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ORIGINAL ARTICLE

# The kinin $B_1$ and $B_2$ receptors and TNFR1/p55 axis on neuropathic pain in the mouse brachial plexus

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## Abstract

Tumour necrosis factor (TNF) and kinins have been associated with neuropathic pain-like behaviour in numerous animal models. However, the way that they interact to cause neuron sensitisation remains unclear. This study assessed the interaction of kinin receptors and TNF receptor TNFR1/p55 in mechanical hypersensitivity induced by an intraneural (i.n.) injection of rm-TNF into the lower trunk of brachial plexus in mice. The i.n. injection of rm-TNF reduced the mechanical withdrawal threshold of the right forepaw from the 3rd to the 10th day after the injection, indicating that TNF1/p55 displays a critical role in the onset of TNF-elicited neuropathic pain. The connection between TNF1/p55 and kinin $B_1$ and $B_2$ receptors ($B_1R$ and $B_2R$) was confirmed using both knockout mice and mRNAs quantification in the injected nerve, DRG and spinal cord. The treatment with the $B_2R$ antagonist HOE 140 or with $B_1R$ antagonist des-Arg$^9$-Leu$^8$-BK reduced both BK- and DABK-induced hypersensitivity. The experiments using kinin receptor antagonists and CPM inhibitor (thiorphan) suggest that BK does not only activate $B_2R$ as an orthosteric agonist, but also seems to be converted into DABK that consequently activates $B_1R$. These results indicate a connection between TNF and the kinin system, suggesting a relevant role for $B_1R$ and $B_2R$ in the process of sensitisation of the central nervous systems by the cross talk between the receptor and CPM after i.n. injection of rm-TNF.

**Keywords** Brachial plexus · Cytokine · TNF · Neuropathic pain · Kinin receptors · Carboxypeptidase

## Introduction

Tumour necrosis factor (TNF) is a pivotal pro-inflammatory cytokine, mainly secreted by immune cells (macrophages, monocytes, neutrophils and lymphocytes), as well as by keratinocytes, fibroblasts, and skin dendritic cells (Aggarwal et al. 2012). Furthermore, Schwann and glial cells also produce TNF in the central nervous system (CNS) in response to inflammation, infection, or tissue damage (Compston et al. 1997; Paul et al. 2006; Olmos and Lladó 2014). It is well established that TNF is critically implicated in the initial degeneration of peripheral nerves, in a process that is dependent on NF-κB activation (George et al. 2004; Bastien and Lacroix 2014). The presence and transport of TNF and its receptors into the injured nerve have been described previously (Shubayev and Myers 2000, 2001; Zeng et al. 2014). It has been well established that rats submitted to a chronic nerve-constriction injury (CCI) model presented accumulated levels of TNF in the mid-axonal region of the sciatic nerve, 5 days after injury. Of relevance, when injected

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intraneurally (i.n.), TNF was capable of reaching the dorsal root ganglia (DRG) within 6 h following the injection (Shubayev and Myers 2001). Andrade et al. (2011) demonstrated that TNF inhibition alleviates the hypersensitivity induced by CCI, and that neuropathic pain recovery was associated with decreased TNF levels in both DRG and spinal cord.

The role of bradykinin $B_1$ and $B_2$ receptors ($B_1R$ and $B_2R$) in mediating nociceptive responses has also been widely investigated and there is a vast literature indicating that kinin effects rely on the production of pro-inflammatory cytokines, such as TNF (Calixto et al. 2004; Leeb-Lundberg et al. 2005; Ferreira et al. 2005). For instance, it was demonstrated that a co-injection of the anti-TNF antibody displays marked inhibitory effects on the hyperalgesia elicited by the injection of $B_1R$ or $B_2R$ selective agonists into the rat paw (Poole et al. 1999). Furthermore, the intraplantar injection of a $B_1R$ agonist resulted in a time-related increase of TNF levels in LPS-primed mouse paws and the $B_1R$-mediated hyperalgesia was greatly reduced by the co-injection with an antibody against TNF (Cunha et al. 2007). Additionally, the pharmacological inhibition of either $B_1R$ or $B_2R$ consistently prevented the increase of TNF mRNA in a model of muscle pain induced by formalin in mice, reinforcing the notion that kinin and TNF receptors likely seem to interact to mediate persistent pain (Meotti et al. 2012). More recently, Podsiadło et al. (2017) demonstrated a crucial role for $B_1R$ in the progression of the neurological symptoms in rats submitted to the experimental autoimmune encephalomyelitis model. The reduction of $B_1R$ activation was accompanied by the decrease of cytokine levels, including TNF.

In a previous study, we described a relevant role exerted by TNF in the mechanical hypersensitivity induced by brachial plexus avulsion (BPA) in mice (Quintão et al. 2006). Furthermore, we provided compelling evidence indicating the participation of both peripheral and central kinin $B_1R$ (but not $B_2R$) in the mouse BPA model (Quintão et al. 2008). With the above information in mind, in the present study, we aimed to unravel whether TNF and kinin receptors interact to induce neuropathic pain, by intraneurally (i.n.) injecting TNF or kinin agonists into the lower trunk of the brachial plexus (LTBP) of mice. Our data provide novel and interesting evidence implicating a possible cross talk between TNFR1/p55 and both $B_1R$ and $B_2R$ in the long-lasting pain processes associated with nerve injury.

## Materials and methods

### Animals and ethical statement

Female 129/J and C57BL/6 wild-type or TNFR1/p55 and either kinin $B_1R$ or $B_2R$ knockout (−/−) mice (20–28 g) were used in this study (total number mice = 311 mice; $N = 6$ mice per group; further details are provided below). Females were used based on previous data, which indicates that they are more susceptible to neuropathic pain (Mogil and Chanda 2005; Quintão et al. 2006, 2008). Animals were housed under conditions of optimum light, temperature, and humidity (12 h light–dark cycle, 22 ± 2 °C, under 60 to 80% controlled humidity), with food and water provided ad libitum. TNFR1 $p55^{-/-}$ were C57BL/6 inbred supplied by the Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, Brazil), and $B_1R^{-/-}$ and $B_2R^{-/-}$ were 129/J inbred and they were kindly supplied by the Department of Biophysics, Federal University of São Paulo (UNIFESP-EPM, São Paulo, Brazil).

The local Animal Ethics Committee approved all the experimental procedures, with Protocol No. 020/2011. The procedures were carried out in accordance with the "Principles of Laboratory Animal Care" from NIH Publication No. 85–23. The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects.

### Induction of neuropathic pain and treatments

The right LTBP was surgically exposed as described elsewhere (Quintão et al. 2006, 2008), under deep anaesthesia after inhalation with 3% isoflurane plus 3% oxygen. Recombinant mouse TNF (rmTNF, 12.5 fg/site; Zelenka et al. 2005), bradykinin (BK; 1, 10 or 100 pmol/site), des-$Arg^9$-BK (DABK, a selective $B_1R$ agonist; 1, 10 or 100 pmol/site) or phosphate-buffered saline (PBS) was injected i.n. into the right LTBP, in a volume of 1 µL, using a 30-G needle coupled to a Hamilton syringe (Zelenka et al. 2005) (Fig. 1a–d). The tissue layers were brought together and the skin closed with 4.0 silk suture strings (Ethicon, Edinburgh).

In separate experimental groups, an anti-murine neutralising antibody anti-TNF (10 ng/site) was co-injected with rm-TNF, or given intravenously (i.v.; 100 µg/kg). The selective $B_2R$ (HOE 140; 1 pmol/site) or the $B_1R$ [des-$Arg^9$-$[Leu]^8$-BK; des-$Arg^9$-$Leu^8$-BK (DALBK); 1 pmol/site] antagonists were co-injected with rm-TNF (12.5 fg/site), BK (100 pmol/site) or DABK (100 pmol/site), or dosed intraperitoneally (i.p.; Hoe 140, 43.69 nmol/kg; DALBK, 1 µmol/kg), 30 min before the i.n. injections (Ferreira et al. 2002, 2004).

