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STEP Regulation of Seizure Thresholds in the Hippocampus

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FULL-LENGTH ORIGINAL RESEARCH

STEP regulation of seizure thresholds in the hippocampus

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Summary

Purpose: To investigate whether striatal enriched protein tyrosine phosphatase (STEP) influences ictogenesis.

Methods: STEP knockout mice were compared to wild-type (WT) mice in pilocarpine-induced seizures. Hippocampal slices were also prepared from these two mouse populations, allowing the examination of ictal-like stimulation in these slices using calcium imaging and electrophysiologic recordings.

Key Findings: To examine seizure thresholds, increasing doses of pilocarpine were administered to adult mice and seizures were scored behaviorally. Significantly fewer STEP knockout mice developed seizures that progressed to the stage of status epilepticus compared to WT mice. To examine potential differences in neural circuits that might account for this finding, seizure-like activity was induced in hippocampal slices. Electrical stimulation of the hippocampal–entorhinal cortex pathway in STEP knockout mice resulted in less activation of the dentate gyrus granule cell layer (GCL), but greater activation of the hilus in STEP knockouts, compared with heterozygous slices.

Significance: STEP deficiency is associated with higher seizure thresholds. The locus of these effects appears to include the dentate gyrus granule cell layer and hilus.

KEY WORDS: Hilus, Inhibitory interneurons, Glutamatergic mechanisms, Pilocarpine, Perforant path, Status epilepticus.

In humans and rodents, a single, severe period of status epilepticus (SE) can result in the emergence of spontaneous epileptic seizures and temporal lobe epilepsy (TLE). The appearance of spontaneous seizures often occurs after a latent period that may range from weeks to years. Epileptogenesis potentially involves the loss of inhibitory synapses onto granule cells, as patients and rodents with TLE often exhibit hippocampal sclerosis and pronounced loss of interneurons in the dentate gyrus (Mathern et al., 1996; Kobayashi & Buckmaster, 2003). In addition, synaptic reorganization in the dentate gyrus appears to contribute to altered network properties causing spontaneous seizures (Thind et al., 2010).

A previous study led to the hypothesis that striatal enriched protein tyrosine phosphatase (STEP) significantly modulates survival of hippocampal interneurons in CA1–3 and the hilus in response to SE (Choi et al., 2007). STEP is enriched in neurons in the striatum, hippocampus, cortex, amygdala, nucleus accumbens and other regions of the central nervous system (CNS) (Lombroso et al., 1993; Boulanger et al., 1995; Pelkey et al., 2002). The current model of STEP function is that it opposes the development of synaptic strengthening by dephosphorylating regulatory tyrosine residues in its substrates, which include proteins involved in synaptic plasticity and intracellular signaling. STEP dephosphorylates and inactivates the key signaling molecules ERK1/2, p38, and Fyn, and has also been shown to dephosphorylate and induce internalization of the ionotropic glutamate receptors GluN1/GluN2B and GluA1/GluA2 (Snyder et al., 2005; Zhang et al., 2008; Kurup et al., 2010a).

In mice and rats, intraperitoneal administration of appropriate doses of the muscarinic agonist pilocarpine can induce SE (Turski et al., 1983a). Depending on the duration and severity of SE, the sequelae can include neurodegeneration and astrogliosis in the hippocampus and temporal lobes. Days to weeks after prolonged SE, an epileptic disease state emerges as spontaneous seizures develop (Turski et al., 1983b; Loscher, 2002). Because these patterns of neuronal damage are similar to those found in patients with TLE, pilocarpine-induced SE is considered a model of severe temporal lobe epilepsy.

STEP-expressing neurons in the hilus and area CA1 undergo excitotoxic cell death following pilocarpine-induced SE. The vulnerability of these neurons to seizures

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was mitigated by disrupting STEP functions in these cells, providing evidence for a possible role for STEP in excitotoxic neuronal cell death (Choi et al., 2007). STEP is expressed in a class of somatostatin-positive, γ-aminobutyric acid (GABA)ergic interneurons in the hilus (Choi et al., 2007). These interneurons are also known as Hilar Interneurons whose axon ramifies in the Perforant Path field (HIPP) (Han et al., 1993), and their main source of excitatory input is the mossy fibers originating from the granule cell layer (GCL) of the dentate gyrus (Acsady et al., 1998). HIPPs produce the neuropeptide somatostatin, which is released from synaptic terminals during bursts of activity and may provide antiepileptic properties (Tallent & Siggins, 1999; Baratta et al., 2002). HIPPs are also characterized by their axonal innervation patterns that target the same field as the perforant path axons, that is, the dendrites of dentate granule cells. Therefore, HIPPs provide substantial feedback inhibition to the granule cells (Buckmaster et al., 2002).

