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Pickpocket is a DEG/ENaC protein required for mechanical nociception in *Drosophila* larvae

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Summary

Highly branched Class IV multidendritic sensory neurons of the *Drosophila* larva function as polymodal nociceptors that are necessary for behavioral responses to noxious heat (>39°C) or noxious mechanical (>30 mN) stimuli. However, the molecular mechanisms that allow these cells to detect both heat and force are unknown. Here, we report that the *pickpocket(ppk)* gene, which encodes a Degenerin/ Epithelial Sodium Channel (DEG/ENaC) subunit, is required for mechanical nociception but not thermal nociception in these sensory cells. Larvae mutant for *pickpocket* show greatly reduced nociception behaviors in response to harsh mechanical stimuli. However, *pickpocket* mutants display normal behavioral responses to gentle touch. Tissue specific knockdown of *pickpocket* in nociceptors phenocopies the mechanical nociception impairment without causing defects in thermal nociception behavior. Finally, optogenetically-triggered nociception behavior is unaffected by *pickpocket* RNAi which indicates that *ppk* is not generally required for the excitability of the nociceptors. Interestingly, DEG/ENaCs are known to play a critical role in detecting gentle touch stimuli in *C. elegans* and have also been implicated in some aspects of harsh touch sensation in mammals. Our results suggest that neurons which detect harsh touch in *Drosophila* utilize similar mechanosensory molecules.

Results and Discussion

Two overlapping deficiencies remove *pickpocket* and *elB*

We have previously found that *ppk* expressing sensory neurons (figure 1A,B) (Class IV multidendritic (md-da)neurons) are required for behavioral responses to harsh (noxious) mechanical stimuli [1]. The *pickpocket* locus is located on the left arm of chromosome 2 at the cytological position 35B1. *ppk* is flanked by *elbow B* (*elB*), a transcription factor involved in tracheal development [2], and *spel1*, a gene involved in DNA mismatch repair [3] (Figure 2A). Two overlapping deficiencies, *Df(2L)b88h49* and *Df(2L)A400*, have been

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shown [4] to overlap and to completely remove *ppk*. Thus transheterozygotes for these deficiencies are DNA nulls for the *ppk* gene. It was previously reported that the 44kb overlapping deficiency specifically removed *ppk;* leaving *spel1* and *elB* intact [4]. However, the *ppk* containing interval between *spel1* and *elB* is only 22kb suggesting that at least one other gene (*spel1* or *elB*) must be removed if these deficiencies overlap by 44kb (Figure 2A). Therefore we examined this interval first to confirm the absence of *ppk*, but also to test which other genes are removed in the transheterozygous combination. Polymerase chain reaction amplification of genomic DNA from the *Df(2L)b88h49/Df(2L)A400* genotype indicated that two genes, *ppk* and *elB*, are removed in the transheterozygote (Figure 2B). Therefore, for the purpose of this discussion, we will refer to transheterozygous larvae of this genotype as *ppk elB*$^{A400/B88}$.

Mechanical nociception responses of *ppk* mutants

Given the nociceptive function of *ppk* expressing neurons [1] combined with the known role of DEG/ENaC proteins in *C. elegans* mechanotransduction, we tested the *ppk elB*$^{A400/B88}$ mutants for defects in mechanical nociception. To examine mechanical nociception behavior, *ppk elB*$^{A400/B88}$ mutant larvae were exposed to mechanical stimuli delivered by 50 mN Von Frey fibers on the dorsal midline at approximately abdominal segment four (Supplemental Video 1). Following such a stimulus, greater than 75% of wild type larvae responded with nocifensive escape locomotion in which the larvae rotate around their long body axis (Supplemental Video 1). Similarly, both *Df(2L)b88h49*/+ and *Df(2L)A400*/+ showed robust nocifensive responses (Figure 3A). In contrast, the *ppk elB*$^{A400/B88}$ mutant larvae showed a significant reduction in nocifensive responses to the noxious mechanical stimuli (Figure 3A) and responded with nocifensive behavior in only 27% of the trials. Notably the mutant larvae were not completely unresponsive to the noxious mechanical stimulus. Instead of nocifensive responses the mutant larvae often inappropriately displayed behaviors that resembled the wild type responses to gentle touch. Indeed, behavioral responses to gentle touch were normal in *ppk elB*$^{A400/B88}$ mutant animals (Figure S1).

