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eIF2alpha phosphorylation controls thermal nociception

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ABSTRACT

A response to environmental stress is critical to alleviate cellular injury and maintain cellular homeostasis. Eukaryotic initiation factor 2 (eIF2) is a key integrator of cellular stress responses and an important regulator of mRNA translation. Diverse stress signals lead to the phosphorylation of the α subunit of eIF2 (Ser51), resulting in inhibition of global protein synthesis, while promoting expression of proteins that mediate cell adaptation to stress. Here we report that eIF2 α is instrumental in the control of noxious heat sensation. Mice with decreased eIF2 α phosphorylation (*eIF2 α ^{+S51A}*) exhibit reduced responses to noxious heat. Pharmacological attenuation of eIF2 α phosphorylation decreases thermal, but not mechanical pain sensitivity, whereas increasing eIF2 α phosphorylation has the opposite effect on thermal nociception. The impact of eIF2 α phosphorylation (p-eIF2 α) on thermal thresholds is dependent on the transient receptor potential vanilloid 1 (TRPV1). Moreover, we show that induction of eIF2 α phosphorylation in primary sensory neurons in a chronic inflammation pain model contributes to thermal hypersensitivity. Our results demonstrate that the cellular stress response pathway, mediated via p-eIF2 α , represents a novel mechanism that could be utilized to alleviate pathological heat sensation.

SIGNIFICANCE STATEMENT

Distinct cellular stresses converge on the translation initiation factor, eIF2 α to modulate the rate of protein synthesis. Increased phosphorylation of eIF2 α has been described in peripheral neurons from neuropathic and diabetic rats. However, the role of eIF2 α phosphorylation in pain has not been reported. Here we show that phosphorylation of eIF2 α controls thermal, but not mechanical sensation, via modulation of the activity of a major heat transducer, TRPV1. We also

find that chronic inflammation-induced eIF2 α phosphorylation contributes to inflammation-induced thermal hypersensitivity. These results demonstrate that eIF2 α phosphorylation plays a major role in controlling noxious heat sensitivity.

INTRODUCTION

Response to stress is a major cellular function involved in many physiological and pathological conditions. Cells respond to various forms of stress by activating specific molecular cascades that orchestrate anti-stress responses or induce apoptosis (Holcik and Sonenberg, 2005; Walter and Ron, 2011). A key effector of cellular stress responses is the eukaryotic initiation factor 2 (eIF2) (Koromilas, 2015; Pavitt and Ron, 2012; Ron, 2002). Phosphorylation of eIF2 causes a reduction in global translation, allowing cells to conserve energy and modify gene expression to effectively manage stress conditions. Diverse stress signals converge onto eIF2 to integrate stress responses through phosphorylation of the α subunit of eIF2.

eIF2 binds GTP and the initiator methionyl-tRNA (Met-tRNA_i) to form the ternary complex (eIF2-GTP-Met-tRNA_i). The ternary complex binds the small ribosomal subunit to form the ribosomal pre-initiation complex, which scans the 5'UTR of the mRNA for the start codon to initiate mRNA translation (Hinnebusch et al., 2016; Walter and Ron, 2011). Upon engagement of the initiation codon GTP is hydrolyzed to GDP (Sonenberg and Hinnebusch, 2009). The recycling of inactive GDP-bound eIF2 to the active GTP-bound form is catalyzed by the guanine nucleotide exchange factor, eIF2B. Phosphorylation of the α subunit of eIF2 at serine 51 converts eIF2 from a substrate to a competitive inhibitor of eIF2B (Sonenberg and Hinnebusch, 2009). Since the amount of eIF2B is lower than eIF2, phosphorylation of a small fraction of the eIF2 in the cell is sufficient to strongly inhibit eIF2B activity and translation initiation.

eIF2 α is phosphorylated by four eIF2 α kinases, each activated in a different stress condition (Donnelly et al., 2013; Raven and Koromilas, 2008; Wek et al., 2006). PKR (double-stranded RNA-dependent protein kinase) is activated by double-stranded RNA during viral infection; PERK (PKR-like ER kinase) by endoplasmic reticulum stress; GCN2 (general control non-derepressible-2) by nutrient deprivation and ultraviolet light; and HRI (heme-regulated inhibitor) by heme deficiency. The eIF2 α kinases, except for HRI, are prominently expressed in the mammalian nervous system (Costa-Mattioli et al., 2009; Trinh and Klann, 2013).

Phosphorylation of eIF2 α blocks general translation, but paradoxically stimulates translation of mRNAs which contain upstream open reading frames (uORFs) in their 5'UTR, such as ATF4 (a cAMP-response element binding protein 2 (CREB-2)) and CHOP (a pro-apoptotic transcription factor) (Harding et al., 2000). ATF4 enhances the expression of a related transcription factor, ATF3, which together with ATF4 contribute to stress adaptation by regulating genes involved in metabolism, the cellular redox status, and apoptosis (Han et al., 2013; Horiguchi et al., 2012; Rutkowski and Kaufman, 2003). In neurons, an activity-dependent decrease in eIF2 α phosphorylation augments long-term potentiation (LTP) and memory via suppression of ATF4 expression (Costa-Mattioli et al., 2007; Costa-Mattioli et al., 2009). Conversely, upregulation of p-eIF2 α is associated with long-term depression (LTD) (Di Prisco et al., 2014; Trinh et al., 2014), and several pathophysiological conditions including viral infection, inflammation and neurodegeneration (Hetz and Mollereau, 2014; Koromilas, 2015; Ozcan et al., 2004; Wang and Kaufman, 2014). Elevated phosphorylation of eIF2 α has been documented in the brain of aged animals (Segev et al., 2013), and Alzheimer's disease patients and model mice (Chang et al., 2002; Kim et al., 2010; O'Connor et al., 2008). Normalization of p-eIF2 α in Alzheimer's disease model mice rescued deficits in protein synthesis, synaptic plasticity, and

