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RESEARCH

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Specific involvement of atypical PKC ζ /PKM ζ in spinal persistent nociceptive processing following peripheral inflammation in rat

Fabien Marchand^{1,4*}, Richard D'Mello², Ping K Yip¹, Margarita Calvo¹, Emilie Muller⁴, Sophie Pezet³, Anthony H Dickenson² and Stephen B McMahon¹

Abstract

Background: Central sensitization requires the activation of various intracellular signalling pathways within spinal dorsal horn neurons, leading to a lowering of activation threshold and enhanced responsiveness of these cells. Such plasticity contributes to the manifestation of chronic pain states and displays a number of features of long-term potentiation (LTP), a ubiquitous neuronal mechanism of increased synaptic strength. Here we describe the role of a novel pathway involving atypical PKC ζ /PKM ζ in persistent spinal nociceptive processing, previously implicated in the maintenance of late-phase LTP.

Results: Using both behavioral tests and *in vivo* electrophysiology in rats, we show that inhibition of this pathway, via spinal delivery of a myristoylated protein kinase C- ζ pseudo-substrate inhibitor, reduces both pain-related behaviors and the activity of deep dorsal horn wide dynamic range neurons (WDRs) following formalin administration. In addition, Complete Freund's Adjuvant (CFA)-induced mechanical and thermal hypersensitivity was also reduced by inhibition of PKC ζ /PKM ζ activity. Importantly, this inhibition did not affect acute pain or locomotor behavior in normal rats and interestingly, did not inhibit mechanical allodynia and hyperalgesia in neuropathic rats. Pain-related behaviors in both inflammatory models coincided with increased phosphorylation of PKC ζ /PKM ζ in dorsal horn neurons, specifically PKM ζ phosphorylation in formalin rats. Finally, inhibition of PKC ζ /PKM ζ activity decreased the expression of Fos in response to formalin and CFA in both superficial and deep laminae of the dorsal horn.

Conclusions: These results suggest that PKC ζ , especially PKM ζ isoform, is a significant factor involved in spinal persistent nociceptive processing, specifically, the manifestation of chronic pain states following peripheral inflammation.

Keywords: atypical PKC ζ , persistent spinal nociceptive processing, inflammatory pain, dorsal horn, Fos

Background

Peripheral nerve damage or inflammation results in the induction of molecular mechanisms within the spinal cord leading to the amplification of the pain signalling ultimately contributing to persistent pain states [1]. Long term potentiation (LTP) is a ubiquitous mechanism throughout the central nervous system underlying a long-lasting, localized increase in synaptic strength and

is believed to be the neuronal substrate of learning and memory [2]. Interestingly, spinal LTP-related phenomena have also been reported in several animal pain models following either nerve damage or inflammation [3-6]. Furthermore, long-lasting enhancement of pain via high frequency stimulation in human subjects, considered to be the perceptual correlate of nociceptive LTP, have been found [7-9]. As a result, it has recently become clear that similarities and probably common intracellular signalling pathways exist between spinal persistent pain processing and LTP in the hippocampus [4,10].

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PKC ζ is an atypical protein kinase belonging to the protein kinase C (PKC) family, consisting of four functional domains, including regulatory domains and a kinase domain at the C-terminus [11,12]. In the brain, not only is the native form of PKC ζ (75kDa) expressed, but also a smaller fragment, PKM ζ (51kDa), which consists solely of the independent catalytic domain of PKC ζ and is therefore persistently active. Importantly, PKM ζ is, for instance, the only kinase involved in the maintenance of the late phase of LTP [12-16]. Perhaps more interestingly, during LTP in the hippocampus, PKM ζ is regulated by several upstream kinases including phosphatidylinositol 3-kinase (PI3K), the mammalian target of Rapamycin (mTOR), Ca²⁺/calmodulin-dependent kinase II (CAMKII) and the extracellular signal-regulated kinase (ERK), all of which are also involved in the establishment of spinal persistent nociceptive sensitization including C-fibre-evoked spinal LTP [17-21]. Together with conventional PKC, the activation of PKC ζ could contribute to the morphine-induced rewarding effect in a neuropathic pain model [22]. Moreover, along with PKC α and PKC ϵ , PKC ζ seems to be involved in sigma-1 activation induced-facilitation of nociception [23]. Finally, a very recent study investigated the role of PKM ζ in a spinal sensitized state promoting pain [24]. These authors used a model consisting in an intraplantar injection of IL-6 which induced short term allodynia. The subsequent intraplantar injection of prostaglandin E2 or intrathecal injection of a glutamate receptor 1/5 agonist precipitated pain behaviors reflecting a state of persistent sensitization of the nociceptive pathway. Spinal inhibition of PKM ζ at the time of intraplantar IL-6 injection or before the subsequent challenge blocked allodynia and/or nocifensive behaviors demonstrating the involvement of spinal PKM ζ in the initiation and also the maintenance of a spinal sensitization state. However, the expression of both PKM ζ , PKC ζ and their activated form (i.e. phosphorylated) in the spinal dorsal horn, the consequence of PKM ζ blockade on neuronal activity in an inflammatory pain context and its role compared to neuropathic pain have not been yet tested. Therefore, the aim of this study was to investigate the involvement of PKC ζ /PKM ζ in spinal persistent nociceptive processing using models of inflammatory and neuropathic pain.

Results

Effect of intrathecal administration of ZIP on mechanical and thermal sensitivity or locomotor function in normal rats

Intrathecal scrambled peptide (10 μ g, $n = 8$) did not modify mechanical and thermal withdrawal responses compared to baseline in normal rats (Figure 1a, b). More interestingly, intrathecal administration of 10 μ g

($n = 8$) of ZIP did not alter mechanical and thermal withdrawal responses compared to baseline and the control scrambled peptide group throughout the whole experiment (Figure 1a, b). Finally, neither the control scrambled peptide nor ZIP had any effect on the locomotor function of rats on the rotarod, assessed at 30 and 60 min post-injection (Figure 1c). We subsequently examined if spinal PKC ζ was involved in pain following peripheral inflammation or nerve injury.

Effect of intrathecal administration of ZIP on pain-related behaviors induced by intraplantar formalin

In control rats pre-treated spinally with either saline ($n = 8$) or the scrambled peptide (10 μ g, $n = 8$), formalin injection induced a similar biphasic response of pain-related behaviors, as expected (Figure 1d,e; saline: 1st phase (0-10 min), = 97 ± 8 s; 2nd phase (10-60 min) = 328 ± 28 s; scrambled peptide: 1st phase = 87 ± 6 s; 2nd phase = 317 ± 13 s). In contrast, pre-treatment with ZIP, especially at the highest dose, significantly decreased pain-related behaviors during both the first and second phases of the response to formalin (Figure 1d,e; 5 μ g: $n = 6$, 1st phase = 59 ± 9 s, $p > 0.05$; 2nd phase = 207 ± 13 s, $p < 0.001$; 10 μ g: $n = 9$, 1st phase = 46 ± 8 s, $p < 0.01$; 2nd phase = 145 ± 14 s, $p < 0.001$) compared to the scrambled peptide group. As the high dose (10 μ g) of ZIP exhibited a clear antinociceptive effect in this inflammatory pain model, this dose was tested in a model of neuropathic pain, the chronic constriction injury (CCI).

Effect of intrathecal administration of ZIP on mechanical allodynia and hyperalgesia in CCI rats

Chronic constriction injury of the sciatic nerve significantly decreased mechanical withdrawal responses and vocalisation thresholds as expected (Figure 1f,g). Intrathecal scrambled peptide (10 μ g, $n = 8$) did not modify mechanical withdrawal responses and vocalisation thresholds compared to baseline post-CCI in CCI rats (Figure 1f,g). More interestingly, intrathecal administration of 10 μ g ($n = 8$) of ZIP did not significantly inhibit mechanical withdrawal responses and vocalisation thresholds compared to baseline post-CCI and also the control scrambled peptide group throughout the whole experiment, indicating a lack of effect of ZIP on both mechanical allodynia and hyperalgesia following nerve injury.

Thus, PKC ζ /PKM ζ seems to be specifically involved in inflammatory pain and not in acute pain and, interestingly, neuropathic pain. Following these behavioral results, we have studied the effect of spinal application of ZIP on deep dorsal horn neurons activity using electrophysiology following formalin administration, and localisation, expression of PKC ζ /PKM ζ .

