Interneurons spark seizure-like activity in the entorhinal cortex

Maxime Lévesque, Rochelle Herrington, Shabnam Hamidi, and Massimo Avoli*

^aMontreal Neurological Institute, McGill University, Montréal, QC H3A 2B4, Canada

^bDepartment of Neurology & Neurosurgery, McGill University, Montréal, QC H3A 2B4, Canada

^cDepartment of Physiology, McGill University, Montréal, QC H3A 2B4, Canada

Abstract

Excessive neuronal synchronization is presumably involved in epileptiform synchronization. However, the respective roles played by interneurons (GABAergic) and principal (glutamatergic) cells during interictal and ictal discharges remain unclear. Here, we employed tetrode wire recordings to establish the involvement of these two cell types in 4-aminopyridine-induced interictal- and low-voltage fast (LVF) onset ictal-like discharges in the rat entorhinal cortex in an in vitro slice preparation. We recorded a total of 90 single units (69 putative interneurons, 17 putative principal and 4 unclassified cells) from 36 slices, and found that: (i) interneurons (66.7%) were more likely to fire during interictal discharges than principal cells (35.3%); (ii) interneuron activity increased shortly before LVF ictal onset, whereas principal cell activity did not change; (iii) interneurons and principal cells fired at high rates throughout the tonic phase of the ictal discharge; however, (iv) only interneurons showed phase-locked relationship with LVF activity at 5–15 Hz during the tonic phase. Finally, the association of interneuron firing with interictal discharges was maintained during blockade of ionotropic glutamatergic transmission. Our findings demonstrate the prominent involvement of interneurons in interictal discharge generation and in the transition to LVF ictal activity in this in vitro model of epileptiform synchronization.

Keywords

Interneurons; Principal cells; 4-Aminopyridine; Interictal discharges; Low-voltage fast onset ictal discharges

1. Introduction

For over a century, excessive glutamatergic synchronization and decreased inhibition in neuronal networks have been postulated to represent the hallmark of epileptic disorders (Ayala et al., 1973; Jackson, 1870; Penfield and Jasper, 1954). However, growing experimental evidence suggests that inhibitory GABA_A receptor signaling is often preserved and may in fact contribute to the generation of seizures (Avoli and de Curtis, 2011; Engel,

*Corresponding author at: Montreal Neurological Institute, McGill University, 3801 University Street, Montréal, PQ H3A 2B4, Canada. massimo.avoli@mcgill.ca (M. Avoli).

Conflicts of interest

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1996). In line with this hypothesis, increased interneuron activity has been shown to occur in patients implanted with depth electrodes (Truccolo et al., 2011) and in animal models of temporal lobe epilepsy (Grasse et al., 2013; Toyoda et al., 2015) before and/or at seizure onset.

Evidence obtained from in vitro models of epileptiform synchronization indicate that the onset of ictal discharges induced by the K⁺ channel blocker 4-aminopyridine (4AP) is preceded by increased interneuron firing, which should lead to GABA_A receptor activation and thus inhibition of principal cell excitability (Uva et al., 2015; Ziburkus et al., 2006). GABA_A receptor mediated Cl⁻ influx also produces a positive feedback that contributes to the initiation of 4AP-induced ictal discharges (Lillis et al., 2012). Finally, optogenetic stimulation of interneurons after 4AP induces ictal discharges that are similar to those occurring spontaneously (Shiri et al., 2014; Yekhlief et al., 2015).

These studies are highlighting a crucial, unexpected role of GABA_A receptor-mediated signaling in the generation of interictal and ictal discharges, but the contribution of interneurons and principal cells remains to be established, and even more so in the in vitro 4AP model of epileptiform synchronization. To this end, we used tetrode wires to record single units in the entorhinal cortex (EC) of rat brain slices maintained in vitro during 4AP application. Our findings highlight the pivotal role of interneurons in the initiation of 4AP-induced interictal and low voltage fast (LVF) onset ictal events, and they suggest that similar mechanisms may underlie the generation of interictal discharges and seizures in animal models in vivo and, perhaps, in epileptic patients.

2. Material and methods

2.1. Slice preparation and maintenance

All procedures were performed in accordance with and approved by the Canadian Council of Animal Care and the McGill Animal Care Committee. All efforts were made to minimize the suffering and number of animals used. Adult male Sprague–Dawley rats (250–275 g; Charles River Laboratories, Saint Constant, Qc, Canada, n = 15) were decapitated under isoflurane anesthesia (Baxter Corporation, Mississauga, ON, Canada). The brain was quickly removed and placed in ice cold, oxygenated artificial cerebrospinal fluid (ACSF) with the following composition: 124 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 1.25 mM KH₂PO₄, 26 mM NaHCO₃, and 10 mM D-glucose. The ACSF was continuously bubbled with an O₂/CO₂ (95/5%) gas mixture to maintain the pH at 7.4. The cerebellum was severed after a rest period of 3 min and the brain was mounted on a vibratome (VT1000S; Leica, Concord, ON, Canada) for slicing. Slices with a thickness of 450 μm were then transferred to an interface chamber where they were maintained between warm (32 ± 1 °C) ACSF (pH 7.4, 305 mOSM/kg) and humidified gas (O₂/CO₂, 95%/5%). Following a recovery period of approx. 1 h, epileptiform activity was induced by continuous bath application of 4AP (50 μM; Sigma-Aldrich, Oakville, ON, Canada) at a flow rate of 2 ml/min. Recordings from the EC were continuously obtained during 4AP application for periods of up to 90 min. The ionotropic glutamatergic antagonists [2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 10 μM; Sigma-Aldrich, Canada) and [3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid] (CPP; 10 μM; Sigma-Aldrich, Canada)

were applied 40 min following the appearance of 4AP-induced spontaneous epileptiform activity.

