CLONING AND CHARACTERIZATION OF A HYPERPOLARIZATION-ACTIVATED, CYCLIC NUCLEOTIDE-GATED CATION CHANNEL IN *APLYSIA CALIFORNICA*

By

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To my parents, Galyna and Mykhaylo Kuzyk

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
Aa	amino acids
acHCN	hyperpolarization-activated, cyclic nucleotide-gated cation channel cloned from *Aplysia californica*
AP	action potential
AS	*Aplysia* saline
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
cNMPs	cyclic nucleotides
CAP	catabolite activator protein
CNBD	cyclic-nucleotide-binding domain
CNG	cyclic nucleotide-gated channel
CNGC	(plant) cyclic nucleotide-gated cation channel
CNS	central nervous system
DIG	dioxigenin
DRG	dorsal root ganglion
E_r	reversal potential
ERG	ether-a-go-go-related channel
EST	expressed sequence tag
HCN	hyperpolarization-activated, cyclic nucleotide-gated cation channel
Hi-Di	high divalent cation solution
I_h	HCN-mediated current
K_a	concentration required for half-maximal activation

K_i	concentration required for half-maximal inhibition
LTH	long-term hyperexcitability
LTP	long-term plasticity
MCC	metacerebral cells
MN	motoneuron
Nt	nucleotide
ORF	open reading frame
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PTW	0.1% Tween 20 in PBS
RACE	rapid amplification of cDNA ends
SN	sensory neuron
τ	activation constant
TEA HCl	TEA HCl
TM	transmembrane domain
UTR	untranslated region
$V_{1/2}$	voltage of the half-maximal activation

Abstract of Dissertation Presented to the Graduate School
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CLONING AND CHARACTERIZATION OF A HYPERPOLARIZATION-ACTIVATED, CYCLIC NUCLEOTIDE-GATED CATION CHANNEL IN *APLYSIA CALIFORNICA*

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Changes in neuronal excitability alter the frequency of neuronal spiking, trigger and modulate associated behaviors and underlie different pathological states. Hyperpolarization-activated, cyclic nucleotide-gated cation (HCN) channels have been suggested to play an important role in modulating neuronal excitability. Most of the properties of HCN channels were studied in vertebrates. However, because neurons of the vertebrate animals are small and neuronal populations are heterogeneous, it is impossible to study the role of the HCN channels on the level of identified individual neurons, neuronal networks and the behaviors they control. In contrast, the marine mollusk, *Aplysia californica*, has many large identifiable neurons integrated into several well-studied networks. Here, *A. californica* has been used as a model animal to determine how the molecular organization of the *Aplysia* HCN channel (acHCN) influences its properties and how the properties of acHCN determine function of the channel on the level of identified individual neurons and neuronal networks.

To characterize the channel, the acHCN transcript was first cloned from the CNS of *A. californica*. The cloned channel is similar to HCN channels from other organisms, but acHCN differs significantly from other HCN channels in its N-terminal region. The first methionine of acHCN is 28 amino acids downstream of the translation start found in other HCN channels indicating that acHCN may be truncated at its N-terminus. However, the region upstream of the

first methionine of acHCN exhibits a weak similarity to other HCN channels implying that it may be important for the channel's functioning. Thus, the question of whether the cloned channel is functional remained. This question was addressed by expressing acHCN in *Xenopus laevis* oocytes and studying the biophysical and pharmacological properties of the channel.

The expressed channel exhibits all major properties of HCN channels, namely, activation by both hyperpolarization and cyclic nucleotides, permeability to potassium and sodium ions and inhibition by Cs^+ and ZD7288.

Knowing the properties of acHCN and confirming that ZD7288 is its specific blocker, allowed studying the role of the channel in *A. californica* neurons. Following localization of the acHCN transcript in the CNS of *A. californica*, three groups of neurons were studied to characterize the functional role of acHCN, i.e., metacerebral cells (MCC) and also buccal motoneuron B3 and pedal locomotory neuron P4, which are part of feeding and locomotory networks, respectively. It was demonstrated that acHCN controls the spiking frequency of these neurons. Together with the fact that spiking of B3 and P4 neurons directly correlates with the contraction of buccal and pedal muscles, respectively (Church and Lloyd, 1994; Hening et al., 1979), this implies a role of acHCN in coordinating feeding and locomotion in *A. californica*.

CHAPTER 1
INTRODUCTION

1.1 HCN Channels and Their Role in Neuronal Excitability

Excitability is an important feature of neurons that allows them to process and transmit information. Changes in neuronal excitability alter the frequency of neuronal spiking, and trigger and modulate the activity of neuronal networks and associated behaviors, e.g., locomotion (Ahlman et al., 1971), reproduction (Conn and Kaczmarek, 1989) and aggression (Keele, 2005). Changes in neuronal excitability also underlie long-term potentiation (LTP) and long-term hyperexcitability (LTH), major processes of synaptic and non-synaptic plasticity, respectively (Kandel, 1976; Weragoda et al., 2004).

In the 1970s, Kandel and his colleagues showed that cyclic nucleotides, particularly cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) contribute to neuronal excitability (Brunelli et al., 1976; Arancio et al., 1995; Lewin and Walters, 1999). It was demonstrated that cAMP and cGMP depolarize neurons and thus increase neuronal excitability through activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) that phosphorylate potassium channels, thus reducing their currents (Klein and Kandel, 1980; Siegelbaum et al., 1982). In parallel, cAMP and cGMP increase neuronal excitability by recruiting different transcription factors that activate expression of genes responsible for the growth of new synaptic connections (Schacher et al., 1988; Dash et al., 1990). However, in addition to the well-studied role of the cyclic nucleotides in controlling neuronal excitability through these indirect pathways, cAMP and cGMP can also regulate neuronal excitability directly by binding to cyclic nucleotide-gated channels, particularly hyperpolarization-activated, cyclic nucleotide-gated (HCN) cation channels (Brown et al., 1979).

HCN channels were first found in the pacemaker cells of the rabbit sino-atrial node (Noma and Irisawa, 1976) where they control cardiac rhythmicity (Brown et al., 1979). Shortly

afterward, these channels were also found in the nervous system of guinea pig (Halliwell and Adams, 1982) and shown to control the spontaneous activity of neurons (Brown and DiFrancesco, 1980; DiFrancesco, 1981; Pape, 1996) and synchronization of the low frequency oscillations in different areas of the brain (Maccaferri and McBain, 1996; Bal and McCormick, 1997; Luthi et al., 1998). In addition to controlling the spontaneous activity of different cells, HCN channels also play a role in LTP (Beaumont and Zucker, 2000) and different pathological processes, such as neuropathic pain (Chaplan et al., 2003), epilepsy (Chen et al., 2001, 2002), seizure and cardiac ischemia (Robinson and Siegelbaum, 2003) (see Table 1-1).

All of the above listed functions are based on the increase of neuronal excitability linked to HCN channels. The ability to increase neuronal excitability, in turn, is determined by the biophysical properties of the channels. HCN channels are activated by hyperpolarization, typically negative to -60 mV and generate a slow inward current (I_h) (Figure 1-1A). This current depolarizes cells towards the reversal potential of the channels, which is between -15 and -40 mV. Thus, the resting potential of HCN-expressing cells is significantly more positive than a typical resting potential of around -60 mV. Therefore, the HCN-expressing cells may need smaller excitatory synaptic inputs to reach a threshold for firing an action potential compared to cells that do not express these channels, i.e., the excitability of the HCN-expressing cells can be higher. In electrically-active cells, low-threshold sodium and calcium channels can act synergistically with HCN channels and further depolarize the cells until they fire an action potential.

In addition to hyperpolarization, HCN channels are also activated by cyclic nucleotides. Cyclic AMP and, to a lesser degree, cGMP directly bind to HCN channels and shift the voltage dependence of the channel gating to more depolarized values (DiFrancesco and Tortora, 1991; Ludwig et al., 1998) (Figure 1-1B). Thus, in the presence of cyclic nucleotides, HCN channels

activate earlier during the after-hyperpolarization following an action potential, more quickly depolarize cells towards firing of the next action potential and, therefore, increase the frequency of neuronal spiking (Figure 1-2).

Concentrations of cyclic nucleotides can be greater in neurons undergoing LTP (Cedar et al., 1972), as well as extracellularly, in the cerebrospinal fluid and different regions of the brain following seizure (Myllyla et al., 1975; Ferrendelli et al., 1980) and nerve injury (Ruis-Morales and Vara-Thorbeck, 1986; Siegan et al., 1996). Thus, during these states HCN channels are expected to be activated and to increase neuronal excitability. Also, during seizure and cardiac ischemia HCN channels may be activated by elevated extracellular K^+ (Robinson and Siegelbaum, 2003) because an increase in extracellular K^+ strongly increases the amplitude of I_h (Figure 1-1C).

1.2 Molecular Organization of HCN Channels

The properties of HCN channels, i.e., activation by hyperpolarization, cyclic nucleotides and elevated extracellular K^+, are determined by the molecular organization of the channels. HCN channels belong to a superfamily of voltage-gated potassium channels (Pongs, 1992) and as such have six transmembrane domains (TM), a voltage sensor in TM4 and a pore with a potassium selectivity filter (Figure 1-3). Similar to classic voltage-gated potassium channels, the voltage sensor of HCN channels contains positively-charged residues, arginines or lysines, separated from each other by two neutral residues (Larsson et al., 1996). However, unlike other potassium channels, which are activated by depolarization, HCN channels are activated by hyperpolarization. This reversed polarity of activation may be determined by differences in the TM4-TM5 linker and C-terminal region that couple channel activation and gating (Prole and Yellen, 2006).

The unusual reversal potential of the channels is determined by their permeability to both K^+ and Na^+. This mixed ionic permeability results from modifications of the potassium selectivity motif TXXTXGYG (Heginbotham et al., 1994) in HCN channels. Whereas the GYG sequence is preserved, the first threonine residue is altered to histidine and the second threonine residue is changed to either serine or cysteine. A conserved aspartate, which follows the GYG motif in most potassium channels, is altered in HCN channels to arginine, lysine, glutamine, alanine, serine or methionine.

Another difference between HCN and classic voltage-gated potassium channels is the presence of a cyclic nucleotide-binding domain (CNBD) at the C-terminus of HCN channels.

As described earlier, all of the changes in HCN channels compared to classic voltage-gated potassium channels result in the ability of HCN channels to regulate neuronal excitability.

1.3 *Aplysia californica* as a Model to Study HCN Channels

The molecular organization and most of the described properties of HCN channels were studied in vertebrates. However, there are limited reports about the role of these channels in network functions or systemic function of the brain. Indeed, neurons of vertebrates are small and neuronal populations are heterogeneous; thus, it is very difficult to study how the activity of the HCN channels in identified individual neurons influences the excitability and spiking of these neurons, and the activity of the neuronal networks and behaviors they control. The CNSs of invertebrate animals have a much simpler organization, with smaller numbers of cells organized into well-defined neuronal circuits. Nevertheless, the majority of neurons in most invertebrate species are also relatively small and difficult to study. In contrast, a marine mollusk, *Aplysia californica*, (Figure 1-4) has many large (up to 1 mm in diameter) identifiable neurons (Figure 1-5) that can be easily isolated and used for cDNA library construction, electrophysiological

recordings, injection, and biochemical analysis, which is a difficult to impossible task in vertebrate and most invertebrate systems (Kandel, 1976; Moroz et al., 2006).

Many neuronal networks controlling identifiable behaviors have been extensively studied in *A. californica*, including memory-forming (Kandel, 1976, 2001), feeding (Kandel, 1976; Jahan-Parwar and Fredman, 1983; Cropper et al., 2004), defensive (Kupfermann et al., 1970; Castellucci et al., 1970; Kandel, 1976; Croll, 2003) and locomotory networks (Jahan-Parwar and Fredman, 1978; Hening et al., 1979; Fredman and Jahan-Parwar, 1983). For example, it is known which pedal neurons control contraction of different parts of the foot (Hening et al., 1979) and which neurons in buccal and cerebral ganglia control different aspects of feeding behavior, i.e., protraction and retraction of buccal mass muscles (Jahan-Parwar and Fredman, 1983; Cropper et al., 2004). Locating a transcript of interest in the cells of a certain network allows predictions about the role the channel plays in the animal's behavior. Subsequent electrophysiological characterization of the channel in these cells provides a means for testing these predictions. Thus, in *A. californica* it is possible to determine the role of the HCN channel on the level of an individual cell, neuronal network and its associated behavior.

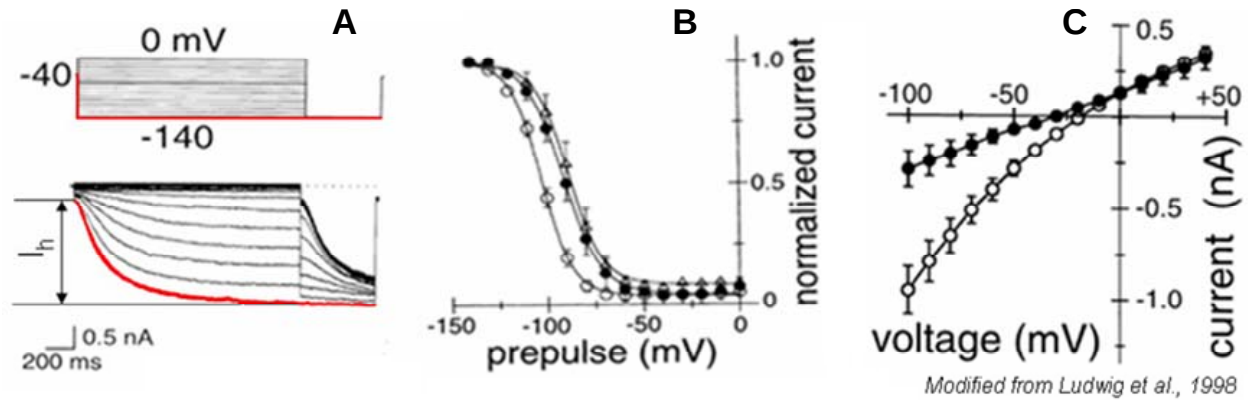

Figure 1-1. Biophysical properties of HCN channels. A) Determination of the voltage dependence of HCN channel activation. Human HCN1 channel was expressed in human embryonic kidney 293 cells; the cells were voltage-clamped from a holding potential of -40 mV to various potentials ranging from -140 mV to 0 mV, in -10 mV increments and then to -140 mV. Following the hyperpolarizing step from -40 mV to potentials negative to -70 mV, slow inward non-inactivating currents developed. Shown in red is the current trace generated upon stepping from the holding potential of -40 mV to -140 mV. B) Voltage dependence of HCN channel activation in the absence of cyclic nucleotides (open circles) or after intracellular perfusion with 1 mM cAMP (filled circles) or 1 mM cGMP (triangles). Cyclic nucleotides shifted the voltage activation of the HCN channel in a depolarizing direction. C) Dependence of Ih amplitude on extracellular potassium concentration. I/V relationship for the fully activated channel was obtained by plotting the tail-current amplitudes measured after stepping from the holding potential of -40 mV to -140 mV for 1.5 sec and then to more depolarized voltages ranging from -100 to +40 mV in 10 mV steps. I/V relationships were determined at 5.4 mM (filled circles) and 30 mM (open circles) extracellular K^+, respectively. Current amplitudes were much larger at 30 mM extracellular K^+. Reprinted by permission from Macmillan Publishers Ltd: Nature Ludwig et al., 1998.

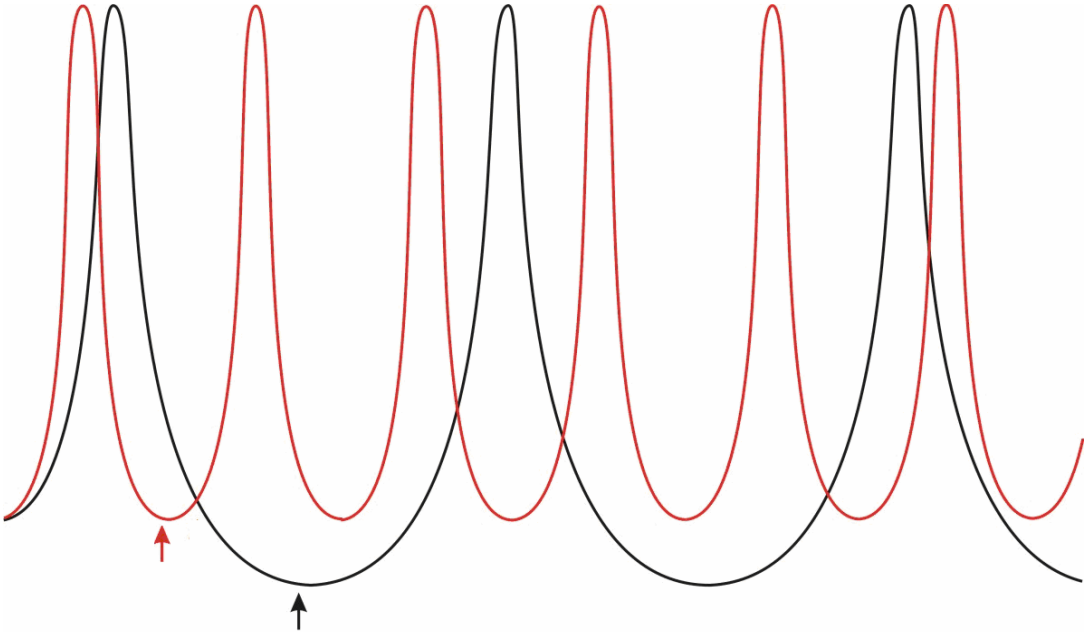

Figure 1-2. Schematic diagram of neuronal spiking in control conditions (black) and in the presence of cyclic nucleotides (red). The potentials of I_h activation are shown by black and red arrows respectively. In the presence of cyclic nucleotides, HCN channels activate earlier during the after-hyperpolarization following an action potential, more quickly depolarize cells towards firing of the next action potential, and therefore increase frequency of neuronal spiking.

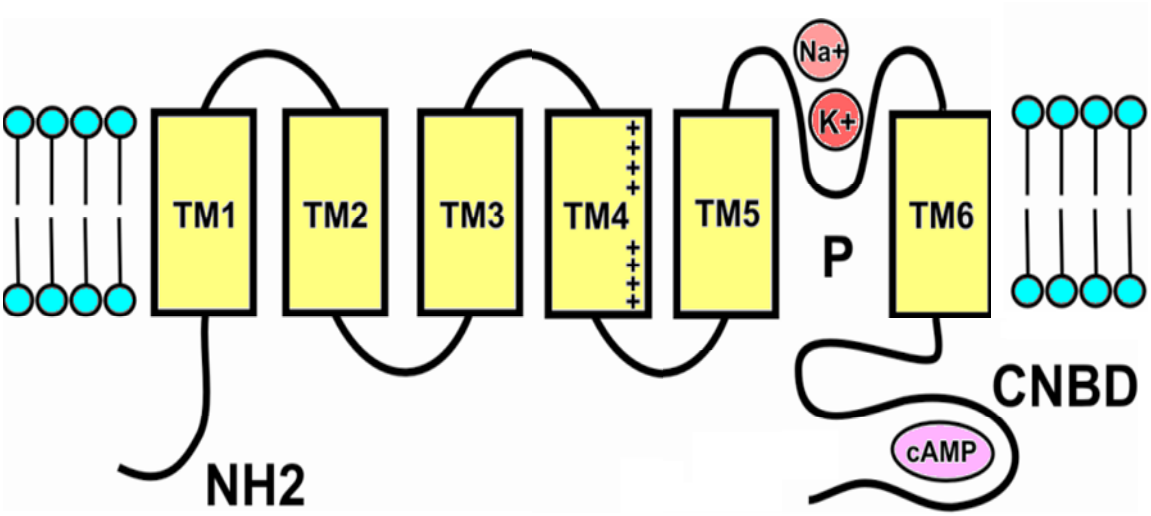

Figure 1-3. Two-dimensional model of an HCN channel. HCN channels have six transmembrane domains (TM1-TM6); an ion pore (P) with a modified potassium selectivity filter; eight positively charged residues in TM4 (+) and a cyclic nucleotide-binding domain (CNBD) at the C-terminus.

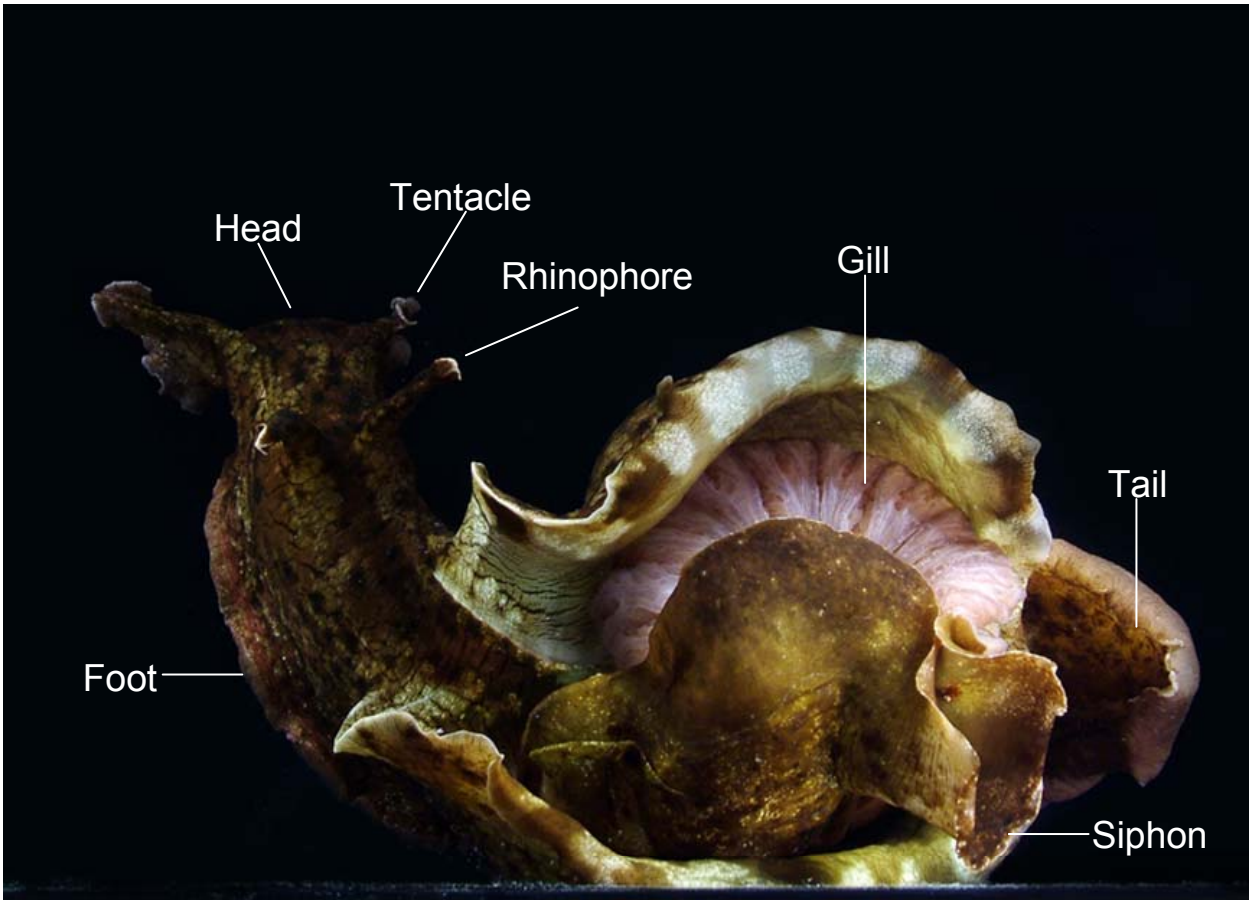

Figure 1-4. Sea slug *Aplysia californica*. *A. californica* has characteristic features of the molluscan body plan: head with tentacles, rhinophores (chemosensitive organs) and eyes, foot and mantle cavity containing gills.

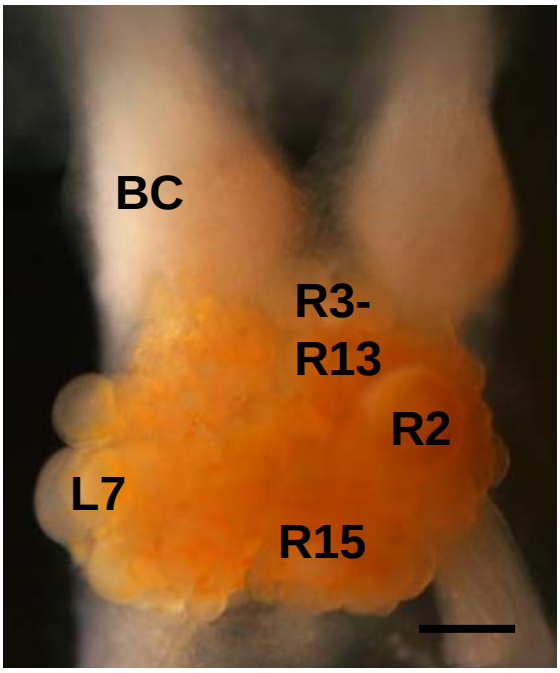

Figure 1-5. Abdominal ganglion of *A. californica*. The abdominal ganglion has many large identifiable neurons with known functions, e.g., L7, which controls gill withdrawal and is a part of a defensive network (Kupfermann et al., 1971), neurosecretory neurons R3-R13 (Gainer and Wollberg, 1974), R2, which controls mucus production (Rayport et al., 1983), R15, which is responsible for respiratory pumping (Alevizos et al., 1991) and bag cluster (BC) cells, which produce egg-laying hormone (Kupfermann and Kandel, 1970). Scale bar = 500 μm.

Table 1-1. HCN channels in different animal phyla.

Taxon/species	Channel name	Accession number	Number of exons	Function and reference
Deuterostomes	HCN1	gi:32698746	8	
Phylum Chordata	HCN2	gi:30580783	8	Epilepsy (Bender et al., 2003), heart contraction (Vaccari et al.,
Class Mammalia*	HCN3	gi:38327037	8	1999)
Homo sapiens	HCN4	gi:4885407	8	
Rattus norvegicus	HCN1	gi:16758108	8	Synchronization in the brain (Maccaferri and McBain, 1996), LTH
	HCN2	gi:50878267	8	following nerve injury (Chaplan et al., 2003), epilepsy (Chen et al,
	HCN3	gi:29840773	8	2001), hippocampal development (Strata et al., 1997), response to
	HCN4	gi:29840771	8	sour stimuli (Stevens et al., 2001), heart activity (Shi et al., 1999)
Class Aves				
Gallus gallus	HCN2	gi:86129554	12	Sound source localization (Yamada et al., 2005), generation of the temporal activity patterns in auditory thalamic relay nucleus (Strohmann et al., 1994)
Class Amphibia				
Ambystoma tigrinum	HCN	Not cloned	?	Adaptation to overly intensive stimuli in rod and cone photoreceptors (Bader et al., 1979; Hestrin, 1987)
Class Actinopterygii				
Oncorhynchus mykiss	HCN1	gi:33312349	?	May impact release of transmitter from hair cells of the saccule (Cho et al., 2003)
Danio rerio	HCN1	gi:94732421	8	Contributes to pacemaking in the heart (Baker et al., 1997)
Phylum Echinodermata	SPIH	gi:3242324	9	
Class Echinoidea				
Strongylocentrotus purpuratus	SpHCN2	gi:69146514	11	Flagellar beating (Gauss et al., 1998; Galindo et al., 2005)
Phylum Arthropoda				
Class Insecta				
Apis mellifera	Amih	gi:52631748	13	May play a sensory role (Gisselmann et al., 2003)
Drosophila melanogaster	Splice form A1B1C1	gi:24653608	13	May play a sensory role (Marx et al., 1999-2000)
Class Crustacea				
Panulirus argus	PAIH	gi:33355925	?	Stabilizes membrane polarization and excitability in varying external conditions in stretch receptor neurons (Edman and Grampp, 1991), may participate in olfactory transduction (Gisselmann et al., 2005b), modifies firing activity of stomatogastric ganglion neurons (Zhang et al., 2003)

Table 1-1. Continued

Phylum Mollusca Class Gastropoda *Hermissenda crassicornis*	HCN	Not cloned	?	Dark adaptation in in B-type photoreceptors (Yamoah et al., 1998)
Lymnaea stagnalis	HCN	Not cloned	?	Modulates activity of central pattern generator underlying rhythmic ingestion movements (Straub and Benjamin, 2001)
Phylum Annelida Class Clitellata *Hirudo medicinalis*	HCN	Not cloned	?	Regulates bursting activity of heart interneurons (Angstadt and Calabrese, 1989)

*Only 8 of 18 cloned mammalian HCN channels are shown.

CHAPTER 2
CLONING AND MOLECULAR CHARACTERIZATION OF THE acHCN CHANNEL

2.1 Introduction

To study the properties and role of the acHCN channel it has first to be cloned from the CNS of *A. californica*.

A. californica belongs to a clade of animals called Lophotrochozoa (Peterson and Eernisse, 2001). Because no HCN channels have been cloned from any species of this group, a question arises about how acHCN differs from HCN channels of other lineages, specifically those belonging to two other clades: Ecdysozoa and Deuterostomia (Gisselmann et al., 2003, 2005a, b; Ludwig et al., 1998). Thus, the major objective of the work presented in this chapter was to clone an HCN channel from the CNS of *A. californica*, characterize the molecular organization of the channel, and compare it to the HCN channels from other organisms.

Since only one HCN gene was found in the CNS of most invertebrates, my hypothesis was that it also holds true for *A. californica.* And because HCN channels of Ecdysozoa and Deuterostomia are highly conserved, I predicted that the general organization of acHCN would be similar to HCN channels of other organisms. However, some characteristics of acHCN might be different as a result of parallel evolution of the lophotrochozoan lineage.

By cloning the *A. californica* HCN channel I could to determine the distribution of the transcript in the CNS of the animal, express the channel in a heterologous system and study the biophysical and pharmacological properties of acHCN in an environment without an interference from other channels, as well as in its native neuronal environment. Obtaining the sequence of acHCN also allowed me to perform comparative analysis of HCN channels across all three clades of the animal kingdom.

2.2 Materials and Methods

2.2.1 Materials and Reagents

All materials and reagents used for the work described in this and subsequent chapters were obtained from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

2.2.2 Cloning and Sequence Analysis

Amplified cDNA was prepared from the whole CNS, buccal, or pleural ganglia, as described previously (Matz, 2002).

Primers (5'-CGGAGTCTACGTCAGAGGATACC-3'; 5'-GACGATGCTGGGGTTCTTGCCGA-3') were designed to the HCN-like cDNA sequence from our *A. californica* expressed sequence tag (EST) database (Moroz et al., 2006). The obtained fragment was extended by multiple 5'- and 3'-RACE (rapid amplification of cDNA ends) reactions (Matz et al., 1999). 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 products of each reaction were run on 1% agarose gel. Distinct bands were excised. 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 sequenced by the Whitney Laboratory molecular core facility or by SeqWright (Houston, TX).

Most of the HCN sequences used for alignments and construction of the phylogenetic trees were obtained from the Entrez Protein database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein). Sequences of HCN transcripts for *Lottia gigantea* (Mollusca, Gastropoda), *Nematostella vectensis* (Cnidaria, Anthozoa), *Capitella sp*.

(Annelida, Polychaeta), and *Ciona intestinalis* (Urochordata, Ascideacea) were downloaded from the Joint Genome Institute (JGI) website (http://www.jgi.doe.gov/) following a search using the keyword "hyperpolarization" and translated using the ExPASy Translate tool (http://www.expasy.ch/tools/dna.html). Each of the output sequences was then blasted against the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) to ensure their identity as HCN channels. The sequences confirmed to correspond to HCN channels were then blasted against the JGI database for a particular organism to find similar fragments and potentially extend the existing sequences.

Alignment of HCN proteins from different organisms was done using ClustalX (ftp://ftp-igbmc.u-strasbg.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).

The bootstrapped neighbor-joining phylogenetic tree was created in ClustalX after removing all gaps in the alignment of HCN proteins (see Appendix A) or CNBD-containing potassium and cation channels (see Appendix B) in GeneDoc. Unrooted trees were generated because creating a rooted tree requires knowing the ancestor of the group of analyzed proteins, and this ancestor is not known for HCN channels. Boostrapping was done to determine the consistency of taxon bipartitions. Bootstrap values were calculated as the number of times that a particular node appeared during 1000 iterations while constructing the phylogenenetic tree. The graphical output was generated using TreeView

(http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Two HCN channels of *N. vectensis* were not put on the tree because they are too divergent and do not align with other HCN channels.

2.2.3 Determination of the Genomic Organization of acHCN

To determine the genomic organization of acHCN, the consensus sequence of the acHCN clone (cDNA) was blasted against the trace archive of *A. californica* genomic sequences (~ 5x coverage). Regions of considerable similarity (alignment scores 80 and higher) between the genomic sequences and the acHCN cDNA were considered to be exons and the genomic sequences flanking exons were considered to be introns.

2.2.4 Northern Blot

2.2.4.1 RNA isolation and blotting

Total RNA was isolated from the whole *A. californica* CNS using the RNAaqueous (Ambion, Carlsbad, CA, USA) isolation protocol. Briefly, the cells were first disrupted in a lysis solution containing guanidinium thiocyanate; RNA was then bound by a silica-based filter and eluted in a small volume of elution solution. mRNA was obtained from the total RNA using a Poly(A) Purist kit (Ambion) based on binding of mRNA by Oligo(dT) Cellulose. RNA quality control was performed using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). RNA samples were denatured by incubation for 30 min at 50°C in Glyoxal Load Dye (Ambion) and then run on a gel made with Ambion's Gel Running Buffer for 1.5 hrs at 5 V/cm. To transfer RNA from the gel to a membrane, transfer material was arranged as follows (from the bottom): 3 cm of paper towels, three dry pieces of filter paper (Whatman International Ltd., Maidstone, England) and two more pieces pre-wet in Transfer buffer (Ambion), nylon membrane (Micron Separations Inc., Westborough, MA) pre-wet in Transfer buffer, gel, and three more pieces of filter paper. RNA was transferred by centrifuging the transfer material for 50 min at 500 rpm. The efficiency of transfer was checked by examining the membrane and gel under UV illumination. RNA was

then cross-linked in a UV crosslinker 1800 (Stratagene, La Jolla, CA) and the cross-linked membrane was kept at -20°C.

