Expression of a neuropeptide similar to allatotropin in free living turbellaria (platyhelminthes)

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A B S T R A C T

Mechanisms coordinating cell–cell interaction have appeared early in evolution. Allatotropin (AT), a neuropeptide isolated based on its ability to stimulate the synthesis of juvenile hormones (JHs) in insects has also been found in other invertebrate phyla. Despite this function, AT has proved to be myotropic. In the present study we analyze its expression in two groups of Turbellaria (Catenulida, Macrostomida), and its probable relationship with muscle tissue. The results show the presence of an AT-like peptide in the free living turbellaria analyzed. The analysis of the expression of the peptide together with phalloidin, suggests a functional relationship between the peptide and muscle tissue, showing that it could be acting as a myoregulator. The finding of immunoreactive fibers associated with sensory organs like ciliated pits in Catenulida and eyes in Macrostomida makes probable that AT could play a role in the physiological mechanisms controlling circadian activities. Furthermore, the existence of AT in several phyla of Protostomata suggests that this peptide could be a synapomorphic feature of this group. Indeed, the presence in organisms that do not undergo metamorphosis, could be signaling that it was first involved in myotropic activities, being the stimulation of the synthesis of JHs a secondary function acquired by the phylum Arthropoda.

1. Introduction

Cell–cell integration is a basic feature to coordinate functions in all living organisms. Then, mechanisms coordinating functions locally as well as systemically are likely to have appeared early in evolution. This is achieved by mean of different ways (e.g. paracrine, endocrine, synaptic) but always involves chemical messengers including molecules with different chemical natures as mineral ions, lipids, aminoacids and peptides. Even in protists (Le Roith et al., 1980), sponges (Lentz, 1966; Weyrer et al., 1999) and cnidarians (Grimmelikhuijzen et al., 1991, 1996, 2002; Hansen et al., 2002), the existence of cellular mediators was documented, and several native neuropeptides were isolated and characterized.

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In Turbellaria, neuropeptides seem to be particularly important as myoregulators, controlling the activity of muscles at the level of both digestive and reproductive organs (Gustafsson et al., 2002). In fact, several neuropeptides have been detected and characterized in Plathyelminthes and Acoela (Gustafsson et al., 2002; Johnston et al., 1995, 1996; Kreshchenko, 2008; Kreshchenko et al., 2008; McVeigh et al., 2009; Mousley et al., 2004; Reuter et al., 1998, 2001; Wikgren and Reuter, 1985).

Allatotropin (AT), a neuropeptide originally isolated from the brain of the Lepidoptera *Manduca sexta* based on its ability to stimulate the synthesis of juvenile hormones (JHs) (Kataoka et al., 1989), has also been isolated and characterized in several other insect species (Abdel-latief et al., 2003; Paemen et al., 1991; Park et al., 2002; Scheng et al., 2007; Truesdell et al., 2000; Veenstra and Costes, 1999) and even in some other invertebrate groups (Elekonich and Horodyski, 2003; Veenstra, 2010), both in nervous as well as in epithelial tissues (Riccillo and Ronderos, 2010; Santini and Ronderos, 2009a, 2009b; Sterkel et al., 2010). Despite the function as a JHs regulator, as other many peptides, AT has proved to be pleiotropic acting as a cardioaccelerator (Koladich et al., 2002; Rudwall et al., 2000; Sterkel et al., 2010; Veenstra et al., 1994), inhibiting ion transport throughout epithelia (Lee et al., 1998), and also acting as a myoestimulator at the level of the digestive system in several insect species (Duve et al., 1999, 2000; Matthews et al., 2007; Santini and Ronderos, 2007; Sterkel et al., 2010). Indeed, a new activity controlling the release of digestive enzymes was recently described in insects (Lwalaba et al., 2009).

On the basis of the multiplicity of functions described for this peptide, including a so specific one for insects (JHs synthesis regulation), and the probability of that this function could not be primitive, we decide to analyze the expression of AT in two groups of Turbellaria (Catenulida, Macrostomida), and its probable functional relationship with muscle tissues.

