

PBAN/pyrokinin peptides in the central nervous system of the fire ant, *Solenopsis invicta*

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Abstract The pyrokinin/pheromone-biosynthesis-activating neuropeptide (PBAN) family of peptides found in insects is characterized by a 5-amino-acid C-terminal sequence, FXPRLamide. The pentapeptide is the active core required for diverse physiological functions, including the stimulation of pheromone biosynthesis in female moths, muscle contraction, induction of embryonic diapause, melanization, acceleration of puparium formation, and termination of pupal diapause. We have used immunocytochemical techniques to demonstrate the presence of pyrokinin/PBAN-like peptides in the central nervous system of the fire ant, *Solenopsis invicta*. Polyclonal antisera against the C-terminal end of PBAN have revealed the location of the peptide-producing cell bodies and axons in the central nervous system. Immunoreactive material is detectable in at least three groups of neurons in the subesophageal ganglion and corpora cardiaca of all adult sexual forms. The ventral nerve cord of adults consists of two segmented thoracic ganglia and four

segmented abdominal ganglia. Two immunoreactive pairs of neurons are present in the thoracic ganglia, and three neuron pairs in each of the first three abdominal ganglia. The terminal abdominal ganglion has no immunoreactive neurons. PBAN immunoreactive material found in abdominal neurons appears to be projected to perisymphatic organs connected to the abdominal ganglia. These results indicate that the fire ant nervous system contains pyrokinin/PBAN-like peptides, and that these peptides are released into the hemolymph. In support of our immunocytochemical results, significant pheromonotropic activity is found in fire ant brain-subesophageal ganglion extracts from all adult fire ant forms (queens, female and male alates, and workers) when extracts are injected into decapitated females of *Helicoverpa zea*. This is the first demonstration of the presence of pyrokinin/PBAN-like peptides and pheromonotropic activity in an ant species.

Keywords PBAN · Neuropeptide · Central nervous system · Immunocytochemistry · Fire ant, *Solenopsis invicta* (Insecta)

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Introduction

Neuropeptides are part of a large group of neurohormones that have important regulatory functions and are found in most animals. A variety of peptide families have been identified from insects (Gäde 1997). One of these families is the pyrokinin/pheromone-biosynthesis-activating neuropeptide (PBAN) family defined by a similar 5-amino-acid C-terminal sequence (FXPRLamide) that is the active core fragment for these peptides (Raina and Kempe 1992). A pyrokinin (leucopyrokinin) from the cockroach, *Leucophaea maderae*, was first identified (Holman et al. 1986) as a myotropin, with subsequent myotropic peptides being

identified from various insect orders (Nachman et al. 1986). All insects are now expected to contain this peptide family, with the peptides having various physiological functions: (1) stimulation of pheromone biosynthesis in female moths (Raina et al. 1989); (2) induction of melanization in moth larvae (Matsumoto et al. 1990; Raina et al. 2003); (3) induction of embryonic diapause in *Bombyx mori* (Suwan et al. 1994); (4) stimulation of visceral muscle contraction (Predel and Nachman 2001); (5) acceleration of puparium formation in several flies (Zdarek et al. 1997); and (6) termination of development of pupal diapause in heliothine moths (Xu and Denlinger 2003). Recently, two PBAN/pyrokinin peptides have been found from genome research in the red flour beetle, *Tribolium castaneum* (Li et al. 2008), and the honeybee (Hummon et al. 2006); however, no physiological function is known in these two insect species. The pyrokinin/PBAN family members are cross-reactive in that each peptide can be active in all physiological functions. In addition, adult male and female insects contain members of this family of peptides.

The immunocytochemical localization of some members of the pyrokinin/PBAN peptides has been examined in several insect orders. In moths, the subesophageal ganglion (SG) contains three groups of neurons that produce PBAN (Kingan et al. 1992). The ventral nerve cord and ganglia also contain PBAN-like activity (Choi et al. 2004; Ma et al. 1996). The SG and the ventral ganglia of other insects contain peptides that belong to the pyrokinin/PBAN family of peptides (Choi et al. 2001; Predel and Eckert 2000a, b; Schoofs et al. 1992; Tips et al. 1993). These investigations indicate that the functional epitope FXPRLamide is widely distributed among Insecta.

