Inflammation and epilepsy: the contribution of astrocytes
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2.4 The inflammatory molecules IL-1β and HMGB1 can rapidly enhance focal seizure generation in rat entorhinal cortex.

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ABSTRACT

Purpose
Inflammatory signalling pathways involving Interleukin-1 β (IL-1β) and high-mobility group B1 (HMGB1) are recognized to increase the susceptibility to epileptic seizures. In the present study, we investigated the role of these proinflammatory molecules in the generation of focal seizure-like discharge in slice preparations from the entorhinal cortex (EC).

Methods
Seizure like-discharges were evoked by either slice perfusion with low Mg²⁺ and picrotoxin or local stimulation with NMDA applied in the presence of the proconvulsant 4-amino-pyridine (4-AP). The effects of local applications with IL-1β or HMGB1 were evaluated by monitoring seizure discharge generation through laser scanning microscope imaging of the Ca²⁺ signal from tens of neurons and astrocytes.

Key findings
we revealed that upon local IL-β or HMGB1 applications a single NMDA pulse, per se ineffective, could evoke a focal ictal discharge (fID). HMGB1 enhanced the direct response of neurons to NMDA stimulation, but only after the neuronal network experienced a sustained epileptiform activity.

Significance
These findings demonstrate that both IL-1β and HMGB1 can rapidly lower focal ID threshold and raise the possibility that targeting these inflammatory pathways may represent an effective therapeutic strategy to prevent seizures.
INTRODUCTION
Epilepsy is a neurological disorder characterized by recurring, unprovoked seizures. It affects about 1% of the population worldwide and often requires lifelong medication (Hauser, Annegers et al. 1993). About 30% of epileptic patients are considered drug resistant as they do not respond to the currently available anti-epileptic drugs (AEDs) (Perucca, French et al. 2007). Increasing evidence supports the involvement of inflammatory and immune processes in the etiopathogenesis of seizures (Vezzani and Granata 2005). Inflammation induced by brain-damaging events such as trauma, stroke, infection, febrile seizures and status epilepticus is associated with acute symptomatic seizures and a high risk of developing epilepsy (Pitkanen and Sutula 2002; Bartfai, Sanchez-Alavez et al. 2007). In particular, high levels of proinflammatory cytokines [e.g. interleukin (IL)-1beta, tumour necrosis factor (TNF)-alpha], danger signals [high-mobility group box (HMGB)1, S100 beta] and downstream inflammatory mediators (e.g. prostaglandins, the complement system) have been measured in epileptogenic tissue from patients affected by epilepsy of various aetiologies (Vezzani, French et al. 2011). The major contributors to the synthesis of these inflammatory mediators are brain-resident cells such as activated microglia, astrocytes and neurons.

Recently, two proinflammatory molecules were found to be proconvulsant in animal models of temporal lobe epilepsy (TLE): IL-1b and HMGB1. These molecules, applied before the induction of experimental TLE, were able to increase the time spent in seizures and reduce the onset time of the first seizure. The effects of HMGB1 and IL-1β seem to be very similar, especially because both are blocked by ifenprodil (Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010), a selective antagonist of NR2B-containing NMDA receptors (Yu, Askalan et al. 1997).

We tested here the hypothesis that IL-1b and HMGB1 act as proconvulsant in two different models of focal seizure-like discharges in rat entorhinal cortex (EC) slices (Gomez-Gonzalo, Losi et al. 2010; Losi, Cammarota et al. 2010). To evoke focal seizure-like, ictal discharges (IDs) we used either slice perfusion with low Mg2+ / picrotoxin or pressure pulses applied to an N-methyl-D-aspartate (NMDA)-containing glass pipette to stimulate an episode of hyperactivity in a small number of layer V–VI neurons, in the presence of the proconvulsant 4-AP and low Mg2+. Fast laser scanning microscope imaging of Ca2+ signals from tens of neurons revealed that local applications with either IL-1b or HMGB1 rapidly favor the generation of an epileptogenic site for focal ID initiation.
MATERIALS AND METHODS

