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Depression of Ca²⁺/Calmodulin-Dependent Protein Kinase II in Dorsal Root Ganglion Neurons after Spinal Nerve Ligation

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Abstract

The enzyme calcium/calmodulin-dependent protein kinase II (CaMKII) is associated with memory and its α isoform is critical for development of activity-induced synaptic changes. Therefore, we hypothesized that CaMKII is involved in altered function of dorsal root ganglion (DRG) neurons after neuronal injury. To test this hypothesis, Sprague–Dawley rats were made hyperalgesic by L5 and L6 spinal nerve ligation (SNL), and changes in total phosphorylated and unphosphorylated CaMKII (tCaMKII) and phosphorylated form of its α isoform (pCaMKII α) were analyzed using immunocytochemistry in different subpopulations of DRG. SNL did not induce any changes in tCaMKII between experimental groups, while the overall percentage of pCaMKII α -positive neurons in injured L5 DRG SNL (24.8%) decreased significantly when compared to control (41.7%). SNL did not change the percentage of pCaMKII α /N52 colabeled neurons but decreased the percentage of N52-negative nonmyelinated neurons that expressed pCaMKII α from 27% in control animals to 11% after axotomy. We also observed a significant decrease in the percentage of small nonpeptidergic neurons labeled with IB4 (37.6% in control vs. 4.0% in L5 SNL DRG), as well as a decrease in the percentage of pCaMKII α /IB4 colabeled neurons in injured L5 DRGs (27% in control vs. 1% in L5 DRG of SNL group). Our results show that reduction in pCaMKII α levels following peripheral injury is due to the loss of IB4-positive neurons. These results indicate that diminished afferent activity after axotomy may lead to decreased phosphorylation of CaMKII α .

INDEXING TERMS

neuropathic pain; spinal nerve ligation; primary sensory neurons; calcium/calmodulin-dependent protein kinase II

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a downstream effector of multiple signaling pathways designed to convert cytoplasmic Ca²⁺ ([Ca²⁺]_c) signals into diverse responses through phosphorylation of various substrates involved in exocytosis,

transcriptional, and translational processes (Fink and Meyer, 2002). Phosphorylated CaMKII (pCaMKII) remains active even after the resolution of the $[Ca^{2+}]_c$ signal decays, making it a molecular memory of activity history (Miller and Kennedy, 1985; Lai et al., 1986) and a frequency detector, particularly in sensory neurons (Eshete and Fields, 2001; Hudmon and Schulman, 2002). By decoding Ca^{2+} oscillations, CaMKII activity also regulates synaptic activity (Colbran, 2004).

There are four different isoforms of CaMKII: α , β , γ , and δ , but only the α and β isoforms are expressed exclusively in nervous system (Ochiishi et al., 1994). The ubiquitous γ and δ isoforms are the most abundant in the developing nervous system (Bayer et al., 1999) but substantial up-regulation of the α isoform is observed postnatally (Pattinson et al., 2006). There is substantial evidence that the α isoform is critical for development of activity-induced synaptic changes (Stevens et al., 1994; Ashraf et al., 2006) and in pain processing at the spinal (Fang et al., 2002; Zeitz et al., 2004; Choi et al., 2006) and supraspinal level (Bruggemann et al., 2000; Ogawa et al., 2005; Wei et al., 2006; Jones et al., 2007). However, there has been only limited investigation of its involvement in pain processing at the level of primary afferent neurons. Our previous work has identified loss of both inward Ca^{2+} current (I_{Ca}) and outward Ca^{2+} -activated K^+ current ($I_{K(Ca)}$) in axotomized sensory neurons as a factor contributing to pain after nerve injury (Hogan et al., 2000; McCallum et al., 2006; Sarantopoulos et al., 2007). Since these currents are also subject to CaMKII regulation in various tissues (Welsby et al., 2003; Liu et al., 2007; Jiang et al., 2008), this kinase can be considered a potential mediator of neuropathic pain.

Our aim was to investigate potential involvement of CaMKII in the genesis of neuropathic pain. We therefore studied changes in levels of total phosphorylated and un-phosphorylated CaMKII (tCaMKII) and separately determined the fraction of CaMKII α that has been activated by phosphorylation (pCaMKII α) in dorsal root ganglion (DRG) neurons after neuronal injury. Sensory neurons are a diverse population, so in addition we determined injury-induced changes of pCaMKII α in specific subpopulations of neurons. Large myelinated neurons are recognized by 200 kD neurofilament protein N52 (Robertson et al., 1991), whereas those lacking N52 are small, slowly conducting nonmyelinated C-type neurons, most of which are nociceptive-specific. Among the small neurons, those that lack peptidergic neurotransmission may be distinguished by binding of isolectin from *Griffonia simplicifolia* (IB4) (Silverman and Kruger, 1990). All other sensory neurons, including myelinated nonnociceptors and peptidergic C-type nociceptors, lack IB4 binding.

MATERIALS AND METHODS

Experimental animals and surgical procedure

All experimental procedures and protocols followed the IASP Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals and were approved by the Ethical Committee of the University of Split School of Medicine and the Medical College of Wisconsin. A total of 57 male Sprague–Dawley rats were randomly assigned to spinal nerve ligation (SNL) ($n = 33$), sham surgery ($n = 11$), and the control group ($n = 13$).

