SUBUNIT-SPECIFIC EFFECTS OF ISOFLURANE ON NEURONAL \( I_h \) IN HCN1 KNOCKOUT MICE

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The ionic mechanisms that contribute to general anesthetic actions have not been elucidated but increasing evidence has pointed to roles for subthreshold ion channels, such as the HCN channels underlying neuronal $I_h$. Here, we used conventional HCN1 knockout mice to test directly the contributions of specific HCN subunits to effects of isoflurane, an inhalational anesthetic, on membrane and integrative properties of motor and cortical pyramidal neurons in vitro. By comparison to wild type mice, residual $I_h$ from knockout animals was smaller in amplitude and presented with HCN2-like properties. Inhibition of $I_h$ by isoflurane previously attributed to HCN1 subunit-containing channels (i.e., a hyperpolarizing shift in $V_{1/2}$) was absent in neurons from HCN1 knockout animals; the remaining inhibition of current amplitude could be attributed to effects on residual HCN2 channels. We also found that isoflurane increased temporal summation of EPSPs in cortical neurons from wild type mice; this effect was predicted by simulation of anesthetic-induced dendritic $I_h$ inhibition, which also revealed more prominent summation accompanying shifts in $V_{1/2}$ (an HCN1-like effect) than decreased current amplitude (an HCN2-like effect). Accordingly, anesthetic-induced EPSP summation was not observed in cortical cells from HCN1 knockout mice. In wild type mice, the enhanced synaptic summation observed with low concentrations of isoflurane contributed to a net increase in cortical neuron excitability. In summary, HCN channel subunits account for distinct anesthetic effects on neuronal membrane properties and synaptic integration; inhibition of HCN1 in cortical neurons may contribute to the synaptically-mediated slow wave cortical synchronization that accompanies anesthetic-induced hypnosis.
INTRODUCTION

Neuronal and ionic mechanisms that underlie behavioral actions of general anesthetics remain to be determined. The actions that define the anesthetic state are immobility, unconsciousness and amnesia (Eger et al. 1997); it is likely that different cellular and molecular substrates contribute to these distinct anesthetic endpoints, and the relevant targets may also be different for individual anesthetic drugs (Franks 2006; Rudolph and Antkowiak 2004; Sonner et al. 2003). There is now good evidence implicating a number of ion channels in anesthetic action, based on their sensitivity to modulation by clinically relevant concentrations of anesthetics and their expression in behaviorally relevant neural systems. This includes a number of ionotropic receptors (e.g., GABA_A, glycine & glutamate receptors) and background K^+ channels (e.g., K_2P2.1, K_2P3.1, K_2P9.1) (Franks 2006; Linden et al. 2007; Rudolph and Antkowiak 2004; Sonner et al. 2003).

Our recent work indicates that general anesthetics also produce a subunit-selective inhibition of HCN channels containing HCN1 and HCN2 subunits (Chen et al. 2005b; Chen et al. 2005a); these channels are expressed in motoneurons and in projection neurons of the thalamus and cortex where they underlie a hyperpolarization-activated cationic current (I_h) that contributes to intrinsic and integrative neuronal properties and to generation of synchronized oscillatory rhythms generated in the thalamus and cortex (Destexhe and Sejnowski 2003; Pape 1996; Pape and McCormick 1989; Steriade et al. 1993b). Depending on HCN subunit composition in heterologous expression systems, we found that inhalational anesthetics primarily inhibit voltage-dependent activation properties (HCN1 homomers) or maximal current amplitude (HCN2 homomers), with combined effects observed in heteromeric HCN1-RCN2 channels (Chen et al. 2005b). However, since there are no HCN subunit-selective antagonists, support
for the conclusion that these particular subunits account for anesthetic effects on neuronal $I_h$ has been indirect and correlative, relying on shared properties of modulation between native currents and cloned channels (Chen et al. 2005b). Also, previous studies have not evaluated the relative contributions of the distinct modulatory mechanisms of anesthetics on neuronal properties that are strongly influenced by $I_h$, such as synaptic integration.

Here, we used HCN1 knockout mice and computer simulations in order to examine HCN subunit contributions to anesthetic effects on intrinsic and integrative properties in motor neurons and cortical pyramidal neurons in vitro. Our results verify HCN subunit-specific effects of isoflurane, and demonstrate that inhibition of $I_h$ by isoflurane causes membrane hyperpolarization and enhanced synaptic summation; the net effect of these changes in cortical neuronal properties is an increase in synaptic excitability. Thus, isoflurane-mediated inhibition of HCN1-containing channels may contribute to synaptically-driven slow-wave oscillations in thalamocortical circuits (McCormick and Bal 1997; Rudolph and Antkowiak 2004; Steriade et al. 1993a), such as those associated with hypnosis at the onset of anesthesia (Antkowiak 2002).
MATERIALS AND METHODS

HCN1 knockout mice

Conventional HCN1 channel knockout mouse lines were used for in vitro electrophysiological experiments investigating the role of HCN1-containing channels in neuronal effects of isoflurane. The HCN1 knockout mice were originally prepared and characterized by the Kandel laboratory (Nolan et al. 2003; 2004); they have been deposited for distribution by Jackson Labs (Stock #005034). The knockout was prepared by ‘floxing’ the exon containing the pore domain and sixth transmembrane segment, followed by Cre-mediated excision of that exon in ES cells. Homozygous HCN1 knockout animals were generated by crossing 129SVEV HCN1−/+ animals to C57BL/6 wild-type mice, and then intercrossing heterozygous progeny to yield the HCN1−/− knockout animals that are maintained as homozygotes by Jackson Labs. As reported in the original publications (Nolan et al. 2003; 2004), mice homozygous for the targeted mutation are viable, fertile, normal in size and longevity and they do not display any gross physical or behavioral abnormalities; functional channel deletion was confirmed by electrophysiological analysis of Ih in cerebellar Purkinje neurons (decreased by ~90%) and hippocampal CA1 pyramidal cells (decreased by ~60−70%) (Nolan et al. 2003). As controls for HCN1 knockout animals, we used age, sex and weight-matched B6129SF2/J mice (Stock #101045). These animals are hybrids of the parental strains C57BL/6J and 129S1/SvImJ; they are recommended by Jackson Labs as an ‘approximate’ control for strains designated B6;129S, including HCN1 knockout mice and are appropriate for the cellular studies reported here (although littermate controls derived from the breeding of heterozygotes are advised for behavioral studies).
**Electrophysiological recordings**

Transverse brain slices from mice of either sex (14-22 days old for cortical cells; 11-15 days old for motoneurons) were prepared, as described previously (Chen et al. 2005a; Talley et al. 2000). Mice were anesthetized (ketamine/xylazine: 200/14 mg/kg, im), decapitated and transverse slices (300 µm) were cut with a microslicer in ice-cold Ringer’s solution (mM): sucrose, 260; KCl, 3; MgCl₂, 5; CaCl₂, 1; NaH₂PO₄, 1.25; NaHCO₃, 26; glucose, 10; and kynurenic acid, 1. Slices were maintained in Ringer’s solution (mM): NaCl, 130; KCl, 3; MgCl₂, 2; CaCl₂, 2; NaH₂PO₄, 1.25; NaHCO₃, 26; and glucose, 10; bubbled with 95% O₂/5% CO₂. Brain slices were submerged in a recording chamber on a Zeiss Axioskop FS microscope and visualized with Nomarski optics; hypoglossal motor neurons and cortical pyramidal neurons were targeted for recording based on location in the slice and characteristic size and shape (Chen et al. 2005a; Talley et al. 2000).

