RESEARCH ARTICLE

Dynamics of Spinal Inotropic Glutamate Receptors During the Development of Prolonged Postoperative Pain in the Rat.

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Abstract

Surgery can cause pain that lasts for weeks to months. Postoperative pain involves an early *induction* phase (hours to l days), and a longer *maintenance* phase. Experimental post-operative pain can be lowered by pre-operative spinal glutamate receptor antagonists as well as inhibitors of certain mitogen activated protein kinases (MAPKs). Here a model of postoperative pain in the rat, leading to 4 weeks of secondary mechanical allodynia-hyperalgesia, was used to examine changes in spinal inotropic glutamate receptors and MAPKs. Western blots show that GluR1subunits of AMPA receptors undergo rapid increases in phosphorylation at 4h post-op (2.3x naive), before tactile hyperalgesia is detectable, change to 1.6x naïve at 2d post-op, when hyperalgesia is clearly present, and fall to sub-baseline levels (0.1x naïve) at 14d, when hyperalgesia is maximum. The NR1 subunit of NMDA receptors are 1.7x, 1.8x, and 1.1x the level of naïve at 4h, 2d and 14d, respectively, whilst the phosphorylated form occurs at 1.7x, 0.9x and 0.5x of naïve at these times. NR2B subunit amounts follow a similar trend; 1.6x, 1.7x and 0.8x. Phosphorylation of the MAPK JNK was elevated to 1.45x, 1.35x and fell to 0.8x of naïve at 4h, 2d and 14d postoperative, whilst $pERK_{1/2}$ phosphorylation remained unchanged. These results suggest that increases in total NMDA subunits and in phosphorylated NMDA and AMPA receptors, paralleled by elevated P~JNK, contribute to the induction phase of persistent post-operative pain, but are not involved in its maintenance.

Key words: Postoperative pain; Glutamate receptors; NMDA receptors; AMPA receptors; receptor phosphorylation; spinal MAPKs.

1.Introduction

Postoperative pain remains a challenging medical problem, with morbidity-related dysfunctions that slow recovery, cause extreme discomfort and lengthen hospital stays.[1] Pain is often a result of nerve injury, with a defined neuropathic component, but certain chronic pain scenarios, such as post-thoracotomy pain, often occur without detected neuropathy.[2] The factors that contribute to the development of chronic, persistent pain that endures beyond the 72-96 hours of acute postoperative pain are not known, but their identity is essential for the development of drugs and procedures to minimize prolonged pain.

The locations that are essential for the development of postoperative pain following surgery appear to move from the periphery to spinal cord, then up the neuraxis to the brainstem, and then more rostral.[3] In the spinal cord, changes in cell signaling, including those in neurons and glia,[4] appear within minutes to hours after cutaneous injury. For example, the activation/phosphorylation of p38 Mitogen Activated Protein Kinase (MAPK) appears in the lumbar dorsal horn 3 days after incision and skin – muscle retraction around the saphenous nerve (SMIR), and inhibitors of p38 activation, delivered peri-operatively into the spinal fluid, can substantially blunt the tactile hyperalgesia that indicates pain in the operated rat.[5] A late delivery of the same inhibitor, however, has only a weak, transiently reversing effect on the already established hyperalgesia.[5] A similar behavioral pattern occurs with intrathecal Resolvins, delivered perioperatively and even several days after experimental thoracotomy.[6] Such results indicate changes of spinal cells and circuits that are critical for the developmental phase of postoperative pain, causing a condition that is no longer present, or essential for the pain that persists many days after surgery.

In the present study we used the SMIR model to examine the role of inotropic glutamate receptors in the development of prolonged postoperative pain (PPP). The SMIR model is noteworthy for the 2-3 days that are required for the first detection of tactile hyperalgesia, which reaches its maximum after about 2 weeks and then has resolved to the baseline sensitivity by 5 weeks.[7] This tactile hypersensitivity is reduced by standard analgesics and so is accepted as a measure of evoked pain.[8] SMIR causes no injury to fibers in the entrapped, purely sensory saphenous nerve, which is stretched and compressed during the 1 hr long retraction.