To assess whether the effects of BK might be mediated by its degradation into DABK, the carboxypeptidase M (CPM) inhibitor thiorphan was injected i.v. (1 mg/kg) (Simard et al. 2009) either at the moment of the neuropathy induction or 4 days later, alone or combined with the kinin agonists or antagonists. The doses of drugs used throughout the study were based on the literature data or pilot study.

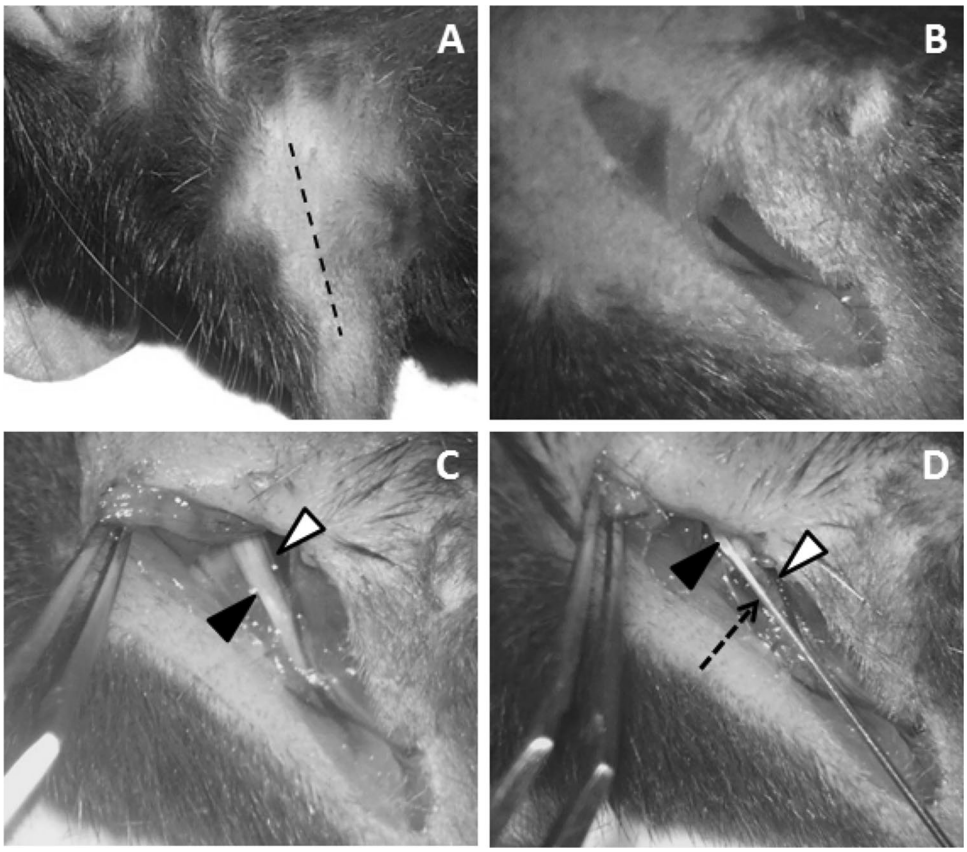


**Fig. 1** Illustration showing the surgical procedure for i.n. injection. **a**, **b** Region of incision, **c** the brachial plexus anatomy, **d** injection into the lower trunk of brachial plexus. Lower trunk of brachial plexus (black arrow); subclavian vessels (white arrow); 30 G needle (dashed arrow)

## Forepaw mechanical withdrawal response induced by Von Frey hair

To assess the mechanical hypersensitivity, mice were individually allocated into clear Plexiglas boxes (9 × 7 × 11 cm) on elevated wire mesh platforms to allow access to the ventral surface of the right forepaw. The animals were acclimatised for 30 min prior to behavioural testing. The mechanical withdrawal response frequency was measured following ten applications (duration of 1 s each) of Von Frey hair filaments (VFH, Stoelting, Chicago, USA). Stimuli were delivered from below to the plantar surface of the right forepaw. The 0.16-g VFH produces a mean withdrawal frequency of about 20%, which is considered an adequate value for the measurement of mechanical hypersensitivity (data not shown). The 0.16-g VFH was used throughout this study. To determine the basal mechanical withdrawal thresholds, all the animal groups were submitted to pre-surgical evaluation and they were re-evaluated at several time points from 3 to 14 days after the surgery (Quintão et al. 2006).

## Mechanical hypersensitivity in TNFR1/p55$^{-/-}$, $B_1R^{-/-}$, or $B_2R^{-/-}$ mice

The relevance of TNFR1/p55 receptor for mechanical hypersensitivity induced by the i.n. injection of TNF or kinins was also analysed by using TNFR1/p55 knockout mice. The animals received an i.n. injection of rm-TNF (12.5 fg/site), BK (100 pmol/site), or DABK (100 pmol/site), as described before. To further investigate the relevance of kinin receptors for TNF-induced neuropathic pain, $B_1R^{-/-}$ and $B_2R^{-/-}$ mice were submitted to rm-TNF injection (12.5 fg/site). The correspondent wild-type mice were used as control. The mechanical hypersensitivity was assessed at the same time point intervals (from 3 to 14 days) after the injection of TNF or kinin agonists into the mouse LTBP.

## RNA extraction and real-time PCR

The expression of $B_1R$, $B_2R$, or TNF mRNA was measured by using quantitative RT-PCR. Separate groups of animals were euthanised on the 7th day following the i.n. injection of TNF or kinin agonists. Control animals received i.n. injections of PBS. The LTBP, the spinal cord (C4–T2), and the respective dorsal root ganglion (DRG; C4–T2 spinal cord segment) were isolated, dissected, and frozen under liquid nitrogen and stored at − 80 °C. Total RNA was extracted using the TRizol® protocol, and its concentration was determined using a NanoDrop 1100 apparatus (NanoDrop Technologies, Wilmington, DE, USA). A reverse transcription assay was performed as described in the M-MLV reverse transcriptase protocol according to the

manufacturer's instructions. cDNA (100 ng) 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-labelled probes for mouse $B_1R$ (Mm00432059_s1), $B_2R$ (Mm01339907_m1) and TNF (Mm00443258_m1), as well as GAPDH (NM_008084.2), which was used as an endogenous control for normalisation. The PCR reactions were performed in 96-well optical reaction plates (Applied Biosystems, Foster City, CA, USA). The thermocycler parameters were as follows: 50 °C for 2 min, 95 °C for 10 min, 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. Fluorescence was measured for each amplification cycle and the data were analysed using the $2^{-\Delta\Delta CT}$ method for relative quantification of expression. The expression of the target genes was calibrated based on the expression detected in control animals, i.e. those that received the vehicle.

### Drugs and reagents

The following drugs and reagents were used: BK, DABK, DALBK, PBS tablets, Tris (Sigma Chemical Company, St. Louis, USA); HOE 140 (icatibant; kindly provided by Aventis, currently Sanofi-Aventis); thiorphan (Peninsula Laboratories International, Inc., San Carlos, USA). Rm-TNF and anti-TNF (R&D Systems Inc., Minneapolis, USA). The stock solutions of the drugs were prepared in PBS in siliconised plastic tubes, maintained at − 20 °C, and diluted to the desired concentration just before use. TRizol® and M-MLV reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). Primers and probes for mouse $B_1R$ (Mm00432059_s1), $B_2R$ (Mm01339907_m1), TNF (Mm00443258_m1), and GAPDH (NM_008084.2), as well as TaqMan® Universal PCR Master Mix Kit were purchased from Applied Biosystems (Foster City, CA, USA).

