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The impact of CREB and its phosphorylation at Ser142 on inflammatory nociception

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Abstract

Peripheral noxious stimulation leads to phosphorylation and thereby activation of the transcription factor CREB in the spinal cord. CREB phosphorylation occurs mainly at serine 133, but the phosphorylation site at serine 142 may also be important. We investigated the impact of spinal CREB protein levels and phosphorylation at Ser142 on the nociceptive behaviour in rat and mouse models of inflammatory nociception. Downregulation of total CREB protein in the rat spinal cord by antisense-oligonucleotides resulted in antinociceptive effects. After peripheral noxious stimulation CREB was phosphorylated in the spinal cord at serine 133 and 142 indicating a potential role of both residues in nociceptive processing. However, Ser142 mutant mice developed equal behavioural correlates of hyperalgesia as wild-type mice in different inflammatory models.

Thus, our data confirm that CREB is essential for spinal nociceptive processing. However, prevention of phosphorylation only at serine 142 is not sufficient to modulate the nociceptive response.

Keywords: CREB; Phosphorylation; Nociception; Hyperalgesia

Persistent peripheral inflammation or tissue injury may lead to long lasting hyperexcitability of spinal sensory neurons resulting in hyperalgesia and allodynia [1]. The noxious stimulation in the periphery induces the release of neurotransmitters in the spinal cord. Particularly, glutamate-mediated NMDA receptor activation causes calcium influx at post-synaptic neurons which in turn triggers the activation of intracellular protein kinases, such as protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}-/calmodulin-dependent kinases (CaMK), and mitogen activated kinases (MAPK) [2,3] all contributing to the development of a state of hyperexcitability. One of the substrates of these kinases is the transcription factor cAMP response element binding protein (CREB) [4,5], which is phosphorylated in dorsal horn neurons after noxious stimulation [6–8] and is supposed to be one of the major players in long term neuronal (mal)adaptive responses [9]. CREB phosphorylation at Ser133 has been identified as an essential trigger for CREB activation leading to transcription of a number of immediate early genes including those coding for the important pain-related proteins c-Fos and cyclooxygenase-2 [10]. CREB is phosphorylated at Ser133 in the dorsal horn of the spinal cord during peripheral inflammation caused by injection of formalin [6,11] or carrageenan [12] into the hindpaw. Ser133 phosphorylation was also observed in response to a traumatic injury of the sciatic nerve [13]. However, besides Ser133, the phosphorylation

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at Ser142 may essentially contribute to CREB regulation as already described by studies in the circadian clock and other neuronal systems [14,15].

In this study, we therefore investigated the impact of CREB-Ser142 phosphorylation in models of acute and persistent inflammatory pain using phosphorylation site mutated CREB S142A mice. The serine phosphorylation site at position 142 is replaced by alanine in these mice allowing us to specifically address the relevance of this phosphorylation site.

Materials and methods

Animals. Experimentally naive male Sprague–Dawley rats (Charles River, Sulzfeld, Germany) weighing 150–200 g at the time of surgery were used for antisense knock down of CREB. Mice with a mutation in the CREB serine 142 phosphorylation site where serine is exchanged for alanine (CREB S142A mice) and wild-type control mice were kindly provided by Prof. Günther Schütz, DKFZ Heidelberg. These mice are viable and fertile and show no obvious abnormalities in size or morphology in comparison to wild-type animals [14]. Animals had free access to food and water prior to the experiments. They were maintained in climate- and light-controlled rooms (22 ± 0.5 °C, 12/12 dark/light cycle). All experiments were performed according to the ethic guidelines for investigations in conscious animals and the procedures were approved by the local Ethics Committee for Animal Research (Regierungspräsidium Darmstadt, Germany). All efforts were made to minimize animal suffering and the number of animals.

Intrathecal administration of oligonucleotides and assessment of thermal hyperalgesia. The antisense-oligonucleotides used in the present study have been previously shown to specifically knock down CREB in the spinal cord after intrathecal (i.t.) injection [16]. Respective sense-oligonucleotides were used in control animals. The sequences are: 5′-TGGTCATCTA GTCACCGGTG-3′ (CREB antisense), 5′-GACCTCAGGTAGTCGTC GTT-3′ (CREB sense).

