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COALESCENCE OF DEEP AND SUPERFICIAL EPILEPTIC FOCI INTO LARGER DISCHARGE UNITS IN ADULT RAT NEOCORTEX

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Abstract

Epilepsy is a disease of neuronal hyper-synchrony that can involve both neocortical and hippocampal brain regions. While much is known about the network properties of the hippocampus little is known of how epileptic neocortical hyper-synchrony develops. We aimed at characterizing the properties of epileptic discharges of a neocortical epileptic focus. We established a multi-electrode-array method to record the spatial patterns of epileptiform potentials in acute adult rat brain slices evoked by 4-Aminopyridine in the absence of magnesium. Locations of discharges mapped to two anatomical regions over the somatosensory cortex and over the lateral convexity separated by a gap at a location matching the dysgranular zone. Focal epileptiform discharges were recorded in superficial and deep neocortical layers but over superficial layers, they exhibited larger surface areas. They were often independent even when closely spaced to one another but they became progressively coupled resulting in larger zones of coherent discharge. The gradual coupling of multiple, independent, closely spaced, spatially restricted, focal discharges between deep and superficial neocortical layers represents a *possible mechanism* of the development of an epileptogenic zone.

Keywords

epileptic discharges; neocortical focus; early development of a focus

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INTRODUCTION

Epilepsy is a disease of recurrent seizures that can develop over months or years after a wide variety of brain insults. Neocortical seizures can be diverse in their clinical and electrical characteristics, depending on where they originate. In epilepsy patients, epileptic brain regions not only produce seizures, but also localized interictal epileptiform discharges (IEDs). In animal models of epilepsy, IEDs can disrupt cortical function and are often present long before seizures start (Staley et al., 2011). IEDs are commonly measured in humans with scalp or brain surface recording electrodes and are thought to be generated by the synchronization of multiple EPSPs over an extended cortical surface (Speckmann et al., 2011).

The neuronal networks generating IEDs and their cortical laminar locations have not been established conclusively. In some *in vitro* models deeper cortical layers have been implicated with discharges originating from layer V (Chagnac-Amitai and Connors, 1989; Telfeian and Connors, 1998; Richardson et al., 2007). Superficial cortical layers at the apical dendrites of pyramidal cells have also been considered a likely source of interictal discharges detected by scalp electrodes (Ebersole, 2003).

Epileptiform discharges detectable in scalp electroencephalography (EEG) recordings from established chronic foci originate from a large cortical surface of at least 6cm² and often reflect surfaces of more than 20cm² (Chang et al., 1991; Ebersole, 2003). An epileptogenic zone in its initial developmental stages may exhibit a limited spatial distribution and be generated by a less complex neuronal network. Indeed, epileptic discharges with a highly focal spatial distribution and limited spread have been observed both in rat brain slices (Tsau et al., 1998, 1999) and in human cortex *in vivo* (Schevon et al., 2008, 2010; Stead et al., 2010) and *in vitro* (Kohling et al., 2000). The relationship between these highly focal discharges and the larger discharges of an established chronic focus has also not yet been determined.

In order to define spatial locations of networks contributing to the IED development in the neocortex, we used multiple electrode arrays (MEAs) on somatosensory cortex slices from a normal adult rat, perfused with 4-Aminopyridine (4-AP) in a magnesium-free solution. MEAs allow simultaneous extracellular recordings from over widespread regions for prolonged periods of time. MEAs have already provided an important insight into the dynamics of epileptiform discharges in the immature mouse cingulate cortex (Chang et al., 2011), and hippocampus (Gonzalez-Sulser et al., 2011, 2012).

Here, we show a gradual coupling of deep and superficial epileptic discharges into a larger columnar discharge zone. This work paves the way to define the layer-specific neurophysiology also of *chronic* epileptic foci (Serafini et al., 2013).

EXPERIMENTAL PROCEDURES

Slice preparation

All studies were carried out at Wayne State University with institutional approval (AIC protocol A01-09-06) in conformity with international guidelines on the ethical use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. We chose to study slices from the somatosensory cortex of 4–5 month-old adult rats to obtain insight into the neurophysiology of mature human epileptic neocortex.

Preparation of slices was performed as previously described (Villalobos et al., 2011). The animal was anesthetized with isoflurane and euthanized by decapitation. The head was immediately placed in a low-calcium high-magnesium saline (NaCl 126 mM; KCl 3.5 mM; CaCl₂ 0.1 mM; MgSO₄ 10.0 mM; NaHCO₃ 26 mM; NaH₂PO₄ 1.25 mM; glucose 10 mM) at 2–3 °C, bubbled with 95% O₂/5% CO₂. The skull was opened and the brain was removed and incubated in a recovery chamber at 2–3 °C for 4–5 min. Parietal lobe brain was affixed to a stage with cyanoacrylate glue. Chamber was filled with ice-cold saline at 2–5 °C.

