Brain Behav Evol 1991;38:7–19

Electroencephalogram *in vitro* and Cortical Transmembrane Potentials in the Turtle *Chrysemys d'orbigny*

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Key Words. Electroencephalogram *in vitro* · Turtle · Sharp waves · TTX · Physostigmine · Hypoxia · Primordium hippocampus · Epilepsy

Abstract. An experimental stable model of an *in vitro* turtle brain *(Chrysemys d'orbigny)* was developed in order to compare electrographic activity (EEG) with transmembrane potentials. Two preparations were used: a whole intact hemisphere and a whole open hemisphere. The latter permitted easier impalement of cortical neurons through the ependymal surface. The EEG characteristics were similar to those described in turtles *in vivo*. The EEG was nonrhythmic (rhythmicity coefficient < 0.40). The power spectrum presented a high energy band between 1 and 3 Hz, decreasing progressively towards the higher frequencies. Total power of the EEG was one order of magnitude greater than the system noise. Random large amplitude sharp waves (22–300 μV, 500–1,900 ms) were recorded spontaneously. Hypoxia produced an increase in frequency and amplitude of the large sharp waves, without modification of either EEG background activity or membrane potentials. Physostigmine provoked the disappearance of the large sharp waves, an effect reversed by atropine. The addition of TTX to the medium provoked the abolition of the EEG, although spikes and plateaus determined by Ca^{2+} conductances persisted. The power spectra band of maximum relative potency was 0.8–2.5 Hz for both EEG and slow membrane potentials.

Introduction

The basic features characterizing brain field activity (EEG) in turtles and other reptiles have been described by several authors [e.g. Bullock, 1984, 1988, 1989; Bullock and Basar, 1988]. Nevertheless, studies of nonmammalian vertebrate EEGs are scarce compared to the numerous reports describing mammalian EEGs [for review, see Bullock and Basar, 1988].

Studies of turtle brains are relevant for attempts to compare both the functional and anatomical organization of vertebrate nervous systems. Turtles, like other reptiles, have a very primitive cortical organization consisting of three layers [Desan, 1984]. This trilaminar structure is distributed throughout the dorsal and medial parts of the hemisphere, which have been named dorsal or general (DC) and medial (MC) cortices, respectively. The study of this relatively simple cortical structure could provide interesting data for the understanding of basic mechanisms in the complex cortex of mammals. Moreover, the general hemispheric organization in reptiles is remarkably similar to that exhibited by mammals [Northcutt, 1981].

Major difficulties are encountered when attempting *in vivo* electrophysiological recordings from a turtle brain. For example, access to the MC, which is contained in the medial aspect of the hemisphere, is difficult, and the great mobility of the brain makes intracellular recordings practically impossible. For these reasons, some authors [e.g. Mori et al., 1980; Connors and Kriegstein, 1986; Shen and Kriegstein, 1986] have resorted to *in vitro* techniques, but only for the analysis of cell properties. We have considered it advantageous to study spontaneous field activity in an *in vitro* experimental model which allows comparison

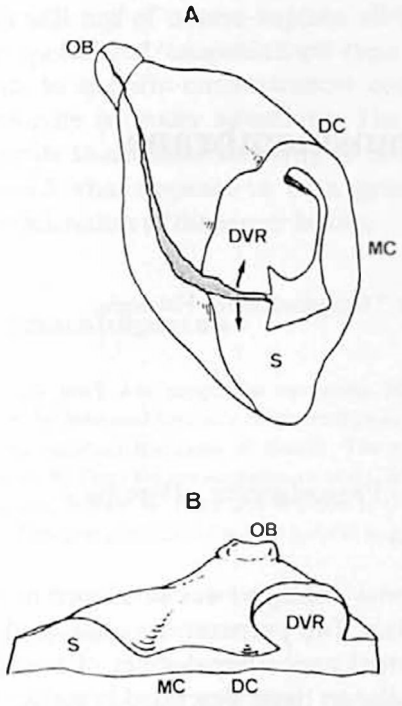

Fig. 1. Preparations for recording brain electrical activity in vitro (schematic drawings). A Dorsocaudal view of the left hemisphere sectioned to show its internal configuration. The WIH was usually recorded with the medial cortex up. Transectioning of the lateral aspects (stippling) allows the hemisphere to be unfolded in the direction of the arrows. B View of the WOH with the ependymal surface up. DVR = Dorsal ventricular ridge; OB = olfactory bulb.

with simultaneously occurring transmembrane potentials. The effects of hypoxia and of some drugs were also analyzed to investigate the genesis of the background EEG and spontaneous epileptiform activity.

Materials and Methods

Experiments were performed in juvenile specimens (caparace length, 3.5–5 cm; total brain length 10 mm) of the fresh water turtle *Chrysemys d'orbigny*. The original research reported herein was performed under guidelines established by the Ministerio de Ganaderia, Agricultura y Pesca, División Fauna, Uruguay.

Tissue Preparation

Animals anesthetized by cold were decapitated, the head was immersed in Ringer solution at 4 °C (see below) and the brain was removed and hemisected. Two preparations were used: a whole intact hemisphere (WIH) and a whole open hemisphere (WOH), obtained by means of a rostrocaudal cut through the lateral brain surface, between the septum (S) and the dorsal ventricular ridge (fig. 1). Hemisected brains were transferred to an open plexiglass recording chamber and superfused with a Ringer solution, flowing at a rate of 1–1.5 ml/min, at room temperature (20–22 °C). The normal superfusate [Mori et al., 1980] contained (in m*M*): NaCl, 96.5; KCl, 2.6; $CaCl_2$, 4.0; $MgCl_2$, 2.0; $NaHCO_3$, 31.5; and glucose, 10.0. The preparation was oxygenated by diffusion, the saline solution being saturated with 5% CO_2 in O_2. With this gas mixture the pH was maintained at 7.6. In some experiments no gasses were added, and the solution was oxygenated by diffusion of atmospheric O_2. In other experiments the medium was bubbled with 100% O_2. In these instances $NaHCO_3$ was replaced by HEPES 20 m*M*, and the osmolarity was compensated by increasing NaCl concentration to 108 m*M*. The same pH (7.6) was consistently maintained.

Drugs

Physostigmine salicylate (10–20 μ*M*) and atropine sulphate (200 μ*M*) were added to the bath to activate the cholinergic systems and to reverse this effect, respectively. The drug TTX (1–2 μ*M*) was added to the normal medium in order to abolish the generation of Na^+ spikes. Osmolarity was not corrected when these drugs were added. Ca^{2+} was substituted by Co^{2+} (3–5 m*M*) in the saline solution to block the Ca^{2+} channels.