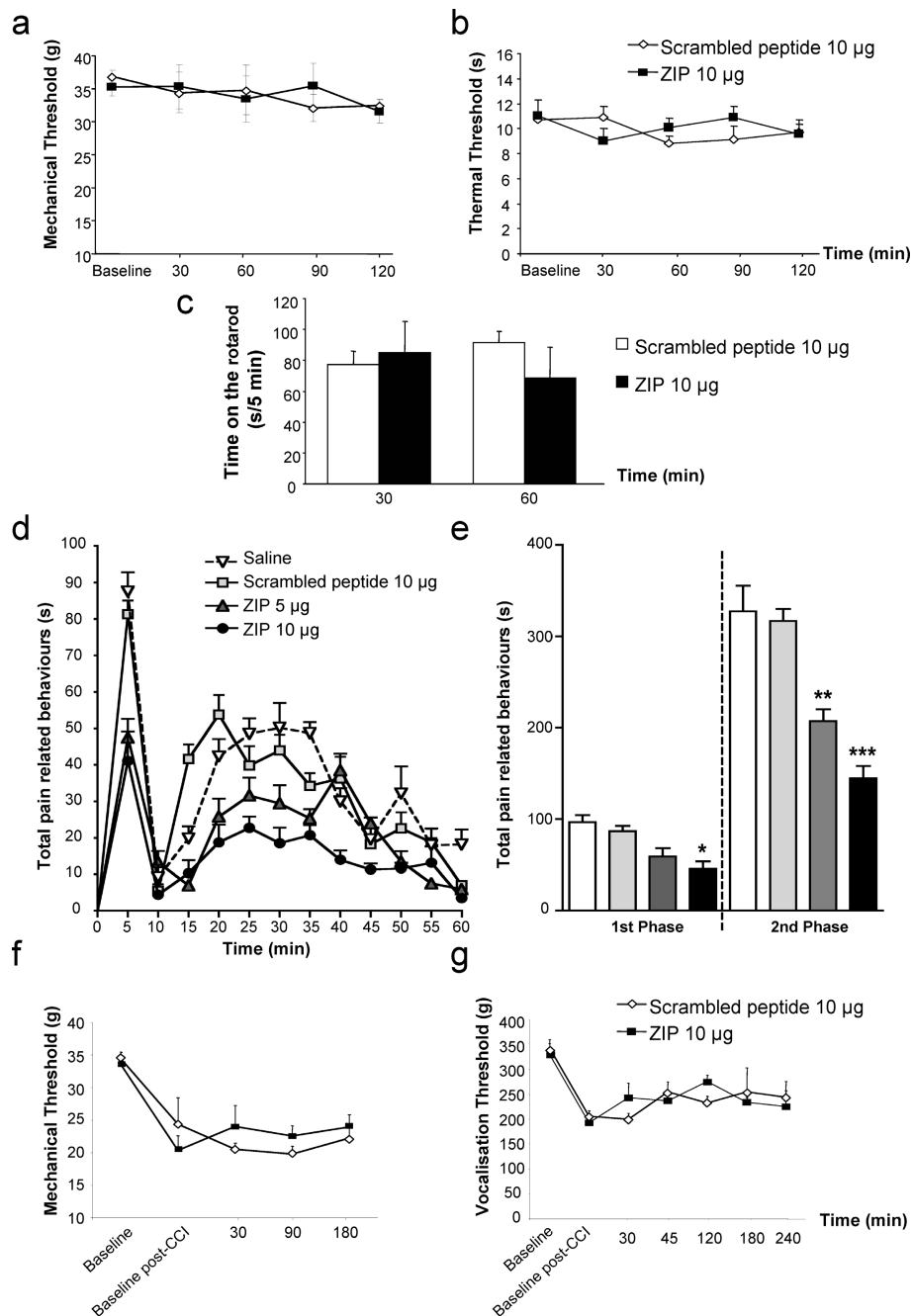


Figure 1 Spinal blockade of PKC ζ /PKM ζ activity specifically reduces pain-related behavior induced by intraplantar formalin. Time-course of (a) mechanical and (b) thermal sensitivity in normal rats following intrathecal administration of the scrambled peptide (10 μ g, $n = 8$) and PKC ζ /PKM ζ pseudosubstrate inhibitor, ZIP (10 μ g, $n = 8$). (c) Time spent on the accelerating rotarod (i.e. latency to fall) after intrathecal injection of the scrambled peptide (10 μ g, $n = 8$) and ZIP (10 μ g, $n = 8$). (d) Time-course of pain-related behaviors following subcutaneous formalin (5%, 50 μ l) injection into the hindpaw following intrathecal pre-treatment with saline ($n = 8$), the scrambled peptide (10 μ g, $n = 8$) or ZIP (5 μ g or 10 μ g, $n = 8$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ versus scrambled peptide). (e) Total pain-related behavior during the 1st (0-10 min) and 2nd (10-60 min) phases of the formalin response with saline, scrambled peptide or ZIP (** $p < 0.01$, *** $p < 0.001$, * $p < 0.05$ versus scrambled peptide). Time-course of (f) mechanical allodynia and (g) mechanical hyperalgesia in CCI rats following intrathecal administration of the scrambled peptide (10 μ g, $n = 8$) and ZIP (10 μ g, $n = 8$). All data presented as mean \pm s.e.m.

Effect of spinal application of ZIP on the formalin-induced firing response of deep dorsal horn WDR neurons

In control rats pre-treated with the scrambled peptide (10 µg, $n = 8$), formalin injected into the hindpaw receptive field produced the characteristic biphasic neuronal firing response of spinal WDR neurons (Figure 2a-c; 1st phase: 0-10 min, total APs = 8743 ± 1778 ; 2nd phase: 10-70 min, total APs = 83358 ± 11531). Spinal pre-treatment with ZIP (10 µg, $n = 8$) reduced both first and second phase neuronal firing, though this effect was only statistically significant on second phase activity (Figure 2a-c; 1st phase: total APs = 4847 ± 979 , $p > 0.05$; 2nd phase: total APs = 29643 ± 11132 , $p < 0.001$).

Expression and localisation of PKCζ and phospho-PKCζ/p-PKMζ in the spinal cord

To our knowledge no specific antibody only against PKMζ is commercially available. Therefore, we used RT-PCR to identify which mRNA isoforms of PKCζ are expressed in the DRG and spinal cord of naïve rats. We found 2 bands at 481 bp and 361 bp as expected for

PKMζ and PKCζ mRNA isoforms, respectively, in our spinal cord and DRG samples but also in our positive control samples (i.e. cortex, hippocampus) suggesting that both mRNA isoforms, and probably proteins, are expressed in the DRG and spinal cord (Figure 3a).

By western immunoblotting, we investigated the expression of PKCζ and PKMζ in the DRG, spinal dorsal horn and hippocampus using a specific anti-PKCζ antibody (Figure 3b). Western blots analysis confirmed that PKCζ protein is detected in the hippocampus, DRG and importantly, spinal dorsal horn (Figure 3b). Using an antibody against PKCζ/PKMζ active form, i.e. phospho-PKCζ/p-PKMζ, for which we have characterized the specificity using the blocking peptide (Figure 4b), we observed that p-PKCζ/p-PKMζ are expressed in all categories of DRG neurons, with a highest intensity of immunostaining in the cytoplasm and membrane of small and medium DRG neurons of naïve rats (Figure 3c). We then investigated if p-PKCζ/p-PKMζ is transported to and/or expressed in primary afferent central terminals within the dorsal horn in formalin animals. Expression of p-PKCζ/p-PKMζ was

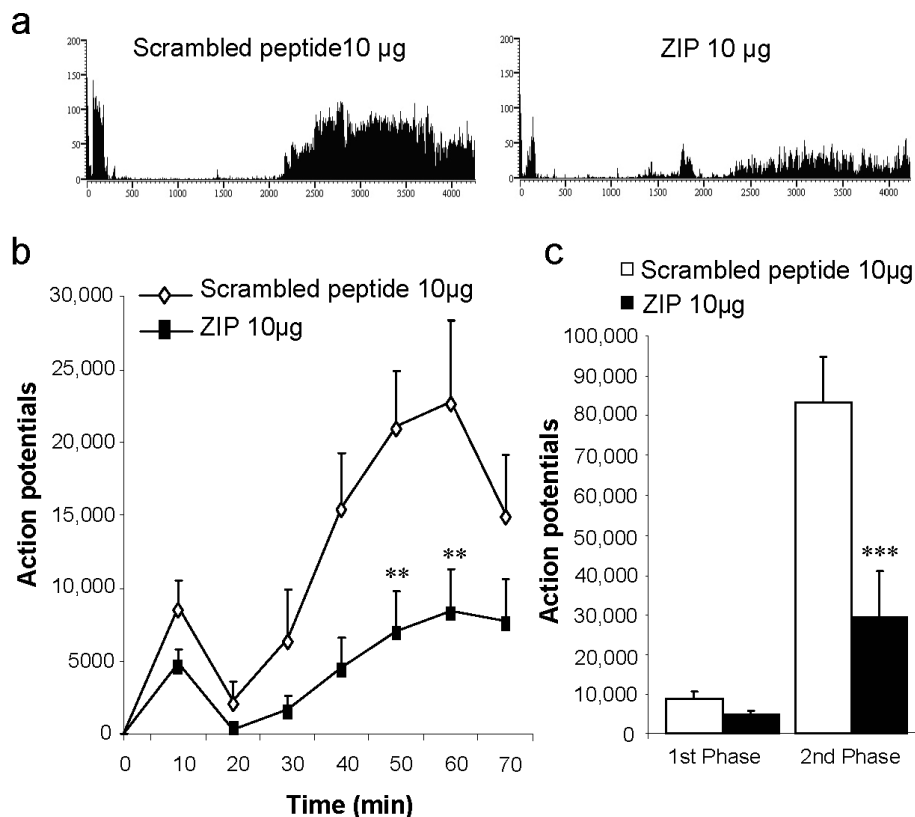
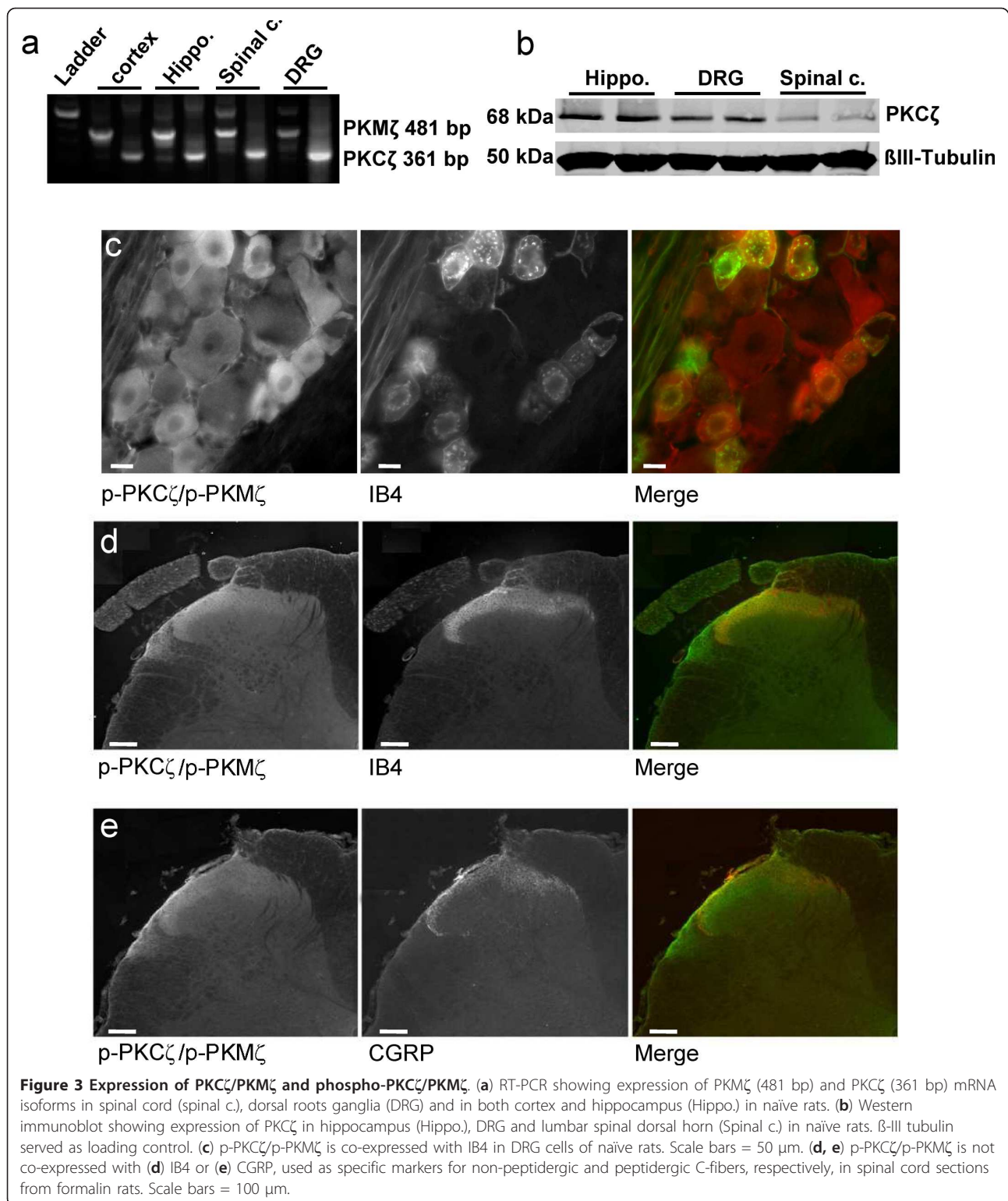


