

A GENETIC SCREEN FOR WNT SIGNALING FACTORS THAT REGULATE
DROSOPHILA MELANOGASTER NOCICEPTION

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By
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Abstract

A GENETIC SCREEN FOR WNT SIGNALING FACTORS THAT REGULATE *DROSOPHILA MELANOGASTER* NOCICEPTION

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The mechanisms that regulate the transduction of noxious stimuli and generation of appropriate behavioral responses in *Drosophila melanogaster* are not fully understood. In larvae, Class IV multidendritic neurons are highly branched sensory neurons that are responsible for detecting noxious chemical, thermal, or mechanical stimuli and generating appropriate behavioral responses. Recent studies have demonstrated involvement of Wnt signaling in regulating nociception and the development of chronic pain in vertebrate models, but the underlying cellular and molecular mechanisms are still not understood. In order to better understand the roles of Wnt signaling in *Drosophila* nociception, I have selected 53 Wnt signaling-related genes and obtained UAS-RNAi lines for each from the *Drosophila* TRIP collection for an RNAi screen for nociception defects. I have crossed these RNAi lines with flies carrying the Class IV-specific *ppk-GAL4* driver and tested the larvae progeny with a well-established thermal nociception assay. We found the Wnt RNAi behavior phenotypes can be divided into three classes: 1) hypersensitivity to noxious stimuli; 2) insensitivity to noxious stimuli; 3) no change in response to noxious stimuli. These phenotypic categories are

established by comparing the response latencies of RNAi animals to the latency of a transgenic control strain. Candidate genes that produce significant hypersensitivity or insensitivity to thermal stimuli have been identified based on their behavioral phenotypes. I have identified the protein tyrosine kinase 7 homolog, *off track2*, as a promising candidate for further analysis based on its reduced sensitivity to noxious thermal stimuli and previously established role in Wnt signaling. This further analysis consists of 1) genetic confirmation with additional RNAi lines and genetic mutants; 2) gain and loss of function studies and epistasis analysis; 3) functional studies to determine the role of *off-track 2* in Class IV neuron morphological development and electrical activity.

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Introduction

According to the Institute of Medicine (IOM), as many as 100 million Americans (33% of the US population) suffer from chronic pain, with treatment costs ranging from \$500-600 billion annually. In this report the IOM not only lays out the cost of treating chronic pain but also the necessity for improving treatment options and making pain-related research a priority. Treating pain with opioids has caused tremendous issues and the IOM suggests getting away from over usage of opioids to the development of new classes of pain medication. To achieve this our understanding of the genes and the molecular signaling pathways involved in chronic pain states will need to be better understood [1].

Nociception is the process of perceiving noxious stimuli indicating that some aspect of an organism's environment is dangerous [3]. Nociceptors are the peripheral sensory neurons that detect noxious stimuli, and the activation of nociceptors can lead to the perception of pain and behavioral responses [2,3]. *Drosophila melanogaster* larvae exhibit a corkscrew roll along the midline of the body when exposed to a noxious stimulus, and this behavior is termed nocifensive escape locomotion (NEL). This behavior provides a quantifiable trait that allows researchers to measure responses noxious stimuli as well as to ask questions about the sensitivity of nociceptor neurons [4,5].

NEL is caused by the activation of the Class IV neurons. The Class IV multidendritic neurons are nociceptors in *Drosophila* larvae [6]. Class IV multidendritic neurons are highly branched and have extensive dendrite arborization [7]. The requirement of Class IV multidendritic neurons' activation during NEL suggests that they are the primary nociceptors and once activated are sufficient to induce NEL responses. Varying stimuli can activate Class IV neurons, such as noxious heat, noxious mechanical, noxious chemical, and UV light stimuli.

A large portion of genes with a known role in the development and function of the *Drosophila* nervous system are conserved in vertebrates [8]. For example, Class IV multidendritic neurons express temperature-sensitive transient receptor potential (TRP) channels that are necessary for nociceptor function [1]. This makes *Drosophila* a good model organism to study nociception and the development of chronic pain [8]. TRPA1 and Painless are required for NEL responses to noxious thermal or mechanical stimuli [1]. Loss of gene function through RNAi knockdown in nociceptors can lead to altering behavioral responses to noxious stimuli [1].

TRPA1 is responsible for detecting noxious stimuli and producing a behavioral phenotype

Drosophila melanogaster TRP channel TRPA1 is activated by elevated temperatures and promotes heat avoidance in both larvae and the adult flies [9,10]. When larvae are placed on a plate with a gradient of temperatures that range from optimal temperature (24 °C) to temperatures as extreme as 35 °C, the wild-type larvae rapidly migrate down the thermal gradient into the cooler, or optimal temperature [10]. To further explore TRPA1's role in heat avoidance, double stranded RNAs (dsRNAs) were injected into fly embryos to induce a knockdown of *TRPA1*. The progeny from these flies were then presented with the same assay and were analyzed for their ability to perform heat avoidance [10]. The larvae that had limited or no function of TRPA1 traveled into the warmer temperatures without eventually making their way to the optimal or cooler temperatures [10]. Thus, TRPA1 is required for heat avoidance and the selection of an optimal environment [10]. This behavior is called thermotaxis.

Not only does TRPA1 play a role in thermotaxis but it also plays a role in *Drosophila* nociception [8]. *TRPA1* mutant *Drosophila* larvae were tested using the thermal nociception assay at 46°C, and the amount of time to perform an NEL was recorded [8]. Many *TRPA1*

mutant larvae failed to initiate the NEL behavior within ten seconds, while the wild-type larvae initiate behavior within three seconds [8]. To test whether TRPA1 is required in nociceptors for thermal nociception, *pickpocket-Gal4*, a nociceptor specific driver, was used to express *TRPA1* RNAi in Class IV neurons of *Drosophila* [8]. These larvae showed significantly reduced responses to noxious thermal temperatures. These results show that TRPA1 is required for nociception and acts in the nociceptors themselves [8].

TRPA1 is conserved across *Drosophila* and mammals and plays a similar role in nociceptors and nociception. Not only does TRPA1 play a role in thermal nociception, it also plays a role in mechanical nociception. When *TRPA1* mutant mice were presented with mechanical stimulus they were less sensitive to the mechanical stimulus than wild-type mice. The mechanical sensitivity was determined by probing the plantar surface of the left hind paw with Von Frey filaments [11]. The sensitivity of the mice was determined as either the measurement of the frequency of responses to forces between 0.04 and 2 g [11]. At higher forces *TRPA1* mutant mice responded half as often as wild-type mice. These results suggest that TRPA1 plays a critical role in detecting specifically mechanical stimuli.

In *Drosophila* sensory neurons, *painless* is essential for thermal nociception

Painless encodes a transient receptor potential ion channel. When *painless* mutants were tested at a temperature of 46°C they produced a defective NEL response [4]. Some of the *painless* mutants failed to perform the behavior even after ten seconds [4]. When presented with a higher temperature, 52°C, *painless* mutants elicited a rapid response, similar to wild-type larvae [4]. This suggests that the role of *painless* in nociception is not due to motor system defects but a defect at the sensory level [4]. To determine the expression pattern of *painless* in

the sensory neurons of *Drosophila* whole-mount in situ hybridization was performed on *Drosophila* embryos [4]. The expression of *painless* was found to be in a small cluster of cells in the central nervous system, but more importantly it was found in the multidendritic neurons of the peripheral nervous system [4]. *Painless* expression in nociceptors suggests that it is required for nociception.

TRPA1 mediates inflammatory response to noxious stimuli

Tissue damage caused by inflammation can lead to increased or decreased sensitivity of nociceptors. TRPA1 is required for nociceptors to detect noxious irritants produced from inflammation [12]. TRPA1 is an important component of the transduction machinery in which environmental irritants and endogenous proalgesic agents depolarize nociceptors to elicit inflammatory pain [12]. TRPA1 channels can be activated by isothiocyanate and thiosulfinate, which constitute the pungent ingredients of mustard oil and garlic, respectively [13,14]. Wild-type neurons containing TRPA1 exhibited an increase of sensitivity when exposed to mustard oil or allicin, the major component in garlic [12]. Neurons of *TRPA1* mutant mice were completely insensitive to mustard oil or allicin elucidating that TRPA1 is the site of action for both isothiocyanate and thiosulfinate [12]. Topical application of mustard oil to the hindpaw of a mouse produces an acute noxious response followed by neurogenic inflammation and increased sensitivity to thermal and mechanical stimuli. These results demonstrate that TRPA1 is responsible for irritant induced inflammation and increased sensitivity. Dorsal root ganglia cultures prepared from *TRPA1* +/- mice that are heterozygous for a *TRPA1* knockout, showed one-third as many mustard oil-sensitive neurons as wild-type cultures of TRPA1 [12]. These neurons generally had smaller responses to mustard oil as compared to wild-type mice [12].

TRPA1 deficient mice show no obvious deficits in capsaicin, menthol, or calcium induced sensitivity [12]. Importantly, functional ablation of the *TRPA1* gene leads to selective sensitivity without affecting cell type specification or sensitivity to other chemical stimuli [12].

Bradykinin in tissue damage and inflammation

Bradykinin (BK) is a peptide that is an inflammatory mediator and can activate TRPA1 [14]. BK receptor B2 is expressed in a subpopulation of capsaicin-responsive nociceptors where TRPA1 is present, suggesting that TRPA1 and B2 are functionally coupled [14,15]. Whole-cell recordings of CHO cells expressing TRPA1 and transfected with B₂ receptor show acute responses to the application of BK [14]. BK activated responses seen in CHO cells is due to TRPA1 activation. BK is known to activate phospholipase C (PLC), a modulator of TRP channel function [15]. Once PLC is activated, it breaks down phosphatidylinositol-4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol triphosphate (IP₃) that binds to the endoplasmic reticulum [14]. IP₃ then releases calcium from internal stores and DAG activates PKC [14]. OAG, a membrane-permeable DAG analog, activates TRPA1-expressing cells [14]. The ability BK to activate PLC and produce DAG and IP₃ from PIP₂, causing a release of calcium, shows that TRPA1 can be activated by BK and is coupled to B₂.

Capsaicin and TRPV1 in peripheral pain development

TRPV1 is an evolutionarily conserved ion channel that is part of the same super family of TRP channels as TRPA1. Both noxious chemicals and noxious heat activate TRPV1. Capsaicin, a phenolic compound, is found in chili peppers and is responsible for their burning and irritant effect [16]. Capsaicin selectively stimulates nociceptors during inflammation and has widely

been used to study pain [16]. The injection of capsaicin induces nociceptor hypersensitivity to both noxious and innocuous stimuli in rats [17]. Once presented with capsaicin, nociceptors become excited by increasing the influx of calcium ions through the transient receptor potential vanilloid receptor (TRPV1) [17]. The activation of TRPV1 can induce pain-like behavior [16]. TRPV1 is part of the TRP family and localized in the peripheral nervous system [18]. TRPV1 is a cation channel expressed in primary sensory neurons and can be activated by vanilloid compounds or heat [19]. Mice lacking *TRPV1* showed an impaired response to noxious heat, and showed thermal hypersensitivity in the setting of inflammation [19].

Substance P in central and peripheral sensitization

Substance P (SP) is an important neuropeptide in nociception that is produced in both peripheral and central nociceptors [20,21]. There are four major receptors for SP and they belong to the G protein-coupled receptor family (GPCRs) [21]. SP has the highest affinity for both the NK1 and substance p receptors (SPR) [21,22].

When SP binds to SPRs located on primary afferent neurons, the sensitization of those neurons occurs. SPR internalization serves as a marker in primary afferent neuron input to acute and persistent pain states [22]. In order for SP to play a role in central and peripheral sensitization, spinal neurons must express SPRs [23]. In rats pretreated with a cytotoxin conjugate of SP, SPR immunoreactivity was observed on cell bodies and dendrites in lamina I neurons [23]. These animals exhibited a decrease in SPR-expressing neurons as compared to animals pretreated with saline [23]. Although nociceptive neurons in animals pretreated with either SP-SAP, a conjugate of SP with the ribosome-inactivating toxin, saporin, or saline exhibited similar degree of excitation, sensitization did not occur in rats pretreated with the SP

conjugate [23]. Complete Freund's adjuvant (CFA) was injected into the hind paw and produced local inflammation, thermal hyperalgesia, and mechanical allodynia [24]. The SP conjugate significantly attenuated thermal and mechanical hyperalgesia as well as nocifensive behaviors produced by capsaicin injection [25]. These results suggest that SPR internalization is essential to the development of central sensitization.

Overview of Wnt signaling

In order to get a better understanding of nociceptor neuron sensitization, cell-signaling pathways must be researched. Wnt signaling plays a major role in the development of the central and peripheral nervous system, in the maintenance of nociceptors, and potentially in the development of chronic pain.

Wnts are cysteine-rich extracellular secreted ligands that play an essential role in various aspects of development and physiological processes [26,27]. In mammals, there are 19 Wnt genes encoding Wnt ligands, each controlling various cellular functions: proliferation, differentiation, cell death, migration, polarity, but most importantly Wnt signaling plays a role in the development and function of the nervous system. The Wnt ligands regulate at least three distinct intracellular signaling pathways: Wnt β -catenin signaling pathway, planar cell polarity (PCP) Wnt signaling pathway, and Wnt calcium signaling pathway. Although these three Wnt signaling pathways have some similarity in the ligands and receptors present, they also have distinct differences in the downstream effectors and also their cellular outcomes. This allows the three Wnt signaling pathways to have differences in the roles they play in nervous system development and function. While Wnt signaling role in development have been studied extensively, the roles in mature neurons are not completely understood [28].

The role of Wnt signaling in the function of nociceptors suggests evidence that Wnt signaling has roles in the pathogenesis of chronic pain [47]. A rat nerve injury model where the sciatic nerve is pinched or cut entirely, shows the accumulation of β -catenin in peripheral neurons. This increase in β -catenin stimulates the biosynthesis and release of substance P, which plays a significant role in pain signaling transmission and the activation of the canonical Wnt signaling pathway [29]. Wnt signaling has also been shown to play a critical role in neuropathic pain after sciatic nerve injury and bone cancer in rats [30]. Wnt signaling stimulates the production of proinflammatory cytokines IL-18 and TNF- α . These cytokines then regulate glutamate receptors and calcium-dependent signaling through Wnt signaling pathways. This suggests that Wnt signaling pathways could be a possible target for treating neuropathic pain and for pain related to bone cancer. Previous research found important roles for TRPA1, TRPV1, and other signaling mechanisms that can potentially activate or deactivate Wnt signaling pathways [14,15,16,17,19]. This can lead to improper nociceptor function and the development of chronic pain states. The previous studies cited have found differing and conflicting roles of specific Wnt signaling pathways in different forms of nociception. I have thus designed my project to determine which pathway or pathways are responsible for the development of chronic pain by systematically testing the roles of the three Wnt signaling pathways in the same experimental paradigm.

Wnt β -catenin signaling pathway

The Wnt β -catenin signaling pathway was named after β -catenin, a cadherin-binding protein involved in cell-to-cell adhesion, now known to function as a mediator of Wnt signaling pathways. The β -catenin pathway is activated through the binding of a Wnt ligand to its

appropriate Frizzled receptor (a G protein-coupled receptor) and co-receptor lipoprotein-related protein 5 and 6 (LRP5/6). LRP5/6 acts through Dishevelled (Dvl) to switch off the destruction complex, which halts phosphorylation of β -catenin (Figure 1) [31]. In the absence of Wnt, β -catenin levels are low because it is ubiquitinated and degraded in the proteasome [31]. Axin, a scaffolding protein, forms a complex with glycogen synthase kinase 3 (GSK3), β -catenin, adenomatous polyposis coli (APC), and casein kinase 1 α (CK1 α). β -catenin is then phosphorylated by CK1 α and GSK-3 and ubiquitinated in the destruction complex. This leads to proteasomal degradation of β -catenin and no gene expression (Figure 2) [32,33].

When Wnt is present and binds to the appropriate Frizzled receptor and co-receptor LRP5/6 and the destruction complex, Axin, APC, GSK-3, and CK1 α , is phosphorylated and becomes inactivated. This allows the levels of intracellular β -catenin to increase. This increase allows β -catenin to make its way into the nucleus, where it associates with transcriptional coactivators such as CBP (CREB-binding protein) where it can then bind to Tcf/LEF (T-cell factor/lymphoid enhancer-binding protein). Once bound to Tcf/LEF, β -catenin displaces Groucho to allow for the transcription of target genes [34,35].

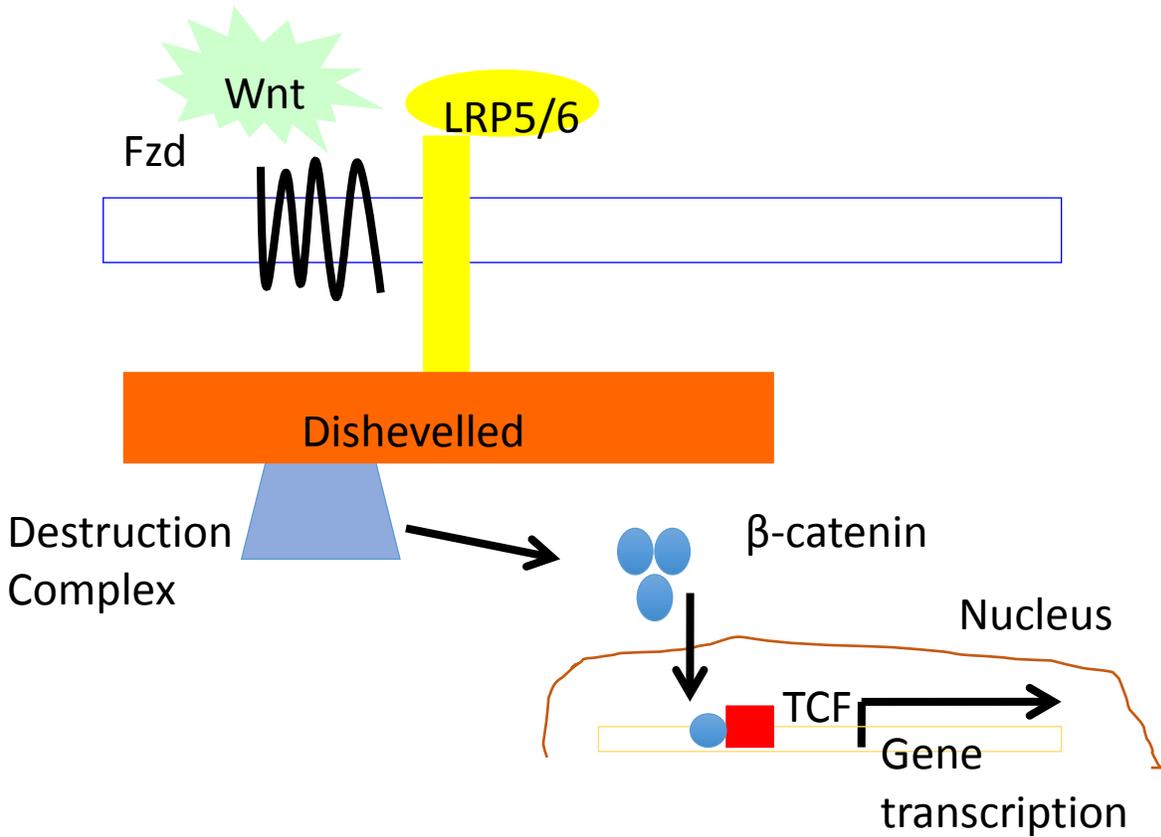


Figure 1. Activated Wnt β -catenin Wnt signaling pathway (21). When a Wnt ligand binds to Frizzled receptor and LRP5/6 co-receptor induces the phosphorylation of the co-receptor. This creates a binding site for Axin, leading to the disruption of the destruction complex. This allows β -catenin to accumulate and make its way into the nucleus and associate with the transcription factors causing the transcription repressors to dislodge and promote transcription.

