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## Abstract

The pyrokinin/pheromone biosynthesis activating neuropeptide (PBAN) family of peptides is characterized by a common C-terminal pentapeptide, FXPRLamide, which is required for diverse physiological functions in various insects. Polyclonal antisera against the C-terminus was utilized to determine the location of cell bodies and axons in the central nervous systems of larval and adult mosquitoes. Immunoreactive material was detected in three groups of neurons in the subesophageal ganglion of larvae and adults. The corpora cardiaca of both larvae and adults contained immunoreactivity indicating potential release into circulation. The adult and larval brains had at least one pair of immunoreactive neurons in the protocerebrum with the adult brain having additional immunoreactive neurons in the dorsal medial part of the protocerebrum. The ventral ganglia of both larvae and adults each contained one pair of neurons that sent their axons to a perisymphatic organ associated with each abdominal ganglion. These results indicate that the mosquito nervous system contains pyrokinin/PBAN-like peptides and that these peptides could be released into the hemolymph. The peptides in insects and mosquitoes are produced by two genes, *capa* and *pk/pban*. Utilizing PCR protocols, we demonstrate that products of the *capa* gene could be produced in the abdominal ventral ganglia and the products of the *pk/pban* gene could be produced in the subesophageal ganglion. Two receptors for pyrokinin peptides were differentially localized to various tissues.

## Keywords

Neuropeptide, immunocytochemistry, Culicidae, pheromone biosynthesis activating neuropeptide, pyrokinin

## Disciplines

Biology | Entomology | Nervous System | Systems Neuroscience

## Comments

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## Pyrokinin/PBAN-like peptides in the central nervous system of mosquitoes

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### Abstract

Pyrokinin/pheromone biosynthesis activating neuropeptide (PBAN) family of peptides is characterized by a common C-terminal pentapeptide, FXPRLamide, that is required for diverse physiological functions in various insects. Polyclonal antisera against the C-terminus was utilized to determine the location of cell bodies and axons in the central nervous systems of larval and adult mosquitoes. Immunoreactive material was detected in three groups of neurons in the subesophageal ganglion of larvae and adults. The corpora cardiaca of both larvae and adults contained immunoreactivity indicating potential release into circulation. The adult and larval brains had at least one pair of immunoreactive neurons in the protocerebrum with the adult brain having additional immunoreactive neurons in the dorsal medial part of the protocerebrum. The ventral ganglia of both larvae and adults each contained one pair of neurons that sent their axons to a perisymphatic organ associated with each abdominal ganglion. These results indicate that the mosquito nervous system contains pyrokinin/PBAN-like peptides and that these peptides could be released into the hemolymph. The peptides in insects and mosquitoes are produced by two genes, *capa* and *pk/pban*. Utilizing PCR protocols we demonstrate that products of the *capa* gene could be produced in the abdominal ventral ganglia and the products of the *pk/pban* gene could be produced in the subesophageal ganglion. Two receptors for pyrokinin peptides were differentially localized to various tissues.

### Keywords

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### Introduction

Neuropeptides are essential neurohormones found in most animals that regulate a variety of physiological and behavioral functions. Pyrokinin (PK)/pheromone biosynthesis activating neuropeptides (PBAN) are a large family of insect neuropeptides characterized by a common C-terminal pentapeptide, FXPRLamide (Raina and G. Kempe, 1992). A variety of physiological functions for this family of peptides have been described including stimulation of pheromone biosynthesis in female moths (Raina et al. 1989), induction of melanization in moth larvae (Matsumoto et al. 1990), induction of embryonic diapause in the silkworm moth (Suwan et al. 1994), stimulation of visceral muscle contraction (Predel and Nachman 2001), and termination of pupal diapause development in heliothine moths (Xu and Denlinger 2003), among others. A highly conserved five amino acid C-terminal sequence has been identified as the active core required for physiological functions (Nachman et al. 1986). The PK/PBAN peptide family is cross-reactive between species and physiological functions and the peptides are expected to be found in all insects (Jurenka and Nusawardani 2011).

