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RESEARCH PAPER

Effects of kinin B_1 and B_2 receptor antagonists on overactive urinary bladder syndrome induced by spinal cord injury in rats

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BACKGROUND AND PURPOSE
Kinin B_1 and B_2 receptors have been implicated in physiological and pathological conditions of the urinary bladder. However, their role in overactive urinary bladder (OAB) syndrome following spinal cord injury (SCI) remains elusive.

EXPERIMENTAL APPROACH
We investigated the role of kinin B_1 and B_2 receptors in OAB after SCI in rats.

KEY RESULTS
SCI was associated with a marked inflammatory response and functional changes in the urinary bladder. SCI resulted in an up-regulation of B_1 receptor mRNA in the urinary bladder, dorsal root ganglion and spinal cord, as well as in B_1 protein in the urinary bladder and B_1 and B_2 receptor protein in spinal cord. Interestingly, both B_1 and B_2 protein expression were similarly distributed in detrusor muscle and urothelium of animals with SCI. *In vitro* stimulation of urinary bladder with the selective B_1 or B_2 agonist elicited a higher concentration-response curve in the SCI urinary bladder than in naive or sham urinary bladders. Cystometry revealed that treatment of SCI animals with the B_2 selective antagonist icatibant reduced the amplitude and number of non-voiding contractions (NVCs). The B_1 antagonist des-Arg^9-[Leu^8]-bradykinin reduced the number of NVCs while the non-peptide B_1 antagonist SSR240612 reduced the number of NVCs, the urinary bladder capacity and increased the voiding efficiency and voided volume.

CONCLUSIONS AND IMPLICATIONS
Taken together, these data show the important roles of B_1 and B_2 receptors in OAB following SCI in rats and suggest that blockade of these receptors could be a potential therapeutic target for controlling OAB.

Abbreviations
BC, urinary bladder capacity; CRC, cumulative concentration-response curves; DALBK, des-Arg9-[Leu8]-bradykinin; DRG, dorsal root ganglion; NVCs, non-voiding contractions; OAB, overactive urinary bladder; RV, residual volume; SCI, spinal cord injury; SSR240612, (2R)-2-((3R)-3-(1,3-benzodioxol-5-yl)-3-[[(6-methoxy-2-naphthyl)sulfonyl]amino] propanoyl)amino]-3-(4-[[2R,6S)-2,6-dimethylpiperidinyl]methyl]phenyl)-N-isopropyl-N-methylpropanamide hydrochloride; VE, voiding efficiency; VV, voided volume

Introduction

Spinal cord injury (SCI) is a devastating condition that affects about 2.5 million people worldwide; it has a profound impact on quality of life and life expectancy, causing a great economic burden to society (Thuret *et al.*, 2006).

The disruption of coordinated control between the brain, spinal cord and peripheral nervous system caused by SCI leads to several secondary pathological conditions, including lower urinary tract dysfunctions. As a consequence, individuals develop overactive urinary bladder (OAB), a syndrome characterized by exacerbated contractions of the urinary bladder during the filling phase, associated with detrusor sphincter dyssynergia (DSD) and inefficient voiding (de Groat *et al.*, 1993; Yoshimura and de Groat, 1997; Yoshimura and Chancellor, 2003).

Overactive urinary bladder syndrome affected approximately 450 million people worldwide in 2008. It has been projected that this number will increase by 100 000 by 2018 (Irwin *et al.*, 2011). Following SCI, OAB is triggered by the emergence of a micturition reflex at the spinal level. In this context, C-fibre urinary bladder afferents present remarkable plasticity and become the predominant afferent route carrying impulses from the spinal tract to control the micturition reflex (de Groat, 1990; Kruse *et al.*, 1995). In addition to C-fibre sensitization to various stimuli, a local effector function of afferent C-fibre endings contributes to neurogenic inflammation (Juszczak *et al.*, 2009). These two phenomena are pivotal for the development of OAB.

Kinins are a group of peptides formed in plasma and tissues during different physiological states and in response to inflammation, infection and tissue trauma. Once formed and released, kinins exert their biological effects through the activation of two G-protein coupled receptors, the kinin B_1 and B_2 receptors (Calixto *et al.*, 2000; Moreau *et al.*, 2005). The B_2 receptor is constitutively expressed in most tissues, whereas the B_1 receptor is generally not constitutively expressed to a great extent under normal conditions but its expression is up-regulated following tissue damage/inflammation (Ni *et al.*, 2003; Calixto *et al.*, 2004; Ferreira *et al.*, 2005; Fox *et al.*, 2005; Leeb-Lundberg *et al.*, 2005; Kayashima *et al.*, 2011). In this context, it was shown that B_1 receptor expression is induced in response to the B_1 ligand Lys-des-Arg^9-bradykinin (BK; Phagoo *et al.*, 1999) and inflammatory cytokines such as IL-1β and TNF-α (Campos *et al.*, 1998; Passos *et al.*, 2004; Fernandes *et al.*, 2005). The expression of B_1 receptors might also be regulated by B_2 receptors through the activation of NF-κB and MAP kinases (Phagoo *et al.*, 1999; Passos *et al.*, 2004).

At the level of the urinary bladder, B_2 receptor expression is firmly established in detrusor muscle and urothelial cells (Chopra *et al.*, 2005). The activation of B_2 receptors present in pelvic afferent fibres stimulates the contraction of the urinary bladder detrusor muscle (Meini *et al.*, 2000). In this regard, it has been shown that the B_2 receptor plays a key role in the excitatory motor innervation of the urinary bladder (Lecci *et al.*, 1995; Patra and Westfall, 1996; Belichard *et al.*, 1999). On the other hand, the constitutive expression of B_1 receptors in the urinary bladder is still controversial. B_1 receptor mRNA was undetectable in control rat detrusor muscle and urothelium (Chopra *et al.*, 2005). These findings are consistent with previous binding studies in control urinary bladder homogenates (Lecci *et al.*, 1999). Nonetheless, Belichard and collaborators (1999) found a basal expression of mRNA coding for B_1 receptors in the rat urinary bladder. Supporting this hypothesis, specific rat B_1 immunoreactivity was observed in nerve terminals innervating the urinary bladder (Wotherspoon and Winter, 2000). Interestingly, stimulation of the B_1 receptor induces the release of chemical mediators that affect afferent fibres, causing urinary bladder contraction (Lecci *et al.*, 1999).

Under pathological states, such as cystitis, both B_1 and B_2 receptors have been reported to be involved in the regulation of urinary bladder functionality (Marceau *et al.*, 1980; Maggi, 1997). In this context, BK can evoke an inflammatory response and changes in the urinary bladder reflex by directly activating B_2 receptors on afferent fibres and/or indirectly by release of ATP and other neurotransmitters of the urothelium (Chopra *et al.*, 2005). With regard to the B_1 receptor, it has been shown that its activation evokes the release of ATP and increases intracellular calcium levels in urothelial cells in the inflamed urinary bladder of the rat (Chopra *et al.*, 2005).

Currently, the treatment options available for OAB syndrome are limited, only modestly effective and have serious side effects that can limit their usefulness (Gulur and Drake, 2010). Hence, there is an enormous and urgent medical need for the development of novel therapeutic strategies that target improvements in OAB, especially following SCI. After considering the data implicating kinin receptors in the pathophysiology of the urinary bladder, we investigated the role exerted by B_1 and B_2 receptors on SCI-induced OAB.

Methods

Animals

Male Wistar rats (270–300 g) from the animal house of the Department of Pharmacology, Federal University of Santa Catarina (UFSC, Florianópolis, Brazil), were used in the experiments. The animals were healthy and were housed in a room maintained under a controlled temperature (22 ± 2°C) and a 12 h light/12 h dark cycle and 60–80% humidity. Food and water were provided *ad libitum*. The experimental procedures were approved by the ethics committee of the Federal University of Santa Catarina (number 016/CEUA/PRPe/2008. Procedure PP00158) and followed the National Institutes of Health Animal Care Guidelines (NIH Publications N°80-23). The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

SCI procedure

The surgical procedure was performed as previously described (Vanický *et al.*, 2001; Andrade *et al.*, 2011). The animals were anaesthetized by an i.p. injection of a 1:1 solution containing xylazine (10 mg·kg^{-1}) and ketamine (70 mg·kg^{-1}), and they also received a wide spectrum antibiotic (oxytetracycline chloride 20%). A dorsal midline incision was made from the T_9-T_{12} spinal process. A laminectomy was performed at the T_{11} vertebra level using an operating microscope and a dental drill followed by the insertion of an embolectomy catheter

(2-French Fogarty, Lemaitre Catheters, Burlington, VT, USA) previously calibrated for a 4.5 mm diameter.

The catheter was introduced into the epidural space and advanced to the rostral direction in such a way that the catheter balloon rested on the T_{10} vertebra level. Subsequently, the catheter was maintained inflated at a diameter of 4.5 mm until the spinal reflexes disappeared, then it was deflated and carefully removed. The muscle and skin were sutured. Sham-operated animals were only subjected to laminectomy.