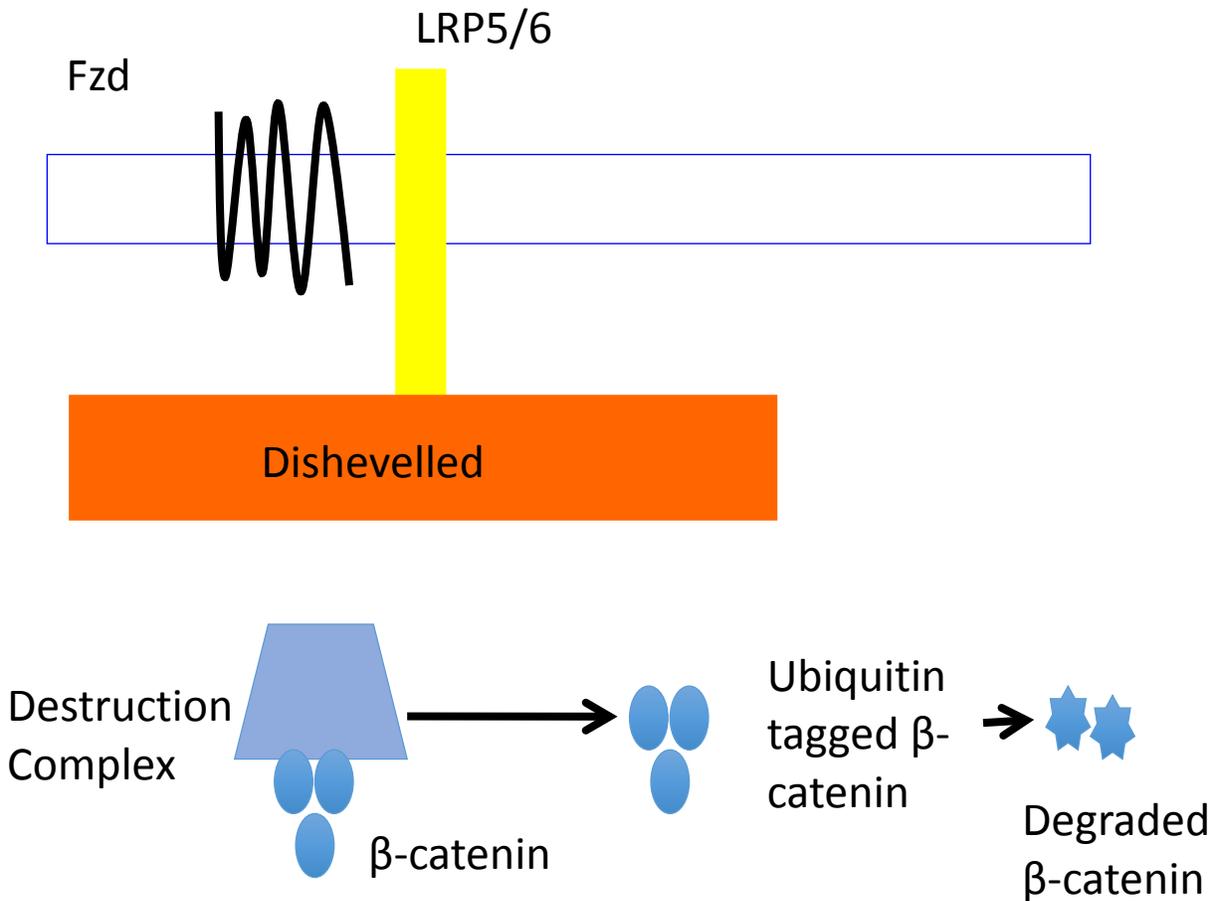


Figure 2. Inactivated Wnt β -catenin signaling pathway (21)

In the absence of the appropriate Wnt ligand the destruction complex binds and phosphorylates β -catenin, targeting it for destruction by the proteasome. The destruction of β -catenin does not allow it to make its way into the nucleus, this allows for transcriptional repressors to be bound the transcription factors

Wnt secreted ligands influence many aspects of neural development and function. Specifically, Wnts are necessary for neural induction, axis formation, axon guidance, and synapse development [36]. Wnt signaling has been shown to regulate the morphogenesis of the neural tube, modulate stem cell proliferation and differentiation, and contribute to neuronal migration [37,38,39]. All of these are essential for normal central nervous system development. When one of these processes goes wrong it can lead to neural tube closure defects and many neuropsychiatric disorders [40].

Adult neurogenesis is the proliferation and specification of neurons [41]. This is the process of maturation and introduction into the existing neural circuitry so it can function appropriately [41]. This process is essential to brain functions including memory, learning new information or tasks, and olfaction [41]. The cells from which new neurons arise are thought to be adult stem cells. Stem cell differentiation can be controlled by internal or external factors. Wnt ligands are part of the external factors that regulate the differentiation of stem cells into specialized neurons [42]. During development Wnt ligands act on the central nervous system (CNS) progenitor cells. This leads to the activation of β -catenin, which leads to proliferation of neural progenitor cells, causing the elongation of the entire neural tube in zebrafish embryos. Wnt3a is expressed in adult hippocampal stem cells, acting as an intrinsic factor of hippocampal neurogenesis. This takes place by controlling the generation of new neurons in the adult brain [43]. In addition, adult hippocampal progenitor cells have also been shown to secrete Wnt ligands. This leads to the activation of the canonical Wnt signaling pathway and an overall increase in the amount of new neurons formed in the adult brain [44].

Wnt7a has been shown to increase the amount of neurotransmitter released in adult hippocampal slices [45]. In mice with decreased levels of Wnt7a and Dvl there was a decrease

in the activity in mossy fiber axons [46]. Wnt7a induces the amount and size of recycling clusters for neurotransmitter release [46]. Dvl is required in mossy fiber terminals for normal synaptic vesicle recycling [46]. These two results indicate that when Wnt7a activates the β -catenin dependent pathway it leads to an increase in neurotransmitter release and ultimately synaptic transmission through the regulation of vesicle control. This regulation of synaptic transmission and neurotransmitter release occurs through some presynaptic mechanism. This increase in synaptic transmission leads to active and healthy neuron connections.

Non-canonical Calcium Wnt signaling pathway

The non-canonical calcium Wnt signaling pathway utilizes Wnt ligands and Frizzled receptors just like the Wnt β -catenin signaling pathway. However, a difference between the two pathways is that the canonical pathway utilizes an LRP5/6 co-receptor while the calcium dependent pathway does not and the non-canonical pathway utilizes Gq-PLC, as an effector while the canonical pathway does not. Gq is a heterotrimeric G protein subunit that associates with Frizzled. Once the Wnt ligand binds to the appropriate Frizzled receptor, Gq-PLC mediates the release of calcium from the intracellular stores of the cell. This occurs through some intermediate steps: 1) the activation of Gq by Frizzled, 2) then Gq activates PLC, 3) the cleavage of PIP₂ into DAG and IP₃. Then IP₃ activates its receptors to release calcium from the endoplasmic reticulum. This now high calcium concentration leads to the activation of the important downstream molecules calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), and the kinase Src [47]. The activation of CaMKII, PKC, and Src leads to long-term transcriptional regulation, the modulation of ion channels, and the activation of other intracellular signaling cascades.

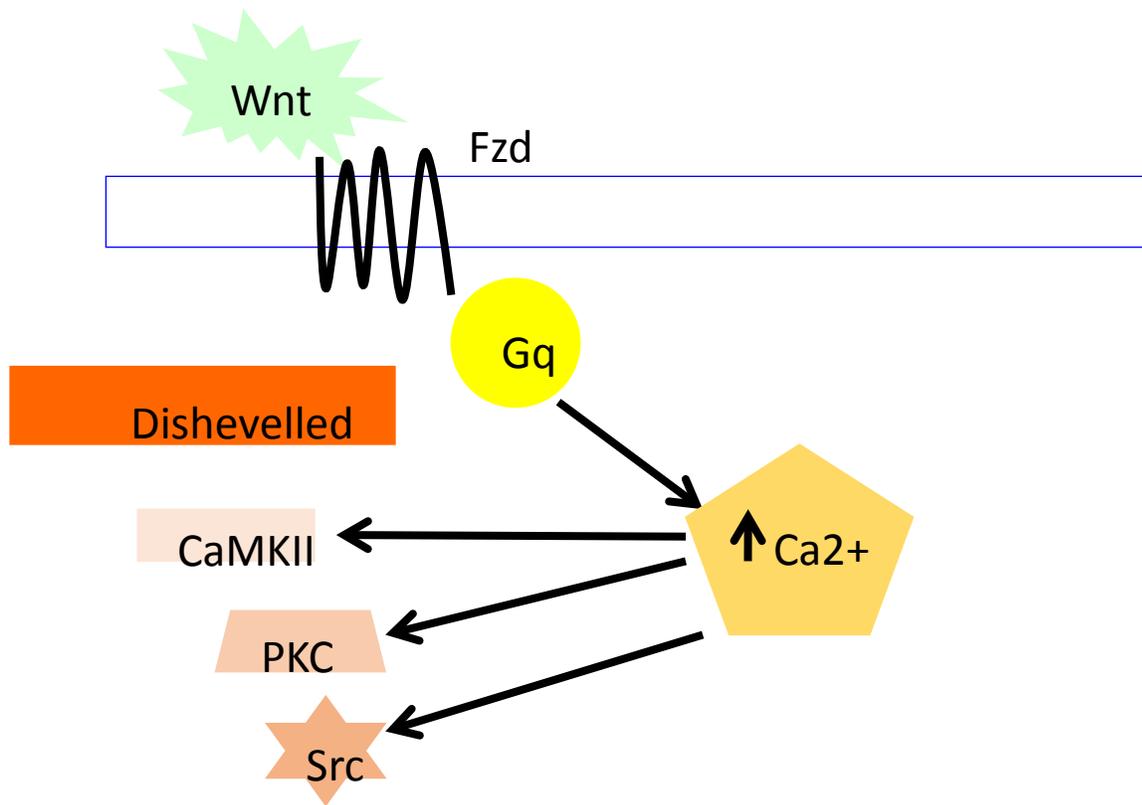


Figure 3. Non-canonical calcium Wnt signaling pathway
 When Wnt is bound to Frizzled it recruits the activation of Dishevelled and acts directly through a G-protein. This leads to the increase in intracellular calcium as well as to the activation of CamKII, PKC, Src. This activation leads to long-term transcriptional regulation.

The Wnt ligands necessary for activating the non-canonical calcium Wnt signaling pathway have been identified. Wnt5a or Wnt11 have been shown to bind to the Frizzled 2 receptor, in the absence of LRP5/6 and the presence of a G-protein. The binding of Wnt to a Frizzled receptor associated with Gq induces higher amounts of intracellular calcium from the endoplasmic reticulum [48,49]. Wnt5a has also been shown to inhibit Wnt 8, one of the Wnts that activate the Wnt β -catenin signaling pathway. The presence of Frizzled 2, Frizzled 3, Frizzled 4, and Frizzled 6 was also shown to increase intracellular levels of calcium in zebrafish embryos when there is co-expression of Wnt5a, suggesting that Frizzled 2 is one of the receptors that Wnt5a binds to [50]. This data suggests that Wnt5a when bound to the appropriate receptor induces the levels of intracellular calcium to increase. This increase of intracellular calcium can then lead to the activation of downstream calcium-sensitive proteins or kinases, such as CamKII and PKC. CamKII has been shown to activate the transcription factor NFAT. Once activated NFAT promotes ventral cell fates [51]. Calcium release by over expression of Wnt5a or Rfz-2 (rat Frizzled 2) in zebrafish embryos is inhibited by pertussis toxin and the α subunit of transducin. They inhibit calcium release by stopping G-protein signaling [52]. This indicates that through Wnt/Frizzled signaling intracellular calcium is released in a trimeric G protein mediated fashion.

The non-canonical calcium Wnt signaling pathway is important for Dorsal Root Ganglia (DRG) function in wild-type mice and once activated leads to the increase of intracellular calcium [47]. Intraplantar injections were performed on mice consisting of Wnt3a or vehicle. Once injected the mice were exposed to infrared heat and recorded the amount of time it took the mice to remove their paw from the hot plate. Mice injected with Wnt3a had a significantly lower latency than both wild type and vehicle injected mice [47]. The activation of DRG neurons plays

a role in the induction of thermal hyperalgesia [47]. When cultured DRG neurons were treated with Wnt3a and analyzed using Fura-2-based imaging they found an increased level of intracellular calcium [47]. During time-lapse analyses researchers found that this increase in intracellular calcium had its first peak at around 4-5 minutes. This delayed response was due to intracellular calcium mediators, such as PKC, that have to be activated or released for sensory neurons upon Wnt3a activated Wnt signaling. This proves that in the presence of Wnt3a the non-canonical calcium Wnt signaling pathway is activated and causes an influx of intracellular calcium in the DRG.

Not only does the non-canonical calcium Wnt signaling pathway play a role in the behavioral responses associated with hypersensitization of neurons at a molecular level, it also plays a role in dendritic arborization (the extension, retraction, and branching of dendrites) [53]. Dendritic arborization is controlled through extrinsic and intrinsic factors, non-canonical calcium Wnt signaling pathway being one of the extrinsic factors [54]. The non-canonical calcium Wnt signaling pathway is essential for dendritic arborization by stimulating the transcription of Wnt2 [55].

Dendrite growth rate in the optic tectum of the *Xenopus* tadpole has been shown to be inversely related to CaMKII activity [56]. The more CaMKII activity in the optic tectum the less dendrite growth [56]. Neurons not only contain CaMKII they also contain the CaMK cascade. This includes CaMK kinase (CaMKK), CaMKI, and CaMKIV [57]. CaMKI is nuclear excluded meaning it is found in the soma, axons, and dendrites of the neuron but not in the nucleus [58]. Recent studies have found roles of CaMKI in mediating axonal growth and cone motility [58]. The presence of the non-canonical calcium Wnt signaling pathway in nociceptors could cause a

change of dendritic arborization or axonal growth. This change could cause nociceptors to become hypersensitive and cause the behavioral phenotype seen in chronic pain states.

The dendrites of Class IV multidendritic neurons cover the entire body wall of *Drosophila* larvae. These dendrites of individual neurons have a very distinct pattern and avoid overlapping each other through repulsive interactions through homotypic dendrites [59,60]. This complete coverage and prevention of growth beyond their boundaries is essential for the correct wiring of neuronal circuitry [60]. Adult abdominal segments are subdivided along the dorso-ventral axis that leads to the formation of a ventral sternite. These sternites are thought to play a role in the development of ventral boundaries [60]. In previous research Wingless-Frizzled signaling were thought to play a role in the development of sternites. Sternites are found in adult flies and is formed during morphogenesis when Class IV multidendritic neurons lose their dendrites. When this signaling is suppressed the sternites become pleura [61]. Sternites are believed to be the source of the production of Wnt5. Wnt5 derived from sternites is essential for the ventral boundaries of Class IV multidendritic neurons to form [61].

Planar Cell Polarity (PCP) Wnt signaling pathway

The PCP Wnt signaling pathway, similarly to the non-canonical calcium Wnt signaling pathway, does not depend on β -catenin and LEF/TCF. The PCP Wnt signaling pathway is activated when the appropriate Wnt binds to the appropriate Frizzled receptor. This interaction activates Dishevelled-associated-activator of morphogenesis 1 (DAAM1) [47]. DAAM1 then stimulates the GTPase RhoA and its downstream molecule RhoA-dependent kinase (ROCK). DAAM1 can also activate a different pathway through the GTPase Rac1 and its downstream target JNK [47]. These two distinct pathways have very diverse cellular targets, including the

regulation of the cytoskeleton [47]. The PCP Wnt signaling pathway regulates tissue morphogenesis and the ability of sheets of cells to polarize [62]. This was seen in *Drosophila* mutants lacking important PCP Wnt signaling genes [63]. This includes the ability to organize cuticle hairs and sensory bristles [64].

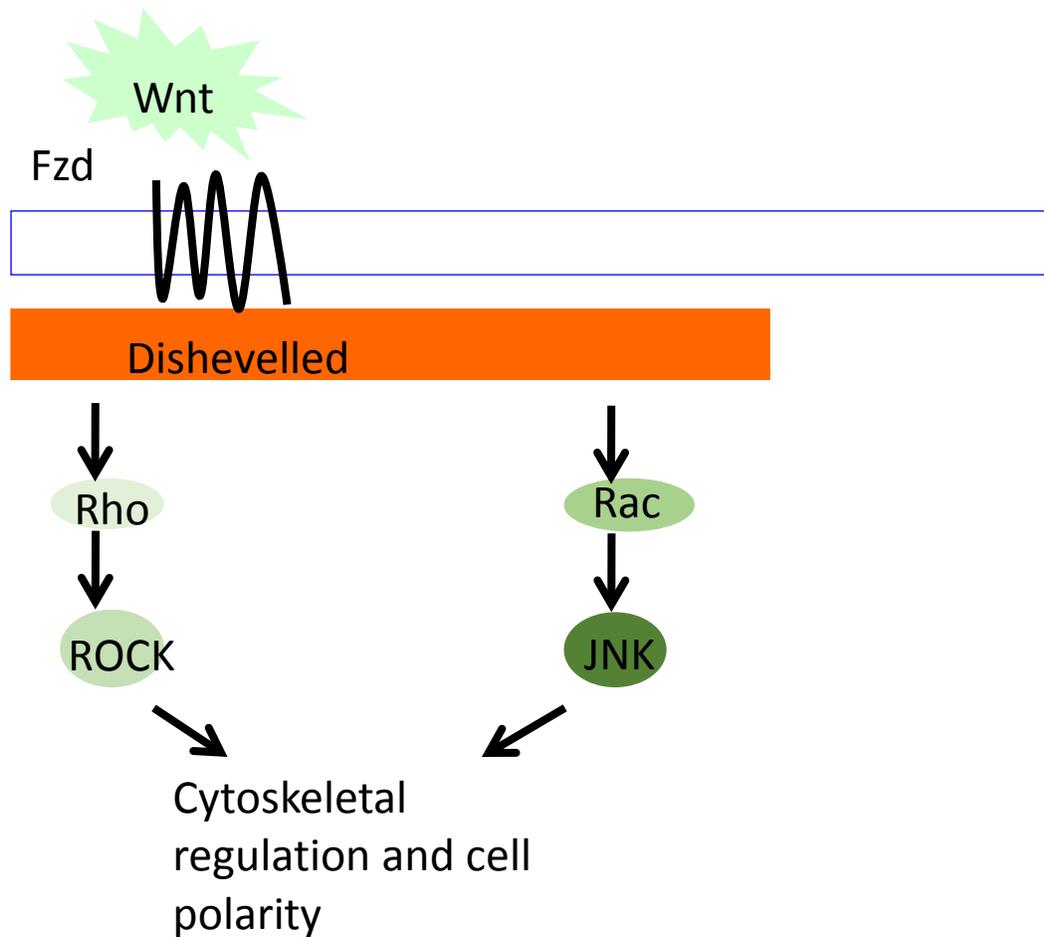


Figure 4. Non-canonical planar cell polarity Wnt signaling pathway
Wnt ligands bind to Frizzled and the recruitment of Dishevelled to the membrane occurs. Directly through Dishevelled either the Rac-JNK pathway can be activated or the Rho-ROCK pathway can be activated. The activation of either of these pathways leads to intracellular signaling, cytoskeletal regulation, and cell polarity.

