

# Fractalkine/CX3CL1 modulates GABA<sub>A</sub> currents in human temporal lobe epilepsy

\*Cristina Roseti, ††Sergio Fucile, †Clotilde Lauro, †<sup>1</sup>Katiuscia Martinello, †Cristina Bertollini, †Vincenzo Esposito, †Addolorata Mascia, ††Myriam Catalano, §¶#Eleonora Aronica, ††Cristina Limatola, and \*†Eleonora Palma

*Epilepsia*, 54(10):1834–1844, 2013  
doi: 10.1111/epi.12354

## SUMMARY

**Purpose:** The chemokine fractalkine/CX3CL1 and its receptor CX3CR1 are widely expressed in the central nervous system (CNS). Recent evidence showed that CX3CL1 participates in inflammatory responses that are common features of CNS disorders, such as epilepsy. Mesial temporal lobe epilepsy (MTLE) is the prevalent form of focal epilepsy in adults, and hippocampal sclerosis (HS) represents the most common underlying pathologic abnormality, as demonstrated at autopsy and postresection studies. Relevant features of MTLE are a characteristic pattern of neuronal loss, as are astrogliosis and microglia activation. Several factors affect epileptogenesis in patients with MTLE, including a lack of  $\gamma$ -aminobutyric acid (GABA)ergic inhibitory efficacy. Therefore, experiments were designed to investigate whether, in MTLE brain tissues, CX3CL1 may influence GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) mediated transmission, with a particular focus on the action of CX3CL1 on the use-dependent decrease (rundown) of the GABA-evoked currents ( $I_{GABA}$ ), a feature underlying the reduction of GABAergic function in epileptic tissue.

**Methods:** Patch-clamp recordings were obtained from cortical pyramidal neurons in slices from six MTLE patients after surgery. Alternatively, the cell membranes from epileptic brain tissues of 17 MTLE patients or from surgical samples and autopsies of nonepileptic patients were microtransplanted into *Xenopus* oocytes, and  $I_{GABA}$  were recorded using the standard two-microelectrode voltage-clamp technique. Immunohistochemical staining and double-labeling studies were carried out on the same brain tissues to analyze CX3CR1 expression.

**Key Findings:** In native pyramidal neurons from cortical slices of patients with MTLE, CX3CL1 reduced  $I_{GABA}$  rundown and affected the recovery of  $I_{GABA}$  amplitude from rundown. These same effects were confirmed in oocytes injected with cortical and hippocampal MTLE membranes, whereas CX3CL1 did not influence  $I_{GABA}$  in oocytes injected with nonepileptic tissues. Consistent with a specific effect of CX3CL1 on tissues from patients with MTLE, CX3CR1 immunoreactivity was higher in MTLE sclerotic hippocampi than in control tissues, with a prominent expression in activated microglial cells.

**Significance:** These findings indicate a role for CX3CL1 in MTLE, supporting recent evidence on the relevance of brain inflammation in human epilepsies. Our data demonstrate that in MTLE tissues the reduced GABAergic function can be modulated by CX3CL1. The increased CX3CR1 expression in microglia and the modulation by CX3CL1 of GABAergic currents in human epileptic brain suggests new therapeutic approaches for drug-resistant epilepsies based on the evidence that the propagation of seizures can be influenced by inflammatory processes.

**KEY WORDS:** Neuroinflammation, Current rundown, Human slices, Oocytes.

Accepted July 30, 2013; Early View publication September 13, 2013.

\*San Raffaele Pisana IRCCS, Rome, Italy; †Pasteur Institute-Cenci Bolognietti Foundation, Department of Physiology and Pharmacology, University of Rome “Sapienza”, Rome, Italy; ††Neuromed IRCCS, Pozzilli, Italy; §Department of (Neuro) Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; ¶Epilepsy Institutes in the Netherlands Foundation (SEIN), Heemstede, The Netherlands; #Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Amsterdam, The Netherlands; and <sup>1</sup>Present address: Department of Pharmacology, UCL School of Pharmacy, London, United Kingdom

<sup>1</sup>Present address: Department of Pharmacology, UCL School of Pharmacy, London, United Kingdom.

E.P. and C.L. contributed equally to this work.

Address correspondence to Eleonora Palma, Istituto Fisiologia Umana, P.le A. Moro 5, 00185 Roma, Italy. E-mail: eleonora.palma@uniroma1.it

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Numerous studies support the hypothesis of the relevance of brain inflammation in the pathophysiology of mesial temporal lobe epilepsy (MTLE). Focal pathologic abnormalities can be observed in patients with MTLE, the most prominent of which is a loss of neurons in the hippocampus termed hippocampal sclerosis (HS; Blümcke et al., 2012). Furthermore, MTLE is a common epilepsy characterized by astrogliosis and microglia activation (Vezzani et al., 2011a, 2012; Aronica et al., 2012; Kan et al., 2012; Sosunov et al., 2012). Seizure activity in epileptic brain rapidly increases the synthesis of inflammatory mediators involved in the initiation and propagation of neuronal hyperexcitability (Vezzani et al., 2011b, 2012). Inflammatory processes, including leukocyte infiltration, activation of microglia and astrocytes, and production of proinflammatory cytokines and chemokines, have all been described in the brains of epileptic patients as well as in experimental models of epilepsy (Ravizza et al., 2008; Fabene et al., 2010; Pernot et al., 2011; Aronica et al., 2012; Kan et al., 2012).

The chemokine fractalkine/CX3CL1 and its G protein-coupled receptor CX3CR1 have been indicated as key players in the modulation of neuronal excitability: CX3CL1/CX3CR1 signaling affects glutamatergic Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid (AMPA)-mediated currents, thereby reducing the amplitude of both synaptic- and agonist-evoked currents (Limatola et al., 2005; Ragozzino et al., 2006), and modulates long-term synaptic plasticity events (Bertollini et al., 2006; Maggi et al., 2009). CX3CL1 also modulates  $\gamma$ -aminobutyric acid (GABA)ergic currents, mediating an increase in postsynaptic GABA activity at serotonin neurons in the raphe nucleus (Heinisch & Kirby, 2009).

CX3CR1, which is present on limited subsets of resident and infiltrating cells, including microglia, monocytes, natural killer (NK) cells, and T lymphocytes (Cardona et al., 2006; Ransohoff, 2009), has been hypothesized to play a role in neuronal damage consequent to status epilepticus (SE) in rats (Yeo et al., 2011), and the expression of CX3CL1 is significantly upregulated in the temporal cortex, in serum, and in cerebrospinal fluid (CSF) of patients with MTLE (Xu et al., 2012).

