EUROPEAN JOURNAL OF ENTOMOLOGY
ISSN (online): 1802-8829
http://www.eje.cz
Eur. J. Entomol. **122**: 189–197, 2025
doi: 10.14411/eje.2025.024
ORIGINAL ARTICLE

Localization of SNAP-29 and Syntaxin 6 in the brain of *Bombyx mori*

Fumika Toryu[1], Yuto Saitoh[1], Kengo Kanamaru[1], Katsuhiko Sakamoto[1], Yuichi Uno[2] and Tomohide Uno[1]*

[1] Graduate School of Agricultural Science, Kobe University, 1–1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan; e-mails: f3104z1@gmail.com (F.T.), ksakamoto@diamond.kobe-u.ac.jp, unotom@kobe-u.ac.jp

[2] Department of Plant Resource Science, Faculty of Agriculture, Kobe University, Nada-ku, Hyogo 657-8501, Japan

Key words. Lepidoptera, Bombycidae, insect, SNAREs, neuropeptide, neurohormone, starvation

Abstract. Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are a family of small conserved eukaryotic proteins that mediate fusion between organelles and the plasma membrane. Prior to fusion, complementary SNAREs, such as syntaxin, synaptosome-associated protein (SNAP), and synaptobrevin, assemble between membranes with the aid of accessory proteins that provide a scaffold to initiate SNARE zippering, pulling the membranes together, and mediating fusion. SNAP-29 and Syntaxin 6 from *Bombyx mori* were expressed in *Escherichia coli* and purified using affinity chromatography. We then produced antibodies against SNAP-29, and Syntaxin 6 of *Bombyx mori* in rabbits, which were used for immunohistochemistry. Immunohistochemistry results revealed that the expression of SNAP-29 was restricted to neurons in the pars intercerebralis (PI) and dorsolateral protocerebrum (DL) of the brain. The amount of SNAP-29 in the brain increases with hunger. Syntaxin 6 was restricted to neurons in the PI, the central complex (CX) and ventro-median protocerebrum (VP). SNAP-29 co-localized with SNAP-25 and Syntaxin 1A in the PI, and with Syntaxin 1A in the DL. Syntaxin 6 co-localized with SNAP-25 and Syntaxin 1A in the PI and with SNAP-25 in the CX. Bombyxin-immunopositive neurons of the brain occurred in SNAP-29 and Syntaxin 6-IRs. PTTH- and period-immunopositive neurons of the brain occurred in SNAP-29-IRs. Syntaxin 6 is present in EH secretory neurons of the brain.

INTRODUCTION

Vesicular transport is a mechanism that causes the transport and secretion of proteins and low molecular weight compounds between organelles or between organelles and the cell membrane via intracellular vesicles. It is also involved in the transport and secretion of neuropeptides and neurotransmitters (Chernomordik & Kozlov, 2005; Houy et al., 2013; Han et al., 2017). In vesicular transport, a section of the membrane of the supplying organelle first 'buds' to form a transport vesicle. The transport vesicle is then 'transported' to a specific organelle or cell membrane, where it 'adheres' to the membrane of the receptor. Finally, 'membrane fusion' occurs and vesicular transport is complete (Cui et al., 2022).

The efficient and controllable fusion of biological membranes is known to be driven by the cooperative action of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which consists of 100–300 amino acids, with an SNARE motif consisting of approximately 60 amino acids within the molecule and constituting the central components of the eukaryotic fusion machine (Pelham, 2001; Südhof & Rothman, 2009; Shin, 2014; Urbina & Gupton, 2020). During exocytosis, vesicle-associated v-SNAREs (VAMP/synaptobrevin) and target cell-associated t-SNAREs (syntaxin and SNAP) assemble into a core trans-SNARE complex. This complex plays a versatile role at various stages of exocytosis, ranging from priming to fusion pore formation and expansion, resulting in the release or exchange of vesicle contents. The exocytic fusion of synaptic vesicles to the plasma membrane is mediated by the formation of SNARE complexes, consisting of v-SNARE synaptobrevin (Syb)/VAMP2 and two t-SNAREs, Syntaxin 1A and SNAP-25 (Takamori et al., 2006).

SNAP-29 is a member of the SNAP-25 family of proteins and is ubiquitously expressed in many tissues (Tian et al., 2021). SNAP-29 localizes to many different internal membranes, including the Golgi apparatus, endosomes, and lysosomes, and binds to a broad range of syntaxins (Steegmaier et al., 1998). In *Drosophila*, the SNARE complex, consisting of Syx17 (Syntaxin 17), ubisnap (SNAP-29), and VAMP 7, is required for the fusion of autophagosomes with late endosomes and lysosomes (Takáts & Juhász, 2013; Xu et al., 2014). In *Drosophila*, when the SNAP-29

* Corresponding author; e-mail: unotom@kobe-u.ac.jp

gene is deleted, starvation-induced autolysosome degradation is inhibited, suggesting that SNAP-29 is involved in starvation-induced autophagy (Takáts et al., 2013)

Syntaxin 6-mediated intracellular cargo transport occurs in various cell types (Jung et al., 2012). Syntaxin 6 is present on the Trans Golgi Network (TGN), the Early Endosome (EE), cargo-specialized vesicles and granules, and the plasma membrane and regulates the intracellular trafficking of cargo molecules. Syntaxin 6 contributes to the post-TGN transport and delivery of membrane microdomain components to the plasma membrane. The SNARE complex (Syntaxin 6, Syntaxin 16, Vti1a, and VAMP 4) participates in membrane trafficking between the EE and TGN. Similarly, Syntaxin 6 interacts with Syntaxin 7, Vti1b, VAMP 7, and VAMP 8 to regulate cargo transport to the Late Endosome in some cell types.

Neuropeptides directly induce various physiological phenomena such as metamorphosis, eclosion, dormancy, appetite, metabolic regulation, and biological clocks (Nassel & Winther, 2010; Hoyer & Bartfai, 2012; Xu et al., 2020; Zeng et al., 2020). And neurotransmitters are secreted from axon terminals in the brain, bind to specific receptors, and transmit various information to control various phenomena. These neuropeptides and neurotransmitters are synthesized, secreted, and degraded in response to stimuli such as light, hunger, and temperature. The aim of this study is to clarify how SNARE, which is involved in vesicle membrane fusion, contributes to the synthesis, secretion, and degradation of neuropeptides and neurotransmitters.

Insect neuropeptides regulate insect-specific phenomena such as metamorphosis, molting, feeding, development, and ecdysis. These neuropeptides are mainly synthesized in the brain and secreted into the hemolymph by neuroendocrine organs, such as the corpus allatum (CA) (Tobe & Pratt, 1974; Roller et al., 2008).

The neurosecretory hormone bombyxin is an insulin-like peptide found in Lepidoptera (butterflies and moths). Bombyxin was first identified in the silkworm *Bombyx mori* (Nagasawa et al., 1986) and later in the tobacco hornworm *Manduca sexta* (Nijhout & Grunert, 2002; Van de Velde et al., 2007). Bombyxin stimulates cell division and is a growth factor in the wing imaginal disks of *Precis coenia* and *M. sexta* (Nijhout & Grunert, 2002; Nijhout et al., 2007).

In all insects, PTTH (Prothoracicotropic hormone) acts on prothoracic glands (PGs), initiating ecdysone synthesis (Nagata et al., 2005). Ecdysone is converted into 20-hydroxyecdysone, which acts on numerous target tissues and induces the expression of genes related to the molting process (Thummel, 2002). PTTH secretion is regulated by the circadian clock. In Lepidoptera, PTTH-IR resides in close proximity to period-IRs in the brain (Sauman & Reppert, 1996).