Anesthesia was induced with 5% isoflurane (Forane, Abbott Laboratories, Queensborough, UK) and then maintained with 2% isoflurane. SNL was performed according to a slightly modified method described by Kim and Chung (1992; Hogan et al., 2004). Briefly, after exposure of the right paravertebral region, the sixth lumbar transverse process was carefully removed. The L5 and L6 spinal nerves were ligated with 6-0 silk suture and transected distally. Sham surgery was a similar procedure except that the nerves were not ligated and transected. Animals in the control group were only anesthetized and their lumbar skin incised in order to blind the observer performing behavioral testing.

Behavioral testing

Behavioral testing was performed on the day preceding the SNL surgery and three times postoperatively (4th, 8th, and 15th day). Testing was performed as previously described (Hogan et al., 2004). Our overall strategy involved measuring differences in responses to noxious punctate mechanical stimulation between paws contralateral and ipsilateral to nerve injury over time. Testing was performed with the rats on a mesh-wire surface (3×3 mm), placed individually in clear plastic enclosures ($10 \times 25 \times 30$ cm). A 22G spinal needle was applied to the mid-plantar surface of the hindpaw of unrestrained rats with a force adequate to indent but not injure the skin. Normal animals withdraw the paw with a brief reflexive flinch with immediate return of the foot to the cage floor. Following SNL, pin testing elicits either the same simple withdrawal as in normal animals, or a complex response with sustained elevation of the paw, shaking, licking, and grooming that only occurs ipsilateral to the injury. The occurrence rate of this hyperalgesia-type response was recorded for each of 10 applications, averaged, and expressed as a probability (Hogan et al., 2004).

Immunohistochemistry

Rats were sacrificed at the end of behavioral testing series 2 weeks following the surgery by anaesthetic exposure (isoflurane; Forane, Abbott Laboratories). The L4 and L5 ganglions were removed and fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS, pH = 7.4). Following overnight cryoprotection in 30% sucrose, ganglia were embedded in Optimal Cutting Temperature (OCT) freezing medium (Tissue Tek, Tokyo, Japan) and sectioned on a cryostat (Thermo Shandon Cryotome, Pittsburgh, PA). Sections 10 μ m thick were cut parallel to the long axis of the ganglia and placed on silane-coated glass slides. All incubations were done overnight at room temperature and 0.3% Triton X-100 in PBS was used for rinsing.

For detection of tCaMKII, sections were treated with rabbit anti-CaMKII polyclonal antibody (Table 1 and Primary antibody characterization section) designed for detection of CaMKII isoforms in phosphorylated and unphosphorylated states. For detection of pCaMKII α , sections were subjected to rabbit pCaMKII α primary polyclonal antibody recommended for detection of the α isoform in phosphorylated form (Table 1 and Primary antibody characterization section).

For simultaneous staining of pCaMKII α and N52 (1:1,000) or IB4 (1:50) (details for both antibodies in the Primary antibody characterization section), sections were incubated

overnight with a mixture of antibodies, or in the case of IB4 with the mixture of primary antibody and IB4 conjugate.

Secondary detection was performed using two detection systems. The first one was based on goat anti-rabbit Texas red secondary antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA; Cat. No. sc-2780, lot G1905) or goat anti-mouse Texas Red secondary antibody (1:100; Molecular Probes, Eugene, OR; Cat. No. T-862, lot 71C3-1). The second consisted of biotinylated goat anti-rabbit secondary antibody IgG-B (1:100, Santa Cruz Biotechnology; Cat. No. sc-2040, lot J0306) and followed by Streptavidin Alexa Fluor 488 conjugate (1:500; Molecular Probes; Cat. No. S-32354, lot 43500A). After final rinsing in PBS, slides were mounted, air-dried, and coverslipped (Immu-Mount, Shandon, Pittsburgh, PA). Staining controls included omission of primary antibody or IB4 conjugate from the staining procedure, which resulted in no staining in DRG tissue. In the SNL group, both L4 and L5 DRG sections were stained and analyzed, while in the control and sham groups, sections of L5 DRG were analyzed only.

Primary antibody characterization

Table 1 lists all primary antibodies used. tCaMKII antibody was purchased from Gorea plus d.o.o. (Sveta Nedelja, Croatia; manufacturer, Santa Cruz Biotechnology). This polyclonal antibody detects all CaMKII subunits of mouse, rat, and human origin (manufacturer's technical datasheet) and has been previously characterized. It identifies a single band of 50 kD protein on Western blot analysis of CaMKII expression in the rat brain extract (manufacturer's technical datasheet) corresponding to the expected molecular weight of CaMKII. An identical band was identified in mouse cholangiocytes (Francis et al., 2008), human astrocytes and neurons (Song et al., 2006), and rat brain extract (Rose and Hargreaves, 2003).

The pCaMKII α antibody (source, Gorea plus d.o.o.; Santa Cruz Biotechnology) is a polyclonal affinity-purified antibody raised in rabbit against a short amino acid sequence (aa 282 HRQET(-phospho) VDCLK 291) containing phosphorylated Thr 286 of CaMKII α of human origin. The sequence of immunizing peptide was determined using the mass spectrometer 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems, Foster City, CA) equipped with a 200 Hz, 355 nm Nd:YAG laser. This antibody has been previously characterized. In mouse brain extracts analyzed by Western blotting this antibody recognizes a single band of $\approx$ 50 kD protein corresponding to the expected molecular weight of CaMKII (manufacturer's technical datasheet). Similar bands were identified in extracts from human astrocytes (Song et al., 2006) and rat hippocampal neurons (Tai et al., 2008). Moreover, specificity of the pCaMKII α antibody was confirmed by preadsorption control with appropriate blocking peptide (source, Gorea plus d.o.o.; Santa Cruz Biotechnology, Cat. No. sc-12886-P, lot D2607).

