Epileptiform synchronization in the cingulate cortex

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Summary

Purpose—The anterior cingulate cortex (ACC)— which plays a role in pain, emotions and behavior— can generate epileptic seizures. To date, little is known on the neuronal mechanisms leading to epileptiform synchronization in this structure. Therefore, we investigated the role of excitatory and inhibitory synaptic transmission in epileptiform activity in this cortical area. In addition, since the ACC presents with a high density of opioid receptors, we studied the effect of opioid agonism on epileptiform synchronization in this brain region.

Methods—We used field and intracellular recordings in conjunction with pharmacological manipulations to characterize the epileptiform activity generated by the rat ACC in a brain slice preparation.

Results—Bath-application of the convulsant 4- aminopyridine (4AP, 50 μ M) induced both brief and prolonged periods of epileptiform synchronization resembling interictal- and ictal-like

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Supporting Information

The following supporting information is available in the online version of this article:

Figure S1. GABA_A receptors contribute to the generation of synchronous epileptiform events recorded after ionotropic glutamatergic blockade. Small, synchronous network events occur during continuous superfusion with 4AP and concomitant application of ionotropic glutamate receptors antagonists (upper traces). Bath-application of the GABA_A receptor antagonist picrotoxin abolishes them (lower traces). The site of recording is indicated on the left of each trace (dACC, dorsal ACC; vACC, ventral ACC).

Figure S2. Effect of ionotropic non-NMDA glutamatergic receptor blockade on 4AP-induced epileptiform activity in the ACC. (A) Epileptiform activity recorded during continuous application of 4AP by three electrodes placed in a dorsal to ventral configuration as illustrated in Fig. 6. The inset shows an example of interictal spike (arrowhead). (B) Bath-application of CNQX by itself abolishes ictal discharges while decreasing the amplitude of interictal events without affecting their duration and interval. The inset shows the expanded trace corresponding to the interictal-like event indicated by the arrowhead. (C) Plot summarizing the effect of CNQX on 4AP-induced interictal activity, where it is possible to appreciate a significant reduction in amplitude. Data values are expressed as normalized to control average. Significance is indicated by the asterisk.

Figure S3. Brief epileptiform discharges are generated by the interaction of principal neurons via glutamatergic receptors. (A) Epileptiform discharges recorded during continuous application of 4AP and GABAergic antagonists (see Fig. 8 for comparison). Three electrodes were placed in a dorsal to ventral configuration as illustrated in Fig. 6. (B) These GABAergic-independent events are no longer generated after ionotropic glutamate receptors blockade by coapplication of CNQX and CPP.

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discharges, respectively. Interictal events could occur more frequently before the onset of ictal activity that was contributed by *N*-methyl-D-aspartate (NMDA) receptors. Mu-opioid receptor activation abolished 4AP-induced ictal events and markedly reduced the occurrence of the pharmacologically isolated GABAergic synchronous potentials. Ictal discharges were replaced by interictal events during GABAergic antagonism; this GABA-independent activity was influenced by subsequent mu-opioid agonist application.

Conclusions—Our results indicate that both glutamatergic and GABAergic signaling contribute to epileptiform synchronization leading to the generation of electrographic ictal events in the ACC. In addition, mu-opioid receptors appear to modulate both excitatory and inhibitory mechanisms, thus influencing epileptiform synchronization in the ACC.

Keywords

Anterior cingulate cortex; Glutamatergic mechanisms; Opioid receptors; Seizures

The anterior cingulate cortex (ACC)—also known as area 24 (Brodmann, 1909; Vogt & Peters, 1981; Jones et al., 2005)—plays several physiological roles that reveal its functional capabilities as an integrated structure of the rostral limbic system (Devinsky et al., 1995; Paus, 2001). The complexity of connections between area 24 and other brain regions, within and beyond the limbic system, accounts for such a wide variety of functional roles. The ACC is mainly an executive region, contributing to goal-directed behavior (Vogt et al., 1992; Devinsky et al., 1995) and to the modulation of autonomic responses (Neafsey et al., 1993; Critchley, 2005). In addition, the ACC receives strong input from lamina I and V nociceptive neurons through the thalamus (centrolateral nucleus and medial dorsal nucleus) and presents with a high density of opioid receptors (Lewis et al., 1983; Mansour et al., 1987; Vogt et al., 1995; Vogt et al., 2001), a characteristic shared with other brain structures involved in pain processing. Although not proved experimentally, activation of the anterior cingulate cortex is generally considered to mediate the manifestation of unpleasant emotional states and the avoidance of noxious stimuli related to chronic pain conditions, the so-called “affective component of pain” (Vogt & Sikes, 2000; Apkarian et al., 2005; Vogt, 2005; Kelly et al., 2007).

Autonomic and behavioral manifestations reflecting the variety of roles played by the ACC become evident during epileptic seizures that are characterized by motor symptoms and manifestations such as sudden fear and anguish and anxiety-related neurovegetative phenomena (Mazars, 1970). In addition, cingulate epilepsy can be accompanied by progressive cognitive impairment and psychiatric disturbances, such as obsessive-compulsive disorder (Levin & Duchowny, 1991; Guarnieri et al., 2005). These clinical signs—which are the hallmark of seizures affecting area 24—have been described by Bancaud and Talairach (1992) as part of the epileptic syndromes of frontal origin. ACC hyperactivity has also been reported in patients with nocturnal frontal lobe epilepsy, a disorder characterized by paroxysmal arousals with motor and autonomic manifestations along with psychiatric disorders (Vetrugno et al., 2005).