2.2.4.2 Chemiluminescent detection

The membrane was pre-hybridized for 1 hr at 68°C in 10 ml of ULTRAhyb buffer (Ambion). Antisense RNA probe (1 µg/µl) was synthesized and labeled with DIG as described in section 4.2.3. 1 µl of the probe was mixed with 1 ml of ULTRAhyb buffer and transferred to pre-hybridization solution. Hybridization proceeded for 14 hrs at 68°C. After hybridization, the membrane was washed once for 10 min with Low Stringency Wash Solution #1 (2x SSC, 0.1% SDS) (Ambion) using agitation and twice for 15 min with High Stringency Wash Solution #2 (0.1x SSC, 0.1% SDS) (Ambion) using agitation. The membrane was washed briefly (1-5 min) in Washing Buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% (v/v) Tween 20, pH 7.5) and incubated for 30 min in Blocking Solution (1x Blocking Reagent) (Roche) in Maleic Acid Buffer – 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and Antibody Solution (Alkaline-Phosphatase-conjugated Anti-DIG antibodies diluted 1:20,000 in Blocking Solution). The membrane was then washed for 5 min in Washing Buffer and equilibrated for 5 min in Detection Buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl). The membrane was placed RNA side facing up on a development folder and CDP-Star (chemiluminescent substrate for alkaline phosphatase) (Roche) working solution (5 µl CDP-Star in 0.5 ml Detection Buffer) was applied. The membrane was immediately covered with another sheet of development folder to spread the substrate evenly over the membrane, exposed to Hyperfilm ECL (high performance chemiluminescence film) (Amersham Biosciences, Piscataway, NJ) for 1-5 min under safe-light illumination and developed in Konika SRX-101 developer.

To strip the membrane of a probe it was incubated in Stripping Solution (50% formamide, 5% SDS, 50 mM Tris-HCl, pH 7.5) two times for 60 min at 80°C, then rinsed in 2x SSC for

5 min at room temperature and stored in 2x SSC before re-using.

2.2.4.3 Radioactive detection

The transcription/labeling reaction was performed as described in the Strip-EZ^{TM} RNA protocol (Ambion) in the presence of 60 μCi of labeled uridine ([α-^{32}P]UTP) for the 533 nucleotide (nt) acHCN probe or 100 μCi for the whole (approximately 2 kb) acHCN probe and 1755 nt FMRFamide probe (corresponding to the whole FMRFamide gene) in 20 μl total volume.

Hybridization was performed in 10 ml of UltraHyb buffer. In each case, 10 μl of probe was added giving a final radioactivity of 30 μCi for the 533 nt acHCN probe and 50 μCi for the whole acHCN and FMRFamide probes. Hybridization proceeded for 15 hrs.

Development was carried out for 1-3 days in a cassette with enhancing screen at -80°C using BioMax XARfilm (Kodak, Rochester, NY).

2.3 Results

2.3.1 Identity and Molecular Organization of acHCN

One of my major goals was to clone the acHCN channel. It proved to be a difficult task due to both the low abundance of the HCN transcripts in *A. californica* neurons and the unusual organization of the 5'- and 3'-regions of acHCN.

By aligning and analyzing 168 cloned cDNA sequences, each one corresponding to an HCN channel, I determined that I have cloned a coding region of the channel from the CNS of *A. californica* (Figure 2-1). The cloned channel is very similar to HCN channels from other organisms, which indicates that it indeed belongs to the HCN channel family (Figure 2-2).

Following identification of a transcript, two major questions arise: how many isoforms of the transcript are present in the organism, and was the whole reading frame obtained ?

I found only one HCN channel in *A. californica*. Existence of a single HCN channel is supported by the fact that all 13 existing HCN ESTs from the *A. californica* transcriptome (Moroz et al., 2006) perfectly align with the consensus sequence of the cloned channel. Also, fragments corresponding to a single HCN channel have been found in the sequenced genome of *A. californica* (~5x coverage).

In addition, several lines of evidence suggest that I obtained a clone containing the entire open reading frame (ORF). First, there is a stop codon (TAG) upstream from the first ATG supported by all nine sequences for this region. Second, there is a stop codon (TAA) at the end of the acHCN transcript. It is also supported by all sequences (n = 16) for this region. Third, the ORF of acHCN ends where HCN channels from most other organisms end (Figure 2-2). Finally, the cloned channel has all the domains of other HCN channels: six TM, a pore region with a modified potassium selectivity filter, a voltage sensor in TM4 and a CNBD.

Although acHCN is very similar to the HCN channels from other organisms, especially in its middle portion, the 5'-region of the cloned channel is different from other HCN channels. acHCN does not start where most other HCN channels start (first black box in the alignment shown in Figure 2-2). It has an isoleucine at this position whereas HCN channels of other organisms have their first methionine (start). The first methionine of acHCN is 28 amino acids (aa) downstream. acHCN also has an in-frame alternative start codon CTG (Tee and Jaffe, 2001; Touriol et al., 2003) 65 aa upstream from its first methionine.

Similar to other HCN channels, acHCN has eight positively-charged residues, seven arginines and a lysine in its voltage sensor (Figure 2-3). Additionally, as in other HCN channels, the potassium selectivity motif (TXXTXGYG) of acHCN is modified. While the GYG sequence is preserved, the first threonine residue is changed to histidine and the second threonine residue

is changed to cysteine. The conserved aspartate, which follows the GYG motif in most of the classic voltage-gated K^+ channels, is altered in acHCN to arginine (Figure 2-4).

Some studies suggest that HCN channel activity can be regulated by phosphorylation and dephosphorylation by a number of protein kinases (Vargas and Lucero, 2002; Wu and Cohen, 1997). I have predicted 14 putative phosphorylation sites for several protein kinases including seven sites for casein kinase II ([ST]-x(2)-[DE]), six sites for protein kinase C ([ST]- x- [RK]), and one site for tyrosine kinase ([RK]-x(6)-Y). One potential N-glycosylation site (N-x-[ST]) and seven N-myristoylation sites (G-{EDRKHPFYW}-x(2)-[STAGCN]-{P}) were also found (see Figure 2-2). However, because proteins are myristoylated only at their N-termini (Maurer-Stroh et al., 2002), the number of predicted N-myristoylation sites in acHCN can be limited to three, which are at the N-terminal region of the protein at the positions 76, 77 and 110. The presence of 14 putative phosphorylation sites may provide ways, in addition to regulation by cAMP/cGMP, to link the activity of the channel to different signaling pathways inside the cells.

Having the sequence of acHCN, I constructed a phylogenetic tree of HCN channels from different vertebrate and invertebrate species (Figure 2-5). It shows that acHCN is closely related to other invertebrate HCN channels, particularly those of a Prosobranch mollusk, *Lottia gigantea* (giant owl limpet), and a polychaete, *Capitella sp*. High bootstrap values support grouping of acHCN with HCN channels of other Lophotrochozoa. Together with molecular data, the phylogenetic tree provides additional evidence that the cloned channel is in fact an HCN channel.

Unlike vertebrates, which have four HCN channels, HCN1-HCN4, most invertebrates have a single HCN channel that may have several splice forms (Gisselmann et al., 2005a; Ouyang et al., 2007). A sea urchin, *S. purpuratus*, has two HCN channels that were cloned from its sperm cells (Gauss et al., 1998; Galindo et al., 2005). I also found two HCN genes in the genome of the

cnidarian *N. vectensis*, and three HCN genes in the ascidian *C. intenstinalis*. Existence of multiple HCN genes in animals of different phyla suggests that duplication of HCN genes occurred in these animals.

2.3.2 Duplication of a Coding Region at the 3'-UTR of acHCN

acHCN has a long 3'-UTR which is almost identical to the coding region of the transcript, but is frame-shifted and thus is not expected to be translated. The "duplicate" sequence in the 3'-UTR was discovered when a fragment produced by one of the 5'-RACEs aligned to the **3'-end** of the existing consensus sequence of acHCN (Figure 2-6A).

To confirm the existence of the duplication I designed a direct primer at the 3'-end of the transcript and a reverse primer at the 5'-end of the transcript corresponding to the 5'-end of the duplicate sequence. PCR with these primers produced a fragment that consisted of the 3'-end of the HCN transcript and the 5'-end of the duplicate sequence (Figure 2-6B).

The same sequence was obtained from three different libraries made from the whole *A. californica* CNS, buccal ganglia or B2 neuron in buccal ganglia. However, despite considerable effort (I performed 173 3'-RACEs), the duplicate sequence was not read to the end and a poly-A sequence was not reached. There are two possible explanations for this. First, a long sequence containing both the coding region of acHCN and the 3'-UTR ending with a poly-A tail, may be very rare or non-existent in the available *A. californica* cDNA libraries. Second, during the production of the cDNA libraries the acHCN fragments might have terminated at the A-rich region at the 3'-end of the transcript (AAACAGAAGAAAAAGACA), downstream of the duplicate sequence, because the poly(T)-containing adaptor, used to make these libraries, bound to this region. The latter explanation is supported by the fact that the 3'-end of the transcript downstream to the A-rich region could not be obtained using conventional 3'-RACEs. It was read only in the experiment shown in Figure 2-6B.

Completion of the *A. californica* genome will help to determine the end of the acHCN transcript. However, having the whole coding region of acHCN I could then proceed to characterize the channel despite not obtaining the full 3'-UTR.

2.3.3 Genomic Organization and Possible Splicing Variants of acHCN

Determining the genomic organization of the channel provides additional means, besides analyzing expressed sequences, for the comparative and evolutionary study of HCN channels. By aligning the consensus cDNA sequence of acHCN and genomic sequences of *A. californica*, I determined that the acHCN gene consists of at least 12 exons (Figure 2-7). The exact genomic organization of acHCN cannot be established at this time because, at the current stage of *A. californica* genome sequencing (~ 5x coverage), there are still gaps in genomic representation of the acHCN transcript, specifically in the 5'- (nucleotides 211-439) and middle (nucleotides 1084-1301) portions of the transcript.

The number of exons in the acHCN gene is similar to that of arthropods that have 13 exons. HCN genes of chordates have fewer exons than invertebrate genes (Figure 2-8). Because the boundaries of most exons in HCN channels of vertebrates and invertebrates coincide, this suggests that exons in Chordata have fused.

Current exon-intron junctions of acHCN are shown in Table 2-1. There are at least seven potential variants of one of the exons, exon 9 (Table 2-2), comprising the beginning of the CNBD. These variants differ in nucleotides in four positions (Figure 2-9). Two variants were found among both genomic and cDNA sequences (variants 1 and 2 in Table 2-2). They differ by the presence or absence of an adenine in one of the positions. The presence of a nucleotide at this position leads to a frame shift and an appearance of a stop codon (TGA) 36 nucleotides downstream. There is a contig (a set of overlapping DNA segments derived from a single genetic source) in the preliminary *A. californica* genomic assembly that contains both of these variants

next to each other. It supports the presence of both of these variants in the *A. californica* genome and suggests that they may be used by alternative splicing.

Interestingly, two splice variants also exist at the beginning of the CNBD in the HCN channel of the spiny lobster, *Panulirus interruptus* (Ouyang et al., 2007), at approximately the same position as in the *A. californica* HCN channel (they start six amino acids downstream compared to acHCN and end two amino acids downstream). These two variants differ by amino acids in seven positions. In the 5'-most of these positions the HCN channel of the spiny lobster has either tyrosine or phenylalanine, while acHCN has either a positively-charged histidine or negatively charged glutamine. This is the only difference between the putative variants of acHCN that is expected to change the properties (charge) of this region of the channel, because three other nucleotide changes are conservative. Also, this is the only position containing alternative amino acids in both *A. californica* and *P. interruptus* suggesting that it may be important in determining the differential properties of these splicing variants.

Alternative splicing in the same region of both *A. californica* and *P. interruptus* HCN channels suggests that different splice variants at this region may be produced to regulate modulation of the channel by cyclic nucleotides. In support of this hypothesis, Ouyang et al.. (2007) showed that the two alternative variants of the spiny lobster are regulated differently by cAMP.

2.3.4 Northern Blot

Because acHCN starts at a different position than HCN channels of other organisms I decided to verify the length of acHCN and determine if forms of the transcript other than the consensus one are synthesized. A traditional method to resolve these issues is Northern blot. I performed 18 experiments using two different probes to acHCN. Probes to a serotonin

transporter (gi:15282076) or FMRFamide (gi:155762) were used as controls since these are among the most abundant transcripts in the *A. californica* CNS.

I performed two types of experiments using either chemiluminescent labeling (with a serotonin transporter probe serving as a control) or radioactive labeling using α-^{32}P-UTP (with an FMRFamide probe serving as a control). Using a standard Northern blot protocol (see section 2.2.4) I did not detect any acHCN bands. To increase the HCN signal I used the following variations of the protocol:

1 Placed on the membrane either the total RNA isolated from the whole *A. californica* CNS or mRNA, obtained from the total RNA. The latter was done to increase the sensitivity of the assay because mRNA makes up only 2 % of the total RNA.

2 Used either a 533 nt probe corresponding to the middle portion of the transcript or a 2 kb probe corresponding to the whole transcript to determine which probe will give a better signal. The shorter probe is expected to bind to its target better, while the longer probe is more specific.

3 Increased probe quantity up to 10 times (for chemiluminescent detection).

4 Increased antibody concentration up to 1:10,000 (for chemiluminescent detection).

5 Extended incubation time in antibodies up to 1 hr (for chemiluminescent detection).

6 Extended hybridization time to either 24 or 41 hrs.

7 Decreased hybridization temperature from +62°C to + 58°C to lower the stringency of hybridization and increase sensitivity.

8 Increased development time up to 3 weeks (for radioactive detection).

9 Carried out development in a phosphoimager that is more sensitive than film (for radioactive detection).

Each time a control band (of the serotonin transporter or FMRFamide) of the expected length was clearly visible, but no HCN bands were detected (Figure 2-10). Possible reasons for this will be discussed in section 2.4.8.

2.4 Discussion

2.4.1 Identity and Molecular Organization of acHCN

acHCN is the first HCN channel cloned from the Lophotrochozoa clade. The presence of HCN channels in all three clades of bilaterian animals: Ecdysozoa, Lophotrochozoa and Deuterostomia, as well as in Cnidaria, suggests that it was present in their common ancestor, and that it was possibly lost in nematodes.

acHCN is very similar to HCN channels from other organisms with the most similarity to the HCN channels cloned from *A. mellifera* (86 % identity), *Heliothis virescens* (tobacco budworm) (84 %), *Aedes aegypti* (mosquito) (84 %) and *P. argus* (83 %). Of human HCN channels, acHCN is most similar to the HCN3 (73 % identity). The similarity of the voltage sensor, pore region and CNBD of acHCN and other HCN channels suggests that acHCN has similar voltage dependence, permeability and regulation by cyclic nucleotides.

The major difference of acHCN is in the length of its 5'-region. In the position where HCN channels of most other animals have their first ATG (start codon) acHCN has an ATT sequence. This difference is most likely due to a single nucleotide mutation (G to T supported by all 12 sequences for this region) resulting in a loss of the first methionine and possible truncation of the channel. However, the region of acHCN between the ATT and the first ATG codon is similar to the corresponding region of other HCN channels. In addition, acHCN has two predicted phosphorylation sites in this region. Both of these facts suggest that this region may be important in the channel's functioning. Conservation of the sequence between the ATT and the first ATG of acHCN suggests that this region is translated because untranslated regions are modified quite rapidly. Translation of acHCN may start from an upstream alternative start codon CTG. In this case all similarity of the 5'-end of acHCN and other HCN channels would be preserved. Otherwise, if translation of the channel starts from the current first ATG codon, the

aforementioned facts can be explained by the recent occurrence of the ATG → ATT mutation. In this case, there was not enough evolutionary time for the untranslated region between the ATT and first ATG sequences to undergo significant changes. To determine the translation start of acHCN, Western blot using antibodies to the acHCN protein has to be performed, but these antibodies currently do not exist.

The similarity of acHCN and HCN channels from other organisms implies that they may have similar biophysical and pharmacological properties, but differences in the 5'-region may result in some unique characteristics of acHCN discussed in more details in Chapter 3.

The similarity of the CNBD of the analyzed HCN channels and other cyclic nucleotide-regulated proteins and the existence of many common structural elements with voltage-gated potassium channels suggests that HCN channels originated from a potassium channel that acquired a CNBD. To test this hypothesis and determine how HCN channels changed in the course of evolution I will examine HCN channels of more basic organisms, starting with invertebrate animals.

2.4.2 HCN Channels in Invertebrates

Of 34 phyla containing invertebrate animals, HCN channels have been cloned and characterized in species belonging to only two phyla: Echinodermata (*Strongylocentrotus purpuratus*) and Arthropoda: *Drosophila melanogaster*, *Apis mellifera*, *Panulirus argus* and *P. interruptus* (see Table 1-1). By analyzing recently emerged genomic and transcriptomic data of different invertebrate animals I found HCN channels in species of four other phyla: Cnidaria (*Nematostella vectensis*), Annelida (*Capitella sp.*), Mollusca (*Lottia gigantea*) and basal Chordata (Urochordata) (*Ciona intenstinalis*). Of the animals with completed genomes only nematodes *Caenorhabditis elegans* and *C. briggsae* lack HCN channels, although they have related cyclic nucleotide-gated (CNG) channels (Figure 2-11).

The presence of two HCN genes in Cnidaria suggests that an ancestral bilaterian organism also had two HCN genes; there was a gene loss before the divergence of Ecdysozoa and Lophotrochozoa, a duplication before the divergence of Urochordata and other Chordata and another one in vertebrates (Figure 2-12B). By a different scenario, an ancestral bilaterian organism had a single HCN gene. Duplications occured independently in Cnidaria, before the divergence of Deuterostomes and Ecdysozoa / Lophotrochozoa, Urochordata and other chordates and another one in vertebrates. This second possibility suggests that a gene loss occurred only once, in nematodes (Figure 2-12A).

Although nothing is known about HCN channels in the other 28 phyla of the animal kingdom, the molecular organization and/or properties of known HCN channels in seven different phyla are very similar. This suggests that HCN channels emerged very early in the evolution of animals or even before the divergence of animals. To determine which scenario is true, I next screened for HCN or related channels in non-animal eukaryotes.

2.4.3 Cyclic Nucleotide-Binding Channels in Non-Animal Eukaryotes

I did not find HCN channels in any non-animal species. However, plants have a similar family of channels, called cyclic nucleotide-gated cation channels (CNGCs, see Figure 2-11). These channels were isolated from thale cress (*Arabidopsis thaliana)* (AtCNGC, Kohler, 1999), barley (*Hordeum vulgare*) (HvCBT1, Schuurink et al., 1998), rice (*Oryza sativa*) and tobacco (*Nicotiana tabacum*) (NtCBP4, Arazi et al., 1999). Like animal CNG channels, plant CNGCs are permeable to Ca^{2+}, blocked by external Ca^{2+}, and have a calmodulin-binding domain. But while animal CNG channels conduct mostly Ca^{2+} and Na^{+} under physiological conditions, plant CNGCs are strongly selective for K^{+} over Na^{+} (Leng et al., 2002). Whereas animal CNG channels are activated by cGMP and only weakly by cAMP, the latter strongly activates plant CNGC channels. In addition, hyperpolarization is required for their activation. These properties

relate plant CNGCs to animal HCN channels. However, plant CNGC channels lack the GYG potassium selectivity filter. Instead, specific amino acids within their pore (Asn416 and Asp417 in AtCNGC2) facilitate K^+ over Na^+ conductance (Hua et al., 2003). This may indicate independent origin of plant CNGC channels and animal CNG / HCN channels or different demands on plant cells.

Plants also have potassium-selective, voltage-gated channels containing a cyclic nucleotide-binding domain, including KAT1 (Anderson et al., 1992), AKT1 (Sentenac et al., 1992) and KST1 (Muller-Rober, 1995) (see Figure 2-11), but contrary to HCN and CNG channels, binding of cyclic nucleotides decreases their current, making them similar to animal ether-a-go-go-related (ERG) channels (Cui et al., 2000) (Table 2-3). Protozoa (*Paramecium aurelia*) have a similar cyclic nucleotide-binding potassium channel, while Fungi lack channels with a CNBD (I checked 15 species with completed genomes, including *Aspergillus fumigatus*, *Candida glabrata*, *Cryptococcus eoformans*, *Debaryomyces hansenii*, *Encephalitozoon cuniculi*, *Eremothecium gossypii*, *Gibberella zeae*, *Kluyveromyces lactis*, *Magnaporthe grisea*, *Neurospora crassa*, *Pichia stipitis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Ustilago maydis*, *Yarrowia lipolytica*).

Absence of HCN channels in Fungi, a sister group of animals, suggests that these channels first evolved in animals. However, to determine what channels gave rise to HCN and animal and plant cyclic nucleotide-gated potassium channels, I screened for potassium channels containing a CNBD in prokaryotes.

2.4.4 Cyclic Nucleotide-Binding Potassium Channels in Prokaryota

I found five putative cyclic nucleotide-binding potassium channels in Prokaryotes (see Figure 2-11). All of these channels have the classic K^+ selectivity motif, which suggests that they conduct predominantly potassium ions.

Archaea do not have cyclic nucleotide-binding channels, but a potassium channel activated by hyperpolarization has been characterized in *Methanococcus jannaschii* (Sesti et al., 2003).

The putative cyclic nucleotide-binding potassium channel of *Rhodopseudomonas palustris* has five regularly interspaced positively-charged residues in its TM4 domain, *Trichodesmium erythraeum* has four, *Magnetospirillum magnetotacticum* has three and *Bradyrhizobium japonicum* and *Mesorhizobium loti* have two. This suggests that these channels are also regulated by voltage, though to a lesser degree than HCN channels. Also, the CNBD of these channels is very similar to the CNBD of bacterial catabolite activator protein (CAP). Thus, prokaryotic cyclic nucleotide-binding potassium channels might have appeared when a potassium channel acquired a CNBD of another protein.

Analysis of HCN and related channels allows making a hypothesis about the origin and potential evolution of these channels.

2.4.5 Origin and Potential Evolution of HCN and Related Channels

Comparative analysis of transcripts containing a cyclic nucleotide-binding domain shows that the CNBD of HCN and related channels from plants, Protozoa and Prokaryota are very similar to the CNBD of bacterial CAP and PKA (Finn et al., 1996; Zagotta et al., 2003). This suggests that these channels evolved by a potassium channel acquiring a CNBD of another protein.

The similarity in the molecular organization of HCN channels and CNBD-containing potassium channels of Prokaryota, including the presence of six TM, the GYG sequence, and a conserved CNBD suggests that HCN channels evolved from cyclic nucleotide-binding potassium channels of ancestral prokaryotic organisms by preserving mutations in the potassium selectivity filter, specifically threonines preceding GYG and aspartate following the GYG sequence. These modifications allow Na^+ to pass through the channel pore along with K^+, setting the reversal

potential of the channels at more depolarized values and thus determining pacemaker properties of spontaneously active cells. Pacemaker cells play a crucial role in coordination of neuronal activity in central pattern generators, which are responsible for rhythmic behaviors such as heartbeat in leech (Cymbalyuk et al., 2002), or feeding in *Lymnaea* (Straub and Benjamin, 2001), as well as in intercellular signaling and synchronization in the brain (Maccaferri and McBain, 1996; Bal and McCormick, 1997; Luthi et al., 1998). Thus, HCN channels might have evolved to control integrative functions of animals.

However, poor bootstrap support of branching of different classes of cation and potassium cyclic nucleotide-binding channels and lack of electrophysiological data from prokaryotes prevents making definite conclusions about the HCN channel origin and evolution. For example, it is not known whether prokaryotic CNBD-containing potassium channels are activated by depolarization or hyperpolarization and whether they are activated or inhibited by cyclic nucleotides like HCN channels or plant voltage-gated potassium channels, respectively. Expression and functional characterization of prokaryotic cyclic nucleotide-binding potassium channels as well as HCN channels from basic eukaryotic organisms will provide a more complete picture of these issues.

2.4.6 Duplication of a Coding Region at the 3'-UTR of acHCN and Genomic Organization of the Channel

The similarity of the 3'-UTR and the coding region of acHCN suggests that the *A. californica* HCN gene has undergone duplication (or multiple duplications). This duplication must have been recent because of the near identity of the coding region and duplicate sequence and the fact that the latter did not acquire any independent regulatory elements and is transcribed together with the coding region. Multiple duplication events might have also resulted in the presence of different variants of exon 9 of acHCN.

2.4.7 Possible Splice Variants of acHCN

The existence of seven variants of exon 9 suggests that up to seven splice forms of acHCN channel are synthesized that may differ in their function. The variant with a frame shift and a putative stop codon may produce a shortened form of the acHCN channel without a CNBD (Figure 2-13). It is recognized that the CNBD has an inhibitory effect on the channel activity and binding of cyclic nucleotides relieves this inhibition. Deletion of the CNBD makes the channel constitutively-active (Wainger et al., 2001), i.e., voltage dependence of I_h activation strongly shifts to a depolarizing direction as in the presence of saturating concentration of cyclic nucleotides. The putative shortened form of the acHCN may be a natural analog of the CNBD deletion. This form is expected to be constantly activated and thus may participate in the development of LTP and LTH. Determining the conditions at which this form is synthesized is part of the future work.

2.4.8 Verification of Channel Length

To determine the exact length of acHCN and how many copies of the coding region sequence are present in the 3'-UTR I performed Northern blot. This method did not detect any HCN bands. The presence of the control bands (serotonin transporter or FMRFamide) eliminates the possibility of a mistake in the design and execution of the experiments since both control and acHCN experiments were performed simultaneously using the same protocol and reagents. Also, I verified that the acHCN probe was functional by its successful use in *in situ* hybridization experiments. Thus, the reason that I did not detect any HCN bands is almost certainly due to the low abundance of the acHCN transcripts that is below the sensitivity limit for Northern blot. *In situ* hybridization provides independent evidence supporting this conclusion. Abundant transcripts (e.g., FMRFamide, serotonin transporter) develop in 5-10 minutes while HCN channel transcripts take approximately 2 hrs. Because development time is considered to be

proportional to the abundance of a transcript, the acHCN RNA is indeed rare in *A. californica* neurons.

In conclusion, verification of the length of acHCN is difficult because of a low abundance of the transcript. Completion of the *A. californica* genome sequencing will help to resolve this issue.

2.4.5 Summary

The evidence presented in section 2.3.1 implies that the whole coding region of acHCN was cloned. This channel is very similar to HCN channels from other organisms. However, possible truncation of the 5'-end of acHCN still leaves a question of whether the channel is functional. This question is addressed in Chapter 3 by expressing the channel in a heterologous system and studying its biophysical and pharmacological properties. Obtaining a sequence of acHCN also allowed localization of the channel transcript that is described in Chapter 4.

Because specific antibodies to acHCN have not been generated, this chapter does not deal with the acHCN protein. In the future, we plan to obtain these antibodies and localize the acHCN protein in the CNS of *A. californica* using immunocytochemistry. We also plan to investigate whether translation of acHCN starts from the first ATG or from the alternative start codon CTG and whether a shortened form of acHCN without CNBD is synthesized in the *A. californica* CNS (and if so, under what conditions). The latter issues will be answered by performing Western blots with antibodies to acHCN.

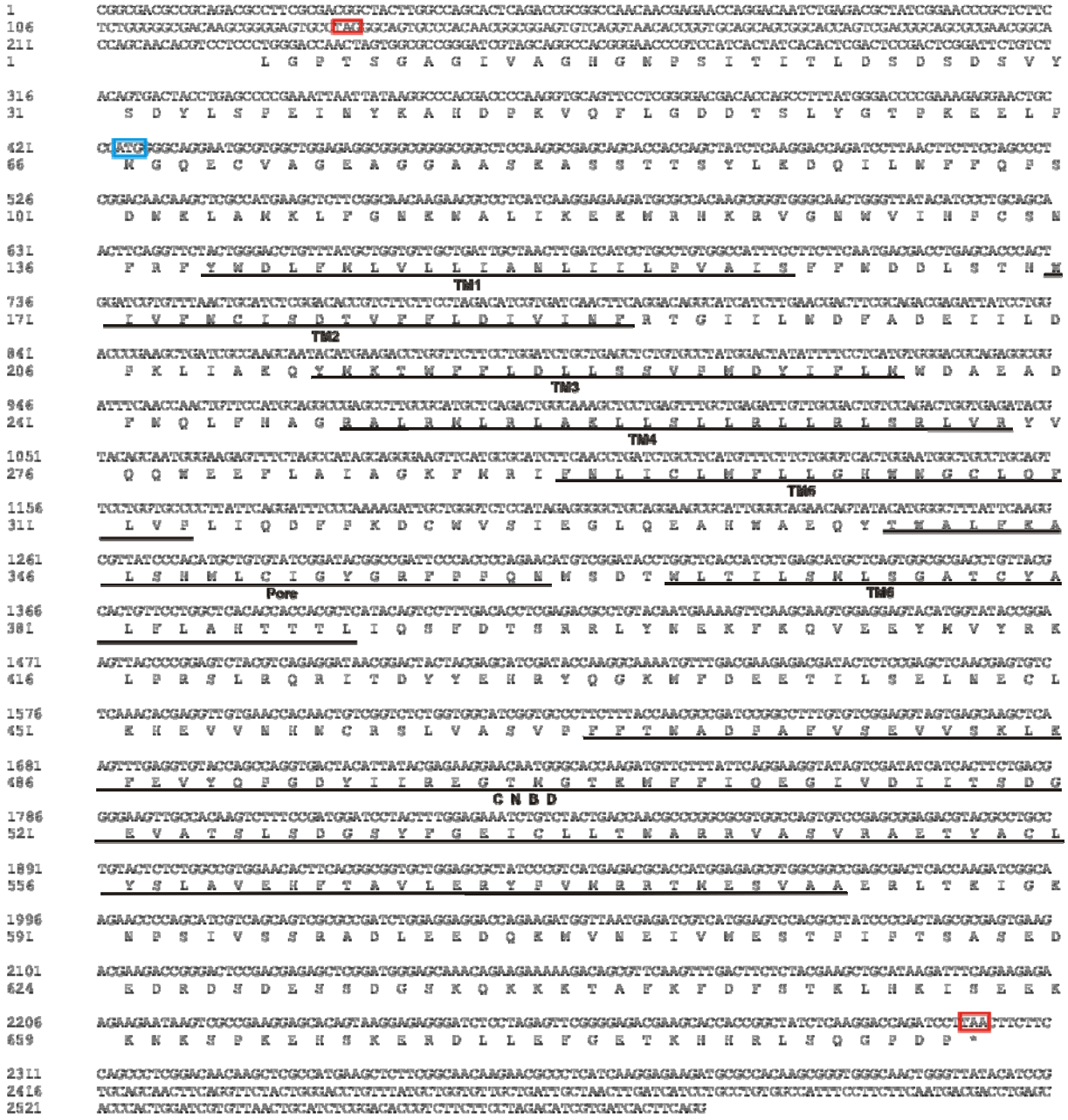

Figure 2-1. The longest cDNA sequence and predicted amino acid sequence of acHCN. The cDNA sequence is a consensus of a 168 clone assembly. The ATG sequence coding for the first methionine (shown in blue box) is supported by 29 chromatograms, the stop codon upstream of the first methionine is supported by nine chromatograms, and the stop codon at the 3'-end of the transcript is supported by16 chromatograms (stop codons are shown in red boxes). The amino acid sequence is shown starting from the alternative start codon CTG and ending at the stop codon. Predicted transmembrane domains (TM1-TM6), a pore and a cyclic nucleotide-binding domain (CNBD) are underlined.

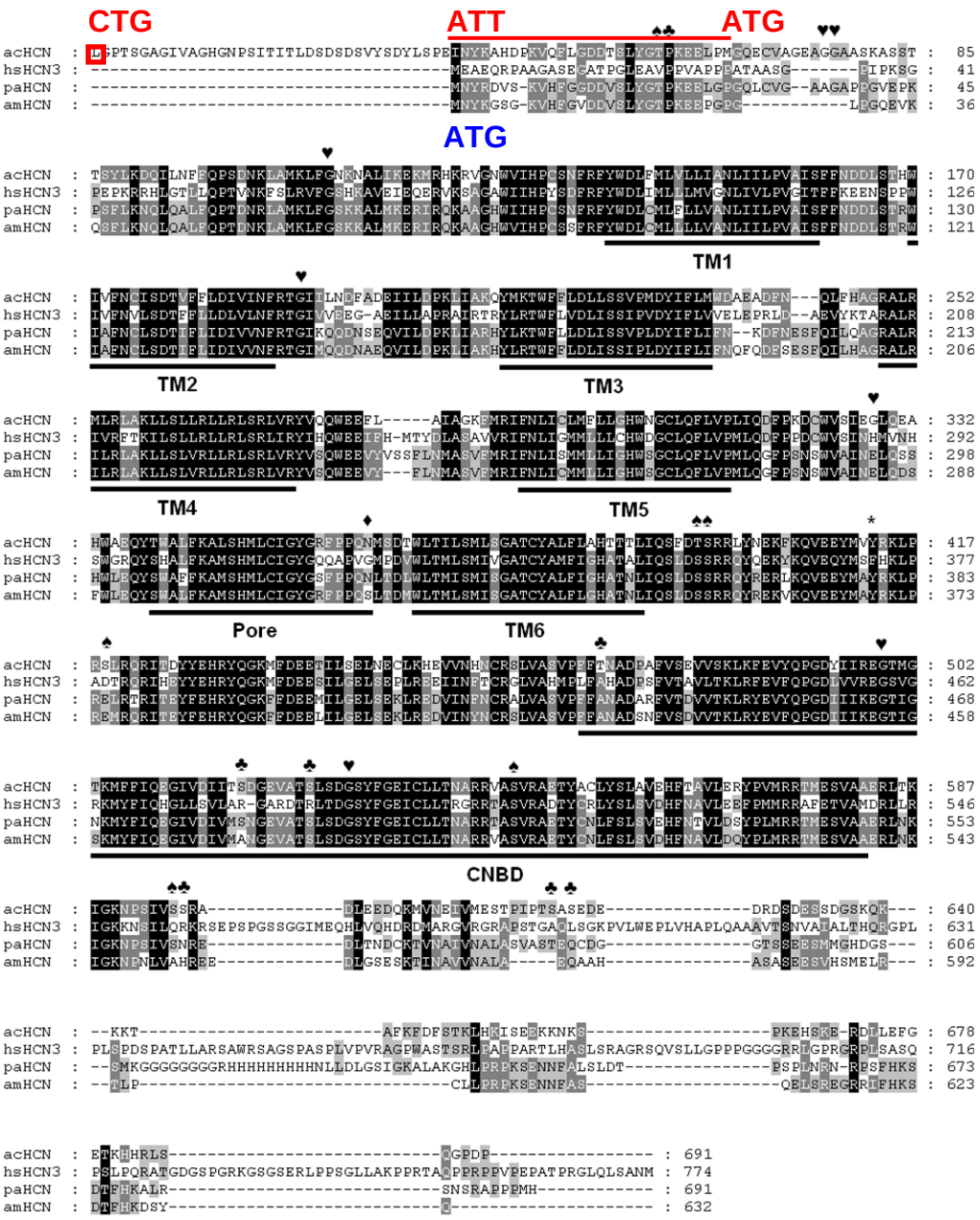

Figure 2-2. Alignment of HCN proteins from different organisms. The alignment shows that HCN channels are highly conserved, especially in their TMs, pore and CNBD. The presence of 14 predicted phosphorylation sites suggests potential regulation of the channel by phosphorylation by protein kinases. The first conventional (ATG) and the alternative (CTG) start codons of acHCN are shown in red and the first ATG codon of other HCN channels is shown in blue. The red bar shows a region of a weak alignment of acHCN and other HCN channels upstream from acHCN's first methionine. Abbreviations: ac –*A. californica*, hs – *Homo sapiens* (human), pa – *Panulirus argus* (lobster), am – *Apis mellifera* (honey bee). Predicted sites of phosphorylation by: ♠ - Protein kinase C; ♣ - Casein kinase II; * - Tyrosine Kinase; ♥ - N-myristoylation site; ♦ - N-glycosylation site

Figure 2-3. Alignment of the voltage sensors from the HCN channels of different organisms. The alignment shows that acHCN, as well as other HCN channels, has eight positively-charged residues (red boxes) which are regularly interspaced. Conserved residues are shown in black boxes. Abbreviations: ac –*A. californica*, hs – *Homo sapiens* (human), pa – *Panulirus argus* (lobster), am – *Apis mellifera* (honey bee).

