PVR, A GROWTH FACTOR RECEPTOR, PLAYS A ROLE IN THERMAL NOCICEPTION

A Thesis
by
ISHANI CHATTOPADHAYAY

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

May 2023
Department of Biology

PVR, A GROWTH FACTOR RECEPTOR, PLAYS A ROLE IN THERMAL NOCICEPTION

A Thesis
by
ISHANI CHATTOPADHAYAY
May 2023

APPROVED BY:

Andrew Bellemer, Ph.D
Chairperson, Thesis Committee

Darren Seals, Ph.D
Member, Thesis Committee

Mark Venable, Ph.D
Member, Thesis Committee

Ava Udvadia, Ph.D
Chairperson, Department of Biology

Marie Hoepfl, Ed.D.
Interim Dean, Cratis D. Williams School of Graduate Studies

Abstract

PVR, A GROWTH FACTOR RECEPTOR, PLAYS A ROLE IN THERMAL NOCICEPTION

Ishani Chattopadhayay
B.S., Appalachian State University
M.S., Appalachian State University

Chairperson: Dr. Andrew Bellemer

Across the globe, chronic pain affects approximately 1.5 billion people. In the United States, it costs approximately $600 billion dollars every year in lost labor, treatments, and healthcare. Therefore, it is important to identify and characterize the molecular pathways and signaling molecules involved in pain to enhance our understanding of why chronic pain occurs and how we can improve the existing treatments. This research project aims to contribute to this goal by determining the role Pvr, a growth factor receptor, plays in thermal nociception in *Drosophila melanogaster,* commonly known as the fruit fly. *Drosophila* is used in this project as they are amenable to transgenesis, allowing for modification of gene function; and they have been widely used for studying nociception. Previous experiments have established that Pvr is required for regulating mechanical nociception; however, there have not been any studies detailing the role of Pvr in thermal nociception. This project aims to knock down and overexpress Pvr in the multidendritic neurons of *Drosophila* and assess the effects produced on thermal nociception. The results of the thermal nociception assay show that knockdown and dominant negative expression of Pvr produce a significant defect in thermal nociception. Moreover, Pvr overexpression and constitutive activation of Pvr caused increased sensitivity to noxious stimuli. The results of this assay showed that Pvr is necessary

and sufficient to promote sensitivity of the neurons to thermal stimuli. This study establishes a novel role for a conserved signaling pathway, Pvf/Pvr in regulating thermal nociception. The results of this study indicate overlap in mechanisms that regulate thermal and mechanical nociception.

Acknowledgments

First and foremost, I would like to thank Dr. Andrew Bellemer for being an amazing mentor and advisor. I am grateful for the opportunity that I was presented with to be a part of the Bellemer lab in my undergraduate and graduate career. Thank you for your guidance, support and endless patience towards me and this project. Thank you for sharing your knowledge and experiences with me and providing me with opportunities that have helped me grow not only as a scientist but also as a person. I would like to thank my committee members, Dr. Darren Seals, and Dr. Mark Venable, for being such amazing professors. Thank you for all your guidance, support, and input that you have provided towards this project. I would also like to thank all the past and present members of the Bellemer lab. I am grateful for having such amazing lab mates that have supported me throughout my undergraduate and graduate career. Thank you for the endless support, feedback, and friendships that you have provided me. I would also like to thank my family and friends who have been nothing short of patient while listening to me practice and go on about my research. I would like to thank the Office of Student Research, the Graduate Student Government Association, and the Cratis D. Williams Graduate school for funding my research. Lastly, I would like to thank the Department of Biology for the amazing community it has provided me with and the wonderful people from whom I have had the opportunity to learn.

Dedication

I would like to dedicate this thesis to my family and friends.

Thank you for the endless support

Table of Contents

List of Tables

List of Figures

Introduction

Chronic Pain

Chronic pain is a prevalent global health concern that affects approximately 1.5 billion people. It is defined as pain that persists beyond normal tissue healing time of approximately three months (Mills et al., 2019). Chronic pain is very common and significantly impacts an individual's quality of life, as it is associated with severe physiological discomfort, functional limitations, and debilitation. In America, healthcare for chronic pain costs billions of dollars, creating a financial burden for most individuals dealing with this disease. There are many treatments available for chronic pain, however many individuals use opioids for long-term pain management. These opioid treatments have led to opioid addiction, overdoses, and an increase in additional treatments. Despite the numerous treatment options available, there are none that have proven to be reliably effective for long term pain management (Rosenblum et al., 2008).

There are several types of pain that individuals can experience, including neuropathic, nociceptive, musculoskeletal, inflammatory, psychogenic, and mechanical. Neuropathic pain is caused by damage or alteration to the nervous system, nociceptive pain is caused by tissue injuries, musculoskeletal pain is caused by injuries to the musculoskeletal system such as fractures (El-Tallawy et al., 2021), inflammatory pain can arise from infections and autoimmune disorders such as rheumatoid arthritis, psychogenic pain is caused by psychologic factors such as headaches or pain caused by emotional and behavioral factors, and mechanical pain is usually caused by stress on bones, discs, or nerves of the spinal column (Will et al., 2018; Dydyk et al., 2023).

Nociception

Nociception is the nervous system's physiological and behavioral response to noxious stimuli. Noxious stimuli can be classified as stimuli that cause or may cause tissue damage and can be categorized as thermal, mechanical, UV radiation, or chemical. Harmful stimuli are detected by specialized sensory neurons called nociceptors (Dubin & Patapoutian, 2010). Nociceptors have an activation threshold, and a stimulus needs to reach that threshold in order to activate the nociceptor, indicating that they are selective and only respond to potentially tissue-damaging stimuli. In vertabrates, nociceptors can be divided into two classes: Aδ fibers and C fibers. Aδ fibers are myelinated medium diameter afferents that mediate acute and well localized pain. Aδ fiber axons are present in nociceptors that detect noxious thermal and mechanical stimulus and transmit action potentials at ~ 5-30 m/s. Class C fibers are unmyelinated small diameter fibers that mediate poorly localized pain. Most Class C fibers act as polymodal nociceptors and are sensitive to both heat and mechanical stimuli as well as other types of noxious stimuli (Basbaum et al., 2009). These nociceptors transmit action potentials at ~ 1 m/s. Nociceptors carry nociceptive signals into the central nervous system through their projection onto the dorsal horn of the spinal cord. The spinal cord then carries this information to the thalamus, located in the brain, where it is further processed (Kandel et al., 2013). The presence of nociceptive stimuli may induce a withdrawal reflex (nociceptive flexion reflex) leading to a withdrawal response. This reflex occurs when sensory nerve fibers detect noxious stimuli and transmit this information to the spinal cord interneurons. The spinal cord interneurons, in turn, stimulate motor neurons that cause the affected muscles to contract, resulting in the movement of the affected tissue away from the source of the noxious stimulus (Derderian & Tadi, 2022). Overall, nociception is crucial for survival and protection again harmful stimuli. With the numerous ways in which pain can develop

and affect individuals, it is important to study the factors that regulate pain to develop new and better therapeutic treatments for long term pain relief.

Drosophila as a Model for Studying Nociception

Drosophila melanogaster has been proven to be an extremely useful model for understanding the mechanisms involved in nociception. The *Drosophila* genome is relatively compact, is fully sequenced, and consists of ~ 13,600 genes located on 4 chromosomes (Adams et al., 2000). It has been found that approximately 75% of genes causing human diseases have a functional fly homolog, suggesting a high level of genetic conservation between humans and flies (Ugur et al., 2016). *Drosophila* also have a simple nervous system that provides a convenient experimental model for studying neural function and dysfunction. With an average lifespan of ~ 90 days, *Drosophila* develop quickly, allowing for generation of large sample sizes for research purposes (Piper & Partridge 2018). They are also economically advantageous due to their low-cost maintenance and upkeep.

Drosophila larvae, under normal undisturbed conditions, move around using a characterized peristaltic motion. When *Drosophila* larvae come in contact with noxious thermal, mechanical or chemical stimuli, they perform a stereotypical behavior termed Nocifensive Escape Locomotion (NEL). This NEL response is characterized by either one or several 360° barrel rolls along the longitudinal axis of the larval body (Tracey et al., 2003; Hwang et al., 2007) (Fig. 1).

NEL is an evolutionarily conserved behavior used to protect *Drosophila* larvae from parasitoid wasps. Small parasitoid wasps in the *Chalcidoidea* and *Ichneumonidae* subfamilies have females that penetrate the *Drosophila* larval body with a sharp ovipositor to lay their eggs. This

causes the wasp larvae to feed on the fly larvae from the inside and emerge as adult wasps instead of adult *Drosophila* (Hwang et al., 2007; Rizki et al., 1990). *Drosophila* larva use the detection of this ovipositor as a signal to avoid being killed.

Upon stimulation with a noxious stimulus, such as temperatures above 39°C (Hwang et al., 2007; Tracey et al., 2003) and mechanical stimulus greater than 30 mN (Zhong et al., 2010), *Drosophila* larva perform NEL behavior. This behavior is experimentally useful, as it allows scientists to have a standard method in which nociception can be quantified, analyzed, and studied. This behavior can be quantified for thermal nociception experiments by measuring the latency, which is the amount of time in seconds between the stimulus exposure to the time it takes the larvae to complete the first 360° roll (Tracey et al., 2003; Hwang et al., 2007). For mechanical nociception experiments, the percentage of larvae that execute the NEL response is recorded. With increased nociceptor sensitivity, response rates are usually faster - indicated by reduced latencies. Similarly, with decreased nociceptor sensitivity, response rates are usually slower which is indicated by a higher latency (Tracey et al., 2003; Zhong et al., 2010).

Figure 1

NEL Behavior in Drosophila Upon Detection of Noxious Stimuli

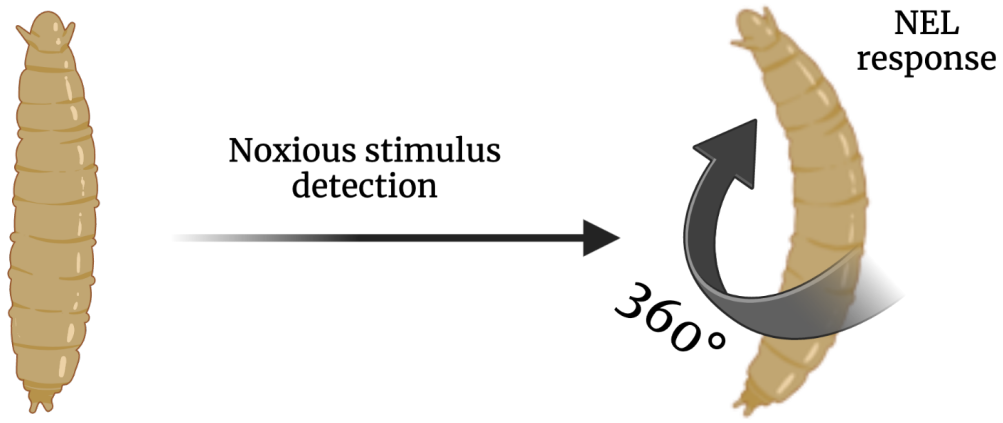

Figure note. Upon detection of noxious stimuli, Drosophila larvae perform a nocifensive escape locomotion (NEL) behavior to avoid the stimulus. (Created with BioRender.com; Adapted from Robertson et al., 2013)

The nervous system consists of the Central Nervous System (CNS) and the Peripheral Nervous System (PNS). The PNS of *Drosophila* larvae contains different types of somatosensory neurons including type I neurons that have ciliated monopolar dendrites and type II neurons that have multi-dendritic projections (md neurons). These md neurons can be further classified according to their dendritic branching complexities where class I has the least complex branching pattern and class IV has the most complex branching pattern allowing it to cover a large surface area and tile the entire larval body (Grueber et al., 2002). Class I md neurons are involved in proprioception (He et al., 2019), class II and III md neurons are gentle touch receptors (Tsubouchi et al., 2012), and class IV md neurons are nociceptors (Hwang et al., 2007) (Fig. 2).

Figure 2

Class 1-IV of Multidendritic Neurons

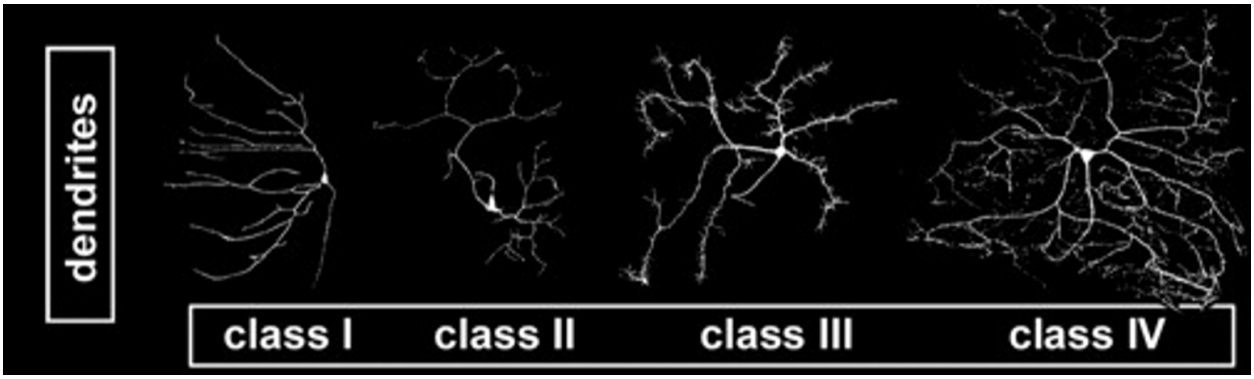

Figure note. Class I-IV multidendritic neurons. Dendritic branching and complexity increases with class (Adapted with permission from Development (Grueber et al., 2007))

In *Drosophila*, class IV multidendritic neurons are required to detect noxious stimuli (Hwang et al., 2007). Nociceptors require a stimulus to reach the activation threshold and activate the nociceptor, indicating their selectivity and responsiveness exclusively to stimuli that may cause tissue damage. (Dubin & Patapoutian, 2010). In a study performed using the neurotoxin tetanus-toxin light chain (TeTxLc) that disrupts neurotransmitter release, it was found that TeTxLc expression in md neurons eradicated the nocifensive response elicited by *Drosophila* upon stimulation by noxious thermal or mechanical stimuli. These findings suggest that md neurons are required for thermal and mechanical nociception (Tracey et al., 2003). In a follow-up study, researchers expressed TeTxLc specifically in each class of md neurons in *Drosophila*. It was observed that TeTxLc expression specifically in class IV md neurons caused a significant defect in nociceptive behavior, therefore it was proposed that class IV md neurons function as nociceptors (Hwang et al., 2007).

