THE ROLE OF MUSCLEBLIND IN NOCICEPTION IN DROSOPHILA MELANOGASTER

A Thesis
by
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Abstract

THE ROLE OF MUSCLEBLIND IN NOCICEPTION IN DROSOPHILA MELANOGASTER

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Chronic pain affects more than 50 million U.S. adults and has an annual economic impact upwards of $650 billion. Chronic pain is often treated with opioids that momentarily reduce pain sensation but can be highly addicting and may not offer a long-term solution to the pain. Nociception is the sensory detection of noxious stimuli by specialized sensory neurons known as nociceptors and is the underlying process through which pain is perceived. Further understanding of the regulatory processes behind sensory neuron sensitivity is important for the development of more effective clinical treatments. The cellular and molecular mechanisms behind nociception and nociceptor sensitivity are highly conserved across the animal kingdom, but the regulatory mechanisms behind this process are not fully understood. The goal of this research is to further the understanding of the roles RNA-binding proteins, specifically muscleblind (mbl), play in the regulation of nociceptor sensitivity. Mbl proteins have been implicated in the regulation of expression of a wide array of genes, particularly in muscle cells, and sequestration of mbl function is known to result in several forms of neuromuscular disease, including muscular dystrophy. Due to mbl role in the pathology of neuromuscular disease, it is
often studied for its role in muscles. However, recent research has demonstrated that mbl knockdown specifically in nociceptors of Drosophila melanogaster results in morphological defects to the nociceptor dendrites, and preliminary data suggests nociceptor-specific mbl knockdown decreases nociceptive response to noxious thermal stimulus.

In this project, nociceptor specific mbl knockdown Drosophila melanogaster larvae were constructed and used to confirm mbl knockdown results in defects in thermal nociceptive response and clV md-da dendrite morphology. Following mbl functional and morphological analysis, a candidate approach was used to analyze possible downstream effectors of mbl involved in regulation of nociceptor sensitivity. Analysis of mbl downstream effectors suggests potential roles for Dystrophin (Dys) and Dystroglycan (Dg), two members of the Dystrophin-Glycoprotein Complex, as well as α-actinin (actn) in thermal nociceptor sensitivity, but not the proneural gene amos. This research confirmed mbl involvement in thermal nociception and identified some downstream effectors involved in the regulation of nociceptor sensitivity. Roles were suggested for Dys, Dg, and α-actinin but further research is needed to confirm the specific functions of these genes in the regulation of nociceptor sensitivity.
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Introduction

Chronic pain impacts more than 50 million U.S. adults and has an annual economic impact upwards of $650 billion dollars that is directly correlated to medical care, lost productivity, and disability services (Pizzo et al., 2012; Dahlhamer et al., 2018). This includes nearly $100 billion spent annually from state and federal budgets (Pizzo et al., 2012). Chronic pain is defined as pain that exists over an extended period, typically longer than three months (International Association for the Study of Pain, 1986). Chronic pain can be influenced by a number of physical, psychological, and social factors, and is one of the most prominent reasons adults seek medical care with total financial expenditure on treatment higher than that of heart disease, cancer, and diabetes combined (Pizzo et al., 2012; Van Hecke et al., 2013).

Chronic pain is most often treated with fast-acting or extended-release pain relievers, like opioids, that temporarily stop pain sensation but may not offer a long-term solution to the pain. Opioids are highly addictive and long-term use has been shown to cause many, often deadly, cardiovascular and pulmonary problems (Rosenblum et al., 2008; Radke et al., 2014; Corbett et al., 2019). The use of opioids to treat chronic pain has contributed to the current opioid epidemic in the United States, where opioids account for 72% of all deadly drug overdoses and 50,000 opioid related overdose deaths occur annually (CDC/NCHS, 2019). Due to the dangers and ineffectiveness of long-term opioid prescription in the treatment of pain, it is the responsibility of the scientific community to explore other treatment options. One obstacle to this exploration is the incomplete understanding of the molecular mechanisms underlying chronic pain. Identification of the underlying cellular and molecular mechanisms that regulate
pain could be influential to the discovery of novel methods for treating sensory disease, like chronic pain, that does not involve opioid prescription.

**Nociception**

Animals use sensory perception to navigate their environment and avoid noxious, potentially tissue damaging, stimuli. Avoiding noxious stimuli is a vital function of the sensory nervous system because tissue damage is detrimental to an organism’s survival (Dafny et al., 2020). Nociception is the sensory detection of noxious stimuli by specialized sensory neurons known as nociceptors and is the underlying process through which pain is perceived (Sherrington, 1906; Bessou and Perl, 1969). The nerve endings of nociceptors innervate throughout the body, including bone, muscle, skin, and internal organs. Nociceptors are pseudounipolar neurons with cell bodies found in the dorsal root ganglia. One end of the axon extends from the cell body in the dorsal root ganglia (DRG) to innervate the target sensory organ, while the other axon ending synapses along the spinal cord (Basbaum et al., 2009; Kendroud et al., 2022).

The process of nociception begins with the detection of noxious stimuli by nociceptors. In vertebrates, nociceptors can be activated by excess thermal, mechanical, UV, or chemical noxious stimuli (Bessou and Perl, 1969). After activation by a noxious stimulus, nociceptors transmit a signal into the grey matter of the dorsal horn where it is passed to a second order neuron via the release of glutamate. This second order neuron then transmits the signal across the spinal cord and up the spinothalamic tract to the thalamus where the signal is perceived by the brain as pain (Figure 1) (Millan, 1999).
In vertebrates, there are two primary classes of nociceptors: those composed of Aδ fibers and those composed of C-fibers. Aδ-fibers are lightly myelinated while C-fibers are non-myelinated. This difference in myelination results in Aδ and C-fiber nociceptors having differing functions. Aδ nociceptors carry acute and highly localized pain and are primarily activated by exposure to noxious mechanical stimuli. C-fibers are smaller in diameter, and most are polymodal, meaning they can detect multiple types of stimuli. The relatively smaller diameter and lower myelination of C-fibers produces a low signal conduction velocity by C-fibers which creates the slow-onset, dull pain sensation associated with chronic pain.

Figure 1. The different types of nociceptive nerve fibers and the stimuli needed to activate each type. The peripheral terminals detect the stimuli that lead to the action potential propagated down the axon of the corresponding fiber. Aβ fibers are activated via innocuous mechanical stimuli such as gentle touch. Aδ fibers respond to both innoxious thermal stimuli and noxious mechanical stimuli. Lastly, C fibers respond to noxious stimuli: thermal, mechanical, and chemical stimuli. (Machen, 2021)
The cellular and molecular mechanisms behind nociception and nociceptor sensitivity are highly conserved across the animal kingdom. For example, the transient receptor potential (TRP) protein family is a highly conserved family of ion channels required for normal nociceptor sensitivity to many forms of noxious stimulus in both vertebrate and invertebrate species. In vertebrates, TRPA1 and TRPV1 channels are found along the membrane of nociceptors and are activated by various noxious stimuli including noxious heat (Caterina et al., 1997; Sinica et al., 2019). Loss of function experiments involving these TRP channels results has been shown to produce defects in thermal nociceptor sensitivity. Many invertebrates including, *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster*, have similar thermosensory TRP channels involved in nociception and knockdown or knockout of these channels also produces defects in thermal nociceptor sensitivity (Glauser et al., 2011; Neely et al., 2011; Zhong et al., 2012; Fowler et al., 2013; Ohnishi et al., 2020). Nociceptor sensitivity to noxious stimulus is important in the sensation of pain. The more sensitive the nociceptor to a stimulus, the more likely it is to transmit the signal to the brain and produce a pain response. While the cellular and molecular mechanisms behind nociception and nociceptor sensitivity are highly conserved, how these mechanisms are regulated has not been fully described. Further understanding of how nociceptor sensitivity is regulated will contribute to a more thorough understanding of nociceptive function and nociceptor sensitivity.

*Drosophila Melanogaster as a Nociceptive Model*

*Drosophila melanogaster* is an effective model for studying nociception and the nervous system. This is due in large part to their rapid life cycle, similarity of sensory neuron structure and function to humans, conservation of genes, and ease of nociceptive analysis (Pandey and
Nichols, 2011). *Drosophila melanogaster* have a life cycle of about 10 days, making it easy for several generation of *Drosophila* to be examined over a short period of time (Pandey and Nichols, 2011). Additionally, 60% of human genes have a homologous gene in the *Drosophila* genome (Mirzoyan et al., 2019). A perfect example of this in relation to nociception is the aforementioned TRPA1 channels. In vertebrates, *TrpA1* encodes proteins that form calcium cation channels in nociceptors that are necessary to nociceptive function (Kwan et al., 2006). Activation of these channels initiates the pain perception pathway. *Drosophila* have a homologous gene known as *dTrpA1* that also encodes proteins that form calcium cationion channels and is required for normal nociceptive function in both adults and larvae (Neely et al., 2010; Zhong et al., 2012).

The ease of conducting nociceptive assays on *Drosophila melanogaster* makes them an ideal model for studying nociception. When the nociceptors of *Drosophila* larvae are exposed to a noxious stimulus they exhibit a reflexive 360° corkscrew maneuver known as nociceptive escape locomotion (NEL) (Tracey et al., 2003). This unique escape mechanism is thought to have evolved as a method for evading parasitic wasp attacks and is distinct from normal *Drosophila* locomotive behaviors (Tracey et al., 2003; Hwang et al., 2007). NEL can be quantified and used as an indicator of degree of nociceptive response by measuring the time it took the larvae to perform NEL after being exposed to a noxious stimulus (Tracey et al., 2003). The time between exposure to noxious stimulus and completion of NEL is known as response latency. The longer the response latency, the lower the nociceptive function of the larvae. The response latency of experimental larvae can be compared to the response latency of wild-type larvae to determine if the experimental larvae exhibit altered nociceptive function. This finding
demonstrates a method for using *Drosophila melanogaster* as a model for nociceptive response to noxious thermal stimuli.

*Drosophila melanogaster* have a simple nervous system compared to humans, making it an excellent model for nociceptive function. The epidermal walls of *Drosophila melanogaster* larvae exhibit a stereotyped patterning by peripheral nervous system (PNS) neurons. This stereotyped patterning allows for the classification of these epidermal PNS neurons (Bodmer and Jan, 1987). The larval peripheral system is composed of segmentally repeating Type 1 and Type 2 sensory neurons. Type 1 are ciliated and have a single dendrite (Hartenstein, 1988). Type 2 are non-ciliated, have multiple dendrites projections, and have naked nerve endings; similar to those seen in mammalian nociceptors. Type 2 neurons are often referred to simply as multi-dendritic neurons (MD) (Gao et al., 1999; Grueber et al., 2002).

MD neurons line the epidermal wall of the individual body segments of *Drosophila* epithelium and can be divided into three classifications: MD neurons that have bipolar dendrites (md-bp neurons), MD neurons that arborize around particular tracheal branches (md-td neurons), and MD neurons that give rise to elaborate dendritic arborizations (md-da neurons) (Bodmer and Jan, 1987). *Drosophila* md-da can be further subdivided into four classes (cI-IV md-da neurons) based on patterning and complexity of their dendritic arbor, with cI md-da having the simplest arbors, and cIV md-da having the most complex (Grueber et al., 2002). Three to five neurons per md-da class are arranged along the dorso-ventral axis of each abdominal hemi segment (Figure 2). The dendrites of cIII md-da and cIV md-da neurons independently innervate the entirety of the *Drosophila* epidermal wall, while cI md-da and cII md-da do not provide complete coverage but still innervate independently (Grueber et al.,
While all classes are found along each segment, not all classes cover the entirety of each hemi-segment. Independent innervation of md-da neurons suggests *Drosophila* md-da neurons act as individual sensory structures instead of responding as a group when exposed to a noxious stimulus.

