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(Citation)
European Journal of Entomology,120:93-104

(Issue Date)
2023-03-29

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100481844

EUROPEAN JOURNAL OF ENTOMOLOGY
ISSN (online): 1802-8829
http://www.eje.cz
Eur. J. Entomol. **120**: 93–104, 2023
doi: 10.14411/eje.2023.012
ORIGINAL ARTICLE

Locations of Rab, Allatotropin, Prothoracicotropic hormone and Period in the larval brain, corpus allatum and frontal ganglion of *Bombyx mori*

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Key words. Lepidoptera, Bombycidae, immunohistochemistry, silkworm, frontal ganglion, insect neurohormone, PTTH

Abstract. Rab, a low molecular weight GTP-binding protein, regulates the transmission of intracellular proteins. Insect neuropeptides that are directly involved in growth, development, reproduction, homeostasis, courtship, feeding, circadian rhythm and many other physiological processes are synthesized in neurons and ganglia in the brain and secreted by specific neurosecretory cells in tissues such as the corpus allatum and frontal ganglion. To clarify the relationship between Rabs, two neuropeptides, Allatotropin (AT) and Prothoracicotropic hormone (PTTH) and the circadian clock protein, Period (PER), were used to determine the locations of nine Rabs (Rab1, Rab3, Rab6, Rab7, Rab11, Rab14, Rab19, Rab21 and RabX4). Rab6-, Rab11-, Rab14- and Rab21-immunohistochemical reactivities (IRs) partially overlapped AT-IR in the brain in *B. mori*. Rab3-, Rab6-, Rab11-, Rab14-, and Rab21-IRs overlapped AT-IR in the frontal ganglion. Of the seven Rabs, only Rab11-IR overlapped PTTH-IR in the brain. Rab1-, Rab3-, Rab11-, Rab14-, Rab19-, Rab21-, RabX4- and NUF (nuclear fallout, an effector of Rab11)-IRs, overlapped PER-IR in the brain. Therefore, Rab may regulate the exocytosis of PTTH, AT and a protein associated with the circadian rhythm.

INTRODUCTION

Small GTPases function as regulators of various cellular events by cycling between two nucleotide-states: a GDP-bound inactive form and a GTP-bound active form (Barr & Lambright, 2010; Müller & Goody, 2018). The largest family of small GTPases is Rab, which in mammals includes around 60 proteins (Stenmark, 2009; Pfeffer, 2017). Rab small GTPases regulate intracellular vesicular transmission (Barr, 2013; Li & Marlin, 2015; Pfeffer, 2017; Jin et al., 2021). Each Rab specifically targets a distinct component of a membrane. For example, Rab11 regulates recycling and secretion of membrane proteins between the plasma membrane and endosomes, Rab6 regulates transport in the Golgi apparatus, Rab7 regulates membrane fusion between vesicles and lysosomes and is involved in autophagy, and Rab11 regulates the traffic and transport of recycling endosomes. Rab14 is located in Golgi/TGN and early endosomes. Rab21 is involved in endocytosis and early endosomal transport and Rab1 regulates transport from the endoplasmic reticulum to the Golgi apparatus (Plutner et al., 1991; Ullrich et al., 1996; Junutula et al., 2004; Simpson et al., 2004; Grigoriev et al., 2007; Wang et al., 2011). These Rabs are also involved in various phenomena in insects, including differentiation of various organs, fertilization, development, dormancy and secretion of neurotransmitters and neuropeptides. (Lighthouse et al., 2008; Uytterhoeven et al., 2011; Ye et al., 2012; Garg & Wu, 2014; Fujita et al., 2017; Elmogy et al., 2018; Caviglia et al., 2019).

Genome analyses indicate that insects have over 40 neuropeptides, for example, there are 42 in *Drosophila* (Nassel & Winther, 2010), 67 in *Apis mellifera* (Boerjan et al., 2010), 36 in *Pteromalus puparum* (Xu et al., 2020) and 67 in *Periplaneta Americana* (Zeng et al., 2021). These neuro-

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peptides are synthesized in specific neurons, mainly in the brain, and are transported along axons to terminal neurosecretory cells in structures such as the corpus allatum (CA) and frontal ganglion (FG). They are then secreted into the haemolymph or their information is transmitted to different neurons (Nassel, 2002; Heuer et al., 2012; Bednar et al., 2017; Schoofs et al., 2017).

The neuropeptide Allatotropin (AT), was isolated based on its activity in stimulating juvenile hormone synthesis in the CA of the lepidopteran *Manduca sexta* (Kataoka et al., 1989). It is expressed in the brain and frontal ganglion (FG), is multifunctional, acting in different insects as a myoregulator and a cardioaccelerator (Duve et al., 1999; Koladich et al., 2002; Villalobos-Sambucaro et al., 2015). AT is synthesized in a number of neurons in the central nervous system and two neurons in the FG (Nagata et al., 2012; Bednar et al., 2017); however, the molecular mechanisms controlling its transport and secretion are unknown.

Prothoracicotropic hormone (PTTH) stimulates the endocrine prothoracic gland to secrete the metamorphic hormone, ecdysone, into the haemolymph. PTTH plays a central role in the endocrine network, controlling insect growth, moulting, metamorphosis and diapause (Goltzené et al., 1992; Nagata et al., 2005). PTTH is released from the CA, which is an organ specific to insects, which together with associated nerves secretes neuropeptides into the haemolymph (Mizoguchi et al., 1987; O'Brien et al., 1988).

Clock cells in the dorsolateral protocerebrum (DL) in the brain express clock genes, such as *Period (Per), Timeless* and *Clock*, which secrete the neuropeptide Pigment dispersing factor (PDF), which regulates the biological clock of insects (Sehadová et al., 2004; Hardin, 2005; Iwai et al., 2008; Inami et al., 2022). PTTH was identified in several lepidopterans as a product of two pairs of brain neurons that are distinct from those expressing *Per* and *Timeless* (Sauman & Reppert, 1996; Wise et al., 2002). Clock neurons can regulate the synthesis and rhythmic release of PTTH into the haemolymph (Vafopoulou & Steel, 2014).

Immunohistochemical analyses using antibodies against several Rab proteins have revealed Rab co-localisation with Bombyxin, an insulin-like peptide, in neurons in the brain and CA of the silkworm (Uno et al., 2013, 2021). However, the localization of Rab using neuropeptides other than Bombyxin is rare. Therefore, to clarify the role of Rab in neuropeptide secretion, an immunohistochemical analysis was carried out using antibodies against multiple Rabs and the neuropeptides, PTTH and AT, and clock protein, PER.

