CELLULAR SIGNALING PATHWAYS REGULATE NOCICEPTOR SENSITIVITY TO NOXIOUS STIMULI IN *DROSOPHILA MELANOGASTER* LARVAE

A Thesis
by
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Abstract

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Chronic pain is a major public health concern that affects about 100 million Americans, generates $600 billion in healthcare costs, and is a major cause of missed work. For these reasons, it is important to research the cellular mechanisms of sensory neuron function in order to develop more effective clinical interventions. To better understand chronic pain, *Drosophila melanogaster* was used to investigate sensory neuron function. The major goal of this thesis was to understand the cellular signaling mechanisms that control sensory neuron sensitivity. In one project, flies lacking the function of the G protein signaling genes, *Gαq* and *norpA,* were studied. These genes are involved in neurotransmitter signaling and electrical excitability, and the results showed that both are required for behavioral responses to harsh thermal and mechanical stimuli. Removal of their function does not result in morphological differences in the sensory neurons that detect harsh stimuli, which suggests that they are involved in signaling in these neurons instead of their development. In a second study, another gene called *off-track 2 (otk2),* which encodes a transmembrane receptor that is

involved in the Wnt signaling pathway in *Drosophila*, was targeted. Loss-of-function of *otk2* showed that it plays a role in sensory neurons in the detection of harsh thermal stimuli but not harsh mechanical stimuli. In addition, loss of *otk2* function results in a tiling defect where the dendrites of sensory neurons overlap with the dendrites of neighboring neurons of the same class. In order to determine if Otk2 and either Frizzled or Frizzled2 are functionally coupled to activate Wnt signaling, loss-of-function experiments on *fz* and *fz2* were conducted. Loss-of-function of *fz* did not result in a defect in either thermal or mechanical nociception, while loss-of-function of *fz2* caused a defect in a noxious thermal behavioral assay. Otk2 and Fz2 have been shown previously to physically interact. The data in this study suggests that *otk2* and *fz2* are functionally coupled in an inhibitory mechanism to regulate sensory neuron sensitivity. A potential mechanism of action for these receptors could be regulating sensory neuron function through the developmental control of dendrite outgrowth.

Acknowledgments

I would like to thank Dr. Andy Bellemer for welcoming me into his lab, and for all that he has pushed me to accomplish. Without his wisdom and guidance, I wouldn't have been as successful in my scientific career. He has provided me the space and resources I needed to become a better researcher, thinker, and teacher. I couldn't have asked for a better mentor, and I am beyond grateful to have worked with him. I want to acknowledge Dr. Ece Karatan and Dr. Annkatrin Rose for serving on my thesis committee and providing feedback on various aspects of my project throughout the program. Their advice and critique helped build my project and advance my writing techniques. I would also like to acknowledge my fellow lab members and other co-graduate students whose antics provided relief from the constant stress of Graduate School. Finally, I would like to thank Appalachian State University for allowing me this opportunity to conduct research and learn through this program, and thank you to the Appalachian State University Office of Student Research for funding for my project.

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Foreword

This thesis covers two research projects, so it is divided into four chapters. The first chapter covers general introduction material for the two projects including chronic pain, nociception, and *Drosophila melanogaster* as a model organism. The second chapter covers the roles of G proteins in *Drosophila* nociception. This chapter has been adapted from the journal article, "Gαq and Phospholipase Cβ signaling regulate nociceptor sensitivity in *Drosophila melanogaster* larvae," written by Joshua A. Herman, Adam B. Willits, and Andrew Bellemer. This article was published in PeerJ in September of 2018 and was cited in the references list. The third chapter covers the roles of Wnt signaling in *Drosophila* nociception. The final chapter covers overall conclusions and future implications of the two projects combined.

Chapter 1: Nociception and Cellular Signaling

Chronic pain and nociception

Chronic pain is a major public health concern that is usually induced by inflammation, nerve damage, or cancer [1]. It is important to research because it affects about 100 million Americans every year, costs around $600 billion in health care, and lowers productivity and quality of life. In addition, one of the few treatments for chronic pain are opioids, which are expensive and highly addictive [2]. As of now, the mechanisms of chronic pain development are not fully understood [1]. For these reasons, it is important to understand the cellular mechanisms of pain and how chronic pain develops. This will allow the advance of the production of innovative therapeutic drugs to treat chronic pain and other neuropathic pain conditions.

One of the approaches by which researchers study chronic pain is by studying nociception. Nociception is the ability of an organism to sense a painful or potentially harmful stimulus and then produce a behavior in response to the stimulus [3]. Sensory neurons extend into the spinal cord through the dorsal horn of the spinal cord. The dorsal root ganglion (DRG) houses the cell bodies of the sensory neurons. The DRG functions to transduce and modulate sensory information moving into the spinal cord [4]. Once the signal enters the spinal cord, the sensory neuron sends it through an interneuron, or multiple interneurons, in the spinal cord to higher order brain regions for processing [5, 6]. Specialized sensory neurons that are directly activated by harsh stimuli are called nociceptors. A noxious stimulus can be presented as thermal, mechanical, or chemical harm. Understanding how nociceptors are activated and dictate behavior in response to these noxious stimuli is important aspect of studying chronic pain.

Drosophila melanogaster as a model organism

Drosophila melanogaster, the common fruit fly, is model organism used to study sensory neuron biology. Recent technical advances in *Drosophila* research including, but not limited to, tissue-specific knockdowns and fluorescent protein expression in cells in an otherwise undisturbed environment have expanded the ability to understand how the nervous system develops and functions [7]. *Drosophila* is inexpensive to maintain and reproduce, has a quick reproduction and development rate, and has a fully sequenced, easily manipulated genome. In addition, the skin of the larva is optically clear, which allows high resolution imaging of neurons. This provides the potential for quantification and analysis of morphology, development, and function of neurons and neural circuits [8]. Nociceptor activation by a noxious stimulus in *Drosophila* larvae results in a reflexive escape behavior called the Nocifensive Escape Locomotion (NEL). The NEL is a 360-degree barrel roll, and this reflexive behavior only occurs in response to noxious stimuli that are of high intensity. Since this response is clear and distinguishable from other forms of locomotor behavior, it allows quantification of nociceptor activity and nociception in *Drosophila* [9]. *Drosophila* is a useful model organism for uncovering the cellular and molecular basis of neuronal morphogenesis and function since the entire peripheral nervous system has been described in the context of individual cells [10]. For all of these reasons, *Drosophila* is a powerful model organism for understanding the cellular and molecular mechanisms that regulate nociceptors and chronic pain.

Drosophila larval somatosensory neurons are named multidendritic (md) neurons based on their highly branched dendrites, which are located just below the epidermal cells in

the larval body wall. The md sensory neurons are organized into four categories, named class I-IV, based on structure and function. They are named in order of increasing complexity of dendrite arborization [11], which implies that class IV neurons are the most branched which is shown in Figure 1 . In addition to morphology differences, the classes of multidendritic neurons also have different functions. Class I multidendritic (md) neurons act as proprioceptors [12, 13], class II and III md neurons are gentle touch receptors [14], and class IV md neurons sense harmful stimuli [15]. Thus, the class IV md neurons act as the nociceptors of the larval nervous system. The dendritic arbors cover the entire body wall of the larva in order to get a full representation of the sensory area. This is important for these neurons since they receive noxious sensory input from the environment. In addition, they send axon projections into the ventral nerve cord to relay signals to the central nervous system for higher order behavioral responses [16-18].

Neuronal dendrites are the primary sites for sensory input in md neurons. The dendrites act through sensory transduction of a noxious stimulus into an electrical signal. For other neuron types in a neural circuit, like interneurons and motor neurons, dendrites are the sites of synaptic connections and receive input by axons of other neurons which determines the way that information is sent and processed within the nervous system [7]. This is why it is fundamental to understand the process of how dendrites transduce a noxious stimulus into an electrical signal to be sent to the CNS and how neural development and morphogenesis of neurons control that process.

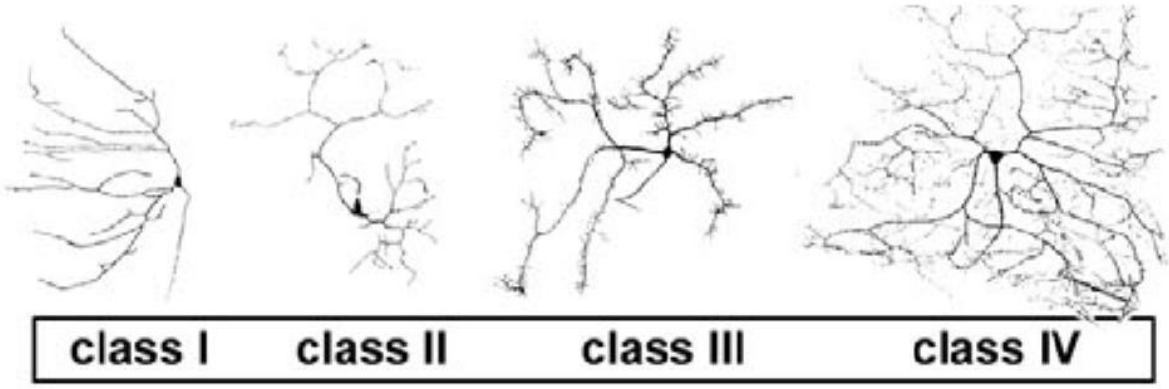

Figure 1. The four classes of multidendritic sensory neurons in *Drosophila.* As shown, sensory neurons are organized based on dendritic arborization with class IV neurons being the most highly branched [16].

TRP channels

Nociceptors use different types of receptors and ion channels such as Transient Receptor Potential (TRP) channels to detect noxious stimuli and other pathological signals caused by inflammation, nerve damage, and cancer [19]. TRP channels are a family of ion channels that act as sensors of the environment and integrate signaling [20], and their expression in neurons determines the activation of nociceptors by different noxious stimuli [21]. This group of channels utilize an ion flux as a result of activation by noxious stimuli. This causes neuronal membrane depolarization activating action potentials. In order to ensure these channels are only activated by noxious stimuli, TRP channels have high thresholds of activation including high-levels of voltage, temperature, and ligands [21].

Ion channels in the TRP family are divided into classes based on sequence homology and function. TRPC channels are closely related to canonical phototransduction channels found in *Drosophila*. The TRPV1 channel is the vanilloid receptor, and TRPN channels are related to the NOMPC channel in *Drosophila* which is a mechanoreceptor [9]. TRPA1 is a heat-activated, cation channel found in *Drosophila* that is permeable to Ca^{2+} [22, 23]. TRPA1 is also found in mice, and it is involved with multiple aspects of nociception. It has been shown in mice to be activated downstream of bradykinin, which is an important substance that is released from tissue following injury and inflammation [20, 24, 25].

In order to understand more about the role of TRPA1 in *Drosophila* nociception, an RNAi knockdown of *TrpA1* in *Drosophila* larvae was conducted. It was found that the knockdown larvae exhibited impaired avoidance of noxious heat, showing it is required for nociception [20, 26]. TRPV1 was also shown in mice to function in transducing the nociceptive signal to noxious heat in the range of $42^{o}C$-$48^{o}C$ [9].

Nociceptor modulation

A major goal in understanding nociception and chronic pain is uncovering which cellular and molecular signaling pathways modulate nociceptor sensitivity. In an injury, cytokines will cause inflammation and other healing mechanisms to develop in the area of injury [27]. After the injury and consequential inflammation, the threshold for pain-sensing neuron activation becomes lower. This means that normal stimuli that wouldn't usually be classified as harmful would activate sensory neuron response. The reduction in threshold of neurons is called allodynia, and a greater response to a supra-threshold stimulus is called hyperalgesia [28]. In cases like opioid-induced hyperalgesia, the hypersensitized state of neurons can cause long-term potentiation of neural activity resulting in chronic pain [27]. It has been shown in *Drosophila* that signaling pathways such as Bone Morphogenetic Protein (BMP) signaling play a role in nociceptor sensitization. RNAi knockdown of crucial proteins in the BMP signaling pathway reduced nociceptor sensitization to UV-induced sensitization [29]. This provides insight into the signaling pathway regulation of nociceptor sensitization in *Drosophila*.

The G protein signaling cascade and Wnt signaling are two particular signaling mechanisms that regulate the sensitivity of sensory neurons [24, 30]. Uncovering the roles of the proteins involved in each pathway and the mechanism by which each pathway modulates neuron sensitivity are important to understand in order to develop better therapeutic drugs. In this study, I started characterizing the roles of crucial proteins in each of the two pathways. My main aim was to determine if the proteins play a role in thermal or mechanical nociception in *Drosophila* larvae. In addition, I aimed to understand if these crucial proteins

play a role in dendrite development to gain insight into how these proteins regulate sensory neuron sensitivity.

Chapter 2: The Roles of G Proteins in *Drosophila* Nociception

Introduction

Chronic pain

Chronic pain and the use of opioids have become such a public health concern that it has been coined the opioid epidemic. Chronic pain severely reduces quality of life and impacts public spending. These are just a few of the reasons for why the molecular mechanisms of sensory neuron modulation should be studied. By using the nociception-induced reflexive behavior (NEL) of *Drosophila melanogaster* larvae, the signaling pathways and cellular machinery regulating neuropathic pain can by uncovered and characterized. This information can be used to develop better clinical therapeutics to treat chronic pain. [1, 2]

Bradykinin

One molecule that has been shown to play a role in sensory neuron sensitivity is called Bradykinin. Bradykinin is a peptide that is a potent pain-inducing molecule [31] and has been linked to inflammatory-induced, neuropathic pain conditions [32]. These peptides are generated during tissue injury and noxious stimulation, and they act as modulators of nociceptors in the perception of nociceptive information in the central nervous system [33]. Bradykinin activates sensory neurons through the activation of two kinin receptors called bradykinin B_1 and bradykinin B_2 receptors which are both known G protein-coupled receptors. This provides a basis for the hypothesis that G protein coupled receptors may act to regulate neuron sensitivity and activation, and they, along with the pathway they activate, could be therapeutic targets in inflammation-induced models [33].

G protein signaling cascade

The G protein signaling cascade utilizes the binding of a ligand to a G protein-coupled receptor (GPCR), which results in the activation of intracellular heterotrimeric G proteins [34]. GPCRs are one of the first lines of sensory reception in some systems since they are cell surface receptors. There are about 850 members of the GPCR gene family, and they are activated by a diverse range of ligands including hormones, neurotransmitters, photons, odorants, tastants, and more [35]. Most importantly for the context of chronic pain, GPCRs can be activated by hormones and neurotransmitters released as a result of inflammation, tissue damage, bradykinin, and even cancer. In mammalian models, around 40 GPCRs contribute to pain reception, which means they are one of the most important targets of therapeutics, specifically in pain [21].

The intracellular domain of a GPCR is the intracellular binding partner of heterotrimeric Guanine nucleotide binding proteins, also called G proteins. The three subunits of the G protein complex are Gα, Gβ, and Gγ. The heterodimer of Gβγ acts to bind to guanosine diphosphate (GDP)-bound Gα and localize it to the plasma membrane. This act of plasma membrane localization by Gβγ is also required for coupling with a GPCR. The Gα subunit functions to tether the heterotrimeric G protein complex to the intracellular domain of a GPCR when simultaneously bound by GDP and Gβγ. When a GPCR is activated by a ligand, the GPCR promotes the release of GDP from Gα and guanosine triphosphate (GTP) then binds to Gα. This in turn results in conformational changes releasing Gα from Gβγ and the intracellular domain of the GPCR. This allows the GTP-bound Gα to initiate signals through downstream effectors. Gα activity causes hydrolysis of GTP to GDP through intrinsic guanosine triphosphatase (GTPase). This returns the Gα back to Gβγ which localizes the complex to the GPCR in an inactive state to be reused again [34].

