

# DIRECTIONAL SPREAD OF ACTIVITY IN SYNAPTIC NETWORKS OF THE HUMAN LATERAL AMYGDALA

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**Abstract**—Spontaneous epileptiform activity has previously been observed in lateral amygdala (LA) slices derived from patients with intractable-temporal lobe epilepsy. The present study aimed to characterize intranuclear LA synaptic connectivity and to test the hypothesis that differences in the spread of flow of neuronal activity may relate to spontaneous epileptiform activity occurrence. Electrical activity was evoked through electrical microstimulation in acute human brain slices containing the LA, signals were recorded as local field potentials combined with fast optical imaging of voltage-sensitive dye fluorescence. Sites of stimulation and recording were systematically varied. Following recordings, slices were anatomically reconstructed using two-dimensional unitary slices as a reference for coronal and parasagittal planes. Local spatial patterns and spread of activity were assessed by incorporating the coordinates of electrical and optical recording sites into the respective unitary slice. A preferential directional spread of evoked electrical signals was observed from ventral to dorsal, rostral to caudal and medial to lateral regions in the LA. No differences in spread of evoked activity were observed between spontaneously and non-spontaneously active LA slices, i.e. basic properties of evoked synaptic responses were similar in the two functional types of LA slices, including input–output relationship, and paired-pulse

depression. These results indicate a directed propagation of synaptic signals within the human LA in spontaneously active epileptic slices. We suggest that the lack of differences in local and in systemic information processing has to be found in confined epileptiform circuits within the amygdala likely involving well-known “epileptic neurons”. © 2017 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

**Key words:** temporal lobe epilepsy, living human brain slices, lateral amygdala, field potentials, voltage-sensitive dyes.

## INTRODUCTION

The amygdala is implicated in emotional modulation of memory formation (LeDoux, 2000; Tovote et al., 2015), and plays a major role in mechanisms of fear, anxiety and related disorders (Quesney, 1986; LeDoux, 2000; Beyenburg et al., 2005). It receives sensory inputs from afferent sources such as thalamus, hippocampal region and entorhinal cortex, integrating them with existing memory, thereby adapting the behavioral and autonomic response of the organism to the environment (Phelps and LeDoux, 2005; Tovote et al., 2015). Of note, the lateral nucleus of the amygdala (LA) is considered to be the main input station of the amygdaloid complex (Amaral and Insausti, 1992) as well as a major output station to the hippocampal region (Pitkänen et al., 2000). Additionally, hippocampal region (Sloviter, 1996; Avoli et al., 2002; Sharma et al., 2007) and LA (Aroniadou-Anderjaska et al., 2008; Graebnitz et al., 2011) are known for their implication in temporal lobe epilepsy (TLE). Alterations in the human LA were found to range from a morphological (Aliashkevich et al., 2003) to a physiological (Graebnitz et al., 2011) and a molecular (Cendes et al., 1994) level and were confirmed in animal models of epilepsy.

The path of afferent signals to the LA has been documented by tracing studies in rats (Pitkänen et al., 1995), cats (Smith and Paré, 1994), and monkeys (Pitkänen and Amaral, 1998), and some results bearing similarities to those obtained in non-human primates were recently reported following measurements of functional connectivity in humans (Stein et al., 2007a,b; Kim et al., 2011; Bickart et al., 2014). Overall, animal studies indicate that, after reaching the LA, processed information is further transferred through a highly developed network

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Abbreviations: LA, lateral amygdala; TLE, temporal lobe epilepsy; VSD, voltage sensitive dyes.

of intra-amygdaloid connections toward the basolateral and basomedial nuclei, and finally toward the central nucleus, which is the main output station of the amygdala (Asede et al., 2015; Tovote et al., 2015).

Abnormal synaptic function in the neocortex together with the LA has been shown to be implicated in the generation of spontaneous interictal-like activity of epileptic patients (Köhling et al., 1998, 2000; Koch et al., 2005; Graebnitz et al., 2011). Therefore, the aim of the present study was to study the functional synaptic network organization of LA in the presence of spontaneous epileptiform activity and compared that to epileptic tissues that did not display these epileptiform discharges. We examined spreading patterns of evoked responses in the human LA in relation to the presence or absence of spontaneous interictal-like activity. Human tissue obtained during therapeutic resection of the amygdala in TLE patients was used and experimentally assessed using a four-step experimental approach. (i) Information on intrinsic epileptiform activity of the tissue was derived from recordings of local field potentials in acute LA slices. (ii) Electrical activity was evoked through electrical microstimulation at systematically varied sites, and the spread of evoked activity was assessed through dual site field potential recordings combined with voltage-sensitive dye imaging. (iii) Physiologically characterized slices were anatomically reconstructed using two-dimensional unitary slices as a reference for coronal and parasagittal planes. (iv) Spatial patterns and spread of activity were assessed by incorporating the coordinates of electrical and optical recording sites into the respective unitary slice.

## EXPERIMENTAL PROCEDURES

### Human amygdala tissue

Tissue containing the amygdala was obtained during therapeutical partial lobectomies from 10 patients with pharmacoresistant TLE. Detailed data of patients concerning seizure history, medication, magnetic resonance imaging, histopathological findings, and number of slices obtained from surgical specimens that were used for electrophysiological recordings are summarized in Table 1. Pathological evaluation of sclerosis was performed considering absence/presence of sclerosis which were encoded as 0 and 1, as previously described (Graebnitz et al., 2011). Pathological examination revealed sclerosis of grade III in the hippocampus, whereas the amygdala was free of sclerosis or, in some cases, at sclerosis level I (Wyler et al., 1992) and this holds true also for the cases with focal dysplasia in contrast to previous reports (Hudson et al., 1993; Aroniadou-Anderjaska et al., 2008). Of note, sclerosis was not detected in resected specimens *in vitro* used for electrophysiological study. Experiments were approved by the local ethics committee (Approval No. 2008-151-f-S, Ethikkommission der Ärztekammer Westfalen-Lippe und der Medizinischen Fakultät der Westfälischen Wilhelms-Universität Münster) and informed consent was obtained from all patients.

