ppk23-Dependent Chemosensory Functions Contribute to Courtship Behavior in Drosophila melanogaster

Beika Lu¹, Angela LaMora¹, Yishan Sun²✉, Michael J. Welsh³, Yehuda Ben-Shahar¹

¹ Department of Biology, Washington University, St. Louis, Missouri, United States of America, ² Neuroscience Graduate Program, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States of America, ³ Howard Hughes Medical Institute, Departments of Internal Medicine, Molecular Physiology, and Biophysics, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States of America

Abstract

Insects utilize diverse families of ion channels to respond to environmental cues and control mating, feeding, and the response to threats. Although degenerin/epithelial sodium channels (DEG/ENaC) represent one of the largest families of ion channels in Drosophila melanogaster, the physiological functions of these proteins are still poorly understood. We found that the DEG/ENaC channel ppk23 is expressed in a subpopulation of sexually dimorphic gustatory-like chemosensory bristles that are distinct from those expressing feeding-related gustatory receptors. Disrupting ppk23 or inhibiting activity of ppk23-expressing neurons did not alter gustatory responses. Instead, blocking ppk23-positive neurons or mutating the ppk23 gene delayed the initiation and reduced the intensity of male courtship. Furthermore, mutations in ppk23 altered the behavioral response of males to the female-specific aphrodisiac pheromone 7(Z), 11(Z)-Heptacosadiene. Together, these data indicate that ppk23 and the cells expressing it play an important role in the peripheral sensory system that determines sexual behavior in Drosophila.

Introduction

Animals have evolved a variety of mechanisms to monitor their chemical environment and to guide their behavior, many of which involve ion channels. For example, in vertebrates and insects, several members of the Transient Receptor Potential (TRP) channel family act as chemosensors for noxious stimuli [1]. Furthermore, insect olfactory receptors likely function as ligand-gated ion channels [2,3]. Recently, a novel family of variant glutamate ionotropic receptors was also identified to contribute to olfactory functions in Drosophila melanogaster [4]. This diversity of ion channels suggested that additional ion channels might also play important chemosensory roles.

Additional ion channels that could potentially contribute to chemosensory-driven behaviors are the degenerin/epithelial Na⁺ channels (DEG/ENaC) [5]. DEG/ENaC genes are animal specific and various subunits are expressed in both the peripheral and central nervous systems of invertebrates and vertebrates. DEG/ENaC channels are activated by diverse ligands including protons (acid-sensing ion channels) [5] and the peptide FMRFamide in Helix aspersa (FaNaCh channels) [6]. They also contribute to salt sensation [7,8], mechanosensation [9], and nociception [10]. However, the ligands and physiological function of the majority of DEG/ENaC family members remain unknown.

The Drosophila genome encodes 31 DEG/ENaC proteins, called Pickpocket (ppk) channels [7]. This is a greater number of DEG/ENaC genes than for any other currently sequenced animal genome, including other insects such as the honey bee, which encodes only five DEG/ENaC subunits [11]. The observations that some DEG/ENaC channels function as receptors for extracellular signals and that some are expressed in chemosensory organs in Drosophila and other species [7], led us to hypothesize that multiple members of the DEG/ENaC family might contribute to chemosensory–driven behaviors in insects.

To test this hypothesis, we used a genetic approach and expression analyses to discover chemosensory specific DEG/ENaC subunits and their possible contributions to behaviors. We have identified pickpocket 23 (ppk23), a member of the DEG/ENaC family, as a gene that is enriched in gustatory sensory neurons that are part of the sex circuit in flies. We demonstrate that ppk23 and the cells expressing it play a role in normal male-female courtship behavior but not feeding.

Results

Identifying potential chemosensory DEG/ENaC channel transcripts

The Drosophila melanogaster Pox neuro (Poxn) mutation causes a developmental fate switch of adult chemosensory bristles into pure mechanosensory bristles [12,13,14]. We reasoned that comparing gene expression in the sensory-rich appendages of wild-type and Poxn adults would reveal novel genes involved in chemosensation.
We primarily focused on members of the DEG/ENaC family, which in flies represents one of the largest ion channel families in the fly genome [7,15]. We used real-time quantitative RT-PCR assays to determine whether the Poxn mutation altered the abundance of several DEG/ENaC and sensory TRP channel transcripts. We also examined the chemosensory-related gene CleB12a as a positive control (Figure 1A). Compared to the wild type, expression of ppk23 and ppk7 were markedly reduced in Poxn appendages, suggesting that the function of these genes is associated with chemosensory bristles. Several other DEG/ENaC subunits and several TRP channels showed a mildly increased expression in Poxn appendages suggesting that they may contribute to mechanosensation. lounge lizard (lz, also called ppk25), which has been shown to be associated with chemosensory functions [16,17,18], showed a very mild reduction in expression that was not statistically significant, likely due to the small sample size used in our screen. In contrast, the dramatic reduction in ppk23 expression in the Poxn genetic background, more than any of the other DEG/ENaC subunits we have looked at thus far, led to our current focus on the role of this specific channel in chemosensory functions. Consistently, northern blot analyses designed to detect all predicted ppk23 transcripts (See Figure S1A for probe design) showed that ppk23 expression was highly enriched in appendages (legs and wings) relative to heads or bodies of both males and females (Figure 1B). Real-time quantitative RT-PCR assays also confirmed these results (Figure S1B).

The ppk23 locus is on the X chromosome and predicted to produce three different transcripts from a single transcriptional start site (FlyBase.org; Figure S1A). A northern blot analysis identified only a single major transcript (Figure 1B). RT-PCR coupled with 5′ and 3′ RACE protocols identified this major transcript as a novel ppk23 transcript (ppk23-RX; NCBI accession number HM026485), (Figure S1A). The northern blot analysis also identified two minor bands. We currently do not know their identity, and whether they represent additional minor ppk23 transcripts or whether this is a non-specific signal. We also have identified an insertional hypomorphic allele of ppk23 ([16,17,18], which has been shown to be associated with chemosensory functions). Consistently, northern blot analyses designed to detect all predicted ppk23 transcripts (See Figure S1A for probe design) showed that ppk23 expression was highly enriched in appendages (legs and wings) relative to heads or bodies of both males and females (Figure 1B). Real-time quantitative RT-PCR assays also confirmed these results (Figure S1B).