The SCI-induced animals had their urinary bladder manually emptied twice a day by means of abdominal compression until the restoration of partial spontaneous micturition. The pre- and post-surgical care followed standard protocols as well as the regulations of the Multicenter Animal Spinal Cord Injury Study (Santos-Benito *et al.*, 2006).

The SCI rats were divided into separate groups: (i) early phase of recovery, 2 days; (ii) intermediate phase of recovery, 7 and 14 days; (iii) late phase of recovery, 28 days, in accordance with a previous study (Lee *et al.*, 2011).

Evaluation of motor behaviour

Motor behaviour of the sham-operated and SCI animals was assessed in an open-field arena using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale (Basso *et al.*, 1995). The animals were evaluated every two days from day 0 to day 28 following the surgical procedure.

Determination of cytokine levels

The urinary bladders were homogenized in phosphate buffer containing 0.05% Tween 20, 0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA and 2 ng·mL^{-1} aprotinin A (all from Sigma-Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at 5000 × *g* for 15 min at 4°C, and the supernatants were stored at –70°C until analysis. IL-1β, IL-6 and TNF-α levels were measured using ELISA kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's recommendations. The amount of protein in each sample was measured using the standard Bradford method (Bradford, 1976).

RNA extraction and real-time PCR

Total RNA from the whole urinary bladder, dorsal root ganglion (DRG, L_6-S_1) and corresponding segment of the spinal cord was extracted using TRizol® reagent (Invitrogen Corp., Carlsbad, CA, USA) and its concentration was determined by NanoDrop 1100 (Nanodrop Technologies, Wilmington, DE, USA) as previously described by Bento *et al.* (2011). The 3′ quencher MGB and FAM-labelled probes for rat B_1 and B_2 receptors (Rn00570261_m1 and Rn01430057_m1, respectively) and the 3′ quencher MGB and VIC-labelled probe for rat β-actin ACTB (Rn00667869_m1), which 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.

Western blot analysis

Urinary bladder and spinal cord tissue samples were removed 2, 7, 14 and 28 days after surgery and homogenized in complete RIPA and the total protein concentration was determined using a NanoDrop 1100 (NanoDrop Technologies, Wilmington, DE, USA). Equivalent amounts of protein for each sample were loaded per lane and electrophoretically separated using 10% denaturing PAGE (SDS-PAGE). After being transferred to a polyvinylidene fluoride membrane, the blots were assembled directly into a Snap i.d. Protein Detection System (Millipore Corporation, Billerica, MA, USA) blot holder for immunodetection. Filters were blocked with 1% BSA – tris-buffered saline-tween 20 buffer and then probed with specific primary antibodies anti-B_1 receptor and anti-B_2 receptor, according to the manufacturer's recommendations (1:200, all from Alomone Labs Ltd, Jerusalem, Israel), for 10 min at room temperature. Control experiments were carried out by pre-absorbing antibodies with the respective peptide antigen according to the manufacturer's instructions (Alomone Labs). After being washed three times with TBS-T buffer, the membranes were incubated with HRP conjugated donkey anti-rabbit IgG secondary antibody (1:800, Promega Corporation, Madison, WI, USA) for 10 min at room temperature. After being washed, the immunocomplexes were visualized using the SuperSignal West Pico Chemiluminescent Substrate Detection System (Thermo Fisher Scientific, Rockford, IL, USA). Band density measurements were made using Scion Image Software (Scion Corporation, Frederick, MD, USA).

Immunohistochemical analysis

On days 2, 7, 14 and 28 after surgery, immunohistochemical detection of B_1 and B_2 receptors was performed in the bladder (5 µm) using rabbit anti-B_1 receptor polyclonal antibody (1:200; Alomone Labs Ltd) and rabbit anti-B_2 receptor polyclonal antibody (1:200; Alomone Labs Ltd) respectively. Control experiments were carried out by pre-absorbing antibodies with the respective peptide antigen according to the manufacturer's instructions (Alomone Labs). We followed the method previously described by Andrade *et al.* (2011). In order to evaluate the difference in B_1 and B_2 receptors staining between urothelial cells and detrusor muscle, digital images of the whole bladder were captured, the specific area of each tissue was delimited, the intensity of receptor staining was measured and the resulting value was divided by the surrounding area. The results are presented as arbitrary units per area.

Isolated tissue preparations

Naive, sham-operated and SCI animals were killed by an overdose of barbiturate i.p. (100 mg·kg^{-1}, pentobarbital sodium). The urinary bladders were carefully removed and set up in 5 mL organ bath as previously described by Andrade *et al.* (2011).

Following stabilization, complete cumulative concentration-response curves (CRCs) were plotted for des-Arg^9-BK (B_1 receptor agonist: 0.001–10 µM) and BK (B_2 receptor agonist: 0.001–10 µM). Only one agonist was tested in each preparation. In a different set of experiments, we assessed the effect of selective kinin receptor antagonists on urinary bladder strips obtained 28 days after the induction of

SCI. The CRCs for des-Arg^9-BK (0.001–10 μM) and BK (0.001–10 μM) were obtained in the absence or presence of the B_1 receptor antagonist des-Arg^9-[Leu^8]-BK (30 μM) or the B_2 receptor antagonist icatibant (0.03 μM). The concentrations of antagonist were chosen on the basis of previous studies (El Sayah *et al.*, 2006; Hara *et al.*, 2008). The results obtained in all experiments are expressed as g tension.

Cystometric parameters

The urodynamic studies were carried out on the 28th day, at which time all of the animals showed partial spontaneous micturition restoration as previously described by Andrade *et al.* (2011).

We assessed the micturition pressure (MP, maximum urinary bladder pressure during micturition), basal pressure (BP, the lowest urinary bladder pressure between micturitions), threshold pressure (TP, urinary bladder pressure immediately before micturition) and the intercontraction interval (ICI). The number and mean amplitude of non-voiding contractions (NVCs) were also measured. The NVCs were defined as rhythmic intravesical pressure increases of greater than 5 mmHg from baseline pressure without the release of saline from the urethra.

Saline voided from the urethral meatus was collected and measured to determine the voided volume (VV). The saline infusion was stopped at the beginning of the voiding contraction and the residual volume (RV) was measured by withdrawing saline through the intravesical catheter and then manually expressing the remaining intravesical contents by exerting pressure on the urinary bladder abdominal wall. The urinary bladder capacity (BC) was calculated as the VV plus the RV. The BC was assessed and the voiding efficiency (VE) was determined as a percentage using the following equation: VE = [(VV/BC) × 100].

When investigating the effects of kinin receptor antagonists, reproducible micturition cycles were recorded before (used as baseline values) and 45 min after administration of the B_2 receptor antagonist icatibant (100 μmol·kg^{-1}, i.v., caudal), the B_1 receptor peptide antagonist des-Arg^9-[Leu^8]-BK (0.3 μmol·kg^{-1}, i.v., caudal), the B_1 non-peptide antagonist SSR240612 (0.4 μmol·kg^{-1}, i.v., caudal) or vehicle (saline). The doses of the antagonists were selected on the basis of previous studies (Meini *et al.*, 2000; Su *et al.*, 2009).

Drugs and chemical reagents

The nomenclature of drugs and molecular targets adopted in this study is that of Alexander *et al.* (2011). The following drugs and reagents were used: pentobarbital sodium, urethane (Sigma Chemical Co., St. Louis, MO, USA), des-Arg^9-[Leu^8]-BK, des-Arg^9-BK and BK (Bachem, Bubendorf, Switzerland), ketamine and xylazine (Virbac, São Paulo, SP, Brazil), oxytetracyclin chloridrate (Terramicin; Pfizer, São Paulo, Brazil), icatibant (Aventis Pharma Deutschland, Frankfurt Main, Germany) and SSR240612 was kindly donated by Sanofi-Synthelabo (France) respectively; des-Arg^9-BK, icatibant, des-Arg^9-[Leu^8]-BK, BK and the SSR240612 solution were prepared in saline. SSR240612 was placed in ultrasom bath until it became soluble. The vehicle used had no pharmacological effects on the tonus of the preparations or on agonist-induced contractions or cystometric parameters.

Statistical analysis

All values are expressed as the mean ± SEM, except for the EC_{50} values (i.e. the concentration of agonist that induced 50% of the response between the baseline and maximum effect) which are given as geometric means accompanied by their respective 95% confidence limits. The EC_{50} values were estimated by using concentrations of each drug that gave an effect between the minimum and maximum using the linear regression for individual experiments with GraphPad Prism® 4 software (GraphPad Software Inc., San Diego, CA, USA). The statistical significance between the groups was assessed using one or two-way ANOVA followed by the Bonferroni test or by Student's paired or unpaired *t*-tests. A *P*-value of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

Results

SCI-induced significant motor impairments

Sham-operated animals showed normal hindlimb motor activity after the surgery. However, all SCI animals developed complete paraplegia corresponding to a BBB score of 0–1 in the first week after surgery, followed by modest motor improvements in the next 3 weeks post-SCI, reaching a final score of 3. In order to guarantee OAB development in all SCI animals, rats that scored over 4 were not used in subsequent experiments (data not shown).

