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Canonical BMP Signaling Is Required For Allodynia In Drosophila Melanogaster

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BMP SIGNALING AND DIET EFFECTS NOCICEPTION AND ALLODYNIA IN THE *DROSOPHILA MELANOGASTER*

BY

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B.S. University of New England, 2013

THESIS

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DEDICATION

I dedicate this thesis to all those who have helped me along my path. Foremost is Dr. Geoffrey Ganter. He extended his role far beyond that of a lab mentor; he nurtured my confidence and sparked a dream that I will fervently pursue for many years to come. I would also like to extend a huge thanks to my fiancé Samantha, who spent countless hours reading my work, listening to my talks and washing my larval preps. Finally, thanks to Michael Anderson who has always been a true friend to share the pains of graduate school and scientific research with. I'll dearly miss our beer and science discussions at Kerrymen's.

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I would like to thank the University of New England CAS for providing me with a teaching assistantship. Furthermore, for funding my final summer of research in Dr. Ganter's research lab. This work would not have been possible if it weren't for the techniques provided by Dr. Michael Galko and Dr. Daniel Cox. Also, Kayla Gjelsvik helped considerably in the troubleshooting of the allodynia methods. I would also like to thank Dr. Ian Meng for providing a research assistantship for my second year. This work was work supported by: NIH/NIGMS award 1P20GM103643-01A1 to I. Meng.

FOREWORD

This thesis describes two major contributions from my Master's Program. The first (chapter II) describes in detail the work discovering that BMP expression in the nociceptor neurons is necessary and sufficient for the formation of allodynia in the *Drosophila melanogaster*. When we decrease expression of a BMP member decapentaplegic (dpp) specifically in the nociceptor neurons we find attenuation in the formation of allodynia. Furthermore, the receptors for dpp are also necessary on the nociceptor neuron in order to produce sensitization. Lastly, this pathway operates through a canonical signaling cascade. This work on the BMP pathway will be submitted to a peer-reviewed journal for publication. The next major finding, described in chapter III, covers the discovery that the type of diet is essential in *Drosophila* for the capacity to form sensitization and it also affects the normal nociception behavior. This work is not getting submitted for peer-review, but is currently being investigated by other members of the lab.

This thesis has been examined and approved.

Thesis Director, Title of Faculty and Discipline

Committee Member, Title of Faculty and Discipline

Committee Member, Title of Faculty and Discipline

Date

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ABSTRACT

CANONICAL BMP SINGALING IS REQUIRED FOR ALLODYNIA IN *DROSOPHILA MELANOGASTER*

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In the United States alone over 100 million people suffer from chronic pain and unfortunately, even still, there is a lack in scientific understanding for the mechanisms of abnormal pain sensitivity. The present study utilized a candidate gene approach to identify novel components required for modulation of the tissue damage induced pain sensitization pathway in *Drosophila melanogaster*. We have shown that RNAi silencing of decapentaplegic (dpp), a member of the Bone Morphogenetic Protein (BMP) signaling pathway, specifically in the class IV multidendritic nociceptor neurons significantly attenuated UV-induced nociceptive sensitization. Furthermore, overexpression of dpp in nociceptor neurons was sufficient to induce sensitization in the absence of tissue damage. We then show that the dpp receptors are required on the nociceptor neuron in order to produce allodynia, demonstrating that dpp is signaling to the very neuron that produced it. Lastly, we show that this BMP pathway is utilizing the canonical signaling SMAD factors to induce allodynia. We show that the effects of BMP signaling were largely specific to the sensitization pathway and not to normal nociception or dendritic morphology. Thus, we have shown that dpp plays a crucial and novel role in sensitization. Because the BMP family is so strongly conserved between vertebrates and invertebrates it seems likely that the genes we have analyzed represent potential therapeutic targets applicable to humans.

CHAPTER I: INTRODUCTION

Pain in the United States has enormous implications within not only the medical field, but also for the economic well being of the country. As stated by the US Pain Foundation, over 100 million people in America are battling chronic pain [1]. As a result, the US economy has been impacted by the 635 billion dollars spent for medications, health care expenses and pain research[1]. With such a large impact on the economy and moreover an even greater impact on the lives of so many suffering humans it is no surprise that many researchers have focused their aims on teasing out the mechanisms behind pain. While there are already some useful pain management drugs such as the opioid analgesics, these drugs have negatively impacted the lives of everyday users with a myriad of side effects including: nausea, constipation, hormonal dysfunction, muscle rigidity, addiction liability and development of drug tolerance [2]. A more thorough understanding of pain and its mechanisms is direly needed to begin developing new and improved drug treatments.

Pain research has made considerable progress in the last couple of decades, and some of the most important discoveries were made utilizing the *Drosophila* model organism. The genetic similarity between the *Drosophila* and vertebrates is striking; the *Drosophila* genome contains 77% of the disease genes found in humans [3]. The study cited utilized a BLAST genome comparison and identified that for most human diseases that involved a mutant Mendelian allele, there were orthologous gene counterparts within the *Drosophila* genome. A few of the disease genes reported were: Huntington's Disease, Saethre Chotzen syndrome and Spino-cerebellar ataxia. The Rubin lab discovered the Transient Receptor Proteins in 1989 while researching the mechanisms involved with the *Drosophila* visual pathway [4]. Little did they know that the TRP protein they discovered was one of many TRP channels that would soon be discovered. Several TRP channels act as transducers for noxious and also non-noxious stimuli. The importance of these proteins, and for pain in general, is to allow avoidance mechanisms for events that could be potentially harmful. When something hot touches human skin, a TRP channel known in vertebrates as TRPV1, expressed in nociceptor neurons, activates and opens a cation channel, inducing an action potential in the neuron. The temperature at which the TRP channel is activated and induces an action potential of the nociceptive neuron is the threshold for that stimulus. Another TRP channel, responsible for sensing heat, was discovered in *Drosophila* as the *painless* gene [5]. Similarly the TRPA1 channel was

shown in *Drosophila* to have a role in chemical nociception, mediating aversion to AITC [6]. Furthermore, dTRPA1 was shown to regulate thermal avoidance through expression in the central nervous system[7] and peripheral nervous system[8]. The TRP channels, discovered in the *Drosophila* system, have provided a cornerstone of research for the entire vertebrate pain community.

The word pain is a general term for the higher cognitive interpretation of a potentially harmful stimulus. Nociception, on the other hand, is the basic response to a potentially tissue damaging stimulus. At first glance the distinction between pain and nociception is vague, however the underling difference is quite clear. Pain is a higher-level cognitive interpretation of a nociceptive stimulus, often coupled with emotional reactions. Thus, usually when researchers intend to study pain, they may actually be studying nociception. Here we plan to study nociception in the fruit fly.

A less known area in the field of nociception research is the mechanism for sensitization. The sensitization pathway is a mechanism distinct from the usual nociception pathway. Instead of seeking to understand the direct pathways of nociception, research in sensitization concentrates on the “control knobs” for pain, the cellular methods that will lower or raise the threshold for perceiving a stimulus as noxious. How does the body effectively lower its threshold to pain as a result of tissue injury? The purpose of this pathway is adaptive. Your body evokes an increased response to stimuli that might inhibit wound healing. Sensitization usually occurs as a result of tissue damage, and when damage occurs the body’s response is to repair the accumulated damages. Thus, if the body can cause avoidance to any further damage of that area, then wound healing can occur unimpeded. It is not yet fully understood how the threshold for noxious stimuli is lowered to evoke allodynia or hyperalgesia in the peripheral neurons. There are two distinct types of sensitization: allodynia and hyperalgesia. Allodynia is defined as pain evoked by a normally non-noxious stimulus. It can be easily imagined if you recall an instance in which you spent too much time outdoors and as a result of the UV exposure, a sunburn formed. The next time that you tried to shower, what was normally a comfortable temperature felt excruciating; the term for this type of sensitization is allodynia. Hyperalgesia is defined as an increase in the perception of pain to an already noxious stimulus.

One group has begun looking at the genes within the *Drosophila* genome necessary for the formation of allodynia and hyperalgesia. In 2009 the Galko lab discovered that the

Drosophila ortholog of Tumor Necrosis Factor alpha (TNFα), eiger, was necessary for the formation of hyperalgesia following UV induced tissue damage. Once they knocked down eiger with a RNA interference manipulation, allodynia would not form after UV induced damage. Two years later the Galko lab made another discovery: hedgehog (Hh), the invertebrate ortholog of sonic hedgehog, was necessary for the formation of allodynia and hyperalgesia. When the Galko lab knocked down Hh production, the result was a lack of allodynia and hyperalgesia formation after UV induced sensitization. The same results were achieved through knocking down the Hh receptor, patched, on the class IV multidendritic neurons. They concluded that Hh was released, along with a multitude of other cytokines, from damaged tissues and were communicating with the primary nociceptor neurons causing increased excitability of these neurons to normally innocuous stimuli (allodynia), and to already noxious stimuli (hyperalgesia). To further explore this pathway, Babcock dove deeper into the Hh pathway and screened for downstream genes that could also be necessary for the formation of allodynia and hyperalgesia. One of those genes was dpp.

Much is already known about dpp's role in development of *Drosophila.* Firstly, *Drosophila* dpp is a functional homolog of mammalian BMP 2/4. In an experiment done by Sampath et al, fly dpp was sufficient to induce endochondral bone formation in mammals [9]. Conversely, human BMP 4 ligand sequences can be inserted into *Drosophila* larvae to rescue dpp deficient larvae [10]. In the fly, dpp is characterized as a graded morphogen. It is produced from a localized source, and diffuses out into the extracellular space, driving development of germline stem cells, wing imaginal discs and even dorsoventral differentiation [11]. The dpp ligand triggers an extensive signaling cascade. dpp is an extracellular ligand that is released from a specific cell; it then diffuses through the extracellular fluid until it binds to a type II receptor, punt. Punt then forms a heteromeric complex with a type I receptor, either thickveins (tkv) or saxophone (sax). punt will phosphorylate the type I receptor, activating serine threonine kinases in the intracellular domain of type I receptor protein. When this occurs, the type I receptor will then trigger the SMAD cascade via activation of receptor-SMADs (R-SMADs). The type I receptor does this first by phosphorylating mothers against dpp (mad). Mad, once activated,

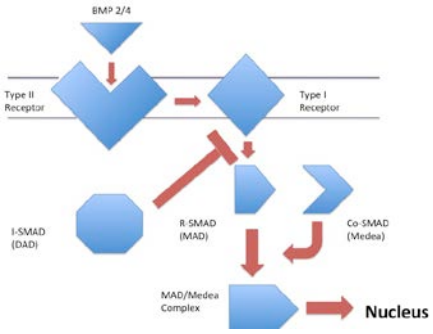

Figure 1.1: The dpp signaling cascade.

forms a complex with medea (med), and is transported to the nucleus where it can execute its transcriptional activation function [12]. There exist inhibitor SMADs, which act to downregulate activity of the SMAD cascade. An example of an inhibitory SMAD is daughters against dpp (DAD). Dad binds with the type I receptor, and blocks activation of the R-SMAD complexes, blocking any further downstream activity.

