Journal of the Anatomical Society of India 66 (2017) 7–14

Contents lists available at ScienceDirect

Journal of the Anatomical Society of India

journal homepage: www.elsevier.com/locate/jasi

Original Article

Role of calcitonin gene-related peptide in nociception resulting from hind paw incision in rats

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ARTICLE INFO

Article history:
Received 23 April 2017
Accepted 8 June 2017
Available online 27 June 2017

Keywords:
Catheter
Intrathecal
Neuropeptide
Pain
Rodent
Spinal cord

ABSTRACT

Introduction: The superficial laminae of the spinal cord are crucial sites for the transmission of incoming noxious information. Calcitonin gene-related peptide (CGRP) is released from the presynaptic nerve terminals in these laminae. One of the objectives was to evaluate the temporospatial pattern of expression of CGRP following paw incision in rats. Paw incision-induced nociception mimics postoperative pain in humans. The next objective was to administer a specific CGRP receptor antagonist directly into the intrathecal space and observe the antinociceptive effect, which was then compared to morphine.

Material and methods: Sprague Dawley rats were subjected to incision on the right hind paw. The related spinal cord segments (L4-5) were isolated at different time intervals after incision and immunostained for CGRP. A different set of rats were implanted with intrathecal catheter and administered saline (control) or BIBN 4096 (CGRP antagonist) or morphine (10 μg/10 μl) and then subjected to paw incision. Nociception was evaluated at different time intervals up to day 7.

Results: Expression of CGRP was observed over laminae I and outer part of lamina II. Synaptic terminals could be discerned containing CGRP. Following incision, the expression decreased abruptly at 2 h. However, at 12 h, the expression had increased. Between days 1–5, the expression decreased again towards basal levels. The antinociceptive effect of BIBN was comparatively less than morphine, which robustly inhibited all three pain parameters at 2 h after incision.

Discussion: Immunohistochemistry revealed that CGRP was involved in the transmission of nociception. However, blocking its action did not produce a robust antinociceptive effect.

1. Introduction

The spinal cord and the brainstem are key sites of transfer of information from the periphery to the central nervous system. Not only that, the neural signals are modulated before onward transmission to the brain by both excitatory and inhibitory interneurons as well as by descending nerve fibers from supra-spinal centers like the rostroventral medulla.[1] For example, interneurons containing γ-Amino butyric acid (GABA) can inhibit the transmission of pain by the "Gate control mechanism". Also, enkephalinergic interneurons can do the same by the release of endogenous opioids. Interestingly, GABAergic neurons constitute almost 25–30% of the neurons in Rexed's laminae I–II, also known as superficial laminae of the spinal cord.[2]

Aδ (thinly myelinated) and C (unmyelinated) groups of peripheral nerve fibers carry nociceptive information to the spinal cord. The central terminals of these nerve fibers contain neuro-transmitters like glutamate and neuropeptides like calcitonin gene-related peptide (CGRP) and substance P (SP).[1] Following tissue damage, these are released into the synaptic cleft, where they bind to specific receptors expressed by the dorsal horn neurons and trigger action potentials, which passes along the lateral spinothalamic tract to the thalamus.

CGRP, a 37 amino acid peptide, is derived from alternative splicing of the mRNA, originating from the calcitonin gene.[3] It is almost exclusively expressed in neurons and referred to as αCGRP or more commonly as CGRP. In contrast, the β isoform is derived from a different gene and is present in the enteric nervous system.[4] CGRP is extensively expressed in perivascular nerve fibers around the cerebral blood vessels, where it produces vasodilatation. Its role in migraine is well established.[5] The corresponding CGRP receptor is a prototypical G protein-coupled receptor (calcitonin-like receptor), which is associated with two other subunits

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http://dx.doi.org/10.1016/j.jasi.2017.06.001
0003-2778/

(Receptor activity-modifying protein 1 and receptor component protein).

CGRP is expressed at high density over the superficial laminae in the rat, monkey and human spinal cords.[6,7] The corresponding CGRP receptor is expressed adjacent to CGRP containing nerve terminals.[8] CGRP expression has been noted to increase, decrease or even remain unchanged under different pain conditions.[9–12] These conflicting results could be due to the different time intervals at which the spinal cords were examined after the noxious injury. For example, Ishida et al. examined the spinal cord at the end of day 1 whereas Wang et al. examined it on day 18.[10–11] Importantly, none of the existing studies have investigated the temporospatial pattern of expression over a period of time.

