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Effect of the brain and suboesophageal ganglion on pupal development in *Helicoverpa armigera* through regulation of FXPRLamide neuropeptides

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Abstract

Recent studies in *Helicoverpa armigera* report a novel role for diapause hormone (DH), pheromone biosynthesis activating neuropeptide (PBAN) and three other FXPRLamide neuropeptides secreted from suboesophageal ganglion (SG) in terminating pupal diapause. In the present paper, we investigated the role of these five FXPRLamide family neuropeptides on pupal development. Although removal of SG could not make nondiapause-destined pupae enter diapause-like status, it did make them eclose approximately 0.6–1.2 days later when compared with the controls. The results of competitive ELISAs showed a high level of FXPRLamide titer in the hemolymph of the SG-removed pupae and this may be due to the expression of the DH-PBAN gene in tissues other than SG. DH-PBAN mRNA and peptides were also detected in the thoracic ganglia (TGs) by RT-PCR and immunocytochemistry. The expression of DH-PBAN gene in the TGs of the SG-removed pupae is significantly higher than that in normal pupae by quantitative PCR and immunocytochemistry. Decerebration experiments proved that the decerebrated pupae could enter diapause-like status through down-regulation of FXPRLamide titer in hemolymph. Our studies confirm that the brain plays an important role in the determination of pupal development by regulating the synthesis and release of FXPRLamide neuropeptides in *H. armigera*. Thus, the function of FXPRLamide peptides in *H. armigera* is closely correlated with pupal development.
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1. Introduction

Insect neuropeptides are involved in various physiological and developmental events such as growth, molting, metamorphosis, reproduction, diapause, metabolism, etc. [1]. The peptides of the FXPRLamide family share a common C-terminal pentapeptide sequence, FXPRLamide (X = G, S, T, or V). This pentapeptide sequence is thought to be the active core required for their activity [2]. The peptides of this family include diapause hormone (DH), pheromone biosynthesis activating neuropeptide (PBAN), melanization and reddish coloration hormone (MRCH), myotropin, and pyrokinin. They regulate various aspects of physiological functions, such as induction of embryonic

diapause in *Bombyx mori* [3], sex pheromone biosynthesis in *Helicoverpa zea* [4], coloration in phase polymorphism in *Pseudaletia separata* [5] and *Spodoptera littoralis* [6], and stimulation of muscle contraction in the hindguts and oviducts in *Locusta migratoria* [7].

Generally, insect growth and development are regulated by ecdysteroids, juvenile hormone (JH), and their regulators: prothoracicotropic hormone (PTTH) [8], prothoracico-static peptide (PTSP) [9], allatotropin (AT) [10], and allatostatin (AST) [11]. In *H. armigera*, a pupal diapause species, the immediate cause of pupal diapause is the failure of the brain to secrete PTTH and the failure of the protho-racic glands to secrete ecdysteroids [12–15]. Recently, Zhang et al. proved that DH and FXPRLamide family peptides secreted from the suboesophageal ganglion (SG) of *H. armigera* could break pupal diapause and promote development by stimulating the prothoracic glands to synthesize and release ecdysteroids (data not shown). Therefore, the FXPRLamide family neuropeptides may have a regulatory role in insect development with the brain and

SG as the central in dictating the developmental fate of *H. armigera*.

To understand the molecular events in FXPRLamide neuropeptide regulating insect development, we report the effects of brain and SG on the synthesis and release of FXPRLamide neuropeptides, and the relationship between the changes of FXPRLamide titer in hemolymph and pupal development. The results show that the SG is a major site for synthesis and release of FXPRLamide neuropeptides, and the thoracic ganglia (TGs) can act as the main site for FXPRLamide neuropeptides in the absence of SG. The brain can determine pupal development through regulation of the expression of DH-PBAN gene, and FXPRLamide neuropeptides might accelerate pupal development in *H. armigera*.

2. Materials and methods

2.1. Animals

H. armigera were kindly provided by Prof. Jin-Liang Shen, Nanjing Agricultural University, Nanjing, and maintained for 4 years at our laboratory. Larvae were reared on an artificial diet at 25 °C, with a L14:D10 photoperiod (nondiapause type) and all the pupae developed without entering diapause. When larvae were reared at 22 °C, with a L10:D14 photoperiod (diapause type), more than 90% of the individuals entered diapause. The developmental stages were synchronized at each molt by collecting new larvae or pupae.

2.2. Surgical procedures for extirpation of the brain and SG

For extirpation, pupae were selected within 4 h after pupation. They were anaesthetized by cooling on ice for 1 h [16]. The brain was removed from the anterior end of the pupal head by making a square incision between the two compound eyes. The SG was removed in a similar manner from the cephalic region just beneath the compound eyes, using a sterilized fine forceps and the wound was sealed with low melting point wax. Pupae with the central nervous system (CNS)-cut between the SG and the first TG, sham-operated pupae, and normal pupae were used as controls. After treatment, all pupae were maintained at 25 °C.