Amygdala tissue for comparing histological studies was obtained at autopsy from the body donor program of the Center of Anatomy and Brain Research, University of Düsseldorf. Subjects ( $n = 5$ ; 2 males) had

**Table 1.** Patients data

| Case/<br>Slice | Gender | Age<br>(y) | Febrile<br>conv. | Seiz.<br>type | s/<br>m | Seiz. for<br>n years | MRI / Pathology                  | AED                                                                                | Slice<br>acti. |
|----------------|--------|------------|------------------|---------------|---------|----------------------|----------------------------------|------------------------------------------------------------------------------------|----------------|
| 1              | f      | 49         | -                | PS,<br>GS     | 12      | 39                   | AHS                              | CBZ, LEV, VPA                                                                      | sp             |
| 2 s.X          | f      | 14         | -                | PS            | 20      | 12                   | Lesion, AHS                      | CBZ, ETX, LEV, LTG, OCBZ, STM                                                      | sp             |
| 3 s.2          | m      | 36         | -                | PS            | 4       | 30                   | Lesion, AHS,<br>focal dysplasia, | CBZ, LEV, LTG, OCBZ, TGB, VPA                                                      | sp             |
| 4 s.3          | m      | 50         | +                | PS,<br>GS     | 1       | 26                   | Focal dysplasia,<br>AHS          | CTH, BROM, CBZ, CZ, DPH, ETX, GPT, LTG, MSX,<br>OCBZ, PHB, PRM, STM, TGB, VGB, VPA | sp             |
| 5 s.4          | f      | 65         | +                | PS,<br>GS     | 7       | 38                   | AHS                              | CBZ, GPT, PHB, PHT, VGB                                                            | nsp            |
| 6 s.1          | m      | 45         | -                | PS,<br>GS     | 2       | 4                    | Ø                                | CBZ, LEV                                                                           | sp             |
| 6 s.2          | m      | 45         | -                | PS,<br>GS     | 2       | 4                    | Ø                                | CBZ, LEV                                                                           | nsp            |
| 7 s.3          | m      | 21         | -                | PS,<br>GS     | 2       | n.g.                 | AHS                              | CBZ, LEV, LTG, VPA                                                                 | nsp            |
| 8 s.3          | f      | 54         | -                | PS            | 2       | 8                    | Lesion, AHS                      | CBZ, LTG, OCBZ                                                                     | nsp            |
| 9 s.3          | f      | 40         | -                | PS,<br>GS     | 8       | 12                   | AHS                              | CBZ, DPH, LEV, LTG, OCBZ                                                           | sp             |
| 10 s.2         | f      | 44         | -                | PS,<br>GS     | 3       | 15                   | Mesial lobe<br>atrophy           | CBZ, GPT, LEV, VPA, PGB                                                            | sp             |

ACTH, adrenocorticotrophic hormone; acti., activity; AED, antiepileptic drug; AHS, Ammon's horn sclerosis; Ø, undetected; bic: bicuculline; BROM, bromide; CBZ, carbamazepine; conv., convulsions; CZ, clobazam; DPH, diphenhydantoin; E-phys, electrophysiology; ETX, ethosuximide; f, female; GPT, gabapentin; GS, generalized seizures; LEV, levetiracetam; LTG, lamotrigine; m, male; MSX, mesuximide; n.g., not given; nb., number; OCBZ, oxcarbazepine; PHB, phenobarbital; PHT, phenytoin; PRM, primidone; PS, partial seizures; RA, receptor autoradiography; s/m, seizures per month; Seiz., seizures; sp, nsp, appearance and non-appearance of spontaneous epileptiform field potentials (spikes and/or sharp waves) in corresponding slice preparation; STM, sultiam; TGB, tiagabine; VGB, vigabatrin; VPA, valproate; y, years.

no known history of neurological or psychiatric diseases. Mean age was  $74 \pm 4$  years and causes of death were cardiac arrest ( $n = 3$ ), cardiorespiratory insufficiency ( $n = 1$ ) and multiple organ failure ( $n = 1$ ).

### Slice preparation

The general techniques for slice preparation (Graebnitz et al., 2011), transport and voltage-sensitive dye staining (Köhling et al., 1996, 2000) have been previously described. Briefly, slices of 400–500- $\mu\text{m}$  thickness were cut from blocks of amygdala within 5 min of tissue resection in the operation room, using a vibratome (MA-752 Motorized Advance Vibroslice, Campden Instruments Ltd, Loughborough, UK). For an hour, slices were placed in a portable incubation chamber with oxygenated (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124; KCl 4;  $\text{CaCl}_2$  2;  $\text{NaH}_2\text{PO}_4$  1.24;  $\text{MgSO}_4$  1.3;  $\text{NaHCO}_3$  26; and glucose 10 (pH 7.4, at 28 °C; (Köhling et al., 1996)). Single slices were then stained with 12.5  $\mu\text{g}/\text{ml}$  of the voltage-sensitive dye RH 795 (Invitrogen, Karlsruhe, Germany, dissolved in ACSF at 33 °C, 1 h incubation followed by 1-h washout both with continuous oxygenation), before being transferred into a recording chamber filled with ACSF at 33 °C. During the experiments, pH, temperature and flow rate (4 ml/min; bath volume 1 ml) were continuously monitored. Following termination of electrophysiological experiments, slices were frozen at  $-40$  °C in isopentane, and stored at  $-80$  °C until further processing for histological verification of recording sites and anatomical reconstruction. Eleven slices were obtained from a total of 10 patients.

### Electrophysiological recordings

The general procedure for field potentials (Graebnitz et al., 2011) and voltage-sensitive dye (Köhling et al., 2000; Broicher et al., 2010) recordings were performed using a submerged chamber, as described previously. Briefly, extracellular field potentials were recorded in the LA using glass microelectrodes (Borosilicate glass capillaries with filament, Code No. 1403512, Hilgenberg, Malsfeld, Germany), pulled to resistances of 0.5–1.5 M $\Omega$  (DMZ Universal Puller, Zeitz Instruments GmbH, Munich, Germany) when filled with ACSF. Stimuli, 150  $\mu\text{s}$  and  $<1$  mA (100% intensity in the text), were applied via custom-made bipolar electrodes (model SNE 200, Science Products GmbH, RMI, Hofheim, Germany). Optimal stimulation intensities of 100%, 50% and 10% were alternately tested. Recorded waveforms were fed through a custom-made amplifier, low-pass filtered at 10 kHz, and stored on a personal computer via an AD/DA interface (Digidata 1322A, Molecular Devices, Sunnyvale, CA, USA). Recordings were visualized online using the software AxoScope 6 (Molecular Devices, Sunnyvale, CA, USA).

Voltage-sensitive dye fluorescence signals were acquired using a 464-element hexagonal photodiode array (WuTech Instruments, Gaithersburg, MD, USA) via a 5 $\times$ , 10 $\times$  or 20 $\times$  objective corresponding to a total diameter of 3.25, 1.575 and 0.8 mm, respectively. These signals are triggered by membrane potential

changes of neuronal population underlying the photodiode array. Sweep duration was 1304 ms, inter-frame interval was 1.274 ms and inter-sweep interval was of 10 s. Three sweeps were averaged for each measurement and were separated by at least 5 min. Data acquisition was performed using the Neuroplex software (RedShirtImaging LLC, Decatur, GA, USA). The position of the photodiode array was systematically changed depending on the electrical stimulation sites (Figs. 1 and 2A).

### Data analysis and statistics of electrophysiological data

Field potential recordings were analyzed off-line using Clampfit 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Amplitudes were measured peak-to-peak on the unfiltered recorded waveforms.