Figure 2-4. Alignment of the pore regions from the HCN channels of different organisms. The alignment shows that the core region (red box) of the potassium selectivity motif (shown above the alignment) is preserved in acHCN, as well as other HCN channels, but the following residue (shown in blue, green or orange) and the threonines preceding the GYG sequence are changed. Conserved residues are shown in black boxes. Abbreviations: ac –*A. californica*, hs – *Homo sapiens* (human), pa – *Panulirus argus* (lobster), am – *Apis mellifera* (honey bee).

Figure 2-5. Phylogenetic tree of HCN channels. The phylogenetic tree was created in ClustalX after removing all gaps in alignment of HCN proteins with GeneDoc. The graphical output was generated using TreeView. Numbers at the nodes represent bootstrap values. Accession numbers: human HCN1 gi:32698746, human HCN2 gi:30580783, human HCN3 gi:38327037, human HCN4 gi:4885407, zebrafish (*Danio rerio*) HCN1 gi:94732421, HCN4 gi:125842901, sea urchin (*Strongylocentrotus purpuratus*) HCN1 gi:3242324, sea urchin HCN2 gi:69146514, *Drosophila melanogaster* HCN (splice form A1B1C1) gi:24653608, lobster (*Panulirus argus*) HCN gi:33355925, honey bee (*Apis mellifera*) HCN gi:52631748. Sequences of HCN channels of the limpet (*Lottia gigantea*), polychaete worm *Capitella sp*., and the sea squirt (*Ciona intestinalis*) were downloaded from the Joint Genome Institute website.

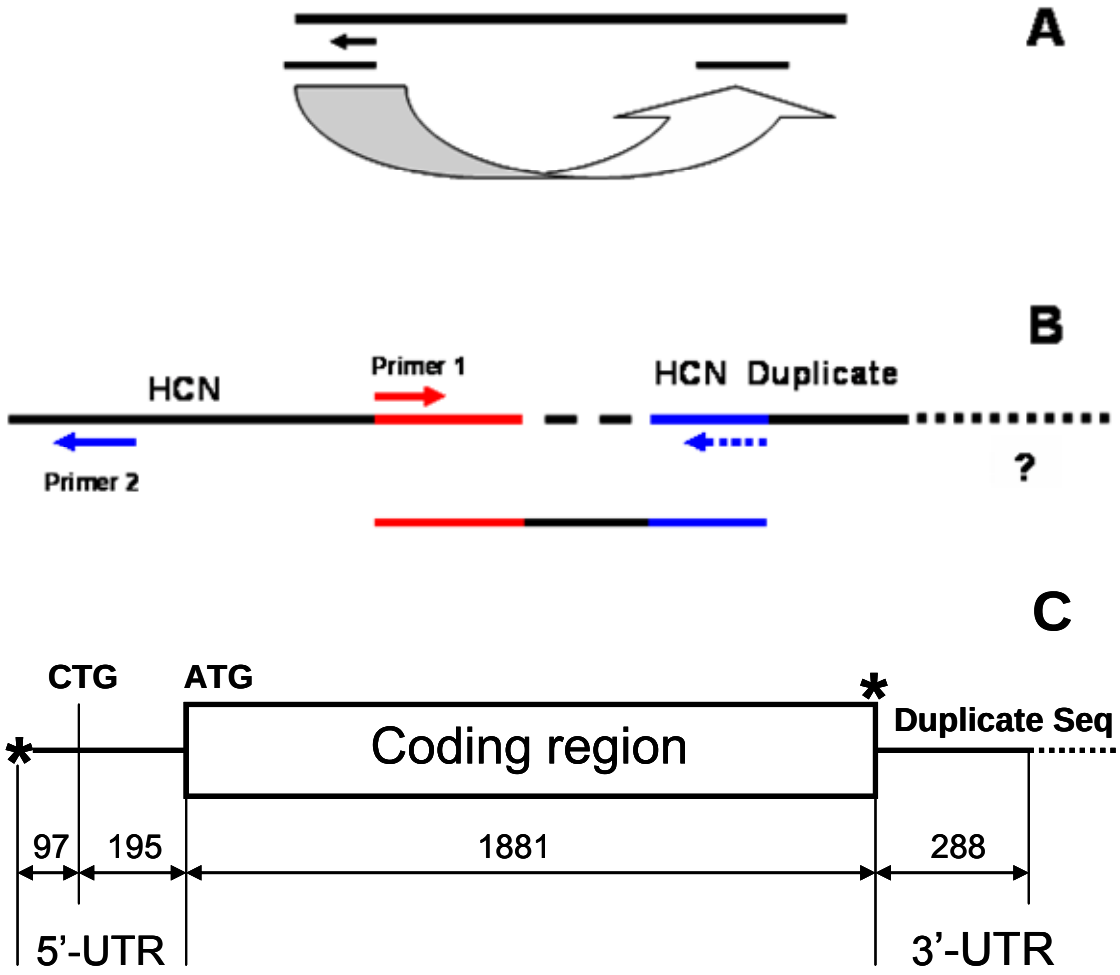

Figure 2-6. Experiments showing the existence of a duplicate sequence at the 3'-end of acHCN. A) 5'-RACE produced a fragment that aligned to the 3'-end of the consensus acHCN sequence. B) A PCR with a direct primer at the 3'-end of the transcript (primer 1) and a reverse primer at the 5'-end of the transcript (primer 2, corresponding to the 5'-end of the duplicate sequence) produced a fragment consisting of the 3'-end of acHCN and the 5'-end of the duplicate sequence. C) Schematic diagram of the chimeric organization of acHCN. * - stop codons, ATG – start codon, CTG – alternative start codon. Numbers indicate numbers of nucleotides.

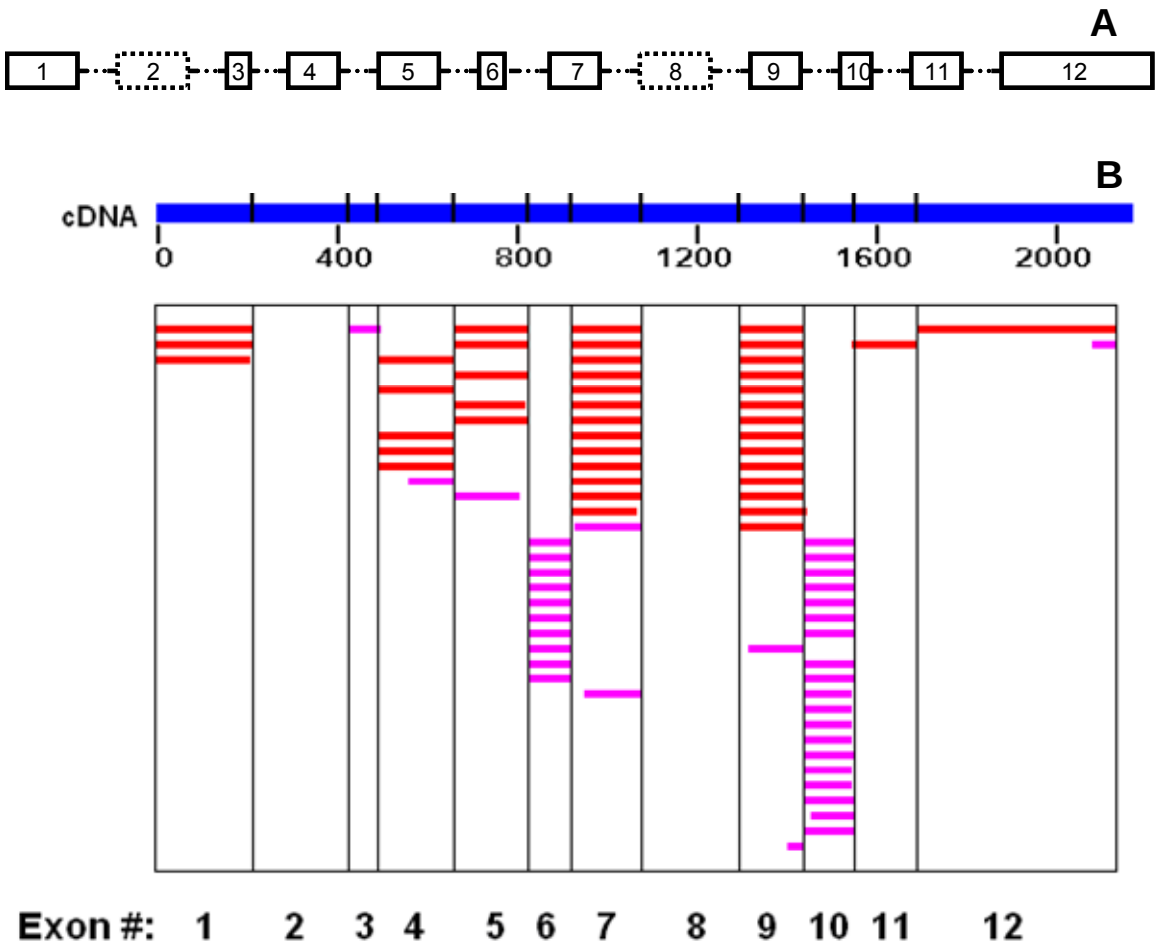

Figure 2-7. Genomic organization of acHCN. A) Schematic diagram of the exon-intron organization of the acHCN gene. B) Alignment of the acHCN cDNA with genomic sequences from the "Trace Archive" database in NCBI BLAST.The acHCN gene consists of at least 12 exons shown in red (alignment score => 200) and pink (alignment score 80-200) bars in the positions corresponding to the consensus cDNA sequence (shown by the blue bar).

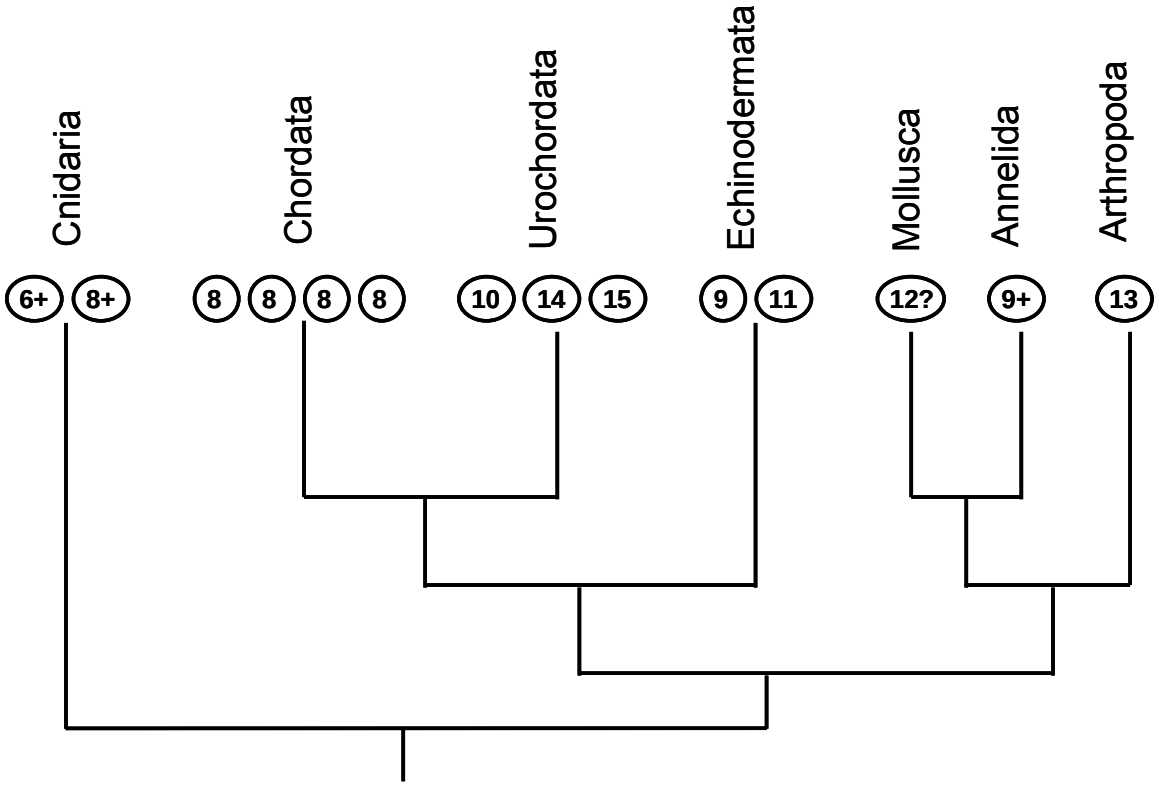

Figure 2-8. Number of exons in HCN genes in different animal phyla. + sign following a number indicates that a gene consists of more than the specified number of exons, but an exact number cannot be determined due to the lack of genomic data. Molluskan HCN genes most probably have 12 exons. This prediction is based on the assumption that the remaining gaps in the genomic representation of the channel transcript contain only one exon because these gaps have approximately the same size as known exons. However, these gaps may contain more than one exon. HCN genes of chordates have fewer exons than invertebrate ones. This suggests fusion of exons in Chordata. Available genomic data does not allow predicting the number of exons in Cephalochordata (*Amphioxus*).

ACACGAGGTTGTGAACCA**1**AACTGTCG**2**TCTCTGGTG
GCATCGGT**3**CCCTTCTTTACCAACGCCGATCC**4**GCCT
TTGTGTCGGAGGTAGTGAGCAAGCTCAAGTTTGAGGT

Figure 2-9. Sequence of exon 9. Nucleotides, which differ between splice variants of acHCN are shown by numbers 1-4.

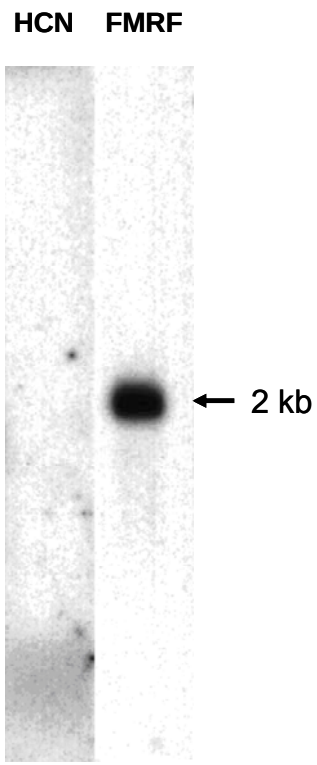

Figure 2-10. Comparison of Northern blots using probes to acHCN and FMRFamide (control). Total RNA isolated from the whole *A. californica* CNS was transfered to a membrane. Probes were labeled by α-P*-UTP. No bands were detected for the HCN channel whereas a single band with the expected length of approximately 2 kb was visible for FMRFamide. The acHCN band was expected to be of approximately the same length.

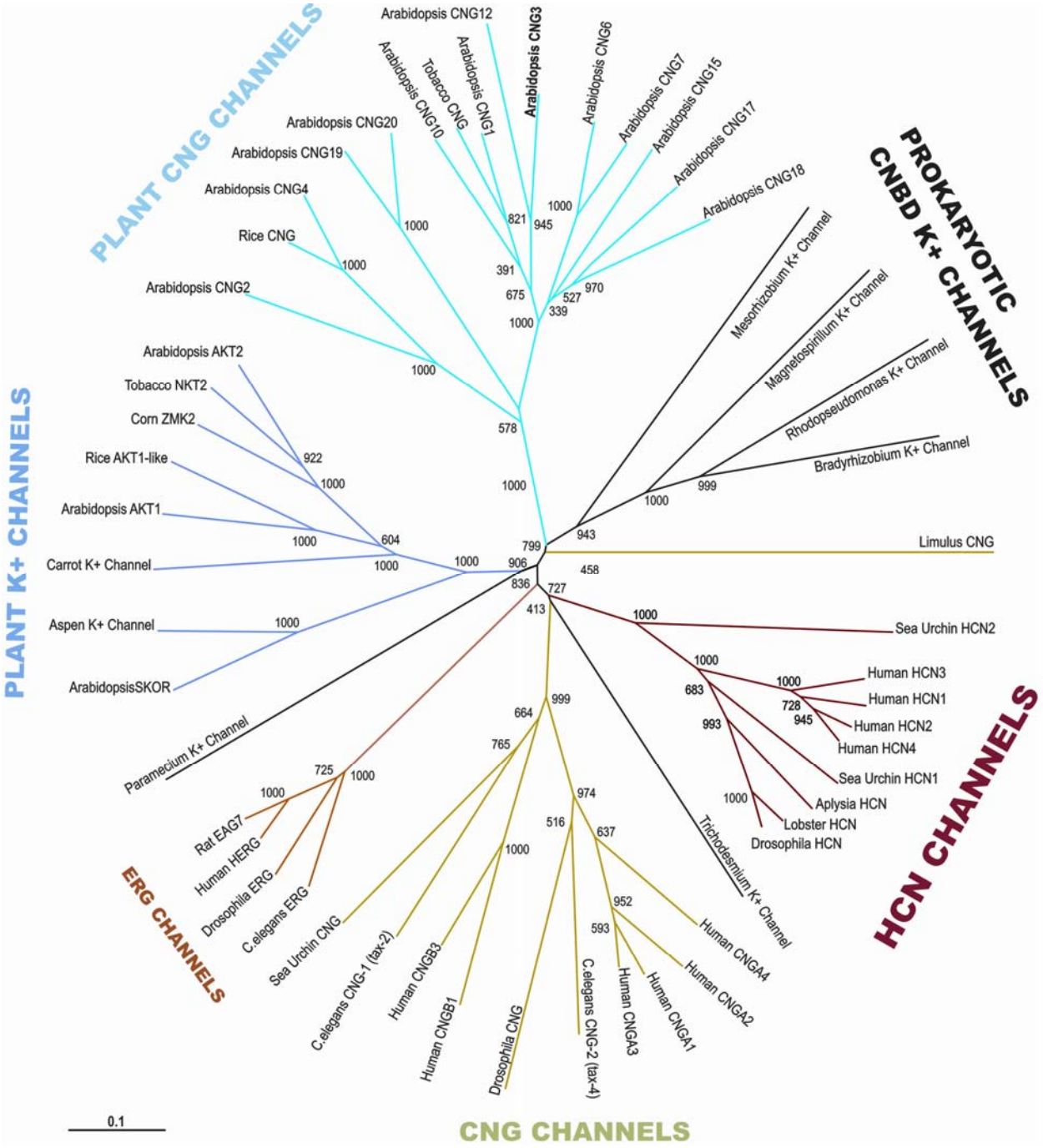

Figure 2-11. Phylogenetic tree of potassium and cation channels containing a cyclic nucleotide-binding domain. The phylogenetic tree was created in ClustalX after removing all gaps in alignment of HCN proteins in GeneDoc. The graphical output was generated using TreeView. Accession numbers: human HCN1 gi:32698746, human HCN2 gi:30580783, human HCN3 gi:38327037, human HCN4 gi:4885407, sea urchin (*Strongylocentrotus purpuratus*) HCN1 gi:3242324, sea urchin HCN2 gi:69146514, *Drosophila melanogaster* HCN (splice form A1B1C1) gi:24653608, lobster (*Panulirus argus*) HCN gi:33355925, human CNGA1 gi:2506302, human CNGA2 gi:2493743, human CNGA3 gi:13959682, human CNGB1 gi:2493750, human CNGB3 gi:48474982, *D. melanogaster* CNG gi:908846, *Caenorhabditis elegans*

Figure 2-11. Continued CNG-1 (tax-2) gi:1805259, *C. elegans* CNG-2 (tax-4) gi:22096336, sea urchin CNG gi:72005966, *Limulus polyphemus* CNG gi:18657056, *Arabidopsis thaliana* CNG1 gi:15238657, *A. thaliana* CNG2 gi:38502856, *A. thaliana* CNG3 gi:18407073, *A. thaliana* CNG4 gi:38503128, *A. thaliana* CNG6 gi:38502863, *A. thaliana* CNG7 gi:15219100, *A. thaliana* CNG10 gi:38503202, *A. thaliana* CNG12 gi:38503031, *A. thaliana* CNG15 gi:38503241, *A. thaliana* CNG17 gi:38503044, *A. thaliana* CNG18 gi:38503201, *A. thaliana* CNG19 gi:38503198, *A. thaliana* CNG20 gi:38503198, tobacco CNG gi:6969231, rice CNG gi:50943023, *A. thaliana* AKT1 (cyclic nucleotide binding / inward rectifier potassium channel) gi:15225768, *A. thaliana* AKT2 gi:18415864, rice AKT1-like gi:17887457, tobacco NKT2 gi:56744187, corn ZMK2 gi:5830781, carrot K^+ channel gi:6562375, aspen (*Populus trichocarpa*) K^+ channel gi:9955728, *A. thaliana* SKOR (cyclic nucleotide binding / outward rectifier potassium channel) gi:15232991, human HERG gi:4156239, rat EAG7 gi:18777774, *D. melanogaster* ERG gi:16197863, *C. elegans* ERG gi:7739757, *Paramecium aurelia* K^+ channel (PAK11) gi:17030694, *Rhodopseudomonas palustris* K^+ channel gi:39651151, *Bradyrhizobium japonicum* K^+ channel gi:27354026, *Magnetospirillum magnetotacticum* K^+ channel gi:46202428, *Mesorhizobium loti* K^+ channel gi:14023572, *Trichodesmium erythraeum* K^+ channel gi:71674753

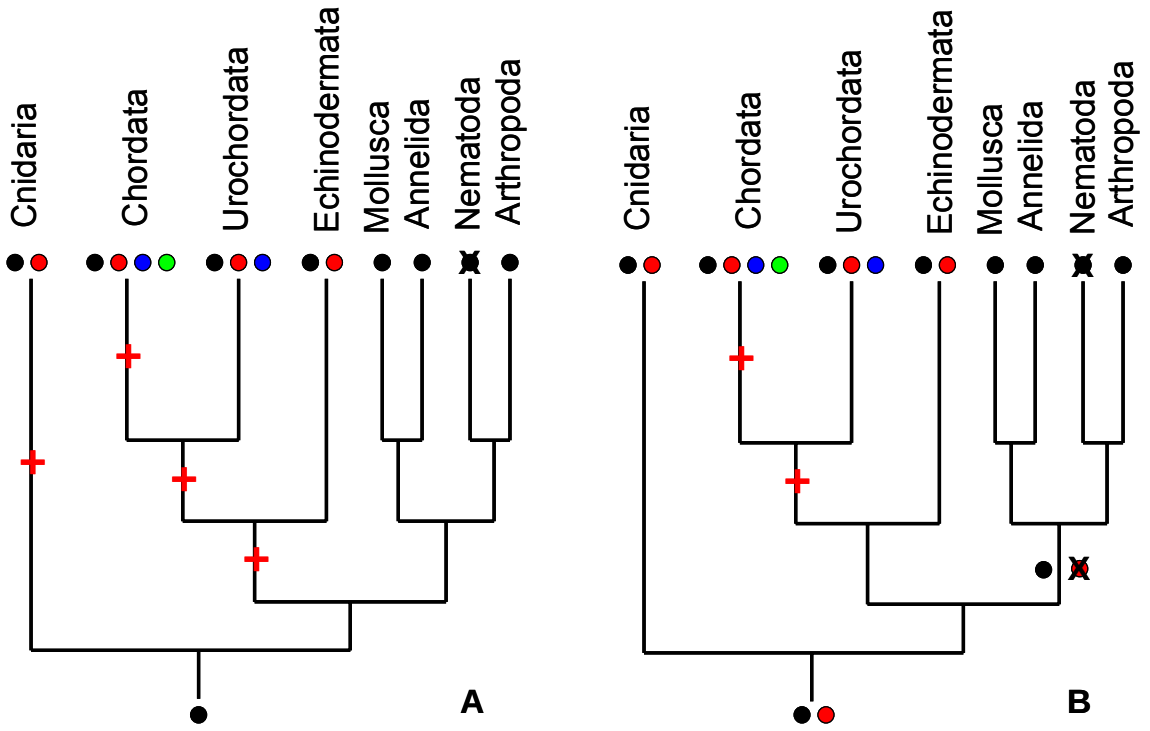

Figure 2-12. Phylogenetic trees showing the number of HCN genes in different phyla of the animal kingdom. These trees are based on the assumption that an ancestral organism had either one (A) or two (B) HCN genes. A) Duplications (shown by +) occured in Cnidaria, before the divergence of Deuterostomes and Ecdysozoa / Lophotrochozoa, Urochordata and other Chordata and in vertebrates. Gene loss (shown by x) occurred in nematodes. B) Duplications occurred before the divergence of Urochordata and other Chordata and in vertebrates. Gene loss occurred before the divergence of Ecdysozoa and Lophotrochozoa and in Nematoda.

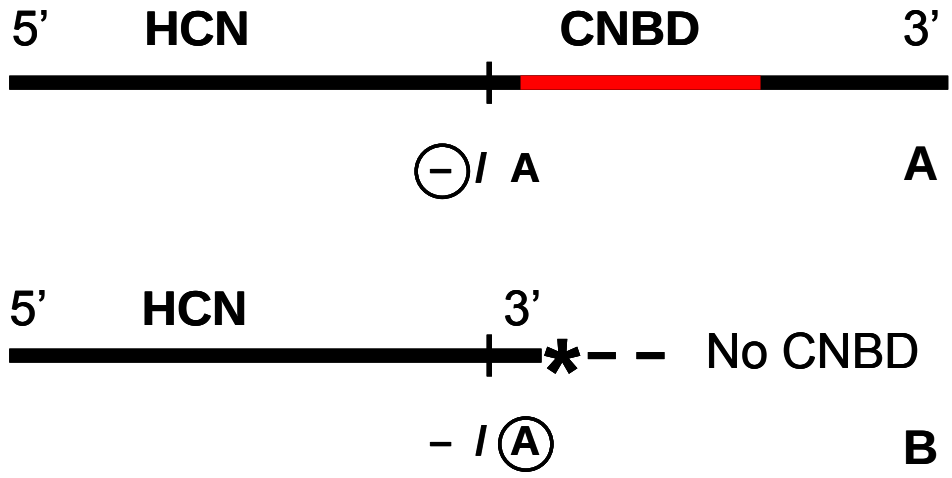

Figure 2-13. Two forms of the acHCN channel that may be synthesized. If there is no nucleotide in position four of exon 9 (see Figure 2-9), then the whole channel, including the CNBD, will be translated (A). The presence of adenine in position four of exon 9 produces a frame shift followed by a stop codon 36 nucleotides downstream. In this case a shortened form of the acHCN channel without the CNBD may be synthesized (B).

Table 2-1. Sequence and position of exon-intron junctions of acHCN gene

Exon #	Exon size, bp	3' intron junction	Exon 5' sequence	Exon 3' sequence	Intron #	5'-intron junction
1	211	-	GTG CCC CAC	AAT TAT AAG	1	**gt** aata
2	228	?	GCC CAC GAC	CTC ATC AAG	2	?
3	61 ?	?	?	CAA CTT CAG	3	?
4	167	tatgcctcattttcttc **ag**	GTT CTA CTG	GGA CAG GCA	4	**gt** gcaa
5	165	atttttttaaatctatgc **ag**	TCA TCT TGA	TCC ATG CAG	5	**gt** acgc
6	98	ttccttttttcggttgac **ag**	GCC GAG CCT	TGG GAA GAG	6	**gt** atgt
7	87	cttgcttgtactctcgc **ag**	TTT CTA GCC	GGG CTG CAG	7	**gt** acgt
8	282	?	TTC CTG GTG	AAT GAA AAG	8	?
9	147	ctctttcttctattttt ag	TTC AAG CAA	CTC AAA CAC	9	**gt** atgc
10	105	tgctgtattgactctac **ag**	GAG GTT GTG	AAG TTT GAG	10	**gt** aatt
11	140	caatgtacatctctctc **ag**	GTG TAC CAG	CTT TGG AGA	11	?
12	441	cactaaccactcgac **ag**	AAT CTG TCT	GGA GAC GAA	12	?

*All shown intron sequences start with "gt" and end with "ag". Sequences that can not be reliably discerned due to the lack of genomic data are substituted by question marks.

Table 2-2. Identities of nucleotides in the positions 1-4 shown in Figure 2-9

Variant #	Nucleotide 1	Nucleotide 2	Nucleotide 3	Nucleotide 4	Presence
1	C	G	A	A	cDNA, genome
2	C	G	A	-	cDNA, genome
3	C	G	G	-	cDNA
4	C	C	G	-	cDNA
5	C	C	G	G	Genome
6	A	G	G	-	Genome
7	A	G	A	G	Genome

Table 2-3. Comparison of properties of HCN channels and other channels containing a CNBD

Channel Class	Voltage Activation	cNMP Regulation	Permeability
HCN	Hyperpolarization	cAMP, cGMP Positive modulation	K^+, Na^+
Animal CNG	-	cGMP, cAMP Activation	Na^+, Ca^{2+}
Plant CNG	Hyperpolarization	cAMP Activation	K^+, Na^+, Ca^{2+}
Plant K^+ channels	Depolarization	cAMP Negative modulation	K^+
Either-a-go-go related	Depolarization	cAMP Negative modulation	K^+
Prokaryotic K^+ channels	?	cNMP ?	K^+ ?

CHAPTER 3
BIOPHYSICAL AND PHARMACOLOGICAL CHARACTERIZATION OF acHCN

3.1 Introduction

The molecular organization of acHCN described in Chapter 2 left a question about whether the cloned channel is functional. Thus, two major objectives of the work presented in this chapter were to answer this question by determining the properties of acHCN in an environment where other channels do not interfere, and to find an inhibitor of the channel for functional studies.

Most characterized HCN channels have similar biophysical and pharmacological properties which reflect the similarity of their molecular organization (see Chapter 2). In response to hyperpolarization these channels generate slow inward currents that do not inactivate. The only exceptions are HCN channels in frog melanotrophs (Mei et al., 1998), and SpHCN1, one of the two HCN channels cloned from sperm flagella of the sea urchin *S. purpuratus* (Gauss et al., 1998). In addition to a slow steady-state current, SpHCN1 and the frog HCN channels have an instantaneous transient component.

Because of modifications in their potassium selectivity motif, HCN channels also conduct Na^+ (Heginbotham et al., 1994). Due to permeability to sodium ions the reversal potentials of HCN channels is more depolarized compared to classic K^+ channels (Table 3-1). Alterations of the potassium selectivity motif may also account for the slow kinetics of HCN channels compared to classic K^+ channels. For example, in humans, the values of time constants of activation (τ) range from 196 ms to 23 s in HCN2 and HCN4 channels respectively (Table 3-1).

Another common feature of all HCN channels is their activation by cyclic nucleotides, specifically cAMP and cGMP (Pape and McCormick, 1989; Pedarzani and Storm, 1995; DiFrancesco and Tortora, 1991; Ludwig et al., 1998). They cause a depolarizing shift in the voltage activation of the channels, which may be as large as +41 mV (produced by 1 mM cAMP in *P. argus*) (Gisselmann et al., 2005b), and, sometimes, increase the current amplitude as in the

mouse HCN1 channel (Santoro et al., 1998; Table 3-1). The only known exception is the human HCN3 channel, which is not activated by cyclic nucleotides (Stieber et al., 2005).

HCN channels are inhibited by low millimolar concentrations of Cs^+ (Glitsch *et al*., 1986) and a specific blocker ZD7288 (4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyridinium chloride) (BoSmith et al., 1993) although in invertebrates the latter was tested only in *P. argus* (Gisselmann et al., 2005b).

Because acHCN has a predicted molecular structure very similar to the HCN channels cloned from other organisms, my hypothesis is that its major biophysical and pharmacological properties, specifically regulation by hyperpolarization and cyclic nucleotides, slow activation and inhibition by Cs^+ and ZD7288, will be similar. However, differences in the length of the N-terminal region of acHCN may confer some unique properties to the *A. californica* channel.

Characterizing the cloned acHCN in a heterologous system will confirm that it is a functional form of the channel and be a basis for identifying and characterizing the channel in *A. californica* neurons.

3.2 Methods

3.2.1 acHCN RNA Synthesis

Two constructs were made from the coding region of a single acHCN transcript (Figure 3-1). Construct 1 started at the 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 (Jespersen et al., 2002) (Figure 3-1). The constructs were transformed into One-Shot competent *E. coli* cells (Invitrogen). The clones were isolated, purified using a MiniPrep kit (Qiagen) and sequenced by the Whitney Laboratory molecular core facility.

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).

3.2.2 Expression in the *Xenopus* Oocytes

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 $MgCl_2$, 1.8 mM $CaCl_2$, 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 below were performed on the oocytes injected with Construct 2 RNA.

3.2.3 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 (Figure 3-2).

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 (Santoro et al., 1998): 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 (Weber, 1999).

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. I did not hyperpolarize oocytes further because at potentials negative to

-110 mV induced currents became too large and damaged the cells. 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. Percent activation by cyclic nucleotides was calculated using the following formula: % activation = $\frac{I_{h\ -\ 110\,mV,\,cNMP}}{I_{h\ -\ 110\,mV} * 100 - 100}$.

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, using the following formulas:

$$\%\ \text{inhibition by } Cs^+ = \frac{100 - I_{h\ -\ 110\,mV}}{I_{h\ -\ 110\,mV,\,Ctrl} * 100} \text{ and } \%\ \text{inhibition by ZD7288} = \frac{100 - I_{h\ -\ 90\,mV}}{I_{h\ -\ 90\,mV,\,Ctrl} * 100}.$$

Experiments were performed on several oocytes from at least two different frogs under each condition.

