Rhythmic Spontaneous Activity in the Piriform Cortex

Slow spontaneous rhythmic activity is generated and propagates in neocortical slices when bathed in an artificial cerebrospinal fluid with ionic concentrations similar to the ones in vivo. This activity is extraordinarily similar to the activation of the cortex in physiological conditions (e.g., slow-wave sleep), thus representing a unique in vitro model to understand how cortical networks maintain and control ongoing activity. Here we have characterized the activity generated in the olfactory or piriform cortex and endopiriform nucleus (piriform network). Because these structures are prone to generate epileptic discharges, it seems critical to understand how they generate and regulate their physiological rhythmic activity. The piriform network gave rise to rhythmic spontaneous activity consisting of a succession of up and down states at an average frequency of 1.8 Hz, qualitatively similar to the corresponding neocortical activity. This activity originated in the deep layers of the piriform network, which displayed higher excitability and denser connectivity. A remarkable difference with neocortical activity was the speed of horizontal propagation (114 mm/s), one order of magnitude faster in the piriform network. Properties of the piriform cortex subserving fast horizontal propagation may underlie the higher vulnerability of this area to epileptic seizures.

Keywords: endopiriform, epilepsy, olfactory, oscillations, up states

Introduction

The olfactory or piriform cortex is a 3-layered structure (paleocortex), with excitatory and inhibitory neurons that are more densely packed in layer II and in the more superficial part of layer III, whereas layer I is mostly formed by axons from the olfactory bulb and other cortical and extracortical areas (for a review, see Neville and Haberly 2004). Deeper to the piriform cortex is the endopiriform nucleus, considered by some authors the fourth layer of this cortex because of their extensive reciprocal connections (O'Leary 1937; Valverde-Garcia 1965; Luskin and Price 1983; Behan and Haberly 1999). We will refer to the piriform cortex and the endopiriform nucleus as the piriform network. The piriform cortex was classically considered a primary sensory area, but it has been recently proposed that it functions as an association area because of its high excitability, which can provoke epileptic discharges in certain conditions (Piredda and Gale 1985; Hoffman and Haberly 1996). Spontaneous interictal spikes in γ-aminobutyric acid (GABA)-blocked slices also spread from the piriform to the neocortex (Rigas and Castro-Alamancos 2004). In addition to the endopiriform nucleus, the deepest part of layer III adjacent to it is also the origin of epileptiform activity (Hoffman and Haberly 1991; Demir et al. 2001). As a consequence of its high seizure susceptibility, the functionality of the piriform cortex has been thoroughly studied in relation to epileptic activity (Piredda and Gale 1985; Racine et al. 1988; Haberly and Sutula 1992; Hoffman and Haberly 1993, 1996; De Curtis et al. 1994, 1996, 1999; Forti et al. 1997; Demir et al. 1999a, 1999b).

Here we describe spontaneous, non-epileptiform physiological activity generated by the piriform network in vitro, when bathed in an artificial cerebrospinal fluid (ACSF) that mimicks the ionic concentrations in situ (Sanchez-Vives and McCormick 2000). This rhythmic activity is similar to that described in the piriform cortex in vivo during ketamine anesthesia (Fontanini et al. 2003) and to some extent analogous to the slow rhythmicity generated by the neocortex during both anesthesia and natural slow-wave sleep (Steriade 1993; Timofeev et al. 2001). This physiological emergent activity from the piriform network can eventually be transformed into epileptiform activity, for example, by decreasing inhibition. Our main interest has been to analyze the distinct properties of the spontaneous rhythmic activity in the piriform network in order to understand its control mechanisms and identify what makes this area more prone to generate epileptiform activity.

Materials and Methods

Ferrets (2-12 months old, either sex) were anesthetized with sodium pentobarbital (40 mg/kg) and decapitated. The entire forebrain was rapidly removed to oxygenated cold (4-10 °C) bathing medium. Horizontal slices (0.4 mm thick) were cut from the ventral side of the temporal lobe. The first 4-5 slices obtained in this way contained the piriform cortex and endopiriform nucleus (Fig. 1). This slicing orientation was chosen because it best preserves rostrocaudal association fibers (Demir et al. 2001). In some experiments, additional slices from the primary and secondary visual cortical areas (areas 17, 18, and 19) were also placed in the bath, and recordings were made for comparison.

During preparation of slices, the tissue was placed in a solution in which NaCl was replaced with sucrose while maintaining the cerebral hemisphere and that arborize extensively within the piriform cortex (Johnson et al. 2000). These long connections are probably one of the factors contributing to the high excitability of these structures (Bhan and Haberly 1999). The deep anterior piriform cortex has been called “area tempestas” because of its high excitability, which can provoke epileptic discharges in certain conditions (Piredda and Gale 1985; Hoffman and Haberly 1996). Spontaneous interictal spikes in γ-aminobutyric acid (GABA)-blocked slices also spread from the piriform to the neocortex (Rigas and Castro-Alamancos 2004). In addition to the endopiriform nucleus, the deepest part of layer III adjacent to it is also the origin of epileptiform activity (Hoffman and Haberly 1991; Demir et al. 2001). As a consequence of its high seizure susceptibility, the functionality of the piriform cortex has been thoroughly studied in relation to epileptic activity (Piredda and Gale 1985; Racine et al. 1988; Haberly and Sutula 1992; Hoffman and Haberly 1993, 1996; De Curtis et al. 1994, 1996, 1999; Forti et al. 1997; Demir et al. 1999a, 1999b).

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osmolarity. After preparation, slices were placed in an interface style recording chamber (Fine Sciences Tools, Foster City, CA). For the first 15 min, cortical slices were superfused with an equal mixture in volume of the normal bathing medium and the sucrose-substituted solution. Following this, normal bathing medium was switched into the chamber and superfused the slices for 1-2 h. The normal bathing medium contained (in mM) NaCl, 126; KCl, 2.5; MgSO4, 2; NaH2PO4, 1.25; CaCl2, 2; NaHCO3, 26; and dextrose, 10 and was aerated with 95% O2 and 5% CO2 to a final pH of 7.4. Following this recovery time, the solution was switched to one of “in vivo-like” ionic composition, which had the same ionic composition except for different levels of (in mM) KCl, 3.5; MgSO4, 1; and CaCl2, 1-1.2 (Sanchez-Vives and McCormick 2000). Bath temperature was maintained at 34.5–36 °C.