Mouse anti-NF200 monoclonal antibody (source, Gorea plus d.o.o.; Chemicon International) was raised against the carboxy terminal tail segment of enzymatically dephosphorylated porcine H-chain of neurofilament 200 kD. The antibody reacts with the phosphorylated and dephosphorylated H-chain of neurofilament 200 kD and shows a broad crossreactivity, including the rat (manufacturer's information). This antibody does not crossreact with the

other intermediate filament proteins (manufacturer's information). It is well established as a marker of myelinated DRG neurons (Lawson and Waddell, 1991; Hammond et al., 2004) and was previously characterized (Harris et al., 1991; Gotow and Tanaka, 1994; Hammond et al., 2004; Amadesi et al., 2009).

Isolectin B4 (IB4) agglutinin was isolated from *Bandeiraea simplicifolia* and highly purified by affinity chromatography. *Bandeiraea simplicifolia* agglutinin has a major affinity for terminal alpha-D-galactosyl residues with a secondary affinity for terminal N-acetyl-alpha-D-galactosaminyl residues. We used isolectin conjugated to fluorescein isothiocyanate (FITC) that allows detection in binding assays by fluorescence (IB4 FITC labeled; source, Biovit d.o.o, Varazdin, Croatia; manufacturer, Sigma-Aldrich, St. Louis, MO; Cat. No. L 2895, lot 103K).

Quantitative analysis for immunohistochemistry

Every fourth section of each DRG was examined under an upright microscope (Olympus, BX61) equipped with a confocal unit (CSU10, Yokogawa, Japan) and argon/krypton laser source (Melles Griot, Irvine, CA) by an observer who was blind to the group of origin for the tissue. Microphotographs were captured using a digital camera (Spot Pursuit, Diagnostic Instruments, Sterling Heights, MI) controlled by Metamorph software (Molecular Devices, Downingtown, PA), using the same magnification (40×), time exposition, binning, and gain for each image.

Each micrograph was divided into $200 \times 200 \mu\text{m}$ squares with at least 80% surface occupied by ganglion neurons. Only the neurons with clearly visible nuclei and those underlying the right and upper borders of the square were counted. The procedure included determination of the total number of ganglion neurons and the number of immunopositively labeled neurons from each area of interest. The percentage was calculated by dividing pCaMKII α -immunopositive or double-labeled neurons (immunostained for both pCaMKII α and IB4 or N52 neurons) by the total number of neurons. To avoid double counting, Abercrombie's correction factor was used (Abercrombie, 1946). In this method, nuclear profiles' counts (n) are multiplied by section thickness (T) and divided by the mean nuclear diameter plus section thickness to yield "true" neuron numbers (N): $n = n[T/(T+H)]$. For calculating the mean nuclear diameter, 10 cells were randomly selected from each neuronal class.

The diameters of immunofluorescently labeled DRG neurons were calculated by summing the length and width of neurons with a visible nucleus and dividing the sum by two.

Microphotograph production

Microphotographs were captured using Metamorph software 7.1.4. (Molecular Devices) under the same magnification (40×), time exposition, binning, and gain for each image. No background subtraction was performed. Images were then imported into Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) where the plates were assembled and text and scale bars were added. All modifications to the microphotographs were performed in an identical fashion for all DRGs on the same slide.

Statistical analysis

Comparisons between control, sham, SNL L4, and L5 neuronal groups were made by *t*-test or one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. Behavioral test scores were analyzed with repeated-measures ANOVA (Statistica 7.0; StatSoft, Tulsa, OK). The data were statistically analyzed and are presented as mean $\pm$ SD. Any difference with $P < 0.05$ was considered statistically significant.

RESULTS

SNL enhanced pain-related behavior

As noted previously (Hogan et al., 2004; Znaor et al., 2007), SNL resulted in a significantly greater probability of hyperalgesia-type response (ANOVA repeated-measurements, $F = 5.12$, $P < 0.001$). Before surgery, the mean difference in hyperalgesia response probability between right and left hind paws was 0.01 ± 0.002 . Following SNL, the probability increased to 0.13 ± 0.17 on the 4th day, 0.13 ± 0.18 on the 8th, and 0.08 ± 0.11 on the 15th postoperative day.

SNL reduced the pCaMKII α in DRG neurons

We observed no differences between the various groups in the percentage of the neurons positive for tCaMKII (ANOVA, $F = 0.79$, $P = 0.5$; Fig. 1). However, the number of pCaMKII α -labeled neurons in injured L5 DRG after SNL decreased significantly in comparison to control, sham, and neighboring L4 DRG neurons (ANOVA, $F = 75.5$, $P < 0.0001$; Fig. 1). The percentage of neurons positive for pCaMKII α was significantly smaller than the percentage positive for tCaMKII in all groups (Figs. 1, 4A,B, 5C,D).

The analyses of cell diameter distribution place tCaMKII and pCaMKII α -positive neurons in the small to medium range (Fig. 2A,B). The mean diameter of tCaMKII-positive neurons was similar in all groups (range, from $23.5 \pm 4.9 \mu\text{m}$ in SNL group to $24.5 \pm 5.0 \mu\text{m}$ in L4 group; $F = 2.94$, $P = 0.053$).

