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Original Research Article

Expression of gap junctions bearing connexin-43 subunits and glial fibrillary acidic protein in the rat dorsal root ganglia following hind paw incision

Vishwajit Ravindra Deshmukh, Pranav Prasoon, Subrata Basu Ray*

Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India

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***Correspondence:**
Dr. Subrata Basu Ray,
E-mail: raysb48@gmail.com

ABSTRACT

Background: Dorsal root ganglion (DRG) neurons mediate the transmission of sensation from the periphery. DRG neurons are pseudounipolar in nature and enveloped by the satellite glial cells (SC). Satellite glial cells have been reported to influence neuronal excitability via gap junctions. Postoperative pain causes induction of various neurotransmitters such as connexin-43 and glial fibrillary acidic protein (GFAP), in the satellite cells surrounding neuronal cell bodies
Objective: To study the expression of connexin-43 and Glial fibrillary acidic protein after hind paw incision.
Methods: Male adult Sprague-Dawley rats (n=12) were used. Rats were randomly divided into two groups. Group I (n=6) and Group II (n=6) for immunohistochemical study with glial fibrillary acidic protein (GFAP) and connexin-43 (Cx-43) respectively. In this study, rats were subjected to noxious stimuli on the right hind paw under general anesthesia. Dorsal root ganglia of both sides (L4 spinal nerves) were isolated after transcardiac fixation with 4% paraformaldehyde. The ganglia from the non-incised side were taken as the control group.
Results: Unipolar neurons in the DRG were surrounded by satellite cells. The satellite cells were positive for GFAP, which showed increased expression on the surgical side after noxious stimuli. Cx-43 immunostaining also showed an increased expression in the periphery of neuronal cell bodies of surgical side representing the location of gap junctions and hyperexcitability of neurons.
Conclusions: Small to medium sized neurons carry pain sensation from the periphery to the central nervous system. Increased gap junctions were noted in small neurons and satellite cells after surgery. Gap junctions might contribute to increased excitability of small neurons in postoperative pain.

Keywords: Neurotransmitter, Pain, Satellite cells, Surgery

INTRODUCTION

Postoperative pain is a common form of acute pain. Pain from a surgical incision occurs at rest and is exacerbated by coughing, ambulation, and change of dressing.[1] Incisional pain in rodents allows assessment of the mechanisms responsible for increased mechanical sensitivity following a surgical incision and also to investigate novel treatments for postoperative pain. Thus, there are two types of postoperative pain, which include the evoked and non-evoked variety.

Non-evoked pain is short lasting, moderate and is the ongoing pain at rest in the patients, which is easier to treat and better relieved by analgesic agents. Evoked pain is intense, long lasting and related to coughing, ambulation, and other related activities.[2] The dorsal root ganglia (DRG) play an important role in the transmission

of pain from the periphery to the central nervous system (CNS). Neurons in the DRG vary in size and can be categorized into small, medium and large depending on the size of their cell bodies. These different sizes are correlated with separate functions like small to medium sized ones are responsible for transmission of nociception while the larger neurons are concerned with proprioceptive and tactile sensations. Sensory neurons in the DRG are ensheathed by specialized glial cells termed 'Satellite glial cells' (SGCs).

Satellite glial cells are peripheral glial cells but share many properties of the astrocytes in the CNS, including the expression of glutamine synthetase. However, satellite glial cells differ in some respects from astrocytes, particularly by the tight sheath they make around the neuronal cell bodies.[3] In the DRG, Schwann cells and the satellite cells are secondarily activated by ischemia, traumatic injury, and inflammation.[4]

Quantitative studies on several species showed that the number of satellite glial cells per neuron increases in proportion to the neuron's volume, consistent with the idea that these satellite glial cells support the neurons metabolically.[5] Application of various cytokines to the exposed DRG resulted in an increase in the discharge rate as well as increased mechanosensitivity and larger peripheral receptor fields.[6]

Recently, there has been considerable interest in these cells as they are profoundly altered by peripheral injuries used to study pain behavior and appear to contribute to pain.[7] Recent studies have demonstrated that the satellite glial cells have the ability to regulate ion concentration and possess mechanisms for the release of cytokines, ATP and other chemical messengers like calcium.[8,9] SGCs are the consistent component of the DRG in all the species, yet their contribution to the basic neuronal functions remains relatively unknown.[10]

SGCs have been reported to influence neuronal excitability the gap junctions.[11] Gap junctions are clusters of intercellular channels that are composed of 12 subunits (connexin proteins), six of which form a connexon or hemichannel contributed by each of the coupled cells.[12] The satellite glial cells undergo major changes as a result of injury to peripheral nerves and appear to contribute to chronic pain.[13]

SGCs are said to undergo activation due to injury. During pathological conditions, SGCs demonstrate an altered phenotype similar to that seen in activated astrocytes, which includes increased expression of the glial fibrillary acidic protein (GFAP) and synthesis of cytokines.[14,15] Increased coupling by gap junctions between SGCs has been observed in several inflammatory pain and axotomy models.[16]

The aim of the study was to see the changes in the neurons and SGCs of the DRG after hind paw incision. The study was mainly performed using the immunohistochemical method.

