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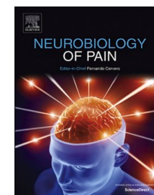

Original Research

eIF4E phosphorylation regulates ongoing pain, independently of inflammation, and hyperalgesic priming in the mouse CFA model

Jamie K. Moy, Jasper L. Kuhn, Thomas A. Szabo-Pardi, Grishma Pradhan, Theodore J. Price*

School of Behavioral and Brain Sciences, University of Texas at Dallas, Richardson, TX 75080, USA

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ABSTRACT

Mitogen activated protein kinase-interacting kinase (MNK)-mediated phosphorylation of the mRNA cap binding protein eIF4E controls the translation of a subset of mRNAs that are involved in neuronal and immune plasticity. MNK-eIF4E signaling plays a crucial role in the response of nociceptors to injury and/or inflammatory mediators. This signaling pathway controls changes in excitability that drive acute pain sensitization as well as the translation of mRNAs, such as brain-derived neurotrophic factor (BDNF), that enhance plasticity between dorsal root ganglion (DRG) nociceptors and second order neurons in the spinal dorsal horn. However, since MNK-eIF4E signaling also regulates immune responses, we sought to assess whether decreased pain responses are coupled to decreased inflammatory responses in mice lacking MNK-eIF4E signaling. Our results show that while inflammation resolves more quickly in mice lacking MNK-eIF4E signaling, peak inflammatory responses measured with infrared imaging are not altered in the absence of this signaling pathway even though pain responses are significantly decreased. We also find that inflammation fails to produce hyperalgesic priming, a model for the transition to a chronic pain state, in mice lacking MNK-eIF4E signaling. We conclude that MNK-eIF4E signaling is a critical signaling pathway for the generation of nociceptive plasticity leading to acute pain responses to inflammation and the development of hyperalgesic priming.

Introduction

New treatments are needed for the treatment of pain that simultaneously attenuate acute pain and prevent the development of chronic pain after injury (Skolnick and Volkow, 2016; Price and Gold, 2017; Volkow and Collins, 2017). Multiple lines of evidence support the contention that changes in nociceptor excitability that drive acute pain and lead to the development of chronic pain are dependent on activity-dependent signaling to translation machinery leading to the synthesis of new proteins that are critical for this form of neuronal plasticity (Khoutorsky and Price, 2018). We have recently focused on the mitogen activated protein kinase (MAPK) interacting kinase (MNK) family (the MNK1 and MNK2 kinases) (Moy et al., 2017; Khoutorsky and Price, 2018; Moy et al., 2018) that are the only known kinases that phosphorylate the mRNA cap-binding protein eukaryotic initiation factor (eIF) 4E (eIF4E) (Waskiewicz et al., 1999). While the entire repertoire of mRNAs whose translation is regulated by eIF4E phosphorylation is not known, eIF4E phosphorylation regulates the translation of a number of mRNA that are involved in nociceptive plasticity, such as matrix metalloprotease 9 (MMP9) (Gkogkas et al., 2014) and brain-derived neurotrophic factor (BDNF) (Moy et al., 2018), in addition to many cytokines and chemokines (Furic et al., 2010; Silva Amorim et al., 2018) that are involved in pain signaling. Mice that harbor a deletion at the MNK1/2 phosphorylation site on eIF4E (*eIF4E*^{S209A} mice) have deficits in development of nociceptive behavioral plasticity in response to many inflammatory mediators, fail to show increased nociceptor excitability in response to these mediators using electrophysiological measures, and have decreased cold hypersensitivity after nerve injury (Moy et al., 2017, 2018). These effects are recapitulated by genetic or pharmacological neutralization of MNK1/2.

Since eIF4E phosphorylation regulates translation of mRNA involved in nociceptive plasticity and inflammation, an open question is whether behavioral effects observed in *eIF4E*^{S209A} mice are due to a loss of nociceptor plasticity *in vivo* or a reduction in inflammation. One way to test this hypothesis is to use a strong inflammatory mediator, such as complete Freund's adjuvant (CFA), in wild-type (WT) and *eIF4E*^{S209A} mice and non-invasively examine inflammation and pain at the same time. In the experiments described here, we set out to address this question using

* Corresponding author at: School of Behavioral and Brain Sciences, University of Texas at Dallas, BSB 14.102G, 800 W Campbell Rd, Richardson, TX 75080, USA.
E-mail address: Theodore.price@utdallas.edu (T.J. Price).