The neurohormonal mode of action of PBAN for pheromone production in lepidopteran moths is well understood (Rafaeli and Jurenka 2003). PBAN is synthesized in the SG and released into the hemolymph to stimulate pheromone biosynthesis in the target pheromone gland. Other than lepidopteran moths, no other insect group has yet been demonstrated to use PBAN for pheromone production or regulation. During the decades of research on social insects, the fire ant is the most studied ant species in the world, and many pheromone components have been identified from their several sophisticated pheromonal communication systems (Vander Meer and Alonso 1998; Vander Meer and Alonso 2002; Vargo 1998). However, virtually nothing is known about the mode of action of neurohormones and/or neuropeptides involved in key physiological and endocrine processes during development or the maintenance of colony social structure and territoriality.

In the present study, we report for the first time that the fire ant, *Solenopsis invicta*, contains pyrokinin/PBAN-like peptides. We demonstrate that neural extracts obtained from fire ant adults stimulate pheromone biosynthesis in moths.

In addition, we have utilized a polyclonal antiserum generated against the C-terminus of PBAN to identify neurons that contain PBAN-like immunoreactivity in the central nervous system (CNS) of *S. invicta*.

Materials and methods

Insects

Fire ants Field-collected monogyne fire ant colonies (*Solenopsis invicta*) from the Gainesville area, Florida, USA were maintained in the laboratory by using standard procedures (Banks et al. 1981).

Moths Pupae of corn earworm (*Helicoverpa zea*) were shipped from North Carolina State University (Department of Entomology) and maintained at room temperature under light/dark conditions of 15 h:9 h until they emerged as adults. Virgin female adults were decapitated when approximately 24 h old, early in their second or third photophase, and were used throughout this study.

Brain-subesophageal ganglia and peptide

Fire ant brain-subesophageal ganglia (Br-SG) were dissected from male and female sexual forms and from workers (sterile). The dissected Br-SG or synthetic Hez-PBAN (Peninsula Laboratories) were dispersed or dissolved, respectively, in a lepidopteran saline (21 mM KCl, 12 mM NaCl, 3 mM CaCl₂, 18 mM MgCl₂, 85 mM trehalose, and 5 mM PIPES, adjusted to pH 6.6 with KOH) and used for subsequent injection into decapitated adult female *H. zea* as described by Raina et al. (1989).

Whole-mount immunocytochemistry

The distribution of pyrokinin/PBAN-like immunoreactivity in the CNS of *S. invicta* was observed by using whole-mount immunocytochemistry as described previously (Choi et al. 2001). A truncated Hez-PBAN, with the sequence Cys-Nle-Asp-Pro-Glu-Gln-Ile-Asp-Ser-Arg-Thr-Lys-Tyr-Phe-Ser-Pro-Arg-Leu-amide, was synthesized and used to generate the polyclonal antisera described previously (Ma and Roelofs 1995). Fire ant Br-SG and ventral nerve cord were dissected in cold hymenopteran saline (130 mM NaCl, 6 mM KCl, 4 mM MgCl₂, 5 mM CaCl₂, 160 mM sucrose, 25 mM glucose, and 10 mM HEPES, adjusted to pH 7.2 with NaOH), fixed for 3 h in 10% formalin in phosphate-buffered saline (PBS: 100 mM NaH₂PO₄, 150 mM NaCl, 3 mM KCl, pH 7.4), and incubated overnight in PBS containing 2% Triton X-100 (PBS-T). Subsequently, the

tissues were incubated for 6–8 h in the PBAN anti-serum (diluted 1:2000), goat anti-rabbit anti-serum-peroxidase (Sigma; diluted 1:2000), and horseradish-peroxidase-conjugated rabbit anti-peroxidase (Sigma; diluted 1:400) in PBS-T after washes with PBS-T overnight with slow agitation at room temperature between each incubation step. Following the last incubation, the tissues were washed with PBS and then incubated in 50 mM TRIS–HCl buffer (pH 7.6) for 10 min. Visualization of the immunoreactivity was performed with 2 ml diaminobenzidine and H₂O₂ (Sigma) for about 1 min. After satisfactory color development was achieved, the tissues were transferred to PBS and dehydrated by being placed sequentially in a graded concentration series of glycerol (40%–80%). Tissues were examined under a Nikon Eclipse E800 microscope equipped with a digital camera. No staining was observed when PBAN and leucopyrokinin were used to preabsorb the antisera.

