLQQA : 735
 HsHCN3 : GKNSILLQRK-RSEPSG---SSGIMEQHLVQHDREMARGVGRAPSTGAQLSGKPVLWEPLVHAPLQAAAVTSNVA : 623
 HsHCN4 : GKNSILLHRVQHDNLNSGVFNQENELIQELIVQHDREMAHCAHRVQAAASATPTPTPVITWPLIQAPLQAAAVTSVA : 794


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AcHCN   : FKDFD-----STRKHK----- : 590
HsHCN1  : LSHSN-LHSPS-----ESTQTPQPSAIIIS----- : 695
HsHCN2  : MSFCQVARPL-----VGPLALG-SPRLVRR----- : 760
HsHCN3  : LTHQR--GPL-----PLSPDSPATLLAR----- : 644
HsHCN4  : LTHHPRLPAALFRPPPGSGLGNLGAQOTPRFLKELQSLIPALGASPASSPSQVDTPESSSFHIQQLAGFSAPAGLSPL : 874

AcHCN   : ----- : -
HsHCN1  : -----PCSYTTAVCSPEVQS-----PLAARTFHYASPTASQLSL-----M : 730
HsHCN2  : -----PPPGPAPAAASEGPP-----PPASPPGA----- : 783
HsHCN3  : -----SAWRSAAGSPASELVP-----VRAGPWASTSRLPAPPAR----- : 677
HsHCN4  : LPSSSSSPPGACGSPSAFTPSAGVAATTIAGFGHFHKALGGSLSSSDSPLLTPLQPGARSPQAAQPSAPPARGGLGL : 954

AcHCN   : ----ISEEKKNSSEKEHS-----KEDLLEFGETKHHRLS----- : 621
HsHCN1  : QQQPQQQVQOSQFPQIQPQQPSQPQTPGSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLEHEVSTLISRPHPTVGE : 810
HsHCN2  : -----PASPAPRQSE--YG-----GLPAAPL--AGPALPARRLSRASRELSASQPSLEHG----- : 830
HsHCN3  : ----TLHASLSRAGRQVSLLGPP-----PGGGRRLLGPRGRPLSASQPSLEQRAT----- : 724
HsHCN4  : PEHFLPPPPSSRSSESSPQQLGQPPGELSLGLATGFL--STPETPPRQPEPPSLVAGASGGASIVGFT----- : 1020

AcHCN   : -----QGEDP----- : 626
HsHCN1  : SLASIQPVTVAVPGTGLQACGRSTVQRVTLFRQMSSGATFPNKGVPPAPPPFAA----- : 865
HsHCN2  : -----APC-----EAASTRPASSSTPRIGETPAARAAAPSDRRDS----- : 866
HsHCN3  : -----GDG-----SPCRK---GSGSERLPPSGLLAKPERTAQPPRPVVEP----- : 762
HsHCN4  : -----PRGGLSPPGHSPCPRTTFESAPPRASGSHGSLLEPPASSPPEQVQRRGTPLTPGRLTQDLKLISASQPAL : 1093

AcHCN   : ----- : -
HsHCN1  : -----ALE-----RESSVINTD----- : 878
HsHCN2  : -----ASE-----GAAGGLDP----- : 877
HsHCN3  : -----ATE-----RGL----- : 768
HsHCN4  : PQDGAQTLRRASEHSSGESMAAFPLFPRAGGGSGGSGSSGGLGPPGRPYGAI PGQHVTLPRKTS S G S L P P P L S L F G A R A T : 1173

AcHCN   : ----- : -
HsHCN1  : -----PDAEKPRFASNL : 890
HsHCN2  : -----QDSARSRLSSNL : 889
HsHCN3  : -----QLSANM : 774
HsHCN4  : SSGGPPLTAGPQREPGARPEPVRSKLFSNL : 1203

```

Fig. S2_A. Alignment of HCN proteins from *Aplysia californica* (1) and *Homo sapiens* (4 isoforms) with major domains indicated shows that HCN channels are highly conserved from molluscs to mammals, especially in their transmembrane domains (S1-S6), pore and CNBD. Seven critical amino acid residues involved in cyclic nucleotide gating are indicated with red stars (★). Predicted sites of phosphorylation are indicated by: ♠ - Protein kinase C; ♣ - Casein kinase II; * - Tyrosine Kinase; ♥ - N-myristoylation site; ♦ - N-glycosylation site. Abbreviations: Ac – *Aplysia californica*, Hs – *Homo sapiens*

Figure S2_B. Comparison of key HCN domains.

S4 Voltage Sensor Residues

```
AcHCN : HAGRALRMLRLAKLLSLLRLLRLSRLVR
HsHCN2 : KTARALRIVRFKILSLLRLLRLSRLIR
MmHCN2 : KTARALRIVRFKILSLLRLLRLSRLIR
```

Alignment of the S4 voltage sensors from HCN channels shows that the *Aplysia* HCN channel, as well as human and mouse HCN channels, have nine positively-charged residues (shown as red boxes) found at every third position and interrupted after the first five positively charged residues by a serine. Conserved residues are shown in black boxes. Abbreviations: Ac – *Aplysia californica*, Hs – *Homo sapiens*, Mm – *Mus musculus*

Pore Region Residues

```
AcHCN : YTWALFKALSHMLCTGYGRFP
HsHCN2 : YSFALFKAMSHMLCTGYGROA
MmHCN2 : YSFALFKAMSHMLCTGYGROA
```

Alignment of the pore regions from HCN channels of human, mouse, and *Aplysia* shows that the core region (red box) of the potassium selectivity motif is preserved in AcHCN, as well as vertebrate HCN channels. Conserved residues are shown in black boxes. Abbreviations are the same as above.

