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ORIGINAL ARTICLE

Comparison of changes in mRNA expression of spinal glutamate transporters following induction of two neuropathic pain models

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Objectives: It is now suspected that different kinds of neuropathic pain syndromes may have significantly different mechanisms. To date, much effort has been made to investigate the function of glutamate transporters (GTs) after nerve injury. The aim of this study is to compare the changes in GTs' mRNA expression levels between two distinct models of peripheral neuropathic pain: chronic constriction nerve injury (CCI) and spared nerve injury (SNI).

Methods: Experiments were performed on animal models of mononeuropathy. Several groups of rats were subjected to behavioral experiments before and 4, 7, and 14 days after the induction of mononeuropathy following the CCI and SNI. Allodynia was assessed by Von Frey filaments, and thermal hyperalgesia was assessed by the paw withdrawal tests. To study molecular experiments, the mRNA expression of (GTs) in CCI and SNI rats, reverse transcription polymerase chain reaction (RT-PCR) were used on days 4 and 14.

Results and conclusion: The maximum responses of mechanical allodynia and heat hyperalgesia in two distinct neuropathic pain models were detected on day 14. CCI and SNI induced upregulation of three GTs on day 4, which were followed by GTs downregulation in CCI and downregulation of glutamate aspartate transporter (GLAST) and glutamate transporter (GLT)1 in SNI when examined on day 14. These results indicate that there is an inverse correlation between pain responses and expression of GTs, and also changes in expression of spinal GTs may have a critical function in both the induction and maintenance of neuropathic pain in independent peripheral neuropathic pain models.

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Introduction

Damage of primary pain afferents in the spinal cord results in abnormal excitability and other pathological changes, including hyperalgesia, allodynia, and spontaneous pain.[1] Hyperalgesia may be due to central hypersensitivity induced by the inputs arising from a peripheral injury.[2] Peripheral neural mechanisms, such as nociceptor sensitization and neurogenic responses, are also likely to contribute to pathological pain.[3,4] In animal models, transection of a major peripheral nerve, such as the sciatic nerve, shows denervation of the distal hindlimb.[5] Recent evidence indicates the existence of interactions between neuropeptides and excitatory amino acids (EAAs) in central nociceptive processing.[2] Glutamate is an excitatory neurotransmitter in the mammalian central nervous system.[6] The concentration of extracellular glutamate in the CNS is controlled by Na^+-dependent transport systems.[7] Glutamate transporters (GTs) have a crucial function in protecting neurons from excitotoxicity by exogenous and endogenous glutamate. Thus, several Na^+-dependent GTs have been cloned, including EAA transporter (EAAT)1, glutamate aspartate transporter (GLAST), EAAT2 (GLT1), and EAAT3 (EAAC1).[8] Glutamate uptake and the expression of GTs in the spinal cord change under pathological conditions.[9] Thus, expression of spinal GTs could also be affected after nerve injury and might contribute to neuropathic pain.[8] In chronic constriction nerve injury (CCI), changes in the expression and glutamate uptake activity of spinal GTs have been observed using immunohistochemistry, western blot, glutamate uptake assays, and pharmacological evaluation.[8,10] Furthermore, there is an ongoing effort to explore the cellular and molecular mechanisms regulating GT function and expression in the context of the pathogenesis of neuropathic pain.[9] It is now suspected that different kinds of neuropathic pain syndromes may have significantly different mechanisms.[11] However, little is known about the expression of GTs after spared nerve injury (SNI), a common model of neuropathic

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pain. The aim of this study is to compare changes in GTs' mRNA expression levels between two distinct models of peripheral neuropathic pain: CCI and SNI.