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infrared imaging to assess inflammation and spontaneous paw guarding to assess ongoing pain (Djouhri et al., 2006), both after CFA injection. Our hypothesis was that pain and inflammation would be dissociated in *eIF4E*^{S209A} mice demonstrating that decreased nociceptor sensitization is the key factor in behavioral phenotypes observed in these mice. Our findings are consistent with this hypothesis.

A second goal of our experimental design was to assess development of chronic pain after CFA injection in *eIF4E*^{S209A} mice using the hyperalgesic priming paradigm. This experimental design is used to model the transition to a chronic pain state where the animal becomes susceptible to a persistent pain state upon injection of a normally sub-threshold inflammatory stimulus (Reichling and Levine, 2009; Reichling et al., 2013; Kandasamy and Price, 2015; Price and Inyang, 2015; Khoutorsky and Price, 2018). While we have previously shown that *eIF4E*^{S209A} mice fail to develop hyperalgesic priming in response to many inflammatory mediators (Moy et al., 2017), we have not previously tested these mice with CFA as the priming stimulus. Our results are consistent with previous observations that MNK-eIF4E signaling is a key signaling factor in the development of hyperalgesic priming.

Methods and materials

Animals

Mice were bred and raised on a 12-h light-dark cycle with lights on at 7:00 AM. Food and water were available *ad libitum* in their home cages. *eIF4E*^{S209A} mice on a C57BL/6 background were from the Sonenberg laboratory at McGill University (Furic et al., 2010). Both C57BL/6 (WT) and *eIF4E*^{S209A} mice were bred at The University of Texas at Dallas to produce experimental animals. Between 3 and 4 weeks old, mice were weaned and ear clipped to verify genotypes. All mice weighed between 20 and 25 g at the time of experimental use. The Institutional Animal Care and Use Committee at The University of Texas at Dallas approved all animal procedures.

Behavior

Male and female mice were habituated for approximately 1hr to acrylic behavior boxes prior to beginning experiments. Guarding scores were evaluated as described in Brennan et al. (1996), Djouhri et al. (2006), Xu and Brennan (2009) Hindpaw mechanical thresholds were determined by using the up-down method as described previously (Chaplan et al., 1994) using calibrated Von Frey filaments (Stoelting Company, Wood Dale, IL). A forward-looking infrared (FLIR) T650SC camera (Wilsonville, OR) was used for thermal imaging. At each time point two pictures were taken and the mean temperature in degrees Celsius across each paw was recorded (Megat et al., 2017; Barragan-Iglesias et al., 2018). The experimenters (*JKM, JLK, TAS-P, and GP)* were blinded to the genotype of the mice.

Chemicals

Complete Freund's Adjuvant (CFA) was purchased from Sigma-Aldrich (St. Louis, MO) at a concentration of 1 mg/mL. For hindpaw injections, CFA was mixed with an equal volume of 0.9% saline and vortexed to create an emulsion. The emulsion was vortexed prior to each injection (10 μL) to ensure equivalent injections between animals. Prostaglandin E_2 (PGE_2) was purchased from Cayman chemicals (Ann Arbor, MI). All other chemicals were attained from ThermoFisher Scientific (Waltham, MA).

Statistics

All data are shown as mean ± standard error of the mean (SEM), with individual samples represented within graphs to depict the n of each group and the distribution of the data points. GraphPad Prism 6 v 6.0 for Mac OS X was used for analysis. Statistical tests, post hoc analyses, and values for each figure are displayed in Table 1.