Inotropic glutamate receptors, particularly the variants of NMDA and AMPA receptors, are well known to contribute to different hyperalgesic states, e.g., from inflammation or nerve injury.[9] Spinal glutamate receptors are also important in the brief (4-5 days) hyperalgesia following paw incision.[10-12] Here, using Western blots, we have examined total and phosphorylated subunits of NMDA and AMPA receptors in homogenates of spinal cord taken from rats early on (4h and 2 days) and 2 weeks after the SMIR procedure. Additionally, we examined the activation by phosphorylation of the MAPKs $ERK_{1/2}$ and JNK, for comparison with the known time course of p38 MAPK activation.

2.Methods

2.1 Animals. Male Sprague-Dawley rats, 200-230 gm, were used in all studies, as approved by the Harvard Committee on Animals and consistent with US national guidelines for the use of laboratory animals.[13] Rats were kept on a 12h dark-light cycle and given water and chow ad libitum. After 1-4 days of handling, for familiarization and to reduce stress-related analgesia, the animals were operated on, with the SMIR procedure. After the designated postoperative time (4h to 14 days) the rats were euthanized by isoflurane, for removal and processing of the spinal cord.

2.2 Surgery

SMIR model was performed as developed by Flatters.[7] In brief, rats were anesthetized with intraperitoneal sodium pentobarbital (Nembutal®; Sigma–Aldrich Chemical Co., St. Louis, MO; 50 mg/ml). A 1.2–1.5-cm skin incision was made to reveal the muscle of the leg. An incision (7–10 mm long) was then made in the superficial muscle layer. A microdissecting retractor with four prongs was inserted into the incision site on the thigh. The skin and the muscle were then retracted 2 cm and was maintained for 1 h. After the retractor was removed, muscle and skin wounds were closed with 4–0 Vicryl® (Myco Medical, Cary, NC) and 3–0 silk sutures (Angiotech; Surgical Specialties Corp., Reading PA), respectively. After recovery from anesthesia, all animals could ambulate normally and rise up on their hind limbs to reach food and water.

2.3 Western blot

Animals were transcardially perfused with PBS. The L5 spinal cord was dissected and homogenized in ice- cold Ringer's solution (NaCl 155 mM, KCl 4.5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, D-glucose 10 mM, HEPES 5 mM, pH 7.4) containing 3% protease inhibitor cocktail (Sigma) and then centrifuged at 14,000 g for 20 min at 4°C. Supernatants were removed and stored at -80°C until time of the assay. Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL). Protein samples (30 μg) were separated on SDS–PAGE gel and transferred to nitrocellulose blots. The blots were blocked with 5% milk and incubated overnight at 4°C with the primary antibodies against following antigens (sources and titers):*GluR1*, Millipore, MAB2263, Mouse, 1:1000; *pGluR1* (ser845), Millipore, 04-1073, Rabbit, 1:1000; *GluR2*, Millipore, MAB397, Mouse, 1:500; *NR1*, Millipore, 05-432,Mouse, 1:5000; *pNR1* (Ser896), Millipore, 06-640, Rabbit, 1:500; *NR2B*, Millipore, AB1557p, Rabbit, 1:1000; *pERK*, Cell Signaling, 9101, Rabbit,1:500; *pJNK*, Cell Signaling, 9251, Rabbit, 1:500; *GAPDH*, Millipore, MAB374, Mouse, 1:20000 These blots were further incubated with HRP-conjugated secondary antibody, developed in ECL solution, and exposed onto Hyperfilm (Millipore) for 1–5 min. Specific bands were evaluated by apparent molecular size. The intensity of the selected bands was analyzed using Imag J software (NIH, Bethesda MD).