Oligonucleotides, synthesized and purified to analytical grade on HPLC by BioSpring (Frankfurt/Main, Germany) were continuously delivered onto the lumbar spinal cord through a spinal catheter (32G Intrathecal Catheters, Recathco Ltd., USA) as described previously [17]. The infusion rate was 1.0 μl/h and lasted for 7 days. Rats were adapted to the test cages during the infusion (day 3 and 5). Six days after starting the infusion two baseline paw withdrawal latencies for heat pain sensitivity (Hargreaves test) were obtained using a Plantar test (Ugo Basile, Comenio, Italy). Complete Freund's adjuvant (CFA) (50 μl) was then injected into the left hindpaw and thermal hyperalgesia was assessed repeatedly up to 24 h. At completion, the position of the catheter was confirmed, the lumbar spinal cord (L4–L5) dissected, snap frozen in liquid nitrogen, and kept at −80 °C until Western blot analysis. Only rats without relevant disturbances of neurological functions and general well being were used for the behavioural experiments.

Formalin test. The formalin test was performed as described previously [18]. Mice were placed on a table top within a Plexiglas cylinder and were allowed to adapt for 60 min. Fifteen microlitres of 5% formaldehyde solution (formalin) was injected subcutaneously into the dorsal surface of the right hindpaw. The time spent licking the formalin-injected paw was recorded in 5-min intervals up to 45 min, starting right after formalin injection.

Assessment of thermal and mechanical hyperalgesia. Unilateral hindpaw inflammation was induced by subcutaneous injection of zymosan A (Sigma–Aldrich, Munich, Germany) suspension in 0.1 M phosphate-buffered saline, pH 7.4 (PBS) into the midplantar region of the right hindpaw (20 μl of 3 mg/ml suspension).

Paw withdrawal latency to radiant heat (Hargreaves model) was assessed using a Plantar Test device (Ugo Basile, Varese, Italy). The cut-off latency was 30 s.

Paw withdrawal latency to mechanical stimulation was assessed with a dynamic von Frey apparatus (Ugo Basile). The maximum force was set at 5 g to prevent tissue damage and the ramp speed was 0.5 g/s. Both assays have been described in detail previously [19].

Western blot analysis. Western blot analysis with CREB antibody (1:1000, Cell Signaling) was performed as described previously [20].

Immunofluorescence studies. Mice were intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in PBS under deep isoflurane anaesthesia. The spinal cord (lumbar enlargement) was removed and post-fixed in the same fixative overnight (4 °C). Fresh-frozen tissue sections (14 μm) of lumbar spinal cord were cut in a cryostat and mounted onto positively charged slides. Slides were washed in PBS and treated for 15 min with PBS containing 0.1% Triton X-100. After blocking in PBS/3% BSA for 1 h, sections were incubated with the primary antibodies against CREB (1:100) (Sigma), phosphorylated CREB Ser133 or Ser142 (both 1:100) (Cell Signaling and [14]) and c-Fos (1:500) (Sigma) in PBS/1%BSA; for 2 h at 37 °C followed by incubation with AlexaFluor488-conjugated (1:800 in PBS/1%BSA; Molecular Probes, Leiden, The Netherlands) and Cy-3 conjugated (c-Fos) (1:1000) (Sigma) secondary antibodies for 1 h at RT.

Image analysis was performed in a blind manner as previously described [21]. Briefly, sections were captured with a fluorescence microscope (Nikon Eclipse E600, Nikon, Germany) equipped with a Kappa DX 20 H camera and Kappa ImageBase software (Kappa, Gleichen, Germany). NIH Image J software was used for counting immunoreactive (IR) cells within the dorsal horn of the spinal cord.

Data analysis. Statistical evaluation was done with SPSS 12.01 for Windows. Data were compared by Student's *t*-test and are presented as means ± standard error of the mean (SEM). Data from CREB immunofluorescence were submitted to univariate analysis of variance (WT control vs WT 10 min formalin vs mutant control vs mutant 10 min formalin) with subsequent *t*-tests employing a Bonferroni α-correction for multiple comparisons. For analysis of inflammatory hyperalgesia the areas under the paw withdrawal latency-versus-time curves over the 8 h observation period were calculated using the linear trapezoidal rule. For all tests, a probability value $p < 0.05$ was considered as statistically significant.

