Cellular/Molecular

Spinal Protein Kinase M ζ Underlies the Maintenance Mechanism of Persistent Nociceptive Sensitization

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Sensitization of the pain pathway is believed to promote clinical pain disorders. We hypothesized that the persistence of a sensitized state in the spinal dorsal horn might depend on the activity of protein kinase M ζ (PKMζ), an essential mechanism of late long-term potentiation (LTP). To test this hypothesis, we used intraplantar injections of interleukin-6 (IL-6) in mice to elicit a transient allodynic state that endured ~3 d. After the resolution of IL-6-induced allodynia, a subsequent intraplantar injection of prostaglandin E_2 (PGE_2) or intrathecal injection of the metabotropic glutamate receptor 1/5 (mGluR1/5) agonist DHPG (dihydroxyphenylglycol) precipitated allodynia and/or nocifensive responses. Intraplantar injection of IL-6 followed immediately by intrathecal injection of a PKMζ inhibitor prevented the expression of subsequent PGE_2-induced allodynia. Inhibitors of protein translation were effective in preventing PGE_2-induced allodynia when given immediately after IL-6, but not after the initial allodynia had resolved. In contrast, spinal PKMζ inhibition completely abolished both prolonged allodynia to hindpaw PGE_2 and enhanced nocifensive behaviors evoked by intrathecal mGluR1/5 agonist injection after the resolution of IL-6-induced allodynia. Moreover, spinal PKMζ inhibition prevented the enhanced response to subsequent stimuli following resolution of hypersensitivity induced by plantar incision. The present findings demonstrate that the spinal cord encodes an engram for persistent nociceptive sensitization that is analogous to molecular mechanisms of late LTP and suggest that spinally directed PKMζ inhibitors may offer therapeutic benefit for injury-induced pain states.

Introduction

The incomplete understanding of the underlying molecular mechanisms that amplify signaling in the pain pathway has impeded the development of novel therapies. Synaptic long-term potentiation (LTP) in nociceptive neurons of the dorsal horn closely resembles mechanisms underlying memory trace formation in other CNS structures (Woolf and Salter, 2000; Ji et al., 2003; Ikeda et al., 2006) and may represent a key point of intervention for preventing the expression of persistent pain. We reasoned that spinal dorsal horn neurons might encode an engram representing a molecular mechanism of central sensitization that may promote pain. The maintenance of late LTP (L-LTP) requires an atypical protein kinase C called protein kinase M ζ (PKMζ) (Ling et al., 2002; Sacktor, 2008). PKMζ is a unique gene product from the *PKCζ* gene that lacks a regulatory region and is therefore autonomously active at the synapse following translation (Sacktor, 2008). PKMζ is sufficient to induce LTP, and inhibition of PKMζ during L-LTP leads to decay of LTP. Critically, PKMζ inhibition *in vivo* leads to the erasure of previously established memories (Pastalkova et al., 2006; Shema et al., 2007). Thus, PKMζ initiates L-LTP formation and persistent PKMζ activity maintains L-LTP, a model consistent with an essential role for PKMζ in the persistence of the long-term memory trace (Sacktor, 2008). We hypothesized that the development and maintenance of a sensitized state promoting persistent pain requires PKMζ in the spinal dorsal horn.

This possibility was tested using an adaptation of a model of "hyperalgesic priming" (Reichling and Levine, 2009) that produces a state of sensitization closely resembling clinical situations with increased risk of development of chronic pain (Aasvang and Kehlet, 2005; Reichling and Levine, 2009). A single injection of interleukin-6 (IL-6) to mice causes a transient acute nociceptive hypersensitivity that resolves within 3 d (Dina et al., 2008; Melemedjian et al., 2010). In this model, persistent sensitization of the nociceptive pathway is revealed by subsequent challenge with injury or stress (Dina et al., 2008; Reichling and Levine, 2009). The underlying mechanisms of this long-lasting sensitization are not known. We reasoned that long-term maintenance of a sensitized state in the spinal cord would require similar mechanisms to those associated with persistent synaptic plasticity and memory maintenance. Our findings demonstrate the requirement for spinal PKMζ in the initiation and maintenance of a spinal

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sensitization state with striking parallels to mechanisms promoting the formation of long-term memory traces in CNS structures.

Materials and Methods

Experimental animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Arizona and were in accordance with International Association for the Study of Pain guidelines. Male ICR mice (20–25 g; Harlan) were used for all studies except where fragile X mental retardation-null (*fmr1*$^{-/y}$) mice were used. Fmr1 mutant mice and wild-type littermates were obtained from Jackson Laboratories on a C57BL/6J background and were bred for these studies at The University of Arizona. Male *fmr1*$^{-/y}$ and wild-type littermates between 12 and 16 weeks of age were used for these studies.

Materials

Human recombinant IL-6 was from R&D Systems; temsirolimus was from LC Laboratories; ZIP, Scrambled ZIP, pep2m, and UO126 were from Tocris Bioscience; 4EGI1 was from Axxora; prostaglandin E_2 (PGE_2) was from Cayman Chemical Company; DHPG (dihydroxyphenylglycol) was from Ascent Scientific; and anisomysin was from Sigma. Stock solutions of U0126, temsirolimus, 4EGI1, and anisomysin were made in 100% DMSO. DHPG stock solution was made in H_2O with 10% DMSO. ZIP, Scrambled ZIP, and pep2m stock solutions were made in distilled H_2O. PGE_2 stock solutions were made in 100% ethanol. All drugs were diluted to final concentrations in saline for injection. Constitutively active PKCζ lentivirus was generated by subcloning myristoylated atypical PKCζ (Chou et al., 1998) IRES mCherry in pBOBi. Viral particles were produced as described previously (Tiscornia et al., 2006).