Our starting assumption was that all pCaMKII neurons are contained within the tCaMKII group. Size determination shows that neurons positive for pCaMKII α overall have a larger diameter than the tCaMKII positive neurons (Fig. 2C,D). Specifically, the mean diameter of pCaMKII α -positive neurons in the control group was $26.0 \pm 5.3 \mu\text{m}$, $26.5 \pm 5.2 \mu\text{m}$ in the sham, and $26.1 \pm 4.7 \mu\text{m}$ in the SNL L4 group (data not shown). The proportion of larger neurons was significantly higher in the SNL L5 group (mean diameter $29.8 \pm 7.4 \mu\text{m}$) in comparison to the merged control and sham group (*t*-test, $t = 9.33$, $P < 0.001$).

SNL decreased the percentage of neurofilament N52-negative neurons that coexpress pCaMKII α

To investigate axotomy-related changes in a subset of myelinated large-diameter neurons, we performed double immunofluorescence staining for N52 and pCaMKII α . There were no significant changes in the percentage of neurons labeled with N52 in either L5 SNL DRG (29.9%) or L4 SNL DRG (35.9%) compared to both control (35.5%) and sham (29.9%) DRGs (ANOVA, $F = 1.8$, $P = 0.16$).

Double labeling of N52-positive DRG neurons with pCaMKII α showed that 40% of N52-labeled neurons (or 14% of the total population) in control group also express pCaMKII α . The proportion of these colabeled (pCaMKII α /N52 positive) neurons in the total neuron population did not differ significantly between groups (ANOVA, $F = 2.25$, $P = 0.09$). Thus, the spinal nerve ligation did not induce any change in the proportion of N52 labeled neurons or proportion of pCaMKII α /N52 colabeled neurons (Figs. 3, 4). The proportion of N52-negative neurons that stained positively for pCaMKII α , however, was diminished by injury (Fig. 3B,C).

Cell-size analysis of neurons that are positively labeled for pCaMKII α and N52 showed that this population belongs to the group of medium-sized neurons (Fig. 2E). The mean diameters of these pCaMKII α /N52-positive neurons were $34.9 \pm 6.4 \mu\text{m}$ in the L5 SNL group, $34.8 \pm 6.2 \mu\text{m}$ in the L4 SNL group, $34.7 \pm 5.1 \mu\text{m}$ in the control group, and $33.2 \pm 4.5 \mu\text{m}$ in the sham group (not significant; ANOVA, $F = 1.4$, $P = 0.25$).

SNL decreases the population of pCaMKII α /IB4-colabeled neurons

SNL caused a marked decrease in the number of neurons labeled with IB4 in L5 ganglia (4.0%) in comparison to the control group (38%; ANOVA, $F = 139.6$, $P < 0.0001$; Fig. 5B). When compared to control, no significant changes in number of IB4-labeled neurons were observed in L4 SNL DRGs (33.1%) or the sham group (35.9%).

Spinal nerve ligation significantly reduced the percentage of pCaMKII α /IB4-colabeled DRG neurons from 26.4% of the total number of neurons in the control group, to 1.4% in the SNL L5 group (ANOVA, $F = 108.3$, $P < 0.001$; Figs. 5, 6). The percentage of all neurons that bind both pCaMKII α and IB4 in the sham group (23.8%) and the L4 SNL group (22.9%) did not differ significantly from control (Fig. 6).

As shown in Fig. 2F, neurons colabeled for pCaMKII α /IB4 were almost all small to medium-sized and we found no statistically significant differences among experimental groups (control group $25.5 \pm 3.5 \mu\text{m}$, SNL L5 group $26.1 \pm 2.3 \mu\text{m}$, sham group $25.4 \pm 4.2 \mu\text{m}$, and SNL L4 $24.8 \pm 3.9 \mu\text{m}$; ANOVA, $F = 1.28$, $P = 0.28$).

DISCUSSION

The main finding of this study is that axotomy of peripheral sensory neurons decreases the level of pCaMKII α in a specific subset of DRG neurons. Injury changed the phenotype of the entire population of non-peptidergic, IB4-binding small neurons, such that fewer neurons staining for both pCaMKII α and IB4 were evident after injury. Since the frequency of pCaMKII α positivity is much higher in the IB4-binding population than in those neurons that do not bind IB4, the loss of this population may explain our other finding of a shift toward a lower percentage of small, N52-negative neurons that are pCaMKII α -positive after injury (11%) compared to controls (27%). Since the level of total CaMKII is not affected by injury, the overall decrease in pCaMKII α indicates diminished kinase activation through phosphorylation of existing CaMKII.

It has been previously reported that a substantial number of DRG neurons that express CaMKII also express putative markers that identify nociceptive neurons (Carlton and Hargett, 2002). Since axotomy in rats induces a rapid decrease of neurons labeled with IB4 (Hammond et al., 2004), the observed reduction in our data was expected. Colocalization of this subpopulation of neurons with pCaMKII α confirmed that this enzyme is present in small nociceptive neurons that are affected by the nerve injury. The population of sensory neurons that convey nociceptive information also includes small, unmyelinated neurons that release peptide neurotransmitters. These do not bind IB4, and in our data are included in the N52-negative group. The loss of CaMKII phosphorylation in the population of nociceptive neurons may contribute to pain generation through regulation of neuronal excitability via effects of CaMKII phosphorylation on channels that convey I_{Ca} and I_{K(Ca)} (Hogan et al., 2000; McCallum et al., 2006; Sarantopoulos et al., 2007). Axotomy did not alter the fraction of myelinated N52-positive neurons that were positive for pCaMKII α , suggesting that CaMKII signaling in the nonnociceptive neuronal population is not affected by injury.

