Neprilysins: An Evolutionarily Conserved Family of Metalloproteases That Play Important Roles in Reproduction in *Drosophila*

Jessica L. Sitnik,* Carmen Francis,[†,‡,1] Korneel Hens,[†,‡] Roger Huybrechts,[§] Mariana F. Wolfner,[*,3] and Patrick Callaerts[†,‡,3]

*Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York 14853, [†]Laboratory of Behavioral and Developmental Genetics, KULeuven, 3000 Leuven, Belgium, [‡]VIB Center for the Biology of Disease, 3000 Leuven, Belgium, and [§]Zoological Institute, KULeuven, 3000 Leuven, Belgium

ABSTRACT Members of the M13 class of metalloproteases have been implicated in diseases and in reproductive fitness. Nevertheless, their physiological role remains poorly understood. To obtain a tractable model with which to analyze this protein family's function, we characterized the gene family in *Drosophila melanogaster* and focused on reproductive phenotypes. The *D. melanogaster* genome contains 24 M13 class protease homologs, some of which are orthologs of human proteases, including neprilysin. Many are expressed in the reproductive tracts of either sex. Using RNAi we individually targeted the five Nep genes most closely related to vertebrate neprilysin, *Nep1-5*, to investigate their roles in reproduction. A reduction in *Nep1*, *Nep2*, or *Nep4* expression in females reduced egg laying. *Nep1* and *Nep2* are required in the CNS and the spermathecae for wild-type fecundity. Females that are null for *Nep2* also show defects as hosts of sperm competition as well as an increased rate of depletion for stored sperm. Furthermore, eggs laid by *Nep2* mutant females are fertilized normally, but arrest early in embryonic development. In the male, only *Nep1* was required to induce normal patterns of female egg laying. Reduction in the expression of *Nep2-5* in the male did not cause any dramatic effects on reproductive fitness, which suggests that these genes are either nonessential for male fertility or perform redundant functions. Our results suggest that, consistent with the functions of neprilysins in mammals, these proteins are also required for reproduction in *Drosophila*, opening up this model system for further functional analysis of this protein class and their substrates.

PROTEASES play key roles in diverse physiological systems. One such family of metalloproteases, the M13 class of neutral endopeptidases, consists mainly of membrane-bound zinc proteases that are involved in the processing of neuropeptides and peptide hormones (reviewed in Turner *et al.* 2000; Turner *et al.* 2001; Bland *et al.* 2008). In mammals, seven members of this family have been identified, of which neprilysin (NEP) and endothelin converting enzyme (ECE) are the best studied. These proteins have been implicated in various diseases including cardiovascular disease (Segura and Ruilope 2011; Wick *et al.* 2011), Alzheimer's disease (Mulder *et al.* 2012; Klein *et al.* 2013), inflammation and inflammatory disorders (Wong *et al.* 2011), and cancer (Smollich *et al.* 2007; Maguer-Satta *et al.* 2011).

In addition to their role in disease, NEPs are essential for development and reproduction in mammals. The mammalian *Neprilysin-2*, called *NL1* in mice, is highly expressed in the testis. *NL1*-deficient males sire fewer pups, even though spermatogenesis appears to be unaffected (Carpentier *et al.* 2004). In females, NEP expression in the uterus is modulated by estrogen treatment in rats (Pinto *et al.* 1999) and during the estrogen/progesterone cycle in humans (Head *et al.* 1993). In female rats and mice, controlled degradation of tachykinins, particularly substance-P, by NEP in the uterus is essential for controlling uterine contractions at different stages of pregnancy; an inability to degrade tachykinins in the uterus is associated with a reduction in litter size

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[1]Present address: Quintiles Belgium SA, 1800 Vilvoorde, Belgium
[2]Present address: Centre for Neural Circuits and Behaviour, University of Oxford, Oxford, OX1 3SR UK
[3]Corresponding authors: Laboratory of Behavioral and Developmental Genetics, KULeuven, VIB Center for the Biology of Disease, Herestraat 49, Box 602, B-3000 Leuven, Belgium. E-mail: patrick.callaerts@cme.vib-kuleuven.be; Department of Molecular Biology and Genetics, Cornell University, 423 Biotechnology Bldg., Ithaca, NY 14853-2602. E-mail: mariana.wolfner@cornell.edu

(Pinto *et al.* 1999; Pintado *et al.* 2003). In rats, tachykinins and their receptors have been implicated in the regulation of luteinizing hormone (LH) release (Sahu *et al.* 1987; Sahu and Kalra 1992; Bonavera *et al.* 1994). In humans, loss of function in either the tachykinin, Neurokinin B (which is preferentially deactivated by neprilysin) , or its receptor NK3-R correlates with a failure to enter puberty (Rance *et al.* 2010; Young *et al.* 2010). The exact ways in which neprilysins act to help regulate these aspects of reproduction are still largely unknown.

While the diverse role of Neps and their substrates in mammals has been the target of intense investigation, in other organisms the functions of neprilysins in reproduction are less clear. Consistent with their mammalian counterparts, neuropeptides and peptide hormones (including tachykinins) play important roles in regulating reproductive success in most organisms studied to date. In the sea squirt, *Ciona intestinalis*, tachykinins regulate oocyte growth (Aoyama *et al.* 2012). Between mammals and the zebrafish, *Danio rerio*, the estrogen-dependent features of tachykinins and their receptors appear to be conserved (Biran *et al.* 2012). In the insects *Drosophila melanogaster* and *Tribolium castaneum*, the tachykinin-like neuropeptide, natalisin, plays a role in regulating mating and reproductive outcomes (Jiang *et al.* 2013). Finally, in both the locust *Locusta migratoria* and the cockroach, *Leucophaea maderae*, the functional cleavage of tachykinins by neprilysins in the brain is conserved (Isaac and Nässel 2003).

To understand the physiological roles of neprilysins in reproduction, and by extension the neuropeptides that they regulate, we focused on this gene family in the genetically tractable model *D. melanogaster*. The *D. melanogaster* genome has 24 NEP-like genes, most of which are actively transcribed (Coates *et al.* 2000; Chintapalli *et al.* 2007; Bland *et al.* 2008). However, little is known about their roles *in vivo*. Neprilysin-like activity has been detected in extracts of larval imaginal discs and of neuronal membranes from larval and adult heads of *Drosophila* (Isaac *et al.* 2002; Wilson *et al.* 2002). At least two *Drosophila* genes, *Nep2* (Bland *et al.* 2007) and *Nep4* (Meyer *et al.* 2009), are active proteases with specific substrate affinities that can be inhibited with the M13-specific peptidase inhibitors thiorphan and phosphoramidon. Nep2 has been shown to cleave locustatachykinin-1 (LomTK-1) and *Drosophila* tachykinins *in vitro* (Thomas *et al.* 2005). Roles for *Drosophila Nep2* in renal function and reproduction have been suggested based on its expression in Malpighian tubules and the reproductive organs of both sexes (Thomas *et al.* 2005; Chintapalli *et al.* 2007).

Here, we examined the phylogeny of *Drosophila* neprilysin proteins and analyzed the function and the expression patterns of a subfamily, containing *Neprilysin1 (Nep1)*, *Neprilysin2 (Nep2)*, *Neprilysin3 (Nep3)*, *Neprilysin4 (Nep4)*, and *Neprilysin5 (Nep5)*, whose expression pattern and sequence homology is most similar to the canonical mammalian neprilysin. Our mutational and RNAi studies revealed that neprilysins are important in males for maximizing egg laying in their mates as well as for regulating egg production and sperm use in mated females.

Materials and Methods

Sequence comparison and tree building

Protein sequences were downloaded from Flybase (Marygold *et al.* 2013) and aligned using Muscle (Edgar 2004), and the alignment was checked by eye in MEGA 5.05 (Tamura *et al.* 2011). The program ProML, part of the Phylip 3.69 suite, was used to make the tree (Felsenstein 2005) and it was visualized for publication using FigTree v. 1.3.1 (Rambaut 2010).

In situ hybridization

Fly culture: *yw* and Canton-S stocks were maintained on a standard diet (6.4% cornmeal, 5.2% molasses, 1.8% dextrose, 1.2% yeast, 1% propionic acid, 0.75% agar, 0.15% methyl-4-hydroxybenzoate in 1.5% ethanol) at 25° in plastic vials.

Egg collection: Flies were allowed to lay eggs for 17 hr on apple juice agar plates (3% agar, 5.5% sucrose, 2.5% EtOH, 1.25% glacial acetic acid in apple juice) supplemented with yeast paste in a 25° incubator. Adult flies were then removed, and the embryos were washed off the original plates with water and transferred to a nylon mesh. To remove remainders of the yeast paste and apple juice agar, the embryos were washed with water. Embryos were dechorionated, permeabilized, and fixed as described in Sullivan *et al.* 2000.

In situ hybridization: DIG RNA labeling: We used cDNA clones GH03315 (*Nep1-RB*), GH07643 (*Nep2*), RE48040 (*Nep3*), LD25753 (*Nep4*), and AT14086 (*Nep5*), from the *Drosophila* Genomics Research Center (DGRC), for preparation of probes. Overnight restriction digest at 37° was done with *Not*I and *Bst*BI for antisense and sense *Nep1* probe, *Eco*RI and *Xho*I for *Nep2*, *Not*I and *Asp*718I for *Nep3*, *Eco*RI and *Xho*I for *Nep4*, and *Sal*I and *Nru*I for *Nep5*.

Linearized template DNA was purified using QiaQuick PCR purification kit (Qiagen). RNA labeling was performed with the DIG RNA labeling kit (Roche) and 1 μg of purified DNA following the manufacturer's protocol. Probes were hydrolyzed to a desired length of 200 bases. The RNA transcripts were analyzed for size by formaldehyde agarose gel electrophoresis and ethidium bromide staining. The labeling efficiency was tested using DIG quantification test strips and control strips (Roche).

Tissue collection, fixation and hybridization: *Third instar larval tissue:* The posterior end of third-instar larvae was removed with forceps and the larvae were inverted to expose the brain, most of the imaginal discs, and parts of the gut and fat body.

Adult tissue: Adult abdomens were removed from the thorax and opened on the ventral side from anterior to posterior to expose all the tissues to the solutions. The thorax was separated from head and abdomen and the dorsal side of the cuticle was removed. For *in situ*

hybridization on adult brains, the proboscis and part of the cuticle and the air sacs were removed from isolated heads.

All dissected tissues were kept in PBT (PBS, 0.1% Tween 20) on ice for maximum 1 hr before fixation. Fixation was done on a shaking platform for 60 min at room temperature in 1 ml of 4% paraformaldehyde containing 0.1% sodium deoxycholate.

Embryos: Embryos were collected and fixed as in Sullivan *et al.* (2000). Before starting the proteinase K treatment, embryos were rehydrated in the following conditions for 10 min each: 25% PBT/75% MeOH; 50% PBT/50% MeOH; 75% PBT/25% MeOH; and 100% PBT.

All tissues were rinsed in 1 ml PBT and washed 5 × 5 min in 1 ml PBT after fixation or rehydration. Different tissues were incubated in a volume of 150 μl proteinase K mixture: inverted third-instar larvae, 15 μg/ml proteinase K for 2 min at 37°; adult abdomen, 15 μg/ml proteinase K for 3 min at 37°; adult thorax, 10 μg/ml proteinase K for 2 min at 37°; adult brain, 10 μg/ml proteinase K for 2 min at 37°; and whole-mount embryos, 40 μg/ml proteinase K for 3 min at RT. Prehybridization, hybridization, and detection were as described in Clements *et al.* (2008).

Fertility/fecundity assays and sperm competition

Fly stocks and media: All flies were raised at room temperature (23° ± 1°) in glass bottles on standard yeast–glucose media (Gligorov *et al.* 2013). Females were aged 3–5 days from eclosion in groups of 5–12 in glass vials with added yeast. Male flies were aged 3–5 days from eclosion in groups of 10–20 in glass vials on standard yeast–glucose media. The RNAi lines used for *Nep1*, *Nep2*, *Nep3*, *Nep4*, and *Nep5* were all obtained from the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl *et al.* 2007) and are identified in Supporting Information, Table S3. Knockdown of transcripts was confirmed by RT–PCR as previously described (Ram and Wolfner 2007) and quantified using Image-J (Schneider *et al.* 2012). A *Nep2* null allele, *Nep2Δ*, was generated by means of a deletion generator compound element as described in (Huet *et al.* 2002). The starting stock was *yw;;P* (Whyteside and Turner 2008) DG19304. Loss of transcript was confirmed by qRT–PCR.

Each RNAi line was crossed to *tubulin*-GAL4/TM3, Sb; the balancer siblings from each cross (UAS–Nep/TM3, Sb) were used as controls to minimize rearing effects. Controls for the other drivers, *n-syb*–GAL4, *slbo*–GAL4, and *Send1*–GAL4 were generated by crossing the VDRC background line w^{1118} to the indicated driver line. In the case of *Nep1* and *Nep4*, whose knockdown was lethal with *tubulin*-GAL4, a *tubulin*-GAL80$^{[ts]}$;*tubulin*–GAL4/TM3, Sb line was used instead to drive knockdown. Flies were raised at room temperature and shifted to 30° 3 days prior to eclosion. Adult flies were collected at room temperature and returned to 30° where they were aged for 3 days.

For *Nep1* and *Nep4*, initial experiments were performed using *hsp70*–GAL4 (HS–GAL4) to drive knockdown. *HS*–GAL4;UAS*Nep1(or Nep4)*RNAi (or control) males and females were aged for 3 days prior to heat shock as previously described. For heat shock, flies were moved to vials without food that contained a wet piece of Whatman paper, after which they were placed in a water bath at 37° for 1 hr. The heat-shocked flies were allowed to recover at room temperature in vials containing fresh food and were then mated 12 hr later for all assays in which they were used.

Fertility/fecundity assays: In all assays involving male fertility, we used 3- to 5-day-old Canton-S virgin females. Females were placed singly in glass vials with food and allowed access to an RNAi (or mutant) male or a control male. Pairs were watched to confirm that mating had occurred. The male was removed upon dismounting. Assays for the effects of the Neps on female fertility were performed the same way using 3- to 5-day-old Canton-S males as mates for either RNAi (or mutant) or control females.

After mating, individual females were housed on yeast glucose media for 24 hr after which each female was transferred to a fresh vial, and the eggs laid in the previous vial were counted as described in Gligorov *et al.* (2013) except that *Nep1* and *Nep4* RNAi and control females were housed at 30° for the entire experiment to ensure adequate suppression of GAL80. The progeny present in each vial were counted after eclosion. Hatchability was calculated as (#progeny/#eggs) except in the case of assays involving *Nep1* (line 1) and *Nep4* (line 1). For these two lines the UAS–RNAi construct is inserted on the second chromosome, allowing for the inheritance of both the UAS–RNAi construct and the *tubulin*–GAL4 driver together in the absence of the GAL80 suppressor. This occurs in 25% of the progeny, resulting in lethality. To calculate differences in hatching rate for all viable progeny the number of observed progeny was compared to the number of expected progeny. The number of expected progeny was calculated by day as ((# eggs) × (0.75, expected survival) × (average hatchability of eggs laid by control females (#progeny/#eggs))). Comparisons of single day and total egg, progeny production, expected progeny, and hatchability between control and experimental females were performed using a Wilcoxon nonparametric test and statistics comparing the overall 10-day trends were performed using a repeated measures ANOVA. All statistical analysis was performed with the Jmp9 software

Sperm competition

After mating, *Nep2Δ* or control females were individually housed for 3 days on yeast–glucose media after which each female was allowed access to a single bw^D male for 12 hr. After the bw^D male was removed, the females were transferred individually to fresh vials and allowed to lay eggs for 4 days before being transferred to fresh food vials and allowed to lay eggs for an additional 4 days. Because the *Nep2Δ* stock is in a *y w* background and the dominant bw^D eye color phenotype (brown) requires the presence of a w^+ allele to be scored, only female progeny who carried the w^+ allele from the male were scored for the presence of bw^D (provided by the second male) or red eyes (provided by the first male). P1 was calculated as (# progeny sired by the first

male)/(total progeny). Comparisons between the P1 of control and experimental females were performed using a one-way ANOVA and by Wilcoxon nonparametric tests.

Embryo collection and staining for development and sperm tails

For assaying the ability of eggs laid by *Nep2Δ* females to develop into embryos, we collected 1.5- to 3.5-hr-old eggs, fixed them using methanol/heptane, and stained them with DAPI, as described in Krauchunas *et al.* (2012). For DAPI staining, fixed embryos were incubated in PBS containing 1 μg/ml DAPI for 5 min and were washed 5 × 15 min in PBST. To assess the presence of sperm tails in eggs laid by *Nep2Δ* and control females we collected eggs laid in a 1-hr window at room temperature and prepared them as previously described except that rat anti-sperm tail antibody was used at a dilution of 1:800 instead (Karr 1991; T. Karr, ASU, personal communication). Images were collected using a Leica CTR5000 microscope (DAPI) (courtesy of Dan Barbash) or a Leica TCS SP2 confocal microscope (sperm tail).

Sperm counts

Nep2Δ or control mated females were frozen in liquid nitrogen at 2 hr ASM or kept in glass vials on yeast–glucose media for 4 days and then frozen. Frozen females were stored at −80° for <2 weeks before counting. Reproductive tracts were dissected and then stained with orcein (Neubaum and Wolfner 1999; Mueller *et al.* 2008; Avila *et al.* 2010). A transillumination microscope was used at 1000× magnification to visualize sperm. Comparisons between the number of sperm present in control and experimental females were performed using Wilcoxon nonparametric tests.

Results

Sequence and phylogenetic analysis of neprilysins

Twenty-four peptidase sequences encoded in the *D. melanogaster* genome are classified as M13 metallopeptidases based on gene prediction, sequence homology, and searches for known active site regions using the MEROPS database (Rawlings *et al.* 2012). We created a tree of all 24 M13 class proteins by comparing their protein sequence similarity (see *Materials and Methods*). Using this analysis, the M13 class proteins fall into three related groups, one of which contains Nep1, Nep3, Nep4, and Nep5, which have been previously shown to be the most closely related to mammalian Neps (Turner *et al.* 2001), and *Nep2*.

CNS and reproductive tract expression in both sexes is characteristic of the canonical mammalian neprilysin (Li *et al.* 1995; Ouimet *et al.* 2000). Thus, we mapped the known expression patterns (Fly Atlas; Chintapalli *et al.* 2007) for either the reproductive tract (RT) (as indicated by genome-wide microarray data determined in females for the ovaries and the spermathecae, and in males for the testes and the accessory glands) or the CNS onto the gene tree (Figure 1). All but two of the 24 genes (CG9507 and CG4580) have some expression in the RT of either sex. Most genes (19/24) show some expression in female reproductive tracts; only 8/24 are detectably expressed in male reproductive tract tissues. Fourteen genes show female RT expression only, 3/24 show male RT expression only, and 5/24 are expressed in both (Table S1). The high frequency of female RT expressed genes in this family suggests that the function of M13 class proteins is likely important in these tissues, but also suggests the possibility of functional redundancy, which could complicate genetic analysis. We decided to focus on the clade containing the candidate genes with the closest homology to mammalian neprilysins. Further, consistent with what is observed in mammals, this clade is also enriched for the somewhat rarer pattern of male RT expression, as well as expression in the CNS.

We characterized the five genes in this clade: *Nep1 (CG5905)*, *Nep2 (CG9761)*, *Nep3 (CG9565)*, *Nep4 (CG5894)*, and *Nep5 (CG6265)*. Figure 2 shows a schematic representation of neprilysin and a sequence alignment of the different functional motifs of *D. melanogaster* Nep1–Nep5, the ECE homolog of *L. migratoria* (LomECE), and *Homo sapiens* ECE-1, ECE-2, and neprilysin. A full sequence alignment and phylogenetic analysis can be found in Figure S1 and Figure S2.

Expression patterns of Nep1–5

FlyAtlas (Chintapalli *et al.* 2007) and RNAseq (Celniker *et al.* 2009) data suggested that *Neps1–5* are expressed throughout development in a variety of tissues. To gain a more precise understanding of the locations and timing of these genes' expression patterns, we performed *in situ* hybridization to look for the expression of each NEP gene in embryos, larvae, and adult flies. Sense-strand hybridizations were used as controls (Figure S3). A summary of the expression data can be found in Table S2.

Embryonic expression of Nep1–5

Two of the genes (*Nep3* and *Nep4*) were expressed before embryonic stage 17 (Figure 3). *Nep4* RNA was detected as early as stage 12 in two patches of cells per hemisegment (Figure 3A). These have been reported independently to correspond to muscle founder cells (Meyer *et al.* 2009). In stage 13, *Nep4* is expressed in two rows of cells that border the amnioserosa (Figure 3B), which we identify as the pericardial cells. These cells flank the aligned cardioblast cells of the dorsal vessel. This staining is visible from stage 13 until stage 16 when dorsal closure is finalized and the cardioblasts of each side fuse. At stage 14 *Nep3* is expressed generally in the central nervous system (Figure 3C). This staining is visible until stage 17. *Nep4* expression can also be detected in the brain and ventral nerve cord of stage 14 to stage 17 embryos (Figure 3, D and K). The staining is localized in cells along the longitudinal connectives and transversal commissures of the ventral nerve cord.

