



Increases in the phosphorylation of cyclic AMP response element binding protein (CREB) and decreases in the content of calcineurin accompany thermal hyperalgesia following chronic constriction injury in rats

Gordana Miletic, Matthew T. Pankratz, Vjekoslav Miletic*

Department of Comparative Biosciences, University of Wisconsin, 2015 Linden Drive, Madison, WI 53706-1102, USA

Received 24 April 2002; accepted 24 June 2002

Abstract

Plasticity in the spinal dorsal horn may underlie the development of chronic pain following peripheral nerve injury or inflammation. In this study, we examined whether chronic constriction injury of the sciatic nerve was associated with changes in the immunoreactive content of cyclic AMP response element binding protein (CREB), protein kinase A (PKA), and calcineurin $A\alpha$ and $A\beta$ in the spinal dorsal horn.

In animals exhibiting thermal hyperalgesia as a behavioral sign of neuropathic pain 7 days after loose ligation of the sciatic nerve (chronic constriction injury), there was a significant increase in the content of phosphorylated (activated) CREB (pCREB). In contrast, following the typical disappearance of thermal hyperalgesia 28 days after loose ligation surgery, there were no differences in pCREB content between control and sciatic ligation animals. The increased CREB activation associated with thermal hyperalgesia was accompanied by significant decreases in the content of both calcineurin $A\alpha$ and $A\beta$. In contrast, there were no differences in the content of non-phosphorylated CREB, and phosphorylated or non-phosphorylated PKA between control and sciatic ligation animals either 7 or 28 days after surgery.

These data established a close association in the expression of thermal hyperalgesia with CREB activation and decreased calcineurin content in the spinal dorsal horn. The data revealed a significant but reversible shift in the manner in which spinal neurons processed sensory information following peripheral nerve injury, and lent further support to the notion that plasticity in the spinal dorsal horn may have contributed to the development of chronic pain. © 2002 International Association for the Study of Pain. Published by Elsevier Science B.V. All rights reserved.

Keywords: Synaptic plasticity; Nociception; Chronic pain; Primary afferents; Spinal cord injury; Kinase; Phosphatase; Nuclear protein

1. Introduction

Plastic changes in spinal nociceptive processing accompany peripheral nerve injury or inflammation to perhaps ultimately contribute to the development of persistent pain (e.g. recent reviews by Millan, 1999; Moore et al., 2000). Using the well-established Bennett and Xie (1988) model of neuropathic pain, we have recently reported that chronic constriction of the sciatic nerve was accompanied by activity-dependent long-lasting synaptic plasticity in the superficial spinal dorsal horn (Miletic and Miletic, 2000), and that there was a close association between the typical disappearance of the behavioral signs of neuropathic pain several weeks after surgery and the loss of long-lasting synaptic plasticity (Draganic et al., 2001).

Recent efforts in understanding the mechanisms underlying long-lasting synaptic plasticity have demonstrated that

activation of protein kinase A (PKA) by cyclic AMP (cAMP) initiates a cascade that ultimately targets the transcription factor cAMP response element binding protein (CREB). The CRE family of transcription factors also includes the cAMP response element modulator (CREM) and activation transcription factor (ATF) groups, and this family is considered critical for induction of gene expression in all eukaryotic cells (Silva et al., 1998; Shaywitz and Greenberg, 1999). The phosphorylation (activation) of CREB by PKA (and other kinases) may initiate gene transcription of target proteins to promote structural subsynaptic changes that increase synaptic efficacy, and ultimately produce long-lasting changes in cellular function (Abel and Kandel, 1998; Silva et al., 1998; Shaywitz and Greenberg, 1999).

PKA may be a critical component of a gate that regulates long-lasting synaptic plasticity by opposing the actions of the phosphatase calcineurin (PP2B). Phosphorylation of CREB (pCREB) by PKA may lead to long-lasting plasticity.

* Corresponding author. Tel.: +1-608-263-1238; fax: +1-608-263-3926.
E-mail address: vam@vetmed.wisc.edu (V. Miletic).

Conversely, activation of calcineurin may initiate the dephosphorylation of target proteins to inhibit the transition from short to long-lasting plasticity (Abel and Kandel, 1998). Recent data suggest that two calcineurin isoforms may play distinct roles in preventing the switch to long-lasting plasticity in the hippocampus. Calcineurin A α may contribute to the depotentiation that follows short-term potentiation of synaptic potentials after high-frequency tetanic stimulation, while calcineurin A β may play an important role in eliciting long-term depression following low-frequency stimulation (Zhuo et al., 1999).

Recent evidence also suggests that changes in the activity of both PKA and CREB contribute to alterations in sensory function in the spinal dorsal horn following peripheral nerve injury or inflammation. On the other hand, virtually nothing is known about the role of calcineurin in the spinal dorsal horn (Millan, 1999; Moore et al., 2000).

