TRANSLATION REGULATION IN CENTRAL AND PERIPHERAL

PAIN PLASTICITY

by

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For my parents, Jack and Chau lan Moy, who have worked hard to give me everything I ever wanted and always encouraged me to do what makes me happy and to work hard to hard to get it. Thank you for all that you have done for me.

TRANSLATION REGULATION IN CENTRAL AND PERIPHERAL PAIN PLASTICITY

by

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TRANSLATION REGULATION IN CENTRAL AND PERIPHERAL

PAIN PLASTICITY

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Pain under normal circumstances serves as a protective mechanism allowing recovery and survival. However, when pain becomes chronic, it no longer provides preservation but rather debilitation. Chronic pain is an important medical problem affecting greater than 33% of the population in America and current treatment options are only effective in a small percentage of the population and often accompanied with side effects. Determining the underlying molecular mechanisms of the maintenance and transition to chronic pain would lead us to developing new therapeutic treatments. Many studies suggest that translational control of gene expression is a key process for the regulation of plasticity in the nervous system. Multiple lines of evidence indicate that translation control plays a critical role in pathological pain plasticity, but precise gene targets have thus far not been elucidated. Brain-derived neurotrophic factor (BDNF) signaling through its cognate receptor, TrkB, is a well-known promoter of synaptic plasticity at nociceptive synapses in the dorsal horn of the spinal cord. However, the downstream effects contributing to the maintenance of a chronic pain state remain elusive. The goal of our research is to further understand the biochemical mechanisms driving chronic pain using pharmacological

techniques and transgenic mice. Our work provides novel insight into mechanisms driving pain chronification and defines MNK regulation of eIF4E phosphorylation as a key target for pain therapeutics.

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CHAPTER 1

GENERAL INTRODUCTION

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Chronic pain is a debilitating disease affecting 100 million Americans. Understanding the underlying mechanisms of chronic pain and the transition to a chronic pain state is crucial in developing new treatments. While it is widely accepted that both the peripheral (Reichling and Levine, 2009a) and central (Latremoliere and Woolf, 2009; Woolf, 2011) nervous systems undergo plastic changes influencing the development of chronic pain, we are only beginning to gain molecular insight into this problem. Here, we will review different molecular mechanisms involved in pain plasticity in both the central and peripheral nervous systems.

CENTRALIZED PAIN PLASTICITY

For decades, central pain plasticity in the dorsal horn of the spinal cord was commonly compared to long-term potentiation (LTP) in the hippocampus and other learning- and memory-associated brain regions. This view is now effectively outdated, as not all mechanisms directly translate from one process to the other, despite their similarities. For example, it has been shown that high-frequency stimulation is necessary to induce LTP in the hippocampus (Figurov et al., 1996), while Ikeda and colleagues found low-frequency stimulation of c-fibers is involved in central pain plasticity via the spinal dorsal horn (Ikeda et al., 2006). This does not translate to LTP in the hippocampus but rather long-term depression (LTD) (Chen et al., 1995; Huerta and Lisman, 1996)**.** Nevertheless, this paradigm has been influential in guiding our thinking and making comparisons to identify several memory-like mechanisms responsible for the development of pain plasticity via the dorsal horn of the spinal cord.

UTILIZING LTP MECHANISMS TO ELUCIDATE THE DEVELOPMENT OF PAIN PLASTICITY

LTP is split into two different phases: an early and a late or maintenance phase. Mechanisms involved in early-LTP require phosphorylation of AMPA receptors by CaMKIIα, PKA, and conventional PKCs (Huganir and Nicoll, 2013). This leads to changes in gene expression, ultimately causing consolidation of early-LTP to late-LTP (Abrahamsen et al., 2008a). A pain model that draws similarities to the phases of LTP was developed by Jon Levine in the early 2000s termed, "hyperalgesic priming". The model dissects nociceptor plasticity into two phases: an initiation state and a late or "primed" state (Reichling and Levine, 2009a). Since then, hyperalgesic priming has been applied to central pain processes in the spinal cord and can be promoted by algogens through peripheral nociceptor stimulation (Ikeda et al., 2006). This example of spinal plasticity is most closely related to late-LTP mechanisms in the hippocampus and other cortical regions (Ikeda et al., 2006; Drdla et al., 2009; Ruscheweyh et al., 2011b; Drdla-Schutting et al., 2012). Very few studies have described mechanisms underlying the maintenance of late-LTP, but are believed to involve the activity of PKMζ, a truncated isoform of PKCζ (an atypical PKC (aPKC) (Sacktor, 2011b). PKCs are divided into 3 subclasses based on their perspective activators: conventional or classic (α, βI, βII, and γ), novel (δ, ε, θ, and η), and atypical (ζ and ι/λ (ι in human, λ in mouse)). Conventional PKCs (cPKCs) require calcium (Ca^{2+}), diacylglycerol (DAG), and phospholipids. As mentioned above, conventional PKCs are required for early-LTP (Huganir and Nicoll, 2013). Like cPKCs, novel PKCs (nPKCs) require DAG, but do not need Ca^{2+} for activation. Pain researchers have focused on one particular

nPKC, PKCε. PKCε is responsible for the development of hyperalgesic priming (Aley et al., 2000; Parada et al., 2003a; Joseph et al., 2010; Bogen et al., 2012b). This mechanism has been shown to be restricted to a subset of small diameter c-fibers called, isolectin B_4-positive (IB_4 +) neurons (Aley et al., 2000; Parada et al., 2003b; Joseph et al., 2010; Bogen et al., 2012b). Levine and colleagues have shown that the transition of acute to chronic pain caused by inflammatory mediators is dependent on PKCε (Aley et al., 2000). Priming was prevented by PKCε inhibitors and prolonged hypersensitivity was triggered by specific PKCε activators (Aley et al., 2000). While it is evident that PKCε plays a role in establishing a chronic pain state, inhibiting PKCε also terminates the mechanism responsible for the "primed" state caused by inflammatory mediator, carrageenan (Parada et al., 2003b). A recent study showed that inhibiting conventional and novel PKCs with staurosporine after a "primed" state is established does not reverse hyperalgesic priming (Asiedu et al., 2011c). Unlike cPKCs and nPKCs, atypical PKCs (aPKCs) do not require Ca^{2+} or DAG second messenger signaling, but are regulated by protein-protein interactions. With this mounting body of literature, it has become evident that PKC signaling has 2 points of contention: 1. The role of aPKCs in a chronic pain state, and 2. The site of action responsible in mediating pain plasticity.

APKCS IN CENTRAL PLASTICITY

Researchers developed what was originally thought as a specific inhibitor of PKMζ, called ζ-inhibitory peptide (ZIP), and discovered that ZIP reversed late-LTP, suggesting that PKMζ is responsible for the maintenance of LTP (Sacktor, 2011b; Sacktor and Fenton, 2012; Sacktor et al., 2012). The specificity of ZIP and role of PKMζ came into question when PKMζ- \

PKCζ-null mice (*prckz)* developed normal late-LTP (Lee et al., 2013b; Volk et al., 2013a). The genetic deletion of *prckz* prevents expression of both PKMζ and PKCζ isoforms. PKMζ is largely recognized to be involved in late-LTP mechanisms versus PKCζ because of its expression in the nervous system versus non-neuronal tissues (Hernandez et al., 2003; Lee et al., 2013b). Interestingly, a recent study showed how these *prckz*-null mice develop a compensatory mechanism for late-LTP that relies on a different aPKC, PKCλ (Tsokas et al., 2016), which is also inhibited by ZIP (Melemedjian et al., 2013b; Volk et al., 2013a; Tsokas et al., 2016). Despite the controversy around these targets, our research has shown that intrathecal ZIP, at the time of or during initiation blocks the development of hyperalgesic priming (Asiedu et al., 2011c; Melemedjian et al., 2013b). Moreover, intrathecal ZIP reverses the established primed state (Asiedu et al., 2011c; Laferriere et al., 2011a; Melemedjian et al., 2013b), similar to its effect on late-LTP (Pastalkova et al., 2006; Sacktor, 2011b). Another important consideration is the mechanism responsible for linking the early- to late- phase of LTP or hyperalgesic priming in pain plasticity. The discovery that brain-derived neurotrophic factor (BDNF) linked the early- to late- phase of LTP in memory-like mechanisms (Lee, 2005) and is also involved in the development of central pain plasticity (Latremoliere, 2009). This represented a breakthrough in how pain neurobiologists approached central pain plasticity and the development of chronic pain.

BDNF

BDNF is a neurotrophin and neurotransmitter that plays a role in plasticity throughout the CNS, including in the dorsal horn of the spinal cord (Lu et al., 2008b) and administration of exogenous BDNF induces central pain plasticity (Zhou et al., 2008a) and hyperalgesic priming

(Melemedjian et al., 2013b). BDNF is released by a subset of cells into the nociceptive synapses in the dorsal horn of the spinal cord and act on pre- and/or postsynaptic tyrosine receptor kinase type B (TrkB) (Zhao et al., 2006a; Zhou et al., 2008a; Melemedjian et al., 2013a) (Figure 1.1) to induce hypersensitivity. The release of BDNF into the nociceptive synapses in the dorsal horn of the spinal cord triggers the translation and phosphorylation of aPKCs, PKMζ and PKCλ (Melemedjian et al., 2013a) **(**Figure 1.1). Both isoforms have been implicated in playing crucial roles for the maintenance of a chronic pain state (Asiedu et al., 2011c). In fact, intrathecal administration of ZIP reverses the primed state caused by BDNF (Melemedjian et al., 2013a), therefore linking downstream signaling of BDNF to aPKCs at central synapses.

BDNF expression is increased following nerve growth factor (NGF) exposure or injury (Kerr et al., 1999a; Mannion et al., 1999). Recent work demonstrated that BDNF/TrkB signaling is required not only for the induction, but also the maintenance of (long-term) pain plasticity in hyperalgesic priming models (Melemedjian et al., 2013b; Price and Ghosh, 2013a; Tillu et al., 2015a). Additionally, blocking BDNF/TrkB signaling after priming, reverses the established pain state (Melemedjian et al., 2013b; Tillu et al., 2015a; Burgos-Vega et al., 2016). However, the source of BDNF release in response to injury has remained controversial. Researchers have shown that BDNF release from microglia contributes to neuropathic pain (Coull et al., 2005b; Sorge et al., 2015), whereas others have shown BDNF deriving from small-diameter, NaV1.8-positive neurons are responsible for acute and inflammatory pain (Zhao et al., 2006a) (Figure 1.1). In an interesting aside, these studies have also reported a sexual dimorphic role for BDNF.

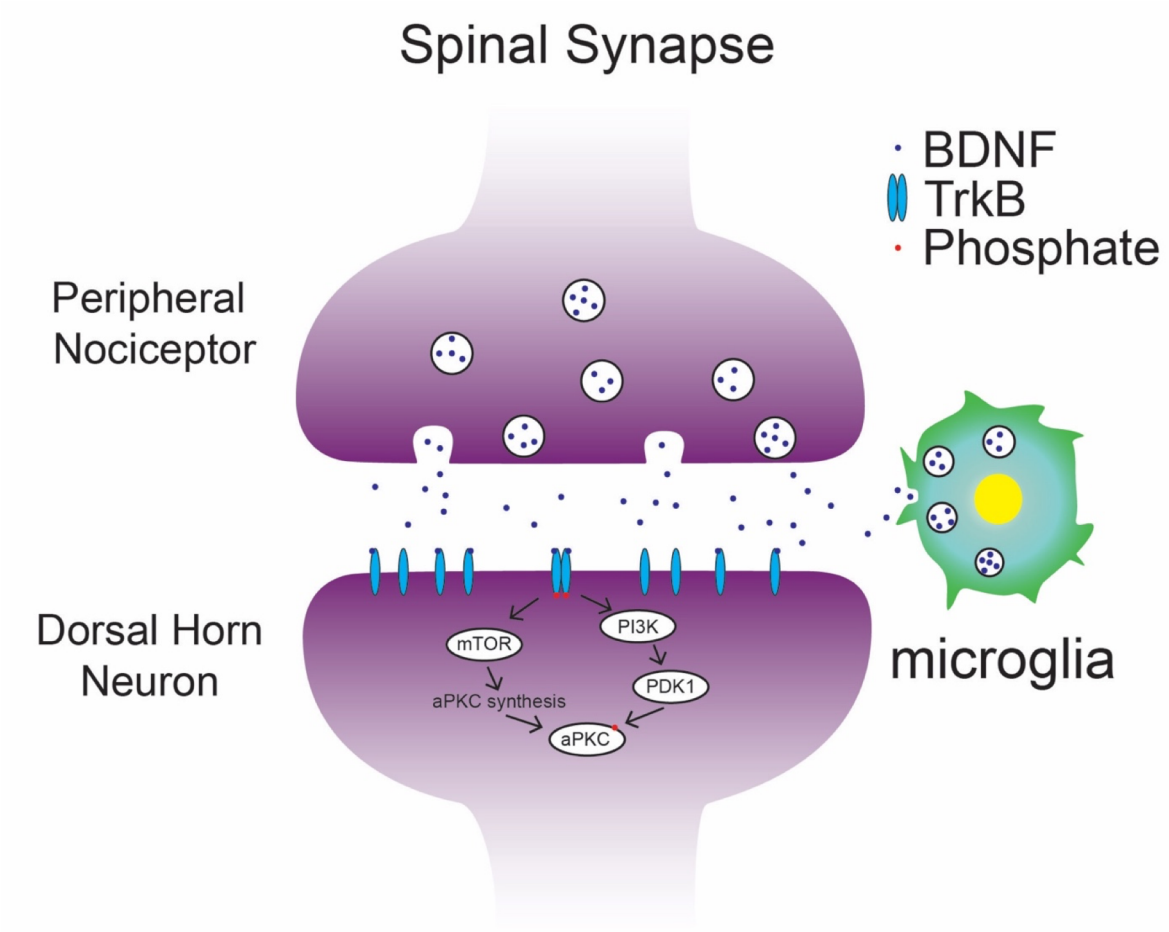

Figure 1.1. Spinal synapse for the induction of hyperalgesic priming. BDNF is released into the spinal synapse from either activated nociceptor terminals or microglia. BDNF then binds to its postsynaptic receptor, TrkB, to induce mTOR-mediated translation of aPKCs. Simultaneously, TrkB activation also triggers the PI3K cascade leading to phosphorylation of the newly synthesized aPKCs by PDK1. The downstream effects of BDNF are believed to be responsible for the initiation of the "primed" state.

In Zhao *et. al.,* it is reported that BDNF released from small-diameter, NaV1.8- positive neurons are required for acute mechanical hypersensitivity in female mice. On the other hand, Sorge *et. al.*, showed that sequestering BDNF with TrkB-fc at central synapses is only effective in male mice in neuropathic pain.

While the cell types responsible for BDNF release remain controversial, the question of

which cell type contains the machinery to create the protein and levels of activation are much less argued. BDNF protein levels are measureable in microglia and nociceptors; however activity-dependent gene expression analysis has only recently become available (Flavell and Greenberg, 2008). Moreover, the level of BDNF transcript only tells part of the story (Baj et al., 2011).

IS MRNA TRANSLATION IMPORTANT FOR THE DEVELOPMENT OF PAIN PLASTICITY?

A large focus has been on gene expression and transcriptional changes that lead to phenotypic and excitability (Woolf and Costigan, 1999; Ji and Woolf, 2001) alterations, as well as single-cell and whole population discrimination of RNA profiling experiments after injury (Chiu et al., 2014; Usoskin et al., 2015). However, recent studies point to translation regulation as an instrumental player in pathological pain plasticity (Weragoda et al., 2004; Price et al., 2007a; Price and Géranton, 2009a; Melemedjian et al., 2010b; Asiedu et al., 2011c; Melemedjian et al., 2011b; Bogen et al., 2012b; Tillu et al., 2012b; Ferrari et al., 2013a; Tillu et al., 2015a).

A well-known example of translation regulation is fragile X mental retardation protein (FMRP). FMRP is a RNA-binding protein that silences (Bassell and Warren, 2008) and traffics (Estes et al., 2008) mRNAs to synaptic sites (Bagni and Greenough, 2005). Absence of this protein leads to mental retardation in humans, and an inability to create synaptic plasticity in FMRP KO mice (Huber et al., 2002; Li et al., 2002). Moreover, while FMRP KO mice display normal pain thresholds, they fail to process induced pain caused by irritants and exhibit deficiencies in neuropathic pain (Price et al., 2007a). FMRP has been shown to play a role downstream of metabotropic glutamate receptors (mGLUR) 1 and 5 activation and mTOR

signaling in both central and peripheral nervous system (Todd et al., 2003; Hou et al., 2006; Muddashetty et al., 2007; Price et al., 2007a).

Recently, it was discovered that several FMRP-binding mRNAs rely on a kinase (MNK) that regulates protein synthesis at the 5' end (Genheden et al., 2015).

YES! THE PROCESS OF MRNA TRANSLATION DOES PLAY A KEY ROLE IN PAIN PLASTICITY

The process of protein synthesis is divided into three steps: initiation, elongation, and termination. Initiation is the rate-limiting step of translational control and will be our primary focus. Initiation is regulated in part, by a group of proteins that bind to the 5'm^7GTP (cap) structure of the mRNA, otherwise known as eukaryotic initiation factor (eIF) 4F (eIF4F) or cap complex. The mRNA 5' cap binds to eIF4F, and consists of three proteins: eIF4G (a scaffolding protein), eIF4E (the cap binding protein), and eIF4A (an RNA helicase that unwinds secondary structures). A key translational regulator, eIF4E binding protein 1/2 (4E-BP1/2) directly interacts with eIF4E to repress interaction with eIF4G. When mechanistic target of rapamycin (mTOR) is activated by a stimulus such as NGF binding to TrkA receptors, mTOR hyperphosphorylates 4E-BP1/2, releasing it from eIF4E, allowing eIF4E (Yanagiya et al., 2012) to bind to eIF4G and eIF4A (Figure 1.2) and the initiation of translation.

Activation of mTOR by noxious stimuli and/or pain promoting endogenous molecules has repeatedly been shown to upregulate mRNA translation, ultimately sensitizing peripheral

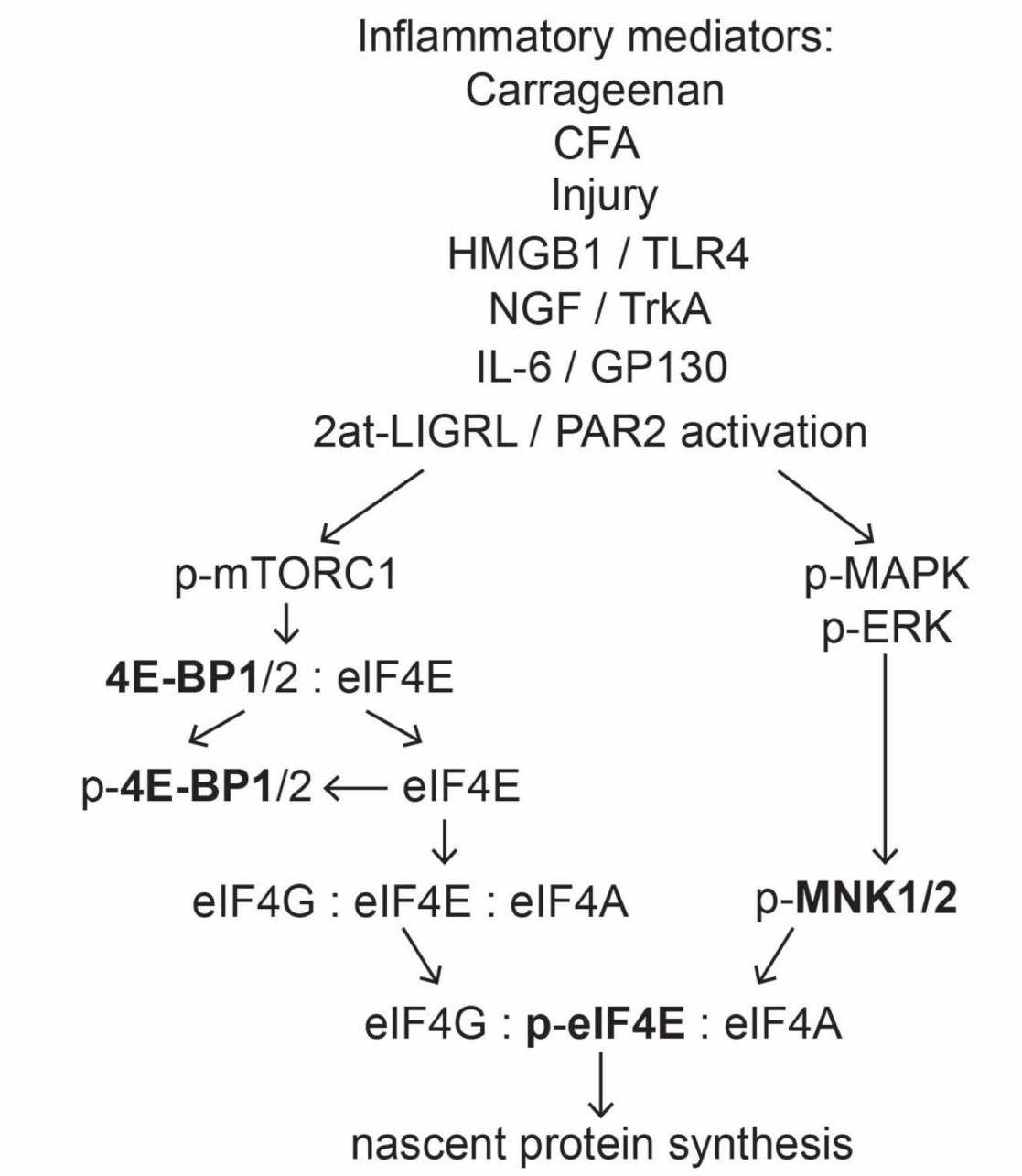

Figure 1.2. Kinase cascades activated by noxious stimuli. Inflammatory mediators and injury induces two main translation regulation pathways mTOR and ERK. Both of these signaling cascades converge onto the eIF4F (cap) complex to induce nascent protein synthesis.

nociceptors and spinal synapses (Jiménez-Díaz et al., 2008; Price and Géranton, 2009a; Melemedjian et al., 2010c; Bogen et al., 2012b; Obara et al., 2012b; Ferrari et al., 2013a). mTOR

is the active subunit of protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Lipton and Sahin, 2014). While both protein complexes contain mTOR, they have very distinctive roles. mTORC1 is responsible for regulating mRNA translation, and mTORC2 is responsible for controlling actin cytoskeleton dynamics (Jacinto et al., 2004; Sarbassov et al., 2004). mTORC1 is rapamycin sensitive, meaning that mTORC1 activity is inhibited by rapamycin, whereas mTORC2 is not. Several studies have shown that inhibiting mTORC1 activity through either systemic or intrathecal administration of rapamycin reduces hypersensitive behaviors in several pain models (Price et al., 2007a; Asante et al., 2009a; Price and Géranton, 2009b; Jiang et al., 2013) including neuropathic pain (Asante et al., 2010a; Zhang et al., 2013a; Zhang et al., 2013b; Cui et al., 2014). mTORC1 regulates translation through p70S6 kinases (S6Ks), and as mentioned above, 4E-BPs. Previous views of translation regulation suggested that the components of the eIF4F/4E-BP complex worked in concert to regulate translation as a single unit. This view has now been supplanted by extensive evidence suggesting that individual components of the eIF4F/4E-BP complex act alone to control translation of subsets of mRNAs (Sonenberg and Hinnebusch, 2009b; Khoutorsky et al., 2015a). Direct phosphorylation of eIF4E by its upstream kinase MNK is thought to enhance cap-dependent translation **(**Figure 1.2**)**. More importantly, both mTOR and extracellular regulated kinase (ERK) signaling converge on eIF4F creating an additional step to initiate mRNA translation (Banko et al., 2006; Gelinas et al., 2007; Tsokas et al., 2007; Melemedjian et al., 2010c). Recent evidence in dorsal root ganglion (DRG) neurons, showed factors inducing inflammation and pain can enhance eIF4F-dependent translation (Melemedjian et al., 2010b; Tillu et al., 2012b). Much of this work has expanded to characterize different translational pathways and their involvement in

pain phenotypic changes (Price et al., 2006; Price and Geranton, 2009; Melemedjian et al., 2010a; Tillu et al., 2012a; Melemedjian et al., 2013e; Melemedjian et al., 2014a; Khoutorsky et al., 2015b; Khoutorsky et al., 2016c) **(**Figure 1.3**)**.

In fact, a recent study demonstrates how the absence of 4E-BP1 promotes central pain plasticity (Khoutorsky et al., 2015a). Khoutorsky and colleagues investigated one of the downstream targets of mTORC1, 4E-BP1, in the context of pain plasticity. 4E-BP exists as three isoforms. 4E-BP3 expression is absent in the nervous system while 4E-BP1 and 4E-BP2 are both expressed in neuronal tissues (Banko et al., 2006; Jiménez-Díaz et al., 2008; Melemedjian et al., 2011b; Cao et al., 2015). First, the authors assessed mechanical and thermal sensitivity in both *Eife4ebp1*$^{-/-}$ and *Eife4ebp2*$^{-/-}$ mice. They saw no changes in *Eife4ebp2*$^{-/-}$ mice, but in *Eife4ebp1*$^{-/-}$ mice found a significant increase in mechanical sensitivity whereas, thermal sensitivity was unaffected by this knock-out. When nociceptive behaviors were induced with chemical irritants, *Eife4ebp1*$^{-/-}$ mice exhibited higher levels of nocifensive behaviors and higher levels of neuronal activity in the dorsal horn of spinal cord compared to their WT littermates. Moreover, intrathecal administration of an eIF4F-complex inhibitor, 4EGI-1, reversed the mechanical hypersensitivity and normalized the nocifensive behavior induced by formalin in the *Eife4ebp1*$^{-/-}$ mice. This data suggests that mRNA translation regulated by 4E-BP1 in the spinal dorsal horn plays an important role in central pain plasticity. Further, biochemical studies measuring translation rates with a puromycin incorporation assay showed no differences in overall translation in *Eife4ebp1*$^{-/-}$ mice compared to WT. This suggested that 4E-BP1 governs translation of a subset of mRNAs in the spinal dorsal horn and one of these mRNAs is neuroligin-1 (Nlgn1) (Tahmasebi et al., 2014). These data illuminate how different components of mRNA translation, originally thought to act

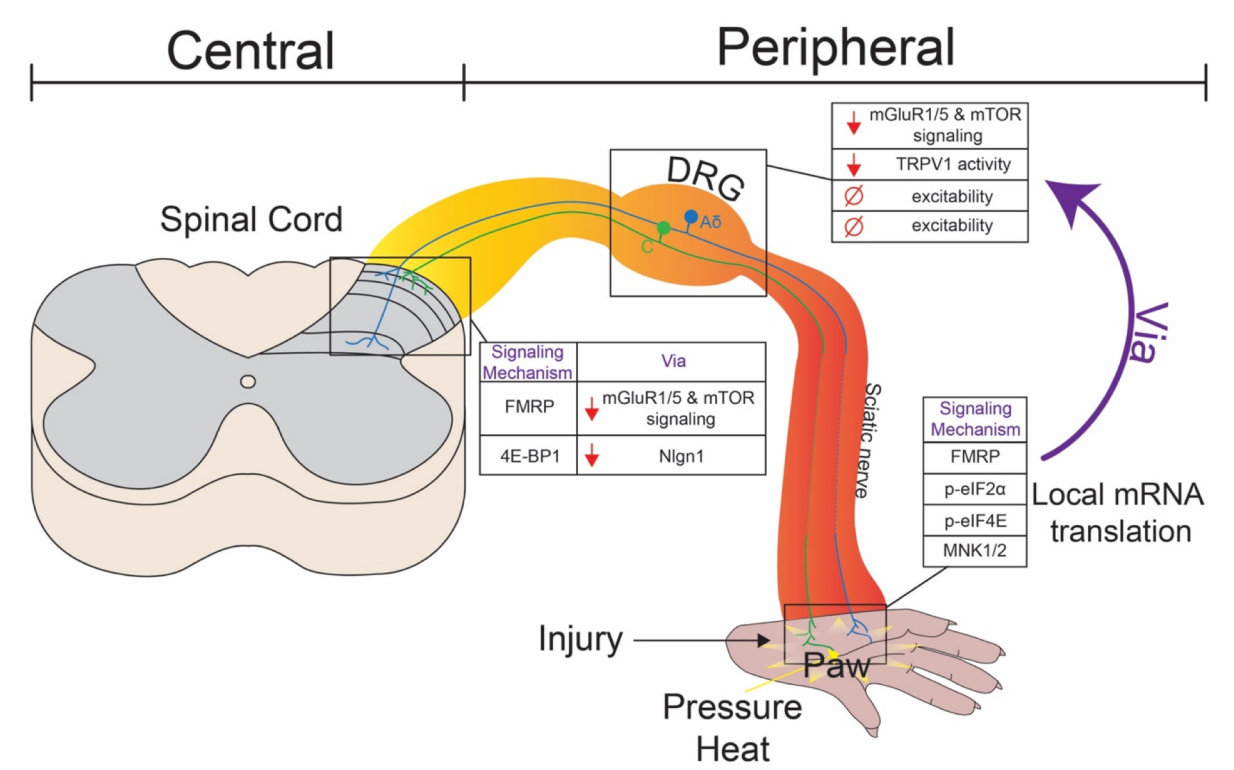

Figure 1.3. mRNA translation regulators influence the development of pain plasticity in both central and peripheral nervous systems. Transgenic mice that lack mRNA translational components, FMRP, p-eIF2α, or p-eIF4E-MNK1/2, fail to develop pain plasticity via a decrease in mGluR1/5 and mTOR signaling, a decrease in functional TRPV1 activity, and an inability to induce changes excitability, respectively, in the DRG. In the spinal cord, FMRP impacts central pain plasticity via mGluR1/5 and mTOR signaling. 4E-BP1 KO mice show increase mechanical sensitivity via a decrease in neuroligin-1 (Nlgn1) protein in the spinal dorsal horn.

as a single unit, regulate translation of different subsets of mRNAs.

This dissertation will investigate different translation regulation mechanisms involved in central and peripheral pain plasticity and how we can use these findings to develop new therapies to treat and prevent chronic pain. Chapter 1 will explore the link between BDNF and PKMζ for maintaining a chronic pain state in the spinal cord. Using behavioral paradigms and pharmacological tools, this study will provide a targeted mechanism to permanently reverse a chronic pain state. Chapter 2 investigates a specific process in translation regulation, eIF4E

phosphorylation and its role in nociceptor sensitization. Using mice with a genetic mutation at the only phosphorylation site on eIF4E and MNK1/2 KO mice and inhibitors, we aim to test if absence of this process is necessary for the development of peripheral pain plasticity and nociceptor hyperexcitability. Completion of these aims will identify a novel mechanism of activity-dependent translation in peripheral sensory neurons driving maladaptive plasticity underlying pain chronification.

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CHAPTER 2

BDNF REGULATES ATYPICAL PKC AT SPINAL SYNAPSES TO INITIATE AND MAINTAIN A CENTRALIZED CHRONIC PAIN STATE

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ABSTRACT

Background: Chronic pain is an important medical problem affecting hundreds of millions of people worldwide. Mechanisms underlying the maintenance of chronic pain states are poorly understood but the elucidation of such mechanisms have the potential to reveal novel therapeutics capable of reversing a chronic pain state. We have recently shown that the maintenance of a chronic pain state is dependent on an atypical PKC, PKMζ, but the mechanisms involved in controlling PKMζ in chronic pain are completely unknown. Here we have tested the hypothesis that brain derived neurotrophic factor (BDNF) regulates PKMζ, and possibly other aPKCs, to maintain a centralized chronic pain state.