MATERIALS AND METHODS

Materials and insect cultures

Oligonucleotides were purchased from Invitrogen (Tokyo, Japan). The other chemicals used were of the purest grade commercially available. We used hybrid (Kinshu × Showa or Daizo, p50 strains) *B. mori* and 5 day-old fourth-instar larvae in all the experiments.

Antiserum production

Antisera against *B. mori* Rab1, 3, 6, 7, 11, 14, 21, X4, and PTTH, PER and AT were obtained as previously described (Uno et al., 2010, 2013, 2016, 2017, 2021). The specificity of the antibodies is shown in Table 1. A fragment of cDNA containing the partial coding sequence of *B. mori Nuf* (*Nuclear fallout*) and Rab19 was generated using reverse transcriptase-PCR (RT-PCR), subcloned and sequenced. This fragment was inserted between the *Bam*HI and *Eco*RI sites of pGEX6P-2 (GE Healthcare UK Ltd, UK) and expressed in *E. coli* (BL21 strain). NUF and Rab19 GST-protein was purified using a glutathione S-Sepharose column as previously described (Uno et al., 2014). After digestion with PreScission protease, the His-tagged protein was purified using a His-accept column.

Antisera were generated in a rabbit by injecting it with a 1:1 (v/v) mixture of each purified *B. mori* protein (1 mg) and Freund's complete adjuvant. The rabbits received three booster injections at 2-weekly intervals. The sera were isolated and tested for the presence of anti-*B. mori* NUF and Rab19 antibodies using western blotting

Western blotting and immunohistochemistry

Western blotting and immunohistochemistry were done as previously described (Uno et al., 2019). For western blotting, purified proteins (1 μg) were transferred to a PVDF membrane after electrophoresis. The membrane was placed in Blocking One solution for 60 min at room temperature [RT]) and then for 60 min at room temperature [RT]) with anti-NUF serum (1:2,000), as the primary antibody, in Tris-buffered saline (TBS; 50 mM Tris·HCl, 50 mM NaCl [pH 8.0]) containing Blocking One solution. The membrane was then washed (3×) with TBS (including 0.05% Tween-20 (v/v)), followed by incubation (60 min, room temperature [RT]) with the secondary antibodies: peroxidase-conjugated goat anti-rabbit IgG (both 1:2000). After the membrane was washed (3× with TBS plus Tween 20), proteins were detected using the peroxidase staining DAB kit (Nakalai Tesque Inc, Kyoto, Japan).

Insect heads were fixed (24 h at 4°C) in Bouin fluid (saturated picric acid, formalin and acetic acid at 15:5:1 by volume). Standard histochemical methods were used for dehydrating tissues and embedding in paraplast, then sectioning (9-μm-thick sections), followed by deparaffinization and rehydration. The insect heads were washed (at RT) in distilled water and in 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 antibody dilution buffer (PBS-Tr containing 1.5% goat serum) and incubated (overnight at 4°C)

Table 1. The relationship between Rab and neuropeptides in terms of the specificity of the antibodies.

	Allatotropin		PTTH	Period	Bombyxin		Reference
	Brain	FG			Brain	CA	
Rab1	n.d.	n.d.	n.d.	+	+	n.d.	(1)
Rab3	n.d.	+	n.d.	+	+	+	(2)
Rab6	+	+	n.d.	n.d.	+	+	(2)
Rab7	n.d.	+	n.d.	–	+	+	(3)
Rab11	+	+	+	+	+	+	(4)
Rab14	+	+	n.d.	+	+	+	(1)
Rab19	n.d.	n.d.	n.d.	n.d.	–	–	(5)
Rab21	+	+	n.d.	+	+	+	(4)
RabX4	n.d.	n.d.	n.d.	+	+	+	(6)

n.d.: not detectable; −: not examined; +: colocalized. (1) Uno et al., 2013; (2) Uno et al., 2016; (3) Uno et al., 2010; (4) Uno et al., 2021; (5) This paper; (6) Uno et al., 2017.