2.3. Production of anti-DH serum and competitive ELISAs

Synthetic Har-DH was conjugated with bovine serum albumin (BSA) using glutaraldehyde. A rabbit was immunized with an initial subcutaneous injection of approximately 1 mg of the conjugated peptide in Freud's complete adjuvant. The injection was repeated 4 times at one-month intervals. Two weeks after the last injection, the anti-DH serum was collected and used without further purification [17,18]. The specificity of the anti-DH serum was determined by dot-immunoblotting assay using various synthetic peptides from the FXPRLamide family peptides [19]. With 1:1000 dilution of the serum, 0.69 pmol of Har-DH was detectable, and there was cross-reactivity among the FXPRLamide family peptides, although the antibody is most specific for DH (data not shown). We thus named the immunoreactivity as FXPRLamide or DH-like immunoreactivity.

Hemolymph (20 µl) was extracted from each pupa, and the hemolymph from 10 pupae (5 males and 5 females) was mixed as a sample for competitive ELISAs. Five samples were tested at each point. Competitive ELISAs were performed according to the method of Ma et al. [18] with modification. All procedures were performed at room temperature. The plates were coated with Har-DH (2 pmol/well) and blocked with 3% nonfat dry milk in a pH 7.25 phosphate buffer containing 0.05% Tween-20 (PBS-$T_{0.05}$). Standard peptides or hemolymph samples were mixed with diluted antiserum (1:5000 final concentration in PBS-$T_{0.05}$ with 3% nonfat milk), added to the blocked plates, and incubated for 1.5 h. Then they were incubated with peroxidase-labeled secondary antibodies (1:5000 in PBS-$T_{0.05}$ with 3% nonfat milk) for 2 h. After the reaction with peroxidase substrate OPD (Sigma), the absorbance was tested at 490 nm on a microplate reader. Nonspecific binding was determined by using normal preimmunized rabbit serum, and the mean absorbance of the background wells was subtracted from the mean absorbance of the standard and test samples. Maximal binding (B_0) was determined in the absence of competing Har-DH. The ratio of the absorbance of each standard and test sample well (B) to the absorbance of B_0 was calculated, and values that coincided with the Har-DH standard curve were used.

2.4. RT-PCR amplification

Brains, SGs, thoracic ganglia (TGs), and abdominal ganglia (AGs) were dissected in lepidopteran saline and stored at −70 °C until use. Total RNA was extracted using the acid–guanidine method [20], and 1 ng of rabbit globin (RG) mRNA/15 tissues was added as an internal standard [21]. The first strand of cDNA was synthesized from 1 µg of total RNA solution at 42 °C for 1 h with reverse transcriptase XL (AMV) (Takara, Japan), and PCR was performed for 22 or 24 cycles in DNA Thermal Cycler 480 (Perkin-Elmer) with primers PX1 (5′-CCCCGAAGTTAGGCA-GAAGC-3′) and PX2 (5′-GAGGTTAGACTAGCTTTG-3′) corresponding to Har-DH-PBAN cDNA sequences 314–333 and 621–638, and with primers RP1 (5′-CACTTC-GACTTCACCCACGG-3′) and RP2 (5′-TCAG-CACGGTGCTCACGTTG-3′) corresponding to RG cDNA sequences 372–391 and 742–761. The number of cycles was determined by our preliminary experiments designed to ensure a linear increase in PCR products (data not shown). The PCR products were electrophoresed on a 1.5% agarose gel, transferred onto a Hybond N$^+$ membrane (Amersham),

and hybridized using radiolabeled Har-DH-PBAN or RG cDNA as a probe, respectively.

2.5. Whole-mount in situ hybridization

The procedure was adapted from Tautz and Pfeifle [22], Hemmati-Brivanlou et al. [23], and Sato et al. [24]. Hybridization probe was generated by in vitro transcription with T7 or T3 RNA polymerase for sense or antisense RNA labeling with digoxigenin-UTP (Boehringer Mannheim).

All procedures were performed at room temperature unless otherwise stated. Tissues (each more than 20) were dissected in 0.75% NaCl, fixed for 2–3 h in fixative [3.7% formaldehyde, 100 mM HEPES (pH 6.9), 2 mM $MgCl_2$, and 1 mM EGTA], and washed with $PBST_{0.2}$ [PBS (20 mM Na-phosphate buffer, pH 7.2, and 130 mM NaCl) with 0.2% Tween-20] for 10 min. After that, tissues were bleached with methanol, 30% H_2O_2 (5:1) for 2 h, rehydrated through a methanol series, and washed with $PBST_{0.2}$. Proteinase K was added to a final concentration of 20 μg/ml and incubated for 10–20 min at 37 °C. The reaction of proteinase K was stopped by washing the tissues with $PBST_{0.2}$ containing 0.2% glycine. The tissues were fixed again for 20 min with 4% paraformaldehyde in PBS. The fixed tissues were washed with $PBST_{0.2}$, then incubated in HS (5 × SSC, 50% formamide, 2% Boehringer blocking regent, 50 μg/ml heparin, 100 μg/ml yeast tRNA, and 100 μg/m salmon sperm DNA) for 2 h at 45 °C, and further incubated overnight with the denatured 100–500 ng/ml probe for hybridization. Thereafter, tissues were washed for 1.5 h with four changes of $PBST_{0.2}$ at 37 °C, kept for 2 h in $PBST_{0.2}$ containing 5% goat serum and 2% BSA (GB-$PBST_{0.2}$), and then the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) was used for hybridization signal visualizing. The specimens were mounted with glycerol for observation with a light microscope (Olympus, BX60).

