Degenerin/Epithelial Sodium Channels (DEG/ENaCs) Required for Mechanical Nociception Responses in *Drosophila melanogaster*

by

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Duke University

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Nina Sherwood

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School of Duke University

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ABSTRACT

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Abstract

*Drosophila* larvae respond to potentially tissue-damaging stimuli with nocifensive escape locomotion (NEL). NEL is a stereotyped withdrawal behavior in which the larva rotates along its long-body axis in a “corkscrew” pattern distinct from normal locomotion and is triggered by noxious heat (>39°C) or noxious mechanical (>30mN) stimuli. The Class IV multidendritic (md) neurons are the polymodal nociceptors responsible for triggering NEL. Recent evidence suggests that the *pickpocket* (*ppk*) gene, a degenerin/epithelial sodium channel (DEG/ENaC) subunit, is involved in the mechanotransduction of these neurons. While removal of this ion channel diminishes the larval response to noxious mechanical force, it does not completely abolish it, which leads us to hypothesize that additional mechanical nociception channels have yet to be identified. To identify these channels, we carried out a tissue specific RNAi screen and knocked down all known and predicted ion channels in the larval Class IV md nociceptors. Knockdown animals for each channel subunit were tested for behavioral responses to noxious mechanical force or noxious heat. Taking this approach, two novel channel genes were specifically required for mechanical responses – we named them *stallone* and *balboa*. Remarkably, both genes are predicted to encode DEG/ENaC subunits and neither is required for optogenetic NEL responses, suggesting that both genes are required near the transduction step, perhaps forming a multimeric channel with PPK. Therefore, my research focused on demonstrating that these two DEG/ENaC
genes, *stallone* and *balboa*, are required for mechanical nociception responses in *Drosophila* larvae.

To extend the RNAi results from the previous mechanical nociception screen and confirm *stallone*’s role in detecting harsh touch responses, I first generated and rigorously analyzed the mechanical nociception phenotype of a Δstallone mutant. Interestingly, *stallone* may have a general mechanosensory function as gentle touch responses were mildly reduced in the knockout mutant. Additionally the mutant allele was used to confirm that thermal nociception responses were normal and mechanical nociception behavioral defects were not a consequence of morphological defects in the nociceptors.

Next I focused my efforts on the *balboa* gene as this locus encodes a DEG/ENaC ion channel subunit similar in amino acid sequence to PPK. Converging lines of evidence strongly support the hypothesis that Balboa and PPK physically interact and likely form a functional ion channel *in vivo*. First, both genes are functionally required for mechanical nociception. Second, both genes show very specific expression in the Class IV nociceptor neurons. Third, *ppk* expression causes a dramatic redistribution of Balboa::GFP fluorescence in the multidendritic neurons. Fourth, Balboa::GFP is altered by PPK knockdown and PPK::Venus is dramatically reduced by Balboa knockdown. Fifth, GFP is reconstituted by co-expression of *balboa::CGFP* and *NGFP::ppk* within neurons of the larval peripheral nervous system.
In summary, two DEG/ENaC genes, *stallone* and *balboa*, were discovered in an unbiased forward, genetic screen to identify genes required for mechanical nociception in *Drosophila melanogaster*. I demonstrated that *stallone* and *balboa* are specifically required for mechanical nociception behaviors documenting the first functional role of these two genes. The body of work outlined in this doctoral thesis has contributed to the overall field of mechanosensation by uncovering two novel genes encoding DEG/ENaCs that are functionally required for animal nociception responses to noxious mechanical force and may shed light on multimeric pain-sensing complexes.
Dedication

To my mentor Dan–for your prevailing enthusiasm for science.

“It’s not as bad as you think!”-Berlin Conversations

To my loved ones–for your unwavering support and never doubting.

To the fruit fly–for without you, this body of work would not be possible.
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1. Introduction

1.1 Pain

Physiological pain is an important submodality of somatosensation that is evolutionarily conserved as a protective mechanism across many species. It rapidly alerts the organism of aversive stimuli and subsequently motivates it to withdraw from the damaging source so as to minimize physical harm. In fact, rare congenital conditions rendering people insensitive to pain can cause serious health issues that result in severe injuries and lead to reduced life expectancies (Rosemberg et al., 1994; Indo et al., 1996; Cox et al., 2006). Such rare cases truly emphasize the benefit of experiencing unpleasant sensations and thus show that the survival of an animal is dependent on its ability to detect harmful environmental changes, protect itself from further damage, and avoid similar experiences in the future.

The concept of pain has been around for millennia. In fact, the etymology of the English word ‘pain’ is derived from Greco-Roman mythology in which the goddess Poena/Poine, who represents retribution, vengeance, and the punishment, would penalize sinners with pain (King, 2013). One of the earliest scientific hypotheses on the origin of pain was proposed in the seventeenth century by the French philosopher René Descartes (La Forge, 1999). He postulated that pain was generated by tissue injury (for example, a boy sticking his foot in the fire) that would result in pulling “a delicate thread” that ran up to the brain via the shortest route(Melzack et al., 1965). Descartes also theorized that reflexive pain and emotional pain were separate. Of course, we know
that these threads are indeed special sensory, afferent nerve fibers in the periphery that transmit their signal to specialized regions of the brain (Sherrington, 1906).

In more recent times, the International Association for the Study of Pain (IASP) has defined pain as, “unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (“Pain terms: a list with definitions and notes on usage. Recommended by the IASP Subcommittee on Taxonomy,” 1979). In humans, a painful stimulus activates sensory neurons in the periphery—nociceptors which detect noxious mechanical, thermal, and chemical stimuli and transduce them via the dorsal root ganglia of the spinal cord to the thalamus and higher order processing centers of the cortex where they are recognized as painful experiences (Raja et al., 1999).

1.2 Nociception

1.2.1 Mammalian nociceptors

Much of the work studying the anatomical and physiological properties of sensory afferents has been carried out in human and nonhuman primates. Mechanoreceptive myelinated fibers are differentiated based on their response to stepwise indentations of the skin and the organ in which they terminate and encode different aspects of touch sensation (Johnson, 2001). A population of afferents with a high threshold of activation are further classified based on their conduction velocity (unmyelinated C-fibers versus myelinated A-fibers), modality of stimulation (thermal, mechanical, or chemical), and temporal characteristics (rapid versus slow response)
The cell bodies of unmyelinated, small-diameter C-fiber mechano-heat nociceptors (CMH) and thinly myelinated, medium-diameter Aδ fibers mechano-heat nociceptors (AMH) are located within the trigeminal or dorsal root ganglia outside of the spinal cord and send two axonal projections: one to the periphery and the other to synapse centrally in the dorsal horn of the spinal cord (Purves et al., 2001). The terminal axonal projection patterns of CMH and AMH nociceptors to the dorsal horn are laminae I and II. Second-order projections continue to laminae IV, V, and VI, cross the midline, and ascend through the spinothalamic tract to the thalamus (Willis, 2004).

Like mammalian systems, Drosophila have specialized sensory afferents in the periphery that relay their information to spatially defined regions of the ventral nerve cord in a stereotyped fashion (Shepherd et al., 1996; Grueber et al., 2007). Many studies detailing the neuroanatomy and function of Drosophila sensory neurons, including nociceptive neurons, has begun to shed light on important conserved sensory mechanisms in neurobiology.

1.2.2 Drosophila as a paradigm for nociception

Drosophila has proven a fruitful model system to identify genes important for detecting noxious stimuli as the larval behavioral responses to high threshold polymodal stimuli serves as a robust paradigm for mutant screening. In a prior forward genetic screen for mutations that affect thermal nociception, the heat sensitive TRPA channel painless was identified as painless mutants showed an increased threshold for thermal nociception (Tracey et al., 2003) and mechanical nociception (Tracey et al., 2003;
Sokabe et al., 2008). This channel was shown to primarily play a role in the multidendritic (md) neurons and opened the door for many questions pertaining to the cellular and molecular mechanisms governing this behavior.

More information pertaining to the nociceptive circuit that encodes the nocifensive responses to noxious stimuli has been elucidated. Hwang et al. (Hwang, 2009) used genetically encoded neuronal silencers and activators to show that the Class IV multidendritic sensory neurons are required and sufficient to elicit nociception induced behavioral responses. Class IV md neurons are non-ciliated sensory neurons with highly branched naked dendrites that tile the larval epidermis (Grueber et al., 2002). These nerve endings interestingly resemble the morphology mammalian nociceptors. Although silencing Class IV md neurons most severely inhibits behavioral responses to noxious mechanical stimuli, silencing of other subtypes of md neurons (Classes I, II, and/or III) also impairs mechanical nociception albeit to a lesser degree (Hwang et al., 2007). The Class I and dbd neurons are required for normal larval locomotion so it is likely their impact on mechanical nociception are an indirect effect of uncoordination—silencing these neurons results in an uncoordinated locomotion (Hughes et al., 2007). Synaptically silencing Class II and Class III md neurons impairs mechanical nociception without affecting locomotion (Hwang et al., 2007). Distinct populations of somatosensory neurons are responsible for detecting innocuous touch and noxious mechanical stimuli and are conserved across humans (Purves et al., 2001), C. elegans (Hall et al., 2008), and recently in Drosophila (Tsubouchi et al., 2012; Yan et al., 2013).
Tsubouchi et al. have shown that Class III neurons are both necessary and sufficient for normal light touch responses in larvae.

### 1.3 Ion channels in mechanosensation

Several mechanosensation studies have shown that ion channels play a critical role in the detection of mechanical force such as stretch or pressure. One of the first mechanosensitive ion channels identified were the Msc channels in bacteria as msc mutants were not able to respond to increasing osmotic pressure (Martinac et al., 1987). Msc channels alone open in response to tension in reconstituted liposomes (without other additional proteins) leading to a model proposing that membrane tension is transduced directly through the cell membrane to the mechanosensitive ion channel (Martinac et al., 1987; Delcour et al., 1989; Sukharev et al., 1993; Saimi et al., 1994; Berrier et al., 1996; Kung, 2005). While these Msc channels have been extensively characterized no metazoan homolog exist (Kung, 2005) leaving open questions on understanding the general mechanosensory processes.

In invertebrates, *in vivo* genetic screens for mutants that are defective in gentle mechanosensation, have suggested a tethered model whereby a mechanosensitive ion channel complex senses force through extracellular or intracellular components. A screen for *C. elegans* mechanosensory mutants identified the MEC-4 and MEC-10 degenerin / epithelial sodium channels (DEG/ENaC) as a putative mechanical transduction complex with potential extracellular matrix (MEC-5, MEC-9) and cytoskeletal (MEC-7 and MEC-12) “tethers” that are also required for wildtype gentle
touch responses (Garcia-Anoveros et al., 1997; Ernstrom et al., 2002; Goodman et al., 2003; O’Hagan et al., 2005; O’Hagan et al., 2006). Recently, two DEG/ENaC genes, deg-2 and unc-8, directly detect harsh mechanical stimuli in the polymodal PVD nociceptive neurons (Chatzigeorgiou et al., 2010a). The Drosophila DEG/ENaC RPK has also been shown to play a role in gentle touch behavioral responses (Tsubouchi et al. 2012) while ppk is required for mechanical nociception (Zhong et al., 2010).

Another ion channel family that has been implicated in mechanosensation is the transient receptor potential (TRP) family. In C. elegans, the TRPV channel subunits osm-9 and ocr-2 genes are important in detecting noxious mechanical stimuli (Geffeney et al., 2011). The Drosophila TRP-N channel Nomp-C is a candidate mechanotransducer that is required for gentle touch behavioral responses in larvae (Tsubouchi et al., 2012; Yan et al., 2013), mechanically activated currents in Drosophila sensory bristles (Walker et al., 2000) and in heterologous cells (Liang et al., 2013), and hearing in Drosophila (Eberl et al., 2000) and zebrafish (Sidi et al., 2003). Additional recent work on Nomp-C’s ankyrin domains has shown that they likely function as membrane microtubule connectors that ‘tether’ the channel to intracellular microtubules suggesting they serve as the ‘gating springs’ in chordotonal neurons (Liang et al., 2013). Gating-springs have been proposed as an elastic element to the channel gate of a transduction channel, whereby increased tension physically pushes the channel to an open state (Markin et al., 1995).

Other TRP channels important for mechanosensation are inactive, nanchung, and painless but no direct measurements of mechanically activated currents mediated by
these channels have been demonstrated. Other potentially pore-forming subunits of the mechanotransduction complex include TMC channels (Pan et al., 2013; Holt et al., 2014; Maeda et al., 2014), and Piezo channels (Coste et al., 2010; Coste et al., 2012; Kim et al., 2012) but further analyses are needed to clarify their roles.

Although previous mechanosensation studies have focused on sensing gentle touch, investigations identifying the molecular components required for detecting high threshold mechanical stimuli have begun to unfold. It appears that several ion channel families are involved in overlapping mechanisms for detection of low and high threshold mechanical stimuli, with individual members showing distinct and different functions with respect to somatosensory neuron subtype (Tracey et al., 2003; Chatzigeorgiou et al., 2010b; Coste et al., 2010; Zhong et al., 2010; Geffeney et al., 2011; Coste et al., 2012; Kim et al., 2012). In Drosophila, the gene pickpocket was recently identified as a required component in mechanical nociception but was expendable for gentle touch responses. However, the C. elegans channel MEC-10 is more general in its mechanosensory role as it is required for detecting both innocuous and noxious stimuli (Huang et al., 1994; Chatzigeorgiou et al., 2010a). Despite recent studies identifying new components of mechanical nociception pathways, the mechanotransducing agent essential for detecting harsh mechanical stimuli remains a mystery.

Therefore Richard Hwang set out to identify this molecular machinery essential for mechanical nociception by carrying out a forward genetic screen in Drosophila to look for mutants exhibiting defects in larval nocifensive escape responses to harsh
mechanical stimuli (Hwang, 2009). Using an RNAi approach he identified two genes encoding DEG/ENaC subunits required for mechanical nociception: CG18110 and CG8546. As these genes were uncharacterized, we named them stallone and balboa respectively.

The scope of my studies, described in this dissertation, has been to further elucidate the potential roles of these channels in mechanical nociception.

2. General Methods

2.1 Fly husbandry

Drosophila used for behavioral or imaging experiments were raised on standard cornmeal molasses fly food medium at 25°C with 75% humidity on a 12 hour light/dark cycle.

2.2 Mechanical nociception behavioral assay

The mechanical nociception behavioral tests were performed by stimulating larvae with a calibrated von Frey fiber as previously described (Tracey et al., 2003; Hwang et al., 2007; Caldwell et al., 2010; Zhong et al., 2010; Honjo et al., 2014), with slight modifications.

Von Frey fibers were made of fishing line mounted onto glass rods (solid, 4mm thick, rounded, fire-polished glass originally intended for spreading bacterial cell cultures on Luria-Bertani agar plates). The glass rods were cut, flame-heated, and bent to a 90° angle with a handle approximately 15-20 cm long and an overhang of
approximately 4-6 cm in length. Omniflex 6 lb test monofilament fishing line
(0.23mm/0.009 in diameter) was adhered to the glass rod with tape to create von Frey
fibers. The filaments were cut to produce overhangs of varying lengths (10mm, 12mm,
or 14mm). These fibers thus generate a distinct amount of maximum force when
depressed. A scale was used to calibrate each fiber by recording the mass in grams
during a series of rapid depressions, such that the fiber began to bend. The average mass
was determined for each von Frey fiber and the force (mN) was calculated using
Newton’s second law of motion, $F=ma$. Unless noted, the 10mm overhang von Frey
probe was used to administer a force of approximately 30mN to larvae for the
mechanical nociception experiments performed in this body of work.

For the initial mutant testing, six females were crossed to three males and
allowed to lay eggs until late third-instar larvae began to wander. These larvae were
collected for behavioral assays between five and seven days post-mating. Since
overcrowding conditions using the above conditions were noted to have an effect on
larval responses (i.e. larvae demonstrated hypersensitivity to weak forces), all remaining
behavioral analyses assays were carried out on larval progeny from the following
crossing method: six females were mated to three males in yeasted vials for two days
and subsequently flipped to a freshly yeasted new food source daily for all remaining
behavior analysis. Five to seven day old larvae at the wandering stage were collected
and transferred to a 60 mm x 15 mm petri dish containing a few milliliters of yeasted
water such that the larvae remained moist in a shallow aqueous environment but were
not entirely submerged. This wet arena is important for larvae to elicit the nocifensive escape behavioral response. Noxious mechanical stimuli were delivered by the rapid depression of a von Frey fiber on the dorsal midline of the larvae (between abdominal segments A3-A6) until the filament began to bend. The stimulus was administered quickly to allow the animal to behaviorally respond. Each larva was stimulated up to three times until a complete 360° nocifensive escape roll along the long-body axis was completed. A positive response was only recorded if the animal responded on the first stimulus. Testing animals from multiple days of transferred crosses was performed from at least three independent trials and all data were pooled together. Each trial consisted of at least 30 animals from a particular cross. The number of larvae showing mechanical nocifensive behavioral responses was tallied and divided by the total number of larvae tested. A corresponding proportion of responders are reported for each genotype and Fisher’s exact test with Bonferroni correction was used to test for statistical significance between multiple genotype comparisons.

2.3 Gentle touch behavioral assay

The gentle touch behavioral assay was performed as previously described (Kernan et al., 1994; Caldwell et al., 2003; Tsubouchi et al., 2012). Assays were performed on arenas that were freshly prepared daily; each arena consisted of 10 ml of 1% agarose in distilled water poured into a 60 mm x 15 mm round petri dish. The stimulation probe consisted of an eyelash taped to the end of a paintbrush.
Crosses were established as described for the mechanical nociception crosses in which parental genotypes were transferred daily. Early third-instar larvae were collected from the top layer of medium, rinsed thoroughly with water to remove any remaining debris, gently transferred to an arena with a soft paintbrush, and allowed to recover for 30-60s prior to touch stimulation. Larvae were laterally touched from anterior segment T1 to T3 with the eyelash probe during normal peristaltic movement. The behavior immediately elicited post stimulus was scored in the following manner, according to Kernan et al (Kernan et al., 1994). If animals did not exhibit a response and continued their normal locomotion, then a score of zero was assigned. If they stopped or hesitated they received a score of one. Those that briefly retracted their anterior region but continued with forward movement were scored a two. Any larva that retracted and turned away from the stimulus <90° was designated a score of three. All animals turning ≥90° or performing reverse peristalsis were given a score of four. Each larva was stimulated four times and was allowed to recover between each stimulation event until normal forward locomotion proceeded; Values were summed to produce final score that ranged from 0 to 16. Data from three independent trials (each trial consisted of ~25 larvae) were pooled and an average gentle touch score was calculated per genotype tested. T-tests for statistical significance were performed for gentle touch scores amongst various genotypes.

The distribution of the gentle touch scores was also determined for each data set. In this approach every gentle stimulation event was treated independently and therefore
the total number of scores corresponds to four times the number of larvae. The proportion of larvae exhibiting each behavioral response immediately following a light touch (i.e. the touch score ranging from 0-4) was calculated and binned appropriately.

2.4 Thermal nociception behavioral assay

The thermal nociception behavioral assay was performed as previously described (Tracey et al., 2003; Hwang et al., 2007; Zhong et al., 2010), with slight modifications.

The thermal probe was comprised of a sharpened soldering iron with a digital thermocouple (Physitemp BAT -12) soldered to the 0.6mm chiseled copper tip. The temperature was regulated by voltage adjustment using a variac and calibrated with the thermocouple that measured the temperature at the tip of the probe. Two temperatures were used for testing larvae: 42°C and 46°C to measure hypersensitive and insensitive phenotypes, respectively.

Six females were crossed to three males in yeasted vials containing standard molasses cornmeal food. Three crosses were set up per genotype. After five to seven days, wandering third-instar larvae were rinsed off the vial walls into a 60 mm x 15 mm petri dish with a few ml of yeasted distilled water that maintains a shallow aqueous environment essential for larvae to elicit a nocifensive escape response. Larvae were kept moist but not submerged throughout testing. Each larva was subjected once to the noxious temperature by gently touching its midline segments (A4-A6) with the thermal probe on the lateral side. All experiments were videotaped and behavior analysis was
performed offline. The latency to response was measured as the time interval (in seconds) it takes a larva to engage in its nocifensive escape locomotion (an entire 360° roll along the long-body axis) upon first contact with the thermal probe. The average latency was calculated for each genotype and a one-way ANOVA followed by a t-test compared the means between genotypes.

