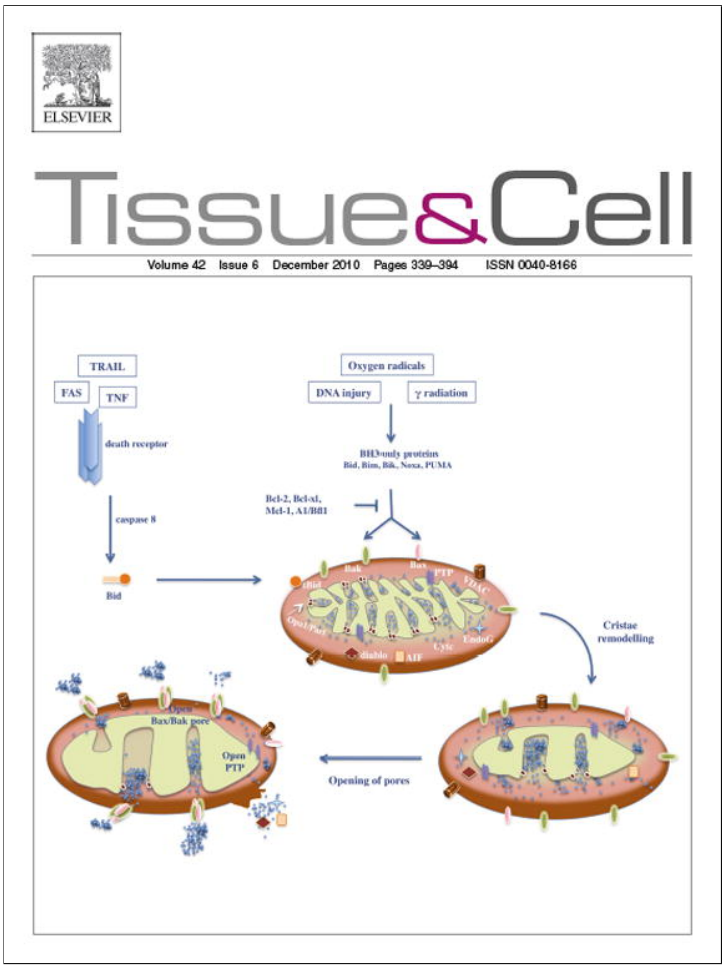
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Tissue and Cell 42 (2010) 355–359

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Allatotropin expression during the development of the fourth instar larvae of the kissing-bug *Triatoma infestans* (Klüg)

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ARTICLE INFO

Article history:
Received 24 March 2010
Received in revised form 13 July 2010
Accepted 20 July 2010

Keywords:
Corpora allata
Triatoma infestans
Nephrocytes
Neuropeptides
Midgut
Aorta

ABSTRACT

Allatotropin (AT) is a neuropeptide originally isolated from the brain of *Manduca sexta* and then characterized in several insect species. It acts as a neurohormone, as well as a neuromodulator. While it was primarily characterized on the basis of its ability to stimulate the secretion of juvenile hormones, it was also found that it acts as a cardioaccelerator and myostimulator. The presence of AT in IV instar larvae of *T. infestans* was previously described at the level of the Malpighian tubules. In the present study we report the presence of the peptide at the level of the brain, retrocerebral complex, as well as in the anterior midgut and aorta. The presence of AT at the corpora allata suggests that the peptide is acting on the gland during the first days of the moulting cycle. Neural processes at the level of the aorta and the anterior midgut suggests that, like in adults, the hormone is acting as a cardioaccelerator and myostimulator. The peptide was also found in open-type cells of the midgut. Finally the presence of allatotropic neurons in the optical lobe of the brain suggests that as in other species, the peptide is related with the control of circadian rhythms.

