ROLES OF eIF4A AND eIF4G IN *DROSOPHILA* NOCICEPTION

A Thesis
by
GITA GAJJAR

Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2019
Department of Biology

ROLES OF eIF4A AND eIF4G IN *DROSOPHILA* NOCICEPTION

A Thesis
by
GITA GAJJAR
August 2019

APPROVED BY:

Dr. Andrew Bellemer
Chairperson, Thesis Committee

Dr. Ted Zerucha
Member, Thesis Committee

Dr. Darren Seals
Member, Thesis Committee

Dr. Zack Murrell
Chairperson, Department of Biology

Michael Mckenzie, Ph.D.
Dean, Cratis D. Williams School of Graduate Studies

Abstract

ROLES OF eIF4A AND eIF4G IN *DROSOPHILA* NOCICEPTION

Gita Gajjar
B.S., Maseno University
M.S., Appalachian State University

Chairperson: Dr. Andrew Bellemer

Nociception refers to the detection of noxious mechanical, chemical, or thermal stimuli by specialized neurons called nociceptors. Sensitization of these nociceptor neurons in response to tissue damage or inflammation is a root cause of chronic pain. Chronic pain is a global health issue with colossal socio-economic impacts, as it is a reality for over 100 million U.S. adults. Chronic pain requires long-term changes in the physiology and function of neurons that normally process painful stimuli. Protein synthesis is a major regulator of neuronal plasticity, and is thus required for changes in nociceptor sensitivity during the development of chronic pain. The goal of this study was to characterize the components of the eukaryotic initiation complex (eIF4F) that regulate protein translational initiation mechanism in the nociceptors. *Drosophila* was used as a model organism to study nociceptor function following manipulation of eIF4A, eIF4G1, and eIF4G2 function. Results show that eIF4A is required for normal thermal and mechanical nociception sensitivity as well as sensitization of the nociceptors following tissue damage. *eIF4A* knockdown larvae showed defects in

dendrite morphogenesis, suggesting eIF4A-dependent mRNAs are involved in dendrite morphogenesis. eIF4G1 and eIF4G2 are required for normal thermal and mechanical sensitivity, but eIF4G1 is not required for hypersensitization, while eIF4G2 is required following tissue damage. This suggests that an eIF4G-independent translational mechanism may function in nociception and in nociceptor sensitization. There were no major defects in morphology following *eIF4G1* or *eIF4G2* knockdown, suggesting eIF4G does not function in morphogenesis. Taken as a whole, these results suggest that the eIF4F assembly function as an important protein translational initiation mechanism in the nociceptors of *Drosophila.*

Acknowledgments

I would like to acknowledge and say hats off to my supervisor, **Dr. Andrew Bellemer** whose advice and mentoring encouragement throughout my stay at Appalachian State and this period of thesis writing has not only impacted on my personal achievements but also on the science that I proudly present. I would also like to acknowledge the members of my thesis committee Dr. Bellemer, Dr. Zerucha and Dr. Seals who tirelessly and patiently guided me in my sludge through the program to become a better person in science.

I would like to acknowledge the contributions of my colleagues in lab. Adam Willits with help with microscopy, Katherine Hoffman who graduated, helped with molecular trouble-shooting and Haley McGuirt for always being a positive personality. I would also like to acknowledge the awesome undergrads of the Bellemer Lab.

I also acknowledge the Appalachian State University for providing the Course, Graduate school and Office of Student research for giving me grants and Office of International Education and Development for giving me scholarship and guiding me through immigration process. I also want to thank the Biology Department for giving me a home away from home.

To you all, I say, thank you very much.

Dedication

To the science community.

Table of Contents

List of Tables

List of Figures

Introduction

Pain is an important, evolutionarily conserved physiological phenomenon that is necessary for survival. It is an unpleasant experience associated with stimuli that have the potential to cause tissue damage (Weary et al., 2006). Pain is thus a valuable defense mechanism. Acute pain may be caused by exposure to a noxious stimulus or by short-term injury, and this may gradually progress towards chronic pain that lasts much longer (Carr and Goudas, 1999). Chronic pain is the number one cause of long-term disability in the United States (NIH, 2010). Chronic pain is defined as any pain lasting more than three months, is persistent, and may limit a person's movement and reduce flexibility, strength, and stamina leading to disability and despair (Treede et al., 2015). The development of chronic pain requires long-term changes in the physiology and function of sensory neurons. Understanding more about how these changes occur could lead to more effective treatment of chronic pain.

The perception of pain is initiated by specialized somatosensory neurons called nociceptors that are present with free nerve endings in the skin, muscle, joints, bone and viscera (Fields, 1987). Nociceptors respond to noxious thermal, mechanical or chemical stimuli. One hundred years ago, Sherrington explained a noxious stimulus as one with an intensity and quality sufficient to trigger reflex withdrawal, autonomic responses, and pain, collectively constituting the nociceptive reaction (Reviewed in (Woolf and Ma, 2007)). Sensing noxious stimuli and responding with appropriate nociceptive responses is essential for avoiding potentially harmful environments, and thus nociceptors are present in organisms ranging from simple invertebrates to complex vertebrates suggesting a conserved response. Nociceptive pain is therefore the pain produced by activating peripheral nociceptors, generally in the soft tissue

like the skin and muscles and helps with organismal survival in different environments (Loeser and Treede, 2008).

Nociceptive sensitization is a response to tissue damage, whereby sensory neurons near damaged tissue enhance their responsiveness to external stimuli by increasing their excitability. When tissue damage occurs, an extracellular exudate rich in inflammatory molecules is formed. This causes peripheral sensitization. Sensitization of nociceptor neurons in response to tissue damage or inflammation is a root cause of chronic pain (Pinho-Ribeiro et al., 2017). Nociceptors neurons have specific thresholds for activation by noxious stimuli. Sensitization can increase nociceptor activity by altering these thresholds. Pain scientists distinguish two aspects of sensitization: allodynia and hyperalgesia (Gold and Gebhart, 2010). Allodynia is pain resulting from a normally innocuous stimulus, and thus associated with a decrease in nociceptor threshold. Hyperalgesia is an enhanced response to a normally painful stimulus (Fein, 2012). Allodynia and hyperalgesia can be thought of as protective adaptations during the healing of injured tissues in order to prevent further damage. Some protein factors that mediate nociceptive sensitization are known. These include nerve growth factor, prostaglandins, bradykinin, and tumor necrosis factor α (Reviewed in (Hucho and Levine, 2007)). A more detailed knowledge of the pathways that lead to nociceptive neuron sensitization may allow for the identification of novel targets for the treatment of chronic pain.

Drosophila as a model organism for the study of nociception

My study used *Drosophila* as a model organism to study nociception. It has been estimated that 75% of known human disease genes have homologs in the *Drosophila* genome (Reiter et al., 2001), and *Drosophila* provides an experimental model for more than 800 human

ailments (Oriel and Lasko, 2018) including neurological disorders (Pandey and Nichols, 2011; Wangler et al., 2015). As is the case with vertebrate animals, *Drosophila* nociceptors function in nociception through expression of molecules that are able to detect and signal the presence of potential danger. Downstream of the nociceptive sensory input, the neural signals trigger protective (nocifensive) behaviors, such that the sensory stimuli that reach the brain are perceived to be painful. *Drosophila* larvae demonstrates such nocifensive behavior in response to parasitoid wasp infestation, and this behavior has been proposed to have evolved as a selective pressure (Hwang et al., 2007). When exposed to noxious stimuli, *Drosophila* larvae curl into a C shape and roll laterally quickly towards or away from the noxious stimuli, which is easily distinguishable from their normal peristaltic motion. This response, called nocifensive escape locomotion (NEL), was used to establish a paradigm for studying nociception in *Drosophila* while researchers in Tracey lab were attempting to study genes involved in nociception (Tracey et al., 2003).

Early studies of *Drosophila* nociception found that when the larval body wall was touched with a heated probe above 39˚C, larvae demonstrated the NEL behavior, which could be quantified based on the time between probe contact and execution of the NEL response (Tracey et al., 2003). The response was recorded as latency and was dependent on the sensitivity of the animal or to the strength of the stimulus; a faster response can be interpreted as a more sensitive animal or stronger stimulus. This experimental paradigm was used to identify the *painless* gene and hypothesized its involvement in the biophysical mechanism underlying both thermal and mechanical nociception. The NEL response provides *Drosophila* researchers with a quantifiable measure they can use to study the mechanisms of nociception in *Drosophila* larvae.

Nociceptors express special receptors called transient receptor potential (TRP) ion channels. TRP channels have roles in many different sensory systems. They were first described in *Drosophila* based on a transient voltage response to continuous light in the photoreceptors of flies harboring *trp* gene mutations. Characterization of *painless* mutants revealed that *painless* mRNA encodes a TRP channel protein of the ankyrin repeat-containing subfamily (TRPA). There are three naturally occurring RNA variants of *painless* that are predicted to encode Painless protein isoforms, which vary in the length of the ankyrin repeat containing N-terminal domain. Individual isoforms confer either thermal or mechanical nociceptive sensitivity, but not both. This could be suggestive that noxious thermal and mechanical sensitivity are being regulated independently by *painless* isoforms (Hwang et al., 2012). *Drosophila* possesses a second homolog of mammalian TRPA1 that is known as *Drosophila* TRPA1 (dTRPA1). Studies of mutant flies lacking dTRPA1 function demonstrate that it is also required for thermal and mechanical nociception (Zhong et al., 2012).

TRPA1 is also activated by noxious chemicals. The activation of TRPA1 by noxious chemicals is seen in TRPA1 channels cloned from the genomes of frogs, zebra-fish and *Drosophila* (Kang et al., 2010; Prober et al., 2008; Saito et al., 2012). In a different study, plants of the genus *Brassica* and *Capsicum* were used to demonstrate similar molecular strategies to produce irritation and inflammation in herbivorous mammals involving activation of TRPA1 on primary sensory nerve endings (Jordt et al., 2004). The study also suggested these ion channels can be activated by the inflammatory molecule, bradykinin, suggesting its role in nociceptor sensitization. Widespread TRPA1 chemical sensitivity hints to its acquisition at an early stage of animal evolution and its importance as a conserved TRPA1 channel function.

Nociceptors also have receptors that are specifically activated by noxious mechanical stimuli. Studies of the *pickpocket (ppk)* gene in *Drosophila* larvae reported that a loss of function mutation in *ppk* produced a defective behavioral response to mechanical stimuli, but not to thermal stimuli. This gene was previously shown to encode a component of a degenerin/epithelial sodium channel (DEG/ENaC) expressed specifically in the class IV multidendritic neurons. The *ppk* mutation was shown to have a minor effect on locomotion, but mostly was involved in nociception (Ainsley et al., 2003). These results were confirmed using RNA interference (RNAi), by expressing *ppk-RNAi* in the nociceptor neurons and observing the same defective behavioral response as the loss-of-function mutant (Zhong et al., 2010). These results indicated that mechanical nociception in these neurons is dependent on *ppk*. Although the effect was not as severe in the RNAi phenotype as in the genetic null mutation, it was still significant and confirmed that *ppk* was important in the mechanical nociceptive response (Zhong et al., 2010).

Piezo I and Piezo II, both sodium ion channels, also respond to mechanical stimuli (Coste et al., 2010; Kim et al., 2012). The effect of Piezo on the nociceptive response of *Drosophila* larvae was characterized using behavioral assays for NEL and by studying behavioral interactions between RNAi knockdown phenotypes of *piezo* and *painless* and *piezo* and *pickpocket*. The first combination produced defects similar to those shown when either gene was knocked down, but knocking down *piezo* and *pickpocket* increased the mechanical nociception defect even further, nearly completely eliminating the response to noxious mechanical stimuli. This suggested *Piezo* and *ppk* were both important components of two different mechanical nociception mechanisms.

Drosophila nociceptor development

The *Drosophila* multidendritic dendrite-arborization (da) neurons are an excellent system to analyze the molecular mechanisms of dendrite differentiation (Gao et al., 1999; Parrish et al., 2007). A study by Hwang et al. (2007) demonstrated that class IV multidendritic neurons function as nociceptors in the sensation of noxious thermal and mechanical stimuli (Hwang et al., 2007). By using optogenetic activation, the study demonstrated class IV multidendritic neuron activation as sufficient and necessary for the NEL response.

Many studies in *Drosophila* have been done to show that transcriptional regulation plays an integral role in the proper development of da neuron dendrites (Brenman et al., 2001; Grueber et al., 2003a; Li et al., 2004; Parrish et al., 2006; Sugimura et al., 2003). The morphological complexity of the dendrites is accomplished under certain organizing principles that govern patterning and spacing of dendrite and axon arbors to fill up space under the larval epidermis. Several transcription factors function in combination to instruct the cells to grow and fill the space, including *Cut*, *Abrupt* (*Ab*) and *Spineless*

Cut is a principle factor involved in the combinatorial regulation of dendrite arborization in da neurons. *Cut* encodes a homeobox gene, and the level of *Cut* expression correlates with neuronal cell type (Grueber et al., 2003a; Parrish et al., 2006). When *Cut* was removed from class IV and III multidendritic neurons, the dendritic arbor was significantly smaller than wild-type neurons. The class III neurons have short protrusions known as dendritic spines. These short actin-based protrusions that are important in mechanotransduction. When the function of *Cut* was removed, these spikes disappeared. When *Cut* was overexpressed in class I neurons, there was a dramatic increase in dendrite length. *Cut* is normally expressed at a higher level in Class III neurons than in Class IV neurons. When

Cut was overexpressed in Class IV neurons, the Class IV neurons showed a morphology closer to Class III neurons. This suggests that *Cut* is being expressed at different levels in da neurons in a persistent and class-correlated pattern. Because loss of function and gain of function manipulations cause reciprocal switches in dendrite branching patterns (Grueber et al., 2003a; Grueber et al., 2003b), it is hypothesized that high *Cut* expression directs the production of more complex arbors, and specifically the dendritic spikes in class III neurons. Low *Cut* levels, in contrast, lead to simple dendrites (Grueber et al., 2003b). Studies using the human homolog of *Cut*, called Cux1, show that *Cut* function is evolutionarily conserved. Like *Cut*, *Cux1* expression exhibited a dose-dependent effect on cortical neuron development (Cubelos et al., 2010).

The *Ab* gene, encodes an evolutionarily conserved transcription factor that is widely expressed during embryogenesis. *Ab* contains a Zinc Finger (BTB/POZ) domain and C2H2 zinc finger motifs (Hu et al., 1995). *Ab* is expressed in class I neurons, but it is absent in other classes of the neurons. This is the opposite of *Cut*, which is absent in class I neurons, but is present in class II, III, and IV neurons. This suggests that transcription codes in different neurons also regulate dendritic morphology (Li et al., 2004). Loss of function mutations in the *Ab* gene increased the dendritic branching of multidendritic neurons in *Drosophila* embryos and altered the arborization of class I dendrites (Parrish et al., 2006). Another study using heterozygous mutant *Ab* embryos reported that Ab^{+}/Cut^{-} neurons, but not Ab^{-}/Cut^{+} neurons show significantly greater dendritic branching in third instar larvae missing one copy of *Ab*. Overexpression of Ab in heterozygous mutants showed a reduction in dendritic branching in Ab^{+}/Cut^{-} neurons. The study suggests *Ab* functions in a dose-dependent manner, meaning that regulation of Ab activity by the neurons could be an effective way to control dendritic

branching complexity (Li et al., 2004). These transcription factors function combinatorially to instruct the growth of cells so that they fill the space during the development of *Drosophila* multidendritic neurons at the embryonic and larval stages.

Spineless (*ss*) is the *Drosophila* homolog of the mammalian aryl hydrocarbon (dioxin) receptor (Ahr) gene, which encodes a basic helix-loop-helix - PAS (bHLH–PAS) transcription factor (Duncan et al., 1998). *ss* is necessary for the formation of distinct morphological features of dendrites in different classes of da neurons. *ss* loss of function mutants exhibited reduced complexity in class IV dendrites. *ss* also regulates the number of dendritic spines in the class III neurons. In *ss* loss of function clones, these spines are lost. The dendritic patterns of different classes of da neurons in *ss* mutants becomes more homogeneous, leading to a reduction in the diversity between the four classes of the multidendritic neurons. *ss* loss of function in class I neurons increases the dendrite arbor complexity, whereas in class III it decreases the dendrite arbor complexity (Kim et al., 2006).

Drosophila tools used to study nociception

Targeting gene expression in a temporal and spatial fashion has proven to be one of the most powerful techniques for addressing gene function in *Drosophila*. To investigate the molecular mechanisms responsible for class IV multidendritic nociceptor function, the *ppk-Gal4* transgene has been widely used, with the 1.9kb promoter fragment of *pickpocket1* driving Gal4 expression to selectively label class IV nociceptive neurons in the periphery (Ainsley et al., 2003). The Gal4/UAS system is a binary expression system, adapted from yeast that allows tissue-specific expression of a gene of interest (Brand and Perrimon, 1993). In this system, expression of the gene of interest, the responder, is controlled by the presence of the UAS

element containing optimized Gal4 binding sites. Gal4 is a transcriptional activator that binds to UAS enhancer sequences found in DNA (Duffy, 2002). It then recruits transcription machinery to the site to induce gene expression. Thus, genes and short interfering RNAs encoded downstream of the UAS sequence in a transgene are only expressed when Gal4 is expressed. This allows gene expression to be targeted to specific tissues. (Caygill and Brand, 2016). Gene knockdown is accomplished by RNAi. The RNase III enzyme Dicer directs an RNA-induced silencing complex (RISC) to cleave mRNA or block its translation by interference (RNAi) thereby lowering the expression of the targeted gene by knockdown. RNAi disrupts the mRNA that is transcribed resulting in reduced levels of the target protein. The resulting phenotypes either are identical to those of genetic null mutants or resemble an allelic series of loss-of-function mutants (Hammond et al., 2001). Thus UAS-RNAi transgenic lines can be used experimentally to define which genes and structures are necessary for nociceptive behaviors.

Nociceptor Sensitization

Following tissue damage and during inflammation, the endothelial cells lining the capillaries contract. This contraction is responsible for the formation of an extracellular exudate rich in inflammatory molecules that cause peripheral sensitization (*Fig 1*). These molecules also activate immune cells and create a neuroimmune interaction both at the site of injury and within the central nervous system (CNS). At the site of tissue injury, these inflammatory molecules maintain a state of inflammation and contribute to nociceptor sensitization.

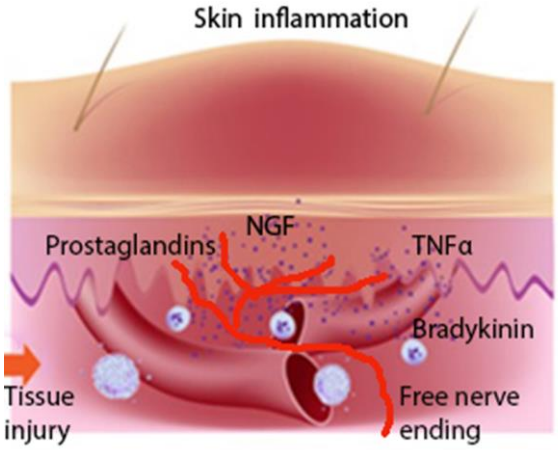

Figure 1: Inflammatory molecules participating in nociceptor sensitization. (*Adapted from ngxaki.com)*

Sensitization of class IV multidendritic neurons gives rise to allodynia and hyperalgesia in *Drosophila* larvae (Babcock et al., 2011). Diverse signaling pathways have been identified that mediate nociceptive hypersensitization in class IV multidendritic neurons, but the underlying mechanisms are not completely understood. In 2009, the Galko lab discovered that the *Drosophila* ortholog of Tumor Necrosis Factor α (TNFα) called *eiger* was necessary for the formation of hyperalgesia and allodynia following UV induced tissue damage (Babcock et al., 2009). Once they knocked down *eiger* via RNA interference, hyperalgesia and allodynia did not develop after UV-induced damage.

Hedgehog (Hh), the invertebrate ortholog of sonic hedgehog, is also necessary for the formation of allodynia and hyperalgesia (Babcock et al., 2011). When Hh was knocked down, the result was a lack of allodynia and hyperalgesia formation after UV-induced sensitization. The same results were achieved through knocking down the Hh receptor, *patched*, in the class IV multidendritic neurons. This suggests that Hh was released from damaged tissues, along with a multitude of other cytokines, and these were detected by the primary nociceptor neurons,

causing increased excitability of these neurons to normally innocuous stimuli (allodynia) and to already noxious stimuli (hyperalgesia).

Further studies have sought to identify genes downstream of Hh that might also be necessary for the formation of allodynia and hyperalgesia. One of those genes was *decapentaplegic* (*dpp)*, which encodes a functional homolog of the mammalian bone morphogenetic proteins (BMP) 2/4. BMPs are a group of signaling molecules that have important roles in the regulation of bone induction, maintenance, and repair. These proteins operate in a critical pathway downstream from Hh in nociceptors that is required for nociceptive sensitization but not for normal nociception or nociceptor development in *Drosophila* (Follansbee et al., 2017). Im et al. (2015) found that tissue injury lowers the threshold at which temperature causes NEL in fruit fly larvae by a signaling peptide called Tachykinin. A molecule called Tachykinin and its receptor is found upstream of Hh, and both are needed to regulate the observed development of allodynia. Another study reports experiments done on ROS-generating Dual Oxidase (*duox*) enzymes in *Drosophila* sensitization that *duox* heterozygous mutant larvae, which have normal basal nociception, exhibit an attenuated hypersensitivity response to heat and mechanical force following UV irradiation. The study further showed that silencing *duox* in class IV multidendritic neurons attenuates UV-induced sensitization, is a novel role for duox in nociceptive sensitization in *Drosophila* larvae (Jang et al., 2018). Together, these findings indicate the underlying mechanisms of nociceptive sensitization in Drosophila. These mechanisms have indeed been shown as evolutionary conserved from insects to mammals as discussed in the next section.

Signaling molecules that regulate nociceptor sensitization

Tumor Necrosis Factor α (TNFα)

The pro-inflammatory molecule TNFα is a mediator released by the immune system during tissue injury (*Fig 1*). TNFα produces local hyperalgesia when injected sub-cutaneously in a mouse model (Woolf et al., 1997). Complete Freund's Adjuvant (CFA) administration, a manipulation known to strongly induce inflammation, results in significant elevation in the levels of TNFα, interleukin-1b (IL-1b), and nerve growth factor (NGF) in the inflamed paw. (Woolf et al., 1997). This suggests that TNFα initiates a cytokine cascade that leads to the upregulation of nerve growth factor (NGF) during peripheral nociceptor sensitization.

Other studies have also shown the function of TNFα in nociceptive sensitization. Application of TNFα along the sciatic nerve results in increased nociceptor activity. When administered subcutaneously, TNFα also decreased the mechanical threshold of nociceptive primary afferent fibers (Sorkin et al., 1997). This suggests a possible role of TNFα in generating ectopic activity in nociceptive afferent fibers and consequently contributing to neuropathic pain states. Another consequence of subcutaneously administered TNFα is a potent increase in vascular permeability, thus allowing for inflammation that leads to peripheral nociceptor sensitization. In glabrous skin, TNFα-induced nociceptor sensitization coincided with TNFα-induced plasma extravasation, both with respect to the onset of the effects and the minimum effective dose (Junger and Sorkin, 2000). The early inflammation evoked by TNFα may be part of the normal immune response and can be accompanied by nociceptor activation.

Prostaglandins

Prostaglandins (PGs) are one type of molecule present in TNFα-induced extravasation. PGs are a group of physiologically active substance with diverse hormone-like effects in mammals. PGs are produced by cells in inflamed tissues (*Fig. 1*) (Ricciotti and FitzGerald, 2011). One of the functions of PG is to maintain the state of inflammation by acting on the smooth muscle cells surrounding the arterioles to continue the dilation of blood vessels (Ricciotti and FitzGerald, 2011). PGs are produced from an arachidonic acid precursor. This precursor is converted first into prostaglandin G2 (PGG2) and then into PGH2 in reactions catalyzed by cyclooxygenase (COX) enzymes (*Fig. 2*). There are two known COX enzymes: COX 1 and COX 2. COX 1 is endogenously expressed by all tissues. COX 2 is upregulated in inflamed tissues. PGH2 gives rise to other types of PGs, including PGE2, which is involved in nociception (Ricciotti and FitzGerald, 2011).

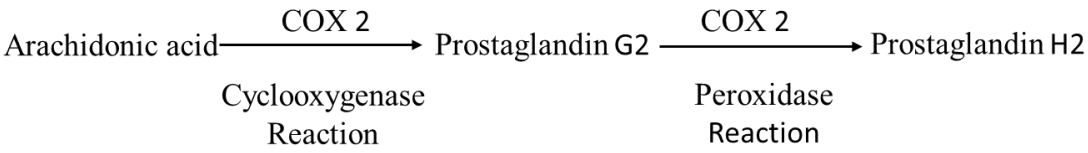

Figure 2: Arachidonic acid conversion to prostaglandins by COX enzymes

Intraplantar injection of PGE2 into the hind paw of mice produced a paw-licking (nociceptive) behavior that was dose-dependent short lived when compared with control animals (Kassuya et al., 2007). Additionally, there was a reduction of paw withdrawal latency (thermal hyperalgesia) following intraplantar PGE2 injection. This was significantly diminished in knockout mice lacking the nociceptor TRP channel, TRPV1 (Moriyama et al., 2005). Currently, the use of Nonsteroidal anti-inflammatory drugs (NSAIDs) to blocks the activity of the COX enzymes may help with pain management.

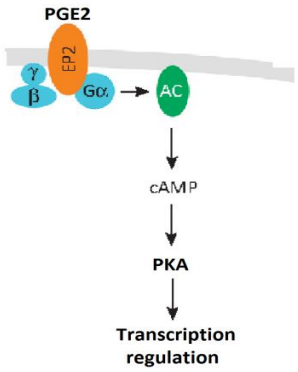

Figure 3: Prostaglandin induced signaling pathway in nociception (*Adapted from Jiang, 2013*)

Nociceptors express G-protein-coupled receptors for PGE2 called EP2 and EP4. PGE2 binding activates the receptors, which in turn activate the G-protein Gαs. This G protein then activates adenylyl cyclase (AC), which makes cAMP from ATP and transduces a signal to Protein Kinase A (PKA). Binding of cAMP activates PKA, leading to the phosphorylation of substrate proteins on serine/threonine residues (*Fig. 3*).

Disruption of the PKA anchoring protein AKAP150 in mice causes an increase in the paw withdrawal latency to thermal stimuli, indicating an decrease in pain (Schnizler et al., 2008). This is because AKAP150 organizes a protein complex between Protein Kinase C (PKC), PKA, AC, and TRPV1 to enhance the phosphorylation efficiency of TRPV1 (Efendiev et al., 2013). These findings strongly suggest that PGE2-induced thermal hyperalgesia is mediated in part by PKA. Another target of phosphorylation by PKA is the α-subunit of the voltage gated sodium channel Nav 1.8. This causes the α-subunit to open at lower threshold potentials and produces more frequent action potentials (Chahine et al., 2005).

Bradykinin

Bradykinin is also another molecule that forms part of the inflammatory milieu (*Fig 1*). Intradermal injection of bradykinin in humans produces a dose-dependent increase in pain and thermal hyperalgesia, indicating that bradykinin both excites and sensitizes nociceptors (Manning et al., 1991). Bradykinin is a polypeptide formed in the blood. It causes contraction of non-vascular smooth muscle, is a potent vasodilator of certain blood vessels, increases vascular permeability, and is involved in the mechanism of pain (Hornig and Drexler, 1997). Intraplantar injection of bradykinin caused a significant thermal hypersensitivity in control mice but not in TRPV1 knockout mice. This demonstrates the role of bradykinin in inducing thermal hypersensitivity *in-vivo* in a TRPV1-dependent fashion (Chuang et al., 2001).

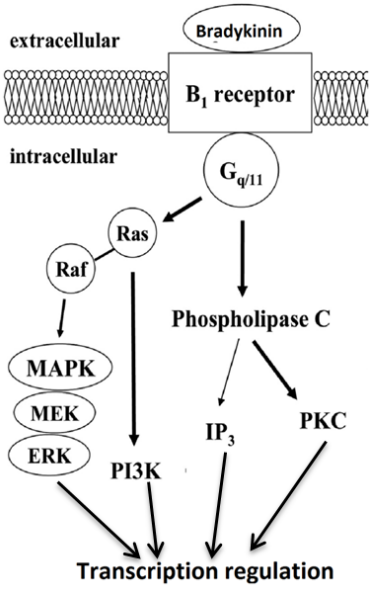

Figure 4: Bradykinin signaling in nociception *(Adapted from Ifuku et. al 2007)*

Bradykinin acts on a G-protein coupled receptor called B2 to activate the G-protein Gαq (*Fig. 4*). B2 receptors are constitutively expressed in the dorsal root ganglion (DRG), the structure that contains the cell bodies of nociceptor neurons (Mathivanan et al., 2016). When

the B2 receptor is in its active state, it activates the Gαq protein. Activated Gαq then activates the enzyme phospholipase C (PLC)-β, which in turn hydrolyzes a component of the cell membrane PIP2 (phosphatidylinositol (4,5)-bisphosphate) to form IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP3 functions as second messenger by binding to an intracellular receptor on the smooth endoplasmic reticulum, causing it to release calcium ions into the cytoplasm. The subsequent increase in cytosolic calcium concentration combined with DAG in the membrane activates PKC, which phosphorylates substrate proteins on serine/threonine residues (Burgess et al., 1989). One substrate of the PKCε isoform that is important in nociception is the TRPVI channel. TRPV1 phosphorylation opens and produces an excitatory current at lower temperatures than 45^0C, thus contributing to thermal allodynia. Inhibition of PKCε is shown to results in a significant decrease in the sensitization of TRPV1 by bradykinin (Bandell et al., 2004).

