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[Original Article]

## LOCAL APPLICATION OF NUCLEUS PULPOSUS INDUCES EXPRESSION OF P2X<sub>3</sub> IN RAT DORSAL ROOT GANGLION CELLS

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**Abstract :** The P2X<sub>3</sub> receptor is a ligand-gated cation channel that is activated by extra cellular adenosine triphosphate (ATP) found in the dorsal root, trigeminal and nodose ganglia. It is one of the receptors transmitting nociceptive information of injuries and inflammation of the periphery by endogenous ATP released from damaged cells. The present study was performed in order to evaluate if there was an increased expression of P2X<sub>3</sub>-immunoreactivity in dorsal root ganglion (DRG) neurons after experimental disc herniation. There were four groups : exposure of the left L4 dorsal root ganglion and incision of the L4-L5 disc, exposure and slight displacement of the left L4 dorsal root ganglion, sham exposure of the L4 dorsal root ganglion, and normal. Seven days after surgery, the DRG's were collected, sectioned and stained immunohistochemically for the P2X<sub>3</sub> receptor. The expression of P2X<sub>3</sub> increased significantly following incision of the L4-5 disc compared to the normal group. Sham surgery induced a minor, although statistically significant increase. Mechanical displacement did not induce any increased expression of the receptors. The study demonstrates that expression of the P2X<sub>3</sub> receptors in the DRG may be induced by local application of nucleus pulposus. This may increase our understanding of the pathophysiologic mechanisms related to disc herniation and sciatica.

**Key words :** P2X<sub>3</sub>, disc herniation, nucleus pulposus, dorsal root ganglion

### INTRODUCTION

Disc herniation is well known to induce pain after rupture of the annulus fibrosus. Symptoms are induced by both mechanical compression and chemical inflammation of the nerve root. The leakage of nucleus pulposus material can induce morphological and functional changes in the nerve root<sup>1)</sup>. The gene expression changes induced by the mechanical and chemical factors differ at 7 days

after surgery in the rat models<sup>2)</sup>. However, the roles of each factor to induce symptoms are still incompletely known.

The P2X receptors were recently suggested to mediate nociceptive information of injury and inflammation, in particular the P2X<sub>3</sub> receptor<sup>3-7)</sup>. The activation of the receptor is based on peripheral adenosine triphosphate (ATP) released from injured cells. ATP has also been suggested to be involved in the induction of mechanical and ther-

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mal hyperalgesia in experimental models<sup>8–10</sup>).

The aim of this study was to investigate the changes in P2X<sub>3</sub> receptor expression in the adjacent dorsal root ganglion (DRG) following experimental disc herniation in rats using immunohistochemical staining.

### MATERIAL AND METHOD

Twenty female Sprague-Dawley rats with an average body weight of 225 g were used. The animals were housed in groups of four with free access to food (B & K Rat/mouse standard, BeeKay feed & beddings, Sollentuna, Stockholm) and tap water. Temperature was kept at 21°C, the light schedule was 12 hours daylight starting at 6:00 AM and 12 hours darkness starting at 6:00 PM, and humidity was kept at 50%. The experimental protocol was approved by the local animal ethics committee.

The animals were divided into four groups ( $n=5$  in each group). The rats were anaesthetized with an intraperitoneal injection of 0.4 ml of diazepam: pentobarbital: saline (1:1:2). Disc herniation was induced according to a previously published protocol as below<sup>11</sup>.

*Disc Incision group* ( $n=5$ ). The left facet joint between L4 and L5 was removed and the L4 DRG and the L5 nerve root, including the intervertebral disc between L4 and L5, were visualized. The L4-L5 intervertebral disc was incised using a 0.4-mm diameter injection needle. Herniation of the nucleus pulposus into the spinal canal was facilitated by injecting a small amount of air into the center of the disc through the needle.

*Nerve Root Displacement group* ( $n=5$ ). After exposing the L4 DRG, a 0.4-mm diameter injection needle was placed between the ganglion and the pedicle of L4, i.e., lateral to the ganglion, and the needle was then gently forced medially to the former location of the center of the ganglion. The needle was then fixed in this position by gently securing it into the underlying vertebral body of L4. This resulted in a slight sustained displacement of the ganglion with a subsequent elongation of the L4 nerve root.

*Sham Exposure group* ( $n=5$ ). After removing the L4/L5 facet joint and visualizing the intervertebral disc, no other procedure was performed in this group. The spinal muscles were sutured and the skin closed by metal clips.

