The role of kinin B₁ and B₂ receptors in the persistent pain induced by experimental autoimmune encephalomyelitis (EAE) in mice: Evidence for the involvement of astrocytes

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A B S T R A C T

Multiple sclerosis (MS) is a progressive, demyelinating inflammatory disease of the human central nervous system (CNS). While the primary symptoms of MS affect motor function, it is now recognized that chronic pain is a relevant symptom that affects both animals and MS patients. There is evidence that glial cells, such as astrocytes, play an important role in the development and maintenance of chronic pain. Kinins, notably bradykinin (BK) acting through B₁ (B₁R) and B₂ (B₂R) receptors, play a central role in pain and inflammatory processes. However, it remains unclear whether kinin receptors are involved in neuropathic pain in MS. Here we investigated by genetic and pharmacological approaches the role of kinin receptors in neuropathic pain behaviors induced in the experimental autoimmune encephalomyelitis (EAE) mouse model. Our results showed that gene deletion or antagonism of kinin receptors, especially B₁R, significantly inhibited both tactile and thermal hypersensitivity in EAE animals. By contrast, animals with EAE and treated with a B₂ selective agonist displayed a significant increase in tactile hypersensitivity. We also observed a marked increase in B₁R mRNA and protein level in the mouse spinal cord 14 days after EAE immunization. Blockade of B₁R significantly suppressed the levels of mRNAs for IL-17, IFN-γ, IL-6, CXCL-1/KC, COX-2 and NOS2, as well as glial activation in the spinal cord. Of note, the selective B₁ antagonist DALBK consistently prevented IFN-induced up-regulation of TNF-α and IL-6 release in astrocyte culture. Finally, both B₁R and B₂R antagonists significantly inhibited COX-2 and NOS2 expression in primary astrocyte culture. The B₁R was co-localized with immunomarker of astrocytes in the spinal cord of EAE-treated animals. The above data constitute convincing experimental evidence indicating that both kinin receptors, especially the B₁ subtype, exert a critical role in the establishment of persistent hypersensitivity observed in the EAE model, an action that seems to involve a central inflammatory process, possibly acting on astrocytes. Thus, B₁ selective antagonists or drugs that reduce kinin release may have the potential to treat neuropathic pain in patients suffering from MS.

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Introduction

Multiple sclerosis (MS), a chronic inflammatory and demyelinating disease of the central nervous system (CNS) (McFarland and Martin, 2007; Sospedra and Martin, 2005), is considered an autoimmune pathology where Th1 and Th17 cells play critical roles (Sospedra and Martin, 2005; Steinman, 2007). The neuroinflammation in the MS is characterized by the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), Fas ligand (Fasl), and interferon-γ (IFN-γ), as well as particular chemokines, namely keratinocyte-derived chemokine (CXCL-1/KC), macrophage inflammatory protein (MIP)-1α and regulated upon activation normal T cell expressed and presumably secreted (RANTES) (Governan, 2009). These mediators are produced by both infiltrating immune cells and CNS-resident glial cells, mainly astrocytes and microglial/macrophages, which orchestrate a pathogenic cascade leading to neuroinflammation and axonal damage (Gerard and Rollins, 2001; Ibrahim et al., 2001; Lock et al., 2002).

Earlier studies have shown that glial cells, mainly astrocytes and microglia, are activated in MS and in animal models of EAE (Volterra and Meldolesi, 2005). It has been reported that astrocytes show pathogenic
pro-inflammatory activity by producing neurotoxic mediators, cytokines and chemokines (Volterra and Meldolesi, 2005). Through the production of these neutromodulators, astrocytes control pain (Gao et al., 2009; Wei et al., 2008); moreover, it has been suggested that activated astrocytes are sufficient to produce chronic pain symptoms (McMahon et al., 2005).

Among the clinical signs affecting most MS patients, persistent pain is a relevant event reported by about 50 to 80% of patients (O'Connor et al., 2008). Meanwhile, this pain has been described as central neuropathic pain (including dysesthetic extremity pain and trigeminal neuralgia), characterized as back pain, painful tonic spasms and headache. Nerve injury is usually accompanied by a local inflammatory reaction in which nerve-associated and immune cells release several pro-nociceptive mediators such as cytokines, eicosanoids and kinins (Bennett, 1999; Tracey and Walker, 1995). Despite intense efforts on the part of basic scientists and pharmaceutical companies, the mechanisms underlying neuropathic pain are currently poorly understood and consequently very difficult to treat. In addition, a recent report showed that in the MOG35–55 EAE model, changes observed in sensory function and the development of neuropathic pain behaviors are apparently independent of symptoms of disease severity and clinical course (Olechowski et al., 2009).

Kinins are a group of peptides involved in a series of pathophysiological processes including control of blood pressure, smooth muscle contraction or relaxation, vascular permeability and pain transmission. Kinins are formed in plasma and tissues in response to infection, tissue contraction or relaxation, vascular permeability and pain transmission. Kinins are involved in several biological effects by activating two G-protein coupled receptors, the B1 and B2 receptors. The B2R is constitutively expressed throughout central and peripheral tissues, and displays a higher affinity for bradykinin (BK) and Lys-BK peptide. In contrast, B1R displays high affinities for the kinin metabolites des-Arg9-BK (DABK) and Lys-des-Arg9-BK and is not present under normal conditions, being up-regulated following inflammatory, infectious or traumatic stimuli. Furthermore, it has been suggested that B1R exerts a critical role in controlling several chronic diseases (Calixto et al., 2004; Campos et al., 2006) and persistent pain responses (Cunha et al., 2007; Ferreira et al., 2005; Fujita et al., 2010; Meotti et al., 2012; Talbot et al., 2010).