Hilar interneurons, including HIPPs, are especially vulnerable to SE (Buckmaster & Jongen-Relo, 1999; Santhakumar et al., 2001), as well as other forms of epileptogenic trauma such as concussion (Lowenstein et al., 1992) and ischemia (Johansen et al., 1987; Freund et al., 1990; Bering & Johansen, 1993). These and other studies suggest that loss of the HIPP subtype of GABAergic interneuron is a causal factor in epileptogenesis, as degeneration of these interneurons removes an important brake on seizure generation, contributing to the development of spontaneous, recurrent seizures. There is much evidence to support this idea, including studies showing that the relative numbers of HIPPs are greatly reduced in humans with TLE (Sloviter, 1987; de Lanerolle et al., 1989; Robbins et al., 1991; Mathern et al., 1995).

Given the prevalence of STEP in hilar interneurons, we tested the hypothesis that a deficiency of the STEP protein would increase resistance to the development of SE in the pilocarpine model. Our results show that a significant number of STEP-deficient mice exhibit a seizure-resistant phenotype. One potential mechanism for the resistance of STEP knockout (STEP KO) mice to pilocarpine-induced SE is reduced excitability of GCL neurons in STEP KO mice. Our analysis of GCL granule cell responses in wild-type (WT) versus STEP KO mouse hippocampal slices suggests larger hyperpolarizing responses in STEP KO versus WT granule cells. These novel findings suggest that STEP deficiency in mice is linked to enhanced feedback inhibition onto the GCL neurons. Taken together, these findings suggest that STEP inhibitors may be novel compounds for seizure resistance therapies.

Methods

Mice

STEP WT and STEP KO mice were maintained on the C57BL/6 inbred strain from Charles River Labs and were obtained from heterozygous breedings in the Wesleyan University Animal Facility, a pathogen-free environment. The KO was originally generated using homologous recombination to remove a portion of the STEP gene, which included the catalytic site, and the knockout mice were generated on the 129 background at Pfizer, Inc. (New York, NY, U.S.A.). The strain was subsequently crossed into the C57BL/6 strain (Charles River Labs, Willimantic, CT, U.S.A.) and backcrossed for seven generations. The WT, KO, and heterozygous (HT) genotypes are fertile and visibly indistinguishable. Furthermore, there were no gross anatomic differences between genotypes in cresyl violet–stained brain sections. Immunoblot analyses showed that no STEP protein is expressed in the KO, whereas the HT expresses 50% of the STEP protein levels for any of our measures. The genotypes of the mice used for all experiments were confirmed by polymerase chain reaction (PCR) using primers for STEP as described previously (Venkitaramani et al., 2009). All experiments involving the use of animals were in compliance with the guidelines provided by the Wesleyan University Institutional Animal Care and Use Committee and the National Institutes of Health (NIH).

Pilocarpine-induced SE

Adult male and female mice, weighing between 18 and 22 g and 6–8 weeks of age, were used. Seizures were pharmacologically induced by systemic injections of pilocarpine (280–320 mg/kg, i.p.; Sigma, St. Louis, MO, U.S.A.) diluted in sterile 0.9% saline. To block the effects of pilocarpine in the peripheral nervous system, atropine methyl nitrite (1 mg/kg, i.p., Sigma) was injected 30 min prior to pilocarpine. Mice displayed seizure activity that was behaviorally scored by a pair of observers using the modified Racine scale (Shibley & Smith, 2002). The measurements included the latency to and number of seizure events before reaching SE. Typically, four to six seizure events were observed before the mice exhibited SE, defined as continuous motor seizures involving head bobbing and full body tremors. Pilocarpine was administered between 10 a.m. and 12 p.m. to minimize differences in hormonal milieu due to circadian rhythms.

Preparation of hippocampal formation slices

Young adult male and female mice (ages postnatal 18–22 days) were injected with ketamine/xylazine (120 mg/kg ketamine + 10 mg/kg xylazine, i.p.). Once unconscious and unresponsive to noxious stimuli, the mice were decapitated and brains were quickly removed and placed in high-sucrose ice-cold artificial cerebral spinal fluid (ACSF). The composition of high-sucrose ACSF is (in mM): 222 sucrose, 27.1 sodium bicarbonate, 1.5 sodium phosphate, 2.6 potassium chloride, 1 calcium chloride, and 3 magnesium sulfate. Hippocampal slices were modeled from a previous study (Rafiq et al., 1993); the brain was removed and blocked on a ramp, so that the dorsal side of

brain was cut at a 12-degree angle (cutting at increasing depth from the rostral to caudal direction). This cut dorsal side of the brain was glued to the vibratome stage. Horizontal slices were cut from this blocked brain with a vibratome (Leica VT1000S, Leica Microsystems Inc., Bannockburn, IL, U.S.A.) at 350 μm thicknesses, while bathed in ice-cold high-sucrose ACSF. Slices were then transferred to warm (37°C), oxygenated ACSF containing high magnesium and low calcium to reduce spontaneous activities and, thus, neurotoxicity [composition of this ACSF (in mM): 126 sodium chloride, 26 sodium bicarbonate, 1.1 sodium phosphate, 10 dextrose, 3 potassium chloride, 3 magnesium sulfate, and 1 calcium chloride]. The slices were then allowed to equilibrate to room temperature. During recordings, the slices were transferred to a recording chamber and perfused with oxygenated recording ACSF (in mM): 1 magnesium sulfate, 1.5 calcium chloride, and 4.5 potassium chloride. This recording ACSF increases excitability of the circuitry by reducing divalent cations and increasing extracellular potassium (Sanchez-Vives & McCormick, 2000).