Electrical recordings were obtained at room temperature in HEPES-based bath solutions containing (mM): NaCl, 140; KCl, 3; HEPES, 10; CaCl₂, 2; MgCl₂, 2; glucose, 10. For voltage clamp recording, pipettes (2-4 MΩ) were filled with (mM): KCl, 120; NaCl, 4; MgCl₂, 1; CaCl₂, 0.5; HEPES, 10; EGTA, 10; MgATP, 3; GTP-Tris, 0.3 (pH 7.2); for current clamp, pipette solutions contained (mM): KCl, 17.5; potassium gluconate, 122.5; HEPES, 10; EGTA, 0.2; NaCl, 9; MgCl₂, 1; MgATP, 3; GTP-Tris, 0.3 (pH 7.2). Bath solutions were bubbled vigorously and inhalational anesthetics were added to the perfusate (~2 ml/min) via calibrated vaporizers (Ohmeda); anesthetic concentrations were determined by gas chromatography of perfusate samples. All data were corrected for a measured liquid junction potential of 8 mV.

In order to block Iₜ in some experiments, CsCl (3 mM) was added to the bath, or ZD-7288 was included either in the pipette solution or the bath (50 µM;
In other experiments, cAMP was added to the pipette solution to activate HCN channels (at 50 µM; from Sigma). In voltage clamp recordings of \( I_h \) from motoneurons, we inhibited the prominent anesthetic-activated TASK-like currents by lowering bath pH to 6.5 (Sirois et al. 2000; Sirois et al. 2002); we also used a pipette solution containing 25 mM HEPES (substituted for KCH\(_3\)O\(_3\)) in order to minimize effects of bath acidification on the pH-sensitive motoneuronal \( I_h \) (Sirois et al. 2002). We also included BaCl\(_2\) (200 µM) in voltage clamp experiments in order to inhibit inwardly-rectifying K\(^+\) currents. Where noted, tetrodotoxin (TTX at 0.5 µM, Alomone Labs) was added to the perfusate to block action potentials and a bicuculline/strychnine cocktail (both at 30 µM; from Sigma) was added in order to block GABA\(_A\) and glycine receptor channels.

Electrophysiological recordings were acquired with an Axopatch 200B amplifier via a Digidata 1322A digitizer using pCLAMP software (all Axon Instruments). For voltage-clamp recording, time-dependent hyperpolarization-activated currents (\( I_h \)) were evoked with incrementing (\( \Delta -10 \) mV) hyperpolarizing pulses (3-4 s) from a holding potential of -48 mV, followed immediately by a step to fixed potential (-98 mV) in order to obtain tail currents. Current amplitude at each potential was taken as the time-dependent current at the end of hyperpolarizing voltage steps; maximal available current was determined at -128 mV. Tail currents were normalized, plotted as a function of the preceding hyperpolarization step voltage and fitted with Boltzmann curves for derivation of half-activation voltage (\( V_{1/2} \)) by using a least squares analysis and the ‘Solver’ add-in of Excel (Microsoft). Time constants (\( \tau \)) were determined by fitting currents evoked during hyperpolarizing steps to a biexponential function (Clampfit). For current clamp analysis, we measured resting membrane potential, input resistance and depolarizing ‘sag’ during rectangular current steps. Because voltage ‘sag’ is influenced by membrane potential and input
resistance as well as the magnitude of $I_h$, we compared voltage ‘sag’ between cells from the same membrane potential (i.e., during current steps in which initial voltage deflections approached ~88 mV, -98 mV and -108 mV). We monitored changes in $I_h$ or membrane potential continuously during isoflurane application and measurements were made after steady state was achieved (usually within 5 min.).

**Synaptic integration & computer simulation**

A stimulating electrode, connected to a Grass stimulator (S48) via a stimulus isolation unit (Grass SIU5), was filled with standard external solution containing Lucifer Yellow and placed in the superficial layers of the cortex. For recordings of evoked EPSPs, the bath solution contained a bicuculline/strychnine cocktail (each at 30 µM). Synaptic responses were evoked by applying 40 Hz, 5-10 V pulses (corresponding to 2 to 10 µA) and CNQX-sensitive EPSPs were recorded under whole cell current-clamp in cortical pyramidal neurons. In order to relate measured effects on EPSP summation to anesthetic-induced changes in $I_h$ properties, a computer simulation was performed using the NEURON software package (Hines and Carnevale 1997) and a previously published pyramidal neuron model (Day et al. 2005) kindly provided by Drs. Joshua Held and Jim Surmeier (Northwestern University). With this computer model, we compared effects on EPSP summation of imposing shifts in $V_{1/2}$ of $I_h$ activation, decreases in current amplitude, or both.

**Neuronal Morphology**

For morphological identification of recorded cells, 0.2% biocytin (Sigma) was included in the patch electrode solution, as described previously (Chen et al. 2005a). In brief, slices were fixed in 4% buffered paraformaldehyde solution and
then rinsed and incubated with 1% H₂O₂ and 0.5% Triton X-100. After incubation with avidin-biotin complex and diaminobenzidine substrate kit (both from Vector Labs, Burlingame, CA), slices were mounted and dehydrated through graded ethanol and xylene and embedded in DPX mounting medium (BDH Laboratory Supplies, England); biocytin-stained neurons were visualized and photographed using an Axioskop microscope (Zeiss), equipped with a digital camera (Retiga 1300C, QImaging) and IPLab software (Scanalytics). A digital camera lucida system was used for reconstruction and quantitative analysis of biocytin-filled cortical neurons (NeuroLucida, MicroBrightfield). In some experiments (e.g., during field stimulation), Lucifer Yellow (0.2%) was included in the pipette solution to identify the soma and primary dendrite of recorded cortical neurons in order to aid in placement of the stimulating pipette.

**Quantitative Real-time PCR**

Cortical HCN subunit expression was determined in wild type and knockout mice by using quantitative real-time PCR (qRT-PCR). The cortex was micro-dissected for RNA isolation from juvenile and adult mice (n ≥ 3 animals per genotype at each age) and qRT-PCR was performed from each sample in quadruplicate, using an iCycler (Bio-Rad, Hercules, CA); each animal contributed a single data point for a given HCN channel subunit. We used the following primer sets: HCN1: GenBank #AJ225123, upper: AGGTTAATCAGATACATACACC, lower: GAGTGCGTAGGAATATTGTTTT, 231 bp amplicon; HCN2: GenBank #AJ225122, upper: CGGCTCATCCGATATATCCA, lower: AGCGCGAACGAGTAGAGCTC, 230 bp amplicon; HCN3: GenBank #NM008227, upper: GATACTGCAGCGGAAACGCTC, lower: AGATACCTGGGAACGCCCTGT;, 482 bp amplicon; HCN4: GenBank #XM287905, upper: CCCGCCTCATTCGATACATC, lower: CCCGCCTCATTCGATACATC, 232 bp amplicon.
bp amplicon; and PCR conditions: 3 min. 95°C; 40 cycles: 10s 95°C, 40 s 60°C, 40s 72°C; that were optimized in preliminary experiments to yield ≥97% efficiency. The identity of PCR products was verified in initial experiments by agarose gel electrophoresis (which yielded amplicons of appropriate size) and in all experiments by melt curve analysis (which yielded a single peak at appropriate Tm). Cyclophilin served as an internal standard (GenBank NM_005038; upper: GGCTCTTGAAATGGACCCTTC; lower: CAGCCAATGCTTGATCATATTCTT; 91 bp amplicon; (Lauritzen et al. 2003) and control samples with no added template were included with every experiment. We analyzed qRT-PCR data by using a modification of the so-called ∆∆Ct normalization procedure in order to obtain HCN subunit mRNA levels for each genotype, relative to cyclophilin (Pfaffl 2001).

