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Original Article

The utility of ionotropic glutamate receptor antagonists in the treatment of nociception induced by epidural glutamate infusion in rats

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Abstract

Background: The authors have previously demonstrated that human herniated disc material contains high concentrations of free glutamate. In an experimental model, elevated epidural glutamate concentrations in the lumbar spine can cause a focal hyperesthetic state.

Methods: Rats underwent epidural glutamate infusion in the lumbar spine by a miniosmotic pump over a 72-hour period. Some rats underwent coinfusion with glutamate and ionotropic glutamate antagonists. Nociception was assessed by von Frey fibers and by assessment of glutamate receptor expression in the corresponding dorsal horn of the spinal cord.

Results: The kainic acid antagonist, UBP 301, decreased epidural glutamate-based hyperesthesia in a dose dependent manner. Concordant with these findings, there was significant decrease in kainate receptor expression in the dorsal horn. The *N*-Methyl-4-isoxazoleproionic acid (NMDA) antagonist Norketamine also significantly diminished hyperesthesia and decreased receptor expression in the dorsal horn.

Conclusions: Both UBP 301, the kainic acid receptor antagonist and Norketamine, an NMDA receptor antagonist, dampened epidural glutamate-based nociception. Focal epidural injections of Kainate or NMDA receptor antagonists could be effective treatments for disc herniation-based lumbar radiculopathy.

Key Words: Glutamate, norketamine, nociception, UBP 301

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INTRODUCTION

Although modest degrees of inflammation have been found in human specimens and in animal models of lumbar disc herniations,[1,3,23] a common belief is that intense sciatic pain is related to inflammation in the disc and nerve root. However, lowering levels of the proinflammatory cytokine tumor neurosis factor alpha (TNFα) alpha in the epidural space did not demonstrate significant reduction in sciatic pain in a human trial.[2,13]

Disc material placed in close approximation to lumbar nerve roots will stimulate neurotransmission.[18] We have

shown that significant concentrations of glutamate are present in herniated discs in humans with disc-based sciatica[6,7,20] (avg 18 mmol/L). Animal experiments have suggested that baseline concentrations of glutamate in the epidural space without disc herniation are very low;[6] other animal experiments have shown that epidural glutamate infusion can create unilateral lower extremity hyperalgesia. Ionotropic kainate, *N*-Methyl-4-isoxazoleproionic acid (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoxaleprionic acid (AMPA) have receptors implicated in peripheral nociception, and are present in the dorsal root ganglion cell bodies.[9,10,21,25] We have performed animal experiments to determine if blocking dorsal root ganglion (DRG) glutamate receptors with epidural ionotropic glutamate receptor antagonists could reduce glutamate induced focal nociception in the lower extremity.

MATERIALS AND METHODS

Testing groups

Retired breeder female Sprague-Dawley rats from Charles River Laboratories weighing between 300 and 400 g were grouped according to the concentration and type of glutamate receptor antagonist infused into the epidural region at the lumbar (L5) spine segment. The L5 nerve root connects to the DH at the L1 level anatomically. The concentration of glutamate infused was 0.02 mM, either with or without the antagonists. In control animals, normal saline solution was infused. A total of 6-15 animals were included for each experimental group. The experimental protocol was approved by the Rhode Island Hospital and Roger Williams Medical Center Animal Care Committee and followed the Laboratory Animal Welfare Act requirements.

Surgical implantation of mini osmotic pump

The animals were initially anesthetized with 5% isoflurane, and anesthesia was maintained with 2.5% isoflurane. The tip of a PE10 tube was inserted into the epidural space on one side of the spinal canal adjacent to the L5 DRG through a small laminectomy at approximately L2, and was then secured by a stitch to the lumbar spinous process. Prior to insertion, the proximal end of the tubing was secured to a mini osmotic pump (model 1003D, Alzet Corp, Palo Alto, CA) containing a given concentration of glutamate antagonist and 0.02 mM glutamate in normal saline. The pump delivered a total volume of 72 μl at a rate of 1.0 μl/hour ± 0.15 μl/hour for 72 hours. Any animals demonstrating gait disturbance consistent with spinal cord injury were euthanized and eliminated from the study.

