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ORIGINAL ARTICLE

Role of neurokinin type 1 receptor in nociception at the periphery and the spinal level in the rat

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Objectives: Noxious stimuli activate small to medium-sized dorsal root ganglion (DRG) neurons. Intense noxious stimuli result in the release of substance P (SP) from the central terminals of these neurons. It binds to the neurokinin type 1 receptor (NK1r) and sensitises the dorsal horn neurons. SP is also released from the peripheral terminals leading to neurogenic inflammation. However, their individual contribution at spinal and peripheral levels to postincisional nociception has not been delineated as yet.

Methods: Sprague–Dawley rats were administered different doses (3–100 μg) of an NK1r antagonist (L760735) by intrathecal (i.t.) route before hind paw incision. On the basis of its antinociceptive effect on guarding behaviour, the 30 μg dose was selected for further study. In different sets of animals, this was administered i.t. (postemptive) and intrawound (i.w.). Finally, in another group, drug (30 μg) was administered through both i.t and i.w. routes. The antinociceptive effect was assessed and compared. Expression of SP was examined in the spinal cord. Intrawound concentration of SP and inflammatory mediators was also evaluated.

Results: Postemptive i.t. administration significantly attenuated guarding and allodynia. Guarding was alone decreased after i.w. drug treatment. Combined drug administration further attenuated all nociceptive parameters, more so after postemptive treatment. Expression of SP in the spinal cord decreased post incision but increased in the paw tissue. Inflammatory mediators like the nerve growth factor also increased after incision.

Conclusion: In conclusion, SP acting through the NK1r appears to be an important mediator of nociception, more so at the spinal level. These findings could have clinical relevance.

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INTRODUCTION

Neurons of the dorsal root ganglion (DRG) are classified on the basis of their size (small, medium or large), functional modalities (pain/temperature or touch/proprioception), neuropeptide content (like substance P (SP), calcitonin gene-related peptide, galanin or somatostatin), diameter of peripheral neurites (Aβ, Aδ or C) or even the expression of molecular markers (like 200 kDa neurofilament protein).[1] In the rat, ~50% of the DRG neurons with unmyelinated C nerve fibres express SP.[2] SP is an 11 amino acid neuropeptide, which is released from both the central and the peripheral terminals of the peptidergic small to medium-sized DRG neurons following noxious stimuli.[3] It binds preferentially to the neurokinin type 1 receptor (NK1r).[4] Release of SP in the spinal cord dorsal horn is associated with enhanced synaptic transmission, whereas its release at the periphery gives rise to neurogenic inflammation.[5,6] Thus, the DRG neurons have been labelled as 'Bidirectional nociceptors'.[7] Also, it has been hypothesised that simultaneous blockade of SP activity at both the central and peripheral terminals of the DRG neurons can produce antinociception.[7]

SP belongs to the tachykinin family, which also includes other members like neurokinins A and B.[3,8] Tachykinin receptors (NK1r, NK2r and NK3r) are G protein-coupled receptors. They are present in the mammalian nervous tissue and mediate functions like nociception, inflammation, memory, depression and epilepsy.[8] Their role in the skin, respiratory tract, gut, urinary system and blood vessels is also important, where they are likely located in primary sensory afferents.[3] Recently, the role of SP in the breakdown of blood–brain barrier and cerebral oedema following stroke has been postulated.[9]

Pain follows tissue damage, which can be due to surgery, accidents, varicose ulcers or burn injuries. However, the treatment of pain continues to remain suboptimal.[10] Previous reports indicate that peripheral release of SP is related to nociception following neuropathy,[11] fractures[12] and hind paw incision.[13] Involvement of SP at the level of superficial laminae (Rexed's laminae I–II) of the spinal cord is well established in animal models of nociception.[4,14,15] Among the various preclinical pain models, the hind paw incision model is representative of postoperative pain.[16] Postincisional nociception is evaluated by guarding behaviour, mechanical allodynia and thermal hyperalgesia. Guarding likely represents ongoing pain (pain-at-rest) following surgery.[16,17] Mechanical allodynia and thermal hyperalgesia (evoked) are also observed following surgery.

The primary hypothesis of this study was that SP contributes to postincisional nociception at both the spinal and peripheral levels. Previously, intra-plantar capsaicin injection in NK1r knockout mice was followed by paw licking behaviour, although mechanical hyperalgesia was completely absent.[18] According to the authors, NK1r is functionally associated with mechanosensitive nociceptors and with related neurotransmission at the spinal level. In a different study, chemo-nociception induced by intraperitoneal injection of capsaicin could be separately attenuated at both the peripheral and spinal levels

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by an NK1r antagonist.[19] However, the role of peripheral versus spinal NK1r in hind paw incision-induced nociception is unknown. Thus, we aimed to block SP-driven neural activity at each of these sites by an NK1r antagonist and observed the resultant effect on nociceptive behaviour. One of the secondary hypotheses was that SP could be more important in the maintenance of nociception rather than its induction at the spinal level. Thus, the comparative difference in nociceptive behaviour between preemptive versus postemptive modes of intrathecal administration of NK1r antagonist was investigated. Another secondary hypothesis was that SP could be secondarily causing the release of proinflammatory cytokines like Interleukin-1β at the incision site, which could contribute to nociception.[20]

MATERIALS AND METHODS

Animals

Prior permission for experimentation was obtained from the Institutional Animal Ethics Committee. Male Sprague–Dawley rats (275–325 g) were randomly allocated to different experimental groups (Figure 1). Food and water were provided *ad libitum*. Light/dark (12:12) cycles were maintained. Animals were acclimatised to laboratory conditions for 3–4 days before commencement of experimental work. Following surgery, rats were housed individually in cages with clean bedding (Alpha-dri, Shepherd speciality papers, Milford, NJ, USA).

Intrathecal catheterization

The procedure of intrathecal catheterization has been reported earlier.[21,22] In brief, under isoflurane anaesthesia, the head of the rat was fixed in a stereotaxic frame. The skin over the scalp was incised. The underlying muscle was detached from the occipital crest to expose the cisternal membrane. A sterile (8.5 cm; PE-5) catheter (ReCath Co, Allison Park, PA, USA) was introduced through a cut in the membrane so that the distal end was located just above the lumbar enlargement. The thicker part (4 cm; PE-10) was placed outside. The wound was closed with 4-0 polyamide (Ethicon, Johnson & Johnson, Mumbai, India) sutures. Animals were allowed to recover for 5 days. Rats, which showed any motor disability, were euthanised. The location of the catheter was confirmed by administration of 15 μl of 2% lignocaine on the third day. There was paralysis of hind limbs for 5–10 min.

Behavioural assessment of nociception

Nociception was assessed by guarding score, mechanical allodynia and thermal hyperalgesia in a sequential manner. The specific time points (for example, 2 h) corresponded with the beginning of behavioural testing. Observers were blinded to the drugs administered to the rats. Postincisional pain behaviour was assessed at 2 h, 8 h and days 1–4 for guarding and days 1–7 for thermal hyperalgesia and mechanical allodynia.

Guarding. Guarding was evaluated as reported earlier.[23] Each rat was placed over a wire mesh (8 × 8 mm) platform and covered with a perspex enclosure (16 × 16 × 16 cm). Following acclimatisation for 15 min, the position of the hind paws was observed from below the mesh. This was done for 1 min in 5 min bins for a total period of 1 h. A magnifying mirror was used for this purpose. If the incised area was off the mesh for maximum period during the 1 min observation period, a score of 2 was given. If it was lightly touching the mesh without weight bearing, a score of 1 and a score of 0 if there was full weight bearing with blanching and distortion of the skin. The left paw was also observed and scored similarly. The 12 scores were summed for each paw. The difference between the scores of the two paws was the cumulative pain score.