In the present study, we examined whether the immunoreactive content of CREB and pCREB, PKA and phosphorylated PKA (pPKA), and the two calcineurin isoforms A α and A β are modified in the spinal dorsal horn of animals after chronic constriction injury of the sciatic nerve. Specifically, we sought to determine whether the timeline of the maximal expression and disappearance of thermal hyperalgesia (as a behavioral sign of neuropathic pain) would closely parallel changes in the activation of CREB, and whether this would be reflected by contrasting changes in the content of PKA, pPKA, and the two calcineurin isoforms.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (Harlan, ~300 g) were used. Water and food were provided ad libitum. 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 Committee of the School of Veterinary Medicine at the University of Wisconsin–Madison.

2.2. Sciatic ligation and hindpaw withdrawal latency

Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and their sciatic nerves loosely ligated as described by Bennett and Xie (1988). This procedure reliably and repeatedly produces thermal hyperalgesia as a symptom of neuropathic pain (Backonja et al., 1995; Miletic and Miletic, 2000; Draganic et al., 2001). The development and disappearance of thermal hyperalgesia was assessed with the well-established hind paw withdrawal latency test to a noxious stimulus (Hargreaves et al., 1988). Baseline withdrawal latencies were obtained for all animals before they were randomly assigned to a control ($n = 8$), 7 days ($n = 8$) and 28 days ($n = 6$) sciatic ligation group. Pilot studies have established that withdrawal latencies of

sham-operated animals (sciatic nerve exposure without ligation) were statistically indistinguishable from unoperated control animals (i.e. like control animals the sham-operated animals do not develop thermal hyperalgesia). At the appropriate time after surgery (7 or 28 days) the hindpaw withdrawal latencies of all animals were obtained again (second behavioral test) before the animals were euthanized, and their spinal dorsal horn tissue harvested for use in Western immunoblots.

2.3. Western immunoblots

The animals were deeply anesthetized with isoflurane, euthanized (Beuthanasia™, 150 mg/kg, intracardially), and their spinal cords exposed around L5. The cord was excised, and divided into a dorsal and a ventral half in ten animals (four each in the control and 7 days sciatic ligation group, and 2 in the 28 days ligation group). In the remaining 12 animals (four in each of the three groups), the dorsal half was further subdivided into ipsilateral and contralateral quadrants. The spinal tissue was weighed, homogenized and centrifuged at 7000 \times g for 15 min. The 50 mM Tris–HCl (pH 7.4) homogenizing buffer also contained 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% NP-40, 0.25% sodium deoxycholate, and 5 μ g/ml of a mixture of protease inhibitors [4-(2-aminoethyl) benzene-sulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucyl-amido(4-guanidino) butane, bestatin, aprotinin, leupeptin]. Total protein content in the homogenates was determined with a commercially available kit (Pierce, #23236).

Proteins of interest were separated by SDS-PAGE electrophoresis (10 μ g of total protein per well), and transferred onto PVDF membranes. The membranes were placed in a blocking solution (Tris-buffered saline with 0.02% Tween and 5% non-fat dry milk) for 1 h, and incubated overnight in rabbit or goat primary antibodies (all from Upstate Biotechnology) to pCREB (1:1000), CREB (1:2000), PKA (1:2000), pPKA (1:1000), calcineurin A α (1:6000) and calcineurin A β (1:6000). After washing, incubation in appropriate peroxidase-conjugated secondary antibodies (1:10,000) for 1 h, and washing again, the proteins were detected by chemiluminescence.

The content of individual proteins was estimated from optical density plots of the scanned images of the bands using the histogram feature in Adobe Photoshop™ (Lehr et al., 1999a,b). This feature calculates the luminosity (staining density) of all pixels within a selected area, and provides a mean value as well as the median and standard deviation. On each gel, samples from a control, 7 days, and 28 days sciatic ligation animal were always loaded adjacent to each other (e.g. Fig. 3B). The average density of the control band was denoted as 100%, and the average density of the corresponding bands in the 7 and 28 days sciatic ligation animals was expressed as a percent change from this control. Analysis of variance (ANOVA) was used for

data analysis, and statistical significance was inferred at the $P < 0.05$ level.