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1. Introduction

Allatotropin (AT) is a neuropeptide originally isolated from the brain of the lepidoptera *Manduca sexta* (Kataoka et al., 1989) and then characterized in several insect species (Abdel-latief et al., 2003; Park et al., 2002; Scheng et al., 2007; Truesdell et al., 2000; Veenstra and Costes, 1999; Yin et al., 2005). It acts as a neurohormone, as well as a neuromodulator. Furthermore, it was recently proposed that this peptide acts in an endocrine as well as in a paracrine way, being secreted by cells of the Malpighian tubules and open-type endocrine cells in the epithelial sheet of the anterior midgut of the Chagas disease vector *Triatoma infestans* (Santini and Ronderos, 2007, 2009a,b; Sterkel et al., 2010). While it was primarily characterized on the basis of its ability to stimulate the secretion of juvenile hormone (JHs) by the corpora allata (CA) in several insect species (Duve et al., 2003; Kataoka et al., 1989; Li et al., 2003; Rachinsky et al., 2003; Weaver and Audsley, 2009), it was also found that this peptide has other relevant physiological functions, acting as a cardioacceleratory (Koladich et al., 2002; Sterkel et al., 2010; Veenstra et al., 1994), and myostimulatory peptide in different species (Duve et al., 1999, 2000; Matthews et al., 2007; Rudwall et al., 2000; Santini and Ronderos, 2007; Sterkel et al., 2010). Furthermore, it was also proposed that AT is involved in the regulation of photic entrainment (Hofer and Homberg, 2006; Homberg et al., 2003; Petri et al., 2002; Würden and Homberg, 1995), ion exchange regulation (Lee et al., 1998) and more recently, in the up-regulation of the secretion of digestive enzymes (Lwalaba et al., 2009). Despite that AT was isolated in insects, members of this family of peptides have also been found in other invertebrate phyla (Elekonich and Horodyski, 2003). While the presence of AT in IV instar nymphs of *T. infestans* was previously described at the level of the Malpighian tubules, in the present study we report the presence of an AT-like peptide at the level of the brain, retrocerebral complex, as well as in the anterior midgut and aorta of the IV instar nymphs of the kissing-bug *T. infestans*.

2. Material and methods

2.1. Insects

T. infestans IV instar insects were obtained from an artificial colony maintained at 28 ± 2 °C, 45% relative humidity and a 12:12 h light–dark period. Insects reaching the 4th instar after only one blood meal in the previous instar were isolated and starved during 21 days before a new blood meal was offered. Only those insects fed *ad libitum* were selected for analysis. Under these conditions,

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doi:10.1016/j.tice.2010.07.011

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the duration of the 4th instar is 13 ± 1 days, reaching apolysis on day 10 (Ronderos, 2009). Insects were fed on live chickens during the light period and sacrificed on different days along the moulting cycle. The corpora allata (CA), together with the brain, the corpora cardiaca (CC) and the surrounding organs (aorta and digestive tube), were dissected under binocular microscope and fixed in formaldehyde–phosphate buffered saline (PBS) (4%) at 4 °C for 12 h. For conventional histological analysis (hematoxylin and eosin and conventional epifluorescence staining), tissues were fixed in formaldehyde–phosphate buffered saline (4%) at 4 °C for 12 h, dehydrated and embedded in paraffin.

2.2. AT-like immunoreactivity in the brain, retrocerebral complex and adjacent organs

After fixation process, tissues were washed in PBS-Tween (0.05%) (PBS-T), permeabilized with Triton (1%) (24 h at 4 °C) and then blocked with 3% bovine serum albumin (BSA) for 2 h. Then the tissue was incubated overnight at 4 °C with a polyclonal antiserum against *Aedes aegypti* AT (Hernandez-Martinez et al., 2005) (1/100 in 3% BSA diluted in PBS-T) which previously proved to be specific in *T. infestans* (Santini and Ronderos, 2007, 2009a). Finally, tissues were incubated with FITC-labelled (laser scan confocal slides) or Cy3-labelled goat anti-rabbit secondary antibody (conventional epifluorescence) (1/200 in blocking-buffer) for 3 h at room temperature. After every step, tissue was washed (3 times × 20 min) with PBS-T (0.05%). As we referred above, primary antibody specificity was previously assayed both for immunohistochemistry, as well as in the whole animal. For secondary antibodies control, primary antibody incubation was replaced with PBS. Both samples, with or without primary antibody incubation, were observed under the same conditions (laser intensity, pinhole and detector gain). When primary antibody was omitted no labelling was evident, showing that the secondary antibody does not cross-react with insect tissues. Finally all the samples were mounted in a media containing DAPI (4′,6-diamidino-2-phenylindole). The resulting material was analyzed with a Laser Scan Microscope Zeiss LSM 510 Meta.