Recurrent seizures in epilepsy can be caused by a reduced efficacy of the GABAergic inhibitory system, and specifically MTLE has been associated with GABA receptor A (GABA<sub>A</sub>R) dysfunction (Pavlov et al., 2013). We have shown previously that the repetitive activation of GABA<sub>A</sub>R produces a use-dependent decrease (rundown) of the GABA-evoked currents ( $I_{\text{GABA}}$ ), which is markedly pronounced in the hippocampus and cortex of patients with drug-resistant MTLE (Palma et al., 2004; Ragozzino et al., 2005). This phenomenon has been also confirmed in pilocarpine-treated rats, a model of MTLE where the increased rundown of  $I_{\text{GABA}}$  is related to an altered expression of  $\alpha 1/\alpha 4$  GABA<sub>A</sub>R subunits (Mazzuferi et al., 2010). To date, no

information is available on the modulation of GABAergic neurotransmission by CX3CL1 and on the expression of its receptor in MTLE. Our study aimed at determining whether CX3CL1 affects  $I_{\text{GABA}}$  in tissue obtained from patients with drug-resistant MTLE. For this reason, we first studied the effect of CX3CL1 on GABA<sub>A</sub>Rs expressed in pyramidal neurons from MTLE slices. Given the limited availability and the complexity of studying fresh human brain tissues, we also took advantage of the “microtransplantation” method, which consists of injecting *Xenopus* oocytes with membranes from surgically resected (fresh or frozen) human brain tissue (Miledi et al., 2002; Eusebi et al., 2009; Li et al., 2011). It was previously shown that the oocyte’s plasma membrane efficiently incorporates the foreign membranes and acquires functional neurotransmitter receptors and channels retaining their native properties (Palma et al., 2003; Miledi et al., 2006; Eusebi et al., 2009). Using this approach, we investigated the effects of CX3CL1 on human GABA<sub>A</sub>Rs transplanted from tissues of MTLE patients and controls (hippocampus and cortex) in *Xenopus* oocytes. Finally, on the same hippocampal tissues used for oocytes recordings, we analyzed CX3CR1 expression in activated microglia. Our results suggest a relation between inflammation, MTLE, and GABAergic function, providing precious information to identify new therapeutic approaches and targets for the treatment of epilepsy.

## METHODS

### Patients

The patients included in this study (Table S1) were selected from the files of the departments of neuropathology of the Academic Medical Center (AMC, University of Amsterdam), the VU University Medical Center (VUMC) in Amsterdam, and the University Medical Center (UMC) in Utrecht. Another group of patients was recruited by Neuro-om, Neurosurgery Center for Epilepsy, Pozzilli-Isernia, Italy. We examined a total of 21 surgical epilepsy specimens (hippocampus and neocortex) from patients who were undergoing surgery for refractory epilepsy. The predominant seizure types were medically intractable complex partial seizures (patients 1–21; Table S1). All MTLE patients showed HS, with appreciable neuronal loss and reactive gliosis. Considering the difficulty in finding “real” controls in human studies, in our experiments we used both autopsies and “healthy” surgical samples from patients with other pathologies. Seizure absence was determined by the patient’s report to the neurologist during the scheduled visits, including 60 min of awake EEG standard recordings, classified according to Engel. Therefore, for comparative purposes we used specimens of nonepileptic tissues from histologic normal specimens (control samples) from patients (26–29; Table S1) undergoing surgery for meningioma (WHO grade III) and from seven control patients without any neurologic diseases (autopsies, patients 30–36). All

autopsies were performed within 12 h after death. The analysis of histologic normal tissues obtained at surgery showed a pattern of immunoreactivity (IR) similar to that observed in control tissues from autopsies, thus arguing in favor of antigen preservation in autopsies. For additional detail see Supporting Information. Informed consent to use part of the biopsy material for our experiments and for access to medical records for research purposes was obtained from all patients. Tissue was obtained and used in accordance with the Declaration of Helsinki; the Ethics Committees of University of Amsterdam and the University of Rome “Sapienza” approved the selection process and surgical procedures. The clinical characteristics derived from the patients’ medical records are summarized in Table S1.

### Immunohistochemistry

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6  $\mu\text{m}$ , mounted on organosilane-coated slides (Star Frost; Waldemar Knittel GmbH, Braunschweig, Germany), and used for immunocytochemistry as described in Supporting Information.

### Immunoblot analysis

Western blot analysis for CX3CR1 expression was performed on protein lysates extracted from hippocampal tissues obtained from three controls (32, 33, 35; Table S1) and from seven MTLE patients (1, 2, 4, 6, 10–12; Table S1) as detailed in Supporting Information.

### Electrophysiology

#### Brain slices recordings

Neocortical slices were prepared from a 1 cc block of surgically resected human inferior temporal gyrus (temporal pole; for patients, see Table S1). Transverse slices (300  $\mu\text{m}$ ) were cut in glycerol-based artificial cerebrospinal fluid (ACSF) with a Leica VT 1000S Vibratome (Leica Microsystems Milan, Italy) immediately after surgical resection. The slices were placed in an incubation chamber at room temperature with oxygenated ACSF and then transferred to a self-constructed glass-acrylic glass recording chamber (volume  $\approx$  1 ml) within 1–18 h after slice preparation. Whole-cell patch-clamp recordings were performed at room temperature on V layer pyramidal neurons exhibiting typical action potential firing and spontaneous synaptic activity. Spontaneous epileptiform activity has been described by field potential measures in these human cells (Köhling et al., 1998); however, when recording from individual cells we did not observe any paroxysmal activity, as described previously (Köhling et al., 1998). Cells were dialyzed with a  $\text{Cl}^-$ -free intracellular solution (see below) eliminating variability due to different  $[\text{Cl}^-]_i$ . GABA-induced currents were recorded at a holding potential of 0 mV, to avoid spurious contributions of inward  $\text{Na}^+$  currents, as described pre-

viously (Ragozzino et al., 2005). Under these experimental conditions, with inactivated voltage-gated channels, cells were stable and healthy for 1–2 h. In some neurons, spontaneous inhibitory postsynaptic currents were present at low frequency, not affecting the quantification of GABA-induced currents. GABA was delivered by pressure applications (10–20 psi for 1 s with a General Valve [Fairfield, NY USA] Picospritzer II) from glass micropipettes positioned above the voltage-clamped neurons. In this way, stable whole-cell currents and rapid drug wash were obtained before the rundown protocol was applied. The following current rundown protocol was adopted after current amplitude stabilization with repetitive applications every 120 s, a sequence of 10 GABA (100  $\mu\text{M}$ ) applications of 1 s duration every 15 s was delivered; then the test pulse was resumed at the control rate (every 120 s) to monitor recovery of the GABA current. In this protocol the reduction in peak amplitude of the 10th current was expressed as percent of the 1st ( $I\%$ ); for more details, see Ragozzino et al. (2005). CX3CL1 was dissolved in  $\text{H}_2\text{O}$ , stored as frozen stock solution (10  $\mu\text{M}$ ), and diluted to the working concentration of 10 nM before each recording session. After rundown, protocol slices were incubated with or without CX3CL1 for 15 min (Bertolini et al., 2006; Ragozzino et al., 2006) before testing again.