Results

CFA-induced inflammation and spontaneous pain are dissociated in eIF4E^{S209A} *mice*

To test if CFA-induced inflammatory responses are regulated by eIF4E phosphorylation, WT and *eIF4E*^{S209A} mice were injected with 5 μg of CFA and a FLIR camera was used to observe changes in temperature in the ipsilateral and contralateral hindpaws. FLIR imaging was used because it allows for non-invasive monitoring of temperature changes that can be paired with behavioral measures. The ipsilateral hindpaw in WT mice displayed a dramatic increase in temperature, indicative of inflammation, compared to the contralateral hindpaw starting at 3 h post CFA injection. This effect lasted for 7 days in WT mice (Fig. 1A & B). Similarly, the injected hindpaw in *eIF4E*^{S209A} mice showed an increase in temperature compared to the contralateral hindpaw at 3 h post CFA injection and this change persisted through the 72 h measurement (Fig. 1A & C). However, on Day 7, CFA-injected paws in WT mice had significantly higher temperatures compared to *eIF4E*^{S209A} mice (Fig. 1D) and ipsilateral hindpaws of *eIF4E*^{S209A} mice were not different from contralateral hindpaws on day 7 after CFA injection. While there is a late difference in inflammation induced by CFA in *eIF4E*^{S209A} mice, the early effects are indistinguishable from WT mice, at least with this measure.

CFA injection causes guarding behaviors in rodents that are linked to ongoing activity in nociceptors (Djouhri et al., 2006; Weibel et al., 2013). These ongoing pain behaviors are present at 24 h after CFA injection but subside rapidly after that, as does the ongoing activity in nociceptors (Djouhri et al., 2006). We hypothesized that ongoing pain would be decreased in *eIF4E*^{S209A} mice given our previous findings that CFA-induced mechanical hypersensitivity is decreased when the MNK-eIF4E pathway is disrupted (Moy et al., 2017). In line with this hypothesis, we observed significantly less guarding in *eIF4E*^{S209A} mice at 24 h after CFA injection (Fig. 1E). Therefore, even though there is no difference in signs of inflammation for at least the first 3 days after CFA injection, evoked and ongoing pain behaviors are reduced in mice where the MNK-eIF4E signaling pathway is disrupted. This strongly suggests that differences in pain behaviors observed when this pathway is disrupted are dependent on decreased nociceptor plasticity and not on decreased inflammatory responses. Male and female mice were used in these experiments and we did not note any sex differences, consistent with our previous work on MNK-eIF4E signaling.

CFA-induced hyperalgesic priming requires eIF4E phosphorylation

Previously, we found that eIF4E phosphorylation is required for the development of hyperalgesic priming using stimuli such as nerve growth factor (NGF), interleukin 6 (IL-6), and protease activated receptor type 2 (PAR2) activation (Moy et al., 2017). Moreover, in MNK1/2 double knockout mice CFA-induced hyperalgesic priming is reduced suggesting that eIF4E phosphorylation also plays a key role in this model. We evaluated hyperalgesic priming with CFA as a priming stimulus using WT and *eIF4E*^{S209A} mice with mechanical sensitivity, guarding and temperature changes as experimental endpoints.

First, we assessed whether PGE_2 also induces changes in hindpaw temperature in mice primed with CFA. Previously primed WT and *eIF4E*^{S209A} mice were injected with 100 ng of PGE_2 and hindpaw temperatures were measured at 3 and 24 h after injection (Fig. 2A). PGE_2 induced a transient increase in temperature at 3 h in WT mice (Fig. 2B; left), whereas, in *eIF4E*^{S209A} mice no changes were observed when comparing the ipsilateral to the contralateral paw (Fig. 2B; right). The ipsilateral paws of *eIF4E*^{S209A} mice displayed a significantly lower temperature compared to WT mice 3 h post PGE_2 (Fig. 2C).

We then assessed whether PGE_2 injection induces guarding

Table 1
Statistical tests and values for all analyses.

