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Rapid loss of dendritic HCN channel expression in hippocampal pyramidal neurons following status epilepticus

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Abstract

Epilepsy is associated with loss of expression and function of hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels. Previously we showed that loss of HCN channel-mediated current (I_h) occurred in the dendrites of CA1 hippocampal pyramidal neurons after pilocarpine-induced status epilepticus (SE), accompanied by loss of HCN1 channel protein expression. However, the precise onset and mechanistic basis of HCN1 channel loss post-SE was unclear, particularly whether it preceded the onset of spontaneous recurrent seizures and could contribute to epileptogenesis, or development of the epileptic state. Here we found that loss of I_h and HCN1 channel expression begins within an hour after SE, and involves sequential processes of dendritic HCN1 channel internalization, delayed loss of protein expression, and later downregulation of mRNA expression. We also found that an *in vitro* SE model reproduced the rapid loss of dendritic I_h, demonstrating that this phenomenon was not specific to *in vivo* SE. Together, these results show that HCN1 channelopathy begins rapidly and persists after SE, involves both transcriptional and non-transcriptional mechanisms, and may be an early contributor to epileptogenesis.

Keywords

HCN channel; epilepsy; channelopathy; dendrites; biotinylation; status epilepticus

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Introduction

HCN channels are voltage-gated ion channels highly expressed in brain regions associated with seizure onset: cortex and hippocampus (principally the HCN1 subtype), and thalamus (primarily the HCN2 subtype). An abundance of evidence has established that HCN1 channels act to diminish the intrinsic excitability of pyramidal neurons in cortex and hippocampus (reviewed in Lewis and Chetkovich, 2011). In these neurons, HCN channels are localized primarily to the apical dendrites where the majority of excitatory synaptic input occurs, and I_h is one of the few ionic conductances continuously active at resting potential, thus exerting an inhibitory influence on synaptic inputs by diminishing dendritic input resistance.

Loss of expression and function of HCN1 channels occurs in epilepsy. Entorhinal cortical neurons had loss of I_h and HCN1 channel protein expression following kainate (KA)-induced SE (Shah et al., 2004), and we found that loss of HCN1 channel function in CA1 hippocampal pyramidal neurons after pilocarpine-induced SE depended on two mechanisms: loss of dendritic I_h amplitude and HCN1 channel expression, and a hyperpolarizing shift in I_h voltage-dependent activation. While the gating change was dependent on spontaneous seizures and mediated by downregulated phosphorylation signaling, the loss of expression was independent of seizures (Jung et al., 2007; Jung et al., 2010). A subsequent study confirmed the I_h loss and gating change, and showed transcriptional downregulation of HCN1 mRNA expression, with onset by 3 d post-SE (Marcelin et al., 2009). Using the KA model, another study showed a similar loss of dendritic I_h in chronic epilepsy, although no loss of dendritic HCN channel expression had occurred by 1-2 d post-SE (Shin et al., 2008). Thus multiple studies describe loss of HCN1 channel expression in chronic epilepsy, but differ somewhat in their characterization of its onset and the underlying mechanisms. To better understand acquired HCN1 channelopathy in epilepsy, we sought to determine its onset after pilocarpine-induced SE, and test the involvement of both transcriptional and non-transcriptional mechanisms.

Materials and methods

Electrophysiology

Hippocampal slices (400 μm) were prepared from 6-10 week-old male Sprague Dawley rats (Jung et al., 2007). I_h amplitudes from dendritic cell-attached patch clamp recordings at maximal voltage-dependent activation (~ −150 mV) are presented without correction for estimated patch area; however, pipette tip diameter (~ 1 μm) was held constant, and each investigator performed their own control series to minimize the effects of differences in pipette fabrication. All supplies were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Pilocarpine model

We administered pilocarpine hydrochloride (385 mg/kg i.p.) to induce SE in male Sprague Dawley rats 6-8 weeks of age (Jung et al., 2007). After 1 hr in SE, in animals studied at 1 d post-SE or longer, seizures were terminated with diazepam (12 mg/kg, i.p.; Hospira, Lake Forest, IL) delivered every 30 min as needed. Animals studied at 1 hr post-SE were sacrificed with ketamine/xylazine terminal anesthesia without administration of diazepam, and brain slices were used after 1 hr incubation at room temperature.