In spite of the role played by the ACC in partial epileptic disorders, little is known about the mechanisms leading to epileptic synchronization in this structure. Therefore, we analyzed

here the electrophysiological and pharmacological characteristics of the epileptiform activity generated in vitro by slices of the rat ACC that were superfused with 4-aminopyridine (4AP). 4AP is known to enhance neurotransmitter release from both excitatory and inhibitory synaptic terminals (Buckle & Haas, 1982) and to elicit epileptiform synchronization (reviewed by Avoli et al., 2002). Since the ACC has a high density of mu-opioid receptors, we also established the effects of a mu-opioid receptor agonist on the network activities generated by this cortical area.

Methods

Brain slice preparation and maintenance

Male, adult Sprague-Dawley rats (250–300 g) (Charles River, St. Constant, QC, Canada) were decapitated under deep isoflurane anesthesia according to the procedures established by the Canadian Council of Animal Care. All efforts were made to minimize the number of animals used and their suffering. Brains were quickly removed and immediately placed in cold (1–3°C), oxygenated artificial cerebrospinal fluid (ACSF). Coronal brain slices including the ACC (450 μm thick) were cut between the rostrum of corpus callosum and the hippocampus using a VT1000S vibratome (Leica, Nussloch, Germany). A schematic of the slice used in this study is shown in Fig. 1A. Slices were then transferred to an interface tissue chamber, lying between ACSF and humidified gas (95% O₂, 5% CO₂) at approximately 32°C and pH 7.4. ACSF composition was 124 mM NaCl, 2 mM KCl, 1.25 mM KH₂PO₄, 2 mM MgSO₄, 2 mM CaCl₂, 26 mM NaHCO₃, and 10 mM glucose. As shown in Fig. 1A, ACC slices included the ventral ACC (vACC) and the dorsal ACC (dACC), also called area 24a and 24b, respectively (Vogt & Peters, 1981; Jones et al., 2005).

Brain slices were superfused with standard ACSF for 1 h before beginning continuous application of 50 μM 4AP. The following drugs were also bath-applied: 4 μM (2S)-3-[[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenyl-methyl)phosphinic acid (CGP5-5845), 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 10 μM 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), 10 μM [D-al², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAGO), 10 μM naloxone, and 50 μM picrotoxin (PTX). Chemicals were acquired from Sigma (St. Louis, MO, U.S.A.) with the exception of CGP55845, CNQX, and CPP, which were obtained from Tocris Cookson (Ellisville, MO, U.S.A.). DAGO was applied for no longer than 30 min to minimize desensitization.

Electrophysiological recordings

Field potential recordings were made with ACSF-filled pipets (tip diameter <10 μm ; resistance, 5–10 M Ω) pulled from borosilicate capillary tubing (World Precision Instruments, Sarasota, FL, U.S.A.) using a P-97 puller (Sutter Instrument, Novato, CA, U.S.A.). Extracellular signals were fed to a Cyberamp 380 amplifier (Molecular Devices, Palo Alto, CA, U.S.A.) connected to a digital interface device (Digidata 1320A; Molecular Devices, Palo Alto, CA, U.S.A.). Two to four electrodes were placed in the ACC deep layers at approximately 700 μm from the pia (Lewis et al., 1983), approximately halfway between the pia and the subcortical white matter (Vogt & Gorman, 1982). Data were acquired at a sampling rate of 5 kHz, using the software Clampex 8.2 (Molecular Devices, Palo Alto, CA,

U.S.A.), stored on the hard drive and analyzed off-line using the software Clampfit 9.0 (Molecular Devices, Palo Alto, CA, U.S.A.).

Sharp-electrode intracellular recordings were performed using 3 M potassium acetate-filled glass pipets (tip resistance, 70–110 MΩ). Intracellular signals were fed to the high-impedance amplifier Axoclamp 2B (Molecular Devices, Palo Alto, CA, U.S.A.). The resistance compensation was monitored throughout the experiment and adjusted as required. Intracellular signals were fed to a computer interface (Digidata 1320A), acquired, and stored on the hard drive using the software pClamp 9.0 (Molecular Devices, Palo Alto, CA, U.S.A.). Subsequent analysis was made with the software Clampfit 9.0.

Measurements and statistical analysis

Measurements in the text are expressed as mean $\pm$ SEM, and n indicates the number of slices or neurons studied under each specific protocol, unless differently specified. Error bars in the graphs represent the SEM. Statistical analysis was performed using a two-tailed Student's *t*-test for paired data or analysis of variance (ANOVA) with Bonferroni post hoc test when appropriate. Results were considered significant for $p < 0.05$. Significance analysis for the patterns of interictal activity was performed by comparing the fitted curves of the cumulative mean percentage of events plotted in regard of their interevent intervals (IEIs). The pattern was described by a sigmoidal curve that was best fitted by the following logistic equation:

$$y = A2 + (A1 - A2) / (1 + (x/x_0)^p)$$

where A1 and A2 are constants set at 0 and 100, respectively, x_0 is the value of IEI corresponding to the 50% of cumulated mean percentage of events, and p represents the slope of the fitted curve.

Power spectral analysis of ictal discharges was windowed with a Hamming function.

Data were obtained from 61 slices, 14 of which were also used for intracellular recordings from 26 neurons. The intrinsic membrane properties of ACC cells ($n = 8$) included in this study were as follows: (1) resting membrane potential (RMP) after cell withdrawal = -71.6 ± 3.3 mV; (2) apparent input resistance (R_i) from the maximum voltage change in response to a hyperpolarizing current pulse (< -1 nA) = 38.4 ± 4.0 MΩ; (3) action potential amplitude (APA) from the baseline = 97.9 ± 5.0 mV; and (4) action potential duration (APD) at half-amplitude = 1.5 ± 0.2 ms. Measurements of the duration of ictal and interictal events were made from field traces. Some ACC slices showed spreading depression-like events that could appear at a variable time after bath-application of 4AP. These slices were not used for pharmacological protocols. Throughout this study, we arbitrarily termed as interictal and ictal the synchronous epileptiform events with durations shorter or longer than 4 s, respectively (cf., Fig. 1C).