Results: We first demonstrate that although other kinases play a role in the initiation of persistent nociceptive sensitization, they are not involved in the maintenance of this chronic pain state indicating that a ZIP-reversible process is responsible for the maintenance of persistent sensitization. We further show that BDNF plays a critical role in initiating and maintaining persistent nociceptive sensitization and that this occurs via a ZIP-reversible process. Moreover, at spinal synapses, BDNF controls PKMζ and PKCλ nascent synthesis via mTORC1 and BDNF enhances PKMζ phosphorylaton. Finally, we show that BDNF signaling to PKMζ and PKCλ is conserved across CNS synapses demonstrating molecular links between pain and memory mechanisms.

Conclusions: Hence, BDNF is a key regulator of aPKC synthesis and phosphorylation and an essential mediator of the maintenance of a centralized chronic pain state. These findings point to

BDNF regulation of aPKC as a potential therapeutic target for the permanent reversal of a chronic pain state.

BACKGROUND

How acute injury transforms to chronic pain remains a long-standing, unresolved question with important medical ramifications. The natural history of most chronic pain conditions suggests that achieving clinically meaningful endpoints requires interventions aimed at targeting or reversing pathological changes that maintain sensitization in these chronic pain states. While studies on plasticity of sensory neurons and CNS structures after injury have led to a wealth of molecular targets implicated in the initiation of pain in preclinical models (Woolf and Salter, 2000; Ji et al., 2003; Sandkuhler, 2007; Latremoliere and Woolf, 2009; Woolf, 2010; Ruscheweyh et al., 2011a), our understanding of molecular mechanisms that maintain chronic pain states remains poor.

Recent advances in understanding how neural circuits maintain long-lasting plasticity may offer insights into how pain becomes chronic (Woolf, 2010; Ruscheweyh et al., 2011a). Analogous to pain, the encoding of memory engrams in CNS structures is separated into initiation and maintenance phases. Initiation of engram encoding requires protein synthesis (Kelleher et al., 2004) and an atypical protein kinase C (aPKC) called PKMζ (Sacktor, 2011a). Maintenance of the engram is has been linked to PKMζ as PKMζ represents the only known kinase whose activity is required for the maintenance of late-long-term potentiation (LTP) and long-term memory (Sacktor, 2011a), although recent studies have called this hypothesis into question (Lee et al., 2013a; Volk et al., 2013b). We have demonstrated that the pharmacology and molecular

mechanism of a chronic pain state in mice parallels memory engram encoding in the CNS wherein the maintenance phase is critically dependent on PKMζ (Asiedu et al., 2011a). These findings have been expanded upon by several groups (Li et al., 2010; Laferriere et al., 2011b; Marchand et al., 2011; King et al., 2012) showing that spinal PKMζ is a crucial kinase for the maintenance of pain states that are no longer dependent on afferent input (Laferriere et al., 2011b). This conclusion is supported by a lack of effect of spinal PKMζ inhibitors in peripheral nerve injury models wherein afferent input is continuous as a result of the nerve injury (Li et al., 2010; King et al., 2012). On the other hand, following peripheral nerve injury, PKMζ in other CNS regions such as the anterior cingulate cortex, plays a key role in spontaneous pain evoked by injury (Li et al., 2010; King et al., 2012).

Hence, PKMζ is a key target for the maintenance of chronic pain states and for the maintenance of long-term memory; however, remarkably little is known about how PKMζ is regulated at CNS synapses. Brain-derived neurotrophic factor (BDNF), like PKMζ, plays a key role in the initiation and maintenance of LTP and long-term memories (Lu et al., 2008a) and is an important mediator of pain in the dorsal horn (Kerr et al., 1999b; Garraway et al., 2003; Zhao et al., 2006b; Zhou et al., 2008b; Zhou et al., 2011). Hence, we hypothesized that BDNF, via its receptor: tyrosine receptor kinase type B (trkB), might play an important role in regulating PKMζ and possibly other aPKCs. Our findings indicate that BDNF acts via trkB to stimulate PKMζ phosphorylation and synthesis of PKMζ and PKCλ via activation of PDK1/AKT/mTOR signaling at spinal and cortical synapses. Moreover, we show that BDNF is required for the initiation and maintenance of a chronic pain state strongly implicating a BDNF/aPKC signaling

module as a key regulator of centralized chronic pain. Therefore, we have elucidated the first neurotransmitter/neurotrophin involved in spinal, synaptic aPKC regulation and linked this system to the initiation and maintenance of a central engram encoding a chronic pain state.

RESULTS

Maintenance of persistent sensitization is independent of CaMKIIα and MEK/ERK signaling:

We have previously used a model of persistent sensitization, based on rat models of hyperalgesic priming (Reichling and Levine, 2009b), to demonstrate a role for PKMζ in maintenance of a chronic pain state (Asiedu et al., 2011a). A key feature of this model is that after the resolution of an initial allodynic state, a subsequent nociceptive hypersensitivity can be revealed by hindpaw injection of a normally subthreshold dose of prostaglandin E_2 (PGE_2), causing a prolonged allodynia, or spinal administration of the mGluR1/5 agonist DHPG, causing pronounced nocifensive behaviors (Reichling and Levine, 2009b; Asiedu et al., 2011a). In naïve animals, PGE_2 and DHPG only elicit transient allodynia or nocifensive behaviors, respectively. Hence, this model establishes a persistent sensitization that can be clearly divided into an initiation (induction of priming with interleukin 6 (IL-6)) and maintenance phase (PGE_2) that persists for long periods of time. Consistent with concepts governing memory encoding and the pharmacology of LTP, our previous findings demonstrate that persistent nociceptive sensitization initiation requires spinal protein synthesis and is reversible by the aPKC inhibitor ZIP whereas maintenance is solely dependent on ZIP reversible process (Asiedu et al., 2011a).

We previously used staurosporine, which inhibits PKC and PKA but not aPKC to demonstrate a specific role for PKMζ in maintenance of persistent sensitization (Asiedu et al., 2011a).

However, these experiments did not assess a possible role of CaMKIIα or MEK/ERK signaling in initiation or maintenance of persistent sensitization. To test this we used the CaMKIIα peptide inhibitor CamKIINTide in a cell permeable myristoylated (myr) form (or the non-myr peptide as a negative control), the small molecule CaMKIIα inhibitor KN93 and the MEK inhibitor U0126. Importantly, CamKIINTide has been previously reported to reverse late-LTP (Sanhueza et al., 2007). Consistent with previous reports suggesting a role of CaMKIIα in the initiation of inflammatory pain states (Zeitz et al., 2004; Jones et al., 2007), myr-CamKIINTide reversed IL-6-induced allodynia when administered intrathecally (i.t.) at the same time as intraplantar (i.pl.) IL-6 (Figure 2.1A). Moreover, this treatment blocked precipitation of persistent sensitization to PGE_2 injection into the same hindpaw 6 days later (Figure 2.1A). Hence, CaMKIIα is involved in the initiation of persistent sensitization. In contrast, when either myr-CamKIINTide (Figure 2.1B) or KN-93 (Figure 2.1C) was injected i.t. after the resolution of the initial IL-6-induced allodynia (maintenance phase), neither compound was capable of reversing persistent sensitization revealed by i.pl. PGE_2 injection. Hence, like protein synthesis inhibitors (Asiedu et al., 2011a), inhibition of CaMKIIα does not reverse an established, centralized pain state. Identical experiments were conducted with U0126 and, while U0126 was capable of inhibiting initiation of persistent sensitization (Figure 2.1D), it had no effect on maintenance (Figure 2.1E). Therefore, we conclude that neither CaMKIIα nor MEK/ERK, but rather a ZIP-reversible process is required for the maintenance of persistent sensitization at dorsal horn synapses.

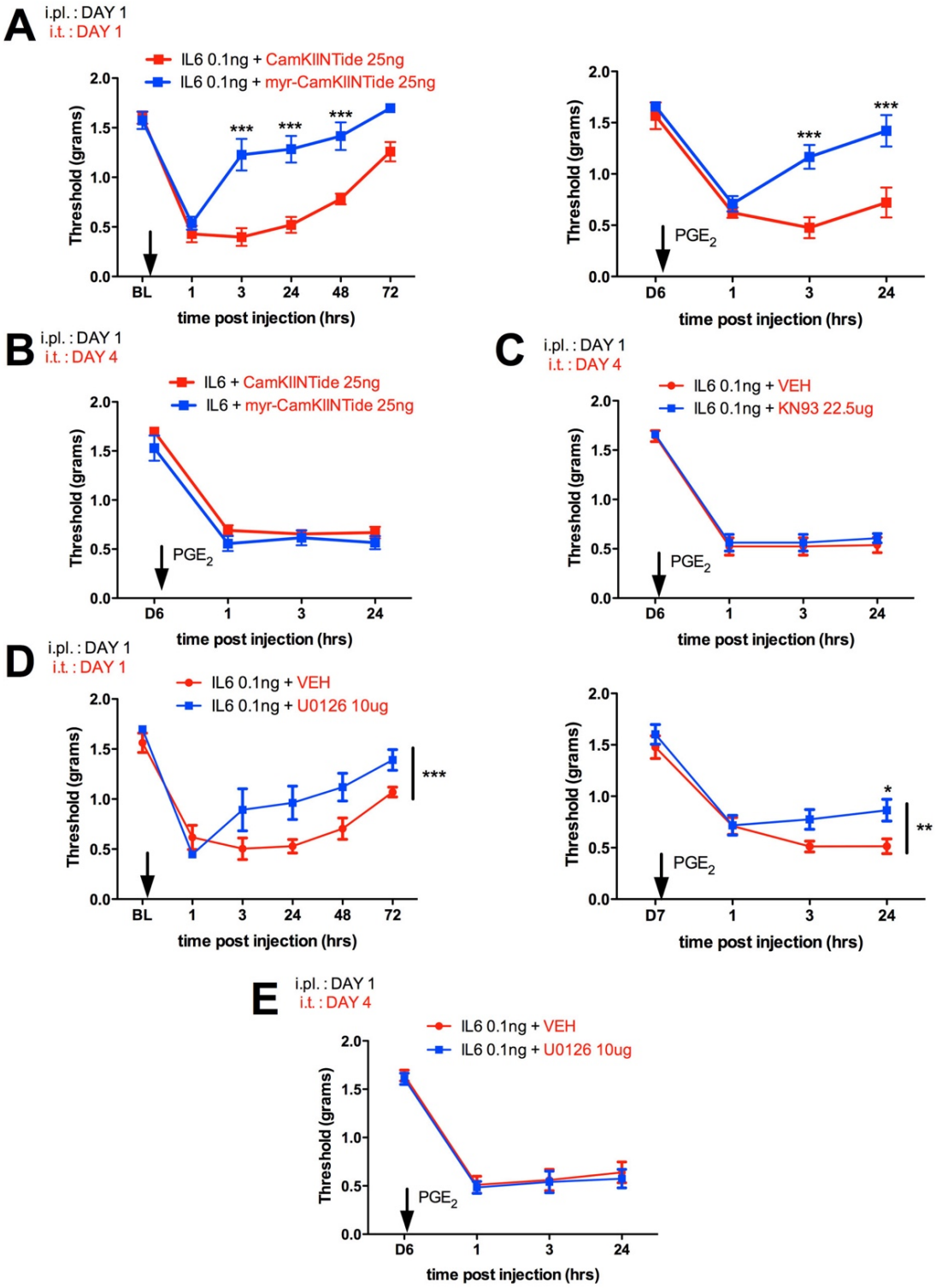
A
i.pl. : DAY 1
i.t. : DAY 1
IL6 0.1ng + CamKIINTide 25ng
IL6 0.1ng + myr-CamKIINTide 25ng
Threshold (grams)
time post injection (hrs)
BL
PGE2
B
i.pl. : DAY 1
i.t. : DAY 4
IL6 + CamKIINTide 25ng
IL6 + myr-CamKIINTide 25ng
C
i.pl. : DAY 1
i.t. : DAY 4
IL6 0.1ng + VEH
IL6 0.1ng + KN93 22.5ug
D
i.pl. : DAY 1
i.t. : DAY 1
IL6 0.1ng + VEH
IL6 0.1ng + U0126 10ug
E
i.pl. : DAY 1
i.t. : DAY 4
IL6 0.1ng + VEH
IL6 0.1ng + U0126 10ug

Figure 2.1. Lack of involvement of CaMKIIα or MAPK in persistent sensitization. A) IL-6 was injected into the left hindpaw (i.pl.) of mice and myr-CamKIINTide (peptide CaMKIIα inhibitor) or the non-myristolyated control were injected i.t. at the same time testing the initiation of persistent sensitization. Hindpaw mechanical thresholds were measured at indicated time points. Myr-CamKIINTide inhibited allodynia induced by IL-6 injection (left) and attenuated PGE_2-precipitated persistent sensitization (right). B) On the other hand, Myr-CamKIINTide administered i.t. on day 4 after IL-6 injection had no effect on PGE_2-precipitated persistent sensitization. C) Likewise, no effect was observed in the same experimental paradigm with the small molecule CaMKIIα inhibitor KN93. D) IL-6 was injected i.pl. and the MEK inhibitor U0126 or VEH control were injected i.t. at the same time testing the initiation of persistent sensitization. U0126 inhibited allodynia induced by IL-6 injection (left) and attenuated PGE_2-precipitated persistent sensitization (right). E) However, U0126 given i.t. 4 days after IL-6 had no effect on PGE_2-precipitated persistent sensitization. All experiments N = 6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two way ANOVA with Bonferroni post hoc test.

BDNF is sufficient to induce persistent sensitization and is required for the initiation and maintenance of persistent sensitization:

I.t. injection of BDNF is known to induce a long-lasting allodynic state in mice (Groth and Aanonsen, 2002) but it is not known if BDNF can induce a ZIP-reversible persistent sensitization as revealed by i.pl. injection of PGE_2. BDNF administered i.t. induced mechanical allodynia in the hindpaws of mice lasting for at least 3 days and resolving within 5 days (Figure 2.2A). 8 days following BDNF injection we injected the aPKC inhibitor myr-ZIP (ZIP) or a myr-scrambled peptide (Scr ZIP) i.t.. Because a previous study had suggested that the effects of ZIP may only last for 2 days (Parsons and Davis, 2011), we waited for 6 days following i.t. injection of ZIP to assess subsequent PGE_2 precipitated persistent sensitization. Mice that received ZIP on day 8 showed only a transient allodynia following PGE_2 injection whereas mice receiving Scr ZIP demonstrated at least 24 hrs of allodynia in response to

PGE_2 injection (Figure 2.2A). Hence BDNF is sufficient to stimulate a ZIP-reversible persistent sensitization.

We then asked whether BDNF sequestration or blockade of TrkB would inhibit IL-6-induced initiation and/or maintenance of persistent sensitization. To test initiation, the BDNF sequestering agent, TrkB/Fc was injected i.t. at the same time as i.pl. IL-6. TrkB/Fc dose-dependently disrupted IL-6-induced allodynia and PGE_2 precipitated persistent sensitization (Figure 2.2B). Importantly, when TrkB/Fc was injected i.t. following the resolution of IL-6-induced allodynia, this treatment significantly reversed the maintenance of persistent sensitization (Figure 2.2C) similar to previous observations with ZIP. If this effect was dependent on BDNF interaction with TrkB, we hypothesized that administration of the small molecule TrkB antagonist ANA-12 should achieve the same effect (Cazorla et al., 2011). ANA-12, which has systemic availability and penetrates the CNS (Cazorla et al., 2011), was injected intraperitoneal (i.p.) at the time of IL-6 injection and again 24 and 48 hrs later. This treatment significantly reversed IL-6 induced allodynia and persistent sensitization revealed by PGE_2 injection on day 7 following IL-6 (Figure 2.2D). Remarkably, when ANA-12 was given i.p. on day 4 and 5 after i.pl. IL-6 injection and persistent sensitization was precipitated with PGE_2 on day 7 (a time where ANA-12 should be cleared from the CNS), persistent sensitization was reversed (Figure 2.2E). Hence, BDNF, acting via trkB, is required for the initiation and maintenance of persistent sensitization.

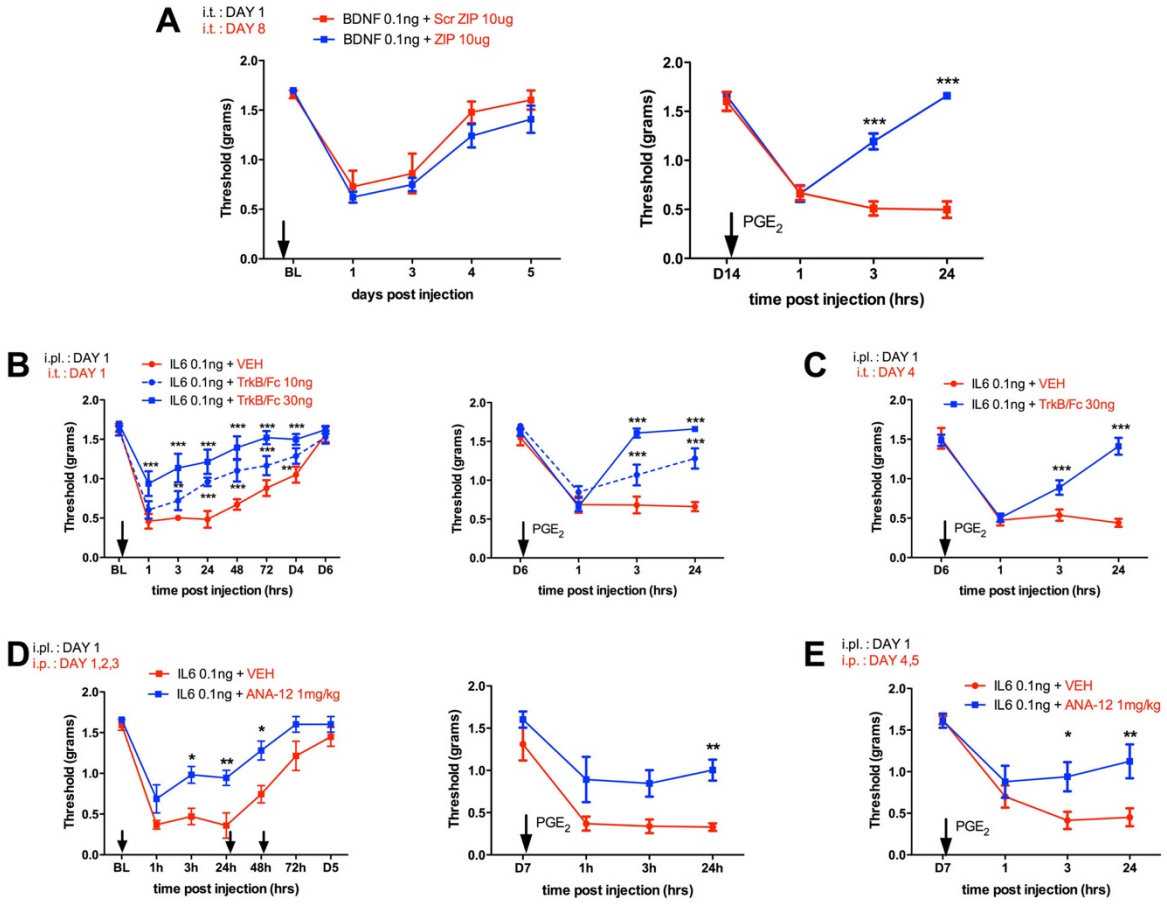

Figure 2.2. BDNF is sufficient to establish a ZIP-reversible persistent sensitization and is required for initiation and maintenance of persistent sensitization. A) BDNF was injected i.t. causing 3 days of allodynia in mice (left) and myristoylated-ZIP or Scr-ZIP were injected i.t. 8 days following BDNF injection. ZIP treatment blocked PGE_2-precipitated persistent sensitization (right). B) IL-6 was injected i.pl. and the BDNF sequestering agent, TrkB/Fc was given i.t. at the same time, dose-dependently blocking IL-6-induced allodynia (left) and PGE_2-precipitated persistent sensitization (right). C) A single i.t. treatment with TrkB/Fc 4 days after i.pl. IL-6 injection was sufficient to attenuate PGE_2-precipitated persistent sensitization. D) The small molecule TrkB antagonist, ANA-12) given i.p. at the same time as i.pl. Il-6 and again 24 and 48 hrs later blocked IL-6-induced allodynia (left) and PGE_2-precipitated persistent sensitization (right). E) Systemic treatment with ANA-12 4 and 5 days after i.pl. IL-6 injection reduced PGE_2-precipitated persistent sensitization. All experiments N = 6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two way ANOVA with Bonferroni post hoc test.

BDNF increases PKMζ protein levels and phosphorylation at spinal synapses:

Having established a role for BDNF in initiation and maintenance of persistent sensitization, we asked if BDNF regulates PKMζ and/or other aPKCs at spinal synapses. We investigated other aPKCs because it has recently been suggested that PKMζ is not required for the maintenance of late-LTP or long-term memory using genetic knockouts (Lee et al., 2013a; Volk et al., 2013b). It has also been shown that ZIP blocks PKMζ and PKCλ (Lee et al., 2013a) indicating that ZIP affects all aPKCs. Finally, ZIP still effectively reverses late-LTP and long-term memory in mice lacking PKMζ suggesting a functional redundancy of aPKCs in plasticity pathways (Lee et al., 2013a; Price and Ghosh, 2013b; Volk et al., 2013b). We first assessed aPKC mRNA expression and protein localization in the mouse spinal cord. As we have shown previously in rat (King et al., 2012), PKCζ mRNA was not expressed in the mouse spinal cord whereas PKCλ and PKMζ were both robustly expressed by qPCR (Figure 2.3B). Likewise consistent with previous findings in rat (Marchand et al., 2011), aPKC protein localized largely to the dorsal horn of the spinal cord and this immunoreactivity was found exclusively in neurons (Figure 2.3A inset). Because the immunostaining does not allow for distinguishing between PKMζ and PKCλ we resorted to isolation of synaptoneurosomes (SNSs, (Muddashetty et al., 2007)) from mouse lumbar spinal cord where PKMζ and PKCλ could be analyzed seperately by Western blot. These SNS preparations were enriched in GluN1 mRNA (Krichevsky and Kosik, 2001), were βIII-tubulin mRNA poor (Figure 2.3B, (Rao and Steward, 1993)) and at least 10 fold enriched in PSD-95 protein consistent with enrichment of spinal synaptic structures using this technique (Figure 2.3B).

To determine if BDNF regulates aPKC protein levels at spinal synapses we stimulated SNSs with increasing concentrations of BDNF. Because previous studies have suggested a role for mTOR in regulating PKMζ formation in LTP (Kelly et al., 2007) and because BDNF is known to regulate mTOR in hippocampus (Takei et al., 2004), we also assessed signaling components of the mTOR pathway in these experiments. BDNF increased mTOR S2481 phosphorylation consistent with activation of mTORC2 (Copp et al., 2009) at spinal synapses with BDNF (Figure 2.3C). Likewise, BDNF increased AKT phosphorylation at T308 (PDK1 site) and S473 (mTORC2 site) and BDNF increased phosphorylation of the mTORC1 target Thr389 residue on p70 S6 Kinase (p70, (Hara et al., 2002) Figure 2.3C). Consistent with engagement of mTORC1-dependent protein synthesis, PKCλ, PKMζ and CaMKIIα protein levels were also increased by BDNF in spinal SNSs (Figure 2.3C). These effects were time dependent with changes in phosphorylation occurring largely at 15 min of BDNF stimulation and resolving by 30 min.

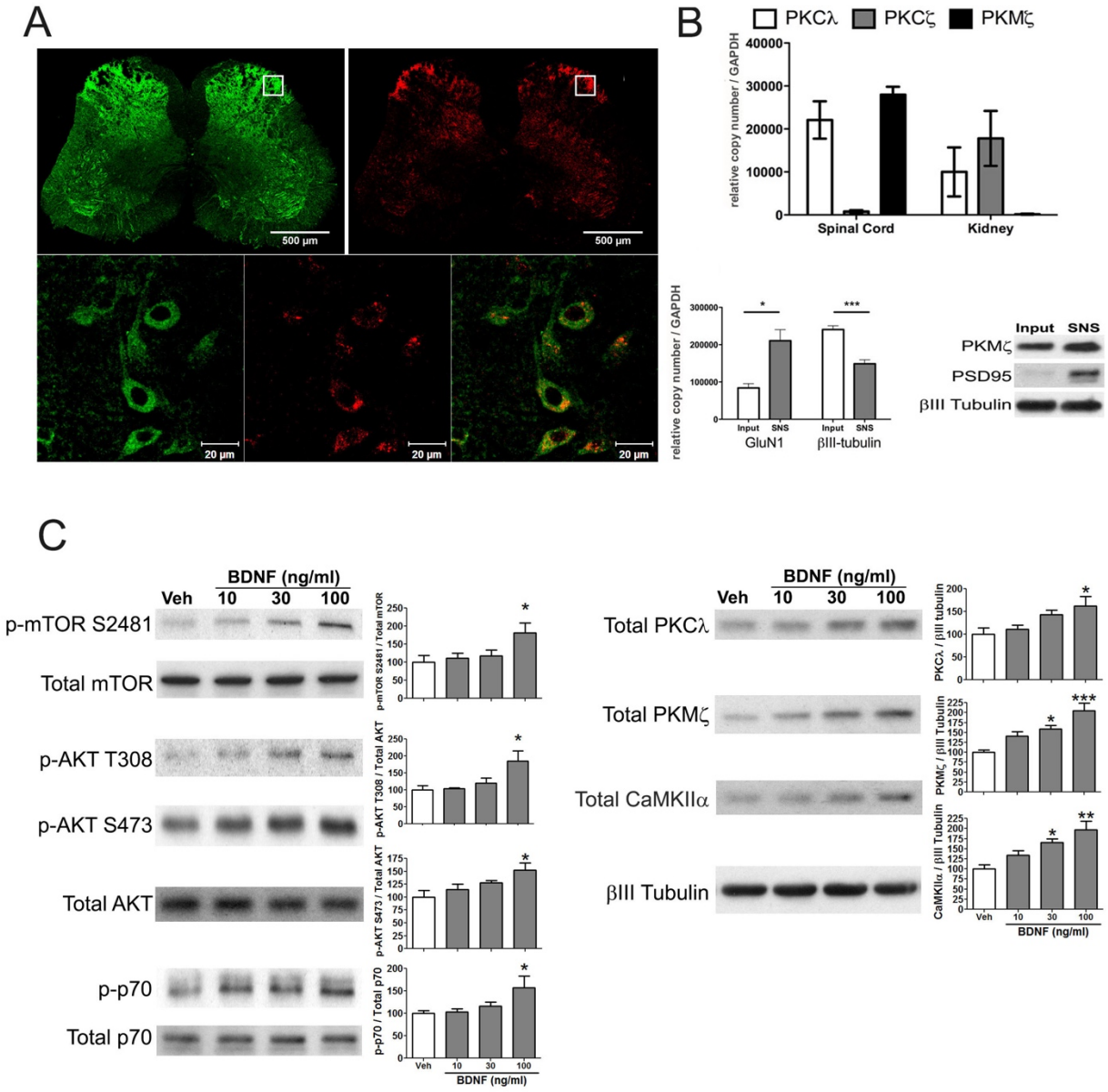

Figure 2.3. PKCλ and PKMζ protein localize to central neurons in the dorsal horn and are increased by BDNF treatment. (A) Slices were prepared from mice and stained with an atypical PKC (aPKC) antibody (red) and the neuronal marker (found in soma and post-synaptic sites) SAP-102 (green). The top panel shows that aPKC immunoreactivity is localized primarily to the spinal dorsal horn. Lower panels (from inset) show that aPKC immunoreactivity is localized almost exclusively to neurons in the dorsal horn. (B) qPCR was used to assess mRNA expression of aPKC isoforms in spinal cord and kidney (N = 3). Spinal SNSs were prepared and mRNA or protein was isolated. Spinal SNSs were enriched for GluN1 mRNA and were βIII tubulin poor, they were likewise enriched in PSD95 protein as shown by Western blotting compared to equal protein concentration of whole spinal cord homogenate (input). C) SNSs

were isolated from mouse lumbar spinal cord and exposed to increasing concentrations of BDNF for 15 min. BDNF dose-dependently increased mTOR, AKT and p70S6 kinase (p70) phosphorylation when standardized to total protein levels. BDNF also increased total levels of PKCλ PKMζ and CaMKIIα when compared to loading control βIII tubulin (N = 6). * $p < 0.05$, *** $p < 0.001$, one way ANOVA with Bonferroni post hoc test compared to Veh.

The exception was T308 phosphorylation of AKT (PDK1 site), which persisted for the full 30 min of BDNF exposure (Figure 2.4A). We also observed long-lasting changes in total amounts of PKCλ, PKMζ and CaMKIIα (Figure 2.4A), again consistent with a protein synthesis-dependent process. These effects are likely not due to aPKC regulation in sensory afferent terminals because exposure of sensory neurons in culture to BDNF led to robust activation of AKT without any corresponding change in aPKC levels (data not shown).

Because total levels of PKMζ were changed by BDNF exposure to SNSs, we performed experiments where protein synthesis could not occur to assess whether BDNF also changed PKMζ phosphorylation in a persistent fashion. In the absence of amino acids, BDNF failed to increase total PKMζ level in spinal SNSs, however, under these conditions, BDNF robustly increased AKT T308 and PKMζ T410 phosphorylation (Figure 2.4B). Because both of these phospho sites are acceptors for PDK1 activity these findings suggest that BDNF stimulates PDK1 to achieve persistent increases in downstream target phosphorylation. Hence, BDNF persistently increases PKMζ protein levels and phosphorylation at spinal synapses.

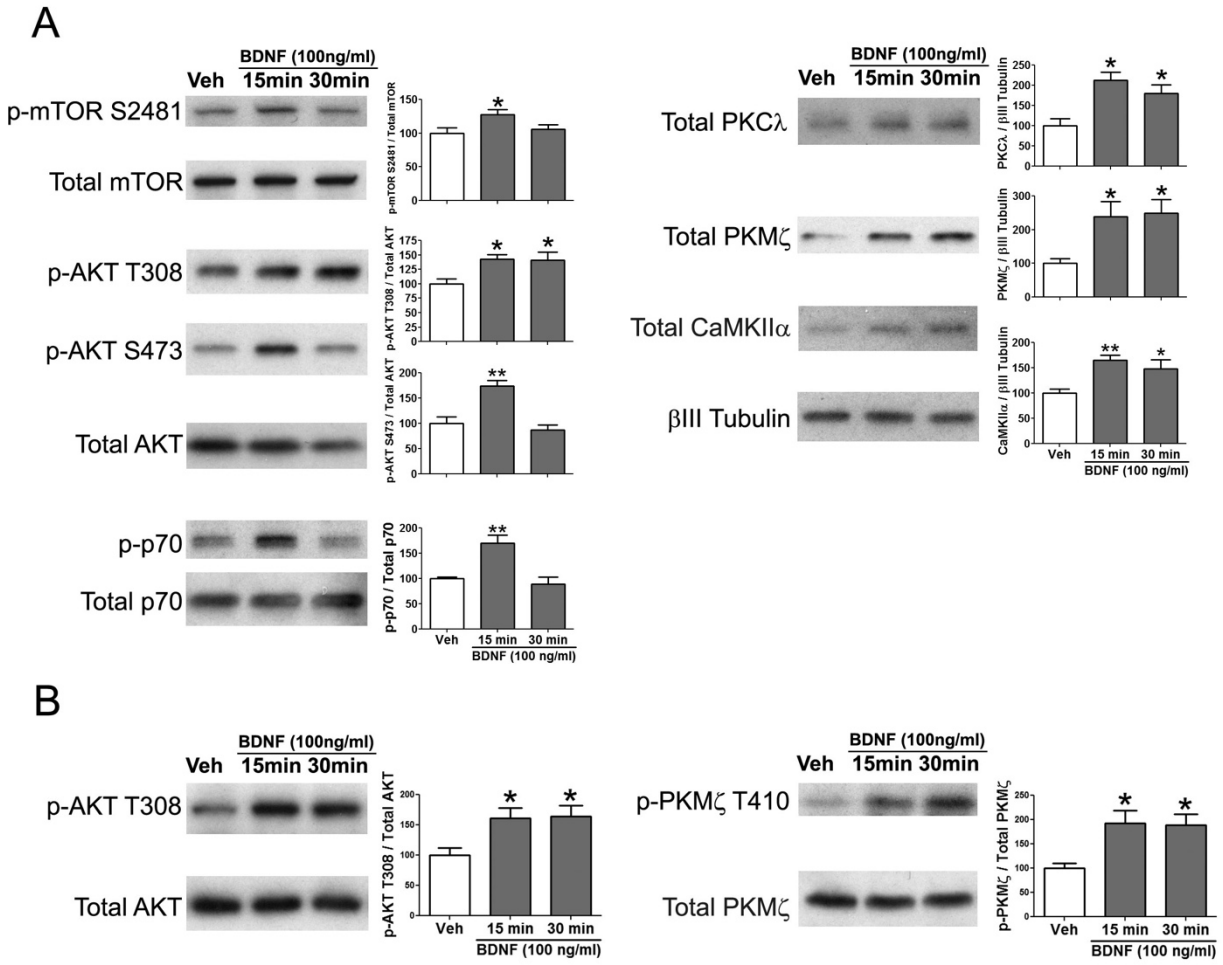

Figure 2.4. Time course of BDNF regulation of aPKCs in spinal SNSs. SNSs were prepared from mice and treated with BDNF for indicated time points. A) BDNF transiently increased mTOR, AKT (S473) and p70S6 kinase (p70) phosphorylation but led to a longer lasting increase in AKT phosphorylation at T308 and in total levels of PKCλ, PKMζ and CaMKIIα (N = 6). B) To assess whether BDNF increased PKMζ phosphorylation, SNSs were treated with BDNF in the absence of amino acids. BDNF treatment led to a persistent increase in AKT (T308, PDK1 site) and PKMζ (T410, PDK1 site) phosphorylation (N = 6). * $p < 0.05$, ** $p < 0.01$, one way ANOVA with Bonferroni post hoc test compared to Veh.