When mice have been injected with Wnt3a the phosphorylation of JNK occurs, leading to behavioral phenotypes. Mice that have been injected with Wnt3a were put through the Von Frey test, a test for mechanical hypersensitivity [47]. The mice injected with Wnt3a had a much higher response rate than the mice injected with vehicle [47]. This indicates that the PCP Wnt signaling pathway promotes the hypersensitivity of sensory neurons to mechanical stimuli. To help elucidate the mechanism of how this pathway induces hypersensitivity, a neuron-specific knockdown of *Rac1*, in a mouse model, was put through the Von Frey test. The induction of sensory hypersensitivity due to mechanical stimuli was lost in the absence of *Rac1* [47]. When Wnt3a is applied to neuron-enriched DRG cultures there is a robust activation of Rac1 in the sensory neurons [47]. This implies that the DAAM1, Rac1-JNK pathway is responsible for inducing mechanical hypersensitivity.

The PCP Wnt signaling pathway plays a significant role in nociceptor sensitivity through membrane translocation of TRPA1. Once the PCP Wnt signaling pathway is activated, specifically the Rac 1-JNK pathway, in peripheral neurons the modulation of molecular machinery and the translocation of TRPA1 can occur. Activating the Rac1-JNK pathway leads to modulation of the molecular machinery involved in transducing mechanical nociceptor sensitization [47]. After exposure to Wnt3a, HEK293 cells transfected with plasmids expressing *Frizzled 3* and myc-tagged TRPA1 showed an increase in anti-myc immunoreactivity at the cell surface [47]. Surface biotinylation experiments on HEK293 cells transfected with *Frizzled 3* and myc-tagged TRPA1 showed a significantly higher abundance of cell-surface biotin-labeled TRPA1 in cells treated with Wnt3a than in cells treated with vehicle [47]. Increased immunoreactivity on the cell surface suggests that the PCP Wnt signaling pathway is responsible

for the translocation of TRPA1 to the cell surface. The translocation of TRPA1 to the surface allows for more channels to become activated, thus leading to the sensitization of the nociceptor.

The PCP Wnt signaling pathway plays a role in axon growth and guidance. In mice that lack either *Frizzled 3* or *Celsr3* genes, neurons lose the major axon tracts that connect the thalamus and cortex and cause spinal cord neurons to freeze at the midline crossing instead of turning [65]. In the *Frizzled 3* mutant, studies have found that in the cortex the projection neurons send their axons into the intermediate zone but these axons fail to extend, or intervene their appropriate targets and eventually degenerate [66]. Neurons in the thalamus extend but fail to leave the thalamus [65]. These results suggest that the PCP Wnt signaling pathway is essential for the appropriate axon guidance and growth. This research has some holes in it: Why are only these areas of the brain affected by inhibition or loss of PCP Wnt signaling pathway, what parts of the pathway are essential for axon guidance and growth?

The Rho-family of small GTPases (Rho, Rac, and Cdc42) has been demonstrated to play a pivotal role in regulating actin during dendrite morphogenesis of *Drosophila* multidendritic neurons [67,68]. The Rho GTPases act as molecular switches and have been shown to play a role in the regulation of neuronal morphogenesis, including migration, polarity, axon growth and guidance, and dendrite growth and plasticity [68]. Rac and Cdc42 function to promote neurite extension, while RhoA/Rho1 activation mediates neurite retraction [69]. Dendrite growth requires the activation of Rac1 and Cdc42, and decreased activity of RhoA [71]. Rho GTPases are regulated by upstream signals through the regulation of guanine nucleotide exchange factors (GEFs) [69]. A strong regulator for both Rac and Rho in Class IV multidendritic neurons is the multi-functional domain protein Trio [70]. *Trio* encodes for a GEF involved in the activation of

small GTPases [71]. The regulation and activation of Rho GTPases by GEFs is essential for dendritic outgrowth.

Trio contains two GEF activation domains, the Rac1-specific GEF1 domain and the Rho1-specific GEF2 domain [70], both of which are expressed in Class IV neurons [70]. Overexpression of Trio-GEF1 led to a dramatic increase in dendritic branching and a reduction in dendritic extensions towards the dorsal midline [70]. In contrast, overexpression of Trio-GEF2 caused a strong reduction in dendritic branching [70]. To further test the interactions between Trio-GEF1/Rac1 and Trio-GEF2/Rho1, epistasis experiments were conducted by simultaneously overexpressing GEF1 and GEF2 domains and RNAi knockdown transgenes for *Rac1* and *Rho1* [70]. Phenotypic analyses revealed that *Rac1* knockdown suppressed Trio-GEF1 induced defects in dendritic branching, whereas *Rho1* knockdown suppressed dendritic defects observed with Trio-GEF2 overexpression [70]. These results confirm that Trio-GEF1 functions through Rac1 to promote dendritic branching and Trio-GEF2 functions through Rho1 to inhibit dendritic branching [70].

Off-track 2 and the PCP pathway

Wnt receptor complexes contain a member of the Frizzled family and a co-receptor [72]. In most cases this co-receptor is a single-pass transmembrane receptor, such as LRP5/6. Protein tyrosine kinase 7 (PTK7) was identified as a Wnt co-receptor in vertebrates that is required for control of the PCP Wnt signaling pathway [72]. The *Drosophila melanogaster* homolog to PTK7 is *off-track*. *Off-track 2* is a paralog to *off-track* [72]. *Off-track* and *off-track 2* bind to each other and form complexes with Frizzled, Frizzled 2, and Wnt 2, suggesting that *off-track* and *off-track 2* are co-receptors for the PCP Wnt signaling pathway [72].

PTK7's role in the PCP Wnt signaling pathway was clearly demonstrated in *Xenopus*. PTK7 binds Wnt ligands and forms a complex with Frizzled 7, LRP6, and Dvl [72]. PTK7 was also found to bind Wnt3a and Wnt8, known Wnt ligands for the Wnt β -catenin signaling pathway [72]. PTK7 did not bind Wnt5a or Wnt11, other well known non-canonical Wnt signaling Wnt ligands [72]. The inability to bind all canonical Wnt signaling Wnt ligands suggests that PTK7 plays a role in the suppression of the Wnt β -catenin signaling pathway [72].

Off-track and off-track 2 have the ability to form both homo and heterooligomers and bind to the Wnt receptor Frizzled [72]. *Off-track* and *off-track 2* are essential during *Drosophila* embryogenesis and have similar expression patterns to one another, and both have the ability to bind Wnt 2 [72]. The ability of off-track 2 to bind to Wnt ligands and Frizzled receptors raises the question, does *off-track 2* play a role in the sensitization of nociceptors. If *off-track 2* is expressed in nociceptors does it affect the sensitization directly or does it interact with other Wnt signaling molecules.

Objectives

My research question is, what Wnt signaling factors play a role in nociceptor function? My study has three primary objectives to answer my research question. The first aim is to create a genetic screen of Wnt signaling genes in *Drosophila melanogaster*. I used Flybase to identify Wnt signaling genes and to find stocks that could be used in my genetic screen. My second aim is to knockdown identified Wnt signaling genes in *Drosophila melanogaster*. I will then test the larvae in the thermal nociception assay and record NEL responses to the noxious thermal stimuli. This will allow for the identification of genes that are important for nociceptor function by comparing the latencies of the RNAi lines to the wild-type phenotype. I will then confirm the

results of the RNAi genetic screen using *Drosophila* genetic tools to support roles for Wnt-signaling genes in nociception. From the validated genes I will select one gene to determine its specific role in the molecular signaling pathways of Wnt signaling and its role in nociception.

Materials and Methods

RNAi Screen

A list of genes targeted was created using Flybase (Table 1). Genes were chosen based on a gene ontology search on Flybase where the terms β -catenin, β -catenin binding, Wnt, Wnt binding, Frizzled, Frizzled binding, and Wnt signaling were used. The search brought up over 200 genes. I narrowed this list down by choosing more of the upstream and more relevant genes that are directly involved in the Wnt signaling pathways. Once the genes were picked, RNAi lines for these genes were ordered from Bloomington Drosophila Stock Center (BDSC) in Indiana.

To look at Wnt signaling role in nociceptor function an RNAi screen was created by using the RNAi lines from BDSC. Wnt signaling genes were targeted in the Class IV neurons. This allowed for the comparison of NEL phenotypes of the knockdown of Wnt signaling genes to a wild-type and positive control in the Class IV neurons. To achieve the appropriate knockdowns I crossed *ppk-GAL4; UAS-dicer2* flies with the RNAi lines for all of the genes tested [73]. The progeny of these crosses express RNA hairpins targeting genes of interest to generate an RNA interference effect, producing a loss-of-function phenotype. GAL4 is expressed specifically in the Class IV neurons in *ppk-GAL4* flies, which ensures that the knockdown occurs in the nociceptors, this helps ensure that the behavioral defects that I find are due to loss of gene function in the nociceptor neurons [74].

Each cross contained a positive and negative control. The positive control is *para* RNAi. *Para* codes for voltage-gated sodium channel, which is necessary for neurons to fire action potentials [75]. If the knockdown of this gene through RNAi is successful the larvae will not exhibit the NEL response. This will produce high latencies to roll (or the lack of rolling)

suggesting a decrease in nociceptor function. The negative control consists of the appropriate wild-type for the RNAi transgenes I tested. Each *UAS*-RNAi line strain was created in slightly different wild-type backgrounds. They both have the docking site for the *UAS*-RNAi transgene, either *attp2, y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03255}attP2* or *attp40, [1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00444}attP40*, with no *UAS*-RNAi inserted. This allows for them to be the closest genotype match possible to a *UAS*-RNAi strain without having RNAi. This is accomplished by crossing the genetic background of the RNAi transgenic strain to the *ppk-GAL4* driver strain.

Table 1. Gene ontology search for Wnt signaling genes and downstream effectors

| Gene Name | Gene Symbol | Gene Ontology Terms | Stock # |
|---|------------------|--|---------|
| <i>Misexpression suppressor of KSR 2</i> | <i>Mesk2</i> | | 29380 |
| <i>Abl tyrosine kinase</i> | <i>Abl</i> | non-membrane spanning protein tyrosine kinase activity, protein binding, protein tyrosine kinase activity | 35327 |
| <i>Ank2</i> | <i>Ank2</i> | Ankryin repeat | 33414 |
| <i>APC</i> | <i>APC</i> | beta-catenin binding | 34869 |
| <i>APC2</i> | <i>APC2</i> | beta-catenin binding | 28585 |
| <i>armidilo</i> | <i>arm</i> | cadherin binding, kinase binding, protein binding, protein complex binding | 35004 |
| <i>arrow</i> | <i>arr</i> | Wnt-activated, Wnt-protein binding | 31313 |
| <i>Axin</i> | <i>Axin</i> | beta-catenin binding, protein kinase binding | 31705 |
| <i>basket</i> | <i>bsk</i> | JUN kinase activity, protein binding and protein kinase activity | 35594 |
| <i>C-terminal Binding Protein carrier of wingless</i> | <i>CtBP</i> | protein binding | 32889 |
| <i>casein kinase 1 alpha</i> | <i>cow</i> | Wnt Protein Binding | 38235 |
| <i>Casein Kinase Iialpha</i> | <i>CK1alpha</i> | protein binding, protein kinase activity | 35153 |
| <i>CDC37</i> | <i>CkIIalpha</i> | Protein binding, protein serine/threonine kinase activity, axon guidance, positive regulation of wnt signaling pathway | 35136 |
| <i>Derailed</i> | <i>CDC37</i> | Hsp90 binding | 34991 |
| <i>Dishevelled</i> | <i>drl</i> | Wnt protein binding | 39002 |
| <i>Exo70</i> | <i>dsh</i> | Wnt signaling pathway | 31306 |
| <i>four-jointed</i> | <i>Exo70</i> | beta-catenin binding | 55234 |
| <i>frizzled</i> | <i>fj</i> | Protein kinase activity, Wnt-protein binding | 34323 |
| <i>frizzled</i> | <i>fz</i> | Wnt-activated, Wnt-protein binding | 34321 |
| <i>frizzled</i> | <i>fz</i> | Wnt-activated, Wnt-protein binding | 31311 |
| <i>Frizzled 2</i> | <i>fz2</i> | PDZ domain and Wnt-Protein binding, Wnt activated receptor activity | 27568 |
| <i>frizzled 3</i> | <i>fz3</i> | Wnt Protein Binding | 44468 |
| <i>gilgamesh</i> | <i>gish</i> | ATP binding, protein serine/threonine kinase activity | 36719 |
| <i>groucho</i> | <i>gro</i> | protein binding | 35759 |
| <i>hipk</i> | <i>hipk</i> | beta-catenin binding, protein kinase activity, protein serine/threonine kinase activity, transcription factor binding | 35363 |
| <i>legless</i> | <i>lgs</i> | beta-catenin binding, protein binding, RNA polymerase II transcription coactivator activity | 41983 |

| | | | |
|--|--------------|---|-------|
| <i>Microtubule star</i> | <i>mts</i> | positivie regulation of wnt signaling pathway | 38337 |
| <i>multiple wing hairs</i> | <i>mwh</i> | Protein binding | 34862 |
| <i>Nek2</i> | <i>Nek2</i> | Protein Serine/Threonine Kinase activity, | 35328 |
| <i>nervous wreck</i> | <i>nwk</i> | Positive regulation of wnt signaling pathway | 27713 |
| | | protein binding | |
| <i>notum</i> | <i>notum</i> | deacetylase activity, interaction with daily, wnt | 35650 |
| <i>off-track</i> | <i>otk</i> | signaling pathway interactions | 55869 |
| <i>off-track 2</i> | <i>otk2</i> | Wnt-protein binding | 55892 |
| | | neurogenesis, positive regulation of both | |
| | | canonical wnt signaling pathway and wnt | |
| <i>opossum</i> | <i>opm</i> | protein sec | 43280 |
| <i>pangolin</i> | <i>pan</i> | regulation of canonical wnt signaling pathway | 26743 |
| | | phosphatidylinositol phosphate binding, rac | |
| | | and rho guanyl-nucleotide exchange factor | |
| | | activity, Rho GTPase binding , negativley reg | |
| <i>pebble</i> | <i>pbl</i> | wnt signaling pathway | 36841 |
| | | beta-catenin binding, positive regulation of | |
| <i>pontiin</i> | <i>pont</i> | gene expression | 50972 |
| | | GTPase activity, kinase binding, protein | |
| <i>Rho1</i> | <i>Rho1</i> | binding | 32383 |
| <i>Sec10</i> | <i>Sec10</i> | beta-catenin binding, neurotransmitter secretion | 27483 |
| <i>secreted Wg- interacting molecule</i> | <i>Swim</i> | Wnt protein binding | 36591 |
| | | kinase activity, protein binding, protein kinase | |
| <i>shaggy</i> | <i>sgg</i> | activity | 35364 |
| | | nucleic acid binding, glial cell fate | |
| | | determination, glial cell migration,axon | |
| <i>split ends</i> | <i>spen</i> | guidance | 33398 |
| <i>starry night</i> | <i>stan</i> | receptor signaling protein activity | 35050 |
| <i>sunspot</i> | <i>ssp</i> | beta-catenin binding | 29358 |
| <i>Twins</i> | <i>tws</i> | Protein serine/threonine phosphatase activity | 36689 |
| <i>Van Gogh</i> | <i>Vang</i> | Frizzled and Protein binding | 34354 |
| | | Frizzled, Glycosaminoglycan, and protein | |
| <i>Wingless</i> | <i>wg</i> | binding | 32994 |
| <i>wingless</i> | <i>wg</i> | Wnt protein | 31310 |
| | | Axon Guidance, Positive regulation of | |
| <i>WNK homolog</i> | <i>WNK</i> | Canonical wnt signaling pathway | 42521 |
| <i>Wnt10</i> | <i>Wnt10</i> | Wnt protein | 31989 |
| <i>Wnt2</i> | <i>Wnt2</i> | Frizzled Binding | 36722 |
| <i>Wnt4</i> | <i>Wnt4</i> | Frizzled and Protein binding | 29442 |
| <i>Wnt5</i> | <i>Wnt5</i> | Receptor binding | 34644 |
| <i>Wnt5</i> | <i>Wnt5</i> | Receptor binding | 28534 |
| <i>Wnt6</i> | <i>Wnt6</i> | Gprotein and Protein Binding | 35808 |

| | | | |
|--------------------|-------------|------------------|----------------|
| <i>WntD (Wnt8)</i> | <i>WntD</i> | Frizzled Binding | 28947 26002 |
|--------------------|-------------|------------------|----------------|

Fly stocks

The strains shown in Table 2 were ordered from BDSC and were used and raised on standard cornmeal molasses fly food at room temperature.

Nociception Assay

A probe was heated to 46°C and made contact with the larval body wall. Once contact was made it was held on the larval body wall until the NEL was completed. Each larva was only tested once. Each behavioral response was recorded using a camera mounted on the top of the dissecting microscope. The videos were then transferred to Adobe Premier where the start and stop of the NEL behavior was marked. A latency to complete the NEL is recorded by measuring the amount of time it takes to complete the NEL from start to finish taking the end of the behavior and subtracting the beginning of the test when the probe is first applied to the larva. Each genotype had 30-40 larvae tested.

Some of the larvae with a hypersensitive phenotype were retested at 42°C. Lowering the temperature to 42°C allows us to distinguish the phenotypes of the RNAi knockdown more easily. The Mann-Whitney U test was used to test for differences between experimental genotypes and their respective negative controls.

Live Imaging

For visualizing the dendrite morphology of *off-track 2* in nociceptors, third instar larvae of *ppk-Gal4-UAS-GFP/Cyo; Sb/Tm3,Ser ; UAS-off-track 2* were paralyzed by tying a strand of

hair just behind the CNS of the larvae. This constricts the larvae and stops action potentials from getting to the rest of the larvae, leading to the paralysis of the larvae. Single Class IV neurons were imaged using an LSM 510 confocal microscope. Z-stacks were obtained using the 488 nm laser line. Image stacks were analyzed using Neuron J, an Image J plugin, to trace and label individual dendrites. This gave the total number of dendrites, total length of dendrites, and total number of branch points.

Over Expression

An over expression line for *off-track 2* was obtained from the BDSC. The over expression strain used was UAS-cDNA for *off-track 2*. Then the UAS-cDNA *off-track 2* line was crossed with *ppk-GAL4* virgins. This will cause more *off-track 2* to be present than in wild-type larvae.

Black Cell is a balancer used to correct non-fertile stocks

Black Cell is a balancer used to allow the maintenance of a lethal mutation as a heterozygous stock. When the double mutant of *off-track* and *off-track 2* is balanced over *Black Cell* it will allow for the production of a homozygous mutant. Double mutant flies containing a *CyO* balancer were crossed to a strain containing the *Black Cell* balancer to produce F1 flies with the double mutant chromosome balanced over *Black Cell*. The flies without curly wings are then selected and used for the cross for testing. Larvae that contain black cells directly below the epidermis, visualized under the dissecting microscope, were not tested. Flies without *Black Cell* phenotype were considered to be homozygous for the double-mutant chromosome and were tested.

