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PERIPHERAL NERVE INJURY ALTERS THE EXPRESSION OF NF- κ B IN THE RAT'S HIPPOCAMPUS

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Abstract

The hippocampus plays an important role in learning and memory and possibly contributes to the formation of pain-related memory and emotional responses. However, there is currently little data linking the hippocampus to neuropathic pain. It has been reported that NF- κ B is an important regulatory factor in memory consolidation within the hippocampus. This study aims to examine a possible relationship between the hippocampal NF- κ B expression and nerve injury-induced thermal hyperalgesia using a rat model of constriction sciatic nerve injury (CCI). Immunofluorescence and Western blot analysis were performed to detect and quantify the hippocampal NF- κ B expression. Thermal hyperalgesia was examined on day 0 and postoperative day 1, day 7 and day 14. The nuclear portion of the p65 NF- κ B expression was significantly increased on the contralateral side on day 7 and day 14 as well as significantly increased on the ipsilateral side on day 14 as compared to the sham control group. Intraperitoneal administration of MK-801, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, reduced hyperalgesia and modulated the NF- κ B expression in the contralateral side of hippocampus. These results suggest an association between the hippocampal NF- κ B expression and the behavioral manifestation of thermal hyperalgesia, which is likely to be mediated through activation of the NMDA receptor.

Keywords

neuropathic pain; NF- κ B; hippocampus

1. Introduction

To date, much of the investigation into the supraspinal involvement in neuropathic pain has focused on the brain regions participating in the nociceptive pathways. Since pain is a multi-dimensional subjective experience, the limbic system has been a subject of research interest in recent years because of its relation to the emotional component of pain. Hippocampus, a

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major element of limbic system, has been shown to play an important role in learning, memory and mood disorders and has been suggested to contribute to the pain-related memory and emotional disorders (McEwen, 2005; Shiryayeva et al., 2008; Zhuo, 2008). Alterations in the morphology and gene expression in the hippocampus have been detected following the onset of persistent inflammatory pain (Duric and McCarron, 2006). More recently, emerging reports point to a possible association between the hippocampus and neuropathic pain. For example, hippocampal long-term potentiation has been shown to be impaired after peripheral nerve injury, suggesting the involvement of hippocampal synaptic plasticity in neuropathic pain condition (Kodama et al., 2007). Furthermore, glucocorticoid receptor mRNA expression was decreased in the hippocampus after chronic constriction injury (CCI) (Ulrich-Lai et al., 2006). These findings suggest an association between a chronic pain state and alterations in the hippocampus. However, the potential mediators associated with these alterations under neuropathic pain condition are yet to be determined.

Nuclear factor kappa B (NF- κ B) is an important transcriptional factor initially studied for its part in the immune response. It includes five subunits, Rel A (p65), RelB, NF- κ B1 (p105/p50), NF- κ B2 (p100/p52) and c-Rel and their expression and regulation in the hippocampus may contribute to learning-associated synaptic reorganization and memory formation (Ahn et al., 2008; Boccia et al., 2007; O'Sullivan et al.). I κ B kinase, an inhibitor of NF- κ B, regulates chromatin structure during reconsolidation of conditioned fear memories (Lubin and Sweatt, 2007), suggesting an important role for NF- κ B in the hippocampal function. Furthermore, evidence also points to the involvement of NF- κ B in both inflammatory and neuropathic pain in the nervous system (Ma and Bisby, 1998; Meunier et al., 2007).

In the present study, we tested the hypothesis that there may be an association between the hippocampal NF- κ B expression and the development of neuropathic pain. Specifically, we examined whether there is altered p65 NF- κ B expression in the hippocampus after peripheral nerve injury and whether the N-methyl-D-aspartate (NMDA) receptor would be involved in this process.

2. Results

2.1 Thermal hyperalgesia following CCI

The threshold for the right hindpaw withdrawal from a radiant heat source (ipsilateral to the ligation of sciatic nerve) was significantly decreased on postoperative day 1 and remained decreased up to at least post-operative day 14 as compared with sham-operated rats (Fig 1A, n=6; $P < 0.05$). There were no postoperative differences in hindpaw withdrawal latencies for the contralateral hind limb of both CCI and sham rats (Fig 1B, n=6; $P > 0.05$).