Materials and methods

Experiments were performed on adult male Wistar rats (180–220 g). Rats were housed in groups of three or four on 12 h light/dark cycle in a room with controlled temperature (22 ± 2 °C). Food and water were available *ad libitum*. Animals were subjected to behavioral and molecular experiments in groups (8 rats per group). All experiments followed the guidelines on ethical standards for investigation of experimental pain in animal.[12] Rats were anesthetized with ketamine–xylazine injected intraperitoneally (i.p). The chronic constrictive injury (CCI) was performed on the sciatic nerve as described earlier.[13] Briefly, the sciatic nerve was exposed at mid-thigh level by blunt dissection through the biceps femoris muscle. On one side, four lose ligatures (4.0 chromic gut) were tied around the nerve. Afterwards, the muscle and skin layers were sutured. The SNI was produced on the sciatic nerve as described earlier.[5,14] First, the sciatic nerve and its three terminal branches, the sural, common peroneal, and the tibial nerves, were exposed. The common peroneal and the tibial nerve were ligated tightly with 4.0 silk. The nerves distal to the ligature were sectioned and 2–4 mm of the nerve stump was removed. Muscle and skin layers were sutured. For the sham operation groups, the sciatic nerve was exposed and handled gently. No ligation was performed.

The behavioral experiments included thermal hyperalgesia and mechanical allodynia before surgery (day 0) 4, 7, and 14 days.[13]

Thermal hyperalgesia was evaluated by the radiant heat method. The rats were placed on an elevated Plexiglas cage and they were allowed 25 min to habituate to the new environment. For testing thermal hyperalgesia, we exposed the plantar surface of a rat's hindpaw to a beam of radiant heat through a transparent Plexiglas surface (Ugobasil, Italy). The paw withdrawal latency was measured repeatedly and averaged across at least three trials separated by 5–10 min intervals. This was done for both the injured (the left) and intact (the right) paw. The cutoff latency was set at 22 s to prevent tissue damage. The values for the left paw were subtracted from those for the right paw, and, if negative, the difference was considered to be a sign of hyperalgesia in the injured paw.[13]

For examining mechanical allodynia, we placed each rat on a metal mesh floor, covered it with a plastic box (20 × 20 × 18), and allowed it for 30 min to habituate. The mechanical stimulation resulting from the bending force of a Von Frey filament was applied to the plantar surface of each hindpaw (bending forces ranged from <2 to 60 g, Stolting Inc., Wood Dale, IL, USA). Each trial consisted of three applications of a Von Frey filament every 5 min, and the cutoff force was 60 g. The Von Frey filaments of increasing forces were delivered to the central region of the plantar surface (in the CCI model) and to the lateral region of the hindpaw (in the SNI model). The withdrawal of the paw in response to Von Frey filament stimulation in at least two out of the three trials was considered a positive result.[11]

We used reverse transcription polymerase chain reaction (RT-PCR) to study mRNA expression of spinal neurons GTs 4 and 14 postoperative days when behaviors of pain were at minimum and maximum levels, respectively. Animals were killed using a CO_2 chamber. Fresh tissue samples of the whole spinal cord were used for the experiments. Spinal cords were removed as quickly as possible. Total RNA was extracted from the spinal cord by using extraction reagent following the manufacturer's protocol (RNX–plus, CinnaGen Inc., Tehran, Iran). cDNA was synthesized using 5 μg RNA with 1 μg (0.5 μg) oligo (dT)18 (Fermentas) in 10 μl DEPC water. The RNA was denatured at 70 °C for 5 min and then placed on ice to cool before adding 4 μl RT Buffer 5 ×, 2 μl dNTP 10 mM, and 0.5 μl RNase inhibitor (CinnaGen). This RNA solution was then incubated at 37 °C for 5 min and later 1 μl (200u) M-MuLV was added. The RNA was then kept at 42 °C for 60 min and the cDNA obtained was heated to 70 °C for 10 min to inactivate the M-MuLV enzyme. The final cDNA was amplified using PCR. All reactions in the PCR were performed in a thermocycler (Techne, UK) using 1.5 μl cDNA, 2.5 μl Taq DNA polymerase buffer, 0.8 μl $MgCl_2$, 0.3 μl Taq DNA polymerase, 1 μl dNTP 10 mM, 0.5 μl forward primer (20 pmol ml^{-1}), 0.5 μl reverse primer (20 pmol ml^{-1}) and 17.9 μl water (25 μl volumes) (primers are listed in Table 1). The DNA was denatured at 94 °C for 5 min before PCR cycling. The amplification protocol was: 45 s of denaturation at 94 °C, 45 s annealing at 53 °C, a 45 s extension at 72 °C, and a final 5 min extension at 72 °C, to optimize ligation conditions for β-actin. The annealing