The neuropeptide eclosion hormone (EH) was first identified as a blood-borne factor released from the nervous system that triggers the emergence of silk moths from their pupal cuticle, a behavioral sequence known as eclosion (Truman & Riddiford, 1970). EH is expressed in two pairs of neurosecretory cells in the *Bombyx* brain (Kamito et al., 1992).

Previous research has shown that SNAP-25 is localized to a wide range of cells in the PI and CX (Sasao et al., 2023). VAMP 2 is localized in cells in the PI, DL and CX, and Syntaxin 1A is localized in cells in the PI and DL. It has been shown that SNAP-25 is localized in Bombyxin-synthesizing cells, VAMP 2 is localized in Bombyxin-synthesizing cells and PTTH-synthesizing cells, and Syntaxin 1A is localized in bombyxin-synthesizing cells and PTTH-synthesizing cells. In this study, we focused on two SNARE proteins (SNAP-29 and Syntaxin 6).

Therefore, the present study aimed to clarify the localization of SNARE proteins (SNAP-29 and Syntaxin 6) and investigate the relationship between SNARE proteins and bombyxin, PTTH, or EH secretion in the brain and the CA of *B. mori*. Antibodies against *B. mori*-derived SNARE proteins (SNAP-29, and Syntaxin 6) were used to identify SNARE protein-expressing cells, locate their regional distribution, and determine their co-localization status with bombyxin, PTTH, period and EH.

MATERIAL AND METHODS

Material

Donkey anti-mouse IgG (H+L)-CF555 (cat. ab150110), and donkey anti-rabbit IgG (H+L)-CF488A (cat. ab150073) was purchased from Biotium Inc. (Hayward, CA, USA). The mRNA purification kit, pGEX6P2 (cat. 28954650), and PreScission protease (cat. 27084301), and glutathione S-Sepharose (cat. 17527901) were purchased from GE Healthcare (UK). Software (Little Chalfont, Buckinghamshire, UK). A pCR2.2 and the oligonucleotides for Syntaxin 6 (5′-GGATCCATGACTTTAGAAGATCCTTTC-TACG-3′) and 5′-TTAGCGATTGGGACTGTC-3′) and SNAP-29 (5′-CCATGTCTGGGCATAAATATTTCACA-3′ and 5′-TTAATTCTTCAGACCATA-3′) was purchased from Invitrogen (Carlsbad, CA, USA). Peroxidase-conjugated goat anti-rabbit IgG antibody (cat. no. 10004301) was purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). Blocking One Solution (cat. 03953-66), and DAB kits (cat. 25985-50) was purchased from Nacalai Tesque (Kyoto, Japan). Taq DNA Polymerase (cat. R001A) and reverse transcriptase (cat. 639522), and a DNA ligation kit (cat. 6023) were purchased from Takara (Kyoto, Japan). Aqua-Poly/Mount medium (cat. 18606) was purchased from Polysciences, Inc. software (Warrington, PA, USA). The DX50 microscope was purchased from Olympus (Tokyo, Japan). All the chemicals used were of commercially available purest grade.

Insect cultures

We raised the hybrids (Kinshu × Showa or Daizo, p50 strains, National BioResource Program, Fukuoka, Japan) of *B. mori*. Twenty larvae in one container were reared on an artificial diet (Silkmate 2M, Nosan Co., Yokohama, Japan) at 25°C and subjected to a 16L : 8D cycle with a relative humidity of 70%. Fourth-instar larvae (5-day old) were used in all experiments.

Purification of *B. mori* SNAP-29, and Syntaxin 6 and the production of antiserum

The cDNA fragments containing the coding sequences of *B. mori* SNAP-29, and Syntaxin 6 were generated using reverse transcriptase-PCR (RT-PCR), and then separately subcloned into pTA2 (cat. No. TAK-101, Osaka, Japan). DNA sequences encod-

ing the His-tag were added to the amino-terminal using PCR. The plasmid pCR2.2 was digested using *Bam*HI and *Eco*RI. The digested fragments containing SNARES of *B. mori* were then independently inserted between the *Bam*HI and *Eco*RI sites of pGEX6P-2. The plasmid pGEX6P2 expresses the target protein as a GST-fusion protein. The identity of the clones containing *B. mori* SNAREs was confirmed using sequence analyses with an ABI Prism 377 DNA Sequencer (Artisan Technology Group, IL 61822, US).

The expression and purification of *B. mori* SNARE proteins in *E. coli* (BL21 strain) were performed as previously described (Uno et al., 2014). GST-proteins, which were independently expressed using pGEX6P2, were purified using a glutathione S-Sepharose column. After digestion with the PreScission protease, the His-tagged protein was purified using a His-accept column.

Antisera were generated in a rabbit by injection of a 1:1 (v/v) mixture of purified *B. mori* protein (1 mg) and Freund's complete adjuvant. The rabbits and rats received three booster injections at 2-week intervals. Sera were isolated and tested for the presence of anti-*B. mori* SNAREs antibodies using western blotting. Antisera against PTTH, EH, period, and bombyxin were obtained as described previously (Uno et al., 2007, 2010, 2014). Antisera against Syntaxin 1A and SNAP-25 were obtained as described previously (Sasao et al., 2023). Antibody against bombyxin was kindly provided by Prof Akira Mizoguchi (Aichi Gakuin University).

Western immunoblotting

One microgram of the purified proteins (His-SNAP-29, His-Syntaxin 6) was transferred to a polyvinylidene difluoride membrane after SDS-PAGE, which was performed according to the method of Laemmli (Laemmli, 1970), using a 4.5% stacking gel/15% separating gel (16 mA for 60 min). The membrane was blocked in Blocking One solution (60 min at room temperature (RT)), and then incubated for 60 min at RT with the appropriate primary antibody, as follows: anti-SNARE serum (1:2,000) in Tris-buffered saline (TBS; 50 mM Tris·HCl and 50 mM NaCl, pH 8.0) containing Blocking One solution. The membrane was then washed three times with TBS (including 0.05% Tween-20 (v/v)), followed by incubation for 60 min at RT with the secondary antibody, as follows; peroxidase-conjugated goat anti-rabbit IgG (1:2,000). The membrane was washed three times with TBS plus Tween-20, and proteins were detected using a peroxidase-staining DAB kit.

Immunohistochemistry

Insect heads of 12–15 brains were fixed (24 h at 4°C) in Bouin's fluid [saturated picric acid, formalin, and acetic acid at 15:5:1 (v:v:v)]. Standard histochemical methods were used for tissue dehydration, paraplast embedding, sectioning (8-µm-thick sections), deparaffinization, and rehydration.

The insect head sections were washed (at RT) in distilled water and phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) containing 0.3% Triton-X100 (PBS-Tr), blocked (30 min at RT) with an antibody dilution buffer (PBS-Tr containing 1.5% goat serum), and incubated overnight at 4°C in dilution buffer with the primary antibodies, anti-SNARE rabbit serum (1:500), and anti-bombyxin, anti-SNAP-25, anti-Syntaxin 1A anti-PTTH, or anti-EH mouse IgG (1:500). After rinsing three times for 10 min each with PBS-Tr at RT, the sections were incubated for 1.5 h at RT with secondary antibody (7.5 µg/mL), donkey anti-mouse IgG (H+L)-CF555, or goat anti-rabbit IgG (H+L)-CF488. After washing in PBS-Tr, the sections were mounted in Aqua-Poly/Mount medium and examined using a BX50 microscope equipped with BX-FLA reflected light fluorescence, WIG, and NIBA mirror/filter units. NIBA is excited in blue and detected in green, and WIG is excited in green and detected in red. Three to five individuals were used in each immunocytochemical experiment. The excitation and emission wavelength ranges used in the WIG mirror/filter units were 520–550 nm and > 580 nm, respectively. The excitation and emission wavelength ranges used in the NIBA mirror/filter unit were 470–490 and 515–550 nm, respectively.

