The hyperpolarization-activated cation current, Ih, plays an important role in regulating intrinsic neuronal excitability in the brain. In hippocampal pyramidal neurons, Ih is mediated by h channels comprised primarily of the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel subunits, HCN1 and HCN2. Pyramidal neuron h channels within hippocampal area CA1 are remarkably enriched in distal apical dendrites, and this unique distribution pattern is critical for regulating dendritic excitability. We utilized biochemical and immunohistochemical approaches in organotypic slice cultures to explore factors that control h channel localization in dendrites. We found that distal dendritic enrichment of HCN1 is first detectable at postnatal day 13, reaching maximal enrichment by the 3rd postnatal week. Interestingly we found that an intact entorhinal cortex, which projects to distal dendrites of CA1 but not area CA3, is critical for the establishment and maintenance of distal dendritic enrichment of HCN1. Moreover blockade of excitatory neurotransmission using tetrodotoxin, 6-cyano-7-nitroquinoxaline-2,3-dione, or 2-aminophosphonovalerate redistributed HCN1 evenly throughout the dendrite without significant changes in protein expression levels. Inhibition of calcium/calmodulin-dependent protein kinase II activity, but not p38 MAPK, also redistributed HCN1 in CA1 pyramidal neurons. We conclude that activation of ionotropic glutamate receptors by excitatory temporospatial pathway projections from the entorhinal cortex establishes and maintains the distribution pattern of HCN1 in CA1 pyramidal neuron dendrites by activating calcium/calmodulin-dependent protein kinase II-mediated downstream signals.

Compartmentalization of voltage-gated ion channels within neurons is critical for integration and transmission of neuronal signals, and disorganization of functional channels among subcellular domains could be a mechanism of pathophysiology in certain neurological diseases (1). Hyperpolarization-activated cyclic nucleotide-gated (HCN)2 channels (h channels) mediate the hyperpolarization-activated current, Ih, in neurons (2, 3). Both Ih and h channel subunit proteins are enriched 6–10-fold in distal apical dendrites compared with the soma of pyramidal neurons in hippocampal area CA1 (2–5), and this enrichment of Ih in distal dendrites profoundly influences neuronal excitability. Along these lines, h channels 1) are active at resting membrane potentials, thereby contributing an inward current that reduces the input resistance at the distal dendrites (6), and 2) close with depolarization, thereby reducing the amplitude and duration of distant synaptic excitatory postsynaptic potentials and normalizing temporal summation (6, 7). Blockade of this nonuniform Ih enhances temporal summation of excitatory inputs, increasing neuronal excitability (7), whereas pharmacological activation of h channels reduces temporal summation of dendritic synaptic inputs and concurrently reduces CA1 excitability (8). Thus, enrichment of h channels in distal apical dendrites serves an important role in providing an antie excitatory influence to hippocampal pyramidal neurons.

Despite the importance of distal dendritic enhancement of h channels for neuronal excitability, molecular factors controlling h channel localization are not well known. Expression and distribution of the principal hippocampal h channel subunits, HCN1 and HCN2, are regulated developmentally. In rodent hippocampus, protein expression levels of HCN1 and HCN2 increase 4-fold from neonatal to young adult animals, and the distally enriched distribution pattern of h channel subunits in CA1 appears in the second postnatal week (9–12). That the onset of the distal dendritic enrichment of h channels coincides with developmental synaptogenesis (13, 14) suggests that synaptic activity could control h channel localization. Interestingly others have reported up-regulation of Ih in CA1 pyramidal neurons by ionotropic glutamate receptor activation (15, 16). Although changes in h channel localization were not evaluated in these prior studies, we wondered whether excitatory neuro-
nal inputs might control h channel localization in CA1 pyramidal neurons, thereby affecting excitability. Apical dendrites of CA1 pyramidal neurons are innervated by the Schaffer collateral pathway from CA3 as well as branches of the perforant pathway often referred to as the temporoammonic (TA) pathway (17, 18). To explore whether excitatory inputs control h channel localization, we evaluated the expression of HCN1 in cultured rat organotypic hippocampal slices. Utilizing pharmacological, immunohistochemical, and biochemical approaches, here we show that the distal dendritic localization of HCN1 in CA1 pyramidal neurons is regulated by excitatory inputs from the TA pathway and specifically requires activation of ionotropic glutamate receptors and CaMKII.

**EXPERIMENTAL PROCEDURES**

**Antibody Generation—**Antibody specific to the C terminus of HCN1 (guinea pig (gp) α-HCN1) was prepared commercially (Affinity Bioreagents, Golden, CO) by immunizing guinea pigs with a fusion protein consisting of amino acids 778–910 of mouse HCN1. cDNA was generated by PCR using primers 5′-CCGGAAATTCAGGAAGCCGGCCGC and 3′-CCGGTCCAGTCAGTACTGATCGG followed by subcloning the PCR product into the EcoRI and BamHI sites of the glutathione S-transferase-producing vector, pGEX-4T1 (GE Healthcare). Fusion protein was expressed in BL21 bacteria (Stratagene, La Jolla, CA) and purified by glutathione-Sepharose affinity chromatography according to the manufacturer’s protocol (Amersham Biosciences).

**Dissociated Hippocampal Neuron Culture—**Hippocampal neuron cultures were prepared as described previously (19, 20). In brief acutely dissociated hippocampal neurons from embryonic day 18 rats were plated at a density of 600/mm² on glass coverslips coated with poly-D-lysine (Sigma) and maintained in Neurobasal medium supplemented with B27 (Invitrogen) with 2 mM sucrose, 25 mM glucose, 0.5 mM CaCl₂, 4 mM MgCl₂, 0.5 mM sodium ascorbate, 0.1 mM kynurenate, 90 mM NaCl, 2 mM KCl, 50 mM HEPES). For maintenance, high serum medium was replaced twice weekly.

**Organotypic Hippocampal Slice Culture—**P7 rats were anesthetized by halothane and decapitated, and then the brain was rapidly removed and placed in ice-cold dissection medium (75 mM sucrose, 25 mM glucose, 0.5 mM CaCl₂, 4 mM MgCl₂, 0.5 mM sodium ascorbate, 0.1 mM kynurenate, 90 mM NaCl, 2 mM KCl, 25 mM NaHCO₃, 1 mM NaH₂PO₄, and 1 mM 2-aminophosphonovalerate (APV)). Hemispheres were separated and individually placed on a Vibratome (World Precision Instruments, Sarasota, FL) and immobilized using superglue. Horizontal sections (350 μm) were generated, and the hippocampal formation including attached entorhinal cortex was gently dissected using 26%-gauge needles. Dissected tissues were placed on culture inserts (Millipore, Billerica, MA) in prewarmed high serum culture medium (50% minimum Eagle’s medium, 25% Earle’s balanced salt solution, 25% horse serum, 36 mM glucose, and 25 mM HEPES). For maintenance, high serum medium was replaced the next day followed by replacement with low serum medium (5% horse serum) every 3 days thereafter. All animal usage in these studies was approved by the Northwestern University Animal Care and Use Committee.

