FULL-LENGTH ORIGINAL RESEARCH

Fractalkine/CX3CL1 modulates $GABA_A$ currents in human temporal lobe epilepsy

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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 γ-aminobutyric acid (GABA)ergic inhibitory efficacy. Therefore, experiments were designed to investigate whether, in MTLE brain tissues, CX3CL1 may influence $GABA_A$ receptor ($GABA_AR$) mediatedtransmission, 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.

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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 γ-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 ($GABA_AR$) dysfunction (Pavlov et al., 2013). We have shown previously that the repetitive activation of $GABA_AR$ produces a use-dependent decrease (rundown) of the GABA-evoked currents (I_{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_{GABA} is related to an altered expression of α1/α4 $GABA_AR$ 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_{GABA} in tissue obtained from patients with drug-resistant MTLE. For this reason, we first studied the effect of CX3CL1 on $GABA_AR$s 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 $GABA_AR$s 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 Neuromed, 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 μm, 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 μm) 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 ≈ 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 Cl^--free intracellular solution (see below) eliminating variability due to different $[Cl^-]_i$. GABA-induced currents were recorded at a holding potential of 0 mV, to avoid spurious contributions of inward Na^+ currents, as described previously (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 μM) 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 H_2O, stored as frozen stock solution (10 μM), 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 (Bertollini 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 μM GABA at 40 s intervals (Palma et al., 2004).

The I_{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°C. To block G-protein–coupled receptors, oocytes were injected with pertussis toxin 50 μg/ml 1 h before CX3CL1 incubation. In other experiments, we performed intranuclear injection of human complementary DNA (cDNAs) encoding the wild-type (WT) α1, β2, and γ2 $GABA_A$ 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 ± 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; $CaCl_2$, 2; NaH_2PO_4, 1.25; $MgCl_2$, 1; $NaHCO_3$, 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; $CaCl_2$, 2.4; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 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 $MgCl_2$, 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; $CaCl_2$, 2.5; $MgCl_2$, 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 $\alpha1\beta2\gamma2$ cDNA was a gift of Dr. Keith Wafford and CX3CR1 (pcDNA3) purchased from cDNA Resource Center.

Results

CX3CL1 decreases I_{GABA} 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_{GABA} rundown in pyramidal neurons in MTLE cortical slices (patients 16–21, Table S1; n = 9). In these cells, I_{GABA} amplitude ranged from 674 to 2,205 pA (mean 1,521 ± 214 pA, n = 9; Fig. 1). In agreement with previous experiments (Ragozzino et al., 2005), in all these cells, repeated applications of GABA (100 μM, 1 s every 15 s; 10 times) induced current of decreasing peak amplitude, so that $I_{\%}$ (amplitude of the 10th current expressed as percent of the 1st; see Methods) was 48 ± 4% (n = 9 cells). This current rundown was significantly limited by a 15-min pretreatment with 10 nM CX3CL1, as $I_{\%}$ became 58 ± 1% (n = 9, $p < 0.05$).

In the absence of CX3CL1, a second rundown protocol had effects similar to the control one, ($I_{\%}$ = 49 ± 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_{GABA} recovery between two rundown protocols. Under control conditions,

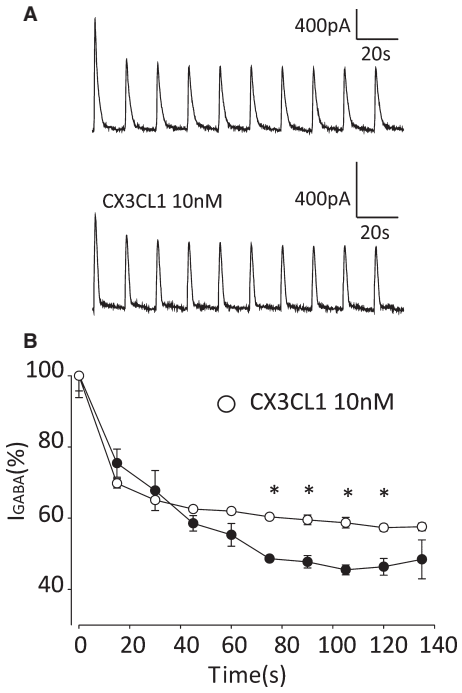

Figure 1.
CX3CL1 induces a decrease of I_{GABA} rundown in pyramidal neurons from human MTLE cortical slices. (**A**) Sample currents elicited by 10 applications of GABA (100 μM) in one neuron in control condition (*Top*) and after a 15-min application of CX3CL1 (10 nM; *Bottom*). Holding potential,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

the I_{GABA} amplitude recorded 15 min after the first rundown protocol was 83 ± 4% (n = 7) of the first I_{GABA} amplitude, possibly because of slow recovery and/or time-dependent current reduction. If CX3CL1 was present during the 15 min interval, I_{GABA} amplitude became 60 ± 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_{GABA} in native pyramidal neurons from patients with MTLE.

CX3CL1 decreases I_{GABA} 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_{GABA} 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 μM) to transplanted oocytes, elicited inward currents (I_{GABA} amplitude range :

−10 nA to −500 nA) blocked by the $GABA_AR$ antagonist bicuculline (100 μM; data not shown).