3. Results

3.1. Presence of the AT-like peptide in the retrocerebral complex

Fig. 1a shows the anatomical relationship between CA and CC. The aorta and part of the serosa covering the gland is absent, showing the microanatomy of the CA. Fig. 1b shows the presence of immunoreactivity at the level of the CA, and its anatomical relationship with the aorta at the level of the head. The presence of the nerve corpora allata, joining the gland with the CC is also evident. The presence of a group of immunoreactive neurosecretory cells forming a neuropile located next to the posteromedial region of the protocerebrum is evident. These neurons send its axons through the nervi corpora cardiaci I (NCCI) to the CC (Fig. 1d).

Fig. 2a shows the continuity between CC and CA and the presence of AT immunoreactivity in both glands. Fig. 2b is a conventional histological slide stained with hematoxylin and eosin showing the existence of two different cell populations in the CA. One of them, a group of peripheral cells which do not stain deeply (arrow), and the second one forming the main portion of the parenchyma of the gland more deeply stained, corresponding to the juvenile hormone secreting cells. Immunohistological analysis of the CA shows the existence of immunoreactive cells also in the gland. These cells are mainly distributed in the periphery of the gland, and present a granular pattern in their cytoplasm (Fig. 2c and d). A confocal 3D projection (Fig. 2e) and a detailed confocal slide (Fig. 2f) of a CA belonging to an insect 4 days after blood ingestion shows that these cells, expressing AT-like immunoreactivity, have their nuclei located beneath the serosa which cover the gland, projecting their cytoplasm into the gland between the juvenile hormone producing cells.

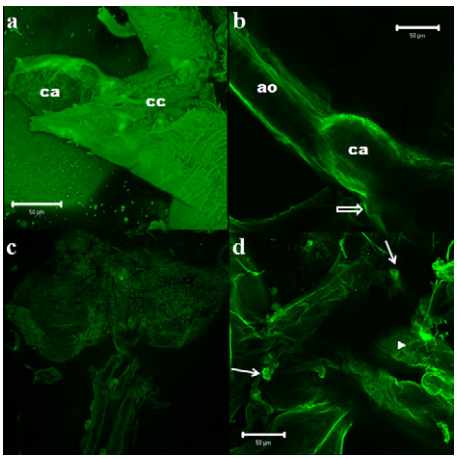


Fig. 1. AT-like expression in the retrocerebral complex of IV instar nymphs of *T. infestans*. (a) 3D reconstruction of the retrocerebral complex showing the anatomical relationship between corpora allata (ca) y corpora cardiaca (cc) in an insect four days after blood meal. (b) Confocal image showing the anatomical relationship between corpora allata and aorta in an insect 8 days after blood ingestion. Note the presence of AT immunoreactivity at the level of the gland and also of the corpora cardiaca (open arrow). (c) Panoramic view of the protocerebrum and retrocerebral complex in a control preparation where primary antibody was omitted. The detector gain was increased respect to positive preparations (from 544 to 843) to increment the background and make more visible the tissue. (d) Panoramic and detailed views of part of the protocerebrum and retrocerebral complex showing the presence of neurons immunoreactives for the AT antibody (arrows) and a neuropile associated with the nervi corpora cardiaci I (NCCI) (arrow head). ao: aorta; ca: corpora allata; cc: corpora cardiaca.

