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Cellular correlates of spontaneous periodic events in the medial entorhinal cortex of the *in vitro* isolated guinea pig brain

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Abstract

Periodic potentials characterized by fast oscillations superimposed on a slow complex event are typically observed in cortical structures during sleep and anaesthesia. In the entorhinal cortex (EC) similar spontaneus periodic events (SPEs) have been described both *in vivo* and *in vitro*. Simultaneous extracellular and intracelluar recordings from superficial neurons of the entorhinal cortex of the isolated Hartley guinea pig brain preparation demonstrated that SPEs recur with a periodicity of 2–10 s and correlate to neuronal firing superimposed on a depolarizing plateau that lasts 0.7–1 s. During SPEs, putative interneurons in all layers discharged high frequency firing (> 100 Hz), whereas no activity was observed in principal neurons of deep entorhinal cortex layers. Linear correlation analysis demonstrated a tight relationship between the fast component of the extracellular SPE and subthreshold oscillatory activity/neuronal firing in both superficial neurons and putative interneurons; firing of deep principal cells was independent from SPEs occurrence. The present study demonstrates that recurrent spontaneous events analogous to periodic activity observed during sleep/anaesthesia are generated in the entorhinal cortex by the interactions between superficial neurons and interneurons.

Introduction

Periodic neuronal activity contributes to the generation of brain signals in different states of vigilance. A typical periodic pattern recorded in the sleeping and/or anaesthetized brain is characterized by the recurrent appearance of slow waves superimposed on fast activity, identified either as K-complexes (Amzica & Steriade, 1997; Halasz, 2005) or as the A-phase of a cyclic alternating sleep pattern (Terzano *et al*., 1985). The cortical circuit responsible for such cortical activity is not defined yet. We recently described the recurrent occurrence of spontaneous periodic events (SPEs) that resemble periodic complexes of sleep in the medial entorhinal cortex (mEC) of the *in vitro* isolated guinea pig brain (Dickson *et al*., 2003). SPEs in mEC are characterized by brief runs of fast activity at 30–80 Hz superimposed on a slow bi/triphasic potential that recur at 0.1–0.5 Hz and are abolished by pharmacological muscarinic activation (Dickson *et al*., 2003). SPEs correlate with membrane potential depolarization associated with fast subthreshold oscillations and sparse neuronal firing of superficial entorhinal cortex (EC) neurons. Similar activity patterns were observed during the up-down states in different structures and in different types of neurons *in vivo* (Buzsaki *et al*., 1992; Steriade *et al*., 1993b; Chrobak & Buzsaki, 1996; Wilson & Kawaguchi, 1996; Lampl *et al*., 1999; Grenier *et al*., 2001), in isolated cortical slabs (Timofeev *et al*., 2000) and on *in vitro* cortical slices (Sanchez-Vives & McCormick, 2000; Wu *et al*., 2002). Periodic up-down states were also reported in the EC *in vivo* (Charpak *et al*., 1995; Collins *et al*., 2001).

SPEs in the EC were previously analysed exclusively in superficial neurons (Dickson *et al*., 2003). To evaluate the possible contribution of different populations of EC neurons to field SPEs, we performed intracellular recordings from principal neurons of deep and superficial layers and from putative interneurons. We demonstrated that SPEs correlate with firing in both superficial neurons and interneurons, but are not correlated with activity in deep layer principal cells. These findings were preliminarily reported in abstract form (Gnatkovsky & de Curtis, 2005).

Materials and methods

Following barbiturate anaesthesia (80 mg/kg sodium thiopental, i.p.), brains of young adult Hartley guinea pigs (150–250 g weight; Charles River, Comerio, Italy) were isolated and transferred to a cooled (15 °C) incubation chamber, according to the previously described technique (Dennis & Kerr, 1968; Llinas *et al*., 1981; Muhlethaler *et al*., 1993; de Curtis *et al*., 1998a). A saline solution composed of (in mM) 126 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, 15 glucose, and 3% dextran (relative molecular mass 70 000), oxygenated with a 95% O_2 and 5% CO_2 gas mixture at pH 7.3, was arterially perfused *in vitro* at 7.5 mL/min. After 1.5 h incubation, recordings were performed at 32 °C. The experimental protocol was reviewed and approved by the Committee on Animal Care and Use and by the Ethics Committee of the Istituto Neurologico, in accordance with the International guidelines on care and use of laboratory animals.

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Extracellular potentials were recorded in the mEC with single glass pipettes filled with 1 M NaCl. A stimulating electrode (insulated twisted silver wire) was positioned on the lateral olfactory tract (LOT) to deliver stimuli (10–25 μA, 300 μs) at 0.2–2 Hz that evoked typical responses in the mEC (see Figs 1–3; Biella & de Curtis, 2000; Gnatkovsky & de Curtis, 2006). Intracellular recordings in proximity to the extracellular pipette were performed with sharp glass microelectrodes filled with 2 M potassium acetate and 1.5% biocytin (input resistance 70–100 MOhm). The position of the electrodes was visually controlled with a stereoscopic microscope (Nikon SMZ-2T, Japan). Three to five penetrations through the entorhinal cortex were made to record blindly from EC neurons in superficial and deep layers; the recording electrode was repositioned > 500 μM apart for successive penetrations. Signals were processed with a multichannel differential extracellular amplifier in DC mode (Biomedical Engineering, Thornwood, US) and with a Neuro Data intracellular amplifier (Neuro Data Instruments Corp, New York, US). Potentials were digitized with a PCI-6071E DAQ board (National Instruments, Austin TX, US) and were acquired and analysed with ELPHO^{TM}, a software developed by Vadym Gnatkovsky at the Istituto Neurologico.

Simultaneous intracellular and extracellular recordings were performed in the mEC. The frequency of action potential firing as a function of time, denoted by FAP(t), was calculated by counting the number of spikes over a 500-ms time window sliding by 60-ms step intervals. Joint time-frequency analysis (JTFA) was applied to study the frequency content of the spontaneous extracellular oscillations.