Brain slices and dye loading

Transverse cortico-hippocampal slices were prepared from postnatal day 14–18 Wistar rats and loaded with OGB1-AM (excited at 488 nm) or Rhod-2 (excited at 543 nm), respectively, as previously described (Losi, Cammarota et al. 2010). Briefly, brain was removed and transferred to ice-cold cutting solution containing (in mM): NaCl, 120; KCl, 3.2; KH$_2$PO$_4$, 1; NaHCO$_3$, 26; MgCl$_2$, 2; CaCl$_2$, 1; glucose, 10; Na-pyruvate, 2; and ascorbic acid, 0.6; at pH 7.4 (with 5% CO$_2$/95% O$_2$). Coronal slices were obtained by cutting with a Leica vibratome VT1000S in the presence of the ionotropic glutamate receptor inhibitor kynurenic acid (2 mM). Slices were recovered for 15 min at 37°C and then loaded with the Ca$^{2+}$-sensitive dye OGB1-AM (Invitrogen) for 60 min at 37°C. Loading was performed in the cutting solution containing sulfinpyrazone (200 µM), pluronic (0.12%), and kynurenic acid (1 mM). After loading, slices were recovered and kept at room temperature in the presence of 200 µM sulfinpyrazone. All experiments were performed at 33–35°C.

Ca$^{2+}$ imaging

Brain slices were visualized with a TCS-SP2-RS or a TCS-SP5-RS confocal microscope (Leica) equipped with a 20× objective (NA, 1.0) and a CCD camera for differential interference contrast images. Time frame acquisitions from 314 ms to 1.24 s (with one to six line averaging) were used. No background subtraction or other manipulations were applied to digitized Ca$^{2+}$ signal images that are reported as raw data. Neurons and astrocytes were distinguished on the basis of the distinct kinetics of their Ca$^{2+}$ response to a stimulation with high K$^+$ extracellular solution (40 mM) obtained by isosmotic replacement of Na$^+$ with K$^+$, applied at the end of the recording session in the presence of 1 µM TTX. Due to the lack of voltage-dependent Ca$^{2+}$ channels in astrocytes, the Ca$^{2+}$ elevation in these cells upon high K$^+$ stimulation occurs with a delay of several seconds with respect to the response in neurons, and appears to be mediated by glutamate release from depolarizing neurons. The onset of the slow Ca$^{2+}$ elevation in astrocytes was determined on the basis of a threshold criterion. The onset was identified by the change in $\Delta F/F_0$ that should be more than two standard deviations over the average baseline and remained above this value in the successive frames for at least 2 s (two to six frames, depending on the frame acquisition rate).
Drugs
TTX (1 µM, Sigma-Aldrich, Milan, Italy), 4-AP (100 µM, Ascent Scientific Avonmouth, Bristol, U.K.) were bath applied. NMDA (1 mM; Sigma-Aldrich, Milan, Italy), IL-1β (1 µg/mL R&D System Minneapolis, USA), and HMGB1 (1 µM, HMGBiotech, Milano, Italy) were pressure applied through a glass-pipette by a PDES Picospritzer (NPI, Tamm, Germany).

Data analysis
The Ca²⁺ signal is reported as ΔF/F₀, where F₀ is the baseline fluorescence. Data are shown as mean ± standard error of the mean (S.E.M.). Unless stated otherwise, the Student t-test was used, with p values ≤0.05 taken as statistically significant.