Behavioral tests: Measurement of static allodynia

Control readings were taken 24 hours prior to and immediately prior to surgery. Animals were also tested at 24 and 72 hours after the initiation of glutamate infusion. Nylon filament Touch Test® Von Frey fibers (VFF) (North Coast Medical, Morgan Hill, CA) were used to examine the pain threshold of both lower extremities. Rats were given 30 minutes to acclimate, and then fibers of increasing grams of force resistance (0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g) were pushed against the plantar region until fiber deformation. Withdrawal was considered to be a positive response. If no response was elicited after three attempts using the 0.6 g of force fiber, the experimenter moved on to the 1 g of force fiber, and so on. Once a response was noted, the experimenter repeated testing in descending order of grams of force to demonstrate reproducibility. The recorded result is the lowest grams of force necessary to elicit lower extremity withdrawal. Each lower extremity was assessed separately. The upper extremities were also measured as an independent control.

Behavioral tests: Evaluation of thermal hyperalgesia

The animal was placed in a plantar testing apparatus designed to measure the response of each hind paw to radiant heat (model number 7370, Ugo Basile Collegeville, PA). The latency of foot withdrawal in response to noxious heat stimuli to the plantar surface was measured in an objective manner. Comparisons were made by subtracting the average latency of the control (contralateral) side from the average latency of the operated (ipsilateral) side.

Tissue harvesting

Following the final behavioral testing, rats were euthanized and intracardially perfused with sterile saline and then buffered with 4% paraformaldehyde. With the aid of a dissecting microscope, the left and right DRG at the L5 level were individually harvested. The spinal cord and proximal filum terminale were harvested from the T8 to the L5 spinal levels. The spinal cord at T13, L1, and L2 were then cut into separate 0.5 cm segments and paraffin embedded.

Immunohistochemistry

Eight-micron thick tissue sections were cut and applied to pretreated slides. Sections were deparaffinized in xylene and gradually hydrated through graded alcohols. Antigen unmasking was performed by microwave heat treatment at 95°C for 10 minutes. Slides were incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, and washed in phosphate buffered Saline (PBS). They were then incubated overnight at 4°C with primary antibody, either Glu-R1, Glu-R5, or NMDA (Santa Cruz Biotechnologies, Santa Cruz, CA). Subsequently, slides were incubated at 37°C for 60 minutes with biotin-conjugated secondary antibody, followed by incubation for 30 minutes with avidin-biotin enzyme reagent (ABC Staining system; Vector Laboratories, Burlingame, CA). Stain was visualized by development in peroxidase substrate (diaminobenzidine, DAB) and counterstaining with 0.05% cresyl violet.

Finally, slides were mounted with Permount, cover slipped and observed by light microscopy at ×40.

Tissue grading

Rat brain sections were used as positive controls for the staining process. Two observers (DPO, WH) scored the samples and were blinded to the concentration of infused glutamate. Receptor expression was analyzed bilaterally at the L5 DRG and in the DH at spinal cord levels corresponding to the nerve root insertion site for the L5 root (L1 anatomically), one level above (T13 anatomically) and one level below (L2 anatomically). In the dorsal horn, an ocular micrometer was used to standardize the counting area as the number of cells per 125 square microns at ×40 with the outer margin of the region of interest based on lamina I and extending internally to lamina III. In the DRG, regions of large and small neuronal cell bodies were measured separately, and four grid measurements were applied to both large and small cell clusters on each DRG. The percentage of stained cells was calculated as the number of stained neuronal cells divided by the total number of neuronal cells times 100. Cresyl violet staining performed prior to counting aided in differentiation of neuronal cell bodies.

Statistical analysis used

Behavioral Studies: A Kruskal–Wallis nonparametric analysis of variance (ANOVA) was used to determine significant differences. A Dunn's multiple comparison test was performed to determine significant differences between two groups using standard error of the mean (SEM).

Immunohistochemical Studies: Same as Behavioral Studies.

RESULTS

Infusion with kainate antagonist: UBP301

Behavioral testing measuring static allodynia with VFFs demonstrated that UBP-301 extinguished significant pre- to postoperative differences in hind paw responses related to the presence of epidural glutamate at 0.006, 0.002, 0.02, and 0.2 mM concentrations [Figure 1]. As seen in our previous study,[11] infusion of glutamate created focal hyperalgesia was not seen in saline infused controls [Figure 1].

Spinal cord sections at the L1 dorsal horn (Laminae I-III) were analyzed for kainite glutamate receptor expression. Significant decreases in receptor expression compared with 0.02 glutamate infusion were found with coinfusion of UBP-301 at 0.02 and 0.00 2 μM concentrations and also with an infusion of UBP-301 alone [Figure 2].