2.4. Immunocytochemistry

In separate experiments designed to more precisely localize the immunoreactive pCREB staining, four control and four animals exhibiting thermal hyperalgesia 7 days after loose ligation of the sciatic nerve were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.), injected intracardially with 0.1 ml heparin (1000 i.u./ml), and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The fixed spinal cords were removed and cryoprotected in 20% sucrose and 5% glycerol in 0.1 M phosphate buffer in the presence of 50 mM NaF as a phosphatase inhibitor. The lumbar spinal cords (~L5) were cut into 50 μ m thick transverse sections, and processed with: (1) rabbit polyclonal antibody to pCREB at 1:4000 (Upstate Biotechnology); (2) biotinylated goat anti-rabbit IgG (Vector Labs) at 1:200; and (3) the Vector ABC standard kit. The reaction product was visualized with 0.04% 3,3 diaminobenzidine hydrochloride (DAB; Sigma) and hydrogen peroxide in phosphate buffer. The DAB-reacted sections were mounted on gelatin coated slides, dehydrated in ethanol, cleared in xylene and cover slipped with permount for light microscopic analysis. As a control, some sections were incubated without addition of primary antibodies. No staining was evident in this situation.

3. Results

3.1. Thermal hyperalgesia accompanied chronic constriction injury of the sciatic nerve 7 days after surgery and disappeared 28 days after surgery.

Sciatic ligation animals exhibited significantly shortened withdrawal latencies 7 days after surgery in the affected leg, 4.8 ± 0.3 (mean \pm SEM) vs. 8.1 ± 0.2 s, $F_{1,7} = 73.9$, $P < 0.001$. This is the time of maximal hyperalgesia for the chronic constriction injury model in our hands (Backonja et al., 1995). In contrast, there were no significant differences between baseline and day 7 latencies in control animals, 8.3 ± 0.2 vs. 8.4 ± 0.3 s, $F_{1,7} = 0.1$, $P < 0.8$. In addition, on the day of the second test, the latencies of 7 days sciatic ligation animals were significantly shorter than those of control or 28 days sciatic ligation animals, $F_{2,19} = 41.9$, $P < 0.001$ (Fig. 1).

Withdrawal latencies of control and sciatic ligation animals did not differ 28 days post-ligation, 8.2 ± 0.4 vs. 7.9 ± 0.3 s, $P = 0.6$. The disappearance of thermal hyperalgesia is a normal feature of this neuropathic pain model (Bennett and Xie, 1988), although the time at which hyperalgesia disappears varies. In our hands, behavioral signs of thermal hyperalgesia typically disappear by 21–24 days after sciatic ligation surgery (Backonja et al., 1995).

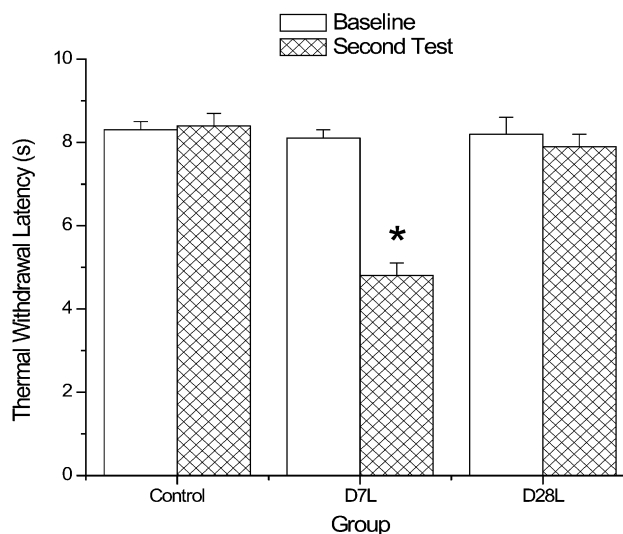


Fig. 1. The development of thermal hyperalgesia accompanied loose ligation of the sciatic nerve in rats. Baseline thermal withdrawal latencies were established before the animals were randomly assigned to control and ligated groups. Latencies were again obtained (second test) before the animals were euthanized 7 or 28 days after sciatic ligation surgery. Note that, as expected, sciatic ligation animals ($n = 8$) exhibited thermal hyperalgesia 7 days after surgery (D7L) as their withdrawal latencies significantly decreased ($*P < 0.001$). This is the time of maximal hyperalgesia for this model in our hands. In contrast, in control animals ($n = 8$) there was no difference between baseline and second test latencies. Note also that as expected 28 days after surgery (D28L) the second test withdrawal latencies of sciatic ligation animals ($n = 6$) were not significantly different from baseline. The disappearance of thermal hyperalgesia (as a sign of neuropathic pain) is a normal feature of the model, and in our hands this typically occurs by 24 days after sciatic ligation surgery. The withdrawal latencies are shown for the ipsilateral (operated) leg. Error bars represent the SEM.

3.2. Increases in pCREB, but not CREB, were associated with thermal hyperalgesia following chronic constriction injury

Immunocytochemical staining demonstrated pCREB immunoreactivity in the spinal dorsal horn in both control and sciatic ligation animals (Fig. 2). The immunoreactivity was confined to cell nuclei (Fig. 2, inset). The immunoreactive pCREB cells appeared more numerous in the chronic constriction injury animals exhibiting maximal thermal hyperalgesia 7 days after surgery.