2.6. Whole-mount immunocytochemistry

The distribution of DH-like immunoreactivity in the SGs and TGs of *H. armigera* pupae was studied by using whole-mount immunocytochemistry, following the method of Ma et al. [18] with modification. All procedures were performed at room temperature. SGs of day 6 diapause pupae, non-diapause pupae, brain-removed pupae, SG-removed pupae and TGs of day 0 nondiapause-destined pupae (each more than 20) were dissected out, desheathed in saline, and fixed for 4–6 h in 4% paraformaldehyde in phosphate buffer (pH 7.4). Then the tissues were soaked in PBS containing 2% Triton X-100 (PBS-T) for 6 h. After incubation with antiserum (1:6000) and the peroxidase-labeled secondary antibodies (1:2000), immunoreaction was developed by DAB. Color development was observed under a dissection microscope. Next, the stained tissues were dehydrated by increasing gradients of ethanol, cleared in xylenes, and mounted in neutral balsam. Tissues were observed under a microscope (Olympus, BX60). In the control experiments, the primary antibodies were replaced by either primary antibodies preabsorbed with 0.02 mg/ml synthetic Har-DH peptide or preimmunized rabbit serum.
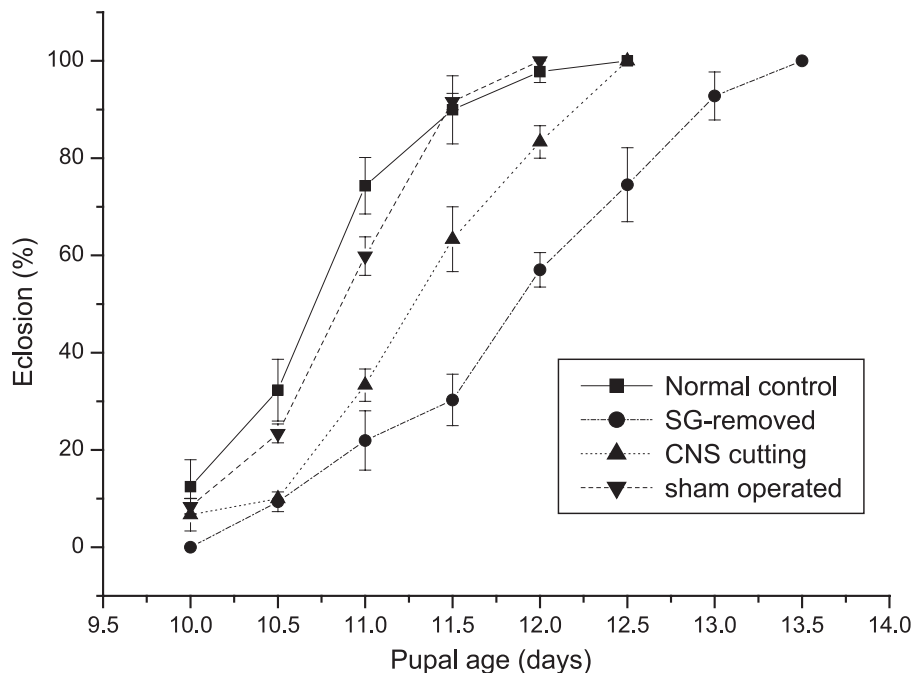

