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# *In vitro* ictogenesis and parahippocampal networks in a rodent model of temporal lobe epilepsy

G. Panuccio [a], M. D'Antuono [a], P. de Guzman [a], L. De Lannoy [a], G. Biagini [b], M. Avoli [a,c,*]

[a] *Montreal Neurological Institute and Department of Neurology and Neurosurgery, McGill University, Montreal, QC, Canada H3A 2B4*
[b] *Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, 41100 Modena, Italy*
[c] *Dipartimento di Medicina Sperimentale, Sapienza Università di Roma, 00185 Roma, Italy*

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ABSTRACT

Temporal lobe epilepsy (TLE) is a chronic epileptic disorder involving the hippocampal formation. Details on the interactions between the hippocampus proper and parahippocampal networks during ictogenesis remain, however, unclear. In addition, recent findings have shown that epileptic limbic networks maintained *in vitro* are paradoxically less responsive than non-epileptic control (NEC) tissue to application of the convulsant drug 4-aminopyridine (4AP). Field potential recordings allowed us to establish here the effects of 4AP in brain slices obtained from NEC and pilocarpine-treated epileptic rats; these slices included the hippocampus and parahippocampal areas such as entorhinal and perirhinal cortices and the amygdala. First, we found that both types of tissue generate epileptiform discharges with similar electrographic characteristics. Further investigation showed that generation of robust ictal-like discharges in the epileptic rat tissue is (i) favored by decreased hippocampal output (ii) reinforced by EC–subiculum interactions and (iii) predominantly driven by amygdala networks. We propose that a functional switch to alternative synaptic routes may promote network hyperexcitability in the epileptic limbic system.



## Introduction

Temporal lobe epilepsy (TLE) is a partial epilepsy disorder involving the hippocampus proper and parahippocampal structures such as entorhinal (EC) and perirhinal (PC) cortices, amygdala, and temporal neocortex (Gloor, 1991; Mathern et al., 1997). TLE patients are often unresponsive to antiepileptic drugs and present with a typical pattern of brain damage known as Ammon's horn sclerosis (Gloor, 1991; Du et al., 1993; Wiebe et al., 2001). Current animal models of TLE are based on local or systemic injection of convulsant drugs (e.g., kainic acid or pilocarpine) or on repetitive electrical stimulation of limbic pathways (reviewed in Pitkänen et al., 2006) to induce an initial status epilepticus (SE) followed by a chronic condition of spontaneous, recurrent, limbic seizures that are also poorly controlled by antiepileptic drugs (Glien et al., 2002; Chakir et al., 2006).

Evidence obtained from humans and rodents indicates that epileptic hyperexcitability results from seizure-induced brain damage leading to (i) synaptic reorganization (Sutula et al., 1989; Mikkonen et al., 1998; Houser, 1999; Gorter et al., 2001), (ii) loss of specific interneuron subtypes (Bernard et al., 2000; Maglóczky and Freund, 2005) and (iii) alterations in GABAergic inhibition (Sloviter, 1987; Brooks-Kayal et al., 1998). The latter changes along with NMDA receptor upregulation have been reported in pilocarpine-treated parahippocampal areas such as the subiculum (Knopp et al., 2005; de Guzman et al., 2006), the EC (Kobayashi et al., 2003; Kumar and Buckmaster, 2006; Yang et al., 2006; de Guzman et al., 2008) and the lateral amygdala (LA) (Benini and Avoli, 2005). In addition, modified $GABA_A$ receptor signaling due to changes in $K^+/Cl^-$ cotransporter has been identified in the epileptic human subiculum *in vitro* (Cohen et al., 2002; Wozny et al., 2003; Huberfeld et al., 2007). It remains however to be defined how these alterations in cellular excitability lead to an epileptic brain capable of producing chronic recurrent seizures. Moreover, the roles played by parahippocampal areas in seizure initiation remain vague. Both aspects are presumably relevant for advancing our understanding of TLE pathogenesis.