Coronal slices (370 µm) of hemispheres were cut between sections corresponding to plate 33 (Bregma 0.00) to plate 47 (Bregma –1.72) of the atlas of Paxinos and Watson (2007). The lateral suture protrusion into the ventricles was a prominent structural landmark easily visible to identify the section at which coronal slices were to be cut. Starting from the posterior part of the septum in each animal we cut only 3–4 coronal slices, each 370-µm thin to facilitate slice oxygenation (Alger et al., 1985; Jiang et al., 1991). The distance between the most anterior and the most posterior slice section was about 1.5 mm along the anterior-to-posterior axis or about 6% of the anterior-to-posterior length of the hemisphere. In our preliminary data we could not identify any obvious differences in patterns of epileptic activity between slices of more anterior level vs those corresponding to a more posterior level. Slices were transferred on a holding chamber at room temperature with physiological saline bubbled with 95% O₂/5% CO₂.

Extracellular field recordings in adult brain neocortical slices by electrode arrays

Recordings were started at least two, but no more than 8 h after the dissection. Discharge amplitudes and rates as well as their degree of spatial spreading were not different between this time period indicating persistent viability even hours after the dissection. The recording solution was: NaCl 126 mM, KCl 3.5 mM, CaCl₂ 1.1 mM, MgSO₄ 1.0 mM, NaHCO₃ 26 mM, NaH₂PO₄ 1.25 mM, glucose 10 mM.

The array was at the bottom of the chamber (like in Steidl et al., 2006) and was a 10 × 6 matrix (10 columns, 6 rows) of planar titanium electrodes, of 30-µm diameter, 500-µm inter-electrode distance and 30–50 kohm impedance (Multichannel Systems, 60MEA500/30iR-Ti, Reutlingen, Germany). Outlet cable was interfaced into a Stellate Harmonie system (25 channels Stellate-Duo amplifier or 64 channels E2 amplifier). Slices were positioned in the chamber. Excess fluid was removed. Slice position was adjusted with a needle. A platinum wire anchor with a suture thread or a mesh kept the slice in position. Solution inflow was 2 ml/min. Adult cortex has a low susceptibility to developing epileptiform activity and the somatosensory cortex is also known to be resistant to developing epileptiform activity.

Accordingly, preliminary data showed that: (i) perfusion of slices from normal animals with the above-mentioned physiological solution did not result in activity different than that of an empty perfusion chamber and (ii) epileptiform discharges were evoked *consistently* by perfusion with *two* pro-convulsant stimuli (a medium without magnesium and with 4-AP at concentrations ranging between 0.1 mM and 3 mM) more than perfusion with a single pro-convulsant stimulus. We proceeded to use two pro-convulsant stimuli because *for this study* our goal was focused in obtaining sustained and consistent discharges rather than distinguishing the activity provided by zero magnesium vs 4-AP.

A digital camera picture of the slice position was taken at the end of the recording. Data acquisition was 0.1–200 Hz. For visual display and analysis, data were filtered to 1–15 Hz, specifically to allow analysis of data with limited signal-to-noise ratio *preventing selection bias*. The typical epileptiform waveforms with established practical clinical relevance (spike-and-slow wave, sharp-and-slow waves, poly-spikes, rhythmic delta) still fall within this bandwidth. In addition, data with highest signal-to-noise ratio were reviewed at a bandwidth of 1–100 Hz: the spatial distribution of discharges appeared very similar to that at 1–15 Hz (not shown). Negative polarity signals were displayed upward.

Operative definition of discharges

On each recording day an initial assessment of at least three hours of the baseline noise was obtained with perfusion with the platinum ring anchor but without a slice. To assess for signal to noise we calculated histograms of all points for epochs of baseline activity and for epochs exhibiting discharges on visual inspection: the noise was the standard deviation of the distribution of all points and the signal amplitude was calculated as the difference between the amplitude of the discharge and the mean of baseline noise.

For the purpose of this study, we define brief electrical signals generated by the slices as “*discharges*”. These are defined operationally as those waveforms that (1) do not appear in the recording of the perfused chamber without the slice; (2) in each recorded slice occur consistently and recurrently in the same channel(s) throughout the recording and when occurring in several channels they do so within a time window of less than 500 ms. These signals exhibit a sustained baseline shift (with/without multiple phases) standing out of high-frequency baseline noise; exhibit an electrographic morphology consistent with epileptic discharges; exhibit a dose-dependent effect on their frequency and are inhibited by perfusion with an anticonvulsant.

Quantitative data analysis

Quantitative analysis is focused on six parameters: (i) spatial location of electrodes with discharges along the lateral convexity of the cortex and (ii) over the cortical layers, (iii) spatial size of foci; (iv) frequency (rate) of discharges, (v) amplitude of discharges; (vi) prevalence or lead from any layers.