BDNF stimulates eIF4F complex formation and aPKC nascent synthesis at spinal synapses:

The results presented above suggest that aPKCs are synthesized as a result of BDNF action on spinal synapses. To pursue this idea with more rigor, we first asked if BDNF increases formation

of the 5' cap binding complex composed of eIF4E, eIF4A and eIF4G, called eIF4F, at spinal synapses. This complex is involved in promoting cap-dependent protein synthesis and occurs downstream of mTORC1 activation (Gkogkas et al., 2010). Using m7-GTP beads, we performed 5' cap pulldown assays on SNSs stimulated with BDNF for 15 min. BDNF increased eIF4A pulldown and decreased 4EBP association with eIF4E, consistent with BDNF inducing formation of the eIF4F complex at spinal synapses (Figure 2.5A). This effect was completely blocked by inclusion of temsirolimus indicating that BDNF promotes eIF4F complex formation in an mTORC1-dependent fashion (Figure 2.5A).

We next asked if BDNF increases nascent synthesis of aPKCs in an mTORC1-dependent fashion. To do this, we first assessed whether aPKC mRNA was found at spinal synapses. SNSs were prepared and mRNA levels were assessed by qPCR. PKMζ and PKCλ, but not PKCζ, mRNA was detected in spinal SNSs demonstrating that these SNSs are capable of supporting nascent synthesis of PKCλ and PKMζ and supporting the notion that PKCλ and PKMζ mRNAs are transported to synapses in the dorsal horn (Figure 2.5B). Having established that PKCλ and PKMζ mRNA are found at synapses, we used azidohomoalanine (AHA), a click-chemistry compatible methionine analogue that does not interfere with other cellular processes (Dieterich et al., 2007), to assess nascent synthesis of PKCλ and PKMζ. The methionine stores were depleted in spinal SNSs by inclublting them in methionine free media for 15 min. This was followed by stimulation of the SNSs with BDNF in the presence of AHA for 30 min. aPKC proteins were immunoprecipitated and labeled with biotin using click-chemistry to label only proteins that had incorporated AHA (e.g. nascently synthesized proteins). Remarkably, BDNF led to a robust increase in nascently synthesized PKCλ and PKMζ that was completely abrogated by mTORC1

inhibition (Figure 5C). Hence, BDNF induces PKCλ and PKMζ nascent synthesis via an increase in eIF4F complex formation downstream of mTORC1 activation at spinal synaptic structures.

BDNF increases mTORC1 activity and aPKC formation at cortical synapses.

Having shown that BDNF regulates PKCλ and PKMζ formation in an mTORC1-dependent fashion at spinal synapses we then asked it BDNF also achieves similar effects at cortical synapses where both BDNF and PKMζ (and possibly PKCλ (Lee et al., 2013a; Price and Ghosh, 2013b; Volk et al., 2013b)) are known to play an important role in LTP and long-term memory maintenance (Lu et al., 2008a; Sacktor, 2011a). By qPCR, PKCλ and PKMζ mRNA localized to cortical SNSs as shown above for spinal SNSs and these cortical SNSs were also enriched for GluN1 mRNA (Figure 2.6A). Likewise identical to observations in spinal SNSs, BDNF stimulated an increase in mTOR S2481, AKT T308 and S473 and p70 phosphorylation (Figure 2.6B). BDNF also increased CaMKIIα, as shown previously (Aakalu et al., 2001), and PKCλ and PKMζ protein levels (Figure 2.6B). Hence, BDNF regulation of PKMζ formation is conserved across CNS synapses.

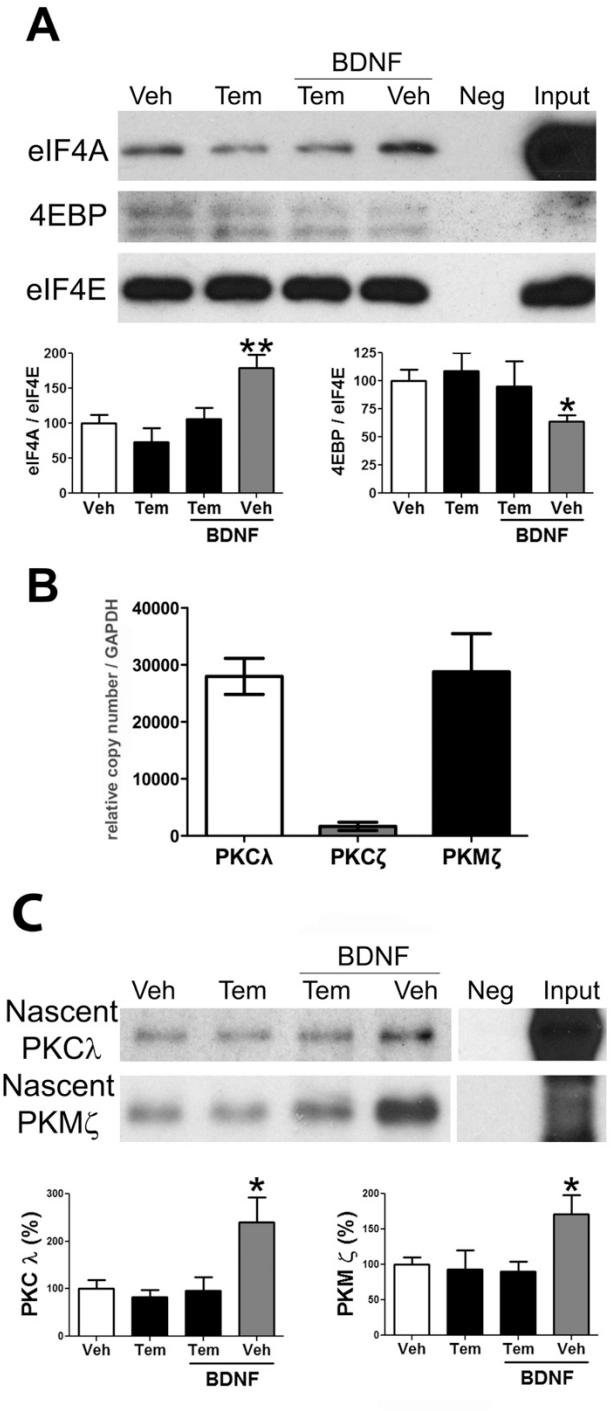

Figure 2.5. BDNF enhances eIF4F complex formation and nascent synthesis of PKCλ and PKMζ in spinal SNSs in an mTOR-dependent fashion. SNSs were prepared from mouse lumbar spinal cord and treated with or without BDNF (100 ng/ml) or the mTORC1 inhibitor temsirolimus (Tem, 100 nM). A) Following 15 min treatment with the indicated conditions, 5'm7-GTP pulldown assays were performed and eIF4A, 4EBP and eIF4E were assessed by

Western blot. BDNF treatment led to an increase in eIF4A pulldown and a decrease in 4EBP pulldown in an mTORC1-dependent fashion (N = 6). B) SNSs were assessed for aPKC mRNA expression by qPCR. SNSs contained PKMζ and PKCλ mRNA but not PKCζ (N = 3). C) SNSs were treated with BDNF +/- Tem in the presence of AHA to tag nascently synthesized proteins for 30 min. AHA aPKC was immunoprecipitated and AHA was labeled with biotin using click-chemistry. BDNF treatment led to an increase in nascently synthesized PKCλ and PKMζ in an mTORC1-dependent fashion (N = 6). * $p < 0.05$, ** $p < 0.01$, one way ANOVA with Bonferroni post hoc test compared to Veh.

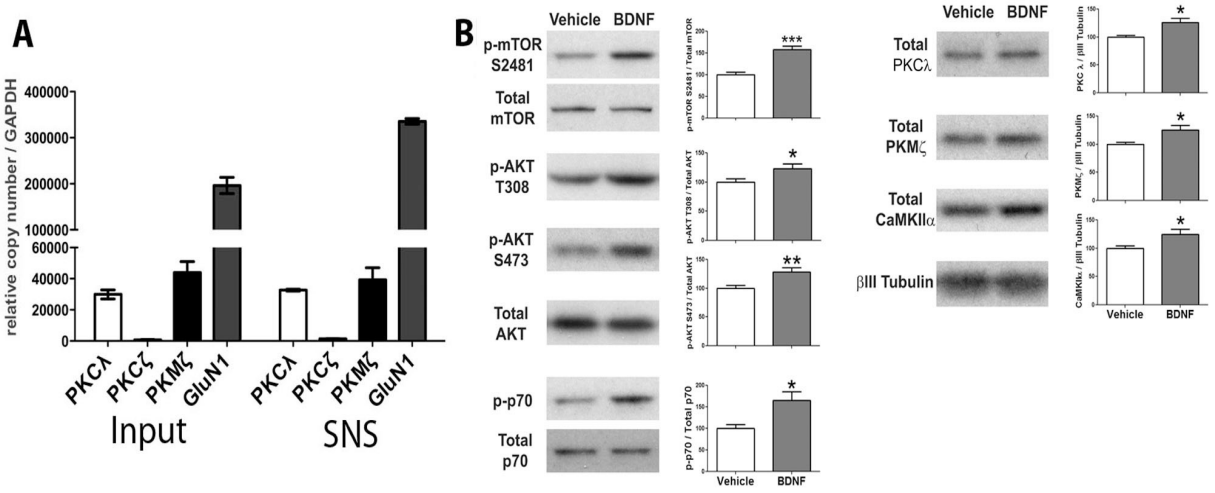

Figure 2.6. BDNF activates mTOR and increases PKCλ, PKMζ and CaMKIIα protein in cortical SNSs. A) qPCR was used to assess aPKC mRNA expression in whole cortex samples (Input) and in cortical SNSs SNSs. PKMζ and PKCλ mRNA were found in cortex and cortical SNSs but not PKCζ. GluN1 mRNA was enriched in cortical SNSs (N = 3). B) SNSs were prepared from mouse cortex and stimulated with 100 ng/ml BDNF for 15 min. Treatment led to an increase in mTOR, AKT and p70S6 kinase (p70) phosphorylation as well as increased levels of total PKCλ, PKMζ and CaMKIIα (N = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, student's t-test.

DISCUSSION

While PKMζ is well-recognized as a potential molecular mechanism for the maintenance of LTP and long-term memory (Sacktor, 2008, 2011a) and its important role in pain plasticity has recently been elucidated (Li et al., 2010; Asiedu et al., 2011a; Laferriere et al., 2011b; Marchand

et al., 2011; King et al., 2012), neurotransmitter systems involved in the regulation of PKMζ have not been described in detail. Moreover, the specific role of PKMζ in CNS plasticity has recently been called into question with PKCλ emerging as a potential redundant mechanism in CNS plasticity (Lee et al., 2013a; Price and Ghosh, 2013b; Volk et al., 2013b). Here we demonstrate that BDNF promotes persistent sensitization via a ZIP-reversible mechanism. Moreover, we show that BDNF is critical for both the initiation and maintenance of persistent sensitization, a role that it may uniquely share with an aPKC-dependent process (Asiedu et al., 2011a; Laferriere et al., 2011b). Linked to these *in vivo* findings, we further demonstrate that BDNF regulates PKCλ and PKMζ synthesis via an mTORC1-dependent pathway and PKMζ phosphorylation via PDK1 at spinal and cortical synapses. Importantly, we show definitively, for the first time, that both PKCλ and PKMζ are synthesized in an activity-dependent fashion at synaptic sites. Therefore, BDNF plays a key role in regulating aPKCs in the pain pathway elucidating a hitherto unrecognized pathway regulating the maintenance of a centralized chronic pain state.

PKMζ is an atypical PKC that was first recognized as a constitutively active kinase that may play a role in maintenance of late-LTP (Osten et al., 1996; Sacktor, 2011a). Because PKMζ lacks a regulatory region, once translated, and phosphorylated by PDK1, the kinase has the potential to maintain autonomous activity over extended periods of time, satisfying theoretical considerations for a kinase-mediated mechanism maintaining late-LTP (Sacktor, 2011a). This hypothesis has been borne-out by a body of subsequent work demonstrating a key role for PKMζ in maintaining late-LTP and also long-term memory (Shema et al., 2007; Shema et al., 2011). While parallels

between molecular mechanisms of long-term memory and pain plasticity have long been recognized, only recently has PKMζ been elucidated as a potential target for maintenance of chronic pain states. PKMζ appears to play different roles in different anatomical locations in the pain pathway. PKMζ in sensory neurons is important for nerve growth-factor mediated hyperexcitability (Zhang et al., 2012). PKMζ in the anterior cingulate cortex plays a key role in regulating tonic-aversive aspects of chronic neuropathic pain (Li et al., 2010; King et al., 2012). Interestingly, a ZIP-reversible process in the spinal cord appears to play little, if any role in maintaining chronic neuropathic pain (Li et al., 2010; King et al., 2012), perhaps because this chronic pain state is critically dependent on ongoing afferent input to the spinal dorsal horn (Campbell and Meyer, 2006). In contrast, in chronic pain states wherein afferent input resolves but hypersensitivity either persists or can be rekindled by a normally subthreshold stimulus (e.g. persistent sensitization (Asiedu et al., 2011a), CFA-induced inflammation (Reichling and Levine, 2009b) or chronic post ischemic pain (Coderre et al., 2004; Laferriere et al., 2011b)) the maintenance of this pain state is reversed by spinal injection of ZIP. Our present findings expand on these previous results demonstrating that while CaMKIIα and MEK/ERK signaling is required for initiation of persistent sensitization, these kinases do not play an active role in the maintenance phase of persistent sensitization. These findings can be viewed as in contrast to other models, such as CFA, formalin, and/or incision, wherein ERK (Ji et al., 1999; Ji et al., 2002) and CaMKIIα (Jones et al., 2007) play an important role in initiation and maintenance of a continuous hypersensitive pain state. Such differences, as mentioned above, may be related to afferent input engaged by these stimuli, which presumably resolves during the maintenance phase of the persistent sensitization model. These results, combined with our previous findings,

strongly implicate aPKCs as the sole family of kinases responsible for the maintenance of persistent sensitization.

Despite the emerging role of PKMζ and potentially PKCλ in pain plasticity, mechanisms involved in aPKC regulation in the pain pathway are nearly completely unknown. We hypothesized that BDNF might play a key role in regulating aPKCs. This hypothesis was based on a known role of BDNF in pain states consistent (Garraway et al., 2003; Zhao et al., 2006b; Zhou et al., 2008b) with the known effects consistent with an involvement of aPKCs. While BDNF can have several sources in the spinal dorsal horn, acutely it is released from nociceptors synapsing in the outer lamina of the dorsal horn (Kerr et al., 1999b; Zhao et al., 2006b) where it regulates inflammatory but not neuropathic pain (Zhao et al., 2006b). BDNF also plays an important role in regulating LTP at dorsal horn synapses (Zhou et al., 2008b) consistent with the known role of BDNF in LTP in other CNS regions (Lu et al., 2008a). These findings, combined with our present results, are consistent with a model wherein BDNF released from nociceptive endings in the spinal dorsal horn initiates signaling cascades that lead to the formation and phosphorylation of aPKCs at these synapses. Although spinal BDNF plays a role in neuropathic pain, this has been linked to release from microglia (Coull et al., 2005a; Trang et al., 2009) and not nociceptor terminals because neuropathic pain develops normally in mice lacking BDNF expression in nociceptors (Zhao et al., 2006b). This finding is consistent with previous findings showing a limited role of a spinal ZIP-reversible process in neuropathic pain. Our findings from spinal SNS experiments clearly demonstrate that BDNF applied exogenously is capable of stimulating synthesis of PKCλ and PKMζ and phosphorylation of PKMζ (Figure 2.7). Whether

BDNF released from microglia is incapable of achieving these effects at spinal synapses will have to await further experimentation.

An important implication of our current findings is that BDNF not only plays a role in initiating a centralized chronic pain state but that it also plays an active role in maintaining such a pain state via regulation of aPKCs. If this is the case, what is the source of BDNF? It is unlikely to be derived from presynaptic release from nociceptors because these sensory neurons are unlikely to be active after the resolution of IL-6-induced allodynia. It is also unlikely that microglia are the source because this would be inconsistent with the neuropathic pain findings for ZIP (Li et al., 2010; Laferriere et al., 2011b; King et al., 2012). Important clues might be gleamed from the LTP literature wherein both pre- and post-synaptic release of BDNF regulates consolidation of late-LTP (Bramham and Messaoudi, 2005; Dean et al., 2009; Jourdi et al., 2009). Interestingly, this likely involves alternatively spliced isoforms of BDNF in hippocampus (An et al., 2008) facilitating the possible recognition of such a mechanism being engaged in the spinal dorsal horn. While these experiments are outside of the scope of the present findings, this is likely to be a fruitful area of future research to gain a better understanding of maintenance mechanisms of a centralized chronic pain state.

Another important question raised by our findings relates to the dependence of maintenance of persistent sensitization on aPKCs but not protein synthesis. If BDNF regulates both PKCλ and PKMζ synthesis and PKMζ phosphorylation and initiation and maintenance of persistent sensitization are dependent on both aPKCs and BDNF but only initiation is dependent on protein synthesis, how is this seeming contradiction resolved? One possible explanation is that in the absence of protein synthesis, BDNF regulation of PKMζ phosphorylation is sufficient to

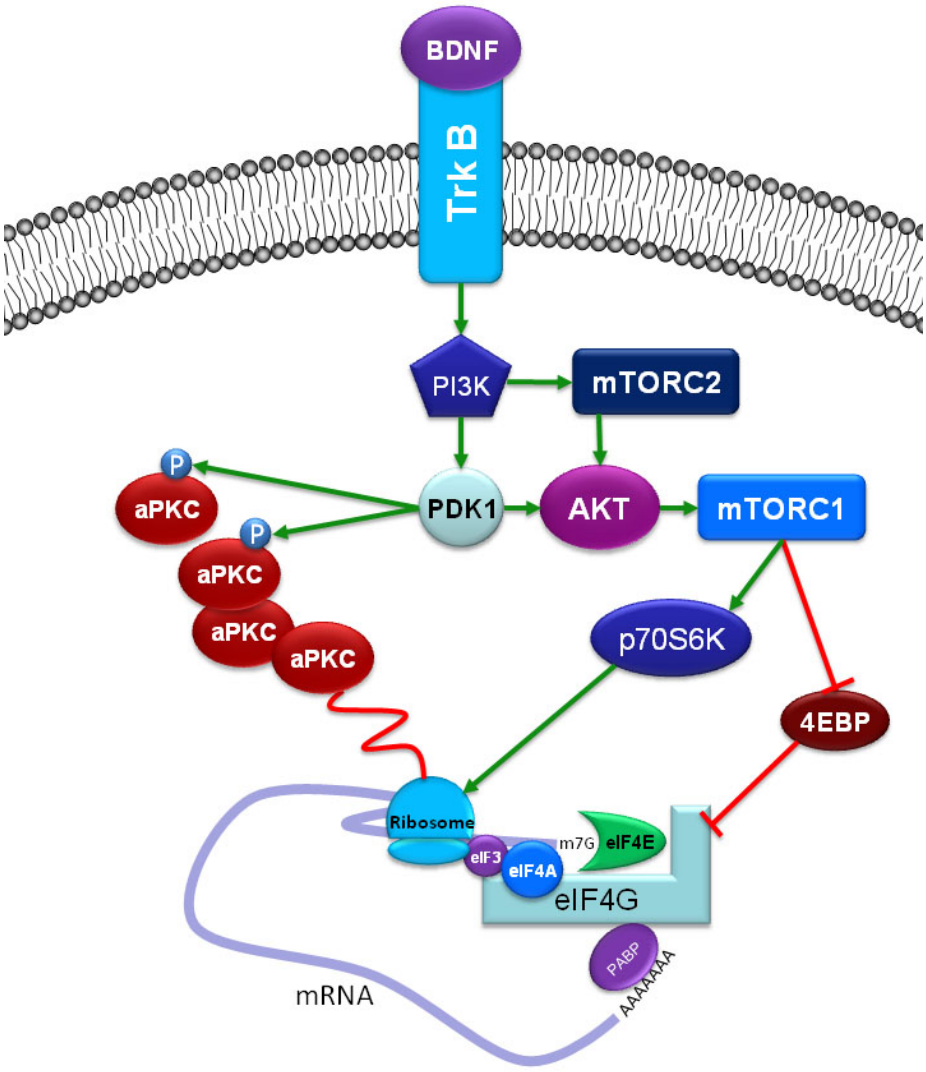

Figure 2.7. BDNF regulation of aPKCs at central synapses. Summary cartoon of major findings presented here. At spinal synapses, BDNF activated trkB leading to increased PI3K and PDK1 activity. This stimulates aPKC phosphorylation at T410 and an increase in AKT activity thereby stimulating mTORC1. This increase in mTORC1 activity leads to an increase in eIF4F complex formation resulting in an enhancement of aPKC translation at spinal synapses. Data from cortical SNS preparations strongly suggest that this signaling complex engaged by BDNF resulting in regulation of PKMζ is present across central synapses.

maintain the chronic pain state. Interestingly, in spinal SNSs, BDNF stimulation of mTORC1 activity was transient whereas PDK1 mediated phosphorylation of both AKT and PKMζ was persistent. Hence, it is physiologically feasible that in the absence of protein synthesis, BDNF-mediated phosphorylation of PKMζ is sufficient to maintain persistent sensitization. Another possibility is that PKMζ, and possibly PKCλ, has an exceptionally long half-life at synapses. In this scenario, despite blockade of protein synthesis over long periods, aPKCs formed via previous protein synthesis would be capable of overcoming a lack of new protein availability due to its long half-life. Our preliminary observations (Melemedjian, Ghosh and Price, unpublished observations) support this model but ultimately require further experimentation. However, it is clear that BDNF can maintain late-LTP when protein synthesis is inhibited via a PKMζ-dependent mechanism (Mei et al., 2011) suggesting that similar mechanisms may be at play in the setting of persistent sensitization.

Importantly, we demonstrate that BDNF regulates aPKC formation in cortical SNSs in an analogous fashion to spinal SNSs. Insofar as both the maintenance of a centralized chronic pain state and long-term memory require both BDNF (Bekinschtein et al., 2007) and PKMζ (Sacktor, 2011a), and considering that we demonstrate that BDNF regulates aPKCs across CNS structures, this illustrates the potential existence of a conserved pathway for the maintenance of synaptic plasticity from pain to memory. We propose that this has profound implications for understanding how mechanisms of plasticity evolved in central nervous systems and we suggest that these mechanisms might have first evolved for the most rudimentary neural function: protecting the organism against potentially lethal tissue injury. An important point moving forward will be to unveil how different aPKC isoforms contribute to pain plasticity through

genetic models, as we have recently reviewed (Price and Ghosh, 2013b). This need is highlighted by the recent findings from the learning and memory literature showing that genetic removal of PKMζ fails to affect learning and memory despite the continued efficacy of ZIP in these animals, suggesting a potential redundant role of PKCλ in these pathways (Lee et al., 2013a; Volk et al., 2013b).

In closing, we reveal that BDNF regulates the formation of PKCλ and PKMζ and phosphorylation of PKMζ and that BDNF/aPKC signaling forms a signaling axis required for the maintenance of a centralized chronic pain state. Our results imply that spinally directed therapeutics targeting BNDF and/or aPKCs might offer disease-modifying effects on certain chronic pain states in humans that are currently only treated by palliative management. The generation of such a class of therapeutics would have profound implications for the treatment of chronic pain.

METHODS AND MATERIALS

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.

Mechanical testing:

Animals were treated as described previously (Asiedu et al., 2011a). In brief, 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 with IL-6, IL-6 was injected into the plantar surface of the left hindpaw in a volume of 25 μl. For day 1 experiments with BDNF, BDNF was injected intrathecally (i.t.) in a volume of 5 μl. For intrathecal treatments on day 1, drugs were injected immediately after intraplantar (i.pl.) injections under brief (<3 min) isoflurane anesthesia in a volume of 5 μl (Hylden and Wilcox, 1980). For day 1 experiments with ANA-12, ANA-12 was injected intra-peritonially (i.p.) on day 0, 1 and 2 following IL-6 injection. For experiments with intrathecal treatments on day 4 or later, mice were tested before i.t. injection to assure that allodynia had completely resolved. I.T. injections were done at the indicated time points under isoflurane anesthesia as described above. For day 4 experiments with ANA-12, ANA-12 was injected i.p. on day 4 and 5 following IL-6 injection. PGE_2 (100 ng) was injected on day 6 or

later 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.

PCR:

Total RNA was extracted from tissue and synaptosomal preparations the RNeasy mini kit (#74104 QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. RNA quantification and purity were tested using a Nanodrop® spectrophotometer. 1 µg of total RNA was used for cDNA synthesis with iScript™ Reverse Transcription Supermix for RT-qPCR kit (#170-8890 Biorad). RT-PCR reactions were performed on an ABI 7500 Fast Real-time PCR System with SYBR Green PCR master mix (#4309155 Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using default two-step (95°-60°) amplification. All primer pairs (Table 2.1) were tested by running 3-4 fold dilution across at least 5 dilution points. Primers only passed if they had a calculated efficiency between 97-103% with an R^2 value greater than 0.98 and had a single, shoulder-free peak upon melt curve analysis. Primer sequences are given in table 1. Reactions were run in triplicate; measurements are based on at least three independent samples. No-RT and Cq dilution controls were routinely performed to check for genomic DNA and inhibitory contamination respectively. Melt curves were performed with each run to insure specific amplification products. Each reaction was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Expression numbers given in the paper were calculated by arbitrarily assigning GAPDH a value of 2^{20} and calculating the expression relative to GAPDH. GAPDH-normalized values were compared with normalization to Eef1A and Rpl29 to ensure controls and comparative data were consistent (data not shown).

Synaptoneurosome preparation and treatment:

Spinal cord and cortical synaptoneurosomes (SNS) were prepared from 3-weeks-old male ICR mice as previously described (Muddashetty et al., 2007). Briefly, dissected spinal cords or cortices were homogenized at on ice in homogenization buffer ((in mM) 118 NaCl, 4.7 KCl, 1.2

Table 2.1. Primer sequences used for qPCR experiments

Gene	Forward primer	Reverse primer	Size
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	95
βIII Tubulin	TAGACCCCAGCGGCAACTAT	GTTCCAGGTTCCAAGTCCACC	127
PKCλ	CCATGTGTACCAGAGCGTCCT	TGTGGCCATTTGCACAATACA	106
PKCζ	CAGGGACGAAGTGCTCATCA	CACGGCGGTAGATGGACTTG	95
PKMζ	AGCAGAGAAAGCCGAGTCCA	TTAAAGCGCTTGGCTTGGAA	96
GluN1	ATGCACCTGCTGACATTCG	TATTGGCCTGGTTTACTGCCT	142

$MgSO_4$, 2.5 $CaCl_2$ and 1.53 $KH2PO_4$, 212.7 glucose pH 7.4], supplemented with Complete protease inhibitors (Sigma, St. Louis, MO) and 40 U/ml recombinant human RNase inhibitor (Life technologies, Grand Island, NY). Samples were successively filtered through three layers of 100 μm and 11 μm nylon mesh filters (Millipore, Bedford, MA) and centrifuged at 1000 × g

for 20 min. The pelleted SNS were suspended in DMEM/F12 (Life technologies, Grand Island, NY) tissue culture media supplemented with Complete protease inhibitors and RNase inhibitor. Some experiments were carried out in homogenization buffer to prevent protein synthesis since this buffer does not contain amino acid. The resuspened SNS were then treated with various concetrations of BDNF (R&D Systems, Minneapolis, MN) for 15 or 30 min at 37ºC. SNS were centrifuged at 20000 x g for 2 min, the pellet was resuspended in lysis buffer (50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA at pH 7.4), ultrasonicated and centrifuged at 20000 x g for 15 min. The supernatant was collected and assayed using Western blot analysis.

Nascent aPKC synthesis assay:

SNS were suspended in methionine-free media (cat # 21013-024, Life technologies, Grand Island, NY) and pretreated with vehicle or temsirolimus (100 nM, LC Labs, Woburn, MA) for 15 min at 37ºC. Azidohomoalanine (AHA) is a methionine analogue that cells can incorporate into nascentlly synthesized protein. AHA (50 μM, Life technologies, Grand Island, NY) was added to the SNS suspension and incubated at 37ºC for 30 min. SNS were then centrifuged at 20000 x g for 2 min and lysis buffer was added to the pellet. Protein was extracted by ultrasonication, centrifugation at 20000 x g for 15 min and collection of the supernatant. PKMζ was immunoprecipitated by incubating the supernatant with 1:50 mouse anti-PKCζ antibody (cat # sc-17781, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4ºC. The samples where then incubated with protein G sepharose beads (Sigma, St. Louis, MO) for 3 hr at 4ºC, followed by centrifugation and wash with lysis buffer 3 times. The pelleted beads were suspended in Tris-SDS buffer (1% SDS and 50 mM Tris-HCl, pH 8.0), centrifuged and the supernatant was collected. At this stage, the supernatant contains the immunoprecipitated PKMζ

where the nascently synthesized form would have incorporated AHA. AHA was biotinylated using Click-it Protein Analysis Detection Kit (Life technologies, Grand Island, NY) according to the manufacturer's instructions. The biotinylated PKMζ was detected by Western blotting.

5'm7-GTP pulldown assays:

After the protein extraction, 50 µg protein was incubated with 7- methyl GTP Sepharose 4B beads (GE Healthcare) in the presence of 100uM GTP for 2 h at 4 °C. Unconjugated sepharose 4B beads were used for the negative controls. The beads were then pelleted and washed twice with lysis buffer. eIF4E, eIF4A and 4EBP bound to the precipitated beads were analyzed by western blotting.