Nerve Growth Factor (NGF)

NGF is a neuropeptide that promotes the survival of nociceptors during development. Its levels also increase in inflamed skin (*Fig 1*). NGF binds to the receptor tyrosine kinase Tropomyosin receptor kinase A (TrkA) (*Fig. 5*), which is expressed in nerve endings comprised of peptidergic nociceptors (Donnerer et al., 1992; McMahon et al., 1995). When NGF binds to TrkA, it causes a conformational change that enables receptor homodimerization and activation of signaling pathways culminating in the increased expression of TRPV1. First, TrkA moves from the nerve ending by endocytosis and retrograde transport up the axon to the cell body where the changes in intracellular signaling and gene expression take place. In this process TrkA undergoes autophosphorylation at several tyrosine residues that selectively

trigger activity along several intracellular signaling pathways. This occurs via binding of specific effector proteins to phosphorylated docking sites on the receptor, especially by the Grb-Sos heterodimer. Attached to the inner leaflet of the cell membrane is the G-protein Ras. When bound by activated Sos, Ras exchanges its GDP for GTP, thereby becoming active. Activated Ras then binds and activates effector proteins, including PLC, the mitogen-activated protein kinases (MAPK), and phosphoinositide 3-kinase (PI3K). Ultimately, there is a change in transcription factor activation and an upregulation in TRPV1 expression (Vetter et al., 1991; Raffioni and Bradshaw, 1992).

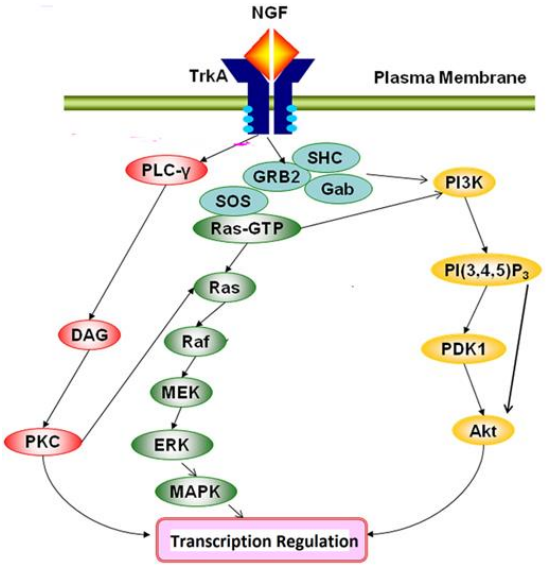

Figure 5: NGF signaling in nociception (*Adapted from Itoh et. al 2011*)

The MAPK family includes extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), and ERK5 (Jin et al., 2003; Widmann et al., 1999). Both ERK and p38 have been shown to have roles in modulating pain sensitivity. Inflammation in the DRG and the dorsal horn of the spinal cord activates ERK, which contributes to the development and maintenance of pain hypersensitivity through both transcription-dependent and -independent

means (Ji et al., 1999; Ji et al., 2002). Thus, inflammatory hyperalgesia can be prevented with ERK inhibitors (Sammons et al., 2000). Activation of p38 in nociceptors has also been implicated in exaggerated pain states (Watkins et al., 2001), in this case by increasing the expression levels of TRPV1 (Ji et al., 2002). As proof of principle, dissociated DRG neurons in culture treated with NGF demonstrated a rapid enhancement of capsaicin-induced current (Shu and Mendell, 1999, 2001). These findings clearly indicate that NGF upregulates TRPV1 ion channels, which are responsible for the neuronal response to capsaicin.

Translational regulation of nociception

The upregulation of nociception effectors such as ion channels during nociceptor sensitization suggests that gene expression is a major regulator of changes in nociceptor sensitivity that may develop into chronic pain. Previous studies have shown that synthesis of new proteins is required for nociceptor hypersensitization and also the production of primary hyperalgesia (Ferreira et al., 1990; Tohda et al., 2001). These experiments suggest that nociception may be regulated post-transcriptionally. This hypothesis is supported by multiple studies characterizing ribonucleic acid (RNA)-binding proteins that regulate RNA processing and translation that control nociceptor sensitivity (Barragan-Iglesias et al., 2018; Dyson, 2017; Jimenez-Diaz et al., 2008; Khoutorsky et al., 2016; Melemedjian et al., 2010; Moy et al., 2017). Regulation of gene expression at the level of translation allows for prompt changes in protein abundance, thus regulating neuronal plasticity during nociceptive sensitization (Hershey et al., 1996; Mathews, 1996).

Translation is a cyclic process with initiation, elongation, and termination stages. Translation initiation can be further subdivided into four different steps: (1) assembly of

initiation factors with the 40s ribosomal subunit to produce the 43s preinitiation complex, (2) attachment of the mRNA with the 43s preinitiation complex, (3) scanning through the mRNA to find the start codon, and (4) assembly with the 60s subunit to form the functional unit for translation.

Translation initiation

Following transcription, mature mRNAs are exported out of the nucleus to the ribosome for translation to occur (Bohnsack et al., 2002; Cioni et al., 2018; Jung et al., 2014). All nuclear transcribed eukaryotic mRNAs contain a 5′ cap, which is a 7-methylguanosine linked to the 5' nucleotide of the mRNA (Sonenberg and Hinnebusch, 2009). The cap is followed by the mRNA's 5′ untranslated region (5′UTR). The 5'UTR is followed by a coding sequence, a 3′UTR, and a poly (A) tail. The cap plays a critical role in protein synthesis by demarcating the 5′ terminus of the mRNA as it interacts with translation initiation factor eukaryotic initiation factor 4E (eIF4E), which, in turn, recruits the translational machinery to the 5′ end of mRNAs. mRNAs lacking this structure were reported to be translated with less efficiency than mRNAs that contained the cap (Zan-Kowalczewska et al., 1977).

The mRNA needs to bind to the ribosome for translation to begin. Ribosomes consist of a small subunit and a large subunit. In eukaryotes the small subunit is 40s and the large subunit is 60s and together they form the 80s ribosome. These subunits are separate prior to the process of translation and they only come together during the process of translation. Four binding sites are located on the ribosome, one for mRNA and three for tRNA. The three tRNA sites are labeled P, A, and E. The peptidyl site (P site), binds to the tRNA holding the growing polypeptide chain of amino acids. The acceptor site (A site), binds to the aminoacyl tRNA,

which holds the new amino acid to be added to the polypeptide chain. The exit site (E site) serves as the final transitory step before a tRNA now bereft of its amino acid is let go by the ribosome.

The initiation phase of eukaryotic translation is promoted by at least ten soluble proteins called eukaryotic initiation factors (eIFs) (Hershey et al., 1996). The initiation process uses a scanning mechanism that allows the AUG start codon of the mRNA to be placed in the P site of the ribosome. An anticodon in the methionyl tRNA (Met-tRNAi) binds to the AUG start codon in the P site during translation initiation. This is facilitated by the multi-protein complex called eIF3. eIF3 first binds to the free 40S subunit, thus blocking association of the 40S subunit with the 60S ribosomal subunit. This allows the smaller 40S subunit to bind to the initiator Met-tRNAi in a ternary complex with GTP-bound eIF2 (Zoll et al., 2002). Together with the help of multiple proteins, including eIF1, eIF1A, and eIF5, the 43S pre-initiation complex (PIC) forms (Asano et al., 2000). The combination of eIF1 and eIF2 blocks the A site on the ribosome and eIF3 and eIF5 block the E site on the ribosome leaving only the P site free for Met-tRNAi binding.

Initiation of cap-dependent translation is thought to depend on the assembly of eIF4F with mRNA. eIF4F is an initiation factor complex and includes the cap-binding protein eIF4E, the scaffold protein eIF4G, and the DEAD-box RNA helicase eIF4A (*Fig. 6*) (Raught and Gingras, 1999; Sonenberg and Hinnebusch 2007). The 43S PIC complex is recruited to the mRNA cap structure by eIF4F and eIF4B, thus generating the 48S PIC. The 48S PIC does not directly bind to the AUG start codon on the mRNA. Instead, it scans the mRNA looking for this sequence.

A short term consequence of eIF4E binding is the recruitment of eIF4A to the 5' UTR (Pestova et al., 2007). This is supported by the interaction between eIF4G and eIF4E, which holds eIF4A in its active conformation to facilitate the unwinding of the mRNA by eIF4A RNA helicase activity. This provides a single-stranded binding site for the 43S PIC near the 5' cap (Hilbert et al., 2010; Schütz et al., 2008). In addition to binding eIF4E and eIF4A, eIF4G also binds poly (A) binding protein (PABP), thus promoting circularization of the mRNA and possibly facilitating re-initiation of translation by ribosomes. There is evidence that the closed-loop mRNA formation via the PABP-eIF4G interaction is nonessential *in vivo* (Tarun et al., 1997), and may serve a redundant function in recruiting eIF4F to mRNA during re-initiation (Park et al., 2011).

The activity of eIF4A ensures the effective translation of mRNAs by unwinding secondary structures in the 5′-UTR during scanning and stabilizing the mRNA (Li, 2002). Thus, eIF4A unwinds the 5′ UTR in an ATP-dependent manner so that the PIC can scan this region base-by-base for an AUG start codon in the optimal sequence context. As ATP hydrolysis is necessary to the unwinding of secondary structures, it also releases eIF4A from the mRNA, thus allowing the recycling of available eIF4A to increase the rate of translation. The mRNA-bound 48S complex facilitates the recognition of the start codon by the anticodon in Met-tRNAi. The correct codon–anticodon pairing stimulates hydrolysis of the GTP bound to eIF2 in a reaction that is catalyzed by the GTPase activating protein (GAP) eIF5. This activity is modulated by eIF1(Nanda et al., 2009). Hydrolysis of GTP triggers the release of eIF2–GDP and other eIFs from the ribosome, creating a 40S initiation complex. This complex combines with the 60S subunit in an eIF5B-dependent manner triggering downstream events in initiation.

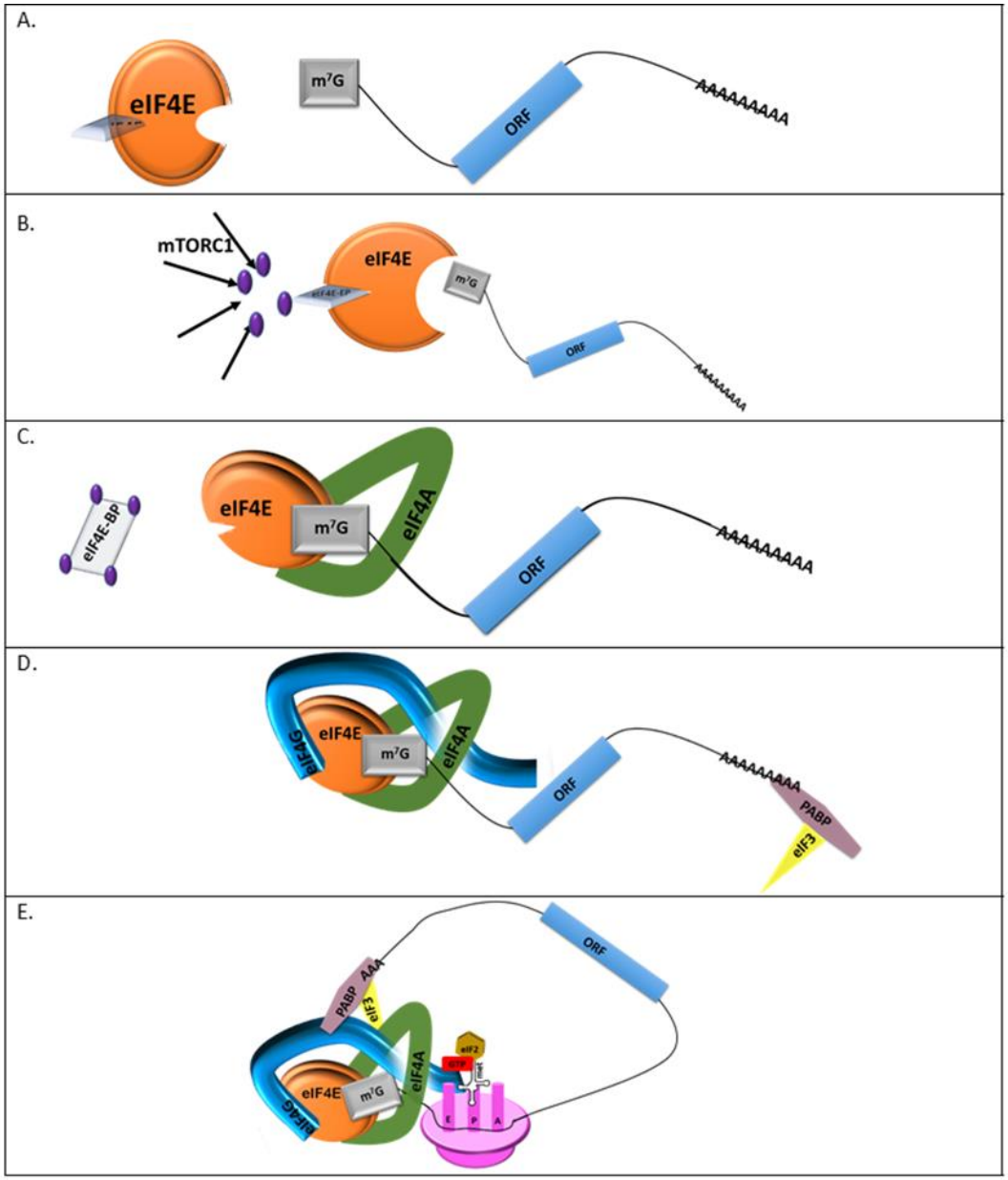

Figure 6: Recruitment of the eIF4F assembly for cap-dependent translation.

A. eIF4E-BP bound to eIF4E regulates translation initiation in the cytoplasm. B. Hyper-phosphorylation of eIF4E-BP by mTORC1 causes release of eIF4E-BP from eIF4E. C. This allows the eIF4E to bind to the cap to start the cap-dependent translation process and recruit eIF4A helicase. D. Both eIF4E and eIF4A are held in position by eIF4G scaffold protein. The poly A tail of the mRNA is bound by PABP and eIF3. E. Circularizing the mRNA by binding eIF4G with PABP and eIF3 and this allows recruitment of 40S and GTP-eIF2^{Met}tRNA. Regulation of translation initiation by the eIF4F complex

The activity of the eIF4F complex (*Fig. 6*) is tightly controlled by its interaction with several proteins, including the eIF4A-binding proteins eIF4B, eIF4H, and programmed cell death 4 (PDCD4). eIF4B and eIF4H stimulate the formation of the eIF4F complex, whereas PDCD4 inhibits interaction of eIF4F with eIF4A. The formation of eIF4F in the cytoplasm is a continuous cyclic process of assembly and disassembly at the 5' ends of mRNAs according to the cell's needs (von der Haar et al., 2004).

An additional complication to the recruitment of eIF4F complex is the number of isoforms for each of the eIF4F subunits (reviewed in Hernández and Vazquez-Pianzola) (Hernández and Vazquez-Pianzola, 2005). There are three isoforms of eIF4A in humans (Li, 2002; Sudo et al., 1995) and two in *Drosophila* (Dorn et al., 1993; Hernández and Vazquez-Pianzola, 2005); three isoforms of eIF4E in mammals (Sonenberg et al., 1979) and eight in *Drosophila* (Hernández and Sierra, 1995; Hernández and Vazquez-Pianzola, 2005); and two isoforms of eIF4G in mammals, (called eIF4G1 (Bradley et al., 2002) and eIF4G2 (Gradi et al., 1998)), and also two isoforms in *Drosophila* (Hernández et al., 1998). With little additional information about concentrations during cell processes, tissue-specific expression, and the biochemistry of the relative affinities for other subunits, the number of combinations between these isoforms is vast. This may explain the diversity in function of the eIF4F complex in translation regulation. Most literature has focused on the biochemical, biological, and structural properties of just one of the possible eIF4F forms (eIF4E1, eIF4A1, eIF4G1). Thus, the interactions with other isoforms remains to be studied in terms of how much each isoform contributes to the overall translation regulation process.

The Cap Binding Protein - eIF4E

eIF4E is implicated in the translation of long and highly structured mRNAs. eIF4E is the only eIF4F complex protein that binds directly to the mRNA cap structure (*Fig. 6*). It is important for the assembly of eIF4F at the 5' cap (Gingras et al., 1999; Raught and Gingras, 1999). eIF4E was discovered more than 40 years ago by cross-linking oxidized reovirus mRNA to initiation factor protein preparations from rabbit reticulocytes (Grifo et al., 1983; Sonenberg and Shatkin, 1977). It was later purified as a 24kDa cap-binding protein using a M^7GDP-Sepharose column and radiolabeled methionine molecules (Marcotrigiano et al., 1997; Sonenberg et al., 1979). Sonenberg co-crystallized the cap binding molecule using mass spectrometry to show the full length of murine eIF4E post protease digestion (Marcotrigiano et al., 1997). Their results also showed a murine eIF4E (28–217)-7-methyl-GDP co-crystal structure that helped explain the structural basis for the cap-binding properties of eIF4E.

The MAPK pathway (*Fig. 4 and Fig. 5*) has been shown to be important in the regulation of translation initiation, as it is known in mammals that phosphorylation of eIF4E at serine 209 by mitogen activated kinase interacting protein kinases (MNKs) decreases the affinity of eIF4E for the 5' cap. The G protein Ras, through the receptor tyrosine kinase effector, Raf, activates the dual-specificity (serine/threonine and tyrosine) mitogen-activated protein kinase kinases (MAPKKs), which in turn phosphorylate and activate the extracellular signal-regulated kinases (ERK1/2) protein kinases, resulting in phosphorylation of MNK. MNK then binds to eIF4G and phosphorylates eIF4E within the eIF4F complex (Scheper and Proud, 2002). MNK1 and MNK2 specifically phosphorylate serine 209 of eIF4E (Flynn and Proud, 1995; Scheper and Proud, 2002; Scheper et al., 2002). MNK and eIF4E interact with eIF4G, bringing them into physical proximity to facilitate eIF4E phosphorylation. Biophysical

studies indicate that phosphorylation of eIF4E decreases its affinity for the 5' mRNA cap. This facilitates the scanning process or it permits the transfer of eIF4E from mRNAs that are undergoing translation to other mRNAs whose translation has not yet to begin (Scheper and Proud, 2002; Scheper et al., 2002).

MNK1 can also be activated by p38 MAPK downstream of cytokine signaling or stressful stimuli as established by phosphorylation screening (Fukunaga and Hunter, 1997). MNK1 and MNK2 knock-out mice as well as knock-in mice in which serine 209 was replaced by an alanine, showed no eIF4E phosphorylation and significantly attenuated tumor growth (Bramham et al., 2016). MNK1 inhibition using cercosporamide gave the same results (Moy et al., 2017). *Drosophila* expressing a mutant eIF4E in which serine 251, the residue which corresponds to serine 209 of mammalian eIF4E, was mutated to alanine produced a growth-arrested phenotype where the larvae failed to reach the third instar or took a substantial longer time to develop. They also reported embryonic lethality in the mutants (Lachance et al., 2002). These results showed that eIF4E phosphorylation is necessary for translation processes in cells *in vivo*.

eIF4E-Binding Protein

The availability of free eIF4E is controlled by eIF4E-binding proteins (eIF4E-BPs) (*fig 6*), which can interact with eIF4E and prevent it from binding to eIF4G. When eIF4E-BPs are phosphorylated they are released from eIF4E, thus allowing eIF4E to form the eIF4F complexes necessary for translation. eIF4E-BP a major target of the mammalian target of rapamycin (mTOR) signaling pathway (mTORC1) (Pause et al., 1994). mTOR is a highly conserved serine/threonine kinase that is present in all eukaryotic cells. mTOR is activated by

PI3K/Akt/mTOR signaling pathways in response to growth factors (*Fig. 7*), cytokines, glucose, and insulin. PI3K phosphorylates membrane-bound phosphatidylinositol 4,5-bisphosphate at position 3 to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 serves as a membrane docking signal for PH domain-containing proteins such as the serine/threonine kinases Akt and PDK1 (phosphatidylinositol-dependent kinase 1). PDK1 activates Akt by directly phosphorylating thronine 308 within the T-loop of the catalytic domain (Alessi et al., 1996). PDK1 can also activate mTOR complex 2 (mTORC2), which can phosphorylate Akt at serine 473 (Sarbassov et al., 2005). Activated AKT then phosphorylates tuberous sclerosis complex 2 (TSC2), thereby inactivating the TSC1/TSC2 complex (Gao and Pan, 2001). Rheb, a small G protein found on lysozyme membrane, is no longer inhibited by the GAP (GTPase-activating protein) activity of TSC2 and the resulting Rheb-GTP activates the mTOR complex1 (mTORC1) by reducing activity of FKBP38, a member of the FK506-binding protein (FKBP) family (Bai and Jiang, 2010). FKBP38 inhibits mTOR activity through direct binding to mTOR. Interaction of the regulatory associated protein of mTOR (Raptor) and mTOR forms the mTORC1 complex.

Activated mTORC1 phosphorylates and inactivates the eIF4E-BP (*Fig 7*), causing the subsequent dissociation of eIF4E-BP from eIF4E. mTOR phosphorylates eIF4E-BP1 on at least two residues, threonine 37 and threonine 46, which leads to its release from eIF4E. eIF4E-BPs are shown to compete with eIF4G for interaction with eIF4E (Haghighat et al., 1995). In its hypophosphorylated form, eIF4E-BPs binds to eIF4E, preventing the eIF4E–eIF4G interaction, thus inhibiting eIF4F complex formation (Adriaensen et al., 1983; Duncan et al., 1987; Sonenberg, 2008). mTORC1 also promotes activation of the translational activator S6K, which phosphorylates the ribosomal protein S6 and other substrates, including eIF4B, which

plays a critical role in recruiting the 40S ribosomal subunit to the mRNA (Shahbazian et al., 2006).

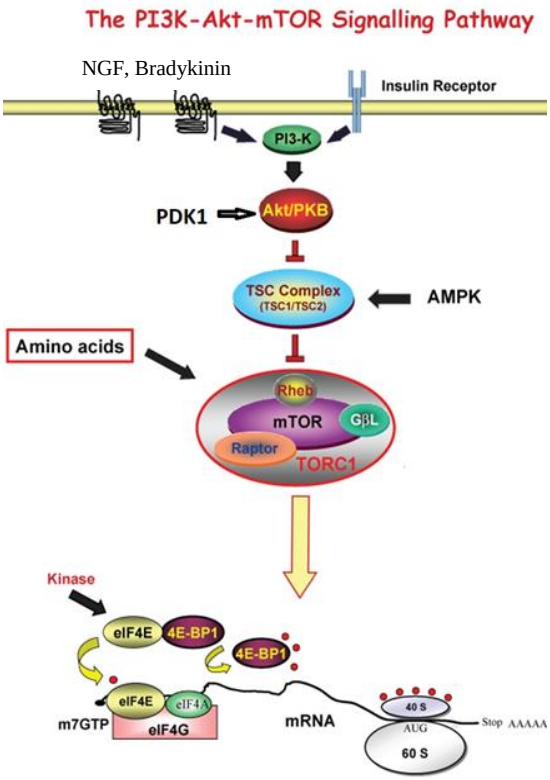

Figure 7: eIF4E-BP phosphorylation by MTORC1 (*Adapted from www.pancrepedia.org, 2010*)

AMP activated protein kinase (AMPK) is a serine-threonine kinase that is highly conserved through evolution. In comparison to mTOR signaling, activation of AMPK results in a suppression of energy-consuming processes while stimulating energy producing processes (Garcia and Shaw, 2017). As mRNA translation is an energy-consuming process in cells, it can be expected that AMPK activation would block protein synthesis. AMPK inhibits cap-dependent translation by indirectly inhibiting mTORC1 activity through phosphorylation of TSC2 at serine 1387 and at two conserved serine residues in the regulatory protein, Raptor (Inoki et al., 2003). This blocks the ability of the mTORC1 kinase complex to phosphorylate

its substrates, including eIF4E-BPs. Thus, hypophosphorylated eIF4E-BPs remains attached to eIF4E, thereby inhibiting eIF4E binding to eIF4G and the formation of the eIF4F complex. This results in reduced translation activity of the eIF4F complex (Williamson et al., 2006).

The Scaffolding Protein - eIF4G

As an essential factor in translation initiation, the eIF4Gs are targets for translation regulation of cellular proteins. There are two isoforms of mammalian eIF4G - eIF4G1 and eIF4G2 which are 46% identical. Mammalian eIF4G has binding sites for poly-A-binding protein (PABP), eIF4E, eIF3, the eIF4E kinase, MNK1, and eIF4A. Thus, it is called the scaffolding protein of the eIF4F complex. eIF4G recruits the 40S ribosomal subunit to the mRNA via its interaction with eIF3. It also binds eIF4B, a protein that aids the RNA helicase function of eIF4A (Rozen et al., 1990). eIF4G undergoes protein-protein interactions with eIF4E and eIF4A to assemble the eIF4F complex (Jackson et al., 2010). Thus, eIF4G facilitates the translation of mRNAs that contain structured 5'UTRs. eIF4G uses a conserved sequence motif of Tyr-X-X-X-X-Leu-φ (where X is variable and φ is hydrophobic) to recognize the eIF4E carboxy-terminal moiety, located in the convex dorsal surface of the eIF4E protein during cap dependent translation (Marcotrigiano et al., 1999).

eIF4E-BP inhibits cap-dependent protein synthesis by binding to eIF4E. Biochemical studies have demonstrated that the eIF4Gs and the eIF4E-BPs occupy mutually exclusive binding sites on the surface of eIF4E (Haghighat et al., 1995). Sequence analyses eIF4E-BPs suggests it to binds to the dorsal surface of eIF4E via Tyr-X-X-X-X-Leu-φ eIF4E recognition motif. Moreover both proteins contain auxiliary eIF4E-binding sequences that help secure the interaction with eIF4E (Grüner et al., 2016; Marcotrigiano et al., 1999). This suggests that

eIF4E-BP is a molecular mimic of eIF4G, that the resulting competition between the two proteins can result in the down regulation of the translation initiation process, and that this down regulation can be relieved by phosphorylation of the eIF4E-BPs.

Eukaryotic mRNA poly A tails have been documented to be enhancers of mRNA translation initiation. Studies show an interaction between eIF4G and PABP brings about circularization of the mRNA and stimulates 40S subunit recruitment. The circularization of the mRNA could be enhancing translation by moving terminating ribosomes directly to the 5' end of the mRNA (*Fig. 6*) (Tarun Jr and Sachs, 1996). Destabilizing the association of eIF4G1 with mRNA affects the formation of a 'closed-loop' structure, which is thought to support efficient translation, though the effect on mRNA is not known. It is hypothesized that eIF4G might undergo conformational changes when bound to PABP making the mRNA more accessible to eIF4G as demonstrated in cells *in vitro* (Haghighat et al., 1996). This increases the translational rate. eIF4E can bind the mRNA cap on its own, but its affinity is dramatically enhanced by the structural change caused by binding to eIF4G that favors of eIF4F interaction with cap-binding proteins (Haghighat and Sonenberg, 1997). mTORC1 in turn phosphorylates eIF4G1 directly, stabilizing its interaction with eIF3 and subsequently loading of ribosomal subunits (Harris et al., 2006). This interaction can be blocked by dephosphorylated eIF4E-BP. The effect of this alteration on the translation of specific mRNAs is unknown.

