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CYSTEINYL LEUKOTRIENE RECEPTOR (CYSLT) ANTAGONISTS DECREASE PENTYLENETETRAZOL-INDUCED SEIZURES AND BLOOD–BRAIN BARRIER DYSFUNCTION

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Abstract—Current evidence suggests that inflammation plays a role in the pathophysiology of seizures. In line with this view, selected pro-inflammatory arachidonic acid derivatives have been reported to facilitate seizures. Kainate-induced seizures are accompanied by leukotriene formation, and are reduced by inhibitors of LOX/COX pathway. Moreover, LTD_4 receptor blockade and LTD_4 synthesis inhibition suppress pentylenetetrazol (PTZ)-induced kindling and pilocarpine-induced recurrent seizures. Although there is convincing evidence supporting that blood–brain-barrier (BBB) dysfunction facilitates seizures, no study has investigated whether the anticonvulsant effect of montelukast is associated with its ability to maintain BBB integrity. In this study we investigated whether montelukast and other CysLT receptor antagonists decrease PTZ-induced seizures, as well as whether these antagonists preserve BBB during PTZ-induced seizures. Adult male albino Swiss mice were stereotaxically implanted with a cannula into the right lateral ventricle, and two electrodes were placed over the parietal cortex along with a ground lead positioned over the nasal sinus for electroencephalography (EEG) recording. The effects of montelukast (0.03 or 0.3 μmol/1 μL, i.c.v.), pranlukast (1 or 3 μmol/1 μL, i.c.v.), Bay u-9773 (0.3, 3 or 30 nmol/1 μL, i.c.v.), in the presence or absence of the agonist LTD_4 (0.2, 2, 6 or 20 pmol/1 μL, i.c.v.), on PTZ (1.8 μmol/2 μL)-induced seizures and BBB permeability disruption were determined. The animals were injected with the antagonists, agonist or vehicle 30 min before PTZ, and monitored for additional 30 min for the appearance of seizures by electrographic and behavioral methods. BBB permeability was assessed by sodium fluorescein method and by confocal microscopy for CD45 and IgG immunoreactivity. Bay-u9973 (3 and 30 nmol), montelukast (0.03 and 0.3 μmol) and pranlukast (1 and 3 μmol), increased the latency to generalized seizures and decreased the mean amplitude of EEG recordings during seizures. LTD_4 (0.2 and 2 pmol) reverted the anticonvulsant effect of montelukast (0.3 μmol). Montelukast (0.03 and 0.3 μmol) prevented PTZ-induced BBB disruption, an effect that was reversed by LTD_4 at the dose of 6 pmol, but not at the doses 0.2 and 2 pmol. Moreover, the doses of LTD_4 (0.2 and 2 pmol) that reverted the effect of montelukast on seizures did not alter montelukast-induced protection of BBB, dissociating BBB protection and anticonvulsant activity. Confocal microscopy analysis revealed that 1. PTZ increased the number of CD45+ and double-immunofluorescence staining for CD45 and IgG cells in the cerebral cortex, indicating BBB leakage with leukocyte infiltration; 2. while LTD_4 (6 pmol) potentiated, montelukast decreased the effect of PTZ on leukocyte migration and BBB, assessed by double-immunofluorescence staining for CD45 and IgG cells in the cannulated hemisphere. Our data do not allow us ruling out that mechanisms unrelated and related to BBB protection may co-exist, resulting in decreased seizure susceptibility by montelukast. Notwithstanding, they suggest that CysLT1 receptors may be a suitable target for anticonvulsant development.

Key words: leukotrienes, pentylenetetrazol, seizure, CysLT1R, montelukast.

INTRODUCTION

Growing clinical and experimental evidence suggests that inflammation plays an important role in the pathophysiology of epilepsy (Vezzani, 2005; Vezzani and Friedman, 2011; Walker and Sills, 2012; Jimenez-Mateos and Henshall, 2013). In line with this view, selected pro-inflammatory cytokines decrease seizure threshold and/or prolong it. For instance, interleukin (IL)-1β receptor antagonist reduces seizures in various experimental models and preventing IL-1β increase blocks kindling development in rats (Balosso et al., 2008; Maroso et al., 2011). Moreover, mice expressing high levels of IL-6 present severe neurologic disease

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Abbreviations: ANOVA, analysis of variance; BBB, blood–brain-barrier; DMSO, dimethyl sulfoxide; EEG, electroencephalography; PBS, phosphate-buffered saline; PGE2, prostaglandin E2; PTZ, pentylenetetrazol; TCA, trichloroacetic acid; TJ, tight junctions.

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characterized by runting, tremor, ataxia, and seizures, supporting the view that cerebral overexpression of IL-6 may precipitate convulsions (Campbell et al., 1993). Interestingly, Nur and colleagues (2012) have shown an association between interleukin-6 single-nucleotide polymorphisms and febrile seizures in children. Pro-inflammatory effects of cytokines include increased expression of rate-limiting enzymes of metabolic pathways involved in the synthesis of inflammatory mediators, such as cyclooxigenase-2, which is the rate-limiting enzyme in the prostaglandin and thromboxane synthesis pathway (Vane et al., 1998). In fact, increasing evidence suggests that COX-2-derived eicosanoids play a role in seizures and epilepsy (Vezzani, 2005; Oliveira et al., 2008), since pharmacological treatments that increase COX-2 expression, such as IL-1β facilitate bicuculline- and pentylenetetrazol (PTZ)-induced seizures (Vezzani et al., 2000; Akarsu et al., 2006). Accordingly, Oliveira and colleagues (2008) have shown that prostaglandin E2 (PGE2) facilitates PTZ-induced seizures, and that selective antagonists for EP1, EP3 and EP4 receptors increase the latency to clonic and generalized tonic–clonic seizures induced by this GABAa antagonist in rats. Also in line with this view, Salvadori and colleagues (2012) have shown that PGE2 may precipitate and exacerbate methylmalonate-induced seizures.

Since EP1, EP3 and EP4 receptor antagonists were more effective than COX-2 inhibitors to decrease PTZ-induced seizures, we have hypothesized that the 5-LOX pathway and its products, leukotrienes, might be involved in the development of seizures. In fact, lipoxygenase pathways, including the 5, 12, 15-LOX pathway of polyunsaturated fatty acid metabolism have been identified either by *in vitro* or by ex vivo studies in cerebral tissue (Simmet and Tippler, 1990; Hedi and Norbert, 2004) and implicated in the pathogenesis of neurodegenerative diseases such as Parkinson's Disease (Chou et al., 2013) and epilepsy (Rehni and Singh, 2011). In the CNS, 5-LOX is expressed in neurons, astrocytes and oligodendrocytes in various regions of the brain, with the most prominent expression in the cortex, hippocampus and the cerebellum (Manev et al., 1998; Hedi and Norbert, 2004; Brock, 2005). The main stable products of the 5-LOX pathway, LTC_4 and LTD_4 are potent chemotactic factors that increase white blood cells' diapedesis (Lewis et al., 1990; Fabene et al., 2008) and vascular permeability, including of the blood–brain barrier (BBB) (Wang et al., 2006). Moreover, it has been shown that kainate-induced seizures are accompanied by time-dependent leukotriene formation. Both leukotriene production and seizures are reduced by phenidone and BW755C, dual inhibitors of LOX/COX pathway, suggesting that leukotrienes play a role in seizures (Simmet and Tippler, 1990; Kim et al., 2000). In line with this view, Rehni and Singh (2011) have shown that montelukast, a CysLT1 receptor inverse agonist (Dupré et al., 2004), and 1,2,3,4, tetrahydroisoquinoline, a LTD_4 synthetic pathway inhibitor, dose-dependently suppress the development of kindled seizures, as well as pilocarpine-induced spontaneous recurrent seizures.

Recent studies have described an increased number of spontaneous seizures in animals with more prominent BBB dysfunction following pilocarpine-induced *status epilepticus*, suggesting a potential direct role for BBB dysfunction in epileptogenesis (Oby and Janigro, 2006; van Vliet et al., 2007; Badawy et al., 2012). Indeed, there is clinical and experimental evidence suggesting that BBB dysfunction triggers corollary events that may lead to epilepsy (Friedman et al., 2009; Friedman, 2011; Heinemann et al., 2012). It has also been shown that montelukast prevents BBB disruption in a rat model of traumatic brain injury (Biber et al., 2009) and focal cerebral ischemia (Zhao et al., 2011). Moreover, in experimental autoimmune encephalomyelitis animals, a model with pathological similarities to multiple sclerosis, montelukast effectively blocks the CNS infiltration of inflammatory cells (Wang et al., 2011). A similar protective effect was observed in a model of bacterial meningitis by *Cryptococcus neoformans*, in which treatment with montelukast prevented penetration of bacteria in to the brain (Kim, 2009). However, no study has systematically investigated whether the anticonvulsant effect of the montelukast is associated with maintenance of BBB integrity. Therefore, in this study we investigated whether montelukast and other CysLT receptor antagonists decrease seizures induced by PTZ, as well as whether these antagonists preserve BBB during PTZ-induced seizures.