Western blotting:

Fifteen micrograms of protein in 1X Laemmli Sample Buffer containing 5 % v/v β-mercaptoethanol were loaded in each well and separated by standard 10% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA) and then blocked with 5% dry milk for 3 h at room temperature. The blots were incubated with primary antibody overnight at 4 °C and detected the following day with donkey anti-rabbit antibody conjugated to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA). Signal was detected by ECL on chemiluminescent films. PKCλ and PKMζ were recognized by a pan-aPKC antibody and differentiated by size. Since PKCζ mRNA was not present in these tissues, the presence of that protein, the only other member of the aPKC family, was excluded. Each phosphoprotein was normalized to the expression of the corresponding total protein on the same membrane. The p-PKMζ antibody does not recognize p-PKCλ and therefore could not be used to determine phosphorylation of PKCλ. This antibody does recognize p-PKCζ but consistent with an absence

of PKCζ in these tissues, no band was observed at the appropriate size for that protein with the p-PKC/Mζ antibody. Densitometric analyses were performed with Image J software (NIH, Bethesda, MD) using the gel analysis tool available as a plugin from McMaster University on the following website: macbiophotonics.ca. Densitometry was done following instructions given for this plugin for ImageJ.

Immunohistochemistry (IHC):

IHC on mouse spinal cord was done as described previously on fresh frozen 20 μm sections of mouse lumbar spinal cord (Price et al., 2007b). Localization of aPKC was assessed with the Santa Cruz sc-216 antibody and SAP-102 (Cell Signaling Technologies) was used to label neuronal structures.

Primary antibodies and chemicals:

The following rabbit antibodies were obtained from Cell Signaling (Danvers, MA): p-AKT (Ser473, cat# 4058 and Thr308 cat# 2965), total AKT (cat# 4691), p-mTOR (Ser2481, cat# 2974), total mTOR (cat# 2983), p-p70 (Thr389, cat# 9205), total p70 (cat# 9202), p-PKC/Mζ (Thr410, cat# 9378), CaMKIIα (cat# 3357), eIF4A (cat# 2424), 4EBP1/2 (cat# 9452 and 2845) and eIF4E (cat# 9742). Total rabbit aPKC was from Santa Cruz Biotechnologies (cat# sc-216, Santa Cruz, CA) and βIII-Tubulin was from Promega (cat# G7121, Madison, WI). Human recombinant IL-6, BDNF and TrkB/Fc were from R&D Systems; myristoyalated CamKIINTide, CamKIINTide and KN-93 were from Calbiochem; ANA-12 was from Maybridge; UO126 was from Tocris Bioscience; and prostaglandin E_2 (PGE_2) was from Cayman Chemical Company. Stock solutions of IL-6, CamKIINTide, KN-93, ANA-12 and UO126 were made in 100% DMSO. BDNF stock solution was made in sterile PBS containing 0.1% BSA and TrkB/Fc stock

solution was made in sterile PBS. PGE_2 stock solutions were made in 100% ethanol. All drugs except U0126 and ANA-12 were diluted to final concentrations in saline for injection. U0126 was diluted to final concentration in 45% cyclodextrin. ANA-12 was diluted to final concentration in 10% polyethylene glycol 300. Matching vehicles (saline + matching amount of stock diluent except for U0126 and ANA-12 where saline with 45% cyclodextrin or saline with 10% polyethylene glycol 300, respectively) were used as a control in all experiments.

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CHAPTER 3

MNK – EIF4E SIGNALING AXIS CONTROLS INJURY-INDUCED NOCICEPTIVE PLASTICITY AND THE TRANSITION TO CHRONIC PAIN

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ABSTRACT

Mechanisms governing the sensitization of nociceptors are widely believed to be responsible for the generation of chronic pain states, but are poorly understood. We investigated the role of phosphorylation of the 5' cap binding protein, eIF4E, by MNK1/2 in nociceptive sensitization and the transition to chronic pain. Using mice harboring a point mutation at the MNK1/2 phosphorylation site on eIF4E, we find that pro-nociceptive and inflammatory factors fail to induce behavioral sensitization, hyperalgesic priming or electrophysiological measures of hyperexcitability in the absence of eIF4E phosphorylation. These effects are recapitulated in MNK1/2 double knockout mice and with the MNK1/2 inhibitor, cercosporamide. Our findings demonstrate that these effects are mediated by a peripheral mechanism of action pointing to a MNK1/2 – eIF4E signaling axis as a pivotal factor in controlling nociceptor plasticity. We conclude that eIF4E phosphorylation regulates nociceptor plasticity and is an integral mediator of the transition to a chronic pain state.

INTRODUCTION

The transition from acute to chronic pain is thought to involve alterations in the phenotype and function of peripheral nociceptors found in the dorsal root ganglion (DRG) or trigeminal ganglion (Reichling and Levine, 2009b). Following injury, DRG nociceptors change expression, localization and/or post-translational modification of ion channels, causing increases in excitability (Waxman and Zamponi, 2014). Moreover, transcriptional changes alter the neurochemical phenotype (Perl, 1996; Woolf, 1996; Koltzenburg, 1999) of these neurons leading to changes in neurotransmission at the first synapse between DRG neurons and the central

nervous system (Pezet and McMahon, 2006) in the spinal or brainstem dorsal horn. The control of protein synthesis has emerged as an important regulatory pathway for the transition to a chronic pain state (Price and Geranton, 2009; Obara et al., 2012a; Melemedjian et al., 2013d; Price and Inyang, 2015). Mechanistic target of rapamycin (mTOR) and mitogen activated protein kinase (MAPK) signaling to eukaryotic initiation factor 4 (eIF4) proteins promotes the sensitization of nociceptors and a transition to a chronic pain state (Jimenez-Diaz et al., 2008; Geranton et al., 2009; Melemedjian et al., 2010a; Melemedjian et al., 2011a; Obara et al., 2011; Melemedjian et al., 2013d; Xu et al., 2014) but the downstream mechanisms associated with these signaling events have not been identified.

Translation of mRNAs is dynamically regulated in cells by upstream signaling factors that respond to cell surface receptor activation. For instance, in DRG neurons nerve growth factor (NGF) and interleukin 6 (IL-6), two extracellular ligands intimately linked to pain across mammalian species, signal via the mTOR and MAPK pathways to induce eIF4F complex formation and promote translation (Melemedjian et al., 2010a). The eIF4F complex is composed of the 5' cap binding protein eIF4E, the deadbox RNA helicase eIF4A, and the scaffolding protein eIF4G. Phosphorylation of 4E-binding proteins (4E-BPs) by mTOR relieves inhibition of eIF4E association with eIF4G and eIF4A (Sonenberg and Hinnebusch, 2009a) while MAPKs act via the MAPK interacting kinases (MNK) 1 and 2 to phosphorylate eIF4E at a single site (Ser209), (Pyronnet et al., 1999; Waskiewicz et al., 1999)). Phosphorylation of eIF4E has been linked to cancer (Furic et al., 2010a), immunity (Herdy et al., 2012a) and certain CNS-mediated behavioral phenotypes (Gkogkas et al., 2014a), but its physiological role in the context of sensory plasticity, including generation of a chronic pain state, is unexplored.

An important theme emerging from recent work on translation control signaling is that specific phosphorylation events control the translation of specific subsets of mRNAs. The mTOR pathway has recently been shown to primarily influence the translation of mRNAs that contain terminal oligopyrimidine tracts in their 5' untranslated regions (UTRs) (Thoreen et al., 2012). Similarly, the RNA helicase eIF4A primarily influences the translation of mRNAs with highly structured 5' UTRs and/or 5' UTRs that contain sequence motifs that form G quadruplexes (Wolfe et al., 2014b). Specific mRNAs regulated by eIF4E phosphorylation have been identified from mouse embryonic fibroblasts (Furic et al., 2010a) but these have not been studied in neurons. In the context of pain, two recent studies have shown distinct phenotypes in 4E-BP1 knockout mice or when eIF2α phosphorylation is genetically reduced. 4E-BP1 knockout mice show increased spinal cord expression of neuroligin 1 and enhanced mechanical sensitivity with no change in thermal thresholds (Khoutorsky et al., 2015b). On the other hand, mice lacking eIF2α phosphorylation on one allele have reduced responses to thermal stimulation and a deficit in thermal hypersensitivity after inflammation but normal mechanical pain (Khoutorsky et al., 2016b). These studies highlight that distinct translation regulation signaling pathways produce diverse sensory phenotypes.

The goal of this study was to test the hypothesis that eIF4E phosphorylation is a central regulator of nociceptive plasticity and the transition to a chronic pain state. We have tested this hypothesis using mice lacking the phosphorylation site for MNK1/2 on eIF4E (*eIF4E*^{S209A}), mice lacking both *Mnk1* and *2* (*Mnk1/2*$^{-/-}$) (Ueda et al., 2004b) and an inhibitor of MNK1/2 kinase activity, cercosporamide (Altman et al., 2013). We find that, different from 4E-BP1 knockouts or eIF2α mutants (Khoutorsky et al., 2015b; Khoutorsky et al., 2016b), eIF4E phosphorylation does not

influence acute pain behavior but is required for nociceptor sensitization to a variety of endogenous factors known to promote pain in humans. Moreover, eIF4E phosphorylation is required for the transition to a chronic pain state in hyperalgesic priming and neuropathic pain models. Our findings elucidate a new pathway regulating plasticity in the nociceptive system with important implications for understanding how pain becomes chronic.

RESULTS

Nociceptive reflexes, acute pain behavior and development of DRG to spinal dorsal horn connectivity is normal in eIF4E^{S209A} mice

To test the hypothesis that eIF4E phosphorylation is a key factor in pain sensitization and a transition to a chronic pain state we used mice harboring a point mutation on the only known phosphorylation site in the eIF4E protein, S209 (Furic et al., 2010a; Herdy et al., 2012a; Gkogkas et al., 2014a). We compared baseline thermal (Figure 3.1A) and mechanical thresholds (Figure 3.1B) between *eIF4E^{S209A}* mice and their wildtype (WT) littermates and noted no differences in tail flick latencies to 55°C water or von Frey stimulation. When 5% formalin, a commonly used noxious irritant, was injected into the hindpaw no differences in pain behaviors were noted between genotypes in either the first (Figure 3.1C, 0 – 10 min) or second (Figure 3.1D, 15 – 45 min) phases of the test. However, when mechanical sensitivity was examined 3 days after formalin administration there was a striking difference between genotypes, with *eIF4E^{S209A}* mice failing to develop mechanical hypersensitivity. The group I metabotropic glutamate receptor (mGluR) agonist, dihydroyphenylglycine (DHPG), promotes tonic pain-related behaviors when injected intrathecally (Karim et al., 2001). These behaviors are decreased

by inhibition of spinal mTOR signaling (Price et al., 2007b). We did not detect any difference in acute pain behaviors upon DHPG intrathecal (i.t.) injection between genotypes (Figure 3.1F), but 6 hr following injection *eIF4E*^{S209A} mice again showed a lack of mechanical hypersensitivity (Figure 3.1G).

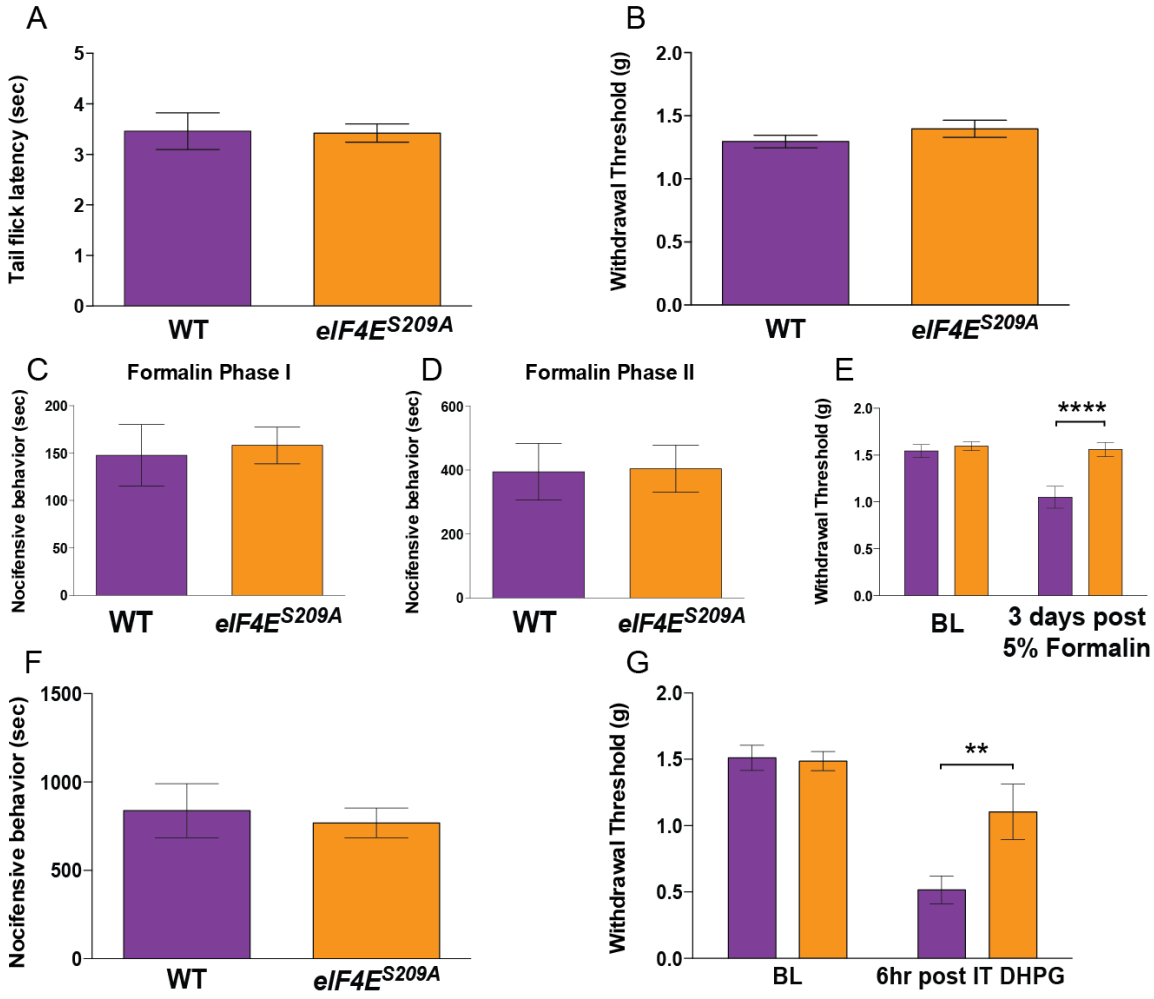


Figure 3.1. eIF4E^{S209A} *mice have normal acute nociceptive responses but fail to demonstrate mechanical hypersensitivity to formalin and a group I mGluR agonist.* A) eIF4E^{S209A} and WT mice show no differences in tail-flick responses (55°C) ($n \geq 6$, t-test) or B) baseline paw withdrawal thresholds ($n \geq 6$, t-test). C, D) Both genotypes were injected with 5% formalin into the hindpaw and observed for nocifensive behavior (licking/shaking) for 45 min. First phase (0–10 min) and second phase (15–45 min) summed responses were not different between

eIF4E^{S209A} and WT mice (n ≥ 10, two-way ANOVA). E) Three days post intraplantar (i.pl.) 5 % formalin injection eIF4E^{S209A} mice exhibited a significantly higher mechanical withdrawal threshold compared to WT mice (n ≥ 10, F(1, 40)=12.56, P=0.001; post-hoc Bonferroni's ****p<0.0001, two-way ANOVA). F, G) The mGlu1/5 receptor agonist, DHPG (50 nmol), was injected intrathecally (i.t.) in both WT and eIF4E^{S209A} mice. Nocifensive behaviors summed during the 30 min after injection were equal in both strains (n = 8, t-test). However, 6 hr post DHPG i.t. injection, WT mice exhibited mechanical hypersensitivity whereas eIF4E^{S209A} mice did not (n = 8, F(1, 28)=4.63, P=0.04; post-hoc Bonferroni's **p=0.007, two-way ANOVA).

We next used a variety of histochemical markers to assess the possibility of developmental differences in sensory anatomy between *eIF4E^{S209A}* mice and WT littermates. In both WT (Figure 3.2A) and *eIF4E^{S209A}* (Figure 3.2B) mice there was a clear delineation between the projections of calcitonin gene-related peptide (CGRP) positive afferents and the isolectin B_4 (IB_4) population that projects to lamina II of the dorsal horn in both genotypes. This was also true for TRPV1-positive and IB_4-positive staining. In contrast, CGRP and TRPV1 afferents overlapped heavily in projections to lamina I and lamina II in both genotypes. Peripherin is expressed predominately in unmyelinated neurons in the DRG, whereas NF200 staining is used to label myelinated, large diameter afferents that are mostly Aβ fibers. These two populations were non-overlapping in both genotypes (Figure 3.2C) and the proportions of DRG neurons expressing these markers were equivalent. We also assessed CGRP, TRPV1 and IB_4 staining in the DRG between genotypes. IB_4-stained neurons in WT were devoid of TRPV1 and CGRP staining while TRPV1 and CGRP very strongly overlapped.

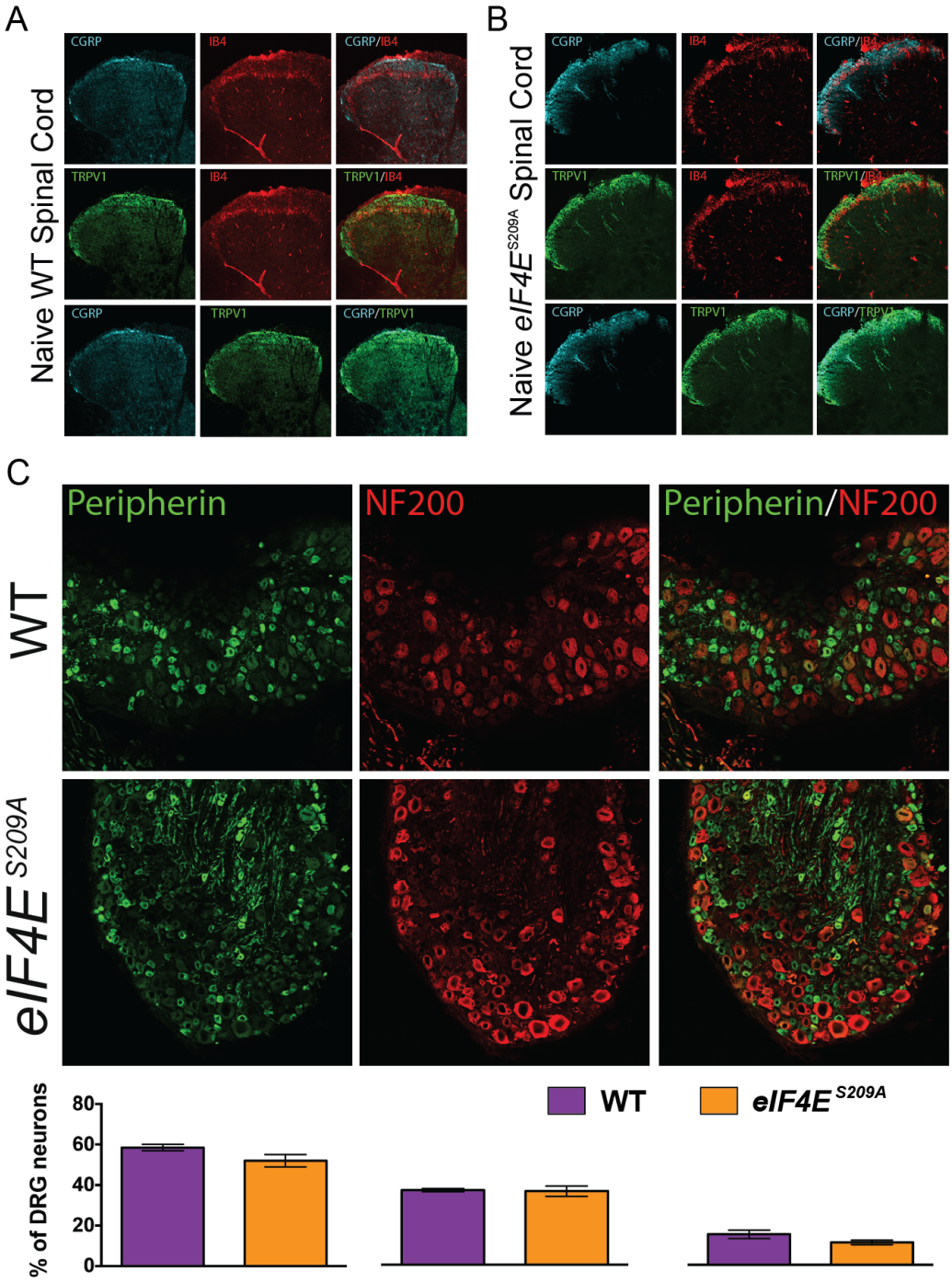


Figure 3.2. Normal development of nociceptive pathways in eIF4E^{S209A} mouse DRG and spinal cord. A, B) WT and eIF4E^{S209A} mouse spinal cords were immunostained with CGRP (cyan), TRPV1 (green), and IB_4 (red). Images show segregation of IB_4 from TRPV1 and CGRP in both

genotypes but overlap of CGRP and TRPV1 in outer lamina of spinal cord (representative images from n = 3 mice). **C)** Immunostaining for peripherin (green) and neurofilament 200 (NF200; red) in DRG from WT and eIF4E^{S209A} mice show no differences in the proportion of peripherin positive neurons per section (n = 3; WT mean = 58.5% ± 1.56% vs. eIF4E^{S209A} mean = 52.0 ± 3.0%), the proportion of NF200-positive neurons per section (n = 3; WT mean = 36.5% ± 0.8% vs. eIF4E^{S209A} mean = 36.1% ± 2.5%) or in overlap between the two markers (n = 3; WT mean = 15.0% ± 2.0% vs. eIF4E^{S209A} mean = 11.0% ± 1.2%).

Identical staining patterns were observed in *eIF4E^{S209A}* mice (Figure 3.2, extended data figure 1).

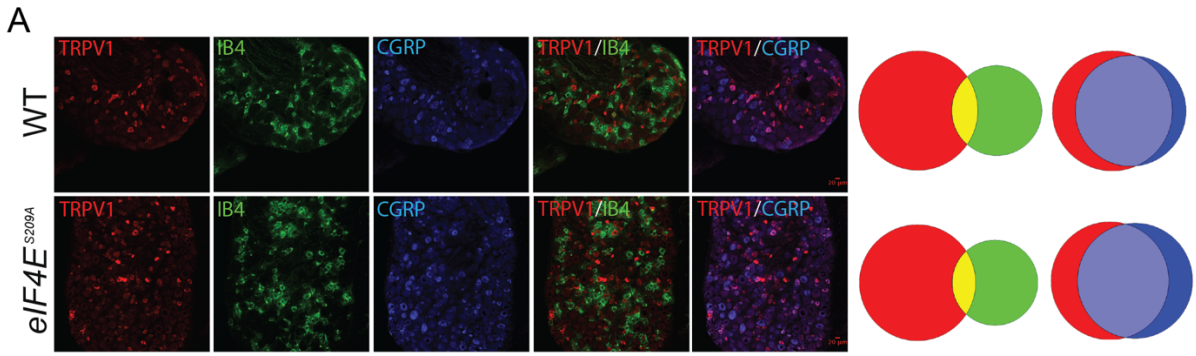


Figure 3.2.1. Normal development of nociceptor populations in eIF4E^{S209A} mouse DRGs. WT and eIF4E^{S209A} DRGs were stained for TRPV1 (red), IB_4 (green), and CGRP (blue), and the proportion of neurons expressing each marker was assessed (TRPV1: WT mean = 34.3% ± 3.1% vs. eIF4E^{S209A} mean = 32.9% ± 1.3%; IB_4: WT mean = 19.6% ± 1.8% vs. eIF4E^{S209A} mean = 17.8% ± 2.0%; WT mean = CGRP: 29.1% ± 1.5% vs. eIF4E^{S209A} mean = 31.5% ± 2.0%). TRPV1 and IB_4 populations were segregated in both mouse strains (TRPV1/IB_4 overlap: WT mean = 3.7% ± 0.1% vs. eIF4E^{S209A} mean = 3.2% ± 0.7%) while TRPV1 and CGRP overlapped substantially (TRPV1/CGRP overlap: WT mean = 74.2% ± 6.3% vs. eIF4E^{S209A} mean = 71.1% ± 2.3%) as demonstrated in the proportional Venn diagrams.

We were concerned about the possibility of feedback signaling that might change activity in upstream signaling pathways (Carracedo et al., 2008a; Melemedjian et al., 2013d) and complicate interpretation of experimental results. We dissected lumbar DRG and dorsal horn spinal cord from WT and *Eif4E^{S209A}* mice and examined Eif4E, extracellular signal regulated protein kinase (ERK) and 4E-BP phosphorylation in both tissues. While Eif4E phosphorylation

was completely absent in *Eif4E*^{S209A} mice, there was no change in either ERK (Figure 3.3A) or 4E-BP (Figure 3.3B) phosphorylation in DRG or spinal cord (Figure 3.3C and D) in *Eif4E*^{S209A} mice. These results rule out the possibility of feedback signaling in the ERK and Mtor pathways in tissues relevant to algesiometric assays.

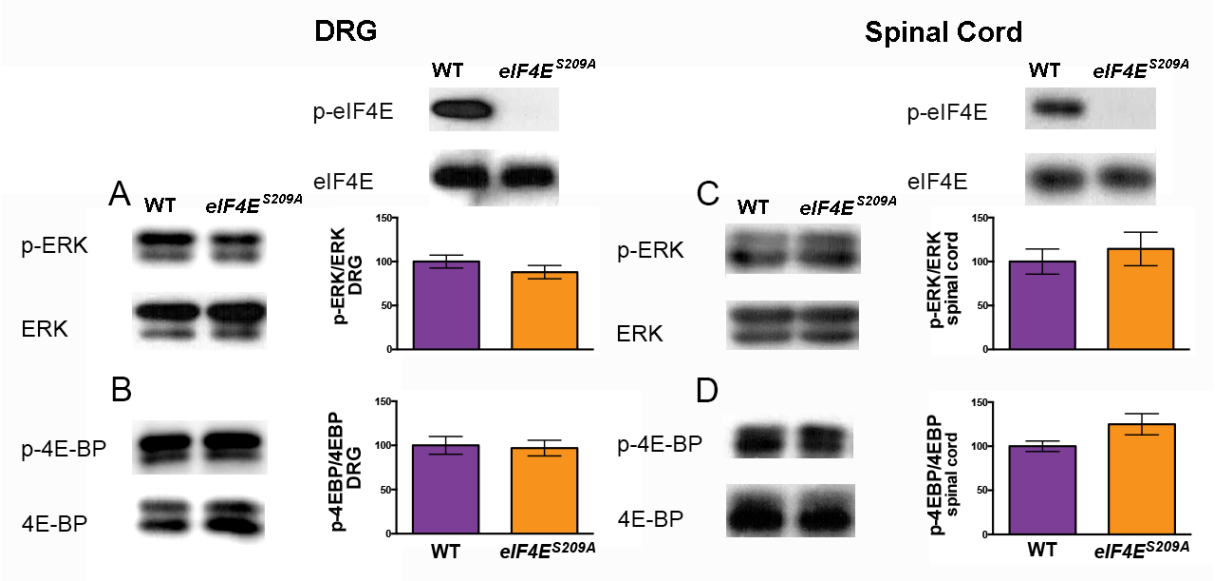


Figure 3.3. Normal ERK and 4E-BP phosphorylation in eIF4E^{S209A} *mouse DRG and spinal cord.* A, B) eIF4E^{S209A} DRG shows equal levels of ERK and 4E-BP phosphorylation while eIF4E phosphorylation is completely absent compared to WT DRG using western blot analysis (n = 6, t-test). **C, D)** Additionally, spinal cord from eIF4E^{S209A} shows similar levels of p-ERK and p-4E-BP compared to WT spinal cord (n = 6, t-test).

From these initial experiments we conclude that Eif4E phosphorylation is not required for the development of normal nociceptive reflexes or acute nociceptive behavior in response to irritants. Moreover, major classes of sensory neurons develop normally in these mice, and at the gross histochemical level have appropriate connectivity to the spinal dorsal horn. However, despite these similarities, mice lacking Eif4E phosphorylation fail to develop mechanical

hypersensitivity in response to formalin and DHPG. Our subsequent experiments focused on understanding the role of Eif4E phosphorylation in nociceptor sensitization.

Deficits in mechanical sensitization, affective pain expression, and the development of hyperalgesic priming in Eif4E^{S209A} mice

Previous studies have shown that IL-6, NGF (Melemedjian et al., 2010a) and activation of protease activated receptor 2 (PAR2) (Tillu et al., 2015b)) promote mechanical hypersensitivity in an ERK-dependent fashion that requires *de novo*, local protein synthesis. To test the role of Eif4E phosphorylation in this process we injected IL-6, NGF and the PAR2 agonist, 2at-LIGRL (Flynn et al., 2011) into the hindpaw of WT and *Eif4E^{S209A}* mice. IL-6 (0.1 ng) injected into the paw evoked mechanical hypersensitivity lasting approximately 72 hr (Figure 3.4A) in WT mice. The magnitude of mechanical hypersensitivity was significantly reduced in *Eif4E^{S209A}* mice 24, 48 and 72 hr after injection (Figure 3.4A). We, and others, have previously shown that activity-dependent translation regulation pathways are required for the full expression of hyperalgesic priming (Melemedjian et al., 2010a; Asiedu et al., 2011b; Bogen et al., 2012a; Melemedjian et al., 2014b; Ferrari et al., 2015a; Ferrari et al., 2015b) but the role of Eif4E phosphorylation in this transition to a chronic pain state has not been addressed. We "primed" WT and *Eif4E^{S209A}* mice with IL-6 (Figure 3.4B) and, after their mechanical thresholds had completely returned to baseline, challenged these mice with a dose of PGE_2 (100 ng) that fails to induce mechanical hypersensitivity in "unprimed" mice. We observed that the response to PGE_2 injection in *Eif4E^{S209A}* mice was blunted compared to WT mice (Figure 3.4B).