To assess the spatial distribution of ppk23 expression, we used the ppk23 gene promoter linked to Gal4 to drive a UAS-nuclear GFP reporter in transgenic flies [19]. We found ppk23 expression enriched in adult appendages (Figure 1C, Figure S1D). When we expressed two copies of UAS-mCD8-GFP by two copies of ppk23 Gal4, a faint signal in few cells in the labellum was detected as well (data not shown), but not in any other sensory structures. We observed similar expression patterns of ppk23 in chemosensory neurons in three independent insertions of the same transgenic construct (data not shown). Expression of a nuclear GFP reporter in legs using the ppk23-GAL4 strain indicated that although the promoter was active in all legs (Figure S1D), expression in the forelegs of males showed significantly more ppk23-positive cells than females (Figure 1C and 1D). Since male forelegs play an important sensory role during courtship [20], these data suggested that the ppk23 locus might be playing a role in sexual behaviors in flies.

ppk23 is enriched in gustatory-like neurons

Higher resolution analyses of membrane-tethered GFP expression in the male foreleg and wing suggested that ppk23-positive cells are chemosensory neurons as evidenced by the projection of their cilium to the base of chemosensory bristles, which were identified by the lack of a bract and a thin, curved morphology [13,21] (Figure 2A and 2B). We also found that the ppk23 promoter is active in pairs of sensory neurons with similar morphology that projected to single bristles (Figure 2A and 2B). Because chemosensory bristles contain at most one pure mechanosensitive neuron with distinct morphology [22], this further suggested that ppk23-positive neurons are chemosensory.

Projections of ppk23-positive neurons in the foreleg are sexually dimorphic and overlap with fru

The chemosensory system in flies plays an important role in regulating social behaviors such as courtship [23]. In addition, recent work indicated that at least some gustatory receptor neurons in fly appendages express the sex-determination gene fruitless (fru) [24,25], and have sexually dimorphic axonal projection patterns to the CNS [26]. These data, in combination with the enrichment of ppk23-positive cells in the forelegs of males relative to females, led us to hypothesize that ppk23 and the cells expressing it might contribute to sex-related behaviors.

We first studied the axonal projection patterns of ppk23-positive neurons by expressing membrane-tethered GFP using a ppk23-GAL4 line and examined GFP patterns in the brain and the thoracic ganglion (Figure 3A and 3B). We did not observe ppk23-positive cell bodies in the brains or the thoracic ganglia of either males or females as evidenced by the lack of positive neuronal cell bodies. In contrast, we were able to clearly observe the axonal projection patterns of ppk23-positive sensory neurons in the thoracic ganglion as well as weak signal in the subesophageal ganglion. We could not resolve whether these axons represent a small population of labellar sensory neurons or extension of axons that originated in appendages. The axonal projection patterns of ppk23-positive sensory neurons from forelegs to the thoracic ganglion were sexually dimorphic, similar to the projections of male specific Poxn-positive gustatory neurons, which require fruM expression for their correct wiring (Figure 3C) [26]; ppk23-positive neurons originating in the male foreleg showed higher incidence of crossing the thoracic ganglion midline relative to females (Figure 3A and 3B respectively). The physiological significance of the midline crossing is still unknown.

The possible overlap of ppk23 expression with the sex circuit was further supported by the co-localization of the ppk23 promoter with the fru” promoter, which is exclusively expressed in the sex circuit (Figure 3C) [24,25,26,27,28,29]. Together, these data suggested that the gustatory sensory system includes a subpopulation of sensory neurons in forelegs that express both fru and ppk23 and is probably not involved in feeding-related taste functions [30].
Figure 1. *ppk23* expression is reduced in appendages of the *Poxn* mutant. (A) Real-time quantitative RT-PCR analysis of total RNA extracted from adult appendages (legs and wings) from a mixed sex population. Analysis compared *Poxn*<sup>642-83</sup>, which do not have external chemosensory bristles, and *CyO* balanced siblings, which develop normal sensory bristles [54]. For illustrative purposes, data are represented as relative expression fold differences in *Poxn* flies relative to controls [51]. Each data point includes the relative expression of a gene in *Poxn* homozygous flies relative to balanced *Poxn/CyO* flies, which develop normal sensory system. *CheB42a* gene was used as positive controls for chemosensory specific genes expressed in appendages [16,17,55]. Statistical analyses were performed on the ΔCt data as previously described [16,17,51]. *, p<0.05; **, p<0.01 (n = 4 per genotype, one-tail paired t-test) (B) Northern blot analysis of *ppk23* spatial expression patterns. Lower panel shows ribosomal bands on the RNA gel, indicating equal sample loading. Using RT-PCR coupled with 5′ and 3′ RACE analyses on RNA extracted from male appendages, we were
ppk23-expressing cells play a role in normal male courtship behavior

The evidence that ppk23 expression is sexually dimorphic suggested the hypothesis that ppk23-expressing cells play a sensory role in courtship behavior. To test this hypothesis, we blocked neuronal activity in ppk23-expressing cells with ectopic expression of TNT using the UAS-GAL4 system. Inhibiting ppk23-expressing neurons increased the proportion of males that failed to demonstrate courtship behavior (Figure 4A). In males that did court, inhibiting ppk23-expressing neurons significantly delayed the initiation of courtship when males were exposed to wild-type virgin females (i.e., courtship latency increased, Figure 4B). The courtship index, which is the proportion of time males spent courting in 10 minutes, also fell (Figure 4C).

ppk23 contributes to normal male courtship

We also asked whether the ppk23 gene was important for normal courtship by utilizing the inserional hypomorphic allele ppk23PB (Figure S1A). Similarly to the TNT-blocking results, a high proportion of ppk23PB mutant males did not exhibit obvious courtship behaviors in 10 minutes (Figure 5A). The ppk23PB mutant males that did court had significantly longer courtship latency and a reduced courtship index towards wild-type mature females (Figure 5B, 5C). The effects of the ppk23 mutation on male courtship were specific to male-female interactions since we did not observe statistically significant effects of the mutations on naturally occurring male-male courtship interactions (Figure 5A–5C).