Three cl md-da neurons are found in each body segment (Greuber et al., 2002). These neurons are proprioceptive and respond to the peristaltic contraction or bending of the larval body wall (Vaadia et al., 2019). Four cII md-da neurons are found in each body segment and they, along with cIII md-da neurons, are involved in gentle touch (Greuber et al., 2002; Tsubouchi et al., 2012; Yan et al., 2013). No distinct functional features for cII md-da neurons have been established. There are five cIII md-da neurons found in each segment and they respond to noxious cold in addition to gentle touch (Greuber et al., 2002; Turner et al., 2016). cIV md-da neurons are the largest and most complex of the md-da neurons and three are found in each hemi-segment (Greuber et al., 2002). cIV md-da neurons are polymodal nociceptors required for sensory response to noxious thermal (Tracey et al., 2003), mechanical (Zhong et al., 2010), UV (Xiang et al., 2010), and chemical (Lopez-Bellido et al., 2019) stimuli and are the focus of this study.
GAL4/UAS System: A Tool for Tissue-Specific Genetic Manipulation

The GAL4/UAS system is a genetic tool utilized for tissue-specific modification of gene expression in *Drosophila*. This genetic system utilizes two transgenic fly lines, a GAL4 “driver” line and UAS “responder” line. The GAL4 “driver line” encodes a transcription factor protein downstream of a tissue-specific promoter. The UAS “responder line” contains a ubiquitously expressed upstream activation sequence (UAS) upstream of a target gene. When conducting nociceptor-specific genetic manipulation, the tissue-specific promoter in the GAL4 driver line is the regulatory sequence from the gene *pickpocket*. *ppk* encodes a subunit of the epithelial sodium channel (ENaC) family found specifically in cIV md-da neurons (Joshua et al., 2003). Using the *ppk* regulatory sequence as the tissue-specific promoter ensures GAL4 is only expressed in nociceptors. When the *ppk*-GAL4 driver line is crossed with the UAS responder line, the GAL4 transcription factor protein is expressed and binds to the UAS specifically in nociceptors. This binding of the GAL4 protein to the UAS results in the recruitment of RNA
polymerase and other transcription factors to the UAS which drives expression of the gene of interest. The tissue-specific nature of this system allows for controlled expression of target genes specifically in cIV md-da neurons (Brand and Perrimon, 1993; Southall et al., 2008).

The GAL4-UAS system can be used to produce both overexpression and knockdown genetic manipulation (Brand and Perrimon, 1993). Overexpression of a gene is accomplished by inserting the gene of interest downstream of the UAS and conducting the cross. GAL4/UAS induced overexpression results in progeny with tissue specific overexpression of a target protein at magnitudes several times larger than endogenous expression levels. The GAL4/UAS system can also be used in unison with RNA interference (RNAi) to produce progeny with tissue-specific genetic knockdowns. In RNAi knockdown, the transgenic insert downstream from the UAS is produced by cloning part of the gene of interest in one direction, inserting a spacer, and cloning the same part of the gene in the opposite direction and inserting downstream of the UAS. Due to the inversely repeating segments of this gene, once the GAL4 protein binds to the UAS and drives tissue-specific expression of the inserted sequence, a double stranded hairpin structure is formed. Dicer and R2D2, two proteins native to drosophila, bind to this hairpin and splice it into short 19-21bp fragments (Tomari and Zamore, 2005). Argo2, another native Drosophila protein, associates with these fragments and cleaves them into single stranded “guide” and “passenger” strands, before expelling the passenger strand. This guide strand is complementary to the mRNA still being produced by the native gene of interest. This guide strands and associated Argo2 proteins, along with other minor protein factors, form RNA-induced silencer complexes (RISC). Once formed, RISC identifies, binds, and degrades all mRNA being produced by the target gene of interest through homology-seeking action, effectively
“knocking down” function of the gene (Iwasaki et al., 2010). The GAL4/UAS system is an example of how scientists can manipulate gene expression to study the roles of specific genes nociceptors and nociceptor sensitivity.

Figure 3: The GAL4/UAS system is a genetic tool used to manipulate the abundance of target proteins within the nociceptors of Drosophila. The combination of the individual components of the GAL4/UAS system through breeding the GAL4 driver line with an RNA line. The resulting offspring contain the ubiquitously expressed transgene and tissue-specific GAL4 protein. The offspring, thus, exhibit cIV md-da neuron-specific transgene gene expression. (Machen, 2021)
The Role of mRNA Processing in the Regulation of Gene Expression

Nociceptor sensitivity is highly dependent on the proteins expressed by the nociceptor both at basal state and in response to tissue damage. The ability of the nociceptor to properly localize and translate specific proteins in response to noxious stimulus is crucial for regulating nociceptor sensitivity (Price and Geranton, 2009). The activation threshold of nociceptors can become reduced at the site of injury/inflammation following exposure to a noxious stimulus. This creates a hypersensitive state in which nociceptors that normally responds to noxious stimuli, now respond to stimulus that are typically innocuous. This hypersensitivity is regulated by the proteins expressed by the nociceptor following peripheral nerve injury. For example, changes in TRPV1 expression and activation threshold are seen following inflammation and have been linked to increased thermal sensitivity (Caterina and Julius, 2001). The ability of a nociceptor to modify itself both functionally and structurally in response to injury is known as nociceptor plasticity (Von Bernhardi et al., 2017). The plasticity of peripheral nociceptors is dependent on the proteins expressed by a nociceptor’s proteome at any given time and that protein expression is conditional to how the mRNA strands transcribed by these genes are processed prior to translation.

There are three primary stages of post-transcriptional mRNA processing: 5’ capping, alternative splicing, and 3’ polyadenylation. 5’ capping is a form of mRNA modification that occurs shortly after the initiation of transcription of a new pre-mRNA strand. The 5’ end of a pre-mRNA strand is capped through the removal of a phosphate and the addition of a methylated guanine nucleotide. This cap identifies the mRNA molecule, prevents the 5’ end of the transcript from being degraded by exonucleases, and serves as a binding site for proteins to
export the mature mRNA strand from the nucleus to the cytoplasm for translation (Hashimoto and Green, 1976; Moss and Koczot, 1976).

3’ polyadenylation is the process of adding a poly(A) tail to the 3’ end an RNA transcript as it is finishing being transcribed. As transcription of the strand is completing at the 3’ end, a group of proteins cleave part of the 3’ end of the transcript and begin to synthesize the poly(A) tail. The poly(A) tail is a long chain of repeating adenine nucleotides with varying length that is transcript dependent. The poly(A) helps protect the 3’ end of the transcript from degeneration in the cytoplasm (Colgan and Manley, 1997; Proudfoot, 2004). All poly(A) tails are degraded slowly in the cytoplasm, so the length of the poly(A) tail determines how long the transcript is available for translation and in turn, how much of the resulting protein is expressed. However, the exact mechanism for length determination is not well understood (Kornfeld et al., 1989).

In order for a pre-mRNA strand to be transformed into a mature mRNA strand, it must first be spliced to remove introns, leaving only exons. Alternative splicing influences cellular function by splicing out all introns and selectively choosing which exon coding regions to include from the transcript. This allows for multiple proteins with differing structure and functions to come from the same gene. This creates protein diversity and allows for differential expression of a gene that is tissue specific and/or developmental stage specific. Alternative splicing is thought to occur in ~95% of the human transcriptome and most proteins are formed by alternatively splicing rather than full transcript translation (Wang et al., 2008). Nociceptor sensitivity is dependent on alternative splicing of specific gene transcripts (Hulse et al., 2014; Jiang et al., 2013; Nakae et al., 2013; Thackeray and Ganetzky, 1994; Zhong et al., 2012), and genes necessary for normal dendrite development and morphology are alternatively spliced.
The best example of this is the *Drosophila* gene Down syndrome cell adhesion molecule (*Dscam*).

In *Drosophila*, the dendrites of individual md-da neurons do not overlap; neither do dendrites of the same class. However, dendrites of different classes exhibit significant overlap, allowing for a complete tiling of the *Drosophila* larval wall (Grueber et al., 2002). *Dscam* encodes a growth cone receptor in developing neurons that recognizes dendritic guidance signals and adjusts the cytoskeleton of the growing dendrites accordingly; this enables proper self-avoidance while still allowing proper tiling by md-da neurons of different classes. *Dscam* is alternatively spliced to form more than 38,000 unique isoforms, more than double the total number of genes in the *Drosophila* genome (Schmucker et al., 2000).

Every class of md-da neurons in *Drosophila* expresses a unique *Dscam* protein isoform expression profile. This unique profile expression allows dendrites from the same md-da neuron or of the same class to avoid themselves while still permitting dendrites to cross over dendrites of other md-da classes. Branches expressing the same *Dscam* protein isoform profile exhibit homophilic repulsion, allowing for self-avoidance in a given neuron and helping md-da neurons of the same class avoid each other. Branches expressing the differing *Dscam* protein isoform profile do not exhibit repulsion, allowing for the crossing over seen between md-da neurons of different classes (Matthews et al., 2007). Misregulation of *Dscam* expression in any class of md-da neuron has been shown to produce defects in self-avoidance and tiling (Soba et al., 2007). The specific expression patterns of *Dscam* isoforms indicate the importance of alternative splicing and regulation of RNA processing to nociceptor development.
RNA Binding Proteins in mRNA Processing

RNA-binding proteins (RBP) are regulatory proteins involved in the regulation of RNA stability, localization, splicing and translation initiation; all of which have significant impact on gene expression. For example, Poly(A) Binding Protein (PABP) are RBPs that increases translation rates of mRNA into proteins by increasing mRNA stability during translation and recruiting ribosomes to the bound mRNA. This increases translation rates significantly compared to transcripts lacking PABP (Kahvejian et al., 2005; Christou-Kent et al., 2020). RNA binding proteins play important roles in the localization of mRNA transcripts for proper temporal and spatial translation. In neurons, synaptic proteins can be synthesized in the cell body before being localized to synapses, or, if it is more energetically favorable, the transcript for these synaptic neurons can be localized to the axons or dendrites for transcription there. During this dendritic localization, the mRNA transcript is translationally repressed by RBPs until it reaches its destination (Tolino et al., 2012).

RBPs are also key modulators of alternative splicing. RBPs regulate alternative splicing by binding to sequence-specific elements within a mRNA template to enhance/repress the inclusion/exclusion of specific exons. The binding of RBPs is what allows for multiple unique proteins to be produced from the same RNA transcript. Some RBPs involved in alternative splicing have been shown to be involved in dendrite morphogenesis and the development of complex arborization patterns in nociceptors. For example, the RBP protein U2af38 is known to be involved in 3′ splice site selection and *U2af38* knockdown produces abnormal splicing of the aforementioned *Down syndrome cell adhesion molecule (DSCAM)*. Both abnormal splicing of *DSCAM* and knockdown of *U2af38* has been demonstrated to produce excessive high-order
dendrite branching, suggesting a role for \textit{U2af38} in the alternative splicing of \textit{DSCAM} (Park et al., 2004; Li and Millard; 2019).

