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Two *Drosophila* DEG/ENaC channel subunits have distinct functions in gustatory neurons that activate male courtship

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Abstract

Trimeric sodium channels of the DEG/ENaC family have important roles in neurons, but the specific functions of different subunits present in heteromeric channels are poorly understood. We previously reported that the *Drosophila* DEG/ENaC subunit Ppk25 is essential in a small subset of gustatory neurons for activation of male courtship behavior, likely through detection of female pheromones. Here we show that, like mutations in *ppk25*, mutations in another *Drosophila* DEG/ENaC subunit, *nope,* specifically impair male courtship of females. *nope* regulatory sequences drive reporter gene expression in gustatory neurons of the labellum, wings and legs, including all gustatory neurons in which *ppk25* function is required for male courtship of females. In addition, gustatory-specific knockdown of *nope* impairs male courtship. Further, the impaired courtship response of *nope* mutant males to females is rescued by targeted expression of *nope* in the subset of gustatory neurons in which *ppk25* functions. However, *nope* and *ppk25* have non-redundant functions, as targeted expression of *ppk25* does not compensate for the lack of *nope* and *vice versa*. Moreover, Nope and Ppk25 form specific complexes when co-expressed in cultured cells. Together, these data indicate that the Nope and Ppk25 subunits have specific, non-redundant functions in a subset of gustatory neurons required for activation of male courtship in response to females, and suggest the hypothesis that Nope and Ppk25 function as subunits of a heteromeric DEG/ENaC channel required for gustatory detection of female pheromones.

Introduction

The study of the elaborate courtship displayed by *Drosophila* males towards females presents a unique opportunity for understanding pheromonal control of a complex behavior at all levels, from the molecular events underlying specific chemosensory detection of pheromones, to the neuronal circuitry that integrates multiple signals, and ultimately controls behavior (Billeter et al., 2006; Villella and Hall, 2008; Siwicki and Kravitz, 2009; Dahanukar and Ray, 2011; Dauwalder, 2011). The advantages of *Drosophila* as a model organism have resulted in a sophisticated understanding of the general mechanisms involved in both taste and smell (Dahanukar and Ray, 2011), as well as the genetic control and brain circuitry underlying male courtship (Billeter et al., 2006; Villella and Hall, 2008; Siwicki and Kravitz, 2009). Furthermore, the chemical structure of a number of pheromones that either stimulate or inhibit male courtship (Ferveur, 2005), and the receptors and neurons involved in both olfactory and gustatory modulation of courtship have been described (Ha and Smith, 2006; Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007; Miyamoto and Amrein, 2008; Grosjean et al., 2011; Wang et al., 2011). Activation of courtship behavior involves both olfaction and taste, but while loss of olfaction decreases male courtship by approximately two-fold (Stockinger et al., 2005; Krstic et al., 2009),

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males with impaired gustatory function display almost no courtship of females (Krstic et al., 2009). However, the neuronal circuitry and molecular mechanisms involved in gustatory activation of courtship have remained elusive. Recently, we reported that *ppk25*, a DEG/ENaC subunit with an essential role in activating male courtship (Lin et al., 2005), functions specifically in a small subset of peripheral taste neurons on the legs and wings of males (Starostina et al., 2012). In addition to identifying for the first time a subset of gustatory neurons required for activation of male courtship behavior, most likely through detection of female pheromones, this work demonstrated that a DEG/ENaC subunit is specifically required for the function of those neurons, but not for gustatory detection of sugars, or of pheromones that inhibit rather than activate courtship behavior. Here, we describe the discovery of *nope*, another DEG/ENaC subunit gene specifically required for male courtship of females. Normal male response to females requires expression of both *nope* and *ppk25* in a common subset of gustatory neurons in legs and wings, indicating that each of these subunits has a unique, non-redundant role. Furthermore, Ppk25 and Nope form specific complexes when co-expressed in cultured cells, suggesting that they also interact in the subset of gustatory neurons in which they are coexpressed. These data provide further evidence that a specific subset of gustatory neurons is required to activate male courtship, support a critical role for DEG/ENaC channels in these neurons, and suggest that Ppk25 and Nope are subunits of a common heteromeric channel required for gustatory activation of male courtship in response to female pheromones.

Materials and Methods

Generation of the *nope-Gal4* and *UAS-nope* transgenes and immunostaining

The *nope-Gal4* construct was generated by cloning a 3020 bp fragment upstream of the *nope* start codon flanked by PmeI and BglII sites into a derivative of the pP{CaSpeR-4} vector (Qian et al., 1991; Starostina et al., 2012) digested with PmeI and BamHI. In the resulting construct, the *nope* ATG is fused in frame with the coding sequences of the yeast transcriptional activator Gal4, followed by SV40 polyadenylation sequences obtained from the *pGATB* vector (Brand and Dormand, 1995). Transgenic lines for *nope-Gal4* were generated using standard procedures for random integration of *P*-element vectors (Rubin and Spradling, 1982). For generation of *UAS-nope* and *UAS-nope*^{alt}, cDNAs were generated from RNA extracted from male front legs. Either the full-length 1496 bp *nope* cDNA, or a 5 nucleotides shorter form in which intron 10 was spliced out using an alternative 5' splice site, was cloned into *pUAS*$_{attB}$ (Bischof et al., 2007) and integrated by Bestgene Inc. (Chino Hills, CA) using site-specific integration at the *attP2* locus on chromosome 3 (Bischof et al., 2007; Markstein et al., 2008). Immunostaining was performed as described in (Starostina et al., 2012) based on a previous report (Laissue et al., 1999). Rabbit anti-GFP (Invitrogen, Carslbad, CA) was used at a dilution of 1:200, and anti-nc82 (Developmental Studies Hybridoma Bank, supported by the NICHD at the University of Iowa) at a dilution of 1:40.

Sequence analysis

Unannotated Nope orthologs were initially identified in most *Drosophila* species by searching genomic sequences using TBLASTN (Johnson et al., 2008). Analysis of the corresponding genomic sequences yielded predicted spliced transcripts encoding proteins with extensive similarity to *D. melanogaster* Nope throughout their sequence, confirming that they are likely Nope orthologs (our unpublished data). Sequences encoding Nope orthologs in *D. ananassae* and *D. grimshawii* overlap putative annotated genes *GF13482* and *GH20163,* respectively. Sequence alignments were performed using ClustalW (Larkin et al., 2007), and displayed using the Boxshade 3.21 server at http://www.ch.embnet.org/.

Cell transfection and co-immunoprecipitation

cDNAs encoding full-length Ppk25 and Nope were cloned into pCMV5 with carboxy-terminal 3xFlag and 3xHA epitopes. A similar b-ENaC-HA construct with a 3xHA epitope was generated using a 1924bp b-ENaC human cDNA from Thermo Scientific/Dharmacon, Lafayette, CO. The Arm-2XHA pCMV5 clone was a kind gift of Hassina Benchabane (Benchabane et al., 2011). Transfections and immunoprecipitations were according to published protocols (Meltzer et al., 2007) with the following modifications. HEK293T human embryonic kidney cells were transfected using Lipofectamine 2000 (Invitrogen/Life Sciences, Grand Island, NY) following the manufacturer's protocol. Transfected cells were lysed at 4°C in Lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH7.4, 1mM EDTA) in the presence of protease inhibitors (Roche Diagnostics, Mannheim, Germany) and 1% Triton X-100 for 1 hour. Cell lysates were collected, clarified by microcentrifugation for 15 min at 4°C and incubated for 1 hour at 4°C with a 1:350 dilution of a 5 micrograms/microliter solution of mouse monoclonal anti-Flag M2 antibody (Sigma-Aldrich, St. Louis, MO). 20 microliters of Protein A/G PLUS Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the extract and incubated for 1 hour at 4°C. The beads were then pelleted and washed 3 times in Lysis buffer with 0.2 % Triton X -100. Immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with rat anti-HA antibody (Roche Diagnostics, Mannheim, Germany).

Behavioral assays

Conditions for fly rearing and courtship assays were as described in (Starostina et al., 2012) with the following modifications. Courtship directed at females was assayed under infrared lights using 5–7 days old w^{1118} virgin females that stood upright and preened after having been anesthetized and decapitated. Courtship directed at males was assayed using intact w^{1118} males under visible laboratory lights. Behavior was recorded with a digital Mini-DV camera for 10 minutes, scored blind and analyzed using the LIFESONG X software (version 0.8) (Villella et al., 2005). The Courtship Index is the fraction of time the male spends performing any courtship behavior x100 (Hall, 1978), and the Total Behavioral Index is the fraction of the same ten minute period during which the male courts, walks or preens x100 (Starostina et al., 2012). For CI and TBI, error bars represent the standard error of the mean, and the Wilcoxon rank-sum test was used to determine statistical significance. For the fraction of males initiating courtship (number of males with non-zero CI divided by the total number of males observed), error bars indicate 95% confidence intervals for binomial distributions, and statistical significance was calculated using a chi-square test for equality of distributions.

Drosophila stocks

Most fly lines used were obtained from the Bloomington Stock Center. The *UAS-nope*^{RNAi} line (stock number 27241) was obtained from the Transgenic RNAi Project at Harvard Medical School (Ni et al., 2009).

Results

A second DEG/ENaC subunit, Nope, is required for male response to females

Our previous work demonstrated that the Ppk25 DEG/ENaC subunit is required specifically in gustatory neurons that activate male courtship behavior in response to females (Lin et al., 2005; Starostina et al., 2012). As DEG/ENaC channels are often composed of several distinct subunits, our findings suggested that other *Drosophila* DEG/ENaCs may also be required for activation of male courtship by females. We therefore tested whether males homozygous for mutations in other *Drosophila* DEG/ENaC subunit genes respond normally

to females (Figure 1A). Male courtship was analyzed in the presence of decapitated females to prevent behavioral feedback, and under infrared light, thereby preventing visual stimulation (Frank and Zimmerman, 1969; Lin et al., 2005). Courtship was quantitated using a Courtship Index (CI), the fraction of a ten-minute observation period in which a male displays any courtship behavior towards a female, multiplied by 100, as well as the fraction of males that initiate courtship during the observation period (Villella and Hall, 2008; Starostina et al., 2012). While most mutants tested court females at normal levels, males homozygous for the *f06838* mutation displayed significant reductions in both CI and fraction of males initiating courtship (Figure 1A). The *f06838* mutation corresponds to the insertion of a transposable element in exon 5 of a previously uncharacterized gene, *CG13568*, which we have named *nope* (for not passionate, Figure 2A), which encodes a 425 amino-acid protein. Interestingly, RT/PCR analysis indicates that, in addition to *nope* transcripts encoding a full-length protein, alternative splicing patterns result in *nope* transcripts that either retain intron 8, or use a different 5' splice site for intron 10, in both cases resulting in shorter open reading frames encoding truncated Nope proteins (Figure 2A and data not shown). A multiple sequence alignment shows extensive sequence similarity between Nope, two likely Nope orthologs from *Drosophila ananassae* and *D. grimshawii*, and other members of the family of DEG/ENaC sodium channel subunits (Figure 3). In particular, clusters of conserved residues are found in and around both transmembrane domains and in a neurotoxin-related cysteine-rich stretch within the long extracellular region (Tavernarakis and Driscoll, 2000; Kellenberger and Schild, 2002). Surprisingly, however, Nope and its orthologs lack a highly conserved amino-terminal motif that is required for the function of ASIC3 (Salinas et al., 2009), of the epithelial sodium channel ENaC (Grunder et al., 1997), and of the *C. elegans* degenerin, Mec-4 (Hong et al., 2000). These data indicate that like *ppk25*, the *nope* gene encodes a DEG/ENaC subunit.