Fig. 1. Differences in rates of development between SG-removed pupae and controls. The controls included nontreated, sham-operated, and central nervous system (CNS)-cut pupae. For each treatment, there are more than 10 pupae (half were female), and each point is represented as mean ± S.E. of three repeats.
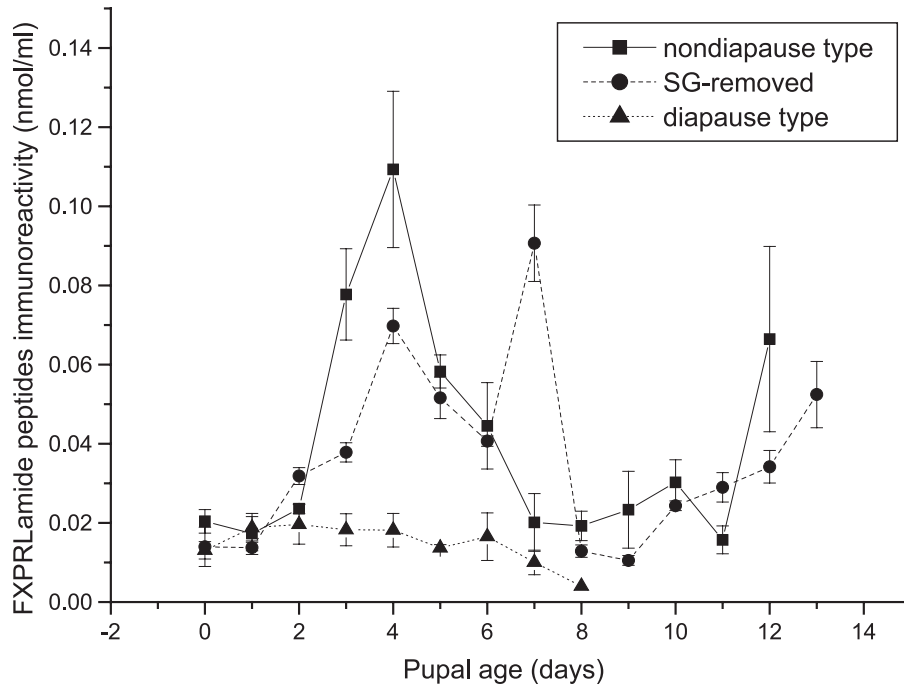
Fig. 2. Changes of FXPRLamide peptides titer in hemolymph. The hemolymph of 10 pupae from diapause-destined, nondiapause, and SG-removed, respectively, was mixed as a sample for competitive ELISAs and each point is represented as mean ± S.E. of five repeats.
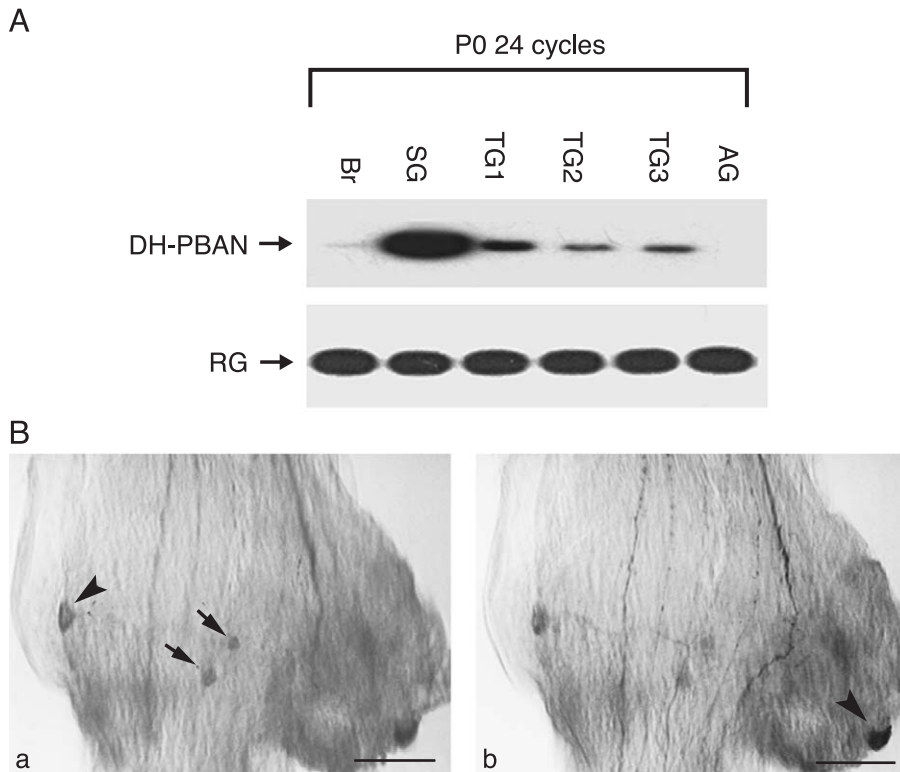

Fig. 3. The expression of Har-DH-PBAN gene in TGs. (A) RT-PCR analyses of Har-DH-PBAN mRNA in the central nervous system of 0-day-old pupae. The results show Southern hybridization of the amplification product with 24 cycles using either radiolabeled Har-DH-PBAN or rabbit globin (RG) cDNA as a probe. The upper panels show the DH-PBAN cDNA, and the lower panels show the RG cDNA. Br, brain; SG, suboesophageal ganglion; TG, thoracic ganglia; AG, abdominal ganglia. B, FXPRLamide peptides immunoreactivity in *H. armigera* TGs of 0-day-old pupae by whole-mount immunocytochemistry. Pre-adsorption of the antiserum with Har-DH abolished all immunostaining of TGs (data not shown). Frontal view of thoracic ganglia (TG$_1$) in nondiapause pupa of 0-day-old showing two (a) ventral midline neurons (arrow) and (a and b) lateral neurons (arrowhead) expressing the FXPRLamide peptides immunoreactivity. Scale bars equal 25 μm.