Finally, significant decreases in receptor expression were found in small neuronal cell bodies (<25 mM) in the L5 DRG with the coinfusion of glutamate and the kainite antagonist at 0.002, 0.06, and 0.2 mM concentrations [Figure 3].

Similarly, large neuronal cell bodies (>25 μm) showed a non significant decrease glutamate expression trend with infusion of antagonist (data not shown).

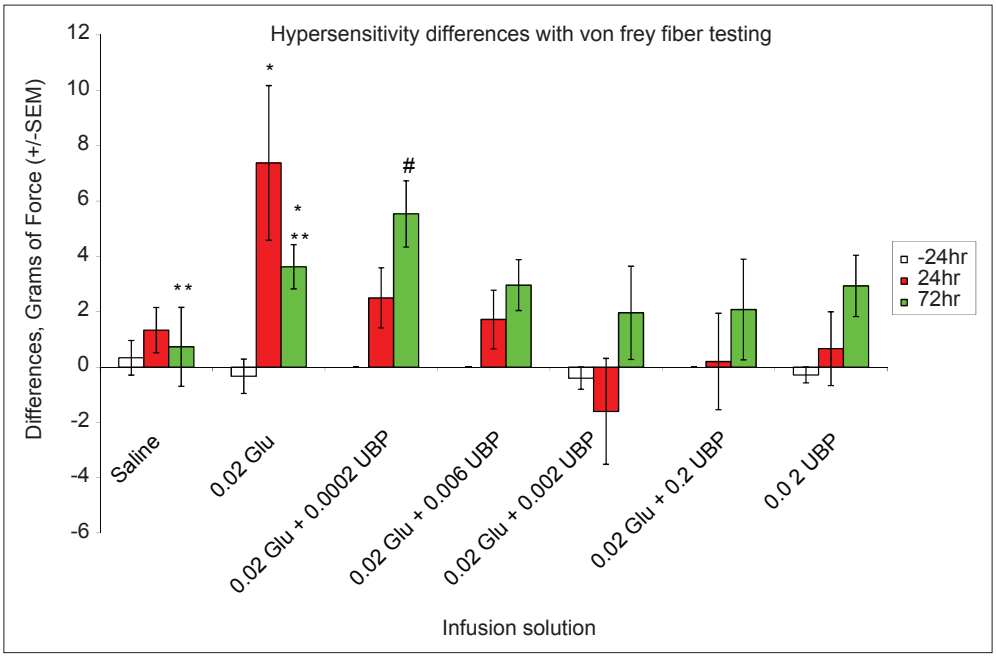

Figure 1: Mechanical hypersensitivity – UBP301: The only treatment group demonstrating pre- to postoperative differences in behavior was 0.0002 mM UBP301 at 72 hours after infusion, #*P* < 0.01 (Dunn's multiple comparison test). Compared with preoperative, glutamate treated animals showed a significant increase in mechanical hypersensitivity at 24 and 72 hours after surgery, **P* < 0.001, (Dunn's multiple comparison test). Postoperative, saline treated animals were significantly less hypertensive than glutamate treated animals at 72 hours after surgery, ***P* < 0.05 (Dunn's multiple comparison test). A Kruskal–Wallis nonparametric ANOVA was significant, *P* < 0.05