To confirm that the defective courtship of males homozygous for the *f06838* insertion results from disruption of *nope*, we tested the behavior of males homozygous for an independent *nope* mutation, *f04205,* in which a transposon is inserted in the fourth exon of *nope* (Figure 2A). Males homozygous for either *f06838* (*nope*^{1}) or *f04205* (*nope*^{2}), and males transheterozygous for either *nope*^{1} or *nope*^{2} and a deletion of the entire locus court females significantly less than control males, confirming that the defect in courtship behavior results from disruption of *nope* (Figure 1B). In contrast, *nope*^{1} or *nope*^{2} heterozygous males court females normally, indicating that both alleles of *nope* are recessive. Furthermore, the Total Behavioral Index (TBI), which is the fraction of the ten minute observation period in which the male performs any of the most frequent behaviors (walking, courting, preening), multiplied by 100, is not significantly different between *nope* mutant males and controls (not shown) indicating that mutations in *nope* specifically affect courtship behavior. Finally, as is the case for wild-type males (not shown), *nope*^{1} or *nope*^{2} homozygous males do not display significant levels of courtship toward other males; even in the presence of visible light, which typically results in higher levels of courtship and has been used in other studies of male-male courtship (Liu et al., 2008), the CI of *nope* mutants is not significantly different from 0 (Figure 1B). Therefore, similar to *ppk25* (Starostina et al., 2012), but in contrast to *fruitless*(*fru*), a master regulator of sex-specific behavior (Billeter et al., 2006; Villella and Hall, 2008; Siwicki and Kravitz, 2009), *nope* is not required for suppression of male-male courtship. Instead, *nope* function is specifically required for activation of male courtship in response to females.

nope is expressed specifically in a small subset of taste neurons that includes taste neurons required for male response to females

In order to determine the expression pattern of the *nope* gene, and given the impossibility of detecting low abundance mRNAs in *Drosophila* legs using *in situ* hybridization, we

generated a transgene in which the open reading frame of the yeast Gal4 transcriptional activator (Elliott and Brand, 2008) is placed at the predicted initiation of translation of the Nope protein (Figure 2B). This transgene contains 3 kb of putative *nope* regulatory sequences upstream of the Gal4 ATG: 700 bp of intergenic DNA between the divergent transcription initiation sites of *nope* and *eIF5a,* the nearest upstream gene, and 2.3 kb of the *nope* 5' UTR, including two large introns, non-coding exons 1 and 2, and a non-coding segment of exon 3 (Figure 2B). Flies containing *nope -Gal4* and a *UAS-mCD8-GFP* transgene express mCD8-GFP specifically in gustatory neurons of the legs, wings and proboscis in both males and females (Figure 4), with a greater number of cells in the front legs of males than females (not shown), consistent with the sexually dimorphic number of taste hairs on that appendage (Possidente and Murphey, 1989). On the legs of males, expression is observed in taste neurons distributed on the tibia and all five tarsal segments, in a pattern very similar to that obtained with the *ppk25-Gal4* driver (Starostina et al., 2012), (Figure 4A–B). Furthermore, as documented previously for *ppk25-Gal4* (Starostina et al., 2012), *nope-Gal4* does not drive expression of mCD8-GFP in the two taste hairs located at the distal tip of the leg, which are the only taste hairs on the legs of males that respond to sugars and salts (Meunier et al., 2000; Meunier et al., 2003). Finally, expression of *nope-Gal4* and *ppk25-Gal4* is not detectably altered in mutants homozygous for *nope* or *ppk25*, respectively, indicating that neither gene is essential for development or survival of the neurons in which it is expressed (data not shown). An important difference between the expression of *ppk25-Gal4* and *nope-Gal4* in gustatory neurons of the legs is that while *ppk25-Gal4* expression is invariably associated with a single neuron per taste hair (Starostina et al., 2012), in some cases the dendrites of two *nope-Gal4-* expressing gustatory neurons project into the lumen of a single taste hair (Figure 4C, "p" indicates a pair of neurons projecting their dendrites into a single taste hair). *nope-Gal4* is also associated with taste neurons on the labellum (Figure 4D), and wings (Figure 4E), while *ppk25-Gal4* is expressed in wings, but not in the labellum (Starostina et al., 2012). Finally, whole-mount staining with anti-GFP allows visualization of the axonal projections of taste neurons in the brain and ventral nerve chord (Figure 4F–I). *nope-Gal4-* expressing gustatory neurons on the labellum project to discrete medial areas in the suboesophageal ganglion, the site of the first synaptic relay for taste neurons in the head (Vosshall and Stocker, 2007)(Figure 4F–G). In contrast, *nope-Gal4* -expressing taste neurons on the legs and wings project to each of the thoracic ganglia in the central nerve chord (Figure 4H–I). Of particular interest, projections from the front legs onto the first thoracic ganglia are sexually dimorphic, crossing the midline in males more frequently than in females (Figure 4H–I). This pattern is identical to that seen using *fru-LexA* (Mellert et al., 2009), a driver expressing the transcriptional activator LexA in the pattern of *fru* (Billeter et al., 2006; Siwicki and Kravitz, 2009), or *ppk25-Gal4* (Starostina et al., 2012). These findings suggest that like *ppk25-Gal4* (Starostina et al., 2012), *nope-Gal4* expression may occur in a subset of *fru* -expressing neurons. Indeed, double-staining of gustatory neurons expressing *nope-Gal4* and *fru-LexA* in the legs with different fluorescent proteins indicate that *nope-Gal4* -expressing nuclei also express *fru-LexA* (Figure 4J–K). In contrast, gustatory neurons that express *nope-Gal4* on the labellum do not express *fru* (Figure 4L). *fru*'s role in courtship is mediated by the male-specific FruM transcription factor (Siwicki and Kravitz, 2009; Dauwalder, 2011). However, FruM is unlikely to directly regulate expression of either *ppk25* or *nope*, since, with the exception of the greater number of *fru-* expressing taste neurons on the front legs of males which is under *dsx* control (Mellert et al., 2009), both DEG/ENaC subunit genes are expressed in similar patterns and at comparable levels in males and females (data not shown). Indeed, real-time PCR indicates that expression of the *ppk25* mRNA is not affected by a mutation in *fru* (our unpublished data). Together, these findings indicate that *nope-Gal4* and *ppk25-Gal4* are expressed in similar subsets of gustatory neurons in the legs and wings.

Given the similar expression patterns of *ppk25-Gal4* and *nope-Gal4* in the legs, we tested for overlapping expression of the two drivers in the front legs of males by counting the number of GFP-expressing cells present in each leg segment for males containing either *ppk25-Gal4*, *nope-Gal4*, or both drivers (Figure 4 M–O, Table 1). Under control of *ppk25-Gal4,* we consistently observe mCD8-GFP expression in 43 gustatory neurons on the tibia and the five tarsal segments. However, in the presence of either *nope-Gal4* alone, or when both drivers are present, mCD8-GFP expression is observed in 54 taste neurons. These results indicate that while all neurons that express *ppk25-Gal4* also express *nope-Gal4*, 11 neurons that express *nope-Gal4* do not express *ppk25-Gal4*. Furthermore, two lines of evidence suggest that in most cases, *nope-Gal4*-expressing neurons that do not express *ppk25-Gal4* are paired within a single taste hair with a neuron that expresses both drivers. First, on each leg, ten taste hairs containing two *nope-Gal4*-expressing neurons account for nearly all eleven *nope-Gal4* -expressing neurons that do not express *ppk25-Gal4* (Table 1). Second, the position of taste hairs containing either one or two *nope-Gal4*-expressing neurons on the surface of the tarsal segment always closely matches that of taste hairs containing a single *ppk25-Gal*-expressing neuron (compare Figure 4M, N, O). In one exception to this rule, a single *nope-Gal4* -expressing taste neuron is typically present on the second tarsal segment at a position where *ppk25-Gal4* is not expressed (not shown). These data reveal that in approximately 33 taste hairs located at stereotypic positions on the tibia and tarsi of male front legs, one of the two *fru*- expressing neurons (Mellert et al., 2009) expresses *nope-Gal4,* and the same neuron also expresses *ppk25-Gal4*. In another ten taste hairs, one of the *fru*-expressing taste neurons expresses both nope-*Gal4* and *ppk25-Gal4*, while the other expresses only *nope-Gal4*.

Nope functions in a small subset of taste neurons

The expression pattern of *nope-Gal4* suggests that, like *ppk25* (Starostina et al., 2012), *nope* may function specifically in gustatory neurons. To test this possibility, we targeted RNAi-mediated knockdown of *nope* using *Poxn-Gal4*, a driver expressed in all taste neurons (Boll and Noll, 2002) (Figure 5). Indeed, gustatory-specific knockdown of *nope* results in significantly lower CI and fraction of males initiating courtship relative to control males in which *Poxn-Gal4* drives expression of GFP, or of an RNAi targeting expression of *CG13895,* a gene with no known role in courtship behavior (Benchabane et al., 2011; Starostina et al., 2012).

To further define the taste neurons in which *nope* functions, we used *nope-Gal4* to drive expression of *nope* in $nope^{1}/nope^{2}$ mutant males (Figure 6). For this purpose, we generated constructs in which *UAS,* the target sequence of Gal4, controls expression of two different *nope* transcripts (Figure 2A and data not shown). *UAS-nope* directs expression of a fully-spliced mRNA with a single open-reading frame encoding the full-length Nope subunit, while $UAS\text{-}nope^{alt}$ expresses a transcript in which intron 10 was spliced out using an alternative 5' splice site and encoding a truncated Nope that lacks most of the extracellular domain, the second transmembrane domain, and the carboxy-terminal intracellular domain. To focus on activation of courtship by gustatory circuits, the courtship response of $nope^{1}/nope^{2}$ males with various combinations of transgenes was tested after removal of the third antennal segment, the main *Drosophila* olfactory organ (Dahanukar and Ray, 2011; Tunstall and Warr, 2012). The courtship levels of $nope^{1}/nope^{2}$ males containing both *nope-Gal4* and *UAS-nope* are significantly higher than in the presence of *UAS-nope* alone, and indistinguishable from that of males with a wild-type *nope* gene. In contrast, pairing *nope-Gal4* with either $UAS\text{-}nope^{alt}$ or *UAS-RFP* does not rescue the courtship of $nope^{1}/nope^{2}$ mutant males. We also find that while $nope^{1}/nope^{2}$ mutant males carrying the *UAS-nope* transgene display somewhat higher levels of courtship relative to $nope^{1}/nope^{2}$ males, or $nope^{1}/nope^{2}$ males with control transgenes, their courtship remains significantly lower than that of control males. In contrast, the presence of $UAS\text{-}nope^{alt}$ at the same genomic site has

no effect. These results suggest that *UAS* constructs inserted at this genomic site result in some *Gal4*- independent expression in cells in which *nope* function is required for courtship. Furthermore, the impaired courtship of *nope* mutants can be rescued by expression of the *nope* transcript that encodes a full-length Nope protein, but not of the alternatively spliced $nope^{alt}$ transcript encoding a truncated Nope. Finally, *nope* expression has no effect on the TBI, further confirming *nope'*s specific role in activation of courtship behavior. Together, these data indicate that expression of full-length Nope protein in the small subset of gustatory neurons identified by expression of *nope-Gal4* is sufficient for normal male courtship behavior.