CNBD Residues

```
AcHCN : FVSEVVS*SKLKFEVYQPGDYIIREGTMG*TKMFFIQEGIVD*IITS*DGEVATS*LS*DGSYFGEICLLTNARRV*ASVRAE*TYAC*LYSLAVEH
HsHCN2 : FVTAMLTKLKFEVYQPGDYIIREGTIGK*KMYFIQ*GVVSVLT*RGNKEMKLS*DGSYFGEICLLTRGRR*IASVRA*TYCRLYSL*SVDN
MmHCN2 : FVTAMLTKLKFEVYQPGDYIIREGTIGK*KMYFIQ*GVVSVLT*RGNKEMKLS*DGSYFGEICLLTRGRR*IASVRA*TYCRLYSL*SVDN

AcHCN : FTA*VLERYPVMRR*TME*SVAAERL*IKIGK*NESIV
HsHCN2 : FNEVLEEYPMMRR*AETVAIDRLDRIGK*KNSIL
MmHCN2 : FNEVLEEYPMMRR*AETVAIDRLDRIGK*KNSIL
```

Alignment of the CNBD regions from HCN channels of human, mouse, and *Aplysia* shows that the CNBD is well conserved among HCN channels. Conserved residues are shown in black boxes. Seven critical amino acid residues involved in cyclic nucleotide gating are indicated with red stars (★). Abbreviations are the same as above.

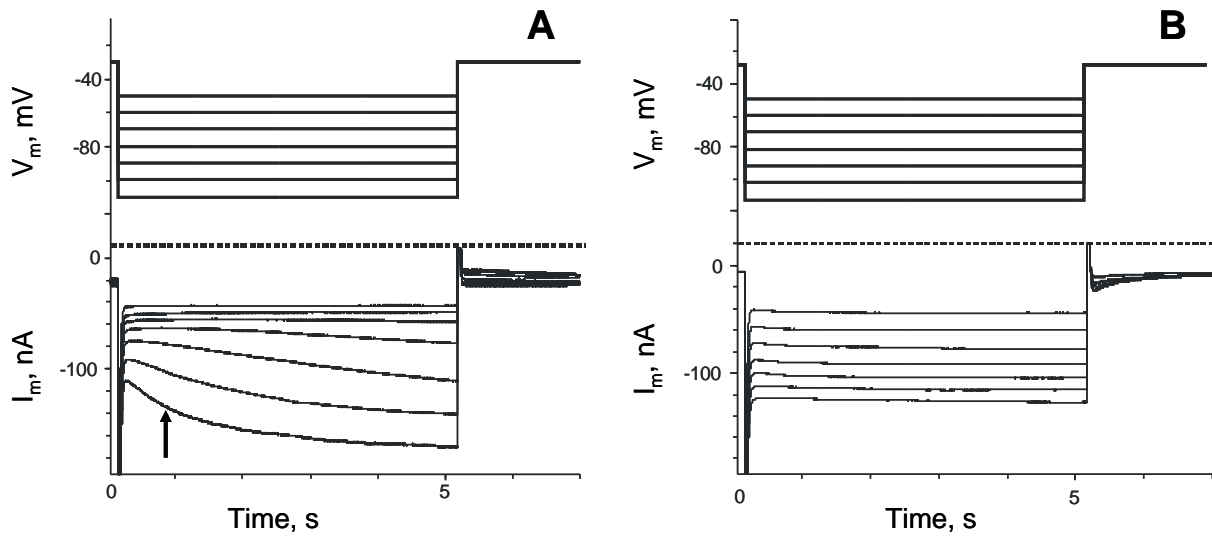


Fig. S3. Ion current (I_h) recordings obtained from the acHCN RNA-injected *Xenopus* oocytes (n=10). Slow inward currents characteristic for HCN channels were generated upon hyperpolarization from a holding potential of -30 mV to potentials negative to -70 mV (A). These currents were not present in water-injected oocytes (B). An arrow in A shows a current generated upon hyperpolarization of an oocyte from a holding potential of -30 mV to -110 mV.

