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Genetic Determinants of Primary Nociceptor Sensitivity in Drosophila melanogaster

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GENETIC DETERMINANTS OF PRIMARY NOCICEPTOR SENSITIVITY

IN *DROSOPHILA MELANOGASTER*

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An Abstract of the Dissertation Presented
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Abnormal pain affects ~50 million adults nationwide. With many of the current treatment options for chronic pain, such as opioid analgesics, carrying side effects such as the threat for addiction**,** research into safer and more effective options for chronic pain relief is crucial. Abnormal alterations in nociceptive sensitivity, which is the sensitivity of peripheral sensory neurons that detect noxious stimuli, can underlie and perpetuate chronic pain. However, much is still unknown about the mechanism of how these abnormal alterations in sensitivity occur. To help elucidate genetic components controlling nociceptive sensitivity, the *Drosophila melanogaster* larval nociception model has been used to characterize well-conserved pathways through the use of genetic modification and/or ultraviolet (UV) irradiation injury to alter the sensitivity of experimental animals. We have continued to build upon this knowledge to reveal a more complete system for how nociceptive sensitivity can be altered, even without injury, by investigation into the potential roles of other novel genes/signaling pathways including, Arm, a component within the Wnt/Wg signaling pathway. Our findings indicate Arm to be a facilitator in controlling nociceptive sensitivity in the absence of injury, by maintaining baseline sensitivity. In an effort to also explore the mechanisms of the primary nociceptors (nociceptors which directly detect noxious stimuli), we conducted bioinformatic analysis of RNA transcripts derived specifically from the nociceptors of larvae after UV injury. Results from this effort led to the discovery of a downregulation in serine proteases during peak allodynia (when something not normally noxious

becomes so) development. Results also led to the hypothesis that upregulated *Rgk1* and *AnxB11* were involved in recovery of the nociceptor from hyperalgesia. This was supported by the knockdown of Rgk1 and AnxB11 having led to nociceptor hypersensitivity in larvae. And in an effort to move the methodology of our field forward, and because the larval stages of fruit fly development are relatively brief, we developed a methodology that allows longer term experimentation of nociceptive sensitization after injury in adult fruit flies. Ultimately, our research uncovered components involved in nociceptive sensitivity, which will hopefully lead to uncovering better treatment options for abnormal pain in the future.

DEDICATION

In loving memory of my father, R.P. Hale.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1°Ab = Primary antibody

ANKTM1= Ankyrin-like with transmembrane domains protein 1

AnxB11= Annexin B11

Arm= Armadillo

Arr= Arrow

ASIC1= Acid-Sensing Ion Channel 1

Aδ= A-Delta fibers

BMP= Bone Morphogenetic Protein

BP= Biological Process

Brk= Brinker

BSA= Bovine Serum Albumin

C2da= Class II Dendrite Arborization Neurons

C4da/CIVda= Class IV Dendrite Arborization Neurons

CB_1= Cannabinoid Receptor Type 1

CB_2= Cannabinoid Receptor Type 2

CC= Cellular Component

CGRP= Calcitonin Gene Related Peptide

ChR2= Channelrhodopsin-2

CK1γ1= Casein Kinase 1 Gamma 1

ClC-3 = Chloride Voltage-gated Channel 3

ClC-c= Chloride Channel-C

CNS= Central Nervous System

COX= Cyclooxygenase

CTCF= Corrected Total Cell Fluorescence

DAPI= 4', 6-diamidino-2-phenylindole

DAVID= The Database for Annotation, Visualization and Integrated Discovery

DEG= Differentially Expressed Genes

DEG/ENaC= Degenerin Epithelial Sodium Channel

DP-ilp7= Dorsal Pair Insulin Peptide 7 Neurons

Dpp= Decapentaplegic

DRG= Dorsal Root Ganglia

DSHB= Developmental Studies Hybridoma Bank

dTk= *Drosophila* Tachykinin

DTKR= *Drosophila* Tachykinin Receptor

dTrpA1= *Drosophila* Transient Receptor Cation Channel A1

EcRA= Ecdysone Receptor A

Eip63E= Ecdysone-induced Protein 63E

FDR= False-Discovery Rate

GABA= γ-aminobutyric acid

GEM= GTP binding protein overexpressed in skeletal muscle

GFP= Green Fluorescent Protein

Gish= Gilgamesh

GO= Gene Ontology

GOrilla= Gene Ontology Enrichment Analysis and Visualization Tool

GPCR= G-protein coupled receptor

Gro= Groucho

GTP= Guanosine Triphosphate

Hh= Hedgehog

HyD= Hybrid Detector

IASP= International Association for the Study of Pain

ICD-11= International Classification of Diseases 11

IR= Inverted Repeat transgene (RNA interference technology)

KEGG= Kyoto Encyclopedia of Genes and Genomes

LRP 5/6= Lipoprotein Receptor-related Protein 5 and 6

Mad= Mothers Against Dpp

mCSIs= Class IV Dendritic Arborization Second Order Interneurons

Md= Multidendritic

Med= Medea

MF= Molecular Function

MiMIC= Minos Mediated Integration Cassette

mRNA= Messenger RNA

NA= Numerical Aperture

NGF= Nerve Growth Factor

NGS= Normal Goat Serum

NIGMS= National Institute of General Medicine Sciences

NIH= National Institutes of Health

NMJ= Neuromuscular Junction

ns= Not Significant

NSAIDs= Non-Steroidal Anti-Inflammatory Drugs

OE= Overexpression

OTC= Over The Counter

p adj= Adjusted *p* value

PAG= Periaqueductal Grey

Pain= Painless

PAR2= Proteinase-Activated Receptor 2

PBS= Phosphate Buffered Saline

PBST= Phosphate Buffered Saline and Triton-X

PCA= Principal Components Analysis Plot

PNS= Peripheral Nervous System

Ppk= Pickpocket

Rem= RRAD and Gem Like GTPase

RGK= Rad, Gem/Kir family proteins

Rgk1= Rad, Gem/Kir family member 1

RNAi= RNA interference technology

RNA= ribonucleic acid

ROI= Region of Interest

RpL10Ab= Ribosomal Protein L10Ab

RT= Room Temperature

Seq= sequencing (high-throughput sequencing of RNA)

Shn= Schnurri

Sna= Segmental Nerve A

tdTomato= Tandem Dimer Tomato

TeTxLC= Tetanus Toxin Light Chain

TG= Trigeminal Ganglia

TGF-β= Transforming Growth Factor Beta

TNF= Tumor Necrosis Factor

TNF-α= Tumor Necrosis Factor alpha

TNT= Tetanus Toxin

TRAP= Translating Ribosome Affinity Purification

TRiP= Transgenic RNAi Project

TRP= Transient Receptor Potential

TRPA1= Transient Receptor Potential Cation Channel A1

TRPM8= Transient Receptor Potential Cation Channel Subfamily M Member 8

TRPV1= Transient Receptor Potential Cation Subfamily V Member 1

UAS= Upstream Activation Sequence

UV= Ultraviolet

v = Vermilion

VNC= Ventral Nerve Cord

w = White

Wg= Wingless

y = yellow

CHAPTER 1

1. INTRODUCTION TO CHRONIC PAIN, NOCICEPTIVE SENSITIZATION, AND THE *DROSOPHILA MELANOGASTER* MODEL FOR CHRONIC PAIN INVESTIGATION

1.1 Introduction and statement of significance

Chronic pain has been estimated to affect ~50 million adults nationwide (Dahlhamer et al., 2018; Yong et al., 2021, 2022; Zelaya et al., 2020), and while we have effective drugs for treating acute pain (such as opioids) these drugs can come with dangerous side effects (including addiction) which has led to the opioid addiction crisis we are currently battling in the US (Benyamin et al., 2008; Buntin-Mushock et al., 2005; Christie, 2008; Eddy et al., 1959; Groenewald et al., 2019; Hay et al., 2009; Vowles et al., 2015). This crisis is also acutely felt here in Maine, where a recent annual report from the Office of the Attorney General in Maine indicated a record increase in overdose deaths in 2020 with 23% of those overdose deaths due to pharmaceutical opioids (Sorg, 2021). Given the scope of this problem, investigation into better drug targets for treating pain is crucial. Despite this need, successful drug development for chronic pain has been laborious, mostly due to a lack of understanding of the multi-faceted mechanisms of chronic pain development and the numerous different pathological manifestations that can result (Kosek et al., 2016; Price et al., 2018; Treede et al., 2019). Included in this lack of understanding are the mechanisms involved in nociceptor sensitization, the sensitivity of peripheral sensory neurons known to detect noxious stimuli, which is involved in the pain signaling pathway and known to underlie and perpetuate chronic pain development (Reichling & Levine, 2009). In an effort to combat the opioid crisis with the discovery of new drug targets, the *Drosophila melanogaster* nociception behavioral model has proven for almost two decades to be both beneficial and translatable in the discovery of genetic components involved in nociceptor sensitivity (Im & Galko, 2012; Khuong & Neely, 2013; Tracey Jr et al., 2003). However, even with the previous discoveries made,

there is still a lack of understanding of the mechanisms involved in all aspects of nociceptive sensitivity: baseline regulation of nociceptive sensitivity, nociceptive sensitization after injury, and recovery of the primary nociceptor from sensitization after injury. By building upon prior research, we believe that further investigation into each of these processes regulating nociceptive sensitivity in different conditions will provide a better understanding of nociceptive sensitivity and provide new drug targets for chronic pain drug formulation in the future. In particular, we hypothesize that by focusing on investigating genetic targets translatable to humans, we may uncover a foundational basis for which further mammalian investigation may be beneficial for pain drug development.

1.2 Chronic pain definition and terminology

1.2.1 Chronic pain definition

Pain after injury is a beneficial biological process to the body of an organism, as it aids in protection. Pain alerts us to the potential threat of danger and guides us in protecting and seeking treatment for our injuries so that they may heal. However, when pain persists or recurs after normal healing, a period of time equal to or greater than three months, the pain is then considered "chronic" (Treede et al., 2015, 2019). This is the part of the definition of chronic pain that was outlined within the recent International Classification of Diseases-11 (ICD-11) in partnership with the classifications that were also described through thorough analysis from the International Association for the Study of Pain (IASP) (Treede et al., 2015, 2019). Once considered only a symptom of disease and injury, some chronic pain disorders, such as those categorized as chronic primary pain, have now been proposed to be seen as physical disorders that stand on their own (Kosek et al., 2021; Nicholas et al., 2019; Treede et al., 2019). For many forms of chronic pain, a known disease or injury can be pinpointed as the trigger for development, but sometimes the trigger is unknown (Kosek et al., 2021; Nicholas et al., 2019).

Also included within the definition for pain, and thus chronic pain, is the detail that pain can also be a source of great emotional distress, and this can negatively affect the quality of life for those burdened by it (Costanza et al., 2021; Rao et al., 2022). Not included in the definition, however, is the reality that chronic pain is also a huge economic burden on the individual, their families, employer, and community (Gaskin & Richard, 2012). As stated previously, chronic pain has been estimated to affect ~50 million adults nationwide (Dahlhamer et al., 2018; Yong et al., 2021, 2022; Zelaya et al., 2020). In regard to financial impact, a recent analysis carried out on the prevalence of chronic pain within the United States suggested the loss in wages due to chronic pain to be ~$79.9 billion when using 2019 average hourly wage data (Yong et al., 2022). Taken together, chronic pain is not just defined by medical terminology for physiological attributes but also by this multilayered social and economic burden that includes concerns of physical, mental, and financial well-being affecting not just the individual but also their surrounding community.

1.2.2 Chronic pain etiology and sub-classifications

As our knowledge of chronic pain and its differences to acute pain have grown, the definition and classification of the term has evolved in hopes of better representing the clinical manifestations of chronic pain so that those afflicted may be better diagnosed and treated properly (Treede et al., 2019). Though all chronic pain is now defined as pain which occurs/recurs for at least 3 months, the ICD-11 has subcategorized seven groups for chronic pain: chronic primary pain, chronic cancer pain, chronic postsurgical and posttraumatic pain, chronic neuropathic pain, chronic secondary headache and orofacial pain, chronic secondary visceral pain, and chronic secondary musculoskeletal pain (Aziz et al., 2019; Bennett et al., 2019; Benoliel et al., 2019; Nicholas et al., 2019; Perrot et al., 2019; Scholz et al., 2019; Schug et al., 2019; Treede et al., 2015). In providing a brief description of these subcategories, primary pain is perhaps the vaguest. Primary pain is described as pain that occurs in at least one bodily

region and that it cannot be better explained by another known chronic pain condition (Nicholas et al., 2019; Treede et al., 2015). Primary pain, like other pain categories can cause disability or distress and examples include conditions with elusive etiology such as fibromyalgia and unexplained back pain (Nicholas et al., 2019; Treede et al., 2015). For the benefit of describing different treatment guidelines, chronic cancer pain has been recently introduced as a subcategory to the ICD-11 and is defined as pain caused by cancer or its subsequent treatment (Bennett et al., 2019; Treede et al., 2015). Chronic postsurgical and posttraumatic pain is detailed as persistent pain occurring after either surgery, such as mastectomy, or bodily trauma, such as severe burn injuries, and can also not be better classified by another chronic pain subclassification (Schug et al., 2019; Treede et al., 2015). For the diagnosis of chronic neuropathic pain, the pain must be caused by an abnormality within the somatosensory nervous system and for definitive diagnosis, involve clarification of the nervous system abnormality through diagnostic measures (e.g., imaging, biopsy, sensory tests) (Scholz et al., 2019; Treede et al., 2015). Chronic neuropathic pain is also known to cause the classic chronic pain symptoms of allodynia and/or hyperalgesia, which are an abnormally painful response to a normally non-noxious stimulus and the heightened response to an already known noxious stimulus, respectively (Scholz et al., 2019; Treede et al., 2015). Chronic secondary headache and orofacial pain that is not better classified under chronic primary pain is described as persistent headaches and orofacial pain stemming from known factors, such as pharmacological withdrawal or dental decay (Benoliel et al., 2019). Chronic secondary headache and orofacial pain also occurs for at least half of the days, for a duration of 2 hours or more a day, during a three-month time frame (Benoliel et al., 2019; Treede et al., 2015). Chronic secondary visceral pain, that is not better classified under chronic primary pain, has been defined by the ICD-11 as pain stemming from the internal organs with mostly known cause, such as ulcerative colitis, and mostly presents as referred somatic pain of known patterns (Aziz et al., 2019; Treede et al., 2015). And finally, chronic secondary musculoskeletal pain that is not better categorized as primary pain is defined as pain that

originates within the musculoskeletal tissues due to known underlying disease and is characterized strictly as nociceptive pain and not referred visceral or neuropathic pain (Perrot et al., 2019; Treede et al., 2015). Some examples of chronic secondary musculoskeletal pain are musculoskeletal pain stemming from autoimmune disorders, spondylosis, or osteoarthritis (Perrot et al., 2019).

1.2.3 Nociplastic pain

A new term used in the description of primary pain in the ICD-11, one which could eventually become important to those investigating nociceptor sensitivity and its involvement in the development and perpetuation of chronic pain in the absence of injury, is nociplastic pain (Kosek et al., 2021; Kosek et al., 2016; Nicholas et al., 2019). The new term arose from the observation that though nociceptive and neuropathic pain are typically regarded as separate, there is some overlap between the two and that overlap may warrant use of its own terminology due to potential differences in treatment (Kosek et al., 2016). In detail, neuropathic pain originates out of injury or disease to the nervous system itself, and nociceptive pain originates from injury to the body outside of the nervous system, which in turn activates the nociceptors (sensory neurons that detect noxious stimuli) (Kosek et al., 2016; Scholz et al., 2019). In comparison, the description of nociplastic pain has been proposed to be used when there is pain originating from abnormal alteration in the sensitivity mechanism of the nociceptors, resulting in symptoms such as hyperalgesia and allodynia, yet there is not an identifiable injury or disease of the somatosensory system or bodily tissue (Kosek et al., 2021; Kosek et al., 2016; Nicholas et al., 2019). Examples of diseases in which the nociplastic pain descriptor are thought to be applicable and beneficial include primary pain syndromes such as: chronic widespread pain, fibromyalgia, and complex regional pain syndrome (type 1) (Kosek et al., 2021; Kosek et al., 2016; Nicholas et al., 2019). It is important to note, however, that the use of this new descriptor is still relatively new and there is still some confusion by the research community as to where and if it can be used as a descriptor of pain (Cohen, 2022; Kosek

et al., 2021; Nijs et al., 2021). For example, the criteria outlined does not account for hyposensitivity, which can also be a dysregulation seen in the nociceptors with some pathologies (Clark et al., 2019; Fairburn et al., 2022; Kosek et al., 2021; Nijs et al., 2021). Nonetheless, the term nociplastic pain, once fine-tuned, may serve to become a central descriptor for some distinct types of pain involving abnormal nociceptor sensitivity in the future.

1.3 Mental health impacts of chronic pain

As stated previously, pain is a beneficial mechanism for the survival of organisms that experience it, yet its psychological effect on the individual can be quite distressing and disabling. The emotional distress from the perception of pain becomes prolonged for an individual in chronic pain conditions. Secondary emotional and psychological effects such as depression, anxiety, and suicidal ideation have been known to increase in manifestation in chronic pain patients when compared to the general population or have been known to be observed concomitantly (Costanza et al., 2021; Gallagher et al., 1995; Narita et al., 2006; Okifuji & Benham, 2011; Racine, 2018). Risk of attempt for suicide and completed death by suicide was also found in one review as being at least doubled for those suffering from chronic pain conditions when compared to the general population (Hitchcock et al., 1994; Magni et al., 1998; Tang & Crane, 2006). A recent study (Costanza et al., 2021) involving a cohort of chronic pain patients within a pain center in Switzerland found that a described psychological construct used in assessing risk for suicide, known as “meaning in life (MiL)” (Frankl, 1985; Heisel & Flett, 2016), may be eroded in individuals experiencing chronic pain. Other epidemiological studies have found that even after factoring in affecting variables such as sociodemographic characteristics or previously diagnosed psychiatric disorders, that there is a substantial increase in those suffering from chronic pain conditions and their risk of suicidal ideation or attempt (Braden & Sullivan, 2008; Racine, 2018; Ratcliffe et al., 2008). Apart from the distress stemming from the perception of continuous pain itself, distress in the

form of anxiety or depression may also arise due to factors surrounding chronic pain. These factors can include financial burden of continuous healthcare costs in treating chronic pain and/or loss of work, the burden of undesirable side effects from chronic pain treatment such as addiction, and societal aspects such as cultural stigmatization and/or the burden taken on by loved ones and the surrounding community (Christie, 2008; Costanza et al., 2021; Gaskin & Richard, 2012; Goldberg & McGee, 2011; Hay et al., 2009; Rao et al., 2022; Treede et al., 2019; Vowles et al., 2015; Yong et al., 2022).

1.4 Pain pathways in humans

1.4.1 Overview of the pain pathway in humans

A critical step in understanding the best way to treat chronic pain, which could be described as irregular or abnormal pain, is to first understand the anatomy and physiology of the pain pathway when it is functioning under normal conditions in humans. The first step in the pain processing pathway begins with the detection of a noxious stimulus by primary nociceptors which are found in the peripheral nervous system (PNS) and are specialized sensory neurons with free nerve endings extended into the periphery (Bessou & Perl, 1969; Burgess & Perl, 1967; Sherrington, 1903; Woolf & Ma, 2007). Cell bodies of the nociceptors are found in the dorsal root ganglia (DRG) or the trigeminal root ganglia (TG) (Woolf & Ma, 2007). Nociceptors are comprised of three key classes: those activated by either thermal or mechanical stimuli, which are two different classes but both comprised as the endings of myelinated Aδ axons, and those which are polymodal, meaning they are activated by thermal, mechanical, and/or chemical, and they are the ends of unmyelinated C-fiber axons (Bessou & Perl, 1969; Burgess & Perl, 1967; Cain et al., 2001; Kandel, 2013; Koltzenburg et al., 1997; Smith & Lewin, 2009; Woolf & Ma, 2007). There is also a fourth, more puzzling class of nociceptors found mostly within the viscera and deep bodily tissues which are activated by inflammation and chemical stimuli and are called the silent or sleeping nociceptors (Häbler et al., 1990; Kandel, 2013; Prato et al., 2017; Schmidt et al., 1995). The

stimuli that activate the nociceptors do so by stimulating associated ion channels found on the nociceptor cell membrane (Cesare & McNaughton, 1996; Giniatullin, 2020; Kandel, 2013). Examples of the ion channels include the large family of transient receptor potential (TRP) ion channels which can individually detect noxious heat, cold, or chemical stimuli, and potentially mechanosensitive ion channels such as the Piezo channels (Bandell et al., 2004; Caterina et al., 2000; Coste et al., 2010; Davis et al., 2000; Dhaka et al., 2007; Giniatullin, 2020; Kandel, 2013; Woolf & Ma, 2007). With enough activation of these ion channels leading to production of a sufficient depolarizing current, an action potential results and travels down the length of the nociceptive sensory neuron axon, ending with neurotransmitter release, such as glutamate (the primary neurotransmitter of the primary sensory neurons of humans) or neuropeptides such as substance P and calcitonin gene related peptide (CGRP), in the axon's terminating region (Figure 1.1) (Basbaum et al., 2009; Kandel, 2013; Liu et al., 1997; Y. Liu et al., 2010; Nagy & Hunt, 1983; Woolf & Ma, 2007; Zhang et al., 2001). For those nociceptors whose cell bodies are found within the DRG, their axons terminate within the dorsal horn of the spinal cord and synapse onto second order neurons in a highly specific manner based on the type of axon fiber (Aδ or C) (Bridgestock & Rae, 2013; Christensen & Perl, 1970; Joseph et al., 2010; Kandel, 2013; Light et al., 1979; Nagy & Hunt, 1983; Réthelyi et al., 1982; Woolf & Ma, 2007). For Aδ fibers, these axons terminate in lamina I, II, and V, and for C fibers, these axons terminate in lamina I and II of the spinal cord dorsal horn (Basbaum et al., 2009; Bridgestock & Rae, 2013; Christensen & Perl, 1970; Dhaka et al., 2008; Hunt & Rossi, 1985; Kandel, 2013; Light et al., 1979; Nagy & Hunt, 1983; Ritz & Greenspan, 1985; Réthelyi et al., 1982; Sugiura et al., 1986; Woolf et al., 1992). Neurons whose cell bodies are found within the laminae of the spinal cord and which respond to and communicate nociceptive information include neurons found within laminae I, II, V, VII, and VIII (Braz et al., 2005; Cervero, 1984; Fernandes et al., 2016; Fields et al., 1995; Fields et al., 1975; Kandel, 2013; Molinari, 1982; Nagy & Hunt, 1983; Ritz & Greenspan, 1985; Réthelyi et al., 1982; Sandkühler et al., 1993; Todd, 2010; Toyooka et al., 1978).

Nociceptive information received in the spinal cord is then transmitted for further processing in the central nervous system (CNS) via five different ascending pathways: the spinoreticular, spinomesencephalic, cervicothalamic, spinohypothalamic, and the spinothalamic tract, which is a primary focus within the pain pathway (Braz et al., 2009; Burstein et al., 1990; Chen & Pan, 2002; Dado et al., 1994; Diaz & Morales, 2016; Giesler et al., 1994; Kajander & Giesler Jr, 1987; Kandel, 2013; Menétrey et al., 1980; Menétrey et al., 1982; Svendsen et al., 2010). In regard to processing of nociceptive signals in the forebrain, there are two networks which are emphasized, the lateral and medial networks, which are included in some of these ascending tracts which relay information to the cerebral cortex (Albe-Fessar et al., 1985; Kandel, 2013; Nicholls et al., 2001; Spreafico et al., 1981; Tracey, 2005). The lateral network includes lateral thalamic nuclei and primary and secondary somatosensory cortices and receives information mainly from laminae I and V of the dorsal horn (Ab Aziz & Ahmad, 2006; Albe-Fessar et al., 1985; Andersson et al., 1997; Casey et al., 1994; Kandel, 2013; Kenshalo et al., 1980; Kenshalo Jr & Isensee, 1983; Mazzola et al., 2006; Nicholls et al., 2001; Stevens et al., 1993; Talbot et al., 1991; Willis et al., 1979). The medial network includes nuclei within the medial thalamus and insular, anterior cingulate, and prefrontal cortices and receives information within dorsal horn mainly from laminae VII and VIII (Ab Aziz & Ahmad, 2006; Casey et al., 1994; Dougherty et al., 2008; Kandel, 2013; Nicholls et al., 2001; Peyron et al., 1999; Rainville et al., 1997; Talbot et al., 1991; Willis et al., 1979). The lateral network is generally regarded as being responsible in tracing the nociceptive stimulus back to a specific location within the body, along with processing other discriminatory characteristics such as intensity (Ab Aziz & Ahmad, 2006; Andersson et al., 1997; Kandel, 2013; Kenshalo et al., 1988; Nicholls et al., 2001; Yam et al., 2018). The medial network is known to be involved in the affective-motivational aspect of pain, which includes the perception of its "unpleasantness" (Ab Aziz & Ahmad, 2006; Desbois & Villanueva, 2001; Kandel, 2013; Nicholls et al., 2001; Peyron et al., 1999; Rainville et al., 1997; Talbot et al., 1991; Wang et al., 2009).

1.4.2 Peripheral nervous system sensitization

Primary nociceptors can activate the pain pathway when responding to a temporary stimulus, however, sensitization of the primary nociceptors can also occur due to the natural inflammatory response to injury or unknown circumstances (Fischer et al., 2010; Kosek et al., 2016; Millan, 1999; Nicholas et al., 2019). This sensitization of the primary nociceptors lowers their threshold for eliciting a response that in turn initiates the signaling mechanism which propagates through the pain pathway of the body (Basbaum et al., 2009; Bessou & Perl, 1969; Fischer et al., 2010; Kandel, 2013). So, the pain is felt more consistently and is prolonged (Millan, 1999). As mentioned previously, evolutionarily this can be an important mechanism, as sensitization of the nociceptors after injury helps to immobilize the injured area so healing can occur (Millan, 1999).

After injury occurs, an "inflammatory soup" of chemicals is released by the damaged cells, by the nociceptors, and by immune cells (Basbaum et al., 2009; Bland-Ward & Humphrey, 1997; Fischer et al., 2010; Kandel, 2013; Kessler et al., 1992; Lang et al., 1990; Liu et al., 1997; Parnavelas et al., 1985; Steen et al., 1995; Wang et al., 2004; Xie et al., 2003). Specifically, the damage caused to cells after injury results in the release of prostaglandins and chemicals such as ATP (adenosine triphosphate), acetylcholine, H+, and serotonin (5-HT), which can stimulate peripheral cells into producing bradykinin and more prostaglandins, well known inflammatory agents that lead to nociceptor sensitivity (Figure 1.1) (Ashton et al., 1986; Basbaum et al., 2009; Bland-Ward & Humphrey, 1997; Dray & Perkins, 1993; Hanada et al., 2012; Kandel, 2013; Kessler et al., 1992; Lang et al., 1990; Millan, 1999; Needleman et al., 1974; Oliveira et al., 2006; Parnavelas et al., 1985; Steen et al., 1992; Steen et al., 1995; Vasko et al., 1994; Wang et al., 2004; Xie et al., 2003). Contributing to the localized inflammatory response at the site of injury also includes the release of neuropeptide substance P and CGRP by the nociceptors of C-fibers, which results in heat and swelling through dilation of blood vessels and post-capillary venules (Figure

1.1) (Gibson et al., 1988; Gold & Gebhart, 2010; Kandel, 2013; Kellstein et al., 1990; Masanori & Mitsuhiko, 1987; McEwan et al., 1986; Mullins et al., 1993; Richardson & Vasko, 2002; Vasko et al., 1994). Due to sensory neuron involvement in the cycle of inflammation and nociceptor sensitization, the process has been called neurogenic inflammation and once started can continue to spread to other healthier parts of bodily tissue and cause sensitization away from the original injury site (Jancsó et al., 1967; Kandel, 2013; Matsuda et al., 2018; Richardson & Vasko, 2002). Substance P release from the sensory neurons also results in histamine release from mast cells, which in turn directly activates sensory neurons (Figure 1.1) (Erjavec et al., 1981; Kandel, 2013). Other chemicals released from the immune system response, in addition to histamine from mast cells, includes nerve growth factor (NGF), triggered by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) (Kandel, 2013; Leon et al., 1994; McMahon, 1996; Safieh-Garabedian et al., 1995; Wagner & Myers, 1996).

Along with the effect of histamine directly on nociceptors, bradykinin release in particular from peripheral cells is known to be powerful in its ability to produce sensitization and pain due to its ability to directly activate both Aδ and C fiber nociceptors (Figure 1.1) (Dray & Perkins, 1993; Kandel, 2013; Koltzenburg et al., 1992; Martin et al., 1987; Vasko et al., 1994). Prostaglandins, lipid mediators that are a product of COX (cyclooxygenase) enzymes which cleave arachidonic acid, are also well-known instigators of peripheral sensitization (Figure 1.1) (Kandel, 2013; Martin et al., 1987; Smith et al., 2000). These COX enzymes are targets for pain relief therapy with drugs such non-steroidal inflammatory analgesics (NSAIDS) which will be described more in the following pain therapy subsection of this background (Section 1.5) (Futaki et al., 1994; Kandel, 2013; Martin et al., 1987; Masferrer et al., 1994; Roth et al., 1975; Seibert et al., 1994). This inflammatory soup of chemicals can stimulate secondary messenger systems via metabotropic receptors, such as G-protein coupled receptors (GPCRs), on the nociceptors (Schaible et al., 2011; Russell et al., 2010; Salzer et al., 2019). These metabotropic receptors then can in turn activate and modulate the activity of ion channels (such as the TRP family) responsible

for propagating action potentials and ascending signaling through the pain pathway, leading to nociceptive sensitization (Gold & Gebhart, 2010; Hucho & Levine, 2007; Russell et al., 2010; Salzer et al., 2019; Schaible et al., 2011; Sugiuar et al., 2004).

Peripheral sensitization is known to frequently lead to and perpetuate central sensitization through proposed mechanisms such as: a reorganization of central terminals in the lamina after peripheral nerve injury, a loss of GABAergic interneurons in the dorsal horn after nerve injury, or through an increase in excitability of central neurons such as dorsal neurons in a process known as "wind-up" (Inquimbert et al., 2018; Kandel, 2013; Koltzenburg et al., 1994; Li et al., 1999; Mendell, 1966; Schaible et al., 2011; Woolf et al., 1992; Yam et al., 2018). Though components upstream and downstream of secondary signaling pathways involved in nociceptor sensitization have been uncovered, the full mechanism of how this sensitization occurs is still unclear, including why nociceptive sensitization continues after an injury has healed or why it occurs in the absence of injury (Hucho & Levine, 2007).

1.4.3 Descending system of endogenous pain modulation

Within mammals there are descending systems of endogenous pain regulation that help to dampen the excitatory signaling stemming from the ascending pain pathways and provide analgesia (Kandel, 2013; Necker & Hellon, 1977). Two main sites in the CNS involved in this descending system of regulation include the periaqueductal grey (PAG) in the midbrain and connections made to the rostral ventromedial medulla (RVM) in the brainstem (Bourne et al., 2014; Bridgestock & Rae, 2013; Kandel, 2013; Mayer & Liebeskind, 1974; McCarberg & Peppin, 2019; Pertovaara et al., 1996; Young & Chambi, 1987). Two major monoaminergic descending pathways for pain modulation associated with these regions include the serotonergic pathway, which involves the activation of serotonergic neurons found in areas of the medulla that inhibit and project to neurons in laminae I, II, and V, and the noradrenergic

pathway originating in areas of the pons and medulla and inhibiting neurons found in laminae I and V (Costa et al., 1994; de Kort et al., 2021; Hagihira et al., 1990; Kandel, 2013; Marlier et al., 1992; McCarberg & Peppin, 2019; Olave and Maxwell, 2004; Yoshimura & Furue, 2006). The CNS produces endogenous peptides called opioids as a method of pain modulation and their receptors, comprised of the four classes: mu, delta, kappa, and orphanin FQ (nociceptin), which are distributed throughout the PNS and CNS, with high concentrations found in the PAG, the medulla, and the dorsal horn of the spinal cord (Anton et al., 1996; Gilbert & Martin, 1976; Henderson & McKnight, 1997; Hughes, 1975; Hughes et al., 1975; Kandel, 2013; Martin et al., 1976; Minami & Satoh, 1995; Mollereau et al., 1994; Pert & Snyder, 1973; Simon et al., 1973). There are four major classes of endogenous opioids involved in analgesia: enkephalins and dynorphins, primarily produced in the spinal cord dorsal horn; β-endorphins, which are produced in the hypothalamus; and orphanin FQ/nociceptin which is distributed within several areas of the CNS (Anton et al., 1996; Bloom et al., 1978; Botticelli et al., 1981; Henderson & McKnight, 1997; Houtani et al., 1996; Kandel, 2013; Merchenthaler et al., 1986; Minami & Satoh, 1995; Nothacker et al., 1996; Reinscheid et al., 2000; Sar et al., 1978). Along with the opioid signaling pathway, another endogenous signaling system that has been shown to have an inhibitory effect in response to pain sensitization mechanisms is the endocannabinoid signaling pathway (Agarwal et al., 2007; Meng et al., 1998; Nicholls et al., 2001; Ogawa & Meng, 2009). Endocannabinoids, such as anandamide and 2-AG (1-arachidonoyl glycerol) are known to target the G-protein coupled cannabinoid receptors, CB_1, one of the most plentiful GPCRs in the brain, and CB_2 (Devane et al 1992; Jansen et al., 1992; Malek et al., 2015; Nicholls et al., 2001; Sugiura et al., 1995; Schatz et al., 1997). Targeting of the cannabinoid receptors, primarily CB_1, via a cannabinoid agonist, results in an antinociceptive effect, shown to occur at least in part through modulation of neuronal transmission within the rostral ventromedial medulla, the medullary dorsal horn, as well as the peripheral nociceptors (Agarwal et al., 2007; Meng et al., 1998; Ogawa & Meng, 2009).

1.5 Treatment options for pain relief

1.5.1 Overview of pain relief treatment options

Due to the unpleasant emotional sensation of pain and the physical and mental disability that chronic pain can inflict on a body, treatment options for pain are sought after fervently by those experiencing it acutely and chronically. Though there are a variety of non-pharmaceutical options for managing pain that can sometimes be effective for mild to moderate pain such as massage therapy, acupuncture, tens unit stimulation, exercise, meditation, herbs and supplements, and cognitive behavioral therapy, the vast majority of pain sufferers turn to pharmaceutical drugs for more potent, reliable, and quick acting pain relief (Buvanendran et al., 2021; Field, 2016; Field et al., 2011; Grover et al., 2018; Henriksen et al., 2014; Loh & Gulati, 2015; Maroon et al., 2010; Molsberger et al., 2002; Nahman-Averbuch et al., 2021; Rudrappa et al., 2020; Zeidan et al., 2011). The majority of pain medications most readily used by pain sufferers over the counter (OTC) in the United States are those which target prostaglandin synthesis by blocking cyclooxygenase (COX) enzymes and include drugs such as aspirin and other such non-steroidal anti-inflammatory drugs (NSAIDS) that help to relieve inflammation, as well as acetaminophen which also acts on COX enzymes, but does not relieve inflammation (Argoff, 2011; Graham et al., 2013; Graham et al., 2001; Mitchell et al., 1993; Moncada et al., 1975; Patrignani & Patrono, 2015; Przybyła et al., 2021; Roth et al., 1975). Though used for both acute and chronic pain alike, medications targeting COX enzymes are considered insufficient on their own for the management of severe pain and also can carry side effects such as liver damage and the development of stomach ulcers when used chronically (Argoff, 2011; Boyd & Bereczky, 1966; Collier & Pain, 1985; Rodríguez & Hernández-Díaz, 2001). Some local anesthetics such as lidocaine, which are partially known to work through blocking voltage-gated sodium channels on sensory neurons, are available both in OTC topical forms and through prescription, though the evidence for significant pain

relief with topical lidocaine is weak (Argoff, 2011; Finnerup et al., 2015; Hermanns et al., 2019; Ho et al., 2008; Scholz et al., 1998).

Commonly prescribed systemic pain medications include drugs such as antidepressants, which work by blocking the reuptake of serotonin and noradrenaline, antiarrhythmics which work by blocking sodium channels, anticonvulsants which work by blocking either neuronal voltage-gated sodium channels or calcium channels, and opiates that mostly target the mu endogenous opioid receptor within the CNS and PNS and increase membrane potassium conductance (Argoff, 2011; Bridgestock & Rae, 2013; Corrodi & Fuxe, 1969; Dogra et al., 2005; Fricker et al., 2020; Gilron et al., 2015; Hoshino et al., 2015; Jensen, 2002; Kandel, 2013; Lees & Leach, 1993; Meuser et al., 2003; North et al., 1987; Pasternak & Pan, 2013; Pert & Snyder, 1973; Ständer et al., 2002; Werz & Macdonald, 1983). Opioids are known to be abundantly prescribed for nociceptive pain (Argoff, 2011; Cavalli et al., 2019; Owusu Obeng et al., 2017). As with most medications, prescribed medications for treating pain come with their own levels of efficacy depending on how the pain is classified and characterized (Section 1.2) as well as a possible host of side effects that can be magnified when applied chronically (Argoff, 2011; Benyamin et al., 2008; Bridgestock & Rae, 2013; Cavalli et al., 2019; Goodman & Brett, 2017; Noori et al., 2019; Owusu Obeng et al., 2017).

1.5.2 Opioid side effects and addiction crisis

Though the effectiveness of opioid administration in short term acute pain relief is very good, the side effects and efficacy of these drugs when applied long-term can include the severe threats of tolerance, physical dependence, and addiction in individuals, which has led to the opioid addiction epidemic in the United States (Benyamin et al., 2008; Groenewald et al., 2019; Imtiaz et al., 2020; Kandel, 2013; Noori et al., 2019; Vearrier & Grundmann, 2021; Vowles et al., 2015). Physiological tolerance to opioid use occurs when dosage must consistently increase over time in order to achieve the

same therapeutic analgesic relief from the drug, leading to a state where relief becomes progressively harder to achieve (Bagley et al., 2005; Kandel, 2013; Lueptow et al., 2018). The proposed mechanisms of opioid tolerance have included: decoupling of mu opioid receptors from G-protein signaling transduction as well as varying cellular changes to both neuronal and non-neuronal cell populations (Bagley et al., 2005; Eidson & Murphy, 2013; Kandel, 2013; Lueptow et al., 2018; Mao et al., 2002; Meuser et al., 2003). Physical dependence from opioid administration is evident by withdrawal symptoms which develop after cessation of opioid use and can include symptoms such as irritability, anxiety, aches, sweating, tremor, and gastrointestinal discomfort (Bradley et al., 1987; Kosten & Baxter, 2019; Vernon et al., 2016; Wesson & Ling, 2003). It has also been shown that opioid use involves associative learning mechanisms and is physiologically rooted in the reward system of the brain which includes mechanisms such as mu opioid receptor effects on dopamine release (Cai et al., 2013; Dai et al., 2016; Garland et al., 2018; Kandel, 2013; Le Merrer et al., 2009; Zarrindast et al., 2002).

Opioid addiction within the United has resulted in a nationwide epidemic with recent provisional data released by the CDC (Centers for Disease Control and Prevention) in July 2021 showing 69,710 opioid overdose deaths in the United States in 2020 alone, an increase from opioid overdose deaths reported at 50,963 for the previous year (Knopf, 2021). Due in part to record increases in prescriptions for opioid analgesics over the years, opioid addiction rates in the United States have resulted in stricter regulations on who receives opioid prescriptions in an effort to curtail the epidemic (Meadowcroft & Whitacre, 2021; Sites et al., 2014). Sadly, though not the intention, drug monitoring programs have also left many turning to non-pharmacological opiates such as heroin (Kim, 2021; Meadowcroft & Whitacre, 2021). In addition, the stricter regulations for prescribing opioids within the United States has also led to many chronic pain sufferers being unable to obtain their rightfully indicated opiate prescription from their doctor for ongoing pain relief which has led some to the costly suffering of severely under-treated pain (Kliuk-Ben Bassat et al., 2019; Pergolizzi et al., 2019). With the current

treatment for chronic pain management not ideal due to side effects and efficacy, researchers are investigating into the still unknown mechanisms surrounding pain and chronic pain development in hopes of uncovering new drug treatment options.

1.6 *Drosophila melanogaster* as a research model in pain research

1.6.1 *Drosophila melanogaster* pain research contribution

At present, investigating the mechanics of pain development, including abnormal pain, is best formulated to an *in vivo* model so that behavior can be observed. However, ethical concerns can sometimes arise with the use of *in vivo* mammalian systems in pain research when there is a need to obtain large sample sizes for screening or to tease out small differences and still achieve a suitable statistical power (Lee et al., 2018; Racine et al., 2012). Fortunately, a *Drosophila melanogaster*, also known as the fruit fly, model circumvents most of the ethical concerns that commonly plague *in vivo* mammalian systems modeling pain. Research using *Drosophila* is also an increasingly attractive model for more broad research into neuropharmacology, as the fruit fly is readily able for high throughput *in vivo* drug experimentation without the same governmental oversight that vertebrate models are under (such as with the Institutional Animal Care and Use Committee (IACUC)), as well as lower cost in comparison to mammalian models (Nichols, 2006). And though humans have 23 pairs of chromosomes, and the fruit fly only has four, the fruit fly still shares over 75% of the genes connected to disease in humans, lending to its role as a useful model for biomedical research (Reiter et al., 2001). The fruit fly also has a life cycle from embryo to adult of only 10-12 days and females can generate upwards of 100 eggs per day, both traits which allow for extremely fast generation time of genetic mutants and transgenic animals for experimentation (Nichols, 2006; Ong et al., 2015). Owing to this ability for translation and ease of experimentation, *Drosophila* have at times been on the very forefront of discoveries later translated to humans (e.g., factors controlling embryonic development or circadian

rhythm) and have garnered six Nobel prizes in Physiology or Medicine involving use of the fruit fly model (Axel, 2004; Baptista et al., 2021; Bargiello et al., 1984; Hardin et al., 1990; Hoffmann, 2011; Huang, 2018; Lewis, 1998; Liu et al., 1992; Morgan, 1916; Muller, 1928; Nichols, 2006; Price et al., 1998; Siwicki et al., 1988; Tolwinski, 2017; Vosshall et al., 1994; Zehring et al., 1984).

In recent years, *Drosophila melanogaster* has also proven to be an exceptional *in vivo* model organism for the investigation of multiple neurological diseases, such as Parkinson's and chemotherapy-induced peripheral neuropathy, made possible due to its relative organismal simplicity and powerful genetic toolkit available (Boiko et al., 2017; del Valle Rodríguez et al., 2011; Feany & Bender, 2000). Examples of some behaviors in which fruit flies have been found to be an excellent model of study include sleep and social behaviors such as: mimicry, courtship, and aggression (Dankert et al., 2009; Dukas, 2020; Klibaite & Shaevitz, 2020; Manoli et al., 2005; Mundiyanapurath et al., 2007; Simões et al., 2021; Szuperak et al., 2018; Versteven et al., 2017). Owing to this outstanding ease of genetic manipulation, fast generation time, and observable behavior phenotypes, discoveries into pathways underlying nociceptive sensitization have also already made considerable progress within the *Drosophila melanogaster* model (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; McParland et al., 2021).

Fruit flies make desirable *in vivo* models for nociceptor sensitivity and its involvement in pain signaling for many reasons, including: nociceptors of the fruit fly have been shown to have similar function and morphology to that of vertebrate counterparts, many genes underlying the perception of pain have been found to be conserved across species, and there is an abundance of evidence that fruit flies, both larvae and adult, respond to noxious stimuli with characteristic escape behaviors (Figure 1.2) (Hwang et al., 2007; Im & Galko, 2012; Khuong & Neely, 2013; Reiter et al., 2001; Sulkowski et al., 2011; Xu et al., 2006). Among some of the important pain discoveries made with this model are the roles of Painless (suggested to be homologous to mammalian ANKTM1 and analogous in function to TRPV1 (Al-

Anzi et al., 2006; Tracey Jr et al., 2003; Xu et al., 2006)), and identification of the *Drosophila* DEG/ENaC channel, Pickpocket (Ppk: similar to vertebrate epithelial sodium channel molecules), which is known for sensing and reacting to harsh mechanical stimulation within the fly (Adams et al., 1998; Zhong et al., 2010). Because of these discoveries, attributes (ex: generation time, nociceptor morphology similar to vertebrates, etc.), and the genetic tools available (described in the following section), using a *Drosophila* model is both relevant and beneficial for investigating genetic mechanisms contributing to pain and chronic pain development stemming from nociceptor sensitivity and/or sensitization.