The animal model for the present study was the same as that of Ishida et al. and involves a surgical incision on the plantar aspect of the right hind paw under anesthesia.[11] This postincisional model was first described in 1996 and has been extensively utilized for understanding the mechanism of postoperative pain. Postoperative nociception is characterized by spontaneously occurring pain during resting condition (guarding behaviour), mechanical allodynia (pain due to non-noxious stimuli) and thermal hyperalgesia (exaggerated response to a mildly noxious stimulus).[13] Despite occurring in a hospital setting, management of postoperative pain continues to remain suboptimal.[14]

2. Material and methods

2.1. Experimental subjects

The experiment was conducted on young adult (9–10 weeks old; 250–300 g) male Sprague Dawley rats (n = 54) (Fig. 1). Permission for experimental work was obtained from the Institutional Animal Ethics Committee (903/IAEC/15 dated 19-2-16). ARRIVE guidelines were followed during the experimental work. Food and water were available *ad libitum*. After intrathecal catheterization, animals were housed singly in each cage, which contained clean bedding (ALPHA-dri, Shepherd Speciality Papers, USA). 12 h light:dark cycles were maintained and temperature varied between 22 and 25 °C. The observer performing the behavioural assessment of nociception was blinded to the exact drug administered to the animals.

2.2. Drugs

BIBN 4096 (henceforth referred to as BIBN), as known as Olcegepant, is a potent and selective antagonist of the CGRP receptor (Tocris Bioscience, UK). It was dissolved in 1 M HCL and then diluted with 1 M NaOH to a pH of 6.8. This was further diluted with isotonic saline to a final concentration of 10 μg/10 μl. Ampoules containing morphine sulphate (15 mg/ml) were purchased from a government agency after obtaining permission from the Drug Controller. It was diluted with isotonic saline to the same concentration as BIBN. Control group was injected saline instead of the drug.

2.3. Immunohistochemistry

The procedure has been reported previously.[15] Briefly, animals (n = 36) were divided equally into six groups. The first group was the control group (without incision) and the remaining was subjected to paw incision (Section 2.6 for details). Among the incised rats, each group was sacrificed at a different time point (2 h, 12 h, day 1, day 3 and day 5 after incision). Rats were anaesthetized with pentobarbitone (100 mg/kg i.p) and perfused with cold 0.1 M phosphate buffer saline (PBS) by the transcardiac route (Masterflex animal perfusion pump, Cole Parmer, USA). It was followed by perfusion with 4% Paraformaldehyde in PBS. The lumbar enlargement of the spinal cord was identified and the region

