Published in final edited form as:

Pain. 2015 December ; 156(12): 2514–2520. doi:10.1097/j.pain.0000000000000323.

PKC γ -mediated phosphorylation of GluA1 in the PSD of spinal dorsal horn neurons accompanies neuropathic pain and dephosphorylation by calcineurin is associated with prolonged analgesia

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Abstract

Loss of calcineurin (protein phosphatase 3) activity and protein content in the post-synaptic density (PSD) of spinal dorsal horn neurons was associated with pain behavior following chronic constriction injury (CCI) of the rat sciatic nerve, and intrathecal administration of the phosphatase provided prolonged analgesia (Miletic et al., Pain 2013;154:2024-2033). In this study we examined if one consequence of the loss of calcineurin was the persistent phosphorylation of the GluA1 subunit of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) in the PSD. This would allow continual activation of AMPAR at the synapse to help maintain a long-lasting enhancement of synaptic function, i.e., neuropathic pain. We also investigated if the phosphorylation was mediated by protein kinase A (PKA), protein kinase C gamma (PKC γ) or calcium-calmodulin dependent kinase II (CaMKII), and if the prolonged calcineurin analgesia was associated with GluA1 dephosphorylation. Mechanical thresholds and thermal latencies were obtained before CCI. Seven days later the behavioral testing was repeated before saline, calcineurin or the specific peptide inhibitors of PKA (PKI-tide), PKC γ (PKC 19-31) or CaMKII (AIP-2) were injected intrathecally. The behavior was retested before the animals were euthanized and their PSD isolated. All CCI animals developed mechanical and thermal hypersensitivity. This was associated with phosphorylation of GluA1 in the ipsilateral PSD at Ser831 (but not Ser845) by PKC γ and not by PKA or CaMKII. Intrathecal treatment with calcineurin provided prolonged analgesia and this was accompanied by GluA1 dephosphorylation. Therapy with calcineurin may prove useful in the prolonged clinical management of well-established neuropathic pain.

Keywords

Central sensitization; Chronic constriction injury; Intrathecal treatment; Kinase; Phosphatase; Rat; Sciatic nerve; Synaptic plasticity

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Conflict of Interest

None of the authors have financial arrangements that could represent a possible conflict of interest.

1. Introduction

Long-lasting enhancement of synaptic function in the spinal dorsal horn is thought to contribute to the development of neuropathic pain following chronic constriction injury (CCI) of the rat sciatic nerve [8,16,19]. The balance between protein kinase and phosphatase activity at the synapse can critically determine overall synaptic strength [9]. As a result the loss of either one of these activities can engender long-lasting changes in synaptic function, i.e., long-lasting plasticity.

We reported recently that pain behavior due to CCI was associated with a loss of calcineurin (protein phosphatase 3) activity and content in the post-synaptic density (PSD) of dorsal horn neurons, and that intrathecal administration of the phosphatase provided prolonged analgesia [12]. We proposed that the loss of calcineurin-mediated dephosphorylation in the PSD may have given rise to neuropathic pain at least in part as a result of the CCI-elicited long-lasting enhancement of dorsal horn synaptic function. This allowed evoked responses by primary afferent activity to be exaggerated and the enhanced sensory input manifested then as neuropathic pain.

We further proposed that calcineurin addition by intrathecal injection reversed the injury-elicited synaptic enhancement through the phosphatase-initiated dephosphorylation of target proteins. This in turn normalized evoked responses to primary afferent activity and provided relief from the well-established pain. Calcineurin-dependent analgesia persisted while the phosphatase was present within the PSD. Once calcineurin levels declined through routine pharmacokinetics the analgesia disappeared as well. This suggested that the reappearance of pain was dependent once again upon the loss of calcineurin dephosphorylating activity in the PSD [12].

In the present study we examined whether one potential consequence of the loss of calcineurin-mediated dephosphorylation was increased phosphorylation (activation) of glutamatergic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) in the PSD. Constitutive expression of AMPAR in the membrane is a normal feature of synaptic function but it is their activity-dependent trafficking that influences synaptic plasticity [4,6]. In general, recruitment of AMPAR into the synaptic membrane enhances synaptic function while their removal weakens synapses. Many details of AMPAR trafficking remain to be elucidated but it is clear that the process is phosphorylation-dependent [4,6].