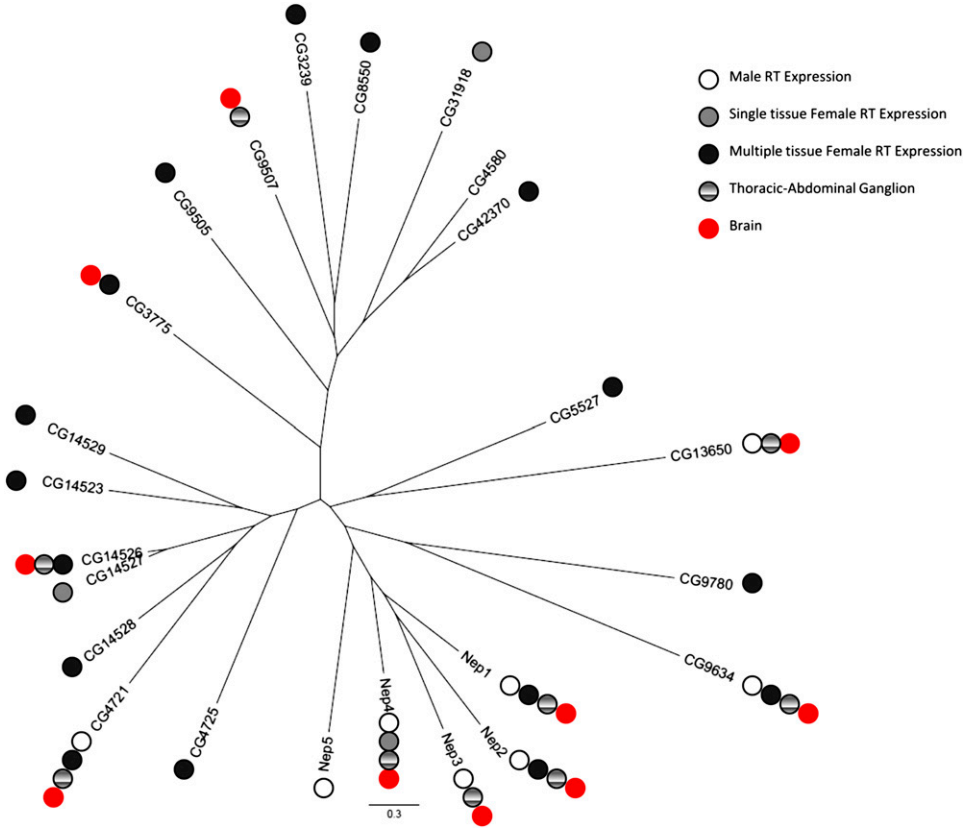

Figure 1 Phylogeny and expression of *Drosophila* neprilysins. A phylogenetic tree of the 24 known *D. melanogaster* M13 class proteases based on protein sequence similarity. The proteins fall into three distinct clades. Mapping the reproductive tract expression of each gene onto the tree reveals broad expression in the female RT (shaded and solid) and enrichment of male expression (open) in the clade that contains Nep1–5. The same clade also demonstrates enrichment for brain (red) and abdominal-thoracic ganglion expression (hatched).

All five of these *Nep* genes are expressed in stage 17 embryos. *Nep1* is expressed in neurons of the peripheral nervous system on the left and right side of the embryo (Figure 3, E and F), and in the antenno-maxillary complex, which is part of the peripheral nervous system and located at the anterior side of the embryo, in front of the first thoracic segment. *Nep1* RNA was also detected in the anterior of the pharynx and in cells of the embryonic midgut. *Nep2* is strongly expressed in the tracheal system including in the dorsal trunk and the dorsal branches (Figure 3G). In the intestinal tract *Nep2* is expressed in the foregut (Figure 3, H and I). *Nep2* expression can also be detected in the hindgut (Figure 3H) and epidermis (Figure 3I). *Nep3* expression remains in the CNS where it becomes more intense in the brain hemispheres (Figure 3J). *Nep4* expression is detectable in the dorsal trunk and dorsal branches of the tracheal system and continues to be detected in the brain and ventral nerve cord (Figure 3K). Expression of *Nep5* is restricted to four small groups of cells at the anterior of stage 17 embryos (Figure 3L).

Larval expression patterns of Nep1–5

Nep1–4 are expressed in the nervous system of third instar larvae (Figure 4). *Nep1* is expressed strongly in the mushroom bodies of the brain, neurons in the *pars intercerebralis*, and neurons in the ventral ganglia (Figure 4A). *Nep2* is expressed in three neurons of both hemispheres of the larval brain and a limited number of six neurons in the ventral ganglia (Figure 4B). Similar to the expression of *Nep3* in embryos, a strong general staining of *Nep3* is detected in the larval brain hemispheres and ventral ganglia. In the hemispheres, the staining is more intense in the central part compared to that in the optic neuropils (Figure 4C). The expression of *Nep4* in third instar larvae is restricted to the central nervous system. Based on the size of the cells that are stained in the brain and ventral ganglia we identify the *Nep4*-expressing cells as glia (Figure 4D), consistent with a previous report of colocalization of Nep4 with the glial marker Repo (Meyer *et al.* 2009).

Outside the CNS, Neps are expressed in the gut and the Malpighian tubules, as well as in developing wing, leg, and eye-antennal discs. More specifically *Nep1* expression is detected in cells of the midgut (Figure 4E), wing disc (Figure 4I), and leg disc (Figure 4J). *Nep2* remains expressed in the foregut, but only in a limited number of cells of the proventriculus (Figure 4F). In the eye-antennal disc *Nep2* is expressed anterior to the morphogenetic furrow in the undifferentiated precursor cells of the eye disc and more generally in the antennal part (Figure 4K). In the leg discs *Nep2* is expressed in the outer concentric ring, giving rise to the first two segments of the fly leg, and in the central part of the disc (Figure 4L). *Nep2* is also expressed in the stellate cells of the larval Malpighian tubules (Figure 4G), which perform excretory and osmoregulatory functions analogous to vertebrate renal tubules (Dow and Romero 2010). *Nep3* expression is detected in a small number of cells in the larval midgut (Figure 4H). We did not detect expression of *Nep5* above background level in third-instar larval tissues.

B

	NAY/FY	HEXXH	EXXXD	CXXW
Dmel_Nep4	NAYY	HELTH	ENIAD	CSVW
Dmel_Nep3	NAYY	HELTH	ENIAD	CEVW
Lmig_ECE	NAYY	HELTH	ENIAD	CEVW
Hsap_ECE1	NAYY	HELTH	ENIAD	CEVW
Hsap_ECE2	NAYY	HELTH	ENIAD	CEVW
Hsap_Nep	NAFY	HEITH	ENIAD	CRVW
Dmel_Nep1	NAYY	HEITH	ENIAD	CSVW
Dmel_Nep2	NAYY	HEITH	ENIAD	CEVW
Dmel_Nep5	NAYH	HELTH	ENIAD	CRIW

Figure 2 Conserved binding motifs in *Drosophila*, human, and locust neprilysins. (A) Schematic representation of neprilysin. Solid, cytoplasmic domain; light shading, transmembrane domain; dark shading, extracellular domain. NAYY/F, important for substrate binding; HE*xx*H, zinc-binding domain; E*xxx*D, zinc-binding domain; C*xx*W: sequence critical for protein folding and maturation of the enzyme. (B) Alignment of NAY/FY, HE*xx*H, E*xxx*D, and C*xx*W sequences of *D. melanogaster Nep1-5*, *L. migratoria* ECE, and *H. sapiens* ECE1-2.

Adult expression patterns for Nep1-5

Consistent with its larval tissue expression pattern, *Nep1* is expressed in the mushroom bodies and neurons of the *pars intercerebralis* of the adult brain (Figure 5A) and in cells of the adult midgut (Figure 5D). In the male reproductive organs *Nep1* is expressed at the end of the testicular tube near and in the seminal vesicles (Figure 5, G and H). *Nep2* is detected in neurons of the *pars intercerebralis* and in a limited number of cells in the optic lobes of the brain (Figure 5B). In the ventral ganglion a few neurons also show expression of *Nep2* (Figure 5C). In the male reproductive organs *Nep2* is expressed in cells at the end of the testicular tube where it meets the seminal vesicle (Figure 5I). In the female gonad, strong staining was detected in posterior polar cells and in border cells of stage 8, 9, and 10 follicles (Figure 5F). By means of immunohistochemistry we also detected *Nep2* expression in the spermatheca (See Figure S4). As in larvae, *Nep2* is expressed in the adult Malpighian tubules and more specifically in the stellate cells, which are located between the principal cells of the Malpighian tubules (Figure 5E). No expression of *Nep3* above background level was detected in adult tissues despite previous reports of broad expression (Chintapalli *et al.* 2007; Celniker *et al.* 2009). The expression of *Nep4* in adult flies is restricted to the male gonads. Expression of *Nep4* is detected in different parts of the testicular tubes (Figure 5, J–L). In the apex of the testis the localization of the staining corresponds to the somatic cyst cells that surround the spermatocytes in this part of the tube. *Nep4* is also expressed at the end of the testis close to the contact with the seminal vesicle, in cells other than the somatic cyst cells. As is true for *Nep4*, the expression of *Nep5* in adult tissues is also restricted to the male gonads, more specifically in the membrane of the seminal vesicles where mature spermatids are stored after transport from the testicular tubes (Figure 5M).

Nep1 plays a role in regulating male fertility

Combined with the previously reported expression patterns from Fly Atlas and modENCODE, our data showed that in adult flies, *Nep4* and *Nep5* are expressed predominantly or exclusively in the male reproductive tract. Conversely *Nep1* and *Nep2* are expressed at high levels in the reproductive tracts of both sexes as well as in the CNS (Table S1). We confirmed *Nep3* expression in the male by RT–PCR (Figure S5). To test if any of these genes are essential for male fertility we generated RNAi-mediated knockdown males. For *Nep2*, *Nep3*, and *Nep5* this was done by crossing the appropriate VDRC line to the ubiquitous driver *tubulin*–GAL4. Since knockdown of *Nep1* and *Nep4* was lethal for both sexes using *tubulin*–GAL4, we used *tubulin*–GAL80$^{[ts]}$ to suppress GAL4 function during development (Elliott and Brand 2008; Southall *et al.* 2008). We confirmed knockdown using RT–PCR (Figure S5 and Table S3). Control or knockdown males, in parallel, were mated to virgin females. Egg production and fertility was measured daily over a 10-day period for each female. Mates of *Nep1* knockdown males laid significantly fewer eggs than mates of control males (Figure 6A). No differences were seen in the total number of eggs produced by females mated to *Nep2*, *Nep3*, *Nep4*, or *Nep5* knockdown males compared to controls (Figure 6A). Mates of *Nep2* knockdown males showed a trend toward reduced fertility. We obtained and tested males from a *Nep2* deletion line to clarify the trend observed in mates of *Nep2* knockdown males. Mates of *Nep2* null males showed no difference in egg laying from controls, suggesting that Nep2 from the male is not essential for stimulating egg laying in females.

While a *Nep1* mutant line exists (Spradling *et al.* 1995; Spradling *et al.* 1999; Bellen *et al.* 2004), it is not a null allele, so we used an alternative RNAi line to verify the egg-laying defects seen in mates of *Nep1* knockdown males (Figure S6A). Similar results for mates of *Nep1*RNAi males were obtained using the conditional ubiquitous driver *hsp70*–GAL4 (HS–GAL4) (Brand and Perrimon 1993) (data not shown). An alternative line for *Nep4* was also tested (Figure S6A). Alternative lines obtained for *Nep5* did not knock down and alternative *Nep3* lines were not available at the time of this study.

The proportion of progeny that eclosed from eggs laid by females (hatchability) mated to *Nep2*, *Nep3*, and *Nep5* knockdown males was comparable to those of controls (Figure 6B). Some small differences in egg laying were observed for *Nep3* knockdown males within the first 24 hr after mating, but these differences were not consistently reproducible.

To determine whether Nep1 or Nep4 is essential for hatchability, we used the *tubulin*–GAL80$^{[ts]}$;*tubulin*–GAL4/

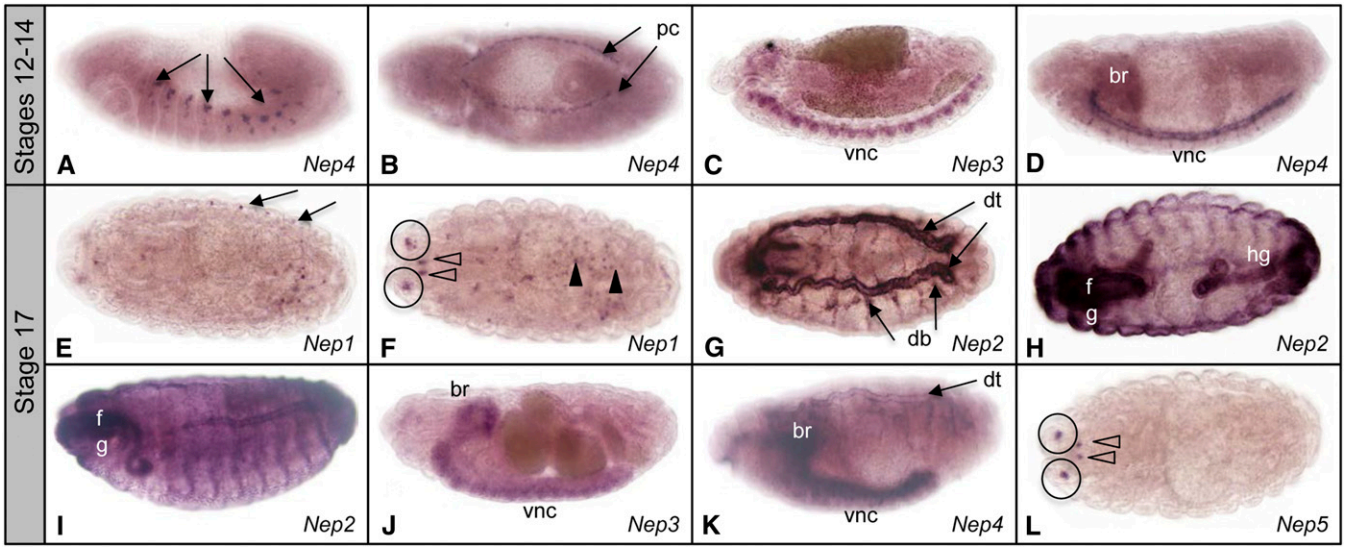

Figure 3 Embryonic expression pattern of *neprilysin* genes. (A and B) Embryonic stage 12 Nep4 expression in muscle founder cells (arrows in A) and in pericardial cells (pc). (C) Embryonic stage 13 *Nep3* expression in the ventral nerve cord (vnc). (D) Embryonic stage 14 *Nep4* expression in brain (br) and ventral nerve cord (vnc). (E and F) Embryonic stage 17 *Nep1* expression in peripheral nervous system (arrows), antenno-maxillary complex (circled), and cells in the pharynx (open arrowheads) and midgut (solid arrowheads). (G–I) Embryonic stage 17 *Nep2* expression in dorsal trunk (dt), dorsal branches (db), foregut (fg), and hindgut (hg). (J) Embryonic stage 17 *Nep3* expression in brain (br) and ventral nerve cord (vnc). (K) Embryonic stage 17 *Nep4* expression in brain (br), ventral nerve cord (vnc), and tracheal dorsal trunk (dt). (L) Embryonic stage 17 *Nep5* expression in antenno-maxillary complex (circled) and in the pharynx (open arrowheads).

TM3,Sb driver used to produce viable *Nep1* and *Nep4* (see *Materials and Methods* for details) and compared the expected progeny ((#eggs laid) × (expected maximum hatch rate (0.75)) × (average hatching rate for controls per day)) to the observed progeny. In both cases, no difference was seen between the expected progeny and those observed (Figure 7C) suggesting that *Nep1* and *Nep4* are not required for hatchability in mates of RNAi males. We used an alternative *Nep4* line to verify these results (Figure S6B). Mates of males knocked down using the alternative *Nep1* RNAi line (Nep1–39759) lay few (most <10) eggs, so we calculated total hatchability over the entire 10-day period as (# total progeny/#total eggs). There was a significant reduction in hatchability for mates of these males (Figure S6B); however, we believe this is due to the insertion site on the third chromosome (see legend to Figure S6).

In summary, *Nep2*, *Nep3*, *Nep4*, and *Nep5* do not appear to be uniquely essential for male fertility. They may not be essential for the specific traits measured here, or they may have redundant functions. Reduced *Nep1* expression in males does consistently affect egg laying in their mates suggesting a role for neprilysins in the reproductive performance of *Drosophila* males.

Nep1, Nep2, and Nep4 are essential for female fertility

Nep1, *Nep2*, and *Nep4* are expressed in the CNS and in the female reproductive tract, based on our expression data (Figure 5) and Fly Atlas (Chintapalli *et al.* 2007). To assess the role of these Neps in female fertility, we knocked down *Nep1* and *Nep4* ubiquitously, using *tubulin*–GAL80$^{[ts]}$;*tubulin*; GAL4 and *Nep2* using *tubulin*–GAL4. We then mated control and knockdown females, in parallel, to Canton-S males and tracked their egg laying over a 10-day period. Knockdown was confirmed via RT–PCR (Figure S5 and Table S3). *Nep1*, *Nep2*, and *Nep4* RNAi females laid significantly fewer eggs than controls laid over the entire 10-day period (Figure 7A). Similar results were obtained using alternate RNAi lines for *Nep1* and *Nep4* (Figure S6A), as well as the *Nep2* null mutant line (Figure 7A). Nep1 results were also confirmed using the conditional ubiquitous driver *hsp70*–GAL4 (HS–GAL4) (data not shown).

To assess whether *Nep1* or *Nep4* are essential for hatchability we compared numbers of expected to observed progeny. In both cases there was no difference in the overall number of progeny across the 10-day period (Figure 7B). However, fewer progeny than expected were observed for *Nep1* RNAi females in the first 2 days after mating (Figure 7B). Similar phenotypes for *Nep1* RNAi females in early hatchability were seen using the *hsp70*–GAL4 driver (data not shown). Similar to our observations in males, the alternative line Nep1–39759 RNAi females showed a more severe phenotype, with a reduction in hatchability across all 4 days measured (Figure S6B). Females knocked down for *Nep4* using the alternative RNAi line Nep4–16668 also showed significant decreases in hatchability (Figure S6B) possibly due to increased knockdown (Table S3) or background effects (see legend to Figure S6).

The proportion of eggs laid by *Nep2* RNAi females that become adult progeny is greatly reduced compared to that of control females (20% in RNAi females *vs.* 80–90% in controls) (Figure 7C). It was not possible to calculate hatchability

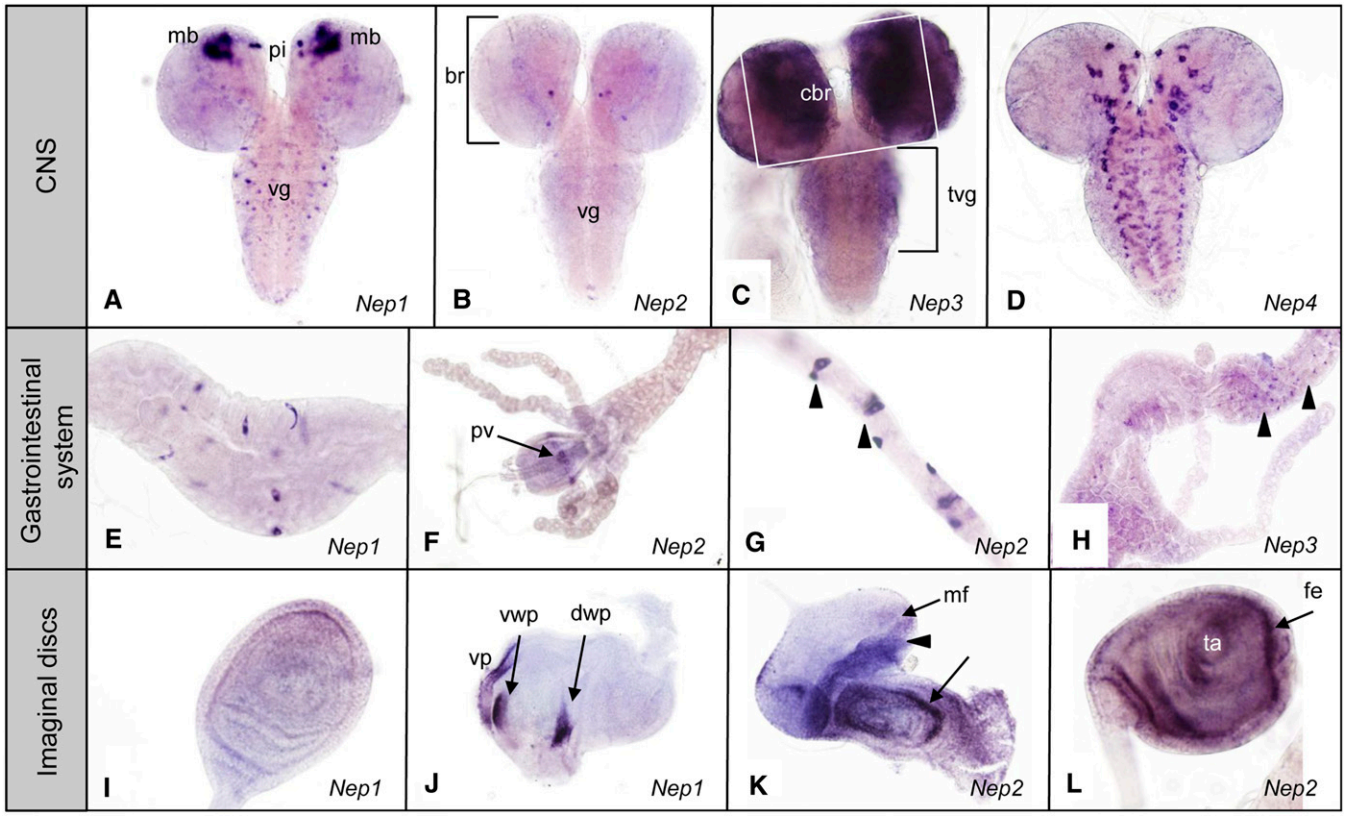

Figure 4 Larval expression pattern of *neprilysin* genes. (A–D) Expression of *Nep1–4* in larval CNS. (A) *Nep1* in mushroom bodies (mb), *pars intercerebralis* (pi), and ventral ganglia (vg). (B) *Nep2* in few cells in the brain (br) and in the ventral ganglia (vg). (C) *Nep3* in central brain (cbr) and thoracic ventral ganglia (tvg). (D) *Nep4* in glia of the larval CNS. (E–H) Expression of *Nep1–3* in the larval gastrointestinal system. (E) *Nep1* in cells in the midgut. (F–G) *Nep2* in the proventriculus (pv) and in the stellate cells of the Malpighian tubules (arrowheads). (H) *Nep3* in scattered cells in the larval midgut (arrowheads). (I–L) Expression of *Nep1* and *Nep2* in larval imaginal discs. (I) Nep1 in leg disc. (J) *Nep1* in dorsal and ventral wing pouch (dwp–vwp) and ventral pleura (vp) of the wing disc. (K) *Nep2* in the eye disc anterior (arrowhead) to the morphogenetic furrow (mf) and in the second antennal segment (arrow). (L) *Nep2* in the leg disc femur (fe) and tarsus (ta).

for the entire 10-day period, since egg laying reached zero for all Nep2 RNAi females by day 5. The reduction in hatchability could reflect roles for *Nep2* in the polar and/or border cells of the follicular epithelium during oogenesis (Figure 5F) and/or roles during embryogenesis where it is also expressed (Bland *et al.* 2007). Similar results were seen using females that were homozygous for a null mutation of *Nep2* (Figure 7C). Together these results indicate that *Nep1*, *Nep2*, and *Nep4* play essential roles in regulating female fertility and fecundity.