In vitro SE model

Hippocampal slices from naive animals were perfused at 30-32 °C with extracellular recording solution (Jung et al., 2007) containing 0 Mg^{2+} and 50 μM bicuculline, and

stimulated extracellularly using bipolar sharpened tungsten electrodes (A-M Systems, Carlsborg, WA) placed in the CA1 stratum lacunosum-moleculare with five constant-current 100 μsec pulses at 50 Hz, delivered every two mins for 1 hr. During perfusion, extracellular recordings were performed in stratum pyramidale with patch pipettes filled with 2 M NaCl solution. Following the perfusion, the bath was switched to normal solution and dendritic I_h recordings were made within an hour.

Western blotting

HCN1 channel protein levels were measured using a modified protocol (Jung et al., 2007). In each experiment, we pooled 6-8 hippocampal slices from a single animal prepared in the same way as for electrophysiology. The CA1 regions were microdissected on dry ice and homogenized in solution containing (in mM): 4 para-nitrophenylphosphate, 1 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (1 tab per 10 ml; Roche, Indianapolis, IN). Homogenates were centrifuged at 15,000× g for 40 min at 4 °C. The pellet was then resuspended with sonication in homogenization solution then run in Laëmmli buffer (with final concentration of 4.5% sodium dodecyl sulfate [SDS]) on a 10% acrylamide gel, transferred to a nitrocellulose membrane, and incubated with HCN1 antibody (1:500; Neuromabs, Davis, CA), followed by incubation in anti-mouse secondary antibody (1:1,000; Invitrogen, South San Francisco, CA), and visualized by enhanced chemiluminescence and film exposure. Three different protein loading amounts were used in each condition so to verify that signal detection was in the linear range, as described previously (Jung et al., 2010). The membrane was then reprobed using anti-β-tubulin III antibody as a marker for neuronal protein content (1:20,000; Sigma-Aldrich), followed by incubation in anti-mouse secondary antibody (1:20,000; Sigma-Aldrich). All reported HCN1 protein levels were normalized to β-tubulin III protein levels.

Surface biotinylation assay

Surface membrane protein expression was measured using a biotinylation protocol (Lugo et al., 2008). We prepared hippocampal slices then incubated them for 45 min on ice in extracellular recording solution containing 1.5 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL), and then in extracellular solution with 1 μM lysine to block all reactive sulfo-NHS-SS-biotin in excess. The CA1 regions were microdissected on dry ice and homogenized in buffer containing: 1% Triton X-100, 50 Tris-HCl, pH 7.5, 150 NaCl, 1 EDTA, 1 sodium orthovanadate, and protease inhibitor mixture. The homogenates were centrifuged at 15,000× g for 15 min at 4 °C, and the supernatant harvested. After the measurement of protein concentration with a BCA protocol (~800 μg in 400 μL) and normalizing the inputs for total protein content, the supernatant was incubated with NeutrAvidin Plus beads (Pierce) overnight at 4 °C, then washed three times with homogenization buffer. After centrifugation at 13,000× g for 5 min, the supernatant was discarded and the beads resuspended in Laëmmli buffer (with final concentration of 6% SDS), and boiled. Total protein (~60 μg in total loaded for gel electrophoresis) and biotinylated surface protein (50% of recovered surface protein in total loaded for gel electrophoresis) levels were analyzed using Western blotting. To confirm the absence of cytoplasmic proteins in the surface fraction, all blots were re-probed using anti-actin antibody (1:10,000; Sigma-Aldrich) followed by incubation in anti-rabbit secondary antibody (1:20,000; Invitrogen).

Real-time reverse transcription-PCR

HCN1 mRNA levels in CA1 hippocampus were determined using relative quantification with β-actin as the reference gene, and forward/reverse primers and hybridization probes as previously described (Chen et al., 2005; Marcelin et al., 2009). Data at each experimental time point post-SE was compared to its own control series. Reaction conditions were 3.125

μl of Maxima Probe/ROX qPCR Master Mix (Fermentas, Glen Burnie, MD) with cDNA dissolved in 1.25 μl of diethylpyrocarbonate (DEPC)-H_2O brought to 6.25 μl with nuclease-free water. Reactions were analyzed in triplets. After preincubation for 2 min at 50 °C and 10 min at 94 °C, 40 cycles of the PCR reaction were performed (15 s at 94 °C followed by 1 min at 59 °C; 7900HT, Applied Biosystems, Carlsbad, CA).