3. Results

3.1. Role of the SG in pupal development

Our previous studies have demonstrated that FXPRLamide neuropeptides (DH, PBAN, β-SGNP, and γ-SGNP) secreted from the SG can break pupal diapause and promote development in *H. armigera* [25]. This finding suggested that the SG might have an important role in the regulation of pupal development. To elucidate the function of FXPRLamide neuropeptides, we investigated the developmental status of nondiapause pupae by SG ablation. As shown in Fig. 1, the SG-removed pupae could not enter diapause-like status, but their rates of development differed significantly from the other three controls. The days of half maximums of eclosion for nontreated, sham-operated, CNS-cut (between the SG and the first TG), and SG-removed pupae were 10.72, 10.86, 11.28, and 11.88, respectively. The time for pupal development in SG-removed individuals is approximately 0.6–1.2 days longer to adult eclose and is significantly different from the three controls (Student's *t*-test, $P < 0.05$), suggesting that SG plays a crucial role in regulating pupal development.

Further, we measured the changes of FXPRLamide titer in hemolymph during the pupal stage using competitive ELISAs (Fig. 2). In diapause-destined pupae, the FXPRLamide titer remained low until day 8, at which time the pupae entered diapause. In nondiapause individuals, the FXPRLamide titer increased rapidly from day 2 to day 4 resulting in a peak and then decreased till day 11, and finally increased at day 12, which was 1 day prior to adult eclosion. The pattern of FXPRLamide in hemolymph of the SG-removed pupae is very similar with that of the nondiapause pupae (Fig. 2), except in having an additional peak at day 7. However, the FXPRLamide titer in hemolymph of SG-removed pupae is somewhat lower and eclosion is a day later (13 days) than the normal nondiapause pupae (Fig. 2).

3.2. The expression of FXPRLamide in thoracic ganglia

The FXPRLamide titer in hemolymph of the SG-ablated pupae is significantly higher than that in diapause-destined pupae, as shown in Fig. 2. This implies that FXPRLamide peptides are expressed in additional tissues other than the SG. We then investigated the distribution of DH-PBAN gene expression in these tissues. The results of RT-PCR showed that the DH-PBAN transcripts were present in three thoracic ganglia (TGs) besides the SG, even though the amount of DH-PBAN mRNA in the TGs is lower than that in SG (Fig. 3A). A pair of ventral midline neurons (Fig. 3B(a)) and lateral neurons (Fig. 3B(a,b)) with DH-PBAN expression was found in each TG by immunostaining with the antiserum.

The expressions of DH-PBAN gene in TGs of normal and SG-removed pupae were measured using quantitative RT-PCR combined with Southern blot analysis. Based on

our preliminary experiments, the range of cycles for a linear increase of PCR products is 20–25, so that 22 cycles were used in this experiment. The amount of DH-PBAN mRNA is significantly higher in the TGs of SG-removed individuals than in those of the normal pupae (Fig. 4A). Along with these studies, the difference in synthesis and release of DH-PBAN between SG-removed and normal pupal TGs was compared by whole-mount immunocytochemistry. The staining of immunoreactive cells were a litter stronger in SG-removed individuals (Fig. 4B(a)) and the axonal projections from lateral neurons (Fig. 4B(c)) were more clear than that in the normal control individuals (Fig. 4B(b,d)). The axonal projections detected in TG of the SG-removed individuals represent that the DH-PBAN is being synthesized heavily as reported in *Antheraea pernyi* PTTH [26]. Therefore, these results were consistent with the result of RT-PCR (Fig. 4A).
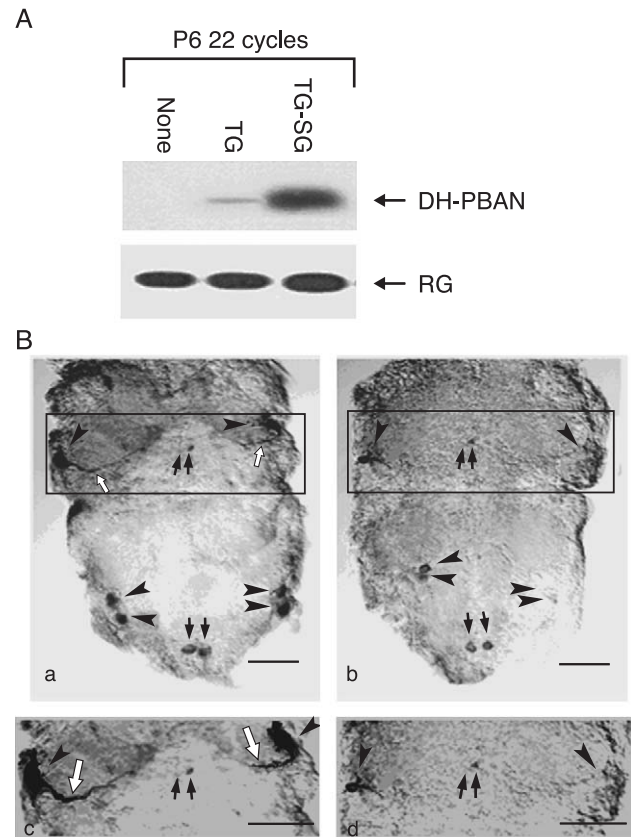