2.5 Optogenetic activation of neurons

The optogenetic activation of nociceptive neurons was performed as previously described (Hwang, 2009; Honjo, K. et al., 2012; Zhong et al., 2012), with slight modifications.

The driver strain ppk-Gal4 was used to express UAS-Channelrhodospin2::eYFP (UAS-ChR2::eYFP), line C specifically in the Class IV neurons. Ten-twenty virgin female flies were crossed to five to ten male flies and allowed to mate for two days on yeasted cornmeal molasses food. The crosses were then transferred to cages and allowed to lay eggs for 24 hours on apple juice agar plates containing a dollop of yeast paste supplemented either with 500uM of all-trans-retinal for experimental larvae or 0.5% ethanol for control larvae. All crosses were kept in the dark at 25°C as all-trans-retinal (atr) is light sensitive and can degrade with excessive light exposure. The seeded plates were exchanged with fresh apple juice plates supplemented with appropriately atr spiked yeast paste and kept in the dark. The larval crossed progeny fed for an additional 96 hours after egg laying were gently transferred with a paintbrush to a 60mm plastic Petri dish containing a couple of ml of yeasted distilled water to keep the larvae moist in
a shallow aqueous environment. Larvae were allowed to acclimate until normal forward peristaltic locomotion resumed. Animals were then photostimulated with 10 klx of blue light (460-500nm) using an Hg light bulb for approximately 5s and resulting behavioral responses were video recorded. A positive nocifensive roll was defined as at least one entire 360° revolution upon photoactivation and all data from a single genotype were pooled to generate a total proportion of rollers. Fisher’s exact test with Bonferroni corrected p-values was used to determine whether a statistically significant difference existed between the tested genotypes.

2.6 Confocal imaging

To observe md neurons, late third-instar larvae were lightly anesthetized with di-ethyl ether for approximately 10 minutes, mounted in 100% glycerol under a 24x55mm #1.5 cover slip, and imaged with an Apochromat 40X N/A 1.3 oil immersion lens on a Zeiss LSM 5 live confocal system. All confocal micrographs were of the dda group from larval segments A3-A6. Confocal Z-stacks were converted to maximum intensity projections using the Zeiss LSM or Zen software package. Negative image conversion and adjustments to brightness and contrast were performed in Adobe Photoshop.
3. *Minos* elements *in trans* generate deficiencies via hybrid element insertion

3.1 Introduction

3.1.1 Genes influence behavior

Behavior, perhaps the most complex phenotype, can be described as an organism’s response to changes within its internal and external environment (Cowie, 1985). The complex interactions between genes and the environment are significant in the development and functioning of behavior. Since the nervous system controls and coordinates an animal’s behavior in response to changing environmental conditions, accurate spatiotemporal expression of specific genes is essential to ensure proper development and function of neurons important for a given behavior.

Much progress has been made towards deciphering the genetic influence on various behaviors by studying animal mutants exhibiting abnormal behavior and subsequently identifying the genes responsible for these anomalous phenotypes. During the 1960s Seymour Benzer pioneered the field of *Drosophila* neurogenetics by dissecting the link between genes and behavior. He demonstrated that single mutant analysis in flies could be “fruitful in tackling the complex structures and events underlying behavior” and could be used to “indicate modifications of the nervous system” (Benzer, 1967). While cognizant of the influences environmental factors have on behavior, his approach was to keep environmental conditions as constant as possible, to induce random mutations, to look for alterations to simple behaviors, and to identify the loci
responsible (Benzer, 1971). Indeed, his discovery of the *period (per)* gene (Konopka et al., 1971) highlighted the power of taking this systematic, forward genetic approach to identify a single gene that affects neural function of fly circadian behavior. Much work elucidating the genetic basis of other fly behaviors, including courtship (Hotta et al., 1976; Hall, 1979), learning and memory (Dudai et al., 1976), and nociception (Tracey et al., 2003) to name a few, was carried out with similar forward genetic approaches.

While the random nature of forward genetic screens has been useful in identifying novel genes essential for behavior, the unpredictable nature of the types of alleles recovered from random mutagenesis may not be sufficient to address gene function. Therefore, to study *in vivo* gene function in any pathway, including behavior, one may need to generate targeted mutations specific to endogenous alleles.

This has been particularly true in *Drosophila melanogaster* as its rich history in mutant analysis has proven value in understanding biological pathways. Over the past 100 years *Drosophila* has served as a great genetic model organism to study eukaryotic gene function due to wide availability of mutations, the annotated genome sequence, the lack of genetic redundancy, and the relative ease with which one can make endogenous genetic manipulations. Moreover, there has been a collective driving force in the *Drosophila* community to develop new methods for generating large collections of mutant alleles and making them available for all researchers. Some of these techniques
utilize the random and insertional nature of mobile DNA elements, known as transposable elements (TEs) or transposons, to perturb gene function.

3.1.2 Transposon-mediated mutagenesis in *Drosophila*

3.1.2.1 Transposable elements

Transposable elements—short sequences of DNA that can excise and insert within the genome—were first noted by Barbara McClintock as mobile DNA elements in maize (McClintock, 1987). They have since been identified in almost all species, including *Drosophila*, and have been extensively characterized at the molecular level. TEs have been broadly classified into two main groups: Class I Retrotransposons that mobilize in a ‘copy and paste’ manner using an RNA intermediate and Class II DNA transposons that move in the host genome via a ‘cut and paste’ mechanism. They can function autonomously, meaning they are self-sufficient in their transposition by encoding their own source of transposase, or non-autonomously, meaning they require other TEs for mobility. One of the most extensively characterized Class II TEs in *Drosophila*, the P element, has led to a greater understanding of transposition biology and which components are critical for transposition (i.e. the minimal transposable unit): specific inverted repeating sequences flanking an encoded source of transposase.

3.1.2.2 P element

In the 1970s, population geneticists discovered a phenomenon in which germline mutagenesis rates varied among certain populations of fruit flies. Importantly male flies
were undergoing high rates of recombination—a phenotype never previously observed in wildtype males (Kidwell et al., 1977). This ‘hybrid dysgenesis’ was linked to a naturally occurring TE that was named the \( P \) element (Kidwell et al., 1977; Sved, 1979; Bingham et al., 1982; Rubin, Kidwell, et al., 1982). When these elements were active, large-scale genomic changes in the genome, such as chromosomal rearrangements and recombination, were occurring at elevated rates (reviewed in Hummel et al., 2008).

Thankfully \textit{Drosophila} researchers had the foresight to appreciate the value of TE-induced chromosomal aberrations as these observations spawned the use of non-autonomous transposons in a controlled manner. The minimal transposable (\( P \) element) unit has been modified for transgenic use as a transgene delivery vehicle ((Rubin & Spradling, 1982). By providing a regulated source of \( P \) transposase \textit{in cis}, \( P \) elements containing a sequence or gene of interest are capable of inserting randomly into the genome.

Indeed, using \( P \) element as a means for transgenesis has been essential to the ever growing number of insertional mutation alleles available (Ballinger et al., 1989; Loukeris et al., 1995; Bellen et al., 2004; Thibault et al., 2004; Venken et al., 2005; Bellen et al., 2011). In principle the short target site of \( P \) elements would allow for saturation of mutagenic insertions in every gene however insertional biases of \( P \) elements to certain loci in the genome limit this. In fact, \( P \) element mutagenesis may have reached its limit to target new genes (Bellen et al., 2004). Therefore, identifying additional TEs and
developing them as tools to make genomic manipulations in *Drosophila* has garnered much interest in recent years (Hacker *et al.*, 2003; Thibault *et al.*, 2004). The most recent superstar in the TE transgenesis field is a member of the mariner/TC1 like transposons, *Minos* elements.

### 3.1.2.3 *Minos* elements

*Minos* elements are a useful tool for transposable element-mediated mutagenesis for many reasons: they are not endogenously found in *D. melanogaster* (Franz *et al.*, 1994; Arca *et al.*, 2000) (originally cloned from *D. hydei* (Franz *et al.*, 1991; Franz *et al.*, 1994)). A trans-acting transposase source is sufficient to induce transposition (Loukeris *et al.*, 1995). They do not need species-specific factors for transposition (Loukeris *et al.*, 1995; Lampe *et al.*, 1996). They randomly integrate at a high rate (Metaxakis *et al.*, 2005; Bellen *et al.*, 2011). They remain stably integrated in the absences of a transposase source (Markaki *et al.*, 2007). They can carry relatively large transgenes (Markaki *et al.*, 2007; Venken *et al.*, 2011). They excise in a precise or imprecise manner (Metaxakis *et al.*, 2005). And, their only target site preference is the TA dinucleotide. Their lack of insertion preference in genomic regions—they do not have cold or hot spots, with the exception of one documented region (Bellen *et al.*, 2011)—makes them particularly useful and unlike other TE-based tools in *Drosophila* (Metaxakis *et al.*, 2005). The above advantages have made them a complementary tool to current transposon-mediated mutagenesis methods.
and have significantly increased the coverage of annotated gene-disrupting insertions (Bellen et al., 2011).

3.1.2.4 Hybrid element insertions

During normal transposition events, \( P \) element mobility is initiated by an excision event that allows the TE to insert elsewhere in the genome (‘cut and paste’ model). However, when two distinct \( P \) elements are inserted at different sites on homologous chromosomes, faulty transposition may occur if the 5’ end of one \( P \) element pairs with a 3’ end of the other \( P \) element to form a temporary ‘hybrid’ element rather than a single TE (Figure 1) (Venken et al., 2005) (Svoboda et al., 1995). This hybrid element reinserts randomly into the genome, but frequently integrates near the site of either original \( P \) element resulting in chromosomal rearrangements (Gray et al., 1996; Preston et al., 1996). In one scenario, HEI may potentially result in a deletion whereby the intergenic region is removed (Gray et al., 1996; Preston et al., 1996). This strong, preferential, inherent bias of the hybrid element to locally insert near one of the original \( P \) element locations allows for deletions to be generated with somewhat defined breakpoints. In addition, the resulting deletions contain one of the original \( P \) elements which therefore provide a convenient molecular identification tag during characterization (Figure 1). Moreover, this type of ‘faulty’ transposition event can be screened for in males from the recombination of visible, flanking markers in a manner that differ from parental strains. In fact, the Bloomington Deletion Project has
widely used the hybrid element insertion (HEI) technique for the construction of large-scale, high-resolution deficiencies with defined breakpoints using two $P$ elements in trans (Parks et al., 2004).

While this HEI approach has been documented as a successful strategy to generate mutants using $P$ elements, it has never formally been tested using other transposable elements. Therefore, I first set out to determine whether HEI- transposition occurs using the new collection of Minos element insertions available to the Drosophila community and whether I could strategically select for deletion recombinants in a manner comparable to previously documented studies with $P$ elements (Svoboda et al., 1995; Gray et al., 1996; Preston et al., 1996). In this chapter, I describe the success of the Minos-based HEI technique to generate DEG/ENaC mutants. The successfully generated alleles were then extensively characterized in Chapter 4 of this work.

### 3.2 Materials and Methods

#### 3.2.1 Fly strains

For all HEI-mediated deletions, the following heat-shock Minos transposase source was used: $w^{118};Sco/SM6aP{hsILMiT}2.4$ (Metaxakis et al., 2005). For the CG18110/stallone locus the following fly strains were used: $y^{1}w^{67c23};Mi(ET1)ppk20^{MB01352}$ (Referred to as Mi[ppk20]) (Bellen et al., 2011), $w^{118}; Mi(ET1)ppk19^{MB05382}$ (Referred to as Mi[ppk19]) (Bellen et al., 2011), $y^{1}w^{118}; PBac[y^{+}-attP-9A]^{VK00020}$ (Referred to as PBac[$y^{+}$] and used as a distal yellow+ marker) (Venken et al., 2006), $w^{118}; Sp/Cyo; Sb/TM3Ser$ (used $Sb$
as a proximal dominant marker). Note that Minos elements were followed with the 3xP3-EGFP marker.

3.2.2 Crossing scheme

For HEI-mediated deficiencies of the stallone locus, visible markers were first recombined with the Minos alleles using standard meiotic crossover crosses to generate the desired marked chromosome. First, the distal $PBac[y^r-attP-9A]^{VK00020}$ marker (located at chromosome position 99F8) was recombined with the most proximal $Mi(ET1)$ $ppk20^{MB01352}$ allele (located at chromosome position 99B6-B7) to establish a stock in which both the yellow$^r$ and 3xP3-EGFP (from the Minos element) markers were present (genotype $y^1w^{67c23};; Mi[ppk20]~PBac[y^r]$). Second, a proximal $Sb$ marker (located at chromosome position 89B4-B6) was recombined with the most distal $Mi(ET1)ppk19^{MB05382}$ allele (located at chromosome position 99B7) to establish a stock in which both the $Sb$ and 3xP3-EGFP marker of the Minos element were present (genotype: $w^{118};;Sb~Mi[ppk19]/TM3~Ser$). Once these balanced stocks were established, the crossing scheme shown in Figure 2 was used to uncover potential deletions. Two days after the G1 crosses were established, the progeny were heat shocked daily in a circulating water bath at 37°C for 1 hour until eclosion (8-9 days post mating). All adult progeny containing both Minos insertions (3xP3-EGFP) and respective markers ($yellow^+$ and $Sb$) plus the helper balancer chromosome containing the heat-shock transposase (SM6a P[hs-ILMiT]) were crossed to a $y^1w^{67c23}; TM3Ser/TM6B~Tb~Hu$ balancer stock and all resulting
male cross progeny were screened for either a) a potential deletion event in which males displayed yellow and $Sb^+$ phenotypes (i.e. loss of both markers) or b) a potential duplication event in which males displayed yellow $^+$ and $Sb$ phenotypes (i.e. gain of both markers). Single males showing HEI-recombination events were established as a balanced stock with the Minos transposase helper chromosome removed, and progeny were further analyzed at the molecular level for the presence or absence of the stallone locus.

### 3.2.3 PCR analysis

To preliminarily screen for stallone deletions, polymerase chain reaction (PCR) was performed on genomic DNA extracted from adult flies homozygous for the putative HEI-mediated deletion. DNA extraction was performed using The Qiagen DNeasy® Blood and Tissue kit following the manufacture’s protocol. PCR amplification (Roche) using the forward primer: 5’-TCC CGT GAT AAT GTG TTC TCC AC-3’ and the reverse primer: 5’-GTT ACA GGT AAG TGT GGG AAC GTG-3’ directly measured the presence or absence of the stallone gene product (near the 3’ end of gene). Control primers targeting Tubulin, were used to confirm integrity of DNA preps. Two additional primer pairs were used to confirm whether flanking genes near the stallone locus were also removed in the HEI-mediated deletion. The first primers targeted the upstream gene, ppk20 and the N-terminal end of stallone (forward primer: 5’-CTC CAA GTT ATG CAG GTG-3’ and reverse primer: 5’-TTA TGC ACG GCC AGA GTC ATG G-3’) and the
second primers targeted the downstream gene Obp99a (forward primer: 5'‑ATA AAC GGC GAC CAG GAC CAG AA TA-3' and reverse primer: 5'‑TGT TCT CCA CGT TGA AAC CGC TCT G-3').

Additional analysis characterizing break points of putative deletions were conducted using various primers targeting neighboring genes. To PCR amplify the ppk20 locus, the following primers were used: the forward primer, 5'-CCA ACT TCG TTC CTT AGG TAG CC-3', the reverse primer 5'-ACT CTT CAG GGT GGA TGT GC-3'. To PCR amplify the ppk19 locus, the following primers were used: 5'-GTT GTG TGG AAG ACC TGC CAG TG-3' and the reverse primer, 5'-TCC CAC GGA CCA GGA AAC TAA AC-3'.

3.2.4 Inverse PCR and plasmid rescue analysis

After PCR confirmation of stallone deletions, a combination of inverse PCR (iPCR) (Bellen et al., 2011) and plasmid rescue (Metaxakis et al., 2005) were performed as described (Huang et al., 2009) to determine the precise breakpoints. For iPCR, genomic DNA (~2 flies equivalent) from PCR confirmed stallone deficient lines were digested with restriction enzyme HpaII (NEB) in appropriate buffer for 4-5 hours, heat-inactivated for 20 min at 64°C, and ~1 fly equivalent was ligated (Invitrogen) in a total volume of 120uL overnight at 4°C to self-circularize. PCR was performed using the primer pair that targeted the 5' flank, forward primer: 5'-CAA AAG CAA CTA ATG TAA CGG-3' and the reverse primer: 5'-TTG CTC TTC TTG AGA TTA AGG TA-3', and the 3' flank, the
forward: 5'-ATG ATA GTA AAT CAC ATT ACG-3' and the reverse primer: 5'-CAA TAA TTT AAT TAA TTT CCC-3'. Each PCR product was further sequenced using the primer: 5'-TTT CGT CGT GAA GAG AAT-3'.

For plasmid rescue, genomic DNA of the candidates were digested with SpeI (for 3' flank), EcoRV (5' and 3' flank), or NheI (5' and 3' flank), diluted, and ligated (Invitrogen) Overnight at 4°C. Ligation products were transformed into TOP10 chemically competent cells (Invitrogen) and plated on Luria Broth plates containing kanamycin (50ug/ml). Single clones were grown in LB medium containing kanamycin and the plasmid DNA was mini-prepped (Qiagen). Sequencing of these plasmids was performed using the following primers: 5'- TTG CTC TTC TTG AGA TTA AGG TA-3' (to assess 5' flank) or 5'- ATG ATA GTA AAT CAC ATT ACG -3' (to assess the 3' flank).

3.3 Results

3.3.1 Minos element-mediated HEI screen yields nine candidate male recombinants

Minos elements flanking the stallone locus were selected based on their close proximity to the locus of interest and their opposite orientation respective to one another. The Mi(pkp20) insertion is in a (+) orientation and is 1806bp upstream of the stallone start site, while the Mi(pkp19) insertion is in a (-) orientation and is 2007bp downstream of the stallone stop codon (Figure 3). Collectively, these two Minos element insertions are separated by 6,142 bp. The orientation and insertion sites of these Minos element alleles were first confirmed using PCR and iPCR techniques (data not shown). Additionally,
two different visible markers (M1 and M2) on third chromosomal arm (3R) flanking both Minos elements were specifically selected to serve as tightly coupled linkage groups that aid in the identification of recombinant progeny differing from parental alleles after HEI. Since males normally do not undergo recombination, any crossover events observed likely occurred at the region of Minos element insertion. Therefore the visible M1 and M2 markers are linked to their respective Minos element and serve as markers for crossover identification at the stallone locus. Thus, these markers were used to track recombinant and parental non-recombinant chromosomes inherited from male founders after HEI.

The first marker (M1), PBac\(y^{-}\text{-attP-9A}\)^{\text{709020}} was selected for the yellow\(^+\) transgene it carried and its far distal location on chromosome arm 3R. Moreover, using a transgene as a visible marker has the advantage of not being limited to visible markers defined to a specific chromosomal region, thus requiring simpler and fewer testcross stocks to build and the broad range and wide coverage of currently available PiggyBacs and P element transgenes. It is important to note that other Mi\{ET1\} or Mi\{MIC\} lines may not be used as visible markers as these TES are obviously mobile in the presence Minos transposase. The second marker (M2), Sb was chosen because of its dominant phenotype and mid-chromosomal location on 3R (i.e. proximal to both Minos elements).

Both of these markers were combined \textit{in cis} in the following manner: the most proximal Mi\{ppk20\} insertion was recombined with the most distal PBac\(y^{+}\) marker (M1).
and the most distal \textit{M}i\textit{ppk}19 allele was recombined with the most proximal \textit{S}b marker (M2) (Figure 1, Figure 2). By recombining the markers and \textit{Minos} insertions in this combination, I was able to select male recombinants that likely underwent a hybrid element insertion event by looking for loss of both \textit{yellow} and \textit{S}b (i.e. identify male progeny had a different genotype from parental alleles). While this approach appears similar to the method Parks, \textit{et al} (Parks \textit{et al}., 2004) employed, it is subtly different in that I selected for progeny displaying marker loss (i.e. both markers were recombined off) rather than retaining a set of recessive markers in the resulting recombinant. This method has the advantage of removing the visible markers which may potentially interfere with future phenotypic analysis of the deletions.