Table 1 PCR primer sets and predicted product size

Genes	*Expected PCR product size (bp)*	*Sequence*	*Gene Bank accession nos.*	*Position*
GLAST	653	F.5′-TCT TGG TTT CGC TGT CTG CCA CG-3′	NM-019225	1830-1808
		R.5′-TCC TCA TTC ATG CCG TCA TCG TCC-3′		1177-1200
GLT1	326	F. 5′-ATG TCT TCG TGC ATT CGT TGT TGG G-3′	NM-017215	1649-1625
		R.5′-AGC CGT GGC AGC CAT CTT CAT AGC-3′		1323-1346
EAAC1	209	F.5′-GAC TGG GAA ATA TTC CGC AAG T-3′	NM-013032	909-930
		R.5′-CGC ACA GCG GAA TGT AAC TGG-3′		1118-1098
β-Actin	849	F.5′-GAA GTA CCC CAT TGA ACA CG-3′	NM-031144	282-301
		R.5′-GAC AGT GAG GCC AGG ATA GA-3′		1130-1111

Abbreviations: EAAC, excitatory amino-acid carrier; GLAST, glutamte aspartate transporter; GLT, glutamte transporter; PCR, polymerase chain reaction.

temperature for the GLT1, GLAST, and EAAC1 primers was 55 °C.[15] Amplified PCR products were analyzed by electrophoresis on 1.5% agarose gels. The primer sets and the expected sizes of PCR products are listed in Table 1.

Data from different experimental groups were analyzed using one-way analysis of variance and Tukey HSD *post hoc*, one independent sample *t*-test, two independent samples *t*-test, and paired sample *t*-test. Data are presented as mean ± s.e.m., and all values $P<0.05$ are considered significant.

Results

Animals that had undergone either CCI or SNI displayed hypersensitivity in the hindpaw ipsilateral to the nerve injury. Hypersensitivity began soon after the injury (postoperative day 4), and it peaked at 14 days after injury. In the CCI group, there was hypersensitivity to innocuous mechanical Von Frey filament stimulation on postoperative day 4. The difference in mechanical allodynia between the CCI and sham groups was significant postoperative days 7 and 14 ($P<0.001$) (Figure 1a). In the SNI group, we also observed hypersensitivity to innocuous mechanical Von Frey filament stimulation postoperative day 4. The difference in mechanical allodynia between the SNI and sham groups was significant postoperative days 4 ($P<0.05$), 7, and 14 ($P<0.001$) (Figure 1b). Comparison of CCI rats vs SNI rats did not reveal a significant difference in mechanical allodynia (Figure 1c).

As regards thermal hyperalgesia, the CCI group showed hypersensitivity postoperative day 4. There were significant differences between the CCI and sham groups on postoperative days 4, 7, and 14 ($P<0.001$) (Figure 2a). The SNI group also showed hypersensitivity to thermal hyperalgesia on postoperative day 4. Furthermore, there were significant differences between the SNI and sham groups on postoperative days 7 and 14 ($P<0.001$) (Figure 2b). There were also significant differences in thermal hyperalgesia between the CCI and SNI groups on postoperative days 4, 7, and 14 ($P<0.01$ and $P<0.001$) (Figure 2c). Across all postoperative days, thermal hyperalgesia was more severe in the CCI than the SNI groups.

The molecular results showed that the CCI and SNI treatments induced upregulation of three GTs by postoperative day 4. This was followed by a downregulation of all GTs in the CCI animals, and the downregulation of GLAST and GLT1 in the SNI animals, as measured on postoperative day 14.