Results

Rapid loss of dendritic I_h post-SE

To examine the time course of HCN1 channel loss post-SE, we performed cell-attached patch-clamp recordings in the apical dendrites of CA1 hippocampal pyramidal neurons (at ~200 μm from the soma) in brain slices of pilocarpine-treated and control animals. Maximal I_h amplitudes were significantly reduced at 1 hr post-SE (control, 43 ± 5.9 pA, $n = 10$; 1 hr, 23.3 ± 3.4 pA, $n = 10$; 53 ± 7.9% of control; $p < 0.05$ by ANOVA). I_h remained decreased 1 d post-SE (12.6 ± 1.9 pA, $n = 5$; 30 ± 4.4% of control; $p < 0.01$ by ANOVA). This decline in dendritic I_h was similar to that previously seen at 1 wk post-SE (Jung et al., 2007) when animals are beginning to experience spontaneous seizures (18 ± 3.9 pA, $n = 10$; compared to a previous control series, 48 ± 4.5 pA, $n = 19$; 38 ± 8.1% of control, $p < 0.01$), and at 1 mo post-SE (Jung et al., 2010) when virtually all animals are chronically epileptic (20 ± 4.7 pA, $n = 8$; compared to its control, 40 ± 6.5 pA, $n = 22$; 50 ± 11% of control, $p < 0.05$). Fig. 1A shows all I_h values post-SE as a percentage of control to facilitate comparison among experiments. This demonstrates that the persistent loss of I_h that occurs in the early and chronic phases of epilepsy begins as soon as 1 hr post-SE, well before the onset of spontaneous seizures. I_h voltage-dependent activation at early time points post-SE was similar to control (half-activation voltage, $V_{1/2}$ in control: −90 ± 1.7 mV, $n = 10$; 1 hr: −87 ± 1.8 mV, $n = 10$; 1 day: −91 ± 5.9 mV, $n = 5$, $p > 0.05$ by ANOVA; Fig. 1B). This is consistent with earlier results demonstrating that the hyperpolarized shift in HCN channel activation that occurs in epilepsy, and is mediated by loss of phosphorylation signaling, depends on the occurrence of spontaneous seizures (Jung et al., 2007; Jung et al., 2010).

Delayed loss of HCN1 channel protein expression post-SE

HCN1 channel protein expression measured with Western blotting in CA1 hippocampus at 1 hr post-SE was unchanged (95 ± 2.9 % of control, $n = 5$, $p > 0.05$, Fig. 2A), but was significantly reduced at 1 d post-SE (54 ± 10 %, $n = 11$, $p < 0.01$). Although we normalized HCN1 channel protein values to those of β-tubulin III expression to control for any changes in neuronal protein content, at 1 d post-SE there was no significant change in β-tubulin III expression compared to control (101 ± 7.0 %, $n = 11$, $p > .05$), suggesting a lack of neuronal death at that time point. The loss of HCN1 protein expression was similar to that previously seen at 1 wk (57 ± 9.6 %, $n = 6$) and 1 mo post-SE (53 ± 7.1 %, $n = 5$; Jung et al., 2007), and of similar magnitude to the loss of I_h. Thus, decreased I_h amplitude at 1 d post-SE and beyond likely reflected a loss of HCN1 channel subunits. We did not examine HCN2 channel expression as these subunits represent only a minority of HCN channel expression in hippocampus (Santoro et al., 2000), and are only transiently downregulated post-SE (Jung et al., 2007).

Rapid internalization of HCN1 channels post-SE

Because I_h amplitude was reduced at 1 hr post-SE while HCN1 protein expression remained intact, we hypothesized that HCN1 channel subunits had become non-functional by internalization from the surface membrane. Surface expression of HCN1 subunits measured with a biotinylation protocol was significantly reduced at 1 hr post-SE (76 ± 4.0 % of control, $n = 5$, $p < 0.01$; Fig. 2B), while total expression of the HCN1 subunit was not significantly altered (111 ± 6.0 %, $n = 5$, $p > 0.05$). Surface normalized to total HCN1 levels

were further decreased (69 ± 1.0 %, $n = 5$, $p < 0.01$). These results suggest that SE rapidly induces internalization of dendritic HCN1 channels, which undergo subsequent degradation by 1 d post-SE.