Table 2. List of Fly stocks acquired from BDSC

| Bloomington Stock Number | FlyBase ID | Gene | genotype |
|--------------------------|-------------|----------------------------|---|
| 26022 | FBst0026022 | starry night | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02047}attP2 |
| 26743 | FBst0026743 | pangolin | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02306}attP2 |
| 27483 | FBst0027483 | Sec10 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02633}attP2 |
| 27568 | FBgn0016797 | Frizzled 2 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02722}attP2 |
| 27713 | FBst0027713 | nervous wreck | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02793}attP2 |
| 28534 | FBst0028534 | Wnt 5 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM05020}attP2 |
| 28585 | FBst0028585 | APC2 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM05073}attP2 |
| 29358 | FBst0029358 | sunspot | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02522}attP2/TM3, Sb[1] |
| 29380 | FBst0029380 | Mesk2 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03312}attP2 |
| 29442 | FBst0029442 | Wnt4 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03378}attP2 |
| 31306 | FBst0031306 | Dishevelled | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01253}attP2 |
| 31313 | FBst0031313 | arrow | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01260}attP2 |
| 31705 | FBst0031705 | Axin | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM04012}attP2 |
| 31989 | FBst0031989 | Wnt 10 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03423}attP2/TM3, Sb[1] |
| 32383 | FBst0032383 | Rho1 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00375}attP2/TM3, Sb[1] |
| 32889 | FBst0032889 | C-terminal binding protein | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00677}attP2 |
| 32994 | FBst0032994 | Wingless | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00794}attP2 |
| 33398 | FBst0033398 | Split ends | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00276}attP2 |
| 33414 | FBst0033414 | Ank2 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00295}attP2/TM3, Sb[1] |

| | | | |
|-------|-------------|-------------------------|---|
| 33923 | FBst0033923 | para | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00868}attP2 |
| 34091 | | dally-like | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00903}attP2 |
| | FBst0034091 | | |
| 34321 | FBst0034321 | Frizzled | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01308}attP2 |
| 34323 | | Four Jointed | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01310}attP2 |
| | FBst0034323 | | |
| 34354 | | VaN Gogh | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01343}attP2 |
| | FBst0034354 | | |
| 34862 | | Mutltiple wing hairs | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00180}attP2 |
| | FBst0034862 | | |
| 34869 | | APC | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00188}attP2 |
| | FBst0034869 | | |
| 35004 | | armidillo | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01414}attP2 |
| | FBst0035004 | | |
| 35050 | | Starry night | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01464}attP2 |
| | FBst0035050 | | |
| 35136 | FBst0035136 | CKIIalpha | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00003}attP2 |
| 35153 | FBst0035153 | CK1alpha | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00021}attP2 |
| 35327 | | Abl tyrosine kinase | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00234}attP2 |
| | FBst0035327 | | |
| 35328 | FBst0035328 | Nek2 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00235}attP2 |
| 35363 | | hipk | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00276}attP2 |
| | FBst0035363 | | |
| 35364 | | shaggy | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00277}attP2 |
| | FBst0035364 | | |
| 35594 | FBst0035594 | basket | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00431}attP2 |
| 35650 | | notum | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GLV21015}attP2 |
| | FBst0035650 | | |
| 35759 | FBst0035759 | groucho | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01506}attP2 |
| 35808 | | Wnt 6 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00457}attP2 |
| | FBst0035808 | | |
| 36303 | FBst0036303 | WT | y[1] v[1]; P{y[+t7.7]=CaryP}attP2 |
| 36591 | FBst0036591 | Swim | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00551}attP2 |
| 36689 | | Twins | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01578}attP2 |
| | FBst0036689 | | |
| 36719 | FBst0036719 | gilgamesh | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01609}attP2 |
| 36722 | FBst0036722 | Wnt2 | y[1] sc[*] v[1]; P{y[+t7.7] |

| | | | |
|-------|-------------|-------------|---------------------------------|
| 38235 | | Carrier of | v[+t1.8]=TRiP.HMS01613}attP2 |
| | FBst0038235 | Wingless | y[1] sc[*] v[1]; P{y[+t7.7] |
| 38337 | | Microtubula | v[+t1.8]=TRiP.HMS01679}attP2 |
| | FBst0038337 | r star | y[1] v[1]; P{y[+t7.7] |
| 41983 | | legless | v[+t1.8]=TRiP.HMS01804}attP2 |
| | FBst0041983 | | y[1] sc[*] v[1]; P{y[+t7.7] |
| 42521 | | WNK | v[+t1.8]=TRiP.HMS02381}attP2 |
| | FBst0042521 | | y[1] v[1]; P{y[+t7.7] |
| 43280 | FBst0043280 | opossum | v[+t1.8]=TRiP.HMJ02087}attP40 |
| | | | y[1] v[1]; P{y[+t7.7] |
| 44468 | FBst0044468 | Frizzled 3 | v[+t1.8]=TRiP.HMC02679}attP2 |
| | | | y[1] sc[*] v[1]; P{y[+t7.7] |
| 50972 | FBst0050972 | pontiin | v[+t1.8]=TRiP.GLC01626}attP2 |
| | | | y[1] v[1]; P{y[+t7.7] |
| 55234 | | Exo70 | v[+t1.8]=TRiP.HMJ21078}attP40 |
| | FBst0055234 | | y[1] sc[*] v[1]; P{y[+t7.7] |
| 55869 | | off track | v[+t1.8]=TRiP.GLC01851}attP2 |
| | FBst0055869 | | y[1] sc[*] v[1]; P{y[+t7.7] |
| 55892 | | Off-track 2 | v[+t1.8]=TRiP.HMC04139}attP2 |
| | FBst0055892 | | y[1] sc[*] v[1]; P{y[+t7.7] |
| 5439 | | Black Cell | v[+t1.8]=TRiP.HMC04171}attP2 |
| | FBst0005439 | | w[1118]; In(2LR)Gla, wg[Gla-1] |
| | | | PPO1[Bc]/CyO |
| | | | ppkGal4-UASGFP/Cyo; Sb/Tum3.Ser |

Results

Thermal nociception assay of Wnt signaling genes produce insensitive and hypersensitive phenotypes

To identify Wnt signaling's role in the development of altered nociceptor function we used RNAi to knock down each gene from the Wnt signaling pathways. Knock down was achieved by using a nociceptor-specific *ppk* driver with *UAS-dicer 2*, in order to enhance RNAi knockdown. A total of 54 *UAS*-RNAi lines were obtained from the BDSC and were used to knock down the genes selected from the gene ontology search (Table 1). The *UAS*-RNAi lines were each tested in parallel to the genetic controls. Progeny from each *ppk-Gal4 UAS-dicer 2 x UAS*-RNAi cross were tested in a thermal nociception assay [4]. The initial screen of Wnt signaling factors were tested at 46°C. An average latency to perform an NEL at 46°C probe stimulation were determined for each RNAi genotype (Figure 5) and was compared to the mean latency of wild-type at 3.4 seconds. Each genotype had at least 30 animals tested.

The cutoff for having a hypersensitive or insensitive phenotype as compared to wild-type was set to 1σ from the appropriate control latency. A hypersensitive phenotype is present if the mean latency of a gene to perform NEL is significantly faster than the wild-type latency. An insensitive phenotype is present if the mean latency to perform NEL is significantly slower than the wild-type latency. Six RNAi lines targeting 6 genes were identified as having a hypersensitive phenotype: *Frizzled 2*, *Frizzled*, *Pangolin*, *Wnt 4*, *Hipk*, and *Wnt 2*. While, 6 RNAi lines targeting six genes were identified in having insensitive phenotypes: *Wnt 10*, *Frizzled 3*, *pontiin*, *Nek2*, *off-track 2*, and *Ank 2* (Figure 5, Table 3,4).

Table 3. Hypersensitive Candidate Genes

| Bloomington Stock Number | FlyBase ID | Gene | Genotype | Latency (s) |
|--------------------------|-------------|------------|--|-------------|
| 27568 | FBgn0016797 | Frizzled 2 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02722}attP2 | 1.5±0.4 |
| 34321 | FBst0034321 | Frizzled | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01308}attP2 | 1.6±0.4 |
| 26743 | FBst0026743 | pangolin | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02306}attP2 | 1.7±0.5 |
| 29442 | FBst0029442 | Wnt4 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03378}attP2 | 1.7±0.6 |
| 35363 | FBst0035363 | hipk | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00276}attP2 | 1.7±0.7 |
| 36722 | FBst0036722 | Wnt2 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01613}attP2 | 1.8±0.6 |

Table 4. Insensitive Candidate Genes

| Bloomington Stock Number | FlyBase ID | Gene | Genotype | Latency (s) |
|--------------------------|-------------|-------------|---|-------------|
| 31989 | FBst0031989 | Wnt 10 | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03423}attP2/TM3, Sb[1] | 3.7±1.5 |
| 44468 | FBst0044468 | Frizzled 3 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GLC01626}attP2 | 3.9±1 |
| 50972 | FBst0050972 | pontiin | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ21078}attP40 | 3.9±1.3 |
| 35328 | FBst0035328 | Nek2 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00235}attP2 | 4±1.5 |
| 55892 | FBst0055892 | off track 2 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04171}attP2 | 4.7±1.9 |
| 33414 | FBst0033414 | Ank2 | y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00295}attP2/TM3, Sb[1] | 5.5±2.5 |

Nocifensive Response Latency 46°C

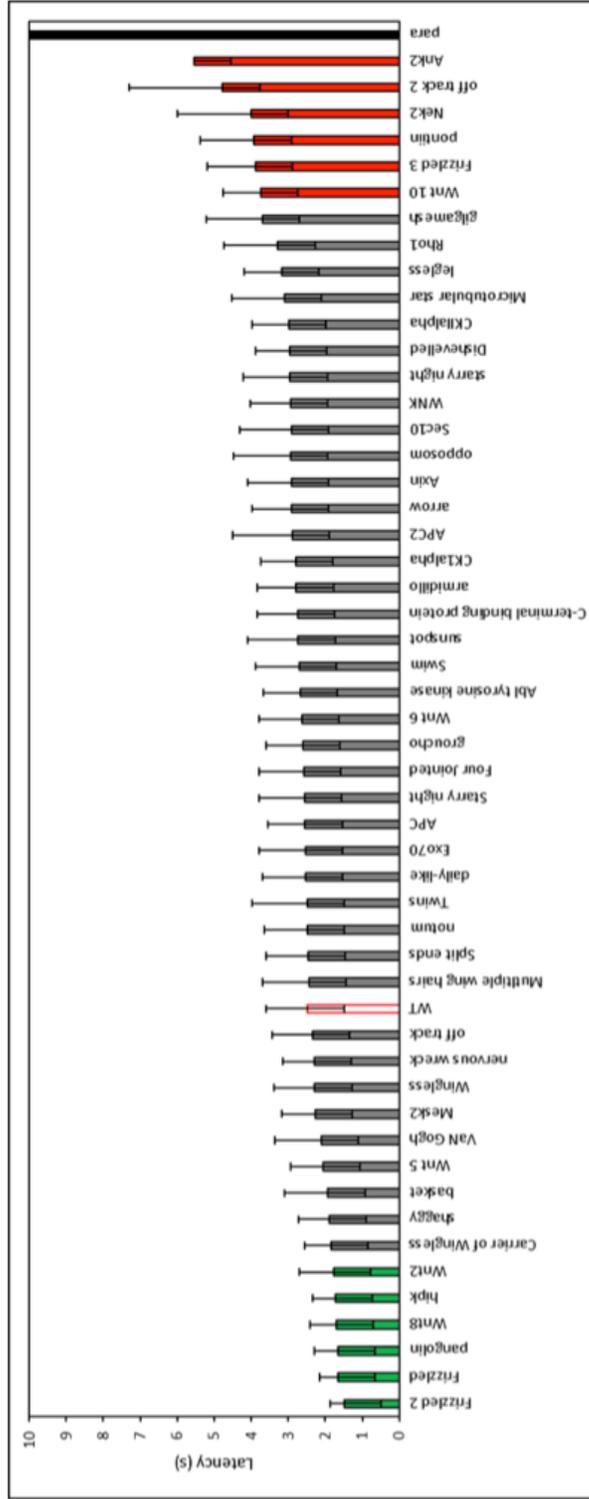


Figure 5. Genetic screen for thermal nociception defects reveals hypersensitive and insensitive phenotypes in response to knockdown of Wnt-signaling genes.

Larval progeny of the listed crosses were tested in a thermal nociception assay at 46°C. Nociceptor-specific knockdown using ppk-Gal4 x UAS-RNAi targeting Wnt signaling genes produced a variety of phenotypes. The error bars show standard deviation. Lines were considered to have a phenotype if they were one standard deviation removed from the population average. Green bars indicate hypersensitive phenotypes, Red bars indicate insensitive phenotypes, gray bars indicate no phenotype was seen and the red outlined bar indicates wild-type

Nociceptor-specific RNAi knockdown of *Frizzled* produces a hypersensitive phenotype

Class IV-specific RNAi knockdown of *Frizzled* produces a decrease in response latency. This decrease in response latency is interpreted as a hypersensitive nociception phenotype (Figure 5, Table 3). *Frizzled* is a member of the *Frizzled* gene family that encodes 7-transmembrane domain proteins that are receptors for Wnt ligands and is essential for both the non-canonical calcium Wnt signaling pathway and the Wnt β -catenin signaling pathway [36,62]. If knocking down *Frizzled* produced a hypersensitive phenotype than this suggests that under baseline conditions *Frizzled* might limit the sensitivity of the Class IV neurons. To test for this the BDSC UAS-RNAi line for *Frizzled* was crossed to *ppk-Gal4 UAS-dicer 2* and the progeny were then subjected to the thermal nociception assay at 42°C [4]. Testing at a lower temperature allows for more separation of the phenotypes between *Frizzled* RNAi knockdown and the wild-type. This allows for the confirmation of statistical differences. The latency of the *Frizzled* RNAi line was then compared to the appropriate wild-type. The knockdown of *Frizzled* produced a hypersensitive phenotype with a latency of 2.7 ± 0.2 as compared to wild-type with a latency of 4.6 ± 0.3 (Figure 6) ($p < 0.001$).

Nociceptor-specific RNAi knockdown of *Frizzled 2* produces a hypersensitive phenotype

Class IV-specific RNAi knockdown of *Frizzled 2* produces a decrease in response latency. This decrease in response latency is interpreted as a hypersensitive nociception phenotype (Figure 5, Table 3). *Frizzled 2* is a member of the *Frizzled* gene family that encode 7-transmembrane domain proteins that are receptors for Wnt ligands and is part of both the non-canonical calcium Wnt signaling pathway and the Wnt β -catenin signaling pathway [36,64,65]. This observation raised the possibility that *Frizzled 2* might play a role in limiting the sensitivity

of Class IV neurons at baseline levels. To test for this the BDSC *UAS-RNAi* line for *Frizzled 2* was crossed to *ppk-Gal4 UAS-dicer 2* and the progeny were then subjected to the thermal nociception assay at 42°C [4]. The latency of the *Frizzled 2* RNAi line was then compared to the appropriate wild-type. The knockdown of *Frizzled 2* produced a hypersensitive phenotype with a latency of 3.7 ± 1.6 as compared to wild-type with a latency of 4.2 ± 1.6 (Figure 7) ($p=0.047$).

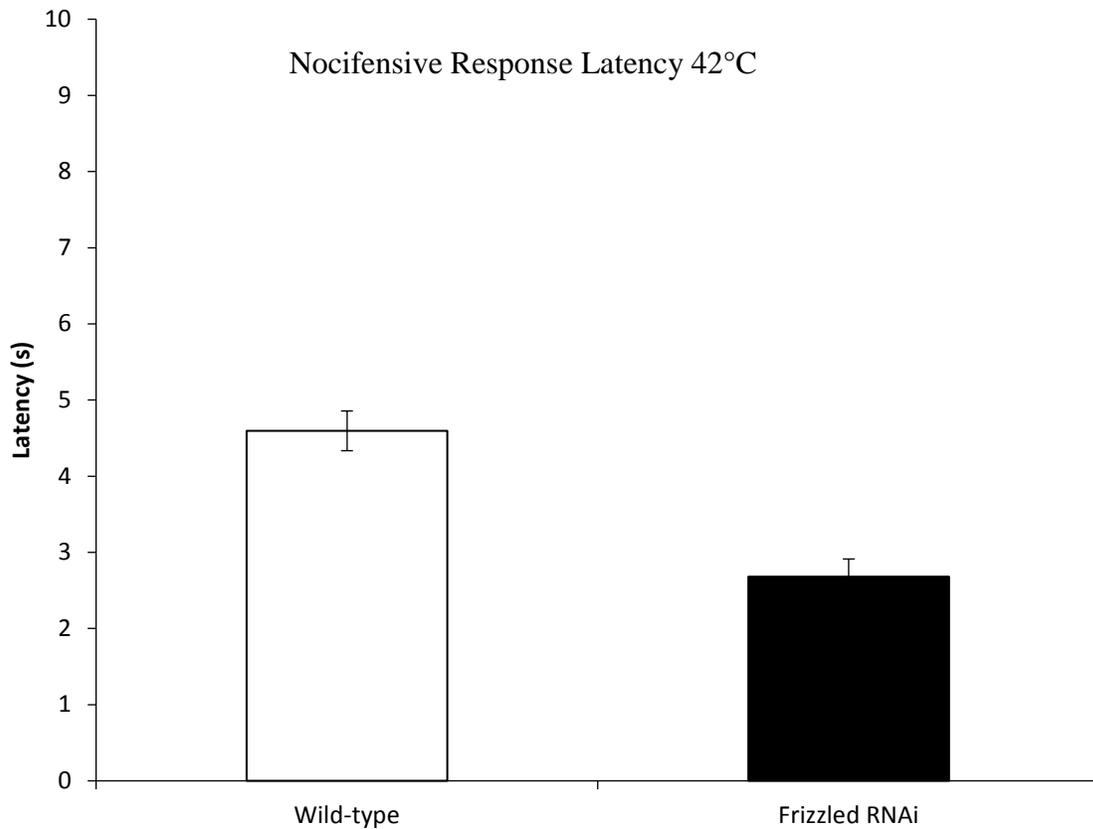


Figure 6. Nociceptor-specific RNAi knockdown of *Frizzled* produces a hypersensitive phenotype.

Larval progeny of the listed crosses were tested in a thermal nociception assay at 42°C. The error bars show standard error. $n=75$, Mann Whitney U test shows significant difference between *Frizzled* knockdown and wild-type ($p<0.001$).

Nocifensive Response Latency 42°C

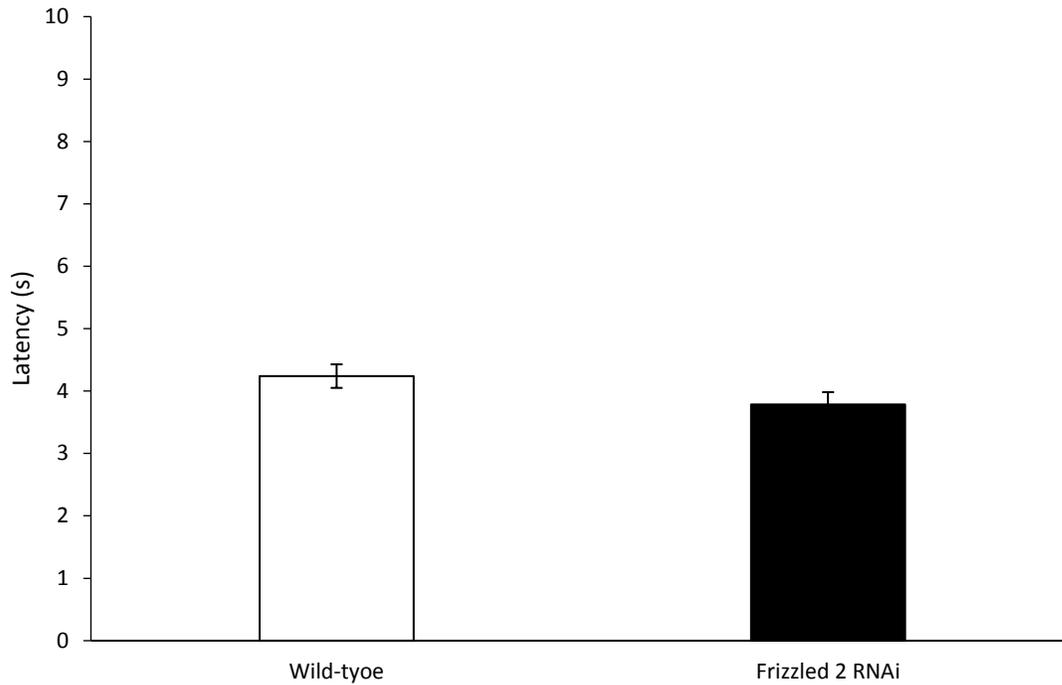


Figure 7. Nociceptor-specific RNAi knockdown of *Frizzled 2* produces a hypersensitive phenotype. Larval progeny of the listed crosses were tested in a thermal nociception assay at 42°C. The error bars show standard error. n=75, Mann Whitney U test shows significant difference between *Frizzled 2* knockdown and wild-type ($p=0.047$).