The Nope and Ppk25 DEG/ENaC subunits have distinct, non-redundant functions

nope mutant males display a severe courtship phenotype despite the presence of the wild-type *ppk25* gene. To test the possibility that this apparent absence of functional redundancy reflects differences in expression of the two genes, we targeted expression of *ppk25* in $nope^{1}/nope^{2}$ males using *nope-Gal4* and a *UAS-ppk25* transgene that, in the presence of *ppk25-Gal4,* rescues the phenotype of *ppk25* mutant males (Starostina et al., 2012). Contrary to expression of *nope* in the same cells, expression of *ppk25* does not rescue the phenotype of $nope^{1}/nope^{2}$ males, indicating that absence of Nope is not compensated by expression of Ppk25 in the cells where Nope normally functions (Figure 6). In a reciprocal experiment, we tested whether expression of *nope* can rescue the function of *ppk25* mutants (Figure 7). Confirming our previous report, expression of *ppk25* under control of *ppk25-Gal4* rescues the deficient courtship of *ppk25* mutant males (Starostina et al., 2012); in contrast, expression of *nope* in the same cells has no effect. Together, these experiments indicate that the lack of redundancy between the *ppk25* and *nope* genes does not result from differences in their expression patterns but from distinct properties of the DEG/ENaC subunits they encode.

Ppk25 and Nope Function in a Common Subset of Gustatory Neurons Required for Male Response to Females

To test whether *nope* expression in the subset of *nope* -expressing taste neurons that also express *ppk25* is sufficient for normal courtship, we used *ppk25-Gal4* to drive *nope* expression in $nope^{1}/nope^{2}$ mutants (Figure 6). As with *nope-Gal4*, *ppk25-Gal4* -directed expression of fully spliced *nope*, but not of $nope^{alt}$, *ppk25,* or RFP rescues courtship response of $nope^{1}/nope^{2}$ mutants. Therefore, expression of *nope* in the small subset of taste neurons on the wings and legs that express both *nope-Gal4* and *ppk25-Gal4* is sufficient for normal male response to females. Since expression of *ppk25* in the same neurons restores the courtship of *ppk25* mutant males (Figure 7)(Starostina et al., 2012), these data indicate that Ppk25 and Nope function in a common subset of gustatory neurons, and confirm that each of these DEG/ENaC subunit has a specific, non-redundant role.

Nope interacts specifically with Ppk25

Their non-redundant functions in a common subset of gustatory neurons suggest that, like other DEG/ENaC subunits (Carattino, 2011; Kashlan and Kleyman, 2011), Nope and Ppk25 may function within a heteromeric channel. To test whether Nope and Ppk25 interact when coexpressed in cultured cells, and given the role of the amino-terminus of other DEG/ENaC subunits in channel function and trafficking (Tavernarakis et al., 2001; Wesch et al., 2012), we performed immunoprecipitations with carboxy-terminal epitope-tagged versions of the two proteins (Figure 8). Cells were simultaneously transfected with multiple plasmids encoding epitope-tagged proteins. In a first set of experiments, we tested whether Ppk25-Flag interacts with Nope-HA using as an internal control HA-tagged Armadillo, a protein that is present in both cytoplasmic and membrane-associated forms (Riggleman et al., 1989) (Figure 8A). Cellular extracts were immunoprecipitated with an anti-Flag antibody, and

extracts, supernatants and precipitates were subsequently analyzed by immunoblotting with an anti-HA antibody (Figure 8A). In the presence of Ppk25-Flag but not in its absence, immunoprecipitation with anti-Flag results in co-precipitation of Nope-HA but not of Arm-HA, revealing a specific interaction between Ppk25-Flag and Nope-HA. Furthermore, Nope-HA levels are depleted in the supernatant relative to the extract, indicating that most Nope-HA in the extract is bound to Ppk25-Flag. Similarly, in the presence of Nope-Flag, Ppk25-HA but not Arm-HA is coprecipitated by anti-Flag antibody, indicating that the interaction between Ppk25 and Nope does not depend on a particular tagged version of either protein (Figure 8B). To further test the specificity of the interaction between Nope and Ppk25, we conducted competition experiments in the presence of another DEG/ENaC subunit, human β-ENaC (Figure 8C). While in the presence of Nope-Flag, some β–ENaC-HA is coprecipitated with anti-Flag, coprecipitation of Ppk25-HA is significantly more efficient (Figure 8C, compare the ratios of β–ENaC-HA and Ppk25-HA in the pellet and supernatant). Similarly, Flag immunoprecipitation of extracts from cells expressing Ppk25-Flag, Nope-HA and β–ENaC-HA, results in preferential coprecipitation of Nope-HA relative to β–ENaC-HA (Figure 8D, compare the ratios of β–ENaC-HA and Nope-HA in the pellet and supernatant). Finally, as assembly of heteromeric DEG/ENaC channels occurs during transit of the newly synthesized subunits to the membrane surface (Butterworth et al., 2008), we tested whether expression of Nope and Ppk25 in the same cells is required for their interaction. Indeed, when Ppk25-Flag and Nope-HA are expressed in different cells, mixing cellular extracts does not result in co-precipitation (not shown), further confirming the specificity of the interaction between these two DEG/ENaC subunits. Together, these data indicate that Nope and Ppk25 form specific complexes when co-expressed in cultured cells, suggesting the hypothesis that, in the courtship-activating gustatory neurons where they are both expressed, these two subunits assemble into a heteromeric DEG/ENaC channel. In an indirect test of this hypothesis, we asked whether the *nope* and *ppk25* genes interact genetically, resulting in lower courtship levels for males heterozygous for both *nope* and *ppk25,* but find that such males court at normal levels (CI=53 −/+ 5 and 89% of males initiate courtship; those numbers are comparable to those of control males, see Figure 1B). Since such a lack of genetic interaction does not necessarily reflect an absence of direct interaction between the two encoded proteins, more direct approaches will be required to test whether the function of Nope and Ppk25 in gustatory activation of courtship requires formation of a specific complex between these two subunits in pheromone-sensing neurons.

In summary, our data indicate that two DEG/ENaC subunits, Nope and Ppk25, are co-expressed and have specific, non-redundant functions in a common subset of courtship-activating gustatory neurons in the wings and legs of males. In addition, Nope and Ppk25 interact specifically when co-expressed in cultured cells. Together, these data suggest the hypothesis that Ppk25 and Nope are subunits of a heteromeric DEG/ENaC channel required for gustatory activation of courtship in the presence of females, most likely in response to female-specific pheromones.

Discussion

Nope and Ppk25 function in a common subset of gustatory neurons in the legs and wings of males that are required for activation of male courtship

We previously discovered that mutations in *ppk25,* a DEG/ENaC subunit gene, specifically impair male courtship of females, but not other behaviors (Lin et al., 2005). Recently, we reported that expression of *ppk25* in a small subset of taste neurons on the legs and wings of males is sufficient for a normal male courtship response to females (Starostina et al., 2012). Here, we describe a second DEG/ENaC subunit gene, *nope*, that is also specifically required for male response to females. Several lines of evidence indicate that both genes are expressed in, and required for the function of a common subset of gustatory neurons that

activate courtship behavior. First, mutations in *ppk25* and *nope* have indistinguishable effects on male courtship. Like mutations in *ppk25* {Lin, 2005 #993}, mutations in *nope* specifically impair the courtship response of males to females, but have no general effect on other behaviors, such as walking or preening. Furthermore, like mutations in *ppk25* (Starostina et al., 2012), but in contrast to mutations in *fru* (Billeter et al., 2006; Villella and Hall, 2008; Siwicki and Kravitz, 2009) or in the *Gr32a* gustatory receptor gene (Miyamoto and Amrein, 2008), mutations in *nope* do not increase homosexual behavior, suggesting that neither *ppk25* nor *nope* is required for gustatory detection of inhibitory pheromones, such as the male-enriched 7-tricosene. Finally, consistent with gustatory function being required not only to initiate courtship but also to progress to late steps (Krstic et al., 2009), mutations in either *ppk25* {Starostina, 2012 #10564} or *nope* result in significant decreases in both the fraction of males that initiate courtship and the total time spent courting. Second, expression of *ppk25-Gal4* and *nope-Gal4* transgenes overlaps in a small subset of taste neurons on the legs and wings of males. In the front legs in particular, both transgenes are expressed in *fru*-expressing taste neurons that make sexually dimorphic projections onto the thoracic ganglia (Mellert et al., 2009), and all neurons that express *ppk25-Gal4* also express *nope-Gal4*. Third, as in the case of *ppk25* (Starostina et al., 2012), targeted RNAi-mediated knockdown of *nope* in gustatory neurons specifically impairs male response to females, indicating that both genes are required for normal gustatory response to females. Finally, and most importantly, using the same *ppk25-Gal4* driver, targeted expression of *nope* and *ppk25* rescues the courtship of *nope* and *ppk25* mutant males, respectively, indicating that for both DEG/ENaC subunits, expression in a common subset of gustatory neurons on the legs and wings of males is sufficient for normal response to females. Coupled with our previous work (Starostina et al., 2012), these findings further identify a specific subset of gustatory neurons on the legs and wings that have an essential role in activating male courtship, starting from the earliest steps.

In addition to the subset of neurons defined by coexpression of *ppk25-Gal4* and *nope-Gal4,* some chemosensory neurons express only one of the two drivers; *ppk25-Gal4* is expressed in two subsets of olfactory neurons implicated in activating courtship (Starostina et al., 2012), and *nope-Gal4* is expressed in gustatory neurons on the legs and labellum. Furthermore, targeted expression of *nope* using the *ppk25-Gal4* driver is sufficient to rescue the courtship of *nope* mutant males. This result indicates that, under our experimental conditions at least, gustatory neurons that express *nope-Gal4* but not *ppk25-Gal4* are not essential for activation of courtship in response to females, and suggest that expression of *nope-Gal4* may occur in functionally distinct subsets of gustatory neurons. In summary, our data indicate that *nope* and *ppk25* function in a common subset of gustatory neurons on the front legs and wings of males that is required for activation of male courtship in the presence of females.

Their non-redundant functions and specific interaction suggest the hypothesis that Nope and Ppk25 function within a heteromeric DEG/ENaC channel required specifically for gustatory activation of courtship

While *ppk25* and *nope* function in the same subset of gustatory neurons, mutants lacking either subunit display severely reduced male response to females, indicating that the *ppk25* and *nope* genes have non-redundant functions. Furthermore, the phenotype of *nope* mutant males is rescued by targeted expression of *nope*, but not of *ppk25,* and *vice versa*, indicating that the lack of redundancy is due to intrinsic properties of the proteins encoded by each gene rather than differences in their expression patterns. In addition, when co-expressed in cultured cells, Nope forms specific complexes with Ppk25 more efficiently than with human β–ENaC, another DEG/ENaC subunit. Together, these data support the hypothesis that Ppk25 and Nope assemble into a heteromeric DEG/ENaC channel with a specific and

essential function in gustatory activation of courtship behavior. The crystal structure of the vertebrate DEG/ENaC acid-sensing channel, ASIC1a, reveals that it is a homotrimer, suggesting that most if not all DEG/ENaC channels are also trimers (Jasti et al., 2007). Furthermore, ENaC, the vertebrate epithelial sodium channel is a heterotrimer containing three types of subunits: a b and g, all which must be present for assembly of a functional channel. Finally, heteromeric assemblies of *C. elegans Mec-4* and *Mec-10* are required for response to gentle touch (Bianchi, 2007). By analogy, Ppk25 and Nope are likely to assemble into a heterotrimeric DEG/ENaC channel, perhaps together with a third subunit yet to be discovered. In addition, while *ppk25-Gal4* and *nope-Gal4* have overlapping expression patterns, each driver is also expressed in cells where the other is not; *ppk25-Gal4* is expressed in two subsets of olfactory neurons involved in activation of male courtship (Root et al., 2008; Grosjean et al., 2011; Starostina et al., 2012), and *nope-Gal4* in gustatory neurons of unknown function on the front legs and labellum of males (this work). Ppk25 may therefore also participate in trimeric channels lacking Nope, and *vice versa*. By analogy with human ASICs, for which channels of different subunit composition have different functional properties (Sherwood et al., 2011), combinatorial subunit composition may confer functional variety to DEG/ENaC channels in gustatory and olfactory neurons.