EXPERIMENTAL PROCEDURES

Reagents

Pentylenetetrazol and sodium fluorescein were purchased from Sigma (St. Louis, MO, USA). Montelukast (CysLT1R inverse agonist) and pranlukast (CysLT1R selective antagonist), Bay-u9973 (CysLT1/2R dual antagonist) and LTD_4 (CysLTR agonist) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-CD45 antibodies were purchased from Biolegend, San Diego, CA, USA and anti-IgG antibodies from Invitrogen, Carlsbad, CA, USA. Alexa Fluor 488 and Alexa Fluor 546 secondary antibodies were purchased from Invitrogen, Carlsbad, CA. Montelukast was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in apyrogenic sterile saline, in such a way that DMSO concentration did not exceed 0.5%. Pranlukast was dissolved in sterile apyrogenic saline. Bay-u9973 and LTD_4, supplied as a solution in ethanol, were diluted in sterile apyrogenic saline in such a way that ethanol concentration did not exceed 1%. All other reagents were of analytical grade and purchased from local suppliers.

Animals

Adult male Swiss mice (from the animal house of the Federal University of Santa Maria, Santa Maria, Brazil) weighing 25 ± 3.5 g were used. The animals were kept under standard conditions of temperature and humidity (12-h light/dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to food and water. Animals were

habituated to laboratory conditions 24 h before each experiment. Experiments were performed between 09:00 and 17:00 hours. The experimental protocols were approved by Institutional Animal Ethics Committee and conducted in accordance with policies of National Institutes of Health Guide for the care and use Laboratory Animals (N° 69/2010). Experimental protocols were designed that aimed to keep the number of animals used to a minimum, as well as their suffering (n = 4–7 per group).

Surgery

Animals were anesthetized with ketamine/xylazine (50/5 mg/kg, i.p.) and a cannula was inserted into the right lateral ventricle (coordinates relative to bregma: AP 0 mm; L +0.9 mm, V = −1.6 mm) (Paxinos and Franklin, 2001) with a stereotaxic device. Two electrodes were placed over the parietal cortex along with a ground lead positioned over the nasal sinus. The electrodes and the cannula were fixed to the skull with dental acrylic cement. Ceftriaxone (100 mg/kg, i.p.) and meloxicam (2 mg/kg, i.p.) were administrated immediately before the surgical procedure. The experiments were performed 7 days after surgery.

Experimental protocol and electroencephalography (EEG) recording

The effect of the CysLTR negative modulators on PTZ (1.8 μmol/2 μL i.c.v.)-induced seizures was investigated by injecting the animals with montelukast (0.03 or 0.3 μmol/1 μL, i.c.v.), pranlukast (1 or 3 μmol/1 μL, i.c.v.), the partial agonist Bay u-9773 (0.3, 3 or 30 nmol/1 μL, i.c.v.) or with its respective vehicle (0.5% DMSO in saline or 0.5% ethanol in saline). The effect of LTD_4 (0.2 nmol/1 μL or 2 nmol/1 μL, i.c.v.) or its respective vehicle (0.5% ethanol in saline) on PTZ-induced seizures was also investigated. Drug doses and time between injections were selected based on previous pilot experiments or dose-effect curves. All i.c.v. injections were performed over a 1-min period by using a 30-gauge needle protruding 1 mm below the guide cannula connected to a Hamilton syringe by PE10 tubing. An additional minute was allowed before removal of the needle to avoid backflow of drug through the cannula. Briefly, the animals were allowed to habituate to a plexiglas cage (25 cm × 25 cm × 60 cm) for at least 20 min before the EEG recordings. The mouse was then connected to the leadsocket in a swivel inside a Faraday's cage. Routinely, a 10-min baseline recording was obtained to establish an adequate control period. The animals were then injected with the antagonist, agonist or vehicle, 30 min before the administration of PTZ (1.8 nmol/2 μL, i.c.v.). After the injection of PTZ, the animals were monitored for additional 30 min for the appearance of seizures, by electrographic and behavioral methods. The behavior of the animals was followed up, and EEG was concomitantly recorded using a digital encephalographer (Neuromap EQSA260, Neuromap LTDA, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, bandpass), digitalized (sampling rate 256 Hz) and stored in a PC for off-line analysis. EEG recordings were visually analyzed for the appearance of seizure activity. This ictal activity was confirmed by visual observation of behavior. Seizures were defined by the occurrence of episodes consisting of the following alterations in the recording leads (McColl et al., 2003): isolated sharp waves ($\geqslant$1.5 × baseline); multiple sharp waves ($\geqslant$2 × baseline) in brief spindle episodes ($\geqslant$1 s, $\geqslant$5 s); multiple sharp waves ($\geqslant$2 × baseline) in long spindle episodes ($\geqslant$5 s); spikes ($\geqslant$2 × baseline) plus slow waves; multispikes ($\geqslant$2 × baseline, $\geqslant$3 spikes/complex) plus slow waves; major seizure (repetitive spikes plus slow waves obliterating background rhythm, $\geqslant$5 s). Rhythmic scratching of the electrode headset by the animal rarely caused artifacts. These recordings were easily identified and discarded. In addition, the mean amplitude (in μV) of EEG traces before and after PTZ injection was calculated. Full baseline traces (obtained in the absence of pharmacological treatment) and full traces obtained after the administration of CysLT ligands were used to quantify mean amplitude during the pre-PTZ period. Data were treated as repeated measures in the statistical analysis. Therefore, each mouse was control of itself, correcting for between-subject variability. However, after PTZ injection, only the mean ictal amplitude was considered in the analysis. Specifically, ictal traces were identified and individually quantified during the whole period after PTZ injection (20 min). If the anticonvulsant activity of montelukast, pranlukast and Bay-u9973 were due to an interaction with CysLTR in the brain, these actions should be reverted by the injection of an agonist (LTD_4). We investigated this possibility by injecting the animals with an effective dose of montelukast (0.3 μmol, i.c.v.) and LTD_4, at doses that caused no effect per se on seizures (0.2 or 2 pmol, i.c.v.), 15 min before PTZ (1.8 μmol/2 μL, i.c.v.). Latency to myoclonic jerks and tonic–clonic generalized seizures were recorded. Mean amplitude and duration of seizure episodes were calculated from EEG recordings.

Blood–brain barrier (BBB) permeability assay

BBB permeability was assessed as described by Morrey et al. (2008). The animals were injected with 10-mg sodium fluorescein in 0.1 mL sterile saline (i.p.). Fifteen minutes thereafter they were injected with montelukast (0.03 or 0.3 μmol, i.c.v.) or LTD_4 (0.2 or 2 pmol). Fifteen minutes after CysLT ligand injection, the mice were injected with PTZ (1.8 μmol/2 μL, i.c.v.), and observed during 20 min for the appearance of seizures. At the end of the observation period, the animals were anaesthetized with ketamine/xylazine (50/5 mg/kg, i.p.) for ventricular blood collection (cardiac puncture). The blood was centrifuged and serum was stored at −70 °C until processing. The brain was perfused with 150 ml of 0.9% NaCl to remove all vascular content, weighed, and each cerebral hemisphere was homogenized in 0.5 ml of sterile phosphate-buffered saline (PBS), and stored at −80 °C until processing. Brain and serum samples were sequentially diluted in sterile PBS (1:10 v/v) and in 20% trichloroacetic acid (TCA) (1:10 v/v). Brain samples were centrifuged at 1250g for 5 min, and the resulting

supernatant was diluted in 20% TCA (1:10 v/v). All samples were incubated at −4 °C for 24 h and centrifuged at 10,000*g* for 15 min to remove precipitated protein. The supernatant was removed and diluted in 1 M Tris (pH 10, 1:2 v/v). Fluorescence was measured in a fluorometer (Molecular Devices, Sunnyvale, CA, USA) using an excitation wavelength of 480 nm, and an emission wavelength of 538 nm. A standard curve for fluorescein quantification in the samples was generated by simultaneously analyzing fluorescein solutions prepared in 10% TCA and 0.5 M Tris. The degree of BBB permeability was measured as the percentage (w/v) of sodium fluorescein in a gram of brain tissue per the amount of sodium fluorescein in a milliliter of serum, in both hemispheres (Morrey et al., 2008).

