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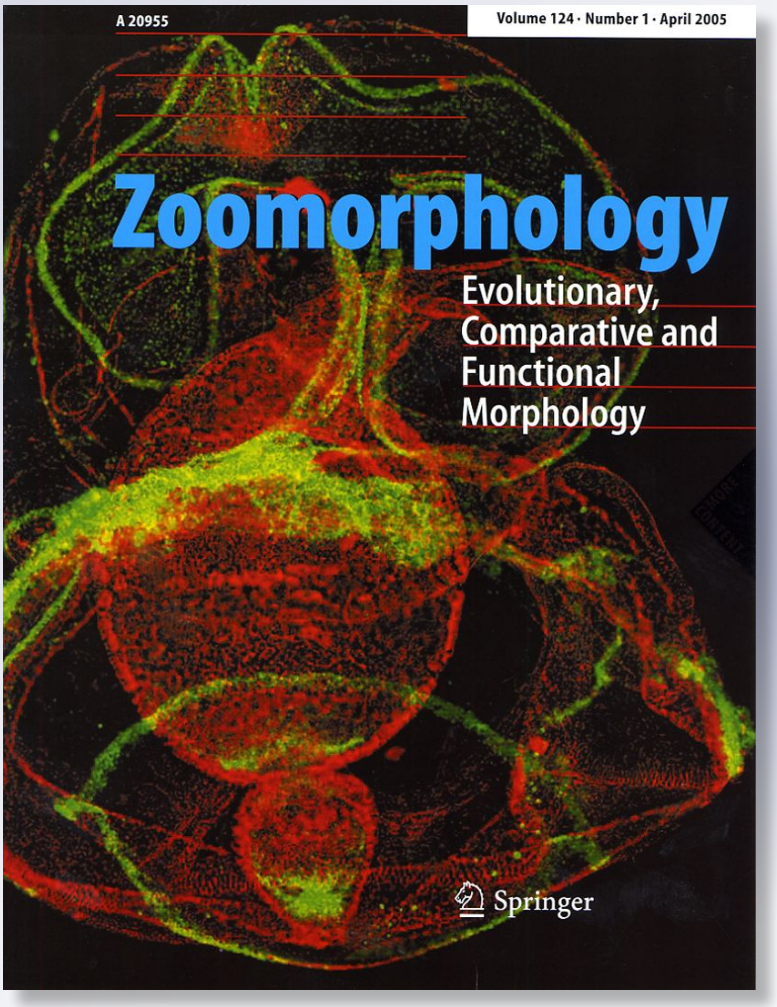

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ORIGINAL PAPER

An allatotropin-like neuropeptide in *Mesostoma ehrenbergii* (Rhabdocoela, Platyhelminthes)

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Abstract Allatotropin (AT), a neuropeptide isolated on the basis of its ability to stimulate the synthesis of juvenile hormones (JHs) in insects, has also been found in other groups. In addition to this function, AT has proved to be myotropic. In the present study, we analyze its expression in the free-living turbellarian *Mesostoma ehrenbergii* (Platyhelminthes: Typhloplanida) and its probable functional relationship with the muscle tissue. The results show the presence of an AT-like peptide in neurons located in different regions of the body of the flatworm. The analysis of the presence of the peptide together with phalloidin labeling suggests a functional relationship between the peptide and the muscle tissue. This is particularly evident at the level of the pharynx, where the peptide induces contractions at concentrations of 10^{-14} and 10^{-12} M, suggesting that it is actually acting as a myoregulator. Detection of AT in several groups of protostomes but apparently not in deuterostomes suggests that this peptide could be a synapomorphic feature of protostomes. Indeed, the presence of AT in organisms that do not undergo metamorphosis could suggest that it was first involved in myotropic activities, being the induction of the synthesis of JHs a secondary function.

Keywords *Mesostoma ehrenbergii* · Neuropeptides · Myoregulators · Platyhelminthes · Allatotropin · Phalloidin

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Introduction

In free-living platyhelminths, neuropeptides seem to be particularly important as myoregulators acting on muscles at the level of digestive and reproductive organs (Gustafsson et al. 2002). In fact, several neuropeptide families have been detected and characterized in Platyhelminthes (Gustafsson et al. 2002; Johnston et al. 1996; Kreshchenko 2008; McVeigh et al. 2009; Mousley et al. 2004; Wikgren and Reuter 1985).

