Forebrain HCN1 Channels Contribute to Hypnotic Actions of Ketamine

Cheng Zhou, M.S.,* Jennifer E. Douglas, B.A.,† Natasha N. Kumar, Ph.D.,‡ Shaofang Shu, B.S.,§ Douglas A. Bayliss, Ph.D.,‖ Xiangdong Chen, M.D., Ph.D.#

ABSTRACT

Background: Ketamine is a commonly used anesthetic, but the mechanistic basis for its clinically relevant actions remains to be determined. The authors previously showed that HCN1 channels are inhibited by ketamine and demonstrated that global HCN1 knockout mice are twofold less sensitive to hypnotic actions of ketamine. Although that work identified HCN1 channels as a viable molecular target for ketamine, it did not determine the relevant neural substrate.

Methods: To localize the brain region responsible for HCN1-mediated hypnotic actions of ketamine, the authors used a conditional knockout strategy to delete HCN1 channels selectively in excitatory cells of the mouse forebrain. A combination of molecular, immunohistochemical, and cellular electrophysiologic approaches was used to verify conditional HCN1 deletion; a loss-of-righting reflex assay served to ascertain effects of forebrain HCN1 channel ablation on hypnotic actions of ketamine.

Results: In conditional knockout mice, HCN1 channels were selectively deleted in cortex and hippocampus, with expression retained in cerebellum. In cortical pyramidal neurons from forebrain-selective HCN1 knockout mice, effects of ketamine on HCN1-dependent membrane properties were absent; notably, ketamine was unable to evoke membrane hyperpolarization or enhance synaptic inputs. Finally, the EC_{50} for ketamine-induced loss-of-righting reflex was shifted to significantly higher concentrations (by approximately 31%).

Conclusions: These data indicate that forebrain principal cells represent a relevant neural substrate for HCN1-mediated hypnotic actions of ketamine. The authors suggest that ketamine inhibition of HCN1 shifts cortical neuron electroresponsive properties to contribute to ketamine-induced hypnosis.

What We Already Know about This Topic

- HCN1 channels are inhibited by ketamine, but the neural substrate for this blockade and a full understanding of the mechanistic basis for ketamine's clinically relevant actions remain to be determined

What This Article Tells Us That Is New

- Using a conditional knockout strategy to selectively delete HCN1, a relevant neural substrate for ketamine's clinical action, HCN1-mediated hypnotic actions of ketamine was found in forebrain principal (excitatory) cells, consistent with the view that ketamine inhibition of HCN1 alters cortical neuron electroresponsive properties

* Ph.D. Student, # Professor, Department of Anesthesiology, Laboratory of Anesthesia & CCM, Translational Neuroscience Center, State Key Laboratory of Biotherapy of Cancer, West China Hospital of Sichuan University, Chengdu, Sichuan, People's Republic of China. † Undergraduate Neuroscience Student, ‡ Research Associate, § Lab Technician, ‖ Professor, Department of Pharmacology and Anesthesiology, University of Virginia, Charlottesville, Virginia.

Received from the Department of Anesthesiology, Laboratory of Anesthesia & CCM, Translational Neuroscience Center, State Key Laboratory of Biotherapy of Cancer, West China Hospital of Sichuan University, Chengdu, Sichuan, People's Republic of China. Submitted for publication May 1, 2012. Accepted for publication January 3, 2013. Supported by GM66181 (to Dr. Bayliss) from the National Institutes of Health, Bethesda, Maryland; and grant 81171274 (to Dr. Chen) from the National Natural Science Foundation of China, Beijing, People's Republic of China; the 2009 Young Investigator Award Program (to Dr. Chen) from the National Alliance for Research on Schizophrenia and Depression, Great Neck, New York; and the Fundamental Research Funds for the Central Universities (to Dr. Chen) from the Ministry of Education of China, Beijing, People's Republic of China.

Address correspondence to Dr. Chen: Department of Anesthesiology, Translational Neuroscience Center, West China Hospital of Sichuan University, Chengdu, Sichuan, People's Republic of China. xiangdong_chen@yahoo.com. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

 Anesthesiology 2013; 118:785–95

MA Presented at the American Society of Anesthesiologists Annual Meeting, October 2012.

◆ This article is accompanied by an Editorial View. Please see: Forman SA: The expanding genetic toolkit for exploring mechanisms of general anesthesia. ANESTHESIOLOGY 2013; 118:769–71.

ANESTHETIC compounds represent some of the most useful drugs in the clinical arsenal. Despite extensive clinical experience and intensive experimental scrutiny, the molecular and neural mechanisms by which most of these drugs mediate their important actions remain elusive.[1,2] Prevailing ideas hold that contributions of individual molecular or neuronal targets depend on the particular anesthetic compound and the specific clinical endpoint examined.[1-3] This implies that understanding actions of any anesthetic drug demands information regarding how modulation of a given target within a defined neural system can yield each clinically relevant outcome.[2,3] In this work, we use conditional knockout mice to test whether inhibition of HCN1 channels specifically in forebrain principal cells contributes to ketamine-induced hypnosis.

In our earlier work, we made the unexpected observation that ketamine inhibited recombinant HCN1 channels at clinically relevant concentrations *via* a decrease in maximal current amplitude and a hyperpolarizing shift in the *V½* of channel activation.[4] These inhibitory effects of ketamine were stereoselective, with greater potency of channel inhibition obtained with the S-(+)-ketamine isomer that is also the more potent anesthetic, and subunit selective, modulating HCN1-containing homomeric or HCN1–HCN2 heteromeric channels but not HCN2 homomeric channels. We validated this action of ketamine on an HCN1-mediated native neuronal hyperpolarization-activated cationic current (I_h) by showing that ketamine-mediated inhibition of neuronal I_h was absent in HCN1$^{-/-}$ mice. We also demonstrated that mice with global deletion of HCN1 were approximately twofold less sensitive to ketamine-induced hypnosis, providing strong evidence that HCN1 channels represent a relevant target for this anesthetic endpoint.[4]

A role for HCN1 channels in constraining oscillatory activity in forebrain pyramidal neurons is predicted based on their depolarizing influence on membrane potential and by virtue of their ability to shunt excitatory synaptic inputs.[5–7] Indeed, we found that inhibition of HCN1 channels by ketamine led to membrane hyperpolarization and improved dendritosomatic synaptic transfer in cortical pyramidal neurons.[4] Thus, insofar as these two effects—membrane hyperpolarization and increased synaptic efficacy—are associated with an enhanced propensity for coherent cortical rhythms,[8,9] such as those accompanying ketamine-induced hypnosis,[10] we proposed that deletion of this ketamine target in cortical neurons could contribute to the diminished hypnotic actions of ketamine we observed in HCN1$^{-/-}$ mice.[4]

Because HCN1 subunits were deleted globally in the ketamine-resistant HCN1 knockout mice used for our previous work, we sought here to use conditional knockout strategies to better localize the neural substrate for HCN1-dependent actions of ketamine. To this end, we took advantage of a line of mice in which HCN1 channels were deleted only from forebrain principal cells, and we focused on the hypnotic endpoint because that was strongly affected in global knockouts.[4] Insofar as we find that forebrain-selective HCN1 knockout mice largely recapitulated the decreased sensitivity to ketamine-induced hypnosis observed previously in global HCN1 knockout animals, these new results reinforce the idea that inhibition of forebrain HCN1 channels can contribute to hypnotic actions of anesthetics.