Western immunoblot analysis confirmed the immunocytochemical data. Control animals exhibited a basal level of CREB phosphorylation, but the immunoreactive content of pCREB was greater in the spinal dorsal horn of 7 days sciatic ligation animals exhibiting thermal hyperalgesia. By 28 days after loose sciatic ligation, the pCREB levels returned to levels comparable to those seen in control animals (Fig. 3A). ANOVA indicated that the difference in pCREB content among the groups was significant ($F_{2,19} = 7.6$, $P < 0.01$). Scheffe's post-hoc analysis established that this was attributable to the increased pCREB levels ($124 \pm 6\%$, mean \pm SEM, $P < 0.01$) in the 7 days sciatic ligation group (Fig. 3B). In contrast, in sciatic ligation

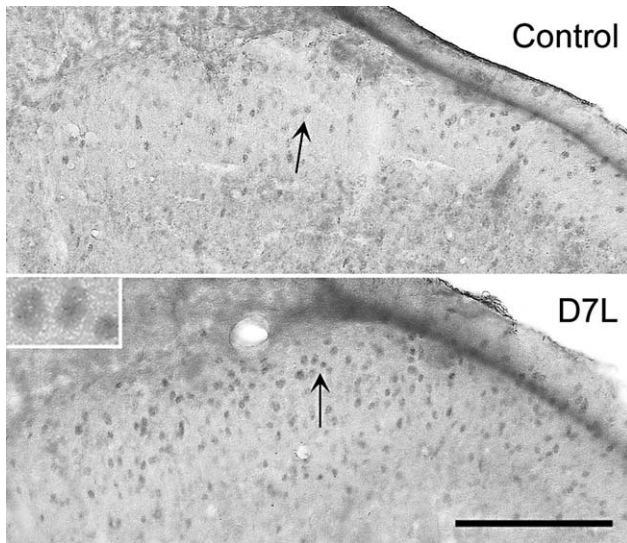


Fig. 2. Staining pattern of immunoreactive pCREB cells in the superficial spinal dorsal horn of control and ligated animals. Immunocytochemistry detected pCREB positive cells in the superficial layers of the spinal dorsal horn in both control and sciatic ligation animals. The immunoreactivity was confined to cell nuclei (inset). Note that in the control animal basal levels of pCREB immunoreactivity were observed, but that the immunoreactive pCREB cells appeared more numerous in the chronic constriction injury animal exhibiting maximal thermal hyperalgesia 7 days after surgery. Dorsal is up, medial is left. Scale bar is 150 μ m for both images except for the inset.

tion animals exhibiting a loss of thermal hyperalgesia 28 days after sciatic nerve injury, the levels of pCREB ($108 \pm 6\%$) were statistically indistinguishable ($P = 0.1$) from those of controls (Fig. 3B). A pilot study established that pCREB activity in 7 days sham-operated animals ($105 \pm 4\%$, $n = 3$) was statistically the same as that in control animals.

Statistical analysis further indicated that there were no differences in the content of non-phosphorylated CREB between control and sciatic ligation animals either 7 or 28 days after loose ligation of the sciatic nerve, $F_{2,19} = 0.7$, $P = 0.5$ (Fig. 3A, B).

Given that the injury was unilateral, we were also interested in examining whether any CREB activation would be confined to the corresponding ipsilateral spinal dorsal horn. As described in Section 2, in these experiments we divided the lumbar spinal dorsal horns into ipsilateral and contralateral quadrants in control, 7 days, and 28 days sciatic ligation animals ($n = 4$ in each group). The results indicated that there were no significant differences in pCREB content between the injured, ipsilateral ($141 \pm 22\%$) and the contralateral ($129 \pm 9\%$) quadrants in the 7 days sciatic ligation animals, $F_{1,3} = 0.3$, $P = 0.7$. This held true also for all of the other examined proteins. This was not surprising given that the sciatic ligation model produces changes in withdrawal latencies of the contralateral leg (Bennett and Xie, 1988). As expected, there were also no differences in pCREB levels between the ipsilateral and contralateral sides in either the control or the 28 days sciatic ligation animals.

3.3. Thermal hyperalgesia was accompanied by decreases in the spinal dorsal horn levels of calcineurin A α and A β

The levels of calcineurin A α and A β decreased in the spinal dorsal horn of animals exhibiting significantly shorter withdrawal latencies 7 days after loose ligation of the sciatic nerve (Fig. 4A). Statistical analysis indicated that these decreases were significantly different from control levels both for calcineurin A α ($68 \pm 8\%$, $F_{2,19} = 12.2$, $P < 0.001$) and calcineurin A β ($70 \pm 9\%$, $F_{2,19} = 8.7$, $P = 0.002$). Interestingly, as with pCREB, following the loss of thermal hyperalgesia 28 days after sciatic ligation the levels of both calcineurin isoforms were similar to those of control animals, $116 \pm 11\%$, $P = 0.3$, and $114 \pm 11\%$, $P = 0.5$, respectively (Fig. 4A, B).