Digital images defined electrode positions on the slice. Each electrode location had bi-dimensional coordinates $C(x,y)$ in the medial-to-lateral and dorsal-to-ventral axis,

respectively. To compare locations of discharges from different slices, relative depth (**rd**) of the electrode(s) with highest negative voltage was calculated as:

$$\text{rd} = \text{Distance from the cortical surface} / \text{Thickness of the cortex.}$$

Positions near the cortical surface and those at white matter interface were 0 and 1, respectively. Estimated cortical layers sizes are (mm): 0.12, 0.58, 0.46, 0.69, 0.46, for layers I, II/III, IV, V and VI, respectively.

Medial-to-lateral, ventral-to-dorsal and cortical depth values were translated onto a standardized coronal section Cresyl Violet stained slice.

Initial recordings were performed with a selection of 20 electrodes. Subsequent studies utilized 60 electrodes. For consistency, discharges spatial locations were derived only from 20 electrode recordings. Similar to human EEG readings a focus is identified as a set of spatially adjacent electrodes *consistently* firing in a coupled fashion. In such cases coupling can be identified unambiguously just by visual inspection of the traces because inter-electrode latencies are typically shorter than a second. Time-locked signals are therefore those that appear coupled within a time interval of a second. The probability of coupling of discharges of two electrodes by chance for waveforms of 1-s duration with an average inter-discharge interval of 30 s can be estimated as 0.03 and would be even lower for synchronization of three or more electrodes. The number of adjacent electrodes reflects the degree of spatial spread and can be utilized as an estimate of the spatial size of foci. Throughout this paper the size of foci is determined by counting the number of adjacent electrodes consistently firing in a coupled way. Local differences in epileptic spread between layers was estimated in two ways: (i) by counting in each layer the number of adjacent electrodes manifesting discharges and normalizing values to the spatial size of the layer; (ii) by comparing the size of foci in areas with equivalent size.

Delays between discharges in coupled electrodes are measured as the time between the voltage peaks of discharges in different electrodes. To assess for development of synchrony between different electrodes we divided recordings into sequential epochs each with 10 consecutive discharges. We plotted the number of coupled discharges in each epoch (vertical axis) as a function of the sequential epoch (horizontal axis) (see Results).

Statistical analysis was performed through a commercial spreadsheet program (Kaleidagraph, Reading, PA, USA).

RESULTS

General overview

The first part of our study is a preliminary identification and definition of discharges by assessing for electrographic morphology consistent with epileptic discharges, by verifying 4-AP concentration-dependent effect and assessing for blockage with an anticonvulsant. The second part is a classification of discharges. The third part is a measurement of the

anatomical spatial distribution of discharges. The fourth part is an attempt to identify cortical layers leading or initiating interictal discharges.

Validating the experimental protocol. Generation and identification of epileptiform discharges—

Perfusion of adult coronal rat brain slices centered over the somatosensory cortex with 4-AP in a magnesium-free extracellular solution evoked small, extracellular voltage transients with features matching those of human EEG recordings (Fig. 1A). Typical discharges appeared as sharp waves with a steep rise, a relatively slower downfall, followed by a baseline shift and by a slow after-wave. These features define an epileptic discharge in clinical EEG practice (Stern and Engel, 2013a). This waveform was observed in one or more adjacent electrodes corresponding to the discharge spread. Discharges occurred also in adjacent electrodes, that is, at a distance of one electrode (500 μ m) from another but without any coupling with each other or one another. Fig. 1B indicating foci with highly restricted spatial spreading and capable of firing independent of one another despite their close spatial proximity. Discharge frequency depended on 4-AP concentrations (Fig. 1C,D). Discharges started with latencies shorter than two minutes after a solution change and the latency to the peak response (highest rate of discharges) was 7.3 ± 0.33 min and 9.8 ± 3.7 min ($\mu \pm$ SE) for 0.3 mM and 1 mM, respectively.

After ~20 min perfusion, 4-AP was washed out. A subsequent higher 4-AP concentration produced a steep further increase in discharge frequency. We assessed whether discharges were inhibited by pharmacological treatment. In three slices discharges were completely blocked by co-perfusion with 0.5 mM kynurenic acid. Complete blockage of discharges developed within 10 min from the co-perfusion with kynurenic acid. Within 10 min of wash out there was a complete recovery of the previous discharge rate.

In these recordings discharges occurred in a semi-periodic fashion, with features resembling periodic epileptiform discharges (Fig. 2A), a well-known clinical EEG pattern (see below) (Stern and Engel, 2013b). We divided the recording into sequential epochs of 60s and we measured the discharges/min throughout the recording. Higher 4-AP doses correlated with increased discharge frequency (histograms of Fig. 1C,D). Higher 4-AP doses showed the pattern of Fig. 2A in six out of ten slices, however, for the other four slices perfusion with 1 mM 4-AP caused a rapid rundown in frequency and in amplitude. While the increase in 4-AP concentration increased the discharge rate, there was no significant change in the discharge area: the number of electrodes exhibiting discharges was $116 \pm 24\%$ ($\mu \pm$ SE; $n = 3$ slices) when increasing 4-AP concentration from 0.3 to 1 mM and changed to $92 \pm 5\%$ ($\mu \pm$ SE; $n = 6$ slices) when changing 4-AP concentration from 1 to 3 mM experiments. Exposure to higher concentrations of 4-AP results in an enhanced rate of discharges but not in an enhanced spatial spread.