Optical data analysis was performed using the Neuroplex software (RedShirtImaging LLC, Decatur, GA, USA). Optical signals are expressed as fractional changes of resting light intensity ( $dI/I = I_{\text{rest}} - I_{\text{recording}}/I_{\text{rest}}$ ). Fluorescence changes were measured from six representative samples from each corner of the hexagonal shaped array (Fig. 2B, C). Each sample consisted of the averaged signal of a selection of six adjacent diodes (Fig. 2C) where the stimulation time point is indicated by the dashed vertical line. Amplitudes were measured as the peak amplitude (within 20 ms of stimulation) minus baseline amplitude (10 ms preceding stimulation). Fluorescence changes were converted to pseudocolor maps. For consistency throughout experiments, all pseudocolor maps were scaled to an amplitude range of 0.08 %  $dI/I$  with warm and cold colors corresponding respectively to depolarization and hyperpolarization.

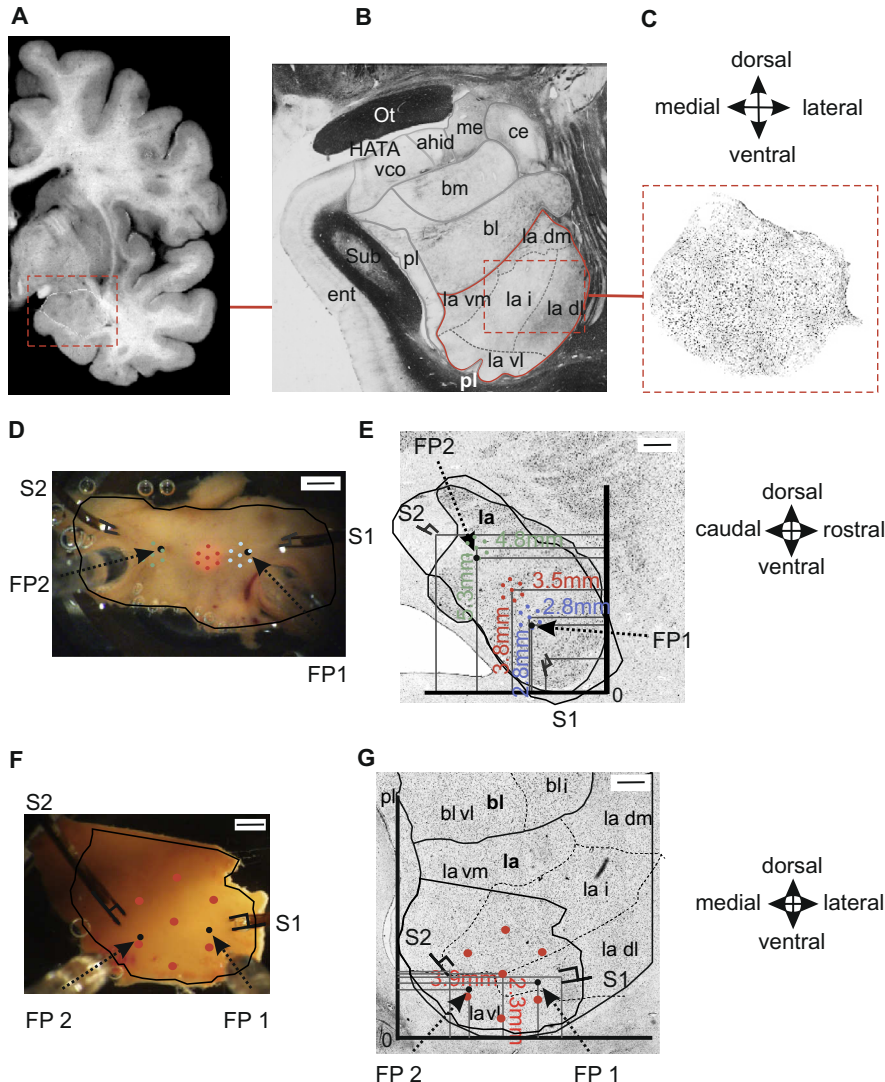
Statistical analysis was done using Prism5 (GraphPad software, La Jolla, CA, USA) and SPSS 20 (IBM Corporation, New York, USA). Input–output and paired-pulse data were analyzed using a two-way ANOVA followed by Bonferroni post hoc tests. Amplitudes of optical signal were tested for normal distribution by using Kolmogorov–Smirnov normality test followed by Mann Whitney U test. Differences are considered significant at  $p \leq 0.05$ .

### Histology and anatomical reconstruction

Slices were processed for histology as described previously (Graebnitz et al., 2011). Briefly, frozen individual slices were serially re-sectioned in 10- $\mu\text{m}$ -thick sections using a cryostat (Leica, Germany) and thaw-mounted. Every 11th section was used for the visualization of cell bodies, every 12th for the visualization of myelin sheaths. Sections were silver stained for cell bodies (Merker, 1983) and for myelin (Gallyas, 1979).

Histologically processed sections were analyzed for cytoarchitectonics and superimposed onto photographs of the corresponding slice taken during electrophysiological recordings. Matching of the landmarks such as fiber tracts, cell density and marks





**Fig. 1.** Anatomical reconstruction and unitary slices. (A) Myelin stained coronal section through a deep frozen, unfixed human hemisphere (section thickness 20  $\mu\text{m}$ ) used as control. The region of the amygdala is highlighted (red dashed rectangle). (B) Cytoarchitecture of the LA was observed in detail (LA outlined with red color). (C) Cytoarchitectonics of slices used for experiments were compared to those from control tissue for two-dimensional orientation. (D, E) Unitary slice for the parasagittal plane. (D) Photograph of an individual slice, outlined using black color, prepared in the parasagittal plane showing two stimulation electrodes (S1 and S2, black forks), two field potential electrodes (FP1 and FP2, dashed arrow line and black dots showing the approximate position of the electrode tip in the slice) and three positions of the photodiodes array used to record fluorescence signals with staining with a voltage-sensitive dye (as represented by colored dotted hexagons). (E) The same slice, represented by its outline, was oriented based on anatomical correlates and superimposed onto the histological photomicrograph of the unitary control section for the parasagittal plane. It illustrates the coordinates system and measurements of the position of the tips of the field potential electrodes, stimulation electrodes, and of the diode arrays. Coordinates for the center of the diode array are indicated with the color matching the one of the arrays. (F, G) Unitary slice for the coronal plane. (F) Photograph of an individual slice, outlined using black color, prepared in the coronal plane showing two stimulation electrodes (S1 and S2, black forks), two field potential electrodes (FP1 and FP2, dashed arrow line and black dots showing the approximate position of the electrode tip in the slice) and one array position (as represented by red dotted hexagon). (G) The same slice, represented by its outline, was oriented based on anatomical correlates and superimposed onto the histological photomicrograph of the unitary control section for the coronal plane. It illustrates the coordinates system and measurements of the position of the tips of the field potential electrodes, stimulation electrodes, and of the photodiode array. Coordinates for the center of the array are indicated in red color. *Abbreviations:* ahid, dorsal amygdalo hippocampal area; bl, basolateral amygdala nucleus; bm, basomedial nucleus; ce, central amygdala nucleus; ent, entorhinal cortex; HATA, hippocampal-amygdala transition area; la dl, lateral nucleus dorsolateral part; la dm, lateral nucleus dorsomedial part; la i, lateral nucleus intermediate part; la vl, lateral nucleus ventrolateral part; la vm, lateral amygdala ventromedial part; me, medial amygdala nucleus; ot, optic tract; pl, paralamina nucleus of the amygdala; Sub, subiculum; vco, ventral cortical nucleus. Scale bar = 1 mm.

left by stimulation electrodes allowed to identify recording sites and to anatomically orient the slice.