Materials and Methods

Animals

We used a Cre-loxP strategy to generate forebrain-selective deletion of HCN1 channels, essentially as described previously.[6,11] In this system, genetic engineering is performed in mice to incorporate two so-called loxP sites flanking a region of the targeted gene (*i.e.*, to "flox" the gene); when mice bearing the floxed gene are crossed to a separate group of mice that express the bacteriophage Cre-recombinase, the enzyme will excise the region between the loxP sites, inactivating the gene.[12] For this work, we obtained mice with loxP sites flanking the fourth exon of the HCN1 gene, the region that encodes the channel pore domain and S6 transmembrane segment, from Dr. Eric Kandel (Columbia University, New York, NY).[6,11] We also obtained the R1ag#5 line of transgenic mice from Dr. Scott Zeitlin (University of Virginia, Charlottesville, VA).[13,14] In these mice, Cre-recombinase is expressed selectively in forebrain principal cells under the control of the CaMKIIα-promoter,[13,14] providing the opportunity to restrict Cre-mediated gene inactivation only to those cells. The CaMK-Cre mice were crossed with HCN1$^{f/+}$ mice and the progeny intercrossed to obtain breeding lines in which CaMKCre:HCN1$^{f/f}$ mice were mated with HCN1$^{f/f}$ mice, yielding littermates homozygous for floxed HCN1 alleles that were either Cre^{+} (forebrain-selective knockouts) or Cre^{-} (control). All procedures involving animals were approved by the University of Virginia Animal Care and Use Committee (Charlottesville, VA).

Immunohistochemistry

To confirm HCN1 channel deletion from HCN1$^{-/-}$ mice and from the forebrain of CaMKCre:HCN1ff mice, HCN1 immunohistochemistry was performed concurrently on tissue obtained from HCN1$^{+/+}$, HCN1$^{-/-}$, HCN1$^{f/f}$, and CaMKCre:HCN1$^{f/f}$ mice (n = 3). Mice were anesthetized with an intramuscular injection of ketamine (ketamine, 200 mg/kg; xylazine, 14 mg/kg) and perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde/0.1 M phosphate buffer. Brains were removed and postfixed in the same solution overnight. Coronal brain sections (30 μm) including the cerebellum, hippocampus, and cortex were cut on a vibrating microtome (VT1000S; Leica Microsystems, Wetzlar, Germany) and stored in cryoprotectant (50% phosphate-buffered saline, 30% ethylene glycol, and 20% glycerol) until they were used for immunohistochemistry. Free-floating sections were washed in 0.1 M Tris-buffered saline before incubation for

30 min in 1% H_2O_2 and again washed in buffered saline. After incubating sections in blocking reagent (Basic M.O.M Immunodetection Kit; Vector Laboratories, Burlingame, CA) for 1 h, tissue was incubated overnight at 4°C in mouse anti-HCN1 (1:1000; clone N70/28; UC Davis/NIH NeuroMab Facility, Davis, CA). Thereafter, sections were incubated for 1 h in M.O.M. biotinylated anti-mouse IgG reagent followed by 1 h with avidin–biotin–peroxidase complex (Vectastain ABC Elite Kit; Vector Laboratories), before visualization with 3,3′-diaminobenzidine (DAB peroxidase substrate kit; Vector Laboratories). The 3,3′-diaminobenzidine reaction was quenched by washing in 0.1 M phosphate buffer. Sections were then mounted onto gelatinized slides, dried, dehydrated in ethanol, cleared in xylene, and cover-slipped using DPX mounting media (VWR International, Radnor, PA). Images were obtained on a Zeiss FS microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) equipped with a PixelFly digital camera (Cooke Corp., Romulus, MI) using IPLab software (Scanalytics; Spectra Services Inc., Ontario, NY).

Quantitative Real-Time Polymerase Chain Reaction

HCN1 expression levels were determined in Cre$^-$ floxed control mice (HCN1$^{f/f}$) and CaMKCre-expressing HCN1$^{f/f}$ mice (CaMKCre:HCN1$^{f/f}$) by using quantitative real-time polymerase chain reaction (qRT-PCR), as described previously.[15] The cortex, hippocampus, and cerebellum were microdissected for RNA isolation from adult mice (with five of more animals per genotype). Total RNA was extracted from all tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. An equal quantity of RNA from each sample was reverse transcribed using Superscript II (Invitrogen) enzyme. The resulting complementary DNA was assayed to quantify the relative abundance of various mRNA species using the SYBR Green Real-Time polymerase chain reaction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The qRT-PCR was performed from each sample in quadruplicate, using an ICycler (Bio-Rad Laboratories, Hercules, CA), a previously validated HCN1 primer set (upper, AGG TTA ATC AGA TAC ATA CACC; lower, GAG TGC GTA GGA ATA TTG TTTT; 231-bp amplicon) and PCR conditions (3 min at 95°C; 40 cycles: 10 s, 95°C; 40 s, 60°C; 40 s, 72°C) that yielded greater than or equal to 97% efficiency.[15] We used cyclophilin as an internal standard (upper, GGC TCT TGA AAT GGA CCC TTC; lower, CAG CCA ATG CTT GAT CAT ATT CTT; 91-bp amplicon), and control samples with no added template were included with every experiment. Each animal contributed a single data point, and qRT-PCR data were analyzed using a modification of the so-called ΔΔCt normalization procedure to obtain HCN1 mRNA levels for each genotype, relative to cyclophilin.[16]

Electrophysiologic Recordings from Mouse Cortical Pyramidal Neurons

Mice of both sexes (14–22 days old) were anesthetized (ketamine, 200 mg/kg; xylazine, 14 mg/kg, intramuscular injection) and transverse brain slices prepared as described previously.[4,15,17] Slices were submerged in a recording chamber on a Zeiss Axioskop FS microscope and visualized with Nomarski optics; pyramidal cells were targeted for recording based on location in the slice and characteristic size and shape. For voltage clamp recording, pipettes (2–4 MΩ) were filled with KCH_3O_3S, 120 mM; NaCl, 4 mM; $MgCl_2$, 1 mM; $CaCl_2$, 0.5 mM; HEPES, 10 mM; EGTA, 10 mM; MgATP, 3 mM; and GTP-Tris, 0.3 mM (pH 7.2); for current clamp, pipette solution contained KCl, 17.5 mM; potassium gluconate, 122.5 mM; HEPES, 10 mM; EGTA, 0.2 mM; NaCl, 9 mM; $MgCl_2$, 1 mM; MgATP, 3 mM; and GTP-Tris, 0.3 mM (pH 7.2). Recordings were obtained with an Axopatch 200B amplifier (Axon Instruments, Inc., Union City, CA) at room temperature (~24°C) in standard bath solution containing NaCl, 140 mM; KCl, 3 mM; HEPES, 10 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2 mM; and glucose, 10 mM; we routinely added tetrodotoxin (0.5 μM; Alomone Labs, Jerusalem, Israel), $BaCl_2$ (200 μM), and bicuculline/strychnine (both at 30 μM; Sigma, St. Louis, MO) to the bath, except where noted. In some experiments, ZD-7288 was used to block I_h (50 μM; Tocris Cookson, Bristol, United Kingdom). Ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) was diluted into the bath solution at the indicated concentrations.

The properties of HCN channel currents and neuronal I_h were determined from whole-cell voltage clamp experiments, essentially as described.[18] Under current clamp, we measured input resistance and the magnitude of depolarizing "sag" from voltage response to hyperpolarizing current injection. We evoked excitatory postsynaptic potentials (EPSPs) in cortical pyramidal neurons using a pipette that was placed in the superficial layers of the cortex and connected to a stimulator (Grass S48; Grass Technologies, West Warwick, RI) *via* a stimulus isolation unit (Grass SIU5; Grass Technologies); the pipette was filled with standard bath solution and synaptic responses were evoked by applying 40-Hz, 5- to 10-V pulses (corresponding to 2–10 μA). The EPSP summation was quantified as the ratio of the amplitudes of the last and first evoked EPSPs in the train (EPSP5:EPSP1).[4,15] All data are corrected (–8 mV) for a measured liquid junction potential.