Findings obtained from animal models of TLE suggest that changes in $K^+$ channel subunit expression may be part of the epileptogenic process leading to neuronal hyperexcitability and the consequent manifestation of spontaneous epileptic seizures (Bernard et al., 2004; Zahn et al., 2008). In addition, contrary to what was reported by us in pilocarpine-treated mice (D'Antuono et al., 2002), treatment with the $K^+$ channel blocker 4-aminopyridine (4AP), a well established model of *in vitro* ictogenesis (Avoli et al., 2002), failed to induce the generation of ictal-like discharges in slices obtained from kainic-

* Corresponding author. 3801 University Street, Montreal, QC, Canada H3A 2B4.
*E-mail address:* massimo.avoli@mcgill.ca (M. Avoli).
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treated epileptic rats (Zahn et al., 2008). We were therefore interested in studying the effects induced by bath-application of this convulsant in a limbic brain slice preparation in the rat pilocarpine model of TLE. Here, we employed field potential recordings to: (i) compare the effect of 4AP application in non-epileptic control (NEC) and pilocarpine-treated tissue; (ii) investigate the role of hippocampal–parahippocampal interactions in the generation of epileptiform discharges and (iii) establish whether the site of epileptiform discharge initiation is modified in epileptic versus NEC tissue.

## Methods

All the experimental procedures were approved by the Canadian Council on Animal Care (cf. de Guzman et al., 2006, 2008). All efforts were made to minimize the number of animals used and their suffering.

### *Preparation of pilocarpine-treated rats*

Adult male Sprague Dawley rats (250–300 g) were injected with the cholinergic agonist pilocarpine hydrochloride (380 mg/kg, i.p.). To prevent discomfort induced through peripheral muscarinic receptor stimulation, rats were treated with scopolamine methylnitrate (1 mg/kg, i.p.) 30 min prior to pilocarpine administration. Animal behavior was monitored for 4–6 h after pilocarpine injection and scored according to Racine's classification (1972). SE, defined as repeated stages 3–5 seizures (Racine, 1972) without recovery in between, was arrested with diazepam (5–20 mg/kg, i.p.) if it did not resolve spontaneously within 2 h. Rats experiencing ≥60 min SE were defined as the experimental group. Previous studies have established that all pilocarpine-treated animals experiencing SE become epileptic (see for example Cavalheiro et al., 1991, reviewed in Pitkänen et al., 2006, and by Curia et al., 2008). We have previously reported that rats exposed to 60–120 min of SE develop spontaneous recurring behavioral seizures within ~3 weeks from treatment (Biagini et al., 2008, 2009). Therefore, animals were randomly monitored for the manifestation of spontaneous behavioral seizures, which was confirmed in all of them, and were used for electrophysiological studies 4–10 weeks following pilocarpine injection. Age-matched NEC rats—injected i.p. with scopolamine and saline—never manifested behavioral seizures.

### *Brain slice preparation and electrophysiological procedures*

Both NEC and pilocarpine-treated animals were decapitated under deep isoflurane anesthesia, the brain was quickly removed and placed in ice-cold (~4 °C) oxygenated (95% $O_2$, 5% $CO_2$) artificial cerebrospinal fluid (ACSF) composed of the following (in mM): NaCl 124, KCl 2, $KH_2PO_4$ 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, and D-glucose 10 (pH = 7.4, 305 mOsm/kg). Horizontal brain slices (450–500 µm thick) were obtained with the use of a vibratome (Leica VT1000S, Leica, Germany). Slices were transferred to an interface tissue chamber and superfused with oxygenated (95% $O_2$, 5% $CO_2$) ACSF at 32–34 °C (pH = 7.4, 305 mOsm/kg). Chemicals were acquired from Sigma-Aldrich Canada, Ltd. (Oakville, Ontario). 4AP (50 µM) was bath-applied.