Allatotropin (AT), a neuropeptide originally isolated from the brain of a lepidopteran *Manduca sexta* Linnaeus, 1763 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). Furthermore, some AT homologous peptides were identified in other groups such as annelids and mollusks (Jing et al. 2010; Veenstra 2011). AT, originally isolated from the nervous system, was also detected in epithelial tissues (Riccillo and Ronderos 2010; Santini and

Ronderos 2009a, b; Sterkel et al. 2010). Despite the function as a JHs regulator, 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), as a regulator of ion transport throughout epithelia (Lee et al. 1998), and as a myostimulator 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). Furthermore, a new activity, controlling the release of digestive enzymes, was recently described for the peptide (Lwalaba et al. 2009).

The existence of a peptide similar to AT was recently demonstrated (Adami et al. 2011) in free-living platyhelminths belonging to two different groups (Catenulida and Macrostomida), suggesting that this family of peptides is present in ancient groups. In the present study, we further analyzed the morphological relationship between nervous and muscle system, analyzing also the functional activity of AT as a myoregulator in *Mesostoma ehrenbergii* (Focke, 1836).

Materials and methods

Animals

The specimens of the free-living platyhelminth, *M. ehrenbergii* (Focke, 1836) (Typhloplanida), were collected at a rainwater pond in Pereyra Iraola Park (34°51′S; 58°03′W), a locality situated near to the city of La Plata (Buenos Aires Province, Argentina). The specimens were obtained during summer and autumn 2010 and were maintained in the water from the original site under a 12:12 h light/dark period until they were processed.

Immunohistochemistry and phalloidin labeling

Specimens were fixed in formaldehyde–phosphate buffered saline (PBS) (4%) at 4°C for 12 h, 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 worms were then incubated overnight at 4°C with a polyclonal antiserum developed against AT of the Diptera *Aedes aegypti* (Linnaeus, 1762) (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 antibody binding was previously demonstrated by Hernandez-Martinez et al. (2005) and Santini and Ronderos (2007, 2009b). Animals were then incubated with FITC-labeled goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) (1/1,000 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) (1/1,000). After every step, the specimens were washed (3 times × 20 min) with PBS-T (0.05%). For secondary antibody control, primary antibody incubation was omitted and replaced with PBS. To reassay primary antibody specificity, polyclonal antiserum was previously incubated overnight at 4°C with pure AT synthesized by Biopeptide Company (San Diego, CA, USA) (kindly provided by Dr Fernando G. Noriega), at a concentration of 20 nmol/ml of diluted antiserum (1/500). Finally, worms were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). The resulting material was analyzed with a Laser Scan Microscope Zeiss LSM 510 Meta. The optical sections were obtained from dorsoventral stacks across all the specimen for panoramic views, and for particular regions (eg. pharynx), taken in a range from 3 to 5 μm in distance between each, corresponding to a number ranging between 10 and 15 optical sections respectively.

Contraction assay

As a first approach to analyze the myoregulatory function of the AT-like peptide found in *M. ehrenbergii*, young/mature specimen were treated with synthetic AT. The experimental individuals were moved to *Mesostoma* sp. saline solution (Koopowitz 1996) and left in there for 10 or 15 min until they showed normal behavior (i.e., the individuals were adapted to saline). Once the experimental specimens were ready, the saline solution was replaced with a new one containing different doses of AT. The concentrations assayed ranged from 10^{-16} to 10^{-8} M. The same individual was used for assays with different doses. Between tests, preparations were washed with the control solution up to basal conditions were restored. The peptide goes into the organisms through the epidermal tissue causing an important delay until the response can be observed. Despite that some individuals showed muscle activity in a shorter period, most of them were maintained in the experimental medium for 30 min until any really visible reaction could be seen in the pharynx. Once the activity started, the number of contractions of the pharynx were recorded for a 3 min period and the frequency was determined as number of contractions/minute. Between the different doses applied, the solutions containing the peptide were replaced by fresh saline solution to restore the individuals to control conditions. Those individuals that did not properly respond to saline solutions were discarded. Some individuals were recorded with a digital camera adapted to a stereoscopic microscope to show the way in which both the pharynx and the body musculature reacted.

Results

The analysis reveals the presence of AT immunoreactive cells in *M. ehrenbergii*. The omission of the primary antibody incubation abolished the labeling, and the preadsorption with the immunizing peptide clearly diminished the staining confirming previous analysis (data not shown).

The expression of AT-like immunoreactivity and the relationships between the neurons producing the neuropeptide and the muscle fibers in *M. ehrenbergii* are seen in Fig. 1.

