The magnitudes of hyperpolarization-activated ($I_h$) and low-voltage-activated potassium ($I_{KL}$) currents co-vary in neurons of the ventral cochlear nucleus

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Running head: Ventral Cochlear Nucleus of HCN1$^{-/-}$, HCN1$^{+/+}$, and ICR Mice

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Abstract

In the ventral cochlear nucleus (VCN), neurons have hyperpolarization-activated conductances ($g_h$) that in some cells are enormous and that contribute to the ability of neurons to convey acoustic information in the timing of their firing by decreasing the input resistance and speeding up voltage changes. Comparisons of the electrophysiological properties of neurons in the VCN of mutant mice that lack the HCN1 $\alpha$ subunit (Nolan et al. 2003) with wild type controls and with outbred ICR mice reveal that octopus, T stellate, and bushy cells maintain their electrophysiological distinctions in all strains. Hyperpolarization-activated currents ($I_h$) were smaller and slower, input resistances were higher, and membrane time constants were longer in mutant than in wild type controls in octopus, bushy and T stellate cells. There were significant differences in the average magnitudes of $I_h$, input resistances, and time constants between wild type controls and ICR mice but the resting potentials did not differ between strains. $I_h$ is opposed by a low-voltage-activated $K^+$ current ($I_{KL}$) in bushy and octopus cells whose magnitudes varied widely between neuronal types and between strains. The magnitudes of $I_h$ and $I_{KL}$ were correlated across neuronal types and across mouse strains. Furthermore, these currents balanced one another at the resting potential in individual cells. The magnitude of $I_h$ and $I_{KL}$ is linked in bushy and octopus cells and varies not only between mutant and wild type controls but also between “wild type” strains of mice raising the question, to what extent the “wild type” strains reflect normal mice.

Keywords: KCNA, hearing, brain stem, electrophysiology, patch-clamp recordings, homeostasis
**Introduction**

In the auditory system, the timing of firing carries sensory information. In mammals that hear low frequencies, neuronal encoding of the phase of sounds is the basis for the ability to resolve differences in pitch and to use differences in the time of arrival of sound at the two ears to localize sounds in the horizontal plane. Making use of high frequencies, too, requires temporal precision. To detect interaural intensity differences for localizing sounds, monosynaptic excitation from the ipsilateral cochlear nucleus must be matched in timing with disynaptic inhibition from the contralateral cochlear nucleus at the lateral superior olive within fractions of a millisecond (Joris and Yin 1995). In mammals that hear only high frequencies, including mice and bats, neurons cannot encode the phase of individual cycles of sounds but they encode the phase of amplitude modulation (Gans et al. 2009).

The auditory pathway is subdivided in the VCN into multiple pathways that differ in the acoustic information they carry and in the routes they take through the brain stem to the inferior colliculus. Bushy cells convey the fine structure of sounds in the timing of their firing that is used to localize sound sources (Yin 2002). Octopus cells detect the presence of broadband transients and convey information to the superior paraolivary nucleus and to the ventral nucleus of the lateral lemniscus (Schofield 1995; Adams 1997; Smith et al. 2005). Individual bushy and octopus cells respond with action potentials that fall within sharper time windows (200 µsec) than their auditory nerve inputs (Joris et al. 1998; Oertel et al. 2000; Smith et al. 2005). The population of T stellate cells conveys information about the spectrum of sounds in the rate and duration of tonic firing that is independent of the fine structure to many brain stem auditory nuclei and to the inferior colliculus (Blackburn and Sachs 1990; May et al. 1998; Oertel et al. 2010).

Each of the classes of principal cells is endowed with $g_h$, a hyperpolarization-activated, mixed-cation conductance. Sharpening and conveying the timing of firing of auditory nerve inputs by bushy and octopus cells depends on the presence of $g_h$ and an opposing $g_{KL}$, a rapid, low-voltage-activated potassium conductance (Manis and Marx 1991; Golding et al. 1995; Golding et al. 1999; Bal and Oertel 2000; Bal and
Oertel 2001; Ferragamo and Oertel 2002; Rothman and Manis 2003; McGinley and Oertel 2006; Cao et al. 2007). T Stellate cells have gh but little or no gKL (Ferragamo and Oertel 2002; Rodrigues and Oertel 2006). gh and gKL are present also in inputs and targets of principal cells of the VCN. gh is prominent in spiral ganglion cells (Mo and Davis 1997), cells in the superior olivary complex, and in the ventral nucleus of the lateral lemniscus (Banks et al. 1993; Wu 1999; Cuttle et al. 2001; Koch et al. 2004; Notomi and Shigemoto 2004; Leao et al. 2005; Scott et al. 2005; Leao et al. 2006; Hassfurth et al. 2009).

The HCN (hyperpolarization and cyclic nucleotide gated) channels that mediate gh are composed of HCN1-4 α subunits (Ludwig et al. 1998; Robinson and Siegelbaum 2003). All four subunits are expressed in the ventral cochlear nucleus with HCN1, HCN2 and HCN4 being expressed at high levels (Moosmang et al. 1999; Koch et al. 2004; Notomi and Shigemoto 2004). HCN channels are tetrameric, voltage-gated, pore-loop ion channels. Homomeric HCN1 channels have the most rapid kinetics, HCN2 channels are slower, and HCN4 the slowest (Santoro et al. 1998; Moosmang et al. 2001); heteromeric channels have properties intermediate between those of the corresponding homomers (Ulens and Tytgat 2001; Altomare et al. 2003; Whitaker et al. 2007). HCN1 is strongly expressed in the octopus cell area, less strongly expressed in the anterior VCN where most bushy cells are located, and least strongly expressed in the multipolar cell area (Bal and Oertel 2000; Koch et al. 2004; Oertel et al. 2008).

The ion channels that mediate gKL also belong to the family of voltage-gated, pore-loop ion channels; specifically they are KCNA (also known as Kv1 or shaker) channels. These channels are formed from KCNA1-4 and/or KCNA6 α subunits, KCNA1 and KCNA2 being the most abundant (Bal and Oertel 2001; Oertel et al. 2008).