The DEAD box RNA Helicase - eIF4A

eIF4A has been biochemically characterized as an RNA-dependent ATPase (Grifo et al., 1983) and RNA helicase. There are two different isomers of eIF4A (eIF-4A1 and eIF-4A2) that are 91% similar in sequence identity (Hershey and Merrick, 2000; Merrick and Pavitt,

2018; Nielsen and Trachsel, 1988). No differential functions are known to exist for these isomers to date, though there is a distinctly different pattern of expression, and both can combine with the eIF4F complex with similar kinetics (Yoder-Hill et al., 1993). eIF4A1 is reported to be generally more abundant than eIF4A2 (Nielsen and Trachsel, 1988). It was noted that eIF4A1 mRNA was synthesized and translated most efficiently in cells that were actively growing, whereas eIF4A2 mRNA was synthesized and translated during growth arrested conditions (Williams-Hill et al., 1997). This variable abundance of the two isoforms is suggestive of different functional roles in cell growth, though the aggregate amount of the two mRNAs appears to be relatively constant. There is a third isoform of eIF4A known as eIF4A3 that does not function in protein synthesis (Li et al., 1999). This isoform is 65% similar to the other isoforms and shares similar activities as eIF4A in RNA dependent ATP hydrolysis and ATP dependent RNA duplex unwinding, but when added to reticulocytes lysates, it inhibited translation. As the relative abundance of eIF4A3 to eIF4A1 is reported to be 1:10, it is not clear that eIF4A3 functions as a negative regulator of translation. eIF4A3 plays a role in nonsense-mediated decay as a component of the exon junction complex (EJC). Using monoclonal antibodies, it was shown that eIF4A3 is found in the nucleus whereas eIF4A1 and eIF4A2 are found in the cytoplasm. Thus eIF4A3 provide a splicing-dependent influence on the translation of mRNAs (Chan et al., 2004).

eIF4A has nine conserved sequence motifs, just like other DEAD-box helicases. These sequences are important for nucleotide and RNA-binding and helicase activity (Cordin et al., 2006). The arrangement of the conserved DEAD-box sequence motifs is functionally important because RNA and ATP are required to bind within the enzyme. The eIF4G HEAT1 domain contacts the N- and C-terminal domains of eIF4A, and thus eIF4G restricts eIF4A's

conformation in such a way that it promotes proper orientation of eIF4A to the RNA strand (Caruthers et al., 2000). This was modeled by the crystal structure of the yeast eIF4A-eIF4G complex (Schütz et al., 2008). The study highlighted eIF4G's Trp-579 interaction with a complementary pocket on the eIF4A surface as essential for complex formation. A W579A mutation inhibited the interaction between these two proteins. In the absence of eIF4A RNA helicase activity, excessive secondary structure in the mRNA 5' UTR impedes both the assembly of additional initiation factors as well as scanning by the 40S small ribosomal subunit (Schütz et al., 2008).

Characterization of the eIF4A by a direct-unwinding assay showed that the helicase activity of eIF4A in combination with eIF4B is bidirectional (Rozen et al., 1990). eIF4A in the presence of eIF4B has a high affinity for single-stranded RNA and unwinding happens in a 5' to 3' direction and is cap-dependent. Unwinding in the 3' to 5' direction, in contrast, is suggested to be cap-independent. The ability of eIF4A to unwind an RNA duplex in a 3' to 5' direction can explain the ability of ribosomes to sometimes reinitiate at upstream AUGs. The obvious function of the helicase activity of eIF4A is to unwind RNA duplex structures at the 5' end of eukaryotic mRNAs. This activity appears to be necessary for threading the mRNA onto the 40S subunit (Svitkin et al., 2001). A second function of the helicase activity of eIF4A is in the process of mRNA scanning, the ATP-dependent movement of the 40S subunit from the cap structure towards the initiating AUG (Hershey and Merrick, 2000).

It is also possible that the helicase activity of eIF4A is required for the RNA structural rearrangement that allow association of other translation factors with the mRNA. eIF4A directs binding of eIF4B to RNA or to ribosomes in an ATP dependent fashion (Hughes et al., 1993). The hydrolysis of ATP by eIF4A may lead to the dissociation of factors from either the mRNA

or the 40S subunit. eIF4F is released from the mRNA upon ATP hydrolysis (Abramson et al., 1987). There is a conformational change in the eIF4E upon ATP hydrolysis that allows it to dissociate from the cap, which may be required for eIF4F recycling. This hypothesis was confirmed by the findings that eIF4F molecules that lacked the eIF4A subunit did not recycle (Ray et al., 1985).

mTOR signaling regulates eIF4A activity through the S6 kinases (S6k), which is a downstream effector of mTORC1. S6Ks phosphorylate eIF4B, which is an eIF4A regulator, and increases eIF4A affinity for ATP. The hydrolysis of the ATP by eIF4A accelerates its helicase activity (Shahbazian et al., 2006). S6Ks phosphorylates PDCD4 at serine 67 and regulates its stability and function as an interacting partner and inhibitor of eIF4A (Dorrello et al., 2006). PDCD4 has been shown to directly bind eIF4A and displace it from eIF4G and RNA, as monitored using the ATP-dependent unwinding assay (Dorrello et al., 2006). Phosphorylated PDCD4 is degraded by the ubiquitin dependent pathway, increasing the free eIF4A for translation. These experiments show that both eIF4E-BP and eIF4A are functionally regulated in translation initiation by the mTor pathway.

Secondary structures in the 5' UTR of mRNAs can influence their translation initiation efficiency. Mammalian mRNAs that have long and structured 5'UTR secondary structures were found to be hyper-dependent on eIF4A for translation *in-vitro* (Svitkin et al., 2001). 5' UTRs of eIF4A-dependent mRNAs were reported to be enriched with G-quadruplex structures rich in guanine nucleotides (Wolfe et al., 2014). eIF4F assembly regulates the translation initiation process in response to numerous signaling pathways. We have established the presence of molecules that sensitize nociceptors by similar signaling pathways and also discussed different ion channels present in nociceptors that are required for nociceptor

sensitization. This suggests that nociceptive molecules such as ion channels may be regulated translationally during processes such as nociception sensitization. There are many gaps in our current understanding of how eIF4F assembly plays a role in the class IV multidendritic neurons of *Drosophila* larvae as a regulator for translation initiation. This study is designed to test the hypothesis that eIF4G, eIF4A, and the eIF4E-BP play important roles in the class IV neurons to regulate nociception and during nociceptive sensitization.

Study Aims

1. Define baseline thermal and mechanical larval sensitivity when eIF4G, eIF4A, and eIF4E-BP genes are knocked down
2. Quantify the effects of eIF4G, eIF4A, and eIF4E-BP gene knockdown on nociceptor morphology
3. Design sensitization assays and characterize the effect of eIF4G, eIF4A, and eIF4E-BP knockdown in hypersensitization
4. Construct transgenic *Drosophila* for assaying localization of fluorescently tagged eIF4E-BP

Methods

Fly stocks and genetics

Gene knockdown was accomplished using the Gal4/UAS system in transgenic *Drosophila* (Brand and Perrimon, 1993). The Gal4/UAS system is a binary expression system primarily used in *Drosophila*, which is used for cell-specific transcription activation. In one transgene, a cell-specific promoter drives the expression of the gene encoding the yeast transcriptional activator, Gal4. In a second transgene, a gene of interest is transcriptionally regulated by an upstream activation sequence (UAS) that provides a binding site for Gal4 (Fischer et al., 1988; Webster et al., 1989). In our experiments, we used the regulatory sequence from the *ppk* gene, which encodes a degenerin/epithelial sodium channel (DEG/ENaC) that is expressed solely in the Class IV multidendritic neurons. Thus the *ppk-Gal4* driver transgene is expressed specifically in the Class IV multidendritic nociceptive neurons, where it binds to the UAS sequence and efficiently drives expression of the downstream gene (Ainsley et al., 2003). A major advantage of this method is that it allows nociceptor-specific expression of UAS effector genes using simple genetic crosses.

For RNAi knockdown, we used flies carrying UAS-RNAi transgenes, which express RNA hairpins that are processed into short interfering RNA sequence that targets the RNA induced silencing complex (RISC) complex to the mRNA of interest and thereby lower expression of the gene of interest. In order to activate this system, the UAS-RNAi lines must be crossed to another line carrying a *UAS-dicer2* transgene and a nociceptor-specific *ppk-Gal4* driver. The progeny of these crosses are expected to have lowered expression of the gene of interest only in the Class IV multidendritic nociceptive neurons. Use of the Dicer2 enzyme improves on efficiency of the Gal4/UAS. This project used *ppk-Gal4; UAS-dicer2* crossed

with UAS-RNAi to increase gene knockdown effectivity (Dietzl et al., 2007). Table 1 shows the RNAi lines used in this project. Use of multiple RNAi line provides for a more robust results thus the more available RNAi line more robust the results are.

For each experiment, crosses were set up to produce two sets of negative control larvae, positive control larvae, and experimental larvae. The negative control larvae contained just one element of the Gal4/UAS system (for example, only the UAS transgene or only the Gal4 transgene). For Bloomington fly RNAi lines, which are from the Transgenic RNAi Project collection, the Gal4-only negative control groups were produced by crossing virgin female *ppk-Gal4; UAS-dicer2* flies to *y v; attP2* males. For Vienna *Drosophila* Resource Center (VDRC) RNAi lines, the Gal4-only controls were generated by crossing virgin female *ppk-Gal4; UAS-dicer2* flies to w^{1118} males. The UAS-only negative control larvae used in all RNAi knockdown experiments were obtained by crossing w^{1118} virgin females to UAS-RNAi males. *Paralytic (para)* is a gene that encodes a voltage-gated sodium channel required for action potential firing. *Para* is used as positive control because knocking down the *para* gene through RNA interference results in an insensitive phenotype to most types of nociceptive stimuli, and this allows for confirmation that the RNAi is functioning as expected. Thus, larvae expressing *para RNAi* in the nociceptors were used for a positive control. The specific cross was set up as virgin female *ppk-Gal4; UAS-dicer2* flies crossed to *UAS-para RNAi.*

Table 1: Fly genotypes used to investigate nociception

Celera Gene #	Gene Name	RNAi line
CG9075	eIF4A	*VDRC 42202*
CG10811	eIF4G1	*BDSC 33049*
CG10192	eIF4G2	*BDSC 35809*
		BDSC 41963
CG8846	eIF4E-BP	*BDSC 9147*
		BDSC 36815

Bloomington *Drosophila* Stock Center – BDSC Vienna *Drosophila* Resource Center - VDRC

To experimentally test for nociceptive behavior, experimental crosses were set up using five *ppk-Gal4; UAS-dicer2* virgin females together with three males from a UAS-RNAi strain. All vials were anonymized during setup for the purpose of blind testing then incubated at 25°C for 48 hours. The flies were flipped to new vials on the third day and again on the fourth in order to establish three sets of vials per cross for testing. Larvae were tested at least on two separate days to account for day-to-day variation. Vials with crosses were kept in the 25°C incubator with a 12-hour light and 12-hour dark cycle. Flies are maintained in 6 oz stock vials on cornmeal molasses yeast medium at a temperature of 25°C with a humidity range of 55%-68%. The same experimental conditions were used for all test crosses. The stock flies (listed in Table 1) for the controls and the RNAi lines were maintained at room temperature on cornmeal molasses and used for cross setup.

Thermal nociception assays

Thermal nociception assays were conducted as previously described (Herman et al., 2018). Five to six days after crosses were established, thermal nociception assays were performed on wandering third instar larvae, the stage at which dendrites and sensory neuronal activity have fully developed (Zwart et al., 2013). Using a fine paint brush, 15-20 larvae were removed and placed into a glass petri dish, that was kept moist using a thin layer of water and baker's yeast. For the assays, a previously developed thermal probe, created by filing the tip of a soldering iron into a chisel shape, was used to deliver the stimulus. A thermocouple was attached to the tip with thermal epoxy in order to constantly monitor temperature, and a voltage regulator was used to control the probe temperature. Assays were conducted at 46 ± 0.5 °C, which is above the heat threshold for normal nocifensive response in *Drosophila* larvae. The

probe was applied as a thermal noxious stimulus laterally, flush with the larval body surface along abdominal segments A1-A3, and held in place until a larval response consisting of a NEL response was observed or until at least eleven seconds had elapsed. Each animal was tested once and then removed from the dish. Assays were performed on a minimum of 50 larvae per genotype in each experiment. Using an existing video analysis protocol, the time in seconds to make one NEL was recorded and then reported as latency in seconds (Dyson, 2017). The measured latency for the positive control was cut off at 11 seconds as an upper limit. Any latencies recorded above 11 seconds was presented as 11 seconds. The latencies were analyzed using a non-parametric Mann-Whitney U statistical test. I used the alpha p value of less than 0.025 as each experimental group was compared to two negative controls.

Mechanical nociception assays

Using a fine paint brush, ten wandering third instar larvae were removed from the cross vial and placed on a glass petri dish moistened with a thin layer of water and baker's yeast. Larvae were allowed to acclimatize for five minutes prior to testing. A Von Frey filament (10mm) calibrated to deliver 50 mN of mechanical force was used to deliver the mechanical noxious stimulus to the third instar larva. The mechanical stimulus is delivered by rapidly depressing the larva with the filament on the dorsal side at segments A4-A6 for approximately 1 second (Hoyer et al., 2018). This result was recorded as Poke 1. The quick release allows the larvae to perform NEL. If the larvae rolled it was recorded and a new larva was tested. If the larva did not roll on Poke 1, then the stimulus was reapplied to the same larva twice more after a pause of 30 seconds each time, and these were recorded as Poke 2 and Poke 3. A positive rolling response is scored as 1 if at least one NEL response occurred in response to the

mechanical stimulus. A negative response is scored as 0 if no NEL response occurred in response to the mechanical stimulus. A minimum of 100 total larvae were tested per genotype per experiment. The data collected was then tested for statistical significance using the Chi square test. I used the alpha p value of less than 0.025 as each experimental group was compared to two negative controls.

Quantification of dendritic morphology

The driver stock *ppk-Gal4 mCD8::GFP; UAS-dicer2* were crossed with the same UAS-RNAi flies used for nociception assays. This generates larvae that have RNAi knockdown and mCD8::GFP expression in the nociceptor neurons. mCD8::GFP is a fusion of Green Fluorescent Protein (GFP) and the transmembrane domain of mouse CD8, which will target fluorescence to the membrane in nociceptors. The third instar larvae were immobilized by ligation by tying them using a human hair near segment A3. They were then mounted on coverslips with 100% glycerol. Larvae were imaged using the Zeiss LSM 880 confocal microscope at 40X magnification. The image was then cleaned to remove background noise, including the clearance of unwanted fluorescence from epithelial cells and neighboring neurons using Photoshop software. Cleaned images were then adjusted for threshold in ImageJ to produce a skeletonized image. The skeletonized image was layered over the original image and checked for accuracy. Sholl analysis was then carried out as a plugin to NeuronJ (Meijering et al., 2004). Sholl analysis creates a series of concentric *shells* (circles) around the center of a neuronal arbor by defining the center of image using a startup radius of image and indicating the ending radius. The plugin then counts how many times connected pixels defining the arbor intersect the sampling shells. The output gives mean dendritic intersections and sum dendritic

intersections which were recorded and analyzed using Student's t test. I used the alpha p value of less than 0.050 to report statistical significance.

Molecular Cloning and Transgenic Fly Construction

For RNA extraction, w^{1118} flies were homogenized using a disposable pestle in 1 ml of TRIzol in pre-chilled microcentrifuge tubes. Total RNA was then isolated according to the *Drosophila* Genomics Resource Center RNA extraction protocol (Bogart and Andrews, 2006). The synthesis of DNA using RNA template, via reverse transcription, produces complementary DNA (cDNA). Reverse transcriptases (RTs) makes use of an RNA template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which is then directly used as a template for the Polymerase Chain Reaction (PCR). This combination of reverse transcription and PCR (RT-PCR) allowed for the detection of low abundance RNAs in my sample, and production of the corresponding cDNA. Primers designed to amplify the full-length open reading frame (ORF) of *eIF4E-BP* as shown in the Table 2.

Table 2: Primer sequences designed to amplify the ORF for eIF4E-BP

Forward Sequence 5'-3'	ACC ATG TCC GCT TCA CCC
Reverse Sequence 5'-3'	C TAC AGA TCC AGT TGG AAC TGT TCC

cDNA for the gene of interest was then PCR amplified from reverse-transcribed cDNA using the Q5 DNA polymerase. This was done using the Q5® High-Fidelity DNA Polymerase (M0491) kit protocol. Then the cDNA was transferred into the pENTR/d-TOPO vector using the pENTR™ /D-TOPO® Cloning Kit and transformed into competent *E. coli*. This entry vector was then used in a Gateway cloning reaction with LR clonase to transfer the cDNA into a pTVW UAS vector containing a fluorescent tag, and the plasmid was then transformed in competent *E. coli*. Sanger sequencing from Eton Biosciences confirmed that the plasmid was

correctly inserted. Constructs were sent to be injected into *Drosophila* embryos by BestGene Incorporated to create transgenic fly lines containing a *UAS-VFP-Thor* transgene through p-element mediated transformation.

Hypersensitization assay

Previous studies have used ultraviolet (UV)-induced tissue damage to sensitize nociceptive neurons in *Drosophila* larvae (Babcock et al., 2011; Im et al., 2015). When a defined dose of UV irradiation was delivered to anesthetized animals, wild-type animals demonstrated an increase in sensitivity to a light touch with a thermal probe starting eight hours after UV exposure (Babcock et al., 2009). Using this as a basis, a thermal hypersensitization model was proposed and optimized, where tissue is damaged with UV and assayed for hypersensitization after eight hours. Crosses were set up the same as for the nociception assays, but in sets of two and vials were flipped every two days. One set of larvae was used for UV irradiation treatment and the other set was used for sham treatment with no UV exposure. Early third instar larvae were used, which should reach the wandering third instar stage for nociception assays eight hours after tissue damage. Apple juice agar plates were used for larval recovery after UV exposure. Freshly prepared yeast paste of 0.5g of yeast mixed in 700ul of distilled water was used as a food source for treated larvae. Apple juice agar plates were brushed with the yeast paste before placing the recovering larvae.

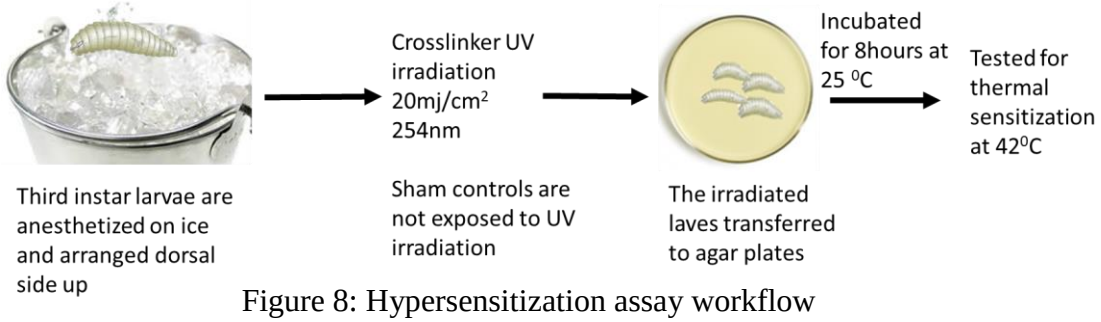

Figure 8: Hypersensitization assay workflow

To prepare larvae for UV exposure (*Fig. 8*), early third instar larvae of similar size were selected and washed out from food onto the Pyrex plate. The plate was then placed on ice for 45 seconds until all the larvae were anaesthetized and immobilized. This was a modifications done to the original assay in that we anaesthetized the larvae before UV exposure (Jang et al., 2018). Larva were positioned dorsal side up in a non-overlapping distribution. The UV crosslinker was warmed up and the all the larvae were exposed to 20mJ/cm^2 at 254nm. This was found to be the setting at which all larvae survived to adulthood (Babcock et al., 2009). Following UV exposure, recovering larvae were transferred to apple cider agar plates coated with yeast paste. Control plates with sham treatment were prepared where the larvae were anaesthetized and placed for five seconds in the crosslinker, but with no UV irradiation treatment. The recovering larvae from sham treatment were then transferred to the apple cider agar plates coated with yeast paste. Prepared samples were then incubated for eight hours at 25°C. After eight hours, the larvae were tested using the previously described thermal nociception assay at 42°C. Testing was conducted over two days, and at least 50 larvae were tested per condition. After testing the latencies were calculated and any latency above 11 seconds was presented as 11 seconds as the upper latency limit. UV^+ vs UV^- latencies per genotype were analyzed and statistically tested for sensitization using the non-parametric Mann-Whitney U statistical test. I used the alpha p value of less than 0.050 as each comparison is pairwise (UV to sham) to report statistical significance.

Results

eIF4A is required for sensitivity in nociception

To examine whether eIF4A is involved in larval nociception, I used a tissue-specific RNAi approach, making use of the Gal4/UAS system available in *Drosophila* as a tool for genetic manipulation. I tested third instar larvae with nociceptor-specific *eIF4A* knockdown using the *VDRC 42202 RNAi* transgene for defects in thermal and mechanical nociception.

eIF4A is required for normal sensitivity to noxious thermal stimuli

For our thermal nociception experiment, I used the *ppk-GAL4; UAS-dicer2* line to drive nociceptor-specific expression of the *VDRC 42202 UAS-eIF4A-RNAi* transgene and tested for defects in thermal nociception. I found that larvae with nociceptor-specific *eIF4A* knockdown showed significantly longer latency to respond to a 46°C probe than controls (*Fig. 9*). *eIF4A* knockdown larvae displayed a mean latency of 5.2 seconds, compared to a mean latency of 1.8 seconds for Gal4-only negative control and 1.7 seconds for the UAS-only negative control. *Para* knockdown larvae were used as a positive control and found to show nearly complete insensitivity to noxious thermal stimuli. The increased latency of *eIF4A* knockdown larval response to noxious thermal temperature was significantly different from the Gal4-only control larval response and UAS-only control larval response at $p \leq 0.001$ by the Mann Whitney U Test. The hyposensitive phenotype of the *eIF4A* knockdown phenotype suggests eIF4A is required for normal sensitivity to noxious thermal stimuli.

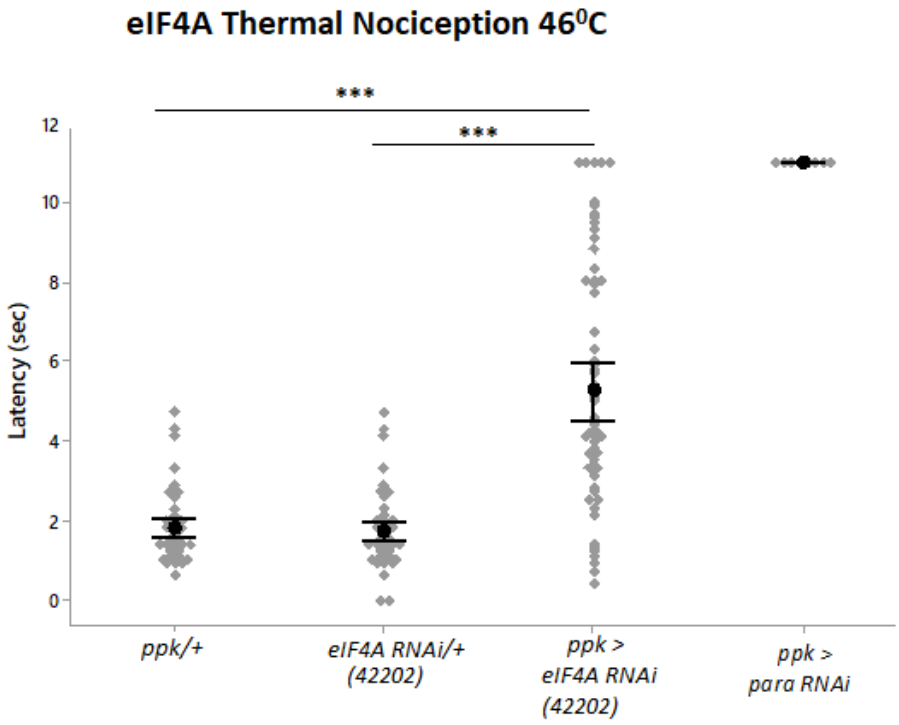

Figure 9: Nociceptor-specific knockdown of eIF4A causes defects in thermal nociception. *Larvae with nociceptor-specific knockdown of eIF4A (VDRC 42202 RNAi line) (ppk > eIF4A RNAi) showed a statistically longer latency response to noxious thermal stimulus (46°C) to both the Gal4-only (ppk/+) control and the UAS-only control (eIF4A RNAi/+). Larvae with nociceptor-specific knockdown of para (ppk > para RNAi) was used as positive control because of its impaired nociceptive responses. Response latencies of individual animals are plotted as points on the graph. The mean for each genotype is indicated with the error bars. (n ≥ 50 for all groups; ***p ≤ 0.001 by non-parametric Mann Whitney U Test)*

eIF4A is required for normal sensitivity to noxious mechanical stimuli

In order to determine whether the hyposensitive *eIF4A* knockdown phenotype is specific to thermal nociception or present for other nociceptive modalities, I tested nociceptor-specific *eIF4A* knockdown for defects in mechanical nociception. I used the same fly cross set up as thermal assay in that I used the *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression of the *VDRC 42202 UAS-eIF4A-RNAi* transgene and stimulated third instar larvae with a 10 mm Von Frey filament calibrated to subject a ~50mN force to induce nociceptive responses. I found that 48% of eIF4A knockdown larvae responded to the first mechanical stimulus, while 65% of Gal4-only larvae responded to the mechanical stimulus (*Fig. 10*). This was found to be statistically significant with $p = 0.011$ by Chi Square Test. In contrast I found that 57% of UAS-only larvae responded to the mechanical stimulus. This was not statistically significant with $p = 0.115$ by Chi Square Test though there was still a noticeably lower percent response recorded for *eIF4A* knockdown larvae than the UAS-only control. Together the results suggest that eIF4A is required for normal sensitivity to noxious mechanical stimuli just as it was required for noxious thermal stimuli.

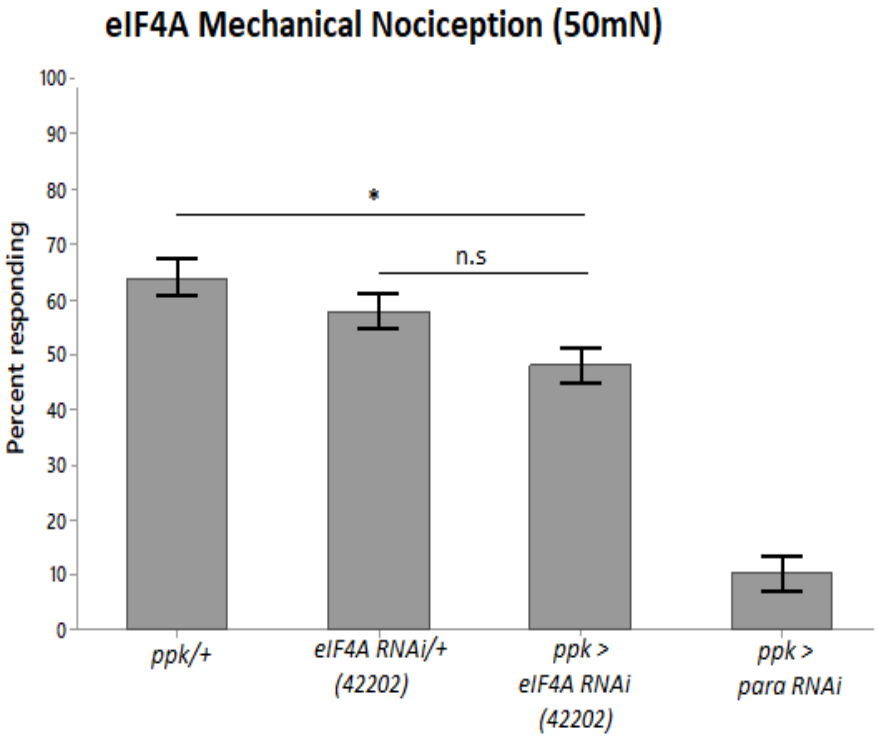

Figure 10: Nociceptor-specific knockdown of eIF4A causes defect in mechanical nociception.