We also tested the courtship responses of ppk23mutant flies under normal white (room) light conditions. Under these conditions, more than 90% of the males of both genotypes showed courtship behaviors towards virgin female targets (Figure 5D) without an observable gene effect on courtship latency (Figure 5E). In contrast to latency, ppk23mutant flies still exhibited a reduced courtship index in comparison to wild type controls even under room light conditions (Figure 5F). No effects were observed on male-male courtship under these light conditions (Figure 5D–5F). In further support of the specific role of ppk23 in courtship behavior, excision of the ppk23 piggyBac insertion completely reverted the mutant courtship phenotype to wild type levels for both latency and index (Figure 5G–5I).

As additional genetic support for the specific role of ppk23 in male courtship behavior, we utilized recently published complete deletion alleles of ppk23 (Δppk23) and ppk28 (Δppk28) in courtship assays. These deletion lines were generated independently of the ppk23PB allele [31]. In agreement with ppk23PB data, males

![Figure 2. ppk23 is expressed in adult chemosensory receptor neurons.](https://www.plosgenetics.org/doi/fig/10.1371/journal.pgen.1002587.g002)
carrying the null ppk23 allele (Δppk23 in CS background) showed comparable effects with significantly longer courtship latency and reduced courtship index (Figure 6A–6C). In contrast, deletion of the ppk28 locus, a DEG/ENaC subunit that is enriched in gustatory neurons implicated in water sensing in flies [31,32], had no impact on male courtship behavior (Figure 6A–6C). Furthermore, overexpressing ppk23 cDNA in ppk23-expressing cells was sufficient to rescue the ppk23-induced deficits in courtship index but not courtship latency, which we observed in animals carrying the Δppk23 null allele alone (Figure 6D–6F). Real-time qRT-PCR analyses of ppk23 mRNA expression in the appendages of Δppk23; UAS-ppk23cDNA flies detected low basal ppk23 expression independent of the presence of GAL4 (Figure S2), which may explain the relatively milder phenotype in this background relative to Δppk23 alone. Furthermore, the presence of the mini white marker in the transgenic constructs has been recently shown to increase overall sexual activity of males [33,34], which may have masked some of the effects of the ppk23 mutation in this background. These data indicate that different DEG/ENaC subunits might contribute to distinct gustatory pathways, some related to feeding and drinking [7,31,32], and some to social behaviors.

Mutations in ppk23 or manipulations of ppk23-expressing sensory neurons did not lead to any obvious feeding related phenotypes (Figures S3 and S4). These data further supported the specific role of ppk23 in mating behaviors. In addition, neither ppk23PM nor TNT-E-expressing flies showed any obvious developmental or locomotion defects, suggesting the observed courtship phenotype was not due to a general lower activity in males (Figure S5). Furthermore, although males carrying the PiggyBac insertion had reduced overall sexual activity, they were not behaviorally sterile. This was supported by the overall viability of the homozygous ppk23PM and the Δppk23 stocks as well as by directly testing for any obvious behavioral phenotypes in ppk23PM females, which showed normal sexual receptivity to males (Figure S6).
ppk23 plays a role in the response to the female-specific pheromone 7(Z), 11(Z)-Heptacosadiene

Our behavioral and anatomical analyses suggested that ppk23 might be directly involved in the sensory perception of sex pheromones. To test this hypothesis, we examined the effects of a ppk23 mutation on the behavioral responses to 7(Z), 11(Z)-Heptacosadiene (7,11-HD), a female specific aphrodisiac pheromone [35]. We first washed the targets flies (male or female CS flies) three times in hexane to remove as much endogenous pheromone as possible. We then applied 7,11-HD or EtOH alone (the solvent for 7,11-HD). Regardless of the sex of the target, wild type males courted pheromone-laced targets significantly more than ppk23 mutant males as measured by the overall percent courtship (Figure 7A and 7B). We also measured the latency and courtship index of males that initiated courtship of perfumed targets. (A) Expression of tetanus toxin light chain (TNT) in ppk23 cells resulted in a high proportion of non-courting males over a 10 min observation time (Chi-square test, \( p < 0.001 \)). Flies expressing TNT in ppk23 cells showed increased courtship latency (B; Kruskal-Wallis rank sum test, \( p < 0.001 \)) and reduced courtship index relative to control crosses (ppk23>GAL4, UAS-TNT-E and UAS-IMP-TNT-V1A [inactive TNT]) (C; Kruskal-Wallis rank sum test, \( p < 0.001 \)). n = 29–36 males per each genotype. Error bars denote the standard error of the means. Letters above bars represent the significantly different groups after post hoc analyses.

doi:10.1371/journal.pgen.1002587.g004

Figure 5. ppk23 is required for normal male courtship behavior. (A) Significantly higher proportion of ppk23\(^{PB}\) males did not show any courtship behavior in a 10-min observation time under dark ("red light") conditions towards females (Chi-square test, *** \( p < 0.001 \)). (B) ppk23\(^{PB}\) males that did court showed increased courtship latency only towards females (Two samples Wilcoxon test; *, \( p < 0.05 \)) and (C) reduced courtship index (Two samples Wilcoxon test; ***, \( p < 0.001 \)). (D) Under white light conditions, proportion of courting ppk23\(^{PB}\) males was not different than wild types (Chi-square test, N.S.). (E) No courtship latency differences were detected either (Two samples Wilcoxon test, N.S.). (F) In contrast, ppk23\(^{PB}\) males exhibited reduced courtship index even under white light conditions (Two samples wilcoxon test; ***, \( p < 0.001 \)). There were no effects of the mutation on any of the measured courtship parameters towards males under white light conditions. (G–I) piggyBac excision rescued the effect of ppk23\(^{PB}\) mutation on male courtship behaviors in all measured parameters under red light conditions (G; Chi-square test, *** \( p < 0.001 \); H; Kruskal-Wallis rank sum test, \( p < 0.05 \); I; Kruskal-Wallis rank sum test, \( p < 0.05 \)). n = 24–38 for each genotype and each condition. N.S. indicates no significant difference. Error bars denote the standard errors of the means.