Mechanical allodynia. This was determined by the 'Up-Down Method'.[24] Nylon von Frey filaments (North Coast Medical Inc., San Jose, CA, USA) of various sizes (3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93 and 5.18) were applied sequentially at the peri-incisional site. The filament was pressed to the skin for 6–7 s in a perpendicular manner until it buckled or resulted in abrupt withdrawal. The size of the first filament was 4.31. If there was no withdrawal, the next heavier filament was applied. In case of withdrawal, the next lighter filament was used. Testing was continued until four filaments were applied (heavier or lighter depending on the exact filament size to which the last response occurred) after the first one that produced a withdrawal. If there was progressive withdrawal up to the lightest filament, the latency was recorded to be 0.4 g. If no withdrawal occurred sequentially up to the heaviest filament, the

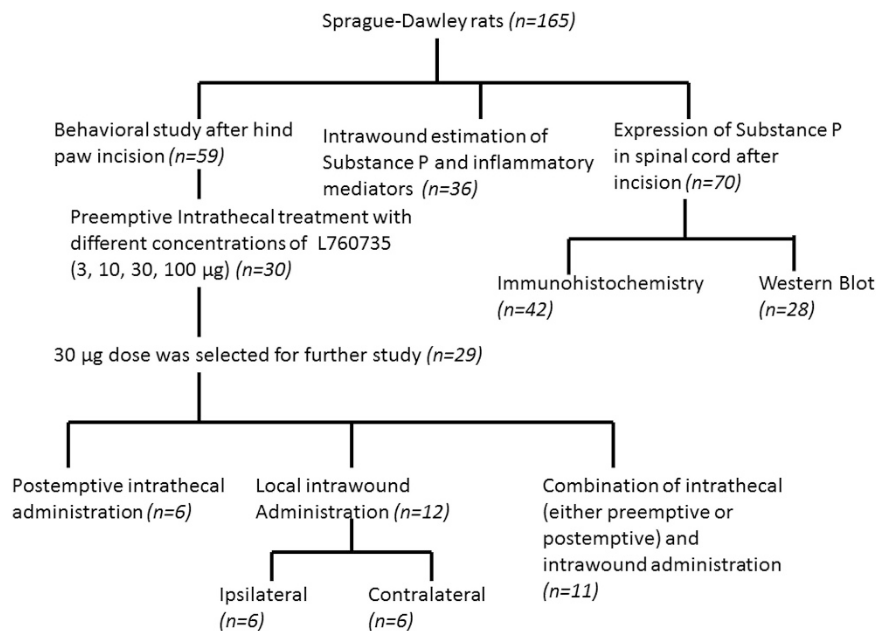


Figure 1 Flow chart of experimental work. Rats were primarily divided into three groups for (a) behavioural evaluation of nociception after administration of L760735, (b) intrawound estimation of substance P (SP) and inflammatory mediators by ELISA and (c) expression of SP in the spinal cord by Immunohistochemistry and western blot methods. Regarding behavioural study, L760735 was initially administered by preemptive intrathecal (i.t.) route, 15 min before incision. On the basis of the result, particularly guarding score, the 30 μg dose was selected for further behavioural study. This was done after postemptive treatment and also after intrawound (i.w.) administration. Then, both i.t. and i.w. treatments were combined. The number of rats in each group has been indicated.

withdrawal latency was noted as 15 g. An interval of 2 min was maintained between two successive applications.

Thermal hyperalgesia. This was determined using the plantar test apparatus (UGO Basile, Varese, Italy).[25] Rats were placed on a glass platform within Perspex enclosures. After acclimatisation for 15 min, an infrared heat source was directed at the incision site and the latency period of withdrawal of the paw recorded. Baseline latency period was between 8 and 10 s. Cutoff time was 20 s. The latency period was determined thrice at intervals of 2 min and the average obtained. The percent maximum possible effect was derived from the following equation: ((Drug induced latency − Baseline latency)/(Cutoff latency − Baseline latency)) × 100.

Drugs and their administration

L760735 (Tocris Bioscience, Bristol, UK) is a high-affinity Neurokinin 1 receptor (NK1) antagonist (IC50 = 0.19 nM at hNK1 receptors), which was dissolved in saline (0.9%) to the required concentration. Intrawound (i.w.) administration was done using a sterile micropipette as reported earlier.[26] Ten microliter of the drug solution (30 μg per 10 μl) was administered just before closure of the wound. The drug solution was left undisturbed in the wound for 30 s.

For preemptive intrathecal administration (i.t.), the NK1r antagonist (3/10/30/100 μg in 10 μl) was administered by a Hamilton syringe using a 30G needle. This was followed by 10 μl of saline flush. The animal was lightly restrained during the drug administration. The control group received physiological saline. Fifteen minutes after drug treatment, plantar incision was performed. Treatment with 30 μg dose resulted in maximum antinociception at days 1–2, and hence this dose was selected for further study. For postemptive administration, rats were subjected to plantar incision. One hour after incision, the animals were anaesthetised by isoflurane inhalation (this became necessary as the rats were in pain) and the drug administered. One-hour period was chosen to sufficiently demarcate the preemptive from the postemptive and also permit sufficient time for the drug to have its effect before starting behavioural testing at 2 h from the time of incision.

Hind paw incision and postincisional pain behaviour

The method of incision has been previously described.[16] In brief, rats were first anaesthetised by inhalation of Isoflurane (2–2.5% in mixture of air and oxygen), and the plantar surface of the right hind paw was swabbed with 10% povidone-iodine solution. A 1 cm long midline incision from heel to toe was made by a No. 11 scalpel blade, starting 0.5 cm from the proximal edge of the heel. The underlying muscle was exposed, which was lifted up with a forceps. It was incised longitudinally for 0.5 cm without damaging the origin or insertion. The tip of the forceps was introduced through the incision and the limbs slightly separated. The muscle was replaced back followed by apposition of the skin edges by two mattress sutures with the knots placed on the lateral side (4-0 polyamide, Ethicon). The sutures were removed at the end of second day.

Antinociceptive effect of NK1r antagonist following i.w. administration and its site of action

To determine whether i.w. NK1r antagonist administration acted peripherally or centrally, guarding of the ipsilateral paw was compared after ipsilateral versus contralateral paw injection (30 μg intra-plantar, subcutaneously). A response to contralateral injection would suggest a centrally acting mechanism. As the guarding score was alone affected by ipsilateral i.w. drug administration, this was only evaluated after contralateral administration.

Estimation of substance P by enzyme-linked immunosorbent assay (ELISA)

Skin and muscle from the incision site (~4–6 mm size) were collected on ice at 2 h, days 1 and 3. Tissue was also isolated from naive rats. None of these rats were subjected to any behavioural study. Tissues were weighed and immediately homogenised in ice-cold 10 mM Tris buffer with 150 mM NaCl, 0.5% sodium deoxycholate, protease inhibitor cocktail (P-8340, Sigma chemicals) and 0.5% Triton-X 100 at 4 °C. Homogenate was centrifuged at 10 000 rpm for 25 min. The total protein concentration of the supernatant was estimated by the Bradford method and then equilibrated using wash buffer. SP competitive enzyme-linked immunosorbent assay (ELISA) was performed using 96-well pre-coated ELISA plate (USCN, catalogue no. CEA393Ra, Wuhan, People's Republic of China) according to the manufacturer's instructions. The minimum detectable concentration of SP was 0.25 pg per 50 μl. No significant cross-reactivity or interference between SP and its analogues (Neurokinin A or B) was present (manufacturer's data sheet).