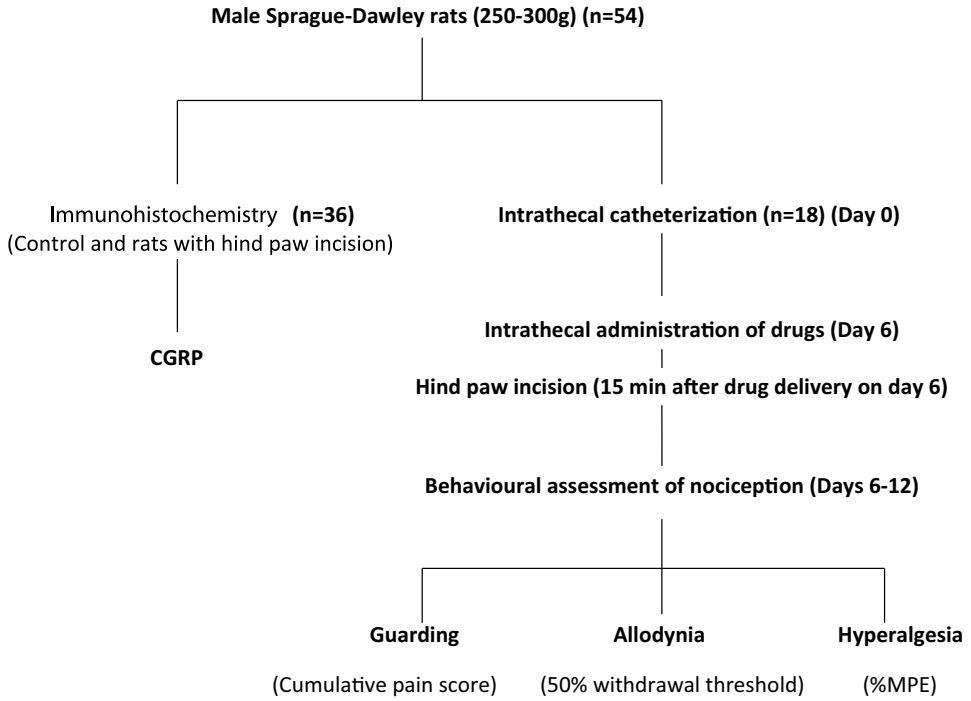

Fig. 1. Flow diagram of the experimental work. The study was divided into two parts – (1) study of the expression of CGRP by immunohistochemistry in the spinal cord and (2) the behavioural assessment of nociception after intrathecal administration of BIBN and Morphine. For the first part, rats were divided into two groups – with or without (control) paw incision. Those with paw incision were examined for CGRP at 2 and 12 h and days 1, 3 and 5. The second part of the study involved the surgical implantation of catheters in the intrathecal space (day 0) followed by drug administration (saline/BIBN/morphine) through the catheter (day 6; 8 a.m.) and paw incision (day 6, 8:15 a.m.). Subsequently, nociception was evaluated by three different tests at 2 and 12 h and days 1–7 (days 1–4 for guarding).

corresponding to the L4-L5 segments was dissected out. These segments were located at the junction of T13 and L1 vertebra.[16] The left side of the specimen was marked with a fine bore capillary tube for side determination. Following cryoprotection in sucrose solution, transverse sections (20 μm thick) of the spinal cord were obtained in a cryostat (CM1950, Leica, Germany) and collected in multicavity trays containing PBS solution. The sections were stored at −20 °C. On the day of immunostaining, the sections were washed with 0.25% Triton X-100 in PBS. Endogenous peroxidise activity was quenched with 0.3% H_2O_2. Tissue sections were exposed to 10% normal goat serum (NGS) in order to block non-specific binding. Sections were incubated with rabbit anti-calcitonin gene-related peptide polyclonal antibody (Calbiochem USA; 1:1000) for 48 h at 4 °C. Sections were then incubated in goat anti-rabbit biotin conjugated IgG secondary antibody (1:200; Vector Laboratories, Burlingame, USA) for 2 h. These were then incubated with avidin-biotin complex solution for 1 h. The antigen-antibody complex was visualised by 0.25% 3, 3′- diaminobenzidine tetrahydrochloride solution in PBS containing 0.025% hydrogen peroxide (Sigma Aldrich, USA). Finally, sections were taken onto gelatin coated slides, dried, dehydrated, cleared and mounted with cover slips. Photomicrographs of the stained sections were acquired with Nikon E- 600 microscope.

2.4. Image acquisition and quantification of immunostaining

Photomicrographs (5–6 sections/animal) were exported to the image J software (NIH, USA) and expression of CGRP was quantified within a user-defined area containing the highest expression within Lamina I-II(outer). The mean intensity (brightness/area) of pixels varied between 0 and 255, where 0 represented maximum darkness and 255 was maximum brightness. Background intensity from an area with no immunoreactivity (white matter) was subtracted from the value of total intensity to correct for nonspecific staining. Quantitation was done on the right side of the spinal cord as the incision was given on the right paw.

2.5. Intrathecal catheterization and drug administration

The procedure of intrathecal (i.t.) catheterization has been previously described.[17] Briefly, rats (n = 18) were anaesthetized with isoflurane inhalation and the head fixed in a stereotaxic frame. The skin over the back of the head and neck was cleaned with 10% povidine-iodine solution followed by 70% alcohol. Later, a ~2 cm long incision was given to expose the interscutularis muscle, which was detached from the occipital crest. The cisternal membrane was exposed and cut with the bevelled end of a 22G needle for 2–3 mm. CSF could be observed at the incision site. An 8.5 cm long sterile polyethylene tube (PE-5; Recath Co, Allison Park, Pennsylvania, USA) was inserted in a caudal direction into the intrathecal space. At the end of the procedure, the distal end of the catheter was placed just above the lumbar enlargement. The wound was closed by 3–4 sutures using 4-0 polyamide (Ethicon®). Antiseptic ointment (Neosporin®) was applied to the incised area. The outer end of catheter (PE-10; 4 cm) was closed with a metal wire to prevent CSF leakage. The rats were administrated ketoprofen (2.5 mg/kg i.m.) dissolved in Ringer's lactate solution. They were kept in a warm recovery chamber till they regained consciousness when they were returned to their cages. Rats were allowed to recover for 5 days.

On day 6, BIBN solution (10 μg) was administered through the catheter using a 30G needle and a sterile Hamilton syringe under gentle restraint (n = 6). The control rats (n = 6) received saline instead of the drug while another set of rats (n = 6) received morphine (10 μg). This dose was selected based upon preliminary experiments involving a range of doses. The total volume injected into the intrathecal space was 10 μl.