Behavioral testing and drug administration

Mechanical testing. The experimental design was based on previous experiments demonstrating "hyperalgesic priming" in rats (Aley et al., 2000; Parada et al., 2003, 2005; Dina et al., 2008; Reichling and Levine, 2009). Animals were placed in acrylic boxes with wire mesh floors, and baseline mechanical withdrawal thresholds of the left hindpaw were measured after habituation for 1 h using the up-down method (Chaplan et al., 1994). The experimenter making measurements was always blinded to the experimental conditions. For day 1 experiments, IL-6 was injected into the plantar surface of the left hindpaw in a volume of 25 μl. For paw coinjection experiments drugs were coinjected with IL-6 and IL-6 plus nerve growth factor (NGF) at doses determined previously (Melemedjian et al., 2010). Plantar incision was performed as described previously (Banik et al., 2006). For intrathecal treatments, drugs were injected immediately after intraplantar injections under brief (<3 min) isoflurane anesthesia in a volume of 5 μl (Hylden and Wilcox, 1980). Drug concentrations for temsirolimus (Price et al., 2007), ZIP (Shema et al., 2007), anisomycin (Nader et al., 2000), and pep2m (Yao et al., 2008) were based on published findings, and concentrations for 4EGI-1 were determined in pilot experiments. For experiments with intrathecal treatments on day 4 or later, mice were tested before intrathecal injection to assure that allodynia had completely resolved. Intrathecal injections were done at these time points under isoflurane anesthesia as described above. PGE_2 (100 ng) was injected on day 6 or 18 in the plantar surface of the left hindpaw in a volume of 25 μl. Allodynia testing was then done at the time points indicated in the text. For viral vector experiments, virus was injected intrathecally after dilution in saline to 10^{14} plaque forming units per milliliter. Transduction was measured 31 d after intrathecal injection by visualizing mCherry in spinal cord slices (20 μm thick) and Neuronal Nucleus marker (NeuN) labeled with donkey anti-mouse Alexa fluor 488 (Invitrogen) with a Zeiss LSM 510 confocal microscope.

DHPG testing. Animals were habituated in a Plexiglas behavior chamber for 1 h before the beginning of the experiment. DHPG (10 nmol) was injected (5 μl, i.t.) under isoflurane anesthesia on day 6 or 10, and caudally directed nociceptive behavior (licking of paw and lower portions of the body, and biting or shaking of paw) was recorded for 30 min postinjection as described previously (Karim et al., 2001).

Statistics

All data are presented as mean ± SEM. Graph plotting and statistical analysis used Graphpad Prism Version 5.03. Differences between groups for caudally directed nociceptive behavior after intrathecal injection of DHPG were assessed using Student's *t* test. Measures of time-dependent allodynia by drug treatment were made by two-way ANOVA with Bonferroni's *post hoc* test. The a priori level of significance was set at 95%.

Results

Local translation is required for the establishment but not maintenance of persistent nociceptive sensitization

We have previously shown that IL-6 injection into the hindpaw of mice leads to allodynia that is dependent on translation regulation via the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase-interacting kinase (MNK)/eukaryotic initiation factor 4 (eIF4E) pathway (Melemedjian et al., 2010). Following intraplantar IL-6 injection, tactile allodynia is observed and endures for ~3 d, with complete resolution by day 4 (Fig. 1*A*). A subsequent injection of PGE_2 6 d following IL-6 injection causes allodynia that lasts for at least 24 h, reflecting the presence of a persistent sensitized state; mice that previously received an injection of vehicle displayed only a transient PGE_2-induced allodynia (≤1 h) (Fig. 1*A*). We determined that expression of sensitization to PGE_2 injection was completely blocked when intraplantar IL-6 was coinjected with the mitogen-activated protein kinase (MEK) inhibitor U0126 (Fig. 1*B*), the protein synthesis inhibitor anisomycin (Fig. 1*C*), or the eIF4F complex formation inhibitor 4EGI-1 (Fig. 1*D*) (Moerke et al., 2007). Our previous findings have shown that IL-6 and NGF signaling in nociceptors converges on the eIF4F complex to promote acute allodynia, a process that can be blocked with the eIF4F complex formation inhibitor 4EGI-1 (Melemedjian et al., 2010); however, it is not known whether local eIF4F complex formation inhibition is also capable of blocking IL-6- and NGF-induced sensitization to subsequent PGE_2 exposure. Coinjection of 4EGI-1 with IL-6 and NGF fully blocked expression of sensitization to PGE_2 injection on day 6 (Fig. 1*E*), demonstrating that convergent signaling to the eIF4F complex is required to induce the establishment of the sensitized state at the time of IL-6 and NGF injection. Hence, IL-6-induced and IL-6 and NGF-induced sensitization requires local signaling to the eIF4F complex at the time of IL-6 or IL-6 and NGF priming.

The fragile X mental retardation protein (FMRP) is involved in multiple facets of translation control and synaptic plasticity (Bassell and Warren, 2008), and we have previously demonstrated the fragile X mental retardation-null mice (*fmr1*$^{-/y}$) fail to show nociceptive sensitization in several preclinical pain models (Price et al., 2007). To further demonstrate a major role of translation regulation in IL-6-induced allodynia and persistent sensitization, we assessed this in *fmr1*$^{-/y}$ mice and their wild-type littermates. IL-6-induced allodynia was strongly reduced in *fmr1*$^{-/y}$ mice (Fig. 1*F*). Moreover, establishment of sensitization to subsequent PGE_2 injection was blunted in *fmr1*$^{-/y}$ mice compared with their wild-type littermates (Fig. 1*G*). Collectively, these results point to an important role for translation regulation via convergent signaling to the eIF4F complex and FMRP-mediated translation control in IL-6-induced allodynia and persistent nociceptive sensitization.