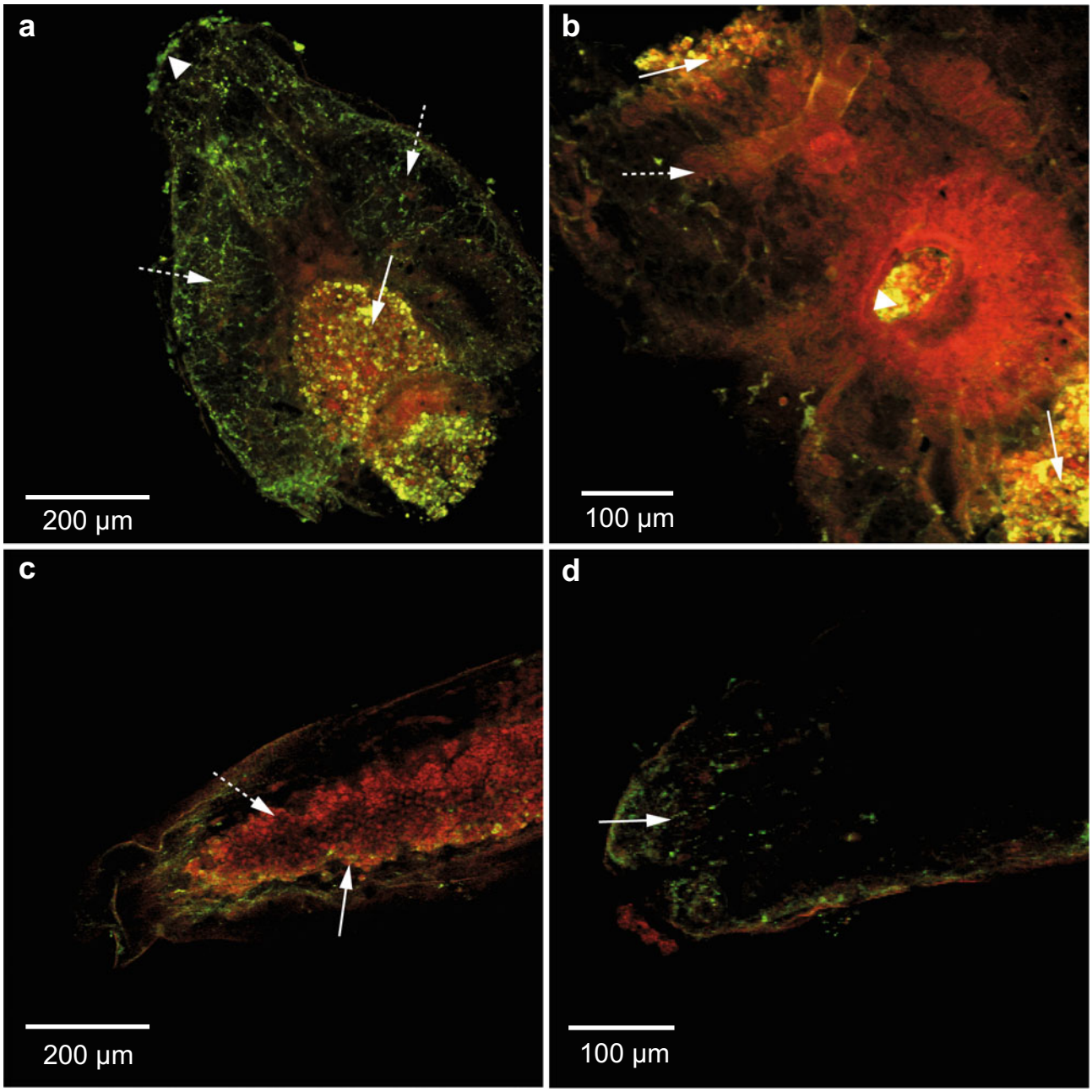


Fig. 1 Presence of the AT-like peptide in *M. ehrenbergii*. Here and in the next figures, note that antibody labeling is in *green* and phalloidin (muscle) in *red*. **a** Anterior region showing the expression of the neuropeptide at the level of the subepidermal dorsal plexus (*dashed arrows*) and immunoreactive intraepidermal cells in the sensory area of the head (*arrowhead*). The presence of the peptide associated with muscle fibers anterior to the pharynx is also evident (*arrow*). **b** View of the medial region of another individual showing neuronal processes reaching the pharynx connecting with muscle fibers at the level of the mouth (*arrowhead*) and immunoreactive processes reaching the uterus (*dashed arrows*). Note the presence of the peptide associated with the anterior and posterior muscle fibers connected to the pharynx (*arrows*). **c** Panoramic view of the posterior region showing immunoreactive nerve processes running along the wall of the intestine (*arrow*). *Dashed arrow* shows muscle fibers at the level of the gut. **(d)** Detailed view of the posterior region showing the existence of an immunoreactive plexus (*arrow*) also at this level

