Evidence for a Role of Na\textsubscript{v}1.6 in Facilitating Increases in Neuronal Hyper-excitability During Epileptogenesis.

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Abstract

During epileptogenesis a series of molecular and cellular events occur culminating in an increase in neuronal excitability, leading to seizure initiation. The entorhinal cortex (EC) has been implicated in the generation of epileptic seizures in both humans and animal models of temporal lobe epilepsy (TLE). This hyperexcitability is due, in part, to pro-excitatory changes in ion channel activity. Sodium channels play an important role in controlling neuronal excitability, and alterations in their activity could facilitate seizure initiation. We sought to investigate whether medial EC (mEC) layer II neurons become hyperexcitable and display pro-excitatory behavior of Na channels during epileptogenesis. Experiments were conducted 7 days after electrical induction of status epilepticus (SE), a time point during the latent period of epileptogenesis and before the onset of seizures. mEC layer II stellate neurons from post SE animals were hyperexcitable, eliciting action potentials (AP) at higher frequencies compared to control neurons. Na channel currents recorded from post SE neurons revealed increases in Na current amplitudes, particularly persistent (INaP) and resurgent (INaR) currents as well as depolarized shifts in inactivation parameters. Immunocytochemical studies revealed increases in Nav1.6 isoform levels. The toxin 4,9-anhydro tetrodotoxin (4,9-ahTTX), which has greater selectivity for Nav1.6 over other Na channel isoforms, suppressed neuronal hyperexcitability, reduced macroscopic Na currents, INaP and INaR current densities and abolished depolarized shifts in inactivation parameters in post SE neurons.

These studies support a potential role for Na,1.6 in facilitating the hyperexcitability of mEC layer II neurons during epileptogenesis.

Keywords: Sodium channels, Status Epilepticus, Entorhinal cortex
**Introduction**

After an epileptogenic insult, epilepsy can develop after a variable latent period, a process often referred to as epileptogenesis. During this period many cellular changes occur, culminating in neuronal hyperexcitability and eventually, seizure activity (Pitkanen and Lukasiuk 2009). The identification and targeting of key cellular events involved in facilitating neuronal hyperexcitability during epileptogenesis could influence disease progression (Blumenfeld et al. 2008).

The entorhinal cortex (EC), which is located within the temporal lobe and provides the main input into the hippocampus (Burwell 2000), is an important site for cellular alterations during epileptogenesis. Patients with epilepsy exhibit spontaneous ictal activity within the EC (Spencer and Spencer 1994; Bartolomei et al. 2005) and have decreased EC volume (Jutila et al. 2001), attributed to a loss of EC layer III neurons (Du et al. 1993). In contrast, EC layer II neurons are spared, and become hyperexcitable, (Bear et al. 1996; Hargus et al. 2011) leading to an excessive excitatory input into the hippocampus (Kobayashi et al. 2003). Increased activity of synaptic networks (Kumar and Buckmaster 2006; Ang et al. 2006; Kumar et al. 2007) and altered functionality of ion channels have been proposed as mechanisms for this hyperexcitability (Hargus et al. 2011).

Na channels play a critical role in controlling neuronal excitability as they are intricately involved in action potential (AP) generation, conduction and the transition between single spiking and bursting in some neurons (Cooper et al. 2005). Pro-excitatory changes in Na channel function have been reported in patients with epilepsy (Whitaker et al. 2001; Vreugdenhil et al. 2004) and in animal models of epilepsy (Aronica et al. 2001; Klein et al. 2004; Blumenfeld et al. 2009; Ketelaars et al. 2001b). Of the multiple Na channel isoforms expressed within the brain (Kress and Mennerick 2008; Candenas et al. 2006), the Na$_v$1.6 isoform has received much attention since it is highly expressed along the axonal initial segment (AIS(Hu et al. 2009), the site of AP generation. Nav1.6 expression is increased in kindling animals (Blumenfeld et al. 2009) and animals with TLE (Hargus et al. 2011). Reducing Na$_v$1.6 levels impairs the initiation and development of kindling (Blumenfeld et al. 2009), inhibits
spontaneous firing and reduces firing frequencies in many neurons (Raman et al. 1997; Royeck et al. 2008; Van Wart and Matthews 2006). Furthermore, a mutation in SCN8A, the gene that encodes Nav1.6, has been associated with infantile epileptic encephalopathy (Veeramah et al. 2012).

In this study we determined whether membrane hyperexcitability and alterations in Na channel activity in mEC layer II stellate neurons appeared before the onset of spontaneous seizure. We determined the role of Nav1.6 by using the toxin 4,9-anhydro-tetrodotoxin (4,9-ah-TTX), which has a greater potency for Nav1.6 over other Na channel isoforms (Rosker et al. 2007). These neurons are hyperexcitable by 7 days post SE. Inhibition of Nav1.6 suppressed neuronal hyperexcitability and reversed the pro-excitatory alterations in Na channel activity. Nav1.6 could be important contributor to the development of epilepsy as the changes occur before the onset of spontaneous seizures.
**Materials and Methods**

*Induction of status epilepticus:* 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 Institute of Animal Care and Use Committee. Adult male Sprague-Dawley rats (250-300 gram) received a bipolar twisted pair of stainless steel electrodes in the posterior ventral hippocampus (coordinates from bregma AP ~-5.3 mm, ML~ 4.9 mm, DV~ 5.0 mm, bite at~ -3.5 mm) (Paxinos and Watson 1996). Electrodes were attached to Amphenol connectors and secured to the skull with jeweler screws and dental acrylic. One week following surgery, rats were stimulated through the hippocampal electrode to induce limbic *status epilepticus* (*SE*) using a protocol previously described (Lothman et al. 1989; Hargus et al. 2011). Animals were stimulated for 90 minutes with 10 second trains of 50 Hz, 1 ms biphasic square waves with a maximum intensity of 400 µA 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. Only animals that exhibited continuous electrographic seizure activity for at least 8 hours after stimulation were maintained because they were at uniform risk for development of limbic epilepsy. Animals were then monitored to ensure absence of seizure activity once the *SE* had stopped. Seven days after the induction of *SE*, animals were used for experimental evaluation. Animals had free access to food and water, as well as a standard 12 hour light-dark cycle during this time.

**Entorhinal Cortex Slices:** Methods for preparing brain slices have been previously described (Hargus et al. 2011). In brief, horizontal brain slices (300 µm) were prepared from either control Sprague-Dawley rats (250 - 350 grams) or rats 7 days post *SE*. All 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 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 0.5 L-Ascorbic
acid, 10 glucose, 25 NaHCO₃, and 2 Pyruvate (oxygenated with 95% O₂ and 5% CO₂). 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 until required. 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. For brain slice experiments mEC layer II stellate 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). 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). For membrane excitability experiments electrodes had resistances of 3.5 - 4.0 MΩ 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). APs 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 (ΔV/ΔI) 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 ≥ 20 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 ± 10 mV from the median value of the AP amplitude. In some experiments APs were evoked synaptically using a bipolar platinum
iridium stimulating electrode (WPI, Sarasota, FL, USA) placed in layer VI of the mEC. 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). To consistently evoke APs the stimulus amplitude was increased 1.5 times from threshold. Duration of the evoked potential 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 potential 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.