RESULTS
IL-1β and HMGB1 favour ictal discharge generation
Picrotoxin/zero-Mg²⁺ entorhinal cortex slice model.
The change in the cytosolic Ca²⁺ signal is a useful tool to study seizure-like, ictal discharges (IDs) in neuronal ensembles since it reflects faithfully the action potential bursts that characterize the epileptic discharges in individual neurons (Aoki, Tajima et al. 2008; Carmignoto and Gomez-Gonzalo 2010). To start to investigate a possible role of inflammatory agents such as IL-1β and HMGB1 in epileptogenesis, we loaded entorhinal cortex (EC) slice preparations from young rats with the Ca²⁺ indicator Oregon Green BAPTA1 (OGB1) and we evoked epileptiform activitie by slice perfusion with the GABA A receptor antagonist picrotoxin in low extracellular Mg²⁺. We found that after the onset of the picrotoxin/low Mg²⁺ perfusion, the first ID occurred with a significantly shorter latency in slices pretreated with IL-1β (applied locally for 15 minutes through an IL-1 b containing glass pipette; see methods) with respect to slices treated with saline applications (Fig. 1B). Furthermore, ID frequency was also increased in IL-1b-treated with respect to saline-treated slices. Differently from IL-1b, HMGB1 affected neither the ID duration nor the latency of the first ID. However, the ID frequency was significantly increased (Fig. 1B).

Focal seizure model.
We next asked whether IL-1b and HMGB1 can also affect the generation of a focal ID. To this aim, we used our recently developed entorhinal cortex (EC) slice model in which focal IDs were reproducibly generated at a restricted site by perfusing the slice with 100 mM 4-aminopyridine (4-AP) and 0.5 mM Mg²⁺, and stimulating a small number of neurons with pressure pulses applied to an NMDA-containing glass pipette. As previously reported {Losi, 2010 #9474;Gomez-Gonzalo, 2010 #9032}, in control, saline treated slices, a double, but not
a single, NMDA pulse triggered a focal ID. The DIC (left) and fluorescence (right) images in Figure 2A shows a representative field in EC layer V-VI, the NMDA- and the IL-1b-containing pipettes. As illustrated by the difference images (Fig. 2B-D) generated by subtracting the fluorescence image captured at basal conditions to that obtained after the NMDA stimulation, a single NMDA pulse induced only a transient Ca²⁺ raise in a limited number of neurons close to the pipette tip, an area that we defined as the focal area (Field A; Fig.2B). In contrast, a double NMDA pulse stimulation evoked a stronger activation of Field A neurons as well as Ca²⁺ elevations in these and the surrounding neurons with the typical pattern of an ID (Fig.2E,F). The ID evoked by a double NMDA pulse was highly reproducible and only one out of fifty single NMDA pulse performed in 16 slices generated an ID within 45 minutes of 4-AP perfusion. We found that if a single NMDA stimulation, per se ineffective, was preceded by IL-1b or HMGB1 applications, a focal ID was evoked in 45 of 90 and 17 of 31 single pulse stimulations, respectively, suggesting that the cytokines can lower the threshold for ID generation (Fig. 2G).

Fig. 1. – IL-1β and HMGB1 effects on ID generation in the 0 Mg/Picrotoxin model. A, Ca²⁺ changes in representative neurons from a rat EC slices perfused with low Mg²⁺/Picrotoxin in presence of saline (upper trace), IL-1β (middle trace) and HMGB1 (lower trace) pulse applications. The pre-treatment with IL-1β reduce the latency time for the first ID, its duration and the ID frequency. HMGB1 application doesn’t influence the first ID, but increases ictal frequency. B, Mean latency and duration of the first ID and ictal and interictal frequency (right) in saline-treated (white bars, n = 11), IL1β-treated (black bars, n = 8) and HMGB1 treated (grey bars, n = 9) slices. *p ≤ 0.05, **p ≤ 0.01
**Fig. 2 - IL-1β and HMGB1 local applications enhance focal ID generation.** (A) DIC (left) and fluorescence (right) images of a cortical region from an EC slice showing the NMDA and the IL-1β pipettes. B,C) Difference images of the neuronal Ca²⁺ increase upon a single ineffective NMDA pulse (B), a double (C) NMDA pulse that successfully evoked a focal ID, and (D) a single NMDA pulse that after IL-1β also evoked a focal ID. E) Ca²⁺ changes in representative neurons of field A (nA) and field B (nB) upon a single, a double NMDA pulses and a single NMDA pulse applied after IL-1β (left) or HMGB1 (right). F) Quantitative evaluation of successful single NMDA pulses in saline-treated (50 pulses, n= 16 experiments) IL-1β (90 single NMDA pulses, n=26 experiments ) and HMGB1 (31 pulses, n=10 experiments).