#### Membrane preparation and *Xenopus* oocytes recordings

Membranes were prepared as described previously (Miledi et al., 2006) and as detailed in Supporting Information, with use of tissues from patients with MTLE (1–12, 16–20; Table S1); from patients with focal cortical dysplasia (FCD; 22–25; Table S1), and from nonepileptic controls (26–32; Table S1). Preparation of *Xenopus laevis* oocytes and injection procedures were performed as detailed elsewhere (Miledi et al., 2006). The use of female *Xenopus laevis* frogs conformed to institutional policies and guidelines.

GABA current rundown ( $I\%$ ) was defined as the decrease (in percentage) of the current peak amplitude after six 10-s applications of 500  $\mu\text{M}$  GABA at 40 s intervals (Palma et al., 2004).

The  $I_{\text{GABA}}$  desensitization was defined as the time taken for the current to decay from its peak to half-peak value ( $T_{0.5}$ ).

CX3CL1 was dissolved as described earlier. In all experiments, the holding potential was  $-60$  mV. In some experiments, oocytes were pretreated with CX3CL1 for 120 min after single application of GABA or after the control rundown protocol. In some experiments, 3 h washout with oocyte Ringer’s solution was performed before initiation of a new rundown protocol. For controls, CX3CL1 was heat-inactivated for 45 min in a water bath at  $90^\circ\text{C}$ . To block G-protein-coupled receptors, oocytes were injected with pertussis toxin 50  $\mu\text{g}/\text{ml}$  1 h before CX3CL1 incubation. In other experiments, we performed intranuclear injection of human complementary DNA (cDNAs) encoding the wild-type (WT)  $\alpha 1$ ,  $\beta 2$ , and  $\gamma 2$  GABA $_A$  subunits and CX3CR1

(pcDNA3 vector) in *Xenopus* oocytes (Miledi et al., 2006). *X. laevis* oocytes and injection procedures were prepared as detailed previously (Miledi et al., 2006). All results are given as mean  $\pm$  standard error of the mean (SEM). Two data sets were considered statistically different when  $p < 0.05$  (analysis of variance [ANOVA] test).

## Chemicals and solutions

### Brain slice recordings

ACSF had the following composition (in mM): NaCl, 125; KCl, 2.5; CaCl<sub>2</sub>, 2; NaH<sub>2</sub>PO<sub>4</sub>, 1.25; MgCl<sub>2</sub>, 1; NaHCO<sub>3</sub>, 26; glucose, 10; and Na-pyruvate, 0.1 (pH 7.35). Glycerol-based ACSF solution contained the following (in mM): glycerol, 250; KCl, 2.5; CaCl<sub>2</sub>, 2.4; MgCl<sub>2</sub>, 1.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 26; glucose, 11; and Na-pyruvate, 0.1 (pH 7.35). Patch pipettes were filled with the following (in mM): 140 K-gluconate, 10 HEPES, 5 1,2-Bis(2-aminophenoxy) ethane-*N,N,N,N*-tetraacetic acid, 2 MgCl<sub>2</sub>, and 2 Mg-ATP (pH 7.35, with KOH).

### Oocyte recordings

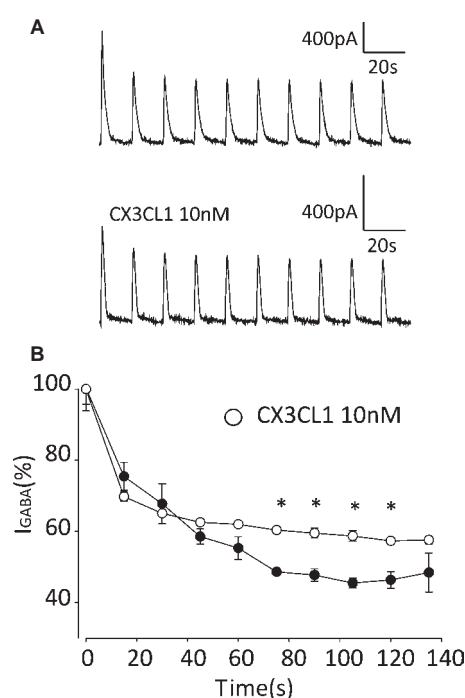
Oocyte Ringer's solution had the following composition (in mM): NaCl, 82.5; KCl, 2.5; CaCl<sub>2</sub>, 2.5; MgCl<sub>2</sub>, 1; HEPES, 5, adjusted to pH 7.4 with NaOH. All drugs were purchased from Sigma Italia with the exception of GABA (purchased from Tocris, Bristol, UK) and CX3CL1 (purchased from Peprotech, London, UK). Human  $\alpha 1\beta 2\gamma 2$  cDNA was a gift of Dr. Keith Wafford and CX3CR1 (pcDNA3) purchased from cDNA Resource Center.

## RESULTS

### CX3CL1 decreases I<sub>GABA</sub> rundown in human epileptic slices from patients with MTLE

To elucidate the role played by CX3CL1 in modulating GABAergic signaling in brain tissue from patients with epilepsy, we tested its effect on I<sub>GABA</sub> rundown in pyramidal neurons in MTLE cortical slices (patients 16–21, Table S1;  $n = 9$ ). In these cells, I<sub>GABA</sub> amplitude ranged from 674 to 2,205 pA (mean  $1,521 \pm 214$  pA,  $n = 9$ ; Fig. 1). In agreement with previous experiments (Ragozzino et al., 2005), in all these cells, repeated applications of GABA (100  $\mu$ M, 1 s every 15 s; 10 times) induced current of decreasing peak amplitude, so that I<sub>0</sub> (amplitude of the 10th current expressed as percent of the 1st; see Methods) was  $48 \pm 4\%$  ( $n = 9$  cells). This current rundown was significantly limited by a 15-min pretreatment with 10 nM CX3CL1, as I<sub>0</sub> became  $58 \pm 1\%$  ( $n = 9$ ,  $p < 0.05$ ).

