Elimination of rat spinal neurons expressing neurokinin 1 receptors reduces bladder overactivity and spinal c-fos expression induced by bladder irritation

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Seki, Satoshi, Kristin A. Erickson, Masako Seki, Osamu Nishizawa, Yasuhiko Igawa, Teruyuki Ogawa, William C. de Groat, Michael B. Chancellor, and Naoki Yoshimura. Elimination of rat spinal neurons expressing neurokinin 1 receptors reduces bladder overactivity and spinal c-fos expression induced by bladder irritation. Am J Physiol Renal Physiol 288: F466–F473, 2005; doi:10.1152/ajprenal.00274.2004.—Substance P (SP) binding to neurokinin 1 receptors (NK1R) in the spinal cord reportedly plays an important role in the micturition reflex as well as in nociceptive responses. We therefore investigated the effect of ablation of NK1R-expressing neurons in the spinal cord using saporin, a ribosome-inactivating protein, conjugated with [Sar9, Met (O2)11]SP, a specific ligand of NK1R (SSP-saporin), on the micturition reflex in rats. In female Sprague-Dawley rats, SSP-saporin (1.0 or 1.5 M) only was injected through an intrathecal catheter implanted at the L5–S1 level of the spinal cord. Three weeks after intrathecal administration of SSP-saporin, NK1R immunoreactivity in lamina I of the spinal cord was significantly reduced, but cystometric parameters in awake rats were not altered. Instillation of capsaicin (15 M) into the bladder of norm rats induced bladder overactivity. This response to capsaicin was significantly suppressed in SSP-saporin-treated animals. SSP-saporin treatment also decreased c-fos expression in the dorsal horn of the spinal cord induced by instillation of capsaicin into the bladder. These data indicate that NK1R-expressing neurons in the superficial layer of the dorsal horn play an important role in transmission of nociceptive afferent information from the bladder to induce bladder overactivity and spinal c-fos expression elicited by bladder irritation. Toxin-induced damage of NK1R-expressing neurons in the lumbosacral spinal cord may provide an effective modality for treating overactivity and/or nociceptive responses in the bladder without affecting normal micturition.

TACHYKININS (TKs) are a family of peptides that include substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) (13). The distribution and functional significance of TKs have been widely investigated in the last decade especially in sensory systems. Although the majority of studies of TKs have been focused on their possible role as pain transmitters in the somatic domain, several studies have also indicated that TKs are involved in the regulation of neural inputs from visceral primary afferents to the spinal cord (20) and that SP and its neurokinin 1 (NK1) receptors (NK1R) in the spinal cord play an important role in the micturition reflex as well as in bladder nociceptive responses (21).

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visceral organs such as the bladder. In addition, while SP has some affinity for other neurokinin receptors, [Sar°,Met (O2)1]SP (SSP) is a specific analog of SP that shows greater selectivity for NK1R and greater potency and duration of action than SP in vivo (35, 40, 43).

We therefore sought to investigate the effects of elimination of NK1R-expressing neurons in the spinal cord on the micturition reflex and bladder nociceptive responses induced by capsaicin using SSP-saporin, a specific NK1R-targeting toxin, in rats to clarify the role of spinal SP-NK1R mechanisms in the control of bladder function.

MATERIALS AND METHODS

Experiments were performed on adult female Sprague-Dawley rats (226–273 g). Care and handling of animals were in accordance with institutional guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Administration of SSP-Saporin

An intrathecal catheter (PE-10, Clay Adams, Parsippany, NJ) was implanted at the level of the L4–L5 spinal cord following a laminectomy at the Th12 vertebra under halothane anesthesia. The proximal end of the catheter was heat-sealed and placed subcutaneously. Three to 4 days after intrathecal catheter implantation, either 8 μl of saporin (1.5 μM, n = 8) or SSP-saporin (Advanced Targeting Systems, 1.0 and 1.5 μM, n = 6 and 7, respectively) was injected through the intrathecal catheter under halothane anesthesia. Both saporin and SSP-saporin were dissolved in artificial cerebrospinal fluid. We used saporin-treated animals as controls as a previous study demonstrated that intrathecal administration of saporin alone has no effects on NK1R staining in the spinal cord (29). In addition, the concentrations of SSP-saporin at 1.0 and 1.5 μM were selected because it has been reported that 5.0 μM SP-saporin injected into the intrathecal space eliminated NK1R-expressing spinal cord neurons to disrupt pain sensation (29) and that SSP-saporin was 6–10 times more cytoxic to NK1R-positive neurons than SP-saporin (43).