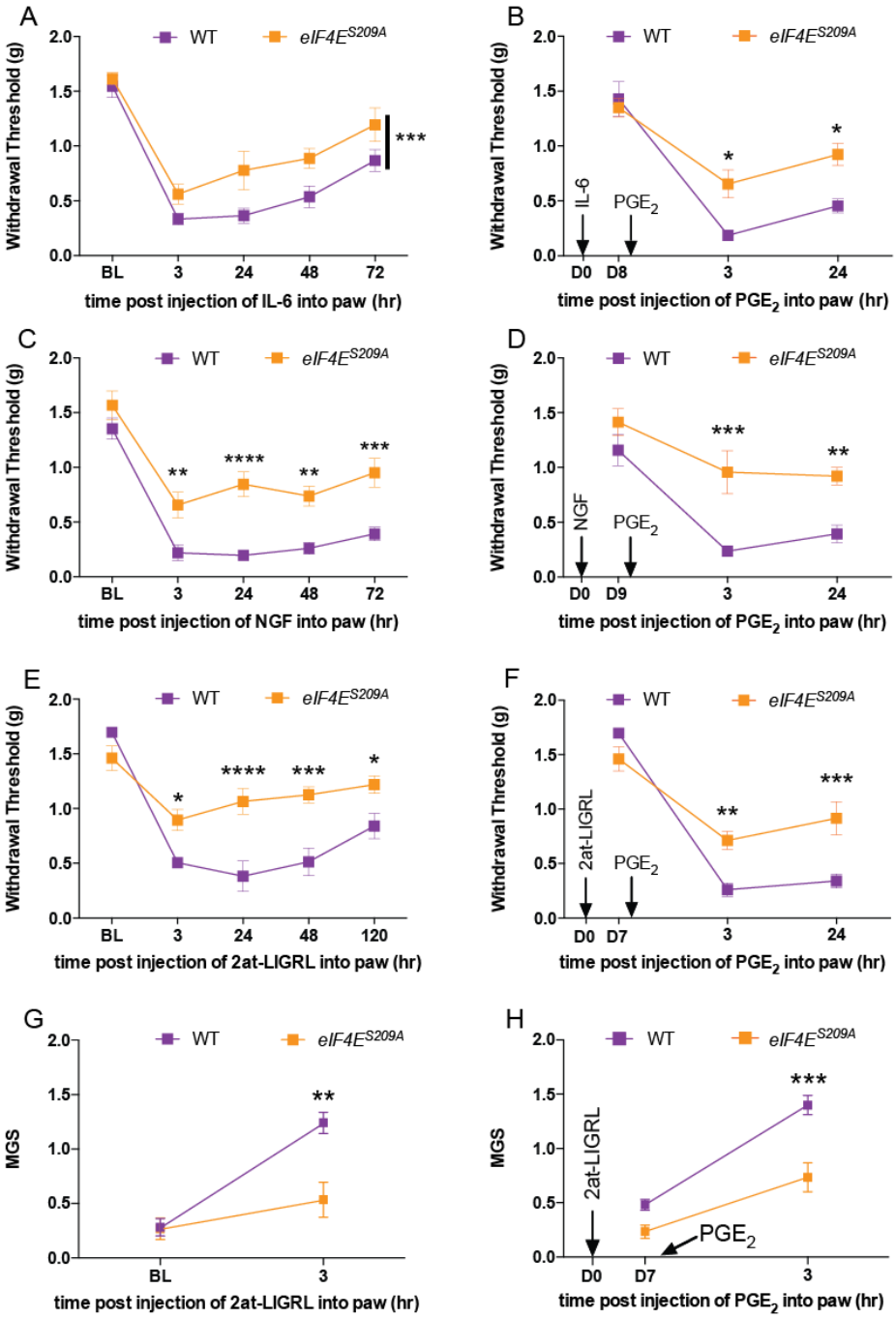


Figure 3.4. Mechanical hypersensitivity, facial grimacing, and the development of hyperalgesic priming are decreased in eIF4E^{S209A} mice. A) IL-6 (0.1 ng) was injected into the hindpaw in both WT and eIF4E^{S209A} mice. Hindpaw mechanical thresholds were measured at 3, 24, 48, and 72 hr. eIF4E^{S209A} mice exhibited reduced mechanical hypersensitivity compared to WT mice (n ≥ 6, F(1, 60)=15.09, P=0.0003, two-way ANOVA). B) eIF4E^{S209A} mice also demonstrated

decreased mechanical hypersensitivity in response to i.pl. injection of 50 ng NGF ($n \geq 6$, $F(1, 60)=67.01$, $P<0.0001$; post-hoc Bonferroni's **p=0.0056, 0.0021, ***p=0.0002, ****p<0.0001, two-way ANOVA). C, D) I.pl. injection of 20 ng PAR2 agonist 2at-LIGRL likewise induced decreased mechanical hypersensitivity in $eIF4E^{S209A}$ mice and reduced facial grimacing 3 hr after injection (MH: $n \geq 6$, $F(1, 50)=33.57$, $P<0.0001$; post-hoc Bonferroni's *p=0.0399, 0.0489 ***p=0.0004, ****p<0.0001, two-way ANOVA; Grimace: $n \geq 6$, $t=0.7933$, $df=18$, **p=0.011, two-way ANOVA). After mice recover from the initial mechanical hypersensitivity from IL-6, NGF, or 2at-LIGRL, hyperalgesic priming is assessed through i.pl. injection of PGE_2 (100 ng). $eIF4E^{S209A}$ mice show a decreased response to PGE_2 reflecting a failure of hyperalgesic priming in response to IL-6 (E, $n \geq 6$, $F(1, 36)=10.83$ $P=0.0022$; post-hoc Bonferroni's *p=0.0109, 0.0108, two-way ANOVA), NGF (F, $n \geq 6$, $F(1, 36)=28.24$, $P<0.0001$; post-hoc Bonferroni's **p=0.0081, ***p=0.0003, two-way ANOVA) and 2at-LIGRL (G, $n \geq 6$, $F(1, 30)=12.16$, $P=0.0015$; post-hoc Bonferroni's **p=0.0049, ***p=0.0004, two-way ANOVA). H) Moreover, $eIF4E^{S209A}$ mice fail to show facial grimacing after 2at-LIGRL priming when subsequently challenged with PGE_2 ($n \geq 6$, $t=5.053$, $df=18$, ***p=0.0002, two-way ANOVA).

Similar experiments were done using a hindpaw injection of NGF (50 ng). NGF evoked robust mechanical and thermal hypersensitivity (Hargreaves et al., 1988) in WT mice, whereas in *Eif4E*^{S209A} mice it was dramatically reduced (Figure 3.4C, mechanical & Figure 3.4, extended data figure 1A, thermal). After the mice returned to baseline mechanical and thermal thresholds, we assessed priming with a hindpaw injection of PGE_2. We observed that similar to IL-6-induced priming, NGF was unable to prime *Eif4E*^{S209A} mice compared to WT mice (Figure 3.4D). We observed transient thermal hypersensitivity during priming in WT mice, but saw no change in *Eif4E*^{S209A} mice (Figure 3.4, extended data figure 1B).

Likewise, the specific PAR2 agonist 2at-LIGRL (20 ng) evoked mechanical hypersensitivity and hyperalgesic priming precipitated by PGE_2 in WT mice but this effect was strongly reduced in *Eif4E*^{S209A} mice (Figure 3.4E and F). These results indicate that Eif4E phosphorylation is a key downstream event for pronociceptive factors that act via the ERK pathway to promote mechanical and thermal hypersensitivity, but whether this also influences spontaneous, non-

evoked components of pain is not known. We used the mouse grimace scale (MGS) (Langford et al., 2010)) to determine this with hindpaw injection of 2at-LIGRL (20 ng).

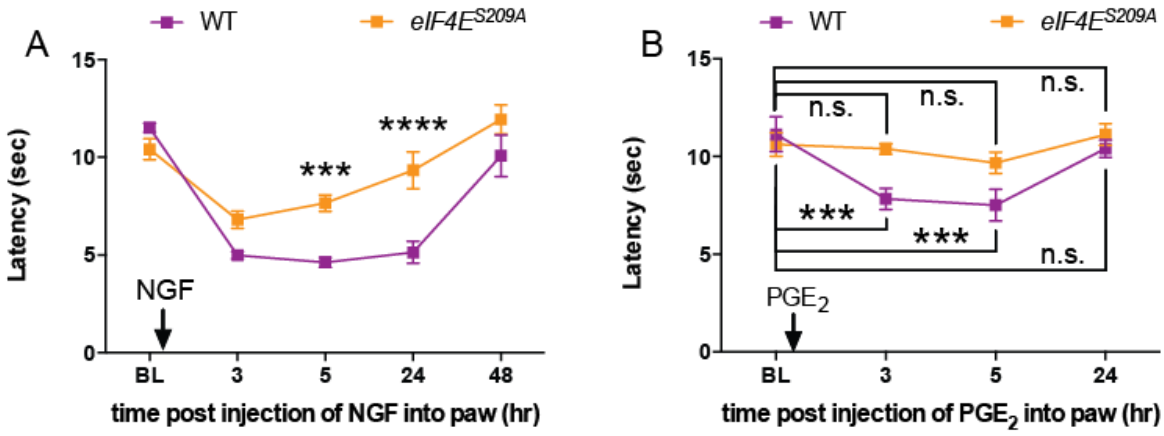


Figure 3.4.1. Thermal hypersensitivity induced by NGF is dependent on eIF4E phosphorylation. A**)** WT and eIF4E^{S209A} mice received a hindpaw injection of NGF (50 ng). Thermal sensitivity was measured using a Hargreaves device with 20% laser intensity. Latency to withdrawal was measured at 3, 5, 24, and 48 hr. eIF4E^{S209A} mice displayed a blunted thermal hypersensitivity compared to WT mice (n = 6, F(1, 50)=26.22, P<0.0001, post-hoc Bonferroni's **p<0.0044, ****p<0.0001). **B)** Additionally, hyperalgesic priming evoked by PGE_2 produced acute thermal hypersensitivity in WT mice, but not in eIF4E^{S209A} mice (n = 6, F(1, 40)=8.187, P=0.0067, post-hoc Uncorrected Fisher's LSD ***p=0.0004, 0.0001).

While PAR2 activation induced an increase in grimacing in WT mice, it failed to do so in *Eif4E^{S209A}* mice (Figure 3.4G) suggesting that this signaling pathway is critical for full expression of affective pain components downstream of ERK activation. Additionally, when we measured facial grimacing in response to PGE_2 injection in mice previously injected with 2at-LIGRL, there was a significant increase in grimacing in WT mice but no change in facial expression scores in Eif4E^{S209A} mice (Figure 3.4H).

Mechanical and thermal hypersensitivity induced by complex inflammatory stimuli are dependent on MNK1/2- Eif4E phosphorylation signaling

While Eif4E phosphorylation is necessary for mechanical and thermal hypersensitivity induced by algogens that signal via ERK, we asked if Eif4E phosphorylation would be required for mechanical and thermal hypersensitivity induced by inflammation. We utilized a hindpaw injection of carrageenan (0.5% w/v) in WT and *Eif4E*^{S209A} mice and measured mechanical (Figure 3.5A) and thermal hypersensitivity (Figure 3.5B). WT mice developed robust mechanical and thermal hypersensitivity whereas no change was seen in *Eif4E*^{S209A} mice (Figure 3.5A and B). When we tested if carrageenan-induced hyperalgesic priming was dependent on Eif4E phosphorylation, we observed that WT mice developed increased long-lasting mechanical hypersensitivity compared to *Eif4E*^{S209A} mice when priming was precipitated with PGE_2 injection (Figure 3.5C).

Additionally, we utilized mice lacking *MNK1* and *2 (MNK*$^{-/-}$ *mice)* and complete Freund's adjuvant (CFA) to further test the role of this signaling axis in inflammatory pain. We injected CFA (0.5 mg/Ml in 10Ml) into the hindpaw of WT and *MNK*$^{-/-}$ mice. While we observed mechanical (Figure 3.5D) and thermal (Figure 3.5E) hypersensitivity in both WT and *MNK*$^{-/-}$ mice at early time points, *MNK*$^{-/-}$ mice recovered faster than their WT littermates. When we tested if *MNK*$^{-/-}$ mice transitioned into the "primed" state with a subsequent injection of PGE_2, we saw a complete lack of mechanical (Figure 3.5F) and thermal (Figure 3.5G) hypersensitivity in *MNK*$^{-/-}$ mice but a robust response in WT mice. These results combined with our previous findings indicate that MNK1/2-mediated Eif4E phosphorylation is required for inflammatory behavioral nociceptive plasticity.

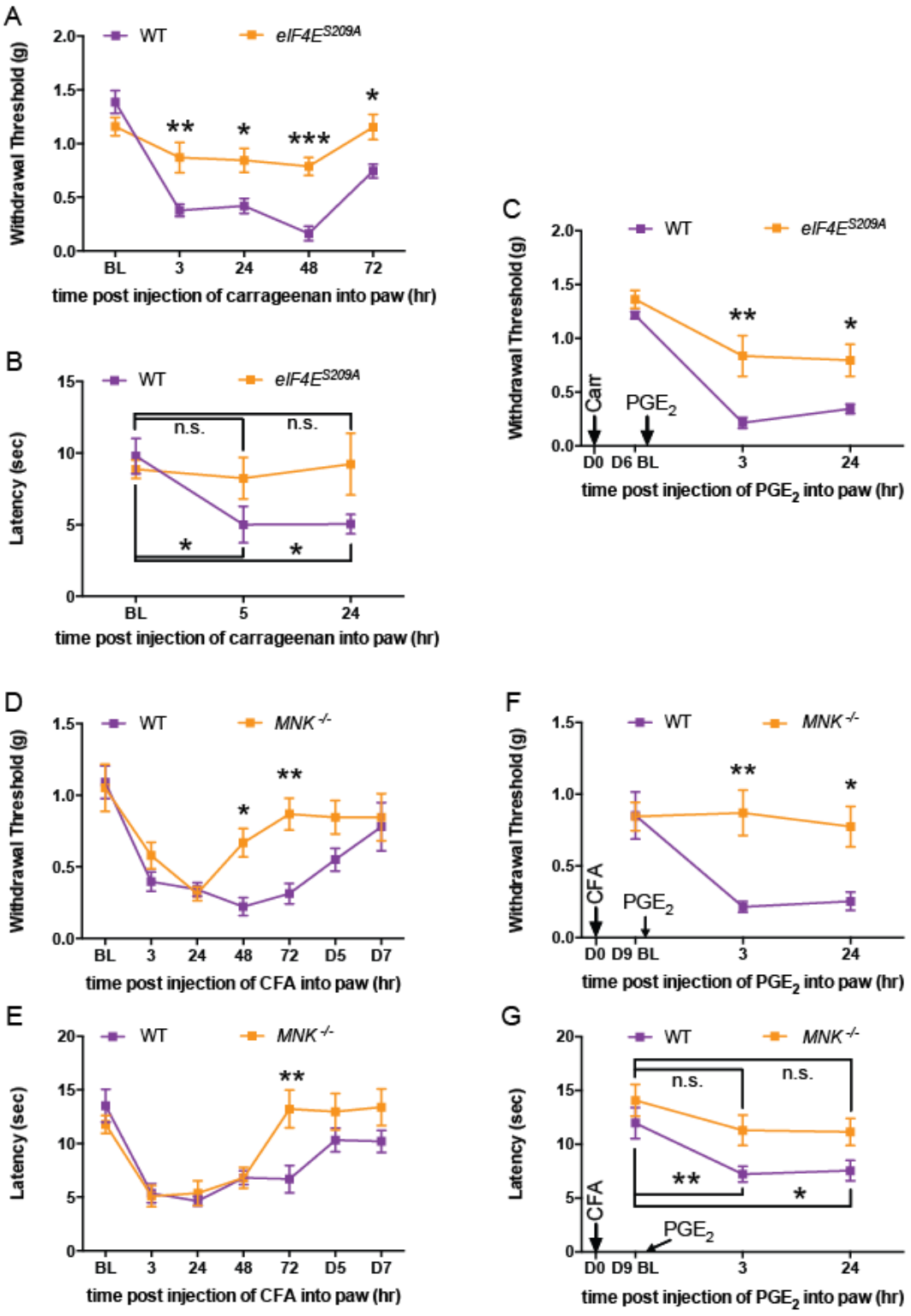


Figure 3.5. MNK1/2 - eIF4E signaling controls the development of mechanical and thermal

hypersensitivity and the development of hyperalgesic priming in response to inflammatory stimuli. A, B) Carrageenan (0.5% w/v) was injected into the hindpaw in both WT and eIF4E^{S209A} mice. Hindpaw mechanical and thermal thresholds show that eIF4E^{S209A} mice exhibited a blunted mechanical and thermal hypersensitivity compared to WT mice (A: $n \geq 5$, $F(1, 45)=30.88$, $P < 0.0001$; post-hoc Bonferroni's *p=0.0185, 0.0246, **p=0.0045, ***p=0.0002 two-way ANOVA; B: $n \geq 5$, $F(1, 27)=3.626$, $P=0.0676$; post-hoc uncorrected Fisher's LSD *p=0.0282, 0.0293, two-way ANOVA). C) eIF4E^{S209A} mice failed to develop hyperalgesic priming when injected with PGE_2 ($n \geq 5$, $F(1, 27)=17.69$, $P=0.0003$; post-hoc Bonferroni's *p=0.0363, **p=0.0029, two-way ANOVA). D, E) Mnk1/2$^{-/-}$ mice injected i.pl. with CFA (0.5 mg/mL, 10μL) recover faster in both mechanical and thermal hypersensitivity compared to WT mice (D: $n = 9$, $F(1, 112)=13.27$, $P=0.0004$; post-hoc Bonferroni's *p=0.0304, **p=0.0031, two-way ANOVA; E: $n = 9$, $F(1, 112)=5.989$, $P=0.0159$; post-hoc Bonferroni's **p=0.0016, two-way ANOVA). F, G) Mnk1/2$^{-/-}$ mice show decreased mechanical and thermal response to PGE_2 after recovering from the initial hypersensitivity from CFA. (F: $n = 9$, $F(1, 48)=15.58$, $P=0.0003$; post-hoc Bonferroni's *p=0.0113, **p=0.0011, two-way ANOVA; G: $n = 9$, $F(1, 48)=10.34$, $P=0.0023$; uncorrected Fisher's LSD *p=0.0156, **p=0.0098, two-way ANOVA).

Pharmacological inhibition of MNK1/2 with cercosporamide recapitulates Eif4E^{S209A} phenotypes

We hypothesized that cercosporamide, which inhibits MNK1/2, should mimic effects seen in *Eif4E^{S209A}* mice, demonstrating that this pathway can be targeted pharmacologically for the alleviation of pain hypersensitivity. To first determine that cercosporamide inhibits Eif4E phosphorylation in DRG neurons these cells were cultured for 5 days and exposed to 10 μM cercosporamide (Altman et al., 2013) or vehicle for 1 hr. Western blot analysis demonstrated a significant decrease of p-Eif4E in treated DRG neurons compared to vehicle (Figure 3.6A). Cercosporamide-treated DRG neurons showed no change in levels of p-4E-BP1 (Figure 3.6B), indicating that cercosporamide does not induce feedback activation of Mtorc1. Levels of p-ERK were also unchanged (Figure 3.6C), demonstrating that upstream regulators of MNK1/2 are unaffected by cercosporamide, and are not activated via a feedback mechanism as we have shown previously for Mtorc1 inhibitors (Melemedjian et al., 2013d). These results show that

cercosporamide blocks MNK1/2-induced Eif4E phosphorylation in DRG neurons without influencing other signaling components that might complicate interpretation of the effects of the compound. We also assessed whether systemic injection of cercosporamide (40 mg/kg) (Gkogkas et al., 2014a)) in mice influences Eif4E phosphorylation. In DRG tissue taken 2 hr after cercosporamide injection we observed a ~50% decrease in Eif4E phosphorylation (Figure 3.6D), whereas no effect was observed in 4E-BP1 phosphorylation (Figure 3.6E).

We then sought to determine the effects of cercosporamide on NGF- and PAR2 activation-induced mechanical hypersensitivity *in vivo*. Similar to observations in *Eif4E*^{S209A} mice, treatment with cercosporamide (10 μg, intraplantar) 15 min prior NGF (Figure 3.6F) or at the time of 2at-LIGRL (Figure 3.6H) injection blunted mechanical hypersensitivity induced by both of these pronociceptive factors. We also tested the ability of cercosporamide, given at the time of the priming stimulus, to prevent the development of subsequent hyperalgesic priming. In mice treated with cercosporamide and primed with NGF, there was no response to injection of PGE_2 15 days later (Figure 3.6G). Similar results were obtained when 2at-LIGRL was used as the priming stimulus (Figure 3.6H and I). Moreover, co-injection of cercosporamide with 2at-LIGRL attenuated grimacing recorded 3 hr after injection (Figure 3.6J), and prevented facial grimacing (Figure 3.6K) in response to PGE_2 injection, again consistent with observations in *Eif4E*^{S209A} mice.

Our demonstration that hindpaw cercosporamide administration profoundly reduces behavioral pain plasticity suggests that p-Eif4E-mediated local translation in peripheral nociceptive fibers contributes to this effect. We therefore sought to evaluate whether p-Eif4E could be observed in nerve fibers innervating the hindpaw. Glabrous skin from both WT and

Eif4E^{S209A} mice were immunostained for TRPV1 and p-Eif4E and imaged. We observed p-Eif4E in TRPV1 positive nerve fibers in WT mice and this staining was completely absent in *Eif4E*^{S209A} mouse skin samples (Figure 3.6L) demonstrating the specificity of this antibody and the presence of p-Eif4E in terminals of TRPV1-positive nociceptors.

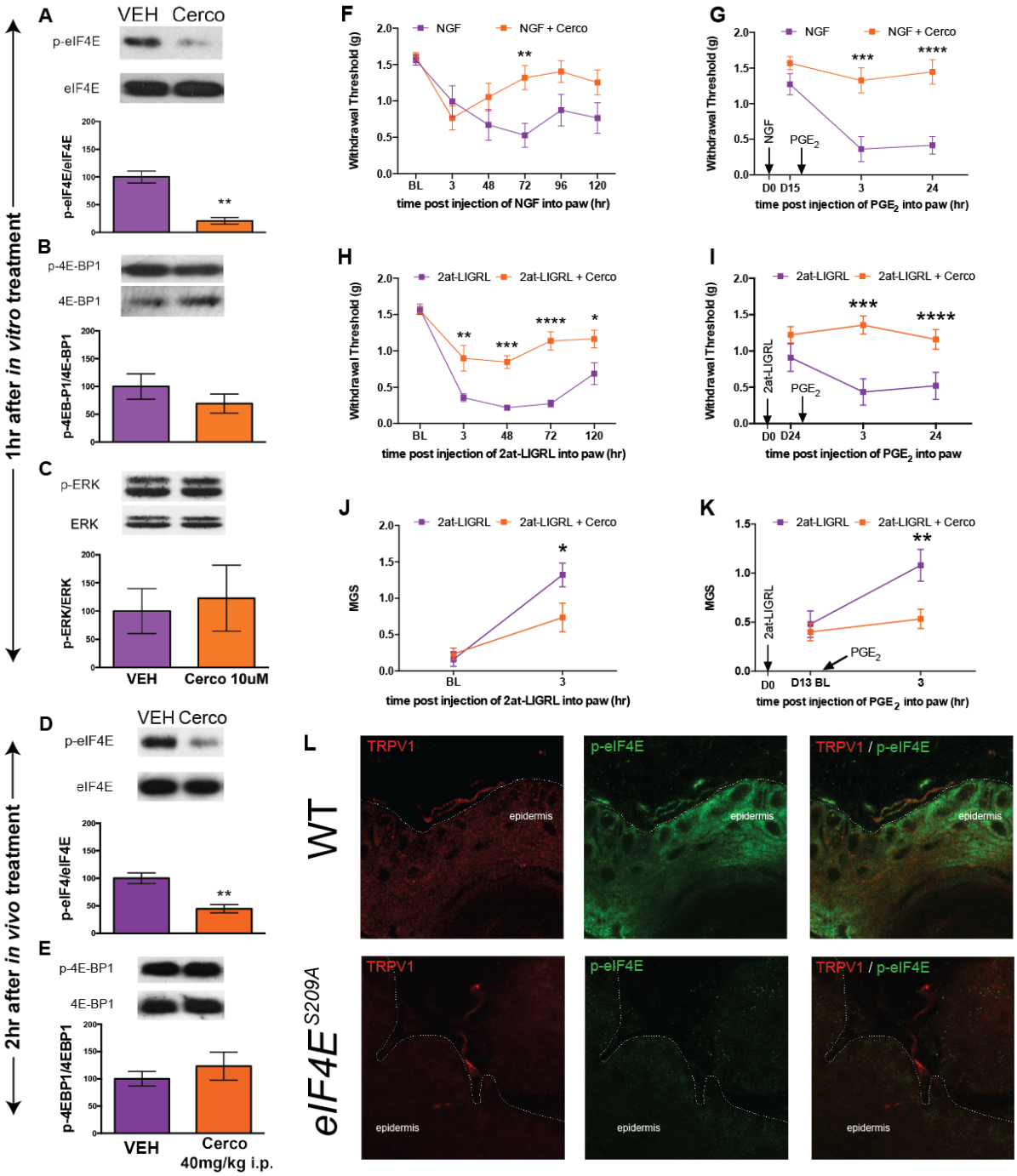


Figure 3.6. Local, hindpaw pharmacological inhibition of MNK1/2 with cercosporamide reduces eIF4E phosphorylation, mechanical hypersensitivity, grimacing, and blocks the development of hyperalgesic priming. A) One hr in vitro treatment of DRG neurons with cercosporamide (cerco, 10 μM) decreased eIF4E phosphorylation (n = 3, t=6.634, df=4,

**p=0.0027, t-test) but did not influence 4E-BP1 phosphorylation (B, n = 3) or ERK phosphorylation (C, n = 3). D) Intraperitoneal injection of cercosporamide (40 mg/kg) results in a decreased eIF4E phosphorylation in DRGs (n = 3, t=4.507, df=10, **p=0.0011, t-test) but does not affect 4E-BP1 phosphorylation (E, n = 3). F, G) Mechanical hypersensitivity induced by NGF (50 ng) or 2at-LIGRL (20 ng) was blocked by cercosporamide (10 μg, given by i.pl. co-injection) (NGF: n ≥ 6, F(1, 114)=11.43, P=0.001; post-hoc Bonferroni's **p=0.009) (2at-LIGRL: n ≥ 6, F(1, 45)=54.46, P<0.0001; post-hoc Bonferroni's *p=0.014, **p=0.0042, ***p=0.0007, ****p<0.0001, two-way ANOVA). H) Facial grimacing induced by i.pl. injection of 2at-LIGRL (20 ng) was also blocked with local cercosporamide (10 μg) treatment (n ≥ 5, t=2.846, df=18, *p=0.0215, two-way ANOVA). Similar to eIF4E^{S209A} mice, pharmacological inhibition of MNK1/2 using cercosporamide blocked the development of hyperalgesic priming induced by NGF (I, n ≥ 6, F(1, 54)=37.72, P<0.0001; post-hoc Bonferroni's ***p=0.0001, ****p<0.0001, two-way ANOVA) and 2at-LIGRL (J, n ≥ 6, F(1, 27)=24.95, P<0.0001; post-hoc Bonferroni's *p=0.0195, ***p=0.0007, two-way ANOVA) when measuring both mechanical hypersensitivity and facial grimacing (K, n ≥ 6, t=3.204, df=18, **p=0.0098). L) Additionally, immunostaining of glabrous skin for TRPV1 (red) and p-eIF4E (green) revealed that eIF4E phosphorylation is present in TRPV1-positive fibers in WT, but absent in eIF4E^{S209A} mice (representative images from n = 2 mice per genotype).

To assess if cercosporamide is specific to MNK1/2 in our behavioral paradigm, we utilized cercosporamide in *Eif4E*^{S209A} mice and measured mechanical hypersensitivity induced by NGF. We found no differences in NGF-induced mechanical hypersensitivity between cercosporamide and vehicle injected *Eif4E*^{S209A} mice (Figure 3.6, extended data figure 1A). Subsequent injection of PGE_2 to precipitate hyperalgesic priming, additionally showed no difference in either *Eif4E*^{S209A} mice previously injected with cercosporamide or vehicle (Figure 3.6, extended data figure 1B). Hence, cercosporamide does not appear to produce additional effects that might be attributed to targets other than MNK1/2. Collectively, these experiments support the conclusion that MNK1/2 can be pharmacologically targeted locally for the alleviation of pain hypersensitivity and for the prevention of pain chronification.

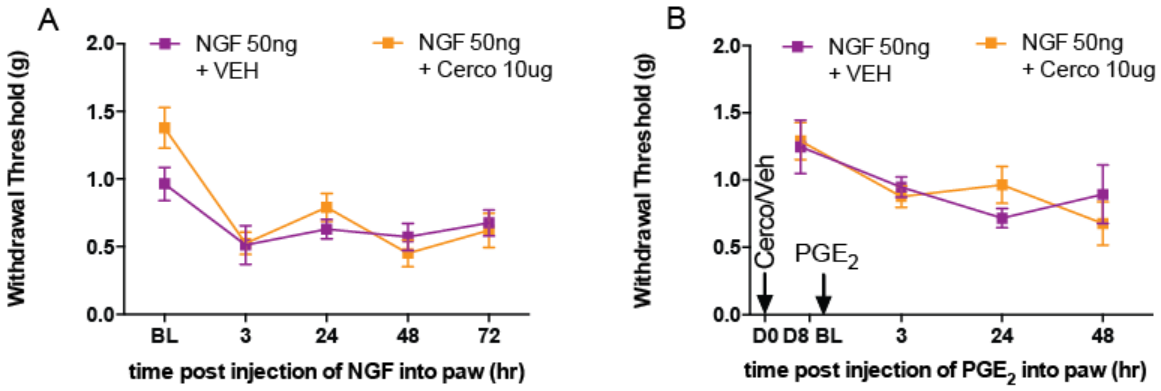


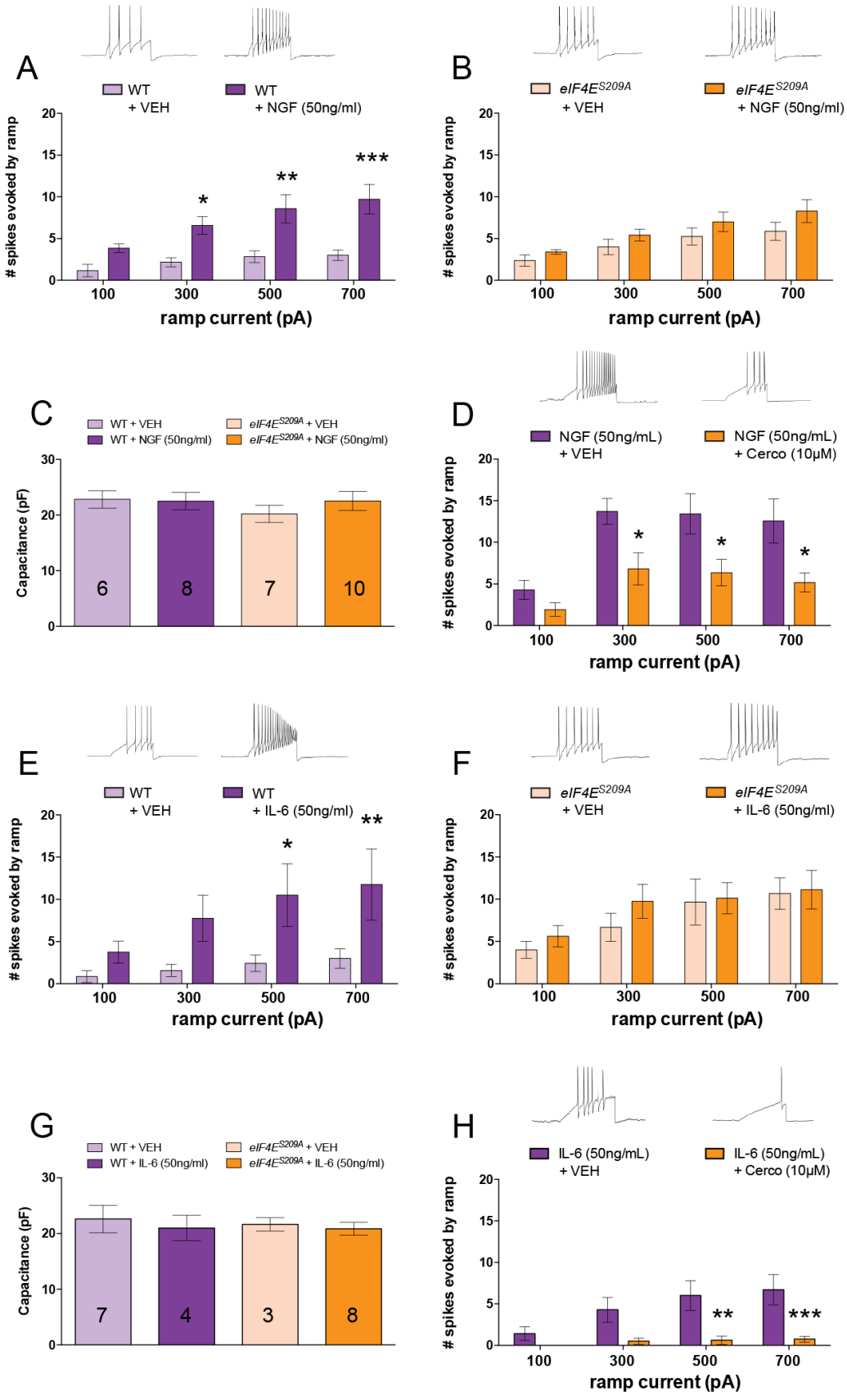
Figure 3.6.1. Cercosporamide has no additional inhibition on NGF-induced mechanical hypersensitivity or hyperalgesic priming in eIF4E^{S209A} mice. A) Mechanical hypersensitivity induced by NGF (50 ng) showed no difference between eIF4E^{S209A} mice that additionally received a hindpaw injection of cercosporamide (10 µg) and eIF4E^{S209A} mice that did not ($n \geq 4$, $p>0.05$, two-way ANOVA). B) Subsequent injection of PGE_2 to precipitate priming also shows no difference between eIF4E^{S209A} mice that previously received cercosporamide and eIF4E^{S209A} mice that did not ($n \geq 4$, $p>0.05$, two-way ANOVA).