Analysis of Anesthetic Action in Mice

Bolus injections of ketamine (5–20 mg/kg) were administered *via* the tail vein of each mouse (2–4 months old). Each animal was injected with only a single concentration of the drug on any given day; some animals received multiple doses (on different days, always separated by at least 1 week), but no individual animal contributed more than one data point for any given dose. Hypnosis was established by loss-of-righting reflex (LORR) that occurred within 10 s after completion of the injection and that persisted for at least 10 s thereafter[19]; we also measured the latency to regain the righting reflex.[19]

To assay ketamine effects on gross sensorimotor function, we used RotaRod (Med Associates Inc., Georgia, VT) and

tail flick assays, essentially as described.[20] For motor function, mice were placed on a rod rotating at constant acceleration (5–40 rpm), and the length of time the animal was able to stay on the rod was recorded (Med Associates Inc., Georgia, VT), under control conditions and then immediately after receiving a subanesthetic dose of ketamine (2.5 mg/kg, IV). To measure sensitivity to painful stimuli, mice were lightly restrained in a commercially available apparatus (Tail-Flick Unit 7360; UgoBasile, Comerio, Italy) while a radiant heat source (intensity and cutoff time were set at 90 and 12 s) was directed onto the tail; the time required for the mice to remove their tails from the heat source was recorded, before and after treatment with ketamine (20 mg/kg, IV, at 30-s intervals for 5 min)

Data Acquisition and Analysis

Data were analyzed statistically with SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Results are presented as mean ± SEM. Comparisons of mean values were performed using two-way ANOVA, with *post hoc* pairwise comparisons used the Tukey correction of the *t* test. For evaluation of neuronal electroresponsive properties, genotype and drug treatment (ketamine, ZD7288) represented principal factors, with a repeated measures design allowing comparisons for each cell under different conditions. For behavioral effects of ketamine analyzed by two-way ANOVA with genotype and dose as principal factors (fig. 5), each observation was treated as an independent data point because not all individual animals were assessed at each dose. Quantal dose–response data of hypnotic effects of ketamine were fitted using logistic equations to obtain EC_{50} values for hypnosis in Cre^+ and Cre^- $HCN1^{f/f}$ mice, and those values were analyzed statistically using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) by ANOVA. Differences in mean values were considered significant for values of $P < 0.05$.

Results

To test the role of forebrain HCN1 expression in anesthetic actions of ketamine, we crossed $HCN1^{f/f}$ mice with a line of CaMKCre transgenic mice that express Cre-recombinase under the control of the CaMKIIα-promoter (see Materials and Methods for details of this conditional knockout strategy).[13,14] Of relevance here, CaMKIIα is expressed only in excitatory (*i.e.*, principal) cells of the forebrain, with expression progressively increasing from barely detectable levels at birth to approximately 10-fold higher levels by postnatal day 21 and a further approximately 2.5-fold increase by postnatal day 90.[21] Thus, this construct drives Cre expression selectively to pyramidal neurons of the hippocampus and neocortex in the postnatal period,[13,14] where it is expected to inactivate the HCN1 channel gene. It is worth noting that forebrain-selective HCN1 knockout mice were described previously to have enhanced spatial memory but otherwise normal sensorimotor function.[6,11]

We first verified forebrain-specific deletion of HCN1 and loss of HCN1-dependent membrane properties in cortical pyramidal cells from CaMKCre:$HCN1^{f/f}$ mice, including ketamine modulation. After confirming HCN1 deletion and absence of ketamine actions on HCN1 channels in those cells, we tested the sensitivity of CaMKCre:$HCN1^{f/f}$ mice to ketamine-induced hypnosis and effects on sensorimotor function.

HCN1 Expression Is Selectively Deleted from Forebrain of CaMKCre:$HCN1^{f/f}$ Mice

To demonstrate that the HCN1 channel gene was deleted selectively from forebrain neurons in CaMKCre:$HCN1^{f/f}$ mice, we performed immunohistochemistry and qRT-PCR to detect HCN1 expression. As shown in figure 1A, immunostaining for HCN1 was observed in the cortex, hippocampus, and cerebellum from mice that were homozygous for either wild-type ($HCN1^{+/+}$) or floxed ($HCN1^{f/f}$) alleles at the HCN1 locus; note the prominent immunostaining in apical dendrites from cortical pyramidal neurons and in the molecular layer of the hippocampal CA1 region (see arrows) (stratum lacunosum-moleculare). In tissue from global HCN1 knockout mice ($HCN1^{-/-}$), HCN1 expression was undetectable in all brain areas. However, consistent with forebrain selective knockout expected for CaMKCre:$HCN1^{f/f}$ mice,[6] HCN1 staining was largely absent from cortex and hippocampus but retained in cerebellum. Corroborating these immunohistochemical results, our qRT-PCR analysis revealed that HCN1 mRNA was reduced by more than 90% in cortex and hippocampus of CaMKCre:$HCN1^{f/f}$ mice (decreased by 93.6% and 96.8%, respectively), with no difference in HCN1 transcript levels in cerebellum (fig. 1B). Together, these data confirm forebrain-specific deletion of HCN1 in CaMKCre:$HCN1^{f/f}$ mice.

I_h Is Reduced and Ketamine Effects Are Diminished in Cortical Pyramidal Neurons from CaMKCre:$HCN1^{f/f}$ Mice

To verify deletion of HCN1 channel subunits functionally, we determined whether HCN1-dependent membrane properties and HCN1-dependent ketamine actions were diminished in cortical pyramidal neurons of CaMKCre:$HCN1^{f/f}$ mice.

In cortical pyramidal neurons, I_h is primarily mediated by HCN1 and HCN2 channels, with HCN1 subunits conferring relatively faster activation kinetics and more depolarized voltage-dependence in homomeric HCN1 channels or heteromeric HCN1–HCN2 channels[17,22]; homomeric HCN2 channels activate with slower kinetics and at more hyperpolarized potentials.[17,22] Accordingly, as shown in the voltage clamp recordings of figure 2, I_h was smaller (I_h amplitude: 316.5 ± 42.6 pA for Cre^- *vs.* 102.0 ± 13.4 pA for Cre^+) and $V½$ of current activation was more negative ($V½$: −92.6 ± 1.6 mV for Cre^- *vs.* −101.2 ± 1.6 mV for Cre^+) in pyramidal neurons from CaMKCre:$HCN1^{f/f}$ mice than in those same cells from control $HCN1^{f/f}$ mice (n = 5 and 7 for Cre^- and Cre^+, $P < 0.05$ for each variable). In addition, the residual I_h in