1.6.1.1 *Drosophila melanogaster* cellular signaling pathways uncovered in regulation of nociceptive sensitivity

Over the past decade or more, a nociceptive sensitization model has been developed using *Drosophila* larvae in which UV injured and/or genetically modified animals become hyper- or hypo-sensitive (Babcock et al., 2009). Fruit flies, like their human counterparts, can develop nociceptive sensitization after injury, allowing for translatable modeling of allodynia and hyperalgesia, both known as possible symptoms in chronic pain (Babcock & Galko, 2009; Babcock et al., 2009; Babcock et al., 2011). And though the use of the post-injury behavioral assays within the fruit fly is relatively new compared to post-injury behavioral assays in mammals, it has already become quite successful in the rapid identification of genes associated with the nociceptive sensitization process after injury (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; Jang et al., 2018; Khuong, Hamoudi, et al., 2019; Khuong, Wang, et al., 2019; McParland et al., 2021). Investigation of genetic components involved in nociceptor sensitivity has also become quite easy through genetic manipulation with the GAL4/UAS system (Brand & Perrimon, 1993). The GAL4/UAS is a system found within yeast, where the transcription factor GAL4 locks onto binding sites, optimized and known as Upstream Activation Sequences (UAS) in its designed use for genetic engineering, which once

bound, leads to transcription of target genes downstream the binding sites (Brand & Perrimon, 1993). This yeast transcriptional enhancer, GAL4, and its binding target sequence are not found within *Drosophila melanogaster*, making it an excellent system for cell/tissue specific gene expression in the fruit fly (Brand & Perrimon, 1993; Fischer et al., 1988). By mating a fruit fly carrying GAL4 downstream a cell specific promoter to another fruit fly carrying the gene/transgene of interest that is designed to be downstream the optimized (for high GAL4 binding affinity) UAS binding site, resultant progeny carries both the cell specific promoter-GAL4 construct as well as the UAS-gene of interest construct, with resultant cell/tissue specific expression of the gene/transgene of interest (Figure 1.3) (Brand & Perrimon, 1993; Fischer et al., 1988; Webster et al., 1988).

Using the Gal4/UAS system, one of the first cellular signaling mechanisms that was uncovered to regulate nociceptive sensitivity after UV injury in *Drosophila* larvae was the TNF signaling system by the *Drosophila* TNF homolog ligand, Eiger (Egr), which activates the TNF receptor, Wengen (Wgn), on the nociceptors and was found to be required for allodynia after UV injury (Figure 1.4) (Babcock et al., 2009; Im & Galko, 2012). Shortly after these findings on the requirement of the TNF signaling pathway in UV injury induced allodynia, the requirement for the Hedgehog (Hh) signaling pathway was also uncovered to be required for injury-induced allodynia and hyperalgesia to occur (Figure 1.4) (Babcock et al., 2011). Components within the Hh signaling pathway found to be involved in the regulation of nociceptor sensitivity included: the Hh receptor Patched (Ptc), the transmembrane protein Patched suppresses: Smoothened (Smo), and downstream of Smo the Hh transcription factor Cubitus interruptus (Ci) and its transcriptional targets: Engrailed (En) and Decapentaplegic (Dpp) (Figure 1.4) (Babcock et al., 2011).

When investigating *Drosophila* Tachykinin signaling, similar to mammalian tachykinin signaling in pain such as substance P (part of the tachykinin neuropeptide family) signaling in mammals, it was found that Tachykinin signaling (Tachykinin (dTk) and the *Drosophila* Tachykinin Receptor (DTKR)) is also required for UV injury induced allodynia in third instar *Drosophila* larvae (Figure 1.4) (Im et al., 2015;

Siviter et al., 2000). *Drosophila* Tachykinin signaling was found to work upstream of Hh signaling and is connected in the nociceptive sensitivity pathway through downstream Hh autocrine signaling via activation of the transmembrane protein Dispatched (Disp), which releases Hh extracellularly from the nociceptor (Figure 1.4) (Im et al., 2015). dTk was found to be necessary pan-neuronally (broadly among neurons) but not specifically within the nociceptors (Im et al., 2015). In contrast, its receptor, DTKR, was found to be necessary specifically in the nociceptors for UV injury induced thermal allodynia to occur and it affects neuronal firing properties (Im et al., 2015). Overexpression of DTKR also resulted in genetically induced allodynia and affected firing properties even in the absence of injury (Im et al., 2015). Through the investigation of several G protein subunits (CG117760, Gβ5 (G protein subunit beta 5), and Gγ1 (G protein gamma 1)) within this same study, it was also found that these G protein subunits bind to DTKR and affect thermal allodynia as well, downstream of DTKR activation (Im et al., 2015).

Building upon the findings by Babcock and colleagues on the Hh pathway and nociceptive sensitization, downstream transcriptional targets such as Dpp, found within the study (Babcock et al., 2011) to be involved in nociceptive sensitization, led to a more thorough investigation into Dpp and multiple other Bone Morphogenetic Protein (BMP) signaling pathway components (part of the Transforming Growth Factor Beta (TGF-β) superfamily of proteins) (Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; McParland et al., 2021; Wang et al., 2014). These investigations were important due to the highly conserved nature of the BMP signaling pathway among mammals and *Drosophila melanogaster*, which could lead to promising new drug targets in humans (Wang et al., 2014). Within these studies, behavioral assays carried out post UV injury with third-instar larvae determined that the BMP signaling ligands, Dpp and Glass Bottom Boat (Gbb), their main receptors, Punt (Put) and Wishful Thinking (Wit) respectively, along with the receptors activated by Punt: Saxaphone (Sax) and Thickveins (Tkv), and two other components known to be transmembrane regulators of BMP signaling, Dally (Dly) and Dally-like (Dlp), are all involved in injury-induced allodynia

(Figure 1.4) (Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018). It was also found that the overexpression of the BMP signaling ligand, Dpp, results in genetically induced allodynia even in the absence of injury, suggesting a mechanism in which Dpp can affect baseline nociception (Follansbee et al., 2017). With regard to other BMP signaling components involved canonically (apart from the components investigated at the nociceptor cell membrane), phosphorylated Mothers Against Dpp (Mad) (known to be phosphorylated by Thickveins and Saxophone) and Medea (Med), which Mad forms a complex with, were also investigated (Follansbee et al., 2017). These SMAD components (named so for being signaling transducers for receptors found within TGF-β superfamily) are known to translocate to the nucleus to activate transcription of BMP signaling target genes and were found to also be required for nociceptive sensitization after injury (Figure 1.4) (Attisano & Tuen Lee-Hoeflich, 2001; Campbell & Tomlinson, 1999; Follansbee et al., 2017).

Through following this canonical known chain of events within the BMP signaling pathway, the investigation then led very recently to uncover nuclear components associated with BMP target gene transcription involved in nociceptive sensitivity regulation (McParland et al., 2021). Transcriptional regulators examined included the transcriptional enhancer, Schnurri (Shn), known to form a complex with Mad-Med, and the transcriptional repressor, Brinker (Brk), whose repression is relieved by the Mad-Med complex (Campbell & Tomlinson, 1999; Jazwinska et al., 1999; McParland et al., 2021). It was found within this study by McParland and colleagues that knockdown of Brk resulted in genetically induced allodynia and hyperalgesia of the nociceptor, in the absence of injury (McParland et al., 2021). It was also found within this study that knockdown of Shn resulted in a failure to develop allodynia after UV injury (McParland et al., 2021). In summary, investigation of these signaling pathways within the *Drosophila* nociceptors has resulted in increased knowledge about the development of nociceptive sensitization, however, a full understanding of this process, including any other cellular signaling

pathways involved in regulating this sensitivity or the recovery of the nociceptor after injury, is still elusive.

Toward this effort, recent investigations included within this dissertation (chapter two) have focused on investigating components within the canonical Wnt/Wg (Wnt in mammals, and Wingless (Wg) in *Drosophila*) signaling pathway since the canonical Wnt/Wg signaling pathway is known to have cross talk with both Hh and BMP signaling pathways. In particular, of the numerous BMP signaling gene candidates previously identified as controlling nociceptive sensitivity in fruit flies, the glypican, Dly, is known to be both a potentiator of both BMP and Wnt/Wg signaling and was shown to be required in the nociceptor for nociceptive sensitization after injury (Tsuda et al., 1999, Brann et al., 2019). Also, the transcription factor, Brk, a negative regulator of BMP signaling in the fly and shown to suppress nociceptive sensitivity, is also known to antagonize Wg/Wnt signaling in the fruit fly (McParland et al., 2021; Saller et al., 2002). These findings on Dly and Brk and their involvement in the regulation of nociceptive sensitivity and contribution to signaling mechanisms in both the BMP and Wnt/Wg signaling pathways contributes foundational support for the hypothesis that the canonical Wnt/Wg signaling pathway possibly also affects nociceptive sensitivity in the fly. To that end, findings from our investigation into canonical Wnt/Wg signaling components, Gilgamesh (Gish), and our published findings on Arm and the transcriptional repressor of canonical Wnt/Wg signaling, Groucho (Gro) (Hale, Moulton, et al., 2022), are shared in chapter 2 of this dissertation. Within the published findings also shared in chapter 2, it was found that Arm does play a role in regulating nociceptive sensitivity in the absence of injury, however, it was unknown if its role in regulating nociceptive sensitivity was directly linked to its function within the canonical Wnt/Wg signaling pathway or if by other functions within the cell (explained further in chapter 2 of this dissertation) (Hale, Moulton, et al., 2022). Within this introduction, however, a summary of the components and mechanism by which canonical Wnt/Wg signaling is activated or inactivated in *Drosophila* is also given as background below.

A main component in the *Drosophila* canonical Wnt/Wg signaling pathway, Arm, is the intracellular transducer of canonical Wnt/Wg signaling when the pathway is activated, whereby its nuclear translocation results in transcriptional activation of Wnt/Wg target genes (Figure 1.4) (Komiya & Habas, 2008; Peifer, Sweeton, et al., 1994; Van de Wetering et al., 1997). In the absence of the Wnt/Wg ligand, Arm protein levels are reduced within the cytoplasm by a two-step kinase-destruction complex, which phosphorylates Arm for subsequent ubiquitination and proteosome degradation (Amit et al., 2002; Komiya & Habas, 2008; Liu et al., 2002; Peifer, Pai, et al., 1994; Stamos & Weis, 2013; Yanagawa et al., 2002). Though homologs also exist in mammals, in *Drosophila*, this Arm destruction complex is made up of the inhibitory binding proteins: Axin (Axn), APC-like (Apc) and Adenomatous polyposis coli 2(Apc2), which form a binding scaffold with the kinases: Casein Kinase I alpha (CkIα), a serine/threonine kinase which phosphorylates Arm initially, and then the glycogen synthase kinase 3, Shaggy (Sgg), which phosphorylates Arm a second time, setting Arm up for ubiquitination and subsequent proteasome degradation when canonical Wg signaling is turned off (Figure 1.4) (Ahmed et al., 1998; Bejsovec, 2013; Hamada et al., 1999; Komiya & Habas, 2008; Peifer, Pai, et al., 1994; Peifer, Sweeton, et al., 1994; Perrimon, 1994; Stamos & Weis, 2013; Waghmare & Page-McCaw, 2018; Welsh et al., 1996; Yanagawa et al., 2000; Yanagawa et al., 2002; Yu et al., 1999). When canonical Wg signaling is turned on by the binding of a Wnt/Wg ligand (Wg) to the Frizzled (Fz) and/or Frizzled 2 (Fz2) cell-surface receptor (not shown), Dishevelled (Dsh) is activated and binds to components within the destruction complex at the same time that Axin within the destruction complex also binds to the LDL receptor-related protein, Arrow (Arr) (which functions as an obligate Wg co-receptor) (Figure 1.4) (Bejsovec, 2013; Cadigan & Nusse, 1997; Doumpas et al., 2013; Kennerdell & Carthew, 1998; Komiya & Habas, 2008; Llimargas & Lawrence, 2001; Noordermeer et al., 1994; Schaefer et al., 2018; Stamos & Weis, 2013; Tolwinski et al., 2003). Gilgamesh (Gish), a plasma membrane associated kinase orthologous to mammalian CK1γ1 and found to positively regulate Hh signaling, also regulates Wnt/Wg signaling by assisting in the inactivation

of the Arm destruction complex through phosphorylation of Arrow (Figure 1.4) (Davidson et al., 2005; Hummel et al., 2002; Li et al., 2016; Verheyen & Gottardi, 2010; Zhang et al., 2006). This inactivation of the destruction complex at the membrane is what allows Arm protein levels to increase in the cytoplasm and then enter the nucleus to displace the transcriptional repressor, Gro (also known to be a transcriptional repressor to BMP target genes), and activate transcription of Wnt/Wg target genes (Figure 1.4) (Cavallo et al., 1998; Hasson et al., 2001; Hsu et al., 1998; Huber et al., 1996; Städeli et al., 2006).

1.6.2 *Drosophila melanogaster* nociceptive signaling neuroanatomy and sensitization

Much like their differences in chromosomes when compared to their human counterparts (to recap *Drosophila melanogaster* only carry four pairs of chromosomes), *Drosophila melanogaster* have a nervous system which is comprised of fewer elements than the human or mammalian mouse model nervous systems (Nichols, 2006). However, its simplicity has been known to help lend to the discovery of complete physiological mechanisms, such as the beforementioned discoveries comprising the mechanism of circadian rhythm that led to the Nobel prize in Physiology or Medicine in 2017 (Bargiello et al., 1984; Callaway & Ledford, 2017; Hardin et al., 1990; Huang, 2018; Liu et al., 1992; Price et al., 1998; Siwicki et al., 1988; Vosshall et al., 1994; Zehring et al., 1984). Fruit flies do have complexity and striking similarities within their PNS and CNS when compared to vertebrates and these similarities facilitate a range of diverse neural molecular mechanisms as well as survival and social behaviors that can be observed in both the larval and adult stages of the fruit fly (Dukas, 2020; Hwang et al., 2007; Nichols, 2006; Wu et al., 2003; Xu et al., 2008).

The CNS of the fruit fly is composed of a brain and a ventral nerve cord (VNC) which connects both to the brain and to the neuromuscular junctions (NMJ) and sensory neurons of the PNS outside of the CNS (Daniels et al., 2008; Freeman, 2015; Hughes & Thomas, 2007; Miyares & Lee, 2019;

Venkatasubramanian & Mann, 2019). The brain of the adult fruit fly is comprised of important structures such as: the optic lobes, the antennal lobes, the protocerebrum, the mushroom bodies, the central body complex containing the fan-shaped body and ellipsoid body, and the suboesophageal ganglia (Nichols, 2006). The VNC is functionally similar to the spinal cord within vertebrates in that information is relayed from the peripheral nervous system to the brain (such as sensory information) and *vice versa* (such as motor output) (Venkatasubramanian & Mann, 2019). The fruit fly VNC also contains its own functional form of a blood nerve barrier, much like the blood brain barrier in vertebrates, by which glial cells ensheath the VNC neuropil and protect the nerves against ions found within the hemolymph, a circulating fluid much like blood in invertebrates (Auld et al., 1995; Limmer et al., 2014; Nichols, 2006).

Like their mammalian counterparts, fruit fly primary nociceptor sensory neurons in the PNS feature sensory nerve endings for detecting noxious stimuli within epithelial tissue (Bessou & Perl, 1969; Gao et al., 1999; Grueber et al., 2002; Im & Galko, 2012, Jiang et al., 2019). Fruit fly nociceptors differ from mammals however, in that the cell bodies of the nociceptors are typically found away from the VNC, out in the periphery, unlike mammalian nociceptor cell bodies which are typically found in ganglia such as the DRG or TG closer anatomically in location to the CNS (Gao et al., 1999; Grueber et al., 2002; Han et al., 2012; Woolf & Ma, 2007). Fruit fly nociceptors also have branch like patterning of dendrites which project off the soma into the periphery and an axon which projects centrally from the soma for afferent signaling to the CNS (Grueber et al., 2002; Im & Galko, 2012; Khuong, Wang, et al., 2019; Shimono et al., 2009; Xu et al., 2006). In contrast, mammalian nociceptors consist of axons which extend both peripherally from the soma to detect noxious stimuli and centrally toward the CNS for afferent signaling (Woolf & Ma, 2007). The nociceptors of fruit fly larvae are also known as class IV multidendritic (Md) sensory neurons, which are a part of a class system of sensory neurons in the fly grouped by diversity in branching and are known to respond to different stimuli for different sensations such as mechanosensation (class II, III, IV), thermosensation (class III and IV), chemosensation (class IV and

possibly I,II,III), and proprioception (class I) (Babcock et al., 2011; Hughes & Thomas, 2007; Hwang et al., 2007; Im & Galko, 2012; Kim et al., 2012; Lopez-Bellido et al., 2019; Neely et al., 2010; Tsubouchi et al., 2012; Zhong et al., 2010). The class IV multidendritic sensory neurons are the most elaborate in dendritic complexity of the classes and are known to be activated by noxious mechanical, chemical, and thermal stimuli (Babcock et al., 2011; Hwang et al., 2007; Kim et al., 2012; Lopez-Bellido et al., 2019; Zhong et al., 2012; Zhong et al., 2010). In fruit fly larvae these class IV multidendritic (cIV md) sensory neurons also are known to form a tiling pattern around the entire body wall of the animal as they innervate between and within all the cells of the epidermis without overlapping each other (Grueber et al., 2002; Im & Galko, 2012). While mostly free or naked, the sensory neurites of fly larvae occasionally are ensheathed by epidermal cells (Han et al., 2012; Jiang et al., 2019; Mauthner et al., 2021; Tenenbaum et al., 2017), as has been reported similarly in mammals (Cauna, 1973; Munger, 1965, Talagas et al., 2020). In regard to what is known about neurotransmitter release of the nociceptors, the excitatory neurotransmitter, acetylcholine, has been shown to be largely involved within synapses of both the CNS and PNS of the fly and can be found in particularly high amounts in sensory neurons (Burgos et al., 2018; Khuong, Wang, et al., 2019; Lee & O'dowd, 1999; Salvaterra & Kitamoto, 2001; Shin et al., 2018). Evidence for acetylcholine requirement in nociceptive signaling of the adult fly has been shown through suppression of fruit fly nociceptive response after inhibition of nociceptor acetylcholine synthesis (Burgos et al., 2018; Khuong, Wang, et al., 2019; Lee & O'dowd, 1999; Salvaterra & Kitamoto, 2001).

In response to a sufficient noxious stimulus (chemical, mechanical, or thermal), receptors responding to these various stimuli are activated on fruit fly larval and adult nociceptors, which leads to an action potential. Once activated and an action potential achieved, these nociceptor axons have been found to release neurotransmitter onto second order interneurons found within the VNC of the fruit fly CNS (Burgos et al., 2018; Chin & Tracey Jr, 2017; Grueber et al., 2007; Hu et al., 2017; Kaneko et al.,

2017; Khuong, Wang, et al., 2019; Lopez-Bellido et al., 2019; Ohyama et al., 2015; Yoshino et al., 2017). In studies with larvae in particular, these interneurons found within the VNC to be in conversation with the axons of the cIV md neurons include: the medial clusters of class IV dendritic arborization second-order (mCSIs) interneurons, A08n neurons, dorsal pair insulin-like peptide 7 neurons (DP-ilp7), Down-and-Back (DnB) interneurons, A05q and A23g interneurons, Basin neurons, and Goro neurons (Burgos et al., 2018; Chin & Tracey Jr, 2017; Grueber et al., 2007; Hu et al., 2017; Kaneko et al., 2017; Lopez-Bellido et al., 2019; Ohyama et al., 2015; Yoshino et al., 2017). Along with nociceptive input, some of these interneurons, like the Basin cells and DP-ilp7, integrate information from other inputs in addition to the cIV neurons, such as mechanosensory chordotonal neurons (Basin cells) or C2da neurons (DP-ilp7) (Hu et al., 2017; Ohyama et al., 2015). This integration of inputs can lead to an enhancement in stimulus response or can become part of a higher order pathway activation in the brain, as is seen with the Basin-Goro brain pathway (Hu et al., 2017; Ohyama et al., 2015). It has also been shown that DP-ilp7 dorsal axons project to a region in the larvae brain as well, specifically a region within the brain lobes called the pars intercerebralis (Hu et al., 2017). Though the complexities of the CNS signaling pathways in nociception have yet to be completely uncovered, the goal of this nociception signaling activity from the class IV neurons to the CNS is ultimately to trigger downstream motor neurons for a "nocifensive escape" response of the animal from a threatening stimulus (Figure 1.2) (Burgos et al., 2018; Hwang et al., 2007; Tracey Jr et al., 2003). This nocifensive response in larvae, which is hypothesized to have evolved as a way to avoid parasitoid wasps, is primarily a 360-degree corkscrew roll along the longitudinal axis of the larva body, but also can include other behaviors such as fast crawling (Figure 1.2 A) (Burgos et al., 2018; Hwang et al., 2007; Tracey Jr et al., 2003). The exact mechanism for how a single motor neuron is activated and the muscle fiber within the larvae body responds is still unknown but some headway has been made recently with the cIVda-mCSIs signaling pathway where it has been shown that mCSIs in the VNC synapse onto segmental nerve a (SNa) motor neurons (Clark et al., 2018;

Gowda et al., 2021; Yoshino et al., 2017). With the various uncovered interneurons and their pathways, such as the cIVda-mCSIs-SNA and Basin-Goro, shown to be involved in the nocifensive response of rolling, there seems to be multiple neuronal circuits producing this response (Chin & Tracey Jr, 2017; Hu et al., 2017; Yoshino et al., 2017).

As stated previously in the above section (1.6.1), it has been found that *Drosophila melanogaster* nociceptors can sensitize after injury, in a way similar to human nociceptors, resulting in a reduced threshold of response to varying stimuli such as thermal, mechanical, cold, and chemical (Babcock et al., 2009; Babcock et al., 2011; Khuong, Wang, et al., 2019; Lopez-Bellido & Galko, 2020; Lopez-Bellido et al., 2019; Massingham et al., 2021; Turner et al., 2018). There also appears to be an element of plasticity found in these sensory neurons and their circuit that can result in a failure to respond to noxious stimuli in mature larvae that have undergone prolonged noxious stimulation during development (Dason et al., 2020; Kaneko et al., 2017). Hyperalgesia, allodynia, or both, have also been characterized in fruit flies via recording response latency to various stimuli after injury (Figure 1.2) (Babcock et al., 2009; Babcock et al., 2011; Lopez-Bellido & Galko, 2020; Lopez-Bellido et al., 2019). Most of this published research in fruit fly larvae has involved use of an ultraviolet (UV) injury on *Drosophila* third instar larvae but has also included puncture wounding (Babcock et al., 2009; Khuong, Wang, et al., 2019; Lopez-Bellido et al., 2019). It has also been shown that adult *Drosophila* possess a CNS sensory inhibition system similar to that found in mammals where when disruption occurs within centrally located GABAergic neurons (by either silencing GABA receptors or triggered by a peripheral amputation injury), a state of chronic sensitization of the nociceptors (even those not involved in injury) follows that is akin to neuropathic pain (Khuong et al., 2019; Moore et al., 2002; Sivilotti & Woolf, 1994). To this end, various cellular signaling pathways and channels (reviewed in section 1.6.1 and 1.6.1.1) have been identified within the nociceptor as contributing to the state of nociceptor hypersensitivity after injury, as well as GABAergic signaling within the CNS of the adult fly, but the complete mechanism has yet to be

fully identified (Babcock et al., 2009; Babcock et al., 2011; Brann, 2018; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; Jang et al., 2018; Jo et al., 2017; Khuong, Wang, et al., 2019; Lopez-Bellido et al., 2019; McParland et al., 2021). And though important studies have uncovered genetic components such as Arm, and channels such as, Painless, dTrpA1, and the Anoctamin Family channel, Subdued, for maintenance in baseline nociceptor sensitivity, even without injury, there are potentially many components of baseline nociceptor sensitivity that are also still elusive and could contribute to chronic pain conditions that result in the absence of any known injury (Hale, Moulton, et al., 2022; Jang et al., 2015; Kosek et al., 2021; Neely et al., 2011; Tracey Jr et al., 2003; Viswanath et al., 2003; Xu et al., 2006).

1.6.3 "Do fruit flies feel pain?" and *Drosophila melanogaster* neuropharmacology

Pain is described by the IASP as an "unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al., 2020) that is subjective to each person based on their own experiences surrounding the idea of pain and also "influenced by varying degrees of biological, psychological, and social factors" (Raja et al., 2020). The IASP 2020 revised definition of pain also indicates that "pain and nociception are two different phenomena" (Raja et al., 2020) with the stimulation of nociceptors in response to noxious stimuli not enough on its own to constitute as pain itself. *Drosophila melanogaster* is not known to possess a complex neural network which combines the awareness of noxious nociceptive stimuli to neural areas within the CNS linking into emotional processing and the subjective distressing experience of pain (Adamo, 2019; Bastuji et al., 2018; Garcia-Larrea & Bastuji, 2018; Nicholls et al., 2001; Raja et al., 2020). Fruit flies are, however, known to exhibit nociception and it has been shown that they can learn from exposure to a repeated noxious stimulus by developing a memory of the stimulus or other sensory cues coupled to the noxious stimulus such as odor coupled with electric shock (Hu et al., 2018; Quinn et al., 1974). This leads to a motivation to avoid the noxious stimulus and this avoidance can be recreated

through stimulation of fan-shaped body neurons in the adult fruit fly brain (Hu et al., 2018). A memory gene found in *Drosophila*, *amnesiac*, has even been found to play a role in thermal nociception in larval and adult *Drosophila* (Aldrich et al., 2010). Notably, memory has been hypothesized to play a part in the development of chronic pain in humans through activation of the hippocampus (Tajerian et al., 2018). So, even though it has not been shown that fruit flies are consciously aware and able to experience pain, there are still aspects of the pain signaling pathway and its dysregulation in humans, such as nociception, hyperalgesia, allodynia, neuropathic sensitization, and the ability to learn and form memories surrounding noxious stimuli/nociceptive input, that constitute fruit flies as a valuable research tool for investigating mechanisms contributing to pain and its relief (Adamo, 2019; Babcock et al., 2009; Babcock et al., 2011; Hu et al., 2018; Kandel, 2013; Khuong, Wang, et al., 2019; Tajerian et al., 2018; Waddell & Quinn, 2001).

Along with a lack of evidence that fruit flies consciously experience pain, however, is the lack of any known descending system of endogenous pain modulation found in *Drosophila melanogaster* such as an opiate system. However, although *Drosophila* do not seemingly possess a highly conserved opiate system, they do seem to carry resemblance to a reward system which is involved in addiction (Koyyada et al., 2018). In detail, adult fruit flies have been shown to seek out and prefer alcohol consumption and odor after repeated exposure (Gilpin & Koob, 2008; Hendershot et al., 2017; Koyyada et al., 2018; Peru et al., 2014). In a study carried out in 2018, it was found that when fruit flies were subjected to naltrexone, a drug used in human alcohol use disorder and which is also an opiate antagonist, they lose that preference for alcohol (Gilpin & Koob, 2008; Hendershot et al., 2017; Koyyada et al., 2018; Peru et al., 2014). Though the exact mechanism of naltrexone and alcohol preference in fruit flies was not uncovered within that experiment, the authors did raise the question of a possible unknown opioid-like system playing a role in the fly (Koyyada et al., 2018).

Fruit flies have also been found to be a translatable and useful model in testing neuropharmacological compounds affecting nociception. For example, it has been found that fruit flies respond to administration of the $GABA_B$ agonist, 3-APMPA (3-aminopropyl(methyl)phosphinic acid), in an antinociceptive manner similar to results found in mammals (Manev & Dimitrijevic, 2004; Thomas et al., 1996). Also, in a study investigating the necessity of the α2δ calcium channel subunit, Straightjacket (*stj*) (orthologous to human CACNA2D3 and 4 (calcium voltage-gated channel auxiliary subunit alpha 2 delta 3)), in the nociceptors of fruit fly larvae and adult for thermosensitivity, it was uncovered that gabapentin and pregabalin, drugs used in treatment of neuropathic pain, were able to relieve thermal nociceptor hypersensitivity after injury in adult fruit flies (Dworkin et al., 2007; Field et al., 2006; Freynhagen et al., 2005; Gee et al., 1996; Khuong, Hamoudi, et al., 2019; Moore et al., 2018; Neely et al., 2010; Wiffen et al., 2017). Uncovered *Drosophila* nociceptor sensitivity signaling pathway components have also inspired mammalian pain pharmacology research. For example, the Hedgehog (Hh) signaling pathway (reviewed in section 1.6.1.1) has been found to be critical for nociceptive sensitization to occur after injury in the fruit fly, a finding that was expanded toward investigation also into morphine tolerance in rats (Babcock et al., 2011). It was found in a study published in 2011 that blocking Sonic Hedgehog (Shh) signaling could induce a synergistic effect with morphine administration in treating inflammatory/neuropathic pain in mammals (Babcock et al., 2011; Milinkeviciute et al., 2012).

1.7 Gaps in knowledge and purpose of research

With the publication of the new ICD-11 listing multiple descriptive terminology for categorizing and addressing different types of chronic pain, which suggests chronic pain to be more of a multifaceted condition with multiple branches, it has become clear that finding better treatment options for pain includes developing a better understanding of what mechanisms underlie and distinguish each of the different pain types (Treede et al., 2015, 2019). Mechanisms underlying chronic pain that involve

nociceptor sensitivity specifically, are still very much elusive. With an expanded and unbiased effort, novel genetic/molecular components involved in the regulation of nociceptor sensitivity can lead to more diverse opportunities for drug development for these chronic pain conditions where underlying nociceptor sensitivity is a main factor in development and progression.

Over the years, *Drosophila melanogaster* has proven to be a fruitful model for pain investigation through modeling of nociceptive sensitivity mechanisms and investigation of molecular components involved in this sensitivity (Figures 1.2 & 1.4) (Babcock et al., 2009; Babcock et al., 2011; Follansbee et al., 2017; Im et al., 2015; Neely et al., 2011; Tracey Jr et al., 2003). However, the complete molecular processes underlying nociceptive sensitivity, which include nociceptive sensitivity before injury, nociceptive sensitivity after injury, and recovery of nociceptive sensitivity after injury, are still largely unknown within the fly and within mammals. Also, most of the nociceptor sensitivity work carried out in the fruit fly has taken place in its immature, larval form (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; McParland et al., 2021; Tracey Jr et al., 2003). This has left little room for chronic experimentation to take place within the fruit fly for pain investigation. And though some adult *Drosophila* thermonociception assays have already been developed, there are various drawbacks to these methods and many improvements that could be made to connect the research progressed in larvae thus far to that which can be carried out chronically in adults (Aldrich et al., 2010; Khuong, Wang, et al., 2019; Manev & Dimitrijevic, 2004; Massingham et al., 2021; Xu et al., 2006).

Therefore, within this dissertation work I carried out a continued investigation into uncovering molecular signaling pathways and genetic components involved in the regulation of nociceptive sensitivity, in varying conditional states, in the ongoing effort to uncover potential drug targets for treating irregular (chronic) pain in humans (Figure 1.5). To do this, in chapter two I investigated molecular components involved in the regulation of nociceptive sensitivity, without the condition of

injury, by using a validated *Drosophila* larval thermonociception model and focusing on the Wnt/Wg signaling pathway. In contrast to chapter two, in chapter three I then investigated the molecular components involved in both nociceptive sensitization after injury (allodynia) and nociceptive sensitization recovery (from hyperalgesia) by bioinformatic analysis of nociceptor-specific RNA sequencing data of a validated *Drosophila* larval UV injury model. Finally, also within this dissertation work we proposed to further the field of pain research using the fruit fly model by contributing to the development of an improved adult *Drosophila* model of injury-induced nociceptive sensitization in chapter four. We proposed that this adult fruit fly model will allow for more chronic experiments to take place in the future and also lead to the investigation and discovery of drug targets to be used in the treatment of chronic pain.

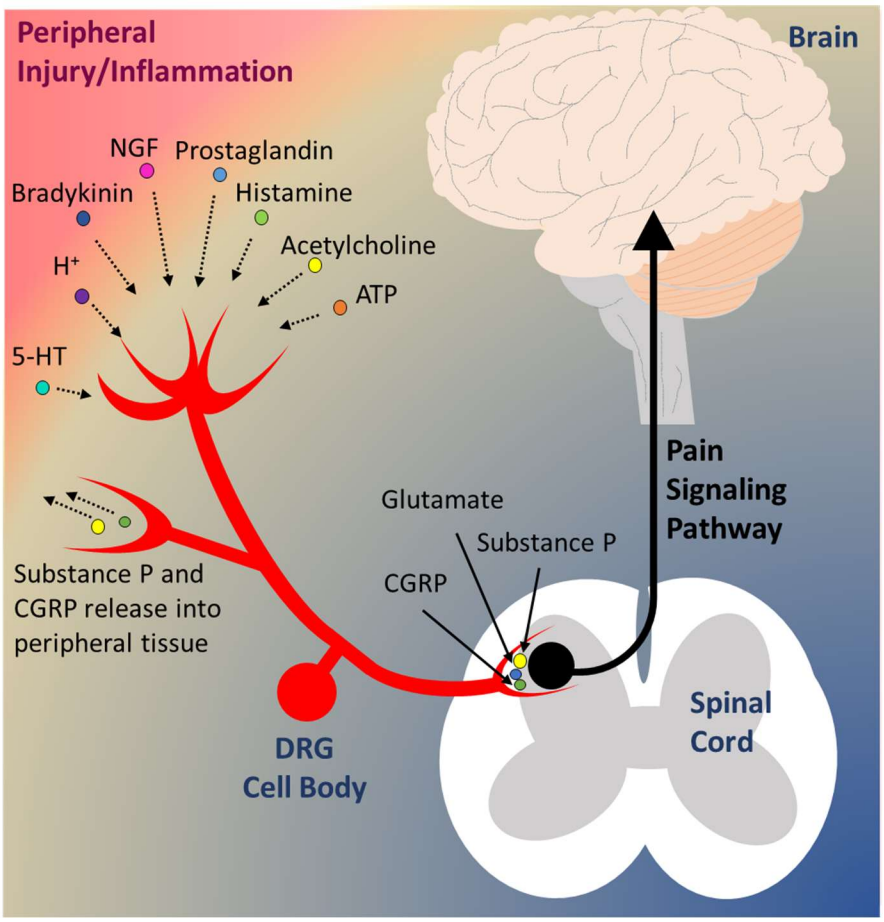

Figure 1.1 Nociceptive sensitization is the first step in the pain signaling pathway

The primary nociceptors detect noxious stimuli from peripheral injury and local inflammation. Some of the inflammatory mediators found in vertebrate peripheral tissue after injury include bradykinin, prostaglandin, histamine, ATP, H+, 5-HT, NGF, and acetylcholine, which can activate the nociceptors and lead to primary nociceptor sensitization. Mammalian primary nociceptors are also known to release substance P and CGRP into peripheral tissue during primary nociceptor sensitization, contributing to neurogenic inflammation within peripheral tissues. The primary nociceptors synapse onto sensory transmission neurons found within the dorsal horn of the spinal cord by neurotransmitter (ex: glutamate, substance P, CGRP) release, resulting in afferent pain signaling through the central nervous system. Graphic by C. Hale.

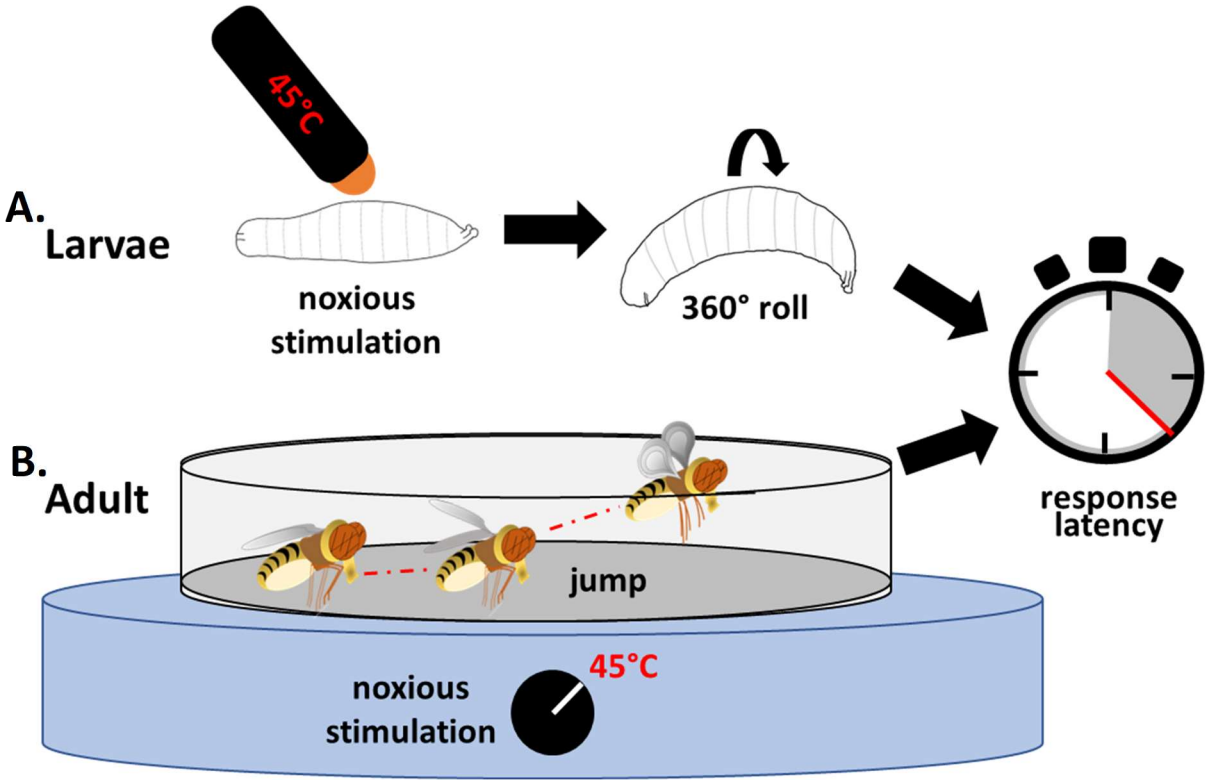

Figure 1.2 *Drosophila* larvae and adult escape response to a noxious thermal stimulus

A. *Drosophila* larvae carry out a 360-degree corkscrew roll when encountering a noxious stimulus. Example shown is a thermal tipped heat probe set to 45 degrees Celsius with latency recorded. **B**. Example of an adult *Drosophila* escape response to a noxious thermal stimulus in the form of a jump when encountering a noxiously heated surface with latency recorded. Graphic by C. Hale.

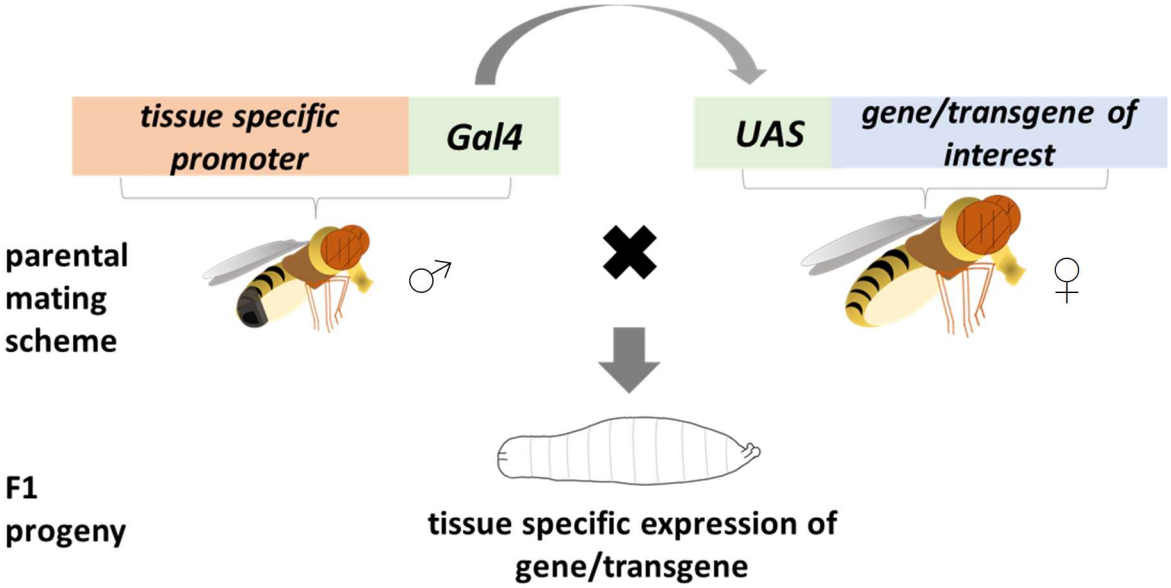

Figure 1.3 Schematic of Gal4/UAS system for tissue specific expression of gene/transgene

Mating of an adult fly carrying the yeast Gal4 (activator) construct driven by a tissue specific promoter to another fly carrying the UAS (Upstream Activating Sequence) (responder) construct upstream a gene/transgene of interest results in F1 progeny having tissue specific expression of the gene/transgene of interest. Graphic by C. Hale.

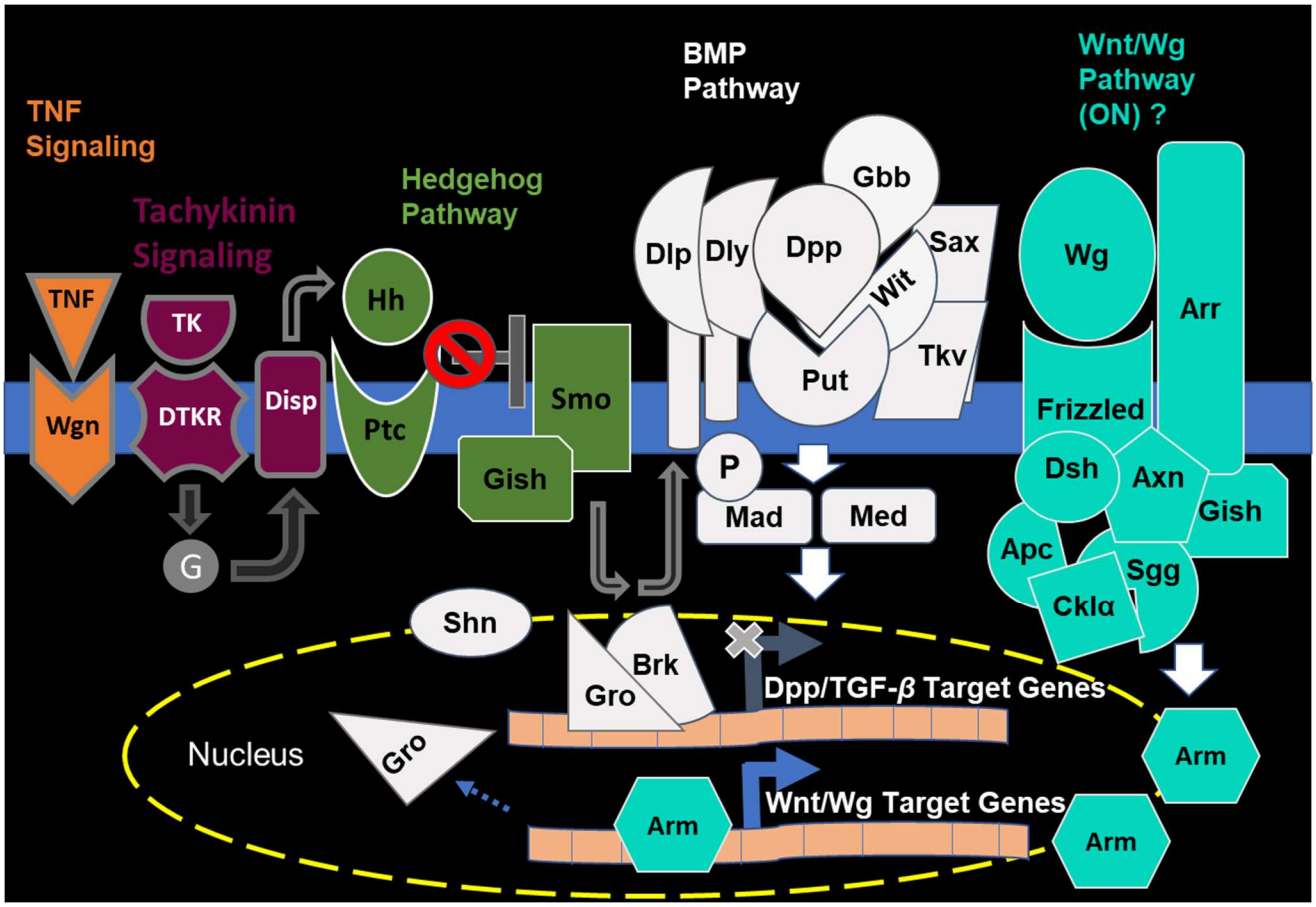

Figure 1.4 Cellular signaling pathways known or hypothesized to be involved in *Drosophila* nociceptive sensitivity regulation

Genetic components found to be involved in the regulation of *Drosophila* nociceptive sensitivity are within the TNF (Tumor Necrosis Factor) signaling pathway (orange), HH (Hedgehog) signaling pathway (green) (except for the component Gish), Tk (Tachykinin) signaling pathway (maroon) and the BMP (Bone Morphogenetic Protein) signaling pathway (light grey) (except for the component, Groucho (Gro)). The BMP pathway components, Dly and Brk (light grey), are also known to also interact with the canonical Wnt/Wg (Wingless) signaling pathway (teal). Due to this crosstalk between the BMP and Wnt/Wg signaling pathways, it is hypothesized that components within the Wnt/Wg signaling pathway may also be involved in nociceptive sensitivity regulation and investigation into this pathway has been included within this dissertation. Components investigated include the canonical Wnt/Wg signaling

Figure 1.4, continued

transcriptional activator, Arm (found to positively regulate sensitivity in the absence of injury), the known BMP and Wnt/Wg signaling repressor, Gro, and the Hh and Wnt/Wg signaling facilitator, Gish. Graphic by C. Hale.

