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# Neuropathic Pain-Like Behavior after Brachial Plexus Avulsion in Mice: The Relevance of Kinin $B_1$ and $B_2$ Receptors

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**The relevance of kinin $B_1$ ($B_1R$) and $B_2$ ($B_2R$) receptors in the brachial plexus avulsion (BPA) model was evaluated in mice, by means of genetic and pharmacological tools. BPA-induced hypernociception was absent in $B_1R$, but not in $B_2R$, knock-out mice. Local or intraperitoneal administration of the $B_2R$ antagonist Hoe 140 failed to affect BPA-induced mechanical hypernociception. Interestingly, local or intraperitoneal treatment with $B_1R$ antagonists, R-715 or SSR240612, dosed at the time of surgery, significantly reduced BPA-evoked mechanical hypernociception. Intrathecal or intracerebroventricular administration of these antagonists, at the surgery moment, did not prevent the hypernociception. Both antagonists, dosed by intraperitoneal or intrathecal routes (but not intracerebroventricularly) 4 d after the surgery, significantly inhibited the mechanical hypernociception. At 30 d after the BPA, only the intracerebroventricular treatment effectively reduced the hypernociception. A marked increase in $B_1R$ mRNA was observed in the hypothalamus, hippocampus, thalamus, and cortex at 4 d after BPA and only in the hypothalamus and cortex at 30 d. In the spinal cord, a slight increase in $B_1R$ mRNA expression was observed as early as at 2 d. Finally, an enhancement of $B_1R$ protein expression was found in all the analyzed brain structures at 4 and 30 d after the BPA, whereas in the spinal cord, this parameter was augmented only at 4 d. The data provide new evidence on the role of peripheral and central kinin $B_1R$ in the BPA model of neuropathic pain. Selective $B_1R$ antagonists might well represent valuable tools for the management of neuropathic pain.**

*Key words:* **mouse brachial plexus; neuropathic pain; hypernociception; kinins; $B_1$ receptor; $B_2$ receptor**

## Introduction

Nerve injury caused by trauma, surgery, or certain pathological states frequently leads to the development of neuropathic pain, a chronic condition that involves sensory abnormalities, including stimulus-independent persistent pain or abnormal sensory perception such as hyperalgesia (exaggerated pain sensations as a result of exposure to mildly noxious stimuli) and allodynia (pain perception on exposure to innocuous tactile stimuli) (Woolf and Mannion, 1999; Ueda and Inoue, 2001). The precise mechanisms underlying neuropathic pain and the relationship among these mechanisms, together with the signs and symptoms exhibited in patients, are not fully understood. Because of this complexity, this pathological state is currently one of the most difficult conditions to treat in clinics (Gordon and Love, 2004).

Peripheral nerve injury is commonly accompanied by local inflammation characterized by the release of pronociceptive mediators such as cytokines, neurotrophins, eicosanoids, and kinins (Bennett, 1999; Cahill et al., 2003; Sah et al., 2003; Marchand et al., 2005). Bradykinin is among the most potent endogenous algogen peptides, and its role in nociceptive processes has been reviewed extensively (Dray and Perkins, 1997; Calixto et al., 2000, 2001; Couture et al., 2001). Most kinin effects are mediated by the activation of two G-protein-coupled receptors named $B_1$ ($B_1R$) and $B_2$ ($B_2R$). The $B_2Rs$ are constitutively expressed, and they are probably involved in the acute phase of inflammatory and nociceptive responses. In contrast, $B_1Rs$ are usually absent in intact tissues and are upregulated during tissue injury. Therefore, they might represent important players in the chronic phase of pain (Couture et al., 2001; Rashid et al., 2004; Leeb-Lundberg et al., 2005; Wang et al., 2005). Recent literature data have implicated kinin $B_1R$ in neuropathic pain mechanisms. For instance, increased levels of $B_1R$ mRNA or protein have been detected in the dorsal root ganglion (DRG) after sciatic nerve constriction injury in rodents (Petersen et al., 1998; Rashid et al., 2004). In addition,

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the systemic administration of $B_1R$ antagonist des-Arg$^9$-Leu$^8$-BK was able to reduce thermal and mechanical hypernociception induced by sciatic nerve constriction in rats (Levy and Zochodne, 2000; Yamaguchi-Sase et al., 2003). Interestingly, the gene deletion of $B_1R$ practically abolished the hypernociception produced by sciatic nerve injury in mice (Ferreira et al., 2005).

Neuropathic pain caused by brachial plexus avulsion (BPA) in humans has important features such as systemic mechanical and thermal hyperalgesia, which appears immediately after the injury and produces long-lasting pain behavior (Anand and Birch, 2002). This nerve lesion may trigger a pathological plasticity of the CNS, which might be responsible for the sensory alterations mentioned above (Carvalho et al., 1997). This model of neuropathic pain was originally described for rats (Rodrigues-Filho et al., 2003), and it has recently been adapted to mice (Quintão et al., 2006, 2007). In the present study, we aimed at investigating the contribution of kinin $B_1R$ and $B_2R$ in the mouse model of BPA. Special attempts were given to determine how the expression of the inducible $B_1R$ could be altered at the periphery and both at the spinal and supra-spinal levels, over the time following BPA.