2.6. Procedure of hind paw incision

Paw incision was done 15 min after the intrathecal drug administration. The procedure was first described by Brennan et al. in 1996.[18] Rats were anesthetized as described earlier. The plantar aspect of right hind paw was disinfected with 10% povidone iodine solution followed by 70% alcohol (Fig. 2). A 1 cm long midline incision was made on the skin, starting 0.5 cm from the proximal end of the heel. The underlying fascia was also incised to expose the flexor digitorum brevis muscle. The muscle was elevated with a curved forceps and incised longitudinally for 0.5 cm with the tip of the scalpel blade. The limbs of the forceps were introduced through the cut and then gently separated. Finally, the skin was apposed by two mattress sutures using 4-0 polyamide (Ethicon®). Antiseptic ointment (Neosporin®) was applied to the incised area. Rats were kept in a warm recovery chamber. Later, they were returned to their cages. Subsequently, behavioural assessment of nociception was performed at 2 h, 12 h and then at the end of every 24 h. Guarding was assessed for 4 days whereas allodynia and hyperalgesia was determined for 7 days.

2.7. Behavioural testing for nociception

2.7.1. Estimation of guarding score

The procedure for determining guarding score was similar to that described earlier.[19] Briefly, rats were placed on an elevated platform made up of metal wire mesh (8 × 8 mm spaces). They were covered with large perspex enclosures (16 × 16 × 16 cm). After acclimatization for 15 min the position of the right hind paw was noted for 1 h from below the platform, using a magnifying mirror. This was done in 5 min bins. During each bin, the first 1 min was used for actually observing weight bearing in the incised paw. A score of 2 was awarded in case the incised paw was completely off the mesh; 1 if the paw was lightly touching the mesh or 0 if the paw was fully on the mesh with complete weight bearing. The non-incised paw was also observed and marked according to its position. The 12 scores of each paw were summed up. At the end, the score of the normal paw was deducted from incised paw to obtain the final "Cumulative pain score." Higher values represented greater nociception.

2.7.2. Mechanical allodynia

Mechanical allodynia was determined, immediately after the evaluation of the guarding behaviour by an earlier describe

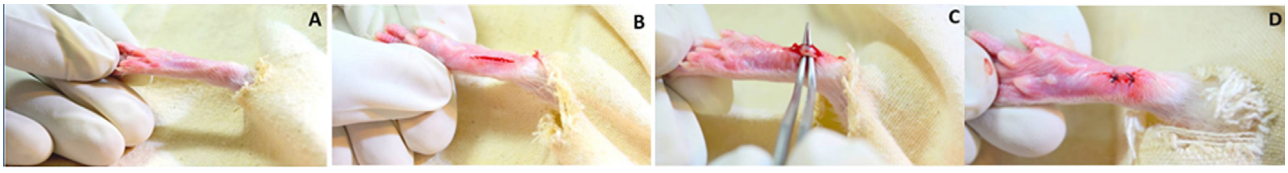

Fig. 2. Steps of paw incision – (A) cleaning the site with iodine and alcohol, (B) making an incision with no. 11 scalpel blade (C) lifting up the flexor digitorum brevis muscle (D) closing the wound with two mattress sutures.

procedure.[20] For this, the rats were allowed to remain on the platform previously used for guarding. This allowed access to the plantar surface of the paw from below the mesh. Fine nylon von Frey filaments of different sizes 3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93 and 5.18 (North Coast Medical Inc., San Jose, USA) were used for the experiment. The filaments were inserted between the mesh and allowed to touch a previously designated spot on the peri-incisional area.[18] Pressure was exerted so that the filament buckled and this pressure was maintained for 7–8 s. Each filament exerted a specific amount of pressure varying between 0.4 to 15 g. The exert size of the filament, which produced paw withdrawal was noted among the succession of filaments applied to the paw. This was used to calculate the 50% withdrawal threshold (g) by an algorithm. Lower values indicated greater nociception. The interval between successive applications of filaments was 2 min.

2.7.3. Thermal hyperalgesia

Rats were transferred to the plantar test apparatus (UGO Basile, Italy) where they were kept over a special glass platform.[19] They were covered as before and acclimatized for 15 min. An infrared heat source was focused on the incision site from below the glass platform. Simultaneously, a timer was started for recording the duration of exposure. Upon paw withdrawal, a motion sensor cut off the heat source and stopped the timer. The time period, which was displayed on a LCD panel was the withdrawal latency (s) of the paw. Baseline latency period was between 8 and 10 s, but decreased after incision. It was determined thrice at intervals of 2 min. Cut off time was 20 s to prevent damage to the paw. The percent maximum possible effect (%MPE) was calculated as follows:

$$[(\text{Latency following incision-baseline latency})/(\text{Cut off latency-baseline latency})] \times 100$$

Higher values indicate greater nociception.