In oocytes injected with cortical membranes (1–12, 16–20; Table S1), we found a consistent I_{GABA} rundown following repetitive applications of GABA ($I_{\%}$ = 50.2 ± 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_{GABA} rundown (10 oocytes; not shown). In contrast, prolonged exposure to CX3CL1 (from 15 min to 5 h) decreased I_{GABA} rundown with a maximal effect obtained 2 h after CX3CL1 treatment (100 nM). In 80% of examined cells, $I_{\%}$ was 45.5 ± 2.3% and 69.2 ± 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 μM; data not shown) confirming that the effect is mediated by $GABA_ARs$.

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_{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 ± 0.2 s, control; 7.8 ± 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 ± 4.1% in control conditions and 77.5 ± 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_{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 ± 1.8% in control conditions and 58.2 ± 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_{GABA} rundown was not modified ($I_{\%}$ = 46.4 ± 1.9% and 43.1 ± 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_{GABA} rundown; and 1 h pretreatment with pertussis toxin abolished CX3CL1-induced effect ($I_{\%}$ = 50 ± 4.5% vs. 48.2 ± 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 $GABA_ARs$, but requires

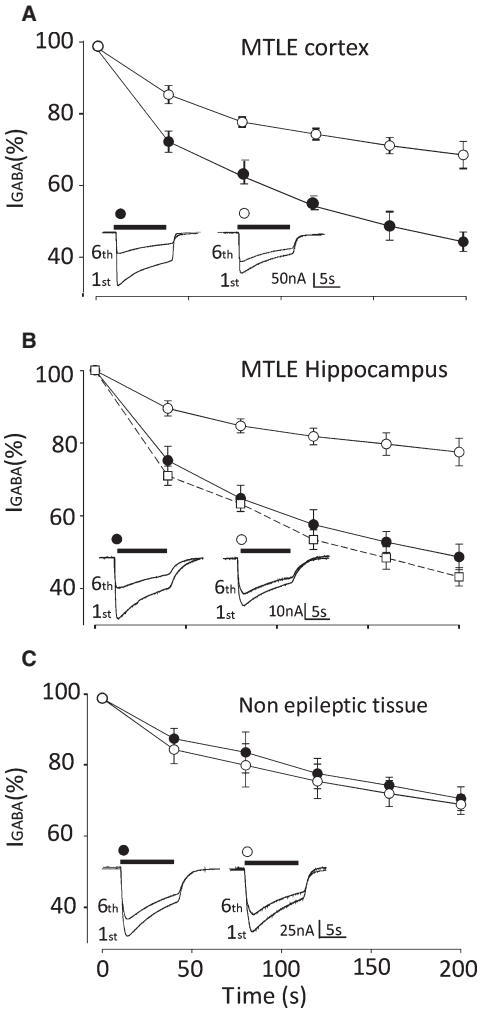

Figure 2.
CX3CL1 reduces I_{GABA} rundown in epileptic brain tissue from patients with MTLE. (**A**, **B**, **C**) Amplitude of consecutive GABA currents (% of first response; 500 μM GABA) in oocytes injected with membranes from different tissues. (**A**) Oocytes injected with membranes from cortex of MTLE patients before (●) and after 2 h treatment with 100 nM CX3CL1 (○; 65 oocytes/15 frogs; 17 patients). Data points show means ± SEM. In this and subsequent figures all currents normalized to the first current; $I_{control}$: (●) 166 ± 12.2 nA; I_{CX3CL1}: (○) 99.3 ± 8.5 nA. (Inset) Superimposed currents elicited by the first and sixth GABA applications (500 μM, 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]) $I_{control}$: (●) 45.3 ± 7.9 nA; I_{CX3CL1}: (○) 32.5 ± 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). $I_{control}$: (●) 96.1 ± 7.6 nA; I_{CX3CL1}: (○) 90 ± 7.3 nA. (inset) Superimposed currents as in (**A**).

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 ± 2.8% and 69.3 ± 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 ± 4.5% and 65.2 ± 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 $GABA_A$ currents is specific for MTLE tissue, we co-injected into Xenopus oocytes the cDNAs encoding $\alpha1\beta2\gamma2$ $GABA_AR$, the predominant $GABA_AR$ 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 $\alpha1\beta2\gamma2$ $GABA_ARs$ 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 μM, 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 ± 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 ± 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

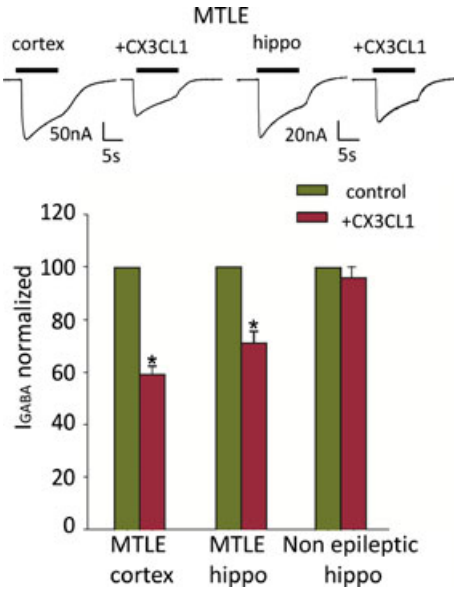

Figure 3.
CX3CL1 affects the recovery of I_{GABA} amplitude from rundown in oocytes injected with epileptic brain tissue from cortex and hippocampus of MTLE patients. The bar-chart represents the mean of I_{GABA} amplitudes (first application of neurotransmitter in the rundown protocol) before or after 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.
Epilepsia © ILAE