2.4 Statistical analyses

All data are expressed as mean ± SEM. The density of specific bands in Western Blot was measured with Image J. The levels of GluR1, pGluR1, GluR2, NR1, pMR1,NR2B, pJNK, and pERK were normalized to loading control GAPDH for each individual sample. Averaged values of each molecule/form were divided by those in separately analyzed, unoperated *naïve* controls. Differences between groups were compared using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1 GluR1. The original SMIR studies (Flatters 2008) showed that no tactile hypersensitivity of the ipsilateral paw was detectable at 4h postoperative, but a significant hypersensitivity was present at 2d and progressed to a maximum vaue at about 13-14 days.[7] Here spinal cord homogenates from SMIR rats taken at 4h, 2d and 14 d after surgery were compared with those from naïve, unoperated animals. Glutamate AMPA receptors were assessed by total GluR1 subunit and its phosphorylated (Ser845) homologue.

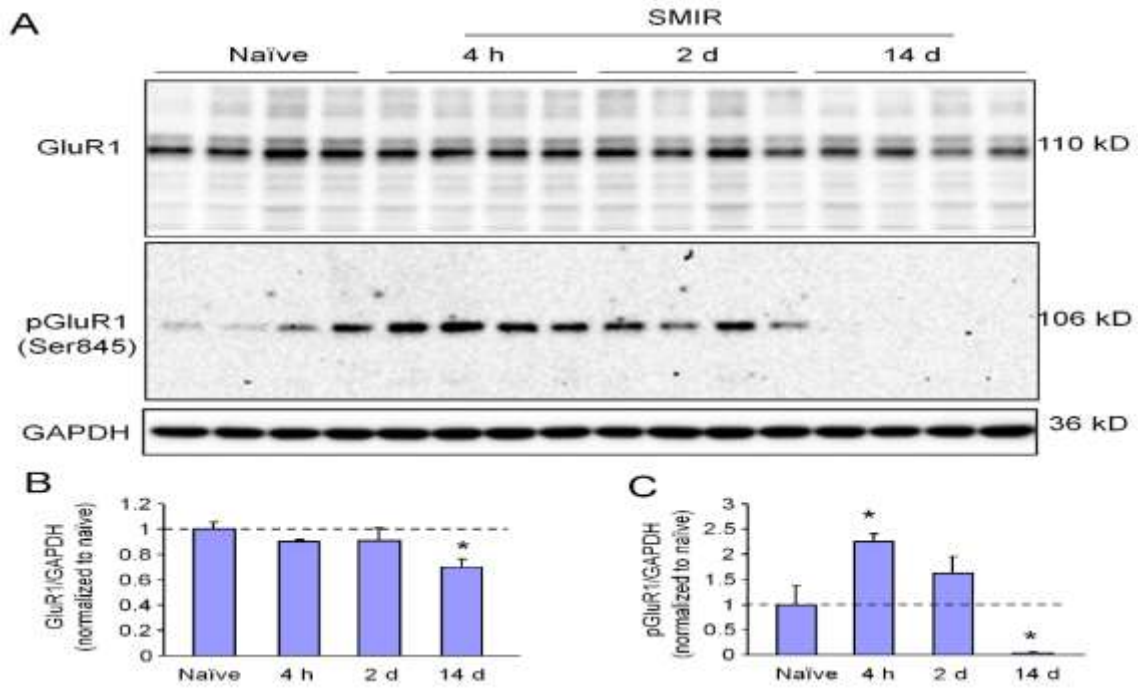

Figure 1. **A**. Western blot of Total GluR1 (upper panel) and p-GluR1 (lower panel) at different times after SMIR surgery. **B**. Averaged values of total GluR1 after SMIR, normalized to GAPDH and then to naïve (pre-operative) levels. **C**. Phospho-GluR1 levels, normalized as in 1.B.

Figure 1 shows Western blots for 4 animals in each group (A) and the mean and SD of these densities (B,C). Total GluR1 was unchanged at up to 4h but decreased from naïve levels by ~20% on day 14. In contrast, phospho-GluR1 more than doubled over naïve at 4h but had declined somewhat by 2d and was virtually undetectable by 14 days.