Estimation of TNF-α, interleukin-1β and NGF in incised tissue by ELISA

Tissues were collected and processed as described for SP estimation. Animals used for ELISA, Immunohistochemistry and western blot studies were parallel sets of rats and were not subjected to behavioural testing. Tissues were harvested from both naive (without incision) and NK1r antagonist-treated (30 μg per 10 μl intrawound) animals. 96-well pre-coated ELISA plates (TNF-α catalogue no. 438207 from Biolegend, San Diego, CA, USA; Interleukin-1Beta, catalogue no. ELR-IL1β-001C and NGF, catalogue no. ELR-BNGF-001 from Raybiotech, Norcross, GA, USA) were used for performing the assay (Sandwich ELISA for TNF-α; Direct ELISA for Interleukin-1Beta and NGF) as per the manufacturer's instructions.

Immunohistochemistry

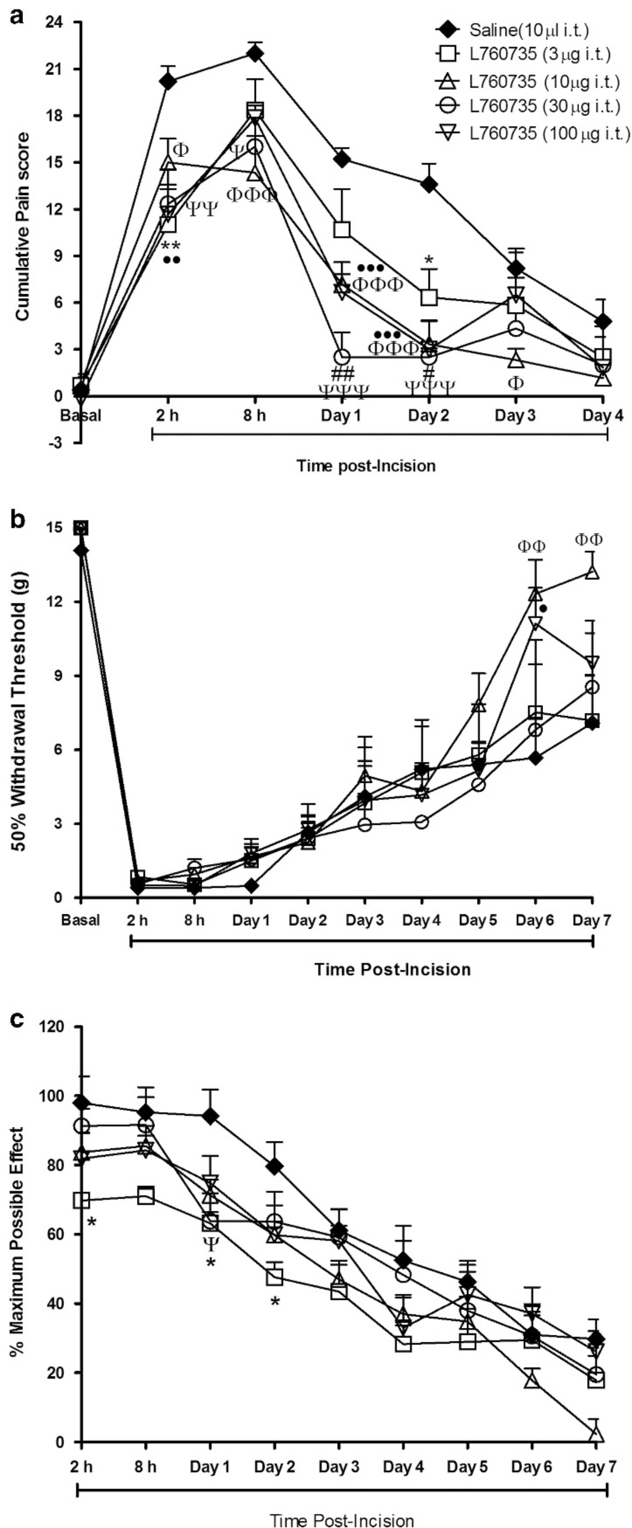
Rats at different time intervals after incision (1, 3, 6 and 12 h and days 1 and 3) along with the naive group were deeply anaesthetised with pentobarbital (100 mg per kg i.p.) and perfused with cold 0.1-M phosphate-buffered saline pH 7.4 through intracardiac route. This was followed by 4% paraformaldehyde in phosphate-buffered saline for fixation. The specific part of the spinal cord (L4–L5 segments) was then dissected out and the side contralateral to the incision marked by a fine-bore capillary tube. It was post fixed for 3 days followed by cryopreservation in sucrose solution. Tissue sections (20 μm thick transverse sections at −20 °C) were cut in a cryostat (CM 1950, Leica, Nussloch, Germany). These were processed for immunostaining by the free-floating method in phosphate-buffered saline. Briefly, sections were quenched for endogenous peroxidase activity with 80% methanol containing 0.3% hydrogen peroxide. Nonspecific binding was blocked with 10% normal goat serum containing 0.25% Triton-X 100. The sections were incubated in primary rabbit anti-SP polyclonal antibody (1:500; Abcam, Cambridge, UK) for 48 h at 4 °C. This antibody reacts with the pure form of SP as well as its precursor forms but not neurokinins A or B. Its specificity was tested by pre-absorption with SP peptide, which completely eliminated immunolabelling (manufacturer's data sheet). Afterwards, sections were exposed to biotin-conjugated IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) followed by the Avidin-Biotin complex for 60 min. Visualisation of SP expression was performed by 0.25% 3,3′-Diaminobenzidine tetrahydrochloride (Sigma) in phosphate-buffered saline. Finally, sections were collected on gelatin-coated slides, dehydrated, cleared and mounted in DPX. Photomicrographs of 5–6 sections per animal were acquired using a E-600 Nikon microscope (Nikon, Tokyo, Japan). Few sections, which were not exposed to the primary antibody but similarly processed, did not show any staining.

Analysis of SP expression was performed using the Image J software (Freely available from website of NIH, Bethesda, MD, USA). The dorsal region of the spinal cord showing SP expression was manually selected, and the mean density per unit area was evaluated (5–6 sections per rat). Nonspecific binding was similarly determined from the adjacent white matter and deducted from the previous value to obtain specific expression (Supplementary Figure 1).

Quantitation of SP by western blot

SP precursors (~12–20 kDa)—SPL1 and SPGKL1—were determined by immunoblotting as SP is a small molecule (~1.5–2 kDa) and difficult to detect.[27] Lumbar spinal cord tissue comprising spinal segments L4–L5 was collected from naive rats and from those after plantar incision (1, 3, 6 and 12 h and days 1 and 3). The ipsilateral half was separated and further processed. Samples were homogenised in ice-cold 1xRIPA buffer using protease inhibitor cocktail (1:100, catalogue No. P8340, Sigma, St Louis, MO, USA). The lysates

were incubated for 45 min and centrifuged at 12 000 *g* for 25 min at 4 °C. Total protein concentration was estimated by the Bradford method. Fifteen percent SDS–polyacrylamide gel (40 μg per well) was used to separate the total protein, which was transferred onto nitrocellulose membrane. After blocking with 5% non-fatty dry milk, the membrane was incubated overnight with primary antibody against SP (1:1000; Abcam). The loading control was incubated with antibody against α-tubulin. Following washing, the membranes were incubated with horseradish preoxidase-labelled secondary antibody for 1 h. Visualisation was performed by DAB prepared in 0.01 M Tris-HCl buffer containing 2 μl hydrogen peroxide. Bands were quantified using the Gel-Documentation system (AlphaImager, Protein Simple, San Jose, CA, USA).



