Published in final edited form as:

Neurobiol Dis. 2011 February ; 41(2): 361–376. doi:10.1016/j.nbd.2010.10.004.

Temporal Lobe Epilepsy Induces Intrinsic Alterations in Na Channel Gating in Layer II Medial Entorhinal Cortex Neurons

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Abstract

Temporal lobe epilepsy (TLE) is the most common form of adult epilepsy involving the limbic structures of the temporal lobe. Layer II neurons of the entorhinal cortex (EC) form the major excitatory input into the hippocampus via the perforant path and consist of non-stellate and stellate neurons. These neurons are spared and hyper-excitability in TLE. The basis for the hyper-excitability is likely multifactorial and may include alterations in intrinsic properties. In a rat model of TLE, medial EC (mEC) non-stellate and stellate neurons had significantly higher action potential (AP) firing frequencies than in control. The increase remained in the presence of synaptic blockers, suggesting intrinsic mechanisms. Since sodium (Na) channels play a critical role in AP generation and conduction we sought to determine if Na channel gating parameters and expression levels were altered in TLE. Na channel currents recorded from isolated mEC TLE neurons revealed increased Na channel conductances, depolarizing shifts in inactivation parameters and larger persistent (I_{NaP}) and resurgent (I_{NaR}) Na currents. Immunofluorescence experiments revealed increased staining of $Na_v1.6$ within the axon initial segment and $Na_v1.2$ within the cell bodies of mEC TLE neurons.

These studies provide support for additional intrinsic alterations within mEC layer II neurons in TLE and implicate alterations in Na channel activity and expression, in part, for establishing the profound increase in intrinsic membrane excitability of mEC layer II neurons in TLE. These intrinsic changes, together with changes in the synaptic network, could support seizure activity in TLE.

Keywords

Sodium channels; Temporal Lobe Epilepsy; Entorhinal Cortex; Axon Initial Segment

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Introduction

Temporal Lobe Epilepsy (TLE) is the most common form of adult epilepsy that involves the limbic structures of the temporal lobe including the entorhinal cortex (EC). The EC receives input from the parahippocampus, prefrontal cortex, and frontal cortex (Apergis-Schoute *et al.*, 2006). This activity is then sent to the hippocampus via the perforant path and the temporoammonic path (TAP) (Burwell, 2000). The EC is subdivided into five main cortical layers with layers I–III superficial and layers IV–V deep layers. Layer II consists of non-stellate and stellate neurons and receives excitatory input from the perirhinal cortex, parasubiculum, olfactory structures as well as structures of the EC (Witter *et al.*, 1989) and form the major excitatory input into the dentate gyrus (DG) and CA3 via the perforant path and the TAP.

In TLE, both animal models and patient studies have shown a decreased volume of the EC (Jutila *et al.*, 2001; Bartolomei *et al.*, 2005), corresponding to substantial loss of layer III neurons within the superficial layers of the mEC (Du *et al.*, 1993). Although mEC layer II neurons are spared, they become hyper-excitable, displaying prolonged excitatory synaptic responses to stimulation of the EC deep layers (Bear *et al.*, 1996). This increase in neuronal activity ultimately leads to an excessive excitatory input onto DG neurons of the hippocampus, further exciting the hippocampal-EC circuit (Kobayashi *et al.*, 2003). Potential mechanisms for the hyper-excitability include reduced inhibitory input onto mEC layer II neurons (Kumar & Buckmaster, 2006), hyper-excitability of remaining mEC layer III neurons, providing enhanced synaptic activity via stimulation of the TAP (Ang *et al.*, 2006), and synaptic re-organization within mEC layer II, although the latter has been recently suggested not to exist (Kumar *et al.*, 2007). In addition to altered synaptic networks, changes intrinsic to the neuron, including modulations in ion channel activity, could also be involved.

In hippocampal neurons from animal seizure models, Na channel gating and expression levels are altered in a manner that would favor an increase in neuronal excitability (Ketelaars *et al.*, 2001; Agrawal *et al.*, 2003; Aronica *et al.*, 2001; Whitaker *et al.*, 2001; Vreugdenhil *et al.*, 1998). Na channels are comprised of an α -subunit, and a variable number of auxiliary β -subunits (Catterall, 2000). Neurons are known to express multiple Na channel isoforms (Kress & Mennerick, 2008; Candenas *et al.*, 2006) with the highest expression density along the axonal initial segment (AIS), a specific region near the start of the axon and the site for action potential (AP) initiation. In view of the importance of Na channels in initiating and propagating AP's it is not surprising that altered activity and expression of Na channels could be pro-excitatory.

In this study we show that mEC layer II neurons from TLE animals continue to be hyper-excitable when devoid of synaptic input. We hypothesize that alteration's in Na channel activity and expression in TLE neurons account, in part, for this continued hyper-excitable. We propose that the changes in Na channel behavior, together with synaptic network changes, contribute to the hyper-excitable of mEC layer II neurons, altering the threshold for seizure initiation and spread throughout the EC-hippocampal circuit.

Materials and Methods

Animals

All animal experiments were conducted in accordance with the guidelines established by the National Institutes of Health guide for the Care and Use of Laboratory Animals and were approved by the University of Virginia's Institute of Animal Care and Use Committee. Fifteen adult male Sprague-Dawley rats (250–300 gram) received a bipolar twisted pair of

stainless steel electrodes to either hemisphere unilaterally in the posterior ventral hippocampus for stimulation and recording (coordinates from bregma AP ~-5.3 mm, ML~ 4.9 mm, DV~ 5.0 mm, bite at~ -3.5 mm) (Paxinos & Watson G, 1996). Electrodes were attached to Amphenol connectors and secured to the skull with jeweler's screws and dental acrylic. One week following surgery, rats were stimulated through the hippocampal electrode to induce limbic *status epilepticus* using a protocol previously described (Lothman *et al.*, 1989). In brief, animals were stimulated for 90 minutes with 10 second trains of 50 Hz, 1 ms biphasic square waves with a maximum intensity of 400 μ A peak to peak delivered every 11 seconds. After 90 minutes, stimulation was stopped and hippocampal activity was recorded for a minimum of 8 hours to ensure that a prolonged period of continuous EEG seizure activity was maintained. Animals that exhibited continuous electrographic seizure activity for at least 8 hours after stimulation were at uniform risk for development of limbic epilepsy. Animals (about 15%) that did not meet the EEG criteria of minimum continuous seizure activity were not maintained, as their chance of developing chronic epilepsy was extremely low.

Following the induction of and recovery from limbic *status epilepticus*, rats were placed in standard laboratory housing. Three months after the induction of *status epilepticus*, animals were evaluated for the presence and frequency of spontaneous temporal lobe seizures, as the seizure pattern and frequency would have plateaued by this time (Bertram & Cornett, 1994). During the monitoring phase, rats were placed in specially designed cages, which allowed full mobility of the animals, good visualization for video monitoring, and a stable recording environment. Animals had free access to food and water, as well as a standard 12 hour light-dark cycle. Seizures were recorded and documented using a commercial computerized EEG program (Harmonie, Stellate Systems). All data were reviewed at an offline reading station connected to the vivarium computers via a local area network. The time of occurrence, behavioral severity (Racine 5 point scale) and duration for all seizures were noted.

Seizure Determination

Electrographic seizures in the rats were characterized by the paroxysmal onset of high frequency (greater than 5 Hz) increased amplitude discharges that showed an evolutionary pattern of a gradual slowing of the discharge frequency and subsequent post-ictal suppression. Seizure duration was measured from the onset of the high frequency activity or initial spike to the cessation of the terminal regular electrographic clonic activity.

