Diversity and Excitability of Deep Layer Entorhinal Cortical Neurons in a Model of Temporal Lobe Epilepsy

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Abstract

The entorhinal cortex (ERC) is critically implicated in temporal lobe epileptogenesis – the most common type of adult-epilepsy. Previous studies have suggested that epileptiform discharges likely initiate in seizure-sensitive deep-layers (V-VI) of the medial entorhinal area (MEA) and propagate into seizure-resistant superficial-layers (II-III) and hippocampus, establishing a lamina-specific distinction between activities of deep- versus superficial-layer neurons and their seizure susceptibilities. While layer II stellate cells in MEA have been shown to be hyperexcitable and hypersynchronous in patients and animal models of temporal lobe epilepsy (TLE), the fate of neurons in the deep layers under epileptic conditions and their overall contribution to epileptogenicity of this region has remained unclear. We used whole-cell recordings from slices of the ERC in normal and pilocarpine-treated epileptic rats to characterize the electrophysiological properties of neurons in this region and directly assess changes in their excitatory and inhibitory synaptic drive under epileptic conditions. We found a surprising heterogeneity with at least three major types and two sub-types of functionally-distinct excitatory neurons. However, contrary to expectation, none of the major neuron-types characterized showed any significant changes in their excitability, barring loss of excitatory and inhibitory inputs in a subtype of neuron whose dendrite extended into layer III where neurons are preferentially lost during TLE. We confirmed hyperexcitability of layer II neurons in the same slices, suggesting minimal influence of deep-layer input on superficial-layer neuron excitability under epileptic conditions. These data show that deep layers of ERC contain a more diverse population of excitatory neurons than previously envisaged that appear to belie their seizure-sensitive reputation.

Key Words: Entorhinal cortex; cell-type classification; TLE; deep-layers; excitatory neurons
Introduction

Temporal lobe epilepsy is the most common form of adult epilepsies (Engel et al. 1998). Yet, the underlying pathophysiology and the reasons why it is often intractable to anticonvulsant therapy are still poorly understood. The involvement of the ERC, especially the medial entorhinal area, in temporal lobe epileptogenesis is supported both clinically and experimentally (Bartolomei et al. 2005; Jutila et al. 2001) with patients and animal models of TLE displaying preferential loss of layer III neurons in MEA (Du et al. 1995; Du et al. 1993; Ribak et al. 1998) and hyperexcitability and hypersynchrony of layer II stellate cells (Bear et al. 1996; Kumar and Buckmaster 2006; Kumar et al. 2007; Scharfman et al. 1998; Tolner et al. 2005) contributing to entrainment of the dentate gyrus via the perforant pathway (Rutecki et al. 1989; Wilson et al. 1990). While mechanisms of superficial layer (II-III) neuronal hyperexcitability and their contribution to the excitability of ERC and downstream structures have been actively pursued, relatively little is known about the status of neurons in the deep layers (V-VI) under epileptic conditions and whether or not they contribute to superficial-layer neuron hyperexcitability.

Previous studies have suggested that epileptiform discharges likely initiate in “seizure-sensitive” deep layers of MEA and propagate into “seizure-resistant” superficial layers and hippocampus, setting up a qualitative distinction between activities of deep- versus superficial-layer neurons that underlie lamina-specific differences in their seizure susceptibilities (Dhillon and Jones 2000; Dickson and Alonso 1997; Jones and Heinemann 1988; Jones and Lambert 1990a; b). Intralaminar interactions alone cannot account for hyperexcitability of neurons within superficial layers given the anatomical evidence that pyramidal neurons in deep layers also provide excitatory synaptic input to the superficial layers (van Haeften et al. 2003) with the potential to not only regulate superficial layer excitability, but mediate reverberation and gating...
of neuronal activity within the entire hippocampal-entorhinal systems. Sensory input to the ERC-
hippocampal system converges either directly or through adjacent cortices onto superficial layers
of the MEA (s-MEA), whose neurons in turn provide the main cortical input to hippocampus via
the perforant (PP: stellate cells: layer II → dentate gyrus) and temporoammonic (TAP: pyramidal
neurons: layer III → CA1) pathways (Fig. 1F). A substantial component of the hippocampal
output is returned to neurons in deep layers of the MEA (d-MEA) via CA1 and subiculum (CA1
→ layer V-VI neurons) en route to the neocortex / association cortices (AC) (Burwell and
Amaral 1998; Buzsaki 1996; Kloosterman et al. 2003; van Haeften et al. 1995; Witter 1993;
Witter et al. 1989). Alternatively, activity in ERC can be transferred from deep layer neurons to
superficial layers (Dickson et al. 2000; Dolorfo and Amaral 1998; Gloveli et al. 2001; Gloveli et
al. 1999; Kohler 1986; Stewart 1999), where it can reenter the ERC-hippocampal loop directly
(Bartesaghi et al. 1989; Deadwyler et al. 1975) or following reverberation between medial and
lateral areas of ERC (Iijima et al. 1996). This reentrant activity, considered a putative mechanism
for information storage (Lopes da Silva et al. 1990), is critically dependent on deep-to-superficial
connectivity (shown in red, Fig. 1F) within ERC and is thought to be gated such that
hippocampal activity returning to d-MEA is either channeled towards superficial layers (where it
reenters the hippocampal-ERC circuit) or the neocortex / association cortices for storage
(Chrobak and Buzsaki 1994; Chrobak et al. 2000). Although anatomical organization of deep-to-
superficial projections has been partially worked out (van Haeften et al. 2003; Wouterlood
2002), neither details of the functional organization nor the mechanisms with which deep-layer
input contributes to the hyperexcitability of superficial-layer neurons are fully known.
Furthermore, deep-layer neurons are deemed epileptogenic on account of their propensity to
become hyperexcitable and hypersynchronous and their ability to initiate spontaneous activity in
MEA (Jones 1993; Jones and Lambert 1990a; b) by themselves or in association with the lateral entorhinal area (LEA) (de Guzman et al. 2008). The mechanisms underlying their intrinsic hyperexcitability or lack thereof have also remained underexplored (Agrawal et al. 2003; Bragin et al. 2009; Yang et al. 2006).

The present study is designed to assess the status of deep layer neurons under chronic epileptic conditions and determine whether deep-layer synaptic input is capable of affecting excitability of neurons in superficial layers of the MEA. A total of 75 neurons in deep layers (V-VI) were recorded using whole cell patch-clamp to characterize their intrinsic electrophysiological properties under normal conditions. Excitatory and inhibitory synaptic drive to these neurons was concomitantly measured by recording spontaneous and miniature post-synaptic currents. Excitability was further assessed in a subset of neurons using local intracortical stimulation. Electrophysiology revealed a heterogeneous set of responses prompting a systematic classification of neurons using an unsupervised hierarchical cluster analysis based on whole-cell patch-clamp recording data. An additional 56 neurons in the deep layers (V-VI) were recorded in slices from rats that were confirmed epileptic based on frank seizures, hyperexcitability of layer II stellate cells and histological criteria, to assess their excitability and measure changes in synaptic drive to these neurons under epileptic conditions.
Materials and methods

Animals. All experiments were performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Florida State University Institutional Animal Care and Use Committee. A previously described pilocarpine treatment protocol (Turski et al. 1989) was modified (Buckmaster et al. 2004). Briefly, male Sprague-Dawley rats (35; 50-65 days old) were treated with pilocarpine (380 mg/kg, i.p.) 20 min after atropine methylbromide (5 mg/kg, i.p.). Approximately 60% of the treated rats experienced status epilepticus. Diazepam (10 mg/kg, i.p.) was administered 2 hr after the onset of status epilepticus and repeated as needed. Following recovery from status epilepticus, rats were video-monitored (40 hrs/week) for spontaneous seizures. Animals used for electrophysiological experiments were confirmed epileptic, displaying spontaneous recurrent seizures, scored 3 or greater on the Racine scale (Racine 1972) on two or more observations during the 40 hours/week video-monitoring. Recordings from epileptic animals were made on average 40 days post status epilepticus (range: 25 to 62 days) with initial seizures observed between 7 to 25 days post-status. Control groups included age-matched naive rats (16) and pilocarpine-treated rats that did not experience status epilepticus (12). Of the 75 neurons that comprised the control group, 56% were from naive rats while the remaining 44% were from the non-responders that were treated with pilocarpine but did not experience status epilepticus, any obvious loss of neurons, or seizures associated with TLE. There were no significant differences in the anatomical or electrophysiological results from the two types of controls although the effects of pilocarpine on the non-responders remain unknown.

