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Neuropathic pain and temporal expression of preprodynorphin, protein kinase C and N-methyl-D-aspartate receptor subunits after spinal cord injury

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ABSTRACT

Central neuropathic pain is refractory to conventional treatment and thus remains a therapeutic challenge. In this work, we used a well-recognized model of central neuropathic pain to evaluate time-dependent expression of preprodynorphin (ppD), protein kinase C gamma (PKCγ) and NMDA receptor (NMDAR) subunits NR1, NR2A and NR2B, all critical players in nociceptive processing at the spinal level. Male Sprague-Dawley rats were subjected to spinal hemisection at T13 level and sham-operated rats were included as control animals. The development of hindpaw mechanical allodynia was assessed using the von Frey filaments test. Real time RT-PCR was employed to determine the relative mRNA levels of NMDAR subunits, ppD and PKCγ in the dorsal spinal cord 1, 14 and 28 days after injury. Our results show that, coincident with the allodynic phase after injury, there was a strong up-regulation of the mRNAs coding for ppD, PKCγ and NMDAR subunits in the dorsal spinal cord caudal to the injury site. The present study provides further evidence that these molecules are involved in the development/maintenance of central neuropathic pain and thus could be the target of therapeutic approaches.

Spinal cord injury is a devastating condition that results not only in the loss of function below the level of the lesion, but also in the development of central neuropathic pain in up to 40% of patients [1]. Neuropathic pain is characterized by altered sensory perception that includes allodynia (painful responses to non-noxious stimuli) and hyperalgesia (exaggerated responses to noxious stimuli). In the majority of these patients, chronic pain is refractory to conventional medical management [3].

Although the precise mechanism of chronic pain after spinal cord injury remains elusive, considerable evidence indicates that N-methyl-D-aspartate receptors (NMDAR) play a critical role in nociceptive processing at spinal level. Behavioral and electrophysiological studies demonstrate that either the blockade or the knock-down of spinal NMDAR inhibits both the allodynic and the hyperalgesic responses after central and peripheral injuries [4,6,11].

The functional NMDAR is a heteromeric complex containing NR1 and NR2 subunits. The NR1 protein is an obligatory component for a functional NMDAR, with at least one of the NR2 subunit family member, of which NR2A and NR2B are the most abundant in adult rat dorsal horn [23]. Previous studies have found that diabetes [31] and excitotoxic injury [8] can induce changes in the expression of NMDAR subunits in the spinal cord contributing to abnormal pain processing.

In addition, dynorphin and the gamma (γ) isoform of the protein kinase C (PKCγ) have been proposed as key players in neuropathic pain signaling by enhancing NMDAR-mediated sensory processing. Dynorphin, an endogenous opioid peptide, was originally identified as a ligand for the kappa opioid receptor with analgesic properties, but it was later found to be required for the maintenance of neuropathic pain [18]. This switch from antinociceptive to pronociceptive properties has been related to non-opioid mediated mechanisms that either directly or indirectly activate the NMDAR [18]. Interestingly, several studies have provided evidence that PKCγ, a crucial mediator of persistent pain behaviors [16,21], enhances the expression of NMDAR and mediates the phosphorylation of the NR1 subunit [30], contributing to central sensitization.

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Thus, all these molecules have been involved in the increased excitatory tone in the spinal cord after peripheral injury, and related to abnormal pain processing and functional motor deficits after spinal contusion or excitotoxic damage [5,8,10,13,16,26]. However, the spinal cord hemisection model, a well-recognized model of central chronic pain [9], has never been used to explore whether the development of neuropathic pain-related behaviors is associated with time-dependent changes in the expression of these molecules.

In order to test this hypothesis, we assessed the development of paw mechanical allodynia and the time-course expression of mRNAs coding for NMDAR subunits NR1, NR2A and NR2B, the dynorphin-precursor preprodynorphin (ppD) and PKCγ in rats subjected to spinal hemisection. Since neuropathic pain after spinal cord injury is the most prevalent and difficult to treat, the contribution of different experimental models may help to understand the complex mechanisms underlying the development/maintenance of chronic pain.

All experimental procedures were reviewed by the local Animal Care and Use Committee (Assurance Certificate N A5072-01) and followed the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Care was taken to minimize animal discomfort and to limit the number of animals used.

Male Sprague-Dawley rats ($n = 27$, 200–220 g) were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.). The T13 spinal cord segment was unilaterally hemisected in a group of rats ($n = 20$), as originally described by Christensen et al. [9]. Post-operative care included control of body temperature and antibiotic administration [17]. Animals with contralateral hindlimb impairment were excluded from the study. Sham-operated rats ($n = 7$) were used as control animals (CTL).