Results

Field potential characteristics of the epileptiform discharges induced by 4AP

First, we performed field potential recordings in 34 slices to characterize the epileptiform activity induced by 4AP. Recording electrodes were placed in the ACC deep layers in a dorsal to ventral configuration (Fig. 1B, left inset). 4AP treatment induced epileptiform activity that was initially characterized by brief events (Fig. 1B, asterisks) and was eventually followed by the appearance of prolonged discharges (Fig. 1B, continuous line). As shown in Fig. 1C, the duration of these synchronous epileptiform events was distributed in two distinct groups: the first ranged from 0.2 to 4.0 s (mean duration = 1.4 ± 0.2 s; mean interval = 17.1 ± 0.8 s; interval range: 0.4 – 77.2 s; $n = 226$ events) defined as interictal; the second included events with duration ranging between 9.3 and 112.1 s (mean duration = 36.0 ± 3.6 s; mean interval = 338.9 ± 32.1 s; $n = 124$ events), hereafter termed ictal. Interictal activity was generated in all slices, while ictal discharges were recorded in 90.2% of them. Both types of epileptiform activity, when not abolished by pharmacological manipulations, persisted up to 6 h.

Interictal events—characterized by one or more negative- going field transients—could be further subdivided in two groups (see inset in Fig. 1C) according to a significant difference in duration ($p = 0.00002$). One group of events had duration shorter than 1.5 s (mean duration = 0.6 ± 0.1 s; $n = 126$ events), and it was defined as short-lasting interictal events; the second group included discharges lasting between 1.5 and 4.0 s (mean duration = 2.8 ± 0.1 s; $n = 100$ events), termed long-lasting interictal events. Fifty-eight percent of the slices generated only short interictal events, while 41.2% of the slices showed both short- and long-lasting interictal discharges. In 65.1% of the cases ($n = 142$ of 218 events from 15 slices), interictal discharges were initiated in the dACC and propagated to the ventral region (Fig. 1Da), while in 29.8% of the cases ($n = 65$ events), they originated in the vACC and spread to the dorsal area (Fig. 1Db). In a minority of cases (5.0% $n = 11$ events) the interictal activity was presumably generated between the two regions and then spread in both directions toward the upper and lower edges of the ACC (Fig. 1Dc).

The onset of ictal activity was usually guided by a negative- going field event with amplitude that was often larger than what seen during the interictal period (Fig. 2A, white circle). In 91.7% of the cases ($n = 22$ out of 24 events from 11 slices), this field potential initiated in the dorsal ACC and spread to the ventral area (Fig. 2Aa). Ictal discharges were characterized by both tonic and clonic electrographic components (Fig. 2A, continuous and dotted line, respectively). The tonic component occurred in the early phase of the ictal discharge and was characterized by fast oscillations at 10.1 ± 0.4 Hz ($n = 11$) (Fig. 2Ab and Ba for power spectral analysis), which were gradually replaced by clonic activity. As shown in Fig. 2A, the clonic discharges slowed down over time (mean frequency = 0.7 ± 0.1 Hz; $n = 10$) and were made of population bursts at 14.6 ± 1.0 Hz ($n = 11$) (Fig. 2Ac and Bb for power spectral analysis). Ictal discharges could be followed by afterdischarges (Fig. 2A, arrowheads) with electrographic features resembling the clonic events (Fig. 2Ad).

As illustrated in Fig. 2A, the rate of occurrence of interictal discharges increased shortly before the onset of an ictal discharge. To quantify this phenomenon, we analyzed the

normalized time courses of interictal events between subsequent ictal discharges ($n = 19$ ictal-to-ictal intervals recorded from 7 slices). Fig. 2C represents a plot of the mean number of events occurring during a normalized interictal period subdivided into 10% time bins, starting from the end of an ictal discharge (normalized time course = 0%) to the onset of the following one (normalized time course = 100%). ANOVA and the subsequent application of the Bonferroni post hoc test revealed significant differences within the time bins, identifying three phases in the interictal time course. An early phase, consisting of the first 10% of the time course, was characterized by an initial silent period of variable duration, presumably corresponding to the postictal depression phase, followed by randomly generated interictal events (mean number of events/bin = 0.6 ± 0.2). The major component of the time course (11%–90%) was represented by the subsequent appearance of interictal events generated at a low, regular rate of occurrence (mean number of events/bin = 1.4 ± 0.1). Finally, interictal events occurred at an increasing frequency until an ictal discharge was generated (mean number of events/bin = 3.6 ± 0.4). This acceleration pattern started before the onset of an ictal discharge, which corresponded to the last 10% of the time course.

Intracellular recordings from ACC neurons during 4AP application

Intracellular recordings from ACC neurons ($n = 26$) showed that field interictal discharges were mirrored by membrane depolarizations that were often capable of triggering action potential activity (Fig. 3). When the interictal activity was characterized by an alternation of short- (range 0.2–1.5 s) and long-lasting (range 1.5–4.0 s) events ($n = 17$ cells from 14 slices), we found a direct relation between the field size/duration and the membrane depolarization amplitude/amount of action potentials generated (Fig. 3A). As shown in the insets in Fig. 3Aa and 3Ab, subthreshold oscillations [presumably representing excitatory postsynaptic potentials (EPSPs)] along with “partial spikes” (presumably reflecting ectopic action potentials; arrow in inset Fig. 3Ab) could occur during these interictal depolarizations. In addition, by changing the membrane potential with steady injection of depolarizing current, we found that small interictal events were associated with slow membrane hyperpolarizations (Fig. 3B, –68 mV trace).