Pheromonotropic activity

Extracts of dissected Br-SG from fire ants were prepared by sonication in lepidopteran saline. The extracts were centrifuged at 12,000g for 10 min at 4°C. One Br-SG equivalent of the resulting supernatant or synthetic Hez-PBAN was injected between the fourth and the fifth abdominal segments of the decapitated *H. zea* females during mid-photophase. The *H. zea* pheromone glands were dissected after 0.5–1 h and extracted with hexane containing 100 ng (Z)-9-tetradecenal as an internal standard. A GC 6890N (Agilent Technologies) equipped with a capillary column (30 m×0.25 mm; DB-23, J&W) was used to measure the amount of pheromone. The oven temperature was programmed at 80°C for 1 min, followed by increases of 10°C/min to 230°C, and held for 8 min. The results were analyzed by non-parametric analysis as ranks (Fisher PLSD, ANOVA) by using Statview 5.0 software.

Results

Pheromonotropic activity in moths

Generally, the *H. zea* female does not produce pheromones during the photophase or when decapitated. However, the amounts of pheromone production after injection with fire ant Br-SG extracts (1 Br-SG equivalent) were all significantly higher than those following saline treatment ($P \leq 0.0019$) but lower than for 3 pmol synthetic Hez-PBAN (*H. zea* true PBAN) injections (Fig. 1). The pheromone amounts produced were similar for queens and female and male alates. The worker Br-SG extract was not as effective as those of the sexual forms ($P \leq 0.038$) but still produced significantly greater amounts than saline injections

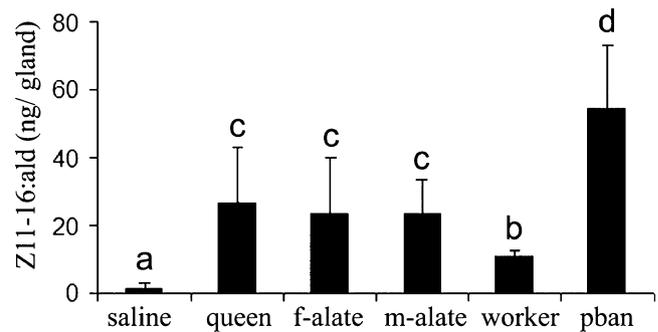


Fig. 1 Pheromonotropic activity of *S. invicta* Br-SG extracts as measured by the amount of (Z)-11-hexadecenal (Z11-16:ald) produced per *H. zea* pheromone gland. Queen, female alate (*f-alate*), male alate (*m-alate*), or worker Br-SG homogenates were injected into decapitated females of *H. zea*. PBAN (3 pmol synthetic Hez-PBAN; *pban*) was injected for comparison. Some moths were injected with control saline. One equivalent Br-SG was injected per female (bars means±SEM of at least 5 replications). Bars with the same letters are not statistically different ($P < 0.05$)