3.2. GluR2. In contrast to GluR1 changes, total GluR2 did not change when all points were included in the analysis (Figure 2). Comparison between naïve and 14 day values showed a small but significant increase in GluR2, opposite that for GluR1.

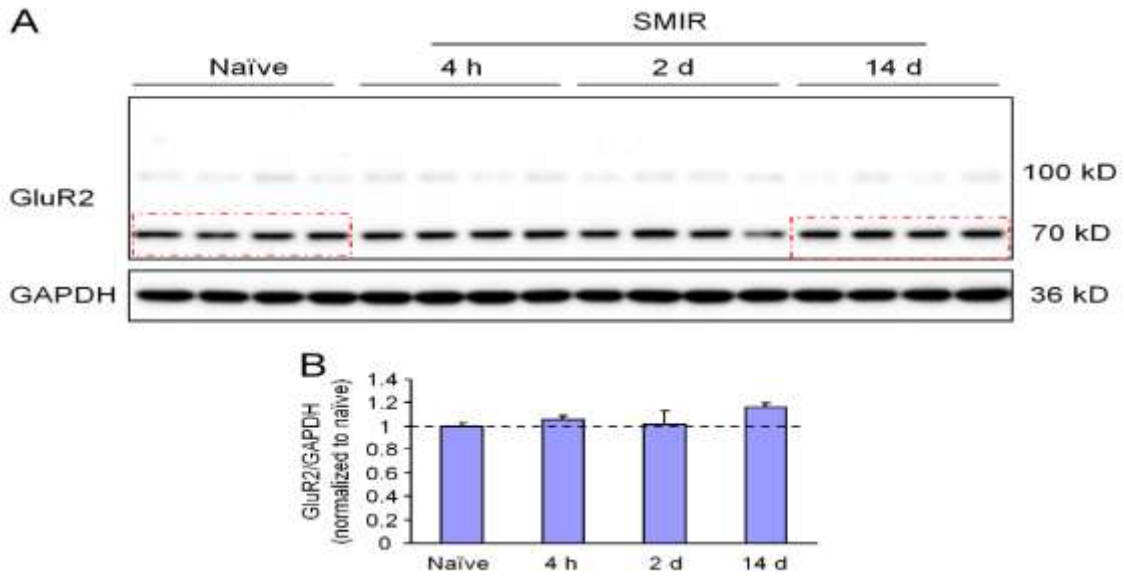

Figure 2. A. Western blots of GluR2, and B. The means (SD) of the densities divided by each lanes respective GAPDH density and then these mean values normalized to the mean levels of naïve, unoperated rats.

3.3 NR1. The NR1 subunit of NMDA receptors was also assessed in SMIR-operated rats. Total NR1 increased at 4h and remained at this level at 2d, but had declined to naïve value by day 14 (Figure 3). Its phosphorylated form, pNR1, peaked at 4h, (although without reaching statistical significance), returned to preoperative levels by day 2 and had fallen below that by day 14 (Figure 3), paralleling the pattern of pGluR1 (Figure 1).