In fact, accumulated evidence suggests the involvement of the kallikrein–kinin system in MS and EAE models (Dos Santos et al., 2008; Gobel et al., 2011; Schulze-Toppshoff et al., 2009). Recent data from our group demonstrated that kinin B1R displays a dual role in the progression of EAE, acting through distinct mechanisms at each stage of the disease, mainly through the modulation of Th1 and Th17-myelin-specific lymphocytes and glial cell-dependent pathways (Dutra et al., 2011). Furthermore, some reports have shown that B1R and B2R are expressed in microglia and astrocytes in rodent (Hosli et al., 1992; Noda et al., 2003; Talbot et al., 2009).

Keeping the above data in mind, the purpose of the present study was to investigate the role of kinin B1 and B2 receptors on the persistent pain induced by EAE and the possible involvement of glial cells in this pain process, by using B1R and B2R knockout mice in combination with selective kinin receptor antagonists/agonists and primary astrocytes culture.

Materials and methods

Experimental animals

Experiments were conducted using female C57BL/6, kinin B1R-knockout (B1R$^{-/-}$) and kinin B2R-knockout (B2R$^{-/-}$) mice (6 to 10 weeks old). Deletion of the entire coding sequence for the kinin B1 and B2 receptors was achieved according to a methodology described previously (Dutra et al., 2011; Pesquer et al., 2000). The mice were kept in groups of six to nine animals per cage, maintained under controlled temperature (22 ± 1 °C) with a 12 h light/dark cycle (lights on at 7:00 a.m.) and were given free access to food and water. All procedures used in the present study followed the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23) and were approved by the Animal Ethics Committee of the Universidade Federal de Santa Catarina (CEUA-UFSC, protocol number 23080038266/2008-43).

Drug treatment protocol

Mouse myelin oligodendrocytes glycoprotein MOG35–55 peptide was synthesized by EZBiolab (Carmel, USA). The selective kinin B1 receptor antagonist-des-Arg$^9$-[Leu]$^7$-BK, the selective kinin B2 receptor agonist des-Arg$^9$-BK (Sigma Chemical Co., St. Louis, MO, USA) and the selective kinin B2 receptor antagonist HOE-140 (Aventis, Frankfurt Main, Germany) were prepared as 1 mM stock solutions in PBS in siliconized plastic tubes, maintained at −20 °C and diluted to the desired concentration just before use. Des-Arg$^9$-[Leu]$^7$-BK (50 nmol/kg), Des-Arg$^9$-BK (300 nmol/kg) or HOE-140 (150 nmol/kg) or their vehicles were administered intraperitoneally (i.p.) twice per day for 5 days (starting on day 0 until day 5 post-immunization — defined as the induction phase), 1 h before immunization and behavioral studies. The choice of dose for each drug was based on pilot experiments (results not shown) or on previous data described in the literature (Costa et al., 2011; Dutra et al., 2011).

EAE induction

Experimental autoimmune encephalomyelitis (EAE) was induced by subcutaneous (s.c.) immunization into the flanks with 200 μl of an emulsion containing 200 μg of MOG35–55 peptide and 500 μg of Mycobacterium tuberculosis extract H37Rv (Difco Laboratories, Detroit, MI, USA) in incomplete Freund’s adjuvant oil (Sigma Chemical Co., St. Louis, MO, USA). This procedure was repeated after 7 days to increase the incidence of EAE, as previously described (Stromnes and Goverman, 2006). In addition, the animals received 300 ng of Pertussis toxin (Sigma Chemical Co., St. Louis, MO, USA) i.p. on day 0 and day 2 post-immunization. Non-immunized (naïve or wild-type) and EAE-group animals were used as control groups. The animals were monitored daily and neurological impairment was quantified using a clinical scale after seven days post-immunization (Stromnes and Goverman, 2006). Mice were weighed and observed daily for clinical signs of EAE for up to 25 days post-immunization. The mice which did not develop the disease were excluded from the study. Clinical signs of EAE were assessed according to the following scores: 0, no signs of disease; 1, loss of tone in the tail; 2, hindlimb paresis; 3, hindlimb paralysis; 4, tetraplegia; and 5, moribund and/or death.

Behavioral experiments

Tactile hypersensitivity

To evaluate tactile hypersensitivity, mice were placed individually in clear Plexiglas boxes (9 × 7 × 11 cm) on elevated wire mesh platforms to allow access to the ventral surface of the right hind paw. The withdrawal response frequency was measured following 10 applications (3 s each) of von Frey hairs (VFH, Stoelting, Chicago, IL, USA). The animals were acclimatized for 30 min before behavioral testing, and tactile hypersensitivity was evaluated at several time points. The VFH of 0.4 g produces a mean withdrawal frequency of about 15%, which is considered to be an adequate value for the measurement of tactile hypersensitivity (Qintao et al., 2005). To determine the basal tactile thresholds, all the groups were evaluated before disease induction. All animals were evaluated at different time points until day 14, since after this day the clinical signs of EAE, such as locomotor deficit, were visible.