Data were only included in the analysis if recording sites had been verified in the lateral nucleus of the amygdala (Heimer et al., 1999).

## RESULTS

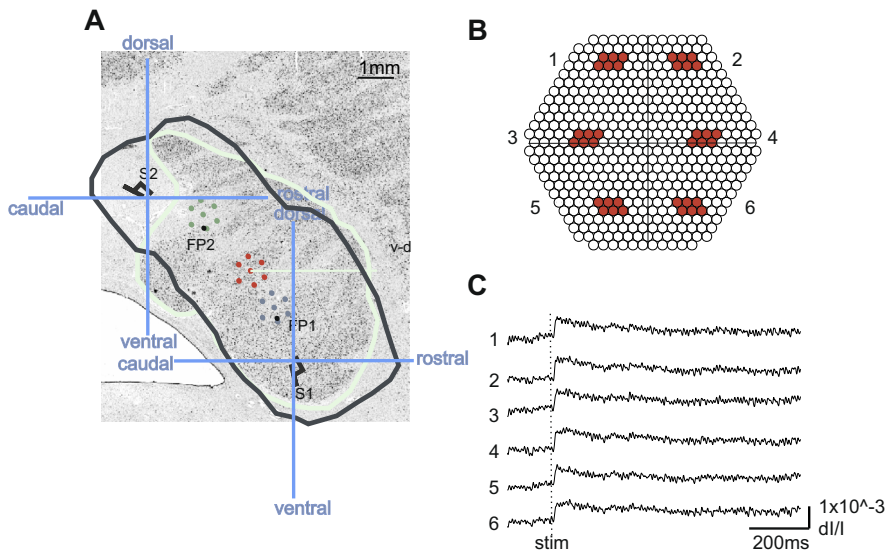
Experiments were performed on 11 human LA slices resected from 10 patients (see Table 1). The slices were anatomically identified on the basis of data from literature (Figs. 1 and 2A; (Amaral and Insausti, 1992; Sorvari et al., 1995)).

Using conventional field potentials, spontaneous epileptiform discharges were detected in seven slices obtained from six patients. The shape and characteristics of spontaneous epileptiform discharges in human slice preparation are described in detail in Köhling et al. (1998, 2000) and Graebnitz et al. (2011). Briefly, sharp potential fluctuations, the amplitude and duration of which were in the range of 20–300  $\mu\text{V}$  and of 50–150 ms, respectively. An exemplary trace is depicted in Fig. 3E (adapted from Graebnitz et al., 2011). The overall electrical activity in each slice was explored by periodically repositioning the field potential recording electrodes in the LA following an imaginary position grid consisting of squares 500  $\mu\text{m}$  across.

Activity evoked by electrical stimulation was recorded in the LA as field potentials and optical signals on the basis of a voltage-sensitive dye. In a given slice, stimulation sites were systematically varied according to the coordinates depicted in Fig. 1D–G. Evoked activity was simultaneously recorded as field potentials from at least two sites and as optical signals by positioning the diode array at three different sites. In order to control for a possible technical contribution to recorded patterns of activity related to the position, recordings were obtained twice in an individual slice, namely before and after rotation of the slices by 180°, along horizontal axis.

## Construction of unitary slices

In order to compare results from electrophysiological and optical



**Fig. 2.** (A) Schematic representation of the outline of the slice on the parasagittal plane. It is oriented based on anatomical correlates and superimposed onto the histological photomicrograph of the unitary control section for the parasagittal plane. It illustrates the coordinates system and measurements of the position of the tips of the field potential electrodes (FP1 and FP2), stimulation electrodes (black forks, S1 and S2), and of the diode arrays (three colored hexagons). Coordinates for the center of the diode array are indicated with color matching that of the arrays. (B) Scheme showing the photodiode array with its 464 honey comb arranged diodes and the position of the family of six diodes chosen at the six corners for averaging of the amplitude of evoked optical signals. (C) Example of typical averaged optical signals for each of the diode family (1–6). Vertical dotted line: Point of time of stimulation (stim).

imaging experiments obtained in the different individual slices, coronal and parasagittal unitary slices were created as reference (Fig. 1). Histological sections were prepared from autopsy tissue, with the sectioning angle set to cover the maximal dimension of the LA in the coronal and parasagittal plane. Thus, the ventro-dorsal axis averaged 7.2 mm and 7.6 mm, and the medio-lateral axis averaged 9.4 mm and 6.6 mm, at the coronal and parasagittal plane, respectively. Each individual acute slice used for physiological recordings (Fig. 1D, F) was then graphically superimposed on the unitary slice of the respective sectioning plane (Fig. 1E, G), using anatomical land marks such as nuclei/subnuclei position, and fiber tracts, and coordinates of stimulation/recording sites. Importantly, coordinates of stimulation sites, positioning of the recording electrodes and optical arrays were measured in the parasagittal plane from ventral to dorsal and from rostral to caudal (Fig. 1D, E), and in the coronal plane from ventral to dorsal and from medial to lateral (Fig. 1F, G), in order to avoid miscalculation and therefore mismatches with the topography (see insets in the figure for orientation).

### Comparison of evoked activity in spontaneously and non-spontaneously epileptiform active slices

LA slices of TLE pharmacoresistant patients have been demonstrated to show spontaneous interictal-like activity in 33.3% of slices (Graebnitz et al., 2011). Therefore, given the often assumed possibility that spontaneous epileptiform activity is linked to a disturbed neuronal network, the question whether the described preferred direc-

tion of activity spread is similar in spontaneously and non-spontaneously active slices arises.