The G protein signaling cascade functions downstream of the G proteins by activating diverse signaling pathways. Different Gα subunits can interact with different downstream effectors, which will activate different types of signaling pathways. The Gαq subunit, in particular, activates phospholipase C (PLC) which cleaves the Phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into Inositol 1,4,5-triphosphate (IP3) and 1,2 diacylglycerol (DAG) which are secondary messengers. DAG activates protein kinase C (PKC) which phosphorylates downstream proteins and ion channels, while IP3 binds to calcium channels on the endoplasmic reticulum resulting in an increase in intracellular Ca^{2+} levels which promotes transcription factors, kinase and ion channel activation, and promotes neurotransmitter release machinery activity (Figure 2) [36, 37].

One of the major consequences of PLC activity is the release of internal calcium stores through the secondary messenger IP3. It has been demonstrated that this release of calcium affects TRP channel activity. In experiments, calcium release was forced in cultured cells not expressing TRPA1 and in cells that expressed TRPA1 using the endoplasmic reticulum Ca^{2+}-ATPase pump blocker, thapsigargin. There was an increase in activation of the cells containing TRPA1 channels, compared to those not expressing TRPA1. Another experiment was conducted where TRPA1-expressing cells were activated with bradykinin while simultaneously inhibiting PLC with a substance called U-73122. In these experiments, there was a lack of cell activation [24, 38]. The data suggests that the calcium released in the presence of PLC signaling modulates TRPA1 channels through calcium from the endoplasmic reticulum [24]. This therefore gives a possible explanation that the G protein signaling cascade increases electrical excitability in nociceptors to stimulate a faster behavioral response [39].

G protein coupled receptors are thought to act in sensory multidendritic neurons by inducing calcium influx to increase TRPA1 channel activity. This increased electrical excitability by TRPA1 modulation would then result in hypersensitivity to noxious thermal stimuli [39]. In HEK cell cultures, B2R was activated, which is a G protein coupled receptor that is typically bound and activated by bradykinin. After B2R activation, they used a voltage-clamp to measure electrical potential in the cells resulting from TRPA1 activation. After confirming that the B2R receptor activated the TRPA1 receptor and induced a voltage change, they conducted the same experiment while inhibiting Phospholipase C, and they found a significantly smaller peak in voltage change [40].

These findings indicate that G protein-coupled receptors and heterotrimeric G protein signaling may act as general mechanisms for adjusting the sensitivity of larval nociception. This could be occurring through their ability to activate nociceptive responses and hyperalgesia via the Gαq-phospholipase Cβ (PLCβ) signaling mechanism which modulates TRPA1 ion channel activity [37, 40]. In this study, we address this hypothesis and use cell-specific RNA interference (RNAi) to demonstrate that Gαq and NorpA signaling, the *Drosophila* homolog of PLCβ, regulate the basal sensitivity of *Drosophila* class IV multidendritic neurons to thermal and mechanical stimuli. I had three specific aims for this project. One, determine if *Gαq* acts in larval nociceptors to affect thermal and mechanical nociception. Two, determine if *norpA* acts in larval nociceptors to affect thermal and mechanical nociception. Three, identify if *Gαq* or *norpA* affect neural development to cause morphological changes in nociceptors.

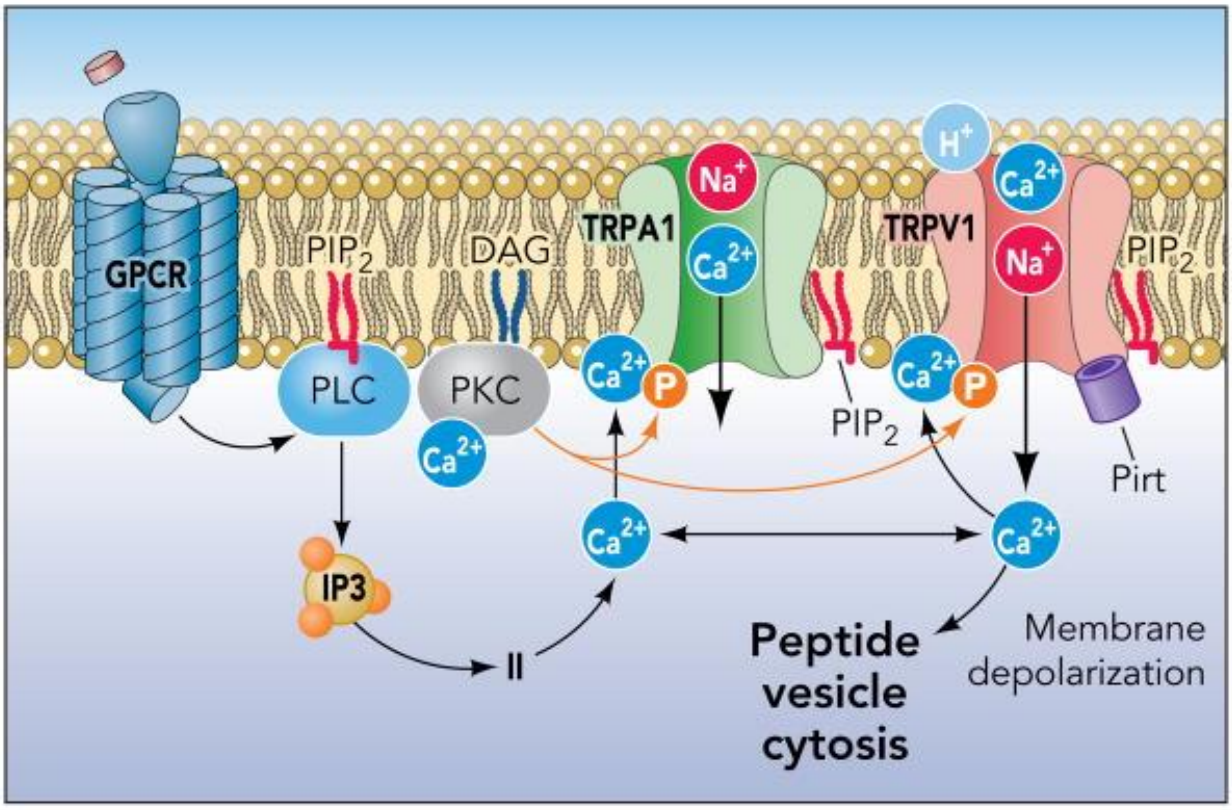

Figure 2. **The activation of a G protein-coupled receptor and the following G protein cascade downstream effectors.** The ultimate result of the cascade is phosphorylation and calcium modulation of TRP channels [41].

Materials and Methods

Cell-specific RNA interference

The GAL4/UAS system is a tissue-specific system used to express desired genes to understand their function [42]. Both the GAL4 driver transgene and the UAS reporter transgene must be present in the cell in order for a desired gene to be expressed. GAL4 binds to UAS in the cells and activates transcription of the gene downstream of the UAS reporter. Using different GAL4 promotors allows for tissue specific expression of the gene downstream of UAS. *pickpocket (ppk)* is an ion channel found exclusively in the nociceptors, so using a *ppk-GAL4* driver expresses the desired gene exclusively in the nociceptors [42].

RNA interference (RNAi) is another technique that is useful in understanding gene function by silencing a gene of interest. RNAi uses short interfering RNA to bind and cleave target mRNA strands, therefore silencing the desired gene. When a fly line with a *UAS-RNAi* transgene inserted into the genome is crossed with a second fly line with a *ppk-GAL4* driver insertion, a short interfering RNA can be expressed in nociceptors. This tissue-specific gene knockdown allows for characterization of the role of a gene in the nociceptors [43].

Fly Stocks

The fly line *w; ppk1.9-GAL4; UAS-dicer2*, was used as a driver line and was crossed with a UAS-RNAi line. In morphology experiments, *w; ppk1.9-GAL4, UAS-mCD8::GFP; UAS-dicer2* was used as the driver line and was crossed with a UAS-RNAi line. The y^1 v^1*; [Py[+t7.7]=CaryP]attP2* (BDSC# 36303) line was crossed with *w; ppk1.9-GAL4; UAS-dicer2* as a negative, GAL4-only control. The w^{1118} control stock was crossed with a UAS-RNAi line as a negative, UAS-only control. The *UAS-para-RNAi* line was crossed with *w; ppk1.9-GAL4; UAS-dicer2* as a positive control in insensitive assays. GL01048 (BDSC#

36820), JF02390 (BDSC# 36775), and JF02464 (BDSC# 33765) were used as *UAS-Gαq-RNAi* lines. JF01713 (BDSC# 31197) and JF01585 (BDSC #31113) were used as *UAS-norpA-RNAi lines.*

Nociception Assays

In thermal behavioral assays, first generation third-instar larvae were washed from the wall of a vial into a petri dish. Yeast was added to the water to break the surface tension, and then a large portion of the water was removed so there was a small film of yeast and water on the bottom of the petri dish to allow larvae to crawl on the dish with ease. A thermal probe heated to 46˚C was touched to the body wall of third instar larvae, and trials were discarded if the temperature waivered outside of ±0.5˚C. A video camera attached to the dissecting microscope recorded the trials for analysis later. Adobe Premiere Pro was used to move frame by frame to measure the latency, which is the time it takes after noxious heat stimulation for the larva to conduct the barrel roll. After calculating the latency of 50 larvae per genotype, the latencies were analyzed for statistical significance using a non-parametric Man Whitney U Test.

Larvae for mechanical assays were prepared the same way as described for thermal assays above. Mechanical assays used a 10mm von Frey filament to stimulate larvae with roughly 50 millinewtons of force. The filament was used to poke the dorsal midline of third instar larvae, and a binary response of whether the larvae rolled or not was marked. After 100 larvae per genotype had been poked, the tabulated data of percent rolling was analyzed using a chi squared test.

Neural Morphology

In order to identify neural defects, *mCD8::GFP* was expressed in nociceptors, which is a fusion between the mouse CD8 protein and GFP which allows membrane localization of the GFP. Use of the *ppk-GAL4* driver allowed nociceptor-specific expression of the *UAS-mCD8::GFP* and UAS-RNAi transgene. Laser Scanning Confocal Microscope (LSM) images were then taken to be analyzed and quantified for morphological differences. The combination of the 40x oil objective lens, 488nm laser, and the z-stack and tiling functions allowed high resolution 3-D images of nociceptors to be captured.

Third instar larvae were washed off the vial wall into a petri dish. In order to take images of the nociceptors using the LSM, the larva had to be ligated in order to block action potential propagation along motor neurons. Thus, muscle twitch could be inhibited allowing for clear images. Ligating the larva involved wrapping human hair around the body of the larva and tightly tying it just below the ventral nerve cord (approximately one third of the body length away from the head). Once the larva was ligated, it was placed in a drop of 100% glycerol between two coverslips for imaging.

After all neuron images were taken, background noise and neighboring neurons were erased using Adobe Photoshop. Fiji, an image processing software [44], was then used to correct the contrast of the image by altering the threshold. Fiji then conducted a Sholl Analysis which creates circles around the dendrites of a neurons as a way of tracing the dendritic arborization to determine if there are significant differences in morphology [45].

Results

Gαq is required for normal sensitivity to noxious thermal and mechanical stimuli

In order to test the hypothesis that *Gαq* plays a role in nociception, *Gαq* RNAi lines were crossed with *w; ppk1.9-GAL4; UAS-dicer2* to induce a tissue-specific knock down of *Gαq,* and the first-generation larvae were tested in a thermal behavioral assay for sensitivity to noxious stimuli. In a 46˚C thermal nociception assay (Figure 3A and 3B), two *Gαq* RNAi lines resulted in a significantly higher latency in comparison to the positive controls while one line (JF02464) was not significantly different. The GL01048 *UAS-Gαq-RNAi* transgene line had a mean latency of 3.0 seconds compared to the GAL4-only control with a mean latency of 1.9 seconds. The JF02390 *UAS-Gαq-RNAi* transgene line had a mean latency of 2.2 seconds compared to the GAL4-only control with a mean latency of 1.7 seconds. The JF02464 *UAS-Gαq-RNAi* transgene line had a mean latency of 1.9 seconds compared to the GAL4-only control with a mean latency of 1.7 seconds. In a follow-up experiment comparing the RNAi line to its respective UAS-only control (Figure 3C), the results reflected the initial experiment where two lines had a significantly higher latency compared to the UAS-only controls (4.1 versus 2.3 s for GL01048; 2.4 versus 1.8 s for JF02390), and the same line as before (JF02464) did not have a significantly different latency compared to the UAS-only control (2.9 s versus 2.6 s).

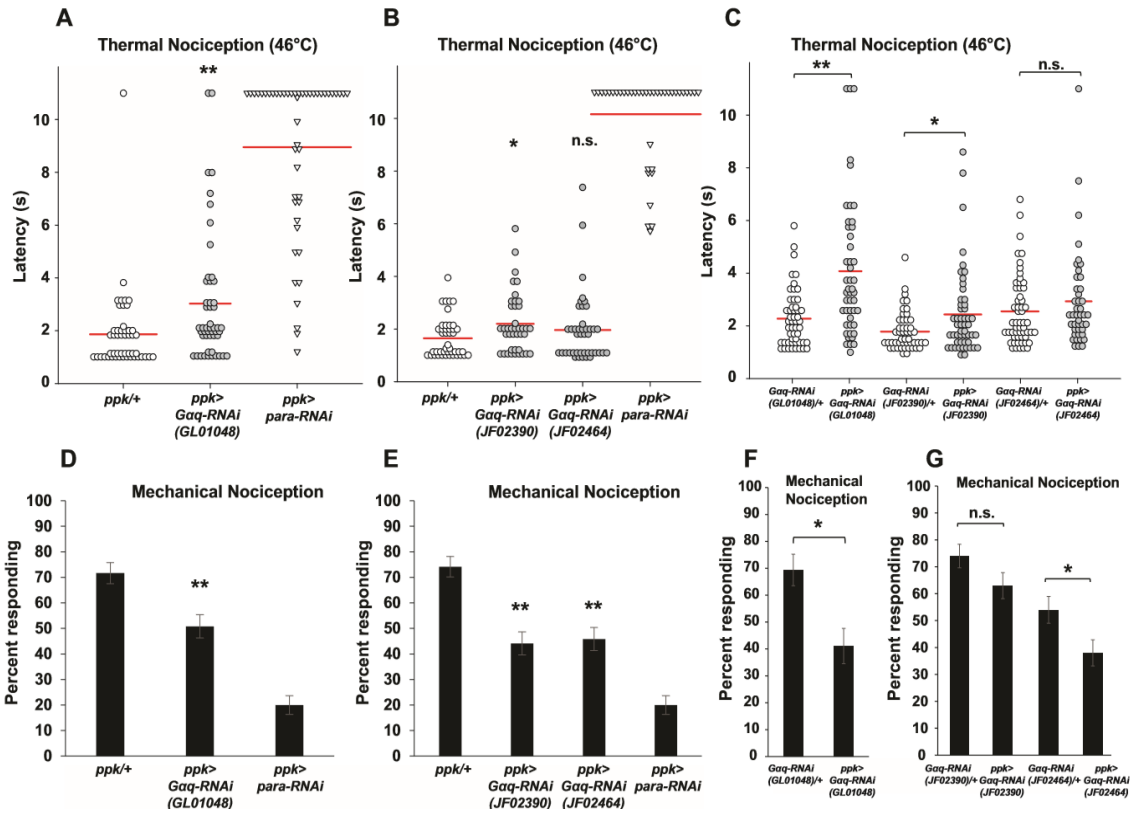

Figure 3. Nociceptor-specific knockdown of *Gαq* causes defects in thermal and mechanical nociception. (A, B). Larvae with nociceptor-specific knockdown of *Gαq* showed a significantly longer latency to respond to a noxious thermal stimulus (46°C) than did GAL4-only controls. Larvae with nociceptor-specific knockdown of *para* showed severely impaired nociceptive responses and were used as a positive control. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a horizontal bar ($n \geq 40$ for all groups; $*p \leq 0.05$ by Wilcoxon Rank-Sum Test; $**p \leq 0.001$ by Wilcoxon Rank-Sum Test). (C) Larvae with nociceptor-specific knockdown of *Gαq* showed a significantly longer latency to respond to a noxious thermal stimulus than did UAS-RNAi-only controls. ($n \geq 40$ for all groups; $*p \leq 0.05$ by Wilcoxon Rank-Sum Test; $**p \leq 0.001$ by Wilcoxon Rank-Sum Test). (D, E) A smaller proportion of larvae with nociceptor-specific knockdown of *Gαq* exhibited nociceptive responses to a noxious mechanical stimulus than did GAL4-only control larvae. Larvae with nociceptor-specific knockdown of *para* showed a very low rate of nociceptive responses and were used as a positive control ($n = 120$ for all groups; $**p \leq 0.001$ by Chi-Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion. (F, G) A smaller proportion of larvae with nociceptor-specific knockdown of *Gαq* exhibited nociceptive responses to a noxious mechanical stimulus than did UAS-RNAi-only control larvae. ($n > 50$ for all groups in *GL01048* graph and $n = 100$ for all other groups; $*p \leq 0.05$ by Chi-Square Test). [46].