Electrophysiological recordings started once in vivo-like ACSF. Drugs were applied either in the bath or locally through the delivery of a brief pressure pulse (10–150 ms; 100–350 KPa) to a drug-containing micropipette (volumes of 1–20 pl per pulse). The following drugs were used: N-2-amino-5-phosphonopentanoic acid (APV, local 500 μM), Bicuculline methiodide (local 200 μM; bath 20 μM), 6-cyano-7-nitroquinoxaline-2,3-dione with 2-hydroxypropyl-β-cyclodextrin (CNQX-HBC complex, local 250 μM), and L-glutamic acid (local 500 μM); all from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany).

Ferrets were cared for and used in accordance with the Spanish regulatory laws (BOE 256; 10/25/1990), which comply with the European Union guidelines on protection of vertebrates used for experimentation (Strasbourg 3/18/1986).

Spike Recording and Analysis

Extracellular multiunit recordings were obtained with 2-4 MΩ tungsten electrodes (FHC, Bowdoinham, ME), amplified, and high passed (>500 Hz) using a Neurolog system (Digitimer, Hertfordshire, UK). All measurements of speed of propagation were obtained from recordings whose exact location was anatomically identified (see below; Figs 1A, 7A, and 9B). Horizontal (same layer) and vertical (across layers) propagation was measured by means of simultaneous recordings from 2 or 3 tungsten electrodes placed on the surface of the slice. Often, one electrode was left in the same position and the others moved to different recording locations along the same vertical (across layers) line, such that up to 8 measurements were done, and the rate of propagation between different layers measured. To analyze the time structure of the multiunit extracellular recordings, the signal was digitized into “events”: upward swings of the multiunit recording (120 s acquired at 10 KHz) that crossed an arbitrary threshold were counted as events (Fig. 1B). Three different thresholds were used: 1) 2 × standard deviation (SD) of the noise, 2) 2 × SD of the same recording that was going to be analyzed, therefore including the activity and the noise, and 3) a threshold chosen by eye at approximately 2 × background noise levels. No significant differences were observed in the duration or frequency of the oscillations measured with any of the methods. All the data and figures presented here correspond to a threshold located at 2 × SD of the noise. The propagation, synchrony, and duration of the oscillations were measured in auto- or cross-correlograms. The Y axes in the auto- and cross-correlograms are expressed as “multiunit firing frequency (Hz)” as a measure of correlation; however, this unit of spikes per second should not be confused with the frequency of firing of a neuron. What multiunit firing frequency (Hz) represents is the total number of accumulated spikes per bin, divided by the number of sweeps, and then divided by the bin width. To calculate the duration of the up states, we obtained autocorrelograms of the spikes (events) occurring during a period of 120 s. The estimated mean frequency—expected value for a Poisson distributed spike train—and the 95% confidence interval were calculated and plotted (Fig. 2A inset). The mean frequency represents spikes per second without taking into account whether those spikes were organized into rhythmic discharges or not. The duration of the up states was taken as the half-width of the central peak in the autocorrelogram (bins = 20 ms) at the point where the mean frequency crossed the peak (Fig. 2A, inset).

The temporal relationship between the neuronal activities at varying recording sites was examined with crosscorrelograms. The speed of propagation was calculated from the offset of the central peak in crosscorrelograms of the activity recorded by electrodes located at a known distance, which was measured with a calibrated grid in the microscope. The main frequency of the oscillations was estimated as the tallest peak in the raw spike train power spectrum (0–20 Hz; Fig. 2B, D) obtained for the same 120 s of spike discharge used for the autocorrelograms. A similar value for the main frequency of the

Figure 1. Oscillatory activity in the piriform cortex. (A) Nissl-stained horizontal slice from the ferret, where the recordings illustrated in (B) were obtained. Anterior is down and the 3 layers of the piriform cortex (I, II, and III) plus the endopiriform nucleus (EP) can be seen. (B) Extracellular multiunit activity recorded simultaneously in the 3 locations of layer III indicated in (A). Spike times of the units that reached the threshold level (2 × SD; see Materials and Methods) are shown in the upper lines (events). (C) Autocorrelograms of the 3 recordings shown in (B) reveal a frequency of oscillation of 1.7 Hz; the horizontal lines are the mean firing frequency. Dashed lines indicate 95% confidence interval (see Materials and Methods for details).
Characteristics of the spontaneous rhythmic activity in the piriform network. (A) Autocorrelogram and mean frequency (horizontal line) of multiunit activity in layer III. The inset shows how duration was measured at the point where the mean frequency line crossed the central peak. The 2 dashed lines represent the 95% confidence interval. The next peak in the autocorrelogram reveals the period of the oscillation (see Materials and Methods for details). (B) Power spectral density for the recording shown in (A), peaking at 2 Hz. (C) Autocorrelogram illustrating an example of multiunit activity in endopiriform nucleus. Note higher frequency of oscillation and mean frequency of firing than in (A). (D) Power spectral density of the same recording shown in (C). Notice that there are 2 peaks in the power spectrum at 2 and 6 Hz. In this case, 6 Hz was taken as the primary frequency because the power was larger at this frequency. (E) Duration of the up states of the extracellularly recorded oscillations in different layers. Because many points overlap, we give here the number of measurements per layer: layer I (n = 6); layer II (n = 12); border I/II (n = 2); layer III (n = 46); border III/endopiriform (n = 5); and endopiriform (n = 21). The black points are the averages for the different layers (some in F and G), and the points in between layers represent the measurements taken at the border between layers (total n = 112). (F) Mean frequency of firing across different layers. (G) Primary frequency of the oscillations in the different layers taken as the main peak in the power spectrum. (H) Average power spectrum for a total of 71 recordings in layers 2, 3, and endopiriform nucleus (recordings between layers have not been included here).