Electrophysiological Recordings

The WIH was placed with its medial surface up, while the WOH was extended horizontally with the ependymal surface facing up. Field activity was recorded with glass micropipettes (1–2 MΩ and 20–100 μm OD tip) filled with 1 *M* NaCl. Micropipettes were visually positioned using a dissecting microscope (50 ×) on the pial surface (WIH preparation) or on the ependymal surface (WOH preparation) of the MC, DC and S. The EEG was amplified with high sensitivity low noise amplifiers (0.3–60 Hz bandwidth) and subsequently low pass 30 Hz filtered.

Transmembrane potentials were obtained from the MC with 4 *M* potassium acetate-filled micropipettes (100–200 MΩ). A conventional amplifier was used for both intracellular recordings and stimulation. Current was injected through the recording electrode via an active bridge circuit, and balance was continually adjusted by cancelling the rapid voltage jump at the onset and offset of the current pulse [Engel et al., 1972]. All signals were simultaneously monitored on a storage oscilloscope, recorded on paper, and stored in a two-channel digital recorder.

Processing

The analog outputs of the stored data were amplified, filtered, and fed into a personal computer. The transmembrane potential was filtered at 6 kHz and sampled at 15 kHz. The EEG and, eventually, the slow membrane potentials (see below) were passed through an antialiasing filter (cutoff 30 Hz) and sampled at 64 Hz. Hard copies of both recordings were obtained with an X-Y plotter. Spike amplitude did not decrease with these procedures. Power spectra (PS) of the analog data were obtained by means of the Fast Fourier Transform (FFT). Periods of 32–48 s of stationary data taken between large sharp waves (LSWs) [Cox and Lewis, 1966; Buño et al., 1978] were processed. The rhythmicity coefficient (RC) was calculated as the quotient of the power of the fundamental and the power of all the spectral components in order to quantify rhythmicity [Gaztelu and Buño, 1982].

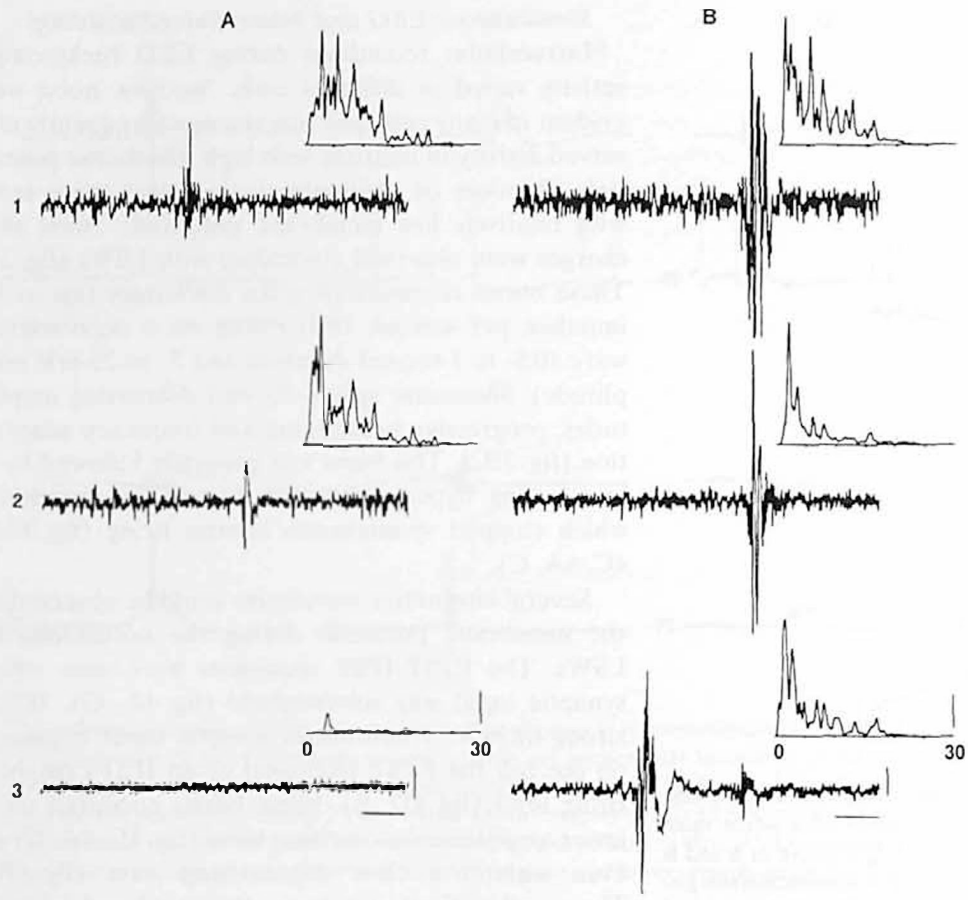

Fig. 2. Spontaneous EEG and noise in the recording system. A EEG of the medial cortex in the WIH (1) and in the whole open hemisphere (2). PS are shown in the inserts. One LSW is present in each recording. Field activity is similar with both techniques. The energy level of noise in the recording system obtained with the electrodes placed in the Ringer solution is one order of magnitude lower (3). B Recordings obtained from the septal region (1), the MC (2) and DC (3) in the WOH. EEGs are similar, although amplitude was higher at the septal area. Calibrations: 2 s, 10 μV, inserts Hz and 2×10^2 $\mu V^2/Hz$.

Data Base

More than 40 stable recordings of field activity were analyzed. Fifty-five stable intracellular recordings were studied concomitantly with the EEG. Neurons selected for this study had resting membrane potentials of at least −50 mV and spike amplitudes of more than 55 mV from the firing level. All neurons were characterized as pyramidal according to their discharge pattern [Shen and Kriegstein, 1986].

Results

The EEG of the MC, DC and S were recorded. Intracellular recordings of MC pyramidal neurons were simultaneously made. The effects of pO_2 and some drugs on field and cellular activity are described.

EEG Characteristics

Background activity was nonrhythmic, with RC < 0.40 and amplitude of 5–15 μV. The EEG was randomly interrupted by LSWs of 22–300 μV (mean = 128.4, SEM = 9.8) appearing at frequencies of 0.5–8/min.