Interictal patterns characterized by more regular field events were associated with robust “bursting” intracellular activity ($n = 9$ neurons out of 26) (Fig. 3C). During injection of steady depolarizing current, the depolarizing envelope sustaining the action potential burst became smaller in amplitude, while the postburst hyperpolarization was enhanced in both amplitude and duration (Fig. 3C, –70 mV, asterisk). Conversely, when the ACC neuron was hyperpolarized with intracellular current injection, the interictal depolarizations increased in amplitude and were followed by a slow depolarizing event presumably representing the reversed postburst hyperpolarization (Fig. 3C, –95 mV, arrow).

Fig. 4A and 4B shows intracellular recordings during ictal events observed in the same cells depicted in Fig. 3B and 3C, respectively. The ACC neuron shown in Fig. 4A displayed at RMP (–88 mV panels) repetitive action potential discharge riding over a depolarization during the tonic (panel b) and clonic (panel c) phases. In addition, the onset of the ictal discharge (asterisk) was mirrored by a slow depolarization. During injection of steady depolarizing (Fig. 4A, –70 mV panels) or hyperpolarizing (Fig. 4A, –104 mV panels)

current, the amplitude of the depolarization sustaining single action potentials decreased or increased, respectively. This pattern of intracellular activity was recorded in 6 out of 12 ACC cells.

In the remaining experiments, ACC neurons ($n = 6$) recorded at RMP generated sustained action potential burst-like activity throughout the ictal discharge (Fig. 4B). As illustrated in Fig. 4Bb (-85 mV), the ictal event onset (asterisk) was associated with a burst of action potentials—which resembled what seen in these experiments during an interictal event (cf., Fig. 3C)—and was followed by sustained firing that eventually turned into recurring discharges during the clonic phase (Fig. 4Bc). In these neurons as well, both the initial (tonic) and late (clonic) components of the ictal depolarizations decreased or increased in amplitude when the membrane potential was depolarized (Fig. 4B, -70 mV) or hyperpolarized (Fig. 4B, -92 mV), respectively, with injection of steady current. As expected from network-driven events, we found in all experiments that changing the membrane potential of ACC neurons did not influence the rate of occurrence of interictal and ictal discharges induced by 4AP (not illustrated).

Ionotropic glutamate receptor antagonists and 4AP-induced epileptiform activity

Next, we characterized the pharmacological mechanisms underlying 4AP-induced epileptiform activity in the ACC by using field potential recordings in conjunction with ionotropic glutamatergic receptor antagonists. In a first series of experiments, we investigated the role played by *N*-methyl-D-aspartate (NMDA) receptors. As illustrated in Fig. 5A, bath-application of the NMDA receptor antagonist CPP ($10 \mu\text{M}$) readily abolished the ictal discharges, while interictal activity continued to occur in all cases ($n = 5$). This effect was reversed by prolonged (~ 30 min) washout (Fig. 5A, Wash). No significant effect was observed on the duration (control = 1.71 ± 0.29 s; CPP = 1.47 ± 0.14 s; $n = 101$ and 99 events from 5 slices, respectively; $p > 0.05$) or on the mean interevent intervals (control = 12.6 ± 0.9 s; CPP = 11.2 ± 0.4 s; $n = 101$ and 99 events from 5 slices, respectively; $p > 0.05$) of the interictal activity during this pharmacological procedure. Moreover, CPP did not affect the pattern of occurrence of interictal events as confirmed by quantifying the normalized distribution of these discharges in control, during bath-application of CPP and after washout of this drug (Fig. 5, B–D).

Next, we tested the effects induced by concomitant application of the AMPA/kainate receptors antagonist $10 \mu\text{M}$ CNQX, and $10 \mu\text{M}$ CPP. This pharmacological procedure abolished ictal discharges (Fig. 6A, +CNQX+CPP). In addition, the interictal activity was reduced in amplitude (control = 1.0 ± 0.2 mV, range: 0.6 – 1.5 mV; +CNQX+CPP = 0.4 ± 0.1 mV, range: 0.1 – 0.6 mV; $n = 5$; $p = 0.02$) and increased in duration (control = 1.4 ± 0.1 s, range: 1.3 – 1.5 s; +CNQX+CPP = 5.7 ± 1.5 s, range: 1.4 – 8.7 s; $n = 5$; $p = 0.02$) while its rate of occurrence demonstrated a not significant decrease (interval in control = 30.7 ± 7.9 s, range: 12.1 – 47.3 s; +CNQX+CPP = 64.0 ± 14.1 s, range: 24.7 – 106.3 s; $n = 5$). As illustrated in Fig. 6A (expanded samples), the interictal events recorded in the presence of CNQX+CPP were characterized by a prominent positive-going component. Finally, these synchronous potentials appeared to be randomly initiated in each area of the slice from where they spread with variable modalities.

We found that application of the mu-opioid receptor agonist DAGO (10 μ M, $n = 5$) markedly decreased the rate of occurrence of these events (mean interval = 104.2 ± 21.3 s, range: 61.0–165.0 s; $n = 5$; $p = 0.005$) (Fig. 6A, +DAGO), while the GABA_A receptor antagonist picro-toxin (PTX; 50 μ M) abolished them ($n = 14$) (see supplementary Fig. S1). Fig. 6B summarizes the data obtained for the event interval duration under control conditions, during subsequent application of CNQX+CPP and after further application of DAGO.