2.8. Statistical analysis

Statistical analysis was performed by Graph Pad prism 5 software (San Diego, USA). Data are expressed as mean ± standard error of mean. The data for behavioral assessment of nociception were compared using two-way analysis of variance (ANOVA) followed by Tukey post-hoc test where the independent variables were the specific drug administered and the specific time interval. Values of image analysis was compared by one-way ANOVA

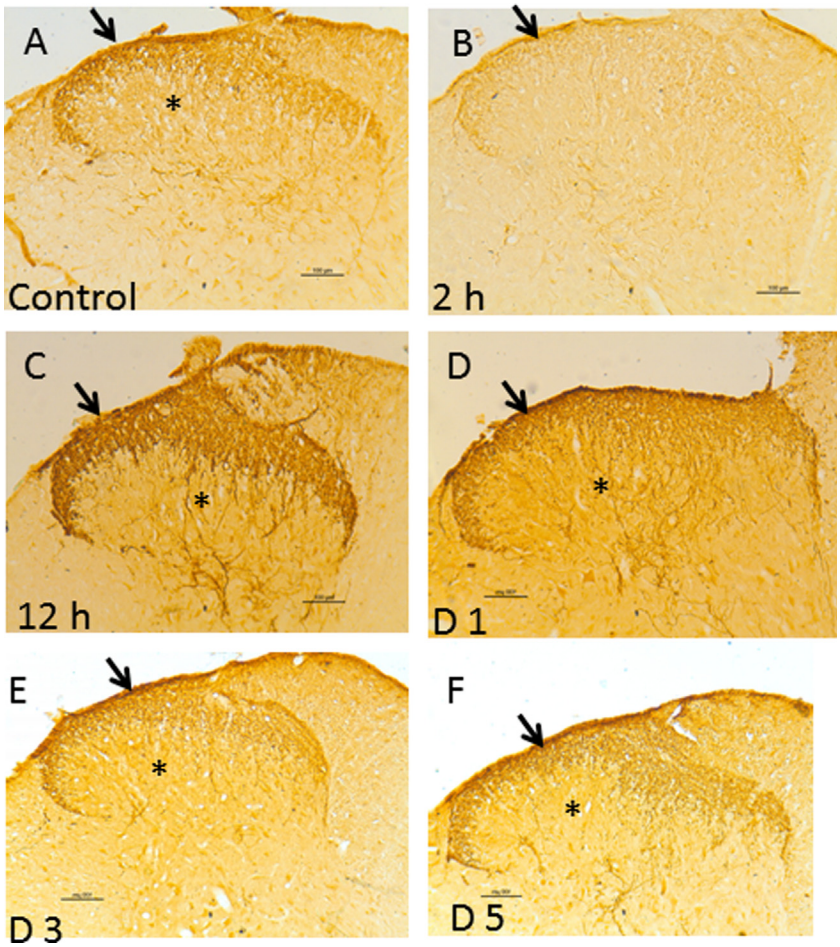

Fig. 3. Expression of CGRP in the rat spinal cord at different stages after incision (A–F). In control group (without incision), CGRP expression was noted over the superficial laminae (black arrow) (A). Following incision, the expression decreased at 2 h (B) but then increased at 12 h (C). Compared to control, expression was still increased as day 1 (D). At days 3 and 5, expression was decreased (E–F). Scale bar = 100 μm.

followed by Bonferroni multiple comparison test. Each experimental group had 6 animals. $P < 0.05$ was considered significant.

3. Results

3.1. Immunohistochemical localization of CGRP

Expression of CGRP was noted as a dense band over the superficial laminae composed of Rexed's laminae I (Marginal layer) and outer part of lamina II (Substantia gelatinosa) in the control group (Fig. 3). CGRP expression was also present in the adjoining deeper part of the dorsal horn (inner part of laminae II and lamina III) to a lesser extent. Under higher magnification using an oil immersion lens, the region of dense expression could be distinctly divided into a lightly-stained background area and scattered foci of intense staining (Fig. 4)., The latter were sometimes arranged in either a linear or arcuate manner. Following incision, the expression decreased abruptly at 2 h producing a washed-out appearance. However, CGRP staining reappeared at 12 h and the density of expression was even greater than that of the control group. The region over which the expression was observed increased to cover the inner part of Lamina II, besides lamina I. Further, extension of this expression was noted in a form of linear streaks into the deeper part of the dorsal horn. At day 1, the density of expression decreased slightly though it was still higher than the control group. Also, the extent of the superficial laminae showing the expression, reverted back to the control group. Finally, the expression further decreased between days 3–5. At day 5, the extent and density of expression was almost similar to the control group. Quantitative image analysis showed a similar pattern of expression, which significantly decreased at 2 h but then increased at 12 h in comparison to the control group (Fig. 5).