Background EEG activity amplitude was always larger than the system's noise (fig. 2A3) within the 0.2- to 25-Hz band, and total EEG power was one order of magnitude greater than noise. The PS always presented a peak between 0.8 and 2.5 Hz, beyond which power decreased steadily with frequencies of up to 25 Hz (fig. 2A1, 2, B1–3, inserts). The power of frequencies above this value did not differ significantly from noise. The EEG showed the same frequency

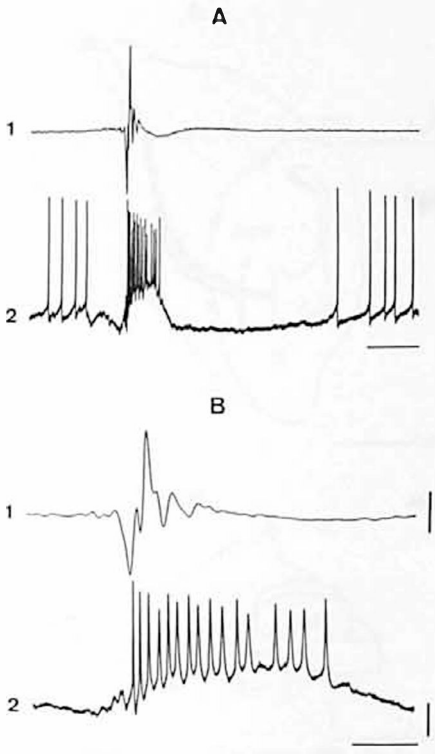

Fig. 3. Simultaneous recordings of field activity and transmembrane potential of a cell in the medial cortex of a whole open hemisphere preparation. The same recording is shown in A and B at two sweep speeds. 1 = Field potential; 2 = transmembrane potential. A burst at neuronal level formed by a depolarizing wave of more than 20 mV amplitude mounted by action potentials is shown in synchrony with the LSW. Calibrations: 1 s for A and 200 ms for B; 100 μV for 1 and 20 mV for 2.

components at the S and MC and DC, both in the intact and open hemisphere preparations (fig. 2). Power was always greater in the S than in either cortex.

The LSWs were simpler, triphasic or, most frequently, polyphasic (fig. 2B1–3, 3, 4) with total duration varying between 500 and 1,900 ms (mean 1,059 ms, SEM 59.8 ms). The simplest ones presented a central wave lasting 50–125 ms with negative or positive polarity, probably depending on the site of origin. The LSWs were frequently synchronous at the DC and MC. They could occasionally be observed asynchronously, when presenting a low amplitude, at the S and cerebral cortex. Sometimes the LSWs were preceded by a discharge of increasing amplitude (fig. 4E, F, H) lasting approximately 1 s.

Simultaneous EEG and Intracellular Recordings

Intracellular recordings during EEG background activity varied in different cells. Synaptic noise was evident in many cells and was the most frequently observed activity in neurons with high membrane potentials. Random or rhythmic single spikes were seen with relatively low membrane potentials. Burst discharges were observed coinciding with LSWs (fig. 3). These bursts consisted of spike discharges (up to 40 impulses per second, IPS) riding on a depolarizing wave (0.5- to 1-second duration and 5- to 25-mV amplitude). Successive spikes showed decreasing amplitudes, progressive broadening and frequency adaptation (fig. 3B2). This burst was generally followed by a long-lasting hyperpolarization (up to 3 s duration), which stopped spontaneous neuron firing (fig. 3A2, 4C, 6A, C).

Several alternative waveforms could be observed in the membrane potential during the occurrence of LSWs. The EPSP-IPSP sequences were seen when synaptic input was subthreshold (fig. 4A, G). When strong or more synchronous synaptic input impinged on the cell the EPSP (followed by an IPSP) reached firing level (fig. 4D, E). Some bursts appeared on a lower amplitude-depolarizing wave (fig. 4C, 6A, C) or even without a clear depolarizing wave (fig. 4F). These spikes fired at a lower threshold and initially showed the same amplitude as isolated spikes. In some instances only a hyperpolarizing wave that stopped the spontaneous discharge was observed (fig. 4B).

Effects of Changes in pO_2

Increasing the pO_2 in the superfusing medium reduced the amplitude and frequency of LSWs. They occasionally disappeared completely without affecting EEG background activity. Figure 5 shows a typical experiment. The EEGs taken at the S and MC are shown from figure 5A to D under different experimental conditions. The two lower traces were obtained with smaller amplification in order to avoid blocking LSWs. Recordings obtained without bubbling the gas mixture in the superfusing medium are shown in figure 5A. In figure 5B, the amplitude of LSWs decreased after perfusion during 11 min with Ringer saturated with O_2 95% and CO_2 5%. This effect was more evident at the MC; some LSWs could still be observed at the S (arrowheads). The LSWs completely disappeared after 35 min. This effect was reversible: after 43 min in unbubbled superfusate (same conditions as in fig. 5A), LSWs reappeared.