## Materials and Methods

*Subjects.* Female Swiss, 129/J and C57BL/6 wild-type, or kinin $B_1R$ and $B_2R$ knock-out mice (20–28 g) were used throughout this study. Females were used on the basis of the literature data, which indicate that they are more susceptible to neuropathic alterations (Mogil and Chanda, 2005; Quintão et al., 2006, 2007). Animals were housed under conditions of optimum light, temperature, and humidity (12 h light/dark cycle, 22 ± 1°C, under 60–80% humidity), with food and water provided *ad libitum*. Swiss mice were obtained from the Department of Pharmacology, Universidade Federal de Santa Catarina (UFSC; Florianópolis, Brazil). 129/J and C57BL/6 wild-type or kinin $B_1R$ and $B_2R$ knock-out mice were supplied by the Department of Biophysics, Universidade Federal de São Paulo (São Paulo, Brazil). The kinin $B_1R$ and $B_2R$ knock-out mice ($B_1R^{-/-}$ and $B_2R^{-/-}$) used in the present study were 129/J and C57BL/6 inbred, respectively. Experiments were conducted in accordance with current guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals laid down by Zimmermann (1983). The Ethics Committee of UFSC approved all the experimental procedures. The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects.

*Surgical procedures for BPA.* The BPA was performed according to the methodology described previously (Quintão et al., 2006, 2007). First, animals were anesthetized with 7% chloral hydrate (6 ml/kg, i.p.). The right brachial plexus was approached through a longitudinal incision parallel to the clavicle, running from the sternum to the axillary region (~1 cm). The subclavian vessels were located, and the lower trunk was dissected. The lower trunks of one group of mice were extorted by traction using forceps. In the sham-operated group, the brachial plexus was exposed and dissected without any lesion to the nerve. The tissue layers were then brought together, and the skin was closed with 4.0 silk suture string (Ethicon, Edinburgh, UK).

*Hindpaw withdrawal response induced by von Frey hairs.* To assess the mechanical hypernociception, 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 hindpaw. The animals were acclimatized for 30 min before behavioral testing. The withdrawal response frequency was measured after 10 applications (duration of 1 s each) of von Frey hairs (VFHs; Stoelting, Chicago, IL). Stimuli were delivered from below to the plantar surface of the right hindpaw. The 0.6 g VFH produces a mean withdrawal frequency of ~15%, which is considered to be an adequate value for the measurement of mechanical hypernociception (Quintão et al., 2005). Therefore, the 0.6 g VFH was used throughout this study. To determine the basal mechanical thresholds, all the animal groups were submitted to presurgical evaluation, and they were reevaluated at several time points after the surgery.

*Tail withdrawal response induced by thermal stimulus (tail flick).* A radiant heat analgesiometer (Tail-Flick Analgesia Meter; Albarsch, Porto Alegre, Brazil) was used to measure latencies for tail withdrawal according to the method described by D'Amour and Smith (1941). All the animals were evaluated to determine the basal thermal threshold, and then they were submitted to the surgery, as described above. The thermal hypernociception was evaluated at several intervals of time after the surgery. Twenty seconds was adopted as the maximal time of reaction to avoid possible tissue damage.

*Mechanical and thermal hypernociception after BPA in kinin $B_1R$ and $B_2R$ knock-out mice.* The relevance of kinin $B_1R$ or $B_2R$ for the mechanical and thermal hypernociception induced by the BPA was analyzed using kinin $B_1R$ and $B_2R$ knock-out mice ($B_1R^{-/-}$ and $B_2R^{-/-}$) and the corresponding wild-type mice (129/J or C57BL/6 strains, respectively). Briefly, the animals were submitted to BPA as described above, and the mechanical and thermal hypernociceptions were evaluated at several time points after the surgery. Each set of experiments used three groups: wild-type operated and sham-operated mice and $B_1R^{-/-}$- or $B_2R^{-/-}$ operated mice.

*Effect of selective kinin $B_2R$ antagonist on the hypernociceptive responses induced by BPA.* In this set of experiments, the involvement of kinin $B_2R$ in the mechanical hypernociception induced by BPA was evaluated by treating mice with the selective $B_2R$ antagonist Hoe 140, at different time periods after the surgery and by different pathways of administration. Initially, operated Swiss mice were treated with the peptidic selective $B_2R$ antagonist Hoe 140 (3 nmol/site, instilled locally; 50 nmol/kg, s.c.), at the moment of the surgical procedures. The mechanical hypernociception was evaluated as described previously, 4 d after the BPA. In other experimental groups, Hoe 140 was administered systemically 4 d after the surgery (50 nmol/kg, s.c.). The doses of the Hoe 140 were selected from literature data (Ferreira et al., 2002, 2004). Under these schedules of treatment, Hoe 140 did not affect the basal threshold response of animals. The results obtained in treated operated mice were compared with those obtained for operated mice that had received the vehicle alone (PBS composition (in mmol/L): 137 NaCl, 2.7 KCl, and 10 phosphate buffer).

*Effect of selective kinin $B_1R$ antagonists on the hypernociceptive responses induced by BPA.* The involvement of kinin $B_1R$ was further evaluated using two selective $B_1R$ antagonists, administered at different intervals of time and routes of administration. First, operated Swiss mice were treated with the peptidic R-715 (60 nmol/site, instilled locally; 438 nmol/kg, i.p.; 25 nmol/site, i.t.; or 25 nmol/site, i.c.v.) or the non-peptidic SSR240612 (60 nmol/site, instilled locally; 390 nmol/kg, i.p.; 25 nmol/site, i.t.; or 25 nmol/site, i.c.v.) $B_1R$ antagonists, given at the time of surgery. The mechanical hypernociception was evaluated as described previously, 4 d after the BPA.

In another set of experiments, the operated Swiss mice received R-715 (438 nmol/kg, i.p.; 25 nmol/site, i.t.; or 25 nmol/site, i.c.v.) or SSR240612 (390 nmol/kg, i.p.; 25 nmol/site, i.t.; or 25 nmol/site, i.c.v.), administered 4 d after the surgery. To gain further insights into the profile of action of the $B_1R$ antagonists, the animals that had received the antagonists by intrathecal or intracerebroventricular routes on the fourth day after the BPA also received another dose on the 10th day after the surgery.