Initial training for recording channel activity from oocytes was done on the cnidarian (*Physalia physalis*) voltage-gated potassium channel cloned in Dr. Peter Anderson's laboratory. This channel was chosen because of its high rate of successful expression. The oocytes were injected with the channel's RNA, incubated for 2 days in ND96 media and the currents generated in response to depolarizing voltage steps in voltage-clamp configuration were recorded. This work was done in Dr. Anderson's laboratory under the supervision of Rebecca Price, a technician in the laboratory who had extensive experience in recording from oocytes.

A typical result of the experiments (n = 6) is shown in Figure C-1 of Appendix C.

3.2.4 Data Analysis

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.

3.3 Results

3.3.1 Voltage Dependence and Kinetics

The first and easiest step in determining whether the cloned acHCN channel is functional is to test its response to hyperpolarization. Stepping the membrane potential from -30 mV to

potentials negative to -70 mV in the acHCN RNA-injected oocytes produced slow inward currents characteristic of I_h (Figure 3-3A). These currents were not present in water-injected oocytes, n = 10 (Figure 3-3B).

The amplitudes of the currents increased with the increase in activating voltage steps. The membrane potential of half-maximal activation ($V_{1/2}$), determined by fitting the I/V curve with the Boltzmann equation, was -83.77 ± 0.79 mV with a slope factor 7.23 ± 0.70 mV, n = 4 (Figure 3-4). Upon return to the holding potential (-30 mV) the currents deactivated, generating decaying, inward tail currents. The time course of activation was strongly dependent on the hyperpolarizing voltage steps with τ values ranging from 6229.76 ± 584.39 ms at -80 mV to 2330.06 ± 156.62 ms at -110 mV, n = 18.

Activation by hyperpolarization and slow kinetics of the recorded current support the prediction that the functional form of the HCN channel was cloned.

3.3.2 Ion Permeability of acHCN

A second major characteristic of a channel, in addition to the voltage dependence, is its permeability to different ions, reflected in the reversal potential (E_r) of the channel.

The reversal potential of acHCN was determined from the I/V curve for tail currents to be -25.84 ± 1.19 mV, n = 17 (Figure 3-5). It was shown in other organisms that HCN channels are permeable only to potassium and sodium ions (Robinson and Siegelbaum, 2003). Because the reversal potential of acHCN is similar to those of other HCN channels, my hypothesis is that acHCN also is permeable only to K^+ and Na^+ with approximately the same ratio of permeabilities for these two ions.

To test this hypothesis I changed the extracellular concentrations of Na^+ and K^+ from the control values (2 mM K^+, 96 mM Na^+) to 16 mM K^+, 82 mM Na^+ and 36 mM K^+, 62 mM Na^+. If the acHCN channel is permeable only to potassium and sodium ions then it is possible to predict

how the reversal potential will change when I change extracellular concentrations of these ions. Otherwise, if the predicted value of the reversal potential does not correspond to the experimentally determined one, it can be inferred that other ion(s) permeate the channel and participate in determining its reversal potential.

In both cases, experimentally determined reversal potentials (-21.11 ± 0.80 mV and -13.04 ± 1.22 mV respectively) closely matched the expected reversal potential calculated for each oocyte (-20.84 ± 1.41 mV and -14.47 ± 0.97 mV respectively, n = 4) determined using the Goldman equation ($V_m = \frac{RT}{F} * \ln(\frac{P_K[K+]_{out} + P_{Na}[Na+]_{out}}{P_K[K+]_{in} + P_{Na}[Na+]i_n})$) based on the assumption that only these two ions contribute to HCN-mediated current (Figure 3-6). This means that the acHCN channel conducts almost exclusively potassium and sodium ions.

Using the reversal potential and concentrations of sodium and potassium ions in extracellular and intracellular solutions, the relative permeability of acHCN to Na^+ and K^+ was determined by the Goldman equation to be 0.40.

As with other HCN channels, elevating the extracellular potassium concentration drastically increased amplitudes of I_h: 4.64 ± 0.62 fold for $[K^+]_{ext}$ = 16 mM and 7.82 ± 0.30 fold for $[K^+]_{ext}$ = 36 mM, n = 4 (Figure 3-7).

The permeability of the cloned channel to both potassium and sodium ions and the dependence of I_h amplitude on extracellular potassium concentration confirm the identity of acHCN.

3.3.3 Activation of acHCN by Cyclic Nucleotides

The third major characteristic of HCN channels is their activation by cyclic nucleotides. cAMP and cGMP activate the acHCN channel by increasing the amplitude of I_h (Figures 3-8 and 3-9). 1 mM 8-Br-cAMP increased current amplitude measured after stepping from -30 to

-110 mV by 18.01 ± 0.71 %, n = 4, and 1 mM 8-Br-cGMP increased current amplitude measured under the same conditions by 35.75 ± 20.31 %, n = 4.

The increase in the current amplitude by cyclic nucleotides was concentration-dependent. The concentration of 8-Br-cGMP required for a half-maximal increase (K_a) was 2.16 ± 0.68 μM, n = 4 (Figure 3-10). Based on the obtained dose-response relationship, the K_a value for 8-Br-cAMP is expected to be much larger. The exact concentration was not determined because at the maximal concentration of 8-Br-cAMP used (10 mM) the dose-response curve still did not plateau (Figure 3-11). An alternative explanation is that the curve plateaus at 100 μM 8-Br-cAMP and the increase in I_h in the presence of higher concentrations of 8-Br-cAMP is due to nonspecific effects.

Unlike in most other cloned HCN channels, cyclic nucleotides did not shift the voltage of the half-maximal activation of acHCN in a depolarized direction (-83.77 ± 0.79 mV, slope factor 7.23 ± 0.70 mV, n = 4, in control; -86.05 ± 0.95 mV, slope 8.47 ± 0.86, n = 4, in the presence of 1 mM 8-Br-cAMP, and -85.34 ± 0.52 mV, slope 6.93 ± 0.46, n = 4, in the presence of 1 mM 8-Br-cGMP).

The observed activation of acHCN by cyclic nucleotides also confirms that the clone used in experiments encodes a functional form of the channel.

3.3.4 Inhibition of acHCN by ZD7288 and Cs^+

Finding inhibitors of acHCN is very important for studying the channel in *A. californica* neurons because blocking the acHCN-mediated current will help to determine functional role(s) of the channel in both neuronal networks and behavior.

As a part of the characterization of the channel I first tested whether acHCN is inhibited by Cs^+ in the millimolar concentrations shown to block other HCN channels (Glitsch et al., 1986). Replacing part of the extracellular sodium with 2 mM Cs^+ led to an almost complete block of

acHCN-mediated currents (79.91 ± 2.72% inhibition at -110 mV, n = 4) (Figure 3-12). The current amplitudes returned to their initial values following 10 min washout.

Although Cs^+ blocks HCN channels, it is not a specific inhibitor of the channels because it also blocks other classes of potassium channels (French and Shoukimas, 1985; Clay and Shlesinger, 1983). Thus, one cannot use Cs^+ to specifically block HCN channels in *A. californica* neurons. Therefore, I also tested ZD7288, a specific inhibitor of HCN channels in mammals (BoSmith et al., 1993). ZD7288 inhibited the acHCN channel by decreasing the amplitude of I_h (Figure 3-13). 100 μM ZD7288 decreased I_h by 83.86 ±2.43 %, n = 4. This concentration of inhibitor was chosen because it is also used in vertebrates, such as guinea pigs and rats (Harris and Constanti, 1995; Kretschmannova et al., 2006).

Inhibition of the acHCN channel by ZD7288 was concentration-dependent (Figure 3-14). The concentration of the blocker required for a half-maximal inhibition (K_i) was 4.68 ± 1.51 μM, n = 4. ZD7288 did not change the voltage of the half-maximal activation of acHCN (-83.77 ± 0.79 mV in the control and -83.93 ± 3.66 mV in the presence of 100 μM ZD7288, n = 4).

Inhibition of the acHCN channel by Cs^+ and ZD7288 additionally supports the identity and functionality of the channel.

3.4 Discussion

3.4.1. Voltage Dependence and Kinetics

The acHCN channel was expressed in *X. laevis* oocytes. The properties of the channel, namely, its activation by both hyperpolarization and cyclic nucleotides, permeability to potassium and sodium ions and inhibition by Cs^+ and ZD7288, confirm its identity as an HCN channel. This is one of the first successful expressions of any *A. californica* channel in oocytes (Pfaffinger et al., 1991; Quattrocki et al., 1994; Collado et al., 2007).

Although acHCN displays the major biophysical and pharmacological properties of HCN channels, it has some unique features. Because the most significant difference between the HCN channels of *A. californica* and other organisms is in the N-terminus, this region can potentially determine some of the differential properties of acHCN, such as its exceptionally slow activation kinetics (Table 3-1).

Pascual et al. (1997) showed that the N-terminal region of voltage-gated potassium channels effects their activation. Deletion of this region led to significantly slower activation times. Because HCN channels belong to a supergroup of voltage-gated potassium channels, the slow activation kinetics of acHCN may result from the truncation of its N-terminal region (if translation of the channel starts from its first ATG codon).

The mechanism of regulating channel activation by the N-terminal region in potassium voltage-gated channels may be charge transfer of the phosphorylated residues to a voltage sensor in TM4 (Perozo and Bezanilla, 1990). Because of the mutation of its first ATG codon, the acHCN channel might have lost both of its two potential N-terminal phosphorylation sites: by protein kinase C and casein kinase II (see Figure 2-2). Therefore, the suggested mechanism may be disrupted in the acHCN channel resulting in its slower kinetics.

TM4 itself is almost identical in all HCN channels, including acHCN, but there are several other regions which may play an important role in determining activation kinetics: TM1 and linkers between TM1 and TM2 (Ishii et al., 2001), TM4 and TM5 (Chen et al., 2001) and TM6 and CNBD (Decher et al., 2004).

TM1 and the TM1-TM2 linker of the vertebrate HCN1 (fast) and HCN4 (slow) channels differ in eight positions. Point mutation studies demonstrated that amino acids of the HCN4 channel in each of these eight positions contribute toward slower activation time constants (Ishii et al., 2001). In three of these positions the acHCN channel has the same amino acids as the

HCN4 channel (L147, I153 and D165) which may contribute to its slow activation. In the other five positions studied by Ishii et al. (F143, V146, N164, D166 and L167), and five additional sites of TM1 and TM1-TM2 linker (L148, I149, A150, A158, S160) acHCN differs from both HCN1 and HCN4 (as well as HCN2 and HCN3). However, it is not known how these differences influence channel activation.

The residues in the linker between TM4 and TM5 (in positions 279, 282 and 290 of the acHCN channel) and in the linker between TM6 and CNBD (in positions 392, 394, 398, 400 and 404), shown to be crucial for gating other HCN channels, do not differ between acHCN and HCN channels cloned from both vertebrates and invertebrates, suggesting that these regions do not determine the slower activation kinetics of acHCN.

The membrane potential of half-maximal activation of acHCN (-83.77 mV) is more positive than $V_{1/2}$ of most other cloned invertebrate HCN channels. Still, it implies that at the potentials of typical after-hyperpolarizations following action potentials in *A. californica* neurons (-50-70 mV), only a small fraction of the acHCN channels are activated. However, in neurons, $V_{1/2}$ of acHCN can be much more positive due to different lipid composition of oocyte membrane and *A. californica* neurons. It was shown that in cardiac and neuronal cells phosphatidylinositol-4,5-bisphosphate (PIP_2) shifts voltage dependence of HCN channel activation by ~20 mV in a depolarizing direction (Zolles et al.; 2006; Pian et al., 2006). The voltage of half-maximal activation of acHCN may be more positive in *A. californica* neurons than in *X. laevis* oocytes due to lower levels of PIP_2 in the membranes of the oocytes. Supporting this hypothesis, there is data that phospholipase C, present in *X. laevis* oocytes, hydrolyzes PIP_2 during maturation of oocytes (Jacob et al., 1993; Han and Lee, 1995).

In conclusion, although acHCN, like other invertebrate HCN channels, is activated by hyperpolarization, its very slow kinetics and rather depolarized $V_{1/2}$ make it more similar to vertebrate HCN channels, especially HCN4 (see Table 3-1).

3.4.2 Ion Permeability of the acHCN Channel

Because the pore region of the HCN channels of *A. californica* and other animals are very similar, I expected the permeability of acHCN to be comparable to that of the other HCN channels. Like them, acHCN conducts only Na^+ and K^+, and elevated extracellular potassium increases the amplitude of I_h by several-fold (Robinson and Siegelbaum, 2003). However, the relative Na^+ / K^+ permeability of acHCN (0.40) is higher than that of the HCN channels of most other animals (but lower than in fish HCN channels, e.g., 0.5 in goldfish; Tabata and Ishida, 1996). This makes the reversal potential of acHCN (-25.84 mV) 4-12 mV more depolarized compared to other invertebrate HCN channels. Similar to $V_{1/2}$, the E_r value of acHCN is closer to that of vertebrate HCN channels (see Table 3-1).

3.4.3 Activation of the acHCN Channel by Cyclic Nucleotides

As discussed in section 3.4.1, the voltage of the half-maximal activation of most invertebrate HCN channels (107-119 mV) lies outside of the physiological potentials (see Table 3-1). Binding of the cyclic nucleotides shifts $V_{1/2}$ by as much as 41 mV in a depolarizing direction (for *P. argus* HCN channel) thus moving it into a physiological range. As a result, cyclic nucleotides may activate invertebrate HCN channels and switch the activity mode of the HCN-expressing cells from silent to spiking. Although $V_{1/2}$ of the acHCN channel is in the physiological range, cyclic nucleotides do not shift it further in a depolarizing direction, but rather increase the amplitude of I_h. This effect may help the cells to recover faster from hyperpolarization following an action potential (AP) and proceed to firing another AP, thus

increasing the frequency of discharge. However, because cAMP does not significantly increase I_h at physiological potentials, this may be true only for cGMP.

The difference between acHCN and both vertebrate and invertebrate HCN channels is that cGMP activates the channel to a larger extent than cAMP. These results cannot be explained based on the available data about the structure-function relationship obtained from the vertebrate channels, since the residues shown to be important for cAMP binding (L526, R543, R584 and I588) are preserved in acHCN (Zagotta et al., 2003).

The modulation of acHCN by cGMP also cannot be explained based on the data obtained from the vertebrate HCN channels. Zagotta et al. (2003) showed that cGMP binds to the CNBD of the vertebrate HCN channels through the threonine in a position corresponding to amino acid 544 of acHCN and suggested that the change of this threonine to valine eliminates activation of an HCN channel by cGMP as in the sea urchin HCN channel (Gauss et al., 1999). However, HCN channels of *A. californica* and insects (*D. melanogaster* and *A. mellifera*) also have valine in this position and they are activated by cGMP. This implies that the mechanism of cGMP binding to the most invertebrate HCN channels may be different than vertebrate HCN channels.

3.4.3 Inhibition of the acHCN Channel by Cs^+ and ZD7288

I showed that both Cs^+ and ZD7288 inhibit the acHCN channel in the concentrations used in other animals.

Inhibition of acHCN by ZD7288 (83.86% by 100 μM ZD7288) is similar to the inhibition of vertebrate HCN channels by the same concentration of the blocker with the maximal percent of inhibition ranging from 84.3 % in the rat pituitary cells (Kretschmannova et al., 2006) to almost complete block in guinea pig substantia nigra neurons (Harris and Constanti, 1995), with the constant of half-maximal inhibition being around 2 μM (Harris and Constanti, 1995). In lobster, the only invertebrate animal where ZD7288 was tested, this blocker inhibited I_h almost

completely at 100-300 μM (Gisselmann et al., 2005b), but the concentration dependence of inhibition and, thus, the constant of inhibition were not determined.

As in mammalian neurons (BoSmith et al., 1993), ZD7288 inhibited the acHCN channel by decreasing the amplitude of I_h, but in contrast to its action in mammalian cells, ZD7288 did not change the voltage of the half-maximal activation of acHCN.

Inhibition by ZD7288 of HCN channels from the animals of all three superclades: Deuterostomia (mammals), Ecdysozoa (spiny lobster) and Lophotrochozoa (*A. californica*) suggests that this is a universal inhibitor of the channels. Additional evidence for that comes from a recent study by Cheng et al. (2007) showing that two residues in TM6 (Ala425 and Ile432 in the mouse HCN2 channel) are the primary determinants for blocking the channels by ZD7288, and these residues are preserved among species (see Figure 2-2).

3.4.5 Summary

Although major biophysical and pharmacological properties of acHCN are similar to those of other HCN channels, a number of characteristics make it different from most other HCN channels. These characteristics include: very slow kinetics, activation by cyclic nucleotides by increasing I_h amplitude rather than shifting $V_{1/2}$ in a depolarizing direction, stronger activation of the channel by cGMP than cAMP, and the relatively depolarized reversal potential and potential of the half-maximal activation. The last two features suggest that the acHCN channel plays a role in controlling spiking frequency of *A. californica* neurons. Testing this hypothesis, which is the subject of Chapter 5, was made easier by the finding that acHCN is inhibited by ZD7288, a specific blocker of the HCN channels. This inhibitor allowed me to remove the HCN-mediated current and determine how it influences neuronal spiking. However, the specificity of ZD7288 in the intact *A. californica* brain and neurons still has to be confirmed to ensure that this blocker does not affect neuronal spiking of the cells that do not express HCN channels. This issue is

addressed in Chapter 5. Also, to study the role of acHCN in *A. californica*, model neurons have to be chosen. To find these cells, I localized acHCN transcript in the CNS of the animal. These experiments are described in the following chapter.

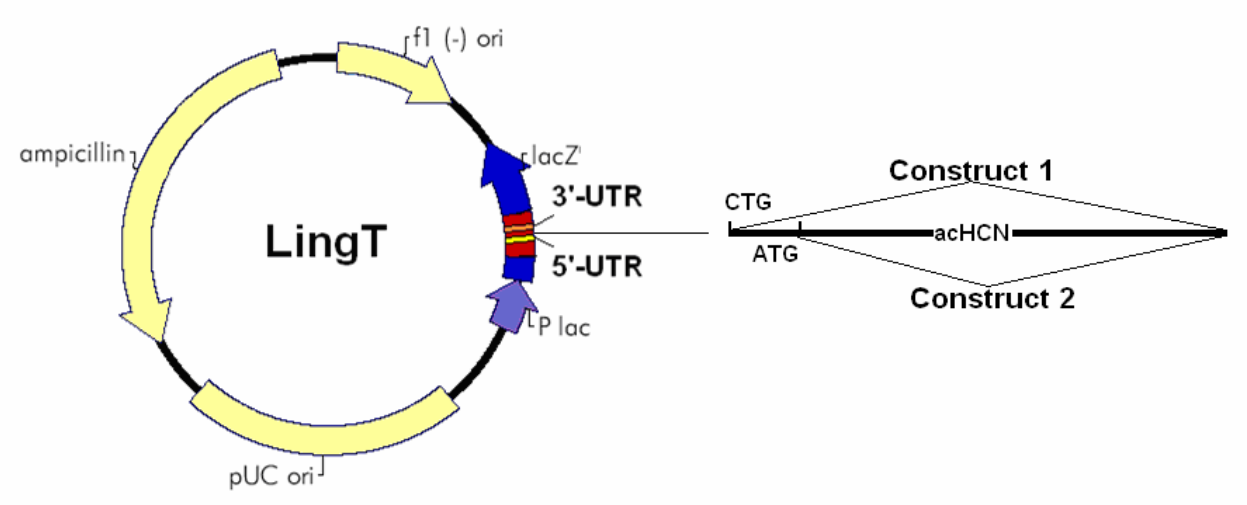

Figure 3-1. Schematic diagram of the constructs used to produce acHCN RNA. LingT plasmid is a modified BlueScript plasmid with added 5'- and 3'-UTRs of *X. laevis* β-globin and T-overhangs. Two constructs made from a single transcript were cloned into the plasmid: Construct 1 starting from the alternative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

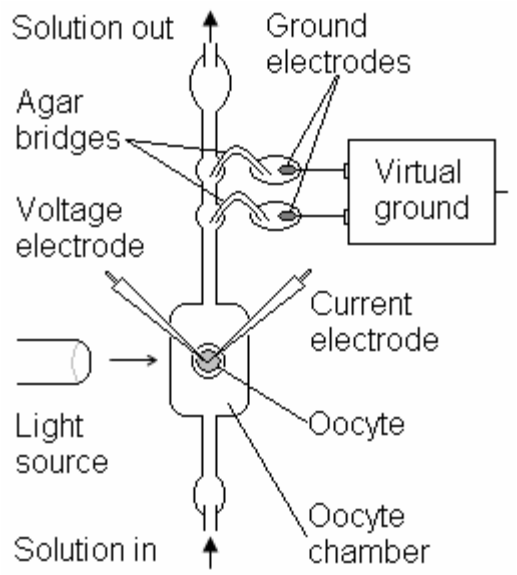

Figure 3-2. Schematic diagram of a two-electrode voltage clamp oocyte recording set-up. Oocytes were placed in a small volume of bathing medium in a plastic chamber which was continuously perfused at a rate of 2 ml/min. The chamber solution was connected through two 2 mM KCl 2 % agar-bridged, Ag/AgCl reference electrodes to a virtual ground of a current monitor head stage.

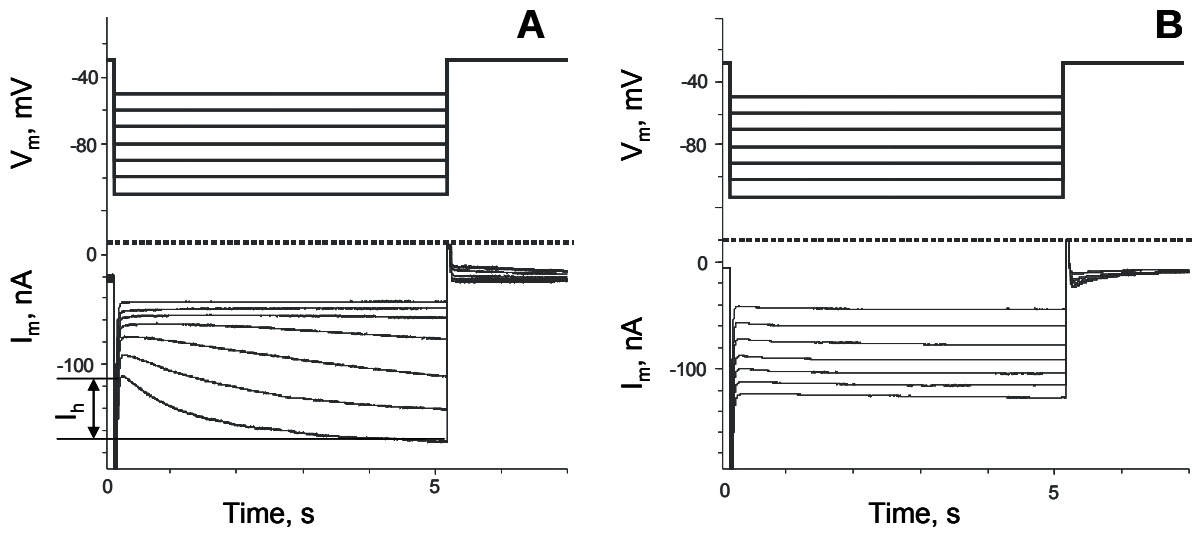

Figure 3-3. Determination of the voltage dependence of the acHCN channel. Slow inward currents characteristic for HCN channels were generated upon hyperpolarization from a holding potential of -30 mV to potentials negative to -70 mV (A). These currents were not present in water-injected oocytes (B). I_h amplitudes were determined as shown in A. N=10.

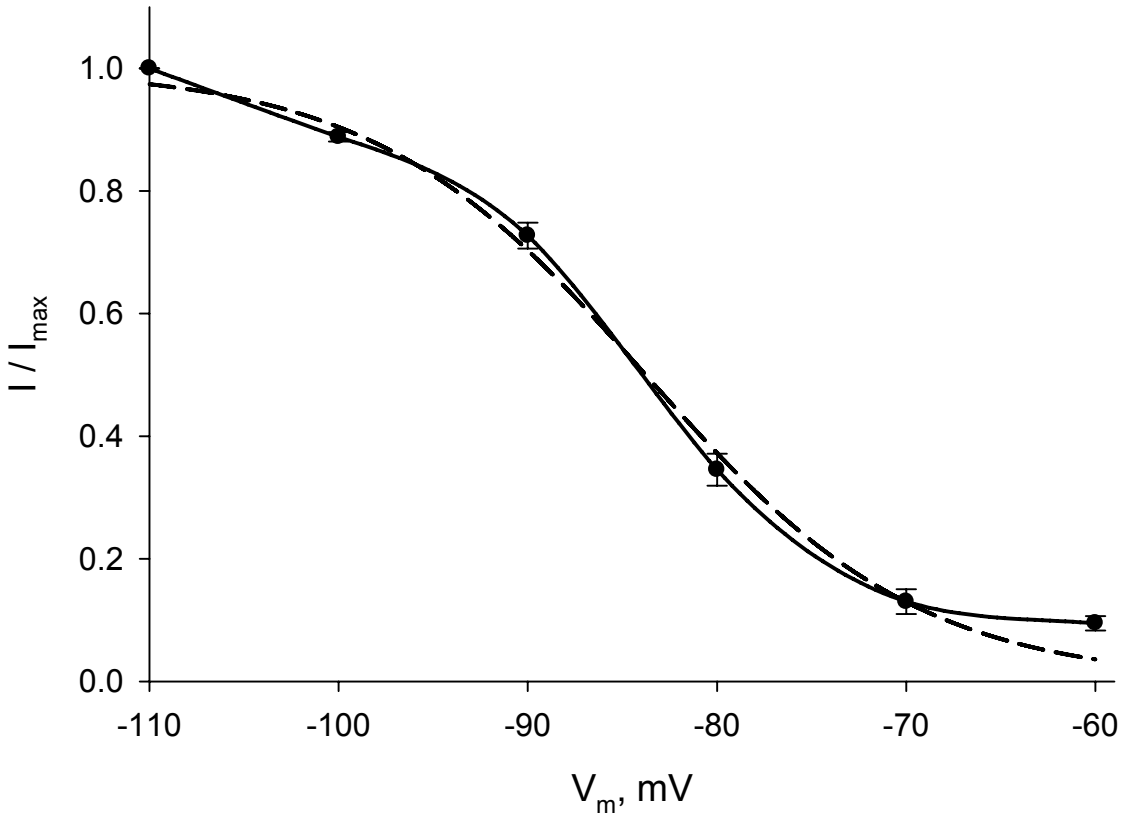

Figure 3-4. Voltage dependence of the acHCN channel activation. The I/V relationship (solid line) of acHCN was determined by hyperpolarizing oocytes (n = 4) for 5 s from the -30 mV holding potential to more negative potentials ranging from -60 mV to -110 mV in steps of -10 mV. $V_{1/2}$ was determined by fitting the I/V curve with a curve calculated by the Boltzmann equation (dashed line) to be ~ -84 mV, n = 4.

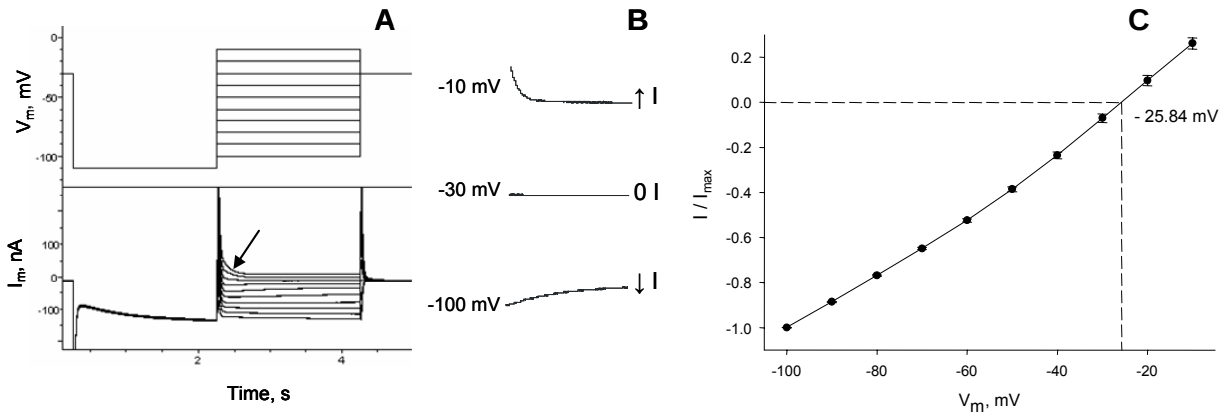

Figure 3-5. Determination of a reversal potential of the acHCN channel. A) Tail currents (arrow) were generated by hyperpolarization of oocytes from a holding potential of -30 mV to -110 mV for 2 s to activate the HCN channels and then stepped to -100 mV and in steps of 10 mV to -10 mV. B) Larger magnification of the inward tail current at -110 mV, outward tail current at -10 mV and approximately zero current at -30 mV. C) Normalized amplitudes of the tail currents were plotted against voltage and the reversal potential of acHCN was determined at a point where the current changes its sign to be ~ -26 mV, n = 17.

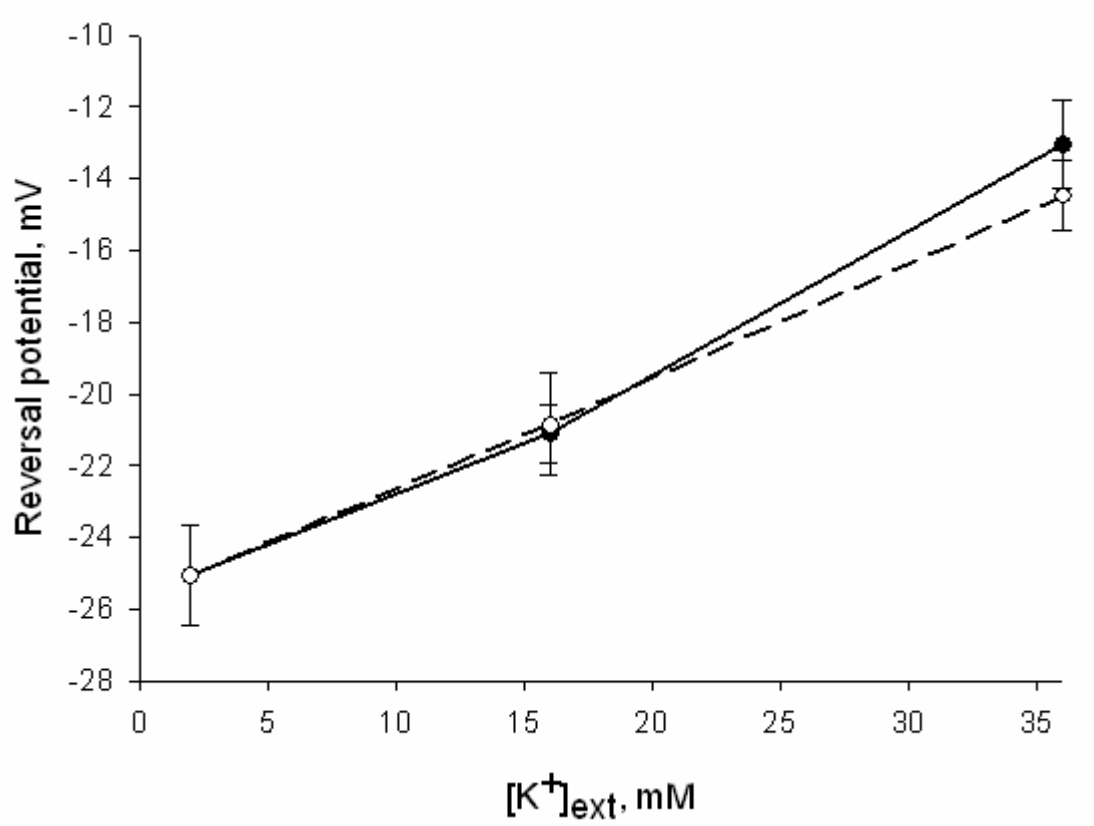

Figure 3-6. Dependence of the reversal potential of the acHCN channel on extracellular potassium concentration. Experimentally determined reversal potential (solid line) closely matched the reversal potential determined using the Goldman equation based on the assumption that the acHCN channel is permeable only to K^+ and Na^+ (dashed line). Error bars in the calculated line reflect that expected reversal potential in 16 and 36 mM K^+ solutions were calculated based on the reversal potential in 2 mM K^+ solution for each individual oocyte.

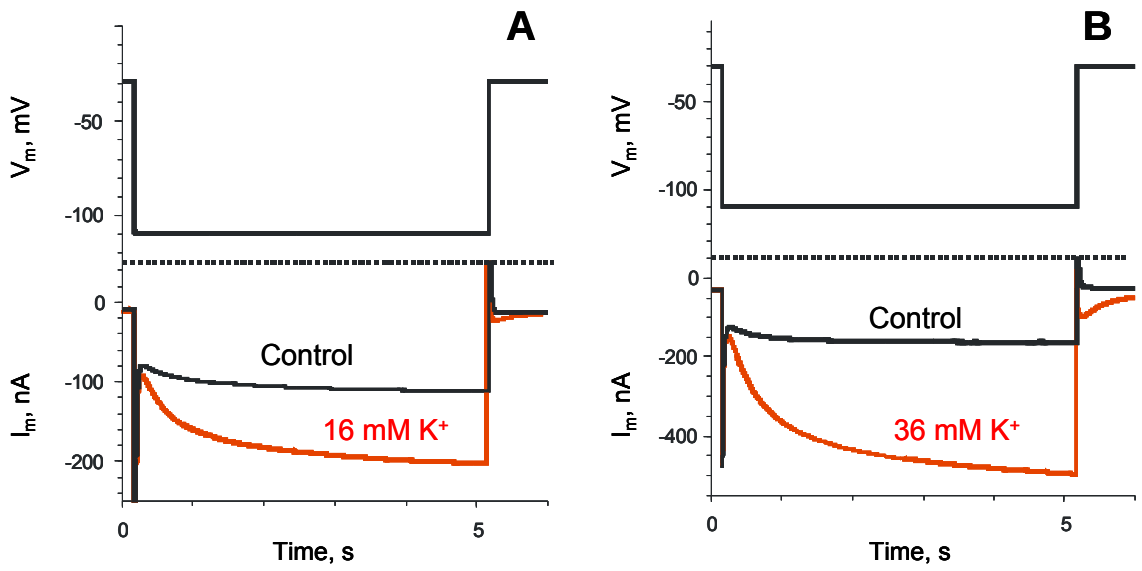

Figure 3-7. Dependence of the I_h amplitude on extracellular potassium concentration. Elevating extracellular concentration of K^+ drastically increases amplitudes of I_h: 4.64 ± 0.62 fold for $[K^+]_{ext}$ = 16mM (A) and 7.82 ± 0.30 fold for $[K^+]_{ext}$ = 36mM (B). Current traces produced by stepping 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.