Statistical analysis

Values are represented as mean ± s.e. of mean. Data were analysed by the GraphPad Prism version 5 software San Diego, CA, USA. All experimental groups for behavioural assessment had 5–6 animals, except for ELISA and western blot studies, which had four animals per group. Repeated measures two-way analysis of variance with the Bonferroni post-test was used for evaluating the data. Western blot data and i.w. concentration of SP were evaluated by one-way analysis of variance. *P*-value < 0.05 was considered statistically significant.

RESULTS

Behavioural assessment of nociception after preemptive i.t. administration

In all the groups, nociception (guarding, thermal hyperalgesia and mechanical allodynia) was highest immediately after incision, which steadily decreased over the next 4–7 days (Figures 2a–c). Treatment with different doses of L760735 (3, 10, 30 and 100 μg) attenuated nociceptive behaviour. However, the response was not dose-dependent. For example, at 2 h, guarding was maximally attenuated by the 3 μg dose (cumulative pain score was 20.2 ± 0.9 for saline vs 11 ± 2.29 for 3 μg L760735) (Figure 2a). Between days 1 and 2, maximum inhibition of guarding was observed with the 30 μg dose. Also, the inhibition of guarding by the different doses of L760735 did not differ significantly from each other, except on days 1 and 2 (30 > 3). Despite this lack of dose-dependent effect, an important observation was the relatively prolonged antinociceptive effect—observable up to day 3 with the 10 μg dose (Saline—8.2 ± 1.02 vs 10 μg L760735–2.33 ± 0.71). A slight reversal of the antinociceptive effect was noted on day 3 for the 30 and 100 μg doses. Allodynia was relatively unaffected in the drug-treated groups, except for a minor antinociceptive between days 6 and 7 (Figure 2b). Thermal hyperalgesia was significantly attenuated ($P < 0.05$) with the 3 and 30 μg doses at 2 h (Saline—98 ± 7.7 vs 3 μg dose 68.8 ± 2.2) and days 1–2 and on day 1, respectively (Figure 2c).

On the basis of the above results, the 30 μg dose was selected for further study (for postemptive i.t. and also combined i.t. and i.w. administration).

Comparison of antinociceptive effect of NK1r antagonist between preemptive and postemptive intrathecal administration

Cumulative pain score for the preemptive or the postemptive i.t. drug-treated group did not differ significantly from each other (Figure 3a).

Figure 2 Antinociceptive effect of i.t. L760735 (3/10/30/100 μg), administered 15 min before hind paw incision (preemptive). (**a**) Guarding behaviour is represented as the cumulative pain score. Different doses produced significant antinociceptive effect compared with saline. However, significant difference in the antinociceptive effect between the different doses was absent, except on day 1 (30 > 3; $P < 0.01$;$^{\#\#}$) and day 2 (30 > 3; $P < 0.05$;$^{\#}$). (**b**) Mechanical allodynia did not show significant antinociceptive effect following drug treatment, except on days 6–7. (**c**) Thermal hyperalgesia did not show any significant difference after drug treatment, compared with saline, except for the 3 μg dose (2 h, days 1 and 2) and 30 μg (day 1). No significant difference was noted between the doses. Explanation for symbols: 3 μg compared with saline (*); 10 μg compared with saline (Φ); 30 μg compared with saline (ψ); 100 μg compared with saline (•). $P < 0.05$—$^{\#}$/*/$^{\Psi}$/$^{\Phi}$, $P < 0.01$—$^{\#\#}$/**/$^{\Psi\Psi}$/$^{\bullet\bullet}$/$^{\Phi\Phi}$, $P < 0.001$—$^{\Psi\Psi\Psi}$/$^{\bullet\bullet\bullet}$/$^{\Phi\Phi\Phi}$.

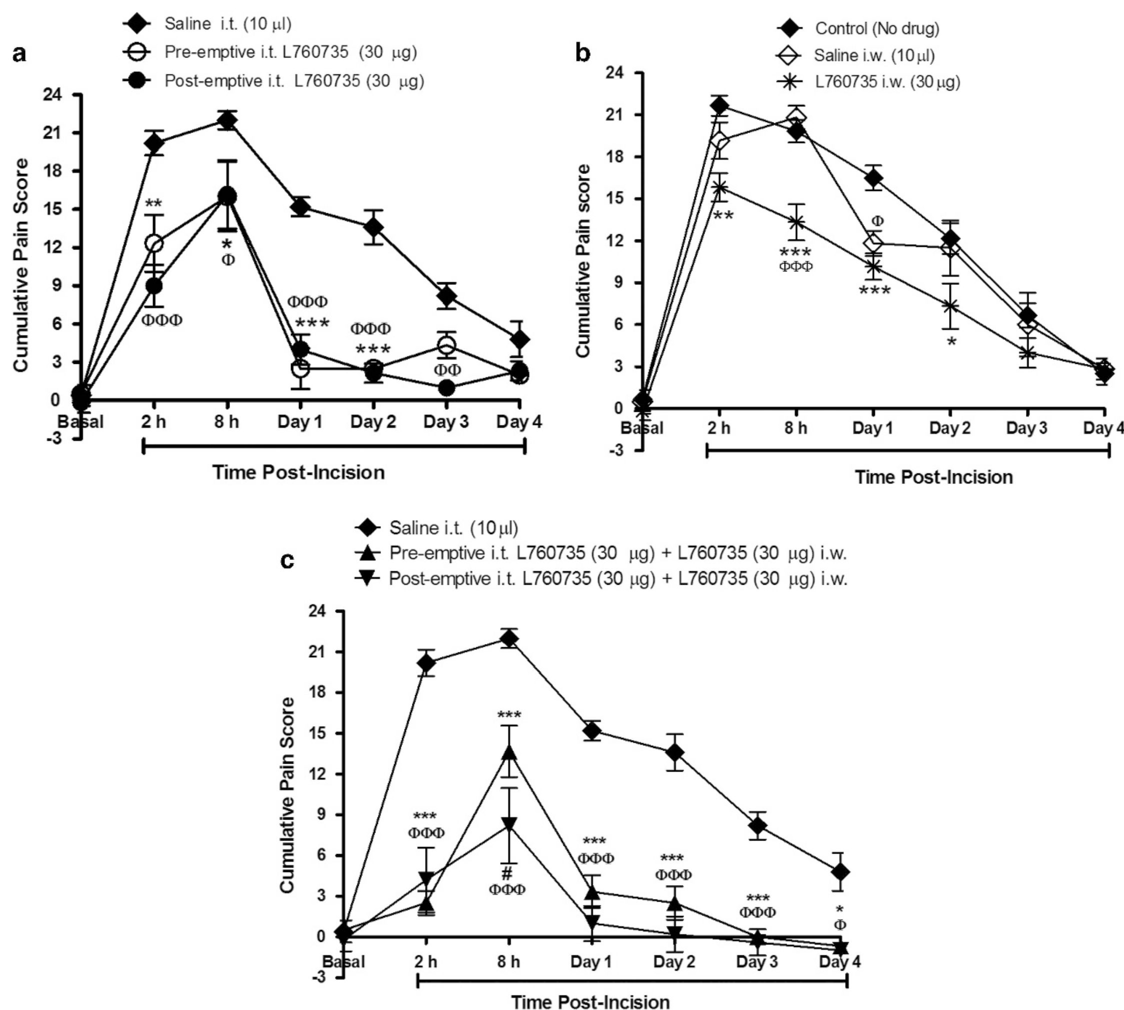


Figure 3 Guarding behaviour following administration of L760735 (30 µg) by (**a**) intrathecal (i.t.), (**b**) intrawound (i.w.) and by (**c**) combined i.t. and i.w. routes. (**a**) The i.t. administration was by either preemptive or postemptive. Comparison of antinociceptive effect following preemptive with postemptive drug administration did not show significant difference. (**b**) Control group was without any drug administration. Saline-treated group received 10 µl saline, whereas drug-treated group received 30 µg L760735. Intrawound drug treatment produced persistently low guarding score compared with both saline-treated (8 h) and control groups (2 h to day 2). Surprisingly, saline treatment of the wound showed a significant decrease at day 1 in comparison with the control group. (**c**) Combined i.w.+i.t. drug administration showed robust antinociceptive effect across the entire experimental period (2 h—day 4) compared with the saline-treated group. Postemptive mode was more effective than preemptive mode at 8 h ($P<0.05$;$^{\#}$). $P<0.05$- $^{\#/*/\Phi/\#}$. $P<0.01$—$^{\Phi\Phi/**}$. $P<0.001$-$^{***/\Phi\Phi\Phi}$.