Cystometry

Three weeks after intrathecal injection of either saporin or SSP-saporin, cystometry in conscious rats was performed according to previously published methods (44). Under halothane anesthesia, a polyethylene catheter (PE-50) was inserted via a midline abdominal incision into the bladder through the bladder dome. After the surgery, rats were placed in a restraining cage and allowed to recover from anesthesia for 1–2 h. The intravesical catheter was connected via a three-way stopcock to a pressure transducer and a syringe pump for recording intravesical bladder pressure and infusing saline into the bladder, respectively. Saline at room temperature was infused at a rate of 0.04 ml/min to elicit repetitive bladder contractions. Before measuring control cystometric parameters, saline infusion was continued for at least 3 h until stable bladder contractions were observed.

Saline voided from the urethral meatus was collected and measured to determine voided volume (VV). After constant VVs were collected, the infusion was stopped and postvoid residual volume (RV) was measured: residual saline was withdrawn through the intravesical catheter by gravity and then the bladder was completely emptied by manual compression through the abdominal wall. Bladder capacity (BC) was calculated as the sum of VV and RV. Based on these values, voiding efficiency (VE, %) = [(VV/BC) × 100] was calculated. Intercontraction interval (ICI), maximal voiding pressure (MVP), pressure threshold for voiding (PT), and baseline intravesical pressure (BP) were also measured.

After a control period with saline infusion, the solution was switched to saline containing 15 μM capsaicin (Sigma, St. Louis, MO) to elicit bladder overactivity, which was evaluated by a reduction in ICI of repetitive bladder contractions. Voiding cycles obtained from 10 to 45 min after switching to the capsaicin solution were measured and used for data analyses. A software package (Windaq, Dataq Instruments, Akron, OH) was used for data collection and manipulation.

Histochemical Staining with NK1R Antibody

After cystometry, animals were deeply anesthetized with pentobarbital sodium (80 mg/kg ip) and perfused through the left ventricle with 200 ml of cold oxygenated Krebs-Ringer solution, followed by a fixative consisting of 4% paraformaldehyde in 0.1 M PBS. After perfusion, the position of the intrathecal catheter tip was confirmed and the L4, L5, L6, and S1 levels of the spinal cord were harvested for histological examination. The tissues were placed in PBS containing increasing concentrations of sucrose (10, 20, and 30%) at 4°C for cryoprotection. These specimens were embedded in OCT compound (Sakura Finetek) and frozen at −80°C. Serial frozen sections, 20-μm thick, were cut with a cryostat (MICROM, Walldorf, Germany) and collected in PBS as free-floating sections. Sections were incubated for 30 min at room temperature in phosphate-buffered donkey Tris serum solution and then incubated for 24 h at 4°C in the primary antiserum [polyclonal rabbit anti-NK1R antibody (1:1,000, Advanced Targeting Systems)]. Sections were washed three times for 10 min in potassium phosphate-buffered saline (K-PBS) and incubated for 90 min at room temperature in the secondary antibody solution [Cy 3-conjugated F(ab’2) fragment of goat anti-mouse IgG (1:600, Jackson Labs, West Grove, PA)]. They were then washed three times for 10 min in K-PBS and placed on Superfrost/Plus Microscope slides (Fisherbrand, Pittsburgh, PA). The sections were placed into coverslips with Gel/Mount medium solution (Biomeda, Foster City, CA).

Histological Analysis of NK1R-Positive Cells

Sections were examined with an epifluorescence microscope, and photographs were taken by a digital imaging camera connected to a microscope (Olympus, Tokyo, Japan).