2.2. Single unit and field potential recording

Eight tungsten tetrode wires with shank diameters of 0.05 mm and impedances of 0.5–1.2 MΩ were inserted into individual microdrives that were mounted into a drive (NLX-18) (Neuralynx, Bozeman, MT). The distance between tetrodes was 0.5 mm. A ground wire was connected to the table and one channel of a tetrode wire placed in the EC was used as reference. Tetrodes were placed above the horizontal slice (Fig. 1A), slowly lowered using a micromanipulator into the EC and left in place for approximately 1 min. If no single unit activity was recorded, tetrodes were moved to another region of the EC. When single unit activity was visible, it was recorded for 10 min after which the tetrode wires were moved to another region of the EC. Recordings were performed with the software Neuroware (2.1) from Triangle Biosystems (Durham, NC, USA). Data were acquired at a sampling rate of 20 kHz, filtered between 300 and 3000 Hz to facilitate the identification of single units and between 1 and 70 Hz for visualizing “slow” field potential signals. After each recording session, raw data were transferred to another computer equipped with Matlab (7.11.0) (The Mathworks, Natick, MA, USA) for clustering and further analysis.

2.3. Clustering and identification of cell types

Filtered signals (300–3000 Hz) were first analyzed with an unsupervised cluster cutting algorithm (Wave Clus) (Quiroga et al., 2004). Briefly, discharges 5 standard deviations above the threshold were considered as putative single units and merged according to selected sets of wavelet coefficients. This step was followed by manual clustering, during which the experimenter selected for further analysis single units with less than 2% of discharges in the refractory period (<3 ms). When more than one unit was detected on a single tetrode, pairs of clusters were merged together and if no discharges occurred during the refractory period, they were considered as a single unit. The action potential of a single unit recorded on a tetrode wire was usually visible on at least 2 channels, and differed in amplitude between channels (Gray et al., 1995).

A set of features from the waveform shape of the action potential was then obtained for each unit in order to classify them as interneurons or principal cells. Then, based on the channel in which action potentials with the highest amplitudes were observed, we calculated the amplitude from the trough to the following peak, the peak amplitude asymmetry, the duration of the repolarization time and the width of action potential at 50% amplitude (Sakata and Harris, 2009). Autocorrelograms were computed with action potentials occurring outside of ictal discharges for each recorded unit.

2.4. Single unit-interictal discharge relationships

We analyzed single unit activity during isolated interictal discharges, the last interictal discharge preceding ictal onset and the “sentinel” spike immediately preceding (and thus appearing to initiate) the LVF onset ictal discharge (Fig. 1B). Perievent time histograms (5 ms bins) were built for each single unit, with time 0 representing the onset (first deviation from baseline) of interictal discharges (Fig. 1Ba). Using Wilcoxon rank sum tests, we

calculated for each single unit the changes in firing rate around the onset of the slow interictal discharges by comparing a time window of 500 ms starting at the interictal discharge onset to another time window 1.5 to 2 s before onset. The level of significance was established at $p < 0.05$.

2.5. Single unit-ictal discharge relationship

The first deflection from baseline associated to the “sentinel spike” that preceded LVF onset ictal discharges was used as time of initiation (i.e., onset) of the ictal activity (Fig. 1Bb). The end of the ictal discharge was considered as the return to baseline activity. A time period of 10 s before the onset was defined as pre-ictal period and a time period of 10 s after the end of the ictal discharge was defined as post-ictal period. In order to evaluate the change in firing rate as a function of time, we compared time periods of 3 s before ictal onset to another time window of 3 to 6 s before onset using Wilcoxon’s sign rank tests. To account for differences in duration, ictal events were transformed into a scale from 0 (beginning of the ictal discharge) to 100% (end of the ictal discharge). The level of significance was established at $p < 0.05$.

During an ictal discharge, the tonic phase was identified based on the occurrence of low-amplitude field oscillations at 5–15 Hz. These periods of activity were identified with the use of custom-built algorithms in Matlab. Briefly, EEG recordings were analyzed using the spectrogram method, and calculated using the discrete short-time Fourier transform. Data were downsampled to 2000 Hz and were low-pass filtered by a 100th order finite impulse response filter. The spectrogram was then elaborated from the data set and separated into 1-s intervals (134 points) to which a Hamming window was applied. The discrete Fourier transforms were evaluated over 4096 points with zero padding. A multiparametric algorithm was used to identify oscillations in the 5–15 Hz band of the spectrogram. For each time slot, the algorithm identified the peak frequency in the band of interest and calculated the energy within a 1-Hz band centered on this peak. An oscillation event was identified by a succession of peaks with a specific track in time and frequency. The relative peak intensities must not have varied in time by $>90\%$ per second, successive peak frequencies must not have varied by more than 7.5 Hz per second, and there must have been a continuous track of candidate peaks at least 2 s long. The power spectral density of the low voltage fast frequency band (5 to 15 Hz) was normalized in amplitude from 0 to 1 and equalized with an exponential function with exponent 0.15. A representative ictal discharge containing a tonic phase with oscillations between 5 and 15 Hz is shown in Fig. 1B.