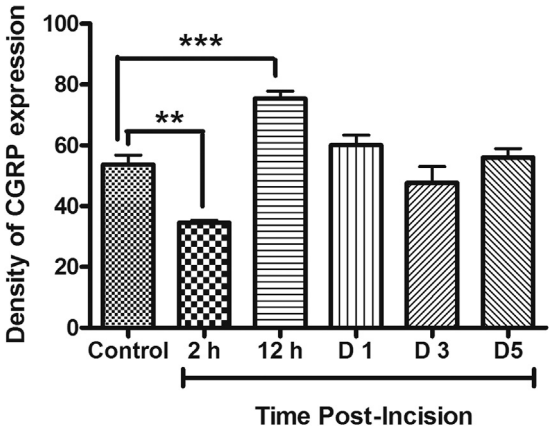

Fig. 5. Bar diagram representing values of quantitative image analysis of the expression of CGRP over the superficial laminae of the spinal cord. Compared to control, there was significant decrease at 2 h after incision but an increase at 12 h after incision. At day 1, expression was still increased but this was not statistically significant. Days 3–5 were associated with minor changes. Data are represented as mean ± sem. n = 6 at each time interval. **P ≤ 0.01. p < 00.001-***.

3.2. Antinociceptive effect of BIBN and morphine

Immediately after paw incision, the control group showed maximum nociception for all the three pain parameters (Guarding, mechanical allodynia, thermal hyperalgesia) (Fig. 6A–C). Subsequently the pain progressively decreased with increasing time intervals although it did not reach basal levels. In comparison to the control group, administration of BIBN decreased guarding behaviour at 12 h ($p < 0.05$) and at day 2 ($p < 0.01$) after paw incision (Fig. 6A). However, morphine produced a robust antinociceptive effect evident from the decrease of cumulative pain score between 2 and 12 h ($P < 0.001$) and a somewhat lesser effect at day 1 ($p < 0.05$) in comparison to the saline treated group. At 2 h, the antinociceptive effect of morphine was significantly greater then BIBN ($P < 0.001$). Subsequently, this antinociceptive effect of morphine disappeared and the values between day 2 to 4 closely followed the control group.

Regarding allodynia, BIBN produced a minor antinociceptive effect at day 7, when compared to the control group (Fig. 6B). However, allodynia was significantly attenuated by morphine at 2 h ($p < 0.01$) and again between days 6–7 in comparison to the control group and at 2 h and at day 6 in comparison to the BIBN treated group.

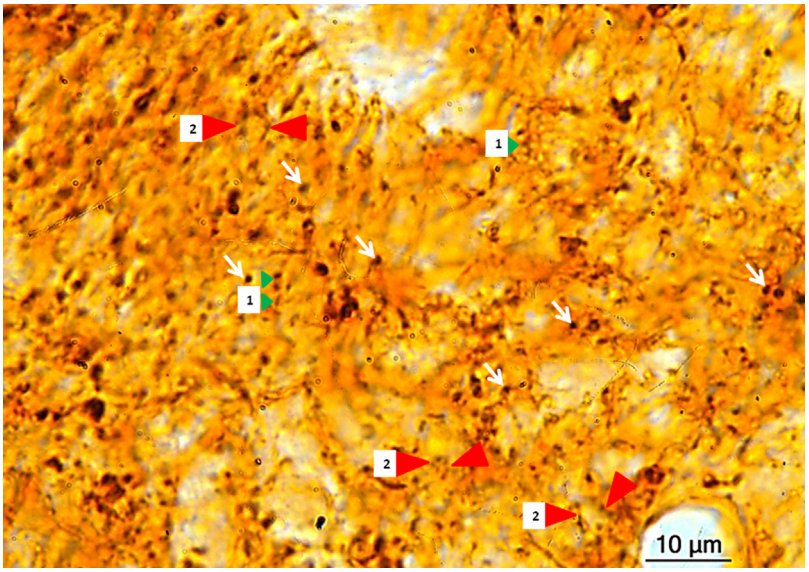

Fig. 4. Higher power view under oil immersion lens of lamina I showing CGRP expression. Several synaptic terminals are observed in the form of focal sites of intense staining (white arrows) scattered throughout the region. Some of them are arranged in a linear manner (green arrowheads labeled 1) whereas other are in the form of arcs (red arrowheads labeled 2). Scale bar is 10 µm.