For the evaluation of NK1R staining in spinal cord sections, we measured relative percent area positively stained with NK1R antibody in lamina I of the dorsal horn in 4–10 randomly selected sections from L4, L5, L6, and S1 spinal cord in saporin- and SSP-saporin-treated (1.0 and 1.5 μM) animals (n = 6–8 in each group). Images were captured with an epifluorescence microscope equipped with UV filters as red-green-blue-tagged image format files and then divided into three colors. After maximal intensity of fluorescence of background in each section was measured, relative percent area, which was stained stronger than background, in the total area of lamina I was calculated using Image-Pro Express software (Media Cybernetics, Silver Spring, MD). The total area of the lamina I was determined by the Nissl’s staining method according to the procedure of Molander et al. (31) in alternate spinal cord sections next to those evaluated for NK1R staining in each animal. Relative percent area expressing NK1R immunoreactivity, total selected area size, and maximal intensity of background area were compared between three groups of animals. In the L4 spinal cord, relative percent area of lamina X and the parasympathetic nucleus was also examined.

Histochemical Staining of Fos-Positive Nuclei

Using a separate group of animals, 15 μM capsaicin solution was instilled into the bladder at a rate of 0.04 ml/min for 2 h followed by animal perfusion. Next, the L4 spinal cord was removed for examination of c-fos immunoreactivity study.

Spinal cord sections were processed by an avidin-biotin complex (ABC) method with Fos antibodies purchased from Oncogene Science (Cambridge, MA), as described previously (2, 16). Briefly, sections were incubated in Fos antiserum (1:15,000) for 30 min at room temperature and then for 72 h at 4°C. Sections were then exposed to
biotinylated secondary antibody (1:600; Vector, Burlingame, CA) and ABC reagent (Vector), each for 1 h at room temperature. Tissue sections were then mounted on gelatin-coated slides, dehydrated in graded ethanol rinses, cleared in xylene, and placed into coverslips with Permount. Control sections in which primary antibody was replaced with 0.4% Triton X-100 were negative.

Histological Analysis of Fos-Positive Nuclei

Analysis was performed on the L6 spinal cord segment, because in the previous studies the largest number of Fos-positive nuclei after bladder irritation was located in this segment (2, 16). The number of Fos-positive nuclei was counted on both sides of the spinal cord and presented as average numbers per section (6–8 sections per animal). The results were compared between saporin- and SSP-saporin (1.5 μM)-treated rats. Randomly selected sections of L6 spinal cord (19 or 22 sections, respectively) in saporin- and SSP-saporin-treated animals (n = 3 and 3, respectively) were used for analysis.

Statistics

Results were expressed as means ± SE. A Kruskal Wallis test with Dunn’s posttest was used to compare cystometric parameters and the percentage of the area positively stained with NK1 in the lamina I, X, and the parasympathetic nucleus. A nonparametric test (Mann-Whitney U-test) was used to compare the number of Fos-positive nuclei in L6 spinal cord between saporin- and SSP-saporin-treated rats. For all statistical tests, P < 0.05 was considered significant.

RESULTS

Cystometry

Control period with saline infusion. Cystometric parameters obtained during saline infusion into the bladder were not significantly different in saporin- and SSP-saporin-treated rats, indicating that the SSP-saporin treatment did not affect normal voiding function (Table 1).

Effects of intravesical capsaicin. When bladder hyperactivity was induced by intravesical instillation of capsaicin (15 μM), bladder overactivity was observed in both saporin- and SSP-saporin-treated rats (Fig. 1). ICI was reduced by 53.5 ± 2.2% in saporin (1.5 μM)-treated rats (n = 8). However, a reduction of ICI after capsaicin instillation was significantly smaller (reduction: 41.6 ± 3.8 and 27.4 ± 4.25%) in SSP-saporin-treated rats [1.0 μM (n = 6) and 1.5 μM (n = 7), respectively] than in saporin-treated rats (Fig. 2). MVP, PT, and BC were not significantly different before and after capsaicin instillation in both saporin- and SSP-saporin-treated rats (Table 1).