doi:10.1371/journal.pgen.1002587.g005
dummies. As shown in Figure 7C and 7D, there were no significant differences in latency between the two genotypes, which is in agreement with our courtship data of live female targets (Figure 5). In contrast, even when they did initiate courtship, \( \Delta ppk23 \) mutant males tended to court the dummies with less intensity than wild type males (Figure 7E and 7F). Although these data do not directly address the issue of whether \( ppk23 \) is directly involved in sensing the pheromone 7,11-HD they do indicate that sensory detection of at least some pheromones requires the function of DEG/ENaC-dependent signaling.

**Discussion**

Flies behave in a complex chemical environment that includes signals for both feeding and social interactions. How these different signals are sensed and coded by the nervous system is still poorly understood. Our identification of \( ppk23 \) expression in chemosensory bristles originally suggested that it might contribute to feeding behaviors. However, we detected no gustatory role for \( ppk23 \) or the cells expressing it. Instead, we found that \( ppk23 \) contributes to processes underlying male sexual behavior; \( ppk23 \) was expressed in sexually dimorphic neurons associated with chemosensory bristles in forelegs, and it was required for normal courtship behavior and attraction to the aphrodisiac pheromone 7,11-HD in males.

Several lines of evidence suggest that \( ppk23 \) and the cells expressing it are chemosensory. These include the similar morphology of the two neurons that express \( ppk23 \) in each chemosensory bristle, which is in contrast to the single distinctive mechanosensory neuron that is present in each chemosensory bristle [22]. Chemosensory functions are also supported by the defective response of \( \Delta ppk23 \) mutant males to the aphrodisiac pheromone 7,11-HD. However, several recent studies also indicated that DEG/ENaC signaling plays a role in mechanosensory functions in the worm and the fly [36,37]. Therefore, we currently cannot completely exclude the possibility that \( ppk23 \) might also play a role in mechanosensation in the context of mating behavior in flies.
Whether ppk23 is specifically playing a role in courtship behavior or whether it might be important for other types of social behaviors in the fly is still unknown. In support of a more general role for ppk23-dependent signaling in mediating chemically-driven social interactions, a ppk23 mutation was recently identified in a p-elements screen for genes associated with male-male aggression [38]. Together, these data suggest that ppk23-dependent signaling might play a general role in social communications, and may affect the response of flies to diverse social-related chemical signals.

Visual cues play an important role in the initiation of courtship behavior in Drosophila males, and typically will mask deficits in other sensory modalities [20]. Our data support this, indicating that ppk23 males showed normal courtship latencies under normal lighting conditions. These findings suggest that mutations in ppk23 did not affect the overall sexual drive of males but rather reduced the ability of males to sustain courtship towards female targets in the absence of visual cues, further supporting a chemo-sensory role for this gene. It also raises an interesting hypothesis; which is that vision (absent under red light condition) is playing a critical role during the initial mate recognition process but is playing a less prominent role in the maintenance of courtship until successful copulation occurs. This is based on our observations that ppk23 males showed reduced courtship index under both light conditions but exhibited prolonged courtship latencies under red light but not white light conditions. These results indicate that visual inputs were able to overcome chemo-sensory deficits during the initial orientation towards a female target but were not enough to overcome such deficits for sustaining courtship. Furthermore, our data also indicate that initial chemo-physical contact between the courting male and the female target represents an important trigger for the release of orientation behavior (which was used to determine courtship latency). This may suggest that PPK23 plays a role in triggering male courtship, which was especially robust under red light conditions in which vision is eliminated.

Figure 7. The behavioral response to the aphrodisiac pheromone 7,11-HD is reduced in ppk23 flies. (A–B) Larger percent of wild type CS flies exhibited courtship behavior in response to male or female targets laced with 125 ng 7,11-HD (Chi-squared test; only significant contrasts are shown; *, p<0.05; **, p<0.01; ***, p<0.001, n = 20–30 for each genotype per treatment). (C–D) Courtship latency was not affected by the ppk23 mutation in flies that did court pheromone-laced dummies. (E–F) Significant effects of ppk23 mutation on courtship index were observed in males that courted pheromone-laced dummies (Two samples t-test or two samples Wilcoxon test, *, p<0.05; **, p<0.01; ***, p<0.001, n = 20–30 for each genotype per treatment). doi:10.1371/journal.pgen.1002587.g007
Although we have established ppk23 as a gene important for male sexual behaviors, we do not know whether it plays a similar role in females. Our promoter and expression studies suggested that the locus is expressed in appendages of females. It is possible that ppk23-expressing cells are playing a role that is relevant to intra-species interactions independent of sex. Our female behavioral data suggested that ppk23 mutation did not alter female sexual receptivity, but they do not exclude a possible role in other behaviors.