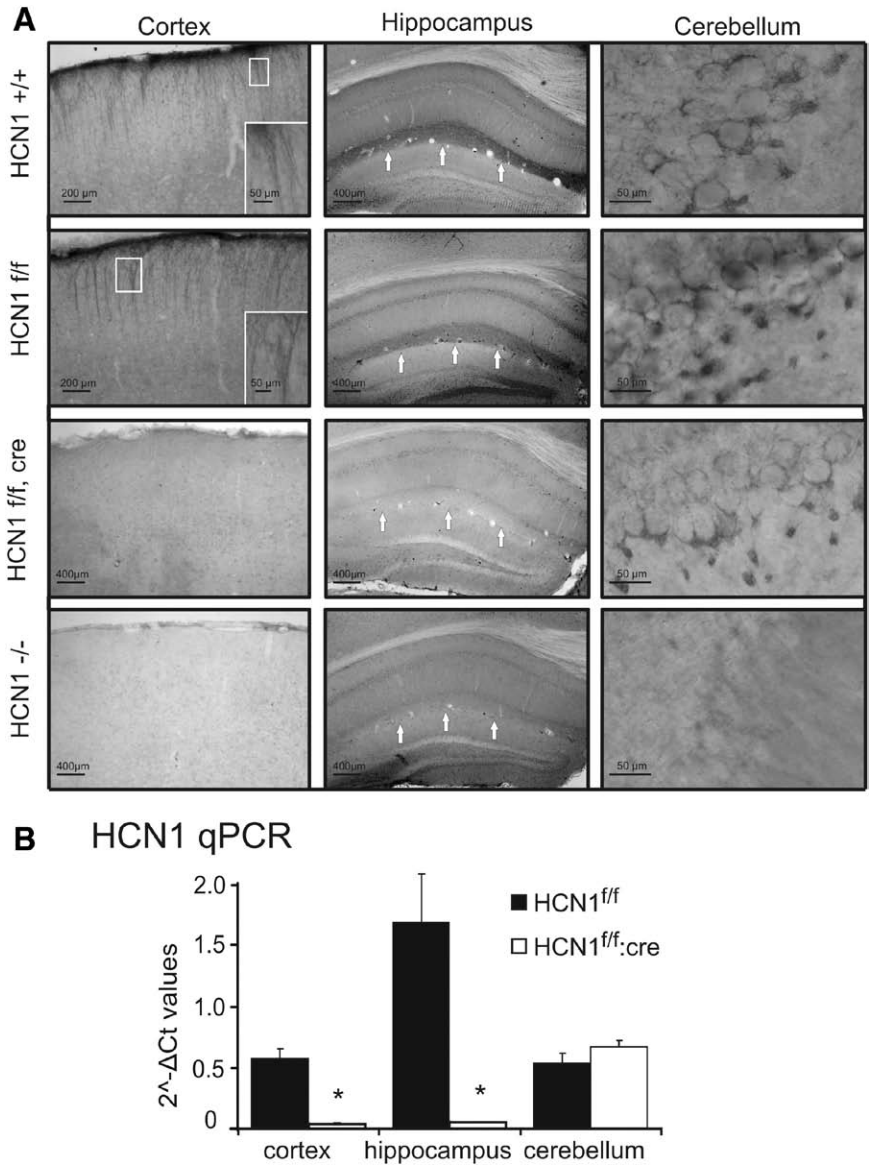

Fig. 1. HCN1 is selectively deleted from forebrain neurons in CaMKCre:HCN1$^{f/f}$ mice. (*A*) HCN1 channel expression was assessed by immunohistochemistry in the cortex, hippocampus, and cerebellum from wild-type (HCN1$^{+/+}$) or floxed (HCN1$^{f/f}$) mice, from global HCN1 knockout mice (HCN1$^{-/-}$) and from floxed mice expressing Cre-recombinase under the CaMKIIα promoter (CaMKCre:HCN1$^{f/f}$). The characteristic HCN1 expression in apical dendrites of the cortex and in stratum lacunosum-moleculare of CA1 in the hippocampus (*arrows*) that is typical of wild-type mice was also present in HCN1$^{f/f}$ mice, but was absent in both HCN1$^{-/-}$ and CaMKCre:HCN1$^{f/f}$ mice. The *insets* highlight dendritic staining, at 4× magnification. Note that HCN1 expression was preserved in the cerebellum of all mice except the global knockout mice. (*B*) Quantitative real-time polymerase chain reaction (qPCR) shows that HCN1 transcript levels were reduced by over 90% in both the cortex and hippocampus from CaMKCre:HCN1$^{f/f}$ mice by comparison with HCN1$^{f/f}$ mice, but HCN1 expression in the cerebellum was not different (n = 5, P < 0.05 by two-way ANOVA, * HCN1$^{f/f}$:cre *vs.* HCN1$^{f/f}$).

pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice showed slower activation kinetics (τ_{fast} at −118 mV: 106.4 ± 24.0 ms for Cre$^-$ *vs.* 358.3 ± 50.0 ms for Cre$^+$; data not shown). These results are consistent with deletion of HCN1 from pyramidal neurons of CaMKCre:HCN1$^{f/f}$ mice, leaving a residual I_h provided primarily by homomeric HCN2 channels.

We previously found that ketamine inhibits recombinant HCN channels that contain an HCN1 subunit in either HCN1 homomeric or HCN1–HCN2 heteromeric conformations; by contrast, ketamine had no appreciable effect on HCN2 homomeric channels.[4] This subunit-selective action of ketamine was also apparent in its effects on native I_h in cortical pyramidal neurons, which express both HCN1 and HCN2: ketamine inhibited I_h in pyramidal neurons from wild-type mice but not from global HCN1 knockout mice.[4] Likewise, as shown in figure 2, we found

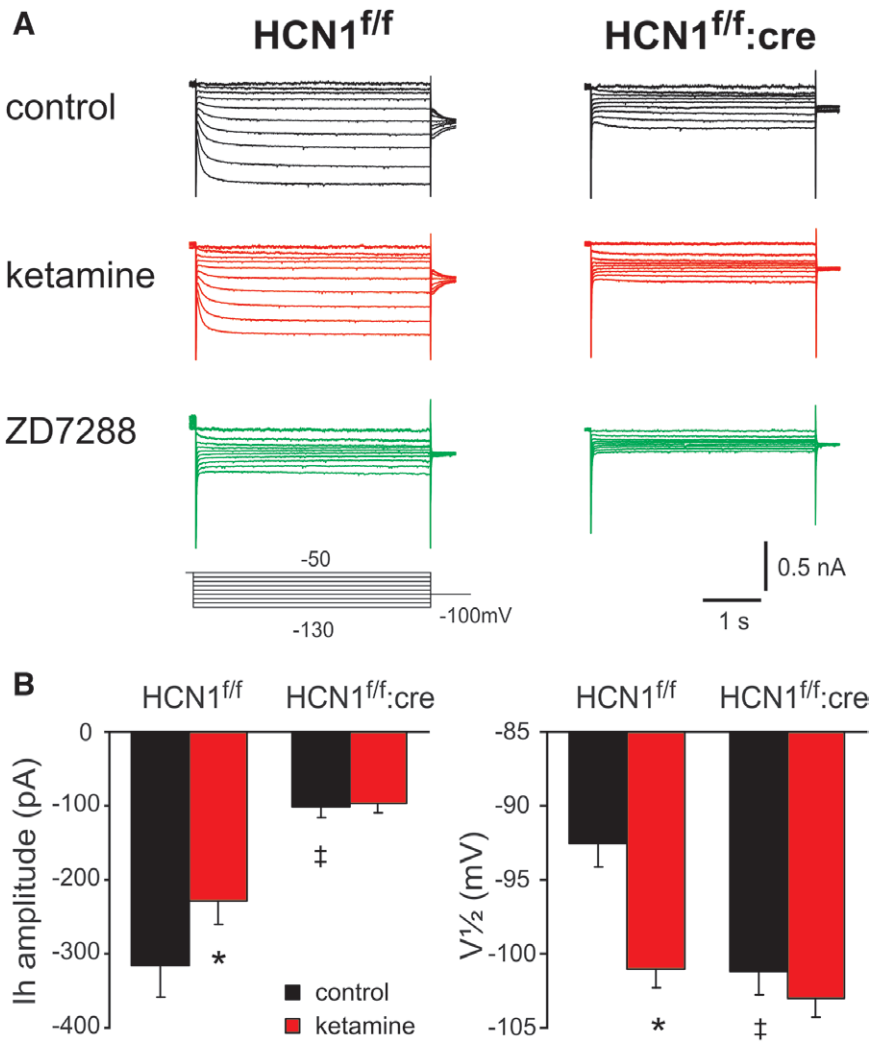

Fig. 2. I_h is diminished and the effects of ketamine on I_h are reduced in cortical pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice. (*A*) Sample voltage clamp recordings of I_h in cortical pyramidal neurons from HCN1$^{f/f}$ and CaMKCre:HCN1$^{f/f}$ mice under control conditions (*above*), during exposure to ketamine (20 μM, *center*) and following treatment with the I_h blocker ZD-7288 (50 μM, *below*). (*B*) Averaged data ± SE depicting I_h amplitude (at –118 mV) and *V*½ of activation of I_h in cortical pyramidal neurons from HCN1$^{f/f}$ mice and CaMKCre:HCN1$^{f/f}$ mice. These data show that I_h was smaller with a more hyperpolarized *V*½ in cortical pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice; they also reveal that the ketamine-induced decrease in current amplitude and hyperpolarizing shift in *V*½ observed in cells from HCN1$^{f/f}$ mice were absent in CaMKCre:HCN1$^{f/f}$ mice. Note that ZD7288 completely blocked I_h in cells from both genotypes; thus, amplitude and *V*½ data after ZD7288 are not presented in *B* (n = 5 and 7, $P < 0.05$, * ketamine *vs.* control; ‡ HCN1$^{f/f}$:cre *vs.* HCN1$^{f/f}$).

that ketamine inhibited I_h in cortical pyramidal neurons from control HCN1$^{f/f}$ mice by evoking a hyperpolarizing shift in voltage dependence of activation ($\Delta V½$: –8.4 ± 1.7 mV, from –92.6 ± 1.6 mV to –101.0 ± 1.3 mV) together with a decrease in maximal current amplitude (% inhibition: 27.6 ± 4.5%, from 316.5 ± 42.6 pA to 228.0 ± 32.8 pA; n = 5, $P < 0.05$ for both variables), whereas ketamine had essentially no effect on the smaller and slower residual I_h in cortical pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice ($\Delta V½$: –1.8 ± 1.0 mV, from –101.2 ± 1.6 mV to –103.0 ± 1.3 mV; % inhibition: 2.9 ± 4.2%, from 102.0 ± 13.4 pA to 97.5 ± 11.8 pA, n = 7, $P < 0.05$) (fig. 2). After ketamine treatment, ZD7288 completely blocked all remaining I_h in cells from both genotypes. Together, these data indicate that the ketamine-sensitive HCN1 subunit was effectively deleted in cortical pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice, leaving a residual I_h that is unaffected by ketamine.