Slice preparation and electrophysiology. Rats were deeply anesthetized with urethane (1.5 g/kg, i.p), decapitated, and horizontal slices (350 μm) were prepared with a microslicer (VT1000S,
Leica, Germany) in a chilled (4°C) low-Ca²⁺, low-Na⁺ "cutting solution" containing (in mM):
230 sucrose, 10 D-glucose, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, and 0.5 CaCl₂
equilibrated with a 95% and 5% mixture of O₂ and CO₂. Slices were allowed to equilibrate in
oxygenated artificial cerebrospinal fluid (aCSF) containing in mM: 126 NaCl, 26 NaHCO₃, 3
KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, and 10 D-glucose (pH 7.4), first at 32°C for 1 hr and
subsequently at room temperature before being transferred to the recording chamber.

Recordings were obtained at 32 ± 1°C from neurons in the MEA under Nomarski optics
(Zeiss, Germany) using a visualized infrared setup (Hamamatsu, Japan). Cell morphology and
location within the various cortical lamina could be identified. Recording electrodes (1.2-2.0 μm
tip diameters, 3-6 MΩ) contained (in mM): 105 potassium gluconate, 30 KCl, 10 HEPES, 10
phosphocreatine, 4 MgATP, 0.3 GTP, and 20 biocytin. Internal solution was adjusted to a pH of
7.3 with KOH and an osmolarity of 300 mOsm. Slices were maintained in oxygenated (95% O₂
and 5% CO₂) aCSF, and drugs and chemicals were applied via the perfusate (2 ml/min).
Tetrodotoxin (TTX, Sigma, St. Louis, MO) was bath applied as required for specific protocols.

Concentric bipolar electrodes (CB-XRC75; Frederick Haer & Co., Brunswick, ME) with
75 μm tip diameters were positioned in layer I in proximity to recorded neurons in layer II or in
layer V off-column to the recorded neurons, and depolarizing constant current pulses, 100-150
μsec in duration and 50-500 μA in amplitude, were applied at low frequencies (0.1-0.3 Hz) to
evoke action potentials. Postsynaptic currents and potentials were recorded with a MultiClamp
700B amplifier and pClamp software (Molecular Devices, Union City, CA), filtered at 1-2 kHz
(10 kHz for current clamp), digitized at 10-20 kHz, and stored digitally. Series resistance was
monitored continuously, and those cells in which this parameter exceeded 15 MΩ or changed by
>20% were rejected. Series resistance compensation was not used. During experiments, putative interneurons were distinguished from principal cells by single action potential waveforms, firing frequency, degree of spike frequency accommodation, and dynamic range, and were subsequently identified by biocytin-labeling. Spike afterhyperpolarization amplitude was measured from the pre-spike baseline to the maximum amplitude of the afterhyperpolarization. Spontaneous and miniature EPSC and IPSC data, obtained from 2-min-long continuous recordings, were analyzed using Mini Analysis (Synaptosoft, Decatur, GA). The threshold for event detection was set at $3 \times \text{root mean square noise level}$. Average root mean square noise levels were similar in control and epileptic groups ($P \geq 0.4$). Software-detected events were visually verified, and their frequency and amplitude was measured. Miniature postsynaptic currents were isolated using TTX (1 μM).

**Electrophysiological analysis.** To classify excitatory cells in deep layers (V-VI) of MEA, an unsupervised hierarchical cluster analysis was performed based on electrophysiological parameters gathered from sixty-five neurons in slices from normal animals. Clustering was performed to determine if the population of deep layer neurons was heterogeneous, and to also establish a schema for their classification. Clustering was carried out independently in SPSS (IBM SPSS Statistics 19.0) and Origin (v8.6; OriginLab, Northampton, MA) using Ward’s method and squared Euclidean distances as the distance measure (Ward 1963). Briefly, neurons were sorted in a multidimensional space based upon similarities of the electrophysiological variables considered. Clustering began with individual neurons in separate clusters with each subsequent stage combining/reducing cluster number by one, based upon the distance measure between neurons, until the final stage when a single cluster contained all of the neurons. Ward’s method used an ANOVA approach in determining between-cluster distances. Cluster
membership was determined by calculating the total sum of squared deviations from the cluster mean, and clusters were combined with the intent of minimizing increases in error sum of squares (Burns 2008). The final number of cluster groups in our analysis was determined using the Thorndike procedure (Thorndike 1953) in which large jumps in within-cluster distances associated with the clustering stages served to indicate salient differences between groups and cluster number.

We considered six electrophysiological parameters in our analysis, five of which were gathered during current-clamp and one during voltage-clamp recordings of each cell considered in the analysis. These included: 1) instantaneous firing frequency (IFF); 2) steady-state firing frequency (SSFF); 3) standard deviation of SSFF; 4) spike amplitude ratio (SAR); 5) sag ratio and 6) spontaneous IPSC (sIPSC) frequency. The choice of parameters was based on empirical observations of the action potential waveforms of a large population of excitatory neurons in the deep layers and consideration of additional variables deemed most valuable in predicting group membership in alternate studies of the electrophysiological classification of neocortical neurons (Cauli et al. 2000; Halabisky et al. 2006). Cellular input resistance was not significantly different between cell groups (Table 1), thereby justifying the use of standard current intensities for measuring action potential waveform parameters. The IFF for cells, a measure of early frequency adaptation, was determined from the averaged instantaneous firing frequencies of the first ten action potentials in a train of action potentials evoked by a 100 pA, 500 ms depolarizing current pulse. SSFF was similarly measured from a period towards the end of the current pulse when firing frequency had reached a steady state. The spike amplitude ratio (SAR), defined as a fraction of the peak amplitudes of the first and fifth spikes in an action potential train, was used to characterize the diminution in action potential amplitude of the initial discharge profile (Cauli
et al. 2000). Sag ratio, a measure of sag conductance ($G_s$), was determined as $\left(\frac{V_{\text{peak}} - V_{\text{ss}}}{V_{\text{peak}}}\right) \times 100$, where $V_{\text{peak}}$ and $V_{\text{ss}}$ are measurements of voltage immediately following a 500 ms current pulse to hyperpolarize the neuron by 200 mV from rest. $V_{\text{peak}}$ is voltage measured at the negative most point in the 500 ms window and $V_{\text{ss}}$ is the measurement at steady-state following the initial voltage excursion.

NeuN – biocytin immunohistochemistry. To visualize biocytin-labeled neurons after recording, slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C for at least 24 hr. Following fixation, slices were stored in 30% ethylene glycol and 25% glycerol in 50 mM PB at -20°C, before being processed using a whole-mount protocol with counterstaining by NeuN-immunocytochemistry. Slices were rinsed in 0.5% Triton X-100 and 0.1 M glycine in 0.1 M PB and then placed in a blocking solution containing 0.5% Triton X-100, 2% goat serum (Vector Laboratories, Burlingame, CA), and 2% bovine serum albumin in 0.1 M PB for 4 hr. Slices were incubated in mouse anti-NeuN serum (1:1000; MAB377, Chemicon, Temecula, CA) in blocking solution overnight. Following a rinsing step, slices were incubated with the fluorophores Alexa 594 streptavidin (5 µg/ml) and Alexa 488 goat anti-mouse (10 µg/ml; Molecular Probes, Eugene, OR) in blocking solution overnight. Slices were rinsed, mounted on slides, and coverslipped with Vectashield (Vector Laboratories) before being examined with a confocal microscope (TCS SP2 SE, Leica, Germany).

