Title
NEUROSENSORY MECHANOTRANSDUCTION IN THE DROSOPHILA LARVA

Author
Gorczyca, David A.

Publication Date
2015

Peer reviewed|Thesis/dissertation

Neurosensory Mechanotransduction in the Drosophila Larva

by

David Gorczyca

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

Acknowledgements:

This work would not have been possible without the generous support of mentors, colleagues, family, and friends.

I first want to thank Lily and Yuh Nung for the training and research opportunities they have offered me over the course of my graduate career. They have taught me to ask creative questions and to think critically about research. I have also been privileged to interact with some outstanding and extremely talented post-docs in their lab. They have encouraged me to pursue my interests and have supported me in every endeavor for which I am ever grateful.

I also thank my colleagues in the Jan lab. The lab would not be the same place without the scientific energy of the superb scientists. In particular I would like to acknowledge Drs. Wei Zhang, Zhiqiang Yan, Susan Younger, Matthew Klassen for their assistance throughout my training. My research has only been possible due to their help over the years and numerous discussions.

My studies would have also not been possible without the help of the Julius Lab, particularly Dr. Alex Chessler who was instrumental in helping me set up the piezo system in the lab. I am also grateful to the help of Dr. Donn Silberman of Physik

Instrumente and Dr. Robert Hodge of Siskiyou Instruments for their feedback and discussions.

This dissertation would also not be possible without the support and advice of my Thesis Committee. I thank Drs. Yuriy Kirichok, Ron Vale, and Su Guo for their time and dedication during my graduate training. It has been a privilege to be advised by such incredible scientists.

Contributions:

Yuh Nung Jan directed and supervised the research that forms the basis for this dissertation. This dissertation contains modifications of previously published materials. Chapter 2 contains material published by Yan et al., Nature 2013. Chapter 3 contains material published by Gorczyca et al., Cell Reports 2014. Individual author contributions are noted at the end of each respective chapter.

This work is comparable to work for a standard thesis awarded by the University of California, San Francisco.

Yuh Nung Jan

Abstract:

Animals contain mechanisms for interacting with their environment that are exquisitely sensitive to particular modalities. Of all our senses, mechanosensation is the least well understood. The mammalian somatosensory system contains several sensory organs specialized for mechanotransduction, however it is difficult to study for a number of reasons. The da neuron system in Drosophila offers a powerful system in which to study the molecular mechanisms of somatosensation. In this work, the Class III neurons are identified as a rapidly adapting mechanotransducers. In addition, the DEG/ENaC channel Ppk26 is found to interact with Ppk1 in Class IV neurons, where it plays a role in mechanical nociception and locomotion behavior.

Table of Contents:

List of Tables:

List of Figures:

Chapter 1. Introduction:

Through of millions of years of evolution animals have developed sophisticated mechanisms for sensing and interacting with their changing environment. These mechanisms are specified at the level of protein biophysics and signal transduction, as well as sensorineuronal integration.

Since the time of Aristotle it has been recognized that there are five senses in humans. Four of these, vision, hearing, taste, and smell, have circumscribed organs dedicated entirely to this function. Aristotle called the fifth sense "touch" but the diversity of cutaneous sensations made him uncertain as to whether the fifth sense should rather be termed "general sensation", a term encompassing corporeal sensation of skin, joints, deep tissues, viscera, etc.

In the mid 1800s Johannes Muller laid out a series of propositions relating to the "specific irritabilities" of the nerves subserving the various senses of Aristotle. Stimulating a given nerve gave rise to the same kind of sensation, regardless of the position of stimulation on the nerve. Based on these findings, Muller suggested that intrinsic differences existed in the nerves subserving each of the senses. Volkmann in 1844 suggested separate nerve endings for each subdivision of cutaneous sensory experience, and Von Helmholtz soon introduced the term "sensory modality" to designate "a class of sensations connected by a qualitative continua". This principle could be applied to most of the Aristotelian senses, but he recognized that cutaneous sensation did not have

the same continuous quality. Each cutaneous sensation, touch, warmth, pain, cold. seemed to have the status of "sensory modality" in that could be experienced on this qualitative continuum (Sinclair et al., 1967).

By the late 1800s the philosophical idea that sensory modality had some anatomical substrate was widely accepted. Even in the 1600s Rene Descartes had proposed animal spirits traveling from the sensory locus through pathways up to the brain (Figure 1.1). The growing field of histology (buttressed by the dye industry) had led to studies of the innervation of the skin and mucosa by pioneering anatomists. It was found that free nerve endings permeated the skin and surrounding hairs, and that in addition, five types of anatomically different nerve endings could be found in addition to the ubiquitous free nerve endings. These were the Vater-Pacini corpuscles (1741, 1834), Meissner Wagner corpuscles (1852), Krause end-bulbs (1859), Merkel's disks (1875), and the Ruffini endings (1894) (Figure 1.2). In 1884 Blix reported mosaic spots in the skin for touch, cold, and warm, but found that the sense of pain could be found throughout the skin, at a density much higher than the mosaic spots. Von Frey suggested in 1895 that under the skin of each of Blix's sensory spot was an endorgan specialized to respond to a particular stimulus (Sinclair et al., 1967).

Unlike the other senses, pain was unique in that it could be produced by *any modality* of stimulus provided that it was sufficiently intense. It was also the most difficult to quantify due to it's subjective nature, and wide variability between individuals. Nociceptors in the skin have been found to be either unimodally or polymodally dedicated to painful sensation of high threshold mechanical stimuli, noxious temperatures, chemical insult or tissue damage. Mammalian nociceptors typically have multiple bare sensory dendrites with a highly elaborate dendritic pattern, with sensory receptors for each modality thought to reside in dendrites (Figure 1.3, 3.3). Nociceptors of similar morphology and function are present in *Drosophila*, and provide evidence for evolutionary conservation of this type of sensory neurons (Tracey et al., 2003).

In the early days of sensory physiology it became recognized that of the mechanical senses, there also must be a "deep sense" of representation of the body's internal position. This so-called sixth sense allows the body to know where it is positioned in space with respect to some coordinate system, and is important for both movement and posture. Although poorly understood, peripheral proprioceptive mechanosensory input provides ongoing information on body position, necessary for tuning central mechanisms driving locomotion and postural control (Proske and Gandevia, 2012). Sensory ion channels are the primary candidates mediating the sensing aspect of this process (Corey, 2006), whereas the central nervous system is responsible for the decoding of this input and the internal representation of body position (Proske and Gandevia, 2012).

Initiation of sensory impulses presents a series of problems: first the transmission of the stimulus from the external source to the receptor cell, second, the mechanism by which the stimulus is transformed into a membrane depolarization, third, the initiation of nerve impulses and their propagation into the afferent axon trunk. Stimuli are detected and encoded by specialized sensory neurons whose signaling mechanisms transduce these signals into depolarizing influences and action potential firing (Katz, 1950). This action potential firing is processed at several levels until it reaches higher order centers of the brain.

The discovery that specifically expressed G-protein coupled receptors were responsible for stimulus sensitivity in the visual, olfactory, and gustatory systems led researchers to suspect that this may be the case in the somatosensory system. In the mid 1990's a heat sensitive cation channel was observed in a subpopulation of nociceptors (Cesare and McNaughton, 1996), which was followed a year later by the cloning of a cDNA of the capsaicin receptor or vanilloid receptor, TRPV1, a Ca2+ permeable non-selective channel belonging to the TRP ion channel superfamily (Caterina et al., 1997), which were first described in Drosophila (Montell and Rubin, 1989). This cation channel could be opened by capsaicin, a selective activator of polymodal nociceptor neurons. In this way, TRPV1 was suggested to be the molecular substrate for sensing painful stimuli in these neurons. In addition, the channel was activated by temperatures above 43 and also activated by protons. It was mainly found in small unmyelinated neurons, thought to be involved in pain and temperature

sensation in the skin. Since the cloning of TRPV1, many more TRP channels have been characterized and expressed in heterologous systems. They are gated by temperature with thresholds from 52 to 18, encompassing the range of noxiuous and innocuous stimuli (Belmonte and Viana, 2008).

In contrast to rapid progress in delineating the molecular mechanism of temperature sensation, the mechanosensory proteins involved in sensing gentle or painful touch have proven more elusive. It is assumed in many cases that a large transduction apparatus composed of multiple proteins is necessary for mechanotransduction, which are often embedded in a tissue or organ. In addition, experimental stimulus delivery is not as straightforward with mechanosensation as it is with temperature or chemicals. Candidates for the touch mechanotransduction channel in mammals include several TRP channels TRPA1, TRPC1, TRPV4, as well as members of the Deg/ENaC family of channels, which are known mechanosensors in c. elegans. In addition, 2-pore potassium channels and P2X purinergic receptors have been implicated in playing modulatory roles during mechanotransduction (Belmonte and Viana, 2008).

A subset of the DEG/ENaC family, the degenerins, also play important roles in touch sensation and proprioception and have lent a great deal to our understanding of mechanosensitivity by ion channels. Specific mutations in MEC-4, MEC-10, UNC-8, UNC-105, and DEG-1 cause swelling and death of the expressing cells. The proteins were nicknamed the degenerins because of the

degenerative phenotype that they caused when mutated. Many of the MEC channels involved in mechanotransduction are co-expressed, such as the DEG/ENaC pore-forming subunits mec-4 and mec-10. These subunits, along with deg-1 act as mechanosensory ion channels for touch sensation *in vivo* (Geffeney et al., 2011; O'Hagan et al., 2004). Although many of the MEC channels are co-expressed and known to be involved in mechanotransduction, very little is known about their trafficking.

ENaC Channels, which are structurally related to the degenerins, are present on the surface of the apical surface of epithelial cells, mediating sodium ion transport across this tight junction bound sheet. The main purpose of the ENaC channel is to allow transepithelial transport of sodium. A strong electrochemical gradient drives the sodium ion through the ENaC channel, and then the Na+K+-ATPase pump acts on the basolateral membrane to pump the sodium out. In the kidney and in the colon, the ENaC channel plays an essential role in maintaining blood sodium and potassium levels, and for their homeostatic regulation. In the lung and salivary glands, the ENaC channel is important for maintaining fluid balance in the alveolar fluid or saliva. In addition, this sodium current can be modulated by shear at the plasma membrane (Fronius 2008).

Mutations in the β and ϒ ENaC subunits lead to hyperactive channels, causing Liddle's syndrome symptoms, with arterial hypertension and intrastitial volume expansion, hypernaturimia and hypokalemia. Unlike in patients with hyperaldosteronism, patient's with Liddle's syndrome have normal levels of

aldosterone in the blood. These mutations are due to a deletion in a PY motif in the intracellular tail of ENaC, leading to a decrease in ubiquitin ligation by the E3 ligase Nedd4. This leads to an increase in the activity of the channel leading to an increase in sodium readsorption.

Another homolog of the Deg/ENaC channels, found in mammals, are the four so-called Acid Sensing Ion Channels (ASIC) that have been cloned based on homology to DEG/ENaC channels, of which 1,2, and 3 are alternatively spliced. ASIC1a, ASIC2a, and ASIC2b, have widespread distribution in the nervous system, with ASIC4 being co-expressed in many areas. In the PNS of mammals ASICs are mainly found in small diameter free nerve endings involved in pain sensation. ASIC2a has been found in taste buds and has been shown to be involved in sour taste perception. ASIC4 has also been localized to the inner ear, but contribution to function is not understood (Belmonte and Viana, 2008).

The physiological role of ASICs is not well understood, but a major role could be in the sensation of painful stimuli, for example in response to tissue acidosis. Significant acidification of the extracellular space has been associated with epilepsy, and ASIC channels could be involved in homeostatic responses to this condition. ASIC2 knockout mice do not show any obvious phenotypes, except for a reduced low threshold rapidly adapting mechanosensor response in a skin-nerve preparation. ASIC2a/b have also been localized to lanceolate nerve endings of the hair follicle. In addition, ASICs are the target of mambalgins,

potent analgesic peptides with comparable in activity to morphine (Diochot et al., 2012).

Another recently discovered family of proteins, Piezo1 and Piezo2, have recently been identified as mechanically activated ion channels in the neuroblastoma cell line Neuro2A (Coste et al., 2010). The Piezo proteins are large membrane proteins with 24 to 40 transmembrane domains, and can induce mechanically activated cationic currents when expressed in a heterologous system. These Piezo proteins are thought to form tetramers, although it is not clear if this complex contains one or more functional pores (Bargiantsev et al., 2014).

Piezo1 appears to function in endothelial cells under static and shear stress conditions, and young knockout mice die in midembryonic stages due to vascular defects in the yolk sac (Liu et al., 2014). Endothelial specific elimination of Piezo1 also caused defects in vessel formation. In addition, Piezo1 also functions as a sensor of shear stress in umbilical vein cells, where cells having diminished Piezo1 showed defects in alignment with shear stress (Ranade et al., 2014).

While Piezo1 plays an important role in the vasculature, Piezo2 appears to function during the recognition of gentle touch as well as noxious stimuli in the somatosensory system. Piezo2 appears to be responsible for a rapidly desensitizing component of the mechanically activated current in Merkel cells,

but there are clearly other channels involved in this system (Maksimovic et al., 2014).

In Drosophila, where only one Piezo protein exists, this protein has been shown to play an important role in mechanical nociception response by Class IV da neurons (Kim et al., 2012). Because Class IV da neurons are thought to be polymodal nociceptors, it is possible that Piezo is functioning as a mechanical sensor for high threshold mechanical force. Although Piezo is a bonafide mechanosensor, it was still not clear whether Piezo would contribute to other mechanosensory behaviors served by Class IV neurons, such as locomotion (Ainsley et al., 2003). This aspect is addressed later in Chapter 3.

In addition to the diversity of ion channels expressed in sensory neurons, one of the most striking features of the somatosensory system is the degree of morphological diversity in the sensory nerve endings. Neurons are each endowed with a characteristic arbor shape as well as often being embedded in accessory tissues or organs. The shape of the neuron is presumably related to the function of the neuron, and is reproducible within neurons belonging to each sensory nerve ending type.

The dendritic arborization (da) neurons in *Drosophila* larvae constitute a useful genetic system for the study of mechanisms underlying dendrite development and function via ion channels. Da neurons are a set of four classes of well-defined segmentally repeated sensory neurons. Each class has dendritic arbors with a unique stereotypic pattern of arborization, presumably reflecting its

functional requirement (Bodmer and Jan, 1987; Grueber et al., 2003). Of these, Class III and Class IV da neurons have dendrites that tile the epidermis. Class I da and bd neurons are thought to express the TRPN channel NompC and are important for coordinating the appropriate timing of peristaltic locomotion; loss of NompC function in these neurons results in profound slowing and paralysis of the animal (Cheng et al., 2010).

The class III da neurons have actin-rich and spine-like filopodial processes and in this work I show that they are sensitive to gentle touch and act as rapidly-adapting mechanoreceptors. With my co-authors, we demonstrated that the class III neurons express the TRPN channel NompC, which is localized to dendrites and is involved in transducing the sensation of gentle touch (Tsubouchi et al., 2012; Yan et al., 2013; Kernan et al., 1994).

Class IV da neurons express the Degenerin/Epithelial Sodium Channel (DEG/ENaC), Pickpocket (Ppk1), and play an essential role in coordinating turning behavior (Adams et al., 1998; Ainsley et al., 2003). The DEG/ENaC or Ppk channel superfamily are voltage insensitive and are assembled as either homomeric or heteromeric trimers. Interestingly, Ppk1 also plays an essential role in mechanical nociception behavior (Zhong et al., 2010), suggesting that these neurons can process multiple stimulus modalities. Class IV da neurons resemble mammalian nociceptors morphologically (Caterina and Julius, 1999; Tracey et al., 2003) and in their polymodal sensitivity to a variety of sensory

stimuli (Ohyama et al., 2013). In addition to sensing mechanical nociception and coordinating locomotion, Class IV da neurons are also sensitive to temperature (Hwang et al., 2012), light (Xiang et al., 2010), and chemical stimuli (Kang et al., 2010; Xiang et al., 2010). Recent studies show that the newly discovered mechanosensory ion channel, Piezo (Coste et al., 2010), functions in mechanical nociception in Class IV da neurons (Kim et al., 2012) in a pathway that is parallel to Ppk1 with respect to mechanical nociception. How Ppk1 functions in parallel to Piezo during this process, is still unclear.

Chapter 2: Identification of Class III neurons as mechanotransducers involved in sensing gentle touch.

introduction

The sensation of touch is vital to the human experience, and is critical to our ability to feel the texture of materials, for appropriate grasping and manual manipulation of objects, as well as for social interaction. Despite the importance of this sensory modality, very little is known about how this signal is transduced in sensory neurons. In humans, several mechanoreceptors of the skin are thought to be involved in the sensation of touch. Each of these mechanosensory nerves have distinct firing properties and stimulus sensitivity, which facilitates effective behavior. Pacinian corpuscles are rapidly adapting mechanoreceptors in the skin that sense very small deflections such as gentle touch. These cells return to their normal firing rate after deflection in less than 100ms. In humans, this mechanoreceptor is located deep inside the skin, and are found at relatively low density. Meissner's corpuscles and hair follicle receptors have mild adaptation properties. They are compatible with sensing static force, and can fire for up to a second before they adapt. Ruffini endings, Merkel's cells, and Tactile disks are examples of mechanoreceptors with slow adaptation properties. This type of receptor is located near the surface of the skin and is thought to sense

static deformation. These are one of the receptors that are responsible for tactile sensation during grip and object manipulation.

The structure of the skin in Drosophila larvae is different from that of humans. A single layer of cuboidal epithelium secretes a layered substance on the apical surface, and is exposed to the Drosophila lumen on the basal surface. It is on the basal surface of the epidermis that sensory neurons of the peripheral nervous system extend many of their dendrites, embedded inside a basal lamina structure.

In the Drosophila larva two neurons are known to tile the basal lamina of the epidermis, the class III and class IV dendritic arborization (da) neurons. When we began this work we had little direct evidence for the mechanosensitivity of da neurons in Drosophila, never mind their adaptation properties. The likely role for the da neurons in facilitating movement was well established based on behavioral studies (Hughes et al., 2007; Song et al., 2007), however little was known with respect to a neuron's direct mechanosensing properties.

We knew that class I and bd neurons expressed NompC, and were important for locomotion, but based on Gal4 expression we did not know NompC was expressed in the Class III neurons. Based on a new Gal4 line (Petersen and Stowers 2011) specially designed to encompass both upstream and downstream enhancers of NompC, NompC-Gal4 could be seen as very highly expressed in

the class III and chordotonal organs. In addition, a new antibody against the ankyrin repeat of NompC showed strong evidence that NompC is expressed in the Class III da neurons (Liang et al., 2011).

Results:

While performing electrophysiological recordings of Class IV neurons, attempting to observe a mechanosensitive response to indentation, which I did not observe, I discovered that the Class III da neurons show a very strong and reproducible firing of action potentials in response to stimulation with a piezoelectric stimulator. This was the first recording of a mechanical response from da neurons. Interestingly the Class III neurons showed a marked adaptation in response to the stimulation, within 100 milliseconds suggesting that they may be best tuned to a rapid or transient stimulus, such as gentle touch. To perform these experiments, I developed a piezoelectric system for stimulation of Class III da neurons, shown in Figure 2.1.