Mechanical sensitivity was assessed with von Frey hairs (Stoelting, WoodDale, IL, USA) [22]. The lowest force at which application elicited a paw withdrawal was taken as the mechanical response threshold. A paw withdrawal reflex obtained with 6 g or less was considered an allodynic response. Mechanical threshold was determined in all animals before surgery (day 0) and at day 1 after surgery and weekly thereafter in CTL animals and those allowed to survive for 14 or 28 days after injury. Results were analyzed using the Friedman repeated measures of analysis of variance followed by multiple comparison test.

After behavioral assessment, animals were deeply anesthetized with chloral hydrate (400 mg/kg) and decapitated. Spinal lumbar segments caudal to the injury site (L3–L5) were immediately removed and the dorsal spinal cord was dissected by cutting through the central canal. Tissues were frozen and stored at −70 °C until gene expression studies were performed. Samples from different experimental groups were run at the same time.

RNA was extracted using TRIzol (Invitrogen, USA) method and subjected to DNAse 1 (Invitrogen) treatment, as previously described [17]. Reverse transcription was performed from 2 μg of total RNA using a SuperScript II Rnase H reverse transcriptase kit (Invitrogen) for 1 h at 42 °C in the presence of random hexamer primers (Promega).

Nucleotide sequences of forward (F) and reverse (R) primers used were as follows: NR1: F: AGA TGG CCC TGT CAG TGT GT, R: GTG AAG TGG TCG TTG GGA GT [2]; NR2A: F: GTG ATC GTG CTG AAC AAG GA, R: GCT CGC AGT CAG AAA AGG AC [2]; NR2B: F: TCC GAA GCT GGT GAT AAT CC, R: TGG TCA TCC TCT TGC TCC TC [2]; PKCγ: F: AGC CTC CTC CAG AAG TTT GGG, R: CCT TTC CCT AGA ACC ATG AGG [27]; ppD: F: GGG TTC GCT GGA TTC AAA TA, R: TGT GTG GAG AGG GAC ACT CA [15]. Cyclophilin (Cyc) (F: GTG GCA AGA TCG AAG TGG AGA AAC, R: TAA AAA TCA GGC CTG TGG AAT GTG; accession number: NM_022536) was chosen as housekeeping gene and designed using the Oligo Primer Analysis software version 6.54 (Molecular Biology Insights Inc., Cascade, Co). Relative gene expression was determined using Syber green master mix and the ABI PRISM 7500 sequence detection system (Applied Biosystems, USA). The change in the target mRNA was calculated using the $2^{-(\Delta\Delta Ct)}$ method [20] and expressed as fold-increase relative to CTL.

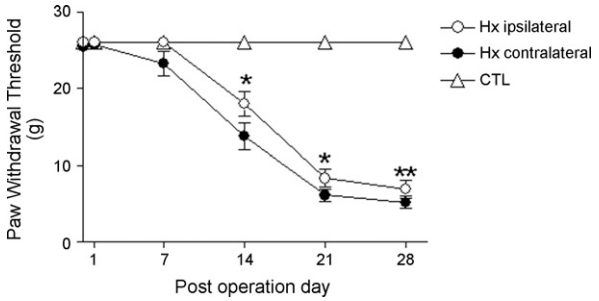

Fig. 1. Paw withdrawal thresholds observed in hemisected rats after mechanical stimulation of both ipsilateral and contralateral hindpaws at different times after injury. The mechanical response of control animals is also shown. The spinal cord injury induced a significant decrease in paw withdrawal threshold in both hindpaws, 14 days after performing the lesion. Nociceptive responses in the allodynic range were detected on days 21–28. Values show mean ± S.E.M ($n = 27$, $n = 6$–7 rats/group). Only statistically significant differences between hemisected and control animals are stated in the graph, using the following symbols to represent p values: * $p < 0.05$; ** $p < 0.01$.

Linearity and efficiency of PCR amplification were validated before quantification as previously described [17]. The correlation coefficients (r) (r: Cyc: 0.997; NR1: 0.991; NR2A: 0.999; NR2B: 0.995; PKCγ: 0.996; ppD: 0.993), and the PCR efficiency values (Ex) (Ex: Cyc: 100.08; NR1: 94.17; NR2A: 91.63; NR2B: 100.92; PKCγ: 99.25; ppD: 110.93, calculated as $Ex = (10^{-1/\text{slope}}) - 1 \times 100$ [25]), allowed an accurate quantification of targeted genes mRNAs. The specificity of PCR amplification and the absence of dimmer formation were confirmed by melting curve analysis and controlled with high resolution gel electrophoresis.

