RNA-PROCESSING GENES CONTROL SENSORY NEURON FUNCTION IN *DROSOPHILA MELANOGASTER*

A Thesis
by
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Abstract

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Chronic pain affects approximately 100 million Americans and generates costs of up to $600 billion per year, according to the Institute of Medicine. Characterization of molecular signaling pathways in sensory neurons is an important step toward development of more effective clinical interventions. The goal of this study was to identify genes involved in regulating the function of class IV multidendritic neurons (nociceptors) integral to the detection of thermal, mechanical, and photic stimuli. We used *Drosophila melanogaster* larvae as a model organism, which exhibit a distinct and quantifiable response to noxious stimuli termed nocifensive escape locomotion (NEL). Recent research has found that the transcripts of key genes necessary for nociceptor function are alternatively spliced, indicating the dependence of function on expression patterns. We systematically knocked down putative RNA-processing genes with a previously identified role in dendrite development and/or alternative splicing by crossing a *ppk-GAL4;UAS-dicer2* fly strain with RNAi lines targeting genes of interest. Using *ppk* as our driver limits expression to nociceptors and *dicer2* promotes increased expression of RNAi transcripts. We then tested progeny for changes in nocifensive response latency relative to wild-type larvae using a thermal nociception assay. Twenty-five genes were identified for which knockdown resulted in either

a significant increase or decrease in response latency, indicating a potential defect. Follow-up assays validated seven genes with an insensitive phenotype and two with a hypersensitive phenotype. Additionally, our results have confirmed that behavioral defects do not correlate with defects in dendrite morphology. Finally, a complex role for translation initiation factors was revealed, suggesting the potential for a nociception-specific and possibly IRES-mediated translation mechanism involving eIFG2 and eIF4E3.

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Dedication

To my children, Connor, Matt, and Ben. You are always my inspiration, my motivation, and the best aspect of my life. Thank you for being willing to share your mom with graduate studies. Your support, patience, and love helped to make this achievement possible.

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Introduction

The Affordable Care Act (2010) included a directive that the Department of Health and Human Services enlist the Institute of Medicine (IOM) in examining the problem of clinical pain (Institute of Medicine, 2011). In their report, the IOM detailed the associated costs and compelling need for improvement in treatment options and explicitly stated the necessity of prioritizing pain-related research. The report also addressed the serious complications and consequences resulting from the broad application of opioids in treating pain. Developing the new classes of pain medication recommended by the IOM will require significant advancement in our understanding of the molecular signaling pathways involved in nociception. Nociception refers to the ability to perceive noxious stimuli, which encompasses all types of stimuli that provoke a pain response, indicating to an organism that an aspect of the environment is dangerous (Bessou and Perl, 1969). Nociceptors are the specific class of sensory neurons integral to sensing pain (Sherrington, 1906).

Drosophila melanogaster serve as an excellent model organism for studying this aspect of the nervous system for several reasons, including similarity of sensory neuron structure and function, conservation of genes, and ease of testing. The multidendritic (md) neurons of the *Drosophila* peripheral nervous system include the dendritic arborization (da) neurons, the dendrites of which spread throughout the epidermis between the epithelium and muscle. *Drosophila* md-da neurons are divided into four groups based on type-specific dendrite morphology, designated Class I, II, III, and IV, which are arranged in a specific design repeated in each abdominal hemisegment (Jan and Jan, 2010). Class IV neurons are polymodal nociceptors (Hwang et al., 2007; Tracey et al., 2003). The stereotyped patterning across segments makes any defects in dendrite morphology readily apparent. From a

behavioral perspective, *Drosophila* perform a unique and specific "rolling" behavior when noxious stimuli are perceived, termed nocifensive escape locomotion (NEL), which gives researchers a distinct marker for obtaining quantifiable data to measure response to noxious stimuli (Hwang et al., 2007; Tracey et al., 2003).

Additionally, a large proportion of genes with a role in the *Drosophila* nervous system encode proteins that are well conserved in vertebrates. For example, *TrpA1* encodes proteins that form ion channels found in class IV neurons that are necessary for nociception function. The transient receptor potential (TRP) family of proteins is conserved with similar functionality in vertebrates, including humans, making *Drosophila* an effective model organism for pain-related research (Zhong et al., 2012). Finally, *Drosophila* are highly amenable to genetic manipulation. Using the UAS/GAL4 system with RNAi for targeted genes allows for effective knockdown of function. A variety of promoters can be used to limit the expression of RNAi to specific cells. For instance, using the *ppk-GAL4* transgene limits expression of RNAi solely to nociceptors (Zhong et al., 2010).

Previous research has established that proper nociception function is affected by alternative splicing of relevant gene transcripts (Hulse et al., 2014; Jiang et al., 2013; Nakae et al., 2013; Thackeray and Ganetzky, 1994; Zhong et al., 2012), and that genes necessary for dendrite development are alternatively spliced (Jan and Jan, 2010), but little is known about possible splicing regulatory proteins involved. Regulation could be occurring at one or more points during the many steps of mRNA processing and subsequent translation, including capping, polyadenylation, alternative splicing, and translation initiation. RNA-processing genes play a variety of important roles throughout these steps, and studying ways in which

NEL behavior is affected by these mechanisms could illuminate the roles of RNA processing in sensory neurons.

The Role of mRNA Processing in Protein Availability and Cell Function

In eukaryotic organisms, any given cell's identity and specific functions are dependent on the gene expression profile unique to a specific cell or tissue type. The first step in characterizing this profile is identifying which genes are transcribed. Of equal importance to shaping a cell's identity are the abundance and specific function of resulting proteins, both of which are controlled to a great extent by additional steps taken with transcripts that produce a mature mRNA strand. The three stages of mRNA processing are 5' capping, alternative splicing, and 3' polyadenylation.

It is important to note that these modifications occur in conjunction with transcription rather than after transcription is complete and that all mRNA molecules are ready for translation prior to export from the nucleus (Bentley, 2005). The RNA polymerase II protein (RNAPII) includes a C-terminal domain (CTD) forming a "tail" that carries proteins and enzymes used to modify pre-mRNA, an association that facilitates the interaction of processing enzymes and proteins with the RNA and increases the efficiency of these modification reactions as the new transcript is synthesized. Each of the three processing stages requires a different assembly of enzymes and proteins. The phosphorylation state of the CTD changes to reflect the processing stage taking place, which serves a regulatory role in recruitment of the appropriate factors for each stage (Phatnani and Greenleaf, 2006). Identification of cell-specific factors affecting variations in mRNA processing and subsequent impact on cell function begins with understanding the role and mechanisms of these modifications.

Capping is a form of mRNA modification that contributes to control of expression levels through facilitation of translation. Soon after transcription of a new pre-mRNA strand has begun, the 5' end of the nascent strand is capped through removal of a phosphate and addition of a methylated guanine nucleotide. Three enzymes associated with the CTD (a phosphatase, a guanyl transferase, and a methyl transferase) are positioned to enable capping as soon as the 5' end exits RNAPII. Many different types of RNA are produced in the nucleus and this cap both identifies mRNA molecules and serves as a binding site for proteins that export mRNA from the nucleus for translation (Hashimoto and Green, 1976; Moss and Koczot, 1976). The 5' cap is also a recognition site for translation initiation factors, along with the 3' poly-A tail described below, helping to ensure that only complete and intact mRNA strands are translated into proteins (Gross et al., 2003; Marcotrigiano et al., 1999).

Polyadenylation helps determine the longevity of a transcript within the cell and interacts with important proteins during translation. This process takes place at the 3' end as transcription of the strand is finishing. The CTD of RNAPII functions to facilitate the process in a manner similar to 5' capping. Two proteins, CstF (cleavage stimulation factor) and CPSF (cleavage and polyadenylation specificity factor) move from the CTD to the mRNA strand once it exhibits the appropriate sequence. CstF and CPSF bind to this sequence along with additional cleavage factors. Synthesis of the poly-A tail is then performed by the enzyme poly-A polymerase (PAP), which ligates adenine nucleotides to the 3' end of mRNA. This process does not require a template; therefore, the length of the poly-A tail for any given transcript is not encoded in its associated gene. Instead, the length is determined at least in part by poly(A)-binding protein (PAB), which forms a complex during RNA processing with CPSF, PAP, and the mRNA strand. The length of the tail is variable between transcripts and

developmental stages, and the mechanism for length determination is not well understood (Colgan and Manley, 1997; Proudfoot, 2004). All mRNA molecules are degraded over time in the cell's cytoplasm, so the length of the poly-A tail helps determine how long the transcript will be available for translation, thereby directly affecting expression levels of the resulting protein (Kornfeld et al., 1989).

Alternative splicing is a modification that helps to shape cell function by enabling the cell to derive a variety of unique gene products from a single gene, all with distinctly different functions. This process involves picking and choosing which coding regions to include (exons) and usually eliminating noncoding regions (introns) from the length of the mRNA strand. The vast majority of eukaryotic genes are alternatively spliced to some degree, and most proteins are the product of alternative splicing rather than straightforward translation of entire gene transcripts. Alternative splicing allows the production of a diverse range of necessary proteins with differing functions specific to the differential inclusion/exclusion of exons and the resulting amino acid composition they code for (Black, 2003; Chow et al., 1977).

One example of an alternatively spliced gene is *slob*, which encodes the protein that binds to and regulates the SLOWPOKE calcium-activated potassium channel. Isoforms SLOB57 and SLOB71 have opposite effects on the potassium channel, depolarizing and polarizing respectively. Additionally, neurons in the *Drosophila* brain express SLOB57, whereas motor neurons in the neuromuscular junction express SLOB71. Alternatively spliced isoforms of the single gene, *slob*, are thus a key component of the differing function of these two neuron types (Jepson et al., 2013).

Many molecular variables contribute to the process of alternative splicing, which is performed by a group of RNA molecules collectively termed the spliceosome. The primary components of this assembly are five small nuclear ribonucleoproteins (snRNPs), each of which is formed by a small nuclear RNA (snRNA) in complex with protein subunits. Each intron on the pre-mRNA strand has a splicing site sequence near each end as well as a branch point, which is the site where the cleaved 5' end of the intron will be bound, producing a lariat shape (Black, 2003; Staley and Guthrie, 1998).

The basic steps of a splicing event to remove an intron begin with the U1 snRNP recognizing the 5' splice site via base pairing while the branch-point binding protein (BBP) and U2 auxilliary factor (U2AF) do the same at the branch-point site. The U2 snRNP takes the place of the BBP and U2AF, forming its own base pairings with the pre-mRNA. This recognition redundancy improves the accuracy of appropriate site selection. U2 binding to the branch-point site causes a single adenine to become unpaired and activated. Then, the "triple snRNP" U4/U6-U5 becomes part of the reaction. The U4 and U6 snRNPs are bound tightly via base pair interactions, whereas U5 is more loosely associated. When the triple snRNP joins the complex, RNA-RNA rearrangements take place that result in U6 breaking away from U4 and replacing U1 at the 5' splicing site, the exit of U1 and U4, the formation of the lariat structure, and cleavage at the 5' site (Black, 2003; Staley and Guthrie, 1998).

These conformational changes create the spliceosome's active catalytic sites and maneuver the pre-mRNA substrate into the appropriate position for the first of two phosphoryl-transferase reactions (Staley and Guthrie, 1998). In this first reaction, U6 binds to U2 and the unpaired adenine binds to the 5' splice site. Additional RNA-RNA interactions now bring the two exons close together for the second phosphoryl-transferase reaction in

which cleavage occurs at the 3’ site, the intron lariat is released for degradation, and the two exons are joined (Black, 2003; Ruskin et al., 1984).

The existence of a splice site consensus sequence alone does not guarantee that the spliceosome will activate and carry out splicing. The most straightforward method of choosing sites is exon definition, in which components of the spliceosome (typically SR proteins) assemble along exons and serve as markers at each 5’ and 3’ splicing site, which recruits U1 snRNP and U2AF, triggering splicing of neighboring introns. In this case, both marking and recruitment are co-transcriptional but the actual splicing events can take place after transcription is complete. This approach is effective because the smaller and more uniform exons have more easily identified boundaries than introns, although it’s unclear how SR proteins are able to differentiate between the two. Enhancer sequences specific to these proteins may play a role (Black, 2003).

Other factors can also play a role in whether a potential splicing event takes place, as well as the specifics of how it is carried out. Many introns include more than one suitable splicing sequence, for instance, and any of these may be the one chosen. Regulatory sequences distinct from splicing sites can be found in both introns and exons and can function as enhancers that stimulate the spliceosome or silencers that repress its activity. Most of these sequences are protein binding sites but some function by forming secondary structures to expose or conceal a potential splice site. Little has been conclusively determined regarding the regulation of these potential factors and sequence data alone is not enough to predict activation of the spliceosome in response to a splicing site (Black, 2003; Matsui et al., 1980).

The structure and composition of the spliceosome is as highly conserved as the process of splicing itself, and a high degree of similarity between *Drosophila* and humans has been shown (Herold et al., 2009). Using mass spectrometry to analyze components of spliceosomal complexes, Herold and colleagues identified more than 120 proteins that are recruited to the spliceosome as snRNP subunits or that serve an accessory role to the process. The need for numerous subunit and accessory proteins is expected when considering the number of variable mechanisms used during splicing to render alternative isoforms, such as multiple 5'- or 3'-splice sites, selection of alternative exons, and the occasional retention of introns (Black, 2003).

The most dramatic example of the importance of alternative splicing in sensory neurons is the gene *Dscam*, a homolog of human *DSCAM*. Dendrites of individual md-da neurons do not cross paths, nor do dendrites of the same class; however, dendrites of different classes show significant overlap, which results in complete tiling of the body wall (Grueber et al., 2002). Mechanisms that control self-avoidance, type-avoidance, and tiling have been the subject of numerous studies (Jan and Jan, 2010). *Dscam* encodes a growth cone receptor in developing neurons that recognizes guidance signals and adjusts the cytoskeleton of the growing axon accordingly. Observed splicing patterns indicate more than 38,000 possible isoforms yielding different proteins, which is more than double the total number of genes in the *Drosophila* genome (Schmucker et al., 2000). Additionally, the uniqueness of these isoforms plays an integral role in the self-avoidance necessary for proper dendrite morphology, forming cell-specific rather than type-specific identities. Expression patterns appear to be generated randomly and are dynamic; expression can adjust as needed to further reduce the risk of sharing isoforms with a nearby neuron (Miura et al., 2013).

Although the pattern a cell will express is apparently chosen at random, the assembly of that pattern is not. The *Dscam* gene has 115 exons. The 95 exons that are alternatively spliced are arranged in four clusters containing 12, 48, 33, and 2 variable exons. Any given transcript will have the ten exons that are not alternatively spliced and then a single exon from each cluster. This system of organized splicing variables is how the gene is able to produce so many unique isoforms (Park and Gravely, 2007).

Expression patterns of *Dscam* isoforms indicate the crucial role of alternative splicing in the ability of neurons to receive and transmit information, and md-da neurons rely on specific isoforms of other proteins as well. The *para* gene encodes proteins for voltage-gated sodium channels necessary for firing action potentials, which neurons require in order to transmit their signal. This gene has thirteen alternatively spliced sites (Park et al., 2004).

Para expression patterns appear to be developmentally regulated, given that eleven isoforms have been identified in embryos compared to eighteen found in adults, and expression rates for several exons shift up or down between embryonic and adult stages (Thackeray and Ganetzky, 1994). For instance, exon f was found present in more than 80% of embryonic clones and less than 7% of adult clones, suggesting there are functional differences in the resulting channels. This possibility is further supported by their identification of isoforms containing either exon c or d but not both. The specific differences in amino acid composition indicate a possible change in the voltage dependence of the gating between the isoforms, creating channels of varying sensitivity.

Isoforms of another highly conserved protein, *Drosophila* TRPA1, have also been found to serve crucial functions in md-da neurons (Zhong et al., 2012). Transient receptor potential (TRP) channels have a well-established role in numerous sensory systems. In their

study, Zhong and colleagues analyzed expression patterns of dTRPA1 in md-da neurons, as well as impact on the ability of Class IV neurons to serve their function as nociceptors, i.e. neurons responsible for detecting noxious stimuli in the environment and prompting an evasive response. The four isoforms evaluated were found to have variable temperature thresholds for heat activation, as well as variable responses to thermal vs. mechanical stimuli. Ability to perceive noxious stimuli (i.e. nociception) was highly dependent on appropriate isoform expression, providing yet another example of the necessity of alternative splicing for survival.

RNA processing plays a key role in shaping cell function. 5' capping and 3' polyadenylation are necessary for ensuring translation produces functional proteins by signaling intact transcripts. These modifications are also primary factors in controlling protein abundance within a cell. Alternative splicing determines which isoform is produced by transcription and, therefore, the specific function of proteins resulting from processed mRNA. As with the transcription process, understanding modification processes, how they are regulated, and the nature of the cell factors involved is a vital aspect of characterizing molecular pathways of interest.

Mechanisms of Translation Initiation in Eukaryotic Cells

Like transcription and mRNA processing, translation of mRNA to functional proteins is fundamental to development of the gene expression profile that shapes the identity and function of any given cell type. Named for their roles in initiating translation, eukaryotic initiation factors (eIFs) guide the process and aid in protecting the integrity of resulting proteins. Although numerous accessory eIFs are involved, the primary factors are eIF4A, eIF4E, and eIF4G, which collectively form the eIF4F complex. As the rate-limiting step of

translation, initiation is an important focal point for regulation of protein production. Translation is regulated primarily by binding proteins that target eIF4F components, controlling activation and repression of these initiation factors (Gingras et al., 1999a). However, a variety of other mechanisms of regulation have been identified, the diversity of which reveal the complexity and versatility of translation control (Jackson et al., 2010). Characterizing the function and regulation of initiation factors is crucial for understanding the nuances of cell-specific gene expression.

eIF activity begins prior to translation as different factors bind to initiator tRNAs, mRNAs, and 40S ribosomal subunits, forming stable molecules ready for the interaction that initiates translation. Met-tRNA$_i^{Met}$ refers to the specific Methionine-bound initiator tRNA that binds to the AUG start codon during translation and includes a sequence recognized by initiation factors. When bound with GTP and eIF2, Met-tRNA$_i^{Met}$ forms a ternary complex ready for acceptance by the 40S subunit (Benne and Hershey, 1978). eIF1, eIF1A, and eIF3 all combine with a 40S subunit, preventing premature binding with the 80S subunit and enabling acceptance of the Met-tRNA$_i^{Met}$ ternary complex to create the 43S complex (Hunter et al., 1977; Majumdar, 2003).