Additional groups of operated Swiss mice were treated with R-715 (438 nmol/kg, i.p.; 25 nmol/site, i.t.; or 25 nmol/site, i.c.v.) or SSR240612 (390 nmol/kg, i.p.; 25 nmol/site, i.t.; or 25 nmol/site, i.c.v.), administered 30 d after the surgery. The mechanical hypernociception was evaluated as described previously at different intervals of time after the drug treatment.

The doses of the R-715 and SSR240612 were selected on the basis of literature data or pilot experiments (Gobeil et al., 1996; Fernandes et al., 2003; Gougat et al., 2004). Under these protocols of treatment, neither the R-715 nor the SSR240612 altered the basal threshold response of animals. The results obtained for treated operated mice were compared with those obtained for operated mice that had received vehicle only (PBS).

*General drug administration procedures.* For the local administration, $B_1R$ or $B_2R$ antagonists were dropped directly into the wound or, more exactly, they were administered onto the exposed plexus immediately after the surgery (Lindenlaub et al., 2000; Sommer et al., 2001; Quintão et

al., 2007). The intrathecal injections were performed in conscious animals to avoid possible anesthetic interference, according to the method described by Hylden and Wilcox (1980) with some modifications. The needle connected to a microsyringe by a polyethylene tubing was introduced through the skin, and a volume of 5 μl of PBS solution alone (control) or containing the drugs was injected between the L5 and L6 vertebral spaces. For intracerebroventricular injections, the animals were slightly anesthetized with ether, and a volume of 5 μl of sterile PBS containing the drugs was injected directly into the lateral ventricle (coordinates from bregma: 1 mm lateral; 1 mm rostral; 3 mm vertical) as described previously by Laursen and Belknap (1986).

*Expression of $B_1$ mRNA.* The expression of $B_1R$ mRNA was measured using reverse transcription (RT)-PCR assay, as described previously by Passos et al. (2004), with some modifications. Separate groups of operated and sham-operated mice were killed on the 2nd, 4th, or 30th day after surgery. After the mice were killed, the spinal cord and different brain structures (hypothalamus, thalamus, hippocampus, and cortex) were isolated, dissected, and frozen under liquid nitrogen and stored at −80°C. Thawed tissue was homogenized, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA were reverse transcribed using oligo(dT) as a primer (0.05 μg), 50 U of reverse transcriptase (Promega, Madison, WI), dNTP (144 μM; Promega), reaction buffer [10 mM dithiothreitol (DTT), 3 mM $MgCl_2$, 75 mM KCl, and 50 mM Tris-HCl, pH 8.3], and 2 U of RNAsin Plus (Promega) in a final volume of 12.5 μl. The cDNA was obtained after incubation of the samples for 5 min at 70°C, 4°C for 5 min, 37°C for 60 min, 70°C for 5 min, and 4°C for 5 min. The cDNA amplification of specific sequences for $B_1R$ and β-actin of mice was performed using the following primers: $B_1R$, sense AACCGTTTCAACTGGCCC and antisense GACATAAATCAGTGGGTTC; β-actin, sense TCCTTCGTTGCCGGTCCACA and antisense CGTCTCCGGAGTCCATCACA. β-Actin cDNA was used for standardization of the amount of RNA. Aliquots of 2 μl of reverse transcriptase were mixed in a buffer containing 10 mM Tris-HCl, pH 9, 1 mM $MgCl_2$, 200 μM dNTP, 300 nM of each primer, and 5 U of *Taq* polymerase (Ludwig Biotec, Porto Alegre, Brazil) in a final volume of 30 μl. The PCR cycling protocols were as follows: 4 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 53°C for $B_1R$, or 45 s at 62°C for β-actin, 60 s at 72°C, and finally, 5 min at 72°C. Aliquots of 5 μl of each sample were analyzed on a 6% Tris/borate/EDTA-polyacrylamide gel stained with silver nitrate. The size of the product was 609 bp for $B_1R$ and 509 bp for β-actin.

*Western blot studies.* Protein extraction was performed as described previously (Ferreira et al., 2005; Medeiros et al., 2007) with minor modifications. In brief, tissues were homogenized in ice-cold 10 mM HEPES, pH 7.4, containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethylsulphonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 50 mM sodium fluoride, and 0.5 mM DTT (all from Sigma-Aldrich, São Paulo, Brazil). The homogenates were chilled on ice and vigorously shaken for 15 min in the presence of 1% Triton X-100. The debris was precipitated by centrifugation at 14,000 rpm for 60 min at 4°C. The supernatant was aliquoted and stored at −70°C until use. Protein concentration was determined using the Bio-Rad (Hercules, CA) protein assay kit. Equal protein amounts were separated on SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon P; Millipore, Bedford, MA). The membranes were saturated by incubation with 10% nonfat dry milk solution and incubated overnight with one of the following antibodies: β-actin or bradykinin $B_1R$ (both from Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the membranes were incubated with adjusted secondary antibodies coupled to alkaline phosphatase. The immunocomplexes were visualized using the BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) color development substrate (Promega). Band density measurements were made using Scion (Frederick, MD) Image software package.

*Drugs and reagents.* The following drugs and reagents were used: PBS tablets and Tris (Sigma, St. Louis, MO); chloral hydrate (Vetec, Rio de Janeiro, Brazil); R-175, kindly provided by Dr. D. Regoli (University of Sherbrooke, Sherbrooke, Quebec, Canada); SSR240612 and Hoe 140 (Icatibant), kindly provided by Sanofi-Synthelabo and by Aventis, respectively (currently Sanofi-Aventis, Bridgewater, NJ). The stock solutions of the drugs were prepared in PBS in siliconized plastic tubes, maintained at −18°C, and diluted to the desired concentration just before use.

