A Drosophila DEG/ENaC channel subunit is required for male response to female pheromones

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Odorants and pheromones as well as sweet- and bitter-tasting small molecules are perceived through activation of G protein-coupled chemosensory receptors. In contrast, gustatory detection of salty and sour tastes may involve direct gating of sodium channels of the DEG/ENaC family by sodium and hydrogen ions, respectively. We have found that ppk25, a Drosophila melanogaster gene encoding a DEG/ENaC channel subunit, is expressed at highest levels in the male appendages responsible for gustatory and olfactory detection of female pheromones: the legs, wings, and antennae. Mutations in the ppk25 gene reduce or even abolish male courtship response to females in the dark, conditions under which detection of female pheromones is an essential courtship-activating sensory input. In contrast, the same mutations have no effect on other behaviors tested. Importantly, ppk25 mutant males that show no response to females in the dark execute all of the normal steps of courtship behavior in the presence of visible light, suggesting that ppk25 is required for activation of courtship behavior by chemosensory perception of female pheromones. Finally, a ppk25 mutant allele predicted to encode a truncated protein has dominant-negative properties, suggesting that the normal Ppk25 protein acts as part of a multiprotein complex. Together, these results indicate that ppk25 is necessary for response to female pheromones by D. melanogaster males, and suggest that members of the DEG/ENaC family of genes play a wider role in chemical senses than previously suspected.

Expression Analysis. Analysis of mRNA and protein levels in various tissues was essentially as described (10). Mass separation of body parts resulted in three fractions: appendages (legs, wings, and third antennal segments), heads (without the third antennal segment), and bodies (without heads, legs, or wings) (12). In Fig. 3b, heads were manually separated from bodies and each fraction was frozen and sieved separately, yielding one sample with third antennal segments and another with legs and wings. Real-time PCR was performed on a DNA Engine Opticon cycler (MJ Research, Waltham, MA), using TaqMan primers that hybridize specifically to ppk25 or rp49 cDNA sequences, as well as appropriate amplification primers. The specificity of the assays was confirmed by the amplification of a single reverse transcriptase-dependent band of the correct size and, for ppk25, by the absence of amplification product or fluorescent signal from Δ5-22 homozygous males. For each sample, the concentration of ppk25 mRNA was obtained by comparison with a standard curve and normalized to that of rp49 mRNA. Sequencing of the cDNA product corresponding to the largest ppk25 hybrid mRNA expressed in Δ5-22 homozygous males showed that it includes intron 3 and lacks intron 4.

Behavioral Analysis. Flies were raised at 25°C, 50% relative humidity, and courtship behavior was recorded and analyzed essentially as described (13). For courtship analysis, virgin yw female flies were aged for 2–5 days and decapitated 1–2 h before the experiment to eliminate female behavior as a source of variation (14). Virgin males of each genotype aged in isolation for 2–5 days were placed in the presence of a decapitated female inside a solid Plexiglas chamber (7 mm diameter × 7 mm deep), and their behavior was recorded for 10 min by using a digital 8-mm camera with infrared capturing capability. Behaviors were scored blind and analyzed by using a recent version of the LIFESONG software (15): LIFESONG X (version 0.51-r2). Statistical significance was calculated by using ANOVA. Geotactic behavior of flies of each genotype was scored by using a geotaxis maze apparatus as described (16). Response to 0.2 mM sucrose was measured in a preference assay that compares ingestion of 0.2 mM sucrose and water (17).