In sum, waveforms with features of epileptiform discharges are evoked by pro-convulsant stimulation; their rate depends on the convulsant dose and they are blocked by a glutamate receptor antagonist. Optimal 4-AP doses to obtain stable recordings are lower than 1 mM. These data taken altogether validate our experimental protocol.

Classifying epileptic discharges—In a second series of experiments, 24 slices obtained from six animals were perfused without magnesium and with 0.1 mM 4-AP for at least 60 min as a means to characterize subtypes of epileptic activity. This concentration of 4-AP has been previously utilized in experiments with arrays of microelectrodes (Gonzalez-Sulser et al., 2011, 2012).

At least two main patterns of epileptiform activity were observed:

A: discharges that were isolated, that is, not progressing into rhythmic repetitive activity (Fig. 1A, B and Fig. 2A) recorded in 14 slices.

B: rhythmic repetitive activity recorded in 10 slices (Fig. 2B,C).

Isolated discharges not progressing into rhythmic repetitive activity (pattern A) occurred at a frequency ranging between eight and 350 events in the 60 min of recording. In 10/14 slices, discharges were quasi-periodic and synchronous (particularly toward the end of the recording session) (Fig. 2A) with inter-discharge intervals ranging between 2 s and more than 20 s. In 7/14 slices discharges exhibited larger amplitudes with higher signal-to-noise-ratio and involved more than two electrodes (see subsequent section for quantitative analyses). More detailed temporal and spatial analysis was performed on these slices. We found that these isolated discharges assumed at least three electrographic morphologies (see later).

There were also rhythmic repetitive discharges (pattern B) a pattern type commonly acknowledged as ictal activity in clinical EEG (Stern and Engel, 2013c). Of the ten slices with rhythmic repetitive discharges, in five there were 1–3-Hz rhythmic delta slow waves with or without superimposition of sharp waves (Fig. 2B). In the other five slices there were rhythmic sharp waves (Fig. 2C). It is not clear why some slices produced isolated discharges while others also produced ictal-like activity. One possibility is that inter-slice differences in the severing of inter-neuronal connections could contribute to this variability.

For the remainder of this study we focused on isolated discharges (pattern A) as these were the initial endpoint of the study and exhibited a simpler, more reproducible electrographic morphology, more amenable to quantitative analysis.

Anatomic distributions of discharges over the somatosensory cortex—A cumulative distribution of all the discharges over a schematic drawing of a slice reveals at least two main distributions (Fig. 3A). Highest discharge densities were over the parasagittal cortex and lateral convexity. These two distributions overlay with the somatosensory cortex and barrel cortex and their gap overlays with the dysgranular zone. The expression of epileptiform discharges across the somatosensory cortex is likely parcellated into zones of heterogeneous excitability and their propagation might be constrained by specific barriers and preferential pathways.

As a first step, to quantify the different cortical layers involved in generating discharges, we counted the number of electrodes involved in producing discharges as a function of cortical depth (Fig. 3B). While we found discharges present at all cortical depths, layer I exhibited the highest count of electrodes with discharges despite its narrow spatial size.

Next, we measured the field size or number of electrodes involved in a given discharge, (providing an estimate of local spread) as a function of layer depth and layer thickness.

First, we counted the number of electrodes with discharges in each layer, divided these values by sizes of layers and averaged data from different slices (Fig. 3C). Layer I exhibits significantly higher numbers of electrodes with discharges ($p < 0.001$, ANOVA) indicating a local broader spreading likely reflecting the specific neurophysiology of this layer.

Second, we divided the cortex into two zones of similar sizes: superficial layers, (comprising layers I, II/III and IV), and deep layers (comprising layers V and VI) (Fig. 3D). In superficial layers the number of adjacent electrodes with discharges ranged between 1 and 6 (2.69 ± 0.39 ; $n = 16$ different clusters of adjacent electrodes with discharges; $\mu \pm \text{SE}$). In deep layers the number of electrodes in clusters of adjacent electrodes ranges between 1 and 3 (1.9 ± 0.17 ; $n = 12$ different clusters of adjacent electrodes with discharges; $\mu \pm \text{SE}$).

