Different Inhibitory Modalities Shape Rhythmic Activity Generated by Anterior Cingulate Cortex Networks

G. Panuccio[1,2], M. D'Antuono[1], A. Colosimo[2], G. Cruccu[3] and M. Avoli[4]

[1]Montreal Neurological Institute and Departments of Neurology & Neurosurgery, and of Physiology, McGill University, Montreal, QC, H3A 2B4, Canada; [2]Dipartimento di Fisiologia Umana e Farmacologia, [3]Dipartimento di Scienze Neurologiche and [4] Dipartimento di Medicina Sperimentale, University of Rome "Sapienza", Rome, Italy

Abstract – Generation of rhythmic activity by neuronal networks may represent the epiphenomenon of pathological conditions that underlie several neurological disorders such as cingulate epilepsy, a type of partial epilepsy affecting the anterior cingulate cortex (ACC), and accompanied by progressive cognitive impairment and psychiatric disturbances. We recently discovered that GABA-mediated neurotransmission plays a pivotal role in the generation and maintenance of epileptiform discharges generated by ACC networks in an acute *in vitro* model of epilepsy. Here we report that the ACC can generate recurrent network events when maintained *in vitro* by perfusion with extracellular medium close to physiological conditions. Field potential and intracellular recordings from the ACC were performed using coronal brain slices obtained from young-adult rats. When slices were superfused with normal medium, we could observe the generation of synchronous network-driven events occurring at ~0.15 Hz. Simultaneous field potential and intracellular recordings demonstrated that this activity corresponded to synaptic depolarizations. We sought to determine the role of GABAergic network activity in the generation of these spontaneous events. Therefore, we performed pharmacological manipulations that interfere with interneuron function and found that activation of *mu*-opioid receptors, which are known to control GABA release from presynatic terminals, significantly decreased bursting activity in ACC neurons while slowing down the occurrence of spontaneous network events. In addition similar effects were observed during application of gap junction decouplers. In conclusion, our findings demonstrate that ACC networks generate synchronous events that result in part from interneuron synchronization through gap junctions. We propose that this inhibitory drive may promote and sustain cortical rhythmic activity.

Key Words: Anterior cingulate cortex; interneurons; gap junctions; *mu*-opioid receptors.

INTRODUCTION

The anterior cingulate cortex (ACC) or area 24 (Brodmann, 1909) is part of the cingulate gyrus, the most extensive anatomical component of the limbic system. The wide variety of roles played by the ACC (Vogt et al., 1992; Devinsky et al., 1995; Paus et al., 2001) becomes evident during epileptic seizures affecting this cortical area (Mazars, 1970). Cingulate epilepsy is a partial epileptic disorder accompanied by progressive cognitive impairment and psychiatric disturbances described by Bancaud and Talairach (1992) as part of epileptic syndromes of frontal origin.

Interneurons appear to play a major role in synchronizing neuronal networks toward the generation of epileptiform recurrent events in several brain regions (e.g., Avoli et al., 1996; Cohen et al., 2002; Perez-Velazquez and Carlen, 2000). We recently reported that GABAergic transmission plays a central role in the generation of epileptiform activity by ACC networks in an acute *in vitro* model of epilepsy (Panuccio et al., 2008). Synchronous inhibitory network activity may be sustained by gap-junction interactions among interneurons (Deans et al., 2001).

In this study we report that ACC slices maintained *in vitro* at physiological condition can generate spontaneous rhythmic activity, even when not challenged by convulsants. In addition, we addressed the role of interneuron activity as a possible mechanism concurring to the generation of these events. To this aim, we employed field potential and intracellular recordings from the mid-deep layers of the ACC using a slice preparation. Some of these findings were presented in abstract form (Panuccio et al., 2006 and 2008).