Stimulation of the Class III neuron resulted in rapid response and adaptation of the neuron, shown in Figure 2.2. This system was used to quantify the contribution of NompC to mechanosensitivity in these neurons (Yan et al., 2013).

The rapid adaptation properties and sensitivity to micron displacement led us to consider if the larva is responding to a gentle and transient signal such as touch. Drosophila larva have a characteristic behavioral response to gentle touch with an eyelash (Kernan et al., 1994), so we tested the role of these neurons in their affect on this behavior. Silencing of the Class III neurons by over-expression of tetanus toxin led to a defect in this gentle touch response of the larva (Figure 2.3).

Discussion:

When we began this work we had little idea for the function of da neurons in Drosophila. The likely role for the da neurons in sensing body position was well established (Song et al., 2007; Hughes et al., 2007), but little was known with respect to their direct mechanosensing properties.

In mammals much is known about the properties of mechanoreceptors in the skin, however these systems are often more complicated than the fruit fly, and less easily amenable to study. Pacinian corpuscles are rapidly adapting mechanoreceptors in the skin that sense very small deflections such as gentle touch. These cells return to their normal firing rate after deflection in less than 100ms. In humans, this mechanoreceptor is located deep inside the skin, and so the density of these receptors is not as high as the free nerve endings; it is not necessary to have such a dense innervation of these receptors because the mechanical indentation is distributed. Meissner's corpuscles and hair follicle receptors have mild or medium adaptation properties. In contrast to the Pacinian corpuscles, they are compatible with sensing static force, and can be firing for up to a second. Ruffini endings, Merkel's cells, and Tactile disks are examples of mechanoreceptors with slow adaptation properties. This type of receptor is located near the surface of the skin and senses static deformation of the skin.

These are one of the receptors that are responsible for tactile sensation during grip and manipulation.

In the Drosophila da neuron system, it is not well understood which plane the neurons are in. The class III and class IV neurons exhibit tiling behavior, and thus it is thought that they occupy the same plane in the epidermis. It is not known however what the association of the dendrites of the Class III neurons, or for that matter, the spines, are with the epidermis.

Using a piezoelectric stimulator and extracellular recording pipette we were able to record action potential firing in the Class III neuron, and observe rapid adaptation of this neuron in response to mechanical stimuli. We were also able to show that the Class III neurons are important for gentle touch behavior. This was the first example of a da neuron sensing mechanical force with a neural response in action potentials. These experiments played a crucial role in our paper Yan et al 2013, whose first authors Zhiqiang Yan and Wei Zhang discovered the heterologous mechanosensitivity of the NOMPC protein, and discovered both based on a new Gal4 line (Petersen and Stowers 2011) and convincing antibody staining (Yan et al., 2013) that NOMPC is expressed in Class III neurons.

It is still not clear how calcium is transduced in the Class III neurons during mechanotransduction. Specifically, it is not known if the spines are responsible for the transduction. It is not clear where the initiation of the mechanical stimulus on the class III neuron occurs. One possibility is that the mechanical response is

initiated in the actin-rich filopodia, and another possibility is that it is initiated in the dendrite, another possibility is that it is initiated in both the spines and the dendrite. Future experiments using dendritic patch can test this.

It is still not clear if NompC is the only mechanosensor present in Class III da neurons. Future work will need to use patch clamp electrophysiology to determine if other mechanosensory ion channels are present in this neuron. Piezo is one possibility, as piezo –Gal4 labels the Class III neruons (Kim et al., 2012). RNASeq approaches may also allow the identification of genes specifically expressed in the Class III neurons.

The mechanism for rapid adaptation of Class III da neurons is not yet understood. One possibility is that the adaptation of the neuron has to do with the adaptation of the mechanosensitive ion channel itself (i.e. NOMPC). Another possibility is that the rapid adaptation is a combination of the properties of NOMPC as well as intrinsic properties conferred by other ion channels. Finally, one cannot exclude the influence of the mechanical shape or nanostructure of the neuron itself contributing to the adaptation of the neuron. Fortunately many of these possibilities can be tested within our system.

Materials and Methods: Materials and methods are described in our paper (Yan et al., 2013). Briefly, a piezoelectric flexure stage was used to deform the larval epidermis (muscles removed) while performing extracellular recordings of Class III neurons using a glass pipette (resistance approximately 500kOhm). Behavioral experiments were performed as previously described (Kernan et al., 1994).

Acknowledgements:

I would like to acknowledge my great colleagues Zhiqiang Yan and Wei Zhang for their encouragement on this work. I would also like to thank Yang Xiang for teaching me the initial recordings of da neurons.

Author contributions:

David Gorczyca, Zhiqiang Yan, and Wei Zhang performed the electrophysiology. David Gorczyca, He Ye, Shan Meltzer, and Zhiqiang Yan performed the behavioral tests.

Chapter 3: Identification of Ppk26, a DEG/ENaC channel functioning with Ppk1 in a mutually dependent manner to guide locomotion and mechanical nociception behavior in *Drosophila.*

David A. Gorczyca, Susan Younger, Shan Meltzer, Sung Eun Kim, Li Cheng, Wei Song, Hye Young Lee, Lily Yeh Jan, Yuh Nung Jan.

Introduction

The somatosensory system serves to integrate multiple modalities, including temperature sensation, mechanical cues, body posture (proprioception), and pain (nociception) (Lumpkin and Caterina, 2007). Stimuli can be either unimodal, responding to only one type of stimulus, or polymodal, responding to multiple stimuli. Nociceptors in the skin are an important class of somatosensory neurons, either unimodally or polymodally dedicated to painful sensation of high threshold mechanical stimuli, noxious temperatures, chemical insult or tissue damage. Mammalian nociceptors typically have multiple bare sensory dendrites with a highly elaborate dendritic pattern, with sensory receptors for each modality thought to reside in dendrites. Nociceptors of similar morphology and function are present in *Drosophila*, providing evidence for evolutionary conservation of this type of sensory neurons (Tracey et al., 2003).

Proprioceptors are another important class of sensory neurons, involved in sensing body position during movement. Although poorly understood, peripheral proprioceptive mechanosensory input provides ongoing information on body position, necessary for tuning central mechanisms driving locomotion (Proske and Gandevia, 2012). Sensory ion channels, primary candidates mediating this process (Corey, 2006), reside in dendrites of multidendritic and ciliated sensory neurons (Desai et al., 2014).

Although some sensory neurons function only as proprioceptors, there is growing evidence that polymodal nociceptive neurons can also convey

information about body position. Patients with congenital insensitivity to pain, arising from deficits in Ad and C fiber function but not from myelinated proprioceptive fibers, also have impaired proprioception (Axelrod and Hilz, 2003; Iijima and Haga, 2009; Rosemberg et al., 1994). “Sleeping” nociceptors in humans do not respond to painful stimuli unless sensitized by chronic injury (Ørstavik et al., 2003), and these could contribute proprioceptive or kinesthetic information in non-sensitized conditions. In *C. elegans*, PVD and FLP neurons are high-threshold mechanosensors mediating mechanical nociceptive behaviors, but also acting to coordinate body posture, locomotion, and temperature sensing (Way and Chalfie, 1989; Chatzigeorgiou et al., 2010; Arnadottir et al., 2011; Liu et al., 2012). An open question is how a single neuron can distinguish between mechanosensory modalities, for example those involved in proprioception and nociception.

The dendritic arborization (da) neurons in *Drosophila* larvae constitute a useful genetic system for the study of mechanisms underlying dendrite development and function via ion channels. Da neurons are a set of four classes of well-defined segmentally repeated sensory neurons. Each class has dendritic arbors with a unique stereotypic pattern of arborization, presumably reflecting its functional requirement (Bodmer and Jan, 1987; Grueber et al., 2003). Of these, Class III and Class IV da neurons have dendrites that tile the epidermis.

Class III da neurons have actin-rich spine-like filopodia and are sensitive to gentle touch. These neurons express the TRPN channel NompC, localized to

dendrites and involved in mechanosensation of gentle touch (Tsubouchi et al., 2012; Yan et al., 2013; Kernan et al., 1994). Class I da and bd neurons also express NompC and are important for coordinating the appropriate timing of peristaltic locomotion; loss of NompC function in these neurons results in profound slowing and paralysis (Cheng et al., 2010).

Class IV da neurons express the Degenerin/Epithelial Sodium Channel (DEG/ENaC), Pickpocket (Ppk1), and play an essential role in coordinating turning behavior (Adams et al., 1998; Ainsley et al., 2003). The DEG/ENaC or Ppk channel superfamily are voltage insensitive and are assembled as either homomeric or heteromeric trimers. Interestingly, Ppk1 also plays an essential role in mechanical nociception behavior (Zhong et al., 2010), suggesting that these neurons can process multiple stimulus modalities. Class IV da neurons resemble mammalian nociceptors morphologically (Caterina and Julius, 1999; Tracey et al., 2003) and in their polymodal sensitivity to a variety of sensory stimuli (Ohyama et al., 2013). In addition to sensing mechanical nociception and coordinating locomotion, Class IV da neurons are also sensitive to temperature (Hwang et al., 2012), light (Xiang et al., 2010), and chemical stimuli (Kang et al., 2010; Xiang et al., 2010). Recent studies show that the newly discovered mechanosensory ion channel, Piezo (Coste et al., 2010), functions in mechanical nociception in Class IV da neurons (Kim et al., 2012) in a pathway that is parallel to Ppk1 with respect to mechanical nociception. How Ppk1 functions in parallel to Piezo during this process, is still unclear.

DEG/ENaC-type channels are often co-expressed in various parts of the nervous system. Of these, Acid-Sensing Ion Channels (ASICs) are gated by protons and are targets of potent analgesics (Diochot et al., 2012). Although gated by protons, this stimulus activates only a small fraction of the maximal ASIC conductance, and mounting evidence suggests the existence of more potent stimuli *in vivo*, acting independently or coincidently to protons (Bagriantsev and Minor, 2010; Bohlen et al., 2011; Yu et al., 2010). During tissue injury and acidosis, ASIC channels function in behavior and physiology directly related to pH change (Ziemann et al., 2009). However, other stimuli may regulate the channels at physiologic pH (Wemmie et al., 2013). In heterologous systems, differential ASIC subunit composition can result in distinct response properties, but heteromutimerization might also affect surface trafficking of these channels *in vivo* (Sherwood et al., 2011). In *C. elegans*, many MEC channels involved in mechanotransduction are co-expressed, such as the DEG/ENaC pore-forming subunits mec-4 and mec-10. These subunits, along with deg-1 act as mechanosensory ion channels for touch sensation *in vivo* (Geffeney et al., 2011; O'Hagan et al., 2004). However, loss of mec-10 does not result in complete loss of touch sensitivity, but rather in an atypical mechanoreceptor current (Arnadottir et al., 2011).

In *Drosophila*, our knowledge about DEG/ENaC hetero-oligomerization is limited to co-expression of Ppk23 and Ppk29 in contact-chemoreceptors (Thistle et al., 2012), Ppk11 and Ppk16 in motor neurons (Younger et al., 2013), and

Ppk11 and Ppk19 in taste bristles (Liu et al., 2003). Ppk23 and Ppk29 play non-redundant and essential roles in contact chemoreceptors, with each mutant showing loss of calcium response to cuticular hydrocarbons and defective courtship. Ppk11 and Ppk16 are co-expressed in motor neurons as a single transcript in an operon-like fashion, and mutations in each or both subunits result in the same defect in homeostatic plasticity (Younger et al., 2013). Disrupting Ppk11 and Ppk19 also affects the ability to taste salt (Liu et al., 2003). Functional dependency on heteromeric channels could explain the non-redundancies observed in these cases, a possibility that remains to be tested.

Here we report that the previously uncharacterized DEG/ENaC channel Ppk26/CG8546 is specifically expressed in Class IV da neurons, which have been implicated in both mechanical nociception and proprioception. We find that Ppk26 and Ppk1 proteins co-localize in dendrites of Class IV da neurons and that they interact biochemically. Analysis of specific Ppk1 and Ppk26 null mutants reveals a reciprocal dependence for plasma membrane localization in dendrites. Behavioral analysis of mutants and knockdowns suggest that Ppk26 and Ppk1 are likely to function together during locomotor behavior, presumably through the transduction or processing of a proprioceptive cue. We also report that both Ppk1 and Ppk26 play a role in mechanical but not thermal nociception behavior. Surprisingly, we found that Piezo null mutants have normal locomotion, suggesting that while Piezo might function in parallel with Ppk1 in mechanical nociception, they participate in a separate pathway during locomotion. The

identification of Ppk26 as a DEG/ENaC subunit that is co-expressed with Ppk1 possibly in the same channel complex, as well as the reciprocal requirement for their dendritic plasma membrane localization, provides insights into the function of DEG/ENaC channels during mechanosensitive behaviors.

Results:

Ppk26 is a member of the DEG/ENaC family

Sensory modality is thought to be in large part determined by the complement of sensory ion channels expressed in a neuron. In a screen for ion channels specifically expressed in larval Class IV da neurons, we identified Ppk26 as a strong candidate. By fusing a 0.8 kB enhancer sequence upstream of the *ppk26* transcriptional start sequence to a minimal promoter and the Gal4 element, we generated the *ppk26-Gal4* driver, which was found to express Gal4 exclusively in Class IV da neurons (Figure 3.1A,B). A similar enhancer of 2.2kB upstream of Ppk26 was independently observed to drive Class IV da neuron-specific expression (Zelle et al., 2013). The *ppk26* gene is predicted to encode a member of the DEG/ENaC family of ion channels, which are thought to be trimeric based on crystallographic evidence (Jasti et al., 2007). Each subunit contains two transmembrane helices flanking a large extracellular domain (Figure 3.1C). The closest relatives of Ppk26 in *Drosophila* are Ripped pocket (Rpk) and Pickpocket (Ppk1), as well as Ppk5, Ppk12, Ppk28, and Ppk29 – all members of the DEG/ENaC family (Figure 3.1D).

A homology model of monomeric Ppk26, based on ASIC1 structure, demonstrates conservation of many structural features, including intracellular N and C termini, of 96 and 20 amino acids respectively, two helical transmembrane domains, and a large extracellular loop structure comprising an arm and hand holding a ball (Jasti et al., 2007) (Figure 3.1C). Key conserved components of

this extracellular loop are the wrist, palm, thumb, finger, knuckle and beta ball domains (Jasti et al., 2007) (Figure 3.1C). Conservation extends to the pore region of the predicted Ppk26 channel, where TM2 lines the pore and TM1 contacts the lipid bilayer (Gonzales et al., 2009, Baconguis et al., 2012) (Figure 3.S1). This conservation includes an alanine residue proximal to the pore that is thought to constitute the Degenerin site, namely, a residue which when mutated from alanine to valine leads to tissue degeneration as well as increased channel open time and open probability in *C. elegans* (Brown et al., 2007). Thus, our screen identified Ppk26 as a DEG/ENaC ion channel expressed in Class IV da neurons, which may form part of a functional channel with Ppk1.

Ppk26 protein is specifically expressed in Class IV da neurons

To confirm that *ppk26-Gal4* reflects the endogenous expression pattern, we generated anti-peptide antibodies to a Ppk26 epitope (amino acids 532-539) located on the exposed and structurally rigid knuckle region (Figure 3.1C) (Jasti et al., 2007, Gonzalez et al., 2009, Baconguis et al., 2012). In third-instar body walls, we found that anti-Ppk26 immunoreactivity is specifically localized to Class IV da neurons in the PNS (Figure 3.1E). anti-Ppk26 signal was found in the cell body as well as in the entire complement of dendritic processes, including higher and lower order branches, and the axon initial segment (Figure 3.1F1, F2). We also raised an antibody against a peptide (amino acids 506-523) inside the knuckle region of Ppk1. Similar to anti-Ppk26, anti-Ppk1 signal was found

exclusively in Class IV da neurons - within the cell body, the entire complement of dendritic processes, and the axon initial segment (Figure 3.1G1-2). The specificity of both antibodies was validated by immunocytochemistry of Ppk1-EGFP and Ppk26-mCherry expressed in heterologous systems (Figure 3.S1), as well as null mutants (Figure 3.3A1-A4).

ENaC channel surface trafficking is important for proper physiological function and mutants affecting trafficking result in aberrant sodium transport, as is the case in the renal tubules of Liddle's syndrome patients (Snyder et al., 1995). However, very little is known about Ppk channel family localization in neurons, besides channel function in sensory bristles (Thistle et al., 2012) and motor neuron presynaptic terminals (Younger et al., 2013). To determine the likely site of Ppk26 function in Class IV da neurons, we ascertained the cell surface localization of the channel under permeabilizing versus non-permeabilizing conditions (Figure 3.1F). Under permeabilizing conditions, Ppk26 was detected throughout the neuron including the dendritic arbor (Figure 3.1 F1, F2). However, under non-permeabilizing conditions, Ppk26 immunoreactivity was absent from the surface of the cell body and segments of primary dendrites proximal to the cell body (Figure 3.1 F3, F4). Instead, Ppk26 immunoreactivity was observed inside more distal regions of primary dendrites as well as in lower order dendrites. Similarly, we found that under permeabilizing conditions, Ppk1 was detectable throughout the neuron including the dendritic arbor (Figure 3.1 G1, G2). However, under non-permeabilizing conditions, Ppk1 immunoreactivity was

absent from the surface of the cell body and segments of primary dendrites proximal to the cell body. Instead, Ppk1 immunoreactivity was observed inside more distal regions of primary dendrites and well as in lower order dendrites (Figure 3.1 G3, G4). Thus, both Ppk26 and Ppk1 are specifically expressed in Class IV da neurons and are inserted in the plasma membrane of dendrites at a distance from the cell body. However, the possibility that glial membrane wrapping of the soma (Han et al., 2011) may prevent antibody penetration to the soma cannot be ruled out.

Ppk26 and Ppk1 likely co-exist in the same protein complex

To test whether Ppk1 and Ppk26 could function together, we first examined if they had overlapping localization in Class IV da neurons. Double labeling *ppk1*>Ppk1-EGFP expressing animals with antibodies against GFP and Ppk26 revealed that both proteins are colocalized in these neurons (Figure 3.1E). We also performed immunoprecipitation experiments to determine if Ppk1 and Ppk26 are found in the same protein complex. We co-transfected HEK293 cells with either Ppk26-mCherry and Ppk1-EGFP or Ppk26-mCherry and EGFP as control, and used anti-EGFP antibodies to immunoprecipitate proteins in complex with Ppk1-EGFP. We found that Ppk26 co-immunoprecipitated with Ppk1-EGFP when Ppk26-mCherry and Ppk1-EGFP were cotransfected (Figure 3.1H, lane 2), suggesting that these channel subunits can form a complex in heterologous cells. This co-immunoprecipitation was specific, since Ppk26 was not detectable in

control immunoprecipitation from cells expressing Ppk26-mCherry and EGFP (Figure 3.1H, lane 1). The finding that Ppk26 can form a complex with Ppk1 and that both proteins colocalize in the same dendritic regions of Class IV da neurons supports the notion that these channel subunits may assemble *in vivo* to form a functional heteromultimeric channel.

Interestingly, examination of RNA seq data through development (Daines et al., 2011) revealed that *ppk26* and *ppk1* transcripts have similar developmental expression dynamics (Figure 3.S1C, bottom) with the absolute *ppk26* RNA levels about twice that of *ppk1* RNA expression (Figure 3.S1C, top).