Immunofluorescence

Mice were injected with montelukast (0.3 μmol/1 μL, i.c.v.) or LTD_4 (2 pmol/1 μL, i.c.v.) and, fifteen minutes thereafter, injected with PTZ (1.8 μmol/2 μL, i.c.v,). The animals were transferred to a clear observation cage and observed during 20 min for the appearance of seizures. At the end of the observation period, mice were deeply anesthetized with ketamine/xylazine (50/5 mg/kg, i.p.), and their brains were sequentially perfused with 150 ml of 0.9% NaCl to remove vascular content, and 4% paraformaldehyde. The brains were removed and immersed in sterile 30% sucrose for at least 72 h and 10-μm sections were incubated with anti-CD45 (Biolegend, San Diego, CA, USA), anti-IgG (Invitrogen, Carlsbad, CA) antibodies. Alexa Fluor 488 or Alexa Fluor 546 secondary antibodies (Invitrogen, Carlsbad, CA) were used. After washes with PBS the slides were stained with DAPI for 5 min at room temperature. The slides were analyzed under a laser scanning confocal fluorescence microscope (Olympus FV300, Tokyo, Japan) and number of CD45 and CD45/IgG positive cells were determined using the software ImageJ (NIH, USA).

Statistical analysis

Kolmogorov–Smirnov (KS) test was used to verify data normality. Latency to myoclonic jerks and to tonic–clonic generalized seizures was analyzed by Kruskal–Wallis test followed by Dunn's multiple comparison test, since data distribution was not Gaussian. Data are presented as median and interquartile ranges. Mean amplitude data were analyzed by a repeated measures analysis of variance (ANOVA) followed by Bonferroni's test, and respective data are presented as mean and standard error of the mean. Sodium fluorescein content data were analyzed by a three-way ANOVA (pretreatment by treatment by hemisphere), considering the "hemisphere" factor as a within-subject factor (repeated measures). Data were subjected to log transformation before analysis in order to meet the assumptions for ANOVA. Immunofluorescence staining data were analyzed by an ANOVA, followed by Bonferroni's test. A probability of $p < 0.05$ was considered significant, and F and H values are shown only if $p < 0.05$.

RESULTS

Effect of CysLT antagonists on the latency to PTZ-induced seizures

Fig. 1 shows the effect of Bay-u9973 (0.3, 3 and 30 nmol, i.c.v.) on PTZ-induced seizures (1.8 μmol/2 μL i.c.v.) measured as latency to myoclonic jerks (isolated myoclonic jerk, with concomitant spike activity in the EEG, Fig. 1A); latency to generalized tonic–clonic seizures (Fig. 1B) and mean amplitude in EEG records (Fig. 1G). Generalized seizures appeared in the EEG recordings as the major seizure activity, and were characterized by 2–3 Hz high-amplitude activity. Statistical analysis (Kruskal–Wallis test followed by nonparametric Dunn's multiple comparisons test) revealed that Bay-u9973 increased the latency to generalized seizures [$H(3) = 20.63$; $p < 0.001$, Fig. 1B; $n = 4$–6 per group], but did not alter the latency to myoclonic jerks (Fig. 1A). PTZ-induced seizures were characterized by the occurrence of multi-spikes plus slow waves and major seizure activity. Multi-spikes plus slow waves coincided with myoclonic jerks. After the ictal discharge, postictal EEG suppression and slow waves were observed, correlating with behavioral catalepsy. Statistical analysis (repeated measures ANOVA followed by Bonferroni's test) revealed that Bay-u9773 decreased the mean amplitude (in μV) of EEG traces after PTZ administration in the two recording leads, in a 20-min observation period [$F(3,20) = 4.17$; $p < 0.01$] (Fig. 1G). Representative ictal EEG recordings of animals treated respectively with vehicle and Bay-u9773 are shown in Fig. 1C and E.

Since the non-selective CysLT receptor antagonist Bay-u9773 decreased PTZ-induced seizures, we investigated whether the selective CysLT1R antagonist pranlukast (1 and 3 μmol), and the CysLT1R inverse agonist montelukast (0.03 and 0.3 μmol, i.c.v.) decreased the convulsive episodes elicited by PTZ. Fig. 2 shows the effect of increasing doses of montelukast on the latency to myoclonic jerks, generalized tonic–clonic convulsions and mean amplitude in EEG records. Statistical analysis (Kruskal–Wallis test followed by nonparametric Dunn's multiple comparison test) revealed that montelukast (0.03 and 0.3 μmol) increased the latency to myoclonic episodes (Fig. 2A) [$H(2) = 11.94$; $p < 0.05$; $n = 5$–7 per group] and to generalized seizures (0.3 μmol; Fig. 2B) [$H(2) = 13.08$; $p < 0.05$]. Fig. 2G shows the effect of montelukast (0.03 and 0.3 μmol) on the mean amplitude (in μV) of the electrographic recordings from animals subjected to PTZ-induced seizures. Repeated measures ANOVA followed by Bonferroni's test of EEG mean amplitude data revealed that montelukast decreased the mean amplitude of ictal EEG recordings [$F(2,15) = 5.8$; $p < 0.05$]. Representative ictal EEG recordings of animals treated respectively with vehicle and montelukast are shown in Fig. 2C and E.

The effect of pranlukast on PTZ-induced seizures is shown in Fig. 3. Statistical analysis (Kruskal–Wallis test followed by nonparametric Dunn's multiple comparison test) revealed that pranlukast decreased seizures

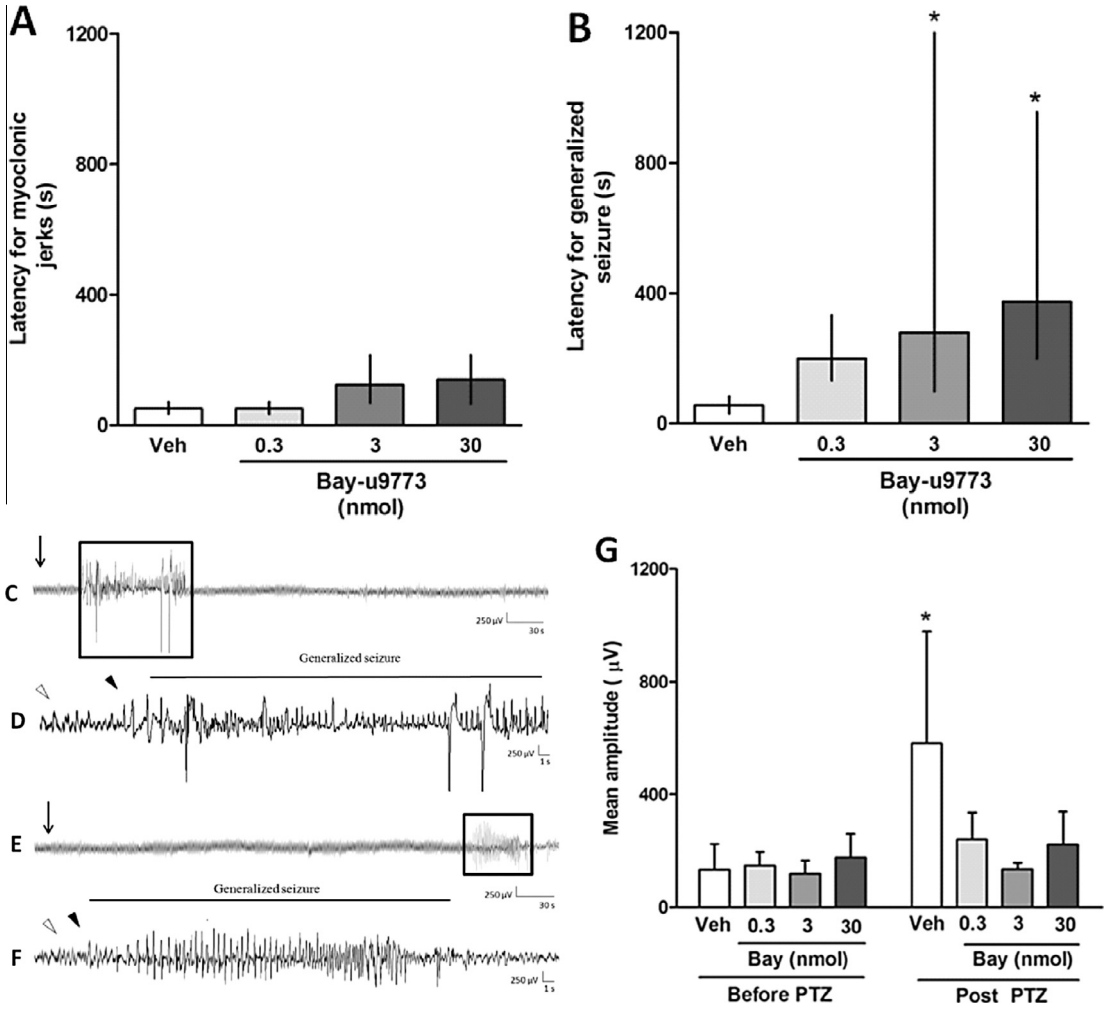

Fig. 1. Effect of Bay-u9973 (Bay) (0.3, 3 and 30 nmol, i.c.v.) on PTZ-induced seizures (1.8 μmol/2 μL, i.c.v.). (A) Latency to myoclonic jerks; (B) Latency to tonic–clonic generalized seizures. Data expressed as median and interquartile range; $^{*}p < 0.05$ vs vehicle. Representative EEG recordings after administration of PTZ (1.8 μmol/2 μL, i.c.v.) in an animal treated with (C) vehicle (0.5% ethanol in saline, i.c.v.) or (E) Bay-u9773 (30 nmol, i.c.v.). The expanded waveforms from the EEG recording outlined by the box in C and E are shown in D and F. The arrow indicates PTZ injection and the black arrowhead indicates the onset of generalized tonic–clonic seizures; G) Mean amplitude of EEG recordings before (basal) and after PTZ. Data expressed as mean and standard deviation; $^{*}p < 0.05$ vs control (before PTZ); (n = 4–6 per group).