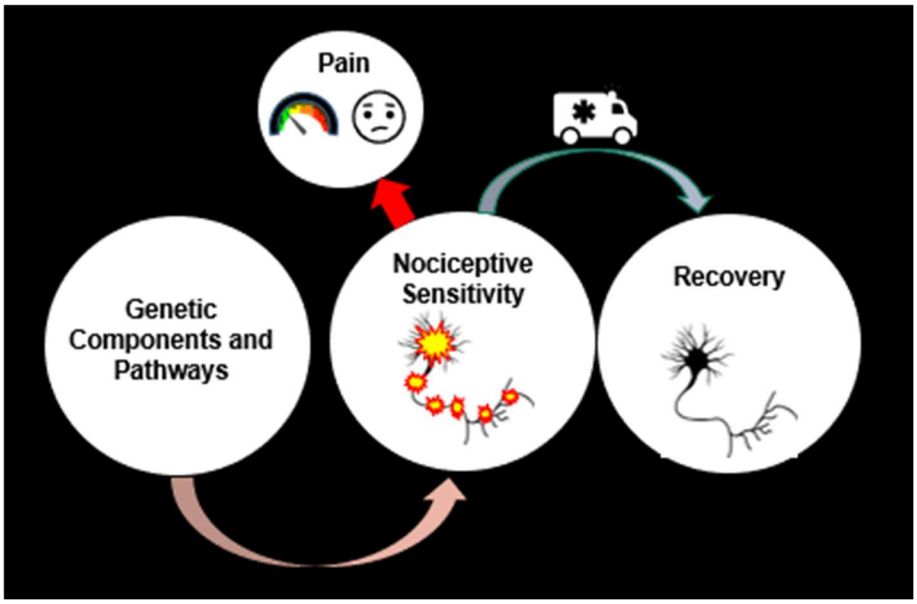

Figure 1.5 Purpose of research

Abnormal nociceptive sensitivity can lead to the heightened perception of pain, but questions remain of the process of this development and its recovery. Graphic by C. Hale.

CHAPTER 2

2. INVESTIGATION OF THE REGULATION OF NOCICEPTIVE SENSITIVITY WITHOUT INJURY: ARMADILLO REGULATES NOCICEPTIVE SENSITIVITY IN THE ABSENCE OF INJURY

** The following chapter includes data and text included in the publication of a primary research article in the Journal of Molecular Pain (Hale, Moulton, et al., 2022). The text has been slightly modified along with the appendage of additional data and text included for the completeness of this dissertation. Corresponding authors with their affiliations and contributions are described within the Acknowledgements section of this chapter and referenced within figure legends.*

2.1 Abstract

Abnormal pain has recently been estimated to affect ~50 million adults each year within the United States. With many treatment options for abnormal pain, such as opioid analgesics, carrying numerous deleterious side effects, research into safer and more effective treatment options is crucial. To help elucidate the mechanisms controlling nociceptive sensitivity, the *Drosophila melanogaster* larval nociception model has been used to characterize well-conserved pathways through the use of genetic modification and/or injury to alter the sensitivity of experimental animals. Mammalian models have provided evidence of β-catenin signaling involvement in neuropathic pain development. By capitalizing on the conserved nature of β-catenin functions in the fruit fly, here we describe a role for Armadillo, the fly homolog to mammalian β-catenin, in regulating baseline sensitivity in the primary nociceptor of the fly, in the absence of injury, using under- and over-expression of Armadillo in a cell-specific manner. Underexpression of Armadillo resulted in hyposensitivity, while overexpression of wild-type Armadillo or expression of a degradation-resistant Armadillo resulted in hypersensitivity. Neither underexpression nor overexpression of Armadillo resulted in dendritic morphological changes that could contribute to behavioral phenotypes observed. A significant behavioral response was also not found in knockdown of

the Wnt/Wg and BMP signaling transcriptional repressor, Gro. Overexpression of the Wnt/Wg signaling pathway facilitator, Gish, however, resulted in hypersensitivity, while underexpression had mixed results. In summary, these results showed that focused manipulation of Armadillo expression within the nociceptors is sufficient to modulate baseline response in the nociceptors to a noxious stimulus and that these changes are not shown to be associated with a morphogenetic effect.

2.2 Introduction/Relevant Background

2.2.1 Chronic pain and the *Drosophila melanogaster* nociceptive sensitization model

Abnormal pain has recently been estimated to affect approximately 50 million adults each year within the United States (Dahlhamer et al., 2018; Yong et al., 2022; Zelaya et al., 2020). With many treatment options for abnormal pain, such as opioid analgesics, carrying numerous deleterious side effects (Benyamin et al., 2008), research into safer and more effective treatment options is crucial. Despite this need, new, successful drug development for abnormal pain has been laborious, mostly due to a lack of understanding into the mechanisms that control pain sensitivity (Reichling & Levine, 2009). Specialized peripheral sensory neurons, referred to here as nociceptors, which detect noxious stimuli, are the first responders to the threat of injury in the pain signaling pathway (Bessou & Perl, 1969; Gold & Gebhart, 2010). Sensitivity of the nociceptors can be increased, for example after injury, by reducing the threshold of activation required to trigger a response. However, if such nociceptive sensitization persists after the injury has healed, symptoms of hyperalgesia and allodynia can take root and give way to abnormal pain (Gold & Gebhart, 2010; Kosek et al., 2021; Nicholas et al., 2019; Scholz et al., 2019). When this type of pain persists/reoccurs for typically three to six months or more, it is commonly referred to as chronic, and can lead to a substantial decrease in quality of life and an increased threat for opioid addiction in these patients (Costanza et al., 2021; Groenewald et al., 2019; International Association for the Study of Pain Task Force on Taxonomy, 1994; Treede et al., 2015; Vowles et al.,

2015). The mechanisms by which nociceptive sensitivity is controlled warrant further study in order to reveal improved treatments for abnormal pain.

In recent years, *Drosophila melanogaster* has proven to be an exceptional *in vivo* model organism for the investigation of mechanisms of neurological diseases, such as Parkinson's or chemotherapy-induced peripheral neuropathy, due to its relative organismal simplicity and powerful genetic toolkit (Boiko et al., 2017; del Valle Rodríguez et al., 2011; Feany & Bender, 2000). Fruit flies, like their human counterparts, exhibit a behavioral nociceptive response to noxious stimuli and can also develop nociceptive sensitization after injury, allowing for translatable modeling of allodynia and hyperalgesia, both known as possible symptoms of chronic pain (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im & Galko, 2012; Im et al., 2015; McParland et al., 2021). The nociceptors of the fruit fly have similar function and morphology to that of vertebrate counterparts; many genes underlying the perception of pain are conserved across species (Im & Galko, 2012; Khuong & Neely, 2013; Reiter et al., 2001). Additionally, there is an abundance of evidence demonstrating that fruit flies exhibit a variety of responses to encountered noxious stimuli, mostly centered around escape behaviors (Hwang et al., 2007; Sulkowski et al., 2011). Important pain discoveries made with this model are the roles of Painless (suggested to homologous to mammalian ANKTM1 and analogous in function to mammalian TRPV1 (Al-Anzi et al., 2006; Tracey Jr et al., 2003; Xu et al., 2006)), and identification of the *Drosophila* DEG/ENaC channel, Pickpocket (Ppk: similar to vertebrate epithelial sodium channels), which is known for sensing and reacting to harsh mechanical stimulation in the fly (Adams et al., 1998; Zhong et al., 2010). Studies have also shown that adult fruit flies possess a GABA-ergic mechanism of central pain regulation, similar to humans (Khuong, Wang, et al., 2019).

A nociceptive sensitization model has been developed utilizing *Drosophila* larvae, in which UV-injured and/or genetically modified animals become hyper or hypo-sensitive (Babcock et al., 2009).

Timepoints for increased sensitivity after UV injury were found to be at 8 hours for the larval hyperalgesia model, where animals were tested at the known noxious temperature of 45°C (Babcock et al., 2009). This peak hyperalgesia was then found to end by 24 hours, however, simultaneously, peak sensitivity for allodynia, where animals were tested at the threshold stimulus of 38°C (or 41°C in similar models), also occurred at the 24-hour mark and ended around 48 hours post UV injury (Babcock et al., 2009; Follansbee et al., 2017).

Using this model, the necessity and sufficiency of several biochemical signaling pathways such as Hedgehog (Hh), TNF-α (named Eiger in *Drosophila*), and BMP signaling pathways, functioning in the nociceptors, were revealed (Figure 1.4) (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; McParland et al., 2021). Of the signaling pathways identified to be involved in nociceptive sensitivity in recent years (Figure 1.4), similarities, and connections standout between the Hh and BMP signaling pathways, beyond the results found for nociceptive sensitivity. The Hh and the BMP signaling pathway are both known to be deeply involved in embryogenesis, development, and cell homeostasis, and form morphogen gradients that help define tissue patterning and cell fate determination (Abe & Tanaka, 2017; Lu et al., 2015; Nakamura et al., 1997; Rahman et al., 2015; Wang et al., 2014). This happens in part by signaling crosstalk with each other, sometimes synergistically and sometimes antagonistically (Abe & Tanaka, 2017; Nakamura et al., 1997; Papathanasiou et al., 2012; Rahman et al., 2015; Zhao et al., 2006). In summary, discovery of each of these pathways has increased knowledge relating to the development of nociceptive sensitization (Figure 1.4). However, a full understanding of this mechanism, including the control of baseline nociceptor sensitivity, meaning the level of sensitivity in the absence of injury, is still elusive.

2.2.2 Wnt/Wg β-catenin/Armadillo signaling and mammalian pain models

Wnt/β-catenin, a highly conserved signaling pathway (Cadigan & Nusse, 1997; Komiya & Habas, 2008) described functionally as much by mammalian research as by research studying its *Drosophila* counterpart, Wg/Armadillo signaling, has been historically investigated for its roles in embryogenesis (Riggleman et al., 1990; van Amerongen et al., 2012) and cancer development (Khramtsov et al., 2010; Kobayashi et al., 2000). A main component in the Wnt/Wg signaling pathway, β-catenin, homolog to *Drosophila* Armadillo (Arm), is the intracellular transducer of canonical Wnt/Wg signaling, whereby its nuclear translocation results in transcriptional activation of Wnt/Wg target genes (Figure 2.1) (Komiya & Habas, 2008; Peifer, Sweeton, et al., 1994; Van de Wetering et al., 1997). In the absence of the Wnt/Wg ligand, β-catenin/Arm protein levels are reduced within the cytoplasm by a two-step kinase-destruction complex, which phosphorylates β-catenin/Arm for subsequent ubiquitination and proteosome degradation (Figure 2.1) (Amit et al., 2002; Komiya & Habas, 2008; Liu et al., 2002; Peifer, Pai, et al., 1994; Stamos & Weis, 2013; Yanagawa et al., 2002). In *Drosophila*, this Arm destruction complex is made up of the inhibitory binding proteins: Axin (Axn), APC-like (Apc) and Adenomatous polyposis coli 2(Apc2), which form a binding scaffold with the kinases: Casein Kinase I alpha (CkIα), a serine/threonine kinase which phosphorylates Arm initially, and then the glycogen synthase kinase 3, Shaggy (Sgg), which phosphorylates Arm a second time, setting Arm up for ubiquitination and subsequent proteasome degradation when canonical Wg signaling is turned off (Figures 1.4 & 2.1) (Ahmed et al., 1998; Bejsovec, 2013; Hamada et al., 1999; Komiya & Habas, 2008; Peifer, Pai, et al., 1994; Peifer, Sweeton, et al., 1994; Perrimon, 1994; Stamos & Weis, 2013; Waghmare & Page-McCaw, 2018; Welsh et al., 1996; Yanagawa et al., 2000; Yanagawa et al., 2002; Yu et al., 1999). When canonical Wg signaling is turned on by the binding of a Wnt/Wg ligand (either Wingless (Wg), Wnt2, or Wnt6) to the Frizzled (Fz) and/or Frizzled 2 (Fz2) cell-surface receptor, Dishevelled (Dsh) is activated and binds to components within the destruction complex at the same time that Axin within the destruction complex also binds to the LDL

receptor-related protein, Arrow (Arr), (which functions as an obligate Wg co-receptor) (Figure 1.4) (Bejsovec, 2013; Cadigan & Nusse, 1997; Doumpas et al., 2013; Kennerdell & Carthew, 1998; Komiya & Habas, 2008; Llimargas & Lawrence, 2001; Noordermeer et al., 1994; Schaefer et al., 2018; Stamos & Weis, 2013; Tolwinski et al., 2003). Gilgamesh (Gish), a plasma membrane associated kinase orthologous to mammalian CK1γ1, has also been found to positively regulate Wnt/Wg signaling and assist in the inactivation of the Arm destruction complex through phosphorylation of Arrow (Figure 1.4) (Davidson et al., 2005; Hummel et al., 2002; Verheyen & Gottardi, 2010; Zhang et al., 2006). This inactivation of the destruction complex at the membrane all allows Arm protein levels to increase in the cytoplasm and then enter the nucleus to regulate transcription (Figures 1.4 & 2.1) (Hsu et al., 1998; Huber et al., 1996; Städeli et al., 2006).

In the rodent, β-catenin expression is upregulated in the spinal cord/ dorsal horn (Itokazu et al., 2014; Yuan et al., 2012; Zhang et al., 2021; Zhang et al., 2020), and dorsal root ganglia (DRG) (Zhang et al., 2021; Zhao & Yang, 2018) in neuropathic pain states. In a study including a mouse model of bone cancer (tumor implantation) pain and rat sciatic nerve injury (chronic constriction injury), Wnt/β-catenin signaling was found to be upregulated in the spinal cord and superficial spinal cord dorsal horn (Zhang et al., 2013). Within this same study, complete Freund's adjuvant (CFA) injection (to induce peripheral inflammation) was found to increase β-catenin expression within the rat spinal cord one day after the onset of mechanical allodynia (Zhang et al., 2013). In mouse model studies using either a partial sciatic nerve ligation (PSL), spared nerve injury (SNI), or multiple sclerosis model of chronic pain (subjects are inflicted with experimental autoimmune encephalomyelitis (EAE)), β-catenin was also found to also be significantly upregulated in its expression in the spinal cord/spinal cord dorsal horn (Itokazu et al., 2014; Yuan et al., 2012; Zhang et al., 2020). When studies focused on the dorsal root ganglia (DRG) specifically, within rat nerve injury models of either a chronic compression of dorsal root ganglion injury or chronic

constriction injury, β-catenin was also found to be significantly upregulated (Zhang et al., 2021; Zhao & Yang, 2018).

In these studies where neuropathic injury has been shown to lead to increased mechanical (Itokazu et al., 2014; Kim et al., 2021; Yuan et al., 2012; Zhang et al., 2021; Zhang et al., 2020) and thermal/cold (Zhao & Yang, 2018) sensitivity in behavioral assays, attenuation of this hypersensitivity was also achieved through local administration of pharmacological Wnt/β-catenin signaling inhibitors. This points toward a therapeutic role for local Wnt/β-catenin pathway blockade in the management of neuropathic pain. Paradoxically, when β-catenin was knocked out in a subset of DRG sensory neurons, the nociceptors, no changes in baseline nociceptive sensitivity were observed (Simonetti et al., 2014).

2.2.3 Aims of this study

The Hh, BMP, and Wnt/Wg signaling pathway are known to carry out extensive crosstalk through their various genetic components in mechanisms such as those involved in development and also those contributing to disease progression, such as cartilage degradation in osteoarthritis and gastrointestinal cancers (Katoh & Katoh, 2006; Okamoto et al., 2014; Papathanasiou et al., 2012; Rahman et al., 2015; Zhao et al., 2006). Given this known crosstalk, the involvement of several components within the Hh and BMP signaling pathways shown to be involved in injury-induced nociceptive sensitization in the fly (Babcock et al., 2011; Brann, 2018; Follansbee et al., 2017; Gjelsvik et al., 2018; McParland et al., 2021), and prior mammalian investigations implicating Wnt/β-catenin involvement in neuropathic pain (Itokazu et al., 2014; Kim et al., 2021; Yuan et al., 2012; Zhang et al., 2021; Zhang et al., 2013; Zhang et al., 2020; Zhao & Yang, 2018), investigation into to the role of Wg/Arm signaling in the fly is warranted.

By capitalizing on the conserved nature of Wnt/β-catenin signaling in the fruit fly (White et al., 1998) and the previously validated fruit fly model for investigating nociception (Babcock et al., 2009;

Babcock et al., 2011; Tracey Jr et al., 2003), this study sought to determine the role of Arm in regulating sensitivity in a specific neuron, the primary nociceptor of the fly, in the absence of injury, using experimental under- and over-expression of Arm in a cell-specific manner. Validation of both BMP and Wg signaling involvement in controlling nociceptor sensitivity was also further investigated through under-expression of their common transcriptional repressor, Groucho (Gro), in the absence of injury (Figure 1.4) (Cavallo et al., 1998; Hasson et al., 2001). Investigation was also conducted in the absence of injury through under- and over- expression of the known Wnt/Wg facilitator, Gish, which is also a known positive facilitator of Hh signaling (Figure 1.4) (Davidson et al., 2005; Hummel et al., 2002; Li et al., 2016; Verheyen & Gottardi, 2010; Zhang et al., 2006).

2.3 Methods

2.3.1 Fly husbandry

Flies were obtained from the Bloomington *Drosophila* Stock Center (BDSC) in Bloomington, Indiana and maintained in 6 oz stock bottles containing sucrose-cornmeal-yeast medium. Bottles were stored at ambient room temperature and kept between 45-60% humidity. Apart from lines used for MiMIC analysis, genotypes used in experiments were prepared using the Gal4/UAS (Brand & Perrimon, 1993; Duffy, 2002) system with the following Gal4 driver lines featuring the *pickpocket* (Adams et al., 1998; Ainsley et al., 2003; Zhong et al., 2010) promoter: *ppk1.9-GAL4* (in w^{1118}) for thermal nociception assays and *ppk1.9-Gal4, UAS-mCD8-GFP (*in *yw*) for neuromorphometric analysis, immunohistochemistry, CTCF and Integrated Density analysis. Transgenic lines included: *UAS-arm.S10* (Pai et al., 1996; Pai et al., 1997) (in y^1w^{1118}) (BDSC_4782), *UAS-arm.S2* (Orsulic & Peifer, 1996; Pai et al., 1997) (in y^1w^{1118}) (BDSC_4783; for behavior experiments, we swapped the balancer within our lab to TM6b for its Tb marker visible in larvae), *UAS-arm-IR-1* (Perkins et al., 2015) (in y^1v^1) (BDSC_35004), *UAS-arm-IR-2* (Perkins et al., 2015) (in y^1v^1) (BDSC_31304), $MiMICarm^{MI08675}$ (Nagarkar-Jaiswal et al., 2015)

(BDSC_44994), *UAS-gro-IR-1* (Perkins et al., 2015) (in y^1v^1) (BDSC_35759), *UAS-gro-IR-2* (Zirin et al., 2020) (in y^1v^1) (BDSC_91407), *UAS-gish-IR-1* (in y^1v^1)(Perkins et al., 2015) (BDSC_28066), *UAS-gish-IR-2* (in y^1v^1) (Perkins et al., 2015)(BDSC_35138), *UAS-gish-myc* (Gish-OE) (in w^*) (Gault et al., 2012) (BDSC_41764), and *ppk1.9-tdTomato*. Wild-type fly lines and control lines for TRiP (Perkins et al., 2015; Zirin et al., 2020) RNAi lines used were: w^{1118} (BDSC_3605), y^1v^1 (BDSC_36303), and y^1w^{1118} (BDSC_6598). Each Gal4/UAS genotype used in thermal nociception assays was compared to two controls: one with the genetic background (w^{1118}) of the Gal4 driver crossed with the UAS transgenic line (No Gal4 control) and one with the Gal4 driver line crossed to the genetic background (either y^1v^1, w^{1118}, or y^1w^{1118}) of the UAS transgenic line (No UAS control). The Gal4/UAS system allows over- or under- expression of a given target gene in a specific cell type, determined by the Gal4 driver (Figure 1.3) (Brand & Perrimon, 1993). In these experiments, the Gal4 driver used was *ppk1.9-Gal4*, which selects the dendritic arborization neurons known as Class IV multidendritic neurons, well characterized as primary nociceptors (Hwang et al., 2007; Im & Galko, 2012).

2.3.2 Ultraviolet-C irradiation injury

Ultraviolet-C irradiation injury was conducted on foraging, 3rd instar *Drosophila* larvae. Around 10-20 larvae at a time were rinsed in water and anesthetized within a wire-mesh bottomed container, placed within a glass Coplin jar with a cotton ball that had been saturated in ~ 1.5 mL of diethyl ether. Animals were kept within the anesthetization chamber ≤ 4 minutes. Anesthetized larvae were then place dorsal side up on a microscope slide (moving larvae were removed before irradiation) and exposed to 14-21 mJ/cm2 of UV-C irradiation using a Spectronics Corporation Spectrolinker XL-1000 ultraviolet crosslinker. UV dosage was measured for each round of irradiation using a Spectronics Corporation Spectroline XS-254 UV-C photometer. After UV exposure, larvae were gently rinsed with water in a petri

dish, collected, and placed in a glass vial containing approximately 1 mL of fly food. Vials were then stored in an incubator for 24 hours at 25°C before the 41°C behavioral assay for allodynia.

2.3.3 Thermal nociception assays

Foraging third instar larvae were assayed by methods validated previously (Babcock et al., 2009; Babcock et al., 2011; Brann, 2018; Follansbee et al., 2017; Gjelsvik et al., 2018; Tracey Jr et al., 2003). In these nociception assays, the dorsal side of the larval epidermis (midline between abdominal segments A4-A6) was lightly touched by a thermal tipped heat probe (ProDev Engineering, Missouri City, Texas) set to the previously determined noxious temperature of 45˚C (Babcock et al., 2009) to assess normal nociception (or 41°C for injury-induced allodynia). The operator was blind to genotype and behavior was evaluated within a 20 s (Babcock et al., 2009) timeframe for latency of larval nocifensive escape behavior, characterized as a corkscrew rolling response, with time of response or no response recorded (Hwang et al., 2007).

2.3.4 Immunohistochemistry

Third instar larvae expressing GFP within their nociceptors (via *ppk-Gal4, UAS-mCD8-GFP*), were filleted as previously described (Follansbee et al., 2017) and immediately fixed by 30-min incubation at room temperature (RT) with ice-cold 4% paraformaldehyde in phosphate buffered saline solution (PBS). Fixation was followed by washes in 0.3%-1.0% PBT (1% Triton X-100 in PBS for anti-c-MYC and anti-Gro experiments and 0.3% Triton X-100 in PBS for anti-Arm experiments), which included two 1-min washes, one 10-min wash, and one 1-hr wash at RT. Washed fillets were then blocked using PBT-B (0.3% Triton X-100 + 1% bovine serum albumin (BSA) + PBS) for at least 1 hr at RT. After initial blocking, fillets were incubated overnight at 4°C using gentle rotation with either mouse anti-Arm (DSHB Hybridoma Product N2 7A1 Armadillo) (Riggleman et al., 1990) for *arm.S2* and *arm-IR-1* experiments at a dilution of 1:200 in

PBT-B, or mouse anti-c-MYC (DSHB Hybridoma Product 9E 10-s) (Evan et al., 1985) for *arm.S10* and *arm.S2* experiments at a dilution of 1:10 in PBT-B, or mouse anti-Gro (DSHB Hybridoma Product anti-Gro supernatant) (Delidakis et al., 1991) for *gro-IR-1* experiment at a dilution of 1:100 in PBT-B. Overnight incubation was followed by two 30-min washes in PBT-B with rotation and then a second blocking for 1 hr using fresh PBT-B + 5% normal goat serum (NGS) at RT. Following the second blocking, fillets were incubated for 2 hrs at RT with the fluorescently conjugated secondary antibody, goat anti-mouse AlexaFluor-647 (Catalog#: A-21236, Invitrogen, Thermo Fisher Scientific, Inc), diluted to 1:500 in PBT-B + 5% NGS. Fillets were then washed three times in 0.3% PBT (0.3% Triton X-100 in PBS) for 30-min, followed by two washes for 2 min with PBS. Fillets were mounted onto slides using Vectashield Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories) for nuclear staining and kept in the dark at 4°C.

2.3.5 Imaging and CTCF Analysis

Nociceptors from third instar larvae fillets prepared for fluorescent analysis by immunohistochemistry were imaged with a Leica TCS SP5 scanning laser confocal microscope using a 40x oil objective and a HyD detector. *Z* stacks were obtained with a 0.38µm step size, a scan format of 1024 x 1024, and, for the channel to be quantified, using uniform acquisition settings across experimental and control samples for smart gain, laser power, zoom, frame averaging, and pinhole. In an effort to comply with previously described ethical and appropriate biological imaging procedures (Cromey, 2010) and to avoid any misrepresentations in fluorescence intensity, significant efforts were taken to avoid the saturation of pixels during image acquisition for the fluorescence channels to be quantified. In this effort to remain below pixel saturation, yet also keep image acquisition settings constant across all samples and treatments within an experiment, fluorescence signal in some samples was obtained at much lower laser power output than if they were imaged independently. For example,

No UAS sample images for anti-Arm in Figure 2.3 were acquired at a scanning confocal laser power output of 14%, however, the No UAS sample images for anti-Arm in Figure 2.4 had to be acquired at a lower laser power output of 4% due to the higher intensity of fluorescence in the *arm.S2* line for anti-Arm. Using Fiji (Schindelin et al., 2012), five z-slices toward the mid-section of each nociceptor z-stack were sum projected and then cropped to remove the majority of dendritic structures and display the nociceptor soma primarily. Additionally, within Fiji (Schindelin et al., 2012), masks were made from these cropped sum projections that corresponded to either the nucleus, visualized by DAPI fluorescence, or the soma, visualized by GFP fluorescence, to obtain regions of interest (ROIs) specific to that portion of the cell. To keep mask generation steps consistent across all samples and eliminate selection bias as much as possible, a macro was recorded in Fiji (Schindelin et al., 2012) for semi-automation and is available upon request. Any overlapping nuclei (visualized by DAPI) surrounding the nociceptor were also masked and made into an ROI which was then cleared from each soma and nuclear mask before obtaining the final ROIs used for measurement, to account for any anti-Arm, anti-Gro, or anti-c-MYC fluorescence that could arise from cells close to the nociceptor. Nuclear, cytoplasm, and soma ROIs were then used to measure area and integrated density in Fiji (Schindelin et al., 2012) for anti-Arm, anti-Gro or anti-c-MYC fluorescence within the cropped sum projections and corrected total cellular fluorescence (CTCF) was calculated using the following calculation described previously (McCloy et al., 2014). The formula for CTCF is: CTCF= Integrated density - (Area of selected cell x Mean fluorescence of background readings) (McCloy et al., 2014). The mean fluorescence of background was the average of three mean fluorescence measurements obtained using images of larval fillet controls that did not receive the primary antibody (anti-Arm or anti-Gro), or those samples that did not express the c-MYC protein endogenously (No UAS samples in the anti-c-MYC experiment). To verify that antibody fluorescence signal was statistically above background levels in anti-Arm and anti-Gro experiments, we compared integrated density (the product of area and mean gray value) measurements obtained in Fiji (Schindelin

et al., 2012) (using the ROIs generated by masks for soma, cytoplasm, and nucleus of each sample) for No 1°Ab controls and No UAS controls. The CTCF and Integrated Density for each sample/group was then averaged and a Student's *t* test with Welch's correction was applied or, in situations where the Shapiro-Wilk test indicated that the data were not normally distributed, a Mann-Whitney *U* test was applied. CTCF and Integrated Density statistical analysis tests were carried out using Microsoft Excel (version 2104 and 2204) with the Real Statistics Resource Pack software package (Release 7.6), Copyright (2013-2021) Charles Zaiontz, www.real-statistics.com) and R statistical coding software (R Core Team, 2021). Representative images used in figures were sum-projected with the same 5 z-slices used in analysis, cropped with uniform area, and adjusted for brightness/contrast uniformly within the channel being quantitated across all conditions since laser level/gain was kept low to prevent saturation during acquisition. DAPI is shown with the lookup table "cyan hot", and anti-Arm, anti-Gro, anti-c-MYC are shown with the lookup table "magenta", in Fiji (Schindelin et al., 2012). Representative CTCF images shown are within one standard deviation of the average soma CTCF calculation/group. Arm::GFP/ppk-tdTomato was imaged using the 63x oil objective on a third-instar larva anesthetized in halocarbon-ether mixture (2:1) and placed on a microscope slide with glass coverslip for live imaging. The z-stack acquired with a 0.34µm step size was then max projected and cropped using Fiji (Schindelin et al., 2012) with brightness/contrast settings adjusted for clarity.

2.3.6 Neuromorphometry

To determine if observed behavioral phenotypes were associated with changes in neuromorphology of the class IV multidendritic neurons targeted, neurons were measured *in vivo* for total dendritic length, dendritic branching, and changes to the dendritic arbor through Sholl analysis (Sholl, 1953). Third instar larvae measuring 4.0 to 4.5 mm in length were anesthetized with ether for up to 4 minutes then placed within a halocarbon-ether mixture (2:1) on a microscope slide and covered

with a 22 x 50 mm glass coverslip for live imaging. Using a Leica TCS SP5 scanning laser confocal microscope and a HyD detector, nociceptors were imaged between abdominal segments 4–6. Z-stacks were collected using the 20x (NA 0.7) dry objective, a resolution of 1024 x 1024, and a z-stack step-size of 0.88 µm. Max projections (carried out in Fiji (Schindelin et al., 2012)) of images were exported as tifs to Jasc® Paint Shop Pro™ (Version 7.04) and/or the Superimpose X Neo for iPad (1.5.2). Within these programs, axons, and background autofluorescence of non-quantifying structures (such as denticle belts) were removed from images and dendrites were traced (Sears & Broihier, 2016) over at disjointed, low intensity pixel areas where due to low signal, decreasing threshold would introduce an excess of noise surrounding the dendrite and increasing threshold would result in the introduction of gaps that would need to be manually reconstructed (Stanko et al., 2015). For analysis of dendritic length and dendritic branching, these images were then thresholded, skeletonized, and measured via the AnalyzeSkeleton (2D/3D) plugin (Arganda-Carreras et al., 2010) in Fiji (Schindelin et al., 2012) as previously described with modification for neuromorphometric quantification (Iyer et al., 2013). Output data from the AnalyzeSkeleton analysis was compiled via Python scripts prior to import into Microsoft Excel (version 2104) for statistical analysis. For Sholl analysis (Sholl, 1953), images were analyzed using the Sholl analysis plugin (T. A. Ferreira et al., 2014) in Fiji (Schindelin et al., 2012) by methods described previously (T. Ferreira et al., 2014;). Representative images have been cropped to the nociceptor of interest, shown without color and the lookup table, “Invert LUT", in Fiji (Schindelin et al., 2012) applied for clearer visualization of dendrites.

2.3.7 Statistics

Thermal nociception assays were plotted as percent accumulated response vs. latency where an end-point cut-off of 20 s was applied and latency in seconds recorded. After applying a binary variable to the data based on ‘response’ or ‘no response’ at the 20 s cut-off time, statistical analysis of latency of

response between all behavioral data groups was completed using log-rank analysis and applying Benjamini and Hochberg procedure for multiple testing. In neuromorphometrical pairwise comparisons, average dendritic branch length and average number of branches were evaluated using a Student's *t* test with Welch's correction. Sholl profile data was found to not be normally distributed via Shapiro-Wilk test and so pairwise comparison was evaluated via the Mann-Whitney *U* test. CTCF and Integrated density analysis was analyzed by a Student's *t* test with Welch's correction, or the Mann-Whitney *U* test as described previously. Log-rank analysis was performed using R statistical coding software (R Core Team, 2021) and applying the 'survival' analysis package (Therneau, 2020). All other statistical tests and plots were carried out using Microsoft Excel (version 2104 and 2204) and the Real Statistics Resource Pack software package (Release 7.6), Copyright (2013-2021) Charles Zaiontz, www.real-statistics.com).

2.4 Results

2.4.1 Arm underexpression within nociceptors

Since Arm is known to activate transcription of canonical Wnt/Wg signaling target genes (Komiya & Habas, 2008; Peifer, Sweeton, et al., 1994; Van de Wetering et al., 1997) and Wnt/β-catenin signaling has been shown to regulate neuropathic pain development in mammals after injury (Itokazu et al., 2014; Zhang et al., 2021; Zhang et al., 2020; Zhao & Yang, 2018), we sought to assess whether underexpression of Arm within the nociceptors would result in a decrease in nociceptive sensitivity in the absence of injury. This assessment can be easily carried out in the fly using validated, thermal nociception assay techniques (Figure 2.2A) (Babcock et al., 2009; Babcock et al., 2011; Hwang et al., 2007; Tracey Jr et al., 2003).

We confirmed that Arm is expressed within third instar larval nociceptors using the MiMIC method (Nagarkar-Jaiswal et al., 2015) (Figure 2.2B), in which GFP is fused with the Arm coding region and subject to the same regulation as the normal gene. The functionality of the resulting Arm::GFP

fusion protein is indicated by the observation that flies homozygous for this construct are viable. Imaged in live anaesthetized larvae, punctate GFP expression is visible in nociceptor somata and neurites, indicated by *ppk1.9-tdTomato* expression, as well as the neurites of other unidentified neurons (Figure 2.2B). Since our analysis using these MiMIC images did not include fluorescence quantification but rather protein localization, efforts to maintain pixel saturation were not stringently followed.

Next, we used Gal4/UAS cell targeting (Figure 1.3) and RNA interference technology (*arm-IR-1* and *arm-IR-2*) to reduce Arm expression, specifically in the nociceptors. Confirmation of Arm expression and knockdown (*arm-IR-1*) within the nociceptors of animals was obtained through immunofluorescent quantification of Arm protein via use of a previously validated Arm antibody (Riggleman et al., 1990) and rigorous comparison techniques. Effort was taken to avoid pixel saturation and image acquisition settings were kept constant in the fluorescence channel quantified across all conditions. Results showed a significant decrease in anti-Arm fluorescent signal in the nociceptor somata of *arm-IR-1* animals, compared to control animals (Figure 2.3A-C). Results also showed a significantly higher anti-Arm fluorescent signal in nociceptor somata of No UAS animals, compared to No 1°Ab controls (Figure 2.3C).

arm-IR-1 animals were then compared to controls for thermal nociception response to a noxious 45°C temperature probe, by evaluation of nocifensive behavior in the absence of injury (Figure 2.3D), within a 20 s timeframe (Babcock et al., 2009). *arm-IR-1* animals showed a significant decrease in nocifensive response when compared to controls (Figure 2.3D). Concerns that off-target effects caused this phenotype are reduced by analysis of a second non-overlapping Arm IR line (*arm-IR-2*) which also showed a significant decrease in nociceptive sensitivity (Figure 2.3E). Though our primary focus was investigation of Arm involvement in nociceptor sensitivity in the absence of injury, we also carried out preliminary experiments, with a smaller sample size in some groups, on third instar larvae for UV injury-induced allodynia when Arm was knocked down within the nociceptors (*arm-IR-1*) (See Appendix 1). *arm-IR-1* animals were injured by ultraviolet (UV) irradiation and behaviorally assayed for nocifensive

response, 24 hours post injury with a thermal tipped heat probe set to 41°C (Babcock et al., 2009; Follansbee et al., 2017). These preliminary experiments did not result in significant findings when comparing injured experimental animals (*arm-IR-1*) to both of the injured control groups (See Appendix 1).

2.4.2 Arm overexpression via *arm.S2*

To compare with the underexpression study described above, we then evaluated Arm's capacity to control nociceptor sensitivity by overexpressing Arm protein. We did this by driving expression of a wild-type Arm protein (Arm.S2) (Orsulic & Peifer, 1996; Pai et al., 1997) within the nociceptors specifically. Resulting overexpression, compared to controls, was confirmed through immunofluorescent quantification using anti-Arm in fixed tissue (Figure 2.4A-C) as above. Effort was again taken to avoid pixel saturation and image acquisition settings were kept constant in the fluorescence channel quantified across all conditions. This effort resulted in lower laser intensity output being used in image acquisition than the previous Arm-IR-1 IHC experiment, due to the increase in fluorescence intensity for the Arm.S2 anti-Arm signal. Results, however, still showed a significantly higher anti-Arm fluorescent signal in nociceptor somata of No UAS animals, compared to No 1°Ab controls (Figure 2.4C), verifying significant anti-Arm signal above background in normal animals. The expression of the Arm.S2 protein significantly increases Arm expression in both the cytoplasm and nucleus of the nociceptor (Figure 2.4A-B). Thermal nociception assays at 45°C were then carried out and showed that the animals expressing increased nociceptor Arm levels (*arm.S2*), and in the absence of injury, responded significantly faster, compared to their specific control lines (Figure 2.4D).

2.4.3 Arm overexpression via *arm.S10*

In an effort to explore the effects of an Arm trafficking environment similar to that produced when Wnt/Wg signaling is activated (Figure 2.1), we carried out experiments (Fig 2.5) using a transgenic line expressing a mutant Arm protein (Arm.S10), in which regions within the N terminus needed for phosphorylation and ubiquitination had been deleted (Pai et al., 1996; Pai et al., 1997). Since these deletions overlap regions of the epitope targeted by the anti-Arm monoclonal antibody used above (Pai et al., 1996; Pai et al., 1997; Riggleman et al., 1990; Tolwinski & Wieschaus, 2001), the expression of the Arm.S10 protein within the nociceptors was confirmed via treatment of fixed tissue with a c-MYC antibody (Evan et al., 1985; Pai et al., 1996; Pai et al., 1997; Tolwinski & Wieschaus, 2001) (Figure 2.5A-B) targeting Arm.S10's c-MYC tag. Since Arm.S2 (Orsulic & Peifer, 1996; Pai et al., 1997) is likewise tagged with c-MYC, we also compared expression levels of these two modified Arm proteins, Arm.S2 and Arm.S10 and found that Arm.S2 is the more highly expressed in this context (Figure 2.5A-B). Both modified protein constructs significantly increased overall Arm levels in the nociceptor nuclei and cytoplasm (Figure 2.5A-B). Thermal nociception assays at 45°C showed that animals expressing *arm.S10* within their nociceptors responded significantly faster than normal controls in the absence of injury (Figure 2.5C).

2.4.4 Arm influence on nociceptor morphology

We investigated the possibility that the nociceptors of larvae with altered expression of Wnt/Wg signaling and Arm activity may also have altered nociceptor morphology and that by this mechanism, adjustment of Arm expression could lead to physiological changes in nociceptor sensitivity, in the absence of injury. Nociceptors within animals expressing either a transgene knocking down expression of Arm within the nociceptors (*arm-IR-1*) or expressing a constitutively active form of Arm within the nociceptors (*arm.S10*), were analyzed for dendritic number, length, and dendritic arbor complexity

(Sholl analysis) (Sholl, 1953) (Figures 2.6 & 2.7). Nociceptors expressing neither *arm-IR-1* (Figure 2.6) nor *arm.S10* (Figure 2.7) showed any statistical difference in dendritic length, number of dendrites, or dendritic arbor complexity when compared to controls.

2.4.5 Gro underexpression within nociceptors

We also knocked down the expression of Gro (Groucho), known to repress the transcription of Wnt/Wg and BMP target genes (Figure 2.8) within the nociceptors of uninjured animals (Cavallo et al., 1998; Hasson et al., 2001). Confirmation of Gro knockdown (via *gro-IR-1*) was obtained through immunofluorescent quantification of Gro protein using a previously validated Gro antibody (Apidianakis et al., 2001; Delidakis et al., 1991) in fixed larval tissues (Figure 2.9A-C). Effort was again taken to avoid pixel saturation and image acquisition settings were kept constant in the fluorescence channel quantified across all conditions. Gro was found to be significantly reduced in expression exclusively within the nuclei of nociceptors in animals expressing *gro-IR-1*, compared to control animals (Figure 2.9A-B). Results also showed a significantly higher anti-Gro fluorescent signal in the nociceptor somata of the No UAS control animals when compared to No 1°Ab controls animals (Figure 2.9C), indicating significant signal against background. *Gro-IR-1* animals, in the absence of injury, were then compared to controls for baseline thermal nociception response to a noxious 45°C temperature and showed no significant differences in nocifensive response (Figure 2.9D). A second, uninjured, non-overlapping Gro-IR (*gro-IR-2*) line was also behaviorally tested and showed similar results (Figure 2.9E).

2.4.6 Gish over- and under- expression in the nociceptors

We continued our investigation of Wnt/Wg signaling influences on nociceptor sensitivity by then also examining whether overexpression of another positive regulator of canonical Wnt/Wg signaling and Hh signaling, the component: Gish, would result in an increase in nociceptive sensitivity, in the absence

of injury. Results of our thermal tipped heat probe assay at 45°C showed there to be a significant increase in nocifensive response of those animals with Gish overexpressed (via *gish-OE*) in their nociceptors when compared to control animals (Figure 2.10A). We then sought to complement this overexpression investigation with Gish underexpression (via *gish-IR*) within the nociceptor. Based on our *gish-OE* results, we hypothesized that animals with *gish-IR* within the nociceptors would result in a decrease in behavioral thermal hypersensitivity. However, results from our thermal tipped heat probe assay at 45°C on un-injured animals where Gish had been knocked down in their nociceptors (via *gish-IR-1* and *gish-IR-2*), showed mixed results (Figure 2.10B-C). *gish-IR-1* was found to show no significant difference in latency when compared to its controls (Figure 2.10B), but *gish-IR-2* showed a significant decrease in response latency over time when compared to both of its controls (Figure 2.10C).

2.5 Discussion

β-catenin, the closest mammalian homolog of *Drosophila* Armadillo (Arm), and the Wnt signaling pathway have been shown to be upregulated during the development of neuropathic pain in mammals (Itokazu et al., 2014; Yuan et al., 2012; Zhang et al., 2021; Zhang et al., 2020), and locally administered blockers of Wnt/Wg signaling produces relief of neuropathic pain (Itokazu et al., 2014; Kim et al., 2021; Yuan et al., 2012; Zhang et al., 2021; Zhang et al., 2020; Zhao & Yang, 2018). However, baseline pain sensitivity is unaltered by nociceptor-specific knockout of β-catenin in a mammalian model (Simonetti et al., 2014). We sought to shed light on the relationship between β-catenin's various functions and baseline pain using a simplified model system, the fly.