2.2 Altered hippocampal p65 expression in CCI rats

Immunostaining showed the expression of active p65 in the hippocampus in both CCI and sham rats (Fig 2). However, the p65 expression was significantly increased on postoperative day 14 on the contralateral hippocampus as compared with the ipsilateral side (Fig 2), which was confirmed by the Western blot analysis (Fig 3B). There were no obvious changes observed in the ipsilateral side of hippocampus compared to sham. The Western blot showed an increase in the nuclear p65 expression in the contralateral hippocampus on postoperative day 7, but not day 1, and again on day 14 as compared with the corresponding side of the sham rats (Fig 3B). In contrast, the cytosolic portion of p65 expression was gradually decreased on postoperative day 7 and day 14 (Fig 3C), suggesting that cytosolic p65 was stimulated and translocated to the nucleus after peripheral injury. In the ipsilateral hippocampus, a smaller but significant increase of nuclear p65 expression was demonstrated on day 14, but not day 1 and day 7, as compared to the sham groups (Fig 3A). These results

indicate that the expression of p65 showed a side and time specific pattern following peripheral nerve injury.

2.3 Effect of MK-801 treatment on CCI rats

In order to determine whether NMDA receptors were involved in the altered hippocampal NF- κ B and thermal hyperalgesia after CCI, the non-competitive NMDA receptor antagonist MK-801 (0.1 mg/kg) was given intraperitoneally for seven postoperative days after CCI. This MK801 treatment regimen significantly reduced hyperalgesia on the ipsilateral hindpaw when tested on post-operative day 7 as compared with the saline-treated CCI rats (Fig 4). The MK-801 treatment did not alter the thermal response in the contralateral hindpaw (Fig 4).

The MK-801 also reversed the increased p65 expression on the contralateral hippocampus in CCI rats when examined on day 7 (Fig 5). Collectively, these results indicate NMDA receptors played a significant role in both the altered p65 expression and thermal hyperalgesia in CCI rats.

3. Discussion

Our data showed a bilateral alteration in the hippocampal NF- κ B (p65 subunit) expression in rats with ipsilateral thermal hyperalgesia after CCI. The nuclear p65 expression was significantly upregulated in the contralateral hippocampus on day 7 and further increased by day 14, whereas the nuclear p65 expression in the ipsilateral hippocampus was not increased until day 14. Both thermal hyperalgesia and the p65 expression were reversed after a systematic MK-801 treatment.

The hippocampus consists of hippocampus proper (CA1, CA2 and CA3), dentate gyrus (DG) and subiculum. The hippocampus is interconnected with many other brain regions, such as the somatosensory cortex, amygdala and periaqueductal gray matter. Several studies using microinjection of drugs, such as lidocaine, NMDA receptor antagonists, 5-HT receptor antagonists, into the hippocampus showed a reduction of nociceptive behavior in laboratory animals (McKenna and Melzack, 1992; Soleimannejad et al., 2006; Soleimannejad et al., 2007). Vanja et al. also conducted studies that demonstrated a decreased expression of hippocampal NK-1 receptors and BDNF mRNA in different pain models (Duric and McCarter, 2005; Duric and McCarter, 2006; Duric and McCarter, 2007). Together, these studies support the notion that hippocampus is involved in the processing of both acute nociception and inflammatory pain. The results from the present study demonstrate changes in hippocampal NF- κ B in association with thermal hyperalgesia, indicating that hippocampus is involved in a persistent pain state such as neuropathic pain as well. As the changes of p65 expression occurred on postoperative day 7 and day 14 after injury, it suggests that NF- κ B is likely to be involved in the chronic phase of neuropathic pain.

NF- κ B, usually forming as a dimer, is retained in the cytoplasm by its inhibitor I Bs. In the central nervous system (CNS), many stimuli, such as NMDA receptor agonists, BDNF, and cytokines, can release NF- κ B complexes from I Bs, which in turn are translocated to the nucleus where NF- κ B binds to DNA resulting in the modulation of target genes (Kaltschmidt et al., 2005). An increasing number of studies have pointed the involvement of NF- κ B in the regulation of neuronal plasticity and memory processing (Boersma and Meffert, 2008; Meffert et al., 2003). Of interest, our present results showed that changes of p65 in the ipsilateral and contralateral hippocampus occurred in different timescales. A significant increase was observed on day 7, but not on day 1, in the contralateral hippocampus, which was again detected on day 14. In contrast, in the ipsilateral hippocampus which is not along with the ascending pain pathway there was no change in the

p65 expression on day 7 but a significant increase on day 14. The increase in the ipsilateral hippocampus is smaller in magnitude as compared with the contralateral side. These results suggested that the level of p65 expression might reflect dual functional changes in the hippocampus after peripheral injury and such changes are time-dependent, which may be contributory to transition from an acute to a persistent nociceptive state after CCI.

