THE ROLE OF NOTCH IN DROSOPHILA NOCICEPTOR FUNCTION AND MORPHOLOGY

A Thesis
by
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Abstract

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In the United States, chronic pain affects approximately 1 in 5 adults, and costs around $600 billion annually. Currently available therapeutics are not able to sufficiently treat chronic pain, indicating that treatment methods must continue to expand. To develop new treatments, novel treatment targets must also be identified. The cellular and molecular mechanisms involved in the sensory neurobiology of chronic pain are complex and not fully understood. This study utilizes the fruit fly, *Drosophila melanogaster*, as a model to investigate the mechanisms of pain sensation. Here, the role of the Notch signaling pathway in pain-sensing neuron function and structure is established. It was found that the *Notch* gene, which encodes the Notch receptor protein, is required for appropriate behavioral responses to noxious mechanical stimuli, but not noxious thermal stimuli or in sensory neuron hypersensitization. Morphological requirements for *Notch* in the dendritic branching of pain-sensing neurons were also found, as neurons with defective *Notch* mRNA were found to have fewer branches, notably higher order branches, than their control counterparts. The

morphological phenotype associated with defective *Notch* mRNA is similar to one found in neurons with a mutation in the *Trio* gene, which encodes the multi-functional Trio protein. Similarity of these phenotypes supports the developing hypothesis that a novel mechanism involving the Notch and Trio proteins mediates dendrite branching.

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Introduction

Pain and Nociception

Acute pain serves to alert an organism to avoid stimuli that could potentially cause tissue damage. These include extreme temperatures, punctate mechanical stimuli, or UV radiation. The ability to recognize environmental hazards through the sensation of pain is vital for survival. However, when pain persists after the noxious stimulus has subsided, or an injury has healed, chronic pain can arise. Chronic pain is a major public health issue in the United States and is defined as pain lasting 3-6 months, or after the original source has healed (Treede et al., 2015). As of 2019, 20.4% of American adults were affected by chronic pain, while 7.4% reported that pain limits their life activities and work (Zelaya, et al. 2020). The annual cost of chronic pain was estimated to be $560-$635 billion in 2010, greater than the cost of cancer and heart disease combined (Gaskin & Richard, 2012). Many cases of chronic pain are treated with multiple medications and a single treatment option has proven elusive (Dydyk & Conermann, 2022). Widespread treatment options include opioids which have mixed efficacy (Chou et al., 2020; McNicol, Midbari, & Eisenberg, 2013) and high potential for abuse, addiction, and overdose (Vowles et al., 2015). Further, existing therapeutics for chronic pain tend to target the symptoms rather than the root cause of pain. It is apparent that new treatment options for chronic pain must be investigated, thus new therapeutic targets must be investigated as well. To find new therapeutic targets, the intricate cellular and molecular mechanisms of chronic pain must be understood.

To study chronic pain, it is sensible to begin by investigating the neural process of encoding and processing damaging or potentially damaging (noxious) stimuli to provoke an avoidance response (Dubin & Patapoutian, 2010). This process is termed nociception (Dubin

& Patapoutian, 2010), and noxious stimuli are detected by specialized sensory neurons called nociceptors (Kandel et al., 2013). In humans, nociceptors are peripheral nerve endings that extend from a sensory neuron cell body in the dorsal root ganglia (for nociceptors in the body) or trigeminal ganglion (for nociceptors in the face) (Basbaum et al., 2009). Peripheral nerve endings of thinly myelinated Aδ axons make up the thermal and mechanical nociceptors, and transmit action potentials at 5-30 m/s (Kandel et al., 2013). Peripheral nerve endings of large, myelinated Aβ fibers also function as non-nociceptive mechanoreceptors (Kandel et al., 2013). Peripheral nerve endings of unmyelinated C axons function as polymodal nociceptors, that is, they detect different modalities of noxious stimuli. These include noxious mechanical, chemical, and thermal stimuli. These polymodal nociceptors transmit action potentials at approximately 1 m/s (Kandel et al., 2013). Nociceptive information is integrated into the central nervous system by sensory neuron projections onto the dorsal horn of the spinal cord, which then transmits information to the thalamus in the brain (Kandel et al., 2013). Then, the information is interpreted, and an actionable response can be evoked. Multiple nuclei of the brainstem, such as the periaqueductal gray, dorsal and medial raphe nuclei, and ventrolateral medulla are also important for processing of noxious stimuli (Napadow, Sclocco, & Henderson, 2019). Nociceptive stimuli can also evoke a withdrawal reflex (nociceptive flexion reflex). In the case of a withdrawal response, noxious stimuli is perceived by sensory nerve fibers, which synapse onto spinal interneurons, which activate motor neurons to flex muscles in the affected tissue and subsequently distance the tissue from the noxious stimulus (Derderian & Tadi, 2022)

Disruptions or damage to components involved in nociception can lead to chronic pain. Nociceptive pain normally arises from the activation of nociceptors in tissue/skin in

response to noxious stimulus or injury (Kandel et al., 2013), and can be considered chronic if the painful sensation persist after an injury has healed or the noxious stimulus has subsided. Chronic pain can also arise when nociceptors are activated in the absence of injury or noxious stimulus. Chronic neuropathic pain is caused by a lesion or disease of the somatosensory system, including neurons in the periphery and central nervous system (CNS) (Colloca et al., 2017). In the CNS, it is common for neuropathic pain to arise following a stroke (Klit, et al., 2011), or from neurodegenerative diseases such as Parkinson's disease (Borsook, 2012). Spinal cord injury (Siddall et al., 2003) and multiple sclerosis (Colloca et al., 2017; Solaro et al., 2004) are other examples of disorders causing central neuropathic pain. Peripheral neuropathic pain is generally attributed to lesion/disease of the sensory neuron fibers (Aδ, Aβ, C fibers) (Colloca et al., 2017; Finnerup et al., 2016), and are often associated with diabetes mellitus, chemotherapy, infectious diseases, and immune diseases (Colloca et al., 2017).

The causes of chronic pain are vast, complex, and differ on an individual basis making it difficult to treat. By investigating nociceptive pathways, the molecular and cellular mechanisms of pain perception can be better understood, and treatment methods can be improved.

Drosophila as a Model for Nociception

Studying nociception in humans can be difficult, as pain is highly subjective. Additionally, large sample sizes can be hard to obtain as many cases (e.g. rare genetic disorders, severe injury) happen unpredictably, in geographically diverse locations, and can be presented differently from one another. Studying nociception in animal models allows for

a much more consistent and comprehensive view than is ethically achievable in humans. The fruit fly*, Drosophila melanogaster* serves as an excellent animal model to investigate nociception. *Drosophila* are genetically tractable and genome manipulation tools are quite accessible. The ability to manipulate the *Drosophila* genome provides a method to precisely and reliably alter expression of a desired gene and observe associated phenotypic changes, thus revealing the role of the desired gene.

Many fundamental characteristics are shared between *Drosophila* and vertebrate nociception. Threshold temperatures that activate both *Drosophila* and vertebrate nociceptors is 39°C-41°C (Tillman et al., 1995; Tracey et al., 2003). Certain homologous ion channels, like those of the Transient receptor potential ankyrin (TRPA) family, are activated by noxious stimuli, and required for nociception in both *Drosophila* (Neely et al., 2011; Zhong et al., 2012) and vertebrates (Kang et al., 2010; Kwan et al., 2006). The highly branched dendrite arbors of *Drosophila* nociceptors resemble the highly branched free nerve endings of vertebrate nociceptors, indicating morphological homology between *Drosophila* and vertebrates (Dubin & Patapoutian, 2010). *Drosophila* larvae exhibit quantifiable aversive behavior to noxious stimuli, presented as a series of 360°-barrel rolls (Tracey et al., 2003). The nociceptive response is termed nocifensive escape locomotion (NEL) and is distinct from the response to innocuous stimuli where, upon stimulation, larvae will pause, retreat, and/or turn away from the stimulus (Tracey et al., 2003). Noxious stimuli are detected by highly branched nociceptors in the epidermal layer of *Drosophila* larvae (Grueber, Jan, & Jan, 2002) which are activated by extreme thermal (Tracey et al., 2003), mechanical (Hwang et al., 2007), and chemical (Lopez-Bellido et al., 2019) stimuli, as well as UV radiation (Xiang et al., 2010). *Drosophila* nociceptors synapse onto Down-and-Back (DnB)

interneurons in the ventral nerve cord (VNC), which synapse onto command neurons that induce NEL (Burgos et al., 2018). Optogenetic activation of nociceptors with the photoactivated cation channel Channelrhodopsin-2 (ChR2) (Boyden et al., 2005; Nagel et al., 2005, 2003; Schroll et al., 2006; F. Zhang et al., 2006) is sufficient to elicit NEL, even in the absence of noxious stimuli (Hwang et al., 2007).

Drosophila nociceptors are also capable of being hypersensitized. Hypersensitization occurs when tissue damage lowers the nociceptive threshold to where innocuous stimuli elicit nocifensive behavior (allodynia) or cause an exaggerated response to noxious stimuli (hyperalgesia) (Babcock, Landry, & Galko, 2009). Hypersensitization of neurons innervating injured tissue occurs to prevent further damage, especially while the injury is healing. Hypersensitivity is valuable to an organism's survival, but when the hypersensitive phenotype persists after the injured cells have healed, or if cells were never damaged, chronic pain can arise. The phenomenon of hypersensitivity can be illustrated by a sunburn, where the affected area is becomes more sensitive. A sunburn can cause noxious stimuli to evoke an exaggerated response and innocuous stimuli to be perceived as noxious. Hypersensitivity can be induced in *Drosophila* through UV irradiation of epidermal cells (Babcock et al., 2009). Following UV-induced epidermal tissue damage, *Drosophila* larvae exhibit allodynia and hyperalgesia in response to thermal stimuli (Babcock et al., 2009). Another reason this paradigm is useful is that the mechanisms responsible for hypersensitization in both flies and mammals are evolutionarily conserved, as they both require the cytokine tumor necrosis factor alpha (TNFα) (Babcock et al., 2009; Woolf et al., 1997).

Drosophila contain multidendritic (md) sensory neurons that are organized into four classes based on dendritic complexity (Grueber et al., 2002, 2003). Class IV md neurons

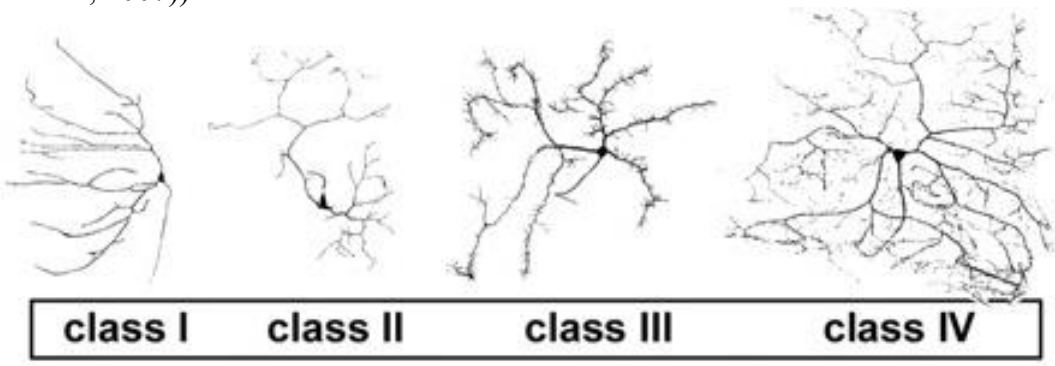
(nociceptors) (Hwang et al., 2007) and Class III md neurons completely line epidermal cells on the interior body wall of larvae through a process called tiling (Grueber et al., 2002). Furthermore, md neurons strictly avoid crossing over dendrites that extend from the same neuron, as well as dendrites from neighboring neurons of the same type (Grueber et al., 2002, 2003). Given the complexity of dendritic arbors in Class IV md neurons, self-avoidance must be tightly controlled. Tiling in this non-redundant fashion facilitates complete body wall coverage and accurate perception of environmental (and in this case, noxious) stimuli. Nociceptors extend dendrites across the epithelial substrate in two dimensions but can become ensheathed by epithelial cells (Han et al., 2012; M. E. Kim et al., 2012). This may occur to restrict branch growth and reduce crossing over events (Tenenbaum et al., 2017). As dendrites extend into the epithelium, a void remains for which other dendrites can now occupy (Tenenbaum et al., 2017). It has been proposed that this ensheathment process is part of the mechanism employed by nociceptors to mediate self-avoidance and ensure complete coverage of the epidermis (Tenenbaum et al., 2017). The stereotyped morphological features of nociceptors provide a consistent baseline for which altered nociceptors can be compared. Further, the characteristic tiling of nociceptors, largely in a two-dimensional plane allows for a simplified image analysis compared to neurons that extend in three dimensions.

The ability to activate nociceptors with known stimuli, quantify NEL, attribute NEL to nociceptor activity, modulate nociceptive function through sensitization in *Drosophila,* and to compare morphological characteristics of nociceptors provides an exceptional paradigm to study nociception and nociceptor development.

