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Expression of IL-1 β in supraspinal brain regions in rats with neuropathic pain

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Abstract

We examined mRNA expression of the pro-inflammatory cytokine IL-1 β in the brainstem, thalamus, and prefrontal cortex in two rat models of neuropathic pain. Rats received a neuropathic injury: spared nerve injury (SNI) or chronic constriction injury (CCI), sham injury, or were minimally handled (control). Neuropathic pain-like behavior was monitored by tracking tactile thresholds. SNI-injured animals showed a robust decrease in tactile thresholds of the injured foot, while CCI-injured animals did not show tactile threshold changes. Ten or 24 days after nerve injury, IL-1 β gene expression in the brain was determined by RT-PCR. IL-1 β expression changes were observed mainly at 10 days after injury in the SNI animals, contralateral to the injury side, with increased expression in the brainstem and prefrontal cortex. The results indicate that neuro-immune activation in neuropathic pain conditions includes supraspinal brain regions, suggesting cytokine modulation of supraspinal circuitry of pain in neuropathic conditions.

Keywords

Cytokines; chronic pain; brainstem; thalamus; prefrontal cortex

There is now convincing evidence that cytokine expression in the peripheral nervous system and in the spinal cord play a critical role in pain behavior in various models of neuropathic injury in rodents [1-3]. Accumulating evidence indicates that IL-1, a major pro-inflammatory cytokine, is involved in the modulation of nociceptive information. Most of the results reported derive from the administration of this cytokine either peripherally, intrathecally or directly into the brain, for review see [4]. Whether IL-1 induces analgesia or hyperalgesia when administered centrally seems to depend on the brain region involved and on the dose injected: lower doses cause hyperalgesia and higher doses are analgesic [5;6]. On the other hand, it has been recently reported that mice in which IL-1 signaling is impaired, show reduce basal pain sensitivity [7], and attenuated neuropathic pain behaviors by reducing mechanical allodynia and thermal hyperalgesia [8].

Increased levels of IL-1 have been observed in the cerebrospinal fluid of chronic pain patients [9]. Cytokine expression in pain states has rarely been studied above the spinal cord. Even

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though cytokine expression has been extensively implicated in inflammatory conditions, there is now emerging evidence for the role of IL-1 and IL-6 in normal synaptic processes as well [10;11], especially in modulating synaptic efficacy. There is also ample evidence that neuropathic pain behavior in rodents is accompanied with re-organization of peripheral and spinal cord nociceptive processing, giving rise to central sensitization [12]. Human brain imaging and cognitive assessment studies indicate that clinical chronic pain conditions, with a substantial neuropathic component, show cognitive abnormalities, and brain morphological and metabolic abnormalities [13-15]. We hypothesized that, at least a portion of these abnormalities, is a secondary supraspinal re-organization of nociceptive circuits mediated by supraspinal cytokine expression, in response to the peripheral and spinal cord re-organization documented in rodents following neuropathic injury. We tested the hypothesis of supraspinal cytokine expression in two models of neuropathic pain, spared nerve injury (SNI) [16] and chronic constriction injury (CCI) [17] that differ in the type and extent of peripheral injury, by examining IL-1 β gene expression in multiple brain regions. The specific choice of brain sites examined is partly motivated by the anatomy of nociceptive transmission, with the thalamus being the main termination site for spinothalamic pathway, and the brainstem containing structures for ascending nociceptive pathways, like the parabrachial nuclei, and important descending modulatory structures, like the periaqueductal grey. It is also motivated by the results of human brain imaging studies pointing to metabolic abnormalities and decreased grey matter density in the prefrontal cortex and thalamus of chronic back pain patients [14;15], as well as by observations showing that neuropathic pain behavior in CCI and SNI rats can be modified by manipulating prefrontal excitability [18]. We compare IL-1 β expression at two time-points after peripheral nerve injury, ipsilateral and contralateral to the peripheral injury. The time points are chosen to be delayed long enough from the date when injury was induced to minimize the contribution of tissue injury related inflammation to our findings as ascertained by tissue healing, and to demonstrate the dynamical changes in IL-1 β expression as a function of time.