AMPA consist of combinations of different subunits (GluA1-4) and specifically in the hippocampus the phosphorylation of GluA1 subunits stabilizes the membrane-inserted AMPAR to increase synaptic strength. In contrast, the dephosphorylation of GluA1 subunits by calcineurin engenders AMPAR internalization to elicit synaptic depression [4,6].

GluA1 subunits are abundant in the spinal dorsal horn and they are highly concentrated in postsynaptic membranes of superficial neurons. Multiple reports have provided compelling evidence that GluA1 activation facilitates the development of pain behavior due to incision [20,21], inflammation [3,10,13,18] or nerve injury [7,10]. In this study we hypothesized that the loss of calcineurin in CCI animals resulted in the persistent phosphorylation of GluA1

subunits in the PSD. This permitted their continual activation at the synapse to help maintain a CCI-elicited long-lasting enhancement of synaptic function, i.e., neuropathic pain.

2. Methods

2.1. Animals, behavioral tests

Male Harlan-Sprague-Dawley rats (200-250g) were assigned to control 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.

Mechanical thresholds were obtained with the Dynamic Plantar Aesthesiometer (Ugo Basile North America, Collegeville, PA) which recorded the force that resulted in an animal's hind limb withdrawal. Thermal hyperalgesia was assessed with the hind limb withdrawal latency test using a plantar analgesia instrument (Ugo Basile North America, Collegeville, PA). The mechanical testing preceded thermal testing.

Animals were acclimated for 15-20min. Each test consisted of four trials which were averaged to obtain a mean value. Each trial was separated by at least 5min. In 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 deeply anesthetized, euthanized, and their lumbar spinal dorsal horns collected for the biochemical assays.

2.2. Anesthesia, sciatic ligation

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 procedure [1]. Briefly, the sciatic nerve was exposed and loosely ligated with 4 simple interrupted 4-0 chromic gut sutures placed about 1mm apart. Control animals were anesthetized but were not subject to surgery.

2.3 Intrathecal injection, tissue collection, immunoblotting

Calcineurin, the specific PKA peptide inhibitor PKI-tide, the specific PKC γ peptide inhibitor PKC (19-31) and the specific CaMKII inhibitor autocamtide-2-related inhibitory peptide (AIP-2) were all purchased from Enzo Life Sciences (Plymouth Meeting, PA) and injected intrathecally in a volume of 10 μ l [11,12]. The same volume of saline served as the vehicle control. For all intrathecal injections animals were briefly anesthetized with isoflurane. Persons performing behavioral tests were blinded to drug treatment.

For tissue collection the animals were anesthetized with isoflurane and while still deeply anesthetized, they were euthanized with an intracardiac injection of super-saturated potassium chloride (400mg/ml). A laminectomy rapidly (<2min) exposed the spinal cord at L5. The cord was excised, cut into dorsal and ventral halves, and the dorsal half divided into ipsilateral and contralateral quadrants. All tissues were stored at -80°C until use.

Tissues were homogenized and sequentially centrifuged in HEPES buffer containing sucrose, NaF, EDTA and a protein inhibitor cocktail at 4°C to yield the PSD-containing LP1 fractions [11]. Briefly, samples were first spun at 1,500g for 10min to yield a pellet (P1) and supernatant (S1) fraction. The P1 fraction was re-suspended in buffer and centrifuged at 1,500g for 10 min to yield the nuclear (P1') and S1' fraction. The S1 and S1' fractions were combined and spun at 15,000g for 15 min to yield the P2 and S2 fractions. The P2 fraction was washed in buffer and centrifuged at 15,000g for 15 min to yield the crude synaptosomal P2' pellet. This pellet was re-suspended in buffer, hypotonically lysed with cold water, and centrifuged at 20,800g for 60 min to yield the synaptosomal vesicles and cytosolic fraction (LS1) and the heavy membrane pellet (LP1).