Fig. 3 shows the CA and surrounding organs pertaining to individuals in three different moments of the moulting cycle. The images acquired under similar conditions show different relative

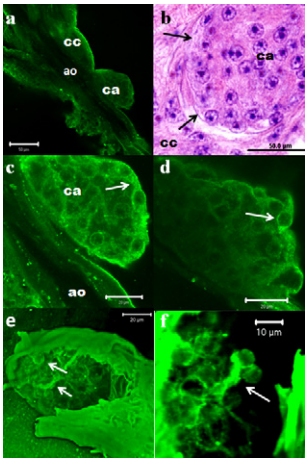


Fig. 2. AT-like expression in the CA. (a) Panoramic view of the retrocerebral complex showing the presence of AT immunoreactivity at the level of the CA. (b) Histological section of the CA (paraffin embedded slide stained with hematoxylin and eosin) showing the existence of two different cell populations in the CA of the IV instar larvae of *T. infestans*. One of them located in the periphery (arrow) with a pale stained cytoplasm and a second cell population deeply stained corresponding to the cells producing JHs. (c and d) Immunoreactive cells in the periphery of the gland in non-fed insects. (e) 3D confocal reconstruction of a CA pertaining to an insect 4 days after blood ingestion. The serosa covering the CA is not present in part of the gland showing the existence of AT immunoreactive cells in the periphery and cytoplasmic processes going inside the gland. (f) Detailed view of the gland showing two cells with a principal cytoplasmic projection and derivates going around of the JHs producing cells. ca: corpora allata; cc: corpora cardiaca; ao: aorta.

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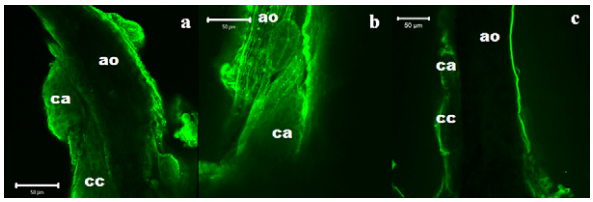


Fig. 3. CA gland at three different time points along the moulting cycle. (a) Non-fed insect. (b and c) 7 and 12 days after blood meal respectively. The confocal images were obtained under similar conditions. Note the differences in the intensity of the presence of the peptide, and also the size of the gland. ca: corpora allata; cc: corpora cardiaca; ao: aorta.

sizes and intensity of immunoreactive. Fig. 3a with intense AT-like expression, shows the CA of a non-fed insect, while Figs. 3b and c show glands with low levels of immunoreactivity, corresponding to insects 7 and 12 days after blood intake (middle of the molting cycle and after de apolysis, next to the time of ecdysis respectively).

3.2. AT-like expression in the aorta, anterior midgut and optical lobe

Fig. 4 shows the presence of AT-like immunoreactivity at the level of the aorta and anterior midgut. In Fig. 4a, the presence of neural processes expressing AT-like, reaching the wall of the cephalic region of the aorta and the wall of the anterior midgut is evident. Fig. 4b shows the external surface of the midgut wall with higher magnification with immunoreactive neural processes at the level of the wall, showing also immunoreactivity associated with muscular fibers. Figs. 4c and d show the existence of epithelial cells expressing the AT-like peptide at the level of the first portion of the midgut. Interestingly, pericardial cells associated to the external surface of the wall of the aorta also show the presence of the peptide (Fig. 5a–c). The cellular distribution of the immunoreactivity shows the accumulation of granules at the level of the cellular membrane, and also surrounding the nucleus (Fig. 5b and c).

At the level of the brain, the presence of neurons expressing the AT-like peptide in the optic lobe is evident (Fig. 5d). The distribution of the immunoreactivity shows the presence of the neuronal somas in the periphery of the lobe, with their axons running downward deeply in the lobe.