Figure 2 Spinal blockade of PKCζ/PKMζ activity reduces the response of deep dorsal horn WDR neurons induced by intraplantar formalin. (a) Representative rate recordings of firing responses of WDR neurons to formalin following spinal pre-treatment with scrambled peptide or ZIP. (b) Time-course of WDR firing response to subcutaneous formalin (5%, 50 µl) injection into the hindpaw receptive field following spinal pre-treatment with scrambled peptide (10 µg, $n = 8$) or ZIP (10 µg, $n = 8$, ** $p < 0.01$ versus scrambled peptide). (c) Total neuronal activity during the 1st (0-10 min) and 2nd (10-70 min) phases of the formalin response with scrambled peptide or ZIP (2nd phase: *** $p < 0.001$ versus scrambled peptide). Results are expressed as mean \pm s.e.m.



observed in the superficial dorsal horn but we did not observe any obvious co-localisation of p-PKCζ/p-PKMζ with IB4 and CGRP, specific markers for non-peptidergic and peptidergic C-fibers, respectively (Figure 3d,e), even at

high power magnification (Figure 4a). In addition, we used lumbar rhizotomy surgery in a separate experiment to see if p-PKCζ/p-PKMζ are expressed in primary afferent fibre terminals as we observed an almost total loss of IB4 and

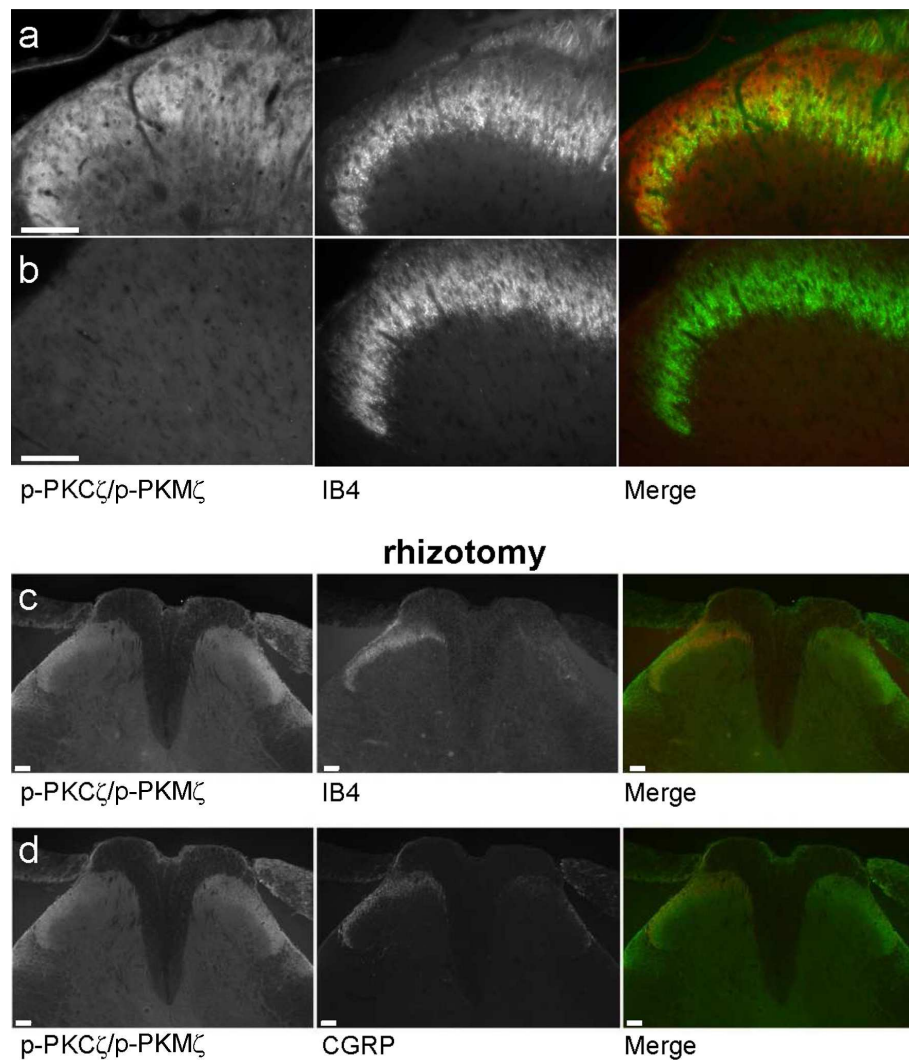


Figure 4 Expression of phospho-PKC ζ /PKM ζ following lumbar rhizotomy. High power magnification of p-PKC ζ /p-PKM ζ expression with IB4 in spinal cord sections from formalin rats (a). (b) The zeta-specificity of the antibody used is shown using the blocking peptide which drastically diminished p-PKC ζ /p-PKM ζ staining (left panel). (c, d) Lumbar rhizotomy caused an almost total loss of (c) IB4 and (d) CGRP staining demonstrating substantial deafferentation. However, p-PKC ζ /p-PKM ζ staining still remained. Scale bars = 100 μ m.

CGRP staining following rhizotomy, illustrating substantial deafferentation (Figure 4c,d). Interestingly, we did not observe any change in spinal p-PKC ζ /p-PKM ζ staining, indicating a lack of expression of p-PKC ζ /p-PKM ζ in primary afferent fibres terminals (Figure 4c,d) and suggesting a predominantly intrinsic spinal source.

Consequently, we attempted to identify the phenotype of p-PKC ζ /p-PKM ζ -expressing cells in spinal cord sections from formalin rats (Figure 5a-c). Phospho-PKC ζ /p-PKM ζ immunoreactivity was observed in NeuN-positive cells, marking neuronal nuclei (Figure 5c). No obvious co-localization was observed with GFAP-positive astrocytes or Iba1-positive microglial cells (Figure 5a, b). In addition, we used immunoprecipitation to reveal a

physical coupling between PKC ζ and NR2B subunits, which are specifically expressed in intrinsic dorsal horn sensory neurons and are also essential for spinal nociceptive plasticity (Figure 5d). PKC ζ protein was co-immunoprecipitated by the anti-NR2B antibody. Normal spinal lysate was run alongside immunoprecipitation samples as positive controls, while CREB and P2X3 were used as two negative control proteins, to verify that the immunoprecipitation step had worked. The P2X3 receptor is located exclusively on the central terminals of primary afferents and CREB is a nuclear protein, therefore these two proteins should not be found in a post-synaptic, membrane-associated NR2B/PKC ζ complex (Figure 5d). Indeed, both P2X3 and