The eIF4F complex of three protein subunits interacts with mRNA molecules, activating them for translation (Benne and Hershey, 1978; Abramson et al., 1987). eIF4E binds to the 5’ 7-methylguanosine cap and eIF4G is a scaffold protein that recognizes the poly(A)-tail of the mRNA strand (Maroto and Sierra, 1989; Tarun and Sachs, 1996). The functions of these two subunits ensure that only intact mRNA molecules will be translated. The third subunit, eIF4A, is an RNA helicase that functions to unwind the secondary

structure of mRNA, enabling accessibility to the ribosome (Abramson et al., 1987; Dorn et al., 1993).

The remaining steps of translation initiation can then take place. The eIF4F-bound mRNA, in conjunction with eIF4B, binds to the 43S complex to create the complete 43S preinitiation complex (Trachsel et al., 1977). The complex proceeds to scan the mRNA in search of an AUG codon flanked by nucleotide sequences that identify a start site for translation (Kozak, 1983). Recognition of the appropriate site requires eIF1 and eIF1A, which discriminate against non-AUG codons and unfavorable AUG codons (Pestova et al., 1998; Pisarev et al., 2006). As the 43S complex binds at the chosen start site, conformational changes occur that displace eIF1 and allow eIF5-mediated hydrolysis of eIF2-bound GTP, forming the "closed" 48S initiation complex (Unbehaun et al., 2004). eIF5B can then mediate the process of joining the 60S ribosomal subunit, as well as releasing factors eIF-1, -3, -4B, -4F, and -5 (Pestova et al., 2000; Unbehaun et al., 2004). In the final step of initiation, GTP hydrolysis by eIF5B enables its release (now GDP-bound) along with eIF1A (Pestova et al., 2000). Initiation is then complete and the elongation-capable 80S ribosome can proceed with translation.

Initiation factors are well conserved across species. The genes encoding each of the eIFs described above have been identified in the genome of *Drosophila* and show a significant similarity to vertebrate orthologs, except eIF4B (Lasko, 2000); however, eIF4B in *Drosophila* has been characterized and was confirmed to function similarly to vertebrate orthologs. It does lack two features found in human EIF4B: the ability to interact with PABP and involvement with IRES-mediated translation (Hernandez et al., 2004b).

As a key component of translation function, eIFs are a common target for regulatory mechanisms of translation initiation. Regulating translation is a crucial component of a cell's ability to adapt to changing conditions and developmental stages. Control of gene expression at this level (as opposed to transcription) allows for rapid yet reversible responses to maintain homeostasis, and targeting the rate-limiting step of initiation maximizes efficiency. The specificities of response necessary are situation-dependent. For instance, acute and transient changes to intracellular conditions require rapid responses that can reverse as soon as conditions have adjusted. On the other hand, shifting between developmental stages and associated expression profiles may call for responses that are rapid and then stabilize indefinitely as the new steady state. To accommodate a variety of situations that require a change in translation, several modes of interactions differing in specificity are employed in regulatory pathways.

One mechanism for regulating translation is phosphorylation. For instance, translation initiation is inhibited when eIF2 or eIF2B subunits are phosphorylated by protein kinases. Given that these factors are necessary for effective cap-dependent translation, this nonspecific method results in global translation reduction throughout the cell. This translation repression mechanism is activated primarily as a reaction to stress that signals the need to conserve resources. Four identified eIF2 kinases function in response to specific situations: deprivation of heme in reticulocytes activates HRI; viral infection, heat, and UV radiation activate PKR; endoplasmic reticulum (ER) stress and/or accumulation of unfolded proteins activate PERK; and amino acid depletion activates GCN2 (Farrell et al., 1977; Harding et al., 2000; Proud, 2005).

When stress prompts a cell to shut down cap-dependent translation, non-translating mRNAs form an RNP complex called a stress granule (SG). mRNAs that were interrupted during the process of initiation are recruited to the SG, along with their bound initiation factors, 40S subunit, and poly(A)-binding protein. Numerous proteins and RNA-binding proteins that function in silencing, stabilizing, or metabolizing mRNA also join this complex. Displaced mRNAs are sorted and targeted for decay or stabilization. SGs disperse once the cell has recovered (Anderson and Kedersha, 2008).

In *Drosophila*, heat shock induces changes in phosphorylation states of eIF4E (decreased) and eIF2α (increased), which is similar to effects observed in mammalian cells but to a much lesser degree. Nonetheless, notable stress response is apparent, manifesting as significant global repression of cap-dependent translation and simultaneous activation of preferential cap-independent translation of heat shock proteins (Anthony and Merrick, 1991; Duncan et al., 1995; Hernandez et al., 2004a). Additionally, phosphorylation of eIF2α in reaction to endoplasmic reticulum stress has been confirmed in *Drosophila* (Williams et al., 2001), as well as the formation of stress granules that include the two *Drosophila* eIF2α kinases, GCN2 and PEK (Farny et al., 2009).

An alternative and less generalized role of phosphorylation is mediated by proteins that bind to eIF4E (4E-BPs). The *Drosophila* genome includes seven genes that encode eIF4E variants, three of which are alternatively spliced, as well as three genes encoding 4E-BPs (*Thor, cup*, and *mextli*), all alternatively spliced (Attrill et al., 2016). Given multiple genes encoding different isoforms of both eIF4E and 4E-BPs, employing the latter as intermediaries allows for more selectivity by limiting the isoform(s) targeted (Hernandez et al., 2005). In an active state, 4E-BPs compete with eIF4G, phosphorylating any eIF4E

proteins to which they bind and rendering them inaccessible for binding with eIF4G, thereby preventing formation of the eIF4F complex (Mader et al., 1995). Translation can be upregulated in the cell by phosphorylation of 4E-BPs, rendering these proteins incapable of binding to eIF4E and freeing the initiation factor for binding with eIF4G (Gingras et al., 1999b). Some mRNAs have a long and structured 5' UTR, rendering them particularly dependent on eIF4E availability for translation and therefore highly regulated by mechanisms of translational control (Richter and Sonenberg, 2005). This is due to a second function of eIF4E, which is the stimulation of eIF4A helicase activity to unwind and restructure these more complicated 5' UTRs. Increased availability of eIF4E is particularly important for translation of this pool of mRNAs (Feoktistova et al., 2013).

Regulation of Initiation Factor Activity

RNA-binding proteins (RBPs) provide a highly selective means of regulating eIF activity due to specificity of binding sequences and are employed in a variety of mechanisms targeting different regions of mRNA. One potential albeit less common target region is the 5' UTR. The most prominent example of regulation through 5' UTR-protein interaction is iron regulatory protein (IRP), which inhibits translation of ferrin and eALAS mRNAs. Ferrin takes up excess iron from a cell for storage and eALAS uses iron for heme biosynthesis. When iron levels in a cell drop too low, IRP binds to a stem-loop structure in the 5' region of both mRNAs known as the iron responsive element (IRE),.When IRP binds to IRE, the resulting structure allows binding of eIF4F but prevents recruitment of the 43S pre-initiation complex thereby preserving iron. Binding eIF4F may be for the purpose of quickly reinitiating translation of these mRNAs when iron levels rise (Gray and Hentze, 1994; Muckenthaler et al., 1998).

Regulation via poly(A)-tail-protein interaction occurs via just one mechanism, which functions to stimulate translation rather than inhibiting the process. Poly(A)-binding protein (PABP) serves as a bridge between the mRNA tail and eIF4G bound to eIF4E at the 5' cap, creating a circular loop of mRNA with both ends tethered to eIF4F. This interaction increases mRNA stability, reducing the risk of losing recruited eIF4F and subsequent need to compete for a replacement. PABP also enhances recruitment of ribosomal subunits, giving their bound mRNAs a significant advantage over mRNAs lacking PABP (Imataka et al., 1998; Kahvejian et al., 2005). A recent study has also identified other binding sites for PABP in addition to the poly(A)-tail, indicating the potential for involvement in numerous gene regulatory pathways and a greater diversity of function than previously supposed (Kini et al., 2016).

The vast majority of identified RBP-mediated initiation control mechanisms function via 3' UTR-protein interactions. The generic model of this interaction involves a protein specific to a binding site in the 3' UTR of the target mRNA, a cap-binding protein, and a third bridging protein binding all three into a closed loop with the mRNA that prevents eIF4F from binding to the 5' cap. This inactive form of mRNA must be derepressed before it can be activated for translation by eIF4F. The protein that binds to the 3' UTR is the only one requiring recognition of a specific sequence so its identity depends on the target mRNA. The bridging and cap-binding proteins are more versatile and can function in more than one pathway. For example, the eIF4E-binding protein Cup in *Drosophila* (homologous to human 4E-T) functions in two regulatory pathways that rely on 3' UTR-protein interactions. In both pathways, Cup serves as the bridging protein. Its ability to bind with eIF4E and inactivate the initiation factor through phosphorylation provides the necessary cap-binding component. During oogenesis, Cup binds with recognition protein Bruno, which binds to a 3' UTR

sequence on *oskar* transcripts, repressing translation until later stages (Nakamura et al., 2004). During embryogenesis, Cup binds with Smaug, which is the recognition protein for repression of unlocalized *nanos* transcripts (Nelson et al., 2004).

miRNAs also utilize the 3' UTR, targeting their complementary mRNAs for repression and ultimately decay. When bound with Argonaute1 (Ago1), miRNAs form the RNA-induced silencing complex (RISC). Scaffolding protein GW182 is also recruited to the target mRNA. RISC silences mRNAs by forcing dissociation of eIF4A and preventing formation of the eIF4F complex necessary for translation. RISC inhibits eIF4A independently of GW182, but its recruitment enables the additional dissociation of eIF4E and subsequent deadenylation and decay of the silenced mRNA (Fukaya et al., 2014). miRNAs bind to the 3' UTR of target mRNA through base pairing with complementary sequences, indicating the high degree of specificity intrinsic to this mechanism, although wobble base-pairing can interfere to some degree. miRNA targets and effects on overall expression levels remain difficult to predict due to the large number of variables involved, e.g. relative levels of miRNA and mRNA, potential introduction of exogenous miRNA, factors specific to developmental stage or tissue type, etc. (Doench and Sharp, 2004).

An additional mechanism has been identified in which decapping proteins involved in the mRNA degradation pathway also function in general repression of translation. Once translation is complete, exiting mRNAs accumulate into mRNPs with other mRNAs targeted for degradation. These mRNP aggregation locations within the cytoplasm are known as P bodies and are very similar to the stress granules described previously. Proteins functioning in the mRNA decay pathway are also present, including decapping activator proteins Dhh1p (*S. cerevisiae*; ortholog of human RCK/p54 and *Drosophila* Me31b) and Pat1p (*S. cerevisiae*;

ortholog of human Pat1b and *Drosophila* Patr-1). Each mRNA exiting translation is either targeted for decapping and degradation or recruited into the P body. Growth of P bodies is associated with increased global repression of translation, but it is unclear how individual mRNA fate is decided or how the size of the P body at any given time is determined. Additionally, Dhh1p, Rck/p54, and Me31b all play an active role in repression of translation. The repressive function of P bodies and associated decapping activators is in constant competition with that of translation machinery, each side keeping the other in check (Coller and Parker, 2005; Nakamura et al., 2001).

Regulation of translation initiation carries important implications for the nervous system. Due to the nature of neuroplasticity, the nervous system is particularly dependent on spatial and temporal control of gene expression. Translational control allows for the rapid and reversible changes necessary for all aspects of nervous system function (Costa-Mattioli et al., 2009). Nociceptor function is dependent on plasticity and therefore mediated by translation control (Woolf and Ma, 2007; Jimenez-Diaz et al., 2008). Elucidating these pathways is crucial to understanding how nociceptors function and improving methods for treatment of pain. It has also been established that intraneuronal differential translation control is a factor, i.e. regulation specific to the cell soma, dendrites, or axons rather than the cell as a whole (Price and Géranton, 2009).

In humans, Interleukin-6 (IL-6) and nerve growth factor (NGF) are primary mediators of acute and chronic pain, modulating translation by initiating signaling cascades along two different pathways. IL-6 signals travel the ERK/Mnk pathway and culminate in phosphorylation of eIF4E. NGF signals via the mTOR pathway to phosphorylate 4EBP and eIF4G. As described previously, phosphorylation of 4EBP renders it inactive and frees eIF4E

to bind with eIF4G, enabling formation of the eIF4F complex. Increased availability of eIF4E also preferentially increases translation of eIF4E-dependent mRNAs. Signals initiated by IL-6 and NGF converge from different pathways to activate all components of eIF4F and initiate translation in the axons of primary afferent neurons. Acute and chronic pain conditions are often associated with manifestation of symptoms at sites distal to ganglia, which is in accordance with the axonal expression resulting from initiation of translation by IL-6 and NGF signaling. Additionally, the mechanical sensitization observed depends on translation of mRNAs already present, confirming the role of translation control in nociceptive plasticity (Melemedjian et al., 2010).

An antagonistic mechanism to this sensitization functions in conjunction with neuronal P bodies, which have characteristics unique to sensory neurons. To decrease sensitivity, AMPK activators reduce mechanical allodynia induced by IL-6 and NGF and downregulate cap-dependent translation. Also, the translation inhibitors associated with P bodies increase formation whereas promoters of cap-dependent translation, such as those activated by IL-6 and NGF signaling, decrease P body formation. As a result, each mechanism regulating P body formation has an opposite effect on cap-dependent translation, suggesting a unique role for P bodies in the PNS and possibly nociceptive plasticity. This potential is further supported by previous studies of P body function in other cells of the PNS indicating association with and functions similar to BDNF, which has a known role in plasticity (Melemedjian et al., 2014).

Although there are no *Drosophila* orthologs of IL-6 or NGF, proteins and pathways analogous to those described above are present. IL-6 is a cytokine signaling protein that activates ERK/MnK, which is a JAK/STAT pathway. In *Drosophila*, Upd cytokines activate

Hopscotch/STAT92E, which is also a JAK/STAT pathway (Panayidou and Apidianakis, 2013). The *Drosophila* neurotrophin Spätzle shares similarities with NGF (DeLotto and DeLotto, 1998). A TOR signaling pathway is also employed (Neufeld, 2004). Although no specific conclusions can be drawn based solely on similarities, the analogous proteins and signaling pathways found in *Drosophila* reinforce its usefulness as a model organism for nociception.

A recent study in mice confirmed that removal of 4E-BP1 also enhanced translation in the spinal cord and resulted in mechanical hypersensitivity. Additionally, these effects were due to increased translation of eIF4E-dependent mRNAs rather than general upregulation. Results also attributed the observed phenotype primarily to increased translation of neuroligin 1 (mouse; ortholog of human NLGN1 and *Drosophila* Nlg3). The mTOR/4E-BP/eIF4E pathway was again found to impact mechanical, but not thermal sensitivity (Khoutorsky et al., 2015).

The ability of cells to maintain homeostasis depends on rapid responses to changing conditions, which is most effectively achieved by altering gene expression without affecting levels of available mRNA. As with regulation of transcription and processing, regulating translation is a vital aspect of cell function and numerous mechanisms are employed with varying degrees of specificity. Additionally, the initiation factors that enable and control translation initiation are a prime target for regulation. Given that plasticity is inherent to neuron function, characterizing translational control mechanisms and targets is particularly vital to understanding nociception. eIF4E function has been revealed as more complex than previously determined and similar complexity of other initiation factors may yet be discovered. As our understanding improves regarding mechanisms transcription, mRNA

processing, and translation, the importance of regulation becomes clear. Understanding regulation necessitates the study of RNA-binding proteins.

The Complex Roles of RNA-Binding Proteins in Regulating Gene Expression

Posttranscriptional regulation of gene expression is of particular importance within the nervous system, given its dependence on rapid response and plasticity for proper functioning. Processing (e.g. capping, splicing, and polyadenylation), transport/localization, translatability (e.g. repression vs. activation), and translation stages (initiation, elongation, and termination) of mRNA are all means of posttranscriptional regulation (Antic and Keene, 1997). RBPs and ribonucleoprotein (RNP) complexes function in a variety of ways to mediate these regulatory processes (Burd and Dreyfuss, 1994). Posttranscriptional regulation is a component of many molecular pathways essential to development and maintenance, particularly within the nervous system. Identification and characterization of associated RBPs are necessary aspects of understanding how key pathways are regulated. Well conserved RNA-binding motifs are used to identify putative RBPs and gain clues to their function (Burd and Dreyfuss, 1994). Hundreds of putative RBPs annotated within the *Drosophila* genome are expressed in sensory neurons and relatively few have been well characterized.

One factor in neuronal plasticity is differential regulation of translation in different parts of the neuron (Price and Géranton, 2009). To enable rapid and specific responses, hundreds of mRNAs are transported through dendrites to synaptic sites (Davis et al., 1987; Moccia et al., 2003). RNA granules are also actively transported to and from dendrites in neurons. These complexes of mRNAs, RNPs, RBPs, ribosomal subunits, and decay enzymes have been identified as mobile centers for mRNA metabolism, including translation

(Knowles et al., 1996; Anderson and Kedersha, 2006), and RBPs mediate this localization (Zhang et al., 2001). Cytoplasmic polyadenylation element binding protein (CPEB) is known to mediate trafficking to dendrites and plays a role in activating translation as a synaptic response (Huang et al., 2002; Shin et al., 2004).