We next assessed whether local translation is required to maintain persistent sensitization to subsequent PGE_2 injection. IL-6 was injected on day 1 and followed on day 4, after initial allodynia had resolved, by intraplantar injection of anisomycin or 4EGI-1. On day 6, persistent sensitization was revealed by PGE_2

injection into the hindpaw (Fig. 1*H*). As opposed to injection of translation inhibitors at the time of IL-6 injection, anisomycin (Fig. 1*I*) and 4EGI-1 (Fig. 1*J*) both failed to reduce PGE_2 precipitated persistent sensitization expression, indicating that local translation is not required to maintain the persistently sensitized state.

IL-6-induced persistent nociceptive sensitization is precipitated by a central stimulus

The failure of local translation inhibitors to reverse the maintenance of persistent sensitization led us to hypothesize that the molecular mechanism responsible for the maintenance of this persistently sensitized state may reside in the CNS. Hence, we next asked whether IL-6-induced sensitization could be revealed with a stimulus directed at the CNS. Intrathecal injection of the metabotropic glutamate receptor (mGLuR) 1/5 agonist DHPG during chronic inflammation causes an enhanced nocifensive response (Adwanikar et al., 2004). It is not known whether this enhanced nocifensive response is maintained even after overt allodynia induced by the initial stimulus has subsided. To assess this possibility, we administered IL-6 or vehicle injections to the hindpaw, and 6 d later, at a time when allodynia was fully resolved, DHPG was injected intrathecally. Mice that had previously received intraplantar injections of IL-6 showed a markedly enhanced response to intrathecal DHPG (day 1 vehicle: 170.5 ± 52.9 s, $n = 6$; day 1 IL-6: 871.5 ± 82.5 s, $n = 6$; $p < 0.001$) consistent with a persistent change in mGluR1/5 responsiveness despite the absence of allodynia. Therefore, the sensitized state can be unmasked as an enhanced response to a peripheral stimulus leading to allodynia as well as increased nocifensive pain behaviors.

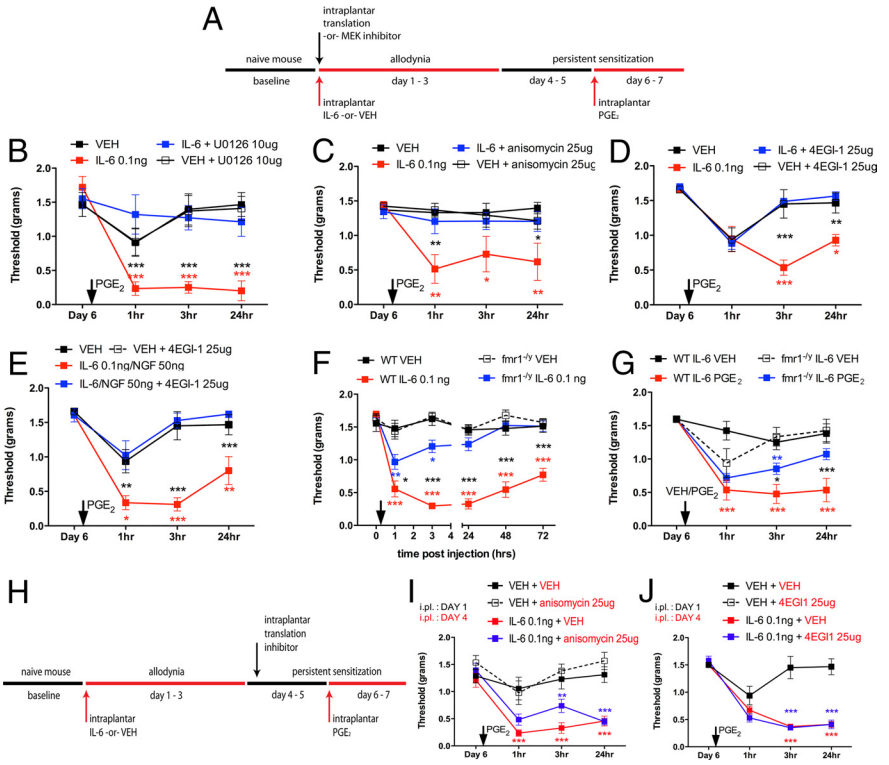

Figure 1. IL-6-induced persistent nociceptive sensitization initiation but not maintenance depends on local translation within the hindpaw at the time of IL-6 administration. ***A***, Experimental design for initiation of persistent sensitization testing. ***B–D***, IL-6 was administered intraplantarly (i.pl.) with or without inhibitors of MEK (U0126, ***B***), general translation (anisomycin, ***C***), or eIF4F complex formation (4EGI-1, ***D***). Six days following IL-6 and inhibitor coadministration, PGE_2 (100 ng) was injected into the same hindpaw to assay persistent sensitization. PGE_2-induced allodynia was abrogated by U0126 (***B***), anisomycin (***C***), and 4EGI-1 (***D***). ***E***, Moreover, 4EGI-1 was also able to block persistent sensitization caused by coinjection of IL-6 and NGF. ***F***, ***G***, Acute allodynia induced by IL-6 injection was decreased in fmr1 $^{-/y}$ mice (***F***), and persistent sensitization to PGE_2 injection on day 6 was also decreased in fmr1 $^{-/y}$ mice (***G***). ***H***, Experimental design for maintenance of persistent sensitization testing. ***I***, ***J***, Anisomycin (***I***) or 4EGI-1 (***J***) injected into the hindpaw 4 d following IL-6 injection into the same hindpaw failed to attenuate PGE_2-precipitated persistent sensitization. $N = 6$ for all experiments. Colored stars denote significant effects compared with vehicle groups, and black stars denote significance between IL-6-treated groups. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. VEH, Vehicle.