Our molecular study of CCI by RT-PCR showed significant changes in the expression of spinal GTs (GLAST, GLT1, and EAAC1) (Figures 3a and 4). The results indicated an upregulation of the three transporters on postoperative day 4 ($P<0.01$, $P<0.001$), followed by a downregulation of these same transporters on postoperative day 14 ($P<0.05$). Molecular study of SNI by RT-PCR showed significant changes in the expression of spinal GTs (GLAST, GLT1, and EAAC1) (Figures 3b and 4). The results indicated an upregulation of the three transporters on postoperative day 4 ($P<0.05$, $P<0.001$), which was followed by the downregulation of GLAST and GLT1 on postoperative day 14 ($P<0.05$). Interestingly, there was no significant downregulation of

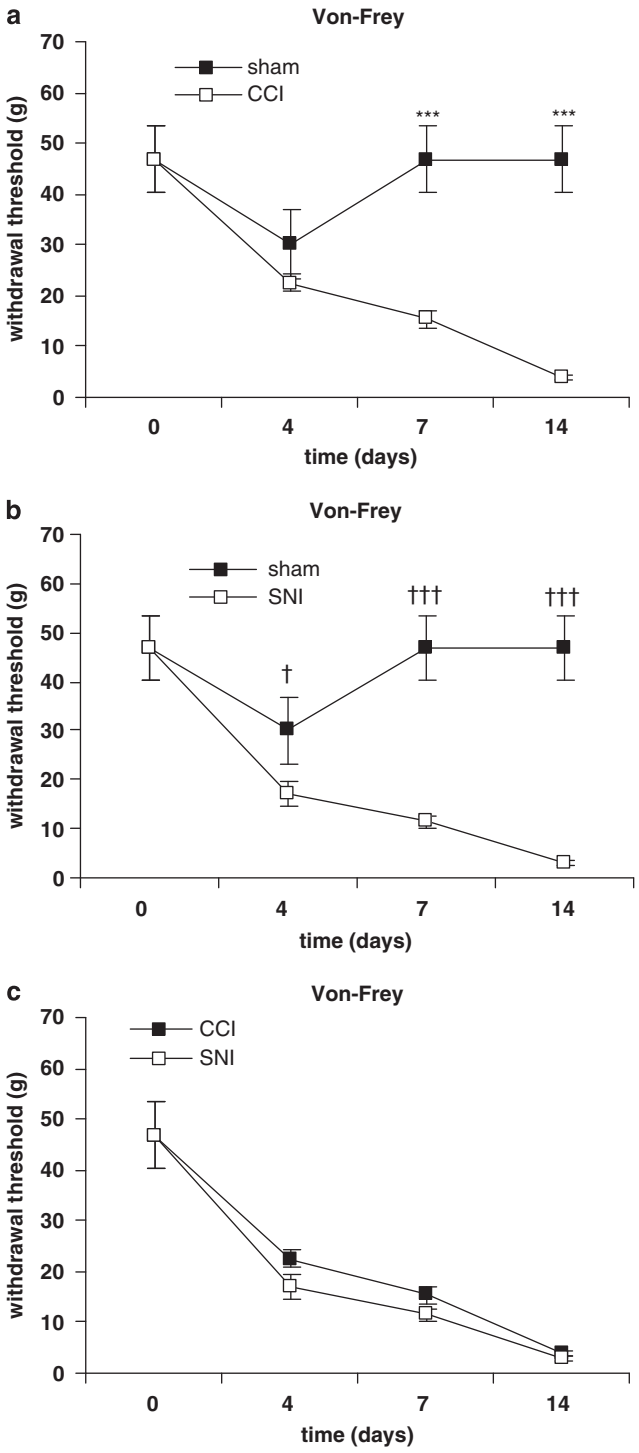

Figure 1 Paw withdrawal threshold (g) with stimuli applied by a series of Von Frey filament. (**a**) Mechanical allodynia in CCI rats vs sham animals. The operated animals showed a significant decline in the withdrawal threshold. (**b**) Mechanical allodynia in SNI rats vs sham animals. (**c**) Mechanical allodynia in CCI rats vs SNI rats. Comparison of CCI rats vs SNI rats did not reveal a significant difference. The operated animals showed a significant decline in withdrawal threshold. $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$, $^{***}P<0.001$.