**Neuron Na Channel Electrophysiology:** All Na channel current recordings, except persistent Na currents (INaP) and resurgent Na currents (INaR) 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 MΩ 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): 20 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 when recording steady-state inactivation parameters. The P/N protocol applies a series of negative pulses to calculate the leak currents. P/N leak subtraction will remove the leak current from the response to a voltage step, leaving only the voltage-activated current.

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:

\[
y = \frac{1}{1 + \exp \left(\frac{(V-V_{1/2})}{k}\right)}
\]

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 \( \tau_1 \) 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 \( \tau_1 \) and \( \tau_2 \) 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 0 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 (\( I_{\text{NaP}} \)) were determined in brain slice preparations using voltage ramps from -100 mV to -10 mV at a rate of 65 mV/s. \( I_{\text{NaP}} \) was recorded in bath solution containing (in mM): 30 NaCl, 120 TEA-Cl, 10 NaHCO\(_3\), 1.6 CaCl\(_2\), 2 MgCl\(_2\), 0.2 CdCl\(_2\), and 5 4-AP (pH 7.4 when oxygenated with 95% O\(_2\) and 5% CO\(_2\); osmolarity adjusted to 310 mosM with sucrose; temperature 32°C) and a pipette solution containing (in mM): 140 CsF, 2 MgCl\(_2\), 1 EGTA, 10 HEPES, 4 Na\(_2\)ATP, 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 \( I_{\text{NaP}} \). To determine the peak \( I_{\text{NaP}} \) current, voltage ramp protocols were repeated in the presence of TTX (1 µM) applied focally to the soma region using of a puffing system consisting of a large diameter (~10 µm) glass microelectrode connected to an air-filled syringe. Traces obtained in the presence of TTX were subtracted from those obtained in its absence.
Resurgent Na currents ($I_{\text{NaR}}$) were also recorded in the brain slice preparation using a bath solution containing (in mM): 100 NaCl, 26 NaHCO$_3$, 19.5 TEA-Cl, 3 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 0.1 CdCl$_2$, 4 4-AP, and 10 glucose (pH 7.4 when oxygenated with 95% O$_2$ and 5% CO$_2$; osmolarity adjusted to 310 mosM with sucrose) using the same pipette solution as that for recording $I_{\text{NaP}}$. 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 $I_{\text{NaR}}$, or by using a series of repolarizing steps from -100 mV to -10 mV to determine the voltage dependance of $I_{\text{NaR}}$. Protocols were again repeated in the presence of TTX (1 µM) to determine $I_{\text{NaP}}$ current amplitudes and gating.

**Cell line sodium channel electrophysiology.** Human embryonic kidney cells (HEK) cells stably expressing either human Na$_v$1.2 or rat Na$_v$1.6 Na channel isoforms were grown in DMEM Glutax media (Invitrogen) supplemented with 10% fetal bovine serum, 1X NEAA, HEPES (10 mM) and G418 (500 µg/ml; Sigma, MO, USA). Cells were grown in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C. Na currents were recorded using the whole-cell configuration of the patch clamp recording technique with 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 and low pass filtered (2 kHz) and sampled at 33 kHz. Borosilicate glass pipettes were pulled using a Brown-Flaming puller (model P87, Sutter Instruments Co, Novato, CA) and heat polished to produce electrode resistances of 0.5-1.5 MΩ when filled with the following electrode solution (in mM): CsCl 130, NaCl 10, MgCl$_2$ 1, MgATP 5, BAPTA 10, HEPES 5 (pH adjusted to 7.4 with CsOH). Cells were plated on glass coverslips and superfused with solution containing the following composition; (in mM) NaCl 130, KCl 4, CaCl$_2$ 1, MgCl$_2$ 5, HEPES 5, and glucose 5 (pH adjusted to 7.4 with NaOH). All experiments were performed at room temperature (20-22 °C). After establishing whole-cell, a minimum series resistance compensation of 75% was applied. Na currents were elicited by a depolarizing step from a holding potential of -100 mV to +10 mV for a duration of 25
ms at 15 s intervals. 4,9 ah-TTX was applied at various test concentrations (10 nM to 3 μM) after a 3 min control period and continued until a steady state current amplitude was observed. All data represent percentage mean block ± standard error of the mean (S.E.M.).

**Immunohistochemistry Experiments:** Brains from three 7 days post SE rats and three age-matched control rats were removed and snap frozen on dry ice. Horizontal cryostat sections (8 μm) 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 with PBS, treated with 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen) nuclei stain for 5 minutes, washed for a final time with PBS and coverslipped using Vectorshield (Vector laboratories). 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). Confocal images were captured of mEC layer II using a Zeiss LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) with a 60 X 1.3 NA oil immersion objective and Zeiss LSM Imaging software. For comparison purposes, all immunocitochemistry 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 post SE preparations and allowed for standardization of the
immunolabelling as a ratio of Na\textsubscript{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-
aminopyradine (4-AP), and tetrodotoxin (TTX) were obtained from Sigma Aldrich (St. Louis, MO,
USA). 4,9-anhydro TTX was obtained from Focus Biomolecules (Plymouth, PA, USA). NBQX, AP5,
strychnine, picrotoxin, TTX and 4,9 anhydro TTX were prepared as 1000 x stock solutions. All were
prepared in DMSO except for TTX, which was prepared in water and 4,9-anhydro TTX which was
prepared in methanol. 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 ± standard error
of the mean (S.E.M). Statistical significance was determined by using 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).
Results

Membrane Properties:

Membrane property recordings from visually identified mEC layer II stellate neurons revealed increased AP discharge rates (figure 1A). APs were elicited by a series of depolarizing current injection steps. When compared at a current injection step of 470 pA, AP firing was increased from 22.2 ± 0.6 Hz (n=32) in control neurons to 29.0 ± 0.7 Hz (n=27; P<0.001: figure 1A, B) in neurons from post SE animals. Analysis of AP properties revealed depolarized resting membrane potentials (RMP), increased input resistances (Ri), AP thresholds, AP widths, AP amplitudes and faster upstroke velocities in neurons 7 days post SE (table 1). Differences in firing rates between control and post SE neurons persisted in the presence of fast synaptic blockers (APV 30 µM, NBQX 10 µM, picrotoxin 50 µM and strychnine 50 µM). At a current injection step of 470 pA, mEC stellate neuron firing frequency in post SE neurons continued to be higher (25.5 ± 1.4 Hz; n = 6; P<0.001) compared to control neurons (12.0 ± 1.5 Hz; n = 12). Firing frequencies were fully reversible upon washout of all synaptic antagonists (control: 22.5 ± 0.8 Hz; n = 4; post SE: 30.1 ± 1.4 Hz; n = 4).