Linear correlation analysis was applied in order to reveal and quantify possible relationships between extracellular and intracellular activity recorded from deep neurons, superficial neurons and interneurons. To proceed, the extracellular activity was filtered in the frequency band of interest (10–120 Hz; Fig. 1) and the time-course of the energy of the filtered signal, denoted by EEA(t), (energy of extracellular activity) was estimated in the classical way (amplitude of the signal squared, averaged over a sliding window of 500 ms). Then, correlation analysis was performed on FAP(t) and EEA(t) processes. For each cell type (deep neuron, superficial neuron and interneuron), such analysis provided the degree to which simultaneously recorded intracellular and extracellular activities are interrelated, expressed by a coefficient, r^2, comprised between 0 and 1. High values of this linear correlation coefficient indicate strong dependence between analysed processes. Existence of a significant correlation between FAP(t) and EEA(t) was systematically assessed using appropriate statistical test, as described in Appendix 1.

At the end of the electrophysiological experiment, brains were removed from the chamber and were fixed by immersion in a 4% paraformaldehyde solution. After at least 24 h in fixative, 75–100-μm slices were cut by vibratome and the morphology of intracellularly recorded neurons was revealed by processing the sections for standard biocytin-horseradish peroxidase visualization (ABC kit, Vector Labor-

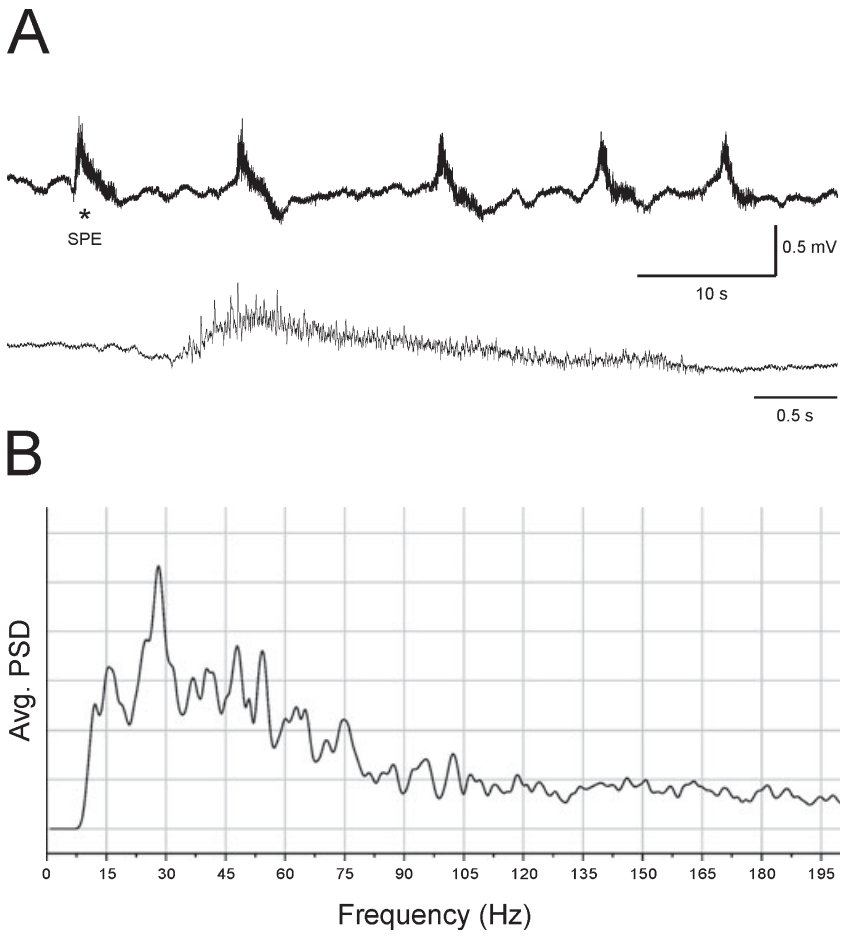

FIG. 1. Spectral content of the extracellular activity recorded in the mEC during SPEs. (A) In the upper trace extracellular SPEs (asterisk) are illustrated. The lower trace shows a single high-pass filtered SPE (cut-off frequency equal to 10 Hz). (B) Averaged power spectral density (PSD) estimated from 15 SPEs filtered at < 10 Hz recorded in different experiments.