The BMP family is largely known for its role in the development of the nervous system. In the developing ectoderm the ectodermal cells produce and excrete BMPs, which then bind to the type II receptors to suppress differentiation of neural cells and promote epidermal cell differentiation[13]. Later, the BMP pathway allows for the differentiation of roof plate cells at the dorsal midline by BMP signal from epidermal cells[14]. Once the neural tube has closed the roof plate cells themselves begin to express BMP and will then induce the differentiation of neural crest cells, followed by several populations of sensory neurons. BMPs produced in the epithelial cells have even been linked with controlling innervation of sensory neurons. Guha overexpressed a BMP inhibitor Noggin from the keratinocytes and showed that when epithelial cell derived BMP signaling was inhibited there was an increase in the number of neurons present in the DRG and an increase in the density of those neurons in the peripheral tissue[15]. Conversely, when they overexpressed BMP4 with the same driver they showed a decrease in the number of sensory neurons in the DRG and a decrease in the peripheral density of neurons.

The role of BMPs has been somewhat connected to the study of pain. Application of BMPs 2,4 or 6 to cultured DRG neurons induces the c-fiber neurons to begin expressing CGRP, which has been implicated for roles in migraine pain [16,17]. Later work showed that BMP 4 acted as a switch to turn on CGRP in DRG neurons[16]. They could inhibit the up regulation of CGRP by applying the BMP inhibitor follistatin. Lastly, Kawakami et al. showed that BMP 7 injected into the nucleus pulposis during a spinal disc compression model reduced the formation of mechanical allodynia[18].

While the role of BMPs in pain has been largely overlooked, the other members of the TGF family have not. The other major branches are the TGF-B subfamily and the activin subfamily, both of which have roles in nociceptive processing and pain sensitization[19]. Intrathecal administration of TGF-B1 during sciatic nerve ligation caused significant attenuation of allodynia and hyperalgesia[20]. The capacity of TGF-B1 to exert anti-nociceptive effects is modulated through control of inflammatory signals such as IL-6. This supports the role of TGF-B1 as an anti-inflammatory. Additionally, knockout of a

native TGF inhibitor, BAMBI, showed a decreased neuropathic pain induced from a sciatic nerve crush model. The results were then reversed with application of naltrexone, indicating that this pathway is connected to the native opioid system[21]. The activin family has implications in the sensitization system by exerting effects on the TRPV1 thermal sensing channel. When activins signal to their receptors a signal transduction cascade phosphorylates the TRPV1 channel causing increased sensitivity to thermal stimuli[17]. Furthermore, activins have been shown be upregulated after tissue damage and are believed to increase CGRP levels in the nociceptor neurons[22].

With so much already known about BMPs, it is intuitive that if dpp is necessary for the formation of allodynia in the fly, it must do so by binding with its receptors and ultimately activating of the SMAD cascade. It is our goal to learn which of dpp receptors are involved and what parts, if any, of the SMAD cascade are involved in pain sensitization. Because the BMP pathway is so conserved, it only follows that research in the field of sensitization should utilize the powerful genetic tools of the *Drosophila* system before extending the study into the vertebrate realm.

GAL4/UAS

The GAL4/UAS system is a genetic tool used for many different genetic manipulations within the fruit fly. GAL4 is a yeast transcription activator protein that binds with a UAS operator sequence within the yeast genome. This genetic system is non-native to *Drosophila*, however scientists have used this it to control gene expression of the fruit fly. The Gal4 sequence is inserted into the genome behind a tissue specific promoter. GAL4 protein gets expressed wherever the promoter is activated. Because of its location dependent effects, the GAL4 is often referred to as the “driver”. Similarly the UAS sequence, being non-native to the fruit fly, must also be inserted into its genome. The UAS sequence is seldom inserted alone. Downstream of the UAS sequence, any of a large variety of different genes can be inserted, such as green fluorescent protein (GFP). The UAS portion of this tool is called the “responder” (fig. 1.2[23]). Usually the Gal4 lines and UAS lines are established homozygously in separate lines of transgenic fruit flies. The lines

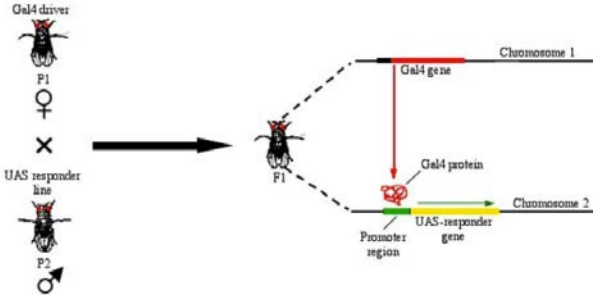

Figure 1.2: Diagram of the Gal4-UAS system[23].

need to be crossed a few days prior to the time of experiment. When crossed the F1 generation carry both the GAL4 tissue specific driver and the UAS reporter protein [24].

RNAi

The RNA interference system utilizes cellular defense machinery to effectively knock down specific mRNAs and therefore proteins from a functional cellular system. Often times it is used in combination with the GAL4/UAS system. The system works by inserting an RNAi allele downstream of a UAS element. Suppose that you wanted to knock down function of a given fly hormone receptor, ecdysone receptor (EcR), you would need to insert a specialized version of the EcR allele into the downstream UAS region. What is specialized about the EcR allele that gets inserted into the genome is that it has the normal genetic sequence of EcR, followed by an inverted copy of the EcR gene. When RNA of this gene is produced it folds back onto itself and forms a hairpin loop, creating double-stranded RNA (dsRNA). Then the cellular defense mechanisms do all of the work. Dicer is a protein designed for cleaving all dsRNA, because dsRNA is often found in viral replication strategies. Thus, Dicer cleaves up the inserted dsRNA and then RNA induced silencing complexes (RISC) get activated [25]. RISC searches for any RNA that matches the cleaved dsRNA dicer produced and destroys it. Because the inserted RNA is the same sequence as the native RNA produced for the EcR protein, all RNA for EcR is destroyed and no new proteins of EcR can form. This causes a drastic reduction in EcR levels and creates a knockdown for EcR. This mechanism is how we will effectively knockdown the dpp protein, and other candidates.

CHAPTER II: CANONICAL BMP SIGNALING IS NECESSARY FOR ALLODYNIA IN *DROSOPHILA MELANOGASTER*

Preamble: This next chapter is written in formal manuscript style because I intend to submit this work to a peer-reviewed journal. This work was all conducted myself with the guidance of my Master's advisor Dr. Geoffrey Ganter.

Abstract:

In the United States alone over 100 million people suffer from chronic pain and unfortunately, even still, there is a lack in scientific understanding for the mechanisms of abnormal pain sensitivity. The present study utilized a candidate gene approach to identify novel components required for modulation of the tissue damage induced pain sensitization pathway in *Drosophila melanogaster*. We have shown that RNAi silencing of a member of the Bone Morphogenetic Protein (BMP) signaling pathway, decapentaplegic (dpp), specifically in the class IV multidendritic nociceptor neurons significantly attenuated UV-induced nociceptive sensitization. Furthermore, overexpression of dpp in nociceptor neurons was sufficient to induce sensitization in the absence of tissue damage. We then showed that the effects of BMP signaling were largely specific to the sensitization pathway and not to normal nociception or dendritic morphology. Thus, we have shown that dpp plays a crucial and novel role in sensitization. Because the BMP family is so strongly conserved between vertebrates and invertebrates it seems likely that the genes we have analyzed represent potential therapeutic targets applicable to humans.

Methods:

Fly Stocks and Genetics:

Experimental flies were purchased through the Bloomington *Drosophila* Stock Center (BDSC) in Bloomington, Indiana. Flies were maintained in 6 oz stock bottles on sucrose-cornmeal-yeast medium at a temperature of 25 °C with a humidity of 50%-60%. Stock bottles were kept in Percival Scientific Incubators (Perry, Iowa) with a 12-hour light, 12-hour dark cycle. The arbitrary dawn time for the incubators was set to 9:00 AM.

We used the GAL4/UAS system to drive expression of RNA interference for specific genes of interest. The driver in all experiments was ppkGAL4, which drives production of GAL4 only in the class IV multidendritic nociceptor neurons[24]. The UAS-RNA*i* lines used are as follows: dppRNA*i* (BDSC#35214, 25782), putRNA*i* (BDSC#35195, 27514),

tkvRNA*i* (BDSC#35166, 35653), saxRNA*i* (BDSC#55865, 36131), madRNA*i* (BDSC#35648, 43183), medRNA*i* (BDSC#31928, 43961). Additionally, we used a UASdpp line to overexpress dpp in the nociceptor neurons (BDSC#1486) and a tkv-active line to constitutively activate the BMP pathway (BDSC#36537).

UV Treatment:

A method of UV induced allodynia was applied[26] 3rd instar larvae 4 days after egg lay were collected, rinsed and anesthetized with diethyl ether. Once anesthetized, the larvae were gently adhered dorsal side up to double-sided tape on a microscope slide and were subjected to 12-15 (mJ/cm^2) of UV light in a Spectronics Corporation Spectrolinker XL-1000 ultraviolet crosslinker. The larvae were then placed in a vial containing ~ 1 ml of sucrose-cornmeal-yeast media. 24 hours after UV exposure the larvae were subjected to a thermal probe assay.

Thermal Nociception Assay:

To test nociceptive behavior the larvae were assayed with a thermal probe. The probe tip was gently applied to the dorsal side of the larvae and held on segments A2-A4 for a maximum of 20 s. To test for allodynia, the larvae were subjected to 41°C, the highest temperature that did not elicit a behavioral response (see Fig 1). The larval response is a nocifensive 360° lateral rolling, that the larvae only exhibit in response to a noxious stimulus[5]. The latency for response was recorded and categorized as follows: fast was 0-6 s, slow was from 6-20 s and none if the 20 s cutoff was reached. For normal nociceptive function the larvae were tested at 45°C and the time to respond was recorded and presented as average response latency. All groups contained a sample size of at least 90 animals.

Quantification of dendritic morphology:

The class IV multidendritic neurons were analyzed for total dendritic length and number of dendritic branches. 3rd instar larvae were anesthetized with CO_2 and placed on a microscope slide with a halocarbon-ether mixture (2:1). Then the larvae were imaged with a Leica SP5 confocal laser microscope using a 20X objective. ddaC neurons, the most dorsal pair of class IV multidendritic neurons, were imaged from abdominal segments 4-6 and z-stacks were taken with a 0.76 µm step size to capture the whole dendritic field. Images were taken with resolution 1024x1024. Images were skeletonized and analyzed for parameters dendritic length and dendritic branching in Fiji.

IHC Conditions and Imaging (must wait until results come in):

Analysis of knockdown was performed using images collected using a Leica SP5 Scanning Confocal Microscope. All images were 1024x1024 on a 40x oil objective. A z-stack was collected to ensure that the entire cell was collected. The Z-stacks were collapsed into maximum projections and the relative intensity of staining was analyzed using Fiji. We stained animals at timepoints 0, 2, 4, 8, 16, 24 hours after UV injury. The antibodies used are as follows: Anti-HRP (Jackson Immunoresearch) at 1:500, and p-SMAD1/5/8 (MMCRI) at 1:500.

Statistical Analysis:

Fishers Exact Test was used to compare population distributions between groups for sensitization results. For normal nociceptive behavior and morphometry Student's t-test was performed. IHC expression was measured using a One-Way Anova. * Represents p= 0.05-0.01, ** represents p<0.01-0.001, *** represents p<0.001.