susceptibility as measured by a significant increase in the latency to PTZ-induced clonic [H(2) = 8.704; $p < 0.05$; Fig. 3A; n = 4–6 per group] and generalized seizures [H(2) = 10.98; $p < 0.01$; Fig. 3B]. Pranlukast also decreased the mean amplitude (in μV) of ictal EEG recordings [$F(2,15) = 6.05$; $p < 0.01$ (ANOVA followed by Bonferroni's test), Fig. 3G]. Representative ictal EEG recordings of animals treated respectively with vehicle and pranlukast are shown in Fig. 3C and E.

Effect of LTD_4 on the latency to PTZ-induced seizures

Fig. 4 shows the effect of LTD_4 (0.2, 2 and 20 pmol, i.c.v.), a selective CysLT1 receptor agonist, on the latency to clonic (A), generalized tonic–clonic seizures (B) induced by PTZ and mean amplitude EEG recordings (C). Statistical analysis (Kruskal–Wallis test followed by nonparametric Dunn's multiple comparison test) revealed that LTD_4 (20 pmol) facilitated PTZ-induced seizures, since it decreased the latency to both the myoclonic behavioral and EEG manifestation (isolated myoclonic jerk, with concomitant spike activity in the EEG) [H(3) = 13.55; $p < 0.01$; Fig 4A; n = 5–7 per group] and to generalized tonic–clonic seizures [H(3) = 12.36; $p < 0.01$; Fig. 4B]. Quantitative analysis of EEG recordings is shown in Fig. 4G. Statistical analysis (ANOVA followed by Bonferroni's test) revealed that LTD_4 increased the mean amplitude (in μV) of EEG recordings of PTZ-induced seizures [$F(3,15) = 3.7$; $p < 0.01$]. Representative ictal EEG recordings of animals treated respectively with vehicle and LTD_4 are shown in Fig. 4C and E. Pharmacological treatments did not alter the duration of seizure episodes (or total time spent seizing).

LTD_4 reverses the anticonvulsant effect of montelukast

If the anticonvulsant actions of the selective and non-selective CysLTR antagonists were due to a decreased LTD_4-CysLT1 receptor activity, their actions should be

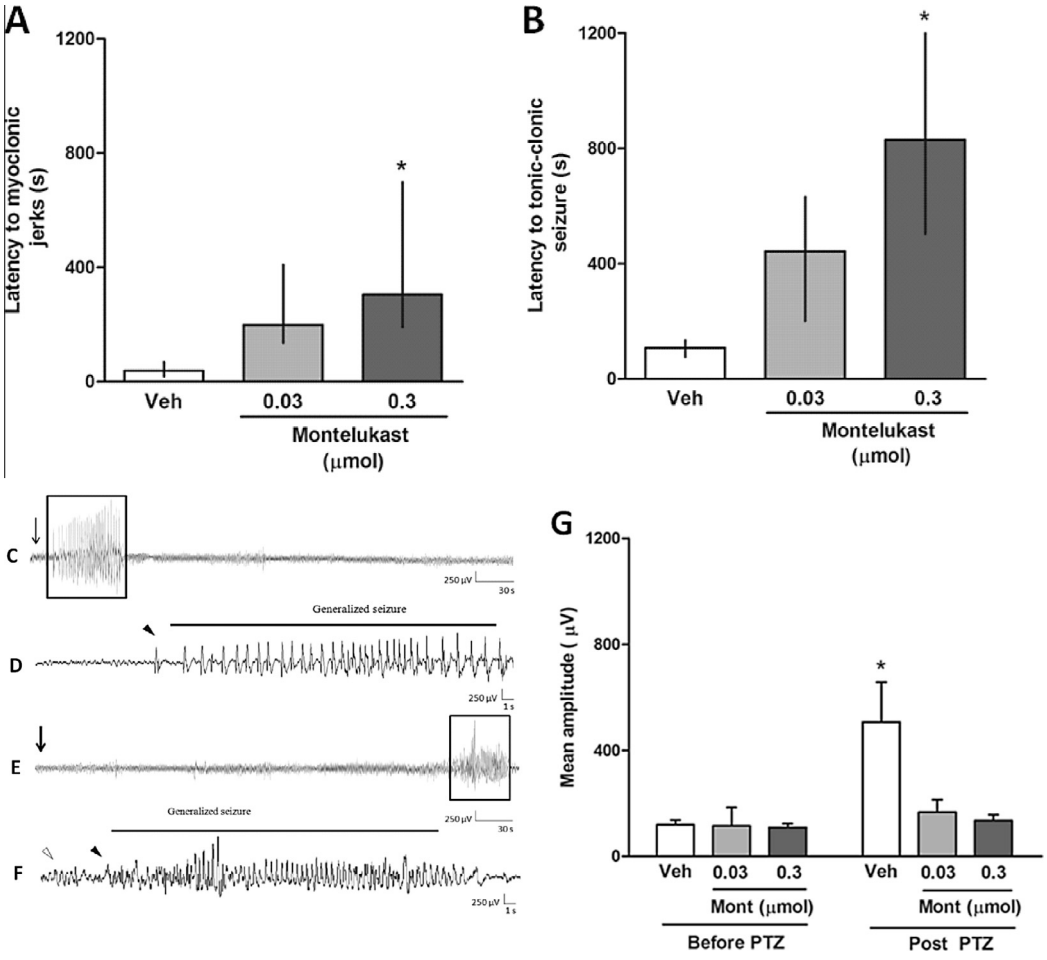

Fig. 2. Effect of montelukast (Mont) (0.03 and 0.3 μmol, i.c.v.) on PTZ-induced seizures (1.8 μmol/2 μL, i.c.v.). (A) Latency to myoclonic jerks; (B) Latency to tonic–clonic generalized seizures. Data expressed as median and interquartile range; $^{*}p < 0.05$ vs vehicle. Representative EEG recordings after administration of PTZ (1.8 μmol/2 μL, i.c.v.) in an animal treated with (C) vehicle (0.5% ethanol in saline, i.c.v.) or (E) montelukast (0.3 μmol, i.c.v.). The expanded waveforms from the EEG recording outlined by the box in C and E are shown in D and F. The arrow indicates PTZ injection and the black arrowhead indicates the onset of generalized tonic–clonic seizures. Data are expressed as median and interquartile range for $n = 5$–6 in each group. $^{*}p < 0.05$ vs vehicle. (G) Mean amplitude of EEG recordings. Data expressed as mean and standard deviation; $^{*}p < 0.05$ vs control (before PTZ); ($n = 5$–7 per group).

reverted by the injection of the respective agonist. We investigated this possibility by injecting the animals with montelukast (0.3 μmol, i.c.v.) or vehicle 30 min before PTZ and LTD_4 (0.2 or 2 pmol, i.c.v.) or vehicle, 15 min before PTZ (1.8 μmol/2 μL, i.c.v.; Fig. 5). Statistical analysis (Kruskal–Wallis test followed by nonparametric Dunn's multiple comparison test) revealed that the administration of LTD_4 (0.2 and 2 pmol, i.c.v.) reversed the anticonvulsant effect of montelukast (0.3 μmol, i.c.v.), measured by both latency to clonic [$H(3) = 15.41$; $p < 0.05$; Fig. 5A; $n = 5$–6 per group] and generalized tonic–clonic seizures [$H(3) = 13.7$; $p < 0.05$; Fig. 5B]. The electrographic pattern of the seizures presented by animals injected with LTD_4 plus montelukast plus PTZ was very similar to that presented by animals treated only with vehicle plus PTZ. Fig. 5C shows that LTD_4 reversed the protective effect of montelukast on mean amplitude (in μV) of ictal EEG recordings [$F(5,32) = 4.17$; $p < 0.01$].

