

Consistent sex-dependent effects of PKM ζ gene ablation and pharmacological inhibition on the maintenance of referred pain

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Abstract

Background: Persistently active PKM ζ has been implicated in maintaining spinal nociceptive sensitization that underlies pain hypersensitivity. However, evidence for PKM ζ in the maintenance of pain hypersensitivity comes exclusively from short-term studies in males using pharmacological agents of questionable selectivity. The present study examines the contribution of PKM ζ to long-lasting allodynia associated with neuropathic, inflammatory, or referred visceral and muscle pain in males and females using pharmacological inhibition or genetic ablation.

Results: Pharmacological inhibition or genetic ablation of PKM ζ reduced mild formalin pain and slowly developing contralateral allodynia in nerve-injured rats, but not moderate formalin pain or ipsilateral allodynia in models of neuropathic and inflammatory pain. Pharmacological inhibition or genetic ablation of PKM ζ also effectively reduced referred visceral and muscle pain in male, but not in female mice and rats.

Conclusion: We show pharmacological inhibition and genetic ablation of PKM ζ consistently attenuate long-lasting pain hypersensitivity. However, differential effects in models of referred versus inflammatory and neuropathic pain, and in males versus females, highlight the roles of afferent input-dependent masking and sex differences in the maintenance of pain hypersensitivity.

Keywords

Sex differences, protein kinase M-zeta, PKC/M ζ knock-out, muscle pain, visceral pain, tactile allodynia, central sensitization, antinociception, analgesia

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Introduction

Although many studies have revealed spinal signaling molecules (protein kinase C [PKC], calcium/calmodulin-dependent protein kinase II [CaMKII], and extracellular signal-regulated kinase [ERK]1/2) underlying the induction of hypersensitivity that accompanies chronic pain, few have identified molecules affecting its maintenance, which is critical for pain chronicity.^{1–3} Although a growing number of studies have implicated spinal PKM ζ as key to the maintenance of pain hypersensitivity, this evidence comes mostly from experiments assessing short-lived nociception,^{4–9} while results from studies of longer lasting pain have been equivocal.^{6–8} Furthermore, all of

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these studies have utilized myristoylated-pseudosubstrate ζ -inhibitory peptide (ZIP), which has questionable specificity as mutant mice lacking PKM ζ still exhibit hippocampal long-term potentiation (LTP) and perform memory tasks that are reversed by ZIP.^{10,11} Surprisingly, these mice have not been tested for a nociceptive phenotype. Notably, all studies examining the nociceptive role of PKM ζ have used male rodents, despite a higher prevalence of chronic pain in females,^{12,13} and evidence for sex differences in hippocampal PKM ζ 's role in spatial memory maintenance,¹⁴ as well as in other critical pain mechanisms.^{15,16} We examine here the contribution of spinal PKM ζ to the maintenance of long-lasting pain, assessing the potential factor of ongoing afferent input to its differential manifestation in inflammatory and neuropathic pain models compared with referred pain after muscular and visceral injury. We also compare the effects of ZIP in rats to genetic disruption of PKM ζ in mutant mice and for the first time examine PKM ζ -dependent sex differences in the maintenance of pain hypersensitivity.

Materials and methods

Animals

Long Evans hooded rats (225–250 g, Charles River, St. Constant, QC) of both sexes were received and acclimatized at our animal housing facility at least seven days before the start of experiments. Methods were approved by the Animal Care Committee of McGill University and conformed to ethical guidelines of the Canadian Council on Animal Care and the International Association for the Study of Pain. Procedures for transgenic mice generation are as follows: While *Prkcz*^{-/-} mice were devoid of exon 9 of the *Prkcz* gene, rendering the transcription of PKM ζ and PKC ζ mRNAs incomplete, heterozygous (*Prkcz*^{+/-}) mice exhibited one wild type (WT) and one *Prkcz* allele. A vector targeting exon 9 of the mouse *Prkcz* gene was generated using a 129S6/SvEvTac mouse BAC clone from the RPC1-22 library and the plasmid pK-11 (Frt-PGKNeo-Frt-Loxp-pBSSK).¹⁷ Following chimera generation, exon 9 was deleted with the help of cre-loxp system and backcrossed for 11 generations into the C57BL/6 background. These breeder mice were obtained from the laboratory of Robert Messing, The University of Texas, Austin, for the development of our experimental mouse colony. *Prkcz*^{-/-} and *Prkcz*^{+/-} male and female mice were bred in-house to produce knockout, heterozygous, and WT off-spring, which were genotyped both at the start and end of experiments. Briefly, for genotyping, a 2 mm sample from the ear of restrained mice was obtained by using a commercially available ear punch that was previously cleaned with 70% ethanol. Ear punches were

incubated overnight at 55°C in 50 μ L of lysis buffer. Proteinase K (20 mg/mL) was added immediately before incubation. After vortexing, the samples were then placed for 10 min at 100°C in a PCR machine to denature the proteinase K. The resulting crude gDNA was subsequently genotyped by PCR amplification using KAPA2G Fast Hot Start Mastermix (Kapa Biosystems, Wilmington, MA) and a Bio-Rad T100 thermal cycler. WT forward primer was used at 300 nM: CCACACCC TCACCAACACT TTTTCC. Knockout forward primer was used at 275 nM: GGAACTTCGTCGACAT AAC TTCGTATAGCATAACAT. A common reverse primer was used at 1000 nM: CCTGCCCAAGACACTTTC AGGGTTC. The reaction was supplemented with betaine HCl (Sigma-Aldrich, St. Louis, MO) at 1 M and bovine serum albumin (Ambion, Foster City, CA) at 1 μ g/ μ L. Total reaction volume was 10 μ L, and 1 μ L of the crude gDNA prep was used. An initial denaturation at 95°C for 3 min was followed by 40 cycles of amplification: 95°C for 15 s, 64°C for 30 s, and 68°C for 1 min. The samples were run on a 1.5% agarose gel in TAE buffer with ethidium bromide and visualized in an Amersham Imager 600. The WT band migrated at 1.9 kb and the knockout band at 1.7 kb.

Pain models

Spared nerve injury. Neuropathic pain was induced in rats and transgenic mice using the SNI procedure of Decosterd and Woolf,¹⁸ by transecting the common peroneal and tibial nerve branches, sparing the adjacent sural and saphenous nerves (see Figure 1(a)). Rats received ZIP intrathecally (i.t.) 10 days or six weeks post-SNI. Paw withdrawal thresholds (PWTs) were measured using von Frey testing (described later) on both contralateral and ipsilateral paws. Transgenic mice were tested for PWTs until six weeks post-SNI.

Complete Freund's adjuvant. Rats were injected i.pl. with 50 μ L of 1 mg/mL complete Freund's adjuvant (CFA) in the left hind paw. We examined the effects of i.t. ZIP on CFA-induced mechanical allodynia and thermal hyperalgesia using von Frey and Plantar tests (see later), respectively, until 48 h postinjection.