We still do not know whether ppk23 interacts genetically or physiologically with other receptors previously implicated in Drosophila courtship behaviors. Nevertheless, several lines of investigation already suggest that ppk23 might represent an independent sensory pathway. Typically, gustatory bristles contain either two or four chemosensory neurons, and each expresses a unique set of sensory receptors dedicated to a single taste modality [22]. In contrast, ppk23 was expressed in a pair of chemosensory neurons innervating a single bristle. Of note, our examination of recently published images of the tarsal expression of Gr32a, a chemosensory-related gene involved in inhibiting male-male courtship [39] and the avoidance of aversive chemicals [40], suggests that Gr32a is also expressed in pairs of tarsal chemosensory neurons projecting to single sensory bristles. However, in contrast to ppk23-expressing neurons, which mostly terminate in the thoracic ganglion, many of the tarsal sensory projections of Gr32a-expressing cells form ascending bundles that terminate in the subesophageal ganglion [41,42]. These data indicate that ppk23 and Gr32a are likely expressed in different sensory neurons, and are playing a role in discrete sensory modalities. This assertion is further supported by previous reports that indicated Gr32a is involved in the inhibition of male-male courtship but not in male-female interactions, while we found that ppk23 plays a role in male-female interactions without any obvious effects on male-male interactions [40,43]. Other gustatory receptors have also been implicated in courtship behavior. A recent study suggested that Gr39a contributes to the male-female courtship and is required in sustaining courtship behavior [44]. These studies suggest a possible relationship between ppk23 and Gr39a, which is currently under investigation. Finally, our data also support a chemosensory role for ppk23. Since DEG/ENaC channels often function as heteromultimeric protein complexes [45,46], these data raise the possibility that ppk23 and ppk7 physically interact to form a chemosensory-related channel.

While we do not yet understand the physiological significance of the differences in organization of typical taste-related and ppk23-containing bristles, this separation is analogous to the mammalian chemosensory system, which includes dedicated sensory neurons for pheromonal sensing that are independent of the general chemosensory system [47].

Finding ppk23 expressed in chemosensory neurons and the lack of behavioral responsiveness of ppk23 males to the aphrodisiac pheromone 7,11-HD suggest that PPK23 is a potential candidate for a pheromone receptor, or for a key component of the pheromonal signaling cascade. In summary, our data indicate that DEG/ENaC signaling contributes to sensory functions underlying sex-related behaviors, and indicate a novel physiological function for this important family of ion channels. Finally, the results presented here further support the idea that insects have evolved separate chemosensory systems for appetitive and pheromonal chemosensory signaling.

Materials and Methods

Drosophila stocks and cultures

Flies were maintained on standard cornmeal medium at 25°C under 12:12 light-dark cycle. A ppk23 promoter-GAL4 line was generated by PCR amplifying a 2.6 kb fragment (X: 17402154-17404754), which included the first intron of ppk23 subcloned into an improved p[GTGAL4] vector [48]. UAS-ppk23 was generated by cloning the ppk23 ORF from male appendages into a pUAST vector [49]. Transgenic flies were generated according to standard procedures (Rainbow Genetics Inc. Ca). UAS-PPK7-E and UAS-IMP-TNT-V1.4 were obtained from C. O’Kane (Cambridge, England). UAS-IR1 and the lines expressing three copies of EGFP under direct control of either Gr30a or Gr66a (Gr30a->3xEGFP and Gr66a->3xEGFP, respectively) were from K. Scott (Berkley, CA). Gr30a-GAL4 and Gr66a-GAL4 were from J. Carlson (Yale, CT). The ppk23PB flies were the piggyBac insertion allele of ppk23 (line c03836, Harvard Exelixis collection), which were outcrossed to the Canton-S (CS) background for six generations. We also used piggyBac transposase to remove the piggyBac insertion in the ppk23 locus according to standard genetic method [50]. The Dppk23 and Dppk28 alleles were from the Wang lab [31]. The original published genetic background that carried these alleles had highly reduced overall male courtship behavior likely due to background mutations. Hence, both published alleles were outcrossed into the wild type CS background for six generations prior to the described behavioral studies. Unless mentioned, other fly strains used in our studies were obtained from the Bloomington Stock Center.

RNA analysis

Flies were separated by sex under CO2 and kept at ~−80°C until processing. To separate body parts, microcentrifuge tubes with flies were dipped in liquid nitrogen and then separated by repeated vortexing. Total RNA from tissues was extracted with the RNeasy mini kit (Qiagen) or Trizol reagent (Invitrogen) according to manufacturer instructions. RT-PCR analysis was performed by using the SuperScript II reverse transcriptase (Invitrogen) with 1 µg total RNA in 20 µl reaction according to manufacturer instructions. PCRs were performed with the ACCUprime pfx supermix (Invitrogen) in 25 µl reactions, and were subsequently separated on a 1.2% agarose gel. Real-time quantitative RT-PCR assays for ppk23 were performed on an “ABI7500 fast” machine with an ABI predesigned probe-based assay (ppk23; Dm01839671_g1) according to manufacturer instructions. For all other assays, gene specific primers were designed with the PrimerExpress package (ABI Inc.) and were analyzed using Power SYBR kit (ABI Inc.). See Table S1 for primer sequences. The housekeeping gene rp49 were used as an RNA loading control (Dm02151827_g1). Data were transformed according to the ΔΔCT method and are represented as relative values [51]. Northern blot analysis was done with the NorthernMax kit (Ambion), using a DIG-labeled ppk23 specific cDNA probe (Roche). The RNA probe was generated via in vitro transcription using a PCR template that included nucleotides 578–1369 of ppk23-RA. The probe was designed to hybridize to all three possible alternatively spliced ppk23 transcripts.

Feeding behavior assay

Groups of 20–30 four to seven day-old flies of mixed sexes were kept overnight in an empty fly vial containing a wet paper towel. On test day, flies were briefly immobilized on ice and then transferred to the test plate. Test plates were 60-well Nunc dishes, which had alternate wells filled with 10 µl of either test compound mixed with 2 mM sucrose or 2 mM sucrose alone (a weak feeding inducer) in 1% agarose. Food dye (1% v/v) was added to each alternative choice (either red or blue). Flies were allowed to feed for 2 h in the dark and were then frozen in a −20°C freezer. Only flies that showed clear dye in their digestive system were further
used for index calculation. Choice index was calculated as (number of flies that consumed tested compound + ½ purple [consumed both]) / total number of flies that had any dye in their abdomen. Choice index of 0.5 indicated that the consumption of the tested compound was not different than 2 mM sucrose. Any deviation towards index of 1 or 0 reflected a positive or negative preference, respectively, of the compound relative to 2 mM sucrose. Concentrations for various compounds are in figure legends, except for in Figure 5D; yeast was 0.16% v/v live Baker’s yeast paste. *E. coli (DH5α strain) was 10% v/v overnight culture in standard LB medium. “Old food” was 10% v/v one-week-old standard *Drosophila* culture food from a high-density population. Monosodium glutamate (MSG) was 30 mM. Bovine serum albumin (BSA) was 2% w/v. No obvious differences between males and females were observed in our assays. Consequently, data were collected from mixed sex assays.