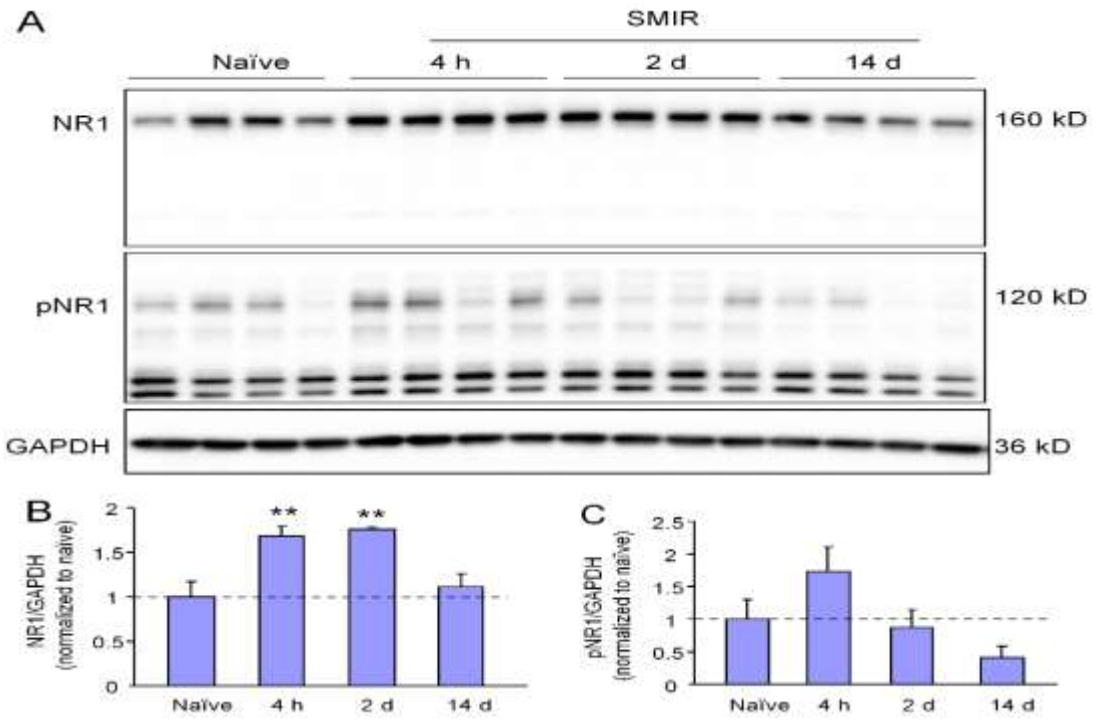

Figure 3. A. Western blots of total NR1 and Phospho-NR1 in naives and post-SMIR spinal cord homogenates. **B**. NR1 levels from individual rats divided by GAPDH levels from the same homogenate, and then averaged and normalized to the levels in naïve rats. **C**. Phospho-NR1 levels, analyzed as in 3.B.

3.4 NR2B. The NR2B subunit of NMDA receptors increased, albeit insignificantly (due to a large variance in the naïve signal), at 4h and 2d, and declined slightly by 14d (Figure 4).

3.5 MAPKs In order to compare other MAPKs in spinal cord with our previously reported analysis of p38, we analyzed Western blots of pJNK and $pERK_{1/2}$ (Figure 5). The activated/phosphorylated form of JNK rose from preoperative (naïve) levels at 4h and 2d, and fell below that level at 14d, whereas the phosphorylated form of $ERK_{1/2}$ was unchanged over that period.