Characterization of the hatchability defects in Nep2 null mutants

To test whether the hatchability defect observed for *Nep2* mutant females was due to a failure of the eggs to be fertilized, we examined early embryos (0–1 hr) laid by these females for the presence of a sperm tail (Karr 1991). There was no difference in the percentage of fertilized embryos laid by *Nep2* null females compared to controls (Figure 8A). We therefore tested whether *Nep2* is important for early embryogenesis by staining 1.5- to 3.5-hr-old embryos laid by *Nep2* null females or controls with DAPI and scoring for their stage of embryonic development. While nearly all of the eggs laid by control females contain developing embryos, significantly more of the eggs laid by *Nep2* nulls (close to 50%) contained only a clear polar body rosette (Figure 8B). The presence of a polar body rosette is typical for activated but unfertilized eggs (Figure 8C) whereas the typical developing embryo at this time range was observed to be at stage 4 (Figure 8D). Since there is no difference in fertilization rate between eggs laid by *Nep2* null females and controls, our data suggest that some of the eggs laid by *Nep2* null females are able to activate and complete meiosis but fail to develop further. This fraction of arrested eggs observed for *Nep2* mutant females is consistent with the magnitude of the hatching defects seen above (Figure 7) and suggests that *Nep2* plays a role in egg laying and that loss of Nep2 in the female has an effect on very early embryogenesis.

Tissue-specific contribution to egg laying and hatchability effects for Nep1 and Nep2 females

The egg-laying defects in *Nep1* and *Nep2* RNAi females suggested that one or more of the tissues in which we detected Nep expression must be essential for egg laying. Both *Nep1* and *Nep2* are highly expressed in the CNS and the

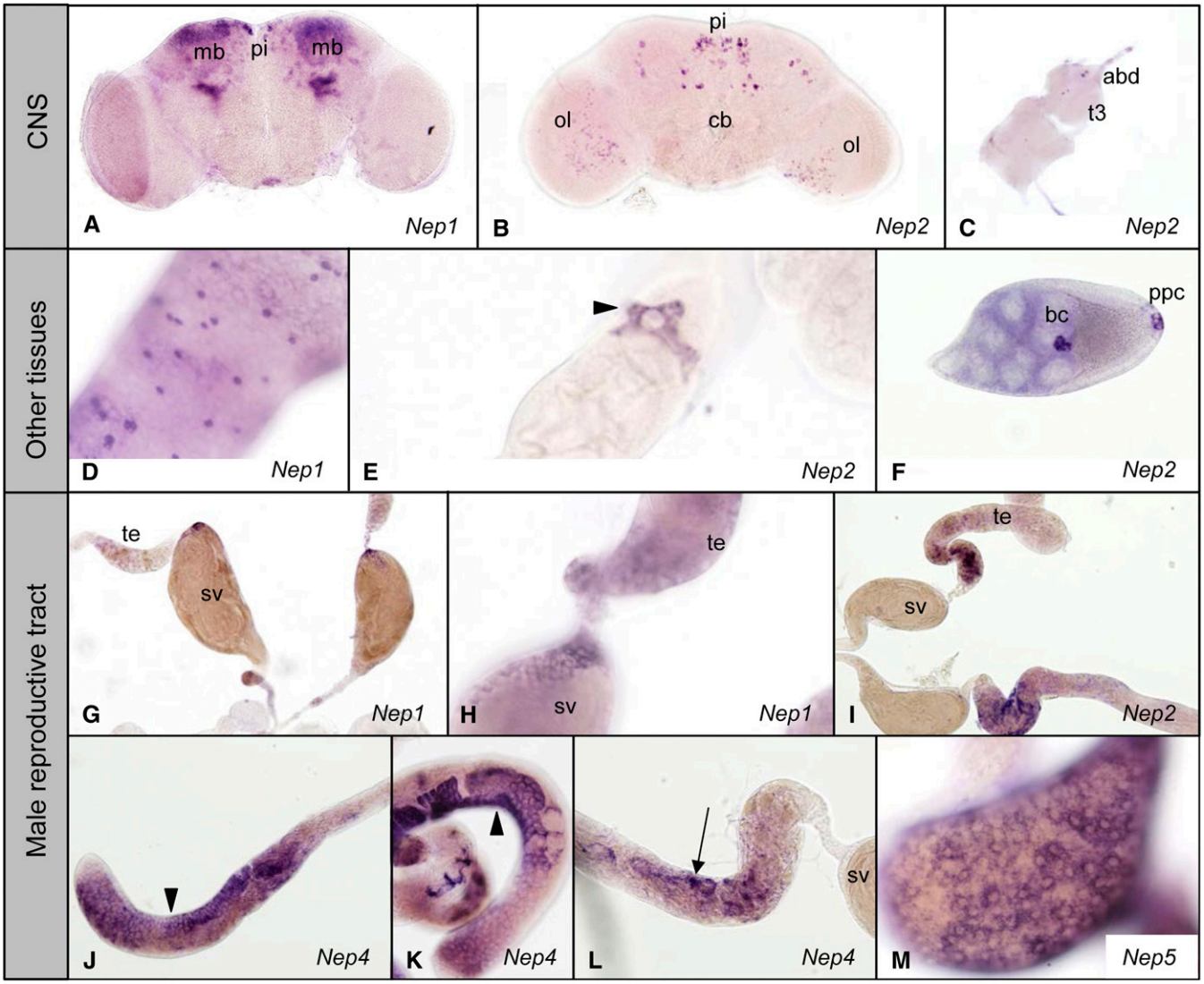

Figure 5 Adult expression pattern of *neprilysin* genes. (A–C) Expression of *Nep1–2* in the adult CNS. (A) *Nep1* in the adult brain mushroom bodies (mb) and *pars intercerebralis* (pi). (B) *Nep2* in cells in the *pars intercerebralis* (pi), central brain (cb), and optic lobes (ol). (C) *Nep2* in the third thoracic (t3) and abdominal (abd) neuromere. (D) *Nep1* in adult midgut cells. (E) *Nep2* in adult stellate cells of the Malpighian tubules (arrowhead). (F) *Nep2* in border cells (bc) and posterior polar cells (ppc) of a stage 10 ovarian follicle. (G–M) Expression of *neprilysin* genes in the male reproductive tract. (G–H) *Nep1* in the testicular tube (te) and the seminal vesicles (sv). (I) *Nep 2* in the part of the testis (te) close to the seminal vesicle (sv). (J–L) Nep4 in the somatic cyst cells (arrowheads) and in other cells (arrows) in the part of the testes close to the seminal vesicle (sv). (M) *Nep5* in the seminal vesicle.

spermathecae, two tissues that are known to influence egg laying (Yang *et al.* 2009; Schnakenberg *et al.* 2011; Sun and Spradling 2013). Nep2 is also expressed in the border cells of the follicular epithelium, which are important for micropyle development and for anterior–posterior polarity in the egg (Furriols *et al.* 2007). To test whether expression of Neps in these tissues are required for normal egg laying or hatchability we individually used *nsyb*–GAL4 (Pauli *et al.* 2008), *Send1*–GAL4 (Schnakenberg *et al.* 2011), and *slbo*–GAL4 (Rorth *et al.* 1998) drivers to locally drive RNAi in the CNS, the spermathecae, and the border cells, respectively.

Females knocked down for either *Nep1* or *Nep2* in the CNS laid fewer eggs than control females (Figure S7 and Figure S8). Similarly, knockdown of *Nep1* or *Nep2* in the spermathecae also reduced egg laying (Figure S7 and Figure S8). However, knockdown in either tissue did not fully recapitulate the egg-laying phenotypes seen in the ubiquitous knockdown of *Nep1* or *Nep2* in females. Contrary to expectations, reduction of *Nep2* expression in the border cells slightly increased egg laying. However, none of the targeted tissues for *Nep2* resulted in a decrease in hatchability. These results suggest that Neps are important in both the CNS and the spermathecae for normal egg laying but not hatchability and that either a combination of both sources or an as-yet untested source of *Nep* expression may be responsible for the majority of the reduction in egg laying observed. One possible source of expression is the seminal receptacle, where

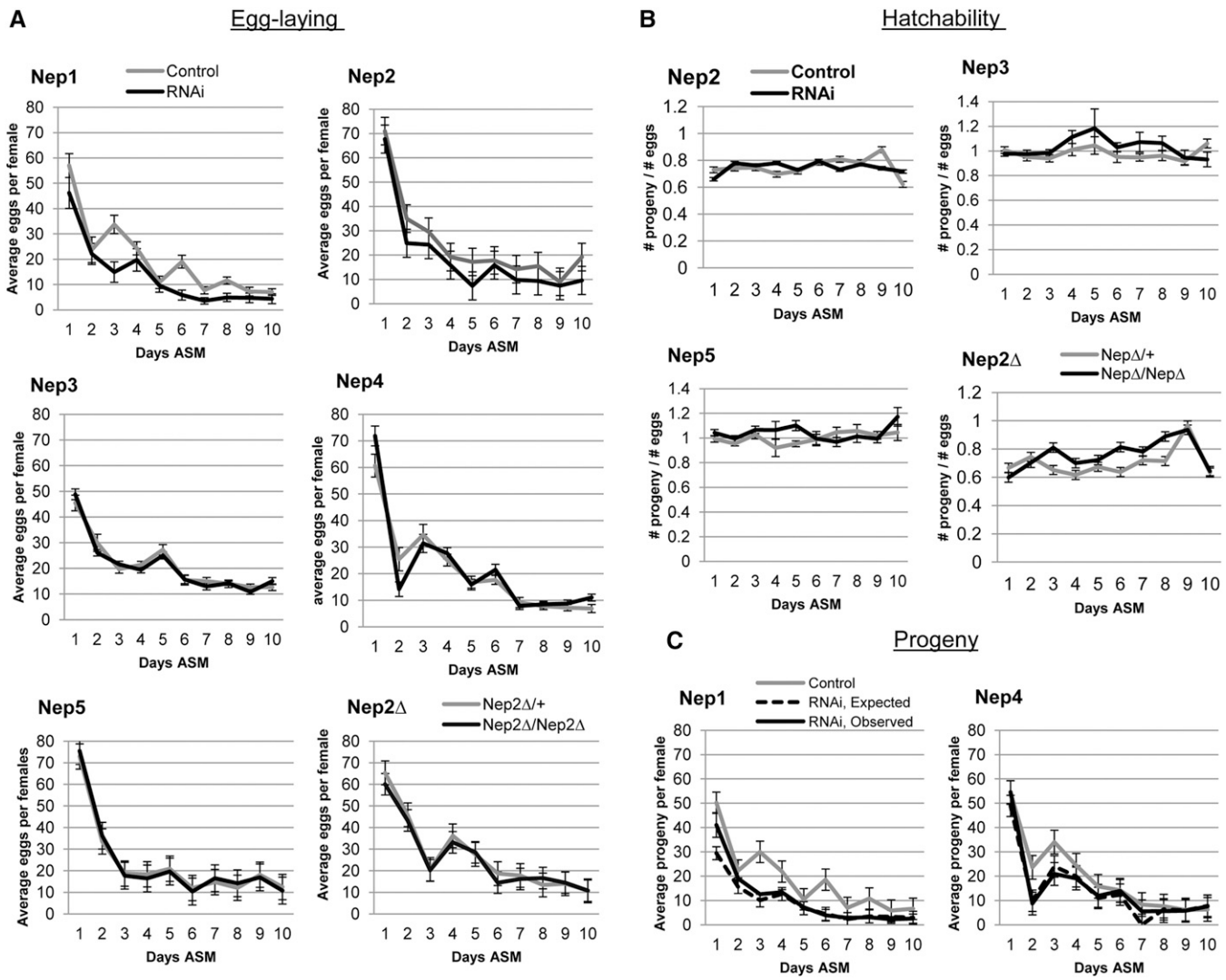

Figure 6 Egg laying in mates of *Nep* RNAi males. (A) The mean number of eggs laid per female mated to either control males (gray line) or RNAi/null males (black line) over a 10-day period. Only mates of *Nep1* RNAi males laid fewer eggs than mates of control males (*Nep1*: rmANOVA $P = 0.0041$, control $N = 16$, *Nep1* RNAi $N = 17$). Mates of *Nep2–5* RNAi laid comparable numbers of eggs as control mated females (*Nep2*—rmANOVA $P = 0.095$, control $N = 11$, *Nep2* RNAi $N = 14$; *Nep3*—rmANOVA $P = 0.7556$, control $N = 17$, *Nep3* RNAi $N = 21$; *Nep4*—rmANOVA $P = 0.6972$, control $N = 16$, *Nep4* RNAi $N = 16$; *Nep5*—rmANOVA $P = 0.8986$, control $N = 22$, *Nep5* RNAi $N = 25$; Nep2 null—rmANOVA $P = 0.3448$, control $N = 18$, Nep2 null $N = 21$). (B) The mean hatchability (#progeny/#eggs) per female for mates of control or RNAi/null males for the egg-laying assays in A. None of the Neps had a significant effect on hatching rate (*Nep2*—rmANOVA $P = 0.4326$; *Nep3*—rmANOVA $P = 0.1494$; *Nep5*—rmANOVA $P = 0.1909$; Nep2 null—rmANOVA $P = 0.3673$). (C) The mean observed (black line) and expected (dashed line) progeny ((# eggs) × (expected survival rate) × (average hatchability of eggs laid by control females)) for mates of *Nep1* and *Nep4* males. No difference in observed *vs.* expected progeny was seen for mates of either *Nep1* or *Nep4* RNAi males (*Nep1*, rmANOVA $P = 0.3714$; *Nep4*, rmANOVA $P = 0.2874$).

Nep1 and *Nep2* transcripts have both been detected (Prokupek *et al.* 2010). However, we cannot test this, as currently no GAL4 drivers target only this organ. Additionally in the case of *Nep2*, which encodes a secreted protein (Thomas *et al.* 2005), it is possible that secreted Nep2 from surrounding tissues may partially compensate for loss of Nep2 expression in our target tissues.

Sperm storage and depletion are abnormal in Nep2 null females

Neprilysins have been implicated in the regulation of muscle contraction in the mammalian uterus (Pinto *et al.* 1999; Pintado *et al.* 2003) and their substrates, the tachykinins, have been shown to induce muscle contraction in the oviduct of locusts (Kwok *et al.* 1999). Muscle contractions are also important in the *Drosophila* uterus, which goes through conformational changes after mating that facilitate sperm storage (Adams and Wolfner 2007; Avila and Wolfner 2009). The spermathecae and the seminal receptacle, which store these sperm, also experience contractions (Middleton *et al.* 2006), the importance of which is unknown but may facilitate the release or storage of sperm. To determine whether *Nep2* is essential for sperm storage or release, we counted the number of sperm stored at 2 hr and 4 days ASM in *Nep2* null females compared to controls.

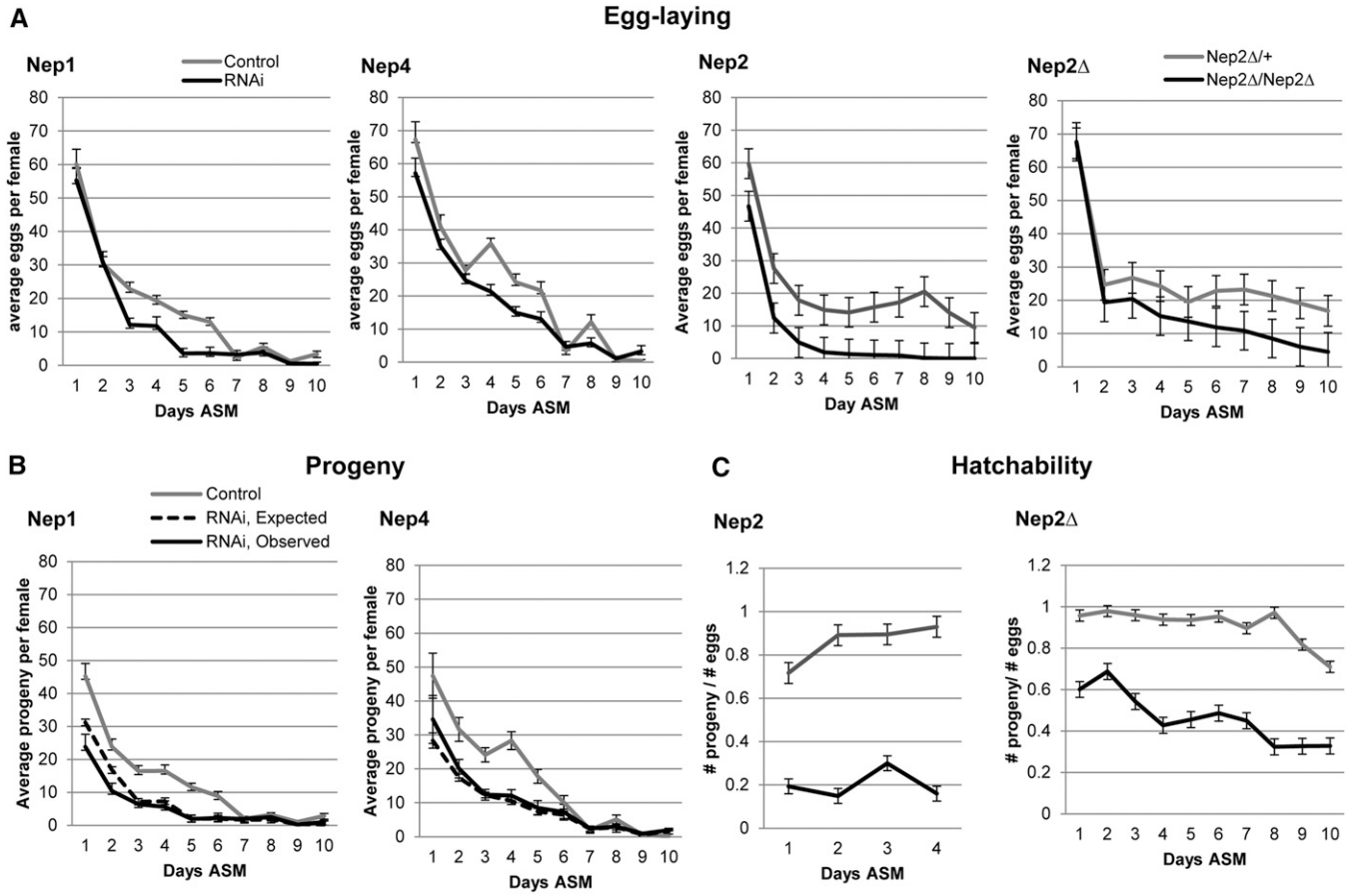

Figure 7 Egg laying in *Nep* RNAi females. (A) The mean number of eggs laid by control (gray line) or RNAi/null females (black line) mated to WT males over a 10-day period. *Nep1*, *Nep2*, and *Nep4* RNAi and Nep2 null females lay fewer eggs than controls (*Nep1*—rmANOVA $P = 0.0015$, control $N = 20$, *Nep1* RNAi $N = 18$; *Nep2*—rmANOVA $P = <0.0001$*, control $N = 12$, *Nep2* RNAi $N = 12$; *Nep4*: rmANOVA $P = 0.0207$*, control $N = 16$, *Nep4* RNAi $N = 16$; *Nep2* null—rmANOVA $P = <0.0001$*, control $N = 18$, *Nep2* null $N = 15$). (B) The mean observed (black line) and expected (dashed line) progeny ((# eggs) × (expected survival rate) × (average hatchability of eggs laid by control females)) for *Nep1* and *Nep4* females. No difference in overall observed *vs.* expected progeny was seen for either RNAi female (*Nep1*, rmANOVA $P = 0.2853$; *Nep4*, rmANOVA $P = 0.4325$). Nep1 RNAi females showed a trend for reduced observed progeny on day 1 ($P = 0.0686$) and a significant reduction on day 2 ($P = 0.0253$) after mating. (C) The mean hatchability (#progeny/#eggs) per female for *Nep2*. Both *Nep2* RNAi and the *Nep2* null females show drastically reduced hatchability (*Nep2*, rmANOVA $P = <0.0001$*; *Nep2* null, rmANOVA $P = <0.0001$*) suggesting that *Nep2* plays an essential role in this process.

After mating to wild-type males, females that were null for *Nep2* stored more sperm overall at 2 hr post-mating (a time when sperm storage has just completed; Bloch Qazi *et al.* 2003) than control females stored (Figure 9A) and had marginally more sperm in the spermathecae. By 4 days ASM, however, *Nep2* null females retained fewer sperm than controls and had marginally fewer sperm stored in their seminal receptacles. This shift from surplus to deficit illustrates that *Nep2* null females are defective in sperm retention. These results suggest that *Nep2* plays a role not only in the initial storage of sperm but also in controlling the release of sperm from the sperm storage organs. Although *Nep2* plays a role in sperm storage and release, the number of sperm stored in the sperm storage organs at the 4-day time point is too high to suggest that these sperm storage differences alone underlie the egg-laying defects seen in *Nep2* null females.

To confirm the role of *Nep2* in regulating sperm release or depletion we performed a sperm competition assay in which we mated *Nep2* null and control females to a Canton-S male and then subsequently to a bw^D male. Loss of *Nep2* function dramatically decreases P1 (the proportion of progeny sired by the first male) (Figure 9B) suggesting that *Nep2* aids in sperm retention and works to help sperm resist displacement by rival ejaculates. This is consistent with the observation that sperm deplete faster in singly mated Nep2 null females. Together these results indicate a role for Nep2 in female regulated sperm use.