Entorhinal Cortex Slices

Horizontal brain slices (300 μ m) were prepared from approximately 15 Sprague-Dawley rats (250–450 grams) with temporal lobe epilepsy (TLE) or approximately 20 age matched controls. TLE animals had a minimum of two spontaneous seizures per day by EEG recordings, 3 months after the induction of *status epilepticus*. TLE animals were used within 48 hours of the last documented seizure. Animals were euthanized with isoflurane, decapitated, and brains rapidly removed and placed in chilled (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgCl_2 , 0.5 L-Ascorbic acid, 10 glucose, 25 NaHCO_3 , and 2 Pyruvate (oxygenated with 95% O_2 and 5 % CO_2). Slices were prepared using a Vibratome (Vibratome 1000 Plus), transferred to a chamber containing oxygenated ACSF, incubated at 37°C for 35 minutes, and then stored at room temperature. In order to preserve slices with the mEC, slices were obtained from 3.9 to 5.9 mm interaural (corresponding to -6.1 to -4.1 mm from bregma), and placed in numbered sub-chambers to maintain anatomical positioning of the slice. For recordings, slices were held in a small chamber superfused with heated (32°C) oxygenated ACSF at 2 mL/min. mEC layer II neurons were visually identified by infra-red video microscopy (Hamamatsu, Shizouka, Japan) using a Zeiss Axioscope microscope (Zeiss, Oberkochen,

Germany). Whole-cell current clamp recordings were performed using an Axopatch 700B amplifier (Molecular Devices). All voltage protocols were applied using pCLAMP 10 software (Molecular Devices) and a Digidata 1322A (Molecular Devices). Electrodes were fabricated from borosilicate glass using a Brown-Flaming puller (model P97, Sutter Instruments Co) and had resistances of 3.5–4.0 M Ω when filled with an intracellular recording solution containing (in mM): 120 Kgluconate, 10 NaCl, 2 MgCl₂, 0.5 K₂EGTA, 10 HEPES, 4 Na₂ATP, 0.3 NaGTP, 20 biocytin (pH adjusted to 7.2 with KOH). AP's were evoked with a series of current injection steps from –20 pA to 470 pA in 10 pA steps for 300 ms at 5 sec inter-pulse intervals. To standardize our tests the resting membrane potential (RMP) was recorded and then maintained at –60 mV by injection of DC current. Cell input resistance (IR) was calculated by dividing the steady-state voltage response evoked by varying current injections ($\Delta V/\Delta I$) from –20 pA until the current pulse just prior to that which evoked an AP. Data points were then fit with a linear line to determine IR values. Threshold was determined as the voltage at which the slope of the AP exceeded $\geq 20 \text{ Vs}^{-1}$. AP amplitudes were measured from threshold to the AP peak. Width was the duration of the AP at the half way voltage between threshold and AP peak. Upstroke velocity was determined as the dV/dt between a range of points that were $\pm 10 \text{ mV}$ from the median value of the AP amplitude. In some experiments AP's were evoked using a bipolar platinum iridium stimulating electrode (WPI, Sarasota, FL, USA) placed in layer III of the mEC approximately 1 mm distant from the mEC layer II neurons. A 400 μs stimulus of varying current amplitude (1 to 3.2 mA) was applied every 15 sec via a digital stimulator (Digitimer Ltd, Hertfordshire, UK). In order to consistently evoke AP's the stimulus amplitude was increased 1.5 times from threshold. Duration of the evoked somatic after depolarizing potential (ADP) was determined as the interval between the start of the deviation from the resting membrane potential to the point at which the response returned to the resting membrane potential. The amplitude of the somatic ADP was measured at the point at which the AP terminated following the fAHP.

mEC Layer II Neuron Isolation

Slices marked for neuron isolation were transferred to a solution containing (in mM): 120 NaCl, 2.5 KCl, 0.2 CaCl₂, 1 MgCl₂, 20 PIPES, 25 glucose and Protease type XIV 1 mg/ml (Sigma) and incubated at 32°C for 35 minutes. The surface of the solution was continuously blown over with 100% oxygen. After incubation the slices were washed in protease free solution several times. Slices used corresponded to –6.1 to –4.1 mm dorsal/ventral from bregma. Under a low power microscope the mEC layer II (the area between 8–9 mm posterior and 4–6 mm lateral from Bregma) was identified as a translucent band and dissected free using a fine dissecting tool, individually triturated with fire-polished glass pipettes of decreasing aperture and plated onto fibronectin-coated glass coverslips for electrophysiological recordings.

Na Channel Electrophysiology

All Na channel current recordings, except persistent Na currents (I_{NaP}) and resurgent Na currents (I_{NaR}) currents, were recorded from isolated neurons using the whole-cell configuration of the patch clamp recording technique and an Axopatch 200 amplifier (Molecular Devices). All voltage protocols were applied using pCLAMP 9 software (Molecular Devices) and a Digidata 1322A (Molecular Devices). Currents were amplified, low-pass filtered (2 kHz), and sampled at 33 kHz. Borosilicate glass pipettes were pulled and heat polished to produce electrode resistances of 2–2.5 M Ω when filled with the following electrode solution (in mM): 140 CsF, 2 MgCl₂, 1 EGTA, 10 HEPES, 4 Na₂ATP, and 0.3 NaGTP (pH adjusted to 7.3 with CsOH, osmolarity adjusted to 310 mosM with sucrose). Isolated neurons were superfused with solution containing the following composition (in mM): 10 NaCl, 3 KCl, 1 CaCl₂, 2 MgCl₂, 0.1 CdCl₂, 10 HEPES, 110

Choline Cl₂, and 30 TEA-Cl (pH adjusted to 7.3 with NaOH, osmolarity adjusted to 310 mosM with sucrose). All experiments were performed at room temperature (20–22°C). After establishing whole-cell, neurons were held at –90 mV for 2–3 minutes to account for equilibrium gating shifts and a minimum series resistance compensation of 75% was applied. Capacitive and leak currents were subtracted using the P/N-4 protocol except during steady-state inactivation.

The current voltage relationship was determined using a 25 ms voltage pulse from –80 to +20 mV in steps of 5 mV from a holding potential of –100 mV at 2 sec intervals. Conductance as a function of voltage was derived from the current-voltage relationship using the equation $g = I_{Na}/(V - E_{Na})$, where V is the test potential and E_{Na} is the reversal potential. The voltage dependence of activation data were fitted by a Boltzmann function:

$$y = 1 / (1 + \exp((V - V_{1/2})/k))$$

where y is the normalized conductance (g/g_{max}) or the normalized current for activation and inactivation respectively, $V_{1/2}$ is voltage of half-maximal activation or inactivation and k is the slope factor. Decay of macroscopic currents were fitted to either a single or a double exponential function and fast time constants determined using the equation:

$$y = A_1(1 - \exp(-t/\tau_1)) - \text{single}$$

where A_1 is the coefficient for the exponential, t is time (ms) and τ_1 is the time constant, or

$$y = A_1(1 - \exp(-t/\tau_1)) + A_2(1 - \exp(-t/\tau_2)) - \text{double}$$

where A_1 and A_2 are the coefficients for the fast and slow exponentials, t is time (ms) and τ_1 and τ_2 are the fast and slow time constants respectively.

For steady-state inactivation, neurons were held at a potential of –100 mV and test potentials from –115 mV to –10 mV for 1 sec at 5 mV increments were applied. The second pulse to +10 mV for 20 ms was used to assess channel availability. For each neuron, currents during the second pulse were normalized so that the largest current was 1.0 and fit to the Boltzmann function.

For recovery from inactivation, neurons were held at –100 mV and then depolarized to a test potential of +10 mV for 1 sec to inactivate the Na channels. Recovery was determined at varying recovery times between 1 ms and 15 sec at a test potential of –90 mV. A 20 ms pulse to +10 mV was subsequently applied to assess the extent of channel recovery. For each neuron, current amplitudes during this test pulse were normalized so that the largest current during the conditioning potential was 1.0. Data were fit with either a single or double exponential function.

Persistent Na currents (I_{NaP}) were determined in brain slice preparations using voltage ramps from –100 mV to –10 mV at a rate of 65 mV/s. I_{NaP} was recorded in bath solution containing (in mM): 30 NaCl, 120 TEA-Cl, 10 NaHCO₃, 1.6 CaCl₂, 2 MgCl₂, 0.2 CdCl₂, and 5 4-AP (pH 7.4 when oxygenated with 95% O₂ and 5% CO₂; temperature 32°C) and a pipette solution containing (in mM): 140 CsF, 2 MgCl₂, 1 EGTA, 10 HEPES, 4 Na₂ATP, and 0.3 NaGTP (pH adjusted to 7.3 with CsOH, osmolarity adjusted to 310 mosM with sucrose). Ramp voltage recordings displayed an inward current that was referred to I_{NaP} . To

determine the peak I_{NaP} current, voltage ramp protocols were repeated in the presence of TTX (1 μ M) applied focally to the axon initial segment (AIS) region using of a puffing system consisting of a large diameter (~ 10 μ m) glass microelectrode connected to an air-filled syringe. Traces obtained in the presence of TTX were subtracted from those obtained in its absence. TTX was reconstituted in ACSF.