Histological Analysis of NK1R-Positive Cells

We investigated the relative percent area positively stained with NK1R antibody (% area) in lamina I in L1, L5, L6, and S1, and lamina X and the parasympathetic nucleus in L6 spinal cord segments. In lamina I of L6 and S1 spinal cord, percent area was significantly decreased by SSP-saporin treatment in a dose-dependent manner compared with saporin treatment (Figs. 3 and 4). However, in the lamina I of L1 (data not shown) or L5 spinal cord, percent area was not significantly changed by SSP-saporin treatment compared with saporin treatment (Fig. 4), indicating that SSP-saporin injected at the L6-S1 spinal cord level did not diffuse to other segments. In addition, in the lamina X (Fig. 4) or the parasympathetic nucleus area (data not shown) of L6 spinal cord, percent area was not altered in SSP-saporin-treated rats compared with saporin-treated rats.

The size of total selected area and the cut-off value (maximal intensity of background area) were not significantly different between three groups of animals in all of the sections examined (data not shown).

Histological Analysis of Fos-Positive Nuclei

After capsaicin infusion for 2 h, nuclei exhibiting Fos immunoreactivity were counted in four regions in the L6 spinal cord: medial dorsal horn (MDH), lateral dorsal horn (LDH), dorsal commissure (DCM), and lateral laminae V-VII containing the sacral parasympathetic nucleus (SPN) (Fig. 5).

The number of Fos-positive nuclei in MDH, SPN, and DCM was significantly decreased in SSP-saporin (1.5 μM)-treated rats (7.0 ± 2.2, 1.5 ± 0.5, and 1.3 ± 0.4, respectively) compared with saporin-treated rats (16.5 ± 4.2, 3.4 ± 1.3, and 2.4 ± 1.0, respectively). However, in LDH the number of Fos-positive nuclei did not decrease in SSP-saporin-treated compared with saporin-treated rats (3.1 ± 0.4 and 4.0 ± 0.6 nuclei/section, respectively).

DISCUSSION

The results of this study indicate that intrathecal treatment with SSP-saporin at the level of L6-S1 spinal cord reduced NK1R immunoreactivity in the superficial layer of the dorsal horn and suppressed bladder overactivity as well as c-fos expression in the spinal cord induced by intravesical capsaicin without affecting normal micturition. Thus targeting NK1R-positive spinal neurons in this area, which receive synaptic inputs from peptidergic C-fiber afferents, may be effective for treating overactivity and/or visceral pain responses in the bladder.

Afferent pathways innervating the urinary bladder consist of myelinated Aδ-fibers and unmyelinated C-fibers. In normal

<table>
<thead>
<tr>
<th>CMG control period</th>
<th>Saporin (n = 8)</th>
<th>SSP-Saporin (1.0 μM) (n = 6)</th>
<th>SSP-Saporin (1.5 μM) (n = 7)</th>
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<tbody>
<tr>
<td>Intercollection interval, min</td>
<td>12.5 ± 0.9</td>
<td>14.4 ± 2.1</td>
<td>15.4 ± 1.5</td>
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<tr>
<td>Maximal voiding pressure, cmH2O</td>
<td>39.7 ± 2.8</td>
<td>33.6 ± 4.6</td>
<td>33.9 ± 2.5</td>
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<tr>
<td>Pressure threshold for voiding, cmH2O</td>
<td>11.1 ± 0.2</td>
<td>12.8 ± 0.7</td>
<td>12.1 ± 0.8</td>
</tr>
<tr>
<td>Baseline intravesical pressure, cmH2O</td>
<td>7.81 ± 0.3</td>
<td>8.35 ± 0.9</td>
<td>7.87 ± 0.5</td>
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<td>Bladder capacity, ml</td>
<td>0.48 ± 0.04</td>
<td>0.56 ± 0.10</td>
<td>0.69 ± 0.96</td>
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<tr>
<td>Voiding efficiency, %</td>
<td>92.0 ± 1.1</td>
<td>93.9 ± 1.3</td>
<td>84.9 ± 8.4</td>
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<tr>
<td>CMG after capsaicin instillation</td>
<td>111 ± 6.6</td>
<td>124 ± 12.7</td>
<td>107 ± 8.4</td>
</tr>
<tr>
<td>Maximal voiding pressure, % of control</td>
<td>91.4 ± 3.2</td>
<td>101 ± 3.5</td>
<td>102 ± 4.9</td>
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<tr>
<td>Pressure threshold for voiding, % of control</td>
<td>105 ± 4.4</td>
<td>104 ± 2.4</td>
<td>100 ± 3.4</td>
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Values are means ± SE; n, no. of rats. SSP, [Sar9, Met(O)11]substance P; CMG, cystometry. Voiding efficiency = voided volume/bladder capacity × 100 (%). Values after capsaicin instillation were expressed as % of control values before capsaicin instillation.
rats, conscious voiding is dependent on Aδ-fiber bladder afferents even though both Aδ-fiber and C-fiber bladder afferents are mechanoreceptive, whereas C-fiber afferents are responsible for bladder nociceptive responses (5, 6, 24, 36). In addition, it has been demonstrated that hyperexcitability of C-fibers in bladder afferent pathways can contribute to bladder overactivity and/or bladder pain under pathological conditions such as spinal cord injury or chronic cystitis (5). It has also been documented that TKs such as SP are expressed in a population of C-fiber afferents (peptidergic C-fibers) (37) and that noxious stimulation reportedly releases SP from these afferent fibers within the superficial dorsal horn (30). Moreover, SP released from afferent terminals in the spinal cord can reportedly facilitate the micturition reflex and has an important role in bladder overactivity induced by bladder irritation (19). Thus an interruption of synaptic input from SP-containing C-fiber afferents to NK1R-expressing spinal neurons seems to be a reasonable approach for the treatment of bladder overactivity and painful responses.