However, compared with saline, postemptive treatment produced greater antinociceptive effect than preemptive treatment at 2 h ($P<0.001$ vs $P<0.01$) and day 3 ($P<0.01$). The slight reversal of antinociception, observed on day 3, in the preemptive group was absent in the postemptive group. Allodynia in the postemptive group was significantly inhibited compared with saline towards the end of the observation period (days 6–7), whereas difference between pre- and postemptive groups was observed at day 4 (Figure 4a). Postemptive drug treatment reduced thermal hyperalgesia in comparison with saline at 2 h and on days 1 and 2 (Figure 5a). Preemptive treatment significantly decreased thermal hyperalgesia in comparison with saline on day 1.

Thus, postemptive intrathecal treatment was more effective than preemptive intrathecal treatment in attenuating nociception.

Behavioural assessment of nociception after intrawound administration of NK1r antagonist

Guarding score after 30 µg i.w. drug administration resulted in a significant decrease (2 h—day 2) compared with both control and the saline-treated (8 h) groups (Figure 3b). Surprisingly, administration of saline appeared to significantly attenuate the guarding score on day 1. Allodynia or thermal hyperalgesia was not affected (Figures 4b and 5b). Contralateral administration of NK1r antagonist did not show any antinociceptive effect with reference to guarding behaviour; there was significant difference from ipsilateral i.w. administration between 2 h and day 2 (Figure 6).

Comparison of antinociception following combined intrathecal (i.t.) and intrawound (i.w.) administration

Combining i.t. (30 µg either preemptive or postemptive) with i.w. (30 µg) drug administration produced robust inhibition of guarding pain, more so after postemptive treatment (Figure 3c). This antinociceptive effect was observable up to day 4. Value of cumulative pain score following postemptive i.t.+i.w. co-administration was lower than preemptive+i.w. mode at 8 h. Also, basal values for guarding were evident by days 2 and 3 for the postemptive+i.w. and the preemptive+i.w. groups, respectively. Similarly, for allodynia,

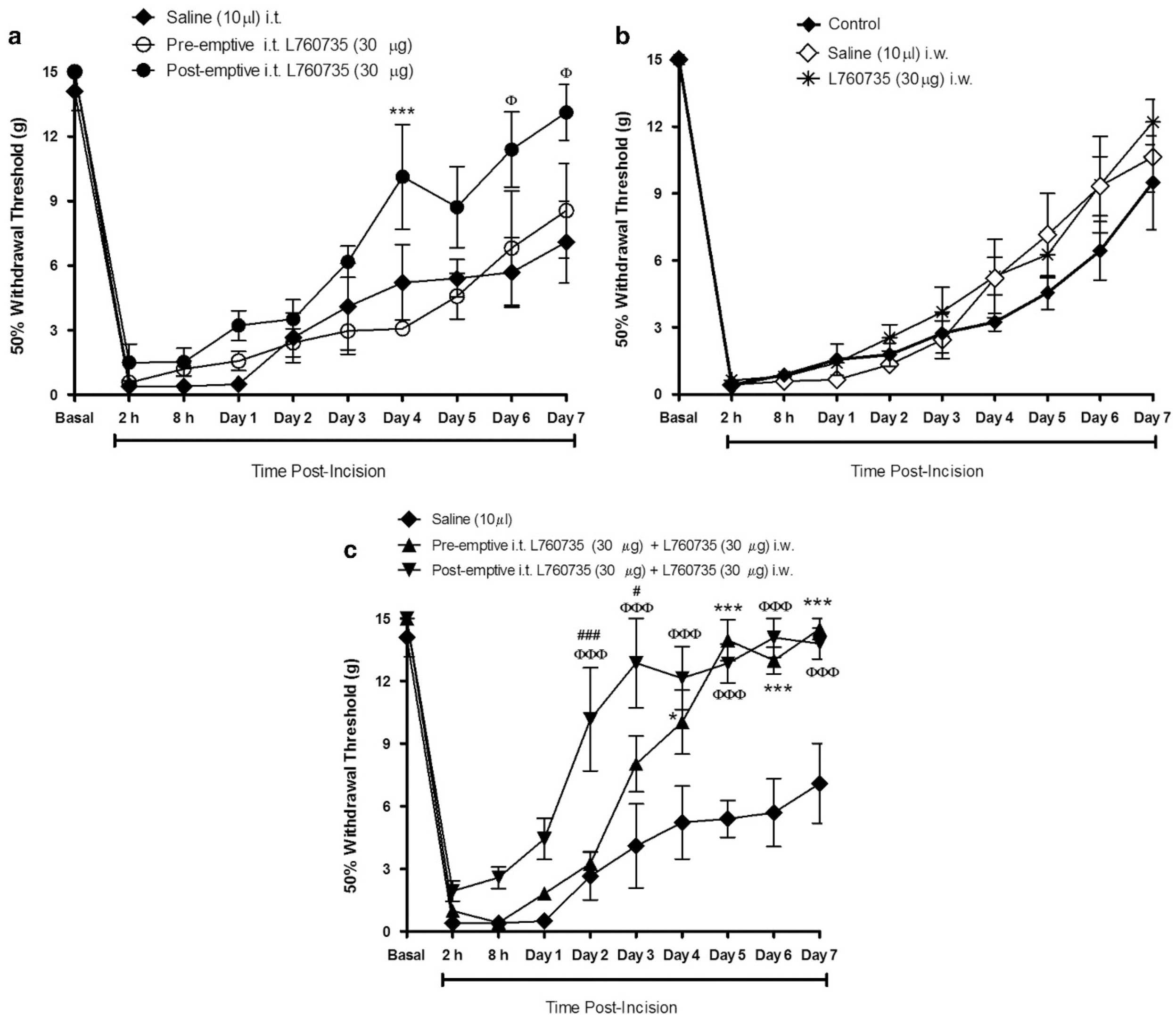


Figure 4 Evaluation of antinociceptive effect of L760735 (30 μg) on allodynia following (**a**) i.t. drug administration (either preemptive or postemptive), (**b**) i.w. drug administration and (**c**) combined i.t. (either preemptive or postemptive)+i.w. drug administration. Saline was administered preemptively. (**a**) Postemptive i.t. treatment showed higher antiallodynic effect compared with the preemptive i.t. drug-treated group (significant difference on day 4) and saline (days 6 and 7). (**b**) I.W. drug administration did not show significant difference. (**c**) Combined i.t. (either preemptive or postemptive)+i.w. administration produced significantly higher antinociceptive effect compared with saline. Almost basal values for mechanical allodynia were observed for i.t. (postemptive)+i.w. administration from day 3 and for i.t. (preemptive)+i.w. administration from day 5. Significant difference between postemptive from preemptive i.t. was noted on day 2 ($P<0.001$;#) and 3 ($P<0.05$;#). $P<0.05$—*/$^{\Phi}$/$^{\#}$. $P<0.001$—***/$^{\#\#\#}$/$^{\Phi\Phi\Phi}$.

postemptive+i.w.-treated group showed greater antinociception than preemptive+i.w.-treated group (days 2 and 3) (Figure 4c). Combined i.t. postemptive+i.w. drug treatment significantly attenuated allodynia with reference to the saline-treated group between days 2 and 7. Preemptive i.t. combined with i.w. drug treatment attenuated allodynia between days 4 and 7. Combined administration (preemptive i.t. mode) reduced thermal hyperalgesia between 2 h and day 3 compared with saline treatment (Figure 5c). Combined (postemptive+i.w.) administration attenuated maximum possible effect between days 1 and 2 compared with saline. However, a significant difference between the preemptive+i.w. and the postemptive+i.w. group was absent.