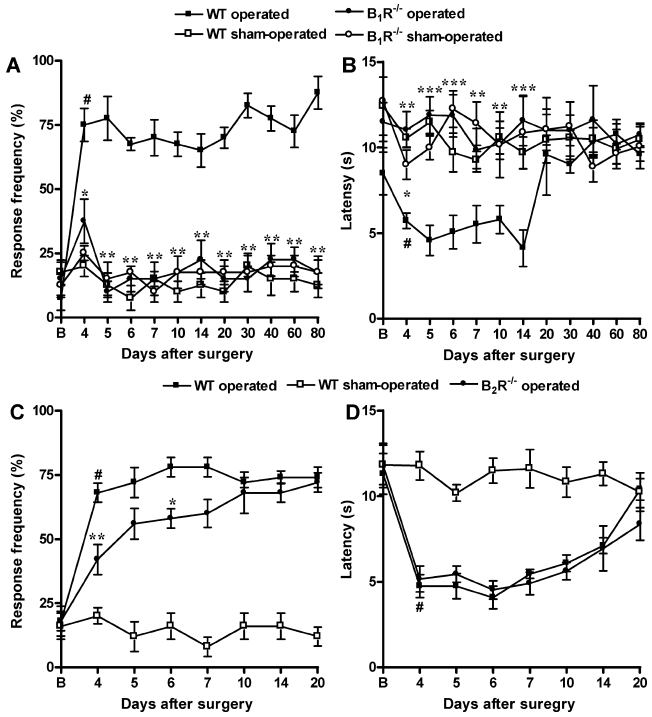


**Figure 1.** Hypernociceptive responses induced by BPA in $B_1R^{-/-}$ and $B_2R^{-/-}$ mice. Mechanical (***A***, ***C***) and thermal (***B***, ***D***) threshold of $B_1R^{-/-}$ and $B_2R^{-/-}$ mice, wild-type (WT) operated mice, and sham operated mice evaluated at different time intervals after BPA. Each group represents the mean of four to six animals, and the error bars indicate the SEM. *$p <$ 0.05, **$p < 0.01$, and ***$p < 0.001$, significantly different from wild-type operated mice; #, significantly different from wild-type sham-operated mice values. B, Baseline withdrawal threshold.

*Statistical analysis.* Results are presented as the mean ± SEM of four to six animals for each experimental group. The percentages of inhibition are reported as the difference (in percentage) between the areas under the time–response curve of the test group in relation to the corresponding control group. The statistical comparison between these values was performed by one-way ANOVA followed by the Newman–Keuls *post hoc* test. Statistical comparison of the data were performed by two-way ANOVA followed by Bonferroni's post-test. $p$ values <0.05 were considered significant.

## Results

The results in Figure 1 demonstrate that BPA induces a significant decrease in both mechanical and thermal withdrawal threshold in wild-type (both 129/J and C57BL/6 strains) mice compared with a sham-operated group, in a manner essentially similar to that described for Swiss mice (Quintão et al., 2006, 2007). When $B_1R^{-/-}$ mice were submitted to the BPA, both mechanical and thermal hypernociceptive responses were almost completely abolished ($p < 0.001$) during the entire period of evaluation (80 d after surgery) (Fig. 1*A*,*B*). However, when $B_2R^{-/-}$ mice were submitted to this surgery, a partial reduction in mechanical hypernociception was observed, but only at the initial time points after BPA (4–6 d after; inhibition of 26 ± 7%), whereas thermal hypernociception remained unaffected compared with corresponding wild-type mice (Fig. 1*C*,*D*),

The participation of kinin $B_2R$ in the mechanical hypernociception induced by BPA in mice was further investigated by using the selective $B_2R$ antagonist Hoe 140. The results presented in

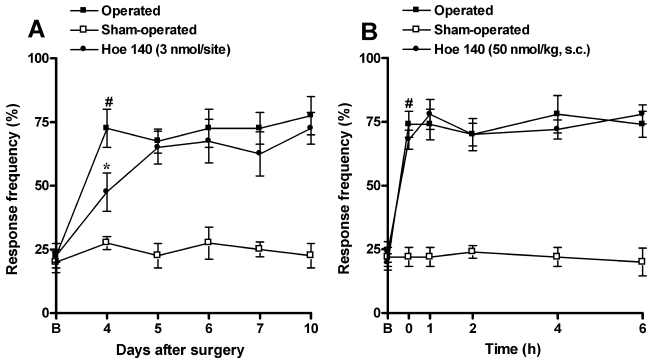


**Figure 2.** Effects of treatment with the $B_2$R-selective antagonist Hoe 140 on mechanical hypernociception induced by BPA in mice. The effect of the treatment with Hoe 140 locally (3 nmol/site; ***A***) at the moment of surgery or systemically (50 nmol/kg, s.c.; ***B***) 4 d after on mechanical hypernociception induced by BPA in mice is shown. Each group represents the mean of four to six animals, and the error bars indicate the SEM. *$p < 0.05$, significantly different from the operated group; #, significantly different from the sham-operated group. B, Baseline withdrawal threshold.