In the absence of CX3CL1, a second rundown protocol had effects similar to the control one, (I<sub>0</sub> =  $49 \pm 2\%$ ;  $n = 7$ , data not shown), indicating that CX3CL1 effect was genuine. CX3CL1 treatment did not affect current decay (not shown), but significantly limited I<sub>GABA</sub> recovery between two rundown protocols. Under control conditions,



**Figure 1.**

CX3CL1 induces a decrease of I<sub>GABA</sub> rundown in pyramidal neurons from human MTLE cortical slices. (A) Sample currents elicited by 10 applications of GABA (100  $\mu$ M) in one neuron in control condition (Top) and after a 15-min application of CX3CL1 (10 nM; Bottom). Holding potential, 0 mV. (B) Normalized time course of the averaged I<sub>GABA</sub> rundown of nine neurons in control condition (●,  $48 \pm 4\%$ ) and after CX3CL1 application (○,  $58 \pm 1\%$ ; nine slices). Data points show mean  $\pm$  SEM. Slices were obtained from temporal cortical tissue surgically resected from six MTLE patients. Current amplitudes normalized to I<sub>1st</sub> of rundown protocol (I<sub>control</sub>: (●)  $1,521 \pm 214$  pA; I<sub>CX3CL1</sub>: (○)  $919 \pm 119$  pA). \* $p < 0.01$ .

the I<sub>GABA</sub> amplitude recorded 15 min after the first rundown protocol was  $83 \pm 4\%$  ( $n = 7$ ) of the first I<sub>GABA</sub> amplitude, possibly because of slow recovery and/or time-dependent current reduction. If CX3CL1 was present during the 15 min interval, I<sub>GABA</sub> amplitude became  $60 \pm 2\%$  ( $n = 9$ ), exhibiting a significantly lower recovery from rundown ( $p < 0.05$ ). These findings, although worthy of further investigation, show a clear effect of CX3CL1 on I<sub>GABA</sub> in native pyramidal neurons from patients with MTLE.

### CX3CL1 decreases I<sub>GABA</sub> rundown in oocytes transplanted with membranes from MTLE brain tissue

To bypass the limited availability of healthy human tissues and the technical difficulties of recording on human MTLE slices, we studied the effects of CX3CL1 on I<sub>GABA</sub> using *Xenopus* oocytes microtransplanted with brain tissues from MTLE patients (1–12, 16–20; Table S1). In agreement with previous results (Palma et al., 2004; Ragozzino et al., 2005), applications of GABA (500  $\mu$ M) to transplanted oocytes, elicited inward currents (I<sub>GABA</sub> amplitude range :

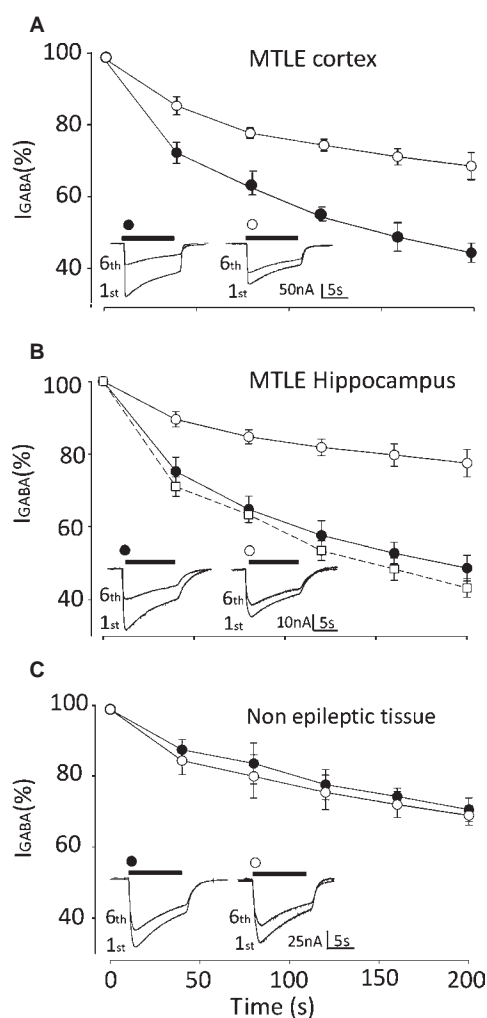


–10 nA to –500 nA) blocked by the GABA<sub>A</sub>R antagonist bicuculline (100  $\mu$ M; data not shown).

In oocytes injected with cortical membranes (1–12, 16–20; Table S1), we found a consistent  $I_{\text{GABA}}$  rundown following repetitive applications of GABA ( $I_{\%} = 50.2 \pm 1.5\%$ ; 95 oocytes; 19 frogs; patients 1–12, 16–20) with a partial recovery (approximately 40%) within 40 min after washout (not shown; see Palma et al., 2004). In these cells, the simultaneous application of CX3CL1 (10–100 nM) and GABA did not alter  $I_{\text{GABA}}$  rundown (10 oocytes; not shown). In contrast, prolonged exposure to CX3CL1 (from 15 min to 5 h) decreased  $I_{\text{GABA}}$  rundown with a maximal effect obtained 2 h after CX3CL1 treatment (100 nM). In 80% of examined cells,  $I_{\%}$  was  $45.5 \pm 2.3\%$  and  $69.2 \pm 2.8\%$ , respectively, before and after CX3CL1 treatment (65 oocytes; 15 frogs;  $p < 0.05$ ; patients 1–12, 16–20; Fig. 2A). Similar results were obtained using muscimol (500  $\mu$ M; data not shown) confirming that the effect is mediated by GABA<sub>A</sub>Rs.

The absence of CX3CL1 effect in 20% of treated cells might be due to variability of receptor expression in patient's tissues or signaling in different oocytes. CX3CL1 effect on  $I_{\text{GABA}}$  rundown was completely reverted after 2 h washout in 60% of oocytes, whereas in the remaining cells, the full recovery was reached after overnight washout (data not shown). In addition, similar to what was observed in slices, CX3CL1 did not modify current decay ( $T_{0.5} = 7.6 \pm 0.2$  s, control;  $7.8 \pm 0.5$  s, treated,  $p > 0.05$ ). Similar results were obtained in oocytes injected with hippocampal membranes from the same MTLE patients (Fig. 2B): during repetitive applications of GABA,  $I_{\%}$  was  $48.7 \pm 4.1\%$  in control conditions and  $77.5 \pm 5.4\%$  after CX3CL1 treatment (45 oocytes, eight frogs;  $p < 0.05$ ; patients 1–12; Fig. 2B). In additional experiments we investigated whether CX3CL1 could affect  $I_{\text{GABA}}$  rundown in oocytes injected with membranes from subiculum of three MTLE patients (Table S1; patients 1, 2, 5), which have been reported to have a positive shift of GABA reversal potential (Palma et al., 2006). In 21 oocytes,  $I_{\%}$  was  $43 \pm 1.8\%$  in control conditions and  $58.2 \pm 2\%$  after CX3CL1 treatment ( $p < 0.05$ ).