Results

Nociceptive behaviour after CREB antisense knock down in the rat spinal cord

The role of CREB in nociceptive transmission has been evaluated by investigation of the CFA-induced thermal hyperalgesia in rats treated either with CREB-sense or -antisense-oligonucleotides. Spinal perfusion with the CREB-antisense-oligonucleotide caused a significant reduction of CREB protein in the lumbar spinal cord as compared to treatment with sense-oligonucleotides (Fig. 1A). At baseline the two groups showed no differences in the paw withdrawal latency (PWL) in response to a thermal stimulus (sense-treated rats 14.1 ± 1.3 s, antisense-treated rats 16.6 ± 1.8 s). After CFA injection into the left hindpaw, rats treated with CREB-antisense-oligonucleotides displayed weaker CFA-evoked thermal hyperalgesia than sense-treated rats indicating that the reduction of CREB protein levels in the spinal cord results in a reduced hyperalgesic response (Fig. 1B). Comparison of the paw withdrawal latency AUCs over 24 h revealed a statistically significant difference between antisense ($AUC_{0–24h}$: 327 ± 28) and sense ($AUC_{0–24h}$: 209 ± 14) ($p < 0.05$) treated animals.

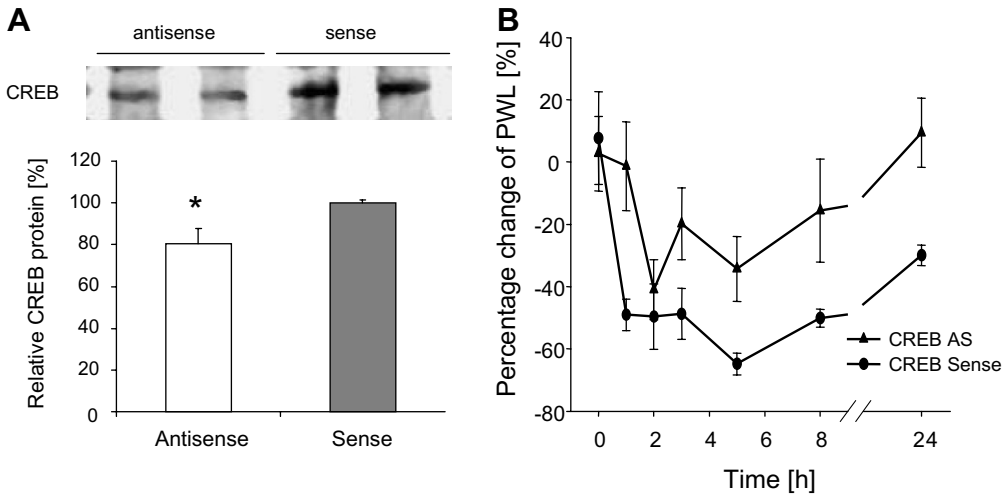

Fig. 1. Knock down of CREB in the spinal cord attenuates zymosan-induced thermal hyperalgesia. (A) Western blot showing the downregulation of CREB after intrathecal infusion of antisense- or sense-oligonucleotides for 7 days. The diagram shows the quantitative analysis of all animals tested ($n = 4$/group, * $p < 0.05$). (B) Time course of thermal hyperalgesia following injection of 1.25 mg zymosan into a hindpaw. Rats were treated as in (A). The diagram shows the paw withdrawal latencies in response to radiant heat (Hargreaves model) ($n = 4$/group).

CREB protein phosphorylation and cFos induction after noxious stimulation in mice

The effect of formalin injection on CREB protein phosphorylation in the spinal cord was assessed using immunofluorescence studies (Fig. 2A and Table 1). Only a very small number of pCREB-Ser133 or -Ser142 immunoreactive neurons were found in the dorsal horn of naïve mice. Ten minutes after formalin injection multiple dorsal horn neurons showed immunoreactivity to pCREB-Ser133 and pCREB-Ser142. In CREB S142A mice we observed a phosphorylation of CREB-Ser133 10 min after formalin injection but, as expected, no immunoreactivity for pCREB-Ser142.

c-Fos is one CREB-regulated protein and a sensitive indicator of neuronal activation after nociceptive stimulation. We investigated c-Fos immunoreactivity in the ipsilateral dorsal horn of the spinal cord in wild-type and S142A mice. c-Fos IR was strongly induced in wild-type and mutant mice 2 h after formalin injection into the hindpaw. This effect was slightly but significantly less pronounced in CREB mutant mice (CREB S142A 47.8 ± 3.4; wild-type 56.9 ± 7.2; $p < 0.05$, $n = 4$ animals/group).