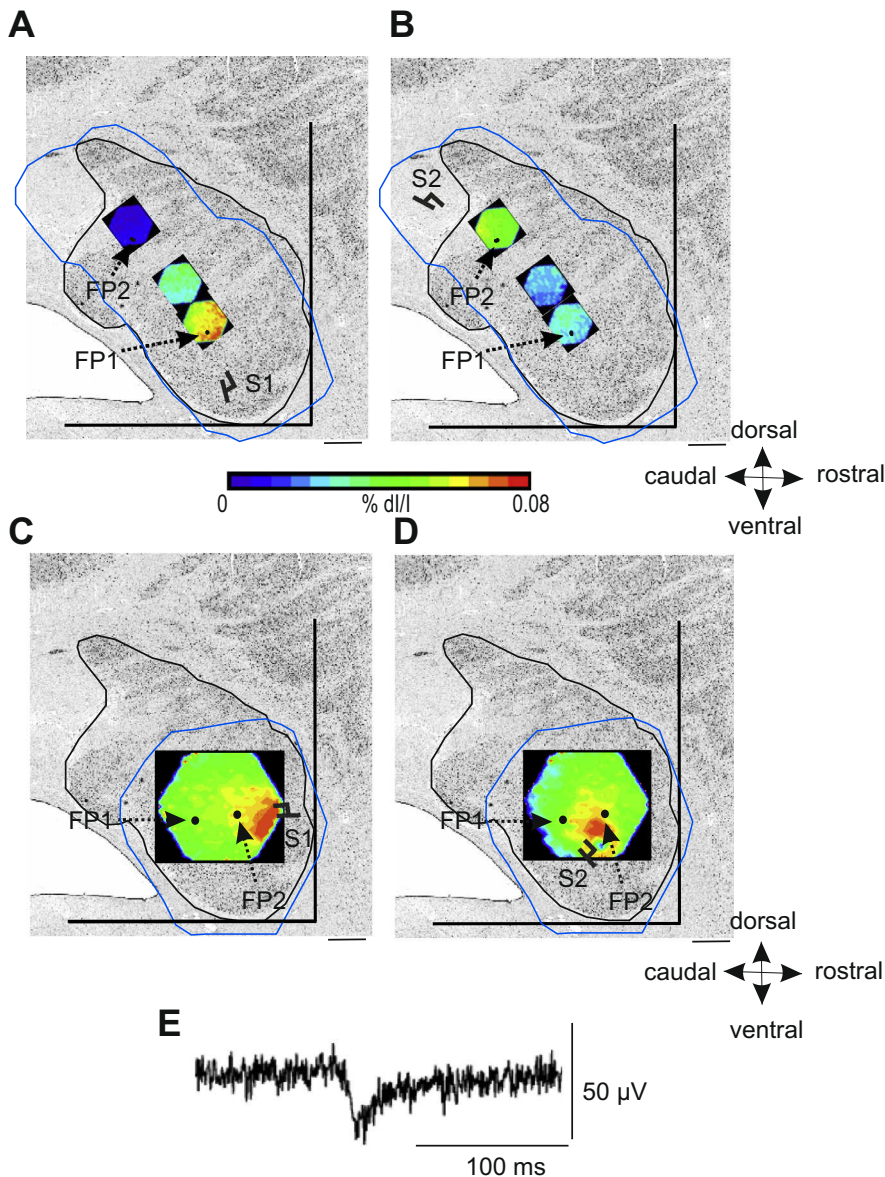
In spontaneously active slices ( $n = 7$ ), the spread of activity in the parasagittal plane was from ventral to dorsal and rostral to caudal (Fig. 3A, B), and in the coronal plane it was from medial to lateral and ventral to dorsal (Fig. 5). This is indicated by the membrane potential peak deflections, analyzed in the form of fluorescence signals by using voltage sensitive dyes (VSD), induced by 100% stimulus intensity, which were not different between the two groups of slices (Mann Whitney  $U = 12,210$ ,  $p = 0.056$ , Fig. 6C). In non-spontaneously active slices, the spread of activity in the parasagittal plane was from ventral to dorsal and rostral to caudal (Fig. 3C, D), and in the coronal plane it was from medial to lateral and ventral to dorsal, as shown by optical signals. This indicates that spread of activity is similar in spontaneous and non-spontaneous slices for both the spatial and temporal domains.

In an attempt to verify the spread of activity suggested by optical signals, electrophysiological analysis was performed in a next step of experiments. Input-output relationships of amplitudes of evoked field potentials, elicited by local microstimulation, indicated an effect of the group (spontaneous and non-spontaneous slices, seven and four slices, respectively) and stimulus intensity (2 way ANOVA: stimulus intensity  $F(2,212) = 4.31$ ,  $p = 0.015$ ; group  $F(1,212) = 6.17$ ,  $p = 0.014$ ; Fig. 6A). However, post hoc tests did not reveal differences in amplitudes of evoked responses between spontaneous and non-spontaneous slices for any given stimulation intensity (FP 100% sp:  $363.8 \pm 73.3$  vs. nsp:  $200.5 \pm 32.5$ ; FP 50% sp:  $269.3 \pm 51.8$  vs. nsp:  $152.9 \pm 27.6$ ; FP 10% sp:  $149.2 \pm 39.6$  vs. nsp:  $68.2 \pm 16.9$ ; Fig. 6B). Furthermore, evoked field potential amplitudes in response to double-pulse stimulation with varying inter-stimuli intervals (paired-pulse ratios) were evaluated for 100% stimulation intensity. Statistical analysis did indicate a group effect (2 way ANOVA: group  $F(1,26) = 7.20$ ,  $p = 0.012$ ), but failed to reveal amplitude differences between the groups for any given inter-stimulus interval (Fig. 6B) indicating an un-altered synaptic structures. Latencies of the peaks of the VSD signals were 10–20 ms and those of the half peak amplitudes 120–150 ms.

### Spread of evoked activity in LA

The slices were systematically superimposed on unitary ones in order for the spatial orientation to be verified during data analysis (see Methods, Figs. 1 and 2A).