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Results

ppk25, a Drosophila Member of the DEG/ENaC Sodium Channel Subunit Family, Is Preferentially Expressed in Male Appendages Rich in Chemosensory Sensilla. We have previously reported that CheB42a, a member of a recently discovered family of Drosophila proteins, is only expressed in a small subset of gustatory sensilla on the front legs of males, suggesting that it may be involved in male-specific gustatory perception (10). Subsequently, Gr68a, a gustatory receptor gene, was found to be expressed in a similar pattern (5). Excitingly, the loss of male response to female pheromones upon either inactivation of Gr68a-expressing neurons or knock-down of Gr68a expression suggests that Gr68a may be a receptor for female pheromones that activate male courtship behavior. More recently, we have found that CheB42a and Gr68a are expressed in the same subset of gustatory sensilla on male front legs (unpublished data), suggesting that CheB42a also plays a role in this process. Intriguingly, ppk25, a gene predicted to encode another protein with a function in chemical senses, is found only 103 nt downstream of the 3′ end of CheB42a (Fig. 1a). The ~30 members of the Drosophila ppk family of genes (8, 18–21) are part of the large family of DEG/ENaC sodium channel subunits that is found in all animals, from Caenorhabditis elegans to humans, and is involved in a wide variety of functions (22, 23). Several Drosophila ppks are expressed in gustatory neurons, and ppk11 and ppk19 are required for gustatory response to salt (8).

To evaluate a possible involvement of ppk25 in male response to pheromones, we tested ppk25 expression in pooled adult appendages that are highly enriched for gustatory (legs and wings) and olfactory (third antennal segment) sensory hairs (24), as well as in body parts that have much fewer chemosensory cells relative to their total mass: heads (without third antennal segment) and bodies (without heads or appendages, see Experimental Procedures). mRNA was isolated from all three types of body parts and analyzed by Northern blot using a full-length ppk25 cDNA probe and exposed for 48 h (Left) or six days (Center Left). The same filter was subsequently boiled and rehybridized with a CheB42a probe. A third hybridization with a probe for the ubiquitous rp49 mRNA (Right) shows that somewhat less “Appendages” mRNA was loaded compared to the other body parts so that preferential ppk25 expression in appendages is underrepresented. A, appendages; B, bodies; H, heads.

Deletion of ppk25, but Not CheB42a, Dramatically Decreases Male Response to Female Pheromones. To test the possible involvement of CheB42a and ppk25 in male response to female pheromones, we generated three homozygous viable deletions in the region by imprecise excision of a P element inserted ~1 kb upstream of CheB42a (9) (Fig. 1b). All three deletions remove part or all of the CheB42a gene, leading to the complete absence of CheB42a mRNA (not shown) and CheB42a protein (Fig. 4a). In contrast, these three deletions have very different effects on ppk25. Males homozygous for Δ5-68, a deletion removing all sequences between the P element insertion site and roughly the middle of the CheB42a gene, have normal or even slightly increased levels of ppk25 mRNA in their appendages (Fig. 4b). The Δ5-2 deletion completely removes the CheB42a gene and terminates only ~59 bp before the ppk25 ATG initiation codon. Although this deletion preserves the predicted ppk25 coding region in its entirety, it significantly impairs transcription of ppk25, such that ppk25 mRNA in male appendages is smaller mRNA of ~700 nt that is present only in the appendages fraction, as reported (10).

To determine which appendages express ppk25 mRNA, we used quantitative real-time RT-PCR (Fig. 3) on total RNA extracted from different types of male appendages. This analysis confirms that, in adults, ppk25 mRNA is most abundant in appendages and also shows that ppk25 expression is approximately three times higher in male than female appendages (Fig. 3a). Interestingly, however, ppk25 mRNA is present at equivalent levels in male legs and wings, appendages that carry many gustatory sensilla, and in the third antennal segment, the main olfactory organ of the fly (Fig. 3b).

Together, these data show that ppk25 expression is highest in olfactory and gustatory appendages of sexually mature males, a distribution consistent with a role in response to female pheromones. In addition, two observations argue that, despite their proximity, CheB42a and ppk25 are indeed two separate genes that are independently transcribed into two separate mRNAs. First, we find no evidence of any transcript containing both CheB42a and ppk25 sequences (Fig. 2). Second, the two mRNAs have related, but not identical, tissue distributions. Both transcripts are present at highest levels in male appendages. However, whereas CheB42a expression is only detectable in male front legs (10), ppk25 mRNA is expressed equally in male legs and antennae and at lower, but significant levels in female appendages as well as bodies and heads of either sex.
undetectable by Northern blot. Finally, in addition to deleting all sequences between the P-element insertion site and the midpoint of ppk25, the Δ5-22 deletion retains part of the original transposon, resulting in a series of hybrid transcripts that originate in P-element sequences but retain the 3’ half of the normal ppk25 mRNA. Characterization of the corresponding cDNAs indicates that these aberrant transcripts are unlikely to produce any Ppk25-related polypeptide and suggest that Δ5-22 is a null mutant for ppk25 (see Fig. 4).