In contrast, the spinal dorsal horn levels of PKA in the sciatic ligation animals either 7 or 28 days after surgery were essentially the same as in control animals, i.e. $106 \pm 3\%$, and $106 \pm 6\%$, respectively, $F_{2,19} = 1.2$, $P = 0.3$ (Fig. 4C, D). Since changes in protein activity can occur in the absence of changes in actual protein amounts, we further examined whether the levels of phosphorylated (activated) PKA (pPKA) were modified by sciatic ligation. Results indicated that there were no significant changes in pPKA levels 7 days ($95 \pm 4\%$) or 28 days ($96 \pm 4\%$) following chronic constriction injury of the sciatic nerve, $F_{2,19} = 0.8$, $P = 0.5$ (Fig. 4C, D).

4. Discussion

These data established a close association in the timeline of the maximal expression and disappearance of thermal hyperalgesia (as a behavioral sign of neuropathic pain) following peripheral nerve injury with CREB activation and a decrease in calcineurin content in the spinal dorsal horn.

The CREB protein family is considered critical for induction of gene expression in all eukaryotic cells, and CREB protein phosphorylation is thought to initiate transcription of several target proteins to ultimately enhance synaptic efficacy. Specifically, CREB activation may serve as a molecular switch to transform short-lasting into long-lasting synaptic plasticity in the hippocampus, and enable long-term memory storage in mammals (Abel and Kandel, 1998). Our data support a similar role of CREB in plasticity in the spinal dorsal horn, and suggest that CREB activation may have contributed to an injury-related but reversible shift in the processing of sensory information in the sciatic ligation animals exhibiting thermal hyperalgesia.

These results confirmed previous findings reporting increases in pCREB immunoreactivity in the spinal dorsal horn following formalin injection (Ji and Rupp, 1997; Anderson and Seybold, 2000), carrageenan injection (Messersmith et al., 1998) or partial sciatic nerve ligation (Ma and Quirion, 2001). Our study extended these findings

