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CHANGES IN CALCINEURIN MESSAGE, ENZYME ACTIVITY AND PROTEIN CONTENT IN THE SPINAL DORSAL HORN ARE ASSOCIATED WITH CHRONIC CONSTRICTION INJURY OF THE RAT SCIATIC NERVE

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Abstract—Plasticity in the spinal dorsal horn is thought to underlie the development of neuropathic pain. Calcineurin (protein phosphatase 3) plays an important role in plasticity in the brain. Here we examined whether chronic constriction injury (CCI) of the sciatic nerve modifies calcineurin expression in the spinal dorsal horn. Male rats were assigned to control (uninjured), sham-operated or CCI groups. CCI animals exhibited both a shift in weight bearing and a reduction in paw withdrawal latencies as signs of pain behavior. At 3 days (3D) the pain behavior was associated with a significant increase in calcineurin gene expression, enzyme activity and content of its Aα isoform in the ipsilateral spinal dorsal horn. In contrast, while the pain behavior persisted at 7 days (7D) calcineurin gene expression returned to control levels and activity and protein content decreased. A single intrathecal injection of MK-801 15 min before the ligation attenuated both signs of pain behavior in 3D but not 7D CCI animals. The same pre-treatment also prevented the CCI-associated increases in calcineurin in these animals. These data suggested an involvement of calcineurin in CCI-elicited neuropathic pain. The time-dependent divergent changes in calcineurin expression may underlie the different phases of neuropathic pain development.

Key words: central sensitization, chronic constriction injury, neuropathic pain, spinal dorsal horn, phosphatase, synaptic plasticity.

It is now well-established that injury-elicited plasticity accompanies peripheral nerve injury and that this significant alteration in sensory processing in the spinal dorsal horn may ultimately contribute to the development of neuropathic pain (Ji and Strichartz, 2004; Latremoliere and Woolf, 2009; Sandkuhler, 2009). The development of neuropathic pain appears dependent upon some of the same mechanisms that give rise to activity-dependent synaptic plasticity in the brain (Citri and Malenka, 2008).

Synaptic plasticity is critically influenced by the actions of protein kinases and phosphatases at a synapse (Lee, 2006). More than a decade ago, Kandel and colleagues (Abel and Kandel, 1998) described how the interplay between protein kinase A (PKA) and calcineurin (protein phosphatase 3, previously 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 AMP, and the subsequent phosphorylation of target proteins, resulted in long-term memory storage. Activation of calcineurin led to the dephosphorylation of these target proteins to prevent the transition from short to long-term memory. Later studies in other brain areas 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 (Yang et al., 2005; Masumura et al., 2007; Baumgartel et al., 2008; Pulipparacharuvil et al., 2008; Wang et al., 2009).

Little is known about the role of calcineurin in the spinal dorsal horn. The phosphatase is highly localized in the superficial spinal dorsal horn with heavy staining in cell bodies and terminals in laminae I and II and only a few labeled neurons in laminae III and IV (Goto et al., 1990; Strack et al., 1996). The terminal staining was judged to be of dorsal horn origin because of the lack of immunoreactivity in sensory axons in the dorsal roots (Strack et al., 1996). In dorsal root ganglia (DRG), moderate staining was associated with DRG neuron cell bodies but not their processes in the ganglia. DRG neuron soma staining was diffuse and appeared to include the nucleus, in contrast to spinal cord neurons where the staining was granular and excluded the nucleus (Strack et al., 1996).

We previously reported that there was a significant decrease in calcineurin content in the spinal dorsal horn of animals exhibiting neuropathic pain 7 days after chronic constriction injury (CCI) of the sciatic nerve (Miletic et al., 2002). In the present study we extended our investigation by examining changes in calcineurin gene expression, enzyme activity, and content of its Aα isoform at two post-ligation periods, 3 days (3D) and 7 days (7D). These times represent the initial and established phases of neuropathic pain development. We chose to examine calcineurin Aα because this isoform is the most abundant in the spinal dorsal horn (Strack et al., 1996). We also investigated whether single intrathecal pre-treatment with MK-801, an NMDA receptor antagonist that blocks synaptic plasticity, would modify pain behavior and any CCI-associated changes in calcineurin expression.

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Abbreviations: CCI, chronic constriction injury; DRG, dorsal root ganglia; 3D, 3 days; 7D, 7 days.

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EXPERIMENTAL PROCEDURES

Animals and behavioral tests

Male Harlan–Sprague–Dawley rats (200–250 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 (Zimmermann, 1983). 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.

A dual channel scale (Incapacitance Meter™, Stoelting, Chicago, IL, USA), which separately measures the weight borne by each hind limb, was used for the weight-bearing test as detailed previously (Miletic and Miletic, 2008). Briefly, a 1 s weighing period was used to average 20 measurements and obtain the weight borne by each limb separately. A weight distribution ratio for each animal was then calculated by dividing the injured leg weight over the uninjured leg weight. While control rats distribute their weight about equally, animals with a unilateral injury will shift their weight from an injured to an uninjured limb. This shift is taken as a measure of the level of discomfort in the injured limb. In other words, the less weight placed on an injured limb, the greater the pain.