It is known that I_h contributes a resting inward current that provides a depolarizing membrane potential bias in cortical pyramidal neurons; in addition, during a step hyperpolarization, voltage-dependent activation of I_h produces a depolarizing "sag" in the membrane potential trajectory.[5,22] Thus, as shown in figure 3, and as expected for deletion of HCN1 channels, cortical pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice presented with a more hyperpolarized resting membrane potential (resting

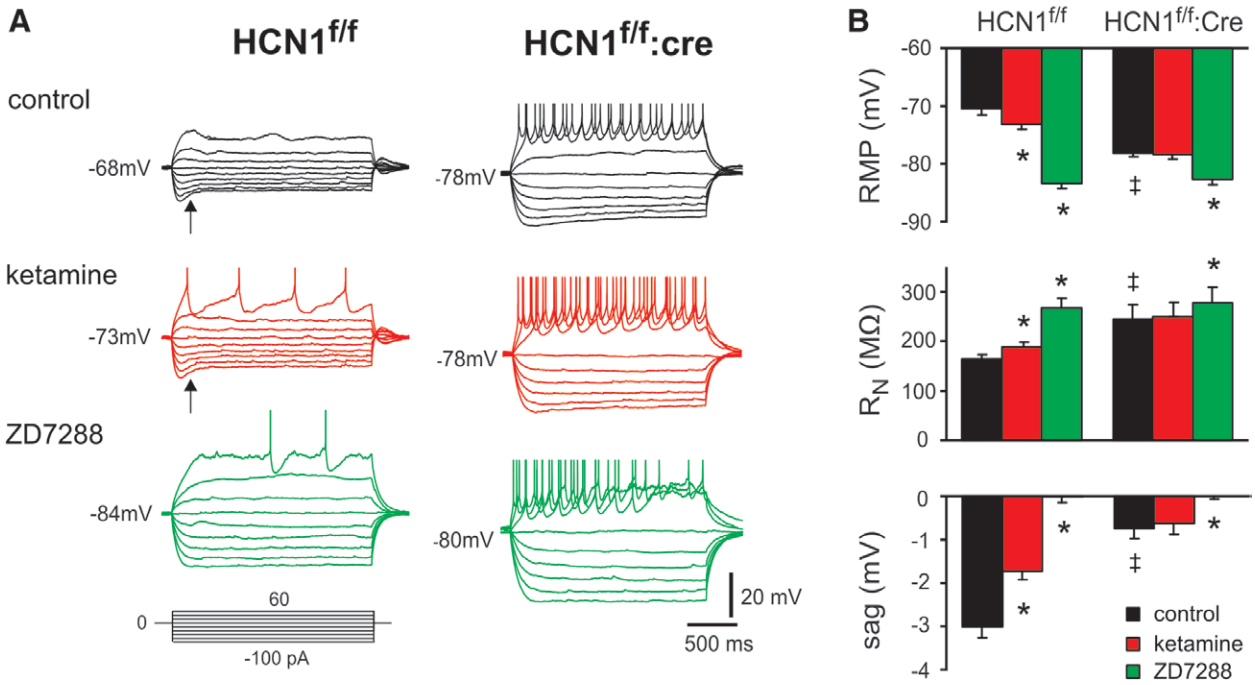

Fig. 3. HCN1 deletion from cortical pyramidal neurons of CaMKCre:HCN1 $^{f/f}$ mice alters basal membrane properties and occludes ketamine modulation. (*A*) Sample current clamp recordings from cortical pyramidal neurons from HCN1$^{f/f}$ and CaMKCre:HCN1$^{f/f}$ mice under control conditions (*above*) and during exposure to ketamine (20 μM, *center*) and ZD-7288 (50 μM, *below*). The depolarizing membrane sag observed during membrane hyperpolarization, a phenomenon attributed to I_h, is indicated by the *arrow*. Tetrodotoxin was not applied in these experiments. (*B*) Averaged data ± SE show that membrane potential was more hyperpolarized, R_N was higher, and sag was reduced in cortical pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice. Ketamine hyperpolarized membrane potential, increased R_N, and decreased sag only in cells from HCN1$^{f/f}$ mice (n = 5 and 5, $P < 0.05$, * ketamine *vs.* control; ‡ HCN1$^{f/f}$:cre *vs.* HCN1$^{f/f}$).

membrane potential: −70.4 ± 1.1 mV for Cre$^-$ *vs.* −78.1 ± 0.7 mV for Cre$^+$), greater input resistance (R_N: 164.1 ± 8.9 MΩ for Cre$^-$ *vs.* 244.3 ± 29.1 MΩ for Cre$^+$), and diminished sag (sag at −90 mV: −3.0 ± 0.3 mV for Cre$^-$ *vs.* −0.8 ± 0.2 mV for Cre$^+$), by comparison with pyramidal neurons from control HCN1$^{f/f}$ mice (n = 5 for Cre$^-$ and Cre$^+$, $P < 0.05$ for each variable). Moreover, whereas ketamine caused membrane hyperpolarization (resting membrane potential: from −70.4 ± 1.1 mV to −73.1 ± 0.9 mV, n = 5, $P < 0.05$), increased input resistance (R_N: from 164.1 ± 8.9 MΩ to 180.5 ± 11.1 MΩ, n = 5, $P < 0.05$), and reduced depolarizing sag (from −3.0 ± 0.3 mV to 1.7 ± 0.2 mV at −90 mV; n = 5, $P < 0.05$) in pyramidal neurons from control HCN1$^{f/f}$ mice, these actions of ketamine were occluded in pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice (resting membrane potential: from −78.1 ± 0.7 mV to −78.3 ± 0.8 mV; R_N: from 244.3 ± 29.1 MΩ to 248.9 ± 27.4 MΩ; sag: from −0.8 ± 0.2 mV to −0.6 ± 0.2 mV at −90 mV; n = 5, $P >$ 0.05 for all variables). Again, blocking the remaining I_h with ZD7288 eliminated all genotype-dependent differences in resting membrane potential, R_N, or sag. Overall, these data are consistent with removal of the ketamine-sensitive HCN1 subunit in cortical pyramidal neurons from CaMKCre:HCN1$^{f/f}$ mice.

Ketamine Modulation of Synaptic Properties Is Abolished in Cortical Pyramidal Neurons of CaMKCre:HCN1$^{f/f}$ Mice

In cortical pyramidal neurons, HCN1 is most prominently expressed in distal dendritic domains (*e.g.*, see fig. 1A) and the corresponding dendritic I_h provides a major current shunt that serves to dampen synaptic inputs onto those cells.[7] We therefore examined whether selective HCN1 deletion in cortical pyramidal neurons was associated with improved dendritosomatic synaptic transfer.