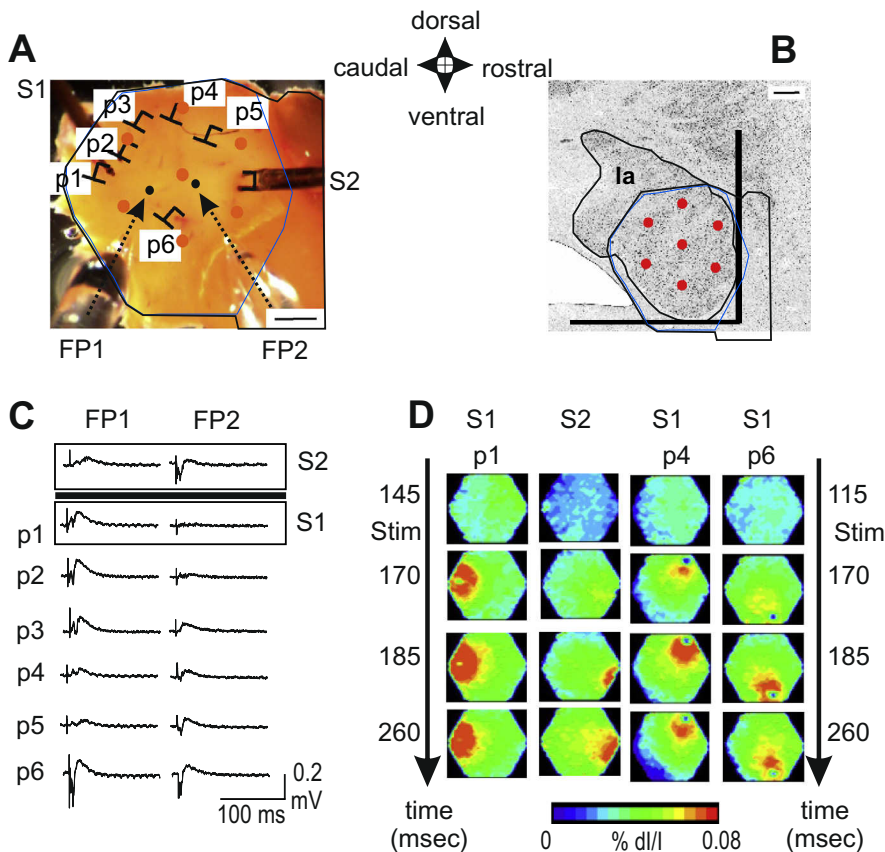
**Fig. 3.** Preferred direction of activity transmission in spontaneously and non-spontaneously active slice. (A, B) Superimposition of the recorded spontaneously active slice (dark blue outline) onto its parasagittal unitary slice (black outline) showing multiple recording sites of optical signals. Color coded optical signals taken at the peak of activity, approximately 10 ms after stimulation (cf. Fig. 2C), confirm that activity spreads preferentially from ventral to dorsal and rostral to caudal (A, stimulation site S1) than from dorsal to ventral and caudal to rostral (B, stimulation site S2). (C, D) Superimposition of the recorded non-spontaneously active slice (dark blue outline) onto its parasagittal unitary slice (black outline) shows recording site of optical signals. Color coded optical signals taken at the peak of activity, approximately 10 ms after stimulation (cf. Fig. 2C), confirm the rostro-caudal (C, stimulation site S1) and ventro-dorsal (D, stimulation site S2) direction of spread of evoked activity. Scale bar = 1 mm. Position of the tips of the field potential electrodes (FP1 and FP2), stimulation electrodes (black forks, S1 and S2). (E) Exemplary trace representing a spontaneous discharge observed in a slice obtained from the resected amygdala of epileptic patients undergoing surgery. The trace was modified from Graebnitz et al., 2011).

Related evoked field potentials to the anatomy of the slices suggested that spread of neuronal activity follows preferential directions. In order to corroborate these conclusions, the site of stimulation (indicated with the letter S in the text and in the figures) was systematically varied while keeping the recording positions consistent in a next series of experiments (n slices = 2; Figs. 4

and 5). In slices cut at a parasagittal plane, the two electrodes for electrical stimulation were positioned at near-maximal caudal and rostral sites of the horizontal meridian (Fig. 4A, S1 and S2). The two electrodes for recording of field potentials were placed along this meridian at a similar distance from the respective stimulation electrode (Fig. 4A, FP1 and FP2). A first measure of transmission quality was taken by the ratio of the amplitude of the evoked field potential far from the stimulation electrode to that evoked close to the stimulation electrode. In this way the fluorescence changes and evoked potential responses were higher from rostral to caudal than from caudal to rostral (Fig. 4A–C, S2 compared to S1 p1), and from ventral to dorsal than from dorsal to ventral (Fig. 4A–C, S1 compared to S2 p1). In slices cut at a coronal plane, stimulation electrodes were positioned at near-maximal ventral and dorsal sites of the vertical meridian (Fig. 5A, S1 and S2), and the two recording electrodes were placed along this meridian at a similar distance from the respective stimulation electrode (Fig. 5A, FP1 and FP2).

Optical signals were analyzed, together with field potential data, in relation to the anatomy of the slices. For this purpose, a photograph showing the placement of the photodiodes array on the slice was transposed onto the appropriate unitary slice and the main directions of the array such as ventral, dorsal, rostral, caudal, medial and lateral could be determined for orientation purposes (Fig. 1). Each slice was analyzed as a separate case. In the parasagittal plane, optical signals demonstrated that rostral and ventral stimulation induced a widespread pattern of activity compared to that induced by caudal and dorsal stimulation (Fig. 4A, D, rostral vs. caudal: S2 compared to S1 p1, ventral vs. dorsal: S1 p6 as compared to S1 p4). In the coronal

plane, ventral or medial stimulation led, respectively, to a stronger and more widespread activation than a more dorsal or lateral one (Fig. 5A, D, ventral vs. dorsal: S1 compared to S2 p1, medial vs. lateral: S2 p3 as compared to S2 p6). Direction of evoked optical activity was in agreement with evoked field potentials data.



**Fig. 4.** Influence of multiple stimulation sites on evoked optical signals and field potential on the parasagittal plane. (A, B) Photograph of the slice showing the photodiodes array position (A, red dotted hexagon) and its superimposition on the parasagittal unitary slice (B, black and dark blue outlines). (C) Evoked field potentials were recorded at the sites FP1 and FP2 in response to stimulation given in S1 (at positions p1 to p6) and S2. Signal propagation was more efficient from rostral to caudal sites (S2, FP1 and FP2, framed recordings), than from caudal to rostral sites (S1 p1, FP1 and FP2, framed recordings). (D) Successive pseudocolor frames taken at different time points, with reference to stimulation sites (position, p1–6), show preferential signal transmission from rostral to caudal (S2) than caudal to rostral (S1p1) and from ventral to dorsal (S1p6) than from dorsal to ventral (S1p4). Abbreviations: la, lateral nucleus of the amygdala. Scale bar = 1 mm. Position of the tips of the field potential electrodes (FP1 and FP2), stimulation electrodes (black forks, S1 and S2, position, p1–6).

Since the VSD responses can only be detected up to 100  $\mu\text{m}$  below the surface of the slice which is 500- $\mu\text{m}$ -thick and the establishment of field potentials could be different in the upper or lower part of the slice, the same experiments described above were repeated after turning the slice upside down and re-arranging the position of the stimulation and recording electrodes. No differences between the two sides of the slice could be observed ( $n$  slices = 9).

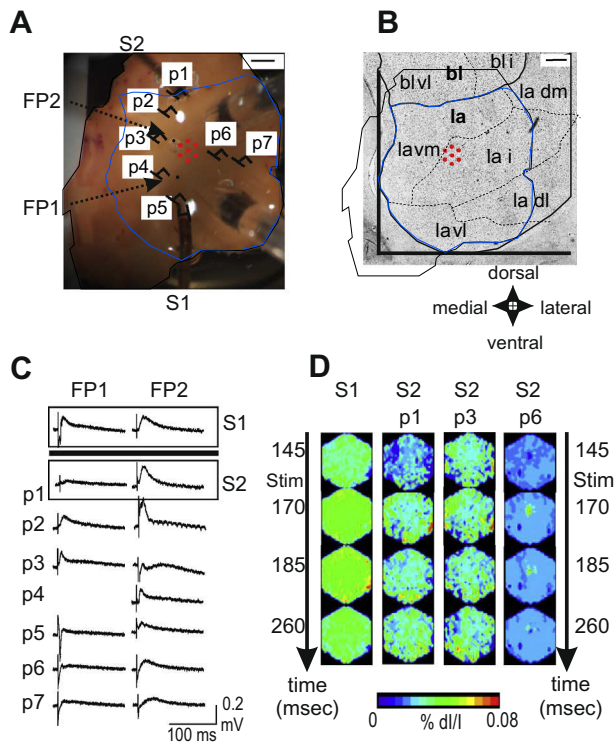
In conclusion, activity in the parasagittal plane spreads preferentially from ventral to dorsal and from rostral to caudal sites (Fig. 6D, E). Consistently with the parasagittal plane, in the coronal plane there is a preferential spreading from ventral to dorsal and an equally strength in the propagation from medial to lateral and vice versa (Fig. 6F). Thus, in the complete intact human LA, the spread of activity is to be expected from ventral to dorsal, rostral to caudal, and medial to more lateral sites as summed up in the scheme in Fig. 6G, H.