The *Drosophila* homolog Orb has been found to function in similar roles during oogenesis (Weil, 2014), localizing mRNAs in conjunction with other proteins and activating translation during oogenesis (Johnstone and Lasko, 2001). Specifically, Orb functions to activate translation of *osk* and *grk* mRNA (Chang et al., 1999; Chang et al., 2001). Interestingly, one of Orb's targets for activation is *orb*; to avoid ectopic expression, *orb*'s positive autoregulatory loop is repressed by another RBP, Cup, until the translation complex has localized properly (Wong and Schedl, 2011).

Orb also functions within the nervous system, although its roles there have not been thoroughly explored. Knockdown of *orb* in mushroom body neurons prevents long term memory formation (Pai et al., 2013), and knockdown in class IV dendritic arborization (da) neurons results in abnormal dendrite branching patterns (Olesnicky et al., 2014).

Pumilio Regulates Expression through Translation Repression

Pumilio (Pum) is a well conserved translation repressor that exhibits diverse means of repression. Pum identifies RNA for repression primarily via an RNA-binding domain (RBD) of "Pum repeats" that binds to the 3' UTR of target mRNA, regulating cap-dependent translation as well as cap-independent IRES-driven (Internal Ribosome Entry Site) translation (Wharton et al., 1998). Pum's RBD antagonizes PABP function and recruits deadenylases to shorten the poly-A tail of the target mRNA, resulting in diminished translation and accelerating mRNA decay. Pum also associates with different context-

dependent cofactors for repression (Weidmann et al., 2014). In addition to its conserved RBD, there are three domains in Pum's N terminus that exhibit repressive activity (Weidmann et al., 2014). Pum is also capable of indirect repression, such as when targeting the transcription activator E2F. When Pum binds to the 3' UTR of E2F3 mRNA, conformational changes occur that expose miRNA seed sequences, allow multiple miRNAs to bind to this region, and enhance repression of E2F3 translation, thereby reducing transcription of this activator's downstream targets (Miles et al., 2012). Pum can function independently or in conjunction with other RBPs, most commonly Nanos (Nos). For example, Pum and Nos function as a protein complex to regulate expression throughout development and also in maintenance of germline stem cells (Parisi and Lin, 1999).

Pum serves numerous and diverse functions within the peripheral nervous system. In muscles, Pum is localized around neuromuscular junction (NMJ) boutons where the axons of motor neurons terminate at muscle cells, forming a synaptic cleft for signaling. Pum is expressed at type Ib and Is boutons, both of which contain glutamatergic synapses, Pum regulates morphology of the terminal for both Ib and Is boutons. Without Pum, Ib boutons are too few and too large relative to wild-type larvae, and restoring Pum in neurons (presynaptic) rescues the phenotype. Is boutons, on the other hand, increase in number, and restoring Pum in muscles (postsynaptic) rescues the phenotype. Also on the postsynaptic side, Pum represses unnecessary accumulation of eIF4E. Additionally, pumilio mutants exhibit an increased number of GluRIIa glutamate receptors and increased frequency of spontaneous release of neurotransmitter at the synapse (Menon et al., 2004). In sensory organ precursor (SOP) cells, Pum inhibits epidermal growth factor receptor (EGFR) signaling to prevent an excessive number of SOP cells and bristles from forming. In this case, Pum binds

to Nanos Response Element (NRE) sequences in the 3' UTRs of numerous transcripts in the signaling pathway, including EGFR, Drk, Sos, and Rl (Kim et al., 2012). In class III and IV da neurons, Pum and Nos function together in dendrite morphogenesis. Mutants carrying loss of function alleles of pumilio or nanos exhibit abnormal higher-order dendrite branching patterns relative to wild-type larvae, and double mutants exhibit the same phenotype as each single mutant (Ye et al., 2004). Pum/Nos regulate normal dendrite density by repressing translation of head involution defective (hid), promoting branch extension and preventing branch retraction (i.e. promoting branch stabilization). Interestingly, this mechanism is dependent on an additional cofactor, Brain Tumor (Brat), and interaction with the eIF4E-like cap-binding protein d4EHP only in class IV da neurons. In class III da neurons, regulation of Hid expression and dendrite branching by Pum/Nos is not dependent on Brat/d4EHP (Olesnicky et al., 2012).

Additional regulatory mechanisms mediated by Pum have been identified in the central nervous system. Like Orb, Pum is also associated with RNA granules. When an event such as behavioral training triggers long-term memory formation, Staufen (RBP) mediates formation of neuronal RNA granules as well as transport of neuronal granules to synapses via dendrite microtubules. Pum (independent of Nos) represses mRNAs in transit to avoid ectopic expression. The granules release transported mRNAs at the synapse and local derepression of translation occurs, possibly activated by phosphorylation of Orb and/or 4E-binding proteins (Dubnau et al., 2003). Pum also plays a role in homeostatic regulation of CNS neuron excitability via regulation of the voltage-gated Na+ channel encoded by paralytic (para). In response to elevated synaptic excitation, such as occurs during embryogenesis or remodeling that accompanies learning and memory formation, Pum binds

to para mRNA, reducing the level of transcripts available for translation and helping to maintain membrane excitability (Mee et al., 2004). Pum requires the cofactor Nos to function in this pathway; however, dependence on Brat is specific to cell type. Repression of para mRNA by Pum/Nos is Brat-dependent in some motoneurons but not in most CNS neurons. Pum also functions to downregulate Nos, which protects neurons from excessive repression of para mRNA if high levels of Pum are present (Muraro et al., 2008). Although, class IV da neurons (nociceptors) also rely on Para channels to fire action potentials, it cannot be assumed that Para activity is regulated in the same way due to the context-dependent nature of Pum repression. For instance, Nos's repressive function is typically as a cofactor with Pum. On the postsynaptic side of the NMJ, however, Pum and Nos act in opposition to regulate glutamate receptor subunits (Menon et al., 2009).

Elav Regulates Expression through Processing

Embryonic Lethal Abnormal Vision (*elav*) and its vertebrate homologs are expressed exclusively in neurons and are essential for development and maintenance of the nervous system. Expression occurs at greatest levels during early development once postmitotic neurons are present and continues at lower maintenance levels (Campos et al., 1985; Robinow and White, 1988). Elav functions primarily in mRNA processing.

One function of Elav is regulation of alternative splicing. The protein Neuroglian has two isoforms, one of which is specific to the nervous system and functions as a neural cell adhesion molecule important for neurite outgrowth, development of dendrites and axons, and plasticity (Hortsch et al., 1990). Elav binds to *neuroglian* pre-mRNA and regulates alternative splicing events that produce the neuron-specific isoform (Koushika et al., 1996; Lisbin et al., 2001), possibly by promoting readthrough of alternative splice sites as it does

with proximal poly(A) signals. Elav also regulates alternative splicing of neuron-specific isoforms of *erect wind (ewg)* and *armadillo (arm)*; however, not all splicing events specific to neurons are regulated by Elav so study of individual events is necessary to determine how they are regulated (Koushika et al., 2000). Knockdown of *elav* in class IV da neurons causes abnormally short dendrite length and inadequate coverage of the larval body well, which may indicate that a splicing target of Elav is involved in dendrite morphogenesis (Olesnickey et al., 2014).

Elav also functions in alternative polyadenylation (APA), a process involving selection between multiple poly(A) signals within a gene during transcription resulting in different isoforms with altered 3' UTRs. Since this region contains binding sites for RBPs and miRNAs, alterations affect how the transcripts can be regulated. Elav binds near proximal ploy(A) signals during transcription, resulting in readthrough of those signals and extension of 3' UTRs enriched with regulatory binding sites. A strong preference for extended 3' UTRs is apparent in the nervous system with hundreds of neuron-specific isoforms exhibiting this trait. RBPs and miRNAs are preferentially selected for 3' UTR extension, indicating a highly complex system of regulation. Interestingly, some of the largest extensions are seen in highly active RBPs, such as *orb* (8.9 kb) and *pumilio* (10.1 kb), indicating the potential for auto- and cross-regulation (Hilgers et al., 2012; Smibert et al., 2012).

The discovery that Elav proteins are also present outside the nucleus in neuronal granules suggests some function in mRNA transport, stability and/or translation. mRNA decay is also a facet of regulation and AU-rich elements (AREs) in the 3' UTR serve as a signal for rapid decay. When Elav binds to AREs, the degradation pathway is disrupted and

these mRNAs are stabilized (Gao et al., 1996; Keene, 1999; Brennan and Steitz, 2001). Additional roles for Elav aside from mRNA processing may yet be determined.

Elav may regulate a given target via any or all of these mechanisms. Elav is involved in multiple regulation pathways of the Hox gene *Ultrabithorax* (*Ubx*). A study by Rogulja-Ortmann et al. determined that Elav regulates alternative splicing and 3' UTR extension of *Ubx* (2014). In addition to abnormal Ubx isoform expression in *elav* mutants, results indicated overall reduced expression levels. The authors suggested this could be due to degradation of improperly processed transcripts but did not report whether they evaluated the 3' UTR of *Ubx* mRNA for AREs. Reduced Ubx expression levels in *elav* mutants could indicate that Elav functions in stabilization of *Ubx* RNA in addition to processing.

Regulation of Alternative Splicing

Numerous RBPs regulate alternative splicing. A recent study identified 56 diverse RBPs as splicing regulators, 12 of which had not been previously identified. Two of these, EIF3GA and Shep, had the 7th and 8th largest effect on splicing events. Both are expressed in the nervous system. Results also supported the multifunctional nature of RBPs. Cross-regulation was observed as well with 26 of the RBPs being affected by another RBP on the list. In other words, 26 of the RBPs that function in splicing regulation result from transcripts that are alternatively spliced, and their expression levels are dependent on other splicing regulators. Additionally, each RBP was capable of either activating or repressing exons depending on the splicing event, and more than half of the splicing events were affected by more than one RBP (Brooks et al., 2015).

What is currently known regarding mechanisms that control alternative splicing is more general than specific. For instance, we know that competitive interaction between

trans-acting elements in binding to splicing enhancers or silencers is a factor in determining which exons are included in the resulting mRNA. The ultimate outcome of a splicing event can thus depend on variations and levels of specific splicing factors and regulators, all of which can be specific to genes, tissues, and/or developmental stage (Cáceres and Kornblihtt, 2002).

Contrasting the nature of splicing patterns between dTRPA1 (four isoforms with specific roles for md-da neuron function) and Dscam (over 38,000 isoforms capable of dynamic expression that are randomly generated from clusters of mutually exclusive alternative exons) demonstrates the likelihood that regulation is variable and dependent on context. The unique splicing patterns of Dscam likely involve unique methods of regulation. Celotto and Gravely explored possible means of regulation by examining expression of cluster 4 containing 12 mutually exclusive exons (2002). They found that regulation was to some degree dependent on development. Specifically, expression of 4.2 increased consistently across developmental stages, whereas exon 4.8 displayed an opposite trend. Alternative exons also exhibited tissue-dependent expression levels.

An excellent example of the gulf between general understanding and specific knowledge is a study by Brooks and colleagues in the Gravely laboratory regarding the role of Pasilla (PS), the *Drosophila* ortholog of human NOVA1 and NOVA2, a human protein known to regulate numerous splicing events. PS expression was reduced with interfering RNA (RNAi) in cultured cells and the resulting transcriptome was analyzed to identify changes in splicing events. 405 altered splicing events were identified, 19% of which included a previously unannotated splice junction. While results provided support for a regulatory role of PS in alternative splicing, they also raised additional questions. PS effects

could be either enhancing or silencing, rate of PS involvement was dependent on the specific mechanism involved (e.g. rarely in intron retention events), and many splicing events were completely unaffected (Brooks et al., 2011). When it comes to specific splicing events, general understanding provides some direction but little is known regarding the intricacies of associated molecular pathways.

Taken together, the results from both studies reinforce the necessity to consider individual splicing events from multiple perspectives. Different RNA-binding proteins may regulate specific steps, tissue types, and/or developmental stages. The well-documented dependence on alternative splicing of the *Drosophila* sex determination pathway introduces yet another variable affecting regulation. In this pathway, the presence of two X chromosomes activates *Sex-lethal (Sxl)*, a gene encoding an RBP that regulates splicing of another splicing regulator, TRA (Black, 2003). With regard to alternative splicing in md-da sensory neurons, the specifics of regulatory pathways remain largely unknown.

Orb, Pumilio, Elav, and Pasilla all exhibit numerous roles in development and maintenance of the nervous system and employ diverse mechanisms of regulation. These are just four of hundreds of RBPs that function within neurons. Most have not yet been characterized. Due to the highly conserved nature of RNA-binding sites, however, putative RBPs have been identified. Given the significant impact of RBPs on neuron structure and function that has been established, a behavioral screen of RBPs expressed in class IV da neurons (nociceptors) should provide new direction for characterizing mechanisms of nociception.

Development of Screen for RNA-Processing Genes Regulating Nociception

With so many varied functions, it is likely that RBPs are regulating nociception in some way. An effective way to test a large number of potential genes of interest is to conduct a loss-of-function screen. With this method, RNAi strains are obtained for a pool of potential genes and used to test for changes in behavior when the function of a targeted gene is lost. These tests are conducted in relatively small sample sizes. Individual results can then be compared to population results to identify the strains worth the time required to test in high enough sample sizes to find statistically significant results. For our purposes, we wanted to develop a pool of genes for RBPs likely to affect nociception, obtain RNAi strains to knock down function, and use thermal nociception assays to test for either increased or decreased response time relative to wild-type larvae.

To assemble our list of likely RBP candidates, we first considered RBPs with an established role in dendrite morphology. The Gavis laboratory at Princeton University conducted a broad screen of genes annotated as RNA-binding proteins or translation factors and identified 88 whose knockdown resulted in some type of abnormal dendrite morphology in the class IV neurons; Olesnicky et al., 2014). All of these proteins are involved in some type of post-transcription regulation, e.g. splicing regulation, translation control, etc. (Table 1). The results of this research identified numerous implications of RNA processing for nervous system developmental pathways. The prevalent role of these proteins in nervous system function indicates a likelihood that at least some may regulate mRNA biogenesis of factors controlling nociception. The focus was on form rather than function, however, and behavioral assays were not included in this study.

Table 1: Molecular functions of genes controlling dendrite morphology (Olesnicky et al., 2014)

Molecular Function	**Number of Genes (%)**
Translation initiation	16 (18)
Translation elongation	6 (7)
Translation repression	5 (6)
Translation termination and release	3 (3)
mRNA splicing	17 (19)
Cell death and engulfment	8 (9)
Cytoskeleton	16 (18)

A study by the Gravely laboratory at the University of Connecticut Health Center also supported the role of RNA-processing factors in nociception, focusing on genes purported to regulate alternative splicing (Park et al., 2004). They made mini-gene reporters of splicing events, added them to cultured *Drosophila* cells, and knocked down each predicted RNA-binding protein. This generated a list of genes that code for RBPs with a confirmed impact on alternative splicing. All three genes studied are expressed in the nervous system. Any of the genes identified in this study could impact nociception but once again, behavioral assays were not performed.

Combining the results from both studies yields a list of RNA-binding proteins with established function in sensory neurons that had not yet been studied on a behavioral level. It is reasonable, therefore, to evaluate them for function specifically in mdIV neurons by assessing impact on nocifensive response, which is the purpose of this RNAi screen to identify RNA-processing genes controlling nociceptor function.

Materials and Methods

Fly Strains

Loss-of-function tests were performed using the GAL4/UAS system to knock down gene expression in *Drosophila* strains (Brand and Perrimon, 1993), crossing *ppk-GAL4; UAS-dicer2* flies with transgenic RNAi lines for each of the 122 genes screened (Appendix A). *GAL4* is a gene for a yeast transcription activator and *UAS* provides a binding site for GAL4, activating transcription of the downstream sequence. When *ppk-GAL4; UAS-dicer2* flies are crossed with *UAS-RNAi* flies targeting a specific gene, GAL4 drives transcription of that RNAi causing transcripts for the associated gene to be targeted for destruction. The *ppk* promoter was used to limit gene knockdown specifically to nociceptors, the only cells which express *ppk* (Zhong et al., 2010), thereby eliminating knockdown in other cells as a variable affecting any phenotype observed. Inclusion of *dicer2* enhances the knockdown of RNAi (Dietzl et al., 2007). The following wild-type strains were used: *y w; attP*, VDRC *isow*, w^{1118}, *y v; attP2*, *y+* and *y v; attP40*, *y+*. When crossed with *ppk-GAL4; UAS-dicer2* flies, no RNAi should be expressed, so these served as negative controls. A lab stock for *para-RNAi* was used as a positive control.

Screening and Validation of RNAi Strains with Thermal Nociception Behavioral Assays

For each set of crosses, six *ppk-GAL4; UAS-dicer2* virgin females and three males from an RNAi strain were added to vials containing food (Genesee Nutri-Fly prepared according to protocol in Appendix B), one vial per genotype, and kept in an incubator at 25 °C and approximately 60-70% humidity. Each set also included one cross each for *para* RNAi males (positive control) and wild-type males corresponding to the background of RNAi strains tested (negative control). To reduce confounding variables, transgenic lines

were tested in groups according to genetic background with a wild-type strain of matching genetic background. All vials were anonymized for the purpose of blind testing and then given 48 hours to establish. 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.