Intracolonic capsaicin. Induction of remote pain in rats using intracolonic (i.c.) capsaicin was as described previously.¹⁹ Briefly, a volume of 200 μ L of 0.1% capsaicin (Sigma; dissolved in a solution consisting of 10% ethanol, 10% Tween 80, and 80% saline) was instilled into the colon via anus using a fine cannula (PE-20 polyethylene tubing) that was inserted up to a maximum length of 7 cm. ZIP or scrZIP effects were tested 20 min before or 1 h after a single i.c. capsaicin instillation. In the case of priming experiments, with two instillations spaced two

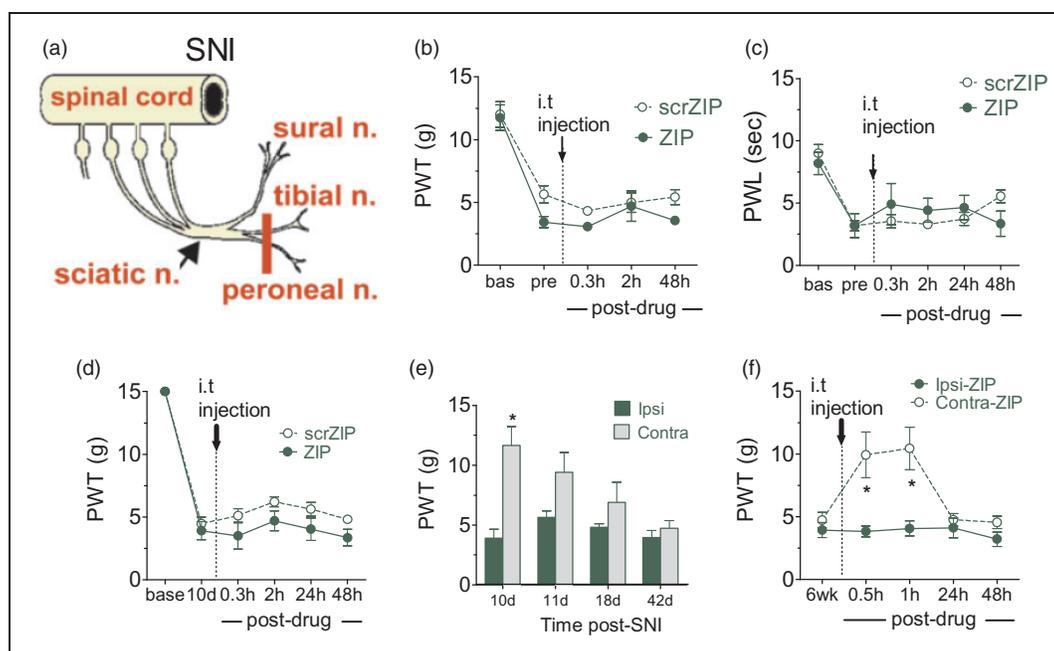


Figure 1. Effects of PKM ζ inhibition in inflammatory and neuropathic pain models. (a) A diagram illustrating the SNI surgical procedure. (b and c) Absence of ZIP effects on CFA-induced thermal hyperalgesia (b) and mechanical allodynia (c). (d) PKM ζ inhibition using i.t. ZIP at 10 days post-surgery does not reverse SNI-induced ipsilateral allodynia. (e) Time course of development of contralateral allodynia in SNI rats. (f) ZIP reduces contralateral allodynia for 60 min when injected at six weeks post-SNI. (b–f) Asterisks denote a significant difference between ipsilateral and contralateral paw withdrawal thresholds ($*p < 0.05$). All of the data were collected in male rats and are presented as mean \pm S.E.M. Note. PKM ζ = protein kinase M zeta; SNI = spared nerve injury; ZIP = ζ -inhibitory peptide; CFA = complete Freund's adjuvant; scrZIP = scrambled ZIP.

weeks apart, drug effects were examined at 20 min prior to the first, two weeks after the first but 20 min before the second, and 24 h after the second challenging stimulus. For mice, 50 μ L of 0.1% capsaicin (Sigma; in the same vehicle as above) was instilled into the colon using a thin cannula (PE-10 polyethylene tubing).²⁰ The perianal area was covered with petroleum jelly prior to insertion of the cannula to avoid pre-injection irritation, and the cannula was inserted for 4 cm. ZIP effects in WT and heterozygous mice were examined until 24 h postinjection.

Intramuscular (i.m.) acidic saline. Two acidic saline (0.9% NaCl in sterile water for injection, pH adjusted to 4.0) injections, spaced five days apart, were administered to the gastrocnemius muscle in rats²¹ (100 μ L) or mice (20 μ L)²² to induce persistent hind paw mechanical allodynia. In rats, we examined the effects of i.t. ZIP, scrZIP, and NPC-15437 (Sigma), 24 h and one week after the second acidic saline challenge. The mice were tested for the time course of expression of mechanical allodynia over a period of four weeks.

Nociceptive testing

von Frey testing. Rats and mice were initially habituated for 20 and 60 min, respectively, to acrylic testing

chambers (12 cm wide, 18 cm long, and 15 cm high) fitted with wire mesh floors (12 \times 12 mm mesh size). Filaments (selected from a Semmes Weinstein Monofilament Kit, Stoelting, Wood Dale, IL) were applied in either ascending (after a negative response) or descending (after a positive response) force as necessary to determine the filament closest to the 50% threshold of response. Each filament was applied for 10 s (for rats) or 4 s (for mice) or until a flexion response occurred. The minimum stimulus intensity was 0.25 g and the maximum was 15.0 g. Based on the response pattern, and the force of the final filament (fifth stimulus after first direction change), the 50% threshold (gram) was calculated as: $(10^{[X_r + k\delta]}) / 10000$, where $X_r = \log(10 \times \text{filament buckling force in mg})$, $k = \text{value for the pattern of positive/negative responses}$, and $\delta = \text{mean difference in log units between stimuli}$ (here $\delta = 0.220$, for more details, see Chaplan et al.²³).

Plantar test. Briefly, rats were placed in clear plastic cubicles mounted on a glass plate and acclimatized for 20 min. A paw withdrawal response was induced by shining a radiant heat source directly under the plantar aspect of the hind paw using a modification of the method of Hargreaves et al.²⁴ The intensity was preset so that a normal withdrawal response happens within a

period of 10 s. To prevent tissue damage, a cutoff time of 20 s was set. The reported thermal paw withdrawal latency (PWL) was determined by calculating the mean of three consecutive PWL measures spaced 10 min apart.

Formalin test. A volume of 50 μ L of 2%, 3.5%, or 5% formalin (Sigma, in 0.9% saline) was injected intraplantar (i.pl.) into the left hind paw of naïve or i.t. catheterized rats (see below). Naïve rats received ZIP or scrZIP by lumbar puncture 20 min prior to formalin; while for catheterized rats, drugs were administered at the peak of the second phase of the formalin test via the i.t. catheter. Sustained nociceptive behaviors (SNBs) were assessed using the weighted means scoring methods of Dubuisson and Dennis²⁵ every 2 min until 46 min. Transgenic mice received 25 μ L formalin to the plantar hind paw at either 1% or 2% concentration. SNB scoring was performed blind to the genotype of the mice.

Drug administration

All drugs were administered in a volume of 20 μ L into the intrathecal space by either lumbar puncture or an indwelling i.t. catheter. ZIP (Tocris Bioscience, Bristol, UK), and its inactive control peptide scrZIP (Tocris Bioscience, Bristol, UK), were dissolved in sterile water for injection and administered in a final dose of 10 nmol. For lumbar puncture, a 26½-gauge needle was inserted between the L5 and L6 vertebrae into the *cauda equina* in rats under isoflurane inhalation anesthesia (4% induction and 2% maintenance). Correct placement of the needle was verified by a brief flick of the tail at initial needle placement and on drug injection. For i.t. catheterization, a 5 cm polyethylene catheter (PE-32) was implanted sub-durally 4 cm to the lumbar enlargement with the help of a 23-gauge needle inserted between the L5/L6 vertebrae in rats under pentobarbital anesthesia. After removal of the needle, the outer end of the PE-32 catheter was glued to a 14 cm piece of PE-10 catheter. The tight connection between PE-10 and PE-32 catheters was verified by flushing saline through the connected catheters. The catheter was anchored in place by stitches over the skin until the time of testing. Rats were given a 24 h recovery period between catheter placement and formalin testing. The correct i.t. placement of the catheter was verified by the observation of a short-lived hind paw paralysis induced by lidocaine injection. Drugs were administered i.t. in a volume of 20 μ L and flushed with the required volume of saline calculated according to length and diameter of the catheter.