Finally, we compared the average amplitudes and morphological features between superficial and deep cortical layers. Superficial layer discharges exhibited slightly lower amplitudes and lower frequency than in deeper layers: amplitudes of discharges were 23 ± 5 and 30 ± 13 μvolts (mean $\pm \text{SE}$ calculated in seven slices with high signal-to-noise ratio; the difference is not statistically significant) in superficial and deep layers, respectively, while the rate of occurrence was 90 ± 30 discharges/hour in superficial layers and 167 ± 62 discharges/hour in deep layers (mean $\pm \text{SE}$ calculated in seven slices with high signal-to-noise ratio; the difference is not statistically significant). While there was nearly a twofold increase in frequency in deep layers, there was also considerable variability between the slices.

In terms of morphology, we noted at least three patterns of activity: (1) a slow rise-time of about 100 ms (10–90%) (Fig. 3E, upper panel, black voltage trace); (2) a steep rise-time of 30 ms (10–90%) (Fig. 3E, upper panel, blue voltage trace); (3) a poly-phasic discharge with overlap of fast activity, multiple low-voltage spikes, sharp waves and slow waves (Fig. 3E, upper panel, blue poly-phasic trace). These complex features did not allow for a reliable measurement of rise-times. Pattern 1 was more common in layer I and II/III and occasionally in other layers. Most layer VI discharges (5/6) are consistent with pattern 2 (Fig. 3E lower panel). Pattern 3 was only in deep layers. Differences between superficial and deep layers suggest distinct generation mechanisms.

Does any cortical layer initiate discharges leading the other layers?—Data shown did not show any confinement of discharges to specific cortical layers but rather expression over most layers. We inquired whether any cortical layers would at least exhibit a prevalent lead in recruiting other layers. The attempt to addressing this question, as we show in the next section, encountered two main unexpected difficulties. First, the ability of any layer to recruit other layers changed throughout the course of the recording: in the initial part of the recording discharges appeared disconnected from one another and during the recording a gradual coupling is established. Second, after cortical layers appear to couple with one another, there is still no consistent lead from any layers, as the triggers and pathways of propagation are constantly shifting.

Development of coupling between superficial and deep neocortical layers. In each recording discharges initially appear independent of one another (Fig. 4A). As the recording proceeds there is gradual increase in their coupling. By the end of the recording, multiple cortical layers fire is coupled in a coherent unit (Fig. 4B).

We divided recordings into sequential epochs each with 10 consecutive discharges. We plotted the number of coupled discharges in each epoch (vertical axis) as a function of the sequential epoch (horizontal axis) (Fig. 4D). The resulting plot illustrates the *gradual* time-dependent increase in coupling. This coupling could be an important mechanism of spike development in neocortical epilepsy.

When superficial and deep discharges became coupled, activation direction varies (Fig. 5A): at times the deep layers focus preceded discharges in layer I or II/III, while at other times the direction of activation was inverted. Latencies between superficial and deep discharges were fairly constant in each slice (Fig. 5C). This flip-flop likely reflect the capacity of each discharge to fire spontaneously by itself, even when coupled, initiating the discharge of the other small foci.

DISCUSSION

We established a multi-electrode array method to record epileptiform activity in the somatosensory cortex of adult rat brain slices. Epileptic activity manifested as highly localized, discharges and could occur independently even in closely spaced zones. Locations of discharges mapped to two clusters over the parasagittal cortex and over the lateral convexity, separated by a gap corresponding to the dysgranular cortex. Discharges may initiate from any cortical layer, but those from superficial layers spread over a larger cortical surface area and this broader spread was significant in layer I, and also exhibited a slower rise-time. A gradual transition toward coupling occurred between deep and superficial layers resulting in highly synchronous vertical firing patterns by the end of the recordings. By the end of the recordings most discharges interconnected with each other or one another into larger discharge zones. While discharges at superficial and deep layers were time-locked with one another, the direction of initiation and spread continuously changed or flip-flopped between superficial and deep layers. The observations of the study define the intrinsic, broad network properties of the adult somatosensory cortex in response to an acute epileptic provocation gradually leading to larger zones of discharge.

Limitations of propagation in the somatosensory cortex

Over the lateral somatosensory cortex a region corresponding to the dysgranular zone showed no discharges. We consider two possible explanations: first, lack of connections of this region with the granular cortex and second, its possible inexcitability.

The dysgranular zone is thought to represent a different functional region than the adjacent barrel field (Rice, 1995). The absence of interconnections between barrel cortex and dysgranular cortex may possibly explain the absence of discharges in this region.

A discharge free zone might also reflect a lower intrinsic excitability or plasticity. Our observations parallel those on long-term potentiation in this brain region (Castro-Alamancos et al., 1995). Granular somatosensory cortex generates LTP (Long Term Potentiation) more easily and reliably than the adjacent agranular cortex. Similarly, the gap in discharges over the dysgranular cortex may reflect a different excitability or synaptic plasticity than in the adjacent cortices.