Intra- and extracellular recordings were digitized, acquired, and analyzed with a CED interface and Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Neuroexplorer software (Nex Technologies, Littleton, MA) was used for some of the spike analysis (autocorrelograms, power spectra). Data are reported as mean ± SD.

Intracellular Recordings
Sharp intracellular recording electrodes were formed on a Sutter Instruments (Novato, CA) P-97 micropipette puller from medium-walled glass (Clark capillaries GC100FS-10, Harvard App. Edenbridge, UK or IB100F-4, WPI, Sarasota, FL) and beveled on a Sutter Instruments beveller to final resistances of 60–100 MOhms. Micropipettes were filled with 2 M potassium acetate, and in some experiments, 50 mM QX-314 was added to prevent action potential generation, allowing us to record the synaptic events at different membrane potentials. Current clamp intracellular recordings were obtained using an Axoclamp 2B Amplifier (Axon Instruments, Foster City, CA). Only intracellular recordings of a stable membrane potential < 60 mV and overshooting action potentials were included in the sample.

Anatomical Reconstructions
Following each experiment, the slices were fixed in paraformaldehyde, Nissl stained, and examined under the microscope to identify the location of the recording sites. Some of these reconstructions have been included (Figs 1A, 7A, and 9B). In some cases, biocytin (1.5–2%) was added to the 2 M potassium acetate in the intracellular electrodes. Biocytin-filled neurons were visualized through standard avidin–biotin–horseradish peroxidase reaction (ABC Vectastain kit) and processed with diaminobenzidine (Sigma) as described by Horikawa and Armstrong (1988).

Results
Spontaneous rhythmic activity was recorded from ferret horizontal brain slices containing piriform cortex and endopiriform nucleus (n = 94 slices; Fig. 1A). The slices were maintained in a modified ACSF that mimics the ionic concentrations in the cerebrospinal fluid (Sanchez-Vives and McCormick 2000). Extracellular multiunit recordings revealed rhythmic bouts of activity that propagated across the slice (Fig. 1B,C).

The primary frequency of this activity was measured as the tallest peak of the spikes power spectrum (see Materials and Methods; examples in Fig. 2B,D) and was 1.8 ± 1.15 Hz (n = 92 measurements in different, anatomically identified locations of the slices; range of frequencies: 0.2–8 Hz). No significant difference was found in the main oscillatory frequency across layers, which was close to 2 Hz in layers II, III, and in the endopiriform nucleus (Fig. 2G,H).

The duration of the bouts of activity, or up states of the network, was measured in autocorrelograms of the extracellularly recorded multiunit activity (Fig. 2A,C; see Materials and Methods). The duration of the up states across different layers is represented in Figure 2E for anatomically identified recording locations. The average duration was 90.0 ± 35.8 ms and spanned between 20 and 238 ms. No significant differences in duration were observed in correlation with the layer. When recorded intracellularly, these bouts of activity appeared as...
barrages of synaptic potentials that could bring the membrane potential to threshold and generate a brisk discharge (Fig. 3).

**Intracellular Recordings**

Intracellular recordings were obtained from 73 neurons in the PC and endopiriform nucleus. In all cases, ongoing multunit rhythmic activity was recorded extracellularly in the vicinity of the recorded cell. Out of 73 neurons, 61 showed barrages of synaptic potentials in synchrony with the extracellularly recorded bouts of activity (Fig. 3A). These bursts had amplitudes between 0.5 and 8 mV and often did not reach threshold (Fig. 3B,C). When they did, usually no more than 2–3 action potentials were generated (Fig. 3A). These characteristics are different from the neocortex, where every recorded neuron seems to participate in the global slow rhythm and reach threshold in each cycle (Steriade 1993; Contreras et al. 1996; Sanchez-Vives and McCormick 2000), although sparse firing has been reported in layers 2/3 of some neocortical areas (Waters and Helmchen 2006). Therefore, the neuronal participation underlying spontaneous rhythmic activity in the PC appeared to be more sparse than in the neocortex, but when the global neuronal network is considered, its emergent activity still consistently produced a regular pattern of activity interspersed with silent periods.

Spontaneous rhythmic oscillations were associated with periodic depolarizations or up states consisting of barrages of

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**Figure 3.** Intracellular and extracellular recordings of rhythmic activity in piriform cortex. (A) Intracellular suprathreshold recording (Vm = −55 mV) from a layer III neuron and extracellular multiunit recording (bottom trace) obtained in the close vicinity of the neuron. Note how the intracellular barrages of synaptic activity and action potentials coincide with the extracellularly recorded bouts of activity. (B) Subthreshold (Vm = −70) recording of the same neuron. (C) Expanded barrages of synaptic activity corresponding to a and b in (B). (D) Intracellular recordings from a different layer III neuron with an electrode containing 50 mM QX-314. Holding the membrane potential at different values unveils the EPSPs and IPSPs concurring during the up states. (E) Intra (top) and simultaneous extracellular recordings in close vicinity (bottom) obtained from a different slice of piriform cortex. At the beginning (left), there were subthreshold barrages of synaptic potentials. Blocking IPSPs with local application of 250 µM bicuculline strongly increased the slope of depolarization and the amplitude of the depolarized state that now reached threshold and generated a burst of action potentials. While still in bicuculline, the glutamatergic blocker CNQX (250 µM) was locally applied and its effect occurred progressively over the course of the next 10 min. At that time, a depolarization of approximately 1 mV in synchrony with a decreased extracellular discharge remained, and it was promptly blocked by 500 µM APV (last trace to the right). The last 3 traces are expanded above.
excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs). This was demonstrated for layer II neurons (Fig. 4A, B), layer III (Fig. 4C), and endopiriform (Fig. 5) neurons. The excitatory and inhibitory components were evidenced by holding the membrane potential at different voltage levels; thus, IPSPs could be observed at depolarized potentials (Fig. 3D, QX-314 in the electrode; Fig. 4A; n = 20). Also, blocking the IPSPs with local application of bicuculline (GABA\(_A\) receptor blocker) strongly increased the amplitude of the depolarized state, which then would invariably reach threshold (Fig. 3E).