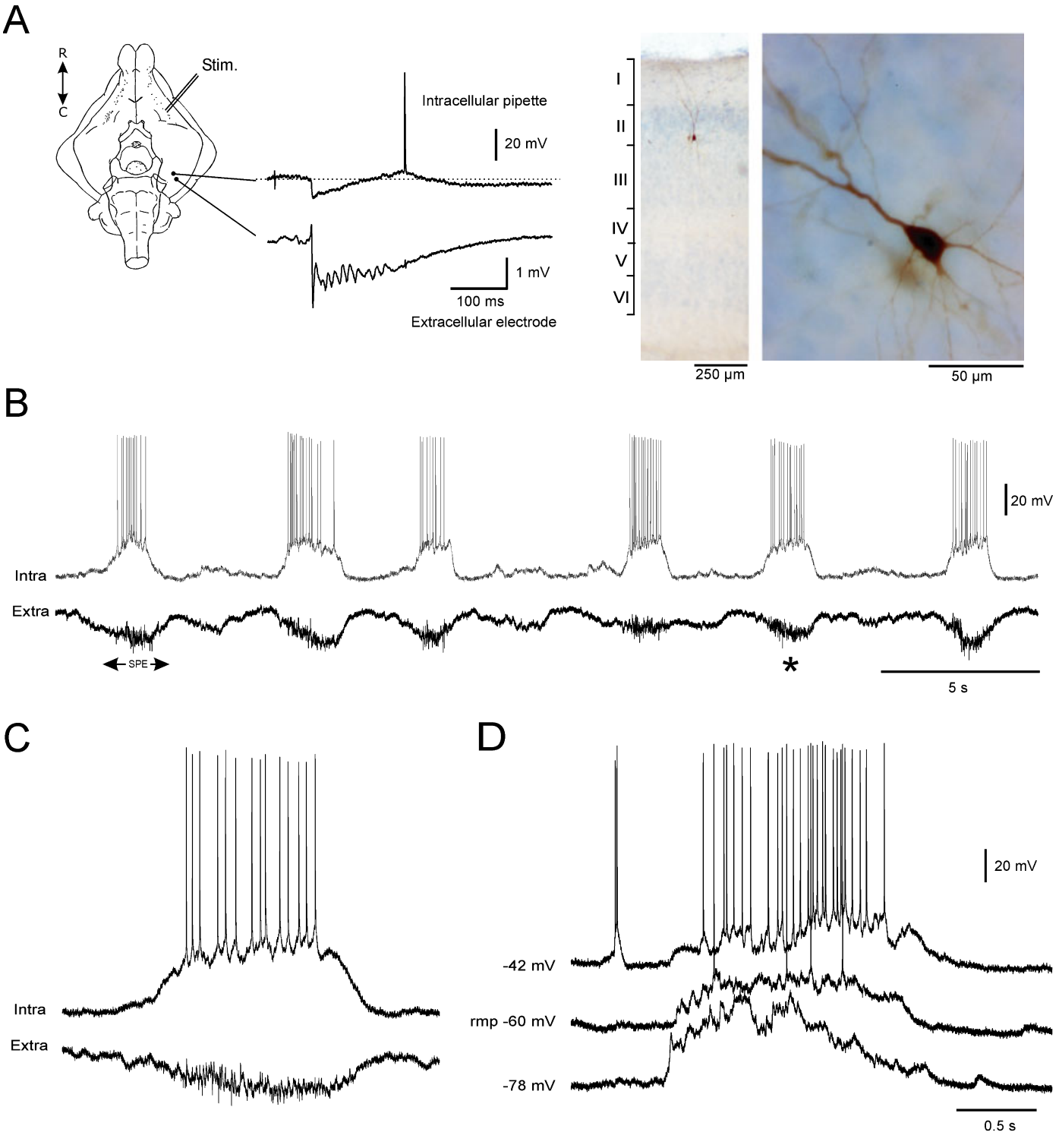

FIG. 2. Intracellular recordings from mEC superficial layer neurons during SPEs. (A) Typical response of a pyramidal cell of layer II to LOT stimulation. The position of the intracellular (upper trace) and extracellular (lower trace) recording electrodes and the stimulation electrode are illustrated in the panel on the left. The morphology of the recorded cell, injected with biocytin at the end of the electrophysiological recording is illustrated in the right panel on a thionine-stained section at two different magnifications. Note dye coupling between the recorded neuron injected with biocytin and a neighbouring cell. (B) Simultaneous extracellular and intracellular recordings during SPEs from the same superficial neuron shown in A. *The SPE marked by the asterisk is illustrated in the expanded trace in C. (D) Sequential SPEs recorded from a different superficial mEC cell at different membrane potentials imposed by injecting steady hyperpolarizing/depolarizing currents via the intracellular recording pipette. Rmp, resting membrane potential (−60 mV).