Nociceptor-specific RNAi knockdown of *Wnt 2* produces no phenotype

Wnt 2 showed a hypersensitive phenotype in the initial screen (Figure 5, Table 3). *Wnt 2* is a ligand that is needed for the activation of the Wnt signaling pathways. However, *Wnt 2* is primarily known for activating the Wnt β -catenin signaling pathway. To validate the hypersensitive phenotype seen in the initial screen the BDSC *UAS*-RNAi line for *Wnt 2* was crossed to *ppk-Gal4 UAS-dicer 2* and the progeny were then subjected to the thermal nociception assay at 42°C [4]. The latency of the *Wnt 2* RNAi line was then compared to the appropriate wild-type. The knockdown of *Wnt 2* produced no phenotype with a latency of 3.7 ± 1.6 as compared to wild-type with a latency of 4 ± 2.1 (Figure 8) ($p=0.075$). In this case our initial screens hypersensitive phenotype was not confirmed and may have been due to a false positive result.

Nociceptor-specific RNAi knockdown of *off-track 2* produced an insensitive phenotype

Class IV-specific RNAi knockdown of *off-track 2* produces an increase in response latency. This increase in response latency is interpreted as an insensitive nociception phenotype (Figure 5, Table 3). To test for this the BDSC *UAS*-RNAi line for *off-track 2* was crossed to *ppk-Gal4 UAS-dicer 2* and the progeny were then subjected to the thermal nociception assay at 46°C [4]. The latency of the *off-track 2* RNAi line was then compared to the appropriate wild-type. The knockdown of *off-track 2* produced an insensitive phenotype with a latency of 4.5 ± 2.1 as compared to wild-type with a latency of 2.5 ± 1.5 (Figure 9) ($p<0.001$).

Nocifensive Response Latency 42°C

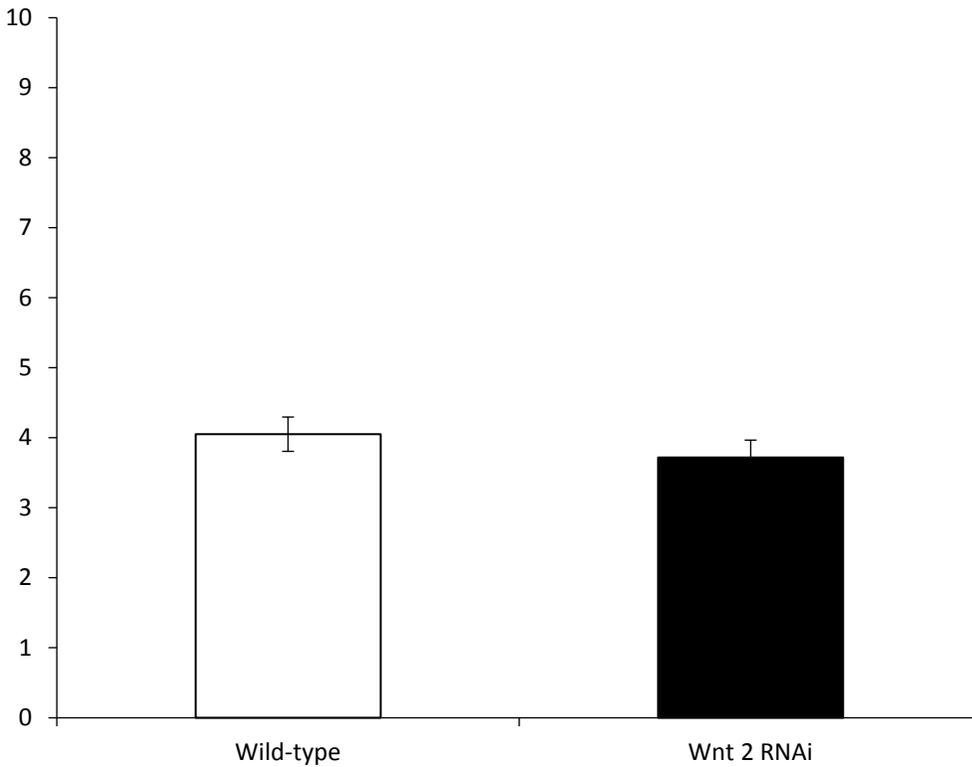


Figure 8. Nociceptor-specific RNAi knockdown of *Wnt 2* produces no phenotype. Larval progeny of the listed crosses were tested in a thermal nociception assay at 42°C. The error bars show standard error. n=75, Mann Whitney U test shows no significant difference between *Wnt 2* knockdown and wild-type ($p=0.0746$).

Nocifensive Response Latency 46°C

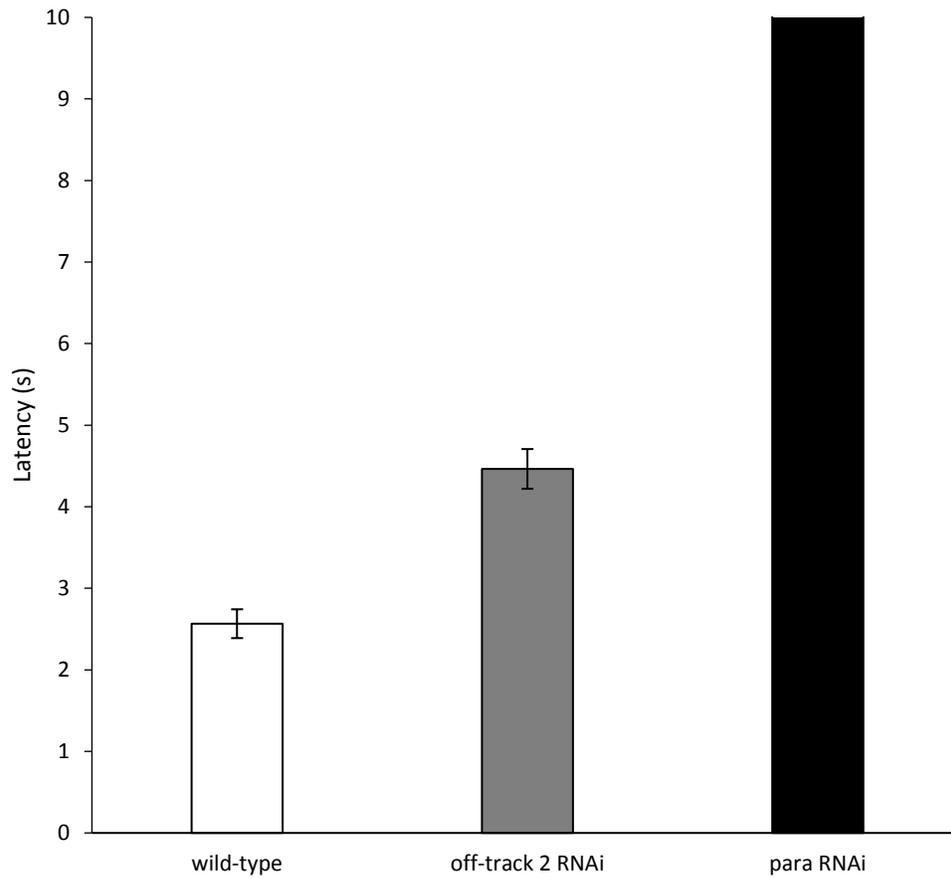


Figure 9. Nociceptor-specific RNAi knockdown of *off-track 2* produces an insensitive thermal nociception phenotype. Larval progeny of the listed crosses were tested in a thermal nociception assay at 46°C. The error bars show standard error. n=77, Mann Whitney U test comparing RNAi knockdown of *off-track 2* to wild-type shows a significant difference ($p < 0.001$).

The overexpression of *off-track 2* causes a hypersensitive phenotype

To help identify the possible role for *off-track 2* in the development of altered nociceptor function we used RNAi to knock down *off-track 2*. This produced an insensitive phenotype (Figure 9). To validate this result a line of UAS- *off-track 2* cDNA was procured from BDSC. The *off-track 2* overexpression line was crossed with *ppk-Gal4* and tested at 46 °C in parallel with the appropriate wild-type. If *off-track 2* does play a role in nociceptor function than the overexpression phenotype should be the opposite of the RNAi knockdown phenotype. Meaning, the overexpression of *off-track 2* should produce a hypersensitive phenotype. This proved to be true in that the overexpression of *off-track 2* had a latency of 2.1 ± 0.6 where wild-type had a latency of 3.1 ± 1.5 (Figure 10) ($p < 0.001$).

Loss of *off-track* and *off-track 2* function produced an insensitive thermal nociception phenotype

A double mutant was tested to see if complete deletion of *off-track* and *off-track 2* would produce a stronger effect than removing the function of *off-track 2* alone. Because the double-mutant chromosome causes sterility when homozygous, the stock was maintained over *Black Cell*. When crossed the homozygous mutant was non-fertile and lethal. To overcome this the double mutant was re-balanced over the *Black Cell* balancer so that homozygous mutant larvae could be tested. Crosses were then set up with the double mutant and w1118 so wild-type, homozygous, and heterozygous larvae could be tested in the thermal nociception assay at 46°C. The homozygous mutant was then compared to both the heterozygous mutant and wild-type strains. The homozygous mutant was significantly different than the wild type with a latency of 7.3 ± 3.2 and 4.8 ± 1 (Figure 11) ($p = 0.002$).

The comparisons of the initial nociceptor-specific RNAi knockdown of *off-track2*, validation of RNAi knockdown of *off-track 2*, overexpression of *off-track 2*, and the double mutant of *off-track* and *off-track 2*, demonstrated that the phenotypes are all consistent with one another. The consistent phenotypes suggest the presence of *off-track 2* in nociceptors is essential for the proper function of nociceptors in *Drosophila melanogaster*.

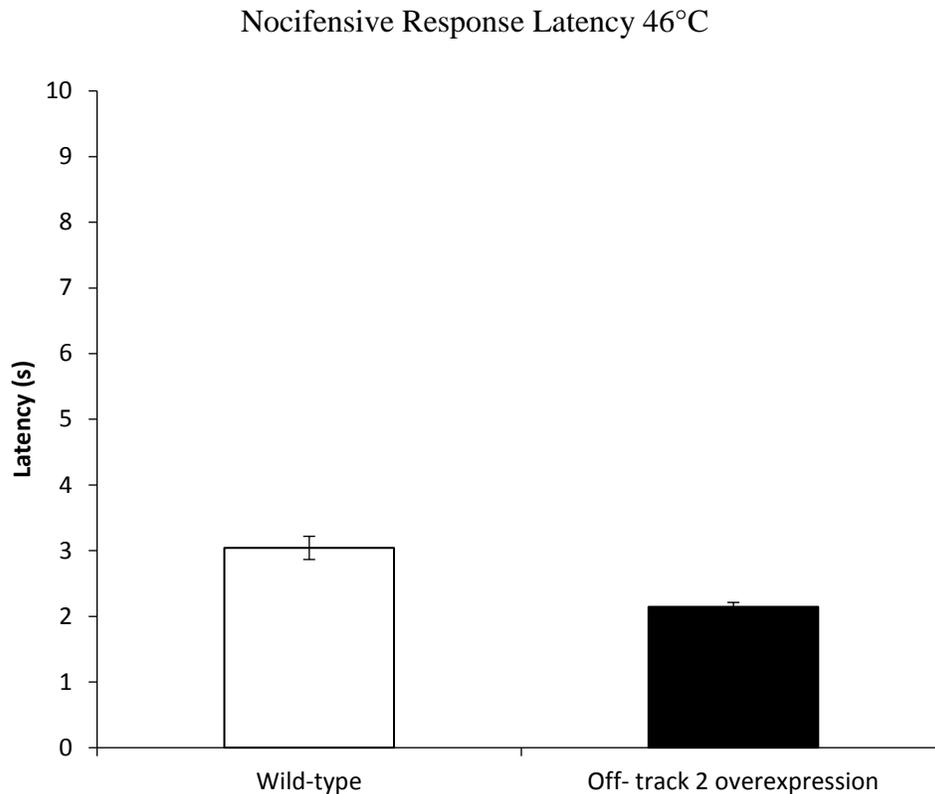


Figure 10. The overexpression of *off-track 2* causes a hypersensitive phenotype

Larval progeny of the listed crosses were tested in a thermal nociception assay at 46°C. The error bars show standard error. n=87, Mann Whitney U shows a significant difference between overexpression of *off-track 2* and wild-type ($p < 0.001$).

Nocifensive Response Latency 46°C

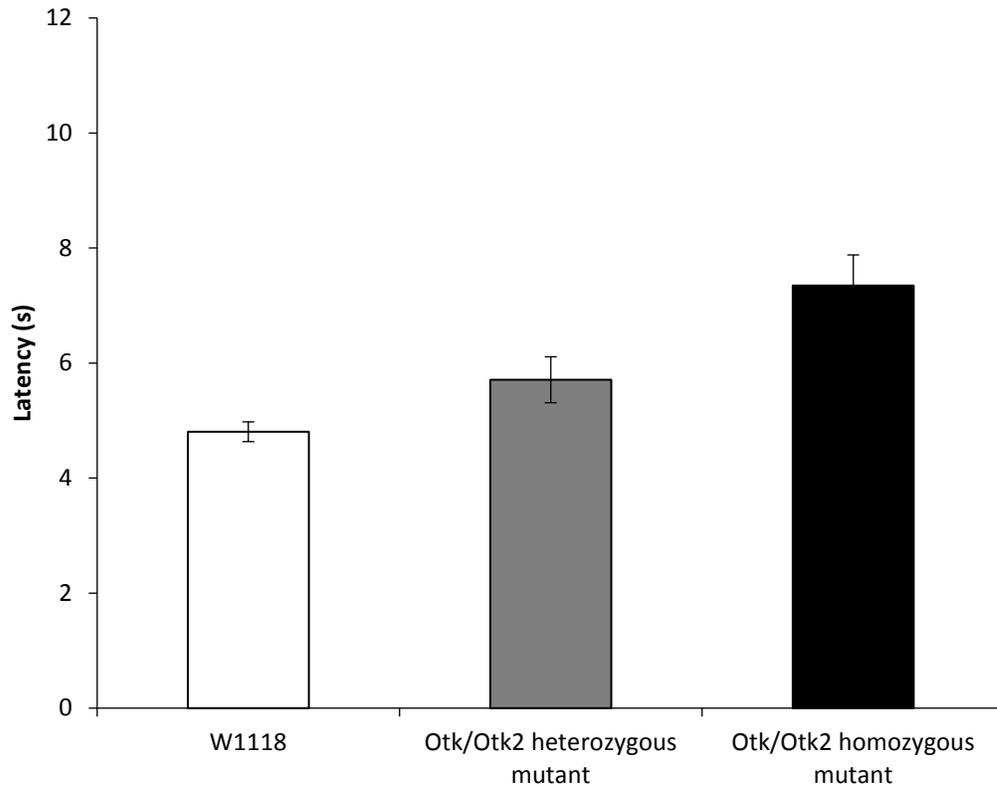


Figure 11. Loss of *off-track* and *off-track 2* function produces an insensitive thermal nociception phenotype

A mutant line for *off-track* and *off-track 2* was crossed to *ppk-dicer 2* and a thermal nociception assay was performed at 46°C. The error bars show standard error. n=80, A Mann Whitney U statistics test was ran on the comparison of *otk,otk2/otk,otk2* to *w1118* and the comparison of *otk,otk2/+* to *otk,otk2/otk,otk2*: ($p=0.002$) and ($p=0.047$) respectively. For multiple comparisons the Bonferroni correction was used. Changing the significance values from 0.05 to 0.025. Which leaves only the *otk,otk2/otk,otk2* comparison to *w1118* with a significant value at ($p=0.002$).

Nociceptor-specific RNAi knockdown of *off-track 2* produced no dendrite morphology phenotype

One of the possible causes for the production of insensitive or hypersensitive phenotypes is a change in dendrite morphology. If dendrite morphology changes the overall body wall coverage of the larvae could be compromised, either having less coverage and potentially producing an insensitive phenotype or having more coverage and producing a hypersensitive phenotype. Crosses were set up using both wild-type and the *off-track 2* RNAi lines. They were both crossed to *ppk-Gal4/Cyo;UAS-GFP/Tm3.Ser*. These crosses provide fluorescence in the Class IV neurons for both the wild-type and the *off-track 2* knockdown, allowing for the dendrite morphology to be analyzed (Figure 12,13).

Nociceptor-specific RNAi knockdown of *off-track 2*, as compared to wild-type, had no significant difference in the total number of dendrite branches (Figure 14). Live images of larval progeny of the listed crosses were using confocal microscopy. These images were analyzed using ImageJ and the plug-in NeuronJ, allowing for the tracing of all dendrites in the field of view for all samples. The production of no morphological phenotype suggests that the insensitive phenotype to noxious thermal temperature was not due to changes in dendritic arborization.

The lack of a difference in total number of dendrite branches in the *off-track 2* RNAi knockdown suggests that it wasn't the total number of branches but it was possibly the total length or coverage provided by the dendrite branches that causes the insensitivity to noxious temperature. To test this, the same samples were used as in Figure 12 but now the total length of dendrites in the field of view was measured in μm . Nociceptor-specific RNAi knockdown of *off-track 2* produces no significant differences in total dendrite length, as compared to wild-type

(Figure 15). The lack of a significant difference in total number of branches or total dendrite length between wild-type and nociceptor-specific knockdown of *off-track 2* indicates that the insensitive phenotype is not due to a change in dendrite morphology.

Lastly to show that dendrite morphology does not play a role in the insensitive phenotype of *off-track 2* the total number of branch points for both conditions were measured. Nociceptor-specific RNAi knockdown of *off-track 2* produced no significant differences in branch points as compared to wild-type (Figure 16). Taking all of the dendrite morphology data together, the hypothesis of dendritic field coverage causing the insensitive phenotype of *off-track 2* was not supported.