All statistical values are presented as mean ± SEM. Statistical differences were measured using the unpaired Student's $t$ test, unless indicated otherwise.
Results

One hundred and thirty one neurons in all were recorded from deep layers V-VI of the MEA in brain slices from normal and epileptic animals. Deep layers in MEA were clearly segregated from superficial layers I-III along the pial-white matter axis by a prominent cell-depleted layer IV or lamina dissecans (Fig. 1A). Neurons were selected randomly (we recorded from the largest cells in the field under x63 magnification) and their laminar location determined visually during recordings and confirmed through biocytin-labeling and counterstaining for NeuN immunoreactivity (Fig. 1B-C). These sections were also used to confirm loss of layer III neurons in all the epileptic animals – a histopathological feature of TLE (Fig. 1C) and hyperexcitability of stellate cells in layer II of MEA (Fig. 1D).

Whole-cell current-clamp recordings were obtained in response to injection of: a) 100 pA of depolarizing current (1-10 seconds in duration) and b) current pulses (500 ms duration) of increasing intensity (100-250 pA) that altered membrane potential incrementally (step size 20 mV) in the range ±100 mV from rest. These data helped determine resting membrane potential ($V_{m}$) of neurons, attributes of single action potentials and parameters associated with the pattern of action potential discharge upon depolarization (IFF, SAR, SSFF, standard deviation of SSFF, sag ratio; see electrophysiological analysis, Material and Methods). We used this information to confirm the identity of recorded neurons (excitatory versus inhibitory) and to classify them using unsupervised hierarchical cluster analysis. We measured excitability of a subset of current-clamped neurons by determining the number of action potentials evoked as a function of intracortical stimulus intensity. Action potential threshold (T) for layer II stellate cells was determined by stimulating fibers in layer I (Fig. 1D), while T for deep layer V-VI neurons was determined by stimulating local intracortical afferents in proximity to the recorded neuron (Fig.
Stimulus intensity was measured as multiples of $T$, which is the current required to evoke a single action potential on ~50% of the trials. Layer II stellate cells in epileptic rats are hyperexcitable and fire multiple action potentials in response to single shocks of increasing stimulus intensity (Kumar and Buckmaster 2006) and we determined whether this was also true of the most prominent type of deep layer neurons in the MEA.

Next, we obtained whole-cell voltage clamp recordings of spontaneous (s) PSCs (postsynaptic currents) in neurons, that were initially current-clamped, to evaluate their excitatory (E) and inhibitory (I) synaptic drive and compare changes under normal and epileptic conditions. EPSC recordings were obtained at a holding potential of -70 mV, and IPSC recordings at 0 mV, the reversal potential for glutamate. EPSCs were recorded without pharmacologically blocking GABA$_A$-receptor mediated events to facilitate obtaining both EPSC and IPSC data from individual neurons. To distinguish between the possibilities that changes in synaptic drive to neurons were mediated by loss/gain of synapses or changes in probability of action potential-dependent neurotransmitter release, we recorded miniature (m) PSCs in the presence of TTX. These data were used to assess the overall status of synaptically-mediated excitation and inhibition within the deep layers. Details of global synaptic interactions between the ERC-hippocampal systems are shown schematically in Fig. 1F.

Excitatory neurons in the deep layers were distinguished from GABAergic interneurons based on action potential discharge profile, attributes of individual action potentials (Bean 2007), and passive membrane properties (Kumar and Buckmaster 2006). Compared with excitatory cells, individual action potentials in interneurons were briefer – average width at half maximal amplitude or half-widths (HW), were $0.98 \pm 0.02$ ms ($n = 78$ cells) for deep layer excitatory cells versus $0.2 \pm 0.01$ ms for type I ($n = 11$) and $0.4 \pm 0.01$ ms for type II ($n = 11$) interneurons in
layer III of MEA. Compared with excitatory neurons, individual action potentials in interneurons were followed by larger spike afterhyperpolarizations – average spike afterhyperpolarization amplitudes were $9.8 \pm 0.4$ mV ($n = 76$ cells) for deep layer excitatory cells versus $31 \pm 1.3$ mV for type I ($n = 11$) and $38 \pm 1.5$ mV for type II ($n = 11$) interneurons. In response to equivalent current injections, excitatory pyramidal neurons generally discharged action potentials slower than GABAergic interneurons – instantaneous firing frequencies ranged between 10-20 Hz for excitatory cells in deep layer (Table 1) versus 90-300 Hz for GABAergic interneurons within the MEA.

As indicated before, recorded neurons were filled with biocytin and counterstained for NeuN-immunoreactivity. Despite our best efforts to optimize conditions for labeling cells completely, we observed a large variability in staining; ranging from partially-filled neurons to cells whose dendritic arborization could be studied in exquisite detail. One feature of biocytin filling of deep layer neurons was the consistent labeling of primary dendrites. A vast majority of neurons in the deep layers had a single primary apical dendrite whose orientation along the pial white-matter axis or the medio-lateral axis correlated well with their physiology. All neurons labeled with biocytin displayed prominent spines. Those few neurons (< 1%) encountered in this region that appeared aspinous had a distinct physiological profile (Kumar and Buckmaster 2006), were deemed GABAergic, and dropped from further analysis. Axons and axonal arborization, though visible, was much harder to follow in the sections and therefore not pursued in the morphological analysis (for more information on axonal projections of deep layer ERC neurons, see (Gloveli et al. 2001)). The classification of deep layer excitatory neurons was based solely on electrophysiological criterion. However, following classification, we assayed for morphological features that were common to neurons in their respective groups. The nomenclature used to
describe the various neuronal classes is based on electrophysiological, not morphological, criteria that best describes the activity of neurons in a particular group. Neuronal subtypes are designated with numerals. Cluster analysis revealed three major types and two subtypes of excitatory neurons in deep layers of the MEA (Fig 2).

**Regular-spiking Cells**

Regular spiking (RS) cells account for 47% of all excitatory neurons recorded in layers V-VI of the MEA (Fig. 3-4) and are characterized by a sustained discharge of action potentials at regular intervals upon current injection or depolarization. Their action potential waveforms had the highest IFF, with moderate to minimum spike-frequency adaptation, and the lowest standard deviations of SSFF amongst all excitatory cell types in the deep layers (Table 1). RS cells could be further subdivided into RS1 (33%; Fig. 3) and RS2 (14%; Fig. 4) cells based on the spike amplitude ratio of the first and fifth action potentials in the initial discharge of action potentials (Fig. 3D-E, 4D-E). RS cells appeared pyramidal-shaped, with a distinct apical dendrite, under high-powered differential interference contrast (DIC) optics (Fig. 3A, 4A). Biocytin-labeling revealed that they typically had a single primary dendrite that was oriented along the pial-white axis with smaller, less prominent branches, ramifying laterally within layers V-VI. The primary dendrite was oriented up, towards the pial surface, in 65% of RS neurons while in the remaining 35%, it faced down, towards the white matter and the angular bundle (Fig. 2C, 3B, 4B). RS cells were the only neurons in the deep-layers that had the unusual downward-facing primary dendrites. Additionally, the projections patterns were similar for both RS1 and RS2 cells.

RS1 cells had low sEPSC and sIPSC frequencies under normal conditions (Table 2, Fig 3F-G). However, the average frequency and amplitude of sEPSCs in RS1 cells from epileptic
rats was significantly elevated to 214% and 130% of controls respectively (p < 0.05, t-test; Fig. 3F-G). More frequent sEPSCs may result from reduced inhibition and subsequent increased spontaneous activity of local excitatory circuits; however, neither sIPSC frequency and amplitude nor mIPSC frequency and amplitude were significantly affected in the epileptic rats (p = 0.2 and 0.8 for frequency and p = 0.1 and 0.7 for amplitude respectively, t-test; Fig. 3G). Alternately, there could be an increase in number of excitatory synapses with RS1 cells and/or increased probability of action potential-dependent neurotransmitter release. However, these possibilities are also inconsistent with the observation that both mEPSC frequency and amplitude are normal under epileptic conditions (p = 0.9 for frequency and 0.1 for amplitude, t-test, Table 2, Fig 3G). The increased spontaneous activity of RS1 excitatory synaptic inputs in the epileptic rats likely reflects enhanced excitability of hippocampal neurons (Sloviter 1991; Smith and Dudek 2001) that project to deep layers of the ERC (Fig. 1F), although it remains to be determined if they synapse specifically with RS1 cells because none of the other cells types in the deep layers followed a similar trend.