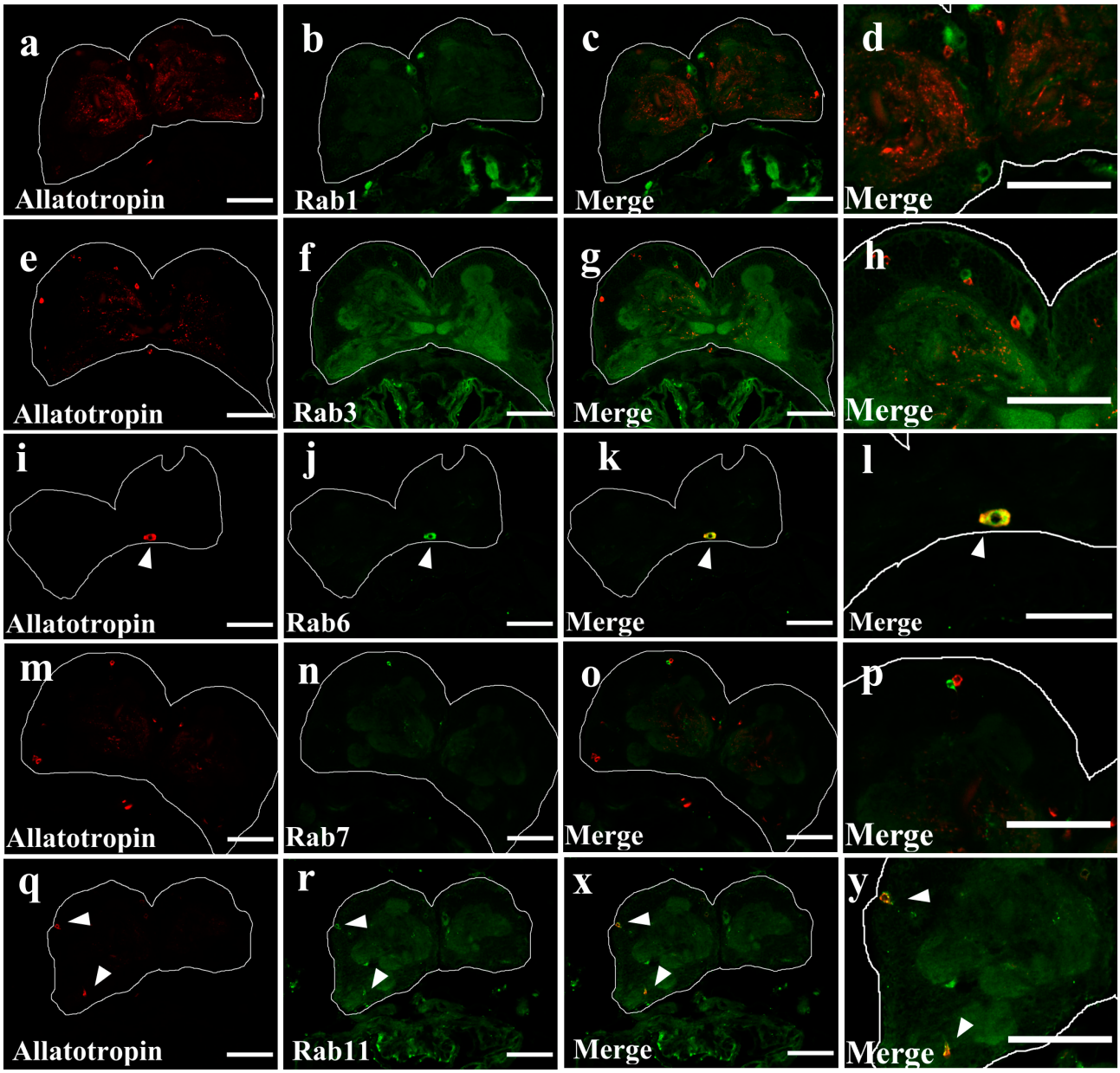

Fig. 1. Co-localization of Rab1-, Rab3-, Rab6-, Rab7- and Rab11-IR with AT-IR in the brain of *B. mori*. Allatotropin (a, e, i, m and q) was visualized using CF™555 (red fluorophore). Rab1 (b), Rab3 (f), Rab6 (j), Rab7 (n) and Rab11 (r) were visualized using CF™488A (green fluorophore). Rab6- and Rab11-IRs partially colocalized in AT-IR neurons (k, l, x and y). Rab1-, Rab3- and Rab7-IRs did not overlap with AT-IR (c, d, g, h, o and p). Scale bar: 100 µm.

in dilution buffer with each of the primary antibodies: anti-Rab rabbit serum (1:500), anti-NUF rabbit serum (1:500), anti-PER mouse IgG (1:500), anti-AT mouse IgG (1:500) and anti-PTTH mouse IgG (1:500). After rinsing (three times for 10 min at RT) with PBS-Tr, the sections were incubated (1.5 h at RT) with 7.5 µg/ml of each of the secondary antibodies: donkey anti-rat IgG (H+L)-CF555 and goat anti-rabbit IgG (H+L)-CF488A. 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 and WIG and NIBA mirror/filter units. Three to five individuals were examined in each immunocytochemical experiment. The excitation wavelength and emission range in WIG mirror/filter unit were 520–550 nm and above 580 nm, respectively. The excitation wavelength and emission range in the NIBA mirror/filter unit were 470–490 nm and 515–550 nm, respectively

In control experiments, the primary antibodies were replaced with pre-immune rabbit serum and no significant staining was observed above the background level. The bleed-through of fluorescence was checked by imaging singly stained samples using both filter/mirror units (WIG and NIBA). In both cases no significant staining was observed.

RESULTS

Location of immunoreactivity of Rabs and AT in the brain of *B. mori*

AT-IR was detected in many neural cells in the brain (Figs 1 and 2). This is consistent with previous findings reported for the silk moth (Bednar et al., 2017). Double-labelling showed that Rab6-, Rab11-, Rab14- and Rab21-IRs were partially colocalized with AT-IR (Figs 1 and 2,