*A significantly smaller proportion of larvae with nociceptor-specific knockdown of eIF4A (VDRC 42202 RNAi line) (ppk > eIF4A RNAi) (48%) exhibited nociceptive responses to a noxious mechanical stimulus than did Gal4-only control larvae (ppk/+) (65%). The UAS -only control larvae (eIF4A RNAi/+) (57%) was not statistically significant from nociceptor-specific knockdown of eIF4A. Larvae with nociceptor-specific knockdown of para (ppk > para RNAi) showed a very low rate of nociceptive responses and were used as a positive control ($n \geq 100$ for all groups; Chi-Square Test, *$p = 0.011$ by Chi Square Test; n.s $p = 0.115$). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.*

eIF4A is required for nociceptor sensitization

Given that eIF4A knockdown larvae are hyposensitive to noxious heat and mechanical stimuli at baseline, I wanted to determine if knockdown larvae are able to sensitize to noxious heat after tissue damage. Thus, I tested whether the *eIF4A* knockdown larvae become sensitized using a thermal hypersensitization assay. I set up crosses of *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression of the *VDRC 42202 UAS-eIF4A-RNAi* transgene. This is the same set of crosses that were used in the thermal assay, but were set up in two sets with the Gal4-only and UAS-only controls. One set of crosses was used for UV irradiation treatment and the other was used for sham treatment. I then used UV irradiation to damage the dorsal epidermis and then allowed UV^+ and UV^- larvae to recover for 8 hours at 25°C before testing for changes in thermal nociception at 42°C. I found that Gal4-only control larvae treated with UV irradiation had a latency of 5.5 seconds compared to the sham treatment with a latency of 7.6 seconds (*Fig. 11*). This was found to be statistically significant at $p = 0.003$ by the Mann Whitney U Test, suggesting the Gal4-only became sensitized as expected. I also found that UAS-only control larvae treated with UV irradiation had a latency of 4.2 seconds compared to the sham treatment with a latency of 5.6 seconds. This was found to be statistically significant at $p = 0.003$ by the Mann Whitney U Test, suggesting the UAS-only control also became sensitized. I found the latency for UV treated *eIF4A* knockdown larvae to be 8.3 seconds and the sham treatment latency at 8.6 seconds. This was statistically not significant at $p = 0.530$ by the Mann Whitney U Test. Thus, the controls were sensitized by the UV irradiation treatment, but as the *eIF4A* knockdown larvae was not sensitized, eIF4A is required for normal sensitization.

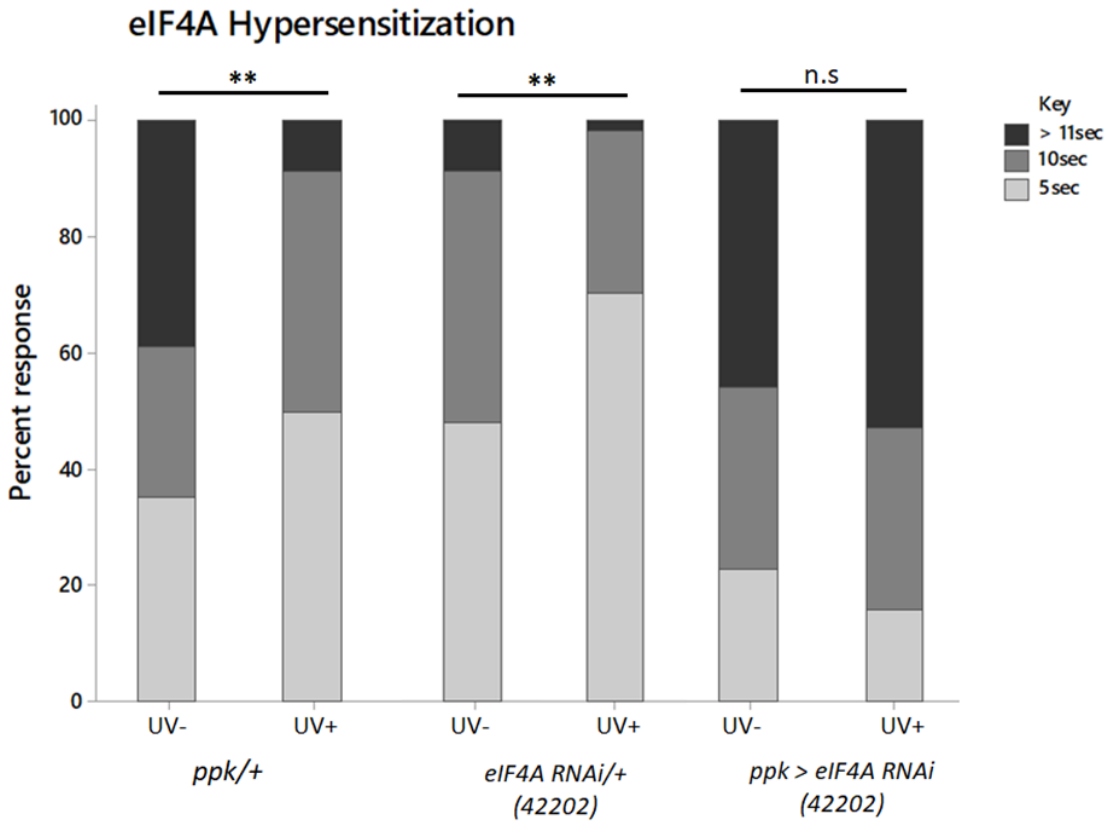

Figure 11: Nociceptor sensitization is blocked when eIF4A is knocked down, post-UV injury.

*Following UV exposure (+) or no UV exposure (-), larvae were assayed to a noxious thermal stimulus (42°C) after 8hrs. Response latencies were recorded and categorized as follows: Light grey ≤ 5sec; Dark grey ≤ 10sec; Black ≥ 11sec. Larvae with nociceptor-specific knockdown of eIF4A (VDRC 42202 RNAi line) when tested for UV sensitization latency response to noxious thermal stimulus (42°C) after 8hrs was not significant. Both the Gal4-only control and the UAS-only control showed significant UV sensitized responses. (n=40; **p ≤ 0.05 by non-parametric Mann Whitney Test)*

eIF4A is required for nociceptor dendrite morphogenesis

Neurons establish diverse dendritic morphologies during development. Defective dendritic arborization of the class IV multidendritic sensory neurons, which display class-specific dendritic morphology with extensive coverage of the body wall, may cause impaired nociception. In order to determine whether manipulation of eIF4A function caused nociception defects via influence on class IV multidendritic neuron arborization, I analyzed the morphology of class IV multidendritic neurons of *eIF4A* knockdown larvae by quantifying the dendritic sum intersection and the average dendritic intersection using Sholl analysis. The overall goal of this computer assisted method is to objectively quantify fundamental characteristics of neuron branching density. This helps to determine if gene knockdown alters neuron development. If there are no differences between a wild type and a gene knockout, then it can be concluded that the gene does not cause morphological defects. To analyze dendrite morphology defects arising from *eIF4A* knockdown, the *ppk-Gal4* driver was used to express *mCD8::GFP* and *eIF4A* RNAi in the Class IV multidendritic neurons. Confocal imaging of ligated third instar larvae was performed to quantify the dendrite arbor. I found that *eIF4A* knockdown (using *VDRC 42202 RNAi* line; n=10) significantly reduced the dendritic sum intersection (the total intersections with all the concentric rings) and the average dendritic intersection (average intersections with a single concentric ring) of class IV multidendritic neurons when compared to no RNAi control (n=10) (*Fig. 12*). The results of the Sholl analysis are tabulated in Table 3 below. These data suggest that *eIF4A* knockdown does produce gross morphological changes in the class IV multidendritic neurons.

Table 3: Sholl analysis statistics for *eIF4A* knockdown in class IV neurons

Genotype	Sum dendritic intersections	Average dendritic intersection
ppk Gal4 > mCD8::GFP, eIF4A RNAi	23971	14
ppk Gal4 > mCD8::GFP	38038	23
p value	0.001	0.001

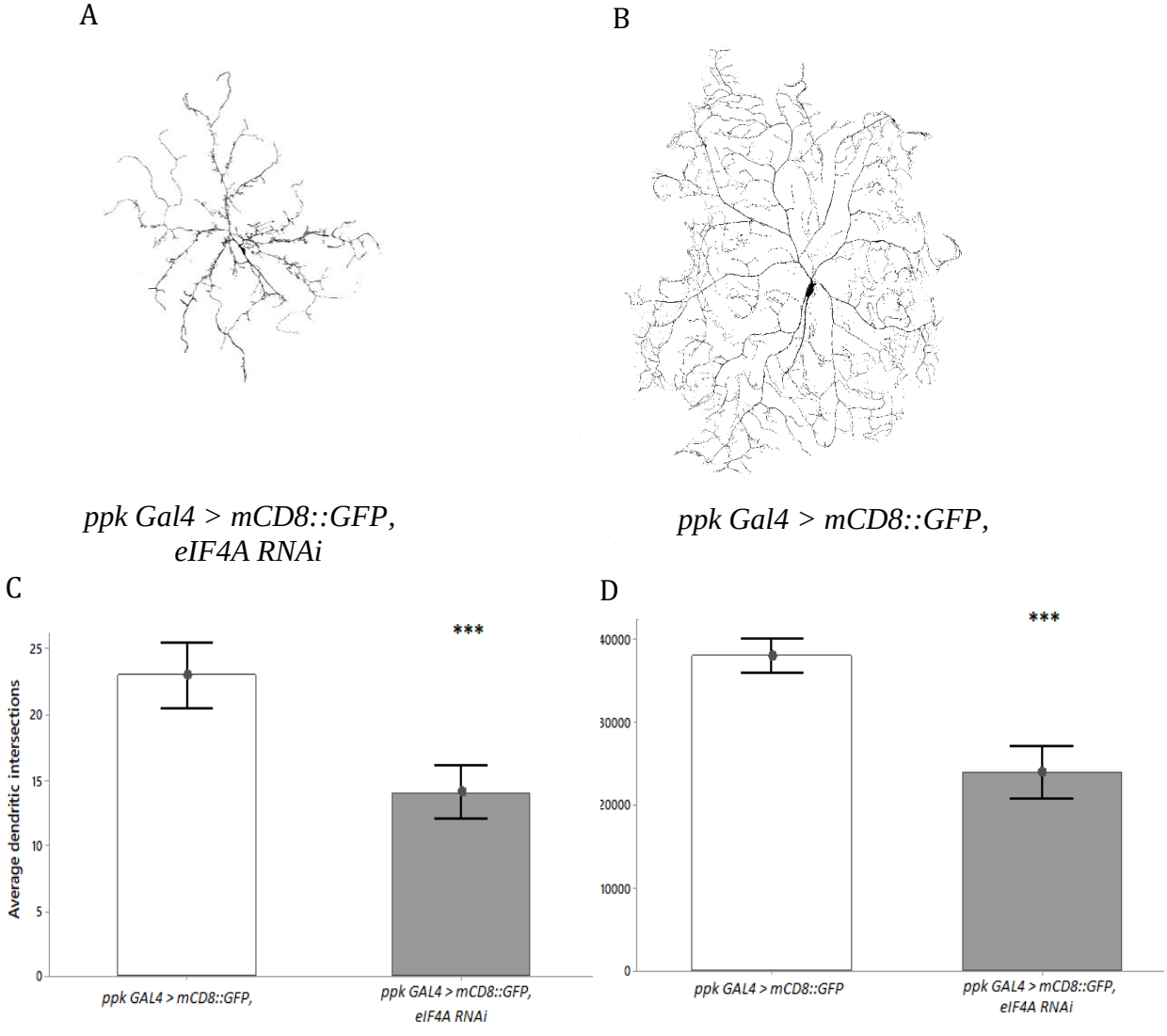

Figure 12: Nociceptor specific knock-down of eIF4A affects class IV multidenderitic neuron morphology.

A) Representative Cconfocal micrographs displaying the dendritic arborization of multidendritic IV neurons expressing GFP protein in the eIF4A RNAi; B) Representative micrographs of wild-type class IV multidendritic nueron morphology; C) Sholl analysis of average dendritic intersections of eIF4A RNAi (VDRC 42202 RNAi line) class IV multidendritic neurons was statistically significant from wild-type (n=10, Students t-test, p= 0.001); D) Sholl analysis of sum dendritic intersections of eIF4A RNAi class IV multidendritic neurons was statistically significant from wild-type (n=10; Students t-test, p=0.001).

eIF4G1 is required for sensitivity in nociception

To examine whether eIF4G1 is involved in larval nociception, I used a tissue-specific RNAi approach, making use of the Gal4/UAS system. I tested third instar larvae with nociceptor-specific *eIF4G1* knockdown using the *BDSC 33049 RNAi* line for defects in thermal and mechanical nociception.

eIF4G1 is required for normal sensitivity to noxious thermal stimuli

For our thermal nociception experiment, I used the *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression of the *BDSC 33049 UAS-eIF4G1-RNAi* transgene and tested for defects in thermal nociception. I found that larvae with nociceptor-specific *eIF4G1* knockdown displayed a significantly longer latency response at 46°C probe than controls. *eIF4G1* knockdown larvae displayed a mean latency of 5.2 seconds, compared to a mean latency of 3.0 seconds for the Gal4-only negative control and 3.4 seconds for the UAS-only negative control (*Fig. 13*). *Para* knockdown larvae were used as a positive control and found to show nearly complete insensitivity to noxious thermal stimuli. The increased latency of *eIF4G1* knockdown larvae was significantly different from the Gal4-only control latency and UAS-only control latency at $p \leq 0.001$ by the Mann Whitney U Test. The hyposensitive phenotype of the eIF4G1 knockdown phenotype suggests that eIF4G1 is required for normal sensitivity to noxious thermal stimuli.

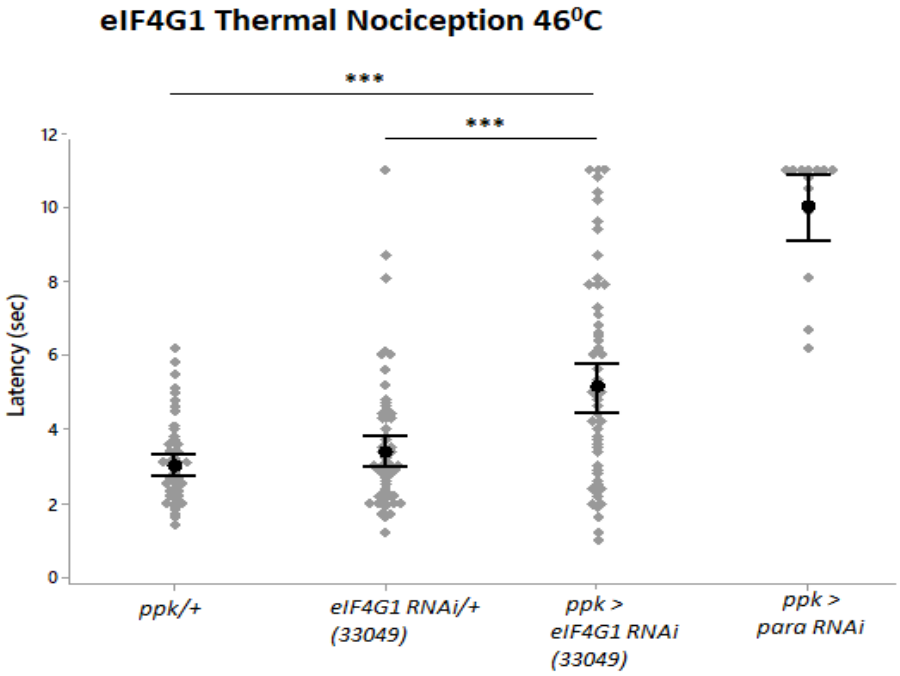

Figure 13: Nociceptor-specific knockdown of eIF4G1 causes defects in thermal nociception.

Larvae with nociceptor-specific knockdown of eIF4G1 (BDSC 33049 RNAi line) (ppk > eIF4G1 RNAi) showed a statistically longer latency response to noxious thermal stimulus (46°C) than both the Gal4-only control (ppk/+) and the UAS-only (eIF4G1 RNAi/+) control. Larvae with nociceptor-specific knockdown of para (ppk > para RNAi) was used as positive control because of its impaired nociceptive responses. Response latencies of individual animals are plotted as points on the graph. The mean for each genotype is indicated with the error bars. ($n \geq 50$ for all groups; $***p \leq 0:001$ by non-parametric Mann Whitney U Test)

eIF4G1 is required for normal sensitivity to noxious mechanical stimuli

In order to determine whether the hyposensitive *eIF4G1* knockdown phenotype is specific to thermal nociception or present for other nociceptive modalities, I tested nociceptor specific *eIFG1* knockdown for defects in mechanical nociception. I used the same fly cross set up as thermal assay in that I used the *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression of the *BDSC 33049 UAS-eIF4G1-RNAi* transgene and stimulated third instar larvae with a 10 mm Von Frey filament calibrated to produce a ~50mN force to induce nociceptive responses. I found that 42% of *eIF4G1* knockdown larvae responded to the first mechanical stimulus, while 64% of Gal4-only larvae responded to the mechanical stimulus (*Fig. 14*). This was found to be statistically significant with $p = 0.001$ by Chi Square Test. I also found that 57% of UAS-only larvae responded to the mechanical stimulus. This was also statistically significant from *eIF4G1* knockdown larvae with $p = 0.015$ by Chi Square Test. The results suggest that eIF4G1 is required also for normal sensitivity to noxious mechanical stimuli.

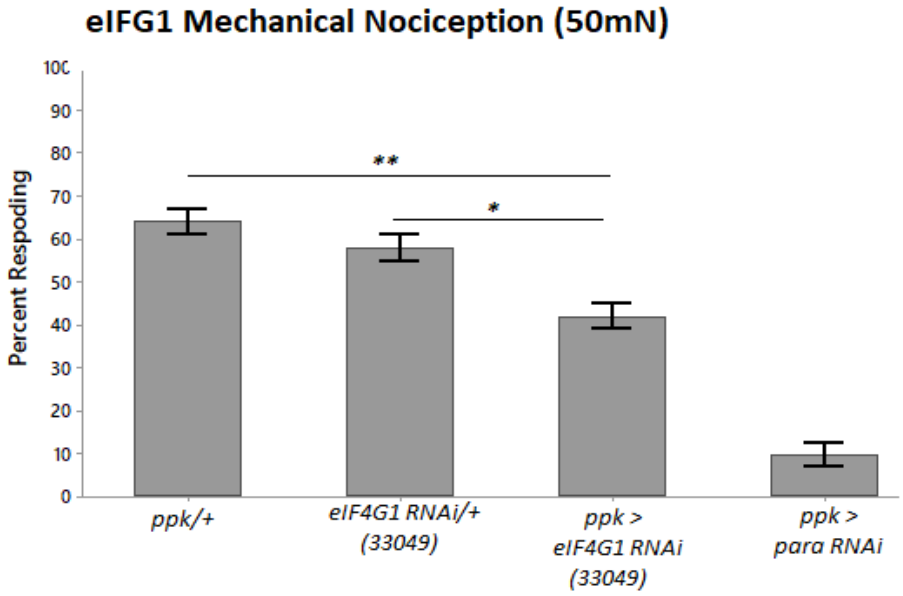

Figure 14: Nociceptor-specific knockdown of eIF4G1 causes defects in mechanical nociception.

*A significantly smaller proportion of larvae with nociceptor-specific knockdown of eIF4G1 (BDSC 33049 RNAi line) (ppk > eIF4G1 RNAi) (42%) exhibited nociceptive responses to a noxious mechanical stimulus than did Gal4-only (ppk/+) control larvae (64%) and the UAS – only (eIF4G1 RNAi/+) control larvae (58%). Larvae with nociceptor-specific knockdown of para (ppk > para RNAi) showed a very low rate of nociceptive responses and were used as a positive control (n≥100 for all groups; Chi-Square Test, **p≤0.001; *p≤0.015 by Chi Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.*

eIF4G1 is not required for hypersensitization

Given that eIF4G1 knockdown larvae are hyposensitive to noxious heat and mechanical stimuli at baseline, I hypothesized that the larvae would also sensitize to noxious heat after tissue damage. I set up crosses of *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression of the *BDSC 33049 UAS-eIF4G1-RNAi* transgene. This is the same set of crosses that were used in the thermal assay, but were set up in two sets with the Gal4-only and UAS-only control. One set of crosses was used for UV irradiation treatment and the other was used for sham treatment. I then used UV irradiation to damage the dorsal epidermis and then allowed UV^+ and UV^- larvae to recover for 8 hours at 25°C before testing for changes in thermal nociception at 42°C. I found that Gal4-only control larvae treated with UV irradiation had a latency of 8.1 seconds compared to the sham treatment with a latency of 9.1 seconds (*Fig. 15*). This was not statistically significant at $p = 0.123$ by the Mann Whitney U Test. I also found that UAS-only control larvae treated with UV irradiation had a latency of 9.8 seconds compared to the sham treatment with a latency of 10.9 seconds. This was not statistically significant at $p = 0.026$ by the Mann Whitney U Test. I found the latency for *eIF4G1* knockdown larvae UV treated at 7.9 seconds and the sham treatment latency at 10.4 seconds. This was statistically significant at $p = 0.001$ by Mann Whitney U Test. Results from the experimental group of eIF4G1 UV irradiation and sham control suggests when the eIF4G1 was knocked down the larvae still became sensitized.

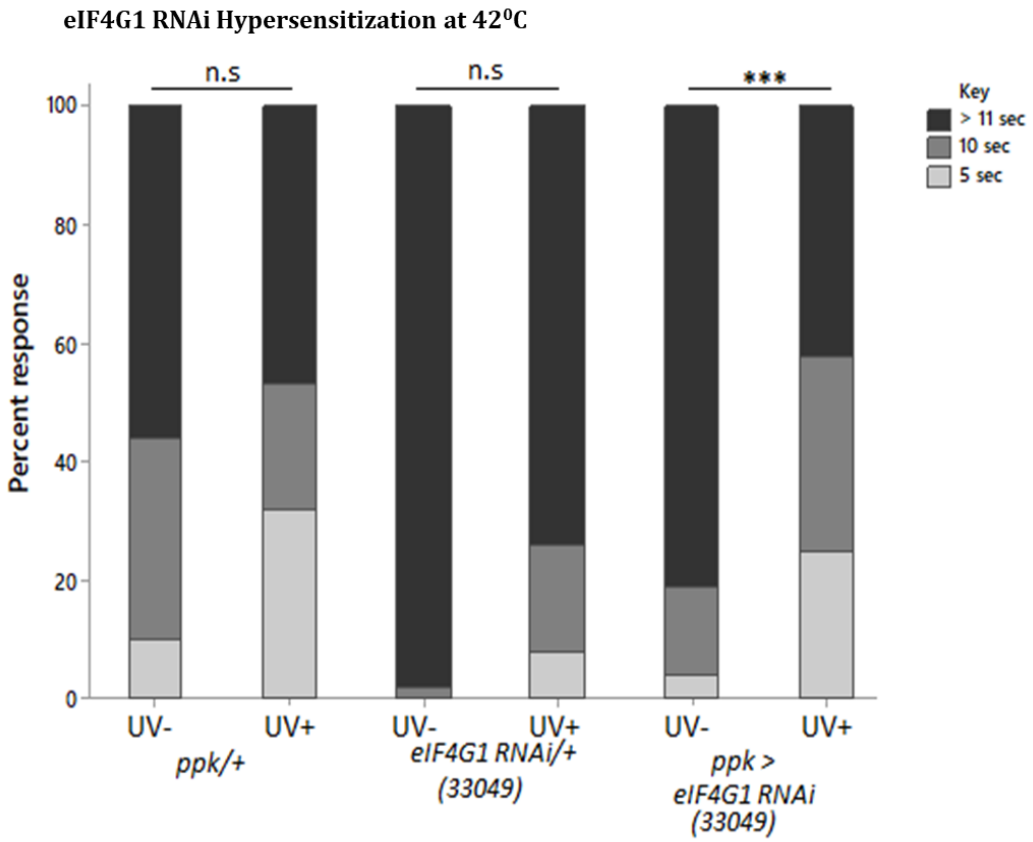

Figure 15: Nociceptor sensitization is still occurring when eIF4G1 is knocked down, post-UV injury.

*Following UV exposure (+) or no UV exposure (-), larvae were assayed to noxious thermal stimulus (42°C) after 8hrs. Response latencies were recorded and categorized as follows: Light grey ≤ 5sec; Dark grey ≤ 10sec; Black ≥ 11sec. Larvae with nociceptor-specific knockdown of eIF4G1 (BDSC 33049 RNAi line) when tested for UV sensitization latency response to noxious thermal stimulus (42°C) after 8hrs was significant with 58% larvae responding within 10sec. Both the Gal4-only control and the UAS-only control showed no significant UV sensitized response. (n=40; ***p≤0.001 by non-parametric Mann Whitney U Test)*

eIF4G1 knockdown affects nociceptor dendrite morphogenesis

It is possible that defective nociception phenotypes arising from eIF4G1 knockdown could be explained by defects in dendrite morphology. Thus, I analyzed the morphology of class IV multidendritic neurons of *eIF4G1* knockdown larvae by quantifying the dendritic sum intersection and the average dendritic intersection using Sholl analysis. To analyze morphology defects arising from *eIF4G1* knockdown, I used *ppk-Gal4* to express mCD8::GFP and eIf4G1 RNAi in the nociceptors. Confocal imaging ligated third instar larvae was performed to quantify the dendrite arbor of Class IV multidendritic neurons. I found that *eIF4G1* knockdown (*BDSC 33049 RNAi* line; n=10) did not significantly affect the dendritic sum intersection, but does significantly affect the average dendritic intersection of class IV multidendritic neurons when compared to Gal4-only controls (n=10) (*Fig. 16*). The results of the Sholl analysis are tabulated in Table 4 below. These data suggest that *eIF4G1* knockdown does produce modest morphological changes in the class IV multidendritic neurons.

Table 4: Sholl analysis statistics for *eIF4G1* knockdown in class IV neurons

Genotype	**Sum dendritic intersections**	**Average dendritic intersection**
ppk Gal4 > mCD8::GFP, eIF4G1 RNAi	31694	19
ppk Gal4 > mCD8::GFP, y v attp2, y +	36692	25
p value	0.063	0.018

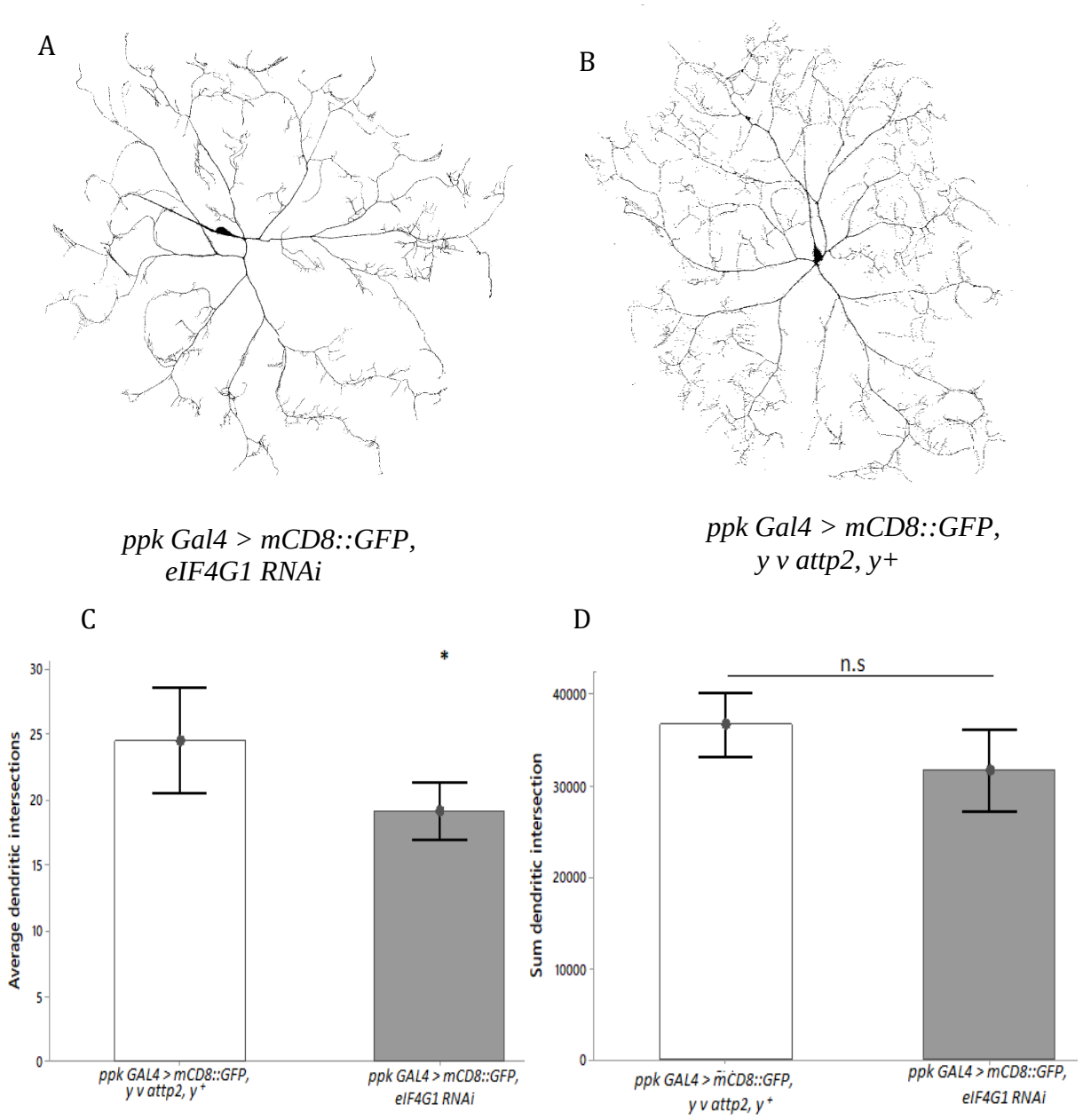

Figure 16: Nociceptor specific knock-down of eIF4G1 slightly affects class IV multidendritic neuron morphology.