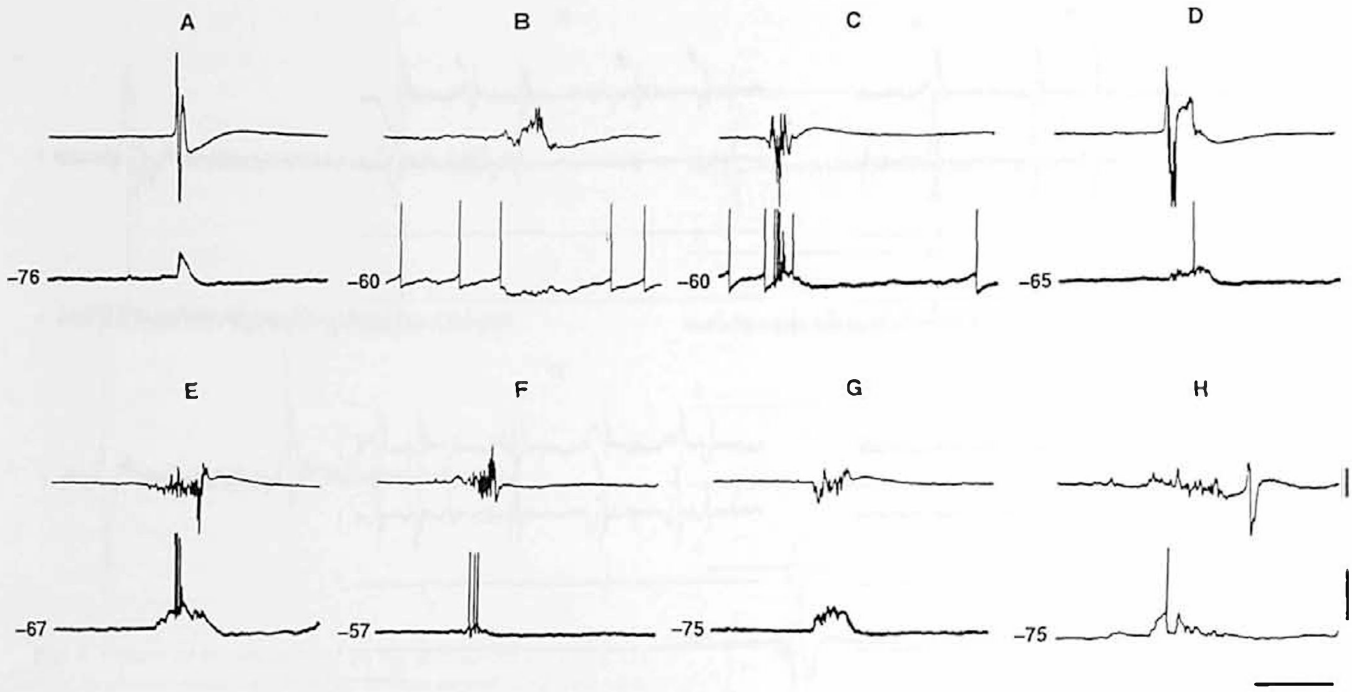

Fig. 4. Some examples of LSWs and corresponding changes in transmembrane potentials. **A–H** Upper records are EEGs obtained at a low amplification in the medial cortex; lower records are intracellular recordings of MC cells (recordings are from different experiments). Figures on the left indicate the resting membrane potential. **A** Simple LSW. **B–H** Complex LSWs. The membrane potential shows different phenomena during LSWs. Simple (**A**) or complex (**D, G**) EPSPs followed by an IPSP, with (**D, H**) or without spikes (**A, G**); IPSP without previous EPSP (**B**) and bursts riding on a depolarizing wave (**C, E**) or not (**F**). Note the apparent lower firing level during the burst in c. Calibrations: 50 μV and 50 mV for upper and lower records, respectively; positive up in both traces; horizontal, 1 s.

Perfusion with 100% O_2, with a constant pH, produced the same effect [data not shown]. This precaution was necessary in order to eliminate the possibility of influence of both CO_2 and pH. Reducing the pH to 7.2 decreased the frequency of LSWs, and a pH increase to 7.8 caused the opposite effect. Nevertheless, no changes in the background EEG could be observed within a broad pH range.

As shown in figure 6, the decrease in pO_2 did not modify either the resting potential or the action potentials. The cell recorded in this experiment showed a spontaneous discharge of isolated spikes and bursts coinciding with the LSWs (fig. 6A, C) when the Ringer solution was not oxygenated. Bursts and LSWs disappeared when the Ringer solution was bubbled with the gas mixture (fig. 6B). This neuron presented a resting potential of –64 mV and a spike amplitude of 69 mV measured from the firing level; these values were not modified during the 40 min the experiment lasted. The shape of the action potential remained constant in both conditions, as can be seen in the superposition of averages of 8 spikes (fig. 6D).

Effects of Drugs and Channel Blockers

The LSWs disappeared when physostigmine salicylate was added to the medium (10–20 μ*M*), while EEG background activity increased in amplitude (fig. 7, 1 and 2). These effects were reversed by atropine sulphate (200 μ*M*; fig. 7, 3).

The addition of TTX (1–2 μ*M*) to the normal Ringer solution resulted in EEG disappearance. Figure 8 shows that the first effect (after 5 min) was the suppression of LSWs, with an unexpected amplitude increase of EEG background activity (fig. 8A2). Concomitantly, intracellularly recorded Na^+ spontaneous spikes reduced their amplitude (fig. 8B2). Fourteen minutes later the EEG trace could not be distinguished from noise. By then Na^+ spikes had disappeared, and only Ca^{2+} spikes and plateaus remained (fig. 8B3) because they could be blocked by the addition of Co^{2+} to the perfusion media [data not shown]. After the normal medium was restored, this effect on both the EEG and the cellular activity was progressively reversed (fig. 8A4, 5, B4, 5).

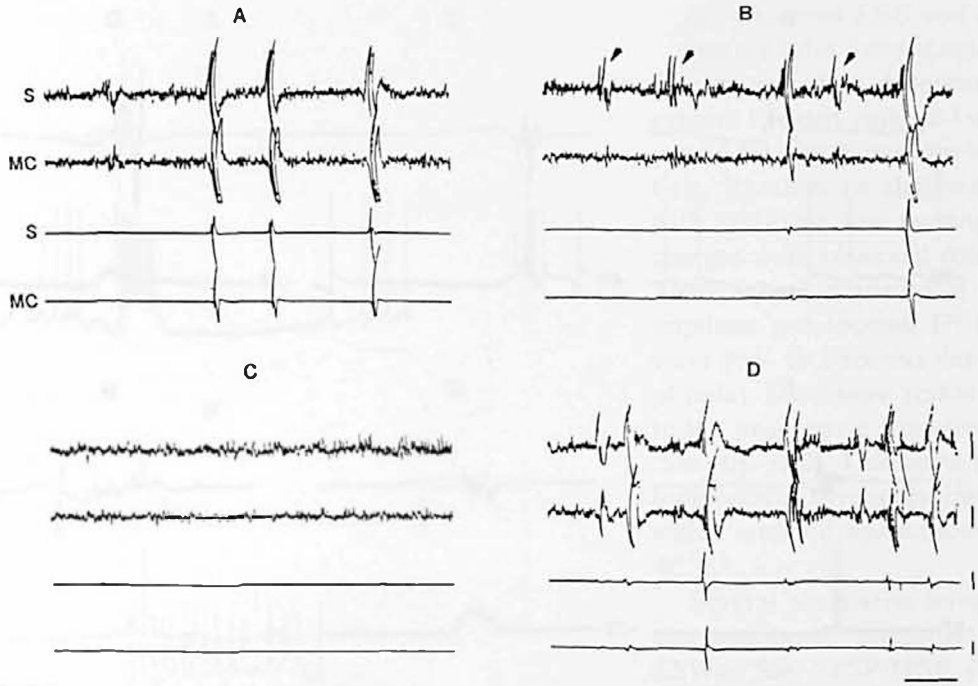

Fig. 5. O_2 effect on the EEG. A–D Upper records, S and the MC EEG at a high amplification; lower traces, same EEGs recorded at a lower amplification. A Recording obtained from the WOH superfused with normal medium without bubbling O_2. Repeated LSWs were observed synchronously in both regions. B After 11 min of passing the same medium saturated with O_2 (95%) and CO_2 (5%): LSWs are reduced in frequency and amplitude, the phenomenon being more evident in the MC; in some instances transient LSWs appear only in the S (arrowheads). C LSWs have completely disappeared after 35 min of passing the same medium saturated with O_2 (95%) and CO_2 (5%). Background electrical activity did not change. D LSWs reappeared after 43 min superfusing without O_2. Calibrations: 20 and 100 μV for upper and lower recordings, respectively; 4 s. Negative up in all traces.