Levels of pro-inflammatory cytokines in the urinary bladder increased after SCI

In order to determine urinary bladder inflammatory conditions in response to SCI, we evaluated the levels of pro-inflammatory cytokines in the urinary bladder. Figure 1 (A and B) shows that the levels of IL-1β and IL-6 were 7- and 42-fold higher respectively, in the SCI urinary bladder compared to the sham-operated urinary bladder, and this increase was only observed on the second day after surgery. The level of TNF-α remained unchanged in both groups (Figure 1C).

B_1 and B_2 receptor mRNA levels increased after SCI

In order to evaluate the involvement of kinins and their receptors in OAB triggered by SCI, we first determined the mRNA levels for B_1 and B_2 receptors by real-time PCR in the urinary bladder, DRG (L_6-S_1), and corresponding segments of spinal cord from naive, sham-operated, and SCI animals taken 2, 7 and 14 days after surgery. The mRNA level of B_1 receptor was increased in the urinary bladder (day 2, 12-fold; day 7, 6-fold), DRG (day 2, 6-fold) and spinal cord (days 2 and 7, 3-fold) after SCI when compared to the respective sham-operated groups (Figure 2 A, C and E respectively). The mRNA levels of B_2 receptor were reduced (day 14, 14%) in DRG (Figure 2 D), but were not significantly altered in the urinary bladder and spinal cord (Figure 2B and F respectively).

SCI induced alterations in B_1 and B_2 receptor protein levels in the urinary bladder and spinal cord

Due to significant alterations in mRNA expression, we next investigated possible changes in B_1 and B_2 receptor protein

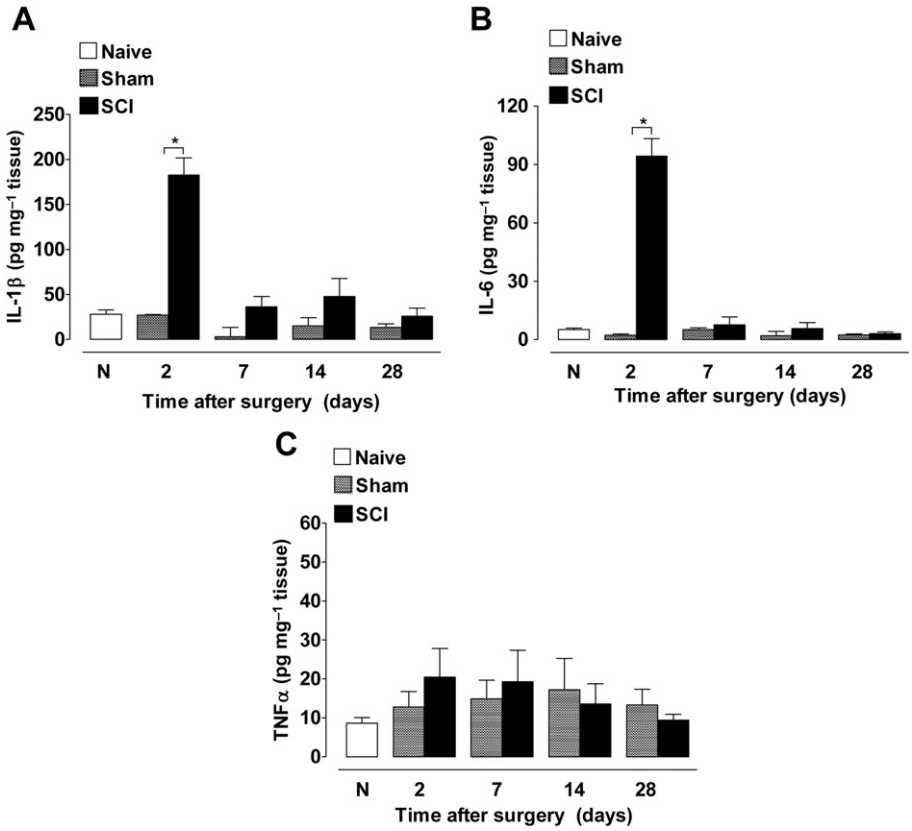

Figure 1

The levels of IL-1β, IL-6 and TNF-α were measured in the urinary bladders of sham-operated, SCI and naive animals. Two days after SCI, the levels of IL-1β and IL-6 (A and B, respectively) were significantly higher in the SCI urinary bladder compared to sham-operated urinary bladders. The levels of TNF-α remained unchanged in both groups (C). Each column represents the mean ± SEM of four animals. The asterisks denote the levels of significance. *$P < 0.05$ compared with the sham-operated group (two-way ANOVA followed by a Bonferroni test).

expression in the urinary bladder collected 2, 7, 14 and 28 days after the surgical procedure. Our results showed a constitutive expression of both B_1 and B_2 receptors in the urinary bladder and spinal cord of naive group (Figure 3). Interestingly, when compared to the respective sham-operated group, SCI induced an up-regulation of B_1 receptors on day 2 after surgery in the urinary bladder (62.3 ± 15.7%; Figure 3A). However, the B_2 receptor expression was unaffected in this tissue in all the periods assessed (Figure 3B). The SCI procedure resulted in an up-regulation of the expression of the B_1 receptor on day 7 (95.5 ± 27.1%) and B_2 receptor on days 2 (36.3 ± 8.3%) and 7 (73.8 ± 4.5%) after the surgery in the spinal cord (Figure 3C and D respectively).

No band was observed in the control experiments (Figure 3).

B_1 and B_2 receptor expression in the urothelial cells and detrusor muscle of the urinary bladder

We next determined the expression of B_1 and B_2 receptors in the detrusor muscle and urothelium after SCI. The immunohistochemical analysis, illustrated in Figure 4, showed that B_1 receptor expression was equally distributed in detrusor muscle and urothelium of naive and sham-operated animals (Figure 4A), while B_2 receptor expression was higher in urothelium that in detrusor muscle of naive (fourfold) and sham-operated animals on days 2 (26-fold), 7 (19-fold), 14 (16-fold), and 28 (570-fold) after surgery (Figure 4C). Moreover, B_1 and B_2 receptor expression were equally distributed in detrusor muscle and urothelium in all the analysed time-points post-SCI (Figure 4B and D respectively). No staining was observed in the control experiments (Figure 4 E and H).

SCI increased the functional contractile response to both B_1 and B_2 agonists in the urinary bladder

Once the overexpression of both B_1 and B_2 receptors was observed in the urinary bladder of SCI animals, we next evaluated the reactivity of isolated urinary bladders to selective agonists of these receptors. Sham-operated urinary bladder strips stimulated with the B_1 receptor agonist des-Arg^9-BK (0.0001–10 μM) showed quite moderate contractile responses compared with the urinary bladder strips of naive animals when assessed on day 2 post-surgery. In addition, on days 7, 14 and 28 after surgery, no significant changes in the contractile response were observed between urinary bladder strips from sham-operated and naive rats (Figure 5). Compared to sham-operated urinary bladders, the SCI urinary