Nociceptors have the capability to become sensitized and increase their excitability. This usually occurs after injury or inflammation and involves a reduction in sensitivity threshold and

an increase in response magnitude. Injuries can cause either allodynia, which is the phenomenon where previously innocuous stimulus becomes noxious, or hyperalgesia, which is where noxious stimuli cause a stronger response to stimulus compared to pre-injury sensitivity (Gold & Gebhart, 2010). In *Drosophila* larvae, increased sensitivity to noxious stimulus can be observed with a faster NEL response and a reduced latency, meaning the response time to the noxious stimulus is significantly faster (Babcock et al., 2009).

Drosophila share many characteristics with vertebrates in regard to nociception such as the same range of threshold temperature, 39°C - 41°C, used to activate nociceptors (Smith & Lewin, 2009). Additionally, they have homologous ion channels belonging to the Transient Receptor Potential Ankyrin (TRPA) family that are required for nociception in *Drosophila* (Tracey et al., 2003; Zhong et al., 2012) and vertebrates (Kang et al., 2010; Saito & Tominaga, 2017; Story et al., 2003). They also share morphological similarities in that the highly branched dendritic arbors in *Drosophila* nociceptors can be considered comparable to the highly branched free nerve endings in vertebrates (Dubin & Patapoutian, 2010).

Drosophila as a Model for Studying Thermal Nociception

Drosophila can be used to study hypotheses regarding thermal nociception and how it is regulated. This includes the characterization of insensitive and hypersensitive phenotypes. In *Drosophila*, temperature above 39°C (Tracey et al., 2003) are noxious and prompt larvae to perform NEL. Thermal nociception experiments can be performed at 42°C and 46°C, which allows for the testing of varying hypothesis. Thermal nociception experiments at 42°C allow us to identify phenotypes that are more sensitive than wild type. The average response time (latency) for control *Drosophila* larvae when exposed to a 42°C thermal stimulus is ~ 5 seconds. However, an experimental group with a shorter latency, which does not overlap with the control curve (Fig.

3A), can suggest that the modifications in the experimental group led to increased nociceptor sensitivity . Comparatively, 46°C is higher up in the noxious stimulus range and allows us to determine whether a gene is required for baseline nociception. The average response time for control *Drosophila* larvae exposed to a 46°C thermal stimulus is ~ 2.5-3 seconds. However, if an experimental group has a response rate of ~ 1.5-2 seconds, there is a lot of overlap with the control curve (Fig. 3B). It can be suggested that the experimental group is required for baseline nociception, however, this temperature range is not ideal for measuring phenotypes more sensitive than the wild type. Moreover, an experimental group with a longer latency, can suggest that there is an obvious defect in detecting thermal noxious stimulus and the gene is not necessary for baseline thermal nociception (Fig. 3B).

Figure 3

Nociception Latency Graphs

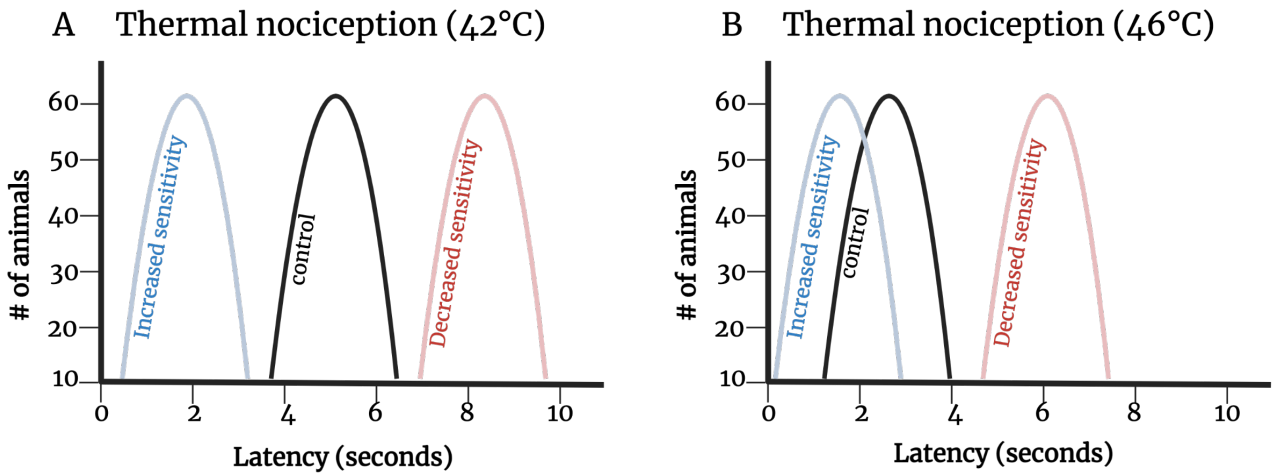

Figure note. Nociception latencies in experimental and control groups at 42°C (A) and 46°C (B) where the blue curve indicates increased sensitivity in nociceptors, the black curve indicates normal sensitivity and the red curve indicates decreased sensitivity in nociceptors.

Ion Channels Involved in Nociception

Nociception is dependent on diverse ion channels that help transduce stimuli and initiate action potential firing. Ion channels are transmembrane proteins that selectively allow charged ions to pass through the cell membrane. This process is mediated by the opening and closing of ion channels that often require specific signals to become activated such as temperature, mechanical force, voltage, and ligand binding. Ion channels in *Drosophila* that mediate nociception signaling include dTRPA1, Painless, Pickpocket, Straightjacket, Piezo and *para*-encoded sodium gated ion channels (Kim et al., 2012; Neely et al., 2010; Tracey et al., 2003; Zhong et al., 2012).

Transient Receptor Potential (TRP) is a superfamily of ion channels that is found in both *Drosophila* and humans (Samanta et al., 2018; Zhong et al., 2012). *Drosophila* TRPA1

(dTRPA1) is a non-selective, calcium-permeable cation channel that is the homolog for the human TRPA1 ion channel and is involved in both thermal and mechanical nociception (Neely et al., 2010; Zhong et al., 2012). In a study assessing the effects of *dTrpA1* mutants, it was found that mutant larvae had a significantly longer latency in response to noxious thermal stimulus (46°C) when compared to control genotypes, suggesting that *dTrpA1* is required for thermal nociception. Similarly, mutant larvae had significantly lower NEL response rates compared to control genotypes when they were exposed to noxious mechanical stimulus, suggesting that *dTrpA1* is also implicated in mechanical nociception (Zhong et al., 2012). Comparatively, human TRPA1 and TRPV1 have been implicated in pain sensation and are expressed in nociceptors where they are required to detect noxious stimuli (Caterina et al., 2000; Saito & Tominaga, 2017; Story et al., 2003).

Painless encodes a protein of the TRP ion channel family that is expressed in larval nociceptors. Through forward genetic screening, *Painless* has been shown to be required for detection of both thermal and mechanical stimuli, as *painless* mutants were defective in sensing both noxious thermal as well as mechanical stimuli. Studies have also shown that the *painless* gene is expressed in md neurons and is required for sensory neurons to detect noxious thermal stimulus (Tracey et al., 2003).

Piezo is a large transmembrane protein that is found in *Drosophila* nociceptors (dPiezo) as well as in human sensory neurons where it is involved in mechanosensation. The human Piezo is encoded by two separate genes *Piezo1* and *Piezo2* and has different functions in different tissues (Coste et al., 2010). Studies have found that tissue specific knockdown of *Piezo* in *Drosophila* nociceptors caused significantly reduced nocifensive response to noxious mechanical stimuli, but no defect was observed in responses to noxious thermal stimuli. Interestingly, these findings

strongly indicate that *Piezo* is involved in mechanical but not thermal nociception, suggesting that thermal and mechanical nociception may be transduced through different signaling mechanisms (Kim et al., 2012).

Another ion channel implicated in *Drosophila* nociception is the Degenerin/epithelial sodium channel (DEG/ENaC) subunit Pickpocket (*ppk*). Ppk has been found to be required for both mechanical nociception and mechanotransduction; however, it is not required for thermal nociception. It is expressed in class IV multidendritic neurons of *Drosophila.* RNAi knockdown of *ppk* in larvae produced a significant reduction in nocifensive responses to noxious mechanical stimuli, but not to noxious thermal stimuli. In *Drosophila, ppk* is expressed specifically in nociceptors, allowing its regulatory sequence to be used as a tissue specific enhancer for targeted genetic manipulations in *Drosophila* nociceptors (Zhong et al., 2010).

Signaling Pathways Involved in Nociception

There are several signaling pathways that have been identified as regulators of nociceptor sensitivity in *Drosophila.* These include the Tumor Necrosis Factor α (TNFα), the Bone Morphogenetic protein (BMP), and the Hedgehog (Hh) signaling pathways.

TNFα is a pro-inflammatory cytokine that regulates nociception (Leung & Cahill., 2010). The *Drosophila* homolog of TNFα is the membrane glycosylated protein Eiger. Eiger is cleaved and released from the surface of the cell as a soluble factor and binds to its receptor Wengen, a receptor tyrosine kinase protein. This Eiger-Wengen complex activates the TNFα signaling pathway that leads to regulation of several processes such as apoptosis and nociceptive sensitization (Kauppila et al., 2003). TNFα signaling is necessary for thermal allodynia in *Drosophila* larvae. To establish this, third instar larvae were exposed to UV radiation and later subjected to a thermal nociception assay at 38°C. The larvae showed increased responses to sub-threshold thermal

stimuli up to 24 hours after UV exposure, suggesting that the Eiger-Wengen complex is important for activating nociception sensitization following injury. However, sensitization gradually decreased after 48 hours due to the healing of the dorsal epidermis (Babcock et al., 2009).

Bone morphogenic proteins (BMPs) are secreted ligands that have been shown to be crucial for axon regeneration, neurogenesis, axon guidance and dendrite growth (Bond et al., 2012; Withers et al., 2000; Zhong & Zou, 2014). BMPs are part of the transforming growth factor beta (TGFβ) superfamily required for normal synaptic growth and stability and are highly conserved (Follansbee et al., 2017). Decapentaplegic (Dpp) is the *Drosophila* ortholog of mammalian BMP 2/4 that binds to the BMP receptor (BMPR). *Dpp,* along with the primary serine/threonine kinase BMP type II receptor (BMPRII) Punt (*put*), and BMP type I receptors Thick veins (*Tkv*) and Saxophone (*Sax*), leads to activation of Mothers against Dpp (*Mad*) and Medea causing nociceptive sensitization. Glass bottom boat (Gbb), another mammalian ortholog of BMP5,6,7, and 8, was shown to work with the type II BMPR Wishful Thinking (Wit) in injury-induced nociceptive sensitization. When Gbb or its receptor Wit were knocked down, sensitization was attenuated following UV injury. These findings indicate that BMP signaling plays an important role in nociceptive sensitization (Gjelsvik et al., 2018).

In *Drosophila,* Hedgehog (Hh) proteins are secreted morphogens that mediate nociceptive sensitization in flies and mice, specifically thermal allodynia and hyperalgesia (Babcock et al., 2011; Collins & Cohen., 2005; Okuda et al., 2022). This Hh signaling that occurs during sensitization occurs in parallel to TNFα signaling and involves multiple TRP channels in nociceptive sensory neurons. Hh-induced thermal allodynia requires the Painless TRP channel, whereas Hh-induced thermal hyperalgesia requires dTRPA1. After UV injury, Hh binds to and inhibits its

receptor Patched (Ptc), which in turn removes inhibition of a transmembrane protein Smoothened (Smo). This leads to a signaling cascade causing activation of the transcription factor Cubitis Interruptus (Ci). This activation turns on expression of *dpp* and *engrailed* (*en)* ultimately leading to thermal allodynia and hyperalgesia (Babcock et al., 2011; Im et al., 2015).

Growth Factor Signaling Involved in Nociception

Growth factor receptors (GFRs) are receptor tyrosine kinase (RTK) proteins that exist on the cell surface and play a role in cellular growth, proliferation, differentiation, and nociception (Barrientos et al., 2008). Each GFR has its own specific ligands that bind and allow for activation. For example, Platelet derived growth factor (PDGF) has 5 different isoforms (PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGF-AB) that can bind to two different PDGF receptors (PDFGRα and PDGFRβ) (Fredriksson et al., 2004). The binding of the growth factor ligand causes two GFR molecules to come together to form a complex through a process known as dimerization. This dimerization then activates either transphosphorylation or autophosphorylations of the kinase domain (Chen et al., 2013). Without phosphorylation of the kinase domain, the growth factor receptor cannot activate and therefore no downstream signaling takes place.

PDGFs play an essential role in many biological processes such as tissue repair, tumorigenesis, wound healing, and nociception (Heldin, 2013; Lynch et al., 1987). These homodimeric and heterodimeric growth factors that act through dimerized RTKs composed of PDFGRα and PDGFRβ. It has previously been reported that PDGF and PDGFR are located in myelinated and unmyelinated sensory neurons (Eccleston et al., 1993) as well as the spinal cord (Heldin & Westermark, 1990). In a recent study, it was found that PDGF-BB, inhibits K_V7/M potassium channels through PDGFR activation and leads to nociceptive hypersensitivity and excitability. In previous studies, K_V7/M channels have been identified as significant regulators of nociceptive

excitability (Brown & Adams., 1980), allowing PDGF-BB induced inhibition to play a key role in nociception regulation (Barkai et al., 2019).

PDGF is also involved in the development of neuropathic pain after nerve injury. Researchers found that following sciatic nerve ligation, mice had decreased thermal and tactile thresholds after repeated intrathecal injections of PDGFRα/Fc compared to controls that did not receive PDGFRα/Fc injections. PDGFR α/Fc is a fusion protein that consists of the extracellular domain of PDGFRα and the Fc region of the human immunoglobulin molecule. This fusion protein acts as an inhibitor by binding to PDGF and preventing the ligand from binding to the PDGFRα. This decrease in threshold suggested that PDGF plays a role in mediating neuropathic pain after injury (Narita et al., 2005). PDGFR and its ligands are also required to induce mechanical sensitivity. Through daily intrathecal injections of PDGF peptides, PDGF AA, BB, and CC were all implicated in mechanical sensitivity. This was done by applying a linearly increasing pressure to the hind paws of rats and analyzing paw withdrawal compared to a vehicle control (Lopez-Bellido et al., 2019).