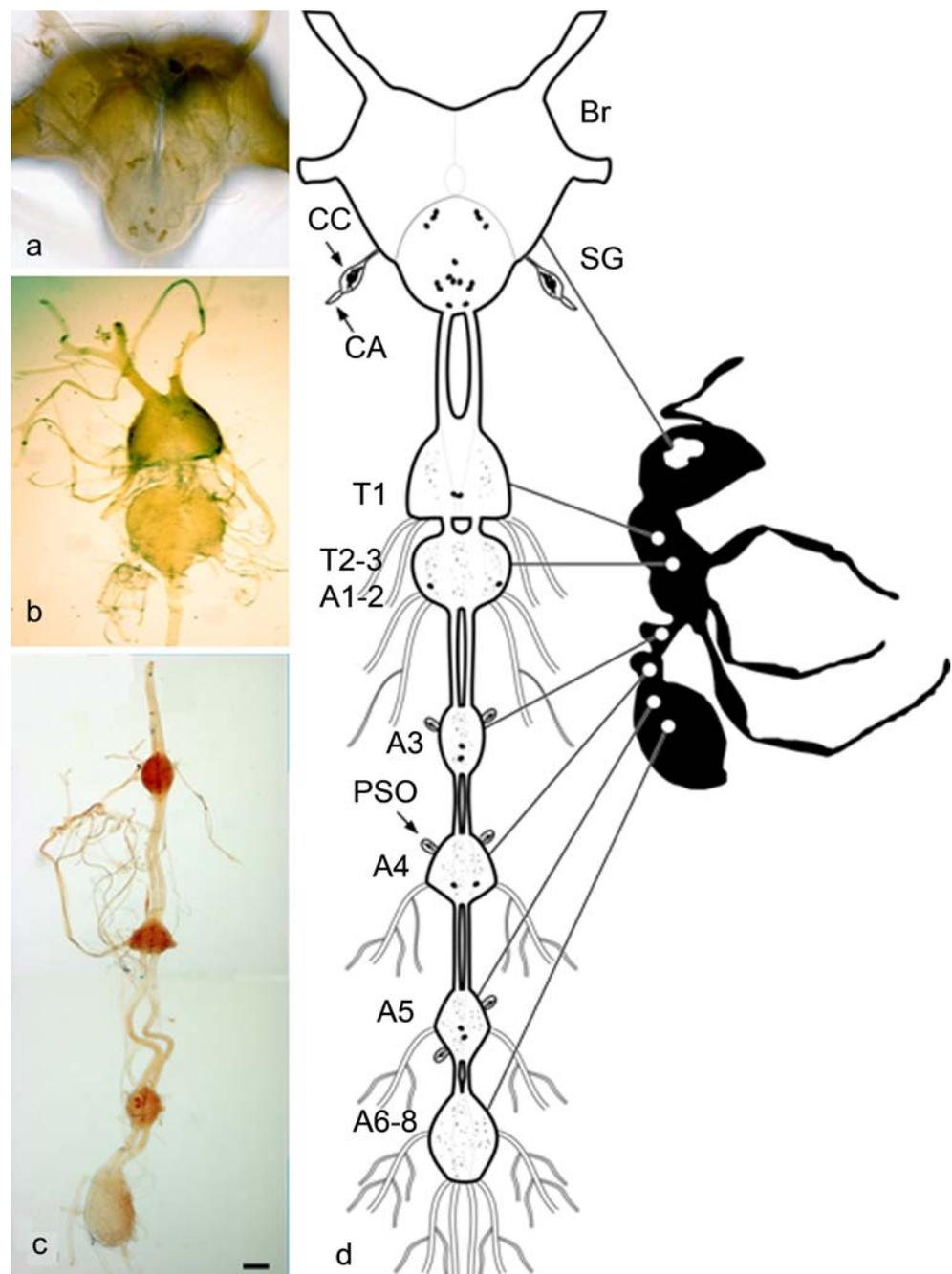
($P \leq 0.0019$). These results suggested that all fire ant adults produced PBAN-like peptides in the CNS.

Localization of PBAN-like immunoreactivity in fire ant

The morphological structure of the fire ant CNS, viz., brain and ventral nerve cord with thoracic and abdominal ganglia, is shown in Fig. 2. Although the neuronal morphology of insects within a group is highly conserved, the CNS morphology of ants has not been defined. The ganglion numerical segment of the fire ant ventral nerve cord has been deduced from the results of anatomical and neuromorphological observations and from insect phylogenetic relationships, since the number of ganglia making up the ventral nerve cord has been investigated for some hymenopteran insects (Niven et al. 2008). The first thoracic ganglion is the pro-thoracic ganglion and the second thoracic ganglion is composed of the meso- and meta-thoracic ganglia and the first and second abdominal ganglia, which are fused in the lower part of the thorax (Fig. 2b,d). The next three abdominal ganglia (the third to fifth) follow in order. The last three abdominal ganglia (sixth to eighth) are probably fused, forming the terminal ganglion (Fig. 2c,d).

The localization of PBAN-like peptides was visualized with an antiserum generated against the C-terminal end of *H. zea* PBAN (Ma and Roelofs 1995). Pre-incubation with synthetic *H. zea* PBAN and with synthetic leucopyrokinin completely abolished the staining (data not shown). PBAN-like immunoreactive material was observed in the dissected CNS of queen, female and male alates, and sterile workers of *S. invicta*. The number and location of PBAN-like immunoreactive neurons showed a similar pattern, although the stain intensities varied depending on the sexual form.