### Statistical analysis

The results are presented as the mean ± standard error mean of three (mRNA expression) to six animals (behavioural assessments) per group. For mechanical hypersensitivity, the inhibition indexes were determined based on the areas under the curve (AUC). The statistical comparison of data in the time-related curves was performed by two-way ANOVA followed by Bonferroni's post-test. For RT-PCR experiments, the statistical comparison was performed by one-way analysis of variance (ANOVA), followed by the multiple comparison Newman–Keuls test. $p$ values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

## Results

### TNF induces neuropathic pain via activation of TNFR1/p55

These experiments were conducted to evaluate the ability of TNF in causing neuropathic pain behaviour and the possible involvement of its receptor TRFR1/p55 in this effect. The i.n. LTBP injection of rm-TNF (1.25–12.5 fg/site) elicited a marked increase in the mechanical withdrawal response frequency, following the application of 0.16-g VFH into the right mouse forepaw, peaking on day 6, with an effect that persisted for up to 10 days (Fig. 2a, b; two-way ANOVA, $F_{(4,140)} = 281.13$, $p < 0.0001$, for treatment). This response was prevented when ab-TNF (10 ng/site) was co-injected with rm-TNF (Fig. 2c, d; two-way ANOVA, $F_{(2,89)} = 150.67$, $p < 0.0001$, for treatment). The gene deletion of TNFR1/p55 also abolished the mechanical hypersensitivity caused by rmTNF i.n. injection into the LTBP (Fig. 2e, f) (two-way ANOVA, $F_{(3,91)} = 74.84$, $p < 0.0001$, for treatment).

### Intraneural administration of kinin agonists into the LTBP elicits mechanical hypersensitivity in mice

In this set of experiments, the animals received an i.n. injection containing BK or DABK to standardise the method and evaluate the involvement of kinin receptors. The results depicted in Fig. 3a–d show that i.n. injection of BK or DABK (1 to 100 pmol/site) into the mouse LTBP reduced the mechanical withdrawal threshold, in a dose-dependent manner (two-way ANOVA, BK: $F_{(3,102)} = 22.93$, $p < 0.0001$, for treatment; DABK: $F_{(3,96)} = 23.80$, $p < 0.0001$, for treatment). For both agonists, the mechanical hypersensitivity peaked as early as 3 days after i.n. injection, persisting for up to 10 days. The response induced by the i.n. injection of BK was partially reduced by the co-treatment with either the selective $B_1R$ or $B_2R$ antagonists DALBK (27 ± 7%) and HOE 140 (28 ± 4%), respectively (Fig. 3e, f; two-way ANOVA, $F_{(2,48)} = 7.69$, $p = 0.0071$ for treatment). Likewise, the mechanical hypersensitivity induced by the i.n. injection of DABK ($B_1$ selective agonist) was consistently reduced by co-injecting DALBK (a $B_1R$ antagonist) and HOE 140 (a selective $B_2R$ antagonist), with inhibitions of 31 ± 4% and 19 ± 3%, respectively (Fig. 3g, h; two-way ANOVA, $F_{(2,48)} = 7.51$, $p = 0.0077$ for treatment).

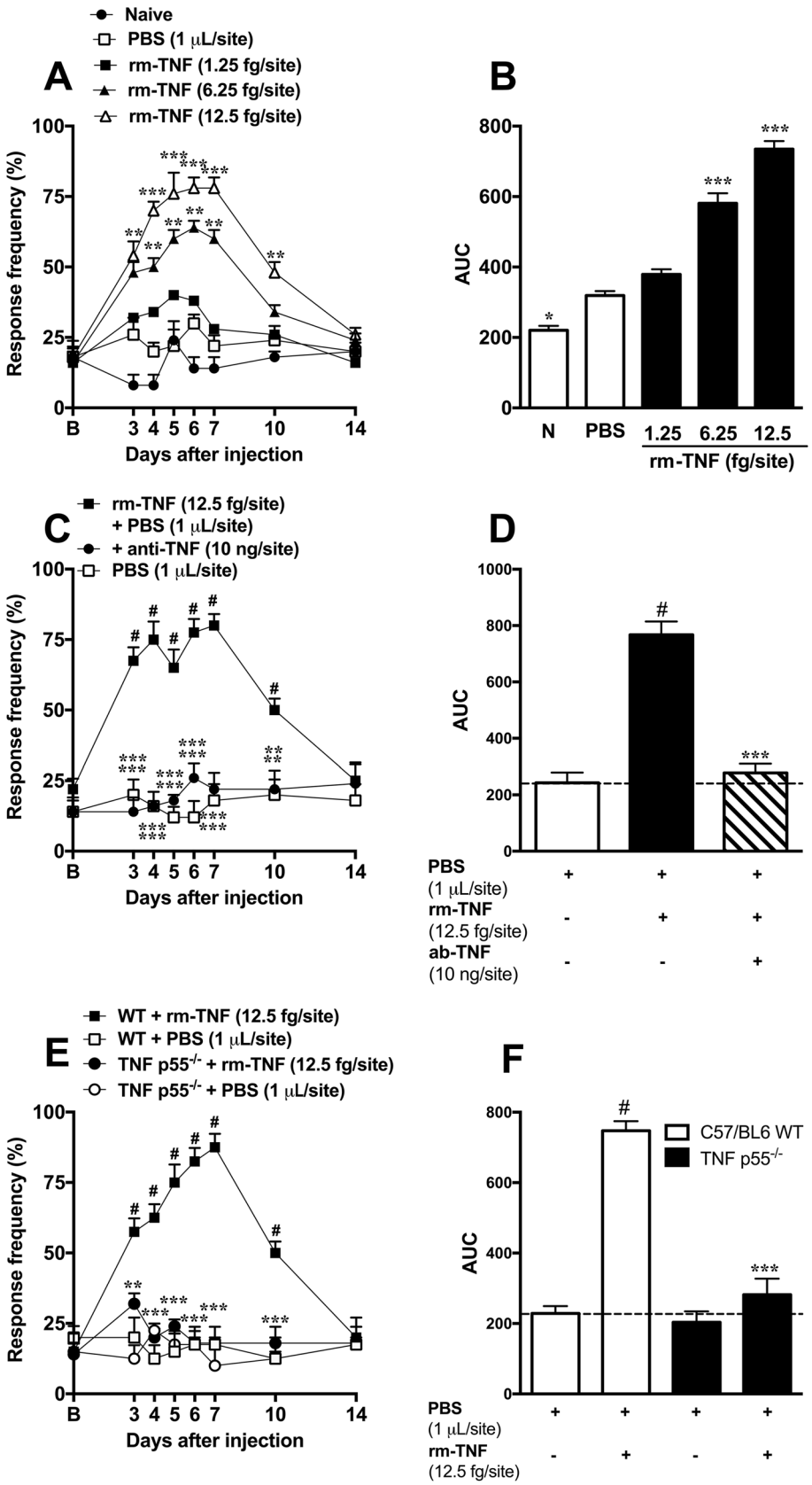


**Fig. 2** Mechanical hyperalgesia of mice injected with rm-TNF into the lower trunk of brachial plexus. **a**, **b** Method standardisation. **c**, **d** Mice co-treated with antibody against TNF; **e**, **f** TNF $p55^{-/-}$ mice injected with rm-TNF. Each group represents the mean of five to six animals and the vertical line bars indicate the S.E.M. Significantly different from rm-TNF group values $^{**}p < 0.01$ and $^{***}p < 0.001$ and significantly different from PBS-injected group $\#p < 0.001$ (one-way ANOVA followed by the Dunnett's post hoc test for column graph and two-way ANOVA followed by Bonferroni's post hoc test for line graph). **b**, **d**, **f** AUC of the respective line graph. 'B'—basal threshold