Data acquisition and analysis

Results are presented as mean ± SEM. Data were analyzed statistically using one-way and two-way analysis of variance (ANOVA) or Students t-test; post-hoc pairwise comparisons used Bonferroni’s correction of the t-test (Excel and/or SigmaStat). Differences in mean values were considered significant if P<0.05.
RESULTS

*HCN1 expression and contribution to membrane properties of cortical pyramidal neurons are diminished in HCN1 knockout mice.*

To verify HCN1 subunit deletion in HCN1 knockout mice, we examined HCN subunit expression by qRT-PCR. As shown in Figs. 1A & 1B, qRT-PCR using primers for HCN1, HCN2, HCN3 and HCN4 revealed a complete deletion of HCN1 channels in cortex from adult HCN1 knockout mice, with little change in expression of other HCN subunits. A substantial expression of HCN2 was evident in both control and HCN1 knockout animals, whereas levels of HCN3 and HCN4 mRNA were ~50 to 100-fold lower in cortex of either wild-type or HCN1 knockout mice. We obtained essentially identical results using cortical tissue from juvenile mice (P20), with the exception that, for both genotypes, HCN2 mRNA levels were approximately two-fold higher in cortex of younger mice than in adults (*data not shown*; n = 3 and 4 for control and HCN1 knockout, respectively). These results are consistent with previous reports that HCN1 and HCN2 are the predominant HCN subunits expressed in cortical neurons (Monteggia et al. 2000; Santoro et al. 2000), and they indicate that there is little change in expression of other HCN subunits to compensate for deletion of HCN1 (Nolan et al. 2003; 2004).

We used whole cell patch clamp recording in brain slices in order to characterize hyperpolarization-activated currents mediated by HCN subunits in cortical pyramidal neurons from wild type and HCN1 knockout mice. We examined properties of HCN currents in cortical pyramidal neurons from juvenile control and HCN1 knockout mice (P14-P22); the age distribution was not different between mouse lines and we found no age-dependent changes in $I_h$ density within the age span considered, in either group of cells (*data not shown*).
Biocytin-filled neurons from wild type and knockout mice, presented in Fig. 1C, display similar morphological features that are typical of cortical pyramidal neurons (i.e., the cells have large apical dendrites that extend upwards toward the pial surface, where they ramify extensively). We reconstructed a subset of biocytin-filled cortical neurons from wild type (n=4) and HCN1 knockout mice (n=3); there were no significant differences in length of the apical dendrites in these neurons (596.2 ± 26.7 µm vs. 648.6 ± 19.9 µm, p=0.20) nor in the number (36.5 ± 7.1 vs. 35.7 ± 3.3, p=0.93) or average length (99.2 ± 9.7 µm vs. 92.4 ± 2.4 µm, p=0.58) of secondary and tertiary dendritic branches, the values for which were generally consistent with previous descriptions of these properties in cortical pyramidal cells (e.g., see Larsen and Callaway 2006).

There were striking differences in voltage- and time-dependent currents evoked by hyperpolarizing voltage steps (i.e., $I_h$) between HCN1 knockouts and control animals, as depicted in Fig. 1D. Specifically, $I_h$ was consistently smaller in cells from knockout animals; in addition, the residual current activated more slowly and at more hyperpolarized potentials. In wild type mice, the maximal time-dependent $I_h$ in cortical neurons was ~320 pA at the end of a hyperpolarizing step to -128 mV (-320.3 ± 26.4 pA, n=24) whereas in cells from HCN1 knockout mice, the residual $I_h$ at the same potential was only -106.5 ± 10.7 pA (n=46, Fig. 1E). In cortical cells from control animals, biexponential fits to $I_h$ revealed a variable slow component and a prominent fast component (accounting for >60% of current amplitude) that activated with a time constant (fast tau, $\tau_f$) of ~80 ms (83.5 ± 6.3 ms at -118 mV, n=18; Fig. 3D). The slower $I_h$ activation in pyramidal neurons from HCN1 knockout mice is apparent in the scaled exemplar traces of Fig. 1F (left), and in the substantially longer $\tau_f$ values (519.2 ± 66.8 ms, n=19; Fig. 1F, middle and right). Finally, the half-activation potential ($V_{1/2}$) for $I_h$ was shifted to more hyperpolarized potentials in pyramidal neurons from HCN1 knockout mice.
knockout mice (-91.2 ± 1.6 mV vs. -106.0 ± 2.0 mV in control and knockout, respectively, n=10 & 12, p<0.05; see Fig. 3D). The differences in amplitude, kinetics and voltage dependence of $I_h$ obtained in cortical pyramidal neurons from control and HCN1 knockout mice are entirely consistent with removal of fast-activating HCN1 subunits, leaving a small residual current mediated primarily by slowly-activating HCN2 subunits that operate over a more hyperpolarized voltage range (Franz et al. 2000b; Santoro et al. 2000).

The differences in $I_h$ characteristics observed in cortical neurons from HCN1 knockout mice were reflected in altered membrane properties of those cells, as determined by voltage recordings obtained under current clamp (Fig. 2). As compared to wild type mice, cortical neurons from HCN1 knockout animals presented with a more hyperpolarized resting membrane potential (RMP), a higher peak input resistance ($R_N$) and they showed less voltage ‘sag’ in response to hyperpolarizing current steps, particularly at more depolarized membrane potentials. Moreover, whereas blocking $I_h$ with CsCl (3 mM) caused a marked hyperpolarization and increased peak $R_N$ of control cells, it had little effect on RMP and peak $R_N$ in pyramidal neurons from HCN1 knockout mouse; furthermore, genotype-dependent differences in RMP, $R_N$ and ‘sag’ largely disappeared after blocking $I_h$ with 3 mM CsCl.

Overall, this analysis reveals differences in $I_h$ characteristics and intrinsic membrane properties of cortical pyramidal neurons that are expected from deletion of the HCN1 subunit. In addition, our results are remarkably similar to those reported previously for hippocampal pyramidal neurons of HCN1 knockout and wild type mice (Nolan et al. 2004).
Effects of isoflurane on $I_h$ attributed to HCN1 subunits were absent in cortical cells from HCN1 knockout animals.

In previous work, we correlated anesthetic actions on cloned HCN channels and native neuronal $I_h$ in order to assign distinct features of anesthetic modulation to particular HCN subunits. Specifically, we found that modulation of homomeric HCN1 channels by inhalational anesthetics was associated primarily with hyperpolarizing shifts in $V_{1/2}$, whereas modulation of homomeric HCN2 channels was associated with decreased current amplitude; with heteromeric HCN1-HCN2 channels, inhibition by inhalational anesthetics included effects on both current amplitude and voltage dependence (Chen et al. 2005b). Our next set of experiments took advantage of HCN1 knockout mice, and the demonstrated absence of HCN1-like currents in cortical neurons, in order to test directly the subunit-specific effects proposed for these anesthetics.