Results:

In order to optimally assay nociceptive behavior of *Drosophila* larvae a thermal response study was performed on a standard wild-type group (w^{1118}). Beginning at 39°C the temperature of the thermal probe was increased in 1°C increments and 90 larvae were assayed at each temperature. The response latency of each larva was recorded. At 39°C most larvae did not respond. However, beginning at 42°C there was a steady increase in responsiveness with temperature, continuing to 50°C (fig. 2.1). Because there were no responders at 41°C and a statistically significant number of responders at 42°C, 41°C was selected as the appropriate temperature at which to test for allodynia, that is to say this is the highest temperature that does not elicit a significant response.

Confirmation that wild type larvae could form allodynia in response to UV damage was necessary. Control flies (w^{1118}) were divided into two groups: a +/- UV irradiation group. The UV dose was between 12-15 mJ/cm^2 with an exposure time of ~6 (s). In the non-UV irradiated group there was only slight response. However, following exposure to UV-irradiation the responsiveness increases to over 60% (fig. 2.2). The animals in the latter group responded to a stimulus that they would normally perceive as non-noxious and thus represent the induction of allodynia, thus supporting the previous findings that *Drosophila* melanogaster larvae can become sensitized in response to UV-irradiation[26].

Using a RNA*i* mediated knockdown of dpp expression in the nociceptor neurons, we confirmed that suppressing the availability of the dpp resulted in changes to the formation of allodynia. As Figure 3.3 shows, compared to controls the dpp-KD group showed a strong attenuation of allodynia ($p<0.001$). Then, instead of knocking down dpp in the nociceptor neurons we drove overexpression of dpp with a UAS-dpp line in the nociceptor neurons. The dpp overexpression animals were assayed at 41°C without any prior tissue damage. The larvae with elevated dpp expression showed significantly higher sensitivity to the 41°C probe than parental controls.

Specifically in the nociceptor neurons, we suppressed the expression of the various receptors for dpp in order to test for their requirement in the formation of allodynia. Larvae in which each receptor: punt (put), thickveins (tkv) or saxophone (sax) was suppressed showed strong attenuation in the formation of allodynia ($p<.001$) (fig. 2.3). Larvae with suppressed put and sax showed a complete failure to sensitize and those with suppressed tkv showed slight formation of sensitization but significantly reduced from controls. Interestingly, even though tkv is necessary for full formation of allodynia, when we expressed a constitutively active form of the tkv receptor in nociceptor neurons there was no change to pain sensitivity at 41°C (fig. 2.3).

To examine the possibility of canonical signaling, we suppressed the expression of mad through GAL4/UAS-RNA*i* in the nociceptor neurons. Larvae with suppressed mad failed to produce sensitization in response to UV damage ($p<.001$) (fig 2.3). Additionally, we stained for p-mad activation at time points 0, 2, 4, 8, 16 and 24 hours after UV damage to determine when p-mad signaling was up regulated. We found that at time point 0 hours there was a basal p-mad expression that strongly increased at 8 hours after injury and returned back to normal p-mad levels by 24 hours after injury (Fig 3.4).

We assessed the normal nociceptive behavior of larvae in which BMP components were suppressed. The time was measured for the larvae to respond to a thermal stimulus and we calculated the average response latency. As shown in figure 2.5, there was no significant change in normal nociceptive functioning when compared to both controls for any of the knockdown groups.

We determined the effect of UV mediated damage on the dendritic architecture of the nociceptor neurons. The morphology of the nociceptor neurons was measured for the parameters dendritic length and dendritic branching in ppk:eGFP-GAL4 animals that express GFP in the nociceptor neurons. We subjected one group to UV irradiation and

the other to a mock UV treatment and analyzed the nociceptor neurons at peak allodynia. There were no statistically significant differences that resulted from UV irradiation (fig. 2.6).

We investigated whether the suppression of BMP signaling components in the nociceptor neurons resulted in any changes to the dendritic arborization of these cells. For neurons with suppressed tkv or sax, there were no statistically significant changes in either dendritic length or in total dendritic branches. In neurons with suppressed put, there was a slight but significant ($p<0.05$) increase in the dendritic branching but no significant change to the dendritic length. In larvae overexpressing dpp in the nociceptor neurons, we measured a statistically significant decrease for both parameters (fig 2.7).

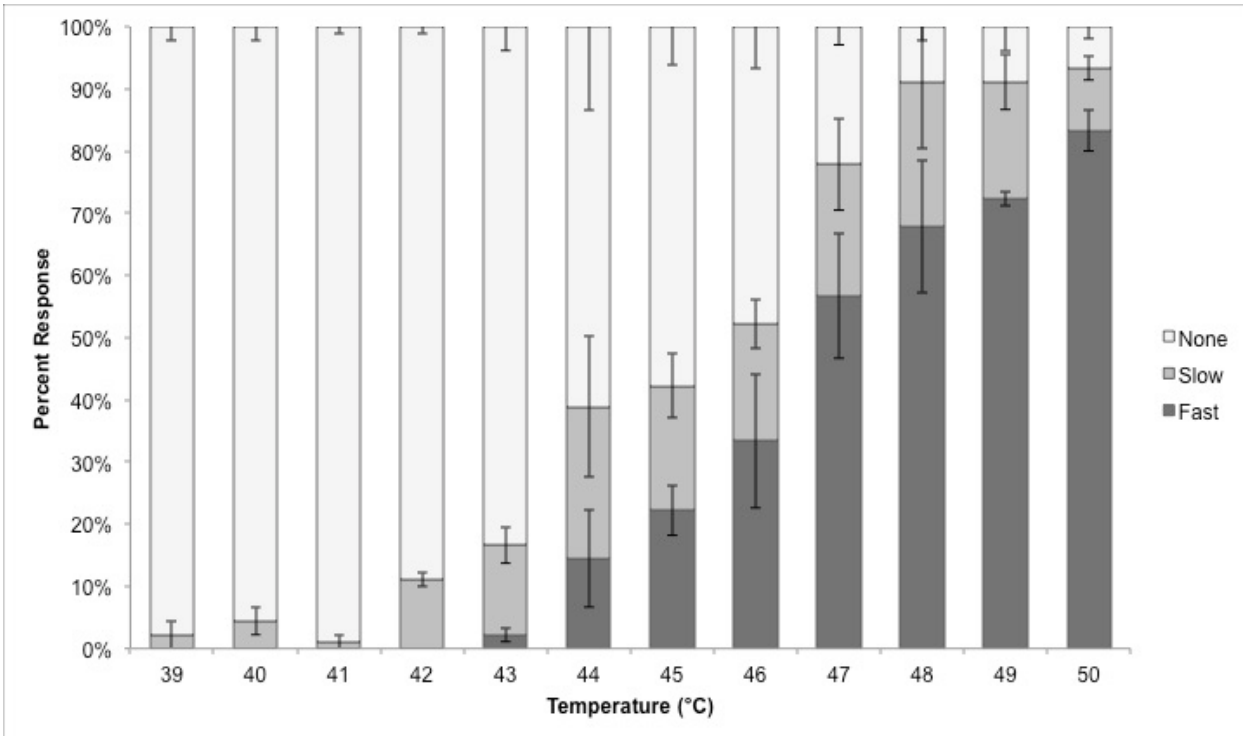

Figure 2.1: Thermal Response of w^{1118}. Shown here are the percentage of animals that responded to a thermal probe stimulus varying in temperature from 39°C to 50°C. 41°C is the lowest temperature for which there is a statistically significant percentage of animals responding. Larvae responding with a nocifensive roll were classified as fast (<6 seconds) or slow (between 6 and 20 seconds), or nonresponders if they did not respond within 20 seconds. Distributions were compared using Fisher's Exact Test. n=90 for all groups.

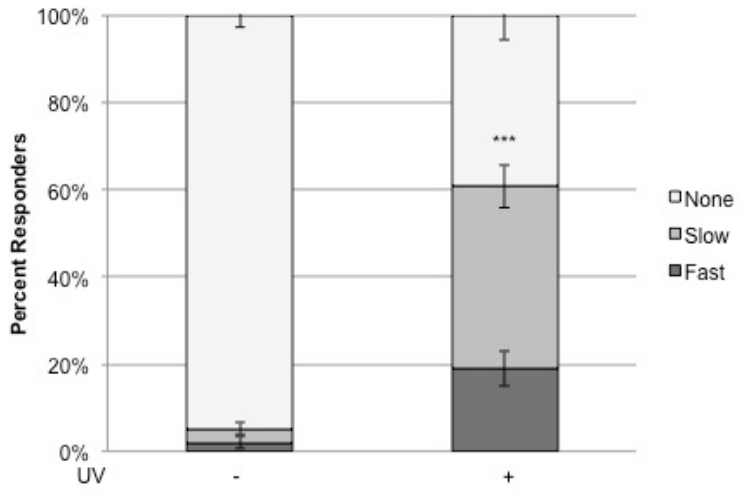

Figure 2.2: Induction of Allodynia in Response to UV Exposure. 24 hours after UV exposure there was a statistically significant increase in the number of animals that respond to a normally non-noxious stimulus. All groups were assayed with a thermal probe set to 39°C. Response latencies were recorded in (s) and categorized as follows: none (>20), Slow (between 6 and 20) and Fast (<6). n=90-117 and distributions were compared using Fisher's Exact Test.

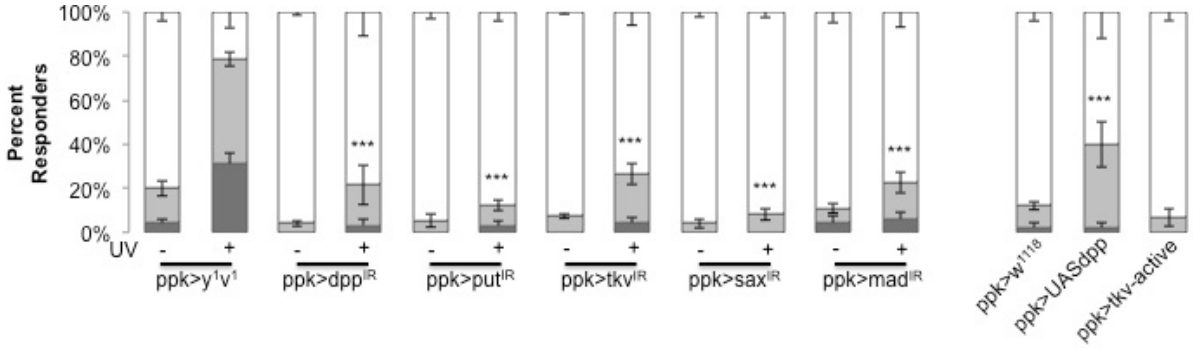

Figure 2.3: RNAi Suppression of BMP Signaling in the Primary Nociceptor Neurons Inhibits the Production of Allodynia. Knock-down of each of the BMP members shown resulted in a failure to produce allodynia, compared to both controls (only 1 control shown). All knockdowns were manipulations made with a ppkGal4>UAS-RNAi genotype to decrease protein levels. The last 2 bars on the right are a ppkGal4>UAS-dpp line to genetically overexpress dpp levels in the nociceptor neurons and a UAS-tkv-active line to express a constitutively active form of the tkv receptor, respectively. All groups were assayed with a thermal probe set to 41°C. Response latencies were recorded in (s) and categorized as follows: none (>20), Slow (between 6 and 20) and Fast (<6). n=90-117, distributions were compared using Fisher's Exact Test.