The PK/PBAN family of peptides has been found in the Diptera including the fruit fly *Drosophila melanogaster* (Baggerman et al. 2002), the blow flies *Protophormia terraenovae* (Shiga et al. 2000) and *Lucilia cuprina* (Rahman et al. 2013), the fleshfly *Neobellieria bullata* (Verleyen et al. 2004), the mosquito *Aedes aegypti* (Predel et al. 2010), the cabbage root fly *Delia radicum* (Zoephel et al. 2012), and the sandfly *Phlebotomus papatasi* (Predel et al. 2013). Acceleration of pupariation formation and cuticular tanning are two functions regulated by PK/PBAN peptides in fleshflies (Nachman et al. 2006; Zdarek et al. 1997; Zdarek et al. 1998). In *D. melanogaster* two genes produce PK/PBAN-like peptides, *hugin* which produces PK-2 and *capa* which produces PK-1 and the periviscerokinins (PVK). The *hugin* gene is expressed in the SEG (Melcher and Pankratz 2005) and the *capa* gene in the SEG and ventral ganglia (Kean et al. 2002). A similar two gene expression pattern for PK/PBAN-like peptides has been proposed for *Solenopsis sp.* (Choi et al. 2009), *Manduca sexta* (Loi and Tublitz 2004), *Bombyx mori* (Roller et al. 2008), and *Periplaneta americana* (Predel and Eckert 2000; Schoofs et al. 1992). A search

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of the *Anopheles gambiae* genome indicates that these same two genes putatively code for peptides in the PK/PBAN family (Riehle et al. 2002) The PKs produced by *capa* and *hugin* have been confirmed by mass spectroscopy in the mosquito *A. aegypti* (Predel et al. 2010).

In this paper we briefly describe techniques to dissect the nervous system of larval and adult mosquitoes. We then describe the localization of neurons that produce PK/PBAN peptides in several species of mosquitoes. Localization of immunoreactive neurons indicates that the peptides are present in the CNS. These peptides may act within the CNS and may also be released into the hemolymph to act at peripheral sites. Results from Culicidae are similar to previously reported insect species, but the physiological roles of these peptides in Culicidae remain to be determined. To begin to determine the functional role of the peptides in mosquitoes we identified the expression of two receptors for these peptides in various tissues of adult female *A. aegypti* mosquitoes.

## Materials and Methods

### Mosquitoes

Mosquitoes were maintained at Iowa State University in controlled conditions ( $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and  $80\% \pm 5\%$  relative humidity) with a 16:8 hour photoperiod. Larvae were fed ground Tetramin<sup>TM</sup> and adults were fed 10% sucrose. *A. aegypti* (Liverpool) were started from a colony at the University of Wisconsin Madison in 2005 which was originally obtained from the University of London in 1977. *Aedes triseriatus* were collected in Iowa and colonized at Iowa State University in 2002. *Anopheles stephensi* line STE2 (MRA-128) were acquired from the MR4 Institute, ATCC<sup>®</sup> Manassas, Virginia, which was donated by William E. Collins, and colonized at Iowa State University in 2009. *Armigeres subalbatus* were started from a colony at the University of Wisconsin Madison in 2005 which was originally obtain from the National Institute of Preventive Medicine, Taipei, Taiwan, in 1992. *Culex pipiens* were collected in Iowa and colonized at Iowa State University in 2002. *Toxorhynchites amboinensis* were acquired from the University of Illinois Natural History Survey from a colony started in 1991. *Ochlerotatus caspius* were collected in Nile Delta, Egypt, and colonized at Iowa State University in 2008.

### Central nervous system dissections

Adult mosquitoes were immobilized by placing at  $-20^{\circ}\text{C}$  for 5 minutes and then were placed on a dissecting tray and legs and wings removed with scissors. The mosquito was then pinned through the abdomen with the right side down. A lateral cut was made in the anterior part of the abdomen, making sure not to cut deeper than necessary to open the abdomen, followed by an incision along the dorsal midline from the lateral cut to the posterior end. The abdomen was then pinned down to expose internal organs and bathed in phosphate buffered saline (PBS) to keep the specimen from drying. The digestive tract, midgut, hindgut, and ovaries were removed. Using probes the ventral nerve cord (VNC) was located and carefully removed. The VNC was transferred to a 24-well microtiter plate containing 4% formaldehyde using ring forceps. To obtain the brain, the mosquito was placed on a dissecting tray and pinned through the thorax with the right side down and the head covered with PBS. The proboscis and maxillary palps were slowly removed from the head capsule with forceps. This technique usually allowed removal of the brain and subesophageal ganglion (SEG). If the brain and SEG were not removed, the head was slowly smashed in a posterior to anterior direction to dislodge the tissues from the head capsule. The brain and SEG were then transferred to a 24-well microtiter plate containing 4% formaldehyde using ring forceps. To obtain the thoracic ganglia mosquitoes were placed on a dissecting tray and legs and wings were removed. They were then pinned through the thorax, dorsal side down. Using probes, the thorax was carefully opened to reveal the thoracic nerve cord. The thoracic nerve cord was removed and transferred to a 24-well plate containing 4% formaldehyde using ring forceps.