Of the numerous gene candidates previously identified as controlling nociceptor sensitivity in fruit flies, the glypican, Dally (Tsuda et al., 1999), a potentiator of Wnt/Wg signaling, was found to be required in the nociceptor for nociceptive sensitization after injury (Figure 1.4) (Brann et al., 2019). Also, the transcription factor, Brinker, a negative regulator of BMP signaling in the fly and known to

antagonize Wg/Wnt signaling (Saller et al., 2002), suppresses nociceptive sensitivity (Figure 1.4) (McParland et al., 2021). The findings reported here contribute further evidence consistent with the idea that β-catenin/Arm signaling affects nociceptor sensitivity in the fly by demonstrating that Arm, demonstrated here to be expressed within class IV da neurons, or nociceptors, of *Drosophila* larvae (Fig 3B), is capable of controlling nociceptive sensitivity in the absence of injury.

Though prior studies have shown the necessity of other pathways such as Hedgehog, TNF-α/Eiger, and BMP pathways for UV injury-induced nociceptive sensitization in the fly (Figure 1.4) (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; McParland et al., 2021), and the TRP channels Painless (Tracey Jr et al., 2003; Xu et al., 2006) and dTRPA1(Viswanath et al., 2003), and Anoctamin Family channel Subdued (Jang et al., 2015) for maintenance of nociceptor sensitivity, there are potentially aspects of the nociceptor sensitivity mechanism that have been largely unexplored. By testing behavioral response latencies at a known noxious temperature of 45°C (Babcock et al., 2009), our results reveal a cell-specific requirement for Arm in maintaining nociceptor sensitivity in the absence of injury or illness. This requirement was demonstrated by significant increase in latencies observed in un-injured animals with Arm knocked down specifically within their nociceptors by two non-overlapping RNAi constructs, compared to normal control animals (Figure 2.3D-E). The results of behavioral testing of animals in which Arm is reduced specifically in the nociceptor indicate that the less Arm is available, the lower the nociceptive sensitivity, compared to normal controls.

Preliminary experiments were also undertaken within this study to investigate Arm and its role in UV injury-induced allodynia. Allodynia is described as nociceptive sensitivity distinguished by when something not normally noxious becomes so and is typically investigated by behaviorally assaying animal models using a stimulus that is just below threshold. In regard to a *Drosophila* UV injury-induced allodynia model, this below threshold temperature used in thermal allodynia nociception assays is 41°C

(Babcock et al., 2009; Babcock et al., 2011; Follansbee et al., 2017). As such, we tested *arm-IR-1* animals 24 hours post UV injury for heat avoidance at the below threshold temperature of 41°C. Results from these preliminary experiments showed no significant difference in injured *arm-IR-1* animals when compared to both injured controls (Appendix 1). Though a lower sample number was used than with other thermal nociception assays and only one IR line was utilized, these results do argue the possibility that Arm in the nociceptors is not involved in injury-induced thermal allodynia within the fruit fly. There are some prior mammalian studies that have uncovered a role for Wnt/β-catenin signaling in mechanical and/or thermal sensitivity after injury, however, manipulations and investigations of the pathway involvement within these studies were not necessarily cell-specific and included multiple tissues/cells (Itokazu et al., 2014; Yuan et al., 2012; Zhang et al., 2021; Zhang et al., 2013; Zhang et al., 2020; Zhao & Yang, 2018). Within a few of these mammalian studies, evidence was uncovered as to the importance of the Wnt/β-catenin pathway and glial cell activation in the development of neuropathic pain (Itokazu et al., 2014; Zhang et al., 2013). It is therefore possible that Wg/Arm signaling manipulation would need to be carried out in cells other than, or in addition to, the nociceptors to achieve significant findings of Arm's involvement in injury-induced allodynia. It is also possible for Wg/Arm involvement in injury induced mechanical allodynia and not necessarily injury induced thermal allodynia, or that Wg/Arm signaling is connected to activation of the TRP channel, dTrpA1, in hyperalgesia development and not activation of the TRP channel, Painless, known for its role in allodynia (Babcock et al., 2009; Babcock et al., 2011). Remarkably, in contrast to the cell *un*-specific mammalian studies referenced, it has also been shown in a mouse model for tumor evoked pain, where β-catenin signaling was targeted specifically in the nociceptors, that β-catenin signaling was not involved in either mechanical or thermal hypersensitivity (Simonetti et al., 2014).

In contrast to our Arm IR experiments carried out in the absence of injury, when additional wild-type Arm (*arm.S2*) was expressed in the nociceptors, animals showed a genetically induced hyperalgesia

response, or enhanced response to a normally noxious stimulus, in the absence of injury (Figure 2.4D). As an additional means of elevating Arm activity, we also employed Arm.S10, in which regions within the N-terminus necessary for phosphorylation and ubiquitination had been deleted, increasing the protein's resistance to degradation (Orsulic & Peifer, 1996; Pai et al., 1996; Pai et al., 1997). This manipulation mimics the reduction in Arm degradation that prevails when Wnt/Wg signaling is activated (Pai et al., 1997; Stamos & Weis, 2013; Yanagawa et al., 2002). Consistent with the results of *arm.S2* expression, expression of *arm.S10* transgene in the nociceptors of uninjured animals also produced behavioral hypersensitivity (Figure 2.5C), despite the lower resulting abundance of Arm.S10 relative to Arm.S2, as compared by immunodetection of the c-MYC tag featured in both constructs (Figure 2.5A-B). Taken together, results of behavioral testing of uninjured animals in which the Arm level was experimentally elevated specifically in the nociceptor indicate that the more Arm is available, the higher the nociceptive sensitivity, compared to normal controls.

When nociceptors either under-expressing Arm (via *arm-IR-1*) or expressing an additional c-MYC tagged form of Arm (via *arm.S10*) were evaluated for changes in dendritic morphology from uninjured animals, no significant morphological changes were found (Figures 2.6 & 2.7). These morphometric analysis results were notable considering that Wnt/Wg signaling is known to be involved in neuronal development and neurogenesis (Ciani & Salinas, 2005; Hirsch et al., 2007; Packard et al., 2002). β-catenin has furthermore been shown in mammalian hippocampal neurons to influence dendrite morphogenesis through its role in the cadherin-catenin complex, influencing actin cytoskeleton stabilization and cell-cell adhesion, a role separate from β-catenin's role in the canonical Wnt signaling pathway (Rosso et al., 2005; Yu & Malenka, 2003). Our findings that Arm manipulation has no detected effect on dendritic morphology are inconsistent with those prior studies in mammals, perhaps due to factors such as species differences, differences in cell type and location (peripheral sensory neurons as

opposed to central interneurons), as well as differences in experimental design (in *vivo* versus *in vitro* (Rosso et al., 2005; Yu & Malenka, 2003)).

Nociceptor dendrites form adhesion structures with overlying epidermal cells known as sheaths. Jiang and colleagues found that manipulations that impair epidermal-dendritic sheath maturation also reduce nociceptive sensitivity (Jiang et al., 2019). Since Arm is a known partner in adherens junction assembly (Pai et al., 1996), these observations are consistent with our results indicating Arm underexpression in the nociceptor, in the absence of injury, leads to reduced nociceptive sensitivity, while overexpression, in the absence of injury, leads to increased sensitivity. Thus, it seems possible that our observations of Arm's effects on nociceptor sensitivity could be at least partially due to its non-transcriptional role in cell adhesion during the maturation of epidermal sheaths.

Supporting this hypothesis is the observation that knockdown of Gro, known to antagonize Arm transcriptional activity (Cavallo et al., 1998), has no significant effect on sensitivity. Animals in which Gro (Groucho), a transcriptional repressor in the Wnt/Wg and BMP pathways, was reduced specifically in the nociceptors (Figure 2.9), showed similar nociceptive sensitivity to controls (Figure 2.9D-E). These results fail to support the hypothesis that Gro is involved in regulating nociceptor sensitivity, despite other reports supporting its role in transcriptional repression of BMP and Wnt/Wg target genes (Cavallo et al., 1998; Hasson et al., 2001). However, it is possible that in this context, Gro's known co-repressors are able to compensate for experimental Gro underexpression, allowing sufficient transcriptional repression of BMP/Wg target genes within the nociceptors.

To further our investigation into canonical Wnt/Wg signaling pathway's role in maintenance of nociceptor sensitivity, we also investigated the casein kinase, Gilgamesh (Gish). Gish is orthologous to mammalian Casein Kinase 1 gamma 1/2/3 (CK1γ1/2/3) and is a known positive regulator of Hh signaling in the fly (See Figure 1.4) (Hummel et al., 2002; Li et al., 2016). Gish is also known to be a positive regulator of canonical Wnt/Wg signaling by its role in phosphorylation of Arrow (mammalian ortholog:

LRP 5/6) (Figure 1.4) (Davidson et al., 2005; Schaefer et al., 2018; Zhang et al., 2006). This phosphorylation of Arrow by Gish in turn helps Arrow to bind components within the Arm destruction complex at the plasma membrane so that the Arm destruction complex activity in the cytoplasm is halted (Davidson et al., 2005; Schaefer et al., 2018; Zhang et al., 2006). Due to this role as a positive regulator of canonical Wnt/Wg signaling and our previous findings on Arm, we hypothesized that we should see an increase in nociceptive sensitivity when Gish expression is increased within the primary nociceptors. By behaviorally testing thermal avoidance latencies of un-injured animals overexpressing Gish in their nociceptors (*gish-OE*), at the known noxious temperature of 45°C, our results indicated that these animals had developed genetically induced hyperalgesia (Figure 2.10A). These results indicated that when there is more Gish available in the nociceptors, the nociceptors become hypersensitive in comparison to controls. In contrast, when we under expressed Gish within the nociceptors by using RNA interference technology (*gish-IR-1* and *gish-IR-2*), we observed mixed results (Figure 2.10B-C). In thermal nociception assays for *gish-IR-1*, animals did not show a significant change in response latencies (Figure 2.10B), however, in nociception assays for *gish-IR-2* animals did show a significant change in response latencies when compared to controls (Figure 2.10C). Though the *gish-IR-1* line had been successfully used in previous studies, it is possible that this line could be insufficient in its knockdown of Gish or that *gish-IR-2* results in off target effects within the nociceptor (Li et al., 2016; Li et al., 2020). Due to the discrepancy in results of these two Gish-IR lines, we will aim to further investigate Gish knockdown in the nociceptors by testing of a third Gish-IR line in the future. Validation of protein knock-down experiments within the Gish-IR lines and investigation into possible morphological changes that could occur due to off target effects of the IR expressed within the nociceptor will also be performed. In short, further investigation into Gish and the canonical Wnt/Wg signaling pathway will be necessary in the future to determine the role of Gish in nociceptor sensitivity, in the absence of injury, before drawing any definitive conclusions.

This report shows that manipulation of Arm expression specifically within the nociceptors is sufficient to modulate behavioral response to a noxious thermal stimulus in the absence of injury and that these changes are not associated with a detectable morphological effect. In contrast, in a similar study carried out in the mouse model, Simonetti and colleagues genetically impaired β-catenin activity specifically in the nociceptor and observed no change in thermonociception (Simonetti et al., 2014). We suggest that the reason for these differing outcomes may lie in the evolutionary relationship of flies and mammals. In vertebrates, another catenin, γ-catenin (plakoglobin) has been shown to be capable of substituting for β-catenin's adhesion function, but not its transcriptional function (Huelsken et al., 2000; Huelsken et al., 2001; Miller & Moon, 1997; Simcha et al., 1998). Similar results were found when mammalian β-catenin and γ-catenin were expressed in *Drosophila* to complement Arm mutants, where both β-catenin and γ-catenin were found to be functional at cadherins complexes, but only β-catenin showed Wg signaling capabilities (White et al., 1998). Knockdown of the fly Armadillo, homologous to both mammalian β-catenin and γ-catenin (Peifer et al., 1992; Peifer & Wleschaus, 1990; White et al., 1998), reduces all β/γ-catenin function; transcriptional, adhesional, or other (Orsulic & Peifer, 1996). So perhaps these findings demonstrate the possibility of further investigation of β-catenin/Arm within nociceptors in a way that could potentially complement mammalian pain investigation in uncovering potential new drug targets for the treatment of clinical pain. Further investigation into β-catenin/Arm's transcriptional and cell adhesion functions is warranted to gain a broader understanding of the mechanism of maintaining baseline nociceptor sensitivity both in flies and mammalian systems.

2.6 Acknowledgements

2.6.1 General acknowledgements

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2.6.2 Acknowledgements in contribution of data acquisition and writing

Contributing authors of submitted manuscript (Hale, Moulton, et al., 2022) included within this chapter:

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Author contribution: Christine Hale and Geoffrey Ganter, PhD, designed research; Christine Hale, Julie Moulton, MS, and Yvonne Otis performed research; Christine Hale statistically analyzed data; Christine Hale and Geoffrey Ganter, PhD, contributed to the writing for the submitted manuscript (Hale, Moulton, et al., 2022) which was included within this dissertation.

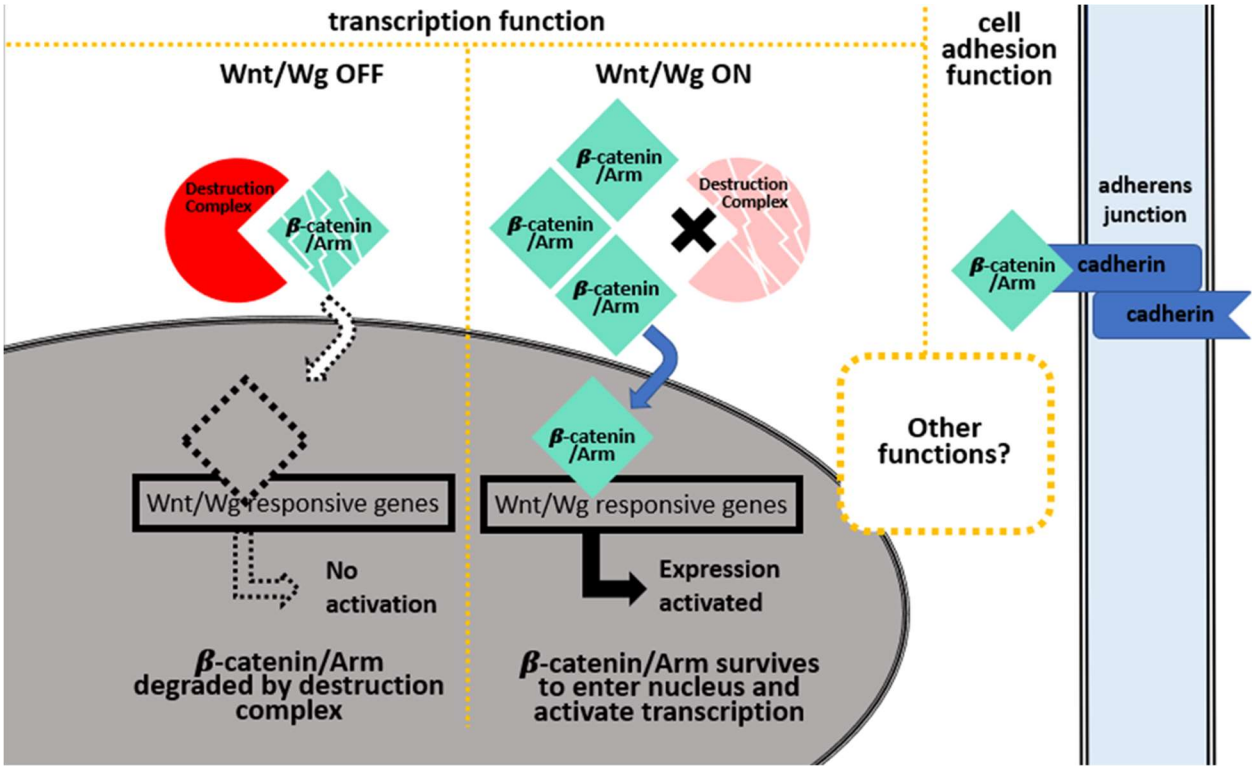


Figure 2.1 Cellular Roles of Armadillo

When Wnt/Wg pathway is off, the destruction complex prevents accumulation of Arm (Armadillo) by proteolysis. When the Wnt/Wg pathway is on (by binding of a Wnt/Wg ligand to a canonical Wnt/Wg receptor), inactivation of the destruction complex allows Arm to accumulate, enter the nucleus, and activate expression of the Wnt/Wg response genes. Arm is also known to play a role in cell adhesion, where it binds to cadherin at the plasma membrane of the cell. Graphic by G. Ganter

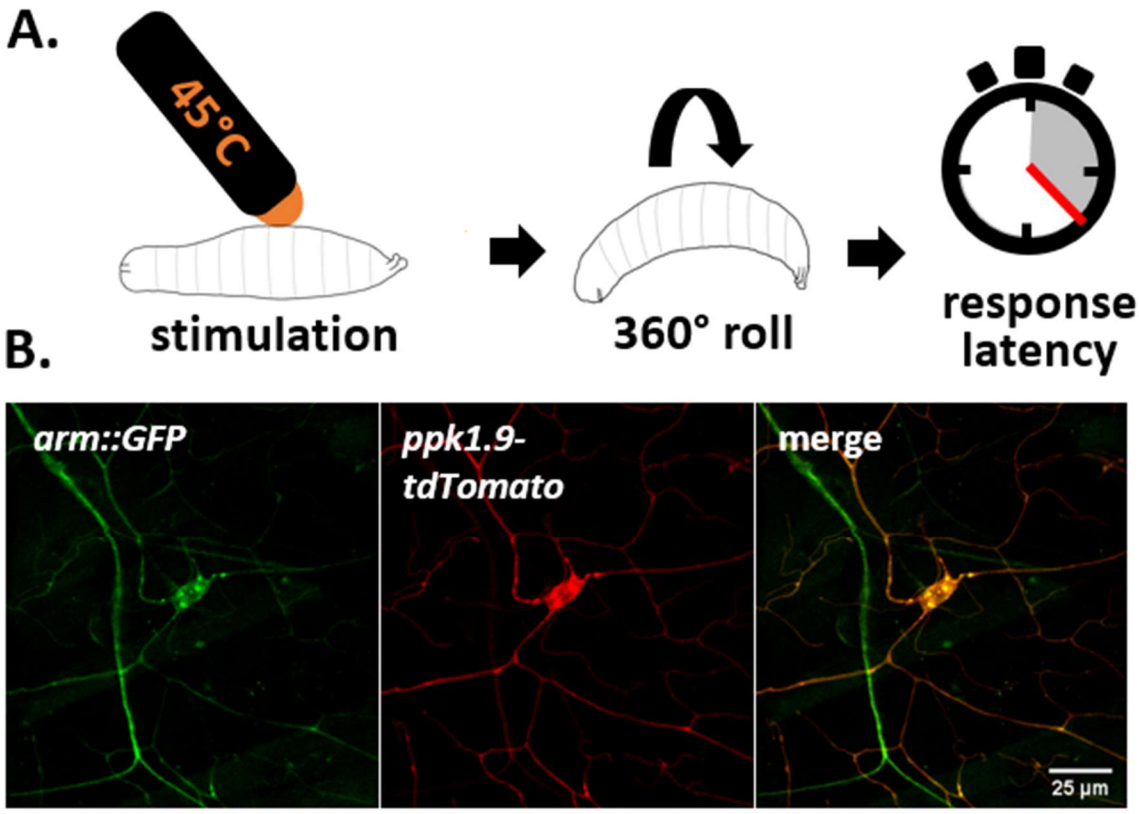


Figure 2.2 Larval thermal nociception assay and MiMIC imaging.

Baseline thermal nociception behavioral assays for transgenic Arm larvae and expression of Arm within nociceptors. **(A)** Schematic of baseline thermal nociception assay of late 3rd instar larvae. Latency of 360° escape roll (or no response) within 20 s is recorded after initiation of thermal stimulus set to 45°C. **(B)** Micrographs of a larva expressing nociceptor specific tdTomato and GFP tagged Arm protein using a 63x objective. Punctate Arm::GFP fluorescence (green) is observed in the soma and neurites of the nociceptor (red), as well as the neurites of other unidentified dendritic arborization neurons**.** Graphic by C. Hale.

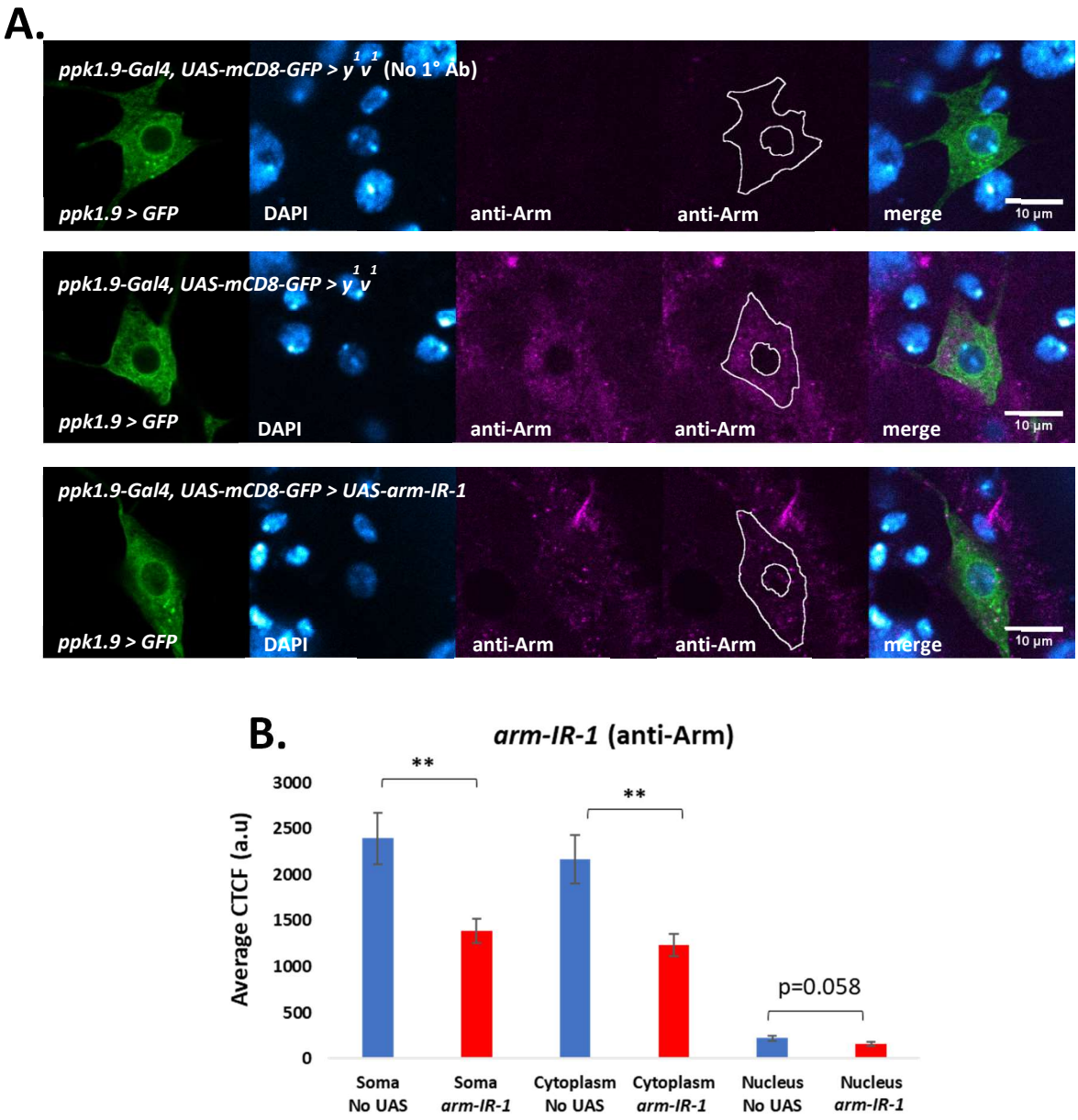


Figure 2.3 Knockdown of Arm within the nociceptors results in behavioral hyposensitivity

(A) Immunofluorescent detection of Arm using anti-Arm monoclonal antibody and confocal imaging (40xmagnification). Top: *ppk1.9-Gal4, UAS-mCD8-GFP > y^1v^1* with no 1° Ab, *n* = 3. Middle: same genotype with anti-Arm, *n* ≥ 10. Bottom: *ppk1.9-Gal4, UAS-mCD8-GFP > UAS-arm-IR-1*, n ≥ 10. A tracing representing an example of an ROI used in measurement of fluorescence has been added to the montage. **(B)** CTCF quantification of Arm immunofluorescence confirms Arm expression in the

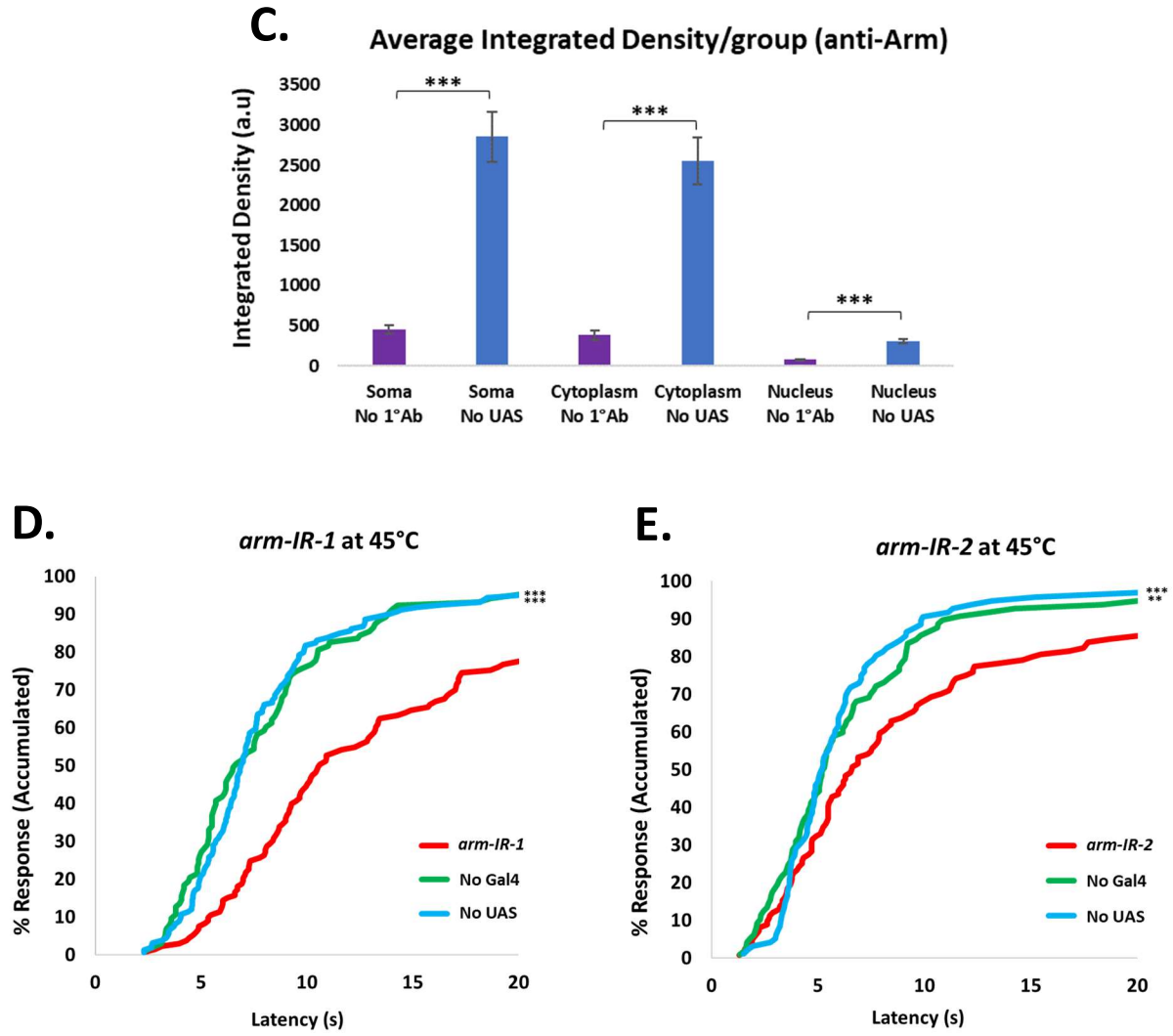


Figure 2.3, continued

nociceptor, and significant knockdown in the cytoplasm, nearly significant knockdown in the nucleus (indicated by co-localization with DAPI), statistically analyzed by Student's *t* test with Welch's correction, ** indicates $p < 0.01$. **(C).** Integrated Density was measured for soma, cytoplasm, and nucleus for "No UAS" (*ppk1.9-Gal4, UAS-mCD8- GFP* > y^1v^1), $n \geq 10$, and "No 1°Ab" (*ppk1.9-Gal4, UAS-mCD8-GFP* > y^1v^1), (where the primary antibody was not added), $n = 5$, samples, averaged per group, and statistically analyzed to verify significant signal over background for anti-Arm fluorescence. Statistical analysis was

Figure 2.3, continued

by Student's *t* test with Welch's correction, *** indicates $p < 0.001$. **(D, E)** Percent response plotted against time in thermal nociception assays at 45° C for *arm-IR-1* (A: *ppk1.9-Gal4 > UAS-arm-IR-1*), and *arm-IR-2* (B: *ppk1.9-Gal4 > UAS-arm- IR-2*) shown in red vs. their controls "No Gal4" (A: w^{1118}*> UAS-arm-IR-1*, B: w^{1118}*> UAS-arm-IR-2)* shown in green and "No UAS" (A: *ppk1.9-Gal4 >* y^1v^1, B: *ppk1.9-Gal4 >* y^1v^1) shown in blue, $n \geq 90$/group. Statistical analysis by log-rank test shows significant hyposensitivity of experimental compared to both controls for both IR lines, ** indicates $p < 0.01$, *** indicates $p < 0.001$. J. Moulton and G. Ganter helped facilitate immunohistochemistry experiments. C. Hale carried out all imaging and analysis, and behavioral experiments.

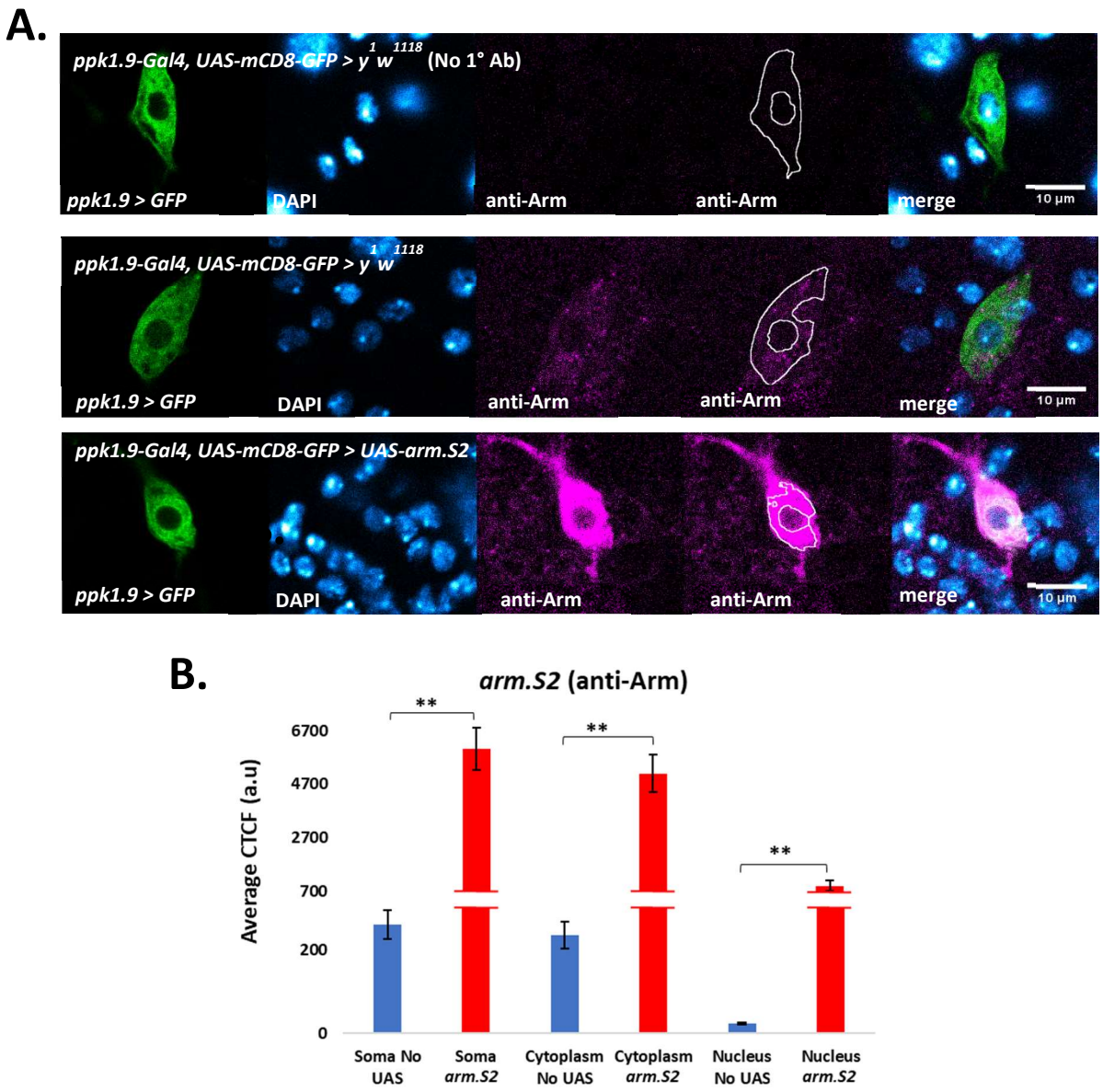


Figure 2.4 Overexpression of Arm via *arm.S2* within nociceptors results in behavioral hypersensitivity

(A) Immunofluorescent detection of Arm.S2 using anti-Arm antibody and confocal imaging (40x magnification). Top: *ppk1.9-Gal4, UAS-mCD8-GFP > y^1w^{1118}* with no 1° Ab, *n* = 3. Middle: same genotype, *n* ≥ 6, with anti-Arm shows the endogenous expression of Arm in the nociceptor in both cytoplasm and nucleus. Bottom: *ppk1.9-Gal4, UAS-mCD8-GFP > UAS-arm.S2*, *n* ≥ 6. A tracing representing an example

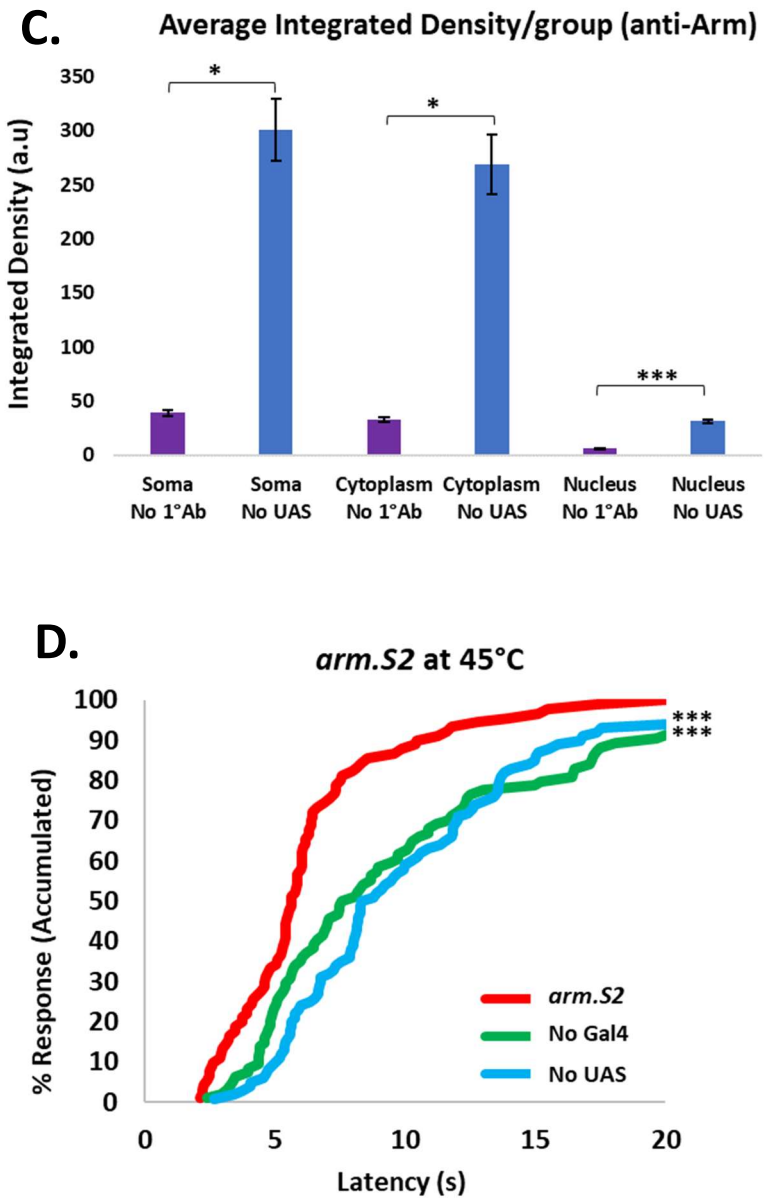
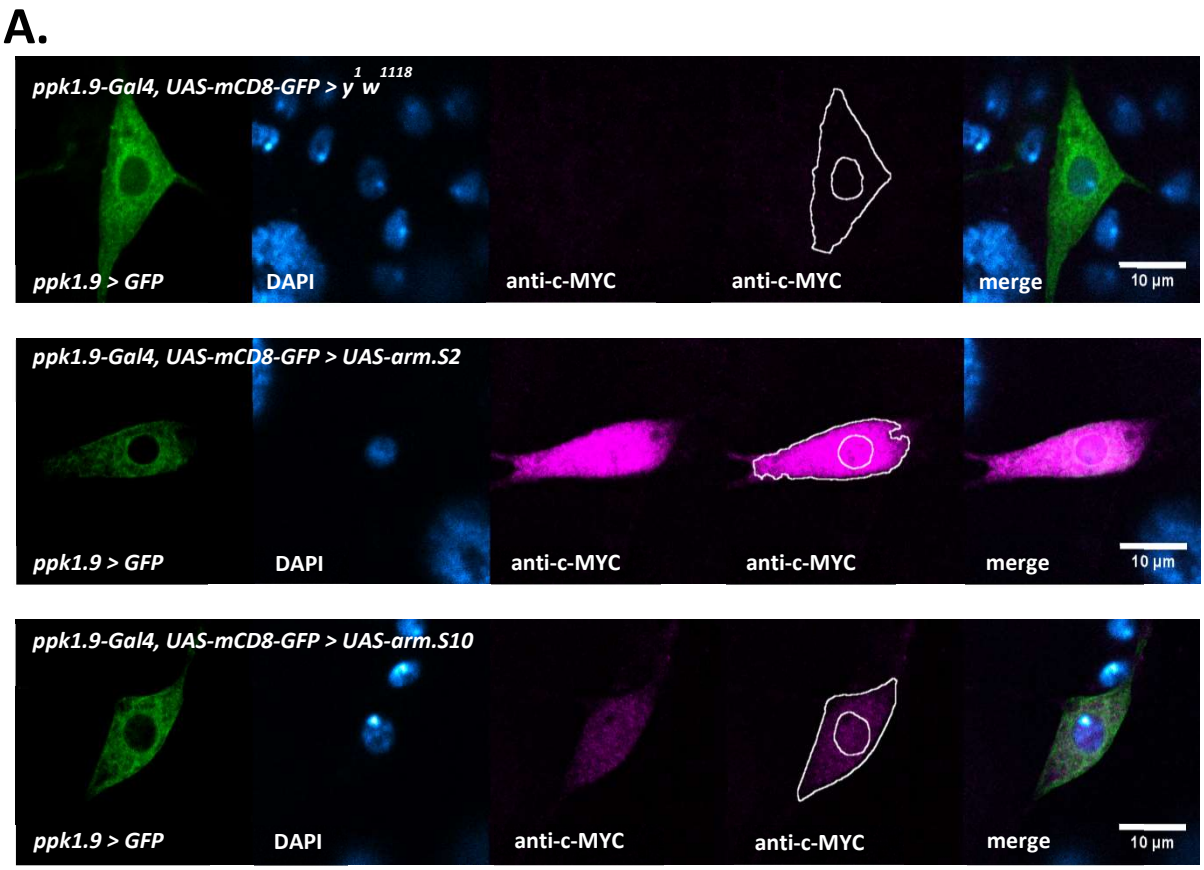


Figure 2.4, continued

of an ROI used in measurement of fluorescence has been added to the montage. **(B)** CTCF quantification of Arm immunofluorescence confirms additional Arm expression in both the cytoplasm and nucleus (indicated by co-localization with DAPI), statistically analyzed by Student's *t* test with Welch's correction or Mann-Whitney *U* test where data was found to not be normally distributed, ** indicates $p < 0.01$. Note the split Y-axis used to represent both the native and elevated Arm levels. **(C)** Integrated Density

Figure 2.4, continued

was measured for soma, cytoplasm, and nucleus for "No UAS" (*ppk1.9-Gal4, UAS mCD8- GFP > y^1w^{1118}*), *n* = 7, and "No 1°Ab" (*ppk1.9-Gal4, UAS-mCD8-GFP > y^1w^{1118}*) (where the primary antibody was not added), *n* = 3, samples, averaged per group, and statistically analyzed to verify significant signal over background for anti-Arm fluorescence. Statistical analysis was by Student's *t* test with Welch's correction or Mann-Whitney *U* test where data was found to not be normally distributed, *indicates $p < 0.05$, ***indicates $p < 0.001$. **(D)** Percent response plotted against time in thermal nociception assay at 45° C for animals expressing additional wild-type Arm in nociceptors via *arm.S2* (*ppk1.9-Gal4 > UAS-arm.S2*) shown in red vs. controls "No Gal4" (*w^{1118} > UAS-arm.S2*) shown in green and "No UAS" (*ppk1.9-Gal4 > y^1w^{1118}*) shown in blue, n ≥ 90/group. Statistical analysis by log-rank test shows significant nociceptive hypersensitivity of *arm.S2* animals, compared to both controls, significance ***indicates $p < 0.001$. J. Moulton and G. Ganter helped facilitate immunohistochemistry experiments. C. Hale carried out all imaging and analysis, and behavioral experiments.



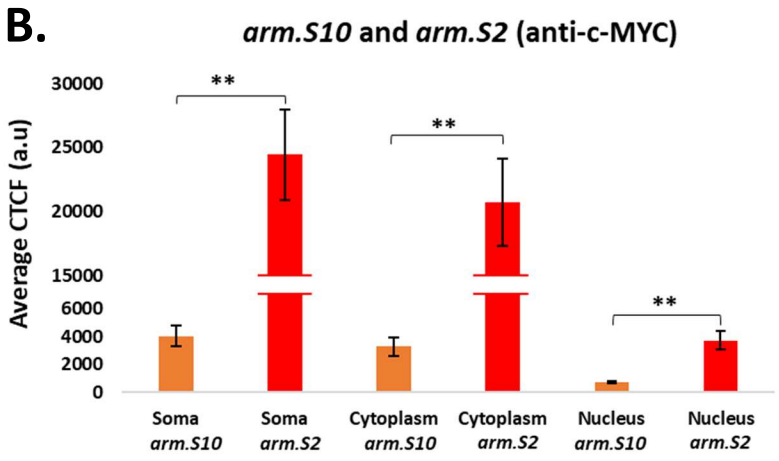


Figure 2.5 Overexpression of Arm via *arm.S10* within nociceptors results in behavioral hypersensitivity

(A) Immunofluorescent comparison of Arm.S2's and Arm.S10's c-MYC tags through confocal imaging (40x magnification). Top: *ppk1.9-Gal4, UAS-mCD8-GFP > y^1w^{1118}*(No 1° Ab), *n* = 3-6, shows absence of c-MYC staining in control animals. Middle*: ppk1.9-Gal4, UAS-mCD8-GFP > UAS-arm.S2*, *n* = 7 shows strong

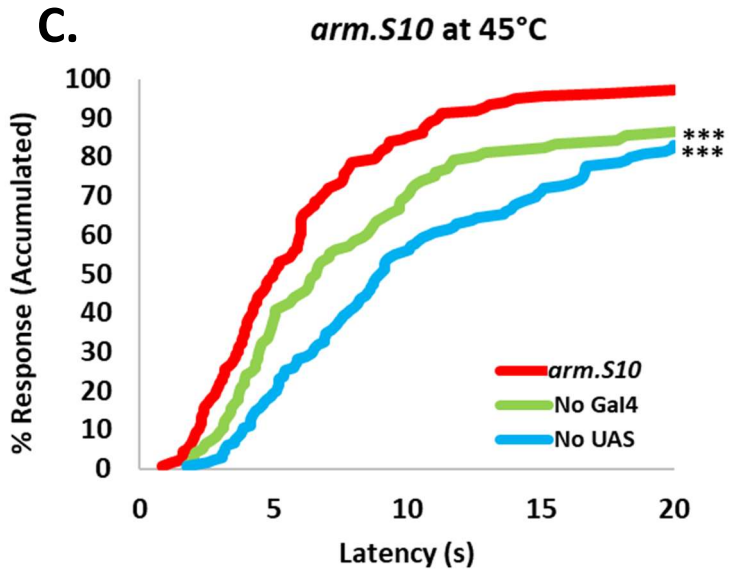


Figure 2.5, continued

expression in the nociceptor. Bottom: *ppk1.9-Gal4, UAS-mCD8-GFP > UAS-arm.S10*, $n = 7$ shows specific expression of Arm.S10 in the nociceptor. A tracing representing an example of an ROI used in measurement of fluorescence has been added to the montage. **(B)** CTCF quantification and comparison of c- MYC immunofluorescence shows that *arm.S2* produces stronger Arm expression than *arm.S10*, in both the cytoplasm and the nucleus, statistically analyzed by Student's *t* test with Welch's correction, **indicates $p < 0.01$. Note the split Y-axis used to represent anti-c-MYC levels. **(C)** Percent response plotted against time in thermal nociception assay at 45° C for *arm.S10* animals (*ppk1.9-Gal4 > UAS-arm.S10*) shown in red vs. control "No Gal4" (w^{1118} *> UAS-arm.S10*) shown in green and "No UAS" (*ppk1.9-Gal4 >* y^1w^{1118}) shown in blue, $n \geq 90$/group. Statistical analysis by log-rank test shows significant nociceptive hypersensitivity of experimental compared to both controls, ***indicates $p < 0.001$. J. Moulton and G. Ganter helped facilitate immunohistochemistry experiments. C. Hale carried out all imaging and analysis, and behavioral experiments.