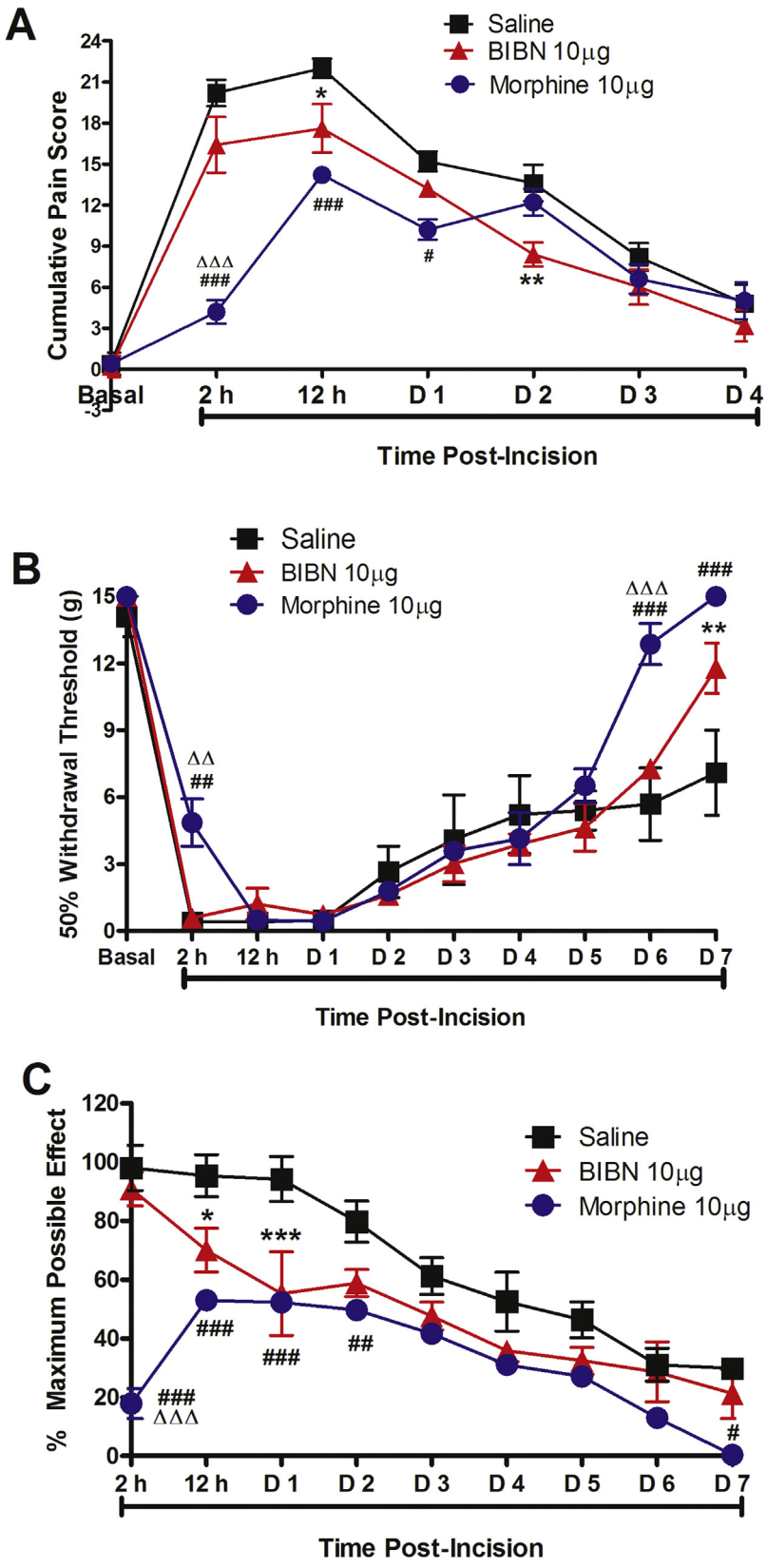

Fig. 6. (A–C): Assessment of nociception after intrathecal administration of saline, BIBN or morphine. The control group received saline. A – BIBN deceased guarding behavior represented by the cumulative pain score (12 h and day 2) in comparison to control although morphine produced a more robust effect (2 h–day 1). In comparison to BIBN, morphine produced significant antinociception at 2 h. B – Higher values indicate comparatively less pain for allodynia in contrast to guarding and thermal hyperalgesia, where higher values indicate more pain. Values of 50% withdrawal threshold (g) was increased at the beginning (2 h) and end of experiment (day 6–7) by morphine with reference to saline. At 2 h and day 6, the values of morphine and BIBN were significantly different. BIBN could only reduce allodynia at day 7. C – Thermal hyperalgesia represented by % Maximum possible effect was decreased by BIBN between 12 h to day 1 though morphine produced a more antinociceptive effect (2 h–day 2) with reference to saline. Compared to BIBN, morphine produced higher antinociception at 2 h. N = 6/group. Values are mean ± sem. * – represents comparison between saline and BIBN; # – represents comparison between saline and morphine while Δ represents comparison between BIBN and morphine. $P < 0.05$ –*/#. $P < 0.01$-**/##/ΔΔ. $P < 0.001$-***/###/ΔΔΔ.