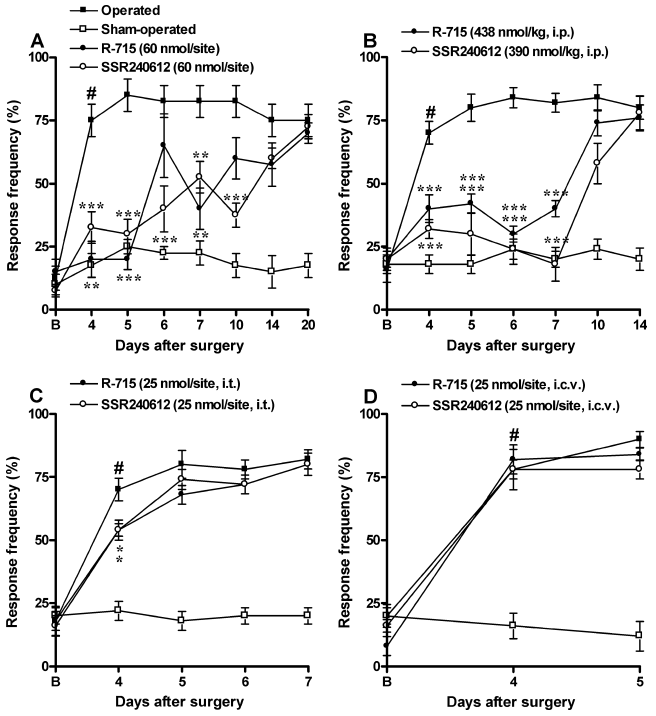


**Figure 3.** Effects of treatment with $B_1$R-selective antagonists, administered at the moment of surgery, on mechanical hypernociception induced by BPA in mice. The effects of local (***A***), intraperitoneal (***B***), intrathecal (***C***), or intracerebroventricular (***D***) treatment with R-715 or SSR240612, administered at the moment of surgery, on mechanical hypernociception induced by BPA in mice are shown. Each group represents the mean of four to six animals, and the error bars indicate the SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, significantly different from the operated group; #, significantly different from the sham-operated group. B, Baseline withdrawal threshold.

Figure 2 demonstrate that neither the local nor the systemic administration of Hoe 140 (3 nmol/site and 50 nmol/kg, s.c., respectively) was able to significantly reduce the mechanical hypernociception induced by BPA when dosed at the time of the surgery or 4 d after.

As can be seen in Figure 3*A*, the selective kinin $B_1$R antagonists R-715 and SSR240612 (both 60 nmol/site) administered locally at the time of surgery were able to prevent the mechanical hypernociception for up to 10 d thereafter, with inhibitions of 57 ± 5 and 59 ± 2%, respectively. When R-715 (438 nmol/kg, i.p.) or SSR240612 (390 nmol/kg, i.p.) was administered systemically at the time of the surgery, both of them were capable of significantly preventing the mechanical hypernociception for up to 7 d after the BPA, with inhibitions of 53 ± 2 and 67 ± 5%, respectively (Fig. 3*B*). In contrast, the administration of R-715 or SSR240612 (25 nmol/site) at the time of surgery, by either the intrathecal or intracerebroventricular route, failed to significantly affect the mechanical hypernociception induced by BPA (Fig. 3*C*,*D*).

In an attempt to further evaluate the involvement of kinin $B_1$R in the maintenance of mechanical hypernociception induced by BPA, operated mice were treated by different routes of administration 4 d after the surgical procedure. The results depicted in Figure 4*A* demonstrate that systemic treatment with R-715 (438 nmol/kg, i.p.) or SSR240612 (390 nmol/kg, i.p.) was effective in inhibiting the mechanical hypernociception for up to 2 h after drug administration, with inhibitions of 32 ± 2 and 51 ± 4%, respectively. The administration of R-715 or SSR240612 (25 nmol/site), by the intrathecal route, markedly inhibited the mechanical hypernociception for up to 6 h after the treatment (inhibitions of 62 ± 4 and 67 ± 6%, respectively). Nevertheless, when the same group of mice received an additional intrathecal dose of R-715 or SSR240612, 10 d after BPA, the inhibitory effects of the antagonists were observed for up to 2 h after (inhibitions of 36 ± 4 and 53 ± 4%, respectively) (Fig. 4*B*). Interestingly, the intracerebroventricular treatment with R-715 or SSR240612 (25 nmol/site) on the fourth day after BPA was able to significantly reduce the mechanical hypernociception induced by BPA, but only for 30 min after the treatment (inhibition of 23 ± 5 and 21 ± 4%, respectively). However, when mice received a second injection of the antagonists 10 d after the surgery, the inhibitory effects were observed for up to 4 h after the drug administration, with inhibitions of 47 ± 2 and 61 ± 3%, respectively (Fig. 4*C*).

To assess how $B_1$R might contribute to the long-term profile of mechanical hypernociception induced by BPA, different groups of mice received the antagonists 30 d after surgery. Figure 4*D* demonstrates that both R-715 (438 nmol/kg, i.p.) and SSR240612 (390 nmol/kg, i.p.) failed to significantly alter the mechanical hypernociception induced by BPA, when administered 30 d after surgery. As can be observed in Figure 4*E*, intrathecal treatment with R-715 (25 nmol/site), but not SSR240612 (25 nmol/site), significantly inhibited the mechanical hypernociception for a mere 30 min after the treatment (inhibition of 12 ± 2%). However, when the antagonists were dosed by the intracerebroventricular route (Fig. 4*F*), the mechanical hypernociception was markedly inhibited for up to 6 h after the drug administration (59 ± 2 and 66 ± 5%, respectively). We applied statistical analysis to confirm how the effects of the antagonists might be significantly different, depending on the time and on the route of administration. This set of results is depicted in Figure 5.