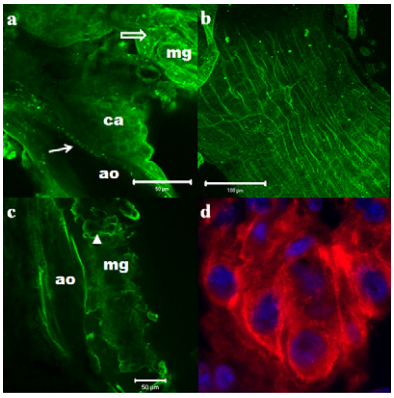


Fig. 4. Presence of AT in the aorta and anterior midgut. (a) Immunoreactive processes running across the wall of the aorta (arrow) and the anterior midgut (open arrow). (b) Detailed view of the wall of the anterior midgut showing AT immunoreactivity in neuronal processes and also in muscle fibers. (c and d) Panoramic and detailed view of the epithelial sheet of the anterior midgut showing the presence of open-type cells producing AT-like peptide (arrow heads in c). ao: aorta; ca: corpora allata; mg: midgut.

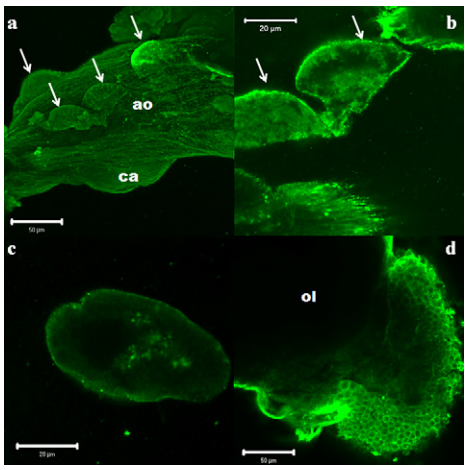


Fig. 5. AT-like expression in pericardial cells and optical lobe of the brain. (a) 3D confocal reconstruction of the aorta showing the presence of pericardial cells closely related to the wall (arrows). (b) Image with a higher magnification showing two pericardial cells (arrows) containing immunoreactive granules at the level of the surface of the cell. (c) Confocal slide of a pericardial cell showing the presence of granules in the nuclei area and across the cytoplasm. (d) View of the brain at the level of the optical lobe showing the presence of several allatotropic neuronal somas and their axons. ao: aorta; ol: optical lobe.

4. Discussion

AT is a peptide originally isolated from the central nervous system of the Lepidopteran *M. sexta*. Then, this neuropeptide was isolated and characterized in several insect species and even in invertebrate phyla other than arthropoda (Elekonich and Horodyski, 2003). Despite the first biological characterization was based on its ability to stimulate the secretion of JHs from the gland CA, AT prove to be pleiotropic acting as a myostimulator, cardioaccelerator and even regulating ion exchange and the activity of digestive enzymes in different insect orders (Duve et al., 1999; Koladich et al., 2002; Lee et al., 1998; Lwalaba et al., 2009; Matthews et al., 2007; Rudwall et al., 2000; Santini and Ronderos, 2007; Sterkel et al., 2010; Veenstra et al., 1994).

In Hemiptera, AT has proved to act as a myostimulator at the level of the anterior and posterior midgut (Santini and Ronderos, 2007; Sterkel et al., 2010) and also as a cardioaccelerator (Sterkel et al., 2010). While the basic function of CA in insect growth and reproduction was formerly analyzed in triatominae insects (Wigglesworth, 1934, 1936, 1948), information about JHs synthesis and the mechanisms of control of the CA activity by allatotropic, as well as allatostatic peptides were not deeply analyzed. Regarding JHs titres, the information is scarce with the only exception of the paper of Baehr et al. (1978). With respect to allatostatic activity, only one of the three families of allatostatins was analyzed in triatominae, showing no anatomical or physiological relationship with the CA (Sarkar et al., 2003). Regarding allatotropic peptides, this study provides the first information about the relationship between AT and CA function in Hemiptera. The presence of a neuropile containing AT-like neurons in the posterior region of the protocerebrum, and also the presence of immunoreactivity at the level of the CC and even in the CA suggest that AT is acting on the gland.