**Spontaneous Rhythmic Activity versus Ictal-Like Activity**

Ongoing network activity has been previously described in the piriform cortex and endopiriform nucleus while in 0 Mg\(^{2+}\), in the presence of GABA\(_A\) blockers, or during modifications of the chloride levels (see Introduction). The resulting activity following those procedures is ictal-like activity. However, the spontaneous emergent activity that we describe in this study had different properties from seizure-like activity, and it was similar to the one described in vivo during xylazine-ketamine anesthesia (Fontanini et al. 2003).

First, it appeared in the absence of any of the aforementioned conditions and as a consequence of ionic modifications in the ACSF that are known to induce slow wave sleep-like oscillations in the neocortex (Sanchez-Vives and McCormick 2000). Second, the characteristics of the activity itself were different from the ictal-like episodes. Figure 6 illustrates the spontaneous activity in a slice before and after the application of the GABA\(_A\) blocker bicuculline. Previous to bicuculline application, there was a rhythmic discharge in the piriform network (Fig. 6C) with bursts of synaptic potentials (Fig. 6D) that evoked action potentials when suprathreshold (Fig. 6A). Following local application of 200 µM bicuculline (n = 4 intracellular recordings; n = 6 extracellular recordings), seizure-like responses appeared (Fig. 6D), consisting of high-frequency bursts of action potentials at 1–20 Hz in epochs that lasted up to several tens of seconds. Intracellular recordings revealed a paroxysmal depolarizing shift followed by fast repetitive bursts of spikes during ictal-like activity (Fig. 6D, E, F). Synaptic activation showed faster rise time and higher
Figure 5. Rhythmic activity in the endopiriform nucleus. (A) Intracellular recording (top trace) from the neuron in (B), and synchronous extracellular multiunit recording in the vicinity of the neuron in the endopiriform nucleus. Note that the extracellularly recorded bursts of activity correspond intracellularly with bursts of synaptic potentials that often remain subthreshold. Inset, autocorrelogram of the extracellular activity showing an 8 Hz oscillation. (B) Reconstruction of a multipolar neuron in the endopiriform nucleus whose intracellular recording is illustrated in (A). The fine trace corresponds to the axon. (C) Reconstruction of an endopiriform multipolar neuron. Note the dense axonal arborization in the vicinity of the neuron. Inset, intracellular recording for the same neuron. Note the rhythmic synaptic potentials corresponding to a 12-Hz oscillation of the membrane potential. (D) Microphotograph (20×) of the same neuron reconstructed in (C). Note the en passant synaptic boutons in the close vicinity of the neuron. An inset at higher magnification illustrates the dendritic spines in this neuron. (E) Autocorrelogram of the multiunit extracellular activity in the endopiriform nucleus from a different slice illustrating the characteristic high frequency, in this case of 10 Hz. (F) Extracellular multiunit recording (bottom trace) from which the autocorrelogram in (G) was obtained. The mean frequency of the spikes that crossed the threshold (see Materials and Methods) is shown in the middle trace. Intracellular recording (top trace) obtained next to the extracellular recording. Note that the high frequency of the extracellular recording is reflected in the membrane potential. (G) Autocorrelogram of the voltage recorded intracellularly (black trace; left vertical axes) showing the rhythmicity in the membrane deflections. Superimposed, autocorrelogram of the multiunit extracellularly recorded activity (right vertical axes). Note the overlap between both traces.
amplitude than synaptic barrages during spontaneous rhythmic activity (Figs 3E and 6F). Following these periods of intense discharge, there were silent intervals that lasted up to several minutes, during which all synaptic activity disappeared (Fig. 6D).

Spontaneous oscillations were blocked by the local applications of CNQX (non-N-Methyl-D-aspartic acid (NMDA) glutamatergic receptor blocker; 250 µM concentration in micropipette) as revealed by both multunit recorded activity (n = 12) and intracellular recordings of rhythmic synaptic barrages (n = 3; see below). However, ictal-like discharges induced by bicuculline were not completely blocked by CNQX (multunit recordings n = 3), the remnant oscillation being blocked by local application of the NMDA blocker, APV (500 µM; n = 2 intracellular recordings; Fig. 3E). This participation of NMDA receptors in epileptiform discharges is consistent with previously published results by other authors (Hoffman and Haberly 1989).

**Activity Propagation across Layers**

Simultaneous recordings with vertically aligned electrodes (see Materials and Methods) revealed rhythmic activity occurring simultaneously across the width of the cortex (n = 16 vertically aligned sets of measurements in 11 slices), although in layer I this activity could be recorded only in its deepest part (Supplementary Fig. 1). In all cases, for each of the bouts of activity, the discharges appeared first in the deepest part of layer III and in the