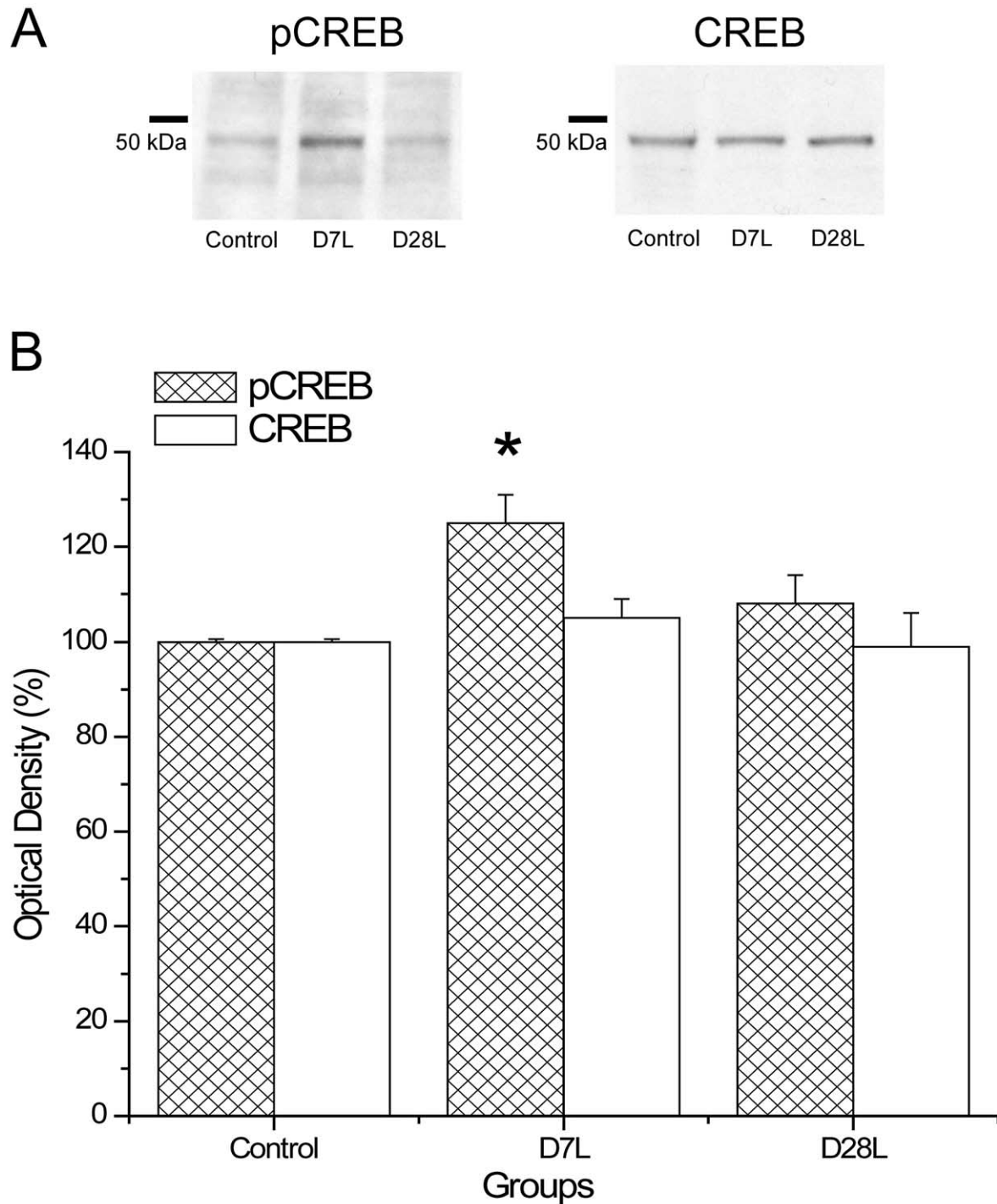


Fig. 3. Increases in pCREB, but not CREB, paralleled the development and disappearance of thermal hyperalgesia in sciatic ligation animals. (A) Representative immunoblots of pCREB and CREB from a control, a 7 days sciatic ligation (D7L), and a 28 days sciatic ligation animal (D28L). (B) Summary plots of the estimated content of pCREB and CREB. Note that there was a significant increase in the content of pCREB in the 7 days sciatic ligation animals (D7L, $n = 8$, $*P < 0.01$), and that there were no differences in the levels of pCREB between control ($n = 8$) and 28 days sciatic ligation animals (D28L, $n = 6$). Note also that there were no significant differences in CREB content among any of the animal groups at any of the times examined. Error bars represent the SEM.

to a different model of chronic pain, and, more importantly, established that the CREB activation was prolonged, reversible, and lasted only as long as the neuropathic pain.

In agreement with these previous studies, we noted that

the increases in pCREB levels occurred bilaterally in the spinal dorsal horn (Ji and Rupp, 1997; Messersmith et al., 1998; Anderson and Seybold, 2000; Ma and Quirion, 2001). This was not completely unexpected given that the sciatic