To the best of our knowledge, only one group using functional imaging study on healthy volunteers reported an increased blood flow in the contralateral hippocampus in response to mild thermal pain (Derbyshire et al., 1997). The authors speculated that the increased activity in the contralateral hippocampus might be related to its connection with the ACC, amygdala, insula and prefrontal cortex (Derbyshire et al., 1997). These brain regions might form a network to process the emotional and contextual information relevant to pain (Derbyshire et al., 1997). In this regard, our data showed a gradual increase of the p65 expression in both contralateral and ipsilateral hippocampus in CCI rats on postoperative day 14. Since the hippocampus has an important role in the consolidation of memories, our finding may be of particular interest because the change in the contralateral hippocampus could be reversed by the systemic treatment with MK-801, an NMDA receptor antagonist known to improve neuropathic pain behaviors in rats (Chizh and Headley, 2005; Mao et al., 1992). Therefore, the increased p65 expression in the bilateral hippocampus might play a role in a formation of chronic pain-related memory, which requires future investigations.

Several studies have indicated that nociception-induced changes in immediate early genes, such as *c-fos* and *Egr1*, occur in the hippocampus. It have been demonstrated that the *c-fos* expression was increased bilaterally after formalin injection (Aloisi, 1997; Ceccarelli et al., 1999). In addition, Wei et al. found that tissue injury caused a rapid increase in the *Egr1* expression in the hippocampus, but not in the spinal cord, and this increase was prevented by the NMDA receptor antagonism (Wei et al., 2000). It would be of interest in future studies to examine a possible relationship between the hippocampal expression of NF- κ B and immediate early genes following peripheral nerve injury.

There are some limitations to this study. The methods for the quantification of NF- κ B expression (western blot) was limited to detect global changes in the hippocampus but not its sub-regions due to the technical feasibility of obtaining tissue from small areas. It has been reported that using MK-801 targeting to the DG area of the hippocampus could alleviate formalin-induced nociception during both acute and tonic phases while targeting the CA1 area could only reduce nociception during the tonic phase, suggesting that the NMDA-sensitive mechanism might be involved in different hippocampal sub-regions in the pain processing (McKenna and Melzack, 2001; Soleimannejad et al., 2007). Similarly, it is possible that NF- κ B may play a different role in sub-regions of the hippocampus during various post-injury periods. Nonetheless, it would be of considerable interest to examine the role of NF- κ B in different hippocampal sub-regions in pain-related memorial or emotional dysfunctions.

In summary, the present study demonstrated that the p65 expression is significantly altered in the hippocampus, which is both time-dependent and side-specific. These changes are also likely to be mediated by the NMDA receptor and associated with the behavioral manifestation of thermal hyperalgesia after CCI. Given the unique expression pattern of p65 in the hippocampus after CCI, our results suggest that the brain mapping of NF- κ B expression may be a useful tool to evaluate the cellular mechanisms contributory to the transition from acute to chronic nociception in preclinical studies of neuropathic pain.

4. Experimental Procedure

4.1 Experimental animals

Male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA, USA) weighing 250–280g were used. The experimental protocol was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were housed under controlled temperature ($21 \pm 2^\circ\text{C}$), relative humidity ($50 \pm 10\%$) and artificial lighting (lights on from 7am until 7pm) with distilled water and food available *ad libitum*. CCI was induced by the method of Bennett and Xie (1988). Under anesthesia (pentobarbital 50mg/kg, i.p.), four chromic gut ligatures were made loosely around the right sciatic nerve. For the sham-operation group, the rats underwent the same procedure without nerve ligation.

4.2 Drug Treatment

MK-801 (Methyl-10, 11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine hydrogen maleate) (Sigma) was dissolved in normal saline. CCI rats received intraperitoneally either saline (n=6) or MK-801 (0.1 mg/kg, n=6) once daily for 7 consecutive days after CCI surgery.

4.3 Behavioral test

After habituation to the test environment, the measurements were made before surgery (baseline) and on postoperative days 1, 7 and 14. The paw withdrawal latency to thermal hyperalgesia was measured using an Analgesia Meter (Model 390, IITC Life Science, Inc.). The radiant heat source was focused on the plantar surface of a hindpaw and light intensity was preset to obtain a baseline latency of approximately 12 seconds. A cut-off time was set at 20 seconds to avoid tissue damage. Each rat underwent two trials with a 5-minute interval and the mean value of two trials was used as the withdrawal latency.