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

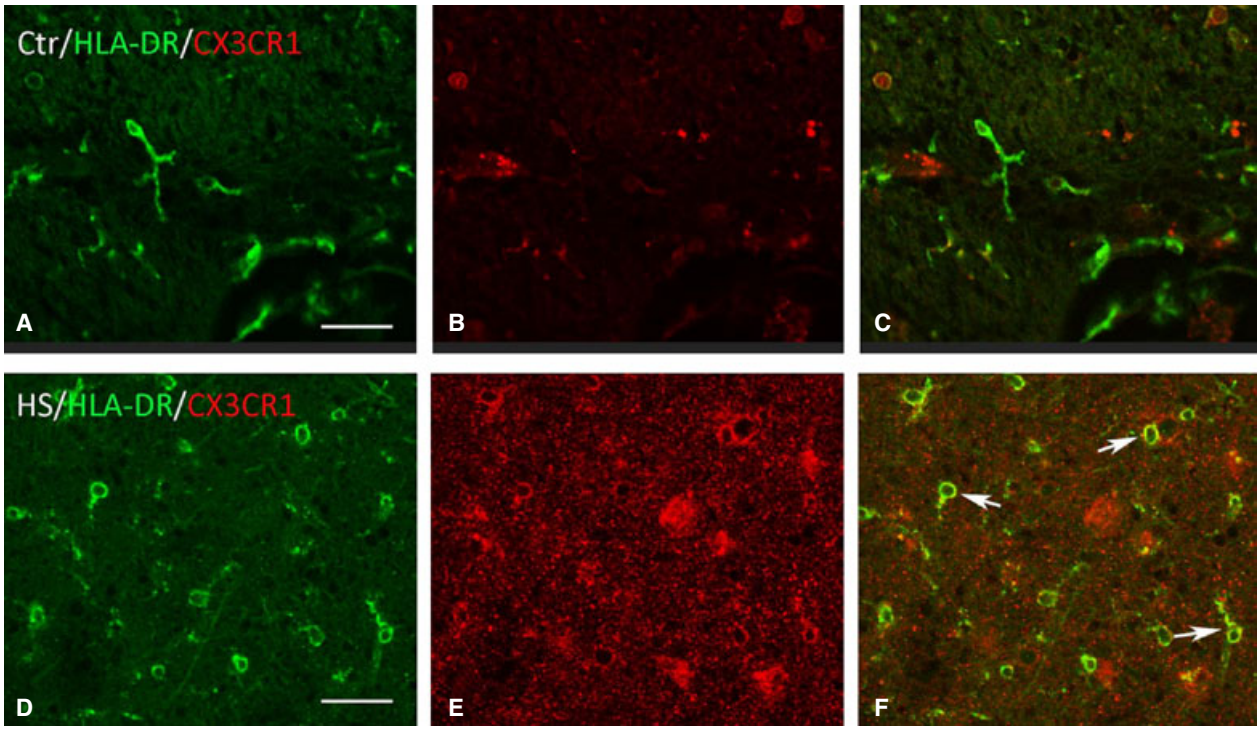

Figure 4.
Cellular distribution of CX3CR1 immunoreactivity increases in hippocampus of MTLE patients. **A**–**F**: (confocal images). **A**–**C**: CX3CR1 immunoreactivity in control hippocampus (hilar region; **A**, HLA-DR, green; **B**, CX3CR1, red; **C**, merged image) showing absence of co-localization 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 μm.
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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 $GABA_AR$ 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 $GABA_AR$ 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-