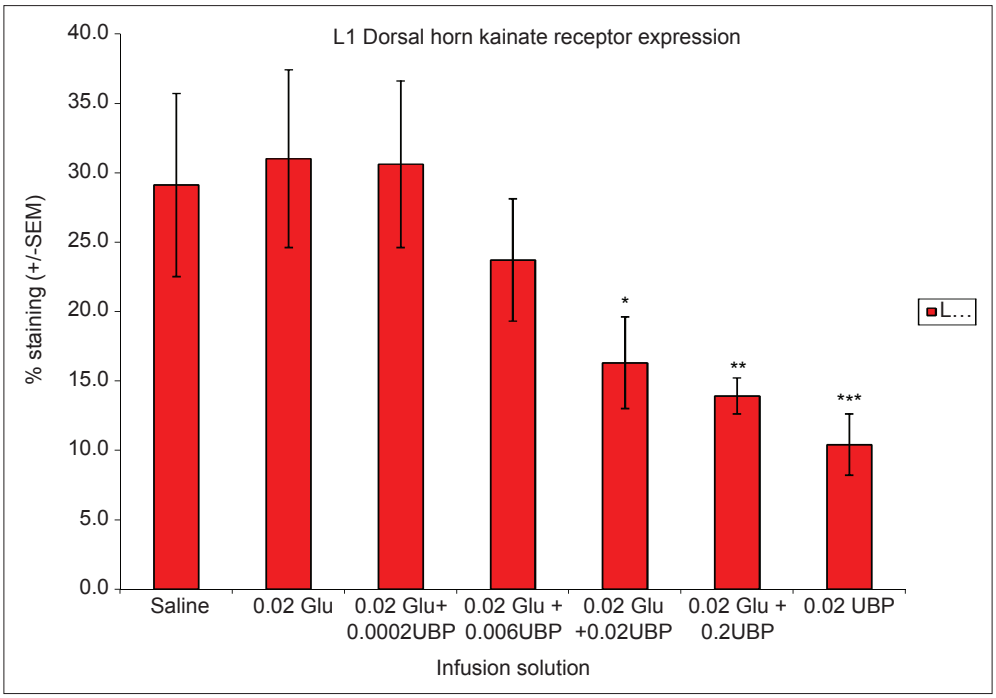

Figure 2: L1 protein expression – UBP301: Significant changes in KA receptor expression were seen between glutamate treated animals and animals treated with glutamate and 0.02 mM, *(*P* < 0.5) or 0.2 mM UBP301, ***P* < 0.01, as well as animals treated with 0.02 mM UBP alone, # *P* < 0.0001 (Dunn's multiple comparison test). A Kruskal–Wallis nonparametric ANOVA was significant, *P* < 0.0001. SEM, standard error of the mean

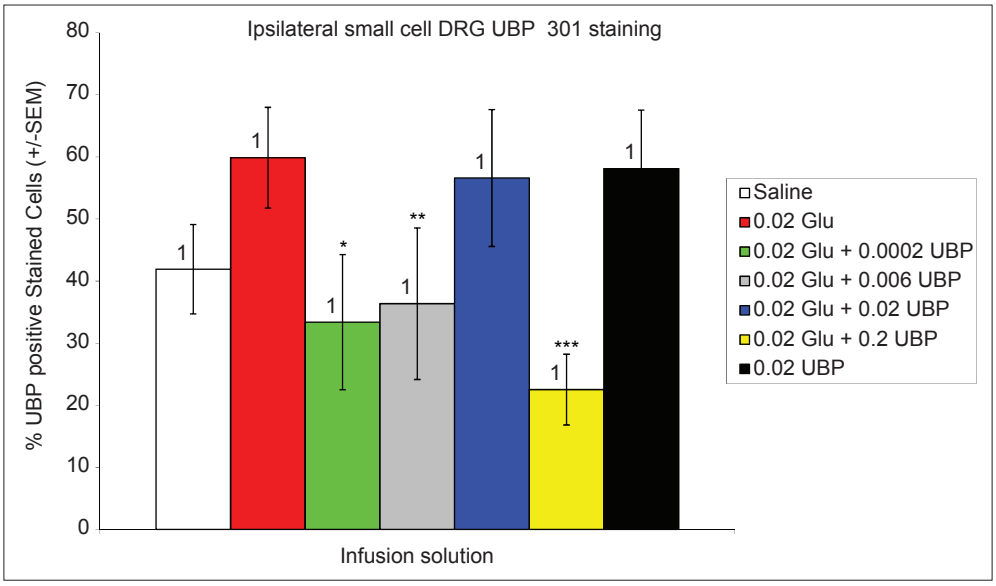

Figure 3: DRG protein expression in small neuronal bodies (<25 μm) – UBP301: KA receptor expression was significantly different in saline versus glutamate treated animals, and significant expression changes were also seen between glutamate treat animals and animals treated with glutamate and 0.0002 or 0.2 mM UBP301, *, ***P* < 0.01 (Dunn's Multiple test). A Kruskal–Wallis nonparametric ANOVA was significant, *P* < 0.001. SEM, standard error of the mean

Infusion with N-Methyl-4-isoxazoleproionic acid antagonist: Norketamine

Von Frey testing showed that an infusion of norketamine extinguished significant pre- to postoperative differences in hind paw responses related to the presence of epidural glutamate at 0.0002, 0.006, 0.002, 0.02, and 0.2 mM concentrations of norketamine see Figure 4 at 24 and 72 hours of coinfusion. Also, there was decreased hypersensitivity with 0.02 mM norketamine alone compared with 0.02 mM glutamate at both 24 and 72 hours of infusion [Figure 5].