The effects of the inhalational anesthetic, isoflurane, on cortical pyramidal neuron $I_h$ are presented in Fig 3. For these experiments, we used supraclinical concentrations of isoflurane (0.9 mM) to ensure full modulation of neuronal currents in cells from both wild type and knockout mice. Isoflurane caused inhibition of maximal current amplitude (-455.5 ± 89.4 pA vs. -238.9 ± 51.0 pA, n=9, p<0.001 by paired t-test) and a negative shift in voltage dependence of $I_h$ activation ($V_{1/2}$: -94.3 ± 2.3 mV vs. -106.0 ± 2.5 mV, p<0.001) in neurons from wild type animals (Fig. 3A & 3B, upper). In cells from HCN1 knockout mice, isoflurane decreased the amplitude of the small residual $I_h$ (see Inset in Fig. 3B, lower left) but had no effect on voltage dependence of activation (Fig. 3B, lower right); current amplitude at -128 mV was reduced by isoflurane from -120.2 ± 18.1 pA to -58.8 ± 19.1 pA (n=6, p<0.05 by paired t-test) but $V_{1/2}$ remained unchanged in the presence of isoflurane (-106.4 ± 1.7 mV vs. -106.9 ± 1.9 mV,
Summary data indicate that whereas isoflurane diminished $I_h$ by ~50% in cells from both wild type and knockout animals (Fig. 3C; $48.2 \pm 3.5\%$ vs. $50.3 \pm 13.5\%$, n=9 & 6) the shift in $V_{1/2}$ induced by isoflurane ($-11.7 \pm 2.1 \text{ mV}$, n=9) in cortical neurons from wild type mice was not observed in cells from HCN1 knockout mice (Fig. 3D).

Inhalational anesthetics modulate HCN2 channels in a cAMP-dependent manner; when intrinsic allosteric inhibition of HCN2 channels is relieved by cAMP, modulation by anesthetics is associated with less amplitude inhibition and a more pronounced shift in $V_{1/2}$ (Chen et al. 2005b). As shown in Fig. 3D, the $V_{1/2}$ of $I_h$ activation was ~10 mV more depolarized in cortical cells from HCN1 knockout mice recorded with 50 µM cAMP in the patch pipette, indicative of relief from intrinsic allosteric inhibition. Anesthetic modulation of the residual, cAMP-stimulated current in cortical cells from HCN1 knockout mice was as expected for HCN2 channels; the anesthetic-induced decrease in current amplitude was reduced to 18.5 ± 6% (vs. ~50% without cAMP; p<0.05) and a clear shift in $V_{1/2}$ was now obtained ($-6.6 \pm 0.7 \text{ mV}$; from $-96.9 \pm 1.3 \text{ mV}$ to $-102.8 \pm 1.6 \text{ mV}$; n= 5, p<0.001).

We also compared effects of isoflurane on membrane potential, input resistance and ‘sag’ in cortical pyramidal neurons from wild type and HCN1 knockout mice under current clamp conditions (Fig. 4). All recordings were performed in the continued presence of bicuculline and strychnine (both at 30 µM), tetrodotoxin (0.5 µM) and barium (0.2 mM). Representative traces from a wild type cell are shown in Fig. 4A, and averaged data from wild type and HCN1 knockout mice are provided in Figs. 4B and 4C. Isoflurane caused membrane hyperpolarization of neurons from wild type animals, with subsequent application of 3 mM CsCl further hyperpolarizing the cells (Fig. 4A, 4B). Anesthetic actions were associated with an increase in $R_N$ (~12% increase, from
197.8 ± 28.7 MΩ to 223.7 ± 24.4 MΩ; n=5, both p<0.05) and a decrease in
voltage ‘sag’ that was evident when compared using current steps that
hyperpolarized the cell to the same peak potential (i.e., to -88 mV; Fig. 4C).
These effects of isoflurane on membrane properties were mediated by $I_h$ since
they were eliminated in neurons from HCN1 knockout animals (Figs. 4B & 4C).

**Deletion of HCN1 also reduces $I_h$ in motoneurons, leaving a residual
HCN2-like current modulation by anesthetics.**

We also verified HCN subunit-selective modulation of $I_h$ by isoflurane in
motoneurons; these cells are implicated in immobilizing anesthetic actions
(Franks 2006; Rudolph and Antkowiak 2004; Sonner et al. 2003) and they
express HCN1 and HCN2 subunits (Monteggia et al. 2000; Santoro et al. 2000).
Hypoglossal motoneurons were recorded in brainstem slices using an acidified
bath (pH 6.5) to inhibit motoneuronal anestheticsensitive TASK-like currents,
and with elevated HEPES in the pipette (25 mM) in order to minimize changes in
intracellular pH (Sirois et al. 2002); $I_h$ properties were not affected by these
manipulations (data not shown; see (Sirois et al. 2002).

Recordings were performed in motoneurons from neonatal control and
HCN1 knockout mice (P11-P15); the age distribution was not different between
mouse lines and we found no age-dependent changes in $I_h$ density within the age
span considered, in either group of cells (data not shown). However, the
magnitude and properties of motoneuronal $I_h$ were different between cells from
the two mouse lines. Sample voltage clamp records in Fig. 5A depict $I_h$ in
representative motoneurons from control (upper) and HCN1 knockout mice
(lower). By comparison to cells from wild type animals, $I_h$ was reduced in
amplitude (by ~32% at -128 mV; Fig. 5B, left) and activated ~5-fold more slowly
in motoneurons from HCN1 knockout mice (Fig. 5B, right). In addition, the
initial $V_{1/2}$ of $I_h$ activation was more hyperpolarized in motoneurons from HCN1 knockout mice ($-108.9 \pm 1.2$ mV vs. $-113.5 \pm 1.4$ mV in control and knockout, respectively; $n=15$ & 14, $p<0.05$). These data are as expected for deletion of fast activating HCN1 subunits from motoneurons and retention of a slower HCN2-like current that activates at more hyperpolarized potentials.

As shown in Figs. 5A & 5C, we also found that anesthetic effects on $I_h$ in motoneurons from control and HCN1 knockout mice were consistent with HCN subunit-selective anesthetic actions (Chen et al. 2005b). Isoflurane (0.9 mM) decreased current amplitude in both control (% inhibition: $40.1 \pm 5.7\%$, $n=6$; $P<0.05$ by paired t-test) and HCN1 knockout motoneurons (% inhibition: $32.1 \pm 5.4\%$, $n=6$; $P<0.05$); however, the shift in $V_{1/2}$ of activation typically evoked by isoflurane in motoneurons from control animals ($\Delta V_{1/2}: -7.0 \pm 1.3$ mV; $P<0.05$) was absent in cells from HCN1 knockout mice ($\Delta V_{1/2}: -0.9 \pm 1.5$ mV; $p=0.56$).

The smaller $I_h$ in motoneurons from HCN1 knockout mice was associated with a more hyperpolarized membrane potential and an increased $R_N$, as determined under whole cell current clamp (Fig. 5D). In motoneurons from wild type mice, isoflurane caused hyperpolarization from the resting membrane potential and increased $R_N$. In cells from HCN1 knockouts, isoflurane had no effect on resting membrane potential, where residual HCN2 currents show little activity; however, an isoflurane-induced increase in $R_N$ was apparent with hyperpolarizing current injection, as expected given the decrease in maximal $I_h$ amplitude with isoflurane that we observed at hyperpolarized potentials.

In sum, the properties and anesthetic modulation of motoneuronal $I_h$ were consistent with removal of HCN1 subunit-containing channels from those cells. In addition, results from motoneurons were generally similar to those obtained in cortical pyramidal cells. It is noteworthy, however, that $I_h$ was considerably larger in motoneurons than in cortical cells from wild type mice ($\sim 470$ pA vs.
HCN1 subunit deletion had a smaller effect on motoneuronal $I_h$ (i.e., in motoneurons of HCN1 knockout mice, residual $I_h$ was ~300 pA, comparable to $I_h$ in cortical neurons from wild type mice). Thus, by comparison to cortical neurons, HCN1 subunits provide a relatively smaller contribution to the larger $I_h$ in motoneurons and the residual isoflurane-sensitive $I_h$ in motoneurons from HCN1 knockout mice remains substantial in amplitude.