VEGF is a growth factor that plays a crucial role in angiogenesis (Niu & Chen, 2010). VEGF family A (VEGF-A) has been widely studied because of its implication in neurodegeneration and neuroprotection and well as its links to several neuronal diseases. VEGF-A has 2 isoform families including VEGF-A_{xxx}a and VEGF-A_{xxx}b, where xxx denotes the number of amino acids that were encoded, and a and b refers to the terminal amino acid sequence. VEGF receptor 2 (VEGFR2) is the main receptor that is activated by both VEGF isoform families and has been shown to be important in nociceptive processing. VEGFR2 efficacy is determined by the C-terminus sequence of VEGF, which differs in VEGF-A_{xxx}a and VEGF-A_{xxx}b. Furthermore, VEGF-A_{xxx}a binding to VEGFR2 results in complete phosphorylation and activation of the receptor

whereas VEGF-A_{xxx}b results in partial phosphorylation and activation leading to receptor degradation (Ballmer-Hofer et al., 2011; Hulse et al., 2014).

A study exploring the therapeutic usefulness of the two isoform families found that VEGF-A_{165}b had neuroprotective effects that were beneficial for neurodegenerative pathologies. This VEGF-A isoform has endogenous expression in the hippocampus and cortical neurons of vertebrate models. In response to multiple harmful injurious stimuli such as glutamatergic excitotoxicity in hippocampal neurons, chemotherapy-induced cytotoxicity of DRG neurons, and retinal ischemia-reperfusion injury in rat retinal ganglion cells *in vivo,* recombinant human (rh) VEGF-A_{165}b elicited neuroprotective effects. Through VEGF2 and mitogen activated protein kinase kinase 1 and 2 (MEK1/2) activation, VEGF-A_{165}b is neuroprotective to central and peripheral neurons (Beazley-Long et al., 2013). Comparatively, VEGF-A_{165}a elicits neuroprotective effects on hippocampal, cortical, and cerebellar granule neurons against various injurious stimuli (Jin et al., 2000) through activation of VEGFR2 and subsequent activation of several intracellular pathways such as PI3K/Akt, MEK1/2, and phospholipase C (Zachary, 2005).

A recent study of the effects of alternatively spliced isoforms of VEGF-A on nociception found that the effect on nociception is dictated by the balance of the VEGF-A_{xxx}a and VEGF-A_{xxx}b isoforms. Even though VEGF-A_{165}a and VEGF-A_{165}b have the same binding affinity for VEGFR2, when both isoforms are equimolar or VEGF-A_{165}b is in surplus, it can reduce VEGF-A_{165}a activity by ~95% through competitive antagonism at VEGFR2. Alteration of RNA splicing that causedVEGF-A_{165}a mRNA reduction in skin caused hypoalgesia in normal animals whereas increased VEGF-A_{165}a using systemic exogenous recombinant protein elicited pro-nociceptive effects on behavior and neurons (Hulse et al., 2014).

VEGFR2 is also found to be implicated in analgesia. When rats were treated with selective inhibitors for VEGFR1, VEGFR2 and VEGFR3, it was found that only the rats treated with Cabozantinib, an inhibitor of VEGFR2, were the only group to develop mechanical analgesia. Interestingly, inhibition of VEGFR2 with Cabozantinib also produced an inhibition of analgesic tolerance. With the involvement of VEGFR2 antagonism in opioid tolerance, VEGFR2 may be an excellent candidate for therapeutic pain treatments (Lopez-Bellido et al., 2019).

Growth Factor Signaling in Drosophila Nociception

In *Drosophila,* Pvr is a cognate receptor that is related to PDGFR and VEGFR. Pvr has three ligands, Pvf1, Pvf 2, and Pvf3. These ligands form a complex with Pvr and mediate physiological processes such as cellular development and differentiation (Mondal et al., 2014), hemocyte proliferation (Munier et al., 2002), and nociception (Lopez-Bellido et al., 2019). In a study exploring how Pvr is regulates in nociception, it was found that Pvr and its ligands Pvf2 and 3 were required to regulate mechanical nociception. This was done by using nociceptor specific knockdown, overexpression, loss of function, and gain of function transgenic lines for *Pvr* and *Pvf.* Larvae that were homozygous for a *Pvf1* null allele did not have a defect in mechanical response compared to larvae homozygous for *Pvf2* and *Pvf3* null alleles. For this reason, *Pvf1* effects were not further tested. Larvae that had knockdown of *Pvr* and *Pvf* showed a decreased response to noxious mechanical stimuli (2346 kPa) suggesting that Pvr is required for response to noxious mechanical stimuli. Similarly, with *Pvr* loss of function groups there was reduced mechanical nociception compared to control. Comparatively, *Pvr* overexpression and constitutive activation were shown to cause mechanical hypersensitivity. Constitutive activation of *Pvr* also caused mechanical allodynia and *Pvr* overexpression caused hyperalgesia (Lopez-Bellido et al., 2019).

The effects of Pvr/Pvf signaling on regulating dendritic branching was also investigated. *Pvr, Pvf2,* and *Pvf3* mutants were used to explore whether defective mechanical nociception behavior had underlying developmental alterations in class IV md neurons. In all mutants, a reduction in total dendritic length was found, however the number and location of the class IV md neurons remained the same. This study also found that Piezo and Painless channels are required to regulate mechanical nociception after *Pvr* activation, suggesting that these channels lie downstream of the Pvr signaling pathway. Using RNAi transgenes that targeted *Piezo* and *Painless,* Pvr-induced mechanical allodynia and hyperalgesia were attenuated, suggesting that mechanical hypersensitivity mediated through Pvr signaling also requires the Piezo and Painless ion channels in class IV md neurons (Lopez-Bellido et al., 2019).

Objectives

Pvf/Pvr signaling has been shown to be important for regulating mechanical nociception and hypersensitivity (Lopez-Bellido et al., 2019). In vertebrates, growth factors related to Pvf, such as PDGF and VEGF, have been observed to have roles in multiple aspects of nociception. However, there is limited research on the role of *Pvr* in *Drosophila* thermal nociception. This study aims to determine whether *Pvr* is necessary for normal thermal nociception activity and if it sufficient for increased thermal nociception sensitivity in *Drosophila*. Furthermore, since growth factors are involved in regulating dendritic branching, this study explore the effects of *Pvr* and Pvr signaling in nociceptor dendrite morphology.

Based on previous research establishing that *Pvr* is required for mechanical nociception (Lopez-Bellido et al., 2019), several predictions regarding larval behavior and alterations to nociception can be made resulting from manipulations of *Pvr*. If Pvr signaling is increased specifically in the nociceptors, it can be expected to result in increased sensitivity in the nociceptors

leading transgenic larvae to respond to noxious thermal stimulus at a higher and faster rate in comparison to control larvae. Comparatively, if Pvr signaling is decreased or removed in the nociceptors, then it can be anticipated that there is decreased sensitivity in nociceptors and transgenic larvae will respond to noxious thermal stimulus at a much reduced and slower rate in comparison to control larvae.

Materials and methods

Drosophila stocks and culture

The flies and larvae that were utilized in this experiment were bred on cornmeal-molasses medium (Nutri-Fly M; Genesee Scientific, El Cajon, CA, USA) and were grown at 25 °C and ~50% humidity under a 12-hour light/12-hour dark cycle (Herman et al., 2018). The driver stock utilized was *w;ppk-GAL4;UAS-dicer 2* and the control stocks were w1118 and 36303 (Control line for TriP RNAi line). PvrRNAi#1 (8222R-3), UAS-Pvr, UAS-PvrDN, and UAS-PvrCA were obtained from the Galko laboratory (Lopez-Bellido R et al., 2019). PvrRNAi#2 (37520) was obtained from Bloomington Drosophila stock center. PvrRNAi#1 (8222R-3) and PvrRNAi#2 (37520) were used for knockdown of Pvr, UAS-Pvr was used for Pvr overexpression, UAS-PvrDN was used for loss of function of Pvr, and UAS-PvrCA was used for constitutive activation of PVR. The GFP stock utilized for dendrite visualization was *w; ppk GAL4, UAS-mCD8::GFP, UAS-dicer2.*

Table 1

Drosophila stock genotypes

Name	BDSC#	Genotype	Manipulation
ppkGAL4	N/A	*w; ppk-GAL4; dicer-2*	GAL4 Driver
w[1118]	5905	*W[1118]*	Control line
36303	36303	*y[1] v[1]; P{y[+t7.7]=CaryP}attP2*	RNAi control line
GFP	N/A	*w; ppk-GAL4, UASmCD8::GFP; UAS dicer-2*	Tissue-specific GFP expression
UAS-Pvr-RNAi$^{\#1}$	37520	*y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01662}attP40*	*Pvr* knockdown
UAS-Pvr-RNAi$^{\#2}$	N/A	*Not provided*	*Pvr* knockdown
UAS-Pvr	58998	*w[*]; P{w[+mC]=UAS-Pvr.D}3*	*Pvr* overexpression
UAS-Pvr^{CA}	58496	*w[1118]; P{w[+mC]=UASp-Pvr.lambda}mP10*	*Pvr* constitutive activation
UAS-Pvr^{DN}	58430	*w[1118]; P{w[+mC]=UASp-Pvr.DN}D1/CyO*	*Pvr* Dominant negative

The objective of this project was to understand the role Pvr plays in thermal nociception. As mentioned above, *ppk* being expressed specifically in *Drosophila* nociceptors allows its regulatory sequence to function as a tissue-specific enhancer for genetic manipulations in the nociceptors. These manipulations are made using the GAL4/*UAS* system (Fig. 4). This system can be used to control tissue-specific gene expression in *Drosophila*. It comprises of two transgenes, the first being GAL4, which encodes a yeast transcriptional regulator protein. The GAL4 gene is inserted downstream of a cell-specific promoter, allowing it to be expressed in a tissue-specific fashion. The second transgene involves the Upstream Activating Sequence (UAS) enhancer present upstream of the gene of interest. In our studies, the regulatory sequence of *ppk* is used to

drive GAL4 expression specifically in class IV multidendritic neurons (Ainsley et al., 2003). *Drosophila* with each transgene of the system are crossed to produce progeny that have both transgenes in the same organism allowing for GAL4 to bind to the UAS enhancer and drive expression of the downstream gene of interest.

Nociceptor-specific knockdown is used to study cell-specific loss of function, which is useful in understanding the role and site of action of genes that control nociception (Neely et al., 2010; Tracey et al., 2003; Zhong et al., 2012). Nociceptor-specific knockdown can be accomplished by combining RNA interference (RNAi) methods with the GAL/UAS system. This approach involves expressing RNA hairpin sequences corresponding to a gene of interest specifically in nociceptors. Dicer-2 then cleaves these hairpins, which combine with Argonaute 2 to form the RNA-induced silencing complex (RISC). Using the RNA molecule complimentary to the gene of interest mRNA, RISC binds to and degrades the mRNA transcript in turn suppressing expression at the translational level in specific cells (Heigwer et al., 2018).

Figure 4

Overview of the GAL4/UAS System

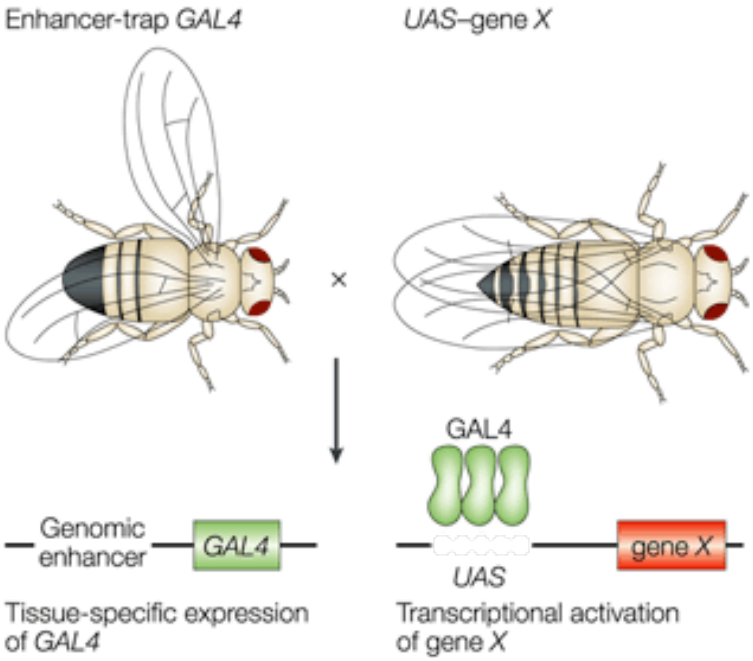

Figure note. Overview of the GAL4/ UAS system utilized for driving tissue specific expression in class IV multidendritic neurons. (Adapted with permission from Nature (St Johnston, 2002))

Experimental and control crosses

For each experiment, three genotypes were tested – the experimental group, the GAL4-only control that carried only the GAL4 transgene, and the UAS-only control that carried only the UAS transgene. For knockdown experiments, an additional positive *para* knockdown control was tested. To knock down Pvr, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with $Pvr^{RNAi\#1}$ *(8222R-3)* male flies. For the GAL4-only control, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with w^{1118} male flies and for the RNAi-only control stock virgin females from the w^{1118} control stock were crossed with $Pvr^{RNAi\#1}$ *(8222R-3)* male flies. The GAL4-only control includes the *ppk-GAL4* driver, however it does not contain a UAS sequence to bind to, and therefore knockdown does not take

place. Similarly, the UAS (or RNAi) -only control has a UAS sequence and the downstream transgene, however it does not contain *ppk-GAL4* to drive expression of the transgene, and therefore knockdown cannot occur. For the positive control, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with *Para RNAi* males. To confirm the results of the knockdown experiment, another RNAi line $Pvr^{RNAi\#2}$ *(37520)* was used. To knock down Pvr, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with $Pvr^{RNAi\#2}$ *(37520)* male flies. For the GAL4-only control, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with *36303* male flies and for the RNAi-only control stock, virgin females from the w^{1118} control stock were crossed with $Pvr^{RNAi\#2}$ *(37520)* male flies. For the RNAi-only control, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with *Para RNAi* males.

To overexpress Pvr, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with *UAS-Pvr* male flies in which UAS drives expression of the Pvr cDNA. For the GAL4-only control, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with w^{1118} male flies and for the UAS-only control stock, virgin females from the w^{1118} control stock were crossed with *UAS-Pvr* male flies. For dominant negative experiments, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with $UAS\text{-}Pvr^{DN}$ male flies. $UAS\text{-}Pvr^{DN}$ flies were used to overexpress the dominant negative form of Pvr, which disrupts the wild-type protein activity as it is lacking the kinase domain. For the GAL4-only control, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with w^{1118} male flies, and for the UAS-only control stock, virgin females from the w^{1118} control stock were crossed with $UAS\text{-}Pvr^{DN}$ male flies. For constitutive activation experiments, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with $UAS\text{-}Pvr^{CA}$ male flies. *UAS-*

Pvr^{CA} was used to overexpress the constitutively active form of Pvr in which the extracellular ligand binding domain was replaced with a constitutive dimerization domain. For the GAL4-only control, virgin females from the GAL4 driver stock (*ppk-GAL4;UAS-dicer2)* were crossed with *w*^{1118} male flies and for the UAS-only control stock, virgin females from the *w*^{1118} control stock were crossed with *UAS-Pvr*^{CA} male flies.