elbowB mutants complement mechanical nociception phenotypes

Although *ppk elB*$^{A400/B88}$ mutant larvae were defective for mechanical nociceptive behavior, it was possible that the mutant defect was due to the absence of *elB* rather than absence of *ppk*. *elB* is a zinc-finger containing protein that is involved in several developmental processes. Expression of *elB* has been detected in a subset of tracheal trunks and *elB* mutants develop aberrant migration of dorsal and lateral tracheal branches in embryos [2]. In addition, *elB* is expressed in the leg, wing, eye, and head primordia where it is involved in appendage formation via the repression of body wall genes [5]. Finally, *elB* plays a role in the regulation of the size of the eye-head primordium [6]. Although *elB*'s expression has not been found in larval nociceptors, an effect on nociception remained possible. In order to test this, we performed complementation tests with three existing alleles of *elB* (*elB*^{3}, *elB*^{6}, *elB*^{8}) and *Df(2L)b88h49*. The *elB*^{6} allele is caused by a chromosomal inversion (*In(2LR)elB*^{6}) with a breakpoint in the first intron of the *elB* locus, but the molecular lesions of the ethyl methanesulfonate induced *elB*^{3} and the γ-ray induced *elB*^{8} alleles are unknown. Nevertheless, the three *elB* alleles complemented the defective mechanical nociception phenotype when placed over *Df(2L)b88h49* (Figure 3B) indicating that the nociception defects of *ppk elB*$^{A400/B88}$ mutant larvae are unlikely to be a consequence of removing *elB*.

RNAi knockdown of *pickpocket* in Class IV multidendritic neurons

In order to further determine whether the mechanical nociception defect observed in *ppk elB*$^{A400/B88}$ larvae was due to the removal of *ppk*, we used the GAL4/*UAS* system [7] to drive tissue specific expression of *ppk-RNAi* in neurons that express *ppk* [8]. With this approach the *UAS* driven *ppk-RNAi* is only expressed in cells that express the *ppk-GAL4*

driver. This allowed us to test if the site of action for *pickpocket* in mechanical nociception is in the same cells that express GAL4 in the *ppk-GAL4* driver line.

With a single copy of *ppk-GAL4* expression of *UAS* transgenes is driven only in Class IV multidendritic neurons (Figure 1A). Indeed, there was a reduction in the larval responses to harsh mechanical stimulation when the RNAi knockdown was performed using a single copy of the *ppk-Gal4* driver and a single copy of the *UAS-ppk-RNAi* (*w; ppk-GAL4*/+; *UAS-ppk-RNAi*/+) (Figure 3C). This result implicates the Class IV neurons as playing a *pickpocket* dependant role in mechanical nociception. However, the reduction of nociception behavior of this genotype was not as severe as in the DNA null allele. The less severe phenotype seen in *w; ppk-GAL4*/+; *UAS-ppk-RNAi*/+ could be a consequence of incomplete knockdown of *pickpocket* in this genotype. To test this possibility, we increased the dosage of RNAi expression by increasing the copy number of the GAL4 driver and *UAS* transgenes that were used in the experiment.

With two copies of the *ppk-GAL4* transgene, *UAS* reporter transgenes are driven in both the Class III and Class IV multidendritic neurons (Figure 1B). We thus used a homozygous driver strain to test whether expanding expression of *ppk-RNAi* to include both Class III and Class IV multidendritic neurons would phenocopy the null mutant situation. Indeed, the defect in mechanical nociception observed in the *w*; *ppk-GAL4; UAS-ppk-RNAi* strain was nearly as severe as what we observed in the *ppk elB*$^{A400/B88}$ null background (Figure 3A,D). The stronger phenotype seen with RNAi knockdown using the homozygous *ppk-GAL4* driver may be due to more highly effective knockdown of *ppk* in the nociceptive Class IV md-da neurons with increased transgene dosage. Alternatively, it is also possible that expression of RNAi in Class III neurons with the homozygous *ppk-GAL4* driver strain (Figure 1B) contributes to the severity of the phenotype. Consistent with the latter possibility, we have previously shown that genetic silencing of Class II and III multidendritic neurons causes a mild impairment in mechanical nociception behavior [3]. Thus, it is possible that expression of *pickpocket* in both Class III as well is in the Class IV multidendritic neurons contributes to its function in mechanical nociception responses.