spatial memory (Ma et al., 2013). Additionally, phosphorylation of eIF2 α is associated with synaptic deficits and neuronal loss in prion-disease model mice (Moreno et al., 2012).

The role of eIF2 in the pain pathway is unknown. An endoplasmic reticulum (ER) stress response is induced in the peripheral nervous system of type I diabetic rats, as phosphorylation of PERK and eIF2 α , along with other ER stress markers, is upregulated (Inceoglu et al., 2015). Induction of ER stress is accompanied by hypersensitivity, and attenuation of ER stress by an inhibitor of soluble epoxide hydrolase (sEH) downregulated ER stress markers, p-PERK, p-eIF2 α , and reversed mechanical hypersensitivity (Inceoglu et al., 2015). Despite these intriguing observations, a direct link between eIF2 α phosphorylation and nociception has not been established. Using a transgenic mouse model with reduced phosphorylation (by ~50%) of eIF2 α (eIF2 α ^{+S51A}), we show that p-eIF2 α controls thermal, but not mechanical sensitivity via the regulation of transient receptor potential vanilloid receptor 1 (TRPV1) activity. Moreover, we find that eIF2 α phosphorylation is induced in primary nociceptors in a chronic inflammation model, and that it contributes to inflammatory pain hypersensitivity.

RESULTS

p-eIF2 α is increased in dorsal root ganglia (DRGs) after chronic inflammation.

First, we examined the distribution of eIF2 α and its phosphorylated form, p-eIF2 α , in DRGs and spinal cord. Immunostaining revealed neuronal expression of eIF2 α and p-eIF2 α in peptidergic (CGRP-positive), non-peptidergic (IB4-positive), TRPV1-positive small diameter and NF200-positive large diameter neuronal cell bodies in DRGs (Figure 1A-B). Colocalization analysis showed that 19.7% of p-eIF2 α -positive cells express CGRP, 36.6% express IB4, 20.8% express TRPV1 and 47% express NF200 (Fig. 1B, bottom panel). In the dorsal horn of the spinal

cord, eIF2 α and p-eIF2 α were found in neurons, as they colocalized with the neuronal marker NeuN, but not with the astrocyte marker, GFAP (Figure S1). However, in the spinal cord p-eIF2 α signal was rather weak and detected only in a small fraction of NeuN-positive neurons (Figure S1).

To determine whether phosphorylation of eIF2 α is affected by chronic inflammation, we injected complete Freund's adjuvant (CFA) subcutaneously into the mouse hind paw (intraplantar injection) and measured the levels of p-eIF2 α in lumbar DRGs, and dorsal horn of the spinal cord. Levels of p-eIF2 α were increased in DRGs, but not in the spinal cord, 1 day after the onset of inflammation and decreased subsequently and returned to normal after 10 days (Figure 1C). The alterations in p-eIF2 α concurred with the inflammation-induced changes in thermal and mechanical thresholds (Figure 1D), raising the possibility that the increase in eIF2 α phosphorylation mediates the inflammatory hypersensitivity.

P-eIF2 α controls thermal sensitivity.

Having established that p-eIF2 α is increased in DRGs in response to chronic inflammation, we investigated its role in nociception. To this end, we used a transgenic knock-in (KI) mouse model (Scheuner et al., 2001), in which serine-51 is mutated to a non-phosphorylatable alanine residue in one allele (*eIF2 α ^{+S51A}*, homozygous KI mice are not viable), leading to a ~50% reduction in basal eIF2 α phosphorylation (Figure 2A). Mechanical sensitivity in the von Frey and tail-clip tests did not differ between *eIF2 α ^{+S51A}* mice and their wild-type (WT) littermates (Figure 2B). However, thermal withdrawal and nocifensive behavior latencies were significantly prolonged in *eIF2 α ^{+S51A}* mice compared to WT mice in the radiant heat paw-withdrawal, hot water tail-withdrawal, and hot-plate tests (40.2 \pm 9.7%, 44.0 \pm 13.1% and

21.6±6.6% increase, respectively, Figure 2C), indicating reduced sensitivity to noxious heat in *eIF2α^{+S51A}* mice. No difference in sensitivity to noxious cold was observed between WT and *eIF2α^{+S51A}* mice (Figure 2D). *eIF2α^{+S51A}* mice also exhibited reduced inflammatory pain in the formalin test. Nocifensive (licking/shaking) behaviour was significantly reduced (by 31.9±6.0%) in *eIF2α^{+S51A}* mice during the late/tonic phase (10-60 min post-formalin injection), as compared to WT mice, whereas no differences were found in the early/acute phase (0-10 min) between these groups (Figure 2E). The behavioral differences occurred despite equal degrees of paw edema in the two genotypes (Figure 2E). Taken together, these results demonstrate that p-eIF2α is upregulated in DRGs in response to chronic inflammation, and mice with reduced eIF2α phosphorylation exhibit decreased heat, but not, mechanical sensitivity.