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**Figure 6.** Differences between oscillatory activity and ictal-like discharges induced by bicuculline. (A) Intracellular recording from a layer III neuron (top trace; Vm = −55 mV) and multunit extracellular recording obtained in the vicinity of the neuron (bottom trace). Note that the bouts of extracellular activity correlate with intracellular deflections that reach threshold and evoke one action potential. Action potentials are truncated. Inset shows the response of this neuron to a square depolarizing pulse (0.8 nA). Scale of inset 25 mV, 50 ms. (B) Subthreshold recording of the same neuron (Vm = −63 mV) in the same electrode configuration. Intracellular voltage trace (top) shows the bursts of EPSPs and IPSPs occurring simultaneously with the bouts of extracellularly recorded discharge (bottom). (C) Autocorrelogram of the extracellular activity recorded in the vicinity of the cell shown in (A and B). Mean firing frequency and 95% confidence interval are displayed. (D) Intracellular recording (top trace; Vm = −64 mV) of the same neuron as in (A, B) and extracellular (bottom trace) recording in the vicinity following application of bicuculline. Observe the long duration (16 s) burst of activity followed by a long period of silence that intracellularly appears as a hyperpolarization. Note the absence of synaptic activity during the period of silence, reflecting the lack of activity in the network and not only in this neuron. Synaptic activity is expanded in 2 insets, pre- and postictal-like burst, and they correspond to the zones indicated by the horizontal lines. The beginning and the end of the ictal-like burst are expanded in (E and F), respectively. (E) Intra- (top) and extracellular recording (bottom) of the beginning of the ictal-like burst. (F) Same as (E) for the end of the burst.
endopiriform nucleus (Fig. 7). In 3 cases in which multiple measures were taken, it was clearly observed that the site of discharge onset for the oscillations was located between the endopiriform nucleus and the border with layer III. In these cases, the activity showed certain latency (0.9--7 ms) toward propagation to superficial layer III probably due to propagation delays. The absolute speed of vertical propagation was difficult to measure because the distances were short (150--1700 μm) and the offset of the crosscorrelograms peaks (Fig. 7B), in many cases, insigificant. Only the cases with a detectable offset of the peak were included in our velocity estimate and, as a consequence, there could be a bias towards slower rates of propagation. Overall, 62 measurements of vertical propagation between different layers were taken (n = 11 slices; 16 vertical alignments; see Materials and Methods), resulting in an average rate of 541.8 mm/s. The distribution of these values was broad, between 18.7 and 4400 mm/s. Such distribution was well fitted by 2 Gaussians (chi squared 1.08), centered at 103 and 532 mm/s, respectively. These 2 values are compatible with 2 different pathways of propagation between layers: a faster one for which the delay could be caused only by axonal conduction and a slower one due to intermediate synapses (Hoffman and Haberly 1993).

More evidence in favor of the activity being originated in the deep layers was provided by experiments in which local applications of CNQX were made (Fig. 8). Simultaneous recordings made in endopiriform and layer II/III of the piriform cortex and local application of 250 μM CNQX showed that CNQX applied in PC did block the oscillations there, but they continued in the endopiriform nucleus (n = 6; Fig. 8B). However, if CNQX was applied in the endopiriform nucleus, the activity in the PC disappeared (n = 4; Fig. 8C).

Additional data obtained in slices in which the endopiriform nucleus had been physically separated from the piriform cortex by a knife cut (Supplementary Fig. 2) also support the origin of the oscillation in the deep layers. In these cases, only in the area of piriform cortex connected with the endopiriform nucleus, the oscillations persisted, whereas in those separated, rhythmic activity disappeared. On the other hand, the endopiriform nucleus did not seem to require the connections with the PC in order to generate rhythmic activity, and those areas that were separated (Supplementary Fig. 2 e and g) still showed a robust oscillation.

**Spontaneous Rhythmic Activity in Deep Layers and Endopiriform Nucleus**

Several differences were observed in the spontaneous activity in deep layers versus superficial layers, all of them suggestive of a higher excitability in deep layers (deep layer III and endopiriform nucleus). The mean frequency of extracellular discharge (Fig. 2F; see Materials and Methods) was significantly higher in deep layers when taken as a whole (III and IV; 258 ± 97 spikes/s; n = 17) than in superficial layers (I and II; 200 ± 64 spikes/s; n = 66; P = 0.02).

No significant difference was found in the main frequency of oscillation (highest peak in the power spectrum) across layers...
Excitation in Ep, the activity is now also vanished in the PC. After the washout of CNQX (right), the activity is recovered in both locations.

Synchronous multiunit recordings in PC and Ep before (left) and after local application of CNQX (250 nM; see Figs 2C,D, 5, and 7C and Supplementary Fig.1C). These higher frequencies often did not propagate toward more superficial layers. Supplementary Figure 1 illustrates a slice in which the recordings in endopiriform nucleus had a primary frequency of 5 Hz that progressively faded away toward the surface. Therefore, the 5 Hz component was restricted to deep layers. A similar situation is shown in the simultaneous multiunit extracellular recordings of Figure 7A. In this case, there was an oscillation in the endopiriform nucleus of 2 Hz that propagated toward superficial layer III and layer II, interleaved with a 6-Hz oscillation of lesser amplitude that was not evident in the superficial layers. These higher-frequency oscillations were sometimes reflected intracellularly as membrane voltage deflections of the same frequency and duration as the extracellularly recorded events. Figure 5 illustrates the intracellular recordings corresponding to 2 reconstructed endopiriform neurons. Both of them (Fig. 5B,C) are multipolar neurons, with numerous spines (Fig. 5D) and axons that form numerous local en passant boutons (Fig. 5D). The recordings from both neurons reveal a high-frequency (8-12 Hz) component that is reflected in the multiunit and in the intracellular recordings (see insets in Fig. 5A,C). A slower component (<1 Hz), similar to the slow rhythm in neocortex (Steriade 1993; Sanchez-Vives and McCormick 2000), was also present in some cases either as the primary or as the secondary frequency. The average power spectra represented in Figure 2H reflect that slow component in the oscillations for values around 0.2 Hz.

To better analyze the activation of superficial layers, we studied the propagation of activity induced by local applications of glutamate (500 µM; n = 4 slices; see Materials and Methods). Glutamate activation of deep layers induced a burst of activity in superficial layers (data not shown). Not every burst of activity induced in deep layers did propagate toward superficial layers: a threshold in the intensity of the discharge in the deep layers had to be reached in order to induce a consistent propagated response in the superficial layers. This finding was coherent with the observation that local rhythms of higher frequencies recorded from deep layers did not propagate toward superficial layers, suggesting that they were not massive enough (Fig 7A). All the findings described in this section are suggestive of a prominent excitability of deep layers along with a higher threshold for recruitment of neurons in superficial layers. These results are in agreement with previous reports of both neuronal and network excitability in deeper layers and with the attribution of a high epileptogenic potential to the border between layer III and endopiriform nucleus (see Discussion).