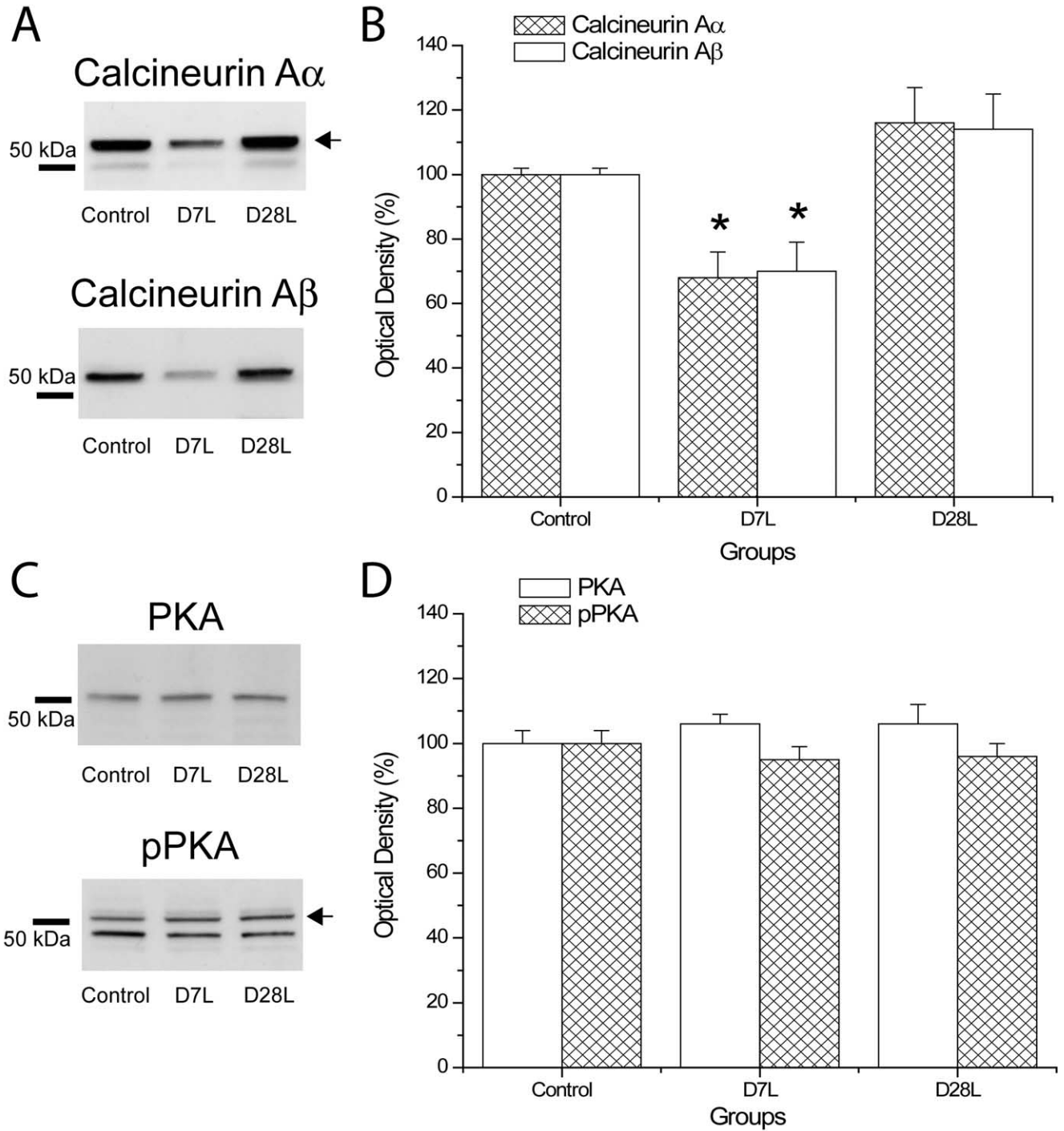


Fig. 4. Decreases in both calcineurin A α and A β accompanied thermal hyperalgesia in animals with chronic constriction injury of the sciatic nerve. (A,C) Representative immunoblots of calcineurin A α (arrow), A β , PKA and phosphorylated PKA (pPKA, arrow) from a control, a 7 days sciatic ligation (D7L), and a 28 days sciatic ligation animal (D28L). (B,D) Summary plots of the estimated content of the two calcineurin isoforms and the non-phosphorylated and phosphorylated PKA. Note that there was a significant decrease in the levels of both calcineurin isoforms 7 days after sciatic ligation surgery (D7L, $n = 8$, $*P < 0.005$), and that there were no differences in the content of either isoform between control ($n = 8$) and 28 day sciatic ligation animals (D28L, $n = 6$). Note also that there were no differences in the levels of PKA or pPKA among control ($n = 8$), 7 days (D7L, $n = 8$), and 28 days (D28L, $n = 6$) sciatic ligation animals. Error bars represent the SEM.

ligation model produces bilateral changes in withdrawal latencies, although typically to a greater degree in the ligated than in the non-affected leg (Bennett and Xie,

1988). A similar bilateral spread of pCREB immunoreactivity following unilateral stimulation was also reported in the hippocampus in vivo (Schulz et al., 1999).

It is unclear why control animals exhibited basal levels of CREB phosphorylation in the spinal dorsal horn. Previous studies similarly noted the presence of some immunoreactive pCREB cells in control animals (Ji and Rupp, 1997; Messersmith et al., 1998; Anderson and Seybold, 2000; Ma and Quirion, 2001). It is possible that the thermal withdrawal latency test by itself (clearly a nociceptive albeit acute stimulus) just prior to the tissue harvesting for immunocytochemistry or Western immunoblots activated CREB in some cells. However, we find it significant that in all animals that were not exhibiting behavioral signs of neuropathic pain, the content of pCREB was statistically indistinguishable from controls. In marked contrast, the levels of pCREB were significantly higher in all animals exhibiting thermal hyperalgesia. This suggests that whatever the underlying reason for a basal level of CREB activation in the spinal dorsal horn, the development of thermal hyperalgesia was closely associated with an additional and significant ongoing increase in CREB phosphorylation.

It is of interest that the increased CREB activation persisted beyond the initial injury, that the activation lasted only as long as the thermal hyperalgesia, and that it was reversible. This suggested that the maintenance of thermal hyperalgesia was dependent upon continual CREB phosphorylation, and that the eventual loss of thermal hyperalgesia resulted from a return to control levels of CREB phosphorylation.

The significant decrease in calcineurin content in the spinal dorsal horn of animals exhibiting thermal hyperalgesia may provide a potential link between the loss of phosphatase activity and the ongoing phosphorylation of CREB. Previous studies have suggested that a loss of calcineurin activity in the hippocampus is necessary for the pCREB-dependent transition to long-term memory storage (Abel and Kandel, 1998). A similar link between the reduction in calcineurin content and prolonged CREB activation may exist in the spinal dorsal horn as well.