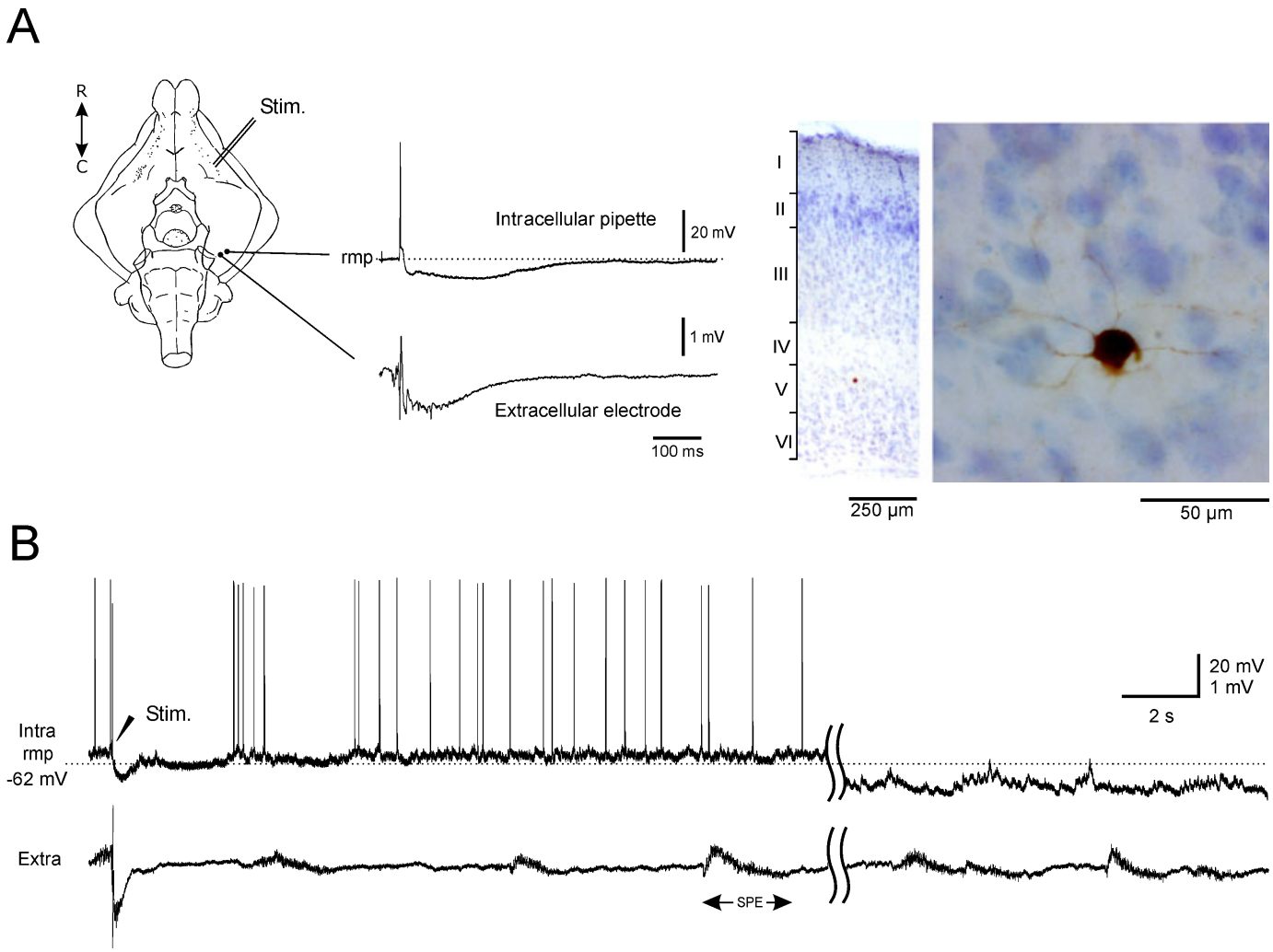

FIG. 3. Intracellular recordings from principal mEC neurons of deep layers during SPEs. (A) Typical deep-layer cell response to LOT stimulation. The morphological features of the recorded cell and its depth location are illustrated in the right part of the panel (section counterstained with thionine) at two different magnifications. (B) Activity during SPEs of the same deep layer neuron illustrated in A. At resting membrane potential (−62 mV; dotted line) background neuronal firing not correlated to SPEs was observed. When the membrane potential of the neuron was hyperpolarized by continuous injection of a negative current via the intracellular electrode (right part of the traces), action potential firing was blocked and no slow depolarizing potential were observed in correlation with SPEs. The response evoked by LOT stimulation is marked by the arrowhead.

atories, Burlingame, CA). Sections were counterstained with thionine to identify cortical layers.

Results

Forty-three and 67 principal neurons were recorded, respectively, in deep layers (800–1100-µm depth) and in superficial layers (200–700-µm depth) of the mEC during SPEs. The morphology of 25 cells was reconstructed after visualization of intracellularly injected biocytin (see Materials and methods). Pyramidal and stellate cells (Germroth *et al*., 1989) with average (Lingenhohl & Finch, 1991; Gloveli *et al*., 1997; Klink & Alonso, 1997; van der Linden & Lopes da Silva, 1998) membrane potential of −60.8 ± 6.8 mV (mean ± SD) were recorded in superficial mEC layers II and III. Biocytin injection in neurons of superficial layers revealed dye coupling with 2–3 neighbouring cells in 30% of the cases. Polymorph-multipolar and pyramidal neurons (Hamam *et al*., 2000) with −61.4 ± 6.4 mV average membrane potential were found in deep mEC layers V and VI. No dye coupling was observed for deep-layer cells. The morphological classification of the neuronal type was based on morphological features of soma and dendrites. LOT stimulation was carried out to identify typical and distinctive responses of principal neurons of both superficial and deep layers in the mEC (Figs 2 and 3; (Gnatkovsky & de Curtis, 2006). The depth of the recording position and the response to LOT stimulation were utilized as criteria to identify those cells that were not stained by biocytin. Intracellular recordings were performed from seven putative interneurons.

As previously demonstrated (Biella & de Curtis, 2000; Gnatkovsky & de Curtis, 2006), LOT stimulation at 1 Hz induced a polysynaptic delayed response in the mEC, mediated by the interposed activation of the hippocampus. The intracellular correlate of the LOT-evoked delayed response in superficial layer mEC neurons was characterized by a pure IPSP, followed by a slow depolarizing component (Fig. 2A; Gnatkovsky & de Curtis, 2006). During extracellularly recorded field SPEs, slow (0.7–1 s) membrane potential depolarizing shifts (18 ± 2.4 mV; mean ± SD) crowned by fast subthreshold oscillations and action potential firing was observed in all principal neurons of layers II and III (Fig. 2B and C; see also