Drosophila Peripheral Neural Substrates for Nociception

The larval *Drosophila* peripheral nervous system contains Type I and Type II neurons. Type I neurons have a single dendrite (Dambly-Chaudiere & Ghysen, 1986; Younossi-Hartenstein & Hartenstein, 1997) and include chordotonal (cho) neurons, which are found in internal stretch receptors (Bodmer, Carretto, & Jan, 1989), and external sensory (es) neurons, which innervate external sense organs and help detect vibration (Singhania & Grueber, 2014). Type II neurons are more structurally complex than Type I neurons and are referred to as multidendritic (md) neurons. The three subtypes of md neurons are tracheal dendritic (td) neurons which have dendritic processes that wrap themselves around tracheal branches, bipolar dendrite (bp) which have two dendrites that extend in opposite directions and can be found at the level of muscles, and dendritic arborization (da) neurons which have elaborated dendritic processes and lie just below the cuticle (Bodmer & Jan, 1987). Type II md-da neurons are further classified (Classes I-IV) based on increasing complexity of dendritic arbors (Figure 1) (Grueber et al., 2002; Sugimura et al., 2003). Class I neurons function as proprioceptors (He et al., 2019; Hwang et al., 2007) and in locomotion (Cheng, et al., 2010). Class II neurons function as gentle touch receptors (Tsubouchi, Caldwell, & Tracey, 2012). Class III neurons function as gentle touch receptors (Tsubouchi et al., 2012; Yan et al., 2013) and noxious cold nociceptors (Turner et al., 2016). Class IV neurons function as the aforementioned nociceptors and detect noxious stimuli. Nociceptors extend dendritic branches into the epidermis and completely line all hemi-segments of *Drosophila* larvae, and a single axon extends to interneurons in the ventral nerve cord (VNC) (Grueber et al., 2007), relaying sensory information to the central nervous system.

Figure 1. Classes I-IV of multidendritic sensory neurons. Dendritic complexity can be observed increasing with class number. (Adapted with permission from *Development* (Grueber et al., 2007)).



When md neuron function is disrupted using the neurotoxin tetanus-toxin light chain (TeTxLC) (Sweeney et al., 1995), *Drosophila* nocifensive response to noxious thermal (46°C) and mechanical stimuli is eliminated (Tracey et al., 2003). This experiment led to the conclusion that md neurons are required for thermal and mechanical nociception (Tracey et al., 2003). It has been observed that disruption of class I and II md neuron function with TeTxLC yields only a slight defect in nociceptive responses to noxious thermal (47°C) and mechanical stimuli, while motor coordination and proprioception were more greatly affected (Hwang et al., 2007). Further, disruption of class II and III neuron function was not associated with any defects in thermal or mechanical nociception (Hwang et al., 2007). Conversely, blocking class IV md neuron function specifically caused significant defects in response to noxious thermal and mechanical stimuli (Hwang et al., 2007). As previously mentioned, class IV md neurons were further implicated as nociceptors when it was observed that optogenetic activation of class IV md neurons is sufficient to induce NEL, even in the absence of noxious stimuli (Hwang et al., 2007). These experiments eliminated the possibility that class I-III md neurons function as nociceptors, and that nociceptive function is dependent only upon class IV md neurons.

Ion Channels in Nociception

Ion channels are transmembrane proteins that allow the passage of ions through membranes. When positively charged ions move into the cell, the cell becomes depolarized. Once a threshold level of depolarization is reached, an action potential is induced. The positively charged ions responsible for the depolarization of neurons are calcium and sodium. Thus, influx of these cations into a neuron cause depolarization of the cell and firing of an action potential. Repolarization is achieved after an action potential through positively charged potassium ions exiting the cell.

One superfamily of ion channels, transient receptor potential (TRP) channels are found throughout eukaryotic organisms including both *Drosophila* and humans (Himmel & Cox, 2020). TRP channels contain six transmembrane domains and allow passage of cations through the membrane. There are a wide array of TRP channel activators, and some channels may respond to multiple mechanisms of activation (Venkatachalam & Montell, 2007). Many TRP channels are activated by multiple sensory modalities, including noxious thermal, mechanical, and chemical stimuli, and are largely important in sensory perception across phyla (Kang et al., 2010; Kwan et al., 2006; Montell, 2005; Peng, Shi, & Kadowaki, 2015). Changes in the cellular environment (i.e. temperature, mechanical force, chemical cues) cause certain TRP channels to open allowing cation influx, which triggers membrane depolarization, action potential firing, and subsequent signaling cascades. There are currently nine known families of TRP channels: TRPA, TRPVL, TRPV, TRPS, TRPM, TRPN, TRPC, TRPY, TRPML, and TRPP (Himmel & Cox, 2020; Himmel, Gray, & Cox, 2020; Peng et al., 2015; Venkatachalam & Montell, 2007). Multiple members of the TRP family are involved in nociception in flies and vertebrates. The TRPA channel, painless, is found in *Drosophila*

nociceptors and is essential for larval thermal and mechanical nociception (Tracey et al., 2003), as well as adult thermal nociception (Neely et al., 2011). *Drosophila* with a mutation in the *painless* gene exhibit a defective response to noxious stimuli, but not to innocuous mechanical stimuli (Tracey et al., 2003). Though a direct ortholog of *painless* does not exist in humans, another member of the TRPA family, TRPA1, shows functional conservation between humans and *Drosophila* (Kang et al., 2010). In humans, a gain-of-function mutation of *TrpA1* has been shown to cause familial episodic pain syndrome (Kremeyer et al., 2010). Mice with a non-functional *TrpA1* allele exhibit defective nociceptive responses to chemical irritants, noxious cold, and noxious mechanical stimuli (Kwan et al., 2006).The *Drosophila TrpA1* ortholog (*dTrpA1*) and its many isoforms are known to be involved in nociception. It has been shown that *dTrpA1* is required for thermal and mechanical nociception in both larval and adult *Drosophila* (Zhong et al., 2012). *dTrpA1* undergoes alternative splicing events to produce the isoforms dTrpA1-A, B, C, D, and E (Gu et al., 2019; Zhong et al., 2012). The dTRPA1-C isoform plays a significant role in nociceptive responses to reactive oxygen species (H_2O_2) and UV radiation, and a lesser role in thermal nociception (Gu et al., 2019). The dTRPA1-D isoform is required for noxious heat nociception and participates in nociception caused by allyl isothiocyanate, a chemical irritant (Gu et al., 2019).

Degenerin/epithelial sodium channels (DEG/EnaCs) are another type of ion channel important for *Drosophila* nociception. The gene encoding the DEG/EnaC subunit pickpocket (*ppk*) is found only in cIV neurons and is required for mechanical nociception in *Drosophila* (Y. Guo et al., 2014; Mauthner et al., 2014; Zhong, Hwang, & Tracey, 2010), mechanotransduction in both *Drosophila* (Ainsley et al., 2003) and *C. elegans* (Goodman & Schwarz, 2003), but a mammalian homolog has not yet been identified. RNAi knockdown of

ppk causes a significant decrease in the percentage of responses to noxious mechanical stimuli, but not to noxious thermal stimuli (Zhong et al., 2010). Interestingly, nociceptors expressing *ppk-RNAi* still elicit NEL when optogenetically activated (Zhong et al., 2010). These results indicated that ppk is required for mechanical nociception but not thermal nociception or general nociceptor electrical excitability, and that ppk contributes to the polymodality of nociceptors (Zhong et al., 2010). Importantly, since *ppk* is found exclusively in nociceptors, its regulatory sequence is commonly used as the tissue-specific enhancer for targeted genetic manipulation in *Drosophila* nociceptors (Ainsley et al., 2003).

The mechanosensory ion channel Piezo is also found In *Drosophila* nociceptors and is required for mechanical nociception (S. E. Kim et al., 2012). The function of Piezo is evolutionarily conserved, as mechanically-activated Piezo homologs are also found in vertebrates (Coste et al., 2010, 2012; Ge et al., 2015). Piezo has been observed to be required for mechanotransduction in human sensory neurons *in vitro* (Schrenk-Siemens et al., 2015), and inactive genetic variants of *Piezo2* are associated with mechanosensory defects in humans (Chesler et al., 2016). Knockdown of *Piezo* in *Drosophila* nociceptors causes defective responses to noxious mechanical stimuli, while thermal nociceptive and gentle touch responses were not affected (S. E. Kim et al., 2012). This experiment provided evidence that Piezo functions in mechanical nociception in *Drosophila*, and that its role as a mechanotransducer is conserved across species.

Interestingly, Piezo has also been found to participate in the inhibition of axon regeneration in multiple *Drosophila* neuron types, including cIV neurons (Song et al., 2019). It is thought that Piezo is activated by mechanosensory forces in the axonal growth cone environment, triggering the inhibition of axon outgrowth (Song et al., 2019).

Mechanosensory ion channels have been observed to direct dendritic branching in other neuron types (Jacques-Fricke et al., 2006; Kerstein et al., 2013), though Piezo may be unique in that it is involved in both mechanical nociception and mechanosensory axon guidance. These qualities present an interesting case where a mechanically gated ion channel can be activated by different stimuli in vastly different processes.

The Notch Signaling Pathway

The *Notch* gene encodes the heterodimeric transmembrane Notch receptor protein and is highly conserved across metazoans (Artavanis-Tsakonas, Rand, & Lake, 1999). The Notch receptor protein is a key component of the Notch signaling pathway and is found to regulate cell fate determination, cell proliferation, and other developmental processes in many cell types, making it a popular therapeutic target in cancer research (Artavanis-Tsakonas et al., 1999; Reichrath & Reichrath, 2020; Siebel & Lendahl, 2017). Evidence suggests that *Notch* mediates md neuron differentiation in *Drosophila* embryonic development (M. Guo, Jan, & Jan, 1996; Vervoort et al., 1997), yet its role in mature *Drosophila* nociceptor function has not yet been uncovered.

Canonical Notch Signaling

The Notch protein forms a heterodimer with an intracellular and extracellular domain (Urbach, Schnabel, & Technau, 2003). The Notch extracellular domain (NECD) acts as a receptor for ligands Delta (Knust & Campos-Ortega, 1989) and Serrate (Fleming et al., 1990) in *Drosophila*, LAG-2 and APX-1 in *C. Elegans*, and Delta and Jagged in humans (Weinmaster, 1997). These ligands are collectively referred to as the DSL family (Spana et

al., 1995). Ligand binding to the NECD induces a conformational change that exposes a cleavage site (Gordon et al., 2015) which is targeted and cleaved by a disintegrin and metalloprotease domain (ADAM metalloprotease) family member, TNFα-converting enzyme (TACE) (Brou et al., 2000). The liberation of the NECD leads to the formation of a transient intermediate molecule called the Notch extracellular truncation (NEXT) (Mumm et al., 2000). The NEXT is cleaved by a gamma-secretase-like protease (De Strooper et al., 1999) to form the Notch intracellular domain, or NICD (Tepass & Hartenstein, 1995). The NICD translocates to the nucleus (Struhl & Adachi, 1998) where it interacts with the transcriptional repressor CBF1 in vertebrates (Henkel et al., 1994; Hsieh et al., 1996), Suppressor of Hairless [Su(H)] in *Drosophila* (Hartenstein & Posakony, 1989), and LAG-1 in *C. Elegans* (Christensen et al., 1996) (termed the CSL protein) to form a NICD-CSL DNA-binding protein (Siebel & Lendahl, 2017). The formation of the NICD-CSL DNA-binding protein recruits the Mastermind (MAM) protein or Mastermind-like (MAML) protein (Siebel & Lendahl, 2017) to form a NICD-CSL-MAML gene transcription complex and transcriptional repression is relieved. The NICID-CSL-MAML complex recruits other co-activators (Co-A), such as PBAF, BRG1, LSD1 (Siebel & Lendahl, 2017; Yatim et al., 2012). The NICD-CSL-MAML complex induces transcription primarily of genes encoding basic helix-loop-helix (bHLH) proteins (*Enhancer of Split (E(spl))* (Bailey & Posakony, 1995), *HES* and *HERP* genes (Iso, Kedes, & Hamamori, 2003)) that go on to repress gene transcription of proneural genes like the *Achaete-Scute* complex and prevent cell differentiation (Heitzler et al., 1996; Oellers, Dehio, & Knust, 1994) (Figure 2).

In the developing *Drosophila* nervous system, a neural precursor expressing Delta ligand activates the Notch receptor in neighboring precursor cells (Bahrampour & Thor,

2020). Upon activation, the Notch signaling pathway prevents proneural gene expression, including *Delta*, forming a feedback loop in which Notch signaling inhibits Delta production and subsequent activation of the Notch receptor in adjacent cells (Collier et al., 1996; Formosa-Jordan et al., 2013). The process in which activation of the Notch receptor causes inhibition of downstream activity (neural differentiation, in this case) is known as lateral inhibition (Artavanis-Tsakonas, Matsuno, & Fortini, 1995) (Figure 3). Activation of Notch directs other neural precursors away from adopting a neural fate, and towards an epidermal cell fate in the CNS (Bahrampour & Thor, 2020; Lehmann et al., 1983), and sensory neuron support cell or Type I neuron (Brewster & Bodmer, 1995) fate in the PNS (M. Guo et al., 1996). In *Drosophila*, md neurons arise from multiple rounds of asymmetric cell divisions of precursor cells (Melnick, Noll, & Perrimon, 1993; Vervoort et al., 1997). The precursor cells can divide to produce chordotonal (cho) neurons, external sense (es) neurons, support cells (sheath cell, socket cell, or shaft cell), or md neurons (Brewster & Bodmer, 1995). These rounds of asymmetric cell division are mediated partially by *Notch*, where *Notch* mutants have been shown to produce excess md neurons, and no es neurons (Brewster & Bodmer, 1995). It has also been observed in *Notch* mutants that precursor cells all develop into md neurons, and support cells are not formed (M. Guo et al., 1996).