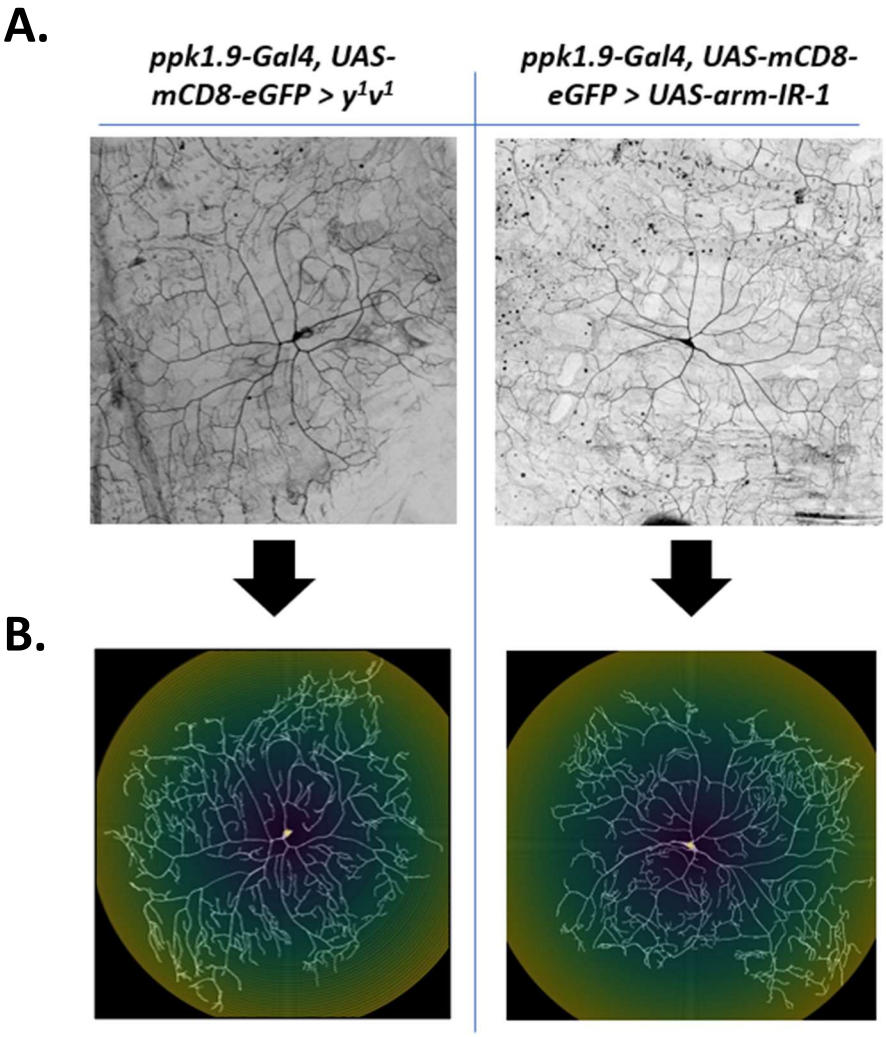


Figure 2.6 Neuromorphometric analysis of nociceptors with Arm knockdown shows no effect on dendritic morphology.

(A) Nociceptors expressing *arm-IR-1* to knock down Arm (*ppk1.9-Gal4, UASmCD8- eGFP > UAS-arm-IR-1*), *n* = 10, were analyzed for **(B)** dendritic arborization by Sholl analysis, **(C)** dendritic number, and **(D)** dendrite length, in comparison to controls (*ppk1.9-Gal4, UAS-mCD8-eGFP > y^1v^1*), *n* = 10. No significant differences in these parameters were observed. Gray area in B represents SEM. ns = no significance found by Student's *t* test with Welch's correction or by the Mann-Whitney *U* test when data was not normally

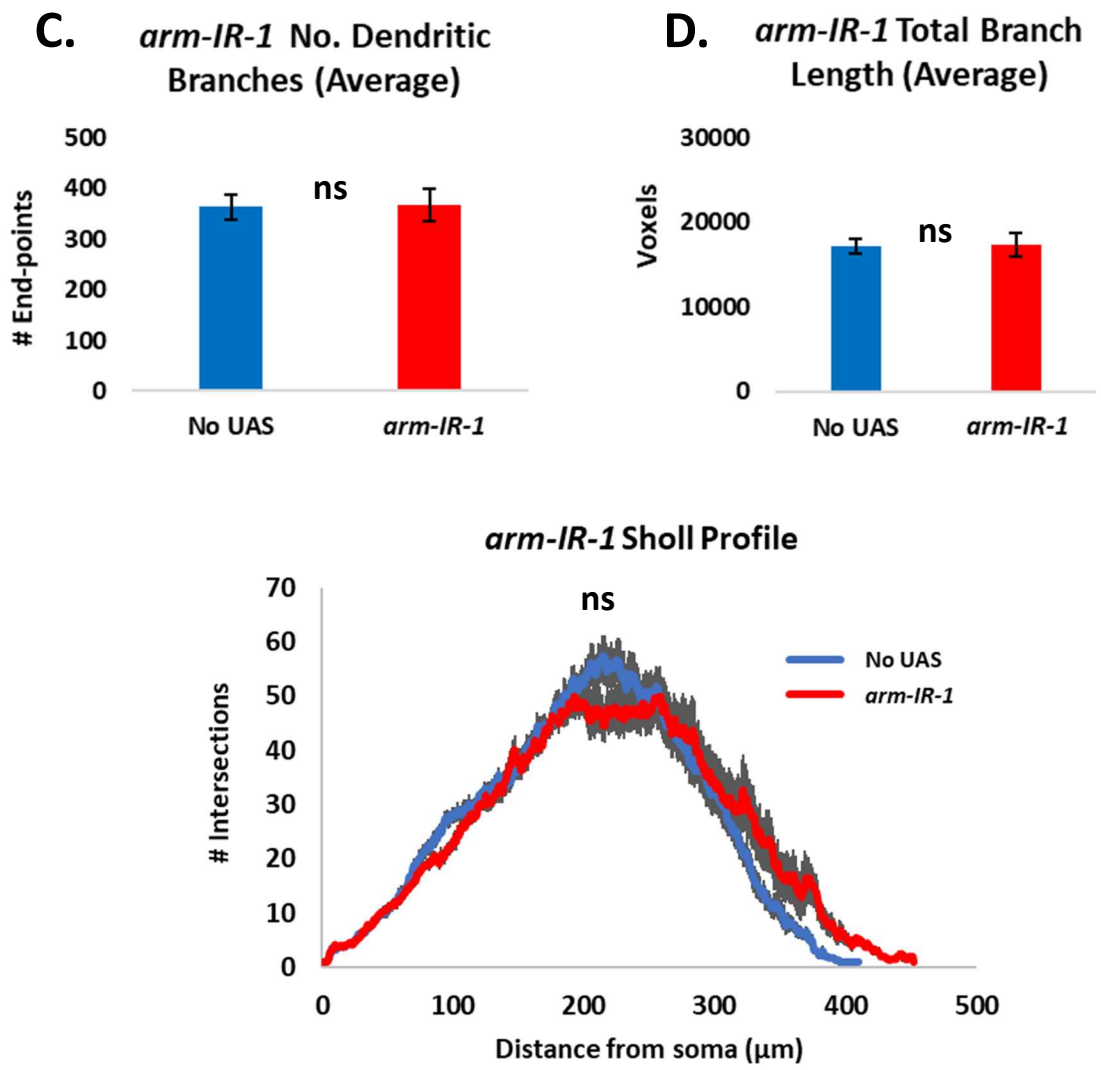


Figure 2.6, continued

distributed. Y. Otis contributed to dendritic tracing of neurons represented by this figure. C. Hale carried out all neuromorphometric analyses of the tracings.

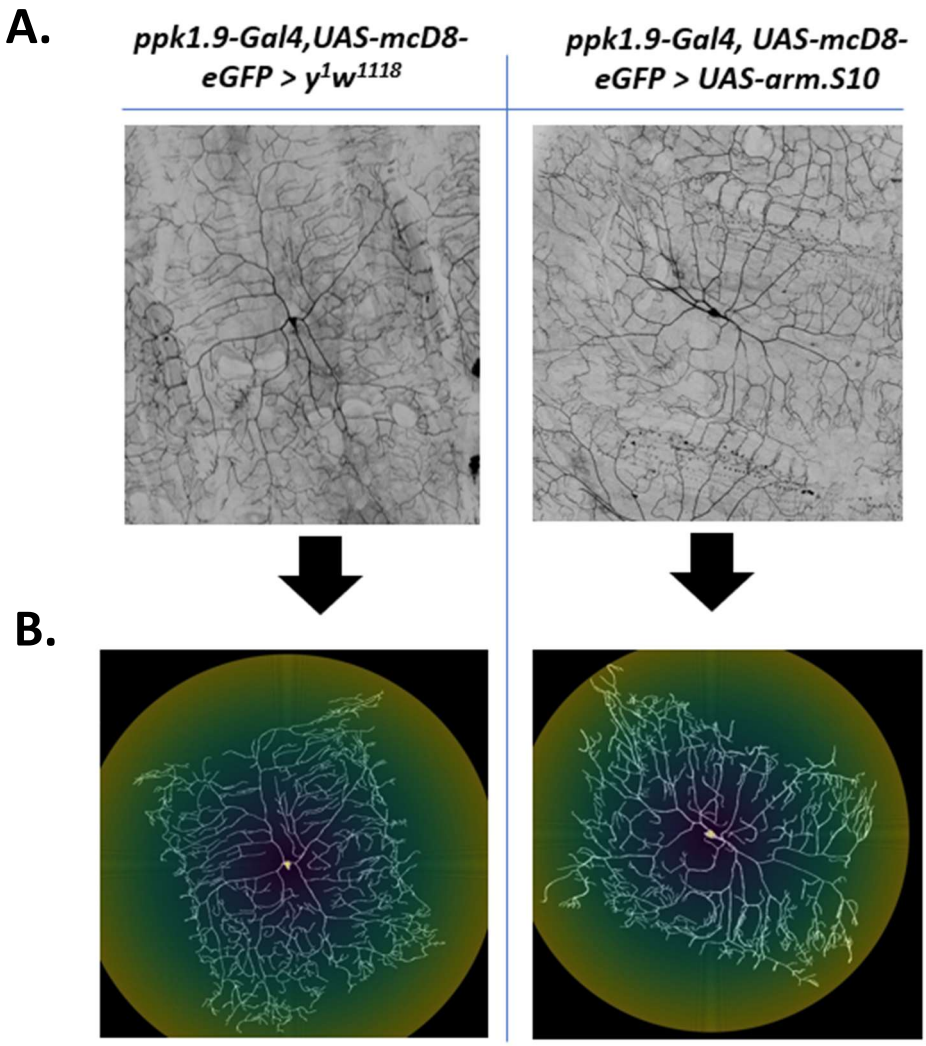


Figure 2.7 Neuromorphometric analysis of nociceptors expressing *arm.S10* shows no effect on dendritic morphology.

(A) Nociceptors expressing *arm.S10* to elevate Arm (*ppk1.9-Gal4, UAS-mCD8-eGFP > UAS-arm.S10*), *n* = 7, were analyzed for **(B)** dendritic arborization by Sholl analysis, **(C)** dendritic number, and **(D)** dendrite length, in comparison to controls (*ppk1.9-Gal4, UAS-mCD8-eGFP > y^1w^{1118}*), *n* = 7. No significant differences in these parameters were observed. Gray area in B represents SEM. ns = no significance

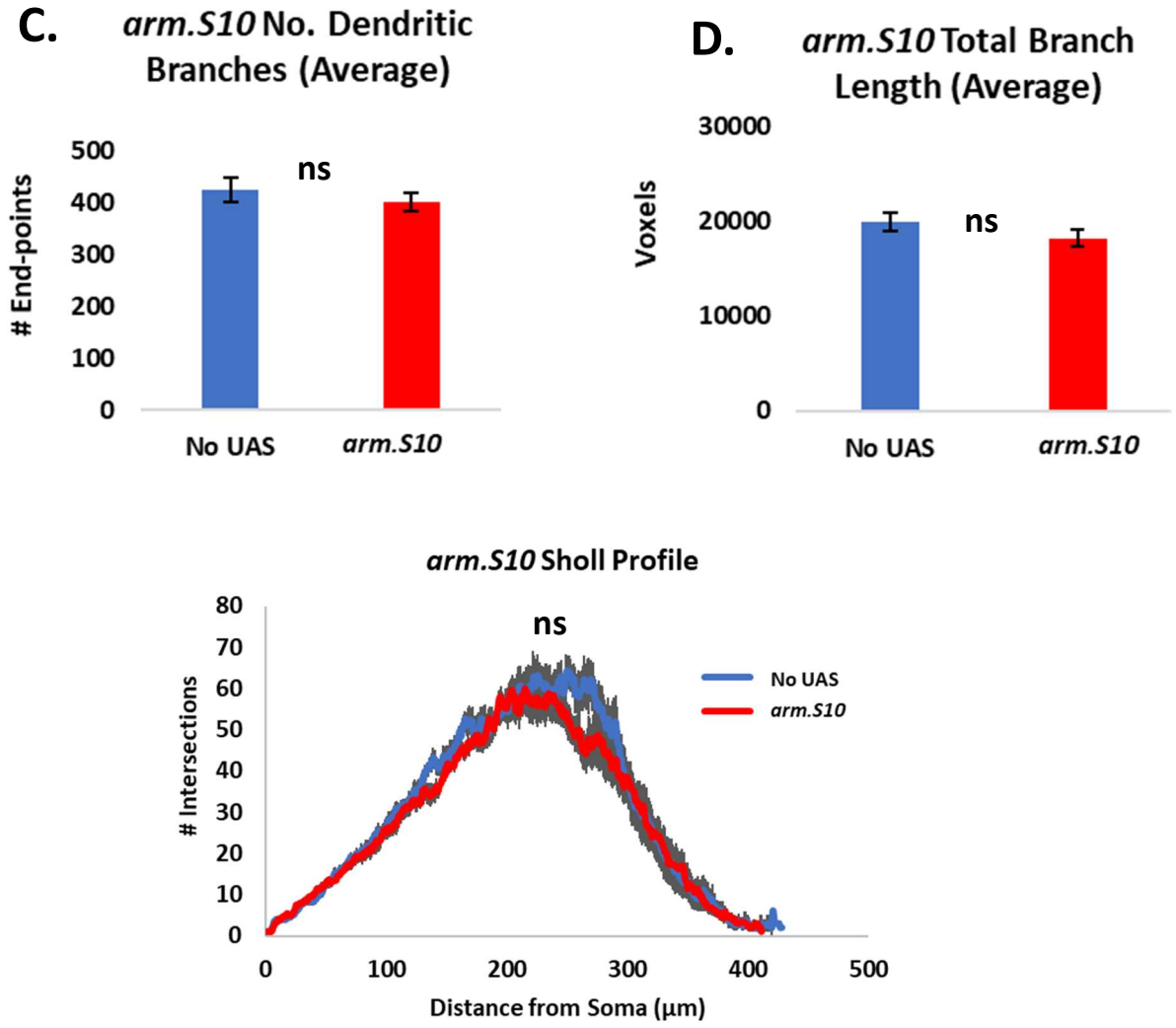


Figure 2.7, continued

found by Student's *t* test with Welch's correction or by the Mann-Whitney *U* test when data was not normally distributed.

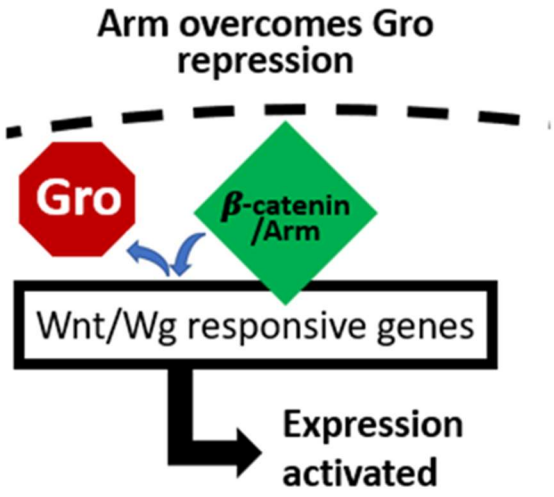


Figure 2.8 Gro (Groucho) is a transcriptional repressor in the Wnt/Wg pathway, downstream of Arm. Wnt/Wg pathway activation leads to Arm accumulation and antagonism with the repressor, Gro, in order to transcriptionally activate target genes. Graphic by G. Ganter.

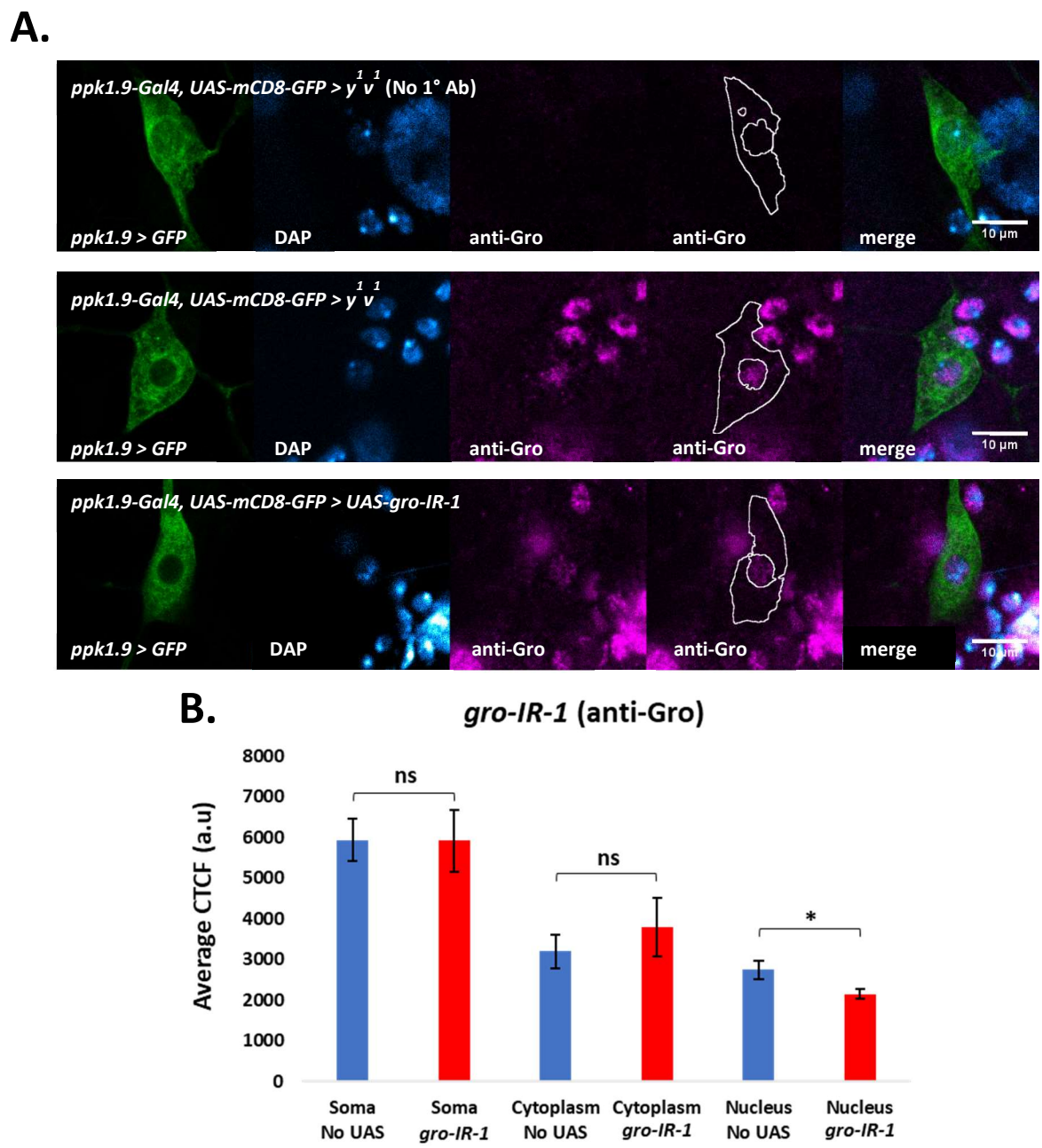


Figure 2.9 Knockdown of Gro within nociceptors does not alter behavioral sensitivity.

(A) Immunofluorescent detection of Gro using anti-Gro monoclonal antibody and confocal imaging (40x magnification). Top: *ppk1.9-Gal4, UAS-mCD8-GFP > y^1v^1* with no 1° Ab, *n* = 3. Middle: same genotype with anti-Gro, *n* = 9. Bottom: *ppk1.9-Gal4, UAS-mCD8-GFP > UAS-gro-IR-1*, *n* = 9. A tracing representing an example of an ROI used in measurement of fluorescence has been added to the montage. **(B)** CTCF

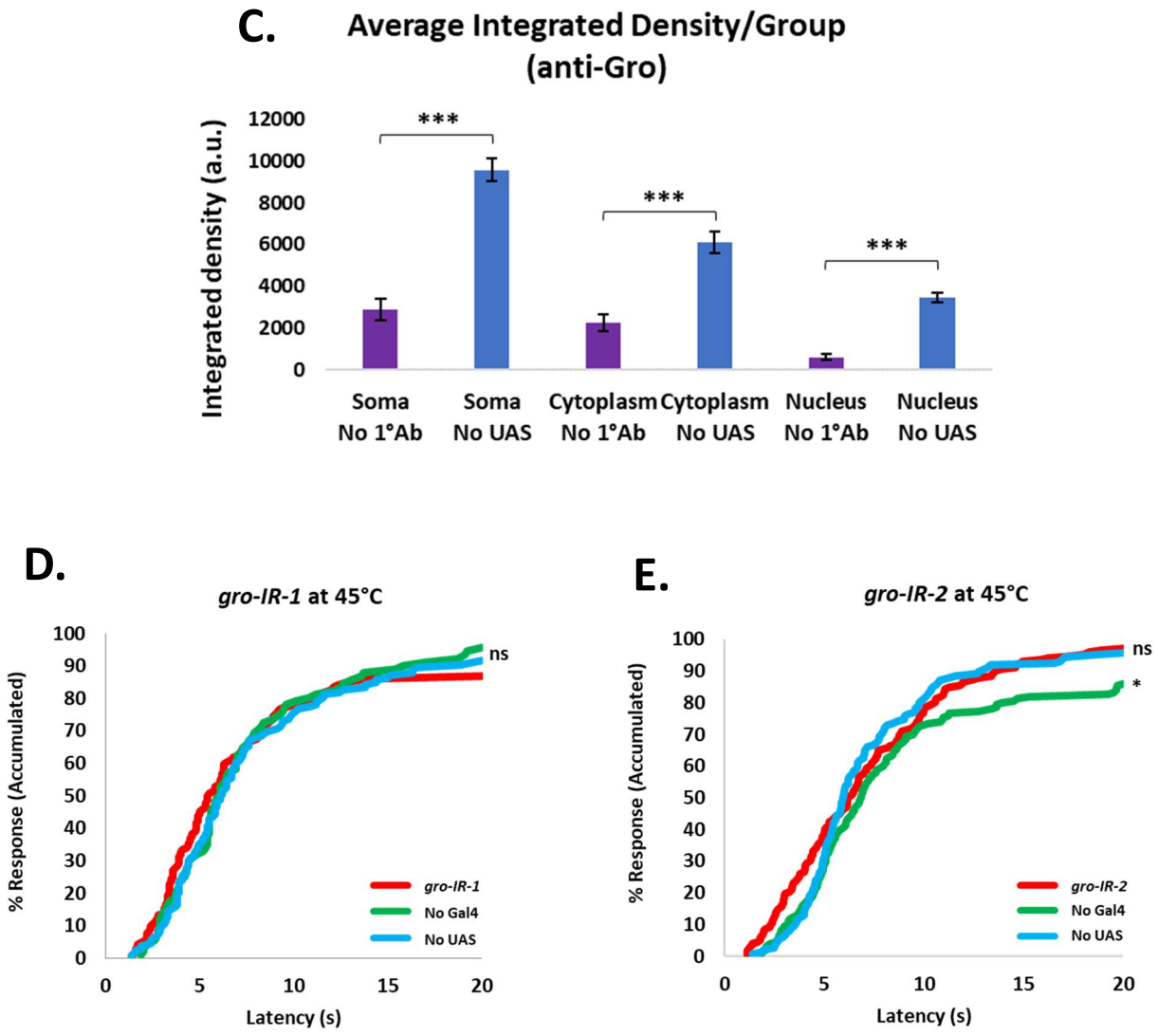


Figure 2.9, continued

quantification of Gro immunofluorescence confirms Gro expression in the nociceptor and significant knockdown in the nucleus (indicated by co-localization with DAPI), statistically analyzed by Student's *t* test with Welch's correction, *indicates $p < 0.05$. **(C)** Integrated Density was measured for soma, cytoplasm, and nucleus for "No UAS" (*ppk1.9-Gal4, UAS-mCD8-GFP* > y^1v^1), $n = 9$, and "No 1°Ab" (*ppk1.9-Gal4, UASmCD8- GFP* > y^1v^1) (where the primary antibody was not added), $n = 3$, samples, averaged per group, and statistically analyzed to verify significant signal over background for anti-Gro fluorescence. Statistical analysis was by Student's *t* test with Welch's correction or by the Mann-

Figure 2.9, continued

Whitney *U* test when data was not normally distributed, ***indicates $p < 0.001$. **(D, E)** Percent response plotted against time in thermal nociception assays at 45° C for *gro-IR-1* (A: *ppk1.9-Gal4 > UAS-gro-IR-1*), and *gro-IR-2* (B: *ppk1.9-Gal4 > UAS-gro-IR-2*) shown in red vs. their controls "No Gal4" (A: w^{1118}*> UAS-gro-IR*-1, B: w^{1118}*> UAS-gro- IR-2*) shown in green and "No UAS" (A: *ppk1.9-Gal4 >* y^1v^1, B: *ppk1.9-Gal4 >* y^1v^1) shown in blue, $n \geq 90$/group. Statistical analysis by log-rank test does not show significant hyposensitivity compared to both controls for both IR lines. ns= not significant, * indicates $p < 0.05$. J. Moulton and G. Ganter helped facilitate immunohistochemistry experiments. C. Hale carried out all imaging and analysis, and also behavioral experiments for *gro-IR-2* (E). J. Moulton acquired behavioral data for *gro-IR-1* (D).

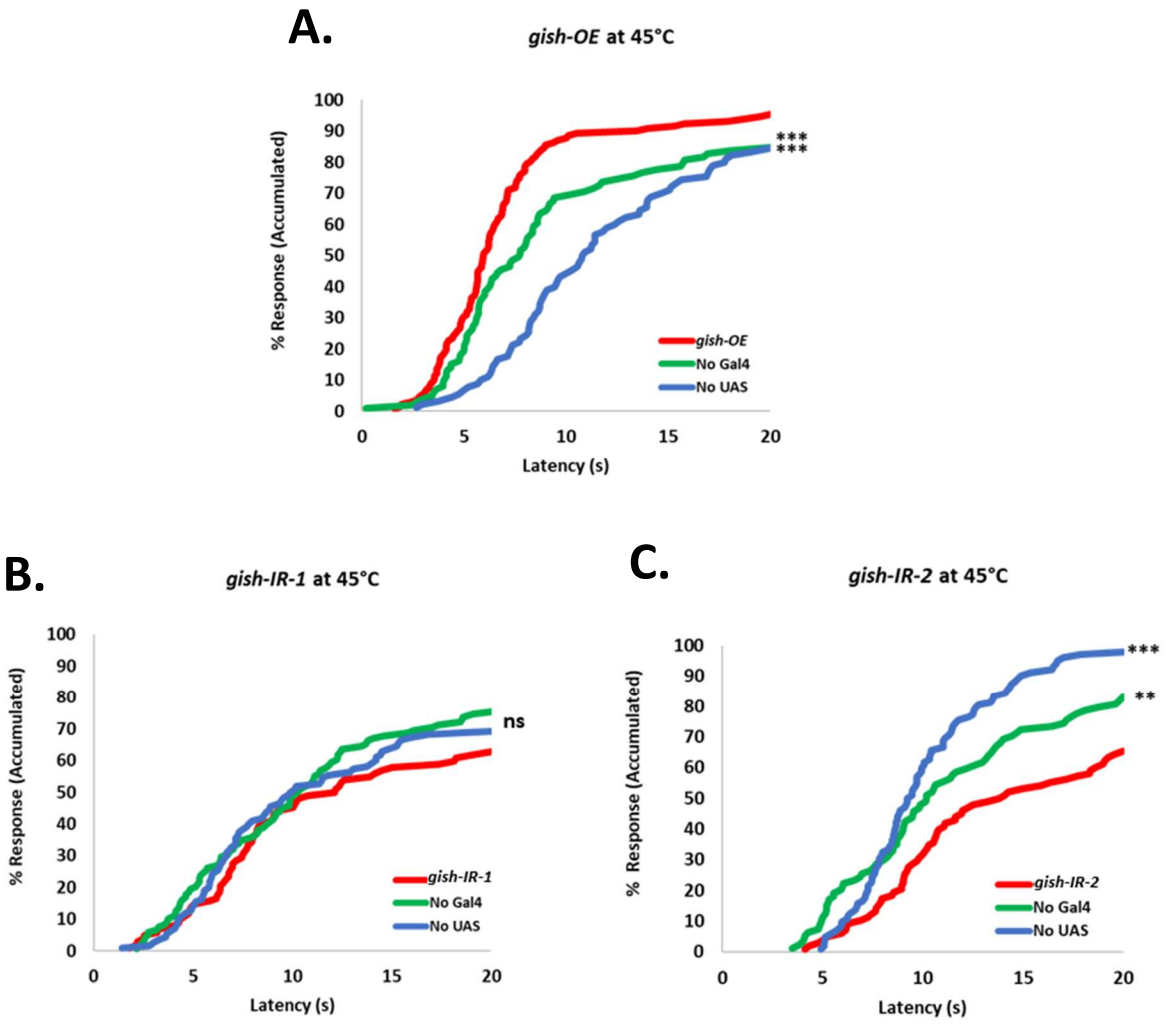


Figure 2.10 Gish overexpression results in behavioral hypersensitivity and Gish underexpression leads to mixed results.

(A-C) Percent response plotted against time in thermal nociception assays at 45° C for *gish-OE* (A: *ppk1.9-Gal4> UAS-gish-OE*), *gish-IR-1* (B: *ppk1.9-Gal4 > UAS-gish-IR-1*), and *gish-IR-2* (C: *ppk1.9-Gal4 > UAS-gish-IR-2*) shown in red vs. their controls "No Gal4" (A: w^{1118} *> UAS-gish-OE*, B: w^{1118}*> UAS-gish-IR-1*, C: w^{1118}*> UAS-gish-IR-2*) shown in green and "No UAS" (A: *ppk1.9-Gal4 >* w^{1118},

Figure 2.10, continued

B: *ppk1.9-Gal4 > y^1v^1*, C: *ppk1.9-Gal4 > y^1v^1*) shown in blue, $n \geq 90$/group. Statistical analysis by log-rank test does show significance for hypersensitivity for Gish overexpression when compared to controls but does not show significant hyposensitivity compared to both controls for both IR lines. ns= not significant, **indicates $p < 0.01$, ***indicates $p < 0.001$. C. Hale acquired behavioral data for *gish-OE* (A) and *gish-IR-2* (C). J. Moulton acquired behavioral data for *gish-IR-1* (B).

CHAPTER 3

3. INVESTIGATION OF NOCICEPTIVE SENSITIZATION AND ITS RECOVERY AFTER UV INJURY: BIOINFORMATIC ANALYSIS OF NOCICEPTOR SPECIFIC RNA SEQUENCING DATA FROM A VALIDATED *DROSOPHILA MELANOGASTER* LARVAL UV INJURY MODEL

**C. Hale performed bioinformatic analysis on the featured RNA sequencing data in this chapter. Experiments conducted for the preparation of the RNA sequencing data used in the bioinformatic analysis were carried out by former and current laboratory members, C. Brann, MS, and J. Moulton, MS, (timed-egg lays and UV injury of larvae) and UNE colleague, R. Geguchadze, PhD, (RiboTag immunoprecipitation and RNA isolation). These preparatory experiments were carried out prior to when the dissertation research of the author was established. Other data contributed by Ganter lab members in addition to the primary author, C. Hale, is referenced within figure legends and outlined within the Acknowledgements section.*

3.1 Introduction/Relevant Background

As previously stated within the introduction of this dissertation, with the publication of the new ICD-11, we were introduced to many new descriptive and categorical terms in characterizing chronic pain, suggesting chronic pain to be more of a multifaceted condition with multiple branches (Treede et al., 2015, 2019). Included within several of the chronic pain conditions outlined in the ICD-11, such as neuropathic pain, was the description of hypersensitivity of the nociceptors after injury (Scholz et al., 2019). Indeed, of the little that is known concerning the mechanism of chronic pain, research has indicated that nociceptive sensitization can underlie and perpetuate chronic pain (Reichling & Levine, 2009). Nociceptors, specialized sensory neurons within the peripheral nervous system (PNS) that detect noxious stimuli, are the first responders to the threat of injury (Bessou & Perl, 1969; Gold & Gebhart, 2010). Sensitization of the nociceptors can be beneficial after injury by reducing the threshold of

activation required to trigger a response, but if nociceptive sensitization persists after the injury has healed, symptoms of hyperalgesia and allodynia can take root and give way to abnormal pain (Bessou & Perl, 1969; Gold & Gebhart, 2010; Hucho & Levine, 2007; Treede et al., 2015). When this type of pain from hypersensitivity persists/reoccurs for typically three to six months or more, it is commonly referred to as chronic, and can lead to a substantial decrease in quality of life and an increased threat for opioid addiction in those being treated for chronic pain (Buntin-Mushock et al., 2005; Christie, 2008; Eddy et al., 1959; Groenewald et al., 2019; Hay et al., 2009; Treede et al., 2015; Vowles et al., 2015). In chapter one and two of this dissertation it was summarized that the thoroughly validated fruit fly larval UV injury and thermal nociception model (Figure 1.2A) has been utilized to uncover signaling pathways such as Hedgehog, TNF-α (named Eiger in *Drosophila*), and BMP as necessary for the formation of nociceptive sensitization after UV injury (Figure 1.4) (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; McParland et al., 2021).

Utilizing the GAL4/UAS system (Figure 1.3), where transgene expression was driven by the nociceptor specific promoter for Pickpocket (Ppk) (Ainsley et al., 2003), coupled with the larval UV injury model, it was discovered that though the epidermis becomes injured by UV irradiation, the nociceptors appear structurally undamaged (Babcock et al., 2009). And in one of these studies that also uncovered the requirement for the Hedgehog (Hh) signaling pathway in injury induced allodynia and hyperalgesia, it was found that two separate TRP channels, either Painless (Pain) in allodynia, or dTrpA1 (dTrpA1) in hyperalgesia, were activated depending upon the type of increased sensitivity of the nociceptors that was observed behaviorally (Babcock et al., 2009; Babcock et al., 2011). Building upon the findings on the Hh pathway and nociceptive sensitization, components within the Bone Morphogenetic Protein (BMP) signaling pathway (part of the Transforming Growth Factor Beta (TGF-β) superfamily of proteins) were also subsequently investigated starting with the Hh signaling transcriptional target and known BMP signaling ligand, Decapentaplegic (Dpp) (Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017;

Gjelsvik et al., 2018; McParland et al., 2021; Wang et al., 2014). Toward this effort, our lab used a candidate gene approach, based on supporting literature, to investigate Dpp and the numerous other BMP signaling pathway components we ultimately found to be involved in UV injury-induced nociceptive sensitization (outlined in chapter one of this dissertation) (Figure 1.4) (Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; McParland et al., 2021). However, even with the discoveries made within recent years, a full understanding of the mechanism involved in nociceptive sensitization is still elusive.

Important in continuing this investigation is the knowledge that two of the BMP signaling pathway components we identified as controlling nociceptive sensitivity after UV injury, are also noted within the scientific literature as having roles as transcriptional regulators: Shn (BMP signaling activator) and Brk (BMP and Wnt/Wg signaling repressor) (Arora et al., 1995; Campbell & Tomlinson, 1999; Jazwinska et al., 1999; Saller et al., 2002). Evidence from our previous study that these transcriptional regulators are required for controlling nociceptive sensitivity and injury-induced sensitization (McParland et al., 2021) strongly suggests that the genetic targets of these regulators represent undiscovered corresponding genetic components that ultimately control normal sensitivity and lead to hypersensitization of the nociceptor after injury. We hypothesize that these targets could lead to new treatment options for treating abnormal pain sensitivity that occurs after injury. Remarkably, in addition to the still unknown process underlying nociceptor sensitization after injury, are also the mechanisms involved in facilitating the recovery of the nociceptor from sensitization after injury (Figure 1.5). We suggest that knowledge of the details of the recovery mechanism from nociceptor sensitization is equally important in the formulation of new drug targets for treating chronic pain development and understanding nociceptive sensitization dysregulation, though it has been largely uninvestigated based on scientific literature published thus far.

3.1.1 Aims of this study

Within this chapter our lab sought to uncover the translatomic, the actively translating mRNAs of a cell, mechanisms by which the nociceptor, after injury to the animal, develops nociceptor sensitization, in the form of allodynia, and also returns to baseline after sensitization, specifically hyperalgesia. In this effort we utilized a nociceptor-specific translating RNA screen obtained from third instar *Drosophila* larvae 24 hours post UV injury (Babcock & Galko, 2009; Babcock et al., 2009; Babcock et al., 2011; Jackson et al., 2015; Thomas et al., 2012). 24 hours post UV injury is the time-point at which *Drosophila* larvae are known to have both peak allodynia, as well as recovery from hyperalgesia (Figure 3.1) (Babcock & Galko, 2009; Babcock et al., 2009; Babcock et al., 2011). I then sought to carry out bioinformatic analysis on differentially expressed genes within this UV injured nociceptor translatome, through pathway and gene ontology enrichment analysis using published online tools (Figure 3.2) (Eden et al., 2007; Eden et al., 2009; Ge et al., 2020; Gillespie et al., 2022; Huang et al., 2009a, 2009b; Kanehisa, 2019; Kanehisa et al., 2021; Kanehisa & Goto, 2000; Mi et al., 2021; Smedley et al., 2015). Through this bioinformatic analysis my aim was to uncover genetic components that could be affecting nociceptor synaptic signaling and/or alterations in electrical physiology. We hypothesized that transcriptional/translational responses to injury lead to the process of nociceptive sensitization and/or recovery therefrom, and therefore can represent novel drug targets for future chronic pain therapies. To preliminarily test this hypothesis, we investigated two gene candidates I found to be significantly upregulated within the RNA screen, *Rgk1* and *AnxB11*, by knocking down their expression within the nociceptors of third-instar larvae and then behaviorally assaying them with a noxious, thermal infrared laser. By building upon our prior research in nociceptive sensitization using fly larvae, we believe that further investigation into the mechanisms underlying nociceptive sensitization development and its recovery will provide a better understanding of the complete nociceptive sensitization mechanism and provide new drug targets for future chronic pain drug formulation.

3.2 Methods

3.2.1 Fly husbandry

Flies were maintained in 6 oz stock bottles containing sucrose-cornmeal-yeast medium. Bottles were stored within Percival Scientific Incubators with a 12-h light/12-h dark cycle and kept between 50-60% humidity and a temperature of 25°C. Incubators were set to an arbitrary dawn time of 9:00 A.M. Genotypes used in experiments were prepared using the Gal4/UAS (Brand & Perrimon, 1993) system with the following Gal4 driver line driven by the *pickpocket* promoter: *ppk1.9-GAL4* (in w^{1118}) for thermal nociception assays and TRAP-seq experiments (Adams et al., 1998; Ainsley et al., 2003; Zhong et al., 2010). Transgenic lines included: *UAS-GFP-RpL10ab* (in w^{*}) (BDSC_42681), *UAS-Rgk1-sh/CyO* (*UAS-Rgk1-IR*) (in y^1v^1) (a gift from Tetsuya Tabata) (Murakami et al., 2017), and *UAS-AnxB11-IR* (in y^1v^1) (BDSC_38311) (Perkins et al., 2015). Wild-type fly lines and control lines for TRiP (Perkins et al., 2015) RNAi lines and other transgenic lines used were: w^{1118} (BDSC_3605) and y^1v^1 (BDSC_36303). Each Gal4/UAS genotype within thermal nociception assays was compared to two controls: one with the Gal4 driver genetic background (w^{1118}) crossed with the UAS transgenic line (No Gal4 control) and one with the Gal4 driver line crossed to the genetic background (y^1v^1) of the UAS transgenic line (No UAS control).

3.2.2 Preparation of larvae for ribotag immunoprecipitation and RNA isolation

**The following details for section 3.2.2 are quoted from C. Brann 2019 and J. Moulton 2020 theses (University of New England) with minor additions of details.*

Flies used for TRAP-seq experiments (Exp: *Ppk.1.9-Gal4* x *UAS-GFP-Rpl10Ab*, Control: w^{1118} x *UAS-GFP-Rpl10Ab*) were allowed to mate for 48 hours prior to the timed egg lay. After two days, the flies were placed in a tube containing solidified grape juice agar along one wall to encourage egg deposition. The grape agar is a mixture of sugar, agar, and grape juice concentrate, allowed to boil and

congeal into jelly-like consistency. The egg-lay period was restricted to two hours, then the adults were removed. Developmentally timed larvae were collected 4-5 days after egg lay. Unlike in other UV experiments, the volume required makes anesthesia unrealistic, and larvae were not given ether. Instead, they were placed onto a dish and allowed to crawl until uniformly distributed. The dish was placed into the UV crosslinker (Spectronics Corporation Spectrolinker XL-1000 ultraviolet crosslinker), and the larvae were exposed to a dosage of UV-C between 12.0-18.0mJ, which was recorded with a UV meter (Spectronics Corporation Spectroline XS-254 UV-C photometer). For mock-treated animals, the identical protocol was performed, including putting the animals into the crosslinker, but without the actual delivery of UV. Although this injury was not restricted to the dorsal side as is the case with anaesthetized animals, it is likely to be unproblematic because we did not perform behavioral assays in which only the dorsal surface is stimulated. The larvae were placed in recovery vials for 24-hours. 24-hours post-injury, larvae were removed from recovery vials and separated into tubes as 100 mg groups. The tubes were flash-frozen in liquid nitrogen and stored in liquid nitrogen to reduce RNA degradation before analysis.