The present study was motivated by a search for mice that hear through biophysically abnormal neurons. The elimination of HCN1 produces mice that are generally healthy but have subtle defects in motor learning (Nolan et al. 2003). We find that these mice do have cochlear nuclear neurons with smaller gh than the wild type but they also differ from the wild type in unexpected ways.
Materials and Methods

Mice: Mice that lack the HCN1 subunit were genetically engineered by Nolan et al. (2003); the mice used for the present experiments were propagated by inbreeding from a breeding pair purchased from the Jackson Laboratory (ME). We routinely test the hearing of mice by looking for a Preyer reflex in response to a click generated by a dog-training clicker. In this crude test of hearing, HCN1-/- mice responded indistinguishably from the wild type controls and ICR mice; mice of all three strains freeze briefly in response to the click.

HCN1-/- mice were constructed from 129S/SvEv-derived MM13 embryonic stem cells that were injected into C57BL/6 mice. The mutant mice are therefore on a hybrid genetic background derived by mixing two different strains for which we could not obtain a perfect control strain. In this study we compare mutant, HCN1-/- mice with two groups of mice. One group comprises F2 hybrids from crosses between 129S and C57Bl/6 parents which we term “wild type controls” or HCN1+/. These wild type control mice were not the same as the wild type mice described by Nolan et al. (2003). We also compare the mutants and wild type controls with outbred ICR mice that we have used in previous biophysical studies (Bal and Oertel 2000; Bal and Oertel 2001; Cao and Oertel 2005; Rodrigues and Oertel 2006; Cao et al. 2007).

Preparation of slices: Coronal brainstem slices from mice between 16 and 19 days after birth and containing the VCN were prepared from ICR mice, from mice that lack the HCN1 α subunit (HCN1-/-), or from hybrid offspring of C57Bl/6 and 129S (HCN1+/-). All procedures were approved by the institutional Animal Care and Use Committee.

Slices were cut in normal physiological saline that contained (in mM): 130 NaCl, 3 KCl, 1.2 KH₂PO₄, 2.4 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, 3 HEPES, 10 glucose, saturated with 95 % O₂/5% CO₂, pH 7.3-7.4, at between 24°C and 27°C. The osmolality, measured with a 3D3 Osmometer (Advanced Instruments Inc, Norwood, MA), was 306 mosm/kg. All chemicals were from Sigma, unless stated otherwise. Slices, 210 μm thick, were cut with a vibrating microtome (Leica VT 1000S). After cutting, slices were transferred to the recording chamber (~0.6 ml) and superfused continually at 5 to 6 ml/min. Slices were mounted on the stage
of a compound microscope (Zeiss Axioskop) and viewed through a 63X water immersion objective. The
temperature was measured in the recording chamber, between the inflow of the chamber and the tissue, with
a Thermalert thermometer (Physitemp) the input of which comes from a small thermistor (IT-23, Physitemp,
diameter: 0.1 mm). The output of the Thermalert thermometer was fed into a custom-made, feedback-
controlled heater that heated the saline in glass tubing (1.5 mm inner, 3 mm outer diameter) just before it
reached the chamber. An adjustable delay in the controller for the heater prevented temperature oscillations.
Recordings were generally made within two hours after slices were cut.

Electrophysiological recordings: Patch-clamp recordings were made with pipettes made from borosilicate
glass that were filled with a solution consisting of (in mM): 108 potassium gluconate, 9 HEPES, 9 EGTA,
4.5 MgCl₂, 14 phosphocreatine (tris salt), 4 ATP (Na salt) and 0.3 GTP (tris salt). The pH was adjusted to
7.4 with KOH; the osmolality was 303 mosm/kg. Resistances ranged between 4 and 6 MΩ. Records were
made with an Axopatch 200A amplifier (Axon Instruments). Records were digitized at 50 kHz and
low-pass filtered at 10 kHz. All reported results were from recordings in which 75~90% of the series
resistance could be compensated on-line with 10 μs lag; no corrections were made for errors in voltage that
resulted from uncompensated series resistance. Series resistances were between 10 and 14 MΩ in each of
the cell types, of which ~90% could be compensated in octopus cells and ~75% - 80% could be
compensated in bushy and T stellate cells. Recordings in which series resistances were >14 MΩ, were
disregarded. The output was digitized through a Digidata 1320A interface (Axon Instruments) and fed into a
computer. Stimulation and recording was controlled by pClamp 8 software (Axon Instruments). The control
solution contained (in mM): 138 NaCl; 4.2 KCl, 2.4 CaCl₂, 1.3 MgCl₂, 10 HEPES, 10 glucose, pH 7.4 and
saturated with 100% O₂. In voltage-clamp experiments to measure Iₘ or IₓL, the voltage-sensitive sodium
current was blocked by 1μM tetrodotoxin (TTX), the voltage-sensitive calcium current was blocked by 0.25
mM CdCl₂, glutamatergic and glycinergic synaptic currents were blocked with 40 μM 6,7-
dinitroquinazoxaline-2,3-dione (DNQX) (Tocris Cookson, UK) and 1 μM strychnine respectively.
Measurements of Iₘ were made in the additional presence of 50 nM α-dendrotoxin (α-DTX) to block IₓL.
(Bal and Oertel 2000). Measurements of $I_{KL}$ were made in the presence of 50 µM ZD7288 to block $I_h$. All reported voltages were compensated for a −12 mV junction potential.

**Data analysis:** Input resistances were measured as the slope of plots of voltage responses to small hyperpolarizing current steps. The slope was measured over the region ~10 mV from the resting potential.

Activation and deactivation time constants of $I_h$ were determined by fitting the current evoked during an activating or deactivating pulse to double or single exponential functions of the form:

$$I_h(t) = I_{ss} + A_f e^{t/\tau_f} + A_s e^{t/\tau_s}$$

where $I_h(t)$ is the current at time $t$, $I_{ss}$ is the steady state current, $A$ is the initial amplitude of exponential component. Statistical analyses were made with Origin software (version 7.5); the results are given as means ± standard deviation, with $n$ being the number of cells in which the measurement was made.