**Pharmacological Treatment—**For pharmacological treatment, 1 μM tetrodotoxin (TTX; Sigma), 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma), or 100 μM APV (Tocris, Ellisisville, MO) was dissolved into the medium either at day in vitro (DIV) 3 for chronic exposure or at DIV14 for acute exposure. During chronic treatment (>3 days), drug-containing medium was replaced every 3 days. Kainic acid (6 μM; Tocris), cell-permeable BAPTA-AM (10 μM; Molecular Probes, Carlsbad, CA), and cell-permeable autacotide-2-related inhibitory peptide II (AIP-II; 30 μM; Calbiochem), 4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino)pyrimidinium chloride (ZD7288; 10 μM; Tocris) were dissolved in distilled H₂O and mixed with medium. The CaMKII inhibitor KN93 (10 μM; Sigma) and its inactive analog KN92 (10 μM; Sigma) as well as the p38 MAPK inhibitor SB203580 (10 μM; Calbiochem) and its inactive analog SB202474 (10 μM; Calbiochem) were dissolved in Me₂SO and mixed in medium to yield the working concentrations listed above. The final concentration of Me₂SO was less than 0.1%.

**Immunohistochemistry—**Immunocytochemistry was performed on dissociated hippocampal neuron cultures at DIV28. Coverslips were removed from wells and placed in fixative (2% paraformaldehyde in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4)) for 10 min at room temperature (RT), then placed in PBS containing 0.1% Triton X-100 (PBST), and washed 3 x 5 min. Coverslips were incubated in PBST containing 1% normal goat serum for 1 h at RT. Primary antibodies gp α-HCN1 (1:1000) and mouse α-MAP2 (1:500, BD Biosciences) were added in this block solution 1 h at RT followed by appropriate secondary antibodies conjugated to Alexa-488 (1:2500; Molecular Probes) or Cy3 (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) fluorophores diluted in block solution for 1 h at RT. Coverslips were washed 3 x 5 min in PBST after each antibody step and then mounted on slides (Fisher) with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL).

For immunohistochemistry of fixed brain tissues, 8–12 week-old rats and mice were perfused with fixative, 4% freshly depolymerized paraformaldehyde in PBS. Brains were removed and postfixed overnight at 4 °C in fixative, and parasagittal or horizontal free floating sections (50 μm) were cut on a microslicer (VT1000 S, Leica, Wetzlar, Germany). Diaminobenzidine staining was performed with primary antibody gp α-HCN1 (1:1000) followed by species-appropriate secondary antibody in an avidin-biotin-peroxidase system (ABC Elite, Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Peroxidase staining was developed using 3,9-diaminobenzidine as the chromogen. For fluorescence staining, Cy3-conjugated α-guinea pig secondary antibody was used. For fluorescence immunohistochemistry of cultured organotypic slices, membranes of tissue inserts surrounding the culture were excised and fixed in 4% freshly depolymerized paraformaldehyde in PBS for 1 h at RT. Tissues were rinsed with PBST twice and then incubated with blocking solution (3% normal goat serum in PBST) at least for 1 h at RT. Then tissues were incubated with the desired primary antibody followed by species-appropriate secondary antibody as described above for 1 h at RT. Slices were washed 3 x 10 min in PBST after each antibody step and then mounted on slides (Fisher) with Vectorshield (Vector Laboratories). Propidium iodide uptake assay was performed by adding 2 μM propidium iodide (Invitrogen)
solution to the culture medium and then incubating at 37 °C for 1 h before fixation.

**Western Blotting**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum, 10 units/ml penicillin, and 10 µg/ml streptomycin. Cells were transfected at 30% confluence in serum-free medium using Lipofectamine reagent according to the manufacturer’s protocol (Invitrogen). After 48 h, cells were washed with ice-cold PBS, and protein extracts were generated in TEEN-Tx (0.1 M Tris, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100). To obtain mouse brain, wild type (C57/B6; Jackson Laboratory, Bar Harbor, ME) and HCN1 knock-out mice (Hcn1tm2Kndl; Jackson Laboratory) were anesthetized by halothane inhalation and decapitated. Brains were rapidly removed and homogenized in 10 volumes (w/v) of buffer containing 10 mM HEPES, pH 7.4, and 320 mM sucrose and centrifuged at 1000 × g to remove nuclei and insoluble material. The post-nuclear homogenate was centrifuged at 50,000 × g for 40 min to yield a cytosolic fraction (S2) and crude membrane pellet, which was then resuspended in TEEN-Tx (S3). Protein extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Primary antibodies including gp α-HCN1 (1:1000) and mouse α-tubulin (clone DM1A, 1:2000; Sigma) were diluted in block solution containing 5% milk and 0.1% Tween 20 in Tris-buffered saline (TBST) and then incubated with membranes overnight at 4 °C or 1 h at RT. Blots were washed 3 × 10 min with TBST, and species-appropriate secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) was added in TBST containing 5% milk at a dilution of 1:2500. Labeled bands were visualized using SuperSignal chemiluminescence (Pierce). Quantification of band intensity was performed by densitometry using NIH Image software, and band intensity of HCN1 in each sample was normalized with the band intensity of tubulin to adjust for loading differences. All chemicals were purchased from Sigma unless specified.

**Microscopy and Data Analysis**—For dissociated hippocampal neuron cultures, images of mounted coverslips were taken under fluorescence microscope with a 63× oil immersion objective (numerical aperture = 1.4) affixed to a Zeiss Axiovert 200M inverted microscope with Axiovision 3.0 software-driven controls equipped with an Axio Cam HRm camera. For organotypic slices and fixed brain tissues, digital images were taken with a 5× objective utilizing the same microscope configuration. Images were analyzed with NIH Image software. h channel distal spatial distribution was quantitated utilizing NIH Image J analysis software. Area CA1 was identified (by thin pyramidal layer and relationship to dentate gyrus blades), and a line beginning with and perpendicular to the pyramidal cell body layer extending to the distal stratum lacunosum molecular (SLM) layer was placed across the middle of area CA1. This line spanning the region of apical dendrites was divided into 10 equal sections, and the average pixel intensity in each subsegment (as well as for the pixels across the soma) was assigned to the distal (with respect to the soma) point of each division. Division in equal subsegments allowed comparisons between different slices that might have been sectioned in slightly different planes or angles and hence have different lengths of dendritic fields.

HCN1 immunoreactivity (pixel intensity) was analyzed across CA1 along the bisection line using the “plot profile” function. The data file was used to graph x as distance from soma and y as intensity of pixels. Data were represented as relative value of pixel intensity (percentage of minimum segmental intensity, normalized to lowest average intensity of all subsegments, minus background signal from an area of the image lacking tissue). For each tissue, five adjacent lines were analyzed, and values from each line were averaged. Statistical analysis involved analysis of variance with posthoc analysis using Tukey’s honest significant difference.

**RESULTS**

**Characterization of Antibody against the C Terminus of HCN1**—We generated guinea pig polyclonal antiserum against HCN1 utilizing a glutathione S-transferase fusion of the C terminus (amino acids 778–910) of HCN1 as antigen. We next sought to characterize the sensitivity and specificity of our custom HCN1 antibody. Western blotting of membrane extracts from transfected COS-7 cells showed that α-HCN1-(778–910) recognizes HCN1 but not another h channel subunit enriched in brain tissue, HCN2 (Fig. 1A). Furthermore this antibody recognized a single 110-kDa protein band on Western blots prepared from wild type mouse brain but not from the HCN1 knock-out mouse brain (Hcn1tm2Kndl) (Fig. 1A), confirming specificity for detecting HCN1 in tissue extracts. Next we evaluated the specificity of α-HCN1-(778–910) for rat brain immunohistochemistry. We found that the expression pattern of our custom α-HCN1-(778–910) in rat brain is similar to that reported in prior studies (Fig. 1B) (5) and is abolished by antigen preabsorption (Fig. 1B). We also performed immunohistochemistry using α-HCN1-(778–910) on control mouse brain (C57/B6) and HCN1 knock-out mouse brain. Whereas we found a staining pattern identical to that observed in rat brain