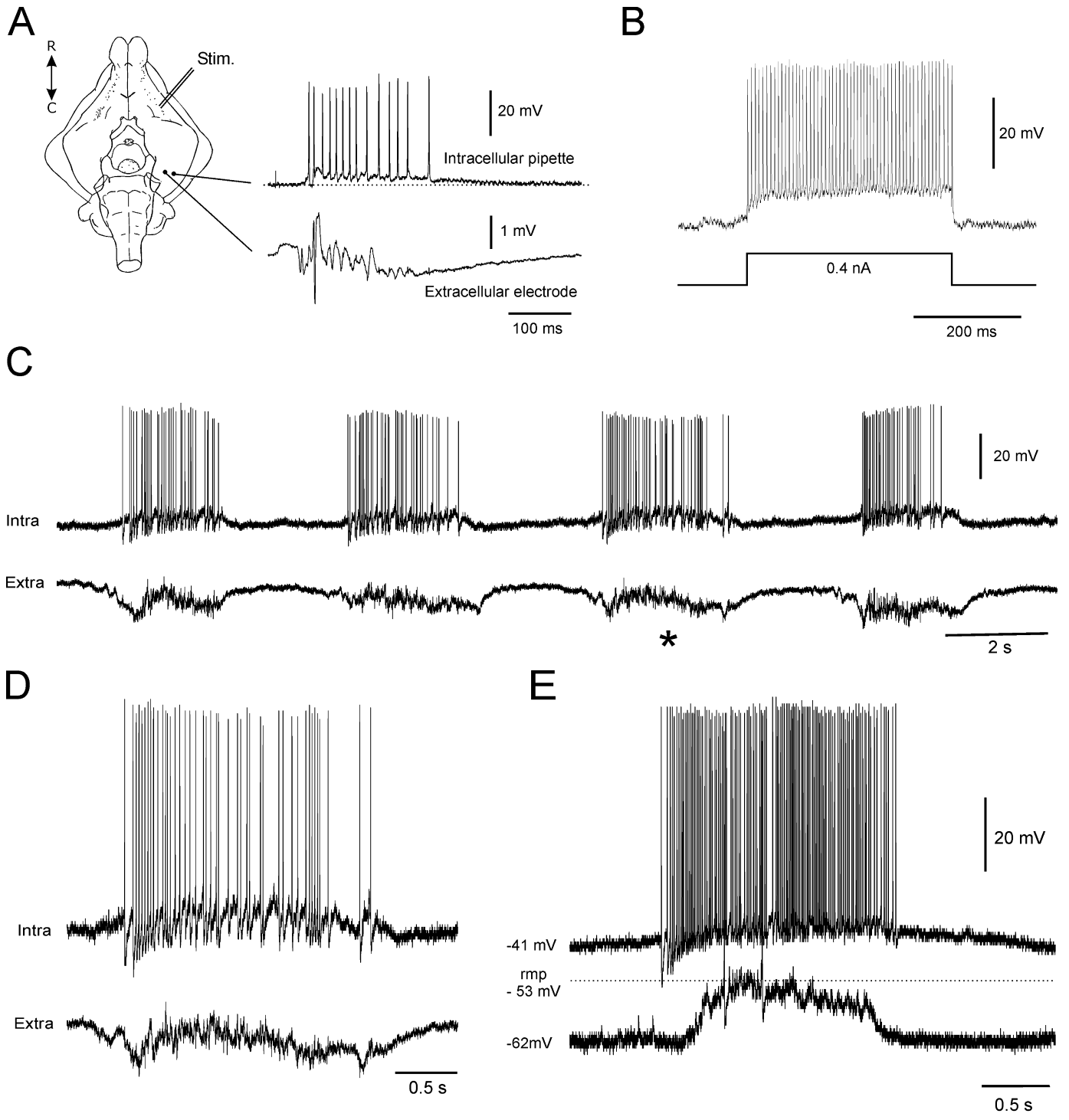

FIG. 4. Intracellular recordings from mEC putative interneurons during SPEs. (A) Typical response of a mEC putative interneuron to LOT stimulation. Simultaneous intracellular and extracellular recordings are shown. (B) Characteristic high frequency, nonadapting firing of the same putative interneuron in A, evoked by intracellular injection of a 0.4-nA depolarizing current pulse (lower trace). (C) The firing discharge of the same putative interneuron during SPEs is illustrated. *The SPE marked by the asterisk is expended in D. Two SPEs recorded at two different membrane potentials are shown in E. Rmp, resting membrane potential −53 mV.

Dickson *et al.*, 2003). In five of the experiments intracellular injection of biocytin into a single layer II–III principal neuron resulted in staining of one or more additional neurons in close proximity to the one injected with the dye (see Fig. 2). Sub-threshold fast oscillations consistently generated action potentials when the membrane potential of neurons was depolarized by a steady intracellular current injection (upper trace at −42 mV in Fig. 2D). In these conditions, action potential firing frequency (20–40 Hz) during SPE was similar to the largest frequency peak of the field potential oscillations (see Fig. 1B). After suppression of action

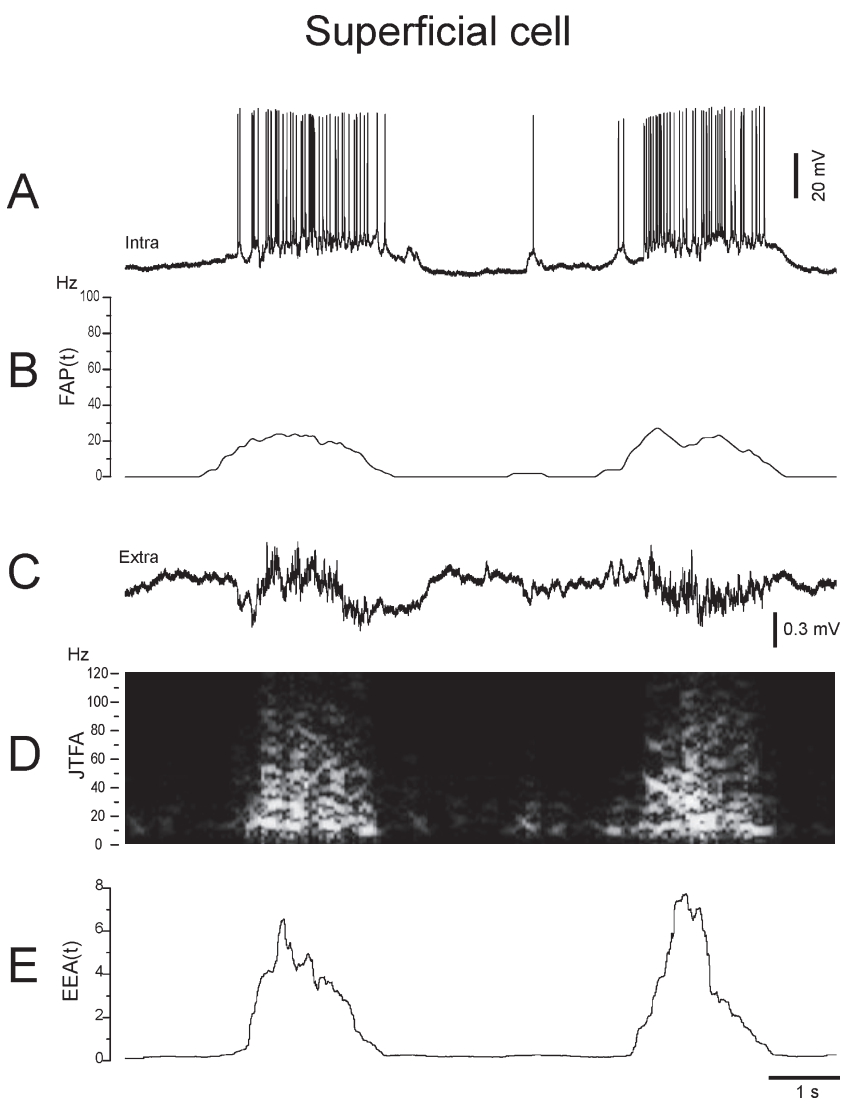

FIG. 5. Correlation between extracellular activity and intracellular potentials of mEC superficial layers principal neurons during SPEs. (A) Intracellular recordings from a mEC superficial layer neurons. (B) Time-course of the cellular firing, FAP(t) shown in trace A, calculated by counting the number of spikes over a 500-ms time window sliding by 60-ms step intervals. (C) Extracellular recordings of SPEs performed simultaneously to the intracellular recording. (D) JTFA of SPE shown in C. (E) Time-course of the extracellular signal energy, EEA(t), in the frequency band of interest (20–120 Hz) estimated as the magnitude of the signal squared, integrated over a sliding temporal window of 500 ms.

potentials by hyperpolarizing current injection, rapid oscillations (15–40 Hz) were observed on the slow membrane potential depolarizing shift (trace at −78 mV in Fig. 2D).