Next, we sought to determine the role of ionotropic non-NMDA receptors in the generation of interictal discharges, since this activity appeared to be affected only when both CNQX and CPP were applied (see Fig. 6A). We therefore investigated the effect of bath-application of CNQX by itself on 4AP-induced interictal-like activity (supplementary Fig. S2). Interestingly, this pharmacological procedure not only abolished ictal discharges, but also yielded a significant decrease in the amplitude of interictal events (control: 1.26 mV; +CNQX: 0.53 mV, 42.1% of control; $p = 0.004$; $n = 5$).

Effect of mu-opioid receptors on 4AP-induced epileptiform activity

Field potential recordings were also used to investigate the effects induced by mu-opioid agonism on the epileptiform discharges in the ACC. DAGO (10 μ M) was bath-applied after induction of robust ictal discharges by 4AP treatment (Fig. 7A, Control). Ictal discharges were abolished in 7 out of 8 slices (Fig. 7A, +DAGO), while a decrease in ictal discharges occurrence was observed in 1 slice. These effects were reversed by bath-application of 10 μ M naloxone in 5 slices (Fig. 7A, +Naloxone).

The interevent intervals of the interictal activity were also analyzed to better assess the effects induced by DAGO on the 4AP-induced interictal discharges. The presence of multiple components in the histogram obtained under control conditions (Fig. 7B) was changed by bath-application of 10 μ M DAGO. Interictal events occurred more regularly, and the most represented components of interevent intervals were 10–15 s (Fig. 7C). These effects were partially reversed by the opioid antagonist naloxone (10 μ M; $n = 4$ slices) (Fig. 7D). To further analyze this phenomenon, we compared the fitted curves of cumulative mean percentage of events plotted in regard of their interval in control conditions and in the presence of DAGO. We found a significant change in the slope of the fitted curves, as illustrated by the inset in Fig. 7C (control = 2.7 ± 0.3 ; DAGO = 17.0 ± 6.8 ; $n = 5$; $p = 0.04$). Interestingly, the mean percentage of events occurring within 5 s interval significantly dropped during bath-application of DAGO (control: $18.0\% \pm 4.9\%$; DAGO: $2.6\% \pm 1.6\%$; $n = 5$; $p = 0.01$). We also investigated whether mu-opioid receptors antagonism by itself was capable of inducing any changes in network excitability. Hence, we bath-applied 10 μ M naloxone during 4AP treatment ($n = 5$). This pharmacological procedure failed to affect both interictal (control: duration = 1.2 ± 0.3 s; interval = 20.5 ± 5.1 s; naloxone: duration = 1.1 ± 0.2 s; interval = 21.0 ± 6.7 s; not significant) and ictal activity (control: duration = 49.5 ± 9.5 s, interval = 340.2 ± 57.3 s; naloxone: duration = 52.3 ± 10.3 s, interval = 320.1 ± 40.2 s; nonsignificant) in all the slices (data not shown).

Effects of GABA receptor antagonists on 4AP-induced epileptiform activity

Finally, we investigated the role of GABAergic transmission on the epileptiform activity generated by ACC networks during application of 4AP. PTX (50 μ M) and CGP55845 (4 μ M) were added to the medium to antagonize GABA_A and GABA_B receptors, respectively. Ictal events occurring during control conditions (duration = 19.6 ± 4.8 s; interval = 323.0 ± 53.4 ; n = 6) were abolished by GABAergic antagonism. Moreover, this pharmacological procedure transformed the epileptiform activity recorded under control conditions into a pattern of recurrent synchronous discharges that were characterized by an initial positive-going event followed by a population afterdischarge (Fig. 8A, +PTX+CGP55845; n = 6). This type of activity was significantly different when compared to ictal discharges recorded under control conditions (duration = 2.6 ± 0.3 s, range: 1.5–3.4 s; interval = 15 ± 0.7 s, range: 12.5–17.2 s; n = 6; p = 0.01 and p = 0.001, respectively) and was thus considered as interictal (see for comparison Fig. 1C). Moreover, these synchronous events were abolished by subsequent application of ionotropic glutamate receptors antagonists (n = 6; see online supplementary material, Fig. S3). Bath-application of the mu-receptor agonist DAGO (10 μ M, Fig. 8A, +DAGO) significantly increased both the duration and the interval of occurrence of the events recorded during application of 4AP and GABA antagonists (duration = 3.1 ± 0.2 s, range: 2.6–3.9 s, n = 6, p = 0.02; interval = 21.0 ± 1.1 s, range: 18.6–25.6 s, n = 6, p = 0.002). In addition, DAGO abolished the initial positive component of the interictal events generated during application of 4AP+PTX+CGP55845. The histograms in Fig. 8B summarize the effect induced by DAGO on the duration and interval of occurrence of the interictal events recorded after GABA receptor blockade.

Discussion

The purpose of this study was to characterize the cellular and pharmacological features underlying epileptiform synchronization of rat ACC networks maintained in vitro in a slice preparation. Epileptic syndromes of frontal origin, including nocturnal frontal lobe epilepsy, are known to involve the ACC (Bancaud & Talairach, 1992). Our findings demonstrate that, when treated with 4AP, ACC networks generate interictal discharges caused by the synergistic activation of glutamatergic and GABA_A receptors; this activity, however, persists when either of these mechanisms is pharmacologically blocked. We have also found that in most experiments interictal discharges accelerate before the onset of ictal events. In addition, NMDA receptors are essential for the occurrence of ictal discharges that are abolished by pharmacological procedures aimed at reducing GABA receptor signalling. Finally, mu-opioid receptors can modulate both GABAergic and glutamatergic network events generated by the ACC during application of 4AP.