Late transcriptional downregulation of HCN1 mRNA post-SE

HCN1 mRNA expression diminishes as soon as 3 d post-SE, reflecting reduced transcription of the *HCN1* gene (Marcelin et al., 2009). We performed real-time quantitative RT-PCR and found that HCN1 mRNA expression in the CA1 hippocampus at 1 hr and 1 d post-SE was unchanged compared to control (1 hr, 124 ± 9.1 %, $n = 6$; 1 d, 90 ± 14.4 %, $n = 6$, $p > 0.05$ by ANOVA, Fig. 3), showing that the loss of I_h and HCN1 protein expression within the first day post-SE was not due to transcriptional downregulation of HCN1 mRNA expression. However, HCN1 mRNA levels at 1 wk post-SE were significantly decreased compared to control levels (45 ± 7.0 %, $n = 6$, $p < 0.01$). HCN1 mRNA levels at 1 mo post-SE were not significantly decreased compared to control (74 ± 8.2 %, $n = 10$, $p = 0.08$). These results show that transcriptional downregulation of HCN1 mRNA after SE follows early non-transcriptional processes of channel internalization and loss of protein expression.

An *in vitro* model replicates rapid loss of dendritic I_h post-SE

We then asked whether the rapid loss of I_h post-SE could be reproduced in an *in vitro* model using naïve tissue. Previously, an *in vitro* model of seizures provoked by extracellular bath perfusate containing 0 Mg^{2+} and 50 μM bicuculline maintained at 35-37 °C yielded extracellularly recorded "seizure-like events (SLEs)" (Jung et al., 2010). During 1 hr of these "*in vitro* seizure" conditions, an average of ~3 spontaneous SLEs occurred, associated with a hyperpolarizing shift in I_h activation but no significant change in maximal I_h amplitude. Since SE consists of nearly continuous seizure activity, we used similar conditions as in the *in vitro* seizure model, but evoked SLEs every 2 min with extracellular stimulation of perforant path afferents to area CA1. This "*in vitro* SE" protocol evoked 30 SLEs per hour manifested by extracellularly recorded outward current sources followed by slow inward current sinks on which were superimposed multiple rhythmic spike discharges (Fig. 4A). Whole-cell current-clamp recordings from CA1 pyramidal neuron somata during these evoked SLEs showed a prolonged depolarization of membrane potential associated with high frequency action potential firing (Fig. 4A).

We measured dendritic I_h after subjecting naïve slices to 1 hr of the *in vitro* SE protocol then returning to normal recording solution. Similar to what was seen 1 hr post-SE *in vivo*, dendritic I_h amplitude was significantly reduced compared to a new control series collected for this experiment (control, 63 ± 7.1 pA, $n = 9$; *in vitro* SE, 29 ± 7.1 pA, 45 ± 11% of control, $n = 6$, $p < 0.01$, Fig. 4B). Interestingly, I_h activation was hyperpolarized as was seen with the *in vitro* seizure protocol ($V_{1/2}$ in *in vitro* SE, −105 ± 2.1 mV, $n = 6$; control, −95 ± 1.7 mV, $n = 9$, $p < 0.01$). Because other studies had demonstrated transient changes in ion channel surface expression with exposure to glutamate (Kim et al., 2007; Noam et al., 2010), we examined whether glutamate receptor activation by itself would provoke loss of dendritic I_h amplitudes. During 1 hr incubation of naïve slices in normal recording solution with 100 μM glutamate added, brief runs of interictal spike-like activity without spontaneous SLEs were observed with extracellular recording ($n = 6$ slices); during whole-cell current clamp recording at the soma, occasional spontaneous EPSPs and action potentials were seen (Fig. 4A). Resting potential during glutamate application (−64 ± 0.91 mV, $n = 4$) was similar to control (−62 ± 3.8 mV, $n = 9$, $p > 0.05$). Incubation with glutamate did not significantly affect dendritic I_h amplitudes when measured after return to normal solution (51 ± 13 pA, 81 ± 21% of control, $n = 8$, $p > 0.05$ vs. control, Fig. 4B). Thus, the early loss of I_h following SE *in vivo* can be replicated using an *in vitro* model of SE, demonstrating that this

phenomenon depends on high levels of network activity and is not specific to pilocarpine-induced SE.