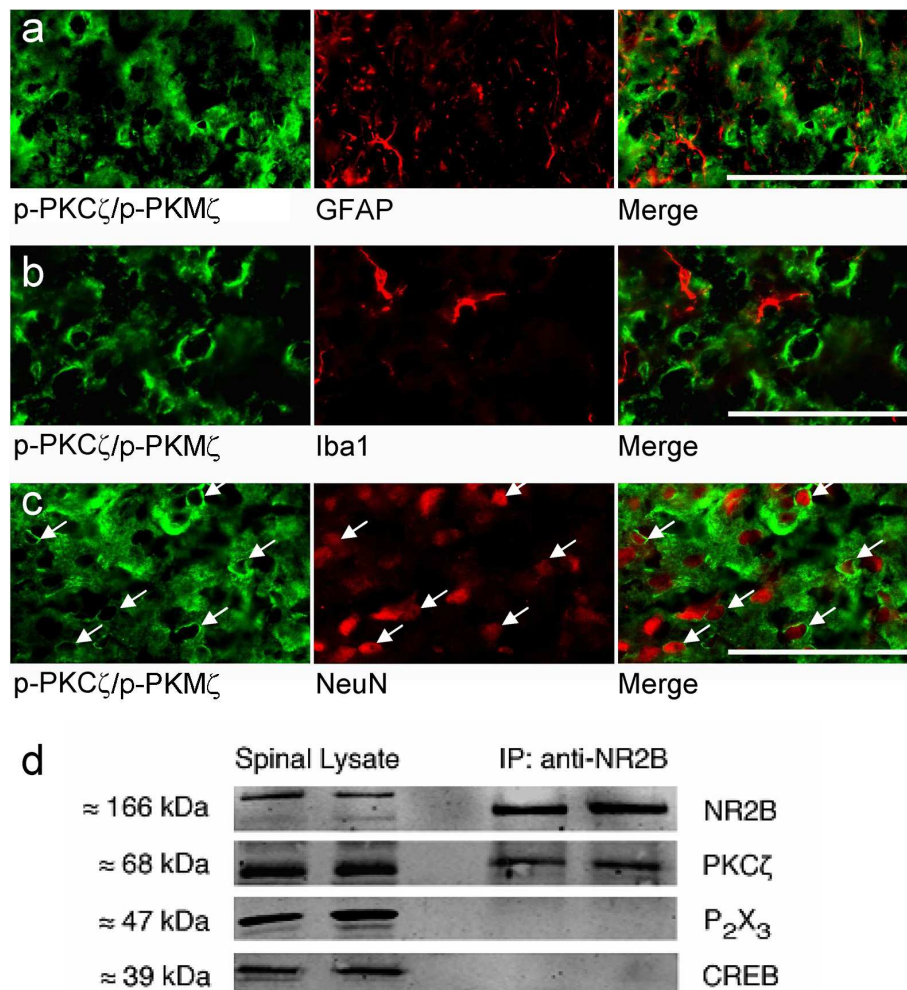


Figure 5 Phospho-PKC ζ /PKM ζ is expressed in spinal dorsal horn neurons. (a-c) p-PKC ζ /p-PKM ζ did not co-localize with (a) GFAP or (b) Iba1 but did co-localize with (c) NeuN. Scale bars = 50 μ m. (d) Western immunoblots of naïve rats' lumbar dorsal horn lysates (left two lanes) and co-immunoprecipitates from lumbar dorsal horn lysates obtained using an antibody against NR2B (right two lanes), probed with antibodies against NR2B, PKC ζ , CREB and P2X3.

CREB were only found in normal spinal tissue lysates, confirming that non-specific detection of proteins in the immunoprecipitation sample had not occurred. Thus, PKC ζ is physically coupled to NR2B-containing NMDA receptors in the spinal dorsal horn.

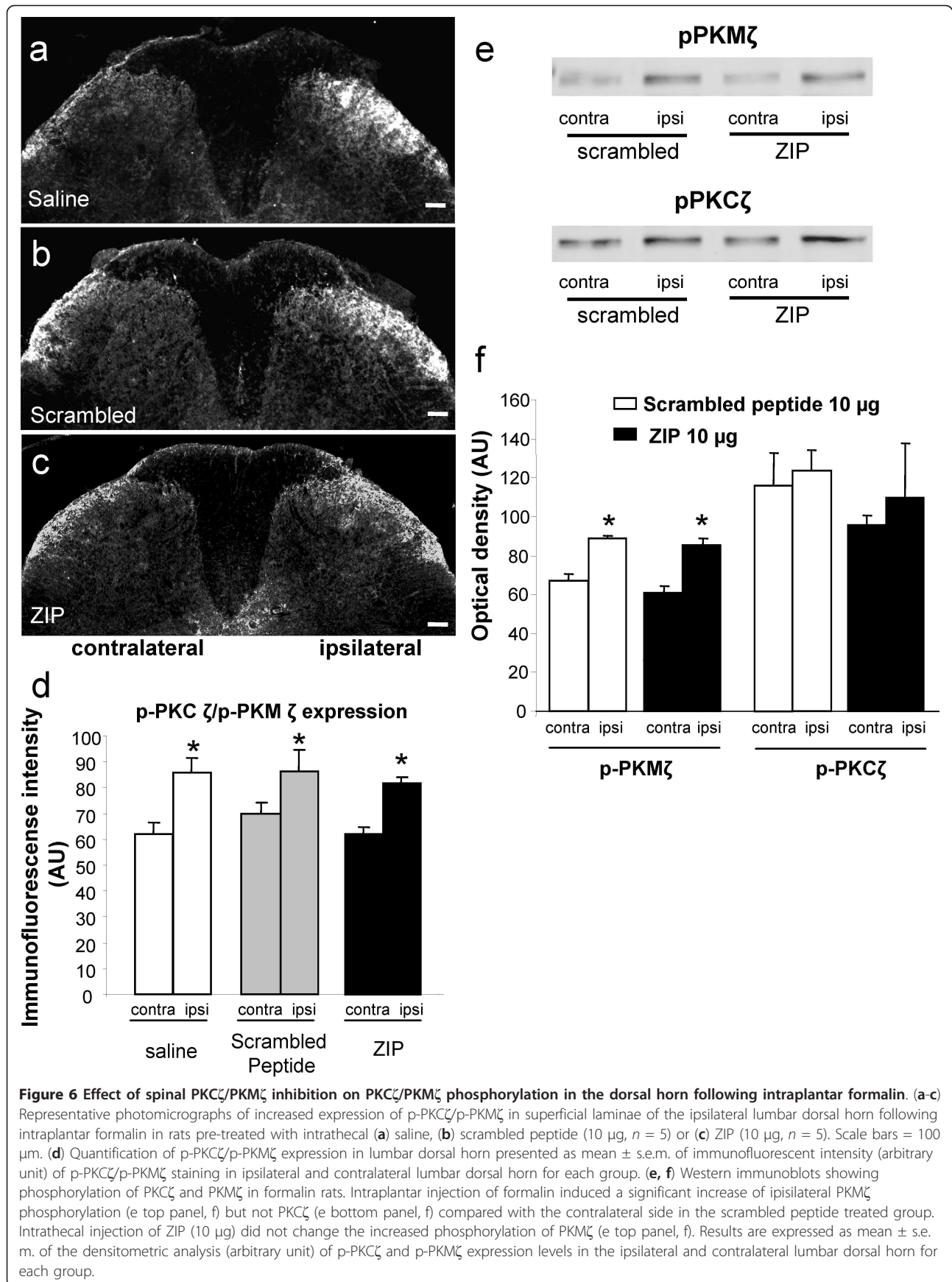
Together, these results suggest that phosphorylation of PKC ζ /PKM ζ following intraplantar formalin is mainly within intrinsic spinal dorsal horn neurons and could occur in NR2B containing NMDA receptors neurons. However, phosphorylation of PKC ζ /PKM ζ needed to be quantified.

Effect of intraplantar formalin on the phosphorylation of PKC ζ /PKM ζ expression in the spinal cord

Intraplantar formalin injection in the saline (10 μ l, n = 5) and scrambled peptide groups (10 μ g, n = 5) produced a significant increase of p-PKC ζ /p-PKM ζ in the

superficial (laminae I-II) layers of the ipsilateral dorsal horn (levels L4-L5) compared to the contralateral side (mean percentage increase = 27% \pm 3.4 and 22% \pm 2, respectively, p < 0.05; Figure 6a,b,d). In addition, intrathecal injection of ZIP (10 μ g, n = 5) did not significantly alter formalin-induced PKC ζ /PKM ζ phosphorylation (mean percentage increase = 25% \pm 2; Figure 6c,d).

In addition to immunohistochemistry quantification, we performed western blot analysis to assess the effect of intraplantar formalin and also PKC ζ /PKM ζ inhibition on the phosphorylation of PKC ζ and PKM ζ in L4-L5 spinal dorsal horn, 60 min after formalin injection. Scrambled-treated rats exhibited a significant increase in p-PKM ζ but not p-PKC ζ expression compared with the contralateral dorsal horn (p < 0.05; Figure 6e,f). Intrathecal administration of ZIP did not alter the phosphorylation of PKM ζ compared with scrambled-treated



rats (Figure 6e,f). As p-PKC ζ expression did not significantly increase in the scrambled group following formalin, ZIP was without any effect (Figure 6e,f).

Effect of intraplantar formalin on Fos expression in the spinal cord

Finally, we evaluated the effect of ZIP on Fos expression which marks activated neurons. The number of Fos positive cells in superficial (I-II) and deep (V-VI) laminae of the lumbar dorsal horn (L4, L5 and L6) was increased at 60 min after intraplantar formalin injection in scrambled peptide-treated rats (10 μ g, $n = 5$; Figure 7a,c). In contrast, intrathecal pre-treatment with ZIP (10 μ g, $n = 5$) resulted in a significant reduction of Fos-positive cells in both the superficial (L4: 45.5% \pm 5.8 reduction; L5: 51.3% \pm 5.9 reduction; L6: 59.3% \pm 1.1 reduction, all $p < 0.001$) and deep dorsal horn (L4: 53.2% \pm 8.4 reduction; L5: 51.6% \pm 9.2 reduction; L6: 52.9% \pm 11.4 reduction, all $p < 0.001$) compared to the scrambled peptide group (Figure 7b,c).

Effect of intrathecal administration of ZIP on mechanical and thermal hypersensitivity induced by intraplantar CFA

Next, we used a more sustained model of inflammation-induced spinal nociceptive plasticity, produced by intraplantar injection of CFA. Twenty-four hours after CFA administration, we observed a significant decrease of mechanical withdrawal thresholds before treatment in all groups (Figure 8a). Intrathecal scrambled peptide administration (10 μ g, $n = 8$) did not modify mechanical withdrawal thresholds, which remained significantly different from baseline throughout the whole experiment. In contrast, intrathecal administration of 10 μ g of ZIP ($n = 8$) significantly increased mechanical withdrawal thresholds 30 min after its administration in comparison to the scrambled peptide-treated group (increase of 41% \pm 14, $p < 0.05$; Figure 8a). However, mechanical withdrawal thresholds of the ipsilateral paw still differed significantly from that of the contralateral paw ($p < 0.05$; Figure 8a). Finally, ZIP had no effect on mechanical withdrawal thresholds at 90 and 180 min post-injection.

CFA administration also reduced thermal withdrawal latencies before treatment in all groups (Figure 8b). Intrathecal scrambled peptide administration did not alter thermal withdrawal latencies from the baseline throughout the test period (Figure 8b). In contrast, intrathecal administration of 10 μ g of ZIP significantly increased thermal withdrawal latencies at 30 and 90 min post-injection compared to the scrambled peptide group (30 min: increase of 82.7% \pm 31, $p < 0.001$; 90 min: increase of 73.5% \pm 21, $p < 0.05$; Figure 8b). Thermal withdrawal latencies of the ipsilateral paw of ZIP-treated group still remained significantly different from those of the contralateral paw at 90 and 180 min.