**Isoflurane enhances temporal summation in cortical pyramidal neurons from wild type mice, but not from HCN1 knockouts**

HCN channel expression and $I_h$ density increase with distance from the soma in pyramidal neurons (Magee 1999; 2000), and it has become clear that dendritic $I_h$ acts to shunt synaptic currents and thereby decrease temporal summation of synaptic inputs (Desjardins et al. 2003; Magee 1999). This suggests the possibility that modulation of dendritic $I_h$ and synaptic integration in cortical pyramidal neurons may also be a consequence of anesthetic action.

We first employed a computer simulation in a cortical pyramidal neuron model (Day et al. 2005) in order to test independently the subunit-specific actions of anesthetics on $I_h$ and assess their effects on EPSP temporal summation, incorporating: [1] 50% inhibition of peak current amplitude (e.g., mimicking effects of isoflurane on HCN2 homomeric channels); [2] -10 mV shift in $V_{1/2}$ of $I_h$ activation (e.g., mimicking effects of isoflurane on HCN1 homomeric channels); or [3] a combination of 50% amplitude inhibition with -10 mV shift in $V_{1/2}$ (e.g., mimicking effects of isoflurane on heteromeric HCN1-HCN2 channels). We performed the computer simulation from two initial membrane potentials. The first approximated resting membrane potential of cortical neurons (~70 mV); the second, from ~90 mV, was chosen to maximize effects of $I_h$ on EPSP
summation, minimize effects of other voltage dependent channels and match conditions used in our experimental tests (see below).

As depicted in Figs. 6A & 6B, simulating anesthetic-induced inhibition of \( I_h \) caused membrane hyperpolarization and enhanced EPSP summation from either starting membrane potential; this was true regardless of whether the inhibition took the form of a decrease in current amplitude, a shift in \( V_{1/2} \), or both. As expected, the initial shunting effect of \( I_h \) on EPSP summation was most pronounced in the simulation performed at the more hyperpolarized potential, where \( I_h \) is most active (EPSP5/EPSP1 was 2.1 at -70 mV and 1.5 at -90 mV); likewise, inhibition of \( I_h \) also had a more prominent effect on membrane potential and EPSP summation in the simulation performed at -90 mV. In that case, simulating decreased \( I_h \) amplitude, a -10 mV shift in \( V_{1/2} \) and both produced a hyperpolarization of the neuron model (from -90.8 mV to -93.4 mV, -95.1 mV and -98.4 mV, respectively) and enhanced temporal summation of EPSPs (EPSP5/EPSP1 increased from 1.5 to 1.7, 2.2 and 2.4, respectively). Note that the shift in \( V_{1/2} \), by itself, produced a large relative enhancement of EPSP summation (48% increase) that was much greater than that accompanying the decrease in current amplitude (15% increase) and only slightly less than that obtained with the combination of both amplitude inhibition and a shift in \( V_{1/2} \) (59% increase). Note, also, that the enhanced EPSP summation accompanying \( I_h \) inhibition was not due to the change in membrane potential since hyperpolarizing the neuron model to those same initial potentials with simulated DC current injection, in the absence of \( I_h \) inhibition, actually depressed the EPSP5:EPSP1 ratio (data not shown).

We next examined, experimentally, the effect of isoflurane on temporal summation of EPSPs evoked in cortical pyramidal neurons by using local field stimulation (40 Hz, 5V-10V) in superficial cortical layers. In cells from wild type
mice, we found that isoflurane (0.4 mM) caused membrane hyperpolarization and enhanced EPSP summation by >60% (Fig. 6C-6E), producing results similar to those obtained with the neuron simulation. Again, the enhanced EPSP summation was observed in spite of the hyperpolarization, which by itself diminished summation (e.g., see effect of DC injection in Fig. 6D). It should also be noted that individual EPSP amplitudes were decreased by isoflurane at these concentrations (Fig. 6C, right), as has been reported by others (Berg-Johnsen and Langmoen 1992; Maclver et al. 1996; Wu et al. 2004); to the extent possible, we adjusted for decreased EPSP amplitudes by increasing stimulus intensity in the presence of isoflurane. The enhanced EPSP summation by isoflurane could be attributed to $I_h$ inhibition since it was mimicked by ZD-7288 (50 $\mu$M), a potent $I_h$ channel antagonist, and diminished in neurons from HCN1 knockout mice (Fig. 6E). Note that although isoflurane decreased maximal $I_h$ in cortical neurons from HCN1 knockout mice (see Fig. 3), this likely had only relatively modest effects on EPSP summation in the knockout since the initial shunting action of the small residual HCN currents would be minimal.

During slow wave sleep and in light planes of anesthesia, a cortically-derived rhythm underlies synchronous neuronal firing at low frequencies (< 1 Hz), with coherent cortical activity driven by synaptic inputs (Steriade et al. 1993b). In order to determine how effects of isoflurane on $I_h$ and EPSP summation influence overall cortical neuron excitability we exposed cortical pyramidal neurons to a low, hypnotic concentration of isoflurane (0.15 mM) while applying the 40Hz stimulus train every second, mimicking a ~1 Hz rhythm of cortical synaptic inputs. An example is shown in Fig. 7. Under control conditions, the cell occasionally fired an action potential triggered by summating EPSPs (Fig. 7A, left & Fig. 7C). Exposure to isoflurane caused a small membrane hyperpolarization (~2-3 mV) and enhanced excitability such that many more
stimulus events were associated with action potential discharge (Fig. 7A, middle & Fig. 7C); after a brief wash, the effect on excitability partially reversed (Fig. 7A, right & Fig. 7C). Examination of individual events revealed that isoflurane caused an increase in EPSP summation that was sufficient to trigger action potential discharge despite the attendant membrane hyperpolarization (Fig. 7B). We obtained similar results in two additional pyramidal neurons. Thus, at concentrations of isoflurane expected to support synaptically driven coherent cortical activity in vivo, we found a net increase in synaptic excitability that could be attributed to effects of isoflurane on $I_h$ (i.e., membrane hyperpolarization and increased EPSP summation).
DISCUSSION

Inhalational anesthetics induce subunit-specific inhibition of HCN channels that is evident at clinically relevant concentrations; this appeared to be reproduced in recordings of $I_h$ from neurons that natively express different HCN subunits (Chen et al. 2005b). In the present study, we used whole cell recording in motoneurons and cortical pyramidal neurons from HCN1 knockout mice to verify subunit-specific actions of inhalational anesthetics on native $I_h$. In these neurons from HCN1 knockout mice, isoflurane did not cause the typical HCN1-like hyperpolarizing shift in $V_{1/2}$ of activation, but it inhibited maximal amplitude of a substantially smaller and slower $I_h$, an HCN2-like action; by comparison to cortical neurons, the larger fraction of residual current in motoneurons suggests a relatively greater contribution from HCN2 channel subunits in those cells. Under current clamp, we found that anesthetic-induced inhibition of $I_h$ caused membrane hyperpolarization and enhanced temporal summation of evoked EPSPs in cortical cells; in NEURON simulations, changes in $I_h$ that were reminiscent of isoflurane actions on HCN1-containing channels (i.e., hyperpolarizing shift in $V_{1/2}$) had the most pronounced effect on synaptic summation and, accordingly, the isoflurane-enhanced EPSP summation was absent in HCN1 knockout mice. In wild type mice, the enhanced synaptic summation evoked by isoflurane increased the probability of cortical neuron discharge. These data indicate that isoflurane acts primarily by inhibition of HCN1-containing channels to cause membrane hyperpolarization and enhanced synaptic summation, conditions that favor generation of synchronized cortical slow wave oscillations that are associated with sleep and anesthetic-induced hypnosis (Bazhenov et al. 1998; Hill and Tononi 2005).
**HCN subunit-specific effects of general anesthetics on \( I_h \)**