Figure 2. Notch signaling pathway. A cell with the Delta/Serrate/Jagged ligand on its surface binds to and activates the Notch receptor protein in a neighboring cell. This binding induces a conformational change in Notch, exposing the S2 cleavage site. Notch is cleaved by an ADAM metalloprotease, the NECD and ligand are endocytosed by the ligand-expressing cell. The NEXT is then cleaved by a gamma-secretase to form the NICD. The NICD translocates to the nucleus where it binds MAM/MAML and CSL, recruits Co-A and induces expression of target genes. (Adapted from "Notch Signaling Pathway", by BioRender.com (2022). https://app.biorender.com/biorender-templates.)

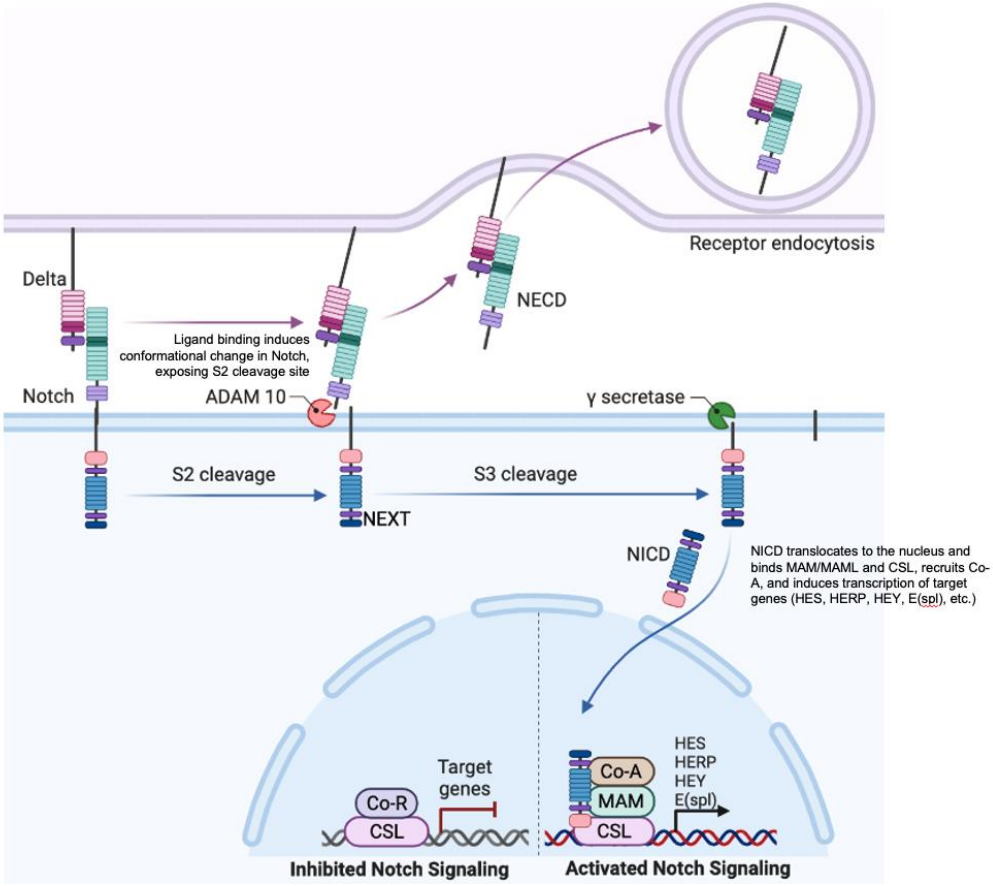
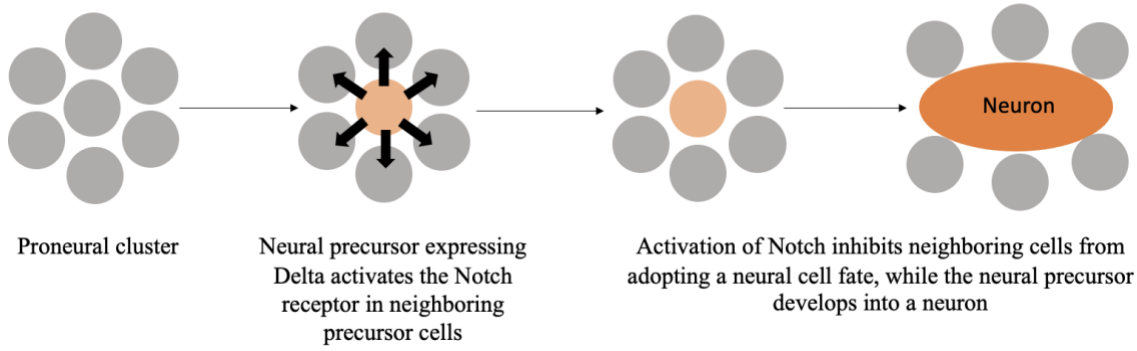


Figure 3. Notch in neural differentiation. Schematic showing the canonical Notch signaling mechanism preventing differentiation of neural precursors through lateral inhibition (Artavanis-Tsakonas et al., 1995; Bahrampour & Thor, 2020; M. Guo et al., 1996; Reichrath & Reichrath 2020).



Aside from cell fate decisions, Notch signaling also participates in neurite extension (Sestan, Artavanis-Tsakonas, & Rakic, 1999). In high density mouse cortical neuron cultures, where contact between neurons occurs more frequently, approximately 7x more Notch activity is observed than in low density neuron cultures (Sestan et al., 1999). Further, when neurons that were actively growing and extending neurites (and lowest endogenous *N* activity was observed) were transfected with human Notch1 and Notch2, neurite extension ceased and even retracted in some cases (Sestan et al., 1999). Additionally, antagonizing *Notch* activity promoted neurite extension and rescued the retraction phenotype observed in neurons transfected with Notch1 and Notch2 (Sestan et al., 1999). These observations provide evidence that Notch signaling functions through contact-dependent interactions in mouse cortical neurons to govern neurite extension.

Another role for *Notch* in the direction of neurite routing is in the routing process of the intersegmental nerve (ISN) in *Drosophila* (Crowner et al., 2003; Giniger, Jan, & Jan, 1993). When *Notch* activity is disrupted during post-mitotic axon extension of the ISN, the ISN branched into adjacent axons and in some cases, axon growth was inhibited altogether

(Giniger et al., 1993). *Notch* is also active in intersegmental nerve b (ISNb) routing (Crowner et al., 2003). The ISNb is a motor nerve that exits the CNS with the ISN, then clearly branches off towards target muscles in the ventrolateral body wall (Crowner et al., 2003; Landgraf et al., 1997). In *Notch* and *Delta* mutants, a bypass phenotype of the ISNb is observed. This is presented as the ISNb continuing to run parallel to the ISN for longer and delaying the extension of its axons to target muscles compared to control flies (Crowner et al., 2003). Delta ligand is expressed on tracheal cells near the ISNb defasciculation points, suggesting the activation mechanism for the Notch signaling pathway in this context (Crowner et al., 2003). Interestingly, axons of the ISNb in *Notch* and *Delta* mutants still reached their target muscles despite the routing bypass phenotype (Crowner et al., 2003). The evident participation of *Notch* and *Delta* in ISN axon routing indicate a post-mitotic role for the Notch signaling pathway.

Non-canonical Notch Signaling

There is evidence for a non-canonical Notch signaling mechanism to direct neurite extension by regulating actin through interactions with the adaptor protein Disabled (Dab) and its effector Abelson tyrosine kinase (Abl) (Crowner et al., 2003; Giniger, 1998) and the RhoGEF Trio and its effector RacGTPase (Kannan et al., 2017, 2018). It is hypothesized that Dab and Trio bind the NICD due to its proximity to their membrane-tethered effectors, Abl and Rac (Hantschel et al., 2003; Hodge & Ridley, 2016; Kannan et al., 2018; Reichrath & Reichrath 2020), though this has not yet been confirmed. Knockout of *abl* and its accessory genes, *neurotactin* and *trio*, in *Notch* mutants suppressed the bypass phenotype of the ISNb observed in *Notch* mutants (Crowner et al., 2003). Conversely, knockout of the *abl*

antagonist *enabled* (*ena*) or overexpression of *abl* in *Notch* mutants enhanced the bypass phenotype (Crowner et al., 2003). The bypass phenotype caused by *abl* overexpression can be suppressed by *Notch* co-overexpression (Crowner et al., 2003). Finally, in the ISNb, *abl* mutant embryos exhibit early termination of axon extension and failure to reach target muscles (Wills et al., 1999). This phenotype can be partially rescued by reducing *Notch* activity (Crowner et al., 2003). Mutations targeting both *Notch* and *abl* fail to show an effect on cell fate decisions (Crowner et al., 2003; Giniger, 1998), indicating the interaction of *Notch* and *abl* is not part of the canonical Notch signaling mechanism that directs cell fate determination. Further, reduction of canonical NICD DNA-binding co-factors Suppressor of Hairless (Su(H)) or mastermind (mam*)* did not affect the bypass phenotype of the ISNb in *Notch* mutants, indicating that the Notch/Abl mechanism for ISNb routing operates separately from the typical Notch signaling pathway (Crowner et al., 2003).

Abl is involved in neurite extension through two separate pathways. These pathways involve actin dynamics that direct dendritic filopodia and dendritic morphology (Reichrath & Reichrath 2020). In one pathway, the adaptor protein Disabled (Dab) stimulates Abl kinase activity, which derepresses the guanine exchange factor 1 (GEF1) domain of Trio (Kannan et al., 2017). The TrioGEF1 domain facilitates GDP/GTP exchange for the RacGTPase (Newsome et al., 2000). The RacGTPase interacts with the WAVE protein (homologous to the *Drosophila* SCAR protein) through IRSp53 (Suetsugu et al., 2006) to activate the Arp2/3 complex, which nucleates actin branches and helps create bundles of branched actin filaments (Blanchoin et al., 2000). RacGTPase also enhances activity of phosphatidylinositol-4-phosphate 5-kinase (PI4P5-K), a potent activator of actin polymerization (Shibasaki et al., 1997). PI4P5-K synthesizes phosphatidylinositol 4,5-

bisphosphate (PIP_2) which functions in actin regulation in multiple ways. One way PIP_2 can stimulate actin polymerization is by inhibiting actin-capping protein, preventing the addition of terminal caps to actin filament (K. Kim et al., 2007). Another way PIP_2 regulates actin is through inhibition of actin-depolymerization-factor Cofilin (Yonezawa et al., 1990). Finally, RacGTPase facilitates p21-activated kinase (PAK) interaction with LIM-kinase (LIMK), which also inhibits Cofilin activity (Edwards et al., 1999). Abl is further involved in neurite extension through a second parallel pathway that involves the Enabled (Ena) protein, but not Trio (Kannan et al., 2017). Abl inhibits Ena, which antagonizes actin capping (Bear et al., 2002) and regulates actin branching and cytoskeletal organization (Gertler et al., 1995; Kannan et al., 2014, 2017). These two pathways illustrate that the Abl-Dab-Trio and Abl-Ena mechanisms are regulators of actin and subsequent neurite extension.

It has been shown that Trio and Dab bind NICD regardless of DSL ligand activation of Notch and stay bound after the S2 and S3 cleavage events (Giniger, 1998; Kannan et al., 2018; Le Gall, De Mattei, & Giniger, 2008). These lines of evidence suggest a mechanism in which Dab and Trio bind NICD to enhance proximity to their membrane-tethered targets Abl and Rac, respectively (Hantschel et al., 2003; Hodge & Ridley, 2016; Kannan et al., 2018; Reichrath & Reichrath 2020). Following DSL ligand activation and proteolytic cleavage, NICD translocates to the nucleus thereby detaching Dab and Trio from Abl and Rac (Kannan et al., 2018). This terminates the TrioGEF-RacGTPase interaction, terminates Abl derepression of the TrioGEF1 domain, and relieves Abl mediated repression of Ena.