Thermal hyperalgesia was significantly decreased at 2 h by morphine, when compared to both saline and BIBN treatment (Fig. 6C). Further, morphine and BIBN attenuated hyperalgesia in comparison to the control group between 12 h to day 2 and 12 h to day 1 respectively. It was also attenuated by morphine at day 7.

4. Discussion

The superficial laminae of the dorsal horn are sites of termination of primary sensory afferents carrying nociceptive stimuli from the periphery.1 Expression of CGRP in this region suggests its participation in the transmission of pain. The scattered foci displaying intense staining are possibly pre-synaptic terminals containing CGRP.21 Their arrangement in a linear manner or an arc are the sites of synaptic junctions on a dendrite or around a neuronal cell body. According to an earlier report, this neuropeptide is primarily derived from the small- and medium-sized dorsal root ganglion neurons through axoplasmic transport along their central terminals.22,23 Transport along peripheral terminals of the dorsal root ganglion neurons is equally important as this forms the basis of neurogenic inflammation – an important causative factor for migraine.24 Immediately after incision at 2 h, CGRP expression decreased significantly. However, it increased again by 12 h. It is likely that tissue damage during incision resulted in the release of CGRP from the presynaptic terminals into the synaptic cleft and to the initial decrease in expression. There is no re-uptake mechanism for neuropeptides at the synaptic terminals.25 Rats with osteoarthritis have a higher basal release of CGRP in the spinal cord.26 The subsequent increase at 12 h was due to a burst in the synthesis and axoplasmic flow of CGRP from dorsal root ganglion neurons. Previously, a similar pattern of expression was observed for substance P after paw incision.27 Expression was also elevated at day 1 which can be correlated with high levels of nociception present at this time. Thereafter, between days 3–5, expression decreased towards basal levels. This indicates that CGRP is particularly involved towards the early part (acute phase) of nociception. Significant levels of nociception, particularly allodynia and hyperalgesia, were present between days 3–5, when CGRP expression was returning to normal. CGRP depolarizes neurons apart from increasing the release of glutamate and increasing its effect on the N-methyl-D-aspartate (NMDA) receptors.28 Compared to previous studies where an up-regulation, status-quo or down-regulation of CGRP was observed in different preclinical models of pain, the current study highlights the need for a continuous assessment of the expression over a period of time as a down-regulation (2 h), up-regulation (12 h and day 1) as well as the relatively unchanged status (days 3 and 5) was observed at different time intervals after incision.9–12,29,30 For example, Ishida et al. (2014) has observed an up-regulation of CGRP expression, one day after paw inflammation.11 Compared to the control group, the current study also observed the same at one day after paw incision.

BIBN was selected based upon it being a non-peptidergic CGRP receptor antagonist, which is quite effective in the treatment of migraine.31 Intrathecal administration of BIBN significantly reduced guarding behavior at 12 h and day 2 after incision. Thermal hyperalgesia was attenuated between 12 h to day 1 though allodynia was unaffected. Similarly, administration of a CGRP antagonist by intrathecal route in mice subjected to paw incision resulted in a short-term decrease of thermal hyperalgesia. 11 On the contrary, the same amount of morphine greatly attenuated all three behavioral tests of nociception, which was most effective at 2 h. Morphine produces analgesia by binding to opioid receptors, particularly the mu-opioid receptor, which is abundantly expressed in the superficial laminae.32 Further studies are required to elucidate the mechanism of this discrepancy between the antinociceptive effect of CGRP receptor antagonist and morphine. which is the gold standard among analgesic drugs.

Guarding is considered to be similar to pain-at-rest in patients in the postoperative period and is a non-evoked type of nociception.13,18 In contrast, allodynia is readily demonstrable in patients (e.g. during change of dressing). It has been proposed that during inflammation, even normal body temperature can excite nociceptors and produce pain.33 Management of postoperative pain continues to remain suboptimal and newer and more effective treatment modalities are required.14

In conclusion, through CGRP expression in the spinal cord demonstrated significant changes after incision, administration of a selective CGRP receptor antagonist could not produce substantial relief of postincisional pain behavior, comparable to morphine. Further studies are required in this direction.

Conflicts of interest

Nil.

Financial support

Intramural research grant of AIIMS, New Delhi to Dr. Saroj Kaler.

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