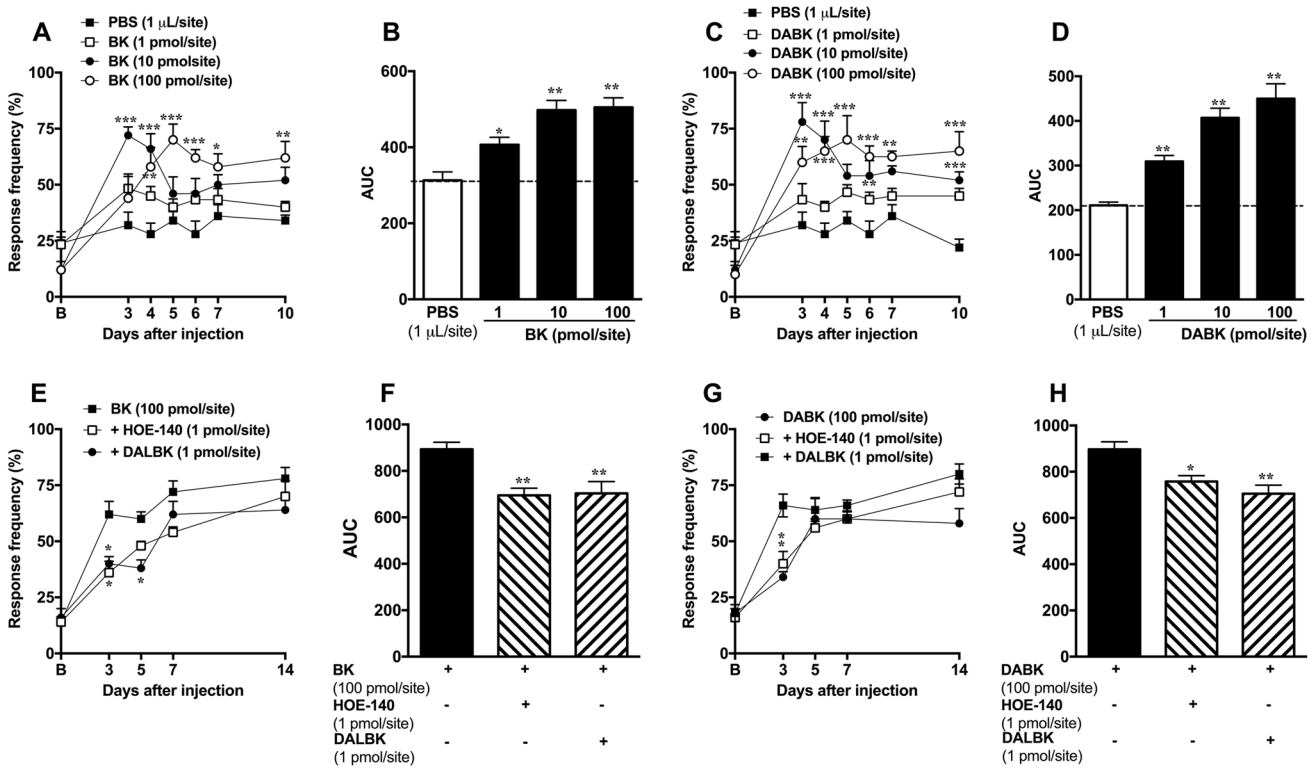


**Fig. 3** Mechanical hypersensitivity of mice injected with BK or DABK into the lower trunk of brachial plexus. **a**–**d** Method standardisation; **e**, **f** Mice co-treated with HOE 140 or DALBK and BK. **g**, **h** Mice co-treated with HOE 140 or DALBK and DABK. Each group represents the mean of five to six animals and the vertical lines indicate the S.E.M. Significantly different from BK or DABK group values $*p<0.05$, $**p<0.01$ and $***p<0.001$, and different from PBS-injected group $\#p<0.001$. (one-way ANOVA followed by the Dunnett's post hoc test for column graph and two-way ANOVA followed by Bonferroni's post hoc test for line graph). **b**, **d**, **f**, **h** AUC of the respective line graph. 'B'—basal threshold

## Possible cross talk between TNFR1/p55 and kinin receptors in LTBP neuropathic pain

The results mentioned below demonstrate the correlation between TNF and kinin receptors in neuronal sensitisation. Figure 4 shows that mice lacking TNFR1/p55 displayed diminished hypersensitivity caused by the i.n. LTBP injection of either BK or DABK, both given at 100 pmol/site (Fig. 4a–c). The inhibitory rates were $50 \pm 4\%$ (two-way ANOVA, $F_{(1,63)} = 67.52$, $p < 0.0001$ for group) and $34 \pm 3\%$ (two-way ANOVA, $F_{(1,63)} = 38.07$, $p = 0.0002$ for group), for BK and DABK, respectively. Of note, mice with deletion of the $B_1R$ or $B_2R$ gene exhibited a pronounced reduction in the mechanical hypersensitivity evoked by an i.n. injection of rm-TNF (12.5 fg/site) into the LTBP, with inhibitions of $70 \pm 5\%$ and $83 \pm 10\%$, respectively (Fig. 4d, e; two-way ANOVA, $F_{(5,126)} = 49.71$, $p < 0.0001$ for group).

The interaction of TNF and kinin receptors was also investigated through analysis of the mRNA expression in different nervous tissues. The i.n. injection of rm-TNF (12.5 fg/site) into the mouse LTBP led to the increased expression of $B_1R$ mRNA when assessed in both the spinal cord and the DRG (Fig. 5a; $p < 0.001$), whereas the $B_2R$ mRNA expression was only increased in the DRG of rm-TNF-treated mice (Fig. 5b; $p < 0.001$). Conversely, animals that received an i.n. injection of BK presented increased TNF mRNA levels in the LTBP (Fig. 5c; $p < 0.001$), whilst the i.n. injection of DABK led to an increase in TNF mRNA expression in both the DRG and the nerve (Fig. 5d; $p < 0.001$).

## Characterisation of the mechanism underlying kinin-induced neuropathy in LTBP

As kinins are clearly involved in neuropathic pain induction and maintenance, the next step was to characterise the role of their receptors using selective antagonists. Data in Fig. 6a show that treatment with the selective $B_2R$ antagonist HOE 140 (43.69 nmol/kg, i.p.), dosed 30 min before the i.n. injection of BK (100 pmol/site), reduced mechanical hypersensitivity, with an inhibitory rate of $39 \pm 5\%$ (two-way ANOVA, $F_{(1,68)} = 37.71$, $p < 0.0001$ for treatment). However, the mechanical hypersensitivity caused by the i.n. injection of BK (100 pmol/site) was clearly inhibited by pre-treatment

