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

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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 (Khutoryansky 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; Khutoryansky and Price, 2018; Moy et al., 2018) that are the only known kinases that phosphorylate the mRNA cap-binding protein eukaryotic initiation factor 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 mRNAs 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 (Puric et al., 2010; Silva Amorim et al., 2018) that are involved in pain signaling. Mice that harbor a deletion at the eIF4E phosphorylation site on eIF4E (eIF4ES209A) 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 eIF4ES209A 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 eIF4ES209A 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...
irradiation to assess inflammation and spontaneous paw guarding
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 hyper-
algesic 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 pre-
vious 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
toxeped to create an emulsion. The emulsion was vortexed prior to each
injection (10 µl) to ensure equivalent injections between animals.
Prostaglandin E2 (PGE2) was purchased from Cayman chemicals (Ann
Arbor, MI). All other chemicals were attained from ThermoFisher Sci-
entific (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. 2A & 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 in-
jection 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 interrupted (Moy et al., 2017). In line with this hy-
pothesis, 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 dis-
rupted 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 PGE2 also induces changes in hindpaw
temperature in mice primed with CFA. Previously primed WT and
eIF4E-S209A mice were injected with 100 ng of PGE2 and hindpaw tem-
peratures were measured at 3 and 24 h after injection (Fig. 2A). PGE2
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 PGE2 (Fig. 2C).

We then assessed whether PGE2 injection induces guarding
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 elf4E$^{S209A}$ mice (Fig. 2D). This finding is consistent with our previous observation that the specific kinase of elf4E 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 elf4E$^{S209A}$ mice after PGE$_2$ injection over a time course of 48 h (Fig. 2E). While both WT and elf4E$^{S209A}$ 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 elf4E$^{S209A}$ mice. These findings demonstrate that elf4E phosphorylation is a key event for the development of neurogenic inflammation, mechanical hyper-sensitivity 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-elf4E 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-elf4E signaling as a core signaling pathway in the generation of mechanical hypersensitivity and ongoing pain in response to inflammation. Second, our findings provide

### Table 1

Statistical tests and values for all analyses.

| Test                              | F (df1,df2) interaction | 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 < 0.0001 |                                       |
|                                   |                          | 3 h = 7.367 | 24 h < 0.0001 |                                       |
|                                   |                          | 48 h < 0.0001 | 72 h < 0.0001 |                                       |
|                                   |                          | D$^7$ = 2.668 | D$^7$ < 0.01 |                                       |
|                                   |                          | Df = 20     |          |                                       |
| Multiple t-tests (Fig. 1C; elf4E$^{S209A}$) | N/A                      |             |         |                                       |
|                                   | Contra vs Ipsi           | BL = 0.3365 | 3 h < 0.0001 |                                       |
|                                   |                          | 3 h = 16.05 | 24 h < 0.0001 |                                       |
|                                   |                          | 24 h = 8.974 | 48 h < 0.01 |                                       |
|                                   |                          | 48 h = 3.577 | 72 h < 0.0001 |                                       |
|                                   |                          | D$^7$ = 2.176 | D$^7$ = 0.79 |                                       |
|                                   |                          | Df = 22     |          |                                       |
| Two-way ANOVA (Fig. 1D)           | F(2, 126) = 1.39         |             |         |                                       |
|                                   | F(2, 126) = 40.13        |             |         |                                       |
|                                   | F(1, 126) = 8.323        |             |         |                                       |
| Nonparametric Mann-Whitney (Fig. 1E) | N/A                      |             |         |                                       |
| Nonparametric Mann-Whitney (Fig. 2B; WT) | N/A                      |             |         |                                       |
| Nonparametric Mann-Whitney (Fig. 2B; elf4E$^{S209A}$) | N/A                      |             |         |                                       |
| Two-way ANOVA (Fig. 2C)           | F(2, 63) = 0.4709        |             |         |                                       |
|                                   | F(2, 63) = 7.552         |             |         |                                       |
|                                   | F(1, 63) = 15.81         |             |         |                                       |
| Two-way ANOVA (Fig. 2D)           | F(2, 63) = 1.263         |             |         |                                       |
|                                   | F(2, 63) = 5.659         |             |         |                                       |
|                                   | F(1, 63) = 15.87         |             |         |                                       |
| Two-way ANOVA (Fig. 2E)           | F(2, 40) = 5.866         |             |         |                                       |
|                                   | F(2, 40) = 19.53         |             |         |                                       |
|                                   | F(1, 40) = 9.969         |             |         |                                       |
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<sup>S209A</sup> mice, the peak of inflammation is not distinguishable from WT mice and at 24 h after CFA injection eIF4E<sup>S209A</sup> 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<sup>S209A</sup> 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
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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\(S^{209A}\) 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
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.

Conflict of interest

The authors declare no competing financial interests.

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