Immunoreactivity was found in neurons located in the anterior region (Fig. 1a), at the level of the pharynx (Fig. 1b) and also in the posterior region of the body (Fig. 1c, d).

The images show the presence of immunoreactive intraepidermal nerve cells (Figs. 1a, 2b). Also, a dorsal subepidermal plexus is widely distributed throughout the anterior region of *M. ehrenbergii* (Figs. 1a, 2a).

In the middle region of the body, the pharynx of *M. ehrenbergii* is closely associated with AT immunoreactive nerve cells. In fact, a connection of AT-like expressing fibers and muscle tissue is found at the level of both the anterior and the posterior gut associated with the pharynx (Fig. 1a, b). Furthermore, some immunoreactive nerve fibers reaching the wall of the uterus were found (Fig. 1b). In the posterior region, toward the caudal end of the gut, two different focal planes also show nerve processes associated with the muscle fibers of the gut (Fig. 1c, d).

In a detailed view of the dorsal anterior region, several neurons with intraepidermal location and two nerve cords running close to the body wall were found (Fig. 2a). Some of these neurons show a big size (Fig. 2b). These intraepidermal neurons are associated with a subepidermal plexus, which seems to innervate the anterior region of the organism (Figs. 1a, 2a). Furthermore, several clusters of intraepidermal immunoreactive neurons were found close to the body wall and also forming a network in the anterior region. In a magnified view of the body wall, at a deeper level, the expression of the neuropeptide was evident in subepidermal groups of neurons connected with the intraepidermal ones (Fig. 2c, d). A dorsal view of the brain shows the presence of an immunoreactive nerve plexus in the two main longitudinal anterior cords (Fig. 2e). At the anterior end of the cords, axons running from the intraepidermal complex of neurons to reach the neuropile, located in the anterior end of the body, are visualized (Fig. 2f).

The association of the pharynx muscles with cells expressing AT in individuals with the pharynx in different positions is evident (Figs. 3, 4). In a confocal 3D reconstruction of *M. ehrenbergii*, a plexus associated with the muscle tissue of the pharynx is evident (Fig. 3a). Furthermore, two allatotropic nerve rings are visualized: one located at the tip and another at the base of the pharynx (Figs. 3a–d, 4a, b). In most of the individuals analyzed, these neurons are also evident, connecting with the muscle tissue at the level of the mouth (Figs. 3c, d, 4a–d). After a 3D confocal reconstruction of the pharynx in one of the specimens under study, both the external and the internal side can be seen showing the relationship between immunoreactive neurons and the muscle fibers of the pharynx (Fig. 4c, d).