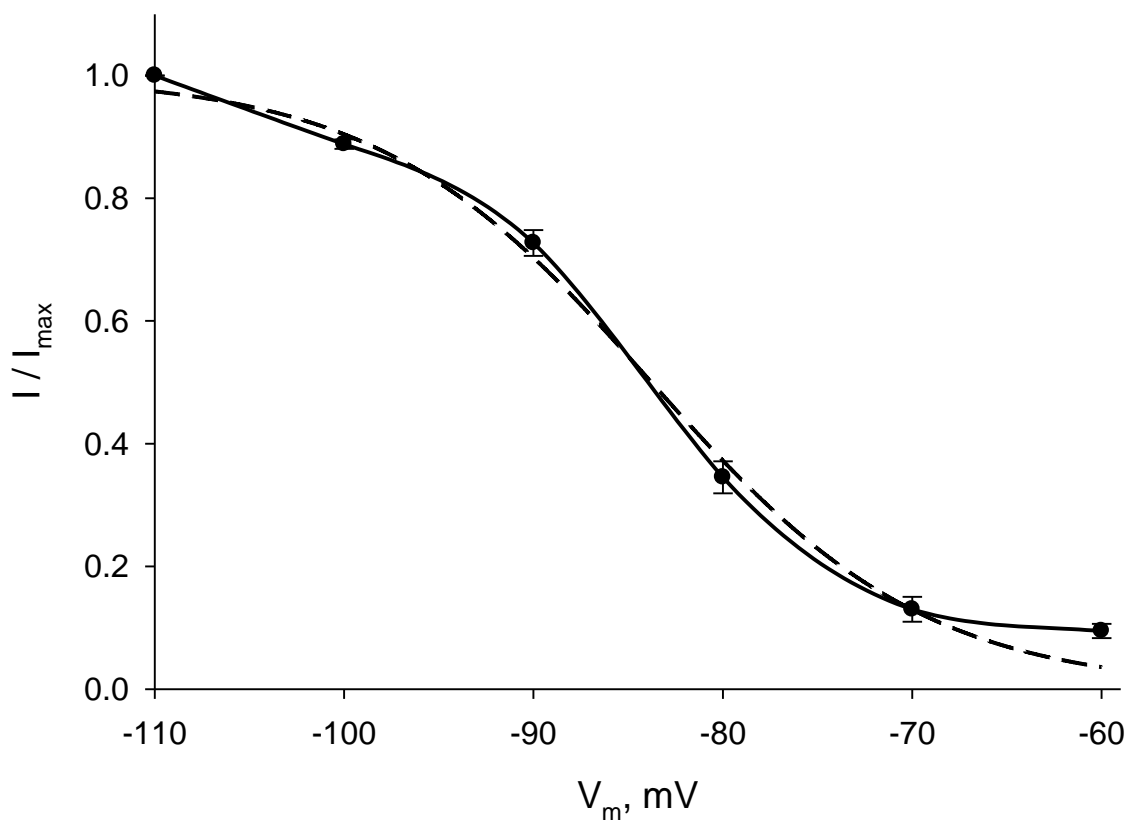


Fig. S4. Voltage dependence of the acHCN channel activation in *Xenopus* oocytes. The I/V relationship (solid line) of acHCN was determined by hyperpolarizing oocytes ($n = 4$) for 5 s from the -30 mV holding potential to more negative potentials ranging from -60 mV to -110 mV in steps of -10 mV. $V_{1/2}$ was determined by fitting the I/V curve with a curve calculated by the Boltzmann equation (dashed line) to be -83.77 ± 0.79 mV, $n = 4$.

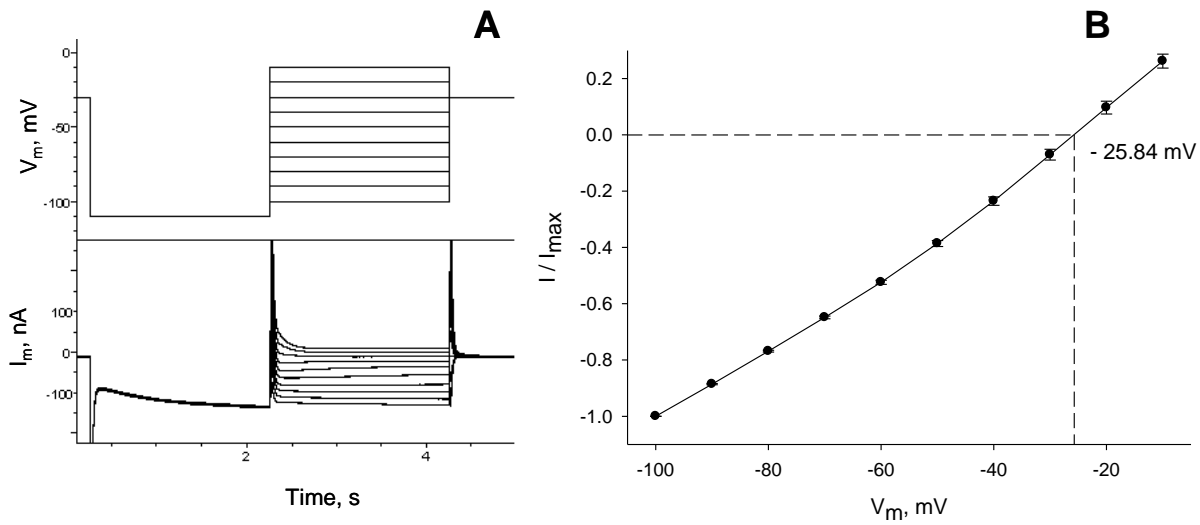


Fig. S5. Determination of a reversal potential of the acHCN channel. A) Tail currents were generated by hyperpolarization of *Xenopus* oocytes from a holding potential of -30 mV to -110 mV for 2 s to activate the HCN channels and then stepped to one of a family of potentials ranging from -100 mV to -10 mV in increments of 10 mV. B) Normalized amplitudes of the tail currents were plotted against voltage and the reversal potential of acHCN was determined as a point where the current changes its sign; $n=17$.