In the present study, immunohistochemical staining of NK1R was consistently found most prominently in the lamina I of the dorsal horn and in an area around the central canal (lamina X) in the lumbosacral spinal cord. This is in line with previous findings that NK1R immunoreactivity was present predominantly in the lamina I, IV-VI, and X, but low in the lamina II, and that the heaviest staining of NK1R was found in lamina I (10). In addition, previous immunocytochemical studies revealed that NK1R immunoreactivity was located on neuronal cell bodies and dendrites in the spinal cord (3, 22, 23).

Although saporin itself is not taken up by neurons, it becomes cytotoxic to induce cell death when internalized in neurons after administrated as saporin conjugates (29). Previous studies also demonstrated that SP-saporin given intrathecally into the spinal cord produced 60 to 85% reduction in the
number of NK1R-positive neurons in the lamina I of the spinal cord, whereas saporin itself had no effects on NK1R immuno-reactivity in the spinal cord (17, 29). Thus a decrease in the spinal cord area stained with NK1R antibodies after intrathecal injection of SSP-saporin in the present study is likely to represent a reduction in the number of NK1R-positive cells in the L₆-S₁ spinal superficial dorsal horn. In addition, because there was not a significant reduction in NK1R staining of the lamina X of L₆ spinal cord, the effect of SSP-saporin was limited to the superficial layer of the dorsal horn. Moreover, a previous study (29) demonstrated that there were no significant changes in SP immunoreactivity in DRG cells after intrathecal

![Fig. 3. Typical immunofluorescence photomicrograph of the lamina I of L₆ spinal cord in saporin (A) and SSP-saporin-treated rats (1.0 (B) and 1.5 (C) μM). Bottom left box: photomicrograph indicates the areas where higher-magnification pictures were taken. Note that the area positively stained with NK1R antibody in the lamina I was decreased after SSP-saporin administration. Scale bar represents 100 μm.](image)

![Fig. 4. Relative % area positively stained with NK1R antibody (% area) in different spinal cord segments. In the lamina I of L₆ (A) and S₁ (B) spinal cord, % area was significantly decreased by SSP-saporin treatment in a dose-dependent manner compared with saporin treatment. However, in the lamina I of L₅ spinal cord (C) or the lamina X of L₆ spinal cord (D), % area was not changed by SSP-saporin treatment compared with saporin treatment. **P < 0.01.](image)
administration of SP-saporin. It has also been reported that NK1R staining was found in spinal cord neurons, but not in primary afferent fibers located in the spinal cord dorsal horn, and that DRG neurons were not stained with NK1R antibodies (4). Thus it is assumed that treatment with the saporin conjugate in the spinal cord does not affect DRG neurons or induce peripheral effects in the bladder.