Horizontal Propagation of Activity

Using multiunit recordings with 2 electrodes, we studied the rate of propagation of the rhythmic bouts of activity. The recording electrodes were placed at the same layer (usually layer III) at distances between 0.6 and 5 mm (Fig. 9B) and the conduction delay measured as the peak offset in the cross-correlograms between the activities recorded by each electrode (Fig. 9A,C; see Materials and Methods). Figure 9D shows the distribution of the measured propagation rates that resulted in an average speed of propagation of 114 ± 54 mm/s (n = 32 measurements in anatomically identified locations from different areas in 13 slices). This value was one order of magnitude faster than the one obtained by the same means in the ferret visual cortex (10.9 mm/s; Sanchez-Vives and McCormick 2000).

Because a minimum delay in the activity recorded with the 2 electrodes was required to calculate the speed of propagation, we did not include those recordings where the peaks of the crosscorrelogram were not clearly separated. Therefore, we cannot rule out a bias in our measurements toward lower values of propagation speed. It should also be considered that horizontal propagation may include discontinuities (Chervin et al. 1988; Chagnac-Amitai and Connors 1989). In any event, a bias toward low propagation speeds would not hinder but
conduction velocity values for 32 measurements as in (C). Persistent epileptiform excitatory activity has been induced in et al. 1994, 1999; Forti et al. 1997; Demir et al. 1999b, 2000). and Sutula 1992; Hoffman and Haberly 1993, 1996; De Curtis mechanism (Piredda and Gale 1985; Racine et al. 1988; Haberly synaptically mediated positive feedback is also the subserving relation to the generation of epileptiform activity, for which its functionality has been thoroughly studied but mostly on neurons in the network. With respect to the piriform cortex, being a result of the intrinsic and synaptic properties of the connectivity of the cerebral cortex, their particular pattern oscillations appear to occur as a result of the recurrent build-up of activity are signs that these rhythmic bursts are the result of recurrent connections in the network. There is a difference between the rhythmic activity generated by the piriform network in the slices and the slow oscillation recorded in vivo (Fontanini et al. 2003). The latter is functionally correlated with the flow of air through the nostrils, therefore following the respiratory frequency (average of 0.97 Hz). However, after tracheotomy, there is still a slow oscillation in the PC with a dominant frequency of 0.8 Hz (Fontanini et al. 2003), which is slower than that one observed in the in vitro network. This could be due to the influence of ketamine-xylazine in the in vivo recordings or to the lack of peripheral

reinforce the remarkable difference in propagation rate between the piriform network and the neocortex.

Discussion
This is, to our knowledge, the first description of spontaneous rhythmic activity in slices of piriform cortex maintained in vitro. Slow oscillations of analogous characteristics have been described in the rat piriform cortex in vivo during ketamine-xylazine anesthesia (Fontanini et al. 2003), but so far, there has been no thorough characterization of spontaneous rhythmic oscillations in the piriform cortex during natural sleep. In vivo olfactory cortex oscillations are functionally correlated with respiration, although they also occur in tracheotomized animals (for differences, see Fontanini et al. 2003), strongly suggesting that they are an emergent property of the neuronal network. Here we have demonstrated that the isolated piriform network does generate per se spontaneous, nonepileptiform rhythmic activity, and we have characterized some of the underpinning mechanisms.

In the neocortex, slow (<1 Hz) rhythmic activity consisting of recurring generation of up and down states has been described through all recorded areas (Metherate and Ashe 1993; Steriade 1993; Cowan and Wilson 1994; Contreras et al. 1996; Stern et al. 1997; Sanchez-Vives and McCormick 2000; McCormick et al. 2003; Shu et al. 2003; Isomura et al. 2006; Volgushev et al. 2006; Luczak et al. 2007). These recurring up states or periods of sustained depolarization consist of concurrent excitatory and inhibitory synaptic events that give rise to outbursts of action potentials. Slow neocortical oscillations appear to occur as a result of the recurrent connectivity of the cerebral cortex, their particular pattern being a result of the intrinsic and synaptic properties of the neurons in the network. With respect to the piriform cortex, its functionality has been thoroughly studied but mostly on relation to the generation of epileptiform activity, for which synaptically mediated positive feedback is also the subserving mechanism (Piredda and Gale 1985; Racine et al. 1988; Haberly and Sutula 1992; Hoffman and Haberly 1993, 1996; De Curtis et al. 1994, 1999; Forti et al. 1997; Demir et al. 1999b, 2000). Persistent epileptiform excitatory activity has been induced in slices of piriform cortex by bursting activity stimulation (Hoffman and Haberly 1989, 1991), with low chloride ACSF (Demir et al. 1999b), low Mg2+ (Hoffman and Haberly 1989), disinhibition in bicuculline (De Curtis et al. 1994, 1999; Halonen et al. 1994; Ebert et al. 2000; Demir et al. 2001; Rigas and Castro-Alamancos 2004), or in slices prepared from previously kindled rats (McIntyre and Wong 1986; Hoffman and Haberly 1991; Haberly and Sutula 1992).

Our aim here was to study if the piriform network maintained in vitro could generate any spontaneous, rhythmic, nonepileptiform activity and to characterize it. Through the analysis of this spontaneous activity, we can identify some of the rules governing the functionality of the piriform network, which is crucial to comprehend both the neuronal ensembles underpinning odor processing and the reason why this area of the brain is prone to the generation of epileptic discharges.