2. Materials and methods

2.1. Animals

Specimens of free-living turbellarian *Macrostomum* sp. (Macrostomida) were collected at a rainwater pond in Pereyra Iraola Park (34°51′S; 58°03′W), while specimens of *Stenostomum* sp. (Catenulida) were collected at another rainwater pond at Berisso (34°57′S; 57°48′W), both localities situated near to the city of La Plata (Buenos Aires Province, Argentina). The specimens were obtained during

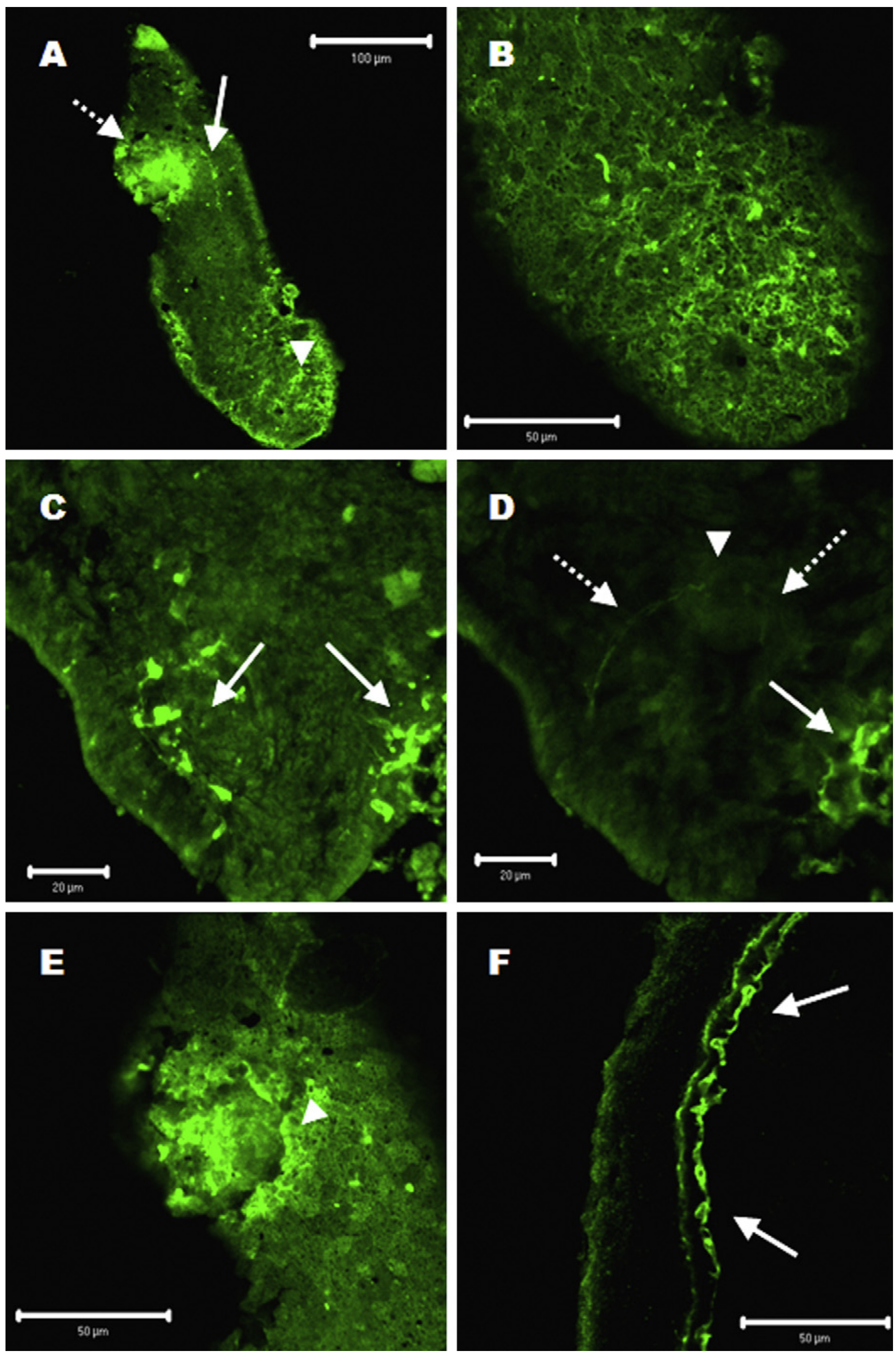

Fig. 1. Confocal images illustrating the general distribution of the AT-like peptide in *Stenostomum* sp. (A) Panoramic view showing AT immunoreactivity at the level of the pharynx (dashed arrow), the lateral longitudinal cord (arrow) and the posterior plexus (arrow head). (B) Detail of the posterior region of the same individual, showing the existence of the AT-like peptide in the posterior (caudal) nervous plexus. (C) Confocal 3D reconstruction of the anterior region of *Stenostomum* sp. (dorsal view) showing the presence of immunoreactive neurons at the level of the ciliated pits (arrows). (D) Optical section at the same level showing one of the ciliated pits (arrow) and axonal fibers (dashed arrows) running from this area to reach the pharynx (arrow head). (E) Lateral view of the anterior area showing the presence of immunoreactive cells associated with the everted pharynx (arrow head). (F) View of the lateral cord showing neurons expressing the AT-like peptide (arrows).