These data showing the strong phenocopy of *ppk elB*$^{A400/B88}$ mechanical nociception defects produced by *ppk* RNAi strongly support the hypothesis that the phenotype of *ppk elB*$^{A400/B88}$ mutants is due to the absence of *ppk*. Furthermore, these tissue-specific RNAi experiments indicate that the site of action for *ppk* is in the Class IV (and possibly the Class III) multidendritic neurons which are specifically targeted by the *ppk-GAL4* driver line.

Optogenetic activation of Class IV neurons *pickpocket* RNAi

Although mechanical nociception was severely impaired by expression of *ppk-RNAi* in nociceptive neurons thermal nociception behavior was unaffected by RNAi knockdown of the gene (Figure S2 F,G). In addition, analysis of *ppk elB*$^{A400/B88}$ mutant larvae did not clearly implicate *ppk* in thermal nociception pathways (Figure S2 A–C). The specific requirement for *ppk* in mechanical nociception behavior suggests a possible role for *ppk* at the mechanotransduction step in the polymodal nociceptive sensory neurons. To further test the possibility that Pickpocket might be required for the general excitability of these neurons we utilized optogenetic activation of the Class IV cells. We have previously shown that expression of Channelrhodopsin-2YFP (ChR2::eYFP) under control of *ppk-GAL4* can be used to activate Class IV md-da neurons with blue light and trigger nocifensive escape locomotion [1]. Blue light activation of the larval nociceptors via ChR2::eYFP should therefore bypass mechanotransduction, but it should still depend on factors that control the general excitability of the neuron. Thus, if *ppk* has a non-specific or general excitability function in Class IV multidendritic neurons then the blue light triggered escape locomotion should be impaired by *ppk* knockdown. For example, the *para* gene encodes a NaV type

sodium channel that is essential for action potential propagation in *Drosophila* [9, 10] and *para* is required in Class IV multidendritic neurons for ChR2::eYFP triggered nociception behavior (Figure 4). In control transgenic larvae that expressed ChR2::eYFP in nociceptors, escape locomotion was seen in response to 81% of blue light pulses and this was reduced to only 4% when *para-RNAi* was targeted to the same cells that expressed ChR2::eYFP (Figure 4). Thus, results of knockdown of *para* showed that optogenetic activation of nociception behavior depends on the generation of action potentials in the nociceptive neurons and upon factors generally involved in neuronal excitability. In contrast, knockdown of *ppk* did not affect the ChR2::eYFP triggered nocifensive escape locomotion (Figure 4). These data indicate that *ppk* is not simply required for the general activation of the multidendritic neurons. Instead, combined with the results described above, our results suggest a specific mechanosensory function for *pickpocket* in larval nociceptors.

In summary, we have shown that the *ppk* gene is required for mechanical nociception in *Drosophila* larvae. The *ppk* gene is specifically expressed in these cells and genetic null mutants of *ppk* showed severely impaired mechanical nociception behavior. In addition, RNAi knock-down of *ppk* gene expression in nociceptive neurons phenocopied this defect. However, RNAi knockdown of *ppk* did not result in a defect in thermal nociception and also had no effect on optogenetic activation of these cells.

The situation for *ppk* is distinct from that of the *painless* gene which is required for both thermal and mechanical nociception responses [11]. In heterologous cells, Painless currents can be activated by heat but not by osmotic pressure [12]. This supports a direct role for Painless in the transduction of thermal nociception stimuli but a direct role as a mechanosensor remains unproven. Interestingly, heat activated Painless currents are strongly affected by intracellular calcium ions. This sensitivity to calcium suggests that the function of Painless in mechanical nociception pathways could feasibly be downstream of Pickpocket. For example, if Pickpocket functions as a direct mechanosensor, calcium influx through downstream voltage gated channels might indirectly sensitize Painless currents. In this model, the function of Painless would serve as an amplifier of mechanically gated Pickpocket currents.