Imaging

A 100-μl solution of calcium dye was prepared prior to slicing: 50 μg of fura-2AM, 2 μl pluronic acid, 48 μl dimethyl sulfoxide (DMSO), and 50 μl ACSF. This solution was divided equally among four to five hippocampal slices and directly applied with a pipette to the surface of each slice. The slices were then left undisturbed to incubate in darkness for 1 h. Fluorescence was observed in loaded neurons using 380-nm wavelength excitation light (ultraviolet), while collecting the emitted 510-nm light. Movies were made with a slit-disk spinning confocal unit (Olympus Disk-Scan and BX51WI upright microscope; Olympus, Nashua, NH, U.S.A.), a Hamamatsu C39100-12 CCD camera, and an acquisition program, SIMPLEPCI (Cimaging, Sewickley, PA, U.S.A.). Frames were captured using a 20× objective at a rate of 5 Hz and spatial resolution of 256 × 256 pixels using SIMPLEPCI software (Compix). Calcium imaging experiments such as these are reliable measures of neuronal action potential activity (Smetters et al., 1999; MacLean et al., 2005; Aaron & Yuste, 2006).

Electrophysiology

Extracellular stimulation of the Schaffer collaterals was performed with a concentric bipolar electrode (Frederick Haer, Bowdoin, ME, U.S.A.) placed directly on the Schaffer collaterals. For each slice, the 60-Hz tetanic stimulation was delivered three times, with 10 min of rest in between each stimulation. For each slice, the tetanic stimulation was repeated twice with a 10-min interstimulus interval, for a total of three tetanic stimuli per slice. Some experiments also included extracellular recordings performed with a 3 MΩ pipette filled with ACSF to verify the large responses seen in the GCL.

Whole-cell patch-clamp recordings

Individual GCL granule cells were identified under infrared differential interference contrast (IR-DIC) optics. Whole-cell voltage clamp recordings were performed using six to nine MΩ pipettes containing a cesium gluconate solution (Cs-gluc) that blocked potassium channels, allowing us to voltage clamp the cell at +10 mV without substantial leak current. This technique reduces excitatory postsynaptic current (EPSC), as this potential is close to the reversal potential of EPSCs, and it increases the driving force for inhibitory postsynaptic current (IPSC), delivering high signal-to-noise recordings of IPSCs. The Cs-gluc solution, contained (in mM): 135 gluconic acid, 135 CsOH, 1 EGTA, 8 MgCl, 0.1 $CaCl_2$, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, 11 biocytin.

Cell-attached recordings

Individual GCL granule cells were identified under IR-DIC optics, and they were recorded with pipette electrodes (6–9 M MΩ) containing 140 mM NaCl. Giga-ohm seals were formed between the electrodes and granule cells. In this configuration, rough measurements of voltage changes in the cell can be achieved in current-clamp mode through the resistance of the patch of membrane between the electrode and intracellular space (Perkins, 2006).

Analyses

Measurement of responses in calcium imaging recordings: For each neuron we defined the fluorescence change over time as $\Delta F/F = (F_1 - F_0)/F_0$, expressed in %, where F_1 is fluorescence at any time point, and F_0 is the baseline fluorescence measured during the first 3 s at the beginning of each trial. Neurons were identified and their respective borders outlined using custom-built macros in Matlab (MathWorks, Natick, MA, U.S.A.). For each identified neuron, the ΔF/F was further adjusted according to the difference between the ΔF/F measured within the neuron's borders minus the ΔF/F measured in the immediate background of the neuron (a halo region around the neuron equal to the area of the neuron). This was done to distinguish the neuronal response from background response.

Mean peak fluorescence

For each neuron, the 2-s interval beginning with the initiation of the 1-s tetanic stimulus is labeled as the stimulus interval. The largest ΔF/F frame during the 2-s stimulus interval was used to center a 1-s interval, the mean of which was taken as the peak fluorescence (as in Fig. 3Bi). Given our sampling rate, this peak fluorescence is the mean of five frames. The mean peak fluorescence from a stimulus in a slice was then taken as the mean fluorescence of each peak measured in each neuron from the group (i.e., GCL or hilus).

Proportion of responses

A neuron qualified as responding to the tetanic stimulation if the mean -ΔF/F during the 2-s stimulus interval was >1 standard deviation of mean of the ΔF/F measured during the baseline. The proportion, defined as the number of responding neurons/all neurons in GCL or hilus, was calculated to allow comparisons of proportions (as in Fig. 3Bii).

Electrophysiologic measurements

For each voltage clamp and cell-attached recording, the mean of the 3-s of baseline before the stimulus was subtracted from the mean of the 1-s stimulus, yielding the mean baseline subtracted 1-s response for each recording.

Both WT mice and STEP KO heterozygote mice were used as the control mouse population in this experiment as no significant differences were found between WT and the heterozygote mice for any of our measures. This result is supported by a previous study (Venkitaramani et al., 2009). All means are listed as the mean ± standard error (SE).