Out of 81 crosses established, I identified eight candidate males showing recombinant loss of both markers and one candidate that possessed both visible markers. Table 3-1 summarizes the genotypes of the candidate aberrations at the \textit{stallone} locus as a result of HEI- transposition and whether or not the \textit{Minos} element was still present based on 3xP3-EGFP expression.

\textbf{3.3.2 Minos element-mediated hybrid element insertion produces six candidates deficient at the \textit{stallone} locus}

Once the eight deletion candidates were identified in this deletion screen, I sought to determine whether the intergenic genes between the \textit{M}i\textit{ppk}20 and \textit{M}i\textit{ppk}19 \textit{Minos} insertions were in fact deleted. Primers targeting the 5’ upstream region encoding the start codon of \textit{stallone} and the 3’ downstream region of \textit{stallone} confirmed that six of
these lines were indeed deficient at the *stallone* locus (Figure 3). Moreover, primers targeting the downstream gene *Opy99a* also showed that these six candidates also lacked this gene (Figure 3) and suggested that the entire intergenic region flanked by these two *in trans* Minos elements was deleted using this approach. Therefore, 75% of candidates indicate that the loss of *Sb* and *yellow* markers phenotype correlates with a Minos-mediated deletion event in males and thus confirms that this approach is a useful screening mechanism for identifying deletions. The remaining 25% are likely events in which non-homologous end joining repair mechanisms of the homologous chromatids occurred (also referred to Hybrid Excision Repair in (Gray *et al.*, 1996)) or were the result of local insertion into the homologous chromatid.

3.3.3 Molecularly defined breakpoints of the deletions produced via Minos-mediated HEI transposition

These six candidates showing a deletion at the *stallone* locus were extensively characterized at the molecular level using a combination of PCR, iPCR, and plasmid rescue analysis. This characterization proved challenging as many candidates were difficult to molecularly define. It appears that this approach yielded many events in which one of the Minos elements had either inserted itself into one of the existing Minos elements or had duplicated itself making resolution of the endpoints difficult to interpret using iPCR and plasmid rescue. Complex Minos element fusions made this especially difficult to interpret. Figure 4 summarizes these data.
While most of the candidates were indeed *stallone* deficient, it was difficult to precisely determine what occurred during transposition. Gray *et al.* (Gray *et al.*, 1996) outlines several possibilities resulting from HEI events. Indeed three candidates showed typical HEI features: 1) two complete deletions (Line 8 and 20) with insertion of the proximal *Minos* element into a nearby region of *ppk20* (highlighted in Figure 1) and 2) one deletion (Line 71) in which both *Minos* elements were present and oriented in a head to head manner (Figure 4). iPCR and plasmid rescue gave the clearest outcome of HEI events for Candidate 20. The breakpoints were identified at a high resolution. Interestingly the distal Minos element re-inserted itself in *ppk20*, but upstream of the original *Mi*(*ppk20*) insertion site and sequencing results confirmed the original *Mi*(*ppk20*) ME was excised and repaired via NHEJ, as a TA-footprint normally left by MEs was observed. Line 20, was thus used for all subsequent behavioral phenotyping experiments outlined in Chapter 3.

The remaining three HEI-mediated deletion events were more difficult to interpret with resolution only available at either the 5' or 3' flanks. Gray *et al.* (Gray *et al.*, 1996) also remarked that their proposed HEI model is not capable of predicting certain observed outcomes as recombinant progeny showed novel fragments that could not have resulted from parental chromosome. Such unpredictable outcomes were also observed in this deletion screen. For example, strange fusions of *Minos* element sequence were present in a manner unexplained by the HEI model. For example, candidate X
displayed 3’ inverted repeats (3’-IR) from the Minos element at both deletion breakpoints suggesting a more complex transposition process than modeled in Gray et al (Gray et al., 1996). Additionally, it appeared that candidate 52 had multiple insertions of the Minos element, suggesting many local hopping events and perhaps an undocumented ‘copy and paste’ mechanism of this TE family. Alternatively this may be a consequence of repeated heat shocks that activate the Minos transposase. Direct sequencing of these regions in these strains may clarify these unexpected outcomes.

3.4 Discussion

In this chapter I have demonstrated that HEI-mediated transposition events resulting in deletions can be recovered using two in trans Minos elements. The high efficiency (10% of crosses contained one male fly that underwent HEI-mediated recombination) of this approach showcases the power of transposable elements in generating chromosomal rearrangements. Moreover 75% of these candidates possessed a complete deletion of the locus flanked by these two in trans Minos elements, thus showing that linked visible markers are sufficient for screening deletion mediated events with high efficiency. The exact breakpoints were difficult to resolve in half of these candidates as the exact mechanism and possible outcomes of all events of HEI-transposition remains unclear. However, this does not detract from the fact that this approach was extremely successful in generating deletions.
With the recent advances made by the *Drosophila* Gene Disruption Project in increasing the number and coverage of available Mi[ET1] and Mi[MIC] insertion collections (Bellen et al., 2011), *Minos* elements are therefore highly attractive tools for HEI-mediated deletions. While this technique has not been explicitly tested using Mi[MIC] insertions, it should in theory follow the same principle outlined in this chapter. Moreover, the increased coverage presented in these collections coupled with this technique could be of great interest to the Bloomington Deficiency Project as a means to potentially increase the number of deficiencies and generate deficiencies that were previously difficult due to *P* element and PBac insertion ‘cold-spots’ of the genome.

One current caveat in using *Minos* elements to facilitate HEI-deletion events is that the *Minos* transposase resources are limited. There is currently only one insert of *P*[hsILMiT] on the second chromosomal balancer, *SM6a* available. This therefore constrains its use for generating deletions to the X or III chromosome. However, this could easily be remedied by hopping the *Minos* transposase source onto another chromosome with *P*-transposase.

Another potential challenge of the *Minos* HEI strategy is that it may be difficult to employ for larger deficiencies (i.e. there may exist a size limit on efficient HEI-deletion events). *Parks et al.* (Parks et al., 2004) reports *P* element HEI based deficiencies ranging
from 80-150kb however the possibility of size constraint using Minos elements remains untested as the deletions generated in this chapter were ~6kb.

My attempts to use the Minos-mediate HEI-deletion approach were unsuccessful in generating a ~23kb deletion at the balboa/CG8546 locus (screened through approximately 600 crosses without any candidates confirming a deletion, S.E.M. unpublished). However it remains unclear if this failure was due to the size of attempted deletion or due to potential lethal effects from mutating or deleting neighboring genes. Indeed, the neighboring CG42458 gene has a predicted role in mRNA binding and mutant alleles have been correlated with pupal lethality (Mummery-Widmer et al., 2009) and could serve as a potential explanation as to why a deficiency was not uncovered. In the event that there is a size limit using Minos-mediated HEI, this method remains beneficial in generating mini deficiencies.

With the recent advances made using the clustered regularly interspaced short palindromic repeats (CRISPRs) technique, it is becoming much simpler to design custom-made genome modifications. While the CRISPR method is preferential for customizing tailored endogenous alleles, it is cloning intensive, time consuming, and rather expensive when used for generating knockout alleles of gene of interest. Therefore, the straightforward approach of the Minos element mediated HEI transposition described in this chapter is a suitable alternative especially with the recent wide coverage of the Mi{ET1} insertion collection. Moreover, if performed on a larger
scale, this simple technique can be used as a non-labor intensive and low-tech method to extend the deficiency collection and increase deletion coverage of the *Drosophila* genome. Additionally, the minimal resources needed to complete such a task could also be suitable to use in a teaching laboratory setting or as an undergraduate research project with relative ease.

In summary, *Minos* elements *in trans* can facilitate HEI-driven deletion events with fairly defined endpoints. This method can generate null mutants that can be subsequently characterized for phenotypic analysis, including genes essential for behaviors. Indeed one of the HEI-mediated *stallone* mutants described this chapter was analyzed for mechanical nociception behavior defects in *Drosophila melanogaster.*
Figure 1: Hybrid element insertions (HEI) generate genetic deletions

Adapted from Venken, K.J.T., and Bellen, H.J., 2005
Figure 1: Hybrid element insertions (HEI) generate genetic deletions.

Two *in trans* Minos elements, indicated as double headed arrows showing 5’ and 3’ polarity, are located in opposite orientation on different chromatids of the same homologous chromosome (labeled as red and blue, with circles designating the centromere). Theoretical dominant markers to track homologous chromosomes are indicated as M1- and M2- and located distally and proximally, respectively to the Minos insertions. During the G2 phase of the cell cycle, Minos transposase cuts at 5’ end of one Minos element and the 3’ end of the other Minos element while the uncut ends remain continuous with the rest of the chromosome. These complementary ends from separate Minos elements synapse together to form a temporary ‘hybrid’ element that inserts elsewhere into the genome (curved arrow) and results in a recombination event. The potential outcomes of an HEI event either delete (shown, loss of M1- and M2-) or duplicate (not shown, gain of M1- and M2-) the intergenic region (pentagon shape).
Figure 2: Genetic crossing scheme to generate HEI-mediated deletions
Figure 2: Genetic crossing scheme to generate HEI-mediated deletions. The genetic scheme used for Minos element mediated deletions in the presence of transposase. The triangles represent two independent Minos insertions in opposite orientations with visible flanking markers (M1 and M2) on the third chromosome. The heat-shock Minos transposase source, P[hsILMiT], is located on the balancer chromosome SM6a and can be activated in premeiotic germ cells by heat-shock (hs) treatment at 37°C for one hour daily. Deletion events are identified as recombination events whereby both flanking markers are lost in test crosses of single males with females carrying proper tester and balancer chromosomes. This final cross establishes a balanced stock.
Table 3-1: Candidate aberrations at the stallone locus as a result of HEI-mediated transposition

<table>
<thead>
<tr>
<th>Candidate HEI line</th>
<th>Genotype</th>
<th>M1</th>
<th>M2</th>
<th>3xP3-EGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>yw/yw; +/SM6a, P(hsilMAT); Δsta/TM3, Ser</td>
<td>x</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>Y</td>
<td>yw/yw;; Δsta/TM3, Ser</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>yw/7; +/SM6a, P(hsilMAT); Δsta/TM3, Ser</td>
<td>x</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>20</td>
<td>yw/7; +/SM6a, P(hsilMAT); Δsta/TM3, Ser</td>
<td>x</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>40</td>
<td>yw/7;; Δsta/TM3, Ser</td>
<td>x</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>52</td>
<td>yw/7; +/SM6a, P(hsilMAT); Δsta/TM3, Ser</td>
<td>x</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>60</td>
<td>yw/7; +/SM6a, P(hsilMAT); Δsta/TM3, Ser</td>
<td>x</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>71</td>
<td>yw/7;; Δsta/TM3, Ser</td>
<td>x</td>
<td>x</td>
<td>✓</td>
</tr>
<tr>
<td>81</td>
<td>yw/yw;; Duplication sta/TM3, Ser</td>
<td>✓</td>
<td>✓</td>
<td>x</td>
</tr>
</tbody>
</table>
Table 3-1: Nine candidate aberrations at the *stallone* locus as a result of HEI-mediated transposition. The genotypes of the nine HEI-transposition candidates showing loss of *PBac(yellow)* (M1) and *Sb* (M2) are reported.
Figure 3: PCR confirms a deficiency at the *stallone* locus in *Minos*-mediated HEI candidates
Figure 3: PCR confirms a deficiency at the *stallone* locus in *Minos*-mediated HEI candidates. Top schematic shows the gene structure of the *stallone* locus and *Minos* element insertions in opposite orientation: *Mi[ppk20]* in (+) orientation and *Mi[ppk19]* is in (-) orientation. Primers targeting the A) 5' *stallone*, B) 3' *stallone*, and C) *Obp99a* genes showed that candidates X, 8, 20, 52, 60 and 71 had a deletion between the original *Mi[ppk19]* and *Mi[ppk20]* *Minos* element insertions. Primers targeting the D) *Tubulin* genes confirmed the presence of high quality DNA for PCR amplification experiments.
Figure 4: Molecularly defined breakpoints of *stallone* deficient candidates
Figure 4: Molecularly defined breakpoints of *stallone* deficient candidates.

The gene structure of the *stallone* locus showing the two *Minos* element insertions, Mi[ET1] ppk20 and Mi[ET1] ppk19, indicated by an unfilled triangle in the (+) orientation (5'-3' *Minos* element polarity) and a filled triangle in the (-) orientation (3'-5' *Minos* element polarity). Molecularly defined breakpoints of the six *Minos* HEI-mediated deletion lines, X, 8, 20, 52, 60, and 71 are depicted in this cartoon. Hatched arrow indicates the inverted repeat endpoints of the *Minos* element with either the 5’ or 3’ polarity denoted above. An asterisk designates the characteristic footprint CGAGTA left behind at the donor site by the *Minos* element following excision during transposition. Question marks indicate that the sequence at the breakpoint remains unclear at this region, but the 5’ region of *ppk20* remains deleted as shown in Figure 4. The shaded box highlighting candidate line 20 is the generated allele used for extensive characterization in Chapter 4 of this work.
4. *stallone* is a DEG/ENaC gene required for mechanical nociception in *Drosophila melanogaster* larvae

4.1 Introduction

A tissue specific knockdown strategy utilizing RNAi lines targeting genes that encode all annotated and putative ion channels in the *Drosophila* genome was previously implemented to specifically reduce ion channel subunits in sensory neurons. This approach provided several advantages over the traditional loss of function mutagenesis screens by specifically targeting genes of interest in a tissue type of interest and avoiding potential pleiotropic effects in whole animal null mutants. Richard Hwang described the results of this screen to identify ion channels that play a role in mechanical nociception (Hwang, 2009). Surprisingly, knockdown of only nine candidates with function in all multidendritic neurons resulted in defective mechanical nociception behaviors. Of these, four candidates specifically functioned within the Class IV nociceptive neurons and two of these were not required for optogenetically triggered escape locomotion, suggesting a role independent of intrinsic excitability. These two candidates, *CG18110* and *CG8546*, were members of the degenerin/epithelial sodium channel (DEG/ENaC) family and were thus suitable candidates for further investigation into their roles in larval mechanical nociception. This chapter focuses on further analysis of *CG18110* which Hwang dubbed *stallone* (Hwang, 2009).
*stallone* is a member of the DEG/ENaC family that shows the canonical predicted topology of two transmembrane domains with a large extracellular domain (Figure 5). *Stallone* shares similarity with other *Drosophila ppk*-related channels (Figure 5). There are currently thirty one *ppk*-family members annotated in the genome. They all share a similar topology, yet have a wide variety of neuronal and non-neuronal functions. The *ppk*-channels have been reported to function in mechanical nociception and locomotion (*ppk*) (Ainsley *et al.*, 2003; Zhong *et al.*, 2010), sensory perception of touch (*rpk*) (Tsubouchi *et al.*, 2012), liquid clearance of the trachea (Liu, Johnson, *et al.*, 2003), salt perception (*ppk19* and *ppk11*) (Liu, Leonard, *et al.*, 2003), tasting water (*ppk28*) (Cameron *et al.*, 2010), regulation of synaptic activity (*ppk11* and *ppk16*) (Younger *et al.*, 2013), male courtship and pheromone sensing (*ppk23*, *ppk25*, and *ppk29*) (Liu *et al.*, 2012; Lu *et al.*, 2012; Toda *et al.*, 2012; Toda *et al.*, 2012; Vijayan *et al.*, 2014), and male aggression (*ppk23*) (Edwards *et al.*, 2009; Yuan *et al.*, 2014).

The *stallone* gene is located within a *ppk*-family gene cluster. It shares a high level of sequence similarity with its neighboring genes, *ppk21*, *ppk20*, and *ppk19* and with two genes that are on the second chromosome *ppk7* and *ppk14* (Figure 5). The closest mammalian homolog predicted by the DRSC Integrative Ortholog Prediction Tool (Hu *et al.*, 2011) is *ASIC4* whose function remains unclear but may play a role in the ubiquitin pathway (Donier *et al.*, 2008).
In this chapter, the mechanical nociception phenotype of my \( \Delta \text{stallone} \) mutant was rigorously analyzed. This was necessary to extend the RNAi results from the mechanical nociception screen and validate our screening approach. Interestingly, \textit{stallone} may have a general mechanosensory function as gentle touch responses were mildly reduced in the knockout mutant. Additionally this allele was used to confirm that thermal nociception responses were normal and mechanical nociception behavioral defects were not a mere consequence of morphological defects in the nociceptors. Additional epistasis analysis was conducted to determine whether \textit{ppk} and \textit{stallone} function in the same pathway and much to my surprise, the \( \Delta \text{ppk} \Delta \text{stallone} \) double mutant displayed wildtype mechanical nociception behaviors.

\textbf{4.2 Materials and Methods}

\textbf{4.2.1 Fly strains}

The hybrid \textit{Minos} element insertion technique described in Chapter 3 was used to generate a null allele of the \textit{stallone} locus. The exact breakpoints of one of those candidates, line 20, was verified (Figure 4) and confirmed that this mutant allele is a genetic null for \textit{ppk20}, \textit{CG18110/stallone}, \textit{Obp99a}, and \textit{ppk19}. This candidate was backcrossed to wildtype, \textit{Canton-S} for six generations and will henceforth be referred to as the \( \Delta \text{stallone} \) mutant allele in this chapter. It is important to note that his mutant allele is actually a quadruple knockout for the aforementioned genes. Unless otherwise noted, this Cantonized allele of \( \Delta \text{stallone} \) was used for all further experimentation.
Additional stocks used for experiments in this chapter include: Canton-S, y\textsuperscript{w\textasciitilde w77c23}, w\textsuperscript{1118}, y\textsuperscript{w\textasciitilde w77c23}; Mi(ET1) ppp20\textsuperscript{MB01352} (Referred to as Mi(ppk20)) (Bellen et al., 2011), w\textsuperscript{1118}; Mi(ET1)ppk19\textsuperscript{MB05582} (Referred to as Mi( ppk19)) (Bellen et al., 2011), isogenic w\textsuperscript{1118} (DrosDel isogenic background for ED deficiencies), w\textsuperscript{1118}; Df(3R)ED13102/TM6C Sb\textsuperscript{i}, isogenic w\textsuperscript{1118} (Exelis isogenic background for BSC deficiencies), and w\textsuperscript{1118}; Df(3R)BSC500/TM6C Sb\textsuperscript{i} cu\textsuperscript{i} for complementation analysis. Minos transposase source: w\textsuperscript{1118}; Sco/SM6a P\textsuperscript{hsILMiT}2.4; Sb/TM3Ser (Metaxakis et al., 2005). For RNAi experiments: md-GAL4;UAS-Dicer2, ppk-GAL4; UAS-Dicer2, isogenic w\textsuperscript{1118} (VDRC isogenic host strain for the GD collection RNAi library) w\textsuperscript{1118}; P\textsuperscript{(GD763)\textasciitilde 51432} (VDRC GD UAS-Obp99a RNAi line) , yw\textsuperscript{1118}; P\{attP y\textsuperscript{v} \textasciitilde w[3\textsuperscript{'}]\} (VDRC integration strain for the KK collection RNAi library) , yw\textsuperscript{1118}; P\{KK104453\textsuperscript{VIE-2608} (VDRC KK UAS-Obp99a RNAi line). Mi[MIC] insertion line: y\textsuperscript{w\textasciitilde w\textasciitilde}; Mi[MIC]ppk30\textsuperscript{MI10852}.

4.2.2 Behavioral assays

See General methods for specifics on mechanical nociception, thermal nociception, gentle touch, and optogenetic activation assays.