Two different slicing procedures were used to partially preserve connectivity between hippocampus and parahippocampal areas. In order to record from brain slices in which hippocampal output activity was unable to spread to the EC (*cf.*, Avoli et al., 1996), the dorsal side of the brain was glued onto the vibratome stage without any prior manipulation of the tissue block. In these brain slices, which included the hippocampal formation throughout its dorso-ventral extent, fast CA3-driven interictal-like activity disclosed by 4AP application was restrained to the hippocampus proper and did not propagate to the EC (see Fig. 1Aa, arrows; *cf.*, Avoli et al., 1996). We will thereafter refer to these slices as *partially disconnected slices*. In a different set of experiments, preservation of CA3 outputs to the EC and other parahippocampal areas was achieved by slicing the brain along a horizontal plane that was tilted by ~10° along a posterosuperior–anteroinferior plane passing between the lateral olfactory tract and the brain stem base (*cf.*, Benini et al., 2003). To this end, only the most ventral slices were used since they present with the highest connectivity between hippocampus and parahippocampal structures (Avoli et al., 2002; Benini et al., 2003). These slices were comprised between −8.6 and −7.6 mm from the bregma (Paxinos and Watson, 1998), and included the most ventral hippocampus, the EC, the PC and the LA; in these experiments fast CA3-driven interictal-like activity induced by 4AP could propagate to the EC and the other parahippocampal structures (see Fig. 3; *cf.*, Benini et al, 2003).

Field potential recordings were made with ACSF-filled glass pipettes (tip diameter <10 µm; resistance 5–10 MΩ) that were positioned in the deep layers of the EC and (i) CA3 (stratum pyramidale or radiatum) or (ii) subiculum, or (iii) the deep layers of the PC and the LA. Signals were fed to high-impedance amplifiers and displayed on a WindoGraph recorder (Gould Instruments, Cleveland, OH, USA) or sent to a computer interface device (Digidata 1322A, Molecular Devices, Palo Alto, CA, USA), acquired and stored in the hard drive using pClamp 8.0 software (Molecular Devices, Palo Alto, CA, USA). Traces were acquired at a sampling rate of 5 kHz and low-pass filtered on line at 2 kHz. Subsequent analysis of these data was performed with Clampfit 9 software (Molecular Devices, Palo Alto, CA, USA). Time-delay measurements for epileptiform discharge onset were obtained by taking as temporal reference the first deflection from the baseline in expanded traces.

Local application of kynurenic acid (5 mM in ACSF) to the EC or subiculum was performed by pressure-ejection through a broken pipette (tip diameter ~15 µm). To avoid diffusion of the drug from the perfused area to distant regions, slices in these experiments were positioned in such a way that the treated area was downstream with respect to the ACSF flow. In addition, we used bipolar, stainless steel electrodes to deliver electrical stimuli (100 µs; <350 µA) in the subiculum or in the EC (Fig. 5), and thus to rule out that the changes in epileptiform activity could reflect decreased network excitability due to kynurenic acid diffusion. The drop-applied solution was visualised by including phenol red to the perfused medium. Cuts within different areas were made with a microknife under visual guidance (*cf.* Benini et al, 2003).

A direct comparison between the pattern of 4AP-induced epileptiform activity in the two animal groups was performed by recording simultaneously from the EC and CA3 subfield of *partially disconnected slices* that were obtained from both NEC and pilocarpine-treated animals; brain slices from these two types of tissue were maintained during the same experiment in a two-well interface recording chamber accommodating 2 to 4 slices from each animal per well. This approach allowed us to implement similar experimental conditions for both animal groups thus ensuring that no bias was introduced by possible subtle differences that may occur during the slice preparation and maintenance.

### *Database and statistical analysis*

In all, we analyzed 4AP-induced epileptiform activity in 29 and 46 brain slices obtained from NEC and pilocarpine-treated epileptic animals, respectively. Within and between each experimental group, all the slices exhibited the same electrographic pattern of 4AP-induced epileptiform activity, regardless of the animal age or the time after SE. Therefore, data were pooled.

Measurements throughout the text are expressed as mean ± SEM, and $n$ represents the number of slices studied. Data were compared with the Student's $t$-test. Results were considered significantly different if $p<0.05$.