In deep mEC principal neurons a characteristic EPSP-spike sequence followed by a biphasic IPSP was recorded following LOT stimulation (Fig. 3A; see Gnatkovsky & de Curtis, 2006). Unlike superficial mEC neurons, no distinctive neuronal firing was observed in deep neurons in correlation with extracellularly recorded SPEs ($n = 43$; Fig. 3B), even though random membrane potential fluctuation occurred. Few neurons ($n = 3$) showed low amplitude membrane oscillation during field SPE (not shown), but depolarizing shifts with action potential firing, as recorded in superficial neurons, were never observed. These results are further supported by linear correlation analysis.

Intracellular recordings throughout the depth of the mEC also led to the identification of seven cells with electrophysiological features different from previous types of cells. They were classified as putative interneurons, according to the following electrophysiological criteria; (i) burst of high rate spikes (> 80 Hz) in coincidence with the hippocampus-driven delayed mEC response generated by LOT stimulation (Fig. 4A), and (ii) fast action potential at high frequency (> 100 Hz) with no frequency adaptation in response to suprathreshold depolarizing pulse injection (Fig. 4B; see McCormick *et al*., 1985; Jones & Buhl, 1993; Kumar & Buckmaster, 2006). We did not succeed in staining interneurons with biocytin, because intracellular recordings in this type of neurons were performed for brief periods (< 10 min). During SPEs, putative interneurons discharged 80–100 Hz action potential barrages at resting membrane potential (Fig. 4C–E). This pattern of firing was never observed in morphologically identified principal neurons.

Figure 5 illustrates the correlation analysis performed by comparing simultaneous extracellular and intracellular recordings from superficial mEC neurons during SPEs. Firing of superficial cells (Fig. 5A) quantified by measuring FAP(t) (Fig. 5B) appeared in coincidence with extracellular SPEs (Fig. 5C) themselves quantified by their time-frequency content and time-varying subband energy values, EEA(t),

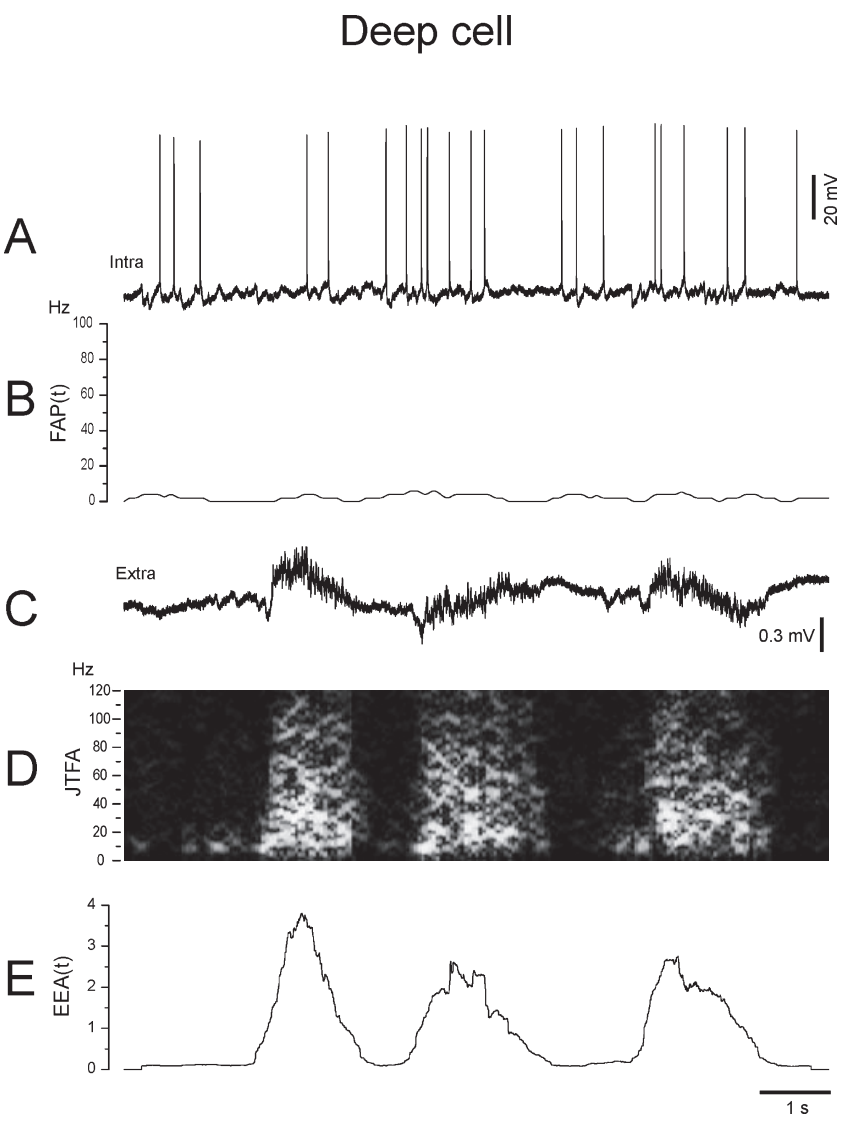

FIG. 6. Correlation between extracellular activity and intracellular potentials of mEC deep layers principal neurons during SPEs. (A) Intracellular recordings from a deep-layer neuron. (B) Time-course of the cellular firing, FAP(t), shown in the trace A. (C) Extracellular recordings of SPEs performed simultaneously to the intracellular recording. (D) JTFA of SPE shown in C. (E) Time-course of the extracellular signal energy EEA(t).

respectively, illustrated in Fig. 5D and E. Linear correlation analysis between FAP(t) (Fig. 5B) and the EEA(t) (Fig. 5E) in pairs of simultaneous intracellular and extracellular recordings, showed a high correlation coefficient r^2 (0.69 ± 0.16, mean ± SD, $n = 67$). Significant correlation was confirmed for all recordings performed in principal neurons in superficial layers.