Since tissue-specific knockdown of *Gαq* resulted in a hyposensitive phenotype to a noxious thermal stimulus, a mechanical nociception assay was conducted on the *Gαq* RNAi knockdown larvae to determine if this gene is involved in other noxious modalities. Larvae were prepared in the same manner as described above in the thermal assay. In the initial mechanical assay (Figure 3D and 3E), all three RNAi lines had a significantly lower response rate to the harsh mechanical stimulus in comparison to the GAL4-only control. The GL01048 *UAS-Gαq-RNAi* transgene line had a mean of 50.8% of larvae responding to first stimulus compared to the GAL4-only control with a mean of 71.7% of larvae responding to the first stimulus. The JF02390 *UAS-Gαq-RNAi* transgene line had a mean percent response of 44.2% and the JF02464 *UAS-Gαq-RNAi* transgene line had a mean percent response of 45.8% both compared to the GAL4-only control with a mean percent response of 74.2%. In a follow-up experiment comparing the RNAi knockdown to the UAS-only control to the respective line (Figure 3F and 3G), two lines showed the same result as previous tested (69.4% versus 41.1% for GL01048; 54% versus 38% for JF02464), while one *Gαq* RNAi line did not have a significantly different percent responding in comparison to the UAS-only control (74% versus 63% responding). Put together, this data shows that *Gαq* acts in nociceptors to regulate both thermal and mechanical nociception.

NorpA is required for normal sensitivity to noxious thermal and mechanical stimuli

In order to test the hypothesis that *norpA* plays a role in nociception, *norpA* RNAi lines were crossed with *w; ppk1.9-GAL4; UAS-dicer2* to induce a tissue-specific knock down of *norpA*, and the first-generation larvae were tested in a thermal behavioral assay for sensitivity to noxious stimuli. In a 46˚C thermal nociception assay (Figure 4A and 4B), one RNAi line (JF01713) had a significantly higher latency compared to both the GAL4-only

control and the UAS-only control (3.8 s versus 1.7 s for GAL4-only and 4.1 s versus 2.2 s for UAS-only); on the other hand, the second RNAi line (JF01585) was not significantly different from neither the GAL4-only control nor the UAS-only control (1.7 s for both genotypes and 2.0 s versus 2.3 s for UAS-only). In order to provide further information to make the results of the RNAi lines more conclusive, a thermal nociception assay was conducted on a *norpA* mutant knockout (Figure 4C). The results of the knockout line confirmed the phenotype of the first RNAi line since the latency of the knockout was significantly higher than the w^{1118} control (4.9 s versus 2.5 s).

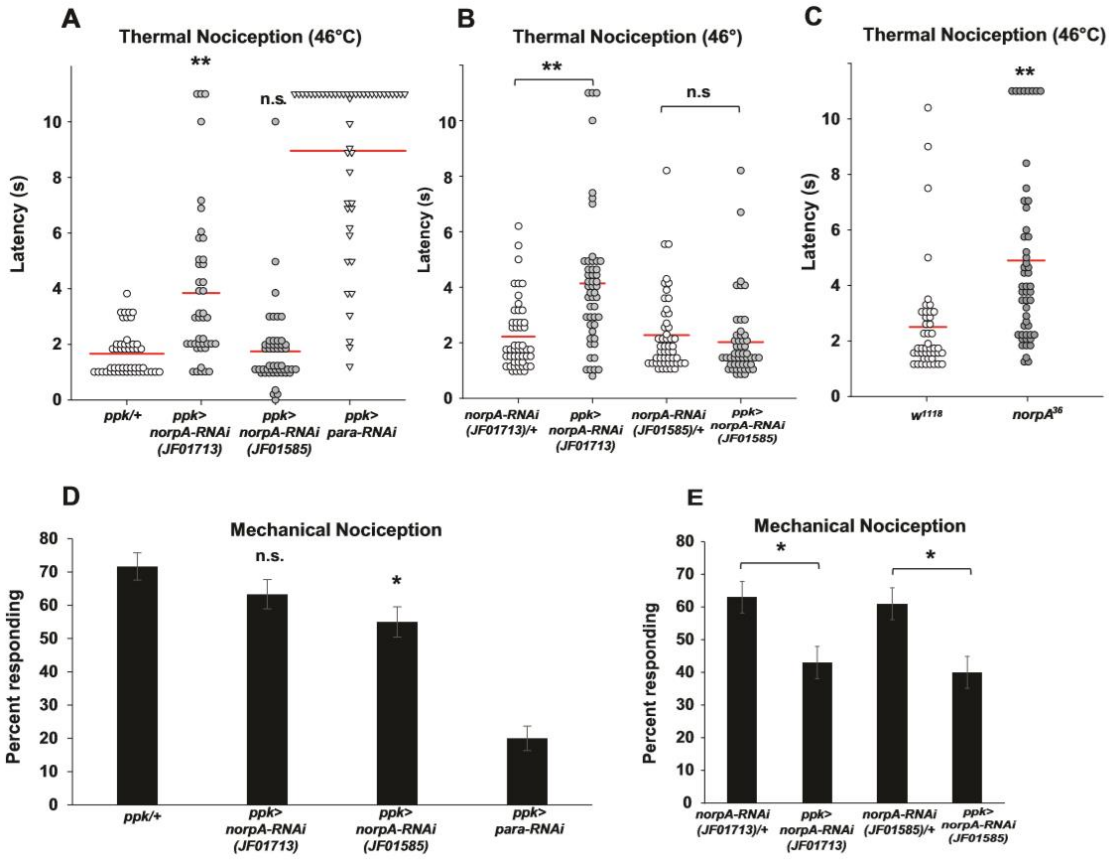

Figure 4. Loss of *norpA* function in the nociceptors causes defects in thermal and mechanical nociception. (A) Larvae with nociceptor-specific knockdown of *norpA* using the JF01713 *UAS-norpA-RNAi* transgene respond to a noxious thermal stimulus (46°C) with a significantly longer latency than do GAL4-only control larvae. Larvae with nociceptor-specific knockdown of *norpA* using the JF01585 *UAS-norpA-RNAi* transgene respond to noxious thermal stimuli with a mean latency that is not distinguishable from the control. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a horizontal bar ($n \geq 40$ for all groups; $^{**}p \leq 0.001$ by Wilcoxon Rank-Sum Test). (B) Larvae with nociceptor-specific knockdown of *norpA* using the JF01713 *UAS-norpA-RNAi* transgene respond to a noxious thermal stimulus with a significantly longer latency than do UAS-RNAi-only control larvae. Larvae with nociceptor-specific knockdown of *norpA* using the JF01585 *UAS-norpA-RNAi* transgene respond to noxious thermal stimuli with a mean latency that is not distinguishable from the UAS-RNAi-only control. ($n \geq 40$ for all groups; $^{**}p \leq 0.001$ by Wilcoxon RankSum Test). (C) norpA36 larvae respond to a noxious thermal stimulus with a significantly longer latency than do w 1118 control larvae. (D) A smaller proportion of larvae with nociceptor-specific knockdown of *norpA* using the JF01585 *UAS-norpA-RNAi* transgene exhibited nociceptive responses to a noxious mechanical stimulus than did GAL4-only control larvae. The proportion of larvae with knockdown of *norpA* using the JF01713 *UAS-norpA-RNAi* transgene that responded to a noxious mechanical stimulus was indistinguishable from that of GAL4-only control animals ($n = 120$ per group; $^{*}p \leq 0.05$ by Chi-Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion. (E) A smaller proportion of larvae with

nociceptor-specific knockdown of *norpA* using the JF01713 and JF01585 *UAS-norpA-RNAi* transgenes exhibited nociceptive responses to a noxious mechanical stimulus than did UAS-RNAi-only control larvae. (n = 120 per group; *$p \leq 0.05$ by chi-square test) [46].

Similar to *Gαq,* tissue-specific knockdown of *norpA* resulted in a hyposensitive phenotype to a noxious thermal stimulus. Following the thermal assay, a mechanical nociception assay was conducted on the *norpA* RNAi knockdown larvae to determine if this gene is involved in other noxious modalities (Figure 4D and 4E). The results of the mechanical assay mirrored those of the thermal assay; the JF01713 line did not have a significantly different percent responding compared to the GAL4-only control (63.3 percent responding versus 71.7 percent responding), while the FJ01585 line had a significantly lower percent responding in comparison to the GAL4-only controls (55 percent responding to the first stimulus versus 71.7 percent responding to the first stimulus). In a follow up experiment, we found that larvae expressing the JF01713 and JF01585 *UAS-norpA-RNAi* transgene lines both responded at significantly lower frequency than their respective UAS-RNAi-only controls (63% versus 43% for JF01713; 61% versus 40% for JF01585). The combination of the thermal and mechanical data suggests that *norpA* play a role in normal nociceptor function.

Gαq and norpA are not required for nociceptor dendrite morphogenesis

Since it was shown that both *Gαq* and *norpA* play a role in nociceptors, a morphology experiment was conducted to understand more about how these genes affect neuron function. The *w; ppk1.9-GAL4, UAS-mCD8::GFP; UAS-dicer2* fly line was crossed with either a control, a *Gαq* RNAi line, or a *norpA* RNAi line. This resulted in larvae with nociceptor-specific expression of both the gene knockdown and GFP expression in the nociceptors allowing for imaging using a Laser Scanning Confocal Microscope. Larvae were prepared and imaged as described in the methods. After images were taken for all three groups (Figures 5A-C show a representative image of each genotype), the neurons were manually

traced using the NeuronJ plugin in the ImageJ software. The plugin used the tracings to quantify total dendrite branches and total dendrite length (Figure 5D and 5E). For both *Gαq* and *norpA* knockdowns (using the JF02390 and JF01713 RNAi lines respectively), there was no significant difference in dendrite branches or length compared to wild-type neurons. This shows that neither *Gαq* nor *norpA* have an effect on morphology of class IV multidendritic neurons.

I conducted the experiments shown in Figures 3C, 3F-G, 4B-C, 4E, and 5A-E. Josh Herman conducted the experiments shown in Figures 3A-B, 3D-E, 4A, and 4D.

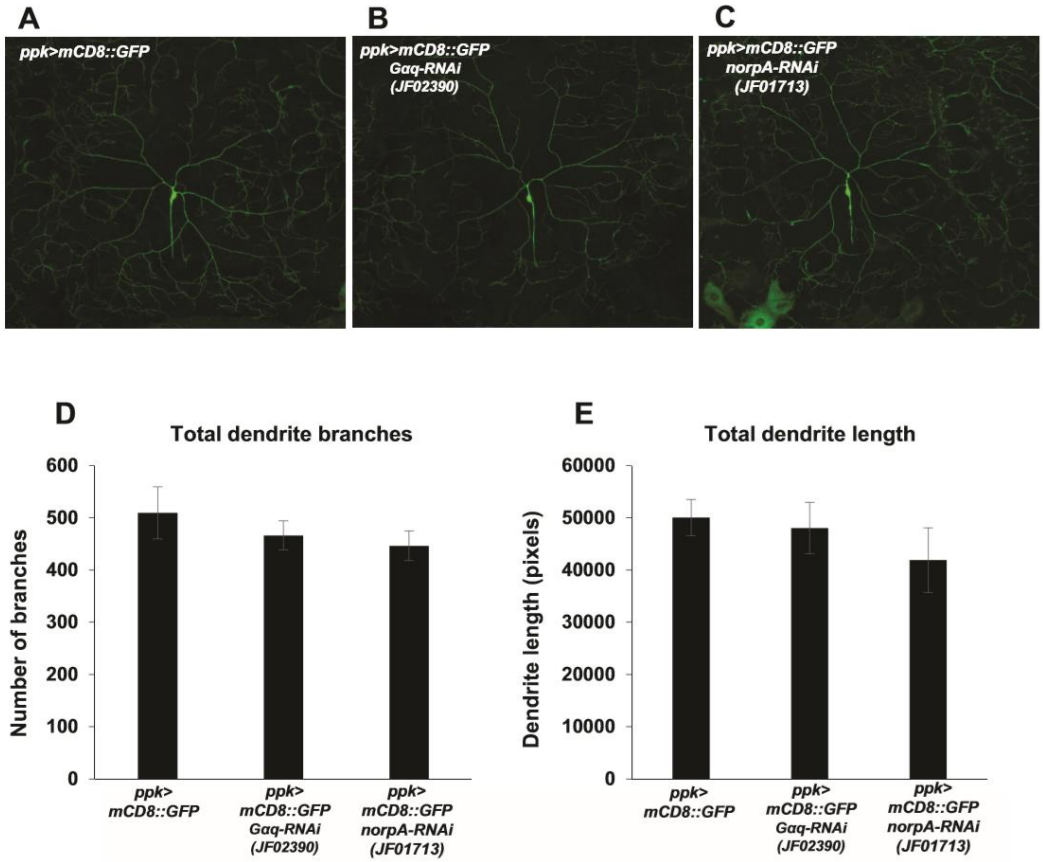

Figure 5. Nociceptor-specific knockdown of *Gαq* and *norpA* does not cause changes in mdIV neuron dendrite length or branch count. (A, B, C) Confocal micrographs displaying the dendritic arborization of ddaC mdIV neurons expressing mCD8::GFP and *Gαq* or *norpA* RNAi. (D) The total numbers of dendrite branches in ddaC dendrites with *Gαq* or *norpA* knockdown were not significantly different (by Student's *t*-test) from those of no-RNAi control ddaC neurons. (E) The total lengths of ddaC dendrites with *Gαq* or *norpA* knockdown were not significantly different (by Student's *t*-test) from those of no-RNAi control ddaC neurons. Bars indicate mean total dendrite length, while error bars indicate standard error of the mean (n = 6 neurons for each group). [46].

Discussion

G protein signaling acts in sensory neurons to regulate electrical excitability and neurotransmitter release. This cascade is activated by a member of the GPCR family which responds to ligands including hormones, neurotransmitters, and peptides. Some of these hormones may be released as a result of inflammation and tissue damage which makes this pathway a promising field of research to understand pain [21, 34, 35].

In our study, we conducted experiments to demonstrate that Gαq and PLCβ signaling regulate the basal sensitivity of *Drosophila* class IV multidendritic neurons to thermal and mechanical stimuli, potentially through modulating electrical excitability. Our results show that *Gαq* regulates nociceptor sensitivity since nociceptor-specific knockdown resulted in a less sensitive behavioral response to noxious stimuli of different modalities. In addition, *norpA* showed similar results to *Gαq* following nociceptor-specific knockdown. Since it has been shown that NorpA is an effector of Gαq in phototransduction in *Drosophila* [37], we hypothesize that NorpA is a downstream effector of Gαq in larval nociceptors. This could be tested by conducting an epistasis experiment with double knockdown of *Gαq* and *norpA* with simultaneous *Gαq* gain-of-function. Finally, we showed that neither loss of function of *Gαq* nor *norpA* have an effect on dendrite development or arborization. This suggests that the behavioral defect shown in thermal and mechanical assays is not arising from defects in multidendritic neuron development. Put together, this data supports the hypothesis that Gαq-PLCβ signaling increase electrical excitability and neurotransmitter release.