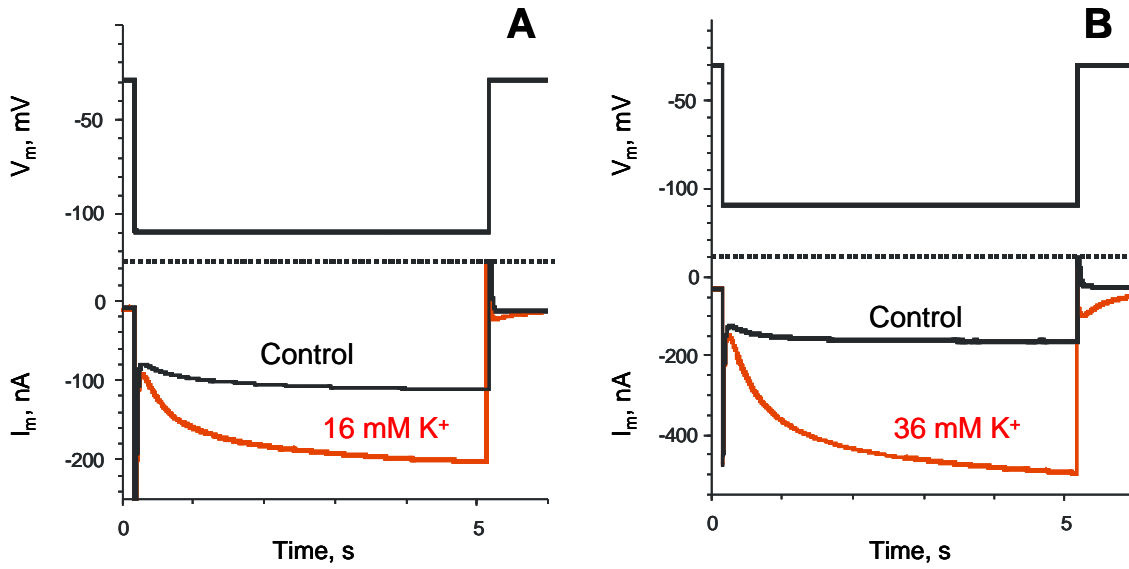


Fig. S6. Dependence of the I_h amplitude on extracellular potassium concentration (*Xenopus* oocytes). Elevating extracellular concentration of K^+ drastically increases amplitudes of I_h : 4.64 ± 0.62 fold for $[K^+]_{\text{ext}} = 16\text{mM}$ (A) and 7.82 ± 0.30 fold for $[K^+]_{\text{ext}} = 36\text{mM}$ (B). Current traces produced by stepping from -30 to -110 mV in 2 mM extracellular potassium (control) are shown in black. Current traces produced under the same conditions in extracellular solution with elevated K^+ were superimposed with control traces and are shown in red; $n = 4$.

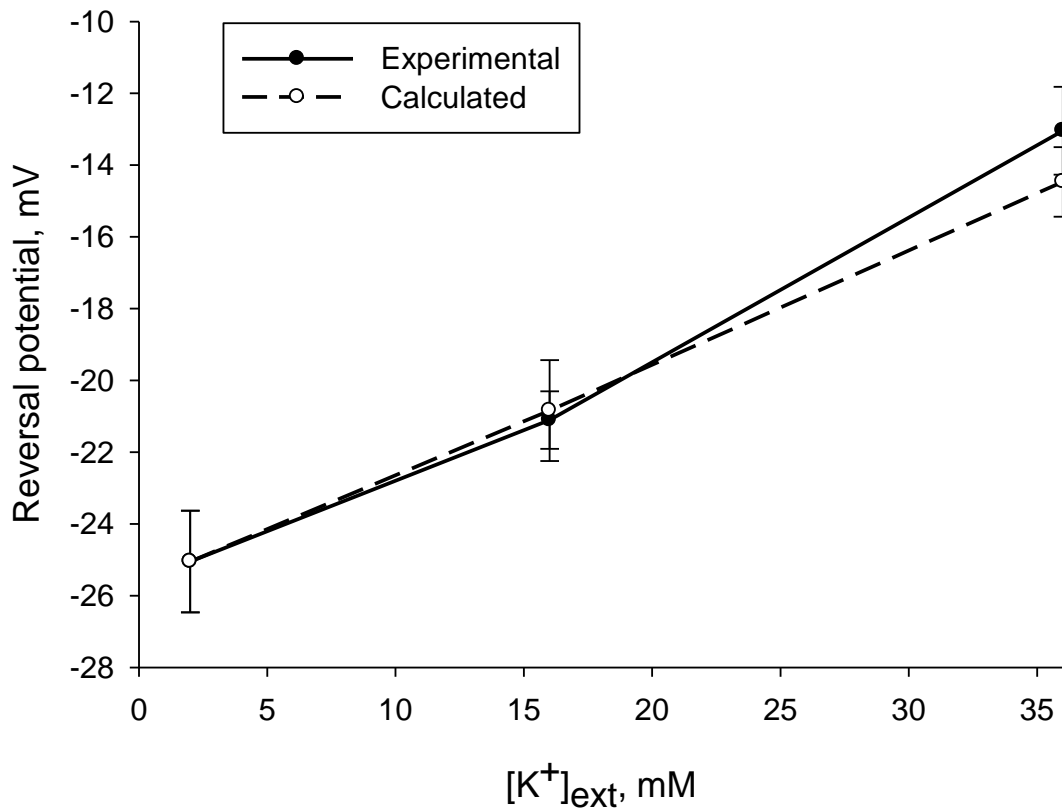


Fig. S7. Dependence of the reversal potential of the acHCN channel on extracellular potassium concentration (*Xenopus* oocytes). Experimentally determined reversal potential (solid line) closely matched the reversal potential determined using the Goldman equation based on the assumption that the acHCN channel is permeable only to K⁺ and Na⁺ (dashed line).