Compared with a 0.02 mM glutamate infusion, significant decreases in L1 dorsal horn receptor expression were seen with coinfusion of 0.06 mM norketamine. Finally,

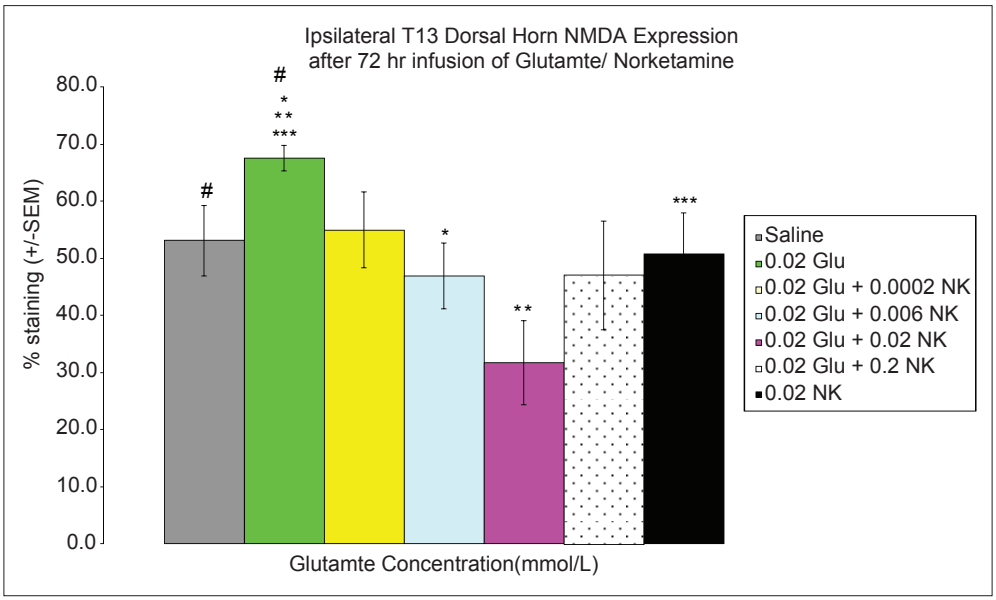

Figure 4: L1 Protein expression – Norketamine: Significant changes in NMDA receptor expression were seen in saline versus glutamate treated animals, #(*P* < 0.01) in addition to animals treated with glutamate and 0.006 mM, *(*P* < 0.01) or 0.02 mM, **(*P* < 0.001) Norketamine. Also, significant expression differences were seen between glutamate and 0.2 mM Norketamine alone, ***P < 0.05 (Mann–Whitney test). A Kruskal–Wallis nonparametric ANOVA was significant, *P* < 0.0001. SEM, standard error of the mean