Forty male Wistar Kyoto rats (250-350 grams) were used. All procedures were approved by the Animal Care and Use Committee (ACUC) at Northwestern University, Chicago, and were in accordance with the NIH for the ethical use of laboratory animals.

The rats were divided into five groups (n = 8 per group): 1) Control animals were not handled, and were only used for measuring IL-1 β expression in the brain; 2) CCI-sham animals; 3) CCI injury animals; 4) SNI-sham animals; 5) SNI injury animals. Groups 2-5 had sham or actual neuropathic injury on the left side, under anesthesia, and their tactile thresholds were monitored prior to and for 3 weeks post-injury.

Animals were anesthetized using ketamine hydrochloride (45 mg/kg, i.p.) and xylazine (10 mg/kg i.p.). After surgery, all wounds were sutured using a non-absorbable surgical suture, and treated with a topical antibiotic ointment. In CCI or CCI-sham animals, the left sciatic nerve was exposed above the level of trifurcation, and in CCI group four loose knots were carefully applied to the nerve using absorbable chromic gut [17], while in CCI-sham group the exposed sciatic nerve was not touched. In SNI or SNI-sham animals, the left sciatic nerve was exposed at the level of its trifurcation into the sural, tibial and common peroneal nerves, and for SNI group each of the tibial and common peroneal nerve was tightly ligated by two knots 4 mm apart using 6.0 silk and then completely severed in between, leaving the sural nerve intact [16], while in SNI-sham group the exposed sciatic nerve was not manipulated.

All operated animals (CCI, SNI and sham) were studied for mechanical thresholds. We decided to limit the behavioral assessment of signs for neuropathic pain only to this test because this procedure is minimally stressful, and unlike e.g. thermal sensitivity testing it cannot induce tissue injuries. Mechanical sensitivity of the hind paw was measured by determining

withdrawal thresholds to Von Frey filaments. All tests were performed on the right (uninjured) and left (injured) hind paws. The 50% threshold for each paw withdrawal was calculated as described by Chaplan et al. [19].

All animals were killed, perfused, and brain tissue extracted for RT-PCR analysis. Half the animals in groups 2-5, with half the control animals, were killed 10 days after neuropathic injury. The other half, together with the remaining control animals, was killed 24 days after injury. To minimize contamination of our measures with stress related IL-1 β changes, animals were not handled for at least 48 hours prior to being sacrificed. They were anesthetized with ketamine and xylazine, and perfused with RNA-ase free saline (Accugene PBS). The brain tissue was then dissected extracting: the upper brainstem, limited primarily to the mesencephalon; the thalamus/striatum separated from the cortex, hippocampus, and hypothalamus; and the prefrontal cortex, encompassing a coronal slice of the most anterior 3 mm of the brain, excluding the olfactory bulbs. The tissue was immediately frozen and stored at -70°C, until processed for the determination of IL-1 gene expression.

Total RNA was extracted from the different rat brain regions using TRIzol Reagent (Invitrogen Life Technologies) according to a standard protocol [20]. The RNA was treated with 2U DNaseI (Epicentre technologies) in 10x Buffer Y⁺/Tango (MBI Fermentas) followed by purification using RNeasy Mini Spin Columns (Qiagen) according to the manufacturer's instruction and eluted in 30 μ l RNase free water. Reverse transcription (RT) was performed from 1 μ g total RNA using 40U MMLV reverse transcriptase (Invitrogen Life Technologies) and 0,5mg/ml oligop(dT) 12-18 primer (Amersham Biosciences) in a total volume of 20 μ l. RT was performed at 42°C for 60 min and 70°C for 15 min. PCR was performed in a volume of 25 μ l with the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) using optical reaction tubes. A master mix was prepared containing 12,5 μ l 2x PCR buffer (100mM KCl, 20mM Tris HCl pH 8,3, 0,02mM EDTA, 0,1% gelatin, 0,02% Tween20), nucleotides dATP, dCTP, dGTP (200 μ M each), 400 μ M dUTP, 1,0 μ l 25mM MgCl₂, 0,625U AmpliTaqGold (PE Applied Biosystems), 0,25U Uracil-DNA-Glycosylase (UDG) (New England Biolabs), 200nM of each primer, 100nM of the corresponding probe, and Rox dye in a final concentration of 300nM (TIB MOLBIOL). 21 μ l of the master mix were added to each well of 96 well-plates followed by addition of 4 μ l cDNA. All PCR reactions were performed 2 to 4 times in duplicates using the following conditions: initial 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The average of the PCR reaction was the outcome measure for each sample.