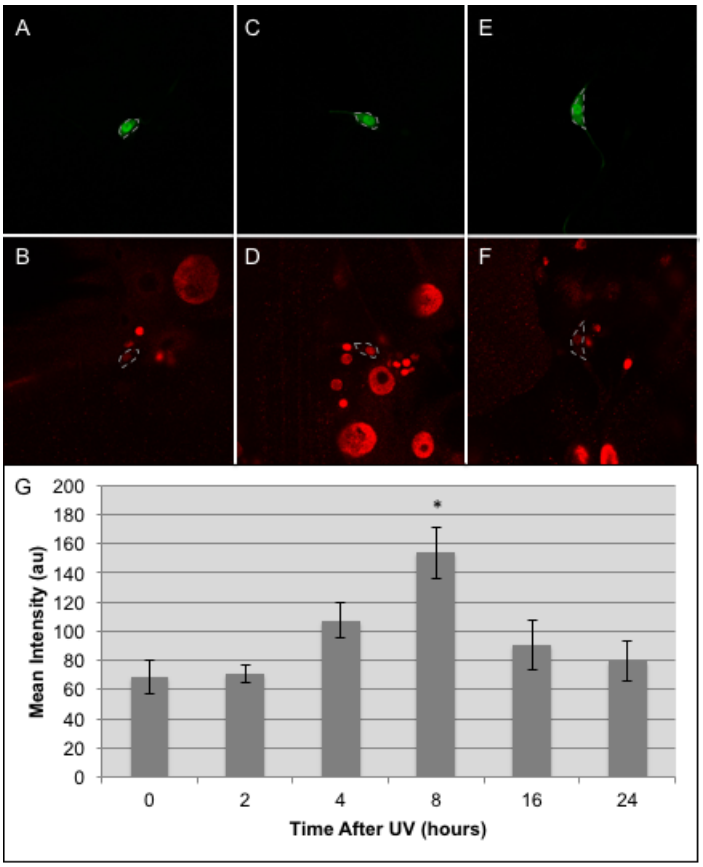

Figure 2.4: p-mad Expression After UV Damage. Staining with anti-p-mad at time points 2, 4, 8, 16 and 24 hours after injury show peak p-mad expression at 4 hours after UV damage. A, C and E show representative nociceptor neurons with anti-p-mad staining (C, D and F) directly below. Cell bodies of the nociceptor neurons have been outlined in white. A and B are representative images of staining immediately following tissue damage. C and D are representative images of staining 8 hours after UV damage. D and F are representative images of staining 24 hours following UV damage. G is the average calculated mean intensity of p-mad signaling in the nucleus of the nociceptor neuron. N=6 for all groups. Data were analyzed with a One-Way ANOVA.

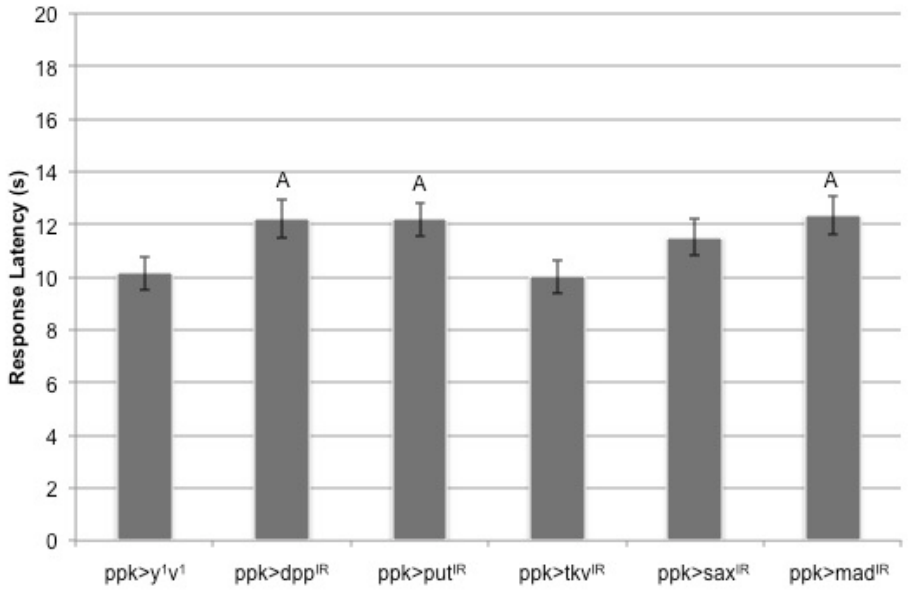

Figure 2.5: Normal Nociception of the Mutants and the Common Control at 45ºC. Normal nociceptive responses were tested to determine the normal nociception behavior of untreated larvae. These data show that the mutants are as capable of responding to normally noxious thermal stimulus as the controls. Probe temperature was set to 45ºC. n= 90 for all groups. (A) indicates difference from one parental control (see appendix 4.7). These data were analyzed by Student's t-test.

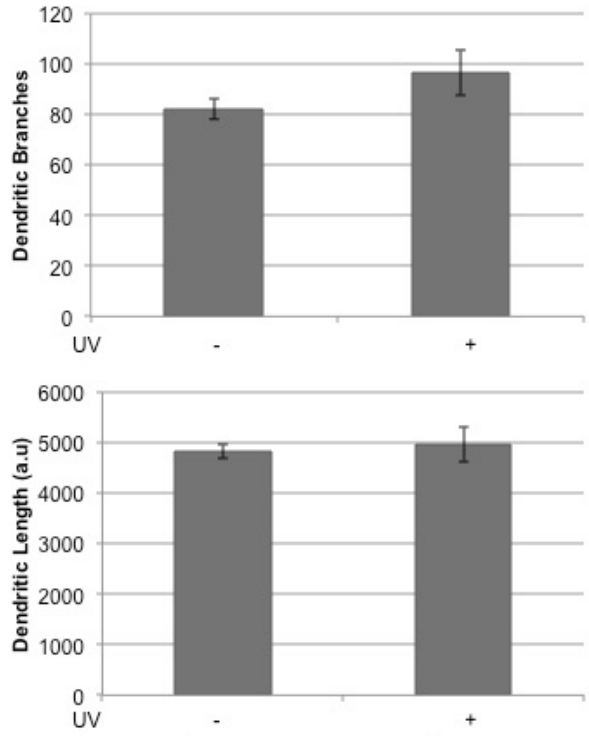

Figure 2.6: Morphological Analysis of Control Animals Exposed to UV Irradiation. Morphological parameters were determined by the total number of dendritic branches (top) and total dendritic length (bottom). Genotype of animals was ppk;eGFP GAL4. Morphometry of control without UV irradiation (left) and with UV irradiation (right) show that there are no significant changes in morphology that result from UV

irradiation. Skeletons were constructed in Fiji and total dendritic length and total the number of branches were calculated. Data were analyzed using Student's t-test. n=18.

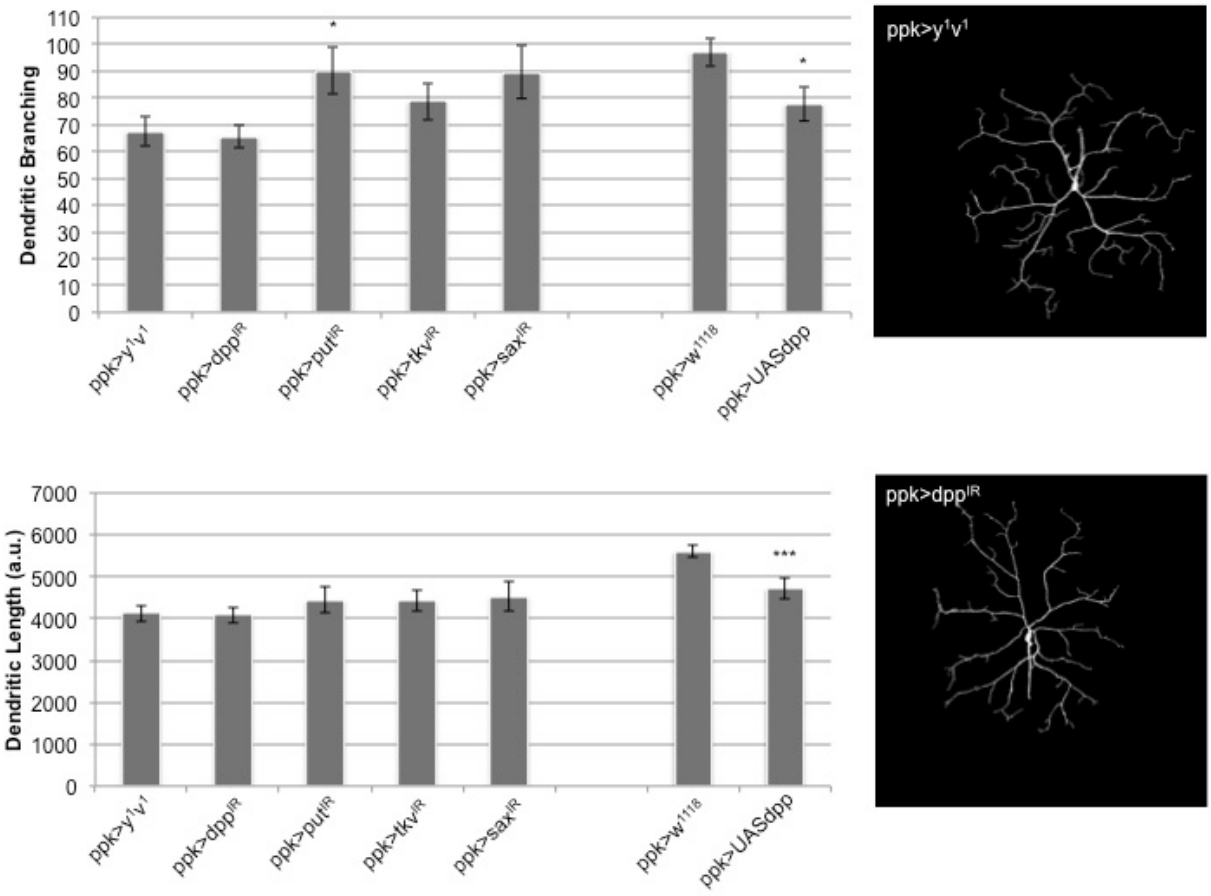

Figure 2.7: Morphological Analysis of Results of BMP Suppression in the Primary Nociceptor Neurons. Morphological parameters were determined by the total number of dendritic branches (top left) and total dendritic length (bottom left). Representative images of parental control (top right) and dpp knockdown (bottom right) show that there are no significant changes in morphology in the BMP manipulations. Up-regulating dpp in the nociceptor neurons significantly decreased the dendritic morphology compared with controls. Skeletons were constructed in Fiji and total dendritic length and total number of branches were calculated. Data were analyzed using Student's t-test. n=18-30.