Previous studies have also suggested that increases in PKA activity parallel the losses in calcineurin activity (Abel and Kandel, 1998). Our observation that PKA content or activity in the spinal dorsal horn was not modified by chronic constriction injury thus appears somewhat surprising. However, it is possible that additional increases in PKA activity in the spinal dorsal horn were unnecessary, given the dramatic reduction in calcineurin dephosphorylating ability because this decrease may have ultimately produced the same outcome, i.e. increased CREB phosphorylation. Moreover, the antibodies we used targeted the regulatory subunit of PKA, and it is possible that novel synthesis of a constitutively active PKA catalytic subunit was responsible for the ongoing CREB phosphorylation. Finally, our data do not exclude the possibility that calcium calmodulin-dependent kinase or mitogen activated kinase pathways alternatively or additionally contributed to CREB phosphorylation in the spinal dorsal horn.

In summary, our study established a close association in

the timeline of the development of thermal hyperalgesia (as a behavioral sign of neuropathic pain) following peripheral nerve injury with CREB activation and a reduction in calcineurin content in the spinal dorsal horn. The data revealed a significant but reversible shift in the manner in which spinal dorsal horn neurons processed sensory information following nerve injury, and lent further support to the notion that synaptic plasticity in the spinal dorsal horn may have contributed to the development of persistent pain.

Acknowledgements

Supported in part by NIH grant NS 34870 and the Christopher Reeve Paralysis Foundation.

References

- Abel T, Kandel E. Positive and negative regulatory mechanisms that mediate long-term memory storage. *Brain Res Rev* 1998;26:360–378.
- Anderson LE, Seybold VS. Phosphorylated cAMP response element binding protein increases in neurokinin-1 receptor-immunoreactive neurons in rat spinal cord in response to formalin-induced nociception. *Neurosci Lett* 2000;283:29–32.
- Backonja M, Miletic G, Miletic V. The effect of continuous morphine analgesia on chronic thermal hyperalgesia due to sciatic constriction injury in rats. *Neurosci Lett* 1995;196:61–64.
- Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988;33:87–107.
- Draganic P, Miletic G, Miletic V. Changes in post-tetanic potentiation of A-fiber dorsal horn field potentials parallel the development and disappearance of neuropathic pain after sciatic nerve ligation in rats. *Neurosci Lett* 2001;301:127–130.
- Hargreaves K, Dubner R, Brown F, Flores C, Joris JA. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988;32:77–88.
- Ji RR, Rupp F. Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. *J Neurosci* 1997;17:1776–1785.
- Lehr H-A, Mankoff DA, Corwin D, Santeusano G, Gown AM. Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. *J Histochem Cytochem* 1999a;45:1559–1565.
- Lehr H-A, van der Loos CM, Teeling P, Gown AM. Complete chromogen separation and analysis in double immunohistochemical stains using photoshop-based image analysis. *J Histochem Cytochem* 1999b;47:119–125.
- Ma W, Quirion R. Increased phosphorylation of cyclic AMP response element-binding protein (CREB) in the superficial dorsal horn neurons following partial sciatic nerve ligation. *Pain* 2001;93:295–301.
- Messersmith DJ, Kim DJ, Iadarola MJ. Transcription factor regulation of prodynorphin gene expression following rat hindpaw inflammation. *Mol Brain Res* 1998;53:260–269.
- Miletic G, Miletic V. Long-term changes in sciatic-evoked A-fiber dorsal horn field potentials accompany loose ligation of the sciatic nerve in rats. *Pain* 2000;84:353–359.
- Millan MJ. The induction of pain: an integrative review. *Prog Neurobiol* 1999;57:1–164.
- Moore KA, Baba H, Woolf CJ. Synaptic transmission and plasticity in the superficial dorsal horn. *Prog Brain Res* 2000;129:63–80.

- Schulz S, Siemer H, Krug M, Holt V. Direct evidence for biphasic cAMP responsive element-binding protein phosphorylation during long-term potentiation in the rat dentate gyrus in vivo. *J Neurosci* 1999;19:5683–5692.
- Silva AJ, Kogan JH, Frankland PW, Kida S. CREB and memory. *Annu Rev Neurosci* 1998;21:127–148.
- Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* 1999;68:821–861.
- Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983;16:109–110.
- Zhuo M, Zhang W, Son H, Mansuy I, Sobel RA, Seidman J, Kandel ER. A selective role of calcineurin $A\alpha$ in synaptic depotentiation in hippocampus. *Proc Natl Acad Sci USA* 1999;96:4650–4655.