Fig. 2 Central nervous system of the adult fire ant. **a–c** Photomicrographs of the brain and subesophageal ganglion (**a**), thoracic ganglia (**b**), and abdominal ganglia (**c**). **d** Representation of CNS and PBAN-like immunoreactivity in adult fire ant (*Br* brain, *SG* subesophageal ganglion, *CC* corpora cardiaca, *CA* corpora allata, *PSO* perisymphatic organ, *T1–T3* first to third thoracic ganglia, *A1–A8* first to eighth abdominal ganglia). *Bar* 50 μ m



Three clusters of neurons were found in the SG (Figs. 2d, 3a) and five pairs of neurons in the ventral nervous system (Figs. 2d, 4a,d,e,f). The somata in the SG and in the ganglia of the ventral nervous system appeared to project axons to various neurohemal organs. One such neurohemal organ was the pair of corpora cardiaca (arrow in Fig. 3d), which release neurohormones into the hemolymph. With respect to the three cell clusters present in the SG (indicated by arrows in Fig. 3a), one pair lay in the most posterior, four pairs in medial, and three pairs in anterior ganglia (Fig. 3a). The medial neurons had axonal projections extending

through the lateral side to the median bundle of the tritocerebrum where they formed a densely stained region of varicosities (Fig. 3b,c).

The ganglia in the ventral nerve cord contained five pairs of PBAN-like immunoreactive neurons (Figs. 2d, 4a–f). One pair was located in the first thoracic ganglion, and another pair in the second thoracic ganglion (Figs. 2d, 4a). Neurites along the midline and lateral side in the thoracic ganglion appeared to descend to the abdominal ganglia (Figs. 2d, 4c). A triangular cuticular structure surrounded the ventral nerve cords of the thoracic ganglia, between T1

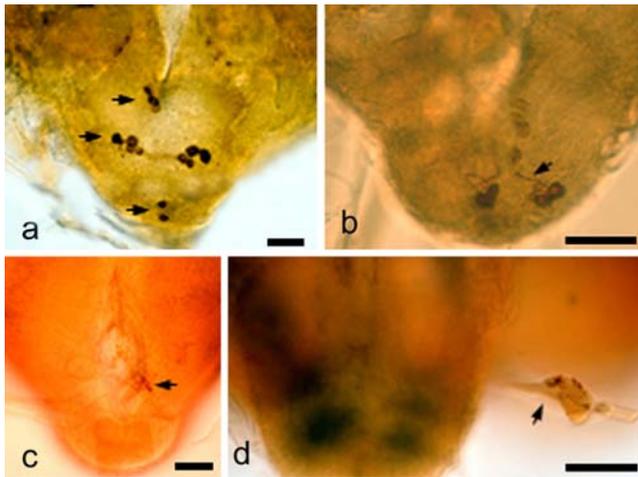


Fig. 3 Photomicrographs of PBAN-like immunoreactivity found in the Br-SG of adult fire ant. Posterior view of an adult brain-SG complex showing somata in the SG and near the esophageal foramen (**a**, arrows). Neurites (arrows) from axons in the SG project in the median bundle of the tritocerebrum where they form a densely stained region of varicosities (**b**). A densely stained region is present in the median bundle (**c**). A neurohemal organ, the corpus cardiacum, contains immunoreactive material (arrow in **d**). Bars 50 μm

and the fused T2–T3 and A1–A2 (Fig. 4b). This unusual structure may protect and stabilize the ventral nerve cord in the thorax. The other three pairs of neurons exhibiting PBAN-like immunoreactivity were found in the third, fourth, and fifth abdominal ganglia where the neurons were associated with a distended neurohemal organ (presumably a perisymphatic organ) located on the surface of each ganglion (see arrows in Figs. 2d, 4d–f). In the fused terminal abdominal ganglia (A6–A8), however, no neurons with PBAN-like immunoreactive material were found. A network of neurite varicosities descended from the somata of the fifth abdominal ganglion (Fig. 4g,h). The immunoreactive material in the last ganglion (arrow in Fig. 4g) could have traveled from the fifth ganglion or from the SG, as found in moths, for release into hemolymph through the neurohemal organs. The origin of this immunoreactive material remains to be determined.