Chemically induced nociceptive behaviour in CREB S142A mice

Injection of formalin into a hindpaw induced the typical biphasic nociceptive licking behaviour of the injected paw in wild-type as well as in mutant mice. The first phase started immediately after formalin injection and lasted for 10 min. The second phase was observed 11–45 min after formalin injection and peaked at 20–35 min. The licking behaviour in CREB S142A mutant mice did not differ from the behaviour of their wild-type littermates (phase 1, $p = 0.687$; phase 2, $p = 0.446$; total flinches, $p = 0.391$) (Fig. 2B).

Mechanical and thermal hyperalgesia following paw inflammation in CREB S142A mice

Zymosan-induced mechanical hyperalgesia was investigated in CREB S142A and wild-type mice using the dynamic plantar aesthesiometer. The baseline latency time for evoking a paw withdrawal reflex upon mechanical stimulation did not differ between CREB S142A and wild-type mice (PWL 7.7 ± 0.3 s and 7.7 ± 0.18 s, respectively). After induction of unilateral hindpaw inflammation the paw withdrawal latency similarly decreased in both strains (AUC wild-type mice 521.4 ± 53.9 and CREB S142A mice 494.3 ± 22.6, respectively; $p = 0.906$) (Fig. 3A).

The development of thermal hyperalgesia was assessed by using the Hargreaves model. At baseline, the paw withdrawal latency to radiant heat was equal in mutant and wild-type mice (PWL 9.14 ± 0.44 s and 9.11 ± 0.37 s, respectively). After zymosan injection paw withdrawal latencies decreased in both groups (Fig. 3B) without any differences between mutant and wild-type mice. (AUC wild-type mice 378.6 ± 53.9 and CREB S142A mice 387 ± 24.7, respectively; $p = 0.819$).

Discussion

Several studies have shown that the activation of CREB in spinal cord dorsal horn neurons plays a central role in the transmission of nociceptive stimuli and long term adaptive responses in the central nervous system [3,5,22]. CREB is a constitutive transcription factor which is activated by phosphorylation through various kinases including PKA,