**Spontaneous Rhythmic Activity in the Piriform Network In Vitro**

Here we have described that horizontal ferret slices containing the piriform network and maintained in an ACSF with an in vivo–like ionic composition (Sanchez-Vives and McCormick 2000) generate a spontaneous and continuous rhythmic activity consisting in a cyclic sequence of up and down states occurring at an average frequency of 1.8 Hz. Underlying the up states were concurrent EPSPs and IPSPs building up a depolarization that not always reached threshold but that was invariably coincident with the extracellularly recorded outbursts of activity. Such concurrency of EPSPs and IPSPs and the build-up of activity are signs that these rhythmic bursts are the result of recurrent connections in the network.

There is a difference between the rhythmic activity generated by the piriform network in the slices and the slow oscillation recorded in vivo (Fontanini et al. 2003). The latter is functionally correlated with the flow of air through the nostrils, therefore following the respiratory frequency (average of 0.97 Hz). However, after tracheotomy, there is still a slow oscillation in the PC with a dominant frequency of 0.8 Hz (Fontanini et al. 2003), which is slower than that one observed in the in vitro network. This could be due to the influence of ketamine-xylazine in the in vivo recordings or to the lack of peripheral
and central afferences in the slices. Given that piriform cortex slices display both faster (6-12 Hz) and slower (<1 Hz) rhythms, it is feasible that those could become more or less prominent in vivo under modulatory influences, given that the substrate for the rhythm generation exists in the network.

Where Is the Spontaneous Rhythmic Activity Generated?
Crosscorrelograms of multunit rhythmic activity along an axis perpendicular to the cortical surface revealed that the oscillations are initiated close to the border between layer III of the piriform cortex and the endopiriform nucleus and propagate toward superficial layers and, eventually, deeper into the endopiriform nucleus. Deep layer III and endopiriform nucleus are highly excitable due to the intrinsic properties of their neurons (Tseng and Haberly 1989; Banks et al. 1996; Magistretti and de Curtis 1998) and to the existence of regenerative feedback loops based on a dense plexus of intrinsic recurrent connections (Hoffman and Haberly 1991, 1993). This is also illustrated by the abundant axonal synaptic boutons that endopiriform neurons have in their close vicinity (Fig. 5B,C). In several studies, the deep layers of the PC have been found to initiate epileptiform discharges. Priedda and Gale (1985) gave the name of area tempestas to the area in the brain with lowest threshold to generate generalized epilepsy by chemoconvulsant injection and identified it as the deep boundary of the PC. There is evidence that epileptiform discharges induced by different methods originate in the endopiriform nucleus and deep part of layer III (Hoffman and Haberly 1991; Demir et al. 1999a, 2000; Ekstrand et al. 2001).

As we describe here, the higher excitability of these areas not only explains their tendency to start epileptic discharges but also contributes to the shaping of their activity in more physiological conditions. In order to start a cycle of regenerative activity in a network with positive feedback connections, it is necessary to reach a certain level of spontaneous activity that brings to threshold a critical number of adjoining neurons, enough to start an up state (Compte et al. 2003). Several lines of evidence indicate that physiological rhythmic activity is initiated in the deep layer III-endopiriform nucleus: 1) the network is more excitable there as shown by a lower threshold in response to glutamate applications, higher spontaneous mean frequency of firing, and a tendency to generate higher frequency rhythms; 2) application of CNQX to the Ep blocks rhythmic activity in the PC; 3) there is a lack of activity in PC when the deep layers are physically isolated by a knife cut; and 4) a leading role in the initiation of the up states when simultaneous vertical measures are taken.

In the neocortex, neurons in infragranular layers, namely in layer V, exhibit some spontaneous firing during the down states and they initiate both physiological and epileptiform activity (Connors 1984) (see Fig. 2 in Sanchez-Vives and McCormick 2000). In the piriform network, this role seems to be played by neurons in deep layer III and endopiriform nucleus, where spontaneously bursting multipolar neurons are present (Tseng and Haberly 1989).

In this study, the rate of propagation from deep to superficial layers varied in different cases, its distribution occurring around 2 means: one, 103 mm/s, of similar values to the ones measured for the horizontal propagation (across the same layer) and a second and considerable faster one at 532 mm/s. This distribution of the vertical speeds of propagation suggests that there are different pathways that can be alternatively used for vertical propagation, the direct connections from endopiriform multipolar neurons to piramidal layer II cells (Haberly and Price 1978; Haberly and Presto 1986) probably underlying the faster rate of propagation. A speed of 500 mm/s is compatible with axonal conduction velocity in the PC (0.2-2.5 m/s; Hoffman and Haberly 1993) and absence of synaptic delays.

Piriform Rhythms and Neocortical Slow Oscillations
Cyclic oscillations resulting from recurrent connectivity are a common outcome in both the neocortex and the piriform cortex. However, as a result of the disparity in their anatomical and functional structure, the emergent activity in each area has different properties. At variance with the neocortex, where the depolarizations occurring during the up states generally reach threshold (Steriade 1993; Sanchez-Vives and McCormick 2000), in the piriform cortex, both in vivo (Fontanini et al. 2003) and in vitro (current study), they often did not reach firing threshold in individual neurons. Still, whereas intracellularly recorded oscillations may consist of none or few action potentials, the extracellularly recorded multunit activity reveals a consistent network discharge, therefore suggesting a more sparse neuronal participation but still reliable enough to maintain a rhythmic ongoing activity.

In the neocortex, the up states are of longer duration (0.5-1 s) and less frequent (0.3 Hz) than the ones reported here. Duration and frequency of the oscillations depend on a constellation of factors related with the synaptic interactions and intrinsic properties of the cortical network. Therefore, different dynamics in different networks reflect their structural and functional uniqueness. At this point, we have no precise explanation for all the factors that give rise to the differences in frequency and duration of oscillations in piriform network and neocortex. A combined experimental and modeling approach is best to analyze heuristically the network mechanisms underpinning differences in emergent activity across different cortices, and we have used that approach in the analysis of one particularly striking difference between neocortex and the piriform cortex: the speed of propagation of the up states along the cortex.