## DISCUSSION

The interplay between amygdala, neocortex and hippocampal regions, i.e. the so-called temporal lobe (TL), is of critical importance for the generation and maintenance of seizures (Bertram et al., 1998; Avoli et al., 2002), next to its impact for learning and memory in particular of aversive encounters (LeDoux, 2000; Phelps and LeDoux, 2005). Thus, alterations of these circuits can lead to broad and serious consequences. For instance, there are frequent reports of TLE patients experiencing excessive anxiety or displaying symptoms of fear during or in between the occurrence of seizures, which is thought to reflect activity in synaptic networks involving the amygdala (Cendes et al., 1994; Biraben et al., 2001). Investigating network activity within the amygdala therefore constitutes a further step in our understanding of the physiology and consequently, the pathophysiology of TLE and its comorbid disorders. In this respect, amygdalar tissue obtained from surgery of patients with medically intractable epilepsy provides an opportunity to explore network properties of the human amygdala. A wealth of information about human amygdala is derived from studies of the intact human brain using fMRI or EEG (Malikova et al., 2015). Cellular activities have been recorded from the human amygdala using depth electrodes *in vivo* (Wieser and Zumsteg, 2008), and in slices obtained from resected amygdala tissue from patients suffering from pharmacoresistant TLE (Graebnitz et al., 2011).

Consistently with previous investigations (Graebnitz et al., 2011), field potential recordings revealed spontaneously and non-spontaneously epileptiform active LA slices. Of note, LA slices obtained from the same individual slices (one patient) contained specimens with and without spontaneous activity, and local electrical microstimulation failed to evoke epileptic discharges in the non-active specimen. Despite being a single observation, this indicates (1) that the epileptogenicity of the two slices is different and (2) that the spatial size of the epileptic area is very small. In light of this finding, we tried to assess whether such differences influence the spreading or propagation of activity within LA synaptic circuits because, after all, an altered information processing could also cause the differences in epileptogenicity. In fact, even despite a small size of epileptic foci, if there are synchronous inputs, a restricted epileptic activity could be assumed to be generalized. Interestingly, we made the





**Fig. 5.** Influence of multiple stimulation sites on evoked optical signals and field potential on the coronal plane. (A, B) Photograph of the slice showing the photodiodes array position (A, red dotted hexagon) and its superimposition on the coronal unitary slice (B, black and dark blue outlines). (C) Evoked field potentials were recorded at the sites FP1 and FP2 in response to stimulation given in S1 and in S2, at positions p1 to p7. Signal propagated more efficiently from ventral to dorsal sites (S1, FP1 and FP2, framed recordings), than from dorsal to ventral sites (S2 p1, FP1 and FP2, framed recordings). (D) Successive pseudocolor frames taken at different time points, with reference to stimulation sites (position, p1–7), showing preferential signal transmission from ventral to dorsal (S1) than dorsal to ventral (S2p1) and from medial to lateral (S2p3) than from lateral to medial (S2p6). *Abbreviations:* bl, basolateral nucleus of amygdala; bl i, basolateral nucleus intermediate part; bl vl, basolateral nucleus ventrolateral part; la, lateral nucleus of the amygdala; la dl, lateral nucleus dorsolateral part; la dm, lateral nucleus dorsomedial part; la i, lateral nucleus intermediate part; la vl, lateral nucleus ventrolateral part; la vm, lateral amygdala ventromedial part. Scale bar = 1 mm.

unexpected observation that the two types of slices were not different from each other, as far as the flow of neuronal activity, as detected with our approach, is concerned. Thus, direction of spreading of evoked activity was preserved in spontaneously active slices. We showed that activity propagates preferentially from more medial to lateral, ventral to dorsal and rostral to caudal sites by using field potentials recordings combined with voltage-sensitive dye functional imaging. These findings indicate the preservation of hierarchical organization in the functional connections within the LA, irrespective of the presence or absence of spontaneous epileptiform activity.

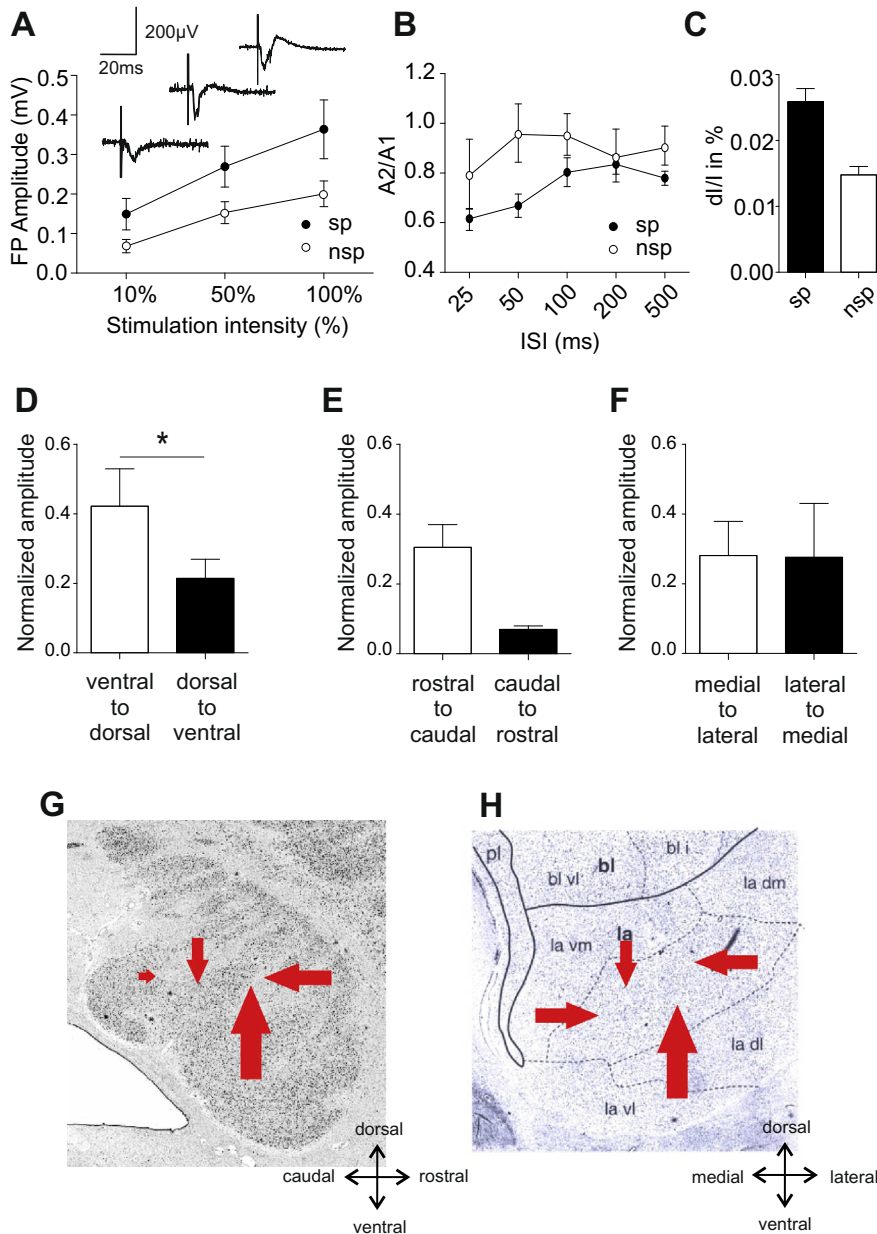
On the background of the above mentioned findings, some suggestions coming from the literature may be considered. Despite the presence of spontaneous activity, but consistently with findings of similar direction