3. Results

3.1. Epileptiform activity induced by 4-aminopyridine

As reported in previous in vitro studies (Avoli et al., 1996, 2013), bath application of 4AP induced interictal and ictal discharges in the EC. In these experiments, interictal discharges consisted of isolated “slow” interictal events (Fig. 1Ba) that lasted on average $0.7 (\pm 0.2)$ s and occurred at an interval of $45.3 (\pm 17)$ s ($n = 142$ interictal events) while ictal discharges were characterized by an initial (also termed “sentinel”) spike (Fig. 1Bb) that resembled an isolated slow interictal event and was followed by LVF activity at 5–15 Hz. This LVF

activity, which corresponded to the tonic phase of the ictal discharge, evolved into an electrographic period of clonic discharges characterized by high amplitude low frequency spikes. Ictal discharges, which terminated with a period of post-ictal depression, lasted 56.5 (± 42.9) s and occurred every 195.7 (± 88.3) s ($n = 21$ ictal events).

3.2. Clustering of single units

Out of a total of 78 recordings obtained from 36 slices, 90 single units were recorded under 4AP alone. Spikes from action potentials are shown on continuous traces from the four channels of a tetrode in Fig. 1C. As specified in the Methods section, several criteria were used to classify single units as putative interneurons or principal cells (Sakata and Harris, 2009), but combining the width of the action potential measured at 50%, the amplitude from the trough to peak and the peak amplitude asymmetry provided the best separation (Fig. 1D). Applying k-means cluster analysis on these values, we identified two groups of units: putative interneurons ($n = 69$) and putative principal cells ($n = 17$), which showed values on the classification criteria that were in the range of those obtained with *in vivo* recordings (Sakata and Harris, 2009) (Fig. 1E). Cells with maximal distances from the centroid on one or more of the three parameters were considered as unclassified ($n = 4$) and were excluded from further analysis.

Clusters of single units selected for analysis had to be clearly distinguished from background noise. Spatial projections of clusters from an interneuron and a principal cell are shown in Fig. 2Aa and Ba, respectively. As shown in detail in Fig. 2Ab, interneurons tended to show a symmetric action potential whereas the asymmetry of the action potential was more pronounced in principal cells (Fig. 2Bb). The shape of the autocorrelograms also differed between each cell type, since interneurons showed a slow decay between 0 and 100 ms lags (Fig. 2Ac), whereas principal cells showed peaks within 0 and 10 ms followed by a rapid decrease (Fig. 2Bc), as previously reported *in vivo* (Csicsvari et al., 1998).

3.3. Single unit activity and interictal discharges

The firing pattern of 69 interneurons was analyzed during the occurrence of interictal discharges. This analysis showed that interneuron firing was tightly coupled to the occurrence of interictal discharges (Fig. 3A), and 46 out of 69 interneurons (66.7%) showed a significant increase in firing rates after the onset of field interictal events (Fig. 3C). The firing rate of the remaining interneurons showed no significant change ($n = 21$) or significantly decreased after interictal discharge onset ($n = 2$). The maximum probability of firing of interneurons was reached at the peak of interictal discharges (Fig. 3C). Principal cells also tended to fire action potentials in correspondence with the interictal discharges (Fig. 3B), but were less likely to show significant increases of firing rates; specifically 35.3% (6/17) of principal cells showed a significant increase in firing rates after the onset of interictal discharges, with a peak in firing that coincided with the peak of the interictal discharges (Fig. 3D).

We then analyzed the firing rate of interneurons associated with all interictal discharges, with the last interictal discharge occurring before an ictal discharge, and with the sentinel spike that preceded the LVF activity occurring during the ictal discharge. We found that

firing rates before the sentinel spike were significantly higher compared to firing rates before the last interictal discharge preceding the ictal discharge and firing rates before isolated interictal discharges ($p < 0.05$) (Fig. 4). Inter-neurons also fired at higher rates before the last interictal discharges preceding the ictal discharge compared to before isolated interictal discharges ($p < 0.05$) (Fig. 4). In contrast, principal cells did not show significant change of firing rates during the sentinel spike. These results suggest that a progressive increase in interneuron excitability leads to the occurrence of interictal discharges (the sentinel spikes) that trigger LVF ictal discharges in the EC.

3.4. Single unit activity and ictal discharges

We selected for analysis interneurons ($n = 35$) and principal cells ($n = 12$) that generated action potentials with waveforms that could be identified throughout the ictal discharge (Fig 5A, a to c panels) (cf., Truccolo et al., 2011). The firing rates of interneurons significantly increased in a time window of 3 s before ictal onset (Wilcoxon's sign rank test, $p < 0.01$), whereas principal cells did not show any significant change (Fig. 5B). This increase corresponded to the time period during which the sentinel spike occurred. The initiation of ictal events was then associated to large increases of activity in both interneurons and principal cells ($p < 0.01$), mainly during the first quartile (25%) of the ictal discharge (Fig. 5B, C); this period corresponded to the tonic phase of the ictal discharge that was characterized by oscillations between 5 and 15 Hz, and was associated to high synchrony in interneuron firing. As illustrated in Fig. 5Ba, cross-correlational analysis ($n = 16$ pairs of interneurons) indicated that at the onset of ictal discharges, interneurons showed synchronized firing, with a significant peak (more than 2 standard deviations) at zero time lag in the cross-correlogram. This was not observed at the end of the ictal discharges (Fig. 5Bb) thus indicating that high coupling between interneurons characterizes the onset of LVF ictal discharges in the EC.

