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Loss of calcineurin in the spinal dorsal horn contributes to neuropathic pain, and intrathecal administration of the phosphatase provides prolonged analgesia

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ABSTRACT

Calcineurin (protein phosphatase 3) regulates synaptic plasticity in the brain. The development of neuropathic pain appears dependent on some of the same mechanisms that underlie brain synaptic plasticity. In this study, we examined whether calcineurin regulates chronic constriction injury (CCI)-elicited plasticity in the spinal dorsal horn. CCI animals exhibited mechanical and thermal hypersensitivity 7 days after ligation of the sciatic nerve. Neither control uninjured nor sham-operated animals exhibited pain behavior. Calcineurin activity and content of its Aα isoform were significantly decreased in the ipsilateral postsynaptic density (PSD) of dorsal horn neurons in CCI animals. Calcineurin activity and content in the contralateral PSD of CCI animals or either side of the dorsal horn in sham animals were not modified. The pain behavior in CCI animals was attenuated by intrathecal application of exogenous calcineurin. The treatment was long-lasting as a single injection provided analgesia for 4 days by restoring the phosphatase's activity and Aα content in the PSD. No signs of toxicity were detected up to 14 days after the single intrathecal injection. Intrathecal application of the calcineurin inhibitor FK-506 elicited pain behavior in control uninjured animals and significantly reduced calcineurin activity in the PSD. CCI may elicit neuropathic pain at least in part as a result of the loss of calcineurin-mediated dephosphorylation in the dorsal horn. Addition of the phosphatase by intrathecal injection reverses the injury-elicited loss and provides prolonged pain relief. Clinical therapy with calcineurin may prove to be a novel, effective, and safe approach in the management of well-established neuropathic pain.

1. Introduction

Injury-elicited plasticity accompanies peripheral nerve injury to ultimately lead to the development of neuropathic pain [11,18]. The development of neuropathic pain is dependent on some of the same mechanisms that give rise to activity-dependent synaptic plasticity in the brain [5]. Like synaptic plasticity, neuropathic pain exhibits early and late phases that may be mediated by different mechanisms [5,11,18].

The balance between protein kinase and phosphatase activity at the synapse can critically determine overall synaptic strength [12]. As a result, the loss of either one of these activities can engender long-lasting changes in synaptic function, ie, long-lasting plasticity.

More than a decade ago, Kandel et al [1] described how the interplay between protein kinase A (PKA) and calcineurin (protein phosphatase 3, also protein phosphatase 2B) was essential in initiating and maintaining long-lasting enhancement of synaptic function in *Aplysia*, *Drosophila*, mice, and rats. Activation of PKA by cyclic adenosine monophosphate, and the subsequent phosphorylation of target proteins, resulted in long-term memory storage. In contrast, activation of calcineurin promoted the dephosphorylation of these target proteins to prevent the transition from short- to long-term memory.

Later studies confirmed the general role of calcineurin in negatively constraining the acquisition of spatial or aversive memory or of long-lasting plasticity in ocular dominance, cocaine addiction, and vestibular compensation [2,13,17,21,22].

Little is currently known about the role of calcineurin in injury-elicited plasticity in the spinal dorsal horn. Somatic and terminal immunoreactive staining for the phosphatase is heavy and highly restricted to the superficial dorsal horn [6,20]. Terminal staining

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is of dorsal horn origin due to the lack of staining in the dorsal root ganglion or dorsal root axons [20].

We reported previously that chronic constriction injury (CCI) of the rat sciatic nerve was associated with the loss of calcineurin content in the spinal dorsal horn [14]. In this study, we extended our investigation by examining changes in calcineurin activity and content of its Aα isoform specifically in the postsynaptic density (PSD) of dorsal horn neurons. We concentrated on calcineurin Aα because this isoform is the most abundant in the dorsal horn [20]. We also focused on the PSD because dynamic remodeling of excitatory synapses appears to play an important role in regulating synaptic efficacy [10], and we reported recently that pain behavior due to CCI was associated with changes in the protein matrix of the PSD [15].

PSD is deserving of attention because its function may represent a final common reflection of the many injury-elicited changes in receptors, pathways, transcription factors, and genes that have been reported to accompany chronic pain. In addition, the localization of changes to the postsynaptic structure reduces difficulties in data interpretation with respect to the origin or site of action of the protein under investigation.

Given the already established role of calcineurin in the transition to long-term memory, we hypothesized a similar association between calcineurin and neuropathic pain, ie, that the development of the pain is a pivotal consequence of the loss of calcineurin activity in the PSD of spinal dorsal horn neurons.

2. Methods

2.1. Animals

Male Harlan-Sprague–Dawley rats (200–300 g) were randomly assigned to control, sham-operated, or CCI groups. All experiments were conducted in accordance with guidelines accepted by the International Association for the Study of Pain [23]. The animal protocol was approved by the Animal Care and Use Committee of the School of Medicine and Public Health at the University of Wisconsin–Madison.