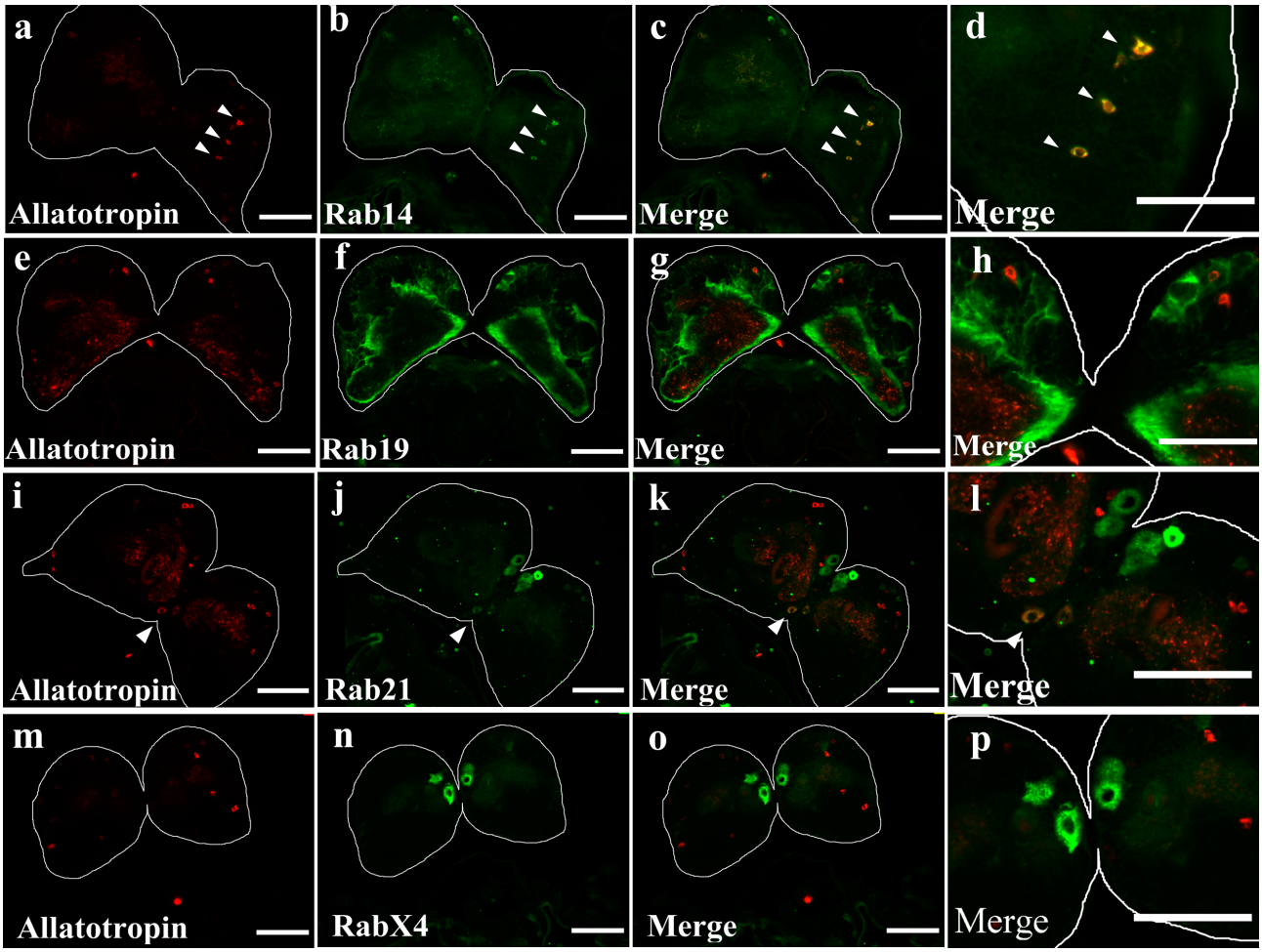

Fig. 2. Co-localization of Rab14-, Rab19-, Rab21- and RabX4-IRs with AT-IR in the brain of *B. mori*. Allatotropin (a, e, I and m) was visualized using CF™555 (red fluorophore). Rab14 (b), Rab19 (f), Rab21 (j) and RabX4 (n) were visualized using CF™488A (green fluorophore). Rab14- and Rab21-IRs partially overlapped with AT-IR (c, d, k and l). Rab19- and RabX4-IRs did not overlap with AT-IR (g, h, o and p). Scale bar: 100 μm.

arrow head). Rab1-, Rab3-, Rab7- and RabX4-IRs did not overlap with AT-IR. Rab6-IR and Rab21-IR were present in the ventral (Figs 1i–l, 2i–l), Rab11-IR in the dorso-lateral (Fig. 1q–y) and Rab14-IR in the central region of the brain (Fig. 2a–d).

Location of immunoreactivity of Rabs and AT in the FG of *B. mori*.

AT-IR was detected in two cells in the FG (Figs 3 and 4). This is consistent with previous findings reported for the silk moth (Bednar et al., 2017). Rab3-, Rab6-, Rab7, Rab11- Rab14- and Rab21-IRs overlapped with AT-IR in the FG (Figs 3 and 4, arrow head). Rab1-, Rab19- and RabX4-IRs did not overlap with AT-IR.

Location of immunoreactivity of Rabs and PTTH in the brain of *B. mori*

In Lepidoptera, PTTH is produced by two pairs of DL neurosecretory cells in the brain (Sauman & Reppert, 1996). PTTH-IR was detected in 1–2 neurons in the DL area (Fig. 5). Double-labelling showed that PTTH-IR colocalized with Rab11-IR in the brain (Fig. 5g–i, arrow-head) but not with other Rab-IR neurons in the brain (Fig. 5). Rab11-immunopositive neurons did not overlap with PTTH-IR in the CA (Fig. 5x).

Location of immunoreactivity of Rabs and PER in the brain of *B. mori*

Rab-IR cells did not overlap with PTTH-IR cells, but many Rab-IR cells were located close to PTTH-IR cells in the DL of the brain (Fig. 5). PER-IR cells were located close to the contralateral PTTH neurosecretory cells (Sauman & Reppert, 1996). Double-labelling showed that Rab1-, Rab3-, Rab11-, Rab14-, Rab19-, Rab21- and RabX4-IRs overlapped with PER-IR in the brain (Fig. 6).

Anti-*B. mori* NUF antibodies and location of NUF immunoreactivity

NUF is a Rab11 effector protein that is important in controlling the transmission of several intracellular cargos (Riggs et al., 2007; Calero-Cuenca & Sotillos, 2018).

As a control for binding specificity, the anti-NUF antibody was pre-incubated with an excess amount of antigen before immunological staining (Fig. 7d–f).

The bleed-through fluorescence was assessed by imaging the single-stained samples using both filter/mirror units (WIG and NIBA) (Fig. 7a–c). In both cases, no significant