Effect of intraplantar CFA on the phosphorylation of PKC ζ /PKM ζ and Fos expression in the spinal cord

Intraplantar CFA injection in the scrambled peptide group (10 μ g, $n = 5$) produced a significant increase of p-PKC ζ /p-PKM ζ in the superficial (laminae I-II) layers of the ipsilateral dorsal horn (levels L4-L5) compared to the contralateral side (mean percentage increase = 19.6% \pm 3.3, $p < 0.05$; Figure 8c,e). As in the formalin experiment, intrathecal injection of ZIP (10 μ g, $n = 5$) did not reduce CFA-induced PKC ζ /PKM ζ phosphorylation (mean percentage increase = 29.4% \pm 6.4; Figure 8d,e).

We also examined the effect of intrathecal administration of ZIP (10 μ g, $n = 5$) on CFA-induced upregulation of Fos expression. In CFA animals treated with the scrambled peptide, we observed a significant increased expression of Fos in superficial (I-II) and deep (V-VI) laminae of the ipsilateral lumbar dorsal horn compared to the contralateral side (Figure 8f,h). In contrast, spinal delivery of ZIP significantly reduced CFA-induced Fos expression in superficial (47.2% \pm 11.2 reduction) and deep (46.5% \pm 19.4 reduction) laminae of the ipsilateral lumbar dorsal compared to the control scrambled peptide-treated group (Figure 8g,h).

Discussion

This study demonstrates that atypical PKC ζ more specifically the PKM ζ isoform is involved in spinal persistent nociceptive processing only following peripheral inflammation. Indeed, a specific pseudosubstrate inhibitor of PKC ζ /PKM ζ , ZIP, injected intrathecally, reduced pain-related behaviors elicited by intraplantar injection of formalin and CFA while it did not modify mechanical and thermal sensitivity or locomotor function in normal rats and interestingly, mechanical allodynia and hyperalgesia in a neuropathic pain model. Furthermore, direct spinal application of this inhibitor reduced the firing response of WDR neurons to formalin administration into the hindpaw receptive field during the second phase. Pain behaviors in both inflammatory models were associated with increased expression of the activated form (i.e. phosphorylated) of PKC ζ , especially phospho-PKM ζ in the ipsilateral dorsal horn of formalin rats, suggested to be specifically within spinal neurons. Finally, inhibition of PKC ζ /PKM ζ decreased Fos expression induced by peripheral inflammation in both superficial and deep laminae of the lumbar spinal dorsal horn.

Numerous studies have shown increased translocation and expression of different forms of PKC in dorsal horn neurons in pain models (for review see [25]). Moreover, inhibition of PKCs using non-specific PKC inhibitors (e.g. chelerythrine) reduces inflammation-induced pain related behaviors [26-28]. PKC ϵ may have a role in neurotransmitter release from primary afferent terminals

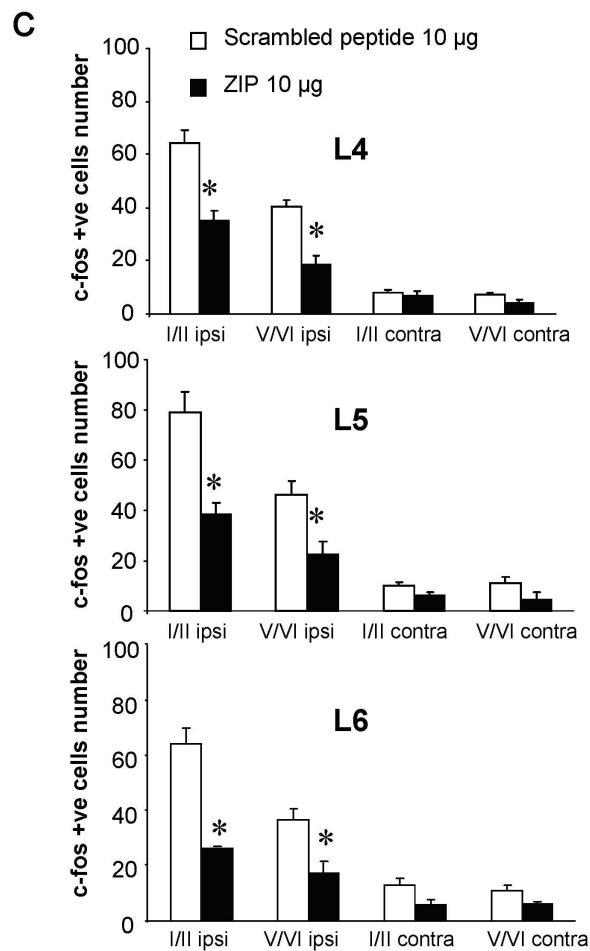
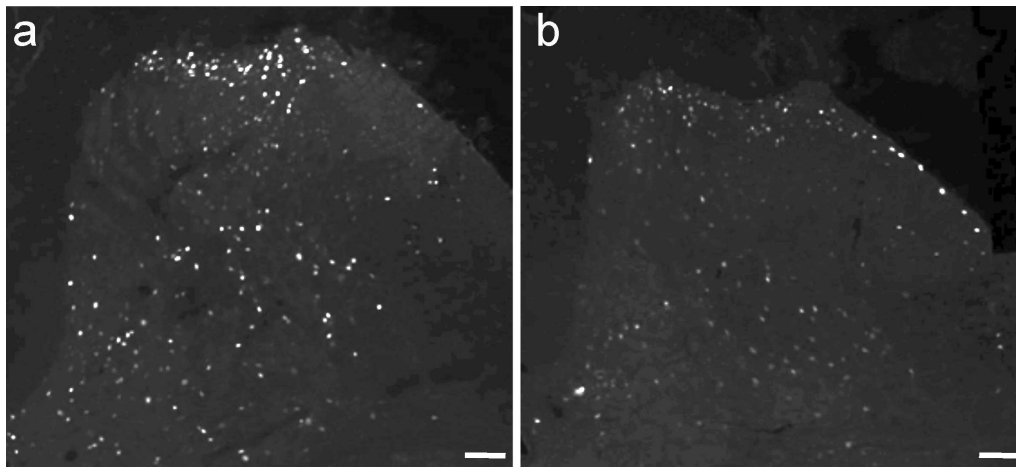
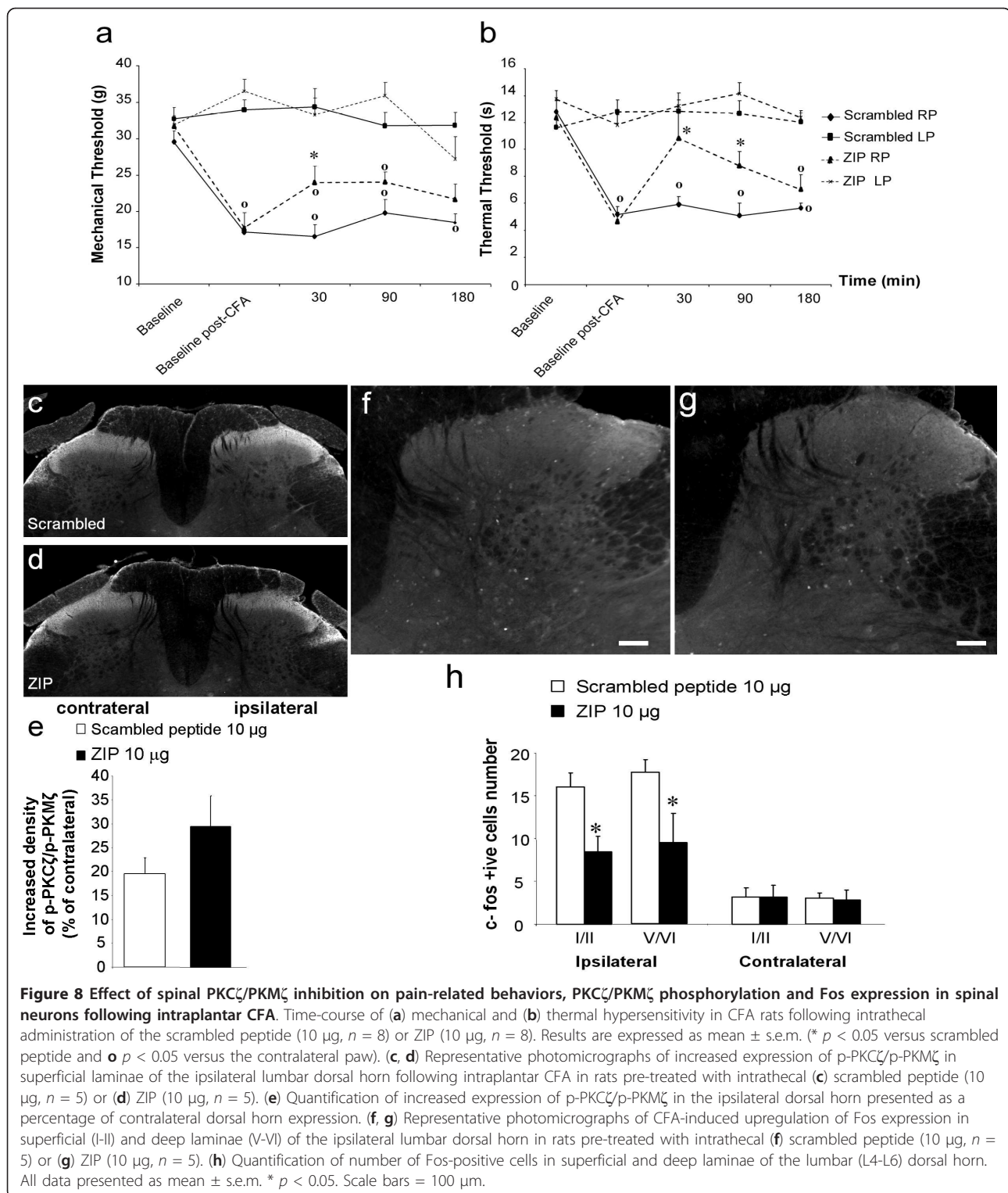


Figure 7 Effect of spinal PKC ζ /PKM ζ inhibition on Fos expression in the dorsal horn following intraplantar formalin. (a, b) Representative photomicrographs of formalin-induced upregulation of Fos expression in superficial (I-II) and deep laminae (V-VI) of the ipsilateral lumbar dorsal horn in rats pre-treated with intrathecal (a) scrambled peptide (10 µg, n = 5) or (b) ZIP (10 µg, n = 5). (c) Quantification of number of Fos-positive cells in superficial and deep laminae of the lumbar (L4-L6) dorsal horn. All data presented as mean \pm s.e.m., * $p < 0.05$. Scale bars = 100 µm.