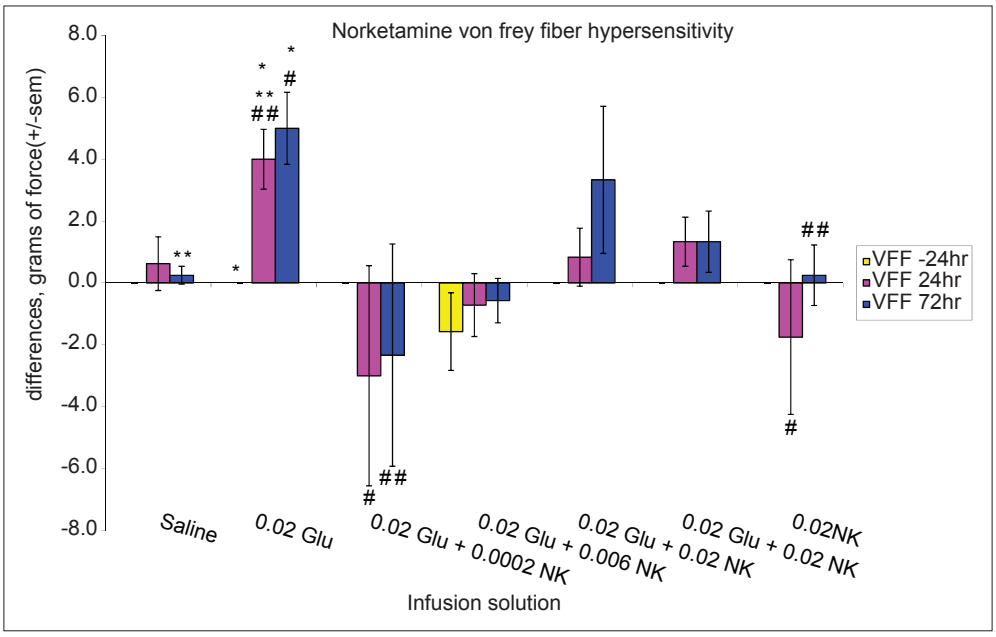

Figure 5: Mechanical hypersensitivity – Norketamine: All treatment groups demonstrated no behavior differences from pre to postoperative conditions. Significant improvement of glutamate induced symptoms was seen after 24 and 72 hours of treatment with 0.006 mM. Treatment with 0.02 mM Norketamine alone was also significantly different from glutamate treatment at 24 and 72 hours. # and ##*P* < 0.05 (Dunn's multiple comparison test). Glutamate treated animals showed a significant increase in mechanical hypersensitivity at 24 and 72 hours after surgery, *P < 0.0001, (Dunn's Multiple Comparison test). Postoperative, saline treated animals were significantly less hypersensitive than glutamate treated animals at 72 hours after surgery, **P < 0.01 (Dunn's multiple comparison test). A Kruskal–Wallis nonparametric ANOVA was significant, *P* < 0.01

significant decreases in receptor expression were found in both large and small neuronal cell bodies in the L5 DRG compared with coinfusion of 0.2 mM norketamine. For both large and small cell clusters of neuronal cell bodies, a significant increase in expression was observed at the 0.02 mM glutamate concentration compared with the saline control group [Figures 6 and 7].

Infusion with a-amino-3-hydroxy-5-methyl-4-isoxaleprionic acid antagonist: SYM2206

When infused with the AMPA antagonist, SYM2206, there was no change in hypersensitivity as measured by

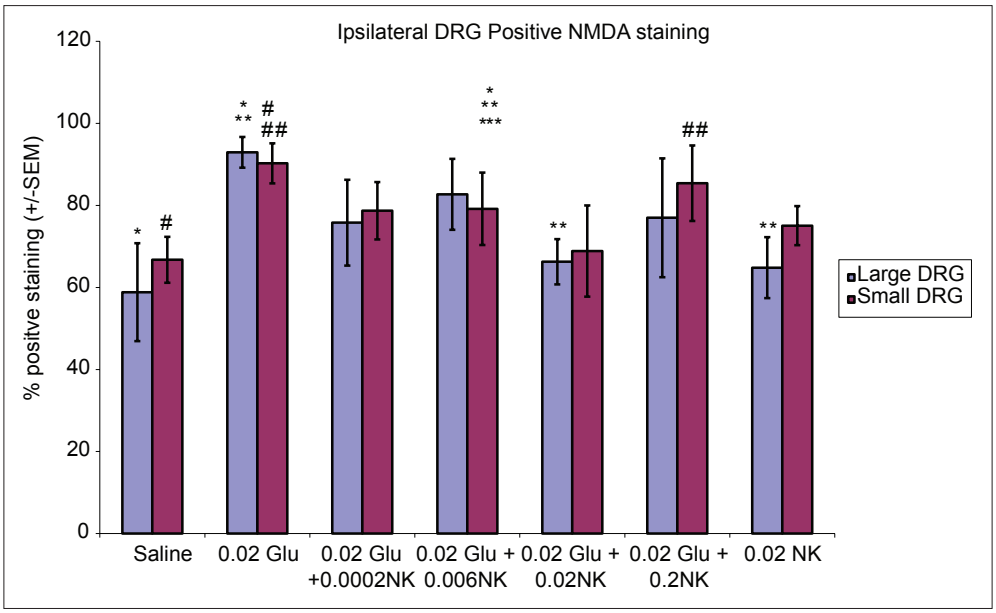

Figure 6: DRG Protein Expression – Norketamine: NMDA receptor expression was significantly different in saline versus glutamate (*, # *P* < 0.05), and significant expression changes were also seen between glutamate treated animals and animals treated with glutamate and 0.02 mM Norketamine (, ## *P* < 0.05) as well as with 0.02 mM Norketamine alone (>25 μm diameter neurons large diameter neurons). A Kruskal–Wallis nonparametric ANOVA was significant, *P* < 0.05. *, **<25 μm diameter neurons; #, ##>25 μm large diameter neurons. SEM, standard error of the mean**