Is the function of either CheB42a or ppk25 required for male response to female pheromones? When placed in the presence of a female, a D. melanogaster male quickly initiates a striking series of stereotyped steps that include following the female, tapping her with his front legs, generating a courtship song by vibrating one of his wings, licking her genitalia, attempting copulation, and copulating (1). Both visual and chemo sensory perception of the female stimulate male courtship behavior. Therefore, we observed the response of males carrying deletions in the CheB42a/ppk25 region to females under infrared lights, which D. melanogaster cannot detect (25), to enhance the contribution of pheromone detection to male behavior. For each male, a courtship index is calculated (26), which represents the fraction of the total observation time spent performing any courtship behavior multiplied by 100 (Fig. 5a). Males homozygous for the Δ5-68 deletion display normal levels of overall courtship. In contrast, males homozygous for either Δ5-2 or Δ5-22 have a much reduced courtship index relative to the G7 controls (P < 9 × 10⁻⁴ and P < 2 × 10⁻⁴ for Δ5-2 and Δ5-22, respectively), suggesting unexpectedly that males require ppk25, but not CheB42a, to achieve normal overall levels of courtship behavior in response to a female. In addition, introduction of a transgenic copy of the genomic region that spans both CheB42a and ppk25 genes rescues the courtship behavior of Δ5-22 homozygous males, whereas an almost identical transgene that lacks ppk25 does not (Tg1 and Tg2, respectively, in Fig. 5b). This result indicates that the courtship deficit of Δ5-22 homozygous males is indeed caused by the loss of ppk25. Importantly, ppk25 is not required for two behaviors unrelated to courtship: walking and preening (Fig. 5). In fact, males homozygous for Δ5-22 walk more than controls or those carrying a transgenic ppk25, whereas Δ5-2 homozygous males display normal levels of this behavior. To further test whether Δ5-2 and Δ5-22 cause generalized brain dysfunction, we measured two other complex behavioral responses to sensory stimuli. Neither the typical climbing response of Drosophila to mechanosensory detection of gravity nor stimulation of food intake by gustatory detection of sucrose is affected by any of the deletions in the region (Fig. 8, which is published as supporting information on the PNAS web site). Together, these results suggest that ppk25 is required specifically for male response to females.

Insertion of a Transposable Element into the Second Intron of ppk25 Causes a Dominant-Negative Decrease of Male Response to Females. Analysis of our deletion lines suggests that the ppk25 gene is required for normal male response to females. As a further test of this possibility, we used males with an independent mutation in ppk25 (27, 28). In this ppk25 mutant, a transposable element is inserted in the second intron of the ppk25 gene, resulting in what we will refer to as the ppk25Δ2 allele (Fig. 1a and c). The presence of 4 kb of extraneous sequences, including a termination site from the miniwhite gene (28), make it unlikely that this modified ppk25 intron 2 can be spliced properly to produce functional ppk25 mRNA. Instead, transcription from the normal ppk25 promoter can be expected to result in an mRNA that retains exons 1 and 2.
followed by part of intron 2, which contains multiple in-frame stop codons. Alternatively, the 5' splice junction of intron 2 may be spliced aberrantly to a cryptic 3' splice site within Piggyback sequences. The protein product of ppk25/PB should therefore be limited to the first transmembrane domain and part of the extracellular domain of Ppk25, perhaps fused to Piggyback sequences (Fig. 1c). Interestingly, for several other DEG/ENaC genes, similarly truncated or fused proteins that retain the first transmembrane domain have dominant-negative properties likely caused by the formation of nonfunctional complexes with other DEG/ENaC subunits or other interacting proteins (8, 21, 29, 30, 45).