Discussion

Rapid onset of HCN1 channelopathy

We found that HCN1 channel loss begins within the first hour post-SE, is persistent, and occurs via three sequential mechanisms: a loss of HCN1 channel surface expression within the first hour; a loss of protein expression within the first day; and transcriptional downregulation that began within a week. Since we had previously shown that spontaneous seizures do not begin in our model until at least 3 d post-SE (Jung et al., 2007), the current study confirms that HCN1 channel loss clearly precedes spontaneous seizure onset. HCN1 channels thus join a limited set of voltage- and ligand-gated ion channels whose expression is dysregulated early in acquired epilepsy models and which cause hyperexcitability, including various $GABA_AR$ subunits (Brooks-Kayal et al., 1998; Goodkin et al., 2008); Cav3.2 channels (Becker et al., 2008); Kv4.2 channels (Bernard et al., 2004; Lugo et al., 2008); and AMPARs (Rakhade et al., 2008). While multiple studies have consistently found loss of I_h and HCN channel expression in chronic phases of epilepsy post-SE, these same changes observed at early time points post-pilocarpine have been only variably observed early post-KA (Shah et al., 2004; Shin et al., 2008). These model-specific differences in the time course of HCN channelopathy may stem from the much slower development of epileptogenesis after KA compared to pilocarpine (Williams et al., 2007).

Non-transcriptional mechanisms underlie onset of HCN1 channelopathy

Transcriptional downregulation of HCN1 mRNA expression had been widely assumed to underlie the loss of HCN1 channel expression in various epilepsy models (Richichi et al., 2008). However, the rapid onset of dendritic I_h loss following SE described here shows that this mechanism cannot be the sole explanation. Indeed, the first step in this process involves a reduction of the surface membrane expression of HCN1 channels, presumably mediated by a post-translational modification of existing channels that alters their membrane stability. Similar processes mediated by altered phosphorylation of channel subunits have been demonstrated for the internalization of $GABA_ARs$ post-SE (Terunuma et al., 2008), and Kv4.2 channels after SE or a glutamatergic stimulus (Kim et al., 2007; Lugo et al., 2008). Following rapid, putatively post-translational mechanisms, it appears that transcriptional downregulation occurs at a delay of at least several days from the initial loss of dendritic HCN1 expression. Determining whether transcriptional downregulation is dependent on or parallel to earlier regulatory processes will be important to understanding the cascade of events the leads to chronic loss of HCN1 channel expression. At a minimum, it appears that HCN1 channel dysregulation in epilepsy involves a combination of both post-translational and transcriptional mechanisms, much as occurs with $GABA_ARs$.

Role of HCN1 channels in epileptogenesis

Dendritic HCN1 channelopathy represents a single ion channel entity that is persistently dysregulated throughout epileptogenesis and chronic epilepsy, as has been corroborated by the time course of spontaneous seizures after pilocarpine-induced SE (Jung et al., 2007; Marcelin et al., 2009). Loss of HCN1 channel expression has also been shown in other animal models of acquired epilepsy, including KA-induced SE, perinatal hypoxia-ischemia (Zhang et al., 2006), and developmental cortical malformation models (Hablitz and Yang, 2010); but see (Chen et al., 2001). Recent reports have confirmed that HCN1 channel loss contributes to epileptogenesis, with deletion of the *HCN1* gene markedly accelerating the rate of epileptogenesis after provoked SE, even though *HCN1* deletion does not by itself cause epilepsy (Huang et al., 2009; Santoro et al., 2010). HCN channel activity is

upregulated by several antiepileptic drugs, possibly contributing to their mechanism of action (Poolos et al., 2002; Surges et al., 2003), thus HCN1 channels may exert an antiepileptic as well as antiepileptogenic action. Because HCN1 channel downregulation occurs so rapidly after SE, study of its mechanisms may provide insight into some of the earliest processes underlying epileptogenesis. The *in vitro* SE model characterized here may allow easier study of the mechanisms underlying the rapid loss of I_h and internalization of HCN1 channel subunits that occurs in the first hour post-SE.