![FIGURE 1. gp α-HCN1 antibody is specific and sensitive in biochemical and immunohistochemical assays. A, protein extracts from COS-7 cells transfected with HCN1-expressing plasmid and mouse brains were separated by SDS-PAGE and blotted with gp α-HCN1 antibody. Our custom antibody detected a single band of ~110 kDa in transfected COS-7 cells and wild type (WT) mouse brain. No band was detected in brain extract prepared from the HCN1 knock-out mouse (Hcn1tm2Kndl). B, parasagittal sections of rat brain were immunolabeled with gp α-HCN1 antibody or antigen-preabsorbed gp α-HCN1 antibody. Antigen preabsorption eliminated immunoreactivity observed with gp α-HCN1, confirming specificity. C, parasagittal sections of wild type or HCN1 knock-out mouse brain were immunolabeled with gp α-HCN1 antibody. No immunoreactivity was found in HCN1 knock-out mouse brain, confirming specificity of gp α-HCN1 antibody. Scale bars, 200 µm. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.](image-url)
(Fig. 1B and Refs. 10 and 21), no staining was observed in the brain of HCN1 knock-out mice using α-HCN1-(778–910) (Fig. 1C). Based on these data, we conclude that gp α-HCN1-(778–910) antibody is specific for detecting HCN1.

**Distal Dendritic Enrichment of HCN1 Is Present in Cultured Organotypic Hippocampal Slices but Not Dissociated Pyramidal Neurons**—To study the basic mechanism controlling h channel localization in dendrites, we sought to develop an *in vitro* assay that would 1) recapitulate enrichment of HCN1 protein observed in distal dendrites of pyramidal neurons in hippocampal area CA1 in fixed brain tissue and 2) allow easy pharmacological manipulation to evaluate the establishment and maintenance of this distal dendritic enrichment. To determine whether h channels are enriched in the distal dendrites of hippocampal neurons maintained in cultures *in vitro*, we sought to develop an assay that would 1) recapitulate enrichment of HCN1 protein observed in distal dendrites of pyramidal neurons in hippocampal area CA1 in *in vivo* and 2) allow easy pharmacological manipulation to evaluate the establishment and maintenance of this distal dendritic enrichment.

**Adult Neurons**—In the hippocampus, HCN1 protein expression levels and distribution patterns change during postnatal development, reaching adult levels and distribution by P28 (11, 22). We wondered whether the expression and distribution pattern of HCN1 showed similar developmental changes *in vitro*. Organotypic hippocampal slice cultures were fixed and immunolabeled with gp α-HCN1 and α-MAP2. Because immunostaining of cultured slices produces an intense staining artifact along the edges of slices containing basal dendrites and because physiologically relevant h channel enrichment in distal dendrites has been best characterized in apical dendrites, we restricted our analysis of HCN1 protein distribution to the soma and apical dendritic fields. To quantitate the changes in HCN1 density, the apical dendritic field of area CA1 was divided into 10 equal segments from the cell body layer (S1) to the distal apical dendritic fields (S10). The average HCN1 immunoreactivity was determined for each segment and normalized to the lowest segmental value to obtain the relative intensity of each segment. At DIV1, HCN1 was distributed predominantly near the somata of CA1 pyramidal neurons with lower immunoreactivity detected in the distal dendrite (137 ± 6% in S1 versus 111 ± 4% in S10; unit, percentage of minimum segmental intensity; p < 0.001 for S1, S9, and S10 compared with DIV14; p < 0.05 for S2 and S3 compared with DIV14; n = 9). At DIV3, HCN1 immunoreactivity was relatively even throughout the dendrite from the soma to the distal dendrite (122 ± 3% in S1, 110 ± 2% in S5, and 110 ± 3% in S10; unit, percentage of minimum segmental intensity; p < 0.001 for S9 and S10 compared with DIV14; n = 13). By DIV6, HCN1 was enriched in SLM layer, consistent with prior reports of the establishment of distal dendritic enrichment of HCN1 in the later 2nd postnatal week *in vivo* (11, 22) (110 ± 2% in S1 versus 149 ± 6% in S10; unit, percentage of minimum segmental intensity; n = 9). From DIV6 to DIV21, the gradient of enrichment from the soma to the distal dendrite remained the same.
Regulation of h Channel Distribution in Hippocampus

FIGURE 3. Developmental changes in distribution and expression of HCN1 in organotypic slice cultures. A, organotypic slices comprising hippocampus and attached entorhinal cortex cultured from DIV1 to DIV21 were immunolabeled with α-HCN1 (green, left panels) and α-MAP2 (red, right panels). HCN1 enrichment in distal dendritic fields of CA1 pyramidal neurons becomes apparent as early as DIV6 (arrows). B, quantification of HCN1 staining in dendritic fields of CA1 pyramidal neurons. The dendritic field from soma to the SLM layer was divided into 10 segments, and the intensities of immunoreactivity of HCN1 subunits was averaged in each segment. The relative intensity of HCN1 staining in each segment was calculated by normalizing to the staining of the lowest intensity segment. At DIV1 and DIV3, HCN1 is localized in the perisomatic region, and no enrichment in distal dendrites was observed. By DIV6, distal dendritic enrichment of HCN1 is present with no significant change in distribution from DIV6 to DIV21 (DIV1, n = 9; DIV3, n = 13; DIV6, n = 9; DIV10, n = 11; DIV14, n = 15; DIV21, n = 11). **, p < 0.01; ***, p < 0.001. C, HCN1 protein expression increases during development in slice culture. Protein expression levels were analyzed by Western blotting of cultured hippocampal area CA1 extracts from DIV1 to DIV21 using gp α-HCN1 antibody. Tubulin immunoreactivity was evaluated as control for protein loading. D, quantification of HCN1 protein expression levels during development reveals that expression of HCN1 increased 3-fold from DIV1 to DIV6 and 5-fold from DIV1 to DIV14 (n = 3). Error bars represent ± S.E. Scale bars, 200 μm.

We also explored developmental changes in HCN1 protein expression by Western blotting extracts prepared from subdissected area CA1 of cultured slices at each developmental stage. Similar to HCN1 protein expression levels in vitro, HCN1 levels increased during development in vitro. Specifically HCN1 expression increased 3-fold (2.7 ± 0.23, n = 3) from DIV1 to DIV6 and 5-fold (5.07 ± 0.25, n = 3) from DIV1 to DIV14 with no significant increase of HCN1 expression level between DIV14 and DIV21 (5.07 ± 0.25 versus 5.23 ± 0.35-fold compared with DIV1, n = 3 each) (Fig. 3, C and D). Because the magnitude and timing of the developmental increase in protein expression as well as the onset of distal dendritic enrichment closely resemble that published for HCN1 in vivo (11), we reasoned that the in vitro slice culture model is a useful tool to study mechanisms controlling HCN1 distribution and protein expression in hippocampus.