Thermal hypersensitivity

The hot-plate test was used to measure response latencies according to a method previously described (Santos et al., 1999), with minor modifications. In these experiments, the hot-plate (Ugo Basile, model-DS 37,
Verese, Italy) was maintained at 50 ± 1 °C. Animals were placed into a glass cylinder 24 cm in diameter on the heated surface, and the time between placement and jumping, shaking or licking of the paws was recorded as the index of response latency. An automatic 30-s cut-off was used to prevent tissue damage. Each animal was tested before disease induction to obtain a baseline. All animals were evaluated at different time points until day 15, since after this day the clinical signs of EAE, such as locomotor deficit, were visible.

**Rotarod test**

In order to evaluate locomotor activity and coordination, the mice were placed on a rotarod apparatus at a fixed rotational speed of 4 rpm. The maximum time for each trial was set at 60 s. Rotarod training was performed prior to disease induction and consisted of three consecutive trials in which the animals became familiar with the task. After disease induction, the mice were tested at different time points for up to 15 days post-immunization.

**RNA extraction and Real-time quantitative PCR**

Total RNA from lumbar spinal cord (L3–L5) (15 days post-immunization) or cultured astrocytes were extracted using the Trizol protocol and its concentration was determined using a NanoDrop 1100 (NanoDrop Technologies, Wilmington, DE, USA). A 50 ng (spinal cord tissue) or 10 ng (astrocyte culture) quantity of total RNA was used for cDNA synthesis. A reverse transcription assay was performed as described in the M-MLV Reverse Transcriptase protocol according to the manufacturer’s instructions. cDNA was amplified in duplicate using the TaqMan® Universal PCR Master Mix Kit with specific TaqMan Gene Expression target genes, the 3′ quencher MGB and FAM-labeled probes for mouse kinin B1R (Mm00432059_s1), kinin B2R (Mm01339907_m1), IL-17 (Mm00439618_m1), IFN-γ (Mm99999071_m1), IL-6 (Mm99999064_m1), CXCL-1/KC (Mm00433859_m1), cyclooxygenase-2 (COX-2, Mm01037334_g1), nitric oxide synthase-2 (NOS-2, Mm1309898_m1), and brain-derived neurotrophic factor (BDNF, Mm00432069_m1). GAPDH (NM_008084.2) was used as an endogenous control for normalization. The PCR reactions were performed in a 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA, USA). The thermocycler parameters were as follows: 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. Expression of the target genes was calibrated against conditions found in control animals or cells (i.e., animals/astrocytes that received vehicle).

**Immunohistochemical analysis**

Fifteen days after EAE induction, animals were sacrificed and each portion of the lumbar spinal cord (L3–L5) was removed and fixed immediately in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) (for the exact amount of time: 1 min). The re-fixation was repeated by using a Sight DS-5M-L1 digital camera connected to an Eclipse 501 light microscope (both from Nikon, Melville, NY). Settings for image acquisition were identical for control and experimental tissues. Four ocular fields per section (4–6 mice per group) were captured and a threshold optical density that best discriminated staining from the background was obtained using the NIH Image 1.36b imaging software (NIH, Bethesda, MD, USA), using a counting grid at ×200 magnification. For B1R, GFAP and Iba1 the total pixel intensity was determined and the data were expressed as optical density (O.D.).

**Immunolabeling protocol**

In another set of experiments, the animals were sacrificed (15 days post-immunization), lumbar spinal cord (L3–L5) was removed and fixed in 4% paraformaldehyde during 15 min following embedding in Tissue-Tek®, and frozen slices of spinal cord (5 μm) were obtained using Cryostat. After PBS wash for three times, the slides were incubated for 30 min with a blocking buffer 1% bovine serum albumin (BSA) dissolved in PBS. Antibodies were diluted in blocking buffer. A solution of mixed primary antibodies was applied: monoclonal mouse anti-GFAP (1:200) and polyclonal rabbit anti-B1R (1:200) following overnight incubation at 4 °C. After wash, secondary antibodies were incubated in a mix solution. In order to target B1R we used goat anti-rabbit Alexa Fluor® 568 (Red) and for GFAP immunolabeling we used donkey anti-mouse Alexa Fluor® 488 (Green) both at the concentration of 1:2500. Nuclei (Blue) were stained with Hoechst (0.5 ml/ml). Images were obtained by using a Fluorescence Bx41 Model Microscopy (Olympus America Inc, Center Valley, PA, USA).

**Western blot assay**

Spinal cord tissue samples were removed 15 days post-immunization and homogenized in complete radio immunoprecipitation lysis buffer (RIPA). Equal amounts of protein for each sample (30 μg) were loaded per lane and electrophoretically separated using 10% denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Afterward, the proteins were transferred to nitrocellulose membranes using a Mini Trans-Blot Cell system (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer’s protocol. Western blot analysis was carried out using polyclonal rabbit anti-B1R (1:1000) incubated overnight. Following washing, the membrane was incubated with secondary antibodies conjugated to horseradish peroxidase (1:25000, Cell Signaling Technology, Danvers, MA, USA). The immunocomplexes were visualized using SuperSignal West Femto Chemiluminescent Substrate detection system (Thermo Fischer Scientific, Rockford, IL, USA) and densitometric values were normalized using monoclonal mouse β-actin antibody (1:500, Cell Signaling Technology, Danvers, MA, USA).

**Determination of cytokine levels**

After the incubation period, astrocyte culture supernatants were collected and stored at −70 °C. The levels of TNF-α, CXCL-1/KC and IL-6 were evaluated using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer’s recommendations.