Anesthetic modulation of \( I_h \) causes membrane hyperpolarization, increased \( R_N \) and a decrease in voltage-dependent ‘sag’. The anesthetic-induced changes in membrane properties were absent in neurons from HCN1 knockout mice, suggesting that those effects primarily reflect inhibition of HCN1-containing channels. This result is consistent with the idea that HCN1 homomeric and/or HCN1-HCN2 heteromeric channels provide a substantial contribution to \( I_h \) near neuronal resting membrane potential by virtue of their relatively depolarized \( V_{1/2} \) of activation (Chen et al. 2001; Franz et al. 2000a; see also Nolan et al. 2004; 2007)

In earlier work, we attributed distinct effects of inhalational anesthetics on neuronal \( I_h \) to specific HCN subunits (Chen et al. 2005b); those conclusions are now strongly supported by this work. We previously found that different forms of \( I_h \) inhibition by inhalational anesthetics – shift in \( V_{1/2} \) and decrease in current amplitude – were associated, respectively, with homomeric HCN1 and HCN2 channels; in heteromeric HCN1-HCN2 channels, inhalational anesthetics caused both a shift in \( V_{1/2} \) and a decrease in current (Chen et al. 2005b). We could attribute the subunit-specific effects of anesthetics to differences in allosteric inhibition imposed by HCN subunit C-terminal domains, as reflected in initial \( V_{1/2} \) of \( I_h \) activation (Chen et al. 2005b). Accordingly, we found here that isoflurane shifted \( V_{1/2} \) and decreased current amplitude in cortical pyramidal neurons from wild type animals, where \( I_h \) included both an HCN1 and HCN2 subunit contribution and presented with a relatively depolarized initial \( V_{1/2} \) (~91 mV). In cells from HCN1 knockout mice, where residual \( I_h \) was slower and activated at more hyperpolarized potentials (\( V_{1/2} \sim -106 \) mV), as expected for HCN2 homomeric channels, the isoflurane-induced inhibition of \( I_h \) included only
a decrease in current amplitude, with no concomitant shift in $V_{1/2}$. In cells from HCN1 knockout animals recorded in the presence of cAMP, the initial $V_{1/2}$ of activation of the residual $I_h$ was shifted to depolarized levels and isoflurane modulation included a prominent hyperpolarizing shift in $V_{1/2}$ and a smaller inhibition of current amplitude. This effect of cAMP on anesthetic modulation is also consistent with previous results obtained with cloned HCN2 channels.

**Anesthetics enhance EPSP summation in cortical neurons by inhibiting $I_h$**

It is now clear that dendritic $I_h$ in cortical pyramidal neurons, which increases in density with distance from the soma, serves as a current shunt to reduce temporal summation of synaptic inputs; inhibition of $I_h$ decreases the dendritic shunt, resulting in enhanced summation of synaptic inputs (Magee 1999; Magee 2000). Our results indicate that isoflurane enhances EPSP summation; these effects were predicted by simulating anesthetic-induced $I_h$ inhibition in a cortical neuron computer model and they were mimicked by application of the $I_h$ blocker ZD-7288. Moreover, the anesthetic-induced increase in temporal summation observed in wild type mice was absent in HCN1 knockout mice, further verifying a role for inhibition of $I_h$. Interestingly, our computer simulation revealed that a shift in $V_{1/2}$ (an HCN1-like effect) induced a relatively larger enhancement of EPSP summation than a decrease in current amplitude (an HCN2-like effect), suggesting that anesthetic modulation of HCN1-containing channels may potentiate synaptic inputs more effectively than inhibition of HCN2 homomeric channels. The combination of synaptic enhancement (via effects on dendritic $I_h$) with membrane hyperpolarization (via effects on somatic $I_h$) provides a background in which isolated synaptic inputs are less effective whereas effects of synchronous excitatory synaptic potentials are enhanced. In general, this
situation would favor coherent oscillatory behavior (Carr et al. 2007), and indeed, decreases in $I_h$ have been observed in a number of epilepsy models in rodents (Jung et al. 2007; Kole et al. 2007; Shah et al. 2004).

**Possible neural mechanism for HCN1-mediated actions of isoflurane**

Could the isoflurane-mediated, HCN1 subunit-dependent membrane hyperpolarization and enhanced synaptic summation that we have described in cortical neurons contribute to behavioral actions of isoflurane?

At low concentrations of isoflurane, the anesthetic induces a hypnosis that is accompanied by EEG oscillations that resemble slow waves of deep sleep (Antkowiak 2002). In thalamic and cortical neurons, $I_h$ contributes to intrinsic mechanisms that contribute to slow rhythms (McCormick and Bal 1997; Rudolph and Antkowiak 2004; Steriade et al. 1993a) and in thalamocortical network models, two major changes in cortical neuron properties are necessary for transitions into synchronized, oscillating cortical activity: membrane hyperpolarization and enhanced excitatory synaptic transmission (Bazhenov et al. 1998; Hill and Tononi 2005). As mentioned above, both these effects accompany isoflurane-induced inhibition of $I_h$ in cortical neurons. Moreover, we showed directly that, at hypnotic concentrations (0.1-0.2 mM), isoflurane acted to increase postsynaptic excitability in cortical neurons by enhancing EPSP summation (see Fig. 7). So, we suggest that inhibition of $I_h$ by anesthetics contributes to the membrane hyperpolarization that supports oscillatory activity in cortical neurons while also reducing dendritic shunt to promote synaptically-mediated synchronization of rhythmic cortical activity. Insofar as oscillations and cortical synchronization are critical components of slow wave EEG activity, we suggest that these effects of isoflurane on HCN1-containing channels may contribute to anesthetic-induced hypnosis in wild type animals.
It is now widely accepted that decreased motoneuronal excitability accounts for immobilizing effects of anesthetics (Franks 2006; Rudolph and Antkowiak 2004; Sonner et al. 2003) and we previously noted that isoflurane-mediated inhibition of $I_h$ plays a part in decreasing motoneuronal excitability via its contribution to membrane hyperpolarization (Sirois et al. 1998). Our results from HCN1 knockout mice indicate that the hyperpolarizing shift in $V_{1/2}$ of $I_h$ caused by isoflurane in motoneurons, as in cortical cells, is due largely to effects on HCN1 subunits; likewise the hyperpolarizing actions of isoflurane mediated by $I_h$ inhibition can also be attributed to HCN1-containing channels. Unlike in cortical cells, however, there is no polarized distribution of HCN1 channels in motoneurons, which instead are expressed strongly on both somatic and dendritic membranes (Milligan et al., 2006). So, it is uncertain if any synaptic enhancement that might result from dendritic HCN1 channel inhibition can overcome the concurrent somatic membrane hyperpolarization to increase excitability in motoneurons, as we observed in cortical pyramidal cells. Moreover, activation of TASK-like background K$^+$ currents by isoflurane largely account for the depressed somatic excitability of motoneurons by causing membrane hyperpolarization and increased input conductance (Sirois et al. 1998; 2000), effects which would further limit any increased excitability due to synaptic enhancement; such a countervailing effect on excitability was not observed in cortical pyramidal cells, where we found little evidence for K$^+$ channel modulation by isoflurane (XC & DAB, unpublished observations). It is also worth noting that we found a more substantial isoflurane-sensitive HCN2-like residual $I_h$ in motoneurons from HCN1 knockout mice (~30% inhibition of a current that remained ~70% of wild type) that could contribute to net effects of isoflurane on motoneuronal excitability. So, if inhibition of $I_h$ plays a role in
immobilizing effects of isoflurane at the level of motoneurons, this likely involves actions on channels including both HCN1 and HCN2 subunits.