As eIF2α is phosphorylated by four different kinases (Figure 3A), it was pertinent to study the effect of each kinase on p-eIF2α and thermal threshold. Since HRI expression is very low in the nervous system (Costa-Mattioli et al., 2009), we examined sensitivity to noxious heat in *Perk^{+/-}* (*Perk^{-/-}* exhibit severe postnatal growth retardation) (Zhang et al., 2002), *Pkr^{-/-}*, and *Gcn2^{-/-}* mice. *Perk* heterozygous had reduced p-eIF2α in DRGs and decreased noxious heat sensation (43.0 ±7.7 % increase in latency to withdrawal in the radiant heat paw-withdrawal test, Figure 3B). Mechanical thresholds in the von Frey test were not altered in *Perk^{+/-}* mice, similar to *eIF2α^{+S51A}* mice. *Gcn2^{-/-}* and *Pkr^{-/-}* mice did not display a significant reduction in p-eIF2α level and sensitivity to noxious heat (Figure 3C and D); however, double knockout mice for *Gcn2* and *Pkr* (*Gcn2/Pkr* DKO) exhibited reduced p-eIF2α levels (Figure 3C) and elevated thermal thresholds (Figure 3E). This finding suggests a redundant role for these two kinases.

Next, we examined whether modulation of eIF2α phosphorylation by drugs alters the thermal threshold. eIF2α phosphorylation was decreased by an inhibitor of eIF2α kinase, PKR

(PKRi) (Jammi et al., 2003). Intraperitoneal (i.p.) administration of PKRi over 3 days reduced noxious heat sensation in a dose-dependent manner, as indicated by the increased withdrawal latency in the radiant heat paw-withdrawal test (Figure 3F), with no effect on mechanical threshold. Conversely, when eIF2 α phosphorylation was increased by i.p. administration of Sal003, an inhibitor of the eIF2 α phosphatase complex, GADD34/PP1 (Growth Arrest and DNA-Damage-Inducible 34/Protein Phosphatase1) (Novoa et al., 2001; Robert et al., 2006) (see Figure 3A), thermal thresholds were decreased (Figure 3G, H), whereas mechanical thresholds were not affected. In summary, using genetic and pharmacological approaches, we show that decreasing eIF2 α phosphorylation reduces noxious heat sensation, whereas increasing p-eIF2 α levels engenders the opposite effect.

TRPV1 activity mediates the effect of reduced p-eIF2 α on thermal thresholds.

The strikingly specific impact of eIF2 α phosphorylation on noxious heat sensation suggests that mechanisms controlling heat transduction might be selectively controlled. TRPV1 channels transduce noxious heat, and are also implicated in inflammation-induced thermal hypersensitivity (Caterina and Julius, 2001; Caterina et al., 2000). TRPV1 activity is tightly regulated via gene expression and post-translational mechanisms (Planells-Cases et al., 2011). We examined the possibility that TRPV1 mediates the effect of eIF2 α phosphorylation on heat sensation by studying the impact of PKRi and Sal003 on thermal thresholds in *Trpv1*^{-/-} mice (Caterina et al., 2000). PKRi increased thermal threshold in WT mice, but not in *Trpv1*^{-/-} mice (Figure 4A). Conversely, Sal003 decreased thermal threshold in WT mice, but not in *Trpv1*^{-/-} mice (Figure 4B). These data demonstrate that eIF2 α phosphorylation controls thermal threshold in a TRPV1-dependent manner. To assess TRPV1 activity we recorded TRPV1-dependent

currents in sensory neurons from $eIF2\alpha^{+/S51A}$ and WT mice. Capsaicin, a specific TRPV1 agonist, elicited significantly smaller currents in dissociated DRG neurons prepared from $eIF2\alpha^{+/S51A}$ as compared to WT mice (92% decrease in $eIF2\alpha^{+/S51A}$ neurons; Figure 4C). For whole-cell recordings, small-diameter (<30 μm) neurons were selected, and only capsaicin-sensitive neurons (~30% of all tested neurons in WT and $eIF2\alpha^{+/S51A}$ groups) were included in the analysis. Resting membrane potential (V_{rest}), input resistance (R_{in}) and membrane capacitance (C_{m}) were not different between WT and $eIF2\alpha^{+/S51A}$ neurons (WT V_{rest} -49.74 ± 3.41 mV, $eIF2\alpha^{+/S51A}$ V_{rest} -46.79 ± 3.99 mV, $p=0.584$; WT R_{in} 686.21 ± 117.41 M Ω , $eIF2\alpha^{+/S51A}$ R_{in} 517.13 ± 38.41 M Ω , $p=0.193$; WT C_{m} 13.67 ± 2.18 pF, $eIF2\alpha^{+/S51A}$ C_{m} 14.68 ± 1.95 pF, $p=0.735$, $n=8/\text{genotype}$). Moreover, using calcium imaging we observed smaller capsaicin-induced calcium transients in cultured $eIF2\alpha^{+/S51A}$ DRG neurons as compared to WT neurons (Figure 4D). The cell body diameter of the responding neurons was not different between the two genotypes (WT 19.18 ± 0.45 μm , $n=53$, $eIF2\alpha^{+/S51A}$ 19.32 ± 0.39 μm , $n=88$, $p=0.81$). Consistent with these results, intraplantar subcutaneous administration of capsaicin induced significantly less nocifensive behaviour in $eIF2\alpha^{+/S51A}$, as compared to WT mice (Figure 4E). Conversely, mice with high p-eIF2 α levels, following Sal003 injections, exhibited increased nociceptive responses to capsaicin (Figure 4F). Despite the reduction in the amplitude of TRPV1-mediated currents in $eIF2\alpha^{+/S51A}$ neurons, Western blot analysis showed that protein levels of TRPV1 in cytosolic and membrane fractions of DRG lysates from $eIF2\alpha^{+/S51A}$ mice were not changed as compared to WT mice (Figure 4G). To examine whether trafficking of TRPV1 to the cell surface is affected by $eIF2\alpha$ phosphorylation, we used a surface biotinylation assay followed by Western blot analysis of TRPV1. We found no differences in TRPV1 amounts on the cell surface (Figure 4H), indicating that TRPV1 activity, but not protein levels or trafficking to the plasma