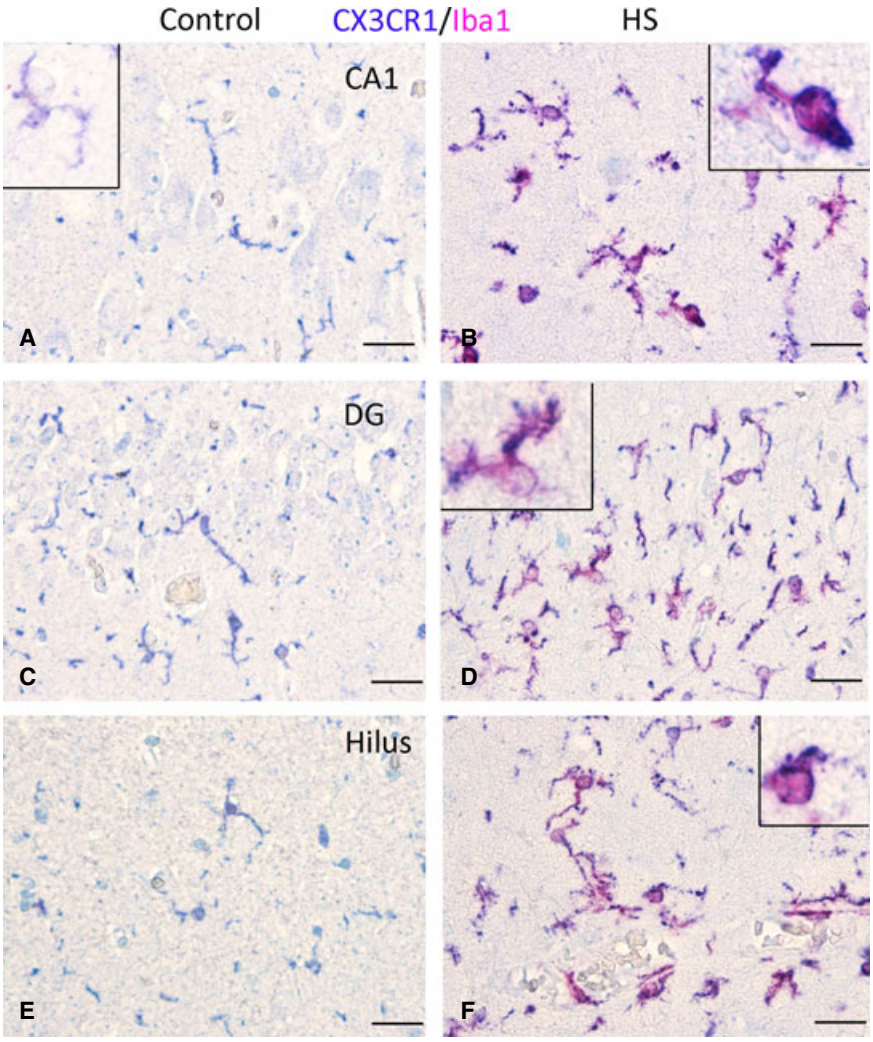

Figure 5.
Cellular distribution of CX3CR1 immunoreactivity increases in activated microglia. **A**–**F**: double-labeling of CX3CR1 (blue) with Iba1 (red) in control hippocampus (**A**, CA1; **C**, dentate gyrus, DG; **E**, hilar region/hilus) and in hippocampus of MTLE patient with sclerosis (HS; **B**, CA1; **D**, dentate gyrus, DG; **F**, hilar region/hilus) showing increased expression of CX3CR1and 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 μm.

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 $GABA_A$ 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 $GABA_A$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 $GABA_A$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 $GABA_A$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 $GABA_A$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 $\alpha1/\alpha4$ $GABA_A$ subunits (Mazzuferi et al., 2010). We can hypothesize that CX3CL1 reduces I_{GABA} rundown, modulating one or more $GABA_A$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 $GABA_A$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 $GABA_A$R subunits (Stell et al., 2003; Mortensen & Smart, 2006). Alternatively, CX3CL1 might exert multiple modulatory effects on current amplitude and $GABA_A$R stability.

The expression level of other chemokines and cytokines like interleukin (IL)-1β, tumor necrosis factor (TNF)-1α, transforming growth factor (TGF)-β, 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-1β (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.

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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 $\alpha1\beta2\gamma2$ $GABA_A$ 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.

SUPPORTING INFORMATION

METHODS

Patients

The predominant seizure types were medically intractable complex partial seizures (patients #1-21; Table 1S). All MTLE patients showed hippocampal sclerosis (HS), with appreciable neuronal loss and reactive gliosis. The HS specimens include 11 cases of classical HS (grade 3), MTS type 1a (Blumcke et al., 2007,2012) and the remaining cases displayed severe HS (grade IV) MTS type 1b (Blumcke et al., 2007,2012). Furthermore, we analyzed surgical specimens of patients with adult and pediatric Focal Cortical Dysplasia (aFCD or pFCD) (patient #22, aFCD; patients #23-25, pFCD; Table 1S). For the FCD specimens we followed the ILAE classification system (Blumcke and Mühlebner 2011).

IMMUNOHISTOCHEMISTRY

Antibodies

Neuronal nuclear protein (NeuN; mouse clone MAB377, IgG1; Chemicon, Temecula, CA, USA; 1:2000); glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000; monoclonal mouse; DAKO; 1:50), vimentin (mouse clone V9, DAKO; 1:1000) and (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark, 1:400) and Iba1 (polyclonal rabbit, WAKO, Richmond, VA, USA; 1:200) were used in the routine immunocytochemical analysis of TLE human specimens. For the detection of CX3CR1, we used a polyclonal antibody (1:50, AB-11012; Immunological Sciences, Rome, Italy).