Next, we established whether the firing of interneurons and principal cells was related to the frequency of 5–15 Hz field oscillations (average duration = $4.9 (\pm 2.6)$ s, mean frequency = $7 (\pm 2.1)$ Hz, $n = 51$ runs) during the tonic phase (Fig. 6A). Twenty-seven interneurons were studied during the tonic phase; the remaining interneurons ($n = 8$) were recorded during ictal discharges which did not contain runs of 5–15 oscillations. This type of analysis revealed that interneuron activity was phase-locked to these field oscillations, with a tendency to fire near the trough of the oscillations (Fig. 6B), at a preferred phase between 90° and 150° (Fig. 6B). We also analyzed the firing pattern of 8 principal cells during the tonic phase but unlike interneurons these neurons did not show phase locking relationships with the 5–15 Hz field oscillations (Fig. 6C, D). The remaining 4 principal cells were recorded in coincidence with ictal discharges that did not contain 5–15 Hz oscillations or did not fire during the tonic phase.

3.5. Single unit activity after blockade of glutamatergic transmission

Interictal discharges induced by 4AP in the EC as well as in other limbic structures continue to occur during blockade of ionotropic glutamatergic transmission and are mainly contributed by GABA_A receptor signaling (Avoli et al., 1996; Avoli and de Curtis, 2011). Therefore, we hypothesized that single unit-interictal discharge relationships would not

change following the application of NMDA and non-NMDA receptor antagonists. To test this hypothesis, we recorded single unit activity under 4AP and during addition of the ionotropic glutamatergic receptor blockers CPP and NBQX. As action potential waveforms were not affected by application of CPP + NBQX (Fig. 7A), we used the same clustering method as the one used for single units recorded under application of 4AP only. A total of 69 interneurons and one principal cell were recorded under CPP/NBQX from 10 slices (Fig. 7B), and nine of them were recorded both under control (4AP) and then under CPP + NBQX treatment.

In keeping with previous studies (Avoli et al., 1996, 2013), ictal discharges were abolished by CPP + NBQX application while interictal discharges lasting $0.7 (\pm 0.3)$ s continued to occur every $35.7 (\pm 7.5)$ s ($n = 148$); these values were not significantly different from those recorded under control (4AP only) conditions. During blockade of glutamatergic transmission, interneurons tended to fire in association with interictal discharges and in 60% of cases ($n = 27/45$), interneurons showed a significant increase of firing rates during interictal discharges; this value as well was similar to what was seen under control conditions (Fig. 7D). The maximum probability of firing of interneurons during interictal discharges was also reached at the peak, as previously observed in the presence of 4AP only (Fig. 7C).

However, compared to the activity patterns observed under 4AP, interneurons recorded under CPP/NBQX had a tendency to show prolonged firing discharges (defined as more than 3 action potentials with inter-spike intervals lasting less than 3 s). Specifically, during application of 4AP only, interneurons fired action potentials in coincidence with interictal spikes for $1.7 (\pm 1.1)$ s, whereas under CPP/NBQX these discharges lasted $19.9 (\pm 21.7)$ s ($p < 0.001$) (Fig. 7D). Interestingly, the first action potential of these prolonged discharges occurred in coincidence with the peak of interictal discharges in the field potential (Fig. 7E) whereas the last action potential occurred randomly (Fig. 7F).

4. Discussion

The main findings of our study can be summarized as follows: (i) interneurons are more likely to fire in association with interictal discharges than principal cells; (ii) the pre-ictal period is characterized by increased interneuron firing that reaches its peak at ictal onset while the activity of principal cells does not change; (iii) the tonic phase of the ictal discharges is associated with high firing from interneurons that fire in a phase-locking relationship with LVF oscillations; and, finally, (iv) interneurons continue to generate action potentials in association with the interictal discharges occurring during blockade of ionotropic glutamatergic transmission.

Our findings challenge the common notion that seizures results from the excessive synchronization of excitatory neuronal networks (Ayala et al., 1973; Jackson, 1870; Penfield and Jasper, 1954). As proposed by us and other investigators (Avoli et al., 1996; Gnatkovsky et al., 2008; Uusisaari et al., 2002) GABA_A receptor signaling can indeed synchronize neuronal networks and sustain the generation of interictal spikes and seizures. We must however emphasize that our tetrode recordings were biased toward interneurons as we could

record only a relatively small number of principal cells. This is probably due to the fact that in-interneurons are active in any environment and are thus more likely to be recorded with a tetrode compared to principal cells (Henze et al., 2000). Also, since interneurons fire at high rates, spike sorting algorithms perform better when clustering single unit activity comprising a high number of action potentials (Pedreira et al., 2012); action potentials from single units with sparse firing rates, like principal cells, are thus more likely to be missed because they are merged with large clusters (Pedreira et al., 2012). Finally, the firing rates recorded in our study from interneurons and principal cells during ictal discharges were lower than what was previously obtained with intracellular recordings in the 4AP model (Lopantsev and Avoli, 1998; Uva et al., 2013). This could be due to the fact that the sorting algorithm excludes some action potentials because it cannot account for changes in their shape occurring during ictal discharges. It is well established that the shape of action potentials during ictal discharges may change as the result of the associated, sustained depolarization (Lopantsev and Avoli, 1998; Trombin et al., 2011) as well as of spatial changes in the relationship between cellular elements and recording electrode due to cell swelling (Lux et al., 1986; Olsson et al., 2006).

4.1. 4AP-induced interictal discharges are sustained by interneuron network activity

We found that slow interictal discharges that are induced by 4AP in rodent brain slices and in the guinea pig whole brain preparations (see for review Avoli and de Curtis, 2011), were associated with action potential firing from both interneurons and principal cells; however, this firing was consistently more robust in the former cell types. This evidence is in line with the view that the slow interictal spikes induced by 4AP mirror GABAergic inhibitory network synchrony as previously shown (see for review Avoli and de Curtis, 2011).