To test the effect of the ppk25/PB allele on male response to females, we generated males that carry the following mutations: (i) ppk25/PB, (ii) △9 a similar Piggyback insertion in an unrelated site on the second chromosome in an otherwise isogenic background to ppk25/PB, (iii) △42E, a deletion of the ppk25 genomic region spanning 100 kb and 20 genes, or (iv) △5, a deletion of similar size in an unrelated area of the second chromosome (Fig. 6). Remarkably, none of the 28 ppk25/PB/△42E males that we tested displayed any detectable courtship behavior during the 10-min observation period under infrared lights, a highly significant decrease relative to control males (compare the courtship index for ppk25/PB/△42E and △5/△42E males in Fig. 6, P = 3 × 10^-5). This result confirms that ppk25 is required for male response to females. In addition, because ppk25/PB homozygous males have normal levels of CheB42a mRNA (data not shown), the result indicates that the requirement for ppk25 is independent of CheB42a. Finally, the complete loss of male response to females in ppk25/PB/△42E males is a significantly more severe phenotype than the reduced courtship observed for △5-22 homozygous males, suggesting that ppk25/PB is indeed a dominant-negative allele. This conclusion is validated by the significantly reduced levels of courtship behavior exhibited by males that carry a single copy of ppk25/PB in the presence of a wild-type ppk25 gene compared to males that only carry one wild-type copy of ppk25 (compare ppk25/PB/C5 to △5/△42E in Fig. 6, P = 0.012).

**Visible Light Completely Alleviates the Block of Mutant Males Carrying the ppk25/PB Allele on the Initiation, but Not Maintenance of Courtship Behavior.** The deficient male response to females observed for ppk25 loss-of-function and dominant-negative alleles under infrared light could be due either to a lack of sensory detection of females or to a more general inability to perform courtship behaviors, regardless of sensory stimulus. To distinguish between these two possibilities, we analyzed the effect of visible light on the response of ppk25/PB mutant males. The two types of males we compared in this experiment carry a single copy of either the wild-type ppk25 gene or the dominant-negative ppk25/PB allele in an otherwise isogenic background that includes the △5-22 deletion. As in Fig. 6, under infrared light and in the absence of any wild-type ppk25, a single copy of the dominant-negative ppk25/PB allele results in the complete loss of male response to females under infrared light, but no decrease in walking or preening (not shown). In sharp contrast, in the presence of visible light, males of the same genotype perform all of the normal steps of courtship, albeit at a significantly reduced rate (Fig. 9, which is published as supporting information on the PNAS web site). This result suggests that the complete inability of males carrying the dominant-negative ppk25/PB allele to respond to females under infrared lights is due to a lack of sensory input rather than an inability to perform courtship behaviors. Furthermore, this experiment provides an indirect test of whether the dominant-negative ppk25/PB mutation blocks pheromone perception through olfaction, gustation, or both chemical senses. Both visual and olfactory inputs can initiate courtship behavior. In contrast, gustatory perception of pheromones may only be required for efficient performance of subsequent steps (5). Because the lag to initiation of courtship behavior and the number of courtship bouts per second displayed by ppk25/PB/△5-22 males are similar to controls in the presence of visible light, the lack of
response of the same males under infrared lights likely results at least in part from their inability to initiate courtship in response to pheromones as would be expected for an olfactory defect. On the other hand, despite the presence of visible lights, the average length of a courtship bout for mutant males is less than half that of controls, suggesting that the ppk25<sup>29<sup>alleles Cause a Subsequent Step, perhaps gustatory detection of pheromones. ppk25<sup>29<sup>'s dominant negative effects on males homologous for the Δ5-2 or Δ5-22 deletions (Fig. 5) also result from a combination of increased lags to courtship, decreased numbers of bouts initiated per second, and shorter bout lengths (data not shown). Together, these results suggest that ppk25 itself is required for both initiation and maintenance of courtship bouts in response to female pheromones.