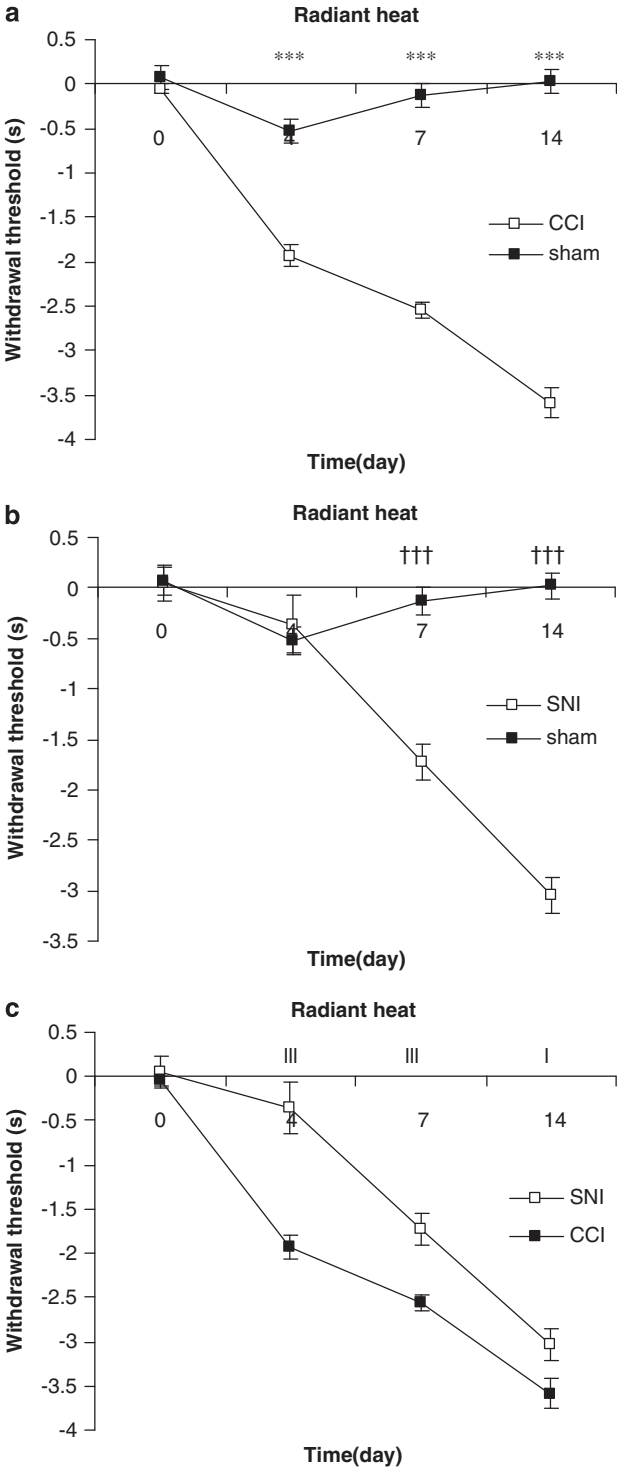

Figure 2 Heat hyperalgesia measured by noxious radiant heat withdrawal reflex at different days after injury (difference of L (left)−R (right paw) = withdrawal latency difference of two paws). Negative differences (below the baseline) indicate a lowered nociceptive threshold on the animals. (**a**) Heat hyperalgesia in CCI rats vs sham animals. The operation led to a significantly increased latency in the 4, 7, and 14 days. (**b**) Heat hyperalgesia in SNI rats vs sham animals. The operation led to a significantly increased latency on 7 and 14 days. (**c**) Heat hyperalgesia in CCI rats vs SNI rats. Comparison of CCI rats vs SNI rats reveals significant difference between the modalities at 4, 7, and 14 days. $^{\dagger\dagger\dagger}P<0.001$, $^{***}P<0.001$, $^{III}P<0.001$ and $^{I}P<0.01$.