**Results**

*Neurons in HCN1−/− mice have higher input resistances and longer time constants than the wild type strains*

In the VCN the intermingled groups of principal neurons have distinct biophysical properties that allow them to be recognized electrophysiologically. Octopus cells have low input resistances that result from the partial activation of $g_h$ and $g_{KL}$ and fire only at the onset of a depolarization (Golding et al. 1995; Golding et al. 1999; Bal and Oertel 2000; Bal and Oertel 2001). In bushy cells, depolarization also produces transient firing but $g_h$ and $g_{KL}$ are smaller than in octopus cells (Cao et al. 2007). T and D stellate neurons fire tonically when they are depolarized but differ in the shapes of their action potentials and in the kinetics of $g_h$; T stellate cells are common excitatory principal cells whereas D stellate cells are more rare inhibitory neurons that innervate the ipsilateral and contralateral cochlear nuclei and will not be considered in this study (Oertel et al. 1990; Fujino and Oertel 2001; Needham and Paolini 2003; Rodrigues and Oertel 2006).

The three major populations of neurons, octopus, T stellate, and bushy cells, remain recognizable in mice that lack HCN1 (Fig. 1). Although their biophysical properties differ, the groups of neurons retain their characteristic features. In whole-cell, patch-clamp recordings, large currents were required to evoke a
single action potential in octopus cells of all three groups of mice. As in mice with HCN1, smaller
depolarizing current pulses evoked tonic firing in T stellate cells and transient firing in bushy cells of mice
that lack HCN1. Currents evoked larger voltage changes in HCN1−/− than in the same cell type from
HCN1+/+ and ICR mice, reflecting their higher input resistances. Hyperpolarizing current pulses evoked
voltage changes that were initially large and then sagged back toward rest. The sag was slower in HCN1−/−
than in the wild type controls or ICR mice.

Input resistances were assessed from steady state voltage changes produced by the injection of small
current pulses that elicited hyperpolarizations <10 mV (Fig. 2A) (Table 1). The input resistances of octopus
cells were significantly higher in mutant HCN1−/− mice (19 MΩ) than in HCN1+/+ mice (14 MΩ) (p<0.05).
There was, surprisingly, a large difference in input resistance between octopus cells in wild type controls
(14 MΩ) and in ICR mice (6 MΩ). In T stellate cells the input resistances around rest were also
significantly higher in mutant (160 MΩ) than in wild type controls (126 MΩ) (p<0.05) and there was a
difference in input resistance between wild type control (126 MΩ) and ICR mice (74 MΩ). In bushy cells
the input resistance was about 70 MΩ in the wild type controls compared with about 100 MΩ in mutant
mice (p<0.05); input resistances were similar in wild type control and ICR mice.

Time constants determine how voltage changes are shaped from synaptic inputs and therefore limit the
temporal precision with which these neurons can signal. The rate at which the voltage falls at the offset of a
depolarizing current pulse is biologically most interesting because that is the physiological voltage range.
The voltage changes could be fit roughly with a single exponential function. As expected from their
elevated input resistances, time constants were longer in mutant than in wild type control neurons (Fig. 2B)
(Table 1). Time constants in octopus cells from mutant mice (0.5 msec) were longer than those in wild type
strains (0.2-0.3 msec). They were also significantly longer in bushy cells from mutant mice (2.6 msec) than
in wild type controls (1.2 msec). For both octopus and bushy cells there were no significant differences in
time constants of wild type controls and ICR mice. Time constants were similar in T stellate cells of all
three strains. Presumably the time constants do not exactly parallel input resistances because the
characteristics of neurons over this voltage range were not passive but rather were affected by gₖ and gₖL.
Resting potentials of neurons are not different in the three strains of mice

In the face of a presumed loss of inward $I_h$ at rest, one might expect the resting potentials to be hyperpolarized but that was not the case. Resting potentials in all three cell types were not statistically different among the three strains (Fig. 2C) (Table 1).

Measurements of $I_h$

Recordings in voltage-clamp show how the loss of HCN1 affects the magnitude and kinetics of $I_h$. Examples of recordings from individual cells to identical voltage steps are shown in Figure 3. To avoid contamination of $I_h$ with other currents, recordings were made in the presence of 40 µM DNQX, 1 µM strychnine, 1 µM TTX, 0.25 mM Cd²⁺ and 50 nM α-DTX. In all neurons, the instantaneous current step was followed by the growth of $I_h$; the rates of activation were slower in the mutant than in wild type and ICR neurons. $I_h$ was largest in octopus cells, intermediate in bushy cells, and smallest in T stellate cells; in mutant mice $I_h$ was smaller than in the wild type and ICR mice in all three groups of cells.

The rates of activation and deactivation of $I_h$ were slower in neurons from the mutants relative to wild type controls and ICR (Fig. 3). This finding is consistent with the observation that homomeric HCN1 channels have the fastest kinetics (Moosmang et al. 2001); in HCN1⁻/⁻ mutants, many of the channels that mediate $I_h$ are formed from subunits that form slower channels. The rate constants were measured and compared for the activation of $I_h$ when the voltage was stepped from –57 mV to –107 mV and for deactivation when the voltage was stepped back from –107 mV to –57 mV (Fig. 4) (Table 1). Activation of $I_h$ was well fit with the sum of two exponential functions with a fast and slow time constant, $\tau_f$ and $\tau_s$ (Fig. 4). In all three groups of neurons both $\tau_f$ and $\tau_s$ were longer and $\tau_s$ was more prominent in the mutants than in the wild type controls (Table 1). Deactivation was assessed with single exponential functions in responses to voltage steps from –107 mV to –57 mV (Fig. 4, right). The time constants of deactivation were between 25% and 30% longer in HCN1⁻/⁻ mutants than in the wild type controls (Table 1). In octopus and bushy cells, but not in T stellate cells, deactivation of $I_h$ was slower in HCN1⁺/⁺ mice than in ICR mice (Fig.
4) (Table 1). The difference in kinetics between ICR and wild type control HCN1<sup>+/+</sup> mice could reflect a difference in the composition of the HCN ion channels between wild type strains.

Adaptation of Ih to changes in temperature in octopus cells was absent in HCN1<sup>−/−</sup> mice

Temperature is an important determinant of the rates of physical processes so it is expected that ion channels open and close more rapidly at 33°C than at 24°C. In octopus cells of ICR mice, but not in bushy or T stellate cells, the amplitude of Ih adapts with changes in temperature. In ICR mice, downward shifts in temperature cause an initial reduction in Ih but the maximal amplitude returns to its original levels within about 15 minutes (Fig. 5, A-D); upward shifts in temperature increase the maximal amplitude transiently and adapt to the original level over minutes (Cao and Oertel 2005). A similar series of measurements was made in octopus cells of HCN1<sup>−/−</sup> mice. Figure 5 (E-I) shows that in an octopus cell of an HCN1<sup>−/−</sup> mouse, a reduction in temperature reduced the amplitude which then remained stable over 40 min. When the temperature was elevated at the end of that period, the amplitude increased to nearly its original value. The regulation of Ih in octopus cells of mutants, therefore, resembles that in bushy and T stellate cells of ICR mice.