**Proboscis extension response**

Proboscis extension was assayed in 2 day old males as previously described [17] with the following modifications. 1 d old flies were starved for approximately 24 hours, then immobilized by chilling on ice and mounted ventral-side-up using myristic acid. Flies were allowed to recover for two hours in humid conditions. To begin the experiment, flies were satediated with water until no proboscis extension was elicited by water stimulation. Next, flies were tested by touching a drop of a solution on a pipette tip to a foreleg and scored for full proboscis extension. Flies were given three trials of same substance in each concentration with water application in between trials; only flies that responded at least 2 out of 3 times were considered ‘responders’ for that concentration. For bitter assays, 100 mM sucrose was included at all concentrations of caffeine. At the beginning and the end of each assay, flies were tested for response to 100 mM sucrose and any fly failing to respond was excluded from the study. The “percent responders” for each genotype at each concentration was calculated by tallying the number of flies responding divided by the total number of flies tested. The responding index represents the cumulative scores (sum of all responses of an individual animal to a specific sequence of tarsal stimuli).

**Courtship behavior**

Courtship behavior was assayed with four to seven day old males as previously described [17] with the following modifications. Newly emerged males were kept in individual vials with food until tested. Courtship assays were done under the red light conditions unless differently stated and the targets were the decapitated flies. Courtship latency was calculated as the time from female introduction until the male showed obvious courtship behavior such as orientation coupled with wing extensions. In wild type males, first orientation was typically concurrent with first contact with a female. In all cases when we observed a delayed latency, the initiation of courtship required multiple contacts between males and females. Males were observed for 10 min. Once courtship began, courtship index was calculated as the proportion of time a male spent in any courtship-related activity during a 10 min period or until mating occurred. For the 7,11-HD experiments, all the targets used were virgin 5 days old flies and freshly cleaned the cuticle hydrocarbon by 3 times 5-min hexane washes, then_PPP washes were applied. 2.5 ul of 50 ng/ul 7,11-HD or the control solvent, 100% ethanol. All assays were performed under normal light conditions in circular courtship arenas, 22 mm in diameter. Tests for general locomotion were performed as previously described [52,53].

**Statistical procedures**

All statistical tests were performed using the R statistical package. Data were tested for normality by using the Shapiro-Wilk test. Two-sample t-tests and one-way ANOVA tests were used for parametric statistics and the two-sample Wilcoxon test and Kruskal-Wallis rank sum test were used for non-parametric tests. Chi-square tests were used for examining frequency-based data.

**Supporting Information**

**Figure S1** The ppk23 locus. (A) Line e03836 from the Harvard Exelixis collection is an insertion of a piggyBac transposon in the ppk23 locus (ppk23<sup>PB</sup>). The piggyBac insertion was verified by plasmid rescue and was found to be in the second intron, shared by all predicted ppk23 transcripts. ppk23-RX represents the main transcript we have identified in appendages, which does not fully correspond to the currently predicted transcripts (RA-RX). Black boxes represent coding exons. Gray boxes represent untranslated regions. Red arrows represent the location of the primers used in real-time qRT-PCR analyses of ppk23. Orange boxes represent the probe used for the northern blot analyses. (B) Real-time quantitative RT-PCR analysis reveals that ppk23 expression is enriched in the appendages in males and females. No expression was detected in heads or bodies. Data are relative mRNA fold differences (n = 4 per group). (C) Real-time quantitative RT-PCR analysis indicates that ppk23<sup>PB</sup> flies have reduced levels of ppk23 transcripts in male appendages (n = 4, t-test, p<0.01), indicating the allele is likely a hypomorph. Data are relative mRNA fold difference in wild-type relative to ppk23<sup>PB</sup> flies. (D) ppk23 expression pattern in each legs (F: foreleg; M: middle leg; H: hind leg. Genotype: ppk23-Gal4/UAS-nls-GFP.) (TIF)

**Figure S2** Real-time quantitative RT-PCR analysis indicates that Δppk23; UAS-ppk23cDNA flies express low levels of ppk23 transcripts independent of the presence of GAL4. Data are relative mRNA fold differences. (TIF)

**Figure S3** Taste responses of ppk23<sup>PB</sup> flies to various tastants. Behavior of adult flies in taste choice assay; choice index is described in Methods section. (A) Response to indicated concentrations of the appetitive sugar trehalose. (B) Response to indicated bitter compounds. Unless noted, all compounds were used at 10 mM. (C) Response to high and low concentrations of salts. (D) Response to a variety of stimuli that either induce or repress feeding response. There were no differences in taste choice behavior between wild type controls and the ppk23<sup>PB</sup> flies in any of the tests (t-test, N.S.; n = 3–10 trials per group across all experiments). (E–G) ppk23<sup>PB</sup> flies showed normal proboscis extension reflex in response to the different concentration of sucrose (E–F) and caffeine (G–H) when compared with the CS wild-type controls. (Chi-square test for the proportion comparison, t-test for the responding index, N.S., n = 30 per genotype). Error bars denote standard errors of the mean. (TIF)