A) Representative confocal micrographs displaying the dendritic arborization of class IV multidendritic neurons expressing GFP protein in the eIF4G1 RNAi (BDSC 33049 RNAi line); B) Representative micrographs of wild-type class IV multidendritic morphology; C) Sholl analysis of average dendritic intersections of eIF4G1 RNAi class IV multidendritic neurons was statistically significant from wild-type (n=10, Students t-test, p= 0.018); D) Sholl analysis of sum dendritic intersections of eIF4G1 RNAi class IV multidendritic neurons was statistically not significant from wild-type (n=10; Students t-test, p=0.063).

eIF4G2 is required for sensitivity in nociception

To examine whether eIF4G2 is involved in larval nociception, I used a tissue-specific RNAi approach making use of Gal4/UAS system available in *Drosophila*. I tested third instar larvae with nociceptor-specific *eIF4G2* knockdown using the *BDSC 35809* and *BDSC 41963* RNAi transgene for defects in thermal and mechanical nociception.

eIF4G2 is required for normal sensitivity to noxious thermal stimuli

For our thermal behavioral experiment, I used the *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression in two of the *BDSC 35809 UAS-eIF4G2-RNAi* and *BDSC 41963 UAS-eIF4G2-RNAi* transgenes. Both the RNAi lines were tested for defects in thermal nociception. I found that larvae with nociceptor-specific *eIF4G2* knockdown displayed significantly longer latency response at a 46°C probe than controls. *eIF4G2* knockdown larvae displayed a mean latency of 6.0 seconds (*BDSC 35809*) and 5.1seconds (*BDSC 41963*) (*Fig. 17*). This compared to a mean latency of 3.0 sec for the Gal4-only negative control and 4.1sec for the UAS-only negative control. *Para* knockdown larvae were used as a positive control and found to show nearly complete insensitivity to noxious thermal stimuli. The increased latency of *eIF4G2* knockdown larval response to noxious thermal temperature was significantly different from the Gal4-only control larval response at $p \leq 0.001$ and the UAS-only control larval response at $p = 0.003$ both by the non-parametric Mann Whitney Test. The hyposensitive phenotype of *eIF4G2* knockdown larvae indicate that eIF4G2 is required for normal sensitivity to noxious thermal stimuli.

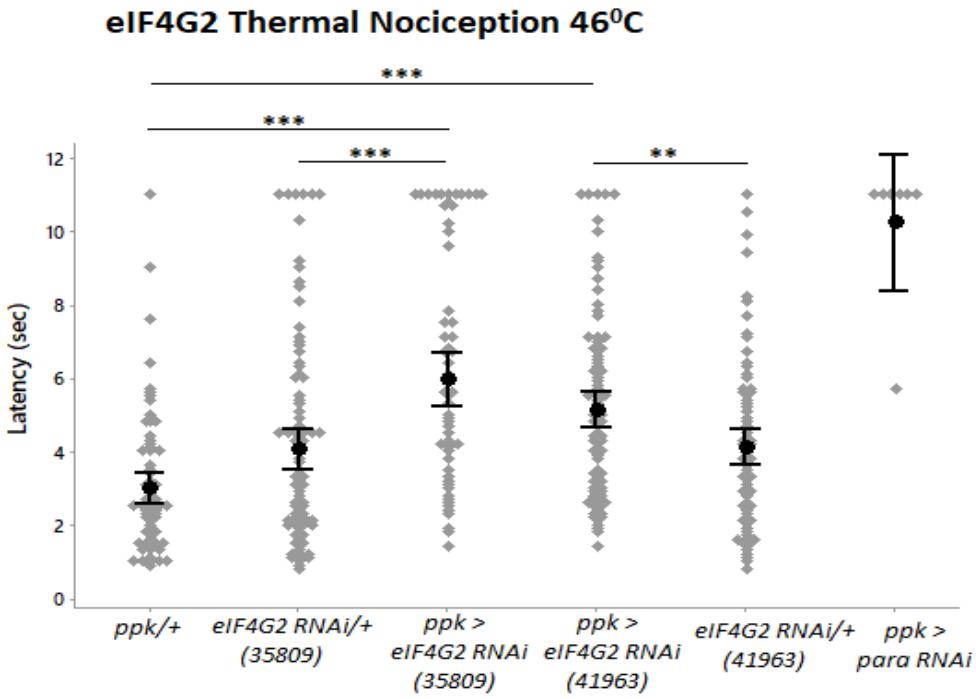

Figure 17: Nociceptor-specific knockdown of eIF4G2 causes defects in thermal nociception.

*Larvae with nociceptor-specific knockdown of eIF4G2 (BDSC 35809 RNAi line and BDSC 41963 RNAi line) (ppk > eIF4G2 RNAi) showed a statistically longer latency response to noxious thermal stimulus (46°C) than both the Gal4-only (ppk/+) control and the UAS-only (eIF4G2 RNAi/+) controls. Larvae with nociceptor-specific knockdown of para was used as positive control because of its impaired nociceptive responses. Response latencies of individual animals are plotted as points on the graph. The mean for each genotype is indicated with the error bars. ($n \geq 50$ for all groups; ***$p \leq 0{:}001$ by non-parametric Mann Whitney Test; **$p = 0{:}003$ by non-parametric Mann Whitney U Test.*

eIF4G2 is required for normal sensitivity to noxious mechanical stimuli

In order to determine whether the hyposensitive *eIF4G2* knockdown phenotype is specific to thermal nociception only or present for other nociceptive modalities, I tested nociceptor specific eIFG2 knockdown for defects in mechanical nociception. I used the *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression in two *eIF4G2* RNAi line *BDSC 35809 UAS-eIF4G2-RNAi* transgene and *BDSC 41963 UAS-eIF4G2-RNAi* transgene, and stimulated third instar larvae with a 10 mm Von Frey filament calibrated to subject a 50mN force adequate to induce nociceptive responses. I found that 50% of both *eIF4G2* knockdown larvae responded to the first mechanical stimulus, while 65% of Gal4-only larvae responded to the mechanical stimulus (*Fig. 18*). This was found to be statistically significant with $p = 0.020$ by Chi Square Test. I also found that 63% of the *BDSC 41963 RNAi* line and 62% - BDCS 35809 RNAi line UAS-only control larvae responded to the mechanical stimulus. This was not statistically significant with $p = 0.055$ by a Chi Square Test though there was still a lower percent response recorded for *eIF4G2* knockdown larvae than the UAS-RNAi-only controls. The results presented in Figure 18 suggest that the *eIF4G2* is required for normal sensitivity to noxious mechanical stimuli.

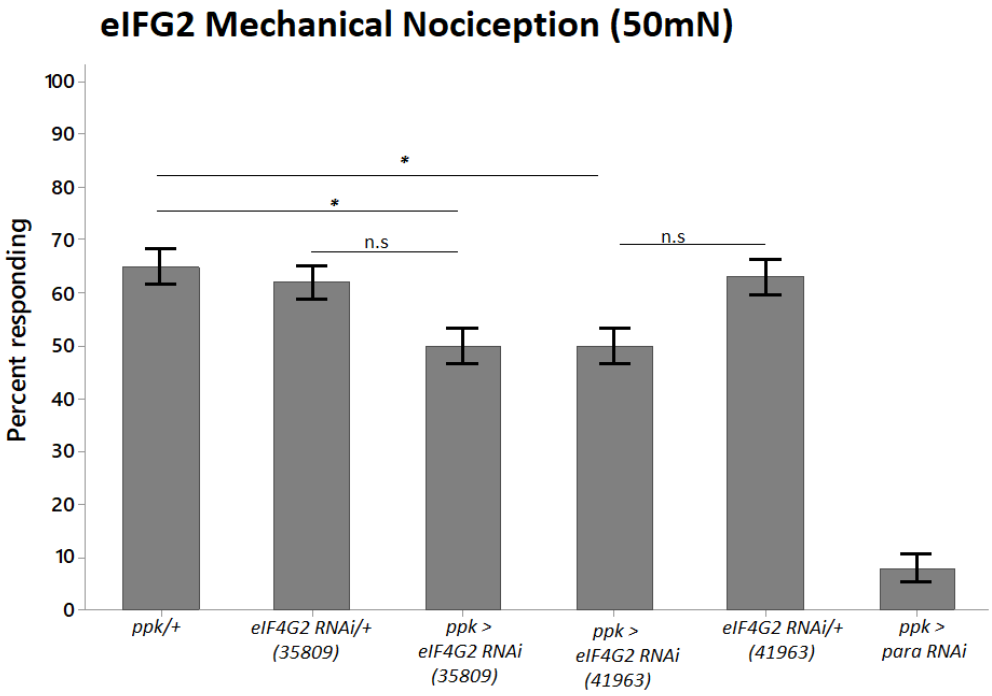

Figure 18: Nociceptor-specific knockdown of eIF4G2 causes defect in mechanical nociception.

A significantly smaller proportion of larvae with nociceptor-specific knockdown of eIF4G2 (BDSC 35809 RNAi line and BDSC 41963 RNAi line) (ppk > eIF4G2 RNAi) (50% for both eIF4G2 RNAi (35809 and 41963)) exhibited nociceptive responses to a noxious mechanical stimulus than did Gal4-only (ppk/+) control larvae (65%). The UAS -only control (eIF4G2 RNAi/+) larvae (63% - 41963; 62% - 35809) was not statistically significant than nociceptor-specific knockdown of eIF4A. Larvae with nociceptor-specific knockdown of para showed a very low rate of nociceptive responses and were used as a positive control ($n \geq 100$ for all groups; Chi-Square Test, $*p \leq 0.020$ by Chi Square Test; n.s $p \leq 0.055$). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

eIF4G2 is not required for hypersensitization

Given that I found *eIF4G2* knockdown larvae hyposensitive to noxious heat and mechanical stimuli at baseline, I hypothesized that the larvae might also sensitize to noxious heat after tissue damage. I set up crosses of *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression in two *eIF4G2* RNAi lines *BDSC 35809 UAS-eIF4G2-RNAi* transgene and *BDSC 41963 UAS-eIF4G2-RNAi* transgene. This is the same set of crosses that was used in the thermal assay but were set up in two sets with the Gal4-only and UAS-only control. One set of crosses was used for UV irradiation treatment and the other was used for sham treatment. I then used UV irradiation to damage the dorsal epidermis and then allowed UV^+ and UV^- larvae to recover for 8 hours at 25°C before testing for changes in thermal nociception at 42°C. I found the latency of larvae with Gal4-only control treated with UV irradiation to be 6.5 seconds compared to its sham treatment with a latency of 8.4 seconds. This was found to be statically significant at $p = 0.004$ by the Mann Whitney U Test (*Fig. 19*). These results suggest that the Gal4-only control were sensitized. I found that UAS-only control larvae treated with UV irradiation had a latency for the *BDSC 41963 RNAi* line at 7.2 seconds and for the BDCS 35809 RNAi line at 8.3 seconds. This compared to the sham treatment with a latency of 9.4 seconds and 9.8 seconds respectively. This was found to be statistically significant at $p = 0.001$ by the Mann Whitney U Test. These results suggest that the UAS-only controls are also getting sensitized. We found the latency for the UV-treated eIF4G2 knockdown larvae to be 8.9 and 10.1 seconds for the *BDSC 41963* and *BDCS 35809 RNAi* lines, and 9.9 and 11.0 seconds for the sham-treated *BDSC 41963* and *BDCS 35809 RNAi* lines, respectively. This was not statistically significant at $p = 0.050$ for the *BDCS 35809 RNAi* line and $p = 0.074$ for the *BDSC 41963 RNAi* line by the Mann Whitney U Test. The results suggest that the controls were

sensitized by the UV irradiation treatment while the eIF4G2 knockdown larvae were not. These results suggest that eIF4G2 is required for normal sensitization.

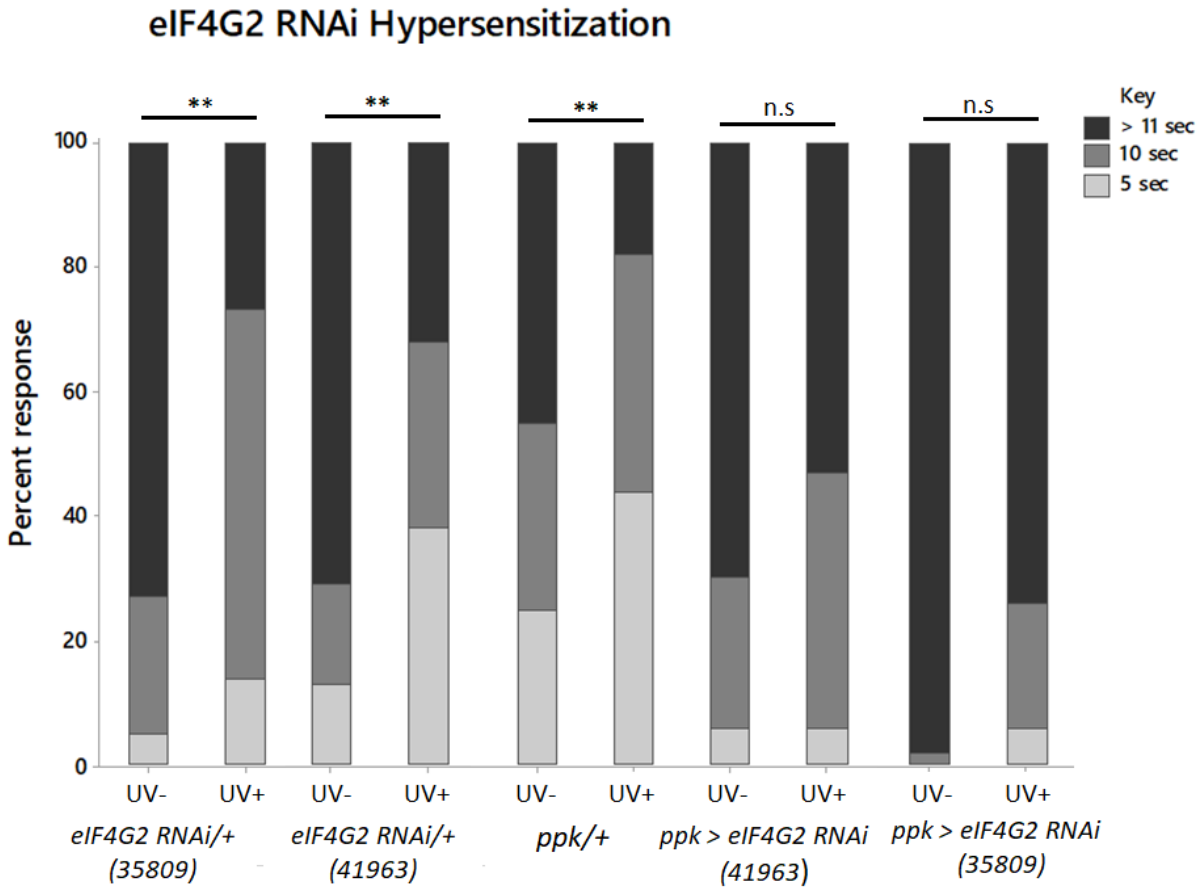

Figure 19: Nociceptor sensitization is not affected when eIF4G2 is knockdown post-UV injury.

*Following UV exposure (+) or no UV exposure (-), larvae were assayed to noxious thermal stimulus (42°C) after 8hrs. Response latencies were recorded and categorized as follows: Light grey ≤ 5sec; Dark grey ≤ 10sec; Black ≥ 11sec. Larvae with nociceptor-specific knockdown of eIF4G2 when tested for UV sensitization latency response to noxious thermal stimulus (42°C) after 8hrs was not significant ($p = 0.050$ for BDCS 35809 RNAi line and $p = 0.074$ for BDSC 41963 RNAi line by Mann Whitney U Test). Both the Gal4-only control and the UAS-only control showed significant UV sensitized response. (n=40; **$p \leq 0.05$ by non-parametric Mann Whitney U Test)*

eIF4G2 is not required for nociceptor dendrite morphogenesis

It is possible that defective nociception phenotypes arising from *eIF4G2* knockdown could be explained by defects in dendrite morphology. Here we went ahead and analyzed the morphology of class IV multidendritic neurons of *eIF4G2* knockdown larvae by quantifying the dendritic sum intersection and the average dendritic intersection using Sholl analysis. To determine the function of eIF4G2 in nociception arising from developmental defects, the expression of mCD8::GFP and *eIF4G2* knockdown in class IV multidendritic neurons was driven by ppk-Gal4. Confocal imaging of the ligated third instar larvae was done to quantify the dendritic arbor. We found that *eIF4G2* knockdown (*BDSC 35809 RNAi* line; n=10) did not significantly affect the dendritic sum nor the average dendritic intersection in class IV multidendritic neurons when compared to no-RNAi control (n=10) (*Fig. 20*). The results of the Sholl analysis are tabulated in Table 5. These data suggest that *eIF4G2* knockdown does not produce gross morphological changes in the class IV multidendritic neurons.

Table 5: Sholl analysis statistics for *eIF4G2* knockdown class IV neurons

Genotype	**Sum dendritic intersections**	**Average dendritic intersection**
ppk Gal4 > mCD8::GFP, eIF4G2 RNAi	37865	20
ppk Gal4 > mCD8::GFP, y v attp2, y +	36692	24
p value	0.610	0.053

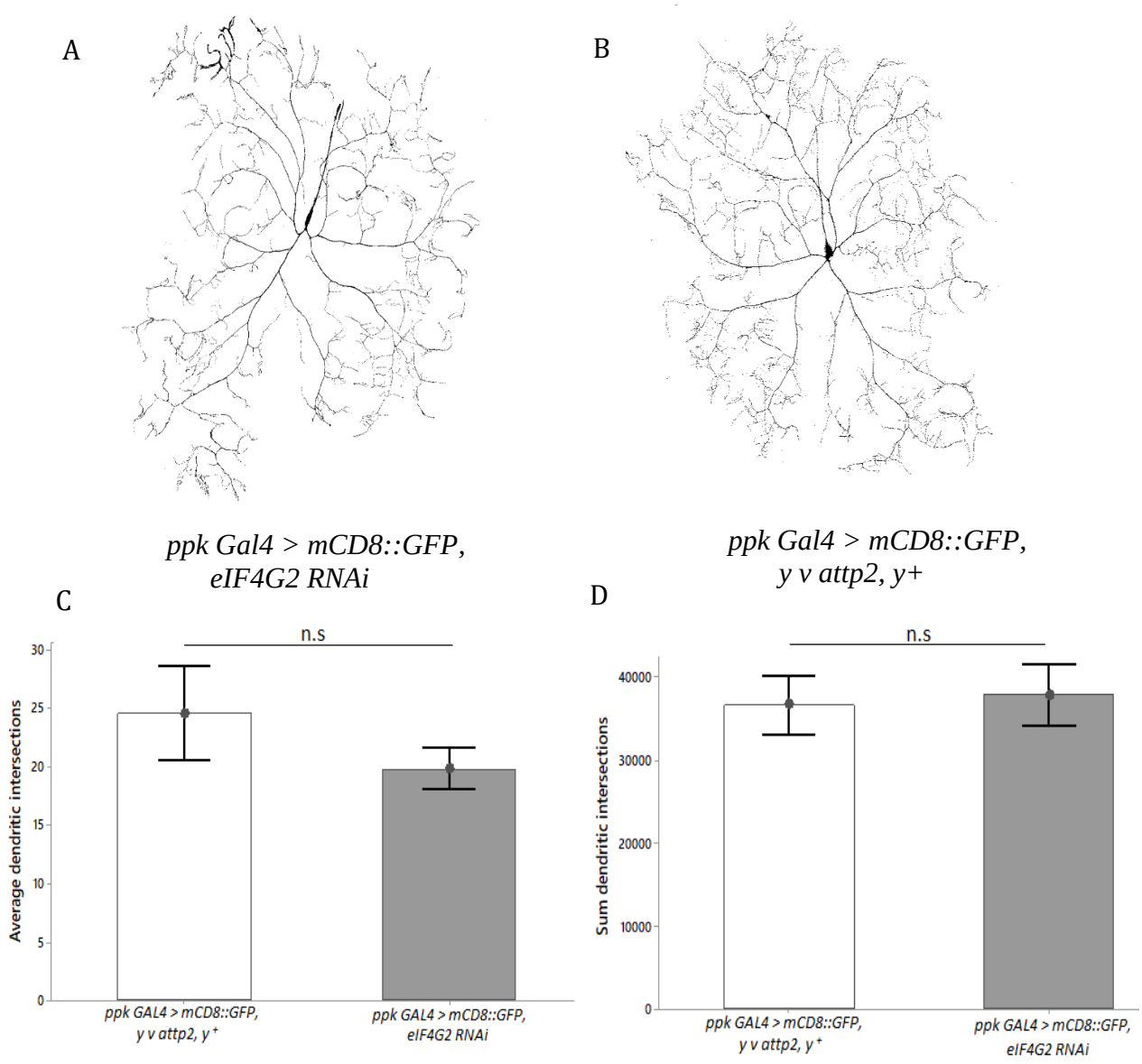

Figure 20: Nociceptor specific knock-down of eIF4G2 does not affect *class IV multidendritic* neuron morphology.

A) Representative confocal micrographs displaying the dendritic arborization of class IV multidendritic neurons expressing GFP protein in the eIF4G2 RNAi (BDSC 35809 RNAi line); B) Representative micrographs of wild-type class IV multidendritic morphology; C) Sholl analysis of average dendritic intersections of eIF4G2 RNAi class IV multidendritic neurons was statistically not significant from wild-type (n=10, Students t-test, p=0.053); D) Sholl analysis of sum dendritic intersections of eIF4G2 RNAi class IV multidendritic neurons was statistically not significant from wild-type (n=10; Students t-test, p=0.610).

Discussion

eIF4A is required for normal thermal and mechanical sensitivity, sensitization, and dendrite morphogenesis.

I have demonstrated that nociceptor-specific knockdown of *eIF4A* knockdown causes *Drosophila* larvae to respond to noxious thermal stimuli with longer response latencies and to noxious mechanical stimuli with reduced frequency. I also observed that *eIF4A* knockdown larvae were not sensitized by UV irradiation in a thermal hypersensitization assay. These results suggest a role for *eIF4A* in positively regulating nociceptor sensitivity. I also observed a gross dendrite morphology defect in the *eIF4A* knockdown larvae, suggesting that eIF4A is required for normal dendrite morphology. The nociception defect may arise from abnormal neuronal morphology or degeneration of the class IV multidendritic neurons.

The role of eIF4A in the eIF4F complex is in the unwinding of secondary structures in the 5′-UTR during scanning and stabilizing the mRNA, all of which promotes efficient translation of mRNAs (Li, 2002). This activity appears to be necessary for threading the mRNA on the 40S subunit (Svitkin et al., 2001). A second function of the helicase activity of eIF4A is in the process of mRNA scanning itself, the ATP-dependent movement of the 40S subunit from the cap structure towards the initiating AUG (Hershey and Merrick, 2000). Another possibility is that the helicase activity of eIF4A is required for is the RNA structural rearrangement to allow association of other translational factors with the mRNA.

The longer latencies observed from the thermal assay results suggest that eIF4A functions in the translation of mRNAs into proteins that are required for thermal nociception. A similar function for eIF4A in mechanical nociception is suggested by the lowered response

frequency observed from *eIF4A* knockdown larvae in the mechanical nociception assay. It is possible that when the function of eIF4A is removed in the *eIF4A* knockdown larvae there is stalled unwinding and scanning of the eIF4A-dependent mRNA needed for the translation of proteins that function in nociception. It is also possible that there is also mis-regulated RNA structural rearrangement that prevents recruitment of other eIFs onto the ribosome, which would affect eIF4A-dependent translation in the nociceptors. I also observed *eIF4A* knockdown larvae were not sensitized after UV irradiation, suggesting that eIF4A functions in the translation of mRNAs of proteins that are required during nociceptor sensitization. When the function of eIF4A is removed in the *eIF4A* knockdown larvae, the translation of eIF4A-dependent mRNAs of proteins required in the nociceptor sensitization was presumably affected, explaining the non-sensitized phenotype observed. Defective dendrite morphology was observed in *eIF4A* knockdown larvae, suggesting the function of eIF4A in regulating translation of eIF4A-dependent genes may also be involved in the class IV dendrite morphogenesis. All together, I suggest that eIF4A is regulating translation by affecting the recruitment of capped mRNAs onto the 40s ribosome so scanning of the 5' UTR can happen. As a result, the protein products that function in nociception, nociceptor sensitization and nociceptor morphogenesis, are no longer synthesized or are synthesized at insufficient levels.

Mammalian mRNA possessing long and structured 5'UTR secondary structures are known to be hyper-dependent on eIF4A for translocation in-vitro (Svitkin et al., 2001). 5′UTRs can contain hairpin structures and pseudoknots such as cap-independent enhancers (CITEs), G-quadruplexes, m^6A induced ribosome engagement sites (MIRES), internal ribosome entry site (IRES) elements (which also can be elsewhere in the mRNA), terminal oligopyrimidine

tract (TOP) motifs, Translation Initiator of Short 5' UTR (TISU), and upstream open reading frames (uORF) that make translation less efficient from the main open reading frame (*Fig. 21*).

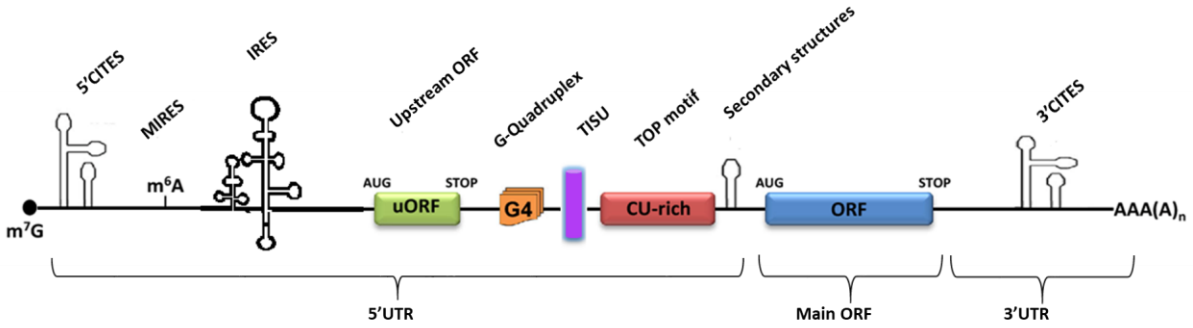

Figure 21: Long and structured 5'UTR secondary structures *(Adapted from Lacerda et. al 2017)*

In the absence of the eIF4A helicase activity, remaining secondary structure can stall the process of translation. As I observed hyposensitive results from *eIF4A* knockdown larvae in nociception, I can hypothesize that the 5' UTRs of the mRNA molecules involved in nociception have at least one of these secondary structures affecting the process of translation. I also observed *eIF4A* knockdown larvae were not sensitized after UV irradiation, which suggests eIF4A functions in translation of mRNAs whose protein products are required in nociceptor sensitizations. Therefore, I also hypothesize that the 5'UTR of the mRNA of the ion channels involved in nociceptor sensitization, the inflammatory molecules, the molecules of the signaling pathway involved in nociceptor sensation, or even the transcription factors regulating the production of inflammatory or signaling pathway molecules have mRNAs that are long and structured, and thus dependent on the eIF4A helicase activity for translation.

Our results suggest that eIF4A is affecting translation of proteins involved in nociception, nociceptor sensitization, and also nociceptor morphology, but I do not know which specific mRNA transcripts in the nociceptors are regulated by eIF4A. A study done in

mice using a specific eIF4A inhibitor and high throughput sequencing approaches identified RNA transcripts that rely on eIF4A activity for efficient translation. The study identified a subset of plasticity-related genes conserved across evolution that contain a sequence motif in the 5' untranslated region that is known to be regulated by eIF4A helicase activity (Moy et al., 2016). Also the 5' UTRs of eIF4A dependent mRNAs were reported to be enriched with G-quadruplex structure sequence rich in 12 or 9 nucleotide guanine quartet (Wolfe et al., 2014). mRNAs of several genes of clinical interest have been experimentally shown to contain the G-quadruplex within their 5' UTR (Bugaut and Balasubramanian, 2012). Thus, I hypothesize that nociceptor mRNAs that require eIF4A for cap-dependent translation in nociception, nociceptor sensitization and nociceptor morphology may also contain these structures.

A genome-wide search for transcripts containing this G-quadruplex motif within 5' UTRs by focusing on a 12-nucleotide sequence identified 3,917 human genes. After eliminating the short 5'UTRs, 2993 candidate genes were identified that were highly enriched in the G-quadruplex motif. RNA sequencing data from human neural tissue, stem cells and hollow organs, suggests that G-quadruplex motif containing genes depending on eIF4A motifs were more likely to display expression enrichment in neural tissues and stem cells than in hollow organs. This suggests a key role for eIF4A activity in regulating mRNA translation in tissues displaying functional plasticity such as neurons involved in chronic pain (Srivastava et al., 2016). This idea helps explain my results that eIF4A silencing affected mRNA translation of proteins involved in nociception, nociceptor sensitization and nociceptor morphology by suggesting these mRNA transcripts are rich in G-quadruplex sequences in their 5' UTRs.