Brief stimulation within the deep layers of the mEC consistently evoked a single AP spike in control mEC layer II stellate neurons (n=11; figure 1C). In contrast, stimulation evoked multiple APs in post SE neurons (2.7 ± 0.2 APs; n=9). Analysis of the evoked response revealed an increased duration of the depolarizing event in post SE neurons (108.5 ± 14.2 ms; n=9: P<0.001) compared with control (47.0 ± 7.7 ms; n=11). The maximum amplitude of the somatic depolarization was also increased in post SE neurons (13.6 ± 1.5 mV; n=11) compared with control (9.5 ± 0.9 mV; n=9), but did not reach significance. Focal application of TTX (500 nM) to the axon initial segment (AIS) region not only abolished the evoked APs, but also reduced the amplitude and duration of the depolarizing event in both control (n = 3) and post SE neurons (n=3). These findings support an important role for Na channels in the generation of the somatic spike ADP in mEC layer II neurons as has been shown for hippocampal CA1 neurons (Yue et al. 2005). Temporal lobe epilepsy is associated with a
decreased volume of the EC due to the loss of layer III neurons (Jutila et al. 2001; Bartolomei et al. 2005; Du et al. 1993). In our electrical stimulation model, a loss of mEC layer III neurons was observed 7 days post SE (post SE), a time point before the onset of spontaneous seizures (figure 1D, E).

**Na channel gating parameters are altered before the onset of spontaneous seizures.**

Na channels play an important role in controlling neuronal excitability and altered Na channel activity could facilitate the increases in firing rates observed in post SE neurons. Na channel currents were recorded from dissociated mEC layer II stellate neurons. Stellate neurons were identified as having polygonal shaped cell bodies with multiple dendrites (Hargus et al. 2011). Cell size was determined using capacitance values and were not different between control (13.4 ± 1.2 pF; n = 29) and post SE neurons (14.0 ± 0.8 pF; n = 26). When normalized to cell capacitance Na current densities were significantly increased in post SE neurons compared with control (control: 191.6 ± 18.4 pA/pF, n=10 and post SE 310.4 ± 35.1 pA/pF, n=25; P<0.01; figure. 2A & B). Decays of the macroscopic currents were fitted to double exponential functions and were not different. Analysis of current voltage curves revealed no change in half maximal activation values (\(V_{1/2}\)) or slope (k) values between the two conditions (figure 2C; table 2). In contrast to activation gating parameters, inactivation curves were shifted in the depolarizing direction for post SE neurons when compared to control neurons and were best fit to a double Boltzmann function (table 2 and figure 2D & E). The second component of the Boltzmann function accounted for approximately 14.3% of the total current and represented persistent Na channel activity. Typical steady state inactivation current traces are shown in figure 2E and illustrate a delay in the inactivation of Na channels in post SE neurons compared with control neurons, indicative of greater Na channel availability. Recovery from inactivation kinetic rates were determined at a test potential of -90 mV and were not different between
control and post SE neurons (figure 2F & G). Time constants for control were 11.4 ± 1.9 ms (n = 9) and 8.8 ± 0.8 ms (n = 9) for post SE neurons.

Synaptic stimulation evoked AP bursts in post SE neurons. Two types of Na currents, persistent (INaP) and resurgent (INaR) Na currents play important roles in generating and facilitating AP bursts and are increased in patients with TLE and in animal models of TLE (Vreugdenhil et al. 2004; Hargus et al. 2011). To determine if INaP and INaR currents were accentuated during the latency period of epileptogenesis, and before the onset of spontaneous seizures, both currents were recorded using brain slice preparations from post SE and control animals (figure 3 A-I). INaP current amplitudes in control neurons were -153.5 ± 18.9 pA (n = 9) and were significantly increased by 51 % to -232.4 ± 6.3 pA (n = 10: P < 0.001) in post SE neurons (figure 3D). Analysis of the INaP conductance revealed no difference in the voltage dependence of the currents. In control neurons V1/2 values were -47.2 ± 3.1 mV (n = 9) and were shifted to -51.6 ± 2.0 mV (n = 10; figure 3E) post SE. Slope of curves were not different between post SE and control neurons (k= -5.1 ± 0.5 mV and -6.3 ± 0.6 mV; respectively).

INaR current amplitudes in control neurons were -688.1 ± 70.3 pA (n = 20) and were also increased in post SE neurons by 50 % to -1032.5 ± 42.1 pA (n = 31: P < 0.001; figure. 3F-H). INaR currents evoked over a series of voltages were used to construct a conductance voltage plot and revealed a small shift in INaR V1/2 values in the depolarized direction for post SE neurons when compared with control (-63.3 ± 1.7 mV; n=12 in control compared with -59.5 ± 1.6 mV; n=11 in post SE neurons; figure 3I). Slope values were also not altered (-4.5 ± 0.4 mV in control neurons compared with -5.3 ± 0.7 mV in post SE neurons).

Nav1.6 is up-regulated before the onset of spontaneous seizures.

Both INaP and INaR currents are thought to arise mainly from activation of the Nav1.6 Na channel isoform (Raman et al. 1997) and increases in Nav1.6 expression levels have been detected in kindled animals (Blumenfeld et al. 2009) and animals experiencing spontaneous seizures (Hargus et al. 2011).
We determined if increases in Na\(_{\text{v}1.6}\) expression levels were initiated during epileptogenesis and before the appearance of spontaneous seizures. Na\(_{\text{v}1.6}\) is thought to be highly expressed along the AIS (Royeck et al. 2008). In agreement, we found robust expression of Na\(_{\text{v}1.6}\) along the AIS in mEC layer II control neurons. Na\(_{\text{v}1.6}\) labeling was co-localized with Ankyrin G labeling, a specific marker for the AIS (figure 4A). Similar expression patterns for Na\(_{\text{v}1.6}\) were also observed in post SE neurons except that intensity of the fluorescent signal was increased (figure 4A & B). Staining intensities were normalized to Ankyrin G for comparison since Ankyrin G expression was not different (\(P = 0.7\) ANOVA) between control (R.O.D. = 111.6 ± 5.4; \(n = 37\)) and post SE slices (105.3 ± 5.5; \(n = 28\)). Fluorescent ratio’s were increased 46 % from 0.49 ± 0.04 (\(n = 37\)) in control to 0.73 ± 0.04 (\(n = 28\)) in post SE slices (\(P<0.001\)). Somatic staining for Na\(_{\text{v}1.6}\) was low in intensity compared to the AIS, but was increased 2 fold compared with control (\(P<0.05\)). In contrast to Na\(_{\text{v}1.6}\) staining, Na\(_{\text{v}1.2}\) staining was not localized with the AIS (data not shown), but was apparent within the somatic regions of the neurons (figure 4C). Normalized Na\(_{\text{v}1.2}\) staining was increased by 39 % in post SE neurons (\(n = 31\) from 3 animals; \(P<0.05\)) compared to control (\(n = 32\) from 3 animals). Low immunolabeling for Na\(_{\text{v}1.1}\) (\(n = 28\) control and \(n = 30\) post SE from 3 animals each) and Na\(_{\text{v}1.3}\) (\(n = 28\) in control and \(n = 30\) in post SE) was only observed within the somatic regions of control brain slices and remained unchanged in post SE slices (figure. 4D).