Larvae were immobilized by placing directly in 4% formaldehyde for 2-3 hours. Several other immobilization techniques were tested including placing larvae on a dissecting tray, pinning down, cutting open and then applying 4% formaldehyde. This technique was not continued as it was much more

difficult and led to very little success. Immobilized and fixed larvae were placed ventral side down on a dissecting tray and pinned through the thorax. A lateral cut was made across the anterior portion of the abdomen. Incisions were then made along the dorsal midline from the lateral cut to the anterior portion of the thorax and from the lateral cut to the posterior portion of the abdomen. Larvae were pinned open and tissues were kept hydrated with PBS. The digestive tract was removed and the VNC in the thorax and abdomen was located and carefully removed using probes. The head capsule was broken apart carefully using probes. Nerves connecting the brain and SEG to the stemmata and muscles in the head were severed and the brain and SEG were removed. Using this technique, if all was done carefully, it was possible to remove the entire nerve cord from the brain to the terminal abdominal ganglion (TAG). The nerve cord was then transferred using ring forceps to a 24 well microtiter plate containing 4% formaldehyde. The larval mosquitoes studied were not distinguished between 3<sup>rd</sup> or 4<sup>th</sup> instar unless otherwise stated. Results under each grouping were similar between multiple individuals unless otherwise stated.

### **Immunocytochemistry**

Mosquito CNS tissues were fixed for 3 hours in 4% formalin in PBS in a 24-well microtiter plate and were subsequently transferred to another well containing PBS with 2% triton X-100 (PBS-T). All incubations were conducted at room temperature (21°C). After incubation overnight, tissues were incubated in PBAN antiserum (Ma and Roelofs 1995; Choi et al. 2001), diluted 1:2000, for 4-6 hours and then washed in PBS-T overnight. This antiserum recognizes PRLamide ending peptides (Choi et al. 2001) and could recognize the PVKs produced by the *capa* gene. The PVKs found in Diptera have a PRVamide C-terminus (Predel and Wegener 2006). Tissues were then incubated with the secondary antibody, goat anti-rabbit Alexa Fluor® 488 (Invitrogen, Carlsbad, CA, USA), diluted 1:200 in PBS-T for 4-6 hours and washed in PBS-T overnight. Following the overnight wash, tissues were washed in PBS for 30 minutes and transferred to a slide in 5 µl of PBS. Tissues were mounted with 15 µl Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and covered with a coverslip and visualized under a Nikon Eclipse 50i fluorescence microscope. Images were captured with a Digital Sight DS-2Mv camera and NIS Elements, version 3.0 software (Nikon, Melville, NY, USA). All images were visualized and figures made with Adobe Photoshop CS5.

Central nervous system (CNS) tissues from larval *A. subalbatus* and adult *A. aegypti* were used for negative controls to test for non-specific binding of the secondary antibody. The same protocol as detailed above was followed except the tissues were not incubated with the PBAN antiserum. Controls showed no binding of the secondary anti-body as indicated in images captured with a 12 second exposure (figure not shown). When tissues were incubated with the PBAN antiserum images were captured with a 2 second exposure or less.

### **RNA extraction, cDNA synthesis, and PCR**

Life cycle and tissue samples were collected from *A. aegypti* larvae and adult females. Whole body, hemocytes, fat body, midguts, abdominal nerve cords, head, and ovaries were collected separately in 500 µl ice-cold PBS. Tissues were then washed three times with ice-cold PBS and then total RNA was extracted in 1 ml of Trizol® reagent according to the manufacturer's protocol (Life Technologies, Grand Island, NY, USA). Oligo-dT primed cDNA synthesis reactions were performed on 1 µg total RNA from each tissue sample by using iScript™ cDNA Synthesis Kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). PCRs were performed in a final volume of 50 µl with the following reagents: 5 µl 10xPCR buffer, 1.5 µl 50 mM MgCl<sub>2</sub>, 1 µl 10 mM dNTP mixture, 1 µl 10 pmol each primer, 0.2 µl *Taq* DNA polymerase (Life Technologies, Grand Island, NY, USA), and 1 µl of cDNA template. The PCR reaction conditions consisted of a denaturation step of 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 1 min, and an extension step of 72°C for 10 min. The following primers were utilized to amplify genes of interest: *actin* – 5'-GAC TAC CTG ATG AAG ATC CTG ACC-3' and 5'-CCG ATG GTG ATG ACC TGW CCG T-3', *pk1-receptor* – 5'-ACA GTT CGG CGT AAC CAA TC-3' and 5'-CCT TGG CTA GTT CCT TGC TG-3', *pk2-receptor* – 5'-CCT GGC CAT

CTG TAT TGT GA-3' and 5'-GCG TTC TAC GGT GAA GGA AG-3', *pban* – 5'-AGC CCG TGT TCT ACC ACA GT-3' and 5'-CAA TGG AAA TGG CCT ACT GC-5', *capa* – 5'-GAA ACG CCA GGG CTT AGT C-3' and 5'-GTT CCT TTG ATC TCG GTG GA-3'. PCR reactions were performed on a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). Gene expression of *pk1-receptor* and *pk2-receptor* was tested in both *A. aegypti* larval and adult female tissues, whereas *pban* and *capa* gene expression was only tested in larval tissues.