Since it has been shown that PLCβ activity affects TRPA1 in other sensory systems [37, 40], it is likely that the G protein signaling cascade is modulating TRPA1 ion channels through calcium influx from the endoplasmic reticulum. The data supports the possibility of

an alteration in electrical excitability of the neuron since there is a change in nociceptor function and no defect in neural development. A follow-up experiment for our study could be conducted to investigate calcium levels in the absence of *Gαq* and *norpA.* A GCaMP calcium indicator could be expressed in the neurons while simultaneously using RNAi to knock down *Gαq* or *norpA* to determine if there is a change in calcium presence with gene knockdown [47]. This could support the hypothesis that calcium influx modulates TRPA1 activity.

How PLCβ activity affects TRPA1 still remains undetermined since PLCβ cleaves PIP2 into two secondary messengers, IP3 and DAG. Inhibition of either of these secondary messengers could determine if the calcium released from IP3 activity or the phosphorylation of downstream proteins and ion channels from DAG activity is the modulating factor of TRPA1 ion channels. It is possible that each contribute modestly to upregulate TRPA1 channel activity; therefore, knockdown of *Gαq* or *norpA* silences both cascades resulting in the hyposensitive phenotype shown in the experimental data.

Our study provides a good basis for clinical relevance of targeting the G protein signaling cascade with therapeutic interventions. Anti-nociception drugs have already been created that bind to G protein coupled receptors and act as inhibitory ligands to dial down GPCR activity lowering electrical excitability. The identity of the GPCR that regulates this pathway in context of pain has yet to be uncovered [35]. It has been shown in *Drosophila* that an sNPF receptor facilitates the G protein signaling cascade in mechanical nociception [48]. This is a possible avenue of clinical intervention, but our project showed that Gαq and PLCβ are both also involved in thermal nociception. This means that there are other GPCRs other than sNPF receptors that mediate this pathway that need to be uncovered.

In addition to targeting GPCRs, therapies could be developed to target important kinases or auxiliary signaling proteins that mediate the G protein signaling cascade [35]. This provides an alternative therapeutic approach for diseases caused by inflammation. Since our study shows that *Gαq* and *norpA* play a role in nociceptor sensitivity to noxious stimuli, Gαq and PLC are potential targets to treat pain conditions. A small-molecule inhibitor of the protein-protein interaction of Gαq on PLC could be a potential avenue, but it has been suggested that it can be difficult to disrupt the interface between interacting proteins [49]. Another possible method of intervention could be using an enzyme inhibitor to reduce the activity of PLC signaling. Either of these two methods of drug intervention would theoretically inhibit the cleaving of PIP2 into IP3 and DAG which would result in less calcium influx from the endoplasmic reticulum. Put together, this would lower TRPA1 activity causing a decrease in pain sensation.

Despite these two possible avenues of drug treatment, it is important to consider that it would be difficult to inhibit PLC activity exclusively in the nociceptors which would result in side effects. Our study has shown that *Drosophila* is a good model for further study of the role of *Gαq* and *norpA* signaling in modulating nociceptor sensitivity. More experiments should be conducted to further characterize Gαq and PLC activity in nociceptors and potential side effects that could arise, but this provides evidence of potential drug interventions for the future.

Chapter 3: The Roles of Wnt Signaling in *Drosophila* Nociception

Introduction

Chronic pain

Opioids are a family of overused drugs that are expensive and highly addictive. As of now, they are one of the few ways to treat chronic pain. The effects of chronic pain range from severely reducing quality of life of the individual to impacting public spending. These are just a few of the reasons why the molecular mechanisms of sensory neuron modulation need to be uncovered. The nociception-induced reflexive behavior (NEL) of *Drosophila melanogaster* larvae can be studied to uncover and characterize the signaling pathways and cellular machinery that modulate chronic pain. The information about these signaling pathways can be used in order to develop better clinical interventions to treat neuropathic pain conditions. [1, 2]

Wnt signaling overview

Wnt signaling is an evolutionarily conserved signaling cascade involved in many cellular processes [50]. It plays an important role in cell fate, proliferation, polarity, cell death during development, and tissue homeostasis in adults [50, 51]. Wnt signaling is broken down into the canonical pathway and non-canonical pathways. The canonical Wnt signaling pathway involves the use of a transcription regulator called β-catenin to promote transcription of Wnt-related genes, resulting in developmental effects. Non-canonical Wnt signaling is further broken down into two pathways which are called the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway. The non-canonical pathways are independent of β-catenin; these pathways regulate cytoplasmic actin dynamics and intracellular calcium instead of altering transcription in the nucleus through β-catenin (Figure 6) [52, 53].

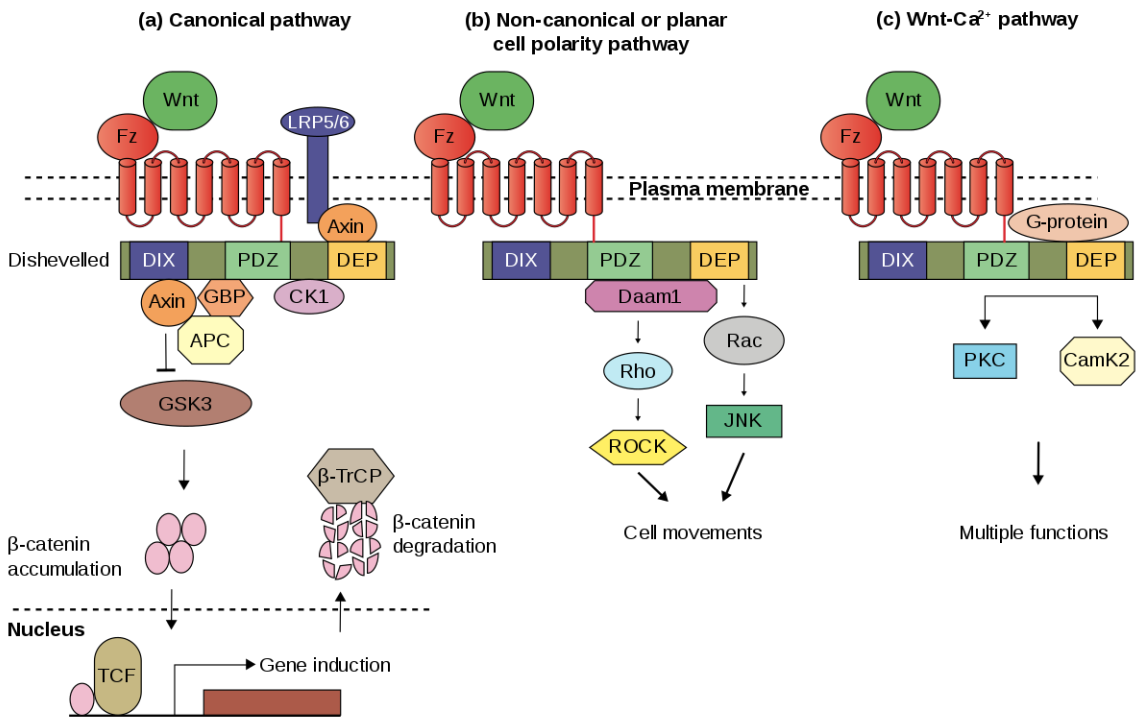

Figure 6. The three Wnt signaling pathways. The three pathways are the canonical pathway, and the two non-canonical pathways which are planar cell polarity and Wnt/Ca^{2+}. The downstream effectors and ultimate outcomes of each pathway are also portrayed [54].

Wnt signaling generally involves a Wnt ligand, a protein secreted from nearby cells, binding and activating a transmembrane receptor complex on the exterior of a cell of interest [51]. The receptor complex contains a member of the Frizzled family of receptors, which are a family of seven-transmembrane domain G protein coupled receptors [53, 55]. The receptor complex also contains a co-receptor to Frizzled which is interchangeable in different Wnt pathways. Examples of Wnt co-receptors are single transmembrane receptors of the low-density lipoprotein receptor-related protein family (LRP5/6) receptor, tyrosine kinase-like orphan receptor (ROR1/2), Protein Tyrosine Kinase 7, and receptor-like TK [56].

Activation of the Frizzled receptor complex recruits Dishevelled, which is a scaffold protein. Dishevelled then transmits the signal to the remaining downstream effectors in the pathway [50]. In the canonical pathway, downstream of Dishevelled is the β-catenin destruction complex which is composed of glycogen synthase kinase 3 beta (GSK3β), axin, and adenomatous polyposis coli (APC). The destruction complex normally functions to degrade β-catenin, but when Wnt is bound to the receptor complex and Dishevelled has been recruited, the destruction complex is inhibited which allows β-catenin to accumulate and translocate to the nucleus to activate gene transcription [50].

The non-canonical Wnt signaling pathways both use similar ligands and receptor complexes to the canonical pathway, but the downstream effectors following Dishevelled diverge, resulting in the different functions of the pathways. In the PCP pathway, the downstream effectors of Dishevelled are broken down into two different pathways. Following Frizzled receptor activation, Dishevelled recruits Dishevelled-associated activator of morphogenesis 1 (Daam1) to phosphorylate Rho-associated protein Kinase (ROCK). This phosphorylation causes ROCK to phosphorylate cytoskeletal proteins like actin, which

results in cytoskeletal arrangement [57, 58]. Another downstream effector of Daam1 is the Rho GTPase, Rac1, and its downstream target, c-Jun amino (N)-terminal kinase (JNK). This alternate branch of the PCP pathway is called the Rac-JNK pathway and contributes to cytoskeleton rearrangement [30, 58]. It has been shown in *Drosophila* that genes like *flamingo/starry night* (a member of the G protein coupled receptor family [59]), *Van Gogh/strabismus* (a candidate co-receptor to Frizzled [60]), and *prickle* (works to localize Dishevelled to Frizzled receptors [61]) act in the planar cell polarity pathway. These genes work to regulate development, cytoskeleton reorganization, and cellular movements including convergent extension movements and neural tube closure [62, 63].

The Ca^{2+}/Wnt pathway is a calcium-dependent pathway that utilizes activation of the G protein cascade as the downstream effector. The Ca^{2+}/Wnt pathway uses Frizzled to activate the G protein signaling cascade to release calcium and activate molecules like calmodulin-dependent protein kinase II (CaMKII) and protein kinase C [30]. This pathway has been shown to be important for development processes, but it also contributes to inflammatory responses [62].

Wnt signaling as a whole has been shown to be important for the development of the nervous system by regulating differentiation and patterning of sensory axons in addition to development of the synapses between sensory and motor neurons in the spinal cord [30]. In addition, Wnt signaling has been shown to play a role in the sensitization of nociceptors [30]. For this reason, it is important to understand the mechanism of how Wnt signaling may affect developmental changes in nociceptors or transcriptional regulation underlying the pathogenesis of neuropathic pain [64].

Wnt3a is a modulator of DRG sensory neuron function

Wnt signaling has been suggested as a modulator of chronic pain because of the presence of Wnt ligands in the DRG and the spinal cord. The presence of Wnt3a, in particular, in the DRG is important to note [65]. Wnt3a is a highly characterized Wnt ligand that activates both canonical and noncanonical pathways and has been shown to be involved in the development and regeneration of the nervous system [64, 66, 67].

Experiments have investigated Wnt3a expression in the DRG and spinal cord in a nerve-injured rat model. It was discovered that there was a rapid and long-lasting increase in the expression of Wnt3a in both the DRG and spinal cord after nerve-injury. This suggests that an increase in tissue damage causes an up-regulation of Wnt3a activation and increased binding of Wnt ligands to the receptor complexes, which could be linked to more long-term pain sensation [64, 65].

Similar experiments have looked at β-catenin expression and translocation in the DRG and spinal cord, since it is the result of canonical Wnt pathway activity. After nerve injury, there was a rapid and long-lasting increase in active β-catenin in the spinal cord. Antibody staining showed that not only was there increased expression of β-catenin, but there was an increased transport of β-catenin into the nucleus for gene transcription. On the other hand, there is no difference in expression or translocation of β-catenin in DRG. These results indicate that Wnt signaling may modulate neuron sensitivity through different mechanisms depending on the neuron type. Wnt signaling may act in the spinal cord through the canonical pathway, and could act noncanonically in DRG [64].

Canonical signaling in nociceptor sensitization

A set of experiments have been conducted to understand more about the mechanism of Wnt signaling in nociceptor sensitization through downstream effectors. In mouse DRG cell cultures, it has been shown that Wnt3a activity stimulates an upregulation of *axin2* and *Dkk1* transcripts, which are known β-catenin transcriptional targets, providing evidence that Wnt3a does act through canonical signaling [30]. In order to understand the role of Wnt3a in neuron sensitization, mouse DRG neuron cultures were treated with Wnt3a. Results showed an upregulation of *axin2* and *Dkk1* which are known to be β-catenin transcriptional targets. In addition, immunocytochemistry on the same cultured neurons showed an increased translocation of β-catenin into the nucleus. This suggests that the downstream mechanisms of Wnt3a are activated during sensory neuron sensitivity modulation in the canonical pathway [30].

The Ca^{2+}/Wnt pathway in nociceptor sensitization

Since the influx of calcium is important for neural excitability and neurotransmitter release, it is important to uncover a role and mechanism for the Wnt/Ca^{2+} pathway in neuron sensitization. Cultured mouse DRG neurons treated with Wnt3a exhibited a peak of intracellular calcium in the neurons after Wnt3 application. This reflects a neuronal calcium response triggered by Wnt signaling mediators which suggest that the Wnt/Ca^{2+} signaling pathway is activated by Wnt3a in sensory neurons [30].

Activation of the cytoplasmic calcium influx by the Wnt/Ca^{2+} signaling pathway can result in additional signaling consequences for a cell. There is an increase in abundance of phosphorylated CaMKII in DRG neuron cultures after Wnt3a application [30], which has been functionally associated with sensitization of TRPV1 channels [28, 68]. Through

immunocytochemistry experiments on cultured DRG cells, Wnt3a exposure resulted in an increased cell surface localization of TRPV1 ion channels. Since there are more heat receptors, the neurons are more able to respond to heat through an increase in excitability of the neurons because of Wnt3a activity. In addition to the increase of CaMKII activation, an ELISA analysis showed that Wnt3a-exposed cultured DRG neurons also released a significant concentration of 7 different cytokines including IL-2, IL-4, IL-10, IL-12, IL-17A, TNFα, and GM-CSF. These cytokines are known to activate and increase the sensitivity of neurons, so this provides another mechanism of neuron modulation based on Wnt signaling activation [30, 69].

The planar cell polarity pathway in nociceptor sensitization

The last arm of Wnt signaling is the planar cell polarity pathway, which is also the second noncanonical pathway. In order to test the effect of this pathway on nociceptor sensitization, Rac1 was knocked down with a validated shRNA in one group of mice and a Rac1 inhibitor was used in another group of mice. Both groups were treated, alongside a control, with Wnt3a, and mechanical hypersensitivity assays were conducted. The mice were stimulated using a von Frey monofilament where the hind paw was treated with Wnt3a. Result showed, in both experimental groups, a decrease in sensitivity; in addition, a JNK blocker completely removed mechanical sensitivity. On the other hand, inhibition of ROCK did not change sensitivity to mechanical force. Putting this all together, this showed that the Rac1-JNK arm of the PCP pathway plays a role in mechanical hypersensitization of nociceptors while the ROCK portion is not involved [30].

Because the Rac-JNK signaling pathway can regulate intracellular trafficking and cell-surface expression of membrane proteins, experiments have been conducted to

determine whether this pathway regulates TRPA1 ion channels, which are needed for mechanical nociception. Cells exposed to Wnt3a were shown to have an increased expression in tagged TRPA1 receptors on the cell surface. In addition, another experiment conducted showed that Wnt3a-induced sensitivity was abolished when TRPA1 channels were pharmacologically blocked. This provides a mechanism of action for the PCP pathway acting through the Rac1-JNK pathway. It appears as though it enhances the membrane expression of TRPA1 in sensory neurons reflecting neuroplasticity changes [30].