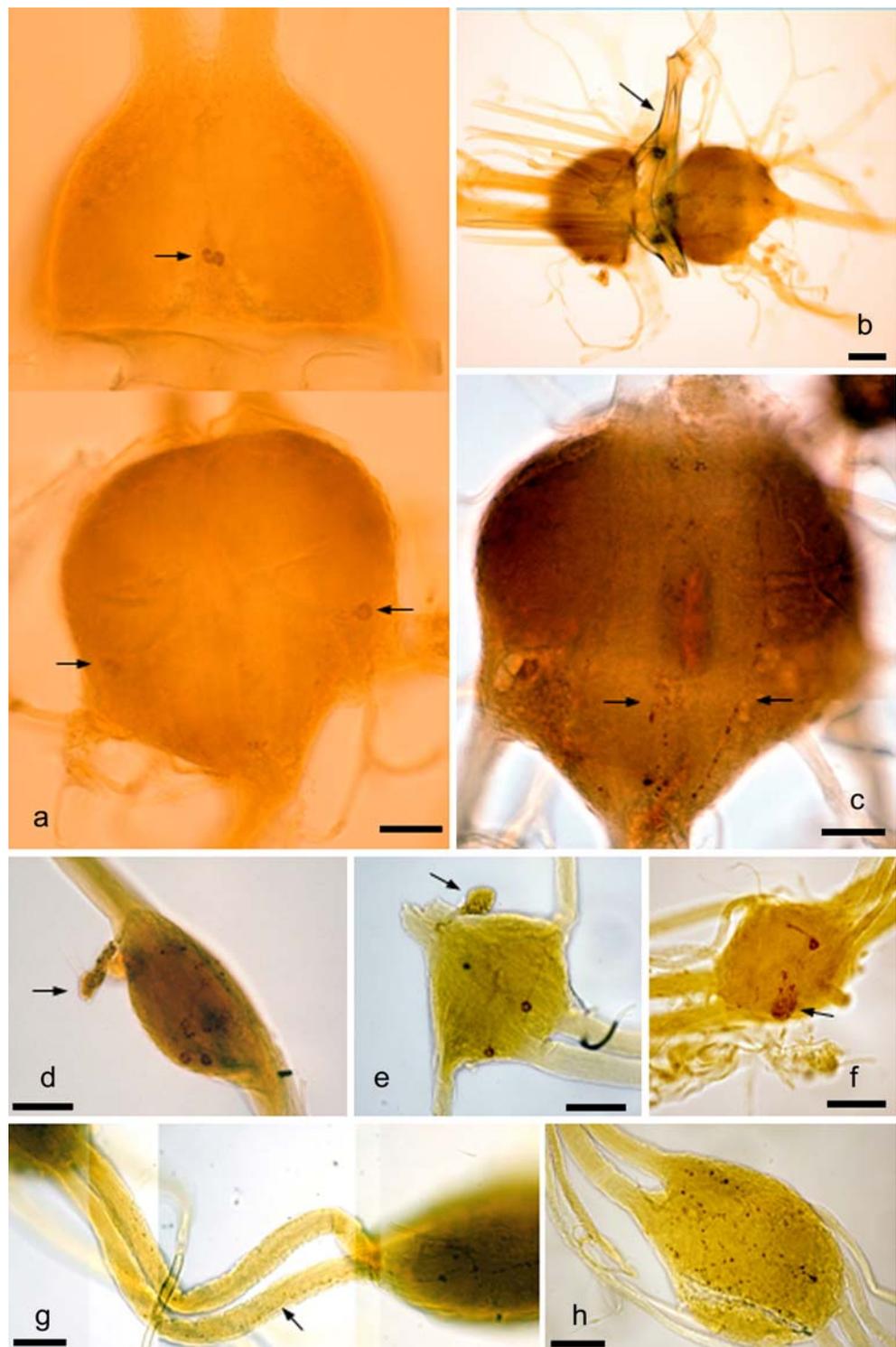
Discussion

The PBAN/pyrokinin family of peptides show cross-species activity and have been found to be important in the development of many insect groups and other invertebrate animals. Here, we have established for the first time in an ant species, *S. invicta*, the presence of the pyrokinin/PBAN family of neuropeptides by using whole-mount immunocytochemical techniques and pheromonotropic activity in moths treated with *S. invicta* Br-SG extracts. The tissue localization and the distribution pattern of