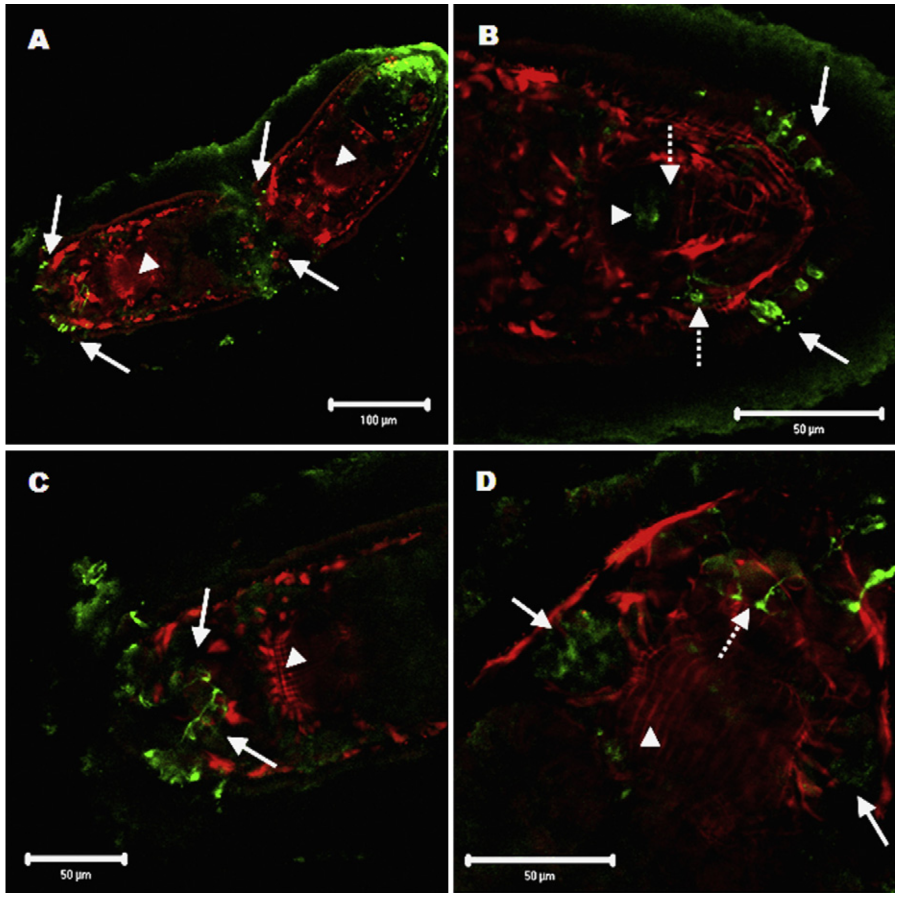

Fig. 2. Presence of the AT-like peptide in *Stenostomum* sp. and its association with the muscle system. Images were obtained by double labeling with the antiserum anti-AT (green) and phalloidin (red). (A) Panoramic view of a single individual showing two zooids. The presence of AT immunoreactive neurons at the level of the anterior region (ciliated pits) in both zooids is evident (arrows). The arrow heads show the pharynx. (B) Detailed view of the region of the head of other individual showing the neurons at the ciliated pits (arrows) establishing synaptic connections with a second level of neurons (dashed arrows) which axons reaching the pharyngeal ring (arrow head). (C) Similar view of another individual showing neurons (with a probable sensory nature), reaching the neuropile at the level of the brain (arrow) before the pharynx (arrow head). (D) Optical section showing in other specimen analyzed, the same two set of neurons associated with the ciliated pits approaching each other at the level of the brain neuropile (dashed arrow), and a cluster of immunoreactive cells (arrows), on each side of the pharynx (arrow head). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