Eif4E phosphorylation regulates DRG neuron excitability following NGF and IL-6 exposure

While the *in vivo* effects of NGF and IL-6 in WT versus *Eif4E^{S209A}* mice suggest that Eif4E phosphorylation is required for changes in excitability in DRG nociceptive neurons, these behavioral experiments do not specifically delineate the cellular target. To directly test the effect of NGF and IL-6 on DRG neuron excitability in the presence and absence of Eif4E phosphorylation we used patch clamp electrophysiology. DRG neurons were isolated from WT and *Eif4E^{S209A}* mice and exposed to 50 ng/Ml NGF or vehicle for 18 – 24 hr prior to recordings. In WT neurons, NGF caused an increase in the number of action potentials fired in response to slowly depolarizing ramp currents of 100 through 700 Pa amplitudes (Figure 3.7A). In contrast, DRG neurons isolated from *Eif4E^{S209A}* mice showed a trend toward elevated baseline excitability

versus WT neurons but did not show a change in their excitability after exposure to the same concentration of NGF over an identical time course (Figure 3.7B). For the DRG neurons sampled from both treatments and genotypes there were no differences in membrane capacitance (Figure 3.7C) or other parameters such as resting membrane potential (WT: -63.34 ± 1.12 Mv, n=10; *Eif4E*^{S209A}: -61.63 ± 1.13 Mv, n =11, $p < 0.05$, t-test). We next examined NGF-induced changes in DRG excitability in the presence of cercosporamide (10 μM) for 1 hr prior to recordings. Analogous to *Eif4E*^{S209A} DRG neurons, cercosporamide blocked NGF-induced hyperexcitability (Figure 3.7D), demonstrating that brief pharmacological inhibition of MNK1/2 reverses augmented excitability in DRG neurons induced by NGF treatment.

Similar experiments were conducted with IL-6 (50 ng/Ml), except that IL-6 was only applied for 1 hr prior to patch clamp recordings. IL-6, as we have observed previously in rat trigeminal ganglion neurons (Yan et al., 2012a), caused an increase in the number of action potentials fired in response to ramp current injection at 300, 500 and 700 Pa amplitudes (Figure 3.7E). As we observed with NGF, IL-6 failed to increase the excitability of DRG neurons from *Eif4E*^{S209A} mice (Figure 3.7F). Again, there were no significant differences in membrane capacitance in the populations sampled for any of these experimental conditions (Figure 3.7G). When we examined the effect of cercosporamide on IL-6-induced hyperexcitability, we found that synonymous to *Eif4E*^{S209A} DRG neurons, cercosporamide inhibited increased neuronal excitability induced by IL-6 treatment (Figure 3.7H). While our behavioral results suggest that cercosporamide's actions are specific to MNK1/2, we wanted to also test for a possible acute effect of cercosporamide on voltage-gated sodium channels (VGNaC) that could lead to a decrease in excitability. To test if cercosporamide acutely affected sodium channels, VGNaC


A
WT
+ VEH
WT
+ NGF (50ng/ml)
# spikes evoked by ramp
ramp current (pA)
B
eIF4E S209A
+ VEH
eIF4E S209A
+ NGF (50ng/ml)
# spikes evoked by ramp
ramp current (pA)
C
WT + VEH
WT + NGF (50ng/ml)
eIF4E S209A + VEH
eIF4E S209A + NGF (50ng/ml)
Capacitance (pF)
6
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7
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D
NGF (50ng/mL)
+ VEH
NGF (50ng/mL)
+ Cerco (10μM)
# spikes evoked by ramp
ramp current (pA)
E
WT
+ VEH
WT
+ IL-6 (50ng/ml)
# spikes evoked by ramp
ramp current (pA)
F
eIF4E S209A
+ VEH
eIF4E S209A
+ IL-6 (50ng/ml)
# spikes evoked by ramp
ramp current (pA)
G
WT + VEH
WT + IL-6 (50ng/ml)
eIF4E S209A + VEH
eIF4E S209A + IL-6 (50ng/ml)
Capacitance (pF)
7
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8
H
IL-6 (50ng/mL)
+ VEH
IL-6 (50ng/mL)
+ Cerco (10μM)
# spikes evoked by ramp
ramp current (pA)

Figure 3.7. MNK1/2 - eIF4E signaling mediates NGF- and IL-6- induced changes in excitability in DRG neurons. A) WT DRG neurons were exposed to NGF or vehicle 18-24 hr prior to patch clamp recordings. Ramp current-evoked spiking demonstrates that NGF exposure increases the excitability of WT DRG neurons (n = 6, 7, $F(1, 44)=40.41$, $P<0.0001$, post-hoc Bonferroni's *p=0.0175, 0.0181, **p=0.0056, 0.0075, two-way ANOVA). **B)** $eIF4E^{S209A}$ DRG neurons showed no difference in the number of spikes evoked by ramp currents between NGF and vehicle-treated DRG neurons (two-way ANOVA). **C)** Membrane capacitance measures between WT and $eIF4E^{S209A}$ DRG neurons show no difference in neuron size between samples demonstrating that small diameter neurons were used for recordings (one-way ANOVA). N for each condition is shown in the appropriate bar. **D)** Pharmacological inhibition of MNK1/2 using cercosporamide (10 μM, n = 11) recapitulated the effect seen in $eIF4E^{S209A}$ DRG neurons blocking NGF-induced hyperexcitability (n = 7, $F(1, 64)=24.07$, $P<0.0001$; post-hoc Bonferroni's *p=0.0234, 0.0192, 0.0131, two-way ANOVA). **E)** IL-6 (50 ng/ml, n = 4) exposure to WT DRG neurons for 1 hr caused an increase in excitability compared to vehicle ($n\geq4$, $F(1, 36)=49.81$, $P<0.0001$; post-hoc Bonferroni's **p=0.0012, 0.0023, ***p=0.0005, two-way ANOVA), but failed to do so in $eIF4E^{S209A}$ DRG neurons **(F**, two-way ANOVA). **G)** Membrane capacitance between these samples was not different (one-way ANOVA). N for each condition is shown in the appropriate bar. **H)** Similar to observations with NGF, cercosporamide blocked enhanced excitability induced by IL-6 in small diameter DRG neurons (50ng/mL) ($n \geq 7$, $F(1, 52)=31.22$, $P<0.0001$; post-hoc Bonferroni's **p=0.0026, ***p=0.0007, two-way ANOVA). Traces shown in all panels are for the 700 pA stimulus.

currents were elicited by 50 msec depolarizing steps and currents were expressed as current density. Acute application of cercosporamide (10μM) had no effect on VGNaC density ruling out this possibility (Figure 3.7, extended data figure 1A and B). These findings show that Eif4E phosphorylation plays a direct role in modulating the excitability of DRG nociceptors in response to NGF and IL-6 treatment.

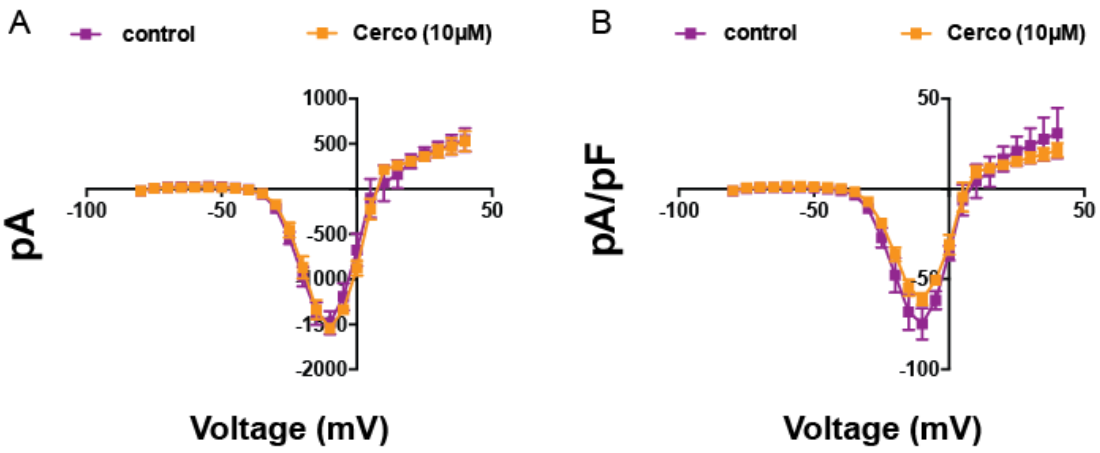

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Figure 3.7.1. Acute application of cercosporamide has no effect on sodium current density. Sodium current **(A)** and sodium current density **(B)** were measured in DRG neurons with cercosporamide (10 μM) perfused acutely over a 5-10 sec period (n = 4, p>0.05, two-way ANOVA) indicating the cercosporamide does not block voltage-gated sodium channels at this concentration.

As an independent assay to support our whole cell patch clamp electrophysiological findings, we assessed excitability with extracellular recordings using microelectrode arrays (MEAs). DRG neurons from both WT and *Eif4E*^{S209A} mice were dissociated and cultured onto MEA devices (Breckenridge et al., 1995; Potter and DeMarse, 2001; Frega et al., 2012; Enright et al., 2016) (Figure 3.8A) for 11 – 15 days prior to recordings. Action potentials were recorded for 1 hr prior to IL-6 exposure, during 1 hr of IL-6 (50ng/Ml) treatment, and again during a 1 hr washout period. WT and *Eif4E*^{S209A} neurons were mostly silent during recordings preceding IL-6 exposure. In WT DRG neurons, spiking was significantly increased during IL-6 treatment and was maintained during washout (Figure 3.8B). In contrast, DRG neurons isolated from *Eif4E*^{S209A} mice, showed a brief increase in spiking in response to IL-6 that rapidly decreased to spiking rates that were significantly less than what was observed in WT neurons (Figure 3.8C).

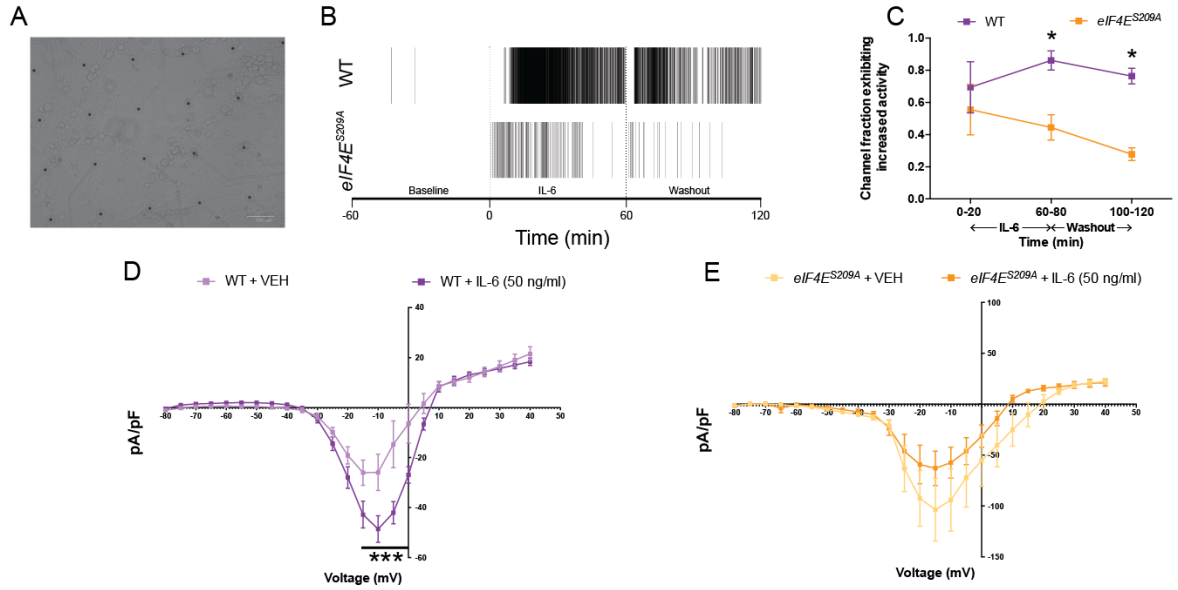

Figure 3.8. IL6-induced sustained spiking and increased voltage gated sodium current density is absent in eIF4E^{S209A} DRG neurons. A) Image of DRG neurons cultured on an MEA device. B) Raster plot showing electrical activity of WT and eIF4E^{S209A} DRG neuronal networks observed using MEAs during BL, IL-6 treatment, and 1 hr wash. C) IL-6 elicited increased spiking in WT DRG cultures during treatment that was sustained throughout the washout period and significantly greater than spiking observed in eIF4E^{S209A} DRG neurons ($n \geq 2$, $F(1, 9)=15.48$, $P=0.0034$; post-hoc uncorrected Fisher's LSD *p=0.0234, 0.0112, two-way ANOVA). D, E) Sodium current density was increased after 1 hr IL-6 exposure in WT DRG neurons, but was decreased in eIF4E^{S209A} DRG neurons (WT: $n \geq 7$, $F(1, 400)=25.78$, $P<0.0001$, post-hoc Bonferroni's ***p=0.0010, ****p<0.0001; eIF4E^{S209A}: $n \geq 12$ $F(1, 725)=7.897$, $P=0.0051$, post-hoc Bonferroni's).

These whole cell patch clamp and MEA experiments indicate that *Eif4E^{S209A}* DRG neurons fail to respond to NGF and IL-6 with increases in excitability. To further investigate the cellular mechanisms response for this effect, we examined the effect of IL-6 treatment on sodium current density. In DRG neurons from WT, there was a significant increase in sodium current density after 1 hr IL-6 treatment compared to vehicle suggesting that IL-6 treatment altered the number of available VGNaCs or changed channel gating properties in WT DRG neurons (Figure 3.8D). In stark contrast to our findings in WT neurons, baseline VGNaC density was higher in

Eif4E^{S209A} neurons and these neurons failed to respond to IL-6 with an increase in VGNaC density (Figure 3.8E). In fact, we observed a trend toward decreased VGNaC density in *Eif4E^{S209A}* mice. From these experiments we conclude that while baseline excitability and VGNaC availability may be higher in *Eif4E^{S209A}* nociceptors, these neurons fail to respond to algogens with an increase in VGNaC-mediated responses. Importantly, this baseline increased VGNaC availability in *Eif4E^{S209A}* DRG neurons does not lead to ectopic activity since no difference in spiking was observed at baseline in our MEA experiments (Figure 3.8B and C) and it does not seem to be recapitulated by brief cercosporamide treatment because we did not observe enhanced excitability in whole cell patch clamp experiments with this drug (Figure 3.7D and H).

Eif4E phosphorylation regulates IL-6-induced enhancements in Ca^{2+} signaling in DRG neurons

To further elucidate the role of Eif4E phosphorylation on DRG excitability, we investigated IL-6-induced changes in Ca^{2+} signaling in DRG neurons by measuring intracellular Ca^{2+} concentration $[Ca^{2+}]_I$ with ratiometric imaging. Dissociated DRG neurons from both WT (Figure 3.9A) and *Eif4E^{S209A}* (Figure 3.9B) mice were loaded with fura-2 and treated with either vehicle or IL-6 (50 ng/Ml) for 1 hr. Kinetic changes in $[Ca^{2+}]_I$ in response to PGE_2 (1 Nm) were measured in all conditions. WT DRG neurons treated with IL-6 displayed a significant decrease in the latency to peak $[Ca^{2+}]_I$ in response to PGE_2 compared to vehicle-treated neurons (Figure 3.9C). In contrast, DRG neurons isolated from *Eif4E^{S209A}* mice showed no differences in the kinetics of PGE_2-induced responses between IL-6 and vehicle treatments (Figure 3.9C).

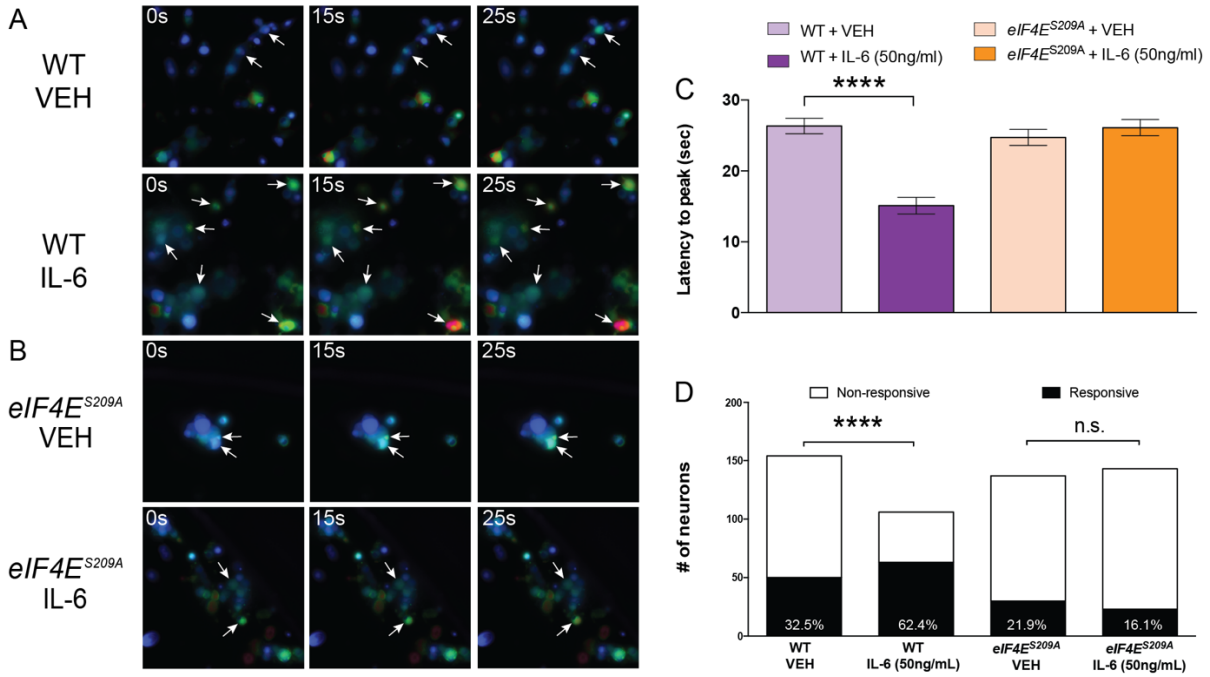

Figure 3.9. IL-6 treatment alters PGE_2 responsiveness in DRG neurons in an eIF4E phosphorylation-dependent fashion. WT and $eIF4E^{S209A}$ DRG neurons were cultured and treated with VEH or IL-6 (50 ng/mL) for 1 hr. PGE_2 (1 nM) was perfused for 30 sec during which Ca^{2+} responses were measured (A & B). C) IL-6 treatment decreased the latency of PGE_2-evoked Ca^{2+} release in WT DRG neurons compared to vehicle-treated WT neurons ($n \geq 50$, $F(3, 162) = 24.65$, $P<0.0001$; post-hoc Bonferroni's ****$p<0.0001$, one-way ANOVA). D) The proportion of neurons responding to PGE_2 was increased after IL-6 exposure in WT DRG neurons but was unchanged in $eIF4E^{S209A}$ DRG neurons (WT: $n \geq 50$, df=18.58, 1, $P>0.0001$, Chi-squared; $eIF4E^{S209A}$: $n \geq 23$, df=1.541, 1, $P=0.2144$, Chi-squared).

In initial experiments in WT DRG neurons we noted that IL-6 treatment revealed PGE_2-mediated responses in a larger subset of neurons than vehicle treatment. We quantified this effect by assessing changes in the proportion of DRG neurons responding to PGE_2 with and without IL-6 treatment in both genotypes. We found that the number of WT DRG neurons responding with a $[Ca^{2+}]_I$ rise above a threshold of 10% was drastically increased after IL-6 treatment whereas no change was observed in *Eif4E*^{S209A} DRG neurons (Figure 3.9D). Interestingly, capsaicin-evoked changes in $[Ca^{2+}]_I$ was unaffected by IL-6 treatment in both WT and *Eif4E*^{S209A} DRG neurons

(Figure 3.9, extended data figure 1A) and the proportion of TRPV1-positive DRG neurons (defined as those neurons responding to the specific agonist capsaicin) was the same in all conditions (Figure 3.9, extended data figure 1B), consistent with our immunostaining results. Responses in $[Ca^{2+}]_I$ evoked by increasing extracellular K^+ from 5 Mm to 50 Mm were also not different across conditions (Figure 3.9, extended data figure 1C). These findings parallel our hyperalgesic priming results, albeit over a very compressed time course compared to the behavioral experiments, and demonstrate a key role for Eif4E phosphorylation in regulating cellular plasticity in response to a key inflammatory mediator.

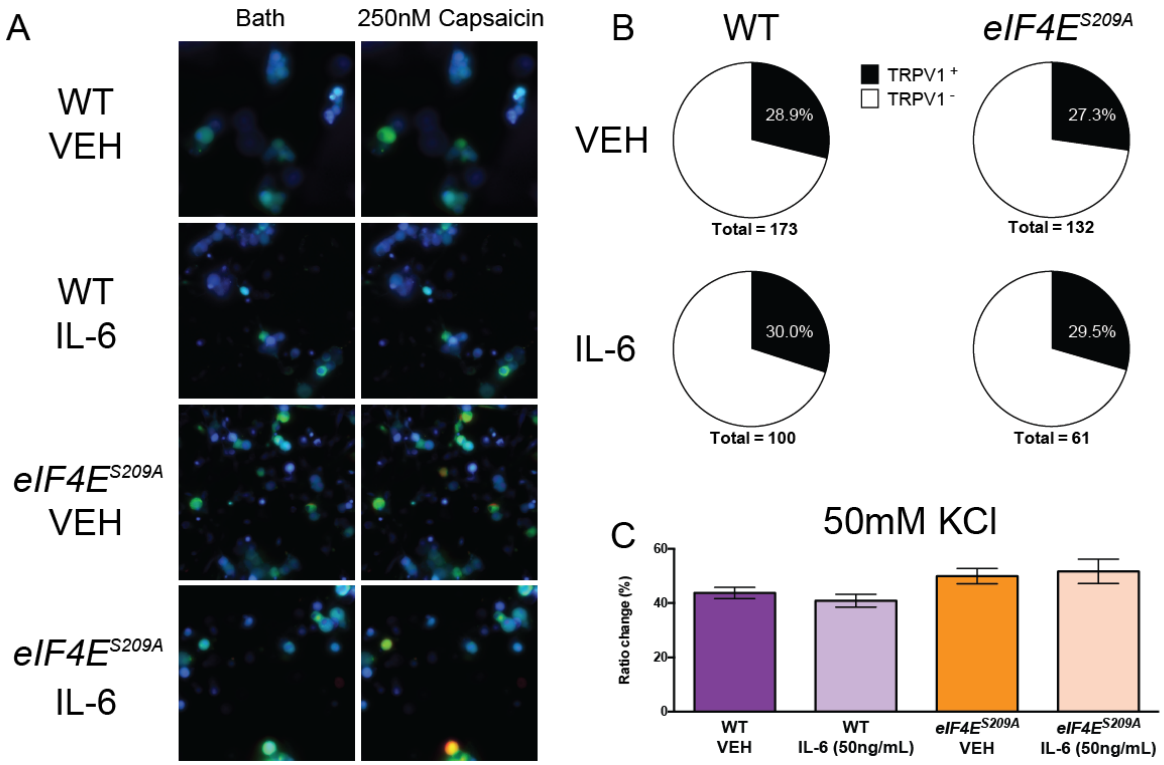

Figure 3.9.1. Normal Ca2+ signaling evoked by KCl and in TRPV1-positive neurons in eIF4E^{S209A} DRG neurons. A) WT and eIF4E^{S209A} DRG neurons were imaged after 1 hr vehicle or IL-6 (50 ng) treatment during vehicle-containing bath solution and capsaicin (250 nM)-

containing bath solution perfusions. B) The proportion of TRPV1-positive neurons were unchanged in WT and eIF4E^{S209A} DRG neurons treated with vehicle of IL-6. C) Moreover, Ca^{2+} signaling evoked by KCl (50 mM) was unchanged in all conditions ($n \geq 57$ neurons per condition, $p>0.05$, one-way ANOVA).

Eif4E phosphorylation is required for cold hypersensitivity after peripheral nerve injury

Compelling evidence supports a key role for translation regulation signaling in the development and maintenance of neuropathic pain although most of this work has focused on Mtor signaling (Price et al., 2007b; Jimenez-Diaz et al., 2008; Melemedjian et al., 2011a; Obara et al., 2011). We evaluated the potential contribution of Eif4E phosphorylation to mechanical and cold hypersensitivity induced by peripheral nerve injury. WT and *Eif4E*^{S209A} mice were subjected to spared nerve injury (SNI) surgery and mechanical and cold hypersensitivity were measured over the ensuing 35 days. Because it was not practical to also treat mice with cercosporamide over this same time course, we also assessed neuropathic pain behaviors in *Mnk*$^{-/-}$ mice. We observed a small delay in the development of mechanical hypersensitivity in *Eif4E*^{S209A} mice but by 14 days these mice developed similar mechanical hypersensitivity to their WT littermates (Figure 3.10A). On the other hand, in *Mnk*$^{-/-}$ mice mechanical hypersensitivity after SNI was blunted in magnitude compared to WT mice (Figure 3.10B). We used the acetone test to measure cold hypersensitivity in both mutant lines; *Eif4E*^{S209A} mice displayed significantly less cold hypersensitivity than WT littermates throughout the time course of the experiment (Figure 3.10C), an effect that was also observed in *Mnk*$^{-/-}$ mice (Figure 3.10D). Finally, we asked if pharmacological inhibition of MNK1/2 with cercosporamide could alleviate cold hypersensitivity in SNI mice. We waited until 4 weeks after SNI (28 days) when mice display very stable cold hypersensitivity and treated mice with vehicle or 40 mg/kg cercosporamide for 3

consecutive days. On the third day we measured cold sensitivity using the acetone test. We observed decreased cold hypersensitivity in the cercosporamide-treated group at 1 hr after the third injection of drug (Figure 3.10E), suggesting that even late after the development of neuropathic pain targeting MNK1/2 signaling to Eif4E is able to alleviate some aspects of neuropathic pain.

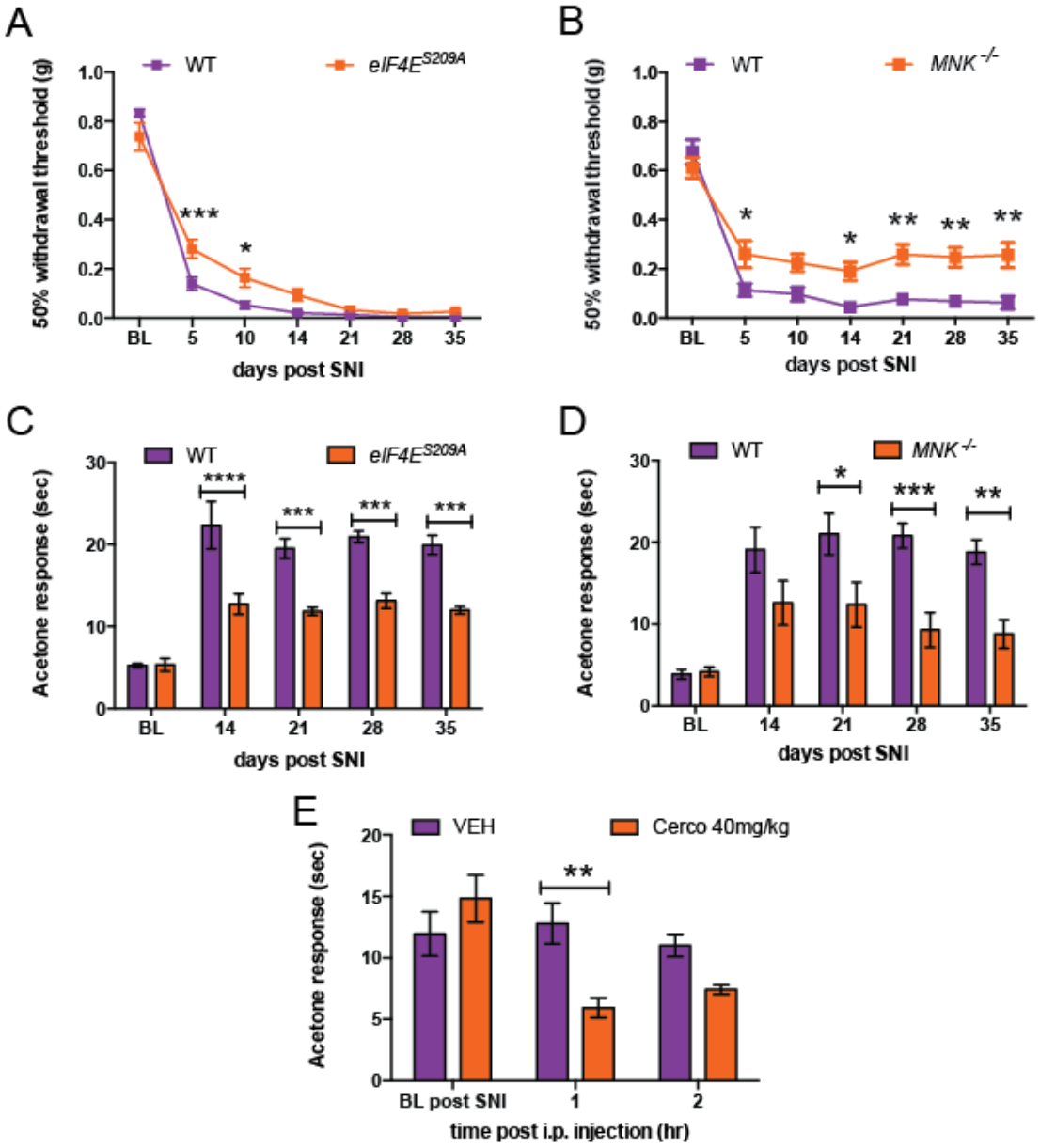

Figure 3.10. Decreased neuropathic pain in eIF4E^{S209A} mice and through MNK1/2 inhibition. A) Following SNI surgery eIF4E^{S209A} mice show a transient decrease in mechanical hypersensitivity that normalizes by day 14 following surgery compared to WT mice (n = 8, F(1, 98)=9.915, P=0.0022; post-hoc Bonferroni's *p=0.0112, ***p=0.0005, two-way ANOVA). B) Mnk1/2$^{-/-}$ mice show a more prolonged decrease in mechanical hypersensitivity after SNI (n = 10, F(1, 126)=43,47, P<0.0001; post-hoc Bonferroni's *p=0.0409, 0.0427, **p=0.0048, 0.0055, 0.0021, two-way ANOVA). C) Following SNI eIF4E^{S209A} mice have a decrease in cold hypersensitivity as measured in the acetone test compared to WT mice (n = 8, F(1, 62)=54.52,

P<0.0001; post-hoc Bonferroni's ***p=0.0004, 0.003, 0.002, ****p<0.0001, two-way ANOVA). D) Mnk1/2$^{-/-}$ mice show a sustained decrease in cold hypersensitivity after SNI compared to WT mice (n = 10, F(1, 90)=31.82, P<0.0001; post-hoc Bonferroni's *p=0.00181, **p=0.0039, ***p=0.0007, two-way ANOVA). E) Following systemic administration of cercosporamide (40 mg/kg) for 3 days, WT mice show a transient decrease in cold hypersensitivity (n = 4, F(1, 18)=5.093, P=0.0367; post-hoc Bonferroni's **p=0.0070, two-way ANOVA).