Fig. 4. The different expression of DH-PBAN gene in TGs of 6-day-old pupae. (A) RT-PCR analyses of the difference at mRNA levels. The DH-PBAN cDNA is amplified with 22 cycles based on our preliminary experiments, and then hybridized with radiolabeled Har-DH-PBAN cDNA as a probe. P6, 6-day-old pupae; TG-SG, thoracic ganglia of SG-removed pupae; TG, thoracic ganglia of normal pupae; None, control of no template; RG, rabbit globin. (B) Whole-mount immunocytochemistry analyses on FXPRLamide peptides of SG-removed pupae (a) and normal pupae (b). Both (a) and (b) are front view of TGs complex showing two clusters of ventral midline neurons (black arrow) and three clusters of lateral neurons (black arrowheads) immunostaining with antiserum. (c) and (d), higher magnification of the rectangle in (a) and (b). White arrow shows the staining of the axonal projections. Scale bars equal 30 μm.
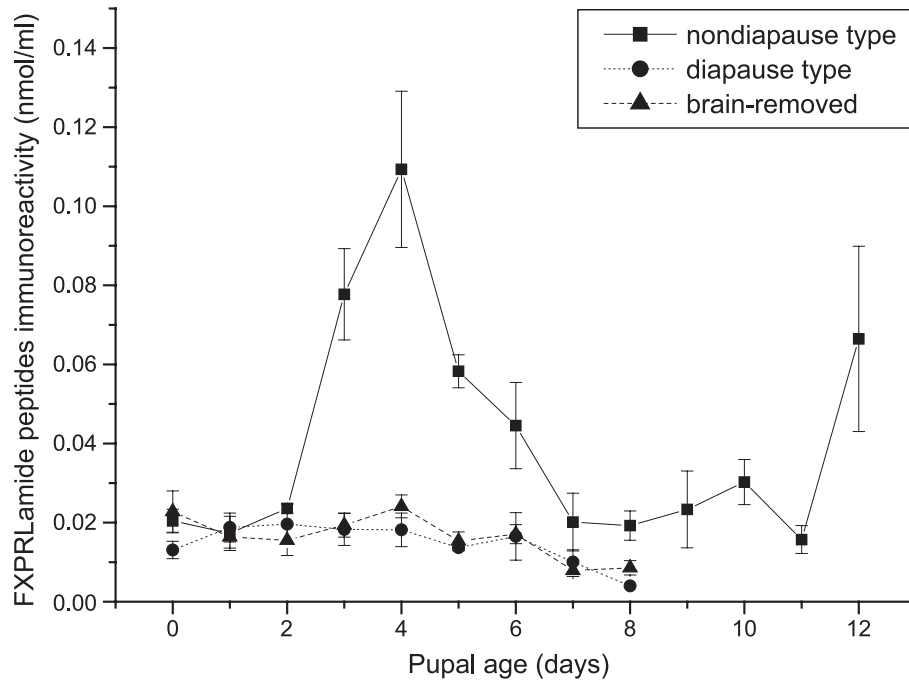
Fig. 5. Changes of FXPRLamide peptides titer in hemolymph. The hemolymph of 10 pupae from diapause-destined, nondiapause, and brain-removed was mixed respectively as a sample for competitive ELISAs, and each point is represented as mean ± S.E. of five repeats.