membrane, is reduced in *eIF2 α ^{+S51A}* neurons. Taken together, these data indicate that TRPV1 is an important mediator of the effect of p-eIF2 α on the thermal threshold, and suggest that TRPV1 activity is modulated by eIF2 α phosphorylation.

PKR-mediated eIF2 α phosphorylation contributes to thermal hypersensitivity.

After establishing that eIF2 α phosphorylation regulates thermal sensation, we studied whether CFA-induced increase in p-eIF2 α contributes to thermal hypersensitivity. eIF2 α kinase, PKR, has been implicated in inflammatory responses (Kang and Tang, 2012). Thus, we assessed PKR activation in DRGs following CFA injection and found that levels of p-PKR were significantly increased (Figure 5A), raising the possibility that the increase in p-eIF2 α is mediated via PKR activation. Consequently, we assessed thermal and mechanical hypersensitivity of *Pkr*^{-/-} mice after CFA-induced inflammation. *Pkr*^{-/-} mice exhibited reduced thermal, but not mechanical pain hypersensitivity after CFA injection (Figure 5B). Moreover, p-eIF2 α increased after CFA injection in DRGs of WT mice, but not in *Pkr*^{-/-} mice (Figure 5C). These data demonstrate that PKR is required for the upregulation of p-eIF2 α following CFA and contributes to thermal hyperalgesia.

Tumor necrosis factor-alpha (TNF- α) induces a robust upregulation of eIF2 α phosphorylation in cultured cells and in the nervous system via activation of PKR (Gilbert et al., 2004; Hu et al., 2007; Lourenco et al., 2013; Mantuano et al., 2011; Sharma et al., 2011). Since TNF- α is a major proinflammatory cytokine (Ohtori et al., 2004; Schafers et al., 2003; Uceyler et al., 2008; Zhang et al., 2011), which plays a critical role in the pathogenesis of inflammatory pain (Cunha et al., 1992; Xu et al., 2010), we examined whether inflammation-induced TNF- α promotes eIF2 α phosphorylation. First, we showed that TNF- α elevates p-eIF2 α in HEK293

cells, replicating previous studies (Figure 5D). Importantly, intrathecally-delivered TNF- α increased p-eIF2 α in DRGs of WT mice, but not *Pkr*^{-/-} mice (Figure 5E), supporting the idea that TNF- α stimulates p-eIF2 α via PKR. In accordance with previous reports, TNF- α (i.t.) induced heat hyperalgesia in WT mice (Zhang et al., 2011), whereas in *Pkr*^{-/-} mice this hyperalgesia was significantly attenuated (Figure 5F). Taken together, these results demonstrate that TNF- α - and PKR-dependent eIF2 α phosphorylation contributes to chronic inflammation-induced thermal hypersensitivity.

DISCUSSION

Here we describe a previously unrecognized role for the cellular stress response pathway in nociception. Transgenic mice with decreased eIF2 α phosphorylation (*eIF2 α* ^{+S51A}) exhibited reduced responses to noxious heat and attenuated nocifensive behaviour in the late phase of formalin test, whereas cold sensitivity and mechanical thresholds were not altered. The noxious heat-specific phenotype was recapitulated in transgenic mice in which the eIF2 α kinases PERK and PKR/GCN2 were reduced or knocked out, as well as in response to pharmacological manipulation of eIF2 α phosphorylation. Reducing eIF2 α phosphorylation with a PKR inhibitor attenuated noxious heat sensitivity, whereas increasing eIF2 α phosphorylation with Sal003 had the opposite effect. Our findings indicate that the effect of p-eIF2 α on thermal nociception is mediated via modulation of TRPV1 activity. First, we show that pharmacological modulation of eIF2 α phosphorylation altered noxious heat sensation in WT mice, but had no effect in mice lacking TRPV1. Second, capsaicin-induced TRPV1-mediated currents and pain behaviour were greatly reduced in *eIF2 α* ^{+S51A} mice. Taken together, these data demonstrate that the eIF2 α