Test	F (df1,df2) interaction F (df1,df2) row F (df1,df2) column	*t*-ratio, Df	P-value	Adjusted p-value (Post-hoc comparison)
Multiple t-tests (Fig. 1B; WT)	N/A	*Contra vs Ipsi* BL = 1.854 3 h = 7.367 24 h = 7.436 48 h = 4.434 72 h = 7.389 D7 = 2.688 Df = 20	*Contra vs Ipsi* BL = 0.08 3 h < 0.0001 24 h < 0.0001 48 h < 0.0001 72 h < 0.0001 D7 < 0.01	N/A
Multiple *t*-tests (Fig. 1C; *eIF4E^{S209A}*)	N/A	*Contra vs Ipsi* BL = 0.3365 3 h = 16.05 24 h = 8.974 48 h = 3.577 72 h = 4.176 D7 = 0.2637 Df = 22	*Contra vs Ipsi* BL = 0.74 3 h < 0.0001 24 h < 0.0001 48 h < 0.01 72 h < 0.0001 D7 = 0.79	N/A
Two-way ANOVA (Fig. 1D)	$F_{i(5, 126)} = 1.39$ $F_{r(5, 126)} = 40.13$ $F_{c(1, 126)} = 8.323$	N/A	$P_i = 0.2326$ $P_r < 0.0001$ $P_c = 0.0046$	WT Ipsi vs *eIF4E^{S209A}* Ipsi BL: p > 0.9999 3 h: p > 0.9999 24 h: p > 0.9999 48 h: p = 0.7019 72 h: p > 0.9999 D7: p = 0.0120
Nonparametric Mann-Whitney (Fig. 1E)	N/A	N/A	*p = 0.0269	N/A
Nonparametric Mann-Whitney (Fig. 2B; WT)	N/A	N/A	*p = 0.0452	N/A
Nonparametric Mann-Whitney (Fig. 2B; *eIF4E^{S209A}*)	N/A	N/A	p = 0.3542	N/A
Two-way ANOVA (Fig. 2C)	$F_{i(2, 63)} = 0.4709$ $F_{r(2, 63)} = 7.552$ $F_{c(1, 63)} = 15.81$	N/A	$P_i = 0.6266$ $P_r = 0.0011$ $P_c = 0.0002$	WT Ipsi vs *eIF4E^{S209A}* Ipsi BL: p = 0.1553 3 h: **p = 0.0091 24 h: p = 0.2194
Two-way ANOVA (Fig. 2D)	$F_{i(2, 63)} = 1.263$ $F_{r(2, 63)} = 5.659$ $F_{c(1, 63)} = 15.87$	N/A	$P_i = 0.2899$ $P_r = 0.0055$ $P_c = 0.0002$	WT vs *eIF4E^{S209A}* BL: p = 0.9542 3 h: *p = 0.0171 24 h: *p = 0.0106 WT: BL vs 3: ##p = 0.0039 BL vs 24: p = 0.775 *eIF4E^{S209A}*: BL vs. 3: p = 0.2865 BL vs. 24: p > 0.9999
Two-way ANOVA (Fig. 2E)	$F_{i(3, 40)} = 5.866$ $F_{r(3, 40)} = 19.53$ $F_{c(1, 40)} = 9.969$	N/A	$P_i = 0.0020$ $P_r < 0.0001$ $P_c = 0.003$	WT vs *eIF4E^{S209A}* D9 BL: p > 0.9999 3 h: p > 0.9999 24 h: *p = 0.0470 48 h: ***p = 0.0004 WT: BL vs 3: ####p < 0.0001 BL vs 24: ####p < 0.0001 BL vs 48: ####p < 0.0001 *eIF4E^{S209A}*: BL vs. 3: ###p = 0.0001 BL vs. 24: p = 0.5984 BL vs 48: p > 0.9999