Resurgent Na currents (I_{NaR}) were also recorded in the brain slice preparation using a bath solution containing (in mM): 100 NaCl, 26 NaHCO₃, 19.5 TEA-Cl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 0.1 CdCl₂, 4 4-AP, and 10 glucose (pH 7.4 when oxygenated with 95% O₂ and 5% CO₂; temperature 32°C) using the same pipette solution as that for recording I_{NaP} . Neurons were held at -100 mV and depolarized to $+20$ mV for 20 ms, followed by either a single repolarizing step to -30 mV for 100 ms to determine the peak I_{NaR} , or by using a series of repolarizing steps from -100 mV to -10 mV to determine the voltage dependance of I_{NaR} . Protocols were again repeated in the presence of TTX (1 μ M) to determine I_{NaP} current amplitudes and gating.

Immunohistochemistry Experiments

Brains from rats with TLE or age-matched control rats were removed and snap frozen on dry ice. Horizontal cryostat sections (8 μ m) were prepared and thaw-mounted onto Superfrost Plus slides (Fisher Scientific) and maintained at -80°C until required. Sections were stored for no more than 1 month prior to use. Slices were fixed in 4% paraformaldehyde for 10 minutes, washed with PBS, permeabilized for 60 minutes in PBS blocking solution (5% fish skin gelatin, 5% serum of the secondary antibody host animal, 0.25% Triton X 100, and 0.65% w/v BSA), and then incubated in blocking solution containing primary antibody overnight at 4°C . Cells were then washed with PBS, incubated with blocking solution for 60 minutes and incubated in secondary antibody for 60 minutes. Cells were then washed for a final time with PBS. Primary antibodies used were rabbit anti-Na_v1.2, anti-Na_v1.6, anti-Na_v1.3 and anti-Na_v1.1 (1:250 Alomone labs) and ankyrin G (1:250 NeuroMab N106/36). Secondary antibodies used were goat anti-rabbit Alexa 488 and Alexa 594 (1:500, Invitrogen). To visualize biocytin labeled neurons, slices were fixed in 4% paraformaldehyde for at least 24 hours and then processed in an identical manner to slices used for Na channel isoform identification. Slices were also stained for NeuN, a neuronal specific nuclear antibody (1:250; MAB377; Chemicon). Secondary antibodies used included Alexa fluor 488 streptavidin (5 μ g/ml; Invitrogen,) to visualize biocytin labeled neurons and Alexa 594 to visualize NeuN staining. Slices were mounted onto slides and coverslipped using Vectorshield (Vector laboratories). Confocal images were captured of mEC layer II using a Zeiss LSM 510 confocal microscope (Zeiss, Oberkochen, Germany) with a 40×1.3 NA oil immersion objective and Zeiss LSM Imaging software. For comparison purposes, all immunohistochemistry experiments were carried out the same time using the same sources of primary and secondary antibodies. Images were taken within one session and within three hours. The following settings were used and not changed. Pinhole was set to 0.99 airy units for all lasers. For 488 nm laser, transmission was set to 8%, detector gain was 780. For 543 nm laser, transmission was set to 90%, detector gain was 923. Quantification and analysis was performed using Image J software (NIH). For analysis of Na_v1.6 expression a line scan representing the length of the AIS, as determined by Ankyrin G staining, was drawn and the mean relative optical density (R.O.D.) determined. The mean R.O.D. for Ankyrin G was unaltered between control and TLE preparations and allowed for standardization of the immunolabelling as a ratio of Na_v1.6 to Ankyrin G staining for each AIS. For quantification of somatic staining a rectangular box of standard area was placed around the somatic regions of each neuron and the average R.O.D. within each box was calculated. Values recorded from control preparations were used to normalize for staining in TLE preparations.

Drugs

2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline -7-sulfonamide (NBQX), D-(-)-2-Amino-5-phosphonopentanoic acid (AP5), strychnine, picrotoxin, tetraethylammonium (TEA), 4-aminopyridine (4-AP), and tetrodotoxin (TTX) were obtained from Sigma Aldrich (St. Louis, MO, USA). NBQX, AP5, strychnine, picrotoxin, and TTX were prepared as 1000 $\times$ stock solutions. All were prepared in DMSO except for TTX, which was prepared in water. Drugs were then diluted to working concentrations directly preceding experiments.

Data Analysis

Electrophysiology data analysis was performed using Clampfit software (v10, Molecular Devices) and Origin (v6, Microcal Software). All values represent means $\pm$ standard error of the mean (S.E.M). Statistical significance was determined by using a students *t*-test (unpaired) or a standard one way ANOVA followed by Tukey's or Dunn's *post hoc* test for parametric data or the Rank Sum test for non-parametric data (SigmaStat, Jandel).

Result

mEC layer II non-stellate and stellate neurons are intrinsically hyper-excitable in TLE

mEC layer II non-stellate and stellate neurons were visually identified using infra red video microscopy and distinguished by their unique AP firing characteristics (Alonso & Klink, 1993; Tahvildari & Alonso, 2005). Although AP's in both neuron subtypes had a fast after hyperpolarization (fAHP) followed by a depolarizing after potential (DAP) and a medium after hyperpolarization (mAHP), non-stellate neurons typically had smaller amplitude fAHPs and DAPs than stellate neurons (fig 1A, 2A). Stellate neurons also exhibited a characteristic "sag" response to a hyperpolarizing current injection, not observed in non-stellate neurons (supplementary fig 1C & D). Neurons were also biocytin labeled and co-labeled with NeuN to confirm location of the neuron within mEC layer II (fig. 1B, 2B, and supplementary fig. 1A & B). In agreement with other animal models of TLE (Du *et al.*, 1995; Kumar & Buckmaster, 2006), there was considerable loss of mEC layer III and a sparing of mEC layer II neurons in our model of TLE (data not shown).

Membrane properties for both mEC layer II non-stellate and stellate neurons were determined in control and TLE brain slices. AP firing rates were similar for both subtypes of mEC layer II neurons under control conditions, but were significantly higher in TLE. For non-stellate neurons rates were increased from 23.5 ± 1.4 Hz (n=15) to 33.3 ± 1.6 Hz (n=13; $P<0.001$; fig 1C–D) in TLE at a current injection step of 470 pA. Stellate neurons also fired at higher rates in TLE (from 21.5 ± 1.0 Hz (n=18) in control to 34.5 ± 1.7 Hz (n=11) in TLE $P<0.001$; figure 2C–D). Analysis of AP properties from control and TLE neurons revealed no changes in resting membrane potentials (RMP), however, AP threshold potentials were significantly hyperpolarized in TLE neurons allowing for lower firing thresholds. AP upstroke velocity rates and fAHP values were also increased in TLE (Table 1). DAP amplitudes were increased in TLE, but did not reach significance. Membrane input resistances were significantly increased in TLE non-stellate neurons ($P<0.05$) when compared to control neurons. Although an increasing trend for input resistances were recorded for TLE stellate neurons compared to control, these values did not reach significance (Table 1, fig 1E and 2E). Increases in input resistance could account for the higher firing rates observed in TLE non-stellate neurons, however, when compared at similar input resistances TLE neurons continued to elicit AP's at higher firing rates, suggesting additional mechanisms for the increases in AP discharge besides increases in input resistance (fig 1E, 2E).

Phase plot graphs of the train of AP's shown in figure 1 and 2Fa & b, together with first and second derivatives of the first AP in each train are shown in fig. 1Fg–Fj (non-stellate) and 2Fg–Fj (stellate). In each phase plot, the asterisk (*) represents the point at which AP threshold was reached and shows a lower firing threshold in TLE neurons. The two inflections along the rising phase of the phase plot indicate the point at which the AP is initiated within the axon initial segment (AIS; arrow) and the point at which it invades the soma (+), recruiting somatic channels (Kress *et al.*, 2008). In agreement with faster upstroke velocity rates, both mEC non-stellate and stellate TLE neurons had larger phase plot amplitudes (fig. 1Fd & 2Fd respectively) and had more pronounced kinks representing spike initiation at the AIS and somal invasion of the spike compared to control plots. The differences in spike initiation and propagation into the soma were more apparent when analyzing the second derivatives of the AP (fig. 1Fi–j and 2Fi–j). In control mEC non-stellate neurons delays between peaks of the second derivative were 0.06 ± 0.01 ms ($n=13$) and were increased to 0.14 ± 0.02 ms in TLE ($n=11$; $P<0.01$). In a similar manner, control mEC stellate neurons had a delay of 0.06 ± 0.02 ms ($n=11$) and were increased to 0.12 ± 0.02 ms ($n=8$) in TLE ($P<0.05$). The presence of a more pronounced axonally derived spike with longer latencies between initiation and invasion into the soma suggests that the site of spike initiation in both TLE mEC non-stellate and stellate neurons is altered when compared to control neurons and likely shifted to a more distal site in TLE.