The elimination of HCN1 reduced not only the inward Ih but also the low-voltage-activated outward K<sup>+</sup> current (IKL)

The resting properties of octopus and bushy cells in ICR mice are influenced by opposing voltage-gated currents. The inward Ih is activated by hyperpolarization whereas the outward IKL is activated by depolarization around the resting potential. Both conductances are larger in octopus than in bushy cells (Bal and Oertel 2000; Bal and Oertel 2001; Cao et al. 2007). Knowing that Ih was reduced in octopus and bushy cells of mutant mice, we therefore examined IKL in these cells.

We found that IKL was reduced in both octopus and bushy cells of HCN1<sup>−/−</sup> mice (Fig. 6). (T Stellate cells have little or no IKL even in the wild type (Ferragamo and Oertel 2002).) Depolarizing voltage steps activated substantial outward currents that inactivated partially and that were sensitive to α-DTX (Fig. 6A-
F) (Bal and Oertel 2001). This current was activated in octopus and bushy cells of ICR mice with depolarizing voltage steps that exceeded -75 mV and is the basis for their being termed “low-voltage-activated” (Bal and Oertel 2001; Cao et al. 2007). The α-DTX–sensitive current is smaller in mutant HCN1−/− mice (Fig. 6G). In octopus and bushy cells of HCN1−/− mice, that outward current was substantially reduced (Fig. 6C, F). There was a large difference in the magnitude of IKL in octopus cells of the mice that have HCN1, ICR mice and wild type controls, showing that differences in IKL between strains parallel differences in Ih.

Magnitudes of IKL and Ih co-vary

The amplitudes of Ih and IKL were measured in populations of neurons and compared. Ih was measured as the steady state inward current at the end of a hyperpolarizing voltage step from -57 to -122 mV, a step from a voltage at which Ih is activated less than 10% to a voltage at which it is activated 100% in all three types of principal cells (Bal and Oertel 2000; Rodrigues and Oertel 2006; Cao et al. 2007). Measurements were made in the presence of TTX, CdCl2, DNQX, strychnine and α-DTX, blockers of potentially contaminating currents. Leak currents were not substracted but these represent less than 10% of the measured current in neurons from ICR mice (Bal and Oertel 2000; Rodrigues and Oertel 2006; Cao et al. 2007). IKL was measured as the peak outward current in response to step depolarizations from -90 mV, a voltage at which IKL is not activated, to -40 mV, the voltage at which the high-voltage-activated K+ conductance begins to activate (Bal and Oertel 2001; Cao et al. 2007). The extracellular presence of TTX, CdCl2, DNQX, strychnine, as well as ZD7288 blocked potentially contaminating currents (Bal and Oertel 2001; Cao et al. 2007).

A plot of IKL as a function of Ih shows that Ih and IKL co-vary and that the relationship between Ih and IKL holds for all types of principal cells of the VCN (Fig. 7). The HCN1−/− bushy cells that have on average the smallest Ih also have the smallest average IKL; the ICR octopus cells that have the largest Ih have the largest average IKL. T Stellate cells have the smallest Ih and have no measurable IKL (Ferragamo and Oertel 2002); the magnitude of Ih in T stellate cells is put into the context of Ih and IKL of bushy and octopus cells (Fig. 7A.)
The relative magnitudes of $I_h$ and $I_{KL}$ vary monotonically in the three types of principal cells of the VCN in the three strains of mice. These results indicate that the expression at the plasma membrane of HCN channels that mediate $I_h$ is somehow tied to the expression of KCNA channels that mediate $I_{KL}$ in each of the principal cells in each of the three genetic environments.

The error bars in Figure 7A are large and average values do not lie perfectly along the regression line. In part this probably results from the variability in the magnitudes of $I_h$ and $I_{KL}$ between cells; comparisons of the isolated $I_h$ and $I_{KL}$ cannot be made within individual cells because the blockers needed to separate the currents are not fully reversible. It is also possible that the measurements of steady state $I_h$ and peak $I_{KL}$ do not reflect the parameters that are regulated directly. At physiological membrane potentials $g_h$ is never maximally activated and the extent of its activation depends in most cells on modulation by cyclic nucleotides and also $g_{KL}$ is partially inactivated. We therefore used a different experiment to determine to what extent $I_h$ and $I_{KL}$ are matched in individual cells; we determined whether $I_h$ and $I_{KL}$ are balanced in individual cells at the resting potential.

We compared $I_h$ and $I_{KL}$ in individual octopus cells on the basis of their drug sensitivity (Fig. 7B). Octopus cells were held at the resting potential under voltage clamp. The application of 50 µM ZD7288, known to block $I_h$ (Bal and Oertel 2000), resulted in an outward holding current in octopus cells in each of the strains of mice; the subsequent application of 50 nM $\alpha$-DTX, known to block $I_{KL}$ (Bal and Oertel 2001), reduced the holding currents to within 10% of the original value in all three octopus cells (Fig. 7B). These experiments indicate that $I_h$ and $I_{KL}$ were balanced near the resting potential in octopus cells in all three strains of mice (Oertel et al. 2000). The resting $I_h$ and $I_{KL}$ determined with such experiments was on average $1.2 \pm 0.2$ nA (n=3) in ICR mice, $0.6 \pm 0.1$ nA (n=3) in wild type control mice and $0.3 \pm 0.1$ (n=5) in the mutant HCN1$^{-/-}$ octopus cells, differences between all groups were statistically significant (p<0.01). In bushy cells the magnitudes of $I_{KL}$ and $I_h$ activated at rest are so small that it is impossible to obtain meaningful measurements. Our results indicate that, at least when $I_h$ and $I_{KL}$ are large, they balance one another at the resting potential.
Discussion

A suite of conductances in membranes along processes of characteristic sizes and shapes give neurons their electrical characteristics. Examining the properties of neurons in which a conductance, or a portion of a conductance, is removed provides a glimpse not only of how that conductance affects the neuron but also of how a perturbation elicits compensatory changes through homeostatic mechanisms. Our study shows that the removal of HCN1 subunits not only reduces I_h but also leads to a reduction of I_KL. Furthermore, even in the presence of the gene for HCN1, the magnitude and kinetics of I_h differ in differing genetic environments.