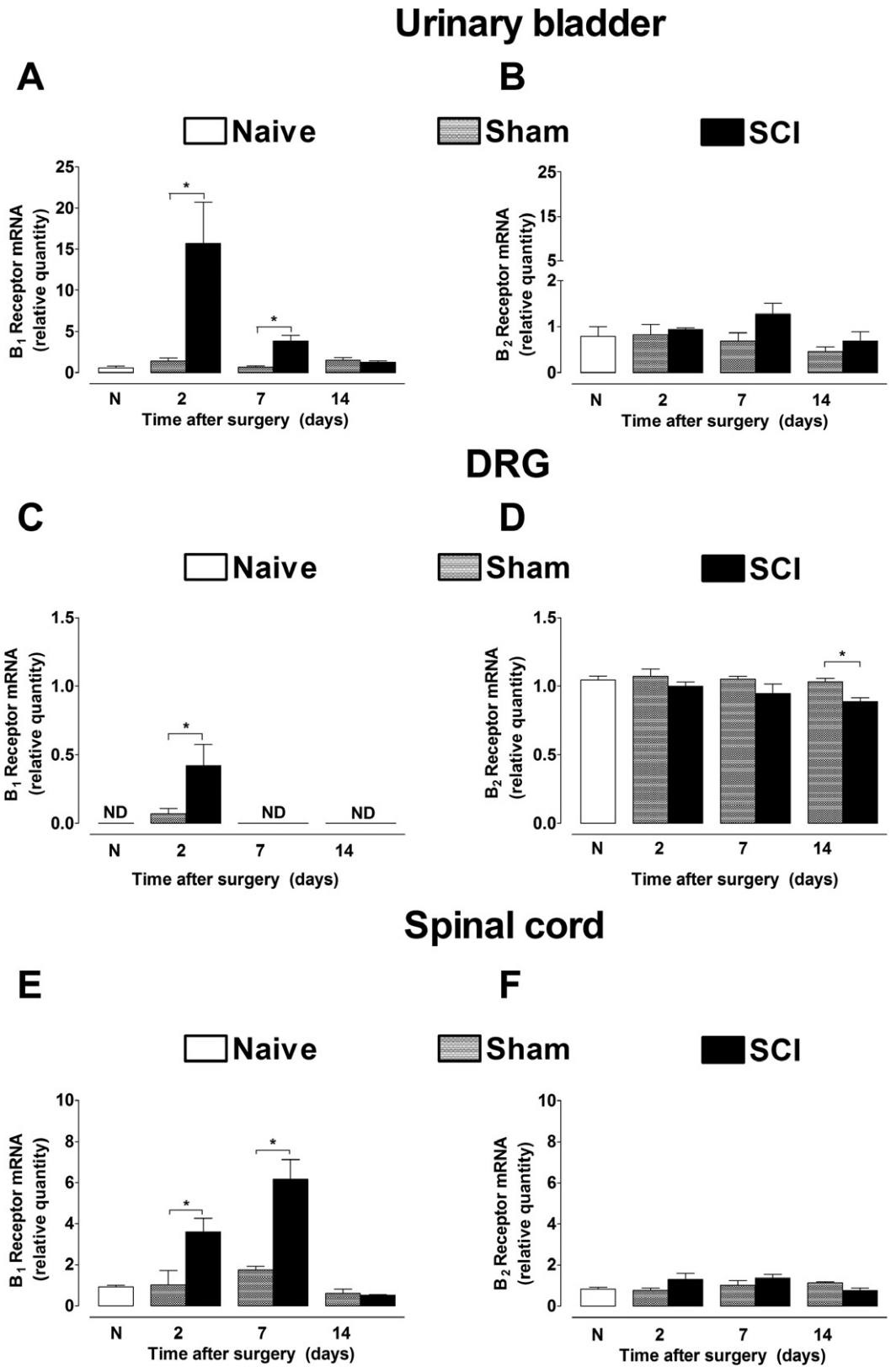

Figure 2

SCI caused a significant increase in B_1 receptor mRNA levels in the urinary bladder and spinal cord on days 2 and 7 (A and E, respectively) and in DRG (C) on day 2 after SCI. B_2 receptor mRNA levels were significantly decreased in DRG on day 14 (D). Each column represents the mean ± SEM of three animals. *$P < 0.05$ compared with the sham-operated group (one-way ANOVA followed by a Bonferroni test).

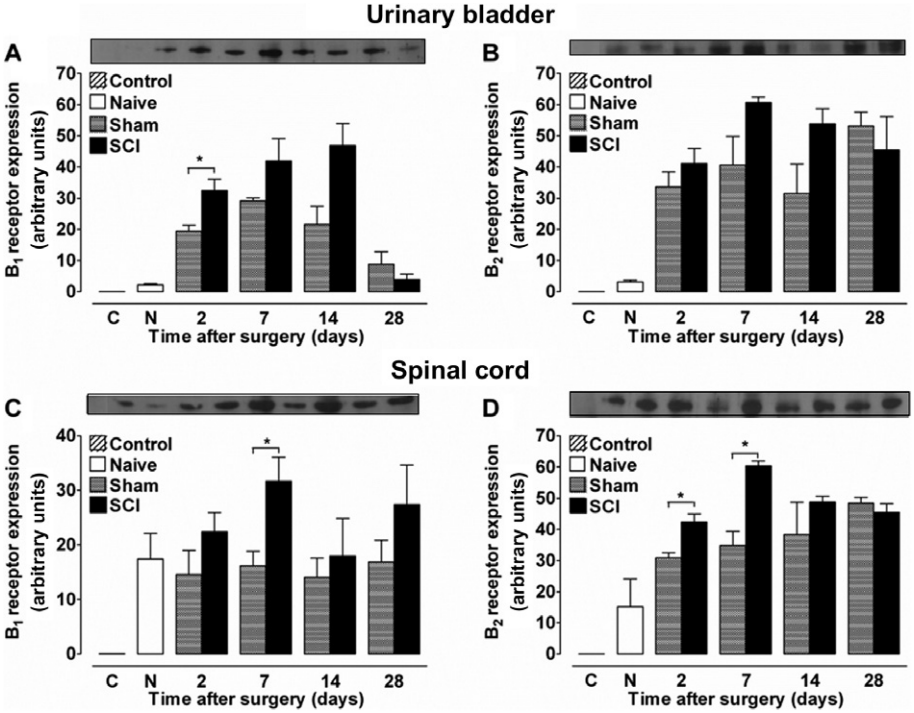

Figure 3

SCI caused a significant up-regulation in B_1 receptor expression in the urinary bladder on day 2 (A) and in the spinal cord on day 7 (C) after SCI. B_2 receptor expression was significantly increased in the spinal cord on days 2 and 7 after SCI (D) but it did not induce any significant alterations in the urinary bladder(B). Total levels of B_1 and B_2 receptors were determined by Western blot analysis using specific antibodies. Each column represents the mean ± SEM of four animals. *$P < 0.05$ compared with the sham-operated-operated group (one-way ANOVA followed by a Bonferroni test).

bladders showed a significantly higher contractile response to des-Arg^9-BK (Figure 5A–D). The calculated mean EC_{50} values obtained for des-Arg^9-BK in the SCI group were 0.3 (0.08–1.2); 0.4 (0.09–2.4); 0.2 (0.009–3.9) and 0.04 (0.02–0.1) μM, and the tension-at-maximum-concentration values were 2.1 ± 0.3, 1.3 ± 0.2, 1.5 ± 0.1, and 1.3 ± 0.5 g for 2, 7, 14 and 28 days after surgery respectively. After SCI, des-Arg^9-BK was more potent on day 28 and more efficacious on day 2 after SCI, according to the EC_{50} and maximal effect analyses respectively. The contractile response induced by des-Arg^9-BK in the SCI urinary bladder strips on day 28 after surgery was significantly reduced (28.6 ± 12.5%) in the presence of the selective B_1 receptor antagonist des-Arg^9-[Leu^8]-BK (30 μM; Figure 5E).

When naive and sham-operated urinary bladders strips were exposed to the B_2 receptor agonist BK (0.0001–10 μM), similar contractile responses were shown in all the periods analysed (Figure 6). In contrast, the SCI urinary bladder strips showed significantly higher contractile responses to BK when compared with sham-operated urinary bladder strips. The EC_{50} values for BK were 0.09 (0.03–0.2), 3.6 (0.8–17) 0.09 (0.03–0.2) and 0.1 (0.06–0.3) μM and the tension-at-maximum-concentration values were 2.3 ± 0.4, 3.3 ± 0.4, 4.3 ± 0.7 and 5.3 ± 0.7 g for 2, 7, 14 and 28 days after SCI respectively. After SCI, BK was more potent on days 2 and 14 and more efficacious on day 28 at inducing the contractile response, according to the EC_{50} and maximal effect analyses respectively.

The BK-induced contractile response in the SCI urinary bladder strips obtained 28 days after surgery was significantly reduced (89.5 ± 2.5%) in the presence of the selective B_2 receptor antagonist icatibant (0.03 μM; Figure 6E).

Effect of the B_1 or B_2 receptors antagonist on urodynamic alterations induced by SCI

Finally, we investigated the urodynamic behaviour of urine voiding by measuring the intravesical pressure and volume in sham-operated and SCI animals. During the urinary bladder filling phase, SCI animals showed more NVCs (10.0 ± 2.6) than sham-operated animals (0.5 ± 0.3). In comparison to sham-operated animals, the SCI animals showed significant alterations such as reductions in the ICI (49.0 ± 7.1%), VV (18.9 ± 1.5%) and VE (2.4 ± 0.3%), as well as an increase in TP (212.7 ± 30.6%) and urinary BC (655.2 ± 72.4%; Table 1). No significant alterations in the urodynamic parameters were observed between naive and sham-operated groups (data not shown).