Primer and fluorogenic probes were designed using the automated primer analysis software, Primer Express (PE Applied Biosystems). Primer and probes were chosen to bind in different exons or to span exon junctions to prevent amplification of genomic DNA. The forward primers (sequence 5'-3') used were: ACC CAA GCA CCT TCT TTT CCT T (amplicon length 106 bp; accession number E05490) for IL-1 β ; and ACG GGA AAC CCA TCA CCA T (amplicon length 64 bp; accession number X02231) for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The fluorogenic internal probes used were TCT TTG AAG AAG AGC CCG TCC TCT GTG ACT for IL-1 β and TTC CAG GAG CGA GAT CCC GTC AAG for GAPDH. The reverse primer used were TGC AGC TGT CTA ATG GGA ACA T for IL-1 β and CCA GCA TCA CCC CAT TTG A for GAPDH. The comparative C_T method, previously described [21] was used to calculate relative gene expression since we have determined in preliminary experiments that the amplification efficiencies of the target gene (IL-1 β) and the reference gene (GAPDH) are approximately the same. Thus, IL-1 β mRNA level was normalized to the GAPDH mRNA level in each sample. The value of untreated control animals was arbitrarily set at 1.0.

Tactile sensitivity changes were tested statistically, for a given test day, between injured and uninjured feet using paired t-test, and between injured and sham-injured animals using independent samples Student's t-test. For expression of IL-1 β , data were analyzed using a three-way analysis of variance (3-way ANOVA) followed by planned comparisons using least squared contrasts. Normality of distributions and homogeneity of variance were tested prior to performing ANOVA. Relationship between behavior and IL-1 β was explored by calculating Pearson correlation coefficients. Differences were considered significant when p was at least < 0.05.

The CCI animals did not show differences in mechanical sensitivity between injured or uninjured paw, and between injured and sham-injured animals (such outcomes have been reported for CCI by other groups, [22-24]). However, gross examination of the ligated sciatic nerve did indicate reduction in diameter of the nerve, implying the presence of some nerve injury. Thus, this group can be viewed as more of a control for presence of nerve injury in the absence of tactile allodynia behavior. In contrast, SNI-injured rats showed a robust and continued decrease in tactile thresholds over the time period of testing, only for the injured limb, and in contrast to the uninjured limb and to sham-SNI animals (see Figure 1).

Expression of IL-1 β mRNA in the brainstem, thalamus/striatum, and prefrontal cortex, at 10 days post-injury and at 24 days post-injury is shown in Figure 2. A 3 factor statistical comparison for IL-1 β expression as a function of animal groups (control, sham, SNI, CCI), time from injury (10 and 24 days) and brain sites (5 conditions: left and right prefrontal cortex and thalami, and brainstem) indicated significant main effects for animal groups ($F_{3, 156} = 9.0$, $p < 0.00001$) and brain sites ($F_{4, 156} = 2.9$, $p < 0.03$); a borderline 2-way interaction between group and time (group*time $F_{3, 156} = 2.5$, $p = 0.06$); and a significant interaction between all three factors (group*brain site*time $F_{12, 156} = 1.9$ $p < 0.04$). Planned comparisons indicated significant changes in IL-1 β expression mainly seen at 10 days post-injury. IL-1 β gene expression was significantly increased in the brainstem ($F_{1, 156} = 9.6$, $p < 0.002$ in contrast to sham; $F_{1, 156} = 10.9$, $p < 0.001$ in contrast to control) and in the right prefrontal cortex ($F_{1, 156} = 5.8$, $p < 0.02$ in contrast to sham; $F_{1, 156} = 5.9$, $p < 0.02$ in contrast to control) of rats of the SNI group. Interestingly, at this time point, IL-1 β gene expression was borderline significantly decreased in the right thalamus/striatum in the SNI group and the CCI group as compared to sham groups. Contrasting between SNI and CCI vs. sham and control indicated a significant decrease ($F_{1, 156} = 4.0$, $p < 0.05$). At 24 days post-injury, the only statistically significant difference observed was an increase in IL-1 β expression in the right thalamus/striatum of the SNI group ($F_{1, 156} = 10.5$, $p < 0.001$ in contrast to sham; $F_{1, 156} = 4.7$, $p < 0.03$ in contrast to control).