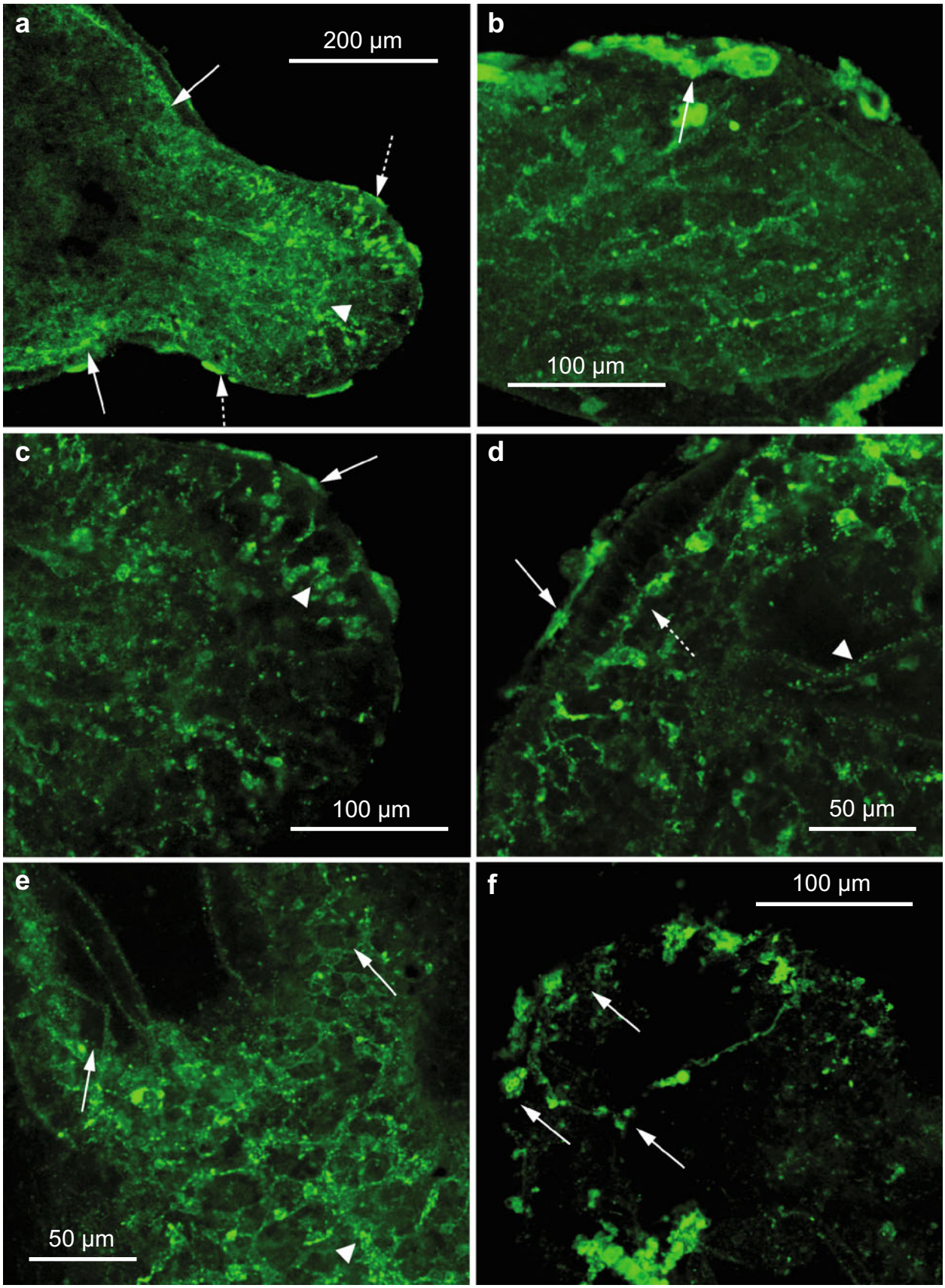
Regarding the contraction assay, despite that all the individuals were maintained in the same medium until the beginning of the experiments and that the specimens used were in a similar stage of maturation, the standardization of these organisms was a hard task, being difficult to perform properly a dose–response curve. In any case, beyond the variation in the time of reaction, and in the response to the different doses applied, none of the treated specimens showed activity at a dose of 10^{-16} M. Nevertheless, doses of 10^{-14} and 10^{-12} M induced contractile activity of the muscle tissue. Contractions were observed in the body wall and were particularly pronounced at the level of the pharynx (see supplementary material). In fact, the frequency, which was null in all the individuals under control conditions (i.e., saline solution) reached an average of 7.50 ± 0.99 contractions/min (mean $\pm$ SEM, $n = 6$), ranging between 5 and 11. With doses higher than 10^{-10} M, the muscles seem to overreact, showing the whole individual in a tetanized state.

Fig. 2 Presence of the AT-like peptide in the anterior region of *M. ehrenbergii*. **a** Presence of immunoreactive neurons associated with the epidermis (*dashed arrows*). Lateral nerve cords (*arrows*) and a nerve plexus are also evident (*arrowhead*). **b** Higher magnification view showing a giant neuron expressing the AT-like peptide in the anterior end of the head (*arrow*). **c** Intraepidermal immunoreactive neurons related to a subepidermal network which also express the peptide (*arrow*). The *arrowhead* shows nerve fibers running across the epidermal layer. **d** Detailed view of a similar area in other specimen showing intra (*arrow*) and subepidermal (*dashed arrow*) neurons expressing the neuropeptide. As shown on Fig. 1c, the presence of allatotropic axons running on the surface of the wall of the uterus is also evident (*arrowhead*). **(e)** Allatotropic plexus in the ventral region of the brain and the anterior nerve cords (*arrows*). A group of neurons converging in the medial line of the brain run backward to the muscle tissue located next to the pharynx (*arrowhead*). **(f)** AT-like neurons located in a neuropile just in front of the anterior nerve cords of the brain (*arrow*) make connection with axons reaching the subepidermal plexus at the anterior end of the head