The analysis of the histology of the CA during the development of the fourth instar nymph of *T. infestans* suggests that the gland develops its activity mainly between days 3 and 8 after blood meal, having a first peak of activity between days 3 and 5. The period of activity of the gland finishes on day 8, previous to the occurrence of the apolysis on day 10 (Ronderos, 2009). Furthermore the application of Precocene II (PII) (which induces the atrophy of the CA)

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(Bowers and Martinez-Pardo, 1977; Pratt and Bowers, 1977), previously or in different days after blood intake, was effective only between days 0 (non-fed insects) and 5. Insects treated with PII on days 6 and 7 did not undergo alterations in growth suggesting that the first peak of activity of the gland (day 4) is the moment in which the pattern of the moult is defined (Ronderos, 2009).

With regards to the AT immunoreactivity in the CA, the results present in this study show that the gland is stimulated during the first days of the cycle, when the intensity of the stain in the gland is highest, decreasing by days 7–8, and being almost indistinguishable on day 12 after blood intake. Interestingly allatotropic cells are not only present at the level of the brain, but also we found the presence of immunoreactive cells inside the CA. These cells expressing AT-like immunoreactivity, are present in the periphery of the gland, and are easily distinguished in classical preparations with hematoxylin and eosin showing a less stained cytoplasm which is projected into the gland. This appearance is congruent with the AT-like peptide expressing cells observed during the first days of the cycle, which project their cytoplasm into the gland, and around the JHs secreting cells. Indeed, in the pioneer study by Wigglesworth (1934), regarding the endocrine function of the CA, the author shows that only cells located in the central part of the gland undergo changes associated with secretory activity, suggesting the existence of more than one cell population in the gland.

Regarding the presence of neural processes expressing the AT-like peptide at the level of the cephalic aorta and the anterior midgut, our laboratory have recently shown that AT is cardioacceleratory and myostimulatory in the adults of *T. infestans*, facilitating the increment of the circulation of the haemolymph during postprandial diuresis (Sterkel et al., 2010). Furthermore, as we have found now in IV instar nymphs, we have previously shown the presence of AT producer open-type epithelial cells also in the midgut of the adults of *T. infestans* which could act in a paracrine way modulating peristaltic contractions of the anterior midgut. The presence of AT at this level could possibly also be related with a myostimulatory activity in juvenile forms of this kissing-bug (Sterkel et al., 2010).

Nephrocytes constitute a cell population closely associated with the wall of the aorta. In triatominae, this population involves two different groups, one of them associated with the external surface of the aorta (pericardial cells), and the other ones located in the inner surface (endocardial cells) (Chiang et al., 1990). It has been proposed that nephrocytes are involved in the filtration of the haemolymph. Furthermore they share anatomical, molecular and functional similarities with podocyte cells present in the vertebrate kidney, which also forms a selective filtration barrier (Weavers et al., 2009). Interestingly, our results show the presence of AT-like immunoreactivity in these cells. The presence of the peptide was evident in the pericardial cells, but not in the endocardial ones. The presence of immunoreactive granules, grouped in the periphery of the cells and also next to the nuclei suggests the secretory nature of the peptide. Regarding the probable function, it was proposed that AT is involved in the regulation of ion exchange in the midgut of the larvae of *M. sexta* (Lee et al., 1998). Taking into account the relevance of the nephrocytes in the regulation of the haemolymph composition, it could be probable that the peptide was involved in the ion exchange regulation also at this level.

The presence of AT associated to the optical lobe has been previously described in other insect species such as the cockroach *Leucophaea maderae* (Hofer and Homberg, 2006; Homberg et al., 2003) and the locust *Schistocerca gregaria* (Würden and Homberg, 1995). Indeed, the probable function of AT in the regulation of the central circadian pace-maker was proposed (Petri et al., 2002). Although than more experiments are necessary, the presence of the AT-like peptide in the optical lobe of *T. infestans* could be related with mechanisms of circadian rhythm regulation in triatominae.

In conclusion, our results confirm the previously reported association of AT with the aorta and midgut in the adult stage of *T. infestans*, and provides information showing, for the first time in Hemiptera that, as in other insect orders, AT is related with the regulation of the CA function and the secretion of JHs.

Acknowledgement

The authors wish to thank Dr. Fernando G. Noriega (Florida International University Florida, USA) for generously supplying us with allatotropin and allatotropin-antibody.

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