[29], while PKCγ only contributes to increased hyperexcitability of a subset of lamina V NMDA-dependent neurons following an inflammatory stimulus, suggesting only a partial contribution of this kinase in spinal

sensitized state, especially in mediating mechanical hypersensitivity [30-32]. Therefore, other PKCs must be involved, with PKMζ a likely contributor since it is involved in the maintenance of hippocampal LTP

[13,14]. Thus, we sought to investigate the function of PKC ζ /PKM ζ in naïve animals and in both inflammatory and neuropathic pain models.

Mechanical and thermal nociceptive thresholds as well as locomotor function in normal rats were unaffected by spinal PKC ζ /PKM ζ inhibition by ZIP as also shown for acute mechanical sensitivity [23,24]. However, pain-related behaviors in the first and second phases of the formalin test were significantly reduced following intrathecal administration of ZIP. This effect observed on the first phase might suggest that PKC ζ /PKM ζ is involved in acute pain but, as demonstrated, ZIP did not affect nociception in normal rats. The biphasic behavioral response seen in freely moving animals is mimicked by the biphasic firing response of WDR neurons to peripheral formalin [26,33]. Using this electrophysiological approach, we observed that the second phase of the formalin test considered to underlie spinal nociceptive sensitization was substantially reduced by spinal ZIP application, indicating an inhibitory effect on neuronal excitability. In contrast, no significant effects were seen during the first phase of neuronal response. Monitoring both the behavioral and neuronal measures, there is a remarkable concordance, yet the second phase of the formalin response was reduced by ZIP but only the first phase in awake animals. There was a clear but insignificant reduction in first phase neuronal firing which may be enough to result in reduced withdrawal responses in awake animals. We subsequently utilized a more sustained model of inflammatory pain induced by intraplantar CFA, which exhibited significant reduction of their nociceptive thresholds within 24 hours, indicating the establishment of spinal persistent nociceptive sensitization. Intrathecal administration of ZIP significantly inhibited both mechanical and thermal hypersensitivity. In contrast to the formalin test, there are few studies on the effect of spinal PKC inhibition on pain behaviors after CFA with mixed results [20,34]. Nonetheless, PKC α or PKC γ are apparently involved in the biochemical modifications of NMDA receptors and/or GluR receptors translocation/internalisation within the lumbar spinal cord of the CFA model [34,35]. Importantly, a very recent study has shown that spinal PKM ζ underlies the initiation but also the maintenance mechanism of persistent nociceptive sensitization [24]. In a model of "hyperalgesic priming", blockage of PKM ζ at the time of intraplantar IL-6 inhibited allodynia. PKM ζ inhibition before subsequent challenge with intraplantar PGE2 or intrathecal mGlu1/5 agonist, when IL-6-induced allodynia was completely resolved, also abolished pain behaviors. Thus, all these data suggest a specific role of PKC ζ /PKM ζ in pain and spinal persistent nociceptive processing, especially following peripheral inflammation. Indeed, intrathecal administration of ZIP did not modify neuropathy-

induced mechanical hypersensitivity. Moreover, Li et al, found that spinal ZIP administration did not modify mechanical allodynia in neuropathic mice, there was no change in lumbar PKM ζ /p-PKM ζ expression and ZIP did not affect neuropathy-induced increased EPSCs from lamina II neurons [36]. However, they demonstrated a role of PKM ζ in the anterior cingulate cortex in neuropathic pain hypersensitivity [36]. This differential involvement of spinal PKM ζ is of crucial importance to understand the difference of the molecular mechanisms of persistent spinal nociceptive processing between inflammatory and neuropathic pain. It is plausible that the type of stimulus at the periphery and the release of specific algogenic substances following an inflammatory stimulus could specifically promote spinal PKM ζ activation. For example, PKC ζ has been reported to be involved in receptor signalling complexes such as TNF- α and IL1- β receptors and activation of NF κ B transcription factor during immune reactions which can occur in inflammatory pain states. Further studies are definitely needed to clarify this differential involvement of PKC ζ /PKM ζ between inflammatory and neuropathic pain.

Next, we assessed the expression of PKC ζ /PKM ζ in naïve animals and whether the phosphorylated forms of PKC ζ /PKM ζ change in the ipsilateral lumbar dorsal horn after peripheral inflammation. In naïve animals, both mRNA isoforms coding specifically for PKC ζ and PKM ζ are present in the DRG and lumbar spinal cord. Using western immunoblotting, we confirmed expression of PKC ζ protein in the lumbar spinal cord but also in the DRG and the hippocampus. However, the antibody used specifically recognized PKC ζ but not PKM ζ . Nonetheless, we found an expression of phospho-PKC ζ /p-PKM ζ mainly in small and medium DRG neurones of naïve animals. We therefore examined if p-PKC ζ /p-PKM ζ are expressed in central terminals of primary afferent fibres within the spinal cord of formalin rats. We did not observe any obvious co-staining of p-PKC ζ /p-PKM ζ with IB4 and CGRP in lumbar spinal cord. In addition, using lumbar rhizotomy, which causes a substantial loss of primary afferent terminals within the spinal cord [37,38], we still observed p-PKC ζ /p-PKM ζ staining in the dorsal horn, suggesting a predominantly intrinsic spinal source. Indeed, cells expressing p-PKC ζ /p-PKM ζ were neuronal as shown by co-expression with NeuN, a marker of neuronal nuclei and PKC ζ is physically linked to NR2B-containing NMDA receptors using co-immunoprecipitation. These results suggest that PKC ζ expression is restricted to intrinsic dorsal horn neurons within superficial laminae and could be an intracellular protein effector of the NMDA receptor complex.

We also conducted experiments to examine possible changes in the levels of phosphorylated (i.e. activated)

PKC ζ and PKM ζ in both inflammatory models. We observed a significant increase of p-PKC ζ and p-PKM ζ in the ipsilateral superficial dorsal horn, predominantly in the medial part, compared to the contralateral side in both models treated either with saline, the scrambled peptide or ZIP using immunohistochemistry. Furthermore, we only detected a significant increase of p-PKM ζ but not p-PKC ζ expression in the ipsilateral dorsal horn compared to the contralateral side in formalin rats using western blot. Finally, ZIP did not affect the phosphorylation of PKM ζ and PKC ζ in both models, as previously demonstrated for PKM ζ in hippocampal slices following electrical stimulation-induced LTP [18]. Therefore, the antinociceptive effect of this specific enzymatic site inhibitor may be due to a reduction of the phosphorylating capacity (i.e. catalytic activity) of mainly PKM ζ , at least following formalin, on downstream targets rather than its ability to be phosphorylated and further activated.

In both inflammatory pain models, we found Fos upregulation in superficial and deep laminae of the dorsal horn, which was reduced by PKC ζ /PKM ζ inhibition, reinforcing the hypothesis of a profound effect on neuronal activity. This also suggests that PKC ζ /PKM ζ is an upstream regulator of Fos expression. PKC ϵ also influences Fos expression in the lumbar dorsal horn following formalin, though its effects are mediated by inhibition of neurotransmitter release from primary afferent fibre terminals [25], while decreased expression of Fos in PKC γ knockout mice following formalin injection only occurs in laminae I-II [30]. Therefore, other PKCs, including PKC ζ /PKM ζ , are certainly involved in Fos expression and neuronal excitability, particularly in the deep dorsal horn. The deep dorsal horn neurons receive nociceptive inputs via interneurons and/or their dorsally extending dendrites. Thus, as PKC ζ /PKM ζ is primarily expressed in superficial dorsal horn, the involvement of this kinase on deep dorsal horn neurons activity is likely to be indirect. Interestingly, PI3K, mTOR, CAMKII, and ERK, expressed by dorsal horn neurons play a crucial role in spinal nociceptive plasticity, while also regulating the expression but probably not the phosphorylation of PKM ζ in LTP [17-19,29,39-41]. Such effectors (e.g. CaMKII, PI3K...) may influence PKC ζ /PKM ζ activity, and ultimately contribute to spinal persistent nociceptive processing. We finally demonstrated a physical link between NR2B-containing NMDA receptors and PKC ζ , therefore atypical PKC ζ /PKM ζ could participate in NMDA receptors activity in a context of chronic inflammatory pain but also through post-synaptic mGlu receptors [24]. Further studies are needed to investigate the upstream regulators (e.g. kinases) and downstream targets of spinal PKC ζ /PKM ζ in chronic inflammatory pain.

Conclusions

In the present study, we have demonstrated that PKC ζ especially PKM ζ isoform may be a significant factor in spinal persistent nociceptive processing following peripheral inflammation. This role of PKC ζ /PKM ζ seems to be restricted to inflammatory pain since there was no involvement of this kinase in acute and neuropathic pain. Pharmacological targeting of PKC ζ /PKM ζ may be of therapeutic benefit in chronic inflammatory pain conditions.

Materials and methods

Animals

All experiments were carried out using adult male Wistar rats (220-250g, Harlan, UK or Central Biological Services, University College London, UK), housed in standard laboratory conditions with free access to food and water. Experiments were approved by the UK Home Office and followed guidelines set by the International Association for the Study for Pain [42].

Behavioral testing

Experiments were performed in a quiet room by a single blinded experimenter using the method of equal blocks with randomization of treatments in order to avoid any uncontrollable environmental influence that might introduce variability in behavioral responses.