Synaptic reorganization within mEC layer III and II is thought to contribute to the increased membrane excitability of EC layer II neurons (Kumar *et al.*, 2007). To determine if these differences in firing rates between control and TLE would remain in the absence of synaptic input, implicating an additional intrinsic component for the increased neuronal activity, neurons were exposed to excitatory (APV 30 μ M and NBQX 10 μ M) and inhibitory (picrotoxin 50 μ M and strychnine 50 μ M) synaptic blockers. Under these conditions firing frequency decreased in both control and TLE neurons. However, both mEC non-stellate and stellate neurons continued to elicit higher frequencies of AP firing in TLE brains slices (fig 3). When compared at a current injection step of 470 pA, mEC non-stellate neurons firing frequency was increased from 13.3 ± 1.3 Hz in control ($n=10$) to 20.3 ± 1.8 Hz ($n=14$; $P<0.01$) in TLE (fig 3A–C). In a similar manner firing frequencies in mEC stellate neurons were increased from 12.5 ± 1.5 Hz in control ($n=12$) to 22.2 ± 3.0 Hz ($n=10$; $P<0.001$) in TLE (fig 3D–F). Firing frequencies were fully reversible on washout of all synaptic antagonists. These findings support the idea that membrane excitability is largely controlled by synaptic activity, but that in the absence of such activity, an increase in the intrinsic membrane excitability is still evident in TLE, suggesting an additional intrinsic component previously not thought to exist in mEC layer II neurons (Kobayashi *et al.*, 2003; Kumar & Buckmaster, 2006).

Synaptically evoked AP's have increased somatic ADPs in mEC Layer II TLE neurons

Brief stimulations within mEC layer III consistently evoked depolarizing events in mEC layer II non-stellate and stellate neurons and evoked single AP spikes in control neurons (fig 4A & D). In mEC non-stellate neurons the duration of depolarizing event was increased from 75.9 ± 9.1 ms ($n = 8$) in control to 138.0 ± 18.6 ms in TLE ($n = 6$; $P<0.01$). The maximum amplitude of the somatic depolarization was also increased from 8.5 ± 0.9 mV (control; $n = 8$) to 18.4 ± 3.4 mV in TLE ($n=6$; $P<0.01$; fig 4B & C) and was coupled to the generation of multiple AP spikes (2.7 ± 0.5). Evoked responses in mEC stellate neurons were also accentuated in TLE. Durations were increased from 68.9 ± 20.3 ms (control; $n = 8$) to 106.4 ± 15.1 ms (TLE; $n = 6$) and amplitudes were increased from 9.5 ± 0.9 mV (control; $n = 8$) to 15.5 ± 2.3 mV in TLE ($P<0.01$; $n = 6$, fig 4E & F). Again, TLE evoked responses evoked multiple AP's (3.0 ± 0.6). Focal application of TTX (500 nM) to the axon initial segment (AIS) region not only abolished the evoked AP's, but also reduced the

amplitude and duration of the depolarizing event ($n = 3$ for control and TLE mEC non-stellate and stellate neurons), suggesting the involvement of Na channels in the generation of somatic spike ADP (Yue *et al.*, 2005). The larger somatic ADP in TLE neurons could be indicative of differences in Na channel activity or density, particularly persistent (I_{NaP}) and resurgent (I_{NaR}) Na currents, contributing to the intrinsic membrane hyper-excitability of TLE neurons. To test this hypothesis, Na channel currents were recorded from isolated mEC layer II neurons. The use of isolated neurons allowed for adequate clamping of Na channel currents and also excluded synaptic involvement.

Na channel gating parameters are altered in TLE non-stellate neurons

Isolated mEC non-stellate neurons were visually identified as having a pyramidal shaped cell body with a single major apical dendrite and two basal dendrites (fig. 5I). Cell capacitance values were not significantly different between control (10.9 ± 1.3 pF; $n=18$) and TLE (13.1 ± 1.6 pF; $n=17$) suggesting no major differences in cell size. Representative current voltage traces recorded from control (fig. 5A) and TLE (fig. 5B) mEC non-stellate neurons show a 3.2 fold increase in peak conductance recorded in TLE neurons (control: 92.2 ± 17.4 nS, $n=9$; TLE: 295.0 ± 41.7 nS, $n=19$; $P<0.01$; fig. 5D). A conductance plot of the current voltage relationship was best fit to a single Boltzmann function and is shown in fig 5C, activation parameters are listed in table 2 and indicate no changes in the half maximal activation voltage ($V_{1/2}$) or the slope (k) of the curves. Decays of the macroscopic current were fit to a double exponential function and revealed slower decay time constants for the fast time constant (τ_1) in TLE mEC non-stellate neurons compared to control neurons (fig 5E–F; $n=10$ for TLE and $n=8$ for control). In fig. 5F normalized current traces recorded at -10 mV are shown superimposed to further emphasize the major differences in the decay of the macroscopic current between control and TLE mEC non-stellate neurons.

In contrast to activation parameters, inactivation parameters were significantly ($P<0.05$) shifted to more depolarized potentials by over 8 mV in TLE mEC non-stellate neurons compared to control (fig 5G; table 2). Slope factors (k) were not significantly altered. Insets in fig. 5G are example steady-state inactivation traces for both TLE and control mEC non-stellate neurons at a pre-pulse potential of -100 mV (largest current amplitude) superimposed with one recorded at a pre-pulse of -60 mV and emphasizes the major differences in Na channel inactivation between the two conditions. In control neurons approximately 15% of the Na channels remained available for opening whereas in TLE approximately 41% of Na channels remained available for opening at -60 mV, a potential close to the RMP of these neurons.

The rate at which Na channels are likely to cycle through from the inactivated state to the closed state would influence their ability to open again and contribute to the generation of APs. To determine if recovery from inactivation rates were altered in TLE, recovery from inactivation kinetics were established for both TLE and control mEC non-stellate neurons. Na channels were subsequently recovered at -90 mV for a variable length of time (1 ms to 15 sec) and then subjected to a test pulse of $+10$ mV to determine the extent of recovery (fig. 5H). In control neurons, recovery from inactivation was best fit with a double exponential function and had a fast time constant (τ_1) of 15.9 ± 2.3 ms ($n=6$) with a slow time constant (τ_2) of $3,216 \pm 761$ ms ($n=6$). In contrast, recovery from inactivation for TLE mEC non-stellate neurons was significantly accelerated compared to control with a fast time constant (τ_1) of 7.5 ± 1.4 ms ($P<0.05$; $n=10$) and a slow time constant (τ_2) of $3,505 \pm 1,049$ ms ($n=10$). Insets in fig. 5H are example recovery from inactivation traces for both TLE and control mEC non-stellate neurons at a recovery time of 100 ms (smaller current amplitude) superimposed with one recorded after 10 sec of recovery. Traces clearly show accelerated recovery for TLE neurons. The faster recovery rates of Na channels in TLE mEC non-

stellate neurons would significantly increase the availability of Na channels for reopening during epileptiform burst activity.

TLE mEC stellate neurons have distinct Na channel gating parameters

Isolated mEC stellate neurons were identified as having polygonal shaped cell bodies with multiple dendrites (fig. 6F). In a similar manner, cell capacitance values for mEC stellate neurons were not altered in TLE (control; 12.9 ± 1.2 pF; $n=29$ and TLE; 15.8 ± 1.2 pF; $n=23$), but peak conductances were increased (control: 156.3 ± 22.7 nS, $n=10$ and TLE 335.4 ± 62.2 nS, $n=11$; $P<0.01$; fig. 6A & B). In contrast to non-stellate neurons, decays of the macroscopic currents were not slowed in TLE mEC stellate neurons compared to control (fig. 6E).

Activation curves remained unchanged in TLE, but steady state inactivation gating was shifted ($P<0.05$) by more than 10 mV to more depolarized potentials when compared to control mEC stellate neurons (Fig. 6G; table 2). Slope factors (k) were unchanged between the two conditions. Example steady-state inactivation traces are shown for both TLE and control as an inset to fig. 6G. Superimposed traces represent the Na current recorded at a pre-pulse potential of -100 mV (largest amplitude current) and -60 mV. In control neurons approximately 22% of Na channels were available for activation whereas in TLE neurons this percentage was increased to 47% at -60 mV. Although activation gating was unchanged, the shifts in inactivation would allow for more Na channels to remain available for opening, contributing to AP bursting activity.