METHODS

Experimental animals

Adult male Sprague-Dawley (SD) rats weighing 200-250 grams were obtained from the experimental animal facility of All India Institute of Medical Sciences after prior approval of the experimental procedure by Institutional Animal Ethics Committee (IAEC). Rats were maintained 3-4 per cage with food and water ad libitum and a 12-hour dark-light cycle. Rats were categorized into two groups: Group I (n=6) and group II (n=6) for immunohistochemical analysis of GFAP and Connexin-43 respectively. Transcardiac perfusion fixation with 4% paraformaldehyde in 0.1M Phosphate buffer solution (PBS) was done for isolating the DRG on the 3rd day post-hind paw incision. All rats underwent plantar incision before immunohistochemistry.

Plantar incision model

Plantar incision was first standardized in 1996.[17] Rats were placed in prone position. They were anesthetized with isoflurane (2%) inhalation via the nose cone in a vehicle of 1:1 mixture of oxygen and air. Plantar aspect of the right hind paw was swabbed with povidone-iodine solution followed by isopropyl alcohol swab. A 1 cm midline incision from the heel towards the base of the toe was made using the tip of a No. 11 scalpel blade. The underlying fascia was also cut. Then small forceps with fine curved tip was used to elevate the flexor digitorum brevis muscle. A 5 mm long incision parallel to the muscle fibers was made leaving the origin and insertion intact. The muscle fibers of either side of incision were further separated introducing the forceps through the cut. The muscle was then repositioned. Using 4-0 silk (Ethicon) two-mattress sutures were used to oppose the skin. Rats were kept singly in cages having soft clean specialized bedding material (Alpha-dri, Shephard, USA).

Tissue collection

After perfusion fixation, vertebral column was exposed and laminae were cut to expose the spinal cord. The location of the lumbar 4 (L4) dorsal root ganglia was identified by counting downwards from the T13 rib. DRG were collected bilaterally. Afterward, the samples were kept in 4% paraformaldehyde for further 3 days before processing for IHC.

Immunohistochemical (IHC) study using GFAP and Connexin-43

Dissected dorsal root ganglia were kept at 4°C. However, before sectioning the DRGs were cryoprotected by the sucrose solution. On the day of cryosectioning the

ganglion were placed on the chuck and embedded in optimum cutting temperature (OCT) medium. Then the specimen was sectioned using cryostat (LEICA CM 1950) at the 10µm thickness at -22°C. For each DRG, the sections were collected in 0.1 M PBS buffer within the multi-cavity tray.

The sections were incubated with H_2O_2, rinsed in PBS and incubated in 10% normal goat serum. Sections were then incubated with GFAP (1:1000) (Abcam) and Anti-Connexin-43 (1:400) (Sigma laboratories, USA) primary antibody diluted in PBS containing TritonX-100 and NGS at room temperature for 2 hours at 4°C. The sections were then processed using Vectastain ABC kit (Vector, Burlingame, CA, U.S.A.) and rinsed with the PBS-TX between each incubation period. Visualization of immune complex- 3, 3 diaminobenzidine complex-0.025% (DAB, Sigma Laboratories) was done. Sections were incubated for 10 minutes.

RESULTS

In the present study, qualitative immunohistochemical analysis for GFAP and Connexin-43 was performed. Alterations in the expression of the glial fibrillary acidic protein (GFAP) and Connexin-43 (Cx-43) after incision in the right hind paw was noted.

Immunohistochemical study for Glial Fibrillary Acidic protein (Figure 1)

GFAP was expressed in the satellite glial cells (SGC) surrounding the neurons within the rat dorsal root ganglia. The satellite glial cells showed less intense staining for GFAP in the control side as compared to the incised side where there was dense intense staining of satellite glial cells under both low and higher magnification.

Examination under higher power revealed more distinct expression in the satellite cells surrounding the neuronal soma in incised side. SGCs surrounding the small to medium sized neurons showed greater GFAP expression compared to the large size neurons.

Immunohistochemical study for Connexin-43(Figure 2)

Expression of Connexin-43 was noted in the junctional areas between neurons and the satellite glial cells as a darkly stained brownish precipitates. Low power magnification for connexin-43 showed higher expression in the plantar incised side as compared to the control side.

Examination under higher magnification also revealed more distinct expression of this protein in the periphery of the ganglion cells. Incised side showed ring-like expression of connexin-43 between satellite cells and neurons. 'Plaque' like staining for gap junctions was specifically noted around the neurons indicating the presence of groups of gap junctions at these locations.