pathway controls noxious heat sensation via TRPV1. Since we found no evidence of alterations in TRPV1 protein levels or trafficking to the membrane, we postulate that the mechanism by which eIF2 α phosphorylation affects thermal sensation involves modulation of TRPV1 activity. Under inflammatory conditions, TRPV1 can be sensitized by numerous inflammatory mediators (e.g. bradykinin, nerve growth factor, prostaglandins, serotonin, and histamine), via multiple agonists and modulators (protein kinase A (PKA), protein kinase C (PKC), metabolites of the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome-P450 (CYP)-pathways, phospholipids, protons, and heat, among others), leading to the reduction in the activation threshold and eventually to pain hypersensitivity (Morales-Lazaro et al., 2013; Sisignano et al., 2014). The modulators, which mediate the sensitization of TRPV1 activity by p-eIF2 α remain to be determined. eIF2 α phosphorylation promotes translation of ATF4 mRNA (Harding et al., 2000; Rutkowski and Kaufman, 2003). Interestingly, ATF4 is increased in DRGs following facet joint distraction (Dong et al., 2011). Moreover, ATF4 transcriptionally activates ATF3 expression, which is a well-known cellular marker of nerve injury (Braz and Basbaum, 2010). This suggests that some of the effects of p-eIF2 α on thermal thresholds could be mediated by ATF4/ATF3 axis.

We show that TNF- α induces p-eIF2 α in WT but not in *Pkr*^{-/-} mice, indicating that TNF- α upregulates p-eIF2 α via PKR. PKR is activated after CFA injection, suggesting that TNF- α -mediated activation of PKR contributes to p-eIF2 α upregulation and thermal hypersensitivity. Our results do not exclude the involvement of other eIF2 α kinases. For example, ER stress induces a robust PERK activation (Segev et al., 2013), which has a strong effect on eIF2 α phosphorylation and thermal sensitivity (Figure 3B).

A recent study found that hyperglycemia, activation of unfolded protein response (UPR), or dysregulation of calcium homeostasis induce ER stress in primary sensory neurons, as evident by the activation of PERK (and eIF2 α), inositol-requiring enzyme 1a (IRE1a), ATF6, MAPK (p38 and JNK), and autophagy (LC3) (Inceoglu et al., 2015). Whether the ER stress-induced mechanical pain is caused by elevated p-eIF2 α or through other mechanisms was not documented. Since elevated p-eIF2 α affects thermal, but not mechanical thresholds, it seems unlikely that the effects of ER stress on nociception are mediated via p-eIF2 α , but could be attributed to the activation of p38 and JNK or to other ER stress-dependent mechanisms. Increased p-eIF2 α was also documented in the sciatic nerve of rats with presumed neuropathic pain (Melemedjian et al., 2011), however the impact of this phosphorylation event on nociception has not been investigated.

Recent preclinical studies concluded that modulators of eIF2 α phosphorylation might have therapeutic potential in treatment of several cellular stress-related pathologies such as Alzheimer's disease (Fullwood et al., 2012; Ma et al., 2013), prion diseases (Moreno et al., 2012), diabetes (Back et al., 2009), Huntington's disease (Reijonen et al., 2008), and amyotrophic lateral sclerosis (Saxena et al., 2009). It will be important to consider the effect of eIF2 α phosphorylation on thermal nociception while developing clinically applicable compounds to the latter maladies.

In summary, we have uncovered a previously unknown role for the cellular stress response pathway in nociception. This knowledge can be used to develop strategies to treat conditions associated with altered heat sensation, most notably burn pain, and should be considered while introducing eIF2 α modulators to clinical practice.

EXPERIMENTAL PROCEDURES

Mice

eIF2 α ^{+S51A} mice were kindly provided by Dr. Kaufman (Sanford-Burnham Medical Research Institute) and were backcrossed for more than 10 generations to a C57BL/6J background. *Gcn2*^{-/-} mice, on a 129/SvEv genetic background and *Perk*^{+/-} mice on a mixed albino Swiss Webster;129/SvEv background were kindly provided by Dr. Ron (University of Cambridge). *Pkr*^{-/-} mice (Abraham et al., 1999) were backcrossed for at least eight generations to 129/SvEv mice. *Trpv1*^{-/-} mice on a C57BL/6J background were obtained from The Jackson Laboratory (B6.129X1-*Trpv1*^{tm1.Jul}/J). Pharmacological experiments were performed on naive, adult outbred CD-1[®] (ICR:Crl) mice. All behavioral experiments were performed on 7–12-week-old mice of both sexes by an experimenter blinded to genotype and drug. Food and water were provided *ad libitum*, and mice were kept on a 12:12 h light/dark cycle (lights on at 08:00 h). All procedures complied with Canadian Council on Animal Care guidelines, and were approved by McGill University's Downtown Animal Care Committee.

Complete Freund's Adjuvant (CFA)

Following baseline testing for mechanical (von Frey) and thermal sensitivity (radiant heat paw-withdrawal) (see below), mice were injected subcutaneously with CFA (50% in 20 μ l) into the left hind paw. Mice were tested every second day (days 1, 3, 5, 7, 9) starting 24 h post-injection for mechanical and thermal sensitivity.