Fig. 6H shows the recovery from inactivation curve for control and TLE mEC stellate neurons. Control neurons were best fit with a single exponential function and had a time constant (τ_1) of 13.4 ± 2.1 ms ($n=6$). In contrast, rates of recovery from inactivation for TLE mEC stellate neurons ($n=9$) were best fit to a double exponential function: $\tau_1 = 16.3 \pm 2.0$ ms and $\tau_2 = 4143 \pm 1161$ ms. The differences in the time constants were not significantly different, but were indicative of a slowing trend for recovery rates in TLE. Insets in fig. 6B show typical recovery from inactivation traces for both TLE and control mEC non-stellate neurons after 100 ms recovery interval (smaller current amplitude) superimposed with one recorded after 10 sec recovery interval and illustrate that TLE neurons have slower recovery from inactivation.

Persistent (I_{NaP}) and resurgent (I_{NaR}) Na currents are accentuated in TLE mEC layer II neurons

Persistent Na currents (I_{NaP}) are thought to be major contributors to the generation of AP bursts and are increased in amplitude in subicular neurons isolated from patients with TLE (Vreugdenhil *et al.*, 2004). To determine if I_{NaP} currents were increased in TLE, accounting for the increased somatic spike ADP in synaptically evoked responses observed in TLE neurons, voltage ramps were applied to visually identified mEC non-stellate and stellate neurons (fig. 7) in brain slice preparations. Ramp voltage recordings displayed an inward current that was completely abolished by focal AIS application of $1 \mu\text{M}$ TTX. The peak I_{NaP} current was determined by subtracting traces obtained in the presence of TTX from those obtained in its absence. Both TLE mEC non-stellate and stellate neurons had larger I_{NaP} current amplitudes when compared to control neurons. In mEC non-stellate neurons control I_{NaP} currents had an amplitude of -121.3 ± 26.1 pA ($n = 9$) and were significantly increased in TLE to -358.0 ± 46.2 pA ($n = 8$; $P<0.001$, fig. 7A–D). Conductance plots revealed a hyperpolarizing shift in the $V_{1/2}$ from -46.18 ± 1.7 mV ($n = 7$) in control to -57.29 ± 3.13 mV in TLE ($n = 7$; $P < 0.01$, fig. 7C). Slopes were not different (control; -7.9 ± 0.5 mV and TLE; -5.8 ± 1.2 mV).

mEC stellate neurons also had increased I_{NaP} current amplitudes in TLE (fig 7E–H). Amplitudes were increased from -153.5 ± 18.9 pA ($n = 9$) under control conditions to -356.8 ± 31.7 pA in TLE ($n = 8$; $P < 0.001$, fig. 7H). Conductance plots were not different in mEC stellate neurons. In control, $V_{1/2}$ values were -47.3 ± 3.1 mV ($n = 9$) and -55.8 ± 3.1 mV ($n = 7$; fig 7G) in TLE. Slopes of curves were not different between TLE and control neurons ($k = -5.2 \pm 1.4$ mV and -6.3 ± 0.6 mV; respectively).

Resurgent Na currents (I_{NaR}) are slowly inactivating Na currents that provide a depolarizing current, enhancing firing frequency or bursting activity (Raman & Bean, 1997). To determine if I_{NaR} current amplitudes were increased in TLE, peak I_{NaR} currents were measured (fig. 8). In TLE, I_{NaR} conductances in mEC non-stellate neurons were profoundly increased from 4.9 ± 1.5 nS ($n = 10$) in control to 9.5 ± 1.6 nS in TLE ($n = 8$; $P < 0.001$; t -test; fig. 8A–C). To further explore the gating parameters of I_{NaR} , families of resurgent currents were evoked to construct a current voltage plot. Example traces of families of I_{NaR} currents are shown in figs. 8D & E for mEC non-stellate neurons. Normalized currents were plotted as a function of voltage and fit with a single Boltzmann function. TLE mEC non-stellate neurons had significantly ($P < 0.05$) hyperpolarized I_{NaR} $V_{1/2}$ values (-61.3 ± 1.9 ; $n = 7$ in control compared with -68.0 ± 2.3 ; $n = 8$ in TLE; figure 8F). Slopes were slowed in TLE, but did not reach significance (-5.0 ± 0.6 in control compared with -6.2 ± 0.5 in TLE).

I_{NaR} conductances in mEC stellate neurons were also significantly larger in TLE compared to controls (fig. 8G–I), increasing from 5.9 ± 0.8 nS ($n = 12$) to 10.9 ± 1.3 nS in TLE ($n = 8$; $P < 0.001$; t -test). In contrast to non-stellate neurons, I_{NaR} $V_{1/2}$ values in stellate neurons were unchanged (-70.1 ± 2.8 ; $n = 10$: in control compared with -65.4 ± 2.9 ; $n = 6$ in TLE; figure 8L). Slope values were, however, slowed in TLE (-4.0 ± 0.4 in control compared with -7.0 ± 0.4 in TLE; $P < 0.05$).

Na_v1.6 and Na_v1.2 Na channel isoforms are up-regulated in TLE mEC layer II neurons

Both I_{NaP} and I_{NaR} currents are thought to arise mainly from activation of the Na_v1.6 Na channel isoform (Raman *et al.*, 1997). To determine if the changes in Na channel current density and gating parameters were due to increases or differential expression patterns of Na channel isoforms in TLE, particularly Na_v1.6, immunofluorescence experiments were performed (fig. 9). Na_v1.6 is thought to be highly expressed along the AIS (Royeck *et al.*, 2008). In mEC layer II neurons robust expression of Na_v1.6 was observed within the AIS and co-localized with Ankyrin G, a specific marker for the AIS (fig. 9A & B). In TLE slices Na_v1.6 staining intensity was increased (fig. 9C). Ankyrin G staining was considered a suitable marker for normalizing between control and TLE brain slices since staining intensities were not different between the two conditions. For comparison purposes we determined a ratio of Na_v1.6 to ankyrin G staining. Ratio's were increased from 0.43 ± 0.06 ($n = 20$) in control to 0.76 ± 0.06 ($n = 18$) in TLE ($P < 0.001$). Somatic staining for Na_v1.6 was low in intensity and not always detected due to the intense staining within the AIS.

In contrast to Na_v1.6 staining, Na_v1.2 staining was not localized with the AIS (data not shown), but was apparent within the somatic regions of the neurons (fig. 9D). Normalized Na_v1.2 staining was increased 2.3 fold in TLE ($n = 10$; $P < 0.001$). Low immunostaining for Na_v1.1 and Na_v1.3 was observed within the somatic regions of brain slices and remained unchanged in TLE brain slices (fig. 9E). The staining observed was consistent throughout mEC layer II and was considered representative of expression profiles for both non-stellate and stellate neurons.

Discussion

Medial entorhinal cortex layer II neurons become hyperexcitable in TLE, leading to potential increased excitatory drive onto the hippocampus (Buckmaster & Dudek, 1997). The mechanisms for this hyper-excitability have focused around alterations in the synaptic network by virtue of the fact that intrinsic changes within the neurons themselves are not thought to occur (Bear *et al.*, 1996; Kobayashi *et al.*, 2003; Kumar *et al.*, 2007). In this study we show that TLE mEC layer II neurons evoke AP's at a higher frequency than control neurons and that this difference in firing rates remains apparent in the presence of excitatory and inhibitory antagonists. This study provides the first report of an additional intrinsic component for the hyper-excitability of mEC layer II neurons in TLE. We provide support for an alteration in the activity of Na channels that could play a role, in part, for establishing the hyper-excitability of the mEC layer II neurons in TLE.

Na channel gating and expression is altered in mEC layer II neurons of TLE animals

Consistent findings between mEC layer II non-stellate and stellate neurons from TLE animals were increases in Na channel conductance. These increases in channel conductance could be due to increases in Na channel expression levels. Our studies suggest that expression levels of Na_v1.6 and Na_v1.2 were increased in TLE. Alternatively, changes in Na channel gating through post-translational pathways could also contribute to increases in Na channel conductance and the altered gating kinetics observed in TLE (Scheuer & Catterall, 2006). The increase in Na conductance coupled with impaired inactivation gating parameters, resulting in half maximal inactivation values shifted in the depolarizing direction, would increase the number of Na channels available for opening at threshold, accounting for the reduced AP threshold, increased conduction velocity and increased AP amplitude observed in TLE neurons. Several studies have now highlighted the importance of Na channel inactivation in regulating neuronal activity, synaptic integration and neuronal spiking. In cortical pyramidal neurons, inactivation of Na channels accounted for the reduction in spike rate during high frequency stimulation (Fleidervish *et al.*, 1996). Development of slow inactivation during high frequency AP discharges was shown to play a major role in attenuating AP back propagation into dendritic regions in CA1 neurons, controlling synaptic integration and postsynaptic firing in the axon (Jung *et al.*, 1997). Not surprisingly disruptions in Na channel inactivation mechanisms alter membrane excitability leading to epileptiform activity and the generation of AP bursts in generalized epilepsy with febrile seizures plus (GEFS+) and severe myoclonic epilepsy of infancy (SMEI) (Spampanato *et al.*, 2001). The depolarizing shifts in inactivation gating parameters we observed in TLE neurons would allow for the accumulation of non-inactivated and available Na channels over a greater range of voltages, resulting in a larger Na channel window current. Consistent with this notion, increases in window currents have been reported for both CA1 and DG neurons isolated from chronically epileptic animals (Ellerkmann *et al.*, 2003; Ketelaars *et al.*, 2001; Remy *et al.*, 2003).