Nociceptors express special receptors called transient receptor potential (TRP) ion channels. The TRP family of proteins is conserved with similar functionality in vertebrates,

including humans. TRP channels have roles in many different sensory systems. During tissue injury there is release of inflammatory molecules. Bradykinin is an inflammatory molecule that acts on a G-protein coupled receptor called B2 to activate the G-protein Gαq. When the B2 receptor is in its active state, it is physically coupled with Gαq protein. Activated Gαq then activates the enzyme PLC-β. TRPA1 (also known as ANKTM1) is reported to be activated at 17^0C in mice as it is involved in cold-sensing (Story et al., 2003). In a study of TRPA1-deficient mice, these mice showed substantially decreased responses to bradykinin at the cellular and behavioral level. These observations indicate TRPA1 as an important component of the signaling machinery that depolarizes nociceptors as a response its activation in inflammation. The same study also shows that PLC is an important signaling component for TRPA1 activation. The study concluded that TRPA1 mediates the inflammatory response by its action with bradykinin (Bautista et al., 2006). The TRPA1 ortholog in *Drosophila* was shown to be activated by temperatures of 24^0C suggesting that this ion channel function in heat sensing (Viswanath et al., 2003), thus the same signaling mechanism maybe happening in *Drosophila*. The *Drosophila* TRPA1 ion channel is also found in class IV neurons (Zhong et al., 2012). Assuming that all of the gene expression changes induced in the nociceptor during sensitization signaling pathways are regulated at the translational level by eIF4F assembly, I hypothesize here that the mRNA coding for ion channels such as TRPA1 as well as the upstream inflammatory signaling molecules are eIF4A-dependent for translation and as a result in *eIF4A* knockdown are not getting optimally translated. This can help explain the results observed in the hypersensitization assay where the *eIF4A* knockdown larvae were not sensitized.

eIF4A knockdown larvae show hyposensitivity latencies in thermal nociception, but not the complete insensitivity as seen in the control *para* knockdown larvae. Also, in

mechanical nociception assays, knockdown larvae show reduced response, but not a complete non-response. These results suggest a translation mechanism functioning in translation of mRNAs of molecules involved in the nociception mechanism (such as *painless*, *TRPA1, ppk*) using an alternate eIF4F-like mechanism in the absence of eIF4A. It is well understood that mRNAs with long 5' UTR mRNAs require eIF4A helicase activity. This suggests the questions of how does the short 5' UTR mRNA get translated? This can be accomplished by the Translation initiator of short 5' UTR (TISU) sequence (*Fig. 22*). This sequence is present in about 4% of all genes, preferentially in those with "housekeeping" functions. The initiating AUG can be preceded by an unusually short 5' UTR with a median length of 12 nucleotides (Elfakess and Dikstein, 2008).

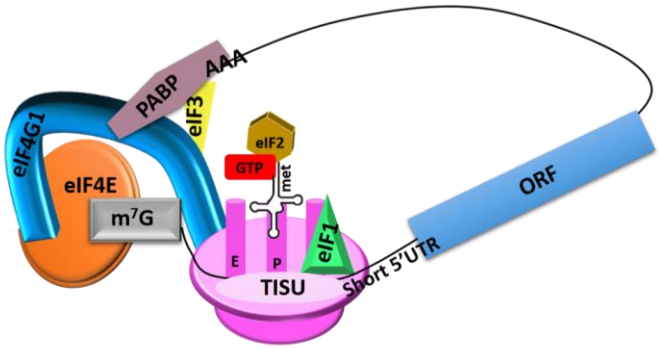

Figure 22: Translation initiator of short 5' UTR (TISU) sequence in eIF4A independent translation

Shorter 5' UTRs generally exhibit leaky translation initiation (Kozak, 1991). TISU sequence allows cap-dependent, but scanning independent, initiation of mRNAs with short 5' UTRs. This process does not require eIF4A, but eIF1 and eIF4G1 cooperate to facilitate TISU-mediated translation initiation (Sinvani et al., 2015). These mechanisms may partially compensate for reductions in the translation process in the absence of eIF4A. With this in mind, I can suggest that in *eIF4A* knockdown larvae this mechanism of mRNA translations

compensates in part as can be seen by the hyposensitivity in thermal nociception and low response in mechanical nociception.

In class IV neurons, ecdysone activates the translation inhibitor eIF4E-BP by inhibiting the insulin and target of Rapamycin pathways (Wong et al., 2013). Thus eIF4E-BP binds to eIF4E in the eIF4F complex and inhibits ribosome recruitment to mRNAs and is able to down-regulate translation during pruning, the process by which dendrites are removed during larval metamorphosis. During pruning, ecdysone activates the *Sox14* transcription factor. *Sox14* is required for apoptosis in type II and III da neurons and for pruning in type I and IV da neurons. In type I and IV da neurons, Sox14 activates expression of dendrite pruning genes, including Mical (Osterloh and Freeman, 2009). Given that ecdysone inhibits eIF4E-dependent translation, a study asked whether there are mechanisms that ensure the translation of ecdysone target mRNAs. The study found that the canonical eIF4F components eIF4E and eIF4G were not required for Mical expression, while an interaction between eIF4A and eIF3 was needed. This specificity is conferred by the 5' UTR of Mical mRNA characterized by different reporter assays (Rode et al., 2018). Both eIF4A and eIF3 interact with the 5'UTRs of their target mRNAs during translation initiation (Hinnebusch, 2014). eIF4A was reported to be regulating eIF3 interactions with the Mical 5' UTR. This suggests that eIF4A/eIF3 constitute a 4E-BP bypass mechanism that ensures the adequate translation of ecdysone-induced genes in class IV multidendritic neurons (Rode et al., 2018). This suggests that eIF4A is involved in the process of translation during the process of pruning. This is suggestive of an alternate translational mechanism to eIF4F assembly. Therefore, the morphological defects that I am seeing in the *eIF4A* knockdown larvae are unlikely to arise from a pruning defect, but may arise from sort of developmental defect using the same mechanism or a similar mechanism described above.

It is interesting to ask how much of the eIF4A nociception and sensitization results are dependent on the morphological defects seen. I suggest that morphological defects are more likely the result of developmental defects, rather than pruning processes. If the morphological genes were rescued during the time window of neural development, would larvae still show the same nociception and sensitization response? It is interesting to note that no adult flies emerged from pupation in the eIF4A crosses that were studied. This result is suggestive that the morphological defects are affecting the dendrite pruning process and causing pupal mortality. With all the data presented, eIF4A appears to be a strong translational regulator for genes involved in nociceptor morphogenesis, and this can be further explored as a future direction.

eIF4G1 and eIF4G2 are required for normal thermal and mechanical sensitivity

I have demonstrated that nociceptor-specific knockdown of eIF4G1 causes *Drosophila* larvae to respond to noxious thermal stimuli with longer response latencies and to noxious mechanical stimuli with reduced frequency. I also observed that *eIF4G1* knockdown larvae become sensitized by UV irradiation in a thermal hypersensitization assay. These results suggest a modest role for eIF4G1 in positively regulating nociceptor sensitivity. Similar results were found for eIF4G2. Nociceptor-specific knockdown of eIF4G2 caused *Drosophila* larvae to respond to noxious thermal stimuli with longer response latencies and to noxious mechanical stimuli with reduced frequency, however these differences in response rate to mechanical stimuli were not statistically significant. These results also suggest a modest role for eIF4G2 in positively regulating nociceptor sensitivity.

I observed increased latency in the thermal nociception assay and lowered response rate in mechanical nociception assay in the *eIF4G1* and *eIF4G2* knockdown larvae. The results suggest that eIF4G is regulating translation in the nociceptors. Studies show an interaction between eIF4G and poly A binding protein (PABP) that brings about circularization of the mRNA by stimulating 40S subunit recruitment. The circularization of the mRNA could be enhancing translation by moving terminating ribosomes directly to the 5' end of the mRNA (Tarun Jr and Sachs, 1996). I hypothesize that in the *eIF4G* knockdown larvae there is impaired circulation of the mRNA resulting in reduced recruitment of the ribosome. I suggest that translation of nociceptor transcripts might be eIF4G-dependent, and thus inefficient translation would be expected to lead to hyposensitive latency in thermal nociception assays and lowered response in mechanical nociception assays.

Initiation of cap-dependent translation is thought to depend on the assembly of eIF4F with mRNA. The availability of free eIF4E is controlled by eIF4E-BPs, which can interact with eIF4E and prevent it from binding to eIF4G. When eIF4E-BPs are phosphorylated they are released from eIF4E, thus allowing eIF4E to form the eIF4F complexes necessary for translation (Adriaensen et al., 1983; Duncan et al., 1987; Sonenberg, 2008). Interaction between eIF4G with eIF4E, holds eIF4A in its active conformation to facilitate the unwinding of the mRNA by eIF4A helicase activity. In *eIF4G* knockdown larvae, eIF4A will not get recruited and this will stall scanning as the 5' UTR sequence needs to be fed into the ribosome after unwinding, a function done by the eIF4A. In absence of eIF4G interaction with both eIF4E and eIF4A, which would be expected to occur in *eIF4G* knockdown larvae, there would be impaired assembly of the eIF4F complex and the nociceptor transcripts required for nociception would not get translated. Translation may therefore eIF4G-dependent. We can see

this effect in the observed hyposensitive latency in thermal nociception and lowered response in mechanical nociception.

The findings of a study done on eIF4G1 depletion suggest the possibility that eIF4G1 might favor translation of partially distinct classes of mRNAs (Ramírez-Valle et al., 2008). Translation of these mRNAs might be eIF4G-dependent, and loss of translation of these mRNAs might cause the nociceptor hyposensitivity in the *eIF4G1* and *eIF4G2* knockdown larvae. I can hypothesize that the mRNA of TRPA1 ion channels that function in nociception is dependent on eIF4G translation, and thus *eIF4G* knockdown larvae would lack a functioning TRPA1. This can explain the observed results associated with the hyposensitive latency in thermal nociception and lowered response in mechanical nociception.

eIF4G1, but not eIF4G2, is required for normal sensitization

From our hypersensitization assay we observed that *eIF4G1* knockdown in UV-irradiated larvae were sensitized when compared to their sham controls, but *eIF4G2* knockdown UV irradiated larvae were not sensitized when compared to their sham controls. This was suggesting that perhaps UV irradiation caused activation of an alternate translation mechanism to function in sensitization in *eIF4G1* knockdown larvae more significantly than in *eIF4G2* knockdown larvae. There are other proteins that could be working in parallel or redundantly with eIF4G1 in nociceptors. Mextli (Mxt) is a novel eIF4E-BP that may be able to function redundantly to eIF4G1 in translation initiation (*Fig. 23*).

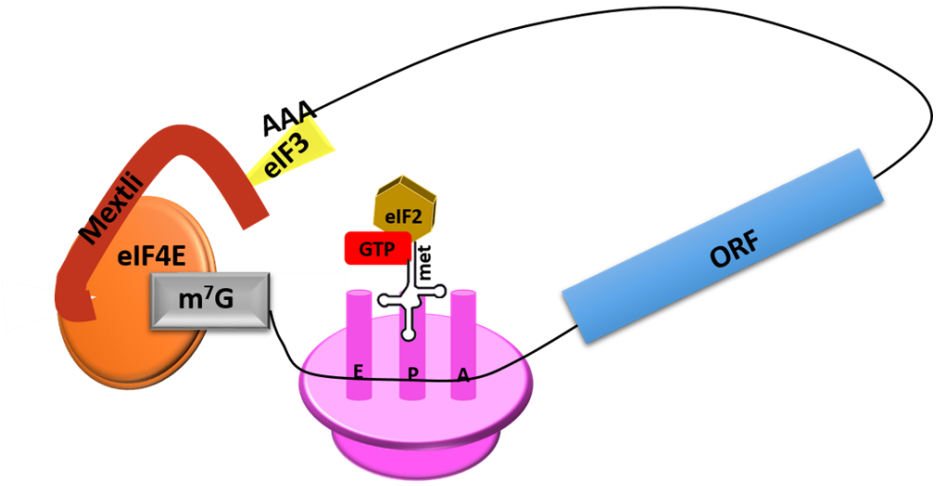

Figure 23: Mextli functions in place of eIF4G in eIF4G independent translation

Like eIF4G, Mxt can binds eIF3 and several eIF4E isoforms and promotes translation, in sharp contrast to other eIF4E-BPs that inhibit translation. Mxt contains a middle segment of eIF4G domain, a canonical homology RNA-binding domain, and a consensus eIF4E binding motif allowing it to function as a scaffolding protein. Mxt may serve as an alternative to the canonical eIF4G1 that coordinates the assembly of translation initiation complexes that serve as an alternative to cap-dependent eIF4F assembly (Hernández et al., 2013). This mechanism can explain the nociceptor sensitized results observed in *eIF4G1* and *eIF4G2* knockdown larvae. Therefore, I can hypothesize that the mRNA of the ion channels involved in nociceptor sensitization, the inflammatory molecules, the molecules of the signaling pathway involved in nociceptor sensation or even transcription factors regulating the inflammatory molecules or the signaling pathway molecules have mRNA that is depending on the eIF4G1 scaffolding activity for translation. Here I hypothesize that when there is *eIF4G1* knockdown the Mxt is picking up the function of regulating translation initiation in the *Drosophila* larvae nociception and sensitization. This might help explain the hyposensitive results seen in thermal nociception

instead of complete insensitivity to thermal noxious stimuli in both *eIF4G1* and *eIF4G2* knockdown larvae. Mxt has no clear mammalian ortholog. Thus, this proposed system can only function in *Drosophila*.

eIF4G recruits the 40S ribosomal subunit to the mRNA via its interaction with eIF3. The function of the eIF3 is to form a bridge between the 40s ribosomal subunit and the mRNA bound to the eIF4G. In absence of eIF4G the ribosome subunit cannot be recruited by the eIF4F assembly and causing a translation deficiency. Thus eIF3 can promote the association of mRNA with ribosomes (Lee et al., 2016). A study in yeast reports eIF3 can recruit mRNA onto the ribosome in absence of eIF4G1 (Jivotovskaya et al., 2006), suggesting that eIF4G may act after 40S ribosome subunit association with mRNA, and is not necessary for ribosome scanning on unstructured mRNAs *in-vitro* (Pestova et al., 2007). eIF3d recruitment to an internal stem-loop structure in the 5' UTR protects the 5' end of the mRNA and allows for cap binding. This binding is postulated to prevent promiscuous mRNA binding prior to assembly of eIF3 (Lee et al., 2016). Thus, translation initiation might proceed on many mRNAs through an alternate mechanism that is cap-dependent and involves little requirement for eIF4GI, but is dependent on eIF3. This observation might explain why the *eIF4G1* knockdown larvae still showed a sensitized phenotype. eIF3 may provide an alternative mechanism to achieve the upregulated translation of nociceptor genes to allow the nociceptor to show a sensitized phenotype in eIF4G1. The mechanism can also allow for the results that were hyposensitive and not insensitive in both thermal and mechanical nociception in both *eIF4G1* and *eIF4G2* knockdown larvae. Nociceptor transcripts might be getting regulated by this eIF4G independent translation mechanism during nociception and sensitization.

Some mRNAs have a mechanism to bypass the need for eIF4E binding. This mechanism uses internal ribosome entry site (IRES) and is independent of cap binding, though it does require scanning for AUG codon. The IRES possesses defined secondary and tertiary structures that account for efficient interaction with the 40S ribosomal subunit (*Fig. 24*). The interactions maybe supported by IRES-trans-acting factors (ITAFs) which assist in recruiting the 40S subunit onto the mRNA through active conformation of the IRES. Hellen and Sarnow demonstrated the presence of IRES in eukaryotic mRNA, and it has been estimated about 10-15% of cellular mRNAs could be translated IRES-dependently (Hellen and Sarnow, 2001; Spriggs et al., 2008). eIF3 functions as an ITAF (Kieft et al., 1999) and replaces the eIF4G1 function as described above. This study shows a cap-independent mechanism of translation initiation using IRES mRNA that function in absence of eIF4G and supports the results of hyposensitivity and not complete insensitivity in nociceptors. This also suggest that the mRNA transcripts that function in nociceptors might use an IRES element in the 5' UTR, which allows it to be translated in an eIF4G-independent manner.

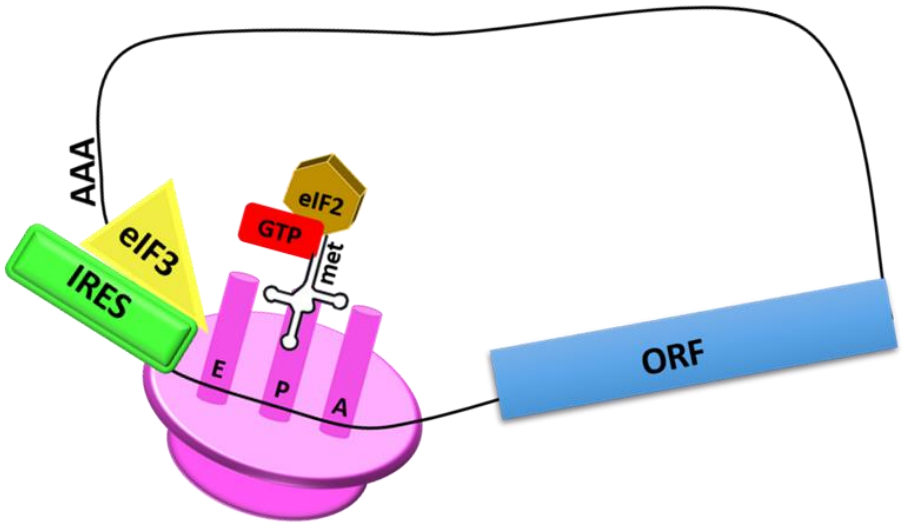

Figure 24: IRES dependent translation initiation

Death associated protein 5 (DAP5) also called p97 or NAT1, lacks the binding site for eIF4E (Hundsdoerfer et al., 2005; Nevins et al., 2003) is an ortholog for human eIF4G2. The homology of DAP5 to eIF4G in *Drosophila* is largely confined to the central segment of eIF4G which contains the binding regions for eIF4A and eIF3 (*Fig. 25*) (Imataka et al., 1997). A study to characterize the molecular functions of DAP5 in translation initiation showed DAP5 interacts with eIF2β and eIF4A1 to drive IRES-dependent translation (Liberman et al., 2015). DAP5 functions in this system as an alternative for eIF4G. This may further help explain the results from our study e. IRES dependent translation initiations occurs mainly when the cell is stressed (Villa-Cuesta et al., 2010). I hypothesize that DAP5 IRES-dependent translation is activated upon the UV irradiation of the larvae and this results in the translation needed for sensitization of *eIF4G1* knockdown larvae. One may expect that DAP5 to be more functional in *eIF4G2* knockdown larvae sensitization but our results suggest that it may be more active in *eIF4G1* knockdown larvae as they are getting sensitized by UV irradiation. This clearly suggests that even though DAP5 is an ortholog to eIF4G2 it is more similar in function to eIF4G1 in the nociceptors.

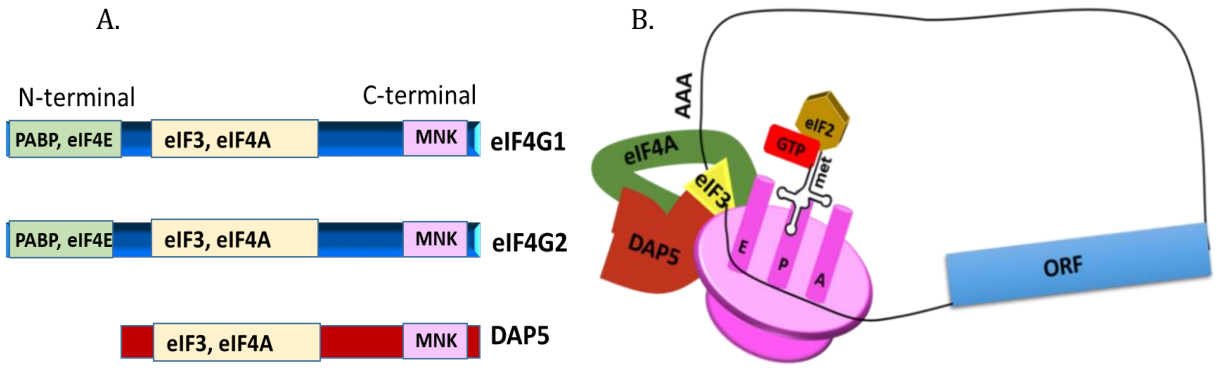

Figure 25: DAP5 has homology to eIF4G1 and eIF4G2 molecules in translation initiation.

A. DAP5 lacks the binding sites for eIF4E and PABP but has the binding sites for eIF3 and eIF4A. B. DAP5 participated in cap-independent translation in absence of eIF4G.

m^{6}A induced ribosome engagement sites (MIRES) are sequences in the 5' UTR that can directly bind to eIF3 and the 43s ribosomal subunit (*Fig. 26*). This allows scanning of the mRNA to take place without recruiting eIF4E in response to cellular stress (Meyer et al., 2015). This study shows, using both *in vitro* reconstitution approaches and translation assays in cellular lysates deficient in eIF4E activity, that m^{6}A in the 5' UTR functions as an alternative to the 5' cap to stimulate mRNA translation. This is another mechanism where the eIF3 might replace the function of the eIF4G1and thus is another potential mechanism by which eIF4G1 function can be compensated in nociceptor sensitization.

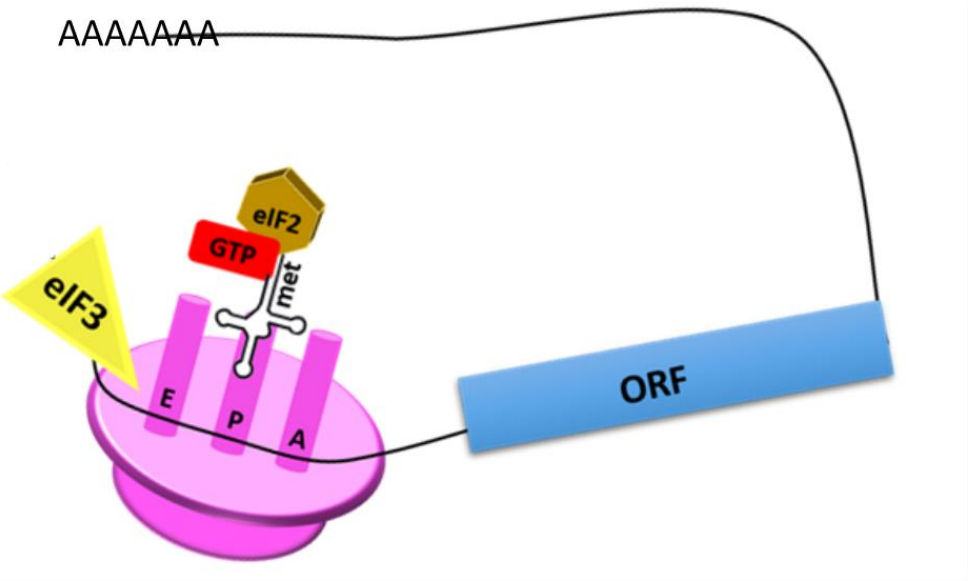

Figure 26: Cellular stresses selectively increase the levels of m6A within 5' UTRs bypassing the cap-dependent translation.

Present in the 5' UTR are cap-independent translational enhancers (CITEs), which can recruit translational components (Shatsky et al., 2010). CITEs have been shown to function independently of cap by recruiting eIF4G and eIF3 (Andreev et al., 2012; Roberts et al., 2015). One can hypothesize here that eIF3 can replace the function of eIF4G1 as it does in cap-dependent translation initiation, as discussed previously in this section. This may still be

another mechanism that comes into action after UV irradiation of the larvae in the hypersensitization assay, which would allow sensitization to take place in the absence of eIF4G1.

Defects in dendritic morphogenesis caused by the absence of eIF4G,1 but not eIF4G2

The dendrites of *eIF4G1* knockdown larvae show reduced arborization with a decreased number of average dendritic intersections, though no changes in the sum dendritic intersections. The total area occupied by the arbor is the same as the control. In contrast there was no significant defect in the dendritic morphology of *eIF4G2* knockdown larvae. With multiple mechanism functioning in translation initiation in absence of eIF4G (*e.g.* Mxt, DAP5, IRES, eIF3, CITES), mRNAs involved in the classIV multidendritic neuron morphogenesis may still be translated, thus allowing these mRNAs to be translated in the absence of eIF4G1 or eIF4G2. With this I can suggest that the mild morphological defects observed in *eIF4G1* knockdown larvae do not affect the nociception phenotype. *eIF4G1* knockdown larvae getting sensitized suggest that nociceptor transcripts involved in the sensitization process are translated via an eIF4G1 independent mechanism. eIF4G can be said to be functioning with gene transcripts that are involved in nociception as the *eIF4G* knockdown larvae had a hyposensitive phenotype.

Future Directions

Having looked at the dynamics of interactions in the assembly of eIF4F and other molecules as well as the mechanisms that seem to be functioning in parallel, it would be interesting to determine their distinct roles in the process of nociception. As both isoforms of

eIF4G showed similar behavioral phenotypes in nociception when knocked down, perhaps a double knockdown experimental design would yield a more insensitive phenotype in nociception. Mxt has been characterized as eIF4G analog in germ line stem cells (Hernández et al., 2013). Thus, it too could be experimentally manipulated during nociception studies. DAP5 has also been characterized mainly through in vitro studies to characterize its interactions with IRES elements in the 5'UTRs. Thus, DAP5 could also be explored relative to nociceptor functioning. eIF3 came up as an interesting point of interaction in translation, as it may provide some compensatory function under conditions when eIF4G levels and function are reduced. It is also a bulky molecule like eIF4G, composed of multiple subunits, and studied in translation in many systems. Thus, it would be exciting to see how it functions in nociceptors, especially in nociceptor sensitization with other eIFs.

Despite the clear behavioral evidence in *Drosophila* larvae that eIF4A and eIF4G are required for nociceptor function, presumably through eIF4F assembly and translational regulation, it remains unclear which mRNAs must be properly repressed or activated to regulate nociceptor function in baseline and sensitized conditions. One methodology that could be used to address this gap in our knowledge is called Translating Ribosomal Affinity Purification (TRAP), which is designed to isolate translating mRNAs from specific types of neurons, such as nociceptors. Using TRAP, an affinity tag is attached to the ribosome and when the ribosome binds an mRNA to translate it, biochemical techniques can be used to purify the tagged ribosome and capture its bound mRNAs. RNA sequencing methods can then be used to reveal what genes are actively translated. With multiple *Drosophila* studies already using this methodology (Pamudurti et al., 2017; Thomas et al., 2012), TRAP can be used to comprehensively characterize up and down-regulated mRNA translation in class IV

nociceptors using the eIF4F complex proteins individually. The results can also shed light also into the signaling pathways involved in activation of nociceptor sensitization process. A recent study reported using TRAP showed that the RagA-mTORC1 network controlling eIF4E cap-dependent translation as a critical generator of neuropathic pain in mouse models (Megat et al., 2018). Similar experiments can be repeated in *Drosophila* to gradually explore the other eIF proteins involved in nociception.

References

Abramson, R., Dever, T., Lawson, T., Ray, B., Thach, R., and Merrick, W. (1987). The ATP-dependent interaction of eukaryotic initiation factors with mRNA. J. Biol. Chem. *262*, 3826-3832.

Adriaensen, H., Gybels, J., Handwerker, H.O., and Van Hees, J. (1983). Response properties of thin myelinated (A-delta) fibers in human skin nerves. J. Neurophysiol. *49*, 111-122.

Ainsley, J.A., Pettus, J.M., Bosenko, D., Gerstein, C.E., Zinkevich, N., Anderson, M.G., Adams, C.M., Welsh, M.J., and Johnson, W.A. (2003). Enhanced locomotion caused by loss of the Drosophila DEG/ENaC protein Pickpocket1. Curr. Biol. *13*, 1557-1563.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. *15*, 6541-6551.

Andreev, D.E., Dmitriev, S.E., Zinovkin, R., Terenin, I.M., and Shatsky, I.N. (2012). The 5′ untranslated region of Apaf-1 mRNA directs translation under apoptosis conditions via a 5′ end-dependent scanning mechanism. FEBS Lett. *586*, 4139-4143.

Asano, K., Clayton, J., Shalev, A., and Hinnebusch, A. G. (2000). A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and initiator tRNAMet is an important translation initiation intermediate in vivo. Genes Dev. *14*, 2534-2546.

Babcock, D.T., Landry, C., and Galko, M.J. (2009). Cytokine signaling mediates UV-induced nociceptive sensitization in Drosophila larvae. Curr. Biol. *19*, 799-806.