Finally, the specific phenotypes of *ppk25* and *nope* mutants coupled with their expression in a small subset of gustatory neurons required for response to females suggest that both DEG/ENaC subunits function specifically in detection of courtship-stimulating pheromones. What is the molecular role of DEG/ENaC channels in pheromone response? In the same way that other DEG/ENaCs channels function as gustatory receptors for water (Cameron et al., 2010; Chen et al., 2010), or sodium ions (Chandrashekar et al., 2010), heteromeric channels containing Ppk25 and Nope may be directly gated by pheromones or by other courtship-activating compounds. Alternatively, heteromeric DEG/ENaC channels containing Nope and Ppk25 may have a less direct role in modulating courtship, similar to the function of *C. elegans* ASIC-1 in learning modulation through presynaptic facilitation of dopamine release (Voglis and Tavernarakis, 2008). Our work lays the foundation for the dissection of the molecular roles of DEG/ENaC channels and the contribution of subunit composition in gustatory activation of male courtship, with relevance to the function of DEG/ENaC channels involved in a number of physiological and pathological processes including hypertension, cystic fibrosis, touch and pain, memory formation, evoked fear, and neuronal cell death in stroke (Kellenberger and Schild, 2002; Bianchi, 2007; Cameron et al., 2010; Chandrashekar et al., 2010; Chen et al., 2010; Qadri et al., 2012).

Note: As this manuscript was in revision, independent findings of *nope*'s expression and role in detection of courtship-stimulating pheromone were published by others (Thistle et al., 2012), and several reports also implicated a third DEG/ENaC channel, *ppk23* in pheromone response (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012), further supporting the central role of DEG/ENaC channels in gustatory detection of pheromones.

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References

Benchabane H, Xin N, Tian A, Hafler BP, Nguyen K, Ahmed A, Ahmed Y. Jerky/Earthbound facilitates cell-specific Wnt/Wingless signalling by modulating beta-catenin-TCF activity. EMBO J. 2011; 30:1444–1458. [PubMed: 21399610]

Bianchi L. Mechanotransduction: Touch and feel at the molecular level as modeled in Caenorhabditis elegans. Molecular Neurobiology. 2007; 36:254–271. [PubMed: 17955200]

Billeter JC, Rideout EJ, Dornan AJ, Goodwin SF. Control of male sexual behavior in Drosophila by the sex determination pathway. Curr Biol. 2006; 16:R766–R776. [PubMed: 16950103]

Bischof J, Maeda RK, Hediger M, Karch F, Basler K. An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc Natl Acad Sci U S A. 2007; 104:3312–3317. [PubMed: 17360644]

Boll W, Noll M. The Drosophila Pox neuro gene: control of male courtship behavior and fertility as revealed by a complete dissection of all enhancers. Development. 2002; 129:5667–5681. [PubMed: 12421707]

Brand AH, Dormand EL. The GAL4 system as a tool for unravelling the mysteries of the Drosophila nervous system. Curr Opin Neurobiol. 1995; 5:572–578. [PubMed: 8580708]

Butterworth MB, Weisz OA, Johnson JP. Some Assembly Required: Putting the Epithelial Sodium Channel Together. Journal of Biological Chemistry. 2008; 283:35305–35309. [PubMed: 18713729]

Cameron P, Hiroi M, Ngai J, Scott K. The molecular basis for water taste in Drosophila. Nature. 2010; 465:91–95. [PubMed: 20364123]

Carattino MD. Structural mechanisms underlying the function of epithelial sodium channel/acid-sensing ion channel. Curr Opin Nephrol Hypertens. 2011; 20:555–560. [PubMed: 21709553]

Chandrashekar J, Kuhn C, Oka Y, Yarmolinsky DA, Hummler E, Ryba NJ, Zuker CS. The cells and peripheral representation of sodium taste in mice. Nature. 2010; 464:297–301. [PubMed: 20107438]

Chen Z, Wang Q, Wang Z. The amiloride-sensitive epithelial Na+ channel PPK28 is essential for drosophila gustatory water reception. J Neurosci. 2010; 30:6247–6252. [PubMed: 20445050]

Coscoy S, de Weille JR, Lingueglia E, Lazdunski M. The pre-transmembrane 1 domain of acid-sensing ion channels participates in the ion pore. J Biol Chem. 1999; 274:10129–10132. [PubMed: 10187795]

Dahanukar A, Ray A. Courtship, aggression and avoidance: pheromones, receptors and neurons for social behaviors in Drosophila. Fly (Austin). 2011; 5:58–63. [PubMed: 20962580]

Dauwalder B. The roles of fruitless and doublesex in the control of male courtship. Int Rev Neurobiol. 2011; 99:87–105. [PubMed: 21906537]

Elliott DA, Brand AH. The GAL4 system : a versatile system for the expression of genes. Methods Mol Biol. 2011; 420:79–95. [PubMed: 18641942]

Ferveur JF. Cuticular hydrocarbons: their evolution and roles in Drosophila pheromonal communication. Behav Genet. 2005; 35:279–295. [PubMed: 15864443]

Frank KD, Zimmerman WF. Action spectra for phase-shifts of a circadian rhythm in *Drosophila*. Science. 1969; 163:688–689. [PubMed: 5762935]

Grosjean Y, Rytz R, Farine JP, Abuin L, Cortot J, Jefferis GS, Benton R. An olfactory receptor for food-derived odours promotes male courtship in Drosophila. Nature. 2011; 478:236–240. [PubMed: 21964331]

Grunder S, Jaeger NF, Gautschi I, Schild L, Rossier BC. Identification of a highly conserved sequence at the N-terminus of the epithelial Na+ channel alpha subunit involved in gating. Pflugers Arch. 1999; 438:709–715. [PubMed: 10555570]

Grunder S, Firsov D, Chang SS, Jaeger NF, Gautschi I, Schild L, Lifton RP, Rossier BC. A mutation causing pseudohypoaldosteronism type 1 identifies a conserved glycine that is involved in the gating of the epithelial sodium channel. EMBO J. 1997; 16:899–907. [PubMed: 9118951]

Ha TS, Smith DP. A pheromone receptor mediates 11-cis-vaccenyl acetate-induced responses in Drosophila. Journal of Neuroscience. 2006; 26:8727–8733. [PubMed: 16928861]

Hall JC. Courtship among males due to a male-sterile mutation in Drosophila melanogaster. Behav Genet. 1978; 8:125–141. [PubMed: 99136]

Hong K, Mano I, Driscoll M. In vivo structure-function analyses of Caenorhabditis elegans MEC-4, a candidate mechanosensory ion channel subunit. J Neurosci. 2000; 20:2575–2588. [PubMed: 10729338]

Jasti J, Furukawa H, Gonzales EB, Gouaux E. Structure of acid-sensing ion channel 1 at 1.9 A resolution and low pH. Nature. 2007; 449:316–323. [PubMed: 17882215]

Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. Nucleic Acids Res. 2008; 36:W5–W9. [PubMed: 18440982]

Kashlan OB, Kleyman TR. ENaC structure and function in the wake of a resolved structure of a family member. Am J Physiol Renal Physiol. 2011; 301:F684–F696. [PubMed: 21753073]

Kellenberger S, Schild L. Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. Physiol Rev. 2002; 82:735–767. [PubMed: 12087134]

Krstic D, Boll W, Noll M. Sensory integration regulating male courtship behavior in Drosophila. PLoS ONE. 2002; 4:e4457. [PubMed: 19214231]

Kurtovic A, Widmer A, Dickson BJ. A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. Nature. 2007; 446:542–546. [PubMed: 17392786]

Laissue PP, Reiter C, Hiesinger PR, Halter S, Fischbach KF, Stocker RF. Three-dimensional reconstruction of the antennal lobe in Drosophila melanogaster. J Comp Neurol. 1999; 405:543–552. [PubMed: 10098944]

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics. 2007; 23:2947–2948. [PubMed: 17846036]

Lin H, Mann KJ, Starostina E, Kinser RD, Pikielny CW. A Drosophila deg/ENaC channel subunit is required for male response to female pheromones. Proc Natl Acad Sci U S A. 2005; 102:12831–12836. [PubMed: 16129837]

Liu L, Leonard AS, Motto DG, Feller MA, Price MP, Johnson WA, Welsh MJ. Contribution of Drosophila DEG/ENaC genes to salt taste. Neuron. 2003; 39:133–146. [PubMed: 12848938]

Liu T, Dartevelle L, Yuan C, Wei H, Wang Y, Ferveur JF, Guo A. Increased dopamine level enhances male-male courtship in Drosophila. J Neurosci. 2003; 28:5539–5546. [PubMed: 18495888]

Lu B, Lamora A, Sun Y, Welsh MJ, Ben-Shahar Y. ppk23-Dependent Chemosensory Functions Contribute to Courtship Behavior in Drosophila melanogaster. PLoS Genet. 2012; 8:e1002587. [PubMed: 22438833]

Markstein M, Pitsouli C, Villalta C, Celniker SE, Perrimon N. Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. Nat Genet. 2008; 40:476–483. [PubMed: 18311141]

Mellert DJ, Knapp JM, Manoli DS, Meissner GW, Baker BS. Midline crossing by gustatory receptor neuron axons is regulated by fruitless, doublesex and the Roundabout receptors. Development. 2009; 137:323–332. [PubMed: 20040498]

Meltzer RH, Kapoor N, Qadri YJ, Anderson SJ, Fuller CM, Benos DJ. Heteromeric assembly of acid-sensitive ion channel and epithelial sodium channel subunits. J Biol Chem. 2007; 282:25548–25559. [PubMed: 17613525]

Meunier N, Ferveur JF, Marion-Poll F. Sex-specific non-pheromonal taste receptors in Drosophila. Curr Biol. 2000; 10:1583–1586. [PubMed: 11137009]

Meunier N, Marion-Poll F, Rospars JP, Tanimura T. Peripheral coding of bitter taste in Drosophila. J Neurobiol. 2003; 56:139–152. [PubMed: 12838579]

Miyamoto T, Amrein H. Suppression of male courtship by a Drosophila pheromone receptor. Nat Neurosci. 2008; 11:874–876. [PubMed: 18641642]

Ni JQ, Liu LP, Binari R, Hardy R, Shim HS, Cavallaro A, Booker M, Pfeiffer BD, Markstein M, Wang H, Villalta C, Laverty TR, Perkins LA, Perrimon N. A Drosophila resource of transgenic RNAi lines for neurogenetics. Genetics. 2009; 182:1089–1100. [PubMed: 19487563]

Possidente DR, Murphey RK. Genetic control of sexually dimorphic axon morphology in *Drosophila* sensory neurons. Dev Biol. 1989; 132:448–457. [PubMed: 2924997]

Qadri YJ, Rooj AK, Fuller CM. ENaCs and ASICs as therapeutic targets. Am J Physiol Cell Physiol. 2012; 302:C943–C965. [PubMed: 22277752]

Qian S, Capovilla M, Pirrotta V. The bx region enhancer, a distant cis-control element of the Drosophila Ubx gene and its regulation by hunchback and other segmentation genes. EMBO J. 1991; 10:1415–1425. [PubMed: 1902784]