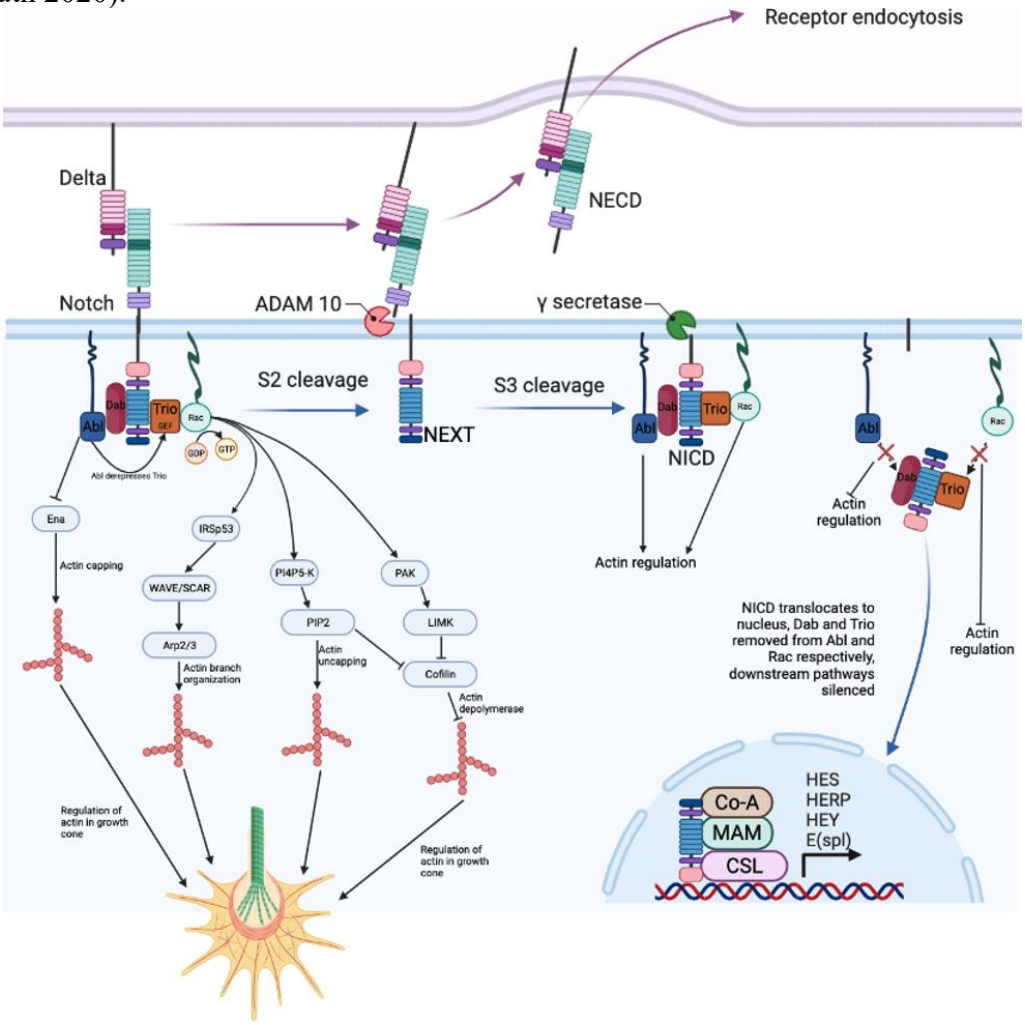
Trio is expressed in *Drosophila* nociceptors and has been shown to mediate dendritic branching (Iyer et al., 2012; Shivalkar & Giniger, 2012). Trio is a large protein with three enzymatically functional domains in vertebrates. Vertebrate Trio possesses two guanine

exchange factor domains (GEF1 and GEF2), and one protein serine/threonine kinase (PSK) domain (Schmidt & Debant, 2014; van Rijssel & van Buul, 2012). *Drosophila* Trio and the *C. elegans* Trio ortholog, UNC-73, contain the two GEF domains, but lack the PSK domain (Newsome et al., 2000; Steven et al., 1998). In *Drosophila*, the TrioGEF1 domain interacts with the GTPase Rac1 to promote dendritic branching, while the TrioGEF2 domain interacts with Rho1 to inhibit dendritic branching (Iyer et al., 2012). *Trio* knockdown and/or mutation yields a decrease in the number of dendritic branches and total dendritic length in nociceptors, (Iyer et al., 2012; Shivalkar & Giniger, 2012). Conversely, *trio* mutants and knockdown larvae exhibit an increase in average length/dendritic branch in nociceptors (Iyer et al., 2012; Shivalkar & Giniger, 2012). Essentially, neurons had longer branches, but fewer of them, and they appear relatively smoother than controls. Further, *trio* knockdown in Class I neurons causes a decrease in higher order branches (3rd order or greater), without affecting branch number of 1st and 2nd order branches (Shivalkar & Giniger, 2012). In nociceptors where *trio* is knocked down, a decrease in higher order branches and increase in lower order branches was also observed (Iyer et al., 2012).

Taken together, these lines of evidence suggest a possible non-canonical signaling mechanism to direct dendritic branching in *Drosophila* nociceptors (Figure 4). In the proposed model, Dab and Trio are bound to the NICD and connections to their membrane-tethered effectors, Abl and Rac, respectively, are lost when ligand binding triggers nuclear translocation of the NICD (Kannan et al., 2017; Reichrath & Reichrath 2020). When these connections are lost during NICD translocation, Dab stimulation of Abl ceases, Abl inhibition of Ena ceases, stopping Ena-mediated actin polymerization (Bear et al., 2002; Kannan et al., 2014, 2017). Additionally, without Dab activity, Abl derepression of Trio is

lost. When Trio (and the TrioGEF1 domain) separates from RacGTPase, TrioGEF facilitated Rac GTP/GDP exchange is halted. This prevents active RacGTPase from interacting with effectors IRSp53, PI4P5-K, and PAK. Since IRSp53 lies upstream of Arp2/3 (Suetsugu et al., 2006), which promotes actin nucleation and elongation (Blanchoin et al., 2000), this separation event causes decreased actin polymerization. Dissociation of Trio and Rac also separates Rac from its target PI4P5-K. Thus, PI4P5-K cannot synthesize PIP_2, which normally inhibits actin capping (Shibasaki et al., 1997) and actin depolymerase Cofilin (K. Kim et al., 2007), allowing actin capping and depolymerization to occur. Finally, separation of Trio from Rac inhibits PAK, which also regulates a Cofilin inhibitory mechanism (Edwards et al., 1999). The net results of NICD translocation and termination of Dab/Abl and Trio/Rac-dependent pathways are an increase in actin capping and depolymerization, causing morphological defects in dendritic branching (Kannan et al., 2017, 2018; Reichrath & Reichrath 2020).

Figure 4. Proposed non-canonical Notch signaling mechanism to direct actin polymerization. (Adapted from "Notch Signaling Pathway", by BioRender.com (2022). https://app.biorender.com/biorender-templates) (Kannan et al., 2017, 2018; Reichrath & Reichrath 2020).



It is unknown if the capability of *Notch* extends beyond cell-fate determination in developing class IV md neurons. This study seeks to elucidate a functional role for *Notch* in the post-mitotic dendritic branching of class IV md neurons, and if *Notch* has any implications in the behavioral responses to noxious stimuli.

Objectives

Notch is highly evolutionarily conserved and is known to mediate cell-fate determination in the developing nervous system. However, little is known about Notch signaling in post-mitotic sensory neurons. Further, a role for *Notch* in UV-induced nociceptor hypersensitization, a revealing experimental paradigm for nociception, has not yet been reported. This study aims to determine the role of *Notch* in *Drosophila* nociceptors through observing changes in behavioral response to noxious thermal and mechanical stimuli following gene manipulation. We seek to establish the role of *Notch* in baseline thermal and mechanical nociception. This study also aims to define the role for *Notch* in UV-induced nociceptor hypersensitization. *Notch* has been implicated in dendritic branch patterning (Sestan et al., 1999) and post-mitotic neurite routing in the periphery (Crowner et al., 2003; Giniger et al., 1993), and here we investigate the effects of *Notch* and Notch signaling on nociceptor dendritic branch morphology.

Methods

Fly Stocks and Genetics

Tissue-specific expression was accomplished through the GAL4/UAS system (Brand & Perrimon, 1993). This system functions through a tissue-specific enhancer which promotes expression of the yeast-derived transcriptional driver, GAL4 (Brand & Perrimon, 1993). GAL4 binds to and induces transcription of a target gene downstream of the upstream activation sequence (UAS). *GAL4* is not endogenously expressed in *Drosophila*, and transcription of the corresponding *UAS-target-gene* cannot be activated in the absence of GAL4. The tissue-specific enhancer used to isolate transcriptional activation in *Drosophila* nociceptors is a regulatory sequence for nociceptor-specific DEG/ENaC subunit, *pickpocket (ppk)* (Ainsley et al., 2003). Progeny of a fly expressing *ppk-GAL4* and a fly expressing the corresponding *UAS-target gene* directly inherit the required genetic sequences for tissue-specific expression. The GAL4 transcriptional activator is expressed in the desired tissue and can then bind the *UAS-target-gene* and drive expression of the target gene (Brand & Perrimon, 1993). *ppk-GAL4/UAS-target gene* can be used to achieve nociceptor-specific overexpression, or, when combined with an RNA interference (RNAi) transgene, can be used to achieve nociceptor-specific knockdown of a desired gene.

The *UAS-target gene* used for knockdown experiments encodes double-stranded hairpin RNA for the target gene, susceptible to RNAi (referred to as *UAS-target gene-RNAi*) (Perkins et al., 2015). The dsRNA is degraded by a viral defense mechanism in which the dicer-2 RNA endonuclease splices double-stranded hairpin RNA into single-stranded RNA (Lee et al., 2004). The single-stranded RNA is taken up by the ribonucleoprotein RNAi silencing complex (RISC). RISC utilizes complementarity between the single-stranded RNA

sequence and target mRNA to identify the target mRNA. Once identified, RISC degrades the mRNA (Fire et al., 1998; Perrimon, Ni, & Perkins, 2010). When the GAL4 driver induces expression of the *UAS-target gene-RNAi*, RNA interference occurs, and translation is blocked (Brand & Perrimon, 1993; Enerly, Larsson, & Lambertsson, 2002; Lee et al., 2004; Perkins et al., 2015; Perrimon et al., 2010). The *Drosophila* stocks used in this experiment are shown in Table 1, organized by name, Bloomington *Drosophila* Stock Center number (BDSC #), genotype, and manipulation.

Table 1. *Drosophila Stock Genotypes*

Name	BDSC #	Genotype	Manipulation
ppkGAL4	N/A	*w*; *ppk1.9-GAL4; dicer-2*	*GAL4* Driver
JF02959 (*UAS-N-RNAi*)	27988	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02959}attP2*	*Notch* knockdown
JF01637 (*UAS-N-RNAi*)	28981	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01637}*	*Notch* knockdown
GFP	N/A	*w; ppk1.9-GAL4, UAS-mCD8::GFP; UAS dicer-2*	Tissue-specific GFP expression
36303	36303	*y[1] v[1]; P{y[+t7.7]=CaryP}attP2*	RNAi control line
w[1118]	5905	*W[1118]*	Control line

In *Notch* knockdown experiments, the *w*; *ppk1.9-GAL4; dicer-2* driver line was crossed with one of two *UAS-Notch-RNAi* lines: *y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02959}attP2* (BDSC# 27988) *or y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01637}* (BDSC# 28981). Experimental crosses were tested against *GAL4-*

only and *UAS-only* controls. Since these control groups only express the *ppk-GAL4* driver sequence, or the *UAS-Notch-RNAi* sequence, gene knockdown does not occur. The '*GAL4-only*' control groups were established by crossing the *w*; *ppk1.9-GAL4; UAS-dicer2* line with the *y[1] v[1] P{y[+t7.7]=CaryP}attP2* (BDSC# 36303) control line. The '*UAS-only*' control groups were established by crossing the w^{1118} line with the *UAS-Notch-RNAi lines.*

All stocks were maintained at room temperature. All crosses were grown and maintained at 25°C and 50% humidity, and on a 12-hour light/dark cycle. Flies were fed a cornmeal-molasses medium (Nutri-Fly M; Genesee Scientific, El Cajon, CA, USA).

Table 2. *Experimental and Control Genetic Crosses*

Driver/enhancer (females)	Responder (males)	Manipulation
w; *ppk1.9-GAL4; dicer-2*	*UAS-N-RNAi* (JF02959, BDSC# 27988)	*Notch* knockdown
w; *ppk1.9-GAL4; dicer-2*	*UAS-N-RNAi* (JF01637, BDSC# 28981)	*Notch* knockdown
w; *ppk1.9-GAL4; dicer-2*	*y[1] v[1]P{y[+t7.7]=CaryP}attP2* (BDSC# 36303)	*ppkGAL4*-only Control
w^{1118}	*UAS-N-RNAi* (JF02959, BDSC# 27988)	*UAS-N-RNAi* (JF02959, BDSC# 27988)-only Control
w^{1118}	*UAS-N-RNAi* (JF01637, BDSC# 28981)	*UAS-N-RNAi* (JF01637, BDSC# 28981)-only Control
w; ppk1.9-GAL4, UAS-mCD8::GFP; UAS dicer-2	*UAS-N-RNAi* (JF02959, BDSC# 27988)	*Notch* knockdown with GFP
w; ppk1.9-GAL4, UAS-mCD8::GFP; UAS dicer-2	*y[1] v[1]; P{y[+t7.7]=CaryP}attP2* (BDSC# 36303)	*ppkGAL4 and GFP*-only Control

Thermal Nociception Assays

Wandering 3rd instar larvae were flushed from vials with deionized water and into a glass petri dish. Enough DI water was added to sufficiently cover the petri dish. Yeast was added to minimize surface tension. The larvae were contacted with a 46°C thermal probe on the lateral surface of abdominal body segments. The thermal probe consisted of a XYtronic 200PHG soldering iron with a Staco Energy Products Co. 12W (0-120V) power supply and Physitemp BAT-12 thermocouple. Assays were recorded on a Canon Vixia HF G20 camera, footage was analyzed in Adobe Premiere Pro. Latency between probe contact and completion of a 360°-barrel roll were recorded. Statistically significant differences between groups were determined by two-sided permutation T-test using estimationstats.com (Ho, et al., 2019) and Microsoft Excel. *Notch-RNAi* transgenic lines JF02959 ($n \geq 80$) and JF01637 ($n \geq 70$) were tested against respective *ppkGAL4-only* and *Notch-RNAi-only* controls.