In deep layers principal neurons, no correspondence between extracellular SPE oscillations (Fig. 6C–E) and neuronal firing (Fig. 6A and B) was observed. Accordingly, FAP(t)–EEA(t) linear correlation coefficient r^2 was very low (0.07 ± 0.05, mean ± SD, $n = 40$) in all but three deep layer neurons.

High frequency firing of putative interneurons at 80–100 Hz (Fig. 7A and B) was associated in time with SPE oscillations (Fig. 7C–E), A high linear correlation coefficient r^2 (0.74 ± 0.11, mean ± SD, $n = 10$) was observed between extracellular SPEs and interneuronal firing.

Finally, for all recordings performed in principal neurons in superficial layers and in interneurons, the test described in Appendix 1 demonstrated a statistically significant correlation between analysed processes FAP(t) and EEA(t). Conversely, the statistical test led to the acceptance of the 'no correlation' hypothesis for all but three deep layer neurons.

Discussion

We confirm in the present study that spontaneus periodic events reminiscent of the periodic activity observed in the sleeping/anaesthetized brain occur in the mEC of the *in vitro* isolated guinea pig brain (Dickson *et al*., 2003). Periodic activity with similar features was described previously *in vivo* in the parahippocampal region of anaesthetized animals (Charpak *et al*., 1995; Collins *et al*., 2001). By correlating the activity of different populations of neurons located in different mEC layers with extracellular SPEs, we conclude that (i) SPEs are coupled with neuronal firing both in principal neurons of superficial layers and in putative interneurons diffusely distributed within the mEC depth; (ii) firing of superficial cells and of putative interneurons coincides and is highly correlated with the fast oscillations recorded during extracellular SPEs, and (iii) firing of principal

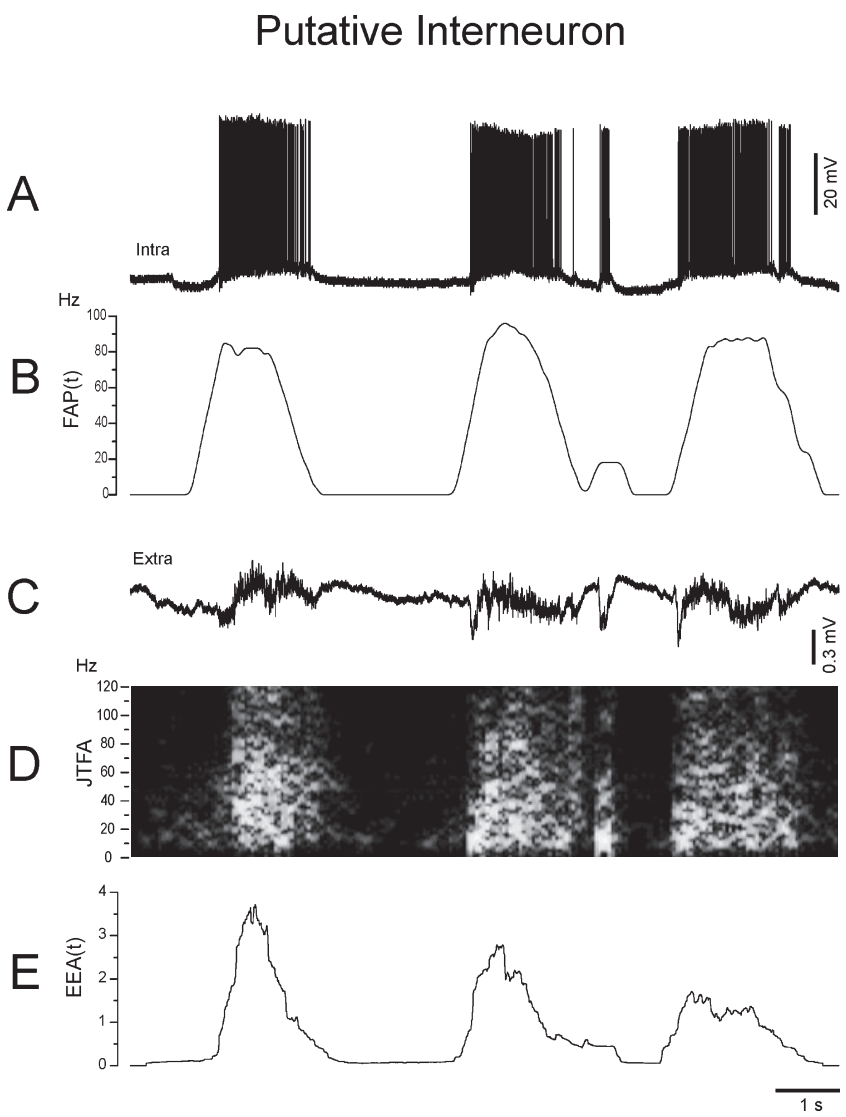

FIG. 7. Correlation between extracellular activity and intracellular potentials of mEC putative interneurons during SPEs. (A) Intracellular recordings from a putative interneuron in superficial mEC layers. (B) Time-course of the cellular firing, FAP(*t*), shown in the trace A. (C) Extracellular recordings of SPEs performed simultaneously to the intracellular recording. (D) JTFA of SPE shown in C. (E) Time-course of the extracellular signal energy, EEA(*t*).

neurons located in the deep layers of the mEC is not associated with the SPE, and therefore do not contribute to its generation.

The large majority of principal neurons in layers V and VI, indeed, do not fire action potentials, neither generate subthreshold oscillations during SPEs. These data are in agreement with a report in entorhinal cortical slices *in vitro* (Cunningham *et al*., 2006). Only three out of 43 deep principal neurons showed low amplitude fast oscillations with sporadic action potential firing in correlation with SPEs. Such activity did not reach threshold for neuronal firing and therefore did not contribute to the generation of SPE. Correlated activity in these three neurons was possibly due to intrinsic connections with the superficial neurons that fired in association to SPEs (Witter & Wouterlood, 2002). Unlike deep-layer principal neurons, firing of putative interneurons strongly correlated to SPE occurrence.