The pivotal role played by interneurons in generating 4AP-induced interictal discharges is further supported by their resistance to ionotropic glutamatergic receptor antagonists. Our tetrode recordings demonstrate that, similar to what is observed in the presence of 4AP only, interneurons continue to increase their firing rate in coincidence with the onset of interictal discharges in the presence of CPP + NBQX. However, their firing pattern changed and consisted of prolonged action potential discharges that initiated simultaneously with the occurrence of interictal discharges. To the best of our knowledge, this is the first demonstration of such an effect that could result from removal of multi-synaptic circuits that control the excitability of interneurons through the activation of excitatory glutamatergic signaling. Therefore, our findings with CPP + NBQX support the hypothesis that reciprocal glutamatergic excitatory inputs in interneuronal networks does not play a fundamental role in the generation of interictal discharges in the 4AP model and that GABAergic transmission and/or gap junction network activity (Draguhn et al., 1998; Gajda et al., 2003; Uusisaari et al., 2002) may sustain epileptiform activity.

4.2. The onset of ictal discharges may reflect high firing rates from synchronized groups of interneurons

It has been proposed that GABA_A receptor-mediated synchronization along with the concomitant increases in K⁺ leading to the recruitment of neuronal networks of increasing size represents a powerful mechanism for ictogenesis in the 4AP in vitro model (Avoli et al.,

1996; see for review Avoli and de Curtis, 2011). In keeping with this hypothesis, the firing rates of interneurons significantly increased before ictal discharges whereas principal cell activity did not change. These findings are in line with those reported by Ziburkus et al. (2006) in hippocampal slices during 4AP treatment and by Gnatkovsky et al. (2008) in the isolated brain following application of low doses of bicuculline. *In vivo*, a decrease in principal cell firing and an increase in interneuron firing has also been observed at the onset of spontaneous seizures (Bower and Buckmaster, 2008; Fujita et al., 2014; Grasse et al., 2013).

It has been proposed that firing from synchronized group of interneurons is responsible for seizure initiation in the 4AP model; in addition, this mechanism is contributed by concomitant elevations in extracellular K^+ that are larger than those occurring during interictal discharges (Avoli et al., 1996; Avoli and de Curtis, 2011). We have indeed found in our tetrode study that the sentinel spike preceding LVF ictal activity is associated with significantly higher firing rates from interneurons compared to the isolated interictal discharges. However, once ictal discharge were initiated, glutamatergic network activity was involved in the maintenance of ictal discharges as clearly suggested by the progressive increase in firing generated by principal glutamatergic cells during ictal discharge progression as well as to the ability of NBQX and CPP to block ictal activity.

Ictal discharges induced by 4AP in our study were similar to LVF onset seizures observed in the whole brain preparation (Gnatkovsky et al., 2008), in animal models of temporal lobe epilepsy (Bragin et al., 2005; Lévesque et al., 2012) and in epileptic patients (Ogren et al., 2009; Velasco et al., 2000). LVF onset seizures have been proposed to depend on inhibitory network activity (Bragin et al., 2007; Gnatkovsky et al., 2008; Lévesque et al., 2013) and their co-occurrence with ripples (80–200 Hz) further supports this hypothesis (Lévesque et al., 2012); ripples are in fact thought to reflect summated inhibitory postsynaptic potentials generated by pyramidal cells in response to inhibitory interneuron firing (Jefferys et al., 2012). Moreover, recent optogenetic studies have shown that LVF seizures rely on the opsin activation of GABAergic interneuronal networks (Shiri et al., 2014; Yekhlief et al., 2015).

4.3. Low-voltage fast activity at seizure onset reflects interneuron firing

Interneuronal activity at ictal onset was also phase-locked to LVF activity (5–15 Hz) as previously shown by Quilichini et al. (2012) in the low Mg^{2+} model of epileptiform synchronization. In addition, 20–30 Hz oscillations coinciding with sustained interneuron firing and principal cell silencing have been identified at ictal onset in the whole brain preparation (de Curtis and Gnatkovsky, 2009; Gnatkovsky et al., 2008). In line with these *in vitro* data, high synchrony between interneurons and LVF activity at 4–30 Hz was reported *in vivo* during the first 10 s following ictal onset (Grasse et al., 2013). Finally, LVF activity at seizure onset in epileptic patients was originally proposed to result from sustained firing from inhibitory interneurons (Wendling et al., 2003), and more recently demonstrated with single unit recordings by Truccolo et al. (2011), although in this last study no correlation was made with LVF activity. Therefore, our findings support the hypothesis that LVF activity at seizure onset reflects the reinforcement of inhibition mediated by the high firing

of interneurons and the high levels of synchrony within interneuronal networks (de Curtis and Gnatkovsky, 2009).

5. Conclusions

Our findings highlight the pivotal role of interneurons in the initiation and maintenance of 4AP-induced epileptiform synchronization. We propose that changes in interneuronal network activity triggers epileptiform discharges that are similar to what is observed in animals and in humans. This may explain the poor efficacy of anti-epileptic drugs that aim at potentiating GABAergic mechanisms. The development of new anti-epileptic drugs should therefore target epileptiform synchronization by modulating rather than enhancing GABA_A receptor-mediated activity. In line with this view, we have recently reported that blocking or enhancing KCC2 activity can respectively abolish or facilitate in vitro ictogenesis (Hamidi and Avoli, 2015).