Previous immunohistochemical studies that examined CaMKII expression in peripheral sensory neurons have also identified CaMKII expression in nociceptors (Carlton, 2002; Carlton and Hargett, 2002; Ichikawa et al., 2004). These results are generally compatible with our findings. The higher overall rate of positive neurons in our data may reflect variability in antibody specificity, staining technique, imaging parameters, and different thresholding during positive cell counting. However, cell size of pCaMKII α -positive neurons is almost the same as in the mentioned studies.

Carlton and Hargett (2002) demonstrated that CaMKII α is present in 44% of small to medium L5 DRG neurons. Among those neurons 38% (or 17% of all DRG neurons) were double stained for IB4. In a similar study, Carlton (2002) also demonstrated that 28% of total DRG neurons are positively stained for CaMKII α . A study of cranial sensory ganglia (Ichikawa et al., 2004) showed that more than 50% of neurons in trigeminal, glossopharyngeal, and vagal ganglia, which are predominantly small to medium-sized, contain CaMKII.

Involvement of CaMKII in the peripheral nervous system after axotomy has not previously been examined. However, axotomy decreases the proportion of CaMKIV-positive DRG neurons and injury effects on CNS (Ji et al., 1996). Changes in CaMKII have been determined in other models of peripheral nerve injury. Specifically, following chronic constriction injury of the sciatic nerve, a large increase in the number of tCaMKII and pCaMKII α -immunoreactive profiles was observed in superficial layers of dorsal horn. This CaMKII α upregulation was N-methyl-D-aspartic acid (NMDA)-dependent and was present up to 14 days after injury, suggesting that CaMKII α is involved in the generation and/or maintenance of Ca²⁺-mediated central sensitization in dorsal horn neurons (Dai et al., 2005). Similarly, inferior alveolar nerve transection increased CaMKII α and pCaMKII α in the superficial laminae of the trigeminal subnucleus caudalis, and behavioral consequences were reversed by a CaMKII α inhibitor (Ogawa et al., 2005). These findings implicate CaMKII in the regulation of pain at central sites.

SNL alters the immunohistochemical phenotype of injured primary afferent neurons in the L5 DRGs, causing a rapid decrease of the number of IB4-labeled neurons and a sustained decrease of CGRP-immunoreactive neurons without any changes in the number of N52-labeled neurons (Hammond et al., 2004). A marked loss of IB4 staining 2 weeks after ligation was present in our study together with an unchanged fraction of N52-positive neurons. However, the number of IB4-binding neurons may recover within 20 weeks, together with the appearance of a novel population of very small-sized neurons labeled by N52 (Neumann et al., 1996; Hammond et al., 2004). These studies demonstrate a loss of expression of neuronal markers in DRGs supplying the transected spinal nerve, confirming that neuronal phenotypes may be quite labile after injury. A switch toward a nociceptor-like electrophysiological phenotype is also observed following SNL (Sapunar et al., 2005). Currently, the molecular mechanisms responsible for these phenotypic changes remain unknown.

The present study also confirmed our previous findings that neighboring L4 DRG neurons are minimally affected by injury (Sapunar et al., 2005). Specifically, axotomy of the L5 DRG neurons did not alter the percent of pCaMKII α or pCaMKII α /IB4-positive neurons in L4 DRG.

Disconnection of axotomized DRG neurons from their receptive field after peripheral nerve injury may reduce activity in these neurons and result in depressed pCaMKII α levels, as noted here. A variety of evidence indicates a potential role of CaMKII in modulation of calcium currents (Hao et al., 2008; Jiang et al., 2008). Altered CaMKII regulation of I_{Ca} (N- and L-type currents) and I_{K(Ca)} may thus link our present results with previous findings that peripheral nerve injury decreases I_{Ca} and I_{K(Ca)} in axotomized neurons (Hogan et al., 2000; McCallum et al., 2003; Sarantopoulos et al., 2007). Since a loss of I_{Ca} and I_{K(Ca)} results in neuronal hyperexcitability (Hogan et al., 2008; Lirk et al., 2008), the diminished activation of CaMKII noted in the present study may in part account for DRG neuron hyperexcitability and pain that follows nerve injury (Sapunar et al., 2005).

CONCLUSION

Our finding that SNL decreases the level of pCaMKII α in sensory neurons may indicate an involvement of this enzyme in transmission of nociceptive input after peripheral nerve injury. This novel mechanism may be a suitable target for therapy in neuropathic pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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LITERATURE CITED