SP concentration in the wound following incision

Concentration of SP at the incision site increased significantly at all the time points examined in comparison with basal state (Figure 7). Maximum increase (about threefold) was noted on day 1.

Estimation of NGF, IL-1β and TNF-α at the incision site following i.w. NK1r antagonist

Tissue concentrations of TNF-α and NGF increased immediately `after incision (Figures 8a–c). IL-1β increased at 8 h. Levels of these inflammatory mediators decreased after i.w. NK1r antagonist treatment. Significant decrease was observed for NGF and IL-1β on day 3.

Expression of Substance P in the spinal cord

In the control group, SP expression was maximum over lamina I-IIo (superficial laminae) of the dorsal horn (Figure 9). Hind paw incision resulted in sudden and marked decrease in SP expression up to 6 h, which was maximally observed at 1 h. Subsequently, the expression increased at 12 h. Afterwards, expression of SP decreased between days 1 and 3. Quantitative image analysis of SP expression showed significant decrease (1, 3 and 6 h) and day 1 compared with the control group (Supplementary Figure 1).

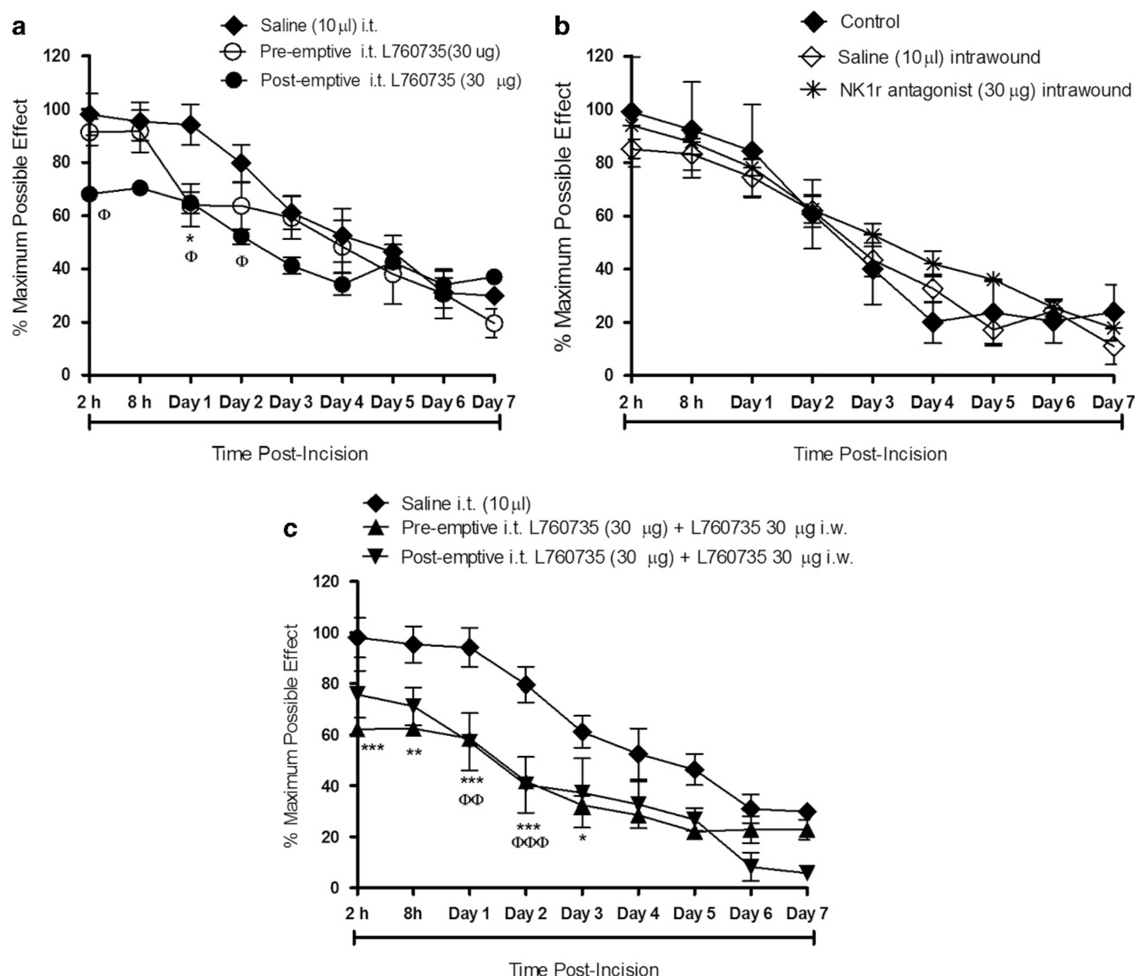


Figure 5 Thermal hyperalgesia following (**a**) i.t. (preemptive vs postemptive), (**b**) i.w. and (**c**) combined i.t. (either preemptive or postemptive)+i.w. drug administration of L760735 (30 µg). (**a**) Postemptive treatment showed higher antinociceptive effect than the saline-treated group (2 h, days 1 and 2). Preemptive i.t. treatment also differed significantly from saline (day 1). No significant difference was noted between preemptive and postemptive treatment groups (**b**) I.W. administration did not show significant difference. (**c**) Postemptive i.t.+i.w. and preemptive i.t.+i.w. did not differ significantly from each other. Combined (preemptive) administration demonstrated higher antinociception compared with control (2 h-day 3), whereas that after postemptive mode showed higher antinociception compared with control (days 1 and 2). $P<0.05$—*/$^{\Phi}$. $P<0.01$—**/$^{\Phi\Phi}$. $P<0.001$—***/$^{\Phi\Phi\Phi}$.

Western blot analysis of SP precursors

There was significant decrease in the precursors of SP after incision (1—6 h) (Figure 10). Expression increased at 12 h to approximately the pre-incisional state. The expression decreased again on days 1 to 3. Statistical analysis showed significant decrease at all postincisional time points, except at 12 h.

DISCUSSION

One of the goals of medicine is the treatment of acute pain. Despite substantial progress in the understanding of its pathophysiological mechanism in animal models, its treatment continues to remain suboptimal.[10,17] SP was thought to have a foremost role in nociception for a long time, particularly at the spinal level. However, clinical trials involving SP antagonists have been disappointing.[28] To our knowledge, few studies have investigated the involvement of SP in the rodent hind paw incision model. The underlying mechanisms for incision-related pain could be different from those observed in other preclinical pain models.[16]

In this study, the NK1r antagonist (L760735) was administered locally into the wound or into the i.t. space or by both routes concurrently in rats. To the best of knowledge, this particular experimental protocol of combined drug administration has never been used for studying the antinociceptive effect of a candidate analgesic drug. The results of the study suggest that administration of an NK1r antagonist by both the local and the i.t. routes needs to be combined for an effective antinociceptive effect.