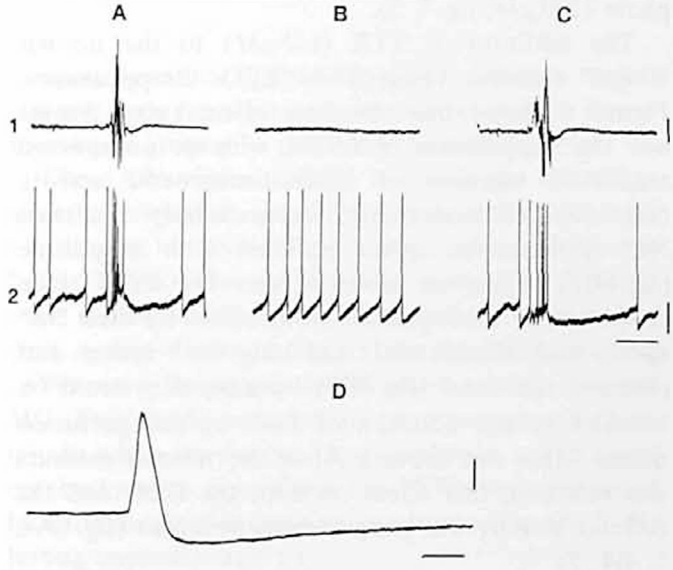

Fig. 6. EEG and intracellular recording of the same medial cortex unit during hypoxia and oxygenation. A WOH superfused with normal medium without bubbling oxygen; a complex LSW is observed in the EEG (1) coinciding with a burst in the cell, followed by a hyperpolarization (2) which stops the firing during approximately 1 s. B After superfusion with bubbled medium (O_2 95% and CO_2 5%), LSWs disappeared, and the cell discharged regularly without bursts. C Complex LSWs reappeared and the discharge pattern was similar to A, after superfusion with unbubbled Ringer. Note the lower level of firing during bursts than isolated ones in A and C. D Superposition of two averages, one during A and the other during B, corresponding to eight action potentials. The AP did not change in either amplitude or duration, indicating that it is not deteriorated by the action of hypoxia. Calibrations: 50 μV and 20 mV for 1 and 2, respectively; 1 s for A–C; 20 mV and 5 ms for D. Positive up in all traces.

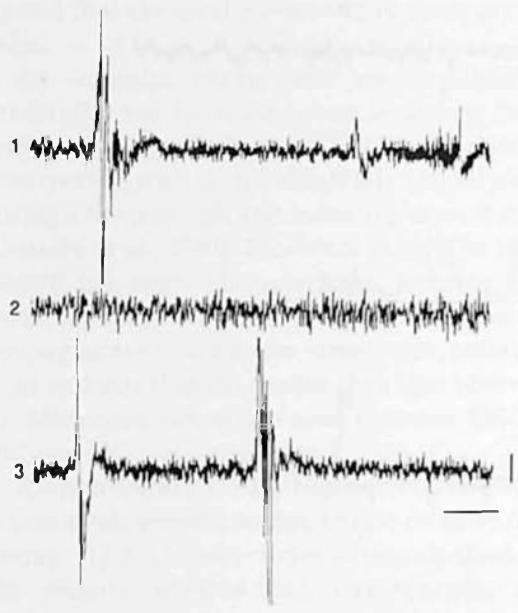

Fig. 7. Effects of physostigmine on the medial cortex EEG. (1) Control in normal medium. (2) After 10 min superfusing with added physostigmine salicylate 10 μM. LSW disappeared and the background activity increased in amplitude. (3) The effect is partially reversed by atropine sulphate 200 μM. Calibrations: 2 s and 10 μV. LSW are truncated.

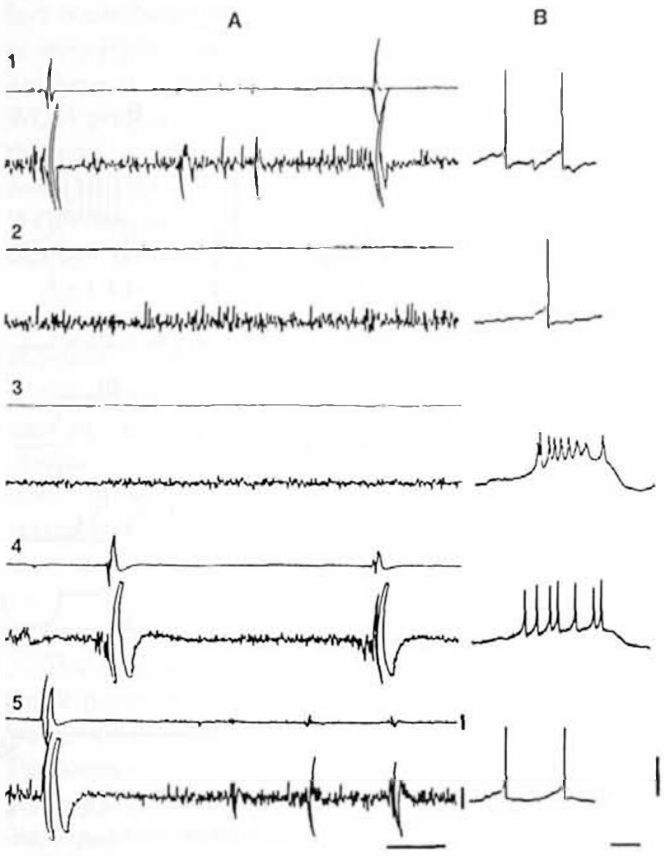

Fig. 8. Effects of TTX on the EEG. **A** EEGs obtained from the MC at low (upper record) and high (lower record) amplification. **B** Spontaneous intracellular activity recorded at the same time as **A** from a medial cortex neuron. **A1**: Control in normal medium without bubbling O_2; typical LSWs are observed. **B1**: Spikes of 60 mV. **A2**: 8 min after adding TTX (1 μM) to the medium, LSWs disappeared and the background activity increased in amplitude. **B2**: Spike amplitude decreased (51 mV). **A3**: Recording amplitude was reduced 17 min after adding TTX. **B3**: Na^+ spikes disappeared, and Ca^{2+} plateaus and spikes were seen. **A4** and **A5**, and **B4** and **B5**, 44 and 55 min, respectively, after suppression by TTX. A progressive normalization can be observed. Calibrations: 50 and 5 μV for **A**, upper and lower records, respectively (negative up), and 30 mV for **B** (positive up); 4 s for **A**; 300 ms for **B1**, 2 and 5; and 40 ms for **B3** and 4.