CA1 area was not significantly different from control, non-sectioned slices (CA3-lacking slices: 112 ± 3% in S1, 108 ± 1% in S5, and 154 ± 8% in S10, n = 13; control slices: 112 ± 3% in S1, 108 ± 2% in S5, and 141 ± 6% in S10; unit, percentage of minimum segmental intensity; n = 15; p > 0.7 for all segments compared with control; Fig. 4, A and B). In contrast, slices with the EC removed showed significant reductions in the distal dendritic enrichment of HCN1 with HCN1 immunostaining displaying an even distribution throughout the apical dendritic fields of CA1 pyramidal neurons (113 ± 2% in S1, 106 ± 2% in S5, and 107 ± 2% in S10; unit, percentage of minimum segmental intensity; n = 19; p < 0.05 for S7 and p < 0.001 for S8, S9, and S10 compared with control; Fig. 4, A and B). These results strongly suggest that afferent inputs from the EC, but not CA3, are required to establish the distally enriched distribution pattern of HCN1 in CA1 dendrites.
Next we tested whether maintenance of the mature distribution of HCN1 in distal dendrites requires EC or CA3. Previous reports have shown that loss of axons of EC neurons from hippocampus begins as early as 1 day after EC lesion (24, 25). Thus we reason that lesioned EC or CA3 neurons will result in loss of excitatory inputs from these areas to CA1 pyramidal neuron dendrites. Because HCN1 immunostaining is enriched in distal dendrites by DIV6 and reaches adult expression levels by DIV14, we cultured slices until DIV14 and then removed EC or CA3 by scalpel at DIV14. The lesioned cultures were maintained in vitro for 14 days and then immunolabeled with α-HCN1 (green, left panel) and α-MAP2 (red, right panel). In slices with both EC and CA3 attached (control) or lacking only CA3 (–CA3), HCN1 is enriched in distal dendritic fields within area CA1. In the slices without EC (–EC), HCN1 is evenly distributed throughout the dendritic field. 

quantitation of HCN1 immunoreactivity in CA1 segments of control slices as well as those lacking EC or CA3 shows distal dendritic HCN1 enrichment requires TA inputs from EC to CA1 (DIV14 control, n = 15; –EC, n = 19; –CA3, n = 13; **, p < 0.05; ***, p < 0.001). C, Western blot of CA1 extracts from DIV14 control and EC- or CA3-lesioned organotypic slice cultures probed with α-HCN1 and α-tubulin. D, intensity of the HCN1 band from Western blotting was quantitated and normalized to tubulin intensity and revealed no significant differences in area CA1 HCN1 expression levels regardless of the absence of CA3 or EC (n = 4). Arrows indicate distal dendritic field of CA1 hippocampus. Error bars represent ±S.E. Scale bars, 200 μm.

FIGURE 4. TA inputs from EC to CA1 are necessary for the establishment of HCN1 distal dendritic enrichment. A, organotypic slices were prepared at P7, and EC or CA3 was removed mechanically at the time of culturing to eliminate TA or Schaffer collateral inputs to CA1, respectively. Slices were maintained in vitro for 14 days and then immunolabeled with α-HCN1 (green, left panel) and α-MAP2 (red, right panel). In slices with both EC and CA3 attached (control) or lacking only CA3 (–CA3), HCN1 is enriched in distal dendritic fields within area CA1. In the slices without EC (–EC), HCN1 is evenly distributed throughout the dendritic field. B, quantitation of HCN1 immunoreactivity in CA1 segments of control slices as well as those lacking EC or CA3 shows distal dendritic HCN1 enrichment requires TA inputs from EC to CA1. (DIV14 control, n = 15; –EC, n = 19; –CA3, n = 13; **, p < 0.05; ***, p < 0.001). C, Western blot of CA1 extracts from DIV14 control and EC- or CA3-lesioned organotypic slice cultures probed with α-HCN1 and α-tubulin. D, intensity of the HCN1 band from Western blotting was quantitated and normalized to tubulin intensity and revealed no significant differences in area CA1 HCN1 expression levels regardless of the absence of CA3 or EC (n = 4). Arrows indicate distal dendritic field of CA1 hippocampus. Error bars represent ±S.E. Scale bars, 200 μm.
Regulation of h Channel Distribution in Hippocampus

A.

B.

C.

D.

FIGURE 5. Maintenance of HCN1 distal dendritic enrichment requires the TA pathway. A, organotypic slice cultures were prepared at P7 and maintained in vitro for 14 days. At DIV14, EC or CA3 was removed mechanically, and then slices were maintained for an additional 48 h. Slices were next immunolabeled with α-HCN1 (green, left panel) and α-MAP2 (red, right panel). Removal of EC, but not CA3, resulted in loss of HCN1 distal dendritic enrichment. B, quantitation of HCN1 immunoreactivity in area CA1 dendritic fields of control, EC-, or CA3-lesioned tissue confirmed a requirement for EC to maintain HCN1 distal dendritic enrichment (DIV16 control: 105 ± 1% in S1, 108 ± 1% in S5, and 111 ± 2% in S10; n = 21; p < 0.05 for S7 and p < 0.001 for S8, S9, and S10 compared with control; n = 14). However, although neurons in CA1 remained viable, with long term exposure of slices to TTX massive cell loss was detected in the EC (data not shown). On the other hand, slice cultures treated with either CNQX or APV showed no apparent cell death (data not shown) but exhibited diffuse dendritic staining of HCN1 consistent with blockade of establishment of distal HCN1 enrichment (CNQX: 112 ± 3% in S1, 108 ± 1% in S5, and 111 ± 2% in S10; n = 21; p < 0.05 for S7 and p < 0.001 for S8, S9, and S10 compared with control; APV: 115 ± 5% in S1, 108 ± 2% in S5, and 114 ± 3% in S10; unit, percentage of minimum segmental intensity; n = 18; p < 0.001 for S8, S9, and S10 compared with control; Fig. 6, A and B). Thus activity of either AMPA- or NMDA-type glutamate receptors appears to be necessary for the establishment of proper HCN1 localization in distal dendrites. To evaluate whether HCN1 protein expression is changed with chronic drug treatment, we performed Western blotting of cultured hippocampal slices. HCN1 protein in TTX-treated slices was reduced to 78 ± 3% of control slices, yet no significant change was found in}

ization in CA1 dendrites, we exposed hippocampal slices to 1 μM TTX, 10 μM CNQX, or 100 μM APV, concentrations known to block sodium channels, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and N-methyl-D-aspartate (NMDA) receptors, respectively, in cultured slices (13). Drugs were added to the culture medium at DIV3, and cultured slices were maintained for 11 subsequent days in vitro and refreshed with medium containing drugs every 3 days. Immunohistochemical and biochemical assays were then performed with cultured slices to evaluate the changes in HCN1 distribution and protein expression levels. To ensure that detected changes in HCN1 distribution were specific to channel or receptor blockade and not affected by cell loss, we performed double labeling with α-MAP2 to evaluate dendritic integrity and health of slices as well as performed propidium iodide uptake assays to detect cell death in drug-treated slices. Similar to the slices in which EC was removed mechanically, HCN1 subunits were redistributed evenly along the dendritic field in hippocampal area CA1 after exposure to TTX (115 ± CNQX- or APV-treated slices (CNQX: 103 ± 8%, APV: 103 ± 7%, p > 0.7, n = 4) (Fig. 6, C and D). We next sought to determine whether glutamatergic activity was required for maintenance of distally enriched dendritic HCN1 after establishment at DIV14. Thus, we treated DIV14 slice cultures with TTX (1 μM), CNQX (10 μM), or APV (100 μM) for 48 h and then evaluated HCN1 localization. In contrast to chronic treatment, treatment of mature slice cultures with TTX for 48 h did not induce cell death nor was cell death noted with acute treatment with APV or CNQX (data not shown). However, treatment of slices for 48 h with TTX, CNQX, or APV eliminated the distal dendritic enrichment of HCN1, producing evenly distributed HCN1 immunostaining throughout the CA1 dendritic field (DIV16 control: 105 ± 1% in S1, 107 ± 2% in S5, and 140 ± 5% in S10; n = 19; TTX: 108 ± 1% in S1, 105 ± 2% in S5, and 113 ± 3% in S10; p < 0.05 for S7 and p < 0.001 for S8, S9, and S10 compared with control; n = 16; CNQX: 110 ± 2% in S1, 106 ± 2% in S5, and 110 ± 2% in S10; p < 0.05 for S7 and p < 0.001 for S8, S9, and S10 compared with control; n = 21; APV: 113 ± 4%
Regulation of h Channel Distribution in Hippocampus