We tested for inter-relationships between the behavioral measure of touch sensitivity and expression of IL-1 β mRNA in all supraspinal sites studied. Only in left prefrontal cortex IL-1 β gene expression showed a significant relationship with touch sensitivity, where IL-1 β levels were positively correlated with left foot mechanical sensitivity, $r = 0.76$, $p < 0.03$ ($n = 8$, combining data from 10 and 24 days after injury). In contrast there was no relationship between IL-1 β and behavior when the same brain region was examined in sham and control animals, $r = -0.04$, $p > 0.8$ ($n = 24$, combining data from control, SNI-sham, and CCI-sham, at 10 and 24 days after injury).

The main observation of the study is that IL-1 β expression does change within the brain of neuropathic injury animals. The change is primarily observed in the SNI-injured animals, which also exhibited robust tactile allodynia. IL-1 β expression is transiently increased 10 days post-injury in the brain stem and in the prefrontal cortex, where it is lateralized to the brain opposite to the injured nerve. The changes detected in the thalamus/striatum are also lateralized but the alterations seem to be bi-phasic. Moreover, IL-1 β expression in the prefrontal cortex

ipsilateral to the injury shows a positive correlation with pain sensitivity only in the SNI animals, even though the magnitude of expression remains unchanged.

The lack of IL-1 β changes in sham animals and the minimal changes observed in the CCI-injured animals are compelling evidence that the observed changes are not due to the stress of the procedure, tissue injury, or persistent inflammation from the initial injury. Instead the data suggests that IL-1 β expression is more related to the presence of the painful condition. It is worthwhile to note, however, that the number and types of fibers undergoing injury and degeneration are substantially different between SNI and CCI injuries. Moreover, since only mechanical responses were tested, we cannot exclude the presence of thermal hyperalgesia in the CCI-injured animals, and we also cannot exclude the possibility that the lack of allodynia in the CCI may be due to the choice of rats, see [25]. Therefore, the present findings demonstrate that when the peripheral injury is extensive enough to induce tactile allodynia, only then brain IL-1 expression is changed.

Animals with chronic constriction injury did not show significant changes in IL-1 β expression, except in the right thalamus/striatum 10 days post-injury, when a trend for decreased IL-1 β was observed. However, 10 days after injury, we observed changes in IL-1 β expression in all regions examined in animals with spared nerve injury. The changes observed in the thalamus are interesting since at this site we observe transient contralateral decrease in IL-1 β expression in SNI group and CCI groups when combined together. This shared pattern of IL-1 β expression between both groups suggests that the thalamic decrease is more attributable to the peripheral deafferentation and its sequelae (e.g. Wallerian degeneration as well as regeneration).

Expression pattern for IL-1 β 24 days post-injury is very different from that seen at 10 days post-injury. Brainstem and prefrontal cortical IL-1 β expressions are now indistinguishable from sham or control levels. On the other hand, IL-1 β in the thalamus/striatum contralateral to the injury is reversed and is now higher than control or sham levels. Therefore, these results indicate a temporal dynamics for endogenous levels of IL-1 β across supraspinal brain regions in animals with neuropathic pain. A similar time dependent change in IL-1 β expression has been noted in the contralateral non-operated nerve following transection of sciatic nerve in the rat [26]. The authors observed high expression of IL-1 β , as well as interleukin-10 and transforming growth factor- β 1, in the endoneurium of intact sciatic nerve, which diminished to control levels at day 7, and was maximally increased at day 35. It is possible that supraspinal IL-1 β expression may also be undergoing such oscillations in time.