Five to six days after initiation of vials, thermal nociception assays were performed on wandering 3rd instar larvae, the stage at which dendrites and sensory neuronal activity have fully developed (Zwart et al., 2013). For these assays, we created a probe by filing a flat side along the tip of a soldering iron. A thermocouple was attached to the tip with solder in order to constantly monitor temperature, and a voltage regulator was used to control the temperature. Assays were conducted at 46 ± 5 °C, which is above the heat threshold for normal nocifensive response (Tracey et al., 2003). Approximately 15-20 animals were gently washed from the vial with distilled water and added to a glass petri dish. Excess water was removed to prevent floating and accidental rolling, and larvae were kept moist enough to allow locomotion. Each trial was performed on a larva actively traveling in a straight path with enough surrounding clearance to prevent interference. The probe was applied laterally, flush with the larval body surface along abdominal segments A1-A3, and held in place until a full roll along the long body axis was observed or until at least ten seconds had elapsed. Each animal was tested once and then removed from the dish. Assays were performed on at least two different days with a minimum of 20 total trials for screen assays and a minimum of 50 total trials for validation assays.

Video Analysis

To measure response latency, which is defined as the time in seconds (s) from initiation of contact to completion of nocifensive behavior (one full roll), I developed a

protocol for analyzing assay videos frame by frame using film editing software (Appendix C). This procedure allowed me to mark time points of contact and roll completion and quickly determine the time between markers. Any trial in which a larva failed to roll within 10 seconds was recorded as 11 s and all trials were rounded to the nearest tenth.

Statistics

Once the screen was completed, wild-type data were grouped by type for determining significant difference between types. The Kruskal-Wallis One Way ANOVA on Ranks was used and a significant difference was found ($p < 0.001$). Dunn's Method for pairwise multiple comparisons was used to isolate differences. *y v; attP2, y+* was different from every other group with p-values ranging from <0.001 to 0.036. For this reason, these data were considered separately to identify genes of interest. There were no other differences between groups found, so data for remaining four wild-type groups were analyzed as a single population. In each of the two groups, the cut-off for identifying genes of interest was one standard deviation above the population mean.

Three strains (*eIF4A-RNAi*, *eIF2Bα-RNAi*, and *pumilio-RNAi*) were validated in crosses testing one RNAi strain against one wild-type strain. For these, the Mann-Whitney Rank Sum Test was used to determine significant difference. All other genes validated were tested in crosses with more than one RNAi strain against one wild-type strain. *eIF4E3-RNAi*, *eIF4G2-RNAi*, and *eIF4AIII-RNAi* were all tested with *y v; attP2, y+*. *eIF2α-RNAi*, *eIF3ga-RNAi*, and *eIF1A-RNAi* were all tested with *y w; attP*. The Kruskal-Wallis One Way ANOVA on Ranks was used to determine significant difference. Dunn's Method for multiple comparisons versus control group was used to isolate differing groups.

SigmaPlot was used to run all tests (see Appendix D for complete output).

Results

Proof-of-Principle Experiment to Establish para-RNAi *as a Positive Control*

In order to demonstrate the effectiveness of the RNAi approach, an initial proof-of-principle experiment was performed comparing *ppk-GAL4; UAS-dicer2* females crossed with *para-RNAi* males and *ppk-GAL4; UAS-dicer2* females crossed with *isoW* wild-type males. *ppk* was used as a promoter to ensure nociceptor-specific knockdown. Thermal nociception assays were performed on wandering 3rd instar larvae at 46°C. Assays were performed on two different days with more than 50 total trials for each cross.

para is a protein-coding gene that encodes a subunit of the voltage-gated Na^+ channels required for neurons to fire action potentials (Ganetzky and Wu, 1986). If gene knockdown with RNAi is effective, larvae with *para* RNAi expressed in nociceptors are predicted to exhibit significantly increased latency in nocifensive response, indicating a decrease in sensitivity of the nociceptor neurons (Thackeray and Ganetzky, 1994). The results from my initial experiment were as expected: none of the *para-RNAi* larvae responded

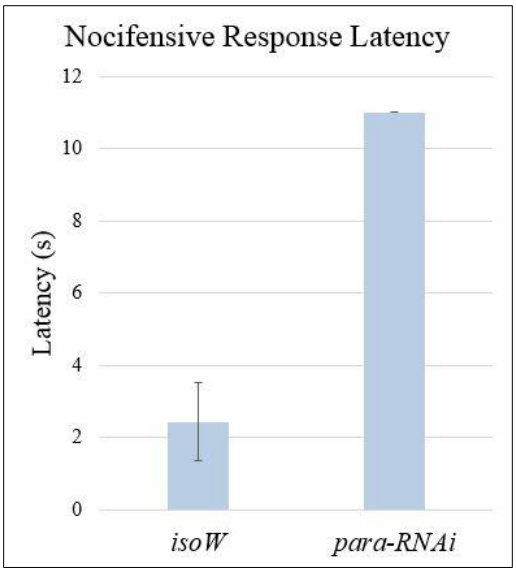


Figure 1: Thermal nociception response latency confirms a nociception defect in *para-RNAi* larvae. *isoW* (wild type) larvae had a mean response time of 2.43 s (n = 50). *para-RNAi* larvae had a mean response time of 11 s (n = 50). Error bars show standard deviation.

within ten seconds (Fig. 1). A *para-RNAi* cross was subsequently used as the positive control for each set of experimental crosses.

Insensitivity Screen of RNA-Processing Genes to Identify Potential Genes of Interest

Repeating the above procedure with the *ppk* promoter to limit RNAi expression to nociceptors, a total of 122 RNAi strains targeting RNA-processing genes were screened using thermal nociception assays at 46°C. Response latency was measured; an increased response time for RNAi larvae relative to wild-type larvae indicates an insensitivity phenotype and potential nociception defect. The RNAi strains that we obtained were created in five different wild-type backgrounds, which necessitated evaluation for differences between wild types. Once the screen was completed, response latency for all wild-type data were compared and statistical tests determined that the data for *y v; attP2, y+* larvae were significantly different from the data for all other wild types. There were no significant differences in response latency among the other four wild types (Appendix D). For this reason, results were separated accordingly into two groups for further analysis. In both analysis groups, one standard deviation above the population mean was used as the threshold for identifying potential genes of interest for additional testing (Figs. 2-4). Between the two analysis groups, a total of 23 such genes were identified.

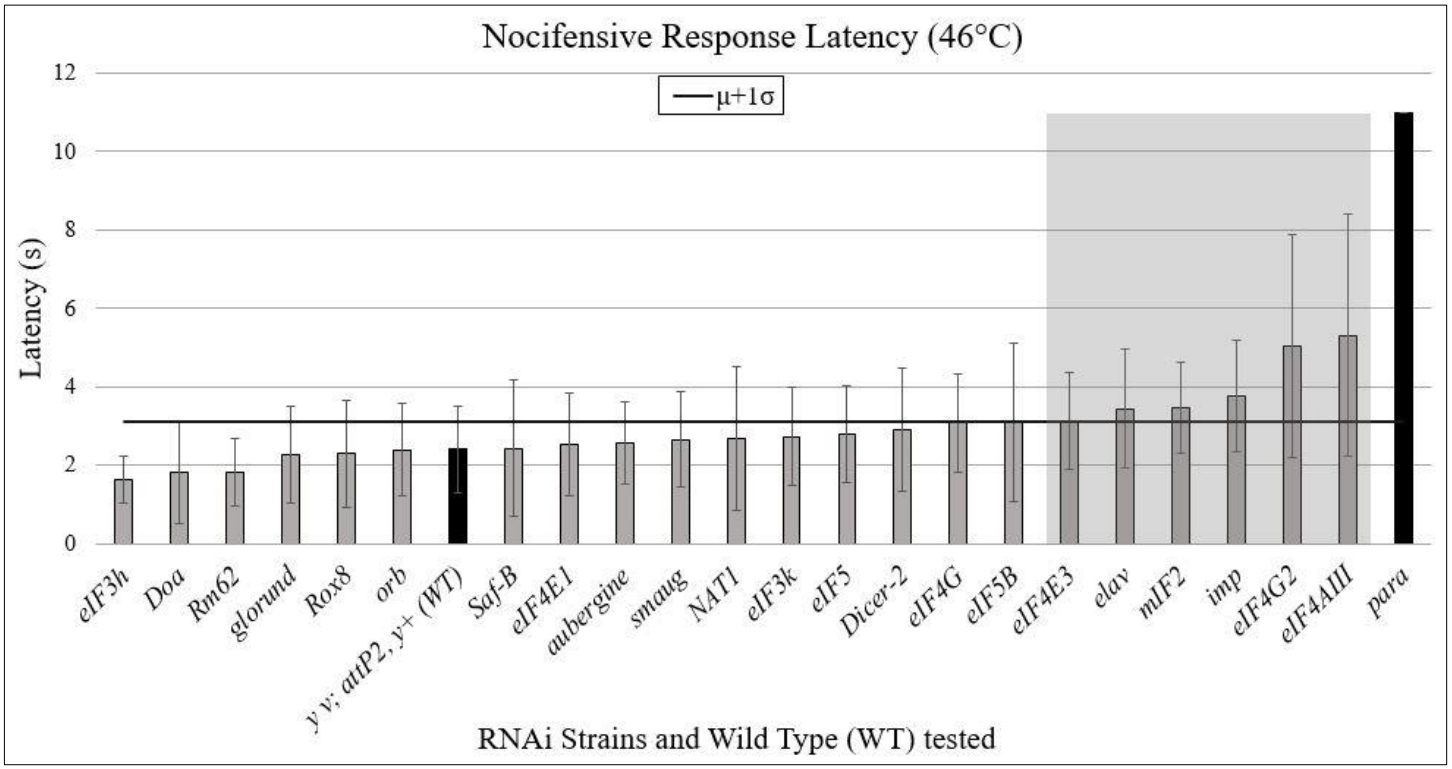


Figure 2: Mean nocifensive response latency indicates six potential candidate genes

RNAi strains with *y v; attP2, y+* genetic background. Horizontal line indicates one standard deviation above the population mean of 2.57 s, the cut-off to identify genes of interest (shaded area). Error bars show standard deviation. N > 20 for each strain tested. Controls in black.

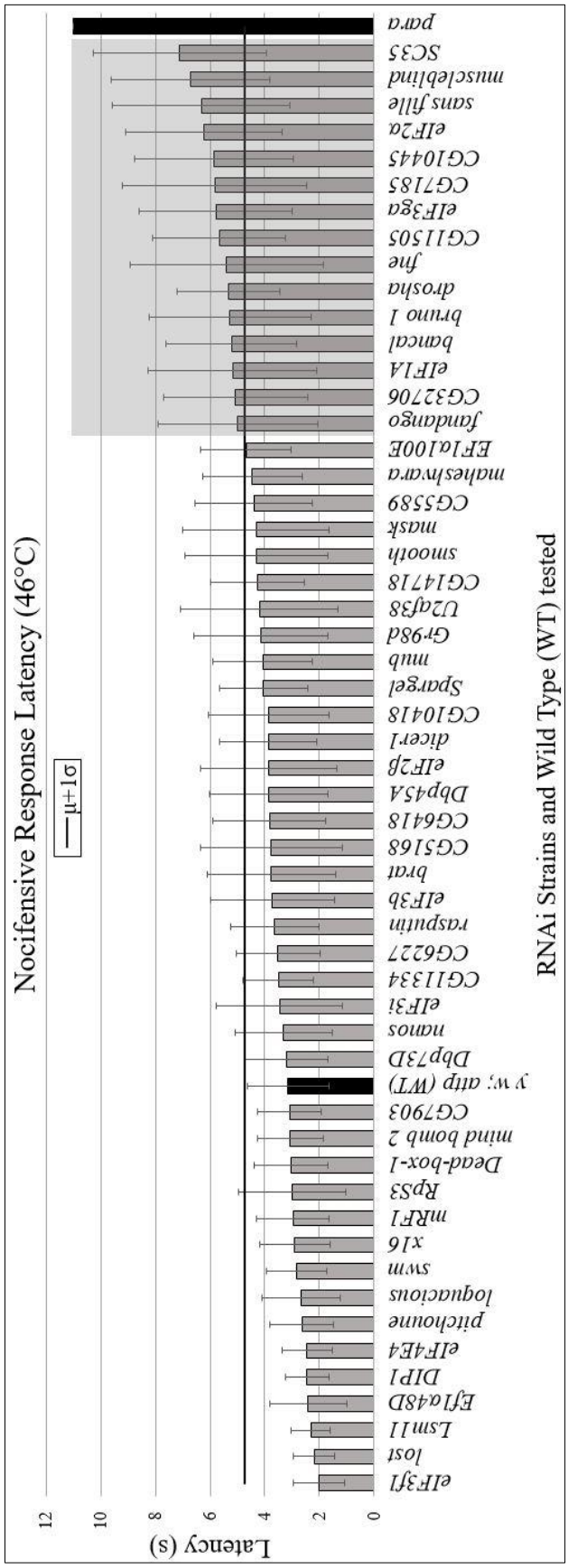


Figure 3: Mean nocifensive response latency indicates 15 potential candidate genes

RNAi strains with *y w; attP* genetic background. Analyzed as a population with strains with *iso W*, W^{1118}, and *y v; attP40, y+* background strains. Horizontal line indicates one standard deviation above the population mean of 3.68 s, the cut-off to identify genes of interest (shaded area). Error bars show standard deviation. N > 20 for each strain tested. Controls in black.

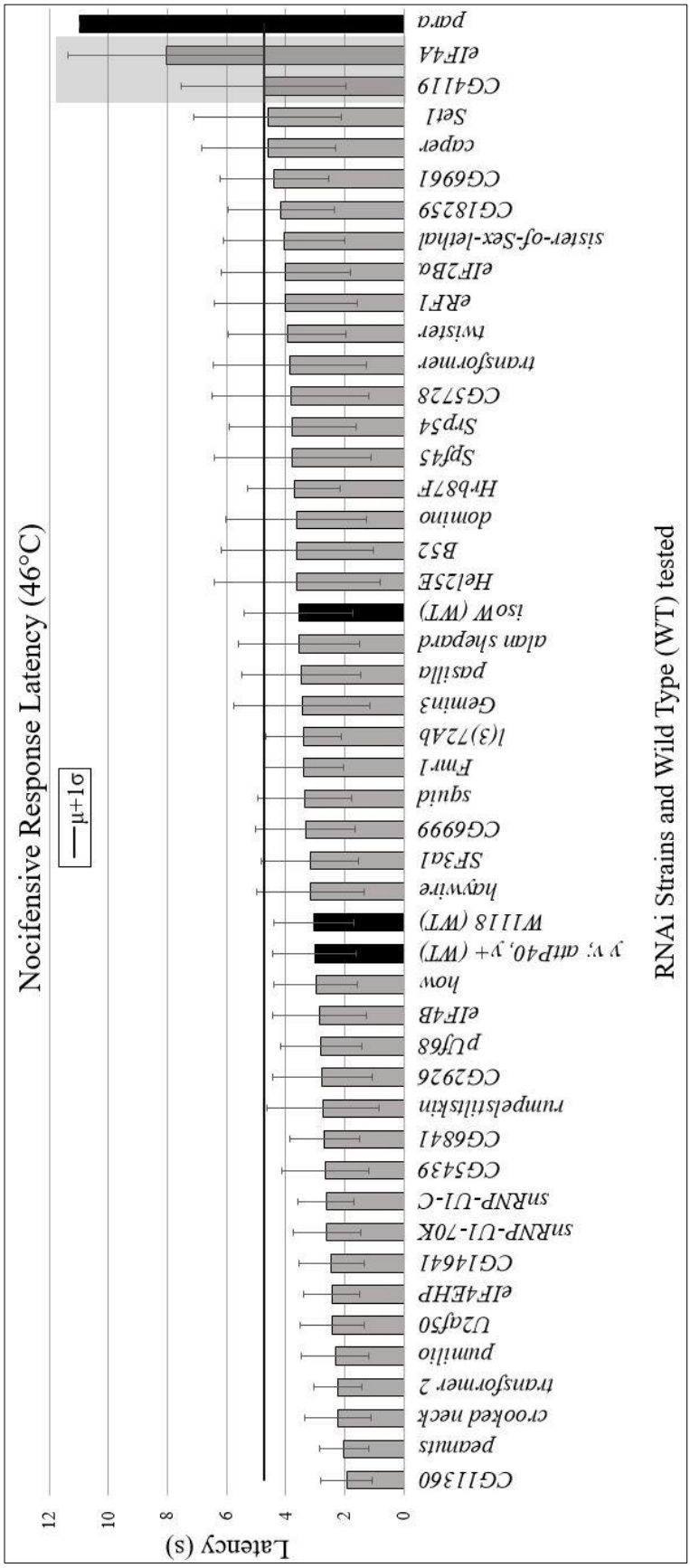


Figure 4: Mean nocifensive response latency indicates two potential candidate genes

RNAi strains with *isoW*, W^{1118}, and *y v; attP40, y+* genetic backgrounds. Analyzed as a population with strains with *y w; attP* background strains. Horizontal line indicates one standard deviation above the population mean of 3.68 s, the cut-off to identify genes of interest (shaded area). Error bars show standard deviation. N > 20 for each strain tested. Controls in black.

In the analysis group with *y v; attP2, y+* wild-type background, six genes were identified with average response latencies ranging from 3.12 to 5.31 s, compared to wild-type response latency of 2.40 s (Table 2, Fig. 5).