Results

We first conducted dose–response experiments to test for differences in thresholds for pilocarpine-induced SE in WT and STEP KO mice. With doses of pilocarpine at 300 or 320 mg/kg, we found that 41 of 55 WT mice entered SE (75%), compared to 10 of 29 STEP KO mice (35%) ($p < 0.001$, chi square test, Fig. 1). These results demonstrated that the STEP KO mice were resistant to the ictogenic effects of pilocarpine.

We next examined whether there were electrophysiologic differences between WT and STEP KO mice that could explain the differences in the required doses of pilocarpine needed to induce SE. We employed the mouse entorhinal cortex–hippocampal slice preparation, where repetitive ictal-like extracellular stimulations of the Schaffer collaterals cause potentiation of neuronal responses in the GCL (Rafiq et al., 1993). The Schaffer collaterals were stimulated with extracellular tetanic stimulation, producing an ictal-like burst of activity that propagated throughout the slice. We measured this activity in the hilus and dentate gyrus simultaneously using calcium imaging (Fig. 2). We obtained these recordings in both STEP KO and WT slices, and included those slices for which a measurable response to the stimulus was observed in the GCL or hilus.

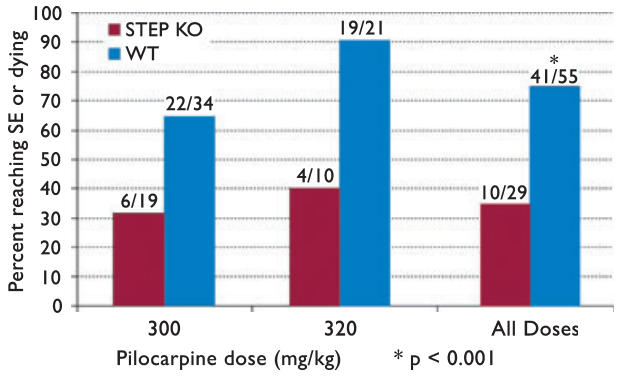

Figure 1.
Percentage of mice reaching SE versus pilocarpine dosage. The number of mice corresponding to the percent values in the y-axis is listed above each bar. The p-value was calculated using a chi square test for the equality of distributions.
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We used calcium imaging to confirm a significant increase among WT slices in the mean peak fluorescence of the GCL measured during stimulus 3 versus stimulus 1 ($p < 0.03$, sign test, Fig. 3Ai,Bi) (Rafiq et al., 1993). In contrast, we observed no such potentiation between stimulus 1 and 3 in the hilus ($p = 0.81$, sign test, Fig. 3Bi) from either the WT or STEP KO slices. Furthermore, the mean peak fluorescence measured in the GCL from STEP KO slices was significantly less than that from WT slices, when comparing responses from stimulus 3 ($p < 0.05$, Mann-Whitney *U*-test, Fig. 3Aii,Bii). These results show that there is less potentiation in the STEP KO slices, such that after three tetanic stimuli, the peak response in the GCL was significantly lower in STEP KO compared to WT hippocampal slices.

Interestingly, the results were markedly different in the hilus of STEP KO slices, where the proportion of neurons responding to stimulus 1 was significantly greater in the STEP KO compared to WT slices ($n = 16$ WT slices and $n = 18$ STEP KO slices, $p < 0.04$, *t*-test, Fig. 3Bii). As shown, there were no apparent differences in the GCL (Fig. 3Bii) with regard to this metric.

These differences were found despite the fact that the numbers of neurons identified with the calcium fluorophore fura-2 were equivalent for the STEP KO versus WT slices. The numbers of neurons imaged in the GCL of WT and STEP KO slices were 137 ± 6.7 versus 148 ± 7.3 imaged neurons, respectively ($p = 0.49$, Mann-Whitney *U*-test). The numbers of neurons imaged in the hilus for WT and STEP KO slices were 118 ± 7.7 versus 118 ± 6.3 neurons, respectively ($p = 0.95$, Mann-Whitney *U*-test; $n = 16$ WT slices and $n = 18$ STEP KO slices). Therefore, the results presented herein demonstrate significantly different levels of responsiveness in both the GCL and hilus of WT versus STEP KO slices, despite no differences in the mean number of neurons that were imaged in these slices.

The data presented are consistent with the hypothesis that HIPP inhibitory input to GCL dendrites is stronger in STEP KO mice. We investigated this hypothesis further by recording IPSCs in single granule cells during the calcium imaging experiments. The IPSCs recorded were most likely $GABA_A$ ionotropic currents, as the electrodes contained a cesium gluconate solution that blocked potassium conductances, including $GABA_B$-activated potassium conductances, and