3.3. Role of the brain in pupal development

Nondiapause pupae can enter a diapause-like status when the brain is extirpated, suggesting that the brain can determinate pupal development (data not shown). The FXPRLamide peptides encoded by DH-PBAN gene can terminate pupal diapause and stimulate decerebrated pupae that have entered a diapause-like status to develop towards adults [25]. These results imply that the brain may regulate pupal development through FXPRLamide peptides. Hence, we measured the FXPRLamide peptides titer in hemolymph of normal and decerebrated *H. armigera* pupae using
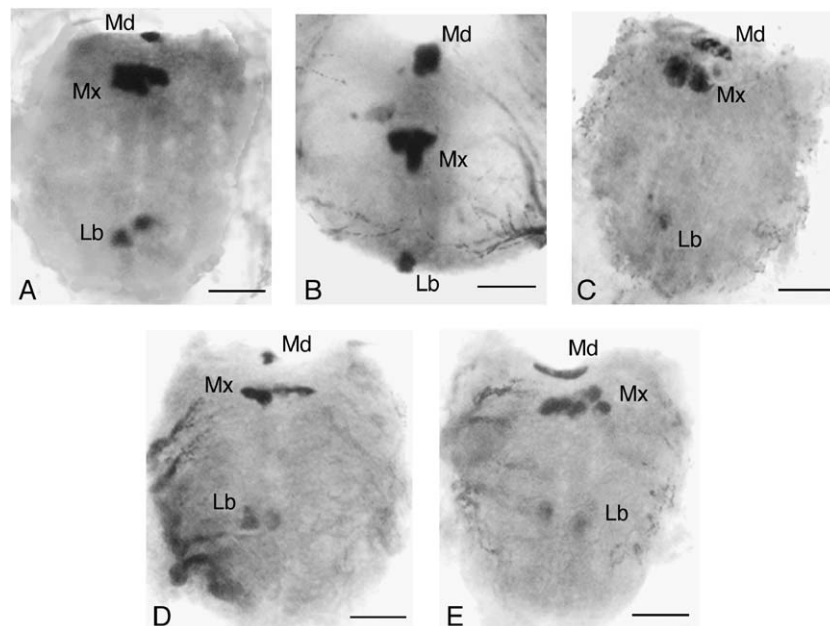

Fig. 6. Localization of DH-PBAN mRNA in the SGs by in situ hybridization. Hybridization signal was not detected in control tissues where the hybridization probe was sense-strand RNA (data not shown). Frontal view of 0-day-old (A) and 6-day-old (B) nondiapause, 6-day-old brain-removed (C), and 0-day-old (D) and 6-day-old (E) diapause-destined SGs of pupae showing hybridization signal in the mandibular (Md), maxillary (Mx), and labial (Lb) cell clusters. Scale bars equal 30 μm.

competitive ELISAs (Fig. 5). The FXPRLamide titer in hemolymph of decerebrated pupae remained at a lower level and is very similar to that of diapause-destined pupae. From this, it is evident that low levels of FXPRLamide titer in decerebrated pupae resulted in pupal diapause. These decerebrated pupae with low FXPRLamide titer could develop into adults when injected DH, one of the FXPRLamide peptides (data not shown).

3.4. Effect of brain on producing of FXPRLamide peptides

Based on the preceding results, it appears that the brain regulates the synthesis and release of FXPRLamide peptides by the SG of *H. armigera*. Therefore, we investigated the expression of DH-PBAN gene in SGs of brain-removed pupae using in situ hybridization and immunocytochemistry methods. In nondiapause pupae, three clusters of mandibular (Md), maxillary (Mx), and labial (Lb) cells were clearly identified in the SG by in situ hybridization (Fig. 6A and B) as reported in *B. mori* and *H. zea* [19,27]. At day 0, the staining pattern is similar in the three cell clusters of nondiapause- (Fig. 6A) and diapause-destined (Fig. 6D) pupae. However, in 6-day-old nondiapause pupae (Fig. 6B) the staining is much stronger than in that of 6-day-old diapause pupae (Fig. 6E). The expression of DH-PBAN gene in SGs of brain-removed pupae, 6 days after brain removal (Fig. 6C), is similar to that of diapause pupae (Fig. 6E), and both of them are weaker when compared with that of nondiapause pupae (Fig. 6B), especially the Lb cell cluster. In *B. mori*, the Lb cell cluster is related to diapause induction [19]. In most SGs from diapause and brain-removed pupae, the Lb cell clusters are much weaker stained. The immunoreactivity detected by DH antibody showed that the staining in the Lb cluster of nondiapause pupae (Fig. 7B) was much stronger than that in the diapause (Fig. 7A) and decerebrated pupae (Fig. 7C). The axonal projects were observed clearly in the Lb cluster of nondiapause pupae whereas no axonal projections were seen from the same cells in diapause and decerebrated pupae. It is evident from these studies that the expression of FXPRLamide neuropeptides in brain-removed pupae has the same pattern as diapause pupae and was obviously lower than nondiapause pupae.

The expression of DH-PBAN gene in TGs of brain-removed pupae was also investigated, but no significant difference when compared with normal controls (data not shown).

4. Discussion

DH is produced by neurosecretory cells of the SG in *B. mori*, and transplanting the SG of diapause type into non-diapause pupa can induce embryonic diapause in *B. mori* [2]. In contrast, Zdarek et al. [28] demonstrated that peptides of the FXPRLamide family could accelerate pupariation in the fleshfly, *Sarcophaga bullata*. Recently, we have demonstrated that the FXPRLamide peptides, DH, PBAN, and SGNPs produced by the DH-PBAN gene, could break pupal diapause and promote development by stimulating the prothoracic glands to secrete ecdysteroids in *H. armigera* (data not shown). Thus, the function of FXPRLamide peptides seems to be related to insect growth and development.