*Normal animal group* ( $n=5$ ). No surgical procedure was performed as the control.

### TISSUE PROCESSING

All rats were deeply anesthetized with an overdose of pentobarbital and were perfused with 200 ml saline followed by 200 ml paraformaldehyde (4% in 0.2 M phosphate buffer) through the aorta on day 7 after the surgery. The L4 DRG and spinal cord were dissected, post fixed in 4% paraformaldehyde (2 h at 4°C), transferred to 10% sucrose in 0.1 M Phosphate buffer (PB) (2 h at 4°C) and then to 20% sucrose in 0.1 M PB (overnight at 4°C). The tissues were then embedded in O.C.T.-compound (Histolab Products AB, Västra Frölunda, Sweden), frozen quickly and stored at -80°C. Transverse sections through the DRG (8 µm thickness) were obtained using a cryostat and thaw-mounted onto Superfrost microscope slides (BDH, Poole, UK).

The sections were incubated with the following reagents, goat anti-P2X<sub>3</sub> receptor antibody (Santa Cruz Biotechnology Inc, California, U.S.A.) overnight, and biotinylated rabbit anti-goat IgG antibody (DACO, Copenhagen, Denmark) for 1 hour (all reagents were diluted in phosphate-buffered saline containing 0.2% Triton X-100 and normal rabbit serum at 10%). Positive signals were obtained by the reaction with avidin-biotin-peroxidase complex reagent (DACO, Copenhagen, Denmark) and 3,3'-diaminobenzidine tetra hydrochloride (Sigma, Stockholm, Sweden).

The number of labeled neurons per section was counted to determine the magnitude of P2X<sub>3</sub> receptor expression. Four sections in each rat of the DRG were selected randomly. The average number of labeled neurons was obtained for each rat across the different tissue sections and recorded as a ratio to the total cell population. The mean value  $\pm$ SD was determined for each group. Only neurons with clearly visible nuclei were counted as positive for P2X<sub>3</sub> receptor. All analyses were performed on coded sections by an unbiased observer. ANOVA with Fisher's Protected Least Significant Difference test were used for the statistical analysis.

### RESULTS

The P2X<sub>3</sub> receptor immunoreactive cells were found in small sized DRG neurons (Figure 1). The expression of P2X<sub>3</sub> receptors increased in the disc incision and sham groups at day 7 after surgery compared with the normal group ( $p<0.05$ ). Especially in the disc incision group, it was about five times higher compared to the normal group ( $p<0.05$ ) (Figure 2). There was no significant dif-

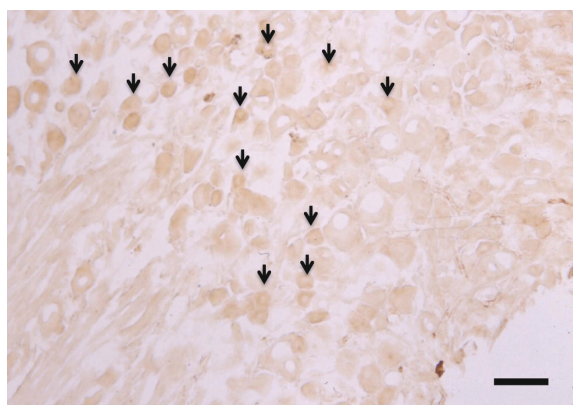


Fig. 1. Expressions of P2X<sub>3</sub> receptors in an L4 DRG from the incision group. A large number of cells (arrows) display a brown staining, indicating expression of P2X<sub>3</sub> receptors. (Scale bar 75  $\mu$ m).

ference of the expression of P2X<sub>3</sub> between the displacement and the normal groups at day 7 after surgery.