Discussion

Drosophila neprilysin genes

We investigated a group of genes encoding M13 class proteases in *D. melanogaster* with expression patterns suggesting that they may play roles in reproduction or the CNS. Sequence analysis of protein sequences of Nep1–Nep5 with

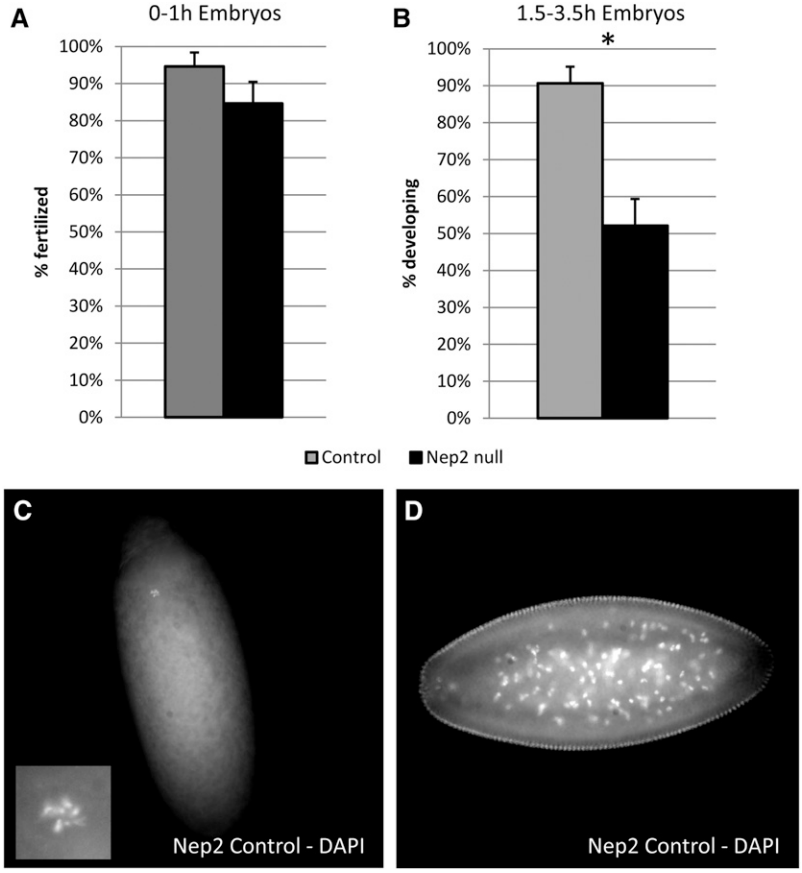

Figure 8 Eggs laid by *Nep2* null females arrest during early embryogenesis. (A) Eggs laid by Nep2 null females are fertilized at the same rate as eggs laid by control females (WRST $P = 0.1593$, control $N = 37$, *Nep2* null $N = 39$) based on sperm tail staining. (B) DAPI staining of 1.5- to 3.5-hr-old eggs laid by $Nep2^{+}$ control or *Nep2* null females were sorted into two categories: developing or nondeveloping. All nondeveloping embryos contained a polar body rosette (C), whereas developing embryos were all at stage 4+ (D) of development consistent with the time point chosen. Eggs laid by *Nep2* null females are significantly more likely to fall into the nondeveloping category than eggs laid by control females (WRST $P < 0.0001$*, control $N = 43$, *Nep2* null $N = 48$). Since the fertilization rate between *Nep2* null and control females is not different this result suggests that *Nep2* may be critical for early embryogenesis.

the sequences of human family members ECE-1, ECE-2, and neprilysin and locust *Lom*ECE and phylogenetic analysis revealed distinct similarities for *Drosophila* neprilysins 1–5. *Drosophila* Nep1 and Nep4 are most closely related to a group of vertebrate neprilysin homologs. Nep3 is most similar to *Lom*ECE and vertebrate ECE and Nep5 clusters in a group with Kell homologs. Nep2 is an invertebrate-specific protein. Overall, our analysis indicates that Nep1–5 are evolutionarily closely related yet representative of the functional divergence that seems to have occurred in this gene family.

Implications of neprilysins 1–5 expression patterns

The strong conservation of domains important for correct protein folding and activity in Nep1–Nep5 suggests that the functional specificity of the enzymes may at least in part depend on their specific spatiotemporal expression patterns, an aspect that has previously also been observed in *Caenorhabditis elegans* (Turner *et al.* 2001). Analysis of the expression patterns of *Nep1–Nep5* by *in situ* hybridization supports this hypothesis. Overall our expression analysis suggests that these five neprilysins may be involved in a range of developmental and physiological processes that in turn may be mediated by numerous bioactive (neuro)peptides.

Additionally, the observed expression patterns for *Nep1* and *Nep2* suggest that neprilysins may affect reproductive behavior at multiple levels. *Nep1* and *Nep2* are expressed in the *pars intercerebralis*. Prominent among the peptidergic neurons in the pars intercerebralis are the insulin-producing cells (IPCs). *Drosophila* insulin-like peptides (DILPs) are necessary for vitellogenesis (Richard *et al.* 2005). DILPs regulate stem-cell division in the ovary (LaFever and Drummond-Barbosa 2005) and mediate sexual attractiveness (Kuo *et al.* 2012). Another neuropeptide expressed in the pars intercerebralis is SIFamide that modulates courtship (Terhzaz *et al.* 2007). The expression of *Nep1* in the mushroom bodies may be significant in several ways. sNPF is expressed in the mushroom bodies and has been implicated in regulating IPCs (Nassel *et al.* 2013) while it also acts as a neuromodulator in olfactory memory (Knapek *et al.* 2013). Mushroom bodies also play important roles in courtship behavior and courtship learning or conditioning (Sakai and Kitamoto 2006; Keleman *et al.* 2012; Zhou *et al.* 2012). Finally, the expression of *Nep1* in abdominal ganglia suggests a possible role in modulating sperm transfer and copulation duration that is regulated by corazonin expressed in the abdominal ganglia (Tayler *et al.* 2012).

Drosophila neprilysins are important for fertility

Nep1, *Nep2*, and *Nep4* are essential for normal female reproductive fitness. *Nep1* and *Nep4* are essential for egg laying. Part of the effects of *Nep1* expression on egg laying can be traced to its role in the spermathecae and the CNS. *Nep2* is also essential for both the post-mating increase in female

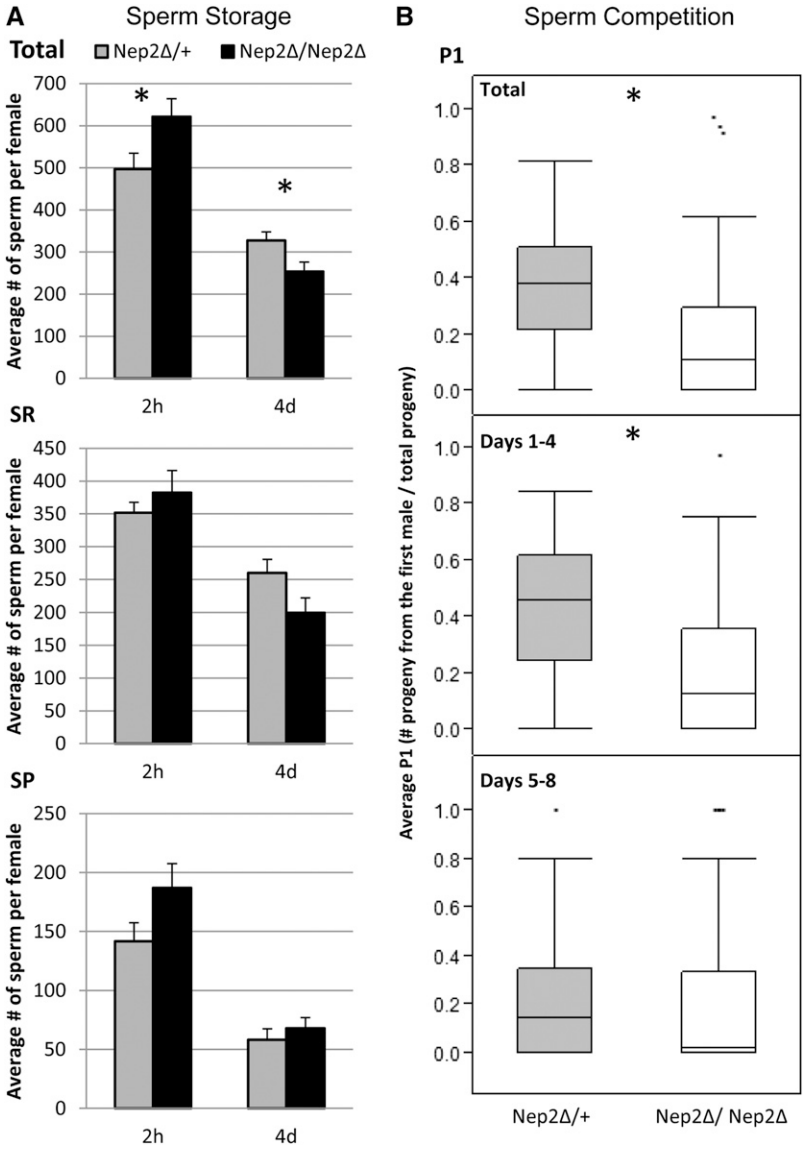

Figure 9 (A) Counts of sperm stored in both sets of sperm storage organs (Total), the seminal receptacle (SR), and the paired spermatheca (SP), of *Nep2* null (solid) *vs.* control females (shaded) at 2 hr and 4 days after the start of mating (ASM). Overall *Nep2* null females store more sperm at 2 hr ASM (ANOVA, *F*= 4.8029, *P* = 0.0398, control *N* = 13, Nep2 null *N* = 10) and fewer sperm at 4 days ASM (ANOVA, *F* = 6.0175 *P* = 0.0215*, control *N* = 13, *Nep2* null *N* = 14) than control females. Within the SR, *Nep2* null females store the same number of sperm at 2 hr ASM (ANOVA, *F* = 0.71 *P* = 0.4061, control *N* = 17, *Nep2* null *N* = 15) and marginally fewer sperm at 4 days ASM (ANOVA, *F* = 3.920 *P* = 0.0580, control *N* = 14, Nep2 null *N* = 15) than controls. Within the SP, *Nep2* null females store the same number of sperm at both 2 hr ASM (ANOVA, *F* = 3.1304. *P* = 0.0901, control *N* = 13, *Nep2* null *N* = 12) and 4 days ASM (ANOVA, *F* = 0.5584 *P* = 0.4614, control *N* = 14, *Nep2* null *N* = 15). (B) For sperm competition assays *Nep2* null or control females were first mated to a Canton-S male and then allowed to mate a second time with a bw^D male. The proportion of female progeny sired by the first male (Canton-S) referred to as P1 (# progeny from first male/total progeny) was significantly reduced in *Nep2* null females compared to control females (WRST *P* = <0.0001*, control *N* = 76, *Nep2* null *N* = 72). This difference is most apparent in the first 4 days ASM (WRST *P* = <0.0001*) compared to days 5–8 (WRST *P* = 0.1886)

egg laying and the hatchability of laid eggs. The hatching defects seen in *Nep2* RNAi and null females are not due to a failure in fertilization but instead manifest in an early embryonic arrest, suggesting that *Nep2* in the female is essential for the development of her progeny. Expression of *Nep2* in both the CNS and the spermathecae contributes to the egg-laying defect but not to the hatchability defect. Surprisingly, even though *Nep2* is present in the border cells of the follicular epithelium, the expression of *Nep2* in these cells is not essential for fertility.

In addition to egg production, *Nep2* influences sperm storage and depletion in females. Loss of *Nep2* in the female also negatively affects retention of sperm from the first mating when a second mating occurs. This suggests that *Nep2* may play a role in sperm retention, helping to insulate stored sperm from displacement by rival ejaculates. Whether this reduction in the presence of the first male's sperm is detrimental to the female is unclear. Together our data paint a broad role for neprilysins, and particularly for insect-specific *Neps* like *Nep2*, in regulating female reproductive success.

Nep1 is also important in male reproductive fitness. Knockdown of *Nep1* in males decreased egg laying in their mates. This finding is consistent with experiments in mice, where loss of NL1 in males caused reduced litter sizes (Carpentier *et al.* 2004). In contrast *Nep2–5* do not appear to have nonredundant, essential roles in male fertility.

The Neps we tested represent only a fraction of the neprilysin-like homologs identified to date in *Drosophila*. The similarity between Nep1 and other vertebrate Neps makes it a good potential model for finding substrates for neprilysins that are conserved throughout female reproduction. *Nep2* offers insight into the insect specific lineages of neprilysin-like genes. Further research on the substrates of Nep2 may reveal divergent or species-specific mechanisms for neprilysins in reproduction. Substrates of Nep2, or Nep2

itself, could also prove to be useful targets for controlling pests and insect disease vectors by reducing fertility.

Distribution and potential targets of neprilysins in D. melanogaster

In this study, we focused on the importance of neprilysins in *Drosophila* fertility. Among the important functions of these proteases, neprilysins are largely responsible for the degradation of neuropeptides (Turner *et al.* 2000; Turner *et al.* 2001). Reduced expression of individual neprilysins should result in an overabundance of their cleavage targets, which could be the ultimate cause of phenotypes such as those that we described here. The cleavage specificities of mammalian neprilysins are well documented: they have a preference for polar residues in the P1 position (G > P > R > S) and hydrophobic residues (F > L > Y > I > V) at the P1' position (summarized from MEROPS; Rawlings *et al.* 2012). In mammals, individual Neps tend to have very specific substrate affinities despite sharing conserved active site residues, suggesting that this specificity is based on other sequence features (Johnson *et al.* 2002; Rose *et al.* 2002; Bland *et al.* 2008; Whyteside and Turner 2008). Although the cleavage specificities of *Drosophila* neprilysins are not as well known, there is evidence that *Drosophila* Nep2 is capable of cleaving locust, human, and fly tachykinins *in vitro*. However, while Nep2 prefers similar polar, hydrophobic residue pairs it does not cleave at the same site used by mammalian neprilysin (Thomas *et al.* 2005; Bland *et al.* 2007). This observation, and the sheer number of Nep-like proteins in *Drosophila*, makes predicting protein-specific neprilysin cleavage sites in *Drosophila* neuropeptides problematic. To date, only Nep2 and Nep4 have been demonstrated to be proteolytically active (Thomas *et al.* 2005; Bland *et al.* 2007; Meyer *et al.* 2009) but their *in vivo* targets are as yet unknown. Neprilysin-like proteins have been shown to be involved in the degradation of the insect neuropeptide pigment dispersing factor (PDF), which has been implicated in mating behavior (Isaac *et al.* 2007); which Nep is responsible for the cleavage of PDF is unclear.

To identify other potential substrates of neprilysins in *Drosophila* that might contribute to the phenotypes we report here, we looked for potential cleavage sites in known *Drosophila* neuropeptides that have been associated with the regulation of physiology and behavior (reviewed in Nässel and Winther 2010) as well as in the newly identified group classified as natalisins (Jiang *et al.* 2013). Using the known cleavage site preferences of mammalian neprilysins and observed *Drosophila* Nep cleavage patterns, we identified potential Nep target sites in 56 of these 68 neuropeptides (Table S4). Of these, several stand out as notable targets for further study in reproduction.

The first candidate, neuropeptide F (NPF), is associated with the regulation of male courtship behavior in *Drosophila* (Lee *et al.* 2006) and with ovarian development in the locust (Cerstiaens *et al.* 1999). NPF has also been implicated in regulating feeding behavior in *Drosophila* (Lee *et al.* 2004), where increases in food intake are correlated with but not completely dependent on post-mating increases in female egg laying (Carvalho *et al.* 2006; Apger-Mcglaughon and Wolfner 2013). Another candidate, allatostatin A (AstA), has been implicated in regulating oviduct muscle contraction in other arthropods (Donini and Lange 2002; Garside *et al.* 2002; Woodhead *et al.* 2003). Further, allatostatins are essential for inhibiting juvenile hormone (JH) biosynthesis (Altaratz *et al.* 1991; Stay and Tobe 2007). JH has been implicated in a wide array of reproductive events including vitellogenesis and egg production (Gavin and Williamson 1976; Soller *et al.* 1999; Dubrovsky *et al.* 2002; Raushenbach *et al.* 2004) and is regulated by mating (Moshitzky *et al.* 1996). If AstA is a neprilysin candidate, then knockdown of that neprilysin in females could result in persistence or more allatostatin, which in turn could decrease egg production in a way similar to what we observed here for females knocked down for *Nep1*, *Nep2*, and *Nep4*. A third candidate, the sex peptide (SP), is a male-derived seminal fluid protein that is transferred to the female during mating and regulates female post-mating behaviors (Kubli 2003). Not only does SP regulate long-term post-mating increases in egg laying (Liu and Kubli 2003), it too has been implicated in changes in feeding and excretion post-mating (Carvalho *et al.* 2006; Cognigni *et al.* 2011; Apger-Mcglaughon and Wolfner 2013). In addition, SP is able to activate JH–B3 biosynthesis (Moshitzky *et al.* 1996). Further, myoinhibitory peptide-1 (MIP-1), part of a family of neuropeptides that are able to bind the sex peptide receptor (SPR) in addition to SP (Kim *et al.* 2010), is a potential target of neprilysin degradation. These latter two examples suggest that Neps could play a role in determining the stability and balance of the mix of neuropeptides that are available to bind SPR after mating and through this might be important in controlling the extent of post-mating changes in behaviors and egg production. Further, SP is known to be cleaved in the hemolymph of females and some of the detected fragments are consistent with a cleavage at one of our predicted neprilysin cleavage sites (R,L) (Pilpel *et al.* 2008).

Based on Fly Atlas (Chintapalli *et al.* 2007) and modENCODE (McQuilton *et al.* 2012; Young *et al.* 2012) data (Table S5) one of these neuropeptides, AstA, has a precursor sequence that is expressed in the female spermathecae, a site where our data suggest that the expression of *Nep1* and *Nep2* is needed for normal female egg laying. However, it is important to note that *Nep4* and *Nep2* encode secreted proteins (Thomas *et al.* 2005; Meyer *et al.* 2009) so their site of action is likely not limited by their expression patterns. Similarly, there is evidence that neuropeptides, particularly tachykinins, from one area of the body can activate receptors elsewhere in insects (Winther and Nassel 2001).

In conclusion, we have shown that *Drosophila* neprilysins play an important role in regulating reproductive success of both males and females. It is likely that they do this by regulating the degradation of one or several neuropeptides substrates in both the female reproductive tract and the

CNS. Future identification of the *in vivo* targets of *Drosophila* Neps will be important for identifying modulators of insect reproduction, as well as serving as a model for the effects of neprilysin action in all organisms.

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Literature Cited

Adams, E. M., and M. F. Wolfner, 2007 Seminal proteins but not sperm induce morphological changes in the *Drosophila melanogaster* female reproductive tract during sperm storage. J. Insect Physiol. 53: 319–331.

Altaratz, M., S. W. Applebaum, D. S. Richard, L. I. Gilbert, and D. Segal, 1991 Regulation of juvenile hormone synthesis in wild-type and apterous mutant Drosophila. Mol. Cell. Endocrinol. 81: 205–216.

Aoyama, M., T. Kawada, and H. Satake, 2012 Localization and enzymatic activity profiles of the proteases responsible for tachykinin-directed oocyte growth in the protochordate, *Ciona intestinalis*. Peptides 34: 186–192.

Apger-McGlaughon, J., and M. F. Wolfner, 2013 Post-mating change in excretion by mated *Drosophila melanogaster* females is a long-term response that depends on sex peptide and sperm. J. Insect Physiol. 59: 1024–1030.

Avila, F. W., and M. F. Wolfner, 2009 Acp36DE is required for uterine conformational changes in mated Drosophila females. Proc. Natl. Acad. Sci. USA 106: 15796–15800.

Avila, F. W., K. Ravi Ram, M. C. Bloch Qazi, and M. F. Wolfner, 2010 Sex peptide is required for the efficient release of stored sperm in mated *Drosophila* females. Genetics 186: 595–600.

Bellen, H. J., R. W. Levis, G. Liao, Y. He, J. W. Carlson *et al.*, 2004 The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. Genetics 167: 761–781.

Biran, J., O. Palevitch, S. Ben-Dor, and B. Levavi-Sivan, 2012 Neurokinin Bs and neurokinin B receptors in zebrafish-potential role in controlling fish reproduction. Proc. Natl. Acad. Sci. USA 109: 10269–10274.

Bland, N. D., J. E. Thomas, N. Audsley, A. D. Shirras, A. J. Turner *et al.*, 2007 Expression of NEP2, a soluble neprilysin-like endopeptidase, during embryogenesis in *Drosophila melanogaster*. Peptides 28: 127–135.

Bland, N. D., J. W. Pinney, J. E. Thomas, A. J. Turner, and R. E. Isaac, 2008 Bioinformatic analysis of the neprilysin (M13) family of peptidases reveals complex evolutionary and functional relationships. BMC Evol. Biol. 8: 16.

Bloch Qazi, M. C., Y. Heifetz, and M. F. Wolfner, 2003 The developments between gametogenesis and fertilization: ovulation and female sperm storage in *Drosophila melanogaster*. Dev. Biol. 256: 195–211.

Bonavera, J. J., A. Sahu, S. P. Kalra, and P. S. Kalra, 1994 The hypothalamic peptides, beta-endorphin, neuropeptide K and interleukin-1 beta, and the opiate morphine, enhance the excitatory amino acid-induced LH release under the influence of gonadal steroids. J. Neuroendocrinol. 6: 557–564.

Brand, A. H., and N. Perrimon, 1993 Targeted gene-expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401–415.

Carpentier, M., C. Guillemette, J. L. Bailey, G. Boileau, L. Jeannotte *et al.*, 2004 Reduced fertility in male mice deficient in the zinc metallopeptidase NL1. Mol. Cell. Biol. 24: 4428–4437.

Carvalho, G. B., P. Kapahi, D. J. Anderson, and S. Benzer, 2006 Allocrine modulation of feeding behavior by the sex peptide of Drosophila. Curr. Biol. 16: 692–696.

Celniker, S. E., L. A. Dillon, M. B. Gerstein, K. C. Gunsalus, S. Henikoff *et al.*, 2009 Unlocking the secrets of the genome. Nature 459: 927–930.

Cerstiaens, A., L. Benfekih, H. Zouiten, P. Verhaert, A. De Loof *et al.*, 1999 Led-NPF-1 stimulates ovarian development in locusts. Peptides 20: 39–44.

Chintapalli, V. R., J. Wang, and J. A. Dow, 2007 Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. Nat. Genet. 39: 715–720.

Clements, J., K. Hens, C. Francis, A. Schellens, and P. Callaerts, 2008 Conserved role for the Drosophila Pax6 homolog Eyeless in differentiation and function of insulin-producing neurons. Proc. Natl. Acad. Sci. USA 105: 16183–16188.