Acute systemic treatment with the B_1 receptor antagonist des-Arg^9-[Leu^8]-BK (0.3 μmol·kg^{-1}, i.v.) only reduced the number of NVCs (54.6 ± 18.8%; Figure 7B–E and Table 2), whereas the acute systemic treatment of SCI animals with the B_1 receptor non-peptide antagonist SSR240612 (0.4 μmol·kg^{-1}) not only reduced the NVCs (77.3 ± 10.8%) and the urinary BC (55.1 ± 3.6%), but it also increased VV (89.3 ± 34.6%) and the VE (223.81 ± 137.1%; Figure 8 and Table 2). On the other hand, the B_2 receptor antagonist

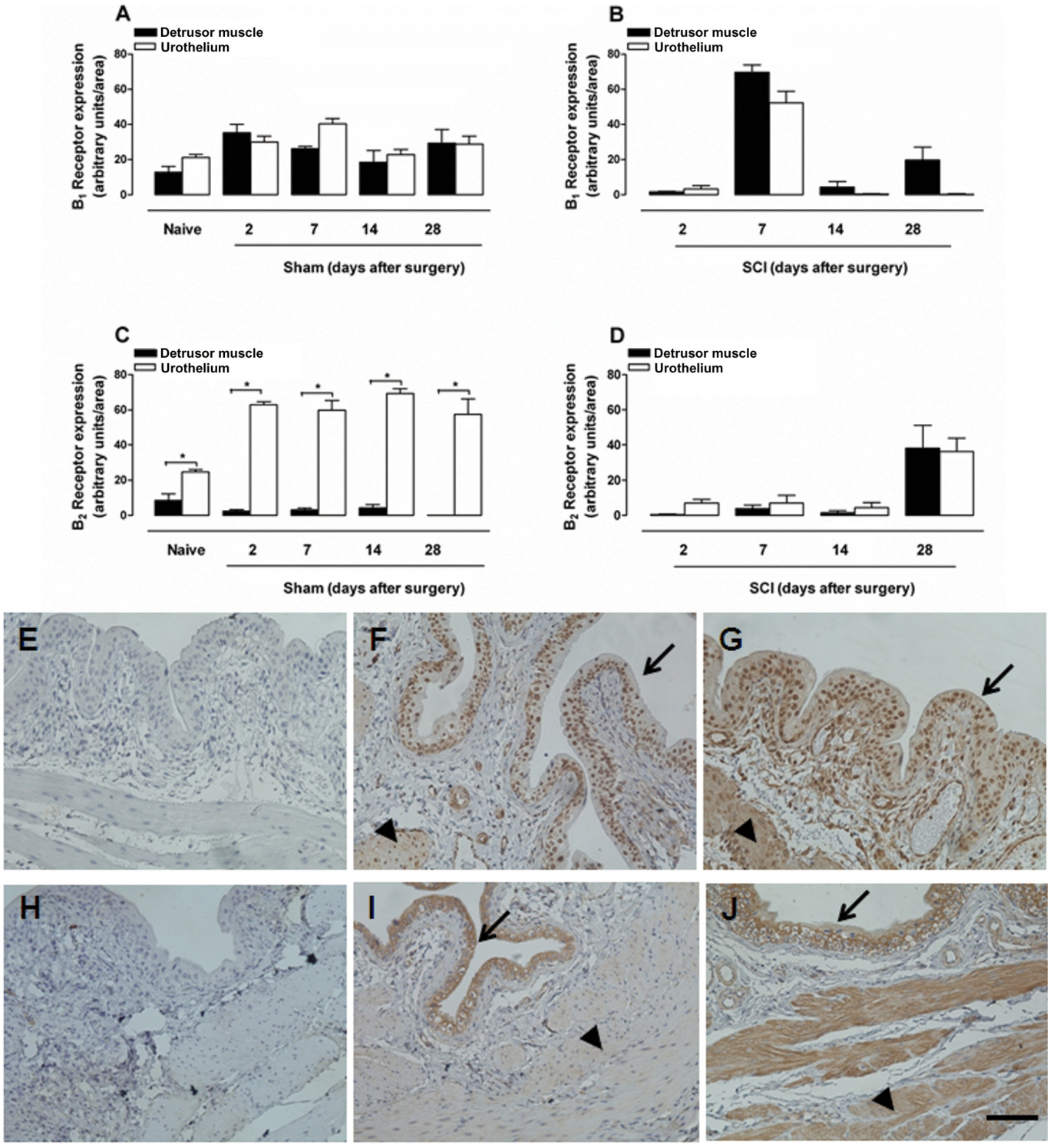

Figure 4

Immunohistochemical analysis of B_1 and B_2 receptor expression in the urinary bladder detrusor muscle and urothelium of naive and sham-operated (A and C, respectively) and SCI (B and D, respectively) rats assessed 2, 7, 14 and 28 days after surgery. Representative images of control group (E and H, respectively) and B_1 (F and G) and B_2 (I and J) receptors immunostaining in the urinary bladder detrusor muscle (arrowhead) and urothelium (arrow) of sham-operated and SCI rats respectively (scale bar = 100 μm). The results are represented as arbitrary units per area. Each column represents the mean and vertical lines show the SEM of three to four animals. *$P < 0.05$ compared with the respective urothelium group (one-way ANOVA followed by a Bonferroni test).

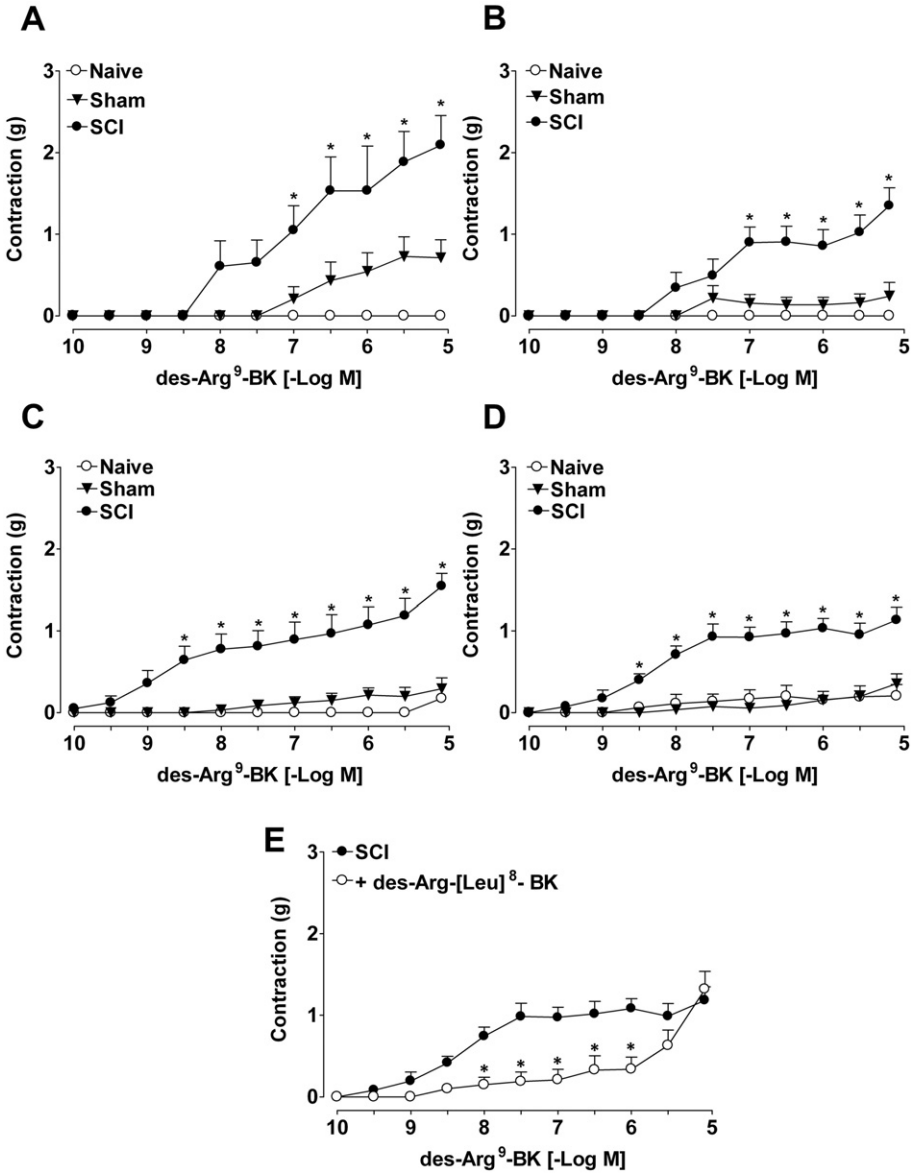

Figure 5

Contractile response induced by the B_1 receptor agonist (des-Arg^9-BK; 0.0001–10 μM) in isolated urinary bladders 2 (A), 7 (B), 14 (C) and 28 (D) days after surgery. The B_1 receptor antagonist (des-Arg^9-[Leu^8]-BK) reduced the contractile response induced by des-Arg^9-BK (0.0001–10 μM) in isolated urinary bladders of SCI animals assessed 28 days after surgery (E). The results are expressed as g of tension. Each point represents the mean ± SEM of six animals for each group. The asterisks denote the significance levels. *$P < 0.05$ compared with the sham-operated-operated group (two-way ANOVA followed by a Bonferroni test).

icatibant (100 μmol·kg^{-1}, i.v., 45 min pretreatment) significantly reduced the amplitude (21.0 ± 9.2%) and the number (59.0 ± 5.8%) of NVCs (Figure 9B–E and Table 2). No significant changes were found in the intercontraction interval, VE, TP or urinary BC before or after the administration of vehicle or kinin receptor antagonists (Table 2).