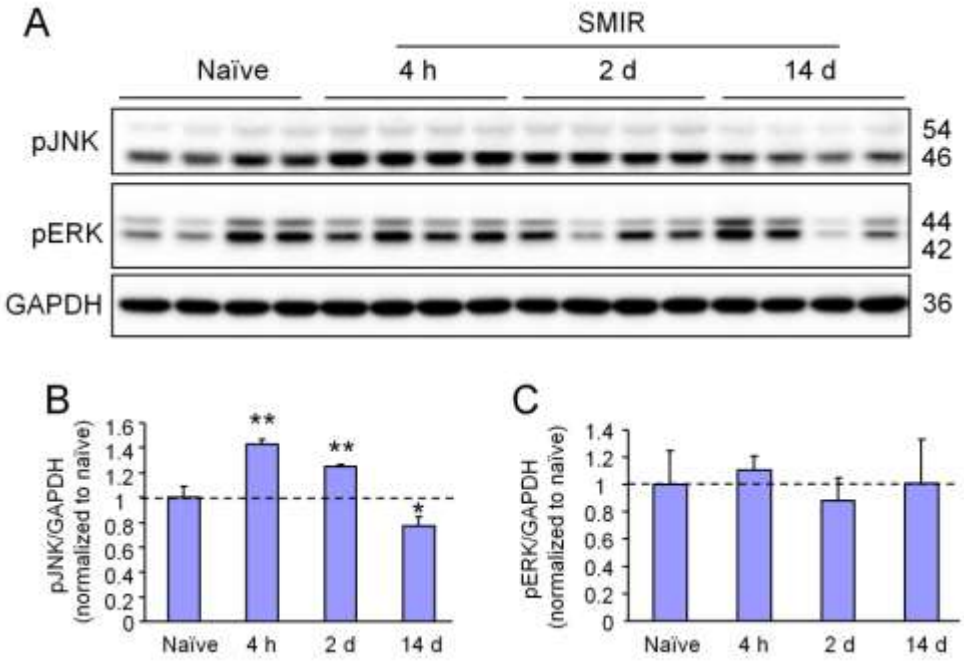

Figure 5. A. Western blots of pJNK and pERK at different times after SMIR. B. The GAPDH normalized densities for pJNK were averaged and then divided by naïve controls, as were C. the levels for both bands of pERK ($pERK_{1/2}$).

4. Discussion

4.1 Characteristics of SMIR-induced pain. Postoperative pain in the SMIR model used in this study is induced by stretching/compressing the saphenous nerve under retraction of the adjacent skin and muscle after an incision. This pain is assessed by examining tactile hypersensitivity of the ipsilateral plantar paw, far removed from the surgical site and so is classified as "secondary hyperalgesia", which involves changes in the spinal cord's dorsal horn, the processing area for sensory input from the periphery.[14] Unlike many other post-surgical pain models, this one is free of nerve damage, eliminating a major contribution via neuropathic pain.

4.2 Glutamate receptors and postoperative pain. Glutamate is the primary excitatory amino acid at synapses in the dorsal horn conveying nociceptive information. Both metabotropic and inotropic glutamate receptors are involved in the transmission driven by glutamate released by excited nociceptive afferents.[10,15-17] A variety of different preclinical models for hyperalgesia have identified important roles for both NMDA and AMPA inotropic glutamate receptors, with increases in specific receptor subunits and phosphorylation that can change the gating of active receptors and the trafficking of different subunits.[18,19] Paw incision is known to increase acutely the glutamate released in spinal fluid[10] and receptor antagonists that discriminate between the inotropic glutamate receptors AMPA and NMDA have been used to differentiate primary hyperalgesia (NMDA-R -dependent), detected at and near the

incision site, from the secondary hyperalgesia (dependent on AMPA-R) that is detected at a distance and that requires spinal responses.[12]

The present paper reports changes in inotropic glutamate receptors that change during the development of SMIR-induced secondary hyperalgesia. NMDA-R NR1 and NR2B subunits are elevated over preoperative (naïve) levels at 4h and 2d, before hyperalgesia is detected and during its initial rise, respectively, and have returned to near naïve levels by day 14, at the peak of hyperalgesia. Phosphorylation of NR1 almost doubles at 4h but equals naïve levels at 2d and half of that at 14d, indicating an active role in protein phosphatases leading into the maintenance phase of hyperalgesia.

In contrast, the GluR1 (GluA1) and GluR2 (GluA2) subunits of AMPA receptors do not change their number but GluR1 phosphorylation is more than doubled at postoperative 4h and declines at 2d, falling to almost undetectable levels at 14d, again evidence for activated phosphatases in the transition to persistent pain.