To evaluate the effects of BPA on the expression of kinin $B_1$R in some of the central structures, both the mRNA and the protein expression of this receptor were evaluated in Swiss mice, by means of RT-PCR and Western blot experimental protocols, respectively. As demonstrated in Figures 6 and 7, a basal kinin $B_1$R mRNA and protein expression was detected in all structures obtained from sham-operated mice. Notably, an expressive enhancement of kinin $B_1$R mRNA was observed in the spinal cord (collected 2 d after the surgery) (Fig. 6*A*); the hypothalamus, hippocampus, thalamus and cortex (collected 4 d after surgery) (Fig. 6*B*); and the hypothalamus and cortex (collected 30 d after

the BPA) (Fig. 6*C*) of operated animals compared with sham-operated mice. Furthermore, the $B_1$ protein expression was found increased in the spinal cord at 4 d and in the hypothalamus, hippocampus, thalamus, and cortex at 4 and 30 d after the surgery (Fig. 7).

## Discussion

We recently reported the relevance of the cytokine tumor necrosis factor-$\alpha$ (TNF$\alpha$) and neurotrophic factors, namely nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor, and neurotrophin-3, in the hypernociception induced by BPA in mice (Quintão et al., 2006, 2007). Evidence points out kinin $B_1$R and $B_2$R as new targets for the actions of TNF$\alpha$ during peripheral nerve injury (Leeb-Lundberg et al., 2005). Lee et al. (2002) have reported that NGF is involved in the upregulation of $B_2$R after peripheral nerve injury. Additional studies on DRG culture cells have demonstrated that GDNF upregulates $B_1$R in ~20% of a total neuronal population (Vellani et al., 2004). The authors suggest that GDNF released by cells of neighboring tissues, together with inflammatory cells, might act on injured peripheral neurons, modulating the expression of $B_1$R. Following these lines of evidence, we decided to analyze the relevance of kinin $B_1$R and $B_2$R in the hypernociceptive responses after BPA in mice. We provide evidence implicating the $B_1$R but not $B_2$R subtype in the nociceptive changes observed in the mouse BPA model. The great novelty of this study is supported by demonstrating that $B_1$R might be distinctly upregulated at the periphery and the spinal and supra-spinal levels, according to the interval of time following BPA. Our data suggest, for the first time, that temporal and regional differences in the expression of kinin $B_1$R could be related to the long-lasting profile of hypernociception observed in BPA.

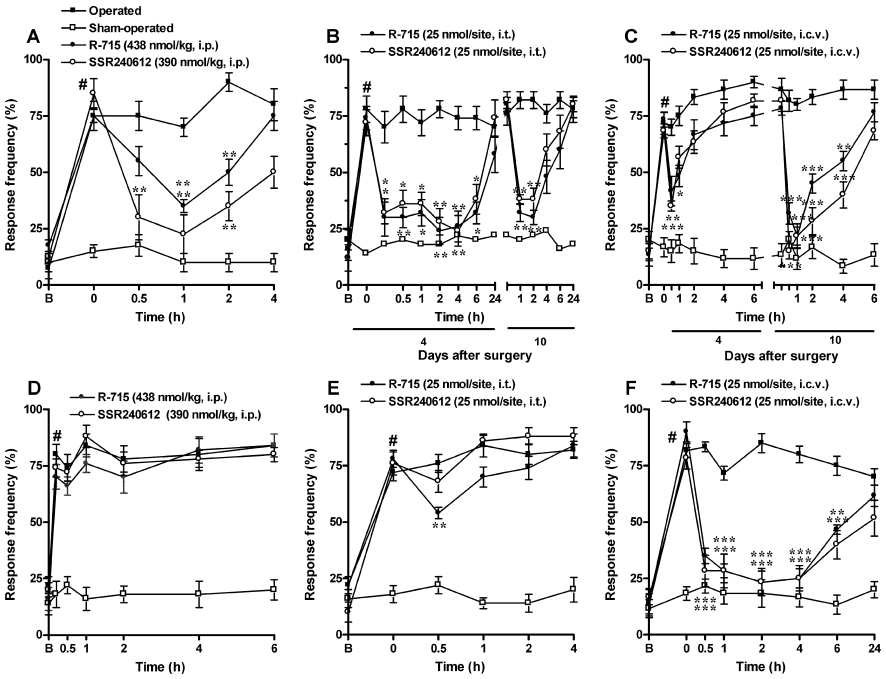


**Figure 4.** Effects of treatment with $B_1$R-selective antagonists, administered on the 4th and 30th days after surgery, on mechanical hypernociception induced by BPA in mice. The effects of intraperitoneal (***A***, ***D***), intrathecal (***B***, ***E***), or intracerebroventricular (***C***, ***F***) treatment with R-715 or SSR240612, administered on the 4th day (***A–C***) or 30th day (***D–F***) after surgery, on mechanical hypernociception induced by BPA in mice. Exceptionally, for intrathecal and intracerebroventricular treatments, mice were retreated 10 d after the surgery. Each group represents the mean of four to six animals, and the error bars indicate the SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, significantly different from the operated group; #, significantly different from the sham-operated group. B, Baseline withdrawal threshold.

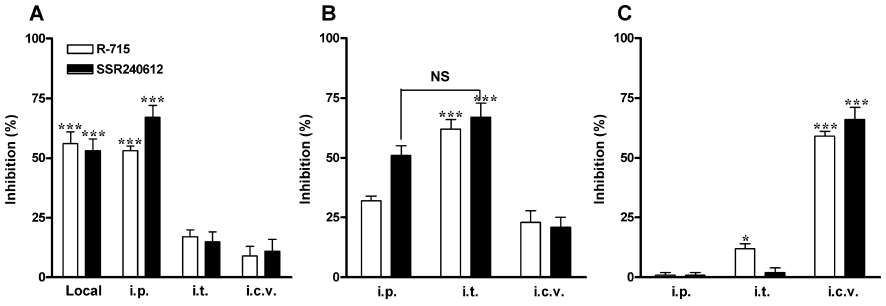


**Figure 5.** Comparison among the percentages of inhibition observed after treatment with the selective $B_1$R antagonists by different pathways of administration, at distinct intervals of time. Percentages of inhibition of R-715 or SSR241612, when administered locally, intraperitoneally, intrathecally, or intracerebroventricularly at the moment of surgery (***A***), on the 4th day (***B***), or on the 30 $^{th}$ day (***C***) after BPA, are shown. Each group represents the mean of four to six animals, and the error bars indicate the SEM. $^*p < 0.05$ and $^{***}p < 0.001$, significantly different from the operated group. NS, Not significant.