In control experiments, the primary antibodies were replaced with pre-immune rabbit serum. No significant staining intensity was observed above the background level.

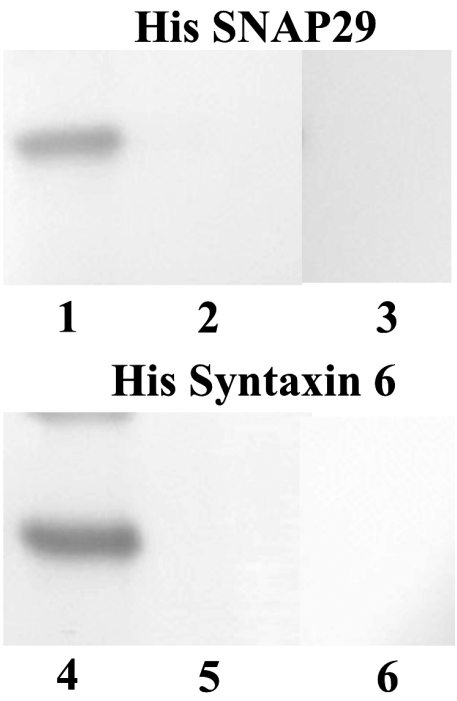

Fig. 1. Immunoblot analysis of the anti-SNARE protein antibodies (SNAP-29, and Syntaxin 6). Lanes 2, and 5 contain preimmune serum instead of the primary antibody (control); Lanes 3, and 6 contain an antibody-positive antigen, which was used as the primary antibody (control); Lanes 1 anti-SNAP-29, antiserum staining; Lanes 4, anti-Syntaxin 6 antiserum staining. Lanes 1–3, His-SNAP-29. Lanes 4–6, His-Syntaxin 6.

RESULTS

Production of antibodies against the SNARE proteins of *B. mori*

To ensure that the antibodies recognized the purified proteins, immunoblotting was performed using purified proteins (Fig. 1, lane 1–6). The antibodies produced against SNAP-29, and Syntaxin 6 of *B. mori* specifically recognized the protein band corresponding to the position of the purified partial protein of *B. mori* (Fig. 1, lanes 1 and 4). In the two control experiments (i.e., addition of pre-immune serum instead of the primary antibody and addition of the antigen and primary antibody together), no bands were detected (Fig. 1, lane 2–3, lane 5–6).

SNARE proteins are present in specific neurons in the *B. mori* brain

First, anti-SNAP-29 detected restricted areas in a set of neurons in the pars intercerebralis (PI) (Fig. 2b and h) and

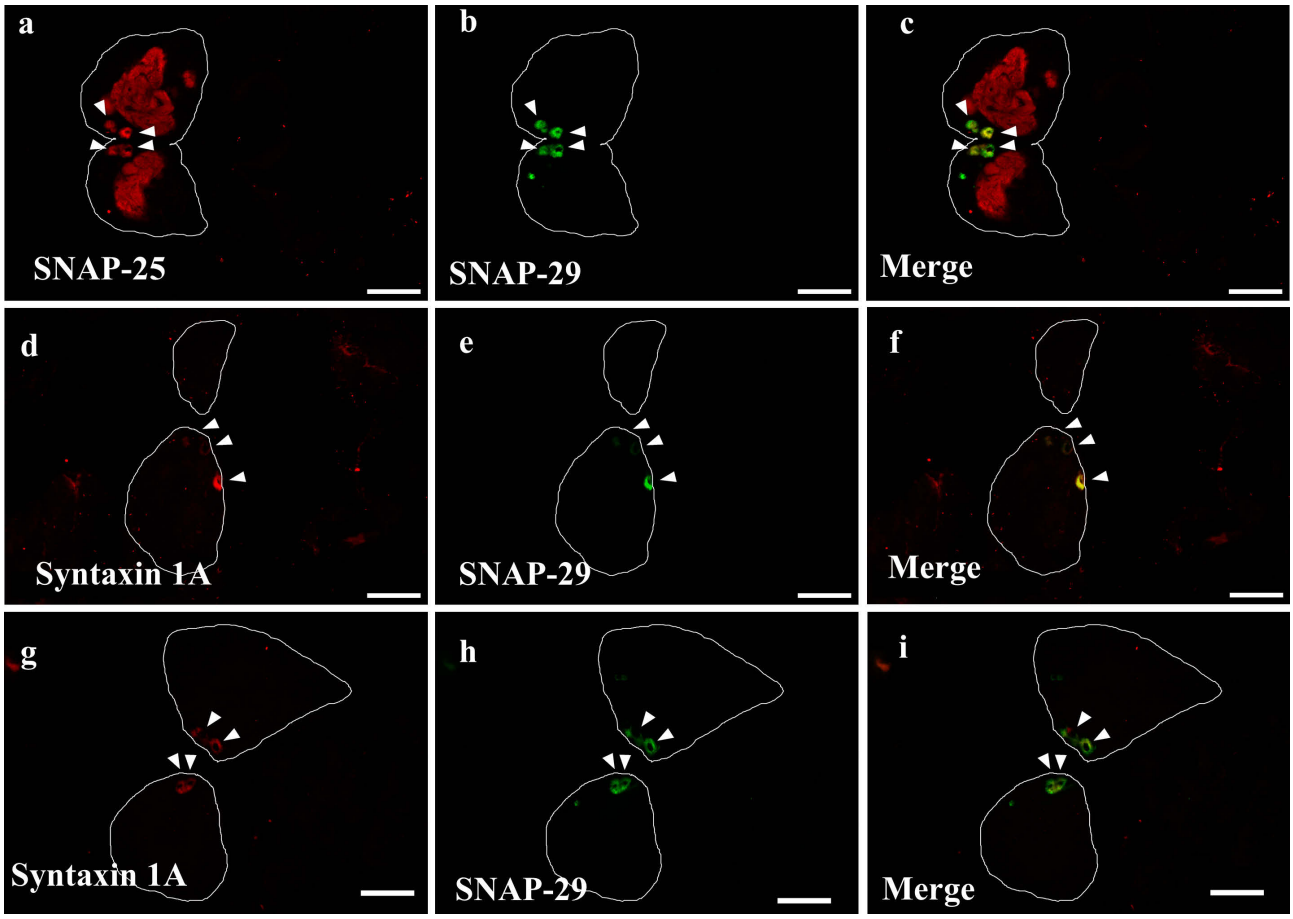

Fig. 2. Co-localization of SNAP-29 and SNAREs in the brain of *Bombyx mori*. Syntaxin 1A (d and g) and SNAP-25 (a) was visualized using CF™555 (red fluorophore). SNAP-29 (b, e and h) were visualized using CF™488A (green fluorophore). Anti-SNAP-29 detected restricted areas in a set of neurons in the pars intercerebralis (PI), and dorsolateral protocerebrum (DL) (b, e and h). SNAP-29-IRs in the PI occurred in SNAP-25-immunopositive neurons (c). SNAP-29-IRs occurred in Syntaxin 1A-immunopositive neurons (f and i). Scale bar: 100 μm.

dorsolateral protocerebrum (DL) (Fig. 2e). One to three neurons were detected in the PI and the DL of a single hemisphere. Double-labeling experiments showed that SNAP-29-IRs (Immunohistochemical Reactivities) in the PI occurred in SNAP-25 immunopositive neurons (Fig. 2c). SNAP-29-IRs in the PI and DL occurred in Syntaxin 1A-immunopositive neurons (Fig. 2f and i). SNAP-29 has been functionally linked to starvation-induced autophagy