Even though we observe changes in IL-1 β in all the brain regions that we studied, the specific nuclei within these regions and the functional significance of changes in IL-1 expression remain to be determined. However, the observed changes most likely are at least linked with changes in local synaptic efficacy [10;11]. Moreover, IL-1 β is only one of a long list of cytokines that usually act in close synchrony with each other. These relationships also remain to be determined in future studies.

There is good evidence for a role of peripheral and spinal cord IL-1 β in the development and maintenance of neuropathic pain through induction of the proinflammatory cytokine cascade. Recent studies demonstrate leukocyte trafficking and astrocyte and glial activation in the spinal cord following peripheral nerve injury, and show that selective cytokine inhibitors and neutralizing antibodies attenuate tactile and thermal sensitivity in animal models of neuropathy [27-32]. Moreover, a recent study showed that disruption of P2X(7), a ligand-gated cation channel expressed by cells of immune origin and which release IL-1 β , abolishes inflammatory and neuropathic hypersensitivity to both mechanical and thermal stimuli [33]. Similarly, genetic manipulation of IL-1 signaling block development of allodynia and hyperalgesia in L5 spinal nerve cutting induced neuropathy, as normally observed in wild-type mice [8]. Since

these experiments were done using knock-out mice, IL-1 β regulation was disrupted throughout the CNS, which may explain the complete blockade to neuropathic behavior in this case in contrast to the above manipulations that targeted the periphery or spinal cord, implicating supraspinal IL-1 β in neuropathic pain states. There is very little information regarding the role of endogenous IL-1 β at supraspinal sites in relation to neuropathic pain behavior, especially outside the hypothalamus. In rat, intracerebroventricularly (ICV) administered IL-1 β when given in a low, non-pyrogenic concentration, induces hyperalgesia through activation of hypothalamic paraventricular and arcuate nuclei [5;6;34;35]. ICV administration of IL-1 β also increases renal and splenic sympathetic nerve discharges [36]. The present study is the first to observe endogenous IL-1 β changes in multiple supraspinal brain sites in neuropathic pain. It suggests a causal relationship between neuropathic pain and expression of IL-1 β in the studied brain regions, implying synaptic re-organization of these regions. A recent study reports expression IL-1 β in the whole brain of rats following L5 spinal nerve transection induced neuropathy [37]. Their data suggests transient increased expression of IL-1 β (perhaps also in TNF α , but not in IL-10, or NF- κ B) in the brain 3 days post injury, which seems sustained for up to 14 days and returns to baseline at 21 days. Thus, the rat neuropathic pain models provide an opportunity to dissect cellular and molecular mechanisms that may link the human observations of brain morphometric changes to cortical re-organization, perhaps directly through cytokine activity.

In summary, we observe supraspinal transient changes in IL-1 β expression in SNI injured animals, with distinct changes occurring in specific brain regions, which shift in time to a new pattern. The functional consequences of the changes observed in IL-1 β expression in the brain in neuropathic pain remain to be determined.