Immunohistochemistry

For single-labeling immunohistochemistry, paraffin-embedded sections were deparaffinized, re-hydrated, and incubated for 20 min in 0.3% H_2O_2 diluted in methanol to quench the endogenous peroxidase activity. Antigen retrieval was performed by incubation for 10 min at 121 °C in citrate buffer (0.01 M, pH 6.0), sections were washed with phosphate-buffered saline (PBS) and incubated for 30 min in 10% normal goat serum (Harlan Sera-Lab, Loughborough, Leicestershire, UK). Sections were incubated with the primary antibodies overnight at 4 °C. Hereafter sections were washed in PBS and we used the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3'-diaminobenzidine (DAB; Sigma) as chromogen. Sections were counterstained with haematoxylin, dehydrated and coverslipped. Sections incubated without the primary antibody were essentially blank.

For double-labeling studies of CX3CR1 with CR3/43 (HLA-DR), sections were, after incubation with the primary antibodies overnight at 4 °C, incubated for 2 h at room temperature with Alexa Fluor® 568-conjugated anti-rabbit and Alexa Fluor® 488 anti-mouse IgG or anti-goat IgG (1:100, Molecular Probes, The Netherlands). Sections were then analyzed by mean of a laser scanning confocal microscope (Leica TCS Sp2, Wetzlar, Germany).

For double-labeling studies of CX3CR1 with Iba1, Iba1 was visualized with a polymer-alkaline phosphatase (AP)-labeled anti-rabbit antibody (Immunologic, BrightVision #DPVR55AP) and liquid permanent red (DAKO, #K0640) as chromogen. To remove the first primary antibody (Iba) sections were incubated at 121 °C in citrate buffer (0.01 M, pH 6.0) for 10 min, as previously described (Aronica, 2007) Sections were then incubated for 1 h at room temperature with the second primary antibody (CX3CR1). The second primary antibodies were visualized with poly-AP anti-rabbit antibody (Immunologic BrightVision #DPVR55AP) and Vector Blue (Vector labs, AP substrate kit III, #SK-5300) as chromogen.

Evaluation of immunostaining

All labeled tissue sections were evaluated with respect to the presence or absence of various histopathological parameters and immunoreactivity for CX3CR1. The intensity of CX3CR1 staining was evaluated using a scale of 0-3 (0: no; 1: weak; 2: moderate; 3: strong staining). All areas of the specimen were examined and the score represents the predominant cell staining intensity found in each case in microglial cells. The frequency of CX3CR1 positive cells [(1) rare; (2) sparse; (3), high] was also evaluated to give information about the relative number of positive cells within the specimen. As proposed before (Vandeputte, 2002; Ravizza, 2006), the product of these two values (intensity and frequency scores) was taken to give the overall score (total score) shown in Fig. 3S.

To analyze the percentage of HLA-DR positive cells that express CX3CR1 within the epileptogenic lesions (controls: n=5; HS, n=5) in each specimens two representative adjacent non-overlapping fields of the areas of interest (CA1, CA3 and hilar region for HS) were captured (magnification 40x; total area of each field: 165.250 μm2) and digitized using a laser scanning confocal microscope. The total number of cells stained with CX3CR1 and HLA-DR, as well as the number of double labeled cells were counted and percentages were calculated (expressed as mean ± SEM) of cells co-expressing CX3CR1 and HLA-DR; as previously described (Iyer et al., 2010).

To analyze the percentage of double-labeled cells positive for CX3CR1 and Iba1, digital photomicrographs were obtained through different regions of the hippocampus (CA1-CA1; DG/hilar region). Images of 8 representative fields per section (magnification 20x) were collected (Leica DM5000B). Images were analyzed with a Nuance VIS-FL Multispectral Imaging System (Cambridge Research Instrumentation; Woburn, MA). Spectra were acquired from 460 to 660 nm at 10-nm intervals and Nuance software version 2.4 was used for analysis (van der Loos et al., 2008; Boer et al., 2008). The total number of cells stained with CX3CR1 or Iba1 as well as the number of cells double labeled with CX3CR1 and Iba1 were counted visually and percentages were calculated (expressed as mean ± SEM) of cells co-expressing Iba1 and CX3CR1.

Statistical analyses were performed with SPSS for Windows (SPSS 11.5, SPSS Inc., Chicago, IL, USA) using two-tailed Student's t-test ($P<0.05$ was considered significant).

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 1S) and from seven MTLE patients (#1,2,4,6,10-12; Table 1S). For each patient, equal amounts (30 μg/lane) of proteins were loaded on SDS-PAGE gel for immunoblot analysis with rabbit anti-CX3CR1 (1:1000; Immunological Science, Rome, Italy). Densitometric analysis was performed with QuantityOne software (Biorad).

MEMBRANES PREPARATION AND XENOPUS OOCYTES RECORDINGS

Briefly, tissues were homogenized using a Teflon glass homogenizer with 2 ml of glycine buffer of the following composition (in mM): 200 glycine, 150 NaCl, 50 EGTA, 50 EDTA, 300 sucrose; plus 20 μl protease inhibitors (Sigma); pH 9 adjusted with NaOH. The homogenate was centrifuged for 15 min at 9.500 x g. The supernatant was collected and centrifuged for 2 h at 105 x g at 4 °C. The pellet was washed, re-suspended in assay buffer (glycine 5 mM) and used directly, or aliquoted and stored at –80 °C for later use.