of evoked activity propagation and receptor expression in spontaneous and non-spontaneous slices (Graebnitz et al., 2011), basic electrophysiological properties of evoked signals, on the basis of field potentials as well as membrane potentials, e.g. input/output relationships, paired-pulse observations, did not differ between the two groups of slices. With particular relevance to TLE, network alterations resulting in an imbalance between excitation and inhibition were shown to be implicated in the generation of spontaneous activity in the human LA (Graebnitz et al., 2011), neocortex (Keller et al., 2010) and hippocampal region (Huberfeld et al., 2007). Indeed, further pharmacological modulation of the GABAergic and glutamatergic systems, exerted using the blockers bicuculline and APV, respectively, reduced the amplitude of the spontaneous discharges. Additionally, somatic synapses contacts from GABAergic neurons onto projection neurons were shown to be reduced in the LA from TLE patients as compared to autopsy controls (Yilmazer-Hanke et al., 2007). Previously observed imbalances between excitation and inhibition can be reconciled with apparently intact intra-nuclear networks by the presence of local microdomains, or foci, in which alterations in neuron type, receptors type, and axonal and dendritic patterns would take place. In accordance with that, the existence of such micro-foci has already been evidenced in the human epileptic cortex (Köhling et al., 1998; Keller et al., 2010).

It has to be acknowledged that these results have to been considered in the light of patients' individuality. We obtained a high number of slices but from different patients suffering from other diseases beside intractable epilepsy. We cannot exclude compensatory mechanisms and we could only use autaptic tissue for morphological controls which could not perfectly match our samples, but this is a basic limitation in human studies. Moreover, as already mentioned, data concerning human amygdalar connections are not numerous and, in many cases, a comparison to primates and non-primates evidence may be useful.

In this context, the results of a first anatomical study in rats (Pitkänen et al., 1995) could be of interest. They indicated dense rostro-caudal fibers' path within the LA, along with either preferential latero-medial or medio-lateral fibers' paths depending on the rostro-caudal level of activation. In that study, ventro-dorsal or dorso-ventral connections were not reported; certainly due to the fact that fibers' directions appeared to be named based on the nuclear subdivisions they connect rather than on the anatomical direction they follow. In non-human primates (Pitkänen and Amaral, 1998), the strongest fibers' paths connected the dorsal, dorsal intermediate and the ventral intermediate divisions to the ventral division. Depending on the rostrocaudal level of the slice, the dorsal intermediate, ventral intermediate and ventral divisions have a more or less ventro-lateral to dorso-medial oblique orientation. Besides rodent studies (Pitkänen et al., 2000; Asede et al., 2015; Fujieda et al., 2015), no study had taken advantage of voltage-sensitive dye imaging in order to appreciate the functionality of the connections within the human LA. Additionally, amygdaloid nuclei have





**Fig. 6.** Strength of directionality of spreading activity and its propagation. (A) Input–output relationships of both spontaneously (sp, filled circles) and non-spontaneously (nsp, open circles) active slices showing that for any given stimulation intensity both types of slices have similar amplitude of evoked responses. Typical examples from a spontaneously active slice illustrate the amplitude of the evoked field potentials for each stimulation intensity. (B) Paired-pulse ratio of both spontaneously (filled circles) and non-spontaneously (open circles) active slices showing that for any given inter stimulus interval (ISI) both types of slices have similar amplitude ratios of the evoked responses when stimulated with 100% intensity. (C) Histogram showing that the average peak amplitude of optical signals evoked for 100% stimulation intensity. No significant difference between spontaneously (filled bar) and non-spontaneously (open bar) active slices ( $p = 0.056$ ) (D–F) The strength of the activity directionality varies depending on the position of the stimulation electrode. The propagation of evoked activity is stronger in the direction ventral to dorsal compared to the direction dorsal to ventral (D,  $p = 0.016$ ) in both coronal and parasagittal planes, as indicated by the normalized amplitude obtained by optical imaging experiments. The direction rostral to caudal allows a stronger propagation in the parasagittal plane (E,  $p = 0.23$ ) and there are no differences when the direction is medial to lateral and vice versa in the coronal plane (F,  $p = 0.29$ ). (G–H) The strength of the directionality in the parasagittal plane (G) and in the coronal one (H) is indicated by the size of the red arrows whose orientation is indicated by the four crossed black arrows.

undergone a large remodeling during primate evolution due to functional adaptation and increase in size of the temporal neocortex (Heimer et al., 1999). Altogether, species specificity as well as changing orientation of the different subdivision according to the rostro-caudal level underline that a direct transposition of anatomical data from animals to humans is difficult.

Living human LA slices are shown to generate spontaneous epileptiform activity. Nevertheless, they still possess the ability to transfer information in similar directions as slices that do not appear to be epileptic. Based on these data, activity of the human LA in patients could be expected to project toward the output nuclei of the amygdala and to spread preferentially to the entorhinal cortex (Pitkänen et al., 1995, 2000; Fujieda et al., 2015). Therefore, we suggest that the reason of the lack of differences in local and even in systemic information processing has to be found in confined circuits within the amygdala likely involving a small pool of well-known “epileptic neurons”.

## AUTHOR CONTRIBUTION

SG performed the experiments and analyzed the data together with EJS, and performed the anatomical reconstruction of the slices together with KZ. MC prepared the final figures and edited the manuscript. SG, EJS, MC, and JL wrote the manuscript, with contribution by HCP. OK performed slice histology. AG helped in providing the human material. HP, VH and KZ performed surgery and helped obtaining the slice specimens. EJS and HCP conceived the study, designed and supervised the project and organized the paper.

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## DISCLOSURE

None of the authors has any conflict of interest to disclose.

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