Because peptidergic afferent fibers containing SP predominantly terminate in lamina I and the outer part of lamina II in the spinal cord (37), it is likely that SP-containing afferent fibers make synapses onto NK1R-expressing spinal neurons in this area. Thus it is assumed that elimination of NK1R-expressing spinal neurons in the lamina I using SSP-saporin can disrupt synaptic inputs from SP-containing afferent neurons. It is also known that in addition to a C-fiber population expressing neuropeptides, there is another population of C-fibers that is largely nonpeptidergic, binds Banderaeae simplicifolia isolectin-B4 (IB4), and projects most heavily to lamina II of the spinal cord (37). We previously found that C-fiber afferents innervating the bladder contain a larger population of IB4-negative, peptidergic C-fiber cells than somatic nerve afferents innervating the distal urethra (80 vs. 51% of C-fiber neurons) (46). The large number of IB4-negative, peptidergic C-fiber cells in bladder afferent pathways has also been reported compared with somatic afferent pathways innervating skin (1). Thus it is assumed that suppression of synaptic inputs from SP-containing peptidergic C-fibers could be more effective in reducing visceral pain responses than in suppression of somatic pain behavior.

It has also been documented that in pathological conditions such as spinal cord injury or cystitis, the contribution of the capsaicin-sensitive, SP-positive afferent neurons to bladder overactivity and painful conditions seems to be increased. For example, NK1R expression has been found to be upregulated in the dorsal horn after spinal cord injury (34, 41) and the density of SP immunoreactivity was increased in the superficial laminae of the L₆ and S₁ spinal segments following chronic cystitis induced by cyclophosphamide (42). Thus it seems reasonable to assume that the role of NK1R-expressing neurons in the superficial dorsal horn becomes more important in inducing bladder overactivity in these pathological conditions and that targeting SP-NK1R mechanisms in the spinal superficial dorsal horn using toxin conjugates such as SSP-saporin may provide a great opportunity to treat these conditions.

Although several studies demonstrated that intrathecal administration of NK1R antagonists does not change bladder function in normal rats under either conscious or anesthetized conditions (15, 19), other studies reported increased BC and/or decreased MVP after intrathecal injection of NK1 antagonists (14, 19). Differences in the permeability and degree of penetration to deeper spinal laminae of NK1R antagonists as well as different types of anesthesia might be responsible for these discrepancies. However, as in our study, elimination of NK1R-expressing neurons at the superficial layer of the dorsal horn by SSP-saporin treatment did not affect normal bladder function under an awake condition, it seems that NK1R-positive spinal neurons in the lamina I of lumbosacral spinal cord are not involved in the normal voiding function.

It should be noted that ablation of NK1R-expressing neurons in the dorsal horn can suppress synaptic transmission through spinal projection neurons to higher brain and/or interneurons activated by capsaicin-sensitive C-fiber afferents but not block the effects of locally released TKs within the bladder wall following capsaicin instillation (20, 25–27). In addition, it has
been reported that some central branches of afferent neurons, which contain SP, enter the Lissauer tract and run through the lamina I area to terminate directly in the area of SPN, thereby inducing bladder contractions (8). Therefore, an incomplete reduction of capsaicin-induced bladder overactivity after SSP-saporin treatment in this study might be related to locally released TKs from peripheral afferent terminals and/or activation of capsaicin-sensitive C-fiber afferents that bypass lamina I, in addition to incomplete elimination of NK1R-expressing dorsal horn neurons after the treatment.

In conclusion, intrathecal administration of SSP-saporin selectively eliminated NK1R-expressing neurons in the superficial dorsal horn of L6-S1 spinal cord and dramatically reduced the development of bladder overactivity after capsaicin instillation without affecting normal micriction reflex. These findings indicate that NK1R-expressing neurons in the superficial layer of the dorsal horn play an important role in transmission ofafferent information from the bladder and in inducing bladder overactivity elicited by bladder irritation. Therefore, elimination of NK1R-expressing neurons in the lumbosacral spinal cord using SSP-saporin may provide a great opportunity for treating detrusor overactivity and bladder nociceptive responses.

GRANTS

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REFERENCES