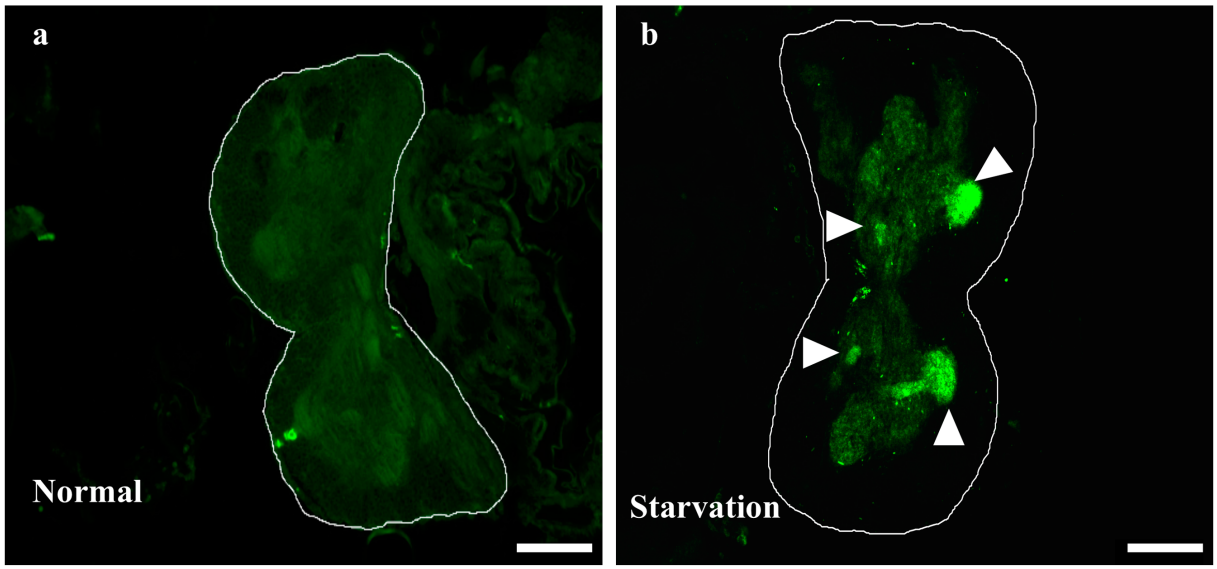

Fig. 3. Localization of SNAP-29 under starvation in the brain of *Bombyx mori*. Scale SNAP-29 (a and b) were visualized using CF™488A (green fluorophore). The amount of SNAP-29 expressed in the brains of silkworms that were starved for two days without being fed increased (b). SNAP-29-positive cells are indicated by arrowheads. Scale bar: 100 μm.

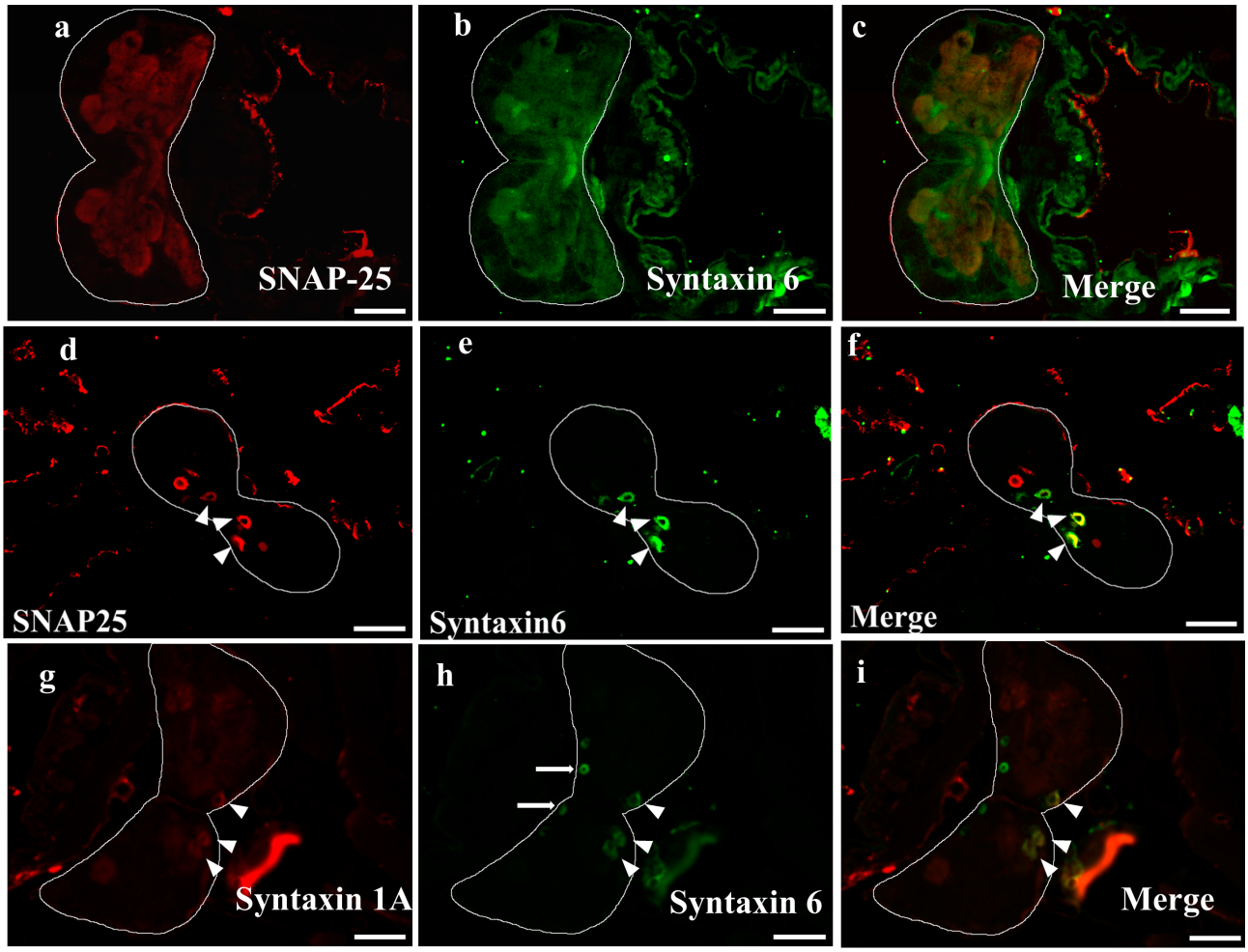

Fig. 4. Co-localization of Syntaxin 6 and SNAREs in the brain of *Bombyx mori*. Syntaxin 1A (g) and SNAP-25 (a and d) was visualized using CF™555 (red fluorophore). SNAP-29 (b, e and h) were visualized using CF™488A (green fluorophore). Anti-Syntaxin 6 detected restricted areas in a set of neurons in the PI, central complex (CX) and the ventro-median protocerebrum (VP) (b, e and h). Two broad areas in CX and 1–2 neurons in PI (arrowhead) and VP (arrow) were detected (b, e and h). The double-labeling experiments showed that SNAP-25-IRs (Immunohistochemical Reactivities) in the CX and PI occurred in Syntaxin 6-immunopositive neurons (c and f). Syntaxin 1A-IRs in the PI occurred in Syntaxin 6-immunopositive neurons (i). Scale bar: 100 μm.

in both humans and fruit flies (Hamasaki et al., 2013; Takáts et al., 2013). An increase in SNAP-29-positive cells was observed in the central part of the brain that were starved for two days without being fed increased (Fig. 3b).