It is important to note that when a control rundown protocol was repeated 2 h after the first,  $I_{\text{GABA}}$  rundown was not modified ( $I_{\%} = 46.4 \pm 1.9\%$  and  $43.1 \pm 1.4\%$ , before and after 2 h, respectively; 15 oocytes/3 frogs;  $p > 0.05$ ; patients 1–3; Fig. 2B) showing that CX3CL1 effect was genuine and not caused by a nonspecific impairment of the phenomenon. Furthermore, in oocytes injected with epileptic hippocampal membranes, heat-inactivated CX3CL1 was ineffective on  $I_{\text{GABA}}$  rundown; and 1 h pretreatment with pertussis toxin abolished CX3CL1-induced effect ( $I_{\%} = 50 \pm 4.5\%$  vs.  $48.2 \pm 3.3\%$ , before and after CX3CL1 treatment; 20 oocytes/3 frogs;  $p > 0.05$ ; patients 2–4) suggesting that this effect was not caused by a nonspecific interaction of CX3CL1 with GABA<sub>A</sub>Rs, but requires



**Figure 2.**

CX3CL1 reduces  $I_{\text{GABA}}$  rundown in epileptic brain tissue from patients with MTLE. (A, B, C) Amplitude of consecutive GABA currents (% of first response; 500  $\mu$ M GABA) in oocytes injected with membranes from different tissues. (A) Oocytes injected with membranes from cortex of MTLE patients before (●) and after 2 h treatment with 100 nM CX3CL1 (○; 65 oocytes/15 frogs; 17 patients). Data points show means  $\pm$  SEM. In this and subsequent figures all currents normalized to the first current;  $I_{\text{control}}$ : (●)  $166 \pm 12.2$  nA;  $I_{\text{CX3CL1}}$ : (○)  $99.3 \pm 8.5$  nA. (Inset) Superimposed currents elicited by the first and sixth GABA applications (500  $\mu$ M, horizontal bar) during rundown protocol before and after drug treatment. In this and subsequent figures, bars indicate the timing of GABA applications. (B) Oocytes injected with membranes from hippocampus of MTLE patients before (●); after 2 h treatment with 100 nM CX3CL1 (○; 45 oocytes/8 frogs; same patients as in [A]; and when a control rundown protocol was repeated 2 h after the first [□; dotted line; 15 oocytes in the same set of experiments])  $I_{\text{control}}$ : (●)  $45.3 \pm 7.9$  nA;  $I_{\text{CX3CL1}}$ : (○)  $32.5 \pm 6$  nA. (Inset) Superimposed currents as in (A). (C) Oocytes injected with membranes from nonepileptic patients before (●) and after 2 h treatment with 100 nM CX3CL1 (○; 40 oocytes/5 frogs, seven patients).  $I_{\text{control}}$ : (●)  $96.1 \pm 7.6$  nA;  $I_{\text{CX3CL1}}$ : (○)  $90 \pm 7.3$  nA. (Inset) Superimposed currents as in (A).

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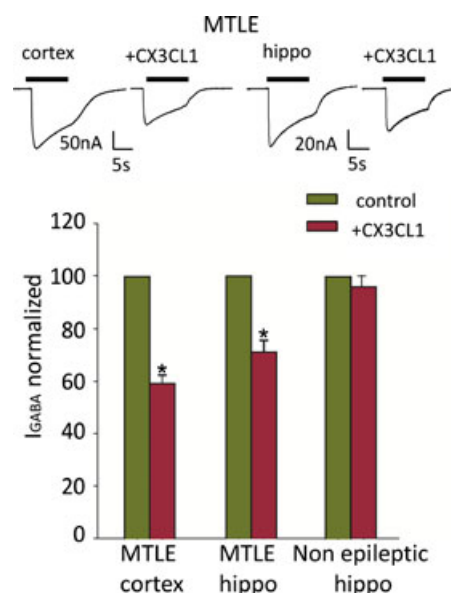
interaction with CX3CR1 and G protein-dependent signaling.

### CX3CL1 does not affect $I_{GABA}$ rundown in oocytes transplanted with membranes from human control tissues or human cortical dysplasia

To investigate whether CX3CL1 could modulate  $I_{GABA}$  rundown in nonepileptic tissue, we injected oocytes with membranes from surgical samples (26–29) and from autopsies (30–32) of healthy nonepileptic subjects (Table S1 and S3). In these oocytes, CX3CL1 did not alter  $I_{GABA}$  rundown ( $I_{\%} = 73.1 \pm 2.8\%$  and  $69.3 \pm 2.5\%$ , before and after CX3CL1, respectively; 40 oocytes; five frogs;  $p > 0.05$  Fig 2C). To address whether CX3CL1 had a peculiar action on MTLE tissues, we analyzed oocytes injected with tissue from patients with epileptic FCD, both pediatric and adult forms (Roseti et al., 2009). In both cases, CX3CL1 did not influence  $I_{GABA}$  rundown ( $I_{\%} = 65.9 \pm 4.5\%$  and  $65.2 \pm 4.8\%$ , before and after CX3CL1, respectively; 42 oocytes; eight frogs  $p > 0.05$ ; adult patient 22, pediatric patients 23–25; Table S1), suggesting that CX3CL1 exerts a specific action on MTLE tissues. To further verify the hypothesis that the effect of CX3CL1 on GABA<sub>A</sub> currents is specific for MTLE tissue, we co-injected into *Xenopus* oocytes the cDNAs encoding  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub>R, the predominant GABA<sub>A</sub>R subtype in the healthy CNS (Macdonald et al., 2010), together with cDNA encoding CX3CR1. In agreement with previous experiments (Palma et al., 2004), the evoked currents due to the activation of  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub>Rs were stable, showing only a weak  $I_{GABA}$  rundown, modified neither by CX3CR1 coexpression (Fig. S1) nor by CX3CL1 pretreatment (20 oocytes/4 frogs; Fig. S1).

### CX3CL1 affects the recovery of $I_{GABA}$ amplitude from rundown in oocytes transplanted with membranes from patients with MTLE

To investigate the CX3CL1 effect on  $I_{GABA}$  amplitude, we analyzed the current evoked by a single application of GABA (500  $\mu$ M, for 5 s) following CX3CL1 treatment. Under these conditions, CX3CL1 (from 100 to 500 nM) did not affect  $I_{GABA}$  amplitude in oocytes injected with cortical or hippocampal MTLE membranes (55 oocytes/7 frogs/4 patients). We then compared  $I_{GABA}$  amplitude between the first GABA applications in two consecutive  $I_{GABA}$  rundown protocols, 2 h interval, in the presence or in the absence of CX3CL1. Results, reported in Figure 3, indicate that when CX3CL1 was present, the first  $I_{GABA}$  amplitude of second rundown protocol was reduced to  $59.5 \pm 3.2\%$  of control in oocytes injected with MTLE cortical membranes (65 oocytes/8 frogs; patients 1–12, 16–20; Table S1) and to  $71.3 \pm 4.5\%$  in oocytes injected with MTLE hippocampal membranes (46 oocytes/6 frogs; same patients;  $*p < 0.05$ ; Fig. 3). By contrast, no significant variations were observed in oocytes injected with membranes from nonepileptic patients or in the absence of CX3CL1 (Table S3). All