Thermal and mechanical nociception tests in normal rats

Naïve rats (180-200g, Harlan, UK) were injected intrathecally with 10 μ g of myristoylated PKC ζ pseudosubstrate inhibitor, ZIP (Myr-SIYRRGARRWRKL-OH; Biosource International Inc., USA) previously used as a specific inhibitor of PKC ζ /PKM ζ in several studies [15,24,36,43] or myristoylated control scrambled peptide (Myr-RLYRK-RIWRSAGR-OH; Genscript, USA). Thermal and mechanical thresholds were assessed 30, 60, 90 and 120 min post-injection using the thermal plantar test and automatic Von Frey test, respectively (for details see below).

Assessment of locomotor function - Rotarod

Locomotor function and motor effects of drugs were assessed using an accelerating rotarod device (Bioseb, France). Naïve rats (180-200 g) underwent a training period over 2 consecutive days (1 trial/day) prior to testing of drug effects in order to ensure that they could remain on the rotarod at 4 revolutions per minute (rpm) for 5 min. On the day of drug administration, the apparatus was set to accelerate from 4 to 40 rpm over 5 min and the latency to fall was monitored. Locomotor function in the accelerating rotarod was assessed 30 and 60 min following intrathecal injection of ZIP or the scrambled peptide (both 10 μ g/rat).

Formalin test

Prior to being placed in a Plexiglas box for 30 min to acclimatize, rats received a direct intrathecal (i.t.) injection of 5 or 10 μg of myristoylated PKC ζ pseudosubstrate inhibitor, ZIP, (Biosource International Inc., USA), control scrambled peptide (Genscript, USA) or 0.9% saline ($n = 8$ in each group). Thirty minutes later rats received 50 μl of 5% formalin injected subcutaneously (s.c.) into the plantar surface of the right hindpaw. Lifting, shaking, biting and licking of the injected paw were recorded by measuring the total duration of the response in seconds during the 60 min period following formalin administration. Data are presented in 5 min time bins. At the end of the test, animals were terminally anaesthetized and perfused (for details see below).

Induction of mononeuropathy: chronic constriction nerve injury (CCI)

Unilateral peripheral mononeuropathy was induced according to the method previously described by [44]. Briefly, rats (150-175 g) were anesthetized with sodium pentobarbital (6%, 1 ml/kg, i.p.). After skin incision, the right sciatic nerve was exposed and 4 polyester sutures (MERSUTURE[®] 3-0, Ethicon, Johnson & Johnson, France) were tied loosely around at 1 mm intervals, so that the nerve was constricted but the circulation was not interrupted. The skin was then sutured (MONO-CRYL[®] 5-0, Ethicon, Johnson & Johnson, Issy-les-Moulineaux, France). The animals were allowed to recover and were routinely monitored to assure good health. To examine mechanical allodynia (i.e. Von Frey test) and hyperalgesia (i.e. paw pressure test), we used two separate groups of animals. The right hindpaw was tested and measurements were taken on two separate days prior CCI surgery (baseline), 14 days after CCI induction (baseline post-CCI) and 30, 90 and 180 min or 30, 45, 120, 180 and 240 min for mechanical allodynia and hyperalgesia, respectively, after administration of a single direct intrathecal injection of ZIP or the scrambled peptide (both 10 $\mu\text{g}/\text{rat}$, $n = 8$ in each group).

Induction of peripheral inflammation with CFA

Rats were injected with 50 μl of Complete Freund's Adjuvant (CFA; 1mg/ml *Mycobacterium Tuberculosis*; Sigma, UK) into the plantar surface of the right hindpaw to induce inflammation. The CFA-treated paw showed marked swelling within 2-3 hrs which persisted for several days. To examine mechanical and thermal hypersensitivity, the left and right hindpaws were tested alternatively and measurements were taken on two separate days prior to CFA administration, 24 hrs after CFA injection (baseline post-CFA) and 30, 90 and 180 min after administration of a single direct intrathecal injection of ZIP or the scrambled peptide (both 10 $\mu\text{g}/\text{rat}$, n

= 8 in each group). At the end of the experiment, animals were terminally anaesthetized and perfused (for details see below).

Thermal and Mechanical hypersensitivity

Each animal was placed in a clear acrylic cubicle (22 \times 16.5 \times 14 cm) on top of a glass floor in a temperature controlled room ($\sim 22^{\circ}\text{C}$) and allowed to acclimatize for 15 min before testing. Thermal responses were tested using the method described by [45]. Withdrawal latencies were averaged over three consecutive tests, at least 5 min apart, in response to the thermal challenge from a calibrated radiant light source (output of 190 mW/cm²). A cut-off of 20 s was imposed to prevent tissue damage.

Mechanical withdrawal thresholds were tested using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). The stimulus was applied via an actuator filament (0.5 mm diameter) which, under computer control, applied a linearly increasing force ramp (2.5 g/sec, maximum force 50 g) to the plantar surface of the hindpaw. The force necessary to elicit paw withdrawal was recorded. The withdrawal threshold was calculated as the average of three consecutive tests, with at least 5 min between each test. A cut-off of 50 g which is the maximum force applied by the apparatus was used.

In order to assess mechanical hyperalgesia, a group of CCI rats were submitted to the paw pressure test previously described by [46]. Nociceptive thresholds, expressed in grams, were measured using an Ugo Basile analgesimeter (Bioseb, France) which applied an increasing pressure to the right hind paw until a squeak (vocalisation threshold) was elicited. The vocalisation threshold was measured 2 or 3 times before (baseline) and after ligatures (baseline post-CCI) in order to obtain two consecutive values that differed no more than 10%, and respecting an interval of at least 10 min between two measures. The maximal pressure (cut-off) applied was 750 g.

Direct intrathecal injection via lumbar puncture

Intrathecal (i.t.) injections were performed under isoflurane anaesthesia (4% induction, 2% maintenance) according to the method previously described [47]. The anaesthetised rat was held firmly at the pelvic girdle and drug was delivered (10 $\mu\text{l}/\text{rat}$) using a 25-gauge \times 1-inch needle connected to a 25 μl Hamilton syringe inserted into the subarachnoid space between lumbar vertebrae L5 and L6, eliciting a tail flick. The syringe was held in position for a few seconds after the injection.

In vivo Electrophysiology - Formalin Test

Preparation

Anaesthesia of rats was induced using 4-5% isoflurane (66% N₂O & 33% O₂) and a tracheal cannula was

inserted, once areflexic. Rats were placed in a stereotaxic frame to ensure stability during electrophysiological recordings, and core body temperature was maintained at 36.5-37°C using a heating blanket connected to a thermal rectal probe. Anaesthesia was reduced to 2.5% isoflurane, and a laminectomy was performed at the L1-L3 vertebral level, exposing the L4-L5 segments of the spinal cord. Anaesthesia was then reduced to 1.5% isoflurane and was maintained at this level for the duration of the experiment.

Identification of different spinal cell types was achieved by tapping at the hindpaw receptive field. Extracellular recordings from single convergent deep dorsal horn (> 600 µm) wide dynamic range neurons (WDRs) were made using parylene coated tungsten electrodes (A-M Systems, Washington USA). Cells were characterised prior to formalin administration. First, cells were stimulated electrically. A train of 16 transcutaneous electrical stimuli (2 ms wide pulses, 0.5 Hz) was applied at three times the threshold current for C-fibres via two stimulating needles inserted under the skin of the hindpaw, in order to assess primary afferent fibre input. Next, the receptive field was stimulated thermally by applying a constant jet of water using a needle and syringe. Both an innocuous (35°C) and noxious (48°C) temperature was applied in order to indicate a strong C-fibre input to the WDR neuron being recorded, which has been shown to be required for the response to subcutaneous formalin [48].

Administration of Drug and Formalin

Following cell characterization, 10 µg of the myristoylated PKCζ pseudosubstrate inhibitor, ZIP (*n* = 8), in a total volume of 50 µl, was applied directly onto the surface of the spinal cord using a Hamilton syringe, 30 min prior to the injection of formalin. A separate group of control animals (*n* = 8) received the control scrambled peptide (10 µg/50 µl).

Formalin (5%, 50 µl) was injected subcutaneously into the centre of the hindpaw receptive field and the firing response of the WDR neuron was followed for the subsequent 70 min. Activity was displayed as a rate recording and quantified in 10 min time bins. Data was captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design, UK; rate function) and presented as number of action potentials (APs).

Lumbar rhizotomy surgery

Lumbar rhizotomy was carried out as previously described by [37] and [38]. In brief, animals were deeply anaesthetized by intraperitoneal (i.p.) injection of a mixture of medetomidine (0.25 mg/kg) and ketamine (60 mg/kg). Skin and muscle incisions were made to expose the vertebral laminae. The intervertebral foraminae were

enlarged and L4, L5 and L6 dorsal roots were exposed and crushed for 10s each. The muscle and skin were then closed with 4.0 sutures. Seven days after surgery, animals were deeply anaesthetised and perfused (see below). Tissue processing (lumbar spinal cord) and immunostaining were conducted as described below.

Immunohistochemistry for phospho-PKCζ and Fos

At the end of the formalin and CFA behavioral experiments, animals were terminally anaesthetised using pentobarbitone and quickly perfused transcardially with saline followed by 4% paraformaldehyde (PFA) with 15% of a saturated solution of picric acid to reduce substantial dephosphorylation. After perfusion, the lumbar spinal cord was excised, post-fixed for 4 hrs in the perfusion fixative, cryoprotected in 20% sucrose in 0.1 M phosphate buffer (PB) overnight at 4°C, and then frozen in O.C.T compound. Transverse sections (30 µm) were cut on a cryostat and thaw-mounted onto glass slides. Sections were stained for p-PKCζ immunohistochemistry as follows: after 3 washes in PBS, sections were incubated for 48 hrs at 4°C with a rabbit primary antibody for anti-phospho-PKCζ (anti-p-PKCζ, Thr 410 sc-12894-R; 1:100; Santa Cruz Biotechnology, USA) followed by a secondary antibody solution for 4 hrs (goat anti-rabbit IgG-conjugated Alexa Fluor 488™; 1:1000; Molecular Probes, USA), as previously described [22]. Slides were washed in PBS and cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA).