RS2 cells had similar sEPSC frequencies and amplitudes as RS1 cells and even lower s- and mIPSCs frequencies (Table 2, Fig 4F-G). However, neither frequencies nor amplitudes of s- or mPSCs were significantly affected under epileptic conditions (Fig 4G), although mIPSCs in these cells were almost undetectable under both control and epileptic conditions. These data show that excitatory and inhibitory synaptic drive to the RS2 subpopulation of regularly-spiking neurons remains unperturbed in the epileptic animals.
Irregular-spiking Cells

Irregular spiking (IR) cells account for 41% of all excitatory neurons recorded in layers V-VI of the MEA (Fig. 5-6) and are characterized by discharge of action potentials at irregular intervals upon current injection or depolarization. Unlike RS cells, the action potential discharge in IR cells either becomes progressively irregular or ceases altogether upon sustained depolarization (Fig. 5D-E, 6D-E). Action potential waveforms of IR cells had the lowest SSFF and the largest standard deviations of SSFF of all excitatory cell types in the deep layers (Table 1). IR cells could be further subdivided into IR1 (12%; Fig. 5) and IR2 (29%; Fig. 6) cells based on the action potential-mediated inhibitory synaptic drive to these neurons under normal conditions (Fig. 2B, 5F, 6F). The sIPSC frequency in IR1 cells was not only higher than IR2 cells (p < 0.0001 for f_{sIPSC} and p = 0.07 for a_{sIPSC}, t-test, Table 2, Fig. 5G, 6G, 2E), it was the highest of all excitatory cells types recorded in deep layers of the MEA. IR cells appeared pyramidal-shaped, with a prominent apical dendrite, under high-powered DIC optics (Fig. 5A, 6A) and biocytin-labeling revealed that they typically had a single primary dendrite oriented along the pial-white axis with smaller, less prominent branches, ramifying laterally within layers V-VI. Unlike RS cells, the primary dendrite in IR cells was oriented up, towards the pial surface in 100% of the neurons labeled (Fig. 2C, 5B, 6B).

Both IR1 and IR2 cells had a high frequency of sEPSCs compared to RS cells under normal conditions (Table 2, Fig. 5F-G, 6F-G), but as mentioned before, the biggest and most conspicuous difference was in the frequency of sIPSCs in these neurons. However, frequencies and amplitudes of sEPSCs and sIPSCs in IR1 cells were not significantly altered in the epileptic animals though their sample size is small under these conditions (Fig 5F-G). In contrast, both s- and mPSC frequencies, but not amplitudes, were significantly reduced in IR2 cells under
epileptic conditions (Fig. 6F-G). Compared with controls, sEPSC and sIPSC frequencies in IR2
cells were reduced by 47% and 80% respectively (p < 0.05 for both, Table 2; Fig. 6G), while the
corresponding reductions in mEPSC and mIPSC frequencies in the same population of cells were
71% and 60% respectively (p < 0.01 for both, Table 2; Fig. 6G). IR2 cells were the only cells in
deep layers of the MEA that were affected in this way in the epileptic animals. Reductions in
mEPSC and mIPSC frequency, but not amplitude, indicate a reduced excitatory and inhibitory
synaptic drive to these neurons under epileptic conditions, likely mediated by a loss of excitatory
and inhibitory synapses. These data also suggest that of the two sub populations of irregularly
spiking neurons in MEA whose primary dendrites project into the superficial layers, the IR2
neurons appear more likely to receive synaptic inputs from layer III cells. Reductions in
excitatory and inhibitory synaptic drive to these neurons likely reflect the loss of excitatory and
inhibitory neurons in layer III under epileptic conditions, a hallmark of TLE.

**Single-spiking Cells**

Single-spiking (SS) cells account for as many as 11% of all excitatory neurons recorded
in layers V-VI of the MEA (Fig. 7) and are characterized by the lack of an action potential
discharge upon current injection or depolarization. Unlike RS or IR cells, SS cells often fire a
single action potential upon depolarization and remain quiescent during the sustained phase of
depolarization (Fig. 7D-E). They have a healthy resting membrane potential (V_m = -66 ± 3 mV)
and increasing the amounts of current injected (> 100 pA) does not alter the responsiveness of SS
cells to fire more action potentials. The single spike most likely is attributable to a low threshold
K^+ conductance that allows these neurons to reset quickly (Brew and Forsythe 1995). Action
potential waveforms of SS cells were therefore stereotypic in which neither IFF nor SSFF could
be assessed (Table 1). SS cells had a low but discernible frequency of sEPSCs, an even lower
frequency sIPSCs and almost no detectable miniature events (Table 2, Fig. 7F-G). Furthermore, none of the spontaneous measurements were significantly affected under epileptic conditions (Fig. 7G). SS cells appeared conical-shaped; with thin but prominent apical dendrites under high-powered DIC optics (Fig. 7A) and biocytin-labeling revealed that they typically had a single primary dendrite that was oriented along the mediolateral-axis and several less prominent branches all of which were confined to layers V-VI in 100% of the neurons labeled (Fig. 7B). SS cells appeared to be the excitatory counterparts of local GABAergic interneurons and just like the other excitatory cells types in MEA remained unperturbed under epileptic conditions. SS cells are also found in other brain regions including the subiculum (Canto and Witter 2011; Hamam et al. 2000; Menendez de la Prida et al. 2003).

**Validation of observations through assessment of LII cell hyperexcitability under epileptic conditions**

Previous studies have shown that layer II stellate cells of the ERC are hyperexcitable in patients and animal models of TLE (Bear et al. 1996; Hargus et al. 2010; Kumar and Buckmaster 2006; Kumar et al. 2007; Scharfman et al. 1998; Tolner et al. 2005) causing downstream granule cells in the dentate gyrus to receive excessive, synchronous, excitatory synaptic input (Buckmaster and Dudek 1997; Scharfman et al. 1998), detected as large-amplitude field excitatory postsynaptic potentials (Bragin et al. 1995) that resemble interictal EEG spikes in patients (Rutecki et al. 1989; Wilson et al. 1990). We used the hyperexcitability of layer II cells under epileptic conditions as a means of validating assessments of excitability in deep layer neurons by recording from layer II stellate cells in the same slices used to gather the deep layer data (Fig. 1D, 8A-B). Whole-cell current clamp recordings were obtained from stellate cells in layer II of the MEA (resting potential: -57 ± 1.4 mV, n = 22 cells). Action potential threshold (T)
was determined by stimulating fibers in layer I, and stimulus intensity was measured as multiples of \( T \) (Fig. 8C). As before (Kumar and Buckmaster 2006), layer II stellate cells from epileptic rats had larger average half-widths and areas compared to controls and fired multiple action potentials in response to single shocks of increasing stimulus intensity (Fig. 8C). Prolonged depolarizations with multiple action potentials also were observed in layer II stellate cells in other models of TLE in which layer III neuron loss was produced by intense and prolonged electrical stimulation of the ventral hippocampus (Bear et al. 1996) or by injection of aminooxyacetic acid into the entorhinal cortex (Scharfman et al. 1998). We repeated the stimulus protocol in RS cells, the predominant excitatory cell type in the deep layers, by stimulating local intracortical afferents in the vicinity of the recorded cells (Fig. 1E). While layer II stellate cells in epileptic rats were hyperexcitable, RS cells in the same animals were not (Fig. 8D), suggesting lamina-specific differences in the excitability of neurons. Next, we obtained whole-cell voltage clamp recordings of sPSCs to evaluate the excitatory and inhibitory synaptic drive to layer II stellate cells under epileptic conditions. In normal aCSF, the average frequencies of sEPSCs and sIPSCs in layer II stellate cells from epileptic rats were 583% and 7% of controls respectively, but average amplitudes were similar (Fig. 8E-F). These data are consistent with previous studies that suggest reduced inhibition and increased excitation as the major contributing factors leading to hyperexcitability of layer II cells (Kumar and Buckmaster 2006; Kumar et al. 2007). Further validation of the assessments of excitability of deep layer neurons under epileptic conditions was through observations of the loss of neurons in layer III of MEA in 100% of the sections from epileptic animals as revealed by NeuN immunohistochemistry (Fig. 1B-C). Collectively, these data suggest that while superficial layers of MEA show changes in excitatory and inhibitory synaptic drive to neurons, hyperexcitability and loss of cells leading to potential reorganization
of circuitry under epileptic conditions, the deep layers of MEA are relatively unaffected and
appear to be resistant to such changes in this model of TLE. Furthermore, the fact that superficial
layer neurons are hyperexcitable under epileptic conditions while deep layer neurons in the same
animals are not, suggests that the latter contribute minimally, if at all, to the hyperexcitability of
neurons in the superficial layers.
Discussion