PCR was performed using 2–4 ng cDNA/μl of reaction under optimised conditions: 95 °C at 10 min followed by 40 cycles at 95 °C for 0.15 s and 60 °C for 1 min. Primers were used in a 0.5 μM final concentration. Samples were run in triplicate and statistical comparisons were made with the use of one-way analysis of variance (ANOVA) and Newmann-Keuls test for post-hoc comparisons. When appropriate, the Pearson (r) or Spearman (rs) correlation coefficients were calculated to test the relationship between the data expressions and/or the behavioral measurements.

As seen in Fig. 1, 14 days after spinal hemisection, all animals displayed a significant decrease in paw withdrawal threshold in both the ipsilateral and contralateral hindlimbs as compared to pre-operative values ($p < 0.05$, Fig. 1). Mechanical responses in the allodynic range (to filaments of 6 g or less) were well-established 21–28 days after surgery ($p < 0.05$ for both hindlimbs vs. CTL). In those animals, a bilateral enhancement of nocifensive and shaking behaviors was observed, indicative of the development of a chronic pain state [9].

Real time RT-PCR was employed to evaluate the relative gene expression for NMDAR subunits, ppD and PKCγ in the caudal dorsal cord of hemisected animals and in an equivalent spinal region of CTL rats, at different time-points after behavioral analysis (i.e. 1, 14 and 28 days after injury). As shown in Fig. 2A, a dramatic down-regulation of NR1 transcription was observed at day 1 and maintained up to 14 days after injury as compared to CTL animals (in both cases: $p < 0.01$ vs. CTL). At those time-points, however, the levels of NR2A and NR2B transcripts remained unchanged and equal to CTL values (Fig. 2B and C). Interestingly, 28 days after injury, mRNA levels of the three major NMDAR subunits exhibited a marked and significant increase in the dorsal spinal cord of the

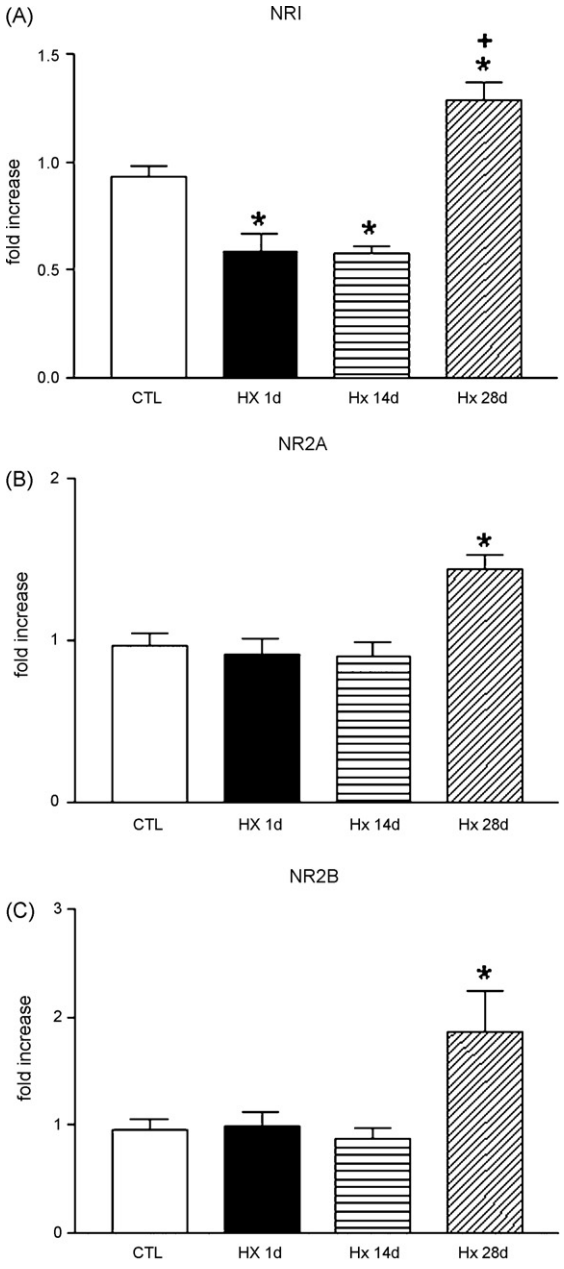

Fig. 2. Expression of (A) NR1, (B) NR2A and (C) NR2B mRNAs in the rat dorsal spinal cord 1, 14 and 28 days after spinal hemisection. NR1 mRNA expression was reduced 1 and 14 days after injury (0.5-fold decrease relative to CTL, $p < 0.01$ in both cases) but exhibited a significant increase 28 days after injury (*$p < 0.01$ vs. CTL; +$p < 0.001$ vs. Hx 1 d and Hx 14 d). NR2A and NR2B only exhibited a significant increase 28 days after injury (*$p < 0.01$ vs. CTL and other time points). Data represent fold-increase relative to CTL levels (mean ± S.E.M.; $n = 6–7$ rats/group). Statistical analysis was made with the use of one-way analysis of variance (ANOVA) and Newmann-Keuls test for post-hoc comparisons. CTL: control, Hx: spinal cord hemisection.