Wild-type

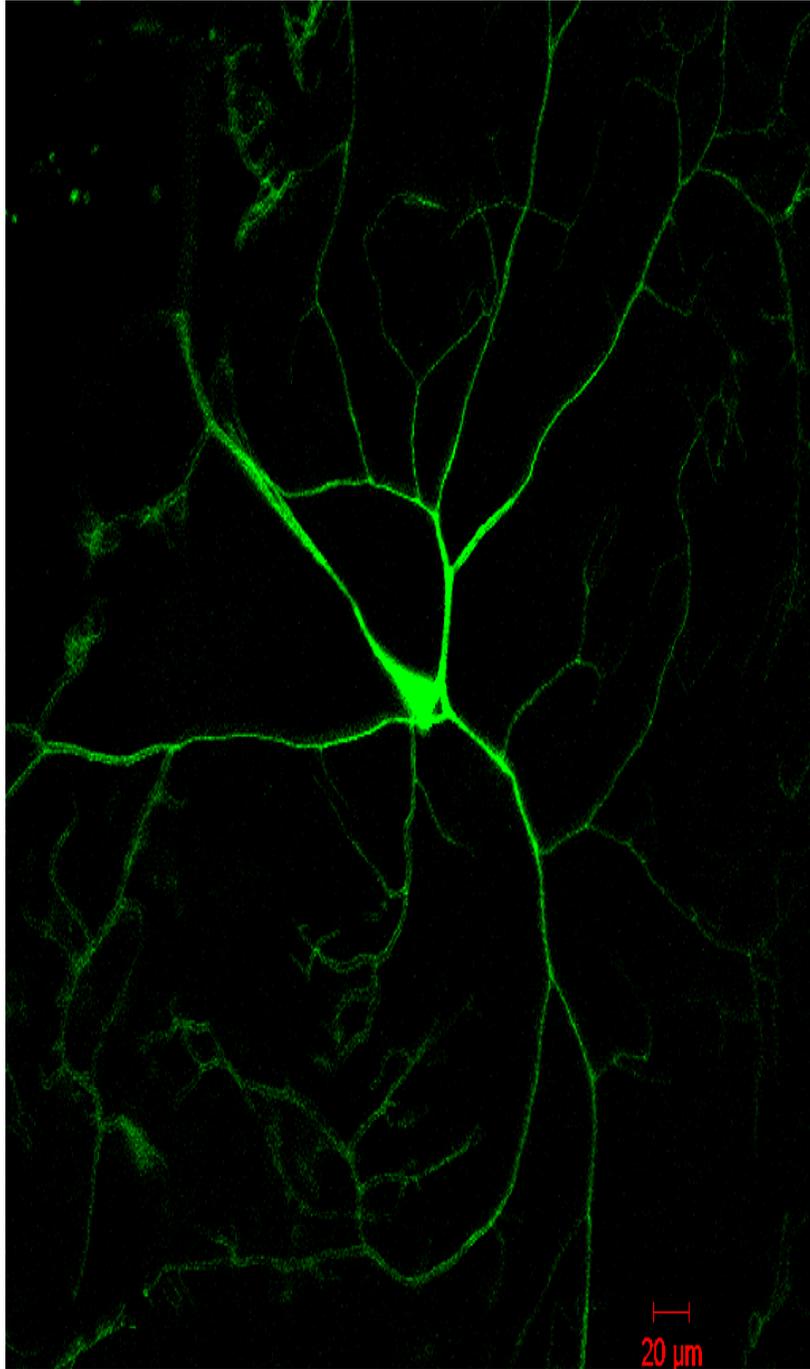


Figure 12. Ppk-Gal4; UAS-GFP/Cyo; sb/Tm3.Ser flies were crossed with wild-type flies and live imaging was performed, n=7.

Off-track 2 RNAi knockdown

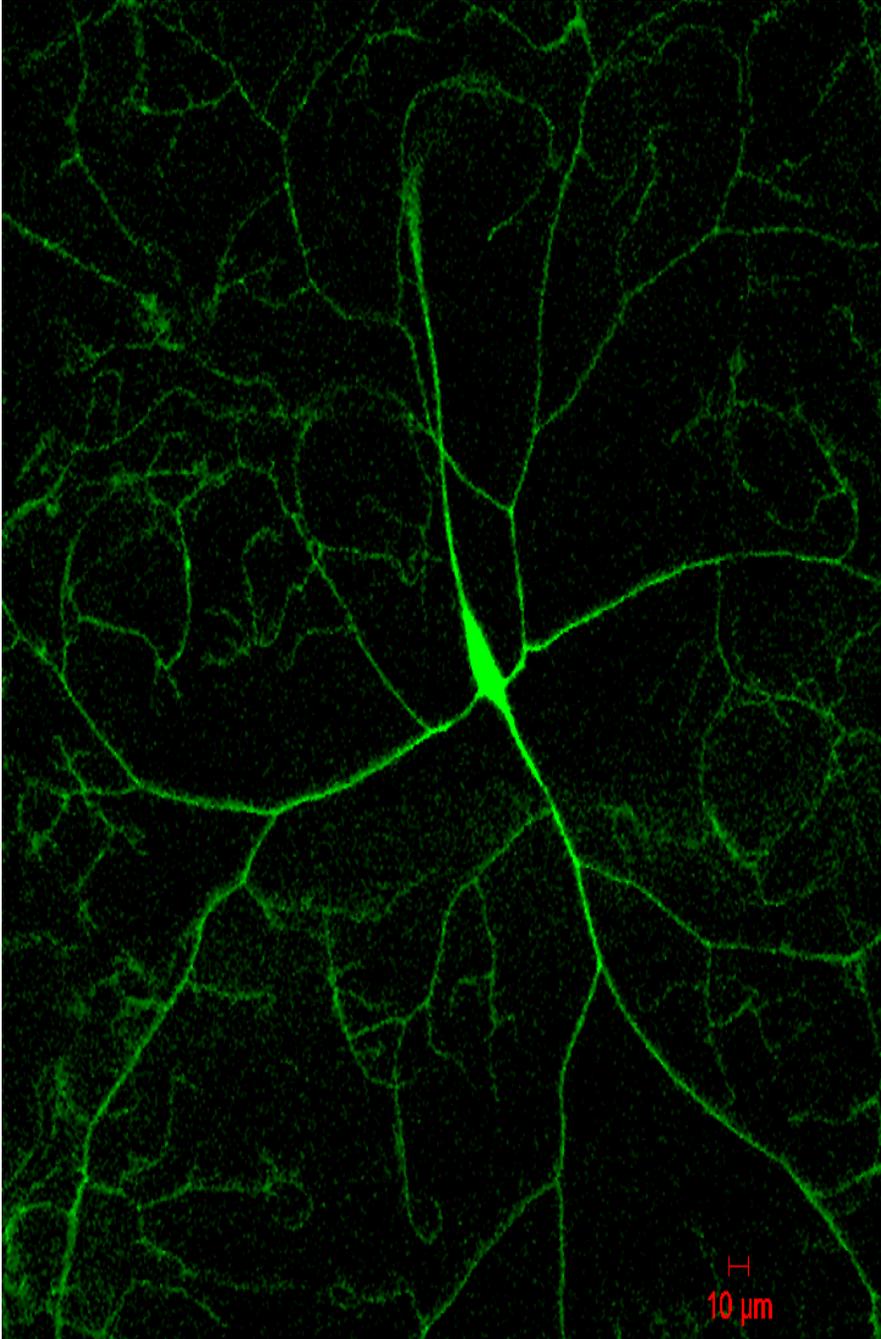


Figure 13. Ppk-Gal4; UAS-GFP/Cyo; sb/Tm3.Ser flies were crossed with *off-track 2* RNAi flies and live imaging was performed, n=4.

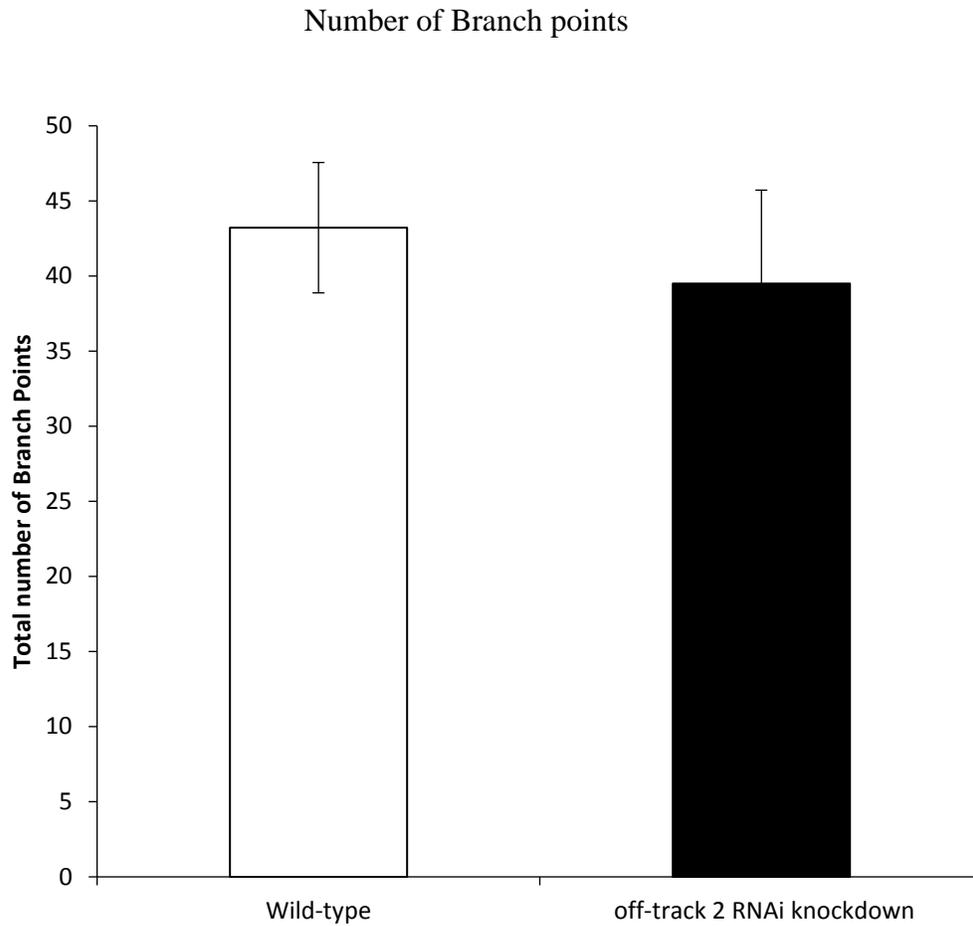


Figure 14. Nociceptor-specific RNAi knockdown of *off-track 2* produces no difference in total number of dendrite branches
 Larval progeny of the listed crosses were live imaged. The error bars show standard error. Wild-type n=9 and *off-track 2* RNAi knockdown n=4. T-test shows no significant differences between off-track 2 knockdown and wild-type total number of dendrite branches, $p=0.606$.

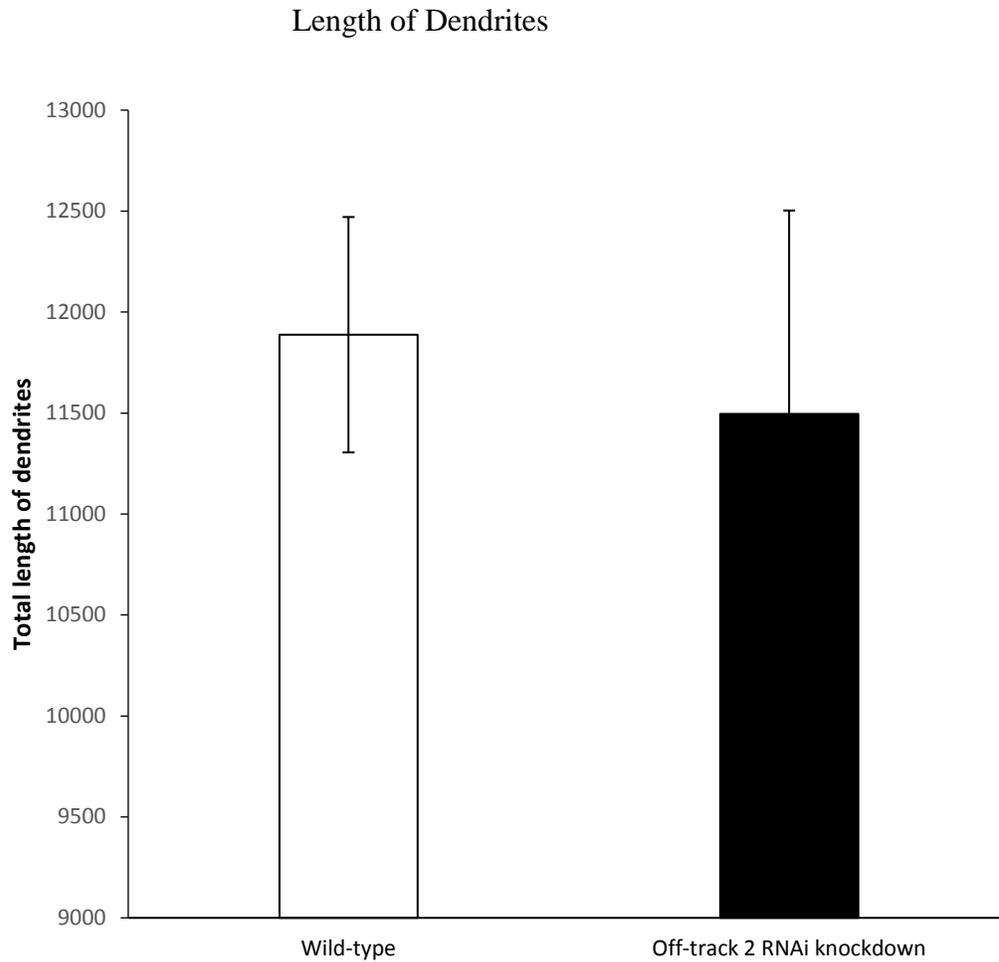


Figure 15. Nociceptor-specific RNAi knockdown of *off-track 2* produces no difference in total length of dendrite branches in µm Larval progeny of the listed crosses were live imaged. The error bars show standard error. Wild-type n=9 and *off-track 2* RNAi knockdown n=4. T-test shows no significant differences between *off-track 2* knockdown and wild-type total length of dendrite branches in µm, $p=0.5309$.

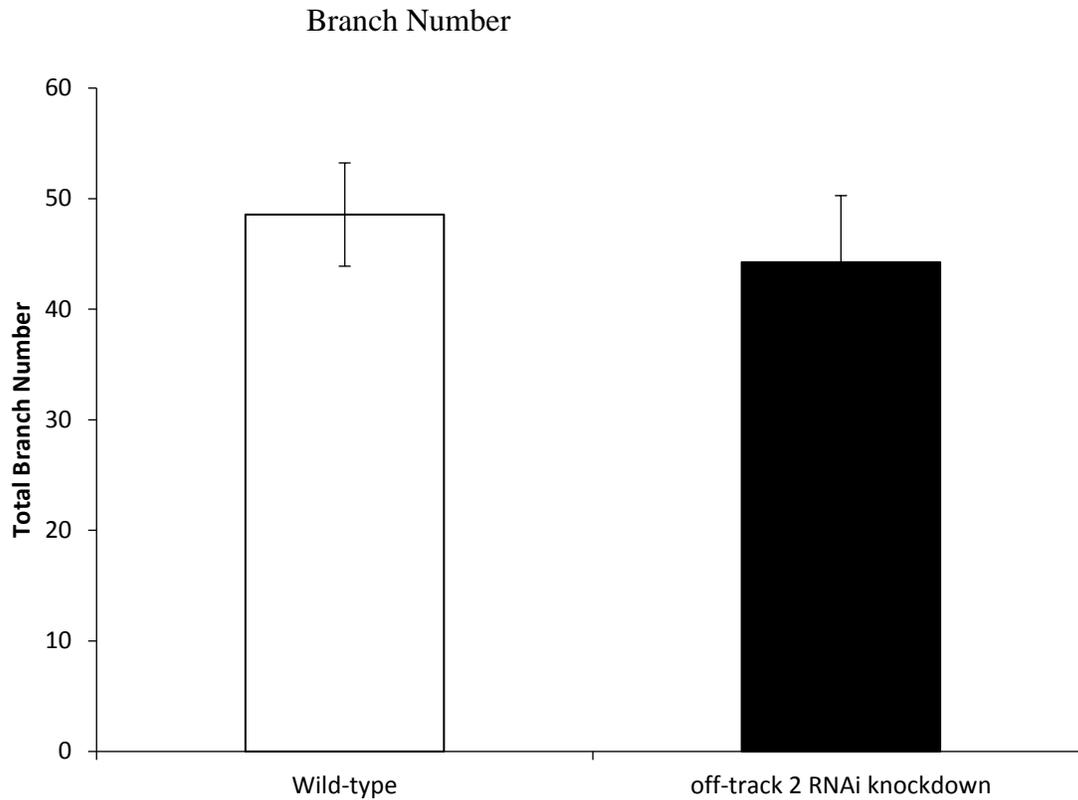


Figure 16. Nociceptor-specific RNAi knockdown of *off-track 2* produces no difference in total branch points of the dendrites. Larval progeny of the listed crosses were live imaged. The error bars show standard error. Wild-type n=9 and *off-track 2* RNAi knockdown n=4. T-test shows no significant differences between *off-track 2* knockdown and wild-type total branch points of the dendrites, $p=0.6394$.

Discussion

Nociceptor-specific RNAi knockdown of Wnt signaling factors produced hypersensitive phenotypes

Hypersensitive phenotypes were produced by the knockdown of six different annotated genes encoding Wnt signaling factors, including: *Frizzled 2*, *Frizzled*, *pangolin*, *Wnt 4*, *hipk*, and *Wnt 2*. After validation it was shown that *Wnt 2* does in fact not have a behavioral phenotype. This hypersensitive phenotype suggests that under normal conditions they play a role in limiting the sensitivity of nociceptors.

Frizzled 2 is a member of the *Frizzled* gene family and encodes a 7-transmembrane domain protein that is a common receptor for the non-canonical calcium Wnt signaling pathway and the PCP Wnt signaling pathway. It has been shown that with coexpression, *Frizzled 2* and *Wnt 5* cause an increase in the release of calcium [42]. In *Drosophila*, the knockdown of *Frizzled 2* leads to a decrease in planar cell polarity-mediated cell polarization in the development of cilia in wing cells [41,82,83].

It has also been shown that *Frizzled 2* calcium signaling is required for determining the final differentiated state of a set of dopaminergic neurons in *Drosophila* [76]. Knocking down or inhibiting *Frizzled 2* signaling during the maturation of the flight circuit affects the maintenance of flight [76]. This is only true when *Frizzled 2* signaling is disrupted in pupal stages. When *Frizzled 2* is knocked down in adult flies the flies will still show a normal flight behavior [76]. These results suggest that *Frizzled 2* serves as a requirement for normal adult behavior. The role *Frizzled 2* has in the development of behavioral phenotypes raises the possibility that it could also be essential for the development of other essential behaviors, such as NEL.

Posap/Shank scaffolding proteins regulate the formation, organization, and plasticity of excitatory synapses [77]. In *Drosophila*, *shank* is enriched at the postsynaptic membranes of glutamatergic neuromuscular junction [77]. *Shank* regulates non-canonical Wnt signaling in the postsynaptic cell by internalizing Frizzled 2 [77]. If Frizzled 2 is internalized in nociceptors this would lessen the amount of possible receptors for Wnt 2 to bind to. This could lead to the limiting of the sensitivity of nociceptors and possibly to a hypersensitive phenotype if presented with a noxious stimulus.

Not only does *Frizzled 2* play a role in synapse formation, CNS organization, and plasticity, it also plays a role in left-right asymmetry in internal organs [78]. Specifically, genes encoding for Frizzled 2 and Wnt4 are required for left-right asymmetric development of the embryonic midgut (AMG) in *Drosophila* [78]. *Arrow*, an ortholog of the mammalian gene encoding LRP5/6 is also essential for left-right asymmetric development of the AMG [78]. Specifically, Frizzled 2, Wnt 4, and *arrow* are present in the visceral muscle of the midgut, where *Drosophila* embryos lacking any of these components had unsuccessful left-right asymmetric rearrangement [78]. Demonstrating that the Wnt β -catenin signaling pathway is essential for asymmetry in internal organs. The development of asymmetry could potentially also be in other tissue types where it is seen as cell specification and or pattern formation. If this occurred in Class IV neurons it could be the cause of the hypersensitivity phenotype, potentially due to a change in pattern formation. This change could be a change in location or number of Class IV neuron cell bodies allowing the larvae to become more sensitive to noxious thermal temperatures.