To evaluate effects of HCN1 deletion on basal and ketamine-modulated synaptic properties, we used a measure of synaptic summation that is known to be influenced by dendritic I_h.[7] For this, EPSPs were evoked in pyramidal neurons by 40-Hz extracellular stimulation of the superficial cortex (fig. 4A), and EPSP temporal summation was calculated as the ratio of the final evoked EPSP (EPSP5) relative to the first EPSP (EPSP5/EPSP1) (fig. 4B); the degree of summation is most clearly evident in the aligned and normalized traces (see lower panels of fig. 4A). Note that temporal summation of evoked EPSPs was enhanced after HCN1 deletion (EPSP5/EPSP1 ratio: 1.9 ± 0.2 for Cre$^-$ *vs.* 3.1 ± 0.2 for Cre$^+$, n = 5, $P < 0.05$). Importantly, whereas ketamine increased EPSP temporal summation by approximately 28% (from 1.9 ± 0.2 to 2.5 ± 0.2, n = 5, $P <$

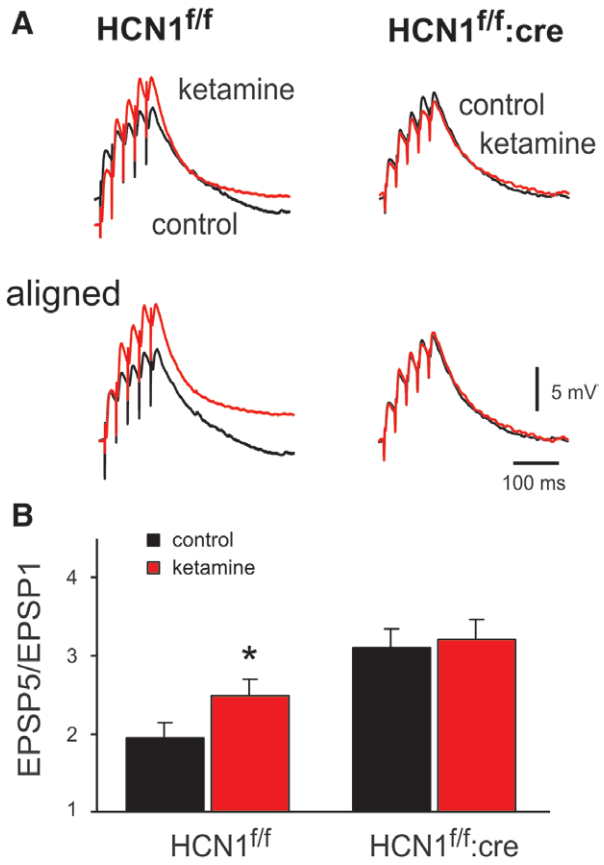

Fig. 4. Ketamine fails to enhance EPSP temporal summation in cortical pyramidal neurons from CaMKCre:HCN1 $^{f/f}$ mice. (*A*) Sample voltage traces show EPSP recordings in cortical pyramidal neuron from HCN1$^{f/f}$ (*left*) and CaMKCre:HCN1$^{f/f}$ mice (*right*) in response to 40-Hz stimulation under control conditions and during exposure to ketamine (20 μM). (*Lower panels*) EPSPs were aligned to initial membrane potential and normalized to the amplitude of the first EPSP in the train to highlight the effects of ketamine on temporal summation. (*B*) Averaged EPSP summation ratio (EPSP5/EPSP1) for HCN1$^{f/f}$ and CaMKCre:HCN1$^{f/f}$ mice under the indicated conditions. Ketamine enhanced EPSP summation in cortical neurons from HCN1$^{f/f}$ animals but not from CaMKCre:HCN1$^{f/f}$ mice (* n = 5 and 5, $P < 0.05$ by two-way repeated measures ANOVA). Tetrodotoxin was not applied in these experiments. EPSP = excitatory postsynaptic potential.

0.05) in HCN1$^{f/f}$ mice, this effect of ketamine was totally eliminated in pyramidal neurons of CaMKCre:HCN1$^{f/f}$ mice (from 3.1 ± 0.2 to 3.2 ± 0.2, n = 5, $P > 0.05$).

CaMKCre:HCN1$^{f/f}$ Mice Are Significantly Less Sensitive to Hypnotic Actions of Ketamine

We previously suggested that HCN1-containing channels may be important molecular substrates for hypnotic anesthetic actions of ketamine[4] because those channels are inhibited by ketamine and because the associated synaptic enhancement could promote cortical synchrony[8,9,23] like that observed during anesthetic-induced hypnosis.[10] Indeed, we showed that HCN1$^{-/-}$ mice showed a marked decrease in sensitivity to ketamine-induced LORR, an established behavioral surrogate in mice for hypnotic anesthetic actions.[1,2] However, those earlier experiments using global knockout mice could not localize that effect to deletion of HCN1 channels within forebrain neurons.[4] Here, we examined whether altered ketamine sensitivity was also observed in CaMKCre:HCN1$^{f/f}$ mice, in which HCN1 subunits were deleted only from forebrain neurons. As shown in figure 5A, the EC_{50} that produced LORR was significantly higher in CaMKCre:HCN1$^{f/f}$ mice (by approximately 31%; 10.4 ± 0.4 mg/kg for Cre$^-$ *vs.* 13.7 ± 0.3 mg/kg for Cre$^+$, n = 31 and 52, $P < 0.0001$); there was no overlap of the 95% CIs for the estimated EC_{50} values between genotypes (9.6–11.3 mg/kg for Cre$^-$ *vs.* 13.0–14.3 mg/kg for Cre$^+$). Also consistent with decreased sensitivity to ketamine, the duration of LORR evoked by ketamine was reduced in CaMKCre:HCN1$^{f/f}$ mice, by comparison with control HCN1$^{f/f}$ mice (fig. 5B) ($F_{1,211} = 10.4$, $P < 0.002$). In contrast to these differences in ketamine-induced LORR, we found no genotype-dependent differences in ketamine actions on gross sensorimotor function. In a tail-flick assay to assess responses to noxious sensory stimulation (fig. 5C), HCN1$^{f/f}$ and CaMKCre:HCN1$^{f/f}$ mice displayed essentially identical latencies to remove their tails from a painful heat source under control conditions, and both showed a similar, transient increase in latency following high-dose ketamine administration (20 mg/kg). In a RotaRod assay to assess motor function (fig. 5D), both control and conditional HCN1 knockout mice were able to remain on an accelerating rod for equal durations initially, and both genotypes showed comparable decreases in duration following a subanesthetic dose of ketamine (2.5 mg/kg). Note that at higher ketamine doses, even those where all animals retained their righting reflex (*i.e.*, 5 mg/kg), the mice were too incapacitated to remain on the RotaRod for more than a few seconds. In sum, these data indicate that actions of ketamine on behaviors that involve spinal nociceptive reflexes or cerebellar function were unaffected in mice with forebrain-selective deletion of HCN1, as expected, whereas those mice were significantly less sensitive to ketamine-induced hypnosis.

Discussion

In this article, we have shown that selective deletion of HCN1 subunits from forebrain principal cells in mice leads to decreased sensitivity to hypnotic actions of ketamine, addressing both the molecular and the neural bases for this anesthetic action. That is, this work provides additional support for our earlier proposition that HCN1-containing channels represent a behaviorally relevant molecular target of ketamine.[4] It also further restricts the potential neural substrate for HCN1-mediated hypnotic actions of ketamine to forebrain principal neurons. In this latter respect, we found that ketamine-mediated inhibition of HCN1-containing channels in cortical pyramidal neurons enhances dendritosomatic synaptic integration, an effect that would support