**Figure 3.**

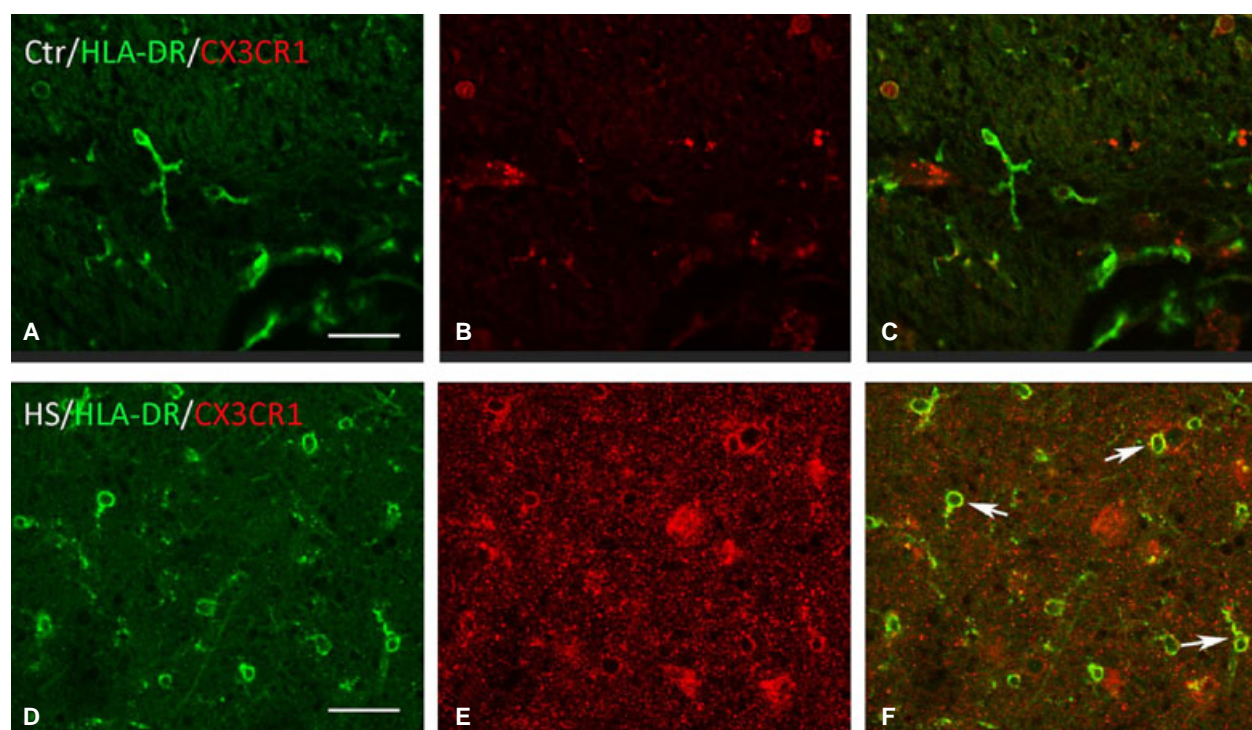
CX3CL1 affects the recovery of  $I_{GABA}$  amplitude from rundown in oocytes injected with epileptic brain tissue from cortex and hippocampus of MTLE patients. The bar-chart represents the mean of  $I_{GABA}$  amplitudes (first application of neurotransmitter in the rundown protocol) before or after the application of 100 nM of CX3CL1 in different tissues. Note that CX3CL1 has no effect in nonepileptic healthy patients (Inset) Sample records of first GABA current in the rundown protocol before and after CX3CL1 treatment in oocytes injected with human membranes from cortex and hippocampus of MTLE patients.

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together these results suggest that the observed  $I_{GABA}$  amplitude decrease after CX3CL1 treatment is due to a reduced recovery from rundown as in MTLE slices.

### The expression of CX3CR1 in MTLE tissues is increased compared to normal brain

There is a general consensus that neuronal damage, gliosis, and inflammation are common features of MTLE hippocampal region (Aronica et al., 2010; Yang et al., 2010; Vezzani et al., 2012). For this reason, we studied the expression of CX3CR1 in the hippocampus of some of the patients described above by immunocytochemistry. In control (from autopsy) hippocampus, CX3CR1 displayed a weak staining in the different hippocampal subfields, including CA1 and hilar regions (Fig. S2A,C). In HS specimens from MTLE, CX3CR1 immunoreactivity was specifically increased in glial cells (Figs. S2B,D and S3). Double labeling confirmed CX3CR1 expression in HLA-DR and Iba1-positive microglial cells (Figs. 4 and 5; Table S2) suggesting that in these tissues the increase of CX3CR1 runs in parallel with the microglia activation. Of interest, we found an increase of CX3CR1 immunoreactivity also in the cortex of one patient with FCD



**Figure 4.**

Cellular distribution of CX3CR1 immunoreactivity increases in hippocampus of MTLE patients. **A–F:** (confocal images). **A–C:** CX3CR1 immunoreactivity in control hippocampus (hilar region; **A**, HLA-DR, green; **B**, CX3CR1, red; **C**, merged image) showing absence of co-localization with the microglial marker HLA-DR. **D–F:** CX3CR1 immunoreactivity in hippocampus of one MTLE patients with hippocampal sclerosis (HS; hilar region; **D**, HLA-DR, green; **E**, CX3CR1, red; **F**, merged image) showing co-localization with HLA-DR (arrows in **F**). Scale bar **A–F:** 40  $\mu$ m.

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(patient 24, Table S1; Fig. S4) for which an increase of microglia reactivity has been reported (Iyer et al., 2010). In addition, Western blot analysis performed on hippocampi from three controls (patient 32, 33, 35; Table S1) and seven patients with MTLE (1, 2, 4, 6, 10–12; Table S1) showed that CX3CR1 is increased (about sixfold) in MTLE versus control tissues (see Supporting Information and Fig. S5).