Western immunoblotting

Spinal cord dorsal horns were quick frozen and homogenized twice for 5 s each in the presence of final sample

buffer containing phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche, Basel, Switzerland). Samples were incubated for 10 min at 95°C. After centrifugation (5 min at 13,000 rpm, room temperature), trichloroacetic acid precipitation was performed on the resulting supernatant. Samples were then incubated for 20 min on ice, centrifuged, and the resulting protein pellet was resuspended in 2 \times gel sample buffer (100–200 μ L depending on size of the pellet). Samples were incubated for 10 min at 95°C and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were blotted onto a nitrocellulose membrane for 86 min. Membranes were incubated overnight with primary antibodies against total PKC ζ , PKC λ , and PKM ζ (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), the phosphorylated forms (1:100, Cell Signaling Technology, Danvers, MA), or actin (1:100,000, Chemicon, Rolling Meadows, IL). Filters were washed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:2,000) overnight. After washing, filters were incubated with enhanced chemiluminescent substrate as recommended by the supplier (Abcam). Films were exposed between 5 s and 60 s and scanned using a Canon N650U image scanner. Relative band intensities were then quantified using Image J (NIH) software.

Statistical analysis

All data are presented as mean \pm S.E.M. Statistical analyses were performed using SPSS. All time course data were analyzed using repeated measures analysis of variance followed by multiple comparisons with Bonferroni's post hoc test. Statistical differences between treatment groups in western blots were obtained by comparisons of ImageJ results using Student's *t*-test. Percent change from baseline was calculated using the formula: $([\text{post-drug PWT} - \text{baseline PWT}]/[\text{baseline PWT}]) \times 100$ for each animal. The resulting percent changes were analyzed for statistical differences using Student's *t*-test. Graphs were constructed using GraphPad Prism (version 6.0).

Results

PKM ζ inhibition in inflammatory and neuropathic pain models

We initially examined spinal PKM ζ -dependent maintenance mechanisms in standard models of inflammatory (hind paw injection of complete Freund's adjuvant [CFA]) and neuropathic (spared nerve injury [SNI], see Figure 1(a)) pain, in which pain hypersensitivity is evidenced by reduced PWLs or PWTs. Male Long Evans rats were treated with ZIP, or the scrambled control peptide scrZIP, i.t. 24 h after CFA and either 10 days or six

weeks after SNI, in an attempt to reverse pain hypersensitivity or allodynia. Neither ZIP, nor scrZIP elevated ipsilateral mechanical PWTs (Figure 1(b)) or thermal PWTs (Figure 1(c)) of CFA rats or ipsilateral PWTs (Figure 1(d)) in SNI rats tested 10–12 days postsurgery. However, at six weeks, when allodynia has developed in the hind paw contralateral to the nerve injury (Figure 1(e)), ZIP produced transient, but highly significant increases in contralateral PWTs at 30 min ($F_{1,10} = 10.627$, $p = 0.009$) and 60 min ($F_{1,10} = 12.438$, $p = 0.005$), without effects on ipsilateral PWTs (Figure 1(f)).

PKM ζ genetic ablation in a neuropathic pain models

Parallel assessment of post-SNI PWTs was performed in WT ($Prkcz^{+/+}$) and mutant mice ($Prkcz^{+/-}$ and $Prkcz^{-/-}$) with deletion of PKC/M ζ (Figure 2(a)). Analogous to ZIP-treated SNI rats, ipsilateral PWTs remained low (i.e., ipsilateral allodynia was present) in both WT and mutant mice for six weeks post-SNI (Figure 2(b)). However, similar to rats given ZIP, contralateral allodynia observed in $Prkcz^{+/+}$ and $Prkcz^{+/-}$ mice at five and six weeks post-SNI was attenuated in $Prkcz^{-/-}$ mice (Genotype \times Time: $F_{10,65} = 2.809$, $p = 0.006$) (Figure 2(c)).

PKM ζ inhibition and genetic ablation in the formalin test

We hypothesized that the inability to demonstrate a role for PKM ζ in ipsilateral neuropathic pain hypersensitivity was due to a masking of ZIP's inhibitory effects by ongoing peripheral inputs from the nerve injury. To test this hypothesis, we examined the effects of posttreatment with i.t. ZIP or scrZIP in the formalin test in separate groups of rats that received increasing concentrations of i.pl. formalin to produce greater afferent inputs. We replicated our previous finding⁷ that i.t. ZIP, but not scrZIP, attenuated formalin-induced nociceptive behaviors when administered 25 min after a 2% formalin injection (Figure 3(a), Treatment \times Time interaction: $F_{20,210} = 3.089$, $p < 0.001$). However, i.t. ZIP produced reduced analgesic effects in rats, given a higher, 3.5% concentration (treatment $F_{1,20} = 13.62$, $p < 0.001$; Figure 3(b)) and no effect after the highest, 5% i.pl. formalin injection (Figure 3(c)). $Prkcz^{-/-}$ mice, when compared with $Prkcz^{+/-}$ or $Prkcz^{+/+}$ animals, showed dramatically reduced nociception after low concentration (1%) i.pl. formalin (Figure 3(d), genotype $F_{2,28} = 94.617$, $p < 0.001$), but only a minor suppression after administration of a higher concentration (2%) i.pl. formalin (Figure 3(e), Genotype \times Time interaction: $F_{88,1144} = 1.894$, $p < 0.001$). Our formalin and SNI data thus suggest

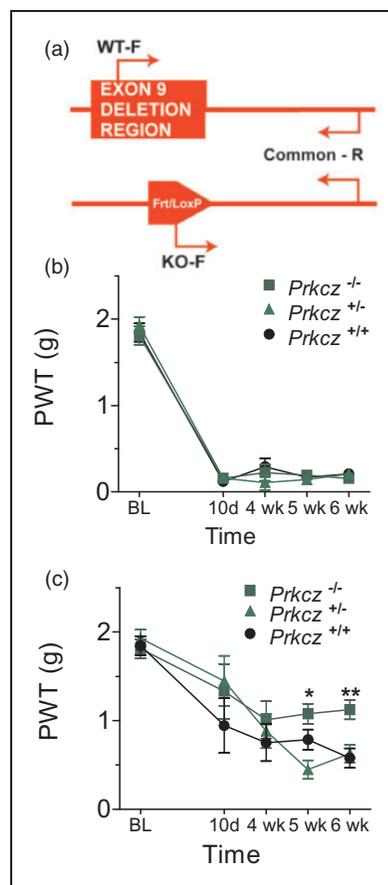


Figure 2. Effects of PKM ζ genetic ablation in inflammatory and neuropathic pain models. (a) Schematic presentation of the *Prkcz* knockout methodology/genotyping strategy. (b) PKC/M ζ knockout ($Prkcz^{-/-}$ and $Prkcz^{+/-}$ mice) did not influence ipsilateral allodynia between 10 days and four weeks post-SNI. (c) $Prkcz^{-/-}$ mice display attenuated contralateral allodynia from five to six weeks post-SNI, whereas $Prkcz^{+/+}$ and $Prkcz^{+/-}$ mice remain persistently allodynic until six weeks postsurgery. (b–c) Asterisks denote a difference between $Prkcz^{-/-}$ and $Prkcz^{+/+}$ animals (* $p < 0.05$, ** $p < 0.01$), daggers denote a difference between $Prkcz^{-/-}$ and $Prkcz^{+/-}$ animals ($\dagger p < 0.05$). All of the data are presented as mean \pm S.E.M. Note. PKM ζ = protein kinase M zeta; PKC = protein kinase C; SNI = spared nerve injury.

that the antinociceptive effect of PKM ζ inhibition or silencing depends largely on the degree of ongoing peripheral inputs.