**Primary astrocyte cultures and treatment**

Mixed glial cultures were prepared according to a method previously described (Cohen and Wilkin, 1995). Neonatal (postnatal days 1 to 3) C57BL/6 mice (n = 8 mice) were decapitated and cortices were dissected and placed into 4 °C Hank’s buffered salt solution (HBSS). Cortical tissue was cut coarsely and incubated in HBSS containing 0.025% trypsin, 0.3% bovine serum albumin (BSA) and DNase (40 mg/ml) for 20 min at 37 °C. The solution was replaced by HBSS containing BSA and DNase and triturated 20 times. After allowing debris to settle, the
supernatant was passed through a 70-μm cell strainer (BD Biosciences). The supernatant was centrifuged at 1000 rpm for 5 min and the pellet was resuspended in 10 ml of Dulbecco’s Modified Eagle’s Medium (DMEM) containing 50 units of penicillin, 50 mg/ml streptomycin and 10% heat-inactivated fetal bovine serum. Suspended cells were divided into 24-well microculture plates, grown for 10 days in vitro and afterward purified by overnight shaking (120 rpm). The purity of the astrocytes was assessed by immunofluorescence using the astrocyte marker glial fibrillary acidic protein (GFAP) (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer’s recommendations. The astrocyte cultures were found to be at least 99% GFAP-positive. The remaining adherent astrocytes were treated with IFN-γ (50 U/ml). DALBK (5 μM) and HOE-140 (5 μM) were added twice, first 18 h before, and then simultaneously with the inflammatory agent. The concentrations of DALBK and HOE-140 (5 μM) used were based on a previous concentration response curve (results not shown) and tested in triplicate. Forty eight hours after addition of the inflammatory agent (IFN-γ, 50 U/ml), culture supernatants and fixed astrocytes were collected to measure the levels and expression of cytokines/chemokines and proinflammatory agents, as described below.

Drugs and reagents

Pertussis toxin, phosphate buffered saline (PBS), incomplete Freund’s adjuvant oil, kinin B1R antagonist des-Arg²-[Leu⁶]-BK (DALBK), kinin B1R agonist des-Arg¹-BK (DABK), HEPES, penicillin, streptomycin, Na-EDTA, trypsin, deoxyribonuclease I (DNase) and BSA were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). The kinin B1R antagonist, HOE-140, was donated by Aventis (Frankfurt Main, Germany). The MODEG₃₅₋₅₅ peptide (MEVGYVRSPSRPHVHYRNC) was obtained from EZBiolab, Carmel, IN 46032, USA and the M. tuberculosis extract H37Ra from Difco Laboratories, Detroit, MI, USA. DMEM and fetal bovine serum were purchased from Gibco (Carlsbad, CA, USA). The anti-mouse-TNF-α, CXCL-1/KC and IL-6 DuoSet kits were obtained from R&D Systems. The monoclonal mouse anti-GFAP was obtained from Cell Signaling Technology (Danvers, MA, USA) and the polyclonal goat anti-Iba 1 from Abcam (Cambridge, MA, USA). The polyclonal rabbit anti-B₁R and polyclonal rabbit anti-B₂R were purchased from Abclonal Labs (Jerusalem, Israel). The secondary antibodies – donkey anti-mouse Alexa fluor® 488 and goat anti-rabbit Alexa fluor® 568 – were obtained from Invitrogen Life Technologies (Grand Island, NY, USA). The primers and probes for mouse B₁R, B₂R, IL-17, IFN-γ, IL-6, CXCL-1/KC, COX-2, NO₃-2, BDNF and GAPDH were purchased from Applied Biosystems (Warrington, UK). The other reagents used were of analytical grade and obtained from different commercial sources.

Statistical analysis

All data are presented as mean ± SEM of six to nine mice per group, and are representative of two or three independent experiments. A statistical comparison of the data was performed by one-way ANOVA followed by Bonferroni or Newman–Keuls testing, depending on the experimental protocol. P-values less than 0.05 (p < 0.05) were considered significant. The statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Blockade and gene deletion of kinin B₁ or B₂ receptors during the EAE induction phase prevent tactile hypersensitivity

As illustrated in Fig. 1, the tactile hypersensitivity produced following EAE induction was characterized by a pronounced and long-lasting increase in the response frequency to stimulation with innocuous von Frey hairs on the right hind paw. This process was observed starting on day 2, reaching a maximum on day 2–4 after immunization and remained elevated for up to 14 days (Fig. 1). Interestingly, treatment with the selective B₁R antagonist (DALBK, 50 nmol/kg, i.p., twice per day) during the induction phase of EAE (days 0–5) (Fig. 1A), significantly suppressed tactile hypersensitivity in EAE animals until day 14, with an inhibition of 66 ± 2% (Fig. 1B), based on the area under the curve (AUC). Moreover, preventive treatment with the B₂R antagonist HOE-140 (150 nmol/kg, i.p., twice per day, days 0–5) during the same phase, significantly reduced tactile hypersensitivity only when assessed on the second day after immunization (Figs. 1A and B), resulting in a transient inhibition. Recently, a study unexpectedly reported that activation of B₁R ameliorates EAE development (Schulze-Topphoff et al., 2009). For this reason, here we assessed whether the B₁R agonist (DABK) could exert a protective action on neuropathic pain induced by EAE. Animals treated with the B₁ receptor agonist DABK (300 nmol/kg, i.p., twice per day, days 0–5), during the induction phase of EAE, displayed a tactile hypersensitivity similar to EAE control animals (Figs. 1A and B).