Riggleman B, Wieschaus E, Schedl P. Molecular analysis of the armadillo locus: uniformly distributed transcripts and a protein with novel internal repeats are associated with a Drosophila segment polarity gene. Genes Dev. 1989; 3:96–113. [PubMed: 2707602]

Root CM, Masuyama K, Green DS, Enell LE, Nassel DR, Lee CH, Wang JW. A presynaptic gain control mechanism fine-tunes olfactory behavior. Neuron. 2008; 59:311–321. [PubMed: 18667158]

Rubin GM, Spradling AC. Genetic transformation of *Drosophila* with transposable elements. Science. 1982; 218:348–353. [PubMed: 6289436]

Salinas M, Lazdunski M, Lingueglia E. Structural elements for the generation of sustained currents by the acid pain sensor ASIC3. J Biol Chem. 1982; 284:31851–31859. [PubMed: 19778905]

Sherwood TW, Lee KG, Gormley MG, Askwith CC. Heteromeric Acid-Sensing Ion Channels (ASICs) Composed of ASIC2b and ASIC1a Display Novel Channel Properties and Contribute to Acidosis-Induced Neuronal Death. J Neurosci. 2011; 31:9723–9734. [PubMed: 21715637]

Siwicki KK, Kravitz EA. Fruitless, doublesex and the genetics of social behavior in Drosophila melanogaster. Current Opinion in Neurobiology. 2009; 19:200–206. [PubMed: 19541474]

Starostina E, Liu T, Vijayan V, Zheng Z, Siwicki KK, Pikielny CW. A Drosophila DEG/ENaC Subunit Functions Specifically in Gustatory Neurons Required for Male Courtship Behavior. J Neurosci. 2012; 32:4665–4674. [PubMed: 22457513]

Stockinger P, Kvitsiani D, Rotkopf S, Tirian L, Dickson BJ. Neural circuitry that governs Drosophila male courtship behavior. Cell. 2005; 121:795–807. [PubMed: 15935765]

Tavernarakis N, Driscoll M. Caenorhabditis elegans degenerins and vertebrate ENaC ion channels contain an extracellular domain related to venom neurotoxins. J Neurogenet. 2000; 13:257–264. [PubMed: 10858823]

Tavernarakis N, Everett JK, Kyrpides NC, Driscoll M. Structural and functional features of the intracellular amino terminus of DEG/ENaC ion channels. Curr Biol. 2001; 11:R205–R208. [PubMed: 11301263]

Thistle R, Cameron P, Ghorayshi A, Dennison L, Scott K. Contact Chemoreceptors Mediate Male-Male Repulsion and Male-Female Attraction during Drosophila Courtship. Cell. 2012; 149:1140–1151. [PubMed: 22632976]

Toda H, Zhao X, Dickson BJ. The Drosophila Female Aphrodisiac Pheromone Activates ppk23+ Sensory Neurons to Elicit Male Courtship Behavior. Cell Reports. 2012

Tunstall NE, Warr CG. Chemical communication in insects: the peripheral odour coding system of Drosophila melanogaster. Adv Exp Med Biol. 2012; 739:59–77. [PubMed: 22399395]

van der Goes van Naters W, Carlson JR. Receptors and neurons for fly odors in Drosophila. Curr Biol. 2007; 17:606–612. [PubMed: 17363256]

Villella A, Hall JC. Neurogenetics of courtship and mating in Drosophila. Adv Genet. 2008; 62:67–184. [PubMed: 19010254]

Villella A, Ferri SL, Krystal JD, Hall JC. Functional analysis of fruitless gene expression by transgenic manipulations of Drosophila courtship. Proc Natl Acad Sci U S A. 2005; 102:16550–16557. [PubMed: 16179386]

Voglis G, Tavernarakis N. A synaptic DEG/ENaC ion channel mediates learning in C. elegans by facilitating dopamine signalling. EMBO J. 2008; 27:3288–3299. [PubMed: 19037257]

Vosshall LB, Stocker RF. Molecular architecture of smell and taste in Drosophila. Annu Rev Neurosci. 2007; 30:505–533. [PubMed: 17506643]

Wang L, Han X, Mehren J, Hiroi M, Billeter JC, Miyamoto T, Amrein H, Levine JD, Anderson DJ. Hierarchical chemosensory regulation of male-male social interactions in Drosophila. Nat Neurosci. 2011; 14:757–762. [PubMed: 21516101]

Wesch D, Althaus M, Miranda P, Cruz-Muros I, Fronius M, Gonzalez-Hernandez T, Clauss WG, Alvarez de la Rosa D, Giraldez T. Differential N termini in epithelial Na+ channel delta-subunit isoforms modulate channel trafficking to the membrane. Am J Physiol Cell Physiol. 2012; 302:C868–C879. [PubMed: 22159085]

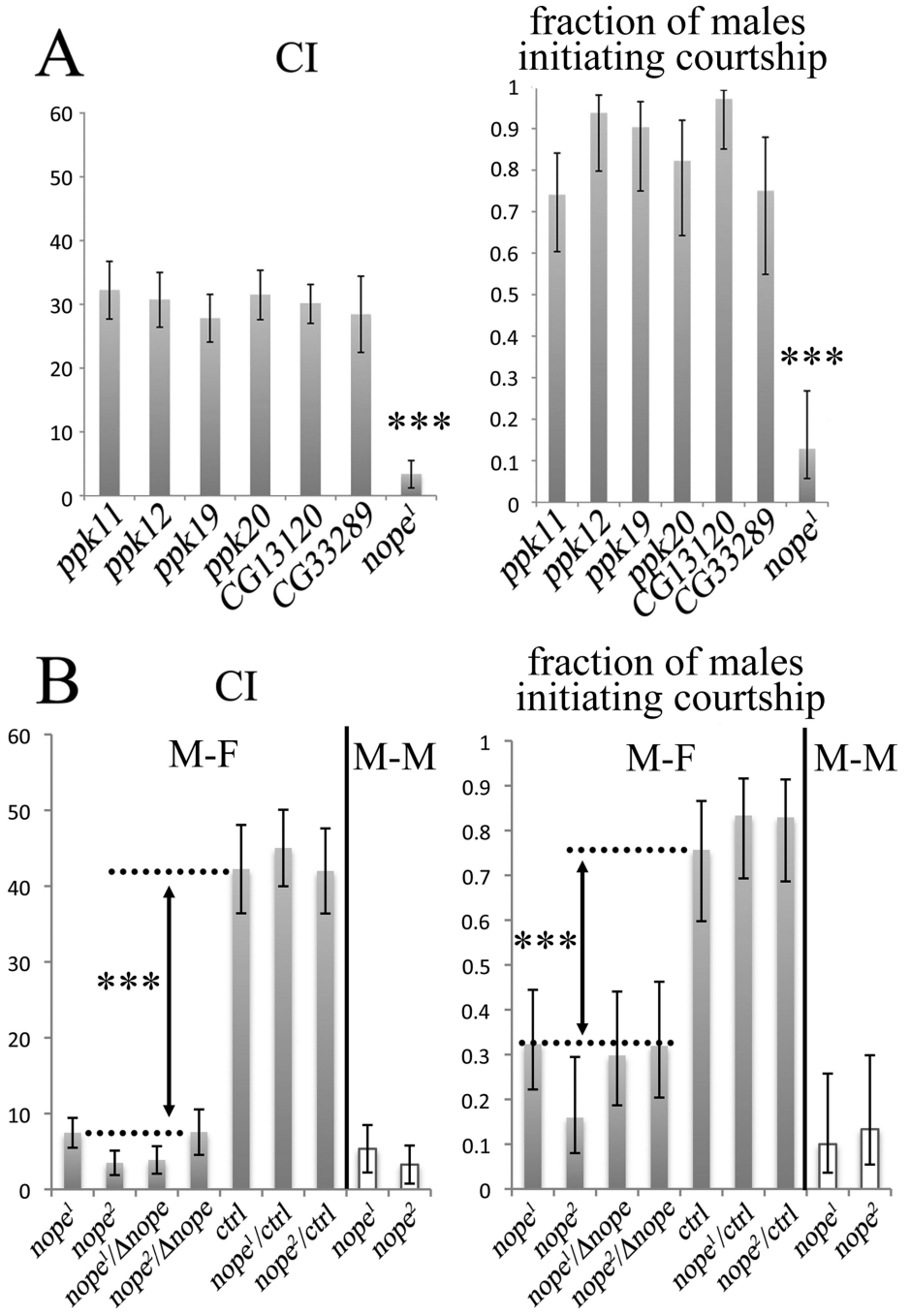

Figure 1. Mutations in the *nope* DEG/ENaC subunit gene block male courtship response to females

A, CI and fraction of males initiating courtship in the presence of decapitated females are shown for males homozygous for mutations in seven different *Drosophila* genes that code for DEG/ENaC subunits. The lines used were: *ppk11*, *f02053*; *ppk12*, *MB11059*; *ppk19*, *MB05382*; *ppk20*, *MB01352*; *CG13120*, *MB07822*; *CG33289*, *EY00388*; *CG13568/nope*, *f06838* (*nope*^{1}) and *f04205* (*nope*^{2}). B, M-F and M-M indicate courtship of test males directed toward females and males, respectively. Male-female courtship was tested under infrared light and in the presence of decapitated females. Courtship directed at other males was measured under visible light using live “object” males {Liu, 2008 #2496}. CI and

fraction of males initiating courtship are shown for the genotypes indicated. *Dnope* is *BSC136*, a deletion that includes *nope* and several neighboring genes. As a control, we used *f02213* males homozygous for a PiggyBac insertion in *CG13563,* a gene neighboring *nope* that has no known role in chemical senses. Error bars show the standard error of the mean (SEM), N>24; ***, $p<0.001$ between (Wilcoxon rank-sum test for CI, and Chi square test for equality of distribution for the fraction of males initiating courtship). In the case of male-male courtship by $nope^{1}$ and $nope^{2}$ mutants neither the distribution of CI values nor the fraction of males initiating courtship are significantly different from 0.