Establishment of persistent nociceptive sensitization requires spinal protein synthesis and PKMζ

The persistence of a spinally mediated amplification mechanism even after the original allodynia has resolved strongly suggests the existence of a molecular storage mechanism for sensitization in the spinal dorsal horn (i.e., an engram that might parallel the molecular mechanisms of LTP). To test this hypothesis, we took advantage of the observation that the consolidation of L-LTP requires nascent protein synthesis [via mammalian target of rapamycin (mTOR) and eIF4F complex formation] during LTP induction, while L-LTP can be maintained even in the presence of translation inhibitors (Kelleher et al., 2004). Conversely, PKMζ is required for L-LTP consolidation, L-LTP maintenance (Ling et al., 2002), and the persistence of memory (Pastalkova et al., 2006; Shema et al., 2007). Injection of IL-6 into the hindpaw was followed directly by intrathecal injection of the mTOR inhibitor temsirolimus, the eIF4F complex formation inhibitor 4EGI-1, or by the PKMζ inhibitor ZIP. Intrathecal injection of temsirolimus (Fig. 2*A*,*B*), 4EGI-1 (Fig. 2*C*,*D*), or ZIP (Fig. 2*E*,*F*) all prevented both IL-6-induced initial allodynia and the expression of sensitization after PGE_2 injection on day 6. It has recently been demonstrated that administration of ZIP reduces synaptic transmission in spinal cord slice preparations (Li et al., 2010); therefore, we asked whether spinal inhibition of PKMζ could lead to a general depression of synaptic transmission that would lead to a long-lasting decrease in nociceptive responses. Intrathecal injection of ZIP 2 d before intraplantar IL-6 injection altered neither IL-6-induced initial allodynia nor PGE_2-mediated sensitization (Fig. 3*A*,*B*).

Maintenance of persistent nociceptive sensitization is erased by inhibition of spinal PKMζ

If the maintenance of persistent sensitization is due to the presence of L-LTP in dorsal horn neurons, we predicted that intrathecal administration of ZIP should erase sensitization to either hindpaw injection of PGE_2 or intrathecal injection of DHPG after IL-6 priming. IL-6 was injected into the hindpaw of mice on day 1, and intrathecal injections of ZIP were performed on day 4, after acute allodynia had resolved. In ZIP-treated animals, the expression of sensitization to hindpaw PGE_2 (Fig. 4*A*) was attenuated, whereas scrambled ZIP had no effect. Critically, this effect was long lasting, such that intrathecal ZIP injection on day 16 following IL-6 priming inhibited the expression of PGE_2 sensitization

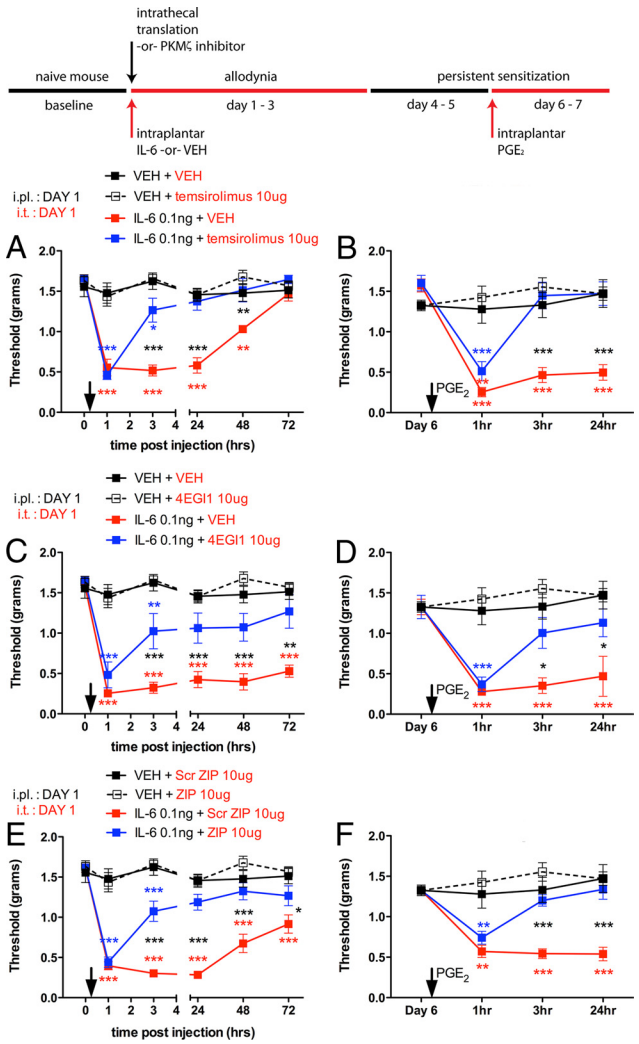

Figure 2. Persistent nociceptive sensitization depends on early, spinal translation and PKMζ activity: intrathecal injection of the mTOR inhibitor temsirlimus at the time of intraplantar (i.pl.) injection. ***A–F***, IL-6 injection blocks allodynia (***A***) and persistent sensitization to PGE_2 (100 ng, ***B***). Allodynia (***C***) and persistent (***D***) sensitization is also blocked by the eIF4F complex formation inhibitor 4EGI1 and by the PKMζ inhibitor ZIP (***E***, ***F***). $N = 6$ for all experiments. Colored stars denote significant effects compared with vehicle groups, and black stars denote significance between IL-6-treated groups. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. VEH, Vehicle.