In order to analyze the effects on dendritic branching patterns in Drosophila melanogaster mutants that had Pvr knockdown and overexpression were analyzed under a confocal microscope. For Pvr knockdown, virgin females from the *w; ppk-GAL4, UAS-mCD8::GFP; UAS-dicer2* line containing Green Fluorescent Protein (GFP) were crossed with PvrRNAi#2 (37520) male flies. For Pvr overexpression, virgin females from the *w; ppk-GAL4, UAS-mCD8::GFP; UAS-dicer2* line containing GFP were crossed with UAS-Pvr male flies. For GFP-only control, virgin females from the *w; ppk-GAL4, UAS-mCD8::GFP; UAS-dicer2* line containing GFP were crossed with control (36303) males.

T**able 2**

Experimental and control crosses

Driver/Enhancer (Females)	**Responder (Males)**	**Manipulation**
w; ppk-GAL4; dicer-2	*y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01662}attP40*	*Pvr* knockdown
W[1118]	*y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01662}attP40*	*UAS-Pvr-RNAi* – only – control
w; ppk-GAL4; dicer-2	*W[1118]*	*ppkGAL4* – only control
w; ppk-GAL4; dicer-2	*y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01662}attP40*	*Para* control
w; ppk-GAL4; dicer-2	*w[*]; P{w[+mC]=UAS-Pvr.D}3*	*Pvr* overexpression
W[1118]	*w[*]; P{w[+mC]=UAS-Pvr.D}3*	*UAS-Pvr* – only control
w; ppk-GAL4; dicer-2	*w[1118]; P{w[+mC]=UASp-Pvr.lambda}mP10*	*Pvr* constitutive activation
W[1118	*w[1118]; P{w[+mC]=UASp-Pvr.lambda}mP10*	*UAS-Pvr^{CA}* – only control
w; ppk-GAL4; dicer-2	*w[1118]; P{w[+mC]=UASp-Pvr.DN}D1/CyO*	*Pvr* dominant negative
W[1118	*w[1118]; P{w[+mC]=UASp-Pvr.DN}D1/CyO*	*UAS-Pvr^{DN}* – only control
w; ppk-GAL4, UASmCD8::GFP; UAS dicer-2	*y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01662}attP40*	*Pvr* knockdown with GFP
w; ppk-GAL4, UASmCD8::GFP; UAS dicer-2	*w[*]; P{w[+mC]=UAS-Pvr.D}3*	*Pvr* overexpression with GFP
w; ppk-GAL4, UASmCD8::GFP; UAS dicer-2	*y[1] v[1]; P{y[+t7.7]=CaryP}attP2*	GFP – only control

Thermal nociception assay

Thermal nociception assays were performed using established protocols (Herman et al., 2018; Tracey et al., 2003). Wandering 3rd instar larvae were washed from vials with DI water and placed on a petri dish with ~1 mL of DI water. In order to break the surface tension, ~ 15-20 yeast pellets were added to the petri dish. The larvae were allowed to wander for a ~ 10 minutes in order to get accustomed to new environmental conditions. A thermal probe with a soldering iron tip was shaved down to have a flatter edge for application to the larval body in order for thermal stimulation along the anterior/posterior axis. The probe temperature were controlled by a Variac Variable Transformer (Part No. ST3PN1210B) (ISE, Inc., Cleveland, OH) and the probe temperature were monitored by a IT-23 thermistor and a BAT-12 digital thermometer (Physitemp, Clifton, NJ) which provides a reading of the temperature to the tenth of a degree. The desired temperatures 46 ± 0.5° and 42 ± 0.5° were maintained during testing. The larval bodies were tested and recorded using a digital camera mounted on the microscope. The sample size consists of ~ 60 larvae for all experiments. Adobe Premier Pro was used in order to analyze the thermal nociception assay video. The video was recorded at 30 frames per second and the latency was recorded by calculating the time from when the thermal probe first came in contact with the larvae to when a NEL response was observed. Larvae with a latency of <10 sec was recorded as 11. This method was replicated for each genotype tested. A statistics estimation site (estimationstats.com) was used to determine significant statistical differences within the control and experimental genotypes using two-sided permutation T-test.

Confocal microscope analysis

In order to analyze dendrite branching and morphology of the nociceptors, a confocal microscope was utilized. Wandering 3rd instar larvae were immobilized by tying a hair around the segment A3 causing circumferential ligation that leads to paralysis of all segments below the ligation. These larvae were mounted between a slide and a coverslip with glycerol. The larvae were viewed under a Zeiss LSM 880 microscope with a 488 nm laser line. Tiled z-stack were obtained to view dendritic arborization.

Results

The purpose of this project was to understand the role of Pvr, a growth factor receptor, in thermal nociception. To do this Pvr was overexpressed (*ppk>UAS-Pvr*), knocked down (*ppk>UAS-Pvr-RNAi*), constitutively activated (*ppk>UAS-Pvr^{CA}*), and made defective (*ppk>UAS-Pvr^{DN}*) in the class IV multidendritic neurons of *Drosophila* using the GAL4/UAS system to either express RNAi or overexpress the gene. For the *Pvr* overexpression, *Pvr* dominant negative, and *Pvr* constitutive activation experiments, two control genotypes were used. The GAL4-only control (*ppk/+)* contained *ppk-GAL4* and *UAS-dicer-2.* The GAL4-only control serves as a comparison of normal latency where Pvr signaling and nociception behavior are expected to be wild type. This can then be compared to genotypes where Pvr signaling is expected to be disrupted to measure the effects on nociception behavior. The UAS-only control (*UAS/+) or* UAS-RNAi-only control *(UAS-RNAi/+)* genotype consisted of only the Upstream activating sequence (UAS) and the larval genotype did not have *ppk-GAL4; UAS-dicer-2.* Similarly, to the GAL4-only control, the UAS-only control also serves as comparison for normal latency in larvae. For the Pvr-RNAi knockdown experiments, there were three controls – the GAL4-only control, RNAi-only control and an additional positive *para-RNAi* control. Since *para* encodes proteins that make up voltage-gated sodium channels, *Drosophila* with *para* knockdown are expected to possess an extremely strong nociception defect and serve as a positive control.

In this study, there were 5 experimental groups. The first two groups were *Pvr* knockdown (*ppk>UAS-Pvr-RNAi$^{\#1}$* and *ppk>UAS-Pvr-RNAi$^{\#2}$*) constructed with two different RNAi lines (#1 & #2). This experimental group is expected to have defects in *P*vr signaling and is expected to have a lower sensitivity (higher latency) to noxious thermal stimuli when compared to the controls. *Pvr* dominant negative (*ppk>UAS-Pvr^{DN}*) was a loss of function experimental

group, where the dominant negative form of Pvr was overexpressed in the nociceptors and was expected to have a reduced response to noxious thermal stimulus. This transgenic line contains the transmembrane and extracellular domains of the Pvr receptor, allowing it to bind to the ligand, but form inactive dimers with the endogenous receptor (Duchek et al., 2001) (Fig. 5). The experimental group *Pvr* overexpression (*ppk>UAS-Pvr*) had Pvr overexpressed in their nociceptors, amplifying Pvr signaling. These larvae were expected to have a faster response (lower latency) to noxious thermal stimulus. A second gain of function experimental group was *Pvr* constitutive activation (*ppk>UAS-Pvr*^{CA}) where the constitutively active *Pvr* was overexpressed in the nociceptors, yielding increased Pvr signaling. The constitutively active form of Pvr was made by exchanging the extracellular ligand binding domain with a constitutive dimerization domain (Duchek et al., 2001) (Fig. 5). These larvae were also expected to have a faster response rate.

Figure 5

Schematic drawing of the Pvr Protein and How it is Modified

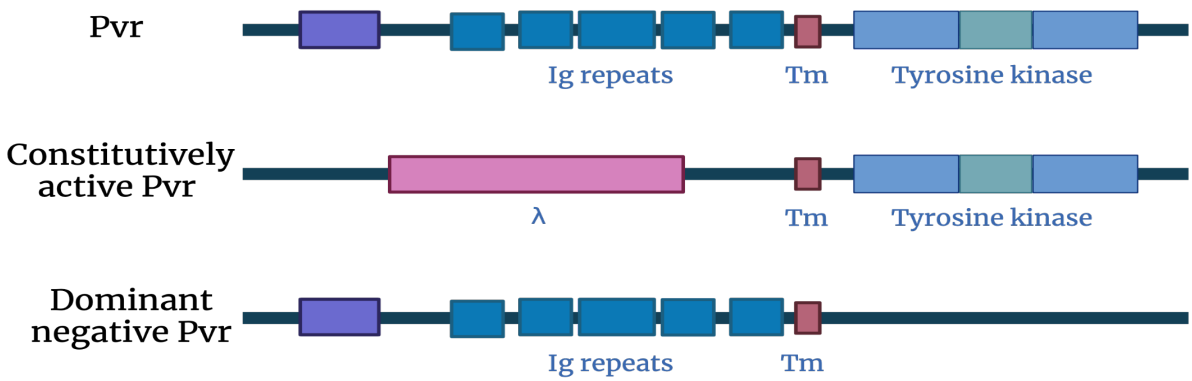

Figure note. Schematic drawing of the Pvr protein and how it was modified to create gain of function and loss of function transgenic fly lines. Tm represents the transmembrane segment. Ig repeats represent the immunoglobulin repeats. λ represents the lambda protein. From top to bottom: Normal Pvr protein; Constitutively activated form of Pvr; Dominant negative form of Pvr. (Created with BioRender.com)

Pvr knockdown has an inconsistent effect on baseline nociceptor sensitization

To understand the role of Pvr in baseline thermal nociception, Pvr was knocked down in *Drosophila* nociceptors using the GAL4/UAS system. Two different RNAi lines (8222R-3 (#1) and 37520 (#2)) were used to investigate this. Wandering 3rd instar *Drosophila* larva from all groups were tested at 46°C.

Pvr knockdown group #1 (*ppk>UAS-Pvr-RNAi*$^{\#1}$) had a mean response latency of 2.4 seconds (s). The *ppk/+* control larvae had a mean response latency of 3.4 s, and the *UAS-Pvr-RNAi*$^{\#1}$*/+* control larvae had a mean response latency of 4.9 s. The negative *para-RNAi* control had an average latency of 10.7 s. The *ppk>UAS-Pvr-RNAi*$^{\#1}$ knockdown group and the *ppk/+* control were statistically different (p= 0.003, n=60, determined by a two-sided permutation t-test). Similarly, the *ppk>UAS-Pvr-RNAi*$^{\#1}$ knockdown group and the *UAS-Pvr-RNAi*$^{\#1}$*/+* control were statistically different (p= 0.000, n=60, determined by a two-sided permutation t-test). Compared to the positive *para-RNAi* control the *ppk>UAS-Pvr-RNAi*$^{\#1}$ knockdown group was also statistically different (p= 0.000, n=60, determined by a two-sided permutation t-test) (Fig. 6).

Figure 6

Pvr Knockdown #1 Shows an Effect in Baseline Thermal Nociception

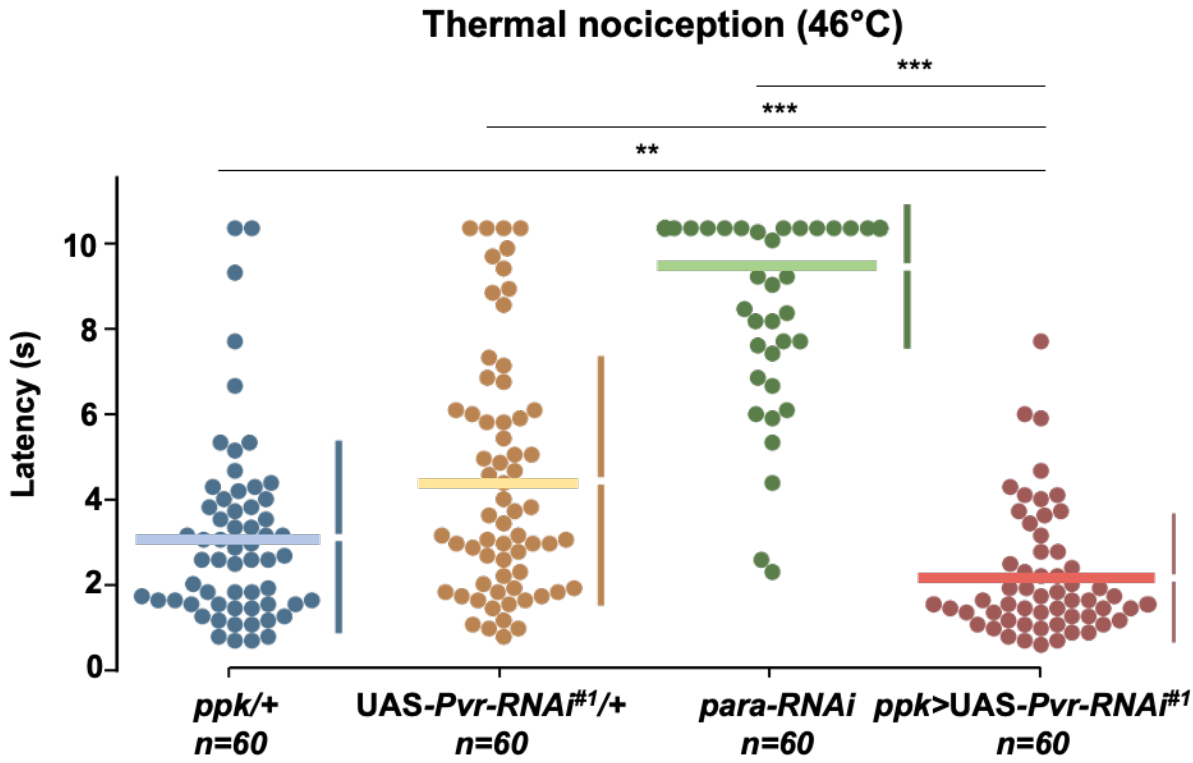

*Figure note. 3rd instar Drosophila larva with nociceptor specific Pvr knockdown (ppk>UAS-Pvr-RNAi#1) shows a statistically different and reduced latency in response to noxious stimuli (46°C) when compared to the ppk/+ control (**, $p \leq 0.001$) and UAS-Pvr-RNAi#2/+ control (***, $p \leq 0.01$). ppk>UAS-Pvr-RNAi#1 shows a statistically different and decreased latency when compared to the negative para-RNAi control group(***, $p \leq 0.001$, determined by two-sided permutation t-test). (n= 60 for all groups) (B)Pvr knockdown shows an effect in baseline thermal nociception.*

Pvr knockdown group #2 (*ppk>UAS-Pvr-RNAi*$^{\#2}$) had a mean response latency of 4.2 seconds (s). The *ppk/+* control larvae had a mean response latency of 2.3 s, and the *UAS-Pvr-RNAi*$^{\#2}$*/+* control larvae had a mean response latency of 3.1 s. The negative *para-RNAi* control had an average latency of 10.6 s. The *ppk>UAS-Pvr-RNAi*$^{\#2}$ knockdown group and the *ppk/+* control were statistically different (p= 0.000, n=82, determined by a two-sided permutation t-test). Similarly, the *ppk>UAS-Pvr-RNAi*$^{\#2}$ knockdown group and the *UAS-Pvr-RNAi*$^{\#2}$*/+* control were statistically different (p= 0.002, n=82, determined by a two-sided permutation t-test). Compared to the positive *para-RNAi* control the *ppk>UAS-Pvr-RNAi*$^{\#2}$ knockdown group was also statistically different (p= 0.000, n=82, determined by a two-sided permutation t-test) (Fig. 7).