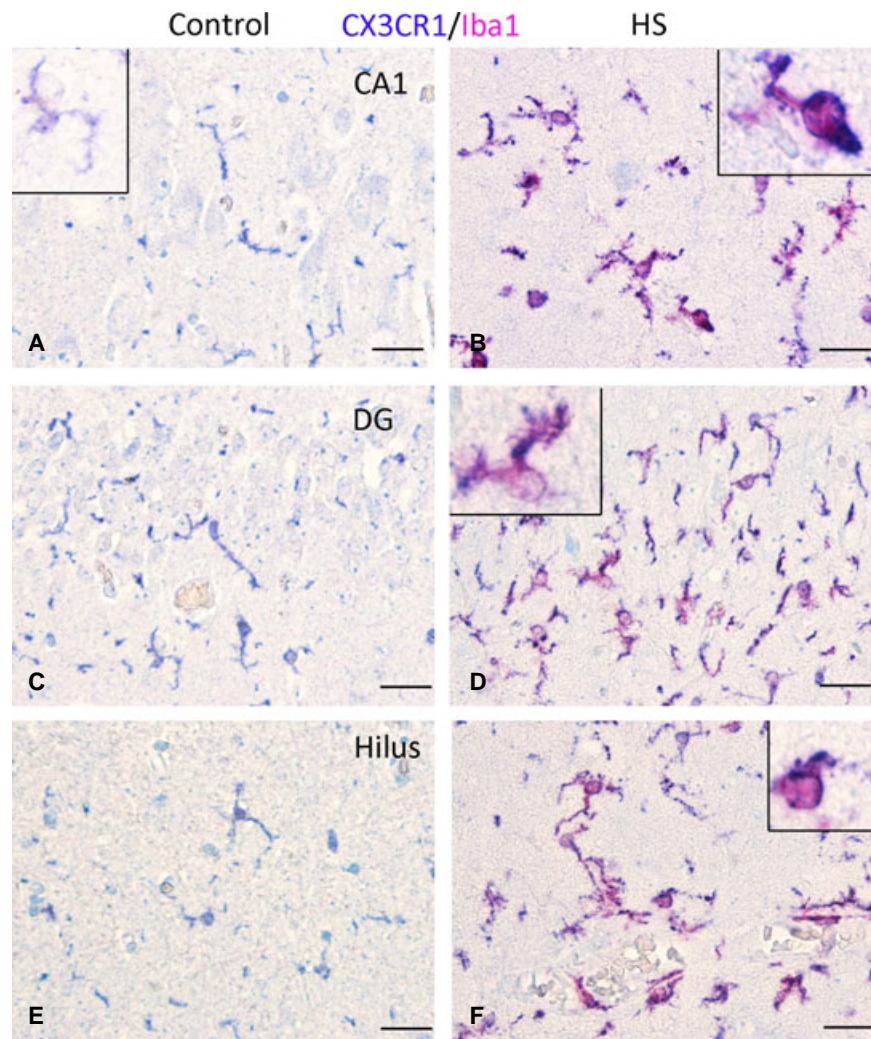
## DISCUSSION

In this work we report for the first time that CX3CL1 modulates  $I_{GABA}$  in human epileptic brain tissue. This result was obtained recording  $I_{GABA}$  both in pyramidal neurons of human cortical MTLE slices and in oocytes transplanted with nervous tissues resected from the same patients with MTLE. We also demonstrated that CX3CR1 expression is increased in microglia of hippocampal regions of patients with MTLE. Both in brain slices and oocytes, CX3CL1 limits the  $I_{GABA}$  rundown and reduces the recovery of current amplitude after repetitive stimulation. All together, these findings indicate that CX3CL1 promotes the stability of  $I_{GABA}$ , which in turn may be expected to impart stability to neural circuits.

We have previously shown that  $I_{GABA}$  rundown caused by repetitive GABA<sub>A</sub>R stimulation is stringently linked to epileptogenesis in MTLE patients (Palma et al., 2004) and in epileptic rats (Mazzuferi et al., 2010) but does not have a key role in human lesional epilepsies (Ragozzino et al., 2005; Conti et al., 2011). This phenomenon is prevented by Brain-Derived Neurotrophic Factor (BDNF), adenosine receptor antagonists, and phosphatase inhibitors (Palma et al., 2004, 2005; Roseti et al., 2009), suggesting that the phosphorylation state of GABA<sub>A</sub>R or associated proteins (Saliba et al., 2012) is likely linked to  $I_{GABA}$  rundown. A similar effect has been described in dissociated neurons from the brain of epileptic patients afflicted with hypothalamic hamartomas and in oocytes microtransplanted with membranes from these same tissues (Li et al., 2011), confirming that  $I_{GABA}$  rundown is a hallmark for impaired GABAergic function contributing to seizures genesis and propagation (Janigro, 2006; Jansen et al., 2008).

In the present paper we demonstrated an increase of CX3CR1 immunoreactivity in glial cells of MTLE hippocampal subfields, in particular in activated microglia. CX3CL1 is abundantly expressed in the nervous system and principally by neurons (Harrison et al., 1998; Xu et al., 2012). Furthermore, CX3CL1 is overexpressed in inflam-





**Figure 5.**

Cellular distribution of CX3CR1 immunoreactivity increases in activated microglia. **A–F:** double-labeling of CX3CR1 (blue) with Iba1 (red) in control hippocampus (**A**, CA1; **C**, dentate gyrus, DG; **E**, hilar region/hilus) and in hippocampus of MTLE patient with sclerosis (HS; **B**, CA1; **D**, dentate gyrus, DG; **F**, hilar region/hilus) showing increased expression of CX3CR1 and co-localization (purple) with the microglial marker Iba1 in HS (**B**, **D**, **F**; inserts: high magnification photographs of double-labeled microglia/macrophages). Scale bar **A–F**: 40  $\mu$ m.

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mation underlying neurodegenerative diseases such as multiple sclerosis and Alzheimer disease (Hulshof et al., 2003), and recent evidence describes an altered expression of CX3CL1 in neurons of the temporal neocortex of patients with epilepsy and in the cortex and hippocampus of epileptic rats (Yeo et al., 2011; Xu et al., 2012). Many recent studies indicate that inflammation is related to the hippocampal remodeling induced by seizures and that inflammatory mechanisms are implicated in MTLE with HS hippocampal sclerosis (Vezzani et al., 2009, 2011b; Aronica et al., 2010; Yang et al., 2010). These findings suggest that CX3CL1/CX3CR1 increase may be part of the inflammatory process present in epileptic hippocampus playing a role in epileptogenesis. Yeo et al. (2011) hypothesized that an increase of CX3CL1/CX3CR1 signaling during epilepsy

could contribute to neuronal damage, being associated with increased microglia activation and neuronal loss. Here, we demonstrated that in native pyramidal neurons of the temporal cortex from MTLE patients, CX3CL1 reduces  $I_{GABA}$  rundown and the recovery of  $I_{GABA}$  amplitude from rundown, suggesting a possible modulatory activity on GABAergic neurotransmission. This is in accordance with data on serotonin neurons of dorsal raphe nucleus (Heinisch & Kirby, 2009), where CX3CL1 modulated both spontaneous and evoked inhibitory post-synaptic current (IPSC) amplitude. Given this CX3CL1 effect, an apparent paradox could arise from the simultaneous increase, in MTLE tissue, of  $I_{GABA}$  rundown and of the CX3CL1/CX3CR1 expression. However, the enhancement of this signaling could represent an attempt to reduce changes induced by epileptic insult, as



shown previously for other mechanisms (Grabenstatter et al., 2012).