Effect of montelukast and LTD_4 on BBB permeability

Since it has been shown that both seizures and leukotrienes increase BBB permeability (Oby and Janigro, 2006; Wang et al., 2006; Qi et al., 2011), we hypothesized that LTD_4 might increase PTZ-induced seizures by causing BBB disruption. Fig. 6A shows the effect of LTD_4 (0.2 and 2 pmol, i.c.v.) on PTZ (1.8 μmol/2 μL, i.c.v.)-induced increase of BBB permeability by the fluorescein method. Statistical analysis revealed a significant pretreatment (vehicle or LTD_4) by treatment (vehicle or PTZ) by hemisphere interaction [$F(2,24) = 4.09$; $p < 0.05$]. Post hoc analysis (Bonferroni's test) revealed that LTD_4 (2 pmol, i.c.v.) increased the brain:serum fluorescence ratio compared with animals injected with vehicle-PTZ in the contralateral hemisphere. This result may have occurred because of a ceiling effect, i.e. because it is easier to increase BBB permeability where the barrier is not disrupted. Moreover, one might interpret a smaller effect of LTD_4 on the ipsilateral hemisphere because of

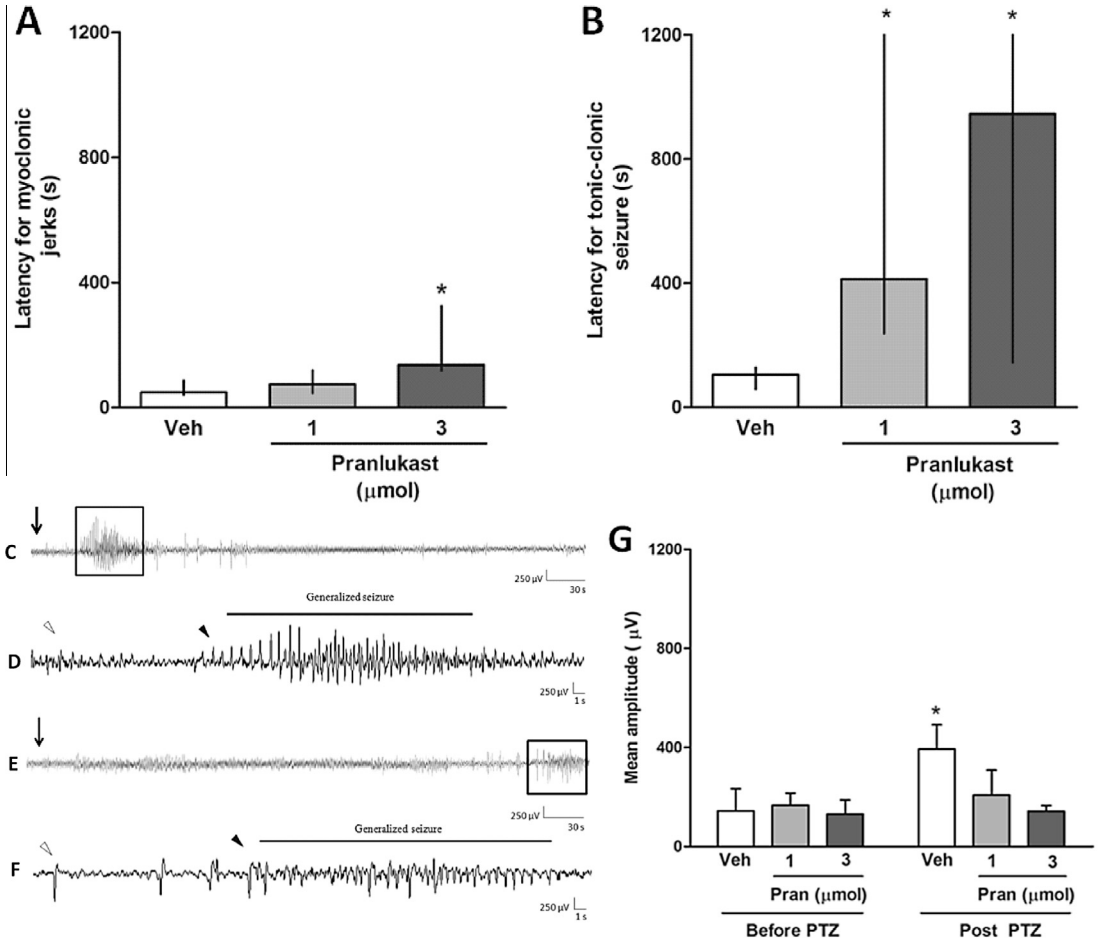

Fig. 3. Effect of pranlukast (1 and 3 μmol, i.c.v.) on PTZ-induced seizures (1.8 μmol/2 μL, i.c.v.). (A) Latency to myoclonic jerks; (B) Latency to generalized tonic–clonic seizures. Data expressed as median and interquartile range; $^{*}p < 0.05$ vs vehicle. Representative EEG recordings after administration of PTZ (1.8 μmol/2 μL, i.c.v.) in an animal treated with (C) vehicle (0.5% ethanol in saline, i.c.v.) or (E) pranlukast (3 μmol, i.c.v.). The expanded waveforms from the EEG recording outlined by the box in C and E are shown in D and F. The arrow indicates PTZ injection and the black arrowhead indicates the onset of generalized tonic–clonic seizures. Data expressed as median and interquartile range for $n = 4$–7 in each group. $^{*}p < 0.05$ vs vehicle; (G) Mean amplitude of EEG recordings in PTZ-induced seizures. Data expressed as mean and standard deviation; $^{*}p < 0.05$ vs control (before PTZ); ($n = 4$–6 per group).

an already established local inflammatory reaction, due to cannula placement.

Fig. 6B shows the effect of montelukast (0.03 and 0.3 μmol, i.c.v.) on PTZ-induced BBB disruption. Statistical analysis (a three-way ANOVA followed by Bonferroni *T*-test) revealed that injection of CysLT receptor inverse agonist at the dose of 0.3 μmol prevented PTZ-induced BBB disruption [$F(2,24) = 28.05$; $p < 0.001$; $n = 6$–7 per group] and a significant effect of hemisphere [$F(1,24) = 95.35$; $p < 0.001$]. This was due to the presence of the cannula in this hemisphere, which increased local BBB disruption.

LTD_4 reverts the protective effect of montelukast on the BBB permeability

Since LTD_4 reversed the anticonvulsant effect of montelukast (measured as latency to myoclonic and generalized seizures), we investigated whether LTD_4 (0.2 and 2 pmol) reversed the protective effect of montelukast on the BBB permeability. However, since LTD_4 at the doses of 0.2 and 2 pmol did not reverse the protective effect of montelukast on BBB (data not shown), we tested whether a high dose of LTD_4 (6 pmol) prevented this effect of montelukast. Fig. 7 shows that LTD_4, at dose of 6 pmol, reverted the protective effect of montelukast on PTZ-induced BBB disruption [significant interaction: $F(1,32) = 16.82$, $p < 0.001$; $n = 4$–5 per group]. Fig. 8 shows a plot of individual ranked data of brain:serum fluorescence ratio of the ipsilateral hemisphere against respective latency to generalized seizure of the experiments shown in Figs. 6 and 7, as well as data not shown from experiments with LTD_4 0.2 and 2 pmol. Spearman correlation analysis of data revealed a highly significant correlation ($r = -0.841$, $p < 0.0001$) between these parameters, on an individual basis.

Effect of LTD_4 and montelukast on the number of infiltrating CD45 and CD45/IgG positive leukocytes in the cerebral cortex

Since BBB permeability experiments with fluorescein showed that LTD_4 increased and montelukast