After assaying for total protein content, the LP1 fraction was processed for Western immunoblots [11,12]. Each blot was a single individual animal sample. Antibodies to pGluA1-831, pGluA1-845, pPKC γ , pPKA and AKAP150 were purchased from Millipore (Billerica, MA) or Abcam (Cambridge, MA). Developed membranes were stripped and re-probed with beta III tubulin (Promega, Madison, WI or Abcam, Cambridge, MA) as the loading control. All of the antibodies were used at a dilution of 1:1000. Protein levels were estimated from optical density measurements using the BioSpectrum 500 Image Analysis System (UVP, Upland, CA). Protein levels within a gel were expressed over the beta III tubulin levels, and then the levels in CCI animals were normalized to those in control animals.

2.4. Statistical analysis

ANOVA was used for the statistical data analysis. The main emphasis was on detecting differences in mechanical thresholds, thermal latencies, or protein content between control and ligated animals, or vehicle and drug-treated animals. Significant effects were further analyzed with Scheffe's post-hoc test. In addition, Pearson correlation coefficients were calculated to detect potential correlations between the pain behavior and degree of GluA1 phosphorylation following treatment with calcineurin or each of the kinase inhibitors. Statistical difference was inferred at $p < 0.05$. All data are expressed as mean $\pm$ SEM.

3. Results

We focused on the 7 days post-ligation period because by this time neuropathic pain is well-established [11,12].

3.1 A single intrathecal injection of calcineurin produced prolonged analgesia in CCI animals

We first sought to confirm that a single intrathecal injection of calcineurin provided prolonged analgesia.

CCI animals exhibited typical pain behavior 7 days post-ligation. After the behavioral testing, saline or calcineurin (100 enzyme units) were injected intrathecally (n=8 in each group) and the animals were behaviorally retested 2 days after the injection. Both the mechanical and the thermal hypersensitivity were significantly attenuated in the calcineurin-treated CCI animals. In other words, mechanical thresholds improved to 27 ± 3 g after having declined to 17 ± 2 g at 7 days post-CCI from a baseline of 31 ± 2 g. Similarly, thermal latencies decreased from 9.4 ± 0.6 s at baseline to 6.0 ± 0.5 s at 7 days post-CCI and then recovered to 9.0 ± 0.6 s after the intrathecal injection of calcineurin. ANOVA confirmed a significant effect of the calcineurin treatment for both the mechanical, $F(2,14)=179.9$, $p<0.001$, and thermal behavior, $F(2,14)=24.3$, $p<0.001$. The pain behavior of CCI animals injected with saline was not attenuated.

3.2. Increased content of pGluA1-831, but not pGluA1-845, was associated with neuropathic pain and calcineurin analgesia restored this content to control levels

Next, we wanted to establish if phosphorylation of the GluA1 subunit of AMPAR in the PSD accompanied CCI-elicited neuropathic pain, and if the prolonged calcineurin analgesia was associated with GluA1 dephosphorylation.

At the completion of the behavioral testing the CCI animals were anesthetized, euthanized and their tissue collected for immunoblot processing. Phospho-specific antibodies were used to detect GluA1 activation at two commonly targeted serine sites, Ser831 and Ser845.

In the saline-treated CCI animals that continued to exhibit pain behavior 2 days post-injection the content of pGluA1-831 in the ipsilateral PSD was greater than in controls, $135\pm 4\%$ (n=8). In contrast, in the calcineurin-treated animals exhibiting analgesia 2 days post-injection the levels of pGluA1-831 were essentially the same as in controls, $94\pm 5\%$ (n=8). ANOVA confirmed a significant difference between the groups, $F(2,21)=41.0$, $p<0.001$ (Fig. 1). Scheffes' post-hoc test revealed that this was due to the significantly higher levels of pGluA1-831 associated with pain behavior, and that calcineurin analgesia normalized this content.

On the other hand, the content of pGluA1-845 in the ipsilateral PSD was not different from controls in either the saline-treated ($90\pm 4\%$, n=8) or the calcineurin-treated ($90\pm 5\%$, n=8) animals, $F(2,21)=4.2$, $p<0.1$ (Fig. 1).