PKM ζ inhibition in referred visceral pain

We have previously shown that i.t. ZIP both prevents and reverses allodynia that extends 24 h following i.pl. injection of capsaicin to the rat hind paw.⁷ We expanded on this cutaneous study to determine whether spinal PKM ζ might also contribute to the maintenance of longer lasting referred pain associated with visceral

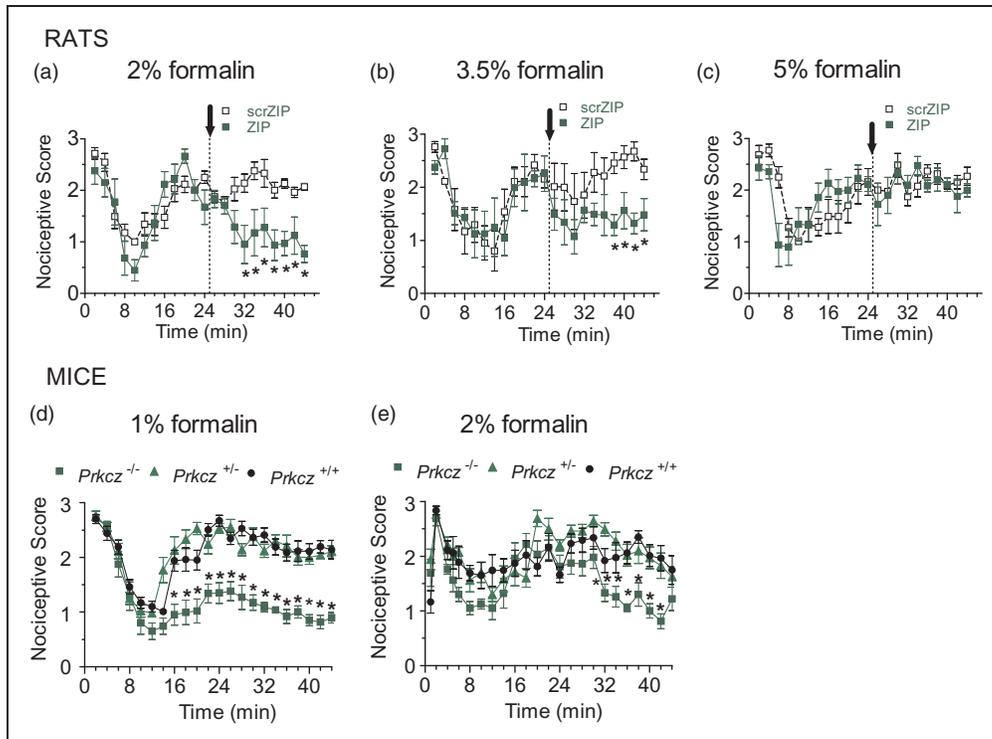


Figure 3. Effects of PKM ζ inhibition and genetic ablation in the formalin test. (a–c). Differential effects of formalin concentration on antinociceptive effects of PKM ζ inhibition in the formalin test. ZIP treatment at 25 min post-2% formalin resulted in a significant reduction of nociceptive scores between 32 and 44 min post-formalin (a), while eliciting shorter effects (38–44 min) against 3.5% formalin (b) and no effects against 5% formalin (c). Asterisks indicate significant differences ($p < 0.05$) between ZIP- and scrambled ZIP-treated groups. (d) $Prkcz^{-/-}$ mice injected with 1% formalin showed a persistent decrease in nociceptive scores beginning 16 min postinjection until 44 min. $Prkcz^{+/+}$ and $Prkcz^{+/-}$ mice exhibited the normally observed increase in formalin-induced nociceptive behaviors in the second phase. (e) $Prkcz^{-/-}$ mice injected with 2% formalin showed a weaker attenuation of formalin-induced nociception from 32 to 42 min. $Prkcz^{+/+}$ and $Prkcz^{+/-}$ mice displayed higher second phase scores as in (d) (* $p < 0.05$ compared to $Prkcz^{+/-}$; † $p < 0.05$ compared to $Prkcz^{+/+}$ in c and d). (a–e) All of the data are presented as mean \pm S.E.M. Note. PKM ζ = protein kinase M zeta; ZIP = ζ -inhibitory peptide.

injury. After rats were given an i.c. instillation of 200 μ L of 0.1% capsaicin (Figure 4(a)), they developed a referred hind paw allodynia, evidenced by lowered PWTs for 24 h (Figure 4(b)). Intrathecal ZIP administered either 20 min before (Figure 4(c)) or 60 min after (Figure 4(d)) i.c. capsaicin prevented and reversed hypersensitivity, respectively, up to 24 h compared with ineffective scrZIP treatment (drug effect: $F_{1,10} = 148.01$, $p < 0.001$ and $F_{1,10} = 102.303$, $p < 0.001$, respectively).

PKM ζ inhibition in referred visceral pain with hyperalgesic priming

As the referred allodynia induced by i.c. capsaicin is short-lived, we employed a method known as hyperalgesic priming^{6,11} to induce longer lasting pain hypersensitivity. Thus, a second i.c. capsaicin instillation was performed two weeks after the first, resulting in referred allodynia persisting for six more days (Figure 5(a), Greenhouse–Geisser (GG) corrected injury \times Time interaction: $F_{2,215, 19,932} = 12.907$, $p < 0.001$). Intrathecal ZIP

and scrZIP effects were then assessed when administered as a 20 min pretreatment (i.e., induction), an intervening treatment (20 min before the second instillation (i.e., maintenance of the priming), or as a posttreatment 24 h following the second instillation (i.e., maintenance of the response to challenge). Significant antiallodynic ZIP effects were observed after pre- (Figure 5(b)), intervening- (Figure 5(c)), or posttreatment (Figure 5(d), GG corrected treatment \times Time interaction: $F_{7,80} = 3.032$, $p = 0.007$; $F_{3,869, 38,692} = 9.401$, $p < 0.001$ and $F_{3,673, 36,727} = 4.994$, $p = 0.003$, respectively).

PKM ζ inhibition in referred muscle pain

We next assessed the effects of i.t. ZIP or scrZIP in another referred pain model in which persistent allodynia develops in the rat hind paw following muscle injury produced by two injections of intramuscular (i.m.) acidic saline to the gastrocnemius muscle in the thigh (Figure 6(a)). We showed that i.t. ZIP, but not scrZIP, at either 24 h (Figure 6(b), $F_{1,3} = 12.93$, $p < 0.001$) or one week

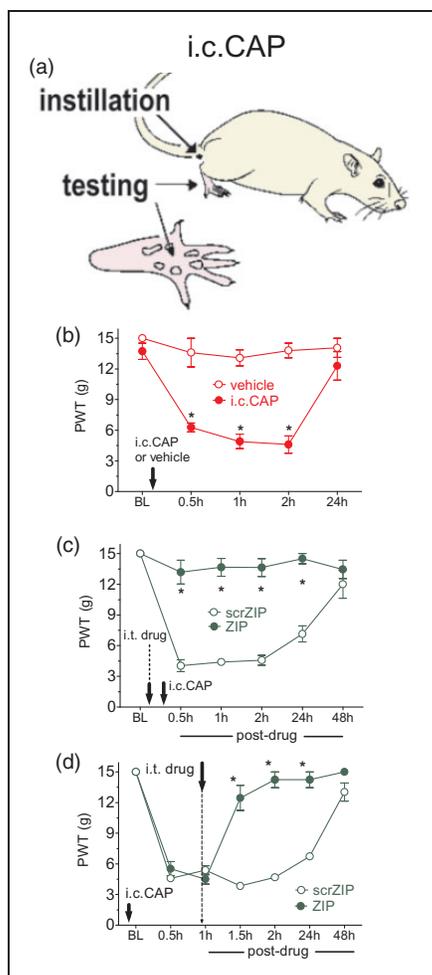


Figure 4. Effects of PKM ζ inhibition in referred visceral pain. (a) Site of capsaicin instillation and testing of hind paw referred allodynia. (b) A single instillation of i.c. capsaicin (i.c. CAP) in male rats resulted in mechanical allodynia, with 50% withdrawal thresholds returning to baseline at 24 h postinstillation. (c and d) Pharmacological inhibition of PKM ζ activity 20 min before (c) and 1 h after (d) i.c. capsaicin resulted in both prevention and reversal of mechanical allodynia, respectively, up to 24 h posttreatment. (b–d) Asterisks indicate a difference between ZIP- and scrambled ZIP-treated groups ($*p < 0.05$). All of the data were collected in male rats and are presented as mean \pm S.E.M. Note. PKM ζ = protein kinase M zeta; i.c. = intracolonic; ZIP = ζ -inhibitory peptide.