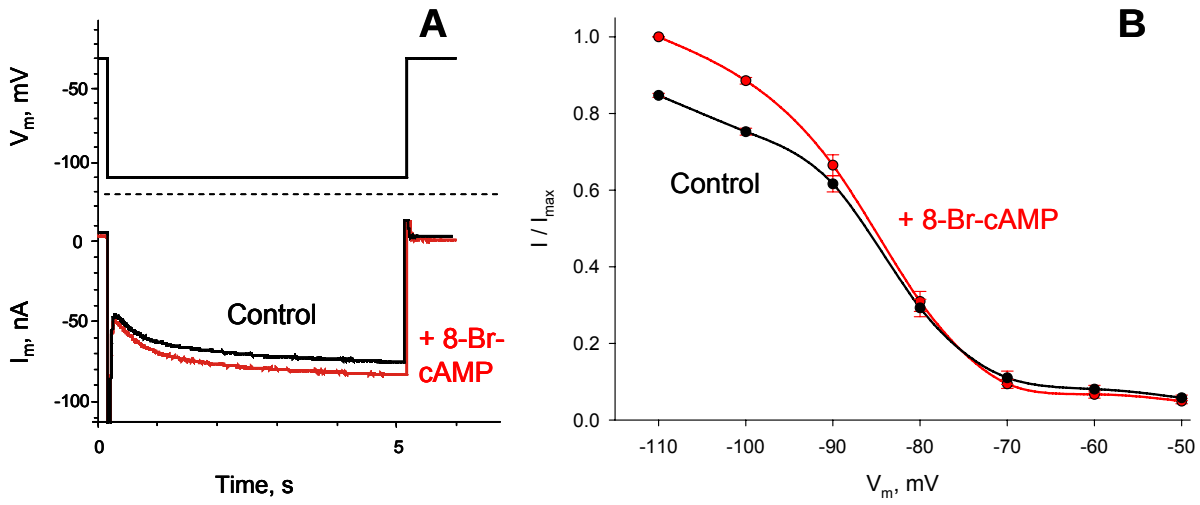

Figure 3-8. Activation of the acHCN channel by cyclic AMP. A) Current trace generated by hyperpolarization from -30 to -110 mV in the presence of 1 mM 8-Br-cAMP (red) was superimposed on a current trace obtained under the same conditions in the absence of 8-Br-cAMP (black) in the bathing solution. B) I/V relationships in control conditions (black) and in the presence of 1 mM 8-Br-cAMP (red). The currents were normalized to the current value at -110 mV in the presence of 8-Br-cAMP. cAMP activates acHCN by increasing the I_h amplitude, with the maximal increase of ~ 18 % at -110 mV, n=4. However, cAMP does not change the acHCN-mediated current at the physiological potentials (-50-70 mV) and does not shift the voltage activation of the channel in a depolarizing direction.

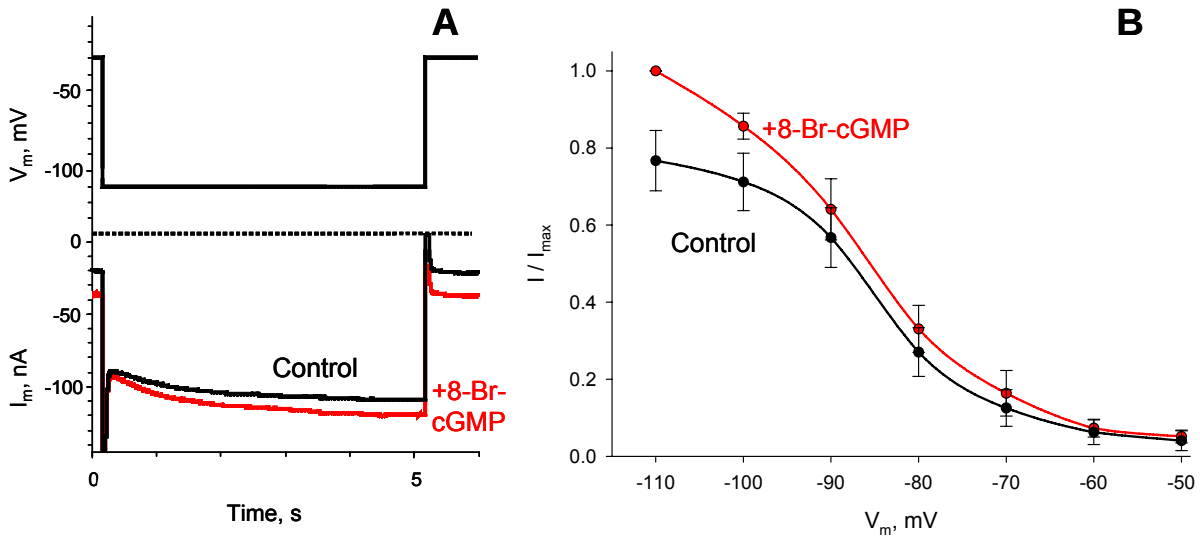

Figure 3-9. Activation of the acHCN channel by cyclic GMP. A) Current trace generated by hyperpolarization from -30 to -110 mV in the presence of 1 mM 8-Br-cGMP (red) was superimposed on a current trace obtained under the same conditions in the absence of 8-Br-cGMP (black) in the bathing solution. B) I/V relationships in control conditions (black) and in the presence of 1 mM 8-Br-cGMP (red). The currents were normalized to the current value at -110 mV in the presence of 8-Br-cGMP. cGMP is more potent than cAMP in activating acHCN, with the maximal increase in the I_h amplitude of ~ 36 % at -110 mV, n=4.

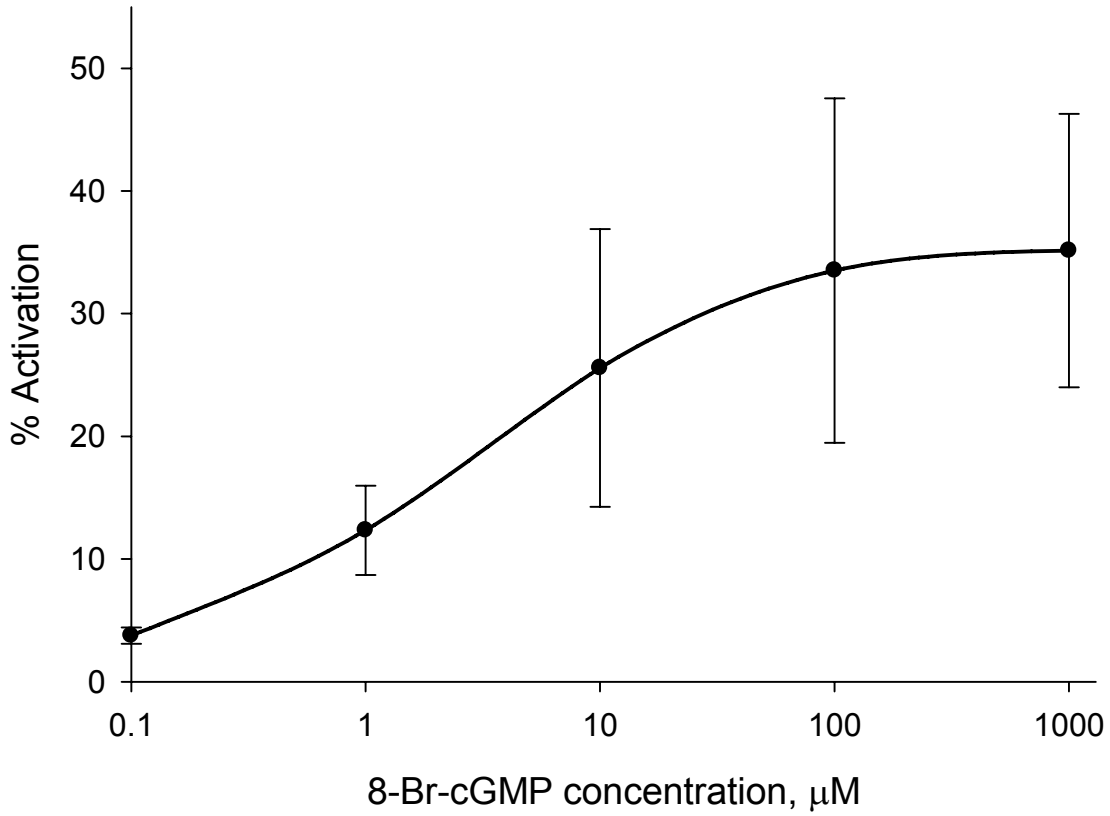

Figure 3-10. Concentration dependence of acHCN activation by 8-Br-cGMP. Percent activation was calculated as an increase in I_h amplitude using the following formula: % activation = $I_{h\text{-}110mV,\ 8\text{-}Br\text{-}cGMP} / I_{h\text{-}110mV} * 100 - 100$. $K_a = 2.16 \pm 0.68$ μM. N = 4 for 0.1, 1 and 10 μM 8-Br-cGMP, n = 6 for 100 μM 8-Br-cGMP and n = 7 for 1000 μM 8-Br-cGMP.

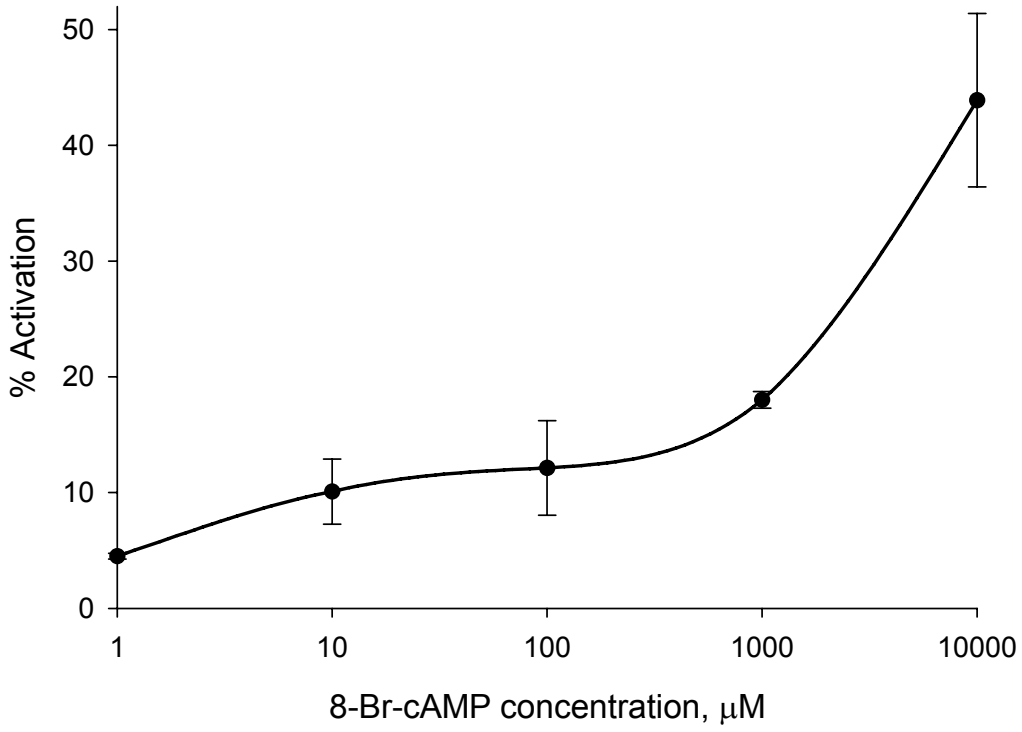

Figure 3-11. Concentration dependence of acHCN activation by 8-Br-cAMP. Percent activation was calculated as an increase in I_h amplitude using the following formula: % activation = $I_{h\text{-}110mV,\ 8\text{-}Br\text{-}cAMP} / I_{h\text{-}110mV} * 100 - 100$. K_a value for 8-Br-cAMP was not determined because at the maximal concentration of 8-Br-cAMP used (10 mM) the dose-response curve still did not plateau. An alternative explanation is that the curve plateau at 100 μM 8-Br-cAMP and the increase in I_h in the presence of higher concentrations of 8-Br-cAMP is due to unspecific effects. N = 4.

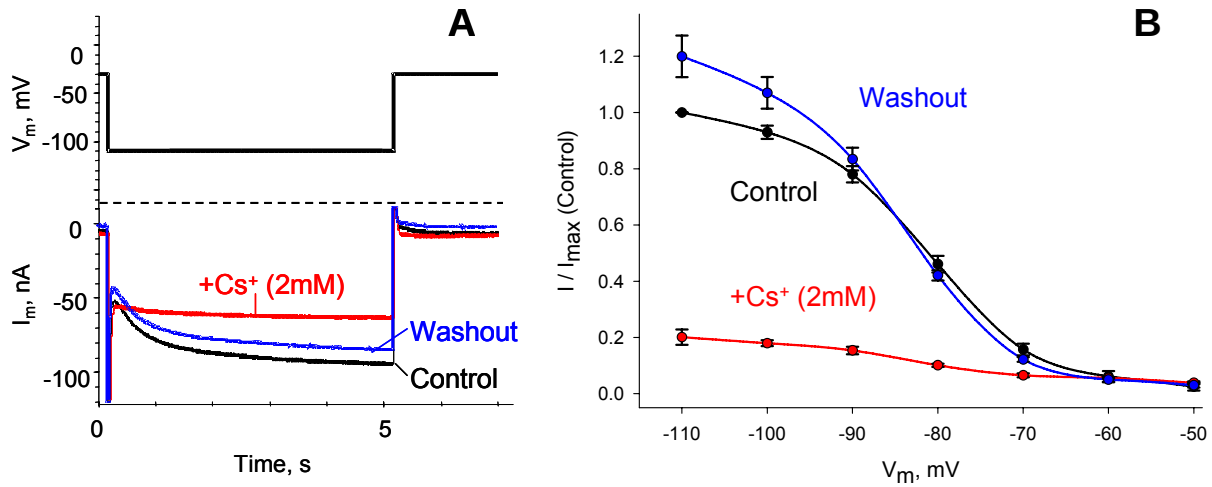

Figure 3-12. Inhibition of the acHCN channel by low concentrations of Cs^+. A) Current traces generated upon hyperpolarization from -30 mV to -110 mV in a media with 2 mM Cs^+ (red) or following washout (blue) were superimposed on the current trace generated under the same conditions in a media without cesium (black). B) I/V relationships for acHCN in control conditions (black), in the presence of 2 mM of Cs^+ (red) or following washout (blue). The currents were normalized to the current value at -110 mV under the control conditions. 2 mM cesium decreased I_h amplitude by 79.91 ± 2.72 %, n = 4.

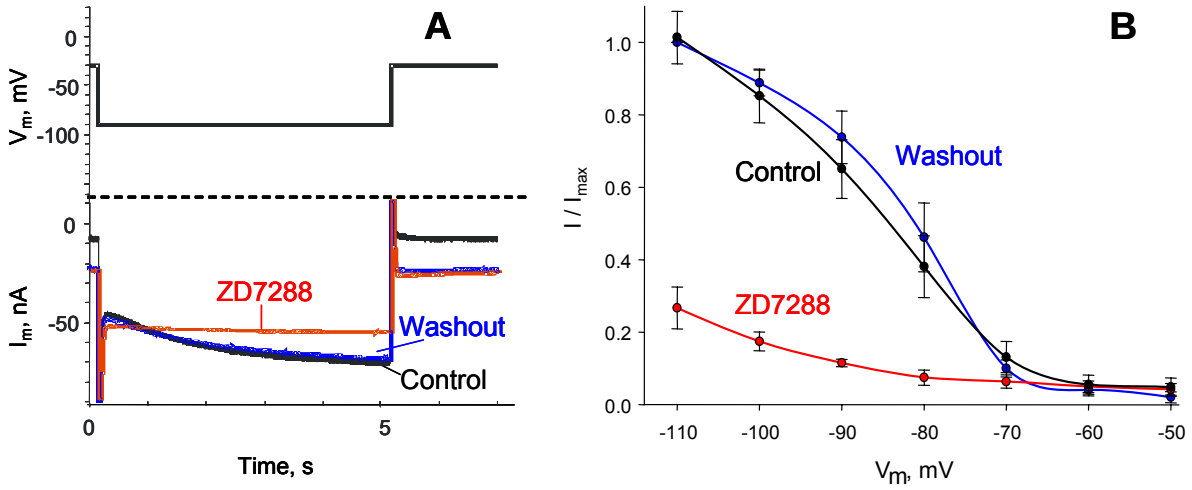

Figure 3-13. Inhibition of acHCN by ZD7288. A) Current traces generated upon hyperpolarization from -30 mV to -90 mV in a media containing 100 µM ZD7288 (red), or following washout (blue) were superimposed on the current trace recorded under the same conditions in the media without the inhibitor (black). B) I/V relationships in control conditions (black), in the presence of 100 µM ZD7288 (red) or following washout (blue). The currents were normalized to the current value at -110 mV under control conditions. 100 µM ZD7288 decreased the amplitude of I_h by ~ 84 %. N = 4.

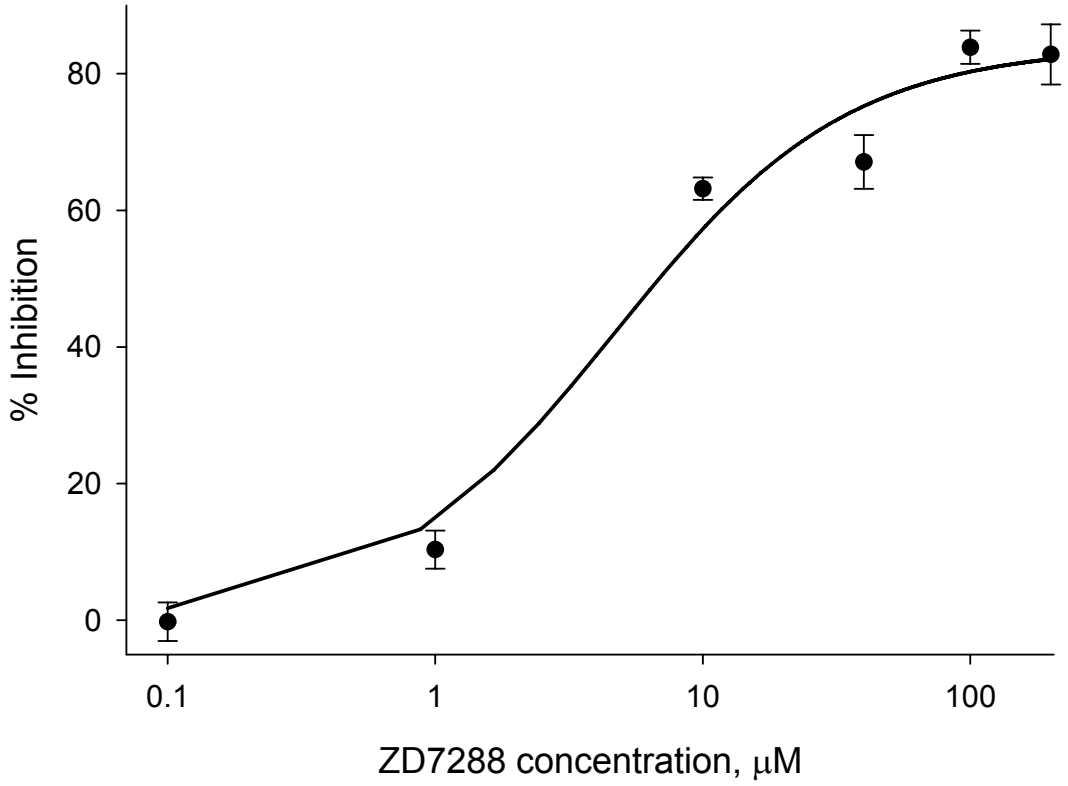

Figure 3-14. Concentration dependence of the acHCN channel inhibition by ZD7288. Percent inhibition was calculated using the following formula:
% Inhibition = 100 – $I_{h\text{-}90\ mV}$ / $I_{h\text{-}90\ mV,\ Ctrl}$ * 100. ZD7288 inhibits acHCN channel in a dose-dependent manner. K_i = 4.68 ± 1.51 μM, n = 4.

Table 3-1. Characteristics and properties of HCN channels cloned from different organisms

Organism	Channel name	Protein length, aa	Ion selecti-vity filter sequence	Hetero-logous expression system	Expression in native organism	$V_{1/2}$, mV	E_{rev}, mV	Time constant, ms	Shift by cAMP (1mM), mV	$K_{1/2}$, μm	Shift by cGMP (1mM), mV	$K_{1/2}$, μm	pK^+/pNa^+	Function	Reference
Human	HCN1	890	CIGYGA	HEK293	-	-70	-21	67	-	-	-	-	-	-	Ludwig et al., 1999; Qu et al., 2002; Seifert et al., 1999; Vaccari et al., 1999; Stieber et al., 2005
	HCN2	889	CIGYGR	-	**Neonatal myocytes*,** brain, heart	-75	-	278	-	-	-	-	-	Rhythmic activity	
				HEK293	-	-93 - 97	-24	196-1192	+15-16	-	-	-	5	-	
	HCN3	774	CIGYGQ	HEK293	-	-77	-21	1244	-	-	-	-	-	-	
	HCN4	1203	CIGYGR	-	**Neonatal myocytes,** brain, heart, testis	-66	-	1271	-	-	-	-	-	Rhythmic activity	
				HEK293	-	-82-109	-22	265-23000	+11-16	-	-	-	4.6	-	
Sea urchin	SpHCN1	767	CIGYGK	-	Sperm flagellum	-	-	-	-	-	-	-	-	May control the waveform of flagellar beating	Galindo et al., 2005
				HEK293		-85	-30	-	-	0.74	-	-	5.6	-	
	SpHCN2	638	SIGFGR	-	Sperm flagellum	-	-	-	-	-	-	-	-	-	Gauss et al., 1998
Lobster	PAIH	692	CIGYGS	-	-	-116	-	-	-	-	-	-	5.6-6.3	Olfactory signal transduction	Gisselmann et al., 2005b
				HEK293	-	-119	-36	297-1495	+41	17.6	+18	120	4.8	-	
Honey bee	AMIH	632	CIGYGR	-	Head, **antennae (ORNs)**, body	-107	-	230-355	-	-	-	-	-	Possibly olfactory signal transduction	Gisselmann et al., 2003
				HEK293	-	-113	-37	50-830	+19	5	+8	75	4.2	-	
Drosophila	DMIH, Isoforms A1B2C2, A3B1C1, A2B1C1, A1B1C1	1319, 627, 618, 844	CIGYGR	-	Antennae, brain, eyes, body	-	-	-	-	-	-	-	-	-	Gisselmann et al., 2005a; Marx et al., 1999-2000
Aplysia californica	acHCN	626 or 693	CIGYGR	*Xenopus* oocytes	CNS, aorta	-84	-26	2330-6230	-	?	-	2.16	2.5	Possible role in locomotion, feeding and memory	Pavlo Kuzyk, unpublished

*Tissues where HCN channels were studied

CHAPTER 4
LOCALIZATION OF acHCN IN *APLYSIA CALIFORNICA*

4.1 Introduction

In Chapter 3, I characterized the biophysical and pharmacological properties of the acHCN channel. Finding that ZD7288, a specific blocker of HCN channels, inhibits acHCN allows removal of HCN-mediated currents and determination of the functional role of the channel in *A. californica*. However, most transcripts are expressed only in specific *Aplysia* neurons. Thus, the major objective for this chapter was to localize the acHCN transcript in the *A. californica* CNS and to identify neurons related to defined neuronal networks. This mapping permitted a study of the role of the channels in these cells. Specifically, because HCN channels depolarize neurons toward firing action potentials, I determined the role of acHCN in controlling the frequency of the neuronal spiking (see Chapter 5). One condition that produces an increase in the frequency of the neuronal spiking is nerve injury. A prolonged increase in spiking frequency is the major manifestation of the LTH, the cellular analog of neuropathic pain (Weragoda et al., 2004). Because HCN channels were suggested to play an important role in nerve injury-mediated responses (Chaplan et al., 2003), I also determined how the expression of the acHCN mRNA changes following nerve injury.

In other organisms HCN channels have been found mostly in sensory cells, such as rod and cone photoreceptors in the tiger salamander (Bader et al., 1979; Hestrin, 1987), newt (Satoh and Yamada, 2002) and lizard (Maricq and Korenbrot, 1990); in B-type photoreceptors in the mollusk *Hermissenda crassicornis* (Yamoah et al., 1998); in the compound eye and chemosensory organs of *D. melanogaster* (Marx et al., 1999-2000); in the antennae of budworm, *H. virescens* (Krieger et al., 1999); in the head and antennae of the honey bee, *A. mellifera* (Gisselmann et al., 2003); and in the stretch receptor neuron (Edman and Grampp, 1989) and

olfactory receptor neurons of spiny lobster, *P. argus* (Gisselmann et al., 2005). In these cells HCN channels play a modulatory role.

HCN channels expressed in inter- and motoneurons often control rhythmic bursting of the neurons in central pattern generators, thus determining repeated activities, such as heartbeat in the medicinal leech, *H. medicinalis* (Angstadt and Calabrese, 1989), contraction of stomach muscles in lobster (Zhang et al., 2003) and rhythmic ingestion movements in *L. stagnalis* (Straub and Benjamin, 2001).

In addition to the nervous system, HCN channels are also expressed in the hearts of vertebrates (Noma and Irisawa, 1976; Goto et al., 1985; Baker et al., 1997) with the strongest expression in the sinoatrial node, the main pacemaker of the heart, where they contribute to the generation of the rhythmic activity. They are also found in the flagella of sperm of the echinoderm *S. purpuratus* (Gauss et al., 1998) and humans (Seifert et al., 1999) where they are proposed to control the waveform of spermatozoan flagellar beating; in the salivary gland cells of the giant Amazon leech, *Haementeria ghilianii* (Wuttke and Berry, 1992) and in rat pancreatic β-cells (El-Kholy et al., 2007). Expression of HCN channels in other tissues, e.g., liver, lung, kidney and skeletal muscle were found by some investigators (Santoro et al., 1998), but not others (Ludwig et al., 1998, 1999). To the best of my knowledge, no HCN channel expression has been demonstrated in the heart of an invertebrate animal.

Because many neurons and neuronal networks in *A. californica* are identified, it is possible to determine the expression of acHCN in the CNS of the animal on the level of not just organs and tissues, but individual cells. And since the role of many neurons and neuronal networks in controlling different behaviors in *Aplysia* is known, localizing acHCN in specific cells will allow predictions about the function of the channel to be made and will identify candidate cells for functional studies.

4.2 Methods

4.2.1 Animals

A. 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 15-18ºC on a 12:12-hour light: dark cycle for up to 3 weeks. Before dissection, animals were anesthetized and killed by injection of 60 % (volume/body weight) isotonic $MgCl_2$ (337 mM).

4.2.2 Surgery

An individual *A. californica* was weighed, anesthetized by injection of 35 % (volume/body weight) of isotonic $MgCl_2$ (337 mM) and placed into the special chamber with sea water. The animal was fixed with hooks so that its dorsal surface was above water level to avoid contamination by bacteria, and a small cut was made to open the central ganglia (Figure 4-1). Then all nerves of the pedal ganglion on one or both sides were crushed, using #4 forceps, by applying firm pressure 2 cm from the ganglion. The incision was sutured and animals returned to the aquaria. Five days after surgery, the CNS of the animals was dissected and *in situ* hybridization performed using a probe to acHCN. This time was chosen because the strongest response to nerve injury, manifested in the increase in neuronal spiking, develops in 5 days following nerve crush (Lewin and Walters, 1999).

4.2.3 Whole-Mount i*n situ* Hybridization Protocol

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.

The *in situ* hybridization protocol (Jezzini et al., 2005) was modified from Bogdanov et al. (1996) and Ono et al. (2000). This method is relatively sensitive as it allows detection of low abundance transcripts, such as *A. californica* two-pore potassium channel AcK2p1 (Jezzini and Moroz, 2004) and fasciclin (Bastiani et al., 1987). 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.

A schematic diagram of the procedure is shown in Figure 4-2.

4.2.4 Imaging

Images were taken with a Digital Sight camera (Nikon, Melville, NY) mounted on an upright SZX12 microscope (Olympus, Center Valley, PA). Figures were prepared using Adobe Photoshop Elements 4.0 and Corel Draw 11.

4.2.5 Densitometry and Cell Counts

Densitometry and cell counts were performed on pedal ganglia stained for acHCN, tryptophane hydroxylase or serotonin transporter on a Nikon Eclipse TE2000 microscope using MetaMorphTM program (Universal Imaging Corp., Downingtown, PA). Brightness of neurons was determined and then converted to the intensity of staining. Cells were considered to be stained if their intensity of staining was two or more times higher than darkest background staining. Intensity of staining of the neurons was determined at the focal plane where they appeared darkest. A region was considered to be background if there was only monotone staining (if any) of neurons and interspersed glia. Graphical output and statistical data have been generated using SigmaPlot.

4.3 Results

4.3.1 Localization of acHCN RNA in *A. californica*

Using whole-mount *in situ* hybridization (n = 16) I have determined the distribution of acHCN transcripts in the CNS of *A. californica* (Figure 4-3, 4-4). As for most *Aplysia* transcripts, this distribution is cell-specific, i.e., only a subset of neurons expresses the HCN mRNA.

In contrast to other organisms, known sensory neurons of *A. californica* do not express detectable amounts of HCN channels (Figure 4-3, 4-4). The strongest staining for acHCN

transcript was in motoneurons of buccal ganglia (20-25 cells, 20-110 μm in diameter) (Figure 4-3A, 4-4, Table 4-1), metacerebral cells (MCC), 8-12 cells in E-clusters (10-40 μm in diameter) and eight-nine cells in G-clusters (10-50 μm) in cerebral ganglion (Figure 4-3B, 4-4, Table 4-1), which are part of feeding network, and up to 100 labeled cells (5-110 μm in diameter) in pedal ganglia (Figure 4-3C, 4-4, Table 4-1), with the highest level of expression between the pedal commissure and the pedal-pleural connective, where locomotory neurons are located (Hening et al., 1979).

The number of cells stained in the abdominal ganglion was variable. The most consistently stained cells were found in the left dorsal semi-ganglion, including two big cells approximately 130 and 200 μm in diameter, presumably L7 and L11. The relative staining of these two cells differed among preparations. Two to six cells stained on the lateral surface of the left side and one to three cells on the medial caudal part of the left side on the ventral surface of the abdominal ganglion. There also was staining in 10-20 cells in the medial and posterior clusters (5-60 μm) and two cells in the anterior cluster of the left pleural ganglion (40-50 μm) (Figure 4-3D, 4-4, Table 4-1).

Also, I found acHCN staining in the aorta, but not in the heart of *A. californica* (Figure 4-5). The staining in other organs, including kidney, esophagus, salivary glands, gonads, gill, buccal mass, rhinophore and visceral complex was not significantly higher than the background. These experiments were done on stage 11-12 animals, n = 4.

Control experiments using sense probes and identical labeling protocols and conditions (n=6) did not produce specific staining in the CNS.

4.3.2 Down-Regulation of the acHCN Transcript Following Nerve Injury

As discussed in Chapter 1, HCN channels are proposed to play a role in the LTH following nerve injury, manifested in increased spiking frequency of neurons (Chaplan et al., 2003). Based

on this finding, I hypothesize that expression of the acHCN transcript increases following nerve injury. To test this hypothesis, I crushed all the nerves of one of the paired pedal ganglia and performed *in situ* hybridization five days following the nerve crush using a probe to acHCN.

Contrary to my prediction, acHCN mRNA is significantly down-regulated following nerve injury as demonstrated by the decrease in both the number of stained cells (30.67 ± 10.07 vs 69.67 ± 13.05, p = 0.0147, n = 3; Figure 4-6A, B) and intensity of staining (76.72 ± 11.31 vs 115.61 ± 14.52 arbitrary units, p < 10 e-10, n = 3; Figure 4-6A, C) in the pedal ganglia with the crushed nerves as compared with the identical cells in the contralateral ganglia (control).

Many pedal neurons, in which expression of the acHCN mRNA changes following nerve injury, are serotonergic (Hernadi et al., 1992, also see staining for serotonin transporter in Figure 4-8). In vertebrates, the concentration of serotonin (5-HT) increases at the site of nerve injury (Sharma et al., 1990; Salzman et al., 1987). Serotonin activates HCN channels through its stimulation of cAMP production (Pape and McCormick, 1989). cAMP, one of the main transcription regulators (Barbas et al., 2003) may, at the same time, down-regulate expression of acHCN mRNA, thus preventing excessive excitability of neurons. Because of these considerations, I decided to test how the transcripts involved in serotonin metabolism change their expression following nerve injury in *A. californica*.

Two processes contribute to the increase in serotonin concentration: serotonin release and serotonin uptake. Therefore, for this study I chose two transcripts that play major roles in these processes, specifically, tryptophane hydroxylase (gi:94434834), the main enzyme in the synthesis of 5-HT, and serotonin transporter, responsible for 5-HT uptake. These transcripts have been cloned in the Moroz laboratory earlier (Moroz et al., 2006)

Tryptophane hydroxylase mRNA is significantly down-regulated following nerve injury in the pedal ganglia with both intact and crushed nerves (from 116 ± 12.73 stained cells to 53 ±

8.49 and 25 ± 4.24, p = 0.01 and 0.03 respectively, n = 2, Figure 4-7). Expression of serotonin transporter RNA also decreased following nerve injury, and only in the pedal ganglia ipsilateral to nerve crush (31 ± 8 stained cells vs 86 ± 5 in contralateral ganglia, p = 7 e-10, n = 10; Figure 4-8B, 4-9A). There was no significant difference between the number of neurons stained for serotonin transporter in left and right ganglia in control preparations (Figure 4-9B).

In addition to HCN, I cloned another channel activated by cyclic nucleotides, cyclic nucleotide-gated (CNG) channel, and determined its localization in the CNS of *A. californica*. The results of these experiments are described in Appendix D.

4.4 Discussion

4.4.1 Localization of the acHCN RNA in *A. californica*

As is the case with other HCN channels, acHCN is most abundantly expressed in the nervous system. The biggest surprise of this study was the lack of the transcript expression in sensory cells. (However, because localization of HCN channels was studied in detail in species of only two phyla (Chordata and Arthropoda), this may also hold true for animals of the other 32 phyla). Instead, the acHCN transcript is expressed in motoneurons of the buccal, cerebral, pedal and abdominal ganglia. The motor output of many of these cells is known. For example, buccal motoneuron B3 innervates intrinsic buccal muscle 3 (I3) that participates in ingestive buccal movements (Church and Lloyd, 1994; Keating and Lloyd, 1999). Because B3 is one of the largest buccal motoneurons and one of the easiest to identify, it was chosen as a target neuron to study the functional role of acHCN.