In S1, 108 ± 2% in S5, and 107 ± 1% in S10; unit, percentage of minimum segmental intensity; p < 0.05 for S7 and p < 0.001 for S8, S9, and S10 compared with control; n = 26) (Fig. 7, A and B). Expression of HCN1 subunit protein was not changed with short term treatment of TTX, CNQX, or APV compared with control (TTX: 97 ± 3%, CNQX: 108 ± 4%, APV: 99 ± 5%, p > 0.7, n = 5) (Fig. 7, C and D), suggesting that the loss of distal HCN1 immunoreactivity reflects redistribution of existing channels rather than a change in total protein. Next we evaluated the distribution of HCN1 with the activation of ionotropic glutamate receptors using kainic acid. DIV14 hippocampal slices were treated with kainic acid (6 μM) for 12 h, and then localization of HCN1 was determined by immunohistochemical analysis. With kainic acid treatment, relative immunoreactivity of HCN1 in the distal dendrites was increased significantly compared with control (kainic acid: 111 ± 4% in S1, 112 ± 4% in S5, 144 ± 3% in S8, 153 ± 3% in S9, and 156 ± 7% in S10; unit, percentage of minimum segmental intensity; p < 0.05 for S8 and S9 compared with control; n = 9) (supplemental Fig. 2). No change in protein expression level or no apparent cell death with kainic acid treatment for 12 h was detected (data not shown). Whereas blockade of ionotropic glutamate receptors redistributes the HCN1 in CA1 pyramidal neuron dendrites, blockade of h channel by ZD7288 (10 μM) for 48 h had no effect on distribution or protein expression level of HCN1 (ZD7288: 105 ± 2% in S1, 110 ± 3% in S5, and 130 ± 7% in S10; unit, percentage of minimum segmental intensity; n = 8; supplemental Fig. 3, A and B; protein expression level: 103 ± 4% of control, p > 0.7, n = 4; supplemental Fig. 3, C and D). Taken together, these data suggest that activation of ionotropic glutamate receptors by TA innervation of CA1 dendrites is necessary to establish and maintain the distal dendritic enrichment of HCN1 in CA1 pyramidal neurons.

HCN1 Redistribution following Activity Blockade Is Reversible—The finding that pharmacological blockade of ionotropic glutamate receptors relocalized HCN1 suggests that trafficking of HCN1 subunits may be dynamically regulated by neuronal activity. We tested whether redistribution of HCN1 during the drug treatment is reversible after drug withdrawal. In these experiments, cultured organotypic slices were treated with TTX (1 μM), CNQX (10 μM), or APV (100 μM) for 48 h from DIV14 to DIV16, and then drugs were washed out by medium exchange for the next 5 days. Interestingly we found that drug washout (TTX, CNQX, or APV) restored the asymmetric, distally enriched distribution of HCN1 in CA1 dendrites to levels that were not significantly different from age-matched control (Fig. 8; DIV21 control: 105 ± 1% in S1, 110 ± 3% in S5, and 142 ± 4% in S10; n = 11; TTX washout: 104 ± 1% in S1, 107 ± 2% in S5, and 131 ± 6% in S10; p > 0.7 for all segments compared with control; n = 9; CNQX washout: 107 ± 2% in S1, 106 ± 2% in S5, and 136 ± 7% in S10; p > 0.7 for all segments compared with control; n = 11; APV washout: 109 ± 3% in S1, 105 ± 2% in S5, and 142 ± 8% in S10; unit, percentage of minimum segmental intensity; p > 0.7 for all segments compared with control; n = 9). These observations suggest that HCN1 localization in CA1 pyramidal neurons is dynamic and mediated by activity-dependent regulation of HCN1 trafficking.

Inhibition of CaMKII, but Not p38 MAPK, Abolishes the Distribution Pattern of HCN1 Subunits—Our studies indicate that localization of HCN1 in CA1 pyramidal neurons can be regulated by activity of ionotropic glutamate receptors. Although others have reported up-regulation of I_h in CA1 pyramidal neurons by ionotropic glutamate receptor activation (15, 16), we found HCN1 protein expression to be unchanged after most mechanical and pharmacological treatments of cultured slices despite large differences in protein localization; thus, we rea-
Regulation of h Channel Distribution in Hippocampus

**A**. organotypic slice cultures were maintained until DIV14 and then treated with TTX (1 μM), CNQX (10 μM), or APV (100 μM) for 48 h. Slices were immunolabeled with α-HCN1 (green, left panel) and α-MAP2 (red, right panel). HCN1 staining was lost from area CA1 distal dendritic fields of slices treated with TTX, CNQX, or APV. Age-matched slices (DIV16) with no treatment were used as control. **B**. quantification of HCN1 immunoreactivity in CA1 dendritic fields confirmed loss of HCN1 staining in drug-treated slices (DIV16 control, n = 19; TTX-treated, n = 16; CNQX-treated, n = 26; **p < 0.05, ***p < 0.001). **C**. Western blot of CA1 extracts from control or drug-treated slices were probed with α-HCN1 and α-tubulin. **D**. intensity of HCN1 band from Western blotting was quantitated and normalized with tubulin and revealed that expression of HCN1 was unchanged in TTX-, CNQX-, or APV-treated samples compared with control (n = 5). Arrows indicate distal dendritic field of CA1 hippocampus. Error bars represent ±S.E. Scale bars, 200 μm.

**FIGURE 7. Maintenance of HCN1 distal dendritic enrichment requires activation of ionotropic glutamate receptors.** A, organotypic slice cultures were maintained until DIV14 and then treated with TTX (1 μM), CNQX (10 μM), or APV (100 μM) for 48 h. Slices were immunolabeled with α-HCN1 (green, left panel) and α-MAP2 (red, right panel). HCN1 staining was lost from area CA1 distal dendritic fields of slices treated with TTX, CNQX, or APV. Age-matched slices (DIV16) with no treatment were used as control. B, quantification of HCN1 immunoreactivity in CA1 dendritic fields confirmed loss of HCN1 staining in drug-treated slices (DIV16 control, n = 19; TTX-treated, n = 16; CNQX-treated, n = 21; APV-treated, n = 26; **p < 0.05; ***p < 0.001). C, Western blot of CA1 extracts from control or drug-treated slices were probed with α-HCN1 and α-tubulin. **D**, intensity of HCN1 band from Western blotting was quantitated and normalized with tubulin and revealed that expression of HCN1 was unchanged in TTX-, CNQX-, or APV-treated samples compared with control (n = 5). Arrows indicate distal dendritic field of CA1 hippocampus. Error bars represent ±S.E. Scale bars, 200 μm.