4.2.3 Minos element excision

I used the following crossing scheme to further remove the Minos element (marked by 3xP3-EGFP) present in the \textit{Astallone} stock and the following primer sequences to perform PCR across the breakpoint of the \textit{Astallone}\textsuperscript{111-3} stock: the forward primer, 5’-GAA CAG
4.2.4 Molecular cloning

stallone cDNA was amplified from the full length cDNA clone IP12342 (Stapleton et al., 2002) using the forward primer, 5’-CAC CAT GAG TGC CAC CGC CTG GAA-3’ and the reverse primer, 5’-CTA GGA GCC ATG GGG ATG CGA C -3’. This PCR amplicon was cloned into pENTR (Invitrogen) and subcloned into pTVW using clonase (Invitrogen). The fully sequenced clone was then midiprepped (Qiagen) according to manufacturer’s protocol and sent to Rainbow Transgenics for transgenesis via standard P element transformation.
4.2.5 BAC recombineering

I performed bacterial artificial chromosome (BAC) recombination-mediated engineering (recombineering) as previously described (Warming et al., 2005; Walcott, 2012), with slight modifications.

A P[acman] BAC (CH321-60N03) was obtained from the BACPAC Resources (http://bacpac.chori.org/). Transformation of BAC DNA into SW102 bacterial strain was performed as described in Recombineering protocol #1: “Recombineering using the modified DH10B strain DY380” (http://web.ncifcrf.gov/research/brb/protocol/Protocol1_DY380.pdf). Modification of BAC DNA in the SW102 strain was performed as described in Recombineering protocol #3: “BAC Recombineering using the modified DH10B strain SW102 and a galK positive/counters election cassette” (http://web.ncifcrf.gov/research/brb/protocol/Protocol3_SW102_galK_v2.pdf). The galK selection scheme is a two-step process. In the first step, the galK cassette, containing homology to a specific position in the BAC, was inserted into the BAC by homologous recombination. Recombinant bacteria were positively selected by growth on minimal media plates with galactose as the carbon source. In the second step, the galK cassette was substituted with an annealed PCR product with homology to either side of the CG18110 portion of the stallone locus, effectively deleting that region. Recombinant bacteria were selected by resistance to minimal media plates containing 2-deoxy-galactose (DOG), as DOG is phosphorylated by functional galK into a toxic intermediate.
In some cases, the DOG⁻ resistant bacteria were background colonies that lost the galK cassette by a deletion. The remaining colonies were true recombinants. The purified Bacterial DNA (CH321-60N032Δsta) was used to create transgenic flies. For general protocol see (Warming et al., 2005).

For amplification of the galK cassette with 50bp homology arms to the desired location in BAC CH312-70F12, a forward primer: 5'-CTA CCA ATT AGC GCG ATT TTC CCA CAG TCG CAG TTC GTC CGC TGA GTG AAC CTG TTG ACA ATT AAT CAT CGG CA-3' and reverse primer: 5'-GAG AAC CCA GCT GCC GAT GGC TGG TAC TTC ATG GTG CCA GCT TGG GCC TAT CAG CAC TGT CCT GCT CCT T-3' were used. For substitution of the inserted galK cassette with a double-stranded DNA oligo to generate a deletion of the region of interest (CG18110), a forward primer: '5'- GCG ATT TTC CCA CAG TCG CAG TTC GTC CGC TGA GTG AAT AGG CCC AAG CTG GCA CCA TGA AGT ACC AGC CAT CGG CAG CTG GGT TCT C-3' and a reverse primer: '5'- GAG AAC CCA GCT GCC GAT GGC TGG TAC TTC ATG GTG CCA GCT TGG GCC TAT TCAC TCA GCG GAC GAA CTG CGA CTG TGG GAA AAT CGC GCT AAT TGG TAG-3' were used. The double-stranded complementary oligos were annealed in vitro according to Step 18 of the Recombineering protocol #3 (http://web.ncifcrf.gov/research/brb/protocol/Protocol3_SW102_galK_v2.pdf). Custom oligos were made by Life Technologies™ (www.invitrogen.com).
Recombineered BAC DNA (CH321-60N032Δsta) was purified using the Qiagen Midi Prep. Transgenic animals were created by PhiC31-mediated chromosome integration of BAC DNA into the P[CaryP]attp40 site (Genetic Services Inc.).

For attempts at generating recombineered alleles to further probe stallone’s expression pattern, I inserted galk immediately upstream of stallone’s start site in the BAC-CH321-26K05 using the following primers: the forward primer using pGalk (Warming et al., 2005) as template, 5’- CTA CCA ATT AGC GCG ATT TTC CCA CAG TCG CAG TTC GTC CGC TGA AGG AGG GTT CTG GCT TCC AGG CGG TGG CAC TCA TTC AGC ACT GTC CTG CTC CTT-3’, the reverse primer, 5’- CAT CAT TAT CCT CGG AGG AGG GTT CTG GCT TCC AGG CGG TGG CAC TCA TTC AGC ACT GTC CTG CTC CTT-3’. To insert the NGFP or GAL4 directly upstream of stallone’s start codon, the following primers were used for amplifying the NGFP or GAL4 gene product from plasmids PL-452 N-EGFP or SM1 for use during the counter selection step: the forward primer, 5’- CCA ATT AGC GCG ATT TTC CCA CAG TCG CAG TTC GTC CGC TGA AGG AGG AGG AG -3’, and the reverse primer, 5’- CAT CAT TAT CCT CGG AGG AGG GTT CTG GCT TCC AGG CGG TGG CAC TTT ACT TGT ACA GCT CGT CCA TG-3’ using PL-452 N-EGFP as template or the forward primer, 5’-CTA CCA ATT AGC GCG ATT TTC CCA CAG TCG CAG TTC GTC CGC TGA GTG AAG CTT GAA GCA AGC CTC CTG AAA G-3’ and the reverse primer, 5’- CAT CAT TAT CCT CGG AGG
AGG GTT CTG GCT TCC AGG CGG TGG CAC TCA TCC GCT CTA GTGG ATC TAA
ACG AG-3’ using SM1 template.

The same galk positive selection protocol detailed above was followed. To
generate the sta^GAL4 allele I used the following primers to amplify GAL4 from the plasmid
SM1 (gift from Hubert Amrein). To generate to NGFP::stallone allele I amplified NGFP
from plasmid pL-452 N-EGFP (Addgene). These PCR products were then negatively
selected for following the same protocol to generate the following clones for
transgenesis: CH321-26K05-NGFP::stallone and CH321-26K05-stallone^GAL4. These BACs
were purified using Nucloebond kit and sent off to Genetic Services Inc. for PhiC
mediated-integration into the lines attp40 and attp-3BV^K000) respectively.

4.3 Results

4.3.1 stallone has a naturally occurring valine residue at the ‘deg’ site

The name degenerin originates from the neurodegenerative phenotype (termed
DEG) of mechanosensory neurons induced by dominant mutations of the deg-1 or mec-4
genes in C. elegans (Chalfie et al., 1990). In addition, dominant effects of these missense
alleles result in a degenerative phenotype of touch sensory neurons upon
overexpression (Hong et al., 2000). Multiple independent missense alleles of mec-4
resulting from single point mutations at the same residue, changing it from an alanine to
a valine, threonine, or aspartate (Driscoll et al., 1991; Hong et al., 2000), all cause
neurodegeneration. This site is thus known as the ‘deg’ site or ‘d’ position. Transgenes
encoding MEC-4 with residues bulkier than alanine at the $d$ position cause dominant mechanosensory (Mec) and Deg phenotypes, while those introducing residues smaller than alanine do not (Driscoll et al., 1991). This led to the hypothesis that that bulky residues at this location prevented channel closure and excitotoxic constitutive activity.

Altering DEG/ENaC function via ‘deg’ mutations is not restricted to *C. elegans* family members as introducing bulky amino acids (like valine and threonine) at this site in mammalian ENaC subunits causes an increase in current amplitude, open probability, and mean open time (Snyder et al., 2000). Similarly, mutating this position in *rpk* also dominantly activates channel function in *Xenopus* oocytes (Adams et al., 1998). These studies show that steric hindrance of bulky side chains like valine, at the $d$ position alter DEG/ENaC gating by stabilizing open states.

Small side chained amino acids, like serine, alanine, and glycine are thus highly conserved at the $d$ position. Therefore it was especially surprising to discover that Stallone in fact had a naturally occurring valine at this exact ‘deg’ site. While scanning the sequence of all other *ppk*-family channels in the *Drosophila* genome, roughly a third show a valine at this position (Figure 6). Interestingly, these channels tend to cluster together on an evolutionary phylogenetic tree (Figure 6) perhaps due to decent by duplication of an ancestral paralog. There have not been any reports of naturally occurring polymorphisms at this site outside of the *Drosophila* genus. Therefore,
determining whether this valine at the ‘d’/‘deg’ site is essential to stallone’s role in
mechanosensation is of great interest.

4.3.2 stallone mutant animals show reduced mechanosensory responses

4.3.2.1 Δstallone genetic mutant phenocopies stallone-RNAi impaired mechanical nociception responses

As noted above, stallone was identified as an important gene for mechanical nociception in an RNAi screen. To confirm observations of impaired mechanical nociception response, I first tested the Δstallone mutant for mechanical nociception defects and indeed found a pronounced impairment in responses (Figure 7), suggesting that a locus within this deletion is important for mechanical nociception responses.

4.3.2.2 Other deficiencies fail to complement the reduced mechanical nociception responses observed in the Δstallone mutant

To confirm that second-site mutations were not a factor in the defective mechanical nociception phenotype, I crossed the Δstallone mutant to two independently generated deficiencies available from the Bloomington Deletion Project, w1118;Df(3R)ED13102 and w1118;Df(3R)BSC574 for complementation analysis. This heteroallelic combination of these two deficiencies with the Δstallone allele failed to complement the mutant phenotype confirming that the locus responsible for defective mechanical nociception responses mapped to the region of interest (Figure 8). One caveat to interpretation of this analysis is that four genes (including stallone) are also genetic null mutants in the
transheterozygous larvae. Therefore, the next step was to determine if the \textit{ppk20}, \textit{Obp99a}, or \textit{ppk19} genes were contributing to the impaired mechanical nociception responses.

### 4.3.2.3 \textit{ppk20}, \textit{Obp99a}, and \textit{ppk19} do not contribute to mechanical nociception defects

While the purpose of generating the \textit{\Delta} \textit{stallone} deficiency (see Chapter 2 for details) was to make a genetic null allele of \textit{stallone}, this deficiency also removed three other genes in the process: \textit{ppk20}, \textit{Obp99a}, and \textit{ppk19}. While the RNAi data suggests that the \textit{stallone} gene itself is contributing to this defect, I needed to rule out that \textit{ppk20}, \textit{Obp99a}, and \textit{ppk19} genes were not contributing to the insensitive phenotype. Since two Minos element insertions from the Mi[ET1] collection disrupted the coding region of \textit{ppk20} and \textit{ppk19} respectively, I used these lines for complementation tests (Figure 4-5). These two insertion lines were also used to generate the \textit{\Delta} \textit{stallone} mutant in Chapter 3. \textit{Mi\{ppk20\}} disrupted exon 4 of \textit{ppk20} and \textit{Mi\{ppk19\}} disrupted exon 2 of \textit{ppk19}. Both of these gene disruptions alleles occur after the first transmembrane domain and based on previous DEG/ENaC work (Liu et al., 2012), these Minos insertions are expected to cause a truncated form of the peptide that would behave in a dominant negative manner if these genes were involved in a phenotype of interest. To determine whether the mechanical nociception defects observed in \textit{\Delta} \textit{stallone} animals are due to the loss of either of these genes, I crossed the \textit{\Delta} \textit{stallone} genetic null line to each Minos insertion, \textit{Mi\{ppk20\}} and \textit{Mi\{ppk19\}}, for complementation analysis. Both Minos insertions complemented the
phenotype indicating that neither loss of \textit{ppk20} nor \textit{ppk19} were sufficient to result in impaired mechanical nociception responses (Figure 9).

The last gene to rule out as a potential source for the mechanical nociception impairments in the \textit{\Delta stallone} mutant allele was \textit{Obp99a}. Since the insertion alleles were not directly disrupting the \textit{Obp99a} gene, I used two independent RNAi lines generated by the Vienna \textit{Drosophila} RNAi Center (VDRC). These lines were produced from two different collections of the Vienna \textit{Drosophila} RNAi Center (VDRC): the GD library and KK library. These two strains contained hairpins targeting different regions of the \textit{Obp99a} gene under the control of UAS. I used the GAL4/UAS system to trigger RNAi pathways that target knockdown of \textit{Obp99a} specifically in Class IV multidendritic neurons using the \textit{ppk-GAL4} driver and \textit{UAS-Dicer}. Neither line from the VDRC’s KK or GD collection showed a defect in mechanical nociception in \textit{Obp99a}-RNAi mutant animals versus the driver control or the UAS-RNAi control (Figure 10). Therefore these findings made a contribution for \textit{Obp99a} in mechanical nociception unlikely.

\textbf{4.3.2.4 \textit{\Delta stallone} mutant displays mild gentle touch defects}

To determine if disrupting \textit{stallone} affects mechanosensation in general, I tested \textit{\Delta stallone} mutant larvae in an established gentle touch assay (Kernan \textit{et al}., 1994; Tracey \textit{et al}., 2003; Hwang \textit{et al}., 2007; Zhong \textit{et al}., 2010; Tsubouchi \textit{et al}., 2012)). In this assay, each larva was gently stimulated four times with an eyelash attached to the end of a small paintbrush and values were summed to produce final score that ranged from 0 to 16. An
average gentle touch score of 8.2 was calculated for wildtype Canton-S animals, 7.6 for heterozygous Astallone mutant animals, and 6.1 for homozygous null animals (Figure 11A). An ANOVA test followed by post-hoc pairwise comparisons indicated that homozygous Astallone mutant animals differed from wildtype and heterozygous animals in a statistically significant manner (p<0.0001 and p<0.005 respectively). The gentle touch score of the mutant was slightly reduced so I examined the distribution of the data to assess whether there were any qualitative differences in behavioral responses in the mutant animals (Figure 11B). Indeed the distribution of scores remains comparable among genotypes with the exception of the number of larvae exhibiting no response to a gentle stimulation (a score of 0). Here, the larvae of the mutant phenotype showing no immediate responses were 1.7 x greater than CS and more than 2x greater than the heterozygote. This noticeable difference suggests that stallone may play a more general mechanosensory role with modular function in detecting both low and high threshold noxious stimuli.

4.3.2.5 Removing the Minos element marker from the Astallone mutant does not affect impaired mechanical nociception responses

To ensure that the Minos marker associated with Astallone mutant was not contributing to the impaired behavioral responses, I performed a series of heat shocks to induce Minos element removal from the mutant strain and molecularly verified the breakpoints by PCR amplifying across the breakpoint (Figure 12). This allele is referred
to as $\Delta$stallone$^{B1-3}$ and it still showed the characteristic impaired responses originally noted in the $\Delta$stallone allele (Figure 12).

4.3.2.6 A stallone specific null allele shows impaired mechanical nociception responses

To further characterize the role of stallone in nociception, I made stallone specific null mutant fly stock using Bacterial Artificial Chromosome (BAC) recombineering (recombination-mediated genetic engineering) which allows for genetic modification of the large pieces of DNA (Warming et al., 2005; Venken et al., 2006) (Figure 13). I used the galk cassette to specifically delete the entire stallone/CG18110 locus, from start to stop codon, while leaving ppk20, Obp99a, and ppk19 intact in the BAC (CH321-60N03).

Therefore, this construct rescues ppk20, Obp99a, and ppk19 without rescuing stallone. This CH321-60N03-$\Delta$sta BAC construct was integrated into the attp40 site on the second chromosome via PhiC31 integrase, generating transgenic $w^{1118};CH321-60N03-\Delta$sta$^{attp40}$ flies. To generate stallone specific genetic null mutant, I crossed $w^{1118};CH321-60N03-\Delta$sta$^{attp40}$ to my $\Delta$stallone mutant to generate a final stock with the following genotype: $+;CH321-60N03-\Delta$sta$^{attp40};\Delta$stallone (Figure 13). These stallone specific mutants were crossed to $\Delta$stallone$^{B1-3}$ and subject to mechanical nociception testing (Figure 13) and indeed confirmed impaired rolling. While the sample size is still small (and consequently not statistically significant) the trend in the data is consistent with the possibility that the stallone gene itself is indeed the gene responsible for observed mechanical nociception defects.
4.3.2.7 Mechanical nociception responses in ∆stallone mutant varies with time

During the course of this project, inconsistencies in the mechanical nociception behavioral phenotype were observed in the ∆stallone over time. In some periods insensitivity was observed but this was followed by periods of more wildtype-like response (Figure 14). The variations in rolling rates exhibited by the ∆stallone mutant to the same noxious 30mN von Frey fiber over time may be explained by the presence of a suppressor floating in the stock. If such a suppressor arose randomly in the population and was selected for over time, one might expect the allele frequency of the suppressor to increase (from a 30% response rate one of roughly 50%) and may explain the above trend of a fluctuating phenotype. Nociception and touch may be important to larval survival in the laboratory and thus may be under natural selection.

Note that this ∆stallone mutant candidate line was backcrossed to Canton-S for six generations to create a stock that could be compared to this wildtype background. While this approach is advantageous as 99% of the linked ∆stallone background is eliminated by the outcrossing procedure, a disadvantage is that Canton-S is not an isogenic strain. Thus, genetic variation present in the stock can be selected over time.

4.3.2.8 A dominant negative allele of stallone impairs mechanical nociception responses

Although the phenotype in the Cantonized ∆stallone mutant stock was increasing over time (i.e. becoming less insensitive) a new reagent then became available for analysis of stallone at this time. With the Gene Disruption Project’s recent increase in
\textit{Minos} transposon insertional allele coverage, the \textit{Minos}-mediated integration cassette (Mi[MIC]) (Venken \textit{et al.}, 2011) line MI10852 was selected for phenotypic analysis of nociception behavior as the Mi[MIC] element disrupted the \textit{stallone} locus. This Mi[MIC] element was inserted into the second exon of \textit{stallone} immediately following the sequence encoding the first transmembrane domain (Figure 15). Previous work on DEG/ENaC truncated proteins retaining the first transmembrane domain displayed dominant negative properties, presumably by forming non-functional complexes with their partnering DEG/ENaC subunit or other interacting protein (Adams \textit{et al.}, 1997; Hong \textit{et al.}, 2000; Bruns \textit{et al.}, 2003; Liu, Johnson, \textit{et al.}, 2003; Liu, Leonard, \textit{et al.}, 2003; Lin \textit{et al.}, 2005). Therefore, the MI10852 insertion is expected to disturb \textit{stallone} function in a dominant-negative manner.

Very few homozygous Mi[MIC] larvae were available for testing. However viability of the transheterozygous combination of the Mi[MIC] insertion and the \textit{Astellone} mutants was present in the expected 50\% ratio. Therefore, the Mi[MIC] insertion was crossed to \textit{Astellone} and \textit{Astellone}\textsuperscript{B1-3} to generate a heteroallelic combination of \textit{stallone} mutants for behavioral testing. Both transheterozygotes displayed impaired mechanical nociception responses (Figure 15) comparable to the null mutant alleles previously described in 4.3.2.1 and 4.3.2.5 (Figured 7 and Figures 12). Although the homozygosity of the Cantonized \textit{Astellone} is losing its phenotype over time, the
phenotype is still detectable in complementation tests with the new Mi[Mic] alleles. This finding aligns with the interpretation of a possible recessive suppressor in the stock.

As anticipated, the heterozygous Mi[MIC] allele also displayed a dominant negative effect (Figure 15). To determine whether this dominant-negative effect is due to Stallone protein truncation, experiments to precisely remove the Mi[MIC] element from the stallone locus are underway. Subsequent analysis of mechanical nociception behavioral responses in these precise excisions will be performed.