When *Frizzled*, a receptor for the Wnt β -catenin signaling pathway, was knocked down using nociceptor-specific RNAi it produced a hypersensitive phenotype. In previous research the

ability of Frizzled to function preferentially in the Wnt β -catenin signaling pathway was shown through Frizzled's ability to induce expression of *Xnr-3*, a TCF/LEF target gene in zebrafish [42]. This suggests that the hypersensitive phenotype could be from the loss of function of the Wnt β -catenin signaling pathway.

Western Blot analysis of *Drosophila* larval lysates shows that Frizzled is required for Vang phosphorylation [79]. Vang is a key component of the Wnt signaling pathways that is essential for regulating planar cell polarity and cell fate [79]. This interaction importantly occurs within the same cell and does not depend on Dvl [79]. Frizzled induced Vang phosphorylation is a mechanism to produce asymmetric localization of PCP Wnt signaling pathway components [79]. It is possible that this interaction could lead to the localization of off-track 2 or even the localization of TRPA1 to the membrane of nociceptors. This localization could lead to the sensitization of Class IV neurons.

Through the PCP Wnt signaling pathway, epithelia-derived wingless regulates dendrite directional growth in *Drosophila* Class IV neurons [80]. *Frizzled* is expressed in Class I neurons in both embryos and third instar larvae [80]. In *Frizzled* mutants a significant decrease in the PD-angle was observed, similarly to *Wg* mutant larvae [80]. The PD-angle is the angle from which the dendrite grows from the branch point. The overexpression of *Frizzled* in the *Frizzled* mutant background showed partial rescue of the decrease in PD-angle seen in the mutant [80]. This demonstrates that *Wg* and Frizzled both play a role in the direction of which dendrites grow. Rac 1 functions downstream in PCP Wnt signaling pathway and when it was knocked down in a *wg* mutant background the PD-angle increased significantly. Thus demonstrating that the PCP Wnt signaling pathway, through Wnt, Frizzled, and Rac1, is responsible for the directionality of dendrite growth in *Drosophila* embryos and third instar larvae. This could

potentially play a role in nociception, in that this same mechanism could be present in Class IV neurons. This would allow Class IV neurons to provide full coverage, and the ability to detect noxious stimuli successfully. But, when loss of function of *Frizzled* occurs, specifically in the nociceptor-specific RNAi knockdown, it could lead to improper directional growth of the Class IV neurons and thus improper coverage leading to the hypersensitive phenotype observed.

Similarly to *Frizzled 2*, *Vang* role in left-right asymmetrical visceral muscle cells orientation in the midgut of *Drosophila*, *Frizzled* and *Vang* define anterior and posterior apical membrane domains prior to sensory organ precursor cells (SOP) division [81]. Genetic live imaging, live imaging of larvae, analyses in *Drosophila* suggest that Dsh restricts the localization of centrosome-attracting activity through *Vang* [81]. *Frizzled* and *Vang* genes act redundantly in SOPs to orient polarity axis through the PCP Wnt signaling pathway [81]. This mechanism could potentially affect the adoption of the distinct identities of the daughter cells. Each SOP cell follows a series of four asymmetrical cell divisions producing four external sense organ cell and one multidendritic neuron. If this mechanism is disrupted the daughter cells could adopt the wrong identity or could not be produced at all. If no multidendritic neuron is produced this would severely affect nociception.

Pangolin (pan) a transcription factor, is activated once β -catenin makes its way into the nucleus. *Pan* has been shown to play a role in the developing wing of *Drosophila melanogaster*. Specifically, in cell proliferation, wing margin specification, and Wg self-refinement [84]. The function of *Pans* is required for all three of these processes [84]. Loss-of-function of *pan* causes a severe reduction in the activity of the Wnt pathway and the target genes *distalless* and *vestigial*. *Distalless* is a homeobox gene that plays a role in development of the peripheral nervous system of *Drosophila melanogaster* [85]. The hypersensitive phenotype of

pan that was produced during the nociceptor-specific RNAi knockdown suggests that *pan* is expressed in nociceptors and plays a role in nociceptor function. The probable cause of the hypersensitive phenotype is that when *pan* is knocked down in Class IV neurons the development of these neurons is altered. Or, with the absence of *pan* the activation of Wnt target genes no longer occurs and it causes the Class IV neurons to lose the Wnt pathway that limits sensitivity and thus the hypersensitive phenotype is seen.

Wnt 4 provides directional input to the PCP Wnt signaling pathway and is necessary for cell polarity and orientation in *Drosophila melanogaster* [66]. *Wnt 4* affects the PCP Wnt signaling pathway by modulating the intercellular interactions between Frizzled and Vang [66]. This interaction is thought to be one of the key steps in establishing directionality in the PCP Wnt signaling pathway [66]. When *Wnt 4* is not present this interaction does not occur and the PCP Wnt signaling pathway can go astray. During the nociceptor-specific RNAi knockdown of *Wnt 4* a hypersensitive phenotype was observed. This could be due to improper activation or function of the PCP Wnt signaling pathway, leading to the improper directionality and polarity of cells in the peripheral nervous system [86,70,89,90,91]. This could in turn cause the hypersensitive phenotype.

Another role for *Wnt 4* is its ability to bind to Frizzled 3 in the PCP Wnt signaling pathway [90]. This binding of *Wnt 4* to Frizzled 3 in mammals has been seen to guide commissural axons along the anterior-posterior axis of the spinal cord [90]. The ability to have proper guidance could be the reason why we see an hypersensitive phenotype in both *Wnt 4* RNAi knockdown as well as for the *Frizzled 3* RNAi knockdown.

Wnt 2 has been shown to be a ligand for the PCP Wnt signaling pathway [72]. Its role in this pathway is to bind to off-track, off-track 2, co-receptors for the PCP Wnt signaling pathway,

Frizzled, and Frizzled 2 [72]. The behavioral result of nociceptor-specific RNAi knockdown of *Wnt 2* implies that *Wnt 2* is in nociceptors, so was *off-track*, *off-track 2*, *Frizzled*, and *Frizzled 2*. Three of these genes produced a phenotype, while *off-track* was the only one where no phenotype was observed. This suggests that the PCP Wnt signaling pathway is located in nociceptors and could potentially play a role in nociceptor function.

Wnt 2 has also been shown to play a role in presynaptic motor neuron morphology and presynaptic protein localization at the neuromuscular junction (NMJ) in *Drosophila melanogaster* [91]. Mutations in *Wnt 2* produce an increase in the presynaptic branch number and a decrease in a presynaptic protein, synaptobrevin [91]. *Wnt2* needs to be tested again to ensure that the lack of phenotype is correct. It is possible that *Wnt2* knockdown does have a hypersensitive phenotype and that could be due to an increase in activation of the PCP Wnt signaling pathway but due to dendrite morphology changes in the NMJ. The increase in branch number in the NMJ could potentially cause the hypersensitization of the nociceptor due to the production of more connections.

Nociceptor-specific RNAi knockdown of Wnt signaling factors produced insensitive phenotypes

Six insensitive phenotypes were produced from the initial genetic screen involving Wnt signaling factors, including: *Wnt 10*, *Frizzled 3*, *Pontin*, *Nek 2*, *Off-track 2* and *Ank 2* (Table 4). *Wnt 10* has been shown to bind to *Frizzled 4* in the CNS and play a role in its development [91]. *Frizzled 4* is a known receptor for the Wnt β -catenin signaling pathway, suggesting that *Wnt 10* is a ligand for the Wnt β -catenin signaling pathway [91]. The production of an insensitive phenotype during the nociceptor-specific RNAi knockdown of *Wnt 10* suggests that *Wnt 10*

could possibly be the main ligand for the β -catenin Wnt signaling pathway. When Wnt 10 is not present the Wnt β -catenin signaling pathway is not activated and the larvae become insensitive to the noxious thermal stimuli. Thus our data demonstrates a role for the Wnt β -catenin signaling pathway in nociceptor function in *Drosophila melanogaster*.

Mutations in *off-track 2* functions produced an insensitive phenotype

The nociceptor-specific RNAi knockdown of *off-track 2* produces an insensitive phenotype when presented with a noxious thermal stimulus (Figures 4, 8). *Off-track* and *Off-track 2* have been shown to interact with Frizzled and Frizzled 2 during *Drosophila* embryogenesis [72]. PTK7 is also known to inhibit the activation of Wnt β -catenin signaling pathway and be a receptor for the PCP Wnt signaling pathway [72]. *Off-track 2* is a homolog to vertebrate PTK7 and is a co-receptor for the PCP Wnt signaling pathway [91]. The overexpression of *off-track 2* produces a hypersensitive phenotype. Taken together, the RNAi knockdown phenotype and the overexpression hypersensitive phenotype show that *off-track 2* plays a role in nociceptor function. Because *off-track* and *off-track 2* are known to dimerize together a double mutant was obtained and tested [72].

The double mutant produced a phenotype for only the homozygous mutant of *off-track* and *off-track 2* (Figure 10.) The double mutant when compared to nociceptor-specific RNAi knockdown of *off-track 2* had a similar insensitive phenotype. This confirms that *off-track 2* is essential for increasing nociceptor sensitivity and baseline levels. This raised the question as to why only the homozygous mutant produced a phenotype and why *off-track* nociceptor-specific RNAi knockdown did not produce a phenotype. The homozygous double mutant could have a

more severe insensitive phenotype due to the loss of function of both *off-track* and *off-track 2*. To validate this *off-track* needs to be retested in nociceptor-specific RNAi.

Dendrite morphology and nociceptor sensitivity

One way that the loss of function of a gene can produce an insensitive or hypersensitive phenotype is by causing a change in the dendrite morphology. Whether that be in total number of dendrite branches, length, or branch points, a defect in any of those categories would potentially cause an issue with nociceptor function. To see if dendrite morphology played a role in the *off-track 2* phenotype live imaging was performed. The comparison of *off-track 2* to wild-type dendrite morphology produced no significant differences. Due to there being no significant defects in dendrite morphology, the possibility of change in field coverage and asymmetrical cell divisions causing the insensitive phenotype seen when knocking down *off-track 2* is not probable. This leads to the possibility that a change in neural physiology or sensory transduction is the probable cause to the insensitive phenotype seen in the nociceptor-specific knockdown of *off-track 2*.

Future directions

The other hypersensitive and insensitive hits from the genetic screen of Wnt signaling factors will be validated. This will be done by retesting the RNAi lines that were originally tested and by acquiring new RNAi lines for the same genes.

The next experiment to be done would be to combine the *Frizzled 2* RNAi line with the *off-track 2* RNAi line. This would knockdown both genes in a nociceptor-specific manner and would clarify how *off-track 2* and *Frizzled 2* interact with each other. There are three possible

outcomes for this experiment: 1) when you knockdown both *Frizzled* and *off-track 2* and *Frizzled* is downstream of *off-track 2* a hypersensitive phenotype would be observed; 2) when you knockdown both *Frizzled* and *off-track 2* and *off-track 2* is downstream of *Frizzled* an insensitive phenotype would be observed; 3) when you knockdown both *Frizzled* and *off-track 2* and there is no interaction between the two so there is no phenotype observed. *Frizzled 2* has a hypersensitive phenotype, it limits nociceptor sensitivity, and *off-track 2* has an insensitive phenotype, it raises nociceptor sensitivity. It would make sense that they antagonize each other under baseline conditions to keep nociceptors at a homeostatic level of sensitivity. Biochemical methods could be used to look at the possible interactions between *Frizzled 2* and *off-track 2*.

The downstream effectors that are activated once *off-track 2* is activated are not known. To characterize this pathway other RNAi lines will be used to knockdown genes in an *off-track 2* RNAi knockdown. *Off-track 2* will be knocked down first and then possible downstream effectors will be knocked down in the same fly. If the downstream effector that is knocked down produces a stronger insensitive phenotype or a similar insensitive phenotype as compared to *off-track 2* knocked down alone then that downstream effector is in fact part of the *off-track 2* pathway leading to the sensitization of nociceptors. Specifically, *Vang* will be re-tested to see if it is a downstream effector of *off-track 2*. This is due to *Vang*'s already known function in directional growth and asymmetrical cell divisions. Based on what is known about *PTK71* I predict that possible downstream effectors of *off-track 2* will be part of the PCP Wnt signaling pathway.

Dendrite morphology can be reassessed by performing dissections of wild-type and nociceptor-specific RNAi knockdown of *off-track 2* larvae. This will provide better images than

live imaging and will increase our sample size to get a better idea of the effects that loss of function of *off-track 2* has on dendrite morphology.

The localization of *off-track 2* can be determined by performing dissections on a cross that has *off-track 2* tagged with GFP. These dissections will then be stained with GFP antibodies and with Class IV neuron specific antibodies. This will allow for the visualization of the subcellular localization of *off-track 2* and demonstrate where *off-track 2* is expressed in Class IV neurons. The expression location of *off-track 2* could determine what specifically *off-track 2* role is in nociceptor function. For instance if it is expressed in the dendrites it could be responsible for detecting noxious stimuli directly, but if it is found in the soma than maybe it receives signals from the periphery and then induces the sensitization of nociceptors.

Conclusion

From the 53 total Wnt signaling genes tested in the original genetic screen six RNAi lines targeting 6 genes were identified as having a hypersensitive phenotype: *Frizzled 2*, *Frizzled*, *Pangolin*, *Wnt 4*, *Hipk*, and *Wnt 2*. While, 6 RNAi lines targeting 6 genes were identified in having insensitive phenotypes: *Wnt 10*, *Frizzled 3*, *pontiin*, *Nek2*, *off-track 2*, and *Ank 2*. The validation confirmed that in nociceptor-specific RNAi knockdown of *Frizzled* and *Frizzled 2* produce a hypersensitive phenotype. This confirms that at baseline levels *Frizzled* and *Frizzled 2* lower sensitivity of nociceptors. The confirmation of *off-track 2* confirms that when knocked down a hypersensitive phenotype is produced. In an *off-track 2* mutant an insensitive phenotype was seen and was more severe than with the RNAi knockdown. To see if this insensitive phenotype was due to a change in dendrite morphology wild-type and *off-track 2* knockdown larvae were imaged using confocal microscopy. No differences were seen in total dendrite length, total number of branch points, and total number of dendrites. Because there was no

change in dendrite morphology that lead us to create a model for what *off-track 2* role is in nociception.

All of my results taken together have developed into a model for *off-track 2* role in increasing nociceptor sensitivity and the roles that *Frizzled* and *Frizzled 2* have in nociceptor function (Figure 17). We now know that *off-track 2* is located in nociceptors and has a insensitive phenotype, where at baseline it is responsible for increasing nociceptor sensitivity. We also know that both *Frizzled* and *Frizzled 2* have hypersensitive phenotypes, where at baseline they limit nociceptor sensitivity. Once the future directions of my project are complete we will have a better understanding of how *off-track 2* is increasing nociceptor sensitivity and what downstream effectors are responsible.

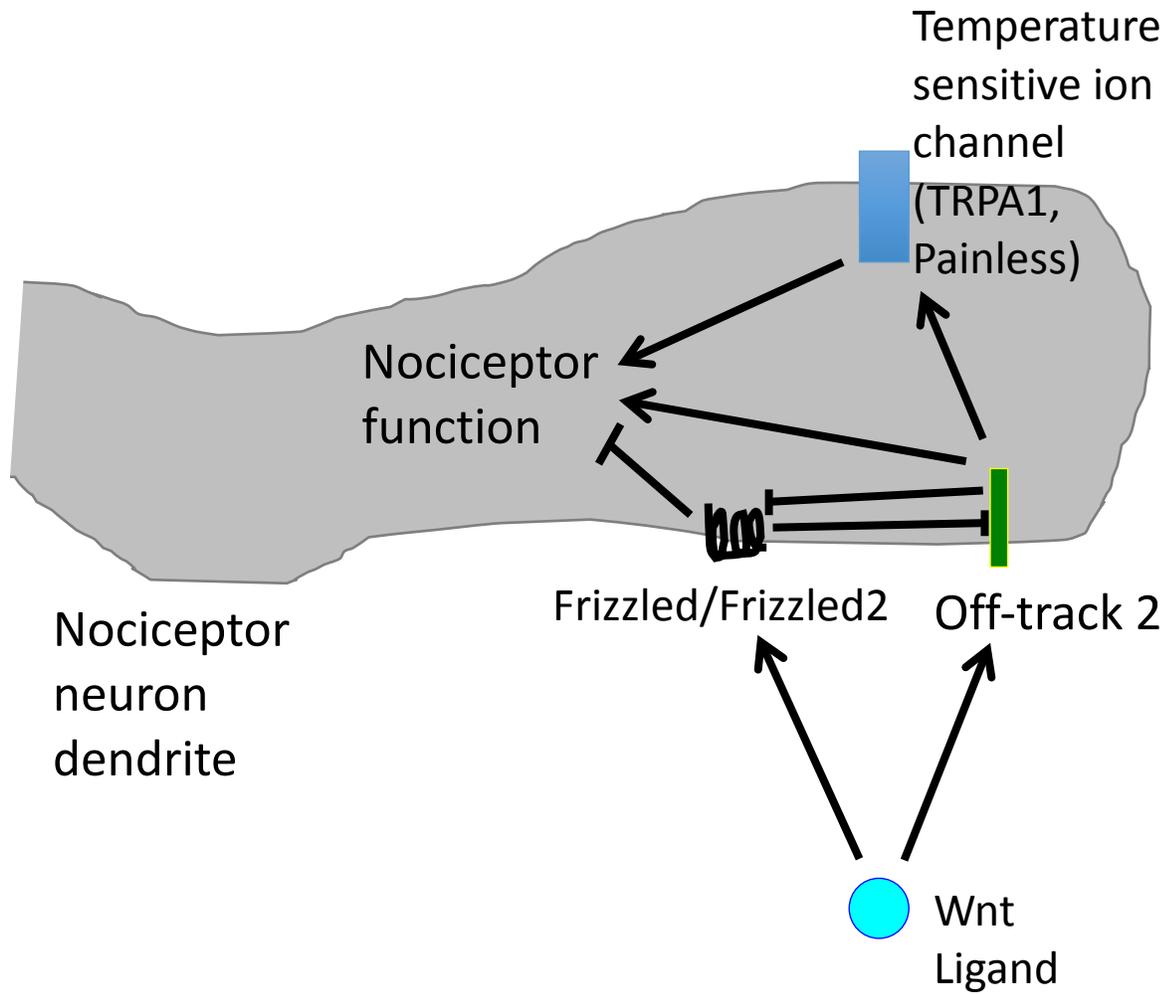


Figure 17. Model for *off-track 2* role in nociceptor function. In the presence of a Wnt ligand *off-track 2* will increase nociceptor function either directly or through a temperature sensitive ion channel. *Frizzled* and *Frizzled 2* limit nociceptor function and potential antagonize *off-track 2*.

References

- [1]. Institute of Medicine. Relieving pain in America, (2011) 1-36.
- [2]. Z. Lixian, A. Bellemer, H. Yan, K. Honjo, J. Robertson, R. Hwang, G. Pitt, W.D. Tracey, Thermosensory and non-thermosensory isoforms of *Drosophila melanogaster* TRPA1 reveal heat sensor domains of a thermo TRP channel, *Cell Rep.* 1 (2012) 43-55.
- [3]. P. Bessou, E.R. Perl, Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli, *J Neurophysiology*, 32 (1969) 1025-1043.
- [4]. W.D. Tracey, J.R. Wilson, R.I. Laurent, G. Benzer, Painless, a *Drosophila* gene essential for nociception, *Cell.* 113 (2003) 261-273.
- [5]. R.Y. Hwang, L. Zhong, Y. Xu, T. Johnson, F. Zhang, K. Deisseroth, W.D. Tracey, Nociceptive neurons protect *Drosophila* larvae from parasitoid wasps, *Curr Biol* 17 (2007) 2105-2116.
- [6]. M. Vervoort, D. Merritt, A. Ghysen, C. Dambly-Chaudiere C, Genetic basis of the formation and identity of type I and type II neurons in *Drosophila* embryos, *Dev.* 14 (1997) 2819-2828.
- [7]. R. Bodmer, Y.N. Jan, Morphological differentiation of the embryonic peripheral nervous system in *Drosophila*, *Roux's Arch, Dev. Biol.* 196 (1987) 69-77
- [8]. F.N. Hamada, M. Rosenzweig, K. Kang, S.R. Pulver, A. Ghezzi, T.J. Jegla, P.A. Garrity, An internal thermal sensor controlling temperature preference in *Drosophila*, *Nature.* 454 (2008) 217-220.
- [9]. M. Rosenzweig, K. Brennan, T. Tayler, P. Phelps, A. Patapoutian, P.A. Garrity, The *Drosophila* ortholog of vertebrate TRPA1 regulates thermotaxis, *Genes Dev.* 19 (2005)

419–424.