summer and autumn 2010 and were maintained in the water of origin under a 12:12 h light/dark period until they were processed. All individuals were fixed during the light phase of the cycle.

2.2. Immunohistochemistry and phalloidin labeling

Flatworms were isolated, and fixed in formaldehyde-phosphate buffered saline (PBS) (4%) at 4 °C for 12 h. Worms were then washed in PBS-Tween (0.05%) (PBS-T), permeabilized in Triton X-100 (1%) (24 h at 4 °C) and blocked with 3% bovine serum albumin (BSA) for 2 h. The flatworms were then incubated overnight at 4 °C with a polyclonal antiserum developed against AT of the mosquito *Aedes aegypti* (1/500 in 3% BSA diluted in PBS-T). The antibody recognizes the following sequence of amino-acids of the *A. aegypti* AT: APFRNSEMMTARGF. The specificity of binding of this antibody to *A. aegypti* AT was previously demonstrated by Hernandez-Martinez et al. (2005) and Santini and Ronderos (2007, 2009b). Worms were then incubated with FITC-labeled goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) (1/1000 in blocking-buffer) for 24 h at 4 °C. To visualize the arrangement of the muscle fibers, and the probable interaction of the neuropeptide with them, samples were co-incubated with a rhodamine-phalloidin solution (Sigma–Aldrich) (1/1000). After every step, flatworms were washed (3 times × 20 min) with PBS-T (0.05%). For secondary antibody control, primary antibody incubation was replaced with PBS. To assay primary antibody specificity, polyclonal antiserum was previously incubated over-night at 4 °C with pure AT as previously described (20 nmol/ml of diluted antiserum (1/500)) (Santini and Ronderos, 2007, 2009b). Finally, worms were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The resulting material was analyzed with a Laser Scan Microscope Zeiss LSM 510 Meta.

3. Results

The analysis reveals the presence of immunoreactive cells in both groups of free living flatworms under study: Catenulida (*Stenostomum* sp.) and Macrostomida (*Macrostomum* sp.). The omission of the primary antibody against *A. aegypti* AT abolished the labeling, and the preadsorption of the primary antibody with the immunizing peptide clearly diminished the staining (data not shown).

3.1. Catenulida

The analysis of *Stenostomum* sp. showed the existence of different kind of cells expressing the AT-like peptide, being evident in all the regions of the body in all the individuals analyzed (Fig. 1A–F). A detailed analysis of the worms at the level of the anterior region showed the presence of the peptide associated both, with the