PBAN-like immunoreactive materials in the CNS of the fire ant is similar to that previously shown in moths and a fly. Three clusters of neurons with PBAN-like immunoreactivity have been detected in the SG of the fire ant, as found in many lepidopteran moths (Blackburn et al. 1992; Choi et al. 2004; Davis et al. 1996; Duportets et al. 1998; Kingan et al. 1992; Ma and Roelofs 1995; Ma et al. 1996; Sato et al. 1994; Sun et al. 2003; Wei et al. 2008), Orthoptera (Tips et al. 1993), and *Drosophila* (Choi et al. 2001). The locations of these neuron clusters correspond to the mandibular, maxillary, and labial neurons, as demonstrated in moths and fruit flies. Some of these neurons probably send processes to the corpora cardiaca, a pair of neurohemal organs that are presumptive release sites for PBAN-like peptides into the hemolymph of fire ants. The corpora cardiaca of the fire ant contain abundant immunoreactive material. Neurohemal organs containing PBAN-like peptides also have been found in moths and *Drosophila*. The maxillary cluster might send processes down the ventral nerve cord and into the terminal abdominal ganglion, as shown in *Drosophila* (Choi et al. 2001) and a moth (Kingan et al. 1992).

Abdominal ganglia can also have one to three pairs of neurons with PBAN-like peptides as shown in moths (Davis et al. 1996; Ma and Roelofs 1995; Sato et al. 1998) and *Drosophila* (Choi et al. 2001). These neurons have been shown to exhibit pyrokinin/PBAN cross-reactivity with antibodies and to have functional activity (Ma et al. 1996). In addition, the abdominal neurons send processes anteriorly to form neurohemal-like varicosities in the transverse nerve. PBAN/pyrokinin immunoreactivity has been found in neurons distributed in both the SG and ventral ganglia of *Drosophila*. These cardioacceleratory peptides were proposed to be encoded by two genes (Choi et al. 2001), viz., hug (PK-2) and Capa (Cab2b-3=PK-1), that were later shown to be expressed in the SG and ventral ganglia, respectively (Melcher and Pankratz 2005). Therefore, the source of the PBAN-like material found in the ventral ganglia of fire ants might be different from that found in the SG. In *Drosophila*, arborizations arising from these paired projections have been found in each abdominal ganglion, indicating that the peptides could be interacting with other neurons, implicating a synaptic receptor (Choi et al. 2001). The immunocytochemical localization of pyrokinin peptides in Orthoptera indicates that they are distributed with a similar pattern to those in moths (Predel and Eckert 2000a; Schoofs et al. 1992). The abdominal ganglia possess several groups of neurons that project into the transverse and median nerves to the neurohemal organs. Although some differences among Hymenoptera, Orthoptera, Diptera, and Lepidoptera are evident, the general pattern of pyrokinin/PBAN immunoreactive neurons is similar in the CNS.

Fig. 4 Photomicrographs of PBAN-like immunoreactivity found in the ventral nerve cord of adult fire ant. Each thoracic ganglion possesses one pair of immunoreactive neurons (**a**, *arrows*). A triangular structure is located between the thoracic ganglia (**b**, *arrow*). A composite photomicrograph shows the projection of neurons down the median and lateral side of the thoracic ganglion (**c**, *arrows*). Each of the third to fifth abdominal ganglia possesses one pair of immunoreactive neurons projecting into one or more perisymphathetic organs (**d-f**, *arrows*). A neurite (*arrow*) between the fifth and terminal ganglia contains PBAN-like immunoreactive material (**g**). The terminal ganglion (sixth to eighth neuromeres) exhibits a densely stained region of varicosities (**h**). *Bars* 50 μ m



Fire ant neurohemal organs attached to the abdominal ganglia produce a dense PBAN-like immunoreactivity. These neurohemal organs are presumably perisymphathetic organs projected from each pair of neurons to release pyrokinin/PBAN-like peptides into the hemolymph. The most distinct neurohemal release sites of the CNS of

insects are the corpora cardiaca and perisymphathetic organ, which are typical neurosecretory storage centers for peptide hormones (Nässel 1996). The abdominal perisymphathetic organs have been well studied in hemimetabolous insects, mainly cockroaches and locusts (Predel and Eckert 2000a), resulting in the discovery of many peptides and

physiological functions. Recently, this type of organ has been demonstrated to be involved in the distribution of neuropeptides from the abdominal neurosecretory system of *Manduca sexta*, a holometabolous insect (Predel et al. 2003). In the present study, we have identified perisymphathetic organs from the ventral nervous system of the fire ant. The terminal abdominal ganglion of the fire ant does not possess PBAN-like immunoreactive neurons, but dense varicosities and a neurohemal organ (perisymphathetic organ, not visible in the presented figures) do indeed show the presence of PBAN-like immunoreactive material, possibly derived from the axon pair of the previous ganglion.