Another RBP family involved in alternative splicing and dendrite morphogenesis is the Muscleblind-like (MBNL) protein family. MBNL proteins are a highly conserved family of RBPs known to be involved in many aspects of RNA processing including alternative splicing (Vicente-Crespo et al., 2008; Kino et al., 2009; Echeverria and Cooper, 2014;), RNA stability (Masuda et al., 2012), and RNA localization (Yair et al., 2005; Wang et al., 2012). In vertebrates, MBNL proteins have been shown to regulate the alternative splicing of \textit{cardiac Troponin T, Insulin Receptor, Chloride Voltage-Gated Ion Channel 1} among many others (Vicente-Crespo et al., 2008; Echeverria et al., 2014; Kino et al., 2009). MBNL proteins are conserved across a number of species including humans, mice, rats, \textit{Drosophila}, and \textit{C. elegans}.

Previous work in the Bellemer lab involved systematically knocked down genes in the nociceptors of \textit{Drosophila melanogaster} that encode RBPs and determining which knockdowns result in decreased nociceptive function. Of the 23 nociception related RBPs identified, knockdown of the \textit{Drosophila MBNL} homolog, \textit{mbl}, resulted in decreased nociceptive response to noxious thermal stimulus (Dyson, 2017). Additionally, the Gavis laboratory at Princeton conducted a genetic screen for RNA binding proteins involved in dendrite morphology of cIV neurons. Of the 88 genes identified, cIV md-da specific \textit{mbl} knockdown was shown to produce an increase in high-order dendritic branching and decrease in total dendrite length of \textit{Drosophila} larvae. The combination of increased high-order branching while still decreasing total dendritic length causes gaps in coverage of the dendritic arbor (Olesnicky et al., 2014). This information taken together suggests a nociceptor specific role for \textit{mbl} in nociception and
highlights the importance of investigating the various roles RBPs play in the modulation of nociception.

*Muscleblind Protein Family*

*Mbl* was first identified in a genetic study intended to isolate and identify novel genes involved in photoreceptor development and differentiation in *Drosophila melanogaster*. In this program, researchers used a transgenic *Drosophila* line that ectopically expressed the gene *Seven-up (Svp)*. *Svp* encodes a nuclear hormone receptor that is required for R3/4 and R1/6 photoreceptor subtype identity. When *svp* is ectopically expressed in R7 cells and cone cell precursors of *Drosophila*, a dose-sensitive rough-eye phenotype is exhibited due to the increased differentiation of cone cells into photoreceptors. In order to identify genes required for proper photoreceptor development and differentiation, scientists took advantage of the rough-eye phenotype (*sev-svp2*) and independently inserted a collection of lethal P-element insertions along the second chromosome of the *sev-svp2* mutants before screening for dominant modifiers of the *sev-svp2* phenotype. One of the lines was identified as being as suppressor of the *sev-svp2* phenotype carried a P-element insertion at chromosomal position 54A1-3. The proteins expressed by the previously unidentified gene associated with the P element insertion 54A1-3 were specifically detected in nuclei of ommatidial pre-cluster cells and imaginal disc cells, as well as in the nucleus of developing muscle cells. Ommatidial pre-cluster cells are the basic optical units of arthropods while imaginal disc cells are epithelial structures found inside arthropod larvae that form external structures such as the head and legs once adult morphogenesis takes place. The localization of these proteins in the nucleus of
developing muscle cells, as well as its implication in photoreceptors development led researchers to name this novel gene *Muscleblind* (Begemann et al., 1997).

In *Drosophila*, the *mbl* locus spans over 150kb of DNA and has 10 exons and several large introns that encode for four different *mbl* protein isoforms (MBLA-D). *Mbl* has a single transcription initiation site with an alternatively spliced primary transcript. Each RNA isoform shares a common N-terminus and contain either one or two Cys\_3\_His (CCCH) tandem zinc fingers but differ at the 3’ end (Begemann et al., 1997). *Mbl* proteins have been demonstrated to be involved in neuromuscular and photoreceptor cell development, terminal muscle differentiation, and self and other cell type avoidance in neurons (Begemann et al., 1997; Kanadia et al., 2003; Li and Millard; 2019). Self and other cell type avoidance refers to the ability of neuronal dendrites to avoid dendrites from the same neuron and neighboring neurons. RNAseq analysis of *Drosophila* embryos lacking functional *mbl* identified 81 genes with 85 unique intron-exon reads that exhibit altered splice levels meaning that *mbl* knockdown in *Drosophila* embryos results in aberrant abundance levels of 85 proteins from 81 genes relative to wild type (Irion, 2012). *Mbl* isoforms MBLA-C contain two Cys\_3\_His (CCCH) tandem zinc-finger binding domains which they use to bind their pre-mRNA targets. MBLD is much shorter and only has one CCCH zinc-finger domain due to the inclusion of exon 3, which contains many stop codons. MBLB and MBLC are enriched in the nucleus while MBLA expression is predominantly cytoplasmic (Vicente et al., 2007). Enrichment of MBLC and MBLB in the nucleus suggest roles in the regulation of transcript splicing (Vicente et al., 2007; Li and Millard; 2019). Enrichment of MBLA in the cytoplasm suggests a role in translational repression and localization. While this function hasn’t been explicitly demonstrated for MBLA, vertebrate cytoplasmic MBNL2
expression has been shown to regulate the localization of specific RNA transcripts (Yair Adereth et al., 2005). It possible that MBLA performs a similar function. The spatial expression of MBLD is currently unknown.

The different mbl protein isoforms are differentially expressed during different stages of Drosophila development (Figure 5). MBLC, the protein thought to be responsible for the vast majority of mbl alternative splicing events, is the most widely expressed isoform and is present at all stages of development except 5-10 hours after egg laying (AEL). MBLC expression peaks at late embryogenesis (12-16 hr AEL) and late third instar-early pupa stages, although strong expression remains throughout all larval stages. It is possible that MBLC is maternally loaded as it was detected in early embryos (0-4 hr AEL) and in adult flies. MBLD was also expressed very strongly and follows the same general expression pattern as MBLC except it is not expressed in late pupa or adult flies. MBLA and MBLB are detected in fewer developmental stages than MBLC and MBLD. MBLA expression is most prominent during late larval and early pupae stages, but is also present to some degree during late embryogenesis and early larval stage. MBLB has the lowest overall expression and is expressed almost exclusively in late larvae and early pupae stages (Figure 4) (Vicente et al., 2006).
Figure 4: Profiling of mbl isoforms during Drosophila development. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed. Data is presented as percentages of the maximum expression of each isoform. Rp49 expression is shown as loading control. Developmental window times from left to right were: 0-4, 5-10, 12-16, 18-24 hr embryos; first, second, early, and late third instar larvae; early and late pupae; 1-6 hr adult and mature adults. (Vicente et al., 2007) (Figure used with permission of Elsevier).
Muscleblind-like Protein Family

*Drosophila* possess a single *mbl* gene whereas vertebrates express three closely related genes *Muscleblind-like 1-3 (MBNL1-3)* (Miller et al., 2000; Fardaei et al., 2002). There is a high degree of conservation between the two tandem zinc fingers of *Drosophila mbl* and human MBNL1 proteins, with an amino acid similarity of ~80% (Irion, 2012). *MBNL1* and *MBNL2* are expressed ubiquitously across all tissue types, with *MBNL1* more prominent in muscle tissue and *MBNL2* levels more prominent in the brain. *MBNL3* is predominately expressed in the placenta and at very low levels in proliferating muscle precursor cells but not differentiated skeletal muscle (Miller et al., 2000; Fardaei et al., 2002).

*MBNL1* is the best studied protein in the MBNL family due to its predominant expression in muscle tissue. *MBNL1* expression levels increase during differentiation, enabling the transition from embryonic-to-fetal and fetal-to-adult splicing patterns in multiple cell types (Miller et al., 2000; Fernandez-Costa et al., 2011; Han et al., 2013). MBNL2 plays a key role in the splicing of pre-mRNAs in the brain (Charizanis et al., 2012) and has been shown, along with MBNL1, to be involved in the development of the chicken retina (Huang et al., 2008). Interestingly, MBNL3 has been shown to be involved in the suppression of muscle differentiation and loss of MBNL3 has shown to accelerate the process of muscle differentiation; suggesting a possible antagonistic relationship between MBNL1 and MBNL3 proteins (Squillace et al., 2002; Lee et al., 2008). However, the specific function of MBNL3 is not well defined. A study on several hundred MBNL associated splicing events found that nuclear MBNL proteins bind introns or exons to activate/repress splicing, whereas cytoplasmic MBNL proteins facilitate transcript localization and translational repression. Nuclear splicing
repression of specific isoforms by MBNL proteins was found to be *generally* associated with binding in the upstream intron and in the alternative exon, with increased MBNL binding resulting in stronger repression. Nuclear splicing activation of specific isoforms was *generally* associated with binding in the downstream intron and also increased in activation with increased binding (Figure 5). This study also showed that MBNL1 and MBNL2 function interchangeably in the regulation of a large set of splicing targets, indicating a degree of functional homology (Wang et al., 2012).
Figure 5: Model for Nuclear and Cytoplasmic Functions of MBNL proteins. MBNL repress or activate splicing, depending on binding location. In the cytoplasm, MBNL binding in 3’ UTRs may facilitate targeting of mRNAs with signal sequences to the rough ER. Alternatively, transcripts may be targeted to membrane-rich organelles for localized translation via actin-, microtubule-, or intermediate filament-based molecular motors. These organelles may include synapses, NMJs, or the plasma membrane, depending on cell type. MBNL may mediate isoform-specific mRNA localization in which MBNL binding sites within distal 3’ UTRs are required for targeting to particular compartments (Wang et al., 2012). (Figure permission granted from Elsevier).
Mbl/MBNL proteins have been implicated as a major factor in the development of a number of neuromuscular genetic diseases including amyotrophic lateral sclerosis (ALS) and myotonic dystrophy (DM) (Machuca-Tzili et al., 2006; Casci et al., 2019). The research of these genetic diseases is where a large portion of our current understanding of these proteins originates. Myotonic Dystrophy (DM) is an autosomal dominant disease that causes progressive loss of muscle mass, hypersomnia, myotonia, ocular cataracts, and in the congenital form of the disease, mental retardation (Grala, 1983). Myotonia is an inability or delayed muscle relaxation due to muscle degeneration and repetitive action potentials in myofibers (Hudson et al., 1995) while hypersomnia refers to excessive daytime sleepiness (Bollu et al., 2018).

There are two main forms of DM, both of which are caused by ectopic expression of toxic DNA microsatellite repeat sequences. DM1 is caused by microsatellite CTG expansions in the 3’ untranslated region (UTR) of the protein kinase encoding gene DMPK (Timchenko et al., 1996). DM2 is caused by microsatellite CCTG expansions in the first intron of zinc finger protein coding gene CNBP/ZNF9 (Liquori et al., 2001). DM has an extreme variability in its degree of symptoms due to high variability in the length of the triplet DMPK and CNBP/ZNF9 CTG/CCTG repeats and differences in their expression levels (Miller et al., 2000; Wenninger et al., 2018).

In vertebrates, when these CTG/CCTG expansions are transcribed, RNA containing CUG/CCUG repeats form a large hairpin structure to which MBNL proteins bind (Mariappan et al., 1996; Miller et al., 2000). This leads to the sequestration of MBNL proteins in nuclear foci, preventing export to the cytoplasm and preventing the proteins from carrying out their splicing regulation function (Miller et al., 2000). MBNL sequestration leads mis-splicing of several
transcripts including *insulin receptor*, and *skeletal muscle chloride channel* and cause the retention of fetal splicing forms in adult tissues (Savkur et al., 2001; Kanadia, et al., 2006; Fernandez-Costa et al., 2011). Research shows that sequestration of MBNL proteins is responsible for the vast majority of the splicing defects seen in vertebrate DM models. 80% of the ~200 alternative isoform changes observed in a CUG repeat expressing mouse model of DM1 are replicated in mice lacking functional MBNL1 proteins (Wang et al., 2012).