The cellular data we obtained from HCN1 knockout mice are consistent with the idea that HCN1 subunits are a viable target for isoflurane action and may contribute to its anesthetic actions in wild type mice. However, this does not imply that HCN1 knockout mice are themselves a good model for assessing the behavioral consequences of HCN1 deletion; indeed, a number of caveats must be considered. First, isoflurane inhibits both HCN1 and HCN2 subunits, and only HCN1 is deleted in these knockouts. As we discuss with respect to effects on motoneurons and immobilization, a substantial residual HCN2-mediated \( I_h \) may continue to provide an effective target. Also, in thalamus, a brain region linked to sleep and hypnosis (McCormick and Bal 1997; Rudolph and Antkowiak 2004; Steriade et al. 1993a), HCN2 is the predominant subunit in both thalamocortical and thalamic reticular neurons (Ludwig et al. 2003; Santoro et al. 2000; Ying et al. 2007); effects of isoflurane on \( I_h \) would be preserved in those cells from HCN1 knockout mice. For cortical neurons, where HCN1 subunits are largely responsible for \( I_h \), a second complicating issue is that of compensation; in those cells, we find a markedly enhanced (~3-fold), isoflurane-activated tonic GABA\(_A\) current (XC & DAB, unpublished observations). The presence of this confounding isoflurane-sensitive current in the knockout mice precludes simple interpretation of behavioral data in terms of HCN1 deletion from those cells.

In conclusion, by using HCN1 knockout mice, we verified HCN1 subunit-specific contributions to effects of isoflurane on \( I_h \) in motoneurons and cortical pyramidal neurons. We also showed that inhibition of HCN1-containing channels largely accounts for isoflurane-induced hyperpolarization and increased temporal summation of EPSPs in cortical pyramidal neurons, two cellular effects
associated with slow wave thalamocortical oscillations similar to those observed in light planes of anesthesia. A definitive test of a behavioral role for inhibition of $I_h$ by isoflurane may require experiments with knockout of both HCN1 and HCN2 and/or development of HCN knockout mice without confounding compensatory currents (e.g., by using GABA$_A$ and HCN1 double knockout mice).
REFERENCES


FIGURE LEGENDS

Figure 1. HCN1 subunit deletion results in a smaller and slower $I_h$ in cortical pyramidal neurons from HCN1 knockout mice.

A. Average traces from quadruplicate determinations by quantitative RT-PCR of HCN1 and HCN2 subunit expression in cortex of control and HCN1 knockout mice. Note that HCN1 subunit mRNA is undetectable and HCN2 expression is not up-regulated in HCN1 knockout mice. 

B. Expression of the indicated HCN subunits in cortex from control and HCN1 knockout animals (n=5 each); for each subunit, data are expressed as averaged fold-difference from a cyclophilin control ($2^{-\Delta Ct}$). (*, significantly different from control, p<0.001 by unpaired t-test).

C. Photomicrograph of biocytin-stained neurons recorded from neocortex in wild type (upper) and HCN1 knockout (lower) mice. Scale bar represents 50 µm.

D. Voltage clamp recordings of $I_h$ in wild type and HCN1 knockout mice (from cells presented in C). The voltage clamp protocol depicted is typical of that used throughout these studies (conditioning voltage steps were always 3-4 s in duration).

E. Time-dependent currents were measured at the end of each voltage step in wild type (WT) or HCN1-KO (KO) mice and plotted as a function of membrane potential (left). The amplitude of $I_h$ (at -130 mV) was determined from individual cells and plotted as a frequency distribution (middle) and averaged (right, ± SEM). $I_h$ was ~70% smaller in cortical cells from HCN1 subunit knockout mice.

F. Sample traces of $I_h$ activation recorded at -120 mV in cells from wild type and HCN1 knockout mice were normalized and overlaid (left); the residual current from the knockout animal activated more slowly than $I_h$ from wild-type mice. Activation kinetics were analyzed by using biexponential fits to currents evoked at -120 mV; time constants ($\tau$) describing the fastest (and largest) current component were obtained from individual cells and plotted as a
frequency distribution (middle) and averaged (right, ± SEM). $I_h$ activation was markedly slower in cortical neurons from HCN1 knockout mice. *, significantly different from wild type, $p<0.001$ by unpaired t-test.

**Figure 2. Cortical neurons from HCN1 channel knockout mice have a more hyperpolarized membrane potential, higher input resistance and display less ‘sag’.**

A. Voltage recordings in cortical pyramidal neurons from wild type (WT) and HCN1 knockout (KO) mice during current injection (from 60 pA to -100 pA, see Inset). Under control conditions (left), the cortical neuron from the HCN1 knockout was more hyperpolarized (-74 mV vs. -66 mV) and had higher input resistance than the cell from a wild type animal; note, also, that the depolarizing ‘sag’ associated with $I_h$ activation was less evident in HCN1 knockout mice, especially at less hyperpolarized potentials (e.g., see indicated traces that reach -88 mV). In the presence of CsCl, the depolarizing sag was essentially eliminated. Note, also, that the membrane hyperpolarization associated with Cs⁺-mediated inhibition of $I_h$ in the wild type cell was not present in the HCN1-KO cell. B. Frequency distributions (upper) and averaged data (lower) for resting membrane potential (RMP, left), input resistance ($R_N$, middle) and voltage ‘sag’ (right, from -88 mV) obtained in cortical neurons from wild type and HCN1 knockout mice. Knocking out the HCN1 subunit or blocking all $I_h$ in cortical neurons by using Cs⁺ induced a hyperpolarization in membrane potential and an increase in input resistance (*, significantly different from wild type control by one-way ANOVA, $p<0.05$). The Cs⁺-sensitive voltage ‘sag’ is plotted against peak membrane potential obtained during the current injection; the sag in HCN1-KO mice was nearly absent at -88 mV (see arrow) and was diminished relative to wild type animals at all potentials. All recordings were performed in
the continued presence of bicuculline (30 µM), strychnine (30 µM), tetrodotoxin (0.5 µM) and barium (0.2 mM).

Figure 3. *Subunit-selective effects of isoflurane on native Iₘ are revealed in cortical pyramidal neurons from HCN1 knockout mice.*

**A.** Sample voltage clamp recordings illustrating Iₘ in cortical pyramidal neurons from wild type (*upper*) and HCN1 knockout (*lower*) mouse under control conditions (*left*) and in the presence of 0.9 mM isoflurane. **B.** Effects of isoflurane on Iₘ properties in cortical neurons from wild type (*upper*) and HCN1 knockout mouse (*lower*). Time-dependent currents were measured at the end of hyperpolarizing voltage steps under control conditions (*squares*) and in the presence of isoflurane (*triangles*) and plotted as a function of membrane potential (*left*). Likewise, normalized tail currents were obtained following incrementing hyperpolarizing steps, averaged and fitted with Boltzmann curves in control and in the presence of isoflurane (*right*). In cells from wild-type animals, isoflurane induced a shift in V½ together with inhibition of maximal current amplitude. In cortical neurons from HCN1 knockout mice, isoflurane retained the ability to inhibit current amplitude (see *Inset* for data on expanded scale in **B, lower**) but was without effect on V½. (*, significantly different from control by paired t-test, p<0.05). **C.** Averaged isoflurane-evoked inhibition of maximal current amplitude (at -130 mV) in cells from wild type and HCN1-KO mice, recorded with or without 50 µM cAMP in the recording pipette. The inhibition of Iₘ amplitude by isoflurane was preserved in cells from HCN1 knockout mice, but amplitude inhibition was reduced by cAMP. **D.** Voltage dependence of Iₘ activation was determined in wild type and knockout cells (with or without cAMP) by using normalized tail currents fitted with Boltzmann curves;
averaged half-activation potentials (\(V_{1/2}; \text{left}\)) and effects of isoflurane on activation gating (\(\Delta V_{1/2}; \text{right}\)) indicate that initial \(V_{1/2}\) was shifted to more hyperpolarized potentials in HCN1-KO cortical neurons and the hyperpolarizing shift in \(V_{1/2}\) typically associated with isoflurane was absent in those knockout neurons. In the presence of cAMP, however, isoflurane caused a shift in \(V_{1/2}\) from a more depolarized initial value in cells from HCN1 knockout mice. (*, significantly different by one-way ANOVA or two-way RM-ANOVA, \(p<0.05\)).