**Fig. 3 – A focal ID can initiate at the site of IL-1β local applications.** A) Schematic view and neuronal Ca²⁺ changes during a focal ID evoked by a double NMDA pulse. The focal ID arose in field A neurons (nA), close to the NMDA pipette, and then propagated to neurons in B (nB). B) In the same slice, after IL-1β applications (red spot in the inset), a focal ID was evoked by a single NMDA pulse. The Ca²⁺ change that marked the onset of the focal ID occurred first in neurons from the site of IL-1β applications and then it propagated to neurons from the site of the single NMDA pulse with a delay of about 20 s. Similar results were observed in 4 out of 26 IDs.
In a few IL-1β experiments (4 out of 26) we also noted that the ID was not generated, as usually, at the original site of NMDA stimulation, but rather at the site where IL-1β was applied (Fig. 3). According to the Ca²⁺ signal change in these experiments, the focal ID initiated, indeed, in neurons from the IL-1β site and then it spread to neurons from the NMDA stimulation site (Fig. 3B).

**The responsiveness of neurons to NMDA pulse increases after IL-1β and HMGB1 local applications**

We then asked whether cytokines could lower the threshold for focal ID generation by enhancing the response of neurons to NMDA stimulation. We measured the number of activated neurons and astrocytes as well as the amplitude of the Ca²⁺ change in these cells in response to a single NMDA pulse that was preceded by either saline or IL-1β (or HMGB1).

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**Fig 4** — *The Ca²⁺ elevations in neurons upon the single NMDA pulse stimulation was amplified following IL-1β and HMGB1 applications.* A, representative Ca²⁺ changes in neurons and astrocytes in field A evoked by a single NMDA pulse in the absence (left traces) and presence (right traces) of IL-1β. B, Bar histograms reporting the significant increase in the responsiveness of neurons and astrocytes to a single NMDA pulse applied after IL-1β (black bars, n = 12 slices, 614 neurons and 356 astrocytes, *p < 0.05; **p < 0.01) or HMGB1 (gray bars, n = 6 slices, 321 neurons and 115 astrocytes).
applications. Since in this latter case the single NMDA stimulation induced a focal ID, we restricted the analysis to the initial phase of the response to NMDA, i.e., the time interval between the NMDA pulse and the Ca\(^{2+}\) rise in neurons surrounding the focus that marked the ID onset (Fig. 4A) (Gomez-Gonzalo, 2010 #9032; Losi, 2010 #9474). As reported in the bar histograms of Fig. 4B, both Ca\(^{2+}\) elevation amplitude (dF/F0) and the number of neurons and astrocytes responsive to a single NMDA pulse were increased significantly after IL-1b and HMGB1 applications.

**IL-1b and HMGB1 action depends on synaptic transmission**

To clarify whether IL-1b and HMGB1 lowered the ID threshold by either affecting synaptic transmission or enhancing the direct response of neurons to NMDA, we performed experiments in the presence of 4 mM tetrodotoxin (TTX). In these experiments the amplitude of the Ca\(^{2+}\) change and the number of neurons activated by five successive single NMDA pulses (applied every two minutes) were measured before and after saline, IL-1b or HMGB1 pulses (applied every 20 s). We found that when synaptic transmission was blocked by TTX, both IL-1b and HMGB1 failed to enhance the NMDA-mediated Ca\(^{2+}\) response of neurons, suggesting that to lower the ID threshold the two cytokines does not act directly on the NMDA receptor activation. However, if TTX was applied to a brain slice that already experienced repetitive IDs, HMGB1, but not IL-1b, increased the responsiveness of neurons to a single NMDA pulse, in terms of both of Ca\(^{2+}\) elevation amplitude and number of activated neurons.