Figure 7

Pvr Knockdown #2 Shows an Effect in Baseline Thermal Nociception

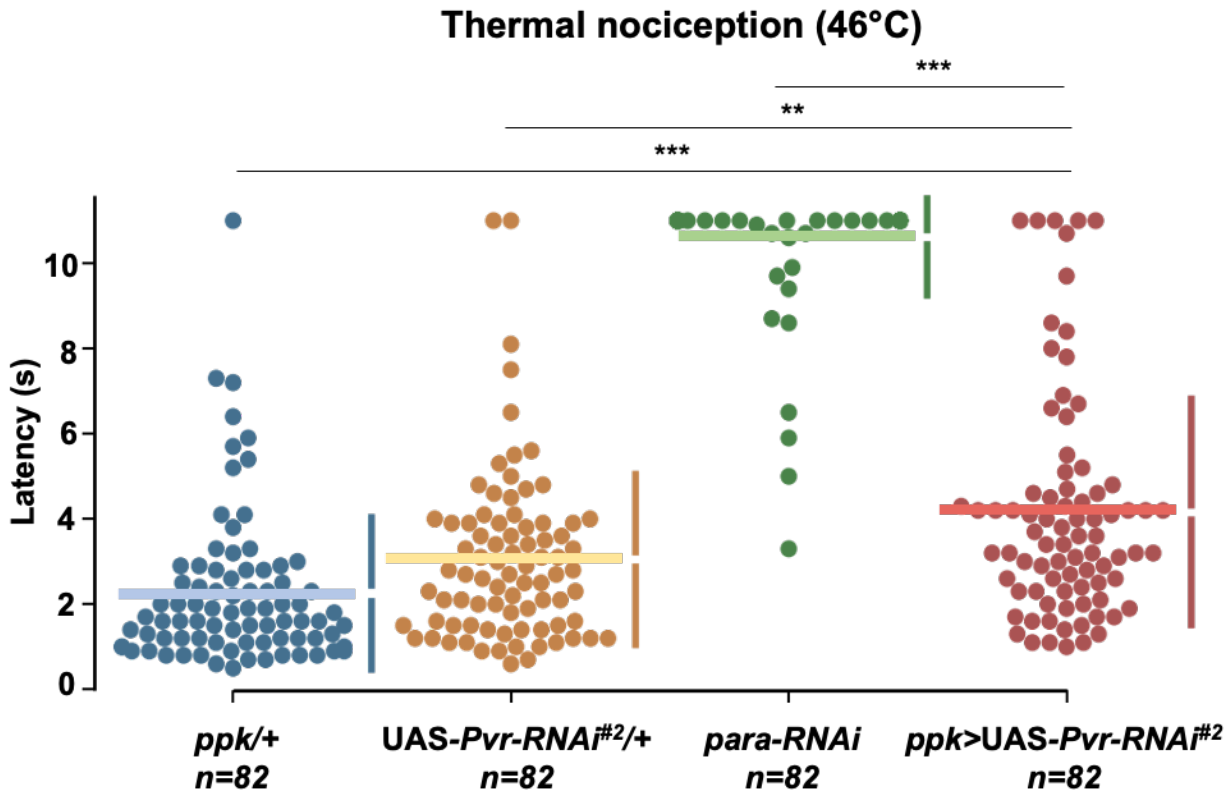

*Figure note. 3rd instar Drosophila larva with nociceptor specific Pvr knockdown (ppk>UAS-Pvr-RNAi#2) shows a statistically different and increased latency in response to noxious stimuli (46°C) when compared to the ppk/+ control (***, $p \leq 0.001$) and UAS-Pvr-RNAi#2/+ control (**, $p \leq 0.01$). ppk>UAS-Pvr-RNAi#2 shows a statistically different and decreased latency when compared to the negative para-RNAi control group(***, $p \leq 0.001$, determined by two-sided permutation t-test). (n= 82 for all groups, horizontal bars indicate means and vertical error bars indicate 95% confidence intervals)*

Dominant negative Pvr activity in nociceptors causes a defect in baseline thermal nociception

To further understand how defects in Pvr causes changes in thermal nociception, Pvr was made defective in the nociceptors by overexpressing a dominant negative form of the receptor that is expected to disrupt Pvr activity. The Pvr dominant negative group (*ppk>UAS-Pvr^{DN}*) had the dominant negative form of Pvr overexpressed in the nociceptors. Thus, the transgenic *ppk>UAS-Pvr^{DN}* line attenuates signaling from the Pvr receptor (Duchek.al, 2001).

All wandering 3rd instar larvae in each group were tested at 46°C. The dominant negative group *ppk>UAS-Pvr^{DN}* had an average latency of 5.0 s, the *ppk/+* control had an average latency of 3.9 s and the *UAS-Pvr^{DN}/+* only control has an average latency of 3.3 s. The *ppk>UAS-Pvr^{DN}* knockdown group and the *ppk/+* control were statistically different ($p= 0.05$, $n=56$, determined by two-sided permutation t-test). Similarly, the *ppk>UAS-Pvr^{DN}* knockdown group and the *UAS-Pvr^{DN}/+* control were statistically different ($p= 0.009$, $n=56$, determined by two-sided permutation t-test) (Fig. 8).

Figure 8

Overexpression of the dominant negative form of Pvr has increased nociceptive hypersensitivity

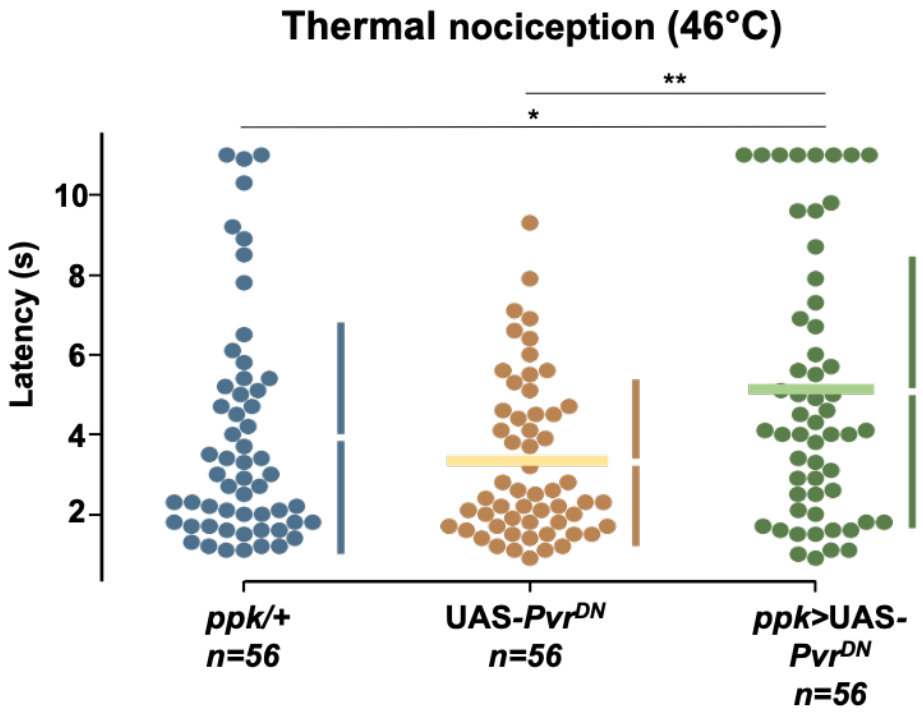

Figure note. Wandering 3rd instar Drosophila larva with defective Pvr receptors shows a statistically different and increased latency in response to noxious stimuli (46°C) when compared to the ppk/+ control (, $p \leq 0.05$) and UAS-Pvr^{DN}/+ control(**, $p \leq 0.01$, determined by two-sided permutation t-test). (n= 56 for all groups, horizontal bars indicate means and vertical error bars indicate 95% confidence intervals)*

Overexpression of Pvr in nociceptors increases nociceptor sensitivity

To understand if increased Pvr signaling increases sensitivity in nociceptors, Pvr was overexpressed in the nociceptors using the GAL4/UAS system. The Pvr overexpression group (*ppk>UAS-Pvr*) had Pvr overexpressed in *Drosophila* nociceptors. Wandering 3rd instar larvae were tested at 42°C and 46°C.

To understand how Pvr contributes to baseline thermal nociception, larvae were tested at 46°C. Analysis of experimental data showed that *ppk>UAS-Pvr* had an average latency of 1.9 s, the *ppk/+* control had an average latency of 4.5 s, and the *UAS-Pvr/+* only control has an average latency of 2.8 s. The *ppk>UAS-Pvr* overexpression group and the *ppk/+* control were statistically different (p= 0.000, n=65, determined by two-sided permutation t-test). Similarly, the *ppk>UAS-Pvr* overexpression group and the *UAS-Pvr* control were statistically different (p= 0.000, n=65, determined by two-sided permutation t-test) (Fig. 9A).

In order to gain insight into how Pvr contributes to increased sensitivity and to better understand the shorter latencies, larvae were tested at 42°C. Analysis of experimental data showed that *ppk>UAS-Pvr* had an average latency of 5.7 s, the *ppk/+* control had an average latency of 9.6 s, and the *UAS-Pvr/+* only control has an average latency of 9.6 s. The *ppk>UAS-Pvr* overexpression group and the *ppk/+* control were statistically different (p= 0.000, n=66, determined by two-sided permutation t-test). Similarly, the *ppk>UAS-Pvr* overexpression group and the *UAS-Pvr* control were statistically different (p= 0.000, n=66, determined by two-sided permutation t-test) (Fig. 9B).

Figure 9

Overexpression of Pvr in Nociceptors

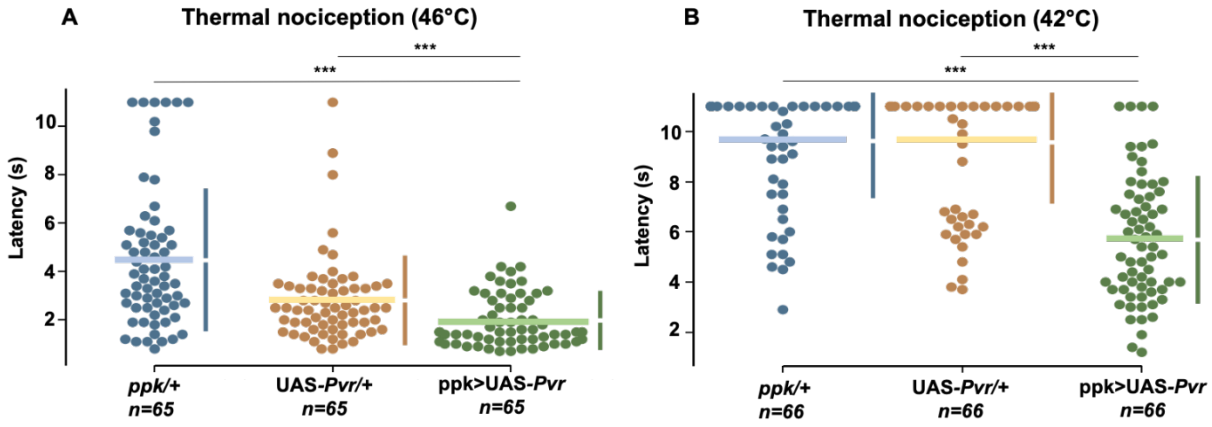

*Figure 8. (A) Overexpression of Pvr in nociceptors causes an increase in baseline nociception. Wandering 3rd instar Drosophila larva with Pvr overexpressed in nociceptors shows a statistically different and decreased latency in response to noxious stimuli (46°C) when compared to the ppk/+ control (***, $p \leq 0.001$) and UAS-Pvr/+ control (***, $p \leq 0.001$). (n= 65 for all groups, horizontal bars indicate means and vertical error bars indicate 95% confidence intervals) (B) Overexpression of Pvr in nociceptors causes an increase in nociceptive hypersensitization. Wandering 3rd instar larvae with Pvr overexpressed in nociceptors show a statistically different and decreased latency in response to noxious stimuli (42°C) when compared to the ppk/+ control (***, $p \leq 0.001$) and UAS-Pvr/+ control (***, $p \leq 0.001$, determined by two-sided permutation t-test). (n= 66 for all groups, horizontal bars indicate means and vertical error bars indicate 95% confidence intervals)*

Constitutive activation of Pvr in nociceptors increases nociceptor sensitivity

To understand if the direct effects of Pvr increased sensitivity in nociceptors, gain of function mutations were investigated, where Pvr was constitutively activated in the nociceptors. This means the Pvr receptor would be activated regardless of the presence of Pvf (ligand). The Pvr gain of function group (*ppk>UAS-Pvr^{CA}*) had the constitutively active form of Pvr overexpressed in class IV multidendritic neurons (Fig. 5). *Drosophila* larva were tested at 42°C and 46°C.