**Figure 4. Isoflurane effects on membrane properties are diminished in cortical neurons from HCN1 knockout mice.**

A. Sample voltage responses to injected current in wild type cortical neurons under control conditions and in the presence of isoflurane (0.9 mM), followed by 3 mM CsCl. B. Isoflurane induced a membrane hyperpolarization in wild type cells whereas in neurons from HCN1 knockout mice, initial membrane potential was more hyperpolarized and isoflurane did not cause further hyperpolarization. C. Voltage dependent ‘sag’ was determined during current steps that reached the same peak voltage (~88 mV). In wild type neurons, isoflurane significantly decreased voltage ‘sag’. In HCN1 knockout animals the initial ‘sag’ was reduced and unaffected by isoflurane. All recordings were performed in the continued presence of bicuculline (30 \(\mu\)M), strychnine (30 \(\mu\)M), tetrodotoxin (0.5 \(\mu\)M) and barium (0.2 mM). (*, significantly different from control, \(p<0.05\) by two-way ANOVA).

**Figure 5. \(I_h\) is reduced in amplitude and isoflurane effects on \(I_h\) are HCN2-like in motoneurons from HCN1 knockout mice.**

A. Sample voltage clamp recordings depict \(I_h\) in hypoglossal motoneurons from a wild type (upper) and HCN1 knockout mouse (lower) under control conditions...
(left) and in the presence of isoflurane (0.9 mM; right). Note that residual $I_h$ in the knockout motoneuron activates more slowly and is reduced in amplitude by comparison to wild type; note, also, that inhibition of $I_h$ by isoflurane remains substantial in HCN1 knockout motoneurons. Cells were recorded in an acidified bath (pH 6.5) with raised intracellular HEPES (25 mM) in order to minimize effects on voltage clamp conditions secondary to anesthetic activation of motoneuronal TASK-like currents. **B. Left:** Averaged amplitude of $I_h$ (at -130 mV) was determined in individual motoneurons from wild type and HCN1 knockout mice; $I_h$ was reduced by ~33% in the knockout cells. **Right:** Averaged fast activation time constants ($\tau_f$ at -120 mV) for $I_h$ in motoneurons from control and HCN1 knockout mice; current activation was ~5-fold slower in knockout motoneurons (*, significantly different from wild type by unpaired t-test, p<0.05). **C.** Effects of isoflurane on $I_h$ in motoneurons from wild type and HCN1 knockout mice. **Left:** Averaged isoflurane-evoked inhibition of maximal current amplitude (at -130 mV) was not different in cells from wild type and HCN1 knockout mice. **Right:** The isoflurane-induced hyperpolarizing shift in $V_{1/2}$ was absent in motoneurons from HCN1 knockout mice. (*, significantly different from wild type by one-way ANOVA, p<0.05). **D.** Basic membrane properties and effects of isoflurane in motoneurons from wild type and HCN1 knockout mice. **Left:** Averaged RMP was more hyperpolarized and isoflurane had no effect on RMP in HCN1 knockout mice. **Right:** Averaged initial $R_N$ was greater in HCN1 knockout mice and isoflurane inhibition of residual $I_h$ led to a further increase in $R_N$. (*, significantly different from wild type by one-way ANOVA, p<0.05).
Figure 6. *Isoflurane enhances EPSP temporal summation in cortical pyramidal neurons from wild type mice, but not from HCN1 knockout mice.*

A & B. Computer simulations in a cortical neuron model from two different initial potentials (-70 mV & -90 mV) illustrate effects on EPSP summation accompanying a 50% decrease in $I_h$ amplitude, -10 mV shift in $V^{1/2}$ of activation, or both. Aligned traces illustrate the enhanced summation observed in the model with both a decrease in $I_h$ amplitude and a shift in $V^{1/2}$, and histogram plots show effects on EPSP summation of independently or coordinately changing these two parameters in the model.

C. Sample voltage traces show EPSP recordings obtained from cortical pyramidal neuron in response to 40 Hz, 7V stimulation under control conditions and after incubation in 0.4 mM isoflurane (*right*). In order to enhance basal dendritic $I_h$, current injection was used to produce an initial somatic membrane potential of -90 mV; EPSPs are aligned to initial membrane potential and normalized to the amplitude of the first EPSP in the train in order to highlight enhanced temporal summation of EPSPs.

D. Summation was quantified as the amplitude ratio of the fifth to first EPSP and plotted with respect to time; isoflurane enhanced temporal summation. Note that DC injection to hyperpolarize the neuron caused a decrease of EPSP summation, consistent with a contribution of $I_h$ to the sub-linear summation under control conditions.

E. Averaged percent increase of EPSP summation (ratio of EPSP5/EPSP1, relative to control) induced by 0.4 mM isoflurane or 50 µM ZD-7288 or in wild type (WT) and HCN1 knockout (KO) mice; isoflurane and ZD-7288 enhanced EPSP summation in cortical neurons from wild-type animals (*, P<0.05 vs. control), but had no effect in cells from HCN1 knockout mice.
**Figure 7. Inhibition of I_h by isoflurane increases synaptic excitability in cortical neurons.**

**A.** Effects on wild type cortical pyramidal neurons of 40 Hz electrical stimulation delivered at a rate of 1 Hz to the superficial cortex under control conditions (*left*), in the presence of 0.15 mM isoflurane (*middle*) and following wash (*right*). (Spikes are truncated in this panel and in B, below). **B.** An expanded view of two stimulus events under control conditions (*blue*) and during exposure to isoflurane (*red*); note the slight hyperpolarization and enhanced EPSP summation in isoflurane, when both events were associated with spike firing. **C.** Quantification of data from this cell, illustrating the increased likelihood that the synaptic stimulation triggered a spike in the presence of isoflurane (as a percentage of all stimulation events in a 2 minute epoch under the indicated conditions). These results are representative of those obtained in the two other cells studied with this protocol.
Figure 1. HCN1 subunit deletion results in a smaller and slower Ih in cortical pyramidal neurons from HCN1 knockout mice.

155x218mm (300 x 300 DPI)
Figure 2. Cortical neurons from HCN1 channel knockout mice have a more hyperpolarized membrane potential, higher input resistance and display less 'sag'.

173x149mm (300 x 300 DPI)
Figure 3. Subunit-selective effects of isoflurane on native Ih are revealed in cortical pyramidal neurons from HCN1 knockout mice.
Figure 4. Isoflurane effects on membrane properties are diminished in cortical neurons from HCN1 knockout mice.

178x50mm (300 x 300 DPI)
Figure 5. Ih is reduced in amplitude and isoflurane effects on Ih are HCN2-like in motoneurons from HCN1 knockout mice.
Figure 6. Isoflurane enhances EPSP temporal summation in cortical pyramidal neurons from wild type mice, but not from HCN1 knockout mice.

169x97mm (300 x 300 DPI)
Figure 7. Inhibition of Ih by isoflurane increases synaptic excitability in cortical neurons.