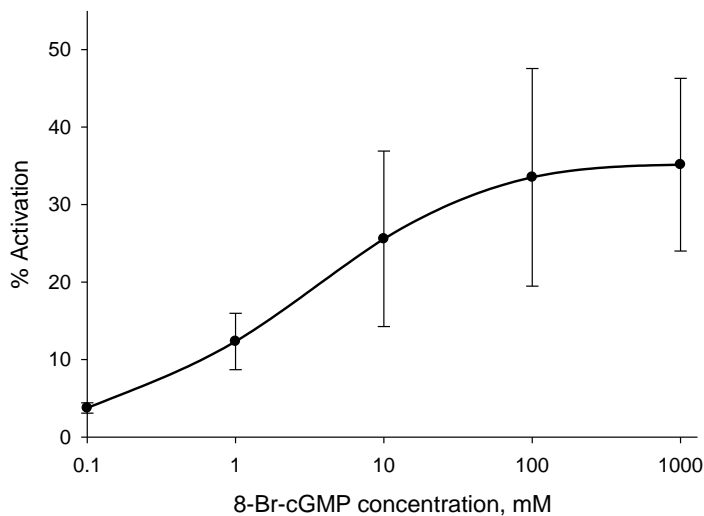
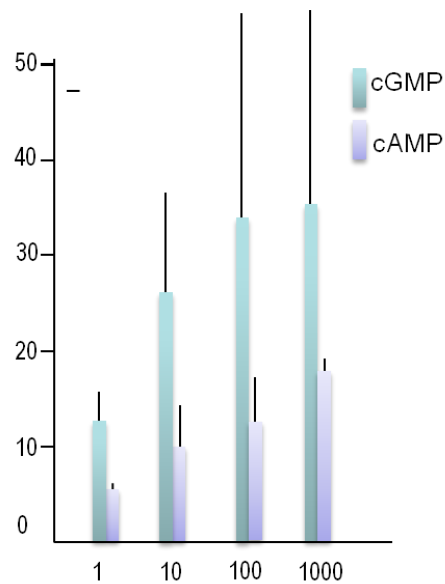
A.**B.**

Fig. S8. A. Concentration dependence of acHCN activation by 8-Br-cGMP. Percent activation was calculated as an increase in I_h amplitude using the following formula: % activation = $I_{h,-110mV, 8-Br-cGMP} / I_{h,-110mV} * 100 - 100$. $K_a = 2.16 \pm 0.68 \mu M$. $N = 4, 4, 4, 6,$ and 7 for 0.1 to $1000 \mu M$ 8-Br-cGMP.

B. The comparison of concentration dependence of acHCN activation by 8-Br-cAMP vs cGMP. Percent activation was calculated as above. K_a value for 8-Br-cAMP was not determined because at the maximal concentration used (10 mM) the dose-response curve still didn't plateau and 8-Br-cAMP induced instability in I_h current recordings. $N = 4$.

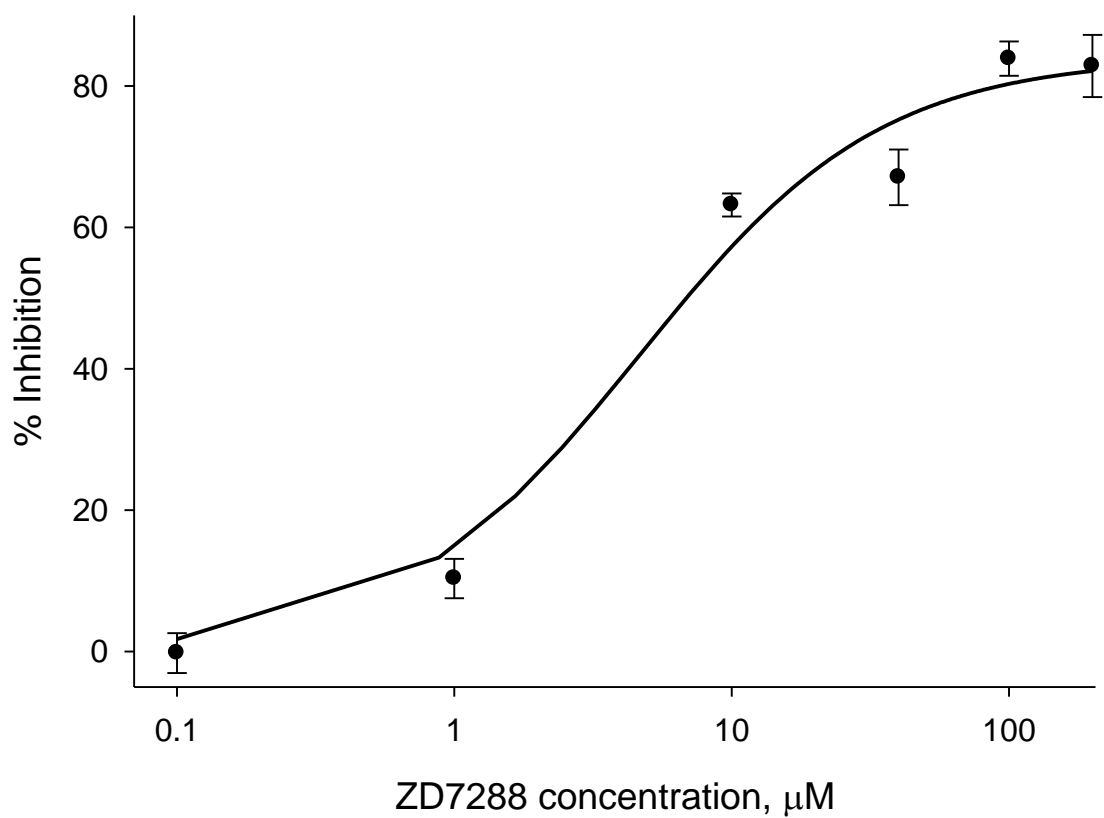


Fig. S9. Concentration dependence of the acHCN channel (I_h) inhibition by ZD7288 (*Xenopus* oocytes). Percent inhibition was calculated using the following formula: $\% \text{ Inhibition} = 100 - I_{h,90 \text{ mV}} / I_{h,90 \text{ mV, Ctrl}} * 100$. ZD7288 inhibits acHCN channel in a dose-dependent manner. $K_i = 4.68 \pm 1.51 \mu\text{M}$, $n = 4$.