Octopus, T stellate and bushy cells retain their characteristic features in different strains of mice

Octopus, T stellate and bushy cells retain their characteristic features and their resting potentials even when the magnitude and kinetics of I_h varies over a four-fold range. The principal cells of the VCN are as distinct electrophysiologically in mutants as in wild type strains so that neurons can be recognized on the basis of responses to depolarizing current pulses. Octopus cells respond to suprathreshold depolarization with only a single, small action potential (Golding et al. 1995; Golding et al. 1999). Bushy cells fire transiently with up to six action potentials that do not overshoot 0 mV when they are depolarized (Wu and Oertel 1984; Manis and Marx 1991; McGinley and Oertel 2006; Cao et al. 2007). T Stellate cells fire tonically when they are depolarized (Oertel et al. 1990; Fujino and Oertel 2001; Oertel et al. 2010). These characteristic properties of neurons are likely common to all mammals, having been described also in puppies (Bal et al. 2009). The resting potentials of all three groups of principal cells varied over about 5 mV and were not significantly different in mutant HCN1^{-/-}, HCN^{+/+}, and ICR mice. As in other neurons, the intrinsic electrical properties that are characteristic of neuronal types can be generated with a varied array of conductances (Grashow et al. 2010; Marder and Taylor 2011).

Biophysical properties differ not only between HCN1^{-/-} mice and wild type controls but also between wild type strains
Input resistances in each of the groups of principal cells were elevated in mutant HCN1−/− mice relative to the wild type controls, as expected, but also differed between ICR and wild type control HCN1+/+ mice in octopus and T stellate cells. In octopus cells of ICR mice input resistances were about 6 MΩ, consistent with earlier measurements (Golding et al. 1999), whereas they were 14 MΩ in hybrid, wild type controls. Differences were also significant in T stellate cells, 74 MΩ in ICR mice compared to 126 MΩ in wild type controls. There were no significant differences in input resistance of bushy cells between the two wild type strains, about 67 MΩ in both.

The removal of HCN1 subunits increases the input resistance but the expression of a single subunit of g_h is governed differently in different groups of principal cells. In the presence of HCN1, the genetic environment affects the input resistance of octopus cells more than the input resistance of bushy cells.

There were unexpectedly large differences in I_h between strains of mice that have HCN1, between the HCN1+/+ wild type controls that are hybrids between C57Bl and 129S strains and ICR mice. ICR octopus cells have extraordinarily large g_h, about 150 nS (Bal and Oertel 2000). In octopus cells of hybrid wild type control mice, I_h was slower and its magnitude on average only about half that of ICR mice, revealing the importance of the genetic background in regulating the expression of HCN1. These findings indicate that octopus cells in ICR mice express more HCN channels and are likely of different subunit composition than those in C57Bl/129S hybrid control mice. Bushy cells have a slower and smaller I_h than octopus cells, in ICR mice on average 30 nS (Leao et al. 2005; Leao et al. 2006; Cao et al. 2007). The magnitude of g_h in bushy cells from ICR mice was similar but the kinetics faster than g_h from hybrid wild type controls. T Stellate cells have relatively still slower and smaller g_h, 19 nS (Rodrigues and Oertel 2006). There was no significant difference in magnitude or kinetics of I_h in T stellate cells of ICR and hybrid wild type control mice. The differences between ICR and wild type controls raise the question whether the cochlear nuclei in the hybrid wild type controls and mutant HCN1−/− mice are biophysically compromised by their hybrid C57Bl and 129S genetic background. Octopus cells of CBA/J and ICR mice were so similar that biophysical measurements across the two strains were pooled in an early study (Golding et al. 1999). It
seems likely that cochlear nuclear neurons in ICR mice resemble those in normal, wild mice more closely than the hybrid HCN1\textsuperscript{+/+} controls.

Elimination of HCN1 results in smaller and lower \( I_h \)

The present experiments show that \( I_h \) was smaller and slower in cells that lack HCN1 than in wild type controls. Antibodies detect high levels of HCN1 in octopus cells, lower levels in the anterior VCN where bushy cells lie and least in the multipolar cell area where T stellate cells are most common (Koch et al. 2004; Oertel et al. 2008). HCN2 is also strongly expressed in the VCN but its distribution is more even (Koch et al. 2004). HCN4 is also strongly expressed in the octopus cell area (Notomi and Shigemoto 2004). Consistent with their known properties, we find that the removal of HCN1, the subunit with the fastest kinetics results in \( I_h \) with slower kinetics (Table 1) (Moosmang et al. 2001; Ulens and Tytgat 2001; Altomare et al. 2003; Whitaker et al. 2007). \( I_h \) in octopus cells of HCN1\textsuperscript{-/-} mice fails to adapt to changes in temperature as it does in ICR mice (Cao and Oertel 2005). Presumably this difference reflects a difference in the trafficking of HCN channels.

The magnitude of \( I_h \) and \( I_{KL} \) co-varies

The ability to encode precise timing of the fine structure of sounds by octopus and bushy cells is thought to be a consequence of \( I_{KL} \) that gives these neurons short time constants and rate-of-depolarization sensitivity and whose rapid activation cuts EPSPs short, giving them a sharp peak whose timing is relatively independent of amplitude (Manis and Marx 1991; Golding et al. 1995; Ferragamo and Oertel 2002; Rothman and Manis 2003; McGinley and Oertel 2006; Cao et al. 2007). T Stellate cells have little or no \( g_{KL} \) (Ferragamo and Oertel 2002). In contrast with \( g_{KL} \) which shapes EPSPs, \( g_h \) acts like a passive conductance. Under physiological conditions, principal cells of the VCN are not hyperpolarized more than -70 mV, the reversal potential of IPSPs, so that \( g_h \) is always only partially activated. Its kinetics are so slow relative to synaptic responses that its activation would be expected to be influenced only when synaptic activity is sustained. Individual EPSPs are only 1 msec in duration in octopus cells (Golding et al. 1995) and about 2
msec in bushy cells (Oertel 1985). In T stellate cells EPSPs can last hundreds of milliseconds but in these cells \( g_h \) is not only small but its voltage range of activation is more negative than in bushy or octopus cells so that only between 4 and 5% of the conductance is activated at the resting potential (Ferragamo et al. 1998; Rodrigues and Oertel 2006; Oertel et al. 2010). Not only neurons in the VCN but also in other brain stem auditory nuclei that are the targets of principal cells of the VCN share these features (Forsythe and Barnes-Davies 1993; Wu 1999; Hassfurth et al. 2009; Mathews et al. 2010).