behaviors in mice primed with CFA. We observed guarding behavior elicited by PGE_2 injection in WT mice at 3 and 24 h post PGE_2 injection (Fig. 2D). No guarding behaviors were observed in *eIF4E^{S209A}* mice (Fig. 2D). This finding is consistent with our previous observation that the specific kinase of eIF4E phosphorylation, MNK1/2, is required for CFA-induced hyperalgesic priming (Moy et al., 2017). To corroborate the guarding behavior findings, we also tested mechanical hypersensitivity in both WT and *eIF4E^{S209A}* mice after PGE_2 injection over a time course of 48 h (Fig. 2E). While both WT and *eIF4E^{S209A}* mice dropped in withdrawal threshold at 3 h, only WT mice displayed a long-lasting mechanical hypersensitivity lasting up to 48 h post-PGE_2 (Fig. 2E). It is notable that although there was mechanical hypersensitivity in both genotypes at 3 h after priming, there were no signs of ongoing pain as measured with paw guarding at this time point in the *eIF4E^{S209A}* mice. These findings demonstrate that eIF4E phosphorylation is a key event for the development of neurogenic inflammation, mechanical hypersensitivity and ongoing pain produced when hyperalgesic priming is revealed by PGE_2 injection into the hindpaw.

Discussion

We reach two primary conclusions based on these experiments. First, our findings suggest that MNK-eIF4E signaling is a key regulatory factor in the production of spontaneous pain produced by inflammation. Combining our findings here with our previous work (Moy et al., 2017, 2018) makes a compelling case for MNK-eIF4E signaling as a core signaling pathway in the generation of mechanical hypersensitivity and ongoing pain in response to inflammation. Second, our findings provide