Local i.w. administration attenuated the guarding score between 2 h and day 2 but not thermal or mechanical hypersensitivity. Again, this was more after postemptive treatment. Compared with evoked pain behaviour, guarding is less intense and is relieved by comparatively low doses of morphine.[16] Also, modality-specific antinociceptive effect has been reported earlier. For example, administration of ketoprofen, a nonsteroidal anti-inflammatory agent, through either i.w. or i.t. route inhibited guarding behaviour alone.[29] Healing of the wound was not grossly affected by local drug administration (data not shown). However, detailed studies are required using specific markers for wound healing like keratins and galectins.

In the current work, i.w. administration, once during incision, could ameliorate the guarding score for up to day 2. Presumably, the antinociception was due to antagonism of ~2–3-fold increase in SP

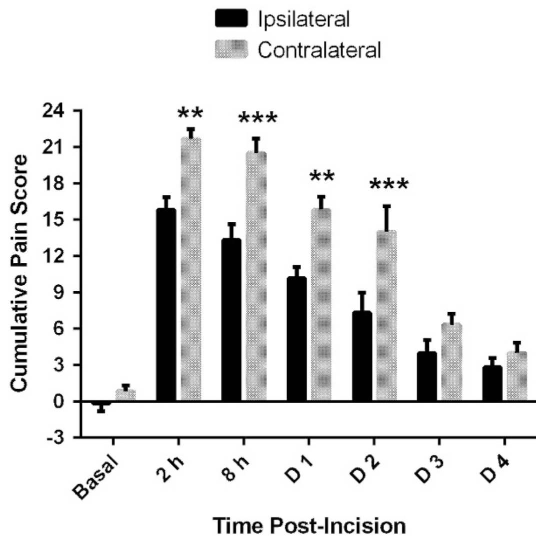


Figure 6 Guarding score represented as a cumulative pain score. Guarding score was persistently low for the ipsilateral drug treatment group. $P<0.01$—**. $P<0.001$—***.

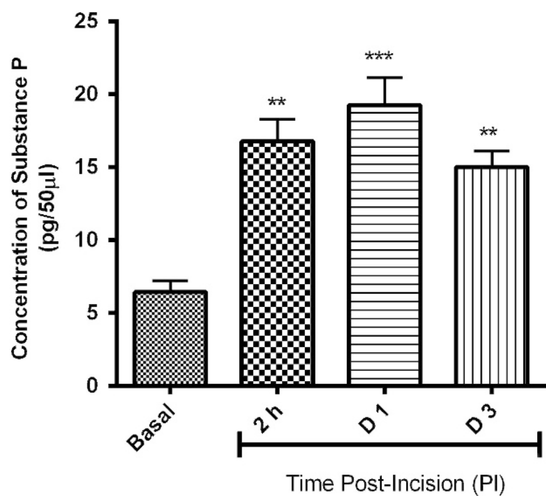


Figure 7 Quantitative estimation of substance P (SP) concentration at the incision site. Significant elevation of tissue SP concentration was observed (2 h—day 3). $P<0.01$ -**, $P<0.001$ -***.

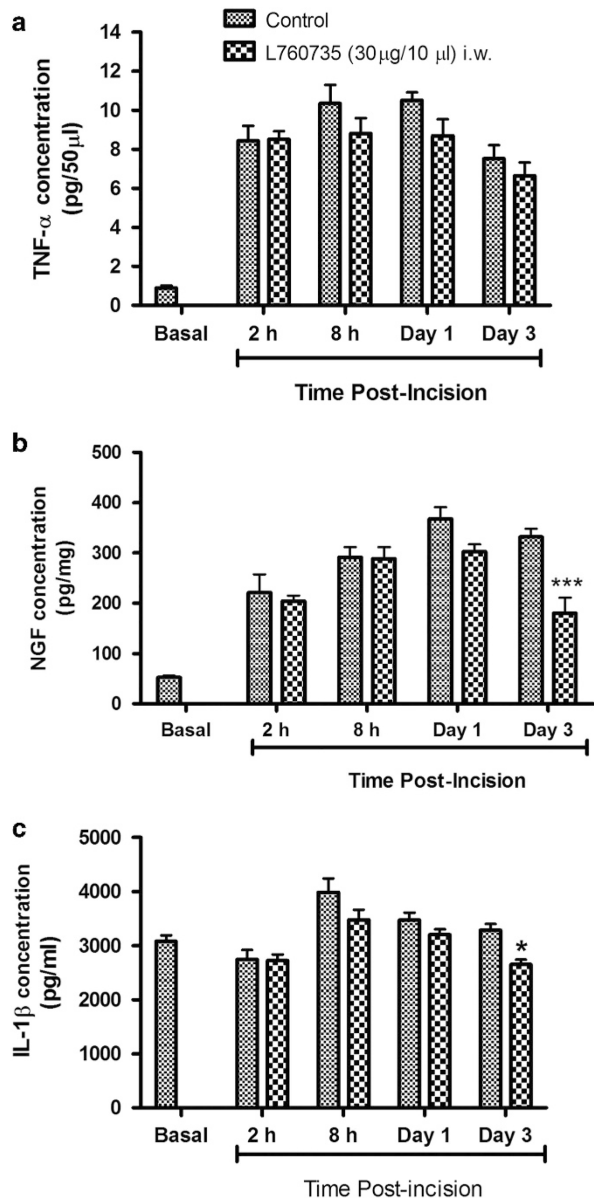


Figure 8 Quantitative biochemical estimation of tissue levels of (**a**) TNF-α, (**b**) NGF and (**c**) IL-1β at the incision site with and without i.w. L760735 (30 μg) administration. Increased tissue levels of TNF-α and NGF were noted at 2 h, whereas that of IL-1β was observed at 8 h after incision. Significant reduction was observed on day 3 for NGF and IL-1β but not for TNF-α. $P<0.05$—*, $P<0.001$—***.

concentration at the incision site. The observed antinociception was not centrally mediated because drug administration in the contralateral paw did not demonstrate an antinociceptive effect. Involvement of both SP and CGRP in the periphery has also been reported in neuropathic pain.[11,30] The increased SP concentration in the present study was possibly due to its release from the peptidergic primary sensory nerve fibres following tissue injury.[31] Recently, keratinocytes were observed to release SP.[32] SP induces mast cells to release histamine and macrophages to release growth factors and interleukins.[33] SP concentration increases rapidly (by 1 h) in the lung after burn injury and in the skin following partial thickness burns (from 4 h).[34,35] The putative antagonism of L760735 in the periphery could also be at the level of unmyelinated axons (~30%) in the skin and deeper tissues, which express NK1r.[36,37] This is corroborated by the finding that subcutaneous injection of SP in the rat results in nociception, which was again blocked by prior intra-plantar injection of NK1r antagonist CP99,994-1.[37] Also, SP induces the biosynthesis of prostaglandins by upregulation of the cyclo-oxygenase-2 enzyme.[38] Inflammatory mediators that have a pronociceptive role in postincisional pain were examined in our study following intrawound administration of NK1r antagonist but showed relatively minor changes.