Pharmacological evidence indicates that \( I_{KL} \) in bushy and octopus cells is mediated by ion channels of the KCNA (Kv1 or \textit{shaker}) family. In octopus cells, \( I_{KL} \) is blocked by \( \alpha\)-DTX, a toxin that blocks channels that contain KCNA1, KCNA2, and KCNA6 \( \alpha \) subunits (Dolly and Parcej 1996; Grissmer et al. 1994; Tytgat et al. 1995; Harvey 1997; Owen et al. 1997). DTX-K, which blocks channels that contain at least one KCNA1 \( \alpha \) subunit (Robertson et al. 1996; Owen et al. 1997; Wang et al. 1999), blocks 75% of \( I_{KL} \); tityustoxin \( \kappa \alpha \), a toxin that is specific for KCNA2 \( \alpha \) subunits (Hopkins 1998; Werkman et al. 1993), blocks 60% of \( I_{KL} \). The ion channel composition must be heterogeneous because differing proportions contain KCNA1 and KCNA2. In bushy cells, too, \( I_{KL} \) is sensitive to \( \alpha\)-DTX and partially sensitive to DTX-K and to tityustoxin \( \kappa \alpha \) (Leao et al. 2004; Cao et al. 2007).

The present results suggest that the magnitude of \( I_{KL} \) depends on the magnitude of \( I_h \). Removal of the HCN1 subunit reduced \( I_h \) relative to the wild type controls in both octopus and bushy cells and resulted in a parallel loss of \( I_{KL} \) in both groups of principal cells (Fig. 7A). It is unclear what genetic difference underlies the difference in the magnitude of \( I_h \) between ICR and wild type controls but the positive correlation in the amplitudes of \( I_h \) and \( I_{KL} \) pertains to octopus cells in that strain too (Fig. 7A). Octopus cells from ICR mice had the largest \( I_h \) and the largest \( I_{KL} \); bushy cells in mutant mice had the smaller \( I_h \) and smallest \( I_{KL} \). The relationship between \( I_h \) and \( I_{KL} \) even holds for T stellate cells that have little or no \( I_{KL} \); a line fit through the data points representing measurements from bushy and octopus cells suggests that neurons can have a small \( I_h \) in the absence of \( I_{KL} \).

It is possible that a role of \( I_h \) is to balance \( I_{KL} \). \( I_h \) is an inward current near the resting potential whereas \( I_{KL} \) is an outward current. The positive correlation between the magnitudes of \( I_h \) and \( I_{KL} \) is evident in
octopus cells that have the largest $I_{KL}$ and those currents balance one another even in individual cells (Oertel et al. 2000) (Fig. 7B). Bushy cells that have a smaller $I_{KL}$ also have a smaller $I_h$ than octopus cells. The fact that $I_{KL}$ is too small at rest to measure with precision also indicates that it could be balanced by small inward currents other than $I_h$. In T stellate cells, $I_h$ at rest is small. Not only is the total $g_h$ small but the voltage sensitivity of $I_h$ is more negative than in bushy or octopus cells so that at rest a relatively smaller proportion of the current is activated than in octopus or bushy cells (Rodrigues and Oertel 2006). Many neurons in the superior olivary complex, including neurons in the medial superior olive, medial nucleus of the trapezoid body (MNTB), the medial (MSO) and lateral superior olivary nuclei (LSO), and ventral nucleus of the lateral lemniscus have the combination of $I_h$ and $I_{KL}$ and likely follow a similar relationship (Banks et al. 1993; Wu 1999; Barnes-Davies et al. 2004; Koch et al. 2004; Notomi and Shigemoto 2004; Scott et al. 2005; Leao et al. 2006; Hassfurth et al. 2009). Indeed the co-variation of $I_h$ and $I_{KL}$ is evident in the parallel gradients in the LSO, both being largest in the lateral limb and smallest in the medial limb (Barnes-Davies et al. 2004; Hassfurth et al. 2009). Furthermore, the correlation between $I_h$ and $I_K$ is not limited to auditory neurons. For example, the magnitude of mRNA for $I_h$ has been found to co-vary with levels of mRNA for KCND potassium channels (Schulz et al. 2006). These observations suggest that these channels could share a common aspect of trafficking or interactions at the membrane as part of a larger complex but these functions are only beginning to be understood (Hegle et al., 2010).

**Homeostasis**

Much has been learned recently about homeostatic regulation of excitability (Turrigiano et al. 1998; Nelson and Turrigiano 2008). Indeed currents through KCNA channels have been implicated in regulating the activity and synchrony in networks of hippocampal CA3 cells (Cudmore et al. 2010). The expression of KCNQ channels, too, is regulated by electrical activity (Kullmann and Horn 2010). Activity influences $I_h$ in the MNTB and LSO. $I_h$ in neurons in the lateral limb of the LSO, where neurons that have the largest and fastest $I_h$, increases at the onset of hearing and after cochlear ablation but $I_h$ in MNTB neurons, which have
smaller and slower $I_h$, changes little with hearing onset and was reduced after cochlear ablation (Hassfurth et al. 2009). In these cells these changes were, however, accompanied by changes in the resting potential.

We have shown that the intrinsic electrical properties of each of the groups of principal cells of the VCN, octopus, T stellate and bushy cells, have common features both within a strain of mice and across strains but that they are also variable as in other neurons (Grashow et al. 2010; Marder and Taylor 2011). It is likely that those conductances are regulated by homeostatic mechanisms. The excitability of neurons as well as the strength of synapses has been shown to be homeostatically regulated to keep the excitability of neurons within the dynamic range (Davis 2006; Pratt and Aizenman 2007; Wilhelm et al. 2009). In cortical neurons it seems that firing rate is regulated by homeostasis (Turrigiano et al. 1998). In some invertebrate neurons it seems to be firing patterns that are regulated (Olypher et al. 2006; Haedo and Golowasch 2006).