animals showing the presence of well-established neuropathic signs (NR1, NR2A and NR2B: $p < 0.01$ vs. CTL; Fig. 2 A–C).

No changes in PKCγ mRNA levels of expression were detected in the dorsal spinal cord 1 or 14 days after spinal hemisection (Fig. 3A). However, we found a significant increase in PKCγ transcripts 28 days after the lesion, a time-point coincident with the over-expression of the NMDAR subunits and the presence of robust tactile allodynia ($p < 0.05$ vs. CTL and other time-points; Fig. 3A).

Linear correlation analysis showed that the expression of NR2A, NR2B and PKCγ exhibited a significant positive inter-relationship ($r_{NR2A/NR2B} = 0.690$; $r_{NR2A/PKC\gamma} = 0.868$; $r_{PKC\gamma/NR2B} = 0.768$; $p < 0.001$ in all cases). In addition, we found a significant correlation between the behavioral measurements and the expression of NR2A, NR2B and PKCγ (rs: NR2A = −0.609; NR2B = −0.592; PKCγ = −0.468; $p < 0.05$ in all cases).

The pattern of ppD expression was quite different (Fig. 3B) and showed no significant covariation with the expression of the other molecules ($p > 0.8$). As early as 1 day after injury, ppD mRNA levels showed an acute and significant increase ($p < 0.05$ vs. CTL), that was maintained 14 days after the lesion ($p < 0.05$ vs. CTL). Twenty eight days after unilateral hemisection, this increase in ppD mRNA levels was still detected in all animals with enhanced pain sensitivity ($p < 0.05$ vs. CTL, Fig. 3B).

Despite the prevalence of pain from direct damage to the cord, it has been less explored than pain resulting from injury to peripheral nerves or tissues [32]. The spinal dorsal horn is the first relay site at which incoming sensory and nociceptive signals undergo convergence and modulation. Thus, after injury, hyperexcitability of dorsal horn neurons may contribute to the development of central neuropathic pain.

The present study shows that spinal hemisection induces time-dependent changes in the expression of ppD, PKCγ and the three major NMDAR subunits in the dorsal spinal cord below the injury site. Furthermore, the expression of NR2A, NR2B and PKCγ showed a significant correlation with pain behavior, suggesting a relationship between their altered expression and pain processing in this neuropathic model. The present data gives further information about the molecular mechanisms underlying the development/maintenance of central pain after direct damage to the spinal cord [14].

Although further studies should be carried out in order to evaluate protein expression and post translational modifications of ppD, PKCγ and NMDAR subunits, we would like to suggest a sequence of events that could explain their contribution to injury induced allodynia in the spinal hemisection model.

Spinal hemisection induced an acute and marked decrease in NR1 subunit expression in the dorsal spinal cord that lasted up to 14 days after injury. NR1 down-regulation has been suggested to represent either a potential neuroprotective mechanism against over stimulation of glutamate receptors or to be related to neuronal death [5,12]. Since we did not observe altered levels of NR2A and NR2B transcripts, the major co-assembly subunits in the dorsal spinal neurons, the strong decrease in NR1 likely represents a transient desensitization of the spinal neurons anti-correlated with the excess of glutamate released after the lesion [12]. It is also worth mentioning that even at low levels of NR1 transcription animals exhibited mild tactile hypersensitivity 14 days after injury. Therefore, other algesic factors may be enhancing spinal sensitization at this time-period, as it has been suggested for mice presenting a partial deletion of the NR1 subunit [6].

In the chronic phase after spinal cord injury (i.e. 28 days), the significant increase observed in the three major NMDAR subunits. mRNAs was coincident with a well-established allodynic state and suggests an enhancement of glutamate transmission related to the maintenance of chronic pain. In addition, NMDAR activation could be enhancing the expression of several genes associated with inflammation, as reported after spinal contusion [24].