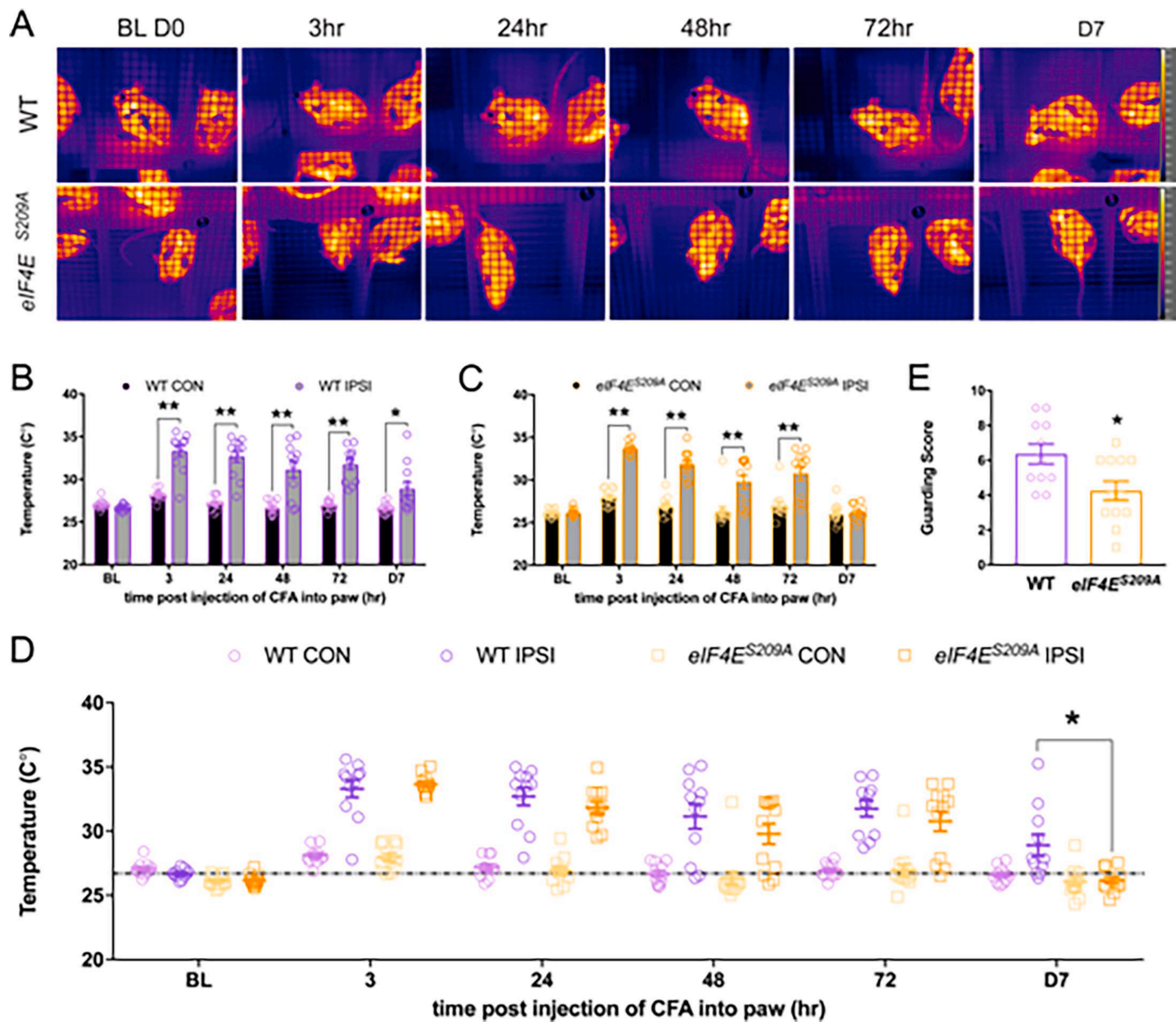

Fig. 1. CFA induces inflammation but with reduced ongoing pain in *eIF4E*^{S209A} mice. A) CFA (5 μg) was injected into the left hindpaw in both WT (B) and *eIF4E*^{S209A} (C) mice and temperatures of both ipsilateral and contralateral hindpaws were recorded through FLIR imaging across 7 days *(WT n = 11; eIF4E*^{S209A} *n = 12,* $^{**}p < 0.01$, $^{*}p < 0.05$, *multiple t-tests with Bonferroni correction)*. D) Both contralateral (CON) and ipsilateral (IPSI) hindpaw temperatures of WT and *eIF4E*^{S309A} mice at BL, 3, 24, 48, 72 h, and D7 after CFA-injection show that will initial changes in temperature are equal in both genotypes, *eIF4E*^{S209A} mice recovered faster than WT mice (*WT n = 11; eIF4E*^{S209A} *n = 12,* $^{*}p < 0.05$, *two-way ANOVA, post hoc Bonferroni test; dotted line represents the average of contralateral paws of both WT and eIF4E*^{S209A} *mice)*. E) WT mice showed more guarding 24 h post CFA-injection compared to *eIF4E*^{S209A} mice (*WT n = 11; eIF4E*^{S209A} *n = 12,* $^{*}p = 0.0269$, *Mann-Whitney test)*.

further support for the hypothesis that MNK-eIF4E signaling is absolutely critical for the generation of plasticity in nociceptors that causes hyperalgesic priming (Moy et al., 2017, 2018). Insofar as this model can be used to understand the transition from acute to chronic pain, targeting MNK-eIF4E signaling should be considered as a prime target for manipulation to block the transition to a chronic pain state after injury. It is notable that interfering with MNK-eIF4E signaling produces a >75% attenuation of hyperalgesic priming using evoked and spontaneous pain measures, in both sexes, with a wide variety of priming stimuli (NGF, IL-6, PAR2 agonists, carrageenan and CFA).

MNK and eIF4E are ubiquitous proteins that play an important role in the regulation of translation in response to a variety of stimuli in diverse cell types. MNK signaling is engaged in immune cells during inflammation leading to the phosphorylation of eIF4E (Joshi and Platanias, 2012; Pashenkov et al., 2017; Silva Amorim et al., 2018). We have shown that many inflammatory mediators stimulate MNK-mediated phosphorylation of eIF4E in DRG nociceptors and this signaling event leads to increased excitability of these neurons (Melemedjian et al., 2010, 2011; Moy et al., 2017). Because this signaling pathway is involved in both inflammation and pain sensitization, it is difficult to know whether the effects of MNK-eIF4E signaling on pain and inflammation can be dissociated. The goal of our experimental design was to use non-invasive methods of pain and inflammation assessment to gain insight into whether the anti-nociceptive effect seen when MNK-eIF4E signaling is disrupted is due to decreased inflammation or due to decreased nociceptor sensitization. While there is a more rapid resolution of inflammation in *eIF4E*^{S209A} mice, the peak of inflammation is not distinguishable from WT mice and at 24 h after CFA injection *eIF4E*^{S209A} mice show decreased signs of ongoing pain but equivalent changes in hindpaw temperature. Our interpretation of this result is that MNK-eIF4E signaling contributes to nociceptor sensitization through signaling events in nociceptors, independently of effects of this signaling pathway in immune cells. A shortcoming of our approach is that assessment of changes in temperature, as we have done here, does not give insight into possible changes in cytokine or other inflammatory mediator production in immune cells of *eIF4E*^{S209A} mice in response to CFA injection.