(Figure 6(c), $F_{3,20} = 21.2$, $p < 0.001$) postinjury resulted in a four-week reversal of allodynia. No antiallodynic effects were observed after administration of the full-length PKC inhibitor NPC-15437 (Figure 6(d)), given as either 24 h (Figure 6(e)) or one week (Figure 6(f)) posttreatment. Furthermore, i.t. ZIP, but not scrZIP, given one week postinjury, produced a significant decrease in the immunodensity of phosphorylated PKM ζ in spinal cord dorsal horn, as determined by

western blotting (two-tailed), $t(6) = 2.521$, $p = 0.045$ (Figures 6(g) and (h)). We conclude that a PKM ζ -dependent mechanism is critical to the maintenance of long-lasting referred visceral and muscle pain, which is possible to demonstrate due to the remoteness between injury and test sites.

Sex differences after PKM ζ genetic ablation in referred visceral pain

We next repeated the referred visceral pain experiments in PKC/M ζ mutant mice. In accordance with the pharmacological results obtained in rats, mutant male mice ($Prkcz^{-/-}$) showed only a transient allodynia 30 min post i.c. capsaicin. In contrast, $Prkcz^{+/+}$ and $Prkcz^{+/-}$ males remained persistently allodynic and recovered only at 24 h. ZIP treatment in both $Prkcz^{+/+}$ and $Prkcz^{+/-}$ males resulted in PWTs similar to those observed in $Prkcz^{-/-}$ mice (Genotype \times Time interaction: $F_{16,210} = 14.12$, $p < 0.0001$) (Figure 7(a)). Allodynia developed fully in i.c. capsaicin-treated female $Prkcz^{-/-}$ mice (Figure 7(b) and (c)), but weakly or not at all in males (Sex \times Time interaction: $F_{4,85} = 13.87$, $p = 0.001$ and Genotype \times Sex interaction: $F_{4,85} = 10.87$, $p < 0.001$, respectively—Figure 7(a)(c)). ZIP treatment reversed visceral referred pain in $Prkcz^{+/+}$ and $Prkcz^{+/-}$ males at 1 and 2 h and additionally in $Prkcz^{+/-}$ males at 24 h (Figure 7(a)). To directly compare ZIP effects in male and female WT mice, one group of each was tested after i.c. capsaicin and given 10 nmol ZIP i.t. Treatment effects were found to differ by sex and time ($F_{4,72} = 7.669$, $p < 0.0001$, Figure 7(d)), males showing less allodynia than females after ZIP at 0.5 and 1 h after i.c. capsaicin (Figure 7(d)).

Sex differences after PKM ζ genetic ablation in referred muscle pain

We next repeated the referred muscle pain experiments in our PKC/M ζ mutant mice. Once again, given the greater prevalence of muscle pain syndromes in females,¹³ and the reported sex differences for PKM ζ 's role in memory maintenance,¹⁴ we again tested both male and female mutant mice. Consistent with referred allodynia after visceral injury, $Prkcz^{-/-}$ male mice displayed higher PWTs than their $Prkcz^{+/+}$ and $Prkcz^{+/-}$ counterparts for four weeks after muscle injury (Genotype \times Time interaction: $F_{8,125} = 2.618$, $p = 0.011$; Figure 8(a)). Persistent allodynia developed fully over four weeks in i.m. acidic saline-treated female $Prkcz^{-/-}$ mice (Figure 8(b) and (c)), but not in males (Figure 8(a) and (c); Sex \times Time: $F_{4,75} = 2.798$, $p = 0.032$ and Sex \times Time: $F_{4,85} = 10.87$, $p < 0.001$, respectively). Overall, our data indicate that PKC/M ζ ablation attenuated referred visceral pain more in male than in female mice.

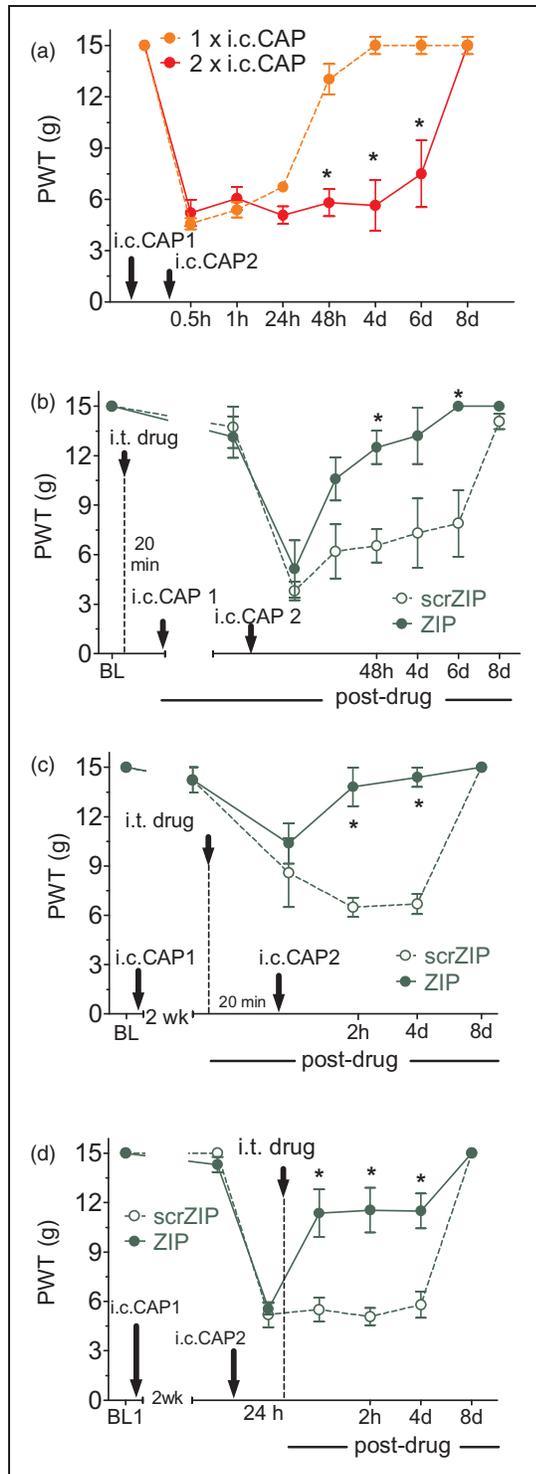


Figure 5. Effects of PKM ζ inhibition in referred visceral pain with hyperalgesic priming. (a) A second i.c. capsaicin (i.c. CAP) instillation administered two weeks after the first resulted in the persistence of referred mechanical allodynia for up to six days post second instillation enabling us to study ZIP effects against long-lasting pain maintenance. (b–d) Long-lasting pain resulting from two repeated i.c. capsaicin instillations (spaced two weeks apart) was effectively attenuated by PKM ζ inhibition, when ZIP was

Sex differences after PKM ζ inhibition in rat formalin and muscle pain

To parallel the sex difference studies in *Prkcz*^{-/-} mice, additional experiments were performed in both male and female rats treated with ZIP. Similar to *Prkcz*^{-/-} mice in other models, ZIP treatment produced less antinociception in the formalin test in female than in male rats (Sex \times Drug treatment interaction, $F_{14,345} = 22.89$, $p < 0.0001$). When compared to the scrambled ZIP control group of the same sex, ZIP reduced nociceptive scores in males from 10 to 37 min post-formalin (Figure 9(a)), but only at the 4- and 33-min time periods post-formalin in females (Figure 9(b)). Pain after ZIP was also significantly less in males than in females at 7 and 10 min post-formalin administration. A similar pattern of results was seen in male and female rats treated with i.m. acidic saline. Thus, males, but not females, that received ZIP after the second of two i.m. acidic saline injections displayed a reduction of allodynia from 1 h to 2 weeks post-ZIP compared with scrambled ZIP (Sex \times Drug interaction $F_{1,21} = 26.772$, $p < 0.0001$, Figure 9(c) and (d)).