Anti-Syntaxin 6 detected restricted areas in a set of neurons in the PI, central complex (CX) and the ventro-median protocerebrum (VP) (Fig. 4b, e and h). Two broad areas in the CX and 1–3 neurons in the PI and VP of a single hemisphere were detected (Fig. 4b, e, and h). Double-labeling experiments showed that SNAP-25-IRs (Immunohistochemical Reactivities) in the CX and PI occurred in Syntaxin 6-immunopositive neurons (Fig. 4c and f). Syntaxin 1A-IRs in the PI were observed in Syntaxin 6-immunopositive neurons (Fig. 4i).

SNARE proteins are present in the bombyxin-secretory neurons of the brain and corpus allatumn (CA)

Anti-bombyxin was detected in 1–2 neurons in the pars intercerebralis area of a single hemisphere (Fig. 5a and d). Bombyxin is produced by four pairs of PI neurosecretory cells in the brain, which are axonally transported to and released from the CA (Mizoguchi et al., 1987). Double-labeling experiments showed that bombyxin-immunopositive neurons occurred in SNAP-29, and Syntaxin 6-IRs (Fig. 5c and f, arrowhead). Anti-SNAP-29 and anti-Syntaxin 6 detected a restricted set of neurons in the CA section (Fig. 5g and j). Further double-labeling experiments showed that SNAP-29-IRs and Syntaxin 6-IRs were present in bombyxin-immunopositive neurons (Fig. 5i and l).

SNARE proteins are present in the PTTH-, Period-, and EH-secretory neurons of the brain

PTTH is released from CA into the hemolymph (O'Brien et al., 1988). Anti-PTTH was detected in 1–2 neurons in the dorsolateral protocerebrum area of a single hemisphere (Fig. 6a). PTTH is produced by two pairs of DL neurosecretory cells in the brain (Sauman & Reppert, 1996). Double-labeling experiments showed that one PTTH-immunopositive neuron was present in four SNAP-29-IRs in the brain (Fig. 6c and d, arrowhead). Period (Per)-IR cells are located close to contralateral PTTH neurosecretory

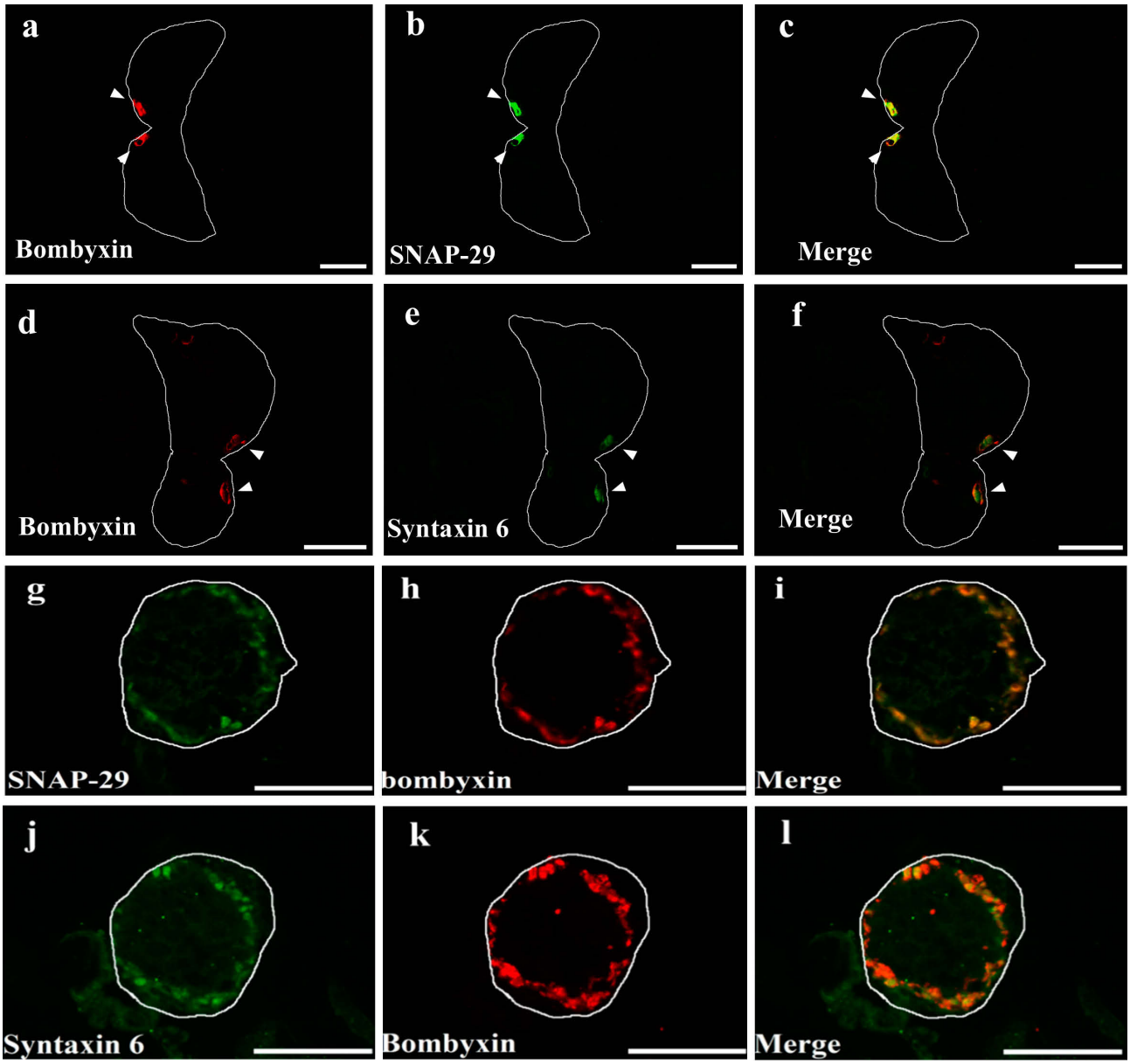

Fig. 5. Co-localization of SNARES (SNAP-29, and Syntaxin 6) and bombyxin in the brain and the corpus allatum of *Bombyx mori*. Bombyxin (a, d, h and k) was visualized using CF™555 (red fluorophore). SNAP-29 (b and g), and Syntaxin 6 (e and j) were visualized using CF™488A (green fluorophore). Anti-bombyxin detected a restricted set of neurons in the pars intercerebralis area (a and d) and a restricted set of neurons in the CA section (h and k). Bombyxin-immunopositive neurons in the brain and CA occurred in SNAP-29, and Syntaxin 6-IRs (c and f, arrowhead; i and l). Scale bar: 100 μm.

cells (Sauman & Reppert, 1996). Double-labelling showed that SNAP-29-IRs overlapped with Period-IR in the brain (Fig. 6g and h). EH-IR cell detected one neuron in the ventro-median protocerebrum of a single hemisphere (Fig. 6i). This result is consistent with previous findings in silk moths (Kono et al., 1990). Furthermore, double-labeling experiments showed that one Syntaxin 6-IR cell was present in one EH-IR neuron (Fig. 6k and l, arrowhead).

DISCUSSION

Neuropeptides and neurotransmitters are synthesized, secreted, and degraded in response to stimuli such as light, hunger, and temperature. Vesicles containing neuropeptides and neurotransmitters eventually fuse with the plasma membrane and are secreted. Membrane fusion of vesicles to the plasma membrane is mediated by the formation of SNARE complexes, consisting of vesicular SNARE and two target SNAREs. The aim of this study is to clarify how SNARE, which is involved in vesicle membrane fusion, contributes to the synthesis, secretion, and degradation of neuropeptides and neurotransmitters.