**Dendritic enrichment of control slices**, we found that HCN1 staining was redistributed evenly throughout the dendrites in the CA1 of hippocampal slices treated with KN93 (KN93: 116 ± 5% in S1, 111 ± 3% in S5, and 109 ± 3% in S10; unit, percentage of minimum segmental intensity; p < 0.001 for S9 and S10 compared with control; n = 13) (Fig. 9, A and B). SB203580 had no effect on distal HCN1 localization (SB203580: 108 ± 2% in S1, 109 ± 3% in S5, and 136 ± 4% in S10; unit, percentage of minimum segmental intensity; p = 0.05 for S9 and S10 compared with control; n = 10) (supplemental Fig. 3). Neither KN92 (10 μM), an inactive analog of KN93, nor SB202474 (10 μM), an inactive analog of SB203580, had any effect on distal HCN1 dendritic enrichment (KN92: 108 ± 2% in S1, 107 ± 2% in S5, and 129 ± 4% in S10; unit, percentage of minimum segmental intensity; p > 0.7 for all segments compared with control; n = 12; SB202474: 104 ± 2% in S1, 106 ± 2% in S5, and 141 ± 7% in S10; unit, percentage of minimum segmental intensity; p > 0.7 for all segments compared with control; n = 6) (Fig. 9, A and B, and supplemental Fig. 3, A and B), suggesting specificity of KN93 for CaMKII inhibition as the mechanism for altering HCN1 localization. To confirm the specificity of CaMKII blockade on HCN1 distribution, we also treated hippocampal slices with the cell-permeable CaMKII inhibitory peptide AIP-II (30 μM) for 48 h. Similar to KN93, treatment with AIP-II redistributed HCN1 along the dendritic tree (110 ± 3% in S1, 109 ± 2% in S5, and 113 ± 3% in S10; unit, percentage of minimum segmental intensity; p < 0.001 for S9 and S10 compared with control; n = 10) (Fig. 9, A and B), suggesting that CaMKII activity is required to maintain the distal dendritic enrichment of HCN1 in CA1 pyramidal neurons. Activation of ionotropic glutamate receptors results in calcium influx, which activates downstream signaling cascades including CaMKII (27). We reasoned that if calcium/calmodulin stimulation of CaMKII activity is important for HCN1 localization then chelation of intracellular calcium should relocalize HCN1. As such, hippocampal slices from DIV14 were incubated with cell-permeable calcium chelator BAPTA-AM (10 μM) for 48 h, and the distribution of HCN1 was determined by immunohistochemical analysis. Similar to CaMKII inhibitors, calcium chelation redistributes HCN1 throughout the dendritic axis more evenly compared with control (111 ± 2% in S1, 108 ± 2% in S5, and 115 ± 4% in S10; unit, percentage of minimum segmental intensity; p < 0.001 for S9 and S10 compared with control; n = 20) (Fig. 9, A and B). None of the pharmacological treatment

**Figure 7**

**A**. organotypic slice cultures were maintained until DIV14 and then treated with TTX (1 μM), CNQX (10 μM), or APV (100 μM) for 48 h. Slices were immunolabeled with α-HCN1 (green, left panel) and α-MAP2 (red, right panel). HCN1 staining was lost from area CA1 distal dendritic fields of slices treated with TTX, CNQX, or APV. Age-matched slices (DIV16) with no treatment were used as control. B, quantification of HCN1 immunoreactivity in CA1 dendritic fields confirmed loss of HCN1 staining in drug-treated slices (DIV16 control, n = 19; TTX-treated, n = 16; CNQX-treated, n = 26; **p < 0.05; ***p < 0.001). C, Western blot of CA1 extracts from control or drug-treated slices were probed with α-HCN1 and α-tubulin. **D**, intensity of HCN1 band from Western blotting was quantitated and normalized with tubulin and revealed that expression of HCN1 was unchanged in TTX-, CNQX-, or APV-treated samples compared with control (n = 5). Arrows indicate distal dendritic field of CA1 hippocampus. Error bars represent ±S.E. Scale bars, 200 μm.

**Figure 7**

**A**. organotypic slice cultures were maintained until DIV14 and then treated with TTX (1 μM), CNQX (10 μM), or APV (100 μM) for 48 h. Slices were immunolabeled with α-HCN1 (green, left panel) and α-MAP2 (red, right panel). HCN1 staining was lost from area CA1 distal dendritic fields of slices treated with TTX, CNQX, or APV. Age-matched slices (DIV16) with no treatment were used as control. B, quantification of HCN1 immunoreactivity in CA1 dendritic fields confirmed loss of HCN1 staining in drug-treated slices (DIV16 control, n = 19; TTX-treated, n = 16; CNQX-treated, n = 21; APV-treated, n = 26; **p < 0.05; ***p < 0.001). C, Western blot of CA1 extracts from control or drug-treated slices were probed with α-HCN1 and α-tubulin. **D**, intensity of HCN1 band from Western blotting was quantitated and normalized with tubulin and revealed that expression of HCN1 was unchanged in TTX-, CNQX-, or APV-treated samples compared with control (n = 5). Arrows indicate distal dendritic field of CA1 hippocampus. Error bars represent ±S.E. Scale bars, 200 μm.

**Figure 7**

**A**. organotypic slice cultures were maintained until DIV14 and then treated with TTX (1 μM), CNQX (10 μM), or APV (100 μM) for 48 h. Slices were immunolabeled with α-HCN1 (green, left panel) and α-MAP2 (red, right panel). HCN1 staining was lost from area CA1 distal dendritic fields of slices treated with TTX, CNQX, or APV. Age-matched slices (DIV16) with no treatment were used as control. B, quantification of HCN1 immunoreactivity in CA1 dendritic fields confirmed loss of HCN1 staining in drug-treated slices (DIV16 control, n = 19; TTX-treated, n = 16; CNQX-treated, n = 21; APV-treated, n = 26; **p < 0.05; ***p < 0.001). C, Western blot of CA1 extracts from control or drug-treated slices were probed with α-HCN1 and α-tubulin. **D**, intensity of HCN1 band from Western blotting was quantitated and normalized with tubulin and revealed that expression of HCN1 was unchanged in TTX-, CNQX-, or APV-treated samples compared with control (n = 5). Arrows indicate distal dendritic field of CA1 hippocampus. Error bars represent ±S.E. Scale bars, 200 μm.
Regulation of h Channel Distribution in Hippocampus

In brain and heart, h channels serve an important role in pacemaker function, contributing to autonomous rhythmicity and network oscillation (29). In distal dendrites of highly polarized pyramidal neurons, h channels also serve a critical role in reducing excitability wherein h channel closure upon depolarization reduces amplitude and kinetic components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound enrichment of h channels in distal apical dendrites that reduces temporal and amplitude components of excitatory postsynaptic potentials and markedly limits temporal summation of synaptic inputs to dendrites (6, 7). As such, controlling the localization of h channels within dendrites could profoundly influence neuronal excitability. The present study demonstrates that subcellular localization of the h channel subunit, HCN1, is regulated by activity. Specifically we found that the distal dendritic enrichment of HCN1 in hippocampal area CA1 pyramidal neurons is established and maintained by 1) direct TA pathway inputs from the EC, 2) synaptic activity mediated by ionotropic glutamate receptors, and 3) CaMKII activity. These biochemical and immunohistochemical studies demonstrate a potential molecular mechanism for prior observations of activity-dependent regulation of Ih in CA1 pyramidal neurons (15, 16).