These findings suggest that PKM ζ may either contribute more strongly to the maintenance of pain in male versus female rats, or in the case of transgenic mice, that a potential compensatory mechanism following constitutive knockout is more efficient in fulfilling the maintenance role in females as has been proposed for LTP.²⁶

Discussion

This study provides novel insights into the role of PKM ζ in models of persistent pain. First, we have shown that pharmacological inhibition of PKM ζ using ZIP did not reverse CFA-induced ipsilateral thermal or mechanical allodynia or SNI-induced ipsilateral mechanical allodynia. Our results support the previous reports of weak or no effects of ZIP on CFA-induced hypersensitivity,^{6,8,27} suggesting PKM ζ activity alone is not responsible for sustaining the underlying nociceptive sensitization. Our findings are also in line with previous studies conducted in rats with various nerve injuries showing PKM ζ

administered 20 min before the first priming stimulus (b), two weeks after the first and 20 min before the challenging stimulus (c), and 24 h after the challenging stimulus (d). In all three treatment scenarios, scrZIP treatment was observed to be ineffective. (a–d) Asterisks indicate a difference between treatment groups ($*p < 0.05$). All of the data were collected in male rats and are presented as mean \pm S.E.M. Note. PKM ζ = protein kinase M zeta; i.c. = intracolonic; ZIP = ζ -inhibitory peptide; scrZIP = scrambled ZIP.

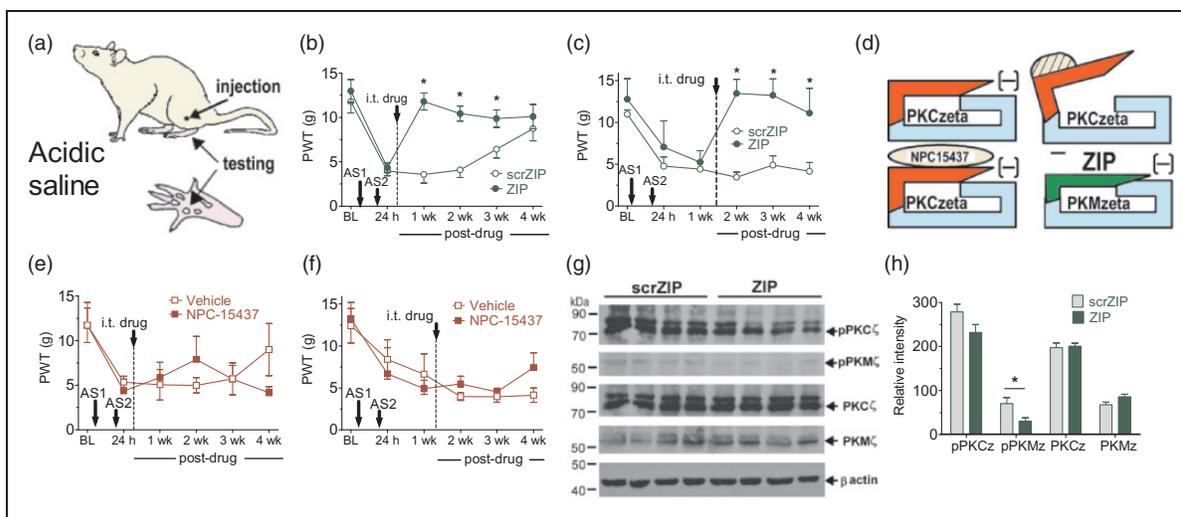


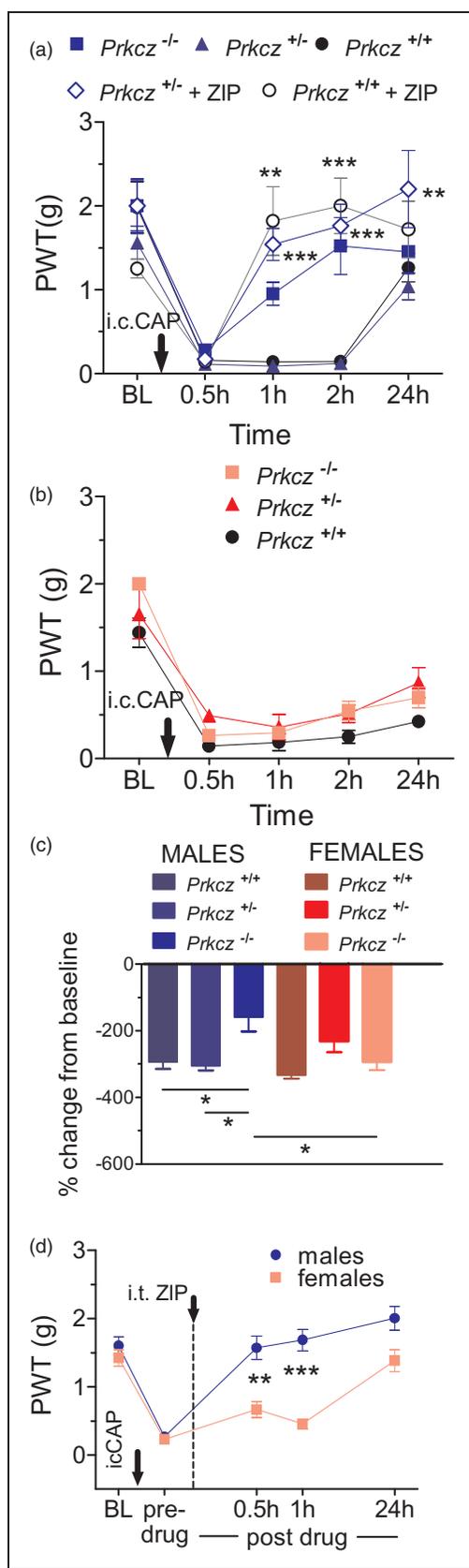
Figure 6. Effects of PKM ζ inhibition in referred muscle pain. (a) Sites of acidic saline (AS) injection and testing of hind paw referred allodynia. (b and c) ZIP treatment 24 h (b) or one week (c) after the second acidic saline injection resulted in a significant reversal of mechanical allodynia up to four weeks posttreatment compared with scrZIP. (d) Separate actions of ZIP versus NPC-15437 on PKM ζ and full-length PKC, respectively. The cyan and red segments represent catalytic and regulatory domains, respectively. The dashed moiety denotes the second messenger. (e and f) NPC-15437 given either 24 h (e) or one week (f) after the second acidic saline injection was without any effect. (g and h) ZIP treatment at one week after the second acidic saline injection resulted in a significant reduction in phosphorylated PKM ζ (pPKM ζ), but had no effect on total PKM ζ , total PKC ζ , or pPKC ζ , compared to the effects of scrZIP. Asterisks indicate a difference between ZIP and scrZIP (* $p < 0.05$). (b, c, e, f, and h) All of the data were collected in male rats and are presented as mean \pm S.E.M. *Note.* PKM ζ = protein kinase M zeta; i.c. = intracolonial; ZIP = ζ -inhibitory peptide; scrZIP = scrambled ZIP.

inhibition using ZIP did not alter ipsilateral hypersensitivity.^{6–8,28} Alternatively, the development of contralateral allodynia in SNI rats at 6 weeks after surgery was transiently reversed by spinal PKM ζ inhibition. As evidence indicates that pain hypersensitivity after nerve injury depends heavily on hypersensitivity or enhanced inputs from damaged and undamaged afferents,^{29–31} we concluded that the inability to demonstrate a role for spinal PKM ζ in the maintenance of ipsilateral neuropathic pain after SNI is masked by the overwhelming contribution of peripheral inputs. This conclusion is supported by our earlier demonstration that in rats with chronic posts ischemic pain, ZIP does not reduce allodynia at an early stage when peripheral pathology is evident but does reduce allodynia at a later stage when peripheral pathology has largely resolved.⁷

Importantly, we also showed that the pharmacological effects of ZIP in neuropathic rats were replicated in mice with genetic ablation of PKM ζ . Thus, while PKC/M ζ knockout mice exhibited normal ipsilateral mechanical allodynia, they also showed significantly reduced contralateral allodynia four weeks post-nerve injury when compared with heterozygous and WT mice, suggesting PKM ζ is required for the development of contralateral allodynia. Importantly, it has been shown that neuropathic contralateral allodynia is insensitive to doses of i.v. lidocaine that reverse

ipsilateral allodynia, suggesting that contralateral allodynia depends on central sensitization.³²

To discern the contribution of central neuroplasticity and ongoing peripheral inputs, we conducted formalin tests with increasing concentrations of formalin: 2%, 3.5%, and 5% in rats posttreated with ZIP. It has been established that the nociceptive responses to higher concentration formalin depend to a greater extent on peripheral inputs, as high, but not low, concentration formalin pain is reduced by anti-inflammatory agents.³³ We reproduced our previous results⁷ showing PKM ζ inhibition largely reversed second phase nociception after 2% formalin and importantly also showed considerably reduced analgesia in rats given 3.5% formalin, and no analgesia in rats given 5.0% formalin. These results are consistent with our earlier demonstration that preemptive spinal anesthesia is capable of reducing nociception induced by lower concentrations of formalin that do not produce plasma extravasation, but has no effects on higher concentrations which produce significant plasma extravasation.³⁴ The results are also consistent with the report that posttreatment with ZIP to the periaqueductal gray area was unable to alleviate second phase nociceptive responses to 5.0% formalin.²⁷ In contrast, spinal pretreatment with ZIP has been shown to reduce subsequent nociceptive responses to 5.0% formalin,⁸



although clearly the maintenance of nociceptive sensitization must be assessed using posttreatments.