Coates, D., R. Siviter, and R. E. Isaac, 2000 Exploring the *Caenorhabditis elegans* and *Drosophila melanogaster* genomes to understand neuropeptide and peptidase function. Biochem. Soc. Trans. 28: 464–469.

Cognigni, P., A. P. Bailey, and I. Miguel-Aliaga, 2011 Enteric neurons and systemic signals couple nutritional and reproductive status with intestinal homeostasis. Cell Metab. 13: 92–104.

Dietzl, G., D. Chen, F. Schnorrer, K. C. Su, Y. Barinova *et al.*, 2007 A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448: 151–156.

Donini, A., and A. B. Lange, 2002 The effects of crustacean cardioactive peptide on locust oviducts are calcium-dependent. Peptides 23: 683–691.

Dow, J. A., and M. F. Romero, 2010 Drosophila provides rapid modeling of renal development, function, and disease. Am. J. Physiol. Renal Physiol. 299: F1237–F1244.

Dubrovsky, E. B., V. A. Dubrovskaya, and E. M. Berger, 2002 Juvenile hormone signaling during oogenesis in *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 32: 1555–1565.

Edgar, R. C., 2004 MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5: 113.

Elliott, D. A., and A. H. Brand, 2008 The GAL4 system: a versatile system for the expression of genes. Methods Mol. Biol. 420: 79–95.

Felsenstein, J., 2005 PHYLIP, Phylogeny Inference Package, version 3.6. Department of Genome Sciences, University of Washington, Seattle.

Furriols, M., G. Ventura, and J. Casanova, 2007 Two distinct but convergent groups of cells trigger Torso receptor tyrosine kinase activation by independently expressing torso-like. Proc. Natl. Acad. Sci. USA 104: 11660–11665.

Garside, C. S., P. M. Koladich, W. G. Bendena, and S. S. Tobe, 2002 Expression of allatostatin in the oviducts of the cockroach *Diploptera punctata*. Insect Biochem. Mol. Biol. 32: 1089–1099.

Gavin, J. A., and J. H. Williamson, 1976 Juvenile hormone-induced vitellogenesis in apterous4, a non-vitellogenic mutant in *Drosophila melanogaster*. J. Insect Physiol. 22: 1737–1742.

Gligorov, D., J. L. Sitnik, R. K. Maeda, M. F. Wolfner, and F. Karch, 2013 A novel function for the hox gene abd-B in the male

accessory gland regulates the long-term female post-mating response in Drosophila. PLoS Genet. 9: e1003395.
Head, J. R., P. C. MacDonald, and M. L. Casey, 1993 Cellular localization of membrane metalloendopeptidase (enkephalinase) in human endometrium during the ovarian cycle. J. Clin. Endocrinol. Metab. 76: 769–776.
Huet, F., J. T. Lu, K. V. Myrick, L. R. Baugh, M. A. Crosby *et al.*, 2002 A deletion-generator compound element allows deletion saturation analysis for genomewide phenotypic annotation. Proc. Natl. Acad. Sci. USA 99: 9948–9953.
Isaac, R. E., and D. R. Nassel, 2003 Identification and localization of a neprilysin-like activity that degrades tachykinin-related peptides in the brain of the cockroach, *Leucophaea maderae*, and locust, *Locusta migratoria*. J. Comp. Neurol. 457: 57–66.
Isaac, R. E., E. T. Parkin, J. N. Keen, D. R. Nassel, R. J. Siviter *et al.*, 2002 Inactivation of a tachykinin-related peptide: identification of four neuropeptide-degrading enzymes in neuronal membranes of insects from four different orders. Peptides 23: 725–733.
Isaac, R. E., E. C. Johnson, N. Audsley, and A. D. Shirras, 2007 Metabolic inactivation of the circadian transmitter, pigment dispersing factor (PDF), by neprilysin-like peptidases in Drosophila. J. Exp. Biol. 210: 4465–4470.
Jiang, H., A. Lkhagva, I. Daubnerova, H. S. Chae, L. Simo *et al.*, 2013 Natalisin, a tachykinin-like signaling system, regulates sexual activity and fecundity in insects. Proc. Natl. Acad. Sci. USA 110: E3526–E3534.
Johnson, G. D., H. R. Swenson, R. Ramage, and K. Ahn, 2002 Mapping the active site of endothelin-converting enzyme-1 through subsite specificity and mutagenesis studies: a comparison with neprilysin. Arch. Biochem. Biophys. 398: 240–248.
Karr, T. L., 1991 Intracellular sperm/egg interactions in Drosophila: a three-dimensional structural analysis of a paternal product in the developing egg. Mech. Dev. 34: 101–111.
Keleman, K., E. Vrontou, S. Kruttner, J. Y. Yu, A. Kurtovic-Kozaric *et al.*, 2012 Dopamine neurons modulate pheromone responses in Drosophila courtship learning. Nature 489: 145–149.
Kim, Y. J., K. Bartalska, N. Audsley, N. Yamanaka, N. Yapici *et al.*, 2010 MIPs are ancestral ligands for the sex peptide receptor. Proc. Natl. Acad. Sci. USA 107: 6520–6525.
Klein, C., C. Patte-Mensah, O. Taleb, J. J. Bourguignon, M. Schmitt *et al.*, 2013 The neuroprotector kynurenic acid increases neuronal cell survival through neprilysin induction. Neuropharmacology 70: 254–260.
Knapek, S., L. Kahsai, A. M. Winther, H. Tanimoto, and D. R. Nassel, 2013 Short neuropeptide F acts as a functional neuromodulator for olfactory memory in Kenyon cells of Drosophila mushroom bodies. J. Neurosci. 33: 5340–5345.
Krauchunas, A. R., V. L. Horner, and M. F. Wolfner, 2012 Protein phosphorylation changes reveal new candidates in the regulation of egg activation and early embryogenesis in *D. melanogaster*. Dev. Biol. 370: 125–134.
Kubli, E., 2003 Sex-peptides: seminal peptides of the Drosophila male. Cell. Mol. Life Sci. 60: 1689–1704.
Kuo, T. H., T. Y. Fedina, I. Hansen, K. Dreisewerd, H. A. Dierick *et al.*, 2012 Insulin signaling mediates sexual attractiveness in Drosophila. PLoS Genet. 8: e1002684.
Kwok, R., D. R. Nassel, A. B. Lange, and I. Orchard, 1999 Locustatachykinin isoforms in the locust: distribution and quantification in the central nervous system and action on the oviduct muscle. Peptides 20: 687–694.
LaFever, L., and D. Drummond-Barbosa, 2005 Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. Science 309: 1071–1073.
Lee, G., J. H. Bahn, and J. H. Park, 2006 Sex- and clock-controlled expression of the neuropeptide F gene in Drosophila. Proc. Natl. Acad. Sci. USA 103: 12580–12585.
Lee, K. S., K. H. You, J. K. Choo, Y. M. Han, and K. Yu, 2004 Drosophila short neuropeptide F regulates food intake and body size. J. Biol. Chem. 279: 50781–50789.
Li, C., R. M. Booze, and L. B. Hersh, 1995 Tissue-specific expression of rat neutral endopeptidase (neprilysin) mRNAs. J. Biol. Chem. 270: 5723–5728.
Liu, H., and E. Kubli, 2003 Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 100: 9929–9933.
Maguer-Satta, V., R. Besancon, and E. Bachelard-Cascales, 2011 Concise review: neutral endopeptidase (CD10): a multifaceted environment actor in stem cells, physiological mechanisms, and cancer. Stem Cells 29: 389–396.
Marygold, S. J., P. C. Leyland, R. L. Seal, J. L. Goodman, J. Thurmond *et al.*, 2013 FlyBase: improvements to the bibliography. Nucleic Acids Res. 41: D751–D757.
McQuilton, P., S. E. St Pierre, and J. Thurmond, 2012 FlyBase 101: the basics of navigating FlyBase. Nucleic Acids Res. 40: D706–D714.
Meyer, H., M. Panz, M. Zmojdzian, K. Jagla, and A. Paululat, 2009 Neprilysin 4, a novel endopeptidase from *Drosophila melanogaster*, displays distinct substrate specificities and exceptional solubility states. J. Exp. Biol. 212: 3673–3683.
Middleton, C. A., U. Nongthomba, K. Parry, S. T. Sweeney, J. C. Sparrow *et al.*, 2006 Neuromuscular organization and aminergic modulation of contractions in the Drosophila ovary. BMC Biol. 4: 17.
Moshitzky, P., I. Fleischmann, N. Chaimov, P. Saudan, S. Klauser *et al.*, 1996 Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. Arch. Insect Biochem. Physiol. 32: 363–374.
Mueller, J. L., J. R. Linklater, K. Ravi Ram, T. Chapman, and M. F. Wolfner, 2008 Targeted gene deletion and phenotypic analysis of the *Drosophila melanogaster* seminal fluid protease inhibitor Acp62F. Genetics 178: 1605–1614.
Mulder, S. D., R. Veerhuis, M. A. Blankenstein, and H. M. Nielsen, 2012 The effect of amyloid associated proteins on the expression of genes involved in amyloid-beta clearance by adult human astrocytes. Exp. Neurol. 233: 373–379.
Nässel, D. R., and A. M. Winther, 2010 Drosophila neuropeptides in regulation of physiology and behavior. Prog. Neurobiol. 92: 42–104.
Nässel, D. R., O. I. Kubrak, Y. Liu, J. Luo, and O. V. Lushchak, 2013 Factors that regulate insulin producing cells and their output in. Front Physiol 4: 252.
Neubaum, D. M., and M. F. Wolfner, 1999 Mated *Drosophila melanogaster* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. Genetics 153: 845–857.
Ouimet, T., P. Facchinetti, C. Rose, M. C. Bonhomme, C. Gros *et al.*, 2000 Neprilysin II: a putative novel metalloprotease and its isoforms in CNS and testis. Biochem. Biophys. Res. Commun. 271: 565–570.
Pauli, A., F. Althoff, R. A. Oliveira, S. Heidmann, O. Schuldiner *et al.*, 2008 Cell-type-specific TEV protease cleavage reveals cohesin functions in Drosophila neurons. Dev. Cell 14: 239–251.
Pilpel, N., I. Nezer, S. W. Applebaum, and Y. Heifetz, 2008 Mating increases trypsin in female Drosophila hemolymph. Insect Biochem. Mol. Biol. 38: 320–330.
Pintado, C. O., F. M. Pinto, J. N. Pennefather, A. Hidalgo, A. Baamonde *et al.*, 2003 A role for tachykinins in female mouse and rat reproductive function. Biol. Reprod. 69: 940–946.
Pinto, F. M., C. P. Armesto, J. Magraner, M. Trujillo, J. D. Martin *et al.*, 1999 Tachykinin receptor and neutral endopeptidase gene expression in the rat uterus: characterization and regulation in response to ovarian steroid treatment. Endocrinology 140: 2526–2532.
Prokupek, A. M., S. I. Eyun, L. Ko, E. N. Moriyama, and L. G. Harshman, 2010 Molecular evolutionary analysis of seminal

receptacle sperm storage organ genes of *Drosophila melanogaster*. J. Evol. Biol. 23: 1386–1398.
Ram, K. R., and M. F. Wolfner, 2007 Sustained post-mating response in *Drosophila melanogaster* requires multiple seminal fluid proteins. PLoS Genet. 3: e238.
Rambaut, A., 2010 FigTree 1.3.1 Available at: tree.bio.ed.ac.uk/software/figtree/.
Rance, N. E., S. J. Krajewski, M. A. Smith, M. Cholanian, and P. A. Dacks, 2010 Neurokinin B and the hypothalamic regulation of reproduction. Brain Res. 1364: 116–128.
Raushenbach, I. Y., N. E. Gruntenko, M. Bownes, N. V. Adonieva, J. Terashima *et al.*, 2004 The role of juvenile hormone in the control of reproductive function in *Drosophila virilis* under nutritional stress. J. Insect Physiol. 50: 323–330.
Rawlings, N. D., A. J. Barrett, and A. Bateman, 2012 MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 40: D343–D350.
Richard, D. S., R. Rybczynski, T. G. Wilson, Y. Wang, M. L. Wayne *et al.*, 2005 Insulin signaling is necessary for vitellogenesis in *Drosophila melanogaster* independent of the roles of juvenile hormone and ecdysteroids: female sterility of the chico1 insulin signaling mutation is autonomous to the ovary. J. Insect Physiol. 51: 455–464.
Rorth, P., K. Szabo, A. Bailey, T. Laverty, J. Rehm *et al.*, 1998 Systematic gain-of-function genetics in Drosophila. Development 125: 1049–1057.
Rose, C., S. Voisin, C. Gros, J. C. Schwartz, and T. Ouimet, 2002 Cell-specific activity of neprilysin 2 isoforms and enzymic specificity compared with neprilysin. Biochem. J. 363: 697–705.
Sahu, A., and S. P. Kalra, 1992 Effects of tachykinins on luteinizing hormone release in female rats: potent inhibitory action of neuropeptide K. Endocrinology 130: 1571–1577.
Sahu, A., W. R. Crowley, K. Tatemoto, A. Balasubramaniam, and S. P. Kalra, 1987 Effects of neuropeptide Y, NPY analog (norleucine4-NPY), galanin and neuropeptide K on LH release in ovariectomized (ovx) and ovx estrogen, progesterone-treated rats. Peptides 8: 921–926.
Sakai, T., and T. Kitamoto, 2006 Differential roles of two major brain structures, mushroom bodies and central complex, for Drosophila male courtship behavior. J. Neurobiol. 66: 821–834.
Schnakenberg, S. L., W. R. Matias, and M. L. Siegal, 2011 Sperm-storage defects and live birth in Drosophila females lacking spermathecal secretory cells. PLoS Biol. 9: e1001192.
Schneider, C. A., W. S. Rasband, and K. W. Eliceiri, 2012 NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9: 671–675.
Segura, J., and L. M. Ruilope, 2011 Dual-acting angiotensin receptor-neprilysin inhibition. Curr. Hypertens. Rep. 13: 74–78.
Smollich, M., M. Gotte, G. W. Yip, E. S. Yong, C. Kersting *et al.*, 2007 On the role of endothelin-converting enzyme-1 (ECE-1) and neprilysin in human breast cancer. Breast Cancer Res. Treat. 106: 361–369.
Soller, M., M. Bownes, and E. Kubli, 1999 Control of oocyte maturation in sexually mature Drosophila females. Dev. Biol. 208: 337–351.
Southall, T. D., D. A. Elliott and A. H. Brand, 2008 The GAL4 System: A versatile toolkit for gene expression in Drosophila. CSH Protoc. 2008: pdb top49.
Spradling, A. C., D. M. Stern, I. Kiss, J. Roote, T. Laverty *et al.*, 1995 Gene disruptions using P transposable elements: an integral component of the Drosophila genome project. Proc. Natl. Acad. Sci. USA 92: 10824–10830.
Spradling, A. C., D. Stern, A. Beaton, E. J. Rhem, T. Laverty *et al.*, 1999 The Berkeley Drosophila Genome Project gene disruption project: single P-element insertions mutating 25% of vital Drosophila genes. Genetics 153: 135–177.
Stay, B., and S. S. Tobe, 2007 The role of allatostatins in juvenile hormone synthesis in insects and crustaceans. Annu. Rev. Entomol. 52: 277–299.
Sullivan, W., M. Ashburner, and R. Hawley (Editors), 2000 *Drosophila Protocols*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sun, J., and A. C. Spradling, 2013 Ovulation in Drosophila is controlled by secretory cells of the female reproductive tract. Elife 2: e00415.
Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei *et al.*, 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739.
Tayler, T. D., D. A. Pacheco, A. C. Hergarden, M. Murthy, and D. J. Anderson, 2012 A neuropeptide circuit that coordinates sperm transfer and copulation duration in Drosophila. Proc. Natl. Acad. Sci. USA 109: 20697–20702.
Terhzaz, S., P. Rosay, S. F. Goodwin, and J. A. Veenstra, 2007 The neuropeptide SIFamide modulates sexual behavior in Drosophila. Biochem. Biophys. Res. Commun. 352: 305–310.
Thomas, J. E., C. M. Rylett, A. Carhan, N. D. Bland, R. J. Bingham *et al.*, 2005 *Drosophila melanogaster* NEP2 is a new soluble member of the neprilysin family of endopeptidases with implications for reproduction and renal function. Biochem. J. 386: 357–366.
Turner, A. J., C. D. Brown, J. A. Carson, and K. Barnes, 2000 The neprilysin family in health and disease. Adv. Exp. Med. Biol. 477: 229–240.
Turner, A. J., R. E. Isaac, and D. Coates, 2001 The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. BioEssays 23: 261–269.
Whyteside, A. R., and A. J. Turner, 2008 Human neprilysin-2 (NEP2) and NEP display distinct subcellular localisations and substrate preferences. FEBS Lett. 582: 2382–2386.
Wick, M. J., E. J. Buesing, C. A. Wehling, Z. L. Loomis, C. D. Cool *et al.*, 2011 Decreased neprilysin and pulmonary vascular remodeling in chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 183: 330–340.
Wilson, C. L., A. D. Shirras, and R. E. Isaac, 2002 Extracellular peptidases of imaginal discs of *Drosophila melanogaster*. Peptides 23: 2007–2014.
Winther, A. M., and D. R. Nassel, 2001 Intestinal peptides as circulating hormones: release of tachykinin-related peptide from the locust and cockroach midgut. J. Exp. Biol. 204: 1269–1280.
Wong, S. S., N. N. Sun, C. D. Fastje, M. L. Witten, R. C. Lantz *et al.*, 2011 Role of neprilysin in airway inflammation induced by diesel exhaust emissions. Res. Rep. Health Eff. Inst. 159: 3–40.
Woodhead, A. P., M. E. Thompson, K. K. Chan, and B. Stay, 2003 Allatostatin in ovaries, oviducts, and young embryos in the cockroach *Diploptera punctata*. J. Insect Physiol. 49: 1103–1114.
Yang, C. H., S. Rumpf, Y. Xiang, M. D. Gordon, W. Song *et al.*, 2009 Control of the postmating behavioral switch in Drosophila females by internal sensory neurons. Neuron 61: 519–526.
Young, J., J. Bouligand, B. Francou, M. L. Raffin-Sanson, S. Gaillez *et al.*, 2010 TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. J. Clin. Endocrinol. Metab. 95: 2287–2295.
Young, R. S., A. C. Marques, C. Tibbit, W. Haerty, A. R. Bassett *et al.*, 2012 Identification and properties of 1,119 candidate lincRNA loci in the *Drosophila melanogaster* genome. Genome Biol. Evol. 4: 427–442.
Zhou, C., H. Huang, S. M. Kim, H. Lin, X. Meng *et al.*, 2012 Molecular genetic analysis of sexual rejection: roles of octopamine and its receptor OAMB in Drosophila courtship conditioning. J. Neurosci. 32: 14281–14287.

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Neprilysins: An Evolutionarily Conserved Family of Metalloproteases That Play Important Roles in Reproduction in *Drosophila*

Jessica L. Sitnik, Carmen Francis, Korneel Hens, Roger Huybrechts, Mariana F. Wolfner, and Patrick Callaerts