Generation of *ppk26* and *ppk1* mutants

Thus far studies of Ppk1 in *Drosophila* have relied on analyses of a combination of RNAi and large deficiencies removing genes in addition to *ppk1* (Ainsley et al., 2003; Zhong et al., 2010; Boiko et al., 2012). To determine the function of Ppk26 and its relation to Ppk1, we generated specific *ppk26* (Figure 3.2B) and *ppk1* (Figure 3.2A) null mutants by "super-sized" Minos element excision, using the Minos-element strains MB01724 and MB10310 respectively to generate specific deficiencies uncovering each gene (Witsell et al., 2009). We excised each of these Minos elements, which were located at the 3' end of the *ppk26* or *ppk1* gene (Figure 3.2). Since imprecise excisions of Minos elements rarely generate large deletions, we combined respective Minos element strains for *ppk1* and *ppk26*, in a mutant background for *mus309*, the *Drosophila* homolog of Bloom

Syndrome Helicase, to generate “super-sized” deletions. This *mus309* mutation interferes with double stranded break repair, resulting in large and frequent deletions upon Minos element excision (Witsell et al., 2009). Using this strategy, we were able to generate large but specific excisions of each gene. Imprecise excision of MB01724 resulted in *Df(3R)ppk26*^{rv11} and *Df(3R)ppk26*^{rv29}, referred to as *ppk26*D*11* and *ppk26*D*29.* The *ppk26*D*11* deletion removes the entire gene but retains a piece of the Minos element (Figure 3.2B), while *ppk26*D*29* removes all except for a piece of the first coding exon. To test for specificity, CG42458 and CG42660 located 1.4kB upstream and 6.9kB downstream respectively were assayed for genomic integrity (Figure 3.S2A). Imprecise excision of MB10310 resulted in *Df(2L)ppk1*^{rv5} and *Df(2L)ppk1*^{rv16}, referred to as *Ppk1*D*5* and *Ppk1*D*16*. The *Ppk1*D*5* deletion removes the gene through exon 7 UTR, and retains a piece of the Minos element (Figure 3.2A), and *ppk1D16* removes the entire gene, also leaving behind a piece of the Minos element. To test for specificity, elbowB and spel1 genes, located 9.4kB upstream and 11.3kB downstream of *ppk1* respectively were assayed for integrity in each case. Both genes were found to be intact (Figure 3.S2B). As genetic background controls, we also isolated precise excisions of MB01724 and MB10310, resulting in the revertants Mi{ET1}MB10724^{rv214} for Ppk26 and Mi{ET1}MB10310^{rv1} for Ppk1. To our knowledge these are the first specific null alleles of *ppk26* and *ppk1*, which will be useful for future functional studies of these co-expressed DEG/ENaC channel subunits, as well as analysis of genetic interactions.

Ppk1 and Ppk26 exhibit mutual dependence for dendritic plasma membrane localization.

Ion channels and receptors composed of multiple subunits are often assembled in the endoplasmic reticulum (ER), and traffic as multimers to their site of function (Heusser et al., 2005, Muth et al., 2003). Thus, lack of one of the subunits may prevent the others from reaching their destination. For example, the vanilloid receptor (TRPV) channel subunits, Nanchung and Inactive, which are critical for hearing in the adult fly (Gong, 2004) and larval sound response (Wu et al., 2011; Zhang et al., 2013) have interdependent trafficking in chordotonal organs. Mutants in either *nanchung* or *inactive* result in the apparent absence of the other subunit in cilia, possibly accounting for the hearing defect in either mutant (Gong et al., 2004), and indicating that expression is either co-regulated or that the proteins become degraded in single mutants. In *C. elegans*, OSM-9 and OCR-2 channels also have mutually dependent transport and function in the ciliated ASH neuron (Tobin et al., 2002), although surface expression has not been assayed.

If Ppk1 and Ppk26 form part of the same ion channel, then their trafficking to the cell surface might be linked. To test this hypothesis, we examined the surface expression of Ppk26 on the dendritic plasma membrane under non-permeabilizing conditions, in both wild type larvae and in *ppk1D16* mutants. Strikingly, in the absence of Ppk1, Ppk26 failed to be inserted at the plasma membrane of dendrites (Figure 3.3B1, B2). Similarly, we found that the removal

of Ppk26 prevented Ppk1 from localizing to the plasma membrane of dendrites (Figure 3.3B3, B4). Thus, Ppk1 and Ppk26 are mutually required for localization to the surface of dendrites – presumably their site of function. Interestingly, although surface localization was mutually dependent, under permeablizing conditions both Ppk1 and Ppk26 immunoreactivities were still found in the dendrites of Ppk26 and Ppk1 mutants, (Figure 3.3C, D) suggesting that it is plasma membrane integration which is affected. Although Ppk1 and Ppk26 are exclusively expressed in Class IV da neurons, we also confirmed that other da neuron classes were present in *ppk26* and *ppk1* mutants by labeling preparations with Futsch/22C10 antibody, which marks a neuronal epitope (Figure 3.3C, D).

Ppk26 degenerin mutation leads to loss of class IV da neuron dendrites

Mutations leading to increased activity of the human ENaC channel result in rare dominant hereditary diseases that impact kidney, colon and lung tissues (Kellenberger and Schild, 2002). In MEC channels it has been suggested that Degenerin mutations (substitution at the Degenerin residue; alanine for threonine, valine, or aspartate) increases calcium permeability (Bianchi et al., 2004), as well as channel open time and open probability (Brown et al., 2007), thereby increasing cytoplasmic calcium levels and leading to excitotoxicity. However, a degenerin phenotype has not been reported for Ppk channels, including animals over-expressing Ppk1-Deg (Wegman et al., 2010). Since Ppk26 contains a conserved Degenerin residue (Figure 3.1C), we sought to ascertain the effects of

this mutation in Class IV da neurons. In particular, we tested a Ppk26-Deg-mCherry transgene containing the Degenerin mutation (A547V) in the peri-TM2 region of the protein for expression in Class IV neurons. Over-expressing a wild type Ppk26-mCherry transgene alone in Class IV da neurons did not result in any obvious abnormal phenotypes in these neurons (Figure 3.4A). In contrast, expressing a Ppk26-Deg-mCherry mutant transgene resulted in loss of dendritic coverage by many of the Class IV da neurons along the body wall (Figure 3.4B), due to a drastic reduction in both primary and secondary dendrites (Figure 3.4C). This diverged from the observation that, as previously reported (Wegman et al., 2010), expressing the Ppk1-Deg mutant did not elicit a strong dendritic arbor phenotype (Figure 3.S4).

Ppk26 regulates locomotor behavior

Class IV da neurons are implicated in locomotor behavior, presumably by acting as proprioceptors that modulate motor output in a Ppk1-dependent manner (Ainsley at el., 2003). To test whether Ppk1 and Ppk26 regulate locomotor behavior via their activity in Class IV da neurons in a cell-autonomous manner, we examined locomotor behavior in larvae expressing either Ppk26-RNAi or Ppk1-RNAi in Class IV da neurons using the *ppk1-Gal4* driver (Grueber et al., 2007). Crawling behavior in *Drosophila* larvae consists of sequential contractions of circumferential and longitudinal muscles in each segment, which propagate from posterior to anterior segments (Fox et al., 2006; Hughes and Thomas, 2007;

Song et al., 2007; Ainsley et al., 2003; Vogelstein et al., 2013). Knockdown of either Ppk1 or Ppk26 resulted in a decrease in turning frequency (Figure 5A), such that locomotion was predominately composed of directional crawling. Similar abnormal behavior was observed in *ppk1* and *ppk26* null mutants (Figure 3.5B, E), but not in their respective revertants. This defect was rescued by expressing a wild type Ppk1-EGFP or Ppk26-mCherry transgene in Class IV neurons of the mutants (Figure 3.5C, E), thus confirming the critical role of these channel subunits in controlling locomotion. To look for any additive effect, we examined the phenotype of *ppk1; ppk26* double mutants, and found that turning was reduced to a similar extent as the single null mutant of either *ppk1* or *ppk26* (Figure 3.5D), supporting the notion that the two genes function together in the same pathway. Silencing the Class IV neurons by over-expressing Kir2.1 showed a reduction in turning, consistent with Class IV sensory neurons themselves contributing to this locomotion behavior (Figure 3.5G). Interestingly, *ppk1* and *ppk26* transheterozygotes did not differ from wild type controls (Figure 5H).

Since Piezo is a mechanosensitive channel required in Class IV da neurons for harsh mechanical touch sensation (Kim et al., 2012), we also examined turning behavior in these mutants. In contrast to *ppk1* and *ppk26* mutants, there was no significant difference in *piezo* mutant turning behavior (Figure 3.5F). Because *painless* and *dTrpA1* have also been implicated in Class IV da neuron function, we also tested mutations in these genes for turning

behavior. Because a bonafide null allele for Painless was not available, we generated *pain*^{pc} and *pain*^{pf} alleles missing the coding region and the entire gene region respectively (Figure 3.S3), to be used in conjunction with existing alleles. While *dTrpA1* mutants showed no significant defect in locomotion, *pain*^{1}, *pain*^{pc} and *pain*^{pf} alleles showed significant defects in turning behavior (Figure 3.5F). While *pain*^{1} showed defects in both thermal and mechanical nociception, *pain*^{pc} and *pain*^{pf} mutants showed less severe phenotypes (Figure 3.S6).

Ppk26 serves mechanical but not thermal nociception behavior

Class IV neurons have been implicated in both proprioceptive as well as multimodal nociceptive behaviors. Since Class IV neurons are important for thermal nociception behavior (Chattopadhyay et al., 2012; Hwang et al., 2012), we examined thermal response in mutant animals. While animals expressing Kir2.1 in Class IV neurons had a significant rightward shift in temperature response to a 46°C heated probe as compared to wild type (Figure 3.6 D-G), *ppk1* and *ppk26* mutants showed no significant difference from wild type (Figure 3.6 A-C). Since Ppk1 as well as Piezo have been implicated in Class IV da neurons for harsh touch sensation (Kim et al., 2012), we also tested harsh touch behavior in *ppk1*, *ppk26* and *piezo* mutants. We found significant defects in harsh touch behavior in all of the above mutants, consistent with previous work, as well as our finding that Ppk26 is the likely partner of Ppk1.

Discussion

We have identified Ppk26, an DEG/ENaC channel subunit that is co-expressed with Ppk1in Class IV da neurons. Consistent with the model that Ppk26 and Ppk1 may be subunits of the same channel, we found that Ppk1 and Ppk26 co-localize in Class IV da neurons, that they form a complex in heterologous expression systems, and that they show non-additive and non-redundant mutant phenotypes *in vivo*. Ppk26 protein was found in somatic, dendritic and axonal compartments, and plasma membrane insertion was observed in terminal dendrites. Ppk1 and Ppk26 were reciprocally required for normal trafficking and/or insertion to the plasma membrane, further supporting the notion that these two channel subunits interact *in vivo*. We show that, as is the case for Mec Degenerin channels, mutations at the Degenerin position of Ppk26 lead to loss of Class IV da neuron integrity. We also found that Ppk26 function plays essential roles in normal larval locomotion, particularly in turning behavior.

Proposed role of Ppk1 and Ppk26 in mechanosensation by class IV da neuron dendrites

Overexpression of EGFP-tagged MEC channels in *C. elegans* has been reported to result in a punctate localization, leading to suggestions that each of these puncta represents a mechanosensory apparatus (O'Hagan et al., 2004). In light of the speculation that contact of dendrites with subcuticular epidermis is

part of the apparatus that senses mechanical stimuli (Chalfie 2009; Chen and Chalfie, 2014; Han et al., 2012), it is intriguing that we find both Ppk1 and Ppk26 on the surface of distal and higher order dendrites (Figure 3.1), consistent with channel function in this compartment. Mutants in Ppk1 and Ppk26 showed defects in the frequency of turning of freely crawling larvae (Figure 3.5). Moreover, loss of function of either or both Ppk26 and Ppk1 had the same effect on larval locomotion. These findings support the notion that Ppk26 and Ppk1 may act in the same pathway – perhaps in the same channel complex – in mechanosensation that is important for proper locomotion.

Is Ppk26 associated with Ppk1 in a multimeric channel?

Class IV da neurons are known to express the DEG/ENaC channel subunit Ppk1 (Adams et al., 1998), an observation that was confirmed in our study. Given that DEG/ENaC channels are trimeric ion channels that typically require the assembly of different subunits for proper function, the specific co-expression of Ppk26 in Class IV da neurons raises the possibility that Ppk1 and Ppk26 may correspond to different subunits of the same mechanosensory channel *in vivo*. This is supported by our findings of biochemical interactions between Ppk1 and Ppk26 (Figure 3.1), their null mutant phenotypes that indicate non-redundancy (Figure 3.5), and by the reciprocal requirements of Ppk1 and Ppk26 for normal trafficking or insertion to the plasma membrane (Figure 3.3). The mechanism for this mutual dependence is currently unknown.

Notably, inspection of the developmental expression profile of *ppk1* RNA and *ppk26* RNA revealed a similar time course, with the absolute levels of RNA for *ppk26* twice as high as those of RNA for *ppk1* throughout development (Figure 3.S1). Although we cannot be certain that this quantitative difference in RNA levels reflects a similar quantitative difference in the levels of Ppk1 and Ppk26 subunits, it is tempting to speculate that two Ppk26 subunits and one Ppk1 subunit may be assembled to form a surface-expressed trimeric channel.

Tendency of the Degenerin mutation to be involved in neuronal degeneration

Studies in many systems have suggested that mutations in the Degenerin domain of DEG/ENaC lead to loss of cell integrity and perhaps degeneration, however this behavior has not yet been observed in *Drosophila* Pickpocket family members. The Degenerin position is localized in the wrist region, close to the mouth of the pore. Thus, it has been suggested that Degenerin mutations change the properties of the channel, increasing open time probability and perhaps shifting its ion selectivity from Na^+ to Ca^{2+} (Bianchi et al., 2004; Brown et al, 2007). Given the tight regulation of Ca^{2+} within cells and its involvement in critical cellular processes, this increase in Ca^{2+} permeability may lead to loss of cellular homeostasis, in a process that has been dubbed excitotoxicity. Consistent with these findings, we also observed that when a Degenerin mutation was introduced into Ppk26 that was overexpressed in Class IV da neurons, it resulted in a marked reduction in dendritic arbor size (Figure 3.5). This suggests that the

Ppk26-Deg mutation leads to toxicity in the sensory neurons, as has been observed in DEG/ENaC channels in *C. elegans*, but not in Ppk1 (Wegman et al., 2010). Whereas it is likely that the function of the pore structure of these channels is evolutionarily conserved, it is unclear why Ppk26-Deg has a more potent effect than Ppk1-Deg. One possibility is that the Ppk26-Deg residue makes a larger contribution to pore structure than Ppk1-Deg, due to its intrinsic structure or due to a potential 2:1 stoichiometry *in vivo*.

Role of Ppk26 and Ppk1 in larval locomotion

Larval locomotion is likely regulated by sensory input provided by sensory neurons in the body wall, which may in turn modulate the motoneurons innervating the body wall. The *C. elegans* mechanosensitive TRPN channel TRP-4 acts in the DVA neuron to coordinate bending behavior and body posture through positive and negative modulation (Li et al., 2006). It seems likely that Class IV da neurons likewise provide some information for sensory modulation of locomotion through a mechanosensory mechanism, but future work will need to determine if this is indeed the case.

Role of Class IV da neurons in proprioception

Proprioception is a mechanosensory process involving sensory neurons that transduce the mechanical information related to body position or characteristics of the environment for the generation of appropriate behavioral

output, such as the turning locomotor behavior that is essential for foraging larvae (Chalfie et al., 2009; Proske and Gandevia, 2012). Here we have identified a member of the DEG/ENaC family of proteins, Ppk26, which acts together with Ppk1 likely as subunits of a channel important for mechanosensation. Our results suggest that perhaps a major site of mechanosensory transduction is located in Class IV da neuron dendritic processes. The behavioral phenotypes of larvae with Ppk1 and Ppk26 knockdown in Class IV da neurons as well as the respective null mutants or double mutants suggest that a deficit in these channels interferes with the ability of the animal to execute proper turning behavior, raising the possibility that the two subunits could be involved in proprioceptively sensing the deformation of the cuticle. Whether Class IV neurons function as proprioceptors still needs to be directly demonstrated, and future experiments will be needed to address the relationship between Class IV neural activity and body position.

Role of Class IV da neurons in nociception

Numerous studies have implicated the Class IV da neurons in both thermal and mechanical nociception behavior (Zhong et al., 2010, Zhong et al., 2012, Kim et al., 2012). We found that while Ppk1 and Ppk26 are important for mechanical nociception behavior, they are dispensable for thermal nociception behavior. While Ppk1 and Ppk26 channels indeed moonlight during two processes in the same neuron, namely mechanical nociception and

proprioception, these channels must be playing a specific role as they are not involved in thermal response by the same neuron (Figure 3.4, Figure 3.6).

Different ion channels may serve different mechanosensory modalities in the same neuron

Whereas the Class I da neurons and bd neurons implicated in proprioception for the regulation of sequential contractions use the TRPN channel NompC as the sensor (Cheng et al., 2010), Class IV da neurons rely on the DEG/ENaC channel likely composed of Ppk26 and Ppk1 for the regulation of turning behavior as well as mechanical nociception, perhaps through sensing a mechanical signal at the cuticle. Interestingly, we found that Piezo, a bonafide mechanotransducing ion channel involved in Class IV da neuron mechanotransduction and required for mechanical nociception, does not appear to be involved in turning behavior, suggesting that different combinations of ion channels may serve different mechanosensory functions in the same neuron.

Materials and Methods:

Molecular Biology and Cloning: Standard cloning techniques were used to generate the C-terminal tagged Ppk26-mCherry and Ppk1-EGFP. Ppk1>Ppk26-mCherry and Ppk1>Ppk1-EGFP lines used for rescue experiments were cloned into an APPHIH vector containing the Ppk enhancer. UAS expression constructs were generated by cloning the tagged genes into pTW and pUAST. UAS-Ppk26 Degenerin-mCherry was generated by site directed mutagenesis [A547V] of Ppk26-mCherry/pmCherry-N1.

Locomotion Behavior Assay:

Wandering 3rd instar larvae were placed on a fresh agarose plate, equilibrated for 2-3 minutes, then transferred to an additional agarose plate for imaging. The plate was illuminated tangentially using 850nm infrared LED strips 120 degree beam angle, #3258 (EnvironmentalLights.com). The LED light was passed through a diffuser to form an arena with high contrast. The larvae were tracked using OpenCV software.

Mechanical and Thermal Nociception Assays:

Mechanical Nociception Assay was performed as in Zhong et al., 2010. Each trial corresponds to the percentage of responding larvae. Thermal Nociception Assay was performed as in Hwang et al., 2012.

Immunocytochemistry and Immunofluorescence Imaging:

Larvae were dissected in PBS and fixed in 4% Paraformaldehyde. For permeablizing conditions, larvae were washed with 0.1% Triton-X 100 in PBS and for non-permeablized conditions, Triton-X 100 was omitted. Rabbit anti-Ppk26 was used at 1:10,000 and Rabbit anti-Ppk1 at 1:3000.