*mbl/MBNL Proteins Regulation of α-actinin*

Due to the functional homology between *Drosophila mbl* and human *MBNL1*, mbl is often used as a model to study the effects of RNA toxicity and the involvement of MBNL proteins in the pathology of DM (Chakraborty et al., 2018; Potikanond et al., 2018). Disruption of the Z-band is exhibited in several forms of human muscular dystrophy. The Z-band marks the lateral boundaries of the sarcomere and functions to transmit the tension generated by contraction/relaxation movements between successive sarcomeres along a muscle (Goldstein et al., 1988).

*mbl* mutant *Drosophila* models exhibit disrupted z-bands similar to those seen in DM *Drosophila* models. They also exhibit altered levels of α-actinin (ACTN) protein isoform expression relative to wild type suggesting a role for *mbl* in the splicing regulation of α-actinin (Machuca-Tzili et al., 2006). This altered isoform expression is rescued by MBLC but not any other *Drosophila mbl* isoforms, indicating functional specialization among *mbl* isoforms. In *Drosophila*, exogenous introduction of MBNL1 rescues normal ACTN isoform expression and computational screening of the alternatively spliced region of the ACTN transcript has demonstrated that ACTN transcripts in *Drosophila* have five perfect matches for human MBNL1
binding sequence, further supporting the conservation of function between MBNL1 and MBLC (Machuca-Tzili et al., 2006; Monferrer and Artero, 2006; Vicente et al., 2007).

In Drosophila, α-actinin is an actin filament crosslinking protein encoded by actn (vertebrate homologs: actn1-4; 70% amino acid similarity) and is alternatively spliced to produce three protein isoforms: one larval supercontractile muscle-specific isoform, one adult fibrillar and tubular muscle-specific isoform, and one non-muscle cell-specific isoform (Roulier et al., 1992). In contrast, vertebrates have 4 different α-actinin genes (actn1-4) that encode for four different α-alpha actinin proteins (ACTN1-4). ACTN1 and ACTN4 are expressed primarily in non-muscle cells while ACTN2 and ACTN3 are primarily expressed in muscle cells (Ebashi and Ebashi et al., 1964; Maruyama and Ebashi, 1965; Beggs et al., 1992; Honda et al., 1998). In muscle cells, α-actinin proteins connect successive sarcomeres by cross-linking actin filaments and other cytoskeleton components, creating a scaffold that increases stability and forms a bridge between the cytoskeleton and signaling pathways (Masaki et al., 1967; Squire, 1997; Otey and Carpen, 2004). In non-muscle cells, α-actinin proteins are calcium sensitive and found in microfilament bundles and at adhesion sites, where they are involved in binding cytoskeletal actin to the membrane (Burridge and Feramisco, 1981; Duhaian and Bamburg, 1984; Edlund et al, 2001; Hsu et al., 2018).

α-actinin associates with a number of cytoskeletal cytoplasmic domains of transmembrane receptors and ion channels, suggesting involvement in extracellular signaling and the structure and regulation of the cytoskeleton. In vertebrates, ACTN2 has been shown to play an important role in the localization, morphogenesis, and anchoring of the NR1 and NR2B subunit of NMDA glutamate receptors (NMDARs) in the dendrites of striatum (Wyszynski et al.,
The assembly of NMDARs is necessary for proper neuronal synaptic function. NMDARs found in the dorsal horn of the spinal cord are important to neuronal plasticity, and inhibition of NMDAR by exogenous introduction of NMDAR antagonists reduces nociceptive function and hypersensitivity following nerve injury (Seltzer et al., 1991; Yamamoto and Yaksh, 1992; Pagadala et al., 2013). While there are no non-muscle interactions established for mbl and α-actinin, these experiments taken together show the broad range that ACTN proteins play in cellular function. It is reasonable to hypothesize that mbl may regulate non-muscle actn in the nociceptors of Drosophila and this regulation may be involved in nociceptor sensitivity by maintaining proper extracellular signaling and/or cytoskeleton integrity. Further understanding of the relationship between mbl and actn could be important for more thorough understanding of the diverse role of RNA binding proteins and nociception.

*mbl/MBNL and the Dystrophin-Glycoprotein Complex*

In addition to altered actn expression and mbl sequestration, muscular dystrophy patients also commonly exhibit deficiencies in the Dystrophin-Glycoprotein Complex (DGC) (Hoffman et al., 1987; Salih et al., 1996; Cote et al., 1999; Sciandra et al., 2003). The DGC is a specialized cell adhesion complex that provides a vital mechanical link between the extracellular matrix (ECM) and cytoskeleton and is found in a variety of tissues including muscle, brain, and the retina (Ervasti and Campbell, 1991; Ervasti et al., 1991; Drenckhahn et al., 1996; Culligan et al., 1998). The DGC is made up of two sub-complexes: the dystroglycan complex and the sarcoglycan complex. The dystroglycan complex main function is to serve as a connector between dystrophin proteins in the ECM and actin cytoskeleton. The role of the sarcoglycan complex is not well defined, but it is thought that the four sarcoglycan proteins that
compose the sarcoglycan complex serve to stabilize the sarcolemma and strengthen the binding of dystrophin and the α- dystroglycan subunit to the β-dystroglycan subunit (Yoshida et al., 2000). The dystroglycan complex is the primary ECM-Cytoskeleton linking component of the DGC. The dystroglycan complex is composed of an extracellular α-dystroglycan protein linked to an intermembrane β-dystroglycan protein. The α-dystroglycan protein links extracellular laminin-2 to the DGC complex while β-dystroglycan links intercellular dystrophin to the DGC complex. Dystrophin binds actin cytoskeleton and the β-subunit of the dystroglycan protein. Mutations in genes encoding for the dystroglycan complex, sarcoglycan complex, dystrophin, and laminin-2 are seen in patients in many forms of muscular dystrophy (Durbeej and Campbell, 2002) (Figure 6). While the DGC has not been shown to be regulated by MBNL proteins in muscular dystrophy patients, abnormalities in both the DGC and MBNL proteins are commonly seen in muscular dystrophy patients. Additionally, mbl has been shown to regulate two components of the DGC, dystrophin and dystroglycan, in Drosophila photoreceptors and flight muscles, which will be discussed in more detail later in this report.
Figure 6: The DGC in skeletal muscle and associated muscular dystrophies. The DGC in skeletal muscle is composed of dystrophin, the dystroglycans (α, β), the sarcoglycans (α, β, γ, δ), sarcospan, the syntrophins (α, β1) and dystrobrevin (α). There is a growing number of proteins reported to be associated with the DGC in the muscle cell. Depicted are nNOS, which interacts with the syntrophin complex and laminin-2, which is one of many extracellular ligands of α-dystroglycan. Several forms of muscular dystrophy arise from primary mutations in genes encoding components of the DGC. Mutations in dystrophin, all four sarcoglycans and the laminin α2 chain are responsible for DMD/BMD, LGMD type 2C-F and CMD, respectively. In addition, several forms of CMD are caused by abnormal glycosylation of α-dystroglycan (not illustrated). (Durbeej and Campbell; 2002) (Figure used with permission of Elsevier).
Due to the role of DGC mutations in DM pathology, DGC's have predominantly been studied in muscle cells, but the role of DGC and its components in non-muscle cells is starting to become more defined. Defects in the DGC in the brain has been shown to cause cognitive impairment and mental retardation (Topaloglu et al., 2003; Godfrey et al., 2007; Waite et al., 2009; Hara et al., 2011). In neurons, DGC has been shown to co-localize post-synaptically at inhibitory synapses with GABA<sub>A</sub> receptors. GABA<sub>A</sub> receptors are specific for Gamma-aminobutyric acid (GABA), an amino acid that functions as the primary inhibitor for the CNS by reducing excitability via inhibition of nerve transmission (Allen et al., 2022). Interestingly, Dystroglycan (Dg) deficient neurons can still form synapses containing GABA<sub>A</sub> receptors, indicating Dg is not required for the development of these synapses (Knuesel et al., 1999; Cote et al., 2002; Lévi et al., 2002). In the brain of mice co-expressing cortical and hippocampal-specific Dg knockdown, impaired long term plasticity is seen in the neurons of the hippocampus, suggesting a role for Dg in neuronal plasticity (Satz et al., 2010). DGC's are also found in many places in the retina, including the outer plexiform layer (OPL), where photoreceptors form synapses with horizontal and bipolar cells, and in the inner plexiform layer (IPL) where bipolar/amacrine cells and ganglion cell synapse, adding further evidence for the role of DGC's at synapses (Claudepierre et al., 2000; Dalloz et al., 2001).

While the DGC’s primary role is structural, the DGC is also thought to be involved in transmembrane signaling by anchoring a variety of signaling molecules. Dg is bound by Grb2, an adaptor protein involved in the activation of the Ras/MapK pathway (Yang et al., 1995). The Ras/MapK pathway transduces signals from the ECM to a cell’s nucleus to activate specific genes involved in a variety of mechanisms such as cell growth, division and differentiation.
(Molina and Adjei et al., 2006). There is also some evidence for Dg in the process of nociception. Schwann cell specific Dg-null mouse models exhibit significantly reduced neuronal conductance and reduced response to noxious thermal and mechanical stimuli (Saito et al., 2003; Masaki and Matsumura, 2010). While there are no nociceptor specific interactions established for mbl/MBNL and the DGC, mbl has been shown to interact with two of its primary components, Dg and Dys in the flight muscles and photoreceptors of Drosophila DM models.

Previous research conducted on Drosophila DM models involved screening for unknown DGC interactors in an effort to identify mechanisms that contribute to muscle degeneration in DM patients. In addition to muscle degeneration and photoreceptor defects, Drosophila with Dg and Dys mutations exhibited visible alterations in the morphology of the fly wing, particularly in the posterior crossvein. Using this visible phenotype, researchers were able to conduct a dominant modifier screen to identify genes that either suppress or notably alter the crossvein phenotype. The gene that demonstrated the strongest suppression of the Dg/Dys mutant wing phenotype was mbl. This means that knockdown of mbl increased the severity of the crossvein phenotype more than any other gene screened. Researchers took this one step further and looked at whether mbl interacts with dystrophin and/or dystroglycan specifically in flight muscles. Both Dys and Dg knockdown mutants exhibit moderate muscle degeneration while Drosophila mbl mutants that have lost one copy of mbl have normal muscle structure. Knockdown of mbl in both Dys mutants and Dg mutants resulted in increased muscle degeneration relative to either Dys or Dg specific knockdown, indicating mbl is required for proper function of both Dg and Dys (Kucherenko et al., 2008).
Mbl has also been shown to interact with members of the DGC in the fly visual system. Heterozygous mutations for either Dys or Dg result in improper photoreceptor axon projections into the laminin and eye neuron elongation defects during development. Heterozygous mbl mutation produces a similar defect axon projection phenotype. Knockdown of mbl in a Dys heterozygous background has been shown to increase the severity of the photoreceptor axon projections. It is worth noting that heterozygous mbl mutations in heterozygous Dg background also appeared to increase axon pathfinding defects just not to a significant degree (Marrone et al., 2011). This supports the idea that mbl and Dys/Dg in interact in nociceptors by providing a neuron specific interaction. Currently, no nociceptor specific interaction has been established for mbl and Dys or mbl and Dg. While the general mechanism by which the DGC functions is understand, how it is regulated is not. Further understanding of the regulatory processes behind the DGC, specifically in sensory neurons, will contribute to a greater understanding of the role of the DGC in sensory neurons and the mechanisms by which this role is regulated.