Dendrite development

Since Wnt signaling plays a significant role in nervous system development, it is important to understand how larval sensory neuron dendrites develop and function. Dendrites are processes of neurons that branch out from the cell body, and are specialized for information input, and the branching morphology of dendrites is important to neuronal function and circuit assembly [70]. During development, multidendritic neurons establish characteristic dendritic branching patterns to cover spatial territories, and dendrites of the same cell subtype do not overlap as shown in Figure 7. This concept is called dendritic tiling.

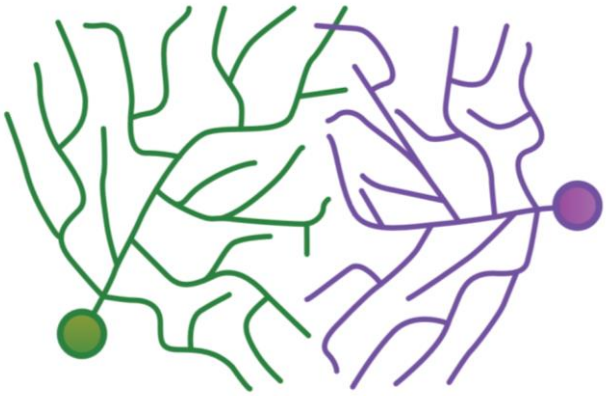

Figure 7. Two sensory neurons displaying tiling their dendrites. The dendrites cover the full space, but there is little to no overlap of dendrites to ensure accurate but non-redundant reception of information [71].

The purpose of tiling is to ensure complete, accurate, and non-redundant reception of sensory input no matter where on the skin the stimulus is targeted [11, 70, 72]. In order to develop the wiring specificity and type-specific morphology of the *Drosophila* peripheral nervous system, dendrite morphogenesis is regulated by the combination of intrinsic transcription factors expressed in the neurons and external factors from glial cells or dendrites from a neuron of the same class [7, 10].

Intrinsic factors

An example of an intrinsic factor controlling dendrite development is a protocadherin involved in the planar cell polarity pathway of Wnt signaling called *flamingo*. Dendrites of class IV neurons from *flamingo* loss of function mutants extended beyond the dorsal midline which is uncharacteristic of multidendritic tiling. A hypothesis proposed is that *flamingo* affects extension and routing of dendrites [73]. Using GFP expression and confocal imaging in the developing neurons, it was hypothesized that wild-type neurons utilize a stop mechanism that inhibit extension beyond the dorsal midline. When *flamingo* mutants were analyzed in a similar manner, an extension of dorsal neuron dendrites beyond the midline into the territory of neurons of the same subtype was seen. This suggests that *flamingo* is intrinsically involved in inhibiting dendrite overextension which shows a manner of intrinsic control of dendrite growth [74]. Even though the results from *flamingo* knockdown provide information about intrinsic Wnt signaling factors, it is not fully clear how Canonical Wnt signaling and the Wnt/Ca^{2+} regulate md neuron morphology.

Another example of an intrinsic factor is a gene called *cut* which encodes a transcription factor. *cut* has been shown to be expressed in all four classes of the multidendritic neurons, but at a higher level in class III md neurons which display truncated

dendrites in wild-type neurons. When *cut* was knocked down in the four classes of md neurons, each class displayed varying levels of complexity in dendrite branching. The classes of md neurons that develop highly branched dendrites have high *cut* expression typically. After *cut* knockdown, these md neurons had a lowered level of dendrite complexity. This suggests that *cut* plays a role not only in increasing dendrite growth, but the level of *cut* expression dictates its functionality. As a confirmation, *cut* was overexpressed in the class I md neurons since there is little expression in this particular class. Overexpression caused an increase in growth of the dendrites which supports the hypothesis that *cut* increases dendrite outgrowth and through different levels of expression [75].

Extrinsic factors

Just as intrinsic factors play a role in dendrite development, neurons must utilize extrinsic cues to ensure there is full, broad coverage of the skin without overlapping dendrites of similar cell types. This extrinsic control of dendrite extension stems from repulsive signaling between dendrites. The role of homotypic repulsion in class IV neurons has been investigated. GFP was expressed in the class IV neurons and images were taken before and after laser ablating a single neuron. Before ablation, there was uniform, full coverage of the body wall with no gaps in the field. After single neuron ablation, neighboring neurons, colored black and blue, extended their dendrites and invaded the region and covered the gap in the field while still not overlapping shown in Figure 8.

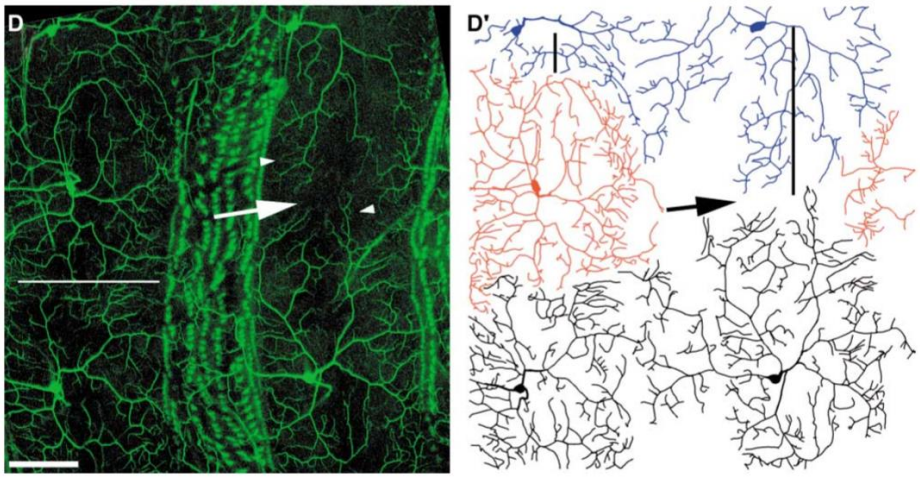

Figure 8. Dendrites of neurons invade empty space to cover full receptive field. D) Class IV multidendritic neurons expressing GFP. The arrow points to the area where a single neuron was ablated, and the ticks show where the neighboring neurons invaded the space. D') Sketch depicts a clear representation of the neurons shown in panel D. The arrow points to the area where the ablated neuron was, and the neighboring neurons invading the space are colored in black and blue [72].

This suggests that there is an ability for dendrites to continue extending until they reach a similar cell type causing a repulsive stop in order to maintain tiling as the larva increases size during development [72].

Dscam

A transmembrane protein in *Drosophila* called Down syndrome cell-adhesion molecule (Dscam) has been shown to be involved in a neuron's ability to recognize dendrites of the same neuron [76]. A GFP tagged to Dscam shows expression on the cell surface of dendrites and axons of multidendritic neurons [77]. Loss-of-function mutants for Dscam showed that dendrites of multidendritic neurons cross extensively with the dendrites of the same cell in comparison to controls which dendrites never touched. Dendrites tended to become so tangled that there were regions of the body wall that were not covered by dendrites. This put together shows that Dscam is a required mechanism to control self-avoidance of dendrites by inducing dendritic repulsion when it makes contact with Dscam expressed on other dendrites.[76].

Wnt signaling genetic screen

In order to get a better understanding of how nociceptors are sensitized, the connections between Wnt signaling, dendrite development, and neuron function must be researched. Despite the vast amount of research on Wnt signaling, it is unclear as to which ligands and co-receptors are activating or inhibiting which Wnt pathway in sensory neurons. The combination of Wnt ligands with different Frizzled receptors and co-receptors in the receptor complex increases the specificity of signaling that can occur. For this reason, it is important to understand which ligands and receptors are involved in which pathways and how these mechanisms regulate nociceptor sensitivity and development.

A previous student in the lab named Paul Freeman conducted a genetic screen of forty Wnt related genes in an effort to determine if Wnt signaling plays a role in sensory neuron function. He knocked down each gene in the nociceptors of *Drosophila* and conducted a thermal nociception assay on the larvae. He found six genes that were insensitive to noxious thermal heat: *wnt10, frizzled3, pontiin, nek2, off-track 2, and ank2*. There were also six genes that were hypersensitive to noxious thermal stimuli: *frizzled2, frizzled, pangolin, Wnt4, hipk, Wnt2.* This provided a basis that Wnt signaling may act to regulate neuron sensitivity in *Drosophila* larvae [78].

Protein Tyrosine Kinase 7 is a Wnt co-receptor that plays a role in nociception

One of the genes of interest from the Wnt genetic screen is *off-track 2 (otk2),* which is the *Drosophila* homolog of Protein Tyrosine Kinase 7 (PTK7). PTK7 has been shown in mouse models to activate the planar cell polarity pathway [79]. Mouse mutant knockout lines of PTK7 showed classic PCP defects in neural tube closure which suggests that PTK7 acts to regulate nervous system development through the PCP pathway [80]. It has also been shown that Otk2 can bind to Wnt2, Frizzled, and Frizzled2 suggesting a role in Wnt signaling similar to PTK7 in vertebrates [79]. This gave rise to the question of if, and to what extent, *otk2* plays a role in nociceptor sensitization and chronic pain. In this study, I address this question and use tissue-specific RNAi to demonstrate that *otk2* regulates the sensitivity of *Drosophila* class IV multidendritic neurons. I had four specific aims for this project. One, verify if *otk2* acts in larval nociceptors to affect thermal and mechanical nociception. Two, determine if *otk2* affects neural development to cause morphological changes in nociceptors. Three, uncover cellular localization of the Otk2 receptor. Four, determine if *otk2* and *frizzled/frizzled2* are functionally coupled.

Materials and Methods

Fly Stocks

The fly line *w; ppk1.9-GAL4; UAS-dicer2*, was used as a driver line and was crossed with a UAS-RNAi line. In morphology experiments, *w; ppk1.9-GAL4, UAS-mCD8::GFP; UAS-dicer2* was used as the driver line and was crossed with a UAS-RNAi line. The y^1 v^1*; [Py[+t7.7]=CaryP]attP2* (BDSC# 36303) line was crossed with *w; ppk1.9-GAL4; UAS-dicer2* as a negative, GAL4-only control. The y^1 v^1*; [Py[+t7.7]=CaryP]attP40* (BDSC# 36304) line was used in the place of BDSC#36303 in experiments where the UAS-RNAi line inserted at the attP40 insertion site. The w^{1118} control stock was crossed with a UAS-RNAi line as a negative, UAS-only control. The *UAS-para-RNAi* line was crossed with *w; ppk1.9-GAL4; UAS-dicer2* as a positive control in insensitive assays. HMC04171 (BDSC# 55892), HMS01889 (BDSC# 38973), HMJ22927 (BDSC# 61206), and HMS04485 (BDSC# 57040) were used as UAS-*otk2-RNAi* lines. HMS01308 (BDSC# 34321) and JF01258 (BDSC# 31311) were used as UAS-*fz-RNAi* lines. JF02722 (BDSC# 27568) was used as the UAS-*fz2-RNAi* line.

Nociception Assays

The nociception assays in this Chapter were conducted as described in Chapter 2.

Neural Morphology

The neural morphology data in this Chapter was collected and analyzed as described in Chapter 2.

Molecular Cloning

An *otk2* cDNA plasmid (Clone RE41180) was ordered from The Drosophila Genome Resource Center. Primers were designed and used to PCR amplify the *otk2* cDNA. The

forward primer was 5’-CACCATGGGGCTGAACGGAAGAC-3’, and the reverse primer was 5’-TCACACAATATCGTAGGFCAC-3’. The amplified *otk2* DNA was ligated into the pENTR/d-TOPO vector using the pENTR™ /D-TOPO® Cloning Kit and transformed into competent *E. coli.* Double digest using NEB high-fidelity enzymes (*BamHI* and *SacII*) followed by Sanger sequencing by Eton Biosciences confirmed the *otk2* sequence insertion into the pENTR/d-TOPO vector.

The Gateway Cloning LR Clonase reaction kit was then used to switch out the *otk2* sequence in the pENTR/d-TOPO vector for the cassette in the pTVW vector, and the plasmid was then transformed in competent *E. coli*. After the Clonase reaction, a digest was performed with the NEB high-fidelity enzyme *KpnI,* which cut twice in the plasmid resulting in two fragments. One fragment was expected to be 1,875 base pairs long, and the second was expected to be 9,313 base pairs long. Both bands were confirmed with gel electrophoresis.

Results

otk2 is required for normal sensitivity to noxious thermal stimuli but not mechanical stimuli

In order to test the hypothesis that *otk2* plays a role in nociception, an *otk2* RNAi line was crossed with *w; ppk1.9-GAL4; UAS-dicer2* to induce a tissue-specific knock down of *otk2*, and the first-generation larvae were tested in a thermal behavioral assay for sensitivity to noxious stimuli. Four *otk2* RNAi lines were tested for sensitivity to a noxious thermal stimulus at 46°C. *UAS-RNAi* transgenes are inserted at either the attP2 or attP40 docking sites, which represent different locations on the chromosomes where the UAS-RNAi transgene is inserted. The *UAS-otk2-RNAi* transgene for the BDSC lines #55892 and #38973 is inserted in the genome in the attP2 insertion site while the *UAS-otk2-RNAi* transgene for BDSC lines #61206 and #57040 is inserted in the attP40 insertion site.

Drosophila larvae were tested at 46°C, and the NEL latencies were calculated for each genotype. The UAS-only controls associated with the RNAi lines inserted in the attP2 site had a significantly longer latency than the GAL4-only control (Figure 9A). The UAS-only control for BDSC #55892 had a mean latency of 3.5 seconds, and the UAS-only control for BDSC #38973 had a mean latency of 2.9 seconds; each were compared to the GAL4-only control which had a mean latency of 2.1 seconds. The *otk2* RNAi knockdown for BDSC #55892 had a mean latency of 3.5 seconds, and the *otk2* RNAi knockdown for BDSC #38973 had a mean latency of 2.9 seconds. The significant different between the GAL4-only control and the UAS-only controls suggests that the insertion of the UAS transgene is causing a defect unrelated to RNAi expression since these genotypes are not expected to have a significantly different behavioral phenotype compared to the GAL4-only control. Since the

two negative controls resulted in significantly different latencies, any behavioral defect shown in the RNAi experimental groups cannot be attributed to *otk2* knockdown.

For the RNAi lines inserted in the attP40 site (Figure 9B), the UAS-only control for BDSC #57040 had a mean latency of 2.2 seconds, and the UAS-only control for BDSC #61206 had a mean latency of 2.6 seconds; each were compared to the GAL4-only control which had a mean latency of 1.8 seconds. The p-values for the UAS-only controls were 0.002 and 0.008, respectively. There was no significant difference in the latencies between the GAL4-only and UAS-only negative controls which shows that the UAS transgenes associated with this insertion site are not affecting behavior. The *otk2* RNAi knockdown using BDSC #54070 had a mean latency of 2.7 seconds, and the *otk2* RNAi knockdown using BDSC #61206 had a mean latency of 3.2 seconds; each were compared to the GAL4-only control which had a mean latency of 1.8 seconds. The p-values for the *otk2* RNAi knockdown genotypes were 0.011 and less than 0.001, respectively. Each of the *otk2* RNAi knockdown using the attP40 docking site showed a significantly longer latency compared to the GAL4-only control.