Distinctive discharges from superficial layers exhibit broader fields than deeper layers and may correspond to layer-specific activation of plasticity genes

The lateral field size of discharges varied as a function of the cortical depth. Layers I–IV exhibited broader fields than in layers V and VI. Traditionally, interictal discharges of scalp electrodes are thought to originate from the apical dendrites of pyramidal cells and are concentrated in superficial cortical layers. Preferential involvement of layer II/III by IEDs was identified using different preparations and techniques (Albowitz and Kuhnt, 1995; Jimbo and Robinson, 2000; Kohling et al., 2000; Borbely et al., 2006). However, Tsau et al. (1998, 1999) found discharges over multiple layers. In laminar analysis of *in vivo* human neocortex, discharges were over distinct cortical layers (Ulbert et al., 2004): those from a distant source propagated to the recording site, exhibiting a sink in layer IV; those from the recording electrode neighborhood exhibited a sink from layers I–III. In other models epileptiform activity relied mostly on layer V (Chagnac-Amitai and Connors, 1989; Telfeian and Connors, 1998; Borbely et al., 2006).

In our data, discharges over superficial layers were not necessarily more frequent or of higher voltages; rather, they *spread over larger surfaces* and this wider spread is significant in layer I. The observation of broader fields in superficial layers of this acute preparation is novel and may provide an electrophysiological correlate to molecular observations indicating MAP-kinase/CREB pathway expression in superficial layers of chronic human epileptic cortex and animal models of neocortical epilepsy (Rakhade et al., 2005, 2007; Barkmeier and Loeb, 2009; Barkmeier et al., 2012; Beaumont et al., 2012; Dachet et al., 2015). MAP-kinase CREB is a candidate to play a role in the development of interictal spikes measured from surface potential and may be due so through the strengthening of synaptic connections (Thomas and Huganir, 2004). In an alternative interpretation, the wider spread of discharges over superficial layers might possibly reflect differences between cortical laminae in viability and thickness after dissection of slices.

We also found that discharges from layers V–VI exhibited faster rise-times and poly-phasic features: discharges in deep layers may rely on different mechanisms than in more superficial layers. Previous observations indicate possible underlying neurophysiological mechanisms. First, in 4-AP perfused hippocampal slices, *slow kinetic* discharges were noted and shown to reflect GABA-activated chloride channels (De Curtis et al., 2012). Second, in 4-AP perfused neocortical slices superficial layer discharges were mediated by GABA-A receptors (Yang and Benardo, 2011). Accordingly, some of the slower discharges of superficial layers of our experiments might also depend on GABA-A receptor channels.

Epileptic foci connect with one another to form larger coherent units

MEA coverage of the neocortex thickness over an extended area allows a birds-eye view of the epileptogenic process. Epileptic activity manifested as focal, spatially restricted, independent discharges at both superficial and deep layers. These evolved into larger coherent units with both columnar and lateral spread: isolated discharges may represent an initial stage of a process leading to larger discharge zones. In an alternative interpretation, the gradual coupling might possibly reflect an accumulated effect of 4-AP rather than of recurrent epileptiform activity. Cortical epileptic discharges with a highly focal and low spread were observed previously, in humans *in vivo* (Schevon et al., 2008, 2010; Stead et al., 2010) and *in vitro* (Kohling et al., 2000) and in the rat *in vitro* (Tsau et al., 1998, 1999). Microelectrode recordings in human cortex demonstrated micro-seizures both in the epileptic foci and in the control cortex though with a lower density in controls (Stead et al., 2010).

Flip-flop of spread direction may reflect the coupling of a smaller discharge zone

The spread direction of time-locked discharges constantly flip-flopped between deep and superficial layers. Previous experimental data support bi-direction or multi-direction propagation, horizontally, between different cortical areas (Trevelyan et al., 2007); but to our knowledge the “vertical” multi-direction propagation, over a cortical column is entirely novel. Our observation closely resembles a theoretical model of epileptiform activity where bursting neurons can trigger network oscillations both between cortical layers and also between cortical areas (Van Drongelen et al., 2007).

The existence of multi-directional propagation paths suggests that, foci, even as they gradually become coupled to one another, retain the capability of independent firing. Hence, each of them can still initiate and lead the firing of adjacent zones, resulting in frequent changes in the discharge initiation and propagation path.

A working hypothesis on the early development of an epileptic focus

Based on our findings here, we hypothesize that the birth and early development of a neocortical epileptic focus consists of three successive phases of interconnection. In the first, under pro-convulsant stimulation, small populations of neocortical neurons recruit nearby cells generating independent discharges. The existence of small foci has been reported by several investigators and Fig. 1A,B show we reproduced this finding. In the second phase, these independently-firing closely spaced discharges become interconnected as shown in Fig. 4. Finally, in the third phase, bi-directional or multidirectional relays between foci become linked (Fig. 5). While this phase has been observed by other investigators, a novel concept here is that the flip-flop of the direction of spread is a reflection of the coupling of several independent small foci.