Additional analysis confirmed a statistical correlation between the expression of pain behavior and degree of GluA1 phosphorylation at Ser831. The content of pGluA1-831 was significantly and negatively correlated with both mechanical ($r=-0.6$, $p<0.01$) and thermal ($r=-0.8$, $p<0.001$) hypersensitivity. This suggested that the pain behavior was reflected in increased GluA1-831 phosphorylation. Calcineurin analgesia restored the content of pGluA1-831 to control values. In contrast, there was no correlation between the degree of phosphorylation of GluA1 at Ser845 and either mechanical ($r=0.1$, $p<1$) or thermal ($r=0.2$, $p<1$) hypersensitivity (Fig. 2).

These data suggested that at least part of the pain behavior in CCI animals could be attributed to the persistent phosphorylation of AMPAR in the PSD and that calcineurin analgesia resulted from the dephosphorylation of these receptors.

3.3 PKC γ but not PKA or CaMKII mediated the pain-associated phosphorylation of pGluA1-831 in the ipsilateral PSD

Phosphorylation of AMPA receptors is dependent on the action of several kinases, most notably PKA, PKC and CaMKII. We next employed specific inhibitors of each kinase to establish whether any of them would block the CCI-associated phosphorylation of GluA1 in the PSD.

CCI animals exhibiting mechanical and thermal hypersensitivity 7 days post-ligation were anesthetized and injected intrathecally with saline, the PKA inhibitor PKI-tide, the PKC γ inhibitor PKC (19-31) or the CaMKII inhibitor AIP-2 (n=6 in each group). At the doses used (100 μ M, 20 μ M or 1 μ M, respectively) these peptide inhibitors were reported to be highly specific for their respective kinase [17]. A 90 min post-injection period was used to evaluate the effects of the injected inhibitors. We have previously shown that this was sufficient to detect changes in biochemistry or behavior [12].

The phospho-specific Ser831 antibody was used again to detect changes in activation of GluA1 because this was the form seen to change with CCI (Fig. 1), and both PKC and CaMKII specifically target this site to phosphorylate GluA1 and promote long-lasting enhancement of synaptic function [4,6].

In the ipsilateral PSD of CCI animals treated with saline vehicle, PKI-tide or AIP-2, the levels of pGluA1-831 were significantly greater than in controls, 138 $\pm$ 4%, 133 $\pm$ 6% and 130 $\pm$ 6%, p<0.001, respectively (n=6 in each group). On the other hand, in the CCI animals treated with PKC (19-31) there was no difference in the ipsilateral PSD content of pGluA1-831, 103 $\pm$ 5%, p<0.1 (n=6), when compared to control animals (Fig. 3).

This outcome established that pGluA1 in the ipsilateral PSD of CCI animals was being phosphorylated at Ser831 by PKC γ but not CaMKII or PKA.

3.4. Intrathecal treatment with the specific PKC γ inhibitor, but not the specific PKA or CaMKII inhibitor, alleviated CCI-associated pain behavior

Did the block of GluA1-831 phosphorylation by the PKC γ inhibitor in the ipsilateral PSD affect pain behavior in these CCI animals? To answer this question we retested the behavior of CCI animals 90 min after the intrathecal injection of saline or one of the specific kinase inhibitors (n=6 in each of the four groups).

Neither mechanical nor thermal hypersensitivity were significantly modified in the CCI animals treated with saline, PKI-tide or AIP-2. On the other hand, the intrathecal injection of PKC (19-31) that blocked pGluA1-831 phosphorylation in CCI animals produced analgesia. In other words, mechanical thresholds recovered to 29 $\pm$ 3g after being reduced significantly to 20 $\pm$ 2g, and thermal latencies recovered similarly to 6.7 $\pm$ 0.8s after decreasing significantly to 4.9 $\pm$ 0.4s (Fig. 4).

Repeated measures ANOVA confirmed an effect of PKC (19-31) treatment for both mechanical thresholds, $F(2,10)=7.5$, $p<0.01$ and thermal latencies $F(2,10)=9.6$, $p<0.005$. The differences were due to the significant recovery in both mechanical and thermal hypersensitivity after administration of the PKC γ inhibitor.