This study characterizes the functionally-distinct classes of excitatory neurons in deep layers V-VI of the MEA. Our study suggests that unlike superficial layers (II-III), that have a homogenous population of excitatory cell types (stellate cells in layer II; pyramidal neurons in layer III), the deep layers contain cells that appear to be morphologically heterogeneous and functionally diverse. We found at least three major types and two sub-types of functionally-distinct excitatory neurons in deep layers of the MEA. A characterization of their functional properties under baseline conditions enabled their formal classification using unsupervised hierarchical cluster analysis. Deep-layer neurons have been dubbed “epileptogenic” (Dhillon and Jones 2000; Jones and Lambert 1990a; b), so we assessed their excitability directly by assaying changes in their excitatory and inhibitory synaptic drive under epileptic conditions. Contrary to expectation, none of the excitatory cell types encountered was hyperexcitable in the epileptic animals. We verified that layer II stellate cells, known to be hyperexcitable under epileptic conditions from previous studies (Kumar and Buckmaster 2006), were indeed hyperexcitable in the same slices used for obtaining deep layer data. Together, these findings suggest that: a) neurons in deep layers are not only diverse morphologically (Hamam et al. 2000), they are functionally heterogeneous as well; b) deep layer neurons are not hyperexcitable under epileptic conditions and thus c) contribute minimally to superficial-layer neuronal hyperexcitability in this model of TLE. Analogous to the role of the dentate gyrus as a gate to keep hyperexcitability of neurons in superficial layers of ERC out of the rest of the hippocampus, the deep layers of the ERC may serve to gate the rebound hyperexcitability of neurons in hippocampus from entraining the rest of the ERC and neocortex, thereby breaking a vicious cycle of reverberations.
Classification of Neurons

There have been several studies of ERC neurons previously, many more focused on superficial layers (Alonso and Klink 1993; Canto and Witter 2011; Dickson et al. 1997; Garden et al. 2008; Giocomo et al. 2007; Gloveli et al. 1997; Klink and Alonso 1997; Lingenhohl and Finch 1991; van der Linden and Lopes da Silva 1998) than on deep layers (Canto and Witter 2011; Egorov et al. 2002; Gloveli et al. 2001; Hamam et al. 2000). ERC neurons have been classified previously using morphological criteria (Hamam et al. 2000) and our study shows that electrophysiological criteria alone may be sufficient to adequately classify neurons in deep layers. However, no single parameter by itself could classify the neurons, and parameters such as sag conductance (G_s) used to distinguish excitatory from inhibitory neurons (Kumar and Buckmaster 2006) or superficial-layer II from deep-layers V neurons (Canto and Witter 2011; Hamam et al. 2000), were ineffective in discriminating deep layer V-VI neurons as their removal from the unsupervised hierarchical cluster analysis did not alter the classification. Indeed, intrinsic spike characteristics of a neuron are deemed an important determinant of its position in the cortical circuit and may have a substantial role in determining its response properties (Agmon and Connors 1992). The heterogeneity among principal neurons observed in the deep layers is consistent with previous studies (Canto and Witter 2011; Gloveli et al. 1997; Hamam et al. 2000; Jones and Heinemann 1988; Lingenhohl and Finch 1991).

Morphological analysis of deep layer ERC neurons can be traced back all the way to Ramón y Cajal and Lorente de No (1933) who described a non-branching primary dendrite (“shaft”) as a conspicuous feature of deep layer pyramidal neurons. Indeed, having sampled a large number of biocytin-labeled neurons, we found orientation of the primary dendrite as a simple yet useful parameter for correlating morphology of deep layer neurons with their
physiology: A mediolateral orientation of primary dendrite/s indicated an SS cell; Cells whose primary dendrites were oriented along the pial-white matter axis but faced down towards the angular bundle were invariably RS cells; Cells whose primary dendrites were orientated along the pial-white matter axis but faced up towards the pial surface were either IR cells (100%) or RS cells (65%). Although, there is no substitute for complete-fills, the qualitative correlation of morphology and physiology was of practical value in cell-identification and determination of excitability of these neurons under epileptic conditions. Consistent with previous studies (Hamam et al. 2000), we observed that the basal dendrites of many of the excitatory cells in deep layers that had pial-white matter-oriented primary dendrites were maintained largely within layer and oriented parallel to the cell layers. However, classification based on morphological criteria yielding three categories of neurons (pyramidal, horizontal and polymorphic cells) in layer V (Hamam et al. 2000) could not be readily reconciled with our electrophysiological or morphological classification despite similarities in observations. The physiological and morphological diversity of excitatory neurons found in ERC and elsewhere in the neocortex calls for a systematic classification system on the lines of the one initiated for GABAergic interneurons (Ascoli et al. 2008).

Excitability of deep-layer neurons in TLE

Deep-layer ERC neurons have been deemed to possess several intrinsic and synaptic properties which confer an extreme susceptibility to generation of sustained seizure activity (Dickson and Alonso 1997; Jones and Heinemann 1988; Jones and Lambert 1990b). Of particular relevance are neurons that are intrinsically bursting like those in layer V of neocortex or the hippocampus (Agmon and Connors 1989; Connors and Gutnick 1990; Connors et al. 1982; Gutnick et al. 1982; Kandel and Spencer 1961; Le Bon-Jego and Yuste 2007) that give rise
to epileptiform discharges in these structures. We did not come across intrinsically-bursting cells in our recordings of deep layer neurons in the MEA either in normal or epileptic animals. The fact that baseline firing patterns of the classified neurons were not altered in the epileptic animals is consistent with the observation that epileptogenic mechanisms in deep layers do not depend on intrinsic cellular bursting mechanisms (Hamam et al. 2000).

We recognize that neuronal categories can be flexible entities as characteristics of firing patterns of cells can be subject to change under a number of conditions including variations in membrane potential, somatic depolarization, behavioral state of the animal and/or in vitro versus in vivo recording conditions (Steriade 2004). We expected deep layer neurons in the ERC to show changes in their baseline action potential waveforms under epileptic conditions precluding their classification into the distinct subgroups obtained under normal conditions. Contrary to expectations, all the major cell types seen in normal animals were also found in the epileptic animals and our random sampling indicated minor variations in the percentages of cells encountered under the two conditions. The percentages of deep-layer V-VI neurons sampled from normal (75 cells) and epileptic (56 cells) animals were ~13% and 7% for SS cells; 13% and 14% for RS2 cells; 16% and 55% for RS1 cells; 37% and 18% for IR2 cells and 17% and 5% for IR1 cells, respectively. We do not know whether these variations were due to sampling or cell loss, as there are fewer Nissl-stained neurons in layer V-VI of MEA in epileptic animals compared with controls (Kumar and Buckmaster 2006). These data coupled with the observation that intrinsic parameters ($R_m$, $C_m$, $V_m$ and input resistance) for the various cell-types do not change under epileptic conditions suggests that baseline electrophysiological properties of deep-layer neurons are not significantly altered under epileptic conditions. The fact that local stimulation of afferents to the predominant cell type in the deep layers, the regular spiking cells,
also fails to mimic the excitability of layer II neurons under similar conditions further argues against hyperexcitability of deep layer neurons under epileptic conditions.