Once again, we showed that the pharmacological effects of ZIP in formalin-treated rats were replicated in mice with genetic ablation of PKM ζ . Thus, protein kinase M (PKM)/C ζ knockout substantially reduced second phase formalin pain at 1%, but the effect was considerably less pronounced at 2% formalin. Conversely, heterozygous and WT mice developed full second phase pain regardless of formalin concentration. These data support our hypothesis that increasing peripheral inputs masks the ability of ZIP to demonstrate a role for spinal PKM ζ in the maintenance of nociceptive sensitization.

We further investigated whether PKM ζ activity plays a role in the maintenance of long-lasting referred visceral pain. Earlier studies have shown that the remote allodynia that is expressed in the hind paw after i.c. capsaicin could be prevented by pretreatment with an inhibitor of ERK,³⁵ implicating this protein kinase (PK) in the induction of the allodynia. Furthermore, plasma membrane-targeted trafficking of GluR1 associated with i.c. capsaicin was found to be blocked by pretreatment with an inhibitor of CaMKII,³⁶ therefore also implicated in allodynia induction. However, studies here are the first to show that remote allodynia after i.c. capsaicin was reversed by *posttreatment* (1 h after capsaicin) as well as pretreatment with a PK inhibitor—in this case, an inhibitor of PKM ζ , for which a single pre- or posttreatment completely prevented or reversed, respectively, remote allodynia for the 24 h duration of testing. This clearly implicates PKM ζ in the maintenance of remote

Figure 7. Sex differences after PKM ζ genetic ablation in referred visceral pain. (a and b) Time course of development of mechanical allodynia in male transgenic mice after i.c. capsaicin (i.c. CAP) instillation. (a) A male-specific reversal of mechanical allodynia was observed after i.c. capsaicin instillation in $Prkcz^{-/-}$ mice. In addition, i.t. ZIP treatment in $Prkcz^{+/+}$ and $Prkcz^{+/-}$ males resulted in the restoration of post-i.c. capsaicin PWTs to baseline values. Asterisks denote significant difference between ZIP-treated and untreated animals of the same genotype (** $p < 0.01$, *** $p < 0.001$). (b) Female knockouts displayed persistent allodynia after i.c. capsaicin throughout the 24 h period of testing. (c) $Prkcz^{-/-}$ male mice showed a smaller change in PWTs from baseline than $Prkcz^{+/-}$ or $Prkcz^{+/+}$ males. $Prkcz^{-/-}$ female mice also show a larger change in PWTs from baseline than $Prkcz^{-/-}$ males (* $p < 0.05$). (d) ZIP treatment also produced a greater antiallodynic effect in $Prkcz^{+/+}$ male, when compared with $Prkcz^{+/+}$ female, mice (** $p < 0.01$, *** $p < 0.001$). (a–d) All of the data are presented as mean \pm S.E.M. Males—blue shaded lines and bars; females—red/pink shaded lines and bars. Note. PKM ζ = protein kinase M zeta; i.c. = intracolonic; i.t. = intrathecal; ZIP = ζ -inhibitory peptide; PWTs = paw withdrawal thresholds.

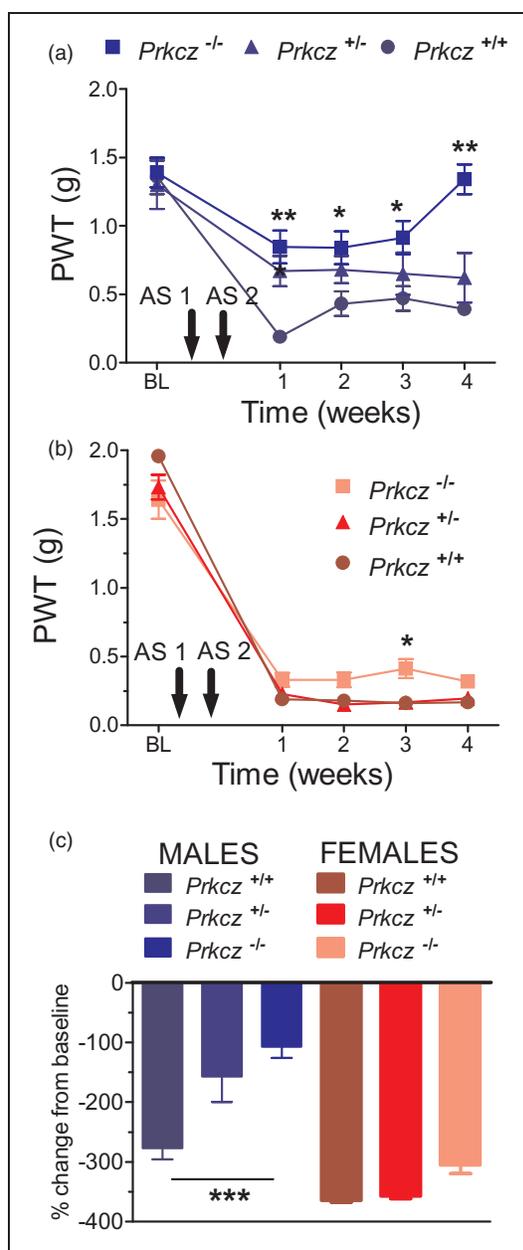


Figure 8. Sex differences after PKM ζ genetic ablation in referred muscle pain. (a and b) Time course of development of mechanical allodynia in male transgenic mice after i.m. acidic saline (AS) injection. (a) Mutant (*Prkcζ*^{-/-}) male mice displayed significantly higher PWTs over a period of four weeks after acidic saline injection compared with their *Prkcζ*^{+/+} and *Prkcζ*^{+/-} counterparts. Asterisks indicate differences between *Prkcζ*^{-/-} and *Prkcζ*^{+/-} (**p* < 0.05, ***p* < 0.01). Dagger indicates difference between *Prkcζ*^{-/-} and *Prkcζ*^{+/+} ($\dagger p$ < 0.05). (b) *Prkcζ*^{-/-} female mice only showed a small reduction in mechanical allodynia compared with wild-type at three weeks. (c) *Prkcζ*^{-/-} male mice show a smaller change in PWTs from baseline than *Prkcζ*^{+/+} males, while female PWTs do not change across genotype (***p* < 0.001). (a–c) All of the data are presented as mean \pm S.E.M. Males—blue shaded lines and bars; females—red/pink shaded lines and bars. Note. PKM ζ = protein kinase M zeta; i.m. = intramuscular; ZIP = ζ -inhibitory peptide; PWTs = paw withdrawal thresholds.

allodynia after visceral injury. These findings are consistent with our earlier finding that ZIP pretreatment or posttreatment alleviated secondary mechanical allodynia induced by i.pl. capsaicin injection.⁷ As in the case of neuropathic contralateral allodynia and low concentration formalin, the antiallodynic effects of pharmacological inhibition of PKM ζ on i.c. capsaicin-induced allodynia were replicated by genetic ablation of PKM ζ . However, in this model, we examined sex-dependent effects of PKM/C ζ knockout and showed that while PKM/C ζ knockout reduced i.c. capsaicin-induced allodynia in males, it had no effect in females.