Pheromotropic activity from moths is dependent on the amount of injected PBAN-like peptide (measured here in Br-SG equivalents) and the peptide sequence similarity to the true moth PBAN, viz., Hez-PBAN in this case, for *H. zea* female moths. The amount of pheromone production from worker Br-SG extracts is significantly lower than that from male or female sexual forms of fire ants, as shown by moths injected with one equivalent Br-SG extract. This result indicates that fire ant workers produce less PBAN-like peptides than sexual forms. Supporting evidence has come from the pyrokinin/PBAN-like immunoreactive signal intensity, which is weak in worker Br-SG compared with that found in female and male sexual forms, even though the neuron distribution pattern is similar (data not shown). In this study, we have selected major workers, which have a relatively large-sized head, in order to dissect Br-SG. Indeed, major worker brains are larger than those of male alates (unpublished data). In moths, the level of PBAN gene expression and the amount of peptide in the SG with pheromotropic activity are not significantly different between female and male adults (Choi et al. 1998; Zhang et al. 2004; Zhao et al. 2002). At this point, the pyrokinin/PBAN-like peptide quantitative differences might be attributable to differences in production, gene expression related to sexual dimorphism, or functional differences between workers and sexuals. Moths are the only insects that have been shown to use a PBAN to stimulate pheromone production. Correlations of moth PBAN stimulating some pheromone biosynthesis have been reported in the Hessian fly, *Mayetiola destructor* (Foster et al. 1991). Head or brain extracts of the Caribbean fruit fly, *Anastrepha suspensa* (Teal 1998), and *Drosophila* (Choi et al. 2001) have been found to stimulate pheromone production in moth surrogates. This activity is not unexpected, because of the cross-reactivity of the pyrokinin/PBAN family neuropeptides. Our results are similar, although fire ants rely heavily on several pheromone systems that require biosynthesis and regulation. Therefore, the fire ant pyrokinin/PBAN family of neuropeptides might be involved in the regulation of pheromone biosynthesis as demonstrated in moths.

Concurrent with the pyrokinin/PBAN studies, we have constructed an anatomical atlas of the entire CNS of the fire ant. This is the first report of a complete CNS atlas of an ant species. The ventral nerve cord in ancestral insects has been shown to consist of eight discrete abdominal ganglia. In evolutionarily advanced insects, the number of abdominal ganglia varies. In most cases, the first part of the abdominal and/or the terminal abdominal ganglia are fused with one or more neuromeres; therefore, the number of discrete ganglia is reduced (Niven et al. 2008). Winged insects (Pterygota) are extraordinarily diverse in terms of reduced segmental ganglia following fusion with one or more neuromeres of the thoracic and/or abdominal ganglia. The fire ant is no exception. The ventral nerve structure of fire ant female alates and queens shows only two thoracic and four abdominal ganglia. The pro-thoracic ganglion (T1) is discrete, but the meso- (T2) and meta- (T3) thoracic, and the first (A1) and second (A2) abdominal ganglia are fused together in the second structurally discrete thoracic ganglia, as found in most insects. Of the four discrete abdominal ganglia, A3, A4, and A5 are distinct ganglia; however, the last three abdominal ganglia may be fused forming the terminal ganglion, which would then account for the expected eight abdominal ganglia. A similar fusion pattern for both thoracic and abdominal neuromeres in the ventral nerve cord has been demonstrated for some lepidopteran and hymenopteran species (Niven et al. 2008).

In conclusion, we have constructed an atlas of the CNS of the fire ant and demonstrated, for the first time, the presence of the pyrokinin/PBAN family of peptides in an ant species, *S. invicta*. The localization of the immunoreactive neurons indicates that the peptides can act within the CNS and can also be released into the hemolymph to act at peripheral sites. The pyrokinin/PBAN-like family of peptides has been detected in all fire ant sexual forms and workers, but the physiological roles of these peptides in *S. invicta* remain to be determined.

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