Table 2: Identified genes of interest with orthologs
(y v; attP2, y+ background strains)

CG #	Name	Mean NEL (s)	N	Human Ortholog(s)
CG8023	*eIF4E3*	3.12	34	EIF4E, EIF4E1B
CG4262	*elav*	3.43	41	ELAVL4, ELAVL2, ELAVL3, ELAVL1
CG12413	*mIF2*	3.47	28	MTIF2
CG1691	*imp*	3.76	43	IGF2BP1, IGF2BP3, IGF2BP2
CG10192	*eIF4G2*	5.05	33	EIF4G3, EIF4G1
CG7483	*eIF4AIII*	5.31	35	EIF4A3

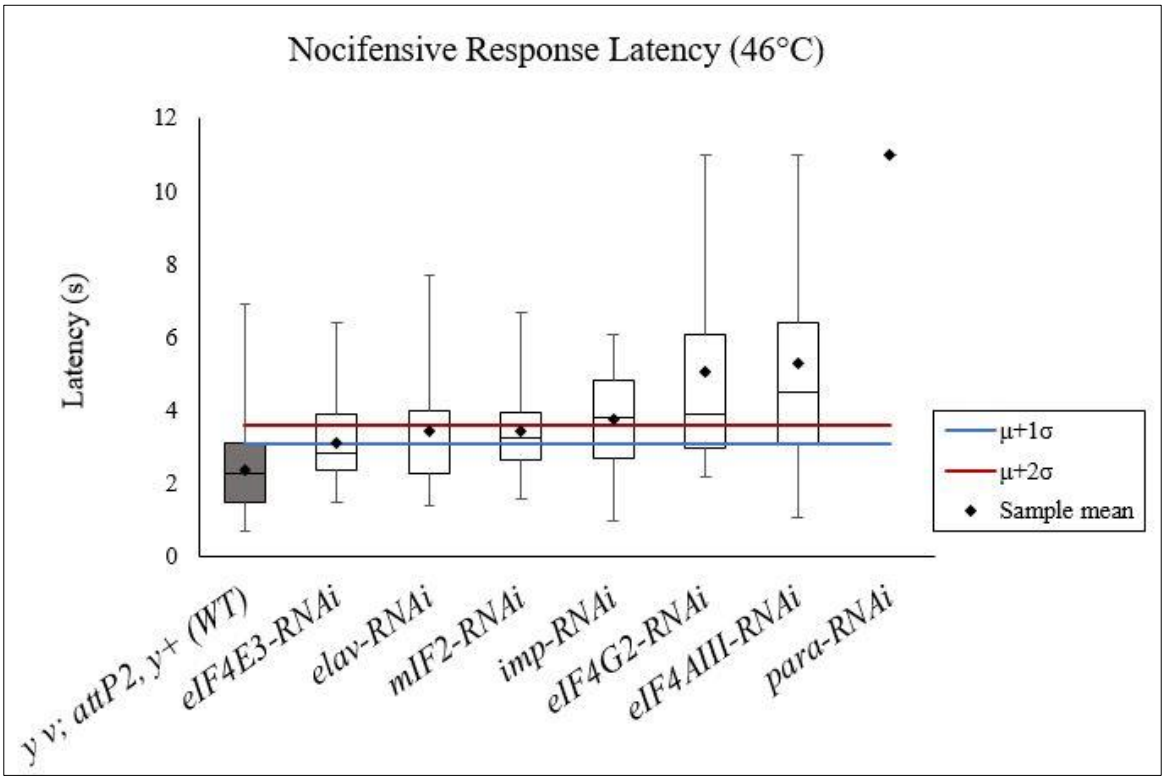


Figure 5: Thermal nociception response latency for identified candidate genes

RNAi strains with *y v; attP2, y+* genetic background. Boxes show 1st quartile, median, and 3rd quartile. Whiskers show minimum and maximum. Diamonds show means. Shaded box is negative control. Horizontal lines are one and two standard deviations above population mean of 2.57 s. N > 20

The remaining strains were evaluated as a second group and included TRiP RNAi lines in *y v; attP40, y+* wild-type background, VDRC RNAi lines in *yw;attP* and *isoW* wild-type backgrounds, and NIG RNAi lines in W^{1118} wild-type background. In this analysis group, seventeen genes were identified with average response latencies ranging from 4.76 to 8.05 s, compared to wild-type average response latencies ranging from 3.02 to 3.57 s (Table 3, Fig. 6).

Table 3: Identified genes of interest with orthologs
(*y w; attP*, *isoW*, W^{1118}, and *y v; attP40, y+* background strains)

CG #	Name	Mean NEL (s)	N	Human Ortholog(s)
CG4119	CG4119	4.76	37	RBM25
CG6197	*fandango*	4.99	25	XAB2
CG32706	CG32706	5.07	30	ABT1
CG8053	*eIF1A*	5.18	29	EIF1AY, EIFIAX
CG13425	*bancal*	5.21	45	HNRNPK
CG31762	*bruno 1*	5.27	24	CELF2, CELF1
CG8730	*drosha*	5.34	35	DROSHA
CG4396	*fne*	5.39	33	ELAVL4, ELAVL2, ELAVL3, ELAVL1
CG11505	CG11505	5.67	41	LARP4, LARP4B
CG8636	*eIF3ga*	5.80	38	EIF3G
CG7185	CG7185	5.83	30	CPSF6, CPSF7
CG10445	CG10445	5.85	21	TTF2
CG9946	*eIF2α*	6.24	45	EIF2S1
CG4528	*sans fille*	6.33	23	SNRPA, SNRPB2
CG33197	*muscleblind*	6.73	49	MBNL2, MBNL1, MBNL3
CG5442	*SC35*	7.10	47	SRSF2
CG9075	*eIF4A*	8.05	23	EIF4A2, EIF4A1

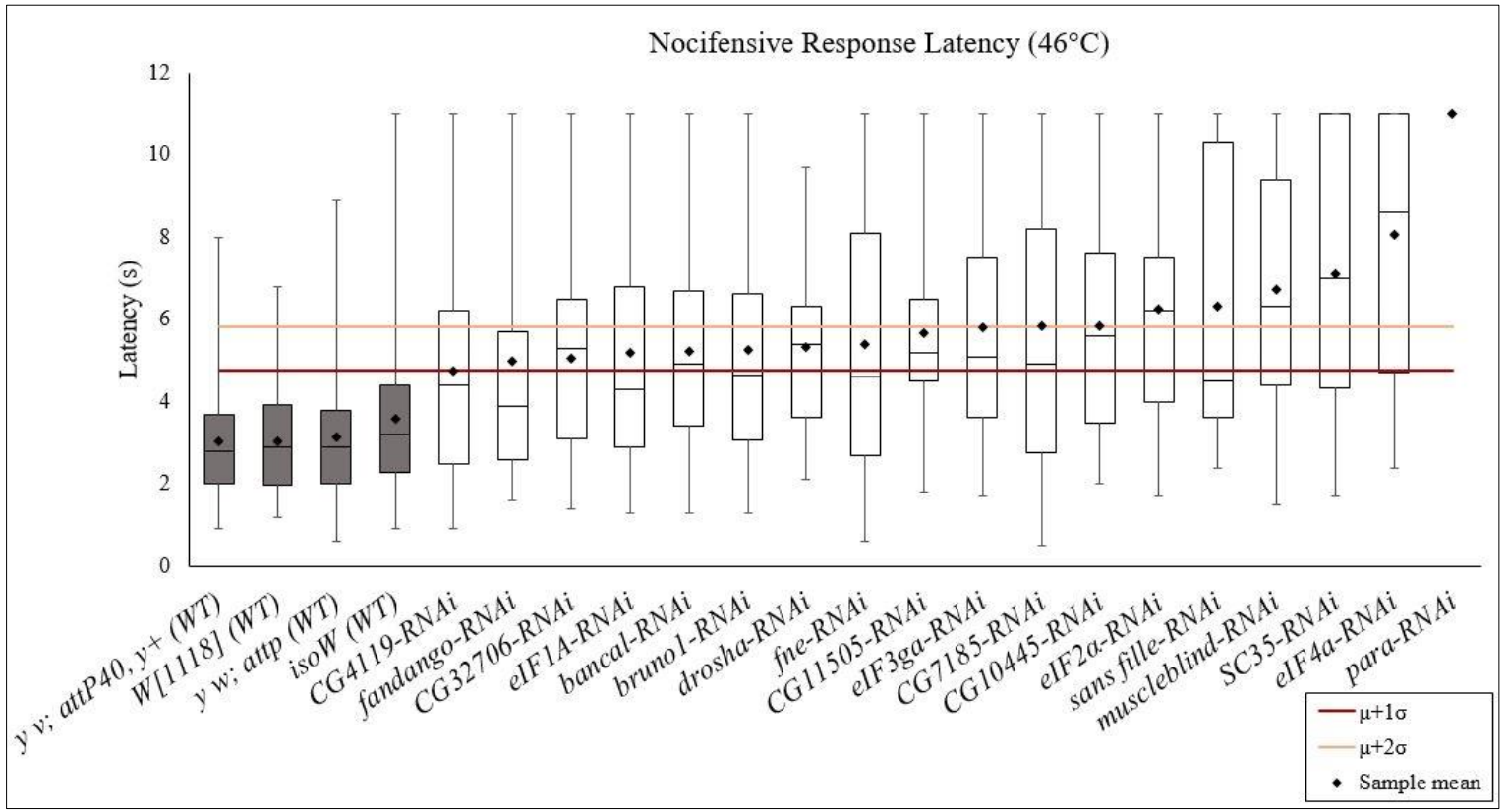


Figure 6: Thermal nociception response latency for identified candidate genes

RNAi strains with *y w; attP*, *isoW*, W^{1118}, and *y v; attP40, y+* genetic backgrounds. Boxes show 1st quartile, median, and 3rd quartile. Whiskers show minimum and maximum. Diamonds show means. Shaded boxes are negative controls. Horizontal lines are one and two standard deviations above population mean of 3.68 s. N > 20

In both analysis groups, a range of outcomes was observed when knocking down different translation initiation factors (Fig. 7). Fourteen factors exhibited normal behavior (response latency within one standard deviation of the population mean), while eight exhibited an insensitive nociception defection (response latency one to two standard deviations above the mean). I found this variation interesting and selected a number of these genes for further testing to validate the insensitive phenotype indicated by the screen.

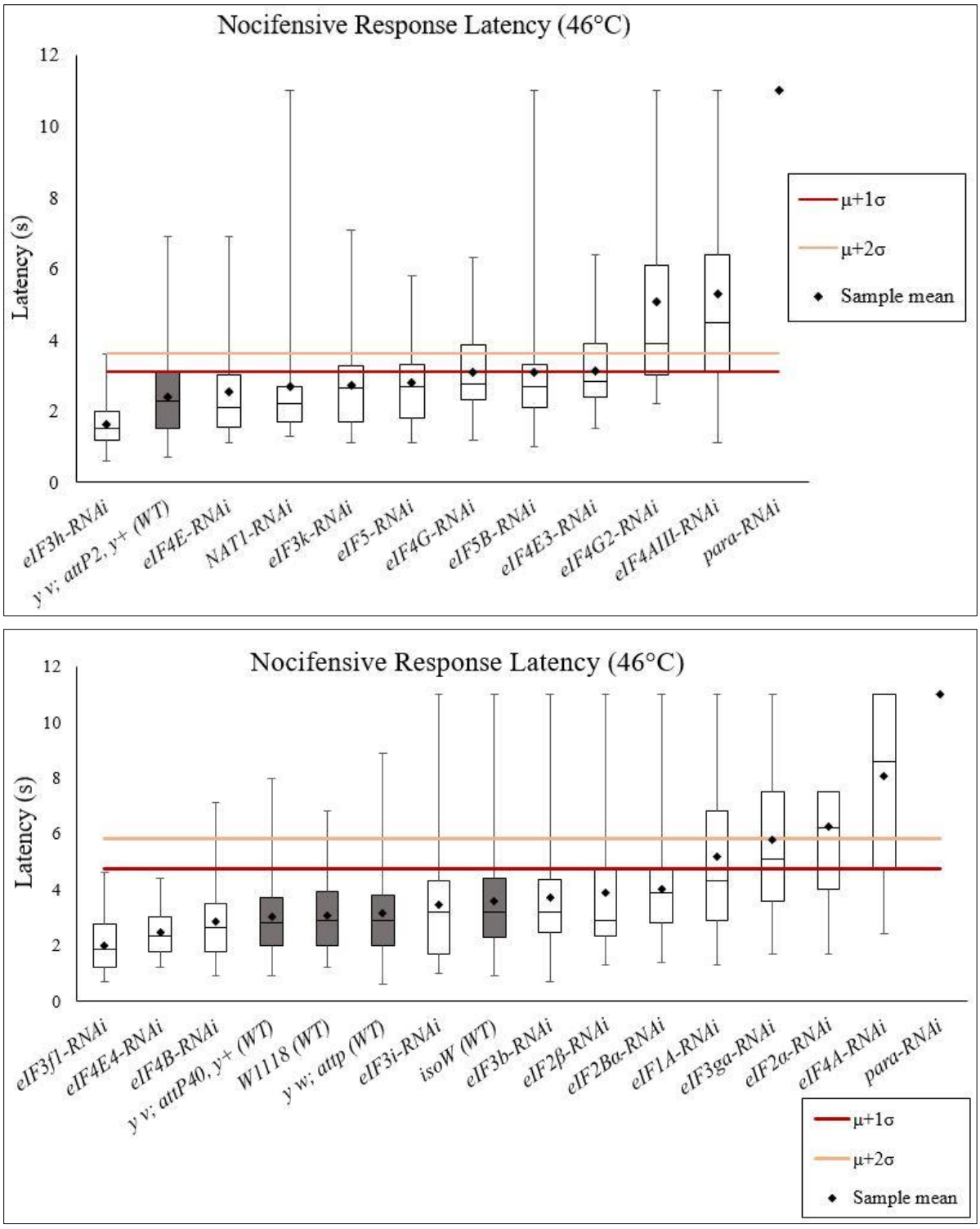
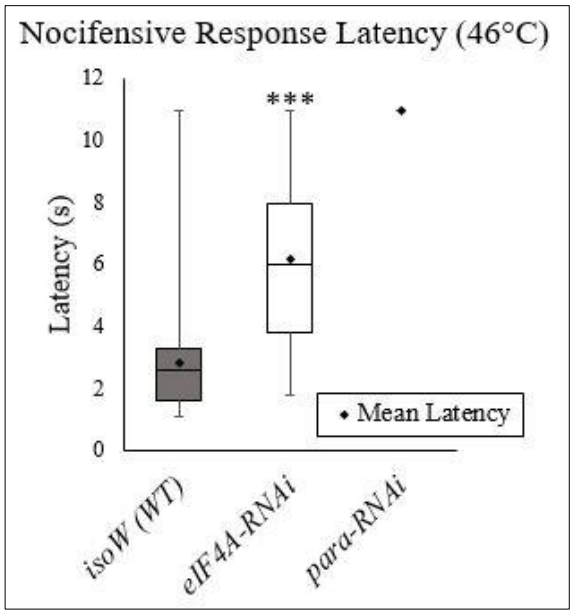


Figure 7: Thermal nociception response latency for initiation factors
Top: *y v, attP2, y+* background strains, population mean 2.57 s.
Bottom: *y w; attP, isoW, W*^{1118}, and *y v; attP40, y+* background strains, population mean 3.68 s
Boxes show 1st quartile, median, and 3rd quartile. Whiskers show minimum and maximum. Diamonds show means. Shaded box is negative control. Horizontal lines are one and two standard deviations above population mean. N > 20

Validation of Insensitive Nociception Phenotypes

As previously discussed, the purpose of a screen is to make efficient use of time and resources by narrowing a large pool of potential genes of interest to a small group of the most promising genes. The small sample sizes preclude statistical analysis and can produce false positives; however, the results are useful for narrowing research focus to the most likely candidates. Further testing is required to statistically confirm a phenotype.

For validation purposes, the steps of the screening process were repeated but with a minimum of fifty trials. Crosses were once again grouped according to genetic background with the associated wild type as negative control and *para-RNAi* as positive control. For all validation crosses, *para-RNAi* larvae performed as expected with an average response latency of 11 seconds. Non-parametric statistical tests were performed on each data set due to the lack of normal distribution.



An insensitive nociception phenotype was confirmed for seven genes. *eIF4A-RNAi* was tested with negative control VDRC *isoW* (n = 77 and 79, respectively) (Fig. 8). The response latency of *eIF4A-RNAi* (6.16 s mean) was significantly greater than the response latency of *isoW* (2.87 s mean), $p < 0.001$.

Figure 8: Thermal nociception response latency validates *eIF4A-RNAi* insensitive phenotype
Boxes show 1st quartile, median, and 3rd quartile. Whiskers show minimum and maximum. Diamonds show means. Shaded box is negative control.
isoW mean latency is 2.87, n = 77.
eIF4A-RNAi mean latency is 6.16 s, n = 79.
***Significant difference ($p < 0.001$)

eIF4E3, *eIF4G2*, and *eIF4AIII* RNAi were tested with negative control *y v; attp2, y+* (n = 55, 52, 54 and 58, respectively) (Fig. 9). The response latencies of *eIF4E3-RNAi* (3.58 s mean), *eIF4G2-RNAi* (3.64 s mean), and *eIF4AIII-RNAi* (4.65 s mean) were all significantly greater than the response latency of *y v; attP2, y+* (2.65 s mean), p = 0.005, p = 0.04, and p < 0.001, respectively.