Centrally mediated pain plasticity is not governed by Eif4E phosphorylation

Brain-derived neurotrophic factor (BDNF) is a well-known mediator of several types of central pain plasticity and is required for the development of hyperalgesic priming (Melemedjian et al., 2013c; Melemedjian et al., 2014b). We predicted that centrally-mediated pain plasticity would also be mediated by Eif4E phosphorylation and that mechanical hypersensitivity and hyperalgesic priming produced by direct injection of BDNF into the CNS would be absent in *Eif4E*^{S209A} mice. BDNF (0.1 ng) was injected intrathecally in WT and *Eif4E*^{S209A} mice and mechanical hypersensitivity was measured over the ensuing 72 hr. Contrary to our hypothesis, while early mechanical hypersensitivity magnitudes were decreased in *Eif4E*^{S209A} mice in response to BDNF, no differences were observed at 48 and 72 hr after injection (Figure 3.11A). Moreover, when mice were later challenged with PGE_2 injection into the hindpaw, full hyperalgesic priming was clearly present in both genotypes (Figure 3.11B) despite the deficit in acute sensitization at early time points in *Eif4E*^{S209A} mice. We then tested pharmacological inhibition of Eif4E phosphorylation centrally by administering cercosporamide (10μg, a dose that was highly efficacious given into the hindpaw) intrathecally at the same time as peripheral injection of NGF (50 ng). In stark contrast to our results when cercosporamide was given peripherally, no differences between cercosporamide and vehicle groups were found when the

drug was given i.t. (Figure 3.11C). Likewise, subsequent injection of PGE_2 elicited hyperalgesic priming equally in both groups (Figure 3.11D). We conclude that MNK-Eif4E signaling plays a specialized role in regulation of DRG neuron plasticity making this signaling axis an important target for peripherally-restricted pain therapeutics.

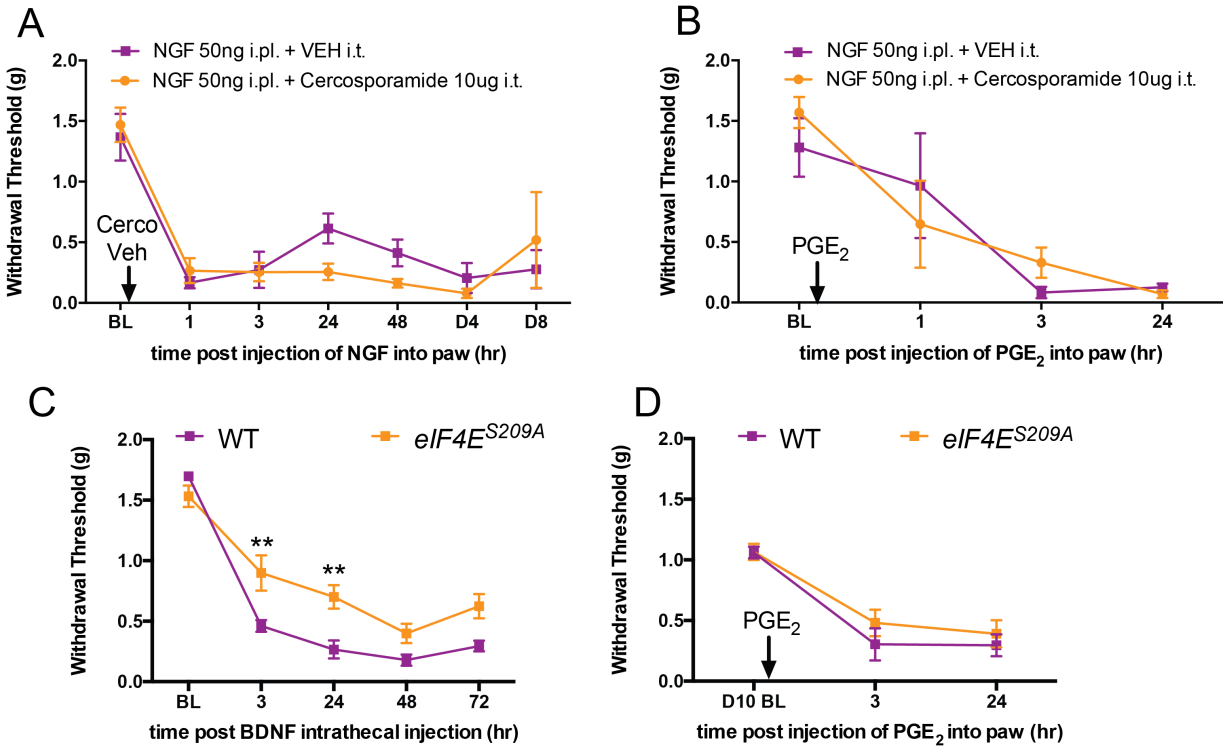

Figure 3.11. Central plasticity is not influenced by MNK1/2-eIF4E signaling. A) WT mice received a hindpaw injection of NGF (50 ng) and an intrathecal (i.t.) injection of vehicle or cercosporamide (10 µg). Mechanical hypersensitivity was measured at 1, 3, 24, 48 hr, D4, and D8. Mice that received an i.t. injection of cercosporamide showed no difference in mechanical hypersensitivity compared to vehicle (n = 4, $p>0.05$, two-way ANOVA). B) Additionally, central inhibition of eIF4E phosphorylation did not prevent the transition to priming precipitated by PGE_2 (n = 4, $p>0.05$, two-way ANOVA). C) I.t. injection of BDNF (0.1 ng) was administered to both WT and $eIF4E^{S209A}$ mice. Acute mechanical hypersensitivity was blunted in $eIF4E^{S209A}$ mice at 3 and 24 hr ($n \geq 6$, $F(1, 60)=18.97$, $P<0.0001$; post-hoc Bonferroni's **p=0.0062, 0.0067, two-way ANOVA), but was equal in both genotypes by 48 hr and beyond. D) PGE_2 was injected i.pl. in WT and $eIF4E^{S209A}$ mice and equal hyperalgesic priming was observed in both genotypes ($n \geq 6$, $p>0.05$, two-way ANOVA).

DISCUSSION

The activity-dependent regulation of protein synthesis is a core mechanism mediating neuronal plasticity (Costa-Mattioli et al., 2009). In the nociceptive system, translation regulation pathways have been shown to contribute to the development and maintenance of pain hypersensitivity in a variety of preclinical models, suggesting that targeting these pathways may lead to new pain therapeutics (Price and Geranton, 2009; Obara et al., 2012a; Melemedjian and Khoutorsky, 2015; Price and Inyang, 2015). Here we report that phosphorylation of Eif4E at S209 is a key biochemical event for sensitization of DRG neurons by pro-nociceptive factors that are known to promote pain in rodents and humans, and that loss of this phosphorylation event does not affect baseline pain behaviors or neurodevelopment in the nociceptive system. Our electrophysiological and Ca^{2+} imaging experiments show that the generation of nociceptor hyperexcitability is critically mediated by Eif4E phosphorylation by MNK1/2. Moreover, Eif4E phosphorylation plays a key role in the transition to a chronic pain state as reflected by deficiencies in peripherally-mediated hyperalgesic priming and a loss of cold hypersensitivity after peripheral nerve injury. Our genetic and pharmacological experiments demonstrate that targeting MNK1/2 recapitulates the phenotype of *Eif4E*^{S209A} mice pointing out that MNK1/2 might be advantageously targeted in the peripheral nervous system for the treatment and/or prevention of chronic pain conditions.

While upstream signaling factors like Mtor and MAPK have been implicated in pain plasticity using pharmacological tools and biochemical measures (Price et al., 2007b; Jimenez-Diaz et al., 2008; Asante et al., 2009b; Codeluppi et al., 2009; Geranton et al., 2009; Asante et al., 2010b;

Melemedjian et al., 2010a; Melemedjian et al., 2011a; Obara et al., 2011; Ferrari et al., 2013b; Melemedjian et al., 2014b), specific downstream mechanisms and Mrna targets linked to these kinase signaling cascades have been elusive until very recently. Recent evidence suggests that different translation regulation targets play strikingly uniquely roles in different aspects of pain sensitivity. For example, genetic loss of a major downstream target of Mtorc1, 4E-BP1, leads solely to changes in mechanical sensitivity via central mechanisms governed by a synaptic adhesion molecule known as neuroligin 1 (Khoutorsky et al., 2015b). In contrast, mice lacking a key phosphorylation site for Eif2α on a single allele have deficits in baseline thermal nociception without any changes in mechanical thresholds (Khoutorsky et al., 2016b). This phenotype can be recapitulated with pharmacological modulation of Eif2α and a deficit in thermal, but not mechanical, hyperalgesia is also associated with this pathway in inflammatory pain. Collectively, these studies suggest that individual translation regulation pathways may target specific subsets of genes that have profound impacts on certain aspects of nociception (e.g. thermal versus mechanical pain; see Table 3.1 for a summary of previous findings compared to the present paper).

Table 3.1. Different translation regulation pathways control different aspects of pain and pain amplification

Mutant mouse	**Thermal**	**Mechanical**	**Thermal hyperalgesia**	**Mechanical hyperalgesia**	**Proposed mechanism**
eIF4E^{S209A}	Normal	Normal	Decreased to inflammator	Decreased to inflammatory	Lack of change in

			y stimuli	stimuli	DRG excitability
MNK$^{-/-}$	Normal	Normal	Decreased to inflammatory stimuli	Decreased to inflammatory stimuli and neuropathic	Lack of change in DRG excitability
Eif4ebp1$^{-/-}$ *(Khoutorsky et al., 2015b)*	Normal	Increased sensitivity	Not tested	Increased hypersensitivity to inflammatory stimuli	Decreased spinal neuroligin 1
eIF2α$^{+/S51A}$*(Khoutorsky et al., 2016b)*	Decreased sensitivity	Normal	Not tested	Decreased hypersensitivity to inflammatory stimuli	Decreased TRPV1 functional activity in DRG
Fmr1 KO (Price et al., 2007b)	Normal	Normal	Not tested	Decreased hypersensitivity to inflammatory stimuli and	Decreased spinal and peripheral mGluR1/5 & mTOR

				neuropathic	signaling

We previously showed that NGF and IL-6 are capable of promoting Eif4E phosphorylation in nociceptors *in vitro*, and that disrupting Eif4F complex formation with 4EGI-1 leads to a blockade of the development of mechanical hypersensitivity and hyperalgesic priming by these pro-nociceptive factors (Melemedjian et al., 2010a; Tillu et al., 2015b). Here we use *Eif4E*^{S209A} and *MNK*$^{-/-}$ mice and pharmacological tools to definitively demonstrate that Eif4E phosphorylation is a core biochemical event modulating how inflammatory stimuli promote mechanical and thermal hypersensitivity, spontaneous pain responses, and the transition to a chronic pain state. These findings distinguish the MNK1/2 – Eif4E signaling cascade from 4E-BP1 and Eif2α signaling which have specific effects on certain pain modalities. Our results support the conclusion that MNK1/2 – Eif4E signaling is key to altering the excitability of a wide population of nociceptors because genetic and pharmacological manipulation of this pathway had profound effects on thermal and mechanical hypersensitivity, as well as spontaneous pain. In our view, this identifies MNK1/2-mediated Eif4E phosphorylation as a crucial target for plasticity in nociceptors that drives the transition to a chronic pain state. Importantly, centrally-mediated pain plasticity remains intact in the absence of Eif4E phosphorylation as shown in our intrathecal BDNF and cercosporamide experiments. This is consistent with a previous study that comprehensively examined CNS phenotypes in *Eif4E*^{S209A} mice finding normal dendritic spine morphology, intact synaptic physiology, unchanged mGluR-dependent long-term depression (LTD), normal protein synthesis rates, and no difference in performance of a variety of anxiety, locomotor and memory tasks (Gkogkas et al., 2014a).

MNK1/2-dependent signaling to Eif4E therefore represents a strong mechanistic target for pharmacological manipulation of chronic pain.

It appears perplexing that mechanical hypersensitivity induced by pro-nociceptive factors was decreased or absent in *Eif4E*^{S209A} mice, but mechanical hypersensitivity developed normally after peripheral nerve injury in these mice. A potential explanation is that mechanisms involved in reflex withdrawal responses after peripheral nerve injury are dependent on centrally mediated effects similar to mechanisms evoked by intrathecal BDNF (e.g., downregulation of KCC2 function (Coull et al., 2003; Price and Prescott, 2015)). Mechanical hypersensitivity following nerve injury persists even when the vast majority of C-fibers are eliminated (Abrahamsen et al., 2008b; Minett et al., 2014). On the other hand, eliminating neurons in the TRPM8 lineage is sufficient to eliminate cold hypersensitivity after peripheral nerve injury (Knowlton et al., 2013). We observed a strong decrease in cold hypersensitivity induced by nerve injury in *Eif4E*^{S209A} and *Mnk*$^{-/-}$ mice as well as following cercosporamide treatment. Therefore, our findings are consistent with the notion that MNK – Eif4E signaling is a key signaling hub for the sensitization of peripheral nociceptive neurons but that this signaling pathway is dispensable for the generation of mechanical hypersensitivity that can be generated exclusively via Aβ-fiber input and disinhibitory mechanisms in the dorsal horn as is observed after BDNF injection (Lee and Prescott, 2015) or with traumatic injury to peripheral nerves (Coull et al., 2005a; Abrahamsen et al., 2008b; Minett et al., 2014).

Despite intact normal acute pain behavior and anatomy in *Eif4E*^{S209A} mice we observed that DRG neurons surprisingly trended toward higher levels of excitability in response to slowly depolarizing ramp currents and a larger baseline sodium current density. On the other hand, these

neurons fail to demonstrate a change in excitability in response to either IL-6 or NGF *in vitro*, an effect that is reflected in a behavioral deficit *in vivo*. A very recent study revealed that Eif4E phosphorylation is mechanistically linked to stress granule formation and Mrna sequestration through an interaction with the Eif4E-interacting protein 4E-T and that this leads to suppression of translation of some mRNAs (Martinez et al., 2015). While this has not been explored in neurons, it is possible that Eif4E phosphorylation can suppress translation of certain Mrna species under some circumstances and promote the translation of the same, or other, mRNAs in different circumstances, for instance in response to inflammatory mediators in DRG nociceptors. Such a mechanism linked to Eif4E phosphorylation could lead to the cellular phenotype of increased basal excitability but a loss of induced hyperexcitability in the complete absence of Eif4E phosphorylation. In fact, similar phenotypes have been consistently observed in *Fmr1* knockout mice (a model of fragile X syndrome) leading to baseline alterations in neuronal excitability or in signaling pathway efficacy coupled with a lack of plasticity (Grossman et al., 2006; Hanson and Madison, 2007; Pfeiffer and Huber, 2007; Bureau et al., 2008) including in the nociceptive system (Price et al., 2007b). Interestingly, recent studies found that Eif4E phosphorylation is enhanced in fragile X syndrome and that crossing mice lacking fragile X mental retardation protein with $Eif4E^{S209A}$ mice or treating *Fmr1* mutant mice with cercosporamide rescues many phenotypes in these mice (Gkogkas et al., 2014a).

An unanswered question arising from this work is what are the key Mrna targets for Eif4E phosphorylation in the context of nociceptor excitability. Our electrophysiology and Ca^{2+} imaging experiments provide some possible clues. The trafficking of channels to the membrane is an important regulatory mechanism for nociceptor excitability (Matsuoka et al., 2007;

Hudmon et al., 2008; Andres et al., 2013). Our findings are consistent with a model where Eif4E phosphorylation enhances the translation of a subset of mRNAs that influence the membrane trafficking of voltage gated channels (e.g., VGNaCs, consistent with our current density experiments) and G-protein coupled receptors (e.g., PGE_2 receptors, consistent with our Ca^{2+} imaging experiments) to enhance nociceptor excitability and increase their responsiveness to inflammatory mediators. While we cannot currently pinpoint the identity of these locally translated mRNAs, we have now identified a precise signaling event that can be manipulated to identify these mRNAs. Discovering these Eif4E-controlled mRNAs will yield significant insight into how nociceptors alter their excitability downstream of initial phosphorylation events that are likely more transient than the synthesis of new proteins that contribute to the maintenance of long-term nociceptor plasticity.

MATERIALS AND METHODS

Experimental animals

Male and female *Eif4E*^{S209A} mice on a C57BL/6 background were generated in the Sonenberg laboratory at McGill University, as described previously (Furic et al., 2010a), and bred at The University of Arizona or The University of Texas Dallas to generate experimental animals. *Mnk1/2*$^{-/-}$ mice on a C57BL/6 background were obtained from Rikiro Fukunaga (Ueda et al., 2004b) and bred at McGill University. Animals were genotyped using DNA from ear clips taken at the time of weaning. All electrophysiological experiments using *Eif4E*^{S209A} and WT mice were performed using mice between the ages of 4 and 6 weeks of age at the start of the experiment. Behavioral experiments using *Eif4E*^{S209A} and WT mice were performed using mice between the

ages of 8 and 12 weeks. Experiments using ICR mice obtained from Harlan Laboratories (Houston, TX) were performed using mice between 4 to 8 weeks of age at the start of the experiment. All animal procedures were approved by Institutional Animal Care and Use Committees at The University of Arizona, The University of Texas at Dallas or McGill University and were in accordance with International Association for the Study of Pain guidelines.

Antibodies and chemicals

The peripherin and neurofilament 200 (NF200) antibodies used for immunohistochemistry (IHC) were obtained from Sigma Aldrich (St. Louis, MO). Isolectin B_4 (IB_4) conjugated to Alexa-Fluor 568 and secondary Alexa-Fluor antibodies were from Life Technologies (Grand Island, NY). Calcitonin gene-related peptide (CGRP) antibody was purchased from Peninsula Laboratories International, Inc. (San Carlos, CA). Transient receptor potential V1 (TRPV1) antibody was procured from Neuromics (Edina, MN). The 105hosphor- and total- Eif4E, 4EBP1, ERK, and GAPDH antibodies were obtained from Cell Signaling Technology (Danvers, MA). The 105hosphor- Eif4E antibody used for immunohistochemistry (IHC) was purchased from Abcam (Cambridge, UK). (RS)-3,5-Dihyroxyphenylglycin (DHPG) was purchased from Tocris Bioscience (Ellisville, MO). Cercosporamide was provided as a generous gift from Eli Lilly and Company (Indianapolis, IN) to the Sonenberg laboratory. Mouse nerve growth factor (NGF) was obtained from Millipore (Billierca, MA). Recombinant human or mouse interleukin-6 (IL-6) and recombinant human/mouse/canine/equine brain derived neurotrophic factor (BDNF) were purchased from R & D systems (Minneapolis, MN). 2-aminothiazol-4-yl-LIGRL-NH_2 (2at-

LIGRL) was synthesized in our laboratory as described previously (Boitano et al., 2011). Prostaglandin E_2 (PGE_2) was purchased from Cayman chemicals (Ann Arbor, MI). β-cyclodextrin (45% weight/volume in H_2O) was purchased from Sigma Aldrich (St. Louis, MO). All other chemicals were attained from ThermoFisher Scientific (Waltham, MA). See Table 2 for additional details on antibodies and chemicals used in this study.

Behavior

Mice were housed on 12-hr light-/dark cycles with lights on at 7:00 AM. Mice had food and water available *ad libitum*. All behavioral experiments were performed between the hours of 9:00 AM and 4:00 PM. Mice were randomized to groups from multiple cages to avoid using mice from experimental groups that were cohabitating. Sample size was estimated as $n < 6$ using a power calculation with 80% power, expectations of 50% effect size, with alpha set to 0.05. Number of animals used in each experiment was based on available animals of the appropriate sex and age but was at least $n = 5$ for experiments (see Table 3.2). Standard deviation was based on

Table 3.2. Sex of animals by genotype in behavioral experiments in this study.

Test	WT Males	WT Females	*eIF4E*^{S209A} Males	*eIF4E*^{S209A} Females
Tail Flick/ von Frey	4	4	7	5
Formalin	6	4	7	5
DHPG	5	3	4	4

IL-6	3	3	4	4
NGF	4	2	4	4
2at-LIGRL (PAR_2)	4	2	4	2
BDNF	4	2	5	3
SNI	4	4	6	2
Grimace [2at-LIGRL (PAR_2)]	4	1	-	7
Cercosporamide (SNI)	4	4	-	-
Cercosporamide + NGF ($eIF4E^{S209A}$)	-	-	4	7
Carrageenan	5	-	6	-
NGF thermal	-	6	-	6
CFA (*$MNK^{-/-}$*)	4	5	4 (*$MNK^{-/-}$*)	5 (*$MNK^{-/-}$*)

previously published data. Mice were habituated for 1 hr to clear acrylic behavioral chambers before beginning the experiment. Mechanical paw withdrawal thresholds were measured using the up-down method (Chaplan et al., 1994) with calibrated Von Frey filaments (Stoelting Company, Wood Dale, IL). Thermal latency was measured using a Hargreaves device ((Hargreaves et al., 1988) IITC Life Science Inc.) with heated glass. Settings of 29°C glass, 20% active laser power, and 20 sec cut-off were used. Facial grimacing was evaluated using the Mouse Grimace Scale (MGS) as described previously (Langford et al., 2010). Nocifensive behavior in the formalin test was defined as licking, biting, or shaking of the affected paw, and

was recorded over an observation period of 45 min. For intraplantar (i.pl.) injections, 50 ng NGF, 0.1 ng IL-6, 10 or 20 ng 2at-LIGRL were diluted in 0.9% saline and injected with a volume of 25 Ml via a 30 ½ -gauge needle. For intrathecal (i.t.) injections, 0.1 ng of BDNF or 50 nmol of DHPG were injected in a volume of 5 Ml via a 30 ½ -gauge needle (Hylden and Wilcox, 1980). Cercosporamide for local injection was made up in 10% DMSO and 45% β-cyclodextrin in water and injected into the paw 15 min prior NGF, and simultaneously with 2at-LIGRL. Cercosporamide was injected i.t. in a volume of 5 μL at the time of NGF i.pl. injection. Mice of both sexes were used in most experiments and no significant differences between sexes were noted for drug or genotype in any experiments. Sex of mice used in all behavioral experiments is shown in Table 3. The experimenter was blinded to the genotype of the mice and drug condition in all experiments. Behavioral experiments were performed by *JKM, AK, MNA, PBI and SM*.

Immunohistochemistry (IHC)

Animals were anesthetized with isoflurane and euthanized by decapitation and tissues were flash frozen in O.C.T. on dry ice. Spinal cords were pressure ejected using chilled 1X phosphate buffered saline (PBS). Sections of spinal cord (25 μm), DRG (20 μm), and glaborous skin (25 μm) were mounted onto SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, MA) and fixed in ice-cold 10% or 4% (skin) formalin in 1X PBS for 1 or 4 hr (skin) then subsequently washed 3 times for 5 min each in 1X PBS. Slides were then transferred to a solution for permeabilization made of 1X PBS with 0.2% Triton X-100 (Sigma Aldrich). After 30 min, slides were washed 3 times for 5 min each in 1X PBS. Tissues were blocked for at least 2 hr in 1X PBS and 10% heat-inactivated normal goat serum. Antibodies for CGRP, IB_4, and TRPV1 were

applied together and incubated with spinal cord and DRG sections on slides at 4° C overnight (see Table 3.3. A combination of TRPV1 and p-Eif4E antibodies were applied to glabrous skin sections and incubated at 4° C overnight. DRG slices were also stained with peripherin and NF200. Immunoreactivity was visualized following 1 hr incubation with goat anti-rabbit, goat anti-mouse, and goat anti-guinea pig Alexa-Fluor antibodies at room temperature. All IHC images are representations of samples taken from 3 animals per genotype except for glaborous skin IHC where 2 animals per genotype were used. Images were taken using an Olympus FluoView 1200 confocal microscope. Analysis of images was done using ImageJ Version 1.48 for Apple OSX (National Institutes of Health, Bethesda, MD).

Table 3.3. List of antibodies used in this study.

Primary antibodies	Catalog number	Incubation	Secondary antibodies
eIF4E	phospho- 9741S phospho (IHC)- ab76256 total- 9742S	p- 1:500; p(IHC)- 1:1000; t- 1:1000; overnight @ 4 °C	Goat anti-rabbit (WB:1:10,000; IHC:1:1,000); 1 hr @ RT
4EBP1	phospho- 9459S total- 9452S	p- 1:500; t- 1:1000; overnight @ 4 °C	Goat anti-rabbit (1:10,000); 1 hr @ RT

ERK	phospho- 9101S total- 9102S	p- 1:3,000; t- 1:3,000; overnight @ 4 °C	Goat anti-rabbit (1:10,000); 1 hr @ RT
GAPDH	2118	1:10,000; overnight @ 4 °C	Goat anti-rabbit (1:10,000); 1 hr @ RT
Peripherin	SAB 4502419	1:500; overnight @ 4 °C	Alexa-fluor goat anti-rabbit 488 (1:1,000); 1 hr @ RT
Neurofilament 200	N0142	1:400; overnight @ 4 °C	Alexa-fluor goat anti-mouse 568 (1:1,000); 1 hr @ RT
Isolectin B_4-568	I21412	Spinal cord- 1:1000; DRG- 1:400; overnight @ 4 °C	none
CGRP	T-4032	Spinal cord & DRG- 1:1000; overnight @ 4 °C	Alexa-fluor goat anti-rabbit 647 (1:2,000); 1 hr @ RT
TRPV1	GP14100	Spinal cord & DRG- 1:1000; Skin-1:3,000; overnight @ 4 °C	Alexa-fluor goat anti-guinea pig 488 (1:2,000); 1 hr @ RT

Western blotting

Male mice were used for all Western blotting experiments and were sacrificed by decapitation following anesthesia and tissues were flash frozen on dry ice. Frozen tissues were homogenized in lysis buffer (50 Mm Tris Ph 7.4, 150 Mm NaCl, 1 Mm EDTA Ph 8.0, and 1% Triton X-100) containing protease and phosphatase inhibitors (Sigma Aldrich), and homogenized using a

pestle. *In vitro* studies consisted of cultured primary DRG neurons from ~ 8 week old mice. Mice were anesthetized with isoflurane and euthanized by decapitation. DRGs were dissected and placed in chilled Hank's Balanced Salt Solution (HBSS, Invitrogen) until processed. DRGs were then digested in 1 mg/Ml collagenase A (Roche) for 25 min at 37 °C then subsequently digested in a 1:1 mixture of 1 mg/Ml collagenase D and papain (Roche) for 20 min at 37 °C. DRGs were then triturated in a 1:1 mixture of 1 mg/Ml trypsin inhibitor (Roche) and bovine serum albumin (BioPharm Laboratories, LLC), then filtered through a 70 μm cell strainer (Corning). Cells were pelleted then resuspended in Dulbecco's Modified Eagle's Medium (DMEM) / F12 + Glutamax (Life Technologies) containing 10% fetal bovine serum (FBS, Life Technologies), 1% penicillin and streptomycin, and 3 μg/ml 5-fluorouridine with 7 μg/ml uridine to inhibit mitosis of non-neuronal cells and distributed evenly in a 6-well plate coated with poly-D lysine (Becton Dickenson [BD]). DRG neurons were maintained in a 37°C incubator containing 5% CO_2 with a media change every other day. On day 5, DRG neurons were treated as indicated in the results section, and cells were rinsed with chilled 1X PBS and harvested in lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich), and then sonicated for 10 sec. To clear debris, samples were centrifuged at 14,000 rpm for 15 min at 4°C. Ten to 15 μg of protein was loaded into each well and separated by a 10% SDS-PAGE gel. Proteins were transferred to a 0.45 PVDF membrane (Millipore, Billierca, MA) at 30V overnight at 4 °C. Subsequently, membranes were blocked with 5% non-fat dry milk (NFDM) in 1X Tris buffer solution containing Tween 20 (TTBS) for 3 hr. Membranes were washed in 1X TTBS 3 times for 5 min each, then incubated with primary antibody overnight at 4 °C. The following day, membranes were washed 3 times in 1X TTBS for 5 min each, then incubated with the corresponding

secondary antibody at room temperature for 30 min to 1 hr. Membranes were then washed with 1X TTBS 6 times for 5 min each. Signals were detected using Immobilon Western Chemiluminescent HRP substrate (Millipore). Bands were visualized using film (Kodak; Rochester, NY) or with a Bio-Rad (Hercules, CA) ChemiDoc Touch. Membranes were stripped using Restore Western Blot Stripping buffer (Thermo Fisher Scientific), and re-probed with another antibody. Analysis was performed using ImageJ v1.48 (NIH).

Ca^{2+} Imaging

WT and *Eif4E^{S209A}* DRG neurons were dissociated and cultured as described above with the exception that cells were plated on glass-bottom poly-D-lysine coated dishes (MatTEK Incorporation). DRG neurons were maintained in a 37°C incubator containing 5% CO_2 with no media changes.

Ca^{2+} imaging experiments began 48 hr after dissociation. Each dish was loaded with 10 μg/Ml of fura-2 AM (Life Technologies) along with IL-6 (50ng/Ml) or vehicle in Hank's Buffered Salt Solution (HBSS, Invitrogen) supplemented with 0.25% w/v bovine serum albumin and 2 Mm $CaCl_2$, for 1 hr at 37°C. The cells were then changed to bath solution (125 Mm NaCl, 5 Mm KCl, 10 Mm HEPES, 1 M $CaCl_2$, 1 M $MgCl_2$, and 2 M glucose Ph 7.4 adjusted with N-methyl Glucamine and ~ 300 mosM) for 30 min in a volume of 2 Ml for esterification. Dishes were then washed with 2 Ml of bath solution prior to recordings.

The perfusion protocol was: bath solution for at least 5 min to obtain a stable baseline, 30 sec of 1 Nm PGE_2 in bath solution, 5 min of bath solution, 10 sec of 50 Mm KCl adjusted bath solution, 1 min of bath solution, and 10 sec of 250 Nm capsaicin in bath solution. Only neurons

were used in the analysis and these were defined as cells with 10% or higher ratiometric change in intracellular Ca^{2+} in response to the 50 Mm KCl perfusion. Maximum Ca^{2+} release was calculated by comparing ratio value change by time compared to baseline. A change of at least 10% intracellular Ca^{2+} in response to 1 Nm PGE_2 or 250 Nm capsaicin was used to classify as neuron as responsive to the stimulus. Experiments were conducted using the MetaFluor Fluorescence Ratio Imaging Software on an Olympus TH4-100 apparatus (Olympus).

Extracellular electrophysiology

MEA-based extracellular recording experiments were performed on dissociated mouse DRG neurons between day-in-vitro 11 and 15 using Ti or ITO 60-channel planar microelectrode arrays (Multichannel Systems, Reutlingen, Germany) equipped with hardware/software from Plexon, Inc. Data were acquired at 40 kHz/channel and digitally filtered (0.1 to 7000 Hz bandpass) during acquisition. An additional 4-pole Butterworth bandpass filter (250 Hz to 7500 Hz) was applied to raw, continuous data, enabling detection of single-event extracellular voltage changes (spikes). Spikes were defined by filtered data crossing a 5σ threshold based on root mean square values calculated for each channel. Active channels were defined by template spike detection resulting in average waveform amplitudes of 40 Mv or higher during any of the three 1 hour experimental intervals: baseline, IL-6 treatment, and wash. Between each interval, recording was paused for ~ 2 min to allow for manual exchange of culture medium for medium + IL-6 (IL-6 treatment, 50 ng/Ml) or fresh culture medium (Wash). Active channel data was exported to NeuroExplorer (Nex Technologies, Madison, AL) for mean spike rate calculations and further analysis. Statistical comparisons of channel activity were carried out using OriginPro

software (OriginLab, Northhampton, MA). MEA cultures were maintained at 37 ˚C, 5% CO_2, and 90% humidity throughout all experiments (OKO Labs, Pozzuoli, Italy). Culture-surface preparation, DRG extraction, dissociation, and cell seeding was carried out as described above.