- Abercrombie M. Estimation of nuclear population from microtome sections. *Anat Rec.* 1946; 94:239–247. [PubMed: 21015608]
- Amadesi S, Grant AD, Cottrell GS, Vaksman N, Poole DP, Rozengurt E, Bunnett NW. Protein kinase D isoforms are expressed in rat and mouse primary sensory neurons and are activated by agonists of protease-activated receptor 2. *J Comp Neurol.* 2009; 516:141–156. [PubMed: 19575452]
- Ashraf SI, McLoon AL, Sclarsic SM, Kunes S. Synaptic protein synthesis associated with memory is regulated by the RISC pathway in *Drosophila*. *Cell.* 2006; 124:191–205. [PubMed: 16413491]
- Bayer KU, Lohler J, Schulman H, Harbers K. Developmental expression of the CaM kinase II isoforms: ubiquitous gamma-and delta-CaM kinase II are the early isoforms and most abundant in the developing nervous system. *Brain Res Mol Brain Res.* 1999; 70:147–154. [PubMed: 10381553]
- Bruggemann I, Schulz S, Wiborny D, Holtt V. Colocalization of the mu-opioid receptor and calcium/calmodulin-dependent kinase II in distinct pain-processing brain regions. *Brain Res Mol Brain Res.* 2000; 85:239–250. [PubMed: 11146127]
- Carlton SM. Localization of CaMKIIalpha in rat primary sensory neurons: increase in inflammation. *Brain Res.* 2002; 947:252–259. [PubMed: 12176168]
- Carlton SM, Hargett GL. Stereological analysis of Ca(2+)/calmodulin-dependent protein kinase II alpha-containing dorsal root ganglion neurons in the rat: colocalization with isolectin Griffonia simplicifolia, calcitonin gene-related peptide, or vanilloid receptor 1. *J Comp Neurol.* 2002; 448:102–110. [PubMed: 12012376]
- Choi SS, Seo YJ, Shim EJ, Kwon MS, Lee JY, Ham YO, Suh HW. Involvement of phosphorylated Ca2+/calmodulin-dependent protein kinase II and phosphorylated extracellular signal-regulated protein in the mouse formalin pain model. *Brain Res.* 2006; 1108:28–38. [PubMed: 16863646]
- Colbran RJ. Targeting of calcium/calmodulin-dependent protein kinase II. *Biochem J.* 2004; 378(Pt 1): 1–16. [PubMed: 14653781]
- Dai Y, Wang H, Ogawa A, Yamanaka H, Obata K, Tokunaga A, Noguchi K. Ca2+/calmodulin-dependent protein kinase II in the spinal cord contributes to neuropathic pain in a rat model of mononeuropathy. *Eur J Neurosci.* 2005; 21:2467–2474. [PubMed: 15932604]
- Eshete F, Fields RD. Spike frequency decoding and autonomous activation of Ca2+-calmodulin-dependent protein kinase II in dorsal root ganglion neurons. *J Neurosci.* 2001; 21:6694–6705. [PubMed: 11517259]
- Fang L, Wu J, Lin Q, Willis WD. Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. *J Neurosci.* 2002; 22:4196–4204. [PubMed: 12019337]
- Fink CC, Meyer T. Molecular mechanisms of CaMKII activation in neuronal plasticity. *Curr Opin Neurobiol.* 2002; 12:293–299. [PubMed: 12049936]
- Francis H, Glaser S, Demorrow S, Gaudio E, Ueno Y, Venter J, Dostal D, Onori P, Franchitto A, Marzoni M, Vaculin S, Vaculin B, Katki K, Stutes M, Savage J, Alpini G. Small mouse cholangiocytes proliferate in response to H1 histamine receptor stimulation by activation of the IP3/CaMK I/CREB pathway. *Am J Physiol Cell Physiol.* 2008; 295:C499–513. [PubMed: 18508907]
- Gotow T, Tanaka J. Phosphorylation of neurofilament H subunit as related to arrangement of neurofilaments. *J Neurosci Res.* 1994; 37:691–713. [PubMed: 8046771]
- Hammond DL, Ackerman L, Holdsworth R, Elzey B. Effects of spinal nerve ligation on immunohistochemically identified neurons in the L4 and L5 dorsal root ganglia of the rat. *J Comp Neurol.* 2004; 475:575–589. [PubMed: 15236238]
- Hao LY, Xu JJ, Minobe E, Kameyama A, Kameyama M. Calmodulin kinase II activation is required for the maintenance of basal activity of L-type Ca2+ channels in guinea-pig ventricular myocytes. *J Pharmacol Sci.* 2008; 108:290–300. [PubMed: 19023178]
- Harris J, Ayyub C, Shaw G. A molecular dissection of the carboxyterminal tails of the major neurofilament subunits NF-M and NF-H. *J Neurosci Res.* 1991; 30:47–62. [PubMed: 1724473]
- Hogan QH, McCallum JB, Sarantopoulos C, Aason M, Mynlieff M, Kwok WM, Bosnjak ZJ. Painful neuropathy decreases membrane calcium current in mammalian primary afferent neurons. *Pain.* 2000; 86:43–53. [PubMed: 10779659]