**Na\(_{\text{v}1.6}\) facilitates increases in neuronal hyperexcitability and alterations in Na channel density and gating.**

The Na\(_{\text{v}1.6}\) isoform plays a critical role in initiating the AP and setting the spike threshold (Royeck et al. 2008). To determine if alterations in Na\(_{\text{v}1.6}\) activity during epileptogenesis could facilitate the increases in neuronal activity and contribute to the increases in Na channel density and depolarized shifts in inactivation parameters, we used the tetrodotoxin (TTX) metabolite 4,9-ahydro-TTX (4,9 ah-TTX), a toxin that has been shown to have greater selectivity for the Na\(_{\text{v}1.6}\) isoform over Na\(_{\text{v}1.2}\)-
Nav1.8 Na channel isoforms (Rosker et al. 2007). To determine if 4,9-ah-TTX remained selective for Na\textsubscript{v}1.6 when tested in mammalian expression systems we evaluated the toxin against the Na channel isoforms Na\textsubscript{v}1.6 and Na\textsubscript{v}1.2 stably expressed in HEK 293 cells (figure 5). Both these Na channel isoforms are abundantly expressed in the brain (Trimmer and Rhodes 2004). Dose response curves revealed a five-fold greater potency of 4,9 ah-TTX against Na\textsubscript{v}1.6 (IC\textsubscript{50} = 122 nM; n = 4 - 5 for each concentration) compared to Na\textsubscript{v}1.2 (IC\textsubscript{50} = 659 nM; n= 4 - 5). At 100 nM, 4,9 ah-TTX inhibited Na\textsubscript{v}1.6 currents by 50.3 ± 1.9 % (n=4), but had no effect on Na\textsubscript{v}1.2 currents (figure 5B & C). In view of these findings the effects of 4,9 ah-TTX at a concentration of 100 nM on membrane properties and Na channel currents were interpreted as being primarily mediated by inhibition of Na\textsubscript{v}1.6. At a higher concentration of 300 nM a small inhibition of Na\textsubscript{v}1.2 currents was observed (by 16.3 ± 2.2 %; n=4). In comparison, Na\textsubscript{v}1.6 currents were inhibited by 70.1 ± 1.2 % (n=4).

When tested on macroscopic Na currents recorded from isolated mEC layer II stellate neurons, 100 nM 4,9 ah-TTX caused a significantly greater block of Na currents in post SE neurons (by 52.4 ± 2.1 %; n=6: P<0.05) compared to control neurons (28.6 ± 4.3 %; n=5: figure 5D & E). At 300 nM, Na current amplitudes were inhibited by similar amounts for both conditions (72.3 ± 2.9 %; n=5 for post SE and 73.1 ± 7.5 %; n=3 for control). These data suggest that there is an increased contribution of Na\textsubscript{v}1.6 channels to the total macroscopic in post SE neurons. However, since 100 nM 4,9 ah-TTX blocked 52.4 % of the macroscopic current in post SE neurons and that these neurons also express Na\textsubscript{v}1.2 channels it is possible that the dose response curve for 4,9 ah-TTX in neurons differs slightly from that recorded in mammalian cell lines.

Epileptogenic injury caused depolarizing shifts in steady-state inactivation that were best fit to double Boltzmann functions (figure 2D). Application of 100 nM 4,9 ah-TTX reversed the shifts in inactivation parameters, shifting V\textsubscript{1/2} values to greater hyperpolarized values (figure 5F & G; table 2). Curves were best fit with a single Boltzmann function. Steady-state inactivation curves were also
shifted in a hyperpolarized direction in control neurons to levels that were indistinguishable from those recorded from post SE neurons.

The Nav1.6 isoform is considered an important contributor to INaR and INaP currents (Raman et al. 1997). Both INaR and INaP currents were larger in amplitude in post SE neurons compared with control (figure 3). Consistent with results obtained on the Nav1.6 isoform (figure 5C), both INaR and INaP currents recorded from either control or post SE neurons were inhibited by 4,9-ah-TTX. At a concentration of 100 nM, 4,9 ah-TTX significantly reduced INaR currents by 43.0 ± 4.8 % (n = 4: P<0.01) in control and by 48.6 ± 7.8 % (n = 8; P<0.01) in post SE neurons (figure 6A & C). At a higher concentration of 300 nM, INaR currents were reduced by 63.6 ± 0.7 % (n = 4; P<0.01) and 79.2 ± 2.9 % (n = 5; P<0.01) in control and post SE neurons respectively.

In a similar manner, INaP currents were also inhibited by 4,9-ah-TTX in both control and post SE neurons (figure 6B & D). At 100 nM 4,9-ah-TTX significantly reduced INaP currents by 50.7 ± 4.8% (n=5) and 58.3 ± 8.7% (n=4) respectively. At a higher concentration of 300 nM, INaP currents were reduced by 72.7 ± 2.7 % (n=5) in control neurons and 84.0 ± 5.4 % (n=4) in post SE neurons. These results further support the hypothesis that INaP and INaR currents are largely mediated by the Nav1.6 isoform. These findings support the hypothesis that increases in INaP and INaR current amplitudes during epileptogenesis are due to increases in the expression levels and/or activity levels of Nav1.6.

Nav1.6 is considered important in controlling neuronal excitability. We determined the effects of inhibiting Nav1.6 on neuronal excitability (figure 7). At 100 nM 4,9 ah-TTX, firing frequencies in post SE neurons were reduced by 38 % (n = 9) at a current injection step of 470 pA to frequencies below that observed in control neurons (from 29.1 ± 0.8 Hz to 18.1 ± 0.9 Hz; P<0.001). In control neurons 4,9 ah-TTX caused a smaller 25 % (n = 8) reduction in AP firing frequencies (from 23.7 ± 1.3 Hz to 17.9 ± 1.7 Hz; P<0.01). At this concentration firing frequencies between control and post SE neurons were the same (figure 7C). Application of 300 nM 4,9 ah-TTX caused a further reduction in AP firing rates in post SE neurons (to 12.5 ± 1.6 Hz; n = 4: P<0.001), and control neurons (to 14.7 ± 3.3 Hz; n
Washout was partial after over 30 mins of washout (23.9 ± 1.3 Hz; n = 5 for control and 23.7 ± 6.6 Hz; n = 7 for post SE neurons). The effects of 4,9 ah-TTX on post SE neurons were more pronounced than in control, particular at the lower concentration of 100 nM, with greater reductions in conduction velocity, threshold potential and decreases in AP amplitudes (table 1). 4,9 ah-TTX (100 nM) also hyperpolarized the RMP and decreased AP widths in post SE neurons. In the presence of 4,9 ah-TTX (100 nM) significant differences in RMP and AP amplitude recorded between control and post SE neurons were abolished. These findings suggest a greater contribution of Na\textsubscript{v}1.6 to the morphology of the AP in post SE neurons.