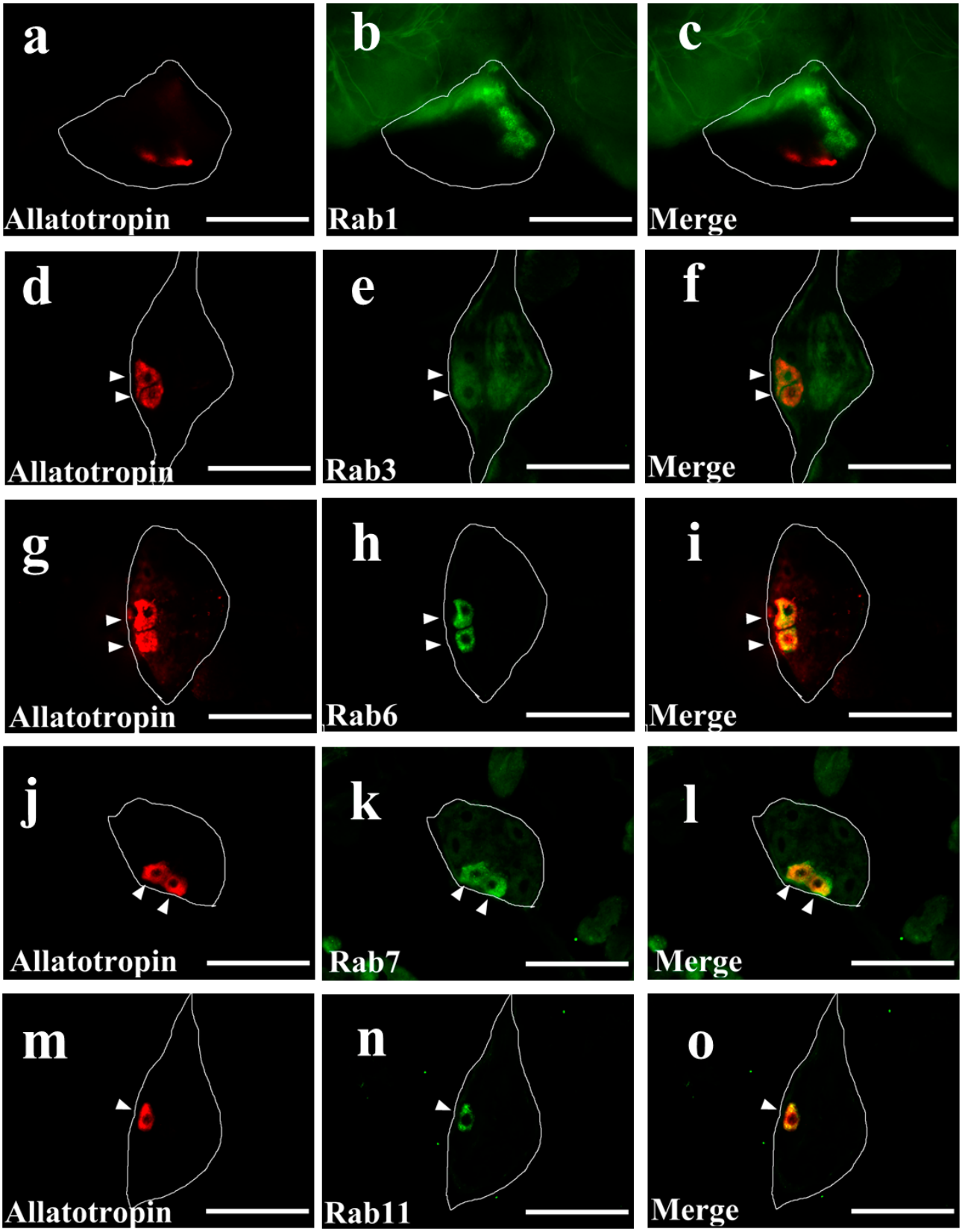

Fig. 3. Co-localization of Rab1-, Rab3-, Rab6-, Rab7- and Rab11-IRs with AT-IR in the frontal ganglion of *B. mori*. Allatotropin (a, d, g, j and m) was visualized using CF™555 (red fluorophore). Rab1 (b), Rab3 (e), Rab6 (h), Rab7 (k), and Rab11 (n) were visualized using CF™488A (green fluorophore). Rab3-, Rab6-, Rab7- and Rab11-IRs overlapped with AT-IR (f, i, l and o). Rab1-IR did not overlap with AT-IR (c). Scale bar: 100 μm.

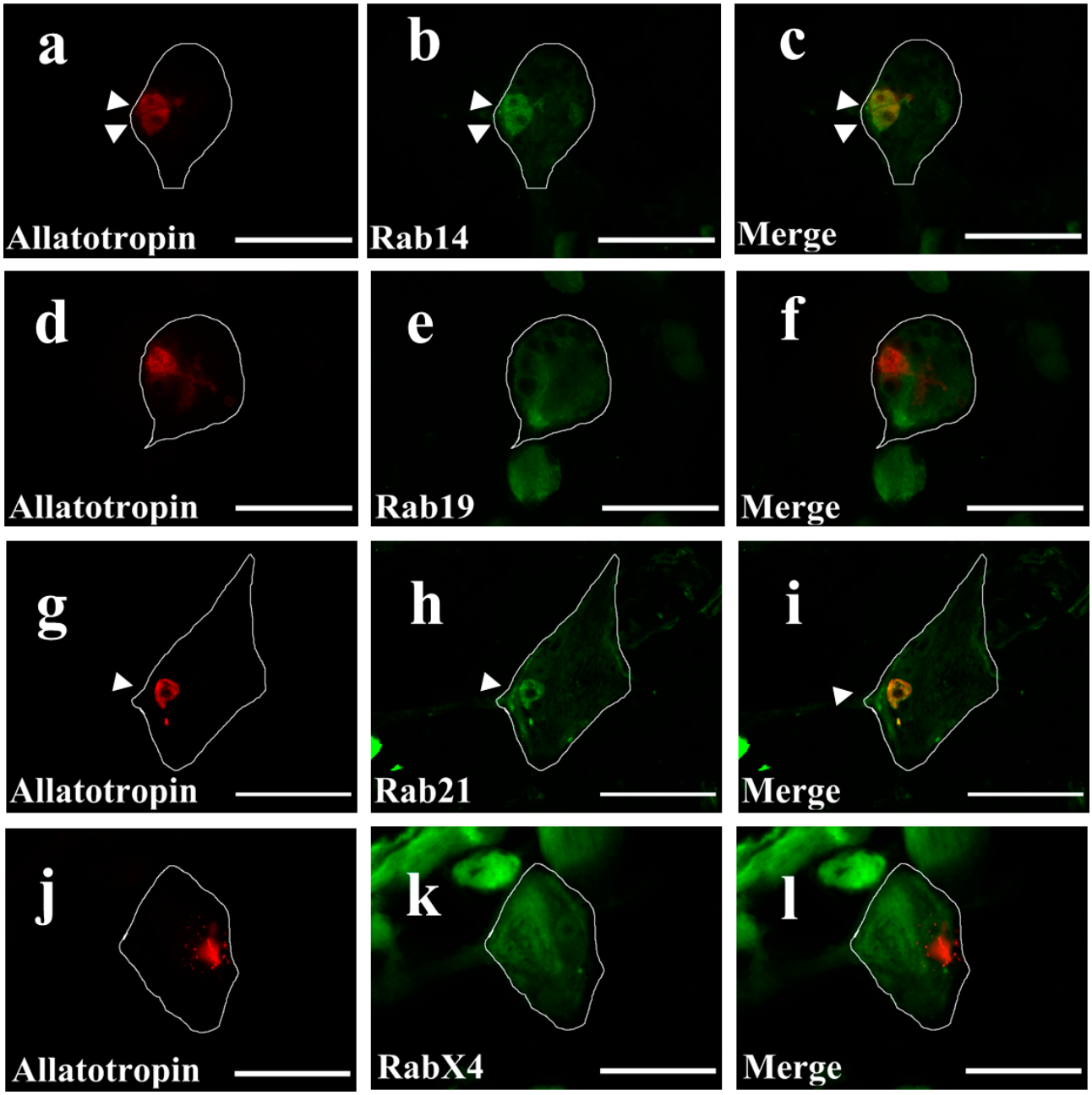

Fig. 4. Co-localization of Rab14-, Rab19-, Rab21- and RabX4-IRs with AT-IR in the frontal ganglion of *B. mori*. Allatotropin (a, d, g, and j) was visualized using CF™555 (red fluorophore). Rab14 (b), Rab19 (e), Rab21 (h), and RabX4 (k) were visualized using CF™488A (green fluorophore). Rab14- and Rab21-IRs overlapped with AT-IR (c and i). Rab19- and RabX4-IRs did not overlap with AT-IR (f and l). Scale bar: 100 μm.

staining was recorded (Fig. 7a, e). Antibodies produced against *B. mori* NUF specifically recognized the protein band corresponding to the position of purified partial *B. mori* NUF (Fig. 7p). Control experiments (the addition of the antigen and primary antibody together) did not result in staining above background levels (Fig. 7p).

Double-labelling showed that NUF-IR colocalized with Rab11-IR in neurons in the brain (Fig. 7g–i), but not with PTTH-IR neurons (Fig. 7j–l). NUF-IR also overlapped with PER-IR in the brain (Fig. 7m–o).