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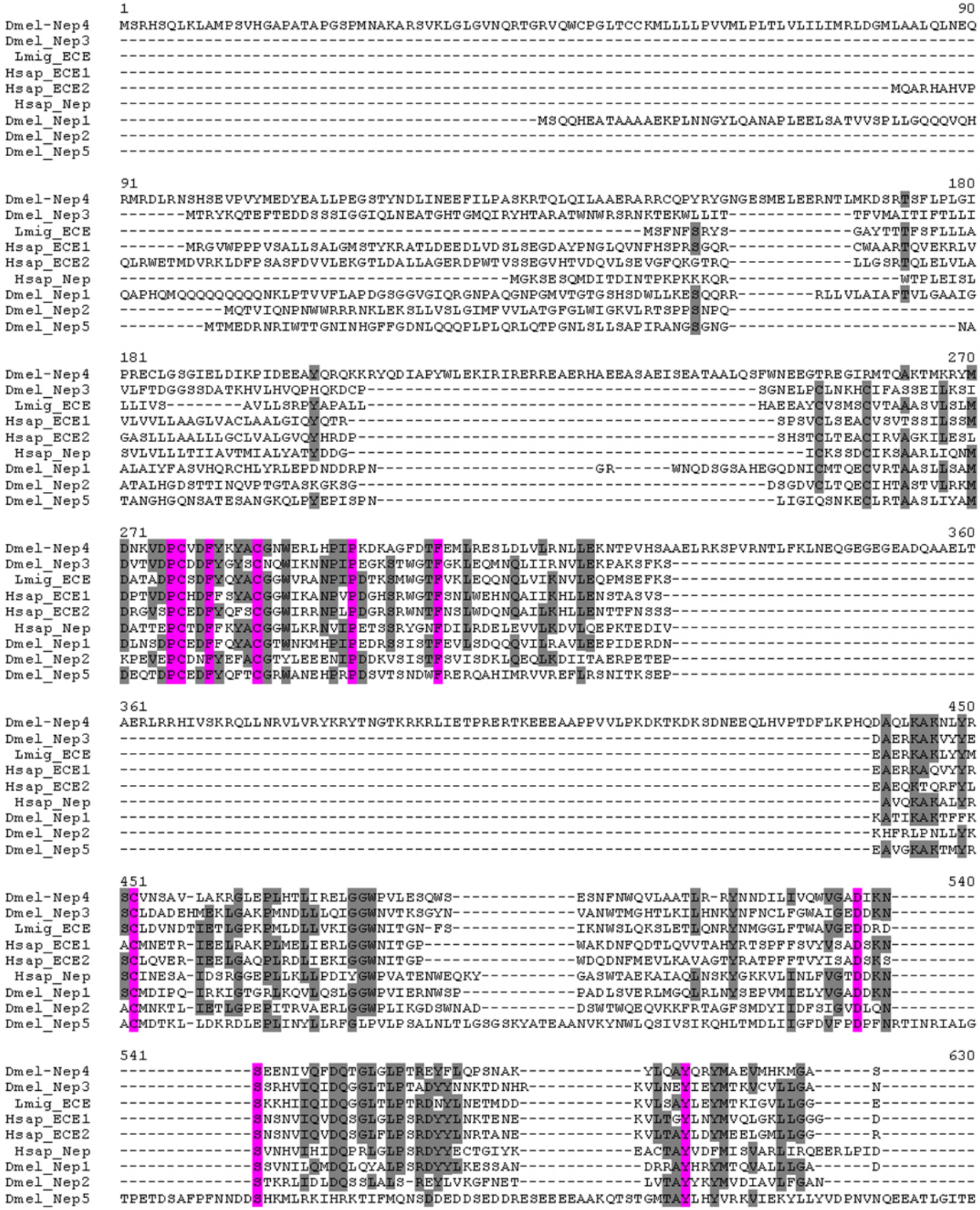
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Dmel_Nep3
Lmig_ECE
Hsap_ECE1
Hsap_ECE2 MQARHAHVP
Hsap_Nep
Dmel_Nep1 MSQQHEATAAAAEKPLNNGYLQANAPLEELSATVVSPLLGQQQVQH
Dmel_Nep2
Dmel_Nep5
91 180
Dmel-Nep4 RMRDLRNSHSEVPVYMEDYEALLPEGSTYNDLINEEFILPASKRTQLQILAAERARRCQPYRYGNGESMELEERNTLMKDSRTSFLPLGI
Dmel_Nep3 -------MTRYKQTEFTEDDSSSIGGIQLNEATGHTGMQIRYHTARATWNWRSRNKTEKWLLIT-------------TFVMAITIFTLLI
Lmig_ECE MSFNFSRYS-------------GAYTTTFSFLLLA
Hsap_ECE1 -------MRGVWPPPVSALLSALGMSTYKRATLDEEDLVDSLSEGDAYPNGLQVNFHSPRSGQR-------------CWAARTQVEKRLV
Hsap_ECE2 QLRWETMDVRKLDFPSASFDVVLEKGTLDALLAGERDPWTVSSEGVHTVDQVLSEVGFQKGTRQ-------------LLGSRTQLELVLA
Hsap_Nep MGKSESQMDITDINTPKPKKKQR-----------------WTPLEISL
Dmel_Nep1 QAPHQMQQQQQQQQQNKLPTVVFLAPDGSGGVGIQRGNPAQGNPGMVTGTGSHSDWLLKESQQRR--------RLLVLAIAFTVLGAAIG
Dmel_Nep2 -----------MQTVIQNPNWWRRRNKLEKSLLVSLGIMFVVLATGFGLWIGKVLRTSPPSNPQ-----------------------
Dmel_Nep5 ---------MTMEDRNRIWTTGNINHGFFGDNLQQQPLPLQRLQTPGNLSLLSAPIRANGSGNG----------------------NA
181 270
Dmel-Nep4 PRECLGSGIELDIKPIDEEAYQRQKKRYQDIAPYWLEKIRIRERREAERHAEEASAEISEATAALQSFWNEEGTREGIRMTQAKTMKRYM
Dmel_Nep3 VLFTDGGSSDATKHVLHVQPHQKDCP---SGNELPCLNKHCIFASSEILKSI
Lmig_ECE LLIVS--------AVLLSRPYAPALL---HAEEAYCVSMSCVTAAASVLSLM
Hsap_ECE1 VLVVLLAAGLVACLAALGIQYQTR---SPSVCLSEACVSVTSSILSSM
Hsap_ECE2 GASLLLAALLLGCLVALGVQYHRDP---SHSTCLTEACIRVAGKILESL
Hsap_Nep SVLVLLLTIIAVTMIALYATYDDG---ICKSSDCIKSAARLIQNM
Dmel_Nep1 ALAIYFASVHQRCHLYRLEPDNDDRPN----------------------GR------WNQDSGSAHEGQDNICMTQECVRTAASLLSAM
Dmel_Nep2 ATALHGDSTTINQVPTGTASKGKSG---DSGDVCLTQECIHTASTVLRKM
Dmel_Nep5 TANGHGQNSATESANGKQLPYEPISPN---------------------------------------LIGIQSNKECLRTAASLIYAM
271 360
Dmel-Nep4 DNKVDPCVDFYKYACGNWERLHPIPKDKAGFDTFEMLRESLDLVLRNLLEKNTPVHSAAELRKSPVRNTLFKLNEQGEGEGEADQAAELT
Dmel_Nep3 DVTVDPCDDFYGYSCNQWIKNNPIPEGKSTWGTFGKLEQMNQLIIRNVLEKPAKSFKS-----------------------------
Lmig_ECE DATADPCSDFYQYACGGWVRANPIPDTKSMWGTFVKLEQQNQLVIKNVLEQPMSEFKS-----------------------------
Hsap_ECE1 DPTVDPCHDFFSYACGGWIKANPVPDGHSRWGTFSNLWEHNQAIIKHLLENSTASVS-----------------------------
Hsap_ECE2 DRGVSPCEDFYQFSCGGWIRRNPLPDGRSRWNTFNSLWDQNQAILKHLLENTTFNSSS-----------------------------
Hsap_Nep DATTEPCTDFFKYACGGWLKRNVIPETSSRYGNFDILRDELEVVLKDVLQEPKTEDIV-----------------------------
Dmel_Nep1 DLNSDPCEDFFQYACGTWNKMHPIPEDRSSISTFEVLSDQQQVILRAVLEEPIDERDN-----------------------------
Dmel_Nep2 KPEVEPCDNFYEFACGTYLEEENIPDDKVSISTFSVISDKLQEQLKDIITAERPETEP-----------------------------
Dmel_Nep5 DEQTDPCEDFYQFTCGRWANEHPRPDSVTSNDWFRERQAHIMRVVREFLRSNITKSEP-----------------------------
361 450
Dmel-Nep4 AERLRRHIVSKRQLLNRVLVRYKRYTNGTKRKRLIETPRERTKEEEAAPPVVLPKDKTKDKSDNEEQLHVPTDFLKPHQDAQLKAKNLYR
Dmel_Nep3 ---DAERKAKVYYE
Lmig_ECE ---EAERKAKLYYM
Hsap_ECE1 ---EAERKAQVYYR
Hsap_ECE2 ---EAEQKTQRFYL
Hsap_Nep ---AVQKAKALYR
Dmel_Nep1 ---KATIKAKTFFK
Dmel_Nep2 ---KHFRLPNLLYK
Dmel_Nep5 ---EAVGKAKTMYR
451 540
Dmel-Nep4 SCVNSAV-LAKRGLEPLHTLIRELGGWPVLESQWS-------------ESNFNWQVLAATLR-RYNNDILIVQWVGADIKN----------
Dmel_Nep3 SCLDADEHMEKLGAKPMNDLLLQIGGWNVTKSGYN-------------VANWTMGHTLKILHNKYNFNCLFGWAIGEDDKN----------
Lmig_ECE SCLDVNDTIETLGPKPMLDLLVKIGGWNITGN-FS-------------IKNWSLQKSLETLQNRYNMGGLFTWAVGEDDRD----------
Hsap_ECE1 ACMNETR-IEELRAKPLMELIERLGGWNITGP----------------WAKDNFQDTLQVVTAHYRTSPFFSVYVSADSKN----------
Hsap_ECE2 SCLQVER-IEELGAQPLRDLIEKIGGWNITGP----------------WDQDNFMEVLKAVAGTYRATPFFTVYISADSKS----------
Hsap_Nep SCINESA-IDSRGGEPLLKLLPDIYGWPVATENWEQKY----------GASWTAEKAIAQLNSKYGKKVLINLFVGTDDKN----------
Dmel_Nep1 SCMDIPQ-IRKIGTGRLKQVLQSLGGWPVIERNWSP------------PADLSVERLMGQLRLNYSEPVMIELYVGADDKN----------
Dmel_Nep2 ACMNKTL-IETLGPEPITRVAERLGGWPLIKGDSWNAD----------DSWTWQEQVKKFRTAGFSMDYIIDFSIGVDLQN----------
Dmel_Nep5 ACMDTKL-LDKRDLEPLINYLLRFGLPVLPSALNLTLGSGSKYATEAANVKYNWLQSIVSIKQHLTMDLIIGFDVFPDPFNRTINRIALG
541 630
Dmel-Nep4 ----------SEENIVQFDQTGLGLPTREYFLQPSNAK-------------YLQAYQRYMAEVMHKMGA------S----------
Dmel_Nep3 ----------SSRHVTQIDQGGLTLPTADYYNNKTDNHR-----------KVLNEYIEYMTKVCVLLGA------N----------
Lmig_ECE ----------SKKHIIQIDQGGLTLPTRDNYLNETMDD-----------KVLSAYLEYMTKIGVLLGG------E----------
Hsap_ECE1 ----------SNSNVTQVDQSGLGLPSRDYYLNKTENE-----------KVLTGYLNYMVQLGKLLGGG-----D----------
Hsap_ECE2 ----------SNSNVTQVDQSGLFLPSRDYYLNRTANE-----------KVLTAYLDYMEELGMLLGG------R----------
Hsap_Nep ----------SVNHVTHIDQPRLGLPSRDYYECTGIYK-----------EACTAYVDFMISVARLIRQEERLPID----------
Dmel_Nep1 ----------SSVNILQMDQLQYALPSRDYYLKESSAN-----------DRRAYHRYMTQVALLLGA------D----------
Dmel_Nep2 ----------STKRLIDLDQSSLALS-REYLVKGFNET-----------LVTAYYKYMVDIAVLFGAN----------
Dmel_Nep5 TPETDSAFPFNNDDSHKMLRKIHRKTIFMQNSDDEDDSEDDRESEEEEAAKQTSTGMTAYLHYVRKVIEKYLLYVDPNVNQEEATLGITE

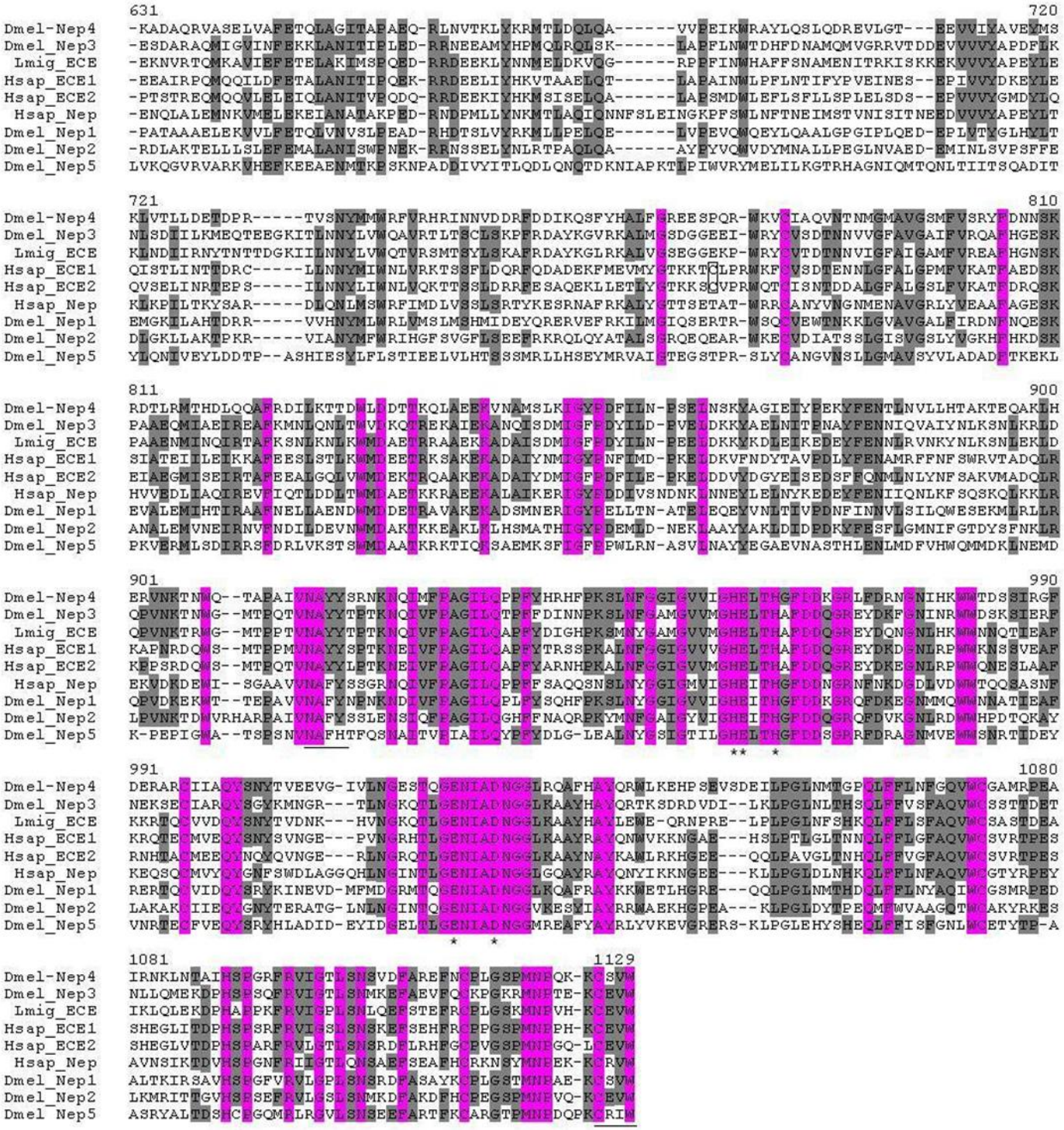


Figure S1 Amino acid sequence alignment of *Drosophila* Nep1-Nep5 with *Locusta migratoria* ECE and *Homo sapiens* ECE-1, ECE-2 and neprilysin. Conserved amino acids are marked in grey, identical amino acids in purple. The zinc binding ligands are indicated with an asterisk, the cysteines in ECE-1 and ECE-2 responsible for dimerization by a rectangle and the NAYY and CEVW sequences are underlined. The HExxH and ExxA/GD zinc binding domains are conserved in all 5 *Drosophila* neprilysins and LomECE. Of the 10 cysteine residues conserved in the predicted extracellular domain in members of this family, the first is not present in Nep4 and Nep5, and the second one is not found in Nep4. The cysteine residue necessary for dimer formation of ECE

(Cys416 in human ECE-1), which is missing in human neprilysin, is not conserved in *Drosophila* Nep1-5 or in LomECE, which suggests that these proteins may exist as monomers. The CEVW sequence, critical for proper protein folding and maturation of the enzyme (MACLEOD *et al.* 2001), is completely conserved in Nep2, Nep3 and LomECE. The glutamate is replaced by serine (CSVW) in Nep1 and Nep4 and by arginine in Nep5, which matches the sequence motif of human neprilysin. In Nep5, the valine is replaced by isoleucine (CRIW). Nep3, Nep4 and the locus ECE sequence contain the NAYY motif, which is important for substrate binding and is characteristic for ECE (SANSOM *et al.* 1998). At the corresponding positions, Nep1 and Nep2 share the sequence NAFY with human neprilysin.

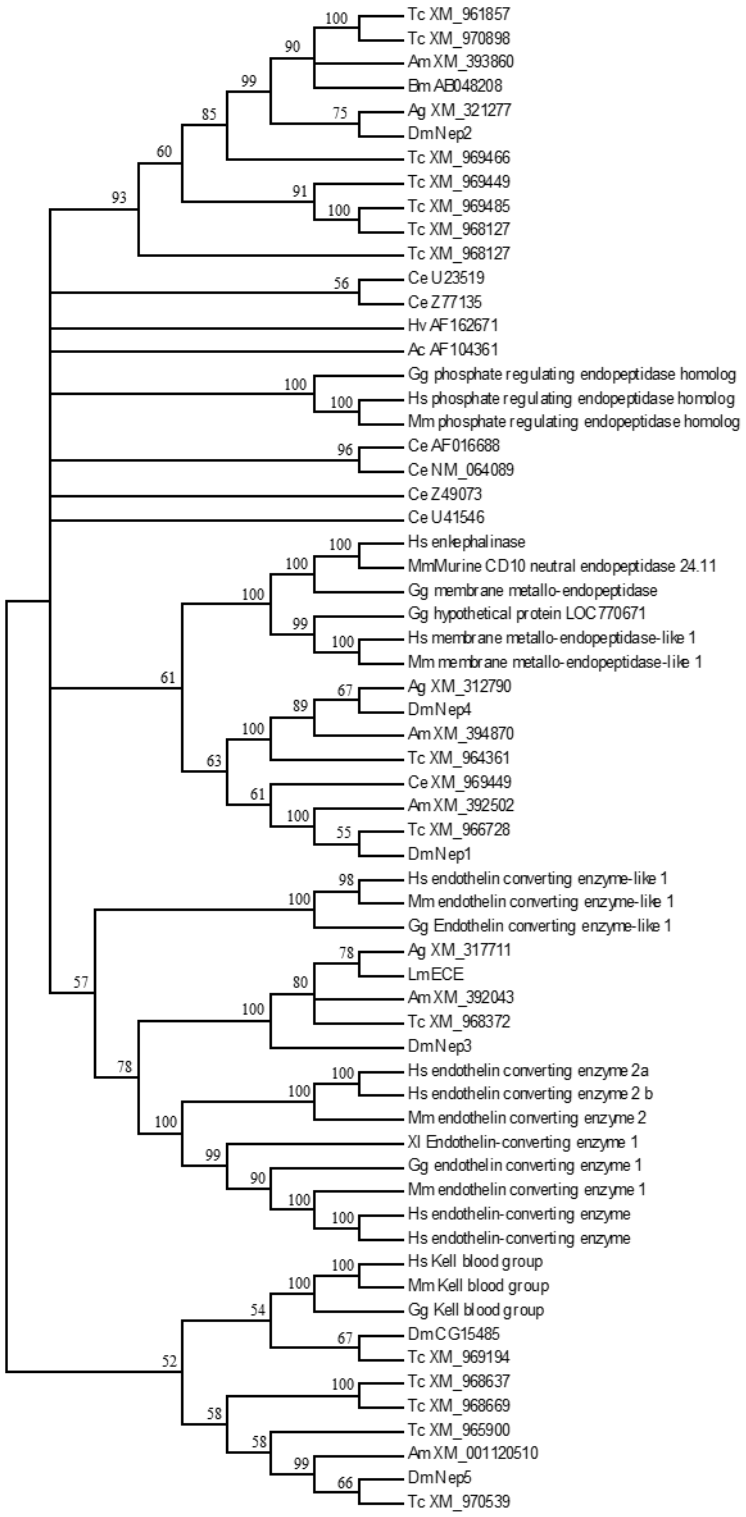


Figure S2 Neighbor-joining tree including *Lom*ECE and *Drosophila* Nep1-Nep5 (Hs: *Homo sapiens*; Mm: Mus *musculus*; Gg*: Gallus gallus*; Xl: *Xenopus leavis*; Tc: *Tribolium castaneum*; Am: *Apis mellifera*; Ag: *Anopheles gambiae*; Dm: *Drosophila melanogaster*; Lm: *Locusta migratoria*; Bm: *Bombyx mori*; Ce: *Caenorhabditis elegans*; Hv: *Hydra vulgaris*; Ac: *Aplysia californica*). A more in depth phylogenetic tree analysis of *Drosophila* Nep1-Nep5 and LomECE is shown in Figure 3. Sequences were aligned in ClustalX (THOMPSON *et al.* 1997) and manually edited in GeneDoc (NICHOLAS 1997). Distance-based neighbor-

joining trees were constructed using the JTT matrix in MEGA 3.1 (KUMAR *et al.* 2004). The reliability of internal branches was evaluated using bootstrap analysis (1000 replicas) and further supported by an analysis of the specific signature sequences of these enzymes. For Nep3 and *Lom*ECE the third zinc binding ligand ExxA/GD is located 61 residues downstream of the active site that corresponds to the 61 amino acid separation in human ECE-1 and ECE-2 (SHIMADA *et al.* 1995). The characteristic ET binding site NAYY (SANSOM *et al.* 1998) and the CEVW motif, critical for enzyme maturation and activity of ECE-1 and ECE-2 (MACLEOD *et al.* 2001), are present in Nep3 and *Lom*ECE. The overall very good conservation between *Drosophila* Nep3, *Lom*ECE and human ECE suggests that Nep3 is the *Drosophila* ortholog of mammalian ECE. Nep1 and Nep4 both cluster in a group with vertebrate neprilysin homologs, although Nep4 possesses the NAYY substrate binding domain characteristic of ECE, and Nep1 shares the NAFY motif with neprilysin. In both Nep1 and Nep4 the E of the CEVW motif is substituted by a different amino acid as is also seen in mammalian neprilysin (replaced by S and R, respectively). In human neprilysin there is a separation of 64 residues between the third zinc binding ligand and the active site. The 63 amino acid separation in Nep1 and Nep4 is not identical to that in neprilysin, but invertebrate neprilysin-like enzymes have a larger distance between the zinc binding residues compared to invertebrate ECE-like enzymes, as do their mammalian counterparts. The phylogenetic analyses suggests that Nep5 is the *Drosophila* homologue of the Kell blood group antigen, though the two lineages show divergent patterns at two key sequence motifs: human Kell contains the NAYY substrate binding domain and a CQLW sequence, while Nep5 possesses a NAFH motif and a CRIW sequence.

Nep2 is grouped into a cluster of insect neprilysin-like genes. The role of this neprilysin-like enzyme might be insect specific or performed by another member of the family in higher organisms. For all neprilysins, orthologs can be found in the genomes of *Anopheles gambiae*, *Apis melifera* and *Tribolium castaneum*.