2.2. Behavioral tests

For assessment of mechanical thresholds, we used the Dynamic Plantar Aesthesiometer (Ugo Basile North America, Collegeville, Pennsylvania) to record the force that resulted in an animal's paw withdrawal. Thermal hyperalgesia was assessed with the hind paw withdrawal latency test using a plantar analgesia instrument (Ugo Basile North America). The mechanical testing preceded thermal testing.

Animals were acclimated for 15 to 20 minutes. Each test consisted of 4 trials that were averaged to obtain a mean value. Each trial was separated by at least 5 minutes. In sham or CCI animals, the ipsilateral injured paw was tested. Mechanical thresholds and thermal withdrawal latencies were obtained for all animals both at baseline and later. After the last behavioral test, animals were anesthetized and euthanized, and their lumbar spinal dorsal horns collected for enzyme activity, Western immunoblot, or real-time reverse transcription polymerase chain reaction (RT-PCR) assays.

2.3. Anesthesia, sciatic ligation, intrathecal drug application, and tissue collection

Animals were anesthetized with isoflurane. Body temperature was kept at 37°C with a homeothermic blanket system. Anesthesia was sufficiently deep to prevent arousal but light enough to permit spontaneous respiration. Adequate anesthesia was assessed by monitoring blink or ear reflexes, withdrawal to toe pinches, respiratory rate, and absence of spontaneous movements.

Loose ligation of the sciatic nerve (CCI) was performed using the Bennett and Xie [3] procedure, as described previously [14]. Briefly, the sciatic nerve was exposed and loosely ligated with 4 simple interrupted 4-0 chromic gut sutures placed ~1 mm apart. In sham-operated animals, the sciatic nerve was exposed but not ligated. Control animals were anesthetized but were not subject to surgery.

Calcineurin (5 or 10 enzyme units/μL; Enzo Life Sciences, Plymouth Meeting, Pennsylvania), FK-506 (1 μg/μL; Sigma–Aldrich, St. Louis, Missouri), or saline solution was injected intrathecally in a volume of 10 μL, as described previously [15]. For all intrathecal injections, animals were briefly anesthetized with isoflurane. Persons performing behavioral tests were blinded to drug treatment.

For tissue collection, animals were anesthetized with isoflurane and while still deeply anesthetized, they were euthanized with an intracardiac injection of supersaturated potassium chloride (>350 mg/mL). A laminectomy rapidly (<2 minutes) exposed the lumbar spinal cord at L5, and ~1 cm of the cord was excised and cut into dorsal and ventral halves and the dorsal half further divided into ipsilateral and contralateral quadrants. All tissues were stored at −80°C until use.

2.4. Fractionation, calcineurin activity assay, immunoblots, and RT-PCR

Tissues were homogenized and sequentially centrifuged to yield the PSD-containing LP1 fraction [15]. After assaying for total protein content, the fractions were used in the biochemical assays. Calcineurin activity was assayed with a commercial kit (Enzo Life Sciences) and expressed as nanomoles of phosphate released per minute per milligram of protein. Western immunoblots were performed as described previously [14]. Antibodies to calcineurin Aα, PSD-95 and green fluorescent protein (GFP, *Aequorea victoria)* were purchased from Millipore (Billerica, Massachusetts) and used at a dilution of 1:1000, 1:1000, and 1:500 respectively. GFP itself was also purchased from Millipore. Developed membranes were stripped and reprobed with β III tubulin (1:1000; Promega, Madison, Wisconsin) as the loading control. Protein levels were estimated from optical density measurements using the BioSpectrum 500 Image Analysis System (UVP, Upland, California). Calcineurin Aα levels within a gel were expressed over the β III tubulin levels, and then the levels in sham-operated or CCI animals were normalized to those in control, uninjured animals. A similar normalization procedure was used for the enzyme activity assays.

Calcineurin mRNA levels were determined by monitoring in real time the increase in fluorescence of SYBR-GREEN dye with the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, California) [16]. Relative expression levels of calcineurin in each sample were determined using a standard curve of 3-fold serial dilutions. Average fold induction relative to control animals was determined after normalizing to the amount of 18S rRNA in each sample. A 2-fold or greater change was considered significant.

Calcineurin primer sequences were GCAGCAATATTCAGTGACCACTTC (forward) and AACATCCAACTGCTGAGATGCA (reverse). The primers were designed with Primer Express software (Applied Biosystems) using the rat calcineurin mRNA sequence NM_017041.1 and were chosen for their uniqueness in the genome. Primers for 18s rRNA (reference controls) were AACGAGACTCTCGGCATGCTAA (forward) and CCGGACATCTAAGGGCATCA (reverse). All primers were purchased from Integrated DNA Technology (Coralville, Iowa). Criteria for primer selectivity included ~20 bases in length, fall within the last 600 bp of the sequence, contained higher GC content, and did not include runs of ⩾4 G bases.