- Hogan Q, Sapunar D, Modric-Jednacak K, McCallum JB. Detection of neuropathic pain in a rat model of peripheral nerve injury. *Anesthesiology*. 2004; 101:476–487. [PubMed: 15277932]
- Hogan Q, Lirk P, Poroli M, Rigaud M, Fuchs A, Fillip P, Ljubkovic M, Gemes G, Sapunar D. Restoration of calcium influx corrects membrane hyperexcitability in injured rat dorsal root ganglion neurons. *Anesth Analg*. 2008; 107:1045–1051. [PubMed: 18713927]
- Hudmon A, Schulman H. Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annu Rev Biochem*. 2002; 71:473–510. [PubMed: 12045104]
- Ichikawa H, Gouty S, Regalia J, Helke CJ, Sugimoto T. Ca2+/calmodulin-dependent protein kinase II in the rat cranial sensory ganglia. *Brain Res*. 2004; 1005:36–43. [PubMed: 15044062]
- Ji RR, Shi TJ, Xu ZQ, Zhang Q, Sakagami H, Tsubochi H, Kondo H, Hokfelt T. Ca2+/calmodulin-dependent protein kinase type IV in dorsal root ganglion: colocalization with peptides, axonal transport and effect of axotomy. *Brain Res*. 1996; 721:167–173. [PubMed: 8793097]
- Jiang X, Lautermilch NJ, Watari H, Westenbroek RE, Scheuer T, Catterall WA. Modulation of CaV2.1 channels by Ca2+/calmodulin-dependent protein kinase II bound to the C-terminal domain. *Proc Natl Acad Sci U S A*. 2008; 105:341–346. [PubMed: 18162541]
- Jones TL, Lustig AC, Sorkin LS. Secondary hyperalgesia in the postoperative pain model is dependent on spinal calcium/calmodulin-dependent protein kinase II alpha activation. *Anesth Analg*. 2007; 105:1650–1656. [PubMed: 18042863]
- Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain*. 1992; 50:355–363. [PubMed: 1333581]
- Lai Y, Nairn AC, Greengard P. Autophosphorylation reversibly regulates the Ca2+/calmodulin-dependence of Ca2+/calmodulin-dependent protein kinase II. *Proc Natl Acad Sci U S A*. 1986; 83:4253–4257. [PubMed: 3012560]
- Lawson SN, Waddell PJ. Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. *J Physiol*. 1991; 435:41–63. [PubMed: 1770443]
- Lirk P, Poroli M, Rigaud M, Fuchs A, Fillip P, Huang CY, Ljubkovic M, Sapunar D, Hogan Q. Modulators of calcium influx regulate membrane excitability in rat dorsal root ganglion neurons. *Anesth Analg*. 2008; 107:673–685. [PubMed: 18633052]
- Liu Q, Chen B, Ge Q, Wang ZW. Presynaptic Ca2+/calmodulin-dependent protein kinase II modulates neuro-transmitter release by activating BK channels at *Caenorhabditis elegans* neuromuscular junction. *J Neurosci*. 2007; 27:10404–10413. [PubMed: 17898212]
- McCallum JB, Kwok WM, Mynlieff M, Bosnjak ZJ, Hogan QH. Loss of T-type calcium current in sensory neurons of rats with neuropathic pain. *Anesthesiology*. 2003; 98:209–216. [PubMed: 12502999]
- McCallum JB, Kwok WM, Sapunar D, Fuchs A, Hogan QH. Painful peripheral nerve injury decreases calcium current in axotomized sensory neurons. *Anesthesiology*. 2006; 105:160–168. [PubMed: 16810008]
- Miller SG, Kennedy MB. Distinct forebrain and cerebellar isozymes of type II Ca2+/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. *J Biol Chem*. 1985; 260:9039–9046. [PubMed: 4019461]
- Neumann S, Doubell TP, Leslie T, Woolf CJ. Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. *Nature*. 1996; 384:360–364. [PubMed: 8934522]
- Ochiishi T, Terashima T, Yamauchi T. Specific distribution of Ca2+/calmodulin-dependent protein kinase II alpha and beta isoforms in some structures of the rat forebrain. *Brain Res*. 1994; 659:179–193. [PubMed: 7820660]
- Ogawa A, Dai Y, Yamanaka H, Iwata K, Niwa H, Noguchi K. Ca(2+)/calmodulin-protein kinase IIalpha in the trigeminal subnucleus caudalis contributes to neuropathic pain following inferior alveolar nerve transection. *Exp Neurol*. 2005; 192:310–319. [PubMed: 15755548]
- Pattinson D, Baccei M, Karadottir R, Torsney C, Moss A, McCutcheon J, Giese KP, Fitzgerald M. Aberrant dendritic branching and sensory inputs in the superficial dorsal horn of mice lacking CaMKIIalpha autophosphorylation. *Mol Cell Neurosci*. 2006; 33:88–95. [PubMed: 16875841]

- Robertson B, Perry MJ, Lawson SN. Populations of rat spinal primary afferent neurons with choleragenoid binding compared with those labelled by markers for neurofilament and carbohydrate groups: a quantitative immunocytochemical study. *J Neurocytol.* 1991; 20:387–395. [PubMed: 1869879]
- Rose AJ, Hargreaves M. Exercise increases Ca²⁺-calmodulin-dependent protein kinase II activity in human skeletal muscle. *J Physiol.* 2003; 553(Pt 1):303–309. [PubMed: 14565989]
- Sapunar D, Ljubkovic M, Lirk P, McCallum JB, Hogan QH. Distinct membrane effects of spinal nerve ligation on injured and adjacent dorsal root ganglion neurons in rats. *Anesthesiology.* 2005; 103:360–376. [PubMed: 16052119]
- Sarantopoulos CD, McCallum JB, Rigaud M, Fuchs A, Kwok WM, Hogan QH. Opposing effects of spinal nerve ligation on calcium-activated potassium currents in axotomized and adjacent mammalian primary afferent neurons. *Brain Res.* 2007; 1132:84–99. [PubMed: 17184741]
- Silverman JD, Kruger L. Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. *J Neurocytol.* 1990; 19:789–801. [PubMed: 2077115]
- Song JH, Bellail A, Tse MC, Yong VW, Hao C. Human astrocytes are resistant to Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *J Neurosci.* 2006; 26:3299–3308. [PubMed: 16554480]
- Stevens CF, Tonegawa S, Wang Y. The role of calcium-calmodulin kinase II in three forms of synaptic plasticity. *Curr Biol.* 1994; 4:687–693. [PubMed: 7953554]
- Tai Y, Feng S, Ge R, Du W, Zhang X, He Z, Wang Y. TRPC6 channels promote dendritic growth via the CaMKIV-CREB pathway. *J Cell Sci.* 2008; 121(Pt 14):2301–2307. [PubMed: 18559891]
- Wei F, Wang GD, Zhang C, Shokat KM, Wang H, Tsien JZ, Liauw J, Zhuo M. Forebrain overexpression of CaMKII abolishes cingulate long term depression and reduces mechanical allodynia and thermal hyperalgesia. *Mol Pain.* 2006; 2:21. [PubMed: 16776832]
- Welsby PJ, Wang H, Wolfe JT, Colbran RJ, Johnson ML, Barrett PQ. A mechanism for the direct regulation of T-type calcium channels by Ca²⁺/calmodulin-dependent kinase II. *J Neurosci.* 2003; 23:10116–10121. [PubMed: 14602827]
- Zeitz KP, Giese KP, Silva AJ, Basbaum AI. The contribution of autophosphorylated alpha-calmodulin-calmodulin kinase II to injury-induced persistent pain. *Neuroscience.* 2004; 128:889–898. [PubMed: 15464294]
- Znaor L, Lovric S, Hogan Q, Sapunar D. Association of neural inflammation with hyperalgesia following spinal nerve ligation. *Croat Med J.* 2007; 48:35–42. [PubMed: 17309137]