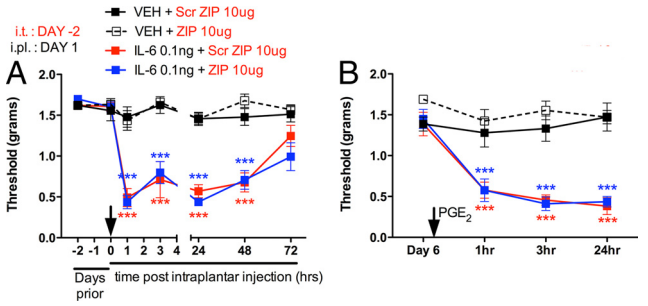

Figure 3. ***A***, ***B***, PKMζ inhibition before IL-6-induced priming does not influence persistent nociceptive sensitization: intrathecal injection of ZIP or Scr ZIP 2 d before intraplantar (i.pl.) injection of IL-6 has no effect on IL-6-induced allodynia (***A***) or on persistent nociceptive sensitization precipitated by i.pl. injection of PGE_2 (100 ng) on day 6 (***B***). $N = 6$ per group. $^{***}p < 0.001$. VEH, Vehicle.

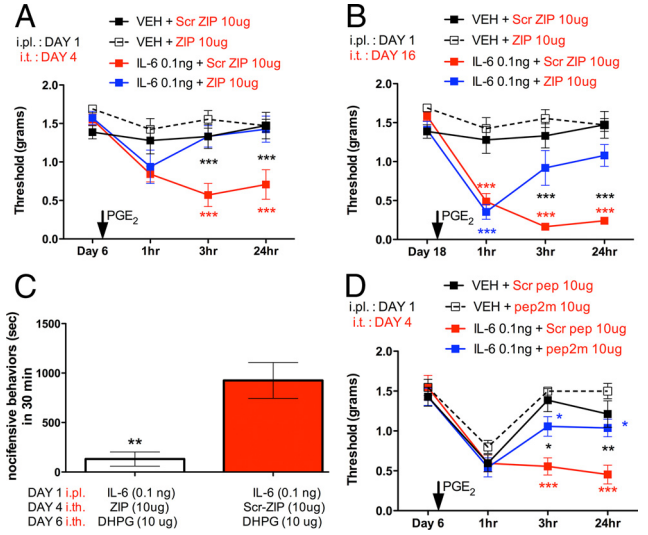

Figure 4. Persistent nociceptive sensitization is maintained by spinal PKMζ. ***A***, Intrathecal injection of the PKMζ inhibitor ZIP on day 4 reverses IL-6-induced persistent sensitization precipitated by intraplantar (i.pl.) PGE_2 injection (100 ng). Moreover, intrathecal ZIP fully reverses persistent sensitization to PGE_2 injection when given up to 16 d following IL-6 injection while Scr ZIP had no effect (***B***). ***C***, Intrathecal injection of the PKMζ inhibitor ZIP on day 4 also reverses IL-6-induced persistent sensitization precipitated by intrathecal injection of DHPG on day 6. ***D***, Intrathecal injection of pep2m on day 4 inhibits persistent sensitization to i.pl. PGE_2 administration on day 6. $N = 6$ for all experiments. Colored stars denote significant effects compared with vehicle groups, and black stars denote significance between IL-6-treated groups. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. VEH, Vehicle.

(Fig. 4*B*). Moreover, intrathecal ZIP injection on day 4 after IL-6 priming profoundly reduced nocifensive behaviors induced by intrathecal DHPG on day 6 (Fig. 4*C*). Intrathecal ZIP injections on day 8 followed by intrathecal DHPG injections on day 10 yielded similar results (scrambled ZIP: 649.2 ± 191 s, $n = 6$; ZIP: 53.0 ± 27.4 s, $n = 5$; $p < 0.05$). PKMζ maintains LTP through persistent *N*-ethylmaleimide-sensitive factor- (NSF)-dependent maintenance of glutamate receptor AMPA subunit 2 (GluA2) to the synapse (Yao et al., 2008; Migues et al., 2010), an interaction that can be disrupted by the peptide pep2m (Yao et al., 2008). Intrathecal pep2m injection 4 d following intraplantar IL-6 injection attenuated the expression of sensitization to intraplantar PGE_2 injection (Fig. 4*D*) and intrathecal DHPG injection (scrambled pep: 925.8 ± 181 s, $n = 6$; pep2m: 268 ± 51 s, $n = 6$, $p < 0.01$). Hence, a PKMζ-dependent mechanism in the dorsal horn of the spinal cord is required to establish and maintain sensitization promoted by IL-6 priming.

Maintenance of persistent nociceptive sensitization does not require spinal protein synthesis

If this mechanism truly parallels L-LTP, then inhibition of spinal mTOR or eIF4F complex formation in the spinal cord on day 4 should have no effect on expression of sensitization. Indeed, intrathecal injection of temsirolimus (Fig. 5*A*) or 4EGI1 (Fig. 5*B*) on day 4 did not influence sensitization to hindpaw injection of PGE_2. Likewise, intrathecal injection of anisomycin also had no effect on sensitization (Fig. 5*C*). Moreover, intrathecal injection of temsirolimus (10 μg) on day 8 had no effect on DHPG-evoked nocifensive behaviors precipitated on day 10 (vehicle: 1038.7 ± 251.7 s, $n = 6$; temsirolimus: 1193.7 ± 219.0 s). We also assessed whether inhibition of other PKCs may contribute to persistent sensitization. ZIP specifically inhibits PKMζ while staurosporine inhibits other PKCs but not PKMζ (Ling et al., 2002). Intrathecal injection of staurosporine (4.67 μg) (Pastalkova et al., 2006) on