Discussion:

This study demonstrates the necessity of the BMP signaling pathway, particularly the components downstream of dpp, for the formation of allodynia in the *Drosophila* larva in response to UV-induced tissue damage. BMP's have been well established to play important roles in developmental contexts ranging from *Drosophila* imaginal discs[12] to bone formation in vertebrates[27]. The BMP pathway has also been implicated in neural development and synaptic development in vertebrates and invertebrates, respectively[28,29]. Furthermore, there is a strong orthology between mammalian BMP 2/4 and invertebrate dpp[9,10]. Here we show that the dpp pathway well known for its role in

development also serves a more acute role in the formation of sensitization following tissue damage.

Previous results showed that upon cutaneous tissue damage, Hedgehog (Hh) is released from apoptotic epithelial cells. Then Hh binds with its receptor patched (ptc) on the nociceptor neurons in order to produce allodynia[30]. In developmental studies, it has been shown that Hh up regulates dpp[31]. Furthermore, previous studies have shown that dpp has a role in the development of allodynia[30]. In this study we have confirmed that dpp is necessary in the nociceptor neurons to produce sensitization following tissue damage (fig 2.3). Up regulating dpp in the nociceptor neurons is sufficient to produce allodynia in the absence of tissue damage, supporting the conclusion that dpp is both necessary and sufficient for the formation of allodynia (fig 2.3, right bar). Furthermore, this effect is specific to the sensitization pathway because the suppression of dpp does not cause any changes to the normal nociceptive function at 45°C. Lastly, this manipulation causes no changes to the morphology of these cells. These studies demonstrate the specificity of dpp pathway in its regulation of neuronal sensitivity following tissue damage.

In previous developmental studies, the dpp signaling pathway has been exhaustively elucidated[12,28]. Exogenous dpp binds with its type II receptor put. put will in turn activate either of two type I receptors, tkv and sax. We explored whether these receptors, in addition to their role in development, also play a more acute role in sensitization. We systematically suppressed the expression of the known receptors for dpp in the nociceptor neurons. We found that the type II, primary receptor punt was necessary for the formation of allodynia (fig 2.3). We then investigated the type I receptors tkv and sax and found that both were necessary on the nociceptor neuron to produce sensitization (fig 2.3). This indicates that the dpp sensitization pathway operates through autocrine signaling. dpp is produced in the nociceptor neuron following epithelial tissue damage, is released and is available to the nociceptor neuron's own dpp receptors. There are examples of BMP's signaling through autocrine mechanisms[29], but a role for autocrine BMP signaling in sensory neurons remains novel. Again, the effects on nociception are specific to the sensitization pathway, as there was no significant difference in normal nociceptive function from both controls resulting from any of the RNAi manipulations. When we tried to activate the BMP signaling cascade by expressing an active form of the receptor tkv, we showed that this did not induce allodynia (fig 2.3, right), indicating

that tkv is necessary but not sufficient to produce allodynia. Because both type I receptors are necessary it may be that the amount of MAD signaling required to engage the allodynia machinery is different than the amount that the active tkv manipulation can produce independently.

The necessity of both type I receptors, sax and tkv, for the formation of allodynia, coupled with prior knowledge about the BMP pathway, suggests that dpp forms a homodimer to trigger this allodynia-producing pathway in this cell. If dpp monomers could induce this signal transduction cascade, then the presence of either sax or tkv alone would be sufficient to induce allodynia, but this is not the case (fig. 2.3, right). Furthermore, expression of tkv-active should have been sufficient to produce the levels of p-mad to activate this pathway if dpp monomers could induce the pathway. However, this is not the case (fig. 2.3, right). Both type I receptors are independently necessary and thus they both need to be active for the formation of allodynia indicating that dpp may signal as a homodimer.

The type I BMP receptors are known to activate the canonical signaling proteins mad and med through phosphorylation to form p-mad. Then p-mad will form a complex with med, translocate to the nucleus and regulate gene transcription. We investigated whether the sensitization pathway could be operating through the canonical MAD pathway. By suppressing the expression of mad (fig 2.3) and med (appendix 4.6) we produce significant attenuation of the formation of allodynia, showing that the canonical pathway is responsible for the manifestation of allodynia. Furthermore, we stained for p-mad, the activated form of mad, following UV induced tissue damage and found that p-mad peaked activation at 8 hours after injury and returned to baseline at 24 hours following tissue damage (fig 2.4). This indicates that the canonical pathway is activated at about 8 hours after injury.

We verified the results of all receptor manipulations with independent RNA*i* genotypes to ensure that the effects were specific to that receptor. The results were consistent with the presented RNA*i* manipulations (appendix 4.2-4.5), suggesting that the observed results are not due to off target effects.

In order to demonstrate that the results of manipulation of BMP signaling are specific to the sensitization pathway and not a general loss of nociceptive functioning in these neurons, we tested larvae with suppressed expression of BMP components at 45°C[26,30]. As demonstrated in figure 2.4, no changes to normal nociception resulted in any case.

Due to the lack of changes in the normal nociceptive function of these cells, it is likely that dpp is mediating its allodynia effects through regulation of transducing ion channels. If the changes caused by activation of the dpp pathway changed the overall excitability of the cell, for example through potassium channel regulation, then there should have been a corresponding change in the normal nociceptive function. Previous studies have linked the painless channel to allodynia[30], and it seems likely that dpp signaling is acutely changing the expression or modulation of the painless thermal sensing channel. The dpp pathway could execute nociceptive sensitization by modulating these channels to open at a lower temperature or by increasing the overall density of the TRP channels to allow more graded currents to reach the axon hillock.

Because UV irradiation induces such a strong effect on sensitivity we tested for an increase in nociceptor dendrite outgrowth. We used morphometric analysis to determine if the induction of allodynia following UV mediated tissue damage was accompanied by changes to the dendritic architecture of the nociceptor cells. We found that there were no significant morphological differences between the neurons of a group of UV-irradiated larvae and those of unirradiated controls (fig 2.6), showing that the allodynia following UV damage is not due to changes in dendritic morphology. This also suggests that the morphological changes observed in some RNAi manipulations (fig 2.7) may be the result of a developmental effect and not related to the acute effect on sensitization.

There were some changes in the neuronal morphology of some our experimental groups. Firstly, put knockdown animals showed a significant increase in the amount of dendritic branching. When dpp was up regulated in the nociceptor neurons we saw a decrease in both dendritic branching and length of these nociceptor neurons. Previously it was reported that BMP is released from epithelial tissues to act as an signal to control the amount of sensory neuron innervation through density of neurons and total neurons in the DRG [15]. If epithelial-derived dpp does act as an inhibitory signal it could explain why the RNAi manipulations of BMP receptors showed an increase in total dendritic branching. Furthermore, dpp knockdown in the nociceptor neuron would not have any effect on the pool of dpp being released from the epithelial cells. The nociceptor neurons still have intact receptor machinery to transduce the epithelial-derived dpp signal and would therefore develop normally, as we observed (fig 2.7, right bars). Lastly, up regulating dpp in the nociceptor neuron would lead to an increase in dpp signaling around that neuron and would increase the inhibitory signal cuing the nociceptor neuron

to stop development and would thus lead to a decrease in dendritic architecture, as observed (fig 2.7).

In summary, we have shown that dpp is both necessary and sufficient for the production of allodynia. dpp likely signals through an autocrine mechanism to the primary type II receptor put, in turn activating both type I receptors tkv and sax which then activate the canonical signaling pathway by phosphorylating mad to ultimately produce allodynia. These components have no effect on the normal nociceptive functioning of these cells and only minor effects on their dendritic morphology.

Due to the high degree of conservation of the BMP system between vertebrates and invertebrates we hope to extend these studies into a rat model to test if BMP signaling mediates sensitization in response to UV exposure.

CHAPTER III: EFFECTS OF DIET ON NOCICEPTION AND SENSITIZATION IN *DROSOPHILA MELANOGASTER*

Preamble

This chapter will cover the results from troubleshooting the techniques used in chapter II. While not yet included in any manuscripts, these are the results from a year of troubleshooting techniques and are now the springboard for future studies within the Ganter laboratory. Due to the nature of troubleshooting, I have chosen the write this chapter in a narrative style, opposed to a traditional manuscript style. This is a story that every scientist will relate to and I hope you enjoy reading about our road to discovery.

And so it begins…

To begin working on my proposed thesis project, investigating the mechanisms of BMPs for sensitization in the fruit fly, it was imperative that we learn a working model of sensitization, specifically allodynia. My first goal was to replicate a published method of inducing sensitization in the fly. Other researchers had shown when larvae are subjected to ultraviolet irradiation (UV), the cutaneous tissue of the larvae is damaged and ultimately leads to thermal sensitization manifested as allodynia. These researchers used a thermal probe set to an innocuous temperature and would then assay a group of UV treated animals and compare with a group of non-UV irradiated animals. The non-UV treated group would have no responders, while 60% of the UV irradiated group would respond to the same thermal stimulus!

We began the process of establishing this approach in our lab. The methodology was relatively straight forward: 4 day old (AEL) larvae were rinsed from their food, anesthetized with diethyl ether and dosed with UV light from a DNA cross linker. After UV treatment the larvae were placed into a recovery vial filled with 1 ml of food and allowed 24 hours to rest before assaying with a thermal probe.

I got my start with performing a response vs. temperature study with our thermal probe. I began at 39°C and increased the temperature in 1°C increments, assaying 90 naïve animals at each temperature. The responses were categorized based upon response the latency for the larvae to execute a 360° roll. If the animals responded within 6 seconds they would be categorized as a fast responder, 6-20 seconds would be slow responder, and if the animal reached the 20 second cut off then it would be marked as a non-responder.