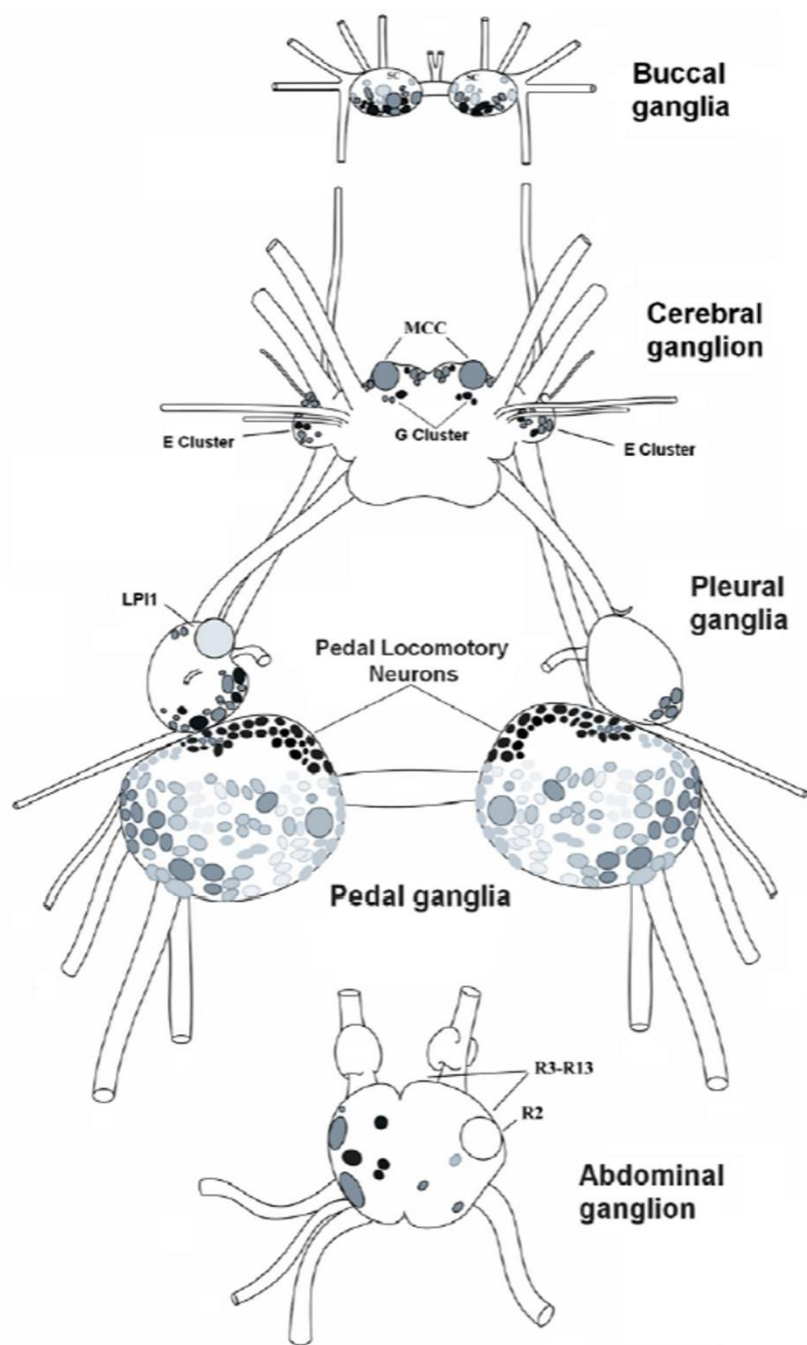


Fig. S10. Schematic diagram of HCN channel expression in the CNS of *Aplysia*.

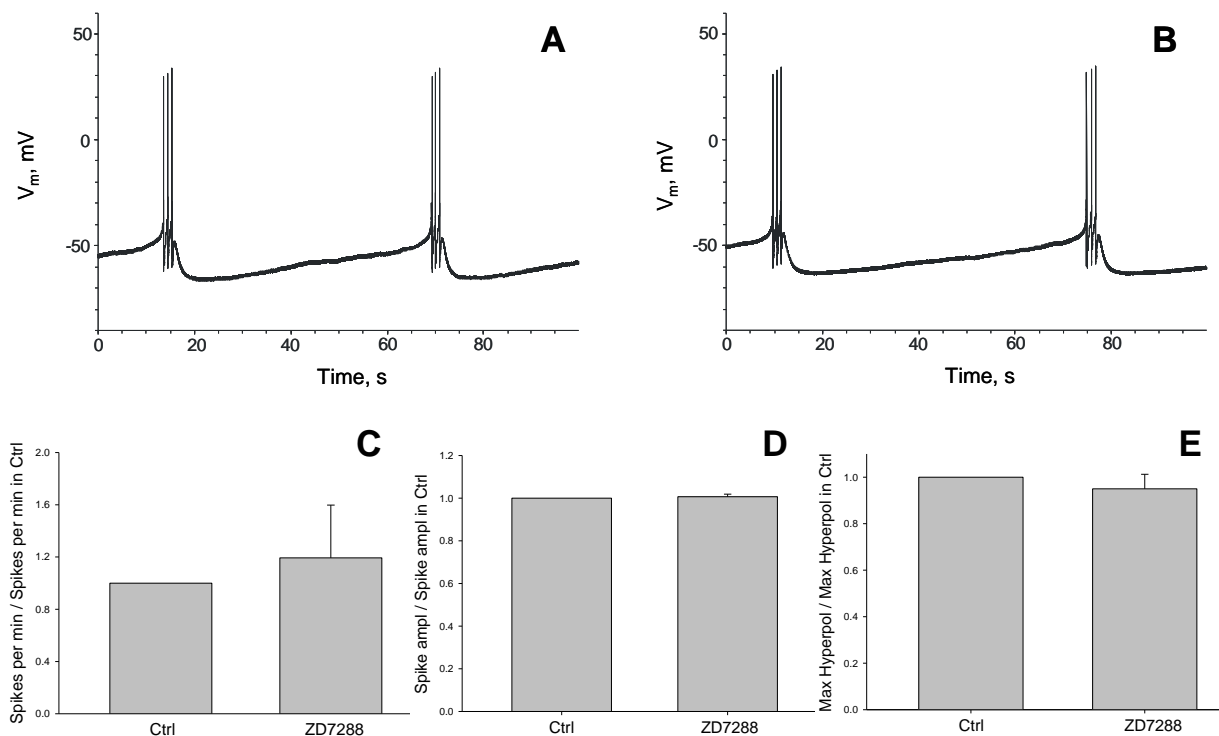


Fig. S11. Determination of the specificity of ZD7288 for acHCN (see also Fig. S12). ZD7288 (150 μ M) did not influence any aspect of spiking of R15 neuron that does not express detectable level of acHCN as shown by *in situ* hybridization tests. **A.** R15 recording in control. **B.** R15 recording following perfusion with AS containing ZD7288. **C.** Number of spikes per minute did not significantly change following perfusion with artificial sea water (AS) containing ZD7288, compared to control (1.19 ± 0.40 fold change, $p = 0.68$, $n = 3$). **D.** Spikes amplitude did not significantly change following perfusion with AS containing ZD7288, compared to control (1.01 ± 0.01 fold change, $p = 0.64$, $n = 3$). **E.** Maximal hyperpolarization did not significantly change following perfusion with AS containing ZD7288, compared to control (0.95 ± 0.06 fold change, $p = 0.51$, $n = 3$).