To examine whether intrathecal treatment with PKC (19-31) would modify the behavior of control, otherwise uninjured animals we obtained baseline mechanical thresholds and thermal withdrawal latencies of six animals, injected them intrathecally with the same dose of the PKC γ inhibitor (20 μ M), and re-tested their behavior 90 min post-injection. Neither thresholds nor latencies were modified in these control animals, i.e., 37 ± 3 g vs. 35 ± 1 g, and 7.8 ± 0.4 s vs. 7.6 ± 0.3 s. Also the content of pGluA-831 in the ipsilateral PSD of these animals ($98\pm 6\%$) was not different from that of control animals that were not injected with the PKC γ inhibitor.

Additional statistical analysis again confirmed that increased phosphorylation of GluA1-831 was significantly and negatively correlated with the expression of both mechanical ($r=-0.7$, $p<0.01$) and thermal ($r=-0.7$, $p<0.01$) hypersensitivity, and that dephosphorylation of GluA1-831 was associated with PKC (19-31)-elicited analgesia (Fig. 5).

These results suggested that, as with calcineurin, the PKC γ inhibitor most likely produced analgesia by deactivating GluA1 in the PSD. Future experiments will need to establish if this analgesia is transient or, as with calcineurin, long-lasting.

3.5 Increased pPKC γ but not pPKA or AKAP150 content in the ipsilateral PSD accompanied pain behavior in CCI animals

Given that the phosphorylation of pGluA1-831 was dependent on PKC γ activity in our next set of experiments we wanted to confirm that the kinase was acting within the PSD.

CCI animals exhibited typical pain behavior 7 days post-ligation. After the behavioral testing, saline or calcineurin were injected intrathecally ($n=8$ in each group) and the animals were behaviorally retested 2 days after the injection. Both the mechanical and the thermal hypersensitivity were significantly attenuated in the calcineurin-treated animals, $F(2,14)=71.0$, $p<0.001$ and $F(2,14)=68.1$, $p<0.001$. In contrast, CCI animals injected with saline continued to exhibit neuropathic pain behavior.

After completion of the behavioral testing the animals were anesthetized, euthanized and their tissue collected for immunoblot assays of the active (phosphorylated) form of PKC γ (pPKC γ). In the saline-treated CCI animals that continued to exhibit pain behavior there was significantly more pPKC γ in the ipsilateral PSD than in controls, $131\pm 5\%$ ($n=8$). In contrast, in the calcineurin-treated CCI animals exhibiting analgesia the content of pPKC γ in the ipsilateral PSD was essentially the same as in controls, $91\pm 6\%$ ($n=8$). ANOVA confirmed a significant treatment-associated difference, $F(2,21)=21.9$, $p<0.001$ (Fig. 6).

Soluble proteins like calcineurin are anchored to the PSD by a family of structural proteins named A kinase anchoring proteins or AKAP [14,15]. At mammalian excitatory synapses three species-specific but highly-related AKAPs have been described, AKAP75 (bovine), AKAP79 (human) and AKAP150 (mouse). AKAPs link calcineurin to other structural

proteins in the PSD (most notably PSD-95) and help keep the enzyme close to its substrates. AKAPs appear principally involved in coordinating the activity of multi enzyme complexes and in addition to calcineurin they typically bind a kinase such as PKA or PKC [14,15].

In the second part of this study we stripped the pPKC γ immunoblots and then re-probed first for the phosphorylated form of PKA (pPKA) (n=8) and then for AKAP150 (n=8). Neither in the CCI animals treated with saline nor in those injected with calcineurin did the levels of pPKA ($104\pm3\%$ and $108\pm8\%$) or AKAP150 ($95\pm6\%$ and $97\pm3\%$) differ from those in the ipsilateral PSD of control, uninjured animals (Fig. 6).

These data confirmed that PKC γ was acting in the PSD and that the prolonged analgesia provided by an intrathecal injection of calcineurin restored PKC γ activity to baseline levels. The data also confirmed the lack of PKA contribution to GluA1 phosphorylation in the PSD of CCI animals (Fig. 1).