Discussion

Local and systemic cell integration is a fundamental process in biology, and in fact, different ways of communication including endocrine and paracrine evolved early during the evolutionary process. Cell messengers of different chemical nature were found in sponges and cnidarians (Grimmelikhuijzen et al. 1991, 1996, 2002; Hansen et al. 2002; Lentz 1966; Weyrer et al. 1999). The cnidarians are the most primitive group characterized by organized nervous system, where peptides seem to act as myoregulators, which is considered a primitive function for this family of messengers (Grimmelikhuijzen et al. 1996).

Beyond the ancient groups, the platyhelminths also show a variety of chemical compounds acting as neuromodulators and myoregulators (Gustafsson et al. 2002; Johnston et al. 1995, 1996; Mousley et al. 2004; Wikgren and Reuter 1985). Fur-



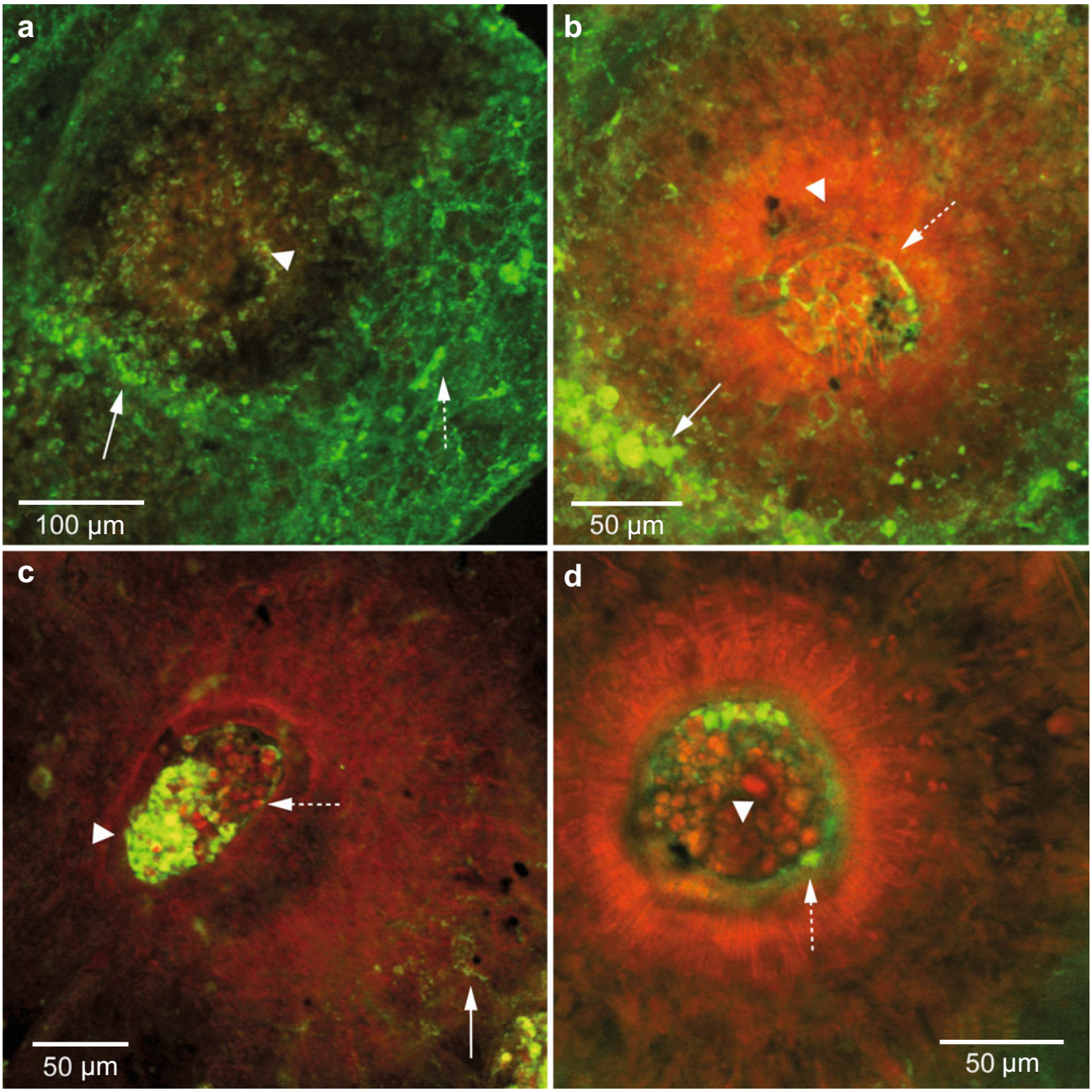


Fig. 3 Different views of several specimens showing the relationship between the AT-like peptide and the muscle fibers of the pharynx. **a** Confocal 3D reconstruction at the level of the pharynx showing immunoreactive neurons located at three levels, forming a net all around the pharynx (*dashed arrow*) and two rings: one at the base (*arrow*) and the other surrounding the mouth (*arrowhead*). **b** Another view at the same level with higher magnification in another individual showing the pharynx retracted and the mouth closed. Note the presence of an immunoreactive net of neurons at this level (*dashed arrow*) and the external inferior ring of AT-like neurons (*arrow*). The *arrowhead* shows muscle fibers of the pharynx. **c** Similar view in other specimen. The pharynx is everted and seems to be opening. Note the immunoreactive ring of neurons around the sphincter of the mouth (*dashed arrow*), some of them connecting with the muscle fibers of the mouth (*arrowhead*), and some of the neurons of the basal ring expressing the neuropeptide (*arrow*). The *arrowhead* shows the fibers of the pharynx as in **b**. **d** View of the pharynx opening in other specimen showing the presence of neurons expressing the peptide associated with the outer ring of the pharynx (*dashed arrow*) and the muscle fibers of the mouth (*arrowhead*)

thermore, peptides have also been involved in regenerative process, inducing mitotic activity of neoblast stem cells (Kreshchenko 2008; Kreshchenko et al. 2008). Our results show the pattern of immunoreactivity of an AT-like peptide in the free-living turbellarian *M. ehrenbergii*. The peptide is present in neurons and localized in different regions of the body. Simultaneous analysis of the expression of the peptide with phalloidin suggests that the neurons producing this peptide