The role of kinins in mediating nociception has been well established (Calixto et al., 2000, 2001, 2004; Pesquero et al., 2000; Ferreira et al., 2005; Leeb-Lundberg et al., 2005; Campos et al., 2006). Walker et al. (1995) affirmed the existence of the kinin system components throughout the CNS. Petersen et al. (1998) demonstrated that, after partial sciatic nerve ligation (PSNL) or axotomy, there are marked changes in the expression of kinin receptors in DRG neurons. In addition, reasonable concentrations of bradykinin, and a moderate density of kinin receptors, have been described in the spinal cord, cerebellum, cortex, and hippocampus, justifying the involvement of kinins in pain control (Kariya et al., 1985; Fujiwara et al., 1989; Couture and Lindsey, 2000). $B_2$R activation has been extensively related with the initial phase of inflammatory pain, whereas $B_1$R stimulation has been associated with long-lasting alterations. Rashid et al. (2004) demonstrated the occurrence of *de novo* synthesis of $B_1$R after PSNL, mainly at large-diameter myelinated DRG neurons, whereas the $B_2$R expression was decreased. Recently, Enquist et al. (2007) demonstrated the occurrence of a rapid $B_2$R internalization after agonist exposure, followed by $B_1$R induction without desensitization.

Our first set of results revealed that both mechanical and thermal hypernociceptions induced by BPA were almost completely abolished in $B_1R^{-/-}$ mice, whereas genetic deletion of $B_2$R produced only a slight reduction in the hypernociception. Rupniak et al. (1997) showed that thermal hyperalgesia induced by intraplantar injection of complete Freund's adjuvant (CFA) was not significantly altered in $B_2R^{-/-}$ mice. Likewise, Ferreira et al. (2001) demonstrated that $B_2R^{-/-}$ mice presented similar hypernociceptive behavior after CFA injection, and these results were confirmed using the selective $B_2$R antagonist Hoe 140. Another

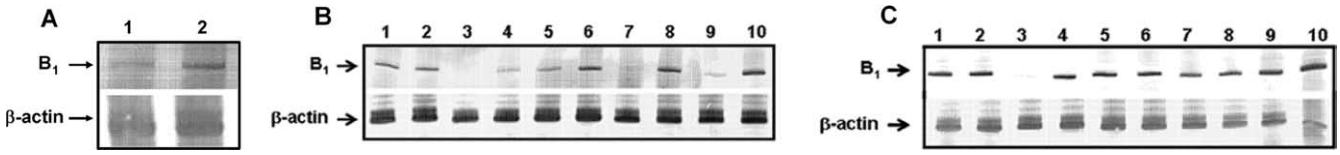


**Figure 6.** Evaluation of $B_1R$ mRNA levels in CNS structures of mice submitted to BPA. $B_1R$ mRNA levels in spinal and supra-spinal structures extracted from operated and sham-operated mice 2 d (***A***), 4 d (***B***), or 30 d (***C***) after BPA are shown. Data were normalized using respective mRNA levels for β-actin. Results were obtained with three individual experiments. Lane 1, Sham-operated spinal cord; lane 2, operated spinal cord; lane 3, sham-operated hypothalamus; lane 4, operated hypothalamus; lane 5, sham-operated hippocampus; lane 6, operated hippocampus; lane 7, sham-operated thalamus; lane 8, operated thalamus; lane 9, sham-operated cortex; lane 10, operated cortex.

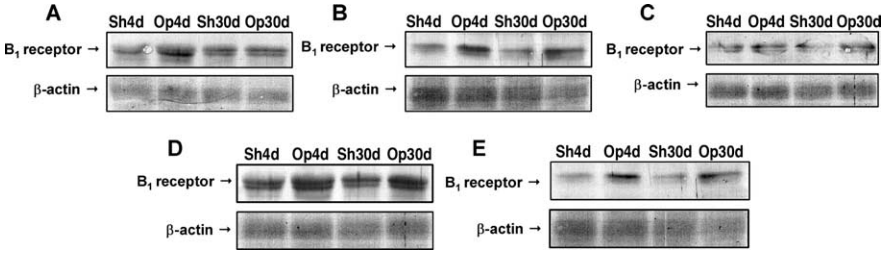


**Figure 7.** Evaluation of $B_1R$ protein expression in CNS structures of mice submitted to BPA. $B_1R$ protein levels in spinal cord (***A***), hypothalamus (***B***), hippocampus (***C***), thalamus (***D***), and cortex (***E***) extracted from sham-operated (Sh) and operated (Op) mice 4 d (4d) or 30 d (30d) after BPA, are shown. Data were normalized using respective protein levels for β-actin. Data represent three individual experiments.

interesting fact is that the hyperalgesic response induced by the selective $B_1R$ agonist des-Arg$^9$-BK was greatly enhanced in $B_2R^{-/-}$ mice (Ferreira et al., 2002). In our study, $B_2R^{-/-}$ mice presented a partial, although significant, reduction in the mechanical hypernociception after BPA, an effect that was observed only at two early time points (fourth and sixth days) after surgery. However, the thermal hypernociception remained virtually unaffected in $B_2R^{-/-}$ mice. These data allow us to imply only a minor role for $B_2R$ in the hypernociceptive changes induced by BPA. Extending this notion, the administration of the selective $B_2R$ antagonist Hoe 140, even at the moment of the surgery or 4 d later, failed to significantly alter the mechanical hypernociception induced by BPA. It is possible to conclude that $B_2R$ activation does not represent an essential step for the nociceptive behavioral changes observed in our model.