Fig. 5—IL-1b and HMGB1 enhanced NMDA-mediated Ca\(^{2+}\) activation of neurons through two distinct mechanisms. A,B) Mean Ca\(^{2+}\) change amplitude (A) and responsive neurons (B) after IL1b (black bars; n = 9, 375 neurons), or HMGB1 (gray bars; n = 5, 367 neurons) with the respect to controls (dashed line) in EC slices that did not experienced any IDs (-IDs) or experienced a sustained epileptic activity (+IDs). * P < 0.05. The white bars expressed the values obtained after saline applications (n = 3, 289 neurons) expressed with respect to the values obtained in control untreated slices.
DISCUSSION

The role of inflammation in epilepsy and epileptogenesis is supported by different observations, in experimental models and in human temporal lobe epilepsy (TLE) (reviewed by Vezzani and Friedman 2011). Astrocytes represent a major source of pro-inflammatory molecules in the brain: they can secrete and sense a large variety of cytokines and chemokines and therefore actively contribute to the inflammatory status of the brain (Aronica, Ravizza et al. 2012).

The focal ictal discharge is an episode of synchronous activity of neurons which is largely influenced by Ca\(^{2+}\) activity in the astrocytes (Gomez-Gonzalo, Losi et al. 2010; Losi, Cammarota et al. 2010). In the present study 2 different models of focal seizures in acute brain slices were used to assess the role of inflammation in triggering epileptiform activity, especially with respect to the involvement of IL-1β and HMGB1. In the first model, EC slices are perfused with a solution containing 0 Mg\(^{2+}\) and Picrotoxin, which leads to spontaneous epileptic activity arising from unpredictable foci (Demir, Haberly et al. 1998). In the second model EC slices are exposed to 0,5mM Mg and 100µM 4AP before receiving focal NMDA application which triggers the ictal discharge (ID) activity (Gomez-Gonzalo, Losi et al. 2010; Losi, Cammarota et al. 2010). The latter model offers the unique opportunity to repetitively evoke an ID from the same restricted site, representing a powerful approach to analyze the contribution of different molecules to the onset of the ictal activity. Here we report that in both models of focal seizures (0 Mg\(^{2+}\)/picrotoxin and 0,5 Mg\(^{2+}\)/4AP/NMDA) IL-1β and HMGB1 were shown to increase the excitability of the network.

IL-1β and HMGB1 enhance ictal discharge generation

We show here that IL-1β and HMGB1 increase the excitability of neurons in EC slices. This fast proconvulsant effect as has been recently reported in an in vivo mouse model in which IL-1β and HMGB1 were effective in enhancing the seizure activity through a mechanism that involves the phosphorylation of the NMDA receptor subunit NR2B (Balosso, Maroso et al. 2008; Maroso, Balosso et al. 2010). Other data obtained from cultured hippocampal neurons support the role of IL-1β in the modulation of activity of NMDA channels by their phosphorylation by Src kinases (Viviani, Bartesaghi et al. 2003). Although we could not show any increase in neuronal calcium response to NMDA after IL-1β application, we can not exclude that phosphorylation is occurring. Interestingly, in our experimental model the IL-1β application not only the reduced the threshold of fID generation, but also shifted the onset of the ictal activity from the NMDA application site to the IL-1β application site. This observation suggests that the cells that had a direct contact with IL-1β are more susceptible to ex-
citation. Since we are investigating an inflammatory pathway, on a cautionary note we also have to consider the inflammatory component triggered by the procedure used to prepare the acute slices. It is known that microglia gets quickly activated during this procedure and that could contribute to the inflammatory status of the slices. Moreover microglia actively participates in the modulation of excitatory neurotransmission by recruiting astrocytes via ATP release (Pascual, Ben Achour et al. 2012).