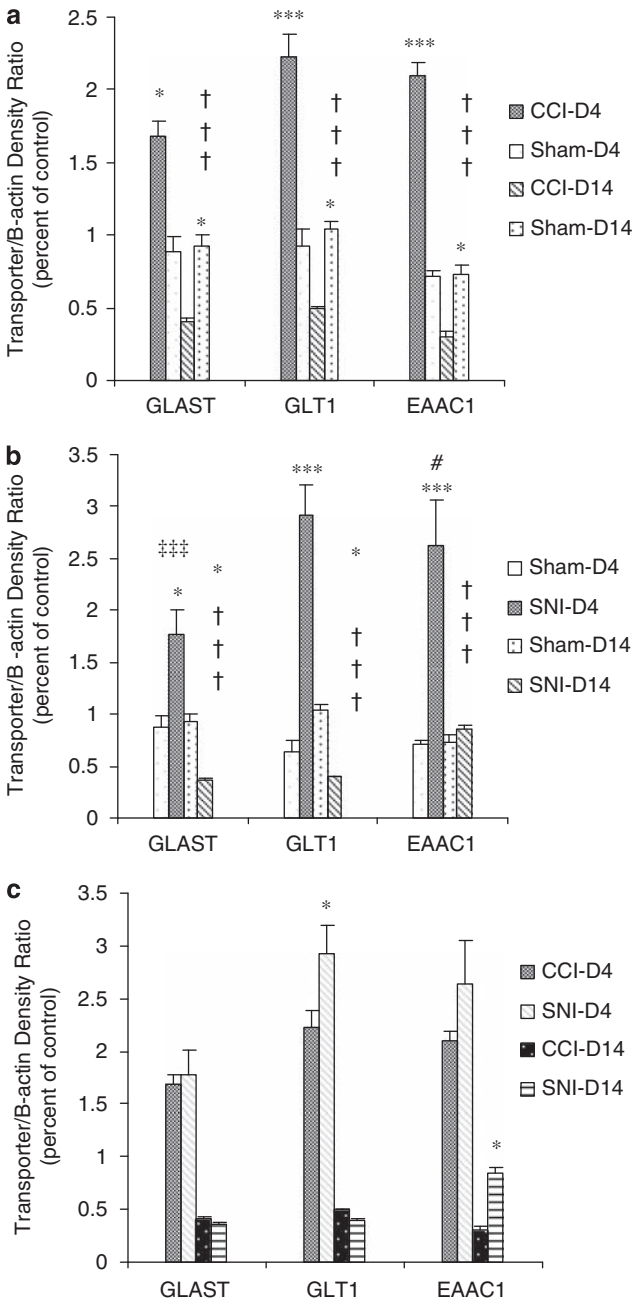

Figure 3 Comparative analysis of glutamate transporters expression (GLAST, GLT1, and EAAC1) in rat spinal cord by RT-PCR. (**a**) The expression of GTs in CCI rats vs sham animals at 4 and 14 days after operation. (**b**) The expression of GTs in SNI rats vs sham animals at 4 and 14 days after operation. (**c**) The GTs expression of CCI rats vs SNI rats. $^{*}P<0.05$ GLAST-D4 vs sham rats, $^{***}P<0.001$ EAAC1- D4 and GLT1-D4 vs their sham rats, $^{\dagger\dagger\dagger}P<0.001$ GTs -D4 vs GTs- D14, $^{\#}P<0.05$ GLAST-D4 vs EAAC1-D4, $^{\ddagger\ddagger\ddagger}P<0.001$ GLAST-D4 vs GLT1-D4.

EAAC1 at postoperative 14 in this experimental group. Comparison between the SNI and CCI groups of the expression levels of the three GTs showed significant differences in GLT1 and EAAC1 on postoperative days 4 and 14, respectively ($P<0.05$) (Figures 3c and 4).