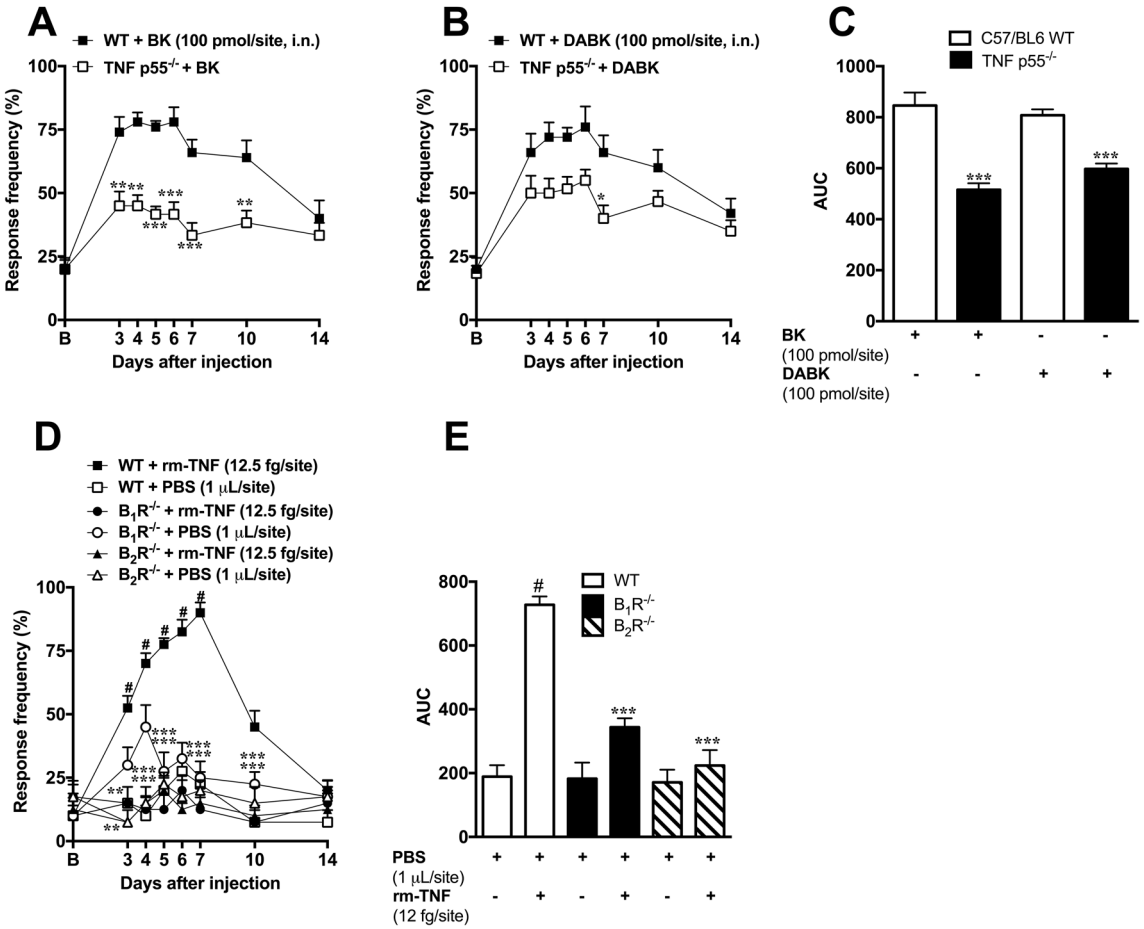


**Fig. 4** Mechanical hypersensitivity of TNF $p55^{-/-}$ mice injected with **a**, **c** BK or **b**, **c** DABK into the LTBP. **d**, **e** Mechanical withdrawal threshold of $B_1R^{-/-}$ and $B_2R^{-/-}$ mice injected with rh-TNF into the LTBP. Each group represents the mean of five to six animals and the vertical lines bars indicate the S.E.M. Different from BK, DABK, or rh-TNF group values $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$, and different from PBS-injected group $\#p<0.001$ (one-way ANOVA followed by the Dunnett's post hoc test for column graph and two-way ANOVA followed by Bonferroni's post hoc test for line graph). **c**, **e** AUC of the respective line graph. 'B'—basal threshold

with the selective $B_1R$ antagonist DALBK (1 μmol/kg, i.p.), given 30 min prior to BK, with an inhibition percentage of 67 ± 4% (Fig. 6b; two-way ANOVA, $F_{(1,68)}=73.98$, $p<0.0001$ for treatment).

DABK-induced neuropathic pain in the LTBP was reduced by treatment with the selective $B_1$ receptor antagonist DALBK (1 μmol/kg, i.p.) (Fig. 6c; two-way ANOVA, $F_{(1,36)}=24.87$, $p=0.0025$ for treatment), with inhibition percentages of 52 ± 6%. However, i.p. treatment with the selective $B_2$ receptor antagonist HOE 140 (43.69 nmol/kg) did not modify the nociceptive response induced by the i.n. injection of DABK (100 pmol/site) (Fig. 6d, two-way ANOVA, $F_{(1,36)}=0.21$, $p=0.662$ for treatment).

## Possible impact of carboxypeptidase (CPM) inhibition on kinin-induced mechanical hypersensitivity

As both $B_1R$ and $B_2R$ selective antagonists were able to interfere with BK-induced mechanical hypersensitivity, and considering their well-known selectivity, the CPM inhibitor thiorphan was used to assess the relevance of BK conversion into DABK in our experimental paradigm. Data depicted in Fig. 7a (schematic presentation of signalling pathway in Fig. 7a) demonstrate that pretreatment with the CPM blocker thiorphan (1 mg/kg, i.v.), 15 min before BK (100 pmol/site) i.n. injection,

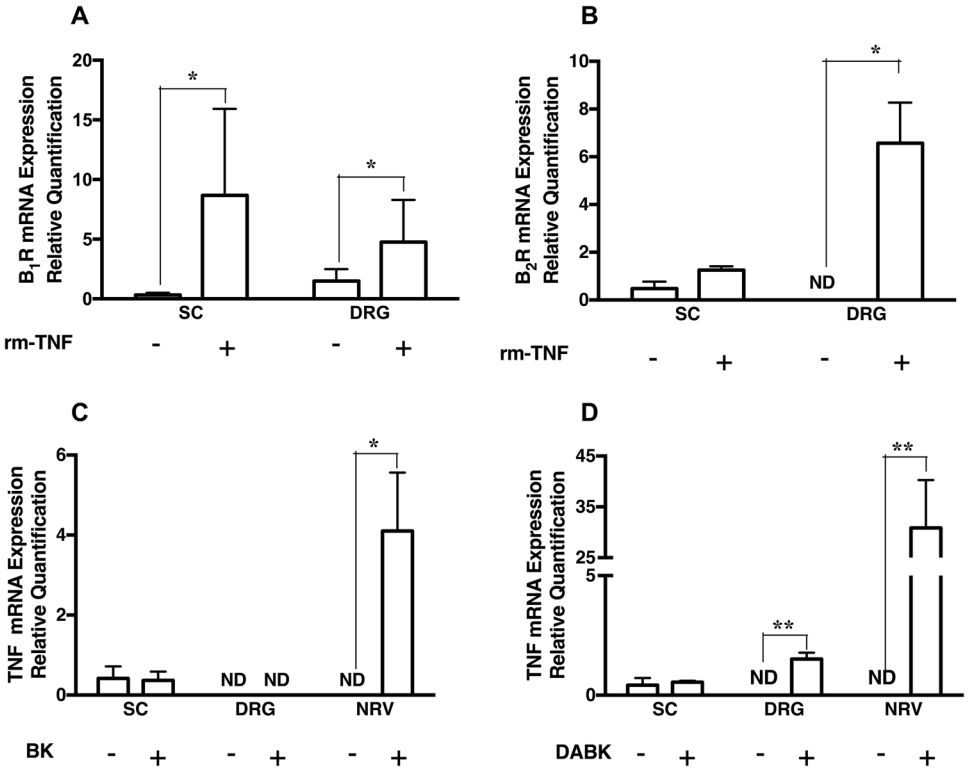


**Fig. 5** Evaluation of **a** $B_1R$ mRNA, **b** $B_2R$ mRNA, and **c**, **d** TNF mRNA levels in the spinal cord, dorsal root ganglia, and LTBP of mice injected with rh-TNF, BK or DABK, collected 7 days after injections. Each group represents the mean of three to five samples and the vertical bars indicate the S.E.M. Different from PBS-injected group, values $*p < 0.05$ and $***p < 0.001$ (Student's *t* test). 'SC'—spinal cord; 'DRG'—dorsal root ganglia; 'LTBP'—lower trunk of brachial plexus