Three findings in the present study support the possibility that the resting potential is homeostatically regulated. First, even when the expression of $I_h$ and $I_{KL}$ span a wide range, the resting potential remains constant. Second, there is a positive correlation between the magnitude of $I_h$ and $I_{KL}$, not only within one type of cell but across cell types. Third, in individual octopus cells, even when the magnitudes of the currents vary over a factor of 4, $I_h$ and $I_{KL}$ are balanced at the resting potential.

Summary

Our findings show that the principal cells of the VCN retain their characteristic properties in strains of mice even when the magnitudes of conductances differ significantly. The finding that $I_h$ and $I_{KL}$ are balanced across widely differing conditions suggests that their relative levels are regulated homeostatically. It is not yet known whether the temporal resolution in hearing parallels the differences in intrinsic characteristics of these neurons.

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Figure Legends

**Figure 1.** Responses to current pulses reflect the intrinsic electrical properties of neurons. A-C. Recordings from octopus, T stellate and bushy cells in ICR mice illustrate the differences that distinguish them. Depolarizing current pulses caused octopus cells to fire transiently with a single action potential, bushy cells to fire transiently with several action potentials, and T stellate cells to fire tonically. Hyperpolarizing current pulses resulted in a polarization that sags back toward rest, reflecting the activation of $I_h$. D-F. Responses to current in neurons from wild type control mice (HCN1$^{+/+}$) are generally similar to those in ICR mice, reflecting the characteristic differences between octopus, T stellate, and bushy cells. G-I. Corresponding recordings from neurons in HCN1$^{-/-}$ mice also show similar overall characteristics. Current pulses evoke larger polarizations in HCN1$^{-/-}$ mice than in the strains that contain HCN1, reflecting their higher input resistances. Amplitudes of current pulses, given in the upper panels, were matched in ICR, HCN1$^{+/+}$ wild type controls, and mutant HCN1$^{-/-}$ neurons.

**Figure 2.** Comparison of characteristics of principal cells from ICR, wild type controls, and mutant HCN1$^{-/-}$ mice. A. Input resistances were significantly higher in HCN1$^{-/-}$ mutants than in wild type controls in all three types of principal cells. The input resistances were also significantly higher in octopus and T stellate cells of wild type controls than in ICR mice. B. The time constants were longer in octopus and bushy cells of HCN1$^{-/-}$ than in the wild type controls. Time constants were not significantly different in ICR and wild type controls in any of the cell types. C. There were no significant differences in the resting potentials of neurons in the three strains of mice. Mean values ± SD are indicated by bars. Statistically significant differences (p<0.05) between mutant HCN1$^{-/-}$ mice and HCN1$^{+/+}$ hybrid wild type controls are illustrated with a * that straddles the bars being compared. Statistically significant differences (p<0.05) between the HCN1$^{+/+}$ wild type controls and ICR mice are indicated by a #.
Figure 3. $I_h$ is smaller and slower in HCN1⁻/⁻ mutant neurons than in wild type controls or ICR neurons. Examples of recordings of $I_h$ evoked by hyperpolarizing voltage steps from the holding potential of −57 mV in 5 mV increments to the levels indicated by numbers at the right of the traces. The steps evoked an instantaneous current that was proportional to the input conductance of the neurons; the inward current then increased gradually as $I_h$ was activated. $I_h$ was largest in octopus cells, intermediate in bushy cells, and smallest in T stellate cells of wild type controls and ICR mice; in HCN1⁻/⁻ mutant mice, $I_h$ was similar in T stellate and bushy cells. Note that $I_h$ activated more slowly in mutant neurons that lack HCN1 (lowest panels) than in wild type controls or ICR neurons (upper panels). Recordings were made in the presence of 40 µM DNQX, 1 µM strychnine, 1 µM TTX, 0.25 mM Cd²⁺ and 50 nM α-DTX.

Figure 4. In all three groups of principal cells of the VCN the kinetics of the activation and deactivation of $I_h$ are slower in HCN1⁻/⁻ than in wild type controls and they are slower in wild type controls than in ICR mice. Left. Traces show responses to voltage steps from −57 mV to −107 mV in examples of each of the three cell types. Traces were fit with the sum of two exponential functions from the beginning of the activation of $I_h$ to near the steady state; panels illustrate only the early parts of those traces and the double exponential fit. Recordings from individual cells from the three strains of mice are superimposed for comparison. The heavy lines are double exponential fits to HCN1⁻/⁻ cells, intermediate lines are fits to wild type controls, and the finest lines are fits to ICR mice. Right. Traces show tail currents when the voltage was stepped from −107 to −57 mV and were fit with single exponential functions. The heavy lines show fits to traces from mutant HCN1⁻/⁻ cells, intermediate lines show the fits to wild type controls, and the finest lines show fits to traces from ICR mice.

Figure 5. Mouse strains differ in regulation of the magnitude of $I_h$ in octopus cells. A-C. In an octopus cell from an ICR mouse, the temperature was shifted from 33°C to 24°C and the cell’s properties were assayed by stepping the voltage from −57 mV to a range of voltages between −57 and −122 mV. Five minutes after the temperature was reduced to 24°C, the same voltage steps evoked currents that were slower and smaller.
Over the next 15 minutes, while the cell continued to be held at 24°C, the amplitude of $I_h$ grew to near its original amplitude at the steady state while remaining slower than at 33°C. D. A plot of the amplitude of the steady-state current in response to a voltage step to –122 mV shows the time course with which the amplitude of the current returns to near the original value. E-H. A recording from an octopus cell of an HCN1$^{-/-}$ mouse that was subjected to a similar temperature change shows that the amplitude of $I_h$ remained reduced for the 40 min after the temperature was reduced from 33°C to 24°C and that the change was largely reversed by elevating the temperature after that period. I. Plot of the time course of the changes in $I_h$ in the same HCN1$^{-/-}$ cell illustrated in E-H.