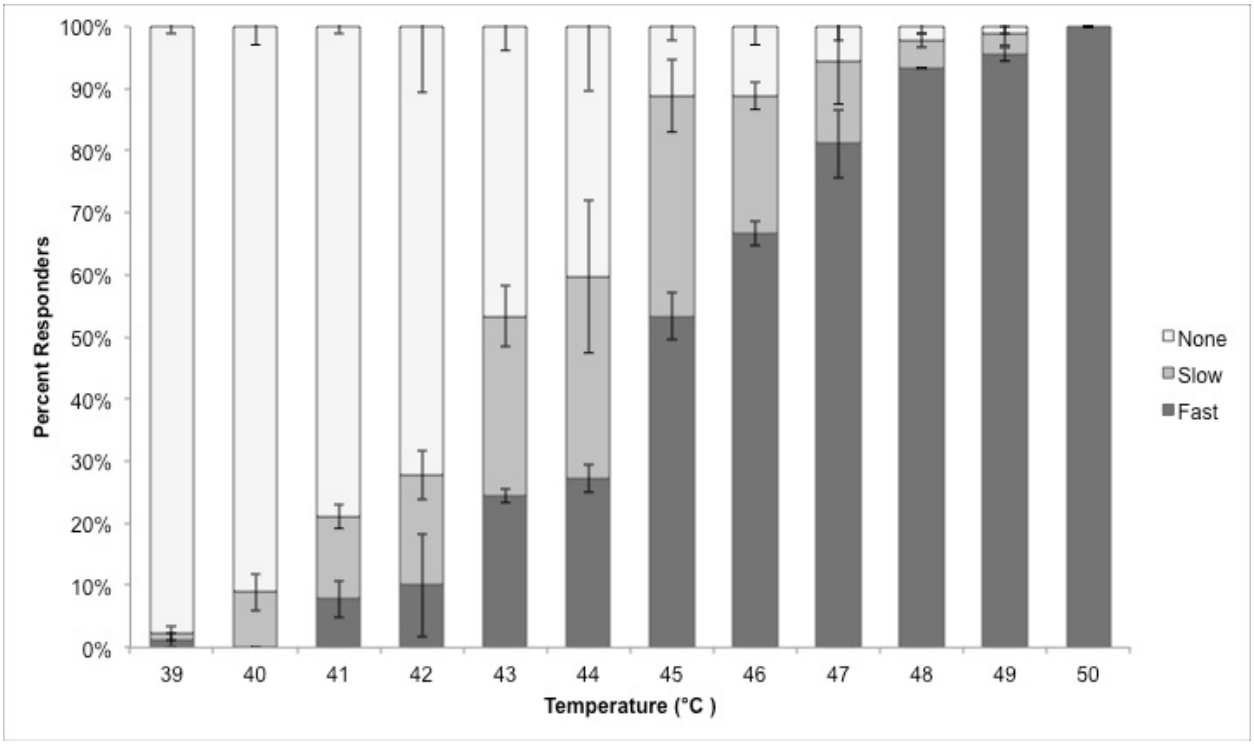

Figure 3.1: Thermal Response of w^{1118}. Shown here are the percentage of animals to respond to a thermal probe stimulus varying in temperature from 39°C to 50°C. 40°C is the first temperature that there is a statistically significant increase in the percentage of animals to respond. Larvae responding with a nocifensive roll were classified as fast (<6 seconds) or slow (between 6 and 20 seconds), or nonresponders if they did not respond within 20 seconds. Distributions were compared using Fisher's Exact Test. n=90 for all groups.

Allodynia is defined as a noxious response to a normally innocuous stimulus. Due to our interest in allodynia, we selected 39°C as the best temperature for measuring allodynia because it is the highest innocuous temperature. After a few weeks of practice I began a blinded experiment. As seen in fig 2.2, after UV irradiation there was an increase from 1.11% slow responders to 15.4% after UV irradiation. This was alarming because the expected results suggested that we would see around 60% response after UV irradiation.

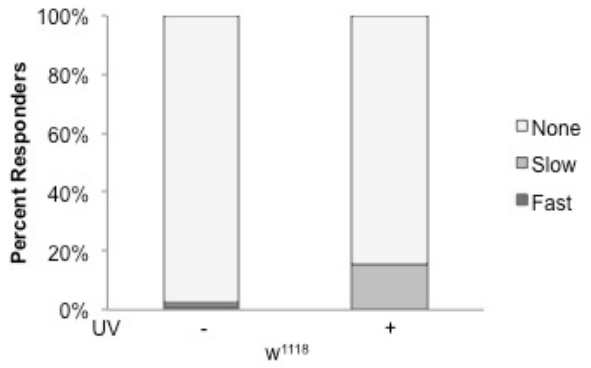

Figure 3.2: Effect of UV Irradiation on a Control Group of Flies (w^{1118}). The non-UV treated group (left) showed almost no response to the 39°C thermal probe. The UV irradiated animals (right) showed only a slight increase in responsiveness to the 39°C thermal probe. n>90 for all groups. Statistical analysis not performed.

After this extremely unsuccessful first attempt at performing the procedure, I began the process of troubleshooting the technique. I tested every variable of the experimental protocol, from the type of UV bulb to the amount of ether and nothing I tried brought our results any closer the standard set by the inventors of the method, Dr. Michael Galko and his lab at the University of Texas Southwestern Medical Center – MD Anderson. Finally, in a desperate attempt to get the project working my mentor, Dr. Geoffrey Ganter, and I traveled to Houston, TX to visit the Galko laboratory and learn directly from the masters. While in Dr. Galko's lab we noticed that the fly food used there was a much different color from the food we use in our own lab. While thinking nothing of it at the time, we continued to unsuccessfully toil away. It wasn't until after we came back to UNE, confident in our technique and thermal probe that we recalled the difference in food. I emailed Dr. Galko asking for his food recipe. He graciously obliged to send us the recipe. I reared animals on the new food and again tried the +/- UV experiment. As seen in figure 3.3 the results were stunning. By changing the recipe to match the Galko lab we now had an increase from 7% slow responders in the –UV group to 7% slow and 33% fast responders in our + UV group. This was the biggest gain we had made in almost a year!

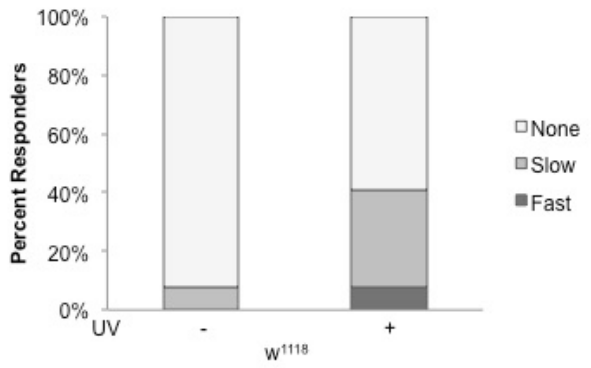

Figure 3.3: Effect of UV Irradiation on a Control Group of Flies (w^{1118}) reared on a Galko Recipe Food. The non-UV treated group (left) showed almost no response to the 39°C thermal probe. The UV irradiated animals (right) showed strong increase in responsiveness to the 39°C thermal probe. n>90 for all groups Statistical analysis not performed.

While these results were certainly a morale boost to the lab, it was still unsatisfying in that we did not achieve the level of sensitization expected. Basking in success just long enough to enjoy a celebration meal at Los Tapatios, I resumed work trying to troubleshoot why our sensitization was sub par. It was striking that even though the new food was made with the Galko food recipe, the color remained unchanged from our normal food. I reached out to Dr. Galko once more and asked if he was willing to identify the sources of the ingredients used to make their food. Once again he obliged and sent me a list of the types of ingredients and their source. Most of the basic ingredients matched ours (sugar, cornmeal, tegosept) others did not (yeast and agar). We immediately purchased the latter ingredients from the same sources used in the Galko lab and set to making a new batch of food with the "Galko Recipe" and ingredients. The color matched the food from the Galko lab and I again tested the formation of allodynia.

As a result of changing the sources of our ingredients to match the food from the Galko lab we saw another marked increase in the amount of sensitization that forms! In the – UV group there was 2% fast and 3% slow, however after UV irradiation the responsiveness skyrocketed to 19% and 42% fast and slow, respectively (fig 3.4). We had finally achieved the level of sensitization that we had set out to obtain!

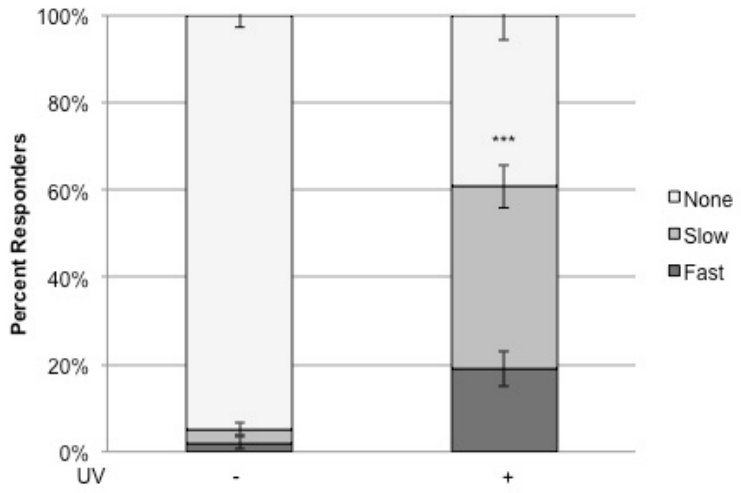

Figure 3.4: Effect of UV Irradiation of w^{1118} Reared on Food with the Galko Recipe/Ingredients. The non-UV treated group (left) showed almost no response to the 39°C thermal probe. The UV irradiated animals (right) showed a significant increase in responsiveness to the 39°C thermal probe. Distributions were compared using Fisher's Exact Test. n=90 for all groups.

Finally, to test if the difference in diet played a role in the normal nociceptive behavior of the *Drosophila* larvae, I performed another temperature response study, measuring the percentage of responders with varying temperature. As seen in figure 3.5 the entire response distribution seemed to be shifted 2°C higher. When I performed the thermal response curve the "allodynia temperature", i.e. the highest innocuous temperature, was at 39°C. However after using the Galko recipe, the best "allodynia temperature was now 41°C.

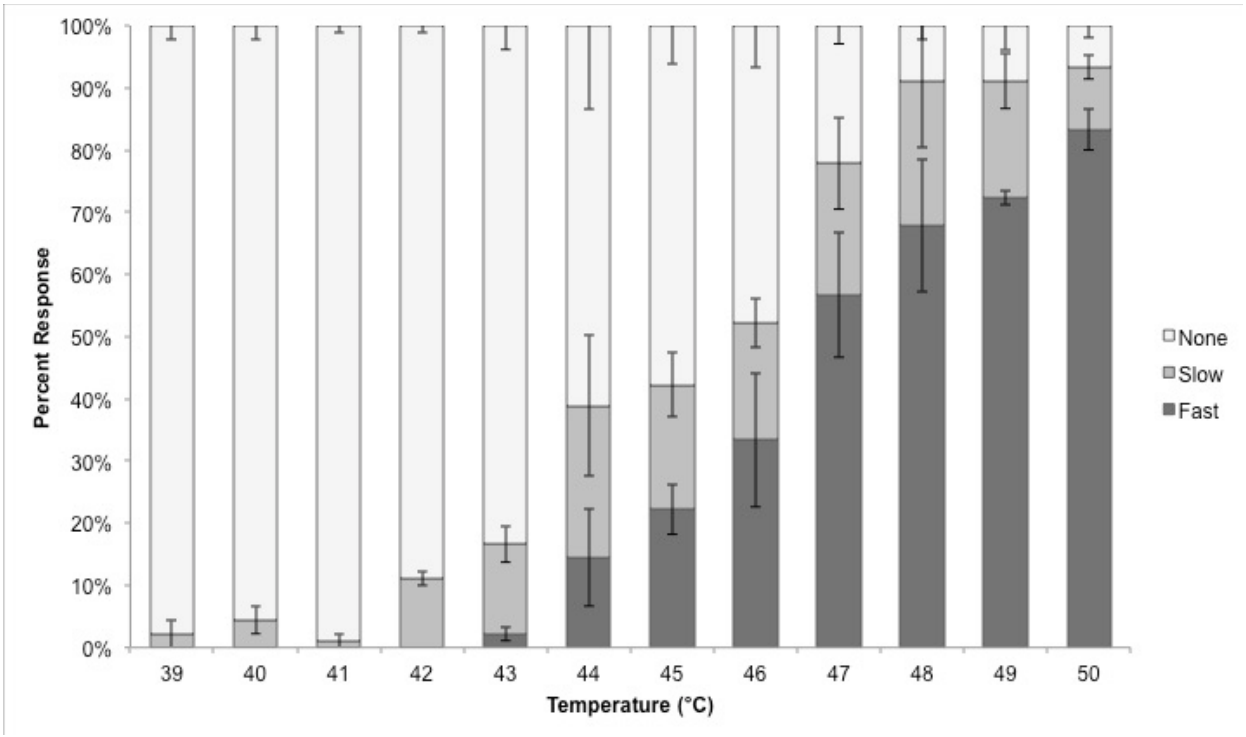

Figure 3.5: Thermal Response Curve of w^{1118} Reared on a "Galko" Based Diet. Shown here are the percentage of animals to respond to a thermal probe stimulus varying in temperature from 39°C to 50°C. 41°C is the first temperature that there is a statistically significant increase in the percentage of animals to respond. Larvae responding with a nocifensive roll were classified as fast (<6 seconds) or slow (between 6 and 20 seconds), or nonresponders if they did not respond within 20 seconds. Distributions were compared using Fisher's Exact test. n=90 for all groups.