Discussion

Herein, we report the novel finding indicating that SCI induces the overexpression of B_1 receptors in the neurogenic urinary bladder of rat. Notably, the *in vivo* inhibition of B_1 or B_2 receptors by selective antagonists provides improvements in urodynamic parameters altered in OAB. Thus, the altered expression and sensitivity of B_1 and B_2 receptors could contribute to the emergence of OAB in SCI rats.

Spinal cord injury leads to important pathologies in the lower urinary tract, including OAB syndrome. SCI-induced OAB is known to produce discomfort in patients and it has become an economic challenge. The current treatment options for OAB syndrome include antimuscarinic agents and β_3-adrenoceptor agonists, which have been associated with moderate to ineffective responses in some patients (Lecci and

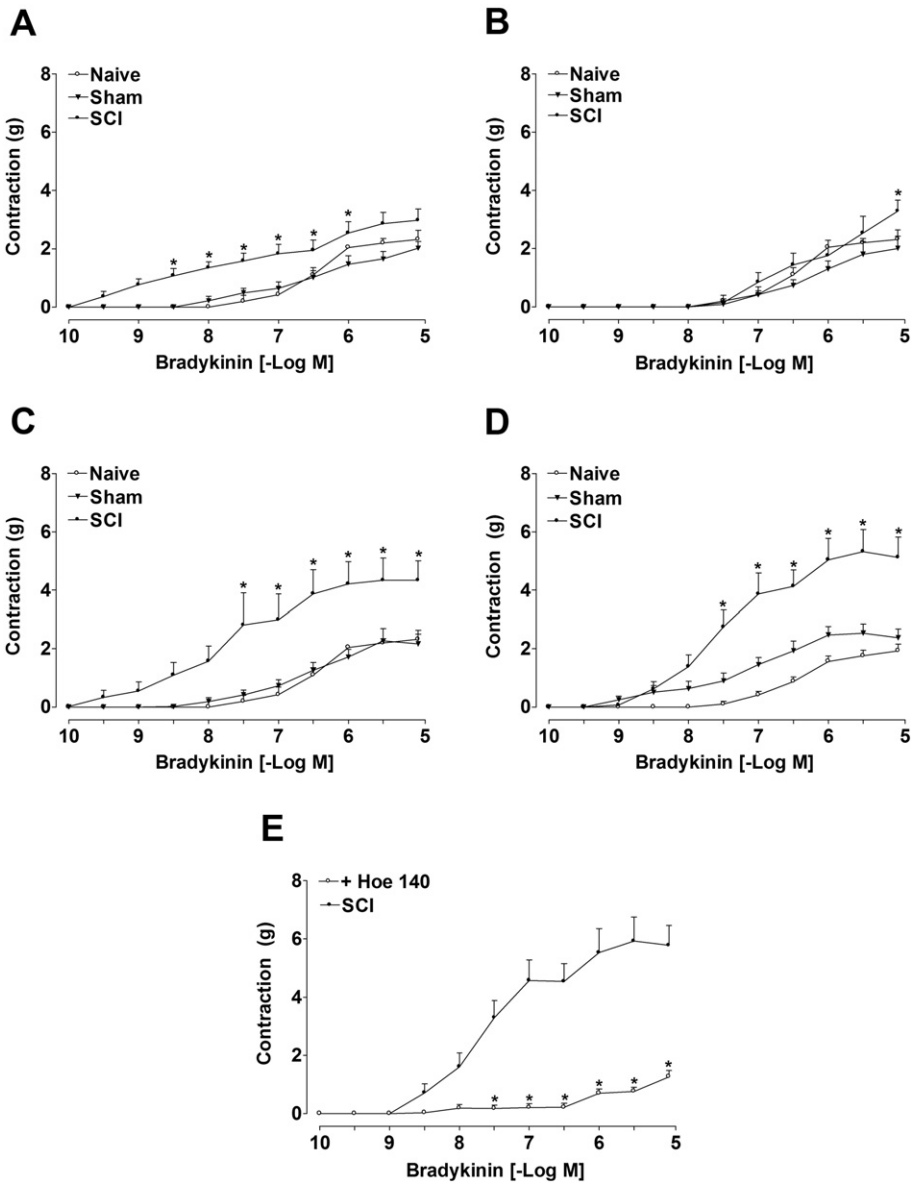

Figure 6

Contractile response induced by the B_2 receptor agonist bradykinin (0.0001–10 μM) in isolated urinary bladders 2 (A), 7 (B), 14 (C), and 28 (D) days after surgery. The B_2 receptor antagonist (icatibant) reduced the contractile response induced by bradykinin (0.0001–10 μM) in isolated urinary bladders of SCI animals assessed 28 days after surgery (E). The results are expressed as g of tension. Each point represents the mean ± SEM of four animals for each group. The asterisks denote the levels of significance. *$P < 0.05$ compared with the sham-operated-operated group (two-way ANOVA followed by a Bonferroni test).

Maggi, 2005; Gulur and Drake, 2010; Limberg *et al.*, 2010; Smith and Wein, 2010). Alterations in the properties, innervations and structure of the detrusor muscle and an increase in the expression and/or sensitivity of urothelial-sensory molecules/receptors that lead to afferent sensitization have been documented as being possible mechanisms for the genesis of SCI-induced OAB (Brading, 1997).

In the present study, we demonstrated the constitutive expression of both B_1 and B_2 receptors in the rat urinary bladder. The existence of the constitutive expression of the B_1 receptor in the urinary bladder is still controversial. Chopra *et al.* (2005) failed to detect B_1 receptor mRNA in the rat urinary bladder, whereas Belichard *et al.* (1999) and our group reported a basal expression of mRNA coding for the B_1 receptor in the rat urinary bladder while Wotherspoon and Winter (2000) detected rat B_1 receptor immunoreactivity in peripheral nerve terminals innervating the urinary bladder. Of note, our present data showed that both B_1 receptor mRNA and protein were overexpressed in SCI urinary bladders in the early phase of recovery, while B_2 receptor mRNA and protein expression were not changed following SCI. Consistent with these observations, an up-regulation of B_1 receptor mRNA levels was reported in the detrusor muscle and urothelium following chemical cystitis in rats (Chopra *et al.*, 2005). In

Table 1

Changes in cystometric parameters induced by SCI

Parameters	Sham operated (n = 6–8)	SCI (n = 6–8)
Mean amplitude of NVCs (mmHg)	8.20 ± 5.30	22.60 ± 1.90*
Number of NVCs	0.50 ± 0.30	10.00 ± 2.60*
Basal pressure (mmHg)	7.20 ± 3.30	15.70 ± 2.70
Pressure threshold (mmHg)	8.30 ± 2.40	17.70 ± 2.50*
Maximum voiding pressure (mmHg)	28.70 ± 6.10	30.40 ± 5.70
Intercontraction interval (min)	1.40 ± 0.20	0.60 ± 0.10*
Voided volume (mL)	0.20 ± 0.02	0.04 ± 0.00*
Urinary bladder capacity (mL)	0.40 ± 0.08	2.60 ± 0.30*
Voiding efficiency (%)	63.70 ± 11.00	1.50 ± 0.20*

*P < 0.05 compared with sham operated (Student's unpaired t-test). Values are expressed as mean ± SEM.

contrast to the B_1 receptor, B_2 receptor expression was higher in urothelium than in detrusor muscle of naive and sham groups, suggesting the significant role exerted by the urothelial B_2 receptor in the BK-induced contractile response of the urinary bladder under physiological conditions. Of particular interest is the finding that both B_1 and B_2 receptors protein expression was equally distributed in the detrusor muscle and urothelium through the different phases of recovery after SCI. Therefore, B_1 and B_2 receptors present in both structures could actively contribute to the exacerbated contractile response of the urinary bladder to des-Arg^9-BK and BK stimulus caused by SCI.

It should be noted that while B_1 receptor expression was not up-regulated on day 28 after SCI, the contractile response to des-Arg^9-BK was exacerbated in this late phase of recovery. Of note, the contractile response to BK was exacerbated in all the analysed periods after SCI, but B_2 receptor expression remained unchanged. Thus, the enhanced functional activity of both B_1 and B_2 receptor in the absence of significant B_1 and B_2 receptor up-regulation could be explained by receptor sensitization following tissue damage/inflammation.