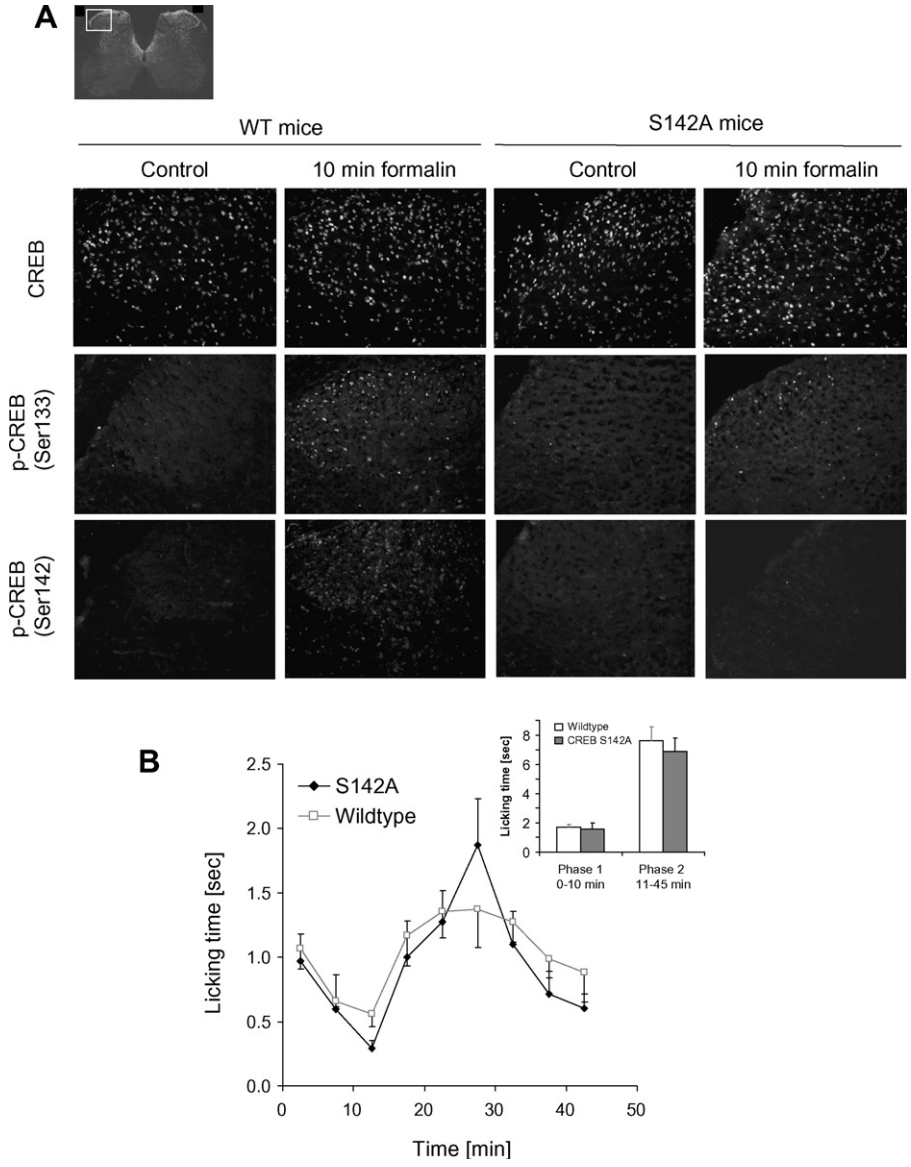

Fig. 2. Formalin assay using wild-type and CREB S142A mice. (A) Immunofluorescence studies showing the level of total CREB and phospho-CREB at serine 133 and serine 142 in the dorsal horn of mouse spinal cord with and without formalin treatment, respectively. Left panels: wild-type (WT), untreated control animals and 10 min after formalin injection; right panels: CREB S142A mice, untreated control animals and 10 min after formalin injection. Scale bar: 100 μm. The rectangle in the spinal cord scheme at the top indicates the area which has been analysed. (B) Time course of the flinching behaviour in CREB S142A mutant (♦, $n = 6$) and wild-type mice (□, $n = 6$) after injection of formalin into one hindpaw. Insert: For statistical comparison of drug effects the total number of flinches in the first (1–10 min) and second phase (11–60 min) of the formalin assay was calculated. No significant differences could be observed.

Table 1
Quantitative analysis of CREB-; p-CREB-Ser133, pCREB-Ser142 immunopositive cells in the dorsal horn of the spinal cord 10 min after formalin injection; $n = 3$ animals in each group

	Total CREB	Phospho 133	Phospho 142
WT control	235 ± 25	20 ± 19	15 ± 11
WT 10 min formalin	301 ± 1	93 ± 38	101 ± 36*
CREB S142A control	293 ± 69	9 ± 6	0
CREB S142A 10 min formalin	270 ± 24	49 ± 9	0

* $p < 0.05$ as compared to control.

PKC, Erk, p38 MAPK, and PKG that all contribute to the immediate adaptation to nociceptive input. Supporting this view we show here, that downregulation of CREB protein levels in the spinal cord by antisense-oligonucleotides reduces inflammatory thermal hyperalgesia in rats. CREB antisense was also found to reduce allodynia in models of