SNAP-29 is a member of the SNAP-25 family of proteins and is expressed in neurons (Pan et al., 2005). SNAP-29 localizes to many different internal membranes, including the golgi apparatus, endosomes, and lysosomes, and binds to a broad range of Syntaxins (Steegmaier et al., 1998; Xu et al., 2014). Overexpression and RNAi studies have indicated that dSNAP-29 mediates an essential process in *Drosophila* development (Xu et al., 2014).

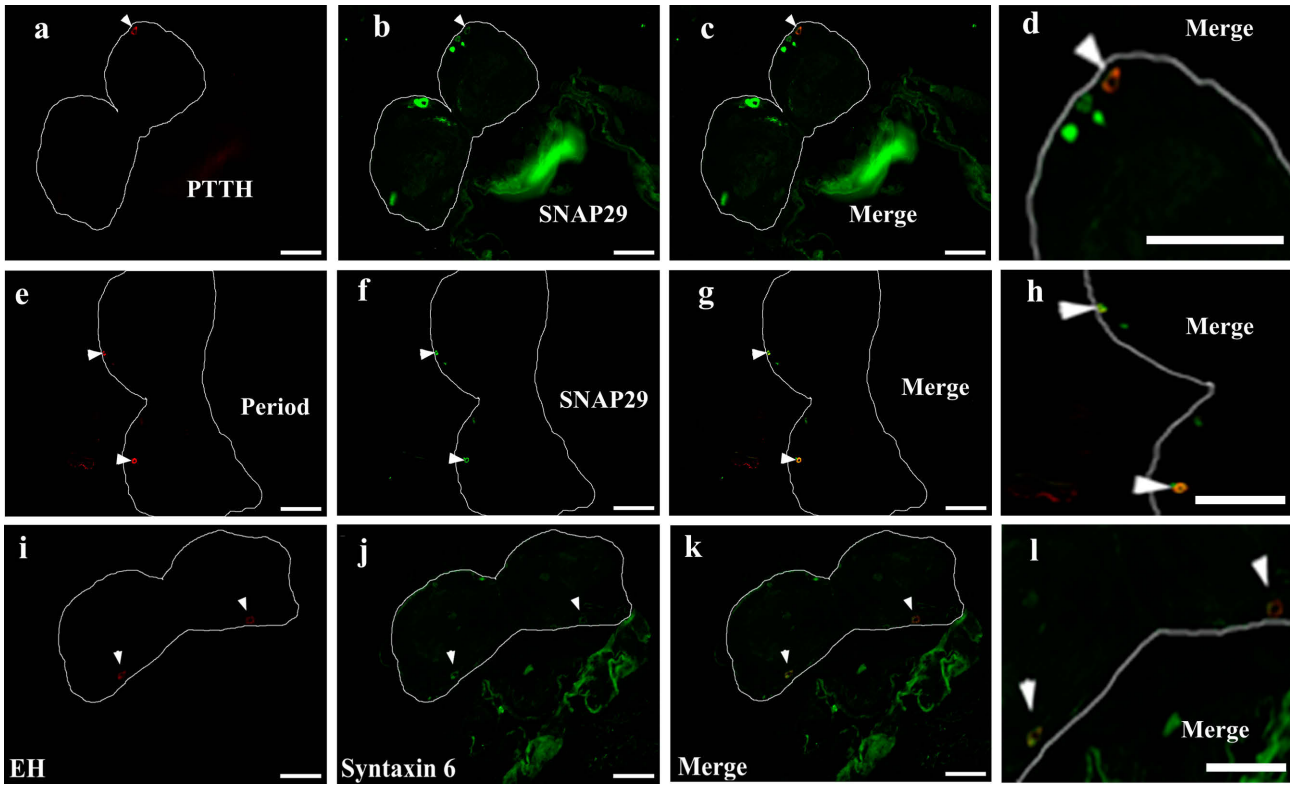

Fig. 6. Co-localization of SNARES (SNAP-29, and Syntaxin 6), PTTH, Period and EH in the brain of *Bombyx mori*. PTTH (a), Period (e) and EH (i) were visualized using CF™555 (red fluorophore) SNAP-29 (b and f), and Syntaxin 6 (j) were visualized using CF™488A (green fluorophore). The double-labeling experiments showed that SNAP-29-IRs (Immunohistochemical Reactivities) occurred in PTTH- and Period-immunopositive neurons (c, d, g and h). Syntaxin 6-IRs occurred in EH-immunopositive neurons (k and l). Scale bar: 100 μm.

Anti-SNAP-29 detected restricted areas in a set of neurons in the PI and DL of the brain of *B. mori* (Fig. 2). Double-labeling experiments showed that SNAP-29-IRs (Immunohistochemical Reactivities) in the PI occurred in SNAP-25 immunopositive neurons (Fig. 2c). SNAP-29-IRs in the PI and DL occurred in Syntaxin1A-immunopositive neurons (Fig. 2f and i). VAMP 2 combined with Syntaxin 1A and SNAP-25 produces a force that induces the formation of fusion pores, thereby mediating the fusion of synaptic vesicles and release of neurotransmitters (Yan et al., 2022). SNAP-29 has been reported to modulate synaptic transmission by interacting with Syntaxin 1A and competing with α-SNAP for binding to the SNARE complex (Su et al., 2001). This interaction results in modulation of synaptic transmission by inhibiting dissociation of the SNARE complex. SNAP29, Syntaxin 1A and VAMP 2 are involved in membrane fusion in the DL, and SNAP-29 modulates membrane fusion by interacting with Syntaxin 1A in place of SNAP-25 in the PI.

SNAP-29 is functionally linked to starvation-induced autophagy in both humans and fruit flies. SNAP-29, Syntaxin 17, and VAMP 7/8 cooperate to mediate the fusion of autophagosomes with late endosomes and lysosomes (Hamasaki et al., 2013; Takáts et al., 2013). The amount of SNAP-29 expressed in the brains of silkworms that were starved for four days without being fed increased (Fig. 3b). It has been suggested that starvation also promotes the fusion of autophagosomes and lysosomes in silkworm brains.

Anti-Syntaxin 6 detected restricted areas in a set of neurons in the PI, central complex (CX) and the ventro-median protocerebrum (VP) (Fig. 4b, e and h). Double-labeling experiments showed that SNAP-25-IRs (Immunohistochemical Reactivities) in the CX and PI occurred in Syntaxin 6-immunopositive neurons (Fig. 4c and f). Syntaxin 1A-IRs in the PI were observed in Syntaxin 6-immunopositive neurons (Fig. 4i). In the PI, Syntaxin 6 may be involved in membrane fusion by interacting with SNAP-25 in the same manner as Syntaxin 1A.

Syntaxin 6 was localized to a wide area of the central part of the brain (Fig.4). The central complex (CX), which is located in the center of the insect brain, is a neural circuit that is involved in directed behavior (such as long-distance migration, homing, searching for food sources, and flight behavior) rather than reflexive behavior (Heinze & Reppert, 2012). The CX forms a complex neural circuit that processes visual information and transmits it to the brain. For this reason, CX is a site of intense release and uptake of neurotransmitters and neuropeptides, and is concentrated in interneurons (Nassel & Homberg, 2006). Since SNAP-25 and VAMP 2 were also localized to the CX in the central part of the silkworm brain (Sasao et al., 2023), it was suggested that SNAP-25, Syntaxin 6, and VAMP 2 form a SNARE complex that is involved in the transport of neuropeptides in the CX.