The cells of cerebral E- and G-clusters, along with buccal motoneurons, are a part of the feeding network controlling buccal muscles responsible for protraction and retraction of the buccal mass during feeding (Jahan-Parwar and Fredman, 1983; Jing and Weiss, 2001). In these cells acHCN channels may play a similar role as in *L. stagnalis*, determining membrane potential

and bursting properties of the cells of a central pattern generator controlling rhythmic ingestion movements (Straub and Benjamin, 2001).

MCCs are a bilateral pair of large serotonergic neurons (Weiss and Kupfermann, 1976). They are among the largest cells of the *A. californica* CNS, they can be easily visually identified and their function is known. The MCCs act centrally to accelerate or trigger bursting in buccal motoneurons, including B3 neuron (Fox and Lloyd, 1998). They also act peripherally, modulating muscle contractions, and contributing to an arousal state induced by food stimuli (Weiss et al., 1978). Specifically, they enhance the speed and strength of biting. Spiking frequency of MCCs correlates with the strength of ingestive movements (Rosen et al., 1989). Therefore, I used these neurons as another model to study the role of acHCN.

In abdominal ganglia, the most consistently labeled neurons are likely to be beating cells L7, L8, $L9_1$ and $L9_2$ and bursting cell L11. acHCN may determine their spontaneous activity (for a mechanism, see Chapter 1). L7, $L9_1$ and $L9_2$ cells control gill contraction and are a part of the defensive network. The large and well-characterized L7 neuron was extensively used in memory-related studies (Kandel, 1976) and is a potential candidate for future study of the role of the acHCN channel in LTP.

In pedal ganglia, the acHCN channel is most strongly expressed in the pedal locomotory neurons (Hening et al., 1979) and it is likely, given the crucial role of the channel in controlling rhythmic movements in other animals, that in *A. californica* the HCN channel may control locomotion. Pedal locomotory neurons are relatively large (80-150 μm in diameter) and four of them, P1 through P4, are identifiable. P4 neuron is the easiest to identify due to its position and size. P4 is the largest neuron (with a size of 80-120 μm) in the region of the dorsal side of pedal ganglia near the pedal commissure (Hening et al., 1979). Therefore, this neuron was chosen as another model for study of the functional role of acHCN. Also, because in *A. californica* pedal

ganglia are used to study the effects of nerve injury on transcript expression, it is possible to determine how expression of acHCN changes following nerve injury in pedal locomotory neurons.

4.4.2 Down-Regulation of the acHCN Transcript Following Nerve Injury

As described in section 4.3.2, my hypothesis about up-regulation of acHCN transcript following nerve injury was not confirmed. Expression of the HCN mRNA following nerve injury also decreases in the rat dorsal root ganglia (Chaplan et al., 2003). However, the I_h density in this system increases after nerve ligation. It is possible that in *A. californica* the HCN protein level also increases following nerve injury and thus the I_h amplitude increases too. In this case, down-regulation of the acHCN mRNA could prevent excessive neuronal excitability. To test this possibility, immunohistochemistry using antibodies to acHCN has to be performed, and the amplitude and voltage-dependence of I_h have to be measured in control preparations and in ganglia with crushed nerves.

Expression of many *A. californica* transcripts increases following nerve injury, e.g., tolloid (Lovell et al., 2005), non-coding RNAs (Bodnarova and Moroz, unpublished observations), etc. This suggests that down-regulation of the acHCN mRNA was not due to a nonspecific reduction of RNAs following nerve injury.

Down-regulation of the serotonin transporter transcript is expected to increase 5-HT concentration around serotonergic cells (due to decreased clearance) and thus induce synthesis of greater quantities of cAMP. Though at physiological potentials cAMP activates the HCN of *A. californica* only to a minimal extent, it may be responsible for the down-regulation of the acHCN mRNA. To determine a role of serotonin in regulation of the acHCN transcript expression (through cAMP), experiments with inhibitors of each stage in the cascade 5-HT → cAMP → acHCN have to be performed.

4.4.3 Summary

Localization of acHCN to motoneurons of the buccal, cerebral, abdominal and pedal ganglia that are part of feeding, defensive and locomotory networks suggests a potential role for the channel in controlling the associated behaviors. Change in expression of acHCN transcripts following nerve injury implies that the channel may also play a role in neuronal responses to nerve injury. However, to clarify what that role is, studies on the level of proteins have to be performed.

Giant interneuron MCC in the cerebral ganglion, buccal motoneuron B3 and pedal locomotory neuron P4 were chosen as the models to study a functional role of the HCN channels, which is a subject of the following chapter.

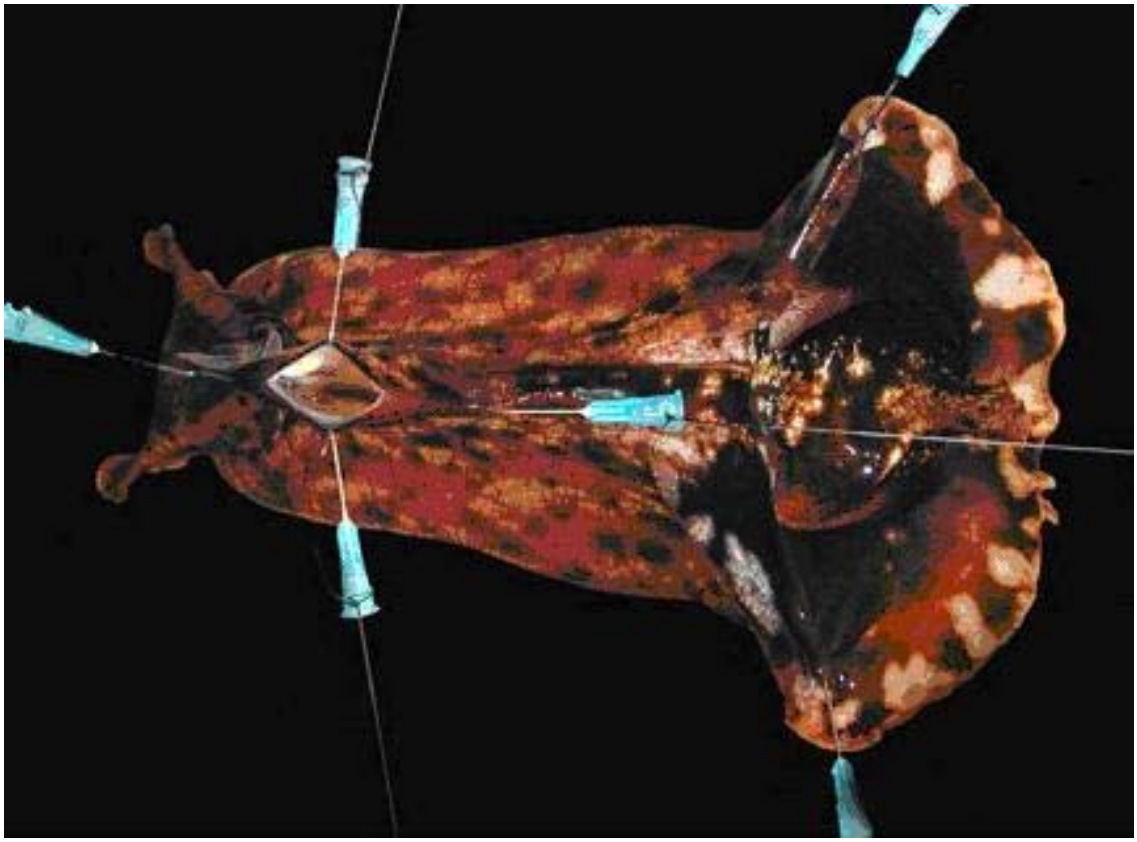

Figure 4-1. Surgery design for pedal nerve crush. *A. californica* is anesthetized, fixed with hooks, and a small incision made in the front dorsal part of the animal, through which the nerves are crushed. The incision is then closed and the animal returned to the tank.

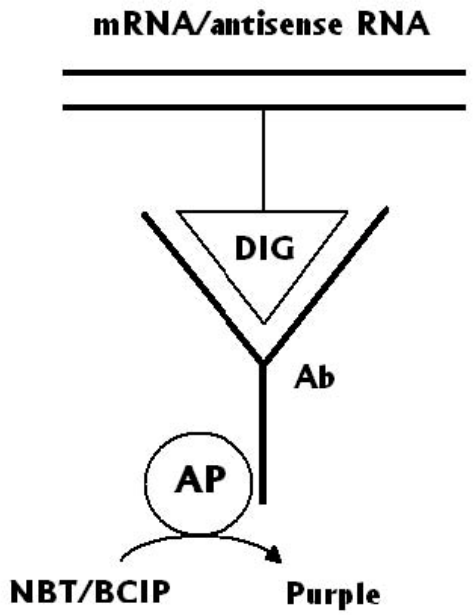

Figure 4-2. Schematic diagram of *in situ* hybridization. RNA labeled with DIG (dioxigenin) probe is hybridized with the complementary mRNA of interest; anti-DIG antibodies (Ab), coupled to alkaline phosphatase (AP), and NBT/BCIP (Nitro-Blue Tetrazolium / 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine, chromogenic substrate of AP) are added. AP reacts with NBT/BCIP solution and turns it purple thus indicating the presence of the mRNA of interest.

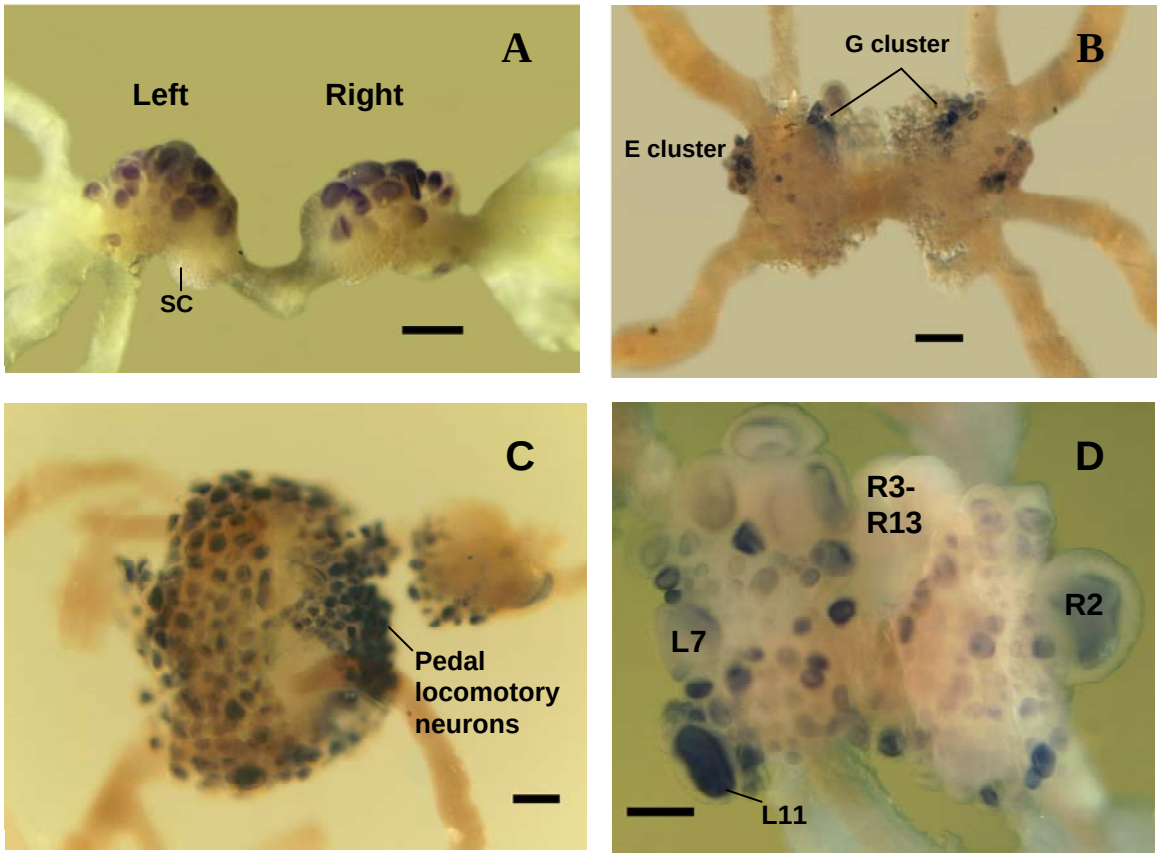

Figure 4-3. Expression of the acHCN channel transcript in the CNS of *A. californica* as determined by *in situ* hybridization. The strongest staining is in the buccal motoneurons and E- and G-clusters of the cerebral ganglion (which are part of a feeding network), pedal locomotory neurons and several individual cells in the abdominal ganglion, some of which may be important in learning and memory. A) Buccal ganglia. SC-sensory clusters. B) Cerebral ganglion. C) Left pedal and pleural ganglia. D) Abdominal ganglion. All scale bars = 500 μm

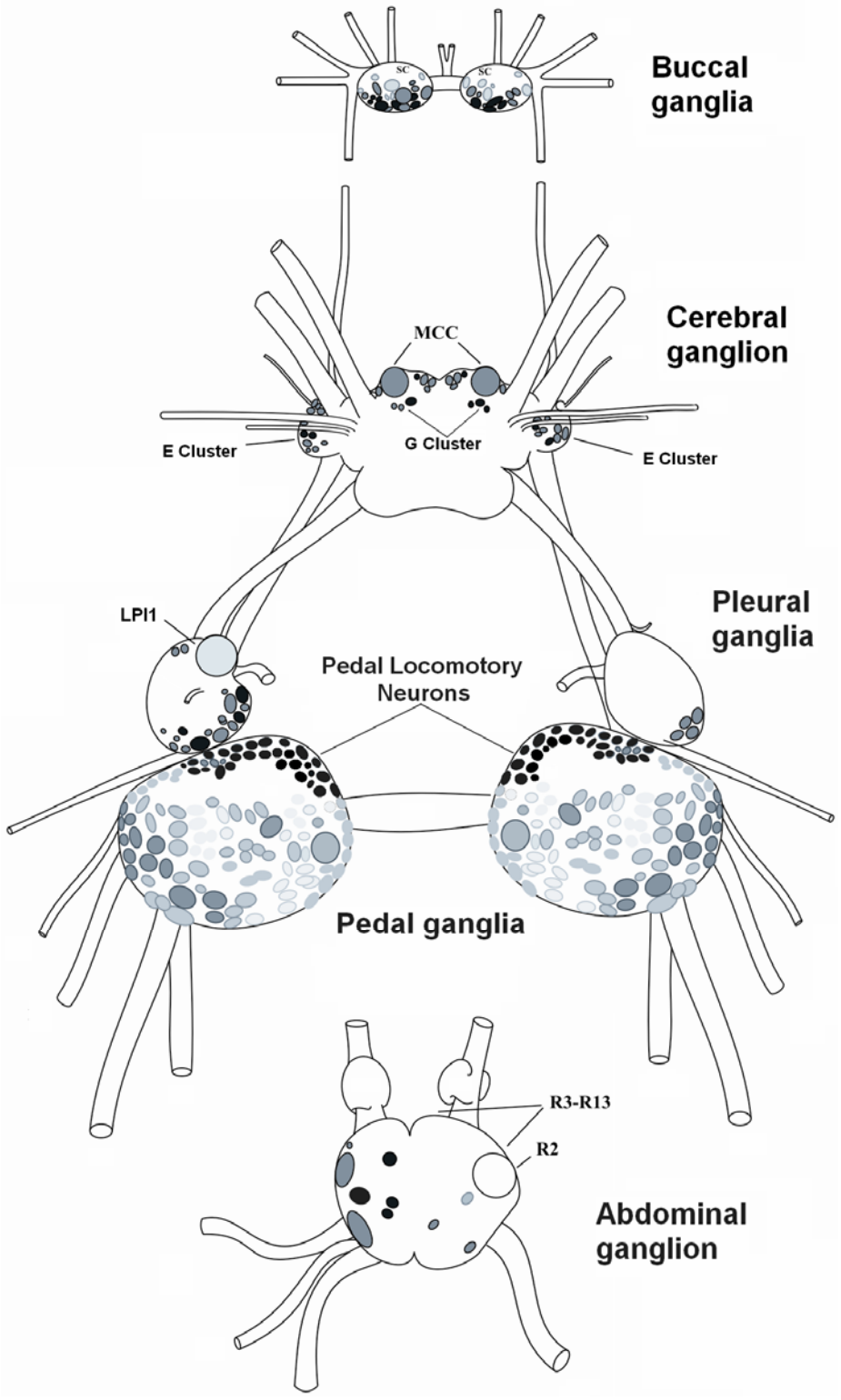

Figure 4-4. Schematic diagram of the expression of the acHCN transcript.

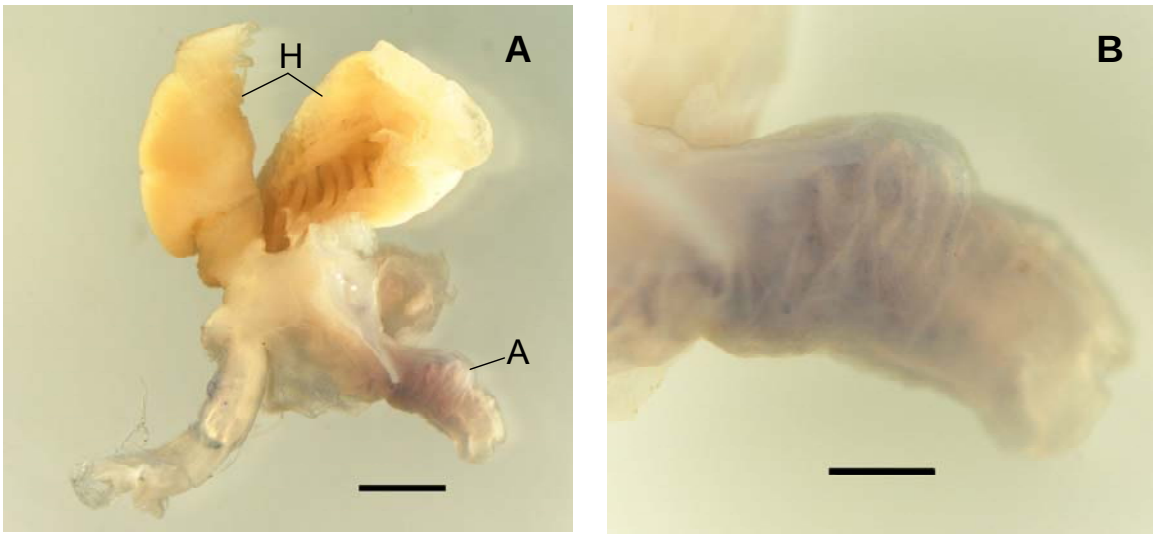

Figure 4-5. Expression of acHCN in the aorta of *A. californica* as determined by *in situ* hybridization. A) Heart (H) and aorta (A). The heart was cut in two to show its internal structure. Scale bar = 2 mm. B) Close-up view of aorta. Scale bar = 1 mm.

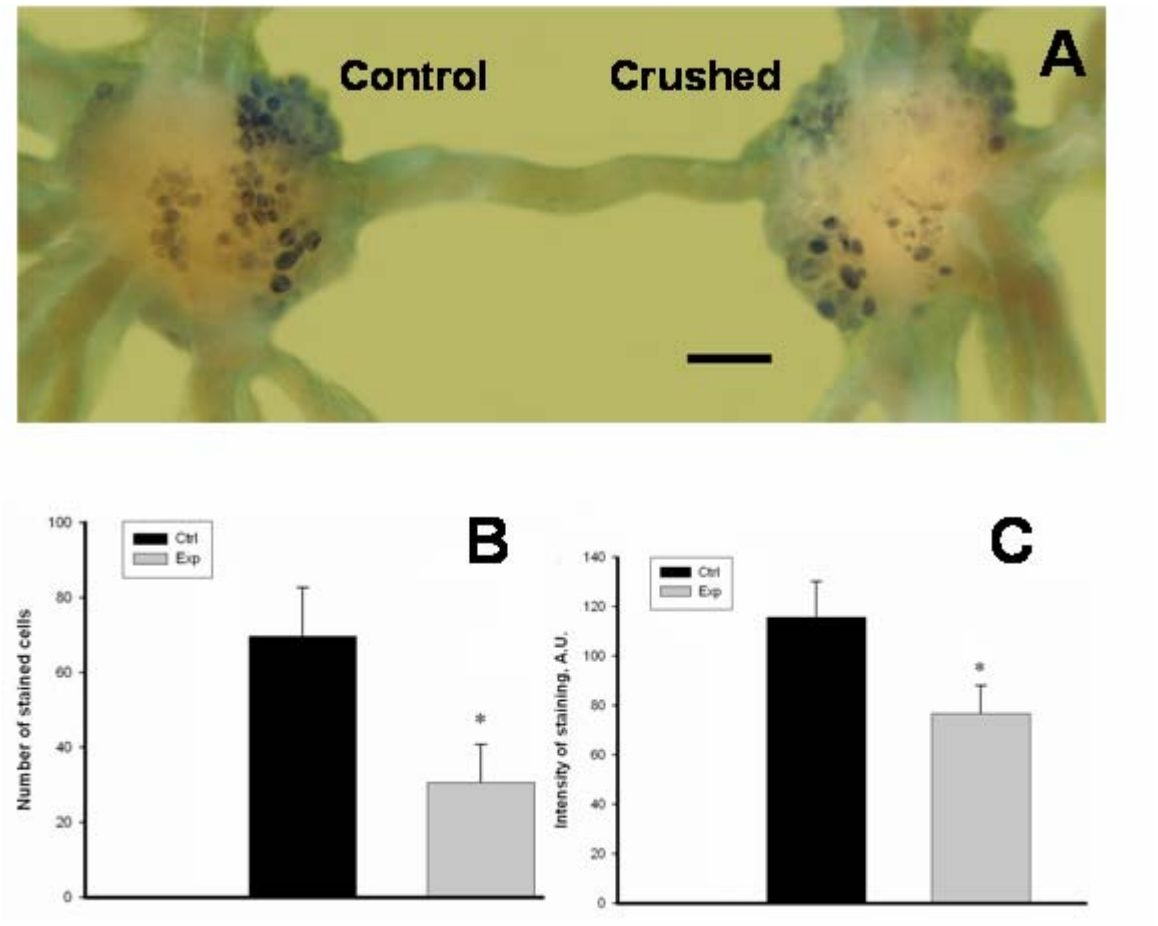

Figure 4-6. acHCN mRNA expression is decreased following nerve injury. A) Staining of pedal ganglia by i*n situ* hybridization using a probe to acHCN. Scale bar = 1 mm. B) Number of the stained cells is decreased in the pedal ganglia with crushed nerves five days after nerve injury ($p = 0.0147$, $n = 3$). C) Intensity of staining is decreased in the pedal ganglia with crushed nerves 5 days after nerve injury ($p < 10$ e-10, $n = 3$). A.U.- Arbitrary units

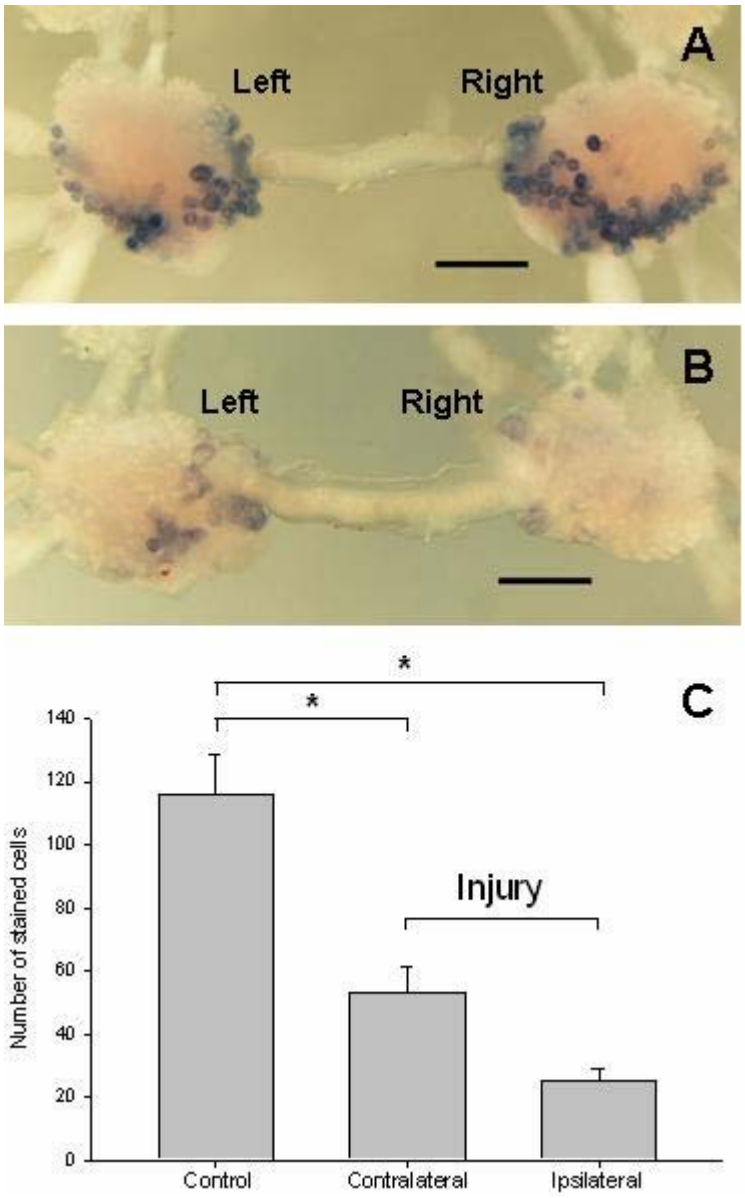

Figure 4-7. Expression of tryptophan hydroxylase mRNA is decreased following nerve injury in pedal ganglia both ipsi- and contralateral to the nerve crush. A) Expression of tryptophan hydroxylase mRNA in control pedal ganglia as determined by *in situ* hybridization. Scale bars in A and B = 500 μm. B) Expression of tryptophan hydroxylase mRNA in pedal ganglia after all nerves of the right gaglion were crushed. C) Number of neurons stained for tryptophane hydroxylase decreased from 116 ± 12.73 in control to 53 ± 8.49 and 25 ± 4.24 in pedal ganglia contra- and ipsilateral to the nerve crush , $p = 0.01$ and 0.03 respectively, $n = 2$).

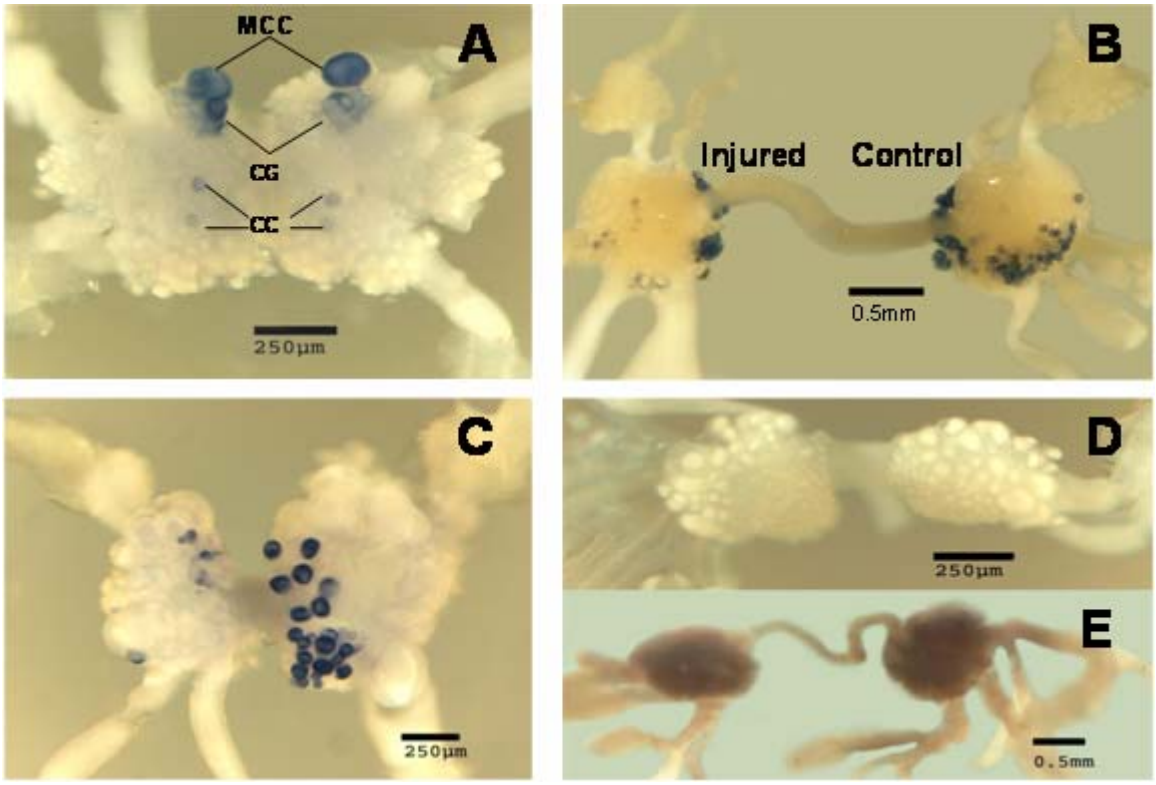

Figure 4-8. Expression of serotonin transporter mRNA in the *A. californica* CNS as determined by *in situ* hybridization. A probe to the serotonin transporter mRNA labels many known serotonergic neurons, including metacerebral cells (MCC), neurons of CG and CC clusters of cerebral ganglion, several cells in the right rostral part of the abdominal ganglion, neurons around the pedal commisure and on the caudal surface of pedal ganglia. A) Cerebral ganglion. B) Pedal and pleural ganglia. All nerves of the left pedal ganglion were crushed while nerves of the right pedal ganglion were left intact. C) Abdominal ganglion. D) Buccal ganglia. E) Staining of pedal ganglia with a sense probe.

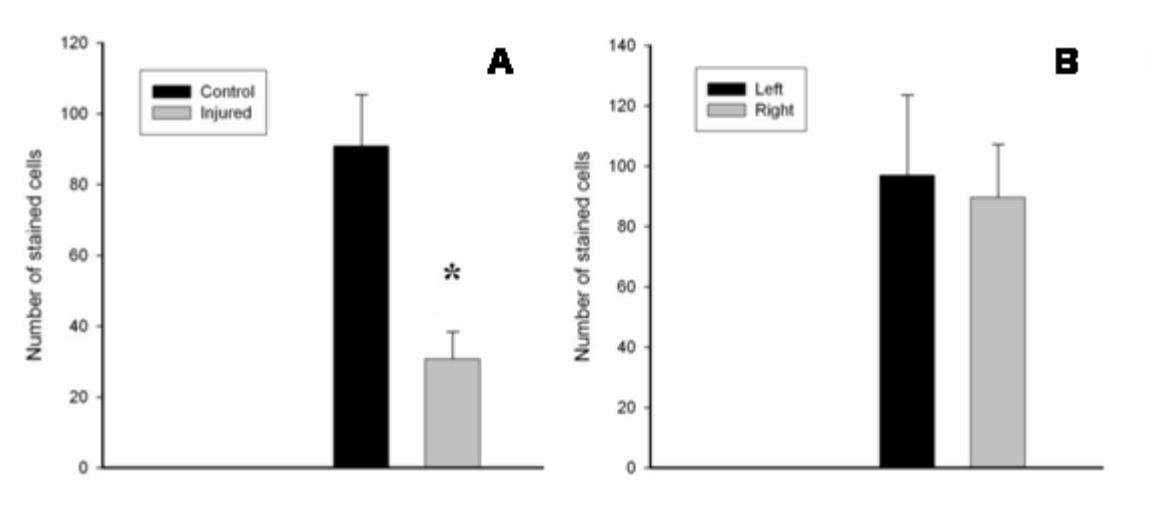

Figure 4-9. Expression of serotonin transporter mRNA is decreased following nerve injury. A) Number of stained cells is decreased in the pedal ganglia with crushed nerves five days after nerve injury ($p = 7e\text{-}10$, $n = 10$). B) Number of neurons stained for serotonin transporter is not significantly different between left and right ganglia in control preparations ($n = 5$).

Table 4-1. Expression of the acHCN mRNA in the CNS of *A. californica*

Ganglia and clusters	Number of stained neurons	Size of stained neurons, μm	Identifiable neurons	Neuronal network	Reference
Buccal	20-25	20-110	B3, B4, B5, etc	Feeding	Church and Lloyd, 1994
Cerebral			MCC		Weiss and Kupfermann,
E cluster	8-12	10-40		Feeding	1976; Jahan-Parwar and
G cluster	8-9	10-50			Fredman, 1983
Pedal	Up to 100	5-110	P1-P4	Locomotory	Hening et al., 1979
Pleural	10-22	5-60	-	?	-
Abdominal	16-30	5-200	L7, L11	Defensive	Kandel, 1976

CHAPTER 5
CHARACTERIZATION OF THE FUNCTIONAL ROLE OF acHCN

5.1 Introduction

The expression of acHCN in *X. laevis* oocytes and biophysical and pharmacological characterization of the channel, described in Chapter 3, confirmed that the cloned channel is functional. Knowing the properties of acHCN and confirming that ZD7288 blocks the channel allowed me to study the role of acHCN in *A. californica* neurons. Thus, the major objective of the work presented in this chapter was to determine the functional role of acHCN in *Aplysia* neurons. There are two goals for this chapter: first, to determine the role of acHCN in spiking of individual neurons and second, to determine the contribution of acHCN in the maintenance of the feeding and locomotion rhythms.

As discussed in Chapter 1, HCN channels depolarize cells and drive them towards the threshold for firing action potentials. Therefore, the initial hypothesis was that acHCN controls the spiking frequency of neurons that express the channel and that blocking acHCN would decrease the frequency of the neuronal spiking.

By localizing the acHCN transcript in the CNS of *A. californica*, described in Chapter 4, target neurons for this study have been selected, i.e., MCCs, arousal interneurons, which activate buccal feeding motoneurons (Weiss and Kupfermann, 1976), buccal motoneuron B3, which controls jaw movement during feeding (Gardner, 1971) and pedal locomotory motoneuron P4, which controls foot muscle contraction during locomotion (Hening et al., 1979). I used synaptically-isolated MCCs to determine the role of acHCN in controlling the spiking frequency on the level of individual neurons because MCCs are the most easily identifiable neurons expressing the acHCN transcript.

To establish the role of acHCN on the systemic level, I determined the role of acHCN in controlling the spiking frequency of B3 and P4 neurons, discharges of which directly correlate

with the contraction of buccal and pedal muscles, respectively, associated with feeding and locomotion, correspondingly (Church and Lloyd, 1994; Hening et al., 1979). Thus, demonstrating how acHCN effects spiking of these neurons will allow predictions to be made about the role of acHCN in the above mentioned behaviors.