Figure 1.

Percentage of tCaMKII and pCaMKIIα-immunopositive neurons within the total DRG neuron population. There were no significant differences in the percentage of tCaMKII-immunopositive neurons between experimental groups. SNL induced a significant decrease in pCaMKIIα expression in axotomized L5 DRG neurons. Data are expressed as mean $\pm$ SD. *Significant difference between SNL L5 and corresponding control group. #Significant differences in percent positive rate for pCaMKIIα in comparison to tCaMKII within each group (*t*-test) ($n = 4-6$ for tCaMKII, $n = 6-15$ for pCaMKIIα).

Figure 2.

Range of diameters for single-labeled tCaMKII positive and negative neurons in (A) control and sham and (B) SNL L5 group. Single-labeled pCaMKIIα-positive and -negative neurons in (C) control and sham and (D) SNL L5 group. Cell size histogram for (E) double-labeled pCaMKIIα/N52 and (F) pCaMKIIα/IB4 neurons. Data are presented as a size frequency of all labeled (for A–D) or all colocalized (for E,F) DRG neurons. Since there were no differences between control and sham group, data were merged.

Figure 3.

A: Percentage of pCaMKIIα/N52 colabeled DRG neurons in total neuron population. There are no significant differences in the percentage of double-labeled neurons between groups. Data are expressed as percentage of neurons that were positive for both markers within the population of all DRG neurons. Data are expressed as mean $\pm$ SD, $n = 4-8$. There were no significant differences between groups. Distribution of N52 and pCaMKIIα-positive and -negative neurons in **(B)** control and **(C)** SNL L5 group.

Figure 4.

Double immunofluorescence of pCaMKIIα (green) and N52 (red) in control (A,C,E) and injured (SNL L5) neurons (B,D,F). Merged microphotograph demonstrates few pCaMKIIα/N52-immunopositive neurons in control (E) and SNL (F) group. Magnification: 40×. The magenta-green microphotographs are available online as Supporting Fig. 1. Scale bar = 100 μm (applies to all). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 5.

Double immunofluorescence of pCaMKIIα (red) and IB4 (green) in control (A,C,E) and injured (L5) neurons (B,D,F) 2 weeks after surgery. Merged microphotograph demonstrates pCaMKIIα/IB4 immunopositivity in few double-labeled neurons in control (E) and no such neurons in L5 SNL neurons (F). The magenta-green microphotographs are available online as Supporting Fig. 2. Scale bar = 100 μ m (applies to all). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 6.

A: Spinal nerve ligation induced a marked decrease of pCaMKIIα/IB4-immunopositive neurons in SNL L5 ganglia. Asterisk denotes difference between SNL L5 and control group (ANOVA, $F = 108.3$, $P < 0.001$, Bonferroni post-hoc test). Data are expressed as mean $\pm$ SD, $n = 4-5$. Distribution of IB4 and pCaMKIIα-positive and -negative neurons in **(B)** control and **(C)** SNL L5 group.

TABLE 1**Primary Antibodies Used**

Antigen	Immunogen	Manufacturer	Dilution used
CaMKII	Aa 303–478 mapping at the C-terminus of CaMKII α of mouse origin, protein accession #P11798	Rabbit polyclonal, Santa Cruz Biotechnology; sc-9035, lot F0304	1:100
pCaMKII α	Aa 282 HRQET(-phospho)VDCLK 291 containing phosphorylated Thr-286 of CaMKII α of human origin, protein accession #Q9UQM7*	Rabbit polyclonal, Santa Cruz Biotechnology; sc-12886-R, lot K2305	1:100
Neurofilament 200 kDa, clone N52	Carboxy terminal tail segment of enzymatically dephosphorylated porcine H-chain of neurofilament 200 kD	Mouse, Chemicon International; MAB 5266, lot 0142	1:1,000

* According to the manufacturer's specifications this antibody is raised against a 270–320 amino acid sequence containing phosphorylated Thr-286 of CaMKII α of human origin. However, the results of our mass spectrometry analysis revealed that blocking peptide sequence correspond to the manufacturer specification but only in a shorter segment of 10 amino acids (282–291). Also, blocking peptide solution contained seven different peptide forms of the same amino acid sequence but with different posttranslational modifications (N-acetylation, C-amidation). Besides monomeric structures we also identified dimeric structures with the same amino acid structure connected with a disulfide bridge.