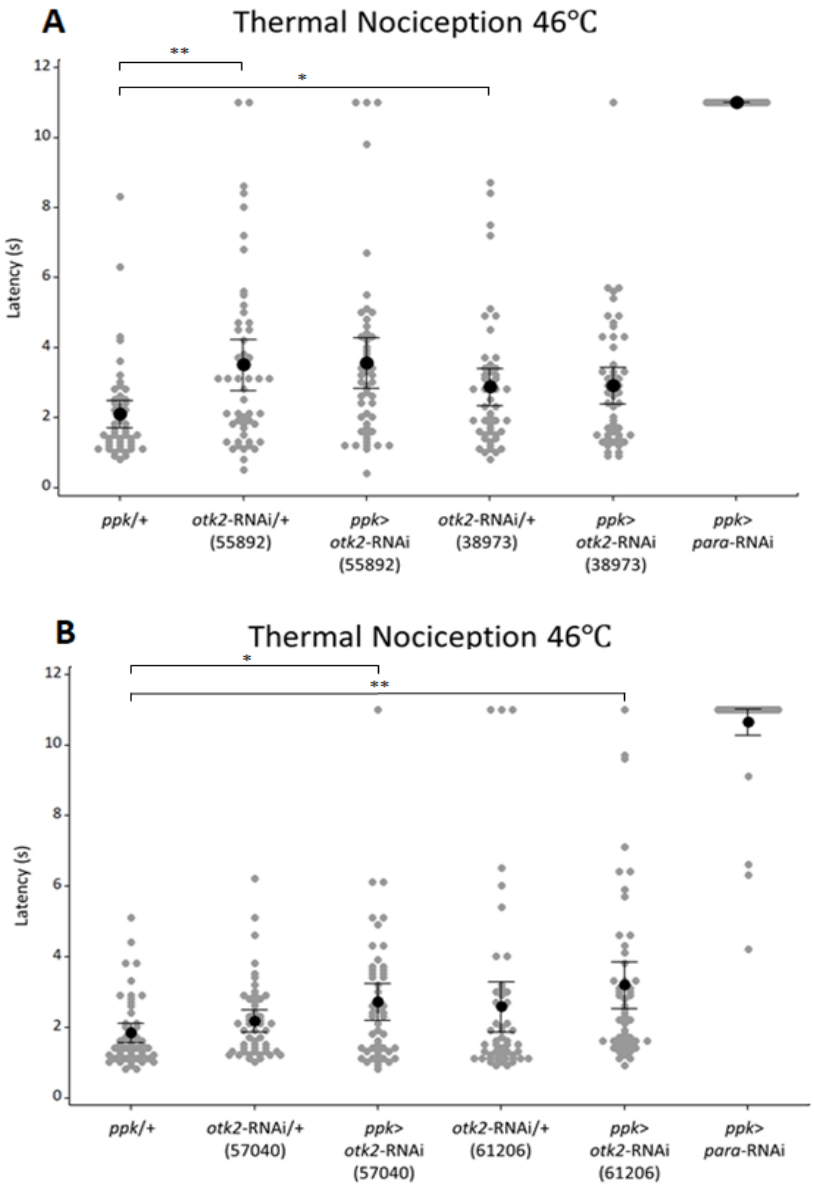

Figure 9. Nociceptor-specific knockdown of *otk2* causes defects in thermal nociception. (A) UAS-only negative control larvae showed a significantly longer latency to respond to a noxious thermal stimulus of 46°C than did GAL4-only controls. Since the negative controls were not functioning as expected, *otk2* lines associated with the attP2 insertion site can't be assessed for behavioral defects. Control p-values: 0.002, 0.008. (B) Larvae with nociceptor-specific knockdown of *otk2* showed a significantly longer latency to respond to a noxious thermal stimulus of 46 °C than did GAL4-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 animals are plotted as points on the graph, while the mean for each genotype is indicated with a black circle ($n = 50$ for all groups; *$p = 0.011$ by Mann Whitney U Test; **$p \leq 0.001$ by Mann Whitney U Test).

Since tissue-specific knockdown of *otk2* resulted in a hyposensitive phenotype to a noxious thermal stimulus, a mechanical nociception assay was conducted on the *otk2* RNAi knockdown larvae associated with the attP40 insertion to determine if this gene is involved in other noxious modalities. Larvae were prepared in the same manner as described above in the thermal assay. In the mechanical assay (Figure 10), for the UAS-only control for BDSC #57040, the proportion of larvae that responded to the stimulus 57%, and the UAS-only control for BDSC #61206 had a percent response of 69%; each were compared to the GAL4-only control which had a percent response of 55%. Neither UAS-only control had a significantly different response rate to the harsh mechanical stimulus in comparison to the GAL4-only control. The percent response after the first stimulus for the *otk2* RNAi knockdown BDSC #54070 was 60%, and the percent response after the first stimulus for the *otk2* RNAi knockdown BDSC #61206 was 56%; each were compared to the GAL4-only control which had a percent response of 55%. The p-values for the two *otk2* RNAi knockdown genotypes were 0.474 and 0.887, respectively. Since the *otk2* knockdown larvae did not respond significantly differently than wild-type larvae in the mechanical assay, it suggests that *otk2* does not play a role in mechanical nociception.

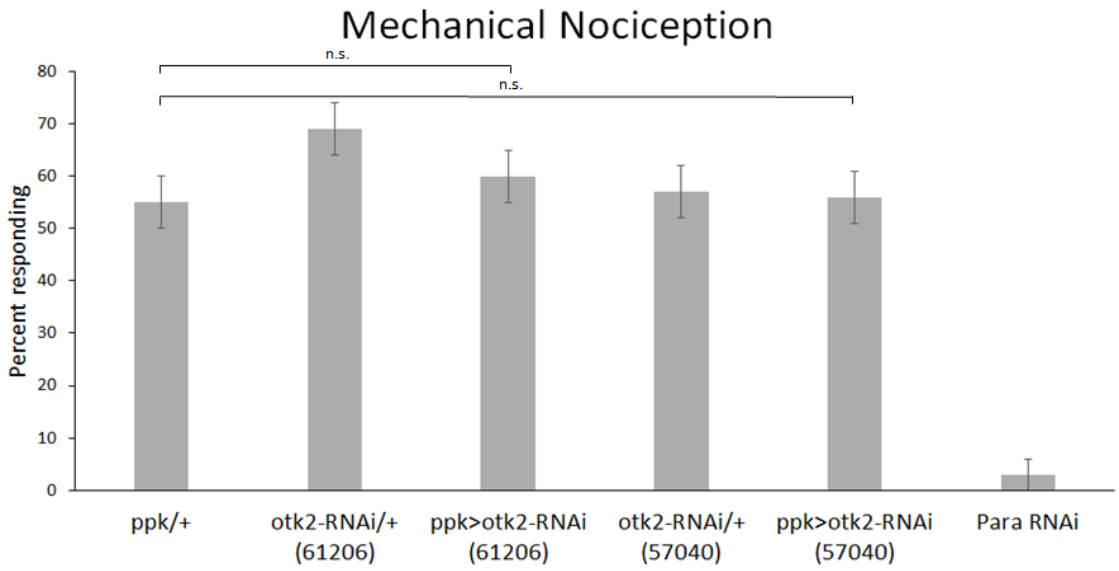

Figure 10. Nociceptor-specific knockdown of *otk2* does not cause defects in mechanical nociception. The proportion of larvae with nociceptor-specific knockdown of *otk2* did not exhibit significantly different nociceptive responses to a noxious mechanical stimulus than GAL4-only control larvae. Larvae with nociceptor-specific knockdown of *para* showed a very low rate of nociceptive responses and were used as a positive control (n = 100 for all groups; $p>0.025$ by Chi-Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

To provide another confirmation of the behavioral phenotype shown with RNAi knockdown, both thermal and mechanical assays were conducted on an *otk2*^{C26} mutant. *otk2*^{C26} is a loss of function mutant where the genomic region of the *otk2* sequence is removed. It was shown that there is no *otk2* protein expression in this line [79]. In the thermal nociception assay, the homozygous mutant had a significantly higher latency to respond to a thermal stimulus than *w*^{1118} control (Figure 11A). The homozygous mutant knockout had a mean latency of 5.3 seconds, while the *w*^{1118} control had a mean latency of 1.8 seconds. The p-value for the homozygous mutant was less than 0.001. In the mechanical assay, the homozygous mutant did not have a significantly different response rate in comparison to the *w*^{1118} control (Figure 11B).. The homozygous mutant knockout had a percent response of 77%, and the *w*^{1118} control had a percent response of 67%. The p-value for the homozygous mutant was 0.115. Both assays confirm the RNAi knockdown phenotype since they reflect similar results. Put together, this data shows that *otk2* acts in nociceptors to regulate thermal nociception, but not mechanical nociception.

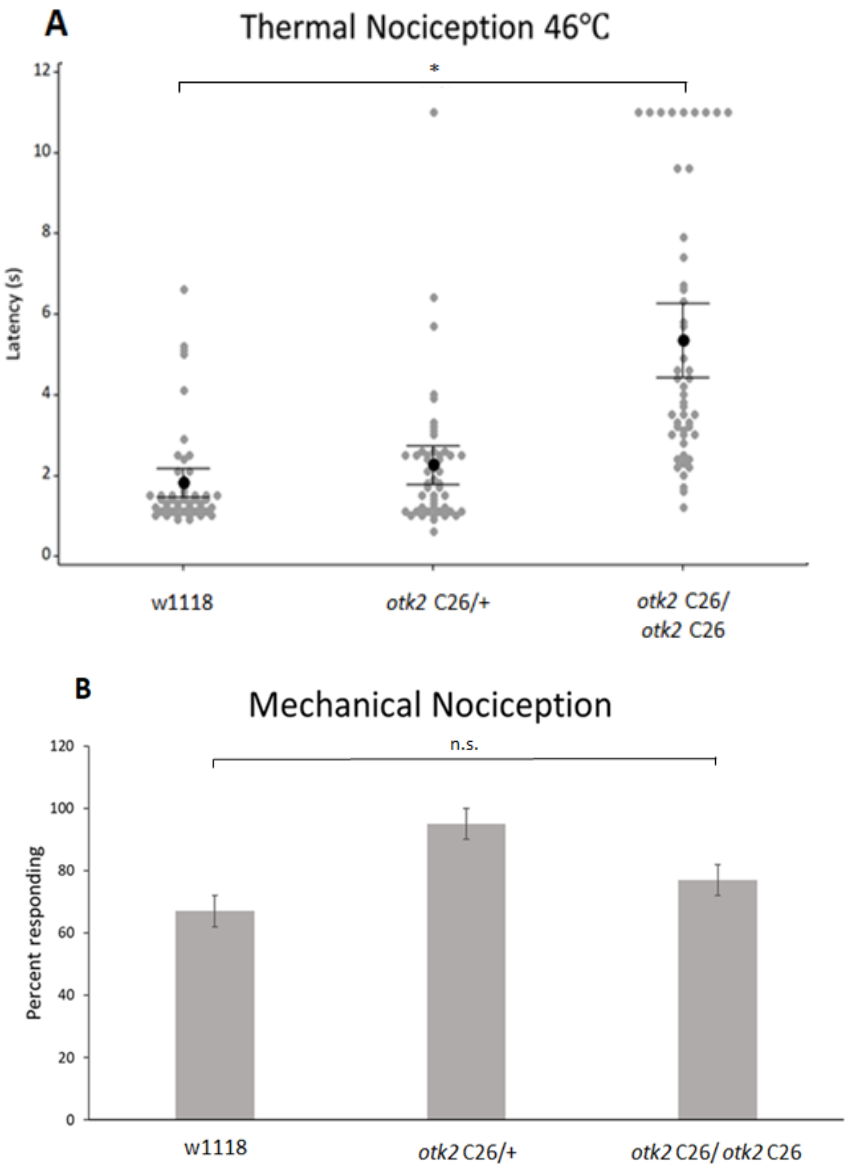

Figure 11. Mutant knockout of *otk2* causes defects in thermal nociception but not mechanical nociception. (A) Larvae with homozygous mutant knockout of *otk2* showed a significantly longer latency to respond to a noxious thermal stimulus of 46°C than did control larvae. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated with a black circle (n = 50 for all groups; *$p < 0.001$ by Mann Whitney U Test). (B) The proportion of larvae with homozygous mutant knockout of *otk2* did not exhibit significantly different nociceptive responses to a noxious mechanical stimulus than control larvae. (n = 100 for all groups; p>0.025 analyzed by Chi-Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

otk2 is required for normal nociceptor dendrite morphogenesis

otk2 is hypothesized to activate the PCP Wnt signaling pathway which regulates cytoskeletal rearrangements. In order to test the hypothesis that *otk2* plays a role in neuronal development, an *otk2* RNAi line (BDSC #61206) was crossed with *w; ppk1.9-GAL4, UAS-mCD8::GFP; UAS-dicer2* to induce a tissue-specific knockdown of *otk2* with simultaneous expression of a green fluorescent protein in the larval nociceptors for visualization and imaging using a Laser Scanning Confocal Microscope. Representative images of a wild-type neuron and an *otk2* RNAi knockdown neuron (Figure 12A and 12B) are shown. The neighboring neurons in the confocal micrographs were traced with red or blue to give better representation of body wall coverage (Figure 12A' and 12B'). The wild-type neuron schematic (Figure 12A') shows full dendritic coverage of the body wall, and there is little to no overlap of dendrites of the same or neighboring neurons. This gives a clear visualization of the concept of dendritic tiling. The schematic of the neuron expressing *otk2* RNAi knockdown (Figure 12B') shows a defect in tiling. There appears to be less coverage of the body wall with dendrites, and there is visible overlap between the dendrites of neighboring neurons.

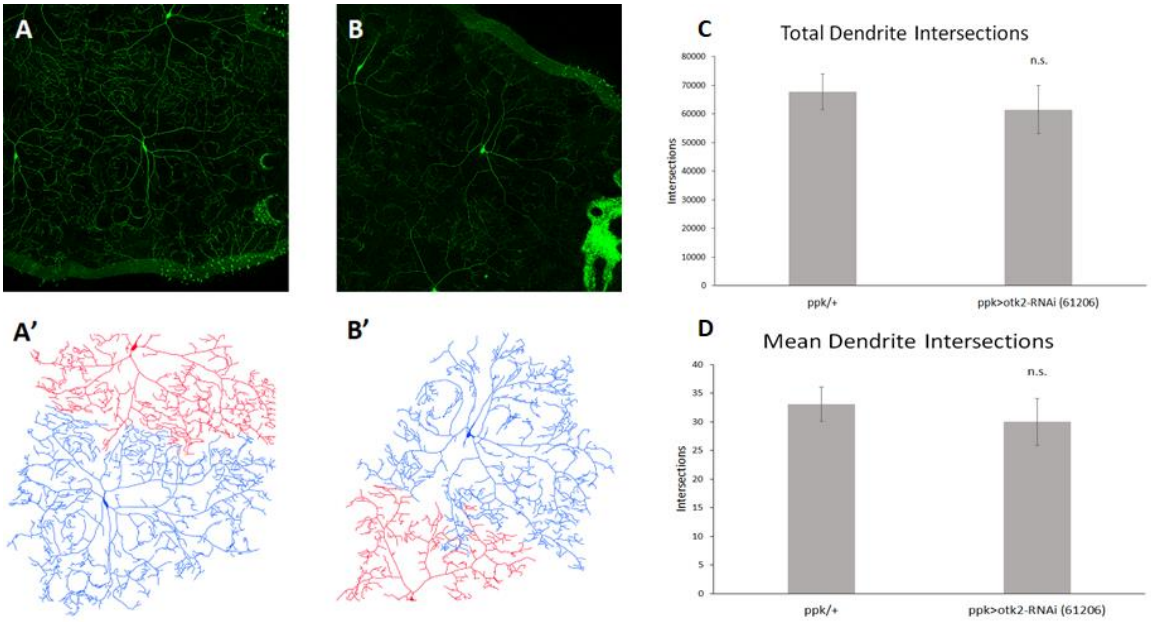

Figure 12. Nociceptor-specific knockdown of *otk2* does not cause changes in mdIV neuron dendrite complexity but causes a defect in dendritic tiling. (A, B) Confocal micrographs displaying the dendritic arborization of ddaC mdIV neurons expressing mCD8::GFP. Panel A is a representative image of a wild-type neuron, and panel B is a representative image of a neuron expressing the *otk2* RNAi. (A', B') Diagrams of neurons from the respective panels where each of the neighboring neurons is colored in red or blue. (C) The total number of intersections between a concentric circle and ddaC dendrites with *otk2* knockdown were not significantly different (by Student's *t*-test) from those of no-RNAi control ddaC neurons. (E) The mean number of intersections with the concentric circles and ddaC dendrites with *otk2* knockdown were not significantly different (by Student's *t*-test) from those of no-RNAi control ddaC neurons. Bars indicate mean total dendrite length, while error bars indicate standard error of the mean (n = 6 neurons for each group).