In summary, discharges with a very limited spatial spread have been reported in *in vivo* human cortex (Stead et al., 2010). Our observations describe, for the first time to our knowledge, how they may spread through interconnections leading to a transition from non-epileptic into epileptic cortex. While further *in vivo* studies will be required, neocortical

slice recordings offer potential insight into the basic underlying mechanisms more easily than *in vivo* recordings and can also be linked to molecular biological studies.

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Abbreviations

4-AP	4-Aminopyridine
EEG	electroencephalography
IEDs	interictal epileptiform discharges
MEAs	multiple electrode arrays

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Fig. 1.

Slice perfusion with 4-Aminopyridine (4-AP) and zero magnesium evokes multiple, distinct closely spaced, spatially restricted foci discharging at a dose-dependent frequency. (A) A 20-s epoch of a slice recording through MEA. When perfused with a solution without Mg and with 0.1 mM 4-AP the slice recording shows epileptiform discharges in three distinct zones, in A1-A3-B3, in B1-C3 and in B9-C7-E5. Of note these three zones of discharge appear independent of one another even as the foci A1-A3-B3 and B1-C3 are in very close proximity. Their locations over the cortical surface correspond to the parasagittal cortex (B9-C7-E5) and to the lateral cortex (A1-A3-B3, and B1-C3). Calibration bars are 30 μ V and 1 s. Negative polarity signals are plotted upward. On the right a digital image of the slice used for precise spatial localization of electrode locations from the MEA recordings. (B) Multiple independent discharges were seen at different cortical depths. This recording was performed with 19 electrodes from which two adjacent columns of four electrodes are displayed. Calibration bars are 60 μ V and 14 s. Negative polarity signals are plotted upward. The tracings show epochs exhibiting discharges. Vertical black lines separate distinct epochs. The most frequent discharges were in E1 and were independent from discharges at D1 in the same column and very close to E1. Two other discharge areas included one at C3, at intermediate layers (approximately layer IV), and a relatively large area of discharge involving multiple electrodes (A1-A3-B1-B3), corresponding to superficial cortical layers (approximately layers I and II/III). (C) Plot on the left shows a dose-dependent effect of 4-AP on the frequency of discharges from a slice perfused with 0.3 and 1 mM. Perfusion with 0.3 mM 4-AP evoked discharges at 2/min. At the end of the stimulation, the rate decreased to 1/min and when the dose was increased to 1 mM the rate was about 3–4/min. Histograms on the right show statistics ($\mu \pm$ SE) of data from three slices. # and * $p < 0.05$ ANOVA vs 0.3 mM for the wash-out and 1 mM groups, respectively. (D) Plot on the left shows a dose-dependent effect of 4-AP on the frequency of discharges from a slice perfused with 1 mM and 3 mM. Slice perfusion with 1 mM 4-AP evoked discharges at 3/min. There was no return to baseline after the wash-out, but when the slice was perfused with 3 mM 4-AP the

spiking increased to 12/min. Histogram on the right shows statistics ($\mu \pm \text{SE}$) of data from six slices. $*p < 0.05$ ANOVA vs 1 mM group.

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Fig. 2.

Slice perfusion with 4-Aminopyridine (4-AP) and zero magnesium evokes quasi-periodic and rhythmic repetitive discharges. (A) Shows an example of discharges with a quasi-periodic occurrence. Calibration bars are 200 μ V and 5.5 s. Negative polarity signals are plotted upward. Isolated discharges were seen with quasi-periodic occurrence every 10–15 s. (B) Shows rhythmic repetitive slow waves occurring A7, B7 electrodes. Toward the end of the trace the pattern evolves manifesting over C7–C9, too. Calibration bars are 150 μ V and 5.5 s. This type of activity was observed in 5/24 slices. (C) Shows rhythmic repetitive sharp waves in E7 and E9, Calibration bars are 150 μ V and 5.5 s. Negative polarity signals are plotted upward. Ictal epileptiform patterns (seizures), similar to those shown here were recorded in 5/24 slices.

Fig. 3.