## Results

The CNS of members of the family of Culicidae consists of a two lobed brain connected to the VNC which contains the SEG, thoracic ganglia, and abdominal ganglia. All species of mosquitoes examined had a similar morphological architecture. Larval mosquitoes have eight abdominal ganglia whereas adults have six. The three thoracic ganglia are very close together but not completely fused together in both the larvae and adult. The adult brain and SEG are very close together in almost a fused fashion with a smaller foramen through which the esophagus fits.

### Larvae

PK/PBAN-like immunoreactivity was observed in the CNS of all larval mosquito species examined. All species had similar positions of immunoreactive neurons in the various ganglia, unless otherwise noted (Fig. 1). The SEG contained three groups of immunoreactive neurons that putatively correspond to the mandibular, maxillary, and labial neuromeres (Fig. 2a). All three neuromeres contained about 6 neurons each in most preparations. In most preparations the neurons of the mandibular and maxillary neuromeres exhibited a stronger fluorescence signal than the neurons of the labial neuromere. Axons originating from the SEG were found to terminate in the corpora cardiaca (Fig. 2a). Although we could not determine specifically from which pair of neurons these axons arose it appears that at least one pair of neurons in each neuromere sent axons to the corpora cardiaca. The brain contained a pair of immunoreactive neurons on the dorsal-medial sides of the protocerebrum (Fig. 2b). In addition immunoreactivity was seen in the anterior-dorsal side of the protocerebrum as an arch-like process that originated from the mandibular and maxillary group of neurons. In most preparations four pairs of axons, originating from the maxillary and mandibular cell bodies, were found leaving the SEG and traveling on the lateral sides of each ventral connective. At least two of these axons were strongly immunoreactive for the entire length of the VNC and terminated in the TAG (Fig. 2c). The labial cell bodies also sent axons down the VNC but these axons were located on the medial side of each connective (Fig. 2c). These immunoreactive axons could only be traced to the prothoracic ganglion. The ends of axons originating from the SEG that terminate in the TAG were associated with multiple varicosities (Fig. 2d). These axons were also associated with varicosities in each thoracic and abdominal ganglion (Fig. 3).

A pair of immunoreactive neurons was observed in the first thoracic ganglion and along the ventral mid-line in each abdominal ganglion except for the first (Fig. 3a, 3b). Axons associated with these pairs of neurons in abdominal ganglia were seen leaving the VNC and terminating in the perisymphathetic organ associated with each ganglion. In some abdominal ganglia of *A. aegypti* and *T. amboinensis* a third, unpaired immunoreactive neuron was observed located along the midline (micrograph not shown). Due to a weak immunoreactive signal, the projection pattern of axons from these neuronal cell bodies could not be determined.

### Adult females

PK/PBAN-like immunoreactivity was observed in the CNS of all adult female mosquitoes (Fig. 1). A pair of immunoreactive neurons was located in the dorsolateral portion of the protocerebrum (Fig. 4a). Groups of immunoreactive neurons, one group on each lobe, were located in the pars intercerebralis where the two lobes of the protocerebrum meet (Fig. 4a). About 24 small cell bodies could be found in this grouping. Axons originating in the mandibular and maxillary neuromeres of the SEG were seen in the circumesophageal connectives and continued to an arch-like process in the protocerebrum. The SEG contained three groups of immunoreactive neurons that putatively correspond to the mandibular,

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maxillary, and labial neuromeres similar to what was found in the larvae (Fig. 4a). Each group had three pairs of neurons with the mandibular and maxillary neuromeres sometimes having four pairs each. Axons originating in the mandibular and maxillary neuromeres of the SEG extended the entire length of the VNC to terminate in the TAG. These axons had many associated varicosities in each ganglion. A pair of immunoreactive neurons was observed in the first thoracic ganglion and in all abdominal ganglia (Fig. 4c, 4d, 4e). The abdominal ganglia neurons sent axons to an associated perisymphathetic organ (Fig. 4d, 4e).