Antibody Generation:

Rabbit anti-peptide antibodies were generated against synthetic peptides representing the knuckle region of PPK1 (AA 506-523) and PPK26 (AA 532-539) (YenZym, South San Francisco).

Fly Genetics and Mutant Generation:

The following fly stocks were used: Mi{ET1}MB10724, Mi{ET1}MB10310, P{hsILMiT}2.4, mus309[N1], and mus309[D2] (Bloomington Stock Center); UAS-Ppk26-mCherry and Ppk1-EGFP (generated via germline transformation); *ppk1-RNAi* (Xu et al., 2004); *ppk26*-RNAi (GD5110; VDRC); was obtained from VDRC; $pain^{pc}$ and $pain^{fc}$ (generated as in Gao et al., 2008); $dTrpA1^{ins}$ (Dr. Paul Garrity); $piezo^{ko}$ (Dr. Ardem Patapoutian). A 1kB *ppk1*-Gal4 was used to label Class IV

neurons (Grueber et al., 2007). Mi{ET1} excisions were performed in a *mus309* mutant background as in Witsell et al,. 2009.

Immunoprecipitation and Westerns

Immunoprecipitations were performed from transfected HEK293 cell homogenates using protein A-conjugated Sepharose beads (Invitrogen). Proteins separated by SDS/PAGE were transferred to nitrocellulose membranes and after immnolabeling examined by chemiluminescence.

Acknowledgements:

We would like to thank Jill Wildonger for her assistance in the painless mutant generation. We would like to thank all members of the Jan Lab for their useful discussion and comments on the manuscript, particularly Smita Yadav and Christian Peters. We would like to thank Matthew Klassen and Wei Zhang for their technical assistance throughout this work. We would like to thank Dr. Miriam Goodman for her helpful discussion. We thank the Bloomington Stock Center, VDRC, as well as Dr. Fen Biao Gao for making fly stocks available for this work. This work is supported by NIHR37NS040929 to YNJ, T32 GM007449 Pre-doctoral Training Grant in Neurobiology to UCSF Neuroscience Graduate Program, and NIGMS Graduate Fellowship to DG. LYJ and YNJ are HHMI investigators.

Author Contributions:

DG YN and LJ designed the experiments. DG, SY, SK, LC, HL, WS, all contributed to the experiments. SY and DG made the ppk1 and ppk26 mutants. SY, SM, and LC made the painless mutants. DG and SM did the behavioral experiments and analyzed the data. WS made ppk1 transgenic lines and ppk26-Gal4. DG made ppk26 transgenic lines. DG and YN wrote the paper.

Chapter 4: Concluding Remarks and Future Directions

Animals contain mechanisms for interacting with their environment that are exquisitely sensitive to particular modalities. Of all our senses, mechanosensation is the least well understood. The mammalian somatosensory system contains several sensory organs specialized for mechanotransduction, however it is difficult to study for a number of reasons, including the diminutive size and overlapping distribution of sensory nerve endings, low abundance of receptor molecules, long distance between sensory specialization and cell body, and relative difficulty to do genome-wide genetic screens. The da neuron system in Drosophila offers a powerful system in which to study the molecular mechanisms of somatosensation in a genetic system amenable to both imaging and electrophysiology. In this work, we showed that the Class III neurons are rapidly adapting mechanotransducers involved in gentle touch response. In addition, we showed that the DEG/ENaC channel Ppk26 can interact with Ppk1 in Class IV neurons, where it plays a role in both mechanical nociception and locomotion behavior.

Although it is clear that NompC is a mechanosensitive ion channel expressed in Class III neurons, and mediating mechanical response of the neuron, it is not clear if Ppk1 and Ppk26 come together to form a mechanosensitive ion channel in Class IV neurons to mediate proprioceptive or

mechanical nociceptive behavior. The fact that thermal nociception, a process dependent on the Class IV neurons, is normal in the mutants suggests that the channels are not required for the basic ability of the neurons to fire action potentials, but rather participate in some specific transduction mechanism contributing to locomotor or mechanical nociceptive behavior. Although we made efforts to test mechanotransduction of Ppk1/Ppk26 in heterologous systems, so far we have not observed any currents. It is possible that a functional channel is not formed in heterologous systems, and that other as of yet unidentified components are necessary, and thus multiple components will need to be reconstituted in this system, or that a current might be isolated from dissociated neurons stimulated mechanically with the appropriate stimulus.

The locomotion phenotype observed in Class IV silenced neurons suggests that there could be some function of these neurons as proprioceptors. In ongoing work we have observed in a fictive locomotion preparation that the Class IV neuron synaptic terminals indeed have periodically active calcium transients that require both muscle contraction and integrity of the nerve bundle. Although this periodic activity may contain information about body position of the animal, a systematic approach must be used to address if indeed the Class IV neurons are sensing the body position, or contractedness of the cuticle. For this, we have built a mechanical device designed to stretch the larval cuticle.

Another open question after this work is whether the Class IV neuron are indeed both proprioceptors and nociceptors, as is our working hypothesis. Since

the Drosophila larva crawls at a frequency of approximately 1Hz, and there are over 10 segments in the animal, it might imply that for a proprioceptive stimulus the moment to moment activation of the Class IV neuron occurs on the order of 100 milliseconds. This is much faster than the time it takes for behavioral response to harsh touch or the light response, which in our observation can be on the order of one second. Ongoing experiments are more precisely timing these behavioral responses as well as recording electrophysiological responses during relevant stimulation.

It is quite interesting that the molecular mechanisms for nociception and locomotion behavior appear to be different in the Class IV neurons. The TRPA1 channel is sensitive to isothiocyanates, and contributes to ability to sense harsh touch and thermal nociception. In addition, the channel piezo is responsible for the response to harsh touch conveyed by the Class IV neurons, and is indeed a mechano-gated channel. On the other hand, TRPA1 and piezo mutants do not have locomotor phenotypes. Furthermore, ppk1 and ppk26 mutants have locomotion phenotypes and harsh touch phenotypes, and painless mutants have only locomotion phenotypes. If all of these channels are expressed in Class IV neurons, it might suggest that TRPA1 and Piezo subserve a pain function, whereas painless subserves a function during locomotion, and Ppk1 and Ppk26 contribute to both (see Table 1).

Future work will need to look at whether mechanoelectric transient is affected in ppk1 and ppk26 mutants. Since piezo is co-expressed in the Class IV

neurons, it would make sense to look at piezo single mutants as well as piezo-ppk26 or piezo, ppk1; ppk26 mutants for these experiments. It is known that free nerve endings in the human knee are likely to be a mixture of both proprioceptor and nociceptor, but this duality has not been demonstrated in any system. Unlike the golgi tendon receptors of muscles, the joint proprioceptors in mammals are composed of Pacinian corpuscles, as well as free nerve endings that are sensitive to both non-nociceptive and nociceptive stimuli. Although the Drosophila larva does not have bones or joints, it is possible that the free nerve endings of Class IV neurons are sensing stretch and position of the body just as the joint proprioceptors of humans.

Figure Legends:

Figure 1.1: The drawing of the kneeling man. In this diagram, Descartes wanted to account for the behavior of a man kneeling next to a fire. He believed that the particles of the fire could excite “animal spirits” in the area of the skin that would pass through conduits in the mans body up to his head, which in turn would cause the eyes to look at it, realize the foot is on fire, and to remove the foot from the fire.

Figure 1.2: In the skin there are several types of nerve endings found, based on morphology. These are the Ruffini Corpuscles, Free Nerve Endings, Merkel’s Disks, Krause’s Bells, Pacini’s Corpuscles, and Hair Follicle Nerve endings

Figure 1.3: modality specificity of the somatosensory system in humans. A, Sensory modalities for touch, temperature, pain, vibration are all present in the finger tip. These project to the spinal chord and then to the hand area of the somatosensory cortex. The finger areas in human contain some of the highest densities of nerve endings. B, Each dorsal root ganglion in humans contains sensory neurons whose dendrites innervate a specific area of the body called a dermatome. Axons from the nerve terminal defasiculate and the nerve endings are spread about the skin surface. Generally the density and relative composition of sensory nerve endings is different in the various dermatomes.

Figure 1.4: Schematic of da neuron classes. Class I, II, III, IV are shown, as well as the bipolar dendrite neuron and chordotonal organ.

Figure 2.1: Schematic of stimulation setup for Class III neuron poking. In this figure, a schematic diagram shows a beveled glass pipette stimulating a Class III neuron. In contrast to mechanical stages driven by magnetic motors, piezoelectric crystals allow movement without the generation of large electric fields, which aid themselves to electrophysiological recording.

Figure 2.2: Class III neurons are rapidly-adapting mechanosensors A, Deflection of the simulating probe during simultaneous recording resulted in a rapid response of the neuron, that was restricted to about 100 milliseconds. B, this system was used to examine the contribution of NOMPC to the respons of this neuron.

Figure 2.3: Behavioral Response to Touch. Animals expressing TNT in Class III neurons showed a defect in touch response (from Yan et al., 2013).

Figure 3.1: Ppk26 is a newly identified DEG/ENaC subunit expressed in Class IV neuron dendrites and interacting with Ppk1. A: Location of Class IV neurons on the larval body wall. B: Expression pattern of *ppk26-Gal4* (Grueber et al., 2007). Scale bar = 5mm. C: Homology model of a single Ppk26 subunit based on ASIC2A crystal structure. Peptide antibodies were raised against the knuckle region. D: Phylogenetic tree showing relationship between members of the Ppk family. E: Endogenous Ppk26 immunoreactivity was colocalized with Ppk1-EGFP immunoreactivity in Class IV dendrites. Scale bar = 10mm. F:

Ppk26 immunoreactivity was present in the cell body and dendrites of Class IV neurons under permeablizing conditions (F1, F2). Surface immunoreactivity was present throughout the dendritic compartment including proximal and distal dendrites. Small arrowheads = terminal dendrites; open arrow = cell body; short arrow = axon. G: Ppk1 immunoreactivity was present in the cell body and terminal dendrites of Class IV neurons (G1, G2), and surface immunoreactivity was present in the cell body and terminal dendrites of Class IV neurons (G3, G4). Scale bar = 20mm. H: Immunoprecipitation of EGFP from cells co-transfected with Ppk26-mCherry and Ppk1-EGFP or EGFP. Coprecipitation of Ppk26-mCherry was observed when co-transfecting with Ppk1-EGFP (lane 2;), but no when co-transfecting with EGFP (lane 1; V= EGFP vector). Note that that IgG heavy chain is observed at ~ 50kD in lanes 1 and 2, and that a EGFP degradation or cleavage product at ~ 27kDa can be observed in lane 2. See also Figure S1.

Figure 3.2: Generation of specific ppk1 and ppk26 null mutants. A: Schematic showing excision of the Mi{ET1}MB10310 element at the 3' of ppk1/CG3478 produced *ppk1D5* and *ppk1D16* mutants, which delete the entire open-reading frame of Ppk1, leaving behind a piece of the Mi{ET1} element. B: Schematic showing excision of the Mi{ET1}MB10724 element at the 3' of *ppk26*/CG8546 gene produced *ppk26D11* and *ppk26D29* alleles. *ppk26D11* deletes the entire open-reading frame of *ppk26* leaving a piece of the Mi{ET1} element behind. *ppk26D29* deletes exons 3 through 6 as well as the 3' UTR and

polyadenylation sequence, leaving intact the non-coding exon 1 and part of the coding exon 2. Dashed lines indicates the region containing the break point, which does not affect upstream or downstream genes. Solid line indicates deletions confirmed by PCR. See also Figure S2.

Figure 3.3: Ppk1 and Ppk26 show mutually dependent surface expression on Class IV neuron dendrites. A: ppk1 and ppk26 null mutants lack immunoreactivity under permeablizing conditions. B: Ppk26 was present on the surface of Class IV neuron dendrites in wild type, and absent or severely reduced in mutants lacking Ppk1 (B1, B2). Ppk1 was present on the surface of Class IV neuron dendrites in wild type, and absent or severely reduced in mutants lacking Ppk26 (B3, B4). C: Immunostaining under permeablizing conditions showed mutants that while lacking Ppk1 still contained Ppk26 in Class IV neuron dendrites. D: Immunostaining under permeablizing conditions showed that mutants lacking Ppk26 still contained Ppk1 protein at Class IV dendrites. In C and D, Futsch/22C10 immunostaining confirmed the presence of the remaining neurons in the da neuron cluster in both in wild type and mutants. Scale bar = 30um.

Figure 3.4: Overexpression of Ppk26-degenerin resulted in a reduction in Class IV dendritic arbors. A: Overexpression of Ppk26-mCherry in Class IV neurons using the Ppk1 promoter resulted in strong localization to dendrites with elaborate primary and higher order arbors. B: Overexpression of Ppk26-degenerin-mCherry resulted in a dramatic reduction in dendritic arbors of mid-

second instar larvae. Arrowheads= cell bodies; arrows= fragmented and reduced dendritic arbors. Scale bar = 300mm. C, D: Over-expression of Ppk26-degenerin-mCherry in a wild type background resulted in reduced arbor complexity of third instar Class IV neurons, as evidenced by the membrane marker CD4-tdGFP. Scale bar = 60mm.

Figure 3.5: Ppk26 and Ppk1 are required for locomotion behavior. A: RNAi mediated knockdown of Ppk26 or Ppk1in Class IV neurons using *ppk1-Gal4* driver (Grueber et al., 2007) results in reduced turn frequency. B*: ppk1* and *ppk26* null mutant, but not revertant strains, have a reduced frequency of turns. C: Expression of Ppk1-EGFP or Ppk26-mCherry in Class IV neurons, using one copy of a direct *ppk1* promoter fusion, rescued the turning defects in *ppk1* and *ppk26* mutants. D: *ppk26* and *ppk1* but not *piezo* mutants had a reduced turning frequency. E: Representative traces of wild type, mutant, and rescue animals for Ppk26 and Ppk1. Arrowheads = turning. F: Locomotor turning behavior of *painless*, *dTrpA1*, and *piezo* mutants. G: Effect of silencing Class IV neurons. Error bars represent ±SEM, *=p<0.01. In violin plots, white circles show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; polygons represent density estimates of data and extend to extreme values. H. Turning behavior of heterozygotes and transheterozygotes. Error bars denote ±SEM, $n \geq 10$; ***= $p < 0.001$, Student's *t-test*. See also Figure S5.

Figure 3.6: Ppk26 and Ppk1 are required for mechanical but not thermal nociception. A-C: Thermal nociception of *ppk1* and *ppk26* mutants were assessed by applying a thermal probe and monitoring the time to nocifestive response ($n \geq 100$). D-G: Silencing of Class IV neurons using ppk-Gal4 (Grueber et al., 2007) resulted in a strong defect in thermal nociception response time ($n \geq 100$). H, I: *ppk1*, *ppk26*, and *piezo* mutant animals showed significant defects in harsh mechanical nociception response. Error bars = ± SEM, $n \geq 10$, ***= $p < 0.001$, Student's *t-test* and Fisher's exact test. See also Figure S6.

Figure 3S1: A. To determine anti-Ppk1 antibody specificity, HEK293 cells were transfected with Ppk26-mCherry (A1) or Ppk1-EGFP (A2) and probed with anti-Ppk1 antibody. B. To determine anti-Ppk26 antibody specificity, HEK293 cells were transfected with Ppk26-mCherry (B1) or Ppk1-EGFP (B2) and probed with anti-Ppk26 antibody. C. Expression levels from RNA Seq data, plotted as absolute expression level through development, and normalized expression level through development. D. Alignment of Ppk1 and Ppk26 protein sequences, as well as the peri-TM2 region of *C. elegans* Mec-4, Mec-10, *D. melanogaster* Ppk1, Ppk26, Rpk, as well as human ENaC a, b and g proteins.

Figure 3S2: A. Genomic locus for *ppk26*, showing upstream (CG42458) and downstream (CG42661) genes as well as the Minos element (m symbol). PCRs of various *ppk26* exons, the first being distal to the Minos element, the last being proximal to the minos element. PCRs of the last exon of CG42458 in wildtype and *ppk26* mutants indicate CG42458 is intact. PCRs of the first exon of

CG42661 in wildtype and *ppk26* mutants indicate CG42661 is intact. A water negative control was used to rule out potential contamination with wildtype DNA in our reagents. B. Genomic locus for *ppk1*, showing downstream (*spe1*) and upstream (*elbowB*) genes as well as the Minos element (m symbol). Several of the candidates from our original screen are shown, with *ppk1D5* and *ppk1D16* showing deletion of the first exon distal to the minos element, indicating the gene is deleted. PCRs of *spe1* last exon show that the gene is intact in the *ppk1D5* and *ppk1D16* mutants. PCRs of *elbowB* last exon show that the gene is intact in the *ppk1D5* and *ppk1D16* mutants. A water negative control was used to rule out potential contamination with wildtype DNA in our reagents.

Figure 3S3:A, B. Morphology of *ppk1D16* (A) and *ppk26D11* (B) mutants were examined by confocaling live animals expressing a Ppk>CD4tdTomato marker in Class IV neurons.

Figure 3S4: A, B. Morphology of Ppk1-Deg over-expressed in wildtype Class IV neurons did not show a dramatic phenotype. Outline of the larva is denoted with a white dashed line (A). Dendrites were marked with the Ppk>CD4tdGFP marker.

Figure 3S5:Schematic diagram of *painless* gene region is shown, with deletion of the coding and full length regions. PCR was used to verify the deletion of *painless* coding and *painless* full length mutants, with primers upstream and downstream of the large locus. NaCP60E 3' UTRs are shown

upstream and CG30427 is shown downstream. Normalized *painless* expression in the mutants was determined using qPCR.

Figure 3S6: A, B. Mechanical nociception response of *painless* mutants to a 50mN Von Frey filament ($n \geq 10$). D-F. Thermal nociception response of *painless* mutants using a 46°C heatprobe (C-F) ($n \geq 50$).

References:

Adams, C.M., Anderson, M.G., Motto, D.G., Price, M.P., Johnson, W.A., and Welsh, M.J. (1998). Ripped Pocket and Pickpocket, Novel Drosophila DEG/ENaC Subunits Expressed in Early Development and in Mechanosensory Neurons. J. Cell Biol. *140*, 143–152.

Ainsley, J.A., Pettus, J.M., Bosenko, D., Gerstein, C.E., Zinkevich, N., Anderson, M.G., Adams, C.M., Welsh, M.J., and Johnson, W.A. (2003). Enhanced Locomotion Caused by Loss of the Drosophila DEG/ENaC Protein Pickpocket1. Curr. Biol. *13*, 1557–1563.

Arnadottir, J., O'Hagan, R., Chen, Y., Goodman, M.B., and Chalfie, M. (2011). The DEG/ENaC Protein MEC-10 Regulates the Transduction Channel Complex in Caenorhabditis elegans Touch Receptor Neurons. J. Neurosci. *31*, 12695–12704.

Axelrod, F.B., and Hilz, M.J. (2003). Inherited autonomic neuropathies. Semin. Neurol. *23*, 381–390.

Baconguis I, Gouaux E. (2012). Structural plasticity and dynamic selectivity of acid-sensing ion channel-spider toxin complexes. Nature. 489, 400-5.

Bagriantsev, S.N., and Minor, D.L. (2010). Small molecule ion channel match making: a natural fit for new ASIC ligands. Neuron *68*, 1–3.