Other studies have reported both pre-synaptic[20,21] and postsynaptic locations and functions for inotropic glutamate receptors.[9,22] Physiological nociception from brief injury relies on AMPA receptors, since NMDA-R are blocked by Mg^{+2} ions at rest, but repeated depolarization, e.g., from a barrage of nociceptive impulses stimulated by the trauma of surgery, will expel the blocking ion and allow Ca^{+2} to enter the cell through the freed receptor.[18] Calcium-activated kinases e.g.,(many isoforms of Protein Kinase C, Calcium-calmodulin-dependent kinase) are then able to act to phosphorylate both NMDA and AMPA receptors, leading to altered trafficking and receptor composition,[23] which, in turn, allows more Ca^{+2} to enter the cell (at pre- and post-synaptic loci) and secures the spinal synapses by a molecular form of neuroplasticity.[22,24]

Other enzymes are also activated by elevated $Ca^{+2,}$ notably MAPKinases that are key regulators of intracellular pathways.[4]

4.3 MAPK responses in post-operative pain. The activation by phosphorylation of specific MAPKs in spinal cord are well known to be involved in the induction of post-injury pain. [25,26] Specific MAPKs are activated in a certain order and localized in different cell types, with $P\sim ERK_{1/2}$ appearing earliest , in microglia, followed by P~p38 in neurons and P~JNK in astrocytes, where it can persist for weeks.[27] Previously we reported that 3 days after SMIR (rapid induction phase) the levels of P~ p38 are elevated and continue to rise to day 12 (peak hyperalgesia), declining, but not to the preoperative baseline value, by day 35, when hyperalgesia has resolved.[5] Also, from 3d to 12d the locus of P~p38 shifts from spinal microglia into spinal neurons. Furthermore, intrathecal (L5-6) injections of an inhibitor of p38 MAPK before pain appears prevents the development of hyperalgesia, but a later injection, at day 9 post-SMIR, results in only a transient, hour long reduction in hyperalgesia.[5,28] Activation of p38 after paw incision has also been reported, along with reduction of the post-incisional pain by p38 inhibitors.[29,30] These results, and very similar ones with the anti-

inflammatory molecule Resolvin (D1, D2) acting on SMIR-[31] and thoracotomy-induced[6] hyperalgesia, confirm the distinction between the spinal mechanisms that drive the induction of post-operative pain and those that determine its persistence.[32,33]

In the present study, P~$ERK_{1/2}$, known to be activated by elevated intracellular Ca^{+2}, is, surprisingly, unchanged after SMIR, even though the changes in glutamate receptor composition would predict an increase in P~$ERK_{1/2}$ and it has been shown to increase in other models of pain, e.g. inflammation and neuropathic pain. Perhaps these other models activate nociceptor populations differently than the stretching-compression of the SMIR model, which causes no consequent nerve injury.

In contrast to P~$ERK_{1/2}$, P~JNK is present above the naïve (pre-operative) value at 4h and 2d and falls slightly below naïve at 14d. This suggests that P~JNK is an important driver for induction of post-SMIR hyperalgesia. However, since the Western blots only report the proteins in spinal cord homogenates, immunocytochemical studies will be required to determine the cell type (glia, neurons) and the pre- or post-synaptic locations for these important modulators of induction of persistent postoperative hyperalgesia.

Summary

These studies show that persistent experimental postoperative pain in rats, resulting from incision and retraction identical to that involved in many clinical surgical procedures, is accompanied by dynamic changes in inotropic glutamate NMDA and AMPA receptors. The NR1 subunit of NMDA receptors is doubled within a few hours after surgery, and its phosphorylation, which enhances its physiological contribution, and may alter its turnover time, is also acutely elevated, but neither of these changes persist during the maintenance phase of pain at several weeks post-surgery. The GluR1 subunit of AMA receptors, whose initial activation by glutamate may be required for depolarization-activation of NMDA receptors, shows an increased phosphorylation in the acute period but actually decreases from pre-operative levels during the pain maintenance phase.

Overall, these inotropic glutamate receptors change rapidly in number or activation state shortly after incision-retraction but are unchanged or decreased while pain is sustained. The potential for clinical application suggests that spinal administration of inotropic glutamate receptor antagonists in the perioperative period may substantially reduce acute postoperative pain, but further animal studies of this effect must be conducted.

Acknowledgements

We thank Dr. Liu Huang for performing the SMIR surgery on rats used in this paper. Partial support for this work was from the BWH Anesthesia Foundation for research support.

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