## DISCUSSION

P2X receptors has been cloned and categorized into seven types (P2X<sub>1</sub>-P2X<sub>7</sub>)<sup>4</sup>. Among these seven subtypes of purinoreceptors, the P2X<sub>3</sub> receptor is one of the receptors transmitting nociceptive information of injuries and inflammation of the periphery by endogenous ATP released from damaged<sup>3,7,12,13</sup>. In inflammatory conditions, the P2X<sub>3</sub> receptor is sensitized and can be evoked by low concentration of ATP<sup>8</sup>. Recently it was seen that administration of ATP and its analogues intra thecally induce mechanical and thermal hyperalgesia as well as pain related behaviors in the rats<sup>9,10,14</sup>. Activation of the P2X<sub>3</sub> receptor at the spinal cord level has also been linked to a release of glutamate with a subsequent induction of the N-methyl-D-aspartate (NMDA) receptor<sup>14</sup>. However, in models on peripheral nerve injury (e.g. sciatic nerve axotomy and sciatic nerve ligation or ligature), both up and down regulation of P2X<sub>3</sub> have been observed<sup>5,15-17</sup>. Tsuzuki *et al.* demonstrated that the P2X<sub>3</sub> receptor in the DRG was up regulated when the injured axons were intact, whereas it was down regulated following axotomy<sup>9</sup>. It is also reported that both spinal and peripheral P2X<sub>3</sub>/P2X<sub>2/3</sub> receptor have contributions to nociception in several animal models (e.g. spinal nerve ligation, sciatic nerve ligation, Freund's adjuvant or carrageenan injection) using a selective P2X<sub>3</sub>/P2X<sub>2/3</sub> receptor antagonist<sup>18</sup>.

In addition, P2X<sub>3</sub>/P2X<sub>2/3</sub> receptors dependent

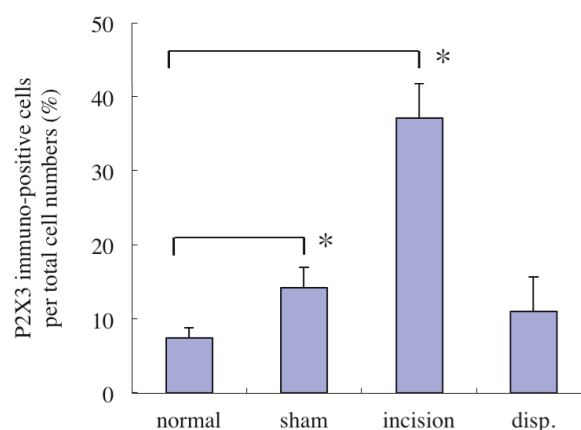


Fig. 2. Histogram of the ratio of P2X<sub>3</sub> receptor immunoreactive cell profiles in the L4 rat DRG in the four experimental groups. Data are presented as mean and standard deviation,  $n=5$  per group. Compared to the normal group, the expression of immunoreactive cells of the sham and the incision groups increased significantly ( $p<0.05$ ).

cytosolic phospholipase A2 activity in primary sensory neurons is a key event in neuropathic pain<sup>19</sup>. In this study, incision of annulus fibrosis induced a significant increase the P2X<sub>3</sub> receptor expression in the DRG adjacent to the disc incision. This result may be interpreted that there are nerve terminals within the spinal canal that conduct pain sensation. Such receptors might probably be found on the surface of the annulus fibrosus, the membranous coverings of nervous structures or periosteum. It seems likely that the herniation of nucleus pulposus may have induced activation of such nerve terminals and that this activation has induced an increase of P2X<sub>3</sub> in the corresponding nerve cell body located in the adjacent DRG. How this activation has occurred, the mechanisms of activation and where such nerve terminals were located cannot be understood from the present study. The limitation of this study was to investigate only at 7 day after surgery because of the preliminary experiment.

Sham exposure of DRG also induced a slight increase in P2X<sub>3</sub> expression that was found to be statistically different to the normal group. However, the increase induced by the sham exposure was not as pronounced as that for the incision group. The mechanisms for this increase cannot be fully understood but may be related to the surgery per se with increased inflammatory activation related to the wound healing process.

When harvesting DRG's for analysis it was apparent that the displacement had induced a mechanical deformation and atrophy of the DRG. It

is known that the membranous coverings of the DRG and nerve roots have nerve endings. We therefore suspected that mechanical stimulation of the DRG might elicit the expression of P2X<sub>3</sub> reactive cells. However one may assume that the synthesis rate in such deformed DRG's may be impaired and that this may be one reason for the absence of an increase of P2X<sub>3</sub> positive cells in the displacement group. This can, however, not be validated by the present study. Another reason for the absent response in P2X<sub>3</sub> activation after mechanical deformation may be the absence of nucleus pulposus. This is, however, also difficult to determine in the present experimental set-up.

In conclusion, it seems evident that the mere incision of annulus fibrosus induces an increase in P2X<sub>3</sub> receptor expression in the adjacent DRG. This may suggest that there are nerve terminals on the intervertebral disc or other structures in the spinal canal and that rupture of the annulus fibrosus with leakage of nucleus pulposus may induce pain. Continued studies in the field may increase our knowledge regarding the basic pathophysiologic events related to the herniation of nucleus pulposus.

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