DISCUSSION

To clarify the relationship between Rabs, two neuropeptides, AT and PTTH, and the circadian clock protein, PER, the colocalization of nine Rabs (Rab1, Rab3, Rab6, Rab7, Rab11, Rab14, Rab19, Rab21 and RabX4) with these proteins was determined. Rab6-, Rab11-, Rab14- and Rab21-IRs partially overlapped with AT-IR in the brain. Rab3-, Rab6-, Rab7-, Rab11-, Rab14- and Rab21-IRs overlapped with AT-IR in the FG. Only Rab11-IR areas overlapped with PTTH-IR in the brain. Rab1-, Rab3-, Rab11-, Rab14, Rab19-, Rab21- and RabX4-IRs and NUF-IR, an effector

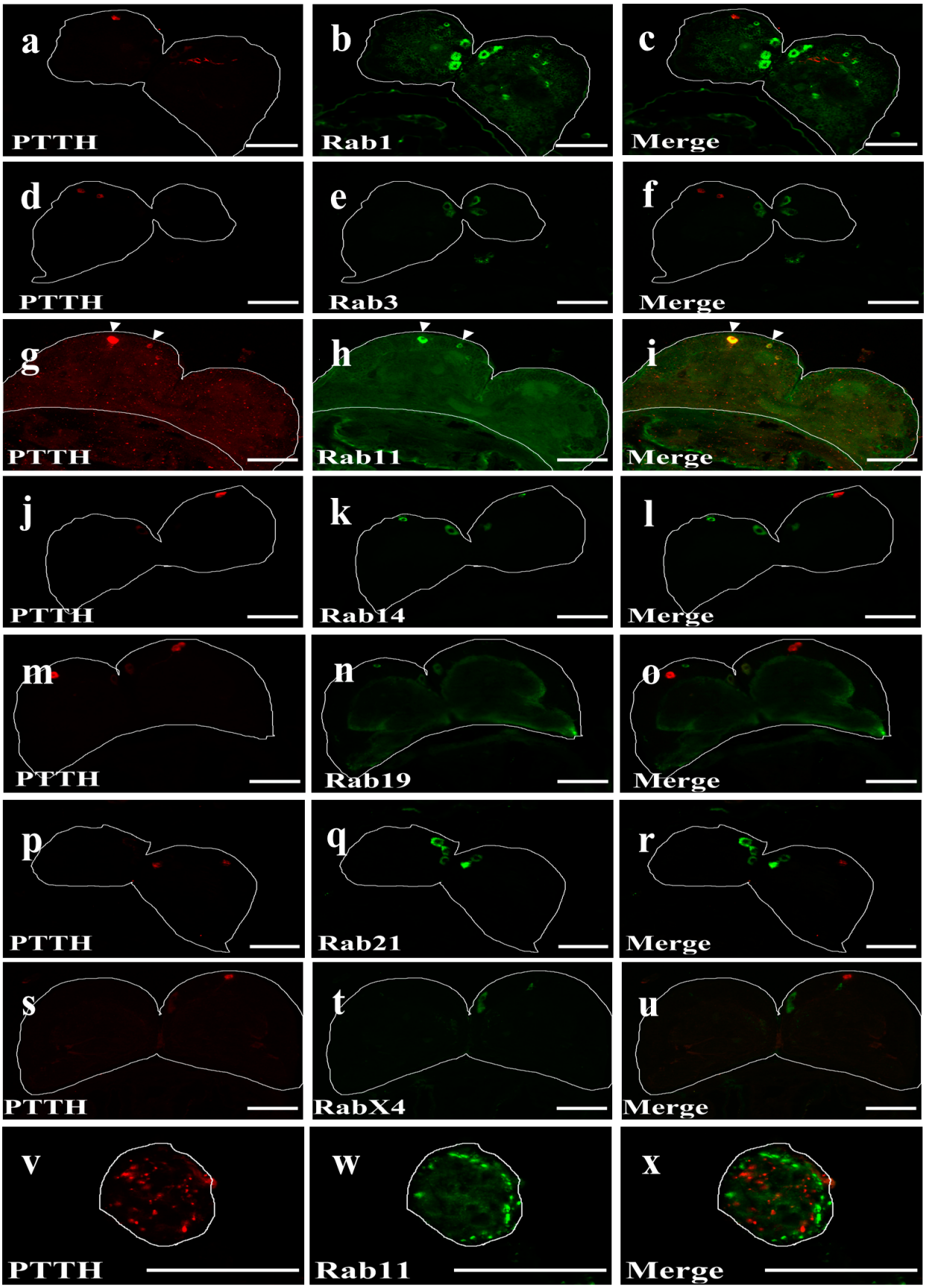

Fig. 5. Co-localization of Rab1-, Rab3-, Rab11-, Rab14-, Rab19-, Rab21-, RabX4-IRs with PTTH-IR in the brain (a–u) and CA (v–x) of *B. mori*. PTTH (a, d, g, j, m, p, s and v) was visualized using CF™555 (red fluorophore). Rab1 (b), Rab3 (e), Rab11 (h and w), Rab14 (k), Rab19 (n), Rab21 (q) and RabX4 (t) were visualized using CF™488A (green fluorophore). Rab11-IR overlapped with PTTH-IR (i). Rab1-, Rab3-, Rab14-, Rab19-, Rab21- and RabX4-IRs did not overlap with AT-IR (c, f, l, o, r and u). Rab11-IR in the CA did not overlap with PTTH-IR (x). Scale bar: 100 μm.