### **PCR products from genes of interest**

PCR products from genes of interest are shown in Fig. 5. The PCR products from larvae show that *capa* mRNA was primarily amplified from the abdominal nerve cord and that *pk/pban* mRNA was primarily amplified from the head but products were also found amplified from the abdominal nerve cord. Two pyrokinin receptors have been characterized from *A. gambiae* (Olsen et al. 2007) and gene sequences identified from *A. aegypti* and *C. quinquefasciatus* (Jurenka and Nusawardani 2011). The PK1-receptor is activated by peptides with a WFGPRLamide C-terminus and the PK2-receptor is activated by peptides with an FXPRLamide C-terminus. The *pk1-receptor* was found primarily in the abdominal nerve cord and head of larvae but was found in all tissues examined of the female adult. The *pk2-receptor* was not found in the larvae and only in the ovaries of adult females.

### **Discussion**

Neuropeptide families have been described in the major insect groups including Diptera. For those Diptera in which the whole genome has been sequenced genes encoding families of neuropeptides have been annotated, including *A. gambiae* (Riehle et al. 2002) and *D. melanogaster* (Hewes and Taghert 2001). The PK/PBAN family of neuropeptides are most likely found in all insects (Jurenka and Nusawardani 2011) including mosquitoes. A recent peptidomic study using *A. aegypti* demonstrated the presence of PK-1 and PVKs produced by the *capa* gene in abdominal ganglia and associated perisymphathetic organ as well as all three PKs produced by the *pk/PBAN* gene (Predel et al. 2010). Peptidomic and immunocytochemical studies have also identified similar PKs and PVKs in the cabbage root fly *D. radicum* (Zoephel et al. 2012), the sandfly *P. papatasi* (Predel et al. 2013) and blow fly *L. cuprina* (Rahman et al. 2013).

Several reports have utilized immunocytochemistry to describe the location of peptides in the CNS of mosquitoes. In some studies whole mosquitoes were used with subsequent embedding and thin sectioning (Meola et al. 1998). In a few studies the nervous system was removed and utilized as a whole mount (Marquez et al. 2011; Riehle et al. 2006; Stanek et al. 2002; Hernández-Martínez et al. 2005). Here we describe techniques utilized to perform a dissection of the nervous system of both larvae and adult mosquitoes for subsequent immunocytochemical localization of neuropeptides in a whole mount. We found that immobilizing adults at -20°C for 5 minutes followed by standard dissection procedures was sufficient to obtain whole mounts of the CNS. With larvae we found that it was best to immobilize directly in 4% formaldehyde for 2-3 hours and then dissect out the CNS to obtain whole mounts for processing.

The location of the PK/PBAN-like peptides in mosquitoes is similar to other insects in general (Sato et al. 1998; Choi et al. 2001; Choi et al. 2011; Predel and Eckert, 2000). The SEG contains three groups of immunoreactive neurons that correspond to the presumptive mandibular, maxillary, and labial neuromeres. Interneurons of the SEG can send axons to the tritocerebrum and protocerebrum. The neurons in the SEG can send axons to the corpora cardiaca for release of peptides into circulation. They also send axons down the length of the VNC to terminate in the TAG. Within each thoracic and abdominal ganglia these axons appear to varicose within the neuropile indicating communication with other neurons. The abdominal ventral ganglia contain a pair of neurons that send an axon to the perisymphathetic organ for release of peptides into circulation. Mosquito larvae were found to have eight abdominal ganglia with all but the first ganglion expressing the PK/PBAN-like peptides. Adult mosquitoes were found to have six abdominal ganglia with all ganglia expressing PK/PBAN-like

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peptides. Most likely the larval first abdominal ganglia has fused with the thoracic ganglia and the last two have fused into the TAG during adult development.

The antiserum used in this study recognizes PRLamide C-terminal ending peptides (Choi et al. 2001) and therefore could recognize the *capa* gene PVK peptides which have a PRVamide C-terminus in mosquitoes (Predel et al. 2010). The *capa* gene produces two PVKs and one PK if not differentially processed. In *A. aegypti* the distribution of PVKs in abdominal ganglia using a PVK antisera (Predel et al. 2010) was identical to what we observed in this study. In addition, both PVK and PK-1 were found in abdominal ganglia by mass spectroscopy (Predel et al. 2010). These results indicate that the PVKs and PK are both processed within the abdominal ganglia of mosquitoes. This is in contrast with results reported from the sandfly *P. papatasi* where the *capa*-PK was found in lower amounts in the abdominal ganglia and in higher amounts in the CC indicating differential processing of the *capa* gene products (Predel et al. 2013). The *capa* gene PK-1 was also found in the CC of *A. aegypti* by mass spectrometry indicating that at least some of the neurons in the SEG could be expressing the *capa* gene in addition to the *pk/pban* PK-2 peptides (Predel et al. 2010).