METHODS

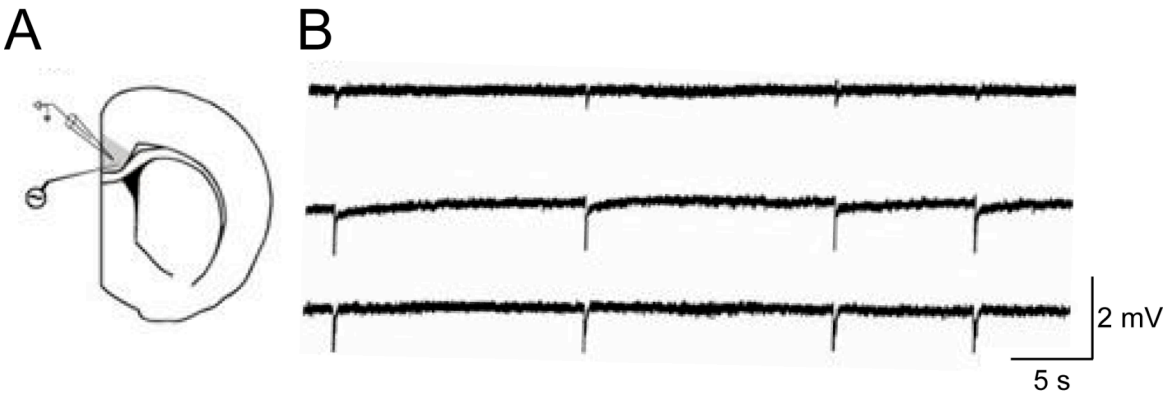
Male, Sprague-Dawley rats, 3-5 weeks old, were decapitated under isoflurane anesthesia according to the procedures established by the Canadian Council of Animal Care. All efforts were made to minimize the number of animals used and their suffering. Brains were quickly removed and placed in cold (1-3^{o}C), oxygenated artificial cerebrospinal fluid (ACSF). Coronal brain slices (450 µm thick) including the (ACC) were cut between the rostrum of corpus callosum and the hippocampus using a VT1000S vibratome (Leica, Nussloch, Germany). Slices were then transferred to an interface tissue chamber, lying between ACSF and humidified gas (95% O_2, 5% CO_2) at 31-32°C and pH 7.4, where they were allowed to recover for ≥ 1 h before beginning any experimental protocol. ACSF composition was (in mM): NaCl 124, KCl 3, KH_2PO_4 1.25, $MgSO_4$ 1-2, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10. The following drugs were also bath applied: carbenoxolone and [D-ala^2, N-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO). DAGO was bath-applied for no longer than 30 minutes to minimize desensitization. Chemicals were acquired from Sigma (St. Louis, MO, USA). Data were acquired at a sampling rate of 5 KHz, using the software Clampex 8.2 (Molecular Devices), stored on the hard drive and analyzed off-line using the software Clampfit 9.0 (Molecular Devices).

Measurements are expressed as mean±SEM and *n* indicates the number of slices or neurons studied. Data were compared with the paired Student's t-test and were considered statistically significant if $p<0.05$.

RESULTS

Spontaneous network events in the ACC.

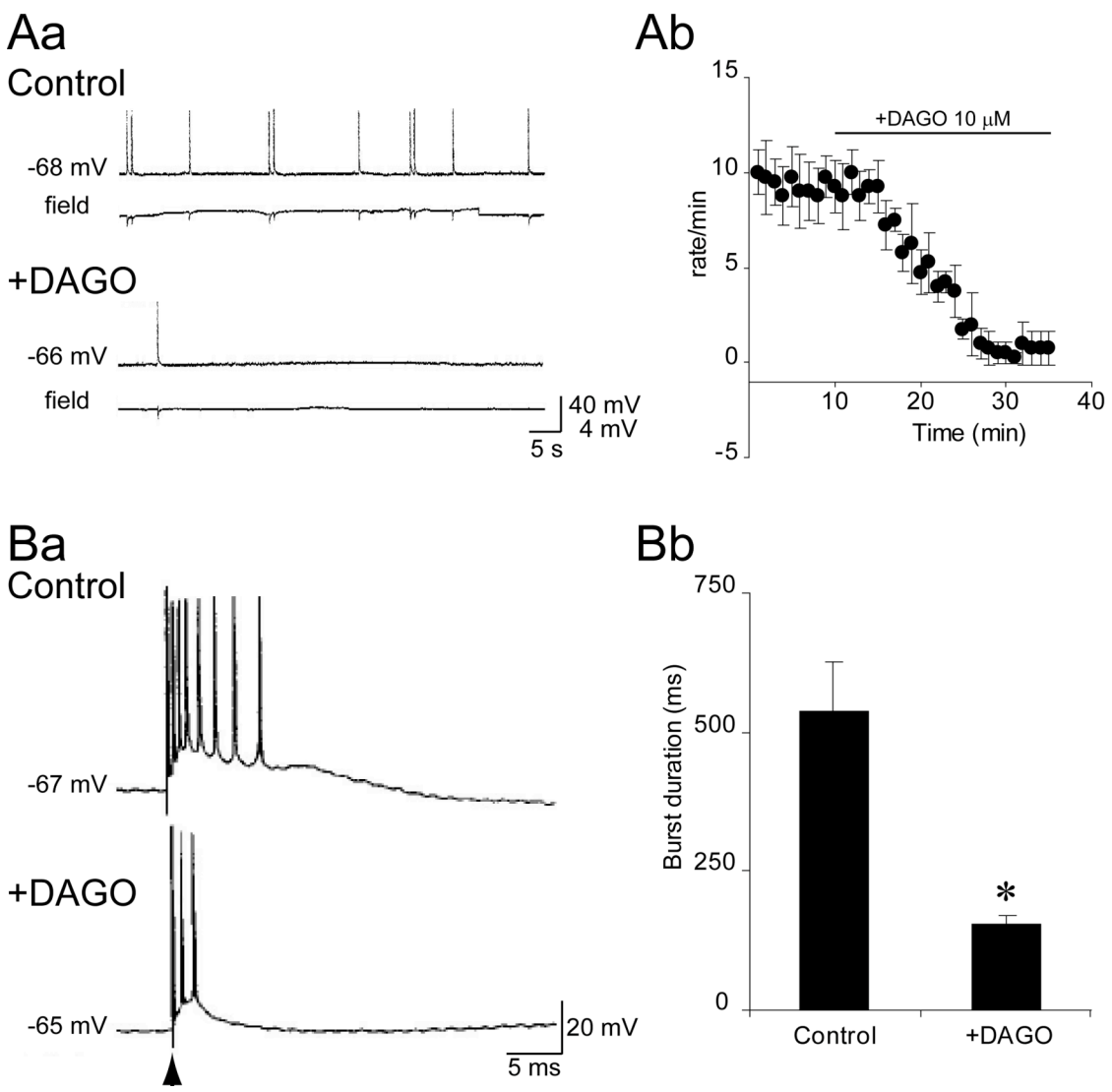
ACC coronal slices superfused with normal medium generated network events that were made of field transients occurring at ~0.15 Hz (mean interval: 6.8±1.1 s; n= 4; Fig. 1).