Bombyxin is an insulin-like peptide that regulates trehalase activity in the silkworm body, and is thought to be involved in growth (Mizoguchi & Okamoto, 2013). It is se-

creted from the brain into body fluids throughout the growth process, from hatching of the larva to molting. Bombyxin is produced by neurosecretory cells and is transported to and released from the CA. Therefore, various SNAREs are thought to be involved in the membrane transport of bombyxin in the brain. Bombyxin-immunopositive neurons occurred in SNAP-29-, and Syntaxin 6-IRs (Fig. 5c and f, arrowhead). In the CA section, SNAP-29-IRs and Syntaxin 6-IRs were observed in bombyxin-immunopositive neurons (Fig. 5i and l). SNAP-25, VAMP 2, and Syntaxin 1A were with bombyxin-IR in the CA and brain (Sasao et al., 2023). When secreted, VAMP2, Syntaxin 1A, and Syntaxin 6 may be involved, and when degraded, SNAP-29, VAMP 7, and Synatxin17 may be involved.

PTTH is a critical neurohormone that regulates postembryonic development in insects by stimulating ecdysteroid secretion from the prothoracic glands (Agui et al., 1979). PTTH is produced by a pair of lateral neurosecretory cells in the protocerebrum of some insects and secreted multiple times at certain times of the day under the control of light and temperature (Mizoguchi et al., 2002; Zavodska et al., 2003). PTTH-IR occurred in VAMP2-IR and Syntaxin-IR neurons in the brain (Sasao et al., 2023). SNAP-29-IRs overlapped with PTTH-IR in the brain (Fig. 6c, arrowhead). Since the secretion of PTTH occurs multiple times at specific times in the fifth instar larvae of the silkworm, it is thought that the secretion and degradation of PTTH occur frequently in the brain. For this reason, it is thought that SNAP29, which is involved in autophagy; and Syntaxin 1A and VAMP 2, which are involved in the fusion of vesicle membranes, are co-localized with PTTH.

In Lepidoptera, PTTH-IR resides in close proximity to Period IRs in the brain (Sauman & Reppert, 1996). SNAP-29-IRs overlapped with Per-IR in the brain (Fig. 6f). Pigment dispersing factor (PDF), the only peptidergic hormone involved in circadian rhythms and a candidate transmitter of behavioral rhythm oscillation, and the clock proteins Timeless and Casein kinase, are localized in these clock cells in the DL area (Iwai et al., 2008). These peptides and proteins are frequently synthesized, secreted, and degraded. SNAP-29 may be involved in these processes.

EH is controlled by the circadian clock and a decrease in ecdysteroids in the brain, and controls ecdysis behavior (Truman & Sokolove, 1972). EH is located in two pairs of neurons in the brain, the VP cells, which project along the entire ventral nerve cord and secreted into the body fluids via the rectal nerves (Zavodska et al., 2003). In this study, Syntaxin 6 was localized in the EH-synthesizing cells in the silkworm brain (Fig. 6g), and its localization was highest in 5th instar larvae before they began to wander (data not shown)**.** Since the blood concentration of EH is highest before molting (Riddiford et al., 1994), we think that the expression level of Syntaxin 6 in the EH-synthesizing cells increased in order to secrete EH before molting. In this study, we did not identify the other SNAREs that bind to Syntaxin 6 and are involved in the secretion of EH, so it is necessary to find these SNAREs.

REFERENCES

Agui N., Granger N.A., Gilbert L.I. & Bollenbacher W.E. 1979: Cellular localization of the insect prothoracicotropic hormone: In vitro assay of a single neurosecretory cell. — *Proc. Natl. Acad. Sci. U.S.A.* **76**: 5694–5698.

Chernomordik L.V. & Kozlov M.M. 2005: Membrane hemifusion: crossing a chasm in two leaps. — *Cell* **123**: 375–382.

Cui L., Li H., Xi Y., Hu Q., Liu H., Fan J., Xiang Y., Zhang X., Shui W. & Lai Y. 2022: Vesicle trafficking and vesicle fusion: mechanisms, biological functions, and their implications for potential disease therapy. — *Mol. Biomed.* **3**(1): 29, 33 pp.

Hamasaki M., Furuta N., Matsuda A., Nezu A., Yamamoto A., Fujita N., Oomori H., Noda T., Haraguchi T., Hiraoka Y. et al. 2013: Autophagosomes form at ER-mitochondria contact sites. — *Nature* **495**: 389–393.

Han J., Pluhackova K. & Böckmann R.A. 2017: The multifaceted role of SNARE proteins in membrane fusion. — *Front. Physiol.* **8**: 5, 17 pp.

Heinze S. & Reppert S.M. 2012: Anatomical basis of sun compass navigation I: the general layout of the monarch butterfly brain. — *J. Comp. Neurol.* **520**: 1599–1628.

Houy S., Croisé P., Gubar O., Chasserot-Golaz S., Tryoen-Tóth P., Bailly Y., Ory S., Bader M.F. & Gasman S. 2013: Exocytosis and endocytosis in neuroendocrine cells: inseparable membranes! — *Front. Endocrinol*. **4**: 135, 6 pp.

Hoyer D. & Bartfai T. 2012: Neuropeptides and neuropeptide receptors: drug targets, and peptide and non-peptide ligands: a tribute to Prof. Dieter Seebach. — *Chem. Biodivers.* **9**: 2367–2387.

Iwai S., Thi Dieu Trang L., Sehadova H. & Takeda M. 2008: Expression analyses of casein kinase 2alpha and casein kinase 2beta in the silkmoth, *Bombyx mori*. — *Comp. Biochem. Physiol. (B)* **149**: 38–46.

Jung J.J., Inamdar S.M., Tiwari A. & Choudhury A. 2012: Regulation of intracellular membrane trafficking and cell dynamics by syntaxin-6. — *Biosci. Rep.* **32**: 383–391.

Kamito T., Tanaka H., Sato B., Nagasawa H. & Suzuki A. 1992: Nucleotide sequence of cDNA for the eclosion hormone of the silkworm, *Bombyx mori*, and the expression in a brain. — *Biochem. Biophys. Res. Commun.* **182**: 514–519.

Kono T., Nagasawa H., Kataoka H., Isogai A., Fugo H. & Suzuki A. 1990: Eclosion hormone of the silkworm *Bombyx mori*. Expression in *Escherichia coli* and location of disulfide bonds. — *FEBS Lett.* **263**: 358–360.

Laemmli U.K. 1970: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. — *Nature* **227**: 680–685.

Mizoguchi A. & Okamoto N. 2013: Insulin-like and IGF-like peptides in the silkmoth *Bombyx mori*: discovery, structure, secretion, and function. — *Front. Physiol.* **4**: 217, 11 pp.

Mizoguchi A., Ishizaki H., Nagasawa H., Kataoka H., Isogai A., Tamura S., Suzuki A., Fujino M. & Kitada C. 1987: A monoclonal antibody against a synthetic fragment of bombyxin (4K-prothoracicotropic hormone) from the silkmoth, *Bombyx mori*: characterization and immunohistochemistry. — *Mol. Cell. Endocrinol.* **51**: 227–235.

Mizoguchi A., Dedos S.G., Fugo H. & Kataoka H. 2002: Basic pattern of fluctuation in hemolymph PTTH titers during larval-pupal and pupal-adult development of the silkworm, *Bombyx mori*. — *Gen. Comp. Endocrinol.* **127**: 181–189.

Nagasawa H., Kataoka H., Isogai A., Tamura S., Suzuki A., Mizoguchi A., Fujiwara Y., Takahashi S.Y. & Ishizaki H. 1986: Amino acid sequence of a prothoracicotropic hormone

of the silkworm *Bombyx mori*. — *Proc. Natl. Acad. Sci. U.S.A.* **83**: 5840–5843.

Nagata S., Kataoka H. & Suzuki A. 2005: Silk moth neuropeptide hormones: prothoracicotropic hormone and others. — *Ann. N.Y. Acad. Sci.* **1040**: 38–52.