Mechanical Nociception Assays

Wandering 3rd instar larvae were flushed from vials with deionized water and into a glass petri dish. Enough DI water was added to sufficiently cover the petri dish in a thin, contiguous layer. Yeast was added to minimize surface tension. Larvae were contacted up to three times or until a response was elicited. The mechanical nociception probe consisted of a 10mm Von Frey filament delivering ~50mN of force. The proportion of larvae exhibiting NEL upon first contact was determined. Statistically significant differences between groups were determined by comparing p-values obtained from Chi-square test. *Notch-RNAi* transgenic lines JF02959 ($n \geq 69$) and JF01637 ($n \geq 113$) were tested against *ppkGAL4-only* and *Notch-RNAi-only* controls, respectively.

Nociceptor Hypersensitivity Assays

Late 2nd instar and early 3rd instar larvae were flushed from vials with DI water into a plastic petri dish. Larvae were thoroughly rinsed to remove all excess particles and allowed to dry. The petri dish was placed on ice for ~15 seconds or until all larvae stopped moving. The petri dish was promptly transported to a Fisher-Scientific 1.6A UV Crosslinker where larvae were exposed to 25mJ/cm^2 of UV radiation. Larvae were then transferred to plastic petri dishes containing apple juice agar and a thin layer of yeast paste. After an 8-hour recovery period at 25°C and 50% humidity, larvae were subjected to the same thermal nociception assay as previously described at 42°C. Statistically significant differences between groups UV-exposed and mock UV-exposed groups were determined by Student's t-test using estimationstats.com (Ho et al., 2019) and Microsoft Excel. *Notch-RNAi* transgenic line JF02959 (n≥42) was tested against *ppkGAL4-only* and *Notch-RNAi-only* controls, respectively.

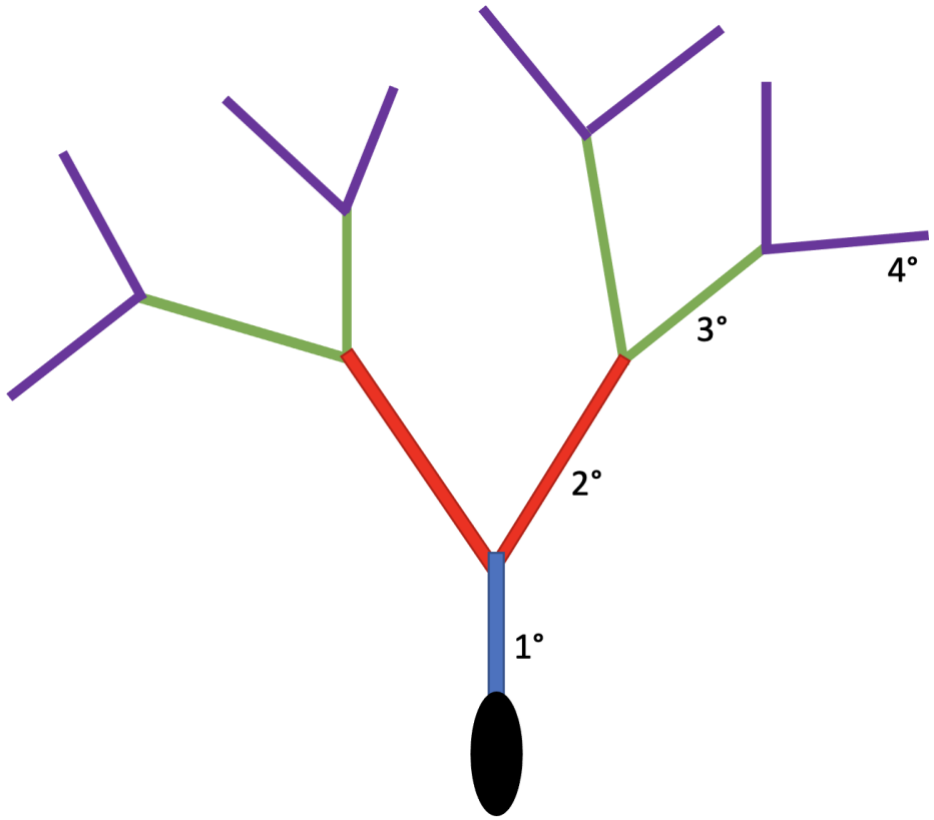
Confocal Microscopy and Analysis

Wandering 3rd instar larvae were flushed from vials with DI water. Larvae were immobilized by tying a hair around them and placing them in glycerol between two ~22x40mm microscope slides. Larvae were imaged at 40x magnification through a Zeiss LSM 880 laser scanning confocal microscope with a 488nm laser line. Stitched Z-stack images were obtained and processed in Adobe Photoshop. Processed micrographs were transferred to Fiji/ImageJ for all further analyses (Schindelin et al., 2012). Images were skeletonized and analyzed using the SkeletonAnalysis (Arganda-Carreras et al., 2010) plugin to determine total branch length. Images were also subjected to Strahler and Sholl (Ferreira et

al., 2014) analyses via the Simple Neurite Tracer (Arshadi et al., 2021) plugin for Fiji/ImageJ (Schindelin et al., 2012). Values from Strahler analyses were inverted in order to match traditional neurite branching nomenclature in which branches with a higher order number are more distal from the cell body (Figure 5). Statistically significant differences were determined by comparing p-values obtained from Student's t-test.

Dendritic branches can be classified by order number based on their relative distance from the cell body. Order number increases as branches get further from the cell body. For instance, a dendritic branch extending directly from the cell body is considered a primary or first order branch. A branch extending from a first order branch is a secondary or second order branch, and so on. This concept can be visualized in Figure 5.

Figure 5. Line illustration displaying dendritic branch order. The cell body is shown in black, first order branches in blue, second order branches in red, third order branches in green, and fourth order branches in purple.



Results

Notch Does Not Play a Significant Role in *Drosophila* Baseline Thermal Nociception

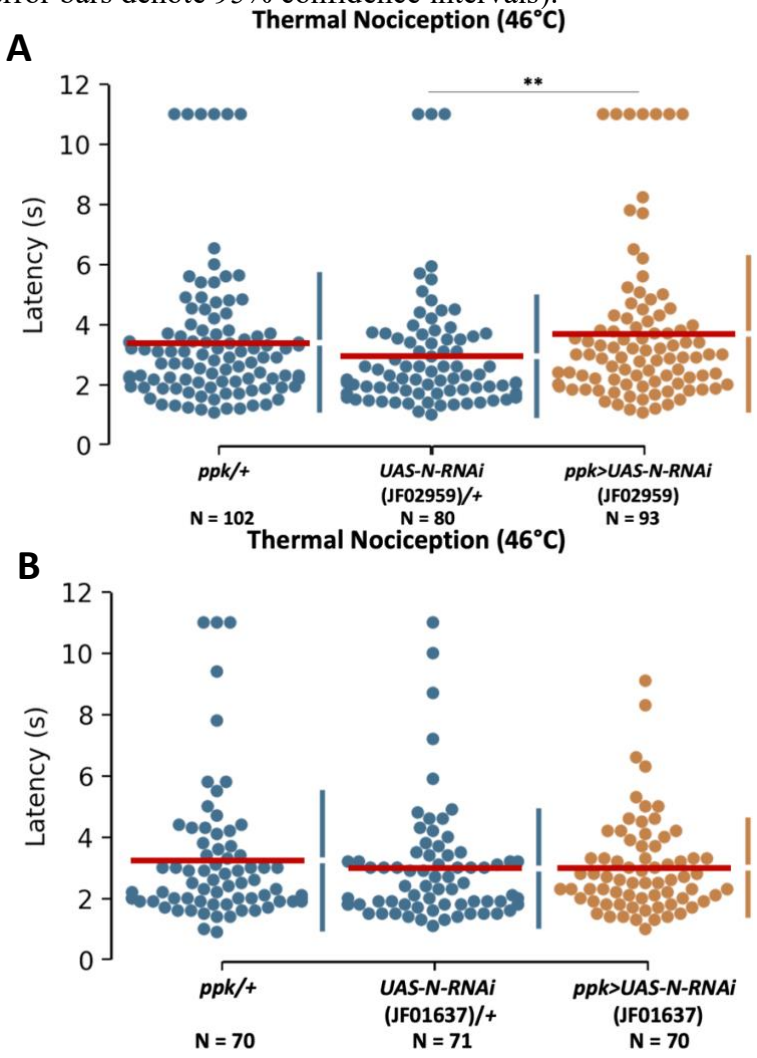
In order to define the role of *Notch* in *Drosophila* thermal nociception, targeted degradation of *Notch* mRNA in the nociceptors was achieved using the GAL4/UAS system and RNAi knockdown. The *pickpocket* (*ppk*) regulatory sequence was used as a nociceptor-specific enhancer, and GAL4 drove expression of *Notch UAS-RNAi* (*ppk>UAS-N-RNAi*). Two different *UAS-N-RNAi* transgenes were used (transgene JF01637, BDSC# 28981, and transgene JF02959, BDSC# 27988). *Notch* knockdown groups were compared to *ppk-GAL4 driver*-only (*ppk/+*) and *UAS-RNAi-only* (*UAS-N-RNAi/+*) groups (Figure 6).

Analyses showed that the mean response latency for the *ppk/+* group was 3.24 seconds (n=70). The mean response latency for the *UAS-N-RNAi/+* group was 2.69 seconds (n=71). The mean response latency for the *ppk>UAS-N-RNAi* group using the transgenic line JF01637 (BDSC# 28981) was 2.99 seconds (n=70). No statistically significant difference was observed between latencies of *ppk>UAS-N-RNAi* and *ppk/+* groups (p-value = 0.921), or between *ppk>UAS-N-RNAi* and *UAS-N-RNAi/+* groups (p-value = 0.427) (Figure 6A).

In experiments involving the transgenic line JF02959 (BDSC# 27988), similar *ppk/+* and *UAS-N-RNAi/+* controls were used. The mean response latency for *ppk/+* larvae was 3.40 seconds (n=102). The mean response latency for the *UAS-N-RNAi/+* group was 2.93 seconds (n=80). The mean response latency in the *ppk>UAS-N-RNAi* group using transgenic line JF02959 was found to be 3.70 seconds (n=93). There was no significant difference between the mean response latencies of the *ppk/+* control group and the *ppk>UAS-N-RNAi* group (p-value = 0.946). The mean latencies of the *UAS-N-RNAi/+* group and the *ppk>UAS-N-RNAi* group were 2.93 seconds and 3.70 seconds respectively, and a slight, but significant

difference between latencies was found (p-value = 0.015) (Figure 6B). These results indicate that *Notch* may have a modest effect on thermal nociception in *Drosophila* larvae.

Figure 6. Knockdown of *Notch* in nociceptors has a modest effect on thermal nociception. (A) Wandering third instar larvae with nociceptor-specific knockdown of *Notch* (JF01637) do not show a significant difference in latency (determined by two-sided t-test) in response to a noxious (46°C) thermal stimulus compared to *ppk-GAL4*-only controls and *UAS-RNAi*-only controls. (n ≥ 70 for all groups). (B) Wandering third instar larvae with nociceptor-specific knockdown of *Notch* (JF02959) show a significant difference in latency between *UAS-N-RNAi*-only controls (**, p ≤ 0.05, determined by two-sided permutation t-test) in response to a noxious (46°C) thermal stimulus. No significant difference was detected between *ppk>UAS-N-RNA* and *ppk-GAL4*-only controls. (n ≥ 80 for all groups, red bars indicate means, vertical error bars denote 95% confidence intervals).