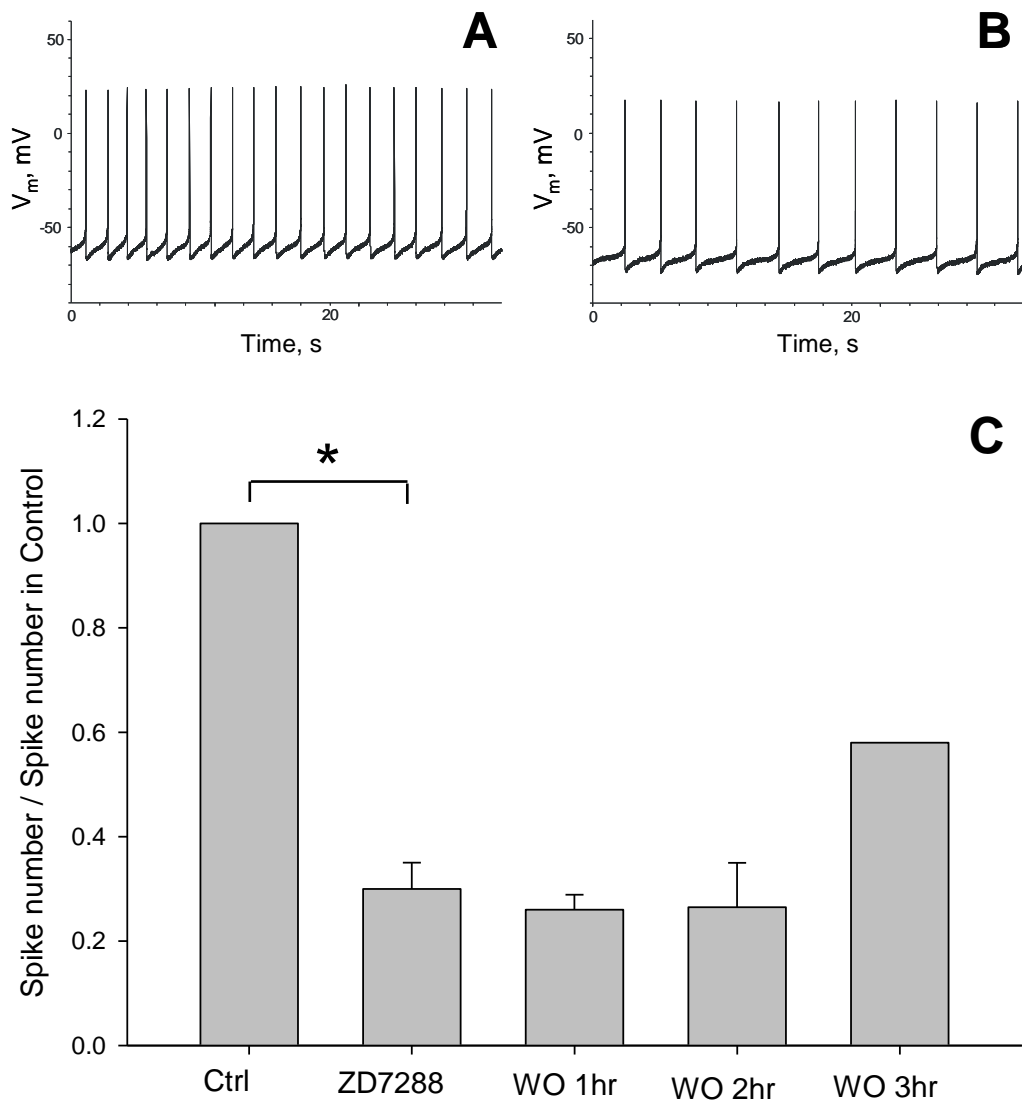


Fig. S12. ZD7288 significantly decreased spiking frequency of the metacerebral cells (MCCs) known to express HCN channels. ZD7288 (150 μ M) significantly decreased spiking frequency of MCCs ($70 \pm 5.03\%$ decrease following perfusion ZD7288, compared to control, $p = 0.005$, $n = 3$). Spiking frequency recovered slowly following washout, with $10.5 \pm 2.5\%$ recovery following 2 hr washout compared to 1 hr washout ($n = 3$). **A.** MCC recording in control. **B.** MCC recording following perfusion with artificial sea-water solution containing ZD7288. **C.** Graphical representation of the effect of ZD7288 on spiking frequency of MCCs. Ctrl – control, WO – washout. Similar results were shown in experiments from buccal and pedal motoneurons known to express high levels of HCN (see **Fig. 3**, main text).