From 12 to 48 h after injection, membrane currents were recorded from voltage-clamped Xenopus oocytes using two microelectrodes filled with 3 M KCl (Miledi, 1982). The oocytes were placed in a recording chamber (0.1 ml) perfused continuously (9-10 ml/min) with oocyte's Ringer solution (OR) at room temperature (20-22oC).

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FIGURE LEGENDS AND TABLES

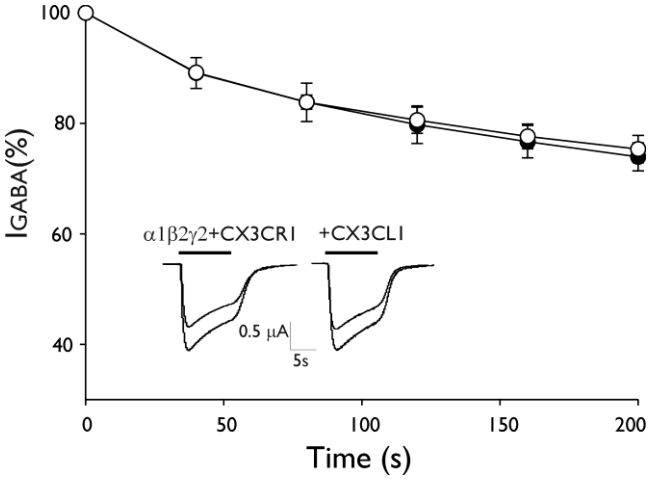

Fig. 1S CX3CL1 does not influence GABA current rundown in oocytes co-injected with human α1β2γ2 $GABA_A$ subunits and CX3CR1 cDNAs. Amplitude of consecutive GABA currents (percent of first response; 500 μM GABA) before (●; 73.9%±2.5) and after CX3CL1 treatment (○; 75.3%±2.5; 25 oocytes/4 frogs; P>0.05). Points represent means (± SEM) of peak GABA currents. Currents normalized to the first response; $I_{control}$: (●)1498nA±224 nA; I_{CX3CL1}: (○)1521±182 nA. (Inset) Superimposed currents elicited by the first and sixth GABA applications (500μM, horizontal bar) during rundown protocol before and after drug treatment.

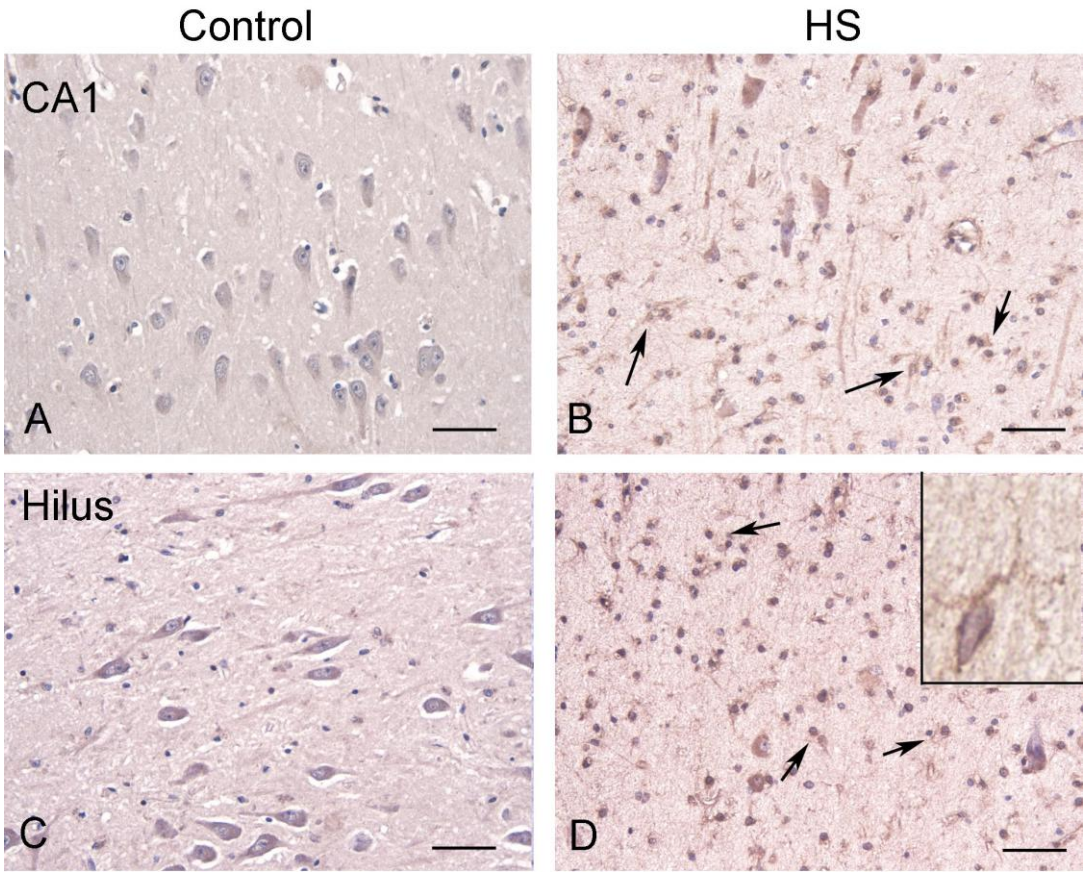

Fig 2S. Distribution of CX3CR1 immunoreactivity in the hippocampus of control and MTLE patients with hippocampal sclerosis . Sections are counterstained with hematoxylin.