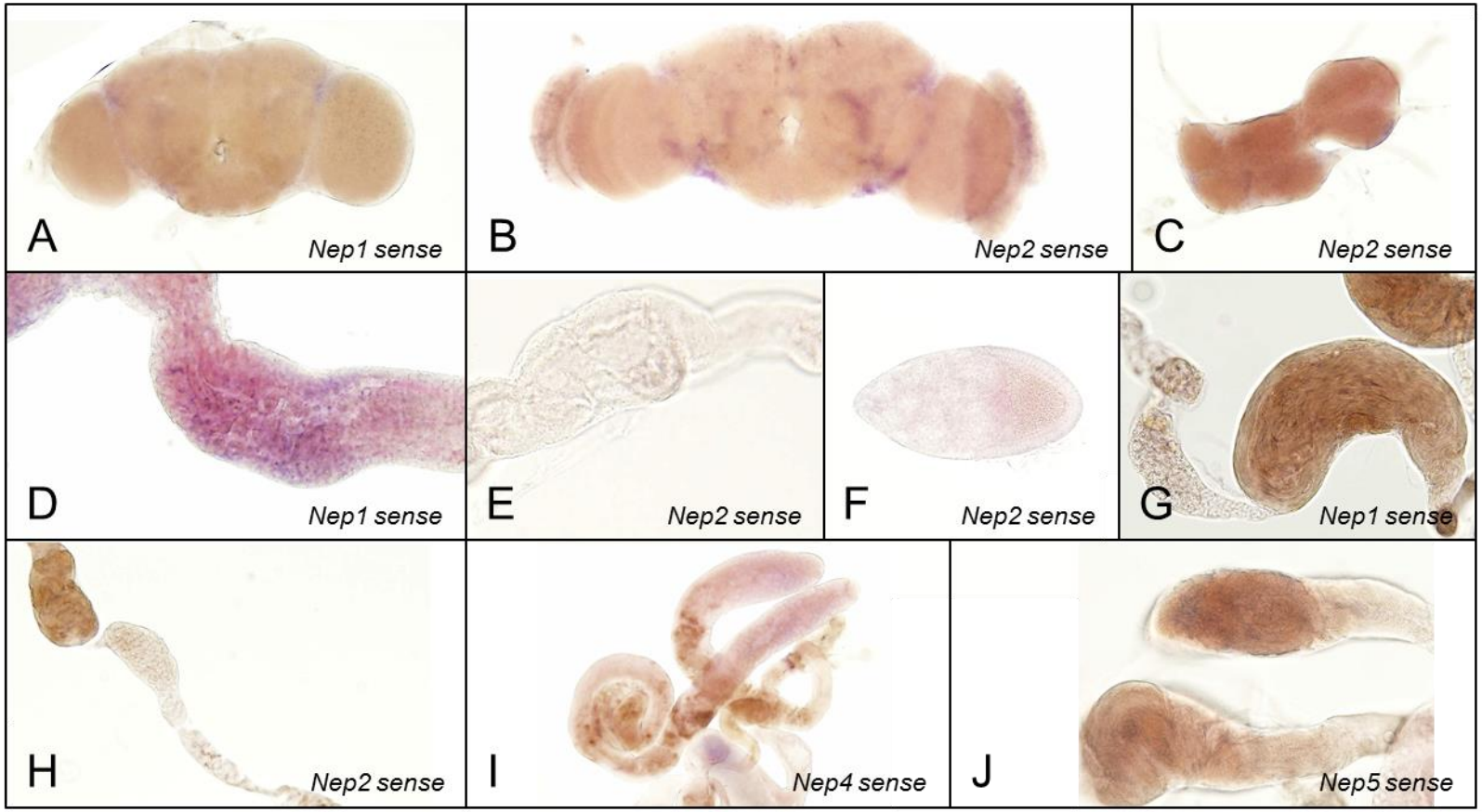


Figure S3 In situ hybridization results with sense probes for Nep1-5 in adult tissues revealed complete absence of signal. (A-B) Adult brain; (C) Thoracico-abdominal ganglion; (D) Midgut; (E) Malpighian tubules; (F) Stage 10 ovarian follicle; (G-J) male reproductive tract. These sense-controls also gave no signal in hybridization to larval tissue or embryos (data not shown).

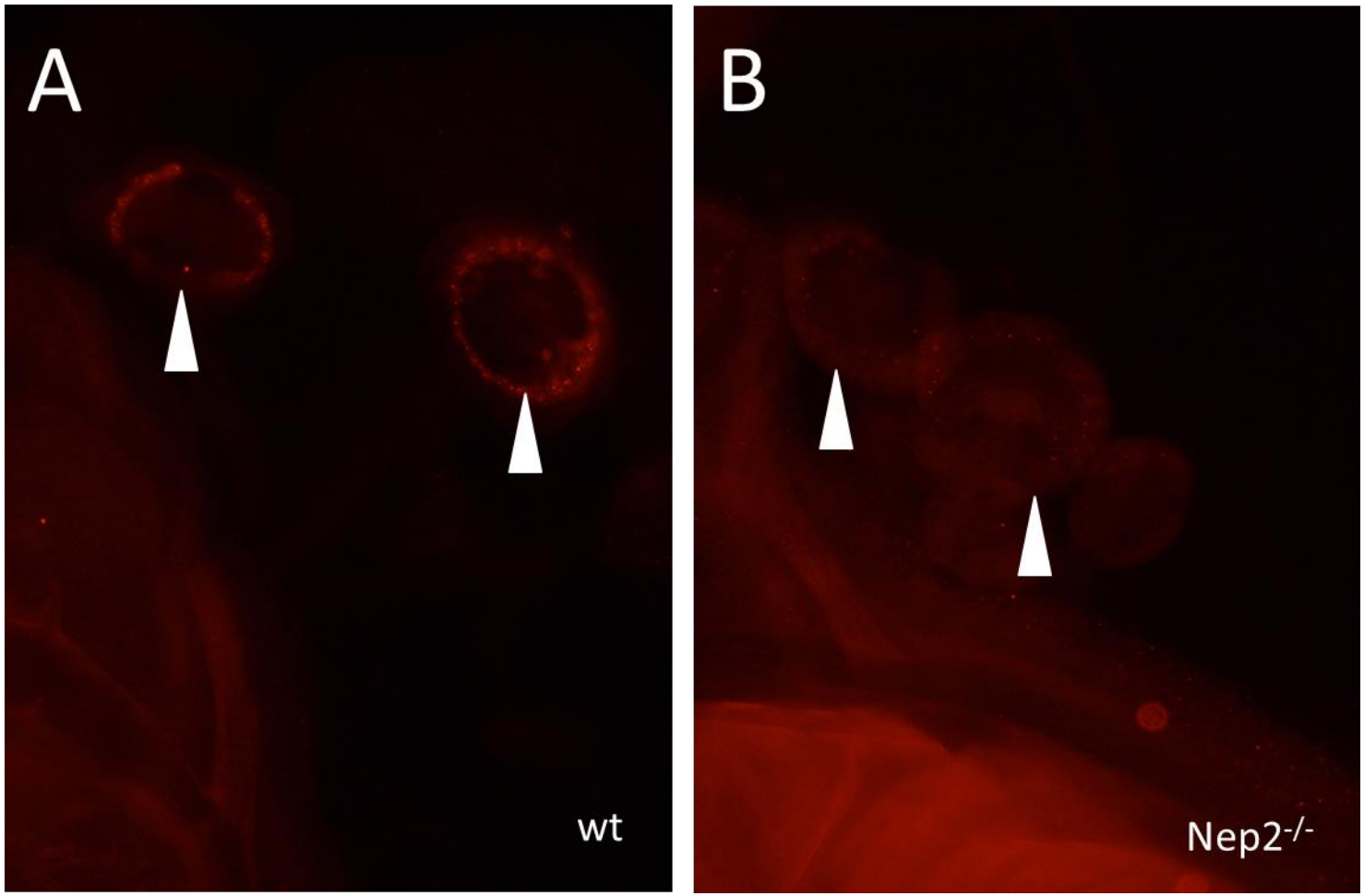


Figure S4 Nep2 expression in the spermathecae. (A) Positive immunolabeling in wildtype spermathecae (arrowheads). (B) Lack of immunolabeling in spermathecae of $Nep2^{-/-}$ females (arrowheads).

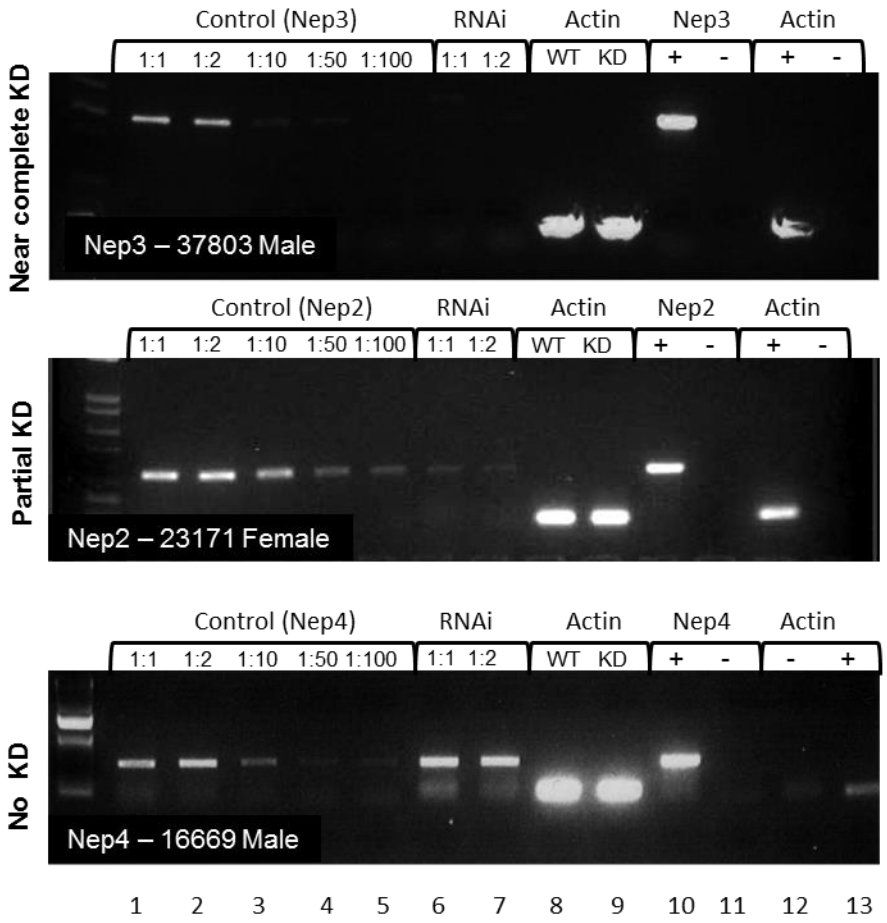


Figure S5 RT PCRs**.** Examples of the RT-PCR results used to generate the integrated optical densities (IOD) for Table S3. The RNA used to generate cDNA for PCR was extracted from whole flies. A dilution series of control cDNA (Lanes 1-5) was amplified with gene specific primers and compared to cDNA from RNAi flies (Lanes 6 and 7). As a loading control for normalization, cDNA at the 1:1 concentration from either Control (WT) or RNAi (KD) flies was amplified with primers specific to Actin (Lanes 8 and 9 respectively). To control for primer and/or solution contamination primer controls were run with (+) or without (-) the presence of cDNA for both gene specific primers (Lanes 10 and 11) and Actin primers (Lanes 12 and 13). We observed near complete knockdown (KD) of *Nep3* in Nep3-37803 RNAi males, partial KD of *Nep2* in Nep2-23171 RNAi females, and no KD of *Nep4* in Nep4-16669 RNAi males.

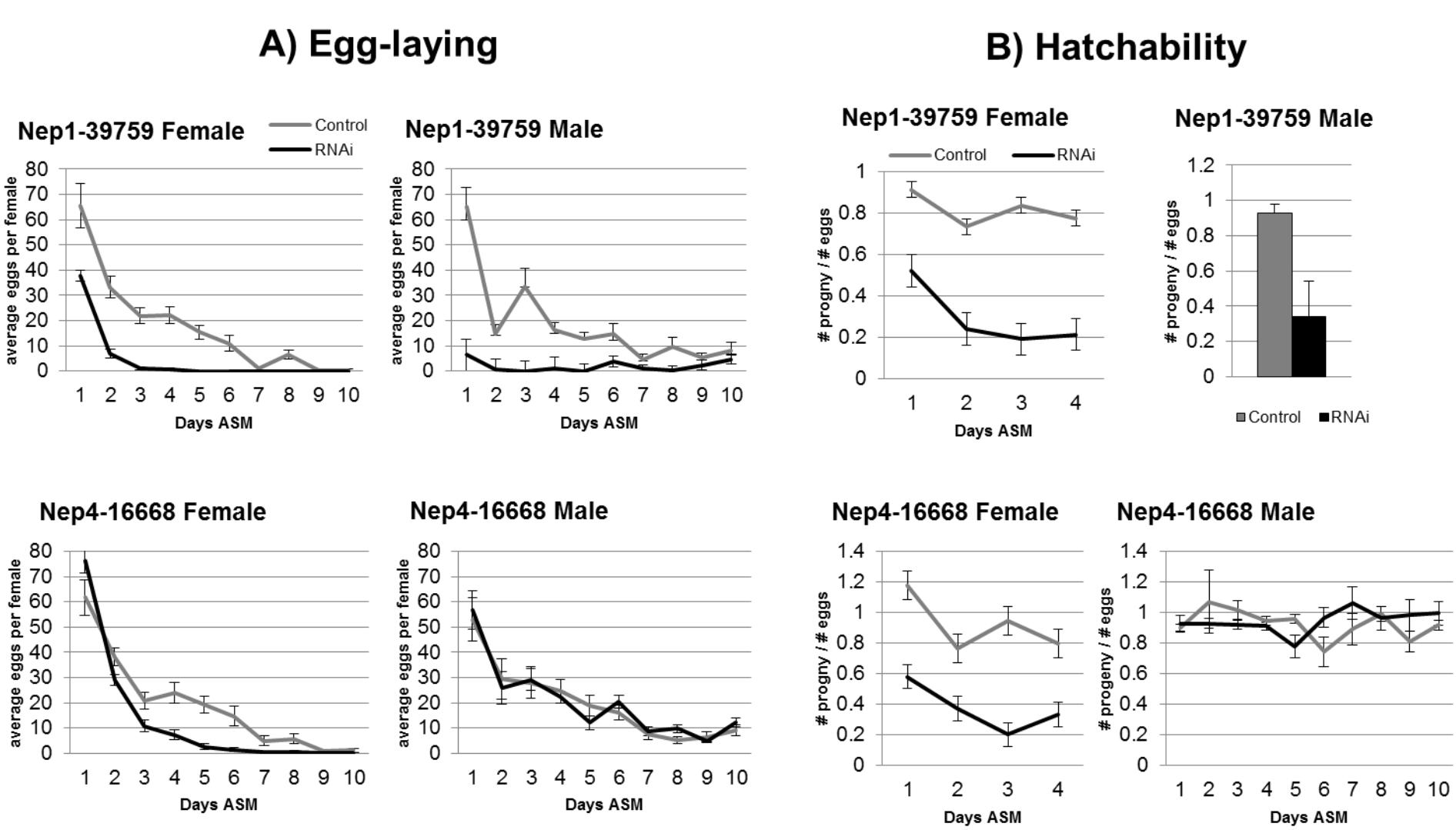
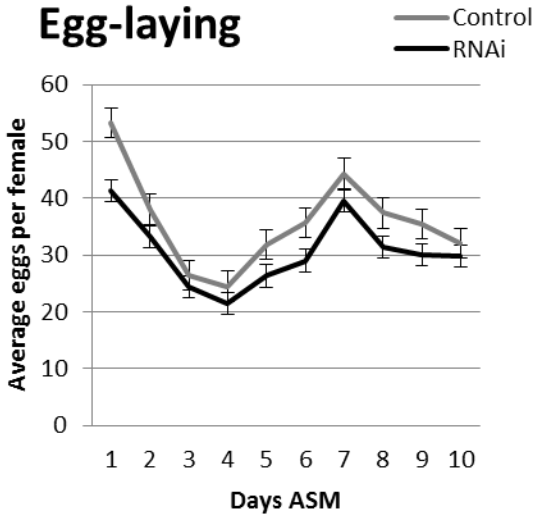


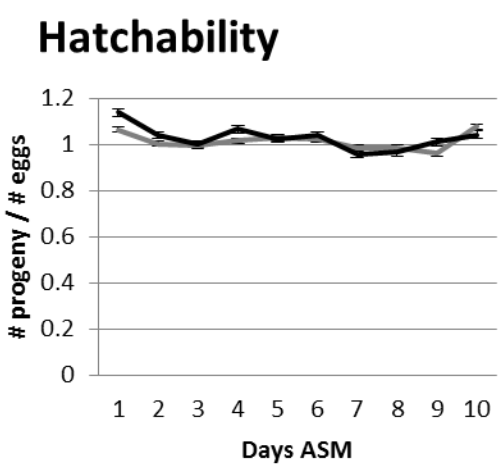
Figure S6 (A) The mean number of eggs laid by Control females or mates of Control males (grey line) or RNAi females or mates of RNAi males (black line) for the *Nep1* and *Nep4* alternative UAS-RNAi lines. A reduction in egg-laying was observed compared to controls when *Nep1* was knocked down using line Nep1-39759 in either males or females (*Nep1* Female: rmANOVA p=<0.0001*, Control N=11, *Nep1* RNAi N=22 ; *Nep1* male: rmANOVA p=<0.0001*, Control N=12, *Nep1* RNAi N=16) consistent with our results using the Nep1-27537 line in Figures 6&7. Reduced egg-laying was also seen for Nep4-16668 RNAi females compared to controls (*Nep4* Female: rmANOVA p=0.0336, Control N=16, RNAi *Nep4* RNAi N=11) but not for mates of Nep4-16668 RNAi males (*Nep4* male: rmANOVA p=0.8985, Control N=13, *Nep4* RNAi N=16) consistent with our results using Nep4-100189 in Figures 6&7. (B) The mean hatchability (#progeny/#eggs) per female for the lines in part A. For both *Nep1* and *Nep4* RNAi Females it was not possible to calculate hatchability for the entire 10 day period, since egg-laying for all or most RNAi females reached zero by day 5. Likewise, mates of Nep1 RNAi males lay few (most fewer than 10) eggs, so we calculated total hatchability over the entire 10 day period as (# total progeny/#total eggs) instead. Consistent with previous results (Figure 6) hatchability in mates of Nep4 RNAi males was comprable to controls (Nep4 Male: rmANOVA p=0.3772). However, in all other cases hatchability was decreased (*Nep1* female: rmANOVA p=<0.0001; *Nep1* male:

WRST p=0.0021 ; *Nep4* female: rmANOVA p=0.0027), inconsistent with previous results. The new Nep1-39759 line uses the same UAS-construct as the original line (Nep1-27537) with an insertion on the 3rd chromosome instead of the 2nd (DIETZL *et al.* 2007). Both the egg-laying and hatchability phenotypes observed in mates of Nep1-39759 RNAi males and in Nep1-39759 RNAi females are more severe than in the original line. This difference is likely not due to improved knockdown, since knockdown appears to be more efficient in Nep1-27537 RNAi males than in Nep1-39759 males (Table S3). It seems likely that the location of the insertion on the 3rd chromosome is responsible for these differences and that this phenotype is not specific to reduced *Nep1* expression. The alternative line Nep4-16668 and the original Nep4-100189 line differ by UAS-construct, method of integration (random v. phiC31 respectively), and chromosomal location (3rd v. 2nd respectively). Unlike in the case of Nep1, the alternative Nep4-16668 line does knockdown slightly better than the original line (Table S3), it is possible that this increase in knockdown, random insertion of the UAS-construct on chromosome-3, and/or a difference in background is responsible for this discrepancy in hatchability measurements. To address the chromosomal insertion possibility, we obtained a second chromosome insertion of the UAS-construct used in Nep4-16668, Nep4-16669, however this line did not knock down (male Figure S5, female not shown). Together these results suggest that Nep1 may play a role in early hatchability in the female (consistent with Figure 7) whereas we cannot currently make definitive conclusions about the function of Nep4 in this process.

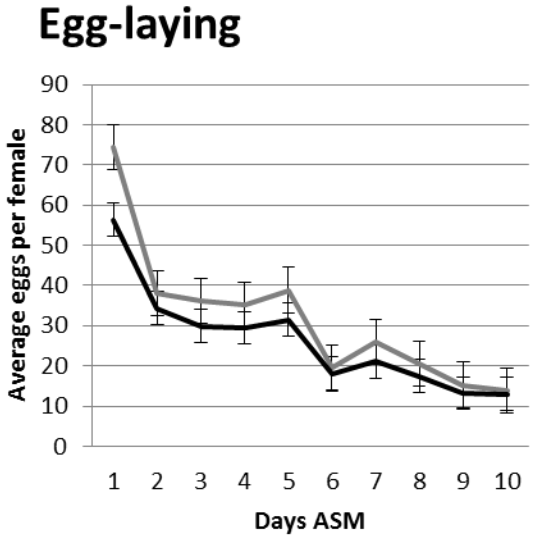
Knockdown of *Nep2* in:

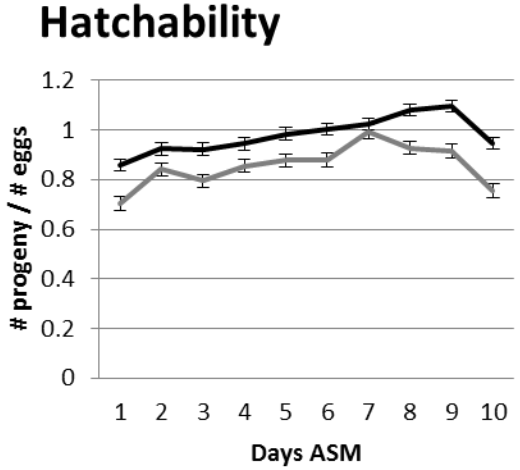
CNS



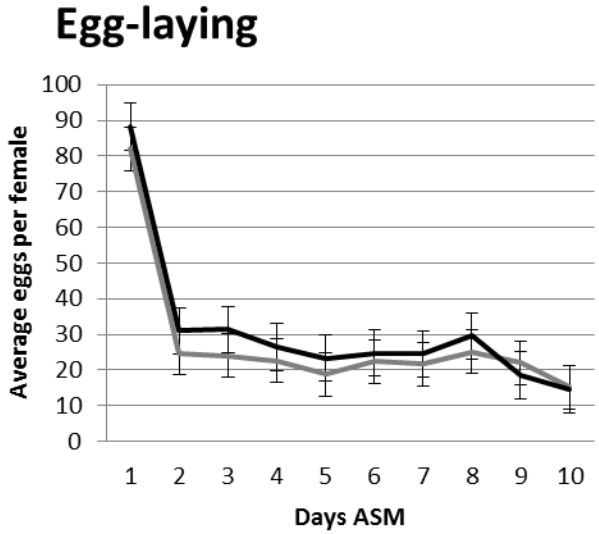


Spermathecae





Border Cells



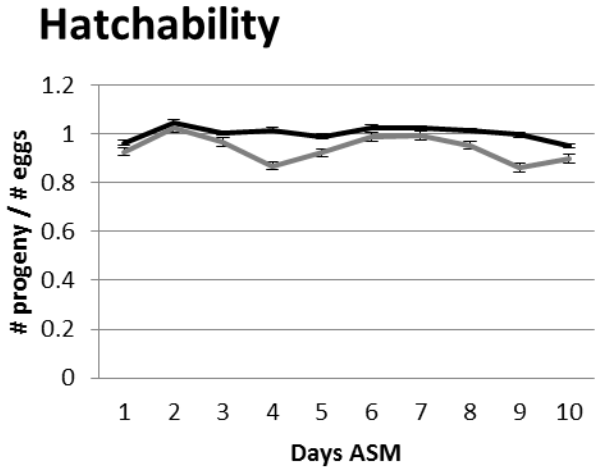
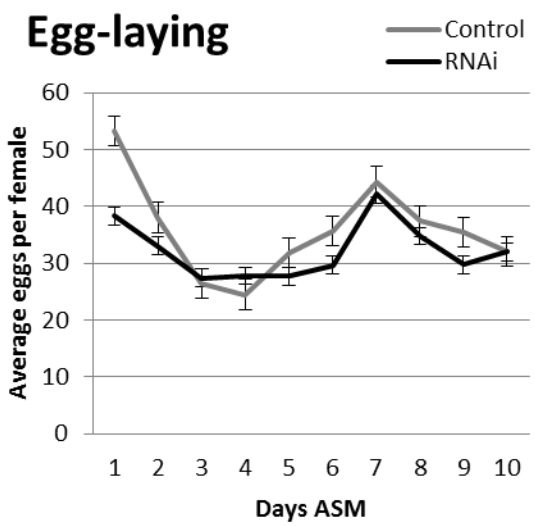
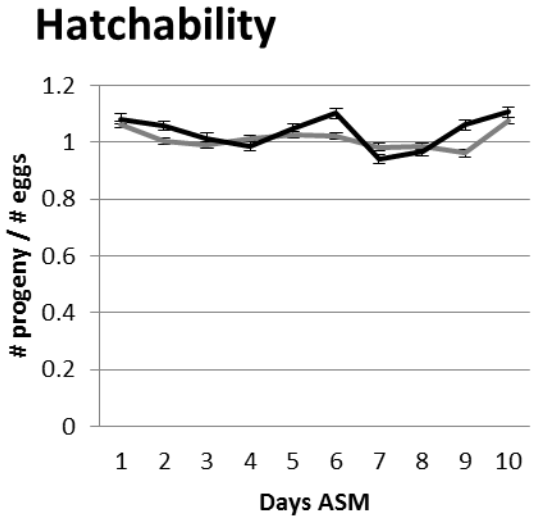


Figure S7 The mean number of eggs laid by Control (grey line) or Nep2 RNAi females (black line) knocked down in one of three tissues (the CNS, spermathecae, or border cells) mated to WT males over a 10 day period. Females with reduced Nep2 expression in either the CNS (rmANOVA p=<0.0001*, Control N= 18, RNAi N=16) or spermathecae (rmANOVA p=0.0002, Control N= 19, RNAi N=15) laid fewer eggs than control females, whereas loss of *Nep2* expression in the border cells slightly increased egg-laying (rmANOVA p=0.0177, Control N= 23, RNAi N=17). None of the tissues recapitulate the hatchability defects seen in Nep2 null females (CNS rmANOVA p=0.1221, Border Cells rmANOVA p=0.1170).