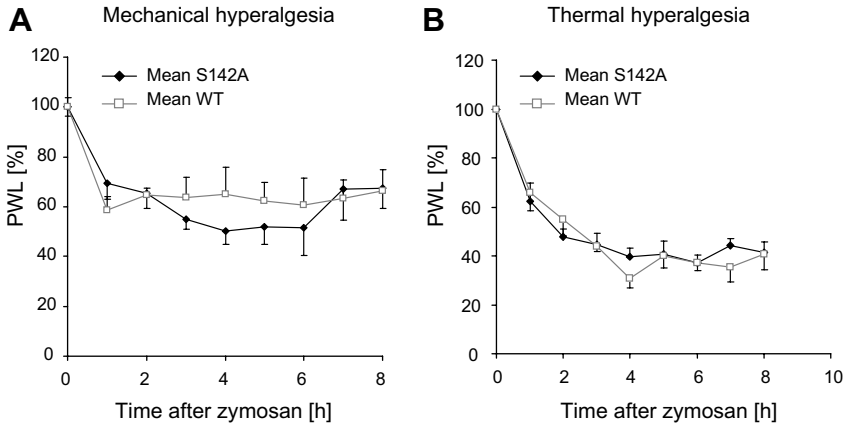

Fig. 3. Zymosan-induced mechanical and thermal hyperalgesia. (A) Time course of paw withdrawal latency (PWL) in response to a mechanical stimulus following injection of 60 μg zymosan into the right hindpaw of CREB S142A mutant (♦, $n = 8$) and wild-type mice (□, $n = 8$). Data are expressed as the difference between the zymosan-treated right and the untreated left hindpaw calculated as: ΔPWL = PWL right paw − PWL left paw. The baseline was set as 100%. (B) Time course of PWL in response to radiant heat following injection of 60 μg zymosan into the right hindpaw of S142A mutant (♦, $n = 8$) and wild-type mice (□, $n = 7$). Data were analysed as described in (A).

neuropathic pain [16,23] suggesting that the reduction of total CREB protein is associated with a reduction of phosphorylation-dependent activation due to the overall reduction of phosphorylation sites. Phosphorylation at Ser133 is considered to be the major regulator of CREB-dependent gene transcription. However, phosphorylation exclusively at Ser133 does not always result in target gene activation [24,25]. Additional phosphorylation sites have been identified in vitro including Ser129 phosphorylated by glycogen synthase kinase III [26], Ser142 phosphorylated by CaM kinase II (CaMKII) [27,28] and Ser142 or Ser143, both targets of casein kinase II (CKII) [29]. Stimulation of Ca^{2+} influx in primary neuronal cultures triggers phosphorylation at Ser133, 142, and 143. Similarly, seizure activity in rat brain caused increased CREB phosphorylation at these three sites. Interestingly, the phosphorylation of Ser142 and Ser143 in neurons was selectively induced by Ca^{2+} influx while Ser133 was phosphorylated upon a variety of stimuli. The triple site phosphorylation however, was necessary for effective Ca^{2+} mediated gene transcription [15]. In addition, light and glutamate evoked phosphorylation of CREB at Ser142 in suprachiasmatic neurons was found to be necessary for entrainment of the mammalian circadian clock [14].

Given the crucial role of glutamate and Ca^{2+} influx for nociceptive signaling we suggested that phosphorylation of CREB at Ser142 might be involved in nociceptive processing. Using immunofluorescence studies we confirmed that peripheral nociceptive stimulation induces CREB phosphorylation at Ser133 and additionally at Ser142 in the dorsal horn of the spinal cord. However, CREBS142A mutant mice did not show any difference in the nociceptive response in inflammatory pain models indicating that phosphorylation at this site is not crucial for development of hyperalgesia in these tests. Following formalin stimulation, CREBS142A mutant mice showed less c-Fos IR in dorsal horn neurons. However, this did not translate into differences in the formalin-evoked licking behaviour, suggesting that Ser142 phosphorylation does contribute to c-Fos-induction but reduction of this immediate early gene is apparently not sufficient to reduce the formalin-evoked pain. In contrast to the activating properties of CREB phosphorylation at Ser133, some studies indicated that phosphorylation at Ser142 may also inhibit CREB activity and may prevent binding of CREB to its binding protein (CBP). While phosphorylation of both Ser142 and 143 enhanced CREB induced transcription in neurons [15], phosphorylation of Ser142 alone attenuated this transcriptional activation [15,27]. Furthermore, mutation of Ser142 to alanine enhanced the ability of Ca^{2+} influx to activate CREB [27]. Another study showed that phospho-Ser-133 stabilizes whereas phospho-Ser-142 disrupts secondary structure-mediated interactions between CREB and CREB binding protein (CBP) [29]. Thus, differential phosphorylation of CREB may form the basis for the precise adjustment of CREB signal transduction pathways. Assuming that the phosphorylation at Ser142 might have an inhibitory effect on CREB activation one would hypothesize that mutation of this site might be associated with increased hyperalgesia. This however, was not the case in the CREB-Ser142 mutant mice. In addition, the reduced number of c-Fos IR neurons in mutant mice rather points to a (co)-activating effect.

Ca-influx and activation of CaMKII or casein kinase II are thus far the only known triggers of phosphorylation at Ser142 in vivo while activation of various kinases converges on the phosphorylation of Ser133 suggesting that the latter activation mechanism is much more robust and likely to be triggered by various stimuli.

In conclusion, our data suggest that CREB activation in dorsal horn neurons contributes to the development of inflammatory hyperalgesia. This however, does not mandatory require phosphorylation at serine 142.

Acknowledgments

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