5.2 Methods

5.2.1 Electrophysiological Recording from *A. californica* Neurons

Animals were dissected as described in section 4.2.1. Treatment time of the isolated CNSs with protease IX (10 μg/ml) was decreased to 20 min to avoid damage to the neurons. Because neurons of the abdominal ganglion were most sensitive and ruptured even following very short digestion times, I did not treat them with protease. The CNS was then desheathed with fine forceps and scissors to expose neuronal somata.

The preparation was put in a sylgard-covered Petri dish (3.5 cm in diameter) and continuously perfused with artificial *Aplysia* saline (AS, 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, 10 mM HEPES, pH 7.6-7.7, adjusted with 10N NaOH).

To determine the role of acHCN at the level of individual neurons, I perfused the preparation with high divalent cation (Hi-Di) solution, containing 1.25x Ca^{2+} and 2.2x Mg^{2+} compared to AS. This solution blocks synaptic transmission between neurons and thus allows one to synaptically isolate them (Liao and Walters, 2002).

Sharp intracellular electrodes (15-20 MΩ) were drawn with a P-2000 puller (Sutter Instrument Co) and filled with 3 M potassium acetate. All recordings were done using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Signals were digitized using a Digidata 1320A analog to digital converter, and analyzed using pClamp software (all from Axon Instruments).

The R15 neuron (Halstead and Jacklet, 1974) was used to determine the specificity of ZD7288 for acHCN because no acHCN expression was detected in this neuron by *in situ* hybridization. R15 was identified by its medial position in the right caudal quarter-ganglion of the abdominal ganglion, its large size and spontaneous spiking. B3 and P4 neurons were identified based on their position and size (see Chapter 4). Spontaneous spiking was recorded for R15 and P4 neurons and spiking initiated by injecting depolarizing current, typically 2 nA, was recorded for MCCs and B3 because these neurons are usually silent except during feeding (Weiss and Kupfermann, 1976; Fox and Lloyd, 1998). Following penetration of neurons with electrodes, neuronal spiking was allowed to stabilize for ~ 30 min, so that spontaneous spiking or spiking in response to the positive current injection was regular for at least 10 min. Control spiking was then recorded. Spiking was recorded again following 1 hr perfusion of the preparation with AS or Hi-Di solution containing 150 μM ZD7288. This concentration of the inhibitor was chosen because it causes maximal inhibition of the heterologously-expressed channel (see section 3.3.4).

All electrophysiological recordings were performed at room temperature (20-22°C).

5.2.2 Data Analysis

Data were analyzed using ClampFit (Molecular Devices) and SigmaPlot 9.0 software (SYSTAT Software Inc). Values depicted in graphs represent the mean ± s.e.m.

5.3 Results

5.3.1 Specificity of ZD7288 in *A. californica*

Because ZD7288 has never been tested in mollusks I verified the specificity of this blocker for acHCN by determining whether ZD7288 changes spiking of the identifiable *A. californica* neurons that do not show detectable expression of the channel with our *in situ* hybridization. By localizing the acHCN transcript in the CNS of *A. californica* two potential targets for this study

have been selected, both in the abdominal ganglion: osmoregulatory neurons R3-R13 (Gainer and Wollberg, 1974) and R15 neuron controlling respiratory pumping (Alevizos et al., 1991). To test the specificity of ZD7288 I selected the R15 neuron, which is large and easily identifiable.

ZD7288 (150 μM) did not significantly change patterns of electrical activity of R15 spiking, including the number of spikes per minute (1.19 ± 0.40 fold change following perfusion with AS containing ZD7288, compared to control, $p = 0.68$, $n = 3$, Figure 5-1A, B, C), spike amplitude (1.01 ± 0.01 fold change, $p = 0.64$, $n = 3$, Figure 5-1A, B, D) or maximal hyperpolarization (0.95 ± 0.06 fold change, $p = 0.51$, $n = 3$, Figure 5-1A, B, E).

These results suggest that ZD7288 is indeed a specific inhibitor of acHCN and can be used to determine the role of the channel in neuronal spiking.

5.3.2 Effect of ZD7288 on the Spiking Frequency of Individual Neurons Expressing acHCN

To determine the role of acHCN in controlling the frequency of neuronal spiking, I blocked the channels by perfusing the preparation with ZD7288. Confirming my hypothesis, stated in section 5.1, ZD7288 significantly decreased spiking frequency of MCCs (70 ± 5.03 % decrease following perfusion with AS containing ZD7288, compared to control, $p = 0.005$, $n = 3$, Figure 5-2). Following washout, the spiking frequency recovered very slowly, with a recovery of 26.50 ± 8.50 % of control value following 2 hr washout ($n = 3$) and 58 % of control value following 3 hr washout ($n = 1$).

To ensure that decreased spiking of MCCs was not due to a decrease in synaptic input from other acHCN-expressing neurons, MCCs were synaptically isolated by perfusing the preparation with Hi-Di solution. Similar to its effect in AS, ZD7288 significantly decreased spiking frequency of the synaptically-isolated MCCs (64.33 ± 5.78 % decrease, $p = 0.008$, $n = 3$, Figure 5-3). Following washout, the spiking frequency of the synaptically-isolated MCCs recovered slowly, to 30 ± 8.08 % of control value following 3 hr washout ($n = 3$).

The difference in decrease by ZD7288 of spiking frequency of synaptically-coupled and synaptically-isolated MCCs was not significant ($p = 0.63$, $n = 3$, Figure 5-4).

Knowing that acHCN controls the frequency of the neuronal spiking in individual neurons allows proceeding to determine the role of the channel on systemic level.

5.3.3 Effect of ZD7288 on Systemic Level

To determine the role of acHCN on systemic level, I blocked the channels by perfusing the preparation with ZD7288 in normal AS and simultaneously recorded from two symmetrical neurons (two B3s or two P4s) in the left and right buccal and pedal ganglia, respectively.

ZD7288 significantly decreased the spiking frequency of B3 neuron (53.33 ± 5.81 % decrease following perfusion with AS containing ZD7288, compared to control, $p = 0.01$, $n = 3$, Figure 5-5). Following washout, the spiking frequency of the B3 neuron recovered slowly, to 41.67 ± 21.48 % of control value following 2 hr washout ($n = 3$).

Similarly, ZD7288 significantly decreased spiking frequency of the pedal locomotory neuron P4 (94 ± 3.67 % decrease following perfusion with AS containing ZD7288, compared to control, $p = 0.0001$, $n = 4$, Figure 5-6). Following washout, the spiking frequency of the P4 neuron recovered slowly, to 23 ± 5.31 % of control value following 2 hr washout ($n = 4$).

ZD7288 also inhibited the rhythmic activity of unidentified buccal neurons representing the feeding central pattern generator (Figure 5-7) and tonic activity of unidentified pedal locomotory neurons (not shown). This indicates a potential role of acHCN in triggering and maintenance of feeding and locomotion.

5.4 Discussion

Lack of the effects of ZD7288 on different aspects of spiking of R15 neuron that does not show detectable expression of acHCN implies that this inhibitor is specific for the acHCN channels. This finding allowed using ZD7288 to block the channels in *A. californica* neurons and

thus determine the functional role of acHCN. As predicted, ZD7288 decreased the spiking frequency of all recorded neurons expressing the channel, i.e., MCCs, B3s and P4s, implying that acHCN controls the frequency of neuronal spiking. This reduction of spiking frequency is not caused by decreased synaptic input from other neurons because in Hi-Di solution ZD7288 decreased spiking frequency by approximately the same amount as in the normal saline.

The recovery of the spiking frequency upon washout was slow, with only a partial recovery following 3 hours of washout. One of the explanations for this is that the volume of the Petri dish containing the preparation is relatively large, much larger than the volume of the oocyte chamber. Consequently, the inhibitor is washed out slowly. The other explanation is that the recovery of the spiking frequency following washout is due to the synthesis of the new channels. I consider this explanation more plausible, because in vertebrates ZD7288 was shown to be irreversible (BoSmith et al., 1993).

Since B3 and P4 neurons belong to feeding and locomotory networks, respectively, the reduction in the spiking frequency of these neurons by ZD7288 implies the role of acHCN in controlling activity of the feeding and locomotory networks. In addition, because spiking of B3 and P4 neurons directly correlates with the contraction of buccal and pedal muscles, respectively (Church and Lloyd, 1994; Hening et al., 1979), the results of the described experiments implicate the role of acHCN in controlling feeding and locomotion in *A. californica*. This role is also supported by inhibition by ZD7288 of the rhythmic activity of different unidentified buccal neurons representing the feeding central pattern generator and tonic activity of unidentified pedal locomotory neurons.

A similar role for an HCN channel in controlling spiking frequency in identifiable feeding motoneurons, specifically, B4/B8 and B4CL (Staras et al., 1998), was suggested in *L. stagnalis*. Serotonin increased the firing frequency of these neurons, and this effect was due to the

activation of a Cs^+-sensitive current and decreased the duration of postinhibitory rebound, membrane depolarization occurring at the offset of hyperpolarization (Straub and Benjamin, 2001). Thus, this current was presumably identified as I_h; however, it was not further characterized. Therefore, it may be a different Cs^+-sensitive current. Similarly, Bertrand and Cazalets (1998) showed that postinhibitory rebound in the rat locomotory motoneurons was abolished by Cs^+ and experimental regulation of the postinhibitory rebound modulated spiking of these neurons. But again, the Cs^+-sensitive current was not further characterized. Also, Chaplan et al. (2003) showed a role for HCN channels in the increased spiking of the rat dorsal root ganglion cells following nerve injury and associated behaviors, i.e., paw-licking, but this work was not done on the identifiable cells. Thus, to the best of my knowledge, my work is the first to show the role of an HCN channel on the molecular level, the level of individual identifiable neurons and a systemic level.

One of the future goals would be to determine under what conditions the activity of acHCN changes. For example, the activity of acHCN and the spiking frequency of *A. californica* neurons are expected to change when the concentration of cyclic nucleotides changes. One such change occurs during nerve injury (Siegan et al., 1996). Elevated cGMP may activate acHCN that would in turn depolarize neurons and lead to the increased frequency of their spiking, one of the most important manifestations of the LTH. Elevated cAMP is not expected to activate acHCN in the same way as cGMP, because in oocyte experiments cAMP did not change acHCN-mediated current. However, since cAMP is one of the main transcriptional regulators it may decrease transcription of acHCN, thus preventing excessive excitability. In addition to being activated by cGMP, the acHCN channel may produce larger currents due to up-regulation of the synthesis of acHCN protein following nerve injury as in the rat dorsal root ganglia (Chaplan et al., 2003). Alternatively, if acHCN protein is down-regulated following nerve injury, similar to

the acHCN mRNA, this may decrease the acHCN-mediated currents and reduce the frequency of neuronal spiking. These possibilities can be tested by performing immunohistochemistry and electrophysiologically recording I_h both under normal conditions and following nerve crush. Also, increased spiking of neurons following nerve injury in A. californica may not be due to activation of HCNs, but other channels, e.g., Na^+ channels.

Based on the properties of acHCN, it may also play a role in classical conditioning. It was long known that this process in *A. californica* is mediated by serotonin and cAMP (Kandel, 2001). However, recently it has been shown that another mechanism of classical conditioning in *Aplysia* is through nitric oxide (Antonov et al., 2007). Nitric oxide in *Aplysia* activates guanylyl cyclase and thus increases production of cGMP (Bodnárová et al., 2005). cGMP, in turn, is expected to activate acHCN, which will increase the excitability of the neurons. HCN channels were also suggested to play an important role in LTP at the neuromuscular junction of the crayfish (Beaumont and Zucker, 2000). In this system, HCN channels were shown to act at the presynaptic level by depolarization of the presynaptic membrane. Because in *A. californica* HCN channels are expressed predominantly in motoneurons and not in the sensory neurons, acHCN may act postsynaptically at the level of sensorimotor connections or presynaptically at the level of neuromuscular connections.

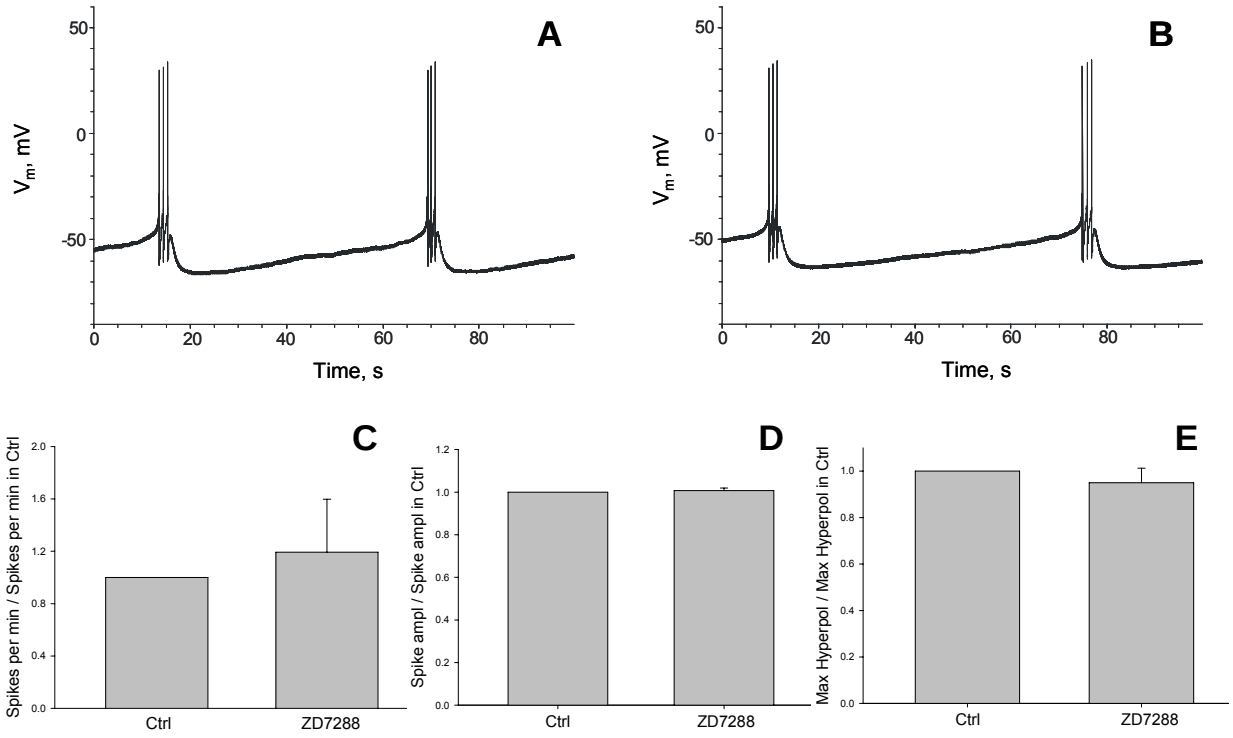

Figure 5-1. Determination of the specificity of ZD7288 for acHCN. ZD7288 (150 μM) did not influence any aspect of spiking of R15 neuron that does not express acHCN. A) R15 recording in control. B) R15 recording following perfusion with AS containing ZD7288. C) Number of spikes per minute did not significantly change following perfusion with 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, $p = 0.51$, $n = 3$).

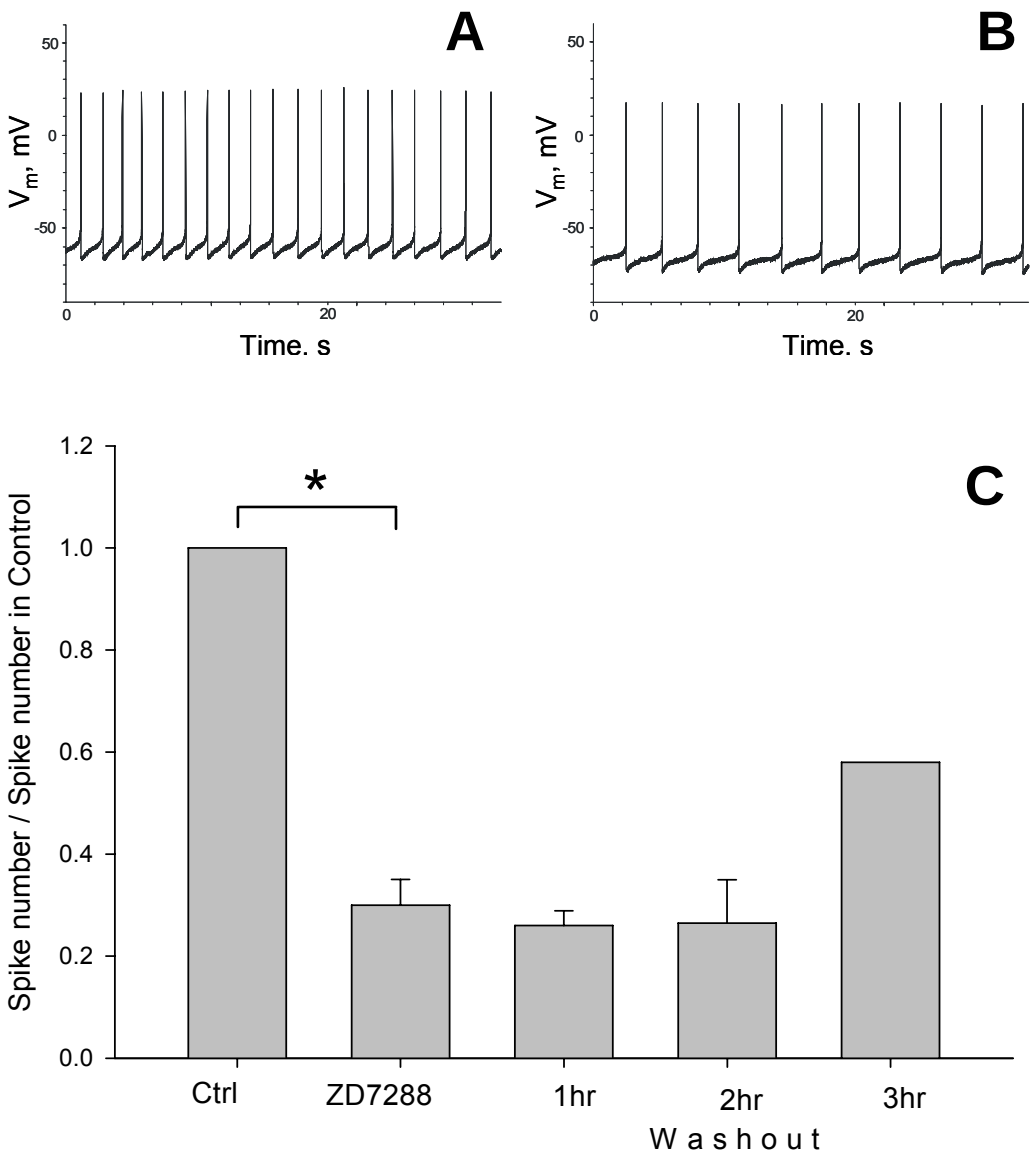

Figure 5-2. Effect of ZD7288 on spiking of the metacerebral cells. ZD7288 (150 μM) significantly decreased spiking frequency of MCCs (70 ± 5.03 % decrease following perfusion with AS containing ZD7288, compared to control, $p = 0.005$, $n = 3$). Following washout, the spiking frequency recovered very slowly, with a recovery of 26.50 ± 8.50 % of control value following 2 hr washout ($n = 3$) and 58 % of control value following 3 hr washout ($n = 1$). A) MCC recording in control. B) MCC recording following perfusion with AS solution containing ZD7288. C) Graphical representation of the effect of ZD7288 on spiking frequency of MCCs.

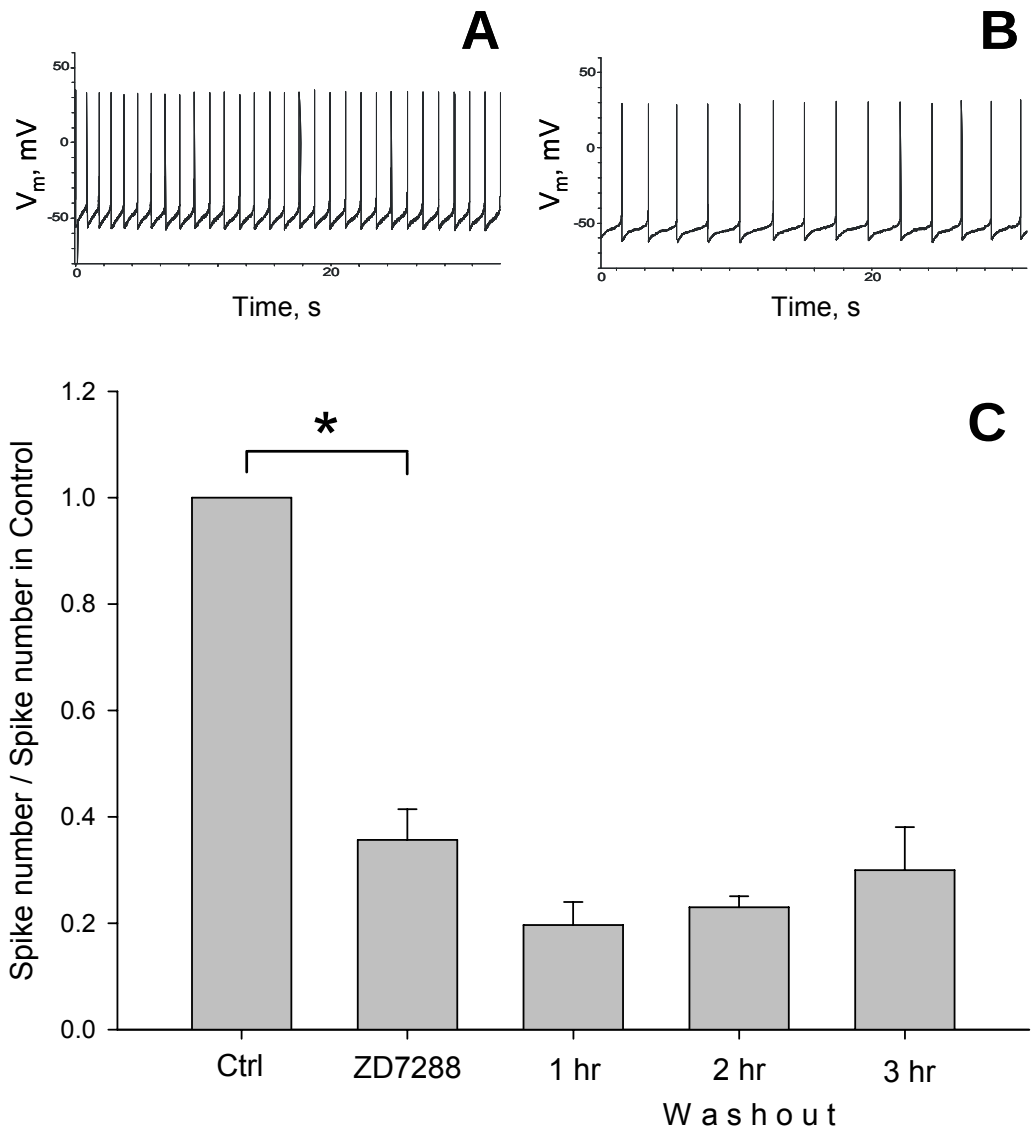

Figure 5-3. Effect of ZD7288 on spiking of the synaptically-isolated metacerebral cells. ZD7288 (150 μM) significantly decreased spiking frequency of the synaptically-isolated MCCs (64.33 ± 5.78 % decrease following perfusion with Hi-Di solution containing ZD7288, compared to control, $p = 0.008$, $n = 3$). Following washout, the spiking frequency recovered slowly, to 30 ± 8.08 % of control value following 3 hr washout ($n = 3$).. A) MCC recording in control. B) MCC recording following perfusion with Hi-Di solution containing ZD7288. C) Graphical representation of the effect of ZD7288 on spiking frequency of the synaptically-isolated MCCs.

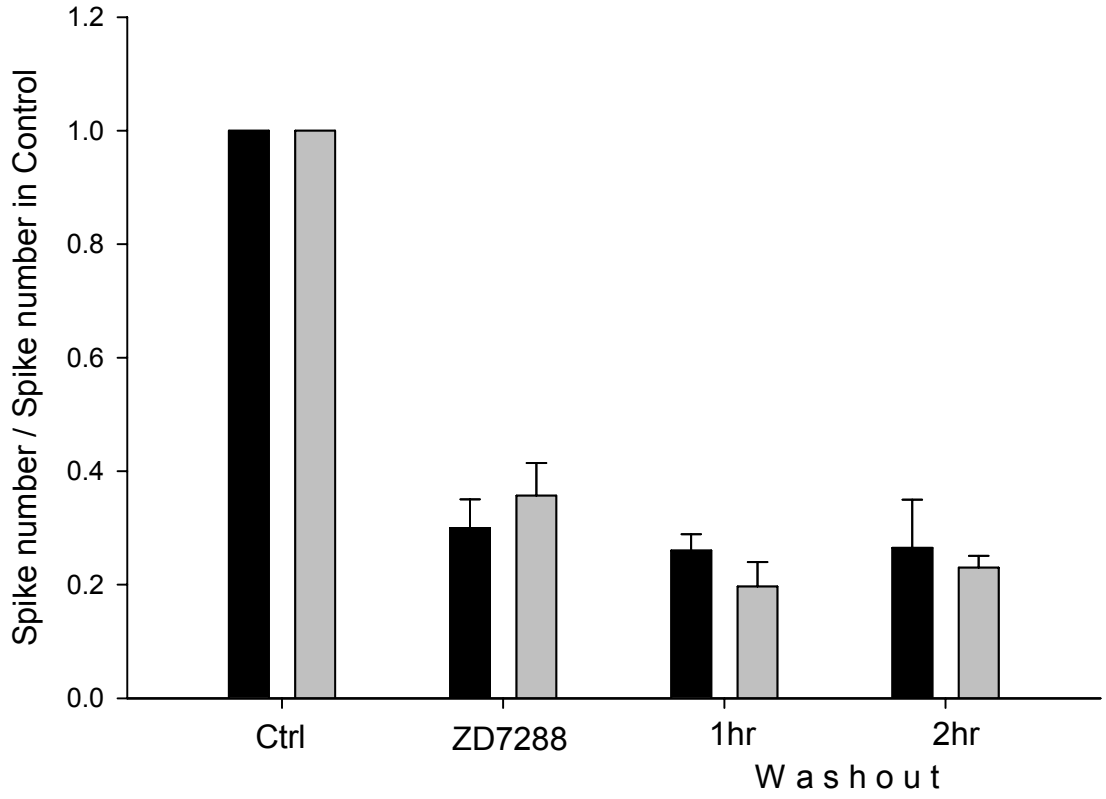

Figure 5-4. Comparison of the effect of ZD7288 on spiking of synaptically-coupled and synaptically-isolated MCCs. The difference in decrease by ZD7288 (150 μM) of spiking frequency of synaptically-coupled and synaptically-isolated MCCs was not significant ($p = 0.63$, $n = 3$).

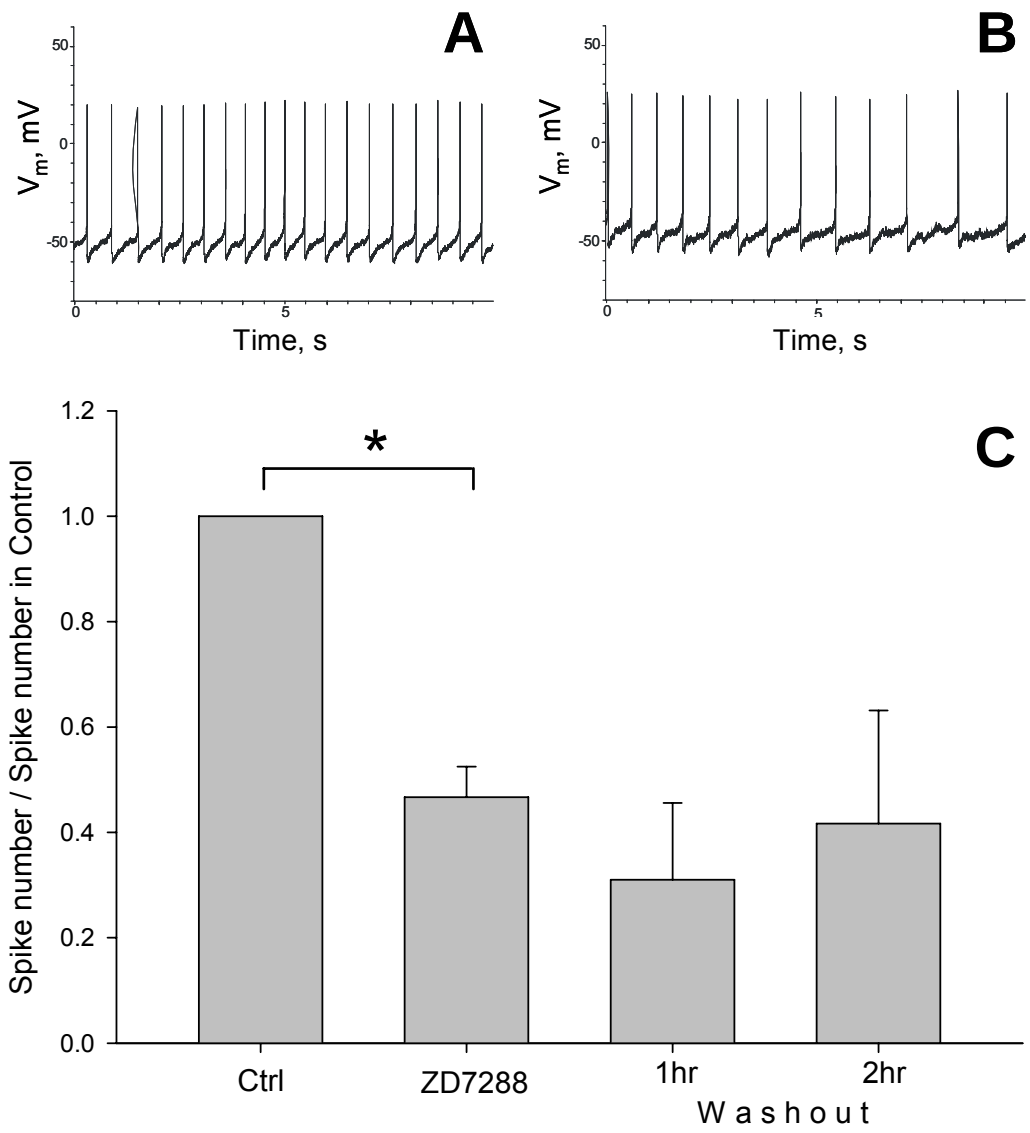

Figure 5-5. Effect of ZD7288 on spiking of B3 neuron. ZD7288 (150 μM) significantly decreased spiking frequency of B3 neuron (53.33 ± 5.81% decrease following perfusion with AS containing ZD7288, compared to control, $p = 0.01$, $n = 3$). Following washout, the spiking frequency of the B3 neuron recovered slowly, to 41.67 ± 21.48 % of control value following 2 hr washout ($n = 3$). A) B3 recording in control. B) B3 recording following perfusion with AS containing ZD7288. C) Graphical representation of the effect of ZD7288 on spiking frequency of B3 neuron.

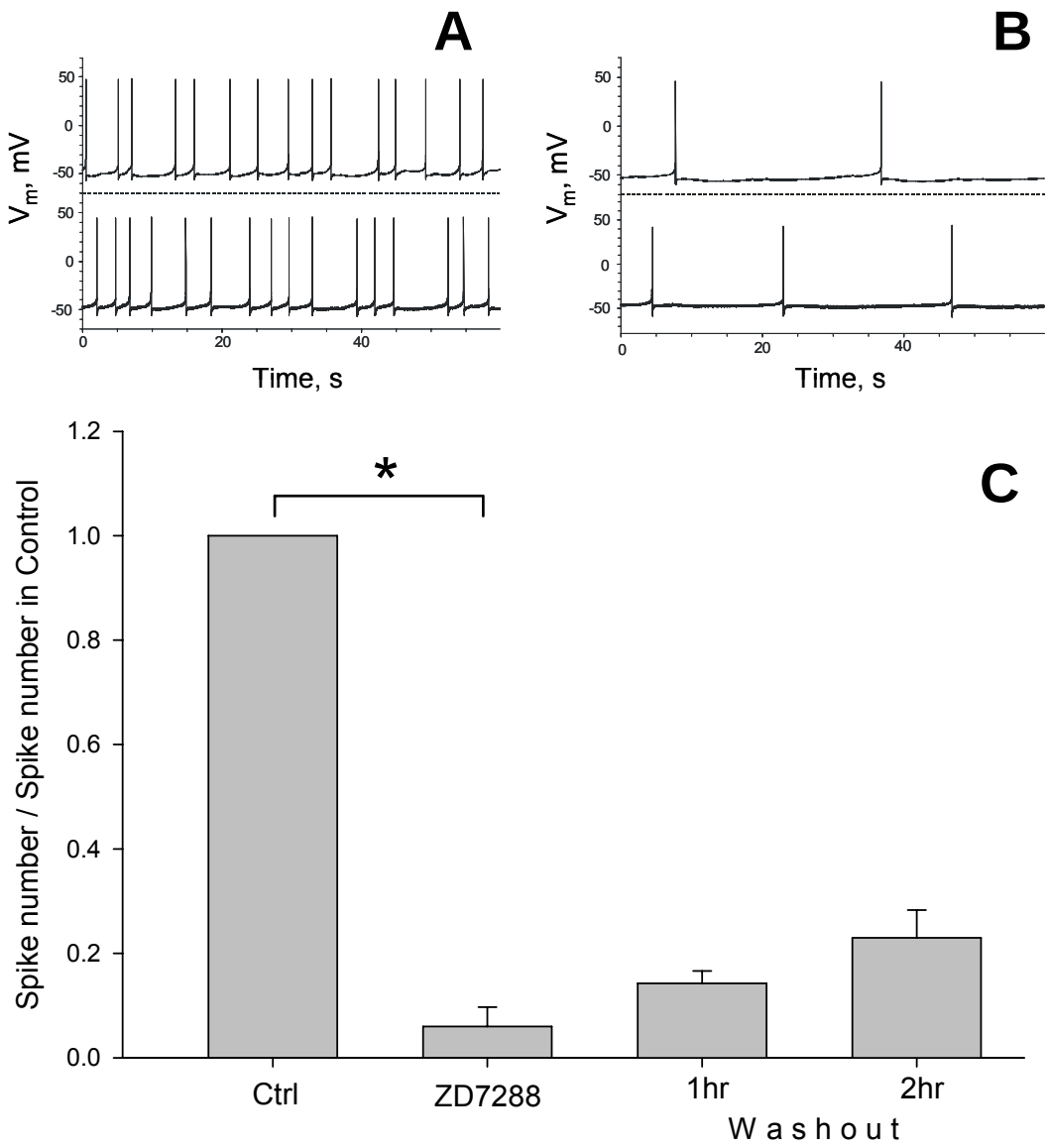

Figure 5-6. Effect of ZD7288 on spiking of the pedal locomotory neuron P4. ZD7288 (150 μM) significantly decreased spiking frequency of P4 neuron (94 ± 3.67 % decrease following perfusion with AS containing ZD7288, compared to control, p = 0.0001, n = 4). Following washout, the spiking frequency of the P4 neuron recovered slowly, to 23 ± 5.31 % of control value following 2 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.