Nassel D.R. & Homberg U. 2006: Neuropeptides in interneurons of the insect brain. — *Cell Tissue Res.* **326**: 1–24.

Nassel D.R. & Winther A.M. 2010: *Drosophila* neuropeptides in regulation of physiology and behavior. — *Prog. Neurobiol.* **92**: 42–104.

Nijhout H.F. & Grunert L.W. 2002: Bombyxin is a growth factor for wing imaginal disks in Lepidoptera. — *Proc. Natl. Acad. Sci. U.S.A.* **99**: 15446–15450.

Nijhout H.F., Smith W.A., Schachar I., Subramanian S., Tobler A. & Grunert L.W. 2007: The control of growth and differentiation of the wing imaginal disks of *Manduca sexta*. — *Dev. Biol.* **302**: 569–576.

O'Brien M.A., Katahira E.J., Flanagan T.R., Arnold L.W., Haughton G. & Bollenbacher W.E. 1988: A monoclonal antibody to the insect prothoracicotropic hormone. — *J. Neurosci.* **8**: 3247–3257.

Pan P.Y., Cai Q., Lin L., Lu P.H., Duan S. & Sheng Z.H. 2005: SNAP-29-mediated modulation of synaptic transmission in cultured hippocampal neurons. — *J. Biol. Chem.* **280**: 25769–25779.

Pelham H.R. 2001: SNAREs and the specificity of membrane fusion. — *Trends Cell Biol.* **11**: 99–101.

Riddiford L.M., Hewes R.S. & Truman J.W. 1994: Dynamics and metamorphosis of an identifiable peptidergic neuron in an insect. — *J. Neurobiol.* **25**: 819–830.

Roller L., Yamanaka N., Watanabe K., Daubnerova I., Zitnan D., Kataoka H. & Tanaka Y. 2008: The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. — *Insect Biochem. Mol. Biol.* **38**: 1147–1157.

Sasao M., Uno T., Kitagawa R., Matsui A., Toryu F., Mizoguchi A., Kanamaru K., Sakamoto K. & Uno Y. 2023: Localization of SNARE proteins in the brain and corpus allatum of *Bombyx mori*. — *Histochem. Cell. Biol.* **159**: 199–208.

Sauman I. & Reppert S.M. 1996: Molecular characterization of prothoracicotropic hormone (PTTH) from the giant silkmoth *Antheraea pernyi*: developmental appearance of PTTH-expressing cells and relationship to circadian clock cells in central brain. — *Dev. Biol.* **178**: 418–429.

Shin O.H. 2014: Exocytosis and synaptic vesicle function. — *Comp. Physiol.* **4**: 149–175.

Steegmaier M., Yang B., Yoo J.S., Huang B., Shen M., Yu S., Luo Y. & Scheller R.H. 1998: Three novel proteins of the syntaxin/SNAP-25 family. — *J. Biol. Chem.* **273**: 34171–34179.

Su Q., Mochida S., Tian J.H., Mehta R. & Sheng Z.H. 2001: SNAP-29: a general SNARE protein that inhibits SNARE disassembly and is implicated in synaptic transmission. — *Proc. Natl. Acad. Sci. U.S.A.* **98**: 14038–14043.

Südhof T.C. & Rothman J.E. 2009: Membrane fusion: grappling with SNARE and SM proteins. — *Science* **323**: 474–477.

Takáts S. & Juhász G. 2013: A genetic model with specifically impaired autophagosome-lysosome fusion. — *Autophagy* **9**: 1251–1252.

Takamori S., Holt M., Stenius K., Lemke E.A., Grønborg M., Riedel D., Urlaub H., Schenck S., Brügger B., Ringler P. et al. 2006: Molecular anatomy of a trafficking organelle. — *Cell* **127**: 831–846.

Takáts S., Nagy P., Varga Á., Pircs K., Kárpáti M., Varga K., Kovács A.L., Hegedűs K. & Juhász G. 2013: Autophagosomal Syntaxin17-dependent lysosomal degradation maintains neuronal function in *Drosophila*. — *J. Cell Biol.* **201**: 531–539.

Thummel C.S. 2002: Ecdysone-regulated puff genes 2000. — *Insect Biochem. Mol. Biol.* **32**: 113–120.

Tian X., Teng J. & Chen J. 2021: New insights regarding SNARE proteins in autophagosome-lysosome fusion. — *Autophagy* **17**: 2680–2688.

Tobe S.S. & Pratt G.E. 1974: Dependence of juvenile hormone release from corpus allatum on intraglandular content. — *Nature* **252**: 474–476.

Truman J.W. & Riddiford L.M. 1970: Neuroendocrine control of ecdysis in silkmoths. — *Science* **167**: 1624–1626.

Truman J.W. & Sokolove P.G. 1972: Silk moth eclosion: hormonal triggering of a centrally programmed pattern of behavior. — *Science* **175**: 1491–1493.

Uno T., Nakada T., Okamaoto S., Nakamura M., Matsubara M., Imaishi H., Yamagata H., Kanamaru K. & Takagi M. 2007: Determination of phosphorylated amino acid residues of Rab8 from *Bombyx mori*. — *Arch. Insect Biochem. Physiol.* **66**: 89–97.

Uno T., Hata K., Hiragaki S., Isoyama Y., Trang le T.D., Uno Y., Kanamaru K., Yamagata H., Nakamura M., Takagi M. et al. 2010: Small GTPases of the Rab family in the brain of *Bombyx mori*. — *Histochem. Cell Biol.* **134**: 615–622.

Uno T., Isoyama Y., Sakamoto K., Uno Y., Sakamoto K., Kanamaru K., Yamagata H., Takagi M., Mizoguchi A. & Takeda M. 2014: Characterization of Rab-interacting lysosomal protein in the brain of *Bombyx mori*. — *Histochem. Cell Biol.* **141**: 311–320.

Urbina F.L. & Gupton S.L. 2020: SNARE-mediated exocytosis in neuronal development. — *Front. Mol. Neurosci.* **13**: 133, 17 pp.

Van de Velde S., Badisco L., Claeys I., Verleyen P., Chen X., Vanden Bosch L., Vanden Broeck J. & Smagghe G. 2007: Insulin-like peptides in *Spodoptera littoralis* (Lepidoptera): Detection, localization and identification. — *Gen. Comp. Endocrinol.* **153**: 72–79.

Xu H., Mohtashami M., Stewart B., Boulianne G. & Trimble W.S. 2014: *Drosophila* SNAP-29 is an essential SNARE that binds multiple proteins involved in membrane traffic. — *PLoS ONE* **9**(3): e91471, 8 pp.

Xu G., Teng Z.W., Gu G.X., Qi Y.X., Guo L., Xiao S., Wang F., Fang Q., Wang F., Song Q.S. et al. 2020: Genome-wide characterization and transcriptomic analyses of neuropeptides and their receptors in an endoparasitoid wasp, *Pteromalus puparum*. — *Arch. Insect Biochem. Physiol.* **103**(2):e21625, 23 pp.

Yan C., Jiang J., Yang Y., Geng X. & Dong W. 2022: The function of VAMP2 in mediating membrane fusion: An overview. — *Front. Mol. Neurosci.* **15**: 948160, 15 pp.

Zavodska R., Sauman I. & Sehnal F. 2003: Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. — *J. Biol. Rhythms* **18**: 106–122.

Zeng H., Qin Y., Du E., Wei Q., Li Y., Huang D., Wang G., Veenstra J.A., Li S. & Li N. 2020: Genomics- and peptidomics-based discovery of conserved and novel neuropeptides in the American cockroach. — *J. Proteome Res.* **20**: 1217–1228.

Received March 17, 2025; revised and accepted June 26, 2025
Published online August 22, 2025