Non-Sensory Disease Associated Interactors of Muscleblind Proteins

In Drosophila, mbl has been shown to alternatively splice DSCAM2 into two protein isoforms, DSCAM2.10A and DSCAM2.10B; named for the differing exon expression/repression between the two isoforms. DSCAM2 encodes a cell recognition molecule that modulates self and other cell type avoidance which is essential for neuronal development (Millard et al., 2007; Millard et al., 2010; Tadros et al., 2016). In the fly visual system, Dscam2.10B is typically expressed in lamina neurons L1 and L4 while Dscam2.10A is normally expressed in L2-L5 lamina neurons (Lah and Millard, 2014). When Drosophila mbl is not expressed in L1 neurons, Dscam2.10B expression is absent, and the Dscam2.10A isoform is expressed solely, causing
morphological and in turn functional defects in the fly visual system. When mbl is overexpressed in L2, L3, and L5 neurons we see ectopic expression of Dscam2.10B, suggesting a role for mbl in the splicing activation of Dscam2.10B in L2, L3, and L5 neurons (Li and Millard, 2019). Prominent expression of DSCAM2.10A but not DSCAM2.10B is observed in mushroom bodies (MB) of the Drosophila brain. Exogenous overexpression of any mbl isoform or MBNL1 results in varying levels of DSCAM2.10B expression in MB neurons that normally express DSCAM2.10A (Li and Millard, 2019). These experiments taken together suggests mbl acts as a splicing repressor of Dscam2.10A and a splicing activator of Dscam2.10B in MB and provides evidence of mbl function in the regulation of a range neuronal transcripts. These results also provide further evidence of functional homology between MBNL1 and mbl proteins.

Mbl has also been shown to interact with absent MD neurons and olfactory sensilla (amos). Amos is a proneural gene that encodes a transcription factor protein involved in the formation of bipolar multi-dendritic neurons and is required for normal olfactory sensilla function (Goulding et al., 2000; Huang et al., 2000). While no research is available on amos and the formation of nociceptors, studies have demonstrated increased expression of amos in the RNA of purified cIV md-da and cI md-da neuron samples relative to genome-wide RNA expression suggesting potential amos involvement in md-da function and/or formation (Iyer et al., 2013). The only established interaction between amos and mbl involves MBLC protein isoform and the fly visual system. Overexpression of MBLC transcript in the photoreceptors of Drosophila produces a rough eye phenotype. This phenotype is enhanced by simultaneous silencing of the amos gene (Vicente-Crespo et al., 2008). Further understanding of the
relationship between mbl and amos will expand on the role of mbl in isoform specification and protein differentiation in sensory neurons.

Previous research has identified mbl as being crucial to both proper clV md-da dendrite morphology (Olesnicky et al., 2014) and normal thermal nociceptive response to noxious thermal stimulus (Dyson, 2017). Taking this information into consideration, I chose to investigate mbl further to better understand the role mbl plays in nociceptor sensitivity. The goal of this research is to confirm the morphological and thermosensory defects seen in clV md-da specific mbl mutants and further the understanding of the role of mbl in nociception using a candidate approach to analyze downstream effectors of mbl possibly involved in nociceptor sensitivity. The study aims to contribute to the growing knowledge of the diverse roles RNA-binding proteins play in nociception and their potential utility as a genetic target for the treatment of sensory conditions in humans.

Methods and Materials

Drosophila Genetics

For each experimental genotype analyzed in this research, four individual crosses were conducted to produce the experimental genotype, two negative controls, and a positive control (Table 1). For the experimental genotypes, a ppk-GAL4; UAS-Dicer2 virgin female driver line was crossed with a male responder line containing UAS upstream of RNAi specific for the target gene (UAS-Targetgene-RNAi). To generate our GAL4-only negative controls, a ppk-GAL4; UAS-Dicer2 virgin female line was crossed with male BDSC#36303, a control line, and used as a negative control. Because there is no UAS in the male responder line for the GAL4 transcription factor protein to bind to, there is no transcription of target gene specific RNAi and therefore it
is used as a negative control. To generate our UAS-only negative controls, a male UAS-
Targetgene-RNAi responder line was crossed with \( w^{1118} \), a separate control line, and used as a
negative control because it contains UAS-TargetGene-RNAi responder but no GAL4 driver. To
generate our positive controls, a \( ppk\)-GAL4; UAS-Dicer2 virgin female line was crossed with a
male UAS-para-RNAi responder line (Table 1). Paralytic (para) encodes a voltage gated sodium
channel in Drosophila nociceptors and knocking para down produces a near complete loss of
nociceptive response (Zhong et al., 2010), making it a good indicator for measuring nociceptive
response in other knockdown lines.

<table>
<thead>
<tr>
<th>Group</th>
<th>Origin</th>
<th>Genotype</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ppk)-GAL4; UAS-dicer2</td>
<td>Dan Tracey Lab</td>
<td>( ppk)-GAL4; UAS-dicer-2</td>
<td>GAL4 Driver</td>
</tr>
<tr>
<td>( w^{1118} )</td>
<td>BDSC# 5905</td>
<td>( w^{1118} )</td>
<td>Negative Control</td>
</tr>
<tr>
<td>TRiP RNAi Control</td>
<td>BDSC# 36303</td>
<td>( y[1] v[1]; P[y[+t7.7]=CaryP]attP2 )</td>
<td>Negative Control</td>
</tr>
<tr>
<td>UAS-para-RNAi</td>
<td>VDRC# 6139</td>
<td>( W1118; P{UAS-para-RNAi} )</td>
<td>Positive Control</td>
</tr>
<tr>
<td>UAS-dys#31553-RNAi</td>
<td>BDSC# 31553</td>
<td>( y[1] v[1]; P[y[+t7.7] v[+t1.8]=TRiP.JF01118]attP2 )</td>
<td>Dys-knockdown Valium 1 Vector</td>
</tr>
</tbody>
</table>

*Table 1*: List of all Drosophila melanogaster fly line genotypes used for mbl analysis
Drosophila Husbandry

*Drosophila* larvae were reared in a 25 °C incubator at approximately 50% humidity while exposed to a 12-hour light / 12-hour dark cycle on a cornmeal molasses medium (NutriFly M; Genesee Scientific, El Cajon, CA, USA). All crosses, regardless of genotype, were crossed with six virgin driver-line females and three RNAi males or control line males (Table 1).

To generate each genotype used in this experiment, six *Drosophila melanogaster* virgin females and three males are placed in a vial with approximately two centimeters of Nutrifly food and yeast. After two days, the *Drosophila* were flipped to another vial, and then flipped daily for two days until there were four vials of offspring of the desired genotypes.

Behavioral Nociception Assays

The larvae were removed from vials by gently rinsing wandering third-instar larvae off the walls of the vials with distilled water. After removal, the third-instar larvae were allowed to acclimate in a glass petri dish with a small amount of distilled water (2 mL) and dry brewer’s yeast (3-5 mg) for 3-5 minutes. The addition of yeast disrupts the surface tension of the water.

For thermal analysis, once the acclimation period was completed, larvae were exposed to a 46 °C heated probe along their dorsal midline while crawling, in the forward direction, along the petri dish for 10 seconds or until NEL was completed. The heat probe used was a modified 6mm soldering iron with a chisel tip and temperature controlled by a Staco Variac Variable Transformer (part number ST3PN1210B) (ISE, Inc., Cleveland, OH). If NEL was completed within ten seconds, then the amount of time between stimulus and completion of NEL was recorded as a response latency. If no response was seen within ten seconds of stimulus, the larvae have a complete loss of nociceptive function and were recorded as 11
seconds. The larvae were recorded on a video camera and then measured via frame-by-frame analysis in Adobe Premiere Pro to accurately determine latency periods. During thermal testing, the temperature of the probe tip was monitored to the tenth degree using a digital real-time temperature display generated by an IT-23 thermistor and a BAT-12 digital thermometer (Physitemp, Clifton, NJ). The probe temperature was monitored both before and after contact with larvae. The trials that saw the probe temperature stray by ± 1 °C from the desired testing temperature were excluded.

For the mechanical analysis, once the acclimation period was completed, larvae actively crawling forward were prodded with an 8mM Von Frey Filament along their dorsal midline. Nociceptive response was determined by the percent of larvae that execute NEL following first poke by the filament.

Confocal Imaging

Wild type nociceptors and mbl knockdown nociceptors were visualized using confocal imaging of Green Fluorescent Protein (GFP) in the dendrites of mbl knockdown larvae in order to determine any morphological changes to the nociceptors caused by the knockdown. Adult males from a UAS-mbl-RNAi line was crossed with virgin ppk-GAL4; UAS-dicer2 line also containing a UAS-mCD8::GFP transgene to generate a fluorescent mbl knockdown sample. A wild type sample was generated by crossing male BDSC#36303 with ppk-GAL4; UAS-dicer2 female containing a UAS-mCD8::GFP transgene and was used as a negative control. mCD8 is a transmembrane protein so when the lines are crossed and the GAL4 transcription protein binds to the UAS upstream of GFP bound mCD8, fluorescence is produced specifically alone the membrane of cIV md-da neurons. Confocal microscopy was used to the observe GFP
fluorescence and determine any morphological changes to the dendrites of the nociceptors caused by mbl knockdown. Virgin ppk-GAL4; UAS-dicer2 were crossed with males to generate a negative control. These images were analyzed using the ImageJ plugin and NeuroJ.

Statistical Analysis

Data from the thermal nociceptive analyses and morphological analysis were plotted and analyzed using a two-sided permutation test to compare mean difference between wild-type and mbl or candidate interactor knockdown larvae. The confidence interval was measured at 95% and the effect size for thermal nociception analysis was measured using Cliff’s delta (Ho et al., 2019). For mechanical nociception analysis, a Chi-square test was used to compare mean difference between wild type and mbl or candidate interactor knockdown larvae. The confidence interval was measured at 95%. The minimum number of trials for the mechanical nociceptive analysis was 100 while the minimum number of trials for thermal analysis was 35. Dendrite morphology was analyzed using the Strahler analysis algorithm from Fiji’s Simple Neurite Tracer plug-in. Significance for morphology analysis was conducted using a two tailed two sample t-test assuming equal variances. Branch length was measured by pixel length.

Results

Analysis of cIV md-da Specific mbl Knockdown Larvae Appears to Confirm Morphological Defects

Previous research conducted by the Gavis lab at Princeton demonstrated that knockdown of mbl produces increased high-order branching while still decreasing total dendritic length. This results in gaps in coverage of the dendritic arbor (Olesnicky et al., 2014). In order to confirm the effect nociceptor-specific mbl knockdown has on nociceptor dendrite morphology, nociceptor membranes of mbl knockdown larvae were fluorescently tagged with
mCD8::GFP and analyzed using confocal microscopy and compared to mCD8::GFP tagged negative controls. mCD8::GFP is a fusion protein of green fluorescent protein (GFP) and mCD8. mCD8 tethers to the membrane, allowing GFP fluorescence to be seen along the membranes of nociceptors. This allows us to visualize the structure of the nociceptor dendrites and overall dendritic arbor (Figure 7). The cIV md-da specific mbl knockdown larvae appear to exhibit a reduced number of branches (1,190) and a reduced total branch length (12,754 pixels) relative to wild type controls average number of branches (1,370) and total branch length (16267 pixels). Unfortunately, the sample size was too small for significance testing so this data can only be compared qualitatively.