Although many lines of evidence indicate that the marked enhancement of h channels in distal dendrites of CA1 pyramidal neurons is important for function, little is known about how this unique localization is established and maintained. To explore this issue, we generated sensitive and specific antibodies with an immunohistochemical staining pattern identical to that of other custom antibodies (4, 5). We found that immunoreactivity of HCN1 was enhanced in distal apical dendrites that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Enrichment of h channels in distal apical dendrites that reduces excitability wherein h channel closure upon depolarization reduces amplitude and kinetic components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. Central to this role in regulating excitability is a profound reduction in Ih that reduces temporal and amplitude components of excitatory postsynaptic potentials and restricts temporal summation. 

with the drugs above induced any apparent cell death as shown by counterstaining with α-MAP2 in the same tissues (Fig. 9A and supplementary Fig. 3A). As another control, Western blotting was performed with cultured hippocampal slices treated with the above inhibitors. CaMKII inhibitors, calcium chelators, and p38 MAPK inhibitors did not affect protein expres-
pyramidal neurons not affected by synaptic innervation from EC. Interestingly a prior study by Brauer et al. (16) demonstrated inhibition of excitatory synaptic transmission by CNQX or APV prevented establishment of HCN1 enrichment in distal dendrites when administered chronically and redistributed HCN1 out of distal dendrites upon acute drug exposure. Interestingly prior studies have implicated ionotropic glutamate receptors in the regulation of h channel function in CA1 pyramidal neurons wherein activation of AMPA receptors (15) or NMDA receptors (16) increased \( I_h \). Although these studies did not evaluate h channel localization, in light of our findings we speculate that AMPA or NMDA receptor activation could influence h channel trafficking among different subcellular domains. In this model, we postulate that AMPA or NMDA receptor activation promotes trafficking from intracellular stores in the soma and proximal dendrites to the surface membrane of distal dendrites (Fig 10). Supporting this model, h channel subunits in distal dendrites of mature subicular pyramidal neurons, which show a distribution pattern of h channels similar to that of CA1 pyramidal neurons, are mostly (>70%) localized on the surface membrane, whereas a significant portion (>90%) of h channel subunits in the soma are found within intracellular pools (4). Future studies evaluating the localization of intracellular and surface-expressed HCN1 protein are planned to further elucidate mechanisms of h channel trafficking.

Interestingly, Fan et al. (16) demonstrated a requirement for protein synthesis for NMDA receptor-mediated increases in \( I_h \) and further observed acute changes in HCN1 protein expression levels with pharmacological NMDA receptor activation in acutely prepared hippocampal slices. In contrast, we found no change in HCN1 expression levels in organotypic slice cultures in response to 48 h of treatment with TTX, CNQX, or APV despite profound and reversible changes in HCN1 distribution. This discrepancy may reflect differences in experimental preparation but might also be explained by the use of different antibodies to detect HCN1. Whereas our antibody preparation is both sensitive and specific for HCN1 as confirmed by testing in HCN1 knock-out animals, we and others (22) have observed that different commercial anti-h channel antibodies have different selectivity. Indeed different aliquots of commercial anti-HCN1 antibody supplied by the same ven-

**Regulation of h Channel Distribution in Hippocampus**

**FIGURE 9.** Blockade of CaMKII activity redistributed HCN1 in area CA1 dendritic fields without affecting protein expression. A, organotypic slice cultures were maintained until DIV14 and then treated with the CaMKII blocker KN93 (10 \( \mu \)M); an inactive analogue of this molecule, KN92 (10 \( \mu \)M); cell-permeable CaMKII inhibitory peptide AIP-II (30 \( \mu \)M); or cell-permeable calcium chelator BAPTA-AM (10 \( \mu \)M) for 48 h. Slices were then immunolabeled with \( \alpha \)-HCN1 (green, left panel) and \( \alpha \)-MAP2 (red, right panel). HCN1 staining was lost from area CA1 distal dendritic fields of slices treated with KN93, AIP-II, or BAPTA-AM, but distribution was not significantly different upon treatment with KN92. Age-matched slices (DIV16) with no treatment were used as control. B, quantitation of HCN1 immunoreactivity in CA1 dendritic fields confirmed loss of HCN1 staining in KN-93-, AIP-II-, or BAPTA-AM-treated slices (DIV16 control, \( n = 19 \); KN93-treated, \( n = 13 \); KN92-treated, \( n = 12 \); AIP-II-treated, \( n = 10 \); BAPTA-AM-treated, \( n = 20 \); \( \ast \ast \ast , p < 0.001 \)). C, Western blot of CA1 extracts from control or drug-treated slices were probed with \( \alpha \)-HCN1 and \( \alpha \)-tubulin. D, intensity of HCN1 band from Western blotting was quantitated and normalized with tubulin and revealed that expression of HCN1 was unchanged in drug-treated slices compared with control (\( n = 5 \) for KN93 and KN92 and \( n = 4 \) for AIP-II and BAPTA-AM). Arrows indicate distal dendritic field of CA1 hippocampus. Error bars represent ±S.E. Scale bars, 200 \( \mu \)M.
Regulation of h Channel Distribution in Hippocampus

FIGURE 10. Schematic model of activity-dependent control of HCN1 localization in the dendrites of CA1 pyramidal neurons. A, before commencement of synaptic activity, HCN1 protein is evenly distributed throughout apical dendrites. B, during development, synaptic activity (vertical arrows) through the direct EC inputs promotes trafficking of HCN1 subunits from proximal intracellular pools to the surface of distal dendrites (horizontal arrows). C, in mature neurons, HCN1 channels are enriched on the surface membrane of distal apical dendrites, and a significant proportion of HCN1 protein in proximal dendrites is intracellular, a distribution that is maintained by direct inputs from the EC. D, blockade of synaptic activity inhibits trafficking to distal dendrites (horizontal arrows), resulting in accumulation of intracellular HCN1 proximally and loss of the HCN1 gradient.

dor show variable specificity for HCN1, recognizing a band of molecular weight similar to HCN1 in HCN1 knock-out mice (supplemental Fig. 1). As such, proper interpretation of data concerning HCN1 subunit expression levels and localization requires confirmation of antibody specificity, which can now be done using commercially available HCN1 knock-out mice.

We found that chronic exposure to TTX prevented establishment of distal dendritic enrichment of HCN1. Of note, chronic treatment with TTX also caused a reduction in total HCN1 protein levels and resulted in marked cell loss in the EC of treated slices. In contrast, acute or chronic exposure to CNQX or APV and mechanical removal of EC eliminated distal enrichment of HCN1 but had no effect on the developmental increases in HCN1 observed in intact slices. Others have shown that chronic TTX treatment in slice culture reduces the number of synaptic spines and produces spines with immature morphology, whereas treatment with APV reduces spine number but has no effect on developmental morphology (13). Thus, the reduction of HCN1 protein with chronic TTX exposure might be due to impairment of neuronal maturation distinct from its acute effects on synaptic transmission. That no change in protein expression was found with EC removal or with acute exposure to TTX or acute or chronic exposure to CNQX or APV suggests that these manipulations have selective effects on HCN1 localization. Thus, we reason that HCN1 protein expression levels and subcellular localization may be governed by distinct mechanisms.

Blocking glutamatergic activity in neurons results in the inhibition of downstream signals, including elevation of intracellular Ca$^{2+}$ and activation of protein kinases such as CaMKII. Similar to h channels, CaMKII is also enriched in distal apical dendrites of pyramidal neurons within the cortex and hippocampal area CA1 (33, 34), consistent with an important functional relationship between these molecules. CaMKII is a critical molecule for mediating changes in synaptic strength and intrinsic excitability in cellular models of activity-dependent plasticity (16, 27). Along these lines, Fan et al. (16) demonstrated that blockade of CaMKII activity inhibits the activity-dependent up-regulation of I_h in hippocampal pyramidal neurons. We now show that CaMKII inhibition or intracellular calcium chelation prevented the establishment and maintenance of distal dendritic HCN1 enrichment. How CaMKII activity might regulate h channel trafficking is unknown. Interestingly in models of activity-dependent plasticity in CA1 pyramidal neurons, CaMKII activation causes changes in the physical location of numerous dendritic proteins, including synapse-associated protein 97, stargazin, and AMPA receptor subunits (35–37). We infer that CaMKII activation could initiate a “homeostatic program” in dendrites wherein increased amplitude of CaMKII-enhanced synapses might be counterbalanced by localized dendritic up-regulation of h channels. In this setting, the antie excitatory influence of relocalized h channels could mitigate effects of synaptic amplification on temporal integration, thereby preserving overall cellular excitability. Future studies will explore the detailed molecular mechanism of how CaMKII regulates the distribution of h channels.