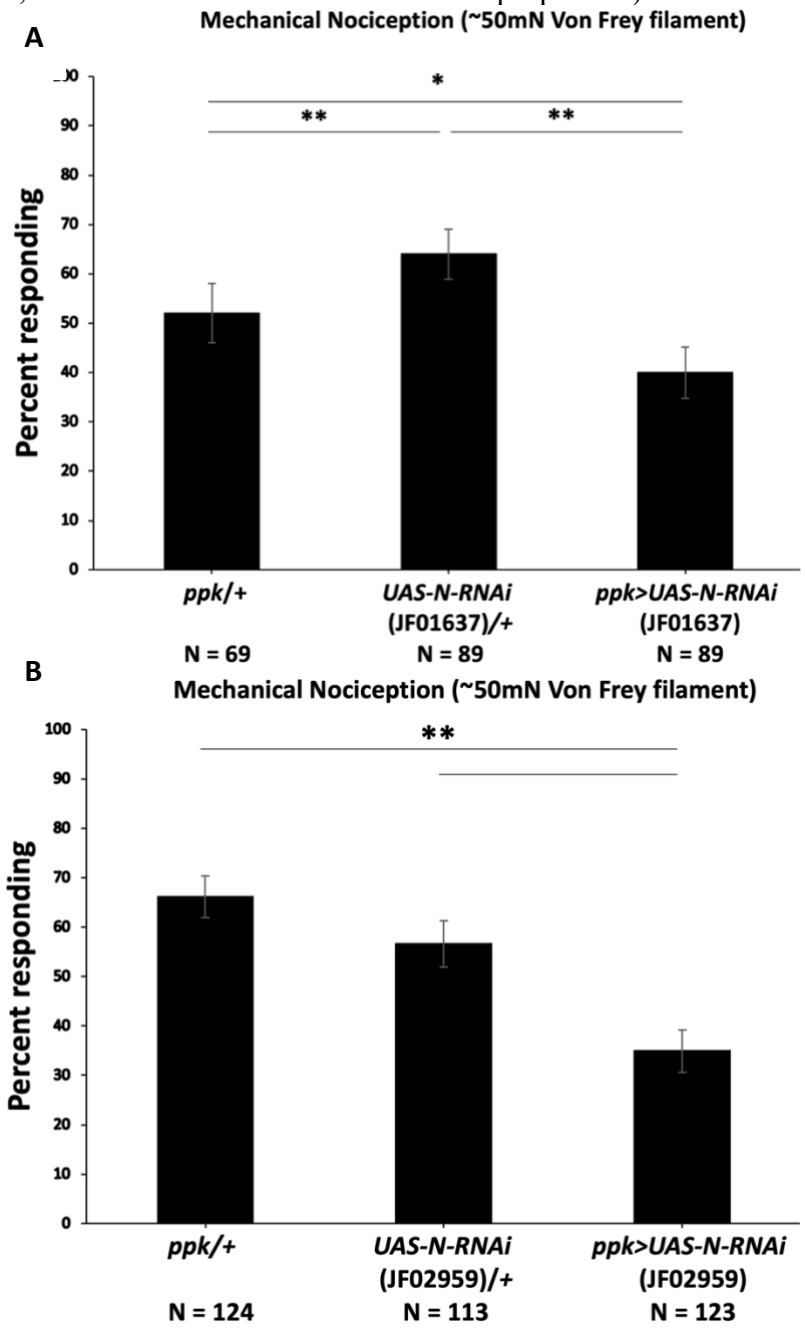
Notch is Required for Baseline Mechanical Nociception

To define the role of *Notch* in mechanical nociception, ppk-GAL4/UAS-RNAi was employed as previously described, using the same *UAS-N-RNAi* transgenes (JF01637 and JF02959), to create larvae exhibiting knockdown of *Notch* RNAi. *Notch* larvae were compared to *ppk-GAL4-only* controls and *UAS-N-RNAi*-only controls. Larvae were exposed to a 10mm Von Frey filament, delivering ~50mN of force (Figure 7).

The response percentage of *ppk/+* larvae was found to be 52% (n=69). The response percentage of UAS-N-RNAi/+ larvae was 64% (n=89). The response percentage for the Notch-RNAi group (transgene JF01637) was found to be 40% (n=89). The response percentage for *Notch-RNAi* larvae was significantly less than *ppk/+* ($p\text{-value} \leq 0.05$, determined by Chi-square analysis) and *UAS-N-RNAi/+* controls ($p\text{-value} \leq 0.001$, determined by Chi-square analysis). These results show that Notch-RNAi larvae respond significantly less frequently to noxious mechanical stimuli than controls (Figure 7A).

In experiments observing effects of *Notch-RNAi* transgene JF02959, similar *ppk/+* and *UAS-N-RNAi/+* controls were used. The response percentage of *ppk/+* larvae was found to be 66% (n=124). The response percentage of UAS-N-RNAi/+ larvae was 57% (n=113). The response percentage for the *Notch-RNAi* group (transgene JF02959) was found to be 35% (n=123). The response percentage for *Notch-RNAi* larvae was significantly less than *ppk/+* and *UAS-N-RNAi/+* controls ($p\text{-value} \leq 0.001$ for both comparisons, determined by Chi-square analysis) (Figure 7B). These results indicate that *Notch* in *Drosophila* nociceptors is required for baseline mechanical nociception.

Figure 7. Targeted RNAi knockdown of *Notch* in nociceptors decreases percentage of responses to noxious mechanical stimuli. (A) Wandering third instar larvae with nociceptor-specific knockdown of *Notch* (JF01637) show a significantly lower percentage response to noxious (~50 mN) mechanical stimulus on first contact compared to *ppk-GAL4*-only controls and *UAS-N-RNAi*-only controls (*denotes p≤0.05, **denotes p≤0.001, determined by Chi-square test, n ≥ 69 for all groups, error bars indicate standard error of proportion). (B) Wandering third instar larvae with nociceptor-specific knockdown of *Notch* (JF02959) show a significant difference in response percentage compared to *ppk/+ and UAS-N-RNAi/+* controls in response to first contact of noxious (~50 mN) mechanical stimulus (**p≤0.001, n≥113 for all groups, error bars indicate standard error of proportion).



Notch Does Not Participate in Nociceptor Hypersensitization

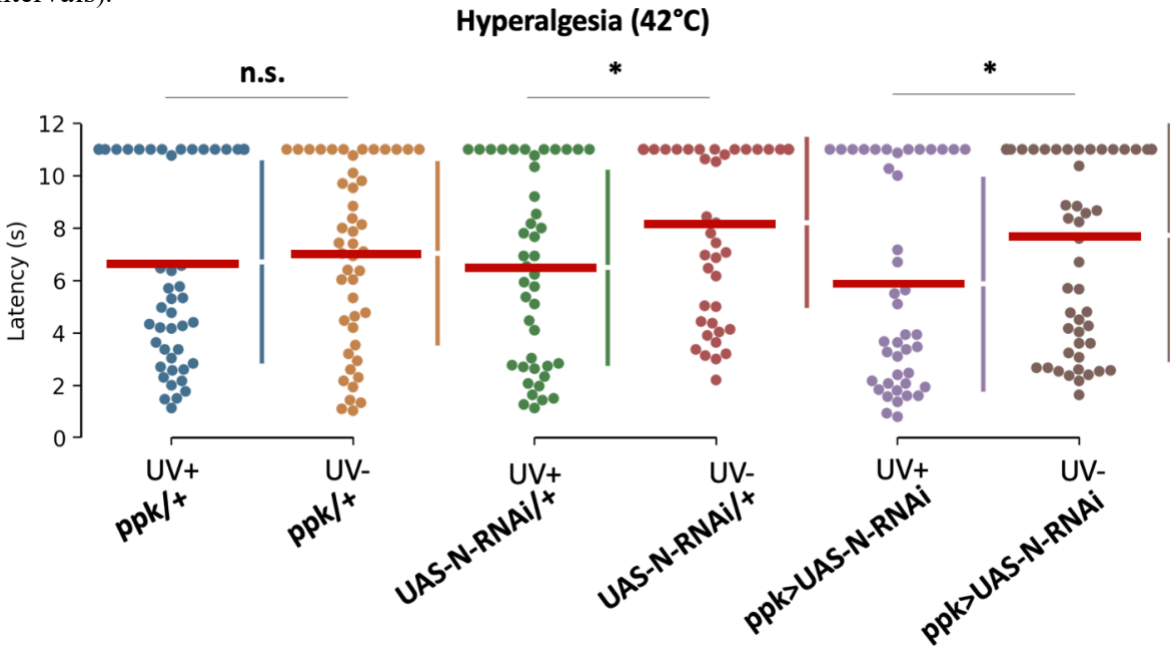
Hypersensitization can occur after injury in order to prevent further tissue damage while the injury heals. Hypersensitization can be presented as hyperalgesia, the exaggerated responses to noxious stimuli, or allodynia, where innocuous stimuli evoke a nociceptive response. If feelings of hyperalgesia or allodynia persist after initial tissue damage has healed, or arise in the absence of tissue damage, chronic pain can arise. A paradigm has been developed where UV-induced tissue damage causes both hyperalgesia and allodynia in response to thermal stimuli in *Drosophila* larvae (Babcock et al., 2009). Comparisons between the response latencies of animals subjected to UV-induced tissue damage and animals not subjected to UV-induced tissue damage can be made to determine if nociceptor hypersensitization developed following injury. This hypersensitization paradigm was used to determine the role of *Notch* in *Drosophila* nociceptor hypersensitization, particularly, in hyperalgesia which appears as UV-exposed larvae responding faster to thermal stimuli than larvae that were not exposed to UV.

Late 2nd and early 3rd instar larvae were exposed to 25mJ/cm^2 of UV radiation and tested for responses to noxious thermal stimuli (42°C) after an 8-hour recovery period. In controls, epidermal tissue damage causes hyperalgesia, or an increase in sensitivity to noxious stimuli which is presented as a decreased response latency to 42°C stimulus (Babcock et al., 2009).

Larvae that were subjected to UV-induced tissue damage (UV+) exhibited shorter latencies in response to 42°C stimulus when compared to their mock UV-exposed (UV-) counterparts of the same genotype (Figure 8, Table 3). UV+ *ppk-GAL4/+* larvae exhibited a mean latency of 6.67s, while UV- larvae exhibited a mean latency of 7.12s, (p-value>0.05,

determined by two-sided t-test). UV+ *UAS-N-RNAi*/+ larvae exhibited a mean latency of 6.43s, while UV- *UAS-N-RNAi*/+ larvae exhibited a mean latency of 8.21s (p-value≤0.05). *Notch-RNAi* larvae (transgene JF02959) exhibited a statistically significant sensitization effect compared to *ppk*/+ and *UAS-N-RNAi*/+ groups. *UV+ Notch* larvae exhibited a mean latency of 5.91s, while *Notch* larvae that were exposed to mock UV exhibited a mean latency of 7.67s (p-value<0.05). The persistence of hyperalgesia following UV-induced tissue damage in *Notch-RNAi* larvae show that the *Notch* not required for nociceptor hypersensitization.

Figure 8. *Notch* RNA silencing in nociceptors does not affect hyperalgesia following UV-induced tissue damage. (A) Wandering third instar larvae exhibit a decreased latency in response to a noxious (42°C) stimulus when comparing UV-exposed (UV+) and Mock UV-exposed (UV) groups. Larvae with nociceptor-specific knockdown of *Notch* (transgene JF02959) do not show a significantly different latency (determined by two-sided permutation t-test) in response to a noxious thermal stimulus following UV-induced sensitization compared to the Mock-UV group. (n≥42 for all groups, *indicates p-value<0.05 by two-sided permutation t-test, red bars indicate means, vertical error bars indicate 95% confidence intervals).



Notch Directs Dendritic Branching in Nociceptors

Notch signaling is involved in morphogenesis and branching of multiple neuron types (Crowner et al., 2003; Giniger et al., 1993; Sestan et al., 1999), so we aimed to determine if Notch signaling plays a morphological role in nociceptors. Based on previous studies, it was hypothesized that *Notch-RNAi* neurons would be less complex and have fewer total branches than controls (Sestan et al., 1999). To investigate this, the *ppk-GAL4* driver was used to drive expression of *mCD8::GFP* and *UAS-N-RNAi* (JF02959) in nociceptors. This group was compared to a control group in which *ppk-GAL4* drove expression of *mCD8::GFP*, but not *UAS-N-RNAi*. Neuron micrographs were quantified to determine total number of branches, total branch length, and average branch length. Images were also subjected to Sholl analysis to observe relative complexity, and Strahler analysis to determine number of branches per order (Arshadi et al., 2021; Ferreira et al., 2014; Schindelin et al., 2012).

In the *ppkGAL4>mCD8::GFP* controls, the number of branches per neuron was found to be 963.9 branches (Figure 9E), and the total dendritic length was 22868.4 pixels (Figure 9F). These values were used to calculate the average length per branch, which was 23.8 pixels (Figure 9G). The average number of intersections from Sholl analysis was 14962.1 intersections (Figure 9H). It was found that the average number of branches by order (1-6) were 11.9, 32, 84.4, 157.0, 237.0, and 440.8 branches, respectively (Figure 9I).

In nociceptors expressing *Notch-RNAi* the mean number of branches was determined to be 862.5 branches (Figure 9E), with a total dendritic length of 20906.0 pixels (Figure 9F). The average length per branch was determined to be 24.3 pixels (Figure 9G). The average number of intersections from Sholl analysis was 13755.4 intersections (Figure 9H). Strahler

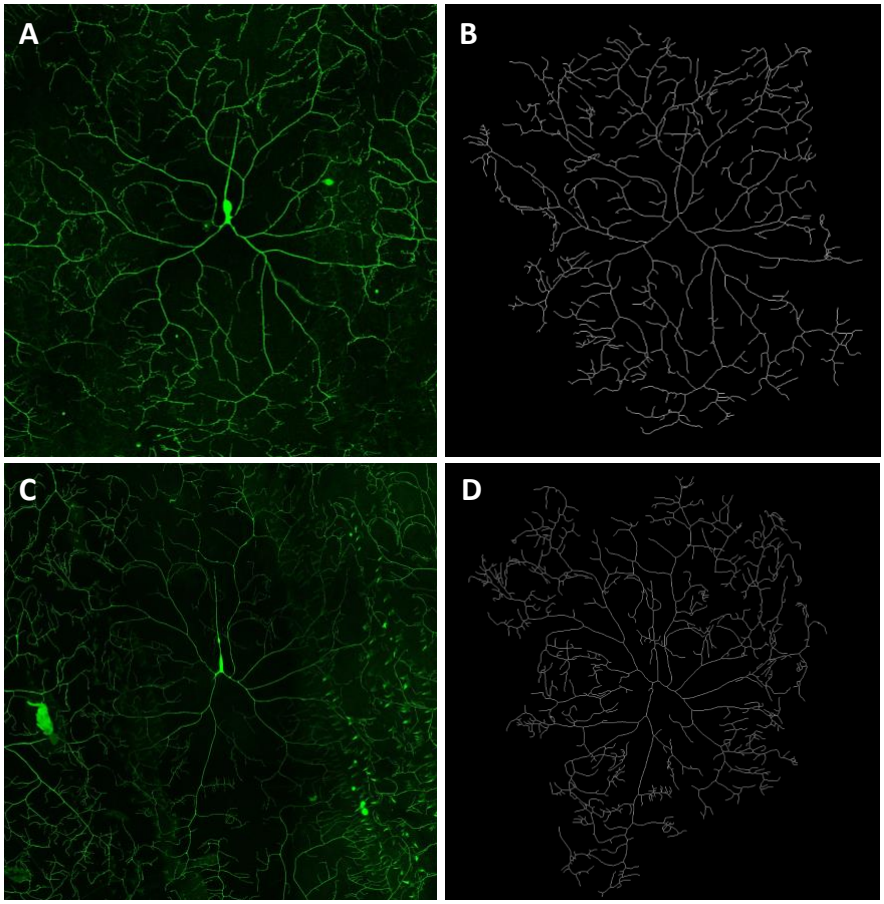
analysis indicated that the average number of branches by order (1-6) was 21.5, 50.9, 113.9, 186.6, 334.9, and 154.8 branches (Figure 9I).