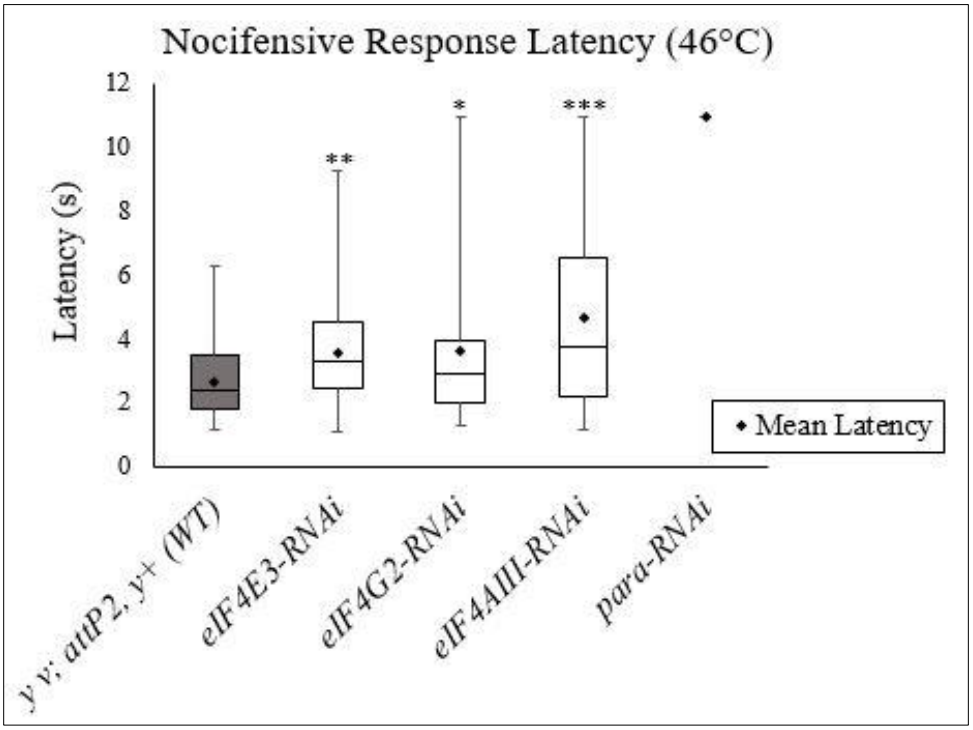


Figure 9: Thermal nocifensive response latency validates insensitive phenotype for *eIF4E3*, *eIF4G2*, and *eIF4AIII* RNAi
Boxes show 1st quartile, median, and 3rd quartile. Whiskers show minimum and maximum. Diamonds show means. Shaded box is negative control.
y v; attP2, y+ mean latency is 2.65, n = 58.
eIF4E3-RNAi mean latency is 3.58 s, n = 52. **Significant difference (p = 0.005).
eIF4G2-RNAi mean latency is 3.64 s, n = 55. *Significant difference (p = 0.040).
eIF4AIII-RNAi mean latency is 4.65 s, n = 54. ***Significant difference (p < 0.001)

eIF2α, eIF3ga, and *eIF1A* RNAi were tested with negative control *y w; attp* (n = 63, 66, 63 and 61, respectively) (Fig. 10). The response latencies of *eIF2α-RNAi* (4.76 s mean), *eIF3ga-RNAi* (4.80 s mean), and *eIF1A-RNAi* (8.22 s mean) were all significantly greater than the response latency of *yw; attp* (2.95 s mean), $p < 0.001$ for each of the three comparisons.

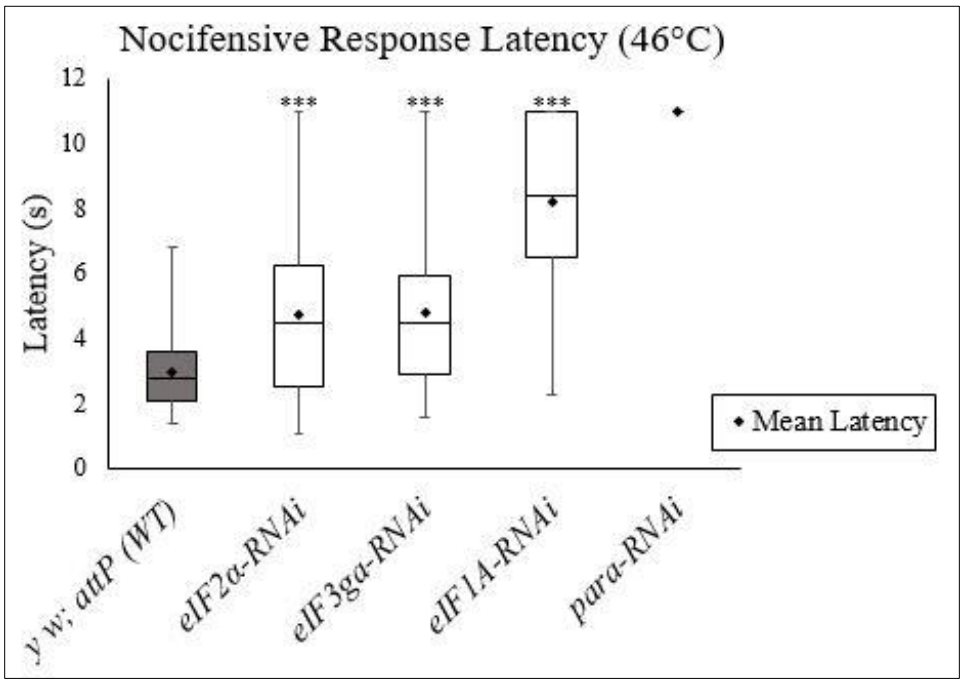


Figure 10: Thermal nocifensive response latency validates insensitive phenotype for *eIF2α*, *eIF3ga*, and *eIF1A* RNAi
Boxes show 1st quartile, median, and 3rd quartile. Whiskers show minimum and maximum. Diamonds show means. Shaded box is negative control.
y w; attP mean latency is 2.95, n = 61.
eIF2α-RNAi mean latency is 4.76 s, n = 63. ***Significant difference ($p < 0.001$).
eIF3ga-RNAi mean latency is 4.80 s, n = 66. ***Significant difference ($p < 0.001$).
eIF1A-RNAi mean latency is 8.22 s, n = 63. ***Significant difference ($p < 0.001$)

Identification and Validation of Hypersensitive Nociception Phenotypes

A number of genes screened had response latencies at or below wild-type, but the response rate at 46°C is too rapid to accurately discern if mutants are truly responding more quickly. A second complete screen was beyond the scope of this project, but we were curious to see if we could develop a procedure for identifying hypersensitive nociception phenotypes. To this end, a "mini screen" for hypersensitivity was conducted for a number of genes with the probe adjusted to 42°C. Since only about half of wild-type larvae respond at this temperature, we had a broader range of response rate to compare with mutants. These tests were limited to one day and only 10-15 trials, so the results are not useful for eliminating genes from consideration for a potential phenotype; however, two genes, *eIF2Bα* and *pumilio (pum),* were identified as potential candidates and validation testing was done in order to confirm the usefulness of screening at 42°C.

eIF2Bα-RNAi was tested with negative control *y v; attP40, y+* (n = 61 and 51, respectively) (Fig. 11). The response latency of *eIF2Bα-RNAi* (7.92 s mean) was significantly lower than the average response latency of *y v; attP40, y+* (9.43 s mean), p = 0.001.

pum-RNAi was tested with negative control *y v; attP2, y+* (n = 51 and 45, respectively) (Fig. 12). The response latency of *pum-RNAi* (5.21 s mean) was significantly lower than the average response latency of *y v; attP2, y+* (6.58 s mean), p = 0.034.

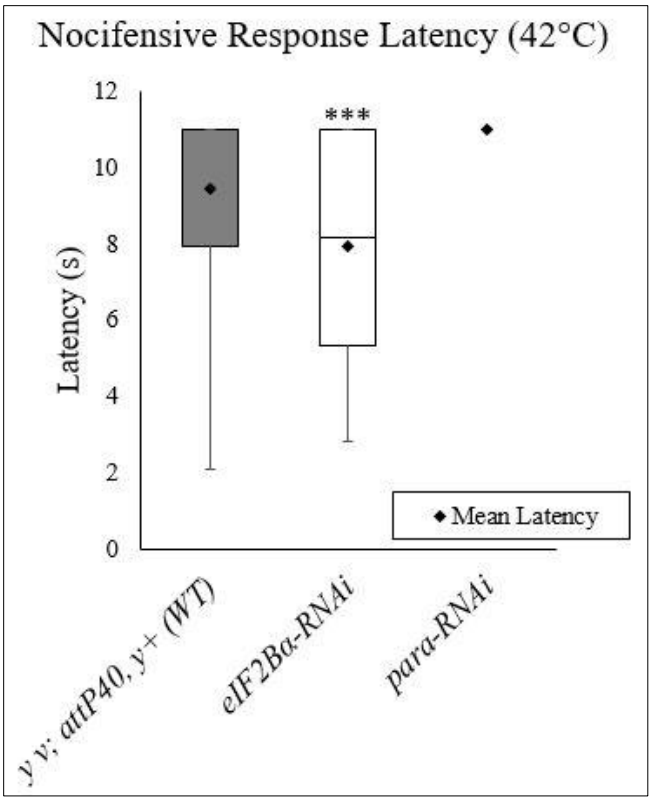


Figure 11: Thermal nocifensive response latency validates *eIF2Bα-RNAi* hypersensitive phenotype

Boxes show 1st quartile, median, and 3rd quartile. Whiskers show minimum and maximum. Diamonds show means. Shaded box is negative control.
y v; attP40, y+ mean latency is 9.43 s, n = 51.
eIF2Bα-RNAi mean latency is 7.92 s, n = 61.
***Significant difference (p = 0.001)

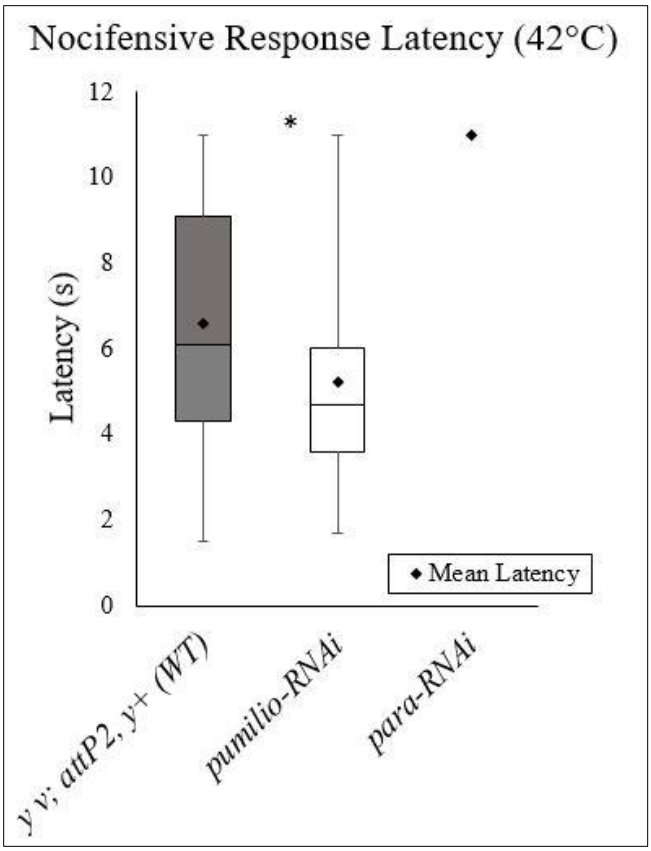


Figure 12: Thermal nocifensive response latency validates *pumilio-RNAi* hypersensitive phenotype

Boxes show 1st quartile, median, and 3rd quartile. Whiskers show minimum and maximum. Diamonds show means. Shaded box is negative control.
y v; attP2, y+ mean latency is 6.58, n = 45.
pumilio-RNAi mean latency is 5.21 s, n = 51.
*Significant difference (p = 0.034)

Lack of correlation between defects in dendrite morphology and nocifensive response

Many of the genes included in this screen were chosen due to confirmed phenotypes for morphological defects in mdIV dendrites, which allowed us to evaluate for a correlation between presence/severity of dendrite defect and nocifensive behavior. When compared with results found by the Gavis lab regarding RNA-binding proteins and dendrite development in nociceptors (Olesnicky et al., 2014), the results from this screen indicate that abnormal dendrite morphology will not necessarily cause a defect in nocifensive response. Knocking down function of some genes caused a defect in dendrite patterning but not nocifensive response; the reverse is also true.

A wide variety of morphological defects were observed by the Gavis lab and these are included in the table below (Table 4). “Reduction in field coverage” refers to defects that result in a failure of dendrites to evenly cover the larval body wall. In this case, number of branch points and branch length may be normal, but the dendrites fail to spread out evenly to achieve normal coverage. Other defects include too many or too few branch points along the dendrites and either an increase or decrease in overall branch length. We found no correlation between the type or severity of morphological defect and nocifensive response.

Table 4: Dendrite morphology phenotypes in candidate nociception genes

CG#	Gene Name	Nociception Defect Identified by Bellemer Lab	Dendrite Defect Identified by Gavis lab
CG8053	*eIF1A*	**Y**	Y; severe reduction in field coverage
CG9946	*eIF2α*		
CG8636	*eIF3ga*		
CG4153	*eIF2β*	N	
CG6779	*RpS3*		
CG8882	*eIF3i*		
CG9769	*eIF3f1*		
CG4878	*eIF3b*		
CG9075	*eIF4A*	**Y**	Y; less severe reduction in field coverage
CG9124	*eIF3h*	N	
CG7883	*eIF2Bα*		
CG10124	*eIF4E4*		
CG4262	*elav*	**Y**	Y; increase in branch number and/or density
CG33197	*muscleblind*		
CG4528	*sans fille*		
CG10203	*x16*	N	
CG3582	*U2af38*		
CG10868	*orb*		
CG31762	*bruno1*	**Y**	Y; decreased length due to branch loss
CG18259		N	
CG4792	*dicer1*		
CG11334			
CG6539	*Gemin3*		
CG9054	*Dead-box-1*		
CG17492	*mind bomb 2*		
CG16901	*squid*		
CG14718			
CG4602	*Srp54*	N	Y; branch points reduced
CG6866	*loquacious*		
CG17686	*DIP1*		
CG6418			
CG5705			

Table 4 (continued)

CG#	Gene Name	Nociception Defect Identified by Bellemer Lab	Dendrite Defect Identified by Gavis lab
CG13425	*bancal*	**Y**	Y; unspecified
CG11505			
CG32706			
CG4119			
CG8730	*drosha*		
CG4396	*fne*		
CG12413	*mIF2*	**Y**	None found
CG7185			
CG7483	*eIF4AIII*		
CG8023	*eIF4E3*		
CG10192	*eIF4G2*		
CG1691	*imp*		
CG5442	*SC35*		
CG10445		**Y**	*not tested*
CG6197	*fandango*		

Lack of correlation between splicing defects and nocifensive response

Alternative splicing is a crucial factor in nociception. As one example, functionality is dependent on appropriate expression of TrpA1 isoforms. For this reason, we also included many genes in this screen that are expressed in neurons and have a confirmed role in alternative splicing. RBPs that are known to regulate splicing events that occur in neurons, such as modification of *Dscam* and *para* transcripts, could be regulating splicing events important to nociception, such as those that occur in TrpA1 transcripts.

There was no correlation between this screen's results and effects on splicing targets observed by the Gravely lab (Park et al., 2004). For genes affecting splicing of *Dscam* exon 4, *para* exons A/I, and *dAdar* exons 3/3a, at least one but not all exhibited a nociception defect. None of the genes affecting *Dscam* exon 17 splicing nor the gene affecting splicing of *para* exons O/N exhibited a nociception defect. This lack of correlation suggests that effects on these splicing targets alone are not enough to explain the observed nociception defects. RBPs encoded by these genes may impact other splicing events necessary for nociceptor function or a different regulatory role may be responsible.

Table 5: Splicing regulation in candidate nociception genes
Identified candidate genes with potential defect in bold

Dscam exon 4	
CG#	Gene Name
CG6197	***fandango***
CG4528	***snf***
CG5442	***SC35***
CG7185	
CG13425	***bancal***
CG31762	***aret***
CG2926	
CG3582	*U2af38*
CG42320	*Doa*
CG4602	*Srp54*
CG5422	*Rox8*
CG5931	*l(3)72Ab*
CG6227	*Prp5*
CG6841	*Prp6*
CG6995	*Saf-B*
CG6999	
CG7269	*Hel25E*
CG7437	*mub*
CG8019	*hay*
CG8144	*ps*
CG8241	*pea*
CG9696	*dom*
CG9998	*U2af50*
CG10210	*tst*
CG10279	*Rm62*
CG10293	*how*
CG10851	*B52*
CG11266	*caper*
CG11360	
CG12085	*pUf68*
CG12749	*Hrb87F*
CG14641	
CG16901	*squid*
CG16941	*SF3a1*
CG17454	

para exons A/I	
CG6197	***fandango***
CG9075	***eIF4A***
CG10445	
CG5728	
CG8144	*ps*
CG10293	*how*
CG10418	
CG11360	
CG12759	*Dbp45A*
CG12924	*Lsm11*

dAdar exon 3/3a	
CG4119	
CG3193	*crn*
CG5422	*Rox8*
CG5454	*snRNP-U1-C*
CG8749	*snRNP-U1-70K*
CG10851	*B52*
CG12085	*pUf68*

Dscam exon 17	
CG#	Gene Name
CG4602	*Srp54*
CG5931	*l(3)72Ab*
CG6999	
CG7269	*Hel25E*
CG10279	*Rm62*
CG11266	*Caper*
CG12085	*pUf68*
CG42320	*Doa*

para exons O/N	
CG8144	*Ps*

Discussion

Out of 122 genes tested in this screen, 25 (20.5%) were identified as potential genes of interest that had not previously been identified as affecting nocifensive behavior. Nine of these were subsequently validated (7.4% of all genes studied). Although there is an expectation that not all hits will be confirmed, none have been invalidated thus far. These results affirm the effectiveness of the screening approach. Screen results are particularly useful when they can be compared and contrasted with data from research on other aspects of the same mechanistic pathway. Confirming the lack of correlation between nocifensive behavior and dendrite morphology or specific splicing events is very useful knowledge. After all, it is the perception of pain that we seek to address with clinical intervention. Coordinating morphological, molecular, and behavioral studies in *Drosophila* will contribute to a more comprehensive understanding of pain mechanisms. For instance, although all seven genes with a validated insensitive phenotype are initiation factors, there is a surprising level of complexity to their potential mechanisms of regulation.