Although our results implicate a specific role for Pickpocket in mechanosensory function, a role for Pickpocket itself as a subunit of a mechanotransduction channel remains unproven. The ultimate proof of this hypothesis would require the detection of mechanically gated currents in a biochemically reconstituted system involving purified Pickpocket protein. However, expression of Pickpocket in heterologous cells has not been found to produce currents [13] and the absence of currents by heterologously Pickpocket proteins likely indicates that additional factors present *in vivo* are needed for the function of this channel subunit [13]. Indeed, co-expression of MEC-4 and MEC-10 is required for expression of these channels in heterologous expression systems [14]; it is thus likely that another DEG/ENaC subunit in *Drosophila* is required for Pickpocket to form a functional channel. In future studies, using the mechanical nociception assay described here, it should be possible to identify additional *Drosophila* DEG/ENaC subunits that are specifically required for mechanical signaling in the multidendritic neurons. These would represent candidates for the additional subunits that may be required for the formation of a functional Pickpocket channel.

Nevertheless, given the specificity of the phenotype, a model where Pickpocket functions as a component of a mechanotransduction complex seems likely especially given the well established functional role of DEG/ENaCs in the *C. elegans* mechanotransduction complex [14–21]. The sub-cellular localization of the Pickpocket protein is found in discrete

varicosities on the dendrites [13] which is reminiscent of the punctate localization of the mechanotransduction complex of the touch receptor neuron processes in *C. elegans* [20].

Although the mechanical nociception stimulus used in our assays seems qualitatively distinct from the gentle touch stimulus used in *C. elegans* studies our results suggest that the molecular machinery involved may be similar. This is somewhat surprising since the *mec-4* and *mec-10* genes of *C. elegans* are required for gentle touch responses in *C. elegans* [14,22,23], but *mec-4* and *mec-10* mutants show normal behavioral responses to harsh touch. This may suggest the existence of additional mechanotransduction pathways in the *C. elegans* touch neurons, or alternatively *C. elegans* may utilize distinct high threshold mechanosensory neurons. Consistent with the former possibility calcium responses to harsh touch in MEC-4 expressing neurons are still observed in *mec-4* mutant ALM neurons [24]. Consistent with the latter possibility worms with laser ablated gentle touch neurons still show behavioral responses to harsh touch [25]. Indeed, although *mec-10* itself is not required for harsh touch responses, it has been proposed that harsh touch detection in the worm may be mediated by high-threshold PVD neurons which express MEC-10[25]. Interestingly, mice that are mutant for the *ASIC3* DEG/ENaC channel show reduced sensitivity to noxious pinch [26]. Thus, it is possible that the function of Pickpocket in *Drosophila* neurons could be similar to that of ASIC3 in mouse nociceptive neurons.

It is clear that distinct mechanosensory pathways are likely to exist within organisms. In addition to the role of Pickpocket in harsh touch responses, gentle touch to the body wall of the *Drosophila* larva is thought to be detected by ciliated chordotonal neurons [27,28] which utilize the TRP channels Nomp-C[29], Iav, and Nan [30] for mechanotransduction[31,32]. Similarly in *C.elegans*, distinct mechanosensory neurons rely on distinct signaling mechanisms [33,34].

Our identification of Pickpocket as a potential mechanotransducer in the *Drosophila* nociceptive neurons suggests a widespread and evolutionarily conserved role for DEG/ENaCs in neurosensory mechanotransduction. Furthermore, the *pickpocket* mutant phenotype genetically separates mechanical nociception from thermal nociception in *Drosophila*.