reduced mechanical hypersensitivity, with an inhibition of 40 ± 9% (two-way ANOVA, $F_{(1,36)} = 21.00$, $p = 0.0038$ for treatment), suggesting an important role of CPM in the genesis of neuropathic pain. As observed in Fig. 7b, systemic treatment with thiorphan (1 mg/kg, i.v.) 4 days after the i.n. BK injection consistently enhanced the mechanical withdrawal threshold for up to 24 h after the treatment, reducing the mechanical hypersensitivity by 57 ± 6% (two-way ANOVA, $F_{(1,48)} = 71.46$, $p > 0.0001$ for treatment; schematic presentation of signalling pathway in Fig. 7b). The results presented in the Fig. 8c also show a comparable inhibition when HOE 140 or DALBK was administered together 4 days after BK injection, with a reduction rate of 63 ± 3% (two-way ANOVA, $F_{(1,36)} = 59.27$, $p = 0.0003$ for treatment; schematic presentation of signalling pathway in Fig. 7c).

In an attempt to verify whether spinal $B_1R$ retained its ability to cause a nociceptive response, even after the treatment with thiorphan, mice that received an i.n. injection of BK (100 pmol/site) 4 days before were systemically treated with thiorphan and, 30 min later, received an i.t. injection of DABK (i.t.; 1 nmol/site). As expected, mice submitted to BK-induced neuropathy and treated with thiorphan had the mechanical threshold restored to the same values as the control group after the i.t. injection of DABK (data not shown).

## Discussion

The present study demonstrates a long-lasting hypersensitivity after an i.n. injection of rm-TNF into the LTBP of mice. This effect seems to be directly related to activation of the TNF p55 receptor, as the co-administration of the anti-TNF antibody or the use of TNF $p55^{-/-}$ largely prevented this neuropathic pain-like behaviour. Zelenka et al. (2005) previously showed that i.n. TNF, injected into the rat sciatic nerve, elicited neuropathic pain in a bell-shaped dose–response curve, similar to the results obtained in this study. The same authors also observed an increase in p38 MAPK and NF-κB activation in the L4 DRG of mice injected with TNF. Besides the involvement of TNF in hypersensitisation mechanisms, this cytokine has been linked to the modulation of early degenerative changes after peripheral nerve injury (Shamash et al. 2002). Furthermore, TNF has also been implicated in other events associated with mechanical hypersensitivity, such as axonal damage, macrophage recruitment, and ectopic activity in injured peripheral nerves (Perkins and Tracey 2000; Schäfers et al. 2003; George et al. 2004). Chronologically, TNF expression was observed as soon as 1 h after nerve injury (Schäfers et al. 2003; Sacerdote et al. 2008; Kato et al. 2009). It has been demonstrated that TNF

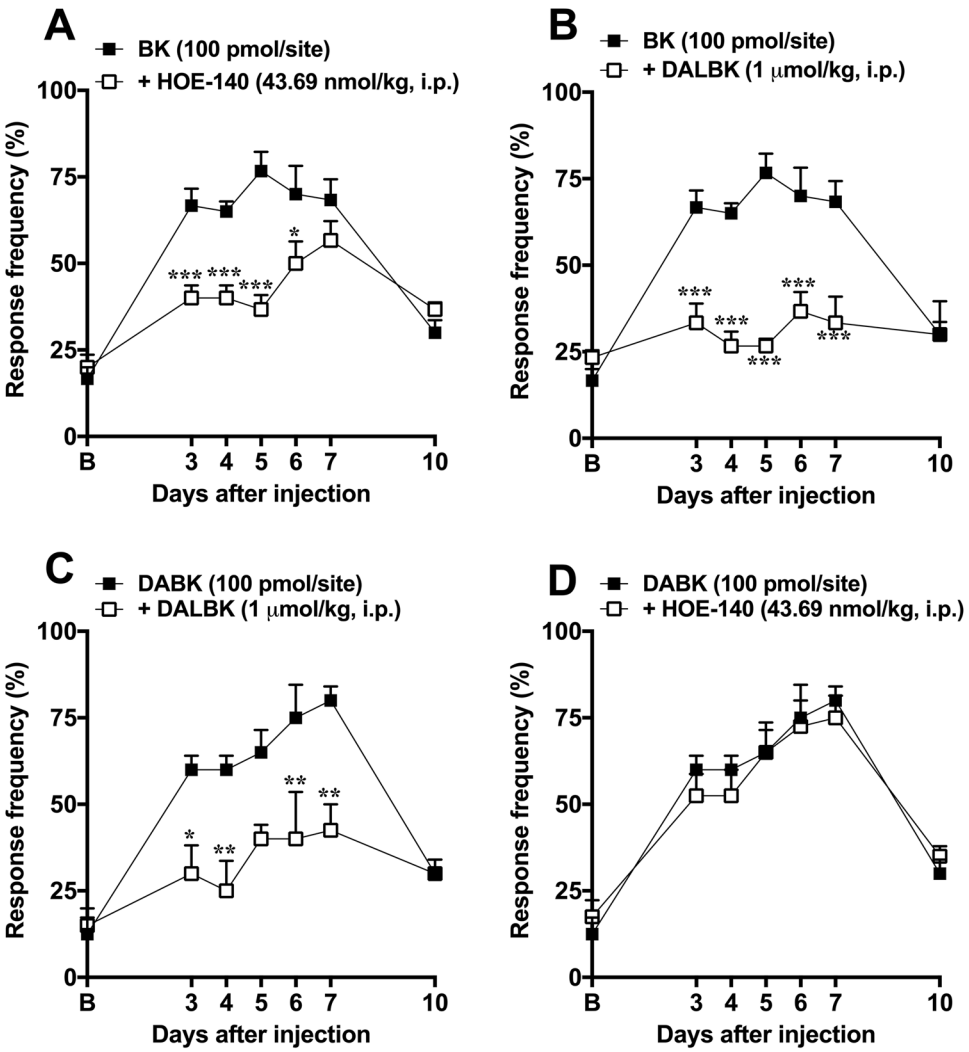


**Fig. 6** Mechanical hypersensitivity of mice injected with **a**, **b** BK or **c**, **d** DABK into the LTBP and treated with HOE 140 or DALBK 15 min before the i.n. injection. Each group represents the mean of five to six animals and the vertical bars indicate the S.E.M. Different from BK or DABK group values, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ (two-way ANOVA followed by Bonferroni's post hoc test for line graph). 'B'—basal threshold

enhances the tetrodotoxin-resistant (TTX-R) $Na^+$ current through p38 MAPK pathway activation (Jin and Gereau 2004). Furthermore, this pro-inflammatory cytokine was demonstrated to play a pivotal role in triggering the production and release of other cytokines and chemokines, such as interleukin (IL)-1β, IL-6, and CXCL1 (Shamash et al. 2002). Our data, in agreement with the literature, reinforce the important role of TNF as a key mediator in the induction of neuropathic pain.