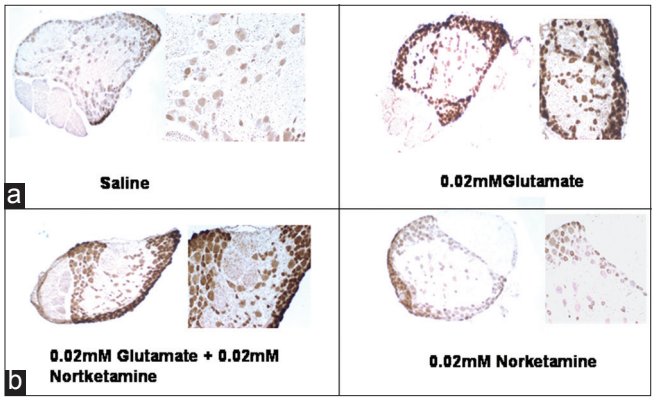

Figure 7: A representative photograph for dorsal root ganglion immunohistochemistry analysis of large and small neuronal cell clusters showing a decrease in NMDA receptor expression from 0.02 mM glutamate to glutamate with 0.02 mM Norketamine and 0.02 mM norketamine alone concentrations. Reduced from (a) ×4, and (b) ×10

VFF testing in the antagonist coinfused group compared with the glutamate alone group as well as no significant changes in AMPA receptor expression at the dorsal horn or at the DRG (data not shown).

DISCUSSION

Glutamate receptors have been demonstrated to exist throughout the peripheral nervous system from the skin to the dorsal root ganglion,[17,30,31] but the role of glutamatergic neurotransmission within the peripheral nervous system is not completely understood. It has been shown that elevated glutamate concentrations in skin and muscle are associated with pain;[27] cell bodies of the dorsal root ganglion have been shown to contain glutamate receptors,[5,14] and an epidural glutamate infusion can create focal hyperalgesia.[10] NMDA receptors have been shown to contribute to hypersensitivity within the temporomandibular joint and the knee.[9,15,29]

Glutamate receptor antagonists placed subcutaneously in regions where pain was induced by raising extracellular glutamate levels can reduce that pain.[12,26,28] Similarly, it now appears that small amounts of a kainate or NMDA receptor antagonists deposited near the DRG can also treat sciatic pain related to the presence of glutamate emanating from disc.

Cartilage matrix protein is located outside the confines of cell membranes, and changes in matrix composition may affect the surrounding environment. Morphologic changes in disc structure by aging or trauma are associated with enzymatic degradation of aggrecan and other matrix components. Enzymatic degradation can liberate significant concentrations of free glutamate from aggrecan.[6,8,20] The potential for suddenly increased glutamate concentration occurs when an aggrecan rich nucleus pulposus extrudes into the epidural space.

Glutamate concentrations building up in the epidural space will remain for some time. Glutamate reuptake systems do not exist in the periphery, whereas in the central nervous system (CNS) extracellular glutamate concentrations are tightly regulated.[4,19] Epidural glutamate can permeate the DRG.[6] Since glutamate can permeate the DRG and remain for considerable periods

of time in the epidural space, sensory neurotransmission, and the pain experience could be affected. In the same sense, a glutamate receptor antagonist also permeable to the DRG basement membrane, able to block key glutamate receptors, could reverse the glutamate effects as nociception.

The kainic acid antagonist UBP301 is highly selectivity for the kainite receptor. The kinetic index for inhibition (KI) of kainic acid receptors is 3.0 μM. Interestingly, our data demonstrate a relatively linear dose response curve beginning one order of magnitude of concentration below the KI. These data suggest that the kainite receptor is an important mediator of pain related to increased glutamate concentrations in the epidural space. Kainate receptors are known to be involved in many painful conditions, so the activity of kainite receptors in this particular type of pain is not surprising. The presence of kainite receptors with both low and high affinities within the DRG could be the reason for the development of the broad and progressive concentration-based changes in nociception related to UBP-301 concentration.[22]

The NMDA antagonist norketamine has a (KI of 3.6 μM) also created significant reductions in glutamate mediated hyperalgesia beginning at a 0.2 μM concentration. NMDA receptor activity has also been associated with many painful conditions. There is a narrower therapeutic range of concentration (0.2 mM to 6 μM). Correspondingly, dorsal horn NMDA receptor expression also showed a narrower concentration-based range of change in receptor expression in the dorsal horn compared with experiments with the kainic acid antagonist. Unlike kainate receptors, NMDA receptors with varying glutamate concentration thresholds may not exist for NMDA receptors in the DRG.