ACC networks generate interictal and ictal discharges in vitro

We have found that 4AP-treated ACC networks are capable of producing interictal and ictal events with electrographic characteristics that are similar to those identified in several limbic areas of adult rodents (cf., Avoli et al., 2002). Both interictal and ictal events originated in most experiments from the ACC dorsal region and eventually propagated to the ventral area. The mechanisms responsible for the propensity of dorsal ACC neurons to initiate epileptiform events remain unclear. It should be, however, noted that in a minority of slices

epileptiform discharges could begin in the ventral area or in between, and then spread to the other regions of the ACC with different modalities. Hence, as highlighted by the morphological study by Jones and coworkers (2005), these results indicate the existence of complex intrinsic connections within the ACC.

The identification of a similar topographic initiation for both interictal and ictal events also suggests that these two types of epileptiform activity are interrelated. In line with this view, we have found that the rate of occurrence of interictal discharges recorded from ACC slices challenged with 4AP increases shortly before the onset of an ictal event. The role of interictal spikes in the initiation of ictal discharges remains contradictory (cf., de Curtis & Avanzini, 2001; Avoli et al., 2002, 2006). However, as discussed below, the possibility that local interictal activity can lead to ictogenesis in the ACC is in line with previous data obtained from cortical structures such as the adult rat entorhinal cortex (Avoli et al., 1996; Lopantsev & Avoli, 1998; Bruckner et al., 1999; Bruckner & Heinemann, 2000), the juvenile hippocampus (Avoli et al., 1993), and the human dysplastic neocortex (D'Antuono et al., 2004). A positive relation between interictal discharges and electrographic seizures was originally identified in vivo in the focal penicillin model (see for review Dichter & Ayala, 1987).

Interictal discharges are contributed by ionotropic non-NMDA glutamatergic and GABA_A receptor-mediated signaling

Interictal discharges induced by 4AP in ACC slices were not influenced by NMDA receptor blockade. Moreover, further application of the AMPA/kainate antagonist CNQX failed in abolishing or significantly reducing their rate of occurrence. This pharmacological procedure, however, decreased the amplitude of the interictal events while modifying their shape. This provides evidence for the role of ionotropic non-NMDA receptor in the generation of interictal events and bath-application of CNQX by itself corroborated our hypothesis. Finally, the events recorded during ionotropic glutamate receptors blockade were abolished by the GABA_A receptor antagonist picrotoxin or reduced by DAGO, which is known to activate mu-opioid receptors, thus decreasing GABA release from interneurons (Madison & Nicoll, 1988; Capogna et al., 1993; Tanaka & North, 1994). Therefore, GABAergic interneuron activation may underlie the generation of interictal discharges, mainly through GABA type A-mediated conductances.

The participation of GABA receptor-mediated mechanisms to the interictal discharges induced by 4AP was also evident in the intracellular recordings obtained from ACC neurons. First, we found that interictal depolarizations associated with small amplitude field events became hyperpolarizing when the neuronal membrane was set to values less negative than -80 mV, which corresponds to the reversal potential of GABA_A-mediated conductances. Second, even the most robust interictal events were often mirrored by depolarizations that were ridden by series of EPSPs and were unable to elicit sustained action potential discharge but only partial spikes. These intracellular characteristics are reminiscent of those reported in the hippocampus (Michelson & Wong, 1991; Perreault & Avoli, 1992), in the entorhinal cortex (Lopantsev & Avoli, 1998), and in the neocortex (Avoli et al., 1994; Benardo, 1997) during 4AP application. The view that GABA receptor-mediated mechanisms can per se

sustain neuronal network synchronization is also in keeping with evidence obtained in neocortical (Köhling et al., 1998) and subicular slices (Cohen et al., 2002) obtained from human epileptic tissue.

NMDA and GABA receptor-mediated mechanisms are essential for ictogenesis in the ACC

We have also found that application of the NMDA receptor antagonist CPP is sufficient to abolish ictal discharges generation induced by 4AP in the ACC, suggesting that ictogenesis in this cortical area primarily depends on NMDA receptor activation. Similar findings have been reported in the guinea pig ACC (Higashi et al., 1991) as well as in limbic areas such as the entorhinal cortex (Avoli et al., 1996; Lopantsev & Avoli, 1998) or the amygdala (Benini et al., 2003). In line with evidence obtained in these studies, we have found that blockade of GABA receptor signaling is also effective in abolishing ictal discharges while unmasking recurrent epileptiform events. In addition, intracellular recordings, performed at different membrane potential values, suggest that GABA_A receptors play an important role in the onset of ictal events. Taken together, our findings indicate that in the ACC glutamatergic conductances are necessary but not sufficient to sustain the generation of ictal discharges and suggest that GABA receptors play a pivotal role in sustaining prolonged periods of epileptiform synchronization and thus ictal activity.

Work performed in limbic networks treated with 4AP has shown that synchronous events contributed by GABA_A receptor-mediated conductances can facilitate the onset of ictal discharges. This paradoxical excitatory effect exerted by GABA_A receptor activation during an interictal discharge may result from: (1) the depolarizing shift of the GABA_A reversal potential caused by the accumulation of $[Cl^-]_i$ (Staley & Proctor, 1999) along with (2) a transient increase in $[K^+]_o$, which, in turn, should contribute to recruiting excitatory and inhibitory neurons through synaptic and nonsynaptic mechanisms (Avoli et al., 1996; Köhling et al., 2000; Voipio & Kaila, 2000; Gigout et al., 2006). Given the presence of GABA receptor-mediated mechanisms during the interictal discharges generated by ACC networks, it is conceivable to conclude that also in this structure interictal activity may contribute to ictal discharge onset via a GABA_A receptor-mediated mechanism.