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References

- Avoli M, de Curtis M. GABAergic synchronization in the limbic system and its role in the generation of epileptiform activity. *Prog Neurobiol.* 2011; 95:104–132. [PubMed: 21802488]
- Avoli M, Barbarosie M, Lücke A, Nagao T, Lopantsev V, Köhling R. Synchronous GABA-mediated potentials and epileptiform discharges in the rat limbic system in vitro. *J Neurosci.* 1996; 16:3912–3924. [PubMed: 8656285]
- Avoli M, Panuccio G, Herrington R, D’Antuono M, de Guzman P, Lévesque M. Two different interictal spike patterns anticipate ictal activity in vitro. *Neurobiol Dis.* 2013; 52:168–176. [PubMed: 23270790]
- Ayala GF, Dichter M, Gumnit RJ, Matsumoto H, Spencer WA. Genesis of epileptic interictal spikes. New knowledge of cortical feedback systems suggests a neurophysiological explanation of brief paroxysms. *Brain Res.* 1973; 52:1–17. [PubMed: 4573428]
- Bragin A, Azizyan A, Almajano J, Wilson CL, Engel J. Analysis of chronic seizure onsets after intrahippocampal kainic acid injection in freely moving rats. *Epilepsia.* 2005; 46:1592–1598. [PubMed: 16190929]
- Bragin A, Claey s P, Vonck K, Van Roost D, Wilson C, Boon P, Engel J Jr. Analysis of initial slow waves (ISWs) at the seizure onset in patients with drug resistant temporal lobe epilepsy. *Epilepsia.* 2007; 48:1883–1894. [PubMed: 17559569]
- Csicsvari J, Hirase H, Czurko A, Buzsáki G. Reliability and state dependence of pyramidal cell–interneuron synapses in the hippocampus: an ensemble approach in the behaving rat. *Neuron.* 1998; 21:179–189. [PubMed: 9697862]
- de Curtis M, Gnatkovsky V. Reevaluating the mechanisms of focal ictogenesis: the role of low-voltage fast activity. *Epilepsia.* 2009; 50:2514–2525. [PubMed: 19674056]
- Draguhn A, Traub RD, Schmitz D, Jefferys JG. Electrical coupling underlies high-frequency oscillations in the hippocampus in vitro. *Nature.* 1998; 394:189–192. [PubMed: 9671303]
- Engel J Jr. Excitation and inhibition in epilepsy. *Can J Neurol Sci J Can Sci Neurol.* 1996; 23:167–174.
- Gajda Z, Gyengési E, Hermes z E, Ali KS, Szente M. Involvement of gap junctions in the manifestation and control of the duration of seizures in rats in vivo. *Epilepsia.* 2003; 44:1596–1600. [PubMed: 14636335]

- Gnatkovsky V, Librizzi L, Trombin F, de Curtis M. Fast activity at seizure onset is mediated by inhibitory circuits in the entorhinal cortex *in vitro*. *Ann Neurol*. 2008; 64:674–686. [PubMed: 19107991]
- Grasse DW, Karunakaran S, Moxon KA. Neuronal synchrony and the transition to spontaneous seizures. *Exp Neurol*. 2013; 248:72–84. [PubMed: 23707218]
- Gray CM, Maldonado PE, Wilson M, McNaughton B. Tetrodes markedly improve the reliability and yield of multiple single-unit isolation from multi-unit recordings in cat striate cortex. *J Neurosci Methods*. 1995; 63:43–54. [PubMed: 8788047]
- Hamidi S, Avoli M. KCC2 function modulates *in vitro* ictogenesis. *Neurobiol Dis*. 2015; 79:51–58. [PubMed: 25926348]
- Henze DA, Borhegyi Z, Csicsvari J, Mamiya A, Harris KD, Buzsáki G. Intracellular features predicted by extracellular recordings in the hippocampus *in vivo*. *J Neurophysiol*. 2000; 84:390–400. [PubMed: 10899213]
- Jackson HH. A study of convulsions. *Trans St Andrews Grad Assoc*. 1870; 3:162–204.
- Jefferys JGR, Menendez de la Prida L, Wendling F, Bragin A, Avoli M, Timofeev I, Lopes da Silva FH. Mechanisms of physiological and epileptic HFO generation. *Prog Neurobiol*. 2012; 98:250–264. [PubMed: 22420980]
- Lévesque M, Salami P, Gotman J, Avoli M. Two seizure-onset types reveal specific patterns of high-frequency oscillations in a model of temporal lobe epilepsy. *J Neurosci*. 2012; 32:13264–13272. [PubMed: 22993442]
- Lévesque M, Salami P, Behr C, Avoli M. Temporal lobe epileptiform activity following systemic administration of 4-aminopyridine in rats. *Epilepsia*. 2013; 54:596–604. [PubMed: 23521339]
- Lillis KP, Kramer MA, Mertz J, Staley KJ, White JA. Pyramidal cells accumulate chloride at seizure onset. *Neurobiol Dis*. 2012; 47:358–366. [PubMed: 22677032]
- Lopotsev V, Avoli M. Laminar organization of epileptiform discharges in the rat entorhinal cortex *in vitro*. *J Physiol*. 1998; 509:785–796. [PubMed: 9596800]
- Lux HD, Heinemann U, Dietzel I. Ionic changes and alterations in the size of the extracellular space during epileptic activity. *Adv Neurol*. 1986; 44:619–639. [PubMed: 3518349]
- Ogren JA, Wilson CL, Bragin A, Lin JJ, Salamon N, Dutton RA, Luders E, Fields TA, Fried I, Toga AW, Thompson PM, Engel J Jr, Staba RJ. Three-dimensional surface maps link local atrophy and fast ripples in human epileptic hippocampus. *Ann Neurol*. 2009; 66:783–791. [PubMed: 20035513]
- Olsson T, Broberg M, Pope KJ, Wallace A, Mackenzie L, Blomstrand F, Nilsson M, Willoughby JO. Cell swelling, seizures and spreading depression: an impedance study. *Clinical Neuroscience*. 2006:140.
- Pedreira C, Martinez J, Ison MJ, Quiroga R. How many neurons can we see with current spike sorting algorithms? *J Neurosci Methods*. 2012; 211:58–65. [PubMed: 22841630]
- Penfield, W., Jasper, H. *Epilepsy and the Functional Anatomy of the Human Brain* Oxford. Little, Brown & Co; England: 1954.
- Quilichini PP, Le Van Quyen M, Ivanov A, Turner DA, Carabalona A, Gozlan H, Esclapez M, Bernard C. Hub GABA neurons mediate gamma-frequency oscillations at ictal-like event onset in the immature hippocampus. *Neuron*. 2012; 74:57–64. [PubMed: 22500630]
- Quiroga RQ, Nadasdy Z, Ben-Shaul Y. Unsupervised spike detection and sorting with wavelets and superparamagnetic clustering. *Neural Comput*. 2004; 16:1661–1687. [PubMed: 15228749]
- Sakata S, Harris KD. Laminar structure of spontaneous and sensory-evoked population activity in auditory cortex. *Neuron*. 2009; 64:404–418. [PubMed: 19914188]
- Shiri Z, Manseau F, Lévesque M, Williams S, Avoli M. Interneuron activity leads to initiation of low-voltage fast-onset seizures. *Ann Neurol*. 2014; 77:541–546.
- Toyoda I, Fujita S, Thamattoor AK, Buckmaster PS. Unit activity of hippocampal interneurons before spontaneous seizures in an animal model of temporal lobe epilepsy. *J Neurosci*. 2015; 35:6600–6618. [PubMed: 25904809]
- Trombin F, Gnatkovsky V, de Curtis M. Changes in action potential features during focal seizure discharges in the entorhinal cortex of the *in vitro* isolated guinea pig brain. *J Neurophysiol*. 2011; 106:1411–1423. [PubMed: 21676935]