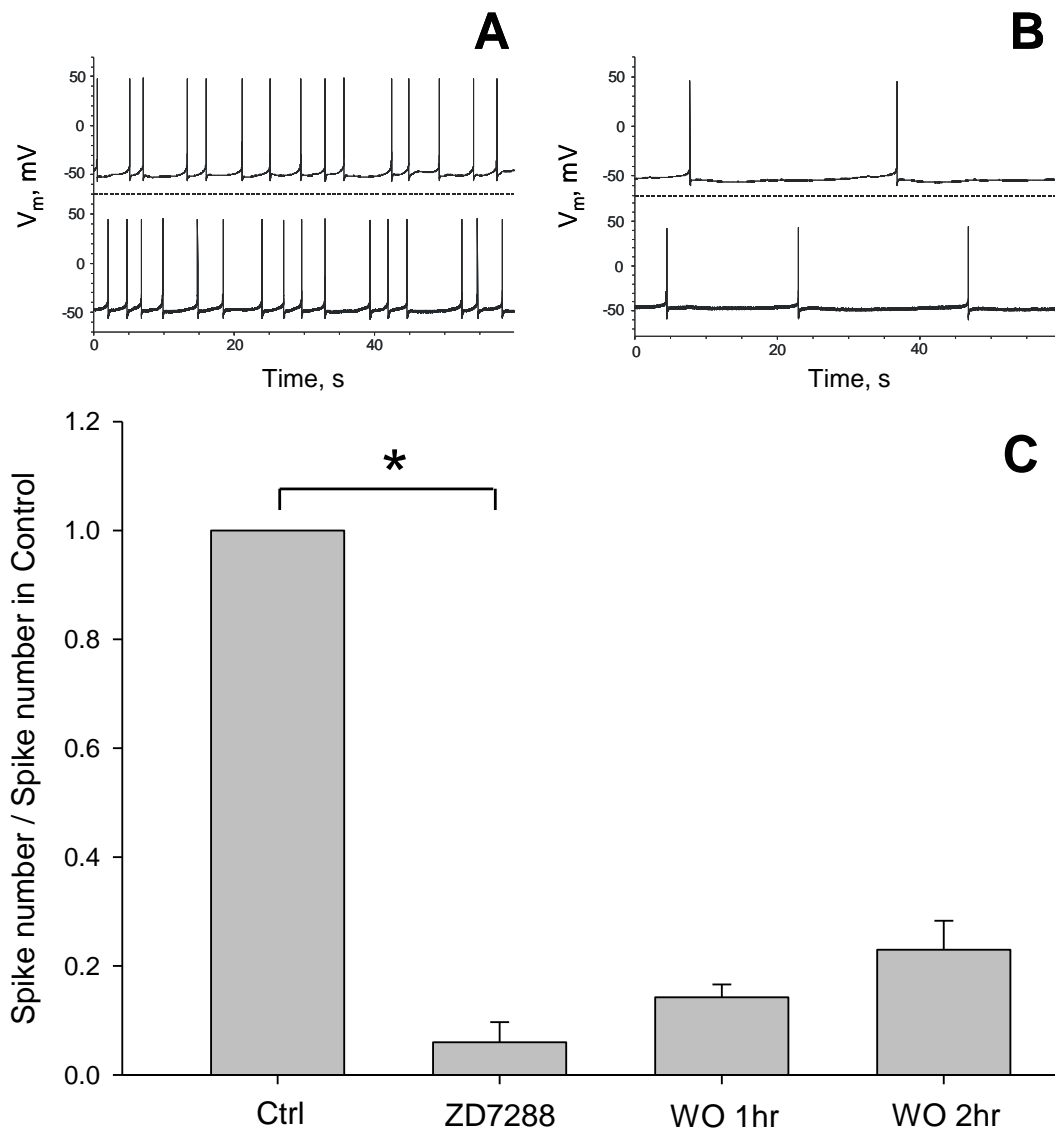


Fig. S13. Effect of ZD7288 on spiking of the pedal locomotory neuron P4. ZD7288 (150 μ M) significantly decreased spiking frequency of P4 neuron ($94 \pm 3.67\%$ decrease following perfusion with AS containing ZD7288, compared to control, $p = 0.0001$, $n = 4$). Spiking frequency slowly recovered following washout, with only minimal recovery following 2 hr washout compared to 1 hr washout ($n = 4$). **A.** P4 recording in control. Upper trace – P4 in the left pedal ganglion; lower trace – P4 in the right pedal ganglion. **B.** P4 recording following perfusion with AS containing ZD7288. Upper trace – P4 in the left pedal ganglion; lower trace – P4 in the right pedal ganglion. **C.** Graphical representation of the effect of ZD7288 on spiking frequency of P4 neuron. Ctrl – control, WO – washout.