Discussion:

These results demonstrate the importance of diet/nutrition on both the normal nociceptive behavior and the capacity for sensitization of the *Drosophila* melanogaster larvae. We have evidence that if the *Drosophila* larvae are reared on a diet of "Ganter" food, they seem to have a minimal ability to develop sensitization, specifically allodynia. Furthermore, their normal nociceptive behavior seems to be affected in that they are generally more responsive to a thermal stimulus. To try and understand the differences in diet I will first discuss the differences in the two compared foods themselves.

The "Ganter" food recipe is derived from a universally comparable recipe to the standard in the field. The "Galko" based recipe differs in many ways. Firstly, there is a 10x decrease in the amount of sugar in the "Galko" food. Furthermore, it has two ingredients that differ in source. The "Galko" food uses inactive brewers yeast instead of the traditional active baker's yeast. Additionally, the "Galko" recipe sources its agar from a different vendor and is ultimately a less refined form of agar than the one we generally

use. Lastly, there is an addition of propionic acid to the Galko food. Interestingly, propionic acid is the main derivative of ibuprofen.

Diet was seen to have an effect on sensitization in two major ways. The first effect was seen when we used the Galko recipe with all the ingredients from our lab. This recipe only had a major change in the amount of sugar used and the addition of propionic acid, with the result that we saw an increase in the magnitude of sensitization that formed. This must have been a result of either the decrease in sugar concentration or the addition of the propionic acid. This result awaits further investigation.

The next major effect of diet on allodynia that was observed was when we switched our ingredient sources to all match those from the Galko lab. This caused a major change in only two ingredients, the type of yeast and agar. When we re-sourced our ingredients there was another increase in our allodynia experiments. Interestingly, the agar is purified from red algae. The newly sourced agar was a lower purity than the agar we had been previously using. This could mean that other compounds from the red algae are in higher concentrations in the new agar. A compound is carrageenan, a highly inflammatory agent that is isolated from red algae.

When we compare the original Ganter recipe to the final “Galko” recipe there was a substantial difference in the normal nociceptive functioning of these animals. The larvae raised on the “Galko” food showed a 2°C shift in temperature sensitivity. These animals now did not respond significantly to 39 and 40°C and only began to start responding at 41°C. This indicates an overall decrease in nociception threshold and could likely be caused by the propionic acid.

One major conundrum in the observed effect of diet on nociception is the difference in sugar. Sugar has been rigorously linked with pain thresholds (REFS), but has been shown to cause an increase in threshold. When provided sugar, rats will have an increase in pain threshold[32]. Here, when we decrease sugar we see an increase in pain threshold. It is difficult to postulate any reason for this difference, and it might not even be the ingredient involved.

Here we report that differences in the diet of *Drosophila* have major effects on both the nociceptive function and the capacity for formation of allodynia. Further testing is necessary to elucidate the potential ingredients. Fortunately, these studies are already underway! Seemingly, it isn’t going to be one ingredient that caused the difference, but

likely a combination of several. When we changed the quantity of sugar and added propionic acid we saw a considerable increase in the formation of allodynia and when we matched the source our ingredients we again saw a dramatic increase. This intrinsically informs us that there are at least two variables that have a dramatic effect on the formation of allodynia and that nothing is as simple as it seems.

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CHAPTER IV: APPENDIX

This section contains the supplemental figures for the previous chapter and any additional work pertaining to the BMP project that hasn't yet been included. The supplemental figures include both relevant genetic controls for each RNA*i* manipulation for both sensitization and normal nociceptive behavior. Additionally, I chose to exclude the SMAD component med from the manuscript.

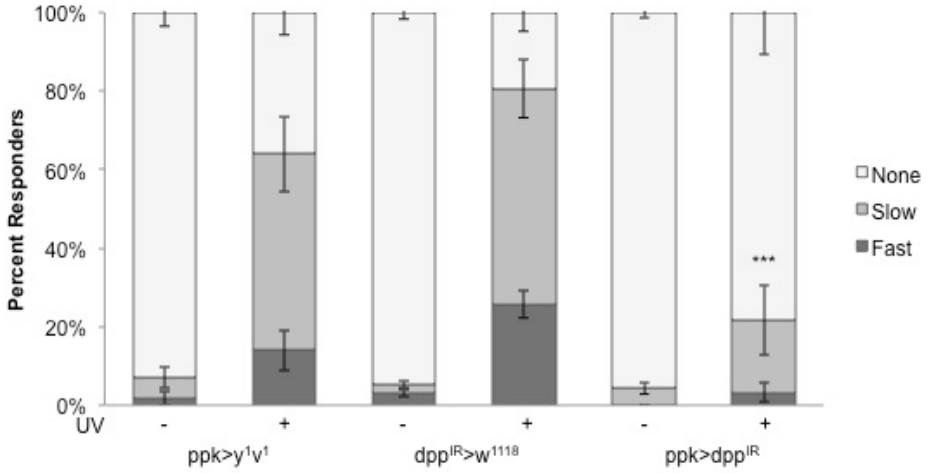

Figure 4.1: RNAi Suppression of dpp Signaling in the Primary Nociceptor Neurons Inhibits the Production of Allodynia. Knockdown of dpp in the nociceptor neurons resulted in a failure to produce allodynia, compared to both controls. Knockdown was a manipulation made with a ppkGal4>UAS RNAi allele to decrease expression level. All groups were assayed with a thermal probe set to 41°C. Response latencies were recorded in (s) and categorized as follows: none (>20), Slow (between 6 and 20) and Fast (<6). n=90-117 and distributions were compared using Fisher's Exact Test.

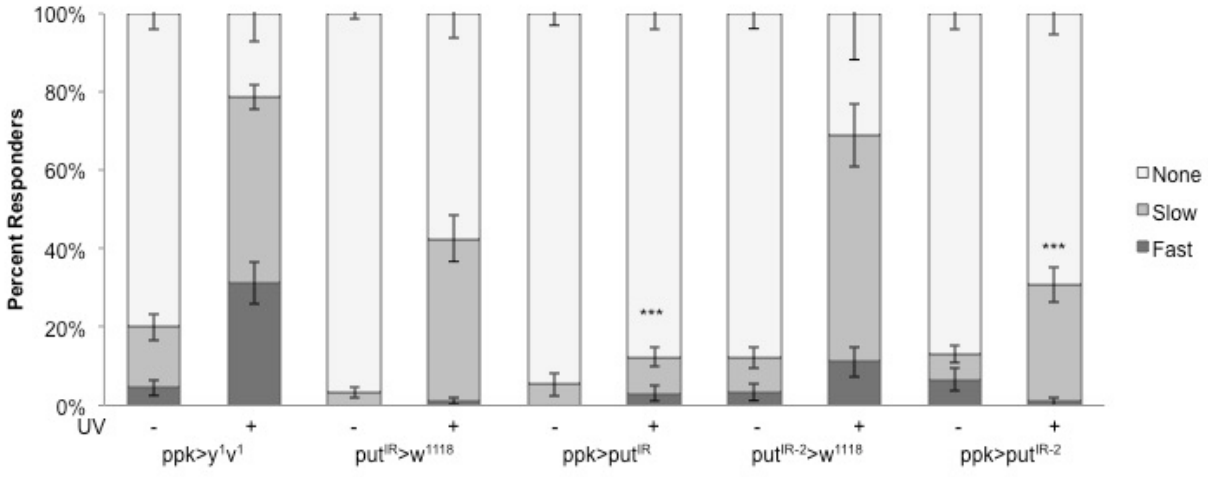

Figure 4.2: RNAi Suppression of put Signaling in the Primary Nociceptor Neurons Inhibits the Production of Allodynia. Knockdown of put in the nociceptor neurons resulted in a failure to produce allodynia, compared to both controls. The effect of put suppression was verified with an independent RNAi knockdown ($put^{IR\text{-}2}$). Knockdown was a manipulation made with a ppkGal4>UAS RNAi allele to decrease

expression level. All groups were assayed with a thermal probe set to 41°C. Response latencies were recorded in (s) and categorized as follows: none (>20), Slow (between 6 and 20) and Fast (<6). n=90-117 and distributions were compared using Fisher's Exact Test.

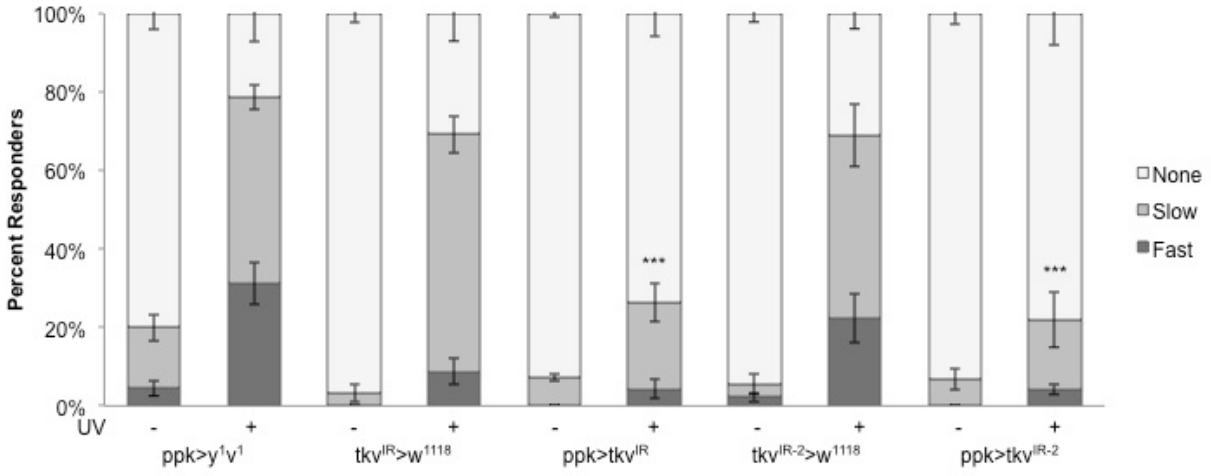

Figure 4.3: RNAi Suppression of tkv Signaling in the Primary Nociceptor Neurons Inhibits the Production of Allodynia. Knockdown of tkv in the nociceptor neurons resulted in a failure to produce allodynia, compared to both controls. The effect of tkv supporession was verified with an independent RNAi knockdown (tkv^{IR-2}). Knockdown was a manipulation made with a ppkGal4>UAS RNAi allele to decrease expression level. All groups were assayed with a thermal probe set to 41°C. Response latencies were recorded in (s) and categorized as follows: none (>20), Slow (between 6 and 20) and Fast (<6). n=90-117 and distributions were compared using Fisher's Exact Test.