- Truccolo W, Donoghue JA, Hochberg LR, Eskandar EN, Madsen JR, Anderson WS, Brown EN, Halgren E, Cash SS. Single-neuron dynamics in human focal epilepsy. *Nat Neurosci.* 2011; 14:635–641. [PubMed: 21441925]
- Uusisaari M, Smirnov S, Voipio J, Kaila K. Spontaneous epileptiform activity mediated by GABA(A) receptors and gap junctions in the rat hippocampal slice following long-term exposure to GABA(B) antagonists. *Neuropharmacology.* 2002; 43:563–572. [PubMed: 12367602]
- Uva L, Trombin F, Carriero G, Avoli M, de Curtis M. Seizure-like discharges induced by 4-aminopyridine in the olfactory system of the in vitro isolated guinea pig brain. *Epilepsia.* 2013; 54:605–615. [PubMed: 23505998]
- Uva L, Breschi GL, Gnatkovsky V, Taverna S, Curtis M. Synchronous inhibitory potentials precede seizure-like events in acute models of focal limbic seizures. *J Neurosci.* 2015; 35:3048–3055. [PubMed: 25698742]
- Velasco AL, Wilson CL, Babb TL, Engel J Jr. Functional and anatomic correlates oftwo frequently observed temporal lobe seizure-onset patterns. *Neural Plast.* 2000; 7:49–63.
- Wendling F, Bartolomei F, Bellanger JJ, Bourien J, Chauvel P. Epileptic fast intracerebral EEG activity: evidence for spatial decorrelation at seizure onset. *Brain J Neurol.* 2003; 126:1449–1459.
- Yekhlief L, Breschi GL, Lagostena L, Russo G, Taverna S. Selective activation of parvalbumin- or somatostatin-expressing interneurons triggers epileptic seizurelike activity in mouse medial entorhinal cortex. *J Neurophysiol.* 2015; 113:1616–1630. [PubMed: 25505119]
- Ziburkus J, Cressman JR, Barreto E, Schiff SJ. Interneuron and pyramidal cell interplay during in vitro seizure-like events. *J Neurophysiol.* 2006; 95:3948–3954. [PubMed: 16554499]

Fig. 1.

Interictal and ictal activity patterns. A: Schematic diagram of a horizontal slice showing the location of tetrodes (squares) in the EC. Each square represents the 4 channels of a tetrode. B: Representative example of an isolated interictal discharge (*, a), the last interictal spike preceding an ictal discharge (**), and the sentinel spike (b). An ictal discharge (dashed line) is also shown with the low-voltage fast (5–15 Hz) at onset, which is identified by a rectangle; arrows represent the time points used as onset to calculate single-unit ictal and interictal relationships. C: Field potentials (filtered between 300 and 3000 Hz) are shown to illustrate multi-unit activity that was used to identify single units. D: Parameters used to cluster single units as putative interneurons or principal cells. The width of the action potential at 50% amplitude and the trough to peak were calculated separately and combined to calculate the peak amplitude asymmetry. E: K-mean clustering analysis showing the two clusters of cells (i.e., putative interneurons and putative principal cells). Red circles indicate the centroid of each cluster.