In an attempt to quantify the developmental defect, a Sholl Analysis was conducted on the wild-type neurons and the *otk2* RNAi knockdown neurons. The analysis showed that there was no significant difference between the two genotypes in either total dendrite intersections with the concentric circles nor in the mean dendrite intersections with the concentric circles (Figure 12C and 12D). This data put together with the visual schematic suggests that *otk2* knockdown is affecting a tiling mechanism instead of simply altering complexity of neurons.

fz2 is required for normal sensitivity to noxious thermal and mechanical stimuli but fz is not required for noxious stimuli

In order to test the hypothesis that *otk2* and a member of the Frizzled family are functionally coupled, a *fz* or *fz2* RNAi line was crossed with *w; ppk1.9-GAL4; UAS-dicer2* to induce a tissue-specific knock down of either gene, and the first-generation larvae were tested in a thermal behavioral assay for sensitivity to noxious stimuli. An initial thermal assay was conducted at 42°C since the behavioral phenotype of these genes was expected to be hypersensitive. At a slightly lower temperature, the negative control is expected to have a higher latency which would allow a faster response to be quantified easier. In the 42°C assay (Figure 13), neither of the *fz* RNAi knockdown lines had a significantly different latency in comparison to the GAL4-only control. The mean latency of the *fz* RNAi knockdown BDSC# 34321 was 9.6 seconds, and the mean latency of the *fz* RNAi knockdown BDSC# 31311 was 9.2 seconds; each was compared to the GAL4-only control which had a mean latency of 9.7 seconds. The p-values for the *fz* RNAi knockdown genotypes were 0.896 and 0.358, respectively. The *fz2* RNAi knockdown line had a significantly shorter latency in comparison to the GAL4-only control. The mean latency of the *fz2* RNAi knockdown BDSC# 27568 was

8.3 seconds and was compared to the GAL4-only control which had a mean latency of 9.7 seconds. The p-value was 0.013.

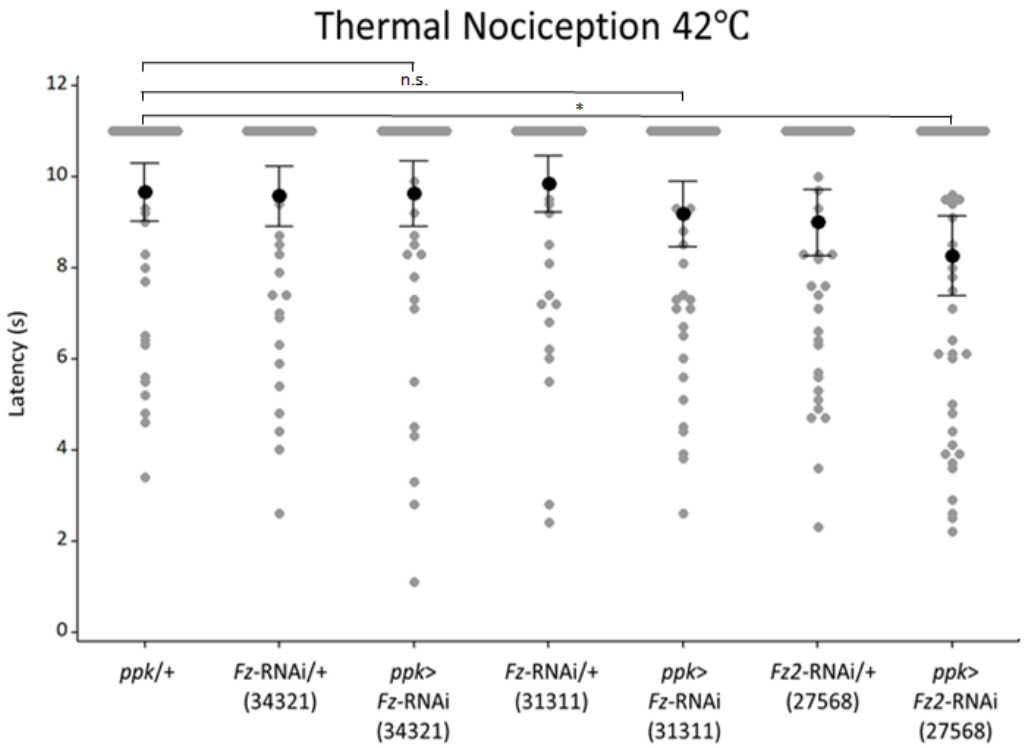

Figure 13. Initial thermal analysis at 42°C shows that nociceptor-specific knockdown of *fz* does not cause defects in thermal nociception, but there is an effect with nociceptor-specific knockdown of *fz2*. Larvae with nociceptor-specific knockdown of *fz* did not show a significantly different latency in response to a noxious thermal stimulus of 42°C in comparison to GAL4-only controls. On the other hand, larvae with nociceptor-specific knockdown of *fz2* showed a significantly shorter latency in response to a noxious thermal stimulus of 42°C in comparison to GAL4-only controls. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated with a black circle (n = 50 for all groups; *p = 0.013 by Mann Whitney U Test).

Since there was a behavioral phenotype for *fz2* knockdown but not *fz* knockdown in the thermal nociception assay at 42˚C, thermal assays at 46˚C and mechanical assays were conducted on the individual genes to confirm the results from the 42˚C assay. There was no significant difference in latency between the *fz* RNAi knockdown and the GAL4-only control for the thermal assay (Figure 14A). The mean latency of BDSC #31311 was 3.8 seconds, and it was compared to the GAL4-only control which had a latency of 4.1 seconds. The p-value was 0.783. There was also no significant difference in response rate when comparing the *fz* RNAi knockdown to the GAL4-only control in the mechanical assay (Figure 14B). The percent response of BDSC #31311 was 71% and was compared to the GAL4-only control which had a percent response of 67%. The p-value was 0.541.

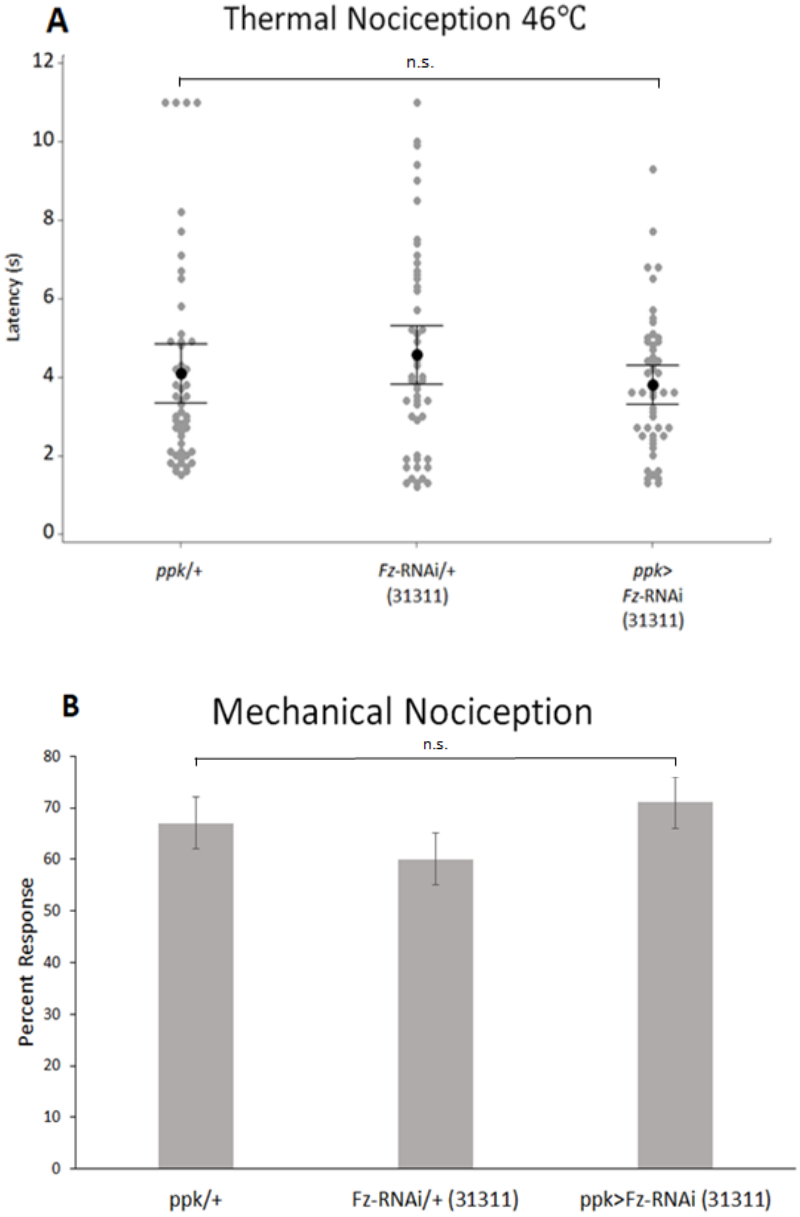

Figure 14. Nociceptor-specific knockdown of *fz* does not cause defects in thermal or mechanical nociception. (A) Larvae with nociceptor-specific knockdown of *fz* did not show a significantly different latency in response to a noxious thermal stimulus of 46°C in comparison to GAL4-only controls. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated with a black circle (n = 50 for all groups; p> 0.025 by Mann Whitney U Test). (B) The proportion of larvae with nociceptor-specific knockdown of *fz* did not exhibit significantly different nociceptive responses to a noxious mechanical stimulus than GAL4-only control larvae. (n = 100 for all groups; p>0.025 analyzed by Chi-Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

Molecular cloning

Molecular cloning was conducted to construct a plasmid containing fusion between *otk2* cDNAand the gene encoding a Venus Fluorescent Protein, VFP. The plasmid was generated using the pTVW vector. After the LR Clonase reaction was completed, I used the *Kpn1* restriction digest enzyme to cut the plasmid, which I named pABW1b. *Kpn1* cut once in the *otk2* sequence, and it cut a second time in the sequence of the gene encoding the VFP. If both sequences were inserted as expected, I anticipated two DNA fragments. One fragment was expected to be 9,313 base pairs, and the second fragment should be 1,875 bases. I performed gel electrophoresis on the pABW1b digest and imaged the gel (Figure 15). The second lane of the gel shows the pABW1b digest, and there are two bands at approximately 9,313 bases and 1,875 bases, as expected. This gives confirmation that *otk2* was inserted appropriately. The next step is to have the pABW1b plasmid sequenced by Eton Biosciences to confirm that there are no mutations in the *otk2* DNA. The pABW1b plasmid can then be injected into a *Drosophila* embryo to construct a fly line expressing an Otk2 receptor fused to a VFP to be used for cellular localization experiments.

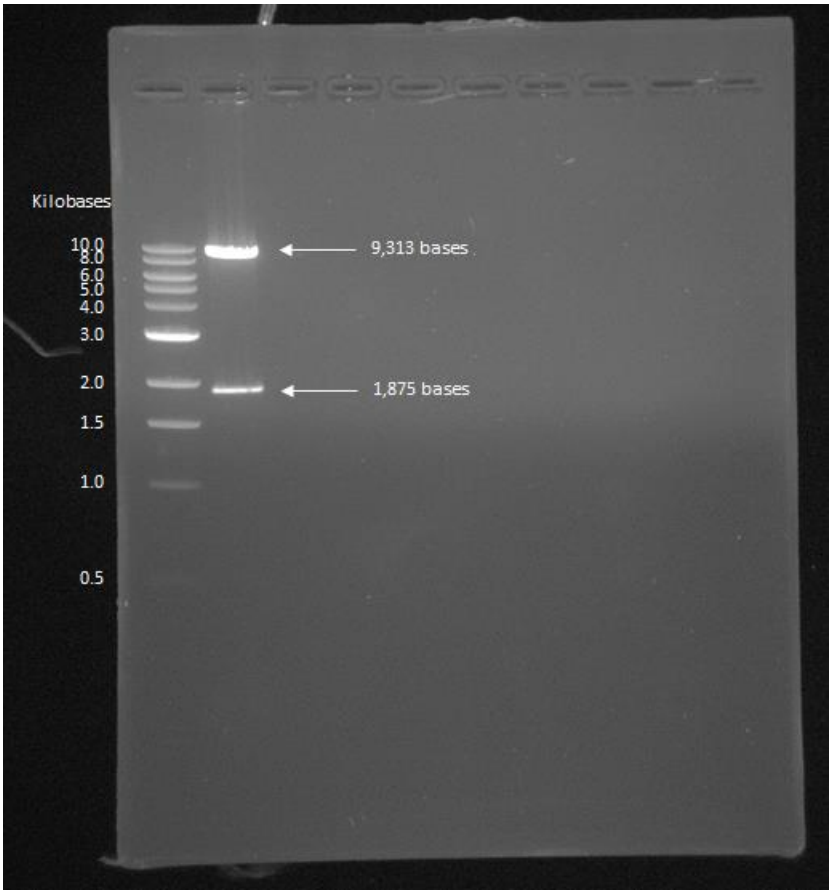

Figure 15. Gel image of a restriction digest of the pABW1b plasmid. The *Kpn1* restriction digest enzyme cut the plasmid at two points in the plasmid. The two fragments that were expected after the digest were 9,313 base pairs and 1,875 base pairs which is confirmed in the second lane.

Discussion

Numerous genetic and environmental perturbations of the Wnt pathways can lead to a large number of human disorders ranging from birth defects to cancers [50]. While elements of Wnt signaling pathways have been identified and examined, the mechanism of how each Wnt signaling pathway regulates nociceptor sensitivity has yet to be fully uncovered [81]. Understanding the basis of human diseases and neuropathic pain conditions resulting from Wnt signaling and further designing therapies targeting aspects of Wnt signaling requires a detailed understanding of the components of each pathway, including the molecular mechanism of the downstream effectors, and how each component could be used in pharmaceutical targeting [50].

Protein Tyrosine Kinase 7 (PTK7) has been shown to control the planar cell polarity pathway in vertebrate models [79]. It has been confirmed that an increase in tissue damage causes an up-regulation of Wnt signaling resulting in more long-term pain sensation, which is dependent on the PCP and Wnt/Ca^{2+} Wnt signaling pathways [64]. For these reasons, my project was designed to uncover more information about the *Drosophila* homolog of PTK7, *off-track 2 (otk2)*. In this study, I conducted experiments to show the *otk2* and *fz2* regulate basal sensitivity of *Drosophila* class IV multidendritic neurons to thermal and mechanical stimuli, possibly through modulating Class IV md neuron dendrite development.

My experimental data showed that *otk2* is required for thermal nociception since larvae expressing RNAi knockdown of *otk2* were less sensitive to the noxious thermal stimulus. Larvae expressing RNAi knockdown of *otk2* showed no defect in sensation of a mechanical stimulus. The results from the *otk2* RNAi knockdown experiments were confirmed with the otk^{C26} mutants which were less sensitive to noxious thermal stimuli but

not mechanical. Since *otk2* is suggested to be a co-receptor to a Frizzled receptor, I conducted behavioral assays on either *fz* or *fz2* RNAi knockdown larvae. I showed that *fz* is not involved in either thermal or mechanical nociception while *fz2* knockdown resulted in a hypersensitive phenotype in a thermal behavioral assay. I did not have enough time to complete a mechanical behavioral assay on *fz2* RNAi knockdown larvae, so that should be the next step in future directions. I anticipate that *fz2* knockdown will result in hypersensitivity to noxious mechanical stimuli.