To further confirm the participation of the kinin receptor in tactile hypersensitivity in EAE, we immunized B₁R−/− or B₂R−/− mice. After immunization with MOG₃₅₋₅₅, mice lacking the gene for the B₁R failed to exhibit tactile hypersensitivity (Figs. 1C and D). On the other hand, similar to what was observed in mice treated with HOE-140, tactile hypersensitivity in B₁R−/− mice was significantly diminished only on day 2 post-immunization (Figs. 1C and D).

Blocking kinin B₁R and B₂R inhibits thermal hypersensitivity induced by EAE

Recently, thermal hypersensitivity has been reported as the most common painful clinical sign associated with central neuropathic pain (Osterberg and Boivie, 2010; Svendsen et al., 2003). Herein, we found a reduction in paw withdrawal latency to heat stimulus when assessed 3 days after EAE immunization, an effect that was stable until day 15 (Fig. 2). In addition, animals treated with the selective B₁ antagonist DALBK or in B₁R knockout mice, thermal hypersensitivity was suppressed compared to the EAE group, showing inhibitions of 33 ± 2% and 42 ± 4%, respectively (Figs. 2A–D). In contrast to the data on tactile hypersensitivity, thermal hypersensitivity was reduced by HOE-140 treatment or in mice lacking B₂R (Fig. 2). Conversely, animals treated with the B₂R agonist DABK (300 nmol/kg, i.p., twice per day, days 0–5) exhibited thermal hypersensitivity similar to EAE control animals (Figs. 2A and B).

Blockade and gene deletion of kinin receptors do not cause any significant reduction in motor coordination until fifteen days after induction of EAE

Analysis of motor disturbances using a rotarod showed that naïve and EAE-control groups did not show any significant changes of locomotor activity on day 15 post-immunization (Table 1), thus not compromising the pain behavioral analysis of this study. In addition, in mice treated with the B₁R antagonist DALBK (50 nmol/kg, i.p.), the B₂R antagonist HOE-140 (150 nmol/kg, i.p.) or the B₁R agonist DABK (300 nmol/kg, i.p.) twice per day (12/12 h) from days 0 to 5, locomotor activity was also unaffected on day 15 (p > 0.05) (Table 1 and Supplemental Fig. 1A). This was also true in B₁R−/− and B₂R−/− mice when assessed 15 days post-immunization (Table 1 and Supplemental Fig. 1B). Nonetheless, clinically relevant the blockade of B₁R by selective antagonist – DALBK (50 nmol/kg, i.p., twice a day, days 0–5) or genetic deletion of B₁R significantly reduced day of disease onset and maximal clinical score when compared to the EAE control group (Table 1), as previously described ( Dutra et al., 2011). Here, our data suggest that animals developed persistent tactile and thermal hypersensitivity in the hind paw prior to any signs of overt neurological dysfunction.
B1 is over-expressed in the spinal cord of EAE mice

To evaluate the effects of EAE on expression of the kinin B1 and B2 receptors, the mRNA and protein level from spinal cords of naïve and EAE-mice (on day 15 post-immunization) were evaluated by means of real-time RT-PCR, immunohistochemistry and Western blot assays, respectively. Basal expression of B1R and B2R was detected in naïve mice (Fig. 3 and Supplemental Fig. 2), while 15 days after EAE induction, mRNA (Fig. 3A and Supplemental Fig. 2A) and protein levels (Fig. 3B) of kinin B1R transcript were markedly increased (seven-fold) in the dorsal horn of the spinal cord. Moreover, B1R−/− mice do not exhibit immunostaining for B1R in the lumbar spinal cord tissue after EAE induction (Fig. 3B). However, no significant difference was observed in kinin B2R both in mRNA (Fig. 3C and Supplemental Fig. 2B) and protein levels (Fig. 3D) between the naïve- and EAE-groups.

Gene deletion of the kinin B1R reduces mRNA expression of inflammatory agents in the spinal cord

Increasing evidence suggests a role of Th17 and Th1 in CNS damage, particularly observations of these subpopulations and their signature cytokines, IL-17 and IFN-γ, in the brains of individuals with MS (Stromnes et al., 2008; Tzartos et al., 2008). In addition, recent studies have shown that glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to particular observations of these subpopulations and their signature cytokines, IL-17 and IFN-γ, in the brains of individuals with MS (Stromnes et al., 2008; Tzartos et al., 2008). In addition, recent studies have shown that glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008). In this set of experiments, we investigated whether the hypersensitivity behavior induced by EAE was linked to glial cells, such as astrocytes and oligodendrocytes, also produce IL-17 (Tzartos et al., 2008).
Blockade of kinin B1R and B2R prevents the expression and release of pro-inflammatory mediators by astrocytes

Since many studies have suggested that astrocytes are key players in neuropathic pain states (Gao and Ji, 2010; Olechowski et al., 2009; Scholz and Woolf, 2007) and these cells express kinin receptors (Talbot et al., 2009), we further investigated whether the blockade of kinin receptors could decrease the levels and expression of pro-inflammatory cytokines/chemokines in primary astrocyte cultures stimulated with IFN-γ, a major Th1 cytokine. In the untreated group, low levels of TNF-α (Fig. 7A), IL-6 (Fig. 7B) and CXCL-1/KC (Fig. 7C) proteins, as well as COX-2 (Fig. 7D and Supplemental Fig. 6A) and NOS-2 (Fig. 7E and Supplemental Fig. 6B) mRNAs were detected. However, stimulation with IFN-γ (50 U/ml) for 48 h markedly increased the TNF-α, IL-6 and CXCL-1/KC levels, as well as COX-2 and NOS-2 mRNA expression (Fig. 7).