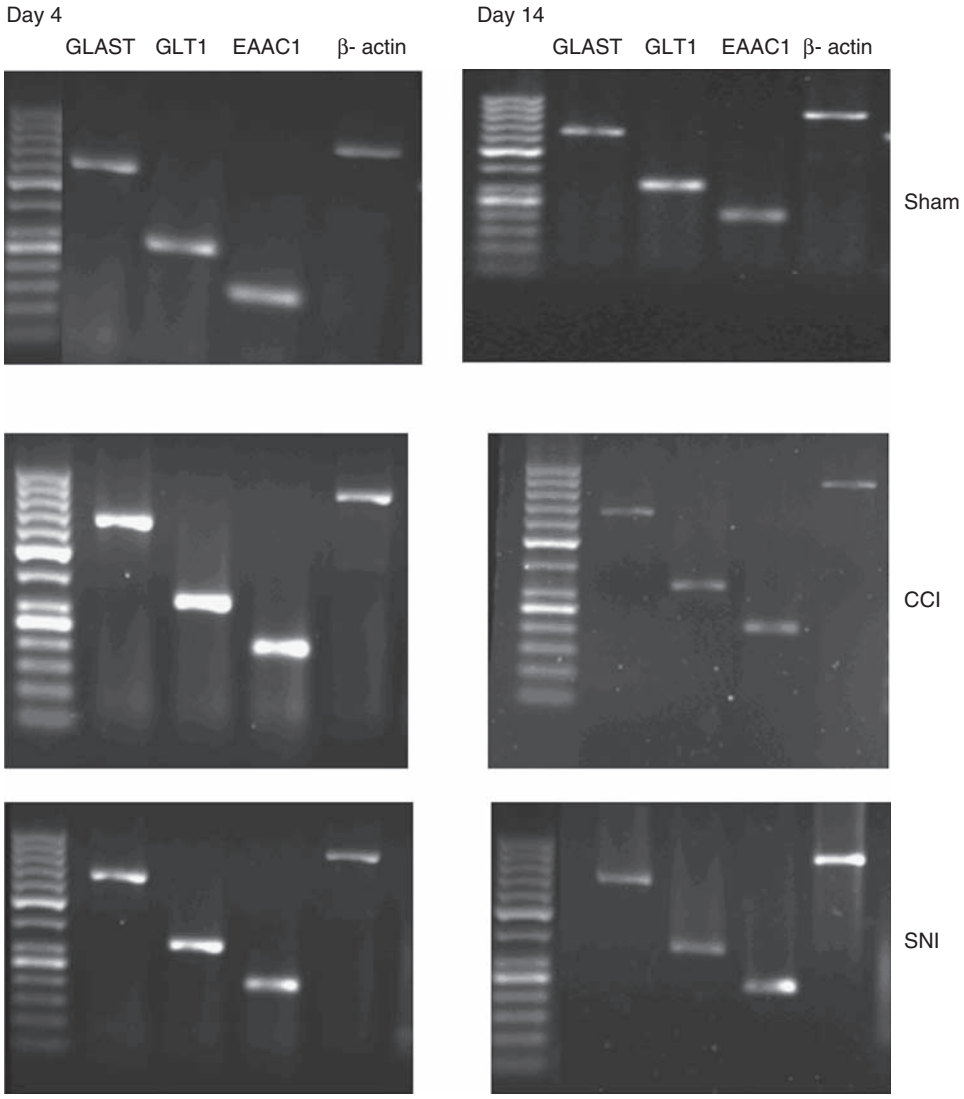

Figure 4 Expression of glutamate transporters (GLAST, GLT1, and EAAC1) and β-actin in the rat spinal cord following SNI and CCI at 4 and 14 days after operation. Electrophoretic analysis of RT-PCR products was performed on a 1.5% agarose gel stained with ethidum bromide. β-Actin is for loading control.

Discussion

This study reveals that both behavioral responses and expression of spinal GTs (EAAC1, GLAST and GLT1) are changed after the induction of peripheral nerve injury. We also find evidence of the existence of an inverse correlation between pain and the expression levels of GTs.