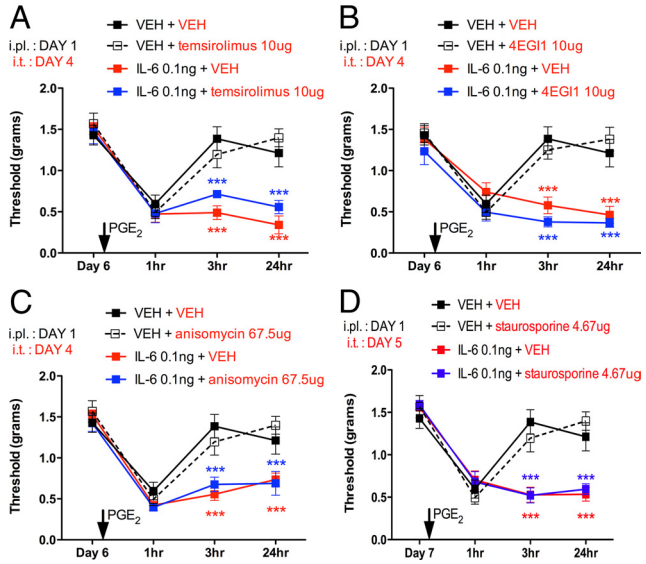

Figure 5. Spinal translation inhibition after the resolution of IL-6-induced allodynia fails to reverse persistent nociceptive sensitization. ***A–C***, Intrathecal injection of the mTOR inhibitor temsirolimus (***A***), the eIF4F complex formation inhibitor 4EGI-1 (***B***), or the general protein synthesis inhibitor anisomycin (***C***) on day 4 does not reverses persistent sensitization caused by intraplantar (i.pl.) IL-6 injection into the paw on day 1 when the persistent sensitization is induced by i.pl. PGE_2 (100 ng) administration. ***D***, Intrathecal injection of staurosporine on day 5 following intraplantar IL-6 injection does not influence PGE_2-precipitated persistent sensitization on day 7. $N = 6$ for all experiments. Colored stars denote significant effects compared with vehicle groups. $^{***}p < 0.001$. VEH, Vehicle.

day 5 had no effect on IL-6-induced persistent sensitization precipitated by PGE_2 on day 7 (Fig. 5*D*), indicating that persistent sensitization is maintained specifically by PKMζ. These results strongly suggest that molecular mechanisms of L-LTP in the dorsal horn underlie the maintenance of nociceptive sensitization.

Spinal PKMζ maintains persistent nociceptive sensitization after plantar incision

Previous studies using the "hyperalgesic priming" model have shown that acute inflammation with carrageenan is sufficient to induce a long-lasting hypersensitivity to subsequent PGE_2 injection (Aley et al., 2000). It is now known, however, whether other procedures, such as plantar incision (a model of postsurgical pain) (Banik et al., 2006), are capable of producing similar priming. Surgery induces chronic pain in a cohort of patients, and this persistent pain state is thought to be mediated, at least in part, by amplification of central pain pathways (Aasvang and Kehlet, 2005; Kehlet et al., 2006). Mice were randomly assigned to plantar incision or naive groups and subdivided into further groups that would receive either ZIP or scrambled-ZIP 15 d following plantar incision. Plantar incision produced mechanical allodynia that resolved within 13 d (Fig. 6*A*). On day 13, all mice received intrathecal injection of either ZIP or scrambled-ZIP. On day 15, PGE_2 was injected into the paw, and mechanical allodynia was measured over the following 24 h. Mice with previous plantar incision that received intrathecal scrambled ZIP displayed mechanical allodynia after PGE_2 injection that lasted for at least 24 h (Fig. 6*B*), demonstrating that plantar incision is capable of producing persistent nociceptive sensitization. Mice that received ZIP intrathecal injection on day 13 failed to develop PGE_2-induced allodynia (Fig. 6*B*), indicating that spinal PKMζ maintains persistent nociceptive sensitization in this model of postsurgical pain.

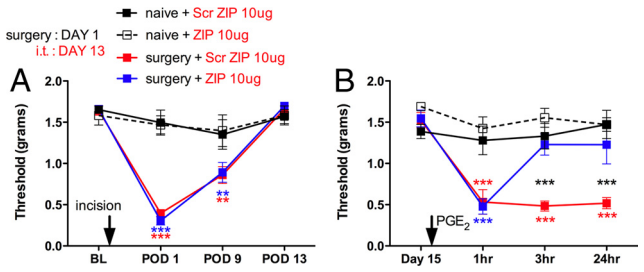

Figure 6. Plantar incision produces a spinal PKMζ-dependent persistent nociceptive sensitization: Plantar incision was done on day 1 (BL) and mechanical allodynia was measured on postoperative day (POD) 1, 9 and 13. ***A***, Mice with plantar incision developed allodynia lasting for 9 d and fully resolved by POD 13. ZIP or Scr ZIP was injected intrathecally (i.t.) on POD 13 and PGE_2 was injected intraplantarly on day 15. ***B***, Intrathecal ZIP abolished persistent nociceptive sensitization induced by plantar incision. Colored stars denote significant effects compared with naive groups that also received intraplantar PGE_2, and black stars denote significance between ZIP- and Scr ZIP-treated groups. $N = 6$ per group. $^{**}p < 0.01$, $^{***}p < 0.001$.

Spinal expression of constitutively active PKCζ recapitulates persistent sensitization

If PKMζ maintains persistent nociceptive sensitization, then virally mediated expression of a constitutively active PKCζ that mimics PKMζ should recapitulate the persistently sensitized state. We used a bicistronic lentiviral vector expressing a modified PKCζ construct, including a myristoylation sequence to induce membrane targeting and constitutive activity of PKCζ (mimicking PKMζ) (Chou et al., 1998), and mCherry to test this hypothesis. A similar approach has recently been used to demonstrate that overexpression of PKMζ enhances, while dominant-negative PKMζ reduces, conditioned taste aversion learning in the cortex (Shema et al., 2011). Intrathecal injection of the lentiviral vector led to stable expression of mCherry for at least 31 d (Fig. 7*A*). Viral transduction in the dorsal horn was prominent in neurons of the substantia gelatinosa (Fig. 7*B*). To test for persistent sensitization, the constitutively active PKCζ or green fluorescent protein control virus was injected intrathecally into mice following baseline mechanical threshold assessment, and mice were tested for mechanical allodynia on subsequent days. Mice receiving the active virus developed mechanical allodynia starting on day 6 after injection, lasting until at least day 12 postinjection (Fig. 7*C*). Thirty-one days after intrathecal virus injection, we tested for persistent sensitization through intrathecal injection of DHPG. Mice harboring spinal expression of constitutively active PKCζ displayed larger nocifensive responses to intrathecal DHPG than mice previously injected with control virus (Fig. 7*D*). Hence, expression of constitutively active PKCζ, mimicking PKMζ activity, in the spinal cord reproduces persistent nociceptive sensitization without prior IL-6 injection or plantar incision.