A basal constitutive expression of the B_1 receptor was demonstrated in DRG ganglia in rats, mice and monkeys (Ma *et al.*, 2000; Wotherspoon and Winter, 2000; Shughrue *et al.*, 2003; Rashid *et al.*, 2004). Herein, we showed a significant amount of B_1 receptor mRNA in the DRG (dorsal root ganglion, L_6-S_1) in the sham-operated group and its up-regulation in the SCI group on day 2 after surgery. Thus, B_1 receptors could have been synthesized in the DRG during the early phase of recovery and moved towards the peripheral afferent terminal, reflecting an increase in B_1 receptor protein on day 2 following SCI in the urinary bladder. However, part of the B_1 receptor synthesis could have been carried out by epithelial and muscle cells of the urinary bladder since that B_1 receptor mRNA levels are increased in urinary bladder in this period.

Table 2

Changes in cystometric parameters induced by SCI and the effect of i.v. administration of vehicle (saline), icatibant (100 μmol·kg^{-1}), DALBK (0.3 μmol·kg^{-1}) or SSR240612 (0.4 μmol·kg^{-1})

	SCI							
	Vehicle (n = 6–8)		Icatibant (n = 6–8)		DALBK (n = 6–8)		SSR240612 (n = 6–8)	
Parameters	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Mean amplitude of NVCs (mmHg)	20.50 ± 3.20	22.90 ± 4.40	20.60 ± 2.66	16.30 ± 1.90*	16.70 ± 2.70	10.90 ± 3.90	11.66 ± 1.99	7.66 ± 1.22
Number of NVCs	10.40 ± 2.60	8.20 ± 2.60	12.20 ± 2.60	5.40 ± 0.50 *	8.30 ± 2.50	3.80 ± 1.50*	11.00 ± 2.27	2.50 ± 1.19*
Basal pressure (mmHg)	17.40 ± 4.10	17.90 ± 4.20	13.50 ± 3.10	11.60 ± 2.00	9.20 ± 3.40	7.90 ± 3.10	1.90 ± 0.44	0.87 ± 0.38
Pressure threshold (mmHg)	20.20 ± 4.20	20.60 ± 4.20	15.50 ± 3.10	13.10 ± 2.00	9.90 ± 3.60	8.60 ± 3.20	3.74 ± 0.42	2.60 ± 0.09
Maximum voiding pressure (mmHg)	27.90 ± 3.60	26.50 ± 2.50	27.30 ± 5.80	20.30 ± 2.40	17.00 ± 3.30	15.70 ± 2.70	16.77 ± 1.81	14.73 ± 3.46
Intercontraction interval (min)	0.50 ± 0.02	0.50 ± 0.04	0.60 ± 0.06	0.50 ± 0.03	0.80 ± 0.10	0.70 ± 0.10	0.71 ± 0.13	0.94 ± 0.24
Voided volume (mL)	0.05 ± 0.06	0.05 ± 0.03	0.04 ± 0.00	0.05 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.46 ± 0.009	0.87 ± 0.016*
Urinary bladder capacity (mL)	2.70 ± 0.30	2.70 ± 0.40	2.80 ± 0.40	2.60 ± 0.20	2.90 ± 0.40	2.80 ± 0.30	2.17 ± 0.16	0.97 ± 0.08*
Voiding efficiency (%)	2.10 ± 0.40	2.15 ± 0.40	1.50 ± 0.20	2.10 ± 0.30	1.10 ± 0.40	1.30 ± 0.40	2.25 ± 0.57	9.17 ± 2.03*

*P < 0.05 compared with pretreatment (Student's paired t-test). Values are expressed as mean ± SEM.

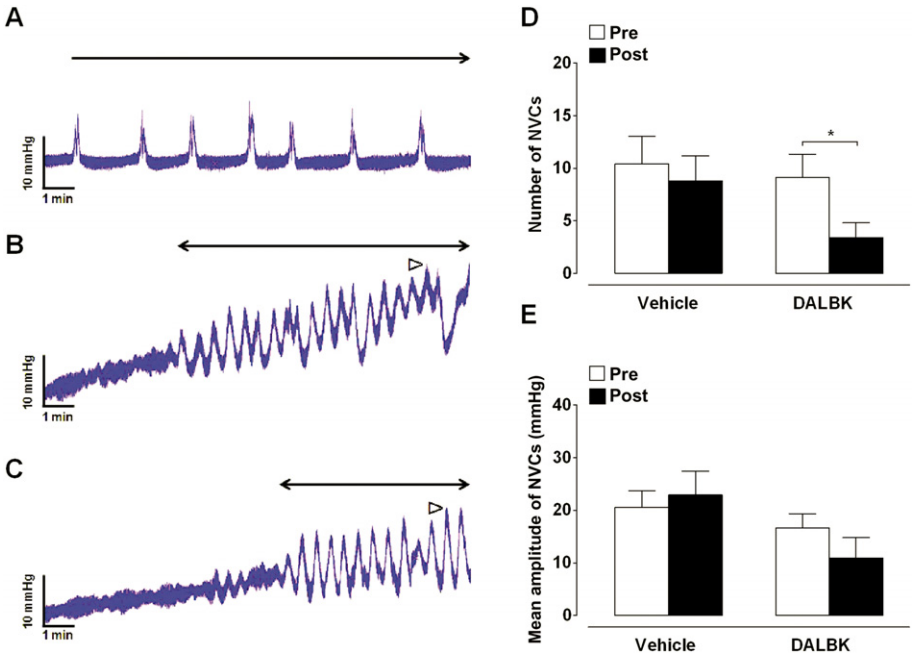

Figure 7

Representative cystometric trace of sham-operated, SCI and DALBK-treated SCI rats on day 28 after surgery. During the filling phase, the sham-operated and SCI rats showed a large number of voiding (A; arrow) and non-voiding (B; double arrow) contractions respectively. The number of NVCs (C and D; double arrow) were reduced by DALBK (des-Arg^9-[Leu^8]-BK (0.3 μmol·kg^{-1}) treatment, but not the amplitude (C and E; arrowhead). Each column represents the mean and the vertical lines indicate the SEM of six to eight animals. *P < 0.05 compared with pretreatment values (Student's paired t-test).

It has been documented that both B_1 and B_2 receptors are constitutively present in the mammalian spinal cord (Calixto *et al.*, 2000; Couture and Lindsey, 2000; Wotherspoon and Winter, 2000; Ma and Heavens, 2001; Shughrue *et al.*, 2003). In accordance with that, we observed the constitutive expression of B_1 and B_2 receptor mRNA and protein in the rat spinal cord. Relatively high levels of B_1 receptor mRNA found in the spinal cord on days 2 and 7 after SCI were accompanied by an increased rate of B_1 receptor protein synthesis on day 7 after SCI. Furthermore, we observed a marked up-regulation of the B_2 receptor protein, but not of mRNA, in the spinal cord on days 2 and 7 after SCI, indicating that the basal levels of mRNA seem to be sufficient for the synthesis of the B_2 receptor protein in the spinal cord. Therefore, these data suggest that spinal B_1 and B_2 receptors significantly contribute to central modulation of the reflex micturition after SCI, as the B_1 and B_2 receptor proteins were up-regulated in the corresponding portion of the spinal cord.

It is now well established that pro-inflammatory cytokines such as IL-1β induce the expression of B_1 and B_2 receptors (Campos *et al.*, 1999; Calixto *et al.*, 2004). Interestingly, IL-1β may induce the up-regulation of B_1 receptors via the MAP-kinase pathways and NF-κB stimulation in several systems (Schanstra *et al.*, 1998; Sjuve *et al.*, 2000; Calixto *et al.*, 2004; Moreau *et al.*, 2005). In addition, Lee *et al.* (2008) have shown that B_2 receptor, PLC and PKC pathways are required for the production of BK-induced IL-6. In accord with these data, we showed increased levels of the pro-inflammatory cytokines IL-1β and IL-6 in SCI urinary bladders 2 days after injury. Even though we did not evaluate the effect of the B_1 and B_2 receptors antagonists on cytokine levels, it is plausible that the overexpression of both B_1 and B_2 receptors in the SCI urinary bladder could be caused, at least in part, by the enhancement of cytokine levels observed in the early phase of recovery, which seems to contribute to the afferent sensitization observed in the OAB.