3.2.3 Ribotag immunoprecipitation and isolation of nociceptor RNA followed by TRAP-sequencing

**The following details for section 3.2.3 are quoted from C. Brann 2019 and J. Moulton 2020 theses (University of New England) with minor additions of details.*

The following methods were carried out by Ramaz Geguchadze, PhD: Following flash-freeze in liquid nitrogen, pooled larvae (pooled by condition: UV injured/Sham in 100 mg groups) were homogenized, and homogenates underwent immunopurification of the eGFP-tagged ribosomes by using magnetic beads (Invitrogen Dynabeads Antibody Coupling Kit), which are bound to two anti-GFP antibodies. RNA was then isolated and purified from these eGFP-tagged ribosomes using a standard RNA isolation protocol. RNA that was purified was then tested for quantity and purity with an Agilent

Bioanalyzer, obtaining an RNA integrity number (RIN). Once obtaining a RIN value deemed suitable for sequencing by the chosen RNA-sequencing vendor, GENEWIZ® (South Plainfield, NJ), RNA was stored in -80 °C before being shipped on dry ice to the vendor.

The RNA-sequencing vendor GENEWIZ® (South Plainfield, NJ), carried out mRNA sequencing via polyA selection with supplied RNA using Illumina HiSeq, PE 2x150 (150 bp paired end). GENEWIZ® (South Plainfield, NJ), trimmed sequence reads via Trimmomatic v.0.36, mapped sequence reads to the *Drosophila melanogaster* BDGP6 reference genome via ENSEMBL using the STAR aligner v.2.5.2b, and determined gene hit counts (calculation of reads/gene/sample) using featureCounts from Subread package v.1.5.2. A total of 6 samples of customer supplied RNA were used for RNA sequencing by the vendor: three control (sham) samples and three experimental (UV-injured) samples, with each sample of RNA being derived from the 100 mg groups of prepared larvae that were pooled by condition. In supplied deliverables by the GENEWIZ® (South Plainfield, NJ), vendor, were original text files of the unique gene hit counts (reads/gene) for each of these six samples. These individual counts files were used as input for our own further quality assessment, differential gene expression, and pathway analysis shown within this chapter.

3.2.4 Differential expression and pathway analysis of TRAP-seq counts files

All six text files of the unique gene counts for each sample were uploaded into R statistical open-source software (version 4.1.2) and R Studio (version 2021.09.1.372) (R Core Team, 2021; RStudio Team, 2021). The counts files were then combined and formatted in R to achieve one text file. This text file, called a counts table, contained the gene counts of all six samples (column data) and gene associated FlyBase IDs (row IDs) for downstream analysis by DESeq2 (version 1.34.0) (Love et al., 2016; Love et al., 2014). DESeq2 is a statistical software package (updated from DESeq) used to determine differentially expressed genes in RNA sequencing count data based on the negative binomial distribution (Love et al.,

2014). Using an experimental sample information text file supplied by GENEWIZ and containing sample ID, condition, and batch information, a metadata table was also prepared on the counts table columns. Factor levels were also set based on "condition" of the samples (either UV injured or control). The two tables were then used to prepare the following DESeq2 data object (dds) to be used in analysis:

'dds <- DESeqDataSetFromMatrix(countData=countdata, colData=sampleData, design = ~ condition, tidy= FALSE)'

Before running DESeq2 analysis for differential gene expression, provided sample gene counts were analyzed preliminarily to determine sample quality, inter- and intra- relationships among groups, read count distribution, and gene dispersion estimation (Koch et al., 2018; Love et al., 2014; Lun et al., 2016). The 'estimateSizeFactors' function in DESeq2 was first carried out to control for differences in library sizes using the "median-of-ratios method" (Anders & Huber, 2010; Love et al., 2014). Inter-/intra-relationships among groups and sample quality was visualized by pairwise scatterplots of all samples in both groups (Figure 3.3) in R, using count data normalized by $\log_{10}$ transformation (McDermaid et al., 2018). Read count distribution and the potential high magnitude of low read counts was investigated through visualization of a histogram of the sum of $\log_{10}$-transformed counts data across all samples, also in R (Figure 3.4B,D) (Lun et al., 2016; R Core Team, 2021). The DESeq2 function 'estimateDispersions', was then used to calculate dispersion estimates across genes for all samples and visualized with the DESeq2 dispersions plot ('plotDispEsts') (Figure 3.4A,C). After preliminary analysis of the counts data for low expression, I set a custom threshold of at least 20 counts per 6 samples via the following code written in R and applied it to the dds object within the DESeq2 pipeline (R Core Team, 2021):

'keep <- rowSums(counts(dds, normalized=TRUE) >= 20) >=6'

'dds <- dds[keep,]'

The dds object, where counts had been thresholded to eliminate lowly expressed genes, was then analyzed for differential gene expression via the 'DESeq' function through DESeq2 with the

previously set design (UV vs Control (Sham)) and under default parameters (Love et al., 2016; Love et al., 2014). Gene symbols were added to the dds results by mapping onto provided Flybase gene IDs using the fly genome annotation package, org.Dm.eg.db (version 3.14) (Carlson, 2021). The DESeq function normalizes counts values before analyzing differential expression and results include the Wald test statistic for *p* value and adjustment for multi-comparison analysis (adjusted *p* value) using the Benjamini-Hochberg procedure as default within an output table (Love et al., 2014). Differential expression analysis was visualized using MA plots through DESeq2, the R CRAN (Comprehensive R Archive Network) package, pheatmap (version 1.0.12), and by the R package, EnhancedVolcano (version 1.12.0) (Blighe et al., 2021; Kolde, 2019; Love et al., 2014; R Core Team, 2021). DESeq output was also visualized for sample clustering analysis using DESeq normalized data and a principal components plot function found within the DESeq2 package (Love et al., 2014).

Genes found to be differentially expressed (DEGs) by DESeq, with an adjusted $p < 0.05$, were used in downstream gene ontology (GO) and pathway enrichment analysis, regardless of the value of the fold change. Gene ontology and pathway enrichment analysis were carried out using the web-based online tools: The Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 2021), Kyoto Encyclopedia of Genes and Genomes (KEGG) (release version 101.0), **G**ene **O**ntology en**RI**chment ana**L**ysis and visua**L**iz**A**tion tool (GOrilla), and ShinyGO (version 0.75) (Eden et al., 2007; Eden et al., 2009; Ge et al., 2020; Gillespie et al., 2022; Huang et al., 2009a, 2009b; Kanehisa, 2019; Kanehisa et al., 2021; Kanehisa & Goto, 2000; Mi et al., 2021). In the web-based analyses, a background gene set consisting of all background genes within the experiment (after the low threshold cutoff was applied) was used as the comparison gene set in the gene ontology and pathway enrichment analysis. A false discovery rate (FDR) of < 0.05 was considered significant in the online analyses for gene ontology and pathway enrichment. Graphs of results not made within the web-based programs used in analyses were made in Microsoft Excel (version 2202) and R (R Core Team, 2021; RStudio Team, 2021).

3.2.5 Larval thermal nociception assay using computer-controlled infrared diode laser

Larvae in which Rgk1 or AnnxB11 was knocked down in nociceptors via Gal4/UAS targeted RNA interference technology were behaviorally analyzed using a novel infrared diode laser methodology. Late third-instar animals of experimental or control genotypes were placed on a transparent silicone test surface moistened with water applied using a small paintbrush. A 0.5mm diameter black plastic radiation absorption disk, punched from heavy duty garbage bag, was affixed to the dorsal surface of the larva, overlaying abdominal segments 4 through 7, using heat-thickened molasses. The larva are prevented from wandering off the test surface by an electrically heated perimeter wire. The test surface is affixed to a motor-driven stage placed below another motor-driven platform that holds the infrared diode laser (300mW at 3.0V, 808-810nm) and video microscope camera. The motion of stage (X dimension) and laser (Y dimension) is coordinated by a Raspberry Pi miniature computer which also serves the camera image to an external monitor. For safety, the device is enclosed in a black acrylic container and features a safety interlock that disconnects laser power if the access door is opened during operation. Watching the external monitor, the operator steers the movement of the stage relevant to the laser using a videogame controller, keeping the laser illumination consistently on the black disk as the larva crawls about, until it executes an escape roll, at which time the laser is switched off and the response latency recorded by the computer.

3.1.6 Nociception assay statistics

Thermal nociception assays were plotted as percent accumulated response vs. latency where an end-point cut-off of 30 s was applied and latency in seconds recorded. After applying a binary variable to the data based on ‘response’ or ‘no response’ at the 30 s cut-off time, statistical analysis of latency of response between all behavioral data groups was completed using Log-rank analysis and applying

Benjamini and Hochberg procedure for multiple testing. Log-rank analysis for thermal nociception assays were performed using R statistical coding software (version 4.1.2) with RStudio (version 2021.09.1.372) (R Core Team, 2021; RStudio Team, 2021) and applying the 'survival' analysis package for statistics output (Therneau, 2020). Percent response plots were carried out using Microsoft Excel (version 2202).

3.3 Results

3.3.1 TRAP-sequencing of larval nociceptors after UV injury results in differential expression of genes

It has been noted within *Drosophila* literature that peak hyperalgesia, a heightened response to already perceived noxious stimuli, and allodynia, in which innocuous stimuli become noxious, occur at different time-points following UV injury (Figure 3.1) (Babcock et al., 2009). Shown in Figure 3.1, peak hyperalgesia can be seen at 8 hrs. after UV injury, hyperalgesia recovery and peak allodynia at 24 hrs., and recovery from peak allodynia at 48 hours after injury (Babcock & Galko, 2009; Babcock et al., 2009; Im & Galko, 2012). Using this knowledge to our advantage, we can look at the 24-hr timepoint after injury and possibly characterize genes involved in both the development of sensitization (peak allodynia) or in the recovery of the nociceptor from sensitization (recovery of hyperalgesia), depending on the response curve of allodynia/hyperalgesia after injury (Figure 3.1). So, to further uncover nociceptive sensitization and recovery transcriptional targets and associated pathways within the nociceptor, we carried out TRAP-sequencing and bioinformatic analysis of the nociceptor translatome within third-instar fruit fly larvae at 24 hours post UV injury (Figure 3.2).

Following TRAP-sequencing of supplied nociceptor mRNA, the vendor, GENEWIZ®, supplied raw counts data text files derived from nociceptor TRAP-sequencing reads (sham and UV-injured conditions) mapped to the *Drosophila melanogaster* genome. An initial quality check for inter-/intra- sample relationships was investigated by visually assessing pairwise scatterplots in R statistical software of the $\log_{10}$ transformed counts data for each sample prior to DESeq2 processing for differential gene

expression (Figure 3.3). Within the scatterplot each dot represents a gene and the mean expression of that gene between the two samples shown by its x-y coordinate placement (McDermaid et al., 2018). Though visually samples within the same condition group (Control/Sham ~ UV) tended to fall more along the diagonal, some intra-relationship noise was apparent by the wider, non-diagonal placement of genes for some pairwise comparisons and so further plots for quality analysis were generated (Figure 3.3). A dispersion plot through DESeq2 of the mean of the normalized counts data was plotted along with a histogram of the $\log_2$ transformed count data vs. number of genes expressed (post removal of 0 count genes) (Figure 3.4A-B). The dispersion plot, which estimates dispersion, or intra-sample variability of a gene's expression within each condition group, displayed a high number of low counts features at the limit of the y-axis for estimated dispersion (Figure 3.4A).

The histogram of the $\log_2$ counts data vs. number or genes expressed also displayed similar results with a high number of low counts features across all samples, even after removal of 0 count genes from the dataset (Figure 3.4B). A conservative pre-threshold for counts across samples was then established (Figure 3.4B) to eliminate possible noise from technical factors or intra sample group quality/variation (Koch et al., 2018; Lun et al., 2016). The counts threshold was applied prior to DESeq2 analysis for differential gene expression between groups and resulted in a reduction of low counts features and lower dispersion among low counts features as shown in Figure 3.4C-D. Quality analysis was once again investigated post application of the DESeq function for differential gene expression analysis on the counts data. Investigation of the DESeq data output showed counts to be normalized across samples (Figure 3.5A) (Love et al., 2016; Love et al., 2014). Clustering of samples per condition was visualized through a principal components analysis (PCA) plot, which breaks down maximum levels of variation into components, of the top 100 differentially expressed genes after regularized-logarithm transformation (rlog) in DESeq2 (Figure 3.5B) (Koch et al., 2018; Love et al., 2016; Ringnér, 2008).

The DESeq results output for differential gene expression resulted in ~8000 differentially expressed genes (DEGs) (however, not all significantly differentially expressed) within the nociceptor translatome from those animals who had been UV injured when compared to control/sham animals (Figure 3.6A). This value of DEGs was after DESeq removed 3 outliers through applying a cutoff for Cook's distance (default) and 496 genes for low counts using independent filtering on the mean of normalized counts (default) (Bourgon et al., 2010; Love et al., 2016; Love et al., 2014). After filtering through the ~8000 DEGs for those with adjusted *p* values less than 0.05, it was found that there were 244 genes that were significant. The top 50 differentially expressed genes were clustered using a heatmap (Figure 3.6B), which displayed a great number of downregulated genes vs. upregulated. In total, 62 genes were found to be significantly upregulated and 182 genes were found to be significantly downregulated.

3.3.2. Gene ontology and pathway enrichment analysis of nociceptor differentially expressed genes

When applying gene ontology (GO) and pathway term enrichment analysis on the significant DEG set (*p* adj < 0.05), I found that only those genes that were significantly downregulated showed enrichment on web-based GO and pathway enrichment sites (Eden et al., 2007; Eden et al., 2009; Ge et al., 2020; Huang et al., 2009a, 2009b; Kanehisa, 2019; Kanehisa et al., 2021; Kanehisa & Goto, 2000). Those that were significantly upregulated were not enriched for any known GO terms or pathways when compared to the thresholded nociceptor background gene set used as comparison. Of the terms found to be significantly enriched within the significantly downregulated gene set using ShinyGO (v0.75), proteolysis was most significantly enriched under GO biological processes (BP) category with a false discovery rate (FDR) of 1.72E-05 and a fold enrichment of 2.504 (Figure 3.7, Appendix 2) (Ge et al., 2020). Under the GO molecular function (MF) category on ShinyGO (v0.75), the terms: Serine-type peptidase activity (FDR = 2.43E-21, fold enrichment= 10.859), serine endopeptidase activity (FDR =

1.64E-20, fold enrichment = 11.287), and serine hydrolase activity (FDR = 2.43E-21, fold enrichment= 10.859) were most significantly enriched (Figure 3.7, Appendix 2) (Ge et al., 2020). Enrichment on ShinyGO (v0.75) for GO cellular component (CC) terms within the significantly downregulated DEG gene set resulted in the extracellular region being most significantly enriched (FDR = 7.56E-14, fold enrichment = 3.815) and enrichment analysis for KEGG pathways resulted in the neuroactive ligand receptor interaction pathway being most enriched (FDR = 3.05E-4, fold enrichment= 13.662) (Figure 3.7, Appendix 3) (Ge et al., 2020; Kanehisa, 2019; Kanehisa et al., 2021; Kanehisa & Goto, 2000). The interactions between these different GO terms were further investigated through a node-edge network plot on ShinyGO (version 0.75), where two nodes (GO terms) were connected by edges (lines) if they shared at least 20% or more genes within the significantly downregulated gene set (Figure 3.8) (Ge et al., 2020). Within this network it was found that the top enriched GO terms: proteolysis, serine hydrolase, serine-type peptidase/endopeptidase, and extracellular region/space, were all connected by their associated genes found within the significantly downregulated DEGs set (Figure 3.8) (Ge et al., 2020).

In complement to these findings, when investigating the significantly upregulated DEG set, there was also found to be three genes without known gene symbols (FBgn0261630, FBgn0033355, FBgn0261634) upregulated that had the “serine-type endopeptidase inhibitor activity” GO annotation and are predicted by FlyBase (version FB2022_01) to have serine-type endopeptidase inhibitor activity (Larkin et al., 2021). Through further analysis of GO terms associated with significantly upregulated genes (p adj ≤ 0.05) within the dataset, several genes of interest were identified by searching for annotations to GO terms involving either “ion activity” or “plasma membrane” terminology (Figure 3.9, Table 3.1) (Huang et al., 2009a, 2009b). A focus on these specific GO terms was utilized in order to narrow down genes possibly involved in altering the electrophysiology of the cell at the plasma membrane during injury induced sensitization and therefore could possibly be utilized as drug targets

due to their role in nociceptor sensitivity. Genes of interest within the significantly upregulated DEGs and their selected annotations included: Annexin B11 (*AnxB11*), Rad Gem/Kir member 1 (*Rgk1*), and Painless (*pain*) which are associated with GO terms that include calcium ion/channel activity (*AnxB11*, *Rgk1*, *pain*) and plasma membrane (*Rgk1*, *pain*), Chloride channel-c (*ClC-c*), which is associated with chloride ion/channel activity and plasma membrane GO terms, and Ecdysone-induced protein 63E (*Eip63E*) which is associated with plasma membrane and Wnt signaling GO terms (Figure 3.9, Table 3.1) (Eden et al., 2007; Eden et al., 2009; Huang et al., 2009a, 2009b).

3.3.3 Knockdown of Rgk1 or AnxB11 results in behavioral hypersensitivity

Of the five significantly upregulated genes of interest shown in Table 1, a literature review on mammalian orthologs and suspected orthologs to the genes *AnxB11* and *Rgk1* resulted in the development of a hypothetical mechanism of recovery from hypersensitivity of the nociceptor by these two genes (Figure 3.10) (Avenali et al., 2014; Charnet et al., 2013; Li et al., 2019; Murakami et al., 2017; Scamps et al., 2015). To initiate investigation into this developing hypothetical mechanism, a behavioral thermonociception infrared laser chamber assay was carried out on third instar *Drosophila* larvae where either Rgk1 (*Rgk1-IR*) or AnxB11 (*AnxB11-IR*) had been knocked down within their nociceptors using RNAi technology. We hypothesized that even without injury, a decrease in Rgk1 or AnxB11 expression within the nociceptor would result in an increase in behavioral thermal hypersensitivity. Results from the laser chamber assay showed a significant increase in nocifensive behavioral response for those animals expressing either *Rgk1-IR* or *AnxB11-IR* within their nociceptors, when compared to control groups (Figure 3.11).

3.4 Discussion

3.4.1 Bioinformatic Analysis of nociceptor mRNA from UV injured larvae

3.4.1.1 Significantly downregulated genes 24 hrs. after UV injury indicate a downregulation in proteolysis within the nociceptors

Along with investigation into mechanisms affecting nociceptor sensitivity without injury (chapter 2), we sought to also shed further light on mechanisms affecting nociceptor sensitization and recovery after injury (Figure 3.1). Prior studies have shown that though the epidermis is severely affected by ultraviolet irradiation injury to larvae, nociceptor morphology tends to remain intact (Babcock et al., 2009; Follansbee et al., 2017). To investigate any still-unknown novel targets and pathways, mRNA transcripts were isolated from the nociceptors of UV injured fruit fly larvae, 24 hrs. after injury (Figure 3.2) (Babcock & Galko, 2009; Babcock et al., 2009; Babcock et al., 2011; Jackson et al., 2015; Thomas et al., 2012).

Bioinformatic analysis of the nociceptor translatome from those animals that had been UV injured when compared to control (sham) resulted in an output of many significant DEGs that were mostly downregulated (~182 DEGs), but also many (~62 DEGs) that were upregulated in their expression as well (Figure 3.6). Through GO and pathway analysis it was found that proteolysis, serine-type peptidase/endopeptidase activity, and serine hydrolase activity were significantly enriched GO terms within the downregulated gene set (Figure 3.7). The KEGG pathway: Neuroactive ligand-receptor interaction (KEGG pathway ID: dme04080) and the term “extracellular region” was also significantly enriched within the downregulated gene set and “extracellular region/extracellular space” was found to share at least 20% or more genes between itself and “serine peptidase activity/serine endopeptidase activity” (Figure 3.8) (Kanehisa, 2019; Kanehisa et al., 2021; Kanehisa & Goto, 2000). Upon further investigation of these components comprised within the enriched Kegg pathway: Neuroactive ligand-receptor (KEGG pathway ID: dme04080), these serine proteases (which consisted of trypsins such as:

alphaTry, *betaTry*, *deltaTry*) were shown within the pathway to be "proteinase-activated like" extracellular interactors in fruit flies (Kanehisa, 2019; Kanehisa et al., 2021; Kanehisa & Goto, 2000). Serine proteases such as thrombin and trypsin for example can hold a wide range of duties within the body including processes such as digestion, inflammation, and blood coagulation (Cirino et al., 1996; Leger et al., 2006). Notable though when investigating nociceptor sensitivity, is that extracellular serine proteases have also been shown to be involved in altering the functional state of certain ion channels (e.g., activation, inactivation, modification) (Kiselyov et al., 2005; Poirot et al., 2004).

In particular, studies have shown that activation of neuronal proteinase-activated receptor-2 (PAR2) (part of a G-protein coupled receptor (GPCRs) subfamily), preferentially by serine proteases like trypsin, leads to pain development through various mechanisms that include sensitization of TRPA1/TRPV1 and/or release of inflammatory peptides such as substance P (Dai et al., 2004; Dai et al., 2007; Lam & Schmidt, 2010; Nystedt et al., 1994; Steinhoff et al., 2000). Given the time-point we collected mRNA within the nociceptors after UV injury, it is possible that significant downregulation of extracellular serine protease activity could be part of hyperalgesia recovery due to halting of an unknown mechanism similar to nociceptor PAR2 sensitization in mammals (see Figure 3.1 for sensitization timeline). In contrast, it could also be hypothesized that proteolytic cleavage by serine proteases of ion channels within the nociceptor could negatively regulate ion channels and that serine protease downregulation is part of the process to reaching peak allodynia (Figure 3.1). This has been seen to occur in the human acid-sensing ion channel 1 (ASIC1) channel by the serine protease matriptase and mammalian ASIC channels have been reported to be homologous to the Pickpocket1 channel in *Drosophila* (Boiko et al., 2013; Boiko et al., 2012; Clark et al., 2010). Overall, however, the downregulated serine proteases and the significantly upregulated serine protease inhibitors within the TRAP sequencing dataset require further investigation as to their role in ion channel regulation after UV injury.

3.4.1.2 Significantly upregulated genes 24 hours after UV injury indicate an increase in nociceptor plasma membrane ion channel activity

Shown in Figure 3.1, hyperalgesia recovery and peak allodynia both occur at 24 hours post UV injury in *Drosophila* larvae (Babcock & Galko, 2009; Babcock et al., 2009; Babcock et al., 2011). As such, this time-point for RNA-sequencing of the nociceptor translatome allows us to possibly characterize genes involved in peak allodynia or in the recovery of the nociceptor from hyperalgesia (Figure 3.2). Within fruit fly nociceptive sensitization studies and the corresponding signaling pathways uncovered thus far (Figure 1.4), there has been evidence that two separate Transient Receptor Potential (TRP) channels regulate allodynia and hyperalgesia individually (Babcock & Galko, 2009; Babcock et al., 2009; Babcock et al., 2011; Im et al., 2015). It was found within these studies that Painless (Pain) is activated during allodynia and Transient receptor potential cation channel A1 (dTrpA1) during hyperalgesia (Babcock et al., 2011; Im et al., 2015). A prominent finding within our TRAP sequencing dataset was the significant upregulation of *painless* within the nociceptors of UV injured animals. This upregulation supports prior findings that peak allodynia occurs at 24 hours after injury and that Painless is the main ion channel involved in allodynia at that time-point (Figure 3.9 and Table 3.1). Another ion channel that was upregulated within the dataset was the chloride channel, *ClC-c* (Figure 3.9 and Table 3.1). *ClC-c* has been found to be orthologous to mammalian *ClC-3* (chloride voltage-gated channel 3) (Cabrero et al., 2014; Larkin et al., 2021). In recent years, chloride voltage gated channel 3 in mammalian studies has been found to both possibly contribute to sensory neuron depolarization as well as inhibit neuropathic pain development within the DRG (Pang et al., 2016; Qi et al., 2018). A primary focus on the role of chloride and chloride channels in nociceptor sensitivity and pain development is still relatively new in mammals, so our findings of *ClC-c* upregulation within our dataset is promising for similar investigations to take place in fruit flies (Figure 3.9, Table 3.1) (Wilke et al., 2020).

Another significantly upregulated gene of interest in the dataset was the *ecdysone-induced protein 63E* (*Eip63E*) (Figure 3.9, Table 3.1). We found upregulation of this cyclin-dependent kinase interesting based on prior research from within our lab showing the involvement of the fruit fly ecdysteroids in neuromodulation (McParland et al., 2015) and our previous findings on nociceptor sensitivity and Arm which is found in the canonical Wnt/Wg signaling pathway (chapter 2). Ecdysone Receptor A (EcRA) was shown in prior studies from within the Ganter lab to be required in nociceptor sensitivity by experiments where *Drosophila* larvae with EcRA knocked down within their nociceptors showed hyposensitivity to thermal and mechanical stimuli (McParland et al., 2015). Eip63E has been shown to have to have at least one transcript (of two) that is induced by ecdysone signaling (D. Liu et al., 2010; Stowers et al., 2000). It has also been shown that Eip63E and its vertebrae homolog, cyclin-dependent kinase 14 (CDK14, also known as PFTK), are positive regulators of canonical Wnt/Wg signaling through their complex development with Cyclin Y (CycY), which leads to phosphorylation of Arrow in the canonical Wnt/Wg signaling cascade (mammalian homolog to LRP5/6) (Davidson et al., 2009; Davidson et al., 2005). This is similar to the function found with Gish and its phosphorylation of Arrow in the canonical Wnt/Wg signaling cascade (Figure 1.4) (Davidson et al., 2005; Schaefer et al., 2018; Zhang et al., 2006). Indeed, in a study carried out in 2009, RNAi technology for both Gish and Eip63E were found to reduce LRP6 (Arrow's mammalian homolog) phosphorylation and canonical Wnt/Wg signaling in an *in vitro* kinome-wide RNAi screen using *Drosophila* cells that had been transfected with mammalian *LRP6* (Davidson et al., 2009). Discovery of the significant upregulation of this gene, *Eip63E*, therefore could serve as a link in describing how both previous findings on EcRA and Arm may be connected in modulation of nociceptor sensitivity through these two different pathways. This finding also suggests later time-points, later than 24 hours after injury, may be warranted in investigation of Wnt/Wg signaling and Ecdysteroid signaling involvement in injury induced nociceptor sensitization. In other words, stimulation of these signaling mechanisms may be just occurring at the 24-

hour time point and the roles of these pathways in nociceptor sensitivity may not emerge fully until past 24 hours after injury.

Finally, when analyzing significant genes upregulated in the dataset, two more genes of interest that emerged were *Rad Gem/Kir family member 1* (*Rgk1*) and *Annexin B11* (*AnxB11*) (Figure 3.9 -3.10 and Table 3.1). *Rgk1* is known to be orthologous to mammalian genes found within the RGK subfamily (*REM*, *REM2*, *GEM/KIR*, *RAD*) of the Ras-related small GTPases superfamily (Murakami et al., 2017; Puhl III et al., 2014). Notably within the Ras superfamily of RGK mammalian orthologs to Rgk1, RGK proteins have been shown to bind to both calmodulin (CaM) and 14-3-3 proteins (Béguin, Mahalakshmi, Nagashima, Cher, Takahashi, et al., 2005; Béguin, Mahalakshmi, Nagashima, Cher, Kuwamura, et al., 2005; Finlin & Andres, 1999; Fischer et al., 1996; Moyers et al., 1997; Ward et al., 2004). 14-3-3 proteins and calmodulin have been shown to play roles in long-term potentiation and memory (Limbäck-Stokin et al., 2004; Qiao et al., 2014). Similarly, Rgk1 has been found in fruit flies to be necessary in memory regarding anesthesia-sensitive and anesthesia-resistant memory (Murakami et al., 2017). Mammalian RGK proteins have also been shown to inhibit high voltage calcium channels (Ca_V1 and Ca_V2) by mechanisms such as: blockade/inhibition of pre-existing channels and decrease of channel density within the membrane in a variety of different cell types (Béguin et al., 2001; Chen et al., 2005; Finlin et al., 2003; Finlin et al., 2005; Yang et al., 2012; Yang et al., 2010). Specifically, the mammalian RGK protein, GEM, has been shown to inhibit voltage gated calcium channel activity following peripheral nerve injury in mice (Scamps et al., 2015). In a study where *Drosophila* Rgk1 was expressed in rat superior cervical ganglion neurons, it was found that expression of the *Drosophila* homolog was sufficient in inhibiting calcium channels (Puhl III et al., 2014). Another mammalian RGK protein, REM2, has been shown to play a role in regulating synaptic development/plasticity, through negative regulation of dendritic arborization and positive regulation of excitatory synapse development, and influences gene

expression involved in morphological changes to the cell (Ghiretti et al., 2014; Ghiretti & Paradis, 2011, 2014; Kenny et al., 2017; Moore et al., 2013).

Regarding AnxB11, it is orthologous to components found within the vertebrate annexins family and some annexins in mammals have been shown to regulate nociceptor channel activity and to have a role in antinociception (Avenali et al., 2014; Ayoub et al., 2008; Chen et al., 2014; Iglesias et al., 2002; Larkin et al., 2021; Li et al., 2019; Pei et al., 2011). Specifically, the mammalian annexin, ANXA2 (Annexin 2), has been shown to inhibit TRPA1 dependent nociception through limiting channel availability within sensory neurons (Avenali et al., 2014), and the mammalian annexin, ANXA1 (Annexin 1), has been shown to be glucocorticoid inducible and facilitate in antinociceptive pain relief in murine and rat models (Ayoub et al., 2008; Chen et al., 2014; Li et al., 2019; Pei et al., 2011). These prior studies investigating the mammalian orthologous families of both of Rgk1 and AnxB11 suggest that Rgk1 and AnxB11 may have similar roles within the fruit fly nociceptor. As such, I hypothesized that both of these candidates may contribute to nociceptive sensitization recovery after injury based on these findings of mammalian orthologs to Rgk1 and genes found within the mammalian Annexin family of proteins (Figure 3.10).

3.4.2 Rgk1 or AnxB11 knockdown results in genetic induced thermal hyperalgesia in *Drosophila* larvae

To test out the hypothesis that either Rgk1 or AnxB11 may be involved in nociceptive sensitization in the fruit fly, we expressed RNA interference technology targeting either Rgk1 (*Rgk1-IR*) or AnxB11 (*AnxB11-IR*) within the nociceptors of third instar *Drosophila* larvae, using the Gal4/UAS system (Figure 1.3) (Brand & Perrimon, 1993). By behaviorally testing thermal avoidance latencies of animals under expressing either Rgk1 or AnxB11 in their nociceptors, our results indicated that these animals had developed genetically induced hypersensitivity even in the absence of injury (Figure 3.11). Though follow-up with a second *Rgk1-IR* line, a second *AnxB11-IR* line, and investigation of dendritic

morphology of these animals with either Rgk1 or AnxB11 knocked down in their nociceptors is needed for further confidence in the roles of Rgk1 and AnxB11 within these behavioral assays, results so far point to both Rgk1 and AnxB11 involvement in reducing nociceptor sensitivity (Figure 3.11).

3.5 Conclusion

In an effort to uncover more of the mechanism involved in nociceptor sensitization and its recovery after injury, GO and pathway analysis of the nociceptor translatome of larvae 24 hours after UV injury revealed a role in the downregulation of serine proteases in either nociceptor peak allodynia development or recovery of the nociceptor from hyperalgesia. Upregulation of *Rgk1* and *AnxB11* also led to the hypothesis that these proteins are involved in the recovery of the nociceptor after sensitization (hyperalgesia). Results of larval hypersensitivity with RNAi technology targeting either Rgk1 or AnxB11 within the nociceptor further supported this possibility. In conclusion, bioinformatic analysis of the nociceptor translatome after UV injury and our investigation into the proteins Rgk1 and AnxB11, give evidence that serine protease activity, and the proteins Rgk1 and AnxB11 play a role in nociceptor sensitization and/or its recovery and warrant further research.

3.6 Acknowledgements in contribution to data acquisition and writing

Research and writing contribution: Christine Hale, Geoffrey Ganter, PhD, and Kyle Beauchmin, PhD, designed research within this chapter; Courtney Brann, MS, and Julie Moulton, MS, prepared *Drosophila* larvae for isolation of nociceptor RNA. Ramaz Geguchadze, PhD, isolated and purified larval *Drosophila* nociceptor RNA for RNA sequencing by GENEWIZ®. Christine Hale carried out bioinformatic analysis on the RNA sequencing results (raw counts files) supplied by GENEWIZ® to determine differentially expressed genes and for gene ontology and pathway enrichment. Josh Smestad performed larval

behavioral nociception assays. Christine Hale wrote this chapter with editing assisted by Geoffrey Ganter, PhD.

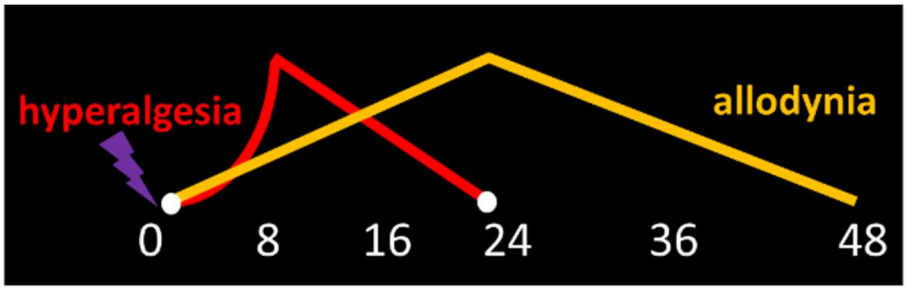


Figure 3.1 Hypersensitivity timeline after UV injury for third instar *Drosophila* larvae.

Third instar larvae reach peak hyperalgesia at 8 hours post UV injury and peak allodynia at 24 hours post UV injury. Larvae recover from hyperalgesia after injury at 24 hours, and from allodynia at 48 hours (Babcock et al., 2011, Babcock et al. 2009). Graphic illustration by G.Ganter.

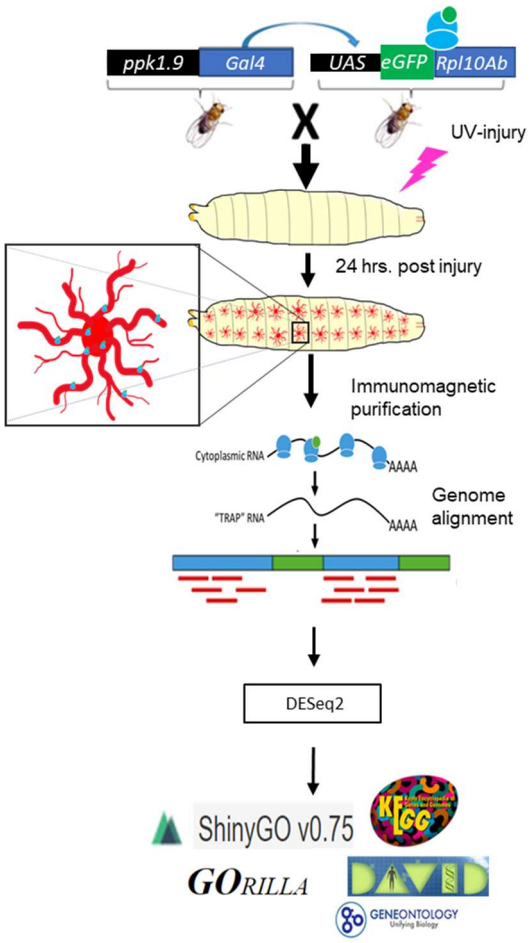


Figure 3.2 Translating Ribosomal Affinity Purification (TRAP)- isolation of nociceptor RNA workflow.

Third instar *Drosophila* larvae expressing *UAS-GFP-Rpl10Ab* within their nociceptors underwent UV injury, were given 24 hours to recover, flash frozen, and then homogenized to undergo immunomagnetic purification of nociceptor GFP tagged ribosomes. RNA was purified by a standard isolation protocol. RNA sequencing vendor GENEWIZ® (South Plainfield, NJ) carried out sequencing, mapped reads to the *Drosophila* reference genome, and determined gene hit counts. Supplied raw counts files were then analyzed for differential gene expression analysis using DESeq2, followed by GO and pathway analysis with published online tools. Further details on the workflow can be found within the methodology of this chapter. C. Hale, K. Beauchmin, and G.Ganter contributed to graphics of this figure.

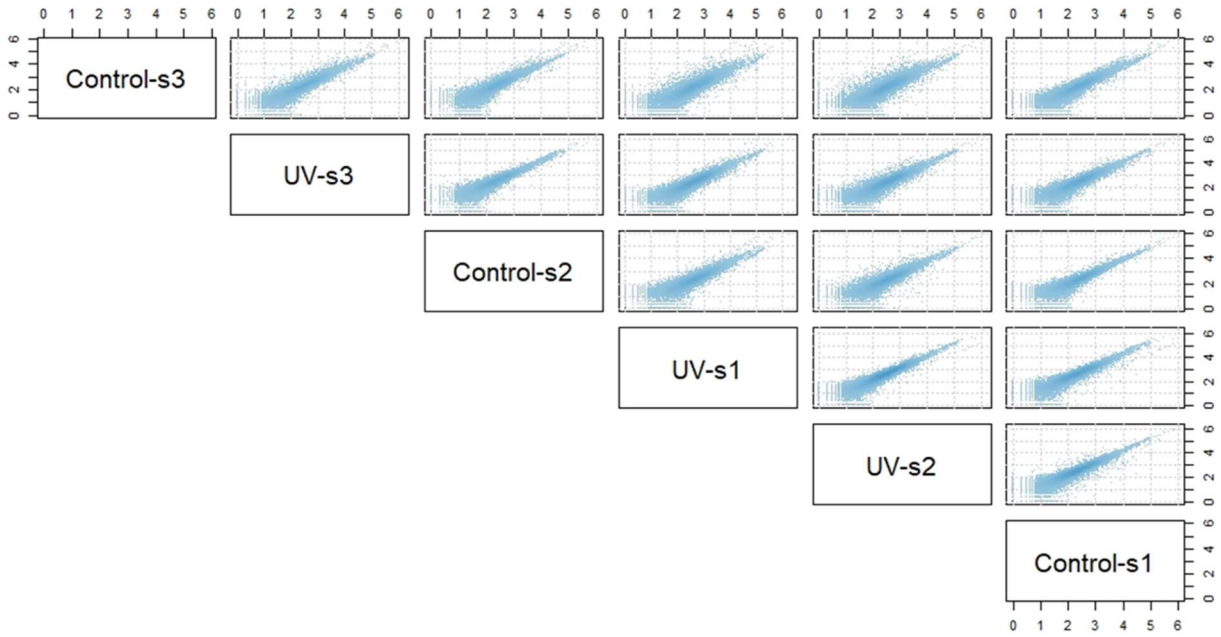


Figure 3.3 Pairwise scatterplots reveal intra/inter sample relationships.

Before differential gene expression analysis, provided sample gene counts from nociceptor transcripts were $\log_{10}$ transformed in R and were analyzed for quality and inter/intra relationships by pairwise scatterplots of all samples in both groups (Control vs. UV injured). Each dot within the scatterplot represents a gene and the mean expression of that gene between the two samples shown by its x-y coordinate placement. *n*= 3 (pooled samples)/group (Control vs. UV injured).

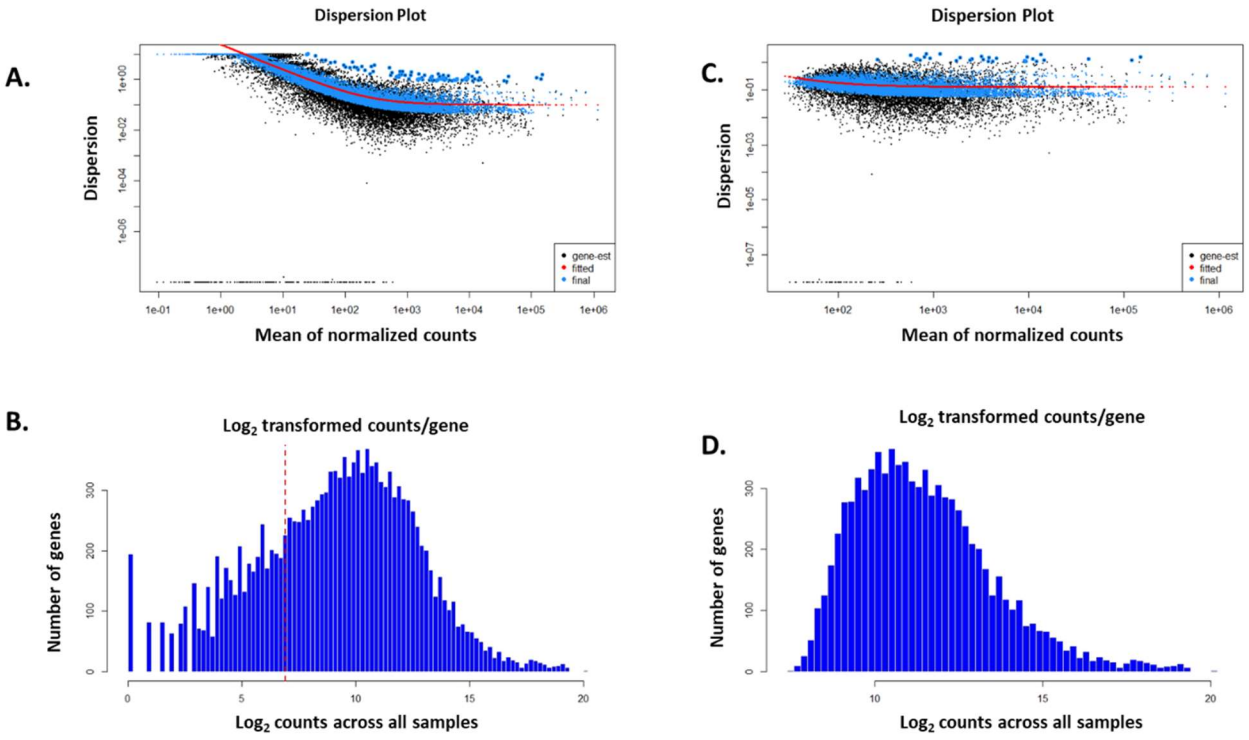


Figure 3.4 Dispersion and histogram plots before and after thresholding of low counts for noise reduction.

Dispersion and histogram plots were generated to visualize sample quality and noise within the data. **(A)** Dispersion plot of the mean of the normalized counts was plotted using DESeq2. The plot estimates dispersion or intra- sample variability of a gene's expression within each condition group (Control vs. UV injured). A preliminary analysis showed a high number of low counts features at the limit of the y-axis for estimated dispersion. **(B)** The histogram of the $\log_2$ counts data vs. number of genes expressed also displayed similar low count features across all samples, even after removal of 0 count genes from the dataset. A conservative pre-threshold limit for counts across all samples (≥ 20 counts for each of the 6 samples (n=3 pooled samples/condition (Control vs. Experimental)) was then established (visualized by the red dotted line). **(C-D)** Dispersion and histogram plots following the established threshold limit for counts across all samples to eliminate noise, shows the removal of low counts features.