Since *otk2* and *fz2* have opposite loss of function behavioral phenotypes to noxious thermal stimuli, I hypothesize that these two receptors are functionally coupled where one protein inhibits the function of the other to regulate the sensitivity of thermal nociceptors. This is further supported by an experiment that showed that Otk2 and Fz2 coimmunoprecipitate together, indicating they interact in the cell [82]. It was also shown that a PCP co-receptor, ROR, interacts with Fz2 and Otk2 [82]. It may be possible that Otk2 binds to Fz2 to regulate thermal nociception while ROR binds to Fz2 in mechanical nociception (Figure 16). Thermal and mechanical behavioral assays on ROR knockdown larvae could be used to test this hypothesis. If it is shown that *ROR* RNAi knockdown does not cause a defect in mechanical nociception, it may be possible that this receptor complex may simply regulate thermal nociception and a different mechanism or signaling pathway regulates mechanical nociception.

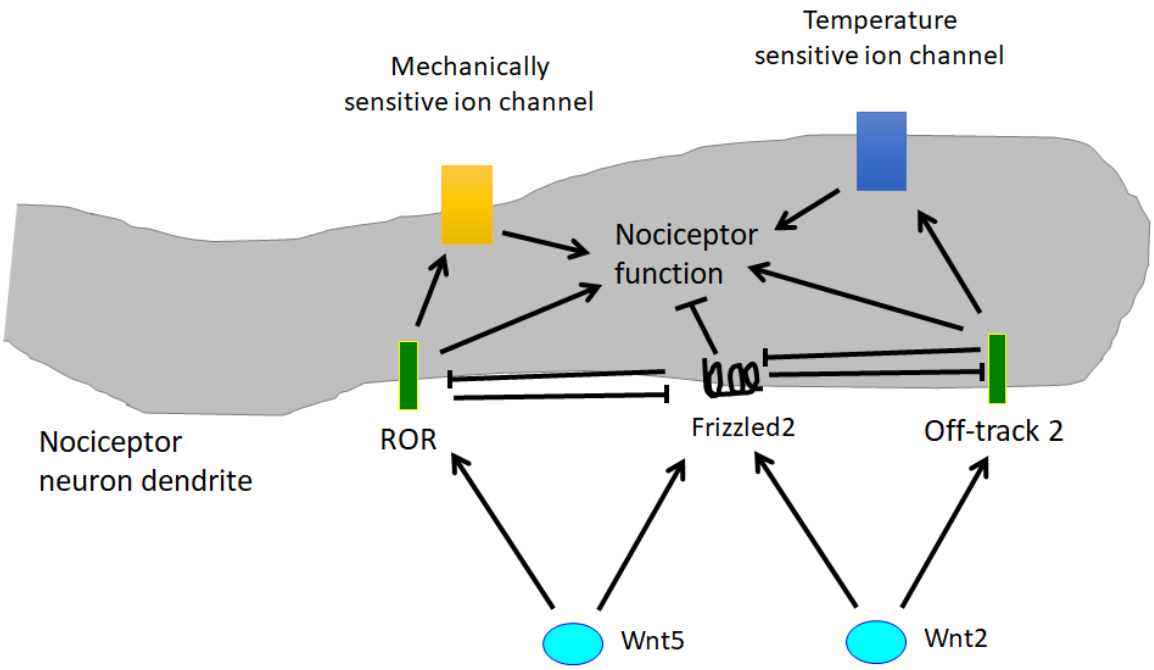

Figure 16. Proposed model for a receptor complex of Otk2, ROR, and Fz2 adapted from Freeman [78]. Fz2 and Otk2 may act on temperature sensitive ion channels regulating thermal nociception while ROR and Fz2 may modulate mechanically sensitive ion channels regulating mechanical nociception.

The Planar Cell Polarity pathway diverges into the Rac-JNK arm and the RhoA-ROCK arm. It has been shown that the Rac-JNK signaling arm contributes to Wnt3a-induced mechanical hypersensitivity, but not thermal hypersensitivity. The RhoA-ROCK arm had no effect on either thermal or mechanical nociception [30]. My data shows that thermal nociception, but not mechanical nociception, is regulated by Otk2, which does not come to the same conclusion on the two arms of PCP signaling that has been previously published [30]. This suggests that nociceptor modulation by Otk2 may not be through PCP activity, assuming this link is evolutionarily conserved.

I originally hypothesized that Wnt3a is the Wnt ligand that binds to the receptor complex involving Otk2, since Wnt3a has been shown to have an effect on nociceptor sensitivity in mice. It has been shown that receptor localization of ROR on the surface of the cell body of nociceptors [82]. Since ROR and Wnt5a both are localized to the cell body and both co-immunoprecipitated with Otk2 [82], it is more likely that Wnt5a is the binding ligand to the receptor complex containing Otk2. In addition, Wnt2 was shown to coimmunoprecipitate with Otk2 [79]. Put together, I hypothesize that Wnt2 binds to Frizzled2 and Otk2 in the complex, and Wnt5a binds to the ROR and Frizzled 2 portion of the complex. This suggests that Otk2 is most likely localized to the nociceptor cell bodies along with ROR and Wnt5a. Tagging the Otk2 receptor with a GFP and conducting fluorescent microscopy would help determine where the Otk2 receptor is localized in the neuron, and it would also show co-localization of Otk2 with other receptors.

To understand more about the activity of Wnt5a, it has been found that Wnt-5a signaling led to the activation of Protein Kinase C and CaMKII while there was no

significant JNK activation was detected [83]. This suggests that Wnt5a binds to a receptor complex and is activating the Wnt/Ca^{2+} pathway and not the PCP pathway. A follow-up experiment showed that Wnt5a works through activating the Ca^{2+} pathway to increase electrical excitability of neurons [84]. This provides a possible mechanism for the role of Otk2 on nociceptor sensitivity. Instead of working the PCP as initially hypothesized, it could bind Wnt5a and activate the Ca^{2+}-dependent pathway which would regulate TRPA1 activity through the G protein signaling cascade.

Another hypothesis could be that instead of simply activating one of the non-canonical pathways to regulate nociception, the Otk2/ROR/Fz2 receptor complex is using a portion of or whole branches of a non-canonical pathway to inhibit the canonical pathway. It has been shown that Wnt5a results in β-catenin degradation independently of downstream effectors of canonical signaling like GSK-3. This suggests that Wnt5a somehow acts through a noncanonical pathway to inhibit canonical activity [83]. It has been shown that loss of PTK7 function activates canonical Wnt signaling which results in PTK7 opposing canonical Wnt signaling in embryonic patterning. A model was proposed where PTK7/Otk2 functions in non-canonical Wnt signaling by turning off the canonical signaling branch [85], which would be consistent with the opposite phenotypes shown in *otk2* and *fz2* knockdown.

We do not fully understand which Wnt ligands and receptors are activating or inhibiting which pathway and how each of the three Wnt pathways work together. In an effort to uncover more about the Otk2/ROR/Fz2 receptor complex, a proteomics experiment on the downstream effectors of each pathway should be conducted, which would tell us which pathways are being activated. A future experiment would be to knockdown *otk2* in nociceptors and look at changes in expression of key downstream effectors in each pathway.

An efficient way to determine effector activity would be to express a fluorescent reporter of important downstream effectors in each Wnt signaling pathway. Important targets would be β-catenin for canonical signaling, Rac or ROCK for each arm of the PCP pathway, and calcium signaling for the Wnt/Ca^{2+} pathway. The decrease or increase in fluorescence in any of these downstream effectors after *otk2* RNAi knockdown would give an idea of which pathways are being affected by Otk2 activity.

Since there is so much controversy in literature about the activity and effects of different ligands and receptor complexes in Wnt signaling, I wanted to understand more about how Otk2 may be regulating sensory neurons. It has been shown that Ryk, another non-canonical Wnt receptor, promoted mouse dendrite development through Wnt5a. With Ryk knockdown, Wnt5a expression inhibited dendrite development; when Ryk is present, dendrites develop normally. This shows that Wnt5a-induced activation of Ryk regulates dendrite growth [86]. This study provided evidence to support my hypothesis that Otk2 regulates sensory neurons using a similar mechanism since they are both non-canonical receptors.

My experimental data showed that there is no significant difference in dendrite complexity of nociceptors expressing *otk2* knockdown in comparison to wild-type nociceptors. In another developmental analysis, I picked a representative image of each of the two genotypes and traced the two neighboring neurons in the segment in either red or blue. The schematic showed a tiling defect in *otk2* knockdown where the dendrites crossed the larval body midline and overlapped with dendrites of the neighboring neurons in the body segment. There seemed to be less of an ability of dendrites to sense neighboring dendrites and inhibit growth as seen in normally functioning tiling mechanisms. It has been shown that

Wnt5a expressed as a gradient along the anterior-posterior axis repelled axon and dendrite growth down the mouse spinal cord [87]. This could provide a possible explanation as to why there appeared to be a tiling defect with *otk2* knockdown. It is possible that Wnt5a binds to a receptor complex containing Otk2 which inhibits dendrite growth, and in the absence of Otk2, it grows past normal boundaries as shown in the experimental data of this study.

My study provided information that has the potential to tie aspects of previous literature together. The behavioral assays provided support for the role of Otk2 and Fz2 in thermal nociception, and the morphology micrographs gave evidence for the activity of Otk2 in neuronal function. In order to establish a connection between the mechanism by which Otk2 regulates thermal nociception and neuron development, more experiments must be conducted.

An important future direction is uncovering the localization of Otk2 in the neuron. This will provide more information as to how *otk2* regulates neurons. If it is localized to the cell body like ROR, it is possible it regulates gene transcription through canonical signaling; if it is localized more to the dendrites, it could be regulating cytoskeletal rearrangements or function in dendrite recognition and guidance. Since it was suggested that ROR may function with Otk2 and Fz2, conducting behavioral assays on ROR knockdown larvae will help uncover more information about the receptor complex. Since Otk2 only functions in thermal and not mechanical nociception, it is possible that ROR will oppositely have an effect on mechanical and not thermal nociception. This would suggest that *otk2* and *fz2* regulate thermal nociception while *ROR* and *fz2* regulate mechanical nociception. A future direction is to conduct thermal and mechanical behavioral assays on ROR RNAi knockdown larvae. Finally, in order to understand how this receptor functions, a proteomics experiment on the

downstream effectors after *otk2* inhibition would determine which of the Wnt pathways this receptor activates.

There is differing evidence as to how noncanonical Wnt co-receptors act to regulate neuron function. Using a GCaMP calcium indicator could determine if there is a change in electrical excitability in the neurons caused by Otk2. This could determine if Otk2 is acting through the Ca^{2+}-dependent pathway. Finally, looking at changes in expression of downstream proteins in each of the three pathways could determine which pathways, and to what extent, Otk2 works to regulate nociceptor sensitivity. These are just a few of the many future experiments that should be conducted on Otk2. Establishing how Otk2 is integrated with Fz2 will greatly expand our understanding of the basic molecular mechanisms regulating neuron function and dendrite growth and guidance. The end results may suggest strategies with which to regulate neuropathic pain conditions after injury or in neurodegenerative disease.

Chapter 4: Conclusions and Future Implications

Each of these projects gives a snapshot of two types of signaling pathways that regulate sensory neuron sensitivity. The G protein signaling cascade is thought to modulate TRP channel activity which would short-term regulate nociceptor sensitivity. Wnt signaling regulates gene transcription and cytoskeletal rearrangements in sensory neurons to provide long-term modulation of nociceptor sensitivity. The Wnt-Ca^{2+} pathway is an intriguing overlap between these two pathways, since Wnt signaling mechanisms are used to activate the G protein signaling cascade. This connection could be a possible avenue of research to determine if there is a way to target the G protein signaling cascade through use of Wnt signaling. This has the potential to combine the specificity of Wnt signaling with the short-term regulation of TRP channels through G protein signaling in one clinical intervention to maintain nociceptor sensitivity and treat chronic pain. Before that can happen, more has to be uncovered about how these pathways function.

In Chapter 2, we demonstrated that Gαq and PLCβ both act to increase the sensitivity of sensory neurons since loss of function of these genes in *Drosophila* larvae resulted in insensitivity to noxious thermal and mechanical stimuli. This supports the hypothesis that G proteins may act to regulate TRP channels through second messengers. A future direction would be to look at calcium influx using a GCaMP calcium indicator. GCaMP is a molecular sensor that utilizes a GFP with a Ca^{2+} binding domain. When Ca^{2+} binds to the domain, it triggers activity of GFP, and the fluorescent signal can be picked up with fluorescent microscopy [47]. We could stimulate wild-type larvae and *Gαq* or *norpA* RNAi knockdown larvae with a noxious stimulus and determine if there is a change in calcium signal in the loss of function mutants.

As suggested in Chapter 2, it could be a potential mechanism of therapeutic intervention to target Gαq and PLCβ. Since it would be challenging to target Gαq and PLCβ exclusively in the nociceptors with a drug, it would be beneficial to uncover the specific GPCR regulating the G protein signaling cascade in nociceptors. A *Drosophila* thermal genetic screen of the 40 GPCRs that have been shown to regulate pain sensation in mammalian models could begin the process of uncovering which GPCRs tissue specifically regulate nociceptors. This future direction would solve the issue of tissue specificity required of clinical intervention to treat neuropathic pain conditions.

In Chapter 3, I demonstrated that Otk2 normally acts to increase the sensitivity of nociceptors since loss of function knockdown larvae displayed an insensitive phenotype in a thermal nociception assay. On the other hand, *otk2* knockdown had no effect on mechanical nociception. It was also shown that *otk2* knockdown nociceptors displayed a tiling defect in the dendrites of the nociceptors. This is potentially possible through a role Otk2 may have in either cytoskeletal rearrangements or inhibiting gene transcription of key genes that regulate tiling mechanisms. Frizzled2 could be functionally coupled with Otk2 since it was shown to regulate thermal nociception as well. An interesting aspect of the results is that Otk2 normally acts to increase the sensitivity of nociceptors, while Frizzled2 decreases the sensitivity of nociceptors since *fz2* knockdown larvae displayed a hypertensive phenotype. It has also previously been shown that Otk2 and Frizzled2 coimmunoprecipitate together [82]. Since the knockdowns display opposite phenotypes and coimmunoprecipitate, it could suggest that they both work together in a receptor complex in an inhibitory interaction. This interaction could be further studied with an epistasis experiment which would determine which receptor is downstream of the other. An epistatic experiment would be to knock down

both genes at the same time and conduct a thermal behavioral assay. The resulting phenotype displayed by the larvae would suggest which receptor is upstream of the other. If the larvae displayed a hypersensitive phenotype after knocking down both genes, then it would provide evidence that *otk2* is upstream of *fz2*, and vice versa.

Understanding more about the function of the Wnt receptor complexes could extend into treating neuropathic pain conditions caused by more than just injury. Experiments have been conducted attempting to understand the role of cancer in nociceptor regulation. Bone cancer cells were injected into mice, and the mice had an increased sensitivity to mechanical noxious stimuli. In a follow-up experiment, the mice with bone cancer were used as a model, and they were injected with neutralizing antibodies against Wnt3a or Wnt5a in the vicinity of the cancer. Inhibition of Wnt3a, but not Wnt5a significantly reduced the mechanical hypersensitivity caused by the cancer [30]. This study showed that cancer cells may be either causing nearby cells to release Wnt proteins or may be releasing Wnt proteins themselves. Either way, this provides insight into how cancer cells may cause hypersensitivity. In the case of cancer, medical doctors could potentially use neutralizing antibodies against Wnt ligands to treat cancer-induced chronic pain or even target the associated receptor complex. Reports suggest that more than 50% of patients with cancer experience moderate to severe pain [88], so neutralizing Wnt ligands could be a promising direction for this significant portion of cancer patients.

The results from the cancer-induced nociceptive hypersensitivity experiment give reason to further investigate the function and mechanisms of Otk2 and Frizzled2. Targeting this receptor complex could be a new therapeutic target to treat tumor-induced hypersensitivity and chronic pain. Experiments should be conducted to further provide

evidence that these two receptors are functionally coupled and to understand how these receptors are regulating nociceptor function. What are the downstream effectors of this receptor complex, and what Wnt ligands activate this pathway? Answering these questions are of utmost importance in order to overcome the obstacle of the opioid epidemic and to increase quality of life among patients suffering for chronic pain and other neuropathic pain conditions.

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Vita

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