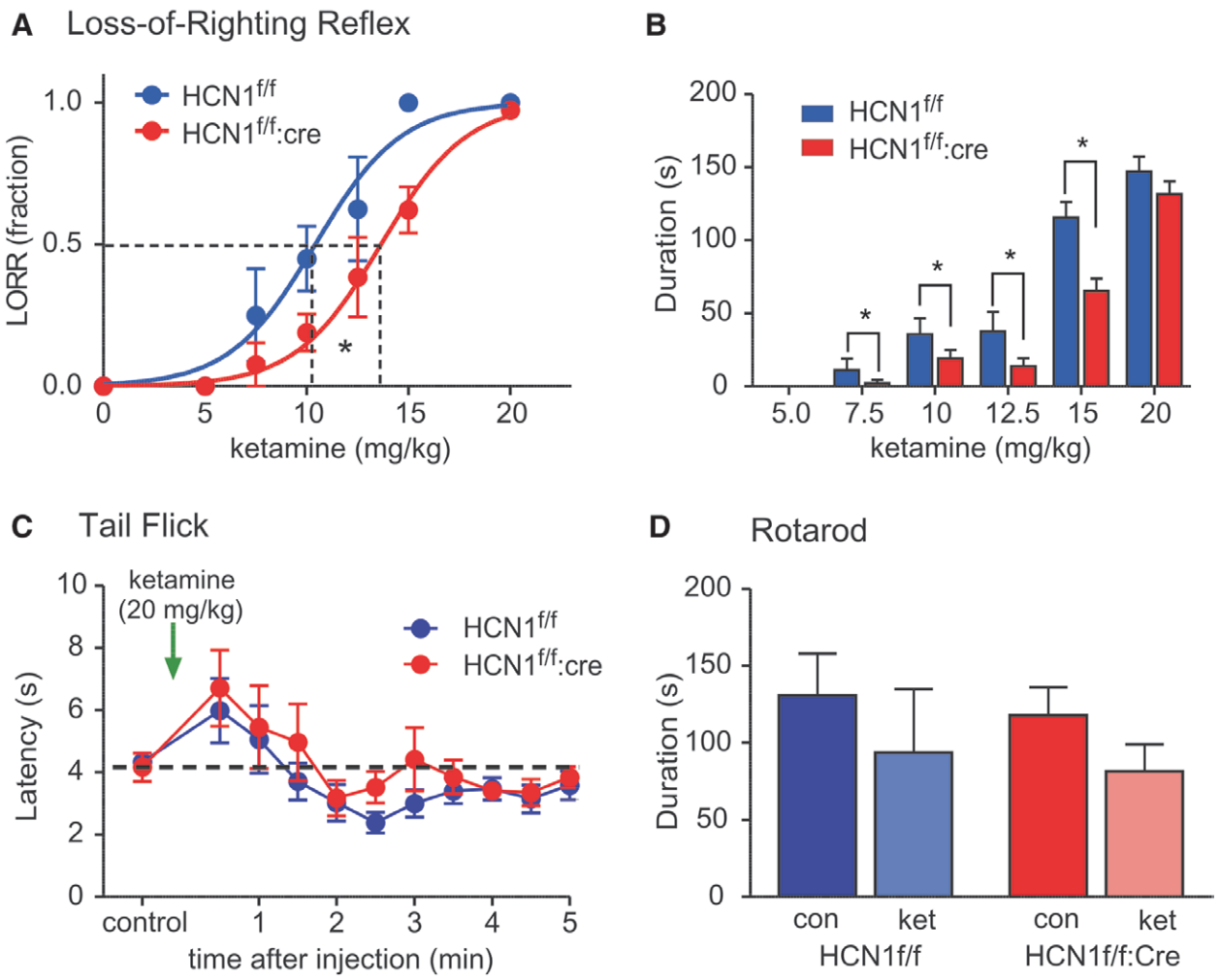

Fig. 5. Deletion of HCN1 channels from forebrain reduced sensitivity of mice to hypnotic actions of ketamine. (*A* and *B*) Mice were injected with incrementing concentrations of ketamine (5–20 mg/kg, IV), and the fraction of HCN1$^{f/f}$ and CaMKCre:HCN1$^{f/f}$ mice that failed to right themselves (loss-of-righting reflex [LORR]) was determined as a measure of hypnosis. CaMKCre:HCN1$^{f/f}$ mice were less sensitive to hypnotic effects of ketamine, as indicated by increased EC_{50} for ketamine-induced LORR (*A*: $F_{1,328}$ = 33.2, $P < 0.0001$ by ANOVA) and reduced duration of the LORR (*B*: $F_{1,211}$ = 10.4, $P < 0.002$ by two-way ANOVA). (*C*) The latency for mice to remove their tail from a radiant heat source was essentially identical in HCN1$^{f/f}$ and CaMKCre:HCN1$^{f/f}$ mice under control conditions, and ketamine (20 mg/kg, IV) evoked a similar transient increase in latency in both sets of mice. (*D*) The duration that HCN1$^{f/f}$ and CaMKCre:HCN1$^{f/f}$ mice were able to stay on an accelerating RotaRod (Med Associates Inc., Georgia, VT) was not different before or immediately after treatment with a subanesthetic dose of ketamine (2.5 mg/kg, IV) (n = 31 and 52, $P < 0.05$, * HCN1$^{f/f}$:cre *vs.* HCN1$^{f/f}$).

the synaptically mediated slow cortical rhythms that accompany the hypnotic state induced by ketamine.[8–10,23] Thus, these data remain consistent with the hypothesis that cortical pyramidal neurons represent a plausible neural substrate for HCN1 contributions to ketamine-induced hypnosis.

As described by others, a number of criteria should be met for a molecular target to qualify as relevant for an anesthetic action.[1,24] These include the following: (1) the target is modulated by the anesthetic at clinically relevant concentrations and with a stereoselectivity that matches the *in vivo* actions of the drug; (2) the target is expressed in a specific anatomical location where its modulation could conceivably mediate the specific behavioral effects of the anesthetic (*i.e.*, plausibility); and (3) elimination of the target (or disrupting its modulation by the anesthetic) diminishes the ability of the drug to achieve a specific anesthetic endpoint *in vivo*. With respect to pharmacology, we previously found a stereoselective, subunit-specific inhibition of HCN1 channels by clinically appropriate concentrations of ketamine[4]; we showed that racemic ketamine shifts the *V½* of HCN1 activation with an EC_{50} of approximately 8 μM, whereas the S-(+)-enantiomer does so with an EC_{50} of approximately 4 μM,[4] consistent with an approximately twofold greater anesthetic potency of S-(+)-ketamine.[25,26] Importantly, we also demonstrated by global elimination of HCN1 channels that HCN1 knockout mice were strikingly less sensitive to the

hypnotic effects of ketamine[4]; this did not appear to represent a nonspecific effect of HCN1 deletion because sensitivity of the mice to the anesthetic effects of etomidate was unchanged.[4] The current study confirms and extends that previous work in terms of plausibility by showing that this relative resistance to ketamine-induced hypnosis was largely recapitulated in mice with deletion of HCN1 channels restricted to forebrain principal cells, a brain region relevant for anesthetic-induced hypnosis. Thus, the accumulated evidence available to this point is consistent with the idea that forebrain HCN1 channels indeed contribute to ketamine-induced hypnosis.

Mechanistically, we favor the idea that the relevant forebrain cells are cortical pyramidal neurons, because inhibition of dendritic HCN1 channels in those cells causes membrane hyperpolarization with enhanced synaptic efficacy and thus yields conditions conducive to coherent cortical activity.[4,8,9] It should be noted that Cre expression is not limited only to cortical pyramidal neurons in this line of mice. However, those cells represent the predominant site of HCN1 expression in the forebrain,[22] and this convergence of both Cre and HCN1 expression thus favors the idea that pyramidal neurons are the likely site of action in this mouse model. Nevertheless, further refinement of the relevant neural substrate, especially between pyramidal neurons of the hippocampus and neocortex, will require mouse lines with even more restricted Cre expression.