To understand how Pvr contributes to baseline nociception, *Drosophila* larvae with the constitutively activated form of Pvr overexpressed in the nociceptors were tested at 46°C. After analysis of the experiments performed at 46°C, the *ppk>UAS-Pvr^{CA}* group had an average latency of 3.0 s, the *ppk/+* control had an average latency of 4.2 s, and the *UAS-Pvr/+* only control has an average latency of 3.3 s. The *ppk>UAS-Pvr^{CA}* group and the *ppk/+* control were statistically different (p= 0.022, n=53, determined by two-sided permutation t-test). However, the *ppk>UAS-Pvr^{CA}* group and the *UAS-Pvr^{CA}/+* control was not statistically different statistically different (p= 0.529, n=53, determined by two-sided permutation t-test) (Fig. 10A).

To study the direct effects of Pvr in increasing nociceptive sensitization, *Drosophila* larvae with the constitutively activated form of Pvr overexpressed in the nociceptors were tested at 42°C. Analysis of experiments performed at 42°C, showed that the *ppk>UAS-Pvr^{CA}* group had an average latency of 5.4 s, the *ppk/+* control had an average latency of 8.9 s, and the *UAS-Pvr/+* only control has an average latency of 8.4 s. The *ppk>UAS-Pvr^{CA}* group and the *ppk/+* control were statistically different (p= 0.000, n=73, determined by two-sided permutation t-test). Similarly, the *ppk>UAS-Pvr^{CA}* group and the *UAS-Pvr^{CA}/+* control was also statistically different statistically different (p= 0.000, n=73, determined by two-sided permutation t-test) (Fig. 10B).

Figure 10

Overexpression of the constitutively activated form of Pvr in nociceptors

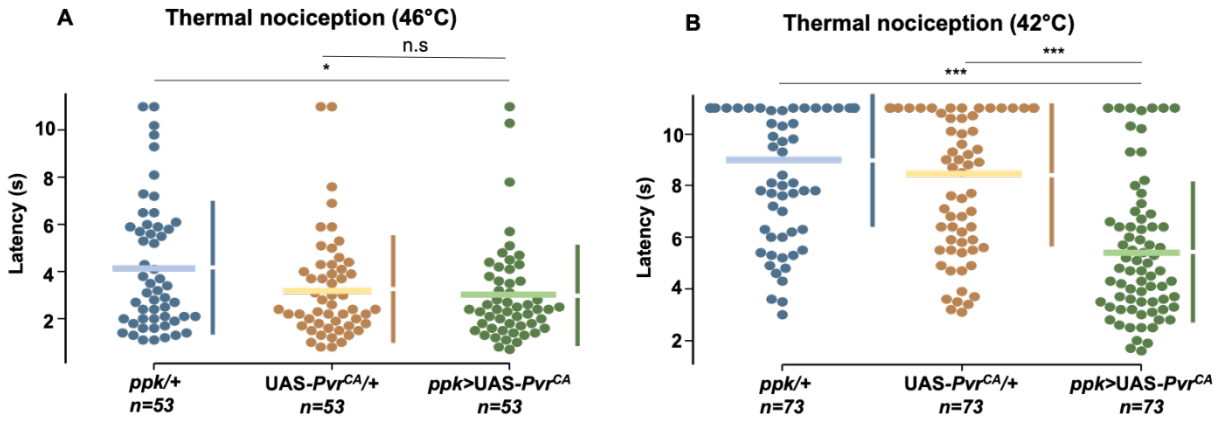

Figure note. (A) Overexpression of the constitutively activated form of Pvr in nociceptors causes an increase in baseline nociception. Wandering 3rd instar Drosophila larva with the constitutively activated form of Pvr overexpressed in nociceptors show a statistically different and decreased latency in response to noxious stimuli (46°C) when compared to the ppk/+ control (, $p \leq 0.001$) and UAS-Pvr/+ control (n.s., $p \geq 0.05$). (n= 53 for all groups, horizontal bars indicate means and vertical error bars indicate 95% confidence intervals). (B) Overexpression of the constitutively activated form of Pvr in nociceptors causes an increase in baseline nociception. Wandering 3rd instar Drosophila larva with the constitutively activated form of Pvr overexpressed in nociceptors show a statistically different and decreased latency in response to noxious stimuli (42°C) when compared to the ppk/+ control (***, $p \leq 0.001$) and UAS-Pvr/+ control (***, $p \leq 0.001$, determined by two-sided permutation t-test). (n= 73 for all groups, horizontal bars indicate means and vertical error bars indicate 95% confidence intervals)*

Increased Pvr Signaling disrupts Dendrite Branching

To determine whether *Pvr* signaling regulates nociceptor dendrite branching in *Drosophila,* the *ppk* GAL4 driver was used to drive expression of *mCD8::GFP* and *UAS-Pvr* (*Pvr* overexpression) in nociceptors, as well as *mCD8::GFP* and *UAS-Pvr-RNAi*$^{\#2}$ (*Pvr* knockdown) in nociceptors. These groups were compared to the control larvae where *ppk* GAL4 drove expression of *mCD8::GFP* in 36303 (RNAi control line). Comparison of dendritic branching between the *Pvr* overexpression group and the control group reveals obvious defects in the *Pvr* overexpression group. It can be observed that the dendritic branching pattern in the overexpression group (Fig. 11 C) is reduced in comparison to the control group (Fig. 11A). However, when comparing the knockdown (Fig. 11B) and control group (Fig. 11A), no obvious defects in dendrite branching can be found. These comparisons and observations are qualitative observations and additional experiments need to be performed in order to understand these effects.

Figure 11

Dendrite morphology observed under a confocal microscope

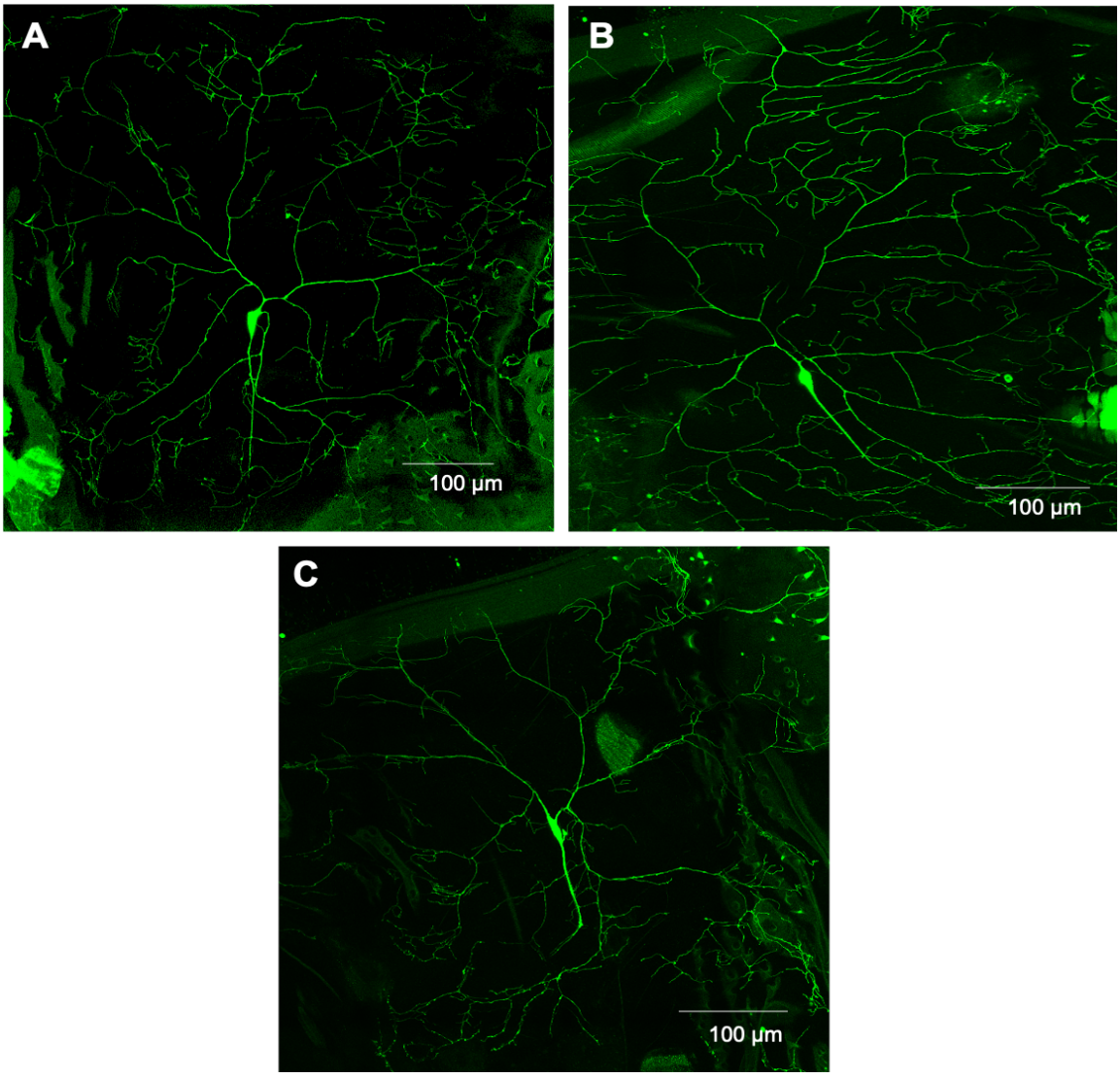

Figure note. (A) Confocal micrograph of Drosophila class IV md neurons expressing GFP. (B) Confocal micrograph of Drosophila class IV md neurons expressing ppk-GAL4>mCD8::GFP, UAS-Pvr-RNAi$^{\#2}$. (C) Confocal micrograph of Drosophila class IV md neurons expressing ppk-GAL4>mCD8::GFP, UAS-Pvr. Scale bar indicates 100 µm.

Discussion

PDGFR and VEGFR are growth factor receptors in vertebrates that have been shown to be heavily implicated in regulating nociception (Narita et al., 2005; Lopez-Bellido et al., 2019; Hulse et al., 2014) . In *Drosophila,* Pvr is an ortholog of human PDGFR and VEGFR. *Pvr* has been shown to be required for mechanical nociception (Lopez-Bellido et al., 2019); however, there are no studies exploring how *Pvr* mediates thermal nociception. This study demonstrates that *Pvr* is implicated in thermal nociception by using *Pvr* knockdown, overexpression, gain of function, and loss of function genotypes. The data presented shows that in baseline thermal nociception *P*vr is increasing sensitivity in the nociceptors.

Pvr is necessary in nociceptors for normal thermal nociception

The results of this study show that *Pvr* is necessary for thermal nociception. In the nociceptor-specific *Pvr* knockdown experiments (*RNAi$^{\#1}$ 8222R-3*) a lower response latency was found in knockdown larvae when compared to the GAL4-only and the RNAi-only controls. This signifies that the knockdown larvae had a behavioral defect in response to thermal stimulus. For this transgenic line, it can be observed that *Pvr* knockdown causes nociceptors to become more sensitive to noxious thermal stimulus causing them to produce a NEL response at a faster rate.

For confirmation, another nociceptor specific *Pvr* knockdown line (*RNAi$^{\#2}$ 37520*) was tested. Interestingly, this line provided results inconsistent with the first knockdown experiment. *Pvr* knockdown larvae (*RNAi$^{\#2}$ 37520*) had a higher response latency when compared to the GAL4-only and the RNAi-only controls. This signifies that *Pvr* knockdown causes a defective behavioral response to noxious thermal stimuli. In this case, nociceptors are less sensitive to noxious stimuli, which aligns with previous experiments exploring the effects of *Pvr* in mechanical nociception.

To further investigate the role of *Pvr* in thermal nociception, a dominant negative mutant was tested. The dominant negative mutants had a higher response latency when compared to the GAL4-only and *UAS*-only control groups. This behavioral defect in response to noxious thermal stimulus, signifies that nociceptors in this mutant line were less sensitive to noxious stimulus in comparison to the control. These results further align with the results from the previous knock-down experiments (*RNAi$^{\#2}$ 37520*) supporting the hypothesis that decreased Pvr signaling would reduce sensitivity in nociceptors to noxious thermal stimuli.

A deeper look into *Pvr* knockdown #1, revealed that knockdown of this RNAi line throughout the larvae using Act5C-GAL4 was lethal and the insertional mutation was semi-lethal according to NIG-fly (https://shigen.nig.ac.jp/fly/nigfly/index.jsp). This may be an effect of strong ubiquitous expression of RNAi. During cross production for larval testing, the RNAi #1 line also failed to reproduce and make progeny several times. These characteristics can signify that this transgenic line may need further testing in order to be used for studying nociception or it might have an off-target effect that makes it inappropriate for nociception studies. Furthermore, the results from the *Pvr* knockdown #2 and *Pvr* dominant negative groups align with previous predictions, suggesting that reduced *Pvr* function should lead to reduced thermal nociception sensitivity. However, the results from *Pvr* knockdown #1 varied from this prediction. This can also suggest that further testing is required in order to confirm the results of this experiment. This experiment suggests that in wild type flies, Pvr signaling is needed under baseline conditions to ensure normal nociceptor activity. Therefore, knockdown or any disruption of *Pvr* activity causes reduced sensitivity in nociceptors.

Pvr is sufficient to produce nociceptive sensitization

Nociceptor sensitization is normally caused by injury. However, for the purpose of this study Pvr is being activated without injury to determine if it produces sensitization on its own. This can provide evidence that Pvr acts to sensitize the neurons, but it does not necessarily indicate that *Pvr* is acting during injury induced sensitization. In order to discern whether *Pvr* is sufficient for producing nociceptive sensitization, *Pvr* was overexpressed and constitutively activated specifically in the nociceptors. In the experiments where *Pvr* was overexpressed, it was observed that the experimental larvae had a significantly reduced response latency in comparison to the GAL4-only and the *UAS*-only controls. This can be interpreted as the experimental larvae being increasingly sensitized to noxious thermal stimulus.

To investigate how *P*vr regulates thermal nociception, larvae with nociceptor specific overexpression of *Pvr* were tested at two different temperatures (42°C & 46°C). Increases and decreases in sensitivity can be measure by both temperatures, however 42°C is used when it can be expected to observe sensitization as there is more room to measure shorter latencies than controls under these conditions.

To gain a deeper understanding of the implications of gain of function in *Pvr,* Pvr was constitutively activated specifically in the nociceptors. This was done by replacing the extracellular ligand binding domain with a constitutive dimerization domain. In this experiment, experimental larvae had a reduced response latency to noxious thermal stimulus in comparison to the controls, further confirming that *Pvr* is regulating in thermal nociception.