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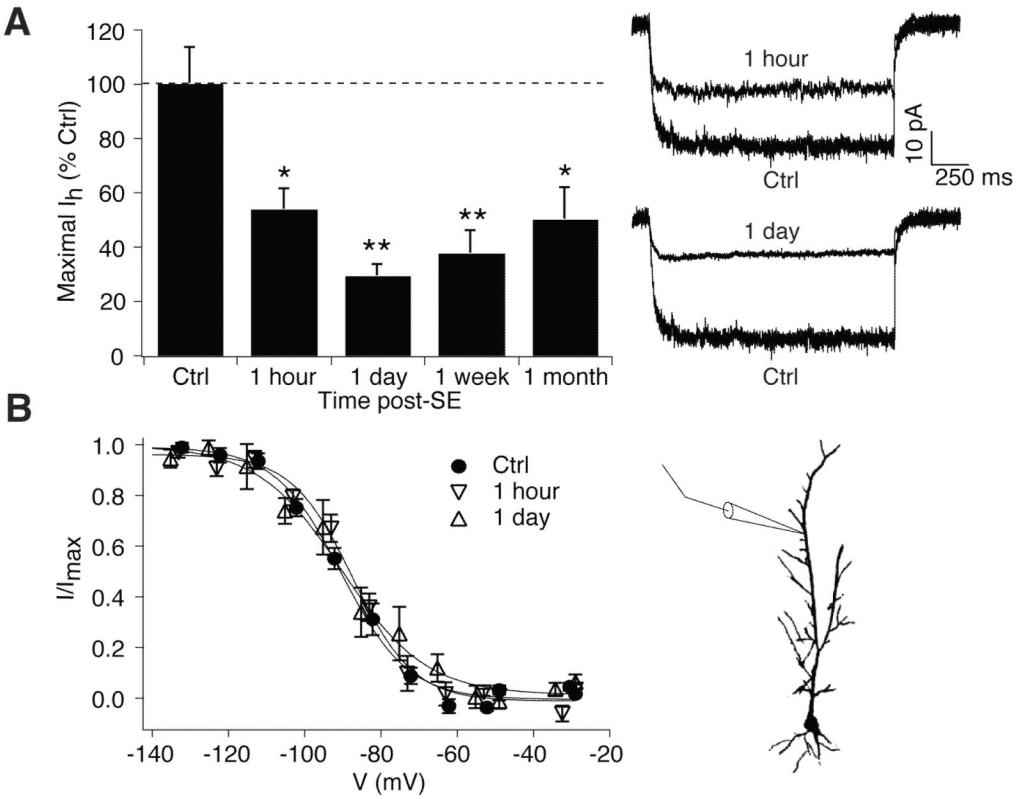

Figure 1.

Dendritic I_h loss begins within 1 hour after SE, and persists for at least 1 month. A, I_h amplitudes at maximal activation obtained from 1 hr and 1 d post-SE animals were significantly reduced compared to control. Representative current traces are shown at voltage commands of ~150 mV. Summary data at 1 wk are from (Jung et al., 2007), and at 1 mo from (Jung et al., 2010). B, Voltage-dependent activation of I_h at 1 hr and 1 d post-SE was similar to control. Diagram shows approximate location of dendritic recordings.