![Wildtype mbl knockdown](image.png)

**Figure 7:** Confocal imaging of a cIV md-da neuron from a cIV md-da specific mbl knockdown larva and wild type control larvae.
Confirming the Involvement of mbl in Thermal Nociception

In addition to morphological defects, nociceptor-specific knockdown of mbl has also been previously shown to produce decreased thermal nociceptive function in Drosophila larvae when knocked down, suggesting a role for mbl in nociceptor sensitivity (Dyson, 2017). To confirm that nociceptor-specific mbl knockdown results in decreased nociceptive function, cIV md-da specific mbl knockdown Drosophila larvae were produced and subjected to a thermal nociception assay. The results of the thermal analysis showed that larvae from the cIV md-da specific mbl knockdown larvae exhibited a significantly longer average response latency (4.169 seconds) than the GAL4-only control larvae (2.083 seconds) and UAS-only control larvae (2.247 seconds). Larvae containing cIV md-da specific mbl knockdown also had a significantly shorter average response latency than the Para-knockdown (10.935 seconds). The statistical difference between the controls and the knockdown was determined via a two-sided permutation t-test after an unpaired Cliff’s delta. The unpaired Cliff’s delta between the GAL4-only control and the mbl knockdown was -0.627; the 95% percent confidence interval bounds were -0.783 and -0.421. The P-value of the two-sided permutation t-test between the GAL4-only control and the mbl knockdown was 0.000. The unpaired Cliff’s delta between the UAS-only control and mbl knockdown was -0.569; the 95% percent confidence interval bounds were -0.736 and -0.354. The P-value of the two-sided permutation t-test between the UAS-only control and the mbl knockdown was 0.000. These results confirmed that mbl knockdown does result in decreased thermal nociceptive function (Figure 8).
The next known interactor of \textit{mbl} investigated for a potential role in thermal nociceptor sensitivity was \textit{Dystroglycan (Dg)}. cIV md-da specific \textit{Dg} knockdown larvae were produced and subjected to a thermal nociception assay. Larvae with cIV md-da specific \textit{Dg} knockdown did have a longer average response latency (3.312 seconds) than the GAL4-only control (2.472 seconds) but not the UAS-only control (2.840 seconds). The \textit{Dg} knockdown mutants exhibited a significantly shorter average response latency compared to the \textit{para}-knockdown positive control (10.904 seconds). The statistical difference between the controls and the knockdown

\textit{Dg Thermal Nociception Assay}

Figure 8: Nociceptor-specific knockdown of \textit{mbl} causes defects in thermal nociception. Larvae with nociceptor-specific knockdown of \textit{mbl} exhibited a significantly longer response latency to a noxious thermal stimulus (46°C) than GAL4-only and UAS-only controls. Larvae with nociceptor-specific knockdown of \textit{para} showed severely impaired nociceptive responses and were used as a positive control. Response latencies of individual \textit{Drosophila} larvae are plotted as points on the graph (\( n \geq 36 \) for all groups; * \( p \leq 0.001 \) by two-sided permutation test).
was determined via a two-sided permutation t-test after an unpaired Cliff’s delta. The unpaired Cliff’s delta between the GAL4 -only control and the \( Dg \) knockdown was -0.326; the 95% percent confidence interval bounds were -0.475 and -0.161. The \( P \)-value of the two-sided permutation t-test between the GAL4 -only control and the \( Dg \) knockdown was 0.00. The unpaired Cliff’s delta between the UAS-only control and \( Dg \) knockdown was -0.155; the 95% percent confidence interval bounds were -0.310 and -0.012. The \( P \)-value of the two-sided permutation t-test between the UAS-only control and the \( Dg \) knockdown was 0.062 (Figure 9).

Figure 9: Nociceptor-specific knockdown of \( Dg \) may cause defects in thermal nociception. Larvae with nociceptor-specific knockdown of \( Dg \) exhibited a significantly longer response latency to a noxious thermal stimulus (46°C) than the GAL4-only control but not UAS-\( Dg \)-only control. Larvae with nociceptor-specific knockdown of \( para \) showed severely impaired nociceptive responses and were used as a positive control. Response latencies of individual \( Drosophila \) larvae are plotted as points on the graph (\( n \geq 37 \) for all groups; * \( p \leq 0.01 \) by two-sided permutation test).
**Dys Thermal Nociception Assay**

Two separate cIV md-da specific *Dys* knockdown larvae genotypes were produced and subjected to a thermal nociception assay: *Dys*\#31553-knockdown larvae and *Dys*\#55641-knockdown larvae. Larvae with *Dys*\#31553-knockdown did not exhibit significantly different response latencies (2.515 seconds) compared to the GAL4-only control (2.512 seconds) or the UAS-*Dys*\#31553-RNAi only control (2.577 seconds). Interestingly, the *Dys*\#55641 knockdown (3.251) larvae exhibited a significantly longer latency than the *Dys*\#31553-knockdown, and the GAL4-only control but not the UAS-*Dys*\#55641-RNAi (2.909 seconds) only control. Both cIV md-da specific *Dys*\#55641 and *Dys*\#31553 knockdown larvae exhibited significantly shorter response latencies compared to the *para* control (10.579 seconds). The statistical difference between the controls and the knockdowns were determined via a two-sided permutation t-test after an unpaired Cliff’s delta. The unpaired Cliff's delta between the GAL4-only control and the *Dys*\#31553 knockdown was -0.028; the 95% percent confidence interval bounds were -0.256 and 0.192. The *P*-value of the two-sided permutation t-test between the GAL4-only control and the *Dys*\#31553 knockdown was 0.8. The unpaired Cliff's delta between the UAS-*Dys*\#31553-RNAi only control and *Dys*\#31553 knockdown was 0.033; the 95% percent confidence interval bounds were -0.199 and 0.257. The *P*-value of the two-sided permutation t-test between the UAS-*Dys*\#31553-RNAi only control and *Dys*\#31553 knockdown was 0.771.

The unpaired Cliff's delta between the GAL4-only control and the *Dys*\#55641 knockdown was -0.262; the 95% percent confidence interval bounds were -0.47 and -0.0344. The *P*-value of the two-sided permutation t-test between the GAL4-only control and the *Dys*\#55641 knockdown was 0.0218. The unpaired Cliff's delta between the UAS-*Dys*\#55641-RNAi only control and
Dys\(^{55641}\) knockdown was -0.094; the 95% percent confidence interval bounds were -0.315 and 0.14. The \( P \)-value of the two-sided permutation t-test between the UAS-Dys\(^{55641}\)-RNAi only control and Dys\(^{55641}\) knockdown was 0.435. The unpaired Cliff’s delta between Dys\(^{55641}\) knockdown and Dys\(^{31553}\) knockdown was -0.252; the 95% percent confidence interval bounds were -0.468 and -0.022. The \( P \)-value of the two-sided permutation t-test between Dys\(^{55641}\) knockdown and Dys\(^{31553}\) knockdown was 0.318 (Figure 10). These results of the experiment show that the cIV md-da specific Dys knockdown constructed using UAS-Dys\(^{55641}\)-RNAi larvae appears to reduce thermal nociceptive function, while cIV md-da specific Dys knockdown constructed using UAS-Dys\(^{31553}\)-RNAi larvae does not appear to reduce thermal nociceptive function.

**Figure 10:** Nociceptor-specific knockdown of Dys may cause small defects in thermal nociception. Larvae with nociceptor-specific Dys knockdown #55641 exhibited a significantly longer response latency to a noxious thermal stimulus (46°C) than the GAL4-only or UAS-only control (#31553). Larvae with nociceptor-specific knockdown of para showed severely impaired nociceptive responses and were used as a positive control. Response latencies of individual *Drosophila* larvae are plotted as points on the graph (\( n = 50 \) for all groups; \* \( p \leq 0.01 \) by two-sided permutation test).
amos Thermal Nociception Assay

The next known interactor of mbl investigated for a role in thermal nociception was amos. cIV md-da specific amos knockdown larvae were produced and subjected to a thermal nociception assay. Larvae with cIV md-da specific amos knockdown did not have a significantly different average response latency (3.105 seconds) when compared to the GAL4-only (3.167 seconds) or UAS-only negative controls (2.914 seconds). amos did exhibit significantly reduced nociceptive response latencies relative to para knockdown larvae (10.773 seconds). The statistical difference between the controls and the knockdown was determined via a two-sided permutation t-test after an unpaired Cliff's delta. The unpaired Cliff's delta between the GAL4-only control and the amos knockdown was 0.084; the 95% percent confidence interval bounds were -0.146 and 0.304. The P-value of the two-sided permutation t-test between the GAL4-only control and the amos knockdown was 0.472. The unpaired Cliff's delta between the UAS-only control and amos knockdown was -0.021; the 95% percent confidence interval bounds were -0.266 and 0.222. The P-value of the two-sided sided permutation t-test between the UAS-only control and the amos knockdown was 0.873. No significant difference in thermal nociceptive response was seen between experimental and negative control groups. These results suggest that amos is not required for normal thermal nociception (Figure 11).
α-actinin (*actn*), a known interactor of *mbl*, was also investigated for its role in thermal nociception. cIV md-da specific *actn* knockdown larvae were produced and subjected to a thermal nociception assay. Larvae with cIV md-da specific *actn* knockdown did have a longer average response latency (3.579 seconds) than both the GAL4-only control (2.749 seconds) and the UAS-only control (2.418 seconds). Nociceptor-specific *actn* knockdown larvae exhibited significantly shorter response latencies compared to *para* knockdown (10.805 seconds). The statistical difference between the controls and the knockdown was determined via a two-sided permutation test.

Figure 11: Nociceptor-specific knockdown of *amos* does not cause defects in thermal nociception. Larvae with nociceptor-specific knockdown of *amos* did not exhibit a significantly longer response latency to noxious thermal stimulus (46°C) than the GAL4-only or UAS-only controls. Larvae with nociceptor-specific knockdown of *para* showed severely impaired nociceptive responses and were used as a positive control. Response latencies of individual *Drosophila* larvae are plotted as points on the graph (*n* ≥ 37 for all groups; p-values determined by two-sided permutation test).
permutation t-test after an unpaired Cliff’s delta. The unpaired Cliff’s delta between the GAL4-only control and the actn knockdown was -0.34; the 95% percent confidence interval bounds were -0.546 and -0.105. The P-value of the two-sided permutation t-test between the GAL4-only control and the actn knockdown was 0.005. The unpaired Cliff’s delta between the UAS-only control and actn knockdown was -0.487; the 95% percent confidence interval bounds were -0.669 and -0.487. The P-value of the two-sided permutation t-test between the UAS-only control and the actn knockdown was 0.00. These results suggest that actn is required for normal thermal nociceptive function (Figure 12).