Herein, we evaluated the effect of the B_1 receptor antagonist des-Arg^9-[Leu^8]-BK (DALBK) and the B_2 receptor antagonist icatibant on the contractile activity 28 days after surgery, a period in which the contractile response to BK and des-Arg^9-BK became stabilized. Icatibant is a known selective and potent B_2 receptor antagonist that exhibits some anti-inflammatory properties such as reducing plasma protein extravasation, as well as reducing the hyper-reflexia in chemically-induced cystitis models (Hock *et al.*, 1991; Lembeck *et al.*, 1991; Giuliani *et al.*, 1993; Ahluwalia *et al.*, 1994; Smith and Wein, 2010). The peptide B_1 receptor antagonist DALBK has a rapid onset of action as well as a rapid reversibility and it has been shown to have a good affinity for the B_1 receptor rabbit urinary bladder (Hall and Morton, 1997; Regoli *et al.*, 1998). Our present results showed that the incubation of the urinary bladder preparations with DALBK reduced the contractile response induced by the lower concentration of des-Arg^9-BK. This phenomenon could be explained by the competitive antagonism exerted by DALBK on the des-Arg^9-BK-induced contractile response, which is reversible on exposure of the preparations to higher concentrations of the agonist. Otherwise, the incubation of isolated SCI urinary bladders with icatibant consistently reduced the contractile response to BK. This finding is in agreement with

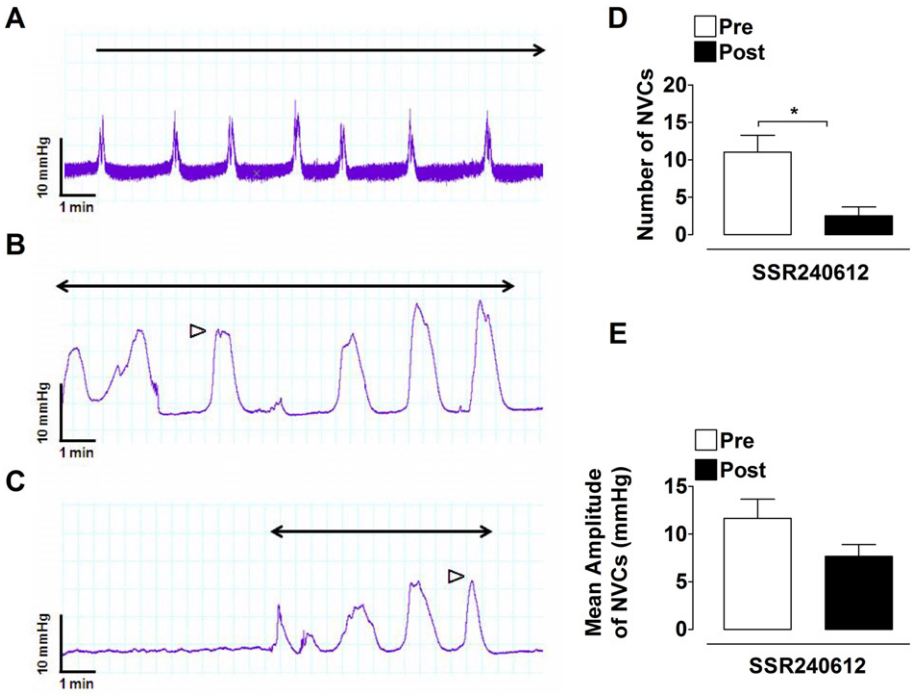

Figure 8

Representative cystometric trace of sham-operated, SCI and SSR240612-treated SCI rats on day 28 after surgery. During the filling phase, the sham-operated and SCI rats showed a large number of voiding (A; arrow) and non-voiding (B; double arrow) contractions respectively. The number of NVCs (C and D; double arrow) were reduced by SSR240612 (0.4 μmol·kg^{-1}) treatment, but not the amplitude (C and E; arrowhead). Each column represents the mean and the vertical lines indicate the SEM of six to eight animals. *$P < 0.05$ compared with pretreatment values (Student's paired *t*-test).

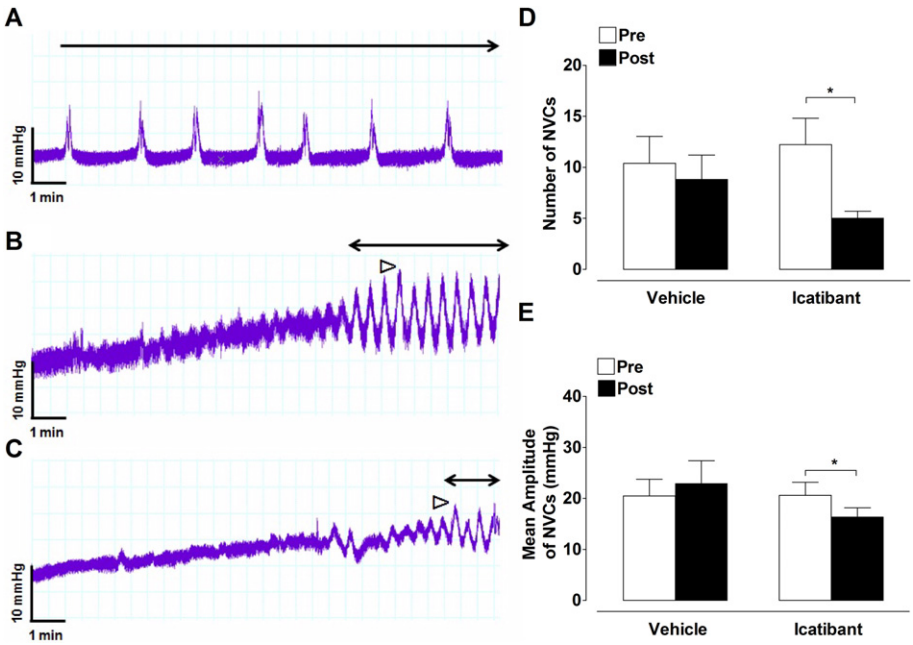

Figure 9

Representative cystometric traces of sham-operated, SCI and icatibant-treated SCI rats on day 28 after surgery. During the filling phase, the sham-operated and SCI rats showed a large number of voiding (A; arrow) and non-voiding (B; double arrow) contractions respectively. The number (C and D; double arrow) and amplitude (C and E; arrowhead) of NVCs were reduced by icatibant (100 μmol·kg^{-1}) treatment. Each column represents the mean and the vertical lines indicate the SEM of six to eight animals. *$P < 0.05$ compared with pretreatment values (Student's paired *t*-test).

the results of several studies showing that icatibant is a non-competitive antagonist of B_2 receptors (Hock *et al.*, 1991; Wirth *et al.*, 1991; Rhaleb *et al.*, 1992; Félétou *et al.*, 1994; Kajekar and Myers, 2000; Levy and Zochodne, 2000).

Similar to the analysis of the effect of kinin receptor antagonists on contractile activity of the isolated urinary bladder, urodynamic analyses of the micturition process were assessed on day 28 after SCI, a period in which the external urethral sphincter (EUS) activity was partially improved, enabling the release and measure of fluid through the urethra. A previous study showed that the urinary bladder is areflexic during the first 4 days after SCI, and that following this period NVCs were present (Takahara *et al.*, 2007). The NVCs are represented by increased spontaneous activity of the detrusor smooth muscle cells during the filling phase, which may cause OAB. Therefore, NVCs are believed to play a central role in the pathophysiology of OAB. It has been shown that the spontaneous phasic activity of the detrusor tissue is myogenic in origin (Liu *et al.*, 1998) and that the urothelium plays a significant role in modulating the nature of these contractions (Buckner *et al.*, 2002). Our results showed that SCI caused a significant increase in the amplitude and number of NVCs during the urinary bladder filling phase. With regard to this parameter, icatibant seems to be more effective than DALBK and SSR240612 since those B_1 receptor antagonists reduced only the number of NVCs while icatibant reduced both the amplitude and number of NVCs. These findings suggest that both B_1 and B_2 receptors expressed in the detrusor muscle and urothelium are involved in the emergence of NVCs.

SCI disrupts the phasic activity of the EUS resulting in decreased VE and increased (RV; Dolber *et al.*, 2007). The dysfunction of the EUS triggers the development of detrusor-sphincter DSD. In our study, we observed marked alterations in the voiding caused by the EUS dysfunction such as reduced VV and VE and greater urinary BC due to the excessive stretching of the detrusor muscle triggered by overloading the urinary bladder. In that sense, acute systemic treatment with SSR240612, a non-peptide B_1 receptor antagonist, was more effective than the treatment with DALBK, a peptide B_1 receptor antagonist, because SSR240612 significantly increased the VV and the VE and reduced the urinary BC. The action of SSR240612 on the VE suggests that B_1 receptor blockade might have an effect on the spinal motor neurons that control the impaired activity of the EUS after SCI. Moreover, the greater efficacy of SSR240612 compared to DALBK on the voiding function could be explained by their long-lasting efficacy and good bioavailability (Gougat *et al.*, 2004). Therefore, B_1 receptor inhibition seems to exert beneficial actions on OAB and DSD.

In summary, we provided evidence for the basal expression of B_2 receptors in the urinary bladder, which suggests that BK may play a role in normal urinary bladder functioning. The up-regulation of B_1 receptors in SCI urinary bladders might be associated with the alterations in urinary bladder reflex pathways in OAB caused by SCI. Moreover, the B_1 and B_2 receptor antagonism improves urodynamic parameters associated with the OAB in SCI animals. Thus, kinin receptor-selective antagonists might constitute an attractive pharmacological tool for the treatment of urinary bladder overactivity in neurogenic conditions such as SCI.

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Conflict of interest

The authors declare no conflict of interest.

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