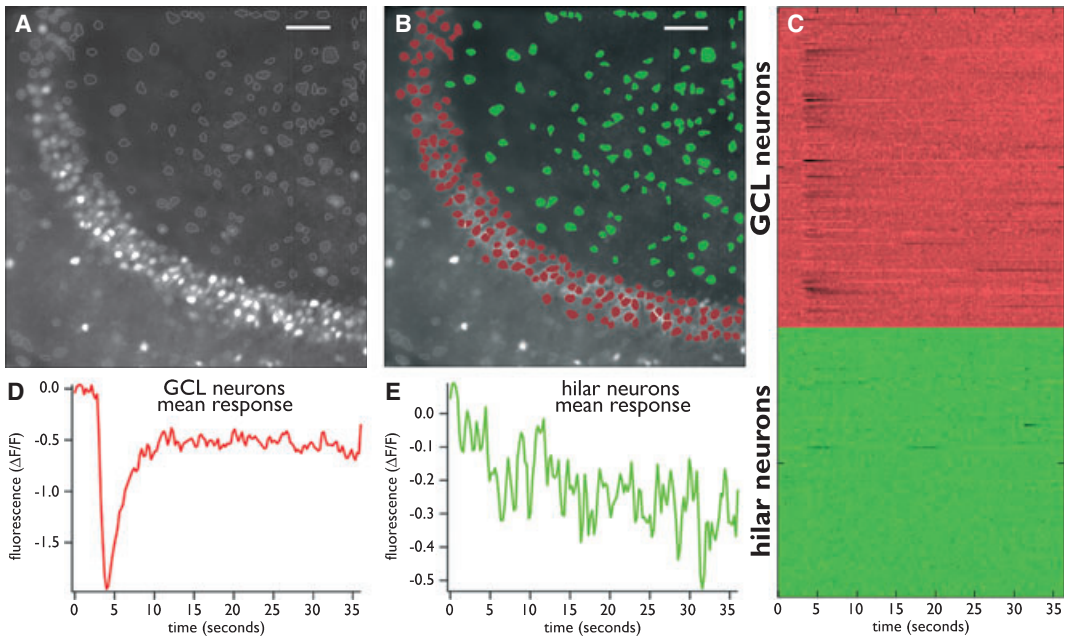

Figure 2.
Calcium imaging in the dentate gyrus: an example. (**A**) Average movie frame from 36-s movie of the GCL and hilus from a single WT slice. Cell bodies labeled with fura-2 were identified by our software. (**B**) The borders of the GCL and hilus were identified manually and the cell bodies are colored red and green, respectively. (**C**) Raster plot of all neuronal calcium activity in these two regions. Each line represents one neuron, and each color represents the corresponding region of the slice. Darker transients indicate increased calcium influx and, thus, high action potential activity. (**D**) Average response of GCL neurons. The response of all identified GCL neurons (labeled red in **B**) is shown as the average response. The response was produced by 60 shocks at 60 Hz, each shock being 0.1 ms and −4.0 mA in amplitude. The stimuli were applied to the Schaffer collaterals after 3-s of baseline. The peak shown in **D** is a measure of the mean response of the observed GCL neurons. (**E**) Same format as **D**, except that the mean response of all hilar neurons is shown. Scale bar in **A** and **B**: 50 μm.
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the voltage clamp was maintained at +10 mV, near the reversal potential for glutamatergic currents. Recordings of IPSCs were made from 23 cells in 11 WT mice, and 26 cells in 10 STEP KO mice. We measured the latencies of IPSCs in those cases in which we could clearly identify the rising phase of the first IPSC in the train. Latencies in these cases were measured from the stimulus artifact from the first shock to the beginning of the IPSC, and there were no difference between STEP KO and WT in these latency measurements (13.7 ± 1.2 ms, n = 12; 13.7 ± 0.5 ms, n = 16; STEP KO and WT, respectively).

In comparing amplitudes of IPSCs between stimulus 3 and stimulus 1, we measured a significant increase from WT mice (Fig. 4), similar to the increase in putative granule cell action potential activity measured during calcium imaging (Fig. 3). As shown in Fig. 4, there was no significant difference in IPSC amplitudes when comparing WT versus STEP KO mice, contrary to the proposed hypothesis of increased GABAergic activity in STEP KO mice.

We then used cell-attached recordings to measure granule cell responses. We were able to record strong responses to the stimulus (Fig. 5), and we were also able to measure potentiation of responses during the three stimulus trials (Fig. 5B). A total of seven cells from four STEP KO mice and 12 cells from four WT mice were successfully recorded with this technique. We observed potentiation (as seen in Fig. 5B) from three WT cells and one STEP KO cell.

Interestingly, the majority of granule cell responses were hyperpolarizing (Fig. 6). Some of these hyperpolarizing responses were large and long-lasting, often outlasting the duration of the stimulus itself. In comparing all hyperpolarizing responses from WT and STEP KO recordings, we found a significantly larger mean hyperpolarizing response in STEP KO versus WT recordings (−8.5 ± 1.7 vs. −3.8 ± 0.53 mV, n = 15 vs. 25 recordings, respectively; $p < 0.04$, Mann-Whitney *U*-test). These results suggest a stronger stimulus-evoked inhibitory response in STEP KO granule cells, in contrast to observations made with voltage clamp experiments.