– Fig. 1 –

Spontaneous network events are generated in the ACC during perfusion with normal medium. A. *Schematic drawing of a coronal brain slice including the ventral ACC (grey area), showing the site of recording and stimulation (when applicable).* ***B.*** *Recurring synchronous discharges generated by ACC networks during perfusion with physiological medium.*

Intracellular recordings (n= 7) demonstrated that each field event corresponded to a membrane depolarization that could trigger action potential firing (Fig. 2Aa, Control). Activation of *mu*-opioid receptors by the exogenous selective agonist DAGO (10 µM, fig. 2Aa, +DAGO) slowed down and eventually abolished these network-driven events. The overall mean interval of occurrence increased to 11.9±2.2 s (p= 0.03; n= 4). Fig. 2Ab shows the time course of the effect of bath-application of DAGO on the frequency of ACC network events. At the cellular level, this pharmacological procedure decreased the duration of stimulus-induced burst responses in ACC neurons (figs. 2Ba and Bb; Control: 536±80.7 ms, +DAGO: 153.3±15.9 ms; p= 0.006; n= 6).

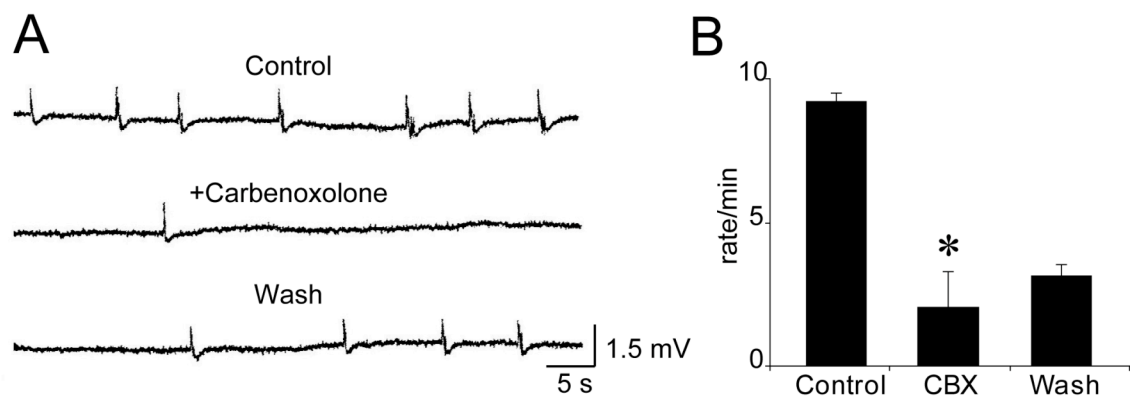


– Fig.2 –

Effect of mu-opioid receptor activation on the activity recorded during perfusion with normal medium.