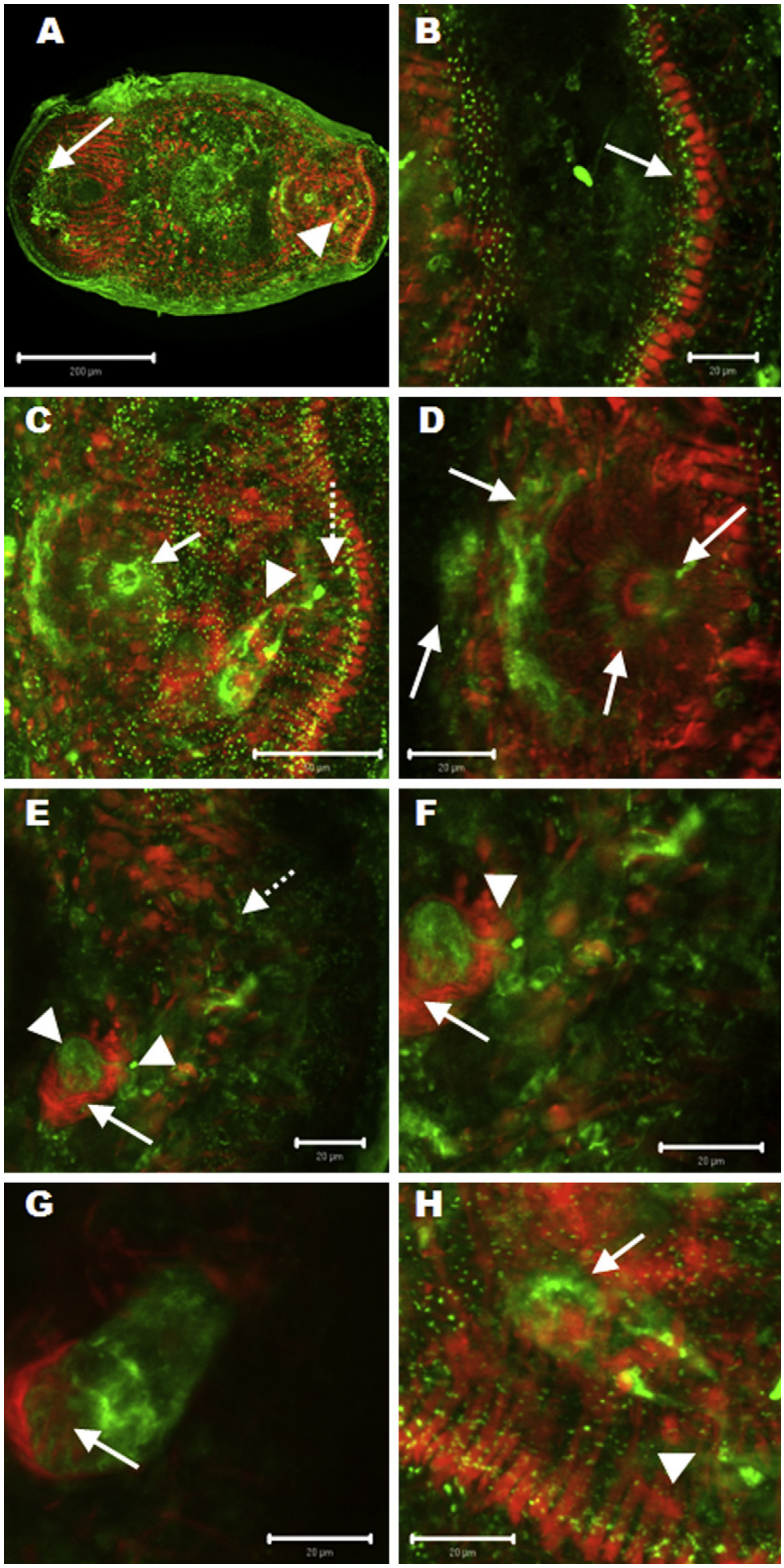

Fig. 3. Presence of the AT-like peptide (green) in *Macrostomum* sp. double labeled with phalloidin to view muscle tissue arrangement (red). (A) Panoramic view of a whole individual showing the presence of immunolabeling next to the pharynx corresponding with the cerebral region at the anterior end (arrow), the reproductive system and wall muscles at the caudal end (arrow head). (B) Panoramic view of the posterior end showing the muscle arrangement at the caudal adhesive plate and its association with AT-like immunoreactive fibers (arrow). (C) Optical section of the same region at a deeper level showing the female gonopore (arrow); the male reproductive system (arrow head) and the fibers innervating the caudal adhesive plate (dashed arrow). (D) Detailed view of the female gonopore showing the relationship between the muscle rings and immunoreactive neuronal cells (arrows). (E–F) Detailed views of the male reproductive system showing the muscular wall of the seminal vesicle (arrows) and the prostatic

pharynx and also with the ciliated pits, where immunoreactive cell were found (Fig. 1C–E). Furthermore, the presence of the peptide in neurons pertaining to the lateral cords was evident along the body (Fig. 1A and F). The images also show the existence of a subepidermal plexus covering all the body from the anterior to the posterior end (Fig. 1B). As we described above, immunoreactive neurons were found in the ciliated pits, associated with the brain, on each side of the anterior region (Fig. 1C and D). Interestingly, some immunoreactive fibers running from these organs, reach the pharynx (Fig. 1D). Also, in a lateral view of one of the individuals analyzed which had the pharynx everted, a network of associated cells expressing the peptide was evident (Fig. 1E).