4.3.3 ΔstalloneBf-3 mutant exhibits normal thermal nociception responses

The polymodal nature of nociceptors may point to shared mechanisms and molecular machinery for transducing both mechanical and thermal nociception responses. Kia Walcott from our lab previously identified stallone/CG18110 from a systematic screen looking for positive regulators of thermal nociception (Walcott, 2012) suggesting a polymodal role for stallone in nociception. When stallone (UAS-stallone-RNAi) was specifically knocked down in Class IV neurons using the md-GAL4 driver and UAS-Dicer2, she observed impaired thermal nociception responses at 46°C (Walcott, 2012). Her results suggested that stallone may play a functionally important role in at least one md neurons subtypes in sensing noxious thermal stimuli. The insensitive thermal nociception responses were not observed in RNAi-animals knocking down stallone specifically in the nociceptors (ppk-GAL4; UAS-Dicer2). Since it has been previously reported that Class IV neurons alone are necessary to elicit nocifensive
escape locomotion in response to a noxious temperature (Hwang et al., 2007), it was important to determine whether this documented insensitive phenotype in RNAi mutant animals described above (Walcott, 2012) was an actual phenotype specific to \textit{stallone} and not some indirect or off-target effect of RNAi.

Therefore, to directly test the contribution of \textit{stallone} in noxious thermal responses, I examined my $\Delta$\textit{stallone}$^{81-3}$ genetic null mutant for any defects with our established thermal nociception assay. In this assay I gently stimulated homozygous mutant animals with a probe heated to 46°C and measured the latency to rolling. The $\Delta$\textit{stallone}$^{81-3}$ larvae did not display a statistically significant difference as compared to wildtype \textit{Canton-S} or heterozygous mutant animals (Figure 16). Thus \textit{stallone} is not necessary for general nociception mechanisms, but specifically required for mechanical nociception responses.

\textbf{4.3.4 $\Delta$\textit{stallone} displays increased Channelrhodopsin2-triggered behavior}

Ion channels functioning at the transduction step in Class IV md neurons should not be required for optogenetically induced rolling behaviors and those that function downstream of Channelrhodopsin-2 are predicted to disrupt ChR2-behavior. Indeed previous work on \textit{ppk} demonstrated that \textit{ppk} mutants did not affect ChR2-triggered nocifensive behavior, while mutants targeting voltage gated sodium channels functioning in nociceptor action potential propagation (\textit{para-RNAi}) drastically impaired optogenetic responses (Zhong \textit{et al.}, 2010). To determine if any of the effects of \textit{stallone} on
nociception were due to transduction independent mechanisms, I used the established optogenetic activation of Class IV md nociceptive neurons assay (Hwang et al., 2007; Hwang, 2009; Zhong et al., 2010; Honjo, K. et al., 2012). I lowered the blue light intensity to ensure the system wasn’t saturated upon nociceptor activation and thus lowered the control responses from previously 80% (Hwang, 2009; Zhong et al., 2010; Honjo, K. et al., 2012) to 60% rolling in positive controls (Figure 17). Interestingly, the \( \Delta \)stallone homozygous null mutant behaved in a hypersensitive manner at 78% (Figure 17). This difference was statistically significant when analyzed with a Fisher’s exact test followed with Bonferroni correction. This finding suggests that a hypersensitive process is caused in the \( \Delta \)stallone mutant downstream of ChR2-dependent neuronal depolarization, perhaps in Class IV projections targeting the ventral nerve cord. This result is counter-intuitive as the \( \Delta \)stallone mutants display insensitivity to noxious mechanical stimuli and normal sensitivity to noxious thermal stimuli, yet hypersensitivity to optogenetically triggered nociception behaviors. These opposing phenotypes may suggest a homeostatic response in general nociceptor excitability that results from reduced mechanosensitivity in animals lacking stallone.

4.3.5 Nociceptor morphology is normal in \( \Delta \)stallone\(^{81-3} \) mutants

The impaired mechanical nociception response and increased rolling response observed in stallone mutants could have been a consequence of altered neuronal morphology. One could imagine that \( \Delta \)stallone might alter dendritic field coverage and
might explain the altered sensitivities to noxious mechanical stimuli. Therefore, class IV md neurons were labeled with ppk-GAL4>UAS-mCD8GFP in a ∆stallone^{B1-3} mutant background. No obvious defects were noted (Figure 18).

4.3.6 ∆ppk ∆stallone double mutant shows wildtype mechanical nociception responses

Since ppk is a member of the DEG/ENaC that also shows severe impairment in mechanical nociception behaviors, I was interested in determining whether ppk and stallone play a role in the same mechanical nociception pathway. Epistasis analysis can predict the order in which the gene products function by describing whether one mutant genotype has the capability to supersede the phenotypic effect of another mutation at a distinct locus. Therefore, if these two genes operate in the same pathway, one would predict to see a response similar to either mutant’s phenotype. However, if a more severely defective response is observed, then these two loci genetically interact and suggest a function within the same pathway.

Since genetic null loss-of-function alleles exist for both genes I therefore conducted epistasis analysis on double mutant larvae. When each gene was individually removed, reduced rolling rates were observed in response to a 30mN von Frey filament. Surprisingly, when both genes were removed ∆stallone ∆ppk exhibited wildtype mechanical nociception responses (Figure 19) indicating a suppressive mechanism. The effects of deleting stallone and ppk are less harmful together than either on its own.
4.3.7 Expression of \textit{stallone} remains undetectable

To investigate the expression pattern of \textit{stallone} I recombineered BACs that either had GAL4 or EGFP inserted directly before \textit{stallone’s} start sequence and generated transgenic flies via PhiC-mediated integration generating the following strains: \textit{w}^{1118}, CH321-26K05-\textit{stallone}^{GAL4-VK0001}, \textit{w}^{1118}, CH321-26K05-\textit{EGFP::stallone}^{attp40}. Fixed brains and fileted third-instar larvae \textit{w}^{1118}, CH321-26K05-\textit{EGFP::stallone}^{attp40} were counter labeled with anti-GFP antibodies, yet no discernable expression was detected (data not shown). As this construct was generated to determine the endogenous expression pattern and levels under the control of proper regulatory elements found in the large BAC, it was possible that the lack of GFP detection was due to a low expression of \textit{stallone}.

To circumvent potential complications of a low expressing gene product, the \textit{stallone}^{Gal4} (\textit{w}^{1118}, CH321-26K05- \textit{stallone}^{GAL4-VK0001}) allele was crossed to UAS-\textit{mCD8::GFP}. Larval brain and epidermal tissues were fixed, immunostained with anti-GFP antibodies, and imaged on the confocal microscope. Again, no discernable levels were detected. Recent line constructs developed by the Rubin lab at Janelia farm with 20xUAS and 40x-UAS-\textit{mCD8::GFP} were crossed to \textit{stallone}^{GAL4} transgenic flies. Again brains and epidermal tissue from larval cross progeny were again immunostained for GFP expression without detectable expression (Data not shown, Ken Honjo).
4.3.8 VFP::Stallone distributes broadly in multidendritic neurons

I generated a tagged construct of stallone to determine where the protein localizes at the subcellular level. When expressed in all multidendritic neurons using the md-GAL4 driver, VFP::Stallone distributed to the cell body, dendrites, and axons broadly (Figure 20). This expression pattern overlaps with the mCD8::RFP membrane labeled pattern suggesting VFP::Stallone localizes to the cell surface of neurons.

4.4 Discussion

DEG/ENaCs have previously been documented to play a mechanosensory role in both harsh and gentle touch responses in flies and worms (Chalfie et al., 1990; Driscoll et al., 1991; O'Hagan et al., 2005; Chatzigeorgiou et al., 2010a; Zhong et al., 2010; Tsubouchi et al., 2012). In this chapter I have described a mechanosensory role for a previously uncharacterized DEG/ENaC, CG18110/stallone. Three different null alleles of stallone confirmed decreased mechanical nociception responses when stimulated with a 30mN von Frey fiber when compared to controls. Additionally, complementation analysis with independently derived deficiencies corroborated a role for the stallone locus in mechanical nociception. Furthermore, the neighboring ppk-family genes, ppk20 and ppk19, did not contribute substantially to rolling responses. Combined these data show that the stallone/CG18110 gene is required for mechanical nociception in Drosophila melanogaster. Additionally, defects in gentle touch responses were observed suggesting a more general mechanosensory function of stallone.
In future experiments I would like to show that the phenotype observed in Mi[MIC] insertion is specifically due to stallone interruption by precisely excising the element and looking for reversion to normal responses. Additionally, tissue specific over-expression of a dominant-negative form of stallone would further prove that stallone is acting in the class IV nociceptors.

I would also like to generate a proper genetic rescue construct to definitively prove that stallone is required for mechanical nociception. Such rescue constructs could include a genomic fragment. Previous attempts at integrating trimmed large P[acman] containing the stallone locus via Phi-C-mediated integration failed. A possible solution would be to clone a 10kb genomic fragment into a vector used for standard P element transgenesis. It would also be ideal to show that stallone cDNA can rescue specifically in nociceptive neurons.

How are the phenotypes that I observed at the behavioral level manifested at the cellular level? Utilizing electrophysiology to better understand ion channel properties will help correlate behavior with function at the molecular and neuronal level. Patch-clamp recordings of ∆stallone mutant Class IV sensory neurons may further uncover fine details about general nociceptor excitability which cannot be resolved with current optogenetic techniques. These experiments may aid in dissecting for which part of the mechanical nociception pathway stallone is crucial. It would also be interesting to explore the hypersensitivity in rolling observed in animals with ChR2-activated
nociceptors. This finding may suggest abnormal function at the synaptic terminals that warrants further examination. Two other ppk-family genes are necessary in presynaptic motor neurons for both the acute induction and long-term maintenance of synaptic homeostasis (Younger et al., 2013). Their findings of a DEG/ENaC role in synaptic plasticity may overlap with the observations of optogenetically-induced hypersensitive behavioral responses. Another possibility would be to perform this ChR2 assay at various development stages of larvae to determine whether early activation has long term effects in wildtype animals and whether this is diminished or enhanced in ∆stallone mutant animals. Additionally, I would like to examine the optogenetic activation of nociceptors in the dominant-negative Mi[MIC] mutant to determine whether a truncated protein would have similar effects on the excitability of the neuron as the null mutant shows.

Moreover, if hypersensitive optogenetic nociception behaviors persist then further examination of mutant nociceptor axon terminal projections to the ventral nerve cord would be warranted. Any patterns deviating from wildtype; for example, overextension of terminal axons, could serve as a possible explanation of nociceptor hypersensitivity.

Since expression remains elusive, I would still like to determine the larval expression pattern. Despite my best efforts to determine the endogenous pattern, I was not able to detect a noticeable expression in the peripheral nervous tissue or in the brain.
Perhaps *stallone* isn’t expressed in these tissue types as I have not looked at other tissues. Embryonic *in-situ* hybridization could also elucidate which cells express *stallone* which could direct me to where *stallone* is expressed in larvae and offer a clue as to its function in mechanical nociception. An alternative reason for not being able to detect *stallone* expression levels with previous BAC approaches may be due to non-permissive position effects of the BAC integration site. Therefore generating a CRISPR allele would be the most straightforward method to reveal *stallone*’s expression pattern. However, in the interim there are now new enhancer GAL4 lines provided by the VDRC, called Vienna Tiles (VT-GAL4). One potential *stallone* VT-GAL4 allele shows adult brain expression and brain regions of the developing embryo (brainbaseimp.ac.at and http://enhancers.starklab.org). Therefore, a key experiment would be to check larval brains and PNS for GFP expression using this VT-GAL4 line.

A surprising finding from the *ppk stallone* epistasis analysis was that the double ∆*ppk* ∆*stallone* showed wildtype mechanical nociception responses. Interestingly, a recent study described an increased frequency of paw-withdrawal behaviors to a range of mechanical stimuli and an increased sensitivity of A fiber-mechano-nociceptors (i.e. a slower mean conduction velocity of Aδ-AMs) of ASIC1a/ASIC2/ASIC3 triple knockout mice, suggesting an overall enhanced cutaneous mechanosensitivity (Kang *et al.*, 2012). Therefore, a logical follow-up experiment would be to test whether the ∆*ppk* ∆*stallone* double mutant in fact displays a similar increased nocifensive rolling rate in response to
a range of weaker forces (i.e. look for hypersensitivity). The current mechanical
nociception assay in which larvae are stimulated with a 30mN fiber does not sufficiently
address this question since this force is likely at the maximum threshold of nociceptor
activation. If hypersensitive behavioral responses are confirmed, it would be interesting
to examine the Chr2-triggered behavior and look for hypersensitivity.

Lastly, to further delve into the epistasis results I would like to look at the
physical interactions of PPK and Stallone to first determine whether the wildtype
phenotype observed in double mutant animals reflects heteromeric interactions between
these two subunits. I have generated split-GFP transgenic larvae to test this hypothesis.
Figure 5: *stallone* is a DEG/ENaC gene located in a *ppk*-family rich cluster and shares homology with *ppk*-genes.
Figure 5: *stallone* is a DEG/ENaC gene located in a *ppk*-family rich cluster and shares homology with *ppk*-genes. *stallone/CG18110* was identified in a previously reported forward genetic mechanical nociception screen (Hwang, 2009). This gene belongs to the degenerating/epithelial sodium channel (DEG/ENaC) family. *stallone/CG18110* is flanked closely by other *ppk*-related genes in a tight cluster (*ppk21, ppk20, and ppk19*). General DEG/ENaC protein topology consists of two transmembrane domains, with a large cysteine rich extracellular domain and small intracellular N- and C-terminus domains. Closely related paralogs sharing high amino acid similarity with *stallone/CG18110* belong to two chromosomal clusters *ppk*-channels: 1) *ppk21, ppk20, and ppk19* on the third chromosome (i.e. genes flanking *stallone/CG18110*) and 2) *ppk7* and *ppk14* on the second chromosome (shares synteny with *ppk20* and *stallone/CG18110* structure).
Figure 6: *stallone* and seven other DEG/ENaC genes possess a valine residue at the ‘*deg*’ site.
Figure 6: *stallone* and seven other DEG/ENaC genes possess a valine residue at the ‘*deg*’ site. A) Sequences from the *ppk* channel family were aligned with the ClustalW algorithm for multiple sequence alignments in MacVector software. A highly conserved region at the second transmembrane region is shown. Grey boxing indicates a region displaying amino acid residue conservation. The ‘*deg*’ residue is highlighted in purple and red boxes denote those genes showing a valine variant at the ‘*d’/*deg*’ position, including stallone. The amiloride selectivity region is in yellow and the strongly conserved channel pore sequence of Glycine-X-Serine is highlighted in blue B) Villela et al. (Villella et al., 2009), generated the maximum likelihood phylogenetic gene trees using TreeBeST software. Refer to Villela for further details describing phylogenetic tree building. Gene tree image was taken from the Ensemble genome browser (www.ensembl.org).
Figure 7: Cantonized ∆stallone mutant shows pronounced mechanical nociception defects
Figure 7: Cantonized ∆stallone mutant shows pronounced mechanical nociception defects. Line 20 from HEI-mediated deletion screen detailed in Chapter 3 was backcrossed to the wildtype strain, Canton-S for six generations to remove possible second-site mutations and provide a genetically diverse background (i.e. not isogenized) more representative of natural populations. This Cantonized mutant is referred to as ∆stallone and removes four genes in total: ppk20, stallone/CG18110 (the gene of interest), Obp99a, and ppk19. Canton-S, ∆stallone/+ heterozygotes, and ∆stallone homozygous null mutants were test for mechanical nociception behavior in response to a 30mN probe and subject to statistical analysis with a Fisher’s exact test followed by a Bonferroni correction for multiple comparisons. No statistically significant differences were found between the control genotypes, Canton-S (74.8% response, n=310) and ∆stallone/+ heterozygotes (70.2%, n=205). However, comparing the ∆stallone homozygous genetic null larvae (n=282) to either control genotypes, a highly statistically significant difference was observed (**p<0.0001). Error bars denote ±standard error of the proportion.
Figure 8: Two deficiencies fail to complement the Δstallone mutant
**Figure 8: Two deficiencies fail to complement stallone mutant.** The \(A^{stallone}\) mutant was crossed to two independent deficiency lines: \(w^{1118};Df(3R)ED13102\) and \(w^{1118};Df(3R)BSC574\) for complementation analysis. The larval crossed progeny were genetic null mutants at the \(ppk20\), \(stallone\), \(Obp99a\), and \(ppk19\) locus and heterozygous for any other genes spanning the deficiency. Fisher’s exact test followed with a multiple comparisons Bonferroni correction showed statistically significant differences between controls and the heteroallelic combination of the transheterozygotes \((A^{stallone}/Df(ED13102)\) and \(A^{stallone}/Df(BSC574)\)). N=145 for all experiments, \(*p<0.05\), \(**p<0.001\), ***\(p<0.05\). Error bars denote ±standard error of the proportion.
Figure 9: *Mi(ppk20)* and *Mi(ppk19)* complement the mutant phenotype.
Figure 9: Mi{ppk20} and Mi{ppk19} complement the mutant phenotype.

Δstallone females and Canton-S females were each independently crossed to

y¹w⁶⁷c²³;;Mi(ET1)ppk20⁷⁰⁰ⁱ³⁵² males, w¹¹¹⁸;; Mi(ET1)ppk19⁷⁰⁰⁵³⁸ males y¹w⁶⁷c²³ males, or , w¹¹¹⁸ males. Larval progeny from each genotype were tested: y¹w⁶⁷c²³/+ (n=118),

y¹w⁶⁷c²³/+;;Δstallone/+ (n=129), y¹w⁶⁷c²³/+;; Mi(ET1)ppk20⁷⁰⁰¹³⁵²/+ (n=118),

y¹w⁶⁷c²³/+;;Δstallone/Mi(ET1)ppk20⁷⁰⁰¹³⁵² (n=82), w¹¹¹⁸/+ (n=150), w¹¹¹⁸/+;;Δstallone/+ (n=50),