A major finding of our studies was the profound increase in persistent (I_{NaP}) and resurgent (I_{NaR}) Na channel current amplitudes in both subtypes of mEC layer II neurons in TLE. I_{NaP} currents are non-inactivating currents that play an important role in establishing repetitive neuronal discharge behavior. In CA1 neurons I_{NaP} currents are known to generate the somatic spike ADP, crucial for controlling the firing mode of a neuron intrinsically (Yue *et al.*, 2005). The Na_v1.6 Na channel isoform is thought to be the main contributor to I_{NaP} (Rush *et al.*, 2005) and is highly expressed at the AIS (Royeck *et al.*, 2008). These two factors make Na_v1.6 an ideal candidate for controlling spike initiation within the AIS. Our immunofluorescence experiments support our electrophysiological studies, showing increased staining of Na_v1.6 specifically at the AIS in TLE. Conductance plots of the resurgent current revealed more hyperpolarized $V_{1/2}$ values (ranging between -70.1 mV to

–65 mV) for I_{NaR} compared to that recorded for macroscopic whole cell Na currents and are in agreement with the hypothesis that threshold for activation at the AIS is lower than that for the soma. In TLE non-stellate neurons the $V_{1/2}$ for I_{NaR} was significantly shifted in the hyperpolarized direction, thereby further lowering the spike threshold in these neurons. These findings suggest that close to 50% of the channels that contribute to I_{NaR} can be activated at the resting membrane potential. Since in TLE we demonstrated an approximately 2 fold increase in I_{NaR} this would significantly influence spike initiation at the AIS and its subsequent invasion into the soma. The increased I_{NaR} and I_{NaP} could account for the presence of two clearly discernable peaks observed in phase plots of TLE neurons, but not in control neurons. These findings support a major role for $Na_v1.6$ in establishing the hyper-excitability of mEC layer II neurons and contribute to growing amount of evidence supporting the importance of this isoform in epileptogenesis. For example, kindling was associated with an increase $Na_v1.6$ expression and increased persistent current in hippocampal CA3 neurons. The initiation and development of kindling was impaired in heterozygote $Na_v1.6^{+/-}$ mice (Blumenfeld *et al.*, 2009). In other studies a loss of spontaneous firing and a reduction in firing frequencies was reported in mice lacking $Na_v1.6$ in cerebellum Purkinje neurons, CA1 pyramidal and ganglion neurons of the retina neurons (Raman *et al.*, 1997; Royeck *et al.*, 2008; Van Wart & Matthews, 2006), further supporting a crucial role for $Na_v1.6$ in establishing and maintaining firing rates. Increases in both I_{NaP} and I_{NaR} recorded in mEC TLE layer II neurons in our study likely contributed to the increased somatic spike ADP and spike number observed in synaptically evoked responses. Increased I_{NaP} currents have been recorded from subiculum neurons isolated from patients with TLE (Vreugdenhil *et al.*, 2004) and from EC layer V neurons in an animal models of TLE (Agrawal *et al.*, 2003). Since these Na currents are important for AP burst firing and overall membrane excitability it is likely that alterations in these currents contribute to the overall increased neuronal hyper-excitability in TLE.

In this study we explored the intrinsic alterations of two subtypes of mEC layer II neurons; namely non-stellate and stellate cells. Besides morphological distinctions, mEC layer II non-stellate and stellate neurons are also electrophysiologically distinct and can be characterized based on their AP waveforms (Alonso & Klink, 1993). Although similar changes in Na channel gating parameters were recorded in both subtypes of mEC layer II neurons, there were also a few differences. For example, TLE mEC non-stellate neurons exhibited significant slowing of macroscopic current decay rates, faster recovery from inactivation rates and greater hyperpolarized I_{NaP} and I_{NaR} conductance plots. In contrast, TLE mEC stellate neurons did not show any delays in macroscopic current decay, exhibited a slowing trend in recovery from inactivation rates and had I_{NaP} and I_{NaR} $V_{1/2}$ values that were not different from control neurons. The significance of these differences in establishing enhanced membrane excitability in TLE is unclear since AP firing frequencies were similar between the two neuron types both in control and in TLE. It is likely that, in addition to the Na channel alterations that we report here, changes in other ion channel sub-types also occur in TLE, thereby contributing to the overall enhancement of AP bursting activity. What is clear is that these two neuron types form the major excitatory input into the DG and CA3 via the perforant path and the temporoammonic pathway. The presence of two distinct populations of neurons within mEC layer II have led some to believe that these two neurons provide two different, parallel input pathways to the hippocampus (Alonso & Klink, 1993). If this is the case, then the subtle differences in Na channel gating parameters described between these two neuron subtypes could influence the excitation of selective inputs to the hippocampus, thereby increasing the membrane excitability of specific subsets of hippocampal neurons, altering the balance of synaptic network connectivity and contributing to the generation and spread of seizures.

Conclusion

In animal models of TLE, mEC layer II neurons are spared and become hyper-excitable leading to an increased net excitatory input into the hippocampus via the DG and CA3 (Kumar & Buckmaster, 2006). Here we report an additional intrinsic component for the hyper-excitability of mEC layer II neurons in TLE; namely a change in Na channel activity and expression levels. These changes would be additive to the extensive changes in synaptic activity reported for the mEC in TLE (Kumar *et al.*, 2007; Kumar & Buckmaster, 2006) and could further amplify the net excitatory input into the hippocampus. The combination of these alterations in TLE identifies mEC layer II neurons as prime candidates for the possible initiation and spread of seizures within the temporal lobe.

Research Highlights

- Layer II medial entorhinal cortex neurons are hyperexcitable in TLE.
- Hyperexcitability is due to network and intrinsic alterations.
- Sodium current inactivation is delayed in layer II mEC neurons in TLE.
- Resurgent and persistent Na current densities are increased in TLE.
- Staining for Nav1.6 and Nav1.2 isoforms is enhanced in TLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health - National Institutes of Neurological Disorders and Stroke grants R21NS061069 (MKP & EHB), The Epilepsy Foundation Predoctoral Research Fellowship & 1F31NS064694 NINDS (NJH). We would like to thank Carl Lynch and Suzanne M. Moenter for useful editorial comments and J. Williamson, Ravi Katari, and Susanna K. Lynch for expert technical assistance.

Abbreviation list

TLE	temporal lobe epilepsy
Na	sodium
mEC	medial entorhinal cortex
TTX	tetrodotoxin
AIS	axon initial segment
AP	action potential
I_{NaP}	persistent sodium current
I_{NaR}	resurgent sodium current

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Figure 1. mEC layer II non-stellate neurons are hyper-excitable in TLE

A: Typical AP waveform of a mEC layer II non-stellate neuron exhibiting a fast afterhyperpolarizing potential (fAHP), depolarizing after potential (DAP), and medium afterhyperpolarizing potential (mAHP). B: Biocytin labeling further confirmed neuron to be within the mEC layer II and with non-stellate morphology. Green labeling is biocytin, red labeling is the neuronal marker NeuN. Scale bar represents 50 μ m. C and D: Membrane properties were recorded from both control and TLE mEC non-stellate neurons in brain slice preparations. Resting membrane potentials were recorded and then maintained at -60 mV by injection of DC current. APs were elicited by a series of DC current injection steps from -20 pA to 470 pA in 10 pA steps for 300 ms at 5 sec inter-pulse intervals. AP frequencies were increased in TLE (D) compared to controls (C). Arrows in top traces represent a membrane potential of -60 mV. E: Voltage deflection graphed as a function of current

injection demonstrates higher input resistances in TLE neurons as compared to controls (left). When compared at similar input resistances, TLE mEC layer II non-stellate neurons continue to evoke higher frequencies of AP than control neurons (right traces). Arrows represent -60 mV. F: Phase plot analysis of TLE mEC layer II non-stellate action potentials. Fa and Fb are the AP trains elicited by a 300 ms depolarizing current injection of 470 pA to control used to generate phase plots shown in Fc (control) and Fd (TLE) non-stellate neurons. AP threshold is denoted by the asterisk (*) and indicates a lower AP threshold in TLE neurons. Phase plots of TLE neurons displayed a more prominent AIS 'kink' as indicated by the arrow, representing initiation of the spike within the AIS. The second peak indicated by the + sign represents invasion of the spike into the soma and recruitment of somatic ion channels. The first AP spike (indicated by arrow in Fa and Fb) on an expanded time scale is shown in Fe for control and Ff for TLE and illustrates the greater hyperpolarized AP threshold in TLE neurons as compared to control neurons. Dashed line represents -40 mV. First derivative of the same AP shows a larger peak amplitude in TLE neurons (Fh) compared with control neurons (Fg) indicating a faster rate of rise of the AP. Second derivatives of same AP reveal two distinct peaks in TLE neurons as a result of spike initiation at the AIS and then propagation and invasion into the soma (Fj; arrow denotes peak 1 and + denotes peak 2). In control neurons two peaks were present, but were not as clearly discernable as those observed in TLE neurons (Fi).