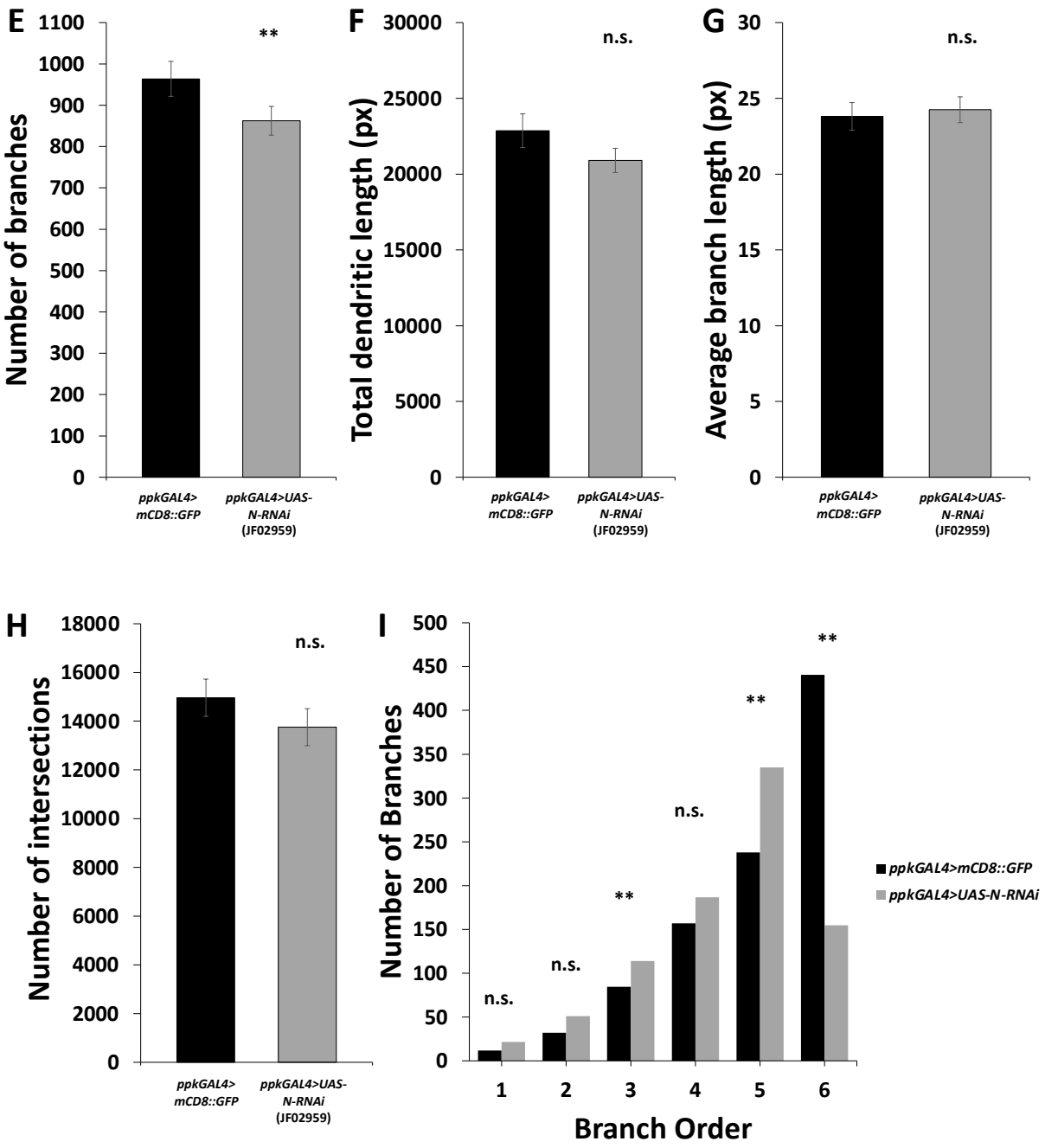
The mean number of branches in the *Notch-RNAi* group was found to be significantly less than that of the control group ($p \leq 0.05$, n = 8) (Figure 9E). There were no significant differences in the total dendritic length ($p > 0.05$, n = 8), though nociceptors expressing *Notch-RNAi* had a slightly lower total dendritic length (20906.0 pixels) compared to controls (22868.4 pixels) (Figure 9F). No difference was found between the average branch length between the *Notch-RNAi* group (mean = 24.3 pixels) and the control group (mean = 23.8 pixels) ($p > 0.05$, n = 8) (Figure 9G). There were no significant differences observed in total number of intersections from Sholl analysis between the Notch-RNAi group (149621.1) and the controls group (13755.4) (Figure 9H). Interestingly, nociceptors expressing *Notch-RNAi* had significantly greater, or equal number of low order branches compared to controls, but significantly fewer branches of the 6th order (Figure 9I) (mean number of 6th order branches in the *Notch-RNAi* group = 154.8 branches, mean number of 6th order branches in the control group = 440.8 branches, $p \leq 0.05$, n = 8).

These data indicate that nociceptors expressing *Notch-RNAi* exhibit fewer dendritic branches compared to controls, entirely attributed to high (6th) order branches (Figure 9I). Though not statistically significant, nociceptors expressing *Notch-RNAi* had slightly lower dendritic length overall, but longer dendritic length per branch. Further, nociceptors expressing *Notch-RNAi* had slightly fewer intersections compared to controls determined by Sholl Analysis suggesting lower complexity, though these values were not statistically different. These results support previous studies indicating that defective *Notch* results in smoother neurons (Sestan et al., 1999), with fewer high-order branches. The phenotype of

smoother, less complex dendrites has also been observed in nociceptors of *Trio* mutants (Iyer et al., 2012; Shivalkar & Giniger, 2012), supporting the idea of a Notch/Trio mechanism to mediate dendritic branching.

Figure 9. *Notch* helps regulate dendritic morphology in nociceptors. (A) Confocal micrograph and skeletonized version (B) of a *Drosophila* mdIV ddaC neuron expressing *ppkGAL4>mCD8::GFP*. (C) Confocal micrograph and skeletonized version (D) of a *Drosophila* mdIV ddaC neuron expression *ppkGAL4>mCD8::GFP, UAS-N-RNAi.* (E) Larvae expressing Nociceptor-specific knockdown of *Notch* exhibit a decrease in the average number of branches per neuron (n = 8, **p-value≤0.05 determined by t-test). (F) Larvae expressing nociceptor-specific knockdown of *Notch* exhibit no change in the total dendritic length per neuron (n = 8, **p-value>0.05 determined by t-test). (G) Larvae expressing nociceptor-specific knockdown of *Notch* exhibit no change in the average distance per branch (n = 8, **p-value>0.05 determined by t-test). (H) Larvae expressing nociceptor-specific knockdown of *Notch* exhibit no change in the average number of intersections from Sholl analysis (n = 8, **p-value>0.05 determined by t-test). (I) Average number of branches by order. Larvae expressing nociceptor-specific knockdown of *Notch* show no difference in branch number for lower order branches, but significantly fewer 6th order branches (p≤0.05determined by t-test, n = 8).




E
Number of branches
1100
1000
900
800
700
600
500
400
300
200
100
0
**
ppkGAL4> mCD8::GFP
ppkGAL4>UAS-N-RNAi (JF02959)
F
Total dendritic length (px)
30000
25000
20000
15000
10000
5000
0
n.s.
ppkGAL4> mCD8::GFP
ppkGAL4>UAS-N-RNAi (JF02959)
G
Average branch length (px)
30
25
20
15
10
5
0
n.s.
ppkGAL4> mCD8::GFP
ppkGAL4>UAS-N-RNAi (JF02959)
H
Number of intersections
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
n.s.
ppkGAL4> mCD8::GFP
ppkGAL4>UAS-N-RNAi (JF02959)
I
Number of Branches
500
450
400
350
300
250
200
150
100
50
0
n.s.
n.s.
**
n.s.
**
**
1
2
3
4
5
6
Branch Order
ppkGAL4>mCD8::GFP
ppkGAL4>UAS-N-RNAi

Discussion

The Notch signaling pathway, and the *Notch* gene which encodes the Notch receptor protein, are conserved across diverse cell types and species. The canonical Notch signaling pathway is documented to direct cell fate through lateral inhibition in the developing nervous system. Little is known about the role of *Notch* after embryonic neural development, much less in the peripheral nervous system. Here, we establish the absence of *Notch* participation in baseline thermal nociception, as well as UV-induced nociceptor hypersensitization, and define the role of *Notch* in mechanical nociception, Furthermore, elucidated here are roles of *Notch* in *Drosophila* nociceptor morphology.

Key Findings

The data presented here show that *Notch* is required for mechanical nociception, but not for thermal nociception or nociceptor hypersensitization. We found that nociceptor-specific knockdown of *Notch* led to a behavioral defect in response to noxious mechanical stimuli. This defect was observed as a lower proportion of larvae responding when compared to controls. In contrast, nociceptor-specific knockdown of *Notch* has no effect on behavioral responses to noxious thermal stimuli. This result was surprising given the ubiquity of *Notch*, but also reflects previous experiments showing that not all mechanisms for detecting noxious thermal and mechanical stimuli overlap (Gorczyca et al., 2014; Y. Guo et al., 2014; S. E. Kim et al., 2012; Mauthner et al., 2014; Tracey et al., 2003; Zhong et al., 2010). We also found that nociceptor-specific knockdown of *Notch* has no effect on the development of injury-induced hyperalgesia to noxious thermal stimuli. Finally, we show that knockdown of *Notch* in *Drosophila* nociceptors causes defects in the morphology of dendritic branches. Given previous experiments implicating *Notch* in neuron morphology (Sestan et al., 1999)

and neurite routing (Crowner et al., 2003; Giniger et al., 1993), hypotheses that morphological defects in nociceptors of *Notch-RNAi* larvae were supported by these data. The association between defective mechanical nociception and decrease in high order branches presents a case where neuron morphology may influence behavior.

The Role of *Notch* in Mechanical Nociception

The data here show that Notch signaling is important for responses to noxious mechanical stimuli, but not noxious thermal stimuli. These results support previous demonstrations that thermal and mechanical nociceptive pathways are distinct from each other (Y. Guo et al., 2014; S. E. Kim et al., 2012; Mauthner et al., 2014; Tracey et al., 2003; Zhong et al., 2010). A key difference in the detection of noxious thermal and mechanical stimuli lies within the activation mechanism of ion channels embedded in the membrane of nociceptors. For instance, the ion channel subunit, ppk, is required for mechanical, but not thermal nociception (Zhong et al., 2010). Similarly, the channel Piezo, is also required for *Drosophila* mechanical nociception, but not thermal nociception (S. E. Kim et al., 2012). Both ion channels are activated by mechanical forces and upon activation they open and allow cation influx. Cation influx depolarizes the cell, and once sufficiently depolarized, an action potential is induced. Though the cellular and molecular mechanisms for initial detection of these two modalities of nociceptive stimuli differ, they are both able to activate nociceptors. As nociceptor activation, even in the absence of stimuli, is sufficient for NEL (Hwang et al., 2007), behavioral responses to either thermal or mechanical stimuli converge on NEL.

The mechanisms of activation for thermal-gated and mechano-gated ion channels are specific to the modality they detect. That is, thermal-gated ion channels are activated by thermal stimuli, and mechano-gated ion channels are activated by mechanical stimuli. Thermal-gated ion channels can be activated by changes in temperature, and mechano-gated ion channels can be activated by changes to the cell membrane. Changes to the cell membrane can be detected by displacement of the membrane in relation to more rigid structural components like microtubules. For instance, in *C. elegans,* some touch receptors require mechanosensitive ion channels to be tethered to the cytoskeleton by a stomatin-like molecule (Fukushige et al., 1999; Huang et al., 1995). This allows changes in the membrane from physical contact to activate the ion channel. In *Drosophila*, the mechanoreceptor no mechanoreceptor potential C (nompC) requires an ankyrin tether to the microtubules to detect membrane displacement caused by mechanical stimuli (W. Zhang et al., 2015).