**Astrocytes Ca\(^{2+}\) elevation**

Astrocytes have been showed to have a pivotal role in the generation of focal ictal discharge (fID) in rat EC slices: they contribute to the neuronal synchronization by signaling back to the neurons in a Ca\(^{2+}\) dependent (Gomez-Gonzalo, Losi et al. 2010). As recently shown (Gomez-Gonzalo, Losi et al. 2010), blocking the Ca\(^{2+}\) signaling of the astrocytes in a focal seizure model in slices increases the threshold of fID, whereas enhancing Ca\(^{2+}\) signaling decreases it, facilitating the onset of fID. In the present study, we saw a strong increase in Ca\(^{2+}\) in astrocytes after the application of NMDA in HMGB1 pretreated slices. Moreover blocking the synaptic transmission with TTX prevented Ca\(^{2+}\) elevation in astrocytes. This evidence suggests that the Ca\(^{2+}\) elevation in astrocytes depends on the synaptic activity and that the proconvulsant effects of IL1β and HMGB1 may reflect a regulation of the neuron-glia communication. Indeed astrocytes have been shown to express a large variety of glutamate receptors, metabotropic an ionotropic (Schipke, Ohlemeyer et al. 2001; Lalo, Pankratov et al. 2006; Verkhratsky and Kirchhoff 2007; D’Antoni, Berretta et al. 2008; Lundborg, Westerlund et al. 2011). Furthermore, increasing number of studies have showed the role of the astrocytic calcium signaling in glia-neuron communication (for rew.: {Zorec, 2012 #580}). The release of gliotransmitters, such as glutamate, ATP or D-serine, can modulate signaling among neurons, leading to synaptic modulation (Yang, Ge et al. 2003; Perea and Araque 2007). Here we propose that the inflammatory molecules IL-1β and HMGB1 increase the excitability of the network, leading to astrocytes Ca\(^{2+}\) elevation which in turn causes the synchronization of more neurons leading to a decreased threshold for fID.

**HMGB1 is pro-ictogenic only after the tissue had experienced a fID**

In our experiments HMGB1 was effective in decreasing the threshold of the fID only after the tissue had experienced one or more episodes of fID. The reason for this has to be searched in the physiological modifications that occur following the fID. The presence of extranuclear staining for HMGB1 in astrocytes and occasionally in neurons after repetitive fID episodes (fig. 6) suggests a translocation of this molecule from the nucleus to the cytoplasm and a possible release in the extracellular space. In a previous study, we showed that
cultured astrocytes can indeed release HMGB1 following IL-1β stimulation (Zurolo, Iyer et al. 2011). Astrocytes, microglia and neurons (expressing TLR4) may respond to HMGB1 stimulation with production of several pro-epileptogenic inflammatory mediators (Andersson, Ronnback et al. 2005; Kim, Choi et al. 2006; Pedrazzi, Patrone et al. 2007) providing a positive feedback loop that amplifies neuronal excitability.

If we consider the acute slices as inflammation primed tissue on account of the slice preparation procedure, it could be speculated that the inflammatory receptors/pathways are somehow already receptive to a stimulatory trigger. Under these conditions, a focal ictal discharge episode could induce translocation of HMGB1. Moreover, its primary receptor, TLR4 could be recruited following an ictal discharge episode. It should be noted that since the time scale of our experiments precludes de novo synthesis of this receptor, an alternative mechanism could involve the accelerated mobilization of pre-synthesised receptor from the Golgi to the membrane (see (Saitoh and Miyake 2009; McGettrick and O'Neill 2010).

Significance: In the present study we showed that IL-1β and HMGB1 induced calcium increase in astrocytes that could promote increased synchronization of the neurons. This enables the neurons to reach the threshold of onset of fID with a less intense stimulus. These findings demonstrate that both IL-1β and HMGB1 can rapidly lower fID threshold and raise the possibility that targeting these inflammatory pathways may represent an effective therapeutic strategy to prevent seizures.

REFERENCES