Discussion
A Member of the Drosophila Family of DEG/ENaC Sodium Channel Subunits Is Required for Male Response to Females. We have found that ppk25, a member of the Drosophila family of DEG/ENaC sodium channel subunits, is required for male response to females. First, we have generated two deletions that inactivate both CheB42a and ppk25: Δ5-2 and Δ5-22. Males homozygous for either deletion display a much reduced response to females but no similar decrease in other behaviors. In contrast, another deletion that results in complete loss of CheB42a expression but has no effect on ppk25 does not reduce male courtship behavior. Second, a genomic fragment that includes both CheB42a and ppk25 rescues the response of Δ5-22 homozygous males to females, whereas an almost identical fragment lacking ppk25 does not. Third, ppk25<sup>29<sup>, an independent mutation resulting from insertion of a transposable element into the second intron of ppk25, affects male response to females more severely than Δ5-22, even though this allele has no detectable effect on CheB42a expression. Indeed, ppk25<sup>29<sup> has dominant-negative effects on male response to females, observable both in the presence or absence of a wild-type copy of ppk25. Fourth, the dominant-negative properties of ppk25<sup>29<sup> are readily interpreted in light of the predicted generation in this mutant of a truncated Ppk25 protein retaining the N-terminal cytoplasmic domain, the first transmembrane domain, and part of the extracellular domain of the normal Ppk25. Similarly truncated variants of various members of the DEG/ENaC family, including several Drosophila ppks, also have dominant-negative properties (8, 21, 29, 30, 45).

Our discovery of a role for ppk25 in male response to female pheromones was the unexpected result of our interest in the neighboring CheB42a. The data in this report show that deletion of CheB42a does not decrease overall male response to courtship-activating pheromones. However, the restricted expression of CheB42a in the same subset of gustatory sensilla that express Gr68a (unpublished data) and are required for response to female courtship-activating pheromones (5) suggest that CheB42a's requirement may be obscured by functional redundancy with one or more of the other 11 Drosophila CheB genes (10) or, alternatively, that CheB42a has a different role in male-specific chemical senses.

Is it a coincidence that two genes implicated in male-specific chemical senses are within <103 nt of each other? These two genes produce mRNAs of different sizes with related, albeit different, expression patterns. Both are preferentially expressed in male gustatory appendages starting late in pupal development and remaining through at least sexual maturity of the adult males. However, whereas CheB42a is only expressed in male front legs (10), ppk25 mRNA is present at similar levels in legs and in the third antennal segment, and at much lower but detectable levels in heads and bodies. The proximity of these two genes may therefore reflect a shared dependence on regulatory elements important for overlapping spatial and/or temporal characteristics of their expression. Indeed, the lack of detectable ppk25 mRNA in males homozygous for Δ5-2 suggests the presence of a regulatory element essential for ppk25 expression within or immediately downstream of the 3' half of CheB42a. Alternatively, the proximity between these two genes may be more a reflection of their involvement in evolutionarily important and related aspects of sexual behavior.

ppk25 Is Required for Chemosensory Activation of Male Courtship Behavior by Female Pheromones. Why can’t ppk25 mutant males respond to females normally? Vision and pheromone detection have both been implicated in the response of Drosophila melanogaster males to females (1, 2). Absence of visible light or mutations that cause partial or complete blindness reduce, but do not eliminate, male response to females. In addition, a number of studies suggest that males detect courtship-stimulating female pheromones by using either gustation, olfaction, or both chemical senses (5, 31–40). Although both vision and olfactory detection of pheromones are important for initiation of courtship behavior, gustatory perception of the same or other pheromones may be required for efficient progression to later steps in the courtship sequence (5). Because both initiation and maintenance of courtship bouts are affected in dominant-negative (Figs. 6 and 7) as well as null