In vitro pre-treatment with DALBK (5 μM), 18 h before and then simultaneously with the inflammatory agent, decreased TNF-α (Fig. 7A), IL-6 (Fig. 7B) and CXCL-1/KC (Fig. 7C) levels, as well as COX-2 (Fig. 7D and Supplemental Fig. 6A) and NOS-2 (Fig. 7E and Supplemental Fig. 6B) mRNA expression in IFN-γ-stimulated astrocytes. However, these outcomes appeared to occur independently of the neuroprotective effects of brain-derived neurotrophic factor (BDNF), since its levels were not affected (Fig. 7F and Supplemental Fig. 6C). Interestingly, the B2 antagonist HOE-140 significantly reduced the mRNA expression of COX-2 (Fig. 7D and Supplemental Fig. 6A) and NOS-2 (Fig. 7E and Supplemental Fig. 6B), but not TNF-α, IL-6 or CXCL-1/KC protein levels, which might explain the minor effect of B2R blockade on neuropathic pain in the EAE model. These results suggest that kinin receptors, mainly the B1 subtype, seem to exert an important role in the neuropathic hypersensitivity induced by EAE through modulation of astrocytes function.

Table 1
Description of clinical features of MOG35–55-induced EAE.

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<th>Groups</th>
<th>Incidence</th>
<th>Mortality (up to day 25)</th>
<th>Day of disease onset</th>
<th>Locomotor activity on day 15</th>
<th>Clinical score on day 15</th>
<th>Maximal clinical score achieved (± day 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>-</td>
<td>-</td>
<td>60</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EAE</td>
<td>89% (8/9)</td>
<td>22% (2/9)</td>
<td>14 ± 0.7</td>
<td>53.2 ± 5.2**</td>
<td>0.5 ± 0.5</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>DALBK</td>
<td>100% (7/7)</td>
<td>0% (0/7)</td>
<td>18 ± 0.5</td>
<td>56.4 ± 2.4**</td>
<td>0</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>HOE-140</td>
<td>100% (9/9)</td>
<td>0% (0/9)</td>
<td>16 ± 0.4</td>
<td>52.0 ± 4.9**</td>
<td>0</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>DABK</td>
<td>100% (8/8)</td>
<td>0% (0/8)</td>
<td>15 ± 0.5</td>
<td>51.0 ± 1.0**</td>
<td>0.5 ± 1.0</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>B1R−/−</td>
<td>100% (8/8)</td>
<td>0% (0/8)</td>
<td>17 ± 0.3</td>
<td>57.2 ± 2.3**</td>
<td>0</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>B2R−/−</td>
<td>78% (7/9)</td>
<td>0% (0/9)</td>
<td>15 ± 0.2</td>
<td>50.4 ± 2.8**</td>
<td>0.2</td>
<td>2.9 ± 0.5</td>
</tr>
</tbody>
</table>

EAE, control group treated only vehicle; DALBK, kinin B1 receptor antagonist des-Arg⁸-[Leu⁹]-BK (50 nmol/kg); HOE-140, kinin B2 receptor antagonist (150 nmol/kg); DABK, kinin B1 receptor agonist des-Arg⁸-BK (300 nmol/kg); B1R−/−, kinin B1 receptor knockout mice; B2R−/−, kinin B2 receptor knockout mice, were evaluated during 25 days. The maximal clinical score corresponds to day 20 after EAE immunization. Locomotor activity (latency in sec) at day 15 after EAE induction. Results are presented as means ± SEM and are representative of three independent experiments (n = 7–9 mice/group), n.s: not significant versus the naïve group (one-way ANOVA with the Newman–Keuls post-hoc test).
EAE induced up-regulation of B1R expression on spinal astrocytes

After all these data, we still had a question to be answered. Does that astrocytes express B1R during the development of EAE? In this context, previous evidence showed that thoracic spinal cord astrocytes bear the B1R in STZ-diabetic rats (Talbot et al., 2009). For this purpose, we measured co-localization of B1R expression with immunomarker of astrocytes (GFAP) in the spinal cord after EAE induction. As shown in Fig. 8, only low level of B1R was detected in the astrocytes from naïve mouse spinal cord tissue, nonetheless these values were markedly increased at 15 days following EAE induction (Fig. 8). These results support the concept that astrocytes express and up-regulate B1R expression after EAE induction. Thus, our data suggest that B1R primarily expressed in the astrocytes after EAE development plays a relevant role in modulating neuropathic pain induced by EAE in mice prior to any overt neurological deficits.