w¹¹¹⁸/+;; Mi(ET1)ppk19⁷⁰⁰⁵³⁸/+ (n=50), w¹¹¹⁸/+;;Δstallone/Mi(ET1)ppk19⁷⁰⁰⁵³⁸ (n=50). There are no statistically significant differences between controls and transheterozygotes using Fisher’s exact test followed by a Bonferroni correction for multiple comparisons. Error bars denote ±standard error of the proportion.
Figure 10: *Obp99a* does not contribute to impaired mechanical nociception behaviors observed in *Astellone* mutant.
Figure 10: *Obp99a*-RNAi does not impair mechanical nociception behaviors observed. *ppk-GAL4;UAS-Dicer2* females were each independently crossed to: isogenic *w^{1118}* (VDRC isogenic host strain for the GD collection RNAi library), *w^{1118};P(GD763)^{51432}* (VDRC GD RNAi line), *yw^{1118};P(attP,y+,w[3`])* (VDRC integration strain for the KK collection RNAi library), *yw^{1118};P(KK104453)^{VIE-260B}* (VDRC KK RNAi line). There are no statistically significant differences between controls (driver alone or *Obp99a*-RNAi alone) and tissue specific knockdown of *Obp99a* from either RNAi collection using Fisher’s exact test followed by a Bonferroni correction for multiple comparisons. Error bars denote ±standard error of the proportion.
Figure 11: *stallone* is required for gentle touch responses
Figure 11: *stallone* is required for gentle touch responses. *Δstallone* mutants show slightly impaired gentle touch responses. A) Data are presented as averages of summed Kernan gentle touch score across genotypes. Error bars denote + SEM. One way ANOVA followed by Student’s t-test was performed for pairwise comparison to wildtype. B) Data are presented as a histogram of the proportion of larvae across genotypes exhibiting a specific behavioral response (a score ranging from 0-4 on the X-axis) immediately after gentle touch stimulation. Genotypes and sample sizes are as follows: wildtype control, Canton-S (n=80), heterozygotes, *Δstallone/+* (n=72), homozygous null mutant, *Δstallone* (n=80).
Figure 12: Δstellone$^{B1-3}$ mutant allele phenocopies impaired mechanical nociception responses
Figure 12: \( \Delta \text{stallone}^{B1-3} \) mutant allele phenocopies impaired mechanical nociception responses. PCR confirms precise Minos element excision from the \( \Delta \text{stallone} \) mutant to generate the new \( \Delta \text{stallone}^{B1-3} \) allele. Primers targeting \( ppk20 \) (purple arrows) and \( ppk19 \) (green arrows) show amplification of a PCR product in genomic DNA extracted from homozygous (hom) and heterozygous (het) \( \Delta \text{stallone}^{B1-3} \) null animals. Additionally, primers spanning the breakpoint confirm the Minos excision and deletion of that locus. Tubulin primers serve as a positive control for DNA quality. The \( \Delta \text{stallone}^{B1-3} \) allele displays impaired mechanical nociception responses similar to the \( \Delta \text{stallone} \) allele. Compared to wildtype Canton-S (n=47) and heterozygous (n=31) animals, homozygous \( \Delta \text{stallone}^{B1-3} \) mutant larvae (n=42) show statistically significant differences in nocifensive escape behavior when stimulated with a 30mN von Frey fiber. Fisher’s exact test with Bonferroni correction for multiple comparisons (**p<0.001). Error bars denote ± standard error of the proportion.
Figure 13: *stallone* specific null allele phenocopies impaired mechanical nociception behavioral responses observed in *Δstallone* mutant.
Figure 13: *stallone* specific null allele phenocopies impaired mechanical nociception behavioral responses observed in Δ*stallone* mutant. The top schematic shows tools available for generating a *stallone* specific genetic null. The 108kb CH321-60N03 BAC was chosen because it overlaps the region deleted in the Δ*stallone* mutant. The BAC was recombineered to precisely delete the *stallone* gene (from start to stop codon) and transformed into flies at the P[Cary]attP40 site via PhiC-mediated integrase transgenesis. These flies were then used to make a stock precisely deleting *stallone* by crossing it to Δ*stallone* and making a stock with the following genotype: +; CH321-60N03- ΔstaattP40; Δ*stallone*. These flies were then crossed to Δ*stallone*Δ1-3 and tested for mechanical nociception defects with a 30mN probe. The *stallone* specific transheterozygous mutant CH321-60N03-ΔstaattP40; Δ*stallone*/Δ*stallone*Δ1-3 showed statistical significance when compared to the controls (p<0.05). However it did not survive the Bonferroni correction for multiple comparisons, presumably because of small sample size. Error bars denote ± standard error of the proportion.
Figure 14: Mechanical nociception behavioral responses in \(\Delta\)stallone mutant increase with time
Figure 14: Mechanical nociception behavioral responses in \textit{\textless}stallone\textgreater mutant vary with time. The proportion of \textit{\textless}stallone\textgreater mutant larvae exhibiting mechanical nociception behaviors in response to a 30mN von Frey probe with each independent trial plotted on the day in which it was performed. The graphs are displayed as either bar graphs or line graphs of proportionate responses over a period of. Error bars denote ±standard error of the proportion.
Figure 15: Heteroallelic combination of \textit{stallone} mutant alleles exhibit mechanical nociception defects
Figure 15: Heteroallelic combination of *stallone* mutant alleles exhibit mechanical nociception defects. The Mi[MIC] insertion line MI10852 crossed to *Astellone* or *Astellone*\textsuperscript{81-3} flies displayed statistically reduced responses to a 30mN probe when compared to heterozygous *Astellone*/+ or *Astellone*\textsuperscript{81-3}/+ controls using a Fisher’s exact test with a Bonferroni correction (*p< 0.05, **p<0.005). When crossed to Canton-S, the heterozygous Mi[MIC] larvae exhibited a slightly defective response rate suggesting a dominant-negative allele of this insertion. Error bars denote ±standard error of the proportion.
Figure 16: \textit{Astallone}^{B1-3} mutant shows normal 46°C thermal nociception responses
Figure 16: \textit{Astallone}^{B1-3} mutant shows normal 46°C thermal nociception responses. A) \textit{Astallone}^{B1-3} was tested for thermal nociception responses when gently stimulated with a 46°C probe and measured the latency to rolling. A Student’s t-test found that a non-significant difference existed between the average latency to response in \textit{Astallone}^{B1-3} mutant animals when compared to the Canton-S wildtype or heterozygous animals. Error bars denote \pm SEM. B) The histogram of the 46°C latency responses in these genotypes shows a slightly right-shifted distribution.
Figure 17: Effects of Δstallone mutant on optogenetic activation of the nociceptors.
Figure 17: Effects of Astallone mutant on optogenetic activation of the nociceptors.

All genotypes tested contain one copy of ppk-Gal4 UAS-ChR2 line C;UAS-Dicer2 for the control (58.0% response, n=81), Astallone mutant het (58.6% response, n=128), and Astallone homozygous null mutant (77.3% response, n=207). The Y-axis is percent response and X-axis is genotype. Data is presented as a proportion ± standard error of the proportion. Fisher’s exact tests with Bonferroni correction were performed to determine significance. * p<0.001.
Figure 18: Nociceptor morphology is normal in \( \Delta \text{stallone}^{B1-3} \) mutants
Figure 18: Nociceptor morphology is normal in \( \Delta \)stallone\(^{B1-3} \) mutants. Representative confocal micrographs of the dorsal cluster Class IV md neuron in A) third-instar wildtype and in B) DstalloneB1-3 mutant larvae. Larvae are heterozygous for ppk-Gal4 and UAS-mCD8::GFP. (Genotypes are +; ppk-Gal-4 UAS-mCD8::GFP/+; + for the control and +; ppk-Gal-4 UAS-mCD8::GFP/+;\( \Delta \)stallone\(^{B1-3} \) for the mutant.)
Figure 19: Δppk Δstallone double mutant shows wildtype mechanical nociception responses
Figure 19: \(\Delta ppk \Delta stallone\) double mutant shows wildtype mechanical nociception responses. When \(ppk\) and \(stallone\) were individually removed, reduced rolling rates of 42.5% and 42.3% respectively were observed in response to a 30mN von Frey filament. Surprisingly, when both genes were removed \(\Delta stallone \Delta ppk\) exhibited wildtype mechanical nociception responses (71.2%). Statistically significant differences were observed between single and double mutants (**p< 0.0005) using a Fisher’s exact test with a Bonferroni correction for multiple comparisons.
Figure 20: VFP::Stallone distributes broadly in multidendritic neurons
**Figure 20:** VFP::Stallone distributes broadly in multidendritic neurons. Representative confocal micrograph of the dorsal cluster of multidendritic neurons in third-instar larvae. When VFP::Stallone is expressed in all multidendritic neurons using the md-GAL4 driver, VFP::Stallone distributed to the cell body, dendrites, and axons broadly. This expression pattern overlaps with the mCD8::RFP membrane labeled pattern suggesting VFP::Stallone localizes to the cell surface of neurons.
5. Balboa binds PPK in vivo and is required for mechanical nociception in Drosophila larvae

Chapter 5 was modified from a manuscript (of the same title) accepted for publication in Current Biology (in press). The authors were Stephanie E. Mauthner, Richard Y. Hwang, Amanda H. Lewis, Qi Xiao, Asako Tsubouchi, Ken Honjo, Jörg Grandl, and W. Daniel Tracey Jr.

5.1 Introduction

The Drosophila gene pickpocket (ppk) encodes an ion channel subunit of the Degenerin/Epithelial (DEG/ENaC) family (Adams et al., 1998). PPK is specifically expressed in nociceptive neurons and is required for mechanical nociception (Zhong et al., 2010; Kim et al., 2012). In a genome-wide genetic screen for other ion channel subunits required for mechanical nociception we identified a gene that we named balboa (a.k.a. CG8546, ppk26)(Hwang, 2009). The balboa locus encodes a DEG/ENaC ion channel subunit similar in amino acid sequence to PPK (Zelle et al., 2013). Laser-capture isolation of RNA from larval neurons and microarray analyses revealed that balboa is highly expressed in nociceptive neurons. Additionally, a ppk26-GAL4 reporter exhibited exclusive expression in larval nociceptors. Balboa::GFP protein distributed uniformly in the dendrites of Class IV neurons (which also express PPK) but localized to discrete punctae when ectopically expressed in Class I, II, and III multidendritic neurons. Interestingly, the subcellular localization of Balboa::GFP and PPK::Venus proteins were
mutually co-dependent. Ectopic co-expression of ppk in Class I, II, and III neurons transformed the localization of Balboa::GFP to resemble the uniform pattern observed in Class IV neurons. Furthermore, ppk-RNAi in Class IV neurons converted the uniform Balboa::GFP pattern to a punctate distribution. Additionally, balboa-RNAi eliminated PPK::Venus from dendrites. Finally, using a GFP-reconstitution approach in transgenic larvae, we directly detected an in vivo physical interaction among PPK and Balboa subunits. Combined, our results indicate a critical mechanical nociception function for heteromeric PPK and Balboa channels in vivo.

5.2 Materials and methods

5.2.1 Fly Strains and Husbandry

*Drosophila* stocks were raised on standard cornmeal molasses fly food medium at 25°C and 70% humidity on a 12/12 light/dark cycle. Fly stocks for RNAi screening were provided by the Vienna *Drosophila* RNAi Center (Dietzl et al., 2007), and TRiP RNAi lines were provided by the Bloomington *Drosophila* Stock Center. The following fly strains were used: w; ppk1.9-GAL4 (ppk-GAL4) (Ainsley et al., 2003), w;; P{ppk26-GAL4.2.2} (Zelle et al., 2013), w;mCD8::GFP (Lee et al., 1999), w;; UAS-Dicer2 (VDRC strain: 60009), isogenic w¹¹¹⁸ for VDRC RNAi control experiments (VDRC strain: 60000), w; P{GD2350} (UAS-balboa-RNAi) (VDRC strain: 5110), y; P{CaryP}attP2 for TRiP control experiments, y; P{TRiP.JF01843}attP2 (UAS-balboa-RNAi) (Magliery et al., 2005), yw; P{KK104185 } VDRC strain v10883 (UAS-ppk-RNAi), w; UAS-ChR2-eYFP line C (Hwang et al., 2007;
5.2.2 Mechanical Nociception Behavior

The mechanical nociception behavioral tests were performed by stimulating larvae with a calibrated von Frey fiber as previously described (Tracey et al., 2003; Hwang et al., 2007; Caldwell et al., 2010; Zhong et al., 2010; Honjo et al., 2014), with slight modifications.

Further mechanical nociception tests additionally characterizing the balboa mutant were conducted using 50mN (data not shown) and 30mN fibers. A positive response was scored if a larva completed at least one full 360° roll along the long-body axis after the first mechanical stimulus.

5.2.3 Optogenetic Activation of Nociception Behavior

The behavioral tests were performed as described previously (Hwang et al., 2007; Zhong et al.; Honjo, Ken et al., 2012). Larvae were exposed to blue light (460–500 nm) for several seconds using the Hg light source of a Leica MZ16 FA stereomicroscope (14,500 lux). Behavioral responses were videotaped and analyzed offline.

5.2.4 Quantitative Real-Time RT-PCR

For Quantitative Real-Time RT-PCR Experiments, total RNA was collected from 30 3rd instar larvae using an RNeasy kit (Qiagen) according to manufacturer’s specifications followed by RNase-free DNase I treatment (Promega). After reverse
transcription with Oligo-dT primed SuperScript III (Life Technologies), 30ng equivalent cDNA was loaded and amplified using a StepOne Plus Real-Time PCR Platform (ABI/Life Technologies) with specific primers for target *ppk* (Forward 5’CCT GCA CTT CGC TGG AAT ATA A3’, Reverse 5’CGT CCG TAT GCT CGT ATT CTT C3’) and *balboa* (Forward 5’GAC CAA ATG TGG CTG TGT TAA G3’, Reverse 5’CTC ATC CTC GGC TTG GTT ATA G3’) gene sequences. qRT-PCR parameters were as follows: denaturing at 95°C for 3 min, 40 cycles of denaturing at 95°C for 10s and annealing/extension at optimal primer temperatures for 60s. Threshold cycles (Ct) were determined by the Platform system. The target RNA values were computed as 0.5^Ct^. Target *balboa* levels in RNAi and control animals are presented as a ratio to levels detected in the corresponding *ppk* animals. The *ppk* mRNA levels among genotypes are presented as ratios of *bba-RNAi* to driver control and *UAS-bba-RNAi* control.

### 5.2.5 Molecular Cloning

The *pUAST-balboa::GFP* and *pUAST-Venus-balboa* plasmids were generated by PCR amplification from Canton-S genomic DNA using the forward primer 5’-CAC CAT GTC GAC GGA CAG CAC ACA AC-3’ and the reverse primer 5’-TTA GGC ATC CTT GTT GAC CGC C-3’. This genomic PCR fragment was cloned into the pENTR/D-TOPO vector (Invitrogen) and fully sequenced. The desired genomic fragment encoding the *balboa* locus or the was then introduced into the *pTWG* vector(Murphy, 2003) and *pTVW* (Murphy, 2003) of the *Drosophila* Gateway Vector Collection with Clonase (Invitrogen).
For generation pUAS-ppk::Venus entry clones were generated by PCR amplification from the EST clone RE19290 using the forward primer 5'-ATG GCC GAG ATC AGG GAG GA-3' and the reverse primer 5'-CTA GTT CTC AGA TTT TTC CTC TGG TAA ATC 3' followed by subcloning into pTWV destination vectors (Murphy, 2003).

Split-GFP constructs (Wilson et al., 2004) needed to generate NGFP (pET11a-link-NGFP) and CGFP (pMRBAD-link-CGFP) fusions for our experiments were kindly provided by the Regan laboratory. balboa mRNA was extracted from embryos collected 24AEL using Qiagen’s RNeasy kit, reverse transcribed using Oligo(dT) primers (First-strand synthesis kit, Invitrogen) and then PCR amplified with the primers indicated above. ppk cDNA was PCR amplified RE19290 (Stapleton et al., 2002). An overlapping PCR extension approach was used to fuse split NGFP and CGFP sequence to ppk and balboa cDNAs. All split-GFP amplified cDNA products were then cloned into Invitrogen’s pENTR/D-TOPO vector using Clonase (Invitrogen) and fully sequenced. All cDNA sequences encoding the split-GFP constructs were sub-cloned into the pTW (Murphy, 2003). In the NGFP clones nucleotides 3-29 encoded a 6X His tag, nucleotides 30-503 encoded NGFP, nucleotides 504-527 encoded an 8 amino acid linker sequence (GGSGSGSS) (Wilson et al., 2004). In the CGFP clones a 6 amino acid spacer sequence (TSGGSGS) preceded the CGFP sequence. The complete sequences of all constructs are available upon request.
For electrophysiology experiments, the *ppk* cDNA was amplified from the EST clone RE19290 and cloned into pCDNA3.1 and *pTW* (Murphy, 2003). Additionally, the *balboa* cDNA was synthesized *de novo* with codon optimization by GenScript and cloned into pCDNA3.1 and *pTW* (Murphy, 2003).

### 5.2.6 Generation of transgenic flies

Microinjections of *pUAST-balboa::GFP*, *pUAST-Venus::Balboa*, *pUAST-NGFP::Balboa*, *pUAST-balboa::CGFP*, *pUAST-NGFP::ppk*, and *pUAST-PPK::Venus* vectors into *w1118* were performed by Rainbow Transgenic Flies, Inc. and BestGene, Inc. Transgenic flies were generated via a standard *P* element mediated transformation.

### 5.2.7 Confocal imaging

To observe md neurons, late third-instar larvae were lightly anesthetized with di-ethyl ether, mounted in glycerol under a 24x55mm #1.5 cover slip, and imaged with an Apochromat 40X N/A 1.3 oil immersion lens. All confocal micrographs were of the dda group from larval segments A3-A6. Confocal Z-stacks were converted to maximum intensity projections using the Zeiss LSM software package. Negative image conversion and adjustments to brightness and contrast were performed in Adobe Photoshop. Imaging of S2R+ cells was performed on a Zeiss LSM 780 with a 63X/1.4 N.A. oil immersion objective. Laser power was significantly lower for images in A than in B-F. Images are maximum intensity projections of confocal Z-stacks. Images were inverted in Adobe Photoshop. Scale bars= 10µm.
5.2.8 Laser Capture Microdissection and Microarray Analyses

Laser Capture Microdissection and Microarray analyses were performed by the NIH Neuroscience Microarray Consortium (W.D. Tracey and K. Honjo, in preparation). Wandering third instar larvae expressing mCD8::GFP under the control of ppk1.9-GAL4 (Class IV md neurons) or 2-21-GAL4 (Class I md neurons) were embedded in O.C.T. compound (TISSUE-TEK) in Cryomold Biopsy Disposable Vinyl Specimen Molds (TISSUE-TEK). The animals were then frozen in O.C.T. over dry ice and temporarily stored at -80°C until processing. 8 µm cross-sections were made through the larvae with a LEICA CM 1850 Cryostat and mounted on RNAse free silane-prep slides. Slides were fixed for 30 seconds in 75% EtOH and then rinsed in RNase-free water to remove residual O.C.T. Slides were then serially dehydrated (75%, 95%, 100%, 100% EtOH) for 30 seconds and soaked in Xylenes for 10 minutes to remove the EtOH. GFP positive cells underneath the epidermis were identified and captured with the Arcturus Veritas Laser Capture Microdissection System (Molecular Devices) onto CapSure HS LCM caps (ARCTURUS LCM). Approximately 40-50 GFP-labeled cells were collected per sample and resuspended in Picopure RNA extraction buffer (ARCTURUS LCM). Total RNA was then extracted using the Arcturus Picopure kit (ARCTURUS LCM), and treated with the Qiagen RNase-Free DNase Set (Qiagen). The concentration and integrity of each total RNA sample was determined using a spectrophotometer and 2100 Agilent Bioanalyzer RNA 6000 Pico Kit (Agilent Technologies), respectively.
Each total RNA sample was converted to double-stranded cDNA using the GeneChip Expression 3’-Amplification Two-Cycle Target Labeling and Control Reagents Kit (Affymetrix). cDNA was then used for in vitro synthesis of cRNA using the MEGAscript T7 Kit (Ambion, Inc.). cRNA was amplified using random primers and the WT-Ovation Pico Kit (NuGen Technologies), fragmented and biotin-labeled using the FL-Ovation cDNA Biotin Module V2 (NuGen Technologies). Biotin-labeled RNA was then hybridized to Affymetrix GeneChip Drosophila Genome 2.0 Arrays for 16 hours at 45°C. The arrays were washed and stained according to the manufacturer’s recommendations (Affymetrix) using the GeneChips Fluidics Station 450 (Affymetrix). Each array was scanned using the GeneChip Scanner 3000 (Affymetrix) and globally scaled using the Affymetrix GeneChip® Operating Software (GCOS v1.4). Normalization and pair-wise comparison of each gene expression were performed in GeneSpring (Agilent Technologies).

5.2.9 Cell Culture and Electrophysiology

HEK293t cells were seeded at ~50,000 cells/ml in DMEM (Invitrogen) on poly-D-lysine- and laminin-coated glass coverslips (Fisher). After 12h cells were transfected using Fugene (Promega) with (per 1 ml): 1.5 µg Mouse Piezo1-IRES-GFP, 1 µg balboa in pCDNA3.1+ 0.5 µg GFP, 1 µg ppk in pCDNA3.1+ 0.5 µg GFP, or 0.5 µg balboa in pCDNA3.1 + 0.5 µg ppk + 0.5 µg GFP. Recordings were performed 24-48 hours after transfection. S2R+ cells were seeded at ~20,000 cells/ml in Schneider’s Medium + FBS
(Genesee Scientific) on glass coverslips for 12 hours and transfected using 8uL Cellfectin (Life Technologies) in 2ml Schneider’s Medium without FBS for 5 hours with: 0.75 µg Ubiquitin-GAL4 + 0.25 µg dmPiezo in pUAST + 0.25 µg mCD8::GFP in pUAST, or 0.75 µg Ubiquitin-GAL4 + 0.25 µg balboa in pUASTW + 0.25 µg mCD8::GFP in pUAST, or 0.75 µg Ubiquitin-GAL4 + 0.25 µg pppk in pUASTW + 0.25 µg mCD8::GFP in pUAST, or 0.75 µg Ubiquitin-GAL4 + 0.25 µg balboa in pUASTW + 0.25 µg pppk in pUASTW + 0.25 µg mCD8::GFP in pUAST, or Ubiquitin-GAL4 + 0.25 µg mCD8::GFP in pUAST. Media was then supplemented with 2 ml of Schneider’s medium + FBS and recordings were performed 48-72 hours post transfection.