Bagriantsev S.N., Gracheva E.O., Gallagher P.G. (2014) Piezo proteins: regulators of mechanosensation and other cellular processes. J. Biol. Chem. 289, 31673-81.

Belmonte, C., and Viana F. (2008). Molecular and cellular limits to somatosensory specificity. Molecular Pain 4, 1-14.

Bianchi, L., Gerstbrein, B., Frøkjær-Jensen, C., Royal, D.C., Mukherjee, G., Royal, M.A., Xue, J., Schafer, W.R., and Driscoll, M. (2004). The neurotoxic MEC-4(d) DEG/ENaC sodium channel conducts calcium: implications for necrosis initiation. Nat. Neurosci. *7*, 1337–1344.

Bodmer, R., and Jan, Y. (1987). Morphological differentiation of the embryonic peripheral neurons in Drosophila. Roux's Arch. Dev. Biol. *196*, 69–77.

Bohlen, C.J., Chesler, A.T., Sharif-Naeini, R., Medzihradszky, K.F., Zhou, S., King, D., Sánchez, E.E., Burlingame, A.L., Basbaum, A.I., and Julius, D. (2011). A heteromeric Texas coral snake toxin targets acid-sensing ion channels to produce pain. Nature *479*, 410–414.

Boiko N, Kucher V, Stockand JD, Eaton BA. (2012). Pickpocket1 is an ionotropic molecular sensory transducer. J Biol Chem. 2012 287, 39878-86.

Brown, A.L., Fernandez-Illescas, S.M., Liao, Z., and Goodman, M.B. (2007). Gain-of-Function Mutations in the MEC-4 DEG/ENaC Sensory Mechanotransduction Channel Alter Gating and Drug Blockade. J. Gen. Physiol.

129, 161–173.

Caterina, M.J., and Julius, D. (1999). Sense and specificity: a molecular identity for nociceptors. Curr. Op. Neurobiol. *9*, 525–530.

Caterina M.J., Schumacher M.A., Tominaga M., Rosen T.A., Levine J.D., Julius D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389, 816-824.

Cesare P, McNaughton P. (1996). A novel heat-activated current in nociceptive neruons. PNAS 93, 15435-9.

Chalfie, M. (2009). Neurosensory Mechanotransduction. Nat. Rev. Mol. Cell. Biol. 10, 44-52

Chattopadhyay A, Gilstrap AV, Galko MJ (2012). Local and global methods of assessing thermal nociception in Drosophila larvae. J Vis Exp. May 18;(63):e3837.

Chatzigeorgiou M, Grundy L, Kindt KS, Lee WH, Driscoll M, Schafer WR. (2010). Spatial asymmetry in the mechanosensory phenotypes of the C. elegans DEG/ENaC gene mec-10. J Neurophysiol. 2010 104,3334-44.

Chen, X., and Chalfie, M. (2014). Modulation of C. elegans Touch Sensitivity Is Integrated at Multiple Levels. J. Neurosci. *34*, 6522–6536.

Chen L, Jeffries O, Rowe IC, Liang Z, Knaus HG, Ruth P, Shipston MJ. (2010) Membrane trafficking of large conductance calcium-activated potassium channels is regulated by alternative splicing of a transplantable, acidic trafficking motif in the RCK1-RCK2 linker. J Biol Chem. *285*, 23265-75.

Cheng, L.E., Song, W., Looger, L.L., Jan, L.Y., and Jan, Y.N. (2010). The Role of the TRP Channel NompCin Drosophila Larval and Adult Locomotion. Neuron *67*, 373–380.

Corey, D.P. (2006). What is the hair cell transduction channel? J. Physiol. *576*, 23–28.

Coste, B., Mathur, J., Schmidt, M., Earley, T.J., Ranade, S., Petrus, M.J., Dubin, A.E., and Patapoutian, A. (2010). Piezo1 and Piezo2 Are Essential Components of Distinct Mechanically Activated Cation Channels. Science *330*, 55–60.

Daines, B., Wang, H., Wang, L., Li, Y., Han, Y., Emmert, D., Gelbart, W., Wang, X., Li, W., Gibbs, R., et al. (2011). The Drosophila melanogaster transcriptome by paired-end RNA sequencing. Genome Research *21*, 315–324.

Desai, B.S., Chadha, A., and Cook, B. (2014). The stum Gene Is Essential for Mechanical Sensing in Proprioceptive Neurons. Science *343*, 1256–1259.

Diochot, S., Baron, A., Salinas, M., Douguet, D., Scarzello, S., Dabert-Gay, A.-S., Debayle, D., Friend, V., Alloui, A., Lazdunski, M., et al. (2012). Black mamba venom peptides target acid-sensing ion channels to abolish pain. Nature *490*,

552–555.

Fox, L.E., Soll, D.R., Wu, C.F. (2006). Coordination and modulation of locomotion pattern generators in Drosophila larvae: effects of altered biogenic amine levels by the tyramine beta hydroxlyase mutation. J. Neurosci. 26, 1486-98.

Geffeney, S.L., Cueva, J.G., Glauser, D.A., Doll, J.C., Lee, T.H.-C., Montoya, M., Karania, S., Garakani, A.M., Pruitt, B.L., and Goodman, M.B. (2011). DEG/ENaC but Not TRP Channels Arethe Major Mechanoelectrical Transduction Channels in a C. elegans Nociceptor. Neuron *71*, 845–857.

Gonzales EB, Kawate T, Gouaux E. (2009). Pore architecture and ion sites in acid-sensing ion channels and P2X receptors. Nature. 460, 599-604.

Gorczyca, D.A., Younger, S., Meltzer, S., Kim, S.E., Cheng, L., Song., W., Lee., H.Y., Jan., L.Y., Jan., Y.N. (2014). Identification of Ppk26, a DEG/ENaC Channel Funcitoning with Ppk1 in a Mutually Dependent Manner to Guide Locomotion Behavior in Drosophila. Cell Reports. 9, 1446-58.

Grueber W.B., Jan L.Y., Jan Y.N. (2002). Tiling of the Drosophila epidermis by multidendritic sensory neurons. Development. 129, 2867-78.

Grueber W.B., Ye B, Yang C.H., Younger S., Borden K., Jan L.Y., Jan Y.N. (2007). Projections of Drosophila multidendritic neurons in the central nervous system: links with peripheral dendrite morphology. Development. 134, 55-64.

Gong, Z. (2004). Two interdependent TRPV channel subunits, inactive and Nanchung, mediate hearing in Drosophila. J. Neurosci. *24*, 9059–9066.

Han, C., Jan, L.Y., and Jan, Y.N. (2011). Enhancer-driven membrane markers for analysis of nonautonomous mechanisms reveal neuron-glia interactions in Drosophila. Proc. Natl. Acad. Sci. U S A *108*, 9673–9678.

Han, C., Wang, D., Soba, P., Zhu, S., Lin, X., Jan, L.Y., and Jan, Y.N. (2012). Integrins regulate repulsion-mediated dendritic patterning of drosophila sensory neurons by restricting dendrites in a 2D space. Neuron *73*, 64–78.

Heusser K, Schwappach B. Trafficking of potassium channels. (2005). Curr Opin Neurobiol. 15, 364-9.

Huang J, Zhou W, Dong W, Hong Y. Targeted engineering of the Drosophila genome. Fly (Austin). 3, 274-7.

Hughes, C.L., and Thomas, J.B. (2007). A sensory feedback circuit coordinates muscle activity in Drosophila. Mol. Cell. Neurosci. *35*, 383–396.

Hwang, R.Y., Stearns, N.A., and Tracey, W.D. (2012). The Ankyrin Repeat Domain of the TRPA Protein Painless Is Important for Thermal Nociception but Not Mechanical Nociception. PLoS ONE *7*, e30090.

Iijima, M., and Haga, N. (2009). Evaluation of nonnociceptive sensation in patients with congenital insensitivity to pain with anhidrosis. Childs. Nerv. Syst.

26, 1085–1089.

Jasti J, Furukawa H, Gonzales EB, Gouaux E. (2007). Structure of acid-sensing ion channel 1 at 1.9 A resolution and low pH. Nature. 449, 316-23.

Kang, K., Pulver, S.R., Panzano, V.C., Chang, E.C., Griffith, L.C., Theobald, D.L., and Garrity, P.A. (2010). Analysis of Drosophila TRPA1 reveals an ancient origin for human chemical nociception. Nature *464*, 597–600.

Katz, B. (1950). Action potentials from a sensory nerve ending. J. Physiol. 111, 248-260.

Kellenberger, S., and Schild, L. (2002). Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. Physiol. Rev. *82*, 735–767.

Kernan, M.**,** Cowan, D. and Zuker, C. (1994). Genetic dissection of mechanosensory transduction: mechanoreception-defective mutations of *Drosophila*. Neuron 12, 1195–1206

Kim, S.E., Coste, B., Chadha, A., Cook, B., and Patapoutian, A. (2012). The role of Drosophila Piezo in mechanical nociception. Nature *483*, 209–212.

Li, W., Feng, Z., Sternberg, P.W., and Shawn Xu, X.Z. (2006). A C. elegans stretch receptor neuron revealed by a mechanosensitive TRP channel homologue. Nature *440*, 684–687.

Li J., Hou B., Tumova S., Muraki K., Bruns A., Ludlow M. J., Sedo A., Hyman A. J.,McKeown L., Young R. S., Yuldasheva N. Y., Majeed Y., Wilson L. A., Rode B., Bailey M. A.,Kim H. R., Fu Z., Carter D. A., Bilton J., Imrie H., Ajuh P., Dear T. N., Cubbon R. M.,Kearney M. T., Prasad R. K., Evans P. C., Ainscough J. F., Beech D. J. (2014). Piezo1 integration of vascular architecture with physiological force. Nature 515, 279-82.

Liu L, Leonard A.S., Motto D.G., Feller M.A., Price M.P., Johnson W.A., Welsh M.J. (2003). Contribution of Drosophila DEG/ENaC genes to salt taste. Neuron. 39, 133-46.

Liu S, Schulze E, Baumeister R. (2012). Temperature- and touch-sensitive neurons couple CNG and TRPV channel activities to control heat avoidance in Caenorhabditis elegans. PLoS ONE. 7:e32360.

Lumpkin, E.A., and Caterina, M.J. (2007). Mechanisms of sensory transduction in the skin. Nature *445*, 858–865.

Maksimovic S., Nakatani M., Baba Y., Nelson A. M., Marshall K. L., Wellnitz S. A., Firozi P., Woo S. H., Ranade S., Patapoutian A., Lumpkin E. A. (2014). Epidermal Merkel cells are mechanosensory cells that tune mammalian touch receptors. Nature 509, 617–621.

Montell C, Rubin GM. (1989). Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction.

Neuron 2, 1313-23.

Muth TR, Caplan MJ.(2003) Transport protein trafficking in polarized cells. Annu Rev Cell Dev Biol. 19, 333-66.

O'Hagan, R., Chalfie, M., and Goodman, M.B. (2004). The MEC-4 DEG/ENaC channel of Caenorhabditis elegans touch receptor neurons transduces mechanical signals. Nat Neurosci. *8*, 43–50.

Ohyama T, Jovanic T, Denisov G, Dang TC, Hoffmann D, Kerr RA, Zlatic M. (2013). High-throughput analysis of stimulus-evoked behaviors in Drosophila larva reveals multiple modality-specific escape strategies. PLoS ONE 20, e71706.

Proske, U., and Gandevia, S.C. (2012). The Proprioceptive Senses: Their Roles in Signaling Body Shape, Body Position and Movement, and Muscle Force. Physiol. Rev. *92*, 1651–1697.

Ranade S. S., Qiu Z., Woo S. H., Hur S. S., Murthy S. E., Cahalan S. M., Xu J., Mathur J., Bandell M., Coste B., Li Y. S., Chien S., Patapoutian A. (2014). Piezo1, a mechanically activated ion channel, is required for vascular development in mice. Proc. Natl. Acad. Sci. U.S.A. 111, 10347–10352.

Rosemberg, S., Marie, S.K., and Kliemann, S. (1994). Congenital insensitivity to pain with anhidrosis (hereditary sensory and autonomic neuropathy type IV). Pediatr. Neurol. *11*, 50–56.

Sherwood, T.W., Lee, K.G., Gormley, M.G., and Askwith, C.C. (2011). Heteromeric Acid-Sensing Ion Channels (ASICs) Composed of ASIC2b and ASIC1a Display Novel Channel Properties and Contribute to Acidosis-Induced Neuronal Death. J. Neurosci. *31*, 9723–9734.

Sinclair, D.C. (1967). Mechanisms of cutaneous sensation. Oxford University Press.

Snyder, P.M., Price, M.P., McDonald, F.J., Adams, C.M., Volk, K.A., Zeiher, B.G., Stokes, J.B., and Welsh, M.J. (1995). Mechanism by which Liddle's syndrome mutations increase activity of a human epithelial Na+ channel. Cell *83*, 969–978.

Song W, Onishi M, Jan LY, Jan YN. (2007). Peripheral multidendritic sensory neurons are necessary for rhythmic locomotion behavior in Drosophila larvae. Proc Natl Acad Sci U S A. 104,5199-204.

Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L., and Scott, K. (2012). Contact chemoreceptors mediate male-male repulsion and male-female attraction during Drosophila courtship. Cell *149*, 1140–1151.

Tobin, D.M., Madsen, D.M., Kahn-Kirby, A., and Peckol, E.L. (2002). Combinatorial Expression of TRPV Channel Proteins Defines Their Sensory

Functions and Subcellular Localization in C. elegans Neurons. Neuron *35*, 307–318.

Tracey, W.D., Jr., Wilson, R.I., Laurent, G., and Benzer, S. (2003). painless, a Drosophila Gene Essential for Nociception. Cell *113*, 261–273.

Tsubouchi, A., Caldwell, J.C., and Tracey, W.D. (2012). Dendritic Filopodia, Ripped Pocket, NOMPC, and NMDARs Contribute tothe Sense of Touch in Drosophila Larvae. Curr. Biol. *22*, 2124–2134.

Vogelstein J.T., Park Y., Ohyama T., Kerr R.A., Truman J.W., Priebe C.E., Zlatic M. (2014). Discovery of brainwide neural-behavioral maps via multiscale unsupervised structure learning. Nature 344, 386-392.

Way JC, Chalfie M. (1989). The mec-3 gene of Caenorhabditis elegans requires its own product for maintained expression and is expressed in three neuronal cell types. Genes Dev. 3,1823-33.

Wemmie, J.A., Taugher, R.J., and Kreple, C.J. (2013). Acid-sensing ion channels in pain and disease. Nat. Rev. Neurosci. *14*, 461–471.

Wegman, L.J., Ainsley, J.A., Johnson, W.A. (2010). Developmental timing of a sensory-mediated larval surfacing behavior correlates with cessation of feeding and determination of final adult size. Developmental Biology 345, 170-179.

Witsell, A., Kane, D.P., Rubin, S., and McVey, M. (2009). Removal of the Bloom Syndrome DNA Helicase Extends the Utility of Imprecise Transposon Excision

for Making Null Mutations in Drosophila. Genetics *183*, 1187–1193.

Wu, Z., Sweeney, L.B., Ayoob, J.C., Chak, K., Andreone, B.J., Ohyama, T., Kerr, R., Luo, L., Zlatic, M., and Kolodkin, A.L. (2011). A Combinatorial Semaphorin Code Instructs the Initial Steps of Sensory Circuit Assembly in the Drosophila CNS. Neuron *70*, 281–298.

Xiang, Y., Yuan, Q., Vogt, N., Looger, L.L., Jan, L.Y., and Jan, Y.N. (2010). Light-avoidance-mediating photoreceptors tile the Drosophila larval body wall. Nature *468*, 921–926.

Xu, K., Bogert, B.A., Li, W., Su K., Lee, A., Gao, F.B. (2004). The fragile X-related gene affects crawling behavior of Drosophila larvae by regulating the mRNA level of the DEG/ENaC protein pickpocket1. Curr. Biol. 12, 1025-34.

Yan, Z., Zhang, W., He, Y., Gorczyca, D., Xiang, Y., Cheng, L.E., Meltzer, S., Jan, L.Y., and Jan, Y.N. (2013). Drosophila NOMPC is a mechanotransduction channel subunit for gentle-touch sensation. Nature *493*, 221–225.

Younger, M.A., Müller, M., Tong, A., Pym, E.C., and Davis, G.W. (2013). A Presynaptic ENaC Channel Drives Homeostatic Plasticity. Neuron *79*, 1183–1196.

Yu, Y., Chen, Z., Li, W.-G., Cao, H., Feng, E.-G., Yu, F., Liu, H., Jiang, H., and Xu, T.-L. (2010). A nonproton ligand sensor in the acid-sensing ion channel. Neuron *68*, 61–72.

Zelle KM., Lu B., Pyfrom SC., Ben-Shahar Y. (2013). The genetic architecture of degenerin/epithelial sodium channels in Drosophila. G3 *3*, 441-50.

Zhang, W., Yan, Z., Jan, L.Y., and Jan, Y.N. (2013). Sound response mediated by the TRP channels NOMPC, NANCHUNG, and INACTIVE in chordotonal organs of Drosophila larvae. Proc. Natl. Acad. Sci. U S A *110*, 13612–13617.

Zhong, L., Hwang, R.Y., and Tracey, W.D. (2010). Pickpocket Is a DEG/ENaC Protein Required for Mechanical Nociception in Drosophila Larvae. Curr. Biol. *20*, 429–434.

Zhong L, Bellemer A, Yan H, Ken H, Jessica R, Hwang RY, Pitt GS, Tracey WD (2012). Thermosensory and nonthermosensory isoforms of Drosophila melanogaster TRPA1 reveal heat-sensor domains of a thermoTRP Channel. Cell Rep. 1, 43-55.

Ziemann, A.E., Allen, J.E., Dahdaleh, N.S., Drebot, I.I., Coryell, M.W., Wunsch, A.M., Lynch, C.M., Faraci, F.M., Howard, M.A., Welsh, M.J., et al. (2009). The amygdala is a chemosensor that detects carbon dioxide and acidosis to elicit fear behavior. Cell *139*, 1012–1021.

Ørstavik, K., Weidner, C., Schmidt, R., Schmelz, M., Hilliges, M., Jørum, E., Handwerker, H., and Torebjörk, E. (2003). Pathological C-fibres in patients with a chronic painful condition. Brain *126*, 567–578.