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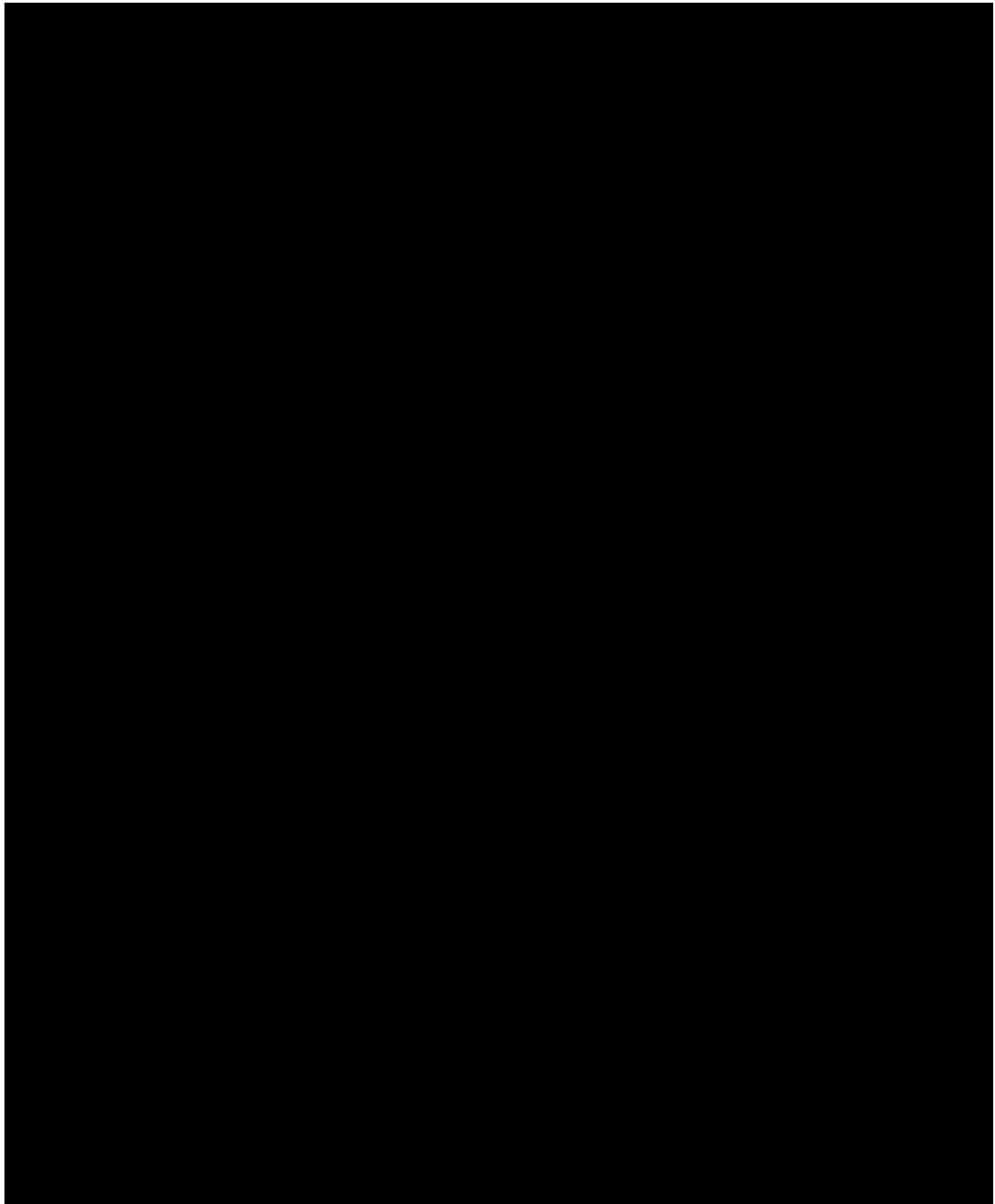
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**Figure 1.**

Tactile thresholds (50% thresholds in grams) after peripheral nerve injury. Top panel: CCI-injury does not result in an observable difference in tactile threshold between CCI-injured foot (left ft, CCI) and contralateral uninjured foot (right ft, CCI), or in contrast to sham-injured foot (left ft, CCI-sham). B. In SNI-injury, there is a statistically highly significant decrease in tactile threshold starting with 4-days post-injury, when SNI-injured foot (left ft, SNI) threshold is compared to contralateral uninjured foot (right ft, SNI), or to sham-injured foot (left ft, SNI-sham) ($p < 0.05$ at 4 days, and becomes $p < 0.0001$ at later test days).