While intrinsic properties of deep-layer neurons seem to have remained unaffected in the epileptic animals, the synaptic drive to these neurons also seems to have followed a similar trend as revealed by concomitant voltage-clamp recordings (Table 2). We actually noted reductions in the excitatory and inhibitory synaptic drive in at least one class of neurons (IR2 cells) under epileptic conditions, while most of the other cell types remained essentially unaffected. This is again contrary to expectation, given that layer II stellate cells in the same tissue are hyperexcitable, and suggests that baseline synaptic activity in deep-layer neurons and circuits remains intact in the epileptic animals. This finding has important implications not only for superficial layers and neocortex, but the dentate gyrus as well, for many deep-layer neurons also have entorhinodentate projections with bifurcating axon collaterals that ascend into the superficial layers of the ERC (Deller et al. 1996; Gloveli et al. 2001; Kohler 1985). Given that these may function as both local circuit and projecting neurons, their hyperexcitability could dramatically influence synchronization between deep ERC layers, superficial ERC layers and the dentate gyrus (Gloveli et al. 2001). We noted that averaged baseline sEPSC frequency tended to be lower in RS and SS cell compared to IR cells and superficial layer II stellate cells in the same tissue. However, SS cells had almost no detectable miniature events under normal and/or epileptic conditions suggesting that the bulk of the activity in these cells is action potential-dependent and likely mediated via low-probability-of-release synapses. The fact that both spontaneous and miniature PSC frequencies, but not amplitudes, are diminished in IR2 cells under epileptic conditions correlates well with the loss of pyramidal and GABAergic neurons in layer III suggesting that of the two subtypes (IR1 and IR2), both of which have primary dendrites
projecting towards the pial surface, the IR2 cells are more likely to receive synaptic inputs from layer III. Changes in excitatory and inhibitory drive to IR2 cells were the only significant changes we observed at the synaptic level in the deep layers under epileptic conditions. Even if the RS1/2 subtypes were IR1/2 subtypes are combined together as two major cell-types (regular- and irregular-spiking), the only significant differences between control and epileptic conditions noted were an increase in sEPSC frequency and amplitude in RS cells, and a decrease in sIPSC frequency (but not amplitude) in the IR cell population (Table 3). Miniature PSC data for both cell-types was similar. Taken together, these data suggest that deep layer neurons are not seizure-susceptible, as previously envisaged, and contribute minimally, if at all, to the hyperexcitability of neurons in superficial layers of the MEA in this model of TLE. Attempts to decipher the increased seizure-resistance or decreased susceptibility of deep layer principal neurons to hyperexcitability and cell loss would invariably have to consider, among other factors, the status of neurons in hippocampal CA1 and the LEA, expression and distribution of ionic conductances, the role of neuromodulators affecting excitability of neurons within ERC, and the states of inhibition and recurrent excitation among deep-layer neurons under normal and epileptic conditions \textit{in vivo}. Our study provides a starting point for addressing these issues and aids in a better understanding of the role of deep-layer neurons and circuits in regulating normal/pathophysiological processes within the ERC.
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Disclosures: None

Author Contributions: JP and SSK designed and conducted experiments together; SA helped with data analysis including clustering and MR with histology; SA and MR together helped SSK bring up the pilocarpine model; SSK analyzed the data and wrote the manuscript.
References


Figure Captions

Figure 1 The entorhinal cortical slice preparation used in the study. A, Low-powered image of a Nissl-stained section identifying the major anatomical landmarks: medial entorhinal area (MEA), lateral entorhinal area (LEA), presubiculum (PrS), subiculum (Sub), parasubiculum (Par), angular bundle (AB), white matter (WM), dentate gyrus (DG) and the hippocampal CA1 area. Black arrowheads indicate the borders of the ERC at the pial surface and the white arrowhead indicates the border of the medial and lateral entorhinal cortex. Dashed lines indicate the borders of the MEA and LEA and layers I-II of the MEA. The superficial layers (I-III) are separated from the deep layer (V-VI) by the lamina dissecans (l.d.). B-C, The MEA in control (B) and pilocarpine-treated epileptic rats (C). Recorded neurons were identified morphologically by biocytin-labeling (red). Counterstaining for NeuN-immunoreactivity (green) revealed the layers of the MEA (L1-LVI and l.d.) and loss of layer III neurons in the epileptic rats (boxed region). Arrowheads in the slices indicate the primary axons of the recorded deep layer excitatory neurons. D-E, Recording paradigms used in the study showing placement of stimulating (S) and recording (R) electrodes in the ERC slice preparation, rendered 3-dimesional through embossing, to assess excitability of neurons in superficial layer II (D) and deep layers V-VI of the MEA (E). F, Schematic of ERC-hippocampal (HIP) interactions via the perforant (PP), temporoammonic (TAP), Schaffer-collateral (SCP) and mossy-fiber (MFP) pathways. Sensory input (SI) to the ERC-hippocampal system converges onto superficial layers of MEA (s-MEA) and exits onto the association cortices (AC) via deep layers of MEA (d-MEA).

Figure 2 Classification of excitatory neurons in deep layer V-VI of MEA using unsupervised hierarchical cluster analysis. A, Classification based on electrophysiological parameters (Table 1). Intersection of dendrogram branches with the x-axis represent individual cells, whereas the y-
Figure 3  Anatomical and electrophysiological features of RS1 cells in deep layers V-VI of the MEA and their excitatory and inhibitory synaptic drive under normal and epileptic conditions. 

A, High-powered image of a typical RS1 neuron (arrowheads point to the proximal dendrite)
under IR-DIC optics. **B**, Morphology of a biocytin-labeled RS1 cell, rendered 3-dimesional through embossing, showing orientation of its primary dendrite (arrowheads) along the pial-white matter axis. **C**, Percentage of RS1 cells amongst all excitatory cell types recorded in the deep layers. **D-E**, Action potential waveforms of RS1 cells in response to current injections (D) and step-increments in voltage (E) at their respective resting membrane potentials. **F**, Spontaneous excitatory PSCs (sEPSCs, inward events recorded at -70 mV holding potential) and spontaneous inhibitory PSCs (sIPSCs, outward events, recorded at 0 mV holding potential) in aCSF from normal (top) and epileptic (bottom) rats. Bottom traces show time-expanded views of the regions indicated by bars in the top traces in these and all subsequent figures. Miniature or mPSCs were recorded in the presence of 1µM TTX. **G**, Summary of the passive parameters (± SEM) of RS1 cells: resting membrane potential ($V_m$), membrane capacitance ($C_m$) and membrane resistance ($R_m$) and the average frequency and amplitude of the events in the indicated number of cells under normal and epileptic conditions. Comparisons of the same parameters between normal and epileptic animals are bolded and boxed if differences between their means are statistically significant ($p < 0.05$) on the unpaired $t$-test, in this and subsequent figures.

**Figure 4** Anatomical and electrophysiological features of RS2 cells in deep layers V-VI of the MEA and their excitatory and inhibitory synaptic drive under normal and epileptic conditions. **A**, High-powered image of a typical RS2 neuron (arrowheads point to the proximal dendrite) under IR-DIC optics. **B**, Morphology of a biocytin-labeled RS2 cell, rendered 3-dimesional through embossing, showing orientation of its primary dendrite (arrowheads) along the pial-white matter axis. **C**, Percentage of RS2 cells amongst all excitatory cell types recorded in the deep layers. **D-E**, Action potential waveforms of RS2 cells in response to current injections (D)
and step-increments in voltage (E) at their respective resting membrane potentials. Note differences in the SAR (filled arrowheads) between RS1 and RS2 cells (D). F, Spontaneous excitatory PSCs (sEPSCs, inward events recorded at -70 mV holding potential) and spontaneous inhibitory PSCs (sIPSCs, outward events, recorded at 0 mV holding potential) in aCSF from normal (top) and epileptic (bottom) rats. Miniature or mPSCs were recorded in the presence of 1µM TTX. G, Summary of the passive parameters (± SEM) of RS2 cells: resting membrane potential (V_m), membrane capacitance (C_m) and membrane resistance (R_m) and the average frequency and amplitude of the events in the indicated number of cells under normal and epileptic conditions. The spontaneous excitatory and inhibitory synaptic drive to RS2 cells is not significantly altered under epileptic conditions. The frequency and amplitudes of mIPSCs could not be determined because the number of events was insufficient for reliable analysis (n.d).