For analyzing gain of function in *Pvr,* experiments with constitutively active *Pvr* were performed at 46°C and 42°C. At 46°C, mutant larvae had a significantly lower response rate in comparison to the GAL4-only control larvae but were not significantly different to the *UAS*-only

control group. This data may seem to be inconsistent with earlier predictions and results suggesting that *Pvr* is involved in regulating baseline thermal nociception, however based on how the data is analyzed at 46°C, this is not the case. Fig. 3B shows that at 46°C, even if there is an overlap with the control curve, reduced latencies not significantly different from control latencies might simply indicate that the assay is not sensitive enough to these changes in behavior at 46°C. Similarly, in the experiment with constitutive activation of *Pvr* at 46°C*,* despite the fact that the UAS-only control and the experimental group were not significantly different, both latencies were on the faster end of the scale suggesting that constitutive activation of *Pvr* is sufficient to cause baseline thermal nociception. The Pvr receptor, without activation from Pvf, is directly mediating thermal nociception, essentially suggesting Pvr modulates thermal nociception in wild type *Drosophila*. At 42°C, a clearer result of increased *Pvr* signaling can be observed. Both the *Pvr* overexpression and *Pvr* constitutive activation groups had a significantly reduced latency compared to controls which translates to a faster response to noxious thermal stimuli and increased sensitivity in nociceptors. These results along with the *Pvr* knockdown results clearly suggest that *Pvr* is necessary for baseline thermal nociception and sufficient for sensitized thermal nociception. The results align with previous predictions indicating that increased *Pvr* signaling causes increased sensitivity in nociceptors as well as previous experiments investigating increased *Pvr* signaling in mechanical nociception.

Pvr is Required for Thermal Nociception

In this study, it was predicted that an increase or decrease in Pvr signaling would cause phenotypic changes that were influenced by increasing sensitivity and decreasing sensitivity in the nociceptors, respectively. From the results of the experiments above, it can be concluded that *Pvr* is necessary and sufficient increase the sensitivity of thermal nociception. The results align

with previous predictions that were made in regard to how *Pvr* regulates mechanical sensitivity in *Drosophila.* Previous studies have found that Painless ion channels are required for mechanical hypersensitivity after Pvr signaling activation (Lopez-Bellido et al., 2019). Painless is an ion channel that is required in the nociceptors for both mechanical and thermal nociception. This suggests that Painless, by some means, is being activated by Pvf/Pvr signaling to increase sensitivity. Since painless has both mechanical and thermal nociception roles, it can be reasonable to hypothesize that the activation of painless by Pvf/Pvr would increase thermal nociception sensitivity.

In previous studies, it has been found that normal cells activate nonapoptotic JNK signaling in response to surrounding oncogenic mutant cells. This JNK activation causes upregulation of Pvr in *Drosophila* which ultimately leads to engulfment of neighboring oncogenic cells (Ohsawa et al., 2011). As JNK signaling is part of the TNF alpha pathway, it can be suggested that the Eiger/Wengen (TNFα/TNFR) signaling pathway upregulates Pvr which forms a complex with its ligand, Pvf, which by some means activates the Painless ion channel. This ion channel then activates nociception signaling by increasing the release of neurotransmitters, ultimately leading to increased nociceptor sensitivity (Fig. 12). However, Pvr/Pvf may also be acting independently of the TNFα signaling pathway. There may be several independent pathways that work to activate thermal nociception in *Drosophila.* A known pathway that works independently from the TNFα signaling pathway is the Hedgehog (Hh) signaling pathway. This Hh pathway works parallel to the TNFα pathway in order to induce thermal allodynia in *Drosophila* (Babcock et al., 2011). The relationship between Pvr signaling and other nociceptive signaling pathways could be determined by genetic epistasis experiments in the future.

Figure 12

Proposed mechanism for Pvr signaling

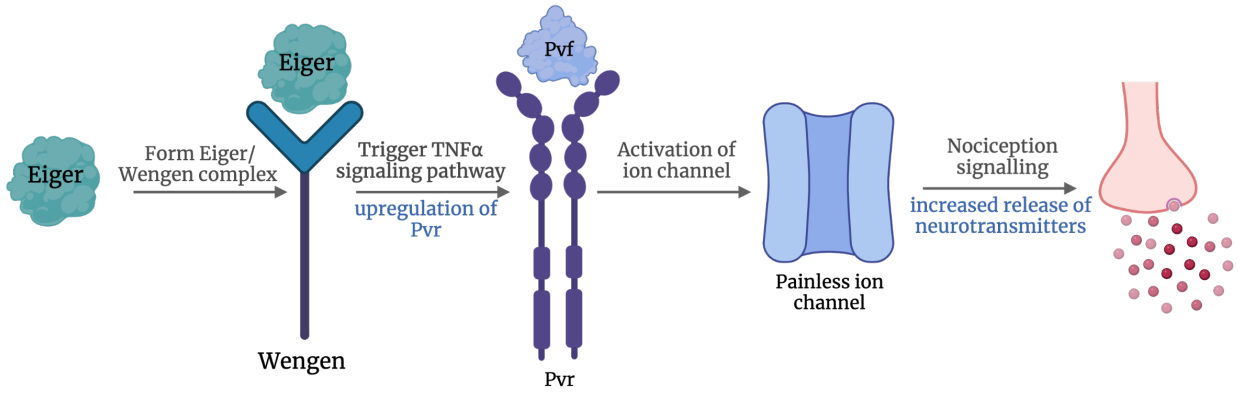

Figure note. From left to right – Eiger binds to Wengen and forms an Eiger/Wengen complex that initiates the upregulation of Pvr. Pvf binds to Pvr to form the Pvf/Pvr complex and triggers a signaling pathway. These signals work to activate the painless ion channel which initiates nociception signaling and leads to the increased release of neurotransmitters.

Pvr is Implicated in Dendrite Branching

Analysis of confocal microscopy revealed that the *Pvr* overexpression group has obvious defects that can be observed in dendrite branching of class IV multidendritic neurons. This defect however was not observed in the *Pvr* knockdown group. Following this data, it can be suggested that knockdown of *Pvr* in nociceptors is not interfering with mechanisms that are involved in dendrite branching, however when *Pvr* is overexpressed in the nociceptors, there might be a significant change in dendrite branching mechanisms which can be observed in Fig. 11.

Previous studies that investigated how dendritic branching is altered due to manipulation of Pvr signaling found that *Pvr* mutants had reduced dendritic arbor complexity which was not observed in this study. This may be due to the reduced sample size and qualitative analysis that

was done as opposed to Scholl analysis with a larger sample size. Previous studies have not investigated the effects of increased Pvr signaling that were found in this study which indicates that further testing and analysis of Pvr overexpression in regulating dendritic branching is required to understand these findings.

Future Directions

This study establishes that *Pvr* is required for thermal nociception and highlights a novel overlap in the mechanical and thermal nociception pathways. To further determine how *Pvr* is involved in nociception, experiments investigating the role of *Pvr* in other pain signaling pathways such as chemical nociception and UV radiation can be studied. This can highlight an overlap of *Pvr* involvement in multiple nociception pathways ultimately allowing for the development of novel and beneficial therapeutic treatments.

Additionally, since *Pvr* has not been widely studied in *Drosophila* thermal nociception, experiments manipulating the *Pvr* ligand, *Pvf,* can be studied. Since *Pvf* is secreted throughout the larval body, there may be a higher magnitude of response or sensitivity observed with *Pvf* defects. Based on previous studies, it can be predicted that *Pvf2* and *Pvf3* knockdown will have strong phenotypic defects in response to noxious thermal stimulus; however, *Pvf1* may not show any significant changes in response to noxious thermal stimulus. Additionally, knockdown of *Pvf2* and *Pvf3* together may also cause a stronger defect than individual knockdown as it has been suggested that they may be functioning in the same tissue and may also be working together (Lopez-Ballido et al., 2019).

To further our understanding of the role of *Pvr* in thermal nociception, we can study its implications in thermal hyperalgesia and allodynia. Previous studies have shown that these path-

ways can function differently from each other and it can be important to understand if *Pvr* is implicated in both. Furthermore, it can be interesting to study whether *Pvr* is involved in maintaining long term hyperalgesia and allodynia. To study thermal hyperalgesia and allodynia, UV sensitization assays can be used, where UV radiation is used to induce tissue injury followed by a recovery phase after which a thermal assay is performed. Moreover, adult flies can be used to explore the maintenance of hyperalgesia and allodynia post amputation (Massingham et al., 2021) and based on our hypothesis, *Pvr* knockdown should prevent UV-induced sensitization.

Epistasis studies can also be performed to determine that the Painless ion channel is being activated by Pvf/Pvr signaling and initiating nociception sensitivity. Since previous studies have established its requirement in mechanical nociception, it can be reasonable to hypothesize that painless is working regulate thermal nociception. Additionally, confirmation studies can also be performed to investigate whether Eiger/Wengen signaling is occurring upstream or downstream of Pvr. Since TNFα plays a major role in nociception, this confirmation can be beneficial for assembling the thermal nociception pathway.

References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F., George, R. A., Lewis, S. E., Richards, S., Ashburner, M., Henderson, S. N., Sutton, G. G., Wortman, J. R., Yandell, M. D., Zhang, Q., Chen, L. X., … Venter, J. C. (2000). The genome sequence of Drosophila melanogaster. *Science (New York, N.Y.)*, *287*(5461), 2185–2195. https://doi.org/10.1126/science.287.5461.2185

Ainsley, J. A., Pettus, J. M., Bosenko, D., Gerstein, C. E., Zinkevich, N., Anderson, M. G., Adams, C. M., Welsh, M. J., & Johnson, W. A. (2003). Enhanced locomotion caused by loss of the Drosophila DEG/ENaC protein Pickpocket1. *Current biology : CB*, *13*(17), 1557–1563. https://doi.org/10.1016/s0960-9822(03)00596-7

Babcock, D. T., Landry, C., & Galko, M. J. (2009). Cytokine signaling mediates UV-induced nociceptive sensitization in Drosophila larvae. *Current biology : CB*, *19*(10), 799–806. https://doi.org/10.1016/j.cub.2009.03.062

Babcock, D. T., Shi, S., Jo, J., Shaw, M., Gutstein, H. B., & Galko, M. J. (2011). Hedgehog signaling regulates nociceptive sensitization. *Current biology : CB*, *21*(18), 1525–1533. https://doi.org/10.1016/j.cub.2011.08.020

Ballmer-Hofer, K., Andersson, A. E., Ratcliffe, L. E., & Berger, P. (2011). Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles thereby specifying signal output. *Blood*, *118*(3), 816–826. https://doi.org/10.1182/blood-2011-01-328773

Barkai, O., Puig, S., Lev, S., Title, B., Katz, B., Eli-Berchoer, L., Gutstein, H. B., & Binshtok, A. M. (2019). Platelet-derived growth factor activates nociceptive neurons by inhibiting M-current and contributes to inflammatory pain. *Pain*, *160*(6), 1281–1296. https://doi.org/10.1097/j.pain.0000000000001523

Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H., & Tomic-Canic, M. (2008). Growth factors and cytokines in wound healing. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*, *16*(5), 585–601. https://doi.org/10.1111/j.1524-475X.2008.00410.x

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell*, *139*(2), 267–284. https://doi.org/10.1016/j.cell.2009.09.028

Beazley-Long, N., Hua, J., Jehle, T., Hulse, R. P., Dersch, R., Lehrling, C., Bevan, H., Qiu, Y., Lagrèze, W. A., Wynick, D., Churchill, A. J., Kehoe, P., Harper, S. J., Bates, D. O., & Donaldson, L. F. (2013). VEGF-A165b is an endogenous neuroprotective splice isoform of vascular endothelial growth factor A in vivo and in vitro. *The American journal of pathology*, *183*(3), 918–929. https://doi.org/10.1016/j.ajpath.2013.05.031

Bond, A. M., Bhalala, O. G., & Kessler, J. A. (2012). The dynamic role of bone morphogenetic proteins in neural stem cell fate and maturation. *Developmental neurobiology*, *72*(7), 1068–1084. https://doi.org/10.1002/dneu.22022

Brown, D. A., & Adams, P. R. (1980). Muscarinic suppression of a novel voltage-sensitive K+ current in a vertebrate neurone. *Nature*, *283*(5748), 673–676. https://doi.org/10.1038/283673a0

Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I., & Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science (New York, N.Y.)*, *288*(5464), 306–313. https://doi.org/10.1126/science.288.5464.306

Chen, P. H., Chen, X., & He, X. (2013). Platelet-derived growth factors and their receptors: structural and functional perspectives. *Biochimica et biophysica acta*, *1834*(10), 2176–2186. https://doi.org/10.1016/j.bbapap.2012.10.015

Collins, R. T., & Cohen, S. M. (2005). A genetic screen in Drosophila for identifying novel components of the hedgehog signaling pathway. *Genetics*, *170*(1), 173–184. https://doi.org/10.1534/genetics.104.039420

Coste, B., Mathur, J., Schmidt, M., Earley, T. J., Ranade, S., Petrus, M. J., Dubin, A. E., & Patapoutian, A. (2010). Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science (New York, N.Y.)*, *330*(6000), 55–60. https://doi.org/10.1126/science.1193270

Derderian, C., & Tadi, P. (2022). Physiology, Withdrawal Response. In StatPearls. Treasure Island (FL).