The experiment described here provides further evidence that MNK-eIF4E signaling is necessary to establish hyperalgesic priming. As mentioned above, our previous work demonstrated a key role for this

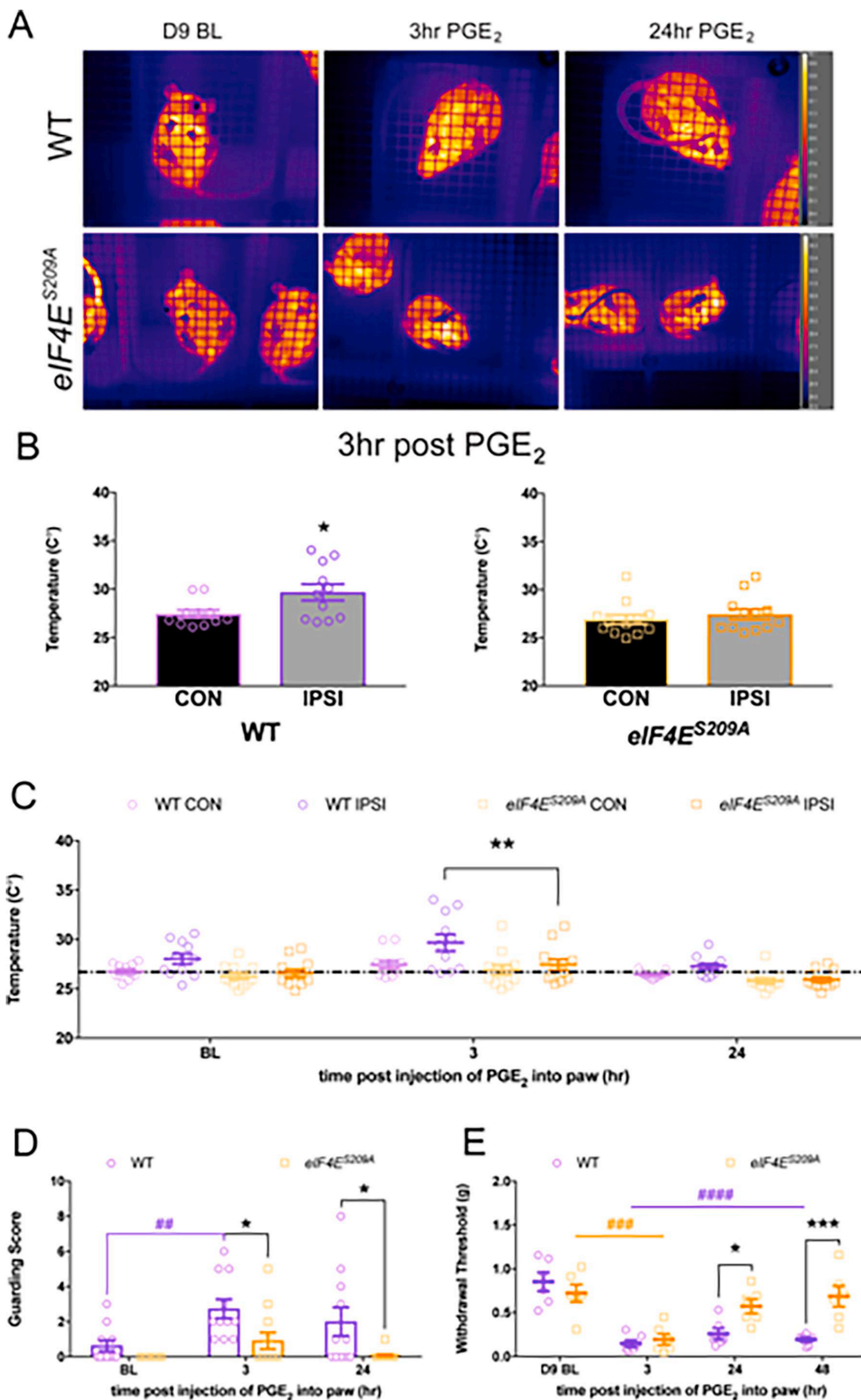

Fig. 2. PGE_2 induces inflammation transiently, guarding, and mechanical hypersensitivity in CFA-primed WT, but not $eIF4E^{S209A}$ mice. Hindpaw images of both WT and $eIF4E^{S209A}$ mice show that 3 h after PGE_2 (100 ng) injection the afflicted paw displayed an increase in temperature in WT mice, but not $eIF4E^{S209A}$ mice (A & B; *WT* $n = 11$; $eIF4E^{S209A}$ $n = 12$, $^{*}p = 0.0452$, *Mann-Whitney test*). C) Hindpaw temperatures (CON and IPSI) of WT and $eIF4E^{S209A}$ mice show that only the ipsilateral paw of WT mice displayed an increase in temperature when compared to $eIF4E^{S209A}$ mice (*WT* $n = 11$; $eIF4E^{S209A}$ $n = 12$, $^{**}p = 0.0091$, *two-way ANOVA, post hoc Bonferroni test; dotted line represents the average of contralateral paws of both WT and* $eIF4E^{S209A}$ *mice*). D) $eIF4E^{S209A}$ mice showed less guarding at 3 and 24 h post CFA-injection compared to WT mice (*WT* $n = 11$; $eIF4E^{S209A}$ $n = 12$, $^{*}p = 0.0171$, $^{*}p = 0.0106$, *two-way ANOVA comparing WT vs* $eIF4E^{S209A}$ *mice, post hoc Bonferroni test;* $^{\#\#}p = 0.0039$, *two-way ANOVA compared to BL, post hoc Bonferroni test*). E) $eIF4E^{S209A}$ mice displayed reduced mechanical hypersensitivity compared to WT mice (*WT* $n = 11$; $eIF4E^{S209A}$ $n = 12$, $^{*}p = 0.037$, $^{***}p = 0.0004$, *two-way ANOVA comparing WT vs* $eIF4E^{S209A}$ *mice, post hoc Bonferroni test;* $^{\#\#\#}p = 0.0001$, $^{\#\#\#\#}p < 0.0001$, *two-way ANOVA compared to BL, post hoc Bonferroni test*).