The Role of *Notch* in Nociceptor Morphology

We have shown that proper dendritic branch morphology of nociceptors is dependent on *Notch*. Nociceptor-specific knockdown of *Notch* causes a decrease in the number of branches per neuron. These results support previous studies suggesting that neurite outgrowth is mediated by Notch signaling (Sestan et al., 1999). Additionally, these results support the idea that a novel Notch signaling mechanism directs dendritic branching (Giniger, 1998; Kannan et al., 2018; Reichrath & Reichrath 2020) . It has been observed that Notch interacts with the membrane-tethered Abelson tyrosine kinase and separately, RacGTPase through Disabled and Trio respectively. These interactions regulate actin in the filopodia of extending neurites (Blanchoin et al., 2000; Crowner et al., 2003; Giniger, 1998; Hantschel et al., 2003;

Hodge & Ridley, 2016; Kannan et al., 2017, 2018; Landgraf et al., 1997; Newsome et al., 2000; Reichrath & Reichrath 2020; Wills et al., 1999). We observed fewer dendritic branches in nociceptors, solely attributed to a decrease in high order branches. Nociceptors expressing *Notch-RNAi* were found to have equal number, or greater number, of low order branches (1-5). Conversely, nociceptors expressing *Notch-RNAi* were found to have significantly fewer 6th order branches. In the case presented here, *Notch-RNAi* would prevent NICD translocation away from the membrane, allowing Dab and Trio to remain bound to their targets and promote actin polymerization. Contact-dependent inhibition of neurite growth has been shown to be mediated by *Notch* (Sestan et al., 1999). In the absence of the Notch receptor, contact with a ligand-expressing cell cannot activate the Notch signaling pathway and inhibit growth. Thus, I propose that Notch is activated by DSL ligand on nearby dendrites and NICD translocation effectively silences Trio-mediated actin polymerization in low order branches. This hypothesis suggests that another mechanism inhibits Trio-mediated high order branching. Interestingly, the phenotype of decreased branch number and decreased high order branches observed in *Notch* knockdown nociceptors mimicked that observed in nociceptor-specific knockdown of *Trio* (Iyer et al., 2012; Shivalkar & Giniger, 2012). Taken together, these data led to the hypothesis that the Notch and Trio proteins may interact to mediate dendrite morphology in *Drosophila* cIV neurons.

The hypothesis stating that Notch activation by DSL ligand in nearby dendrites and/or neurons inhibits dendrite growth by silencing Trio-mediated actin polymerization could be validated by conducting an epistasis experiment in which both *Trio* and *Notch* are knocked down in nociceptors of *Drosophila* larvae. This would require construction of a *Drosophila* strain expressing both a *UAS-Trio-RNAi* transgene and a *UAS-Notch-RNAi* transgene.

Confocal micrographs could then be obtained and quantified for samples exhibiting both *Notch* and *Trio* knockdown. Comparing these micrographs to samples expressing only *Notch-RNAi* would allow us to determine if *Trio* knockdown can rescue the morphological defects observed in *Notch-RNAi* nociceptors.

The validation of the hypothesis suggesting that a mechanism separate from a Notch/Trio interaction is responsible for termination of high order dendritic branching could be approached by first ruling out the possibility of a Notch/Trio interaction. This would require conducting a similar experiment to the one described above in which both *Notch* and *Trio* are knocked down in *Drosophila* nociceptors and subjected to confocal analyses. Absence of morphological differences between neurons expressing double knockdown (*UAS-Notch-RNAi* and *UAS-Trio-RNAi*) and neurons expressing a single knockdown (*UAS-Notch-RNAi*) would support the hypothesis stating that high order branching in nociceptors is Notch/Trio independent. To define the mechanism by which Trio-mediated high order dendrite branching is terminated a genetic screen could be conducted to identify Trio interactors in high order branches and epithelial cells. Antibody staining of candidate interactors could confirm interactions, and morphological parameters of neurons expressing candidate knockdown (*UAS-candidate-RNAi*) could be quantified. Previous studies have found that *Trio* knockdown in nociceptors causes a decrease in the percentage of higher order branches compared to wild-type neurons (Iyer et al., 2012). It has also been observed that *UAS-Trio-RNAi* expression in the Class I md neurons causes a decrease in high order branches (Shivalkar & Giniger, 2012). Expressing both *UAS-Trio-RNAi* and *UAS-candidate-RNAi* in nociceptors and comparing morphological parameters to neurons expressing just

UAS-Trio-RNAi could identify the Trio interactors that mediate high order dendritic branching.

Neuron Morphology and Behavior

This study indicates that *Notch* is required for both mechanical nociception and proper dendritic branching in nociceptors, suggesting an association between morphology and behavior. In the context of a non-canonical Notch signaling mechanism, knock down of *Notch* through RNAi causes inappropriate actin regulation. The change in actin regulation causes fewer high order branches, which may reduce the functional surface area of nociceptors and prevent the transduction of some nociceptive signals. One way this may happen is that reduction of high order branches effectively reduces ensheathment. High order branches often apically migrate toward the epithelium and become ensheathed by epithelial cells (Han et al., 2012; Jiang et al., 2019; M. E. Kim et al., 2012), and preventing ensheathment causes defects in mechanical nociception (Jiang et al., 2019). Interestingly, PIP_2 is partly synthesized through RacGTPase and RhoGTPase (which is in the same family of GTPases as RacGTPase). PIP_2 and a RhoGTPase are enriched in ensheathment areas (Jiang et al., 2019), suggesting that ensheathment may be partly regulated by GEF domains of Trio. Other studies have investigated the morphological role of *Notch* in ISNb motor nerve routing in developing *Drosophila* (Crowner et al., 2003; Giniger et al., 1993; Kannan et al., 2017). During ISNb routing, the Notch receptor is activated by Delta expressed on non-neuronal cells near the "choice points", or locations where ISNb axons defasciculate (Crowner et al., 2003). Reduction of *Notch* may disrupt the Notch activation by DSL ligand-expressing epithelial cells at nociceptor dendritic branch choice points when routing along

the epithelium. This may implicate both canonical and non-canonical Notch signaling mechanisms in the process of ensheathment of nociceptors by epidermal cells and may help explain the association of morphological defects and behavioral defects.

Notch-mediated actin regulation may also affect microtubule-tethered mechanosensory channels. Mechanoreceptors may rely on a gating spring mechanism, where mechanical force is detected by displacement of the membrane relative to the microtubule cytoskeleton. This force is mediated by a tether, linking the ion channel to the microtubule. In *Drosophila*, the mechanosensory TRP channel nompC has the largest ankyrin repeat (AR) domain out of all Drosophila TRP channels (Montell, 2004). nompC is tethered to microtubules via the ARs, and the ARs are required for mechanotransduction (W. Zhang et al., 2015). The ARs act as a spring, able to detect compression of the membrane due to mechanical force (Wang et al., 2021). This suggests that in the absence of *Notch* due to *Notch-RNAi,* actin polymerization is disrupted thereby preventing extension of lamellipodia and filipodia in the growth cone. Without these extension events, subsequent microtubule formation does not occur, and microtubule-tethered mechanosensory channels are no longer able to detect membrane displacement.

Notch Receptor Activation in cIV Neurons

During the 3rd instar stage of larval *Drosophila* development, canonical Notch signaling has taken place to guide cell fate decisions. During these cell fate decisions, the Notch receptor on neural precursors is activated by DSL ligand on a neighboring cell, and they are directed away from a neural cell fate. However, it appears that in post-mitotic events, Notch still influences cIV neuron activity, though it is unclear how the Notch receptor

is activated in this context. *Drosophila* nociceptors are known to tile epithelial cells of the larval body wall and display strict self-avoidance (Grueber et al., 2002). Given the increase seen in many lower orders of dendritic branches associated with decreased *Notch*, I hypothesize that Notch is activated by ligand being expressed on neighboring dendrites, likely from the same neuron.

Nociceptors largely occupy a two-dimensional space on the basal epithelium, and this position is maintained through neuronal integrins (Han et al., 2012; M. E. Kim et al., 2012). Reduction of integrin-mediated prevention of neuron ensheathment by epidermal cells allows for dendrites to extend toward the periphery to be ensheathed by epithelial cells (Han et al., 2012; M. E. Kim et al., 2012). It is thought that ensheathment may occur, at least partially, as a mechanism to vacate space for neighboring nociceptors to expand their dendritic field while maintaining self-avoidance between neurons (Tenenbaum et al., 2017). It has been observed that blocking epidermal sheath formation of dendrites causes excessive dendritic branching (Jiang et al., 2014, 2019; Tenenbaum et al., 2017) Additionally, epidermal ensheathment of nociceptors has been found to increase nociceptor sensitivity (Jiang et al., 2019). Since the nociceptors contact, extend into, and have a functional relationship with epidermal cells, changes in the external environment (i.e. mechanical stress or concentrated pressure) for which epidermal cells are the first to be affected, may cause DSL-ligand activation of the Notch receptor on sensory neurons and trigger subsequent signaling cascades. Additionally, growth cone interactions with external cues help mediate neurite outgrowth, and epithelial DSL ligand may activate Notch in the nociceptor membrane and influence neurite growth. In the ISNb, Delta expressed on tracheal cells near the first routing choice point is required for proper routing (Crowner et al., 2003), suggesting that ligand

expressing cells, even of a different cell type, can direct Notch-mediated neurite outgrowth. I hypothesize that in high-order dendrite branches of nociceptors, Notch is activated by ligand expressed by epidermal cells.

Notch May Act as a Secondary Mechanotransducer

A model has been proposed where activity of the mechanosensitive ion channel, Piezo, secondarily influences the Notch signaling pathway (Caolo et al., 2020). Mechanical forces activate Piezo and allow Ca^{2+} influx across the membrane (Coste et al., 2010; Wu, Lewis, & Grandl, 2017). Increased Ca^{2+} levels relieve Calmodulin-mediated inhibition of the ADAM10 metalloprotease (Nagano et al., 2004), which is required for the S2 cleavage event during Notch signaling (Brou et al., 2000).

Many attractive and repulsive cues exist to mediate neurite extension and outgrowth. One of these cues is mechanical forces in the environment encountered by growth cones. These mechanical cues can serve to prevent self-contacting and crossing over events in neurons, contain neurite outgrowth, maintain appropriate branch patterns, and ensure proper neurite routing to target tissues. Information about mechanical forces is communicated in part by mechanosensitive ion channels. The mechanosensitive ion channel Piezo has been shown to inhibit axon regeneration and outgrowth through the detection of mechanical cues (Song et al., 2019). Though practically quite different from our research, previous studies may support the idea that Notch can act as secondary mechanotransducer.

Future Directions

To investigate the possible Notch/Dab/Trio relationship in cIV neurons, mechanical nociception assays could be performed on larvae expressing *Trio-RNAi.* The predicted phenotype would be defective response to mechanical stimuli, through the loss of Trio-mediated guanine exchange for the RacGTPase, which would no longer activate PI4P5-K, thus decreasing levels of PIP_2. Since PIP_2 is TRP channel regulator (Brauchi et al., 2007; D. Kim, Cavanaugh, & Simkin, 2008) defective response to multiple modalities of noxious stimuli could be possible. Disrupting *Trio* activity would presumably affect actin polymerization and may cause defects in microtubule-tethered mechanosensory channels as well. To address the morphological interactions of Notch and Trio, epistasis experiments could be conducted in which both *Trio* and *Notch* are knocked down, and antibody staining could be used to determine Trio interactors in or around high order branches. To broadly determine the interaction of Notch/Dab/Trio in mechanotransduction, it would be interesting to perform experiments observing locomotion and proprioception on larvae expressing *Notch-RNAi* and/or *Trio-RNAi* in Class I md neurons, as this class of neuron is involved in proprioception (He et al., 2019; Hwang et al., 2007) and locomotion (Cheng et al., 2010). To continue the discussion on Notch/Dab/Trio as mediators of nociceptor morphogenesis, investigating the role of these molecules in nociceptor regeneration would provide further insight into their roles as morphological directors. Another question that would be interesting to follow up on is, what other mechanisms do Dab and Trio use to maintain proximity to Abl and Rac, respectively? It would seem that Dab and Trio bind the NICD out of convenience, and there must be other ways for these molecules to reach their targets. Further, it would be interesting to "follow" Dab and Trio after NICD nuclear translocation by fluorescently

tagging these proteins and capturing micrographs in real time. Is it possible that they also interact with DNA binding proteins once inside the nucleus, and if so, what are those targets? Or do Dab and Trio detach from Notch upon nuclear entry? The results of this study provide an intriguing opportunity to further investigate novel cellular and molecular processes in nociceptors, as well as their role in detecting stimuli and influencing behavior.

Concluding Remarks

The results from this study show that *Notch* is involved in nociceptor function and implicate *Notch* as a potential therapeutic target for the treatment of chronic pain. Additionally, the results obtained here show that *Notch* participates in directing dendritic morphology of nociceptors making it a potential therapeutic target for conditions that arise from structurally defective neurons in the periphery. These data also suggest a hypothesis stating that Notch interacts with the adaptor protein Disabled and its effector Abelson tyrosine kinase, and the RhoGEF Trio and its effector RacGTPase to direct dendritic branching. The morphological defects presented as decreased high order branching through a non-canonical Notch signaling mechanism to direct actin polymerization are associated with defects in behavior upon noxious mechanical stimuli exposure. This association suggests a mechanism where cytoskeletal regulation affects behavior, possibly through interactions of the nociceptor with epidermal cells through ensheathment, or by preventing microtubule formation and subsequent anchoring for microtubule-tethered mechanosensory channels. This study establishes that *Notch* in *Drosophila* nociceptors has post-mitotic roles in detection of noxious mechanical stimuli and dendritic branch morphology.

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Vita

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