Figure 5 Anatomical and electrophysiological features of IR1 cells in deep layers V-VI of the MEA and their excitatory and inhibitory synaptic drive under normal and epileptic conditions. A, High-powered image of a typical IR1 neuron (arrowheads point to the proximal dendrite) under IR-DIC optics. B, Morphology of a biocytin-labeled IR1 cell, rendered 3-dimensional through embossing, showing orientation of its primary dendrite (arrowheads) along the pial-white matter axis. C, Percentage of IR1 cells amongst all excitatory cell types recorded in the deep layers. D-E, Action potential waveforms of IR1 cells in response to current injections (D) and step-increments in voltage (E) at their respective resting membrane potentials. F, Spontaneous excitatory PSCs (sEPSCs, inward events recorded at -70 mV holding potential) and spontaneous inhibitory PSCs (sIPSCs, outward events, recorded at 0 mV holding potential) in aCSF from normal (top) and epileptic (bottom) rats. Miniature or mPSCs were recorded in the presence of 1µM TTX. G, Summary of the passive parameters (± SEM) of IR1 cells: resting
membrane potential ($V_m$), membrane capacitance ($C_m$) and membrane resistance ($R_m$) and the 
average frequency and amplitude of the events in the indicated number of cells under normal and 
epileptic conditions. Although mPSCs were not determined in epileptic rats because the number 
of cells was insufficient for reliable analysis (n.d), the spontaneous excitatory and inhibitory 
synaptic drive to IR1 cells is not significantly altered under epileptic conditions.

Figure 6  Anatomical and electrophysiological features of IR2 cells in deep layers V-VI of the 
MEA and their excitatory and inhibitory synaptic drive under normal and epileptic conditions. 
A, High-powered image of a typical IR2 neuron (arrowheads point to the proximal dendrite) 
under IR-DIC optics. B, Morphology of a biocytin-labeled IR2 cell, rendered 3-dimensional 
through embossing, showing orientation of its primary dendrite (arrowheads) along the pial- 
white matter axis. C, Percentage of IR2 cells amongst all excitatory cell types recorded in the 
deep layers. D-E, Action potential waveforms of IR2 cells in response to current injections (D-E) 
and step-increments in voltage (E) at their respective resting membrane potentials. Note 
differences in steady-state firing frequency SSFF (filled arrowheads) between IR1 and IR2 cells 
(D). F, Spontaneous excitatory PSCs (sEPSCs, inward events recorded at -70 mV holding 
potential) and spontaneous inhibitory PSCs (sIPSCs, outward events, recorded at 0 mV holding 
potential) in aCSF from normal (top) and epileptic (bottom) rats. Bottom traces show time-
expanded views of the regions indicated by bars in the top traces in these and all subsequent 
figures. Miniature or mPSCs were recorded in the presence of 1µM TTX. G, Summary of the 
passive parameters ($\pm$ SEM) of IR2 cells: resting membrane potential ($V_m$), membrane 
capacitance ($C_m$) and membrane resistance ($R_m$) and the average frequency and amplitude of the 
events in the indicated number of cells under normal and epileptic conditions. Comparisons of
the same parameters between normal and epileptic animals are bolded and boxed if differences between their means are statistically significant ($p < 0.05$) on the unpaired $t$-test, in this and subsequent figures.

**Figure 7** Anatomical and electrophysiological features of SS cells in deep layers V-VI of the MEA and their excitatory and inhibitory synaptic drive under normal and epileptic conditions. 

*A*, High-powered image of a typical SS cell (arrowheads point to the proximal dendrite) under IR-DIC optics. *B*, Morphology of a biocytin-labeled SS cell, rendered 3-dimesional through embossing, showing orientation of its primary dendrite (arrowheads) along the medio-lateral axis. *C*, Percentage of SS cells amongst all excitatory cell types recorded in the deep layers. *D-E*, Action potential waveforms of SS cells in response to current injections (D) and step-increments in voltage (E) at their respective resting membrane potentials. *F*, Spontaneous excitatory PSCs (sEPSCs, inward events recorded at -70 mV holding potential) and spontaneous inhibitory PSCs (sIPSCs, outward events, recorded at 0 mV holding potential) in aCSF from normal (top) and epileptic (bottom) rats. Miniature or mPSCs were recorded in the presence of 1µM TTX. *G*, Summary of the passive parameters (± SEM) of SS cells: resting membrane potential ($V_m$), membrane capacitance ($C_m$) and membrane resistance ($R_m$) and the average frequency and amplitude of the events in the indicated number of cells under normal and epileptic conditions. The excitatory and inhibitory synaptic drive to SS cells is not significantly altered under epileptic conditions and miniature events are not detectable (n.d).

**Figure 8** Stellate cells in superficial layer II of MEA are hyperexcitable in the same animals in which deep layer neurons are not. *A*, Morphology of a biocytin-labeled stellate cell, rendered 3-
High-powered image of a typical stellate neuron under IR-DIC optics (R: recording electrode).

Current clamp recordings of layer II cells (at the indicated mean resting membrane potentials, \( V_m \)) in normal and epileptic animals. Action potentials were evoked by stimulating fibers in layer I with a single brief current pulse at the threshold (T) for evoking an action potential and at a multiple of T (a failure at T is indicated in gray). Scatter plots of the mean number of action potentials that could be evoked as a function of stimulus intensity in superficial layer II cells (left) and RS cells in deep layer V-VI (right) of MEA in the same brain slices from rats under the indicated conditions. Numbers by the symbols indicate cells tested in the respective groups and error bars indicate SEM.

Spontaneous excitatory PSCs (sEPSCs, inward events recorded at -70 mV holding potential) and spontaneous inhibitory PSCs (sIPSCs, outward events, recorded at 0 mV holding potential) in aCSF from normal (top) and epileptic (bottom) rats. Bottom traces show time-expanded views of the regions indicated by bars in the top traces. Note the significant increase in sEPSC frequency and reduction in sIPSC frequency under epileptic conditions.

Summary of the passive parameters (± SEM) of layer II stellate cells: resting membrane potential \( V_m \), membrane capacitance \( C_m \) and membrane resistance \( R_m \) and the average frequency and amplitude of the events in the indicated number of cells under normal and epileptic conditions. Comparisons of the same parameters between normal and epileptic animals are bolded and boxed if differences between their means are statistically significant \( (p < 0.05) \) on the unpaired \( t \)-test.
Table 1. Action potential waveform properties of excitatory cells types in deep layers V-VI of the MEA in normal rats.