Synaptically evoked APs were also abolished by 100 nM 4,9-ah-TTX in 8 out of 11 control neurons tested (figure 7E). In post SE neurons synaptically evoked AP bursts were abolished by 100 nM 4,9-ah-TTX in 3 out of 6 neurons tested and reduced to single APs in the remaining 3 neurons (figure 7F). These findings support the hypothesis that increases in Na\textsubscript{v}1.6 channel currents facilitate neuronal hyperexcitability of layer II mEC stellate neurons during epileptogenesis.
Discussion

The principal findings of this study are that 1) increased excitability of mEC layer II stellate neurons develops before the appearance of spontaneous seizures, 2) pro-excitatory changes in Na channel activity are evident at this early time point, 3) inhibition of Na\(_{\alpha}1.6\) channel currents can reverse these pro-excitatory alterations in Na channel activity and inhibit neuronal hyperexcitability. These findings suggest a potential role for the Na\(_{\alpha}1.6\) isoform, in part, in facilitating increases in neuronal excitability of mEC layer II neurons during epileptogenesis.

Hyperexcitability of mEC layer II stellate neurons preceeds the onset of epileptic seizures.

In animals models of spontaneous seizures, mEC layer II stellate neurons become hyperexcitable leading to an excessive, synchronized excitatory output into the dentate gyrus (Buckmaster and Dudek 1997; Kobayashi et al. 2003; Hargus et al. 2011). Our previous studies demonstrated that 3 months post SE, when the animals were experiencing spontaneous limbic seizures, increases in neuronal excitability and pro-excitatory alterations in Na channel activity were apparent (Hargus et al. 2011). Since seizure activity itself can induce neuronal changes it is unclear if these alterations in Na channel physiology observed in chronically epileptic animals contributed to the development of epilepsy, or were merely a consequence of the seizures. In the present study we found that increases in neuronal excitability and pro-excitatory changes in Na channel activity were present 7 days post SE, a time point within the latency phase of epileptogenesis, before the onset of spontaneous seizures. This observation suggests that increased activity of mEC layer II stellate neurons may be a contributor to the development of epilepsy and is not a consequence of ongoing seizure activity. Early pre-seizure increases in EC global network output have been previously reported (Scharfman et al. 1998; Kobayashi et al. 2003). Since AP discharge frequencies remained elevated in post SE neurons in the presence of fast synaptic antagonists, increases in the intrinsic electrophysiological properties of mEC layer II neurons, in addition to network changes, also occur
during epileptogenesis. A common factor amongst these studies and the current study was the loss of mEC layer III neurons early after epileptogenic injury (3-7 days). mEC layer III principal neurons provide an excitatory drive onto inhibitory neurons within layer II, which in turn, synapse onto mEC layer II stellate neurons. It is unclear if in animal models of epilepsy, inhibitory neurons within the EC are spared or lost (Kobayashi et al. 2003; Kumar and Buckmaster 2006), but the loss of mEC layer III neurons could still lead to uncoupling from the excitatory input, reducing inhibitory control of mEC layer II neurons and increasing neuronal activity.

**Pro-excitatory alterations in Na channel behavior precede the onset of spontaneous seizures.**

In addition to changes in the synaptic network organization with the mEC, alterations in Na channel activity contribute to hyperexcitability of layer II stellate neurons post SE. Such changes include increases in whole cell macroscopic currents as well as increases in persistent ($I_{NaP}$) and resurgent ($I_{NaR}$) currents. Steady state inactivation parameters were also shifted in the depolarizing direction and were best fit with a double Boltzmann function. This additional slowly inactivating second Boltzmann function represented approximately 14.3 % of total inactivation curve and persisted throughout the AP threshold voltage range and beyond. This inactivation resistant Na current component would increase the Na channel window current providing a greater availability of Na channels during the upstroke of the AP, increasing conduction velocity and promoting high frequency AP firing. Increases in Na channel window currents have been reported in CA1 and DG neurons from animals with spontaneous seizures and may be a common feature amongst hyperexcitable neurons in epilepsy (Ellerkmann et al. 2003; Ketelaars et al. 2001a; Remy et al. 2003). Since timely inactivation of Na channels is important for controlling neuronal activity, synaptic integration and neuronal spiking (Fleidervish et al. 1996), any disruptions in this process would increase membrane excitability leading to epileptiform activity and the generation of AP bursts. Mutations in Na channel genes that result in the disruption in Na channel inactivation properties have
been associated with generalized epilepsy with febrile seizures plus (GEFS+) and severe myoclonic
epilepsy of infancy (SMEI) (Spampanato et al. 2001). Most recently, a mutation in SCN8A, the gene
that encodes Na\textsubscript{v}1.6, has been associated with severe infantile epileptic encephalopathy (Veeramah
et al. 2012). Biophysical analysis of the mutation revealed a 5 fold increase in $I_{\text{NaP}}$, an 11 fold
increase in $I_{\text{NaR}}$, and a 13 mV depolarizing shift in steady-state inactivation that was accompanied by
a non-inactivating component over the voltage range of the AP threshold and upstroke. Expression of
the mutant into hippocampal neurons led to the generation of spontaneous firing in about 18% of
neurons, presence of paroxysmal depolarizing shifts and a two fold increase in firing frequencies.
Alterations in the biophysical properties of Na\textsubscript{v}1.6 as a result of the mutation followed a strikingly
similar pattern to those observed in our post SE acquired channelopathy model.

**Na\textsubscript{v}1.6 contributes to increases in neuronal excitability and pro-excitatory alterations in Na channel activity.**

To test the contribution of Na\textsubscript{v}1.6 in facilitating increased neuronal activity and alterations in Na
channel gating parameters we used the TTX metabolite, 4,9 ah-TTX which has a <200 fold greater
selectivity of 4,9 ah-TTX against Na\textsubscript{v}1.6 over the other TTX sensitive isoforms when tested on Na
channels expressed in Xenopus Oocytes (Rosker et al. 2007). Using mammalian HEK 293 cells we
found the difference in potency between Na\textsubscript{v}1.6 and Na\textsubscript{v}1.2 to be attenuated and likely attributable to
the fact that pharmacological properties of antagonists and toxins vary between mammalian and non-
mammalian expression systems. However, we did see a 5.4 fold difference in IC\textsubscript{50} values and of
particular importance was the fact that at a concentration of 100 nM, 4,9 ah-TTX had no effect on
Na\textsubscript{v}1.2 currents, while inhibiting Na\textsubscript{v}1.6 currents by almost 50%. In support, 100 nM 4,9 ah-TTX
inhibited both $I_{\text{NaP}}$ and $I_{\text{NaR}}$ currents to levels that were similar to those recorded for inhibition of the
Na\textsubscript{v}1.6 isoform expressed in HEK 293 cells. $I_{\text{NaP}}$ and $I_{\text{NaR}}$ currents are attributed to the Na\textsubscript{v}1.6 isoform
(Rush et al. 2005). An increased contribution of Na\textsubscript{v}1.6 to the macroscopic current in post SE neurons
likely accounted for the greater inhibition of these currents by 100 nM 4,9 ah-TTX compared with control neurons (52.4 % in post SE compared with 28.6 % in control). The high percentage block in post SE neurons would indicate that post SE neurons only express Na\textsubscript{v}1.6 channels, however, since these neurons also express Na\textsubscript{v}1.2 (figure 4), it is likely that the dose response curve for 4,9 ah-TTX between mammalian cell lines and neurons differs slightly.