In vivo models as well as the isolated brain preparation offer the unique advantage to preserve functional connections compared to slice models. However, in these experimental models, the counterpart is that morphological identification of interneurons is not easily achieved. As we were not able to verify the morphology of the putative interneurons, we utilized widely accepted electrophysiological criteria (McCormick *et al*., 1985; Jones & Buhl, 1993; Kumar & Buckmaster, 2006) for their identification. In the isolated guinea pig brain intracellular recordings with sharp electrodes are performed by penetrating the cortex from the pial surface. Because of both the unfavourable tissue approach and the small size of the cells, intracellular recording from interneurons in our preparation was consistently lasting less than 10 min. Brief impalements may not allow for intracellular diffusion of enough biocytin into interneurons during the electrophysiological recording, therefore hindering the possibility of reconstructing their morphological features.

Slow periodic patterns associated with slow-wave sleep and/or the anaesthetized state are maintained by local cortical generators. Spontaneus periodic events at < 0.1 Hz characterized by rhythmic cycles of slow depolarizing waves superimposed on fast oscillations (up state), followed by membrane hyperpolarization and cessation of firing (down state) have been demonstrated during slow-wave sleep

in vivo and during anaesthesia in cortical and diencephalic regions (Metherate & Ashe, 1993; Steriade *et al*., 1993a; Cowan & Wilson, 1994; Timofeev *et al*., 2000). The intracellular mechanisms that sustain such on–off states have been detailed further during an intracellular study performed in tissue slices maintained *in vitro*, obtained from cortical and subcortical structures. According to one report, the up state of the slow periodic event can be attributed to a tonic condition of excitation that is transiently interrupted by the activation of intrinsic conductances during the down state (Contreras *et al*., 1996). More recently, the role of synaptic network interactions in initiating an up state has been emphasized. Grenier *et al*. (2001) proposed that fast activity during up states in the neocortex could be sustained by recurrent connections between excitatory and inhibitory neurons. Studies by the group of David McCormick (Haider *et al*., 2006) confirmed that the up state of the periodic slow activity is the result of active local excitatory and inhibitory networks that spontaneously maintain a depolarized membrane potential initiated by excitatory influences, therefore generating self-sustaining activity that can be turned on and off by synaptic inputs (Shu *et al*., 2003).

Previous studies on ferret neocortical slices *in vitro* (Sanchez-Vives & McCormick, 2000; Hasenstaub *et al*., 2005) reported that putative interneurons (fast spiking cells) generate action potentials in phase within the occurrence of on states similar to SPEs. High frequency firing of putative interneurons during up states was also reported *in vivo* in the neocortex of the ferret anaesthetized with xylazine–ketamine (Haider *et al*., 2006). Our findings on the generation of SPEs in the mEC are in line with these reports and extend to a limbic cortical structure the critical role played by inhibition in controlling periodic fast activities associated with sleep and anaesthesia.

Several reports indicate that gap junctions between cortical neurons (Nadarajah *et al*., 1996) may be responsible for functional coupling of large subsets of cells during the generation of synchronous fast oscillations in cortical structures (Traub *et al*., 1999; Schmitz *et al*., 2001). The contribution of gap junctions in network oscillations (Kano *et al*., 2005) and in bicuculline-induced spontaneous interictal spikes (de Curtis *et al*., 1998b) has been demonstrated in the limbic cortices. Gap-junction blockers disrupt kainite mediated gamma oscillation in superficial mEC neurons on *in vitro* EC slices (Cunningham *et al*., 2004). The functional link between neurons through gap-junctions can be responsible for intercellular diffusion of small molecules, such as biocytin, and results in dye coupling when intracellular biocytin is revealed by immunoreaction. In our experiments, neuronal dye coupling was observed between layer II–III principal superficial mEC neurons injected with biocytin (Fig. 2). Dye coupling was never observed to occur between deep layer neurons. The presence of functional gap-junctions, as revealed by the presence of dye coupling may predispose superficial mEC neurons to generate synchronous oscillations during SPE and in conditions of hyperexcitability, such as during the generation of epileptiform discharges (Gnatkovsky *et al*., 2006).

Appendix 1

Statistical test for the existence of a significant correlation between variables of interest

Because of the variability of correlation estimates, it is required to verify if a sample non-zero value r_{xy} of the linear correlation coefficient ρ_{xy} corresponds to a statistically significant correlation between the variables of interest. This analysis can be performed by testing the hypothesis H_0 that $\rho_{xy} = 0$, where statistically high correlation is indicated if this hypothesis is rejected. In order to perform this test, a particular function w of r_{xy} is introduced

$$w = 1/2 \ln[(1 + r_{xy})/(1 - r_{xy})] \quad (1)$$

It can be shown that the random variable w is normally distributed with mean

$$m_w = 1/2 \ln[(1 + \rho_{xy})/(1 - \rho_{xy})] \quad (2)$$

and variance

$$\sigma_w^2 = 1/(N - 3) \quad (3)$$

under H_0.

Therefore, the acceptance region at the $(1 - p)$ level of confidence for hypothesis H_0 is given by:

$$-z_{p/2} \leq [0.5\sqrt{(N-3)}] \times \log_e[(1 + r_{xy})/(1 - r_{xy})] \leq z_{p/2} \quad (4)$$

where z is the normalized normal variable. It follows that values outside the interval [−2.576, +2.576] constitute evidence for the existence of a correlation between variables of interest at 99% level of confidence (P-value = 0.01, $z_{0.005} = -2.576$). This statistical test was applied to FAP(t)- and EEA(t)-values obtained from simultaneous intracellular and extracellular recordings for the three populations of neurons, as mentioned in the last paragraph of the Results section.

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Abbreviations

EC, entorhinal cortex; EEA(t), energy of extracellular activity as a function of time; FAP(t), frequency of action potential firing as a function of time; JTFA, joint time-frequency analysis; LOT, lateral olfactory tract; mEC, medial entorhinal cortex; SPEs, spontaneus periodic events.

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