Sections were visualized under a Zeiss Axioplan 2 fluorescent microscope running Axiovision 3.1 image analysis software and classified according to spinal level (L4, L5 and L6). Quantitative assessment of p-PKCζ/p-PKMζ staining was carried out by determining the immunofluorescence intensity using grey scale within a fixed area of the dorsal horn of the spinal cord (ipsilateral and contralateral to injury), as described by [19]. A box measuring 10⁴ µm² (100 × 100) was placed over the medial part of the superficial dorsal horn and the mean intensity of each area recorded. The measurement protocol was carried out on L4-L6 spinal sections of each animal (at least 5 sections per animal). The background fluorescence intensity of each tissue section was also determined and subtracted from recorded values. Data are presented as mean ± s.e.m. of immunofluorescence intensity (arbitrary unit) of p-PKCζ/p-PKMζ staining in ipsilateral and contralateral dorsal horn for each group.

To determine the cellular distribution of p-PKCζ/p-PKMζ, sections from formalin rats were counterstained with primary antibodies against markers for neurons [mouse anti-neuronal nuclei (anti-NeuN); 1:500; Chemicon, Hampshire, UK], astrocytes [mouse anti-gial fibrillary acidic protein (anti-GFAP); 1:1000; Abcam, Cambridge, UK], microglial cells [rabbit anti-ionized

calcium binding adaptor molecule 1 (anti-Iba1); 1:100; Wako Pure Chemical Industries Ltd, Japan], peptidergic primary afferent fibres [sheep anti-calcitonin gene related peptide (anti-CGRP); 1:800; Biomol International LP, USA], and non-peptidergic primary afferent fibres, [biotinylated lectin IB4 (anti-IB4); 1:1000; Sigma, UK], followed by the appropriate secondary antibody solution [goat anti-rabbit IgG-conjugated Alexa Fluor 546™; (1:1000; Molecular Probes, USA) for NeuN, GFAP and Iba1 or anti-sheep Cy3 (1:400; Stratech Scientific Ltd, UK) and extra avidin-TRICT [1:200; Sigma, UK; for CGRP and IB4].

Double immunostaining for p-PKCζ/p-PKMζ with CGRP or IB4 (see protocol above for CGRP or IB4 staining), was also conducted on lumbar spinal cord sections from a group of animals which had rhizotomy surgery (see 2.5). Following CGRP or IB4 staining, sections were incubated with rabbit anti-p-PKCζ (Thr 410, sc-12894-R; 1:100; Santa Cruz Biotechnology, USA) followed by secondary antibody solution (goat anti-rabbit IgG-conjugated Alexa Fluor 488™; 1:1000; Molecular Probes, USA). Slides were washed in PBS, cover-slipped and visualised under a Zeiss Axioplan 2 Microscope (Zeiss, Hertfordshire, UK).

The specificity of p-PKCζ/p-PKMζ immunostaining was verified by preabsorption of the primary antibody (Thr 410, sc-12894-R; 1:100; Santa Cruz Biotechnology, USA) with a 5 times excess (weight/weight) of the peptide used to generate the antibody (sc-12891 P, blocking peptide) overnight at 4°C. The pre-absorbed peptide was then centrifuged 20 min at 10 000 rpm and the top half of the liquid was collected and applied on tissue sections. A positive control (antibody alone at the same dilution) and a negative control (lack of primary antibody) were run in parallel and applied on sister sections. The antibody against IB4 was added to all tubes include in this specificity test. Immunostaining was revealed by incubation with secondary antibody (goat anti-rabbit IgG-conjugated Alexa Fluor 546™; 1:1000; Molecular Probes, USA) for 2 hours.

Sections were processed for Fos staining as follows: following 3 washes in PBS, sections were incubated overnight, at room temperature with the primary rabbit anti-Fos antiserum (1:2500 in PBST-Azide, AB5; Oncogene Science, Uniondale, NY, USA). Next, sections were incubated for 4 hrs with secondary antibody (Alexa-Fluor™ 488 goat anti-rabbit IgG; 1:1000; Molecular Probes, USA). Slides were washed in PBS and cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA). From each animal 4-6 sections (L4-L6) were randomly selected for counting Fos positive cells by a blinded investigator and an average of these counts was taken.

Western immunoblotting and immunoprecipitation

Naïve rats anaesthetized with urethane were sacrificed by decapitation and fresh dorsal roots ganglia (DRG), spinal cord (dorsal horn only) and hippocampus were dissected out and snap frozen. Tissue samples were subsequently homogenized in RIPA (Radioimmunoprecipitation assay) buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS) + 0.5% DOC (Deoxycholic acid) + Complete protease inhibitor cocktail using a glass homogenizer. Homogenate was then centrifuged at 14000 rpm for 10 min at 4°C and supernatant was collected. DRG, spinal dorsal horn and hippocampus whole cell lysates were next titrated to determine their protein concentrations using a BCA Protein Assay kit (Pierce, UK).

In a separate experiment, ipsilateral and contralateral sides of superficial spinal dorsal horn from scrambled peptide-treated animals (n = 4) and ZIP-treated animals (n = 4) were dissected out 60 min after intraplantar formalin 5%. Following the dissection, tissues samples followed the same procedure as above.

For western immunoblotting, lamselli loading buffer was added to DRG, dorsal horn and hippocampus protein lysates (30 µg) from naïve animals and samples were incubated at 70°C for 30 min. Samples were then loaded onto 8% gels and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After protein transfer to nitrocellulose membranes, membranes were incubated with rabbit anti-PKCζ primary antibody (1:500, ab59364, Abcam, UK) and rabbit anti-neuronal βIII-tubulin (1:3000, ab18207, Abcam, UK), which served as a loading control, overnight at 4°C, in order to identify PKCζ/PKMζ expression in either DRG, lumbar spinal cord or hippocampus in naïve rats.

Samples from ipsilateral and contralateral sides of superficial spinal dorsal horn from formalin rats were submitted to the same procedure as above and finally transfer to nitrocellulose membranes. Membranes were then incubated with rabbit anti-pPKCζ primary antibody (Thr 410 sc-12894-R; 1:500; Santa Cruz Biotechnology, USA) overnight at 4°C, in order to quantify p-PKCζ and p-PKMζ expression between ipsilateral and contralateral dorsal horn of formalin animals.

Following incubation with dye-linked donkey anti-rabbit IR800 or goat anti-mouse IR600 secondary antibody, proteins were revealed using the Odyssey fluorescence detection system (Licor, UK). For p-PKCζ and p-PKMζ expression levels, bands were quantified by densitometric analysis using Image J software. Results are expressed as mean ± s.e.m. of the densitometric analysis (arbitrary unit) of the phosphorylation of p-PKCζ and p-PKMζ expression levels in the ipsilateral and

contralateral dorsal horn for each group (both ipsilateral and contralateral samples were run on the same gel).

For immunoprecipitation of NR2B subunits, Dynabeads[®] Protein G (Invitrogen Ltd, UK) were washed and coupled with 5 µg of rabbit anti-NR2B antibody (06-600, Upstate, USA). Next 500 µg of spinal dorsal horn lysate from naïve animals was added to the antibody-dynabeads complex and allowed to incubate overnight for capture of target antigen. Captured protein was eluted from the dynabeads by resuspension in 40 µl of Lamaelli loading buffer and heating at 70°C for 30 min. Samples were then separated onto 8% gels by SDS-PAGE. Normal spinal lysates samples were run alongside IP samples as positive controls. Following electrophoresis and protein transfer to nitrocellulose membranes, membranes were incubated with primary antibody overnight at 4°C. Antibodies used were mouse anti-NR2B (1:500, 75-101, Neuromab, USA), rabbit anti-PKCζ (1:500, ab59364, Abcam, UK). Membranes were also probed with rabbit anti-CREB (1:500, ab5803, Abcam, UK) and rabbit anti-P₂X₃ (1:500, ab10269, Abcam, UK) antibodies used as negative controls. Following incubation with dye-linked donkey anti-rabbit IR800 or goat anti-mouse IR600 secondary antibody, proteins were revealed using the Odyssey fluorescence detection system (Licor, UK).

RT-PCR

Total RNA from cortex, hippocampus, spinal cord and DRG from naïve rats was used to synthesize cDNA with the SuperScript[™] II Reverse Transcriptase kit (Invitrogen). 1 µg of cDNA was used for PCR (50 µl final reaction volume) and amplified according to [11]. Cycle parameters were set as 34 cycles at 94°C for 30 s, 60°C for 1 min and 72°C for 1 min, with a final step of 72°C for 10 min. For amplification, F 5'-CCATGCCAG-CAGGACCACC-3' and 5'-CCTTCTATTAGATGCTGCTCTCC-3' were used as specific forward primers for PKCζ and PKMζ, respectively and R 5'-TGAAGGAAGGTCTACACCATCGTTC-3' was the reverse primer for both.

Statistical analysis

All data are presented as mean ± s.e.m. Cell characteristics from PKCζ/PKMζ pseudosubstrate inhibitor or scrambled peptide groups were compared by one-way analysis of variance (ANOVA), followed by Bonferroni's post-test. Formalin neuronal and behavioral response time-course data for formalin and CFA rats were compared between treatment groups by two-way repeated measures (RM) ANOVA, followed by Bonferroni's post-test. Total activity in the first and second phases was compared between treatment groups by one-way ANOVA, followed by Bonferroni's post-test.

Immunostaining and western immunoblotting of p-PKCζ/p-PKMζ and Fos was analyzed by one-way and two-way ANOVA, respectively, followed by Bonferroni post-tests. Statistical analyses were carried out using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA).

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Authors' contributions

FM and RM overall experimental design, collected the behavioral data, performed RT-PCR, participated to immunohistochemistry and western blotting experiments. RM performed the electrophysiology experiment. PY realised lumbar rhizotomy surgery and contributed to RT-PCR. MC, EM and SP performed immunohistochemistry and western blotting experiments. FM, RD and SP performed analysis and drafted the manuscript. AH and SB overall supervision of the experiments and writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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