A subset of HEK293t cells were cultured in the presence of 100 µM amiloride, which did not affect current responses. For pressure activation, cell-attached patches were made using a pipette solution (in mM): 130 NaCl, 5 KCl, 10 HEPES, 1 CaCl₂, 1 MgCl₂, 10 TEA-Cl, pH 7.3 with NaOH and a bath solution of (in mM): 140 KCl, 10 HEPES, 1 MgCl₂, 10 glucose, pH 7.3 with KOH. When filled with buffer solution pipettes pulled from borosilicate glass (Sutter) had a resistance of 2-5 MΩ. Pipette pressure was controlled with a high-speed pressure clamp (HSPC-1; ALA Scientific). Cell-attached patches were held at -80 mV and stimulated with a 500 ms pressure step from 0 to -60 mmHg in -10 mmHg increments, with 10 s between successive sweeps to allow for recovery from inactivation. For whole-cell recordings, the pipette solution was (in mM): 133 CsCl, 10 HEPES, 5 EGTA, 1 CaCl₂, 1 MgCl₂, (pH 7.3 with CsOH). The bath
solution was (in mM): 130 NaCl, 3 KCl, 1 MgCl₂, 10 HEPES, 2.5 CaCl₂, and 10 glucose. Cells were held at -100 mV and series resistance (<10 MΩ) was compensated >50%. A fire-polished glass pipette was positioned at an angle of ~80 degrees relative to the coverslip and advanced by a piezo-electric driver (E-625 LVPZT Controller, Physik Instrumente) in 1 µm increments for 250 ms and then retracted to its initial position. The indicated position of 0 µm refers to the step just prior to touching the cell, and 1 µm refers to the first step that touched the cell. 10 s were allowed for recovery from inactivation between stimulations. All recordings were made from gigaseal patches and at room temperature using an EPC10 amplifier and Patchmaster software (HEKA). Currents were sampled at 5 kHz and filtered at 2.9 kHz. Baseline currents before mechanical stimulations were subtracted off-line from the current traces. Data were analyzed with Igor Pro 6.22A (Wavemetrics).

5.3 Results

5.3.1 Balboa is highly expressed in nociceptive neurons and is required for mechanical nociception

Using a set of UAS-mediated RNA interference (RNAi) lines that covers the majority of ion channels in the genome (Tsubouchi et al., 2012), we carried out a tissue-specific RNAi screen to identify channels required for behavioral responses to noxious mechanical force (Hwang, 2009). Noxious force is detected by Drosophila larvae primarily through activation of the nociceptive Class IV multidendritic (md) neurons, but other classes of multidendritic neurons also contribute to noxious force detection.
albeit to a lesser degree (Hwang et al., 2007). Thus, to identify ion channels required for noxious force detection we first crossed our UAS-RNAi collection (Tsubouchi et al., 2012) to the md-GAL4 driver (Tracey et al., 2003), which drives expression in all classes of multidendritic neurons, and then tested the larval progeny for normal or abnormal nocifensive rolling responses to stimulation with a 50 mN von Frey fiber (Tracey et al., 2003). This primary screen yielded defective responses in UAS-RNAi lines targeting nine channel subunits (Hwang, 2009). In parallel studies, we investigated the genome-wide expression levels of mRNAs isolated from Class IV and Class I multidendritic neurons by performing laser capture microdissection of the target fluorescently-labeled neurons from cryosectioned larvae (Supplemental Procedures). The Class IV samples showed high enrichment of ppk, Gr28b, and knot, transcripts that are known to have enriched expression in these cells (Adams et al., 1998; Jinushi-Nakao et al., 2007; Thorne et al., 2008) (Figure 21A). As expected, the transcript for the pan-multidendritic neuron marker multiple (Wang et al., 2011) was seen in both Class IV and Class I samples. Remarkably, we found highly enriched expression in the Class IV neurons (relative to the Class I neurons) for a messenger RNA that encoded one of the ion channel subunits that was identified in our forward genetic screen (CG8546/ppk26) (Figure 21A). In addition to the microarray evidence, a ppk26-GAL4 reporter gene (Zelle et al., 2013) drove expression of UAS-mCD8::GFP exclusively in the Class IV multidendritic neurons (Figure 21B,C). Interestingly, CG8546 is highly similar in amino acid sequence to PPK, a DEG/ENaC
previously shown to be expressed in Class IV neurons that is required for mechanical nociception (Zelle et al., 2013).

Given the highly enriched expression in the nociceptive Class IV neurons, we tested the effects of knocking down CG8546 specifically in these cells. To do so, we crossed the UAS-RNAi lines targeting CG8546 to the Class IV-specific ppk-GAL4 driver. Consistent with the highly enriched Class IV expression pattern that was detected in our microarrays, RNAi targeting of CG8546 with the Class IV driver nearly eliminated the transcript from whole animal RNA isolates (Figure 22A, B). In addition, we found that CG8546 knockdown in the Class IV neurons resulted in profound mechanical nociception defects (Figure 21D). Thus, to reflect this defective mechanical nociception phenotype, we named the gene balboa (in honor of the pain-resistant prize fighter hero-Rocky Balboa) (Hwang, 2009). As previously described for ppk (Zhong et al., 2010), expressing balboa RNAi in the Class IV neurons did not cause a defect in optogenetically-triggered nociception behaviors (Figure 21 E) (Hwang, 2009). Therefore, the effects of balboa-RNAi are unlikely to be explained by a non-specific effect on the intrinsic excitability or general health of the Class IV neurons. Indeed, balboa-RNAi did not cause a noticeable change in Class IV neuron morphology (Figure 22C, D). Given the well-established role for ion channels of this gene family in C. elegans mechanotransduction, an interesting possibility is that Balboa is also involved in force sensing by the Class IV cells.
5.3.2 The subcellular localization of Balboa::GFP varies among multidendritic neuron subtypes

We next investigated the subcellular localization of the Balboa protein by creating transgenic flies expressing a GFP-tagged version of Balboa (UAS-balboa::GFP). When crossed to the md-GAL4 driver, the subcellular localization of Balboa::GFP was observed to differ between the various classes of multidendritic neurons (Figure 23A). Within the Class IV neurons, we observed uniform Balboa::GFP fluorescence throughout the dendritic arbor, but in the Class I, II, and III neurons (where balboa may not normally be expressed) Balboa::GFP localized to bright punctae within the dendritic arbors (Figure 23B). Although these punctae appeared similar to those previously described for expression of MEC-4 in C. elegans mechanosensory neurons (Chelur et al., 2002), it is likely that these ectopic punctae are distinct as they could represent an intermediate stage of Balboa::GFP tracking that is not surface localized or is aggregated. Using drivers specific to different classes of neurons, we confirmed that the uniform distribution of Balboa was due to its presence in the Class IV neurons where it is endogenously expressed (Figure 23C, D) and that the highly punctate distribution was due to its ectopic expression in Class I (Figure 23E, F), II, and III neurons. These distinct patterns for Balboa::GFP localization were unlikely to be a consequence of disruption of the normal protein distribution by the GFP tag, as N-terminally tagged Balboa transgene (UAS-Venus::balboa) showed an identical distribution (Figure 24A). In addition, the uniform pattern that was seen in Class IV neurons was observed even with very low
expression levels of the transgene (Figure 24B) suggesting that the uniform pattern was not an abnormal consequence of a very high expression level.

5.3.3 Ectopic expression of PPK alters the subcellular localization of Balboa::GFP

We reasoned that the distinct subcellular localizations for Balboa::GFP proteins in the different neuronal classes might be due to differential PPK expression among multidendritic neurons. *ppk* is expressed at high levels in the Class IV neurons but it is expressed at low or undetectable levels in the Class I (Figure 21A), II, and III neurons.

Indeed, we found that simultaneous co-expression of *ppk* and *balboa::GFP* resulted in a Balboa::GFP distribution that was essentially uniform in all the multidendritic neurons (Figure 25A-C). These data were strikingly different from what we observed with Balboa::GFP alone (Figure 26A, B). In the absence of additional PPK, the Balboa::GFP foci appeared evenly spaced, like beads on a string (Figure 25D-F). This dramatic change in localization was particularly evident when we specifically examined the pattern within the Class I neurons using the 2-21-GAL4 driver (Figure 25G-K). In the presence of PPK, this Balboa::GFP localization pattern was converted to an even and uniform one where the fluorescence evenly filled the dendrites (Figure 25G-I,K).

If co-expression of PPK and Balboa are indeed necessary for each other’s stability and/or localization then removing PPK or Balboa from the Class IV neurons should result in alterations in the distribution of the cognate partner protein. Indeed, when we tested this prediction through knockdown of *ppk* in the Class IV neurons, the normally
uniform distribution of Balboa::GFP was converted to a strongly punctate distribution (Figure 26A-D). Thus, reducing ppk expression caused a mislocalization of the Balboa::GFP protein to punctae within the Class IV neurons.

In converse experiments, we generated transgenic animals for expression of fluorescently tagged PPK::Venus protein (UAS-ppk::Venus) (Figure 26E). We then used this transgene to test whether PPK localization was Balboa dependent through RNAi knock-down of balboa in the Class IV neurons. This manipulation caused a dramatic reduction in the expression levels of PPK::Venus to the point that it became undetectable in the dendrites but remained weakly present in vesicles of the Class IV cell bodies (Figure 26F). Importantly, balboa-RNAi did not reduce expression of ppk mRNA (Figure 24B) or the strength of ppk-GAL4 expression (Figure 25C,D) indicating that these effects on PPK::Venus were not at the level of transcription or mRNA stability.

5.3.4 Balboa and PPK physically interact in vivo

The above observations provided strong suggestive evidence that PPK protein subunits and Balboa protein subunits may physically interact in vivo to form a functional DEG/ENaC channel. Cells in which Balboa and PPK were co-expressed showed a uniform distribution of Balboa::GFP, whereas cells that expressed only Balboa::GFP displayed the punctate or aggregated distribution pattern. In addition, the expression of dendritic PPK::Venus was also dependent on the presence of Balboa. Nevertheless, it remained possible that these mutually dependent effects were indirect. For example,
expression of PPK and/or Balboa within neurons might have regulated another protein (or proteins) that then in turn caused changes in the distribution of the cognate subunits.

Thus, to test for a direct physical interaction between Balboa protein subunits and PPK protein subunits we utilized the split-GFP technique (Ozawa et al., 2001; Wilson et al., 2004). In this approach, GFP is split into an N-terminal fragment (NGFP) and a C-terminal fragment (CGFP). The cleavage is performed in such a way that neither GFP protein fragment is able to form a radiative fluorophore upon cellular co-expression. However, when the NGFP and the CGFP fragments are co-expressed as fusions to other proteins that physically interact, the two GFP fragments come into sufficiently close proximity to permit reconstitution of GFP fluorescence (Ozawa et al., 2001).

To apply this approach to Balboa and PPK, we generated flies containing UAS transgenes that encoded either a balboa::CGFP transcript, an NGFP::balboa transcript, or an NGFP::ppk transcript. As expected, neither the balboa::CGFP transcript (Figure 27A-C) nor the NGFP::ppk (Figure 27D-F) transcripts produced detectable GFP fluorescence when individually expressed in neurons. However, co-expression of the two transcripts together in all four classes of multidendritic neurons (Figure 26G) or specifically in Class IV neurons (Figure 27H, I) caused clear reconstitution of GFP in the dendrites (Figure 25I), soma, and axon. These results are consistent with a model where the PPK
dependent redistribution of Balboa::GFP observed above is due to direct physical interactions between the proteins.

We next used the split-GFP protein approach to further test for homophilic in vivo interactions among Balboa subunits. Remarkably, upon co-expression of balboa::CGFP and NGFP::balboa in all four classes of multidendritic neurons we observed GFP reconstitution that was restricted to Class IV multidendritic neurons (Figure 26H). In surprising contrast, co-expression of balboa::CGFP and NGFP::balboa did not result in detectable GFP reconstitution in the Class I, II or III multidendritic neuron cell types. These findings further suggest that homophilic interactions among Balboa subunits do occur, but these interactions are confined to the Class IV neurons that also express PPK. Combined with the findings above, that Balboa::GFP distribution in Class IV neurons also depends on PPK, these results suggest that homophilic Balboa interactions are likely to also depend on the presence of PPK. Interestingly, the punctate structures seen with Balboa::GFP in Class I, II, and III neurons are not observed with reconstituted GFP. This may be due to a relatively long maturation time for the split-GFP fluorophore (Magliery et al., 2005) and might indicate that Balboa::GFP present in punctae is less stable relative to the uniformly distributed Balboa::GFP. Alternatively, Balboa proteins in punctae do not form homophilic interactions.

In addition to these in vivo experiments we used the split-GFP approach to further investigate interactions among Balboa and PPK subunits in transiently expressed
S2R+ cells (Figure 28A-F). As in multidendritic neurons, reconstitution of GFP was detected at the plasma membrane with heteromeric channels (NGFP::PPK + Balboa::CGFP). Interestingly, homophilic interactions among PPK subunits (Figure 28C) or Balboa subunits (Figure 28E) were confined to intracellular secretory structures in the absence of the heteromeric partner. In contrast, the hemophilic interactions were clearly detectable at the plasma membrane upon co-transfection with the untagged heteromeric partner (Figure 28D,F). Combined, these data suggest that plasma membrane localization of both Balboa and PPK each depend on the heteromeric partner protein. Furthermore, as both homophilic Balboa and PPK interactions were detected at the plasma membrane, our results suggest that both types of heterotrimeric channel (i.e. both BBA/PPK/BBA and PPK/BBA/PPK) may be formed in S2R+ cells.

Are Balboa and PPK heteromeric channels sufficient to form a mechanosensitive channel? To test for this possibility we co-expressed the subunits heterologously both in HEK293t cells and in Drosophila S2R+ cells (Figure 29A-D). While the known mechanosensitive channel MPiezo1 produced robust currents both when stimulated with a blunt glass pipette (poke) and with negative pressure (stretch), (Figure 29A-C) cells transfected with Balboa, PPK, or with Balboa and PPK together did not exhibit currents above background. Lack of currents from co-expression of ppk and balboa may be explained by the need for other accessory proteins. This is exemplified by observations indicating that the MEC-2 stomatin protein dramatically increased the
currents generated from mutant versions of MEC-4 and MEC-10 in *Xenopus* oocytes (Goodman *et al.*, 2002). Similarly, mammalian stomatin domain proteins also regulate acid sensing ion channels in the DEG/ENaC family (Moshourab *et al.*, 2013).

### 5.4 Discussion

In summary, our study again demonstrates the power of forward genetics in the identification of novel components important in nociception signaling pathways. Converging lines of evidence strongly support the hypothesis that Balboa and PPK physically interact and likely form a functional ion channel *in vivo*. First, both genes are functionally required for mechanical nociception. Second, both genes show very specific expression in the Class IV nociceptor neurons. Third, *ppk* expression causes a dramatic redistribution of Balboa::GFP fluorescence in the multidendritic neurons. Fourth, Balboa::GFP is altered by PPK knockdown and PPK::Venus is dramatically reduced by Balboa knockdown. Fifth, GFP is reconstituted by co-expression of *balboa::CGFP* and *NGFP::ppk* within neurons of the larval peripheral nervous system.

It is noteworthy that although many DEG/ENaCs have been shown to form homotrimeric or heterotrimeric channels (Jasti *et al.*, 2007; Stewart *et al.*, 2011), our findings using the split-GFP approach provide the first direct measurement of such a physical interaction between heteromeric DEG/ENaC subunits *in vivo*. Interestingly, GFP reconstitution measurements of homophilic Balboa interactions were confined to the Class IV neurons. Thus, it is highly likely that these *in vivo* homophilic interactions
depend on the presence of PPK. Indeed, results in S2R+ cells suggest that the presence of PPK is needed for plasma membrane localization of Balboa.

A surprising aspect of our findings is that Balboa::GFP, Venus::Balboa and PPK::Venus show uniform labeling throughout the dendritic arbor of the nociceptive Class IV neurons. This pattern differs from the previously described punctate localization pattern of MEC-4/MEC-10 in C. elegans mechanosensory neurons. Indeed, our results suggest that the uniform dendritic localization pattern for the Balboa::GFP protein is the functionally relevant pattern for mechanical nociception. Perhaps, the uniform pattern might contribute to the high mechanosensory threshold of these cells. Further experimentation will be needed to identify the structures marked by the ectopic punctate pattern seen in Class I, II and III neurons. As Balboa plasma membrane localization depends on the presence of PPK, these structures likely represent an unknown component of the secretory pathway, or sites for protein degradation. To our knowledge, similarly labeled intracellular structures in multidendritic neurons have not been previously observed. Future analyses of these structures may thus provide important insight into the regulation of mechanosensory ion channel biosynthesis.
Figure 21: *balboa* is highly expressed in nociceptive neurons and is required for mechanical nociception.
Figure 21: balboa is highly expressed in nociceptive neurons and is required for mechanical nociception. (A) Affymetrix microarrays performed on mRNA isolated Class IV and Class I neuron cell bodies show high expression of balboa, ppk, Gr28b and knot mRNA in nociceptors. The pan-multidendritic marker multiple is present in both Class I and Class IV neurons. (B) ppk26-GAL4 > UAS-mCD8::GFP exclusively labels the Class IV md neurons (ddaC neuron is shown, scale bar = 50 µm). (C) Within the CNS, ppk26-GAL4 > UAS-mCD8::GFP exclusively labels projections of Class IV neurons and is absent from central neurons (3rd instar ventral nerve cord, dorsal view, scale bar = 20 µm). (D) Knockdown of balboa severely impairs the NEL of larvae stimulated with a 30mN von Frey fiber. The proportion of larvae exhibiting NEL was 67% of control animals without a driver (w/yv; CG8546[01843][n=67]), 59% of control animals with the driver (w/yv; ppk-GAL4/+; UAS-dicer2/attp2[n=64]), and 21% of animals in balboa RNAi targeted to Class IV md neurons (w; ppk-GAL4/+; UAS-dicer2/C8546[01843][n = 62]) (**p < 0.001, Fischer’s Exact test with Bonferroni correction). (E) Knockdown of balboa did not affect ChR2-triggered nociception behavior. Optogenetic rolling was seen in 84% of control animals (w;ppk-GAL4 UAS-ChR2:eYFP Line C/+; UAS-dicer-2/+[n = 111]). Knockdown of balboa did not reduce the frequency of nociception responses to blue light (w; ppk-GAL4 UAS-ChR2:eYFP Line C/+; UAS-dicer-2/G8546GD2350[n = 113, mean = 78%, Fisher’s Exact test p=0.31]). Error bars in A denote ±SEM, and in D,E denote ±SEP.
Figure 22: Experiments on the effects of balboa-RNAi
Figure 22: Experiments on effects of balboa-RNAi. (A) Quantitative Real-Time RT-PCR measurements of balboa mRNA relative to pickpocket mRNA. balboa and pickpocket mRNA was expressed at similar levels in the driver control (ppk-GAL4; UAS-dicer-2 x y v; P{CaryP}attP2) and no driver control (w^{1118} x yv; UAS-balboa-RNAi^{F01843}). In contrast, balboa mRNA was dramatically reduced in crossed progeny of ppk-GAL4; UAS-dicer-2 x yv; UAS-balboa-RNAi^{F01843}. The difference among the means was significant (1 way ANOVA (p<0.000005), (B) The levels of pickpocket were not reduced in balboa-RNAi animals as the expression of ppk mRNA was slightly elevated relative to the driver alone in balboa-RNAi larvae (1.6 +/- 0.24 a.u.). The no driver control larvae (w^{1118} x yv; UAS-balboa-RNAi^{F01843}) showed reduced pickpocket relative to the driver control animals. (C) Confocal micrograph of ddaC neuron showing dendrites of driver control animals (w; ppk-GAL4 UAS-mCD8::GFP; UAS-dicer-2 x y v; P{CaryP}attP2). (D) Confocal micrograph of ddaC neuron showing dendrites of balboa-RNAi animals (w; ppk-GAL4 UAS-mCD8::GFP; UAS-dicer-2 x yv; UAS-balboa-RNAi^{F01843}). Scale bars = 20 µm.
Figure 23: The subcellular localization of Balboa::GFP varies among multidendritic neuron subtypes
Figure 23: The subcellular localization of Balboa::GFP varies among multidendritic neuron subtypes. (A-F) The subcellular distribution of Balboa::GFP in the dorsal cluster of md neurons. Expression of Balboa::GFP under the control of (A, B) md-GAL4 (C,D) ppk-GAL4 and (E,F) 2-21-GAL4 shows a broad, diffuse localization pattern throughout the arbor of Class IV ddaC neurons but a punctate expression pattern in the dendrites and axons of Class I, II, III neurons. (B, D, F) High magnification images of insets of A, C, and E, respectively. Scale bars = 50 µm. In this and all subsequent confocal micrographs, anterior is to the left, dorsal is at the top, and all images are of the dorsal peripheral md-neuron cluster of third instar larvae. Genotypes shown are w; md-GAL4/+; UAS-balboa::GFP/+ (A and B), w; ppk-GAL4/+; UAS-balboa::GFP/+ (C and D), and w; 2-21-GAL4/UAS-balboa::GFP (E and F).
Figure 24: Additional experiments on tagged Balboa proteins *in vivo*
Figure 24: Additional experiments on tagged Balboa proteins in vivo. (A)