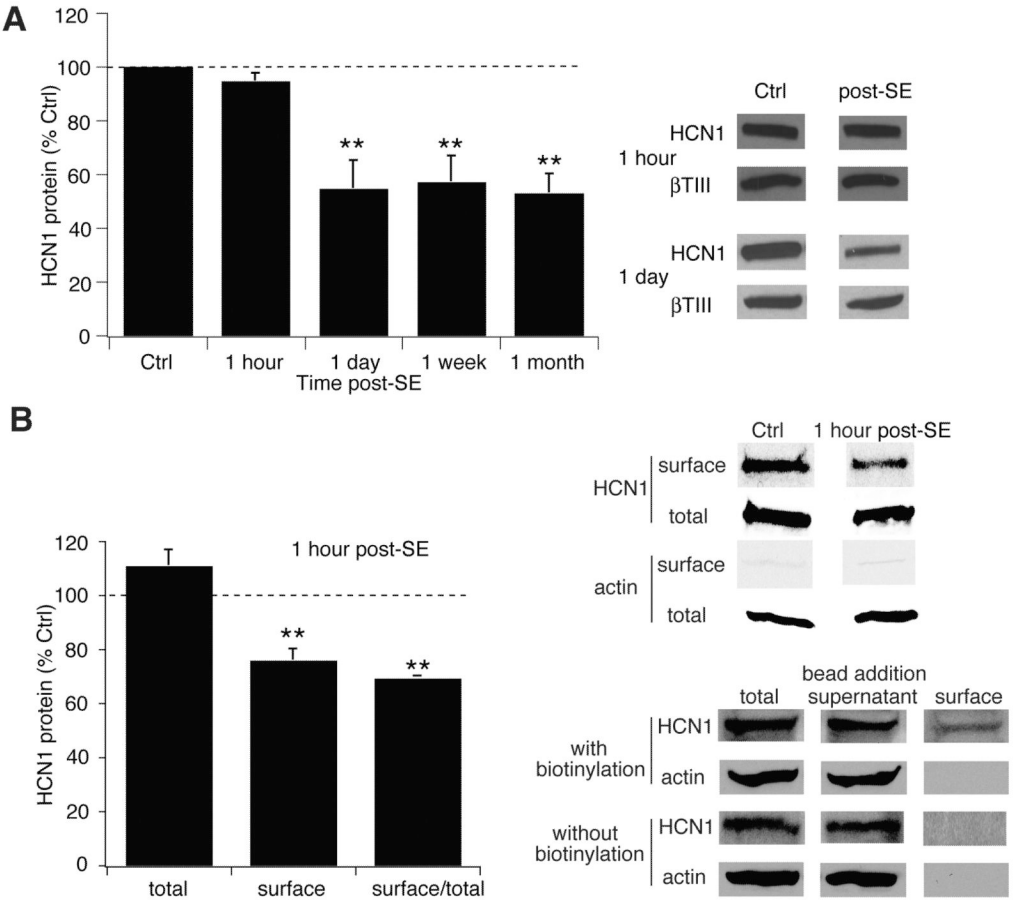

Figure 2.

HCN1 channel protein expression falls after a delay following SE, while surface expression declines immediately at 1 hr post-SE. A, HCN1 channel protein expression at 1 hr post-SE was unchanged, while at 1 d post-SE was decreased and remained reduced for at least 1 mo. Representative blots are shown in each condition, along with blots of β-tubulin III as a marker of neuronal protein content. Summary data at 1 wk and 1 mo are from (Jung et al., 2007). B, Expression of surface HCN1 channel protein was decreased at 1 hr post-SE compared to control. Control and post-SE samples were processed in the same gel to enable accurate comparison; total and surface samples were processed in separate gels and thus cannot be directly compared. Lack of actin staining in the surface fraction confirmed the absence of cytoplasmic proteins. Bottom panels from a single representative gel show that no surface protein was recovered with avidin-complexed beads when biotinylation was omitted.

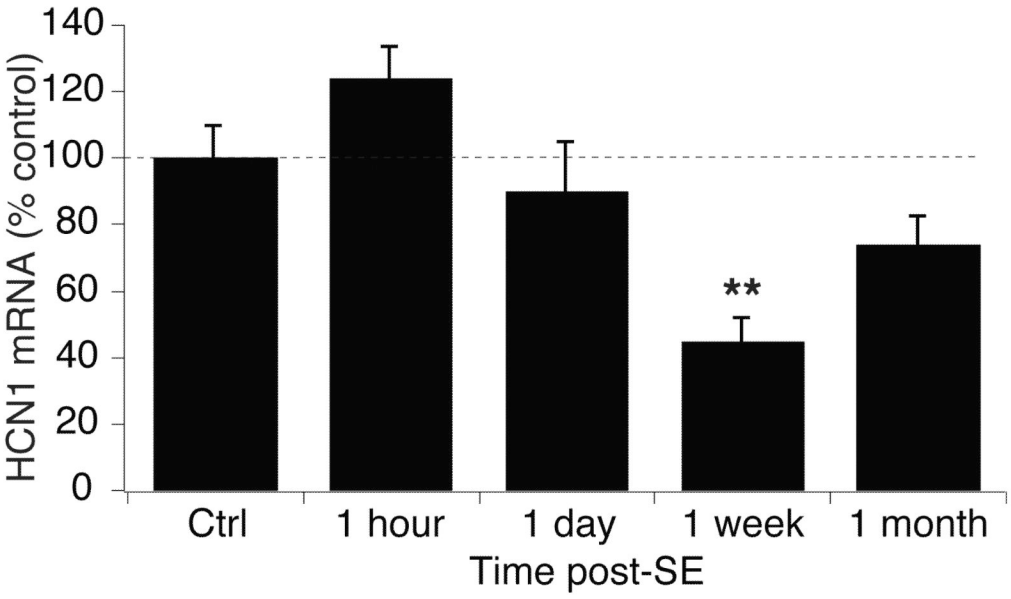

Figure 3.
HCN1 mRNA levels are reduced within 1 week post-SE. HCN1 mRNA levels were unchanged at 1 hr and 1 d post-SE, but were significantly reduced at 1 wk post-SE. Control data from the 1 hr time point is shown.