Dubin, A. E., & Patapoutian, A. (2010). Nociceptors: the sensors of the pain pathway. *The Journal of clinical investigation*, *120*(11), 3760–3772. https://doi.org/10.1172/JCI42843

Duchek, P., Somogyi, K., Jékely, G., Beccari, S., & Rørth, P. (2001). Guidance of cell migration by the Drosophila PDGF/VEGF receptor. *Cell*, *107*(1), 17–26. https://doi.org/10.1016/s0092-8674(01)00502-5

Dydyk, A. M., & Conermann, T. (2023). *Chronic Pain*. StatPearls Publishing LLC. https://www.ncbi.nlm.nih.gov/books/NBK553030/

Eccleston, P. A., Funa, K., & Heldin, C. H. (1993). Expression of platelet-derived growth factor (PDGF) and PDGF alpha- and beta-receptors in the peripheral nervous system: an analysis of sciatic nerve and dorsal root ganglia. *Developmental biology*, *155*(2), 459–470. https://doi.org/10.1006/dbio.1993.1044

El-Tallawy, S. N., Nalamasu, R., Salem, G. I., LeQuang, J. A. K., Pergolizzi, J. V., & Christo, P. J. (2021). Management of Musculoskeletal Pain: An Update with Emphasis on Chronic Musculoskeletal Pain. *Pain and therapy*, *10*(1), 181–209. https://doi.org/10.1007/s40122-021-00235-2

Follansbee, T. L., Gjelsvik, K. J., Brann, C. L., McParland, A. L., Longhurst, C. A., Galko, M. J., & Ganter, G. K. (2017). *Drosophila* Nociceptive Sensitization Requires BMP Signaling via the Canonical SMAD Pathway. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *37*(35), 8524–8533. https://doi.org/10.1523/JNEUROSCI.3458-16.2017

Fredriksson, L., Li, H., & Eriksson, U. (2004). The PDGF family: four gene products form five dimeric isoforms. *Cytokine & growth factor reviews*, *15*(4), 197–204. https://doi.org/10.1016/j.cytogfr.2004.03.007

Gjelsvik, K. J., Follansbee, T. L., & Ganter, G. K. (2018). Bone Morphogenetic Protein Glass Bottom Boat (BMP5/6/7/8) and its receptor Wishful Thinking (BMPRII) are required for injury-induced allodynia in Drosophila. *Molecular pain*, *14*, 1744806918802703. https://doi.org/10.1177/1744806918802703

Gold, M. S., & Gebhart, G. F. (2010). Nociceptor sensitization in pain pathogenesis. *Nature medicine*, *16*(11), 1248–1257. https://doi.org/10.1038/nm.2235

Grueber, W. B., Jan, L. Y., & Jan, Y. N. (2002). Tiling of the Drosophila epidermis by multidendritic sensory neurons. *Development (Cambridge, England)*, *129*(12), 2867–2878. https://doi.org/10.1242/dev.129.12.2867

Grueber, W. B., Ye, B., Yang, C. H., Younger, S., Borden, K., Jan, L. Y., & Jan, Y. N. (2007). Projections of Drosophila multidendritic neurons in the central nervous system: links with peripheral dendrite morphology. *Development (Cambridge, England)*, *134*(1), 55–64. https://doi.org/10.1242/dev.02666

He, L., Gulyanon, S., Mihovilovic Skanata, M., Karagyozov, D., Heckscher, E. S., Krieg, M., Tsechpenakis, G., Gershow, M., & Tracey, W. D., Jr (2019). Direction Selectivity in Drosophila

Proprioceptors Requires the Mechanosensory Channel Tmc. *Current biology : CB*, *29*(6), 945–956.e3. https://doi.org/10.1016/j.cub.2019.02.025

Heldin C. H. (2013). Targeting the PDGF signaling pathway in tumor treatment. *Cell communication and signaling : CCS*, *11*, 97. https://doi.org/10.1186/1478-811X-11-97

Heldin, C. H., & Westermark, B. (1990). Platelet-derived growth factor: mechanism of action and possible in vivo function. *Cell regulation*, *1*(8), 555–566. https://doi.org/10.1091/mbc.1.8.555

Herman, J. A., Willits, A. B., & Bellemer, A. (2018). Gαq and Phospholipase Cβ signaling regulate nociceptor sensitivity in *Drosophila melanogaster* larvae. *PeerJ, 6*, e5632. https://doi.org/10.7717/peerj.5632

Heigwer, F., Port, F., & Boutros, M. (2018). RNA Interference (RNAi) Screening in *Drosophila*. *Genetics*, *208*(3), 853–874. https://doi.org/10.1534/genetics.117.300077

Hulse, R. P., Beazley-Long, N., Hua, J., Kennedy, H., Prager, J., Bevan, H., Qiu, Y., Fernandes, E. S., Gammons, M. V., Ballmer-Hofer, K., Gittenberger de Groot, A. C., Churchill, A. J., Harper, S. J., Brain, S. D., Bates, D. O., & Donaldson, L. F. (2014). Regulation of alternative VEGF-A mRNA splicing is a therapeutic target for analgesia. *Neurobiology of disease*, *71*, 245–259. https://doi.org/10.1016/j.nbd.2014.08.012

Hwang, R. Y., Zhong, L., Xu, Y., Johnson, T., Zhang, F., Deisseroth, K., & Tracey, W. D. (2007). Nociceptive neurons protect Drosophila larvae from parasitoid wasps. *Current biology : CB*, *17*(24), 2105–2116. https://doi.org/10.1016/j.cub.2007.11.029

Im, S. H., Takle, K., Jo, J., Babcock, D. T., Ma, Z., Xiang, Y., & Galko, M. J. (2015). Tachykinin acts upstream of autocrine Hedgehog signaling during nociceptive sensitization in Drosophila. *eLife*, *4*, e10735. https://doi.org/10.7554/eLife.10735

Jin, K. L., Mao, X. O., & Greenberg, D. A. (2000). Vascular endothelial growth factor rescues HN33 neural cells from death induced by serum withdrawal. *Journal of molecular neuroscience : MN*, *14*(3), 197–203. https://doi.org/10.1385/JMN:14:3:197

Kandel, E. R., Schwartz, J. H., Jessell, T. M., Siegelbaum, S., & Hudspeth, A. J. (2013) Principles of neural science (5th ed.).

Kang, K., Pulver, S. R., Panzano, V. C., Chang, E. C., Griffith, L. C., Theobald, D. L., & Garrity, P. A. (2010). Analysis of Drosophila TRPA1 reveals an ancient originfor human chemical nociception. Nature, 464(7288), 597-600. doi:10.1038/nature08848

Kauppila, S., Maaty, W. S., Chen, P., Tomar, R. S., Eby, M. T., Chapo, J., Chew, S., Rathore, N., Zachariah, S., Sinha, S. K., Abrams, J. M., & Chaudhary, P. M. (2003). Eiger and its receptor, Wengen, comprise a TNF-like system in Drosophila. *Oncogene*, *22*(31), 4860–4867. https://doi.org/10.1038/sj.onc.1206715

Kim, S. E., Coste, B., Chadha, A., Cook, B., & Patapoutian, A. (2012). The role of Drosophila Piezo in mechanical nociception. *Nature*, *483*(7388), 209–212. https://doi.org/10.1038/nature10801

Leung, L., & Cahill, C. M. (2010). TNF-alpha and neuropathic pain--a review. *Journal of neuroinflammation*, *7*, 27. https://doi.org/10.1186/1742-2094-7-27

Lopez-Bellido, R., Puig, S., Huang, P. J., Tsai, C. R., Turner, H. N., Galko, M. J., & Gutstein, H. B. (2019). Growth Factor Signaling Regulates Mechanical Nociception in Flies and Vertebrates. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *39*(30), 6012–6030. https://doi.org/10.1523/JNEUROSCI.2950-18.2019

Lynch, S. E., Nixon, J. C., Colvin, R. B., & Antoniades, H. N. (1987). Role of platelet-derived growth factor in wound healing: synergistic effects with other growth factors. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(21), 7696–7700. https://doi.org/10.1073/pnas.84.21.7696

Massingham, J. N., Baron, O., & Neely, G. G. (2021). Evaluating Baseline and Sensitised Heat Nociception in Adult *Drosophila*. *Bio-protocol*, *11*(13), e4079. https://doi.org/10.21769/BioProtoc.4079

Mills, S. E. E., Nicolson, K. P., & Smith, B. H. (2019). Chronic pain: a review of its epidemiology and associated factors in population-based studies. *British journal of anaesthesia*, *123*(2), e273–e283. https://doi.org/10.1016/j.bja.2019.03.023

Mondal, B. C., Shim, J., Evans, C. J., & Banerjee, U. (2014). Pvr expression regulators in equilibrium signal control and maintenance of Drosophila blood progenitors. *eLife*, *3*, e03626. https://doi.org/10.7554/eLife.03626

Munier, A. I., Doucet, D., Perrodou, E., Zachary, D., Meister, M., Hoffmann, J. A., Janeway, C. A., Jr, & Lagueux, M. (2002). PVF2, a PDGF/VEGF-like growth factor, induces hemocyte proliferation in Drosophila larvae. *EMBO reports*, *3*(12), 1195–1200. https://doi.org/10.1093/embo-reports/kvf242

Narita, M., Usui, A., Narita, M., Niikura, K., Nozaki, H., Khotib, J., Nagumo, Y., Yajima, Y., & Suzuki, T. (2005). Protease-activated receptor-1 and platelet-derived growth factor in spinal cord neurons are implicated in neuropathic pain after nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *25*(43), 10000–10009. https://doi.org/10.1523/JNEUROSCI.2507-05.2005

Neely, G. G., Hess, A., Costigan, M., Keene, A. C., Goulas, S., Langeslag, M., Griffin, R. S., Belfer, I., Dai, F., Smith, S. B., Diatchenko, L., Gupta, V., Xia, C. P., Amann, S., Kreitz, S., Heindl-Erdmann, C., Wolz, S., Ly, C. V., Arora, S., Sarangi, R., … Penninger, J. M. (2010). A genome-wide Drosophila screen for heat nociception identifies α2δ3 as an evolutionarily conserved pain gene. *Cell*, *143*(4), 628–638. https://doi.org/10.1016/j.cell.2010.09.047

Niu, G., & Chen, X. (2010). Vascular endothelial growth factor as an anti-angiogenic target for cancer therapy. *Current drug targets*, *11*(8), 1000–1017. https://doi.org/10.2174/138945010791591395

Okuda, H., Ishikawa, T., Hori, K., Kwankaew, N., & Ozaki, N. (2022). Hedgehog signaling plays a crucial role in hyperalgesia associated with neuropathic pain in mice. *Journal of neurochemistry*, *162*(2), 207–220. https://doi.org/10.1111/jnc.15613

Ohsawa, S., Sugimura, K., Takino, K., Xu, T., Miyawaki, A., & Igaki, T. (2011). Elimination of oncogenic neighbors by JNK-mediated engulfment in Drosophila. *Developmental cell*, *20*(3), 315–328. https://doi.org/10.1016/j.devcel.2011.02.007

Piper, M. D. W., & Partridge, L. (2018). Drosophila as a model for ageing. *Biochimica et biophysica acta. Molecular basis of disease*, *1864*(9 Pt A), 2707–2717. https://doi.org/10.1016/j.bbadis.2017.09.016

Rizki, T. M., Rizki, R. M., & Carton, Y. (1990). Leptopilina heterotoma and L. boulardi: strategies to avoid cellular defense responses of Drosophila melanogaster. *Experimental parasitology*, *70*(4), 466–475. https://doi.org/10.1016/0014-4894(90)90131-u

Robertson, J. L., Tsubouchi, A., & Tracey, W. D. (2013). Larval defense against attack from parasitoid wasps requires nociceptive neurons. *PloS one*, *8*(10), e78704. https://doi.org/10.1371/journal.pone.0078704

Rosenblum, A., Marsch, L. A., Joseph, H., & Portenoy, R. K. (2008). Opioids and the treatment of chronic pain: controversies, current status, and future directions. *Experimental and clinical psychopharmacology*, *16*(5), 405–416. https://doi.org/10.1037/a0013628

Saito, S., & Tominaga, M. (2017). Evolutionary tuning of TRPA1 and TRPV1 thermal and chemical sensitivity in vertebrates. *Temperature (Austin, Tex.)*, *4*(2), 141–152. https://doi.org/10.1080/23328940.2017.1315478

Samanta, A., Hughes, T. E. T., & Moiseenkova-Bell, V. Y. (2018). Transient Receptor Potential (TRP) Channels. *Sub-cellular biochemistry*, *87*, 141–165. https://doi.org/10.1007/978-981-10-7757-9_6

Smith, E. S., & Lewin, G. R. (2009). Nociceptors: a phylogenetic view. *Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology*, *195*(12), 1089–1106. https://doi.org/10.1007/s00359-009-0482-z

St Johnston D. (2002). The art and design of genetic screens: Drosophila melanogaster. *Nature reviews. Genetics*, *3*(3), 176–188. https://doi.org/10.1038/nrg751

Story, G. M., Peier, A. M., Reeve, A. J., Eid, S. R., Mosbacher, J., Hricik, T. R., Earley, T. J., Hergarden, A. C., Andersson, D. A., Hwang, S. W., McIntyre, P., Jegla, T., Bevan, S., & Patapoutian, A. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, *112*(6), 819–829. https://doi.org/10.1016/s0092-8674(03)00158-2

Tracey, W. D., Jr, Wilson, R. I., Laurent, G., & Benzer, S. (2003). painless, a Drosophila gene essential for nociception. *Cell*, *113*(2), 261–273. https://doi.org/10.1016/s0092-8674(03)00272-1

Tsubouchi, A., Caldwell, J. C., & Tracey, W. D. (2012). Dendritic filopodia, Ripped Pocket, NOMPC, and NMDARs contribute to the sense of touch in Drosophila larvae. *Current biology : CB*, *22*(22), 2124–2134. https://doi.org/10.1016/j.cub.2012.09.019

Ugur, B., Chen, K., & Bellen, H. J. (2016). Drosophila tools and assays for the study of human diseases. *Disease models & mechanisms*, *9*(3), 235–244. https://doi.org/10.1242/dmm.023762

Will, J. S., Bury, D. C., & Miller, J. A. (2018). Mechanical Low Back Pain. *American family physician*, *98*(7), 421–428.

Withers, G. S., Higgins, D., Charette, M., & Banker, G. (2000). Bone morphogenetic protein-7 enhances dendritic growth and receptivity to innervation in cultured hippocampal neurons. *The European journal of neuroscience*, *12*(1), 106–116. https://doi.org/10.1046/j.1460-9568.2000.00889.x

Zachary I. (2005). Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. *Neuro-Signals*, *14*(5), 207–221. https://doi.org/10.1159/000088637

Zhong, J., & Zou, H. (2014). BMP signaling in axon regeneration. *Current opinion in neurobiology*, *27*, 127–134. https://doi.org/10.1016/j.conb.2014.03.009

Zhong, L., Bellemer, A., Yan, H., Ken, H., Jessica, R., Hwang, R. Y., Pitt, G. S., & Tracey, W. D. (2012). Thermosensory and nonthermosensory isoforms of Drosophila melanogaster TRPA1 reveal heat-sensor domains of a thermoTRP Channel. *Cell reports*, *1*(1), 43–55. https://doi.org/10.1016/j.celrep.2011.11.002

Zhong, L., Hwang, R. Y., & Tracey, W. D. (2010). Pickpocket is a DEG/ENaC protein required for mechanical nociception in Drosophila larvae. *Current biology : CB*, *20*(5), 429–434. https://doi.org/10.1016/j.cub.2009.12.057

Vita

Ishani Chattopadhayay was born in Fremont, California, to Biru and Kakoli Chattopadhayay. She graduated from Appalachian State University in May 2021 and was awarded a Bachelor of Science degree in Biology. The following semester, she entered the Cratis D. Williams Graduate School at Appalachian State University to pursue a Master of Science in Biology. Following completion of the Master of Science degree, she will relocate to Bloomington, Indiana, where she will Pursue a Ph.D. in Cell, Molecular, and Cancer Biology at Indiana university.