4. Discussion

In this study we first examined if phosphorylation of the GluA1 subunit of AMPAR in the PSD was one potential consequence of the loss of calcineurin dephosphorylating activity in the rat CCI model of neuropathic pain and whether the prolonged analgesia produced by intrathecal administration of the phosphatase was associated with GluA1 dephosphorylation. Once we determined that GluA1 was phosphorylated at Ser831 (but not Ser845) and that dephosphorylation of GluA1 accompanied calcineurin analgesia we investigated if the phosphorylation was mediated by PKA, PKC γ or CaMKII. We also examined if specific inhibitors of each kinase would provide analgesia and like calcineurin reverse the GluA1 phosphorylation. After we established that the phosphorylation was mediated by PKC γ (but not PKA or CaMKII) and that a specific inhibitor of this kinase provided at least transient pain relief we confirmed that the site of action of PKC γ was the PSD.

To our knowledge this is the first demonstration of how intrathecal administration of calcineurin may provide prolonged analgesia by dephosphorylating persistently active AMPAR in the PSD of spinal dorsal horn neurons.

Phosphorylation of the long-tailed subunits of AMPAR like GluA1 leads to the recruitment of these receptors to the synaptic membrane and to enhanced synaptic function. In contrast, the dephosphorylation of GluA1 engenders loss of synaptic strength by removal of AMPAR from the synapse [4,6].

AMPA are also a substrate of calcineurin so our previous [12] and present data suggested that CCI-elicited neuropathic pain resulted at least in part from the loss of calcineurin-mediated dephosphorylation in the PSD of spinal dorsal horn neurons. This loss would allow for the persistent phosphorylation and activation of AMPAR in the PSD membrane which could then help maintain the injury-elicited enhancement of synaptic function, i.e., neuropathic pain. Addition of the phosphatase by intrathecal injection provided pain relief in CCI animals because the phosphatase dephosphorylated AMPAR. This then led to their removal from the synapse and allowed a return to baseline (pre-pain) synaptic function.

Several kinases phosphorylate GluA1 at different sites, most notably PKA which targets Ser845, and PKC γ and CaMKII both of which target Ser831. Our data demonstrated that in the rat CCI model the phosphorylation of GluA1 was mediated by PKC γ rather than PKA or CaMKII. These data were in agreement with many studies reporting the specific contribution of GluA1 phosphorylation to pain due to incision, inflammation or nerve injury [3,7,10,13,18,20,21].

The present data provided further support for our previous suggestion that CCI-elicited neuropathic pain may develop at least in part as a result of the loss of calcineurin-mediated dephosphorylation in the spinal dorsal horn [12]. Together with the recent demonstration that calcineurin also critically influenced the phosphorylation of NMDA receptors [2,5] our data indicate that the phosphatase may play a pivotal role in the regulation of the overall activity of ionotropic glutamate receptors at synapses in the spinal dorsal horn.

The outcome of the experiments employing kinase inhibitors suggested that phosphorylation of AMPAR in the PSD was dependent on continual PKC γ activity because intrathecal treatment with PKC (19-31) provided pain relief presumably by blocking ongoing AMPAR phosphorylation by PKC γ . Otherwise, if the phosphorylation had been initiated by injury but was then maintained without further kinase action only a subsequent dephosphorylating action by phosphatases would be able to provide pain relief.

Our data confirmed an important contribution of PKC γ to CCI-elicited neuropathic pain and this was in agreement with multiple studies reporting a critical role of this PKC isoform in several types of chronic pain [22].

It is of interest that in our study the content of AKAP150 in the PSD was constant irrespective of whether or not the animals exhibited neuropathic pain behavior. Coupled with our previous data [12] this implied a fixed binding relationship between calcineurin and AKAP150 in the PSD. Addition of exogenous calcineurin by intrathecal injection did not increase the content of the phosphatase in the PSD of control uninjured animals [12]. This suggested that all of the calcineurin binding sites on AKAP150 were occupied. On the other hand, after calcineurin was lost due to CCI, the content of calcineurin in the PSD could be replenished by intrathecal injection of the phosphatase [12]. This suggested that the intrathecally added exogenous calcineurin was now able to access the binding sites freed by the CCI-elicited loss of endogenous calcineurin.

In summary, the present study established that persistent PKC γ -mediated phosphorylation of GluA1 at Ser831 in the PSD of spinal dorsal horn neurons was one potential consequence of the loss of calcineurin dephosphorylating activity in the rat CCI model of neuropathic pain. A single intrathecal injection of calcineurin provided prolonged analgesia by dephosphorylating GluA1. A similar dephosphorylating action may have also allowed the specific inhibitor of PKC γ to provide pain relief. Overall, our study suggested that therapy with calcineurin may prove useful in the prolonged clinical management of well-established neuropathic pain.