Fig. 2 shows the relationships between cells expressing the AT-like peptide with muscle fibers in *Stenostomum* sp. Furthermore, as we described above, in Fig. 2A a panoramic view of an individual with two zooids revealed the presence of immunoreactive cells at the level of the ciliated pits. These neurons are present in both zooids, being evident also in the developing zooid, just at the split zone (Fig. 2A). Confocal images of different individuals, either with pharyngeal muscle fibers contracted or not, show that the intraepidermal neurons located at the ciliated pits establish nerve connections at different levels. Some of them reach the neuropile at the level of the brain while others innervate the nerve ring of the pharynx (Fig. 2B–D). Furthermore, another group of cells expressing the AT-like peptide is found on each side of the pharynx (Fig. 2D).

3.2. *Macrostomida*

The existence of cells expressing the AT-like peptide was also detected in the flatworm *Macrostomum* sp. (see Figs. 3 and 4).

Fig. 3A shows a panoramic view of a whole individual showing the presence of immunoreactive tissues at the level of the anterior end (pharynx and cerebral neuropile), as well as the posterior one, where reproductive systems and the adhesive caudal plate are located. The adhesive caudal plate shows the presence of a muscle arrangement associated to immunoreactive cells (Fig. 3B and H). When different confocal planes of the posterior region were analyzed, the female gonopore and the structures which compose the male reproductive system were evident. The female gonopore, formed in Macrostomida by glands and surrounding muscle fibers, appears associated with several neuronal rings expressing the AT-like peptide (Fig. 3C and D). With a higher magnification, the presence of 4 rings of immunoreactive cells around the gonopore is evident (Fig. 3D). Fig. 3E–H shows the male reproductive system formed by the seminal vesicle, which presents a wall formed by muscle fibers, having an anatomical relationship with the prostatic vesicle. The prostatic vesicle wall presents AT-like neurons innervating both, the seminal vesicle and the stylet (Fig. 3E, F and H). Finally, in another confocal plane, the existence of immunoreactive neurons innervating the region of the ciliated chamber of the prostatic vesicle is also evident (Fig. 3G).

A panoramic view of the anterior region shows the arrangement of the muscle fibers, and the nervous cells expressing the peptide. Two groups of subepidermal immunoreactive neurons, placed on either side of the body run towards the posterior region (Fig. 4A). In a magnified view, groups of fibers of neurons located next to the anterior end converge at the brain neuropile, just in front of the eyes (Fig. 4B). As in *Stenostomum* sp., a relationship between intraepidermal neurons expressing the peptide and clusters of subepidermal neurons was found (Fig. 4C). Indeed, as Fig. 4D and E shows, the presence of allatotropic processes reaching the eyes is also evident. Furthermore, a colocalization of the peptide with the muscle fibers of the pharynx is also evident (Fig. 4E and F) including the presence of immunoreactive fibers at the level of the pharyngeal ring (Fig. 4F).

4. Discussion

Our results show the expression of an AT-like peptide in both groups of free living Turbellaria analyzed. The peptide is present in cells resembling neurons, and distributed in different regions of the body of the flatworms. The simultaneous analysis of the expression of the peptide with phalloidin, suggests that the neurons producing this peptide have a functional relationship with muscle fibers, particularly at the level of the pharynx in the two turbellarian groups. Indeed, in Catenulida, in which individuals in different moments of the feeding process could be observed, the intensity and number of cells expressing the AT-like peptide at the level of the pharynx, and also the colocalization of immunoreactive cells with muscle tissue correlates, suggesting that as in insects, this peptide could be acting as a myoregulator.

The morphological relationship between the cells expressing the AT-like peptide and the muscle tissue associated with the reproductive system was evident at the level of both, the female and male reproductive organs in *Macrostomum* sp.