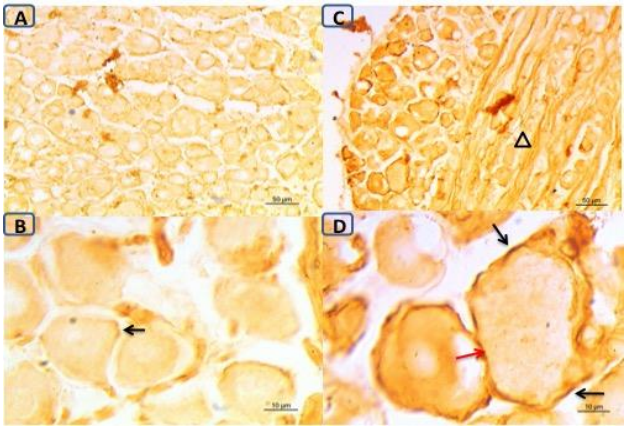

Figure 1: Expression of GFAP in control (A, B) and surgical side of rat (C, D). Low magnification images (A, C) showed more immunostained neurons on plantar incised side. (C) Nerve fibers were located in the center of the ganglion (arrowhead). High magnification images (B, D) showed darkly staining rim formed by satellite glial cells around cell bodies on the surgical side compared to control side. (D) Two neurons lie adjacent to each other with intervening satellite glial cells (red arrow). Black arrows marked the location of satellite glial cells.

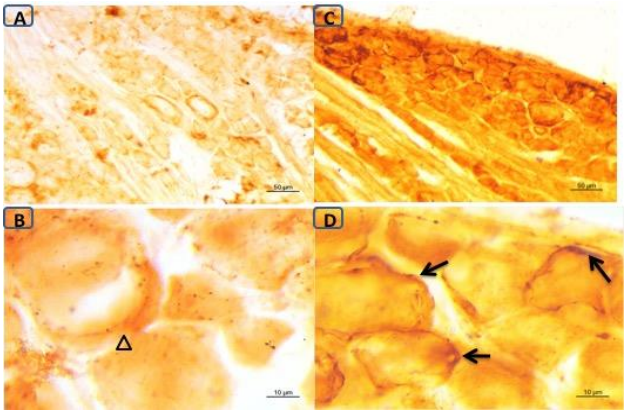

Figure 2: Expression of Connexin-43 in control (A, B) and surgical side (C, D) of the rat. Low magnification images showed that expression of antibody is comparatively more on the plantar incised side. High magnification images demonstrated irregular ring like formation around the neurons. Dot like (Punctate) expression (black arrowhead) surrounding the neurons represents the location of gap junctions between satellite glial cells and neurons (B) on the control side, compared to plaque-like appearance (Black arrows) on plantar incised side (D).

DISCUSSION

Post-operative pain is most common in surgical wards of hospitals. The aim of this study was to observe the changes in the expression of GFAP and Connexin-43 after a surgical incision. Altogether, it was noted that there was increased expression of GFAP in satellite glial

cells and Connexin-43 in margins of small to medium sized neurons of dorsal root ganglia in comparison to the contralateral side. GFAP was present at low levels under basal conditions, making this particularly useful marker for noting their activation.

Satellite glial cells are unique in that they usually form a tight sheath around the neurons which is still permeable to many large molecules.[13] The gap between the neurons and SGCs, which is about 20nm wide constitutes the extracellular space of the neurons, and its small volume allows SGCs to control the neuronal environment.[18] Work from the other labs confirmed that injury increases SGC coupling by means of gap junctions in rats.[19] Studies involving chronic pain models such as infraorbital nerve compression model showed that expression of the gap junctional protein Connexin-43 increased in SGCs in rat trigeminal ganglia. This finding is similar to our observation. The type of Connexin that is upregulated depends on specific pain model. For example Garret and Durham (2008) detected an increase in expression of Connexin-26 bearing subunits between the neurons and satellite glial cells of rat trigeminal ganglia after injecting complete Freund adjuvant (CFA) into the temporomandibular joint.[20]

During the development of the nervous tissue, Connexin-26 is the most common subtype of gap junctions. However with the maturation, Connexin-43 becomes more prevalent while Connexin-26 is limited to leptomeninges, ependyma, and pineal gland. One of the hallmarks of most types of glial cells is that they are interconnected by gap junctions which enable them to function as electrical syncytium.[21] Gap junctions are clinically relevant. Chemotherapy-induced neuropathic pain, which occurs in about 1/3rd of the patients could be due to the gap junctions. Oxaliplatin and taxol were able to increase the gap junctions between the satellite glial cells by up to five folds.[22]

The earlier authors also noted that submandibular inflammation results into the increased signaling between the satellite cells themselves and also with the neurons. Furthermore, this signaling decreased after administration of carbenoxolone, a well-established gap junctional blocker. Gap junction blockers abolished the inflammation-induced changes in SGCs and neurons, and significantly reversed the pain behavior. It is also proposed that inflammation induces augmented cell coupling in DRGs that contributes to neuronal hyperexcitability leading to visceral pain.[22]

CONCLUSION

In conclusion, the result of the present study shows that gap junctions (Cx-43) subunits were widely expressed between satellite cells and neurons during postoperative pain. Changes were also observed in the satellite cells as an overexpression of GFAP. Pharmacotherapy for reducing the functional status of gap junctions may be helpful in the treatment of postoperative pain.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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