NR1 is essential for NMDAR activity and interaction with NR2A and NR2B subunits confers functional variability. In particular, blocking the activity of NR2B subunit with antagonists [29], vaccination strategies [33] or small interfering RNAs [28] prevents the development of neuropathic pain. Recently, morphine exposure [19] and diabetes [31] have been shown to induce an up-regulation of NMDAR subunits within the spinal dorsal horn, contributing

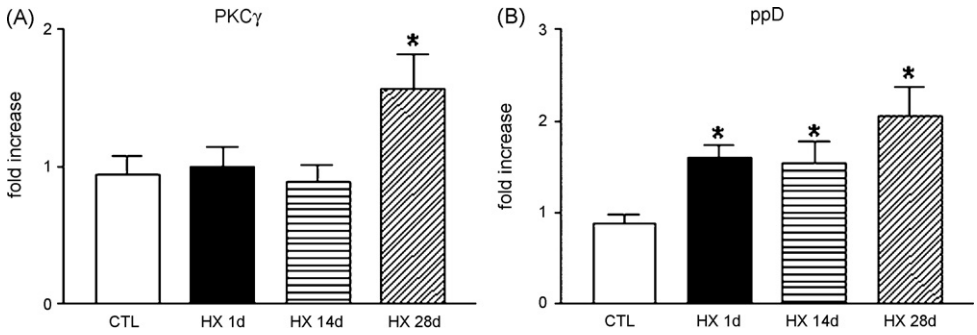

Fig. 3. (A) PKCγ and (B) preprodynorphin (ppD) mRNAs expression in the rat dorsal horn 1, 14 and 28 days after spinal cord hemisection. No changes in PKCγ mRNA levels of expression were detected in the dorsal spinal cord 1 or 14 days after spinal hemisection but a significant increase in PKCγ transcripts was observed 28 days after the lesion (*$p < 0.05$ vs. CTL and other time-points). One day after injury, ppD mRNA levels showed a significant increase ($p < 0.05$ vs. CTL), that was maintained 14–28 days after the lesion (*$p < 0.05$ vs. CTL, in both cases). Data represent fold-increase relative to CTL levels (mean ± S.E.M.; $n = 6–7$ rats/group). Statistical comparisons were performed by one-way ANOVA, followed by Newman-Keuls post-hoc test. CTL: control, Hx: spinal cord hemisection.

to reinforce the role of NMDAR in pain and tolerance mechanisms.

Regarding ppD, we found an early and significant increase in its expression in the pre-allodynic period that was maintained over time. This steady pattern of expression could explain its lack of covariation with the other molecules. Our results agree with previous observations showing that several chronic pain states are associated with increased levels of ppD in the spinal cord [18] and are in accordance with the mechanism proposed to explain the paradox of dynorphin actions [7,18]. Immediately after injury, the increased levels of dynorphin could be binding to opioid receptors in order to suppress nociceptive input [7]. However, if the release of dynorphin continues, it could enhance the activity of NMDAR and increase nociceptive hypersensitivity [7]. Accordingly, the increased levels of ppD mRNA observed in the chronic phase after spinal hemisection, coincident with the enhanced transcription of NMDAR subunits, could result in the stimulation of receptor activity and the induction of pain-related behaviors.

Activation of NMDAR is known to stimulate activation of PKCγ which in turns triggers NR1 phosphorylation [30], increasing NMDAR responsiveness to subsequent stimulation by glutamate. Interestingly, we found that PKCγ expression is up-regulated in the chronic but not in the early phase after injury. PKCγ is known to mediate the enhanced expression of NMDAR and the potentiation of NMDA-mediated currents that contribute to central sensitization. Furthermore, PKCγ knock-out mice fail to develop neuropathic pain syndromes [21]. Our findings strengthen the hypothesis of a relevant role of this enzyme in central neuropathic pain after spinal cord injury.

However, it must be emphasized that some of these molecules are expressed in a laminae-specific manner in the normal spinal cord and thus, may have a differential expression after spinal hemisection. Since we have analyzed the dorsal spinal cord as a whole, further single-cell real time RT-PCR, immunohistochemical and/or in situ hybridization studies will help to determine the contribution of the different dorsal horn layers to the mRNA changes observed in this study, as well as the cellular and subcellular localization of the different molecules.

In summary, we demonstrate time-dependent alterations in the expression of NMDAR subunits, PKCγ and ppD in the dorsal horn that could underlie the development/maintenance of mechanical allodynia after spinal hemisection. The uncovered sequence of events may be crucial for the molecular mechanisms that contribute to central neuropathic pain after spinal cord injury. Thus it could become a potential target for the development of new and more effective therapies for central chronic pain management.

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