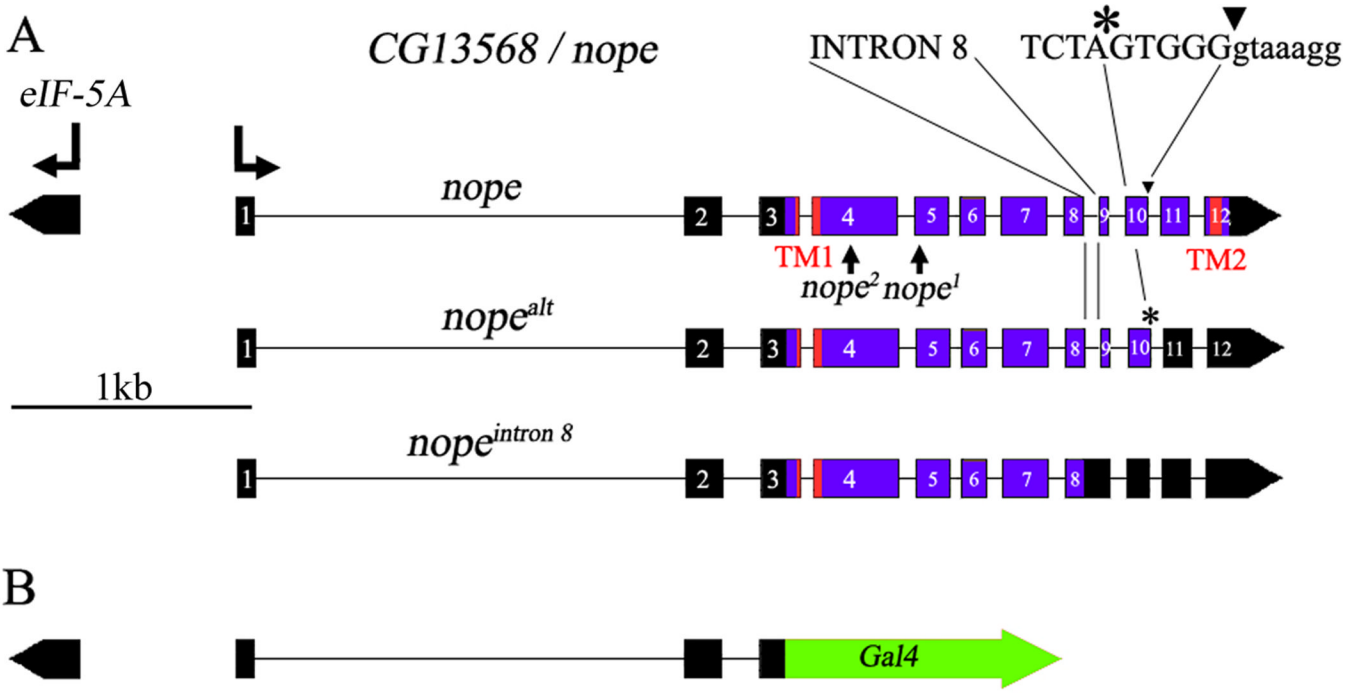

Figure 2. Schematic representation of the *nope* gene

A, The genomic region that includes *nope* and the divergently transcribed *eIF5A* is shown. A fully spliced *nope* transcript encodes a DEG/ENaC subunit of 425 amino acids with two conserved transmembrane domains (TM1 and TM2){Kellenberger, 2002 #960} {Tavernarakis, 2000 #10573}. Two other *nope* transcripts have alternative splicing patterns resulting in shorter open reading frames that encode truncated proteins lacking part of the extracellular domain, the second transmembrane domain and the carboxy-terminal intracellular domain (Berkeley Drosophila Genome Project and data not shown). In $nope^{alt}$, intron 10 is spliced to an alternative 5' splice site, 5 nucleotides upstream of the normal 5' splice junction, and intron 8 is retained in the $nope^{intron\ 8}$ transcript. The $nope^{1}$ and $nope^{2}$ alleles contain insertions of PiggyBac elements in exons 5 and 4, respectively, and are therefore also predicted to encode truncated Nope proteins.

B, The *nope-Gal4* transgene was generated by fusing the open reading frame of the yeast Gal4 transcriptional activator gene at the *nope* ATG. It contains approximately 3 kb of genomic sequences: 0.7 kb of intergenic region between *eIF5A* and *nope,* and 2.3 kb of 5' untranslated sequences of the *nope* precursor mRNA, up to the *nope* ATG, followed by the *Gal4* open reading frame and the polyadenylation site of SV40 (Elliott and Brand, 2008).

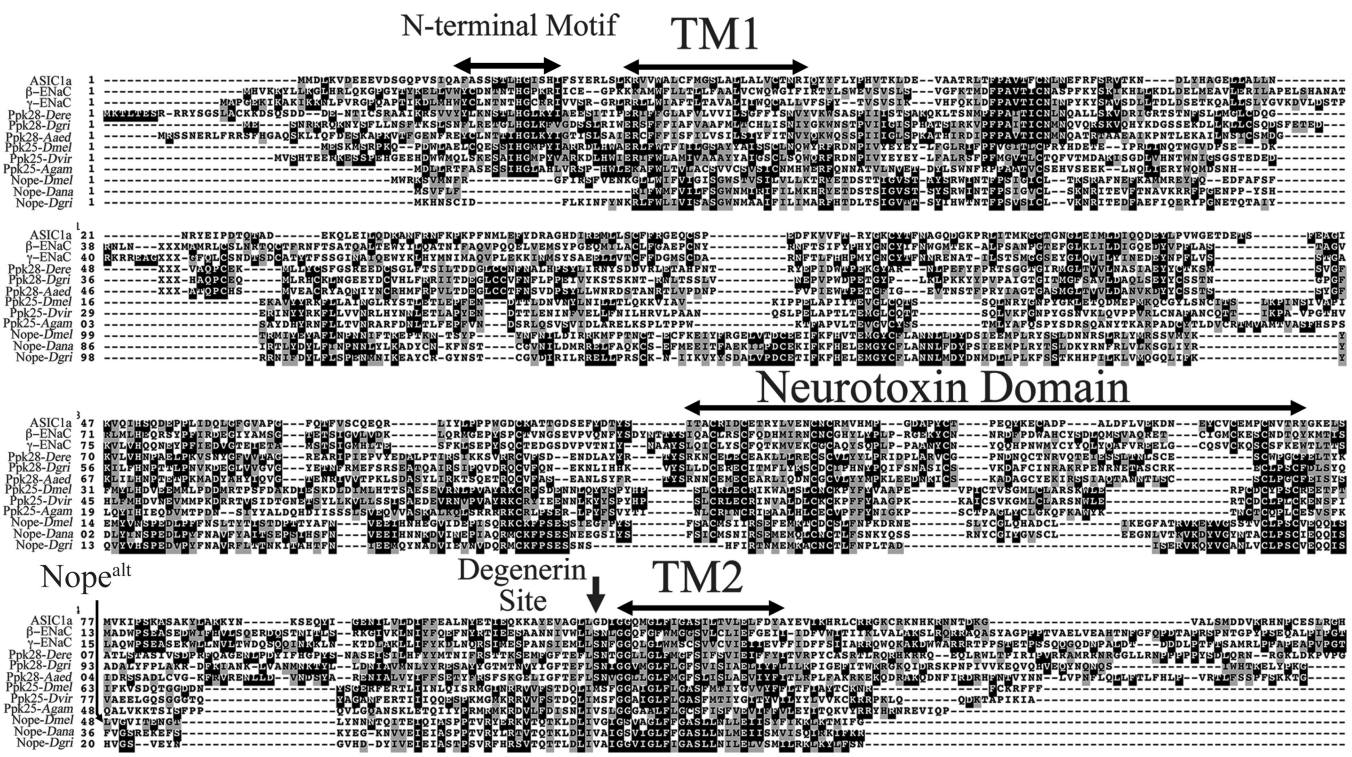

Figure 3. Nope is a member of the DEG/ENaC family of sodium channel subunits

Multiple sequence alignment displaying sequence conservation between Nope from *Drosophila melanogaster,* predicted Nope orthologs from other *Drosophila* species and other members of the family of DEG/ENaC sodium channel subunits from *Drosophila* and vertebrates. Identical and similar residues present in three or more of the aligned sequences are shaded in black and gray, respectively. Sequence conservation between Nope and other DEG/ENaCs is present throughout the protein, particularly in the first and second transmembrane domains (TM1 and TM2), and in a cysteine rich extracellular domain related to neurotoxins (Tavernarakis and Driscoll, 2000; Kellenberger and Schild, 2002). However, Nope and its orthologs in other *Drosophila* species lack a highly conserved and functionally important amino-terminal domain found in other DEG/ENaCs (Grunder et al., 1997; Coscoy et al., 1999; Grunder et al., 1999; Salinas et al., 2009; Carattino, 2011). Interestingly, as was observed for some other *Drosophila* DEG/ENaC subunits (Liu et al., 2003), Nope and its orthologs, as well as Ppk25 and its orthologs have bulky amino acid (Valine or Methionine) at the functionally important degenerin site, just amino-terminal to TM2. In vertebrate and *C. elegans* DEG/ENaC subunits, as well as in other *Drosophila* DEG/ENaCs such as Ppk28, this position always encodes residues with a small side-chain such as Gly, Ala or Ser, and mutations to amino acids with a larger side-chain, such as Leu, Thr or Lys result in constitutively open channels (Kellenberger and Schild, 2002). Nope^{alt} indicates the last amino acid of Nope that is retained in Nope^{alt}, a translation product resulting from alternative splicing of intron 10 that lacks TM2 and some preceding extracellular sequences, including the degenerin site. Sequences and accession numbers: ASIC1a, gi|94957760; β-ENaC, gi|8928561; g-ENaC, gi|6755414|. Ppk28-Dere, Ppk28-Dgri and Ppk28-Daaed correspond to annotated Ppk28 orthologs in *D. erecta* (GG19083), *D. grimshawii* (GH11924) and *Aedes aegypti* (AAEL000582). Ppk25-Dvir and Ppk25-Agam are annotated Ppk25 orthologs in *D. virilis* (GJ17577), and *Anopheles gambiae* (AGAP005516). Nope-Dana and Nope-Dgri are previously unannotated Nope orthologs encoded by the genomes of *D. ananassae* and *D. grimshawii*, respectively.

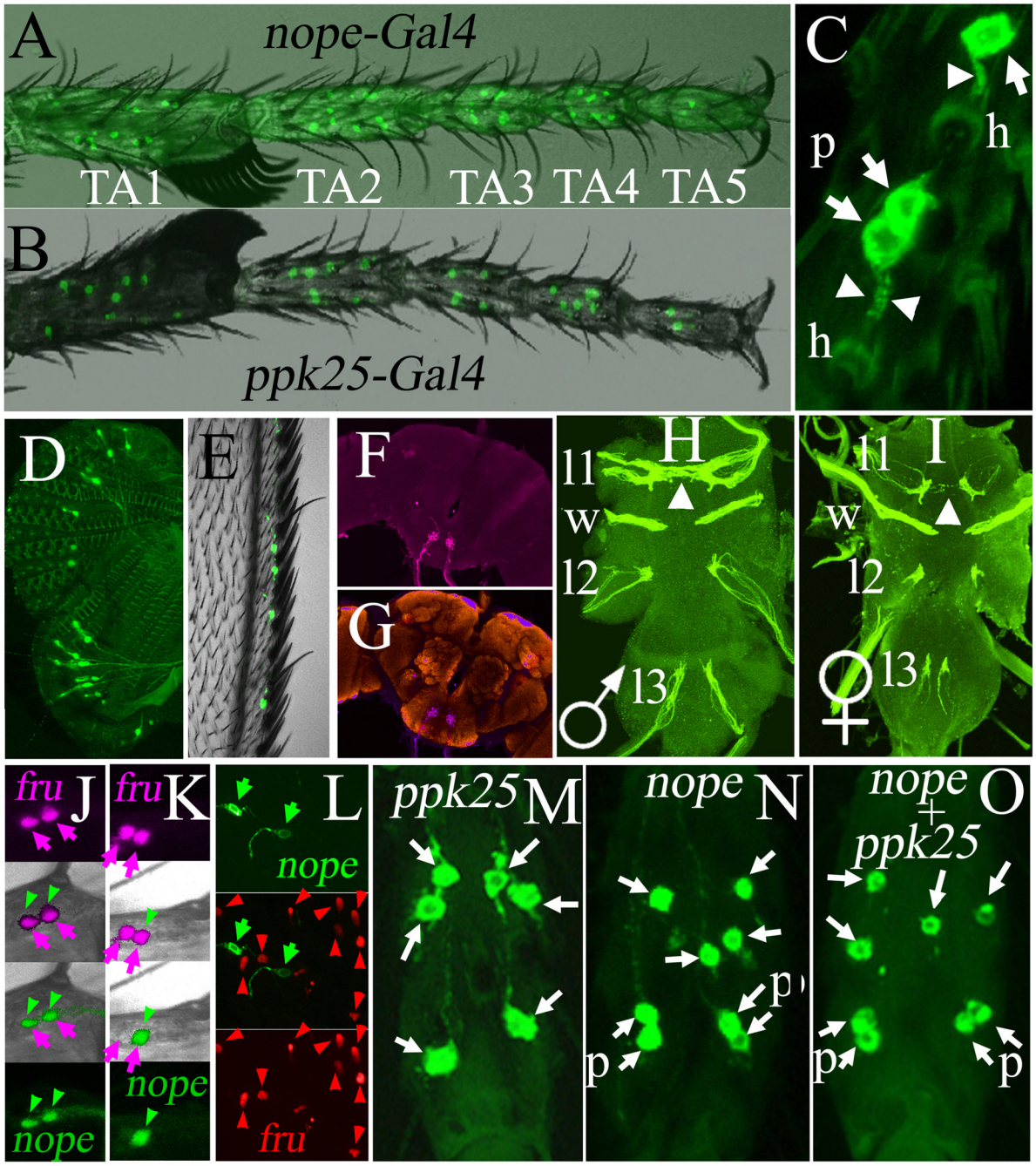

Figure 4. The *nope-Gal4* transgene is expressed specifically in gustatory neurons that also express *fru*, including all gustatory neurons that express *ppk25-Gal4*

GFP fluorescence was analyzed in males expressing mCD8-GFP under control of *ppk25-Gal4*, *nope-Gal4* or both drivers as indicated.