VFF and plantar heat testing demonstrate inherent variability. In previous publications, we have been able to show significant unilateral differences in the absolute value of hindpaw responses in glutamate infused animals. Related to variability factors, relatively large number was required. Given the larger number of conditions (antagonist concentrations) that required surgical procedures in this antagonist study, effects of baseline variability were minimized by calculating the difference in hindlimb responses between sides. Using this measure, we found significant differences postinfusion related to treatment with UBP-301 and norketamine compared with glutamate infused animals. Also, the therapeutic potential of UBP-301 and norketamine was demonstrated by a second comparison that showed antagonist coinfusion extinguished significant pre and postinfusion differences in lower extremity nociception for most concentrations of UBP-301 and norketamine.

These experiments in rats did not involve disc material, the putative source of epidural glutamate in the human condition of sciatica associated with a lumbar disc herniation. Instead, a low volume epidural glutamate and/or glutamate receptor antagonist solution was used. If we had modified the model to include placing rat disc material in the epidural space, experimental variables would have included the placement within the epidural space, the size and shape of the disc fragment, and variability of its glutamate content. These additional variables could have made effects of epidural glutamate and glutamate receptor antagonists on glutamate receptors in the region more difficult to determine. This straightforward model we have employed has clearly demonstrated the importance of glutamate concentration in the vicinity of the DRG.

In this study, patterns of neurotransmission related to the presence of epidural glutamate and ionotropic glutamate receptor antagonists were not directly tested, nor did we test whether ionotropic glutamate receptor antagonists permeated the DRG. SYM-2202 may have failed to affect AMPA-based nociception related to a lack of bioavailability at the DRG rather than for a lack of antinociceptive effects.

These behavioral and semiquantitative immunohistochemistry patterns can define a nociceptive state.[24] The reversal of these nociceptive parameters by coinfusion with kainite and NMDA receptor antagonists is strong evidence for the efficacy of these compounds to treat glutamate-based sciatic pain.

Epidural and intrathecal ionotropic glutamate receptor antagonists have already been shown to have some analgesic effects in other conditions not associated with elevated extracellular glutamate levels.[11,16,24] This is not surprising, since glutamate neurotransmission is essential to nociceptive processing. However, effectiveness of glutamate antagonists' compounds may be even greater in painful conditions such as disc-based sciatica where elevated extracellular glutamate concentrations from cartilage may be driving the process.

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Commentary

This is a very important article for the clinical neurosurgeon and will eventually change the way we treat both acute and chronic nerve pain. Their previous study, which demonstrated the release of high levels of free glutamate from the ruptured disc, especially the extruded disc, is critical for our understanding of painful nerve conditions. As the authors state, in the recent past inflammation and the accumulation of proinflammatory cytokines were considered to play a major role in chronic pain. Yet, some follow-up studies in which inflammatory cytokines (TNF-a) were blocked, failed to produce significant pain relief.

Previous studies have shown that other glutamate receptors also play a significant role in pain syndromes, including AMPA receptors and metabotropic glutamate receptors. The pathophysiology involved is quite complex and is still not completely understood. As with other neurological conditions there exist significant evidence to suggest that there is an important link between inflammatory pathway signaling and amplification of glutamate receptor function, immunoexcitotoxicity, which may involve an interaction of the various glutamate receptors via neuronal cell signaling in the DRG and dorsal root entry zone.

Triggering of chronic immunoexcitotoxicity has been shown to stimulate trafficking of calcium-permeable (GluR2-lacking) AMPA receptors, NMDA receptors and to promote endocytosis of GABA receptors, producing a state of chronic hyperactivity of

nerve pain pathways. The kainite receptors are intimately linked to AMPA receptors as well as NMDA receptors via cell signaling pathways.

Because of the hyperactivity of these glutamate pain receptors, one should also consider the patient's diet, as most processed foods contain significant levels of free glutamate, which can raise human blood glutamate levels from 11- to 20-fold higher than normal for prolonged periods. Many foods served in hospitals contain high levels of added glutamates and should be discouraged.

Another contributing factor would be ischemia of the nerve root produced by constricting scar tissue, which also increases the release of tissue glutamate. A number of natural compounds reduce inflammation as well as excitotoxicity. These include curcumin, quercetin, resveratrol, hawthorne extract, magnesium, DHA, R-lipoic acid, acetyl-L carnitine, epigallocatechin gallate (EGCG), apigenin, and luteolin, to name just a few.

This paper should be read by all neurosurgeons and hopefully will lead to further studies on this mechanism and ways to utilize this knowledge to treat this debilitating condition.

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