Initiation Factors with Insensitive Phenotype: Regulation via Translational Control

Six genes confirmed to have an insensitive phenotype are translation initiation factors: *eIF1A, eIF2α, eIF3ga, eIF4A, eIF4E3,* and *eIF4G2*. Given the dependence of nociceptor function and sensitivity on differential protein synthesis in the cell body, dendrites, and axons (Price and Géranton, 2009), these results may indicate the specific genes involved in translational control necessary for nocieptor sensitivity.

As previously discussed, the eIF4F translation initiation complex includes the subunits eIF4A, eIF4E, and eIF4G. The *Drosophila* gene *eIF4G* encodes the 4G subunit for this complex. Two other genes, *eIF4G2* and *NAT1*, encode proteins highly similar to eIF4G.

eIF4G2 has been established as a homolog of eIF4G (Franklin-Dumont et al., 2007), whereas NAT1 lacks the eIF4E binding site and plays a role in translation suppression (Imataka et al., 1997). All three genes were tested in this screen and only *eIF4G2* displayed an insensitive phenotype. Seven *Drosophila* genes encode isoforms of the 4E subunit (Hernandez et al., 2005), four of which had RNAi lines available for this screen. Of those four, *eIF4E3* was the only gene to display an insensitive phenotype.

The specific 4E and 4G subunits identified in this screen are non-canonical. eIF4G2 has been established as a homolog of eIF4G and its necessity in some translation processes has been confirmed (Baker and Fuller, 2007), but its verified role in translation has been considered somewhat puzzling because it lacks the domain necessary to bind eIF5 (Ghosh and Lasko, 2015). eIF4E3 also differs from the canonical 4E subunit, lacking both the high affinity for eIF4G found with eIF4E-1/2 and eIF4E-4 (Hernandez et al., 2005). 4E subunit activity is normally regulated via a serine residue that can be phosphorylated (Scheper and Proud, 2002) or sequences allowing for interaction with 4E-BPs, as previously discussed. eIF4E3 lacks both of these regulatory features (Hernandez et al., 2012).

If eIF4G2 and eIF4E3 function as 4G and 4E subunits integral to translational control of nociceptor sensitivity, they could be potential targets for clinical intervention in patients with chronic pain conditions due to the high degree of function similarity between human and *Drosophila* subunits (Grüner et al., 2016). Interaction between eIF4G2 and eIF4E3 has been established in the testis and eIF4G2 was found to colocalize in the testis to a significantly greater degree with 4E3 than did eIF4G. Additionally, a variety of eIF4F complexes with differing 4G and 4E subunit combinations are involved in spermatogenesis, suggesting a potential mode of translational control involving mRNA-specific 4F complexes

(Hernandez et al., 2012; Ghosh and Lasko, 2015). It is possible that eIF4E3 and eIF4G2 function similarly in md-da neurons to create an eIF4F complex specific to nocifensive response. Isoform-specific functions have been observed in human eIF4G and eIF4GII as well (Pyronnet et al., 2001).

Initiation Factors with Insensitive Phenotype: Regulation via Alternative Splicing

The importance of translational control to nociceptor function is well established; however, it must be considered that one or more eIFs may affect nociception via regulatory targets other than translation initiation. This is particularly true for eIF4AIII, the only "eukaryotic initiation factor" (eIF) identified by this screen that is not actually involved with translation initiation. Although *eIF4AIII* is named for its sequential similarity to *eIF4A*, its length is only 1656 base pairs compared to *eIF4A*'s length of 4145 base pairs, it is not redundant for *eIF4A* (Lasko, 2000), and translation initiation activity has not been observed.

eIF4AIII does serve important functions, however, such as its role in alternative splicing as a core component of the exon junction complex (EJC) (Tange et al., 2004). For instance, eIF4AIII is required for *MAP kinase* splicing and expression and ultimately, function of the RAS/MAPK signaling pathway (Roignant and Treisman, 2010; Ashton-Beaucage et al., 2010). Knockdown of eIF4AIII expression has been shown to reduce neuronal hyperexcitability in seizure mutants, which correlates with the insensitive phenotype validated here; however, *pasilla* RNAi produced similar results in seizure mutants yet did not display an insensitive phenotype in this screen (Lin et al., 2015). Therefore, eIF4AIII may be involved in regulating alternative splicing of other genes important for nociception function. Involvement of eIF4AIII in the EJC is a regulated process that only

applies to some spliced mRNA so further testing would be necessary to determine if any nociceptor-specific spliced mRNAs are a target (Sauliere et al., 2010).

eIF4A also plays a role in regulating alternative splicing, affecting splicing of *para* exons A and I (Park et al., 2004) *TAF1* exons 12a and 13a (Katzenberger et al., 2009). As with eIF4AIII, additional testing would be necessary to determine whether eIF4A is affecting other targets in nociceptors.

Regulation of alternative splicing is apparently a primary function for eIF3ga, which affected more than 300 splicing events in a recent study (Brooks et al., 2015). As previously discussed, eIF3ga shared a great deal of overlap in targets with alan shepard (Shep), which was also tested in this screen but had no phenotype. Given that eIF3ga was considered one of the more high-impact genes in this study, it would be worth testing loss-of-function mutants for splicing changes relevant to nociception (e.g. *para, TrpA1* transcripts).

Initiation Factors with Insensitive Phenotype: Regulation via Localization

As previously discussed, an important requirement for neuroplasticity is the ability of differential expression within the neuron, which necessitates localization of transcripts at axons and dendrites. Two RBPs with validated phenotypes are known to be involved in the localizing process. Although eIF4AIII is primarily known for its EJC role, this RBP also functions in oocytes as a component of an RNP complex responsible for the localization of *oskar* mRNA (Palacios et al., 2004) *oskar* mRNAs are also transported to md-da dendrites and the resulting regulatory proteins are necessary for normal development (Olesnicky et al., 2014). It is possible that eIF4AIII could be involved in mRNA localization in other cell types, such as sensory neurons. eIF3ga has been found to associate with cytoskeletal proteins (Hou et al., 2000). Since interaction between mRNA and the cytoskeleton is a necessary

factor in localization (Bassell and Singer, 1997), eIF3ga may be serving as a link to the cytoskeleton for transport of mRNA, possibly in complex with other initiation factors or subunits.

Initiation Factors with Insensitive Phenotype: Regulation via Less Common Mechanisms

Sometimes, the regulatory activity of initiation factors is itself regulated and knockdown yields surprising results. For instance, one study found that *eIF1A* mutations reduced general translation yet increased expression of yeast GCN4 (Fekete et al., 2005). The latter study is particularly interesting because yeast cells normally increase GCN4 translation when eIF2 is phosphorylated as a stress response to amino acid depletion, which renders GTP/eIF2/ $tRNA_i^{Met}$ ternary complexes (TCs) inactive. Under normal conditions, regulatory short open reading frames (ORFs) in the 5' UTR of *GCN4* transcripts slow down translation of functional GCN4 proteins. When the level of TCs is low due to inactivation, ribosomes are more likely to bypass 5' UTR ORFs beyond the first as they wait for rebinding with TC, resulting in preferential binding to the start codon for GCN4 and increased expression (Hinnebusch, 1993). eIF1A guides the recruitment of the TC to the 40S subunit, and reduction of eIF1A may result in the same slowed scan rate that causes the 40S subunit to bypass regulatory UTR ORFs (Fekete et al., 2005; Li et al., 2014). This counter-intuitive effect of *eIF1A* knockdown could also be the cause for the observed insensitive phenotype.

eIF4A also plays additional roles within cells. For instance, this RBP could be affecting a signalling pathway with a role in nociception. For instance, eIF4A was found to negatively regulate Dpp/BMP signalling through degradation of necessary proteins (Li and Li, 2006). eIF4A functions differently in ovarian germ stem cells, however; rather than regulating BMP signalling, it is able to bind directly with and inactivate BAM, thereby

promoting cell maintenance over differentiation (Shen et al., 2009). As an RNA helicase, eIF4A likely has additional roles in RNA metabolism to be discovered (Linder, 2006; Schwer and Meszaros, 2000)

Initiation Factors with Insensitive Phenotype: Regulation via Stress Response and Alternative Translation Mechanisms

Identifying six initiation factors with validated insensitive phenotypes supports the potential for translational control of nociception, but the number of necessary factors that apparently do not impact nociception must be considered. Additionally, one validated gene suggests an unexpected mechanism for translational regulation. As previously discussed, serine kinases are able to phosphorylate the alpha subunit of eIF2 when a cell is under stress, shutting down cap-dependent translation and activating cap-independent translation. The beta subunit provides eIF2 with the binding sites for eIF5 and eIF2B, necessary components for cap-dependent translation (Asano et al., 2000; Asano et al., 2001). Interestingly, RNAi knockdown of eIF2α resulted in an insensitive phenotype, but knockdown of eIF2β did not cause any abnormal nocifensive behavior. In other words, the subunit necessary for eIF2's function in cap-dependent translation is apparently unnecessary for normal nociception. On the other hand, loss of the subunit necessary for deactivating eIF2 and shutting down cap-dependent translation resulted in a nociception defect, indicating the potential for regulation of nociception via cap-independent translation, possibly as a previously unidentified stress response.

Given that at least 3% of mRNAs in humans potentially contain an internal ribosome entry site (IRES) sequence and many of those identified are involved with stress responses (Johannes et al., 1999), it is certainly possible for cap-independent translation to be a factor

in nociception. This would also be consistent with the lack of phenotype found in knockdown of eIF5 and the presence of insensitive phenotype in only noncanonical 4G and 4E subunits. It would also explain eIF4G2's lack of eIF5-binding domain. If eIF4G2 is involved in cap-independent translation that does not require eIF5, the relevant binding domain would not be necessary. The noncanonical characteristics of eIF4E3 make more sense in this context as well. Involvement in atypical or stress-dependent translation would explain the lack of canonical regulation mechanisms.

eIF1A and eIF4A are both linked to somewhat minor roles in stress response. For instance, when genes encoding DNA repair enzymes are mutated in oocytes, a checkpoint activates that halts the process of meiosis in part by repressing Grk translation, yet mutating to *eIF1A* in these mutants restores Grk translation. This suggests eIF1A plays a role in this stress response pathway functioning appropriately, the mechanism of which has not been determined (Li et al., 2014). It was proposed that the presence of eIF1A may be necessary for repression of normal translation activity. eIF4A is an active component of the granules that form as part of stress response, possibly for the purpose of mRNA metabolism (Anderson and Kedersha, 2008), but the mechanisms of stress granules are still largely a mystery. It is possible that the helicase activity of eIF4A would be useful in cap-independent translation. IRESes have been found in the 5' UTR of mRNAs localized in dendrites (Pinkstaff et al., 2001), and eIF4A may play a role in unwinding these regions to read the mRNA for binding with its IRES.

Although its specific function is unknown, one study has shown that eIF4A is vital to IRES-mediated translation (Thoma et al., 2004). The same study also established the poly-A tail and the requirement of a 4G subunit for binding. Moreover, cleavage of 4G subunits

impacted cap-dependent translation but stimulated IRES-mediated translation. This supports the potential for an IRES-specific subunit like eIF4G2 that can bind to eIF4E3 and the poly-A tail but not eIF5, and therefore likely to be preferentially involved in cap-independent translation. It is also worth noting that human EIF4G1 and EIF4G2 contain IRES sequences (Johannes and Sarnow, 1998).

One characteristic that has already been demonstrated as shared by sensory neurons and IRES-mediated translation is the need for dendrite localization. In one study, five mRNAs were identified that were regularly translated via IRES in addition to cap-dependent mechanisms, and that IRES-mediated translation was more efficient in the dendrites than cell body. These mRNAs equipped with IRES sequences in their 5' UTR were continuously expressed, even in conditions that caused general expression levels to fall (Pinkstaff et al., 2001). In *Aplysia californica*, an egg-laying hormone increases expression in response to the physiological signal to lay eggs. It was determined that this happens via IRES-mediated translation (Dyer et al., 2003). This study provides a basis for physiological stimulus activating IRES-mediated translation, and underscores the importance of this translation method at synapses due to a limited ribosome population. The same has been found true in axons, where recovery of sensory neurons from the physiological distress of photobleaching was accomplished via IRES-mediated translation occurring in axons (Pacheco and Twiss, 2012).

There is a great deal of support for the possibility that there is a nociceptor-specific means of translation that functions independently of the 5' cap. All seven validated RBPs could be playing either a direct role in this mechanism or an indirect role (e.g. localization).

Further research is needed, but if this is indeed the case, it presents eIF2α as a potential treatment target (Kim et al., 2012).

Validated Hypersensitive Phenotype: eIF2Bα

eIF2B is an interesting RBP made up of 5 different subunits, each one serving as a target for one or more different regulators, making eIF2B a component of a wide range of diverse mechanisms (Pavitt, 2005). The α subunit serves as a target for eIF2α. When kinases such as GCN2, PERK, PKR, or HRI are activated, these stress-response kinases and translation inhibitors phosphorylate eIF2α, which increases its affinity for binding with eIF2Bα, rendering eIF2B inactive.

It is surprising that knockdown of this gene produced the opposite phenotype from knocking down eIF2α. One possible explanation is that the two inactivations serve different purposes. For instance, nociception may be dependent on eIF2α phosphorylation to start the cascade of signaling events that make conditions more favorable for cap-independent translation. If inactivation of eIF2B is the end of that particular chain and not needed to trigger any other reaction, the opposite phenotypes could make sense. Nociception may be dependent on eIF2α phosphorylation as a trigger for other events and would be inhibited by lack of eIF2α expression. If nociception function is enhanced by inactivation of eIF2B, knocking down eIF2Bα expression could also enhance nociception if the lack of that subunit interferes with eIF2B function. Active eIF2α binds with eIF2B via its alpha unit in the course of normal cap-dependent translation, so lacking eIF2Bα could cause repressed cap-dependent translation and enhance IRES-mediated translation.

Validated Hypersensitive Phenotype: Pumilio

Pumilio (Pum) is a known translation repressor, regulating both cap-dependent and IRES-mediated translation, depending on cell type and context (Wharton et al., 1998). One known Pum function is regulation of neuronal excitability through repression of *para* translation. In one study, *para* overexpression and *pum* knockdown both resulted in hyperexcitability, but this term was described as a faster onset of sensitization following prolonged stimulation (Mee et al., 2004; Schweers et al., 2002). Given the experiments described in the study, it seems unlikely that latency in a thermal nociception assay would trigger this type of hyperexcitability.

Additionally, Pum repression of *para* mRNA in neurons apparently requires Nanos in all types of neurons and Brat in some types (Muraro et al., 2008), but neither *Nanos* nor *Brat* exhibited a phenotype in this screen. Thermal nociception assays on mutants overexpressing *para* could be illuminating if a hypersensitive phenotype results, but interaction with *para* may not be reason for *pum*'s phenotype. Pum also has a known role in regulating synaptic function in the neuromuscular junction but its activity in that context is postsynaptic (Menon et al., 2004) and may not be relevant.

Hundreds of genes are subject to regulation by Pum (Gerber et al., 2006) and finding the target resulting in this phenotype could be difficult. One possible target that would be particularly useful if identified is TrpA1-A. This isoform activates at 24-29°C, well below normal nociception threshold (Viswanath et al., 2003). Ectopic expression of this isoform in nociceptors causes a nocifensive reaction in the lower temperature range (Zhong et al., 2012). It is possible that TrpA1-A transcripts are present in nociceptors but repressed under normal circumstances (e.g. by Pum) and that injury activates their translation. This type of

mechanism could explain the development of allodynia, in which skin becomes more sensitive to stimuli following injury.

Future Directions

The results from this screen have opened up numerous avenues for potential future research. Hits that have not been validated will need to be tested more thoroughly. Additionally, completing a full screen at 42°C of all genes that did not exhibit a phenotype could identify additional hits with a hypersensitive phenotype. It would also be beneficial to pursue molecular experiments with validated hits from either or both screens.

Using Channelrhodopsin-2 from green algae to produce light-activated nociceptors in target gene mutants would be the next step to discern defects in signal reception versus transmission (Hwang et al., 2007), particularly when nociception defects occur in conjunction with dendritic defects. It is important to note that these thermal assays involved stimulation of 2-3 segments of the body wall; therefore, it is likely that in nociceptors with morphological abnormalities and normal nocifensive response, the dendrites are still functioning normally and were stimulated by the probe. In cases of dendrite abnormalities combined with a nociception defect, it is possible that the defect causing abnormal morphology also resulted in dysfunctional behavior. However, the lack of correlation indicates the potential for defect causes unrelated to dendrite functionality.

Given the previously discussed lack of correlation with identified splicing targets, it would also be worthwhile to evaluate potential splicing regulators for impact on splicing events affecting nociception. PCR could be used to identify any changes in splicing targets of interest. *eIF4A, eIF4AIII*, and *eIF3ga* are particularly strong candidates to evaluate for

effects on alternative splicing. *eIF2Bα* and *pumilio* should also be evaluated due to their hypersensitive phenotype, which indicates potential expression of *TrpA1-A*.

The role of Pum will also require additional testing to discover its target for repression. Larvae overexpressing Para could be tested for hypersensitivity. Testing for binding of Pum with *TrpA1* mRNA, isoform A in particular, would also be useful. Other translation repressors expressed in sensory neurons could also be considered. If there are repressed *TrpA1-A* transcripts maintained in nociceptors, a low-temperature screen of RNA-binding proteins could help to identify the repressor involved.