A. 10 days post-injury **B. 24 days post-injury**

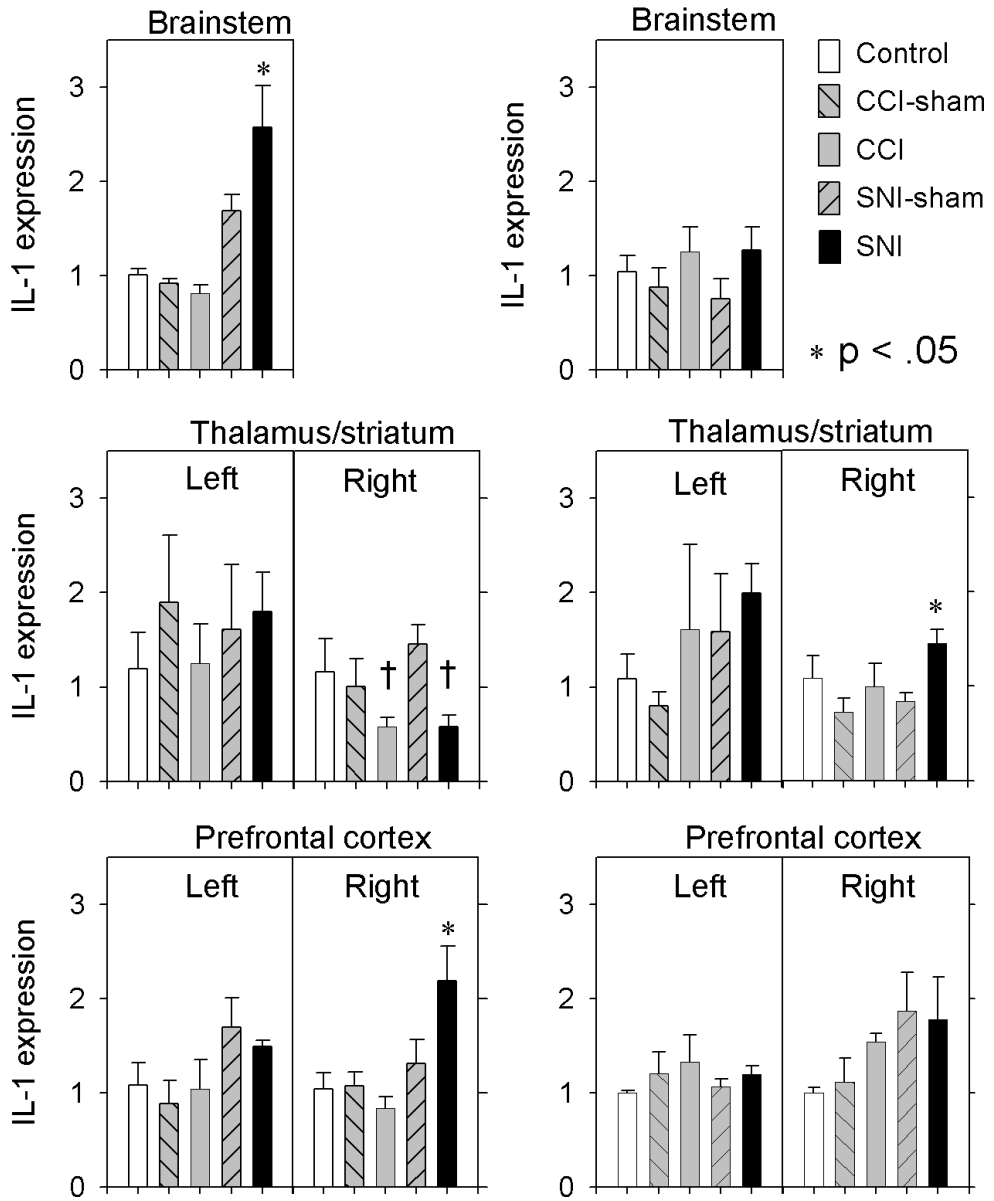


Figure 2. Expression of IL-1 β mRNA in various brain regions at two times from neuropathic injury (fold change relative to GAPDH): A. is at 10 days post-injury, and B. at 24 days post-injury. Results for 5 groups of animals are shown for 5 brain areas. Mean and standard error is plotted, and statistically significant differences as compared to either control, or corresponding sham, are indicated (*) († = only the combination of both CCI and SNI results in significant decrease in expression from sham + control).