Table 1: Null mutant phenotypes of Class IV expressed ion channels:

	Ppk1/Ppk26	Painless	Piezo	dTRPA1
Locomotion Behavior	X	X		
Mechanical Nociception	X		X	X
Thermal Nociception				X

Figure 1.1:

Figure 1.2:

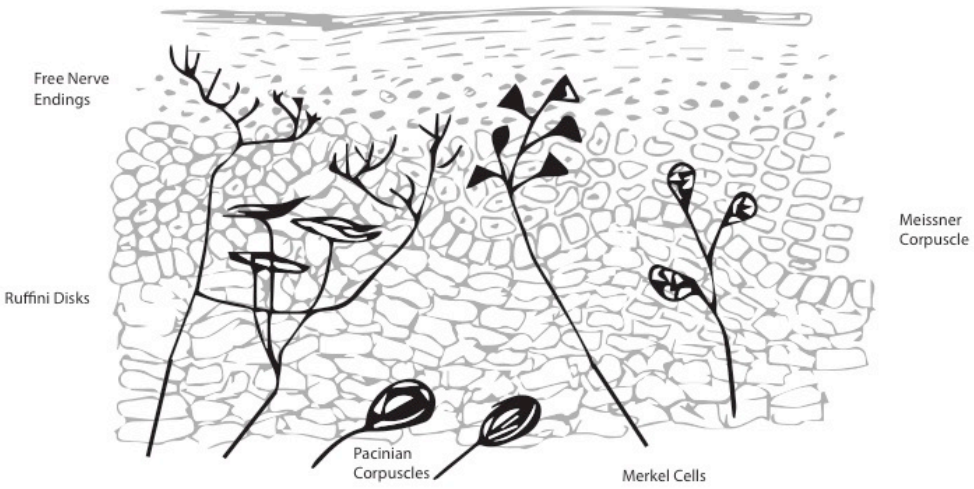
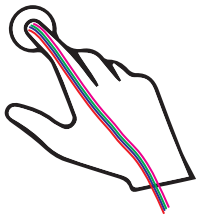

Free Nerve Endings
Ruffini Disks
Meissner Corpuscle
Pacinian Corpuscles
Merkel Cells

Figure 1.3:

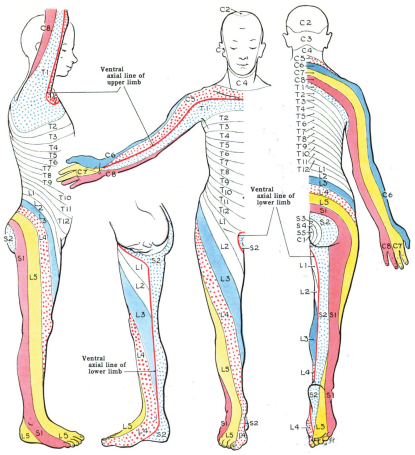
A



Touch
Temperature
Pain
Vibration

Cutaneous Sensory Modalities

B



Dermatomes in Human

Figure 1.4:

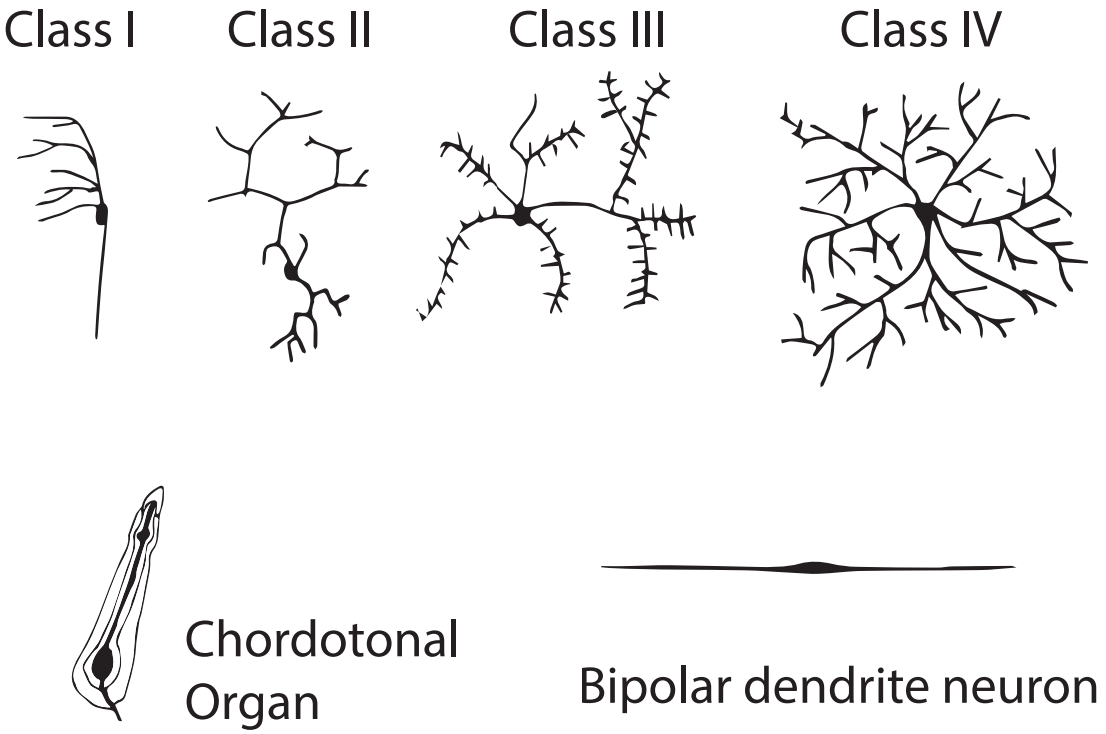

Class I
Class II
Class III
Class IV
Chordotonal Organ
Bipolar dendrite neuron

Figure 2.1:

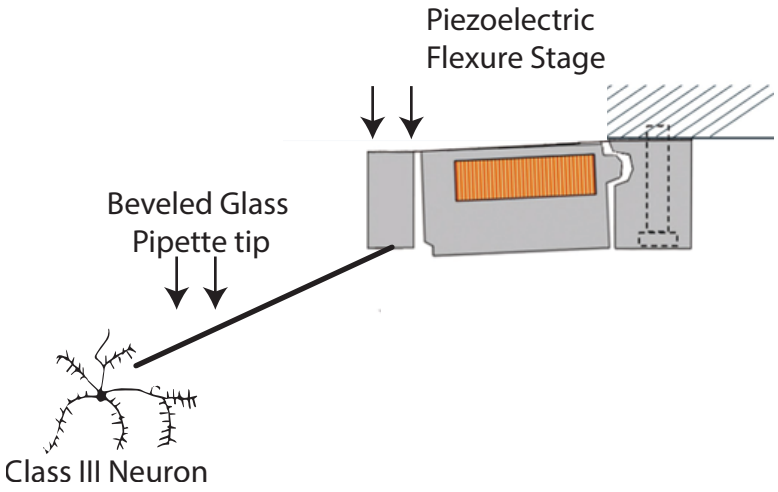

Piezoelectric
Flexure Stage
Beveled Glass
Pipette tip
Class III Neuron

Figure 2.2:

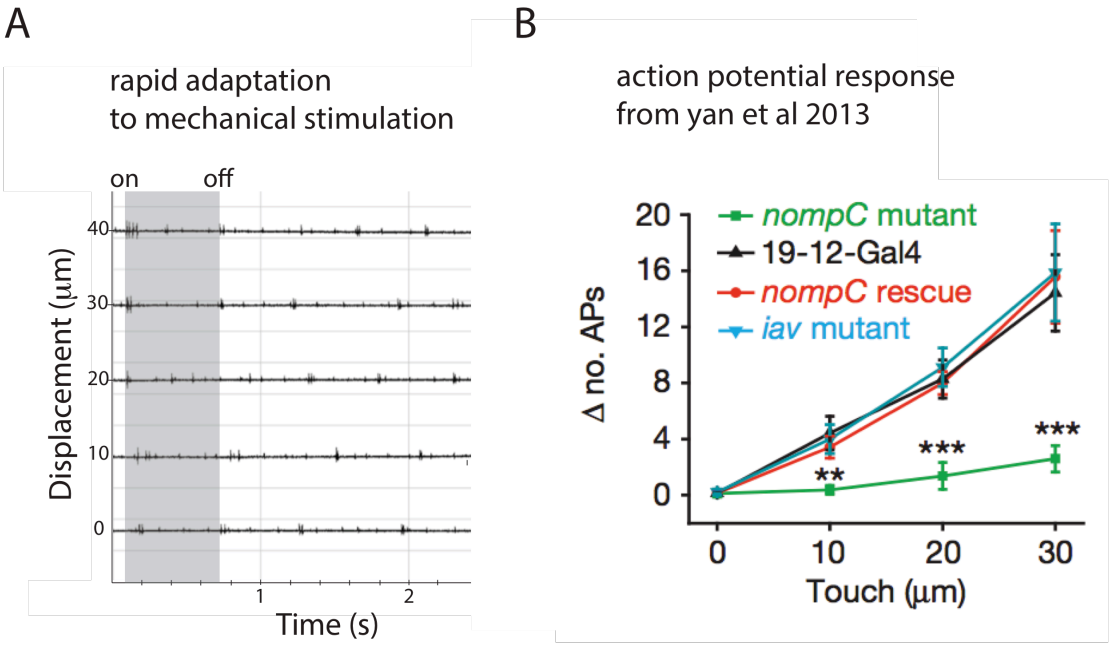

A
rapid adaptation
to mechanical stimulation
on
off
40
30
20
10
0
Displacement (µm)
1
2
Time (s)
B
action potential response
from yan et al 2013
nompC mutant
19-12-Gal4
nompC rescue
iav mutant
20
16
12
8
4
0
Δ no. APs
**
***
***
0
10
20
30
Touch (µm)

Figure 2.3:

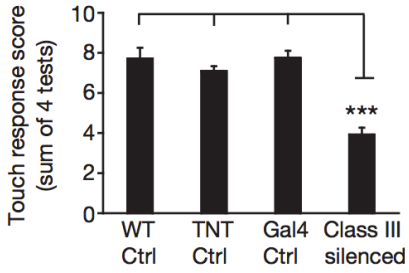

Touch response score
(sum of 4 tests)
10
8
6
4
2
0
***
WT
Ctrl
TNT
Ctrl
Gal4
Ctrl
Class III
silenced

Figure 3.1:

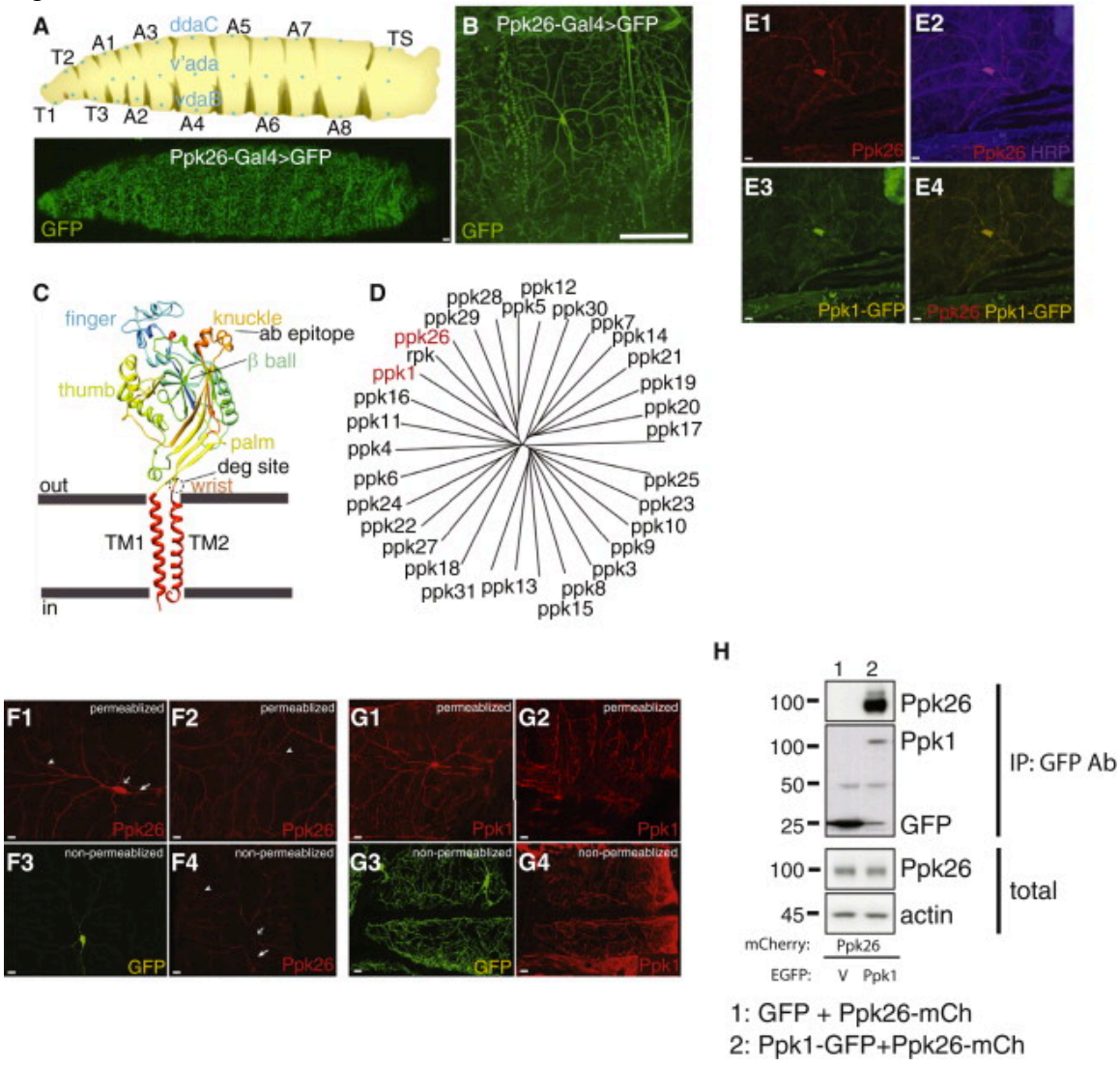

A
T1
T2
T3
A1
A2
A3
ddaC
A4
A5
A6
A7
A8
TS
v'ada
vdaB
Ppk26-Gal4>GFP
GFP
B
Ppk26-Gal4>GFP
GFP
E1
Ppk26
E2
Ppk26 HRP
E3
Ppk1-GFP
E4
Ppk26 Ppk1-GFP
C
finger
knuckle
ab epitope
β ball
thumb
palm
deg site
wrist
out
TM1
TM2
in
D
ppk28
ppk12
ppk29
ppk5
ppk30
ppk7
ppk26
ppk14
rpk
ppk21
ppk1
ppk19
ppk16
ppk20
ppk11
ppk17
ppk4
ppk25
ppk6
ppk23
ppk24
ppk10
ppk22
ppk9
ppk27
ppk3
ppk18
ppk31
ppk13
ppk8
ppk15
F1
permeablized
Ppk26
F2
permeablized
Ppk26
G1
permeablized
Ppk1
G2
permeablized
Ppk1
F3
non-permeablized
GFP
F4
non-permeablized
Ppk26
G3
non-permeablized
GFP
G4
non-permeablized
Ppk1
H
1
2
100
Ppk26
100
Ppk1
50
25
GFP
IP: GFP Ab
100
Ppk26
45
actin
total
mCherry:
Ppk26
EGFP:
V
Ppk1
1: GFP + Ppk26-mCh
2: Ppk1-GFP+Ppk26-mCh

Figure 3.2:

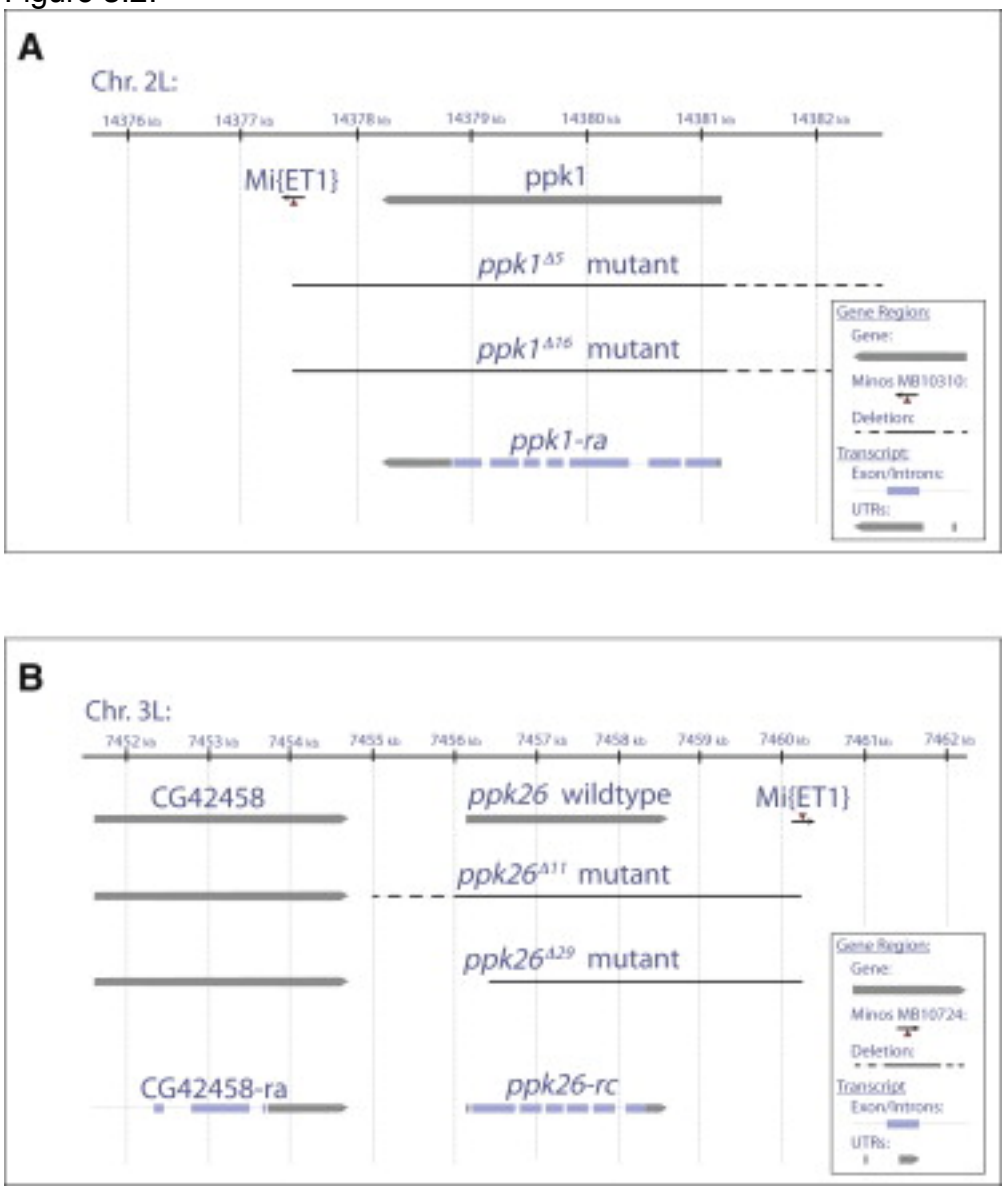

A
Chr. 2L:
14376 kb
14377 kb
14378 kb
14379 kb
14380 kb
14381 kb
14382 kb
Mi{ET1}
ppk1
ppk1Δ5 mutant
ppk1Δ76 mutant
ppk1-ra
Gene Region:
Gene:
Minos MB10310:
Deletion:
Transcript:
Exon/Introns:
UTRs:
B
Chr. 3L:
7452 kb
7453 kb
7454 kb
7455 kb
7456 kb
7457 kb
7458 kb
7459 kb
7460 kb
7461 kb
7462 kb
CG42458
ppk26 wildtype
Mi{ET1}
ppk26Δ11 mutant
ppk26Δ29 mutant
CG42458-ra
ppk26-rc
Gene Region:
Gene:
Minos MB10724:
Deletion:
Transcript
Exon/Introns:
UTRs:

Figure 3.3:

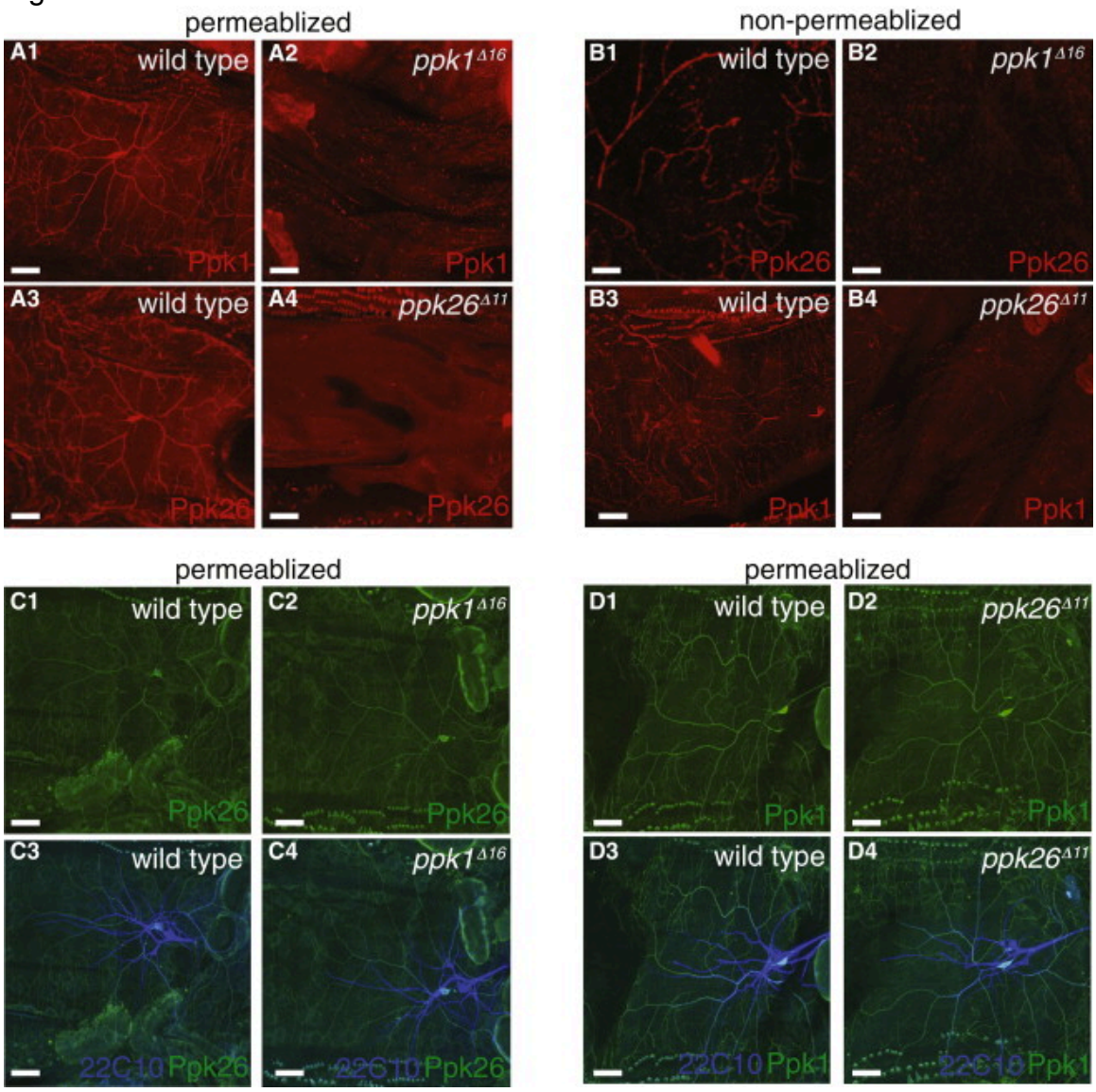

permeablized
non-permeablized
A1
wild type
Ppk1
A2
ppk1Δ16
Ppk1
B1
wild type
Ppk26
B2
ppk1Δ16
Ppk26
A3
wild type
Ppk26
A4
ppk26Δ11
Ppk26
B3
wild type
Ppk1
B4
ppk26Δ11
Ppk1
permeablized
permeablized
C1
wild type
Ppk26
C2
ppk1Δ16
Ppk26
D1
wild type
Ppk1
D2
ppk26Δ11
Ppk1
C3
wild type
22C10 Ppk26
C4
ppk1Δ16
22C10 Ppk26
D3
wild type
22C10 Ppk1
D4
ppk26Δ11
22E18 Ppk1

Figure 3.4:

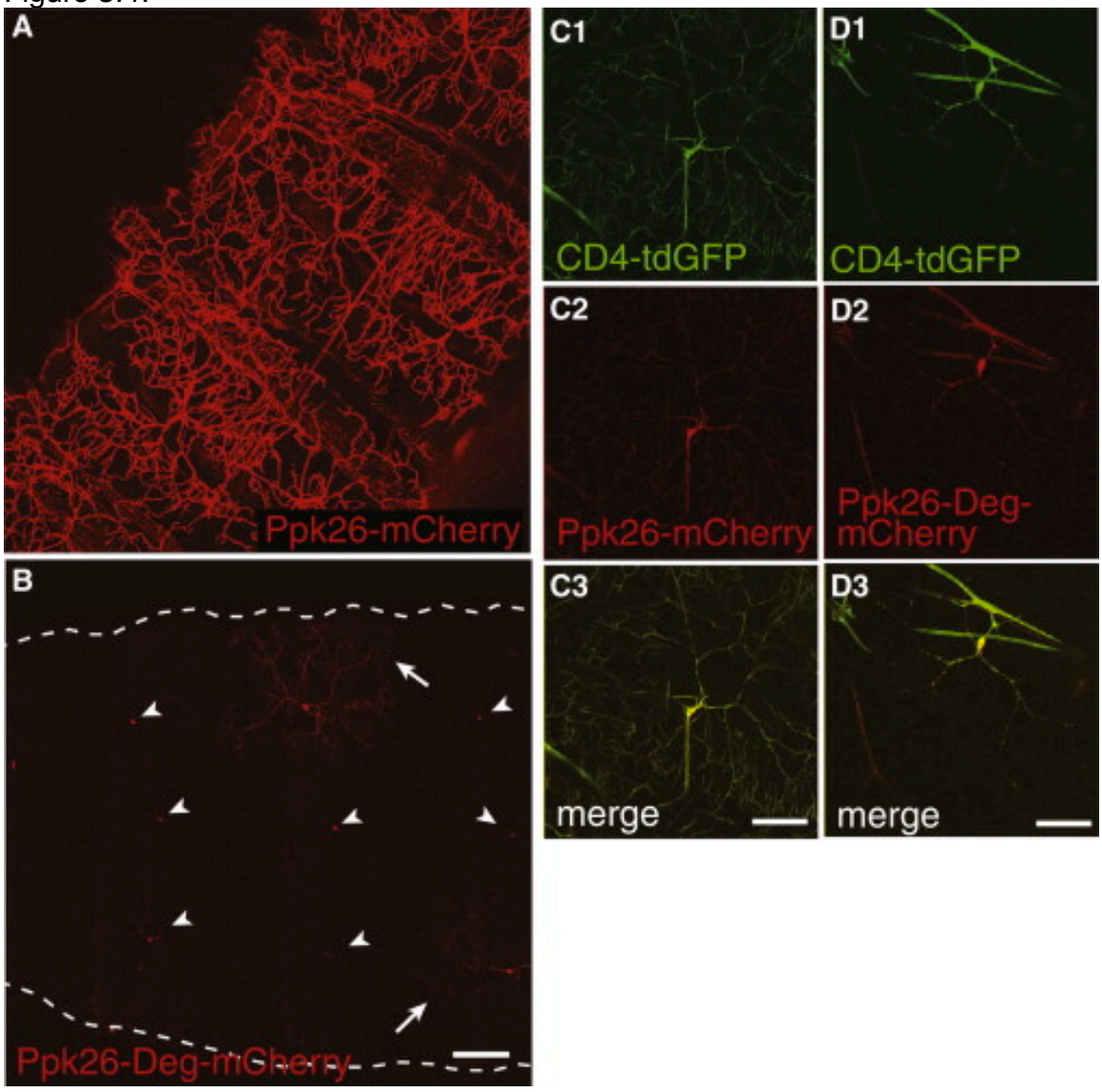

A
Ppk26-mCherry
B
Ppk26-Deg-mCherry
C1
CD4-tdGFP
C2
Ppk26-mCherry
C3
merge
D1
CD4-tdGFP
D2
Ppk26-Deg-mCherry
D3
merge

Figure 3.5:

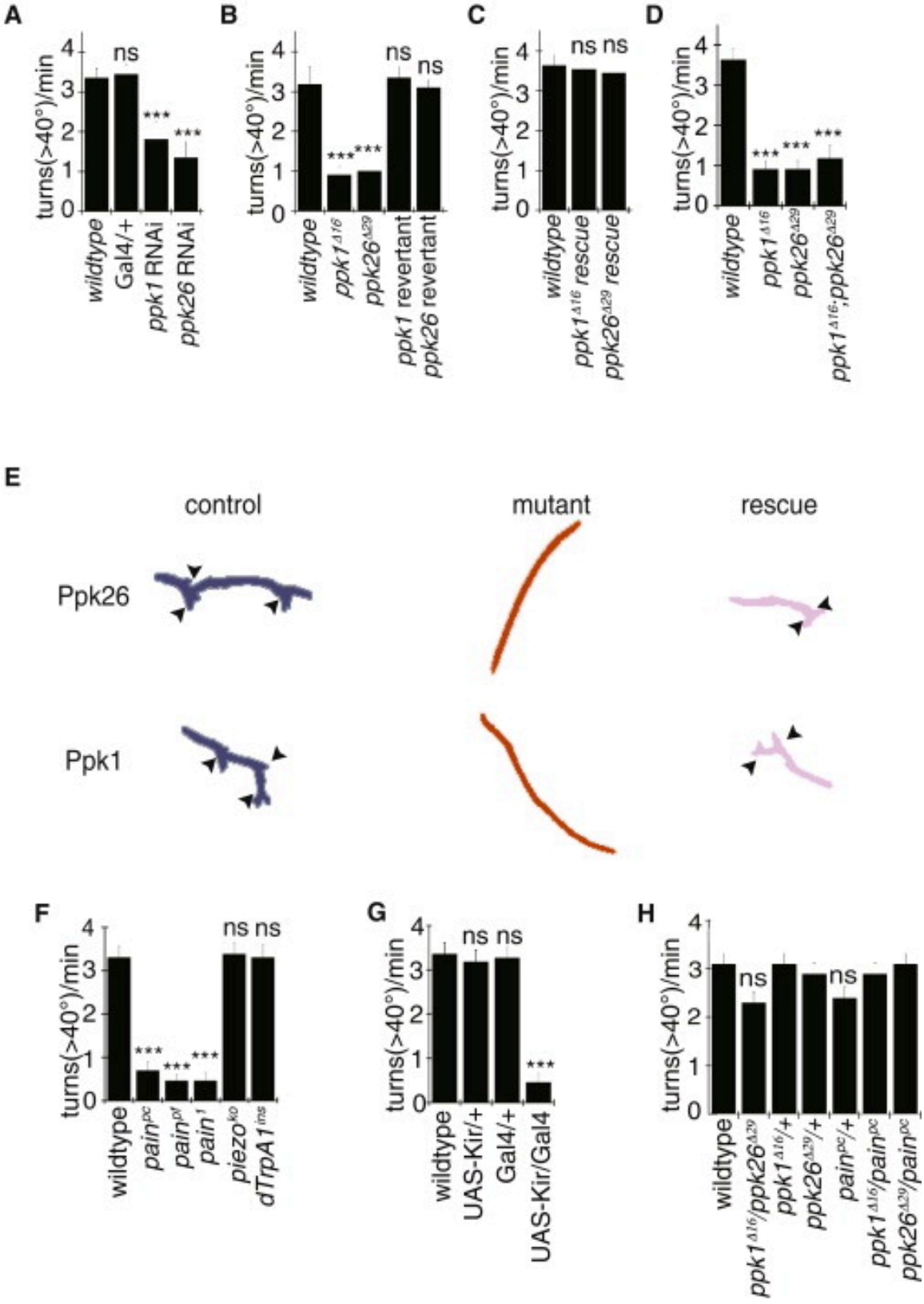

A
B
C
D
turns(>40°)/min
ns
***
wildtype
Gal4/+
ppk1 RNAi
ppk26 RNAi
ppk1Δ16
ppk26Δ29
ppk1 revertant
ppk26 revertant
ppk1Δ16 rescue
ppk26Δ29 rescue
ppk1Δ16;ppk26Δ29
E
control
mutant
rescue
Ppk26
Ppk1
F
G
H
wildtype
painpc
painpf
pain1
piezoko
dTrpA1ins
UAS-Kir/+
Gal4/+
UAS-Kir/Gal4
ppk1Δ16/ppk26Δ29
ppk1Δ16/+
ppk26Δ29/+
painpc/+
ppk1Δ16/painpc
ppk26Δ29/painpc

Figure 3.6:

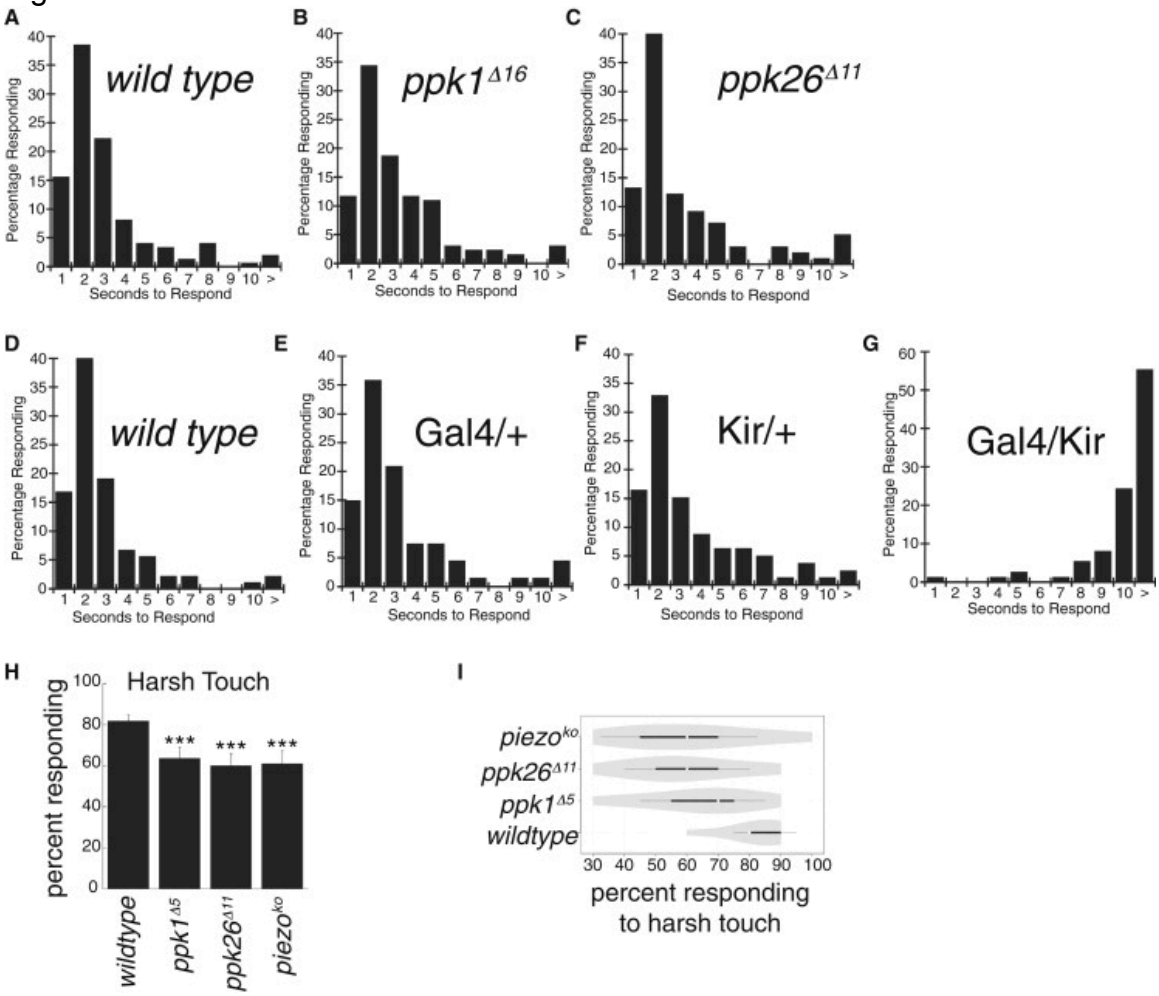

A
wild type
B
ppk1Δ16
C
ppk26Δ11
D
wild type
E
Gal4/+
F
Kir/+
G
Gal4/Kir
Percentage Responding
Seconds to Respond
H
Harsh Touch
percent responding
***
wildtype
ppk1Δ5
ppk26Δ11
piezoko
I
piezoko
ppk26Δ11
ppk1Δ5
wildtype
percent responding
to harsh touch

Figure 3S1:

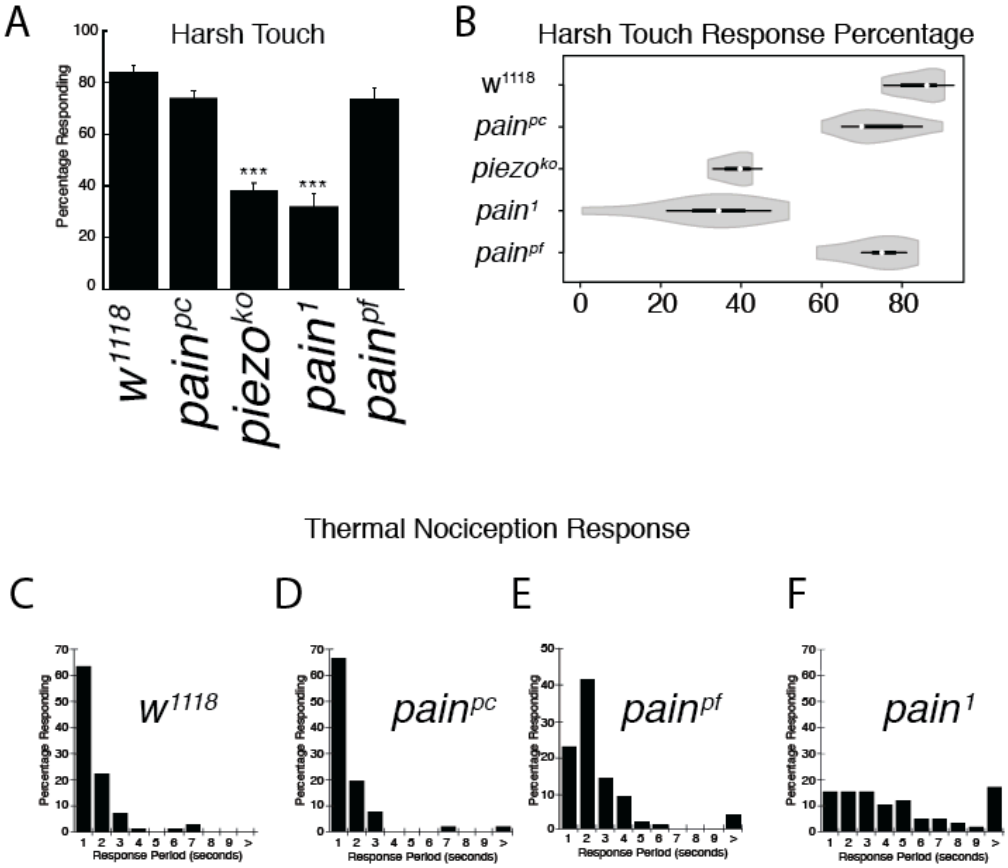

A
Harsh Touch
Percentage Responding
100
80
60
40
20
0
***
***
w1118
painpc
piezoko
pain1
painpf
B
Harsh Touch Response Percentage
w1118
painpc
piezoko
pain1
painpf
0
20
40
60
80
Thermal Nociception Response
C
D
E
F
w1118
painpc
painpf
pain1
Percentage Responding
Response Period (seconds)
1 2 3 4 5 6 7 8 9 >
70
60
50
40
30
20
10
0