TNF is well known to activate the NF-κB signalling pathway, which in turn causes the modulation of several inflammatory proteins (Mitchell et al. 2016). Also of relevance are the findings implicating this pathway in the deep regulation of kinin receptor expression, mainly the kinin $B_1R$ subtype, both in vitro (Phagoo et al. 2001; Sabourin et al. 2002) and in vivo (Campos et al. 1999; Fernandes et al. 2003; Passos et al. 2004; Medeiros et al. 2004). BK is recognised as a potent endogenous algogen peptide and its role in nociceptive processes has been extensively reviewed (Calixto et al. 2000, 2001; Couture et al. 2001; Campos et al. 2006; Kuduk and Bock 2008). Evidence also indicates that $B_2R$, constitutively expressed in most tissues, is probably involved in the acute phase of inflammatory and nociceptive responses (Ferreira et al. 2004). In contrast, $B_1R$ is commonly absent in intact tissues, being significantly up-regulated after injury or under some inflammatory processes, and is responsible for the long-lasting nociceptive kinin effects (Couture et al. 2001; Rashid et al. 2004; Leeb-Lundberg et al. 2005; Wang et al. 2005). Petersen et al. (1998) observed changes in neuropeptide and kinin receptor expression in the DRG after partial ligation or axotomy of a sciatic nerve. Levy and Zochodne (2000) also described an increase in both kinin $B_1R$ and $B_2R$ mRNA expression in the ipsilateral and contralateral DRG of mice submitted to peripheral nerve injury.

The present study shows, for the first time, that the i.n. injection of BK (a $B_2R$ selective agonist) or DABK (a $B_1R$ selective agonist) elicited a dose-dependent hypersensitivity. The above-mentioned effect was significantly diminished by the co-administration of HOE 140 (selective $B_2R$ antagonist) or DALBK (selective $B_1R$ antagonist), in both BK- and

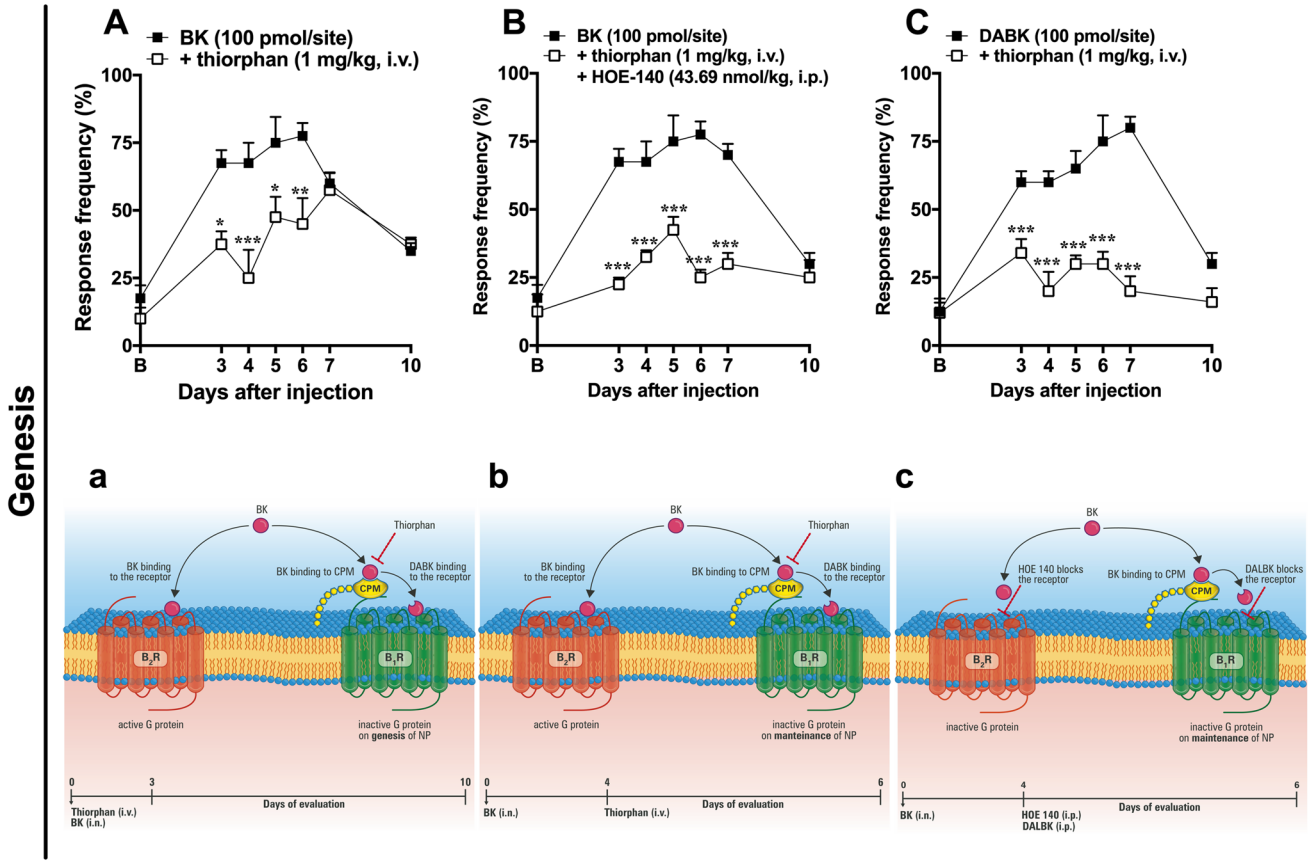


**Fig. 7** Role of BK on **a** *genesis* and **b**, **c** *maintenance* of mechanical hypersensitivity in mice. Mice were i.n. injected with BK and systemically treated with **a**, **b** thiorphan or **c** HOE 140 plus DALBK. **d** Mice pre-treated with thiorphan and submitted to BK i.n. injection were then challenged with i.t. injection of DABK to certify the $B_1R$ functionality. Each group represents the mean of five to six animals and the vertical bars indicate the S.E.M. Different from BK or DABK group values, $*p<0.05$, $**p<0.01$ and $***p<0.001$ (two-way ANOVA followed by Bonferroni's post hoc test for line graph). **a**–**d** Schematic hypothesis of the drug effect based on the results observed in (**a**–**d**), respectively. **a** Thiorphan treatment blocks the conversion of BK into DABK and also inhibits the allosteric $B_1R$ activation by CPM; **b** Thiorphan treatment blocks the conversion of BK into DABK and also inhibits the allosteric $B_1R$ activation by CPM even when administrated after the neuropathic-pain setting; **c** DALBK and HOE 140 antagonising their, respective, $B_1R$ and $B_2R$. 'B'—basal threshold; 'NP'—Neuropathic pain

DABK-injected mice. Of relevance, the interaction between TNF and kinin further receptors was confirmed by using mice lacking kinin receptors ($B_1R^{-/-}$ or $B_2R^{-/-}$ mice) or the TNF receptor ($TNFR1^{-/-}$ mice). Thus, TNF seems to be relevant for $B_1R$- and $B_2R$-induced neuronal sensitisation, whereas kinins also appear to be essential for the sensitisation elicited by TNF.

The connection between TNF and kinin receptors was also evidenced by the enhancement in $B_1R$ mRNA in the DRG and spinal cord, while the $B_2R$ mRNA levels were enhanced only in the DRG of TNF-injected mice. On the other hand, BK i.n. injection increased TNF mRNA levels only in the injected nerve, while DABK i.n. injection increased the TNF levels in both DRG and injected nerves. Of note, only $B_1R$ mRNA had increased levels in the spinal cord, suggesting a key role for its receptor in central sensitisation. Rashid et al. (2004) demonstrated that $B_1R$ was expressed 7 days after partial sciatic nerve ligation, mainly in the medium-to-large-sized DRG neurons, while $B_2R$ expression was markedly reduced. Ferreira et al. (2005) also observed that $B_1R$ mRNA was constitutively expressed in several structures that are important for the detection, transmission, and modulation of pain, including the peripheral nerve (sciatic nerve), spinal cord, and brain cortex in mice, and was found to be enhanced several days after nerve injury (Ferreira et al. 2005).