Discussion

The results shown in this series of experiments lead to a number of conclusions: (1) STEP KO mice are less sensitive

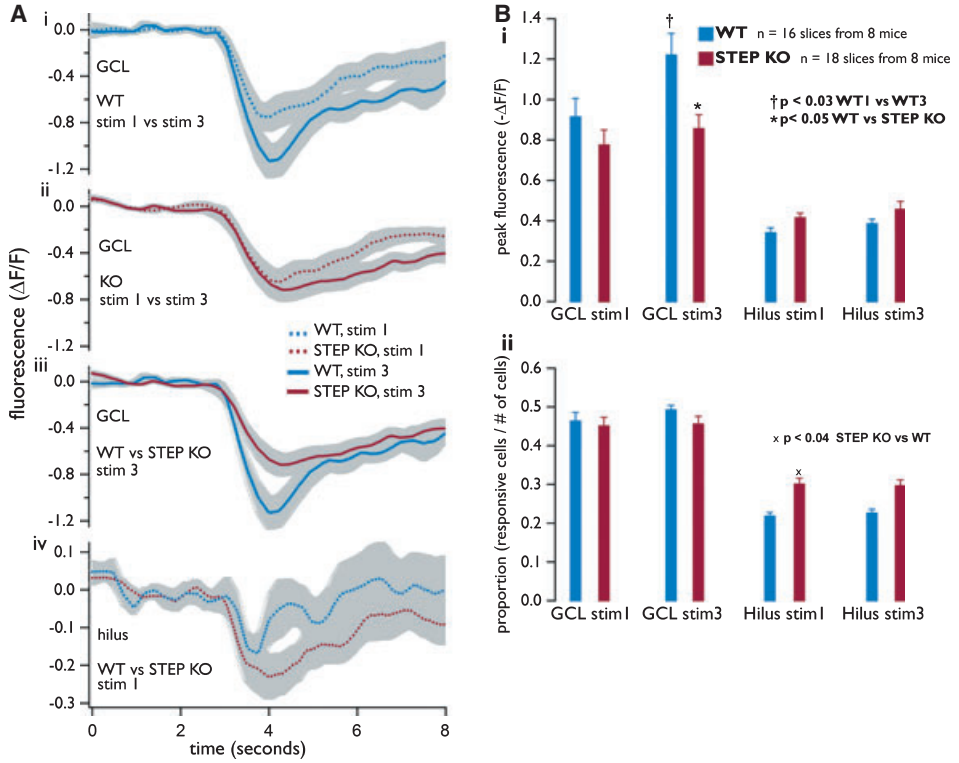

Figure 3.

GCL and hilus calcium transients evoked during ictal-like stimulation of the hippocampal slice are significantly different. As in Fig. 2, the Schaffer collaterals were stimulated with tetanic stimulation. For all panels, plots are mean ± SE; n = 16 slices from eight WT mice and n = 18 slices from eight STEP KO mice. All WT results are shown in red; STEP KO results are shown in blue. (**A**) Mean 8-s–long calcium transients from all slices. Each colored trace represents the mean transient from each indicated group (i.e., GCL, WT, stim1 as one group), and each group is composed of the mean ΔF/F transients recorded from each slice (as in Fig. 2D,E). Gray shadings show the standard error of the mean traces. (**B**) Peak 1-s calcium transients. (i) The mean 1-s peak difference of GCL transients between stimulus 1 and stimulus 3 was significant in WT slices ($p < 0.03$), and the same measured difference between WT and STEP KO slices was significant at stimulus 3 ($p < 0.05$). (ii) Mean responsiveness is displayed, measured as the proportion of neurons responding to a stimulus. A statistically significant difference between WT and STEP KO responsiveness in the hilus is shown ($p < 0.04$).

to the ictogenic effects of pilocarpine as they require higher doses to reach SE; (2) the GCLs of STEP KO mice have a reduced excitability, especially as measured during repetitive ictal-like stimulations; and (3) neurons in the hilus of STEP KO mice demonstrate enhanced excitability during repetitive ictal-like stimulation, compared to WT mice. These neurons may be the source of enhanced inhibition to the dendrites of GCL granule cells in STEP KO mice.

STEP

STEP has been shown previously to regulate synaptic strengthening, glutamate receptor trafficking, and cell death (Braithwaite et al., 2006a,b). The novel finding reported herein is that STEP deficiency is correlated with a seizure-resistant phenotype, both in the pilocarpine model of TLE and in hippocampal slices subjected to tetanic stimulation. Substrates of STEP include the extracellular-signal regulated kinase 1/2 (ERK1/2), the stress-activated protein kinase p38, and the Src kinase family member Fyn (Nguyen et al., 2002; Munoz et al., 2003; Paul et al., 2003).