4.4 Immunohistochemistry

At each respective time point, rats were anesthetized with pentobarbital (50mg/Kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.2–7.4, 4°C). The brain was dissected, post-fixed overnight and changed to 30% sucrose solution at 4°C . Tissues were mounted in OCT compound and frozen at -18°C . Coronal brain sections ($35 \mu\text{m}$) were cut using a cryostat. The sections were washed three times (5 min each) with $1\times\text{PBS}$, blocked in 0.1 M PBS containing 8% goat serum, 1% bovine serum albumin (BSA) and 0.3% Triton X-100 for 1 hour at room temperature and then incubated for 24 hours at 4°C with first primary antibody NF- κB (p65 active form, 1:200, from CHEMICON). The sections were rinsed three times and then incubated with CyTM3-conjugated Goat Anti-mouse secondary antibodies (1:400, Jackson ImmunoResearch) for 1 hour at room temperature in the dark. Sections were washed subsequently and mounted onto chrome alum-coated slides, and covered with Vectashield Mounting medium (Vector Laboratories). Brain sections were examined using an Olympus fluorescence microscope, recorded with its digital camera, and processed with Adobe Photoshop 7.0 (©Adobe System Incorporated).

4.5 Western blot

Western blot was used to semi-quantify the expression of p65 in the hippocampus. Rats were rapidly decapitated on postoperative day 1, day 7 or day 14 under pentobarbital anesthesia (50 mg/kg, i.p.). Hippocampus was removed and immediately placed on dry ice and stored at -80°C until use. The nuclear extracts and cytosolic extracts were prepared

using Nuclear Extract Kit (Active Motif). Protein extracts (3 μ g) were then separated by SDS-PAGE gels (4%–15% gradient gel; Invitrogen) and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% milk for 1 hour at room temperature, and then incubated overnight at 4°C with primary p65 antibody (1:1000, Santa Cruz). This was followed by three washes in PBS and incubation for 1 hour with HRP-conjugated secondary antibody (1:7000, Amersham Biosciences). The blots were visualized in ECL solution (Thermo Scientific) and exposed onto X-ray films (Kodak) for 1–10 minutes. The membranes were then incubated in a stripping buffer (Thermo Scientific) for 15 minutes in room temperature and re-probed with β -actin antibody (1:12000, mouse monoclonal, Abcam) as a loading control. The density of each band was measured by Quantity One software (Bio-Rad) and normalized against a corresponding loading control band.

4.6 Data and statistical analysis

All results are expressed as mean $\pm$ standard error (SEM). For Western blot analysis, the protein expression in the sham group was normalized as 1 and the relative density of the other groups was calculated proportionately within the same side. There were no significant differences between the ipsilateral and contralateral side in the sham group before adjusting. Differences in the values were compared using one-way analysis of variance (ANOVA) followed by a post hoc test (Turkey) using SPSS Software.

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Figure 1. Behavioral changes following CCI

The values for paw withdrawal latency (PWL) in response to thermal stimuli are shown for the ipsilateral (A) and contralateral (B) hindpaw. The data for days 0, 1, 7 and 14 are displayed as mean $\pm$ SEM. * $P < 0.05$ (n=6).

Figure 2. Immunohistochemical staining of hippocampal NF- κ B

The p65 expression was shown in both ipsilateral and contralateral hippocampus areas in a representative sham-operated and CCI rat on postoperative day 14.

Figure 3. NF-κB expression in the hippocampus

The nuclear p65 expression (Western blot) in the ipsilateral hippocampus (A) was significantly increased on day 14 as compared with sham group while the p65 expression was significantly increased in the contralateral hippocampus (B) on both postoperative day 7 and day 14. Each bar represents the mean $\pm$ SEM of 6 rats. * $P < 0.05$, as compared to the sham group on each corresponding time point. The expression of p65 in the nucleus and cytosol after chronic constrict injury on the contralateral hippocampus was demonstrated (C).

Figure 4. Effects of MK-801 on thermal hyperalgesia

CCI rats were treated intraperitoneally with saline or MK-801 (0.1mg/kg) once daily for seven consecutive postoperative days. The hindpaw withdrawal latency (PWL) to thermal stimuli is shown for the ipsilateral (A) and contralateral (B) hindpaw. * $P < 0.05$, as compared with the baseline (Day 0; $n=6$). # $P < 0.05$, as compared with the saline group on each respective time point ($n=6$).

Figure 5. Effect of MK-801 on the hippocampal P65 expression

The histograms show the Western blot analysis of p65 expression of the ipsilateral and contralateral side of the hippocampus in CCI rats treated with either saline or MK-801 (0.1 mg/kg) on postoperative day 1 and day 7. * $P < 0.05$, as compared with the sham group ($n=6$); # $P < 0.05$, as compared with the CCI+ saline group ($n=6$).