Mu-opioid receptors modulate epileptiform activity in the 4AP-treated ACC

In line with the evidence that mu-opioid receptor activation exerts an inhibitory effect on interneurons, we have found that bath-application of the mu-opioid receptor agonist DAGO exerts a significant depressant effect on the rate of occurrence of pharmacologically isolated GABAergic events. Accordingly, pharmacological procedures aimed at decreasing GABAergic signaling, such as mu-opioid agonism, should affect the generation of ictal discharges. Our observations have confirmed this hypothesis. Moreover, mu-opioid receptor activation was capable of changing the pattern of occurrence of interictal events recorded during 4AP treatment, restraining the acceleration of interictal spikes observed before the onset of an ictal discharge.

We have also found that DAGO modified the pattern of the recurrent, interictal epileptiform discharges recorded in the ACC during concomitant application of 4AP and GABA receptor antagonists. These events are presumed to reflect the interaction of principal neurons via

glutamatergic receptors, as confirmed by further application of ionotropic glutamate receptors antagonists. Hence, our findings suggest that mu-receptor activation can influence glutamatergic mechanisms in this cortical area. The role of mu-opioid receptors in the ACC is indeed contradictory. It has been demonstrated that inhibitory postsynaptic potentials (IPSPs) but not EPSPs recorded from layer V neurons are affected by mu-opioid receptor activation (Tanaka & North, 1994). However, morphological experiments performed by Vogt and colleagues (1995) have lead to hypothesize an input/output model for the regulation of ACC excitatory activity through pre- and postsynaptic mu-opioid receptors located in superficial and deep layers respectively. Consistently, Ostermeier et al. (2000) have hypothesized a presynaptic inhibition of glutamate release in layer II/III neurons, and recently Hao and coworkers (2005) have reported that morphine decreases extracellular glutamate concentration in freely moving rats. In support of the latter findings, taken together, our results indicate that, at least in our slice preparation, mu-opioid receptors may modulate both excitatory and inhibitory network activity.

The analgesic function of the opioid system is well established in patients affected by chronic pain condition (Inturrisi, 2002), where ACC hyperactivity has been proved to occur (Wei & Zhuo, 2001; Baliki et al., 2006; Gao et al., 2006). The high density of opioid receptors in the ACC has traditionally linked this cortical area to the study of pain. On the other hand, the involvement of area 24 in pain-related affective responses shares similar mechanisms of hyperexcitability with epileptiform synchronization (Coull et al., 2003). However, the role of opioid receptors in epileptic disorders still remains controversial. Recent evidence suggests that changes in transmission mediated by endogenous opioid occur during spontaneous seizures in patients affected by TLE (Hammers et al., 2007). Whether opioid system activation may lead to or control epileptic seizures still returns ambiguity. The effect of opioids differs among cortical regions, exhibiting anticonvulsant effects on neocortical structures and mu-opioid receptors have been proposed to act postictally as a self-limiting strategy to terminate the seizure and induce refractoriness (Tortella et al., 1988). Here, we have corroborated this hypothesis, showing that mu-opioid receptor activation can control seizures in the 4AP model of epilepsy in the ACC.

Conclusive Remarks

Our findings indicate that network-driven epileptiform events generated by the ACC are remarkably similar to those identified in other limbic structures, such as the entorhinal and perirhinal cortices and the amygdala, when challenged with 4AP. On the other hand, 4AP-induced epileptiform activity generated by ACC networks is unique in that interictal events consistently exhibit an acceleration pattern culminating with the generation of an ictal-like discharge. This aspect supports the hypothesis that interictal events may sustain the synchronization that leads to the generation of ictal-like activity. Interestingly, this typical pattern of occurrence was disrupted by application of a mu-opioid agonist, which also abolished ictal discharges. Therefore, our findings indicate that activation of mu-opioid receptors may represent a future perspective in the control of cingulate epilepsy.

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Figure 1.

Epileptiform activity induced by 4AP in the ACC. **(A)** Drawing of the medial surface of the rat brain (left) depicting the tissue block from which ACC slices were obtained (black); the two ACC subregions included in the slice are also indicated (dACC, dorsal ACC; and vACC, ventral ACC). On the right is the schematic of a coronal slice including the ACC region (black solid outline). **(B)** Typical pattern of epileptiform activity recorded during bath-application of 4AP with four extracellular electrodes that were placed in the ACC deep layers in a dorsal to ventral configuration (left inset). Interictal and ictal discharges are identified with asterisks and solid line, respectively. **(C)** Histogram of the duration of the epileptiform events ($n = 350$) recorded from 34 experiments. The gap between 4 and 9 s suggests that epileptiform discharges could be categorized in two main groups: interictal (up to 4 s) and ictal discharges (longer than 9 s), the latter spreading across a wide range of duration. The histogram in the inset, which provides the distribution of the interictal events duration at a higher resolution, indicates the presence of short (less than 1.5 s) and long-lasting (1.5 and 4 s) interictal discharges. **(D)** Expanded traces obtained from the experiment shown in panel A suggest different modalities of initiation. The arrows shown on the left of each panel indicate the spread modality illustrated by the traces.

Figure 2.

Ictal activity exhibits multiple components and is preceded by an acceleration of interictal events. **(A)** Ictal discharge recorded with four extracellular electrodes placed in a dorsal to ventral fashion as indicated on the left inset in Fig. 1B. The upper trace illustrates the ictal discharge at slow time base, while selected portions of the recording are expanded in panels **a** to **d**. The ictal onset coincides with a slow negative-going event (white circle) that originates from the dorsal ACC (panel **Aa**); the ictal discharge is characterized by an initial tonic phase (solid line), consisting of oscillatory activity (panel **Ab**), followed by clonic events (dotted line) characterized by population bursts (panel **Ac**). The arrowheads in the slow time base recording identify afterdischarges that could follow the ictal episode and resembled the clonic events (panel **Ad**). **(B)** Power spectral analysis of the tonic (**Ba**) and clonic (**Bb**) events demonstrated peak amplitudes corresponding to approximately 10 and 15 Hz, respectively. **(C)** Normalized time course of the occurrence of interictal events recorded between two subsequent ictal discharges. Data were obtained from 19 intervals in 7 slices. Note that the number of events is significantly different during the interictal period and suggests the presence of an initial phase (1), presumably corresponding to postictal depression and characterized by the appearance of randomly generated interictal events, a middle phase (2), during which interictal events occurred regularly, and a preictal phase (3), characterized by interictal acceleration and corresponding to the last 10% of the normalized time course.