In our study, we focused on two models of peripheral nerve injury: SNI and CCI. In both models, behavioral responses showed the occurrence of thermal hyperalgesia and mechanical allodynia, although the models differed somewhat in their specific behavioral responses. In the SNI and CCI models, both thermal hyperalgesia and mechanical allodynia started on postoperative day 4 and lasted up to day 14. However, we saw a greater degree of allodynia in the SNI model, and a greater degree of hyperalgesia in the CCI model. Earlier study mentioned that myelin degeneration can consider hyperalgesia and allodynia as important factors during neuropathic pain. Also, myelinated peripheral nerve fibers degeneration elicits the release of several pronociception factors.[1]

The RT-PCR studies showed changes in the expression of GTs in CCI and SNI neuropathies. In both models, we found a significant increase in GT mRNA expression on postoperative day 4, followed by a reduction in mRNA expression for all three GTs in CCI, and for GLAST and GLT1 in SNI (postoperative day 14). There were no significant changes in the mRNA levels for EAAC1 in SNI on postoperative day 14. However, there were significant differences between CCI and SNI in GLT1 mRNA levels on postoperative day 4 and in EAAC1 mRNA levels on postoperative day 14.

Mechanical allodynia has also been observed 3 days post-CCI neuropathy, but no thermal hyperalgesia was described in these studies.[11] Other studies have found mechanical allodynia by day 2 post-CCI neuropathy, and thermal hyperalgesia by days 2–12 post-CCI neuropathy, reaching a

maximum in the second week and lasting up to 8 weeks.[3] It is believed that maximum thermal hyperalgesia occurs on post-CCI days 7–14.[16] Our results showed mechanical allodynia and thermal hyperalgesia and mechanical allodynia on post-CCI day 14. Mechanical allodynia and thermal hyperalgesia have also been observed in the SNI model.[5] The CCI model showed behavioral changes that were similar to neuropathic syndromes in humans, including mechanical allodynia on postoperative days 4–15.[13] Thus, it has been suggested that hypersensitivity of uninjured afferent fibers is involved in the production of mechanical allodynia.[14] Studies of SNI models showed that mechanical allodynia began earlier than thermal hyperalgesia.[5] Other studies showed that thermal hyperalgesia was accompanied by a reduction in the density of myelinated fibers.[17] Production of allodynia depends on A_β fibers, as blocking these fibers results in the disappearance of allodynia.[18] Central sensitization may also be the outcome of a large variety of changes in the CNS at both the molecular and circuit levels. When glutamate is released from primary afferent neurons, it binds to the AMPA and NMDA receptors, allowing for an influx of calcium into the post-synaptic neuron. This initiates cascades of biochemical processes, including the induction of gene expression, which contribute to the strengthening of synapses and central sensitization.[19]

We explored the molecular mechanisms of GTs function and expression in relation to the pathogenesis of neuropathic pain. The GT system is the main mechanism for removal of synaptically released glutamate, and the maintenance of glutamate homeostasis. Although GTs have a crucial function in protecting neurons from excitotoxicity by exogenous and endogenous glutamate, however, the exact function of GTs in pathological pain is not completely understood.[8,9] In this study, we showed the function of the three spinal GTs during pathological pain and the correlation of this function with GTs expression in the spinal cord. We found significant upregulation of GTs expression on postoperative day 4 in both CCI and SNI models. The results also showed a significant downregulation of GLAST, GLT1, and EAAC1 in the CCI model and of GLAST and GLT1 in the SNI model on postoperative day 14.

Earlier studies using western blots showed that CCI induced an initial upregulation in GLAST, GLT1, and EAAC1 expression within the ipsilateral dorsal horn of the spinal cord. This upregulation took place primarily on the first postoperative days and was then followed by a downregulation on postoperative days 4–14.[8] Similar studies, also using western blots, showed expression of the GTs GLAST (EAAT1), GLT1 (EAAT2), and EAAT1 (EAAT3) in the rostral, caudal, and epicenter areas of the spinal cord, 1 and 6 h after spinal cord injury (SCI). This expression gradually decreased after 24 h.[10] There were no changes in the expression of EAAT1, EAAT2, and EAAT3 proteins in an oxidative stress model.[20] It has been reported that nociceptive input can cause induction of GLT1 transcription.[9] Our results also showed the downregulation of GT expression concurrent with the appearance of hyperalgesia. Most earlier studies have also emphasized that neuropathic pain can be generated by a reduction in the expression of GTs.[8] Given that there is an inverse correlation between pain responses and expression of GTs, it is possible that changes in the expression of spinal GTs can have a critical function in both the induction and maintenance of neuropathic pain after nerve injury.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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