Babcock, D.T., Shi, S., Jo, J., Shaw, M., Gutstein, H.B., and Galko, M.J. (2011). Hedgehog signaling regulates nociceptive sensitization. Curr. Biol. *21*, 1525-1533.

Bai, X., and Jiang, Y. (2010). Key factors in mTOR regulation. Cell Mol. Life Sci. *67*, 239-253.

Bandell, M., Story, G.M., Hwang, S.W., Viswanath, V., Eid, S.R., Petrus, M.J., Earley, T.J., and Patapoutian, A. (2004). Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron *41*, 849-857.

Barragan-Iglesias, P., Lou, T.F., Bhat, V.D., Megat, S., Burton, M.D., Price, T.J., and Campbell, Z.T. (2018). Inhibition of Poly(A)-binding protein with a synthetic RNA mimic reduces pain sensitization in mice. Nat. Commun. *9*, 10.

Bautista, D.M., Jordt, S.E., Nikai, T., Tsuruda, P.R., Read, A.J., Poblete, J., Yamoah, E.N., Basbaum, A.I., and Julius, D. (2006). TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell *124*, 1269-1282.

Bogart, K., and Andrews, J. (2006). Extraction of total RNA from *Drosophila*. CGB Tech. Rep. *10*, 1-4.

Bohnsack, M.T., Regener, K., Schwappach, B., Saffrich, R., Paraskeva, E., Hartmann, E., and Görlich, D. (2002). Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. EMBO J. *21*, 6205-6215.

Bradley, C.A., Padovan, J.C., Thompson, T.L., Benoit, C.A., Chait, B.T., and Rhoads, R.E. (2002). Mass spectrometric analysis of the N terminus of translational initiation factor eIF4G-1 reveals novel isoforms. J. Biol. Chem. *277*, 12559-12571.

Bramham, C.R., Jensen, K.B., and Proud, C.G. (2016). Tuning specific translation in cancer metastasis and synaptic memory: control at the MNK–eIF4E Axis. Trends in Biochem. Sci. *41*, 847-858.

Brand, A.H., and Perrimon, N. (1993). Targeted gene-expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Brenman, J.E., Gao, F.B., Jan, L.Y., and Jan, Y.N. (2001). Sequoia, a tramtrack-related zinc finger protein, functions as a pan-neural regulator for dendrite and axon morphogenesis in *Drosophila*. Dev. Cell *1*, 667-677.

Bugaut, A., and Balasubramanian, S. (2012). 5′-UTR RNA G-quadruplexes: translation regulation and targeting. Nucleic Acids Res. *40*, 4727-4741.

Burgess, G.M., Mullaney, I., McNeill, M., Dunn, P.M., and Rang, H.P. (1989). Second messengers involved in the mechanism of action of bradykinin in sensory neurons in culture. J. Neurosci. *9*, 3314-3325.

Carr, D.B., and Goudas, L.C. (1999). Acute pain. Lancet *353*, 2051-2058.

Caruthers, J.M., Johnson, E.R., and McKay, D.B. (2000). Crystal structure of yeast initiation factor 4A, a DEAD-box RNA helicase. Proc. Natl. Acad. Sci. U. S. A. *97*, 13080-13085.

Caygill, E.E., and Brand, A.H. (2016). The GAL4 system: a versatile system for the manipulation and analysis of gene expression. Drosophila *1478* 33-52.

Chahine, M., Ziane, R., Vijayaragavan, K., and Okamura, Y.J. (2005). Regulation of Nav channels in sensory neurons. Trends in Pharmacol. Sci. *26*, 496-502.

Chan, C.C., Dostie, J., Diem, M.D., Feng, W., Mann, M., Rappsilber, J., And Dreyfuss, G. (2004). eIF4A3 is a novel component of the exon junction complex. RNA *10*, 200-209.

Chuang, H.H., Prescott, E.D., Kong, H.Y., Shields, S., Jordt, S.E., Basbaum, A.I., Chao, M.V., and Julius, D. (2001). Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P-2-mediated inhibition. Nature *411*, 957-962.

Cioni, J.-M., Koppers, M., and Holt, C.E. (2018). Molecular control of local translation in axon development and maintenance. Curr. Opn. Neurobiol. *51*, 86-94.

Cordin, O., Banroques, J., Tanner, N.K., and Linder, P.J.G. (2006). The DEAD-box protein family of RNA helicases. Gene *367*, 17-37.

Coste, B., Mathur, J., Schmidt, M., Earley, T.J., Ranade, S., Petrus, M.J., Dubin, A.E., and Patapoutian, A. (2010). Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. Science *330*, 55-60.

Cubelos, B., Sebastian-Serrano, A., Beccari, L., Calcagnotto, M.E., Cisneros, E., Kim, S., Dopazo, A., Alvarez-Dolado, M., Redondo, J.M., Bovolenta, P., and Walsh, C. A. (2010). Cux1 and Cux2 regulate dendritic branching, spine morphology, and synapses of the upper layer neurons of the cortex. Neuron *66*, 523-535.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., and Scheiblauer, S. and Cuoto, A. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature *448*, 151.

Donnerer, J., Schuligoi, R., and Stein, C. (1992). Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience *49*, 693-698.

Dorn, R., Morawietz, H., Reuters, G., and Saumweber, H. (1993). Identification of an essential *Drosophila* gene that is homologous to the translation initiation factor eIF-4A of yeast and mouse. Mol. Gen. Genet. *237*, 233-240.

Dorrello, N.V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E., and Pagano, M. (2006). S6K1-and ßTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science *314*, 467-471.

Duffy, J.B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis *34*, 1-15.

Duncan, D.M., Burgess, E.A., and Duncan, I.J.G. (1998). Control of distal antennal identity and tarsal development in *Drosophila* by spineless–aristapedia, a homolog of the mammalian dioxin receptor. Genes Dev. *12*, 1290-1303.

Duncan, R., Milburn, S.C., and Hershey, J.W. (1987). Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F. J. Biol. Chem. *262*, 380-388.

Dyson, A.D. (2017). RNA-Processing genes control sensory neuron function in *Drosophila melanogaster* ("Masters Thesis" Appalachian State University).

Efendiev, R., Bavencoffe, A., Hu, H.Z., Zhu, M.X., and Dessauer, C.W. (2013). Scaffolding by A-Kinase Anchoring Protein Enhances Functional Coupling between Adenylyl Cyclase and TRPV1 Channel. J. Biol. Chem. *288*, 3929-3937.

Elfakess, R., and Dikstein, R. (2008). A translation initiation element specific to mRNAs with very short 5′ UTR that also regulates transcription. PLoS One *3*, e3094.

Fein, A. (2012). Nociceptors and the perception of pain. Uni. of Connecticut Health Ctr. *4*, 61-67.

Ferreira, S.H., Lorenzetti, B.B., and De Campos, D.I. (1990). Induction, blockade and restoration of a persistent hypersensitive state. Pain *42*, 365-371.

Fields, H. (1987). Analgesic drugs. Pain *1*, 272.

Fischer, J.A., Giniger, E., Maniatis, T., and Ptashne, M.J.N. (1988). GAL4 activates transcription in *Drosophila*. Nature *332*, 853.

Flynn, A., and Proud, C.G. (1995). Serine 209, not serine 53, is the major site of phosphorylation in initiation factor eIF-4E in serum-treated Chinese hamster ovary cells. J. Biol. Chem. *270*, 21684-21688.

Follansbee, T.L., Gjelsvik, K.J., Brann, C.L., McParland, A.L., Longhurst, C.A., Galko, M.J., and Ganter, G.K. (2017). *Drosophila* nociceptive sensitization requires BMP signaling via the canonical SMAD pathway. J. Neurosci. *37* 3458-3416.

Fukunaga, R., and Hunter, T. (1997). MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J. *16*, 1921-1933.

Gao, F.B., Brenman, J.E., Jan, L.Y., and Jan, Y.N. (1999). Genes regulating dendritic outgrowth, branching, and routing in Drosophila. Genes Dev. *13*, 2549-2561.

Gao, X., and Pan, D. (2001). TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev. *15*, 1383-1392.

Garcia, D., and Shaw, R.J. (2017). AMPK: Mechanisms of cellular energy sensing and restoration of metabolic balance. Mol. Cell *66*, 789-800.

Gingras, A.C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. *68*, 913-963.

Gold, M.S., and Gebhart, G.F. (2010). Nociceptor sensitization in pain pathogenesis. Nat. Med. *16*, 1248-1257.

Gradi, A., Imataka, H., Svitkin, Y.V., Rom, E., Raught, B., Morino, S., and Sonenberg, N. (1998). A novel functional human eukaryotic translation initiation factor 4G. Mol. Cell Biol. *18*, 334-342.

Grifo, J.A., Tahara, S.M., Morgan, M.A., Shatkin, A.J., and Merrick, W.C. (1983). New initiation factor activity required for globin mRNA translation. J. Biol. Chem. *258*, 5804-5810.

Grueber, W.B., Jan, L.Y., and Jan, Y.N. (2003a). Different levels of the homeodomain protein cut regulate distinct dendrite branching patterns of *Drosophila* multidendritic neurons. Cell *112*, 805-818.

Grueber, W.B., Ye, B., Moore, A.W., Jan, L.Y., and Jan, Y.N. (2003b). Dendrites of distinct classes of Drosophila sensory neurons show different capacities for homotypic repulsion. Curr. Biol. *13*, 618-626.

Grüner, S., Peter, D., Weber, R., Wohlbold, L., Chung, M.-Y., Weichenrieder, O., Valkov, E., Igreja, C., and Izaurralde, E. (2016). The structures of eIF4E-eIF4G complexes reveal an extended interface to regulate translation initiation. Mol. Cell *64*, 467-479.

Haghighat, A., and Sonenberg, N. (1997). eIF4G dramatically enhances the binding of eIF4E to the mRNA 5′-cap structure. J. Biol. Chem. *272*, 21677-21680.

Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995). Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. EMBO J. *14*, 5701-5709.

Haghighat, A., Svitkin, Y., Novoa, I., Kuechler, E., Skern, T., and Sonenberg, N. (1996). The eIF4G-eIF4E complex is the target for direct cleavage by the rhinovirus 2A proteinase. J. Virol. *70*, 8444-8450.

Hammond, S.M., Caudy, A.A., and Hannon, G.J. (2001). Post-transcriptional gene silencing by double-stranded RNA. Nat. Rev. Genet. *2*, 110.

Harris, T.E., Chi, A., Shabanowitz, J., Hunt, D.F., Rhoads, R.E., and Lawrence, J.C. (2006). mTOR-dependent stimulation of the association of eIF4G and eIF3 by insulin. EMBO J. *25*, 1659-1668.

Hellen, C.U., and Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev. *15*, 1593-1612.

Herman, J.A., Willits, A.B., and Bellemer, A. (2018). Gαq and Phospholipase Cβ signaling regulate nociceptor sensitivity in Drosophila melanogaster larvae. Peer J. *6*, e5632.

Hernández, G., and Sierra, J. (1995). Translation initiation factor eIF-4E from *Drosophila*: cDNA sequence and expression of the gene. Gene Struc. Exp. *1261*, 427-431.

Hernández, G., and Vazquez-Pianzola, P. (2005). Functional diversity of the eukaryotic translation initiation factors belonging to eIF4 families. Mech. Dev. *122*, 865-876.

Hernández, G., del Mar Castellano, M., Agudo, M., and Sierra, J.M. (1998). Isolation and characterization of the cDNA and the gene for eukaryotic translation initiation factor 4G from Drosophila melanogaster. Eur. J. Biochem. *253*, 27-35.

Hernández, G., Miron, M., Han, H., Liu, N., Magescas, J., Tettweiler, G., Frank, F., Siddiqui, N., Sonenberg, N., and Lasko, P. (2013). Mextli is a novel eukaryotic translation initiation factor 4E-binding protein that promotes translation in Drosophila melanogaster. Mol. Cell Biol. *33*, 2854-2864.

Hershey, J., Mathews, M., and Sonenberg, N. (1996). Translational Control. Cold Spr. Har. Lab. Press.

Hershey, J.W., and Merrick, W.C. (2000). 2 The Pathway and Mechanism of Initiation of Protein Synthesis. Cold Spr. Har. Mon. Arch. *39*, 33-88.

Hilbert, M., Kebbel, F., Gubaev, A., and Klostermeier, D. (2010). eIF4G stimulates the activity of the DEAD box protein eIF4A by a conformational guidance mechanism. Nucleic Acids Res. *39*, 2260-2270.

Hinnebusch, A.G. (2014). The scanning mechanism of eukaryotic translation initiation. Annu. Rev. Biochem. *83*, 779-812.

Hornig, B., and Drexler, H. (1997). Endothelial function and bradykinin in humans. Drugs *54*, 42-47.

Hoyer, N., Petersen, M., Tenedini, F., and Soba, P. (2018). Assaying mechanonociceptive behavior in *Drosophila* larvae. Bio-Protoc. *8*, 4.

Hu, S., Fambrough, D., Atashi, J.R., Goodman, C.S., and Crews, S.T. (1995). The Drosophila Abrupt Gene Encodes a Btb-Zinc Finger Regulatory Protein That Controls the Specificity of Neuromuscular Connections. Genes Dev. *9*, 2936-2948.

Hucho, T., and Levine, J.D. (2007). Signaling pathways in sensitization: toward a nociceptor cell biology. Neuron *55*, 365-376.

Hughes, D.L., Dever, T.E., and Merrick, W.C. (1993). Further biochemical characterization of rabbit reticulocyte eIF-4B. Arch. Biochem. & Biophy. *301*, 311-319.

Hundsdoerfer, P., Thoma, C., and Hentze, M.W. (2005). Eukaryotic translation initiation factor 4GI and p97 promote cellular internal ribosome entry sequence-driven translation. Proc. Natl. Acad. Sci. U. S. A. *102*, 13421-13426.

Hwang, R.Y., Zhong, L., Xu, Y., Johnson, T., Zhang, F., Deisseroth, K., and Tracey, W.D. (2007). Nociceptive neurons protect Drosophila larvae from parasitoid wasps. Curr. Biol. *17*, 2105-2116.

Hwang, R.Y., Stearns, N.A., and Tracey, W.D. (2012). The ankyrin repeat domain of the TRPA Protein Painless is important for thermal nociception but not mechanical nociception. PLoS One *7*, e30090.

Im, S.H., Takle, K., Jo, J., Babcock, D.T., Ma, Z., Xiang, Y., and Galko, M. (2015). Tachykinin acts upstream of autocrine Hedgehog signaling during nociceptive sensitization in *Drosophila*. Elife *4*, e10735.

Imataka, H., Olsen, H.S., and Sonenberg, N. (1997). A new translational regulator with homology to eukaryotic translation initiation factor 4G. EMBO J. *16*, 817-825.

Inoki, K., Zhu, T., and Guan, K.L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.

Jackson, R.J., Hellen, C.U., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nat. Rev. Mol. Cell Biol. *11*, 113.

Jang, W., Baek, M., Han, Y.S., and Kim, C. (2018). Duox mediates ultraviolet injury-induced nociceptive sensitization in Drosophila larvae. Mol. Brain. *11*, 16.

Ji, R.R., Baba, H., Brenner, G.J., and Woolf, C.J. (1999). Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat. Neurosci *2*, 1114-1119.

Ji, R.R., Samad, T.A., Jin, S.X., Schmoll, R., and Woolf, C.J. (2002). p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. Neuron *36*, 57-68.

Jimenez-Diaz, L., Geranton, S.M., Passmore, G.M., Leith, J.L., Fisher, A.S., Berliocchi, L., Sivasubramaniam, A.K., Sheasby, A., Lumb, B.M., and Hunt, S.P. (2008). Local translation in primary afferent fibers regulates nociception. PLoS One *3*, e1961.

Jin, S.X., Zhuang, Z.Y., Woolf, C.J., and Ji, R.R. (2003). P38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. J. Neurosci. *23*, 4017-4022.

Jivotovskaya, A.V., Valášek, L., Hinnebusch, A.G., and Nielsen, K.H. (2006). Eukaryotic translation initiation factor 3 (eIF3) and eIF2 can promote mRNA binding to 40S subunits independently of eIF4G in yeast. Mol. Cell Biol. *26*, 1355-1372.

Jordt, S.E., Bautista, D.M., Chuang, H.H., McKemy, D.D., Zygmunt, P.M., Hogestatt, E.D., Meng, I.D., and Julius, D. (2004). Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature *427*, 260-265.

Jung, H., Gkogkas, C.G., Sonenberg, N., and Holt, C.E. (2014). Remote control of gene function by local translation. Cell *157*, 26-40.

Junger, H., and Sorkin, L.S. (2000). Nociceptive and inflammatory effects of subcutaneous TNFα. Pain *85*, 145-151.

Kang, K., Pulver, S.R., Panzano, V.C., Chang, E.C., Griffith, L.C., Theobald, D.L., and Garrity, P.A. (2010). Analysis of Drosophila TRPA1 reveals an ancient origin for human chemical nociception. Nature *464*, 597-600.

Kassuya, C.A., Ferreira, J., Claudino, R.F., and Calixto, J.B. (2007). Intraplantar PGE2 causes nociceptive behaviour and mechanical allodynia: the role of prostanoid E receptors and protein kinases. J. Pharmacol. *150*, 727-737.

Khoutorsky, A., Sorge, R.E., Prager-Khoutorsky, M., Pawlowski, S.A., Longo, G., Jafarnejad, S.M., Tahmasebi, S., Martin, L.J., Pitcher, M.H., Gkogkas, C.G., and Sharif-Naeni., R. (2016). eIF2alpha phosphorylation controls thermal nociception. Proc Natl Acad Sci U S A *113*, 11949-11954.

Kieft, J.S., Zhou, K., Jubin, R., Murray, M.G., Lau, J.Y., and Doudna, J.A. (1999). The hepatitis C virus internal ribosome entry site adopts an ion-dependent tertiary fold. J. Mol. Biol. *292*, 513-529.

Kim, M.D., Jan, L.Y., and Jan, Y.N. (2006). The bHLH-PAS protein Spineless is necessary for the diversification of dendrite morphology of Drosophila dendritic arborization neurons. Genes Dev. *20*, 2806-2819.

Kim, S.E., Coste, B., Chadha, A., Cook, B., and Patapoutian, A. (2012). The role of Drosophila Piezo in mechanical nociception. Nature *483*, 209-212.

Kozak, M. (1991). Effects of long 5′ leader sequences on initiation by eukaryotic ribosomes in vitro. Gene Express. *1*, 117-125.

Lachance, P.E.D., Miron, M., Raught, B., Sonenberg, N., and Lasko, P. (2002). Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth. Mol. Cell Biol. *22*, 1656-1663.

Lee, A.S., Kranzusch, P.J., Doudna, J.A., and Cate, J.H. (2016). eIF3d is an mRNA cap-binding protein that is required for specialized translation initiation. Nature *536*, 96.

Li, Q. (2002). Eukaryotic Translation Initiation Factor EIF4AIII is Functionally Distinct from EIF4AI and EIF4AII.

Li, Q., Imataka, H., Morino, S., Rogers, G.W., Richter-Cook, N.J., Merrick, W.C., and Sonenberg, N. (1999). Eukaryotic translation initiation factor 4AIII (eIF4AIII) is functionally distinct from eIF4AI and eIF4AII. Mol. & Cellul. Biol. *19*, 7336-7346.

Li, W., Wang, F., Menut, L., and Gao, F.B. (2004). BTB/POZ-zinc finger protein abrupt suppresses dendritic branching in a neuronal subtype-specific and dosage-dependent manner. Neuron *43*, 823-834.

Liberman, N., Gandin, V., Svitkin, Y.V., David, M., Virgili, G., Jaramillo, M., Holcik, M., Nagar, B., Kimchi, A., and Sonenberg, N. (2015). DAP5 associates with eIF2β and eIF4AI to promote Internal Ribosome Entry Site driven translation. Nucleic Acids Res. *43*, 3764-3775.

Loeser, J.D., and Treede, R.D. (2008). The Kyoto protocol of IASP Basic Pain Terminology. Pain *137*, 473-477.

Manning, D.C., Raja, S.N., Meyer, R.A., and Campbell, J.N. (1991). Pain and hyperalgesia after intradermal injection of bradykinin in humans. Clin. Pharmacol. Ther. *50*, 721-729.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. (1997). Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell *89*, 951-961.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. (1999). Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. Mol. Cell *3*, 707-716.

Mathews, M.B. (1996). Origins and targets of translational control. Cold Spr. Har. Mon. Arch., *30* 1-29.

Mathivanan, S., Devesa, I., Changeux, J.P., and Ferrer-Montiel, A. (2016). Bradykinin Induces TRPV1 Exocytotic Recruitment in Peptidergic Nociceptors. Front. Pharmacol. *7*, 178.

McMahon, S.B., Bennett, D.L., Priestley, J.V., and Shelton, D.L. (1995). The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. Nat. Med. *1*, 774-780.

Megat, S., Ray, P., Moy, J., Lou, T.-F., Barragan-Iglesias, P., Li, Y., Pradhan, G., Wangzhou, A., Ahmad, A., and North, R. (2018). Nociceptor translational profiling reveals the RagA-mTORC1 network as a critical generator of neuropathic pain. Cold Spr. Har. Mon. Arch., *10* 336784.

Meijering, E., Jacob, M., Sarria, J.C., Steiner, P., Hirling, H., and Unser, M. (2004). Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cyto. A: J. Int. Soc. Analyt. Cyto. *58*, 167-176.

Melemedjian, O.K., Asiedu, M.N., Tillu, D.V., Peebles, K.A., Yan, J., Ertz, N., Dussor, G.O., and Price, T.J. (2010). IL-6- and NGF-induced rapid control of protein synthesis and nociceptive plasticity via convergent signaling to the eIF4F complex. J. Neurosci. *30*, 15113-15123.

Merrick, W.C., and Pavitt, G.D. (2018). Protein synthesis initiation in eukaryotic cells. Cold Spr. Har. Perspec. Biol. *10*, a033092.

Meyer, K.D., Patil, D.P., Zhou, J., Zinoviev, A., Skabkin, M.A., Elemento, O., Pestova, T.V., Qian, S.-B., and Jaffrey, S.R. (2015). 5′ UTR m6A promotes cap-independent translation. Cell *163*, 999-1010.

Moriyama, T., Higashi, T., Togashi, K., Iida, T., Segi, E., Sugimoto, Y., Tominaga, T., Narumiya, S., and Tominaga, M. (2005). Sensitization of TRPV1 by EP 1 and IP reveals peripheral nociceptive mechanism of prostaglandins. J. Mol. Pain *1*, 3.

Moy, J., Ray, P., Walters, E., Pelletier, J., and Price, T. (2016). Exploring the potential for an evolutionarily conserved role for eIF4A in pain plasticity. J. Pain *17*, S61.

Moy, J.K., Khoutorsky, A., Asiedu, M.N., Black, B.J., Kuhn, J.L., Barragan-Iglesias, P., Megat, S., Burton, M.D., Burgos-Vega, C.C., Melemedjian, O.K., and Boitano, S. (2017). The MNK-eIF4E signaling axis contributes to injury-induced nociceptive plasticity and the development of chronic pain. J. Neurosci. *37*, 7481-7499.

Nanda, J.S., Cheung, Y.-N., Takacs, J.E., Martin-Marcos, P., Saini, A.K., Hinnebusch, A.G., and Lorsch, J.R. (2009). eIF1 controls multiple steps in start codon recognition during eukaryotic translation initiation. J. Mol. Biol. *394*, 268-285.

Nevins, T.A., Harder, Z.M., Korneluk, R.G., and Holčík, M. (2003). Distinct regulation of internal ribosome entry site-mediated translation following cellular stress is mediated by apoptotic fragments of eIF4G translation initiation factor family members eIF4GI and p97/DAP5/NAT1. J. Biol. Chem. *278*, 3572-3579.

Nielsen, P.J., and Trachsel, H. (1988). The mouse protein synthesis initiation factor 4A gene family includes two related functional genes which are differentially expressed. EMBO J. *7*, 2097-2105.

Oriel, C., and Lasko, P. (2018). Recent Developments in Using Drosophila as a Model for Human Genetic Disease. Int. J. Mol. Sci. *19*, 2041.

Osterloh, J.M., and Freeman, M.R. (2009). Neuronal death or dismemberment mediated by Sox14. Nat. Neurosci. *12*, 1479-1480.

Pamudurti, N.R., Bartok, O., Jens, M., Ashwal-Fluss, R., Stottmeister, C., Ruhe, L., Hanan, M., Wyler, E., Perez-Hernandez, D., and Ramberger, E. (2017). Translation of circRNAs. Mol. Cell *66*, 9-21. e27.

Pandey, U.B., and Nichols, C.D. (2011). Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. Pharm. Rev. *63*, 411-436.

Park, E.-H., Zhang, F., Warringer, J., Sunnerhagen, P., and Hinnebusch, A.G. (2011). Depletion of eIF4G from yeast cells narrows the range of translational efficiencies genome-wide. Genomics *12*, 68.

Parrish, J.Z., Emoto, K., Jan, L.Y., and Jan, Y.N. (2007). Polycomb genes interact with the tumor suppressor genes hippo and warts in the maintenance of Drosophila sensory neuron dendrites. Genes Dev. *21*, 956-972.

Parrish, J.Z., Kim, M.D., Jan, L.Y., and Jan, Y.N. (2006). Genome-wide analyses identify transcription factors required for proper morphogenesis of Drosophila sensory neuron dendrites. Genes Dev. *20*, 820-835.

Pause, A., Belsham, G.J., Gingras, A.C., Donze, O., Lin, T.A., Lawrence, J.C., Jr., and Sonenberg, N. (1994). Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature *371*, 762-767.

Pestova, T.V., Lorsch, J.R., and Hellen, C.U. (2007). The mechanism of translation initiation in eukaryotes. Cold Spr. Har. Mon. Arch. *48*, 87.

Pinho-Ribeiro, F.A., Verri Jr, W.A., and Chiu, I.M. (2017). Nociceptor sensory neuron–immune interactions in pain and inflammation. Trends in Immunol. *38*, 5-19.

Prober, D.A., Zimmerman, S., Myers, B.R., McDermott, B.M., Kim, S.H., Caron, S., Rihel, J., Solnica-Krezel, L., Julius, D., and Hudspeth, A.J. (2008). Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function. J. Neurosci. *28*, 10102-10110.

Raffioni, S., and Bradshaw, R.A. (1992). Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. Proc. Natl. Acad. Sci. U. S. A. *89*, 9121-9125.

Ramírez-Valle, F., Braunstein, S., Zavadil, J., Formenti, S.C., and Schneider, R.J. (2008). eIF4GI links nutrient sensing by mTOR to cell proliferation and inhibition of autophagy. J. Cell Biol. *181*, 293-307.

Raught, B., and Gingras, A.C. (1999). eIF4E activity is regulated at multiple levels. Int. J. Biochem. & Cell Biol. *31*, 43-57.

Ray, B.K., Lawson, T.G., Kramer, J., Cladaras, M., Grifo, J., Abramson, R., Merrick, W., and Thach, R.E. (1985). ATP-dependent unwinding of messenger RNA structure by eukaryotic initiation factors. J. Biol. Chem. *260*, 7651-7658.

Reiter, L.T., Potocki, L., Chien, S., Gribskov, M., and Bier, E. (2001). A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res. *11*, 1114-1125.

Ricciotti, E., and FitzGerald, G.A. (2011). Prostaglandins and inflammation. Arterioscler Thromb. Vasc. Biol. *31*, 986-1000.

Roberts, R., Zhang, J., Mayberry, L.K., Tatineni, S., Browning, K.S., and Rakotondrafara, A.M. (2015). A unique 5′ translation element discovered in triticum mosaic virus. J Virol. *89*, 12427-12440.

Rode, S., Ohm, H., Anhäuser, L., Wagner, M., Rosing, M., Deng, X., Sin, O., Leidel, S.A., Storkebaum, E., and Rentmeister, A. (2018). Differential Requirement for Translation Initiation Factor Pathways during Ecdysone-Dependent Neuronal Remodeling in *Drosophila*. Cell Rep. *24*, 2287-2299. e2284.

Rozen, F., Edery, I., Meerovitch, K., Dever, T., Merrick, W., Sonenberg, N. (1990). Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Mol. Cell Biol. *10*, 1134-1144.

Saito, S., Nakatsuka, K., Takahashi, K., Fukuta, N., Imagawa, T., Ohta, T., and Tominaga, M. (2012). Analysis of Transient Receptor Potential Ankyrin 1 (TRPA1) in Frogs and Lizards Illuminates Both Nociceptive Heat and Chemical Sensitivities and Coexpression with TRP Vanilloid 1 (TRPV1) in Ancestral Vertebrates. J. Biol. Chem. *287*, 30743-30754.

Sammons, M.J., Raval, P., Davey, P.T., Rogers, D., Parsons, A.A., and Bingham, S. (2000). Carrageenan-induced thermal hyperalgesia in the mouse: role of nerve growth factor and the mitogen-activated protein kinase pathway. Brain Res. *876*, 48-54.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Scheper, G.C., and Proud, C.G. (2002). Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? Eur. J. Biochem. *269*, 5350-5359.