As was seen with global HCN1$^{-/-}$ mice, ketamine remained capable of inducing hypnosis in CaMKCre-HCN1$^{f/f}$ mice, albeit at higher concentrations. Thus, other molecular targets must also be able to contribute to ketamine-induced hypnosis. In this respect, it is important to emphasize that ketamine is well known to be a potent modulator of *N*-methyl-D-aspartic acid (NMDA) receptor channels.[1,26] However, at present there remains no evidence from NMDA receptor knockout mice to support a role for NMDA receptor modulation in anesthetic actions of ketamine. Moreover, MK801 is a more potent and selective NMDA receptor blocker than ketamine but is actually a poor anesthetic.[27,28] Thus, despite widespread recognition of NMDA receptors as a ketamine target, the contributions of NMDA receptor antagonism to specific anesthetic actions of ketamine remain to be delineated. It is possible that inhibitory effects on NMDA receptors underlie other nonanesthetic central actions of ketamine (*e.g.*, antidepressant or psychotomimetic)[26,29–31]; it is also possible that modulation by ketamine of other molecular targets (*e.g.*, activation of tonic γ-aminobutyric acid type A receptors containing α6 or δ subunits) may contribute to its other anesthetic actions.[32–34]

In conclusion, these results indicate that forebrain principal cells represent a relevant neural substrate for HCN1-mediated hypnotic actions of ketamine. As knowledge of the molecular determinants for ketamine inhibition of HCN1 channels becomes available, it may be possible to develop knock-in mice that express ketamine-insensitive mutant HCN1 channels, preferably in more restricted neuronal populations, to even more rigorously define the molecular and neuronal targets for this specific clinical endpoint of ketamine.

References

1. Franks NP: Molecular targets underlying general anaesthesia. Br J Pharmacol 2006; 147: S72–81
2. Franks NP: General anaesthesia: From molecular targets to neuronal pathways of sleep and arousal. Nat Rev Neurosci 2008; 9:370–86
3. Grasshoff C, Rudolph U, Antkowiak B: Molecular and systemic mechanisms of general anaesthesia: The 'multi-site and multiple mechanisms' concept. Curr Opin Anaesthesiol 2005; 18:386–91
4. Chen X, Shu S, Bayliss DA: HCN1 channel subunits are a molecular substrate for hypnotic actions of ketamine. J Neurosci 2009; 29:600–9
5. Pape HC: Queer current and pacemaker: The hyperpolarization-activated cation current in neurons. Annu Rev Physiol 1996; 58:299–327
6. Nolan MF, Malleret G, Dudman JT, Buhl DL, Santoro B, Gibbs E, Vronskaya S, Buzsáki G, Siegelbaum SA, Kandel ER, Morozov A: A behavioral role for dendritic integration: HCN1 channels constrain spatial memory and plasticity at inputs to distal dendrites of CA1 pyramidal neurons. Cell 2004; 119:719–32
7. Magee JC: Dendritic integration of excitatory synaptic input. Nat Rev Neurosci 2000; 1:181–90
8. Hill S, Tononi G: Modeling sleep and wakefulness in the thalamocortical system. J Neurophysiol 2005; 93:1671–98
9. Bazhenov M, Timofeev I, Steriade M, Sejnowski TJ: Computational models of thalamocortical augmenting responses. J Neurosci 1998; 18:6444–65
10. Amzica F, Steriade M: Electrophysiological correlates of sleep delta waves. Electroencephalogr Clin Neurophysiol 1998; 107:69–83
11. Nolan MF, Malleret G, Lee KH, Gibbs E, Dudman JT, Santoro B, Yin D, Thompson RF, Siegelbaum SA, Kandel ER, Morozov A: The hyperpolarization-activated HCN1 channel is important for motor learning and neuronal integration by cerebellar Purkinje cells. Cell 2003; 115:551–64
12. Rossant J, Nagy A: Genome engineering: The new mouse genetics. Nat Med 1995; 1:592–4
13. Dragatsis I, Zeitlin S: CaMKIIalpha-Cre transgene expression and recombination patterns in the mouse brain. Genesis 2000; 26:133–5
14. Dragatsis I, Levine MS, Zeitlin S: Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. Nat Genet 2000; 26:300–6
15. Chen X, Shu S, Kennedy DP, Willcox SC, Bayliss DA: Subunit-specific effects of isoflurane on neuronal Ih in HCN1 knockout mice. J Neurophysiol 2009; 101:129–40
16. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29:e45
17. Chen X, Shu S, Bayliss DA: Suppression of ih contributes to propofol-induced inhibition of mouse cortical pyramidal neurons. J Neurophysiol 2005; 94:3872–83
18. Chen X, Sirois JE, Lei Q, Talley EM, Lynch C III, Bayliss DA: HCN subunit-specific and cAMP-modulated effects of anesthetics on neuronal pacemaker currents. J Neurosci 2005; 25:5803–14
19. Garfield JM, Bukusoglu C: Propofol and ethanol produce additive hypnotic and anesthetic effects in the mouse. Anesth Analg 1996; 83:156–61

20. Crawley JN: Behavioral phenotyping strategies for mutant mice. Neuron 2008; 57:809–18
21. Colbran RJ: Regulation and role of brain calcium/calmodulin-dependent protein kinase II. Neurochem Int 1992; 21:469–97
22. Santoro B, Chen S, Luthi A, Pavlidis P, Shumyatsky GP, Tibbs GR, Siegelbaum SA: Molecular and functional heterogeneity of hyperpolarization-activated pacemaker channels in the mouse CNS. J Neurosci 2000; 20:5264–75
23. Antkowiak B: In vitro networks: Cortical mechanisms of anaesthetic action. Br J Anaesth 2002; 89:102–11
24. Hemmings HC Jr, Akabas MH, Goldstein PA, Trudell JR, Orser BA, Harrison NL: Emerging molecular mechanisms of general anesthetic action. Trends Pharmacol Sci 2005; 26:503–10
25. Franks NP, Lieb WR: Where do general anaesthetics act? Nature 1978; 274:339–42
26. Kohrs R, Durieux ME: Ketamine: Teaching an old drug new tricks. Anesth Analg 1998; 87:1186–93
27. Irifune M, Katayama S, Takarada T, Shimizu Y, Endo C, Takata T, Morita K, Dohi T, Sato T, Kawahara M: MK-801 enhances gabaculine-induced loss of the righting reflex in mice, but not immobility. Can J Anaesth 2007; 54:998–1005
28. Stabernack C, Sonner JM, Laster M, Zhang Y, Xing YL, Sharma M, Eger EI: Spinal N-Methyl-D-aspartate receptors may contribute to the immobilizing action of isoflurane. Anesth Analg 2003; 96:102–7
29. Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS, Krystal JH: Antidepressant effects of ketamine in depressed patients. Biol Psychiatry 2000; 47: 351–4
30. Zarate CA Jr, Singh JB, Carlson PJ, Brutsche NE, Ameli R, Luckenbaugh DA, Charney DS, Manji HK: A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. Arch Gen Psychiatry 2006; 63: 856–64
31. Wolff K, Winstock AR: Ketamine: From medicine to misuse. CNS Drugs 2006; 20:199–218
32. Hevers W, Hadley SH, Lüddens H, Amin J: Ketamine, but not phencyclidine, selectively modulates cerebellar GABA(A) receptors containing alpha6 and delta subunits. J Neurosci 2008; 28:5383–93
33. Rudolph U, Antkowiak B: Molecular and neuronal substrates for general anaesthetics. Nat Rev Neurosci 2004; 5:709–20
34. Schnoebel R, Wolff M, Peters SC, Bräu ME, Scholz A, Hempelmann G, Olschewski H, Olschewski A: Ketamine impairs excitability in superficial dorsal horn neurones by blocking sodium and voltage-gated potassium currents. Br J Pharmacol 2005; 146:826–33

ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Giessen's Most Famous Chemist: Professor Justus von Liebig

After studies at Bonn and Erlangen led to his eventual doctorate, Justus Liebig (1803–1873) apprenticed with Parisian chemist Gay-Lussac. In 1824 on Alexander von Humboldt's recommendation, 21-year-old Liebig assumed a professorship for the next 28 years at the University of Giessen. There he founded the world's premiere school of chemistry, codiscovered chloroform (1831), founded the journal *Annalen der Chemie* (1832), and assumed his baronetcy as Justus von Liebig (1845). In 1946 in his honor, the University was renamed Justus-Liebig-Universität Gießen. (Copyright © the American Society of Anesthesiologists, Inc.)

George S. Bause, M.D., M.P.H., Honorary Curator, ASA's Wood Library-Museum of Anesthesiology, Park Ridge, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.