- [10]. K.Y. Kwan, A.J. Allchorne, M.A. Vollrath, A.P. Christensen, D.S. Zhang, C.J. Woolf, D.P. Corey, TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction, *Neuron*. 50 (2006) 277-289.
- [11]. D.M. Bautista, S.E. Jordt, T. Nikai, P.R. Tsuruda, A.J. Read, J. Poblete, E.N. Yamoah, A.I. Basbaum, D. Julius, TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents, *Cell*. 124 (2006) 1269-1282.
- [12]. D.M. Bautista, P. Movahed, A. Hinman, H.E. Axelsson, O. Sterner, E.D. Hogestatt, D. Julius, S.E. Jordt, P.M. Zygmunt, Pungent products from garlic activate the sensory ion channel TRPA1, *Proc Natl Acad Sci USA*. 102 (2005) 12248-12252.
- [13]. M. Bandell, G.M. Story, S.W. Hwang, V. Viswanath, S.R. Eid, M.J. Petrus, T.J. Earley, A. Patapoutian, Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin, *Neuron*. 41 (2004) 849-857.
- [14]. B. Minke, The TRP channel and phospholipase C-mediated signaling, *Cell Mol Neurobiology*. 21 (2001) 629-643.
- [15]. V. Fattori, M. Hohmann, A. Rossaneis, F. Ribeiro, W. Verri, Capsaicin: Current understanding of its mechanisms and therapy of pain and other pre-clinical and clinical uses, *Molecules*. 21 (2016) 844.
- [16]. K.A. Sluka, W.D. Willis, The effects of G-protein and protein kinase inhibitors on the behavioral responses of rats to intradermal injection of capsaicin, *Pain*. 71 (1997) 165-178.
- [17]. B. Frias, A. Merighi, Capsaicin, Nociception and pain, *Molecules*. 21 (2016) 6.
- [18]. M.J. Caterina, A. Leffler, A.B. Malmberg, W.J. Martin, J. Trafton, K.R. Peterson-

- Zeit, M. Koltzenburg, A.I. Basbaum, D. Julius, Impaired nociception and pain sensation in mice lacking the capsaicin receptor, *Science*. 5464 (2000) 306-313.
- [19]. D.G. Snijdelaar, R. Dirksen, R. Slappendel, B.J. Crul, Substance P, *Eur J pain*, 2 (2000) 121-135.
- [20]. K. Marzena, M. Ewa, L. Andrzej, Substance P and its receptors- a potential target for novel medicines in malignant brain tumor therapies, *Folia Neuropathologica*. 3 (2007) 99-107.
- [21]. P. Honore, P.M. Menning, S.D. Rogers, M.L. Nichols, A.I. Basbaum, J.M. Besson, P.W. Mantyh, Spinal substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states, *J Neurosci*. 17 (1999) 7670-7678.
- [22]. S. Khasabov, J. Rogers, J. Ghilardi, C. Peters, P. Mantyh, D. Simone, Spinal neurons that possess the substance P receptor are required for the development of central sensitization, *J Neurosci* 20 (2002) 9086-9098.
- [23]. M. Nichols, B. Allen, S. Rogers, J. Ghilardi, P. Honore, N. Luger, M. Finke, J. Li, D. Lappi, D. Simone, P. Mantyh, Transmission of Chronic nociception by spinal expressing the substance P receptor, *Science*. 286 (1999) 1558-1561.
- [24]. L. Koehler, International review of Neurobiology, *Pain*. 35 (1987) 299.
- [25]. A. Kohn, R.T. Moon, Wnt and Calcium signaling: β -catenin-independent pathways, *Cell Calcium*. 38 (2005) 439-446.
- [26]. C.Y. Logan, R. Nusse, The Wnt signaling pathway in development and disease, *Annu. Rev. Cell Dev. Biol.* 20 (2004) 781-810.
- [27]. V. Amerongen, R. Nusse, Towards an integrated view of Wnt signaling

- in development, *Dev.* 19 (2009) 3205-3214.
- [28]. Y.S. Li, Y. Xi, X.J. Li, C.L. Leng, M.M. Jia, W. Zhang, H.B. Tang, Up-Regulation of the Biosynthesis and Release of Substance P through Wnt/ β -Catenin Signaling Pathway in Rat Dorsal Root Ganglion Cells, *PLoS ONE*. 10 (2006) 6.
- [29]. Y.K. Zhang, Z.J. Huang, S. Liu, Y.P. Liu, A. Song, X.J. Song, Wnt signaling underlies the pathogenesis of neuropathic pain in rodents, *JCI*. 123 (2016) 5.
- [30]. H. Aberle, A. Bauer, J. Stappert, A. Kispert, R. Kemler, **b**-Catenin is a target for the ubiquitin–proteasome pathway, *EMBO J.* 16 (1997) 3797-3804.
- [31]. E.H. Jho, T. Zhang, C. Domon, C.K. Joo, J.N. Freund, F. Costantini, Wnt/beta-catenin/tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway, *Mol Cell Biol.* 22 (2002) 1172-1183.
- [32]. P. Polakis, The many Ways of Wnt in cancer, *Cur Opin Genet Dev.* 17 (2007) 45-51.
- [33]. Y. Komiya, R. Habas, Wnt signaling transduction pathways, *Organogenesis*. 4 (2008) 68-75.
- [34]. A. Hurlstone, H. Clevers, T-cell factors: turn-ons and turn-offs, *EMBO J.* 21 (2002) 2303–2311.
- [35]. C. Kiecker, C. Niehrs, A morphogen gradient of Wnt/beta-catenin signaling regulates anteroposterior neural patterning in *Xenopus*, *Development*. 128 (2001) 4189-4201.
- [36]. M. Carter, X. Chen, B. Slowinska, S. Minnerath, S. Glickstein, L. Shi, F. Campagne, H. Weinstein, M.E. Ross, Crooked tail (Cd) model of human folate-responsive neural tube defects is mutated in Wnt coreceptor lipoprotein receptor-related protein

- 6, Proc Natl Acad Sci U S A. 102 (2005) 12843–12848.
- [37]. M.E. Dickinson, R. Krumlauf, A.P. McMahon, Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system, *Development* 120 (1994) 453–1471
- [38]. C.E. Clark, C.C. Nourse, H.M. Cooper, The tangled web of non-canonical Wnt signalling in neural migration, *Neurosignals*. 20 (2012) 202–220
- [39]. N.D. Okerlund, B.N. Cheyette, Synaptic Wnt signaling-a contributor to major psychiatric disorders, *J Neurodev Disord*. 3 (2011) 162–174.
- [40]. T. Shimogori, J. VanSant, E. Paik, E.A. Grove, Memebers of the Wnt, Fz, and Frp gene families expressed in postnatal mouse cerebral cortex, *J. Comp. Neurol*. 473 (2004) 496-510.
- [41]. R. Nusse, Wnt signaling and stem cell control, *Cell Res*. 18 (2008) 523-527.
- [42]. C.J. Zhou, C. Zhao, S.J. Pleasure, Wnt signaling mutants have decreased dentate granule cell production and radial glial scaffolding abnormalitites, *J. Neurosci*. 24 (2004) 121-126.
- [43]. E.M. Wexler, A. Paucer, H.I. Kornblum, T.D. Plamer, D.H. Geschwind, Endogenous Wnt signaling maintains neural progenitor cell potency, *Stem Cells*. 27 (2009) 1130-1141.
- [44]. W. Cerpa, J.A. Godoy, I. Alfaro, G.G. Farias, M.J. Metcalfe, R. Fuentealba, C. Bonansco, N.C. Inestrosa, WNT-7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons, *J. Biol Chem*. 283 (2008) 5918-5927.
- [45]. A. Ahmad-Annur, L. Ciani, I. Simeonidis, J. Herreros, N. Fredj, S. Rosso, A. Hall, S. Brickley, P. Salinas, Signaling across the synapse: a role for Wnt and

- Dishevelled in presynaptic assembly and neurotransmitter release, *J. Cell Biol.* 174 (2006) 27-139.
- [46]. M. Simonetti, N. Agarwal, S. Stosser, K.K. Bali, E. Karaulanov, R. Kamble, B. Pospisilova, M. Kurejova, W. Birchmeier, C. Niehrs, P. Heppenstall, R. Kuner, Wnt-Fzd signaling sensitizes peripheral sensory neurons via distinct noncanonical pathways, *Neuron.* 83 (2014) 104-121.
- [47]. A. Kohn, R.T. Moon, Wnt and calcium signaling: β -catenin-independent pathways, *Cell Calcium.* 38 (2005) 439-446.
- [48]. D.C. Slusarski, F. Pelegri, Calcium signaling in vertebrate embryonic patterning and morphogenesis, *Dev Biol.* 307 (2007) 1-13.
- [49]. D.C. Slusarski, V.G. Corces, R.T. Moon, Interaction of Wnt and a Frizzled homologue triggers a G-protein-linked phosphatidylinositol signaling, *Nature.* 390 (1997) 410-413.
- [50]. L.C. Sheldahl, D.C. Slusarski, P. Pandur, J.R. Miller, M. Kuhl, R.T. Moon, Dishevelled activates Ca^{2+} flux, PKC, and CamKII in vertebrate embryos, *J Cell Biol.* 161 (2003) 769-777.
- [51]. D.C. Slusarski, J. Yang-Snyder, W.B. Busa, R.T. Moon, Modulation of embryonic intracellular Ca^{2+} signaling by Wnt-5a, *Dev Biol.* 182 (1997) 114-120.
- [52]. A.K. McAllister, L.C. Katz, D.C. Lo, Neurotrophin regulation of cortical dendritic growth requires activity, *Neuron* 17 (1996) 1057–1064.
- [53]. S.B. Rosso, D. Sussman, A. Wynshaw-Boris, P.C. Salinas, Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development, *Nat. Neurosci.* 8 (2005) 34–42.

- [54]. G.A. Wayman, S. Impey, D. Marks, T. Saneyoshi, W.F. Grant, V. Derkach, T.R. Soderling, Activity-dependent dendritic arborization mediated by Cam-Kinase I activation and enhanced CREB-dependent transcription of Wnt-2, *Neuron*. 50 (2006) 897-909.
- [55]. G.Y. Wu, H.T. Cline, Stabilization of dendritic arbor structure in vivo by CaMKII, *Science*. 279 (1998) 222–226.
- [56]. A.R. Means, Regulatory cascades involving calmodulin dependent protein kinases, *Mol. Endocrinol.* 14 (2000) 4–13.
- [57]. G.A. Wayman, S. Kaech, W.F. Grant, M. Davare, S. Impey, H. Tokumitsu, N. Nozaki, G. Banker, T.R. Soderling, Regulation of axonal extensions and growth cone motility by calmodulin dependent protein kinase I, *J. Neurosci.* 24 (2004) 3786–3794.
- [58]. B.J. Gavin, J.A. McMahon, A.P. McMahon, Expression of multiple novel Wnt-1 /int-1 related genes during fetal and adult mouse development, *Genes Dev.* 4 (1990) 2319-2332.
- [59]. K.I. Yasunaga, A. Tezuka, N. Ishikawa, Y. Dairyo, K. Togashi, H. Koisumi, K. Emoto, Adult *Drosophila* sensory neurons specify dendritic territories independently of dendritic contacts through Wnt5-Drl signaling pathway, *Genes and Development*. 29 (2015) 1763-1775.
- [60]. A.D. Shiras, J.P. Couso, Cell fates in the adult abdomen of *Drosophila* are determined by wingless during pupal development, *Dev. Biol.* 175 (1996) 24-36.
- [61]. M. Simons, M. Mlodzik, Planar cell polarity signaling: from fly development to human disease, *Annu Rev Genet.* 42 (2008) 517-540.

- [62]. H. Mcneil, J.R. Woodgett, When Pathways collide: collaboration and connivance among signaling proteins in development, *Nat Rev Mol Cell Biol.* 1 (2015) 1-21.
- [63]. J.R. Seifert, M. Mlodzik, Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility, *Nat Rev Genet.* 8 (2007) 126-138.
- [64]. Y. Wang, J. Nathans, Tissue/planar cell polarity in vertebrates: new insights and new questions, *Dev.* 134 (2007) 647-658.
- [65]. Y. Wang, J. Zhang, S. Mori, J. Nathans, Axonal growth and guidance defects in Frizzled 3 knock-out mice: a comparison of diffusion tensor magnetic resonance imaging, neurofilament staining, and genetically directed cell labeling, *J. Neurosci.* 26 (2006) 355-364.
- [66]. Luo L, Hensch T, Ackerman L, Barbel S, Jan L, Jan Y, Differential effects of the Rac GTPases on Purkinje cell axons, dendrites, and dendritic spines, *Nature.* 379 (1996) 837-840.
- [67]. L. Luo, Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity, *Ann Rev Cell Dev Biol.* 18 (2002) 601-635.
- [68]. L. Luo, Rho GTPases in neuronal morphogenesis, *Nature Reviews.* 1 (2000) 73-180.
- [69]. S.C. Iyer, D. Wang, E.P. Iyer, S.A. Trunnell, R. Meduri, R. Shinwari, M.J. Sulkowski, D.N. Cox, The RhoGEF trio functions in sculpting class specific dendrite morphogenesis in drosophila sensory neurons, *PLoS ONE.* 3 (2012) 1-15.
- [70]. A.Y. Nakayama, M.B. Harms, L. Luo, Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons, *J Neurosci.* 20 (2000) 5329-5338.

- [71]. K. Linnemannstons, C. Ripp, M. Honemann-Capito, K. Bretchel-Curth, M. Hedderich, A. Wodarz, The PTK7-related transmembrane proteins off-track and off-track 2 are co-receptors for *Drosophila* Wnt 2 required for male fertility, PLOS. 10: (2014) 7.
- [72]. D. Busson, A.M. Pret, GAL4/UAS targeted gene expression for studying *Drosophila* Hedgehog signaling, Methods Mol Biol. 1 (2007) 161-201.
- [73]. L. Zhong, R. Hwang, W.D. Tracey, Pickpocket is a DEG/ENaC protein required for mechanical nociception in *Drosophila* larvae, Curr Bio. 20 (2010) 429-434.
- [74]. B. Ganetzky, C.F. Wu, Neurogenetics of Membrane Excitability in *Drosopholia*, Annu Rev. Ganet. 20 (1986) 13-44.
- [75]. T. Agrawl, G. Hasan, Maturation of central brain flight circuit in *Drosophila* requires Fz2/Ca²⁺ signaling, Elife. 4 (2015) 1-10.
- [76]. K. Harris, Y. Akbergenova, R. Cho, M. Baas-Thomas, T. Littleton, Shank modulates postsynaptic Wnt signaling to regulate synaptic development, J Neurosci. 21 (2016) 5820- 5832.
- [77]. J. Kuroda, M. Nakamura, M. Yoshida, H. Yamamoto, T. Maeda, K. Taniguchi, N. Nakazawa, R. Hatori, A. Ishio, A. Ozaki, S. Shimaoka, T. Ito, H. Lida, T. Okumura, R. Maeda, K. Matsuno, Canonical wnt signaling in the visceral muscle is required for left-right asymmetric development of the *Drosophila* midgut, Mechanisms of Devel.1 28 (2012) 625-639.
- [78]. L. Kelly, J. Wu, W. Yanfeng, M. Mlodzik, Frizzled-induced Van Gogh phosphorylation by CK1e promotes asymmetric localization of core PCP factors in *Drosophila*, Cell Rep. 2 (2016) 344-356.

- [79]. X. Li, Y. Wang, H. Wang, T. Liu, J. Guo, W. Yi, Y. Li, Epithelia-derived wingless regulates dendrite directional growth of *Drosophila* ddaE neuron through the Fz-Fmi-Dsh-Rac1 pathway, *Mol Brain*. 9 (2016) 46.
- [80]. J.E. Gomes, M. Corado, F. Schweisguth, Van Gogh and Frizzled act redundantly in the *Drosophila* sensory organ precursor cell to orient is asymmetric division, *PLoS One*. 4 (2009) 2.
- [81]. H.C. Huang, P.S. Klein, The Frizzled family: receptors for multiple signal transduction pathways, *Genome Biology*. 5 (2004) 1-7.
- [82]. M.T. Veeman, J.D. Axelrod, R.T. Moon, A second canon: functions and mechanisms of beta-catenin-independent Wnt signaling, *Dev Cell*. 5 (2003) 367-377.
- [83]. L. Schweizer, D. Nellen, K. Basler, Requirement for Pangolin/dTCF in *Drosophila* Wingless signaling, *Pnas*. 100 (2003) 5846-5851.
- [84]. M. Galindo, D. Fernandez-Garza, R. Phillips, J. Couso, Control of Disal-less expression in the *Drosophila* appendages by functional enhancers, *Dev Biol*. 353 (2011) 396-410.
- [85]. J. Wu, A.C. Roman, J. Carvajal-Gonzalez, M. Mlodzik, Wg and Wnt 4 provide long-range directional input to planar cell polarity orientation in *Drosophila*, *Nature Cell Bio*. 15 (2013) 1045-1055.
- [86]. L. Luo, Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity, *Ann Rev Cell Dev Biol*. 18 (2002) 601-635.
- [87]. S.C. Iyer, D. Wang, E.P. Iyer, S. Trunnell, R. Meduri, R. Shinwari, M. Sulkowski, D. Cox, The RhoGEF trio functions in sculpting class specific dendrite morphogenesis in *Drosophila* sensory neurons, *PLoS ONE*. 3 (2012) 1-15.

- [88]. L. Luo, Rho GTPases in neuronal morphogenesis, *Nature Reviews*.
1 (2000) 173-180.
- [89]. A. Lyuksyutova, C.C. Lu, N. Milanesio, L. King, N. Guo, Y. Wang, J. Nathans, M. Tessier-Lavigne, Y. Zou, Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling, *Science*. 302 (2003) 1984-1988.
- [90]. F. Liebl, C. McKeown, Y. Yao, H. Hing, Mutations in Wnt 2 alter presynaptic motor neuron morphology and presynaptic protein localization at the *Drosophila* neuromuscular junction, *Plos*. 5 (2010) 9.
- [91]. K. Janson, E.D. Cohen, E. Wilder, Expression of DWnt6, DWnt10, and DFz4 during *Drosophila* development, *Science Direct*. 103 (2001) 17-20.

Vita

I was born in 1992 in Saginaw, Michigan. I moved to Wilkesboro, North Carolina at the age of five with my parents and my twin sister. I attended Wilkes Central High School and graduated in 2010. I then went on to pursue a bachelor degree in Biomedical Sciences at the University of North Carolina at Pembroke, where I played college soccer. I graduated college in 2013 and moved to Wilmington, North Carolina. I married Nicole Andreski on June 6th, 2014 and soon after accepted a job at the University of North Carolina at Wilmington as a lab technician. I then went on to pursue my Masters degree at Appalachian State University where I will then attend the University of Georgia to pursue a PhD.