Scheper, G.C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D.J., and Proud, C.G. (2002). Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J. Biol. Chem. *277*, 3303-3309.

Schnizler, K., Shutov, L.P., Van Kanegan, M.J., Merrill, M.A., Nichols, B., McKnight, G.S., Strack, S., Hell, J.W., and Usachev, Y.M. (2008). Protein kinase A anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and prostaglandin E2 in mouse sensory neurons. J. Neurosci. *28*, 4904-4917.

Schütz, P., Bumann, M., Oberholzer, A.E., Bieniossek, C., Trachsel, H., Altmann, M., and Baumann, U. (2008). Crystal structure of the yeast eIF4A-eIF4G complex: An RNA-helicase controlled by protein–protein interactions. Proc. Natl. Acad. Sci. U. S. A. *105*, 9564-9569.

Shahbazian, D., Roux, P.P., Mieulet, V., Cohen, M.S., Raught, B., Taunton, J., Hershey, J.W., Blenis, J., Pende, M., and Sonenberg, N. (2006). The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. EMBO J. *25*, 2781-2791.

Shatsky, I.N., Dmitriev, S.E., Terenin, I.M., and Andreev, D. (2010). Cap-and IRES-independent scanning mechanism of translation initiation as an alternative to the concept of cellular IRESs. Molec. & Cells *30*, 285-293.

Shu, X., and Mendell, L.M. (1999). Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neurosci. Lett. *274*, 159-162.

Shu, X., and Mendell, L.M. (2001). Acute sensitization by NGF of the response of small-diameter sensory neurons to capsaicin. J. Neurophysiol. *86*, 2931-2938.

Sinvani, H., Haimov, O., Svitkin, Y., Sonenberg, N., Tamarkin-Ben-Harush, A., Viollet, B., and Dikstein, R. (2015). Translational tolerance of mitochondrial genes to metabolic

energy stress involves TISU and eIF1-eIF4GI cooperation in start codon selection. Cell. Met. *21*, 479-492.

Sonenberg, N. (2008). eIF4E, the mRNA cap-binding protein: from basic discovery to translational research. Biochem. Cell Biol. *86*, 178-183.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell *136*, 731-745.

Sonenberg, N., and Shatkin, A. (1977). Reovirus mRNA can be covalently crosslinked via the 5'cap to proteins in initiation complexes. Proc. Natl. Acad. Sci. U. S. A. *74*, 4288-4292.

Sonenberg, N., Rupprecht, K.M., Hecht, S.M., and Shatkin, A.J. (1979). Eukaryotic mRNA cap binding protein: purification by affinity chromatography on sepharose-coupled m7GDP. Proc. Natl. Acad. Sci. U. S. A. *76*, 4345-4349.

Sorkin, L.S., Xiao, W.H., Wagner, R., and Myers, R.R. (1997). Tumour necrosis factor-alpha induces ectopic activity in nociceptive primary afferent fibres. Neuroscience *81*, 255-262.

Spriggs, K.A., Stoneley, M., Bushell, M., and Willis, A.E. (2008). Re-programming of translation following cell stress allows IRES-mediated translation to predominate. Biol. Cell *100*, 27-38.

Srivastava, S., Potla, S., Torck, A., Zhang, M., Dussor, G., Ray, P., and Price, T. (2016). (278) A potential role for eIF4A in regulation of nociceptor plasticity: a motif-based genome-wide search for eIF4A targets. J. Pain *17*, S45.

Story, G.M., Peier, A.M., Reeve, A.J., Eid, S.R., Mosbacher, J., Hricik, T.R., Earley, T.J., Hergarden, A.C., Andersson, D.A., and Hwang, S.W. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell *112*, 819-829.

Sudo, K., Takahashi, E., and Nakamura, Y. (1995). Isolation and mapping of the human EIF4A2 gene homologous to the murine protein synthesis initiation factor 4A-II gene Eif4a2. Cytogen. Gen. Res. *71*, 385-388.

Sugimura, K., Yamamoto, M., Niwa, R., Satoh, D., Goto, S., Taniguchi, M., Hayashi, S., and Uemura, T. (2003). Distinct developmental modes and lesion-induced reactions of dendrites of two classes of Drosophila sensory neurons. J. Neurosci. *23*, 3752-3760.

Svitkin, Y.V., Pause, A., Haghighat, A., Pyronnet, S., Witherell, G., Belsham, G.J., and Sonenberg, N. (2001). The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5′ secondary structure. RNA *7*, 382-394.

Tarun Jr, S.Z., and Sachs, A.B. (1996). Association of the yeast poly (A) tail binding protein with translation initiation factor eIF-4G. EMBO J. *15*, 7168-7177.

Tarun, S.Z., Wells, S.E., Deardorff, J.A., and Sachs, A.B. (1997). Translation initiation factor eIF4G mediates in vitro poly (A) tail-dependent translation. Proc. Natl. Acad. Sci. U. S. A. *94*, 9046-9051.

Thomas, A., Lee, P.J., Dalton, J.E., Nomie, K.J., Stoica, L., Costa-Mattioli, M., Chang, P., Nuzhdin, S., Arbeitman, M.N., and Dierick, H.A. (2012). A versatile method for cell-specific profiling of translated mRNAs in *Drosophila*. PloS one *7*, e40276.

Tohda, C., Sasaki, M., Konemura, T., Sasamura, T., Itoh, M., and Kuraishi, Y. (2001). Axonal transport of VR1 capsaicin receptor mRNA in primary afferents and its participation in inflammation-induced increase in capsaicin sensitivity. J. Neurochemi. *76*, 1628-1635.

Tracey, W.D., Jr., Wilson, R.I., Laurent, G., and Benzer, S. (2003). Painless, a *Drosophila* gene essential for nociception. Cell *113*, 261-273.

Treede, R.-D., Rief, W., Barke, A., Aziz, Q., Bennett, M.I., Benoliel, R., Cohen, M., Evers, S., Finnerup, N.B., and First, M.B. (2015). A classification of chronic pain for ICD-11. Pain *156*, 1003.

Vetter, M.L., Martin-Zanca, D., Parada, L.F., Bishop, J.M., and Kaplan, D.R. (1991). Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene. Proc. Natl. Acad. Sci. U. S. A. *88*, 5650-5654.

Villa-Cuesta, E., Sage, B.T., and Tatar, M. (2010). A role for *Drosophila* dFoxO and dFoxO 5′ UTR internal ribosomal entry sites during fasting. PloS one *5*, e11521.

Viswanath, V., Story, G.M., Peier, A.M., Petrus, M.J., Hwang, S.W., Patapoutian, A., and Jegla, T. (2003). Ion channels - Opposite thermosensor in fruitfly and mouse. Nature *423*, 822-823.

von der Haar, T., Gross, J.D., Wagner, G., and McCarthy, J.E. (2004). The mRNA cap-binding protein eIF4E in post-transcriptional gene expression. Nat. Struc. & Mol. Biol. *11*, 503.

Wangler, M.F., Yamamoto, S., and Bellen, H.J. (2015). Fruit flies in biomedical research. Genetics *199*, 639-653.

Watkins, L.R., Milligan, E.D., and Maier, S.F. (2001). Spinal cord glia: new players in pain. Pain *93*, 201-205.

Weary, D.M., Niel, L., Flower, F.C., and Fraser, D. (2006). Identifying and preventing pain in animals. Appl. Anim. Behav. Sci. *100*, 64-76.

Webster, N., Green, S., Tasset, D., Ponglikitmongkol, M., and Chambon, P. (1989). The transcriptional activation function located in the hormone-binding domain of the human oestrogen receptor is not encoded in a single exon. EMBO J. *8*, 1441-1446.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. *79*, 143-180.

Williams-Hill, D., Duncan, R., Nielsen, P., and Tahara, S. (1997). Differential expression of the murine eukaryotic translation initiation factor isogenes eIF4AIand eIF4AIIIs dependent upon cellular growth status. Arch. Biochem. Biophy. *338*, 111-120.

Williamson, D.L., Kubica, N., Kimball, S. R., & Jefferson, L. S. (2006). Exercise-induced alterations in extracellular signal-regulated kinase 1/2 and mammalian target of rapamycin (mTOR) signalling to regulatory mechanisms of mRNA translation in mouse muscle. J. Physiol. *573*, 497-510.

Wolfe, A.L., Singh, K., Zhong, Y., Drewe, P., Rajasekhar, V.K., Sanghvi, V.R., Mavrakis, K.J., Jiang, M., Roderick, J.E., and Van der Meulen, J. (2014). RNA G-quadruplexes cause eIF4A-dependent oncogene translation in cancer. Nature *513*, 65.

Wong, J.J.L., Li, S., Lim, E.K.H., Wang, Y., Wang, C., Zhang, H., Kirilly, D., Wu, C., Liou, Y.-C., and Wang, H. (2013). A Cullin1-based SCF E3 ubiquitin ligase targets the InR/PI3K/TOR pathway to regulate neuronal pruning. PLoS Biol. *11*, e1001657.

Woolf, C.J., and Ma, Q.J.N. (2007). Nociceptors—noxious stimulus detectors. Neuron *55*, 353-364.

Woolf, C.J., Allchorne, A., Safieh-Garabedian, B., and Poole, S. (1997). Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. J. Pharmacol. *121*, 417-424.

Yoder-Hill, J., Pause, A., Sonenberg, N., and Merrick, W. (1993). The p46 subunit of eukaryotic initiation factor (eIF)-4F exchanges with eIF-4A. J. Biol. Chem. *268*, 5566-5573.

Zan-Kowalczewska, M., Bretner, M., Sierakowska, H., Szczesna, E., Filipowicz, W., and Shatkin, A.J. (1977). Removal of 5'-terminal m7G from eukaryotic mRNAs by potato nucleotide pyrophosphatase and its effect on translation. Nucleic Acids Res. *4*, 3065-3081.

Zhong, L., Bellemer, A., Yan, H., Ken, H., Jessica, R., Hwang, R.Y., Pitt, G.S., and Tracey, W.D. (2012). Thermosensory and nonthermosensory isoforms of *Drosophila melanogaster* TRPA1 reveal heat-sensor domains of a thermoTRP Channel. Cell Rep. *1*, 43-55.

Zhong, L.X., Hwang, R.Y., and Tracey, W.D. (2010). Pickpocket Is a DEG/ENaC Protein Required for Mechanical Nociception in Drosophila Larvae. Curr. Biol. *20*, 429-434.

Zoll, W.L., Horton, L.E., Komar, A.A., Hensold, J.O., and Merrick, W.C. (2002). Characterization of mammalian eIF2A and identification of the yeast homolog. J. Biol. Chem. *277*, 37079-37087.

Zwart, M.F., Randlett, O., Evers, J.F., and Landgraf, M. (2013). Dendritic growth gated by a steroid hormone receptor underlies increases in activity in the developing Drosophila locomotor system. Proc. Natl. Acad. Sci. U. S. A. *110*, E3878-E3887.

Appendix

eIF4E-BP results

To examine whether eIF4E-BP is involved in larval nociception processing, I used a tissue-specific RNAi approach making use of Gal4/UAS system available in *Drosophila*. I tested third instar larvae with nociceptor-specific *eIF4E-BP* knockdown for defects in thermal and mechanical nociception. I found that there was a significant difference in the two negative controls that were used: the Gal4-only negative control and UAS-only negative control. I also wanted to determine if larvae also sensitize to noxious heat after tissue damage and find that *eIF4E-BP* knockdown does show sensitized phenotypes in the thermal hypersensitization assay.

For our thermal behavioral experiment, I used the *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression in two eIF4E-BP RNAi line *BDSC 9147 UAS-eIF4E-BP-RNAi* line and *BDSC 36815 UAS-eIF4E-BP-RNAi* line. Both the RNAi lines were tested for defects in thermal nociception. I found that there was a significant difference in the two negative controls used ($p<0.022$). These results are graphically presented in Figure 27. Thus the data gives inconclusive results for the thermal assay. The same results are to be reported for the mechanical assay. The two negative controls used were significantly different ($p<0.020$). These results are graphically presented in Figure 28. This makes the data from the mechanical assay inconclusive as well.

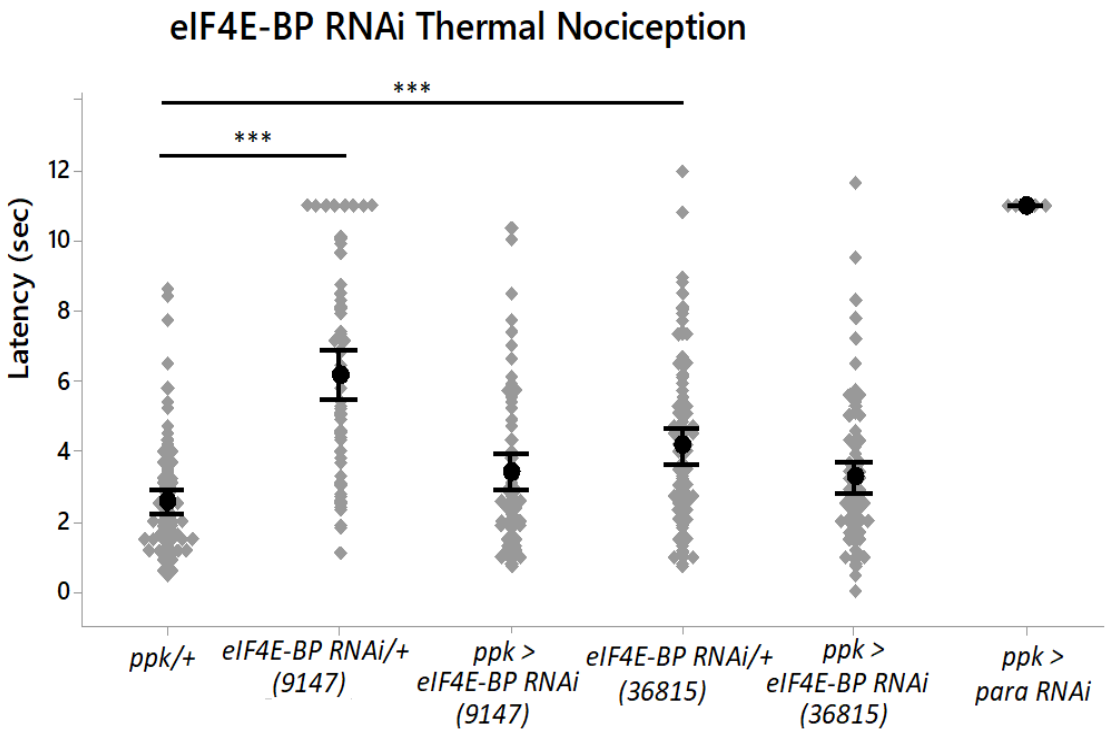

Figure 27: Nociceptor-specific knockdown of eIF4E-BP is inconclusive in thermal nociception.

*Larvae with nociceptor-specific knockdown of para was used as positive control because of its impaired nociceptive responses. Latency response to noxious thermal stimulus (46°C) for both the Gal4-only control and the UAS-only control was significantly different. Response latencies of individual animals are plotted as points on the graph. The mean for each genotype is indicated with the error bars. (n≥50 for all groups; ***p ≤ 0:001 by non-parametric Mann Whitney Test.*

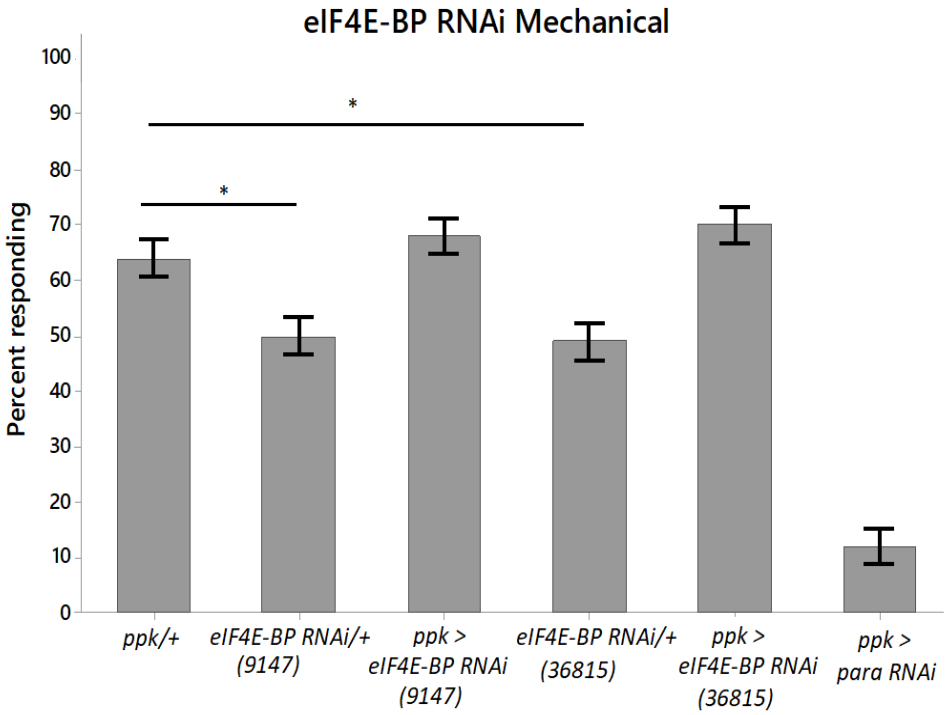

Figure 28: Nociceptor-specific knockdown of eIF4E-BP is inconclusive in mechanical nociception.

*Larvae with nociceptor-specific knockdown of para showed a very low rate of nociceptive responses and were used as a positive control. A significant proportion of larvae exhibited nociceptive responses to a noxious mechanical stimulus between Gal4-only control larvae (64%) and the UAS -only control larvae (50% - eIF4E-BP RNAi (9147); 49% - eIF4E-BP RNAi (36815)) (n≥100 for all groups; Chi-Square Test, *p≤0.025 by Chi Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.*

eIF4E-BP knockdown larvae sensitization is increased after tissue damage by UV irradiation

We tested *eIF4E-BP* knockdown larvae in thermal hypersensitization assay. We used the *ppk-Gal4; UAS-dicer2* line to drive nociceptor-specific expression in two eIF4E-BP RNAi lines, *BDSC 9147 UAS-eIF4E-BP-RNAi* and *BDSC 36815 UAS-eIF4E-BP-RNAi*. We used UV irradiation to damage the dorsal epidermis and then allowed sham treated or irradiated larvae to recover for 8 hours at 25°C before testing for changes in thermal nociception at 42°C. The latencies were recorded and there was a significant change in the response latency of the UV-irradiated *eIF4E-BP* knockdown larvae (58% by 10 seconds) relative to the sham-treated *eIF4E-BP* knockdown larvae (19% by 10 seconds) (Figure 15). This indicates that UV-induced tissue damage does increase the behavioral response threshold of UV-treated larvae in the *eIF4E-BP* knockdown by exhibiting a lowering of their nociceptive threshold.

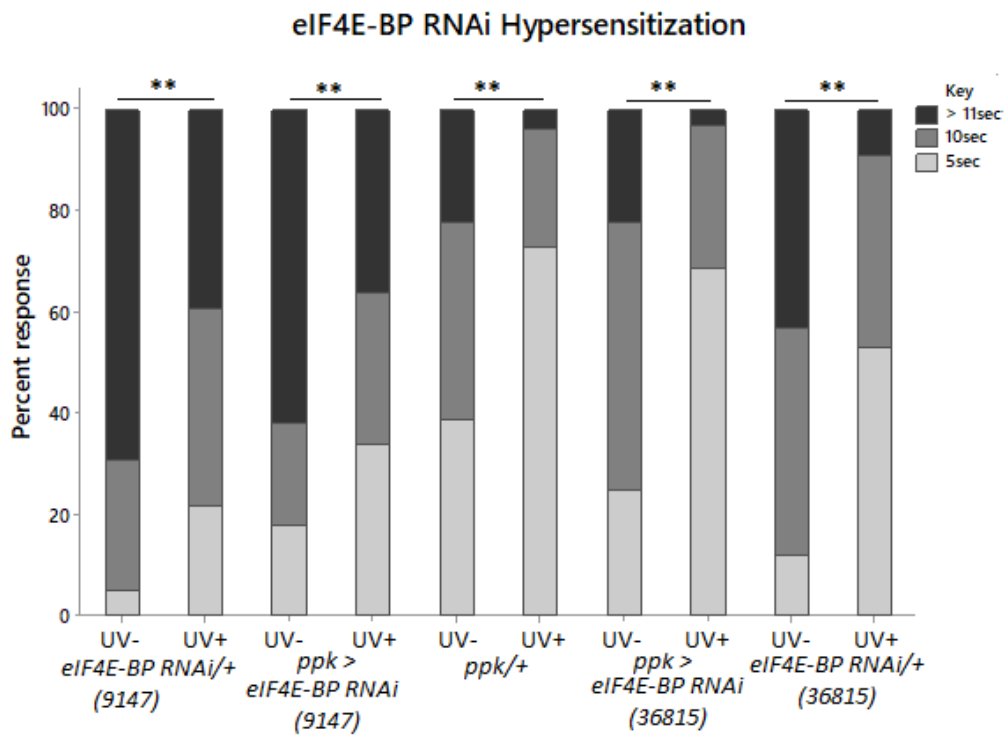

Figure 29: Nociceptor sensitization is increased when eIF4E-Bp is knocked down post-UV injury.

*Following UV exposure (+) or no UV exposure (-), larvae were assayed to noxious thermal stimulus (42°C) after 8hrs. Response latencies were recorded and categorized as follows: Light grey ≤ 5sec; Dark grey ≤ 10sec; Black ≥ 11sec. Larvae with nociceptor-specific knockdown of eIF4E-BP when tested for UV sensitization latency response to noxious thermal stimulus (42°C) after 8hrs was significant. Both the Gal4-only control and the UAS-only control showed significant UV sensitized response. (n=40; **p≤0.05 by non-parametric Mann Whitney Test)*

eIF4E-BP not required for nociceptor dendrite morphogenesis

To see the effect of eIF4E-BP on nociceptor morphology we analyzed the morphology of class IV multidendritic neurons of *eIF4E-BP* knockdown larvae by quantifying the dendritic sum intersection and the average dendritic intersection using Sholl analysis. To determine the function in nociception of eIF4E-BP arising from developmental defects, the expression of GFP and *eIF4E-BP* knockdown in class IV multidendritic neurons was driven by ppk-Gal4 to express mCD8::GFP and *eIF4E-BP* knockdown. Confocal imaging of the ligated third instar larvae was done to quantify the dendritic arbor. We found that *eIF4E-BP* knockdown (*BDSC 36815 RNAi* line; n= 10) did not significantly affect the dendritic sum intersection, nor did it affect the average dendritic intersection of class IV multidendritic neurons when compared to no-RNAi control (n=10) (Figure 16). The results of the Sholl analysis are tabulated in Table 6. These data suggest that *eIF4E-BP* knockdown does not produce gross morphological changes in the class IV multidendritic neurons.

Table 6: Sholl analysis statistics for *eIF4E-BP* knockdown class IV neurons

Genotype	**Sum dendritic intersections**	**Average dendritic intersection**
ppk Gal4 > mCD8::GFP, eIF4E-BP RNAi	35603	21
ppk Gal4 > mCD8::GFP, y v attp2, y +	36692	24
p value	0.651	0.171

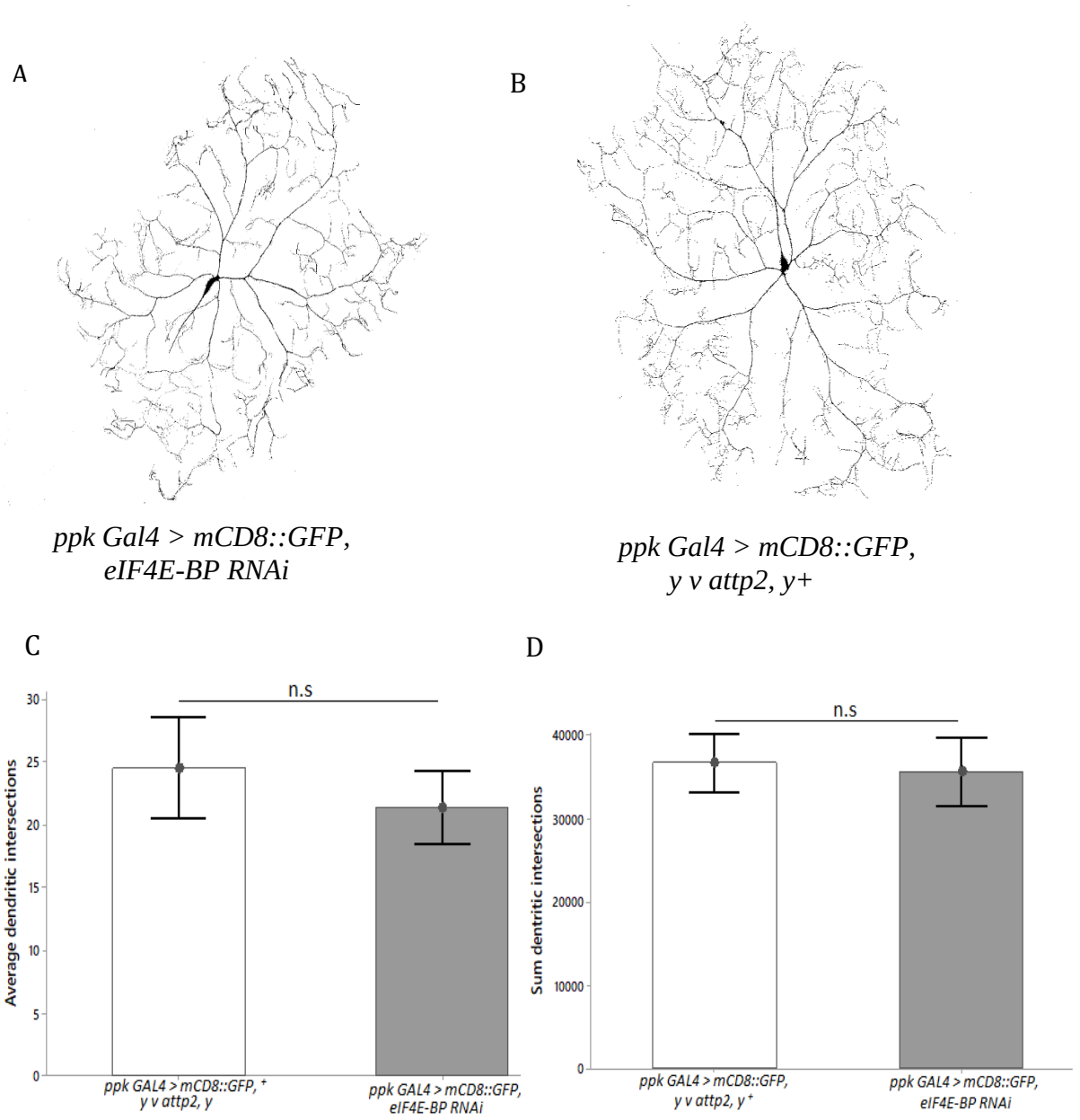

Figure 30: Nociceptor specific knock-down of eIF4E-BP does not affect class IV multidendritic neuron morphology.

A) Representative micrographs displaying the dendritic arborization of class IV multidendritic neurons expressing GFP protein in the eIF4E-BP RNAi; B) Representative micrograph of wild-type class IV multidendritic morphology; C) Sholl analysis of average dendritic intersections of eIF4E-BP RNAi class IV multidendritic neurons was statistically not significant from wild-type (n= 10, Students t-test, p= 0.171); D) Sholl analysis of sum dendritic intersections of eIF4E-BP RNAi class IV multidendritic neurons was statistically not significant from wild-type (n= 10; Students t-test, p= 0.651).

Removed eIF4E-BP function does not affect the gross morphology of dendrites.

There was no significant defect in the dendritic morphology of *eIF4E-BP* knockdown class IV neurons. As the eIF4E-BP protein functions in cap-dependent translation initiation and since multiple cap-independent translation mechanisms might exist in class IV multidendritic neurons, some morphogenesis may simply be not eIF4E-BP-dependent. We would need more information from the nociception sensitivity assay to make a more affirmative conclusion.

Conclusion

As there were significant differences between the Gal4 only controls and UAS-only controls in nociception the results from the two RNAi lines were deemed inconclusive.

Vita

Gita Gajjar was born in Mombasa, Kenya, to Arvind and Urmila Gajjar. She enrolled to Maseno University to study Biomed, and in August 2007 she was awarded the Bachelor of Science degree. In the fall of 2017, she accepted a research assistantship in Biology department at Appalachian State University and began study toward a Master of Science degree. The M.S. was awarded in July 2019. In August 2019, Gita commenced work towards her Ph.D. with the Department of Biochemistry & Molecular Biology at East Carolina University.

Gita is an international student and resides in Greenville, N.C. as she does science.