Discussion

Data from the present experiments support several conclusions, as follows: (1) local translation plays a key role in the initiation, but not maintenance, of IL-6 priming-induced persistent nociceptive sensitization; (2) IL-6-induced priming promotes sensitization that can be precipitated by either a peripheral (PGE_2) or central (DHPG) stimulus; (3) spinal protein synthesis- and PKMζ-dependent mechanisms are required for the initiation of persistent sensitization; (4) the maintenance mechanism of persistent nociceptive sensitization following IL-6 injection or plantar incision requires spinal PKMζ; and (5) spinal expression of a constitutively active PKCζ, mimicking PKMζ activity, is sufficient to induce persistent nociceptive sensitization. Collectively,

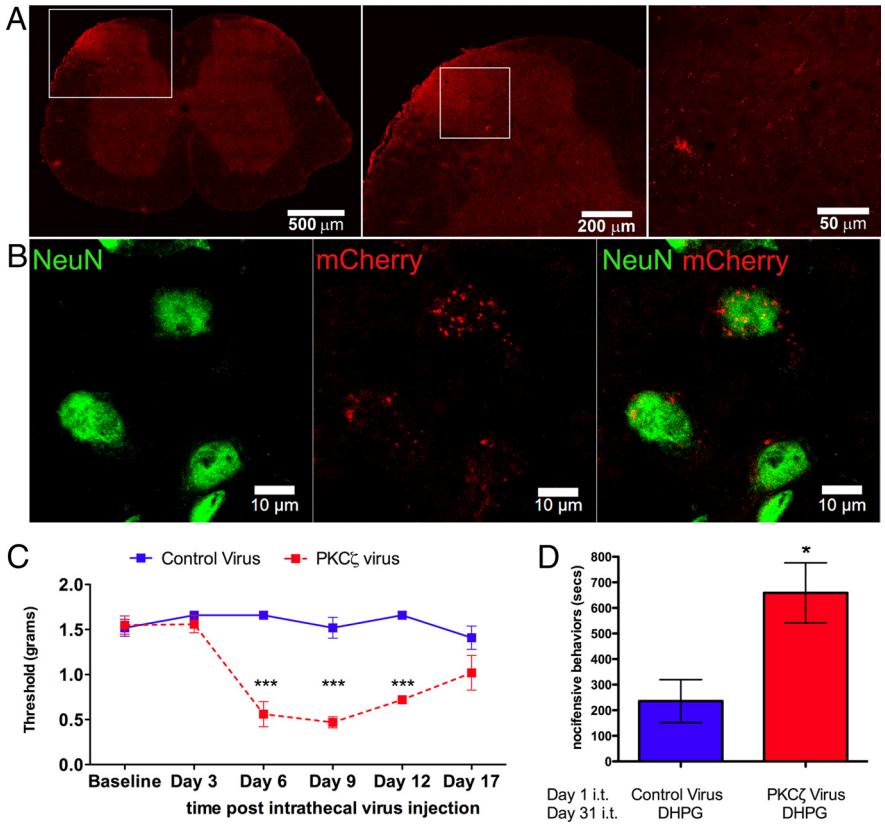

Figure 7. Spinal expression of constitutively active PKCζ recapitulates persistent sensitization. ***A***, mCherry expression in dorsal horn neurons, 31 d following intrathecal (i.t.) injection of lentiviral construct bicistronically expressing constitutively active PKCζ and mCherry. ***B***, Neuronal viral transduction was measured with NeuN colabeling with mCherry. Prominent mCherry expression was noted in the cytoplasm of neurons (nucleus labeled with NeuN antibody) throughout the substantia gelatinosa. ***C***, Mice receiving intrathecal injection of the active virus developed mechanical allodynia lasting until at least 12 d postinjection. $N = 6$ per group. ***D***, Expression of constitutively active PKCζ increased nocifensive behaviors induced by intrathecal injection of DHPG on day 31 after virus injection. $N = 5$ per group. $^{*}p < 0.05$, $^{***}p < 0.001$.

these findings point to a spinally encoded mechanism for the persistence of nociceptive sensitization with molecular underpinnings that closely resemble those involved in L-LTP and the maintenance of long-term memory traces.

The present experiments demonstrate a clear role for translation control via mTOR signaling to the eIF4F complex in the spinal dorsal horn in the initiation of IL-6-induced sensitization but not in its maintenance. Previous findings show that spinal mTOR signaling is important for the full expression of formalin-induced pain (Price et al., 2007; Asante et al., 2009), inflammatory pain (Norsted Gregory et al., 2010; Xu et al., 2011) and neuropathic pain (Asante et al., 2010). Our experiments demonstrate that IL-6-induced allodynia is decreased by spinal inhibition of mTOR or eIF4F complex formation. Moreover, when these pathways are inhibited at the time of IL-6 injection into the hindpaw, persistent nociceptive sensitization fails to develop. It is likely that this effect is mediated postsynaptically in dorsal horn neurons because mTOR immunoreactivity in primary afferents fails to invade central terminals of these fibers past the dorsal root entry zone (Géranton et al., 2009). Importantly, however, inhibition of mTOR, eIF4F complex formation, or general protein synthesis after initial IL-6-induced allodynia has resolved has no effect on persistent nociceptive sensitization. The time dependence of these effects is reminiscent of the maintenance of L-LTP, which is not sensitive to translation inhibition (Kelleher et al., 2004), leading us to search for other spinal mechanisms that may be responsible for the persistence of the sensitized state.