Although the mechanism underlying CX3CL1 effect on native pyramidal neurons needs further investigation, we could hypothesize that the improvement of  $I_{GABA}$  rundown is caused by an interaction between neurons and microglia (Gao & Ji, 2010), where the activation of CX3CR1 by CX3CL1 may lead to an involvement of a phosphorylation cascade and to a “stabilization” of neuronal GABA<sub>A</sub> receptors. An important task for future studies will be to investigate the signaling activated by CX3CL1.

To strengthen the data obtained on human slices, we took advantage of the microtransplantation technique confirming that CX3CL1 specifically limits  $I_{GABA}$  rundown and the recovery of  $I_{GABA}$  from rundown both in the cortical and hippocampal tissues obtained from patients with MTLE. This gave us the opportunity to overcome the difficulty to record on human MTLE slices, due to the high degree of gliosis and neuronal loss (Blümcke et al., 2007), and we were able to compare the results on MTLE with those obtained in control tissues from patients without neurologic diseases and without inflammatory processes occurring.

With this approach, the exact cellular origin, glial or neuronal, of the membrane patches transplanted on oocytes surface is not known. However, the microtransplantation is a good technical approach to investigate the “whole” GABA evoked currents, since the patches of membranes from native cells seem to maintain most the receptors in their native conformation (Palma et al., 2003), and in this study it fully reproduced the CX3CL1-mediated  $I_{GABA}$  rundown observed in MTLE slices. Obviously, it is unlikely that CX3CL1 could affect GABA<sub>A</sub>Rs in human slices and in oocytes by the same mechanism. One hypothesis may be that, since oocytes can incorporate both glial and neuronal membranes (Eusebi et al., 2009), CX3CL1/CX3CR1 system can act on GABA<sub>A</sub>Rs by signaling endogenous to either cell types or even to oocytes, as previous reported for other substances (Palma et al., 2005, 2007).

In addition, we found only a small  $I_{GABA}$  rundown in oocytes injected with membranes from nonepileptic patients or from patients with FCD. In these experiments rundown was not significantly different between fresh surgical samples and postmortem tissues, as previously demonstrated (Conti et al., 2011), and it was not affected by CX3CL1.

Our observation that CX3CL1 exerts modulatory effects on  $I_{GABA}$  in both oocytes and native neurons is indicative of a specific action of the CX3CL1 on MTLE GABA<sub>A</sub>Rs and could be the consequence of an increased expression of CX3CR1 underlying the disease. However, our observation that CX3CR1 expression is increased in cortical dysplasia where it has been reported a strong microglial activation (Iyer et al., 2010) suggests that the effect of CX3CL1 is specific for MTLE tissues paralleling the presence of  $I_{GABA}$

rundown and not necessarily the increase of receptor expression.

We have previously shown that the altered  $I_{GABA}$  rundown in MTLE is due to GABA<sub>A</sub>Rs formed by subunits with a low sensitivity to  $Zn^{2+}$  antagonism (Palma et al., 2007) and that in epileptic rats the occurrence of  $I_{GABA}$  rundown is related to an altered ratio of  $\alpha 1/\alpha 4$  GABA<sub>A</sub> subunits (Mazzuferi et al., 2010). We can hypothesize that CX3CL1 reduces  $I_{GABA}$  rundown, modulating one or more GABA<sub>A</sub>R subunits involved in this mechanism. Consistent with this hypothesis, we found that in MTLE tissues, the recovery of  $I_{GABA}$  amplitude after rundown was impaired by CX3CL1, both in human slices and oocytes, suggesting that CX3CL1 may abolish a fraction of  $I_{GABA}$  due to the activation of highly desensitizing GABA<sub>A</sub>Rs. This hypothesis is in line with the idea that some modulators, like  $Zn^{2+}$  or the neurosteroid tetrahydrodeoxycorticosterone THDOC, can affect  $I_{GABA}$  selectively acting on specific GABA<sub>A</sub>R subunits (Stell et al., 2003; Mortensen & Smart, 2006). Alternatively, CX3CL1 might exert multiple modulatory effects on current amplitude and GABA<sub>A</sub>R stability.

The expression level of other chemokines and cytokines like interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF)-1 $\alpha$ , transforming growth factor (TGF)- $\beta$ , and chemokine (C-C motif) ligand 4, CCL4 increases in epilepsy and evidence has demonstrated their involvement in epileptogenesis, in neuronal hyperexcitability, seizure frequency, and duration (Wu et al., 2008; Fabene et al., 2010; Vezzani et al., 2011, 2013; Vezzani, 2012; Vezzani et al., 2012; Kan et al., 2012).

Although we cannot demonstrate from our data if the increase of CX3CR1 precedes or follows the onset of epilepsy, our results on  $I_{GABA}$  stability in epileptic hippocampus and cortex would suggest a potential antiepileptogenic role for this chemokine in MTLE. This hypothesis would be in line with a common view of CX3CL1 as a protective chemokine in several neuropathologies (Cardona et al., 2006; Lee et al., 2010; Cipriani et al., 2011), and with the observations that CX3CL1 reduces the production of the proepileptogenic IL-1 $\beta$  (Cardona et al., 2006). In contrast, the action of CX3CL1 could increase excitability of subiculum, where it reduces the excitatory  $I_{GABA}$  rundown (Palma et al., 2006). Therefore, several questions are still open at this stage and further experiments will be necessary to better elucidate this point.

## ACKNOWLEDGMENTS

This work was supported by PRIN 2009 projects (to C.L. and E.P.) and Ministero della Sanità Antidoping (to C.L. and E.P.), Cenci Bolognietti (to C.L.) and National Epilepsy Fund NEF 05-11; NEF 09-05 (to E.A.). We are grateful to Jasper Anink for the expert technical assistance and to W. M.G. Spliet (UMC) and Johannes C. Baaijen (VUMC) for the selection of the cases. We thank Dr. Francesca Grassi for reading the manuscript, and Prof. Ricardo Miledi for the valuable discussion and suggestions. We are grateful to the patients for allowing us to carry out this work.

## DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Methods.

**Figure S1.** CX3CL1 does not influence GABA current rundown in oocytes co-injected with human  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> subunits and CX3CR1 cDNAs.

**Figure S2.** Distribution of CX3CR1 immunoreactivity in the hippocampus of control and MTLE patients with hippocampal sclerosis.

**Figure S3.** Evaluation of CX3CR1 immunoreactivity in control hippocampus and in hippocampal sclerosis.

**Figure S4.** CX3CR1 immunoreactivity in FCD.

**Figure S5.** CX3CR1 expression in hippocampal tissues of MTLE patients.

**Table S1.** Clinical characteristics and neurophysiologic findings of patients.

**Table S2.** CX3CR1 expression in glial cells in control hippocampus and in hippocampal MTLE patients.

**Table S3.** GABAergic characteristics and rundown in control nonepileptic patients.