Aa. *Control: ACC slices superfused with normal medium generate recurring field events that are mirrored by membrane depolarization and action potential firing. Further application of the mu-opioid receptor agonist DAGO significantly decreases the rate of occurrence of these network events as well as the frequency of action potential generation in principal cells (+DAGO).* ***Ab.*** *Plot showing the time course of the effect of mu-opioid agonism on field activity. The time of application of DAGO is indicated by the solid line.* ***B.*** *Intracellular recording showing the effect of mu-opioid agonism on an intrinsically bursting neuron. DAGO decreases the burst duration (**Ba,** +DAGO) as summarized in panel **Bb**. Significance is indicated by the asterisk.*

Next, we sought to determine the role of gap junctions in the generation of these synchronous discharges. Therefore, we bath applied the gap junction decoupler carbenoxolone (200 µM, n= 4). Interestingly, spontaneous field events not only slowed down over time (mean interval - control: 6.6±2.2 s, +Carbenoxolone: 17.9±12.4 s; p= 0.002; n= 5), but also appeared to be desynchronized, as reflected by the random generation of these field potentials (not shown). Finally, this pharmacological procedure abolished them (Fig. 3A, +Carbenoxolone). This effect was partially recovered by washout of the drug (mean interval: 14.4±7 s; n=5; Fig. 3A, Wash). These data are summarized in Fig. 3B.



– Fig. 3 –

The gap-junction decoupler carbenoxolone exerts an inhibitory effect on ACC network activity.

A. *Spontaneous network events generated by ACC slices superfused with normal medium are initially desynchronized and eventually abolished by bath-application of the gap-junction decoupler carbenoxolone. The middle trace shows the final effect of gap junctions decoupling, when network events are eventually abolished. The pattern of generation of these network discharges partially recovered after prolonged washout of the drug.* ***B.*** *Plot summarizing the results reported in panel A, showing an overall decrease in the average frequency of the events. Significance is indicated by the asterisk.*

DISCUSSION

We have reported here that ACC networks can generate spontaneous rhythmic activity in physiological condition. Moreover, our findings indicate that the occurrence of these spontaneous network events: (i) is modulated by *mu*-opioid receptors (ii) involves gap-junction interactions. We conclude that GABAergic interneurons may play a major role in synchronizing ACC networks towards the generation of spontaneous activity under physiological experimental conditions.

Over the last decade, several studies have extensively addressed the paradoxical role of the inhibitory neurotransmitter GABA in the generation and maintenance of epileptiform discharges (Avoli et al., 1996; Cohen et al., 2002; Voipio and Kaila, 2000 ;

see Avoli et al., 2002 for review). Consistently, we recently reported that ACC networks challenged with the convulsant 4-aminopyridine (4AP) are incapable of generating prolonged periods of epileptiform synchronization when devoid of the GABAergic drive (Panuccio et al., 2008). In the present study, we provide convergent evidence for a possible role of interneuron synchronization as an underlying mechanism for the generation of spontaneous synchronous discharges in this cortical area, in the absence of convulsants.

The role of GABA receptor-mediated conductances is supported by the ability of *mu*-opioid agonism to slow down and eventually abolish the spontaneous synchronous events recorded in the ACC. Such a procedure is indeed known to diminish GABA release from cortical interneurons (*Madison and Nicoll 1988; Capogna et al., 1993*), although a modulatory effect on glutamatergic transmission in the ACC cannot be excluded (*Panuccio et al., 2008*). In addition, a major role for inhibitory interneurons is further supported by the effect of carbenoxolone on these spontaneous recurrent events. Gap junctions are known to be highly expressed in interneurons (Deans et al., 2001), where electrical coupling plays a pivotal role in sustaining synchronous firing (Yang and Michelson, 2001).

Recent work by Yang and Ling (2007) has reported that carbenoxolone increases the frequency of sEPSCs generated by somatosensory cortex slices. These authors also reported that this effect is prevented by pretreatment with the $GABA_A$ receptor antagonist picrotoxin, supporting the view that gap junction decoupling may release excitatory neurons from GABAergic inhibition, confirming that electrical synapses are mainly present at the inhibitory network level. In the present study we show that carbenoxolone decreases the rate of occurrence of spontaneous network events in the ACC. Therefore, in light of these findings, we are more inclined to hypothesize that GABAergic interneurons, rather than glutamatergic cells, play a primary role in the generation of spontaneous network discharges in the ACC by providing the necessary drive to the excitatory network. It appears however that the role of gap-junctions may differ from that of *mu*-opioid receptors. In support of this view we have found here that decoupling electrical synapses initially desynchronizes ACC network activity, as reflected by the random occurrence of spontaneous events, which eventually disappear.

Our observations sustain the hypothesis that different modalities of inhibitory network modulation work in cooperation to shape rhythmic activity generated by the ACC. This region - which serves several physiological functions including motor and autonomic responses as well as affection and behavior (Devinsky et al., 1995) - works as a bridge between emotion and action. The generation of spontaneous events in brain cortical slices when not challenged by convulsants may represent the manifestation of basal activity generated by a resting network. This, in turn, may serve as a fine-tuning system towards more complex cortical functions. Changes in functional connectivity within and among cortical networks may underlie the manifestation of pathological patterns.

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