**Figure 6.** The magnitude of low-voltage-activated currents ($I_{KL}$) are reduced in neurons of HCN1$^{-/-}$ mice relative to wild type controls in octopus and bushy cells. A. In a bushy cell from an ICR mouse, depolarizing voltage steps from –90 mV to –40 mV in 5 mV steps (shown in C) activated a voltage-sensitive outward current. B. In a bushy cell from a wild type control mouse, similar voltage steps evoked similar outward currents. C. In a bushy cell of an HCN1$^{-/-}$ mouse, outward currents evoked by similar voltage steps were smaller. Inset: voltage protocol applies to panels A-F. D: In an octopus cell from an ICR mouse, depolarizing voltage steps evoked large outward currents. E. Similar voltage steps applied to an octopus cell of a wild type control mouse, evoked smaller outward currents. F. In an octopus cell from an HCN1$^{-/-}$ mutant mouse, outward currents evoked by the same protocol were smaller still. Inset: More than half of the outward current was blocked by the application of 50 nM α-DTX. G. Current/voltage relationship of average peak outward current in HCN1$^{-/-}$ octopus cells (n=4) as a function of voltage under control conditions (○) and in the presence of 50 nM α-DTX (●). Recordings were made in the presence of 50 μM ZD7288, 1 μM TTX, 0.25 mM CdCl$_2$, 40 μM DNQX, and 1 μM strychnine.

**Figure 7.** The magnitude of the low-voltage-activated currents ($I_{KL}$) is correlated with the magnitude of the opposing hyperpolarization-activated inward currents ($I_h$) across cell types and across strains of mice. A. The magnitude of $I_h$ was assessed from the size of maximal inward currents evoked at the end of a 2-second
voltage step from -57 mV to -122 mV in the presence of 40 µM DNQX, 1 µM strychnine, 1 µM TTX, 0.25 mM CdCl₂ and 50 nM α-DTX. This current reflects the sum of $I_h$ and a small leak current. The magnitude of $I_{KL}$ was measured in octopus and bushy cells as the peak outward current in responses to voltage steps from -90 mV to -40 mV, measured in the presence of 40 µM DNQX, 1 µM strychnine, 1 µM TTX, 0.25 mM CdCl₂, and 50 µM ZD7288. This measurement reflects the sum of $I_{KL}$ and a small leak current. The cell type is designated by the shapes of symbols and the strain of mice is indicated by the relative size of symbols. Octopus cells of ICR mice have the largest $I_h$ and also have the largest $I_{KL}$ whereas bushy cells of HCN1⁻/⁻ mutants that have a small $I_h$ and also a small $I_{KL}$. A regression line, $R = 0.95$, was fit to measurements of $I_h$ and $I_{KL}$ in octopus and bushy cells in all three stains of mice (dashed line). T Stellate cells have no measurable $I_{KL}$ (Ferragamo and Oertel 2002); the values of $I_h$ measured in T stellate cells in the three strains are shown with diamonds. B. In individual octopus cells, $I_h$ and $I_{KL}$ are balanced at the resting potential. An octopus cell from each of the three strains of mice was initially held at the cell’s resting potential. Resting potentials of the three octopus cells were: ICR -64 mV, HCN1⁺/⁻ -66 mV, and HCN1⁻/⁻ -66 mV. Upon blocking $g_h$ with 50 µM ZD7288, an outward holding current was observed. The further addition of 50 nM α-DTX in the continued presence of ZD7288 reduced the outward holding current to 0.1 nA. The similarity of the magnitudes of currents blocked by ZD7288 and α-DTX in individual cells indicate that in each cell the inward $I_h$ balanced the outward $I_{KL}$ at the holding potential.
Table 1. Comparison of properties of neurons in ICR, HCN1<sup>+/+</sup> and HCN1<sup>-/-</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>Octopus</th>
<th>T stellate</th>
<th>Bushy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICR</td>
<td>HCN1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>HCN1&lt;sup&gt;-/-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Input Resistance (MΩ)</td>
<td>6 ± 2 (10)</td>
<td>14 ± 2 (8) *</td>
<td>19 ± 3 (19) *</td>
</tr>
<tr>
<td>Time Constant (msec)</td>
<td>0.2 ± 0.1 (9)</td>
<td>0.3 ± 0.2 (8) *</td>
<td>0.5 ±0.1 (5) *</td>
</tr>
<tr>
<td>Resting Potential (mV)</td>
<td>-64.5 ± 1.3 (8)</td>
<td>-64 ± 1 ( 5)</td>
<td>-64 ± 1.3(13)</td>
</tr>
<tr>
<td>Activation of g&lt;sub&gt;a&lt;/sub&gt;: τ&lt;sub&gt;τ&lt;/sub&gt; = -57→ -107 mV</td>
<td>23 ± 4 (7)</td>
<td>100 ± 19 (6)</td>
<td>270 ± 34 (5)</td>
</tr>
<tr>
<td></td>
<td>81%</td>
<td>63%</td>
<td>60%</td>
</tr>
<tr>
<td>Activation of g&lt;sub&gt;a&lt;/sub&gt;: τ&lt;sub&gt;τ&lt;/sub&gt; = -57→ -107 mV</td>
<td>1131 ± 396 (6)</td>
<td>2120±760 (5)</td>
<td>770±200 (5)</td>
</tr>
<tr>
<td></td>
<td>19%</td>
<td>37%</td>
<td>40%</td>
</tr>
<tr>
<td>Deactivation of g&lt;sub&gt;a&lt;/sub&gt;: τ&lt;sub&gt;τ&lt;/sub&gt; = -107→ -57 mV</td>
<td>107±21 (7)</td>
<td>307±79 (6)</td>
<td>430±41 (5)</td>
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Table 1. Summary of the differences in the properties of neurons in mice of different strains. Numbers are given as means ± SD, with the number of cells from which measurements were made given in (). A significance of difference, p<0.05, between HCN1<sup>-/-</sup> and HCN1<sup>+/+</sup> wild type controls is indicated with *. A significant difference, p<0.05, between ICR and HCN1<sup>+/+</sup> wild type controls is indicated with a #.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

ICR

33°C

0
0.5
1.0
1.5
sec

nA

-8
-4
0
-122

24°C (5 min)

24°C (15 min)

nA

-10
-8
-6
-4
-2
0

min

33°C

24°C

-57 mV

-127 mV

HCN1−/−

33°C

0
1
2
3
4
5
sec

nA

-3
-2
-1
0
-127 mV

24°C (6 min)

24°C (40 min)

33°C

nA

-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0

min

33°C

24°C

33°C

-127 mV

-57 mV
Figure 6
Figure 7