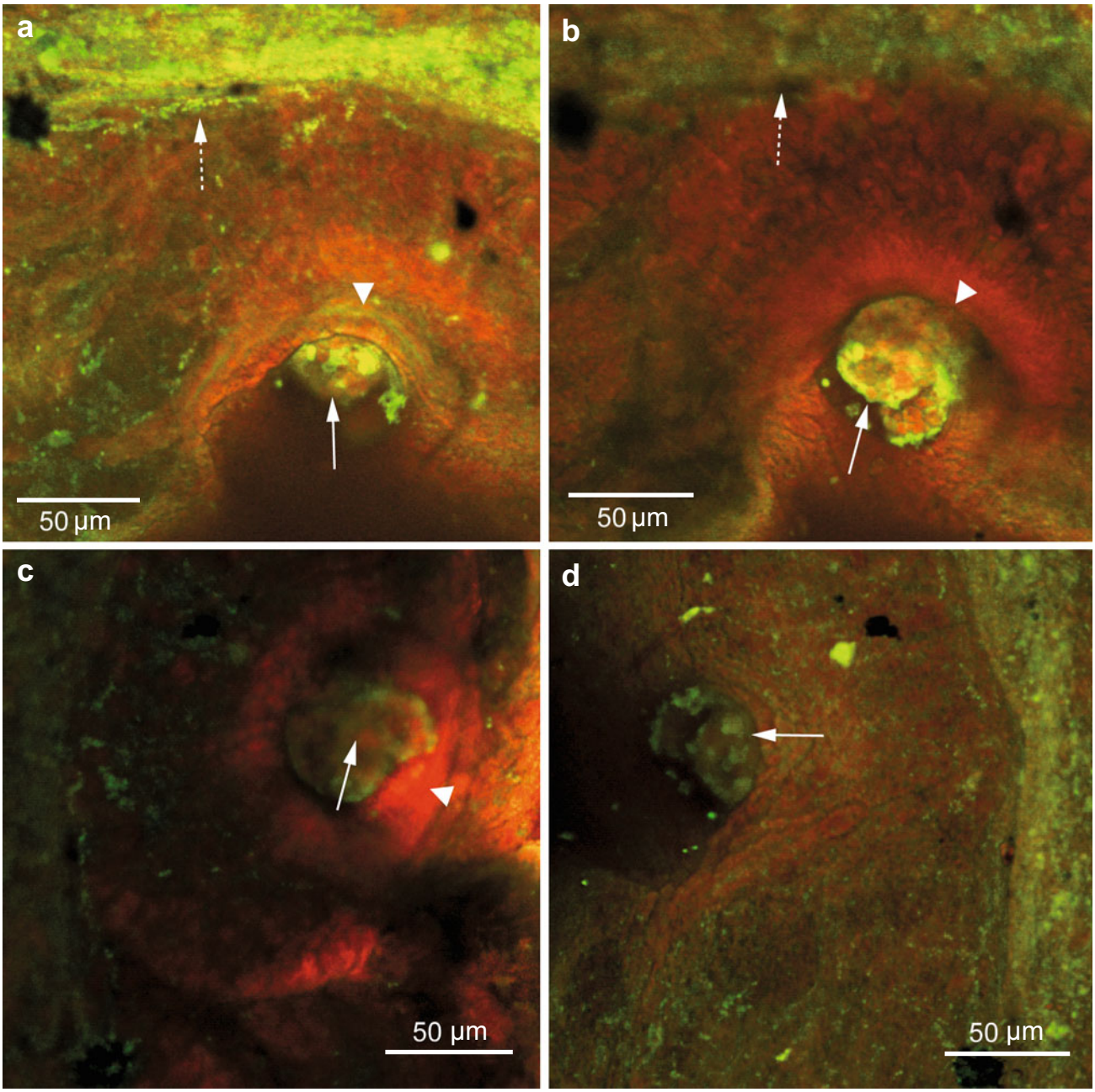


Fig. 4 Different views of several specimens showing the relationship between the AT-like peptide and the muscle fibers of the pharynx. **a**, **b** Views of the contracted pharynx of the same organism at two different levels showing the relationship between allatotropic processes and the muscle fibers of the pharynx (*arrowheads*) and the mouth (*arrows*). Furthermore, the presence of nerve processes running along the external ring of the pharynx is also evident (*dashed arrows*). **c**, **d** Two views of the pharynx after a 360° confocal 3D reconstruction showing the relationship of allatotropic neurons and the muscle tissue of the pharynx, corresponding to the external view **c** and the internal one **d**. *Arrows* as in previous images

have a functional relationship with muscle fibers, particularly at the level of the pharynx. Indeed, a spatial coincidence of the immunoreactive nerve cells with muscle fibers has been observed, suggesting a myoregulatory role of the AT-like peptide, as is the case in insects. As a first approach to analyze the myoregulatory activity of the AT-like peptide observed in *M. ehrenbergii*, we have decided to apply the pure peptide to some young/mature individuals. Interestingly, treatments with pure AT showed that *M. ehrenbergii* responds to the exogenous administration of 10^{-14} and 10^{-12} M, which causes contractions of the muscle tissue, including the pharynx musculature.

The images also showed the presence of AT-like immunoreactive fibers at the level of the wall of the uterus.