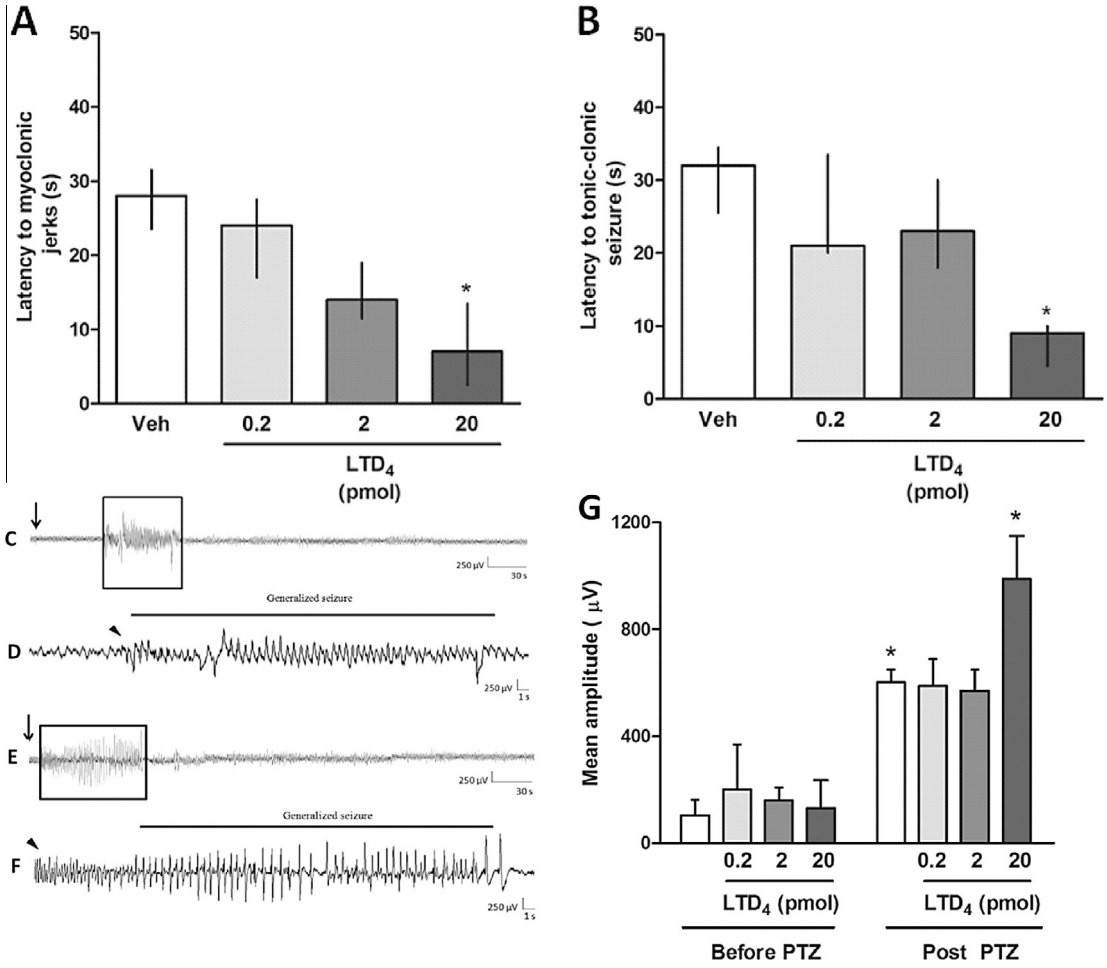

Fig. 4. Effect of LTD_4 (0.2, 2 and 20 pmol/μL i.c.v.) on PTZ-induced seizures (1.8 μmol/2 μL, i.c.v.). (A) Latency to myoclonic jerks; (B) Latency to generalized tonic–clonic seizures. Data expressed as median and interquartile range; $^*p < 0.05$ vs vehicle. Representative EEG recordings after administration of PTZ (1.8 μmol/2 μL, i.c.v.) in an animal treated with (C) vehicle (0.5% ethanol in saline, i.c.v.) or (E) LTD_4 (20 pmol, i.c.v.). The expanded waveforms from the EEG recording outlined by the boxes in C and E are shown in D and F. The arrow indicates PTZ administration; the black arrowhead indicates the onset of generalized tonic–clonic seizures; (G) Mean amplitude of EEG recordings on PTZ-induced seizure. Data expressed as mean and standard deviation; $^*p < 0.05$ vs control (before PTZ); (n = 5–7 per group).

decreased BBB leakage, we decided to investigate whether CD45-positive cells increased in the cerebral cortex (that had the cannula implanted), as a measure of leukocyte infiltration. In addition, double staining for CD45 and IgG was performed to label leukocytes and IgG in the cortical parenchyma. Fig. 9A–G shows representative images of CD45+ cells in the cerebral cortex. Consistent with BBB permeability experiments, quantitative immunohistochemistry analysis showed a significant reduction in PTZ-induced leukocyte infiltration in mice treated with montelukast, when compared to those that received vehicle. Moreover, LTD_4 significantly increased the number of CD45+ cells in the cerebral cortex, confirming that LTD_4 facilitates PTZ-induced BBB rupture (Fig. 9H) [$F(6,21) = 20.67$; $p < 0.001$]. Quantitative analysis of double labeled CD45/IgG cells (Fig. 10A–G) by confocal microscopy also revealed, as expected, that montelukast decreased leukocyte infiltration and IgG spreading [$F(6,21) = 15.99$; $p < 0.001$] (Fig. 10H). No difference between groups was found in the hemisphere without cannula (data not shown).

DISCUSSION

In the current study we showed that a selective CysLT1 receptor antagonist and a CysLT1 receptor inverse agonist decrease PTZ-induced seizures in mice. In addition, the administration of LTD_4, a CysLT1 receptor agonist, decreased the latency to PTZ-induced seizures and reversed the anticonvulsant effect of montelukast. Montelukast and LTD_4 respectively reduced and increased the BBB disruption induced by PTZ. Notwithstanding, the dose of LTD_4 required to antagonize the protective effects of montelukast on the BBB was thirty times higher than the dose required to antagonize the anticonvulsant effect of montelukast.

Leukotrienes, first identified by Samuelsson (Samuelsson, 1979), are eicosanoid inflammation mediators that have been implicated in lesion/injury resolution, airway anaphylaxis and, more recently, in chronic degenerative diseases (Haeggstrom and Funk, 2011; Wang et al., 2011). Experimental evidence supports that 5-lipoxygenase is the rate-limiting enzyme in the conversion of arachidonic acid to cysteinyl- and noncysteinyl-leukotrienes

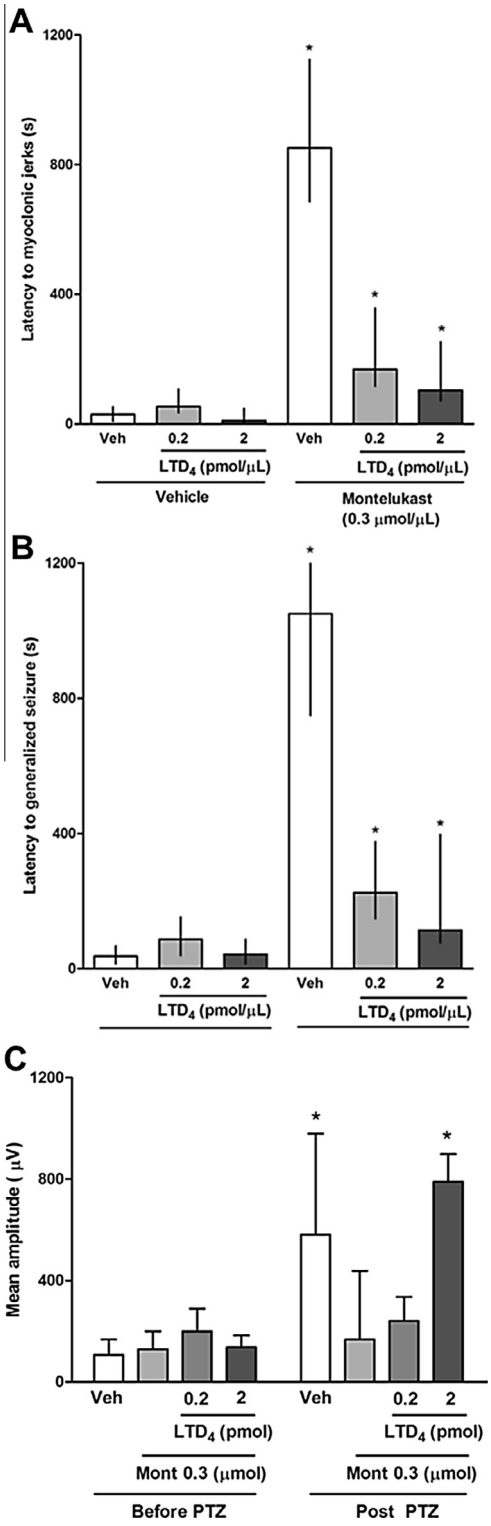

Fig. 5. LTD_4 (0.2 and 2 pmol, i.c.v.) reverts the protective effect of montelukast (0.3 μmol, i.c.v.) on PTZ-induced seizures (1.8 μmol/2 μL, i.c.v.). (A) Latency to myoclonic jerks; (B) Latency to generalized tonic–clonic seizures. Data are presented as median and interquartile range; $^{*}p < 0.05$ vs vehicle. (C) Mean amplitude of EEG recordings. Data are presented as mean and standard deviation; $^{*}p < 0.05$ vs respective control (before PTZ); (n = 5–6 per group).