Nociceptive Assays

Nociceptive assays are described in detail in Mogil et al. (2006). A brief description is as follows:

von Frey Test: Mice were placed individually in transparent Plexiglas cubicles (5 x 8.5 x 6 cm) set on a perforated steel floor and habituated for 1 h prior to testing. Nylon monofilaments (Stoelting #2-#9) were firmly applied to the plantar surface of each hindpaw for 0.5 s. The up-down method of Dixon (Chaplan et al., 1994) was used to estimate the 50% withdrawal threshold.

Radiant Heat Paw-Withdrawal Test: Mice were placed in cubicles (described above) on a glass floor and a focused beam of high-intensity light was aimed at the plantar surface of the hindpaw. The intensity of the light was set to 15% or 20% of maximum (IITC Model 390) with a cut-off value of 40 s. The latency to withdraw the hindpaw was measured to the nearest 0.1 s.

Hot-plate Test: Mice were placed into a clear Plexiglas cylinder atop a metal surface (Columbus Instruments) maintained at 50°C. The latency to lick or shake either hind paw was measured with a stopwatch to the nearest 0.1 sec. Only one measurement was made.

Tail Clip Test: A small alligator clip (force 700 g) was applied at 1 cm from the base of the tail. The latency to attack/bite the clip was measured with a stopwatch to the nearest 0.1 sec. Upon attack, the clip was removed and the animals were returned to their cages. Only one measurement was made.

Tail Withdrawal Test: Mice were lightly restrained inside a cloth/cardboard “pocket” with their tail maintained outside. The distal half of the tail was immersed into 47°C water and the latency

to vigorously withdraw the tail from the water was measured with a stopwatch to the nearest 0.1 sec. Two measures were taken at 30-min intervals for a total of eight measures per mouse (4 time points).

Cold Water Tail Immersion Test: For cold water tail-immersion/withdrawal test mice were lightly restrained inside a cloth/cardboard “pocket” and the distal half of their tail was immersed in ethanol maintained at -15 °C. The time to vigorous withdrawal of the tail was measured to the nearest 0.1 sec with a stopwatch. Each mouse was tested twice at 5-min intervals.

Formalin Test: Mice were placed into Plexiglas cylinders on a glass floor and habituated for at least 30 min. Following habituation, all mice were given intraplantar injections of formalin (4%) into the left hind paw and placed back into the cylinders. Cameras recorded the licking behavior over the next 60 min. Video files were sampled at 1-min intervals for the presence or absence of licking behavior in the first 10 sec of each interval. Data are expressed as the percent of positive (licking) samples. The early phase was defined as minutes 0-10 and the late phase as minutes 10-60 post-injection.

Drugs

PKR inhibitor (PKRi) and Sal003 were purchased from Calbiochem and dissolved in 30% polyethylene glycol in saline. Capsaicin was purchased from Sigma and dissolved in ethanol. TNF- α was purchased from Kamiya Biomedical Company (Seattle, WA USA).

DRG cultured neurons

Electrophysiological recordings and calcium imaging experiments were performed on dissociated dorsal root ganglion (DRG) neurons. Briefly, DRGs were isolated from 6-8-week-old WT and *eIF2 α ^{+S51A}* littermate mice and incubated in a solution containing dispase (1.37 mg/ml) and collagenase (1.08 mg/ml) for 45 min at 37 °C. After 2 washes in complete medium (Ham's F12 Nutrient Mixture, 10% fetal bovine serum, 2 mM L-Glutamine and 1% Pen-Strep), DRGs were mechanically dissociated using three fire-polished Pasteur pipettes with sequentially decreasing diameters. The cells were concentrated by centrifugation (3000 rpm for 1 min), resuspended in culture medium and plated on the glass bottom of the 35-mm dish pre-coated with a mixture of 100 μ g/ml poly-D-lysine and 10 μ g/ml laminin in Hanks' Balanced Salt Solution (HBSS). Calcium imaging experiments and patch clamp recordings of capsaicin-evoked responses were performed 18–48 h after cells plating.

Whole-cell recordings from DRG cultured neurons

Cells were patch-clamped in voltage clamp mode using glass pipettes containing a solution (pH 7.2) comprising (in mM): K-Gluconate 120, MgCl₂ 1, EGTA 10, HEPES 10, ATP 4, adjusted to 285 mOsmol/kg. Recordings were done in the whole cell mode using an Axopatch-200B amplifier (Axon Instruments Inc.). Membrane current (d.c. - 2 KHz) was digitized at 10 kHz via a Digidata 1322A interface coupled to a PC running Clampex 8 (Axon Instruments Inc.). Whole-cell capacitance and series resistance were compensated electronically and values of cell input capacitance (C_i) were noted. During recordings cells were perfused with HEPES solution consisting of 150 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose and mannitol to adjust the osmolality of the solution to 312 mosmol/kg. Capsaicin (1 μ M final concentration) was applied by rapidly switching the fluid delivery tube facing the cell using a

piezoelectric stepper device (SF-77B; Warner Instruments). DRG cultured neurons with a diameter smaller than 30 μm were used for recordings and about 30% of those showed responses to capsaicin. To measure input resistance (R_{in}) and membrane capacitance (C_{m}) a small square current pulse was injected into cells under the current clamp mode, and R_{in} and C_{m} were computed. Only cells that responded to capsaicin were included in the calculations.