Experimental Procedures

Fly Strains and husbandry

The following fly strains were used: *w; pickpocket1.9-GAL4* (*ppk-GAL4*), *w; UAS-ppk-RNAi* (III) [8]. *Df(2L)A400 T(2;3;4)CA4* / *Cy*O *P{ActGFP}JMR1, Df(2L)b88h49*/ *Cy*O P{ActGFP}JMR1, w^{1118} ;P{*UAS-dicer-2, w*[+]} (*UAS-dicer-2* on III, VDRC stock number 60009), w^{1118};P{*UAS-para-RNAi*} (VDRC stock number 6132), elB^{8}. Transheterozygous larvae (*Df(2L)A400T(2;3;4) / Df(2L)b88h49*) were identified as GFP negative progeny from the cross *Df(2L)A400 T(2;3;4) / CyO P{ActGFP}JMR1 X Df(2L)b88h49*/*CyO P{ActGFP}JMR1*. *elB* mutant larvae were identified as GFP negative progeny from the cross of *elB[3,6 or 8]* / *CyO P{ActGFP}JMR1* X *Df(2L)b88h49*/ *CyO P{ActGFP}JMR1*. Channelrhodopsin experiments were performed with the YFP positive progeny from the cross of *ppk-Gal4 UAS-ChR2::eYFP* /*Cy*O; *UAS-dicer-2* to males of the corresponding *UAS-RNAi* line or to an isogenic w^{1118} control (VDRC stock number 60000). Flies were raised on standard cornmeal medium on a 12hour light dark cycle. All stocks were maintained at 25°C and 75% humidity.

Behavioral Assays

The thermal nociception behavioral tests were performed as described previously [1,11]. The mechanical nociception assays were performed as described previously, with slight

modifications[1,11]. Wandering third-instar larvae were stimulated with a 50mN calibrated Von Frey filament. Omniflex monofilament fishing line Shakespeare (6 lb test, diameter 0.009 inch [0.23 mm]) was cut to a length of 18 mm and attached to a glass pipette such that 8 mm of the fiber protruded from the end of the pipet and 10 mm anchored the fiber. Von Frey fibers were calibrated by using them to depress a balance until the fishing line was seen to bend. The force (in grams) was recorded and converted to millinewtons by multiplying the measured grams by a factor of 9.8. Noxious mechanical stimuli were delivered by rapidly depressing the larva with the fiber on the dorsal side. The stimulus was delivered and released as quickly as possible. The quick release allows the larvae unrestrained freedom to perform escape locomotion behavior. The stimulus was delivered to abdominal segments four, five, or six. A positive response was scored if at least one 360 degree revolution around the A/P axis occurred in response to the mechanical stimulus. Each larva was tested only once.

For each genotype, three to seven trials were performed. For each trial, five vials of crosses were established using six virgin females and three males. These vials were kept at 25°C, 75% humidity and a 12 hour light/dark cycle. Third instar wondering larvae were tested in behavioral assays on the sixth, seventh and eighth day after setting up the crosses. The results of tests on the animals from the five vials were pooled with the goal of obtaining a sample size of approximately 30 larvae for a given trial. The averaged results from the trials for a given genotype were used to obtain a behavioral score and to generate the standard error of the mean. Statistical analyses were performed using the Microsoft Excel one-way Analysis of Variance (ANOVA) tool. In experiments where the between group ANOVA reported a significant p-value post hoc pair-wise comparisons of the means were performed using Sheffe's test.

The optogenetic activation of *ppk-GAL4* neurons was performed as described previously[1]. However, the strain utilized here (*ppk-Gal4 UAS-ChR2::eYFP* /*Cy*O; *UAS-dicer-2*) harbored the *UAS-dicer-2* trangene which enhances RNAi[35]. When crossed to the *UAS-ppkRNAi* strain only half of the progeny inherit the *ppk-Gal4 UAS-ChR2::eYFP* chromosome. These larvae were identified following exposure to blue light by visualizing their YFP fluorescence. The YFP negative (*Cy*O) larvae were used as in internal negative control since they never produce nocifensive escape locomotion in response to blue light.