Acknowledgments

Supported in part by National Institutes of Health grant NS075917.

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Summary

PKC γ -mediated persistent phosphorylation of AMPAR in the PSD of spinal dorsal horn neurons may elicit neuropathic pain because of the loss of calcineurin dephosphorylating activity.

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pGluA1-831 and pGluA1-845 Content in the Ipsilateral PSD of CCI Animals

Figure 1. Neuropathic pain was associated with greater content of pGluA1-831 but not pGluA1-845 in the ipsilateral PSD of CCI animals

Pain behavior in CCI animals 2 days post-saline injection was accompanied by significantly greater levels of pGluA1-831 in the ipsilateral PSD when compared to control uninjured animals. In contrast, calcineurin analgesia 2 days post-treatment restored the pGluA1-831 levels in CCI animals to control values. On the other hand, ipsilateral PSD levels of pGluA1-845 in CCI animals were not different from those in control uninjured animals either in CCI animals exhibiting pain behavior 2 days post-saline injection or in CCI animals displaying analgesia 2 days post-calcineurin treatment. (n=8 in each group). *p<0.001.

Figure 2. Mechanical and thermal hypersensitivity were correlated with increased phosphorylation of GluA1 at Ser831 but not at Ser845

Plot of individual animal data from Fig.1 illustrates how the expression of pain behavior (lower mechanical thresholds and shorter thermal latencies) correlated with increased content of pGluA1-831 in the PSD. The calculated Pearson correlation coefficients were $r = -0.6$ for the mechanical and $r = -0.8$ for the thermal data. These were significant at $p < 0.01$ and $p < 0.001$ respectively. On the other hand, there was no correlation between mechanical ($0.1, p < 1$) or thermal ($0.2, p < 1$) hypersensitivity with GluA1 phosphorylation at Ser845.

Figure 3. PKC γ but not PKA or CaMKII mediated the CCI-associated activation of GluA1 in the ipsilateral PSD

The levels of pGluA1-831 in the ipsilateral PSD were significantly elevated in the CCI animals treated with saline, the specific CaMKII inhibitor AIP-2 or the specific PKA inhibitor PKI-tide ($n=6$ in each group). In contrast, the injection of the specific PKC γ inhibitor PKC (19-31) ($n=6$) was associated with pGluA1-831 levels that were not different from controls. * $p<0.001$ when compared to the pain-associated increased levels.

Figure 4. Intrathecal treatment with PKC (19-31) but not AIP-2 or PKI-tide alleviated the CCI-associated pain behavior

CCI animals exhibited mechanical and thermal hypersensitivity 7 days post-ligation. Intrathecal treatment with saline, AIP-2 or PKI-tide did not modify the pain behavior 90 min post-injection (n=6 in each group). In contrast, intrathecal injection of the PKC (19-31) (n=6) attenuated significantly both the mechanical and the thermal hypersensitivity elicited by CCI. #p<0.01, *p<0.005.

Figure 5. Expression of pain behavior was correlated with increased pGluA1-831 content in the PSD of CCI animals treated with kinase inhibitors

The plot of individual animal data from Figs.3 and 4 again demonstrated a significant correlation between lower mechanical thresholds and shorter thermal latencies with increased content of pGluA1-831 in the PSD. The calculated Pearson correlation coefficients were $r=-0.7$ for the mechanical and $r=-0.7$ for the thermal data. Both were significant at $p<0.01$.

Figure 6. Increased content of pPKC γ , but not of pPKA or AKAP150, in the ipsilateral PSD accompanied pain behavior in CCI animals

Pain behavior in 7 days post-CCI animals (n=8) was accompanied by a significantly greater content of pPKC γ in the ipsilateral PSD of spinal dorsal horn neurons. Calcineurin analgesia (n=8) restored this content to control values. On the other hand, the content of pPKA or AKAP150 in the ipsilateral PSD was not different from controls in either saline-treated or calcineurin-injected CCI animals (n=8 in each group). *p<0.001.