Calcium imaging

Calcium imaging was performed using dual-wavelength fluorescent calcium indicator FURA-2AM (Invitrogen). Isolated DRG neurons were loaded with 1 μM FURA-2AM mixed with pluronic acid (Sigma) in serum free DMEM for 30 min at room temperature (RT), followed by a 10 min wash. Experiments were conducted at RT. Cells were visualized using Imaging workbench software (Photometrics, Tucson, AZ) connected to an Olympus IX71 microscope attached to a Lambda DG-4 fluorescence unit (Sutter) and fluorescence images were captured with a cooled CCD camera (Coolsnap HQ2 Photometrics, Tucson, AZ). Neurons were tested with 1 μM of capsaicin. At the end of each experiment, 50 mM KCl solution was applied to depolarize neurons, thereby allowing to distinguish viable neurons from non-neuronal cells or non-functioning neurons. No differences in KCl-induced responses were observed between *eIF2 α ^{+SS1A}* DRG neurons as compared to WT neurons. For analysis, only neurons with a diameter of less than 30 μm that responded to capsaicin were considered.

Western Blotting and Immunohistochemistry

Proteins were resolved on SDS-polyacrylamide gels using standard techniques. See Extended Experimental Procedures for details of the experimental procedures and antibodies used.

To separate the membrane and cytosolic fractions DRGs were homogenized in ice-cold homogenization buffer containing (in mM): 30 Tris-HCl, pH 7.4; 1 EGTA; 0.1 Na₃VO₄; 10 NaF; and protease inhibitor cocktail (complete, EDTA-free, Roche Applied Science), as described (Gong et al., 2014). Following centrifugation at 23,000 × g for 10 min the supernatant, containing the cytosolic fraction, was collected. Pellets were re-suspended in the homogenization buffer containing 1% triton. Following sonication (20 pulses of 5 seconds) and centrifugation at 23,000 × g for 10 min at 4°C, the supernatant, containing the membrane fraction, was collected. Proteins in the membrane fraction were normalized to N-cadherin (1:1000, Cat#610920, BD Biosciences).

Cell surface biotinylation assay

DRG neurons, cultured for 24 h, were subjected to cell surface biotinylation using Cell Surface Protein Isolation Kit (Pierce, Cat#89881), according to the manufacturer's instructions. Briefly, cells were washed with PBS and biotinylated with Sulfo-NHS-SS-Biotin [sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] in PBS for 30 min at 4°C. After quenching, cells were lysed and labeled proteins were then isolated by incubating the lysate with NeutrAvidin™ Agarose beads for 60 min at room temperature. After washing, proteins were eluted by heating the beads for 5 min. at 95°C in SDS-PAGE Sample Buffer containing 50mM DTT. The amount of the TRPV1 was analyzed by Western blot with an anti-TRPV1 antibody (Alomone, Cat #: ACC-030).

Statistical Analyses

All results are expressed as mean \pm SEM. All statistical comparisons were made with either the Student's *t*-test or a one-way ANOVA followed by between-group comparisons using Bonferroni post-hoc test, unless otherwise indicated, with $p < 0.05$ as the significance criterion. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Power analyses were not possible since we had no *a priori* expectation of effect size, but rather were informed by normative practices in the pain field (Mogil et al., 2006).

SUPPLEMENTAL INFORMATION

Supplemental information includes Extended Experimental Procedures and one figure.

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FIGURE LEGENDS

Figure 1. eIF2 α is expressed in DRG neurons and its phosphorylation is increased in an inflammatory pain model.

The distribution of total and p-eIF2 α in mouse lumbar dorsal root ganglia was examined using immunostaining. Total (A) and p-eIF2 α (B) were co-stained with calcitonin gene-related peptide (CGRP), IB4, TRPV1, and NF200. Percent of p-eIF2 α -positive neurons expressing the markers is shown in B, bottom panel. (C) Mice were injected (intraplantar) with complete Freund's adjuvant (CFA), and levels of eIF2 α phosphorylation were measured in DRGs at different time points post injection using Western blot analysis (n=4 mice/condition). (D) CFA induces thermal (left) and mechanical (right) hypersensitivity as assessed in radiant heat paw withdrawal and von Frey assays, respectively (n=5 males and 4 females/assay). Data are presented as mean \pm SEM. *p < 0.05, **p < 0.01 by Bonferroni post-hoc test following one-way ANOVA. Scale bar: 100 μ m. For distribution of eIF2 α and p-eIF2 α in the spinal cord see Figure S1.

Figure 2. Noxious heat sensation is reduced in *eIF2α*^{+S51A} mice.

(A) eIF2α phosphorylation is reduced in DRGs and spinal cord of *eIF2α*^{+S51A} mice. *eIF2α*^{+S51A} mice demonstrate no alterations in mechanical sensitivity (B, n=4 males and 4 females/genotype, p>0.05), whereas noxious heat sensation is significantly attenuated (C, n=4 males and 4 females/genotype/assay, p<0.05). (D) Sensitivity to cold is not changed in *eIF2α*^{+S51A} mice (n=4 males and 6 females/genotype). (E) Nocifensive (licking/shaking) behaviour is significantly reduced in formalin test during the late/tonic phase (10-60 min post-formalin injection, p<0.05), whereas no differences are found in the early/acute phase (n=4 males and 4 females/genotype). Changes in paw weight, indicative of formalin-induced inflammation, are not different in *eIF2α*^{+S51A} mice (E, right panel, p>0.05). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ns - not significant by Student's *t*-test.