Immunoreactive fibers were found associated with the eyes in *Macrostomum* sp. Regarding catenulids, neurons expressing the AT-like peptide, were found at the level of the ciliated pits in *Stenostomum* sp. These organs closely associated with the anterior lobe of the brain and the root of the lateral nerve cord have sensorial functions (Wikgren and Reuter, 1985). In insects, AT was found in neurons of the optical lobe in several species pertaining to different orders (Hofer and Homberg, 2006; Riccillo and Ronderos, 2010; Würden and Homberg, 1995). Furthermore, it was found colocalizing with GABA at the level of the accessory medulla, a neuropile located in the optical lobe where the central circadian pacemaker is located in cockroaches, and it was involved in the regulation of photic entrainment of the circadian clock (Petri et al., 2002). The function of the groups of neurons expressing the AT-like peptide in association with sensorial structures associated with the brain in the two groups of turbellarians under study remain unknown but, on the basis of the information obtained in insects, it is probable that this peptide plays a relevant role in the physiological mechanisms controlling circadian activities in flatworms.

AT was first isolated based on its ability to induce JHs secretion by the gland corpora allata, but several other functions were confirmed later, showing that this neuropeptide is pleiotropic. Although it was originally found in the nervous system, it is also produced by cells of the epithelial sheet of the digestive system and even by the excretory organs (Riccillo and Ronderos, 2010; Santini and Ronderos, 2009a, 2009b; Sterkel et al., 2010). Moreover of its presence in insects, members of this family of peptides were also found in other invertebrate phyla like mollusks and annelids (Ukena et al., 1995; Veenstra, 2010). JHs are a particular family of hormones which is only present in Arthropoda. This is an interesting fact due that, in spite that several genomes of deuterostomata have been completely sequenced or are under sequencing process, AT seems to be not present, suggesting that this peptide evolved only in protostomates. The presence of AT in organisms which

vesicle showing the presence of AT-like immunoreactive cells in its wall, some of them innervating the seminal vesicle (arrow heads), and fibers projecting to the stylet. (G) Optical section showing the anatomical relationship between the seminal and prostatic vesicles at the level of the ciliated chambers of the prostatic vesicle. (H) 3D confocal reconstruction of the posterior end of *Macrostomum* sp. showing the AT-like innervation of the prostatic vesicle and the stylet (arrow) and the AT-like innervations of the caudal adhesive plate (arrow head). The color of the cells expressing the AT-like peptide and the muscle fibers suggest a functional relation between the peptide and the muscle fibers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