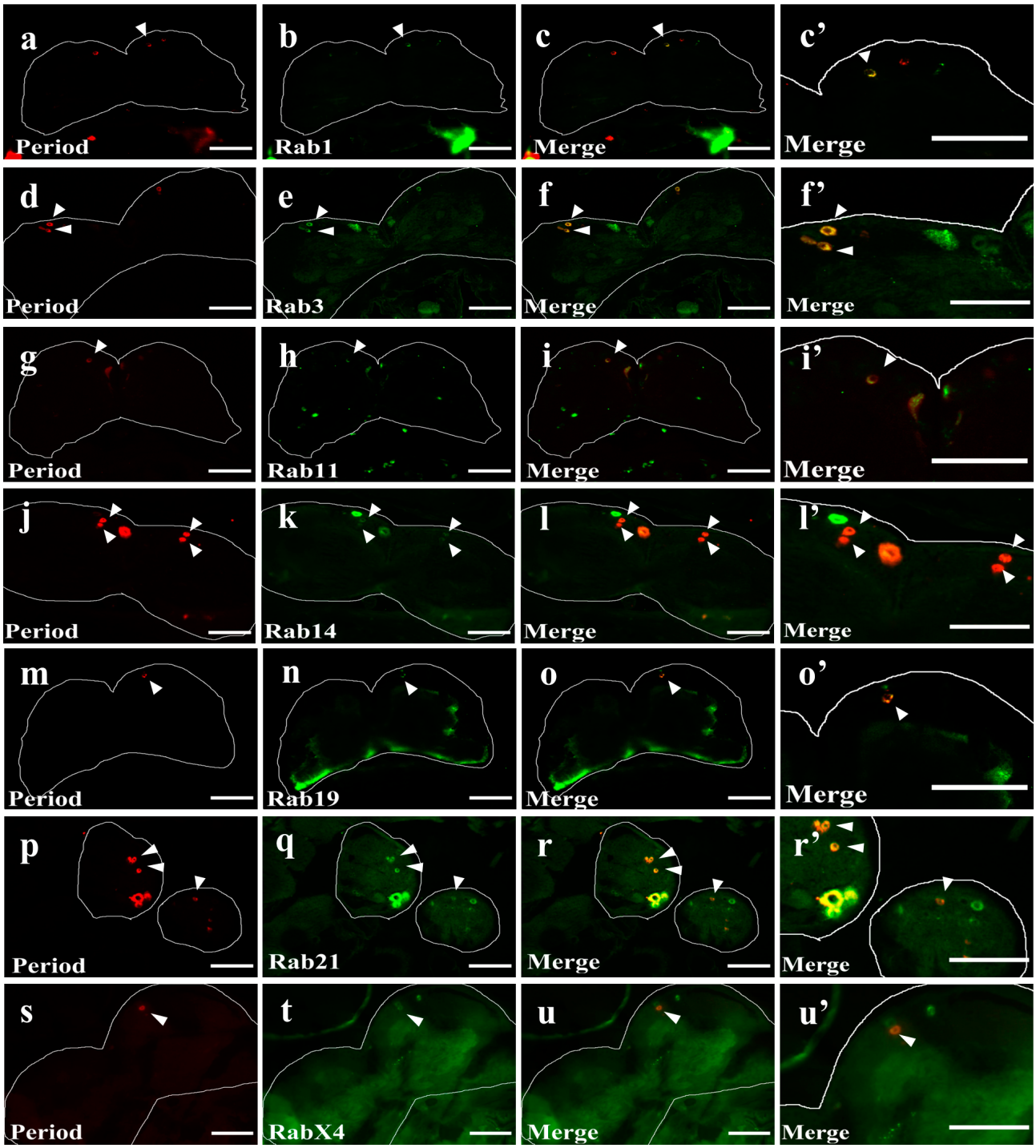

Fig. 6. Co-localization of Rab1-, Rab3-, Rab11-, Rab14-, Rab19-, Rab21- and RabX4-IRs with PER-IR in the brain of *B. mori*. PER (a, d, g, j, m, p and s) was visualized using CF™555 (red fluorophore). Rab1 (b), Rab3 (e), Rab11 (h), Rab14 (k), Rab19 (n), Rab21 (q) and RabX4 (t) were visualized using CF™488A (green fluorophore). Rab1-, Rab3-, Rab11-, Rab14-, Rab19-, Rab21-, and RabX4-IRs overlapped with PER-IR (c, c', f, f', i, i', l, l', o, o', r, r', u, and u'). Scale bar: 100 μm.

of Rab11, overlapped with PER-IR in the brain. Table1 shows the relationship between Rabs and neuropeptides and the specificity of the antibodies.

AT-IR is present in many neurons in the central nervous system and in two neurons in the FG in *B. mori* (Nagata et al., 2012). Rab6-, Rab11-, Rab14- and Rab21-IRs partially overlapped AT-IR in the brain (Fig. 1 and 2). AT-IR was detected in two cells in the FG (Fig. 3 and 4). Rab3-, Rab6-, Rab7-, Rab11-, Rab14- and Rab21-IRs overlapped AT-IR in the FG (Fig. 3 and 4). Rab6, Rab11, Rab14 and Rab21 in the brain may regulate the transport and secretion of AT.

Rab6 regulates transport in the Golgi apparatus and Rab11 the recycling and secretion of membrane proteins between the plasma membrane and endosomes. Rab6 and Rab11 may regulate the transport of AT in the brain and FG. Rab14 functions in Golgi/endosome transport process-