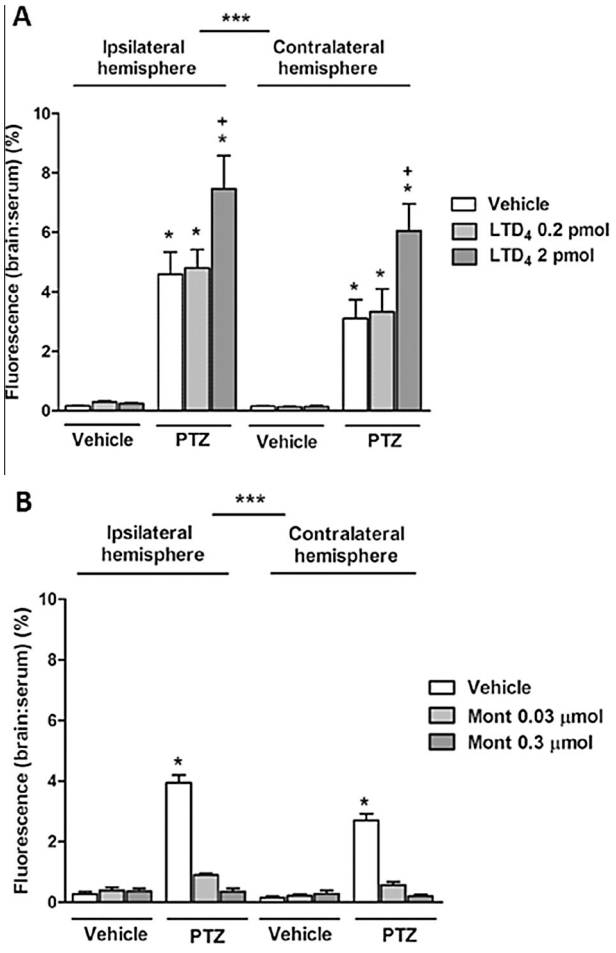

Fig. 6. Effect of LTD_4 (0.2 and 2 pmol, i.c.v., A) and montelukast (0.03 and 0.3 μmol, i.c.v., B) on PTZ-induced disruption of BBB (1.8 μmol/2 μL, i.c.v.). Data are presented as mean and S.E.M. $^{*}p < 0.05$ vs respective vehicle; $^{+}p < 0.05$ vs vehicle-PTZ; $^{***}p < 0.05$ ipsilateral vs contralateral hemispheres; (n = 6–7 per group).

(Denzlinger, 1996; Hedi and Norbert, 2004), since inhibition of 5-LOX with nordihydroguaiaretic acid abolishes the release of LTC_4-like material from both gray and white matter (Simmet et al., 1988). There is also increasing evidence suggesting a role for 5-LOX and leukotrienes in acute seizures and both chemical and electrical kindling (Simmet et al., 1988; Simmet and Tippler, 1990, 1991; Rehni and Singh, 2011). Accordingly, kainic acid-induced seizures are accompanied by time-dependent cerebral cysteinyl-leukotriene formation, which is inhibited by the 5-LOX inhibitor phenidone, in a dose-dependent manner (Simmet and Tippler, 1990). The currently reported anticonvulsant effect of montelukast is in agreement with these early studies by Simmet and Tippler (1990) and the study by Rehni and colleagues (2011), who have shown that montelukast attenuates the development of kindling and pilocarpine-induced spontaneous recurrent seizures (Rehni and Singh, 2011). Moreover, the current findings that the i.c.v. injection of LTD_4 facilitates the convulsant effect of PTZ and reverts the anticonvulsant action of

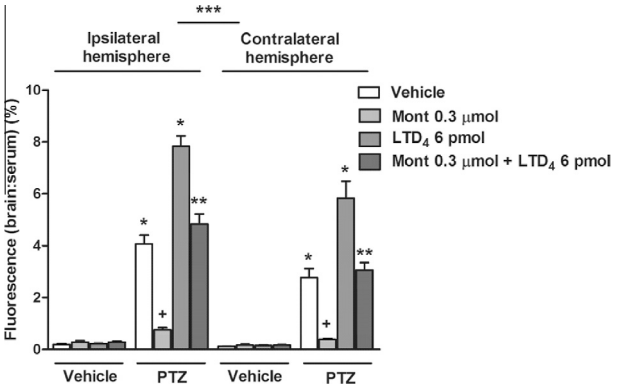

Fig. 7. LTD_4 (6 pmol, i.c.v.) reverses the protective effect of montelukast (0.3 μmol, i.c.v.) on BBB after PTZ-induced seizures (1.8 μmol/2 μL, i.c.v.). Data are presented as mean and S.E.M. $^{*}p < 0.05$ vs respective control (vehicle-vehicle); $^{\#}p < 0.05$ vs LTD_4-PTZ group; $^{**}p < 0.05$ vs montelukast-PTZ group. $^{***}p < 0.05$ ipsilateral vs contralateral hemispheres; (n = 4–5 per group).

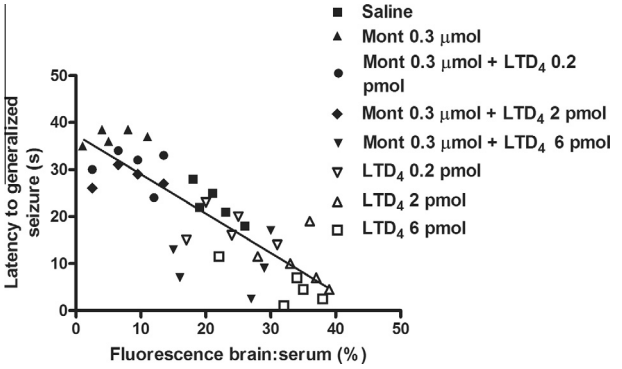

Fig. 8. Correlation plot of ranked data of brain:serum fluorescence ratio of the ipsilateral hemisphere against respective latency to generalized seizure of the experiments shown in Figs. 6 (dose–effect curve for LTD_4 and montelukast) and 7 (effect of LTD_4 on montelukast-induced protection against seizures). Spearman $r = -0.841$ $p < 0.0001$.

montelukast constitute converging pharmacological evidence for the involvement of CysLT receptors in seizures. The finding that two other CysLT receptor antagonists (pranlukast and Bay-u9973) also decreased PTZ-induced seizures indicates that this a common feature of CysLT antagonists, and strongly support a role for CysLT receptors in seizures. Several forms of brain injury trigger leukotriene production, including ischemia (Zhao et al., 2011), trauma (Farias et al., 2009), stroke (Hartiala et al., 2011) and seizures (Simmet and Tippler, 1990), which are associated with increased BBB permeability (Goldstein and Betz, 1986, Denzlinger, 1996, Di Gennaro et al., 2004, Wang et al., 2006). Although the biological role of brain damage-associated leukotriene production remains unclear, it has been suggested that cysteinyl leukotrienes underlie injury-associated BBB disruption and, by these means, exacerbate secondary injury processes. In fact, it has been shown that LTC_4 temporarily increases capillary permeability in pathological conditions (Cloughesy and Black, 1995). Since CysLT1R and CysLT2R are constitutively expressed in the human brain