Fig. 7. Visible light enables courtship behavior in males carrying the dominant-negative ppk25<sup>29<sup> allele. Male response to females was measured as in Fig. 5 except for the presence of visible light. For this experiment, three separate parameters of male behavior are shown to demonstrate the differential effect of the dominant-negative ppk25<sup>29<sup> allele: lag to courtship, number of courtship bouts per minute, and length of courtship bouts (see text). The males tested carry a Δ5-22 deletion on one copy of the second chromosome and are completely isogenic except for the presence on their other second chromosome of either (i) the dominant-negative ppk25<sup>29<sup> allele, or (ii) the normal ppk25 gene and another Piggyback insertion at an unrelated site. In the presence of visible light, replacement of the normal ppk25 gene by the ppk25<sup>29<sup> allele causes a statistically significant decrease in the average length of a courtship bout (P < 0.02) but no change in the lag to courtship or in the number of courtship bouts per minute.

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mutations in ppk25 (not shown), this gene may be required for detection of pheromones by both sensory modalities, a possibility supported by the expression of ppk25 in both olfactory (antennae) and gustatory (wings and legs) appendages.

Is a Ppk25-Containing Sodium Channel Involved in the Peripheral Detection of Female Pheromones? Our data strongly support the requirement for ppk25 in the male's ability to respond to female courtship-activating pheromones. In addition, mutations in ppk25 do not similarly impair other behaviors that are either largely independent of sensory inputs, such as walking and preening, or sensory-driven such as geotaxis and chemosensory response to sugars. Most importantly, these mutations have no effect on the initiation of courtship behavior in the presence of visible light. Therefore, ppk25's requirement for male response to pheromones likely reflects a specific role in the sensory detection of pheromones or subsequent processing within the central nervous system rather than a more general requirement for neural function or even for performance of courtship behavior. Finally, ppk25 expression is first detectable during late pupal stages, after determination of all of the various types of chemosensory cells and as they undergo the final stages of differentiation (41, 42), suggesting that ppk25 is required for the function, rather than the development of chemosensory organs.

Is ppk25 required in peripheral olfactory or gustatory neurons that sense and respond to female pheromones in the environment, or in central nervous system neurons that receive and process the information coming from the periphery? Although these alternatives remain to be tested, the former hypothesis is supported by ppk25's preferential expression in male chemosensory appendages as well as by the established roles of other DEG/ENaC subunits in peripheral sensory responses to mechanical stimuli (43) and salt (8). ppk25's putative role in pheromone detection may not involve direct participation in the primary molecular response to pheromones. However, recent imaging of the electrophysiological response in mechanosensory neurons indicate that the C. elegans DEG/ENaC gene mec-4 is specifically required for the mechanosensory function rather than the general physiology of the neurons in which it is expressed (44). Similar questions arise regarding the role ppk25 plays in male detection of female pheromones and in particular, whether it interacts, directly or indirectly, with the G protein-coupled signal transduction pathways that underlie chemical senses in Drosophila as in other arthropods (4).

Finally, the dominant-negative properties of the ppk25Δp allele most likely reflect the participation of the Ppk25 protein in a multisubunit protein complex. Proteins of the DEG/ENaC family are thought to interact in the formation of heteromeric sodium channels (22, 23). Several truncated versions of DEG/ ENaC proteins have dominant-negative properties that most likely result from their ability to form partial and inactive complexes with other DEG/ENaC subunits (8, 29, 30, 45). By analogy, our results suggest that one or more of the ~30 other Ppk proteins encoded in the Drosophila genome (8) interacts with Ppk25 within a heteromeric sodium channel.

In conclusion, our data demonstrate a role for a member of the DEG/ENaC family of sodium channel subunits in the peripheral detection or central processing of a pheromonal signal. This finding opens the door to the dissection of ppk25's role in pheromone response and its relationship with other proteins involved in pheromone detection in the central nervous system. Finally, this work suggests that other members of the Drosophila ppk family, as well as DEG/ENaC subunits in other organisms, play more complex roles in chemical senses than previously suspected.

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