A-D: CX3CR1 immunoreactivity. A, C: Control hippocampus showing non detectable glial immunoreactivity (A, CA1 hilar region/hilus, C). B, D: Hippocampal sclerosis (HS) showing increased expression of CX3CR1 in glial cells (arrows) in CA1 (B) and hilar region (D). Insert in D shows high magnification of a positive microglial cell. Scale bar: A-D: 80 µm.

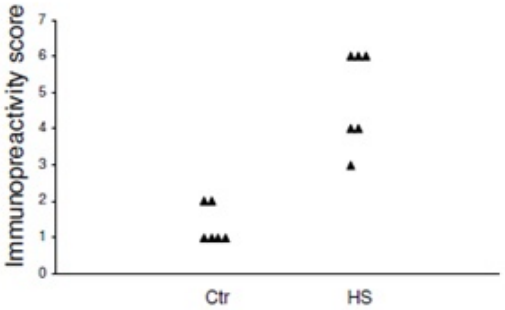

Fig. 3S. Evaluation of CX3CR1 immunoreactivity in control hippocampus and in hippocampal sclerosis. Distribution of CX3CR1 immunoreactivity score (total score; see for details Methods section) in microglia of normal control autopsy hippocampus (Ctr; n= 6), and hippocampal sclerosis (HS; n= 6).

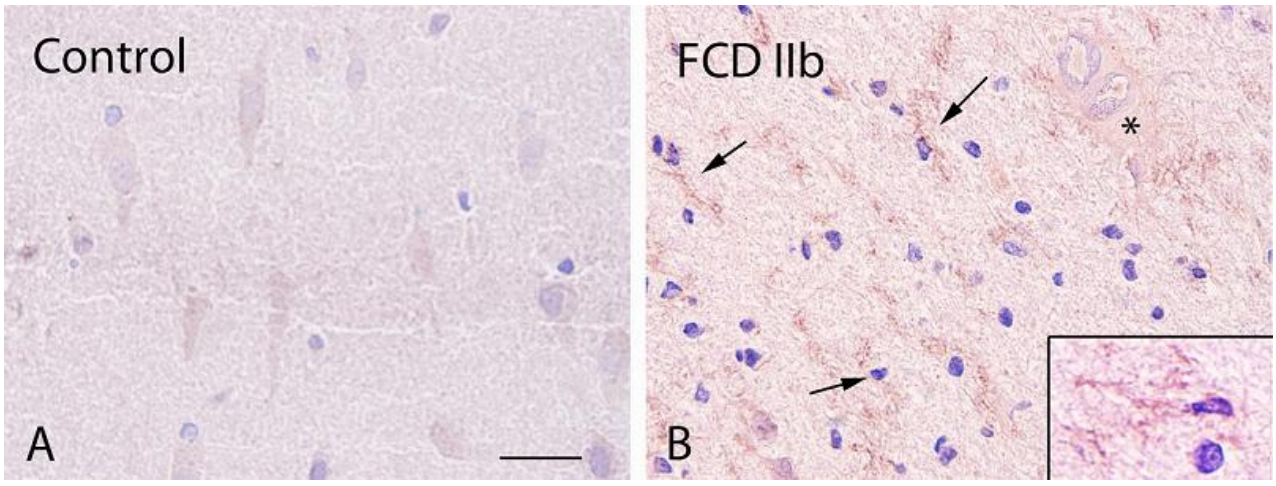

Fig. 4S. CX3CR1 immunoreactivity in FCD.

A-B: CX3CR1 immunoreactivity (IR). A: control cortex not detectable glial IR. B: FCD IIb (#24, Table 1S) showing increased expression of CX3CR1 IR in glial cells (arrows; asterisk, negative balloon cells). Insert in B shows high magnification of a positive microglial cell. Sections are counterstained with hematoxylin. Scale bar in A: A-B: 40 µm.

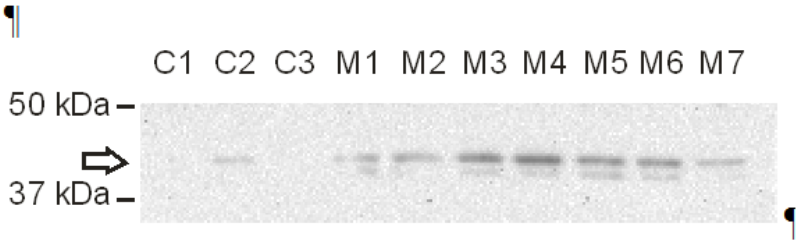

Fig. 5S CX3CR1 expression in hippocampal tissues of MTLE patients.
Western blot analysis of CX3CR1 expression in hippocampi obtained from three controls (C1–3; #32,33,35; Table 1S) and seven epileptic MTLE patients (M1-7; #1,2,4,6,10-12; Table 1S). Fractalkine receptor was detected as a band of 42 kDa which is none or weakly detectable in control and increased in MTLE tissues.