A, *nope-Gal4* and B, *ppk25-Gal4* are expressed in very similar patterns in gustatory neurons in the front legs of males (only the tarsi are shown, TA1-TA5 indicate tarsal segments 1–5). C, higher magnification image of three gustatory neurons expressing mCD8-GFP under control of *nope-Gal4* (arrows). Each neuron has a single sensory dendrite (arrowhead) that can be traced to the base of a taste hair (h). In the case of two neighboring neurons (labeled "p" for paired), the two dendrites run in parallel towards the base of the same taste hair.

D, E, expression of UAS-mCD8-GFP under control of *nope-Gal4* shows gustatory neurons in the labellum (D) and wings (E, superimposed to bright-field image).

F, G, whole-brain staining of male expressing mCD8-GFP under control of *nope-Gal4* using anti-GFP shows axonal projections of taste neurons onto the subesophageal ganglion. In G, anti-GFP staining is superimposed to staining with nc82, a monoclonal antibody that allows visualization of brain structures {Laissue, 1999 #575}.

H and I, whole mount staining of the ventral nerve chord with anti-GFP shows the axonal projections labeled by UAS-mCD8-GFP under control of *nope-Gal4* in males and females, respectively. Gustatory neurons on all three pairs of legs (l1, l2, l3) and on the wings (w) project to specific thoracic ganglia. Projections from the front legs onto the first thoracic ganglia cross the midline much more frequently in males than females (arrowheads).

J, K, imaging of taste neurons on the front legs of males expressing two nuclear fluorescent proteins shows that all *nope-Gal4* expressing neurons express *fru*; expression of nuclear GFP is controlled by *nope-Gal4* (green arrowheads), and expression of nuclear tdTomato is directed by *fru-LexA* (Mellert et al., 2009) (blue arrows). While *fru* is expressed in two gustatory neurons per taste hair (Mellert et al., 2009), *nope-Gal4* is expressed in both *fru*-expressing neurons in some hairs (J), or only one in others (K). L, double-labeling of the neurons on the labellum with membrane-bound mCD8GFP (green) under control of *nope-Gal4,* and nuclear tdTomato (red) under control of *fru-LexA* (Mellert et al., 2009). In contrast to the male front legs, expression of the two drivers on the labellum does not overlap.

M–O, Comparison of the number of taste neurons expressing mCD8-GFP under control of *ppk25-Gal4* (L), *nope-Gal4* (M), or both transgenes (N) on the third tarsal segment of male front legs (“p” indicates a pair of mCD8-GFP-expressing neurons associated with a single taste hair).

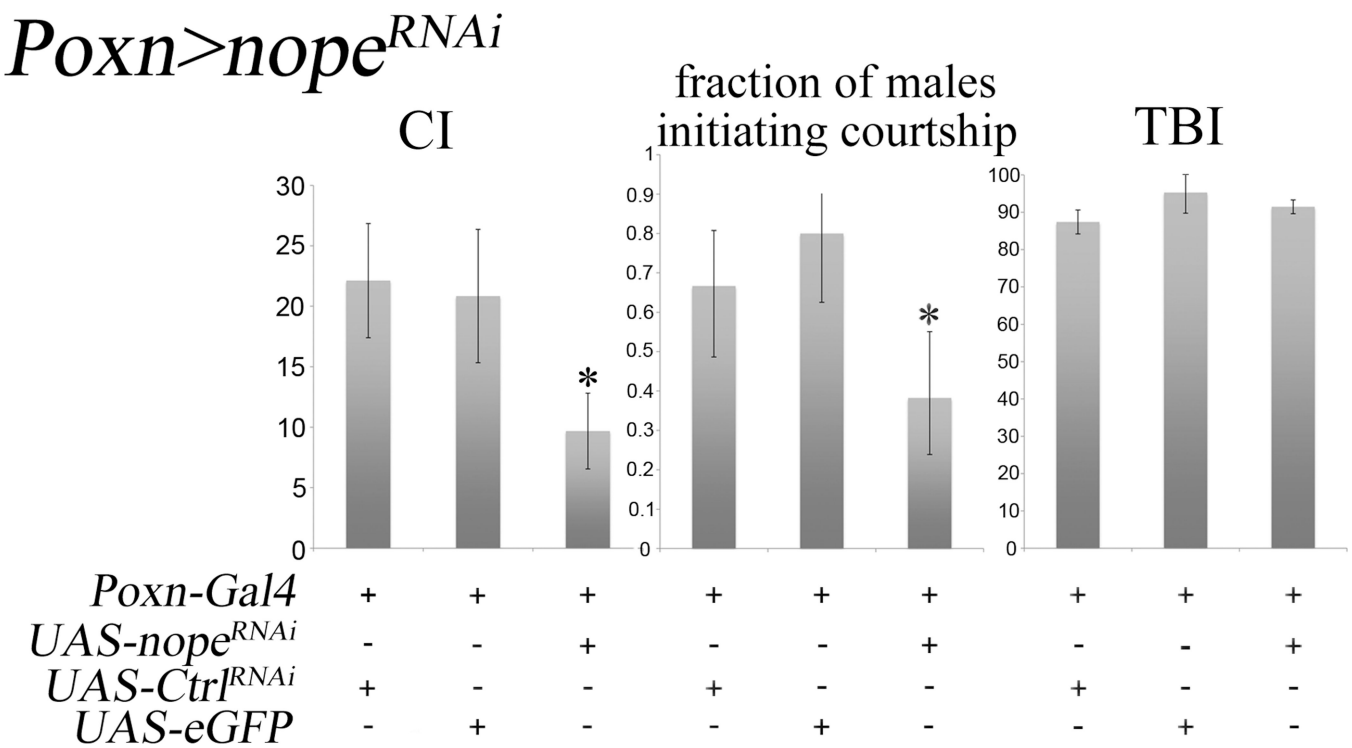

Figure 5. Gustatory-specific RNAi knockdown of *nope* impairs male courtship response to females

The gustatory-specific driver *Poxn-Gal4* (Boll and Noll, 2002) was used to drive expression of GFP, of an RNAi targeting *nope* (*nope*^{RNAi}), or of a control RNAi (*ctrl*^{RNAi}) targeting *CG13895*, a gene with no known involvement in courtship behavior or chemical senses (Benchabane et al., 2011). Error bars are SEM; N>30; *, $p<0.05$, (Wilcoxon rank-sum test for CI, and Chi square test for equality of distribution for the fraction of males initiating courtship).

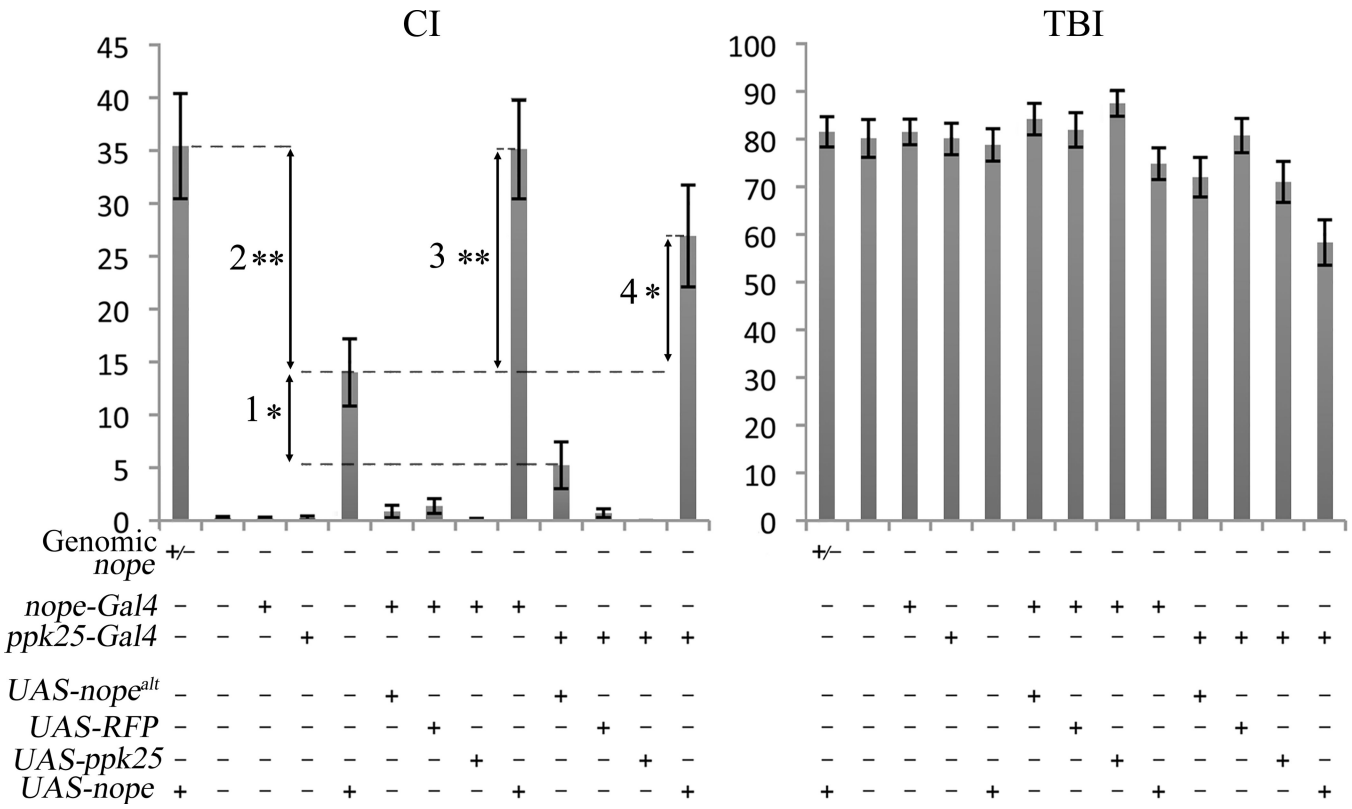

Figure 6. Targeted expression of *nope* but not of *ppk25* in a small subset of taste neurons in the legs and wings rescues the courtship defect of *nope* mutant males

The CI and TBI are shown for antennaless males containing the transgenes indicated and with the following genotypes at the endogenous *nope* gene: heterozygous males carrying one mutant and one wild-type copy of the *nope* gene, +/−; and $nope^1/nope^2$ mutant males, -. *nope* mutant males carrying the *UAS-nope* transgene display significantly more courtship than *nope* mutant males with other transgenes, but significantly less than male wild-type for *nope*. *nope* mutant males expressing *nope* under control of either *nope-Gal4* or *ppk25-Gal4* have significantly higher CIs relative to *nope* mutants with any other combination of transgenes. Vertical arrow 1 represents a statistically significant difference in CI between $nope^1/nope^2$ males carrying the *UAS-nope* transgene and other $nope^1/nope^2$ mutant males, while vertical arrow 2 represents a statistically significant difference between the latter and $nope^1/+$ controls. Vertical arrows 3 and 4 represent statistically significant differences in CI between $nope^1/nope^2$ males carrying the *UAS-nope* transgene and males of identical genotype except for the additional presence of either *nope-Gal4,* or *ppk25-Gal4*, respectively. Error bars are SEM, N>30, **, $p<0.01$, *, $p<0.05$ (Wilcoxon rank-sum test).