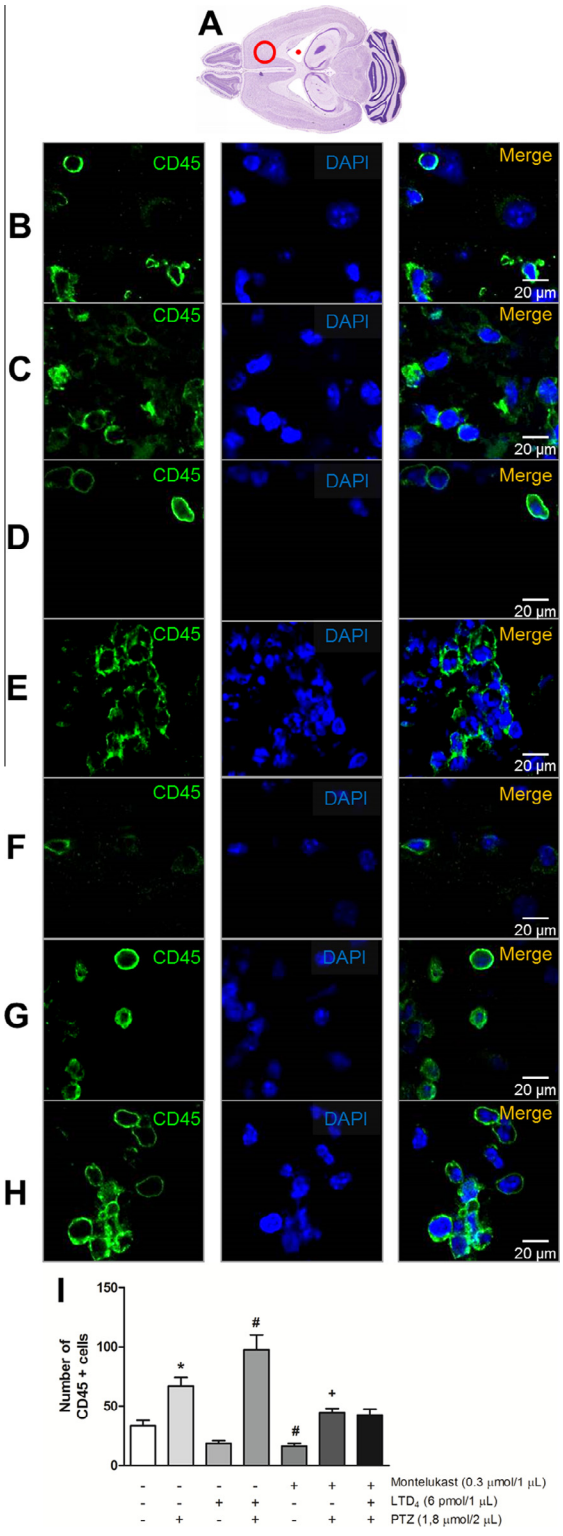

Fig. 9. Effect of PTZ, LTD_4 (6 pmol, i.c.v.) and montelukast (0.3 μmol, i.c.v.) on the number CD45+ cells in the cerebral cortex. Representative image of the cerebral cortex region used for making slides for immunohistochemistry analysis (A). Representative images of brain slices (10 μm) stained with anti-CD45 and DAPI of mice injected with vehicle (line B); vehicle/PTZ (line C); LTD_4/vehicle (line D); LTD_4/PTZ (line E); vehicle/montelukast (line F); montelukast/PTZ (line G) and montelukast/LTD_4/PTZ (line H). The bar graph shows data quantification (H). Data are presented as mean and SEM. $^{*}p < 0.001$ vs vehicle; $^{\#}p < 0.001$ vs vehicle/PTZ; $^{+}p < 0.001$ vs LTD_4/PTZ (n = 4–5 per group).

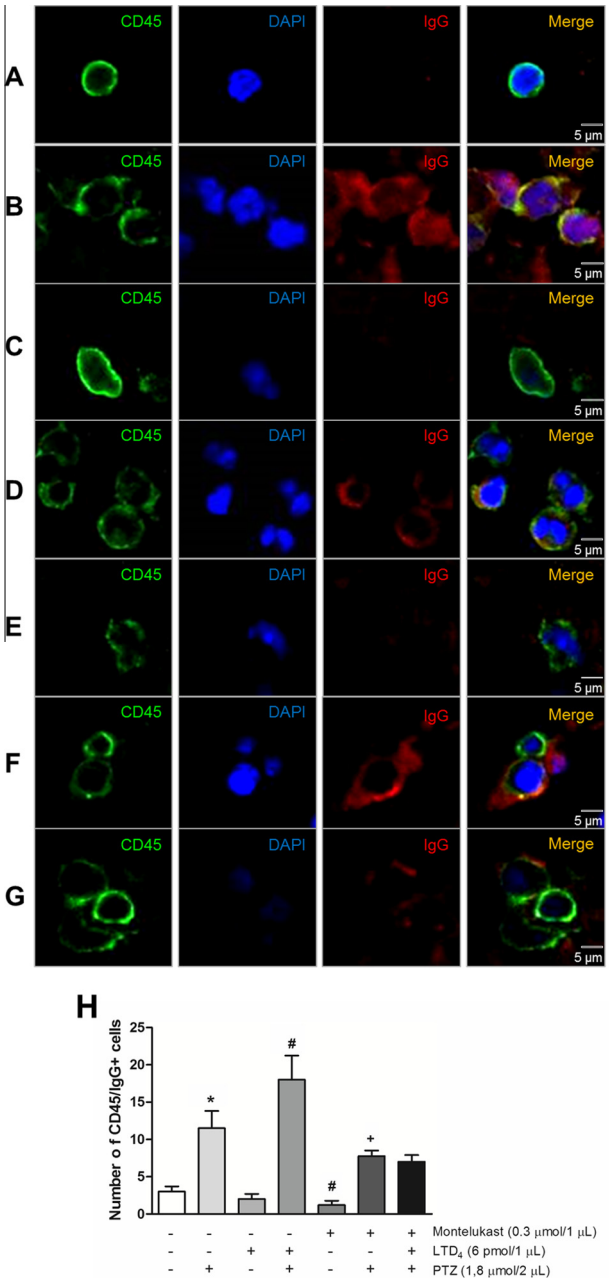

Fig. 10. Effect of PTZ, LTD_4 (6 pmol, i.c.v.) and montelukast (0.3 µmol, i.c.v.) on the number of double-labeled CD45/IgG cells in the cerebral cortex. Representative images of brain slices (10 µm) stained with anti-CD45, anti-IgG and DAPI of mice that received vehicle (line A); vehicle/PTZ (line B); LTD_4/vehicle (line C); LTD_4/PTZ (line D); vehicle/montelukast (line E); montelukast/PTZ (line F) and montelukast/LTD_4/PTZ (line G) are shown. The bar graph shows data quantification (H). Data are presented as mean and S.E.M. $^{*}p < 0.001$ vs vehicle; $^{\#}p < 0.001$ vs vehicle/PTZ; $^{+}p < 0.001$ vs LTD_4/PTZ (n = 4–5 per group).

vasculature and increase after injury, it has been suggested that LTC_4 binds to these receptors on endothelial cells and opens tight junctions, facilitating paracellular diffusion (Dupré et al., 2004). The processes by which leukotrienes induce the formation of interendothelial gaps probably involves two simultaneous processes: changes in adhesive properties of tight and adherens junction proteins and reorganization of the actin cytoskeleton (Stanimirovic and Satoh, 2000; Stamatovic et al., 2008; Abbott et al., 2006). Our confocal microscopy data support this view, since while the treatment with the CysLT1R agonist increased, montelukast reduced PTZ-induced leukocyte infiltration. Moreover, PTZ increased leukocyte infiltration in the hemisphere that had been implanted with the cannula, but not in the contralateral hemisphere, suggesting that cannula implantation has an important effect on the tissue response to the convulsant agent. Since all animals used in the current study were implanted with cannulas for i.c.v. injection, there was an established inflammatory process when PTZ was injected. Therefore, a plausible interpretation of the data obtained is that cannula implantation caused initial local inflammation and BBB disruption, and that montelukast prevented further PTZ-induced BBB disruption by inhibiting paracellular diffusion and leukocyte infiltration, slowing the onset of PTZ-induced seizures. Interestingly, magnetic resonance imaging studies in patients with post-traumatic epilepsy demonstrated that permeability of BBB to contrast agents co-localized with the presumed epileptic focus (Tomkins et al., 2011). In animal studies, a role for BBB opening was suggested in the progression of temporal lobe epilepsy based on the finding of serum albumin presence in brain parenchyma following SE, and a positive correlation between the extent of BBB opening and the number of seizures (van Vliet et al., 2007). Our leukocyte infiltration findings, to some extent, also agree with the data from Fabene and colleagues (Fabene et al., 2008), who have shown a role for leukocyte infiltration in the seizures induced by pilocarpine, in the sense that leukocyte infiltration may play a central role in the induction of hyperexcitability states.

While the current study shows that montelukast decreases seizure susceptibility, defining whether the anticonvulsant effect of the blockade of CysLT1 receptors is due to a stabilizing effect on the BBB, or not, requires a thoughtful discussion of the whole data obtained, including those obtained with the CysLTR agonist, LTD_4.

The following experimental findings indicate that the maintenance of BBB integrity may underlie the currently reported anticonvulsant effect of CysLT1R antagonists: 1. intracerebroventricular injection of montelukast prevented seizures and maintained the BBB integrity at the same range of doses; 2. increased latency to seizures correlated with low BBB permeability scores (Fig. 8). Notwithstanding, one might argue that if the anticonvulsant effect of montelukast depended on BBB integrity, these effects should not be dissociable by any means. Low doses of LTD_4 (0.2 and 2 pmol), prevented the anticonvulsant, but not the protective effect of montelukast on BBB. Such a dissociation, according to this view, suggests that BBB maintenance does not determine the anticonvulsant effect of montelukast. Considering the exposed above, it seems unlike that there is a direct cause–effect relationship between the anticonvulsant activity of montelukast and its ability to protect the BBB, at least in what concerns the BBB permeability to fluorescein and fluorescein-bound molecules. Moreover, it suggests that montelukast

decreases seizures by mechanisms other than decreasing BBB permeability to fluorescein and fluorescein-bound molecules, which remain to be identified. It is worth mentioning, however, that our pharmacological data do not allow ruling out that mechanisms unrelated and related to BBB protection may co-exist, and contribute for the montelukast-induced decreased seizure susceptibility, particularly in chronic models, such as pilocarpine-induced epilepsy, in which inflammation is clearly engaged (Fabene et al, 2008; Rehni and Singh, 2011).

In summary, this study shows that intracerebroventricular injection of montelukast decreases PTZ-induced seizures and BBB disruption, suggesting that CysLT1 receptors may be a suitable target for anticonvulsant development. This view is corroborated by recent clinical evidence that add-on therapy with pranlukast reduces seizure frequency in patients with intractable partial epilepsy (Takahashi et al, 2013).

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