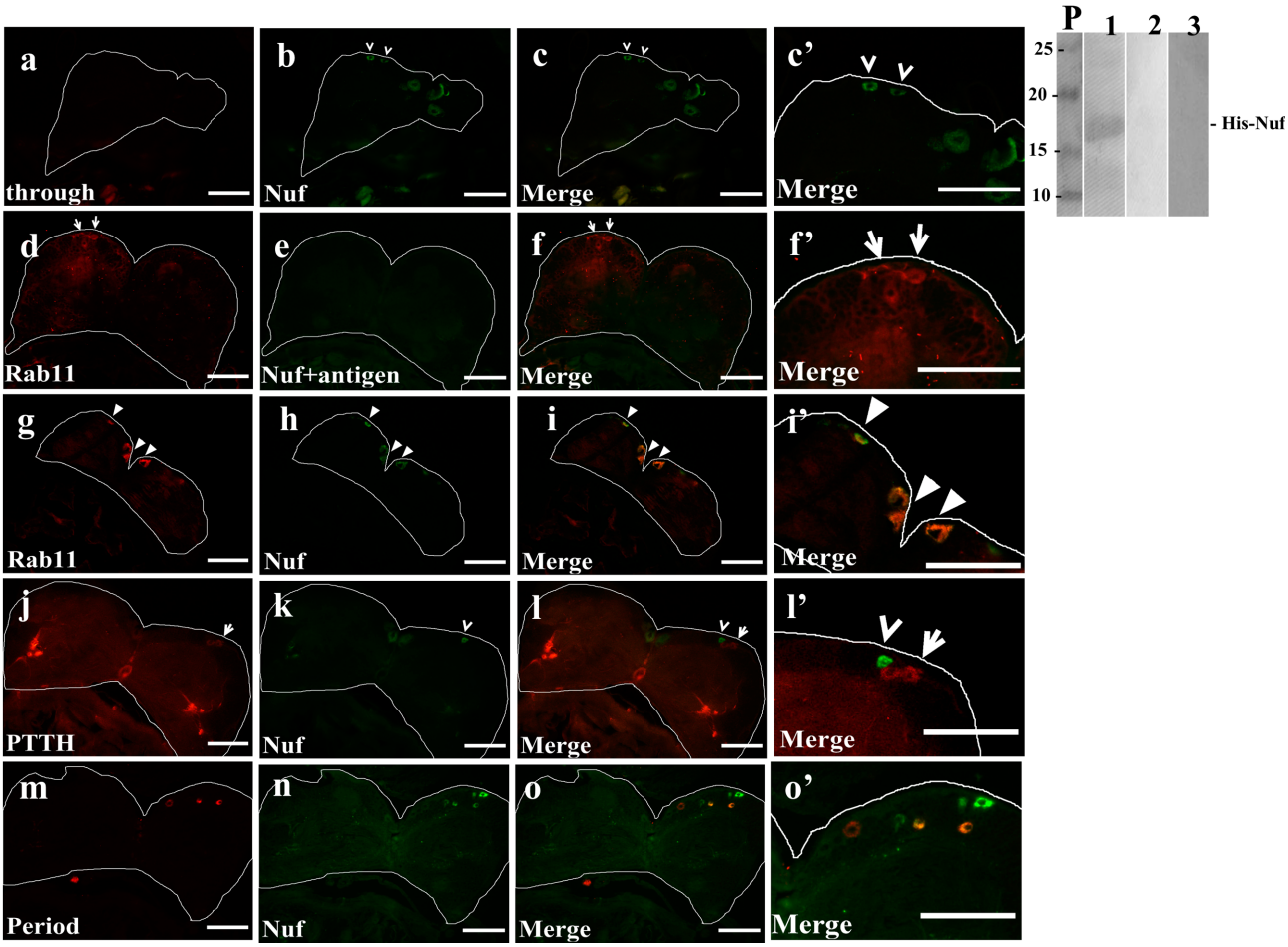

Fig. 7. Location and Immunoblot analysis of NUF in the brain of *B. mori*. Bleed-through fluorescence (a–c`) and Control (d–f`). Single stained samples were imaged through both filters (a–c`). Nuf-IR was visualized using CF555 (red fluorophore, a) and CF™ 488A (green fluorophore, b). Control (d–f`); (antibody + antigen, used as a primary antibody). Nuf-IR was visualized using CF™ 488A (green fluorophore, e). Rab11-IR was visualized using CF555 (red fluorophore, a). In both cases, no significant staining was observed (c, c`,f and f`). Rab11 (g), PTTH (j) and PER (m) were visualized using CF™555 (red fluorophore). NUF (h, k and n) was visualized using CF™488A (green fluorophore). NUF-IR colocalizes with Rab11-IR in neurons in the brain (i and i`), but not with PTTH-IR neurons in the brain (l and l`). Rab11-IR colocalized with PER-IR in neurons (o and o`). Scale bar: 100 μm. Panel p, lanes 1–3 were loaded with His-NUF. Lane 2 was probed with pre-immune serum as a negative control. Lane 3 contains an antibody-positive antigen as a positive primary antibody control.

es and Rab21 in endocytosis and early endosomal transport (Plutner et al., 1991; Junutula et al., 2004; Simpson et al., 2004; Grigoriev et al., 2007; Wang et al., 2011). In addition, *Drosophila* Rab21 and Rab14 are associated with autophagy. *Drosophila* Rab21 promotes endosomal sorting of VAMP8 required for autophagosome-lysosome fusion and Rab14 is necessary for autophagosome growth, positioning and fusion (Jean et al., 2015; Mauvezin et al., 2016). Rab21 and Rab14 may be involved in autophagy-related degradation of AT in the brain and FG. Multiple Rabs may regulate transport and secretion of AT in the brain and FG. Further study is necessary to determine whether Rab effector-IR is present in AT-IR cells and whether the inhibition of Rabs by dsRNA interference inhibits AT secretion.

Of the Rabs, only Rab11 was present in cells synthesizing PTTH in the DL (Fig. 5). Rab11 is involved in membrane transmission of recycling endosomes. Rab11 proteins are involved in a great variety of cellular transmission pathways, such as, the regulation of cell polarity, ciliogenesis, integrin recycling, neuritogenesis, oogenesis, receptor/adhesion protein recycling and cytokinesis (Simon & Prekeris, 2008; Jing & Prekeris, 2009; Eva et al., 2010; Knödler et al., 2010). As Rab11 is the only Rab present in PTTH-IR cells, PTTH secretion may be regulated by controlling vesicular membrane recycling. Rab11 may be involved downstream of the neurotransmitter serotonin because receptors for this neurotransmitter are also present in PTTH-IR cells (Wang et al., 2013).

NUF is a Rab11 effector protein that is important in controlling the transmission of several intracellular cargos (Riggs et al., 2007; Calero-Cuenca & Sotillos, 2018). NUF-IR cells did not colocalize with PTTH-IR (Fig. 7). Rab11 has multiple effectors (Dusty-like protein, Rip11) in addition to NUF (Nagaraj & Adler, 2012; Calero-Cuenca & Sotillos, 2018). These other effectors may be involved in PTTH secretion.

PTTH-IR was not present in Rab11-IR cells in the CA (Fig. 5). In the CA, another Rab may be involved in PTTH secretion. Alternatively, Rab may not be required at the site of PTTH secretion in the CA.

Except for Rab11-IR cells, Rab-IR was not present in PTTH-IR cells, but many Rab-IR cells in the DL were in close proximity to PTTH-IR cells (Fig. 5). Clock cells where PER is located are adjacent to PTTH synthesizing cells in DL. Rab1-, Rab3-, Rab11-, Rab14-, Rab19-, Rab21- and RabX4-IRs and NUF-IR overlapped with PER-IR in the brain (Figs 5 and 6).

Pigment dispersing factor (PDF), the only peptidergic hormone involved in circadian rhythms and a candidate transmitter of oscillations in behaviour and the clock proteins Timeless and Casein kinase, are located in these clock cells in DL (Iwai et al., 2008). In addition, synthesis and secretion of PTTH are regulated by circadian rhythms (Vafopoulou et al., 2007). Several Rabs in the clock cells in the brain may regulate the secretion and transport of PDF and circadian rhythm-related proteins.

To clarify the role of Rab in the secretion of neuropeptides, genetic methods, such as, direct introduction of dsRNA into the brain and gene editing need to be used to determine whether suppressing Rab reduces neuropeptide secretion or alters the biological clock. In addition, it is also necessary to determine whether effectors that specifically bind to Rab are located in the same cells in the brain.

CONFLICT OF INTERESTS. The authors declare that there are no conflict of interests.

ACKNOWLEDGEMENTS. We thank L. Benyon, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript. This work was supported by the Hyogo science and technology association.

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Received November 23, 2022; revised and accepted March 14, 2023
Published online March 29, 2023