Fig. 2. Single unit sorting. Representative features of an interneuron (A) and of a principal cell (B). Action potential waveforms recorded on the four channels of a tetrode are shown for an interneuron (Aa) and a principal cell (Ba). Note that the amplitude of action potentials differs between channels and that interneurons show a more symmetric action potential whereas in principal cells, a peak during action potential repolarization was not observed. Three dimensional representations of the clusters obtained with the cluster cutting algorithm are shown in Ab and Bb. Multiunit activity (gray circles) are separated from single units (black circles). Note in Bb that another single unit (principal cell) is visible (gray circles). Auto-correlograms for each unit are shown in Ac and Bc. Note that interneurons tended to show a slow decay between 0 and 100 ms lags whereas this decay was faster in principal cells.

Fig. 3.

Single unit activity during interictal discharges. A: Spike train of an interneuron (A) and of a principal cell (B) and their corresponding field potential. The firing of interneurons was tightly coupled to the occurrence of interictal discharges, although they could also fire outside of them. Principal cell firing was also tightly coupled to the occurrence of interictal discharges, but compared to interneurons, they were less likely to fire outside of interictal discharges. C: Raster plot of an interneuron showing activity 2 s before and after slow interictal discharges ($n = 20$ interictal discharges). The perievent time histogram of interneurons ($n = 46$) is also shown. Note the increase in firing rates at the peak of the spike component of interictal discharges. D: Raster plot of a principal cell at the onset of interictal discharges ($n = 20$ interictal discharges). Note that action potentials from principal cells mainly occurred during interictal discharges. The perievent time histogram of principal cells ($n = 17$) is also shown.

Fig. 4.

Single unit activity during isolated interictal and pre-ictal spikes. A: Firing rates of interneurons during isolated interictal discharges, during the last interictal discharge that precedes an ictal discharge and during the “sentinel” spike that leads to LVF activity. Note that the “sentinel” spike is associated to significantly higher firing rates before its onset compared to the last interictal spike preceding ictal discharges and isolated interictal discharges (* $p < 0.05$) (a). The last interictal discharge that preceded ictal discharges was also associated to significantly higher firing rates from interneurons compared to isolated interictal discharges (* $p < 0.05$) (a).

Fig. 5.

Single unit activity during ictal discharges. A: Field potential recording obtained from EC and filtered between 300 and 3000 Hz shows single unit activity. Note that the amplitude and waveforms of action potentials did not change significantly throughout the ictal discharge. B: Single unit activity from interneurons ($n = 35$) and principal cells ($n = 12$) during the pre-ictal, ictal and post-ictal periods. Note the increase in firing rate of interneurons before ictal onset ($*p < 0.01$). After ictal onset, both interneurons and principal cells showed increased firing rates ($*p < 0.01$). This period was associated to high synchrony between pairs of interneurons (a). Such effect was not observed at the end of ictal discharges (b) ($n = 16$

pairs). Note the high synchrony near zero time lag (*). The solid line represents the average of the cross-correlogram and the dashed line the threshold for significance (2 SD). C: Spike trains of two interneurons (INT 1 and INT 2) recorded on the same tetrode and the corresponding field potential showing an ictal discharge. Note that interneurons (INT) fired at high rates before and at the onset of the ictal discharge, during the tonic phase. The bottom trace shows a principal cell (PC) and two interneurons recorded during an ictal discharge. Principal cells were less likely to fire during ictal discharges.

Fig. 6.

Single unit relationship with low-voltage fast onset activity A: Spike train of an interneuron and its corresponding field potential with power spectral analysis. Low-voltage fast oscillations during the tonic phase (5–15 Hz) are highlighted (gray square). Note the progressive decrease in frequencies over time. B: Interneurons show a phase-locking relationship with the 5–15 Hz oscillations recorded during the tonic phase, and tend to fire closed to the trough of field oscillations (peak at 0°, inset), between 90° and 150° (polar plot). C: Spike train of a principal cell and its corresponding field potential with power

spectral analysis. D: Principal cells do not show any preferred phase during low voltage fast oscillations (5–15 Hz) occurring during the tonic phase.

Fig. 7.

Single unit activity during blockade of ionotropic glutamatergic transmission. A: Action potential waveform of an interneuron recorded under 4AP and CPP/NBQX. No difference was observed between the two conditions. B: K-mean clustering analysis performed with values obtained for the trough to peak, the halfwidth duration and the peak amplitude asymmetry, for single units recorded after the addition of CPP/NBQX. Single unit features were similar as the ones obtained under 4AP (see Fig 1E). C: Raster plot of an interneuron recorded under CPP/NBQX. Note the increase in firing rates after the first deflection (onset) of interictal discharges. The perievent histogram for interneurons ($n = 27$) recorded under CPP/NBQX is also shown. D: Spike train of an interneuron and its corresponding field potential under 4AP and under CPP/NBQX application. Note that CPP/NBQX abolished ictal discharges, leaving only interictal discharges to occur. Interneurons under CPP/NBQX also tended to show prolonged discharges of action potentials. The inset (a) shows the

initiation of a long-lasting discharge in coincidence with the occurrence of an interictal spike. E Average field potential centered on the first action potential of discharges recorded under CPP/NBQX ($n = 7$ interneurons, 93 discharges). Note that the first action potential of a discharge was associated to the occurrence of an interictal discharge. F: Average field potential centered on the last action potential of long-lasting discharges. Note that the last action potential of a discharge was not related with the occurrence of an interictal discharge.