**Figure S4** ppk23 chemosensory receptor neurons do not contribute to appetitive behaviors. (A) Behavior of adult flies in taste choice assay; choice index is described in Methods section. Controls (Cont) were ppk23-GAL4 x UAS-TNT<sup>active</sup> flies and experimental flies were of ppk23-GAL4 x UAS-TNT-E (TNT). Compounds were at the following concentration: trehalose, 50 mM; caffeine, 10 mM; KCl, 100 mM; citric acid, 1 M; monosodium glutamate (MSG), 30 mM. Twenty to thirty flies were used in each trial per test plate. Three to ten trials per
compound were analyzed. (B) Z-stack confocal image of last tarsal segment from fly carrying ppk23-GAL4 x UAS-DrRed (red soma, solid arrows) and EGFP directly driven by the Gr66a promoter (Gr66a>3xEYFP, green soma, dashed arrows). (C) Z-stack confocal image of last tarsal segment from fly carrying ppk23-GAL4 x UAS-DiRed (red soma, solid arrows) and EGFP directly driven by the Gr66a promoter (Gr66a>3xEYFP, green soma, dashed arrows). In both (B) and (C), green and red cells do not project to the same sensory bristles. (D) Behavioral response to capsaicin. All promotersX-GAL4 lines were crossed to UAS-VR1 flies. Gr66a-GAL4 (bitter receptor neurons) and Gr5a-GAL4 (sweet receptor neurons) were positive controls for repulsion or attraction to capsaicin, respectively. a1118 controls were the same background used for producing ppk23-GAL4 transgenic flies and were used for the wild-type control cross. Capsaicin test plates were 2 mM sucrose versus 2 mM sucrose+0.01 mM capsaicin [56], *, p < 0.05 [N = 4–7 groups of 20–30 flies per genotype]. (E–H) Proboscis extension reflex responses of flies after blocking ppk23 expressing cells with tetanus toxin (TNT). Flies expressing the inactive form of TNT (IMP-TNT) were used as wild type control. (E–F) Induction of the proboscis extension reflex in response to increasing concentrations of sucrose. One parental line, ppk23-Gal4, showed higher sensitivity to the low concentration of sucrose (E, Chi-squared test, p < 0.01, **, [sucrose] = 1 mM; p < 0.05, *, [sucrose] = 5 mM) and high responding index relative to the other parental genotypes (ANOVA, p < 0.01, **). Nevertheless, there were no differences in sucrose responsiveness between the two experimental groups that either expressed the active or inactive forms of TNT in ppk23 expressing neurons (n = 30 for each genotype per treatment). (G–H) Inhibition of the proboscis extension reflex in response to increasing concentrations of the bitter chemical caffeine. (G) Overall, inhibition of ppk23-expressing neurons did not alter the responsiveness to caffeine except for a mild effect at the 10 mM concentration (Chi-squared test, p = 0.05 for total 5 groups, p < 0.05 for ppk23–TNT-E flies and ppk23-Gal4/UAS-TNT-E flies, n = 30 for each genotype per treatment). No differences were found in the overall caffeine responding index (ANOVA, N.S.). Error bars denote standard error of the means.

**Figure S5** ppk23 mutation or TNT expression in ppk23 cells have no effect on general locomotion. (A) Climbing score of ppk23B flies and matched CS wild type control (see methods). There was no effect of the ppk23 mutation on male climbing (t-test, n = 10 groups, 10 flies per group). (B) Walking score of ppk23B flies and CS wild type flies. The number of times that a male flies crossed the bisecting line of the test chamber in a minute was recorded. There was no effect of the ppk23 mutation on male general locomotion (t-test, n = 12 flies per genotype). (C) There was no effect of TNT expression in ppk23 cells on male climbing. All experimental and controls genotypes are as in Figure 4. (D) There was no effect of the TNT expression in ppk23 cells on male general locomotion. (Kruskal-Wallis rank sum test, p < 0.001, n = 12–15 flies per genotype). N.S. indicates no significant difference. Error bars denote the standard error of the mean.

**Figure S6** ppk23 mutation has no effect on male copulation success or female sexual receptivity. Courtship responses of CS wild type males to ppk23B and CS wild type females. (A) There were no effects of the ppk23 mutation in target females on courtship index of CS wild type males flies (t-test, n = 15 per target genotype). (B) There was no effect of ppk23 mutations on female receptivity measured by her copulatory success. (Chi-square test, N.S. indicates no significant difference. Error bars denote the standard error of the means.

**Table S1** List of real-time q-RT-PCR primers.

**Acknowledgments**

We thank Judith Tigha and Lorenzo Katin-Grazzini for help with fly husbandry, behavioral assays, and general laboratory assistance. We thank Mahmood Panahi for general technical assistance. We thank Whitney Pennington and Helene Bars for help with courtship behavior data analyses. We thank Bruce Baker and Dave Melleti for helpful comments on the manuscript.