<table>
<thead>
<tr>
<th>Electrophysiological Parameter</th>
<th>RS1 (n = 11)</th>
<th>RS2 (n = 10)</th>
<th>IR (n = 9)</th>
<th>SS (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instantaneous firing frequency (IFF, Hz)</td>
<td>15.2 ± 2.0</td>
<td>12.0 ± 1.1</td>
<td>9.6 ± 1.4</td>
<td>n/d</td>
</tr>
<tr>
<td>Steady-state firing frequency (SSFF, Hz)</td>
<td>13.1 ± 1.5</td>
<td>10.2 ± 1.0</td>
<td>7.2 ± 1.0</td>
<td>n/d</td>
</tr>
<tr>
<td>Standard deviation of SSFF (Hz)</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>2.3 ± 0.3</td>
<td>n/d</td>
</tr>
<tr>
<td>Spike amplitude ratio (SAR)</td>
<td>1.02 ± 0.01</td>
<td>1.07 ± 0.01</td>
<td>1.06 ± 0.02</td>
<td>n/d</td>
</tr>
<tr>
<td>Sag ratio (%)</td>
<td>1.1 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>1.8 ± 0.7</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Spike afterhyperpolarization amplitude (mV)</td>
<td>10.4 ± 1.1</td>
<td>7.5 ± 0.9</td>
<td>8.0 ± 1.1</td>
<td>4.0 ± 1.3</td>
</tr>
<tr>
<td>Half-width (ms)</td>
<td>1.1 ± 0.06</td>
<td>1.1 ± 0.03</td>
<td>0.9 ± 0.03</td>
<td>0.8 ± 0.03</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>197 ± 39</td>
<td>198 ± 22</td>
<td>185 ± 18</td>
<td>185 ± 48</td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. n/d = not determined, because number of action potentials were insufficient for reliable analysis. Electrophysiological parameters were measured as described in Materials & Methods. Refer to Fig. 2 for statistical analysis of differences.
Table 2. Summary of the frequency and amplitude of PSCs in various excitatory cell types in deep layers V-VI of the MEA in normal and epileptic rats.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Control</th>
<th>Epileptic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>sEPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency (Hz)</td>
<td>0.7 ± 0.2</td>
<td>1.5 ± 0.2 **</td>
</tr>
<tr>
<td>amplitude (pA)</td>
<td>19.7 ± 1.7</td>
<td>26.4 ± 1.4 **</td>
</tr>
<tr>
<td><strong>mEPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>0.7 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>amplitude</td>
<td>23.4 ± 4.3</td>
<td>27.9 ± 1.8</td>
</tr>
<tr>
<td><strong>sIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>0.8 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>amplitude</td>
<td>42.7 ± 4.4</td>
<td>52.3 ± 3.1</td>
</tr>
<tr>
<td><strong>mIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>0.4 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>amplitude</td>
<td>51.3 ± 1.1</td>
<td>56.0 ± 4.5</td>
</tr>
<tr>
<td><strong>RS2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>sEPSCs</strong></td>
<td>(n = 10)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>frequency (Hz)</td>
<td>0.8 ± 0.4</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>amplitude (pA)</td>
<td>26.0 ± 2.5</td>
<td>31.0 ± 2.0</td>
</tr>
<tr>
<td><strong>mEPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>amplitude</td>
<td>37.2 ± 14.6</td>
<td>27.5 ± 2.7</td>
</tr>
<tr>
<td><strong>sIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>amplitude</td>
<td>56.9 ± 8.9</td>
<td>58.3 ± 7.8</td>
</tr>
<tr>
<td><strong>mIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>amplitude</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td><strong>IR1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>sEPSCs</strong></td>
<td>(n = 13)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>frequency (Hz)</td>
<td>3.1 ± 0.5</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>amplitude (pA)</td>
<td>27.3 ± 2.5</td>
<td>26.1 ± 2.5</td>
</tr>
<tr>
<td><strong>mEPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>0.7 ± 0.4</td>
<td>n/d</td>
</tr>
<tr>
<td>amplitude</td>
<td>21.2 ± 1.4</td>
<td>n/d</td>
</tr>
<tr>
<td><strong>sIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>2.6 ± 0.6</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>IR2</td>
<td>SS</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td><strong>mIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>amplitude</td>
<td>21.6 ± 1.9</td>
<td>42.0 ± 6.0</td>
</tr>
<tr>
<td><strong>sEPSCs</strong></td>
<td>(n = 28)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>frequency (Hz)</td>
<td>3.6 ± 0.4</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>amplitude (pA)</td>
<td>26.7 ± 2.0</td>
<td>27.4 ± 3.0</td>
</tr>
<tr>
<td><strong>mEPSCs</strong></td>
<td>1.4 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>amplitude</td>
<td>22.7 ± 2.6</td>
<td>22.3 ± 1.9</td>
</tr>
<tr>
<td><strong>sIPSCs</strong></td>
<td>0.5 ± 0.1</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>amplitude</td>
<td>48.5 ± 4.6</td>
<td>42.3 ± 4.9</td>
</tr>
<tr>
<td><strong>mIPSCs</strong></td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>amplitude</td>
<td>42.0 ± 6.0</td>
<td>46.3 ± 7.6</td>
</tr>
<tr>
<td><strong>sEPSCs</strong></td>
<td>(n = 10)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>frequency (Hz)</td>
<td>0.9 ± 0.3</td>
<td>1.7 ± 1.2</td>
</tr>
<tr>
<td>amplitude (pA)</td>
<td>27.4 ± 3.0</td>
<td>31.1 ± 4.4</td>
</tr>
<tr>
<td><strong>mEPSCs</strong></td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>amplitude</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td><strong>sIPSCs</strong></td>
<td>(10)</td>
<td>(4)</td>
</tr>
<tr>
<td>frequency</td>
<td>0.1 ± 0.02</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>amplitude</td>
<td>61.3 ± 5.1</td>
<td>47.0 ± 16.1</td>
</tr>
<tr>
<td><strong>mIPSCs</strong></td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>amplitude</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. n/d = not determined, because number of events or measurements were insufficient for reliable analysis. The total number of cells tested (n) is indicated. Statistically significant differences between epileptic and normal groups are indicated: **p < 0.05, ***p < 0.01, t-test.
Table 3. Summary of the frequency and amplitude of PSCs in regular- and irregular-spiking excitatory cells in deep layers V-VI of the MEA in normal and epileptic rats.

<table>
<thead>
<tr>
<th>Major Cell Type</th>
<th>Control</th>
<th>Epileptic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>sEPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>0.8 ± 0.2</td>
<td>1.5 ± 0.2 **</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>22.6 ± 1.6</td>
<td>27.3 ± 1.2 **</td>
</tr>
<tr>
<td>mEPSCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Amplitude</td>
<td>28.9 ± 6.2</td>
<td>27.8 ± 1.5</td>
</tr>
<tr>
<td>sIPSCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>0.5 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Amplitude</td>
<td>49.8 ± 5.1</td>
<td>53.6 ± 2.9</td>
</tr>
<tr>
<td><strong>mIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Amplitude</td>
<td>38.5 ± 12.8</td>
<td>58.8 ± 4.2</td>
</tr>
<tr>
<td><strong>IR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>sEPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>3.4 ± 0.3</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>26.9 ± 1.6</td>
<td>24.0 ± 1.0</td>
</tr>
<tr>
<td>mEPSCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>1.3 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Amplitude</td>
<td>22.3 ± 2.0</td>
<td>22.0 ± 1.7</td>
</tr>
<tr>
<td>sIPSCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>1.2 ± 0.3</td>
<td>0.2 ± 0.1 **</td>
</tr>
<tr>
<td>Amplitude</td>
<td>54.0 ± 4.7</td>
<td>52.9 ± 11.2</td>
</tr>
<tr>
<td>mIPSCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Amplitude</td>
<td>37.3 ± 5.2</td>
<td>51.4 ± 8.0</td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. The total number of cells tested (n) is indicated. Statistically significant differences between epileptic and normal groups are indicated: **p < 0.05, t-test.
**Figure D** shows the typical current recordings from a neuron. The y-axis represents the voltage (20 mV scale), and the x-axis is labeled as 1 s. The current is indicated as 100 pA, and the voltage is shown as 100 mV. The voltage is held at -64 mV.

**Figure F** illustrates the comparison between normal and epileptic sEPSCs and sIPSCs. The recordings are labeled as follows: normal sEPSCs, normal sIPSCs, epileptic sEPSCs, and epileptic sIPSCs. The amplitude and frequency are shown in the legend, with units of pA and Hz, respectively.

**Table G** summarizes the data for normal (nor) and epileptic (epi) conditions. The table includes columns for n (cells), V_m (mV), C_m (pF), and R_m (MΩ). The sEPSCs and sIPSCs have frequency and amplitude values. The mEPSCs and mIPSCs have n.d. for amplitude.
**A**

Micrograph images of neuronal structures with labels for WM, M, P, and L.

**B**

Magnified view of a neuronal structure labeled R.

**C**

Graphs showing action potentials under normal and epileptic conditions for T, 5T, and 8T.

**D**

Bar graphs comparing mean evoked action potentials for L2 cells under normal and epileptic conditions.

**E**

Waveforms of sEPSCs and sIPSCs for normal and epileptic conditions.

**F**

Table summarizing neural parameters:

<table>
<thead>
<tr>
<th>Condition</th>
<th>n (cells)</th>
<th>V_m (mV)</th>
<th>C_m (pF)</th>
<th>R_m (MΩ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>22</td>
<td>-57 ± 1.4</td>
<td>135 ± 13</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>epileptic</td>
<td>6</td>
<td>1.4</td>
<td>0.1</td>
<td>75</td>
</tr>
</tbody>
</table>

- Frequency (Hz)
- Amplitude (pA)