Figure 3.

Intracellular characteristics of 4AP-induced interictal events in ACC. **(A)** Field and intracellular recordings in a slice generating short- and long-lasting interictal events. **(Aa)** The short-lasting discharge corresponds to an intracellular depolarization sustaining one action potential along with low-amplitude events (expanded trace in the inset) presumably representing EPSPs. **(Ab)** The prolonged interictal discharges are associated with repetitive action potentials along with series of EPSPs and ectopic action potentials (arrow head in the inset where calibration bars correspond to 3 mV and 22 ms). **(B)** Low amplitude field interictal events correspond to depolarizations at RMP that become bigger or invert in hyperpolarizations when intracellular hyperpolarizing or depolarizing steady current is injected, respectively. **(C)** Regular interictal field events correspond to intracellular action potential bursts which are followed by a hyperpolarizing phase (asterisk) when the neuron is recorded at depolarized potentials (left). Depolarizing postbursts become larger (arrow) when the neuron is hyperpolarized by current injection (right).

Figure 4.

Intracellular characteristics of 4AP-induced ictal discharges in ACC. **(A)** ACC neurons displayed a weak intracellular activity during field ictal events. This neuron never performed a burst at the onset of the ictal event (expansions **b**) nor showed action potential in correspondence of the preictal event (asterisks) (see for comparison panel **(B)**). The depolarizing envelope is bigger at more hyperpolarized potentials. During the clonic phase (expansions **c**) the neuron shows single action potentials without depolarizing after potentials at each membrane potential tested (see for comparison expansion **c** in panel **(B)**). In the bottom trace, the dotted line indicates an afterdischarge represented by sets of burst. **(B)** Fifty percent of the neurons recorded presented with a stronger activity. Ictal field events triggered a burst (asterisks), followed by a long lasting depolarization sustaining action potentials when the neuron was recorded at resting membrane potential (middle panel, expansion **b**), as well as at depolarized (upper panel, expansion **b**) or hyperpolarized (lower panel, expansion **b**) membrane values. The depolarizing envelope sustaining action potentials was larger at more hyperpolarized membrane potentials than at more positive values. Sets of action potentials with a pronounced depolarizing after potential occurred during the clonic phase of ictal events (expansions **c**).

Figure 5.

NMDA receptors are involved in ictal discharges generation. (A) Field potential recordings obtained from the ACC (same recording configuration as in Figs. 1 and 2) under control conditions, in the presence of 10 μ M CPP and after wash. This pharmacological procedure abolishes in a reversible manner the ictal activity without any apparent effect on interictal discharge occurrence. (B–D) Normalized distribution of the interevent intervals (IEIs) of the interictal activity recorded under control conditions (B), during CPP application (C), and after wash (D). NMDA receptor antagonism does not affect the IEI variability. This is better illustrated by the fitted curves of cumulative mean percentages of events obtained for control conditions and subsequent bath-application of CPP (panel C, inset).

Figure 6.

Effects of ionotropic glutamate receptor blockade on epileptiform discharges. **(A)** Field potential recordings obtained with three electrodes under control conditions, in the presence of 10 μ M CNQX and 10 μ M CPP, and after additional application of DAGO. Asterisks indicate the interictal events that are expanded in the right panels. Interictal discharges decrease in amplitude, increase in duration and change in shape during glutamatergic receptor blockade, showing a prominent positive-going component. Further application of DAGO slows down the rate of occurrence of the glutamatergic-independent events. **(B)** Summary of the effect induced by ionotropic glutamatergic receptor antagonists and subsequent application of DAGO on the rate of occurrence of interictal events. Note that a significant decrease in interictal activity frequency is caused by DAGO only (asterisk).

Figure 7.

Epileptiform activity in the ACC is modulated by mu-opioid receptors. **(A)** Ictal activity induced by 4AP (Control) no longer persists after bath-application of the mu-opioid agonist 10 μ M DAGO; this effect is reversed by the opioid antagonist 10 μ M naloxone. **(B–D)** Normalized distribution of the interevent intervals (IEIs) of the interictal activity recorded under control conditions **(B)**, during DAGO **(C)** and after further application of naloxone **(D)**. Interestingly, when mu-opioid receptors are activated by DAGO, a major IEI component, ranging 10 to 15 s, emerges. This effect is reversed by the opioid antagonist naloxone. The inset in panel **C** shows the fitted curves of cumulative mean percentage of events obtained for control conditions and subsequent application of DAGO, revealing a significant change in the slope as the consequence of mu-opioid receptor activation.

Figure 8.

Effects of mu-opioid receptor agonism on the synchronous events recorded in presence of GABAergic antagonists. **(A)** Interictal and ictal activity induced by 4AP (Control) is transformed into recurring synchronous discharges during GABA receptor antagonism (+PTX+CGP55845). Subsequent mu-opioid receptor activation (+DAGO) decreases the rate of occurrence of these GABA-independent events. The events indicated by the asterisks are expanded in the right inserts; the recurrent epileptiform discharge seen in the presence of PTX+CGP55845 consists of an initial positive-going event, followed by an afterdischarge.

(B) Plots of the effects induced by DAGO on the duration (left) and interval of occurrence (right) of the GABA-independent events.