Our PCR data indicates that the gene encoding *pk/pban* is most likely expressed in the SEG with some expression in the abdominal ganglia. The abdominal ganglia are most likely expressing the *capa* gene in addition to some neurons in the SEG. This is a similar pattern that has been described for *D. melanogaster* (Kean et al. 2002) and in the moth *M. sexta* (Loi and Tublitz 2004). In mosquitoes the PVKs are involved in regulating water balance as they are in *D. melanogaster* (Pollock et al. 2004; Ionescu and Donini 2012). The function of the PK/PBAN peptides is unknown in mosquitoes. In higher Diptera, PK/PBAN peptides are involved in regulating puparium formation and cuticular tanning (Verleyen et al. 2004; Zdarek et al. 1997), however mosquitoes do not form a puparium. In *D. melanogaster* PK/PBAN peptides are also implicated in controlling feeding behaviors (Bader et al. 2007). To address possible functions of the PK/PBAN peptides in mosquitoes we determined tissue localization of two PK-receptors. We found the *pk1-receptor* expressed in nerve tissue of larvae and all tissues examined from adult females. In contrast the *pk2-receptor* was not found in larvae but only in ovaries of adult females. Tissue expression patterns using microarrays have been conducted for *D. melanogaster* tissue (Chintapalli et al. 2007). The *pk2-receptors* were found mostly in the CNS and carcass of larvae, and the CNS, crop, and accessory glands of adults; the *pk1-receptor* was found in the head, crop, and carcass. Tissue expression patterns for PK/PBAN receptors have been determined in several moths including the silkworm, *B. mori* (Watanabe et al. 2007), and the European cornborer, *Ostrinia nubilalis* (Nusawardani et al. 2013). The *pk1-receptor* and *pk2-receptor* were found in the pupal ovaries of *B. mori* (Watanabe et al. 2007) where the PK-1 receptor is involved in inducing embryonic diapause. Both receptors were found in pheromone glands of *B. mori* pupae at one day before eclosion (Watanabe et al. 2007) where they are involved in inducing pheromone biosynthesis. In *O. nubilalis* similar expression patterns were found where both receptors were also found in the pheromone gland (Nusawardani et al. 2013). The *pk1-receptor* was also found in other tissues of *A. aegypti* and moths indicating multiple functions for the PK/PBAN peptides. One of the many physiological functions is controlling molting and metamorphosis (Watanabe et al. 2007). The role PK/PBAN neuropeptides play in mosquito physiology remains to be determined but this study indicates one site of action to be the ovaries.

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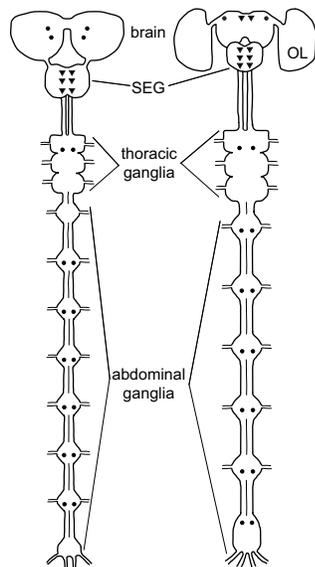
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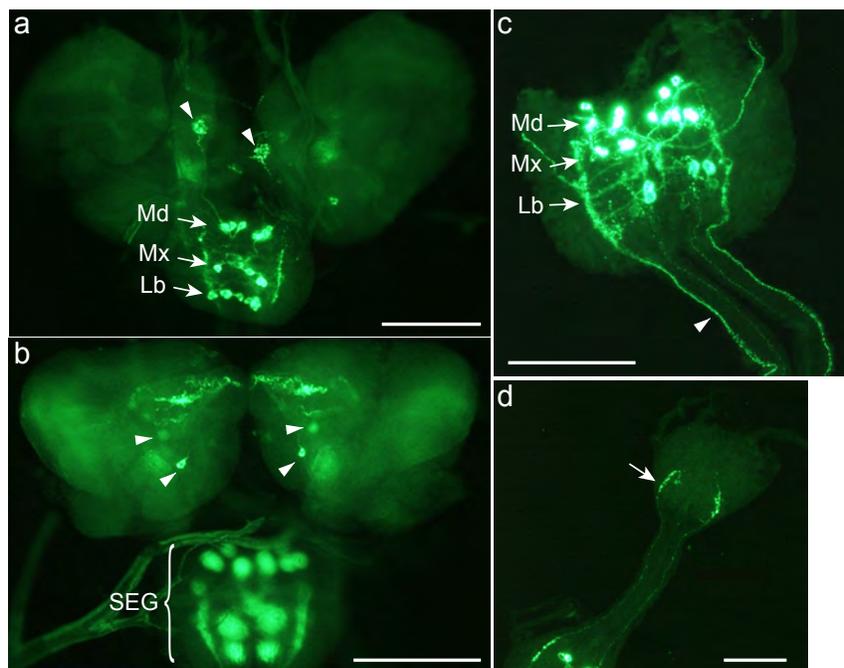
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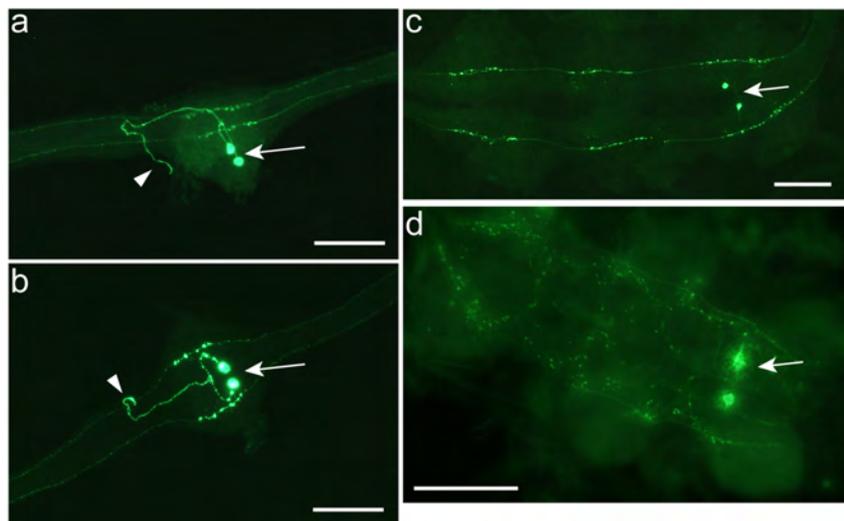