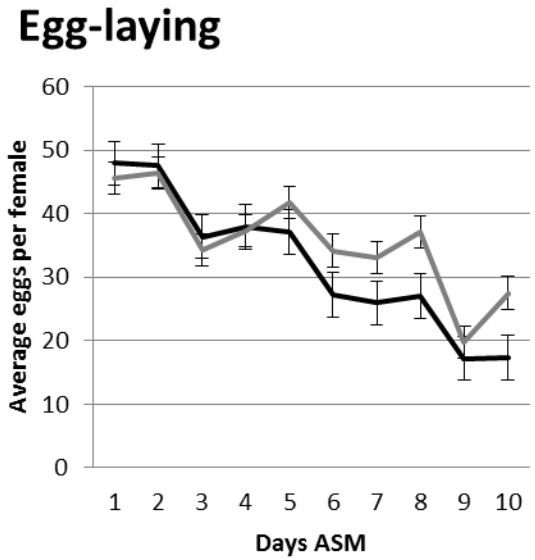
Knockdown of *Nep1* in:

CNS





Spermathecae



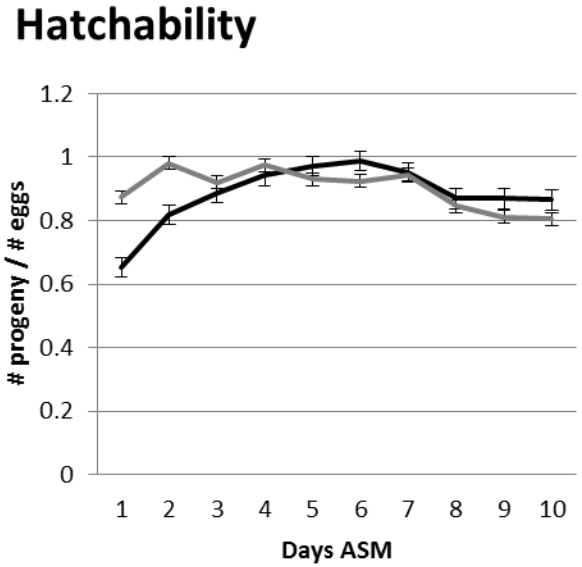


Figure S8 The mean number of eggs laid by Control (grey line) or *Nep1* RNAi females (black line) knocked down in one of two tissues (the CNS or the spermathecae) mated to WT males over a 10 day period. Females that have reduced Nep1 expression in either the CNS (rmANOVA p=0.0042, Control N=18 , RNAi N=18) or spermathecae (rmANOVA p=0.0078, Control N= 17, RNAi N=13) lay fewer eggs than control females. There was no overall effect of reduction in *Nep1* expression on hatchability in the case of the CNS (rmANOVA p=0.0853) or the spermathecae (rmANOVA p=0.4419) although loss of *Nep1* in the spermathecae can reduce initial hatchability on Days 1 and 2 (Day 1 WRST p=0.0161, Day 2 WRST p=0.0005)

Table S1 Gene expression profiles for *Drosophila* Neprilysin family genes in reproductive organs. A summary of the gene expression profiles of Drosophila neprilysins in both male and female reproductive organs. Expression data were obtained from Fly atlas (CHINTAPALLI *et al.* 2007), positive expression is denoted (+) and lack of expression is denoted with (-). Total tissues refers to the number of reproductive tract tissues in which the gene is found. Mated verses Virgin spermatheca samples were treated separately, since it is known that mating causes differential gene expression in the female spermatheca (SCHNAKENBERG *et al.* 2011).

Gene	Total Tissues	Virgin Spermatheca	Mated Spermatheca	Ovary	Testis	Male AG	RT Specificity	CNS Expression
CG13650	2	-	-	-	+	+	Male	+
CG14523	2	+	+	-	-	-	Female	-
CG14526	3	+	+	+	-	-	Female	+
CG14527	1	-	+	-	-	-	Female	-
CG14528	2	+	+	-	-	-	Female	-
CG14529	1	+	-	-	-	-	Female	-
CG31918	1	-	-	+	-	-	Female	-
CG3239	2	+	+	-	-	-	Female	-
CG3775	2	+	+	-	-	-	Female	+
CG42370	2	+	+	-	-	-	Female	-
CG4580	0	-	-	-	-	-	None	-
CG4721	3	+	+	-	-	+	Both	+
CG4725	2	+	+	-	-	-	Female	-
CG5527	2	+	+	-	-	-	Female	-
CG8550	2	+	+	-	-	-	Female	-
CG9505	3	+	+	+	-	-	Female	-
CG9507	0	-	-	-	-	-	None	+
CG9634	4	+	+	+	+	-	Both	+
CG9780	2	+	+	-	-	-	Female	-
Nep1	3	+	+	-	+	-	Both	+
Nep2	4	+	+	+	+	-	Both	+
Nep3	1	-	-	-	+	-	Male	+
Nep4	2	+	-	-	+	-	Both	+
Nep5	1	-	-	-	-	+	Male	-

Table S2 ***In situ*** **summary.** Summary of expression patterns as detected by in situ hybridization. The Nep2 expression in spermathecae was detected with antibody staining. Embryonic stages are listed as SX with X being the appropriate stage number.

	Embryo (all in stage 17 except otherwise indicated)	Larva (third instar)	Adult (sex-specific expression indicated where appropriate)
Nep1	PNS (incl. ant-max complex) Pharynx Midgut	 Midgut Brain + ventral ganglion Imaginal discs (wing, leg)	 Midgut Brain Testes (male) Seminal vesicles (male)
Nep2	Tracheal system Foregut Hindgut Epidermis	 Foregut Malpighian tubules (stellate cells) Brain + ventral ganglion Imaginal discs (eye-antennal, leg)	 Malpighian tubules (stellate cells) Brain + ventral ganglion Testes (male) Ovaries (female)
Nep3	CNS (S14-17)	Brain + ventral ganglion Midgut	-
Nep4	Muscle founder cells (S12) Pericardial cells (S13) CNS (S14-17) Tracheal system	 Brain + ventral ganglion (glia)	 Testes (male)
Nep5	Sparse anteriorly located cells	-	 Seminal vesicles (male)

Table S3 A summary of the new findings presented in this paper. For each line expression data is reported as the normalized integrated optical density (nIOD) from RT-PCR results. Signals were normalized to the IOD of Actin from the same samples (gene specific IOD/Actin IOD) and knockdown (KD) as % of WT expression calculated as (nIOD RNAi/nIOD Control). Experimental results are reported as either n/a (not applicable), n.s. (non significant), n.d. (not done), or as p-values where significant. Black p-values indicate a significant decrease in the measured value whereas red p-values indicate an increase. In the case of Nep1 Line2 (VDRC ID-16668) we were unable to determine level of knockdown in females (denoted as n.d.). Two other lines, Nep4 Line3 (VDRC ID-16669) and Nep5 Line2 (VDRC-ID 27570) did not knock down and thus were excluded from this table.

Gene	Sex	Line			Expression - RT-PCR			Egg-laying		Hatchability		Other Notes
		#	Type	VDRC transformant ID	nIOD Control	nIOD RNAi	KD as % of WT expression	Ubiquitous KD	Tissue specific KD	Ubiquitous KD	Tissue specific KD	
Nep1	Male	1	RNAi	27537	0.6743	0.0272	4.0%	**p=0.0041**	n.d.	n.s.	n.d.	
Nep1	Male	2	RNAi	39759	2.2610	1.1156	49.3%	**p=<0.0001**	n.d.	**p=0.0021**	n.d.	
Nep1	Female	1	RNAi	27537	0.3786	0.0776	20.5%	**p=0.0015**	**CNS: p=0.0042** **Spermathecae: p=0.0078**	n.s. **Day2 p=0.0253**	CNS: n.s **Spermathecae n.s** **Day1 p=0.0161** **Day2 p=0.0005**	
Nep1	Female	2	RNAi	39759	n.d	n.d	n.d	**p=<0.0001**	n.d.	**p=<0.0001**	n.d.	
Nep2	Male	1	RNAi	23171	0.3988	0.0085	2.1%	n.s.	n.d.	n.s.	n.d.	
Nep2	Male	2	Mutant	n/a	n/a	n/a	n/a	n.s.	n.d.	n.s.	n.d.	
Nep2	Female	1	RNAi	23171	0.4214	0.0413	9.8%	**p=<0.0s01**	**CNS: p=<0.0001** **Spermathecae: p=0.0002** **Border cells: p=0.0177**	**p=<0.0001**	CNS: n.s Spermathecae: n.s Border cells: n.s	

Nep2	Female	2	Mutant	n/a	n/a	n/a	n/a	**p=<0.0001**	n/a	**p=<0.0001**	n/a	**sperm storage: 2h p=0.0398 4d p=0.0215** **(P1) p= <0.0001** Fertilization: n.s **Embryogenesis: p=0.0001**
Nep3	Male	1	RNAi	37803	0.2445	0.0169	6.9%	n.s.	n.d.	n.s.	n.d.	
Nep4	Male	1	RNAi	100189	0.1220	0.0066	5.4%	n.s.	n.d.	n.s.	n.d.	
Nep4	Male	2	RNAi	16668	1.0094	0.0406	4.0%	n.s.	n.d.	n.s.	n.d.	
Nep4	Female	1	RNAi	100189	0.1046	0.0135	12.9%	**p=0.0207**	n.d.	n.s.	n.d.	
Nep4	Female	2	RNAi	16668	0.8031	0.0478	6.0%	**p=0.0336**	n.d.	**p=0.0027**	n.d.	
Nep5	Male	1	RNAi	107430	0.0670	0.0007	1.1%	n.s.	n.d.	n.s.	n.d.	

Table S4 Nep cleavage sites in *Drosophila* neuropeptides. A summary of the predicted cleavage sites for Neps in *Drosophila* neuropeptides associated with the regulation of physiology and behavior (reviewed in (NASSEL and WINTHER 2010) as well in the newly identified group classified as Natalisins (JIANG *et al.* 2013). Human Nep cleavage sites are based on predictions from the MEROPS database (RAWLINGS *et al.* 2012) and Drosophila cleavage sites are based on *in vitro* experiments (BLAND *et al.* 2007; MEYER *et al.* 2009; THOMAS *et al.* 2005). The presence of at least one potential cleavage site is denoted as (+) absence of a cleavage site is denoted as (-). Predicted cleavage sites based on Human Nep are denoted in red, predicted *Drosophila* Nep cleavage sites are denoted in green, shared sites are denoted in blue, and prior experimentally confirmed *Drosophila* cleavage sites (*in vitro*) are denoted in black.

Neuropeptide name	Acronym	Predicted Cleavage Sites		Sequence
		Drosophila NEP site	Human NEP site	
Adipokinetic hormone	AKH	-	-	pQLTFSPDWa
Allatostatin A (AstA)	AstA-1	+	+	VERYAFGLa
	AstA-2	+	+	LPVYNFGLa
	AstA-3	+	+	SRPYSFGLa
	AstA-4	+	+	TTRPQPFNFGLa
Allatostatin B (AstB; MIP)	MIP-1	+	+	AWQSLQSSWa
	MIP-2	-	-	AWKSMNVAWa
	MIP-3	-	-	pEAQGWNKFRGAWa
	MIP-4	-	-	EPTWNNLKGMWa
	MIP-5	-	-	DQWQKLHGGWa
Allatostatin C (AstC)	AstC	-	+	pEVRYRQCYFNPISCF
CAPA-PVK/PK	CAPA-PVK-1	+	+	GANMGLYAFPRVa
	CAPA-PVK-2	+	+	ASGLVAFPRVa
	CAPA-PK	+	+	TGPSASSGLWFGPRLa
	CPPB	-	-	GDAELRKWAHLLALQQVLD
CCAP	CCAP	-	-	PFCNAFTGCa
CCHamide	CCH1	-	-	SCLEYGHSCWGAHa
	CCH2	-	-	GCQAYGHVCYGGHa
Corazonin	CRZ	-	-	pQTFQYSRGWTNa
Diuretic hormone 44	DH44	+	+	NKPSLSIVNPLDVLRQRLLLEIARRQMKENSRQVELNRAILKNV
Diuretic hormone 31	DH31	+	+	TVDFGLARGYSGTQEAKHRMGLAAANFAGGPa
dFMRFamides	dFMRFa-1	-	+	SVKQNDFMHFa
	dFMRFa-2	+	+	DPKQDFMRFa
	dFMRFa-3	+	+	TPAEDFMRFa

	dFMRFa-4	+	+	SDNFMRFa
	dFMRFa-5	+	+	SPKQDFMRFa
	dFMRFa-6	+	+	PDNFMRFa
	dFMRFa-7	-	-	SAPQDFVRSa
	dFMRFa-8	+	+	MDSNFIRFa
Drosulfakinins	DSK-0	+	+	NQKTMSFa
	DSK-1	+	+	FDDYGHMRFa
	DSK-2	+	+	GGDDQFDDYGHMRFa
Dromyosuppressin	DMS	+	+	TDVDHVFLRFa
Ecdysis-triggering hormone	ETH-1	+	+	DDSSPGFFLKITKNVPRLa
	ETH-2	-	+	GENFAIKNLKTIPRIa
Hugin-pyrokinin	hug-PK	-	+	SVPFKPRLa
	hug-g	-	+	LRQLQSNGEPAYRVRTPRLa
Leucokinin	LK	+	+	NSVVLGKKQRFHSWGa
Natalisin (CG34388)	Dm01	-	+	EKLFDGYQFGEDMSKENDPFIPPRa
	Dm02	+	+	HSGSLDLDALMNRYEPFVPNRa
	Dm03	-	-	DKVKDLFKYDDLFYPHRa
	Dm04	-	+	HRNLFQVDDPFFATRa
	Dm05	-	+	LQLRDLYNADDPFVPNRa
Neuropeptide F	NPF	+	+	SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFa
Neuropeptide F (short NPF)	sNPF-1	+	+	AQRSPSLRLRFa
	sNPF-1[4-11]	+	+	SPSLRLRFa
	sNPF-2	+	+	WFGDVNQKPIRSPSLRLRFad
	sNPF-2[12-19]	+	+	SPSLRLRFa
	sNPF-3	-	+	KPQRLRWa
	sNPF-4	-	+	KPMRLRWa
NPLP1	MTYamide	+	+	YIGSLARAGGLMTYa
	IPNamide	-	+	NVGTLARDFQLPIPNa
	APK	+	+	SVAALAAQGLLNAPK
	VQQ	-	+	NLGALKSSPVHGVQQ
NPLP2	NEF	-	-	TKAQGDFNEF
NPLP3	SHA	-	+	VVSVVPGAISHA

	VVIamide	+	+	SVHGLGPVVIa
NPLP4	YSY	+	+	pQYYYGASPYAYSGGYYDSPYSY
Pigment-dispersing factor	PDF	+	+	NSELINSLLSLPKNMNDAa
Proctolin		-	+	RYLPT
Sex peptide	SP	-	+	WEWPWNRKPTKFPIPSPNPRDKWCRLNLGPAWGGRC
SIFamide	SIFa	-	-	AYRKPPFNGSIFamide
Tachykinin-related	DTK-1	+	+	APTSSFIGMRa
	DTK-2	+	+	APLAFVGLRa
	DTK-3	+	+	APTGFTGMRa
	DTK-4	+	+	APVNSFVGMRa
	DTK-5	+	+	APNGFLGMRa
	DTK-6	+	+	QQRFADFNSKFVAVRa

Table S5 Neuronal precursor expression. A summary of tissue expression and cleavage potential for the neuronal precursors in *Drosophila melanogaster*. Potential cleavage is based on Table S4 and is divided into those precursors whose derived mature neuropeptides contain at least one potential Nep cleavage site (+) and those precursors whose derived mature neuropeptides contain none (-). Expression data were obtained from FlyAtlas (CHINTAPALLI *et al.* 2007), positive expression is divided by FlyAtlas category (low, moderate, high, very high) and lack of expression is denoted with (-). Mated vs. Virgin spermatheca samples were treated separately, as in Table S1.

Neuropeptide name	Potential Cleavage Site	Malet RT		Female RT			CNS	
		Testis	AG	Ovary	Virgin SP	Mated SP	Brain	TAG
Adipokinetic hormone	-	-	-	-	-	-	high	high
Allatostatin A (AstA)	+	-	-	-	low	low	high	very high
Allatostatin B (AstB; MIP)	+	low	-	-	-	-	high	moderate
Allatostatin C (AstC)	+	-	-	-	-	-	very high	high
CAPA-PVK/PK	+	-	-	-	-	-	high	very high
CCAP	-	low	-	-	-	-	low	moderate
CCHamide-1	-	-	-	-	low	-	low	moderate
CCHamide-2	-	-	-	-	very high	very high	moderate	moderate
Corazonin	-	-	-	-	-	-	very high	-
Diuretic hormone 44	+	low	-	-	-	-	high	moderate
Diuretic hormone 31	+	-	-	-	-	-	high	very high
dFMRFamides	+	-	-	-	-	-	high	very high
Drosulfakinins	+	-	-	-	-	-	very high	-
Dromyosuppressin	+	-	very high	-	low	low	very high	moderate
Ecdysis-triggering hormone	+	-	-	-	-	-	-	-
Hugin-pyrokinin	+	-	-	-	-	-	very high	-
Leucokinin	+	-	-	-	-	-	moderate	very high
Natalisin (CG34388)	+	-	-	-	-	-	moderate	-
Neuropeptide F	+	-	-	-	-	-	high	moderate
Neuropeptide F (short NPF)	+	-	low	-	-	-	high	moderate
NPLP1	+	-	-	-	low	low	very high	very high
NPLP2	-	low	moderate	low	very high	very high	very high	very high
NPLP3	+	-	n/a	-	n/a	n/a	n/a	low
NPLP4	+	-	-	-	low	low	low	-
Pigment-dispersing factor	+	-	-	-	-	-	very high	high

Proctolin	+	-	-	-	-	-	moderate	high
Sex peptide	+	-	very high	-	-	-	-	-
SIFamide	-	-	-	-	-	-	moderate	-
Tachykinin-related	+	-	-	-	-	-	high	moderate

Supporting Information References

Kumar, S., K. Tamura, and M. Nei, 2004 MEGA3: Integrated software for molecular evolutionary genetics Analysis and sequence alignment. Brief. Bioinform. 5: 150–163.

MacLeod, K. J., R. S. Fuller, J. D. Scholten, and K. Ahn, 2001 Conserved cysteine and tryptophan residues of the endothelin-converting enzyme-1 CXAW motif are critical for protein maturation and enzyme activity. J. Biol. Chem. 276: 30608–30614.

Nicholas, K. B., H. B. Nicholas, Jr., and D. W. Deerfield, II, 1997 GeneDoc: analysis and visualization of genetic variation. EMBNEW 4: 14.

Sansom, C. E., M. V. Hoang, and A. J. Turner, 1998 Molecular modelling and site-directed mutagenesis of the active site of endothelin-converting enzyme. Protein Eng. 11: 1235–1241.

Shimada, K., M. Takahashi, M. Ikeda, and K. Tanzawa, 1995 Identification and characterization of two isoforms of an endothelin-converting enzyme-1. FEBS Lett. 371: 140–144.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins, 1997 The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876–4882.