Patch-clamp Electrophysiology

Mice were anesthetized with isoflurane (Vedco Inc., St. Joseph, MO) and sacrificed by decapitation. DRG neurons were aseptically removed from all levels and placed in HEPES (10 Mm) buffered Hank's Buffered Salt Solution (HBSS, Invitrogen) on ice. Ganglia were incubated for 15 min in 20 units/ml Papain (Worthington) followed by 15 min in 3 mg/ml Collagenase Type II (Worthington). After trituration through a fire-polished Pasteur pipette, dissociated cells were resuspended in Liebovitz L-15 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 10 Mm glucose, 10 Mm HEPES and 50 units/ml penicillin/streptomycin and plated on poly-D-lysine and laminin (Sigma) – coated dishes. Cells were allowed to adhere for several hours at room temperature in a humidified chamber and covered with media described above. DRG neurons were treated with 50 ng/ml NGF 18 – 24 hr prior to recordings or with 50ng/ml mouse IL-6 for 1 hr.

Whole cell patch-clamp experiments were performed on isolated mouse DRG neurons within 24 hr of dissociation using a MultiClamp 700B (Axon Instruments, Sunnyvale, CA) patch-clamp amplifier and Pclamp 9 acquisition software (Axon Instruments). Recordings were sampled at 2 kHz and filtered at 1 kHz (Digidata 1322A, Axon Instruments). Pipettes (OD: 1.5mm, ID: 0.86mm) were pulled using a P-97 puller (Sutter Instrument, Novato, CA) and heat polished to 1.5-4 MΩ resistance using a microforge (MF-83, Narishige, East Meadow, NY). Series

resistance was typically <7 MΩ and was compensated 60-80%. Data were analyzed using Clampfit 10 (Molecular Devices, Sunnyvale, CA) and Origin 8 (OriginLab, Northhampton, MA). All neurons included in the analysis had a resting membrane potential (RMP) -60 Mv or lower. The RMP was recorded 1–3 min after achieving whole-cell configuration. In current-clamp mode, action potentials were elicited by injecting slow ramp currents from 0.1 to 0.7 Na with Δ = 0.2 Na over 1 s to mimic slow depolarization. The pipette solution contained (in Mm) 140 KCl, 11 EGTA, 2 $MgCl_2$, 10 NaCl, 10 HEPES, 1 $CaCl_2$ Ph 7.3 (adjusted with N-methyl glucamine), and was ~ 320 mosM. External solution contained (in Mm) 135 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 KCl, 10 Glucose, 10 HEPES, Ph 7.4 (adjusted with N-methyl glucamine), and was ~ 320 mosM. For neuronal VGNaC current recordings, pipette solution contained (in Mm) 120 CsCl, 10 EGTA, 2 $MgCl_2$, 5 NaCl, 10 HEPES, 2 $CaCl_2$, Ph 7.3 (adjusted with N-methyl glucamine) and osmolarity was 320 mosM. External solution contained (in Mm) 95 Choline, 20 Tetraethyl ammonium (TEA), 20 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 KCl, 0.1 $CdCl_2$, 0.1 $NiCl_2$, Ph 7.3 (adjusted with N-methyl glucamine) and osmolarity was 320 mosM. In whole-cell configuration, cells were voltage-clamped and VGNaC currents were evoked by a 50 ms depolarizing steps (from -80 to +40 Mv in 5 Mv increments) from a holding potential of -120Mv. Sodium currents were normalized to whole-cell capacitance and expressed as current density (Pa/Pf).

Data and statistics

All data are represented as mean ± standard error of the mean (SEM). All analysis was done using GraphPad Prism 6 v 6.0 for Mac OS X. Single comparisons were performed using Student's *t*-test and multiple comparisons were performed using a two-way ANOVA with

Bonferroni posthoc tests for across group comparisons or Uncorrected Fisher's LSD for within group comparisons. Outliers were determined using the Grubbs' test with alpha set to 0.05, and then excluded. The only outliers that were excluded from the data presented in the manuscript were in the Ca^{2+} imaging experiments. Variances were not different between groups in this study. This was assessed using an F test for two sample comparisons and Bartlett's test for multiple comparisons. Statistical tests, *p*-values and number of replicates per experiment are shown in the figure legends.

Data Availability

Raw data from all experiments is available as a supplemental prism file.

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CHAPTER 4

GENERAL DISCUSSION

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MRNA TRANSLATION REGULATION IN PERIPHERAL PAIN PLASTICITY

Hyperalgesic priming was originally developed as a peripheral mechanism stating that nociceptor stimulation by inflammatory mediators develop a long-lasting state of hyperresponsiveness to subsequent inflammatory stimuli (Aley et al., 2000; Parada et al., 2003b; Reichling and Levine, 2009a). Specifically, we have demonstrated how pain plasticity induced by algogens such as NGF requires engagement of translation machinery: mTOR and ERK (Melemedjian et al., 2010c; Tillu et al., 2012b; Melemedjian et al., 2013f; Melemedjian et al., 2014c) (Figure 1.2). In addition to mTORC1, ERK is also activated by inflammatory mediators to regulate mRNA translation and sensitize nociceptors (Yan et al., 2012b). Our evidence strong supports that factors involved in inflammation and injury, such as mTOR and ERK activation induce pain plasticity by promoting translation initiation in sensory neurons, but until now we were unable to separate out these pathways.

Injury- and inflammatory- related signals cause sensitization of the sensory system in a translation-dependent fashion, and are associated with increased activation of ERK and MNK (Weragoda et al., 2004; Melemedjian et al., 2010c). Studies show that the phosphorylation of eIF4E by MNK is increased in mouse brain 24H post NGF-infusion (Salehi and Mashayekhi, 2007) and after BDNF stimulation of cortical neurons (Genheden et al., 2015). Current studies (Chapter 3) show that an inhibition of eIF4E phosphorylation suppresses pain behavior and pain plasticity, suggesting that a key step in the transition to pathological pain plasticity is the phosphorylation of eIF4E by its upstream kinase MNK.

Many translational pathways that play an important role in pain plasticity originated from

cancer studies and how inhibition of mRNA translation prevents tumorigenesis. For example, the transgenic mouse used in the current study, *eIF4E*^{S209A}, in which the single phosphorylation site on serine 209 on eIF4E was mutated to alanine, showed that mouse embryonic fibroblasts (MEFs) isolated from these mice displayed a resistance to oncogenic changes and *eIF4E*^{S209A} mice failed to develop tumors (Furic et al., 2010b).

In eIF4E^{S209A} MEFs, a variety of mRNAs are excluded from translating polysomes (Herdy et al., 2012b). These eIF4E-dependent mRNAs code for plasticity-related genes such as matrix metalloproteinases, cytokines, and chemokines (Herdy et al., 2012b; Gkogkas et al., 2014b). Many of these eIF4E-dependent mRNAs are predicted to have secondary structures in their 5' untranslated region (5'UTR) and may rely on the activity of eIF4A to initiate translation (Manzella et al., 1991; Koromilas et al., 1992; Svitkin et al., 2001; Cencic et al., 2009; Cawley and Warwicker, 2012). Previous studies indicate that translation of mRNAs containing long, highly structured 5'UTRs are influenced by signaling to the eIF4F complex (Svitkin et al., 2001; Sonenberg and Hinnebusch, 2009b). Recent evidence suggests that highly conserved secondary structures in the 5'UTR of mRNAs suppresses gene expression (Boussemart et al., 2014; Wolfe et al., 2014c). These secondary structures are energetically stable and make it difficult for ribosomes to scan the start codon in mRNAs, causing inefficient production of the protein product. The RNA helicase, eIF4A, is recruited during the formation of the eIF4F complex to unwind secondary structures such as G-quaduplexes (Ray et al., 1985; Bordeleau et al., 2005; Wolfe et al., 2014c). It is possible that eIF4E phosphorylation enhances eIF4A helicase activity to unwind G-quaduplexes in plasticity-related mRNAs. Or the motifs that require eIF4E phosphorylation could identify a separate pool of mRNAs. A recent study identified a 15-

nucleotide motif termed 'CERT'-gene signature in the 5'UTR for mRNAs dependent on eIF4E in oncogenic translation (Truitt et al., 2015). One way to identify if eIF4E phosphorylation governs mRNA translation of a motif is to utilize a new technique called ribosome foot-printing profiling (RPF). This technique was developed by Ingolia and colleagues to identify mRNA sequences that are actively being translation (Ingolia et al., 2012; Ingolia, 2014). Using a combination of ribosome inhibitors, RNases, and RNAseq, RPF allows for the identification mRNA sequences protected by the ribosomal subunits. Looking at the RPF in eIF4E^{S209A} DRG neurons would provide insight into mRNAs whose translation is influenced by eIF4E phosphorylation and could potentially reveal sequence motifs that identify such transcripts.

EIF2ALPHA

Another critical pathway that regulates translation initiation separate of MNK-eIF4F is eIF2α. In its nonphosphorylated form, it aids in translation by recruiting the small ribosomal subunit, 40S, to form the ribosomal preinitiation complex. On the other hand, when eIF2α becomes phosphorylated general translation is decreased (Dever et al., 1992). Surprisingly, phosphorylated eIF2α increases translation of upstream open-reading frames (uORF) (Young and Wek, 2016). There are 4 main kinases that phosphorylate eIF2α, PKR-like ER kinase (PERK), double-stranded (ds) RNA-dependent protein kinase (PKR), general control non–derepressible-2 (GCN2), and heme-regulated inhibitor kinase (HRI) (Trinh and Klann, 2013). Each of these kinases are activated via different states of cellular stress: ER stress (PERK), presence of dsRNA (PKR), amino acid deprivation (GCN2), and heme deficiency (HRI). Researchers found that genetic manipulation of an important phosphorylation site on eIF2α (S51)

or deletion of one of its upstream kinase, GCN2, led to an increased state of memory (Costa-Mattioli et al., 2005; Costa-Mattioli and Sonenberg, 2006; Costa-Mattioli et al., 2007; Costa-Mattioli et al., 2009); while increased phosphorylated-eIF2α is associated with LTD and other pathological conditions (Di Prisco et al., 2014). A recent study discovered that mice with decreased eIF2α phosphorylation (*eIF2*$\alpha^{+/S51A}$) exhibited lower sensitivity to thermal pain (Khoutorsky et al., 2016a). Using a hypomorph phospho-mutant (*eIF2*$\alpha^{+/S51A}$), *Khoutorsky et. al.* found that a 50% reduction in eIF2α phosphorylation decreased thresholds of transient receptor potential vanilloid 1 (TRPV1) functional activity in DRG neurons.

TOOLS TO ASSESS CELL-SPECIFIC MRNA TRANSLATION

Translating ribosomal affinity purification

Even though these proteins which are responsible for mRNA translation are ubiquitously expressed, it is tempting to relate their effects on pain plasticity to changes in the nervous system. However, the subset of mRNAs governed by eIF4E-phosphorylation or eIF4A in neurons remains unknown. Utilizing a novel transgenic mouse technique, translating ribosomal affinity purification (TRAP) will allow us to tag actively translating ribosomes and identify subsets of mRNAs that are actively being translated. TRAP mice contain a flox-stop-flox eGFP sequence fused to a 60S ribosomal subunit protein, L10a (Zhou et al., 2013). When these mice are crossed with a cell-specific CRE recombinase expressing mutant mouse, only mRNAs being translated in that particular cell-type will be tagged with eGFP (Zhou et al., 2013). It is possible to identify these mRNAs through eGFP pull-down and RNA sequencing. We could utilize several cre-lines that are driven in peripheral nociceptors, from NaV1.8(Akopian et al., 1996),

Pirt (Kim et al., 2014), TRPV1(Cavanaugh et al., 2011), or peripherin (Zhou et al., 2002).

Targeted activation of specific tissues with opto-genetics

While eIF4E phosphorylation has its own specific kinase, MNK1 and MNK2 play differential roles in eIF4E phosphorylation (Ueda et al., 2004a). MNK2 is found to phosphorylate eIF4E under basal conditions while MNK1 plays a constitutively active role (Ueda et al., 2004a). It would be interesting to look at different expression levels of MNK1 and MNK2 in peripheral vs central nervous systems and if/and/or which MNK isoform plays a key role in the development of pain plasticity by utilizing MNK1 KO and MNK2 KO mice (Ueda et al., 2004b) Additionally, determining the tissue distribution of MNK1 vs MNK2, i.e. peripheral nerve vs DRG, would give us further insight into the location of activity-dependent translation (Copits et al., 2016). A new emerging therapy to not only identify mechanisms of pain plasticity, but also treat chronic pain is optogenetics. Recently, Zhou *et. al.* published in Science the development of photoswitchable kinases. They were able to switch off kinase activity with a 500nm cyan light, and switch on the activity using a 400nm violet light. In combination with the new soft-stretchable implantable device to deliver the optics (Il Park et al., 2015; Il Park et al., 2016), the development of a photoswitchable- MNK1 or MNK2 would be able to answer several questions on how, where, and even when translation control is required for pain plasticity.

IS MRNA TRANSLATION IN OTHER CELL TYPES IMPORTANT IN THE DEVELOPMENT OF PAIN PLASTICITY?

While utilizing TRAP technology identifies mRNAs regulated by eIF4E phosphorylation in specific cell-types, the question of which cell-type is responsible for the development of

peripheral pain plasticity remains. Evidence continues to accumulate showing that the peripheral nervous system is able to detect alterations in inflammatory signals (Aley et al., 1998; Dina et al., 2005; Abrahamsen et al., 2008a). The intracellular mechanisms of these plastic changes, consequential sensitization, and promotion of chronic pain are not completely understood. Danger-associated molecular patterns (DAMPs), are molecules expressed by native tissues in response to injury or inflammation and can activate the peripheral nervous system leading to plasticity that contributes to chronic pain (Kato and Svensson, 2015) (Honda et al., 2000; Allette et al., 2014; Agalave and Svensson, 2015; Krock et al., 2016). These changes can lead to the plasticity that promotes the development of chronic pain. However, the exact cell type(s) expressing these translation factors and intracellular mechanisms that regulate the initiation and progression of chronic pain remains poorly understood.

Several studies have demonstrated that translation control in response to injury or specific algogens, is a key signaling mechanism required for sensitivity of nociceptors and the development and maintenance of pain plasticity in a range of preclinical pain models (Price and Géranton, 2009b; Melemedjian et al., 2010c; Melemedjian et al., 2011b; Price and Ghosh, 2013a; Khoutorsky et al.). This is important because translation signaling is controlled by a specific set of kinases that provide therapeutic opportunities and pharmacological intervention. Notably, current inhibitors of prototypic kinase pathways (ex. mTORC1) lead to negative feedback-mediated nociceptive hypersensitivity, contributing to their failure in clinical trials (Carracedo et al., 2008b). The signaling mechanism involving MNK is found in every subset of cells pertinent to pain plasticity (Wolfe et al., 2014a).This altered translation process causes nociceptor hypersensitivity and hyperexcitability, potentially involving regulation of voltage

gated sodium channels (VGNaC) (Matthews et al., 2006; Wilson-Gerwing et al., 2008; Yan et al., 2012b; Hockley et al., 2017). Direct action on both neurons and immune cells (and perhaps other cell types) can play an important and potentially disparate role on nociceptive hypersensitivity and the development of pain. The evident need for MNK1 floxed and MNK2 floxed mice becomes glaringly strong. Once these mice are developed we could possibility identify whether translation regulation contributes to pain plasticity in an exact cell-type or if it is a combination of several cell-types.

CONCLUSIONS

Translation control has repeatedly been shown to play a role in pain plasticity in both central and peripheral nervous systems. Identifying the mechanisms that drive the transition from injury to chronic pain is critical to the design of new treatment options for patients. Thus far, mechanisms involving translation control in the spinal cord include the roles of PKMζ (Asiedu et al., 2011d), mTOR (Melemedjian et al., 2013e), and FMRP (Price and Melemedjian, 2012). While it is clear that manipulating these pathways affect the development of pain plasticity, we believe that these mechanisms mediate synaptic plasticity in the central nervous system. Synaptic formation is important for higher brain functions such as learning or working memory (Neves et al., 2008). This is not something we as pain researchers want to target. On the other hand, we have identified a translational pathway, MNK – eIF4E, that modulates intrinsic excitability in peripheral nociceptors and that inhibiting this pathway blunts the transition to chronic pain. We believe that targeting mechanisms involved in intrinsic excitability will produce more reliable

therapeutics. We strongly believe that the MNK - eIF4E pathway provides an ideal mechanism for new therapeutic treatments for chronic pain.

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BIOGRAPHICAL SKETCH

Jamie Katherine Moy was born in Mesa, Arizona to Jack and Winnie Moy. She attended Corona del Sol high school in Tempe, Arizona and graduated in 2007. After completing, she went on to join The University of Arizona in Tucson, Arizona. In 2011, she received a Bachelor of Science degree majoring in biochemistry and molecular biophysics and minoring in chemistry. Jamie next joined the Smithsonian Tropical Research Institute in Panama City, Panama as a Conservation, Research and Education Opportunity (CREO) research assistant. In the summer of 2012, Jamie began her graduate work at The University of Arizona in the department of medical pharmacology. In the spring of 2014, she transferred to The University of Texas at Dallas under the department of cognition and neuroscience.

CURRICULUM VITAE

Jamie Katherine Moy

EDUCATION

The University of Texas at Dallas
Doctorate of Philosophy in Cognition and Neuroscience
Projected Graduation – May 2017
Dissertation – Translation regulation in central and peripheral pain plasticity
Cumulative GPA: 3.835

The University of Arizona, Tucson, Arizona
Bachelor of Science in Biochemistry and Molecular Biophysics
Minor in Chemistry, awarded May 2011
Senior Thesis – Quantification of Symbiotic Bacteria in Apis mellifera
Cumulative GPA: 3.34, Major GPA: 3.28
Awards: Dean's List (Spring '08/Spring '10)

RESEARCH GOALS

To investigate the role of protein translation in mechanisms of pain plasticity, discovering new therapeutics to treat chronic pain. Ultimate goals: to become a tenure track professor at a research university to be able to inspire science just as my professors did for me.

SCHOLARSHIPS

- First place in UT-Dallas 3-minute Thesis (2016)
  – $1,000.00
- People's choice in UT-Dallas 3-minute Thesis (2016)
  – $500.00
- Award recipient in Graduate Professional Student Council: Student Showcase poster fair (2013)
  – $125.00
- Recipient of the Hank Yamamura Endowed Fellowship in Pharmacology (2012)
  – Recipient 1 out of 2, awarded $250.00
- Recipient of The University of Arizona Graduate Fellowship (2012)
  - Awarded $230.00
- Recipient of Initiative for Maximizing Student Diversity for Ph.D.s in Biomedical Sciences (2012)
  - Funded by the National Institute of Minority Health and Health Disparities, awarded to minority student by funding the 1st year ($24,000).
- Award recipient in Chemistry Biochemistry poster fair (2011)
  - $100.00
- Undergraduate Affiliated Network (UAN) Travel Award recipient (2011)

- Was awarded $400 to travel to Experimental Biology
- Wildcat Excellence scholarship (2007-2011)
  - Awarded $5,000 for 4 years

RESEARCH EXPERIENCE

University of Texas at Dallas, Pain neurobiology group

Dr. Theodore Price's laboratory, August 2012 – Present

- Research focuses on discovering novel mechanisms to treat the initiation and transition to chronic pain. My main project focuses on mRNA translation machinery, specifically the cap-complex (eIF4F) of mRNAs. We hypothesize that each component of eIF4F regulates the translation of a subset of mRNAs that play key roles in pain plasticity.
- **In lab:** Protein extraction, western blots, immunohistochemistry, confocal imaging, behavioral assays, dissections (mouse and rat), (see Laboratory Skills section for further information)
- Several papers published with Dr. Price (see below).

Conservation, Research and Education Opportunity (CREO) Assistantship, National Institute of Minority Health and Health Disparities, Smithsonian Tropical Research Institute

International Cooperative Biodiversity Group laboratory, Dr. Kevin Tidgewell, November 2011-April 2012

- Research focuses on natural products drug discovery from marine cyanobacteria. My goal will be to collect, identify, extract, isolate, and identify secondary metabolites around Panama. The extractions will then be screened for activity against 3 parasitic diseases (Malaria, Chagas' disease, and Leishmaniasis) as well as cancer cells.
- **In lab:** 16S sequencing, purification and structure elucidation, flash/vacuum liquid chromatography, HPLC and NMR (see Laboratory Skills section for further information).

Undergraduate Researcher, University of Arizona Department of Pharmacology

Dr. Todd Vanderah's laboratory, September 2010 – November 2011 (including summer of 2011)

- Research focuses on cannabinoids and their importance in cancer metastasis. My goal is to successfully eliminate a functional pathway associated with cannabinoid receptor 2 as part of a larger study to understand the pathway and its importance.
- **In lab:** western blots, transfections, mouse surgeries/dissections, cell culture, SRB staining, protein extraction, prepared selection medium, cloning (see Laboratory Skills section for further information).
- Poster presented in 2011 Chemistry and Biochemistry poster fair: First Author: "Cannabinoid receptor 2 knockdown in breast cancer cells: an impossible feat?"- Award Recipient
- Paper published (see below).

Undergraduate Researcher, University of Arizona School of Plant Sciences, Department of Plant Pathology

Dr. Anne Elizabeth Arnold's laboratory, June 2010 – November 2011 (including summers of 2010 and 2011)

- Research focuses on microbial biodiversity, evolution, and systematics, and on applications of newly discovered symbiotic microbes in medicine, industry, and agriculture. I am assisting with a large project assessing the diversity and secondary metabolites of endophytic fungi associated with Sonoran Desert plants, and conducting an independent project to examine the effects of surface-sterilants on leaf chemistry and associated microbial yields.
- **In lab:** media preparation, DNA extraction, PCR, sequence assembly and editing, fungal isolation and vouchering, sterile technique, BLAST (see Laboratory Skills section for further information).
- Poster presented in 2011 Research Insights in Semiarid Ecosystems (RISE) Symposium: First Author: "Effects of microbial communities on in vitro and in situ degradation of plant material in an arid ecosystem"

Undergraduate Researcher, University of Arizona Department of Molecular and Cellular Biology, Department of Biochemistry, and Department of Ecology and Evolutionary Biology

Drs. Howard Ochman/Nancy Moran's laboratory, February 2009 – May 2010 (including summer of 2009)

- Research focuses on bacterial communities living symbiotically within insects. My work has focused on gene expression in aphids and their diet as well as bacterial communities in the gut of *Apis mellifera* (European honeybee).
- **In lab:** RNA extraction, PCR, quantitative PCR, DNA recombination, insect dissection (see Laboratory Skills section for further information).
- Senior Thesis: Research focused on where and when certain bacterial communities developed in the gut throughout the lifetime of *Apis mellifera* (European Honeybee).
- Paper published with Mr. Martinson and Dr. Moran (see below).

Laboratory Assistant, University of Arizona School of Plant Sciences:

Dr. Rod Wing laboratory, August 2007 – May 2008

- Research focuses on plant genomics. My work consisted of aiding a graduate student in assessing sensitivity of different genotypes of rice to different environmental conditions.
- **In lab:** making solutions, preparing gels, operating fill machines, spreading plates (see Laboratory Skills section for further information).

LABORATORY SKILLS

Nuclear magnetic resonance, gas chromatography, column chromatography, DNA extraction, RNA extraction, protein extraction, Polymerase Chain Reaction (PCR), Quantitative PCR (qPCR), sequence assembly and editing, Primer designs, BLAST analysis, mouse surgeries (Spared nerve injury, Brennan model), dissection, DRG and TG primary cell culture, western blot, gel electrophoresis, histology, dual luciferase, florescence, calcium imaging, pain behavioral assays: Hargreaves, Von Frey, and grimace. Confocal imaging, live-cell imaging, Aplysia electrophysiology, immunohistochemistry, etc.

PUBLICATIONS

- **Moy, JK.** Khoutorsky, A. Asiedu, MN. Black, BJ. Kuhn, JL. Barragán-Iglesias, P. Megat, S. Burton, MD. Burgos-Vega, CC. Melemedjian, OK. Boitano, S. Vagner, J. Gkogkas, CG. Pancrazio, JJ. Mogil, JS. Dussor, G. Sonenberg, N. Price, TJ. eIF4E phosphorylation regulates behavioral pain plasticity and sensory neuron excitability through sodium current density. The Journal of Neuroscience. *In revision.*
- Ganzer, P. Meyers E. Solorzano, B. Robertson, N. Adcock, K. James, J. Ruiz, A. Becker, A. Goldberg, M. Pruitt, D. **Moy, J.** Hassler, S. Price, T. Gluf, W. Kilgard, M. Rennaker, R. Enhancing plasticity and recovery following spinal cord injury. eLife. *Submitted.*
- Hanlon, KE. Lozano-Ondoua, AN. Umaretiya, PJ. Symons-Liguori, AM. Chandramouli, A. **Moy, JK**. Kwass, WK. Mantyh, PW. Nelson, MA. Vanderah, TW. Modulation of breast cancer cell viability by cannabinoid receptor 2 selective agonist JWH-015 is calcium dependent. Breast Cancer: Targets and Therapy. PMID: 27186076
- Kim, JV. Megat, S. **Moy, JK.** Asiedu, MN. Mejia, GL. Vagner, J. Price, TJ. Neuroligin-2 regulates spinal GABAergic plasticity in hyperalgesic priming, a model of the transition from acute to chronic pain. Pain. PMID: 26859820
- Burgos-Vega, C. **Moy, J**. Dussor, G. Meningeal afferent signaling and pathophysiology of migraine. Progress in Molecular Biology and Translation Science. PMID: 25744685
- Melemedjian OK, Tillu DV, **Moy JK**, Asiedu MN, Mandell EK, Ghosh S, Dussor G, Price TJ. Local translation and retrograde axonal transport of CREB regulations IL-6-induced nociceptive plasticity. Molecular Pain. PMID: 24993495
- Parker SS, Mandell EK, Hapak SM, Maskayakina IY, Kusne Y, Kim JY, **Moy JK**, St. John PA, Wilson JM, Gothard KM, Price TJ, Ghosh S. Competing interactions of PKMζ and aPKCλ with Par complex regulates polarity. Proceedings of the National Academy of Sciences. doi: 10.1073/pnas.1301588110. PMID: 23940317
- Melemedjian OK, Tillu DV, Asiedu MN, Mandell EK, **Moy JK**, Blute VM, Taylor CJ, Ghosh S, Price TJ. BDNF PKMζ at spinal synapses to initiate and maintain a centralized chronic pain state. Molecular Pain. doi: 10.1186/1744-8069-9-12. PMID: 23510079.
- Martinson VG, **Moy J**, Moran NA. April 2012. Establishment of characteristic gut bacteria during development of the honey bee worker. Applied and Environmental Microbiology. doi: 10.1128/AEM.07810-11. PMID: 22307297.

RESEARCH PRESENTATIONS

1. **16th World Congress: International Association for the Study of Pain,** September 30, 2016 *Moy JK*, *Khoutorsky A, Asiedu MN, Black BJ, Kuhn JL, Barragán-Iglesias P, Megat S, Burton MD, Burgos-Vega CC, Melemedjian OK, Boitano S, Vagner J, Gkogkas CG, Pancrazio JJ, Mogil JS, Dussor G, Sonenberg N, Price TJ. "eIF4E phosphorylation regulates behavioral pain plasticity and sensory neuron excitability" (poster)*
2. **American Pain Society,** May 12, 2016 *Moy JK, Ray P, Walters ET, Pelletier J, Price TJ. "Exploring the potential for an evolutionary conserved role for eIF4A in pain plasticity" (poster)*

3. **Society for Neuroscience,** October 20, 2015 *Moy JK, Khoutorsky A, Asiedu MN, Gkogkas CG, Dussor G, Sonenberg N, Price TJ. "eIF4E phosphorylation links BDNF mRNA translation to pathological pain plasticity" (poster)*
4. **Society for Neuroscience,** November 18, 2014 *Moy JK, Asiedu MN, Khoutorsky A, Tillu DV, Melemedjian OK, Mejia GL, Kim JY, Sonenberg N, Dussor G, Price TJ. "An essential role for eIF4E phosphorylation in peripherally mediated pain plasticity" (poster)*
5. **Graduate Professional Student Council Student Showcase,** November 8, 2013 *Moy JK, Asiedu MN, Khoutorsky A, Sonenberg N, Price TJ. "Inhibition of eIF4E phosphorylation prevents acute and persistent sensitization in a mouse model of hyperalgesic priming"* (poster)
6. **Research Insights in Semiarid Ecosystems (RISE) Symposium,** October 29, 2011 *Moy JK, Arnold AE, Predick K, Levi EM, Archer, SR. "Effects of microbial communities on in vitro and in situ degradation of plant material in an arid ecosystem"* (poster)
7. **Chemistry and Biochemistry Senior Thesis/Capstone Poster Session,** May 3, 2011 *Moy JK, Martinson V, Moran N. "Quantification of Symbiotic Bacteria in* Apis mellifera*."* (poster)
8. **Experimental Biology 2011 Annual Meeting,** April 9-12, 2011
   Moy JK, Hanlon KE, Vanderah TW. "Cannabinoid receptor 2 knockdown in breast cancer cells: an impossible feat?" (poster)
9. **University of Arizona Chemistry and Biochemistry Department Poster Fair,** April 6, 2011
   Moy JK, Hanlon KE, Vanderah TW. "Cannabinoid receptor 2 knockdown in breast cancer cells: an impossible feat?" (poster)
10. **University of Arizona Ecology and Evolutionary Biology Student Research Symposium,** April 20, 2010
    Moy JK, Martinson V, Moran N. "Quantification of Symbiotic Bacteria in Apis mellifera*."* (poster)
11. **Biological, Engineering, and Chemical Undergraduate Research Meeting (BECUR),** February 26-27, 2010
    Moy JK, Martinson V, Moran N. "Quantification of Symbiotic Bacteria in Apis mellifera*."* (poster)

TEACHING EXPERIENCE

- **Graduate Teaching Assistant** The University of Texas at Dallas (2014-present)
  - Run review sessions for neuroscience courses, grade exams, as well as give occasion lectures.

- **Laboratory training** The University of Texas at Dallas (2014-Present)
  - Trains undergraduates and masters students in the research laboratory to do hands on research. Training includes basic problem solving skills as well as expanding their scientific knowledge based off of current research.

REFERENCES

- Theodore J. Price, PhD., Associate Professor, School of Behavioral and Brain Sciences, Department of Cognition and Neuroscience, The University of Texas at Dallas, Richardson, TX 75080. Theodore.price@utdallas.edu, (972) 883-4311

- Gregory Dussor, PhD., Associate Professor, School of Behavioral and Brain Sciences, Department of Cognition and Neuroscience, The University of Texas at Dallas, Richardson, TX 75080. Gxd14130@utdallas.edu, (972) 883-2835.

- Edgar T. Walters, PhD., Professor, Department of Integrative Biology and Pharmacology, The University of Texas Health Science Center at Houston, Houston, TX 77030. Edgar.T.Walters@uth.tmc.edu. (713) 500-6314

- Kevin J. Tidgewell, PhD., Assistant Professor, Department of Medicinal Chemistry, Duquesne University, Pittsburgh, PA 15282. tidgewellk@duq.edu, (412) 396-5279