Venus::Balboa shows a uniformly distributed pattern throughout the dendrites of the Class IV neurons and a punctate distribution in the Class I, II and III neurons (w; md-GAL4/+; UAS-Venus::balboa/+). (B) The 2-21-GAL4 driver causes weak expression in Class IV neurons yet Balboa::GFP still shows the uniformly distributed pattern in the Class IV dendrites. Scale bars = 20 µm.
Figure 25: Ectopic expression of PPK alters the subcellular localization of Balboa::GFP
Figure 25: Ectopic expression of PPK alters the subcellular localization of Balboa::GFP. (A-C) Balboa::GFP was smoothly distributed throughout the dendritic arbors of all four classes of multidendritic neuron when PPK was ectopically co-expressed under the control of the md-GAL4 driver (w; md-GAL4/+; UAS-balboa::GFP/UAS-ppk). (A) Balboa::GFP channel. (B) Counter-label of the neuronal plasma membrane (mCD8::DsRed). (C) Merge of A and B. (D-F) Expression of Balboa::GFP alone is punctate in Class I and II neurons, although dendrites show normal morphology. (D) Balboa::GFP, (E) mCD8::DsRed, (F) Merge of D and E. (G-I) Co-expression of PPK in Class I and II shows uniformly localized Balboa::GFP expression (G) Balboa::GFP, (H) mCD8::DsRed, (I) Merge of G and H. (J) Ectopically expressed Balboa::GFP in Class I neurons (2-21-GAL4) shows punctate expression. (K) Balboa-GFP with co-expressed PPK under control of 2-21-GAL4. Scale bars for A-C = 50 µm; D-K = 20 µm. Genotypes are (A-I) w; md-GAL4 UAS-mCD8::DsRed/+; UAS-balboa::GFP/UAS-ppk. (J) w; UAS-balboa::GFP/+; 2-21-GAL4/UAS-balboa::GFP. (K) w; UAS-balboa::GFP/+; 2-21-GAL4/UAS-ppk.
Figure 26: Balboa and PPK physically interact in vivo
Figure 26: Balboa and PPK physically interact in vivo. (A) Balboa::GFP shows a broad distribution pattern in dendrites and axons of the Class IV ddaC neuron (boxed area shown in more detail in B). (B) Zoomed in view of the boxed area in A. (C) Balboa::GFP is distributed in discrete bright foci in ppk RNAi animals (Boxed area is shown in more detail in D). (D) Zoomed in view of the boxed area in C. (E) PPK::Venus is distributed uniformly in the dendrites of Class IV neurons (arrow indicates cell body) (F) balboa-RNAi eliminates PPK::Venus from dendrites of Class IV neurons but the cell body remains weakly visible (arrow). (G) GFP is reconstituted in the dendrites of Class I-IV neurons when NGFP::PPK and Balboa::CGFP are co-expressed. (H) When Balboa::CGFP and NGFP::Balboa are co-expressed in Class I-IV neurons reconstitution of GFP is limited to the Class IV neurons. Scale bars are 50 µm. Genotypes were w;ppk-GAL4 UAS-dicer2/+; UAS-balboa::GFP/+ (A and B), w;ppk-GAL4 UAS-dicer2/+; UAS-balboa::GFP/ppkKK104185 (C and D), w;ppk-GAL4 UAS-dicer-2/ UAS-PPK::Venus (E), w;ppk-GAL4 UAS-dicer-2/ UAS-PPK::Venus; UAS-balboa-RNAi{F01843}/+ (F), and w;md-GAL4/+;UAS-NGFP::ppk/UAS-balboa::CGFP (G), w;md-GAL4/+; UAS-balboa::CGFP/UAS-NGFP::Balboa(H).
Figure 27: Additional experiments on split-GFP Proteins
Figure 27: Additional experiments on split-GFP Proteins. (A-H) Controls for Split-GFP tagged protein experiments. (A-C) Balboa::CGFP does not fluoresce on its own when expressed under the control of the $md$-$GAL$ driver ($w; md$-$GAL4$, $UAS$-$mCD8$-$::$DsRed$+$/+; 
$UAS$-$balboa$-$::$CGFP$+$/+$). (A) Balboa::CGFP channel (B) Counter label of the neuronal plasma membrane ($mCD8$::DsRed) (C) Merge of A and B. (D-F) NGFP::Pickpocket does not fluoresce on its own when expressed under the control of the $md$-$GAL$ driver ($w; md$-$GAL4$, $UAS$-$mCD8$-$::$DsRed$+$/+; $UAS$-$NGFP$-$::$ppk$+$/+$). (D) NGFP::Pickpocket channel (E) Counter label of the neuronal plasma membrane ($mCD8$::DsRed) (F) Merge of D and E. (G) Balboa::CGFP and NGFP::Pickpocket show reconstitution of GFP in Class IV neurons ($w; ppk$-$GAL4$+$/+; UAS$-$balboa$-$::$CGFP$/$UAS$-$NGFP$-$::$balboa$). (H) Zoomed in view of GFP reconstitution in distal dendrites of the segmental boundary. Scale bars = 20 mm.
Figure 28: Heteromeric Balboa/PPK channels localize to the plasma membrane of S2R+ cells but homomeric channels do not
Figure 28: Heteromeric Balboa/PPK channels localize to the plasma membrane of
S2R+ cells but homomeric channels do not. (A) The fluorescent marker mCD8::GFP shows the typical plasma membrane morphology of Drosophila S2R+ cells. This morphology is easily apparent in cells that are spread out against the cover glass and is characterized by many fine filopodia-like protrusions. Intracellular vesicles are also darkly labeled with this marker. (B) Heteromeric Balboa/Ppk channels labeled by reconstitution of GFP (NGFP::PPK + BBA::CGFP) are clearly localized to the plasma membrane of S2R+ cells as seen by comparison to mCD8::GFP. (C) Homomeric PPK channels labeled by GFP reconstitution (NGFP::PPK + PPK::CGFP) are confined to the perinuclear secretory pathway. The cellular morphology that is typically seen with plasma membrane markers in S2R+ cells is clearly absent. (D) Homophilic interactions between PPK subunits (NGFP::PPK + PPK::CGFP) are localized to the plasma membrane in the presence of co-transfected BBA. (E) Homomeric BBA channels are confined to the perinuclear secretory pathway (NGFP::BBA + BBA::CGFP). The plasma membrane is not labeled. (F) Homophilic interactions between BBA subunits (NGFP::BBA + BBA::CGFP) are localized to the plasma membrane in the presence of co-transfected PPK.
Figure 29: Heterologous expression and electrophysiological recordings of PPK and Balboa
Figure 29: Heterologous expression and electrophysiological recordings of PPK and Balboa. (A) Left: Representative currents from cell-attached recordings of HEK293t cells transfected with Mouse Piezo 1, Balboa, PPK, Balboa and PPK or pcDNA. Recordings were performed at -80 mV and cells were stimulated with negative pressure (0 to -60 mmHg) in the patch-pipette. Right: Mean peak inward current in response to negative pressure pulse. N=10 (pcDNA, MPiezo1, PPK), n=14 (Balboa), n=9 (Balboa+PPK). Error bars are ± SEM. (B) Left: Representative currents from cell-attached recordings of S2R+ cells transfected with Mouse Piezo 1, Balboa, PPK, Balboa and PPK or UAS-mCD8::GFP. Recordings were performed at -80 mV and cells were stimulated with negative pressure (0 to -60 mmHg) in the patch-pipette. Right: Mean peak inward current in response to negative pressure pulse. N=6 (UAS-GFP), n= 9 (DmPiezo), n=10 (Balboa), n=9 (PPK), n=10 (Balboa+PPK). Error bars are +/- SEM. (C) Left: Representative currents from whole-cell recordings of HEK293t cells transfected with Mouse Piezo 1, Balboa, PPK, Balboa and PPK or pcDNA. Recordings were performed at -100 mv and cells were stimulated by probing with a fire-polished glass pipette. The indicated position of 0 µm refers to the step just prior to touching the cell, and 1 µm refers to the first step that touched the cell. Right: Mean peak inward current in response to the stimulus. N=8 (pcDNA), n=12 (MPiezo1), n=9 (Balboa), n=10 (PPK; Balboa+PPK). Error bars are +/- SEM. (D) Left: Representative currents from whole-cell recordings of S2R+ cells transfected with Balboa and PPK or UAS-GFP. Cells were held at -100 mV for 50 ms.
**Figure 29 continued:** before applying a ramp from -100 mV to +100 mV at 1 mV/ms, then held at +100 mV for 50 ms. Right, current at -100 mV and +100 mV for each cell and mean values. For each recording, currents from segments of 30 ms at +/-100 mV were averaged. N=8 (UAS-GFP), n=10 (Balboa+PPK). Error bars are +/- SEM.
6. Thesis summary and future directions

6.1 Conclusions and Future Directions

Understanding the pathways underlying mechanotransduction has remained a major challenge in sensory neurobiology. The anatomy and physiological properties of somatosensory neurons that relay mechanosensory messages have been extensively characterized across many organisms but the actual transduction mechanisms remain a mystery. By uncovering new molecular components that function in mechanosensory neurons we can begin to dissect how this complicated and elusive process might work.

Specific somatosensory afferent neurons—nociceptors—are selectively tuned to rapidly respond to significantly intense mechanical, thermal, or chemical stimuli that may potentially cause tissue damage and transmit this information via the spinal cord and brain for further processing as pain. However, the process by which nociceptors discriminate various stimuli and their intensity remains unclear. Much work has shown that numerous types of ion channels play a considerable role in granting nociceptors their highly specialized sensory capabilities and are also important for the progression of nociception signaling.

To further explore mechanisms relying on distinct ion channels required for high-threshold mechanical nociception responses, we selected *Drosophila* as our model system for study. *Drosophila* larvae are an ideal system to examine nociception signaling mechanisms as they have polymodal nociceptors (Class IV multidendritic neurons),
elicit a unique and stereotyped behavior in response to noxious stimuli (>30mN force or >39°C temperature), have readily available tools for neurogenetic studies of nociception behaviors, and many of their genes are evolutionarily conserved. An in vivo, forward tissue specific RNAi screen was employed to reduce expression of all known and predicted ion channels specifically in the Class IV multidendritic neurons and identify genes required for mechanical nociception but not for thermal nociception. To identify key factors in transducing nociceptive stimuli, candidates showing no changes in optogenetically activated nociception responses were selected for study. This led to the identification of two uncharacterized genes, CG18110 and CG8546, and based on their insensitive mechanical nociception phenotype, were named stallone and balboa respectively in honor of the pain-resistant prize fighter hero-Rocky Balboa played by Sylvester Stallone (Hwang, 2009). These genes belong to an evolutionarily conserved protein family known as the degenerin/epithelial sodium channels (DEG/ENaCs) that have been shown in other invertebrate systems to have a mechanosensory function (Chalfie et al., 1989; Driscoll et al., 1991; O'Hagan et al., 2005; Chatzigeorgiou et al., 2010a). Therefore, my research has focused on demonstrating the role these two DEG/ENaC genes, stallone and balboa, play in the specific modality of somatosensation, mechanical nociception.

During the initial stages of my dissertation project I focused my efforts on characterizing stallone. I first generated a null allele of stallone to validate the mechanical
nociception impairments observed in \textit{stallone}-RNAi mutants. Since there were no available genetic tools to easily generate a \textit{stallone} mutant —such as overlapping deficiencies, insertional mutagenic alleles, neighboring \textit{P} elements PiggyBac elements for local hopping or FLP/FRT mediated deletions—I turned to the newly available collection \textit{Minos} elements insertions (Metaxakis \textit{et al.}, 2005; Bellen \textit{et al.}, 2011), in which two Mi\{ET1\} alleles inserted in neighboring genes flanking \textit{stallone} were available. Other groups (Parks \textit{et al.}, 2004) had generated many deficiencies with fairly defined breakpoints using an HEI-based approach with two \textit{P} elements \textit{in trans}. Therefore to determine whether this method could be used with the new \textit{Minos} element collection I applied this technique as outlined in Chapter 3 using the two Mi\{ET1\} aforementioned alleles. By strategically using visible markers to track recombinant chromosomes inherited from male founders after HEI, I was able to select for candidates with potential deletions. Excitingly, multiple lines showed a deletion at the \textit{stallone} locus and one mutant line was extensively characterized in Chapter 4 for \textit{stallone’s} role in mechanical nociception.

Not only did the \textit{Minos}-mediated HEI method produce a genetic mutant for my project, it also documents and details the first male recombination events that occurred as a result of mispaired hybrid \textit{Minos}-elements. This suggests that \textit{Minos} and \textit{P} elements employ similar mechanisms for during ‘faulty’ HEI transposition, and provides further insight into general transposition biology.
In Chapter 4, I generated and rigorously analyzed the mechanical nociception phenotype of my ∆stallone mutant. This was important to extend the RNAi results from the previous mechanical nociception screen and validate our screening approach. Interestingly, stallone may have a general mechanosensory function as gentle touch responses were mildly reduced in the knockout mutant. Additionally this allele was used to confirm that behavioral defects were not a mere consequence of morphological defects in the nociceptors. Rescue alleles are still needed to confirm stallone defect and ideally, rescue would be targeted specifically to the nociceptors using the GAL4/UAS system. Additional follow-up experiments include determining stallone’s endogenous expression pattern. Recent release of the Vienna-Tiles offers a resource to potentially determine tissue-specific expression. However the most-straightforward approach that truly recapitulates spatiotemporal expression of stallone uses CRISPR to insert a native tag like GFP or GAL4/LexA sequence at the endogenous locus.

One interesting finding to follow-up would be to explore the potential function of the valine at the ‘deg’ residue in Stallone proteins by performing a whole-cell and single channel electrophysiology recordings in Xenopus oocytes. These approaches would allow for further characterization of ion selectivity mechanisms and give insight into general gating properties to determine whether this subunit is a functional part of the ion channel (i.e. not an accessory subunit). Additionally, electrophysiology would determine whether this valine residue is part of the pore domain and whether mutating
the valine to serine, glycine, or alanine (typically the amino acid found at this position) will have an effect on any of these properties. Perhaps Stallone’s ‘dig’ valine residue gives the DEG/ENaC complex its unique properties that may align with mechanical properties of the nociceptors. This would be especially novel if true.

Experiments testing for protein-protein interactions between Stallone and PPK are warranted as epistasis analysis in the double Appk Δstallone mutant background gave surprising results: wildtype mechanical nociception responses. It would be interesting to test whether these two subunits physically interact and thus act in the same pathway, or if the suppression-like phenotype is due to parallel pathways. I have generated the Split-GFP constructs necessary for these experiments.

For the second part of my project, I focused on balboa’s role in mechanical nociception. Previous studies in our lab performing laser capture microdissection of mRNA from Class IV and Class I found high enrichment of balboa in comparative microarray analyses (Hwang, 2009). This corroborated recent work exhibiting exclusive Class IV multidendritic neuron expression of a CG8546 promoter fusion reporter. Removing balboa did not have an effect on morphology and thus excludes balboa serving as some structural role in the nociceptors. To determine the subcellular localization of Balboa and PPK, tagged constructs were generated to visualize their distribution within the Class IV cells. I demonstrated that Balboa and PPK require each other for proper localization within the Class IV md neurons. An interesting observation from ectopic
Balboa::GFP expression in non-nociceptive multidendritic neurons (Class I, II, and III neurons), are the structures marked by the punctae. As Balboa plasma membrane localization depends on the presence of PPK, these structures likely represent an unknown component of the secretory pathway, or sites for protein degradation. To our knowledge, similarly labeled intracellular structures in multidendritic neurons have not been previously observed. Future analyses of these structures may thus provide important insight into the regulation of mechanosensory ion channel biosynthesis.

Additional findings from Split-GFP experiments I conducted, showed reconstitution of GFP by co-expressing balboa::CGFP and NGFP::ppk within neurons of the larval peripheral nervous system. This result definitively confirms that Balboa and PPK physically interact, presumably in a heteromeric ion channel complex. I have recently generated a deletion allele of balboa using CRISPR targeting methods. This mutant will be essential for epistasis analysis. While Chapter 5 clearly shows that Balboa and Ppk proteins physically interact in the nociceptors, it will be interested to test double ∆balboa ∆stallone mutants and ultimately triple knockout animals removing all three balboa, ppk, and stallone genes.

Overall, I concentrated my efforts on two genes that were discovered in an unbiased forward, genetic screen to identify genes required for mechanical nociception in Drosophila melanogaster: CG18110/stallone and CG8546/CG8546. I demonstrated that these two DEG/ENaC genes are required for mechanical nociception in Drosophila larvae,
the first documented functional role of these two genes. Another *Drosophila* DEG/ENaC gene—*ppk*—is required for sensing noxious mechanical stimuli in the Class IV multidendritic neurons, making *stallone* and *balboa* the second and third DEG/ENaC reported to function in high-threshold mechanosensory mechanisms in *Drosophila* larvae. Importantly, the above genes aren’t required for general processing of noxious stimuli as the impaired nociception behavioral responses observed were specific to mechanical forces and not noxious thermal stimuli. Moreover, these three DEG/ENaC subunits (or combination) may therefore come together to form a functional nociception channel.

The experiments outlined in this doctoral thesis have contributed to the overall field of mechanosensation by uncovering two novel genes encoding DEG/ENaCs that are functionally required for animal nociception behavioral responses to noxious mechanical force but not noxious thermal stimuli. Additionally these subunits shed light on the stoichiometry of multimeric pain-sensing complexes. This discovery also opens the door for potential, pharmacological targets useful in the treatment of pain associated with many human conditions and diseases.
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Biography

Stephanie Mauthner was born on July 22, 1983 in Gainesville, Florida. She attended the University of Florida from 2001-2005 and received her Bachelor of Science (B.S.) in Molecular Biology and Biochemistry. She was awarded highest honors for her thesis work on “In vivo and in vitro identification of molecular domains utilized by arrestin during dark adaptation in rod photoreceptors” and thus graduated Summa Cum Laude.

In the fall of 2006 she entered the Ph.D. program, University Program in Genetics and Genomics (UPGG) at Duke University and joined the laboratory of Dan Tracey in 2009. Stephanie has presented posters on her thesis work at the following conferences: Neurobiology of Drosophila Meeting, Cold Spring Harbor Laboratory, NY (October 2011); Behavioral Neurogenetics of Drosophila larva: the Maggot Meeting Series. HHMI Janelia Farm Research Campus, VA (October 2012); Behavioral Sensory Signaling in Model Organisms Meeting. HHMI Janelia Farm Research Campus, VA (April 2013); Neurobiology of Drosophila Meeting, Cold Spring Harbor Laboratory, NY (October 2013); The 55th Annual Drosophila Research Conference in San Diego, CA (March 2014); Sensory Transduction Symposium, Marine Biological Laboratory in Woods Hole, MA (September 2014).

She has given oral presentations at two internationally recognized conferences: the 53rd Annual Drosophila Research Conference in Chicago, IL (March 2012) and the
Force transduction and Emerging Ion Channels Conference at the Max Delbrück Communications Center in Berlin, Germany (May 2012).

Stephanie has one first-author paper in press at Current Biology titled, “Balboa (PPK-26) binds to Pickpocket in vivo and is required for mechanical nociception in Drosophila larvae” (Accepted October 10, 2014).