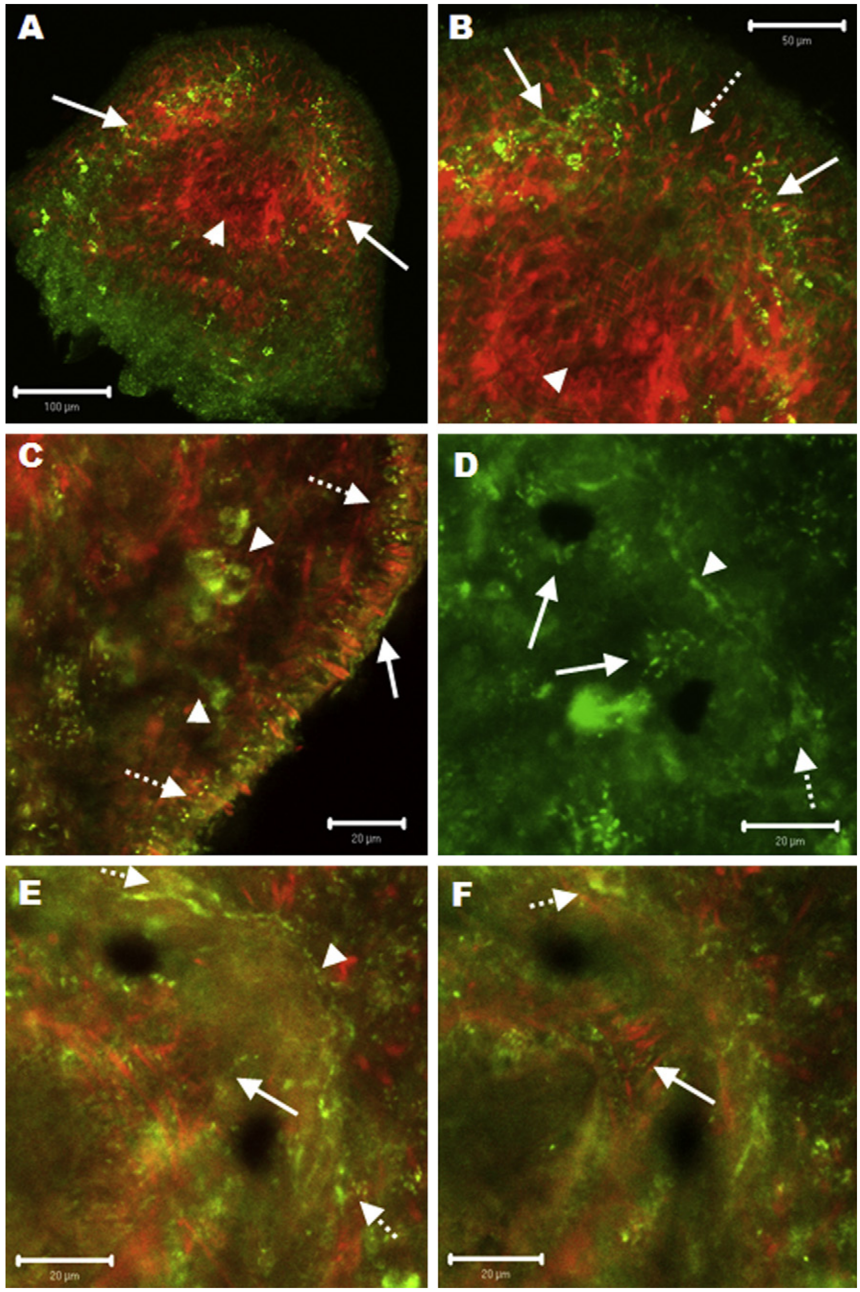

Fig. 4. Presence of the AT-like peptide in *Macrostomum* sp. (A) Panoramic view of the anterior region of *Macrostomum* sp. showing the presence of a symmetric chain of allatotropic neurons running laterally beneath the surface (arrows), and the pharynx (arrow head). (B) Detail of the same region showing two groups of neurons located symmetrically at the anterior area (arrows), reaching the neuropile at the level of the cerebral ganglion (dashed arrow), and the pharynx (arrow head). (C) Higher magnification of the lateral anterior region showing intraepidermal neurons expressing the peptide (arrow), a cluster of subepidermal neurons (arrow heads), and immunoreactive processes going throughout the epidermal layer (dashed arrows). (D) Detailed view at the level of the brain showing the presence of a group of neurons on each side (dashed arrow) and immunoreactive processes reaching the middle line just in front of the ocelli (arrow head), and processes expressing the neuropeptide at the level of the ocelli (arrows). (E–F) Similar views showing the relationship between neuronal processes expressing the peptide and the muscle fibers of the pharynx (arrows). Note that the color of the fiber muscles has changed, suggesting the colocalization of the peptide with the muscle tissue at the level of the pharynx. The rest of the references as in the previous image.

neither produce JHs, nor undergo metamorphosis, could be signaling that, as it was previously hypothesized (Elekonich and Horodyski, 2003), this neuropeptide was first involved in myotropic activities, being the stimulation of the synthesis of JHs a synapomorphic feature in the phylum Arthropoda. If the peptide is only present in Protostomata phyla, and not present in Deuterostomata, it would be an interesting clue to find out the probable presence of AT in organisms like cnidarians and acoels, which might be close of the common ancestor of these groups (Egger et al., 2009). In fact, preliminary results obtained in our laboratory suggest that a peptide with some homology could be present in neuroepithelial cells of *Hydra* sp. (data not shown).

Several chemical messengers have been highly conserved throughout evolution. Some authors have suggested that, the presence of a given regulatory peptide in different groups, could be considered of phylogenetic significance (Wikgren and Reuter, 1985; Reuter and Halton, 2001). Finally, the presence of an AT-like peptide in Turbellaria, could be of interest from an evolutionary point of view about the origin of this and other families of peptidic messengers.

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