It has been shown that $B_1R^{-/-}$ mice show hypoalgesia in chemical models of nociception, probably related with a reduction in dependent activity facilitation (wind-up) of spinal nociceptive reflexes (Pesquero et al., 2000). The present results clearly suggest that $B_1R$ has a critical role in the hypernociceptive behavior caused by BPA, as evidenced by an almost complete absence of both mechanical and thermal hypernociceptive responses in $B_1R^{-/-}$ mice. Ma et al. (2000) and Wotherspoon and Winter (2000) described the presence of constitutive $B_1R$ in rat and mouse sensory neurons, justifying the reduction in nociceptive responses in $B_1R^{-/-}$ mice at the early time points after BPA. When evaluated in a persistent inflammatory model of pain (Ferreira et al., 2001), $B_1R^{-/-}$ mice presented a marked reduction in ipsilateral and contralateral thermal sensitivity induced by CFA. A comparable inhibitory effect was observed using the selective $B_1R$ antagonist des-Arg$^9$-[Leu$^8$]-BK (Ferreira et al., 2001). In addition, selective antagonists for $B_1R$ effectively reduce thermal hyperalgesia in streptozotocin–diabetic mice (Gabra and Sirois, 2002, 2003). When analyzed in concert with literature data, our results reinforce the importance of $B_1R$ in the modulation of persistent pain.

We have in mind that knock-out animals might display some compensatory differences in relation to the corresponding wild-type strain. For example, the occurrence of a compensatory increase in $B_1R$ expression has been suggested in $B_2R^{-/-}$ mice (Madeddu et al., 1997; Duka et al., 2001). Moreover, experiments conducted in $B_1R^{-/-}$ mice do not completely permit us to define whether $B_1R$s are involved in the early and/or the long-term hypernociceptive changes caused by BPA. Thus, we performed additional experiments in which selective peptide (R-715) and nonpeptide (SSR240612) $B_1R$ antagonists were dosed by different pathways of administration, at distinct intervals of time before and after BPA. These experiments supplied very interesting data concerning the role of $B_1R$ under nerve injury: (1) both the local and the systemic (intraperitoneal) administration of R-715 and SSR241206 (but not the intrathecal or intracerebroventricular) significantly prevented the mechanical hypernociception induced by BPA, when dosed at the moment of the surgery; (2) both $B_1R$ antagonists produced a marked and long-term decrease in mechanical hypernociception induced by BPA when dosed on the fourth day by the intrathecal route; and (3) the intracerebroventricular administration of R-715 and SSR240612 on the 30th day after BPA greatly reduced the mechanical hypernociception. We might infer that peripheral $B_1R$s are probably involved in the establishment of hypernociceptive alterations in this model. Alternatively, $B_1R$ might well be implicated in the maintenance of hypernociceptive processes after BPA, probably by activating spinal and supra-spinal pathways of pain control. This conclusion is reinforced by data in Figure 5, which show a statistical comparison among the percentages of inhibition after administration of $B_1R$ antagonists by different routes and at distinct intervals of time. It appears that $B_1R$s are expressed and/or activated in a different manner depending on the period after the BPA.

Ferreira et al. (2005) demonstrated that $B_1R^{-/-}$ mice had the nociceptive hypersensitization abolished when submitted to PSNL. The authors also observed that $B_1R$ mRNA was significantly enhanced in mouse plantar surface tissue, sciatic nerve, and spinal cord 7 d after PSNL. It is reasonable to suggest that the upholding of the neuropathic state in the BPA model might be dependent on the increased $B_1R$ expression at central structures related to the pain control. Therefore, we assessed the levels of $B_1R$ mRNA and the protein expression in certain central structures by means of RT-PCR and Western blot experiments, respectively, at distinct time points after BPA. First, kinin $B_1R$ mRNA expression was slightly, but visibly, enhanced in the spinal cord of operated animals at 2 d after the surgery. Second, a marked increase in $B_1R$ mRNA was found in the hypothalamus, hippocampus, thalamus, and cortex at 4 d after BPA, and in the hypothalamus and cortex at 30 d. Last, an enhancement of $B_1R$

protein expression was found in all the analyzed brain structures at 4 and 30 d, whereas it was increased only at 4 d in the spinal cord. These pieces of evidence substantiate the pharmacological experiments using the intrathecal and intracerebroventricular routes for the administration of the $B_1R$ antagonists. Whereas both the spinal and the supra-spinal upregulation of $B_1R$ seems to be related to the hypernociceptive changes observed at 4 d after BPA, only the supra-spinal modulation of this receptor is likely associated with the painful alterations at 30 d after the neuropathic lesion. Certainly, additional experiments using GTP$\gamma$[S] autoradiography to evaluate G-protein activation at central anatomical structures after $B_1R$ agonist stimulation might be useful to confirm the present molecular data. However, this remains to be investigated in the future.

Altogether, our results indicate a relevant role for peripheral and central kinin $B_1R$ (but not $B_2R$) in the mouse BPA model and call attention to the attractive potential of $B_1R$ as a new target for neuropathic pain management. Selective $B_1R$ antagonists, especially those of a nonpeptidic and orally active nature, might be useful for the control of long-lasting neuropathic states that are refractory to the currently available therapy.

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