Although increases in input resistance observed in post SE neurons would contribute to increases in neuronal excitability our findings provide support for an additional role for Na channels. Inhibition of Na\textsubscript{v}1.6 channel by 4,9 ah-TTX (100 nM) suppressed neuronal hyperexcitability in post SE neurons by 38%, reducing AP firing rates to below control neurons. The effects of 4,9 ah-TTX on firing rates in control neurons were not as pronounced and rates were reduced by only 25%. These findings suggest that 1) partial inhibition of Na\textsubscript{v}1.6 channels is sufficient to normalize the high firing rates in post SE neurons and 2) the contribution of Na\textsubscript{v}1.6 currents in establishing AP firing rates is accentuated in post SE neurons. The reductions in firing rates in post SE neurons were accompanied by significant decreases in AP amplitude and conduction velocity and increases in AP threshold and AP width and could be account for by the increases in persistent (I\textsubscript{NaP}) and resurgent (I\textsubscript{NaR}) in post SE neurons we observed over the voltage range for AP initiation. 4,9 ah-TTX also reversed the depolarizing shifts in steady state inactivation curves in post SE neurons suggesting that alterations in Na\textsubscript{v}1.6 biophysical properties account for depolarizing shifts in post SE neurons. These findings, together with our immunolabelling data, support the notion that during epileptogenesis, Na\textsubscript{v}1.6 is up-regulated and has a greater contribution to the whole cell macroscopic Na current and to spike generation.

Both I\textsubscript{NaP} and I\textsubscript{NaR} currents have delayed inactivation properties and continue to provide a net depolarizing current, allowing repetitive neuronal discharge behavior. Within mEC layer II stellate neurons, the number of neurons with detectable I\textsubscript{NaP} and I\textsubscript{NaR} currents increases during development with all neurons exhibiting these currents by postnatal day 7 (Nigro et al. 2012). The increase in
current incidence is also coupled with an increase in current densities, particularly $I_{NaR}$ currents which reach amplitudes approaching 500 pA as early as postnatal day 22. In CA1 neurons $I_{NaP}$ currents are known to generate the somatic spike ADP and are crucial for controlling the firing mode of a neuron intrinsically (Yue et al. 2005). Increased $I_{NaP}$ currents have been recorded from subiculum neurons isolated from patients with epilepsy (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, alterations in these currents would lead to neuronal hyperexcitability in epilepsy. As such, inhibition of $I_{NaP}$ and $I_{NaR}$ currents by 4,9 ah-TTX likely contributed to the profound effects on synaptically evoked burst firing in post SE neurons, reducing them from bursting neurons to either single spiking neurons, or in some cases, completely abolishing the AP.

Our studies suggest an early role for Nav1.6 in the development of mEC neuronal hyperexcitability during epileptogenesis. Previous studies using kindled rats have reported an increased Nav1.6 expression and increased $I_{NaP}$ in hippocampal CA3 neurons. In mice lacking Nav1.6 a loss of spontaneous firing and a reduction in firing frequencies was recorded from a number of distinct brain nuclei (Raman et al. 1997; Royeck et al. 2008; Van Wart and Matthews 2006). Kindling of heterozygote Nav1.6+/- mice was also impaired (Blumenfeld et al. 2009).

During the latent period of epileptogenesis molecular and cellular changes occur and result in a net increase in neuronal network excitation. We report here that mEC layer II stellate neurons become intrinsically hyperexcitable before the appearance of spontaneous seizures, possibly influencing the development of increased net global excitability important for the generation and spread of epileptic seizures. We show for the first time that inhibition of Nav1.6 currents can reduce neuronal hyperexcitability and reduce post SE mediated alterations in Na channel gating. These studies implicate a role for Nav1.6, in part, in the facilitating an increase in neuronal hyperexcitability of mEC layer II stellate neurons. Early intervention strategies may include the targeting Nav1.6 for delaying the development of epilepsy after brain injury.
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Figure Legends

**Figure 1.** mEC layer II stellate neurons are hyper-excitable 7 days post SE. Recording of mEC layer II stellate neurons in brain slices from control and post SE animals (A). 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 post SE neurons (right panel) compared with control neurons (left panel). Arrow indicates a potential of -60 mV. Discharge frequency (f) versus injected current (I) plot (f-I) illustrate higher firing rates in post SE neurons (n = 27) compared with control (n = 32) neurons over a range of depolarizing current injection steps (B). Data points represent means ± S.E.M. (C) Brief stimulation of mEC layer VI elicited a small somatic depolarizing potential and a single AP in control mEC layer II neurons (left trace). In contrast, brief stimulation evoked a broader and larger amplitude response in post SE neurons and was associated with a burst of AP spikes (right trace). Superimposed gray lines show effects of focal application of TTX (500 nM) to the AIS via a wide bore perfusion pipette to the synaptically evoked responses. In each case the somatic depolarizing potential was almost completely inhibited and AP spikes abolished. Immunolabeling of brain slices obtained from post SE animals using a primary antibody against NeuN revealed loss of mEC layer III neurons in post SE brain slices (E) compared to control (D). Scale bar represents 500 µm.

**Figure 2.** Macroscopic Na currents recorded from dissociated mEC layer II stellate neurons were increased in amplitude and had delayed inactivation parameters in post SE animals. Representative traces of families of Na channel currents recorded from (A) control and (B) post SE illustrate differences in current amplitude. 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. Mean Na channel current density is shown in (B; control: n = 10; 7 day post SE: n = 25). (C) Conductance plot
shows no difference in activation gating parameters between control (n = 10) and post SE neurons (n = 25). Smooth lines correspond to the least squares fit when average conductance data were fit with a single Boltzmann equation. Gating parameters are listed in table 2. 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. (D) post SE neurons (n = 7) had steady-state inactivation curves that were shifted in a depolarizing direction compared with control neurons (n = 11) and best fitted using a double Boltzmann equation (smooth lines). Control neurons were fit with a single Boltzmann equation (parameters listed in table 2). Representative steady state inactivation traces shown in (E) emphasize the delay in Na channel inactivation at -60 mV, -70 mV and -100 mV pre-pulse voltages in post SE neurons compared with control neurons. (F) 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 fully inactivate Na channels. Cells were then held at -90 mV for variable lengths of time (1 ms – 10 s) to allow for channels to recover. Rates of recovery from inactivation were not different between 7 day post SE and control mEC stellate neurons (control: n = 6; 7 day post SE: n = 9). Representative recovery from inactivation traces are shown in (G). Smooth lines correspond to the fits using a double exponential function. All data points represent means ± S.E.M.,* P<0.01.