Takano and co-workers (2008) showed that lipopolysaccharide (LPS)-induced TNF release led to the increased secretion of kininogen, with the consequent formation of BK by kallikreins in the CNS. Furthermore, TNF was demonstrated to enhance kallikrein activity on the cell surface (Akita et al. 2002). On the other hand, Podsiadło et al. (2017) recently presented evidence of $B_1R$ overexpression followed by an increase in TNF levels in the CNS of mice

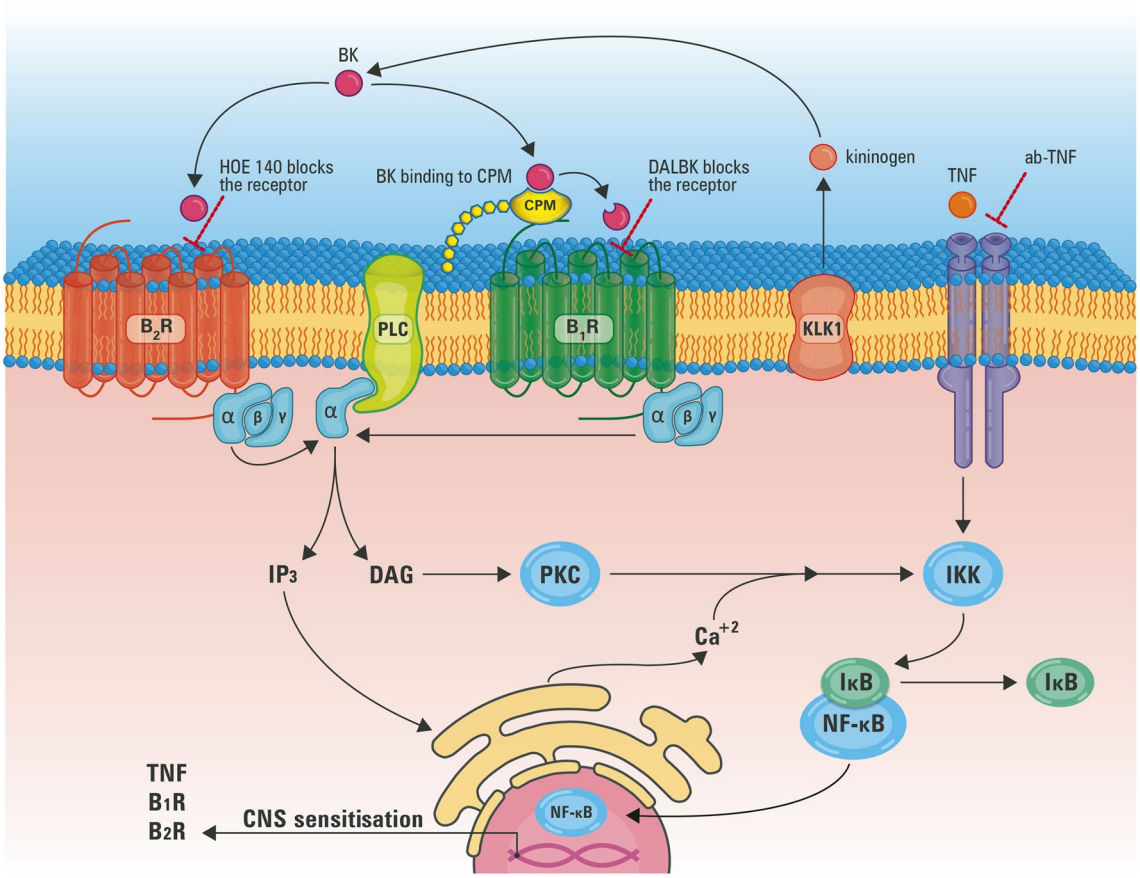


**Fig. 8** Mechanism hypothesis for the cross talk between TNF and kinins receptors to induce neuropathic pain. The i.n. injection of TNF activates its receptor (TNF p55), that induces kallikrein to cleave kininogen into BK which directly activates the $B_2R$. On the other hand, BK is cleaved into DABK by the action of CPM and activates $B_1R$, which also can be allosterically activated by CPM. TNF is an important cytokine to induce the neuronal sensitisation, but it seems that $B_1R$ activation is the main pathway of neuropathic pain maintenance. Bradykinin (BK); des-Arg$^9$-BK (DABK); des-Arg$^9$-Leu$^8$-BK (DALBK); carboxypeptidase M (CPM); $B_1$ receptor ($B_1R$); $B_2$ receptor ($B_2R$); tumour necrosis factor (TNF); phospholipase C (PLC); protein kinase C (PKC); IκB kinase (IKK); kallikrein-1 (KLK1); central nervous system (CNS)

submitted to the EAE model. Our data suggest that the i.n. injection of TNF induces the production of BK, which in turn seems to contribute to the central sensitisation elicited by this cytokine.

Considering the results observed for the kinin antagonists on the mechanical hypersensitivity elicited by either BK or DABK, it is tempting to suggest that BK effects involve the activation of both $B_2R$ and $B_1R$. Kallikrein-related serine proteases, namely kininases I and II (ACE), are responsible for the cleavage of BK into the active peptide DABK (which has a high affinity for $B_1R$), or by generation of inactive metabolites (Joedicke et al. 2018). The type-1 kininases, also named carboxypeptidase M (CPM), are constitutively expressed and linked to the cell membrane; after cell stress, these enzymes can cleave BK into DABK. They are also expressed to some degree in the PNS and CNS (Deiteren et al. 2009). BK has a short half-life of less than 30 s in the plasma, being promptly hydrolysed by ACE. Of note, Zhang et al. (2008, 2011, 2013) proposed a cross talk between CPM and $B_1R$. The authors described three main consequences of the allosteric interaction of kinin $B_1R$ agonists with CPM and its co-expression with $B_1R$: (1) the $B_1R$ conformational activation by CPM; (2) an increased affinity of the $B_1R$ orthosteric agonist DABK; and (3) the classic generation of DABK from BK cleavage. These three possibilities change the classical concepts on kinin effects, and give greater emphasis on $B_1R$. The interaction of CPM with $B_2R$ was excluded (Marceau et al. 2018).

Our data demonstrate that the treatment of mice with thiorphan, a CPM inhibitor, 15 min before the i.n. injection of BK, significantly reduced the genesis and maintenance of hypersensitivity. Furthermore, a similar result was observed for co-treatment with $B_1R$ and $B_2R$ antagonists, leading us to suggest the crucial role of CPM in converting BK into DABK, which then would sensitise nociceptive neurons causing and maintaining neuropathic pain.

Altogether, the present results, allied to the literature data, indicate the following (Fig. 8): TNF seems to be responsible for the genesis of neuropathic pain by inducing BK production associated with an enhancement of both kinins $B_1R$ and $B_2R$. Once produced, BK can activate $B_2R$ directly, or can be converted into DABK by CPM, finally leading to $B_1R$ activation. Also relevant are the results supporting the relationship between TNF and kinins, demonstrating the role of kinins in neuropathic pain-like behaviour induced by i.n. TNF, a cytokine that also amplifies the neuronal sensitisation induced by kinins. Eventually, inflammatory processes, including the one implicated in neuronal injury, usually presents an increase in cytokines, kinins and protease activation; this triad might compromise the treatment of both inflammation and pain. This hypothesis denotes TNF/$B_1R$/$B_2R$/CPM as a relevant target for drug development to treat inflammatory chronic pain.

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