Discharges are located over two distributions and over both superficial and deep layers. (A) The cumulative locations of discharges were mapped on a single coronal section to compare results from 24 different slices. Discharges were concentrated into two clusters, over the parasagittal and lateral convexity. Each unique symbol shows data of an individual slice. The gap between the two clusters corresponds to the dysgranular zone of the primary somatosensory cortex (SIDZ) separating the barrel field (SIBF) from the forelimb (SIFL) fields. The atlas picture was redrawn from Paxinos and Watson (2007). (B) Microdischarges are recorded over both superficial and deep layers. This figure shows frequency histograms of discharge locations for isolated discharges. Data show scattering over all cortical layers. There are peaks over layer I and layer VI. (C) In each cortical layer we counted the number of adjacent electrodes manifesting epileptic discharges. As different cortical layers exhibit different sizes (i.e. layer I and IV are smaller in size) we normalized the number of adjacent electrodes to the size of each layer, assuming a cortical thickness of 2.3 mm. VIa and VIb refer to the superficial and deep layer VI, respectively. Reported values are the number of adjacent electrodes per millimeter. Foci in layer I exhibits the highest number of adjacent electrodes even as layer I is small in size. *** $p < 0.001$ ANOVA. (D) Discharges of deep layers exhibit a limited spread. This figure shows the number of electrodes of microfoci in superficial layers (layers I, II/III and IV) represented in the black histogram and in deep layers (layers V and VI) represented in the blue histogram. Discharges occur in clusters of spatially contiguous electrodes and each cluster corresponds to a focus: the number of electrodes in each cluster correlates with the spatial extension of the focus, that is, with spatial size of the correspondent field potential. Numbers of contiguous electrodes were counted toward the end of the recording. The smallest foci manifest in only one to two electrodes while the largest foci manifest in three to six contiguous electrodes. Data show over superficial layers wide zones of discharge, involving often more than three adjacent electrodes. In contrast, in deep layers (layers V and VI) most discharges spread only to 1–2 adjacent electrodes. (E) At least three patterns of interictal discharges are recorded. The upper panel overlays a discharge from layer I (black trace) on a discharge from layer VI

(blue trace). The discharge from layer VI appears with a delay and exhibits a steeper rise-time. Calibration bars are 0.35 s and 25 μ V. Negative polarity signals are plotted upward. Below there is a discharge type observed only in deep layers. It exhibits a poly-phasic character. Calibration bars are 1 s and 50 μ V. Negative polarity signals are plotted upward. The lower panel shows that distinct patterns of discharge prevail in superficial and deep layers. The plot shows rise-times of discharges recorded at different cortical layers. In layers I and II/III six of fourteen discharges exhibit rise-times longer than 80 ms. In layer VI 5/6 discharges exhibit rise-times shorter than 60 ms. Data suggest that in layer VI the mechanism of generation of discharges might be different than in the other layers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Independent small-scale foci become coupled over time. (A) After perfusion with 0.1 mM 4-AP and no Mg, discharges occurred every 10–40 s. Green arrows point to discharges from deep cortical layers and pink arrows point to those from superficial layers. The upper panel shows a sample of the majority of 15 consecutive discharges after about 5 min of 4-AP were centered at E3, corresponding to deep cortical layers. There were also independent discharges, including ones centered at A3/B3, directly above the E3 discharges in superficial layers. The voltage gain is 3 μ V/division. Calibration bars correspond to 60 μ V and 3 s. Negative polarity signals are plotted upward. (B) At later time points of the recording, these two independent foci became coupled between superficial and deep layers. In a selection of 10 slices with higher voltage discharges involving multiple electrodes, this gradual increase in coupling was seen in five slices. Of note discharges from deeper layers (E3) involved fewer electrodes than those over superficial layers (A3/B3). The picture shows epochs exhibiting discharges. Vertical black lines separate distinct epochs. (C) Digital image of the slices used for precise spatial localization of electrode locations from the MEA recording. (D) The percentages of coupled discharges (vertical axis, synchrony (%)) for each 10 successive discharges (horizontal axis) are shown as a function of time. Each discharge was given a sequential number corresponding to the order in which it had occurred. Each recording was divided in intervals corresponding to these 10 successive discharges. In each we counted the number of times at least two independent foci appeared coupled. Each line corresponds to a different slice that was recorded for 60 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Superficial and deep small-scale discharges become coupled and can propagate in opposite directions. (A) A time-locked pattern of discharge propagation was commonly noted between superficial and deep cortical layers in a single vertical column. The arrows indicate the observed pathways of propagation of epileptic activity that occurred in both directions from superficial to deep layers (red arrows) and from deep to superficial layers (green arrows). On the top left, the thin vertical lines correspond to the time of the voltage peaks for a discharge in superficial layers (electrode A3) and in deep layers (electrode E3). The time interval between the two thin vertical lines is the delay between the superficial layer and deep layers. Measurements of delays were performed for all time-locked discharges. In a selection of eight slices with higher voltage discharges involving multiple electrodes, this flip-flop phenomenon was seen in five slices. Calibration bars correspond to 100 mV and to 1 s. Negative polarity signals are plotted upward. The picture shows epochs exhibiting discharges. Vertical black lines separate distinct epochs. (B) Digital image of the slice used for precise spatial localization of electrode locations from the MEA recording. (C) Discharges from superficial and deep layers were time locked with delays between 200–500 ms. Delays were measured between the voltage peaks of superficial and deep layers as shown in left of panel A. Data are from four different slices and time-delay values are plotted as a function of the sequential discharge number. A positive value was given to the delay value when the superficial layers led and the deep layers and a negative value to the delay when deep layers led and superficial layers followed. To each discharge we have given a sequential number corresponding to the order in which it had occurred and plotted the values of delay as a function of the sequential number of the discharge. Data demonstrate a recurrent inversion of the direction of propagation throughout the recording. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)