Another experiment with TTX (1 μM) in the perfused medium is shown in figure 9. In this case the cell was silent and spike discharges were elicited by a depolarizing pulse. The EEG (fig. 9A) the PS (fig. 9B), and cell activity (inserts in fig. 9B) are shown. After 7 min of TTX superfusion, LSWs disappeared, the PS shifted towards higher frequencies, and the total power increased, while the Na^+ spikes decreased in amplitude (fig. 9A2, B2). After 13 min (fig. 9A3), the recording had the same noise level, as indicated by the PS (fig. 9B3), similar to that seen in figure 2A3. Intracellular recordings showed only low amplitude deflections (fig. 9B3), probably Ca^{2+}-dependent. These experiments showed that the blockade of Na^+ spikes abolishes the EEG.

Slow-Membrane Potentials and EEG

Background EEG activity devoid of LSWs and simultaneously recorded transmembrane potentials were both low-pass-filtered at 30 Hz in order to block the action potential and to establish a relationship between both activities. Raw data and the PS corresponding to each recording may be observed in figure 10. The filtered membrane potential (fig. 10B) probably contained the known slow potentials, i.e. postsynaptic potentials, hyperpolarizing and depolarizing afterpotentials, Ca^{2+} slow spikes and plateaus, and hyperpolarizing potentials. The EEG and the slow

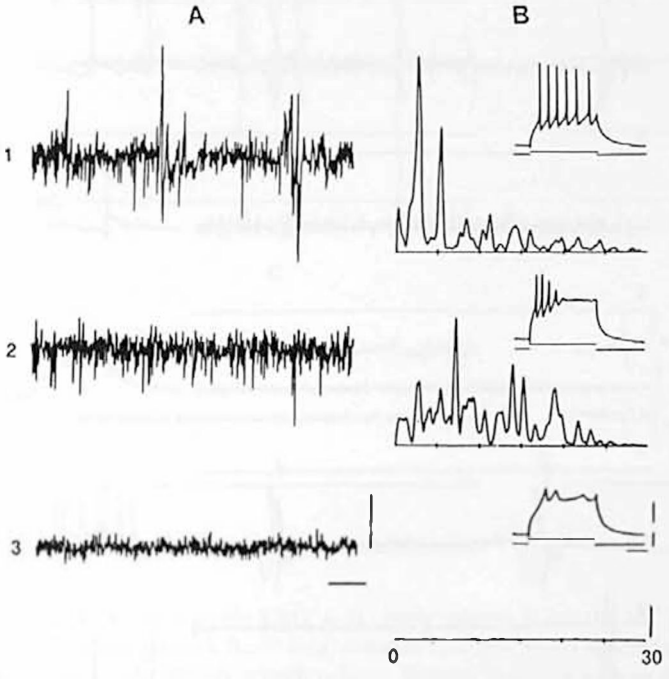

Fig. 9. Effects of TTX on the medial cortex EEG. **A** EEGs. **B** PS; inserts: intracellular recordings from an MC cell (upper record) during a depolarizing current pulse (lower record). (1) Control in normal medium. (2) After 10 min superfusion with TTX 1 μM, LSWs disappeared, EEG total power increased, the PS shifted towards higher frequencies, and Na^+ spikes were partially blocked although the depolarizing current was increased. (3) 20 min after TTX superfusion, the total power of the record is reduced, and Na^+ spikes have disappeared; only Ca^{2+} spikes and plateaus remain. After adding TTX, the recording could not be distinguished from the noise of the recording system (see fig. 2). Calibrations: 2 s and 10 μV for **A**; Hz = abscissae, and 2×10^2 μV^2/Hz = ordinates, for **B**; 200 ms, 20 mV and 1 nA for inserts.

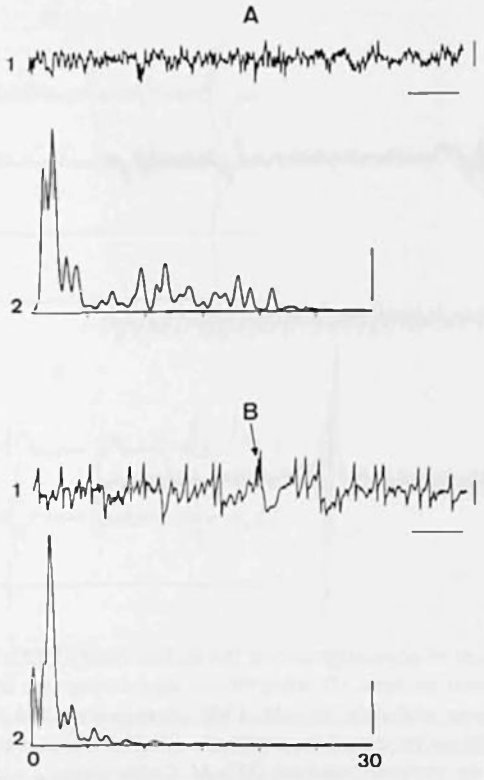

Fig. 10. Simultaneous recordings of EEG and SMP. **A** MC EEG (1) and the corresponding power spectrum (2). **B** SMP from a medial cortex cell (1) and the corresponding power spectrum (2). Fast spikes were filtered and the arrow shows a truncated burst of action potentials. Both power spectra were similar. The EEG power spectra, however, show relatively more energy in the lower and higher frequencies. Calibrations: 1 s for **A1** and **B1**, 5 μV for **A1** and 10 mV for **B1**; Hz = abscissae and 2×10^2 μV^2/Hz = ordinate for **A2**, and 4×10^2 mV^2/Hz ordinate for **B2**.

membrane potentials (SMP) power spectra coincided in the maximum relative power band (0.8–2.5 Hz). However, SMP power spectra showed more relative energy at the lower frequencies (peak at 0.3 Hz) and less relative energy in the 9- to 22-Hz band.