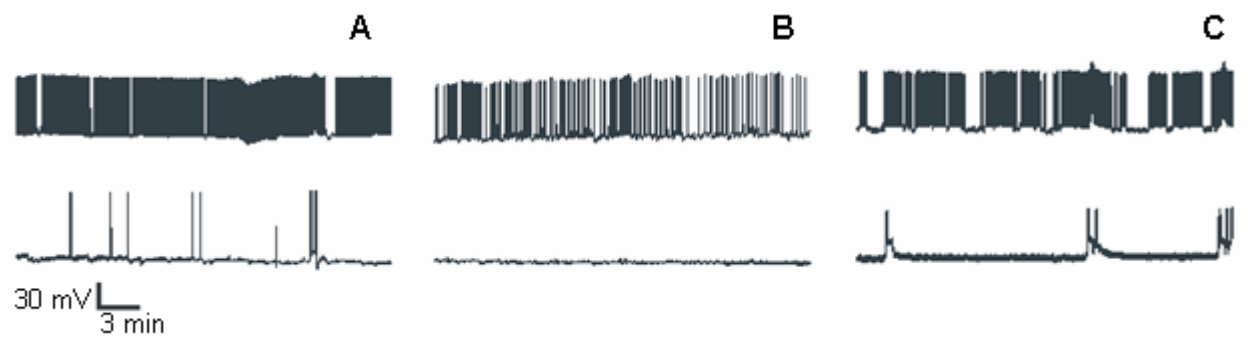

Figure 5-7. Effect of ZD7288 on the rhythmic activity of two unidentified buccal neurons expressing acHCN transcript. ZD7288 (150 μM) inhibits rhythmic activity of the unidentified buccal neurons. A) Control. B) Perfusion with ZD7288. C) 3 hr washout.

CHAPTER 6
SUMMARY AND FUTURE WORK

The main objective of my PhD work was to determine the role of acHCN in neuronal excitability. As the first step for achieving this goal, I have cloned a coding region of the channel from the CNS of *A. californica*. The evidence presented in Chapter 2 suggests that *Aplysia* has only one HCN channel and that the whole coding region was obtained. I did not reach poly-A tail at the end of the transcript because existence of the long 3'-UTR, which is almost identical to the coding region of acHCN, made cloning of the 3'-UTR very difficult. Completion of the *A. californica* genome will help to determine the end of the acHCN transcript and to fill the gaps in the current genomic representation of the transcript. The latter will allow determining the number of exons in the acHCN gene and comparing it with HCN genes from other organisms.

The available sequence of acHCN, however, is sufficient to compare this channel with the HCN channels from other organisms. This analysis, described in section 2.3.1, shows that the 5'-region of the cloned acHCN channel is different from other HCN channels. Due to a single-nucleotide mutation, acHCN has an ATT sequence at the position where HCN channels of other organisms have their first ATG (start codon). The first ATG codon of acHCN is 28 aa downstream, thus the channel is possibly truncated at its N-terminus. When in evolution the ATG → ATT mutation occurred and whether HCN channels of other invertebrate phyla are also mutated in this position can be determined when genomic sequencing and annotation for species from other invertebrate phyla will be completed.

The existence of two potential phosphorylation sites and a weak alignment between acHCN and other HCN channels just upstream of the first ATG sequence of acHCN (see Figure 2-2) suggest that translation of acHCN may start from the alternative start codon, CTG, upstream of the first ATG codon (see section 2.3.1). To determine the translation start of acHCN, Western blot using antibodies to the acHCN protein has to be performed. This Western blot could also

demonstrate whether a shortened form of acHCN (see section 2.3.3) without the CNBD is synthesized. Having antibodies to the acHCN channel will also allow localizing the protein in the CNS of *A. californica* and determining how the expression of the protein changes under different conditions, e.g., following nerve injury. Therefore, we are planning to obtain these antibodies and perform the above listed experiments.

Because of the possible N-terminal truncation of acHCN, the question arose of whether the cloned channel is functional. To answer this question, the acHCN channel was expressed in *X. laevis* oocytes. The properties of the channel, namely, its activation by both hyperpolarization and cyclic nucleotides, permeability to potassium and sodium ions and inhibition by Cs^+ and ZD7288, confirm that a functional form of acHCN was cloned. Finding that acHCN is inhibited by ZD7288 allowed me to remove the acHCN-mediated current and determine how the channel influences neuronal spiking. Model cells for this study, cerebral interneurons MCC, buccal motoneurons B3 and pedal locomotory neurons P4 were chosen following localization of the acHCN transcript in the CNS of the animal.

Electrophysiological recording of acHCN in the *A. californica* neurons demonstrated that the channel controls the frequency of neuronal spiking in the target neurons and thus may control the associated behaviors, i.e., feeding and locomotion. However, it remains to be determined, under what conditions acHCN is activated or inhibited. One of the possible conditions is nerve injury. We will determine how acHCN-mediated current changes following nerve crush in the neurons of feeding and locomotory networks.

Properties of HCN channels from different animal phyla are summarized in Table 6-1. As most other HCN channels, acHCN is activated by both hyperpolarization and cyclic nucleotides and passes both K^+ and Na^+. However, unlike the HCN channels of vertebrates, arthropods and echinoderms, the voltage of acHCN activation is not shifted in depolarized direction by cyclic

nucleotides. Another unique feature of acHCN is that cGMP is more effective than cAMP in activating the channel. In addition, unlike vertebrates and arthropods, acHCN is not expressed in sensory neurons.

Table 6-1. Comparison of acHCN and other HCN channels

Property	Vertebrates	Arthropods*	Echinoderms**	*Aplysia*
Activation by hyperpolarization	+	+	-	+
Permeability to K^+ and Na^+	+	+	+	+
Activation by cNMPs	+	+	+	+
Shift of $V_{1/2}$ by cNMPs	+ (except HCN3)	+	+	-
More effective cNMP	cAMP	cAMP	cAMP	cGMP
Localization in CNS	SN, MN	SN, MN	?	MN

* *D. melanogaster*, *A. mellifera*, *P. argus* and *P. interruptus*. ** *S. purpuratus*

APPENDIX A
SEQUENCES OF THE HCN CHANNELS USED TO CONSTRUCT PHYLOGENETIC TREE AND THEIR ALIGNMENT

>acHCN
LGPTSGAGIVAGHGNPSITITLDSDSDSVYSDYLSPEINYKAHDPKVQFLGDDTSLYGTPK
EELPMGQECVAGEAGGAASKASSTTSYLKDQILNFFQPSDNKLAMKLFGNKNALIKEK
MRHKRVGNWVIHPCSNFRFYWDLFMLVLLIANLIILPVAISFFNDDLSTHWIVFNCISDT
VFFLDIVINFRTGIILNDFADEIILDPKLIAKQYMKTWFFLDLLSSVPMDYIFLMWDAEAD
FNQLFHAGRALRMLRLAKLLSLLRLLRLSRLVRYVQQWEEFLAIAGKFMRIFNLICLMF
LLGHWNGCLQFLVPLIQDFPKDCWVSIEGLQEAHWAEQYTWALFKALSHMLCIGYGRF
PPQNMSDTWLTILSMLSGATCYALFLAHTTTLIQSFDTSRRLYNEKFKQVEEYMVYRKL
PRSLRQRITDYYEHRYQGKMFDEETILSELNECLKHEVVNHNCRSLVASVPFFTNADPAF
VSEVVSKLKFEVYQPGDYIIREGTMGTKMFFIQEGIVDIITSDGEVATSLSDGSYFGEICLL
TNARRVASVRAETYACLYSLAVEHFTAVLERYPVMRRTMESVAAERLTKIGKNPSIVSS
RADLEEDQKMVNEIVMESTPIPTSASEDEDRDSDESSDGSKQKKKTAFKFDFSTKLHKIS
EEKKNKSPKEHSKERDLLEFGETKHHRLSQGPDP
>hsHCN1
MEGGGKPNSSSNSRDDGNSVFPAKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSV
CFKVDGGGGGGGGGGGGEEPAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMF
GSQKAVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTP
WIIFNVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWSVVDFISSIPVDYIF
LIVEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVR
IFNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMS
HMLCIGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLDSSRRQYQEKYK
QVEQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLRGEIVNFNCRKLVA
TMPLFANADPNFVTAMLSKLRFEVFQPGDYIVREGAVGKKMYFIQHGVAGVITKSSKE
MKLTDGSYFGEICLLTKGRRTASVRADTYCRLYSLSVDNFNEVPEEYPMMRRAFETVAI
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PLAARTFHYASPTASQLSLMQQQPQQQVQQSQPPQTQPQQPSPQPQTPGSSTPKNEVHK
STQALHNTNLTREVRPLSASQPSLPHEVPTLISRPHPTVGESLASIPQPVTAVPGTGLQAG
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PPEAADEGGPRGRLRSRDSSCGRPGTPGAASTAKGSPNGECGRGEPQCSPAGPEGPARG
PKVSFSCRGAASGPAPGPGPAEEAGSEEAGPAGEPRGSQASFMQRQFGALLQPGVNKFS
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EMKLSDGSYFGEICLLTRGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVA
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ASPPGAPASPRAPRTSPYGGLPAAPLAGPALPARRLSRASRPLSASQPSLPHGAPGPAAST
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SLFKSTSHMLCIGYSEVIPIGLIDLWMTMLSQIVGAILFAVFIGNAINLMEEMDASKNAYK
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DCWVSLNKMVNDSWSELYSFALFKAMSHMLCIGYGRQAPESMSDIWLTMLSMIVGAT
CYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYMSFHKLPADFRQKIHDYYEHRYQGK
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REGTIGKKMYFIQHGVVSVLTKGNIGMKLSDGSYFGEICLLTRGRRTASVRADTYCRLY
SLSVDNFNEVLEEYPMMRRAFETVAIDRLDRIGKKNSILMHKVQHDLNSGVFNNRENE
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MSFCPQMGNPLMGSGSVQSPRMVRRFQVVQTQTPPGSQYSTLARASALASTPVHSPLA
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QDFPADCWVAKNKMVNDTWGQQYSYALFKAMSHMLCIGYGMYPPVGMTDVWLTILS
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PFFQTFPPDCWVVINGLENAGKLEQYSWSFFKAICHMISIGFGRFPPMNVTEMWMTTFSI
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>*Nematostella vectensis* HCNa

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LASSLPFDYAYFIASSTTEEQTLLRASRALRILKLAKLLSLLRLLRVSRLVRYMTRFEELL
NIAKGQLRIMKLICCMLVLSHWNGCIQFFVPYLQEFPDDSWVSTSNLKTASPGEQYSWS
LFRALSHMLCIGYGHYPPQNLTDLWLTVCSMTAGATFYAVFIGIMSSLIMSIDSSGRLFN
EKLNQVEEYMRYRKLPLRIRLQVQDFYEHRFHHKLFDEDAILTELSKNLRETILVHNIKP
LLTTVPFFSGASISFITDIVTKLKFEVFLNGDYICRSGHRGDKMYFIQKGIVDILTREGALA
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KERLSRLGLKPKL

>*Nematostella vectensis* HCNb

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IIAKRYLKTWFFIDLISSLPIDYIVEAATQSSTKSLIGATRTLKLLRFAKLLGLLKLLRLSRL
VRYVQQYEEILNVTRSVIRFINLVSIILLIAHWNGCLQFLVPYLQDFPETSWVSIHNLMDN
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QQLDSPGRNYREKIQQIEEYMSYRRLPVELRDRMTKYYDHRYQGRMFDEEKILHEISKP
LREQIINYNCRDLVQSVPFFTEAEPDFVSAIITRLSFEVYLEGDIIVREGELGTEMYFLREG
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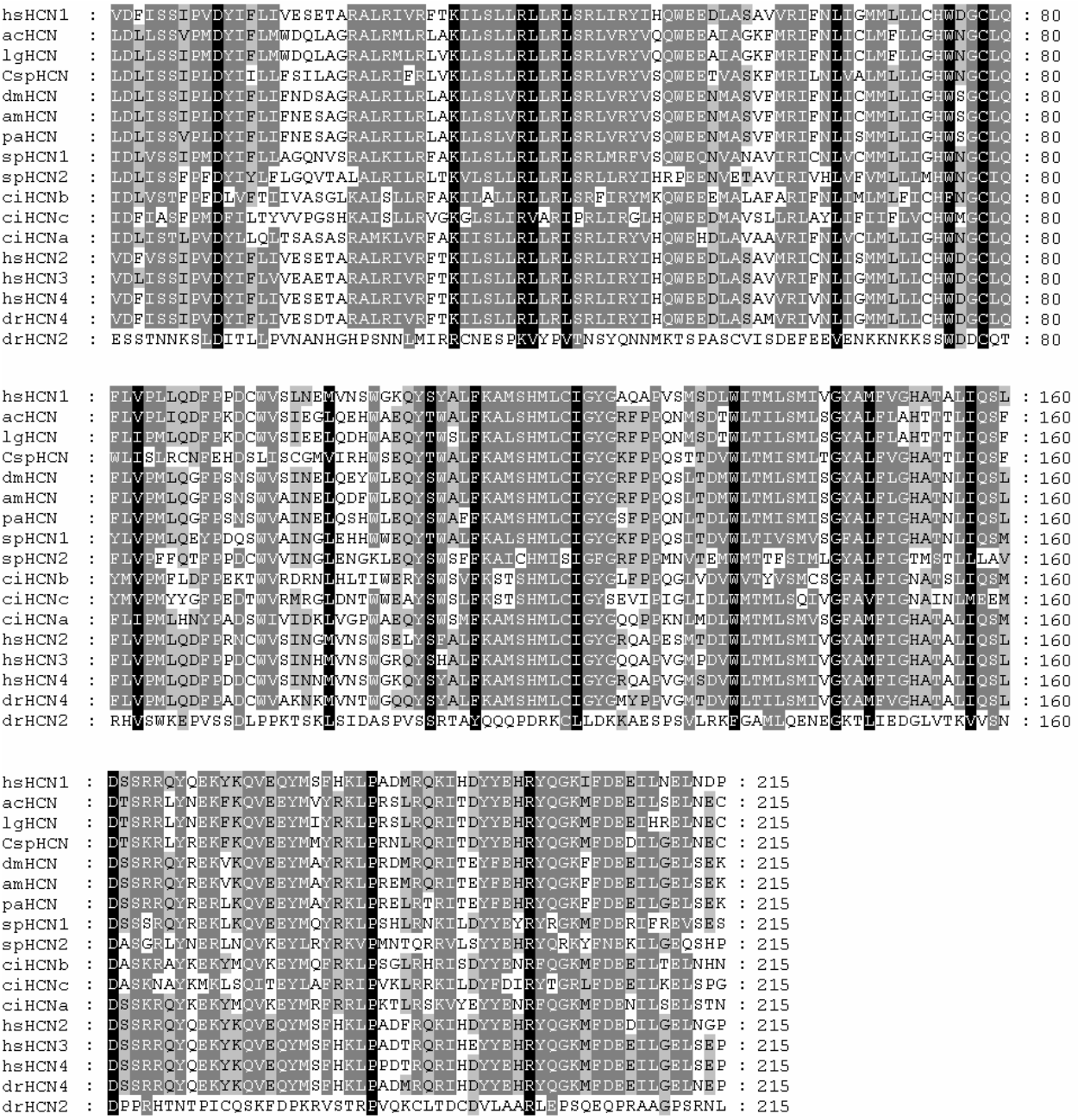

Figure A-1. Alignment of HCN proteins used for construction of the phylogenetic tree. This alignment was obtained using ClustalX followed by the removal of all gaps in GeneDoc. Abbreviations: hs – *Homo sapiens* (human), ac – *Aplysia californica*, dr - *Danio rerio* (zebrafish), sp – *Strongylocentrotus purpuratus* (sea urchin), dm - *Drosophila melanogaster*, pa – *Panulirus argus* (lobster), am – *Apis mellifera* (honey bee), lg – *Lottia gigantea* (limpet), Csp – *Capitella sp*. (polychaete worm), ci – *Ciona intestinalis* (sea squirt).

APPENDIX B
SEQUENCES OF THE CNBD-CONTAINING POTASSIUM AND CATION CHANNELS USED TO CONSTRUCT PHYLOGENETIC TREE AND THEIR ALIGNMENT

>hsHCN1
MEGGGKPNSSSNSRDDGNSVFPAKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSV
CFKVDGGGGGGGGGGGGEEPAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMF
GSQKAVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTP
WIIFNVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWSVVDFISSIPVDYIF
LIVEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVR
IFNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMS
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QVEQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLRGEIVNFNCRKLVA
TMPLFANADPNFVTAMLSKLRFEVFQPGDYIVREGAVGKKMYFIQHGVAGVITKSSKE
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DRLDRIGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAPINYPQMTTLN
SASSTTTPTSRMRTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQS
PLAARTFHYASPTASQLSLMQQQPQQQVQQSQPPQTQPQQPSPQPQTPGSSTPKNEVHK
STQALHNTNLTREVRPLSASQPSLPHEVPTLISRPHPTVGESLASIPQPVTAVPGTGLQAG
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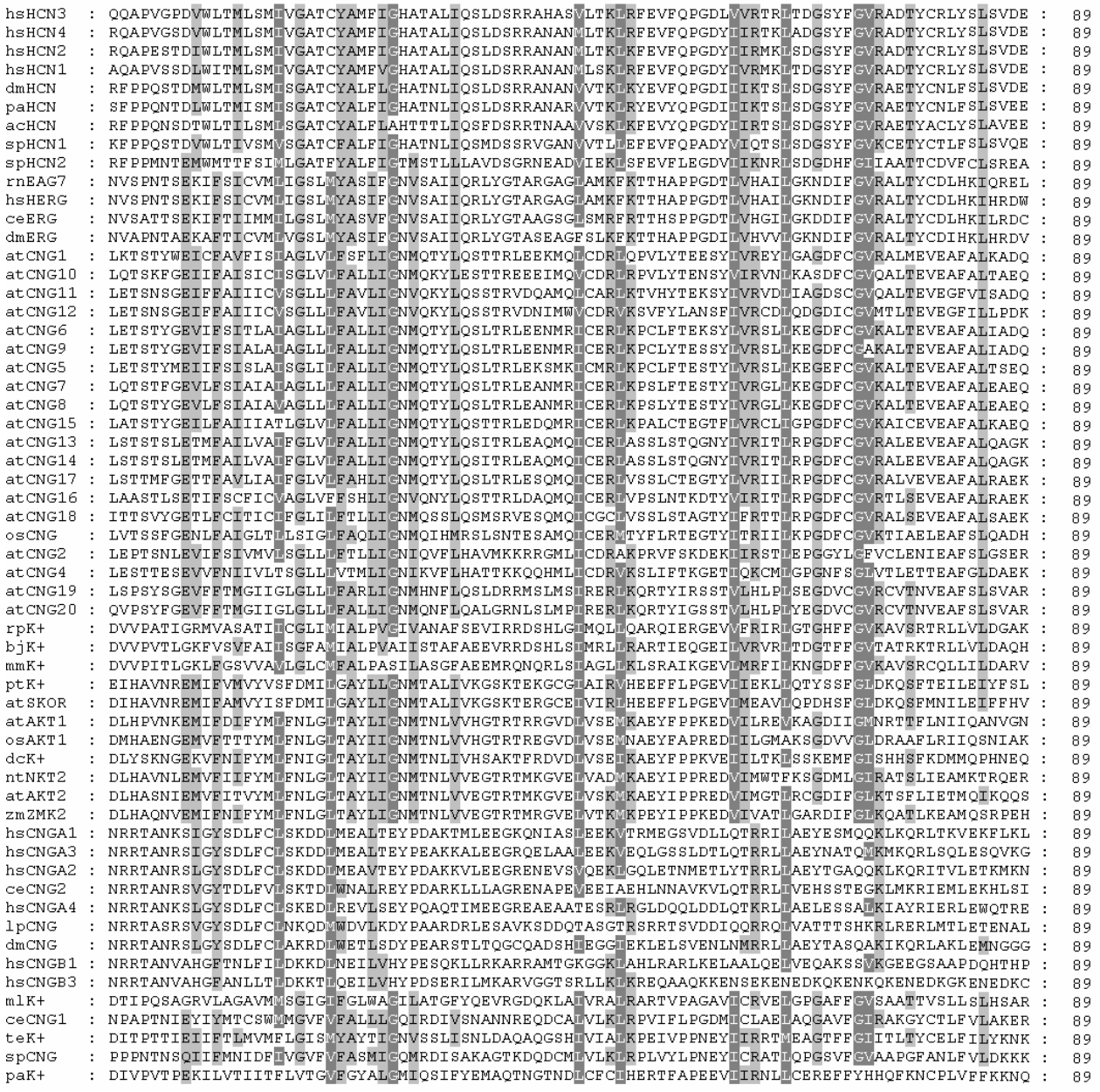

Figure B-1. Alignment of CNBD-containing potassium and cation channels used for construction of the phylogenetic tree. This alignment was obtained using ClustalX followed by the removal of all gaps in GeneDoc. Abbreviations: hs – *Homo sapiens* (human), dm - *Drosophila melanogaster*, pa – *Panulirus argus* (lobster), ac – *Aplysia californica*, sp – *Strongylocentrotus purpuratus* (sea urchin), rn – *Rattus norvegicus* (rat), ce - *Caenorhabditis elegans*, at - *Arabidopsis thaliana*, os – *Oryza sativa* (rice), rp - *Rhodopseudomonas palustris*, bj - *Bradyrhizobium japonicum*, mm - *Magnetospirillum magnetotacticum*, pt - *Populus trichocarpa* (aspen), dc – *Daucus carota* (carrot), nt – *Nicotiana tabacum* (tobacco), zm – *Zea mays* (corn), lp - *Limulus polyphemus* (horseshoe crab), ml - *Mesorhizobium loti*, te - *Trichodesmium erythraeum*, pa - *Paramecium aurelia*.

APPENDIX C
RECORDING OF THE PHYSALIA VOLTAGE-GATED POTASSIUM CHANNEL EXPRESSED IN OOCYTES

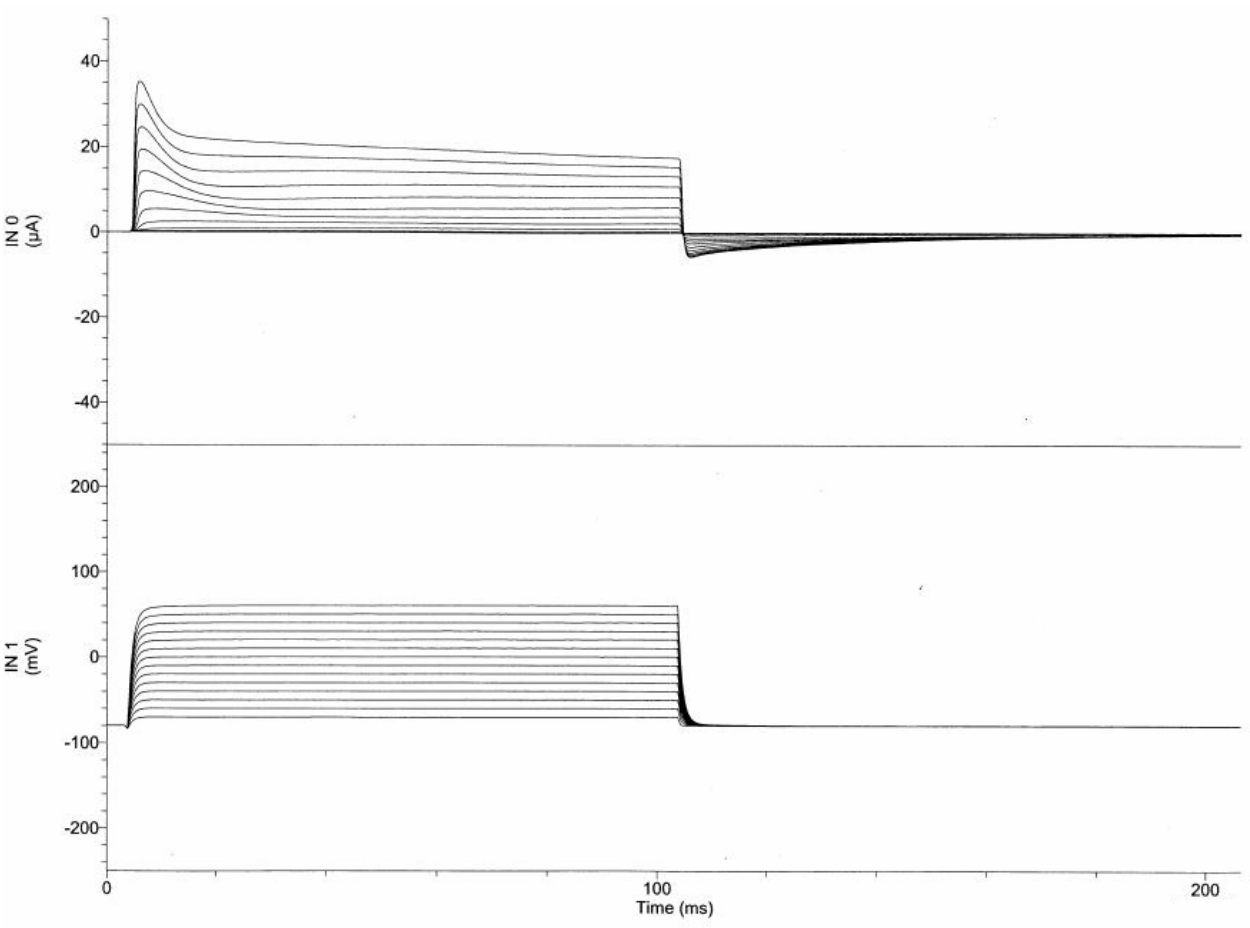

Figure C-1. Recording of the *Physalia* voltage-gated potassium channel expressed in oocytes. In response to depolarization from the holding potential of -80 mV to +50 mV (in +10 mV steps) an instantaneous outward current which decreased to a smaller steady-state current was observed. This is the characteristic current expected of the *Physalia* voltage-gated potassium channel.

APPENDIX D
CLONING AND LOCALIZATION OF A CYCLIC NUCLEOTIDE-GATED CHANNEL IN *APLYSIA CALIFORNICA*

CNG Cloning and Probe Synthesis

Analysis of the *A. californica* transcriptome and preliminary genomic data showed the existence of six fragments corresponding to α–subunits of a CNG channel. Comparison with the CNG channels of other organisms showed that these fragments would overlap if they belonged to the same transcript, but assembly of these fragments in SeqMan (DNASTAR) did not produce any alignment. This suggests that potentially up to six α–subunits and one or two β-subunits of the CNG channel exist in *A. californica*. However, most of these fragments have a low E-value (e-6 or less).

Primers (5'-CTACAGACCTCTTTTACTTCCTCAAC-3' and

5'-CTTGATCACCCTTTCCTCGAGCTC-3') were designed to the only CNG fragment with a good E-value (e-66). A 545 nucleotide fragment was obtained by PCR from the whole CNS library. This fragment was then ligated into pCR4-TOPO vector (Invitrogen), and transformed into One-Shot competent *E. coli* cells (Invitrogen). The clones were isolated, purified using a Qiagen Kit and sequenced by the Whitney Laboratory molecular core facility. A probe for *in situ* hybridization corresponding to the whole obtained fragment was then synthesized.

Localization of the CNG RNA in the CNS of *A. californica*

Similar to acHCN, the cloned CNG channel transcript expressed most abundantly in presumable motoneurons, specifically in 11-14 neurons (90-120 μm in diameter) in the A cluster of the cerebral ganglion (Figure D-1, n=3). Also, six-nine neurons (15-45 μm in diameter) stained between cerebropleural and pleuroabdominal connectives of left and right pleural ganglia. The transcript did not express in sensory cells or serotonergic neurons.

There was only very weak staining in pedal ganglia, a model to study nerve injury in *A. californica*. Because the CNG transcript expressed consistently only in the cerebral ganglion, I did not determine how expression of this transcript changes following nerve injury, because the cerebral ganglion in *A. californica* is unpaired, thus there is no control for this study.

Discussion

Similarly to HCN, CNG channel is also activated by cyclic nucleotides. In vertebrates, CNG channels are most strongly expressed in sensory cells, such as rod and cone photoreceptors (Yau and Baylor, 1989), extraretinal photoreceptors of pineal gland (Dryer and Henderson, 1991) and parietal eye (Finn et al., 1997) and olfactory neurons (Frings et al., 1995) where they participate in signal transduction. In invertebrates, CNG channels were found exclusively in sensory organs and associated neuronal structures: eyes and antennae of *D. melanogaster* (Baumann et al., 1994) and several of its neuronal regions responsible for processing of visual and olfactory information, including the medulla, lobulla and lobulla plate, the antennal lobe glomeruli, and mushroom bodies (Miyazu et al., 2000); olfactory, gustatory, and thermosensory neurons of *C. elegans* (Coburn and Bargmann, 1996); hyperpolarizing and depolarizing photoreceptors of some molluskan species (McReynolds and Gorman, 1974, Johnson et al., 1986); and (4)ventral eye nerves of *Limulus polyphemus* (Chen et al., 1999). Thus, it came as a surprise to find expression of the cloned CNG channel in the putative motoneurons of the A cluster in the cerebral ganglion and not in the sensory cells. These motoneurons were shown to control pedal and parapodial movements in *A. californica* (Jahan-Parwar and Fredman, 1978). Therefore, in mollusks, CNG channels might have evolved to perform different functions than in chordates or arthropods, e.g., they may play a role in controlling locomotion.

However, the possibility remains that all or some of the other potential *A. californica* CNG channels do express in sensory neurons. This possibility can be tested after more genomic and

transcriptomic data on the rest of the potential CNG channels in *A. californica* becomes available.

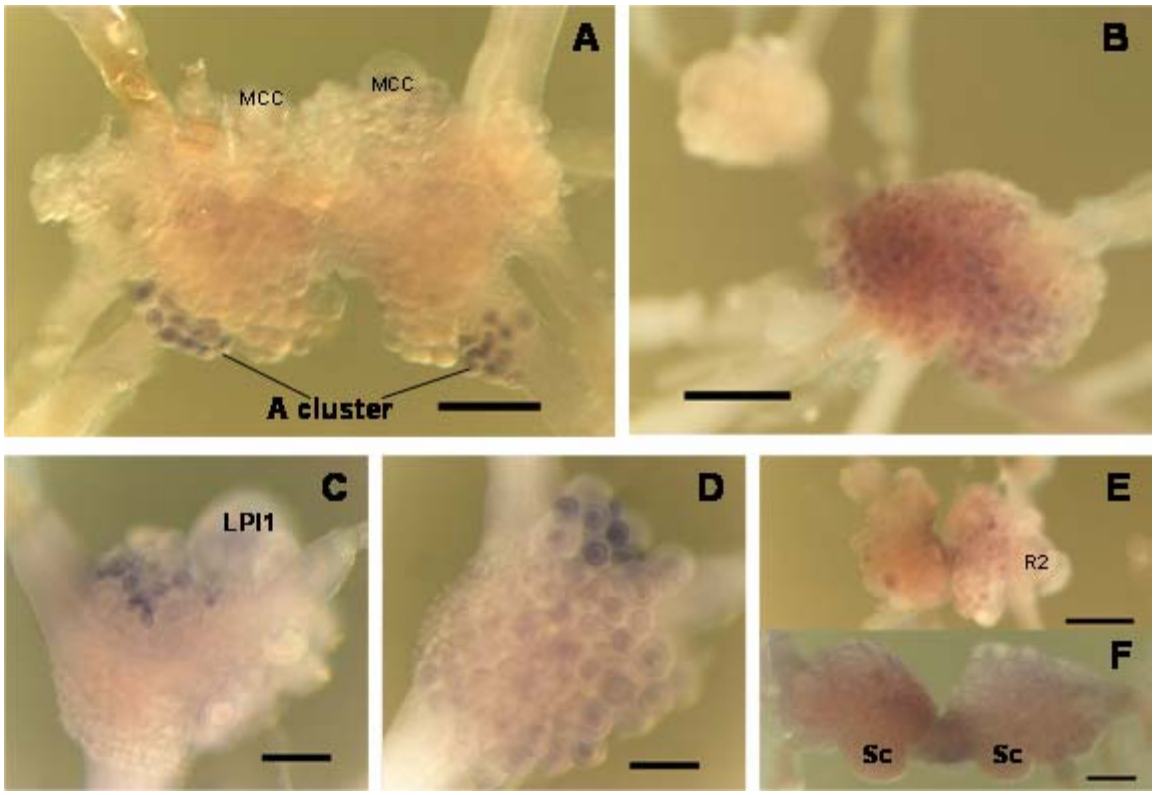

Figure D-1. Expression of an *A. californica* CNG channel transcript as determined by *in situ* hybridization. The strongest staining is in the A-cluster neurons of the cerebral ganglion, which control pedal and parapodial movements. There was also staining of several cells in left and right pleural ganglia and weak staining in pedal ganglia. A) Cerebral ganglion. MCC-metacerebral cells. B) Left pedal ganglion. C) Left pleural ganglion. D) Right pleural ganglion. E) Abdominal ganglion. F) Buccal ganglia. Sc-sensory clusters. Scale bars in A, B and E= 500 μm; scale bars in C, D and F=200 μm

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BIOGRAPHICAL SKETCH

Pavlo Kuzyk was born in Rostov-on-Don in 1979. He graduated from Experimental School-Laboratory in Ivano-Frankivs'k, Ukraine, in 1996. Biology was his passion since his childhood and already in the middle school Pavlo wrote a manuscript about local birds and successfully defended it earning the second place in the republican Olympiad. His other passion was drawing and in 1995 he graduated with a diploma cum laude from the Art School in Ivano-Frankivs'k, Ukraine. However, he chose biology as his career and following graduation from the High School, he studied in the Lviv National University, Ukraine, majoring in microbiology. Pavlo graduated in 2001 with a diploma cum laude. He then moved to US and for the next year worked in the laboratory of Dr. Ronald Calabrese at the Emory University, Atlanta, studying the neuronal network controlling heartbeat in the leech. In the fall of 2002 he joined Interdisciplinary Program in the Biomedical Sciences at the University of Florida and proceeded to work with Dr. Leonid Moroz in the Whitney Laboratory for Marine Bioscience, Saint Augustine, Florida.