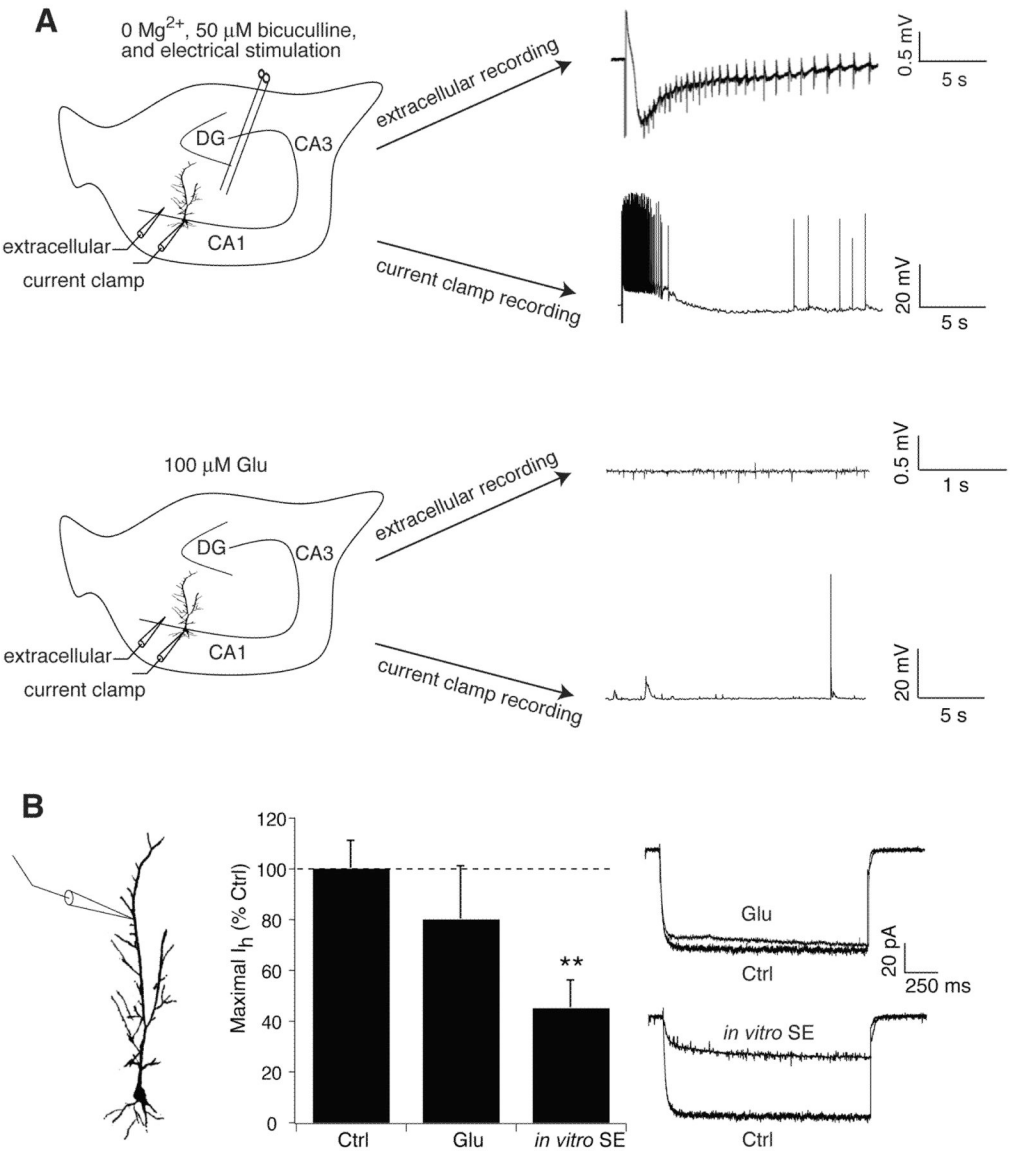

Figure 4.

Dendritic I_h is reduced in an *in vitro* model of SE. A, Representative traces show an evoked seizure-like event in both extracellular and whole-cell current-clamp recordings in the presence of bath solution with 0 Mg^{2+} and 50 μM bicuculline with extracellular stimulation of perforant path afferents. In the presence of 100 μM glutamate alone, only modest spontaneous excitatory activity is seen. Example traces were not obtained simultaneously. B, *In vitro* SE conditions for 1 hr caused a significant decrease in dendritic I_h amplitude compared to control, while 100 μM glutamate application for 1 hr had no significant effect.