**Figure 12:** Nociceptor-specific knockdown of actn causes defects in thermal nociception. Larvae with nociceptor-specific knockdown of actn exhibited a significantly longer response latency to a noxious thermal stimulus (46°C) than both the GAL4-only and UAS-only controls. Larvae with nociceptor-specific knockdown of para showed severely impaired nociceptive responses and were used as a positive control. Response latencies of individual Drosophila larvae are plotted as points on the graph (n ≥ 40 for all groups; * p ≤ 0.01 by two-sided permutation test).
**mbl and Dg Mechanical Nociception Assay**

Due to the clear involvement of *mbl* in thermal nociception and possible involvement of *Dg*, I thought it reasonable to investigate whether *mbl* and/or *Dg* may play a role in mechanical nociception. To do this, cIV md-da specific *mbl* knockdown larvae and cIV md-da specific *Dg* knockdown larvae were independently constructed and subjected to the same mechanical nociception assay. Larvae from the *Dg* knockdown (44%) exhibited significantly decreased nociceptive response to mechanical stimuli relative to the UAS-*Dg*-RNAi only control (68%) but not the GAL4-only (53%). Larvae from *mbl* knockdown (44%) did not exhibit significantly different response compared to UAS-*mbl*-RNAi only controls (57%) or GAL4-only control (53%). Larvae with cIV md-da specific *mbl* knockdown and larvae with cIV md-da specific *Dg* knockdown exhibited increased nociceptive response to noxious mechanical stimulus relative to *para* knockdown (3%). Both *mbl* and *Dg* knockdown larvae had a standard error of 0.0496. UAS-*Dg*-RNAi only control had a standard error of 0.047. UAS-*mbl*-RNAi only control had a standard error of 0.0495. GAL4-only control had a standard error of 0.0499. The p-value of the Chi-Square test between either *mbl* or *Dg* knockdown larvae and the GAL4-only control was 0.216. The p-value of the Chi-Square test between both *Dg* knockdown larvae and the UAS-*Dg*-RNAi only control was 0.004. The p-value of the Chi-Square test between the *mbl* knockdown larvae and the UAS-*mbl*-RNAi larvae was 0.085 (Figure 13).
Figure 13: Nociceptor-specific knockdown of Dg may cause small defects in mechanical nociception. Larvae with nociceptor-specific knockdown of Dg exhibited a significantly longer response latency to a noxious mechanical stimulus than UAS-Dg-only controls but no difference was seen when compared to the GAL4-only control. Mbl knockdown larvae did not exhibit significantly different results from GAL4-only control or UAS-mbl-only control. Larvae with nociceptor-specific knockdown of para showed severely reduced nociceptive responses and were used as a positive control. Bars indicate the proportion of Drosophila larvae from each genotype that responded to application of the 10mM Von Frey filament mechanical stimulus. Error bars indicate the standard error of the proportion. (n = 100 for all groups; *p ≤ 0.01 determined by Chi-Square Test)
Discussion

The work detailed in this report sought to further the understanding of the role RNA-binding proteins play in the regulation of nociceptor sensitivity by investigating the role of the RNA-binding protein mbl in nociceptors. Previous research involving the *Drosophila melanogaster* gene mbl found that mbl is required for normal thermal nociceptive function (Dyson, 2017) and normal nociceptor morphology (Olesnicky et al., 2014), but the specific role mbl plays in thermal nociception has not been identified. mbl has been investigated as a regulator of the alternative splicing of pre-mRNA transcripts in a number of tissues, and a large number of splicing targets have been identified. However, no alternative splicing targets have been identified in relation to nociception. cIV md-da specific mbl knockdown larvae were produced to confirm the thermal nociception defects previously observed in nociceptor-specific mbl knockdown larvae and identify any possible morphological defects in the nociceptors caused by the mbl knockdown. To further describe the role mbl in nociceptor sensitivity, a literature search was conducted to identify candidate effectors of mbl previously known to interact with mbl in other systems that may also interact with mbl in nociceptors. Nociceptor-specific knockdown larvae of these downstream effecters were generated and exposed to nociceptive behavioral assays to see if they too are involved in regulating nociceptor sensitivity. The results of this experiment confirm mbl is required for normal thermal nociception, identify potential morphological changes to nociceptors caused by mbl knockdown, and provide several potential possible downstream effectors through which mbl knockdown may disrupt thermal nociceptive function.
**mbl Knockdown May Result in Morphological Defects in the Dendrites of Nociceptors**

Previous work has shown that cIV md-da specific knockdown of *mbl* produces increased high-order branching in dendrites while still decreasing total dendritic length, resulting in gaps in coverage of the dendritic arbor (Olesnicky et al., 2014). In order to confirm the effect nociceptor-specific *mbl* knockdown has on nociceptor dendrite morphology, nociceptor membranes of cIV md-da specific *mbl* knockdown larvae were fluorescently tagged with Green Fluorescent Protein (GFP) and analyzed using confocal microscopy and compared to mCD8::GFP tagged negative controls. While the sample size was too small to demonstrate significance, cIV md-da specific *mbl* knockdown larvae appear to exhibit bunched terminal branches and loss of field coverage of the dendritic arbor relative to wild-type. An example dendritic arbor from a cIV md-da specific *mbl* knockdown larvae is shown below (Figure 7). This analysis of nociceptor-specific *mbl* knockdown dendrite morphology is supported by research from the Gavis lab which also showed cIV md-da specific *mbl* knockdown larvae exhibit clustering of terminal branching and gaps in coverage within the dendritic arbor. It is possible that this loss of field coverage results in decreased innervation of the *Drosophila* larval body wall by cIV md-da neurons and in turn, decreased nociceptor sensitivity to noxious stimulus.

**Nociceptor-specific mbl Knockdown Drosophila Larvae Exhibit Defects in Thermal Nociception**

Preliminary research identified *mbl* as potentially being required for normal response to noxious thermal stimulus (Dyson, 2017). Before the role of *mbl* in thermal nociception could be further investigated, these preliminary results had to be confirmed. cIV md-da specific *Mbl* knockdown larvae were constructed and exposed to a thermal nociceptive assay. The *mbl* knockdown larvae exhibited significantly longer response latencies than both negative control
larvae genotypes and had a shorter response latency than the \textit{para}-knockdown larvae which represent complete loss of nociceptive function. This difference in thermal nociceptive response between cIV md-da specific \textit{mbl} knockdown larvae and the negative controls, indicates \textit{mbl} has an unidentified function in nociceptors that is involved in the regulation of thermal nociceptor sensitivity. The mean response latency of \textit{mbl} knockdown was also less than that of \textit{para} knockdown larvae, indicating that nociceptor-specific \textit{mbl} knockdown results in a \textit{partial} loss of nociceptive function. To better determine \textit{mbl} function in nociceptors, cIV md-da specific knockdown larvae were generated for downstream effectors of \textit{mbl} involved in other systems to see if they too played a role in nociceptor sensitivity.

\textit{Dys} and \textit{Dg} May Be Required for Normal Thermal Nociceptive Function

The first candidate \textit{mbl} interactors investigated for their role in \textit{mbl} were two members of the Dystrophin Glycoprotein Complex (DGC): \textit{Dystroglycan (Dg)}, and \textit{Dystrophin (Dys)}. The DGC functions to form a strong mechanical link between intracellular actin cytoskeleton to the ECM. Intracellular Dys proteins bind intracellular actin on one end and the membrane integrated β subunit of the Dg protein on the other. Dg’s extracellular α subunit binds the β Dg subunit on one end and laminin of the ECM on the other; forming the mechanical link between actin cytoskeleton and the ECM (Durbeej and Campbell, 2002). \textit{Mbl} knockdown has been shown to increase the severity of muscular degeneration seen in the flight muscles of both \textit{Dys} and \textit{Dg} mutant \textit{Drosophila} and increase the severity of the crossvein phenotype seen in \textit{Dg/Dys} dual knockdown \textit{Drosophila} mutants (Kucherenko et al., 2008). \textit{Mbl} is also thought to interact with \textit{Dg} and \textit{Dys} in the fly visual system. Heterozygous mutations for either \textit{Dys} or \textit{Dg} result in defects in photoreceptor axon path finding. Heterozygous \textit{mbl} mutations produce a similar
phenotype. Additionally, knockdown of \textit{mbl} in a \textit{Dys} heterozygous background has been shown to increase the severity of the photoreceptor axon path finding defects. It is worth noting that heterozygous \textit{mbl} mutations in heterozygous \textit{Dg} background also appeared to increase axon pathfinding defects just not to a significant degree (Marrone et al., 2011).

Using this information, I hypothesized that \textit{Dg} and/or \textit{Dys} may interact with \textit{mbl} proteins in the regulation of nociceptor sensitivity. Two separate clV md-da specific \textit{Dys} knockdown larvae genotypes were used to investigate the role \textit{Dys} play in noxious thermal nociceptor sensitivity: \textit{Dys}#55641 and \textit{Dys}#31553. Nociceptor-specific \textit{Dys}#55641 knockdown larvae exhibited longer response latency to the noxious thermal stimulus compared to the GAL4-only and UAS-\textit{Dys}#31553-only control but not UAS-\textit{Dys}#55641-only control. clV md-da specific \textit{Dys}#31553 knockdown did not produce a significantly different thermal response latency relative to wild type. Interestingly, \textit{Dys}#55641 knockdown larvae exhibited significantly longer response latencies than the \textit{Dys}#31553 knockdown. This is likely because the UAS-\textit{Dys}#31553-RNAi line uses a VALIUM1 vector to incorporate the transgenic cloned repeats of the \textit{Dys} transcript into the fly construct while the UAS-\textit{Dys}#55641-RNAi line uses a VALIUM20 vector. VALIUM20 vectors are much more effective at RNAi knockdown than VALIUM1 vectors, particularly in somatic cells, because they incorporate shorter hairpin repeats than VALIUM1 vectors (Hu et al., 2021). It is likely that there is a more complete knockdown of \textit{Dys} in the nociceptors of \textit{Dys}#55641 – knockdown larvae compared to \textit{Dys}#31553 – knockdown larvae and this is the reason we are seeing differences in their response latencies. The thermal nociception assay results for the clV md-da specific \textit{Dys}#55641 knockdown larvae indicate that a more complete knockdown of \textit{Dys} function in nociceptors may result in small defects in thermal nociception while a weaker knockdown of
Dys does not. Further research is needed to confirm that the differences in vectors is the reason for the disparity seen in nociceptive response between $Dys^{55641}$ and $Dys^{31553}$. cIV md-da specific $Dg$ knockdown larvae also exhibited longer response latencies to noxious thermal stimulus than the $ppk$-GAL4-only control but not the UAS-$Dg$-RNAi only control. However, because cIV md-da specific $Dg$ knockdown larvae were only significantly different from one negative control but not the other, it is difficult to have full confidence in these results.

While there is no direct evidence of $Dg$ or Dys expression in nociceptors, DGC’s have been identified in a number of other neuron types suggesting DGC’s may also be present in cIV md-da neurons. The DGC serves as a crucial mechanical link between the actin cytoskeleton and laminin of the ECM and adhesion of the cytoskeleton is important for cell-to-cell signaling and membrane integrity. It is possible that the reduced nociceptive responses seen in cIV md-da specific $Dg$-knockdown larvae and cIV md-da specific $Dys^{55641}$-knockdown larvae is due to a disruption of this mechanical link between the nociceptor cytoskeleton and surrounding ECM. Disruption of this connection could be causing defects in nociceptor morphology leading to improper nociceptor innervation resulting in reduced nociceptive response to noxious thermal stimulus. This is supported by research that shows knockdown of $Dys$ or $Dg$ in the photoreceptors of Drosophila results in defects in photoreceptor axon guidance and rhabdomere elongation (Marrone et al., 2011). Defects in morphology can also have a significant impact on cIV md-da neurons interactions with surrounding epidermal cells. The dendrites of cIV md-da neurons are ensheathed by epidermal cells allowing for innervation of the epidermis by nociceptor nerve endings. Loss of this ensheathment has been shown to produce excessive dendrite branching and branch turnover, as well as reduced nociceptive
sensitivity in *Drosophila* (Jiang et al., 2019). It is possible that *Dg/Dys* knockdown results in the loss of the mechanical link between intracellular actin cytoskeleton and the ECM. Loss of this connection could produce decreased nociceptor sensitivity by preventing clV md-da neurons from communicating with epidermal cells resulting in a loss of ensheathment of nociceptors by epidermal cells and in turn, defect morphology and innervation.