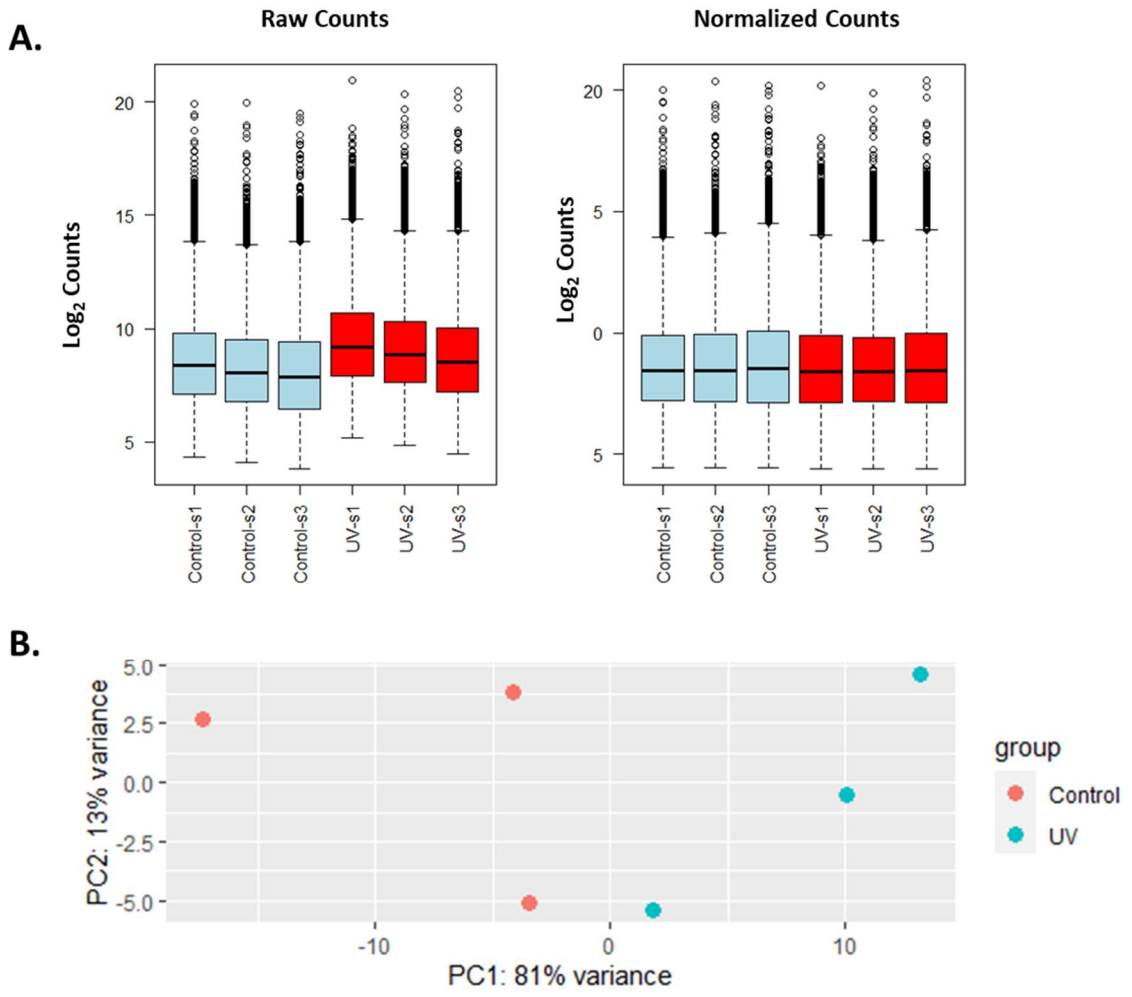


Figure 3.5 Analysis of normalization of gene count data across all samples and Principal Component Analysis (PCA) of sample relationship post DESeq2 normalization in R.

(A) Quality analysis was investigated by comparing bar plots of the raw (un-normalized) log_2 counts data across all samples to the normalized log_2 counts data after the DESeq2 function for differential gene expression analysis had been applied across all samples. **(B)** Clustering of samples per condition was visualized through a principal components analysis (PCA) plot, which breaks down maximum levels of variation into components, of the top 100 differentially expressed genes after regularized-logarithm transformation (rlog) in DESeq2.

A.

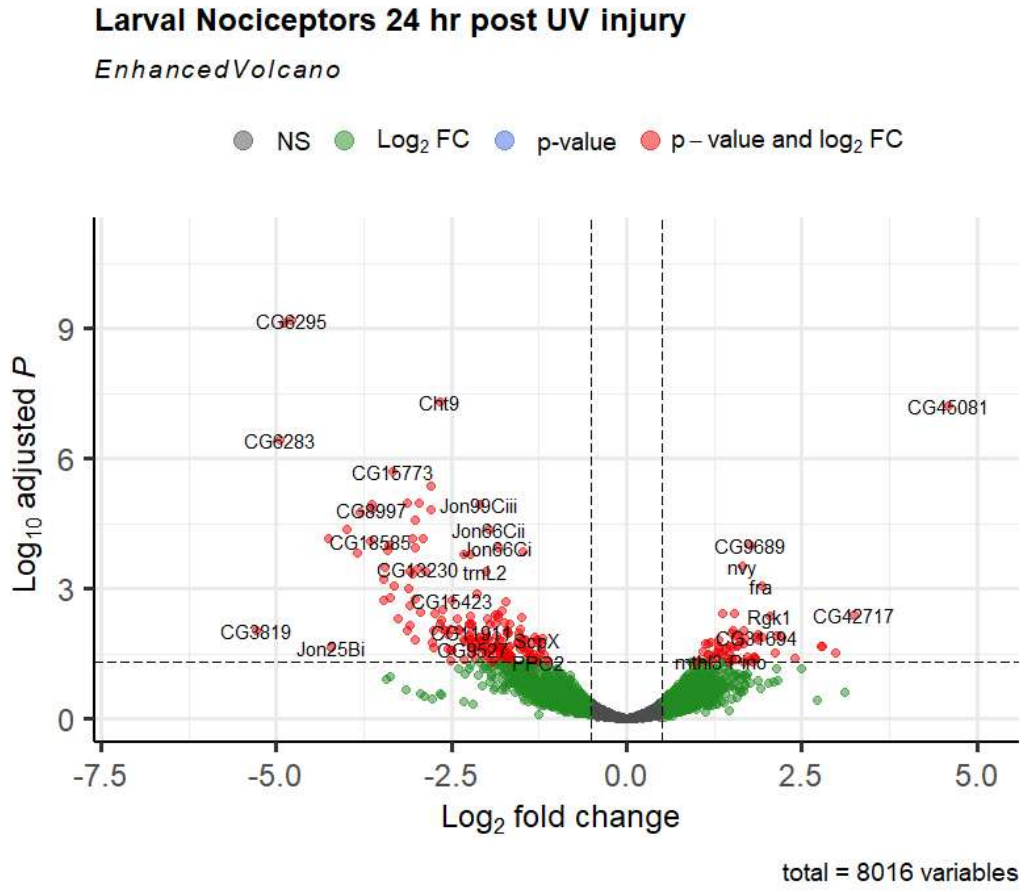


Figure 3.6 Nociceptor translatome 24 hours after UV injury results in differentially expressed genes. **(A)** Enhanced Volcano plot shows differentially expressed genes by adjusted *p* value (log_{10}) and log_2 fold change. Each dot represents a gene. Those genes in red are significant based on the adjusted *p* value cutoff of 0.05 and have a log_2 fold change of at least 0.5. Those genes in green have a log_2 fold change of at least 0.5 but were not found to be significant. **(B)** The top 50 differentially expressed genes in the nociceptor RNA-seq dataset, based on a significant adjusted *p* value of less than 0.05, were clustered by Pearson correlation for rows (genes expression) and columns (samples) data, and displayed using a heatmap. The figure legend shows correlated relative expression colors extrapolated from normalized

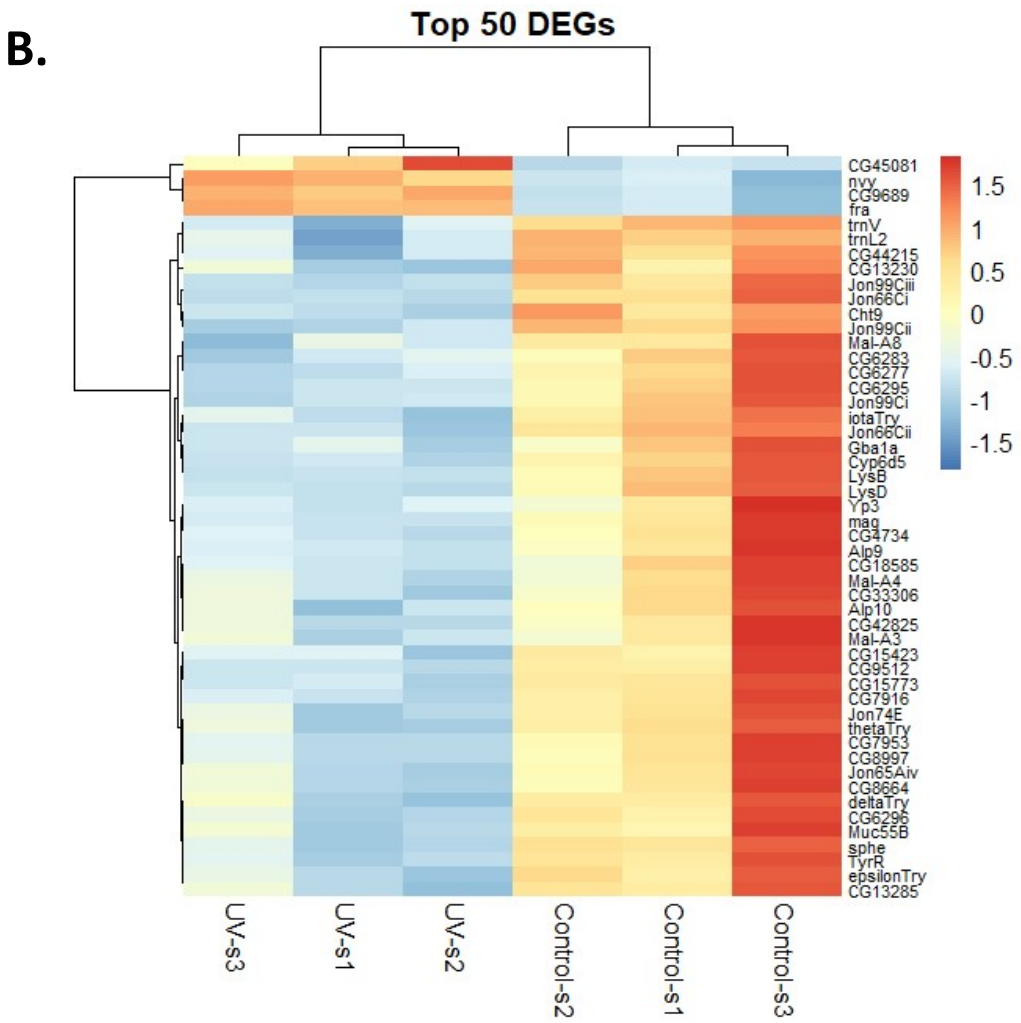


Figure 3.6, continued

log_2 transformed counts data using the DESeq2 "rlog" function. In total, 62 genes were found to be significantly upregulated and 182 genes were found to be significantly downregulated.

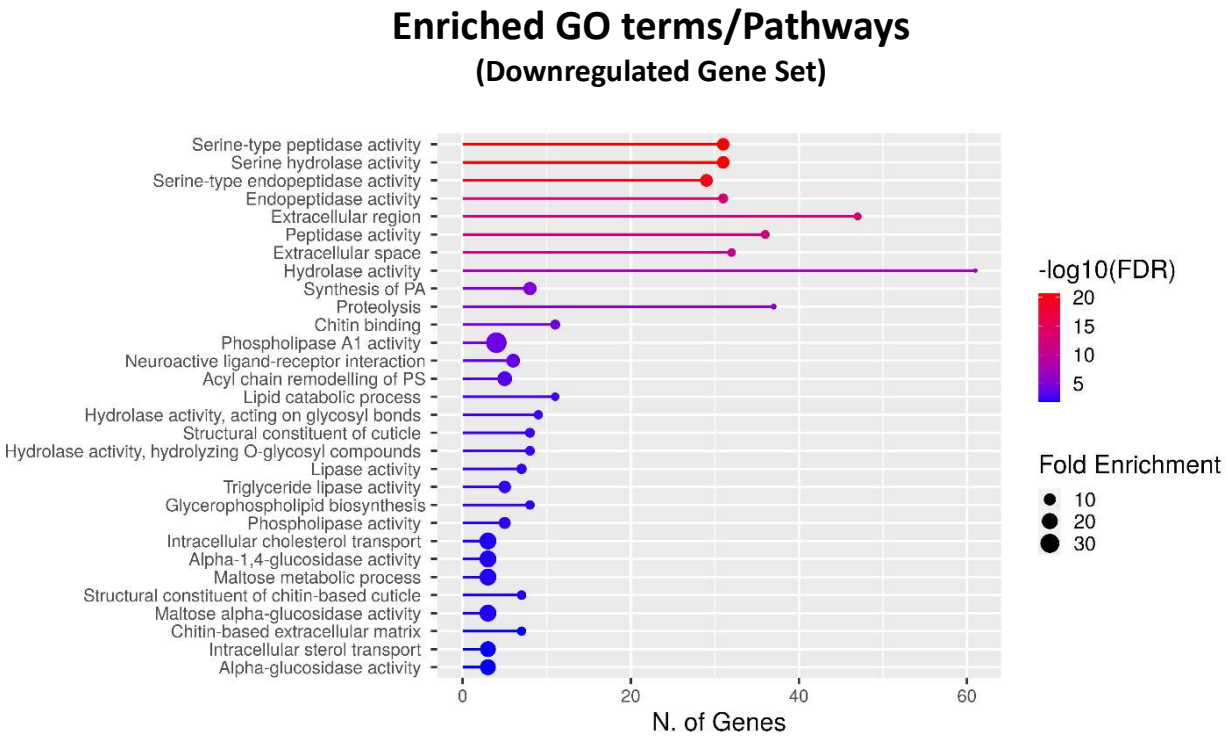


Figure 3.7 Enriched Gene Ontology (GO) terms and pathways found for the genes significantly downregulated in the nociceptor RNA sequencing dataset 24 hours after UV injury.

False discovery rate (FDR) was calculated in ShinyGO and based on nominal p-values from the hypergeometric test. Fold enrichment in ShinyGO was detailed as the percentage of genes in your list belonging to a pathway and then divided by the corresponding percentage in the background (which was a gene list of all genes within the nociceptor that met the pre-cutoff threshold of at least 20 counts for each sample).

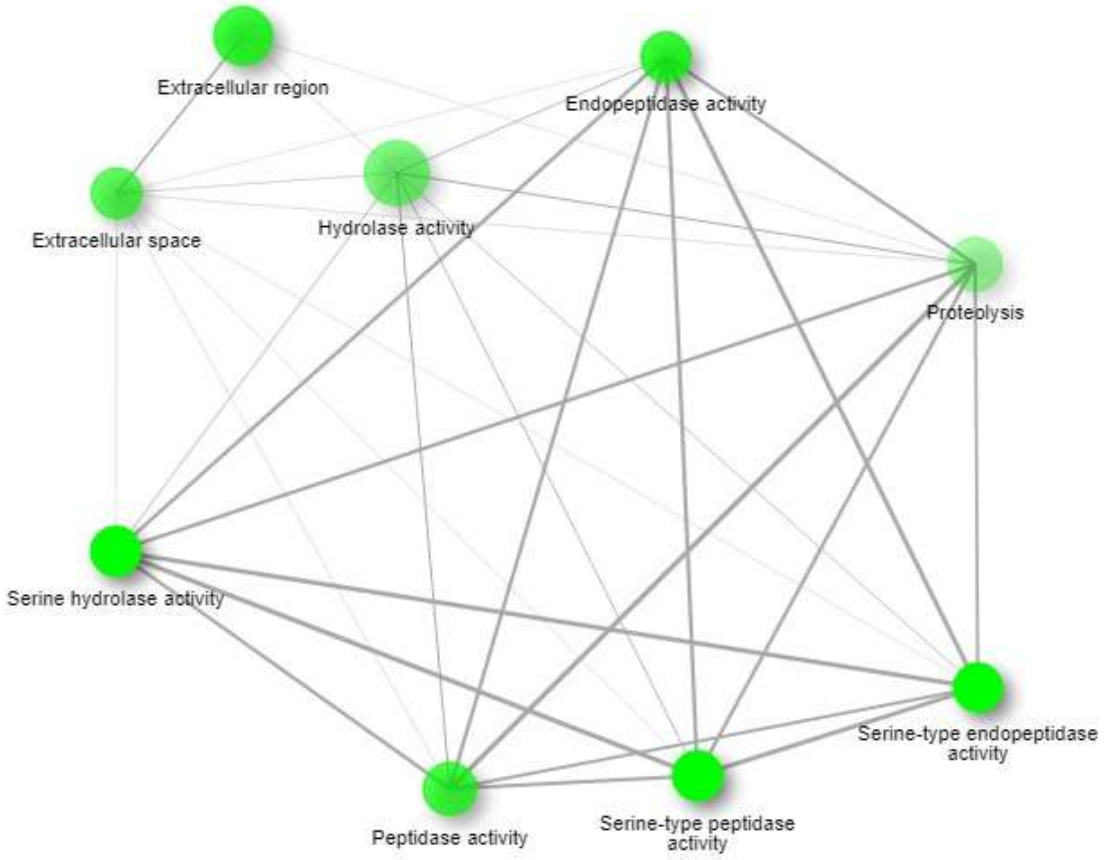


Figure 3.8 ShinyGO interaction network between Gene Ontology (GO) terms and pathways enrichment terms for the genes significantly downregulated in the nociceptor RNA sequencing dataset 24 hours after UV injury.

Each node (circle) is representative of a GO and Pathway enrichment term found within the downregulated gene set and the size of the node is correlated to the size of the gene set (bigger node= bigger gene set) and darker nodes are more significantly enriched. The thickness of the lines connecting the nodes is representative of more overlapped genes between the two enriched terms. Two pathways are connected using this plot in ShinyGO if they share at least 20% (default) of their genes with each other.

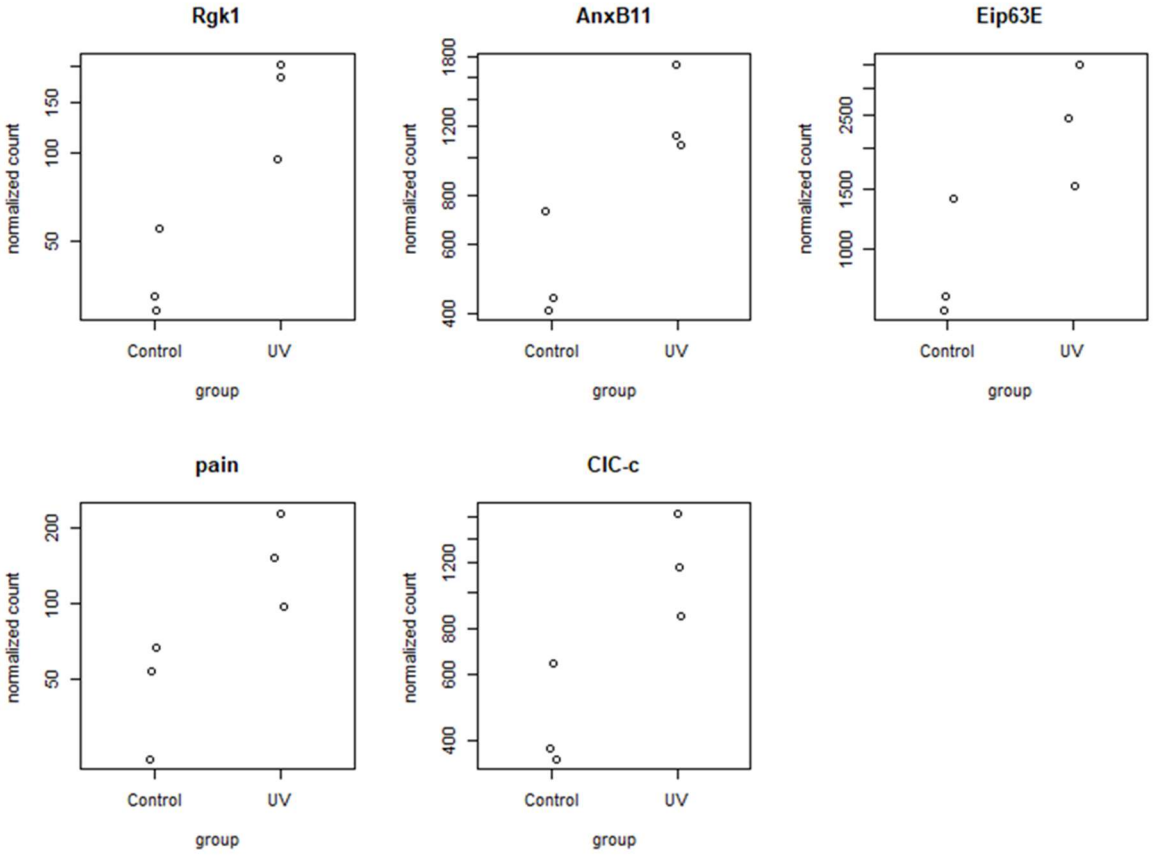


Figure 3.9 DESeq2 output for upregulated genes of interest.

Counts of five upregulated genes of interest within the nociceptor RNA sequencing data 24 hours after UV injury were plotted in DESeq2 using R Studio after applying the "DESeq" function, which in brief controls for library size differences, gene dispersion, and fits to a generalized linear model. The DESeq function is used in preparation to analyze the count data for differential gene expression. Each circle within each plot is representative of the normalized counts for one sample from the associated sample group on the x-axis (Control or UV injured), *n* = 3 samples (pooled)/group (Control vs UV injured).

Table 3.1. Gene Ontology (GO) enriched terms for upregulated genes of interest from nociceptor RNA sequencing data 24 hours post UV injury.

		Flybase ID	Gene Symbol	Gene Ontology ID ~ Annotation	Log_2 FC	Adj *p* value
Plasma Membrane	Ion activity	FBgn0030749	*AnxB11*	GO:0051592~response to calcium ion, GO:0005509~calcium ion binding, GO:0005544~calcium-dependent phospholipid binding	1.320	0.030
		FBgn0264753	*Rgk1*	GO:1901386~negative regulation of voltage-gated calcium channel activity, GO:0005886~plasma membrane, GO:0009898~cytoplasmic side of plasma membrane, GO:0016020~membrane, GO:0098793~presynapse	2.046	0.004
		FBgn0060296	*pain*	GO:0006816~calcium ion transport, GO:0070588~calcium ion transmembrane transport, GO:0005886~plasma membrane, GO:0016021~integral component of membrane, GO:0034704~calcium channel complex	1.733	0.037
		FBgn0036566	*ClC-c*	GO:1902476~chloride transmembrane transport, GO:0005887~integral component of plasma membrane, GO:0016020~membrane, GO:0005247~voltage-gated chloride channel activity, GO:0005254~chloride channel activity, GO:0015108~chloride transmembrane transporter activity	1.402	0.026
		FBgn0005640	*Eip63E*	GO:0090263~positive regulation of canonical Wnt signaling pathway, GO:0005886~plasma membrane	1.429	0.050

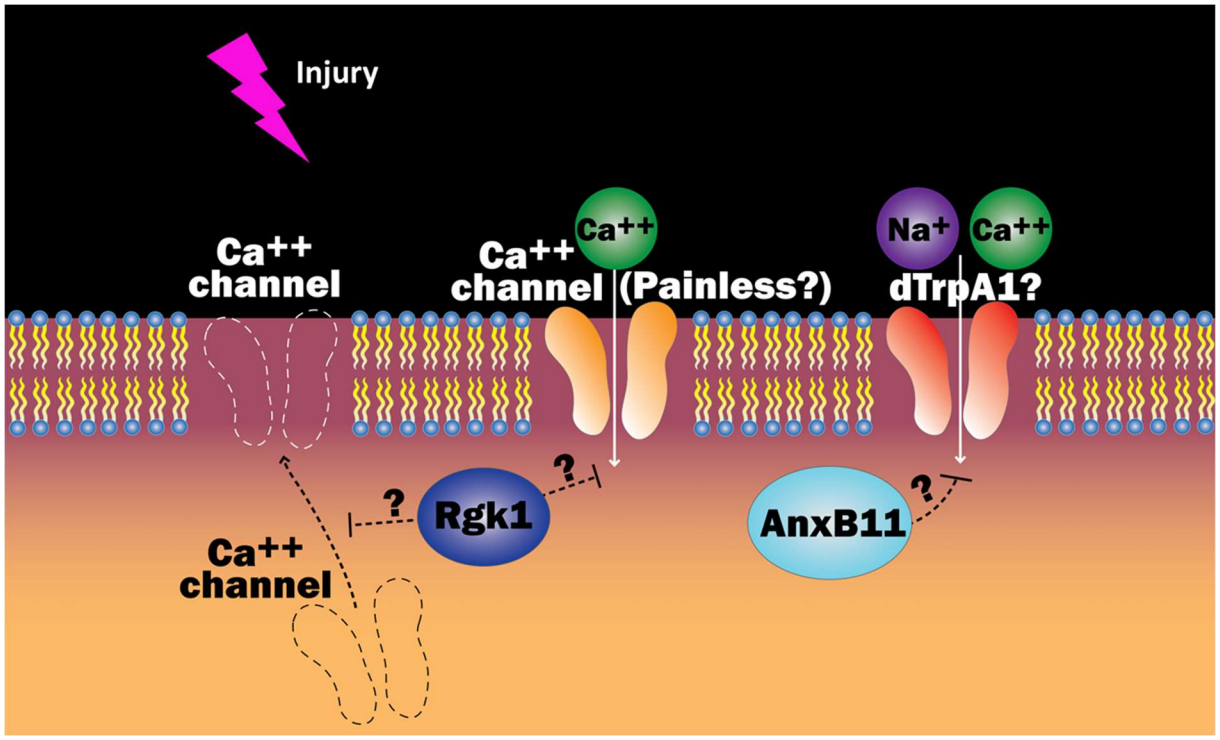


Figure 3.10 Illustration of Rgk1 and AnxB11 hypothetical mechanism of recovery from nociceptive sensitization.

In TRAP-seq analysis data from the nociceptors of UV-injured larvae: *Rgk1* $\log_2$ fold-change= increase of 2.05; *AnxB11* $\log_2$ fold-change= increase of 1.32. Hypothetically (based on mammalian literature of orthologs), Rgk1 blocks distribution of Ca^{++} channels into the nociceptor membrane and/or their action, and AnxB11 blocks the action of the dTrpA1 receptor within the nociceptor membrane. By blocking these channels producing neuronal excitability, the nociceptor is more likely to recover to a more normalized state.

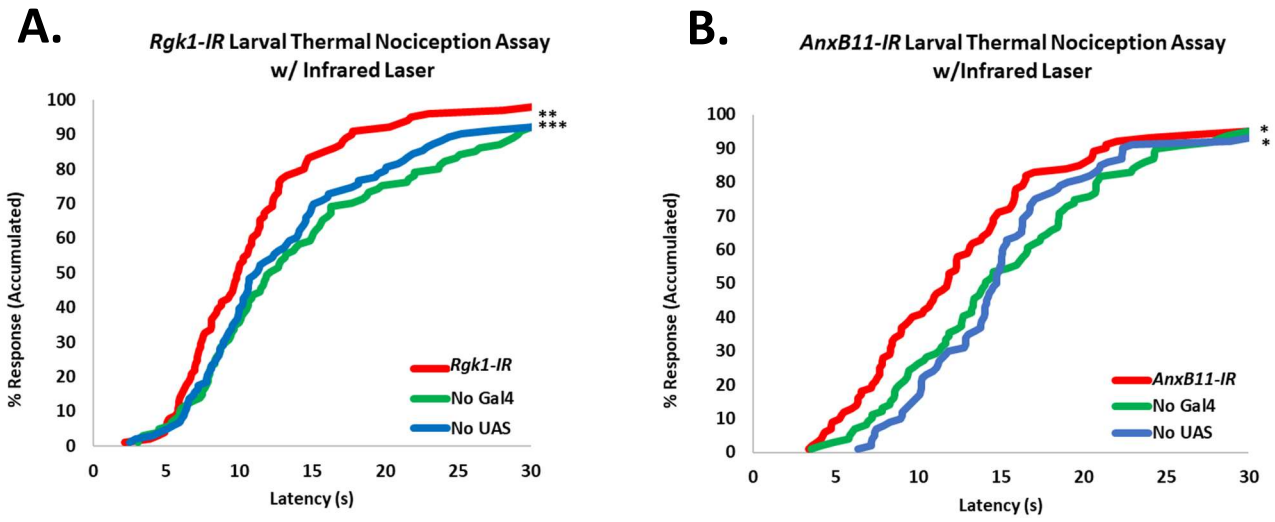


Figure 3.11 Knockdown of Rgk1 and AnxB11 with larval nociceptors results in thermal hypersensitivity.

Percent response plotted against time in larval noxious thermal infrared laser stimulation assays for *Rgk1-IR* (A: *ppk1.9-Gal4 > UAS-Rgk1-IR*), and *AnxB11-IR* (B: *ppk1.9-Gal4 > UAS-AnxB11-IR*) shown in red vs. their controls "No Gal4" (A: w^{1118}> *UAS-Rgk1-IR*, B: w^{1118}> *UAS-AnxB11-IR*) shown in green and "No UAS" (A: *ppk1.9-Gal4 >* y^1v^1, B: *ppk1.9-Gal4 >* y^1v^1) shown in blue, $n \geq 90$/group. Statistical analysis by log-rank test shows significant nociceptive hypersensitivity compared to both controls, ***indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$. All larval nociception data shown was acquired by J. Smestad.

CHAPTER 4

4. INVESTIGATION OF NOCICEPTIVE SENSITIZATION AFTER UV INJURY IN AN ADULT *DROSOPHILA MELANOGASTER* MODEL: ADULT *DROSOPHILA* INFRARED THERMONOCICEPTION ASSAY AND UV INJURY-INDUCED NOCICEPTIVE SENSITIZATION

** The following chapter includes data and text included in preparation of a submission of a primary research article to the Journal of Behavioral Processes (Hale, Pratt, et al., in preparation 2022). The text has been slightly modified for the completeness of this dissertation. C.Hale performed channelrhodopsin experiments, all microscopy imaging, and statistical analyses of all data acquired within this chapter. J. Herbert conducted TNT/TNTi thermonociception assays, and S. Pratt conducted UV injuries followed by subsequent thermonociception assays. Corresponding authors with their affiliations and contributions are also described within the Acknowledgements section of this chapter and referenced within figure legends.*

4.1 Abstract

Nociceptive sensitization underlies and perpetuates chronic pain, a condition that affects ~50 million adults nationwide. With many treatment options for chronic pain, such as opioid analgesics, carrying numerous deleterious side effects, research into safer and more effective treatment options is crucial. Research using *Drosophila melanogaster* larvae has led to the discovery of numerous factors that affect nociceptive sensitivity. However, because the larval stages of fruit fly development are relatively brief, a methodology that allows longer term experimentation in adult fruit flies, for example to study long term effects of nociceptive sensitization after injury, is crucial. Using a thermonociception assay employing infrared diode laser stimulation, we have developed a method in which to harmlessly investigate nociceptive sensitivity in adult flies. We are now using the method to investigate

involvement of nociceptor genes critical to the injury-induced sensitization process, potentially leading to identification of new drug targets useful in the treatment of chronic pain in humans.

4.2 Introduction

Nociceptive sensitization underlies and perpetuates chronic pain, a condition that is estimated to affect ~50 million adults each year in the United States (Yong et al., 2021; Zelaya et al., 2020). With many treatment options for chronic pain, such as opioid analgesics, carrying numerous deleterious side effects (Benyamin et al., 2008), research into safer and more effective treatment options is crucial. Despite this need, successful drug development for chronic pain has been laborious, mostly due to a lack of understanding of the mechanisms of chronic pain. Though little is known about chronic pain mechanisms, research indicates that injury induced nociceptive sensitization may perpetuate chronic pain (Kosek et al., 2021; Nicholas et al., 2019; Reichling & Levine, 2009). Primary nociceptors, specialized sensory neurons within the peripheral nervous system that detect noxious stimuli, are the first responders to the threat of injury (Bessou & Perl, 1969; Gold & Gebhart, 2010). After injury, sensitization of nociceptors can be beneficial by reducing the threshold of activation required to trigger a response. However, if nociceptive sensitization persists after the injury has healed, symptoms of hyperalgesia and allodynia can take root and give way to abnormal pain (Gold & Gebhart, 2010; Kosek et al., 2021; Nicholas et al., 2019; Reichling & Levine, 2009; Scholz et al., 2019). When this type of pain persists/reoccurs for typically three months or more, it is referred to as chronic, and can lead to a substantial decrease in quality of life and an increased threat of opioid addiction (Costanza et al., 2021; Groenewald et al., 2019; International Association for the Study of Pain Task Force on Taxonomy, 1994; Treede et al., 2015, 2019; Vowles et al., 2015).

In recent years, *Drosophila melanogaster* has proven to be an exceptional *in vivo* model organism for investigating the mechanisms of neurological diseases, such as chemotherapy-induced peripheral neuropathy or Parkinson's disease, due to its relative organismal simplicity and powerful genetic toolkit

(Boiko et al., 2017; del Valle Rodríguez et al., 2011; Feany & Bender, 2000). Fruit flies, like their human counterparts, exhibit a behavioral response to noxious stimuli and can develop nociceptive sensitization, allowing for translatable modeling of allodynia and hyperalgesia (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im & Galko, 2012; Im et al., 2015; McParland et al., 2021). The fruit fly system is a desirable *in vivo* model because nociceptors of the fruit fly have been shown to have similar function and morphology to those of vertebrates, and because many genes underlying the perception of pain are conserved across species (Im & Galko, 2012; Khuong & Neely, 2013; Reiter et al., 2001). The majority of pain research using *Drosophila melanogaster* has capitalized on assays performed in the larval life stage, in which an observer measures the latency of the rolling escape behavior in response to noxious stimuli (Figure 1.2A) (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Hwang et al., 2007; Im et al., 2015; McParland et al., 2021; Sulkowski et al., 2011; Tracey Jr et al., 2003). The use of thermal and mechanical nociception behavioral assays using *Drosophila* at this immature life state has brought the rapid identification of genes (Figure 1.4) associated with baseline nociception and/or the nociceptive sensitization process triggered by injury, such as ultraviolet (UV) radiation injury (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Hale, Moulton, et al., 2022; Im et al., 2015; Jang et al., 2018; Lopez-Bellido & Galko, 2020; McParland et al., 2021; Tracey Jr et al., 2003).

Some important pain discoveries translatable to mammals made employing *Drosophila* third instar larvae include the roles of the transient receptor potential (TRP) channel Painless (whose suggested mammalian homolog is ANKTM1 and is analogous in function to TRPV1 (Al-Anzi et al., 2006; Tracey Jr et al., 2003)), and identification of the *Drosophila* DEG/ENaC channel, Pickpocket (Ppk: similar to vertebrate epithelial sodium channel), which is known for sensing and reacting to harsh mechanical stimulation in the fly (Adams et al., 1998; Zhong et al., 2010). Though much has been learned about pain sensitivity using larvae, there are drawbacks to using the *Drosophila* model at this immature life stage, such as the

brevity (2-3 days) of the third instar stage of the *Drosophila* larvae required for most of these assays. The immaturity of the sex organs at the larval life stage could also potentially limit investigation of the role of sex differentiation in pain sensitivity. Due to these considerations, the refinement of adult *Drosophila* nociception behavioral assay methods is desirable and necessary for continued successful contribution to the pain research field.

There have, in fact, been a number of distinct assays developed for studying nociceptive response to a noxious stimulus in adult *Drosophila*. Some paradigms include: measuring adult fly movement, such as jumping, in response to a noxiously heated surface (Figure 1.2B) (Khuong, Wang, et al., 2019; Massingham et al., 2021; Ohashi & Sakai, 2018; Xu et al., 2006), measuring locomotion away from a heated surface (Neely et al., 2010; Neely et al., 2011), measuring adult fly locomotion past a noxious heat barrier (Aldrich et al., 2010; Manev & Dimitrijevic, 2004), and assays that include measurement of latency of an immobilized fly to throw an object when heated by a laser beam (Aldrich et al., 2010; Xu et al., 2006). These published methods for studying adult fly nociception have contributed positively to the field of nociception and nociceptive sensitization research, however, some drawbacks are noted. For methods in which a mobile adult fly navigates among heated and unheated surfaces (Aldrich et al., 2010; Manev & Dimitrijevic, 2004; Neely et al., 2010; Neely et al., 2011), it is not clear what temperature is acutely perceived by the fly as it decides where to move. It is possible that the freely behaving animal rarely if ever encounters the hottest surfaces presented, in which case it is not clear if the effects are due to nociception or thermotaxis. In regard to the method where adult fly jumping behavior in response to a noxiously heated surface in a closed chamber is measured (Figure 1.2B) (Khuong, Wang, et al., 2019; Massingham et al., 2021), it is presumed that thermonociceptors in the legs are most at play, but it has yet to be demonstrated which tissues are important in triggering the escape behavior. In contrast, targeting an infrared laser beam to heat a particular tissue of a restrained fly is an improvement in this regard. In acute laser-based strategies, the fly must be restrained so that it may be accurately targeted by

the beam, and this has previously been accomplished by the use of adhesives (Aldrich et al., 2010; Xu et al., 2006).

Here we present methodology for analyzing adult *Drosophila* nociception that includes both new elements and modifications of existing adult fly nociception assays (Aldrich et al., 2010; Khuong, Wang, et al., 2019; Manev & Dimitrijevic, 2004; Massingham et al., 2021; Neely et al., 2010; Neely et al., 2011; Ohashi & Sakai, 2018; Xu et al., 2006). We coupled the diode laser first reported by Aldrich et al, which is less dangerous and costly than a CO_2 laser previously used, with a novel vacuum immobilization method, which is chemical-free and reversible, in contrast to the use of adhesives (Aldrich et al., 2010; Xu et al., 2006). The narrow laser beam allows targeted acute stimulation of lateral abdominal segments, and the multidendritic, ppk-expressing neurons, which potentially serve as nociceptors, situated directly beneath the lateral abdominal epidermis (Shimono et al., 2009).

We also present a method of triggering injury-induced nociceptive sensitization that is novel in adult flies. It has been previously indicated that leg amputation leads to central sensitization via perturbation of GABA-ergic processing of nociceptive input to the ventral nerve cord of adult flies (Khuong, Wang, et al., 2019). In contrast, we induced nociceptive sensitization in adult flies by using ultraviolet (UV) to injure presumably superficial tissues, allowing direct comparison with peripheral sensitization identified using UV injury of larval flies (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; McParland et al., 2021).

4.3 Materials and methods

4.3.1 Fly husbandry

Flies were obtained from the Bloomington *Drosophila* Stock Center in Bloomington, Indiana and maintained in 6oz stock bottles containing sucrose-cornmeal-yeast medium. Bottles were stored in Percival Scientific incubators at 50-60% humidity and a temperature of 25°C. Fly lines were placed on a

12h light/12h dark cycle except for adult flies that were to be used in optogenetic behavioral experiments, which were placed in continuous darkness. Genotypes were prepared using the Gal4/UAS (Brand & Perrimon, 1993; Duffy, 2002) cell targeting system (Figure 1.3), using *yw;;ppk1.9-Gal4, UAS-mcD8-GFP* line (Figure 4.2), or *w;;ppk1.9-Gal4* (Figure 4.3), both driven by the *pickpocket* (Adams et al., 1998; Ainsley et al., 2003; Hwang et al., 2007; Zhong et al., 2010) promoter. Channelrhodopsin experiments included a No Gal4 control: w^{1118} crossed with the *UAS-ChR2* line. To reduce ppk cell neurotransmission, we used the 'weakly expressing' *UAS-TeTxLC.tnt* (BDSC_28837) and used the inactive *UAS-TeTxLC.(-)Q* (BDSC_28839) as normal control (Sweeney et al., 1995). To allow optogenetic activation of ppk cells, we used *UAS-ChR2* (BDSC_9681) (Nagel et al., 2003; Schroll et al., 2006). To visualize the ppk-expressing neurons, we used *ppk1.9-eGFP*. Flies used in all other behavioral assays were w^{1118} (BDSC_3605).

4.3.2 Ultraviolet C (UVC) irradiation injury

Adult flies of both sexes, aged 15-16 hrs., were lightly anesthetized with carbon dioxide, and arranged on a glass microscope slide with the bodily side to be stimulated by the laser stimulation assay post injury facing the overhead UV source inside of a Spectronic Corporation Spectrolinker XL-1000 ultraviolet crosslinker. Approximately 10 flies at a time were then briefly exposed to 138.7-147.8 mJ of UVC irradiation, measured by a Spectronics Corporation Spectroline XS-254 UVC photometer. Once irradiation was complete, the flies were transferred carefully into sucrose-cornmeal-yeast medium filled stock vials and allowed to recover under general fly husbandry conditions for at least 24 hours prior to performing behavioral assays.

4.3.3 Infrared thermonociception assay

In methods adapted from Xu et al. 2006, adult flies (24-48h post-eclosion) were harmlessly restrained by a vacuum tube (27G hypodermic needle filed blunt, delivering negative pressure of 22 Hg) placed on the anterior-dorsal surface of the thorax (notum) without interfering with movement of head, wings, or legs (Figure 4.1). The fly was positioned with its lateral abdomen in the path of and 3 cm away from a pinhole-restricted (1mm) infrared (808-810nm) diode laser (3V, 300mW) inside a light-tight safety cabinet equipped with an interlock switch that interrupts the circuit if the cabinet is opened. Beam targeting and noxious temperature range was confirmed using thermochromic film. Each fly held a sucrose-coated cotton thread (1x5 mm) with its legs. Thermal stimulation resulted in a presumed fictive jumping behavior such that the string was thrown, as observed using a digital camera (Arducam Lens Board for Raspberry Pi Camera). Latency was measured using a stopwatch. Operators were blinded to treatment and genotype.

4.3.4 Optogenetic blue light stimulation assay

Newly emerged *ppk1.9-Gal4> UAS-ChR2* adult flies and their controls were passed onto sucrose-cornmeal-yeast medium supplemented with 100 uM all trans retinal (ATR: Fisher #18-600-415) and stored under dark conditions for 3 days (+/- 2-3 hours) before carrying out optogenetic blue light stimulation assays. Optogenetic blue light stimulation assays were delivered by the same method and conditions as the previously detailed infrared thermonociception assays, except that a blue light (360-480 nm) laser (with a current of 0.08-0.09 A) was substituted for the infrared diode laser. The operator was blinded to genotype and all assays were performed in a room darkened but weakly illuminated by dim, red light.

4.3.5 Fluorescent immunohistochemistry

Adult *Drosophila* expressing an eGFP transgene under control of the ppk1.9 promoter (*ppk1.9-eGFP*), which, in larvae produces nociceptor-specificity (Hwang et al., 2007), were used 24-48 hours after eclosion, briefly anesthetized with CO_2, and whole abdomens were dissected from the remainder of the animal while being submerged in Grace's Insect Medium (Gibco™) as described previously with modifications (Bailey et al., 2020). Extracted abdomens were then filleted by longitudinally cutting down the dorsal surface of the entire abdomen, leaving the ventral abdominal surface intact. The fillet with the exposed ventral abdomen was then pinned to a silicone (Sylgard) filled dish, and any remaining abdominal contents were removed, leaving the cuticle and muscular wall intact. Filleted abdomen samples were then fixed 30 minutes in 4% PFA at room temperature, washed in PBS containing 0.3% Triton X-100 (wash buffer), and blocked in PBS with 0.3% BSA, 0.3% Triton X-100 (blocking buffer). Alexa Fluor-488 conjugated antibodies to GFP (ThermoFisher, A-21311, final concentration 5ug/ml) mouse anti-fasciclin III (Developmental Studies Hybridoma Bank, 7G10) (Patel et al., 1987), diluted 1:10 in blocking buffer, were incubated with the samples overnight with rocking. After washing in wash buffer, samples were incubated with 5 µL of Alexa Fluor 594 conjugated goat anti-mouse secondary antibodies (Abcam 150116) diluted in blocking buffer (final concentration 10ug/ml) for 3 hours at room temperature with rocking. After removing secondary solution, filets were washed in wash buffer, then PBS, and mounted onto slides using Vectashield Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories) for nuclear staining, oriented with cuticle side toward the coverslip. Slides were kept in the dark at 4°C until imaging on a Leica TCS SP5 scanning laser confocal microscope. Z-stacks were obtained by using a 40x oil objective and a scan format of 1024 x 1024. Z-stacks were max projected and cropped in Fiji (Schindelin et al., 2012).

4.3.6 Statistics

Behavioral assays were performed on samples sizes of $n \geq 179$. Data acquired from behavioral assays was plotted as percent accumulated response vs. latency where an end-point cut-off of 60 s was applied and latency in seconds recorded. After applying a binary variable to the data based on 'response' or 'no response' at the 60 s cut-off time, statistical analysis of latency of response between all behavioral data groups was completed using log-rank analysis, performed using R statistical coding software (R Core Team, 2021) and applying the 'survival' analysis package (Therneau, 2020). All other statistical tests and plots were carried out using Microsoft Excel (version 2104).