Finally, the possibility of a mode of translation specific to proteins that enable sensory transduction in nociceptors, possibly IRES-mediated, should be explored. Binding between eIF4G2 and eIF4E3 in nociceptors should be tested. Loss-of-function behavioral testing of Gcn2 and PEK kinases in nociceptors is another option. If nociception is dependent on phosphorylation of eIF2α, eliminating the kinase responsible for phosphorylating eIF2α in nociceptors should produce the same phenotype as *eIF2α-RNAi*. 3’ and 5’ UTRs of mRNAs relevant to nociception could also be analyzed for IRES sequences.

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Appendix A: Stocks Tested

VDRC

CG #	Stock #		CG #	Stock #		CG #	Stock #
CG3193	39335 GD		CG4528	104334 KK		CG9809	103355 KK
CG5454	22132 GD		CG12759	104183 KK		CG4396	101508 KK
CG12924	108336 KK		CG9696	7787 GD		CG5637	108900 KK
CG8241	47782 GD		CG7185	107147 KK		CG9755	45815 GD
CG10418	105940 KK		CG4119	26395 GD		CG9373	44658 GD
CG12085	20144 GD		CG9075	42202 GD		CG6203	8933 GD
CG7437	105495 KK		CG4602	51088 GD		CG14648	110736 KK
CG9998	24176 GD		CG33197	105486 KK		CG11334	109672 KK
CG8144	24214 GD		CG31061	106079 KK		CG5589	108642 KK
CG7269	22557 GD		CG9218	108351 KK		CG14718	105543 KK
CG5931	43962 GD		CG10084	105950 KK		CG32706	109212 KK
CG14641	38790 GD		CG17492	108947 KK		CG6961	109951 GD
CG6841	34253 GD		CG17686	108186 KK		CG6418	108552 KK
CG2926	33589 GD		CG9680	108310 KK		CG18259	50094 GD
CG10445	104753 KK		CG10203	100226 KK		CG40351	40683 GD
CG6227	110778 KK		CG6866	108358 KK		CG5168	110451 KK
CG3582	110075 KK		CG6375	106078 KK		CG5705	108376 KK
CG31762	107459 KK		CG13425	105271 KK		CG10777	109465 KK
CG16941	20338 GD		CG5605	45027 GD		CG7903	106475 KK
CG12749	51759 GD		CG10719	105054 KK		CG11505	105949 KK
CG11360	38491 GD		CG6539	49506 GD		CG8053	100611 KK
CG8749	23150 GD		CG32423	37863 GD		CG9946	104562 KK
CG6197	104186 KK		CG33100	38399 GD		CG4153	105291 KK
CG10851	38860 GD		CG16901	32395 GD		CG8882	103141 KK
CG10210	38356 GD		CG9054	103365 KK		CG8636	105325 KK
CG5442	104978 KK		CG6779	106321 KK		CG9769	101465 KK
CG10128	8868 GD		CG4792	106041 KK		CG4878	107829 KK
CG10851	38860 GD		CG33106	103411 KK		CG10124	107595 KK
CG16724	2560 GD		CG8730	108026 KK		CG8280	104502 KK
CG8019	41023 GD		CG9412	109911 KK		CG1873	102736 KK
CG6999	41828 GD						

TRiP (Bloomington)

CG #	Stock #		CG #	Stock #		CG #	Stock #
CG6946	36066		CG17454	43199		CG10279	31395
CG5263	35477		CG5422	28035		CG12413	41839
CG10868	25843		CG9177	34841		CG4035	34096
CG6493	27486		CG10840	44418		CG10192	35809
CG4262	28371		CG10837	57305		CG3845	32357
CG6137	35201		CG10306	44493		CG7883	55624
CG1691	34977		CG7483	32444		CG8023	42804
CG10293	55665		CG10811	33049		CG6995	51759
CG42320	55908		CG5728	55916		CG9124	55603

NIG

CG #	Stock #		CG #	Stock #
CG11266	11266R-3		CG5439	5439R-1
CG17540	17540R		CG3056	3056R-1

Appendix B: Fly Food Protocol

The maintenance of a healthy fly colony absolutely depends on consistent fly food quality and production. We have previously found that nociception-related behaviors (and likely other behaviors as well) are highly dependent on food composition and quality. Thus it is essential maintain a rigorous protocol for cooking fly food of standard composition, and continuously monitor that food for quality and consistency. We are currently using the Nutri-Fly Molasses Formulation produced by Genesee Scientific in 1L pouches (#66-116). Using this formulation, one pouch produces enough food for 4-6 bottles and 75-90 vials. We are cooking one pouch of food a week currently, but this protocol should be easily scaled to two or three pouches.

1. Obtain **1L deionized water** from purification system in 3rd floor autoclave room. Empty one pouch of Nutri-Fly MF into a two-liter Erlenmeyer flask and add ~500ml of deionized water. Swirl flask vigorously to fully suspend solids and break up any large chunks.

2. Add a large stir bar to the flask as well as the remaining ~500ml of deionized water. The water can be used to wash off any solids stuck around the walls of the flask.

3. Set stirring hot plate to **~300°C** and stirring speed to 6 on the dial. **Cover** the flask with aluminum foil and place it on the hot plate and make sure that you are getting vigorous stirring (i.e. a big whirlpool in the center of the flask). The speed will need to be adjusted upward as it thickens (up to 7.5).

4. Cook the food until it reaches a rolling boil, which takes approximately 35 minutes. Watch closely during the last 5-10 minutes to prevent the food from boiling over.

5. When food reaches boiling, turn the temperature of the hot plate to **135°C**, remove the foil cover, and set timer for **15 minutes (simmer time)**. If covered, the food will threaten to boil over. Once food has settled down, replace cover. Continue simmering with foil cover in place until timer is finished. Remove from heat when finished and turn the hot plate temperature to zero.

6. When flask has **cooled for 10 minutes**, return the flask to stir plate and stir at speed 6. Add **4.8ml of 99% propionic acid** [and **10 mL Tegosept when applicable**]. Replace cover, set stir speed to 7-7.5, and **stir** for an additional **5 minutes**. During this time, fill food dispenser tank to appropriate water level, which is marked inside the tank (~500 ml).

7. Pour the liquid food into the dispenser, put pump/cover in place, and turn on dispenser. Flask should be washed immediately with very hot water. Dispense food into vials and bottles. Spacers can be used on the pump handle such that one pump will dispense approximately 3-4cm of food into a polypropylene narrow vial, which is a good target amount. Several pumps will be required to dispense a similar height into a glass bottle. When you have finished dispensing, there will be enough food left in the dispenser to pour into 2 additional bottles (if prepared food can be used for stocks).

8. The inner tank and pump/cover of the food dispenser must be washed immediately with very hot water. Any remaining food must be significantly diluted in very hot water to prevent solidification of the food, which will clog pipes. Be sure to dispense hot water through the pump until it runs clear.

9. **Label boxes clearly with date and purpose, i.e. "for crosses" or "for stocks" and "+tego" for any food containing Tegosept.** Food should be allowed to dry at room temperature for approximately 24 hours. Cover food with cheesecloth to prevent infestation by escaped flies.

10. Plug food and store at 4°C once drying time is complete. Most, if not all, condensation should be evaporated from the walls of the vials and bottles at this point.

Notes: Food humidity is often the biggest source of variation in food quality. Water levels and drying time can be adjusted if batches of food are noticed to either be peeling away from the walls of their containers or accumulating water on their surface.

Materials and equipment:
Nutri-Fly MF packets (Genesee Scientific #66-116)
Stirring hot plate
Water-heated hot fudge dispenser (for dispensing food)
99% propionic acid in water (Genesee Scientific #20-271)
Cheesecloth (Genesee Scientific #53-100)
Polypropylene narrow vials with cotton ball plugs (Genesee Scientific #32-120BC)
Glass fly bottles

Appendix C: Video Analysis Protocol

1. Open Adobe Premiere Pro.
2. Choose "New Project."
3. Enter a file name if desired, then click "OK."
4. Click "OK" on the "New Sequence" window that opens.
5. Click on the File menu and choose "Import…" (Shortcut: Ctrl+I).
6. Choose video for analysis and click "Open."
7. The video will appear in the "Project" pane in the bottom left corner. Drag the video to the "Video 1" track in the "Sequence" pane (to the right of the Project pane). A "Clip Mismatch Warning" will appear; click "Change sequence settings."
8. If running the program for the first time, the video will appear in the top right corner. Click on the menu icon in the top right corner of the video panel and select "Undock Frame." You can now drag the frame to the left-hand monitor and resize it to fit screen.
9. Click on the time scroll bar at the bottom of the video to make it active.
10. Using the left and right arrow keys will advance or reverse the video one frame at a time. Shift+arrow will advance or reverse five frames at a time (useful for moving quickly to next trial).
11. Create an Excel spreadsheet to enter information for the video being analyzed.
    a. Enter each trial as a separate row with two columns for start/stop times. The moment of probe connecting with larva is recorded as "start time." The moment of roll completion (or >10 sec) is "stop time." Record data for each larva in the spreadsheet as you progress through your video. Label each cross appropriately to keep data separate.
    b. Video time is shown as hh:mm:ss:ff. "ff" indicates number of frames. There are 30 frames per second, so 3 frames = 0.1 sec. Round to the nearest tenth. Examples:
       - 00:00:03:10 is 3 seconds and 10 frames, which can be rounded to 3 seconds and 9 frames, or 3.3 seconds.
       - 00:00:03:29 is 3 seconds and 29 frames, which can be rounded to 3 seconds and 30 frames, or 4.0 seconds.
    c. Create an additional column to automatically calculate latency between start and stop time (subtract start time from stop time).
    d. Only input time point data required to calculate latency (i.e. don't include minutes). Although a start time greater than 50 seconds will require a stop time greater than 60 seconds to calculate properly, enter the time as seconds, not minutes (i.e. 58:06 - 01:16 becomes start time: 58.2; stop time: 61.5; and 61.5 - 58.2 = 3.3 seconds latency).
    e. See Page 2 for example of video data spreadsheet.

Sample: data collection and spreadsheet

Video Start/Stop times:	Start/Stop time converted to seconds:
1. 00:00:31:06, 00:00:38:13	[31.2, 38.4]
2. 00:01:58:29, 00:02:04:07	[59, 64.2]
3. 00:03:21:06, 00:03:34:16	[21.2, 34.5]
4. 00:04:56:11, 00:05:06:17	[56.4, 66.6]
5. 00:06:46:04, 00:06:49:12	[46.1, 49.4]
6. 00:07:31:02, 00:07:42:22	[31.1, 42.7]
7. 00:0848:24, 00:08:54:28	[48.8, 54.9]
8. 00:9:49:28, 00:10:02:13	[49.9, 62.4]
9. 00:11:19:13, 00:11:32:03	[19.4, 32.1]
10. 00:13:29:14, 00:13:38:06	[29.5, 38.2]
11. 00:14:27:08, 00:14:37:07	[27.3, 37.2]
12. 00:16:03:11, 00:16:09:11	[3.4, 9.4]
13. 00:17:34:16, 00:17:46:06	[34.5, 46.2]
14. 00:18:14:08, 00:18:24:04	[14.3, 24.1]

Corresponding spreadsheet layout:

	A	B	C	D
1	Assay Date: 7/9/15			
2	Cross Date: 7/2/15			
3	Gene tested: CG2926			
4	Filename: 20150709b.mts			
5				
6	Trial	Start	Stop	Latency
7	1	31.2	38.4	7.2
8	2	59	64.2	5.2
9	3	21.2	34.5	13.3
10	4	56.4	66.6	10.2
11	5	46.1	49.4	3.3
12	6	31.1	42.7	11.6
13	7	48.8	54.9	6.1
14	8	49.9	62.4	12.5
15	9	19.4	32.1	12.7
16	10	29.5	38.2	8.7
17	11	27.3	37.2	9.9
18	12	3.4	9.4	6
19	13	34.5	46.2	11.7
20	14	14.3	24.1	9.8

(Column D contains formulas, e.g. “=C7-B7” in D7, “=C8-B8” in D8, etc.)

Appendix D: Statistics

Wild-Type Data Comparison

Kruskal-Wallis One Way Analysis of Variance on Ranks:

Group	N	Missing	Median	25%	75%
isoW	278	0	3.2	2.3	4.4
y w; attp	440	0	2.9	2	3.8
y v; attP2, y+	155	0	2.3	1.5	3.1
y v; attP40, y+	54	0	2.8	2	3.725
w^{1118}	72	0	2.9	1.925	3.975

H = 53.911 with 4 degrees of freedom. ($p < 0.001$)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p < 0.001$)

All Pairwise Multiple Comparison Procedures (Dunn's Method):

Comparison	Diff of Ranks	Q	P	P < 0.050
isoW* vs *y v; attP2, y+	211.169	7.301	<0.001	**Yes**
isoW vs *attP40*	78.498	1.829	0.673	No
isoW vs *W[1118]*	70.968	1.86	0.629	Do Not Test
isoW vs *yw; attp*	61.63	2.788	0.053	Do Not Test
y w; attp* vs *y v; attP2, y+	149.54	5.549	<0.001	**Yes**
y w; attp vs *y v; attP40, y+*	16.868	0.405	1	Do Not Test
y w; attp vs w^{1118}	9.338	0.255	1	Do Not Test
w^{1118}* vs *y v; attP2, y+	140.202	3.407	0.007	**Yes**
w^{1118} vs *y v; attP40, y+*	7.53	0.145	1	Do Not Test
y v; attP40, y+* vs *y v; attP2, y+	132.672	2.91	0.036	**Yes**

Validations

Kruskal-Wallis One Way Analysis of Variance on Ranks (*y v; attP2, y+* wild-type background)

Group	N	Missing	Median	25%	75%
y v; attP2, y+	58	0	2.35	1.775	3.5
eIF4G2 RNAi	55	0	2.9	2	4
eIF4AIII RNAi	54	0	3.8	2.15	6.625
eIF4E3 RNAi	52	0	3.35	2.35	4.575

H = 18.972 with 3 degrees of freedom. ($p < 0.001$)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p < 0.001$).

Multiple Comparisons versus Control Group (Dunn's Method)

Comparison	Diff of Ranks	Q	P	P < 0.050
eIF4AIII RNAi vs y v; attP2, y+	49.7	4.148	**<0.001**	**Yes**
eIF4E3 RNAi vs y v; attP2, y+	37.998	3.14	**0.005**	**Yes**
eIF4G2 RNAi vs y v; attP2, y+	29.481	2.472	**0.040**	**Yes**

Kruskal-Wallis One Way Analysis of Variance on Ranks (*y w; attp* wild-type background)

Group	N	Missing	Median	25%	75%
y w; attp	61	0	2.8	2.05	3.65
eIF1A RNAi	63	0	8.4	6.5	11
eIF2α RNAi	63	0	4.5	2.5	6.3
eIF3ga RNAi	66	0	4.5	2.875	6.1

H = 92.884 with 3 degrees of freedom. ($p < 0.001$)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p < 0.001$).

Multiple Comparisons versus Control Group (Dunn's Method)

Comparison	Diff of Ranks	Q	P	P < 0.050
eIF1A RNAi vs y w; attp	125.152	9.521	**<0.001**	**Yes**
eIF3ga RNAi vs y w; attp	52.965	4.075	**<0.001**	**Yes**
eIF2α RNAi vs y w; attp	48.43	3.684	**<0.001**	**Yes**

Mann-Whitney Rank Sum Test (*eIF4A RNAi*)

Group	N	Missing	Median	25%	75%
eIF4A RNAi	77	0	6	3.7	8.1
isoW	79	0	2.5	1.6	3.3

Mann-Whitney U Statistic= 847.500
T = 8238.500 n(small)= 77 n(big)= 79 ($p < 0.001$)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference ($p < 0.001$).

Mann-Whitney Rank Sum Test (*eIF2Bα RNAi*)

Group	N	Missing	Median	25%	75%
eIF2Bα RNAi	61	0	8.1	5.2	11
y v; attP40, y+	51	0	11	7.9	11

Mann-Whitney U Statistic= 1037.000
T = 3400.000 n(small)= 51 n(big)= 61 ($p = 0.001$)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference ($p = 0.001$).

Mann-Whitney Rank Sum Test (*pumilio RNAi*)

Group	N	Missing	Median	25%	75%
pumilio RNAi	51	0	4.7	3.6	6.4
y v; attP2, y+	45	0	6.1	4.2	10.05

Mann-Whitney U Statistic= 858.000
T = 2472.000 n(small)= 45 n(big)= 51 ($p = 0.034$)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference ($p = 0.034$).

Vita

Amber Dawn Dyson (née Cavanaugh) was born in 1977 in Valladolid, Spain. Her father is George Cavanaugh. She moved stateside in 1981. She graduated from Girls Preparatory School (Chattanooga, TN) in 1995 and was accepted to Georgia Institute of Technology with a full scholarship. She left after one quarter to pursue a nomadic lifestyle. She married Spencer Dyson in 2001 and became subject to the whims of the U.S. Navy, of which her husband was a member. She attended Dalton State College (GA) and Hawaii Pacific University before earning her Associate of Science degree in Math and Science from Cayuga Community College (NY). She continued her studies in biochemistry at University of North Florida, but her family was transferred before she graduated from UNF. Due to a lack of available science programs in commuting distance, she earned her Bachelor of Arts in Psychology from Thomas Edison State College (NJ) via their distance program serving military members and families.

In 2013, Ms. Dyson accepted a Chancellor's Fellowship at Appalachian State University (NC) Graduate School for study in Marriage and Family Therapy. She transferred to the Biology department the following year and became the first graduate student member of the Bellemer molecular neurobiology research laboratory. In 2017, she was awarded her Master of Science in Biology with a concentration in Cell and Molecular Biology.

After completing her research, Ms. Dyson moved with her children to Morgantown, WV where her husband had gained employment as a civilian. She plans to continue her career in research as a molecular biologist; however, she does not consider herself "settled" with regard to location or future pursuits.