A dose-dependent effect was absent after preemptive i.t. administration of different doses of the NK1r antagonist (3–100 μg). Previously, repeated local administration of SP in the rat paw also did not show a dose-dependent effect.[37] The authors speculated that this could have been due to receptor desensitization. Moreover, in our study, the number of animals in each group was small ($n=6$); the results need to be confirmed in larger number of animals. Intrathecal administration in mice of NK1r but not NK2r or NK3r

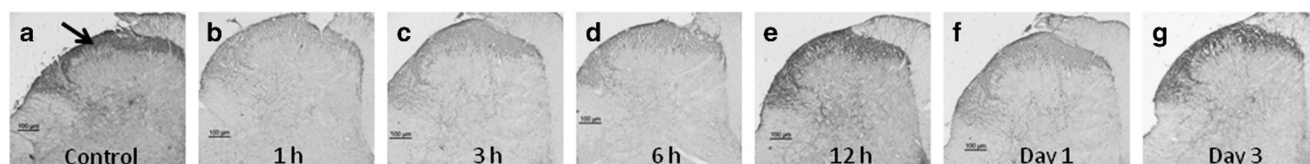


Figure 9 Immunohistochemical localisation of substance P in the spinal cords of both control and incised rats at different time intervals (1 h—day 3). Under basal conditions, selective expression of substance P was noted over the superficial laminae (I-IIo). (**a**) The expression decreased after hind paw incision, particularly at 1 h (**b**–**d**). An increase was observed at 12 h (**e**). This was followed by decreased expression between days 1 and 3 (**f**–**g**). Scale bars, 100 μm. A full color version of this figure is available at the *Spinal Cord* journal online.

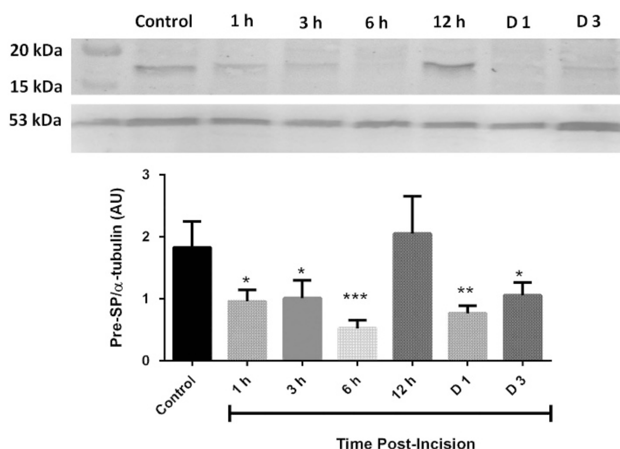


Figure 10 Western blot analysis of precursors of substance P in the spinal cord of control and the incised group of rats. Expression of Substance P decreased between 1 and 6 h after incision. Decrease was also noted between days 1 and 3 (D1–3). A transient increase was noted at 12 h. α-tubulin (53 kDa) was the loading control. $P<0.05$ -*, $P<0.01$-** and $P<0.001$- ***.

agonist resulted in a dose-dependent biting and scratching responses.[39] This was inhibited again in a dose-dependent manner by LY303870, a highly selective NK1r antagonist administered by both i.t. or i.p. routes. Besides, i.t. administration of SP was associated with acute agitation and thermal hyperalgesia.[40] One of the mechanisms of nociception following intrathecal SP is to promote prostaglandin E2 synthesis in a NK1r-dependent manner, and this could be blocked by nonspecific COX inhibitor or COX-2 inhibitor or even by inhibiting calcium-dependent phospholipase A2.[40–42] In the current study, combining i.t. and i.w. routes resulted in further inhibition of nociception, particularly the guarding behaviour. Importantly, postemptive mode of combined drug administration showed greater antinociception, except for thermal hyperalgesia. The latter finding suggests that SP is more involved in the maintenance rather than induction of postincisional pain. Importantly, these data provide ‘proof of concept’ validation of the hypothesis that blocking the activity of DRG neurons at both the peripheral and central terminals will effectively relieve nociception.[7]

Previous studies, which had explored the role of NK1r in postincisional nociception, had administered the NK1r antagonist systemically. For example, in one such study in rats, preemptive but not postemptive subcutaneous administration of PD 154075 (30–100 mg kg^{-1}) attenuated thermal hyperalgesia and mechanical allodynia for a prolonged period (49–72 h).[43] In a different study, LY303870 (40 mg/kg i.p.) could relieve allodynia for 6 h following hind paw incision.[13] Also, Preprotachykinin A (ppt-A$^{-/-}$) gene knockout mice showed persistently low level of allodynia after hind paw incision (up to day 2), although thermal hyperalgesia was transiently decreased. However, the authors did not delineate the precise contribution of the spinal or peripheral NK1r in their work. It could be important because therapeutic measures directed specifically at the appropriate site would be beneficial. Some related studies appear to indirectly indicate that the major part of the antinociceptive effect is centrally mediated. For example, pain following intra-plantar capsaicin injection was noted to be mediated predominantly by central NK1r.[18] Also, reflex changes in mean arterial pressure subsequent to intraperitoneal injection of capsaicin were more effectively inhibited by intrathecal rather than systemic administration of an NK1r antagonist.[19] Similarly, we show that spinal NK1r is somewhat more important than peripherally expressed NK1r.

SP immunoreactivity in the superficial dorsal horn (Laminae I-IIo) markedly decreased, immediately after incision.[44] Subsequently, expression of SP transiently increased at 12 h. Intra-articular injection of a mixture of kaolin and carrageenan in rats led to decreased SP immunoreactivity in the dorsal horn, which subsequently increased by 8 h.[45] The authors suggested that the initial decrease was because of exocytosis of SP from presynaptic terminals in the superficial laminae, which was corroborated by other reports noting high SP concentration in the dorsal horn/cerebrospinal fluid, soon after noxious insults.[46,47] Further, the subsequent increase supposedly resulted from increased synthesis and axoplasmic transport of SP from cell bodies of DRG neurons and even brain stem neurons. The result of western blot experiment only represents the precursor forms of SP, as the molecular weight of SP is extremely small and difficult to detect (~1.56 kDa).[27] Despite this, the results of western blot and immunohistochemistry were broadly similar.

Noxious stimulation reportedly elicits a slow and prolonged excitatory postsynaptic potential in dorsal horn neurons, which is inhibited by NK1r antagonist.[48] Blockade of spinal NK1r not only attenuated spinothalamic neuron activation following capsaicin injection in the paw but also prevented sensitization to post-capsaicin mechanical stimuli.[49] Similarly, multibarrel electrode recording form dorsal horn neurons showed that the activity of SP-sensitised dorsal horn neurons receiving afferent noxious input from the periphery was reduced by specific NK1 r antagonist—the study reports on the critical role played by SP and NK1r in pain from joints.[50] Recently, the intrathecal SP-saporin complex has been observed to relieve facet joint pain and bone cancer pain.[51,52] Furthermore, a clinical trial on intrathecal SP-saporin has been initiated for the pain associated with terminally ill cancer patients unrelieved by opioids (ClinicalTrials.gov Identifier NC T02036281 accessed on 25 June 2014).

The present study examined the role of NK1r in postincisional pain using a combination of i.t and i.w. routes for drug administration. Intrathecal administration alone of NK1r antagonists (CP-96,345 and FK888) resulted in an antiallodynic effect only at a very high dose (200 μg per 10 μl).[53] In contrast, our results show that there was significant attenuation of the guarding score and allodynia for 4/7 days

after combined drug administration. The result could be of clinical relevance. However, further studies are necessary to precisely assess dose responsiveness after intrathecal administration and also to investigate potentially harmful effects on wound healing. In conclusion, the role of NK1r in various acute pain conditions like postoperative pain and burn pain needs to be re-assessed as much evidence has accumulated recently, regarding its involvement.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Supplementary Information accompanies this paper on the Spinal Cord website (http://www.nature.com/sc)