**Author Contributions**

Conceived and designed the experiments: BL, MJW YB-S. Performed the experiments: BL, AL, YS YB-S. Analyzed the data: BL, YB-S. Wrote the paper: BL, MJW YB-S.
20. Ejima A, Griffith LC (2007) Measurement of Courtship Behavior in Drosophila melanogaster. Cold Spring Harb Protoc 2007: pdb.prot4847–.
21. Awasaki T, Kimura K (1997) fus-neuro is required for development of chemosensory bristles in Drosophila. J Neurobiol 32: 707–721.
22. Vosshall LB, Stocker RF (2007) Molecular architecture of smell and taste in Drosophila. Annu Rev Neurosci 30: 305–333.
23. Amrein H (2004) Pheromone perception and behavior in Drosophila.Curr Opin Neurobiol 14: 435–442.
24. Demir E, Dickson BJ (2005) fruitless specifies male courtship behavior in Drosophila. Cell 121: 783–794.
25. Manoli DS, Foss M, Villella A, Taylor BJ, Hall JC, et al. (2005) Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. Nature 436: 395–400.
26. Mellert DJ, Knapp JM, Manoli DS, Meissner GW, Baker BS (2010) Midline crossing by gustatory receptor neuron axons is regulated by fruitless, drosomes and the Roundabout receptors. Development 137: 325–332.
27. Yu JY, Kanai MI, Demir E, Jeffers GS, Dickson BJ (2010) Cellular organization of the neural circuit that drives Drosophila courtship behavior. Curr Biol 20: 1602–1614.
28. Swirski KK, Kravitz EA (2009) Fruitless, drosomes and the genetics of social behavior in Drosophila melanogaster. Curr Opin Neurobiol 19: 200–206.
29. Stockinger P, Kvitsoi D, Rotkopf S, Tiran I, Dickson BJ (2005) Neural circuitry that governs Drosophila male courtship behavior. Cell 121: 795–807.
30. Marella S, Fischer W, Kong P, Asgarian S, Rueckert E, et al. (2006) Imaging channels in a synthetic aphrodisiac. Journal of Chemical Ecology 11: 1617–1629.
31. Chen Z, Wang Q, Wang Z (2010) The amiloride-sensitive epithelial Na+ channel PPK23 is essential for Drosophila gustatory water reception. J Neurosci 30: 6247–6252.
32. Cameron P, Hiroi M, Ngai J, Scott K (2010) The molecular basis for water taste in Drosophila. Nature 463: 91–95.
33. Anaka M, MacDonald CD, Barkova E, Simon K, Rostom R, et al. (2008) The white gene of Drosophila melanogaster encodes a protein with a role in courtship behavior. J Neurogenet 22: 243–276.
34. Hing AL, Carlson JR (1996) Male-courtship behavior induced by ectopic expression of the Drosophila white gene: role of sensory function and age. J Neurobiol 30: 454–467.
35. Antony C, Davis TL, Carlson DA, Pechine JM, Jallon JM (1985) Compared Behavioral-Responses of Male Drosophila-Melanogaster ( Canton-S) to Natural and Synthetic Aphrodisiacs. Journal of Chemical Ecology 11: 1617–1629.
36. Geffeney SL, Cueva JG, Glauser DA, Doll JC, Lee THC, et al. (2011) DEG/ENaC but Not TRP Channels Are the Major Mechanoelectrical Transduction Channels in a C. elegans Nociceptor. Neuron 71: 845–857.
37. Zhong L, Hwang RY, Tracey WD (2010) Pimpocket is a DEG/ENaC protein required for mechanoeception in Drosophila larvae. Curr Biol 20: 429–434.
38. Edwards AC, Zwart L, Yamamoto A, Callaerts P, Mackay TF (2009) Mutations in many genes affect aggressive behavior in Drosophila melanogaster. BMC Biol 7: 29.
39. Miyamoto T, Amrein H (2008) Suppression of male courtship by a Drosophila pheromone receptor. Nat Neurosci 11: 874–876.
40. Lee Y, Kim SH, Montell C (2010) Avoiding DEET through insect gustatory receptors. Neuron 67: 555–561.
41. Contreras-Castaneda M, Haba D, Matou T, Yamamoto D (2010) The shaping of male courtship posture by lateralized gustatory inputs to male-specific interneurons. Curr Biol 20: 1–8.
42. Nojima T, Kimura K, Kosugezawa M, Yamamoto D (2010) Neuronal synaptic outputs determine the sexual fate of postzygotic targets. Curr Biol 20: 836–840.
43. Moon SJ, Lee Y, Jiao Y, Montell C (2009) A Drosophila gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. Curr Biol 19: 1623–1627.
44. Watanabe K, Toba G, Kosugezawa M, Yamamoto D (2011) G63R, a highly diversified gustatory receptor in Drosophila, has a role in sexual behavior. Behav Genet 41: 746–753.
45. Benson CJ, Xie J, Wemmie J, Price MP, Hems JM, et al. (2002) Heteromultimers of DEG/ENaC subunits form Hv-gated channels in mouse sensory neurons. Proc Natl Acad Sci U S A 99: 2339–2343.
46. Eskandari S, Snyder PM, Kremen M, Zampiglia GA, Welsh MJ, et al. (1999) Number of Subunits Comprising the Epithelial Sodium Channel. J Biol Chem 274: 27281–27286.
47. Brennan PA, Zufall F (2006) Pheromonal communication in vertebrates. Nature 444: 308–315.
48. Sharma Y, Cheung U, Larsen EW, Ebert DE (2002) PPTGAL, a convenient Gal4 P-element vector for testing expression of enhancer fragments in drosophila. Genesis 34: 115–118.
49. Phelps CB, Brand AH (1998) Ectopic Gene Expression in Drosophila Using GAL4 System. Methods 14: 367–379.
50. Thabault ST, Simer MA, Miyazaki WY, Milash B, Dompe NA, et al. (2004) A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat Genet 36: 203–207.
51. Ben-Shahar Y, Robichon A, Sokolowski MB, Robinson GE (2002) Influence of gene action across different time scales on behavior. Science 296: 741–744.
52. Sun Y, Liu L, Ben-Shahar Y, Jacobs JS, Ebert DF, et al. (2008) TRPA channels distinguish gravity sensing from hearing in Johnston’s organ. Proc Natl Acad Sci U S A 105: 13606–13611.
53. Villella A, Gailey DA, Berwald B, Ohshima S, Barnes PT, et al. (1997) Extended reproductive roles of the fruitless gene in Drosophila melanogaster revealed by behavioral analysis of new fru mutants. Genetics 147: 1107–1139.
54. Boll W, Mull N (2002) The Drosophila Ppm gene: control of male courtship behavior and fertility as revealed by a complete dissection of all enhancers. Development 129: 5667–5681.
55. Xu A, Park SK, D’Mello S, Kim E, Wang Q, et al. (2002) Novel genes expressed in subsets of chemosensory sensilla on the front legs of male Drosophila melanogaster. Cell Tissue Res 307: 381–392.
56. Fischer W, Keng P, Marella S, Scott K (2007) The detection of carbonation by Drosophila gustatory system. Nature 448: 1054–1057.