STEP dephosphorylates these kinases at regulatory tyrosine residues within their activation loop and inactivates them. STEP dephosphorylates the *N*-methyl-D-aspartate receptor (NMDAR) subunit GluN2B at tyr^{1472}, which promotes internalization of the GluN1/GluN2B receptor complex (Snyder et al., 2005; Kurup et al., 2010b). STEP also mediates internalization of GluA1/GluA2-containing α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR) following activation of group I metabotropic glutamate receptors, although whether this is due to a direct dephosphorylation of GluA2 remains to be determined (Zhang et al., 2008). STEP is expressed in select groups of hippocampal neurons, including the HIPPs in the hilus (Choi et al., 2007), a group of neurons that provide

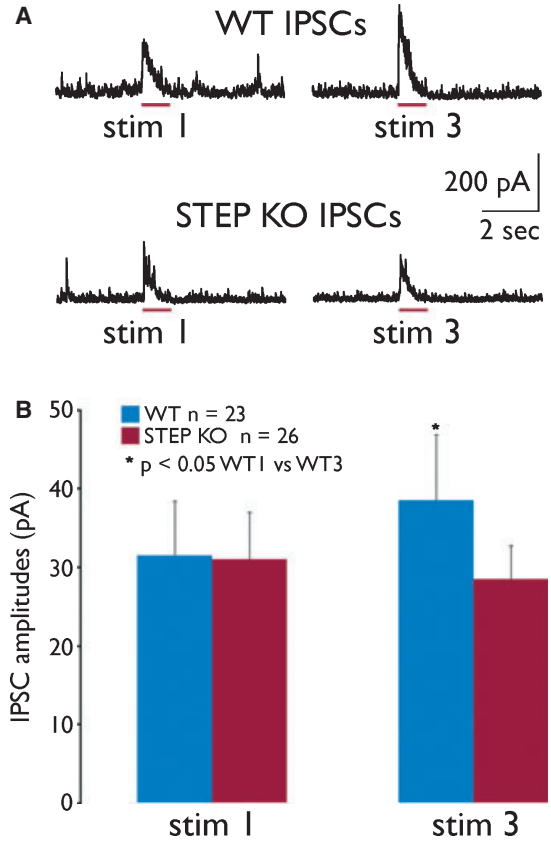

Figure 4.
IPSCs recorded from GCL granule cells during Shaffer collateral stimulation. (**A**) Representative examples of IPSCs evoked from WT (top) and STEP KO slices. Granule cells were voltage-clamped at +10 mV with a cesium-gluconate intracellular solution to block potassium conductances. All outward (positive) currents are most likely due to $GABA_A$ ionotropic currents in these recordings. The timing of the 1-s stimulus is indicated by the red bar for each trace. (**B**) Mean IPSC amplitudes were calculated from 23 WT and 26 STEP KO recordings. Mean IPSC amplitudes increased significantly between stimulus 1 and 3 for the WT recordings ($p < 0.05$, paired *t*-test).

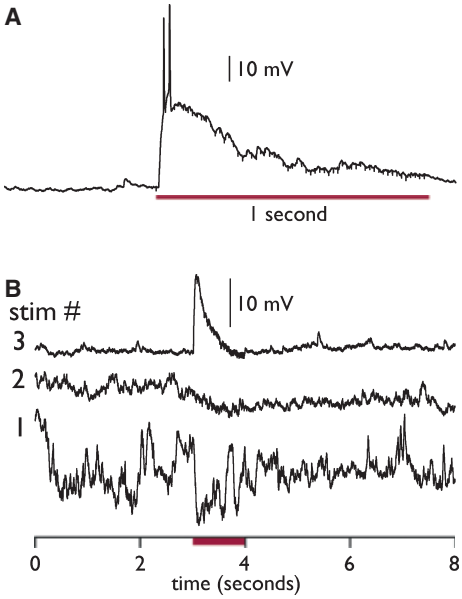

Figure 5.
Cell-attached recordings of GCL granule cells during Shaffer collateral stimulation. Tight-seal cell-attached recordings allow some measure of the changes in membrane potential that occur in a single granule cell. (**A**) A large depolarization with action potentials in a single STEP KO granule cell is evoked by the same stimulus applied in the calcium imaging experiments (60 Hz train, 1-s duration). (**B**) This WT granule cell recording shows a hyperpolarizing response in stimulus 1 that becomes a strong depolarizing response in stimulus 3. The timing of the stimulus is indicated by the red bar.

feedback inhibition to the GCL. Therefore, removal of STEP from the HIPPs was predicted to increase glutamatergic responsiveness in the HIPPs and increase their inhibitory output. Our data are consistent with this prediction by showing that STEP deficits led to reduced potentiation of excitatory responses in the GCL.

Calcium imaging of responsiveness in GCL and hilus

These results were supported by the imaging data. When we measured peak fluorescence in the GCL from WT neurons, we found significantly larger values compared to those from the GCL of STEP KO neurons. Peak fluorescence, defined as the mean of the largest 1-s deflection in the stimulus interval, seemed to represent the responsiveness that could be seen in the raw data (compare Fig. 3A to Fig. 3Bi). However, for cases for which the numbers of neurons responding to the stimulus were very low compared to the total number of neurons, these measurements were marred by overwhelming noise from nonresponders. This was almost always the case when measuring responses from the hilus, especially from WT slices, where very low numbers could be seen as responding in the hilus (Fig. 3C). We, therefore, added a measure of the proportion of responders, and we used criteria that would produce no false negatives, as there seemed to be little danger of a ceiling effect whereby all neurons might be classified as responding. Indeed, the highest measured proportion for any experiment was 0.79, found in one STEP KO slice in the GCL.