Indeed, in some specimens containing developing worms, the growing organisms were expulsed during a series of contractions of the body wall musculature induced by the exogenous administration of the peptide (data not shown).

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 layer of the digestive system and even by the excretory organs (Riccillo and Ronderos 2010; Santini and Ronderos 2009a, b; Sterkel et al. 2010). In addition to its presence in insects, members of this family of peptides were also found in other groups like Mollusca and Annelida (Jing et al. 2010; Veenstra 2011). JHs are a particular family of hormones present only in Arthropoda. As for deuterostomes, despite the fact that several deuterostome genomes have been completely sequenced or are under sequencing process, after a search in several databases, AT seems to be absent, suggesting that this peptide may have evolved only in protostomes. The presence of AT in organisms that neither produce JHs nor undergo metamorphosis could indicate, as it was previously hypothesized (Elekonich and Horodyski 2003), that this neuropeptide was first involved in myotropic activities, while the stimulation of the synthesis of JHs could be a synapomorphic feature of the Arthropoda. If the peptide is indeed only present in protostomes and absent in deuterostomes, it would be an interesting clue to search for AT in organisms like cnidarians and acoels, which might be close to the common ancestor of these groups (Egger et al. 2009). In fact, preliminary studies developed in our laboratory strongly suggest that a peptide similar to AT is produced by neuroepithelial cells acting as a myoregulator in Cnidaria (unpublished results).

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