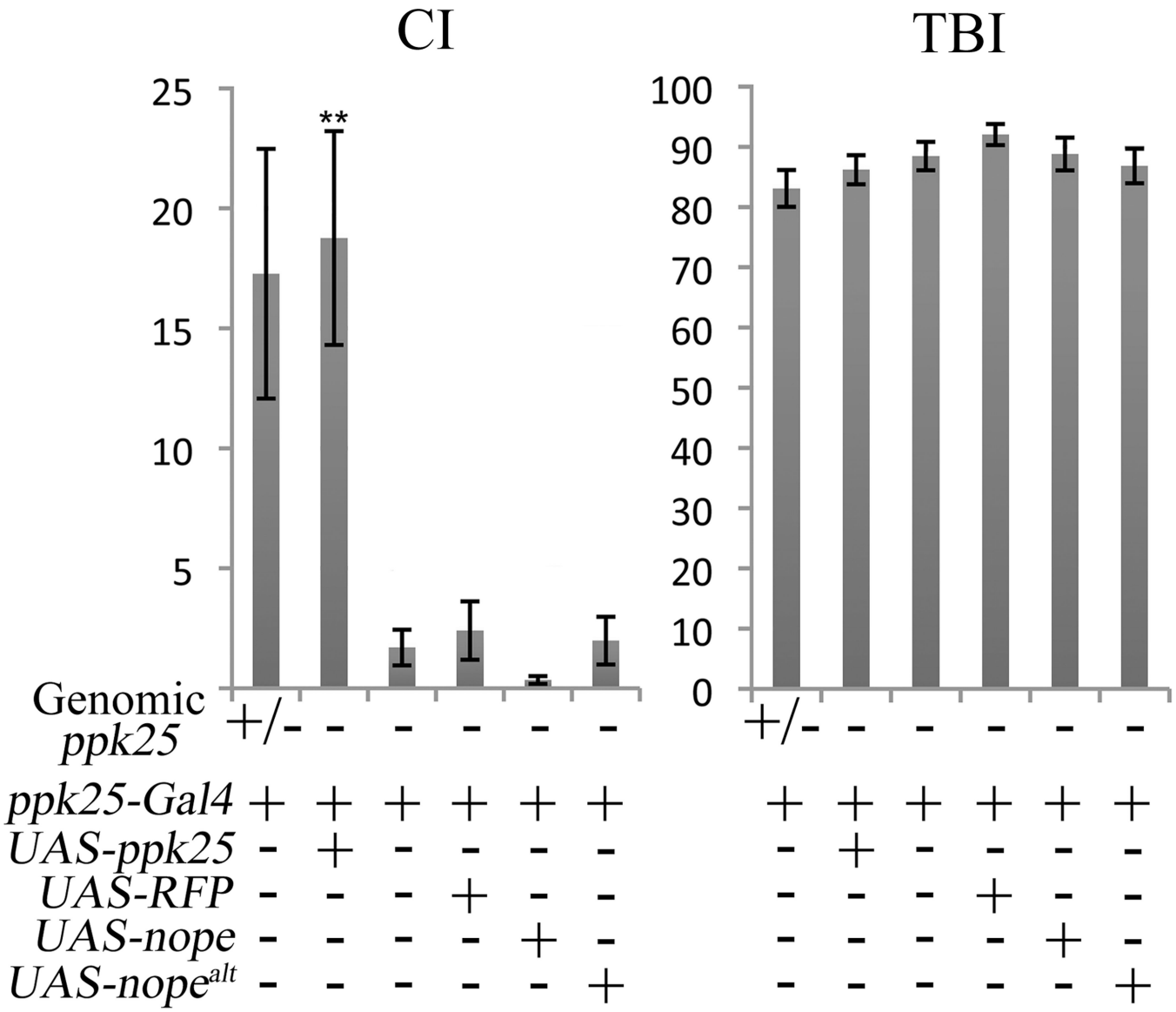

Figure 7. Targeted expression of *ppk25* but not *nope* rescues the courtship defects of *ppk25* mutant males

CI and TBI of antennaless males containing the transgenes indicated and with the following genotypes at the endogenous *ppk25* gene: heterozygous males carrying one mutant and one wild-type copy of the *ppk25* gene, +/−; and males heterozygous for two different deletions of the *ppk25* gene, -. As shown previously, (Lin et al., 2005; Starostina et al., 2012), *ppk25* mutant males display significantly lower CIs than controls, and the presence of both *ppk25-Gal4* and *UAS-ppk25* restores normal courtship levels. In contrast, the courtship of *ppk25* mutant males is not improved in the presence of both *ppk25-Gal4* and *UAS-nope* transgenes. N>30. Statistical significance relative to the CI of *ppk25* mutant males calculated with the Wilcoxon rank-sum test: **, $p<0.01$.

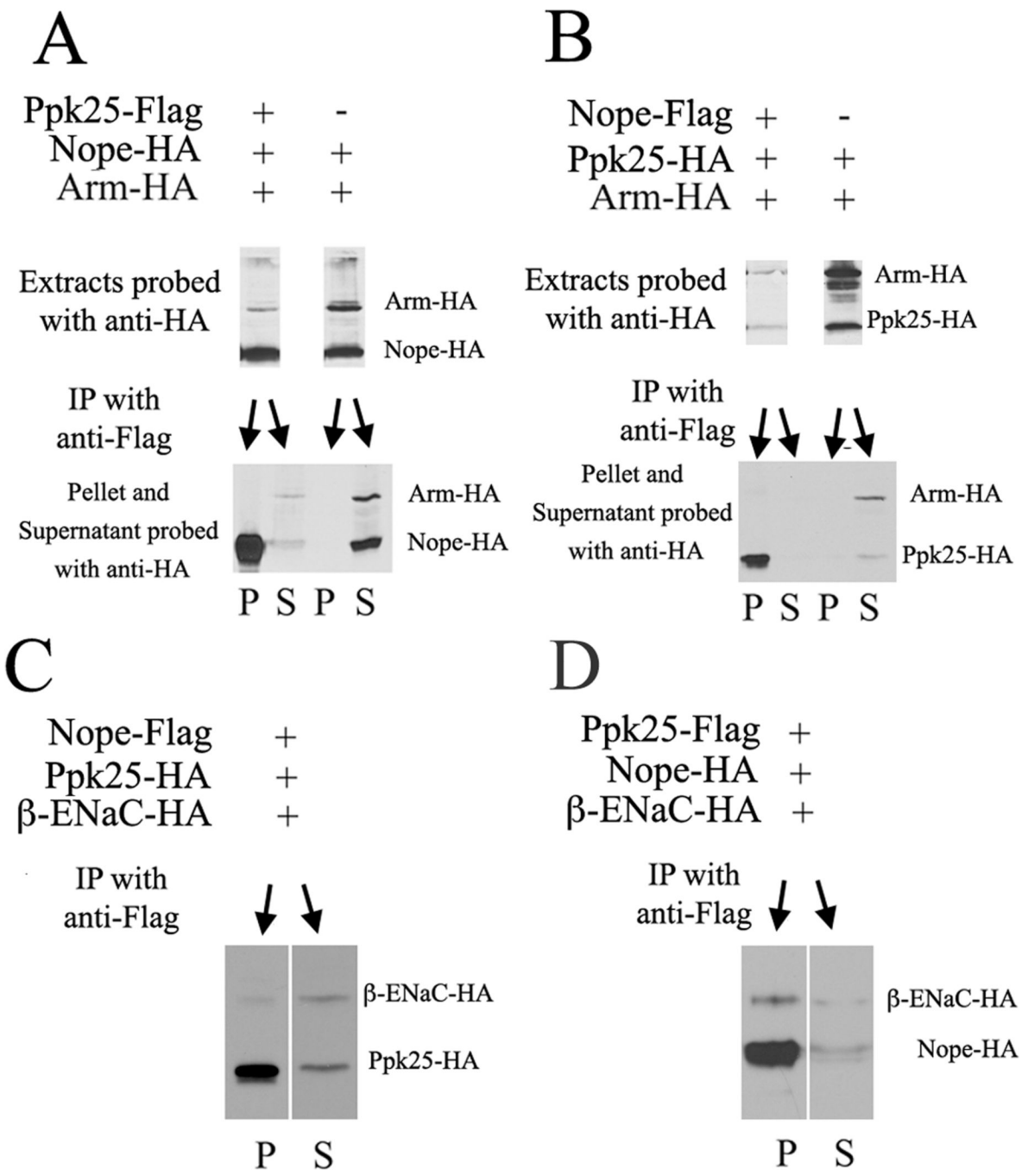

Figure 8. Nope and Ppk25 interact specifically in cultured cells

A, HEK293 cells were transfected with plasmids encoding Nope-HA, Arm-HA and, where indicated, Ppk25-Flag and immunoprecipitated using anti-Flag antibody. Extracts, supernatants (S), and precipitates (P) were analyzed on western blots with anti-HA antibody. B, Same as in A, except that Nope was tagged with Flag and Ppk25 with HA. C, Extracts of cells expressing Nope-Flag, Ppk25-HA and human β-ENaC-HA were immunoprecipitated with anti-Flag, and both supernatant and pellet were immunoblotted with anti-HA. The increased ratio of Ppk25-HA to β-ENaC-HA in the pellet compared to the supernatant indicates that Nope-Flag interacts preferentially with Ppk25-HA.

D, Same as in C, except that Ppk25 was tagged with Flag and Nope with HA. Here also, the ratio of Nope-HA to β-ENaC-HA is higher in the pellet than in the supernatant.

Table 1

Number of gustatory neurons in the legs of males expressing the *UAS-mCD8-GFP* transgene under control of either *nope-Gal4, ppk25-Gal4* or in the presence of both drivers

For males of each genotype, the total number of taste neurons as well as the number of neuron pairs associated with a single taste hair is shown for the tibia and tarsal segments 1 through 5. The numbers shown are typical for the majority of legs examined for each genotype (N>0), with occasional exceptions displaying an extra cell at a variable location.

Driver	***ppk25-Gal4***		***nope-Gal4***		***ppk25-Gal4 + nope-Gal4***	
	Neurons	**Paired neurons in a single hair**	**Neurons**	**Paired neurons in a single hair**	**Neurons**	**Paired neurons in a single hair**
Tibia	10	0	11	1	11	1
TA1	11	0	13	2	13	2
TA2	7	0	11	3	11	3
TA3	6	0	8	2	8	2
TA4	7	0	9	2	9	2
TA5	2	0	2	0	2	0
Total	43	0	54	10	54	10