In taking the proportion data and mean peak fluorescence data together, we believe that these results are convincing given the following observation: compared to STEP KOs, the responses were generally greater in GCL for WT slices, whereas they were less in the hilar regions of WT slices—all from the same stimuli in the same slices. Therefore, these results could not be explained by simple differences in the

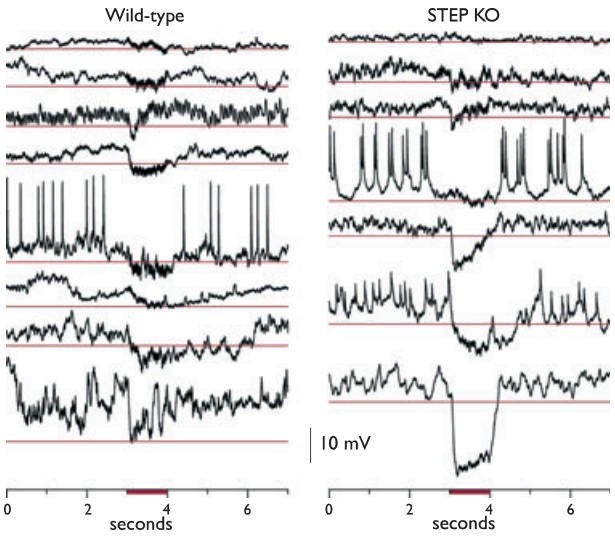

Figure 6.
Cell-attached recordings of GCL granule cells demonstrate large hyperpolarizing responses in STEP KO slices. Only stimulus 1 responses that displayed a putative hyperpolarizing response to the stimulus are shown here. Examples from WT slices are shown on the left, and STEP KO slices are shown on the right. The thin red lines for each recording represent the 99% confidence interval calculated from the 3-s of baseline potentials before each respective stimulus (not including presumed action potentials). The timing of the stimulus is indicated by the red bar.

strength of stimulation or overall responsiveness, as these differential responses were seen in the hilus and GCL within the same slices.

Electrophysiologic measurements from single GCL granule cells

Voltage clamp results

To measure inhibition of granule cells directly, we repeated the same stimulation protocol and measured responsiveness in individual GCL granule cells using voltage clamp recordings. The latencies of these IPSCs were identical for WT and STEP KO slices, and the durations of these latencies (>12 ms) are consistent with a multisynaptic circuit connecting the stimulus site in CA1 to the evoked IPSCs.

To isolate GABAergic responses, the cells were voltage clamped at +10 mV, and the electrodes contained a cesium gluconate solution in order to block potassium conductances, which would otherwise produce large leak currents. In contrast to what we expected, the size of the evoked IPSCs were similar in the STEP KO and WT mice, whereas the WT mice demonstrated a significant increase in stimulus 3 versus stimulus 1 (Fig. 4). This result supports the calcium imaging experiments in that there is an increase in responsiveness in WT slices during the stimulation protocol; the greater action potential firings of GCL granule cells drive local GABAergic interneurons more strongly, providing the potentiated IPSCs that are measured here. However, this finding does not support the idea that the lack of potentiation seen in STEP KO slices is due to increased GABAergic inhibition relative to WT slices.

Cell-attached results

We performed cell-attached recordings to verify that the granule cells in STEP KO and WT slices were responding to the electrical stimuli with excitatory responses, consistent with what we measured in the calcium imaging experiments. Several recordings with large depolarizations and action potentials evoked by the stimulus provided this verification (Fig. 5). In a majority of responses, however, we measured hyperpolarizations during the stimulus (Fig. 6). In comparing all hyperpolarizing stimulations between STEP KO and WT cells, we found the STEP KO responses to be significantly more hyperpolarized than the WT responses ($p < 0.04$).

The cell-attached results contrast with the voltage clamp recordings, where no significant differences between the WT versus STEP KO granule neurons were found. One explanation for the differences in the two approaches to measure inhibition is that the voltage clamp recordings precluded measurements of $GABA_B$-mediated potassium currents. $GABA_B$ receptors are expressed strongly in the dendrites of GCL granule cells (Sloviter et al., 1999). Blocking $GABA_B$ responsiveness in the voltage clamp recordings may have reduced a large component of dendritic GABAergic inhibition, resulting in recordings that were biased toward somatic GABAergic inhibition.

Conclusions

We propose the hypothesis that HIPP inhibitory input to GCL dendrites is stronger in STEP KO mice. With regard to the STEP KO mice versus WT mice, this hypothesis can explain the larger seizure thresholds shown in the pilocarpine studies, the lack of potentiation in excitatory responses demonstrated in the calcium imaging studies, the lack of potentiation in $GABA_A$ inhibitory responses shown in the voltage clamp data, and the larger putative $GABA_B$ component shown in the cell-attached recordings. Future studies should confirm whether HIPP neurons are indeed the source of greater inhibitory input in STEP KO mice, and confirm that the larger hyperpolarizations seen in STEP KO GCL neurons are derived from $GABA_B$ responses.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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