Figure 3. Persistent Na channel currents (I_{NaP}) and resurgent Na channel currents (I_{NaR}) are increased in post SE mEC stellate neurons. Voltage ramps were applied at a rate of 65 mV/s to elicit I_{NaP} currents (shown as inset). I_{NaP} currents (black traces) recorded from post SE neurons (B) were larger in amplitude than those recorded from control neurons (A) and were abolished in the presence of TTX (1 µM; depicted as gray traces). Resulting TTX subtracted traces are superimposed in C to emphasize the significant increase of I_{NaP} post SE. (D) Averaged I_{NaP} amplitudes for control (n = 9) and post SE neurons (n = 10) show a 51 % increase in I_{NaP} in post SE neurons (P < 0.001). (E)
Conductance plots revealed no differences in the voltage dependence of $I_{NaP}$ currents. Average control conductance curve is depicted in black with the post SE curve depicted in gray. $I_{NaR}$ currents were elicited by a depolarizing step to $+20 \text{ mV}$ for $20 \text{ ms}$ from a holding potential of $-100 \text{ mV}$, followed by either a single repolarizing step to $-30 \text{ mV}$ for $100 \text{ ms}$ to determine the peak $I_{NaR}$ (F & G), or by using a series of repolarizing steps from $-100 \text{ mV}$ to $-10 \text{ mV}$ in $10 \text{ mV}$ increments to determine the voltage dependence of $I_{NaR}$ (I). $I_{NaR}$ amplitudes were determined by subtracting traces obtained in the presence of TTX (1 µM). TTX subtracted $I_{NaR}$ currents were larger in amplitude in post SE neurons (G) compared to control neurons (F). (H) Averaged peak amplitudes of $I_{NaR}$ recorded from control ($n = 20$) and post SE neurons ($n = 31$) show more than a 50 % increase in $I_{NaR}$ in post SE neurons ($P < 0.001$). Conductance plots of $I_{NaR}$ currents showed no significant change in conductance curves between the two conditions (I). Smooth lines correspond to the least squares fit when average conductance data were fit with a single Boltzmann equation. Data represent means ± S.E.M.

**Figure 4.** $Na_v,1.6$ expression along the axon initial segment (AIS) of control and 7 day post SE brain slice sections show increased fluorescence in post SE tissue (A). Green staining for the $Na_v,1.6$ isoform is seen in left panels for each group with red staining for the AIS specific marker Ankyrin G seen in the right panels. Middle panel represents the merge of the two panels. $Na_v,1.6$ fluorescent signals were normalized to Ankyrin G fluorescent signals for comparison purposes (B). Fluorescent ratio's were increased by 46 % in post SE slices ($n = 28; P<0.001$) compared to control ($n = 37$). Example somatic fluorescence images for $Na_v,1.2$ (top panels) and $Na_v,1.6$ (bottom panels) are shown in C. For each image shown, left panel represents corresponding nuclei staining by DAPI. D) Bar charts show an increased normalized relative optical density (R.O.D.) for $Na_v,1.2$ ($P<0.05$) and $Na_v,1.6$ ($P<0.05$) isoform fluorescence in post SE tissue localized to the soma ($n = 31$ for $Na_v,1.2$ and $n = 15$ for $Na_v,1.6$) as compared to control tissue ($n = 32$ for $Na_v,1.2$ and $n = 16$ for $Na_v,1.6$). Expression patterns for $Na_v,1.1$ ($n = 28$ control; $n = 30$ post SE) and $Na_v,1.3$ ($n = 28$ control; $n = 30$ post SE) were
also determined and revealed similar levels of somatic fluorescence for control and post SE sections. Data represent means ± S.E.M. Sections were obtained from 3 animals from each of the two conditions. Scale bars represent 10 µm.

**Figure 5.** 4,9 ah-TTX exhibits greater potency for Na\(_v\)1.6 channels and exhibits greater block of macroscopic Na channel currents recorded from isolated 7 day post SE neurons. (A) Dose response curve showing greater potency of 4,9 ah-TTX for Na\(_v\)1.6 over Na\(_v\)1.2 Na channels stably expressed in HEK 293 cells. Na currents for both isoform subtypes were elicited by a step depolarization to +10 mV for 12 ms from a holding potential of −100 mV. Smooth lines represent the least squares fit when data were fitted with the Hill Equation. IC\(_{50}\) values for Na\(_v\)1.6 were 122 nM and 659 nM for Na\(_v\)1.2 (n= 4 - 5 for each concentration). Representative current traces showing inhibition of Na\(_v\)1.2 currents by 4,9 ah-TTX are shown in (B) and for Na\(_v\)1.6 in (C). Values represent means ± S.E.M. (D) example traces of macroscopic Na currents recorded from isolated layer II mEC stellate neurons (note difference in scale bar amplitude). Left traces represent stellate neurons from control animals, right traces represent neurons isolated from post SE animals. Mean normalized data shown in (E) illustrates greater inhibition of macroscopic Na currents in post SE neurons (n = 6; P<0.05) by 100 nM 4,9 ah-TTX compared to control (n = 5) suggesting an increased contribution of Na\(_v\)1.6 channels to the total macroscopic current in post SE neurons. Application of 300 nM 4,9 ah-TTX further inhibited macroscopic Na currents, but were not different between control (n = 5) and post SE neurons (n = 3). (F) 4,9 ah-TTX (100 nM) reversed the depolarized shifts in steady state inactivation in post SE neurons (open symbols). 4,9 ah-TTX (100 nM) also shifted inactivation curves in control neurons in a hyperpolarizing direction (closed symbols). Smooth lines correspond to the least squares fit when average data were fit with either a double (post SE) or single Boltzmann equation. Representative steady state inactivation traces shown in (G) illustrate the effects of 4,9 ah-TTX (100 nM) on channel
availability at -60 mV, -70 mV and -100 mV pre-pulse voltages in post SE neurons (note differences in amplitude scale bar). Data represent means ± S.E.M.

**Figure 6.** 4,9 ah-TTX inhibits both resurgent (I_{NaR}) and persistent (I_{NaP}) Na channel currents in a dose dependent manner. Effects of 4,9 ah-TTX (100 nM and 300 nM) on (A) I_{NaR} and (B) I_{NaP} Na channel currents. Traces shown were obtained after subtraction of recordings obtained in the presence of TTX (1 µM). Averaged percent inhibition of I_{NaR} and I_{NaP} currents are shown in (C) and (D), respectively, for both control and post SE neurons. Numbers shown in brackets represent the sample size. Data represent means ± S.E.M.