Molecular biology

Genomic DNA was extracted from adult flies using QIAGEN DNeasy Tissue Kit. The following primers were used to amplify ppk and its neighboring genes. *Spel:* forward 5' - TCA AGC AAC TGG ACC TAA ACC G - 3', reverse 5' - GTT ATC TCG TGA AAA TGA GTG GCG - 3'. *ppk:* forward 5' - AGC ACG ACC ATT CAC GGC ATA C - 3' reverse 5' - CCA AAG TTC ACT CAC TGG GCA TC - 3'; *elB* C-terminus: forward 5' - CGC AAT ACG ACG CTT TTC CG - 3', reverse 5' - TTG ATA GTT CCC CCT GTG AGG TC - 3'; *elB* : Forward primer 5' - AGC AGT CAT TCA TCC AGC GTT AG - 3', reverse primer 5' - TTC ATT CGG GCG TAG TTC AGA TAC - 3'; *Pburs* : forward primer 5' - CAA CCA TCG GTG ATA ACG CC – 3', reverse primer 5' - TCG CCA CAT TTG AAG CAC TTG - 3'.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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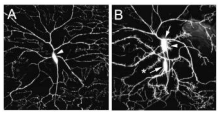

Figure 1.

Expression of *pickpocket* in multidendritic neurons (*ppk-GAL4* driving *UAS-mCD8::GFP*). Confocal micrographs of dorsal cluster peripheral neurons in a third instar larva. (A) When heterozygous for *ppk-GAL4* and heterozygous for *UAS-mCD8::GFP* the driver directed target gene expression solely in Class IV multidendritic neurons (arrowhead indicates the ddaC neuron). (B) When homozygous, the *ppk-GAL4* driver targeted both Class IV (arrowhead indicates ddaC) and Class III multidendritic neurons (arrows indicate ddaF and ddaA). The Class III neurons were unambiguously identified by spine like protrusions from the dendrites (asterisk).

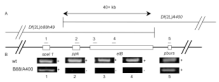

Figure 2.
Genomic region containing the *pickpocket* gene. (A) Schematic representation of *Df(2L)B88h49* and *Df(2L)A400*. The deleted region in the transheterozygous deficiency genotype is indicated by a double arrow (as determined by results Polymerase Chain Reaction (panel B)). The results indicated that *Df(2L)B88h49/ Df(2L)A400* animals are deficient for both *pickpocket* and *elbow B*. (B) Results of PCR on genomic DNA from wildtype (wt) *or Df(2L)B88h49/Df(2L)A400* (B88/A400). On the distal side of *pickpocket*, *spel1* was not deleted. On the proximal side, *pburs* was present. Within the overlap of the deficiencies, *pickpocket* and *elbow B* were both deleted.

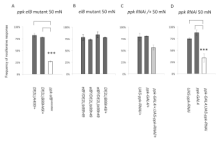

Figure 3.

pickpocket is required for mechanical nociception (A) Response of deficiency lines (*Df(2L)A400*/+ (5 trials, n=181), *Df(2L)b88h49*/+ (5 trials, n=186), and *Df(2L)A400/Df(2L)B88h49* (*ppk* null mutant, 3 trials, n=156)). *Df(2L)A400/Df(2L)B88h49* animals showed a severe reduction in nocifensive responses ($p<0.001$ (for both one-way ANOVA and Sheffe's post-hoc test). (B) Nociception responses in *elB* mutants and control (elB^3/*Df(2L)B88h49* (3 trials, n=86) , elB^6/*Df(2L)B88h49*(3 trials, n=122), elB^8/*Df(2L)B88h49* (3 trials, n=98) and *Df(2L)B88h49*/+ (5 trials, n=181)) ($p=0.39$ one-way ANOVA). (C) Nociception responses with heterozygous *ppk-RNAi* strains (*UAS-ppk-RNAi*/+ (3 trials, n=92), *ppk-Gal4*/+ (3 trials, n=132) and *ppk-GAL4*/+; *UAS-ppk-RNAi*/+ (3 trials, n=223)). Although a statistically significant difference was found among the groups ($p<0.05$ one-way ANOVA) Sheffe's post-hoc analysis did not detect a significant difference among the means in pair-wise comparisons. (D) Nociception behavior with homozygous *ppk-RNAi* strains (*UAS-ppk-RNA*i (7 trials, n=415), *ppk-GAL4* (5 trials, n=219), *ppk-GAL4*; *UAS-ppk-RNAi* (3 trials, n=96)). ($p<0.001$ (for both one-way ANOVA and Sheffe's post-hoc test).