Our results indicate that spinal PKMζ underlies the maintenance mechanism of persistent nociceptive sensitization. Inhibition of PKMζ, either at the time of IL-6 injection into the hindpaw or at multiple time points after this allodynia has resolved, leads to a complete resolution of sensitization precipitated by both hindpaw PGE_2 injection and intrathecal DHPG injection. We attribute this enhanced DHPG-mediated effect to postsynaptic sites because (1) previous studies have linked mGluR-mediated ERK activity in dorsal horn neurons to increased excitability (Karim et al., 2001; Adwanikar et al., 2004; Hu et al., 2007); (2) >90% of mGluR1/5-immunoreactive sites in the dorsal horn are dendritic (Pitcher et al., 2007); and (3) pep2m, which acts via disruption of postsynaptic GluA2 trafficking, inhibited persistent sensitization to intrathecal DHPG. On the other hand, transient receptor potential vanilloid-1 (TRPV1)/mGluR5 signaling has been described on the central terminals of primary afferent neurons, and *TRPV1*$^{-/-}$ mice show a small but significant decrease in DHPG-evoked nocifensive behaviors (Kim et al., 2009). Hence, we cannot completely exclude the possibility of presynaptic effects in persistent sensitization to intrathecal DHPG. Inhibition of PKMζ also led to a resolution of persistent sensitization following plantar incision, indicating that spinal PKMζ is likely to play a key role in postsurgical pain conditions. Chronic pain presents in a large cohort of patients following surgery, and the incidence of chronic pain in these patients is linked to presurgical pain status and previous surgeries (Aasvang and Kehlet, 2005; Kehlet et al., 2006; Aasvang et al., 2010). It is tempting to speculate that spinally directed, PKMζ-based therapies may be able to reduce the incidence of chronic postoperative pain by resolving central amplification mechanisms responsible for its persistence, or even emergence in the case of pre-existing pain conditions (Kehlet et al., 2006; Aasvang et al., 2010) or multiple surgeries at the same site (Aasvang and Kehlet, 2005).

PKMζ is necessary and sufficient for the maintenance L-LTP (Ling et al., 2002), and the inhibition of PKMζ with ZIP causes the decay of previously established memories (Pastalkova et al., 2006; Shema et al., 2007; von Kraus et al., 2010). The molecular substrate for PKMζ in the setting of L-LTP appears to be regulation of GluA2 localization to the potentiated synapse in an NSF-dependent fashion (Yao et al., 2008; Migues et al., 2010). Consistent with this, we found that pep2m, a peptide that disrupts GluA2–NSF interaction, was capable of suppressing persistent sensitization after IL-6-induced allodynia had resolved. We also demonstrate that virally mediated spinal expression of constitutively active PKCζ, thereby mimicking PKMζ activity, recapitulates persistent nociceptive sensitization. This demonstration suggests that, like L-LTP (Ling et al., 2002), PKMζ is sufficient for persistent nociceptive sensitization. While our results show a striking similarity to maintenance mechanisms of L-LTP in terms of pharmacology, the role of spinal LTP in persistent pain states

remains unclear (Latremoliere and Woolf, 2009, 2010; Sandkühler, 2010). Further electrophysiological work is needed to definitively link PKMζ to LTP initiation and maintenance in dorsal horn neurons of the spinal cord.

A role for anterior cingulate cortex (ACC) PKMζ has recently been demonstrated in the setting of neuropathic pain. This work suggests that ACC PKMζ phosphorylation is increased following peripheral nerve injury and that inhibition of PKMζ in the ACC leads to a transient (at 2 h) reversal of neuropathic mechanical allodynia (Li et al., 2010). In contrast, neuropathic allodynia was not alleviated by spinally directed PKMζ inhibition at the time points examined after peripheral nerve injury, although PKMζ inhibition was capable of suppressing synaptic transmission in spinal slice preparations (Li et al., 2010). We rule out a general suppression of synaptic transmission in the dorsal horn as a causative factor in the present findings because PKMζ inhibition before the priming stimulus had no effect on subsequent IL-6-induced allodynia or persistent nociceptive sensitization. Nevertheless, differences between our findings and those of Li et al. (2010) are likely to be important for fully understanding the mechanisms that maintain sensitization and that could contribute to chronic pain. We have used models where afferent discharge is expected to resolve after stimulation with an acute stimulus (IL-6) or where afferent spontaneous activity is known to last for a short period of time (~1 d, plantar incision) (Banik and Brennan, 2004, 2008). It is tempting to speculate that this type of stimulus leads to PKMζ-maintained plasticity that does not invade CNS structures outside the dorsal horn of the spinal cord. While this hypothesis clearly requires further investigation, gaining a better understanding of these processes may lead to considerable insight into differential molecular mechanisms of persistent pain states.

In conclusion, the present results reveal the encoding of a spinal cord engram for nociceptive sensitization promoting enhanced responses that facilitate the development of a persistent pain state (Aasvang and Kehlet, 2005; Kehlet et al., 2006; Reichling and Levine, 2009). Our data strongly suggest that spinal PKMζ is essential for the storage of persistent nociceptive sensitization. The findings point to a model in which spinal LTP, one form of central sensitization (Ikeda et al., 2006; Sandkühler, 2007; Latremoliere and Woolf, 2009; Woolf, 2011), is responsible for the amplification of subsequent noxious stimuli following a priming event to promote persistent pain. Hence, we have demonstrated a novel, PKMζ-mediated mechanism for the maintenance of a sensitized state promoting persistent pain that points to the potential value of spinal PKMζ inhibitors as pain therapeutics.

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