4.4 Results

Infrared laser stimulation of the lateral abdomen of a vacuum-restrained adult fly, aged 24-48 hours, holding a segment of cotton string causes a fictive jumping behavior manifested by its throwing of the string (Figure 4.1). The latency of this fictive jump/no fictive jump was recorded during a 60 s timeframe and compared between groups. As a control measure, flies were tested for average latency of this fictive jumping behavior without infrared laser stimulation. Without stimulation, only ~ 7% of adult flies were found to respond with a fictive jump behavior before the 60 s cutoff (data not shown). To demonstrate that the ppk cells of adult flies were necessary for the fictive jump observed, neurotransmission was suppressed within the ppk cells using genetically targeted expression of tetanus toxin. Adult flies were caused to express either a "weakly expressing" tetanus toxin (*UAS- TeTxLC.tnt*) or an inactive form of the same toxin (*UAS- TeTxLC.(-)Q*) as a control, specifically within the *ppk*-expressing cells using the Gal4/UAS system (Figure 1.3) (Brand & Perrimon, 1993; Duffy, 2002; Sweeney et al., 1995). Those flies expressing *UAS- TeTxLC.tnt* within their ppk cells were significantly less likely ($p < 0.01$) to carry out a fictive jump in under 60 s when compared to control (*UAS-TeTxLC.(-)Q*) during the thermonociception assay (Figure 4.2).

To demonstrate that the *ppk*-expressing cells in the adult flies were sufficient to trigger the fictive jump observed, *Chlamydomonas* blue light-activated Channelrhodopsin-2 was expressed (*UAS-ChR2*) specifically within the adult fly nociceptors using the Gal4/UAS system (Brand & Perrimon, 1993; Duffy, 2002; Nagel et al., 2003; Schroll et al., 2006). In contrast to the thermonociception assays which include a targeted infrared laser, adult flies were instead targeted with a blue light laser while held under otherwise dark conditions. Flies expressing *UAS-ChR2* within their ppk cells were observed to carry out the fictive jump significantly more frequently ($p < 0.05$) when compared to their No Gal4 controls (Figure 4.3).

To test for the development of nociceptive hypersensitivity after injury, adult flies aged 16-24 hours were exposed to a defined dose (~700 mJ) of UVC radiation on one side of their body and then allowed 24 hrs. to recover. Flies were then tested for nociceptive sensitization using the infrared thermonociception assay, where latency of the fictive jump was recorded within a 60 s timeframe. UV injured flies were observed to carry out the fictive jump behavior at a significantly lower latency ($p < 0.001$) when compared to sham control animals (Figure 4.4).

4.5 Discussion

Fruit flies, like their human counterparts, exhibit injury-induced nociceptive sensitization and this process has been characterized most widely in a larval thermonociception model (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im & Galko, 2012; Im et al., 2015; McParland et al., 2021). Because the larval stages of fruit fly development are relatively brief, however, a methodology that allows longer term experimentation of nociceptive sensitization after injury in adult fruit flies is desired for investigation of a state more representative of chronic pain. In contrast with a previously reported adult fly injury model involving amputation-induced hypersensitivity (Khuong, Wang, et al., 2019; Massingham et al., 2021), the use of UV injury allows

comparison with the body of literature generated in studies of larvae (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im et al., 2015; McParland et al., 2021).

In the presented nociceptive behavioral assay method, comparable to the Hargreaves assay used in rodents (Deuis et al., 2017; Hargreaves et al., 1988), animals are stimulated by an infrared radiation source, and latency of their escape response, in this case a fictive jump, is recorded. As was employed in a previous report (Aldrich et al., 2010), the infrared diode laser also used here is less expensive and less dangerous than the CO_2 lasers used in other previous studies (Xu et al., 2006). The beam can be restricted by a pinhole aperture, allowing selective acute stimulation of various body parts, such as the abdomen, in contrast with hot-surface methods which presumably heat primarily the legs (Figure 1.2B) (Aldrich et al., 2010; Khuong, Wang, et al., 2019; Manev & Dimitrijevic, 2004; Massingham et al., 2021; Neely et al., 2010; Neely et al., 2011; Ohashi & Sakai, 2018; Xu et al., 2006). This stimulus can be directed toward the lateral abdominal surface of the adult fly, a region tiled with multidendritic neurons, which, in larvae, have been known for decades to detect noxious stimuli (Figure 4.1) (Hwang et al., 2007; Shimono et al., 2009).

The novel use of vacuum pressure on the anterior-dorsal surface of the fly thorax, called the notum, allows effective, reversible restraint, and also allows for sufficient movement of the wings and legs so that the animal may carry out an escape response, in which it throws the cotton string it has been offered and takes fictive flight (Figure 4.1). When the vacuum device used in this method is placed on the notum, only about 7% of adult flies carry out an unstimulated, fictive jump behavior (data not shown). The use of light vacuum is reversible, nonlethal and, in contrast to previous thermonociception assays using adult fly restraint where either a metallic hook (Xu et al., 2006) or a pipette tip (Aldrich et al., 2010) is glued to the head and/or thorax of the fly, requires neither chemicals, recovery period, nor

anesthesia before the assay. Flies restrained in this manner typically "fly" until offered the cotton string, indicating that they are capable of normal behavior.

To demonstrate that the *ppk*-expressing cells of adult flies are necessary for the fictive jump behavior observed in this assay, neurotransmission was suppressed within these cells using a low-activity tetanus toxin (Sweeney et al., 1995), targeted by Gal4/UAS (Brand & Perrimon, 1993; Duffy, 2002). Tetanus toxin is a neurotoxin produced by *Clostridium tetani* and its mechanism of action involves cleaving the synaptic vesicle protein, synaptobrevin, from a complex necessary for excitatory neurotransmission (Link et al., 1992). Prior studies have found that targeted expression of tetanus toxin within nociceptors of *Drosophila* larvae reduces the behavioral response to a noxious heat stimulus (Tracey Jr et al., 2003). Adult flies expressing, within the ppk cells, either a low-activity tetanus toxin (*UAS- TeTxLC.tnt*) or an inactive form of the same toxin (*UAS-TeTxLC.(-)Q*), as a control (Sweeney et al., 1995), were assessed using the infrared laser thermonociception assay. Those flies expressing *UAS-TeTxLC.tnt* within their *ppk*-expressing cells were significantly less likely ($p < 0.01$) to carry out a fictive jump in under 60 s when compared to control (*UAS-TeTxLC.(-)Q*) during the thermonociception assay (Figure 4.2). This demonstrates the necessity of *ppk*-expressing cells in triggering the fictive jump behavior upon stimulation by the infrared laser, suggesting that at least some *ppk*-expressing cells are nociceptors.

To demonstrate that activation of the Ppk cells is sufficient for the fictive jump behavior observed in this assay, Channelrhodopsin-2 was expressed within the Ppk cells specifically (Nagel et al., 2003; Schroll et al., 2006) and flies were stimulated by blue light instead of infrared thermal stimulus. Channelrhodopsin-2 is a light-gated, cation-selective ion channel found in *Chlamydomonas reinhardtii* and it has been shown to elicit action potentials after stimulation with blue light in *Drosophila* neurons that have been in the presence of all-trans retinal (Nagel et al., 2003; Schroll et al., 2006). Flies expressing *UAS-ChR2* within their ppk cells were observed to carry out the fictive jump significantly

more frequently ($p < 0.05$) when compared to the No Gal4 controls after stimulation with blue light (Figure 4.3). This demonstrates the sufficiency of Ppk neurons in triggering fictive jump behavior, again suggesting that at least some adult Ppk cells are nociceptors.

Next, we used the assay to investigate the effects of a treatment known to elevate nociceptive sensitivity in larval flies: cutaneous injury by UV radiation. While an adult *Drosophila* injury model for investigating chronic pain has previously been developed, involving amputation of the right middle leg of the adult fly (Khuong, Wang, et al., 2019; Massingham et al., 2021), it was not previously known if UV injury produces sensitization in adults. In our study, 24 hours after UV injury, flies were observed to carry out the fictive jump in response to thermal stimulation significantly more frequently than sham controls ($p < 0.001$, Figure 4.4). These results suggest that adult *Drosophila* injured by ultraviolet C exposure exhibit behavioral hypersensitivity, as is known to occur in larvae. Applying the UV injury model to adult flies offers the option to build upon the wealth of knowledge already acquired regarding the mechanisms of UV injury-induced nociceptive sensitization in larvae (Babcock et al., 2009; Babcock et al., 2011; Brann et al., 2019; Follansbee et al., 2017; Gjelsvik et al., 2018; Im & Galko, 2012; Im et al., 2015; McParland et al., 2021).

Moving forward, this UV injury model and vacuum-assisted infrared laser thermonociception assay can be used to investigate nociception in adult *Drosophila*, including the involvement of nociceptor genes necessary for the sensitization process after injury, leading to the identification of new drug targets and clarification of mechanisms of normal and abnormal pain.

4.6 Acknowledgements

4.6.1 General acknowledgements

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4.6.2 Acknowledgements in contribution of data acquisition and writing

Contributing authors of the manuscript in preparation (Hale, Pratt, et al., 2022) included within this chapter: Christine Hale [1,2], Samia Pratt [3], Joel Herbert [4], Geoffrey Ganter [1,2]

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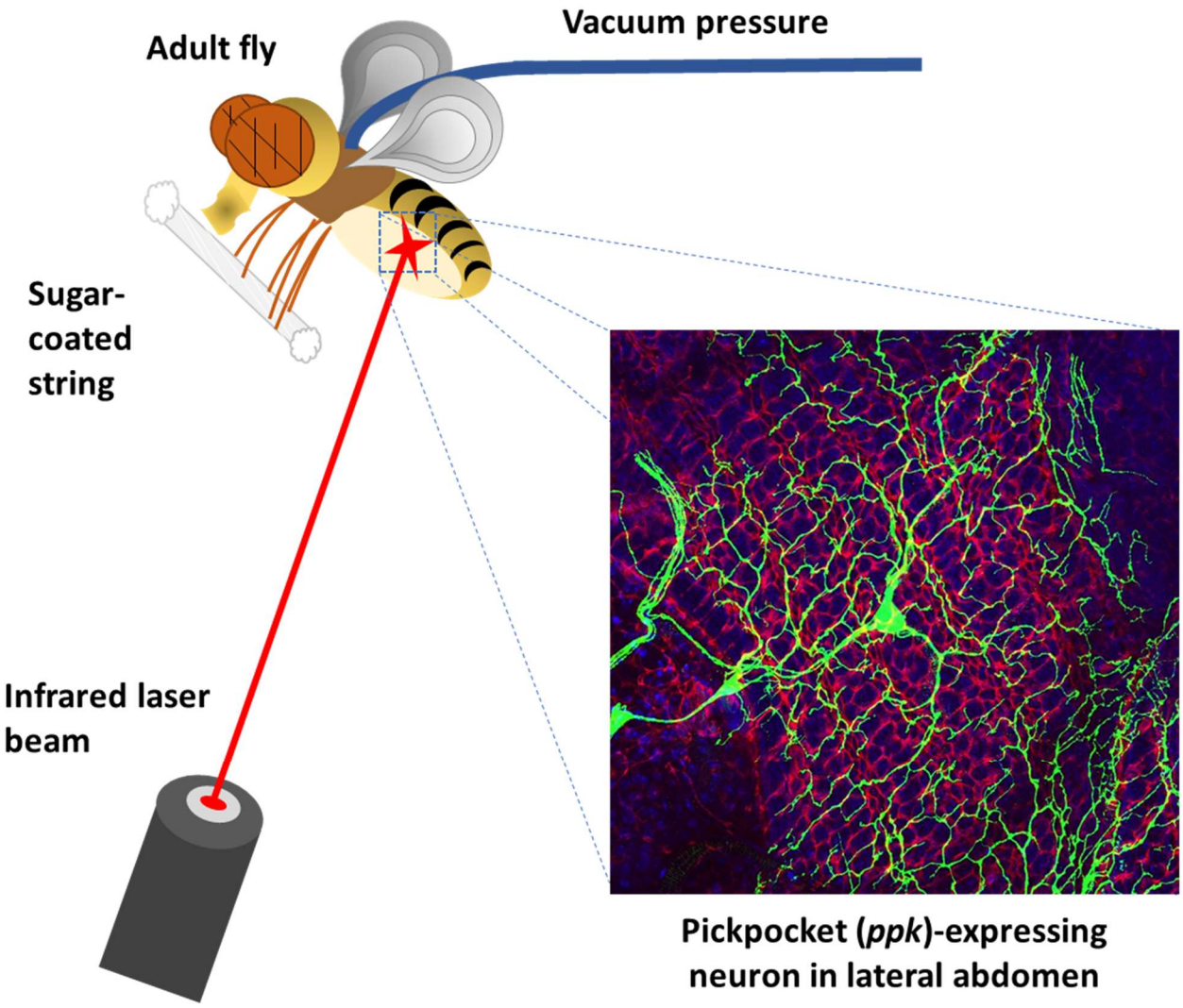

Figure 4.1 Acute thermal nociception assay.

An adult fly is restrained by vacuum provided through a steel tube (top) placed on the notum. The fly holds a sugared cotton string while thermal stimulation via infrared laser aimed at its abdomen heats tissues. A fictive escape jump is triggered, represented by throwing of the cotton string, and the latency of the response recorded. A micrograph of a fly abdomen taken with a 40x objective shows a ppk neuron (green) in the adult lateral abdomen, visualized by *ppk-Gal4 > UAS-mCD8::GFP*. Anti-fasciclin reveals the boundaries of epidermal cells (red) and nuclei are visualized by DAPI (blue).

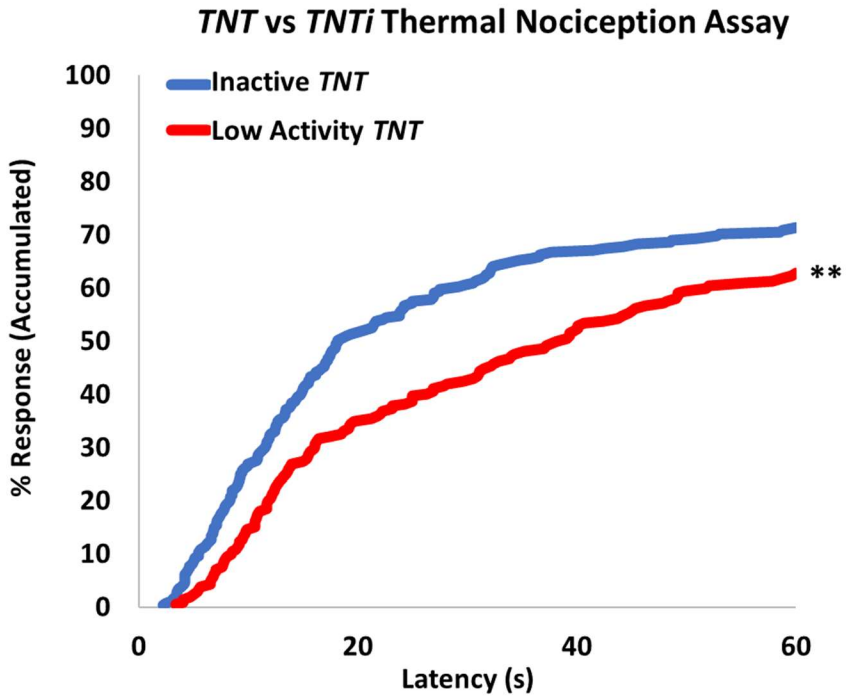

Figure 4.2 Jump response can be inhibited by tetanus toxin.

Adult flies expressing a low-activity tetanus toxin (TNT) (*UAS-TeTxLC.tnt*) within their Ppk cells (shown in red) respond to infrared laser thermal stimulation with significantly less frequent fictive jump behavior when compared to animals expressing the inactive form (shown in blue) of TNT (TNTi) (*UAS-TeTxLC.(-)Q*). $n \geq 212$. ** indicates $p < 0.01$ using log-rank analysis. Data acquired by J. Herbert.

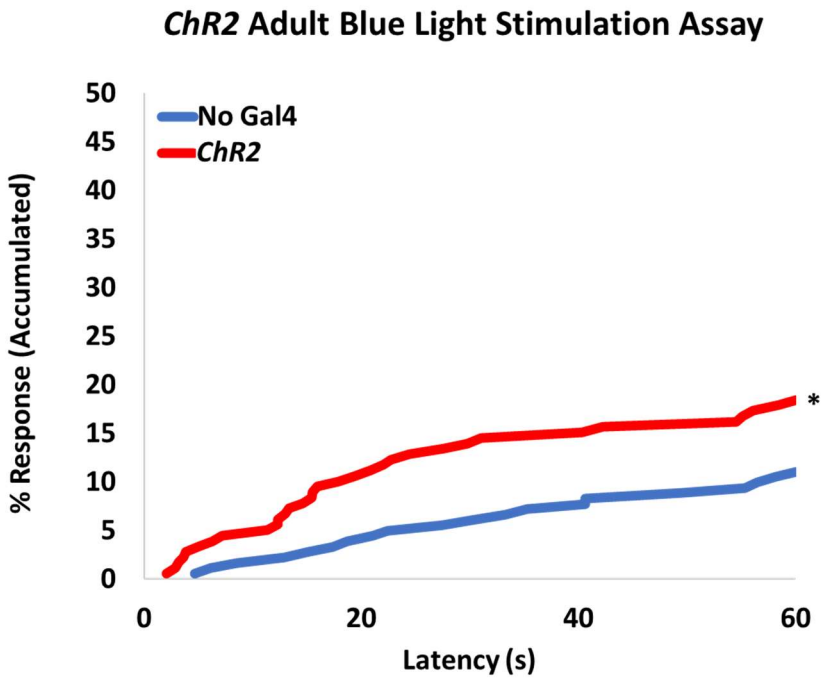

Figure 4.3 Jump response can be activated by Channelrhodopsin.

Adult flies expressing Channelrhodopsin within their ppk cells (*ppk1.9-Gal4 > UAS-ChR2*) (shown in red) respond to blue light stimulation with significantly more frequent fictive jump behavior when compared to their No Gal4 (w^{1118} *x UAS-ChR2*) controls (shown in blue). $n \geq 179$. * indicates $p < 0.05$ using log-rank analysis. Data acquired by C. Hale.

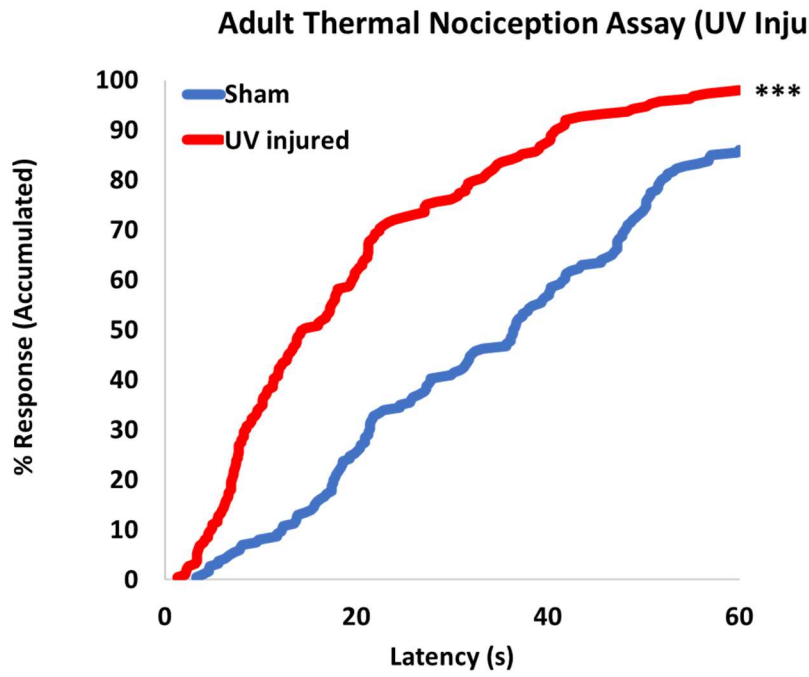

Figure 4.4 Demonstration of injury-induced sensitization

24 hours after UV injury (shown in red), adult flies are significantly hypersensitive to thermal stimulation, as compared to sham treated animals (shown in blue). $n \geq 186$. *** indicates $p < 0.001$ using log-rank analysis. Data acquired by S.Pratt

CHAPTER 5

5. OVERALL DISCUSSION OF FINDINGS

5.1 Brief summary of overall findings

The body of work detailed within this dissertation reveals components involved in nociceptive sensitivity, under various conditions, thereby reflecting the different conditions of the nociceptor (such as with or without injury) where its sensitivity has been implicated to encourage chronic pain pathologies. As stated within chapter 1, the new descriptors and terminology used for treating and discussing chronic pain have recently become more detailed and complex, in an effort to better treat patients suffering from these conditions (Treede et al., 2015, 2019). From these new medical terminology descriptors and guidelines for their use, such as when to determine if chronic pain is neuropathic, primary, nociplastic, etc., there are also varying states of nociceptive sensitivity described (Treede et al., 2015, 2019). These descriptions include whether nociceptive sensitization could be described as injury induced or if the sensitivity of the nociceptor has arisen seemingly without just or known cause. As such, we have sought within this body of work to investigate the various homeostatic mechanisms underlying sensitivity of the nociceptor in different conditions and to progress the field of nociceptive sensitivity research in the fruit fly further by also developing an improved adult model for more chronic investigations. To uncover the discoveries outlined in this dissertation, four different questions have been investigated:

1. What unknown signaling pathways and/or their genetic components may be involved in the regulation of (baseline) nociceptive sensitivity? (chapter 2)
2. What molecular responses in the nociceptor to injury lead to the process of nociceptive sensitization (allodynia) 24 hours after injury? (chapter 3)
3. What molecular responses in the nociceptor are involved in the recovery of nociceptive sensitization (hyperalgesia) after injury? (chapter 3)

4. How can we improve upon existing adult *Drosophila* nociception assays for nociceptor sensitivity investigation in a way which builds upon prior research and allows for more chronic experimentation? (chapter 4)

In response to the first question, in chapter 2 we described our findings on the canonical Wnt/Wg positive regulator, Arm, and showed evidence that Arm is involved in baseline nociceptor sensitivity maintenance without injury (Hale, Moulton, et al., 2022). Our findings on Arm, homologous to mammalian β-catenin, within our larval fruit fly model uncovers a molecular component within the cell that facilitates the somewhat still unknown baseline nociceptor sensitivity mechanism. This finding is a meaningful starting point into the exploration of how this mechanism of baseline nociceptive sensitivity may become dysregulated in humans, especially where injury is not known to preclude the symptoms of hyperalgesia. For our next two questions, which dive into the investigation of molecular responses involved in nociceptive sensitization after injury as well as in responses in recovery after injury, we responded by asking what transcriptional/translational responses occur in the nociceptor 24 hours after UV injury (chapter 3). The investigation of both of these questions within the same experiment was made possible by seizing upon the uniqueness of the larval fruit fly UV injury model and its ability to show both nociceptor sensitization aspects (allodynia) as well as aspects involved in nociceptor sensitization recovery (specifically recovery from hyperalgesia) at one, common time-point (24 hours post UV injury) (Figure 3.1) (Babcock & Galko, 2009; Babcock et al., 2011). Even though prior *Drosophila* research has concluded that UV injury does not change the morphology of the nociceptor after injury (Babcock et al., 2009; Follansbee et al., 2017), our results demonstrated that there are changes occurring in the nociceptor even 24 hours after injury that involve activation/alteration of ion channels and downregulation of proteolysis events within the nociceptor. These transcriptional/translational changes that occur reveal pieces of the molecular response that occurs in the cell during peak allodynia as a result of this seemingly superficial injury. By understanding more of these

transcriptional/translational changes that are occurring within the nociceptor, we can begin to investigate their potential to also become dysregulated after injury and learn how that may lead to chronic pain development. This unbiased effort of investigation was also able to reveal potential hypothetical mechanisms of recovery in the cell through upregulation of components, *Rgk1* and *AnxB11*. These results and hypothesis for their involvement in recovery of hyperalgesia were bolstered further by thermal nociception assays of animals with Rgk1 or AnxB11 protein knockdown displaying hypersensitivity (chapter 3). The molecular responses of the cell to nociceptive sensitization recovery after injury, a process by which the nociceptor returns to a homeostatic state after a period of sensitization, are still not fully understood. Uncovering novel genetic components involved in this process could lead to even more diverse opportunities for drug development and further our knowledge into why pain sometimes persists after injury has healed.

Our final question was one asked due to the great need we have seen in the development of an adult fruit fly nociceptor sensitization model which is affective, safe, and allows the ability to build upon the multitude of prior research carried out in larvae. Though there have been adult fruit fly injury models for nociception investigation by other research teams (Khuong & Neely, 2013; Khuong, Wang, et al., 2019; Manev & Dimitrijevic, 2004; Massingham et al., 2021; Neely et al., 2010; Neely et al., 2011; Ohashi & Sakai, 2018; Xu et al., 2006), our fruit fly nociception model outlined in chapter 4 features several improvements upon those behavioral assay methods and is the first that we know of which also includes a UV injury. By utilizing the UV injury model described in chapter 4, we are able to build upon our prior findings that were uncovered using *Drosophila* larvae and build upon those findings by replicating in a model better equipped for chronic investigation. Our adult UV injury model coupled with a thermonociception assay, using a cost friendly infrared diode laser and harmless fly restraint, gives evidence that it has the potential to be an excellent means of screening genes involved in injury-induced nociceptive sensitization. We hypothesize that its effectiveness in producing injury induced nociceptor

sensitivity that can be applied chronically will prove useful for either individual investigation or in collaboration with mammalian research teams where ethical concerns can sometimes limit sample size and power.

5.2 Future directions and closing statement

In an effort to continue this investigation into nociceptive sensitivity until a more complete mechanism is known, there are several directions that could be built upon the findings of this body of work. The first of these directions is to further delineate the genetic components and pathways involved in baseline nociceptor sensitivity. We have demonstrated through our research on Arm, and continued research into other Wnt/Wg signaling components such as Gish, that *Drosophila* is a useful tool for this investigation, but the full role of how Arm regulates nociceptor sensitivity is not clear. Arm, like its mammalian orthologs, is known to play roles both within the canonical Wnt/Wg signaling pathway and cell-to-cell adhesion (Orsulic & Peifer, 1996). However, Arm is the only known ortholog in the fly, and so both of these actions are carried out by the same protein, unlike in mammals, and we have yet to tease out which role is responsible for the alterations in baseline nociceptor sensitivity we saw in chapter 2 (Huelsken et al., 2000; Huelsken et al., 2001; Miller & Moon, 1997; Simcha et al., 1998). We hypothesize that these dual roles by Arm in the fly will allow for greater ease in further teasing out these questions, in comparison to mammals, as to its particular role in baseline nociceptor maintenance in the future, furthering both fly and mammalian pain research. Future directions in Arm investigation include investigating its role within the canonical Wnt/Wg signaling pathway specifically. A possible course of action in this investigation (besides the investigation of other canonical Wnt/Wg signaling components) could be to knock down Armadillo specifically in the nociceptors of *Drosophila* larvae, which has previously been shown to result in hyposensitivity (chapter 2) and then an attempt to rescue the hyposensitive phenotype with nociceptor expression of mammalian *plakoglobin*. Mammalian

plakoglobin (ortholog to Arm and a paralog to β-catenin) has been shown in previous studies to function in the role of Arm at *Drosophila* cadherin complexes but not for Arm within the canonical Wnt/Wg signaling pathway, and so failure to rescue the hyposensitive phenotype could lead evidence to its role in canonical Wnt/Wg signaling for sensitivity regulation (White et al., 1998).

Another course of action in investigating the other known roles of Arm in nociceptive sensitivity could be to target the catenin-cadherin complexes (which both Arm and β-catenin are both part of) within the nociceptor specifically through cadherin knockdown within the nociceptor, followed by thermal nociception assays for determining sensitivity. Catenin-cadherin complexes are known to be present at synaptic junctions near neurotransmitter release sites (Arikkath & Reichardt, 2008; Uchida et al., 1996) and this could indicate a role for Arm at the synapse site of the *Drosophila* nociceptor when it synapses onto secondary nociceptors within the *Drosophila* ventral nerve cord (ex: secondary neurons such as the DnB neurons) (Figure 5.1). However, there could also be another role of Arm in the cadherin-catenin complex in the cell-cell adhesion of the nociceptors with the surrounding epidermal cells (Figure 5.1). As far as we know from the literature, evidence of Arm within the catenin-cadherin complex between the nociceptors and epidermal cells in *Drosophila* has not been investigated specifically in nociceptive sensitivity regulation, but it has been shown in prior studies that epidermal cells do ensheath nociceptor neurites (similar to recent discoveries on neuronal cells and keratinocyte tunnels in humans (Talagas et al., 2020)) and this ensheathment can play a role in regulating nociceptor sensitivity (Figure 5.1) (Griffin & Thompson, 2008; Jiang et al., 2019). In order to investigate the role of Arm in catenin-cadherin complexes between nociceptors and epidermal cells, we could target Arm and associated cadherins for knockdown in the epidermal cells specifically and evaluate nociceptive sensitivity again through our larval thermonociception assays (and possibly morphology as well).

Another future direction in building upon this research is an investigation of the complete translatomic timeline of events after injury. By gathering evidence in chapter 3 from our nociceptor

specific RNA sequencing data, from animals 24 hours post UV injury, that changes within the cell are occurring at that time-point after injury, it becomes recognizable that the implementation of investigating multiple time-points after injury would be greatly beneficial. By uncovering a fuller timeline of translatomic events in the cell, these results could help to elucidate and piece together the unknown puzzle of how the nociceptor achieves sensitization after injury and fully recovers in both allodynia and hyperalgesia. This knowledge could then contribute toward the expansion of new drug targets but would also provide fundamental knowledge of these vital roles of the cell that are currently unknown.

Finally, another future direction for building upon this dissertation research is exploration into the different nociceptor sensitization molecular responses involved after different types of injuries that lead to chronic pain development. Using our improved adult *Drosophila* thermal nociception assay outlined in chapter 4, forward progress can be made in uncovering genes involved in UV injury-induced nociceptive sensitization in a chronic setting. This tool, however, also opens up the opportunity to test other injuries, such as thermal burn or viral induced neuroinflammation, that could have different molecular mechanisms that lead to chronic pain development and hence warrant different drug targets in treatment.

The compilation of data within this dissertation adds understanding to areas of possible dysregulation of pain known to occur in chronic pain conditions, where nociceptor sensitivity is described as arising in various states (both in the presence of injury and without). It has been made clear through the more detailed terminology and classification recently brought in by the release of ICD-11 on chronic pain (Treede et al., 2015, 2019), that more focused effort is needed in developing better ways to both diagnose and treat different forms of chronic pain. Through investigation within this body of work on baseline nociceptive sensitivity maintenance, nociceptive sensitization after injury, and nociceptive sensitization recovery after injury, we gain more knowledge into some of these specific nuances. This knowledge could in the future lead to better tailored drugs and clinical behavioral/genetic tests for

diagnosing varying forms of chronic pain involving nociceptive sensitivity. Though finding drug alternatives to alleviate the national opioid crisis is of the utmost importance, our investigations into chronic pain development are also of the utmost importance to the societal stigma and individual anguish afflicted on chronic pain sufferers. Every genetic component, signaling pathway, molecular function, cell, and biological process discovered to be a part of the chronic pain process, brings more credibility and understanding of the plight experienced by those affected.

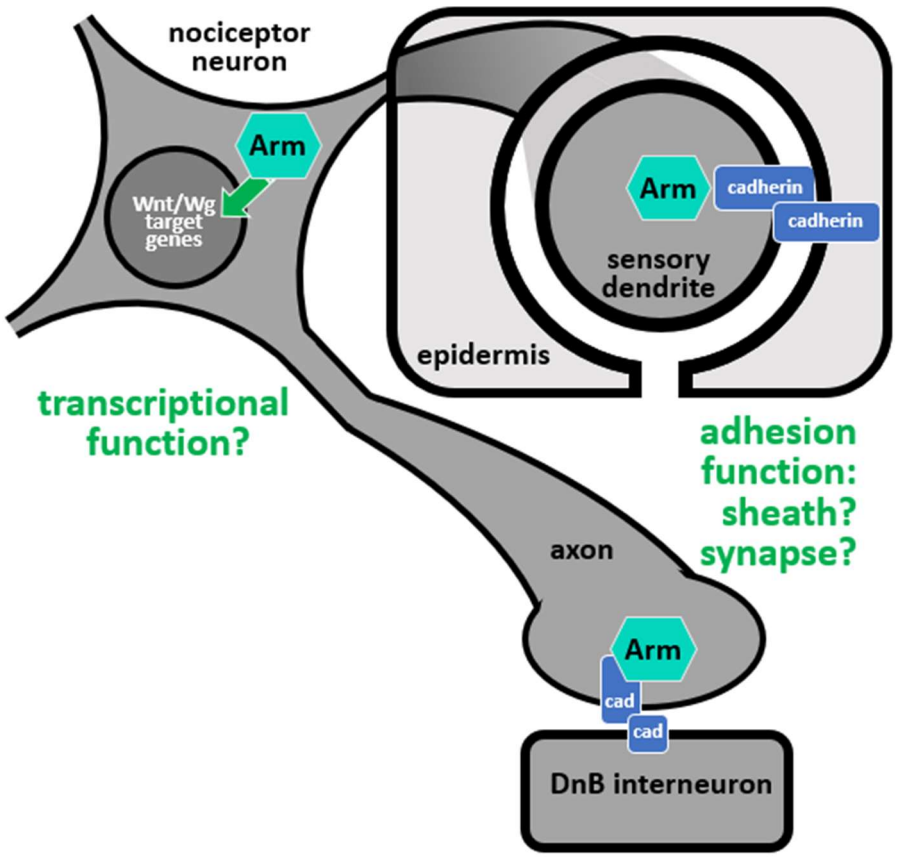

Figure 5.1 Alternative roles for Arm in nociceptive sensitivity regulation

Arm is a moonlighting protein that is known to have more than one function in the cell. One of these functions is as the main component in transcriptional activation of canonical Wnt/Wg signaling target genes. The other function of Arm is its role in the catenin-cadherin complex at adherens junctions of cells. Due to the duality of function of this protein, further investigation into its role either as a canonical

Figure 5.1, continued

Wnt/Wg signaling transducer and/or component at the catenin-cadherin complex within the axon terminal of nociceptors and/or possible role in other cell-cell adhesion complexes such as with epidermal cells, is necessary to deduce its mechanism in nociceptive sensitivity regulation. Graphic by G. Ganter.

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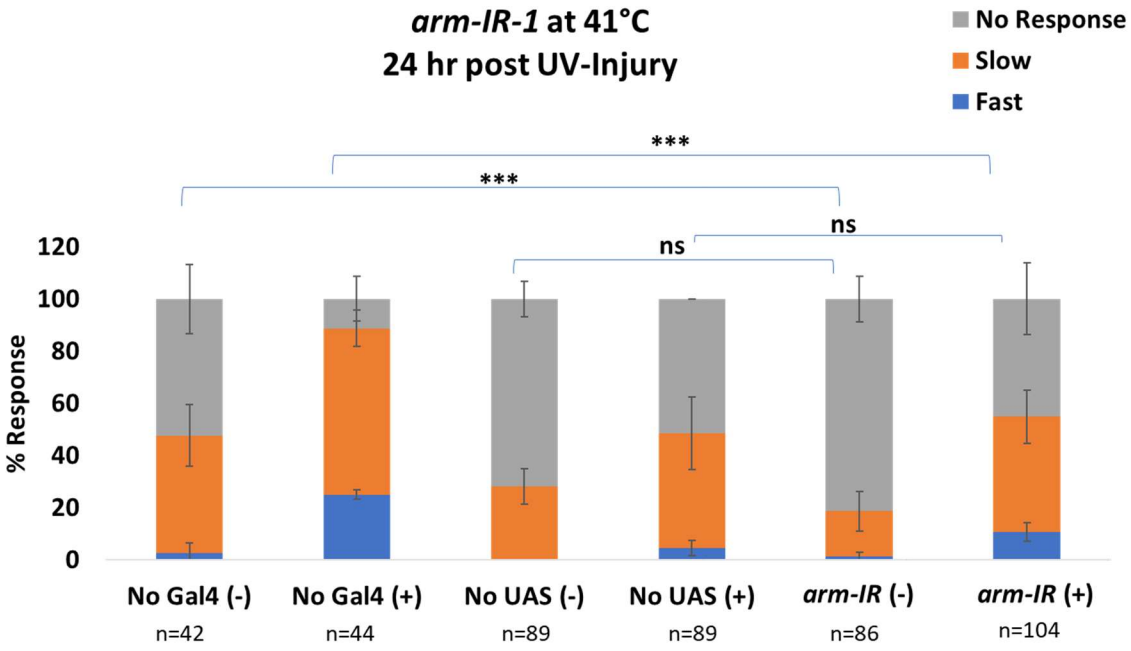
APPENDICES

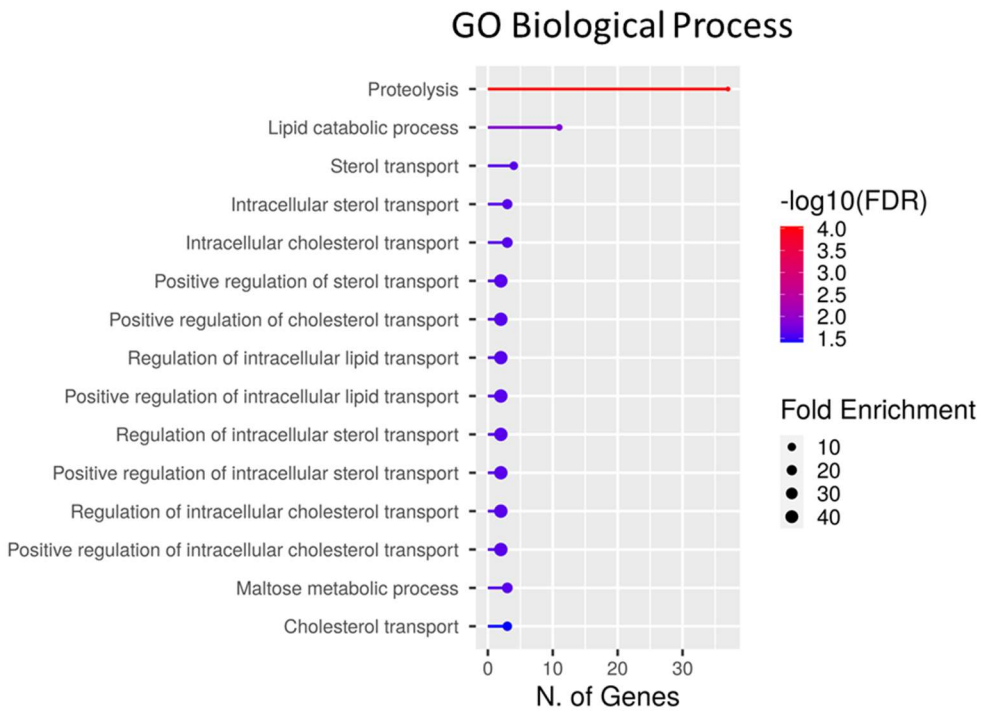
APPENDIX 1



*** indicates $p < 0.001$

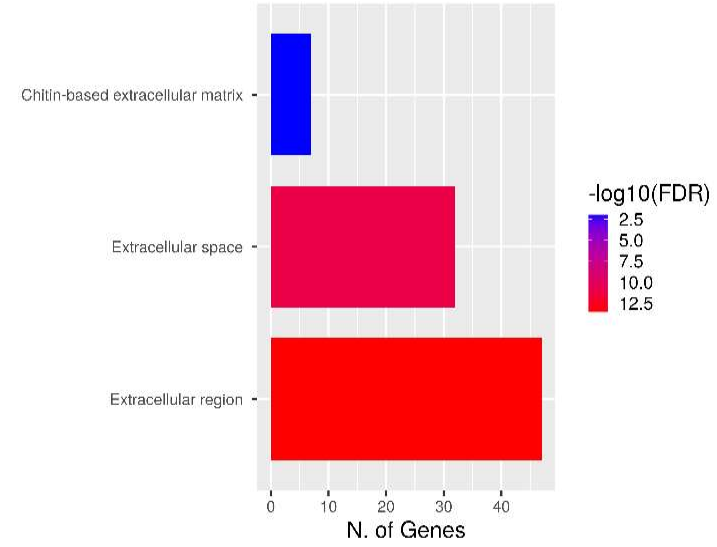
APPENDIX 2


GO Molecular Function
Phospholipase A1 activity
Alpha-1,4-glucosidase activity
Maltose alpha-glucosidase activity
Alpha-glucosidase activity
Alkaline phosphatase activity
Glucosidase activity
Serine-type endopeptidase activity
Serine-type peptidase activity
Serine hydrolase activity
Triglyceride lipase activity
Phospholipase activity
Lipase activity
Chitin binding
Endopeptidase activity
Structural constituent of cuticle
Structural constituent of chitin-based larval cuticle
Hydrolase activity, hydrolyzing O-glycosyl compounds
Structural constituent of chitin-based cuticle
Hydrolase activity, acting on glycosyl bonds
Peptidase activity
Hydrolase activity
Catalytic activity, acting on a protein
Catalytic activity
0
20
40
60
80
N. of Genes
-log10(FDR)
20
15
10
5
Fold Enrichment
10
20
30

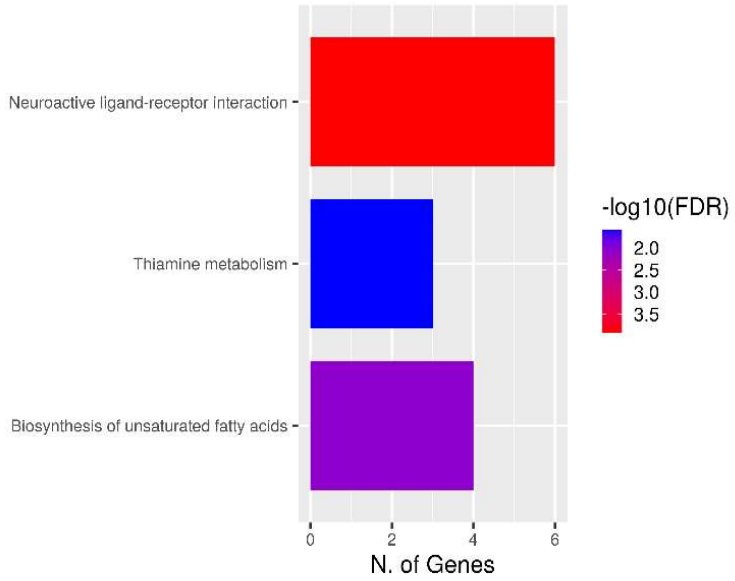

GO Biological Process
Proteolysis
Lipid catabolic process
Sterol transport
Intracellular sterol transport
Intracellular cholesterol transport
Positive regulation of sterol transport
Positive regulation of cholesterol transport
Regulation of intracellular lipid transport
Positive regulation of intracellular lipid transport
Regulation of intracellular sterol transport
Positive regulation of intracellular sterol transport
Regulation of intracellular cholesterol transport
Positive regulation of intracellular cholesterol transport
Maltose metabolic process
Cholesterol transport
0
10
20
30
N. of Genes
-log10(FDR)
4.0
3.5
3.0
2.5
2.0
1.5
Fold Enrichment
10
20
30
40

APPENDIX 3

GO Cellular Compartment



KEGG Pathway



BIOGRAPHY OF THE AUTHOR

Christine Hale was born in Newport News, Virginia where she spent the early years of her life before moving to the beautiful state of Tennessee. It was in Tennessee that she completed her high school degree in December 2002 and then also completed massage therapy school in 2007. After having worked full-time as a licensed massage therapist for many years, a period of time she was very grateful to have spent with clients, learning, and growing as a skilled professional in treating pain relief through massage and bodywork, she decided to go back to school to further her education. She first attended Jackson State Community College for her Associate of Science degree, where she was inspired by her encouraging Organic Chemistry professor, Dr. Karen Carey, to contemplate a life in scientific research. Following graduation in 2015, she continued her education at the University of Tennessee at Chattanooga (UTC) where she obtained a BS degree in Preprofessional Biology with Honors. It was at UTC where she experienced her first role in conducting independent, scientific research in the genetics laboratory of her former mentor and current friend, Dr. Margaret Kovach. She graduated from UTC in the summer of 2017 and within the same month had travelled to Maine to become part of the Graduate School of Biomedical Science and Engineering (GSBSE) program at The University of Maine. She made a comfortable home for herself to diligently explore the questions she had in scientific research in the welcoming laboratory of her current mentor, Dr. Geoff Ganter, at the University of New England. Christine is a candidate for the Doctor of Philosophy degree in Biomedical Science from the University of Maine in August 2022.