Speed of Propagation in Piriform Cortex versus Neocortex In Vitro
The horizontal rate of propagation (across the same layer) of oscillations in the piriform cortex in vitro was 114 mm/s, with a distribution of values reaching speeds faster than 230 mm/s. These values imply in general the participation of synaptic connections and not only axonal conduction of which the slowest value for unmyelinated axons in the PC is 0.2 m/s (Hoffman and Haberly 1993). The speed of propagation in PC is therefore 10 times faster than the one found in slices of neocortex (10.9 mm/s; Sanchez-Vives and McCormick 2000). Several of the known properties of the piriform cortex could determine this difference, and we have explored them in a cortical network model. The first one is the weaker inhibition in the piriform network. Both experimentally and in computer models, inhibition is known to slow down the propagation of activity in the cortical network, whereas the lack of inhibition accelerates it (Flint and Connors 1996; Sanchez-Vives and McCormick 2000, Wu et al. 2001; Buzhenov et al. 2002; Golomb and Ermentrout 2002; Compte et al. 2003). Lesser inhibition in the piriform network as evidenced by very low GABA transporter-1 immunoreactivity, near absence of GABAergic cartridge endings on axonal initial segments (Ekstrand et al.
and a significant lesser inhibitory/excitatory neuronal rate for calbindin-, calretinin-, and parvalbumin-positive neurons in the piriform network versus visual cortex of the ferret (our own unpublished observations) is probably one contributing cause for the high propagation speeds. We confirmed this quantitatively in a computational network model of the cortical circuitry, indicating that indeed a decrease in inhibition produced an augmented propagation speed (Supplementary Fig. 3).

A second potential mechanism that may influence the rate of propagation is the existence of long excitatory connections in the PC that extend up to distances of 4 mm (Johnson et al. 2000). The lack of a columnar organization, the predominance of horizontal connectivity, and the continuous distribution of axonal boutons on the horizontal connections, rather than contacts on restricted spots (Johnson et al. 2000), are also factors that may contribute to increase propagation speed. In our model simulations, doubling the footprint of horizontal excitatory connections (leaving inhibitory connections unchanged) resulted in a several fold increase in propagation speed (Supplementary Fig. 3). In the model, propagation accelerates not only because excitation reaches farther but also because this far-reaching excitation is not correspondingly mitigated by far-reaching inhibition.

A third factor that can contribute to the fast propagation speed in the piriform cortex is the more positive inhibitory reversal potential in piriform cortex than in neocortex (Kapur et al. 1997 and our unpublished observations). The computational model suggests that the reversal potential of GABA A synapses could be a critical factor governing the propagation speed of activity in a cortical module (Supplementary Fig. 3).

The speed of propagation of the oscillations that we have described in this study does not differ markedly from the propagation rates described for epileptiform discharges (Hoffman and Haberly 1993; Demir et al. 2001) induced by a variety of methods in vitro. This suggests that in the piriform network, its lesser inhibition may play a weaker role in the determination of the actual propagation speed of physiological activity, whereas in neocortical studies, GABAergic blockade increased the rate of propagation by one order of magnitude (Sanchez-Vives and McCormick 2000). This poorer influence of inhibition on propagation speed in the piriform cortex implies the lack of one control mechanism over activity with respect to the neocortex and may result in a higher tendency to generate epileptic discharges.

In this heuristic exploration of the mechanisms of activity propagation in piriform cortex, we have restricted our analysis to the primary factors that are currently thought to subserve the physiological differences between the piriform cortex and neocortical circuitry. These are not, however, the only mechanisms that influence the propagation of activity waves in the cortical tissue. Indeed, it has been shown that other elements that contribute markedly to the neuronal integration time (spiking threshold, membrane time constant, synaptic dynamics, etc.) also have a profound impact in propagation speeds in cortical modules (Golomb and Amitai 1997; Pinto and Ermentrout 2001). We want to emphasize at this point that we have used a computational network model that reproduces adequately the phenomenology of the visual cortex (Compte et al. 2003), but none of our manipulations brings us to a simulation that reflects all phenomenological aspects of slow oscillatory activity in olfactory cortex. As we were concerned with a heuristic exploration of the factors intervening in the velocity of propagation, we did not attempt to simulate faithfully either the more sparse involvement of neurons of piriform cortex in this emerging oscillatory activity or the duration and interval properties of these oscillations. A thorough modeling of piriform cortex oscillatory activity will be undertaken separately.

Epileptiform Activity and Slow Oscillations
Persistent epileptiform excitatory activity has been induced in slices of piriform cortex by stimulation in low Mg 2+ (Hoffman and Haberly 1989, 1991), incubation in low chloride ACSF (Demir et al. 1999b), disinhibition in bicuculline (De Curtis et al. 1994; Halonen et al. 1994; Ebert et al. 2000; Demir et al. 2001; Rigas and Castro-Alamancos 2004), pilocarpine (Chen et al. 2006), or previous kindling (McIntyre and Wong 1986; Haberly and Sutula 1992; Hoffman and Haberly 1996). In our preparation, application of bicuculline produced the appearance of epileptiform discharges characterized by paroxysmal depolarizing shifts and repetitive synchronous discharges (Fig. 6), similar to those evoked by several maneuvers in neocortex and piriform cortex. The transition from spontaneous rhythmic activity to ictal-like discharges was progressive, with low concentrations of bicuculline producing an increase in the amplitude and a faster depolarization rate (Fig. 6) of the recorded oscillations. Inhibition in the piriform network, thus, shapes the characteristics of the slow oscillations and restrains the network tendency to produce highly synchronized activity. Thus, as it occurs in the neocortex, the balance between excitation and inhibition is crucial for the generation of physiological, in vivo–like rhythmic activity and to control the tendency of the recurrent network to generate epileptiform discharges. In the case of the piriform network, this balance may be more delicate than in other areas, given the lower proportion of inhibitory versus excitatory neurons, high neuronal excitability, more positive GABA A reversal potential, and long-range excitatory connections.

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Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

Notes
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