**Fig. 1** Schematic representation of a larval (left) and adult (right) CNS. Triangles represent locations where multiple cell bodies were found to contain PK/PBAN-like peptides. Circles represent locations where single cell bodies were found to contain PK/PBAN-like peptides. SEG – subesophageal ganglion, OL – optic lobe.



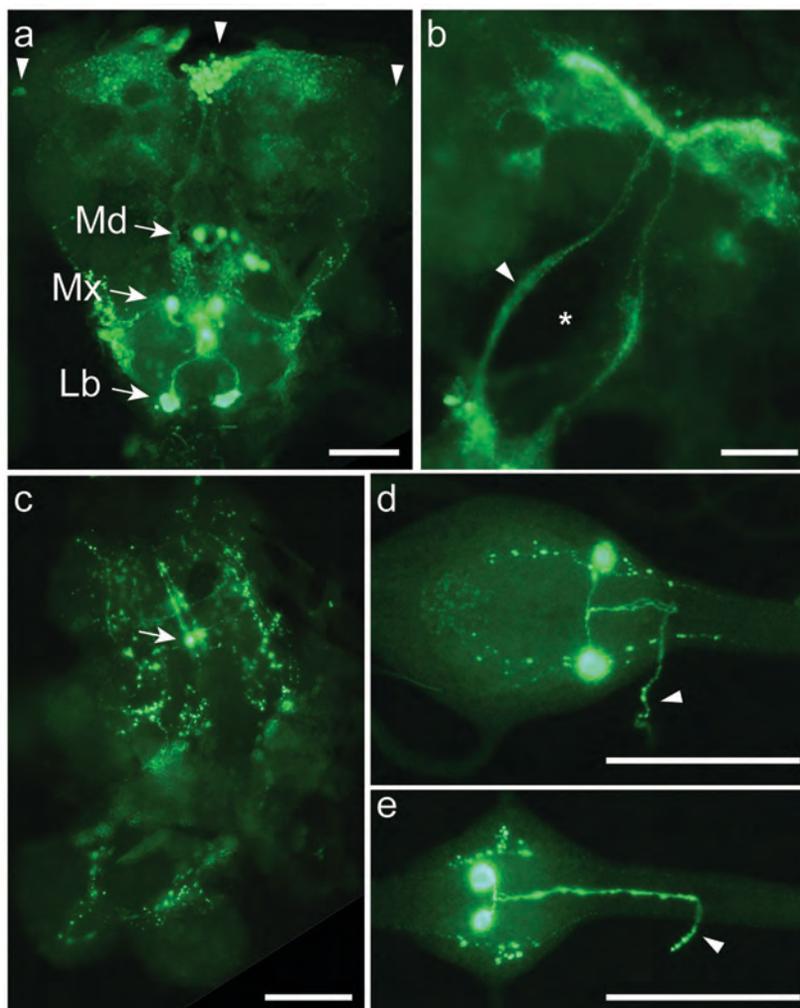
**Fig. 2** Fluorescent micrographs showing the immunolocalization of PK/PBAN-like peptides in the brain (a and b), SEG (c), and TAG (d) of 4<sup>th</sup> instar larval mosquitoes. (a) Arrows point to three groups of immunoreactive neurons in the SEG corresponding to the mandibular (Md), maxillary (Mx) and labial (Lb) neuromeres. Arrowheads point to the paired corpus cardiacum. This is a posterior view. (b) Arrowheads point to two pair of immunoreactive cell bodies in the protocerebrum near the esophageal foramen. The dorsal pair are out of focus in