Discussion

The *in vitro* turtle brain preparations presented here demonstrated that it is possible to record the ongoing EEG during periods of 24 h or more. Moreover, simultaneous recording of intracellular activity in cortical neurons may be performed during many hours (up to 5). Spontaneous field activity has not been described in other *in vitro* turtle preparations, such as the olfactory bulb [Mori et al., 1980], the cerebellum [Hounsgaard and Midtgaard, 1988], DC [Connors and Kriegstein, 1986] and MC slabs [Shen and Kriegstein, 1986], nor in isolated guinea pig brainstem cerebellum *in block* preparation [Llinás and Mühlethaler 1988a, b].

Background EEG

Background EEG was of low amplitude but always above the noise level of the system. The PS demon-

strated that the total power was 10 times greater than noise.

An irregular background low-amplitude activity (10–25 μV) has been described *in vivo* in the MC of turtles during periods of up to 12 s. This activity was interspersed with larger amplitude (35–60 μV) epochs lasting a few seconds and including several slow waves [Gaztelu et al., 1990]. Electrical activity in the DC exhibited the same characteristics, but amplitude was lower. In our *in vitro* recordings the EEG activity was not segmented in epochs, amplitude remained constant and was slightly smaller than that observed *in vivo*. Moreover, no differences between EEGs at the MC and DC were observed.

Amplitude differences between our *in vitro* and the *in vivo* models could be due to one or more of the following. (1) An *in vitro* brain obviously does not have any sensory afferent and, consequently, does not process peripheral information. It, therefore, would present a very low level of activity. Indeed, under these conditions of reduced sensory input, the EEG presents a lower amplitude than during periods of activity in turtles [Karmanova and Churnosov, 1972; Walker and Berger, 1973; Patching, 1974], in lizards [Tauber et al., 1968], in caimans [Parsons and Huggins, 1965a] and chameleons [Tauber et al., 1966]. (2) The effect of temperature might also be influential. Although experiments were performed at room temperature, it is probable that in the same conditions the *in vivo* brain may have a higher temperature owing to greater energy consumption. In this context, a decrease in EEG amplitude has been described in turtles [Walker and Berger, 1973], alligators [Parsons and Huggins, 1965b] and lizards [Hunsaker and Lansing, 1962] with decreased temperature of the environment. The assumption that a smaller amplitude of the *in vitro* EEG results from a lower brain temperature must be further tested experimentally. (3) Finally, damage resulting from *in vitro* conditions might be responsible for these differences, although the persistence of a nonmodified EEG and the absence of membrane potential variations over a period of several hours make this less probable.

The PS demonstrated that EEG frequency components were similar to those observed *in vivo* [Gaztelu et al., 1990], with a peak around 2 Hz and a progressive decrease towards the higher frequencies. However, frequencies over 25 Hz were not detected *in vitro*, whereas components of up to 40 Hz could be recorded *in vivo* [Patching, 1974; Gaztelu et al., 1990]. This fact could be explained by the lower amplitude of the *in vitro* EEG, which increases the difficulty of distinguishing it from the system's noise. The WIH and WOH preparation EEGs were similar, indicating that the section of hemispheric connections does not affect the EEG. We therefore chose to work with the WOH because impalement of the cortical neurons through the ependymal surface was easier.

An EEG rhythmicity was never observed, not even after adding physostigmine to the medium. The nonrhythmicity of the EEG was confirmed by the RC, which always presented values under 0.45. In the presence of sustained rhythmic activity, such as the hippocampal theta rhythm, the RC varied between 0.45 and 0.80. An RC above 0.50 corresponded to EEG recordings with continuous rhythms as observed by the naked-eye examination [Gaztelu and Buño, 1982].

Large Sharp Waves

The presence of spontaneous LSWs in the *in vitro* turtle preparation merits special consideration. These waves have been described in a fresh water turtle, *Pseudemys scripta elegans* [Gaztelu et al., 1990] and also in a land turtle, *Testudo denticulata* [Walker and Berger, 1973] in *in vivo* preparations. They were simple or polyphasic, presenting values similar both in amplitude and duration to those observed in our *in vitro* preparation. These transient waves were recorded in both waking, curarized, artificially respirated animals and in free animals [Gaztelu et al., 1990]. Walker and Berger [1973] demonstrated that these LSWs – which they called 'spikes' – were present during behaviorally inactive periods and disappeared upon arousal and movements. They discriminated two distinct electrophysiological states according to the polygraphic recordings: (1) the 'nonspike' active state (i.e. without LSWs), characterized by a high EMG associated with low-voltage EEG activity and a heart rate of 20–30 beats/min, and (2) the 'spiking' state during behavioral quiescence, with a reduced EMG, EEG 'spikes' and a heart rate of 10–20 beats/min. The presence of LSWs during inactive states has been described in other reptiles [Pyrethon and Dusan-Pyrethon, 1968; Tauber et al., 1968; Flanigan et al., 1973]. It is debated whether this inactive state in reptiles corresponds to behavioral sleep or simply to a resting state. Independent of the interpretation adopted, and considering the EEG characteristics, it seems logical to think that the *in vitro* brain would present a spiking inactive state.

The effect of physostigmine supports this interpretation. In mammals this drug induces an EEG activity similar to that present during wakefulness, i.e. a conspicuous hippocampal theta rhythm associated with neocortical low-voltage fast activity. This effect is blocked by muscarinic cholinergic antagonists such as atropine [Stumpf et al., 1962; Stumpf, 1965; Kramis et al., 1975; Vanderwolf et al., 1978; Leung, 1985]. The administration of physostigmine in turtles *in vivo* [Gaztelu et al., 1990] results in the disappearance of LSWs and an amplitude increase in background EEG activity – an effect reversed by atropine. These changes correspond to electrographic modifications produced when passing from the 'spiking' to the 'non-spiking' state [Walker and Berger, 1973]. We have demonstrated that physostigmine exerts the same effect in *in vitro* brains.

The LSWs were observed in the three regions studied: DC, MC and S. In conditions resulting in the decrement or abolishment of LSWs, however, they always persisted longest at the S. They likewise presented greater amplitude and complexity in the MC than in the DC, coinciding with data described in the *in vivo* model [Gaztelu et al., 1990]. These facts suggest that the S presents a greater capacity for LSW generation, followed in decreasing order by the MC and the DC.