Loss of the mechanical link between the ECM and intracellular actin cytoskeleton could also be producing defects in cell-to-cell signaling. *Dg* and *Dys* have been shown to associate with certain signaling molecules. *Dys* has been shown to associate with neuronal nitric oxide synthase (nNOS). nNOS synthesizes nitric oxide, a nociceptive signaling protein, and is expressed in nociceptors and their terminals in the spinal cord (Maihofner et al., 2000). In neurons, nitric oxide activates cGMP-dependent protein kinase (PKG) which regulates neurite outgrowth and collapse and neurotransmitter release (Tegeder et al., 2011). While this exact mechanism has not been identified in fly nociceptors, PKG is known to be expressed in human nociceptors (Luo et al., 2012), and nNOS expression has been observed in the rat and monkey nociceptors (Zhang et al., 1993; Qian et al., 1996), suggesting this role for *Dys* and nNOS signaling may be applicable to nociceptors and fly nociceptor sensitivity. It is possible that loss of *Dys* function prevents the recruitment of nNOS to the developing nociceptor resulting in decreased PKG activation and partial loss of nociceptive response due to decreased ability to transmit neurotransmitters to second-order neurons (SONs).

β-Dystroglycan is known to be bound by *Grb2*, a signal transduction adapter protein involved in *Ras/MapK* signaling pathways. The function of this MAPK pathway is to transduce signals from the ECM to the inside of the cell. *Grb2* is known to serve as an adapter for the
binding of Ras, the first component of Ras/MapK signaling cascades (Molina and Adjei, 2006). The Ras/Mapk signaling pathway consists of a RAS signaling protein and three protein kinases: RAF, MEK, ERK. Extracellular signal-regulated protein kinase (ERK) is a mitogen-activated protein kinase (MAPK) that mediates intracellular signal transduction in response to a variety of stimuli (Obata and Noguchi, 2004). ERK is activated by phosphorylation via MEK protein kinases in response to noxious stimulation of peripheral tissue. Research involving rats has demonstrated that exogenous introduction of capsaicin, a known activator of C-fiber nociceptors, induces ERK phosphorylation in nociceptors of the DRG and SON of the dorsal horn (Dai et al., 2002). Activation of ERK is important for the development and maintenance of nociceptor sensitivity (Obata and Noguchi, 2004; Ji et al., 2009). It is possible that loss of Dg function in nociceptors could be preventing the binding of Grb2 to Dg in the nociceptor membrane inhibiting the formation of the Ras/ERK signaling pathway. Loss of this pathway would prevent ERK activation in response to noxious stimuli and in turn produce defects in nociceptor sensitivity. Grb2 is not the only adaptor protein associated with the Ras/ERK signaling pathway. It is possible that other adaptor proteins, not bound by Dg, are still incorporated in the cell membrane by other intermembrane proteins, allowing Ras/ERK pathway to still occur, albeit at a lower level. It is also known that Grb2 interacts with many different transmembrane receptors so it is possible that some other receptor can activate the Ras/Erk through Grb2. Both hypothesis could explain why Dg knockdown does not result in complete loss of nociceptive function.

While there is no direct evidence of clV md-da specific relationship between Dg or Dys and mbl, it is possible that the defects seen in mbl knockdown are caused by a dysregulation of
*Dg* or *Dys*. This potential misregulation of *Dg/Dys* by *mbl* could result in a disruption in the mechanical link formed between ECM and cytoskeleton by the DGC resulting in the loss of the *Dg-Grb2* interaction and the *Ras/MapK*, loss of the nNOS-PKG signaling pathway and/or some other yet-to-be identified interaction. More research needs to be conducted to determine the direct cause of the thermal nociceptive deficiencies seen in cIV md-da specific *Dg* and *Dys* mutants.

**Dg but not mbl May Play a Role in Mechanical Nociception**

After confirming that *mbl* is required for thermal nociceptive sensitivity and finding that *Dg* may also be involved, I decided to investigate if *mbl* or *Dg* has a role in mechanical nociception. cIV md-da specific *mbl* knockdown larvae did not exhibit altered mechanical nociceptor sensitivity relative the negative controls. Interestingly, cIV md-da specific *Dg* knockdown larvae exhibited significantly reduced nociceptive response to the noxious mechanical stimuli relative to UAS-*Dg*-only control but not the *ppk*-GAL4 only control. This finding suggests *Dg* knockdown may cause small deficiencies in mechanical nociceptive function. However, because cIV md-da specific *Dg* knockdown larvae were only significant from one negative control but not the other, this makes it difficult to have full confidence in these results. If nociceptor-specific *Dg* functions in mechanical nociception, it is likely a mechanism that does not involve regulation by *mbl* as cIV md-da specific *mbl* knockdown larvae did not exhibit alternative mechanical nociceptive function relative to either negative controls. It all suggests potential polymodal nociceptive function for *Dg* and possibly the DGC but further research is needed to confirm this.
Nociceptor-specific actn Knockdown Results in Defects in Thermal Nociception

*actn* was also investigated for its role in nociception in *Drosophila melanogaster*. *actn* encodes a cytoskeletal actin filament cross-linking protein (α-actinin) with muscle and non-muscle-specific isoforms produced via alternative splicing (Larkin et al., 2021). Previous research in *Drosophila melanogaster* has shown that α-actinin pre-mRNAs require MBLC for normal expression and that α-actinin transcripts contain putative MBNL1 binding sites (Vicente et al., 2007). Using this information, I decided to investigate whether *actn* is a downstream effector of *mbl* in nociceptors involved in thermal nociceptor sensitivity. The results of the thermal nociception analysis showed that cIV md-da specific *actn* knockdown larvae exhibited the greatest defects in thermal nociception relative to other investigated interactors and was the only interactor to have longer response latencies than both the GAL4-only and UAS-only controls. This indicates that nociceptor specific *actn* is required for normal thermal nociceptive function.

While there are no nociceptor-specific functions established for *actn*, non-muscle isoforms of *actn* have been identified as being involved in the attachment of actin microfilaments into the cell membrane of numerous cell types (Burridge and Feramisco, 1981; Duhaiman and Bamburg, 1984; Edlund et al, 2001; Hsu et al., 2018). It is possible that reduced thermal nociceptive response seen in nociceptor-specific *Actn* knockdown larvae is due to the reduced actin attachment along the membrane of nociceptors, resulting in decreased cytoskeletal and membrane connection and stability. This reduced cytoskeletal/cell membrane connection could be causing morphological defects via a loss of nociceptor ensheathment in
the larval body wall as was described for Dys and Dg previously in this discussion. Loss of this connection could also be causing defects in nociceceptor motility and dendrite elongation.

Actinin proteins have also been shown to be required for proper cell migration and adhesion. Actinin proteins are a primary component of the actin network in the lamellipodia of migrating cells and are incorporated into focal adhesions involved in the migration process. Focal adhesion form mechanical links between intracellular actin bundles and the ECM. These mechanical links between the cell membrane and ECM assemble and disassemble in successive fashion allowing cells to “crawl” during migration (Choi et al., 2008; Liu and Chu, 2017). In humans, focal adhesions are composed of ~10-30 actin filaments cross-linked by α-actinin proteins (Masuda et al., 2009; Legerstee and Houtsmuller, 2021). Neuronal motility is required for directed migration of neuronal precursor cells, dendrite development, and guided elongation of axons to their target cells (Zheng and Poo, 2007). It is possible that loss of α-actinin in cIV md-da neurons results in reduced migration of nociceptor precursor cells and/or defects in dendrite elongation along the Drosophila larval wall in turn producing defects in innervation and reduced nociceptive response to noxious thermal stimulus. This is supported by research that showed genome-wide ACTN1 knockdown results in the failure of focal adhesions to form and produces severely inhibited motility rates in migrating cells (Choi et al., 2008).

α-actinin has also been implicated in the localization, morphogenesis, and anchoring of the NR1 and NR2B subunit of the NMDA glutamate receptors in vertebrates (Wyszynski et al., 1997; Dunah et al., 2000). Glutamate is released by peripheral nociceptors following activation by a noxious stimulus and is bound by NMDARs along the post-synaptic terminal of SONs found in the dorsal horn of the spinal cord, allowing for nociceptive signal propagation. Due to the
high expression levels of NMDARs in dorsal horn neurons, NMDARs are traditionally associated with post-synaptic neuron endings. Interestingly, presynaptic NMDARs have also been identified in the terminal nerve endings of nociceptors (Shigemoto et al., 1992; Liu et al., 1994). Presynaptic nociceptive NMDARs are involved in the hypersensitization of nociceptors. In the case of inflamed peripheral tissue, enhanced calcium influx through NMDARs is observed relative to basal nociceptive calcium influx (Li et al., 2016). These enhanced calcium levels activate the PKG-1 signaling cascade which results in increased glutamate signaling from nociceptors to SON. Activation of this pathway has been shown to convert nociceptive C-fiber synaptic depression into potentiation via a positive feedback loop following SON signal transmission, resulting in increased nociceptor sensitivity and signaling in inflamed tissue (Xie et al., 2022). The role of NMDARs in vertebrates is established but further research is needed to confirm if this same mechanism occurs in Drosophila nociceptors.

Regardless of the mechanism by which α-actinin regulates thermal nociceptor sensitivity, it is possible that mbl has a role in the regulation of actn in nociceptors, and that the defective thermal response seen in nociceptor specific mbl mutants is due to altered splicing patterns of actn by mbl but further research is needed to confirm this. This is partially supported by previous research demonstrating that actn has five perfect nucleotide matches for the MBNL1 binding domain (Monferrer and Artero; 2006).

Future Directions

Now that confirmation of mbl role in thermal nociceptor sensitivity has been established, and several notable downstream effectors of mbl have been identified as potentially having roles in nociceptor sensitivity, research is needed to confirm if the thermal...
nociceptive defects seen in mbl knockdown larvae is due to misregulation of the mRNA of candidate interactors also shown to effect nociceptor sensitivity (Dg, Dys, α-actinin) or if it is due to some other mbl mechanism. Antibody staining could be conducted on isolated nociceptor cells to confirm the presence of Dg, Dys, α-actinin proteins in nociceptors in control and mbl knockdown genotypes. Following confirmation, RNA-sequence analysis could be conducted to determine the presence of aberrant splicing patterns of Dys, Dg, or α-actinin in the nociceptors of cIV md-da specific mbl knockdown larvae.

In order to confirm the role of mbl in nociceptor dendrite morphology, more samples of cIV md-da specific GFP tagged, mbl knockdown larvae analyzed so the sample size is large enough for statistical comparison to negative controls. Following confirmation, knockdown larvae for GFP-tagged Dys, Dg, or α-actinin knockdown larvae could also be constructed and subjected to confocal microscopy to determine if similar morphological defects are observed relative to cIV md-da specific mbl knockdown larvae. If aberrant isoform expression is detected for Dys, Dg, or α-actinin proteins in mbl knockdown mutants and if these aberrantly spliced genes show similar defects to mbl knockdown larvae when knocked down, this would strongly suggest a nociceptor specific regulation of that gene by mbl.
References


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