To demonstrate this, we tried to ablate the SG of non-diapause pupa with the expectation that this might induce pupal diapause. However, we failed to induce diapause. However, to our surprise, a delay of 0.6–1.2 days for adult eclosion was observed in SG-removed pupae in comparison to the three controls (Fig. 1). We suspected a high level of FXPRLamide peptides titer present in the hemolymph of SG ablated pupae. To confirm this, we measured the FXPRLamide titer in hemolymph of SG-removed pupae using competitive ELISAs. This showed that the expression pattern of FXPRLamide peptides in hemolymph of SG-removed pupae is similar to, but a little lower than, that of nondiapause pupae. The SG-removed pupae were able to develop because of their higher level of FXPRLamide peptides titer when compared with that of the diapause-destined pupae (Fig. 2). The delayed development in the SG-removed pupae probably results from the decrease of the FXPRLamide peptides titer, such as a lower peak on day 4. Additional peak present on day 7 is most likely to supply more FXPRLamide peptides for pupal development, since lower FXPRLamide titer is present in early–middle stage of
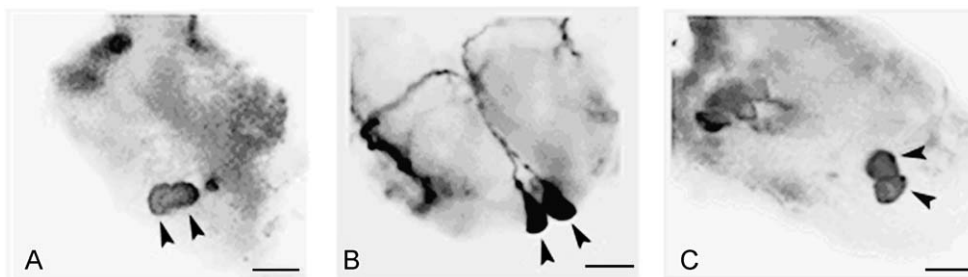

Fig. 7. FXPRLamide peptides immunoreactivity in the SGs. Frontal view of diapause (A), nondiapause (B), and decerebrated (C) SGs of 6-day-old pupae showing Lb cell cluster immunostained with antiserum (arrowheads). Scale bars equal 30 μm.

SG-removed pupae compared to that of normal pupae. The competitive ELISAs result suggests that tissues other than the SG may also be involved in the expression of FXPRLamide peptides, since their hemolymph titer was high in SG-removed pupae. We further investigated the tissue distribution of the DH-PBAN gene expression using a more sensitive RT-PCR method. In the TGs, a specific band clearly appeared at the expected size and could be hybridized by a DH-PBAN cDNA probe (Fig. 3A). However, the DH-PBAN gene expression is not observed in the AGs and nonneural tissues, such as midgut, fat body, and epidermis (data not shown). Immunocytochemical data proved positive with DH antibodies in all the TGs (Fig. 3B). DH-PBAN mRNA has been detected in the TG by RT-PCR and immunocytochemistry in *H. zea* [18,27]. So that, our finding that expression of Har-DH-PBAN mRNA in the TG, tissue other than the SG, is not unique. These lead us to conclude that the TGs express DH-PBAN and are responsible for the development of the SG-removed pupae. The amounts of both DH-PBAN mRNA and FXPRLamide peptides in the TGs of SG-removed individuals are significantly higher than in that of controls (Fig. 4). This might be caused by some other compensatory mechanism, though the mechanism is yet unknown.

The brain, as the receptor and repository of environmental stimuli, plays a central role in regulating the growth and development of insects [12,13]. In *B. mori*, the brain promotes or inhibits the secretion of DH in the SG determining the diapause or the development of the embryos [2]. In *H. armigera*, brain extirpation can induce pupae to enter a diapause-like status, and injection of DH could restore pupal development (data not shown). The competitive ELISAs showed that the FXPRLamide peptides titer in hemolymph of decerebrated pupae is much lower than that in nondiapause pupae, but is similar to that of diapause pupae (Fig. 5). The results from in situ hybridization indicated that the brain regulates the pupal development by inhibiting the expression of DH-PBAN gene in the SGs of diapause-destined pupae (Fig. 6). There was no axonal projection in the SGs of diapause and brain-removed pupae (Fig. 7), which suggests a low immunoreactivity in hemolymph of the diapause and decerebrated pupae along with a decreased expression of the DH-PBAN gene in the SGs. Thus, the brain regulates pupal development by controlling the SG through regulating of synthesis and release of FXPRLamide peptides, which can activate the prothoracic glands to synthesize and release ecdysone, either directly or indirectly (data not shown). The classic experiments elegantly demonstrated that the PTTH is required to stimulate the prothoracic glands to produce the ecdysone needed to regulate insect growth, molting, metamorphosis, and diapause [12,14,29]. Most probably, our conclusion that FXPRLamide regulate the synthesis and release of ecdysone is another pathway for insect development. Thus, the DH-PBAN gene products, the five FXPRLamide peptides may not only affect sexual behav-

iors, diapause, and melanization, but also be closely related to insect development.

Acknowledgements

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