Discussion

Painful neuropathies are a worldwide health problem caused by traumatic injury, metabolic challenges and some chemotherapeutic agents (Canton et al., 2004; Polomano and Bennett, 2001; Seltzer et al., 1990). The precise mechanisms underlying these syndromes are poorly understood, and for many patients, currently available therapies do not provide satisfactory pain relief. It is becoming increasingly apparent that kinins and their receptors play a critical role in neuropathic pain (Cunha et al., 2007; Ferreira et al., 2005; Fujita et al., 2010) through the release of prostanoids, nitric oxide (NO) and pro-inflammatory cytokines, activation of immune cell influx and increased vascular permeability (Calixto et al., 2004; Marceau and Bachvarov, 1998). Patients with MS suffer persistent pain (O’Connor et al., 2008), and indeed high levels of kinin components, namely des-Arg9-bradykinin (DABK), bradykinin, kallikrein-1, kallikrein-6 and low-molecular-weight kininogens (KNGL) have been found in...
CNS tissue and cerebrospinal fluid from MS patients (Germain et al., 1988; Schulze-Topphoff et al., 2009). In addition, the B1R has been found to be up-regulated not only in brain endothelial cells (Prat et al., 2000), but also in the peripheral T lymphocytes (Prat et al., 2005) of these patients. Also of relevance, recent reports have demonstrated the involvement of kinins in experimental models of MS in mice (Dos Santos et al., 2008; Dutra et al., 2011; Gobel et al., 2011; Prat et al., 2005; Schulze-Topphoff et al., 2009). However, to our best knowledge, thus far, there are no studies reporting the role of kinins and their receptors in persistent pain in the EAE mouse model. Our present study showed that both tactile and heat hypersensitivity are major sensory disturbances that can be detected early in the course of disease in the MOG35–55 model of EAE. This behavioral hypersensitivity threshold to both heat and tactile stimuli is not consequent of the specific degree of neurological deficits that arise in the disease. Of relevance, the present results indicate that the tactile and thermal hypersensitivity caused by EAE seem to be mediated through the activation of kinin B1 and B2 receptors, demonstrating that endogenous kinin production may contribute to progressive EAE symptoms, including chronic pain.

Fig. 5. Kinin B1R deletion reduced glial activation during EAE pathology in the dorsal horn of the spinal cord. The lumbar spinal cords (L3–L5) obtained on the 15th day after immunization from the naïve group, EAE group and from mice deficient in B1R were processed for immunohistochemistry assay. Representative sections of astrocytes activation, GFAP (A) and microglial reactivity, Iba 1 (B). (A and B) Graphical representation of the immunostaining for GFAP and Iba1 expression evaluated in dorsal horn of the spinal cord. Specifically, four 5-mm sections of lumbar spinal cord white matter (four to six mice/group) < 150 mm apart were obtained between L3 and L5. Scale bar corresponds to 100 μm and applies throughout. Each column represents the mean ± SEM of 4 to 6 mice per group and are representative sections from two independent experiments. *p < 0.05 and **p < 0.001 versus wild-type naïve (+/+) group, #p < 0.05 and ##p < 0.001 versus EAE-control group (one-way ANOVA with Newman–Keuls post hoc test).

Fig. 6. Gene deletion of kinin B1R inhibited mRNA expression of inflammatory mediators. At the end of the 15th day, lumbar spinal cord (L3–L5) tissue was collected and processed for IL-6, CXCL-1/KC, COX-2 and NOS2 mRNA expression. Total RNA was extracted from the lumbar spinal cords of mice in the wild-type naïve (+/+) group, the control group (EAE), B1R−/− mice and B2R−/− mice on day 15 p.i. The mRNA levels of IL-6 (A), KC (B), COX-2 (C) and NOS2 (D) were measured in lumbar spinal cord tissue. All data have been normalized for levels of GAPDH expression within the same sample. The data are presented as mean ± SEM of six to nine mice/group and are representative of two independent experiments. **p < 0.001 versus wild-type naïve (+/+) group, *p < 0.05 and **p < 0.001 versus EAE-control group (one-way ANOVA with Newman–Keuls post hoc test).
It has been noted that in a large proportion of MS patients, neuropathic pain can be a major symptom just prior to or immediately at the onset of clinical deficits in the disease (Osterberg and Boivie, 2010; Osterberg et al., 2005). Our data also show that the mechanisms involved in kinin receptor-mediated hypersensitivity during EAE appear to be primarily associated with the ability of these peptides to induce the release of pro-inflammatory cytokines and increase the expression of important proteins involved in the inflammatory response, namely COX-2 and NOS-2. However, to date, we are not able to confirm whether the hypersensitivity induced by EAE is a direct effect of MOG-induced nerve injury or if the disease induction induces the activation of astrocytes or microglia cells, which through the production/release of several inflammatory mediators sensitizes primary and secondary afferent neurons in dorsal horn of spinal cord (see proposed scheme in Fig. 9). For this reason, future studies are needed to clarify this hypothesis.

In agreement with our data, other authors have shown that neuropathic pain responses are associated with kinin receptor regulation, both in peripheral tissue, such as dorsal root ganglia (DRG), and in the CNS (Ferreira et al., 2005; Quintao et al., 2008; Talbot et al., 2010; Werner et al., 2007). Once formed in the periphery, kinins have been shown to activate Aδ and C fibers in sensory nerves, producing pain or hypersensitivity in both humans and experimental animals (Calixto et al., 2004). Therefore, it may be possible to infer that blockade or deletion of kinin receptors can downregulate Aδ and C fiber activation, thus preventing the tactile and thermal hypersensitivity induced by EAE. Whether kinin receptors directly affect different fibers and consequently pain processing in the EAE model is an important question that remains to be further addressed.