this micrograph. Note the varicosities on the dorsal side of the protocerebrum. The SEG is located below and out of focus. This is an anterior view. (c) Arrowhead indicates axons originating in the SEG that travel the length of the VNC. Note the fainter axons towards the medial side of the paired connectives. (d) Arrow indicates the location of varicosities in the TAG from axons originating in the SEG. This is a dorsal view. Micrographs a and b are from *A. subalbatius* and c and d are from *A. aegypti*. Bar 0.1 mm



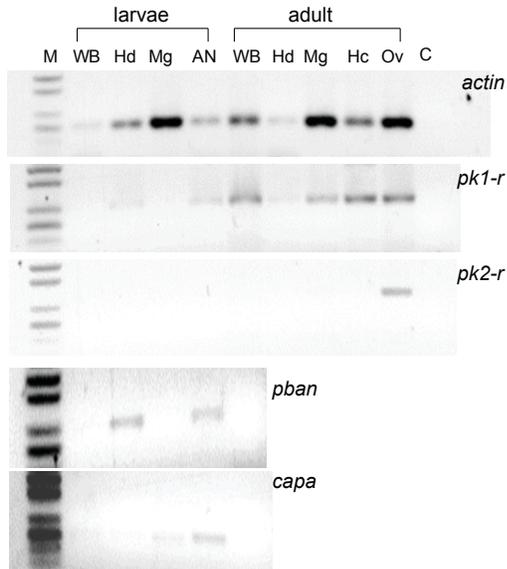
**Fig. 3** Fluorescent micrographs showing the immunolocalization of PK/PBAN-like peptides in the abdominal and thoracic ganglia of 4<sup>th</sup> instar larval mosquitoes. (a and b) Arrows point to a pair of immunoreactive neurons in the abdominal ganglia, which send an axon to a perisymphatic organ (arrowhead) that exits the CNS. Micrograph a is a lateral view of the 4<sup>th</sup> abdominal ganglion from *C. pipiens*.

Micrograph b is a dorsal view of the 6<sup>th</sup> abdominal ganglion from *A. aegypti*. (c and d) Arrows point to two immunoreactive cell bodies in the first thoracic ganglion. Note the extensive varicosities associated with the axons originating in the SEG in micrograph d. Micrograph c is from *A. subalbatus* and d is from *A. aegypti* and both are dorsal views. Bar 0.1 mm



**Fig. 4** Fluorescent micrographs showing the immunolocalization of PK/PBAN-like peptides in the brain, SEG, thoracic and abdominal ganglia of adult female mosquitoes. (a) Arrows point to three groups of immunoreactive neurons in the SEG of *T. amboinensis* corresponding to the mandibular (Md), maxillary (Mx), and labial (Lb) neuromeres. Arrowheads point to cell bodies located in the protocerebrum. Two faint cell bodies are located laterally with many cell bodies centrally. (b) Arrowheads point to axons originating in the SEG that travel through the circumesophageal connective to the protocerebrum of *A. aegypti*. Note the arch-like process in the dorsum of the protocerebrum. The SEG is located in the lower left and out of focus. The asterisk indicates the esophageal foramen. (c) Arrow indicates a pair of cell

bodies in the first thoracic ganglion of *A. aegypti*. Note the varicosities throughout the three thoracic ganglia from axons originating in the SEG. (d) Arrowhead indicates the perisymphetic organ associated with the TAG that contains axons coming from paired neurons in *A. triseriatus*. (e) Arrowhead indicates the perisymphetic organ associated with the 4<sup>th</sup> abdominal ganglion and associated paired neurons of *A. triseriatus*. Bar 0.1 mm



**Fig. 5** PCR products of the indicated genes from larvae and adult tissues from *A. aegypti*. WB = whole body, Hd = head, Mg = midgut, AN = abdominal nerve cord, Hc = hemocytes, Ov = ovaries, M = molecular weight marker, C = control, r = receptor

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