Figure 3. eIF2α kinases control thermal threshold.

(A) eIF2α kinases and phosphatase GADD34/PP1 (Growth arrest and DNA-damage-inducible 34/protein phosphatase1). (B) *Perk*^{+/-} mice show a decrease in p-eIF2α (n=4 mice/genotype, p<0.05) and in noxious heat sensitivity (n=4 males and 4 females/genotype, p<0.001). *Gcn2*^{-/-} and *Pkr*^{-/-} mice show no alteration in thermal latencies (C and D, respectively, n=4 males and 4 females/genotype, p>0.05). (E) *Gcn2*^{-/-} *Pkr*^{-/-} double knockout mice show reduced noxious heat sensitivity (n=3 males and 4 females/genotype, p<0.05). eIF2α phosphorylation in DRGs of *Gcn2*^{-/-}, *Pkr*^{-/-}, and *Gcn2*^{-/-} *Pkr*^{-/-} double knockout mice is shown at C (n=3 mice/genotype). PKR inhibitor (PKRi) (F) and Sal003 (G) were injected intraperitoneally (i.p.) for 3 days daily, and thermal and mechanical thresholds were measured 30 min after the last injection (n=4 males and

4 females/condition). (H) eIF2 α phosphorylation in DRGs of mice injected with PKRi (1 mg/kg) and Sal003 (1 mg/kg) is shown (n=5 mice/drug). Data are presented as mean \pm SEM. *p < 0.05. **p < 0.01, ***p < 0.001, ns - not significant by Student's *t*-test and Bonferroni post-hoc test following one-way ANOVA.

Figure 4. eIF2 α phosphorylation regulates thermal threshold via TRPV1.

Modulation of eIF2 α phosphorylation in *Trpv1*^{-/-} mice does not alter heat sensation. PKRi (1 mg/kg for 3 days daily, i.p.) elevates thermal threshold in WT but not in *Trpv1*^{-/-} mice (A, n=4 males and 4 females/genotype-drug condition). Sal003 (1 mg/kg for 3 days daily, i.p.) decreases thermal threshold in WT but not in *Trpv1*^{-/-} mice (B, n=4 males and 4 females/genotype-drug condition). For radiant heat paw withdrawal test the light beam was set to 20% of the maximal intensity in A and to 15% in B. Capsaicin (1 μ M) evokes smaller currents (C, n=12 cells for *eIF2 α* ^{+S51A} and 10 for WT, from 3 different neuronal cultures per genotype) and smaller calcium transients (D, n=72 cells for *eIF2 α* ^{+S51A} and n=46 cells for WT, from 4 different neuronal cultures per genotype, using Fura-2 340/380 nm ratio) in cultured DRG neurons prepared from *eIF2 α* ^{+S51A} as compared to WT mice. Capsaicin (2.5 μ g), injected subcutaneously into the plantar surface of the hindpaw, elicited less nocifensive behaviours in *eIF2 α* ^{+S51A} as compared to WT mice (E, n=4 males and 4 females/genotype or drug), whereas in mice injected with Sal003 (1 mg/kg for 3 days daily, i.p.) capsaicin-induced pain behaviour is increased (F). Western blot analysis shows that the TRPV1 protein levels are not altered in membrane and cytosolic fractions as well as in total lysates of DRGs of *eIF2 α* ^{+S51A} mice (G, n=4 mice/genotype). TRPV1 surface levels were measured in DRG cultured neurons prepared from *eIF2 α* ^{+S51A} and WT mice using surface biotinylation assay (H, n=3/group). Data are presented as mean \pm SEM. *p < 0.05. **p <

0.01. *** $p < 0.001$ by Student's *t*-test and Student's *t*-test following two-way (genotype x drug) ANOVA.

Figure 5. TNF- α mediated PKR activation elevates p-eIF2 α .

(A) Phosphorylation of PKR (Thr451) was measured at different time points after CFA injection (n=4 mice). (B) CFA-induced thermal hyperalgesia is attenuated in *Pkr*^{-/-} mice as compared to WT mice, whereas mechanical hyperalgesia is not changed (n=4 males and 4 females/genotype). (C) P-eIF2 α in DRGs was measured at different time points after CFA injection in WT and *Pkr*^{-/-} mice. (D) HEK293 cells were treated with TNF- α (100 ng/ml) for 15, 30 and 60 min. and p-eIF2 α was measured (n=3, $p < 0.05$). (E) TNF- α (20 ng in 5 μ l of PBS+0.5% BSA) was injected i.t. 3 times every 3 h. in WT and *Pkr*^{-/-} mice, and the p-eIF2 α was measured in lumbar DRGs 30 min. after the last injection. TNF- α induced an increase in p-eIF2 α in WT but not in *Pkr*^{-/-} mice (n=5 females/genotype/drug). (F) TNF- α (20 ng, i.t., 3 injections at 3 h intervals) elicited bigger thermal hyperalgesia in WT mice, as compared to *Pkr*^{-/-} mice (n=4 males and 4 females/genotype/drug, $p < 0.05$). Data are presented as mean \pm SEM. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$ by Bonferroni post-hoc test following one-way ANOVA and Student's *t*-test following two-way (genotype x drug) ANOVA.