signaling pathway in the development of hyperalgesic priming using a variety of other priming stimuli. All of these stimuli (NGF, IL-6, PAR2 agonists, CFA) are capable of increasing the excitability of nociceptors and this effect is strongly decreased in nociceptors from $eIF4E^{S209A}$ mice or when MNK activity is blocked pharmacologically. Moreover, we have recently shown that priming stimuli induce increased translation of brain derived neurotrophic factor (BDNF) in DRG neurons that is also dependent on eIF4E phosphorylation. Therefore, MNK-eIF4E signaling is involved in changing the excitability of DRG nociceptors in response to priming stimuli and this signaling pathway also controls enhanced translation of a key neurotransmitter for hyperalgesic priming, BDNF, in DRG neurons (Moy et al., 2017, 2018). Importantly, another recent

study demonstrated that genetic deletion of BDNF from DRG neurons leads to a loss of hyperalgesic priming in response to inflammation (Sikandar et al., 2018).

The conclusion we reach from these studies is that MNK-eIF4E signaling is a critical signaling pathway in DRG neurons for the development of hyperalgesic priming because it regulates phenotypic changes in these cells that support long-term nociceptor plasticity. This highlights the utility of targeting MNK1/2 for the prevention of chronic pain.

Editorial disclosure

Given his role as Associate Editor, Theodore J. Price had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Editor-in-Chief, Fernando Cervero.

Conflict of interest

The authors declare no competing financial interests.

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