Earlier studies revealed that systemic administration of kinin receptor antagonists for either the B1R (DALBK) or B2R (HOE-140) significantly inhibited cold, heat and tactile hypersensitivity in rats subjected to unilateral spinal nerve ligation injury (Werner et al., 2007). Moreover, another relevant study demonstrated that gene deletion or pharmacological inhibition of the B1R abolished the nociceptive hypersensitivity associated with sciatic nerve injury (Ferreira et al., 2005). Our data confirm and greatly extend these previous data, by demonstrating that kinin B1R seems to have a dominant role in preventing the tactile and thermal hypersensitivity induced by EAE in mice. In addition, our data also show that B1R, but not B2R, might be distinctly upregulated at the spinal level after MOG35-55 immunization. Taken together, these findings are in full accordance with previous data from our own and other groups that have shown both functional and molecular upregulation of B1R in peripheral as well as central tissues after induction of neuropathic pain in rodents (Costa et al., 2011; Fujita et al., 2010; Talbot et al., 2010; Werner et al., 2007).

Besides kinins, many other inflammatory mediators are involved in neuropathic pain; recently the cytokine IL-17, which is involved in a wide range of inflammatory and autoimmune diseases, was also shown to be involved in pain (Kim and Moalem-Taylor, 2010). The main cellular sources of IL-17 are Th17 lymphocytes (Steinman, 2007), although many other cellular types such as CD8+ lymphocytes, neutrophils (Ferretti et al., 2003) and eosinophils (Mole et al., 2001) can produce it. In addition, it has been shown that glial cells such as astrocytes and oligodendrocytes are also able to produce IL-17 (Tzartos et al., 2008). Accordingly, our data also demonstrate a direct relation between IL-17 and pain behavior. As published previously, the EAE model can be divided into three distinct phases; the induction, acute and chronic phases. The induction phase is characterized by activation and accumulation of MOG-reactive T cells in regional lymph nodes; later in the acute phase the blood brain barrier (BBB) is disrupted and
lymphocytes start to migrate to the CNS. At the end, in the chronic phase, these cells can be found accumulating in the spinal cord (Dutra et al., 2011). In this context, until the fifteenth day following EAE induction, it would be unlikely to observe large populations of Th17 lymphocytes within the CNS. Therefore, it is possible to infer that the increased level of IL-17 observed in spinal cord of EAE-wild type mice is due to activation of glial cells, such as astrocytes, which together could modulate neuropathic pain induced by EAE.

Primary cultures of astrocytes and microglial cells from postnatal rat cortex are known to express both B1 and B2 receptors (Hosli et al., 1992; Noda et al., 2003; Talbot et al., 2009). Indeed, BK has been reported to indirectly activate rat DRG neurons in culture by activating non-neuronal satellite cells (Hieblich et al., 2001). Moreover, others have demonstrated that B1R expression has been found on brain endothelial cells (Prat et al., 2000), on T lymphocytes from individuals with MS (Gobel et al., 2011; Prat et al., 2005), and on parenchymal CD3+ T cells within perivascular lesions (Schulze-Topphoff et al., 2009), as well as astrocytes and microglia activation have been found on spinal cord from animals with EAE (Dutra et al., 2011). Several lines of evidence now suggest that activated astrocytes are linked to enhanced pain states and produce chronic pain symptoms. These conditions include the following: (1) nerve injury (Wei et al., 2008; Zhuang et al., 2006), (2) inflammation (Raghavendra et al., 2004), and (3) tumor growth (Hald et al., 2009; Zhang et al., 2005). Furthermore, it is generally believed that astrocytes can modulate pain conditions by producing neuromodulators and pain mediators, such as cytokines, chemokines and growth factors such as IL-1β, IL-6, TNF-α, prostaglandin E2 (PGE2), COX-2, NO, substance P, kinins and adenosine triphosphate.

![Co-localization of B1R in spinal astrocytes before and after EAE immunization.](image-url)}
On the other hand, recently some authors have explored the participation of microglia in neuropathic pain induced by EAE (de Lago et al., 2012; Olechowski et al., 2009; Olson, 2010), and here our data also show that gene deletion of kinin B1R inhibits microglial activation in the spinal cord during development of EAE. For this reason, the complete relationship between kinin receptors, mainly B1R subtypes, and microglial activation during neuropathic pain induced by EAE must be further investigated.

In addition, reactive glial cells can modulate spinal excitability and mediate pain hypersensitivity through their production and release of growth factors such as BDNF (DeLeo and Yezierski, 2001), and here our data also show that gene deletion of kinin B3R inhibits microglial activation in the spinal cord during development of EAE. For this reason, the complete relationship between kinin receptors, mainly B3R, and microglial activation during neuropathic pain induced by EAE must be further investigated.

In summary, data from the present study reveal, for the first time, that kinins acting mainly through B3R play a relevant role in modulating neuropathic pain induced by EAE in mice prior to any overt neurological deficits, an event also reported in some patients with MS (Osterberg et al., 2005). At the cellular level, we have identified significant changes in astrocytes reactivity in the spinal cord as a potential underlying mechanism for the hypersensitivity observed in these mice (Fig. 9). Altogether, it is attractive to hypothesize that a selective antagonist for kinin B3R, administered in the earlier stages of clinical MS, might be useful in reducing neuropathic pain induced by EAE, and consequently might represent a potential therapeutic target to treat the often neglected symptoms of neuropathic pain in MS patients.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2013.02.007.

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