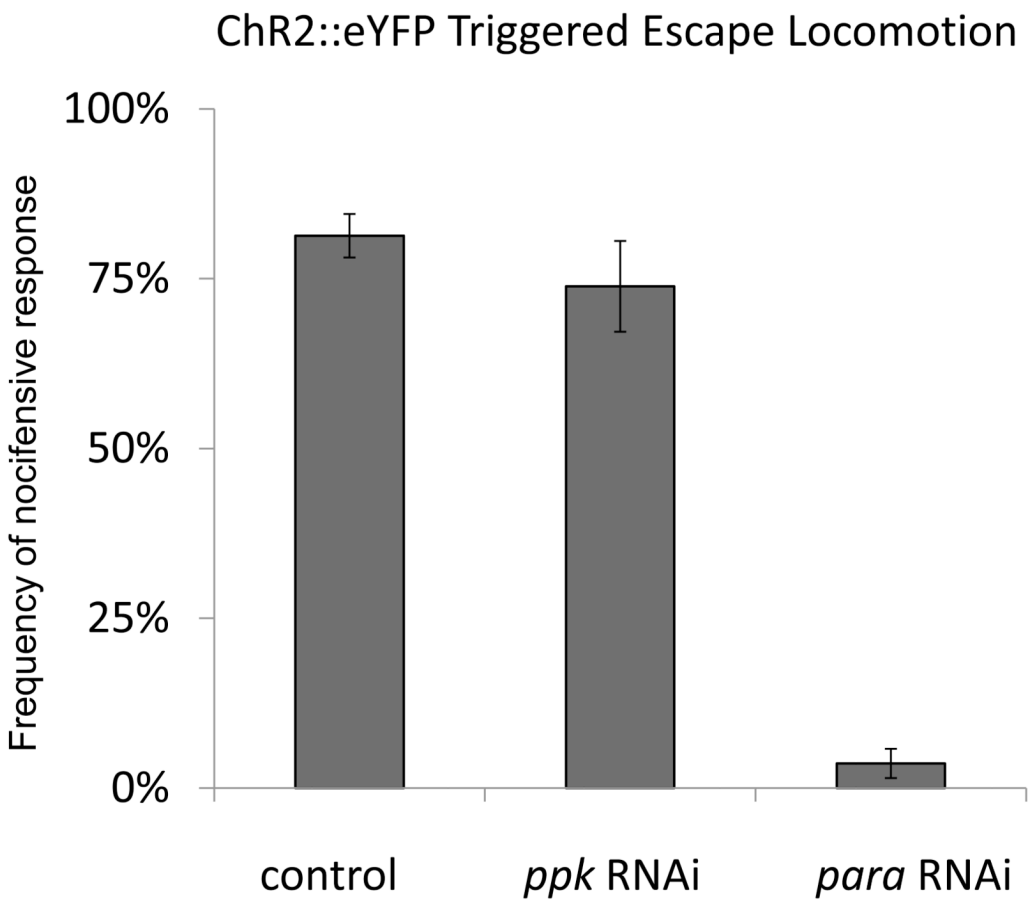

Figure 4.

Knockdown of *ppk* did not affect ChR2 triggered nocifensive behavior. Blue light triggered activation of nociception behavior was seen in 81%(SEM 3%) of animals of the Control optogenetic activation genotype (*ppk-GAL4 UAS-ChR2::eYFP* Line C / +, *UAS-dicer-2*/+ (8 trials, n=245)). Knockdown of *ppk* did not significantly reduce the frequency of escape locomotion in response to blue light (note that *UAS-dicer-2* [35]was utilized in this experiment to enhance the effectiveness of RNAi) (*ppk-GAL4, UAS-ChR2::eYFP* Line C / *UAS-ppk-RNAi* ; *UAS-dicer-2* / +(3 trials, n=103) ((74% SEM 7%) t-test relative to control, p=0.32). In contrast, knockdown of *para* dramatically reduced the response to blue light (*ppk-GAL4 UAS-ChR2::eYFP* Line C / *UAS-para-RNAi* ; *UAS-dicer-2* / +, 4 trials, n=115).