Figure 2. Distinct AP Firing Properties of mEC layer II stellate neurons in TLE

Recording of mEC layer II stellate neurons in brain slices were confirmed by the distinct AP waveform of a fAHP, DAP, and mAHP (A) and by biocytin labeling (B). Green labeling is biocytin, red labeling is the neuronal marker NeuN. Scale bar represents 50 μ m. Biocytin labeling confirms the distinct morphology of stellate neurons as compared to non-stellate neurons. Note the differences in amplitude of the fAHP, DAP, and mAHP of mEC stellate neurons compared to mEC non-stellate neurons. Membrane properties were recorded from both control (C) and TLE (D) mEC stellate neurons in brain slice preparations. Resting membrane potentials were recorded and then maintained at -60 mV by injection of DC current. A series of 300 ms DC current injection steps from -20 pA to 470 pA in 10 pA steps at 5 sec inter-pulse intervals were applied to elicit APs. Spike frequencies were increased in TLE (D) neurons compared to control (C) neurons. E: Voltage deflection graphed as a function of current injection demonstrates no difference in input resistances in TLE neurons as compared to controls (left). Right panel emphasizes the difference in firing frequency in TLE mEC layer II stellate neurons compared to control neurons at the same input resistance. Arrows represent -60 mV. Phase plot analysis of the train of APs evoked by a 300 ms depolarizing current injection of 470 pA to control (Fa) and TLE (Fb) stellate neurons is shown for control (Fc) and TLE (Fd). AP threshold is denoted by the asterisk (*) and indicates differences in AP threshold between control and TLE neurons. In a similar manner to mEC non-stellate neurons, phase plots of mEC stellate TLE neurons also displayed a more prominent AIS 'kink' as indicated by the arrow, representing initiation of the spike within the AIS. The second peak indicated by the + sign represents invasion of the spike into the soma and recruitment of somatic ion channels. An expanded trace of the AP is shown in panel Fe & Ff. Dashed line represents -40 mV and demonstrates the more hyperpolarized AP threshold in TLE neurons as compared to control neurons. The peak of the first derivative was larger in amplitude in TLE (Fh) compared with control (Fg) indicating a faster rate of rise of the AP. Second derivatives of APs in control (Fi) and TLE (Fj) neurons also revealed two distinct peaks in the TLE neurons as a result of spike initiation at the AIS and then propagation and invasion into the soma. Again, in control neurons the two peaks were not as clearly discernable.

Figure 3. TLE mEC layer II neurons are intrinsically hyper-excitable

Membrane properties and AP firing frequencies were recorded in the presence of both excitatory (APV 30 μ M, NBQX 10 μ M) and inhibitory (picrotoxin 50 μ M and strychnine 50 μ M) synaptic blockers. A series of 300 ms DC current injection steps from -20 pA to 470 pA in 10 pA steps at 5 sec inter-pulse intervals were applied to elicit APs. In mEC non-stellate neurons, AP firing frequencies remained higher in TLE neurons (B) as compared to control neurons (A) in the presence of synaptic blockers. C: Discharge frequency (f) versus injected current (I) plots (f-I) illustrate higher firing rates in TLE (n=14) compared with control (n=10) neurons. In mEC stellate neurons, AP firing frequencies also remained elevated in TLE neurons (E) as compared to controls (D). F: Discharge frequency versus injected current plots confirm increased AP firing rates in TLE (n = 10) as compared with controls (n = 12). Data points represent means $\pm$ S.E.M.

Figure 4. Somatic ADPs were longer in duration with greater amplitudes and evoked more APs in TLE mEC layer II neurons than in control neurons

Brief stimulation elicited a small somatic ADP and a single AP in control mEC neurons (A; non-stellate: D; stellate). In TLE, somatic ADP's were broader and of larger amplitude and were associated with a burst of AP spikes (B; non-stellate: E; stellate). Superimposed gray lines show effects of focal application of TTX (500 nM) to the AIS to evoked responses. In each case the somatic ADP was reduced and AP spikes abolished. Insets shown are of expanded timescales to illustrate effects of TTX. Morphological differences in synaptically evoked responses between control and TLE mEC neurons are shown superimposed in (C) for non-stellate and (F) for stellate mEC neurons.

Figure 5. TLE mEC layer II non-stellate neurons have altered Na channel gating

Macroscopic Na channel currents were larger and decayed slower in TLE non-stellate neurons. Representative traces of families of Na channel currents recorded from (A) control and (B) TLE mEC layer II non-stellate neurons. Currents were elicited by depolarizing steps of 25 ms from a holding potential of -100 mV to test pulses in the range of -80 to $+20$ mV in 5 mV increments. (C) Conductance plot shows no difference in activation gating parameters between control and TLE. Smooth lines correspond to the least squares fit when average conductance data were fit with a single Boltzmann equation (control: $n=17$; TLE: $n=18$). Gating parameters are listed in table 2. (D) Peak Na channel conductance was increased in TLE ($P<0.01$). (E) Decays of the macroscopic current were fit to a double exponential function and revealed slower decay time constants for the fast time constant (τ_1) for TLE mEC non-stellate neurons when compared to control mEC non-stellate neurons ($n=10$ for TLE and $n=8$ for control). (F) Example current traces recorded at -10 mV are shown superimposed to illustrate slowing in the decay of the macroscopic current as a result of TLE. (G) Steady-state inactivation parameters were determined from a holding potential of -100 mV using conditioning pulses of 1 sec at voltages ranging from -115 mV to -10 mV. A test pulse of $+10$ mV was then used to assess channel availability (control: $n=9$, TLE: $n=12$). Smooth lines correspond to the average of least squares fits when data were fitted with a single Boltzmann equation. Insets show representative steady state inactivation traces at -100 mV and -60 mV pre-pulse voltage steps for comparison purposes. (H) Recovery from inactivation was assessed at -90 mV using a two-pulse protocol. A pre-pulse from -100 mV to 0 mV was applied for 1 sec to inactivate Na channels. Cells were then held at -90 mV for variable lengths of time (1 ms – 15 s) to allow for channels to recover. TLE neurons showed significantly faster rates of recovery from inactivation compared to control. Insets show a representative trace recorded at a recovery interval of 100 ms superimposed with one recorded at 10 s. Smooth lines correspond to the fits using a double exponential function. All data points represent means $\pm$ S.E.M. (I) DIC image of an isolated non-stellate neuron. Horizontal scale bar represents $10 \mu\text{m}$.