**Figure 7.** Inhibition of Na_{v1.6} currents by 4,9 ah-TTX suppresses neuronal hyperexcitability of mEC layer II stellate neurons. Recording of mEC layer II stellate neurons AP discharge rates in brain slices from control (A) and post SE animals (B) under toxin free conditions are shown in left panels. Effects of 4,9 ah-TTX (100 nM) on firing frequencies is shown in right panels. 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. Arrow indicates a resting membrane potential of -60 mV. Discharge frequency versus injected current plot (f-I; C) reveals suppression of higher firing rates of post SE neurons by 4,9 ah-TTX (100 nM; n = 9; closed symbols) to levels below those recorded for control neurons (open symbols). Firing rates were also suppressed in control neurons (n = 8) over a range of depolarizing current injection steps. Mean reductions in firing frequencies by 4,9 ah-TTX are shown in (D). 4,9 ah-TTX (100 nM) inhibited synaptically evoked, single AP’s in 8 out of 11 control neurons tested (E). In post SE neurons, AP bursts evoked by brief stimulation evoked AP burst firing which was completely abolished by 4,9 ah-TTX (100 nM) in 3 out of 6 neurons and reduced to a single AP in the remaining neurons.
3 neurons (F). The actions of 4,9 ah-TTX were reversible after 30 mins washout. Data points represent means ± S.E.M. * P<0.01, #P<0.001.

**Table 1.** Membrane properties of mEC layer II stellate neurons are altered in post SE neurons and are also modulated by 4,9 ah-TTX. RMP; Resting membrane potential. R_i; input resistance. Significance determined using an analysis of variance (ANOVA) with Tukey’s post hoc test when testing control vs post SE neurons is indicated by *P<0.05. Difference in testing between control parameters and parameters in the presence of 4,9 ah-TTX (100 nM) are indicated by $P<0.05. Values represent means ± S.E.M.

**Table 2.** Steady state activation and inactivation parameters in control and post SE neurons. Modulation of parameters by 4,9 ah-TTX application. Analysis of variance (ANOVA) testing revealed differences between control parameters and parameters in the presence of 4,9 ah-TTX (100 nM); * P<0.05; $P<0.01; # P<0.001. Values represent means ± S.E.M.
A Current Injection APs

Control  20 mV  Post SE  50 ms

20 mV

470 pA

250 pA

150 pA

C Synaptically Evoked APs

Control  20 mV  Post SE  25 ms

D. V/VI  III

II

LEC

E. V/VI

II

III

LEC

Control

Post SE

Figure 1. Hargus et al
Figure 2. Hargus et al.
Figure 2. Hargus et al.
Figure 3. Hargus et al.
Figure 3. Hargus et al
Figure 4. Hargus et al.
Figure 5. Hargus et al

A

- Fraction Blocked
- 4,9-ah TTX (nM)

B Na\textsubscript{v} 1.2

- 500 pA
- 1 ms
- 600 nM
- 300 nM
- control

C Na\textsubscript{v} 1.6

- 500 pA
- 1 ms
- 100 nM
- control
Figure 5. Hargus et al
Figure 5. Hargus et al.
Figure 6. Hargus et al

A

Post SE

100 nM 4,9 ah-TTX

300 nM 4,9 ah-TTX

250 pA

20 ms

B

Post SE

100 nM 4,9 ah-TTX

300 nM 4,9 ah-TTX

100 pA

100 ms

C

INaR Inhibition (%)

Control

Post SE

100 nM

300 nM

4,9 ah-TTX

(4)

(5)

D

INaP Inhibition (%)

Control

Post SE

100 nM

300 nM

4,9 ah-TTX

(4)

(5)

Figure 6. Hargus et al
Figure 7. Hargus et al

A  Control  + 4,9 ah-TTX (100nM)

B  Post SE  + 4,9 ah-TTX (100nM)
Figure 7. Hargus et al
TABLE 1. Membrane properties of mEC layer II stellate neurons are altered in post SE neurons and are also modulated by 4,9 ah-TTX.

<table>
<thead>
<tr>
<th></th>
<th>RMP (mV)</th>
<th>R&lt;sub&gt;i&lt;/sub&gt; (MΩ)</th>
<th>Threshold (mV)</th>
<th>Width (ms)</th>
<th>Amplitude (mV)</th>
<th>Upstroke velocity (mV/ms)</th>
<th>n</th>
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<tbody>
<tr>
<td>Control</td>
<td>-60.8 ± 0.7</td>
<td>46.6 ± 3.6</td>
<td>-44.1 ± 0.4</td>
<td>0.87 ± 0.03</td>
<td>88.0 ± 1.9</td>
<td>237.3 ± 5.5</td>
<td>32</td>
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<tr>
<td>+ 4,9 ah-TTX (100 nM)</td>
<td>-61.0 ± 0.7</td>
<td>44.1 ± 13.5</td>
<td>-41.6 ± 1.2$</td>
<td>1.14 ± 0.14</td>
<td>90.8 ± 2.9</td>
<td>196.8 ± 9.5$</td>
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<tr>
<td>Post SE</td>
<td>-57.7 ± 0.4*</td>
<td>67.1 ± 5.3*</td>
<td>-46.0 ± 0.5*</td>
<td>0.99 ± 0.02*</td>
<td>96.0 ± 1.3*</td>
<td>271.4 ± 9.2*</td>
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<tr>
<td>+ 4,9 ah-TTX (100 nM)</td>
<td>-60.3 ± 0.5$</td>
<td>62.2 ± 11.4</td>
<td>-40.4 ± 0.8$*</td>
<td>1.51 ± 0.06$</td>
<td>90.9 ± 1.9$</td>
<td>184.5 ± 8.0$</td>
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</tbody>
</table>
TABLE 2. Steady state activation and inactivation parameters in control and post SE neurons. Modulation of parameters by 4,9 ah-TTX application.

<table>
<thead>
<tr>
<th></th>
<th>Activation</th>
<th>Inactivation</th>
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<tr>
<td></td>
<td>(V_{1/2}) (mV)</td>
<td>(k) (mV)</td>
<td>(n)</td>
<td>(V_{1/2}) (mV)</td>
<td>(k) (mV)</td>
<td>(V_{1/2}) (mV)</td>
<td>(k) (mV)</td>
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<tr>
<td>Control</td>
<td>-29.9 ± 1.4</td>
<td>-6.7 ± 0.2</td>
<td>22</td>
<td>-69.5 ± 1.3</td>
<td>6.6 ± 0.4</td>
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<td>+ 4, 9 ah TTX</td>
<td>-37.5 ± 3.8*</td>
<td>-6.9 ± 0.7</td>
<td>5</td>
<td>-81.1 ± 3.2</td>
<td>7.2 ± 0.5#</td>
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<td></td>
</tr>
<tr>
<td>(100 nM)</td>
<td></td>
<td></td>
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<tr>
<td>Post SE</td>
<td>-34.4 ± 1.3</td>
<td>-5.8 ± 0.2</td>
<td>19</td>
<td>-66.1 ± 1.1</td>
<td>6.5 ± 0.4</td>
<td>-28.3 ± 0.4</td>
<td>10.2 ± 1.6</td>
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<tr>
<td>+ 4, 9 ah TTX</td>
<td>-46.8 ± 3.8#</td>
<td>-5.6 ± 0.9</td>
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<td>-82.9 ± 5.4</td>
<td>6.7 ± 0.6§</td>
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<td>(100 nM)</td>
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