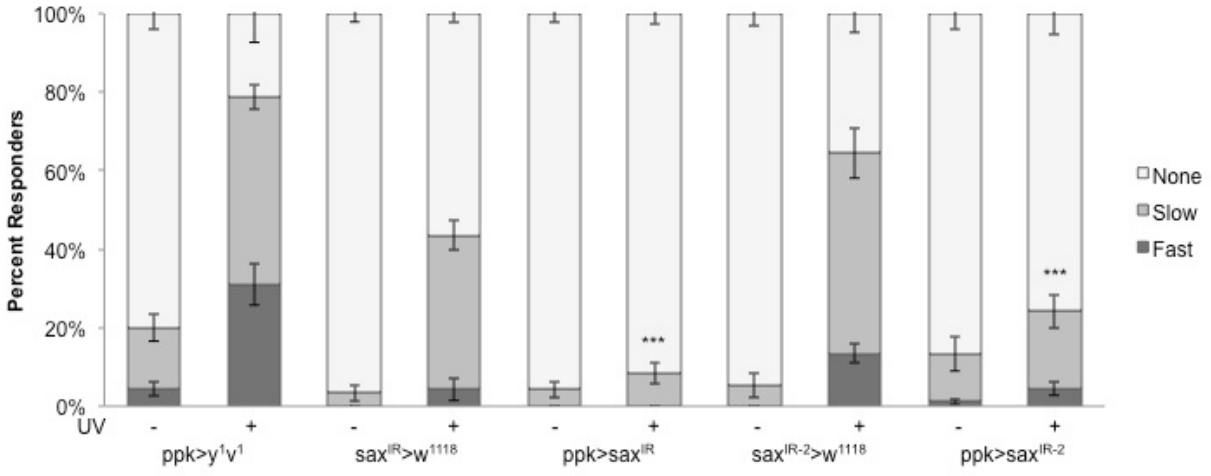

Figure 4.4: RNAi Suppression of sax Signaling in the Primary Nociceptor Neurons Inhibits the Production of Allodynia. Knockdown sax in the nociceptor neurons resulted in a failure to produce allodynia, compared to both controls. The effect of sax suppression was verified with an independent RNAi knockdown (sax^{IR-2}). Knockdown was a manipulation made with a ppkGal4>UAS RNAi allele to decrease expression level. All groups were assayed with a thermal probe set to 41°C. Response latencies were recorded in (s) and categorized as follows: none (>20), Slow (between 6 and 20) and Fast (<6). n=90-117 and distributions were compared using Fisher's Exact Test.

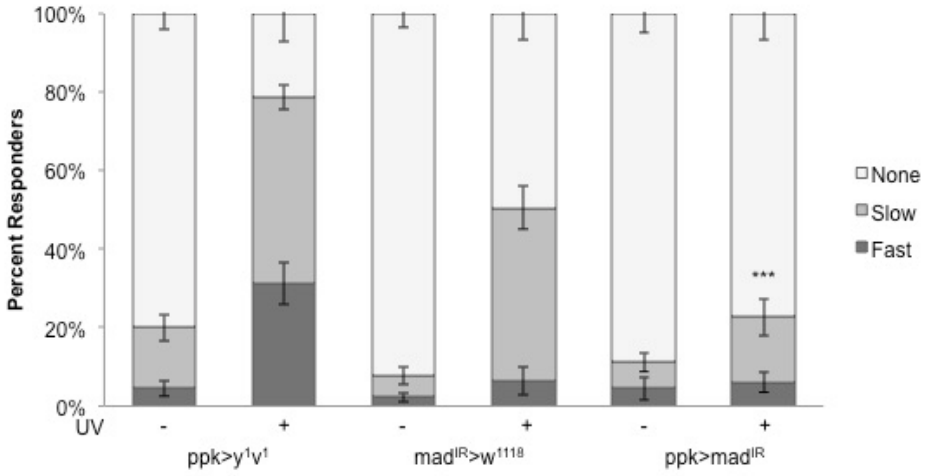

Figure 4.5: RNAi Suppression of mad Signaling in the Primary Nociceptor Neurons Inhibits the Production of Allodynia. Knockdown of mad in the nociceptor neurons resulted in a failure to produce allodynia, compared to both controls. Knockdown was a manipulation made with a ppkGal4>UAS RNAi allele to decrease expression level. All groups were assayed with a thermal probe set to 41°C. Response latencies were recorded in (s) and categorized as follows: none (>20), Slow (between 6 and 20) and Fast (<6). n=90- and distributions were compared using Fisher's Exact Test.

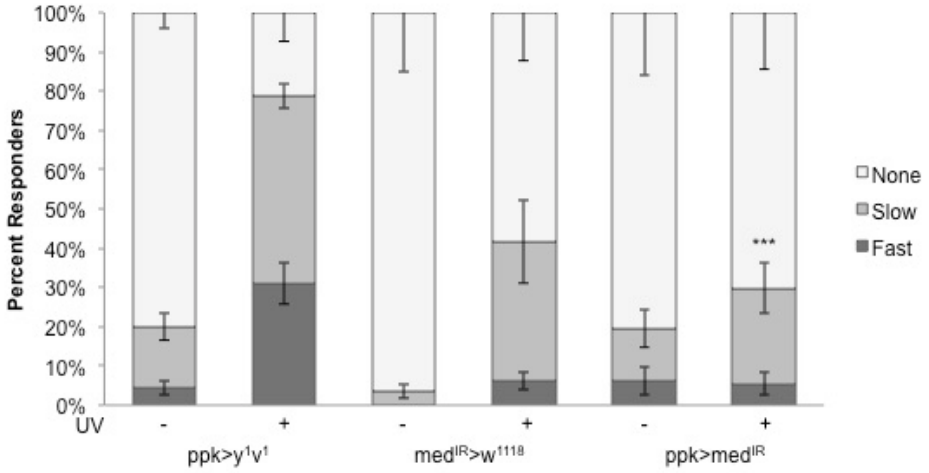

Figure 4.6: RNAi Suppression of med Signaling in the Primary Nociceptor Neurons Inhibits the Production of Allodynia. Knockdown of med in the nociceptor neurons resulted in a failure to produce allodynia, compared to both controls. Knockdown was a manipulation made with a ppkGal4>UAS RNAi allele to decrease expression level. All groups were assayed with a thermal probe set to 41°C. Response latencies were recorded in (s) and categorized as follows: none (>20), Slow (between 6 and 20) and Fast (<6). n=90-117 and distributions were compared using Fisher's Exact Test.

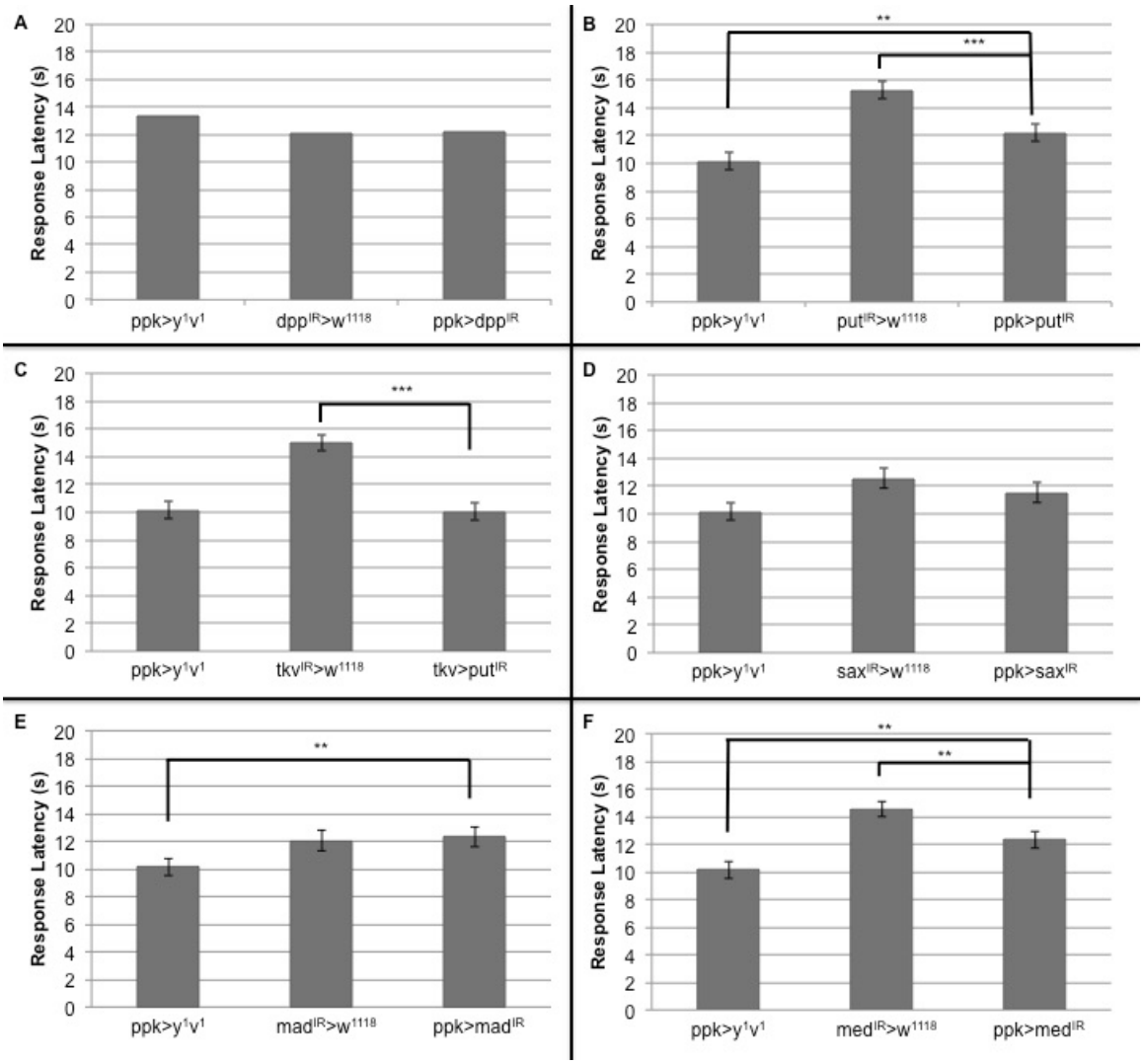

Figure 4.7: Normal nociception of the mutants and controls at 45ºC. Normal nociceptive responses were tested to determine the normal nociception behavior of untreated larvae. These data show that the mutants are just as capable of responding to normally noxious thermal stimulus as the controls. Probe temperature was set to 45ºC. (A) shows no difference between dpp^{IR} and either control. (B) shows that the put^{IR} manipulation group is significantly less responsive than one control but significantly more responsive than the other. (C) shows that the tkv^{IR} manipulation group is significantly more responsive than one parental group. (D) shows that the sax^{IR} manipulation group is not different from either control. (E) shows that the mad^{IR} manipulation group is only less responsive than one control. Finally (F) shows that the med^{IR} manipulation group is significantly less responsive than one control but significantly more responsive than the other. n= 90 for all groups. These data were analyzed by Student's t-test.