Removal of the TA pathway and Schaffer collateral inputs to CA1 increases the excitability of CA1 pyramidal neurons in organotypic slice cultures (38). Furthermore chronic exposure of organotypic hippocampal slices to TTX results in abnormal excitability in CA1 pyramidal neurons (39). Our findings of redistribution of HCN1 away from distal dendrites following deafferentation or activity blockade strongly suggest that changes in h channel localization could contribute to changes in cellular excitability observed by others following similar manipulations. Taken together with the observation of activity-dependent establishment of HCN1 distal dendritic enrichment during development, it is evident that control of h channel localization within dendrites may be an important homeostatic mechanism regulating neuronal excitability. To wit, increased activity enhances h channels in distal dendrites where they may reduce excitability by reducing temporal summation of distal synaptic inputs, whereas reduced activity diminishes distal h channels and increases temporal summation. Interestingly aberrant I_h has been suggested as a pathophysiological mechanism underlying the abnormal excitability seen in neurological diseases such as temporal lobe epilepsy (31, 32, 40–42). Whether the normal distribution of h channels in hippocampal dendrites is disrupted or whether homeostatic control of h channel localization is disordered in temporal lobe epilepsy is unknown but will be an area of intense focus for future studies.

Acknowledgments—We thank Thomas Jaramillo, Won Joon Choi, and Daniel Johnston for comments on the manuscript. We are also grateful to Sung Jae Kim and Daniel Johnston for providing data concerning specificity of commercial anti-HCN1 antibodies.
Regulation of h Channel Distribution in Hippocampus

REFERENCES

1. Lai, H. C., and Jan, L. Y. (2006) Nat. Rev. Neurosci. 7, 548–562
2. Ludwig, A., Zong, X., Jeglics, M., Hofmann, F., and Biel, M. (1998) Nature 393, 587–591
3. Santoro, B., Liu, D. T., Yao, H., Bartsch, D., Kandel, E. R., Siegelbaum, S. A., and Tibbs, G. R. (1998) Cell 93, 717–729
4. Lorincz, A., Notomi, T., Tamas, G., Shigemoto, R., and Nusser, Z. (2002) Nat. Neurosci. 5, 1185–1193
5. Notomi, T., and Shigemoto, R. (2004) J. Comp. Neurosci. 471, 241–276
6. Magee, J. C. (1998) J. Neurosci. 18, 7613–7624
7. Magee, J. C. (1999) Nat. Neurosci. 2, 508–514
8. Poolos, N. P., Migliore, M., and Johnston, D. (2002) Nat. Neurosci. 5, 767–774
9. Bender, R. A., Brewster, A., Santoro, B., Ludwig, A., Hofmann, F., Biel, M., and Baram, T. Z. (2001) Neuroscience 106, 689–698
10. Vasilyev, D. V., and Barish, M. E. (2002) J. Neurosci. 22, 8992–9004
11. Brewster, A. L., Chen, Y., Yeh, A., Shigemoto, R., and Baram, T. Z. (2007) Cereb. Cortex 17, 702–712
12. Bender, R. A., Galindo, R., Mameli, M., Gonzalez-Vega, R., Valenzuela, C. F., and Baram, T. Z. (2005) Eur. J. Neurosci. 22, 2669–2674
13. Collin, C., Miyaguchi, K., and Segal, M. (1997) J. Neurophysiol. 77, 1614–1623
14. Baizley, D. E., and Smith, S. J. (1996) J. Neurosci. 16, 2983–2994
15. van Welie, I., van Hooft, J. A., and Wadman, W. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 5123–5128
16. Fan, Y., Fricker, D., Brager, D. H., Chen, X., Lu, H. C., Chitwood, R. A., and Johnston, D. (2005) Nat. Neurosci. 8, 1542–1551
17. Amaral, D. G., and Witter, M. P. (1989) Neuroscience 31, 571–591
18. Witter, M. P. (1993) Hippocampus 3, 33–44
19. Chetkovich, D. M., Bunn, R. C., Kuo, S. H., Kawasaki, Y., Kohwi, M., and Bredt, D. S. (2002) J. Neurosci. 22, 6415–6425
20. Chetkovich, D. M., Chen, L., Stocker, T. J., Nicoll, R. A., and Bredt, D. S. (2002) J. Neurosci. 22, 5791–5796
21. Santoro, B., Grant, S. G., Bartsch, D., and Kandel, E. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14815–14820
22. Surges, R., Brewster, A. L., Bender, R. A., Beck, H., Feuerstein, T. J., and Baram, T. Z. (2006) Eur. J. Neurosci. 24, 94–104
23. Noraberg, J., Kristensen, B. W., and Zimmer, J. (1999) Brain Res. Brain Res. Protoc. 3, 278–290
24. Brauer, A. U., Savaskan, N. E., Plaschke, M., Ninnemann, O., and Nitsch, R. (2001) Neuroscience 102, 515–526
25. Brauer, A. U., Savaskan, N. E., Kole, M. H., Plaschke, M., Monteggia, L. M., Nestler, E. J., Simburger, E., Deisz, R. A., Ninnemann, O., and Nitsch, R. (2001) FASEB J. 15, 2689–2701
26. Remondes, M., and Schuman, E. M. (2002) Nature 416, 736–740
27. Lisman, J., Schulman, H., and Cline, H. (2002) Nat. Rev. Neurosci. 3, 175–190
28. Poolos, N. P., Bullis, J. B., and Roth, M. K. (2006) J. Neurosci. 26, 7995–8003
29. Kaupp, U. B., and Seifert, R. (2001) Annu. Rev. Physiol. 63, 235–257
30. Santoro, B., Chen, S., Luthi, A., Pavlidis, P., Shumyatsky, G. P., Tibbs, G. R., and Siegelbaum, S. A. (2000) J. Neurosci. 20, 5264–5275
31. Brewster, A., Bender, R. A., Chen, Y., Dubé, C., Eghbal-Ahmadi, M., and Baram, T. Z. (2002) J. Neurosci. 22, 4591–4599
32. Brewster, A. L., Bernard, T. A., Gall, C. M., and Baram, T. Z. (2005) Neurorobol. Dis. 19, 200–207
33. Ouimet, C. C., McGuinness, T. L., and Greengard, P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5604–5608
34. Erdou, N. E., and Kennedy, M. B. (1985) J. Neurosci. 5, 3270–3277
35. Mauceri, D., Cattabeni, F., Di Luca, M., and Gardoni, F. (2004) J. Biol. Chem. 279, 23813–23821
36. Tomita, S., Stein, V., Stocker, T. J., Nicoll, R. A., and Bredt, D. S. (2005) Neuron 45, 269–277
37. Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C., and Malinow, R. (2000) Science 287, 2262–2267
38. Cai, X., Wei, D. S., Gallagher, S. E., Bagal, A., Mei, Y. A., Kao, J. P., Thompson, S. M., and Tang, C. M. (2007) J. Neurosci. 27, 59–68
39. Niesen, C. E., and Ge, S. (1999) Dev. Neurosci. 21, 328–338
40. Shah, M. M., Anderson, A. E., Leung, V., Lin, X., and Johnston, D. (2004) Neuron 44, 495–508
41. Bender, R. A., Soleymani, S. V., Brewster, A. L., Nguyen, S. T., Beck, H., Mathern, G. W., and Baram, T. Z. (2004) Science 287, 6826–6836
42. Chen, K., Aradi, I., Thon, N., Eghbal-Ahmadi, M., Baram, T. Z., and Soltész, I. (2001) Nat. Med. 7, 331–337