Neuronal Basis of LSW

The MC neurons evidenced several modifications in the membrane potential during the LSWs, the most important of which were the bursting discharges. High frequency spike discharges riding on large-amplitude slow depolarizations are similar to the 'paroxysmal depolarization shifts' described by Matsumoto and Ajmone-Marsan [1964]. 'Bursting cells' have been described in a discrete strip of the dorsal MC region of turtles [Shen and Kriegstein, 1986]. Bursts generated with a low firing level and a lower amplitude depolarizing wave have probably originated at different electrotonic distances from the recording electrode – perhaps in a dendritic shaft – and secondarily propagated to the soma.

The LSWs are similar to EEG interictal epileptiform sharp waves. Their production requires the synchronization of burst activity in a large group of neurons. This synchronization is produced by an excitatory synaptic circuitry organized so that the output of a group of bursting cells feeds back onto itself and its neighbors [Prince, 1985]. The anatomical basis for this feedback excitation in the cortex of turtles could be provided by recurrent pyramidal cell axons [Trujillo-Cenòz, pers. commun.]. As found by Russo et al. [unpubl. observations] most MC pyramids are capable of generating plateaus, due to their high threshold Ca^{2+} conductance, which surely must participate in burst generation.

Effects of Hypoxia

The LSWs increased in amplitude and frequency when O_2 was not bubbled in the Ringer solution. These effects were produced by the O_2 per se but not by either CO_2 or pH modifications under these experimental conditions. The preparation maintained its properties unchanged during many hours in conditions of hypoxia, with the exception of an increase in LSWs and bursting discharges. No changes were observed in either background EEG activity, resting membrane potential spikes or afterpotentials. Present results do not agree with those obtained *in vivo* in fresh water turtles by Lutz et al. [1985]: EEG amplitude decreased, and LSWs were not present. This discrepancy may be caused by the complete anoxia to which turtles were submitted in experiments by Lutz et al.

There is evidence that fresh water [Ultsch and Jackson, 1982] and salt water [Berkson, 1966; Felger et al., 1976] turtles can survive during prolonged periods (weeks and months) in an N_2 environment. Indeed, these animals possess an efficient anaerobic mechanism characterized by increased glycolysis, the depletion of creatine phosphate stores and a decrease of ATP consumption [Lutz et al., 1985] which enables the compensation of the prolonged anoxia, and characterizes them as 'facultative anaerobes'. Because of this metabolical duality, aquatic turtles do not need to breathe regularly when out of the water.

We may assume that the increase in bursting discharges and the later synchronization giving rise to LSWs are due to the relative hypoxia and consequent anaerobiosis. The appearance of these transient waves during behaviorally inactive periods might be determined by the decrease in cerebral blood flow, given that cardiac output is reduced by a decrease in heart rate [Walker and Berger, 1973]. Nevertheless, it is not known whether the compensatory processes promoting survival during anaerobiosis are present in land turtles and other reptiles.

Activity remained stable in *in vitro* brain preparations in spite of important pH variations. Nevertheless, LSW frequency increased with alkalosis and de-

creased with acidosis. These findings are in agreement with data obtained in mammals, including humans, which state that epileptiform discharges and cortical excitability vary in accordance with pH variations [Millichap, 1969].

EEG Genesis

Suppression of Na^+ spikes by TTX causes the EEG to disappear in spite of the persistance of potentials generated by Ca^{2+} inward currents. This effect of TTX on the EEG could be due to two mechanisms: (1) Na^+ spikes could participate directly in the genesis of the field potentials, and (2) the disappearance of Na^+ spikes could determine the disappearance of synaptic activity through the nondepolarization of axon terminals. It has been found that Na^+ spikes may contribute to the production of field potentials only if they are highly synchronized [Creutzfeldt et al., 1966a]. Therefore, although it cannot be discarded, it is not likely that TTX could act through this mechanism. On the other hand, the contribution of postsynaptic potentials suggested by some authors [Eccles, 1951; Bremer, 1958] has been demonstrated experimentally [Jasper and Stefanis, 1965; Creutzfeldt et al., 1966a, b]. Calcium potentials do not determine synaptic activity because they are mainly generated in dendrites and do not propagate through the axon. Obviously, voltage-dependent Ca^{2+} conductances that trigger neurotransmitter release are not activated because they require the depolarization of the presynaptic terminal determined by Na^+ spikes.

The power spectra corresponding to slow membrane potentials in the MC cells coincide in the 0.8- to 2.5-Hz maximum power band of the EEG, indicating that their contribution to EEG genesis is fundamental compared to that of rapid phenomena (Na^+ spikes). Apart from postsynaptic potentials, other slow phenomena could be in the basis of EEG genesis, namely hyperpolarizing and depolarizing afterpotentials, slow Ca^{2+} spikes, plateau potentials and long duration hyperpolarizations. The 0.3-Hz peak frequency band, however, does not coincide with an important band in the EEG power spectra. In the summation of SMPs of the neuronal aggregate, the power of the 0.3-Hz band would decrease due to the predominance of faster potentials. Likewise, the power increase in the 9- to 22-Hz band of the EEG power spectra with respect to the SMP power spectra might result from the summation of asynchronic potentials of neuronal aggregates, which would tend to increase the power of the higher frequency band.

In addition to the advantages provided by *in vitro* preparations – media modifications, mechanical stability, easy electrode positioning – the whole hemisphere has none of the disadvantages of slices [see for example Schwartzkroin, 1981] or slabs, because most neural connections are preserved intact. Synaptic activity was prominent and neurons presented spontaneous firing – which cannot be attributed to injury because it can last for many hours. Nevertheless, impalement and electrode positioning was more difficult than in slices.

The model proved to be stable for several hours, probably due to the high anaerobic brain capacity of aquatic turtles. The simple atmospheric diffusion of O_2 in the medium was sufficient to maintain the preparation without requiring intravascular perfusion. The fact that we used young specimens with small brains (measuring about 400 μm between the pia and the ependymary epithelium) must have contributed towards this stability.

The *in vitro* preparation of turtle brains constitutes a natural model of epilepsy, with spontaneous LSWs which can be correlated with cellular activity. The model is useful in furthering our understanding of generation mechanisms and study discharge propagation in septocortical circuits – and eventually in others. The simultaneous study of intracellular activity and the EEG might enable us to approximate the mechanisms intervening in complex phenomena such as kindling, long-term potentiation and basic aspects of learning and memory.

Acknowledgements

We thank Dr. O. Trujillo-Cenóz and Dr. O. Macadar for the critical reading of the manuscript, Mr. S. Basalo for computer aid and Dr. I. Trabal for the English version of the text. This research was supported by grants CII.0165 U(H) and CII.0211-4 (EL) from the Commission of European Communities.

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