Figure 6. mEC layer II stellate macroscopic Na channel currents are larger in TLE neurons
 Representative traces of families of Na channel currents recorded from (A) control and (B) TLE stellate neurons. Currents were elicited by depolarizing steps of 25 ms from a holding potential of -100 mV to test pulses in the range of -80 to $+20$ mV in 5 mV increments. (C) Conductance plot shows no difference in activation gating parameters between control and TLE. Smooth lines correspond to the least squares fit when average conductance data were fit with a single Boltzmann equation (control: $n=14$; TLE: $n=14$). Gating parameters are listed in table 1. (D) Mean Na channel conductance was increased in TLE ($P<0.01$). (E) Decays of the macroscopic current were fit to a double exponential function and revealed no difference in decay time constants for the fast time constant (τ_1) between TLE or control mEC stellate neurons ($n=9$ for TLE and $n=10$ for control). (F) DIC image of an isolated mEC non-stellate neuron. Scale bar represents $10\ \mu\text{m}$. (G) Steady-state inactivation parameters were determined from a holding potential of -100 mV using conditioning pulses of 1 sec at voltages ranging from -115 mV to -10 mV. A test pulse of $+10$ mV was then used to assess channel availability. Smooth lines correspond to the average of least squares fits when data were fitted with a single Boltzmann equation. Insets show representative steady state inactivation traces at -100 mV and -60 mV voltage step for comparison purposes. (control: $n=11$, TLE: $n=7$). (H) Recovery from inactivation was assessed at -90 mV using a two-pulse protocol. A pre-pulse from -100 mV to 0 mV was applied for 1 sec. Cells were then held at -90 mV for variable lengths of time ($1\ \text{ms} - 15\ \text{s}$) to allow for channels to recover. Rates of recovery from inactivation were not different between TLE and control mEC stellate neurons, although means suggested a slowing trend in recovery rates for TLE neurons (control: $n=6$, TLE: $n=9$). Inset shows a representative trace recorded at a recovery interval of $100\ \text{ms}$ superimposed with one recorded at $10\ \text{s}$. Smooth lines correspond to the fits using a double exponential function. Data points represent means $\pm$ S.E.M.

Figure 7. Persistent Na channel currents (I_{NaP}) are increased in both TLE mEC non-stellate and stellate neurons

Voltage ramps were applied at a rate of 65 mV/s (inset to A.), using recording solutions designed to reduce other types of inward and outward currents. (A) I_{NaP} currents recorded from control mEC non-stellate neurons (depicted as black traces) were abolished in the presence of TTX (1 μ M; depicted as gray traces). Resulting TTX subtracted traces are shown below in each case. (B) I_{NaP} currents were significantly larger in amplitude in TLE mEC non-stellate neurons. (C) Conductance plots for non-stellate neurons revealed a hyperpolarizing shift in the $V_{1/2}$ value in TLE mEC non-stellate neurons. In each case the control conductance curve is depicted in black with the TLE curve depicted in gray. (D) Averaged I_{NaP} amplitudes for control ($n = 9$) and TLE ($n = 8$) mEC non-stellate neurons show a 2.5 fold increase in I_{NaP} in TLE ($P < 0.001$). I_{NaP} currents were also increased in TLE mEC stellate neurons (F) when compared with control stellate neurons (E). I_{NaP} currents are shown as black traces with current traces recorded in the presence of TTX (1 μ M) shown as gray traces. TTX subtracted I_{NaP} currents were larger in amplitude in TLE stellate neurons compared to control stellate neurons (E and F, lower traces). (G) Conductance plots for control (black trace) and TLE (gray trace) mEC stellate neuron shows no significant change in conductance curves between the two cell types. (H) Averaged peak amplitudes of I_{NaP} recorded from control ($n = 9$) and TLE neurons ($n = 8$) showing more than a 2 fold increase in I_{NaP} in TLE ($P < 0.01$). Data represent means $\pm$ S.E.M.

Figure 8. Analysis of the resurgent Na current (I_{NaR}) in mEC non-stellate and stellate neurons
 In recording solutions designed to reduce other types of inward and outward currents, I_{NaR} currents were recorded from control (A,D) and TLE mEC non-stellate (B,E) neurons using the voltage protocol shown. Traces shown were obtained after subtraction of recordings obtained in the presence of TTX (1 μ M). (C) Averaged I_{NaR} conductances for mEC non-stellate neurons show an almost 2 fold increase in I_{NaR} in TLE neurons ($n=7$; $P < 0.05$) compared with control ($n=6$). Conductance plots for I_{NaR} are shown in F and reveal a hyperpolarized shift in the $V_{1/2}$ of I_{NaR} activation in TLE ($n=8$) compared with control ($n=7$). Analysis of the resurgent Na current (I_{NaR}) in mEC stellate control neurons is shown in G–L. Peak I_{NaR} currents were also increased in TLE mEC stellate neurons (H) compared with control (G). Traces shown were obtained after subtraction of recordings obtained in the presence of TTX (1 μ M). (C) Averaged I_{NaR} conductances for control ($n = 12$) and TLE neurons ($n = 8$) mEC stellate neurons show a 2 fold increase in I_{NaR} in TLE neurons ($P < 0.05$). Conductance plots for I_{NaR} are shown in L and indicate no alterations in I_{NaR} activation gating parameters between control ($n=10$) and TLE mEC stellate neurons ($n=6$). Values represent means $\pm$ S.E.M.

Figure 9. Altered Na channel expression in TLE mEC layer II neurons

Na_v1.6 expression in the axon initial segment (AIS) of control (A) and TLE (B) tissue showing an increased staining in TLE tissue ($P < 0.01$). Green staining for the Na_v1.6 isoform is seen in left panels and red staining for the AIS marker Ankyrin G is seen in the right panels. Merge of the two is seen in the middle panels. (C) Bar charts showing an increased normalized relative optical density (R.O.D.) for Na_v1.6 isoform staining in TLE tissue ($n=18$) as compared to control tissue ($n=20$) while Ankyrin G levels remain constant. (D) Expression of the Na_v1.2 isoform was restricted to the soma for both control (left panel) and TLE (right panel) neurons. Somatic staining of the Na_v1.2 isoform was increased in TLE slices ($n=10$) compared to control slices ($n=10$). Expression patterns for Na_v1.1 and Na_v1.3 were also determined and revealed somatic staining. Staining intensities for Na_v1.1 ($n=8$ control; $n=7$ TLE) or Na_v1.3 ($n=8$ control; $n=6$ TLE) were not different in TLE. (E) Bar charts showing the normalized R.O.D. for the Na_v1.1, 1.2, and 1.3 isoforms in mEC layer II from control and TLE slices. Data represent means $\pm$ S.E.M. Scale bars represent 10 μ m.

TABLE 1

Membrane properties of mEC non-stellate and stellate neurons.

	RMP (mV)	R _i (MΩ)	Threshold (mV)	Width (ms)	Amplitude (mV)	Upstroke (mV/ms)	fAHP (mV)	DAP (mV)	mAHP (mV)	n
Control Non-stellate	-61.5 ± 0.9	49.3 ± 3.0	-41.9 ± 0.6	0.84 ± 0.04	79.4 ± 2.4	255.7 ± 13	7.9 ± 0.5	0.3 ± 0.1	11.9 ± 0.6	14
TLE Non-stellate	-59.3 ± 1.0	66.8 ± 4.4 [‡]	-45.2 ± 0.9 [*]	0.85 ± 0.04	86.6 ± 2.8	302.8 ± 17 [‡]	9.9 ± 0.7 [‡]	0.7 ± 0.3	12.9 ± 1.0	13
Control stellate	-60.2 ± 1.1	53.5 ± 3.1	-42.9 ± 0.5	0.79 ± 0.03	81.7 ± 2.1	248.9 ± 11	6.1 ± 0.6	2.2 ± 0.3	10.9 ± 0.5	18
TLE stellate	-59.8 ± 1.1	62.9 ± 5.4	-46.9 ± 1.6 [‡]	0.81 ± 0.03	95.5 ± 4.5 ^{\$}	303.4 ± 22 [‡]	8.7 ± 0.6 [‡]	2.8 ± 0.3	10.2 ± 0.4	11

RMP; Resting membrane potential. R_i; input resistance. fAHP; fast after-hyperpolarizing potential. DAP; depolarizing after-potential. mAHP; medium after-hyperpolarizing potential.

^{\$}P < 0.01,

[‡]P < 0.05,

^{*} P < 0.001, one way analysis of variance using Tukey's *post hoc* test when tested against control neurons.

TABLE 2

Steady state activation and inactivation parameters.

	Activation			Inactivation		
	$V_{1/2}$ (mV)	k (mV)	n	$V_{1/2}$ (mV)	k (mV)	n
Control Non-stellate	-31.1 ± 1.8	-5.9 ± 0.3	17	-70.4 ± 0.8	6.2 ± 0.2	9
TLE Non-stellate	-29.4 ± 1.1	-6.1 ± 0.3	18	-62.1 ± 1.1^a	7.1 ± 0.3	12
Control stellate	-31.2 ± 1.6	-6.6 ± 0.3	14	-69.0 ± 1.4	6.9 ± 0.5	11
TLE stellate	-35.0 ± 1.7	-6.6 ± 0.4	14	-60.5 ± 1.2^b	6.6 ± 0.3	7

^aP < 0.05 vs control, Mann-Whitney Rank Sum Test;

^bP < 0.05 vs control, Kruskal-Wallis One Way Analysis of Variance on Ranks when tested against control neurons.