Thermal hyperalgesia was assessed with the well-established hind paw withdrawal latency test using a plantar analgesia instrument (Stoelting, Chicago, IL, USA). Animals were acclimated for 15–20 min. The ipsilateral, injured, paw was tested four times to obtain an average latency. Each test was separated by 5 min. Thermal withdrawal latencies were obtained both at baseline and 3 or 7 days after sciatic nerve exposure or ligation (second test latency). Animals that served as the corresponding control groups were tested at the same times. The weight-bearing test preceded the second latency test. Whenever possible, persons performing behavioral tests were blinded to treatment.

Anesthesia, sciatic ligation, intrathecal drug application, 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 (chronic constriction injury) was performed using the Bennett and Xie (1988) procedure. The sciatic nerve was exposed and loosely ligated with four simple interrupted 4-0 chromic gut sutures placed about 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.

In some experiments MK-801 (2 μg/μl, Sigma-Aldrich, St. Louis, MO, USA) or saline vehicle were injected intrathecally 15 min before sciatic exposure or ligation in a volume of 10 μl as described previously (Miletic and Miletic, 2008).

For tissue collection animals were anesthetized with isoflurane and while still deeply anesthetized they were euthanized with an intracardiac injection of super-saturated potassium chloride (>350 mg/ml). A laminectomy rapidly (<2 min) exposed the lumbar spinal cord at L5, and the tissue was excised, 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.

RT-PCR, enzyme activity assay and immunoblots

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, CA, USA) as detailed previously (Miyabe et al., 2006). Relative expression levels of calcineurin in each sample were determined using a standard curve of three-fold serial dilutions. Average fold induction relative to control animals was determined after normalizing to the amount of 18S rRNA in each sample. A two-fold or greater change was considered significant. Primer sequences are available upon request.

Calcineurin activity was obtained with a commercial kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) and expressed as nmol of phosphate released/min/mg of protein. Immunoblots were performed as described previously (Miletic et al., 2002). Calcineurin Aα (Millipore, Billerica, MA, USA) was used at a dilution of 1:1000. Developed membranes were stripped and re-probed with beta III tubulin (1:1000; Promega, Madison, WI, USA) as the loading control. Protein levels were estimated with the BioSpectrum 500 Image Analysis System (UVP, Upland, CA, USA). The calcineurin Aα content within a gel was expressed over the beta III tubulin content, and then the content in sham-operated or CCI animals was normalized to those in control animals. The same normalization procedure was used for the enzyme activity assay.

Statistical analysis

ANOVA was used for the statistical data analysis. The main emphasis was on detecting differences in behavior, calcineurin message, activity or protein content between control, sham-operated and CCI animals, or vehicle and drug-treated animals. Significant effects were further analyzed with Scheffe's post hoc test. Statistical difference was inferred at $P \leq 0.05$. The analysis was based on six animals in each group. All data are expressed as mean±SEM.

RESULTS

Signs of pain behavior accompanied CCI

Both 3D and 7D CCI animals placed less weight on their injured, ipsilateral limb as their ipsilateral to contralateral ratio was reduced to 0.55±0.03 and 0.35±0.03, respectively (Fig. 1). In contrast, uninjured control (0.98±0.01), 3D sham (0.91±0.02) or 7D sham (0.88±0.02) animals did not show a similar shift in weight distribution suggestive of pain behavior. ANOVA indicated a significant difference among groups, $F(4,25)=137.1$, $P<0.001$. Scheffe's post hoc test confirmed that the difference was due to the weight-bearing ratio in CCI animals.

In the same 3D and 7D CCI animals there was also a significant reduction in the post-ligation thermal withdrawal latency (second test latency) of the injured, ipsilateral limb (5.9±0.2 s and 5.4±0.2 s) when compared to the pre-surgery baselines (8.2±0.2 s and 7.8±0.2 s, respectively). In contrast, there was no significant reduction in the second test thermal withdrawal latency in control (8.0±0.3 s vs. 8.3±0.3 s), 3D sham (7.5±0.3 s vs. 7.9±0.3 s) or 7D sham (7.6±0.4 s vs. 7.6±0.4 s) animals. ANOVA confirmed no difference among groups at baseline, $F(4,25)=0.8$, $P<1$, but a significant difference later, $F(4,25)=16.5$, $P<0.001$. This difference was due to the second test latencies in the 3D and 7D CCI animals.

Time-dependent differential changes in calcineurin message, activity and Aα protein content were associated with CCI

After the last behavioral test, the animals were anesthetized, euthanized and their tissues collected for the biochemical assays.