As the remote allodynia induced by i.c. capsaicin is relatively short-lived (24 h), we used two i.c. capsaicin instillations to produce a more prolonged remote allodynia in the rat hind paw. This method replicated previous demonstrations of what has been called hyperalgesic priming, a term first used to describe the prolonged hyperalgesia induced by intradermal prostaglandin E₂ in animals that were earlier primed by carrageenan injection.³⁷ Here, two injections of i.c. capsaicin increased the length of remote hyperalgesia from 24 h after a single instillation up to six days after the second instillation. As in the case of a single i.c. capsaicin instillation, pretreatment or posttreatment with ZIP prevented or reversed, respectively, the allodynia for the duration of testing (six days) following a second i.c. capsaicin instillation. Our study is consistent with earlier studies which have shown that PKM ζ inhibition reduces hyperalgesic priming elicited with hind paw IL-6, BDNF, or carrageenan injections followed by PGE₂ injection or plantar incision or plantar incision followed by PGE₂ injection.^{4,5,9} In these studies, however, PKM ζ was inhibited prior to the priming stimulus or prior to the challenging stimulus. Thus, we present novel evidence that ZIP reversed established long-lasting allodynia when administered either prior to priming, as well as prior to, or following, the challenging stimulus.

Additional studies examined whether PKM ζ activity plays a role in the maintenance of long-lasting referred muscle pain. Once again, earlier studies had shown that remote allodynia that is expressed in the hind paw after two injections of i.m. acetic saline could be reduced by pretreatment with an inhibitor of protein kinase A (PKA),³⁷ but not by posttreatment with inhibitors of either PKA³⁸ or PKC.³⁹ When injected at either 24 h or one week post-i.m. acetic saline, the PKM ζ inhibitor ZIP, but not the full-length PKC inhibitor NPC-15437, reversed the established mechanical allodynia. Importantly again, a single injection of ZIP at either of these posttreatment time points reversed allodynia for its duration (three to four weeks); thus strongly implicating PKM ζ in the maintenance of remote allodynia after muscle injury. The lack of effect of the PKC inhibitor on remote allodynia after i.m. acetic saline supported our

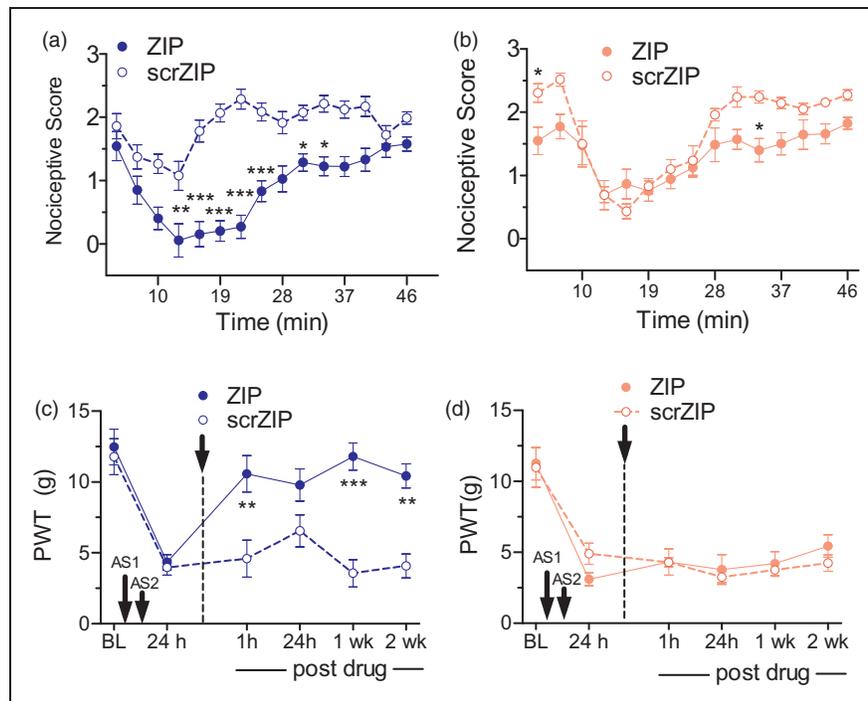


Figure 9. Sex differences after PKM ζ inhibition in formalin and muscle pain in rats. (a) Effects of ZIP or scrambled ZIP treatment on the response to 2% formalin in male (a) and female (b) rats. Relative to scrZIP, ZIP administration had a larger effect in male vs. female rats. For each sex, asterisks indicate a difference between ZIP- and scrZIP-treated groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). ZIP treatment after the second of two intramuscular injections of acidic saline (AS) resulted in a significant increase in PWTs up to two weeks posttreatment in male rats (c), but was without comparable effect in female rats (d). Asterisks indicate a difference between ZIP- and scrZIP-treated groups (** $p < 0.01$, *** $p < 0.001$). (a–d) All of the data are presented as mean \pm S.E.M. Males—blue shaded lines and bars; females—red/pink shaded lines. Note. PKM ζ = protein kinase M zeta; ZIP = ζ -inhibitory peptide; scrZIP = scrambled ZIP; PWTs = paw withdrawal thresholds.

earlier effects demonstrating no contribution of PKC to the maintenance of nociceptive sensitization after cutaneous injury.⁷ Concordant with a reversal of i.m. acidic saline-induced remote allodynia following ZIP, the western blot data highlighted a decrease of pPKM ζ , but not pPKC ζ/λ , nor total PKM/C ζ/λ levels, compared to that produced by scrZIP. These data further indicate that a single injection of ZIP is sufficient for the disruption of PKM ζ 's persistent activity. As in the case of allodynia after i.c. capsaicin, the anti-allodynic effects of pharmacological inhibition of PKM ζ for i.m. acetic acid-induced allodynia were replicated by genetic ablation of PKM ζ . However, once again, we found sex-dependent effects of PKM/C ζ knockout, showing that although PKM/C ζ knockout reduced i.m. acetic acid-induced allodynia in males, it had almost no effect in females.

Concerning sex differences, we generally show that female mice of all genotypes develop greater allodynia than their male counterparts—which is in line with previous reports on sex differences regarding pain behavior.^{40,41} However, the sex discrepancy in allodynia was most profound in *Prke2* knockout mice—where females developed robust persistent allodynia, while their male counterparts did not. This finding suggests PKM/C ζ may

contribute more strongly to the maintenance of pain and remote allodynia in male mice than in female mice, as has been shown for PKM ζ 's role in long-term spatial memory.⁴² Alternatively, a potential compensatory mechanism which develops following constitutive knockout may be more efficient in fulfilling the maintenance role in female mice than in males, as has been shown the role of GluR1 and nitric oxide for hippocampal-dependent contextual conditioning.⁴³ However, the former may be more likely, as our further studies on ZIP effects in both male and female rats indicated that PKM ζ inhibition had greater effects in males than females both on nociception in the formalin test and on remote allodynia after either i.c. capsaicin or i.m. acetic acid.

Overall, our results indicate that, unlike findings with hippocampal LTP and memory maintenance, a consistency exists between the pharmacological antinociceptive effects of ZIP and the effects of genetic ablation, which implicates significant PKM ζ -dependent mechanisms in the maintenance of long-term spinal nociceptive sensitization. Differences between models of referred pain versus inflammatory and neuropathic pain stress the importance of accounting for masking effects of afferent inputs on the modulation of central sensitization when

assessing nociceptive maintenance. Importantly, there is also a differential contribution of PKM ζ to pain maintenance between sexes. We propose that the maintenance of spinal nociceptive sensitization in females may rely more heavily on factors than PKM ζ .

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Author Contributions

TJC and HN designed the study; HN, AL, SD, and AM conducted behavioral experiments; HM and HN performed protein assays; SG and HN bred and genotyped the mice; HN performed statistical analysis; HN, AL, and JVR prepared figures; HN, JVR, and TJC wrote the paper; TJC and US supervised experiments and edited the paper.

Declaration of Conflicting Interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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