Table 1S. Clinical characteristics and neurophysiological findings of patients

P #	gender	age (years)	epileptogenic zone	surgery	epilepsy onset (years)	histopathology
1* □	M	43	L-T	ETL	28	HS
2* □	M	41	R-T	ETL	20	HS
3*	F	39	R-T	ATL	12	HS
4* □	M	19	L-T	ETL	6	HS
5 *o	F	29	R-T	ETL	15	HS
6*o□	M	34	L-T	ETL	4	HS
7*o	F	36	R-T	ETL	6	HS
8 *o	M	27	R-T	ETL	10	HS
9*o	F	54	R-T	ETL	12	HS
10*o□	F	31	R-T	ETL	23	HS
11*o □	M	56	L-T	ETL	36	HS
12*o □	M	19	L-T	ETL	10	HS
13o	M	33	R-T	ETL	23	HS
14o	F	34	R-T	ETL	18	HS
15o	F	33	R-T	ETL	19	HS
16●*	F	29	L-T	AMTL	15	HS

17●*	F	37	R-T	AMTL	6	HS
18●*	M	42	L-T	ETL	17	HS
19●*	M	36	R-T	AMTL	9m	HS
20●*	M	19	R-T	ETL	4	HS
21●	M	55	L-T	AMTL	42	HS
22○*	M	48	L-T	LES	6	FCDIIA
23○*	F	3m	L-T	LES	1m	FCDIIA
24○*	M	2	R-T	LES	0.5m	FCDIIB
25○*	M	7	R-T	LES	3	FCDIIB
26○*	F	47	none	LES plus	-	-
27○*	M	52	none	LES plus	-	
28○*	M	38	none	LES plus	-	
29○*	F	32	none	LES plus	-	
30○ *	F	47	none	-	-	-
31○ *	M	64	none	-	-	-
32○* □	M	26	none	-	-	-
33○ □	F	54	none	-	-	-
34○	F	48	none	-	-	-
35○ □	M	53	none	-	-	-
36○	M	36	none	-	-	-

Patients1-21: afflicted with MTLE; Patients 22-25 afflicted with adult and pediatric FCD; Patients 26-29: surgical specimens from **non epileptic patients afflicted by meningioma III WHO;** Patients 30-36: non epileptic tissue from autopsies.

T, temporal; F, frontal; L, left; R, right; AMTL, anteriomesial temporal lobectomy; ETL, extensive temporal lobectomy; LES, lesionectomy; HS, hippocampal sclerosis; FCD, focal cortical dysplasia. Tumor grading was according to the histological classification (WHO, Louis et al., 2007); classification system FCD (Blumcke 2011)

*patient tissues used for oocyte injection; ○patient tissues used for immunohistochemistry; □ **patient tissues used for immunoblot**; ●patient tissues used for human slices.

***Surgery*. All operations were performed by the same neurosurgeon (V.E). Both ETL and AMTL included microsurgical resection of the amygdala and en bloc excision of the hippocampal formation and parahippocampal gyrus. These interventions differed in the extent of the neocortical resection. Nondominant ETL included excision of 4-4.5 cm of the superior temporal gyrus and the middle temporal gyrus and 5-6 cm of the inferior temporal gyrus, whereas dominant ETL included excision of 4-5 cm of the middle and inferior temporal gyrus, whereas the superior gyrus was left intact. On the other hand, in AMTL, the extent of the neocortical excision was 3 cm for all of the first three temporal gyri (sparing the superior gyrus in the dominant hemisphere). Lesionectomy (LES) consisted of complete removal of the foreign epileptogenic focus.**

Table 2S. CX3CR1 expression in glial cells in control hippocampus and in hippocampal MTLE patients

	HLA-DR	Iba1
Controls (n= 5)	4.3 ± 2.3	8.1 ± 3.3
HS (n= 5)	88.6 ± 4.3*	95.8 ± 5.5*

Data represent percentages of cells immunoreactive for HLA-DR, or Iba1 and co-expressing CX3CR1. Data are expressed as mean ± SEM. ND, not determined; P<0.05 vs control. Hippocampal sclerosis (HS).

Table 3S. GABAergic characteristics and rundown in control non epileptic patients

patients	**I_{GABA} (nA)**	**$I_{\%}$**	**$T_{0.5}$**
surgical(#26-29)	**−98.1 ± 9.0**	**71.0 ± 3.0%**	**8.1±0.5 s**
autopsies (#30-32)	**−89.1 ± 12.0**	**76.1 ± 2.7%**	**7.6±0.6 s**

Data represent the I_{GABA} mean amplitude (GABA 500 μM), the percent of I_{GABA} rundown ($I_{\%}$) and the I_{GABA} decay expressed as $T_{0.5}$ in oocytes injected with membranes prepared from fresh surgical or post- mortem samples of control patients as indicated (see Table 1S for patients). Data are expressed as mean ± SEM. P>0.05 surgical *vs* autopsies for all the parameters.