Figure 3S2:

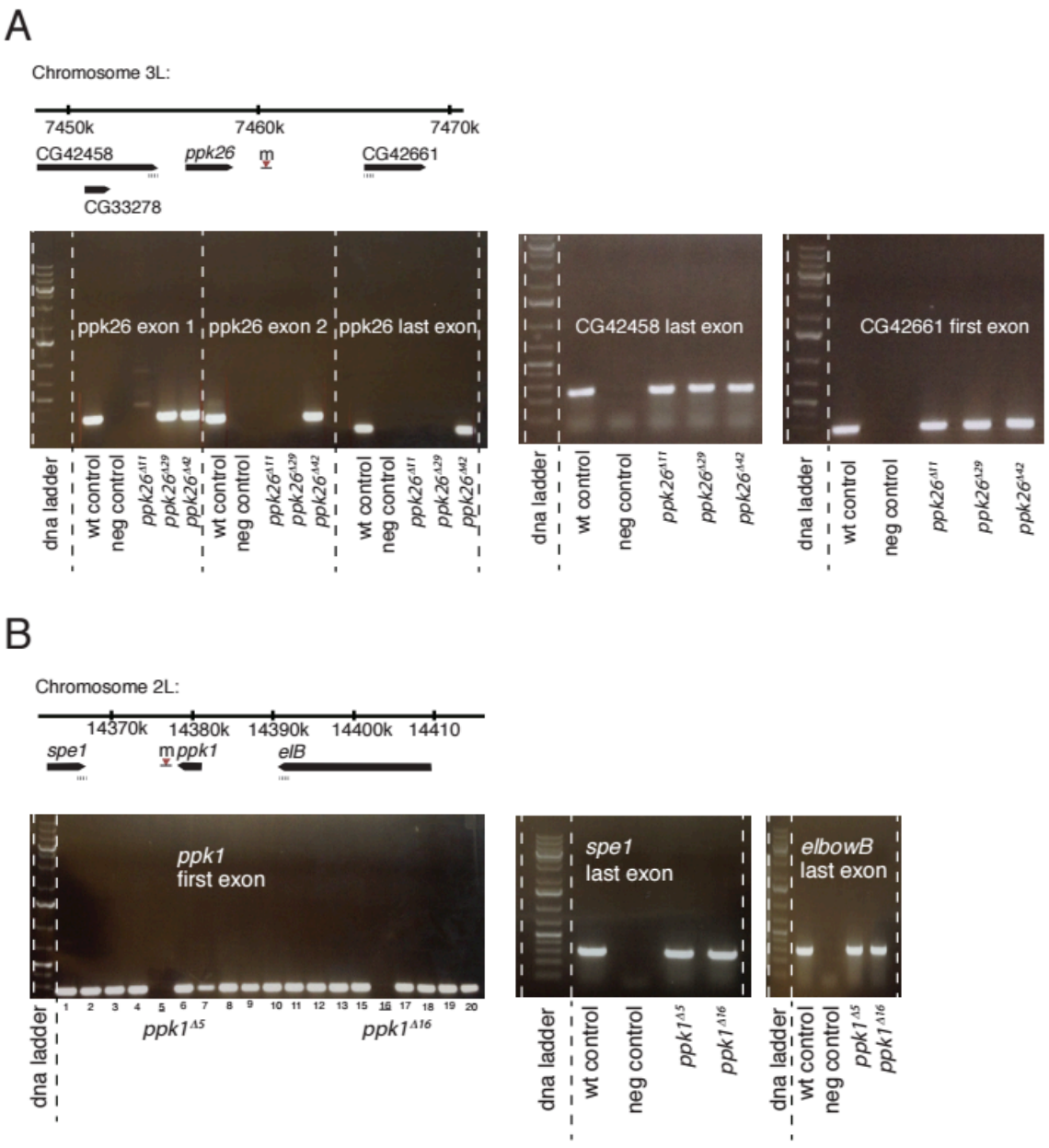

A
Chromosome 3L:
7450k
7460k
7470k
CG42458
ppk26
m
CG42661
CG33278
ppk26 exon 1
ppk26 exon 2
ppk26 last exon
CG42458 last exon
CG42661 first exon
dna ladder
wt control
neg control
ppk26Δ11
ppk26Δ29
ppk26Δ42
B
Chromosome 2L:
14370k
14380k
14390k
14400k
14410
spe1
m ppk1
elB
ppk1 first exon
spe1 last exon
elbowB last exon
1 2 3 4 5 6 7 8 9 10 11 12 13 15 16 17 18 19 20
ppk1Δ5
ppk1Δ16

Figure 3S3:

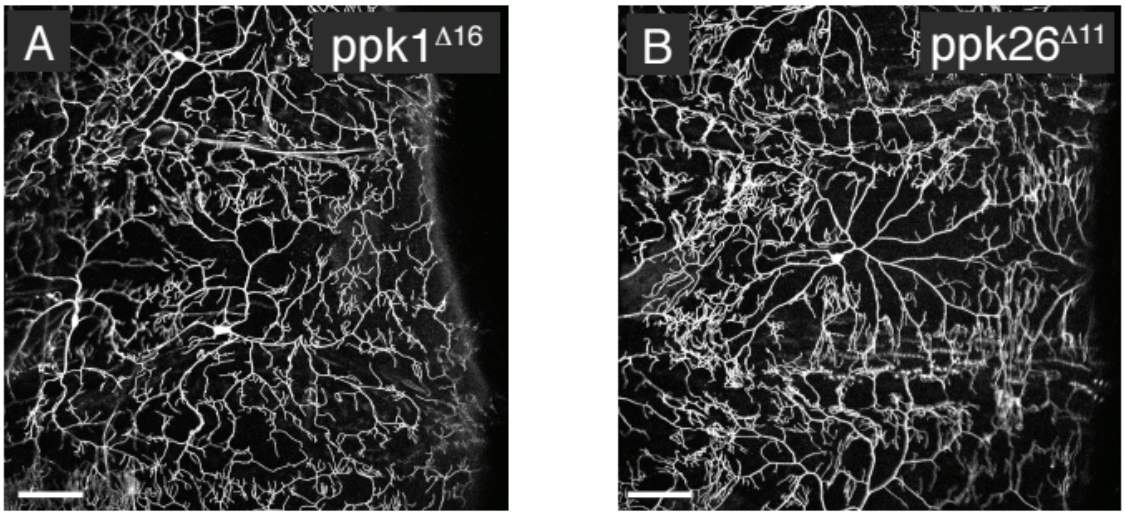


Figure 3S4:

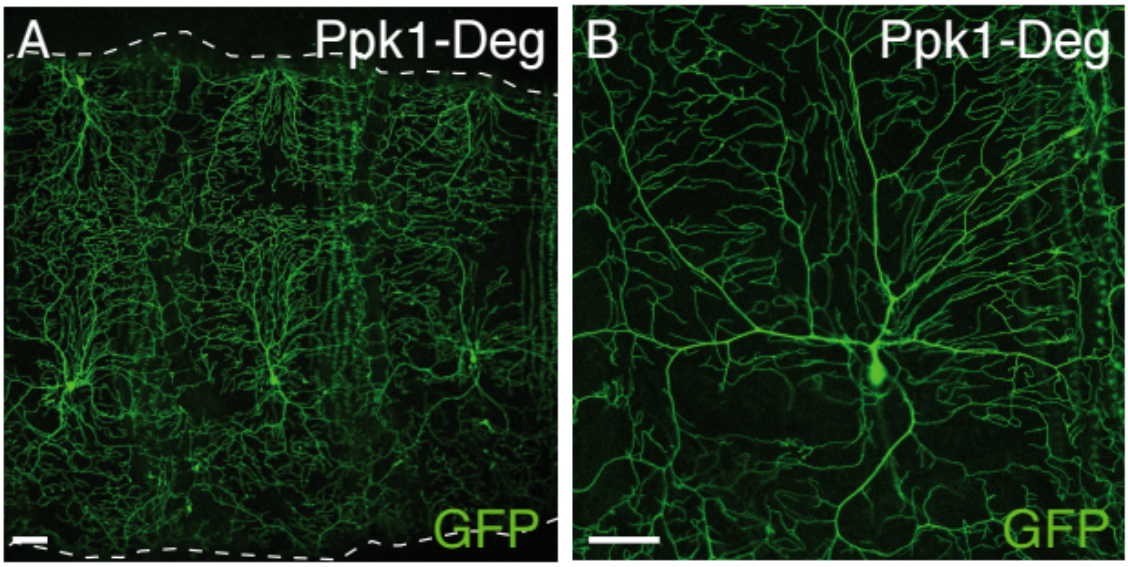


Figure 3S5:

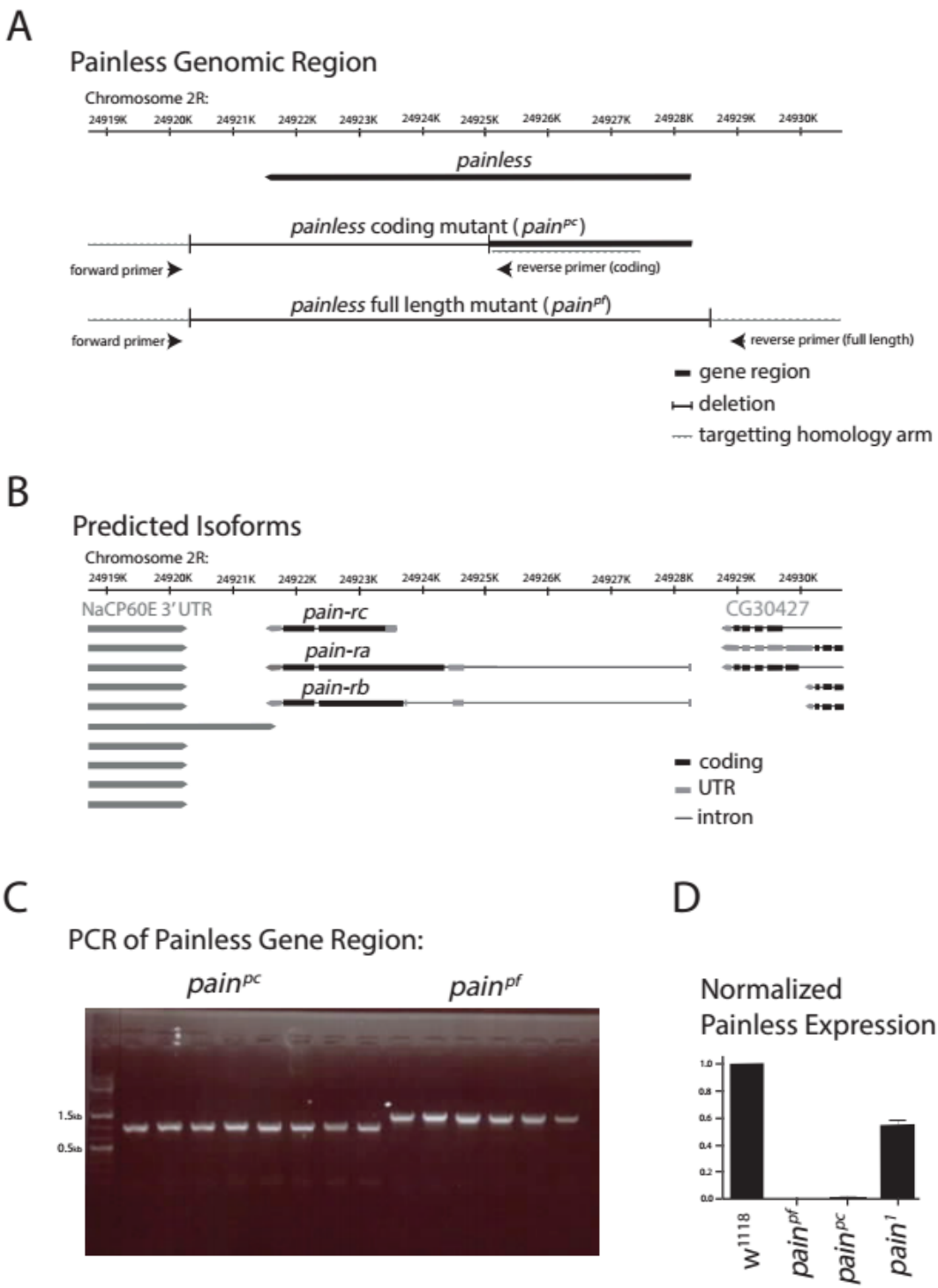
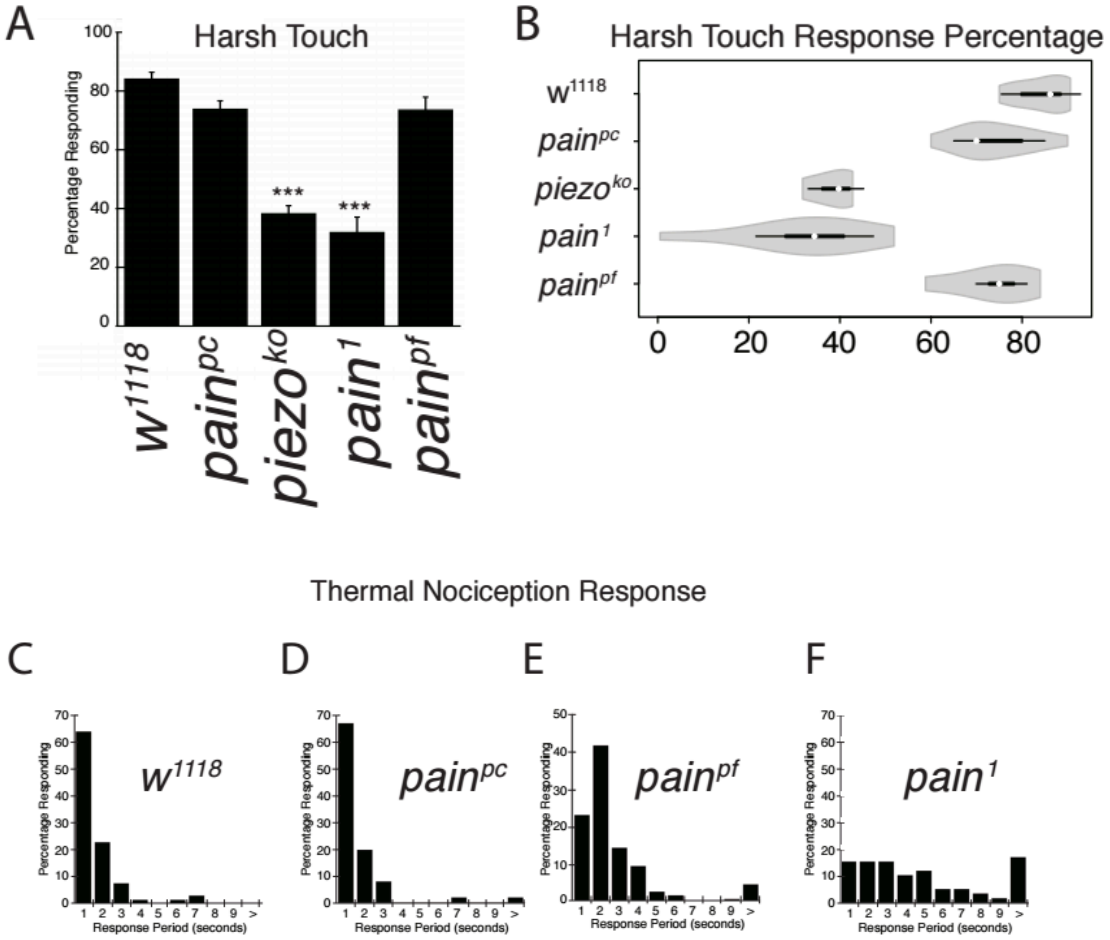

A
Painless Genomic Region
Chromosome 2R:
24919K 24920K 24921K 24922K 24923K 24924K 24925K 24926K 24927K 24928K 24929K 24930K
painless
painless coding mutant (pain^pc)
forward primer
reverse primer (coding)
painless full length mutant (pain^pf)
forward primer
reverse primer (full length)
gene region
deletion
targetting homology arm
B
Predicted Isoforms
Chromosome 2R:
24919K 24920K 24921K 24922K 24923K 24924K 24925K 24926K 24927K 24928K 24929K 24930K
NaCP60E 3' UTR
pain-rc
pain-ra
pain-rb
CG30427
coding
UTR
intron
C
PCR of Painless Gene Region:
pain^pc
pain^pf
1.5kb
0.5kb
D
Normalized
Painless Expression
1.0
0.8
0.6
0.4
0.2
0.0
w^1118
pain^pf
pain^pc
pain^1

Figure 3S6:

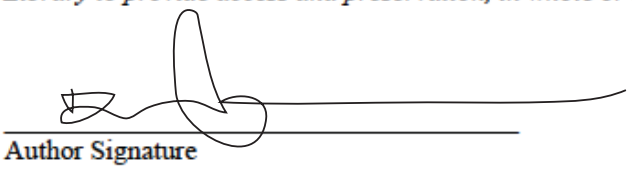

A
Harsh Touch
Percentage Responding
100
80
60
40
20
0
***
***
w1118
painpc
piezoko
pain1
painpf
B
Harsh Touch Response Percentage
w1118
painpc
piezoko
pain1
painpf
0
20
40
60
80
Thermal Nociception Response
C
D
E
F
w1118
painpc
painpf
pain1
Percentage Responding
Response Period (seconds)
1 2 3 4 5 6 7 8 9 >

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.



Author Signature

3/11/15

Date

UCSF Publishing Agreement Form Instructions

On the UCSF Publishing Agreement Form, enter the number for this page (should be the last page of your thesis / dissertation / manuscript document) in one of the 6 highlighted areas on the form (this should be consistent with the pagination formatting properties of the remainder of your thesis / dissertation / manuscript).
For example, if the pagination occurs in the bottom center of the rest of your document, then you should either click in the bottom center highlighted box (or use your TAB key on your keyboard to advance to this location) and enter in the final page number - then click on the PRINT button on the document.

Once you have the UCSF Publishing Agreement Page printed, you will need to sign and date it - then it must be scanned and combined with the rest of your document as the final page in your PDF that you will upload to Proquest/UMI when submitting your work to the Graduate Division.