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Research paper

Allatoregulatory-like systems and changes in cytosolic Ca^{2+} modulate feeding behavior in *Hydra*

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ABSTRACT

Allatotropin (AT) and allatostatin-C (AST-C) are neuropeptides originally characterized by their ability to modulate the secretion of juvenile hormones in insects. Beyond the allatoregulatory function, these neuropeptides are pleiotropic acting as myoregulators not only in insects, but also in other groups of invertebrates. We have previously proposed the existence of AT and AST-C like systems in *Hydra* sp., a member of the phylum Cnidaria, which is a basal group of Metazoa, sharing a common ancestor with Bilateria. In the present study we analyze the regulatory effects of both peptides on the activity of the hypostome during feeding in *Hydra* sp. Furthermore, the importance of changes in the cytosolic Ca^{2+} levels involved in the response of the hypostome were analyzed. Physiological assays showed that while the presence of food or treatment with AT stimulates the extrusion of the hypostome, AST-C has an inhibitory effect on the behavior induced by both, food and AT. These facts suggest that both systems participate in the regulatory mechanisms associated with feeding and, as in insects, AST-C and AT may exert opposite effects. The use of thapsigargin (TG) and nifedipine, two compounds that modify the levels of cytosolic Ca^{2+}, showed that changes in the levels of this ion are involved in the regulation of the activity of the hypostome. Indeed, these results suggest that the two basic mechanisms operating to increase the cytosolic levels of Ca^{2+} (i.e. the influx from the extracellular space and the release from endoplasmic reticulum) are relevant for the extrusion of the hypostome. Like in insects, the treatment with TG counteracted the effect of AST-C, suggesting that this peptide acts by reducing cytosolic Ca^{2+} levels. Furthermore, nifedipine prevented the myostimulatory effect of AT, showing that the effect of this peptide depends on the influx of Ca^{2+} throughout voltage-gated calcium channels. Altogether, these results suggest that the Allatotropin/Orexin and Allatostatin/Somatostatin regulatory systems could represent an ancestral mechanisms regulating hypostome activity and feeding behavior in Cnidaria.

1. Introduction

The coordination of physiological mechanisms requires precise communication between cells. On the basis of these cellular interactions, tissues and organs are functionally related, allowing the organisms the accomplishment of integrated functions. Peptidic molecules acting as messengers for intercellular communication, are widely distributed in Metazoa, playing regulatory roles in a variety of physiological processes. These molecules are ubiquitous and pleiotropic, acting as neurotransmitters and neuromodulators in the nervous system, and as hormones in endocrine and neuroendocrine ways. Moreover, it has been proposed that neuropeptides are the most ancient myoregulatory messengers (Grimmelikhuijzen and Hauser, 2012).

Allatotropin (AT) and allatostatin-C (AST-C) are neuropeptides originally characterized in insects, based on their regulatory function on the activity of the *corpora allata* (CA), modulating the synthesis of juvenile hormones (JH) (Kataoka et al., 1989; Kramer et al., 1991). In addition to their allatoregulatory function, both neuropeptides proved to be pleiotropic being secreted not only by the nervous system, but also by endocrine epithelial cells (Riccillo and Ronderos, 2010; Santini and Ronderos, 2007, 2009a, b; Sterkel et al., 2010). In fact, it has been proposed that AT is involved in midgut ion transport and digestive enzymes synthesis regulation in lepidopterans (Lee et al., 1998; Lwalaba et al., 2009) and more recently with the immune response in mosquitoes (Hernández-Martínez et al., 2017). Beyond these functions, AT has been

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widely characterized as a myostimulatory and cardiostimulatory peptide (Veenstra et al., 1994; Duve et al., 1999, 2000; Rudwall et al., 2000; Koladich et al., 2002; Matthews et al., 2007; Sterkel et al., 2010; Villalobos-Sambucaro et al., 2015), while AST-C showed myo- and cardioinhibitory functions in several insect species (Duve et al., 1999, 2000; Matthews et al., 2007; Price et al., 2002; Villalobos-Sambucaro et al., 2016).

AT and AST-C exert their functions by binding to receptors pertaining to the family of rhodopsin-like G protein-coupled receptors (GPCRs), causing the activation of different signaling cascades. The AT receptor (ATr) has been characterized in several insect species (Horodyski et al., 2011; Lismont et al., 2015; Nouzova et al., 2012; Verlinden et al., 2013; Villalobos-Sambucaro et al., 2015; Yamanaka et al., 2008) and is considered as orthologue of the orexin (Ox) receptor of Chordata (Horodyski et al., 2011). Similarly to ATr, the AST-C receptor was characterized in several holo and hemimetabolous insect species (Mayoral et al., 2010; Vuerinckx et al., 2011; Audsley et al., 2013; Villalobos-sambucaro et al., 2016), showing homology with the somatostatin (SST) family of receptors in vertebrates (Auerswald et al., 2001; Mayoral et al., 2010). Although these peptides and their receptors were mainly studied in insects, the existence of AT and AST-C-like systems has been proposed in other groups of Arthropoda and even in other phyla of invertebrates like Annelida and Mollusca (Christie, 2015a, b; Christie et al., 2015; Veenstra, 2010, 2011). Indeed, it has been proposed that AT and AST-C-like systems have myoregulatory activity in Platyhelminthes and Cnidaria (Adami et al., 2011, 2012; Alzugaray et al., 2013, 2016).

Hydra sp. is a fresh water member of the phylum Cnidaria, which is considered a basal metazoan group, sharing a common ancestor with Bilateria. Previous studies in *Hydra* sp. using AT and AST-C conjugated with nano-crystals, showed that these two neuropeptides are recognized by different myoepithelial cell populations, suggesting the existence of distinct receptors for both peptides (Alzugaray et al., 2013, 2016). Moreover, physiological assays showed that both peptides act as myoregulators, inducing different behaviors. The application of AT caused the extrusion of the hypostome, resembling the behavior observed during feeding (Alzugaray et al., 2013). On the other hand, AST-C caused changes in the shape and length of the tentacles, peduncle and gastrovascular cavity (Alzugaray et al., 2016). Furthermore, *in silico* search of the putative AT and AST-C receptors in *Hydra vulgaris* showed that the genome predicts the existence of GPCRs sharing homologies with both, AT/Ox and AST-C/SST receptors families respectively (Alzugaray et al., 2013, 2016).

The cnidarian myoepithelial cells are considered the most primitive type of contractile cell; they share the actin-myosin machinery with bilaterians groups (Chapman et al., 2010). It is also known that muscle contraction depends on the increment of the cytosolic Ca^{2+} levels. This increment occurs by the release from the sarcoendoplasmic reticulum and/or the influx through voltage-dependent calcium channels located in the plasma membrane. It has been proposed that in striated muscle of the jellyfish *Polyorchis penicillatus*, both mechanisms are involved (Lin and Spencer, 2001a,b), but the role of these processes in *Hydra* are not completely understood. AT and AST-C might be acting by modifying the cytosolic concentration of this ion, and activating different signaling cascades. In fact, it has been proposed that the activation of both AT and Ox receptors generate increments in the levels of cytosolic calcium (Rachinsky et al., 2003; Wu et al., 2013). Although there is no information about the *in vivo* mechanism of AST-C, it is known that SST receptors act mainly decreasing the cytosolic levels of calcium (Barbieri et al., 2013; Farrell et al., 2014).

In the present study we further analyzed the evolutionary origin and roles of AT and AST-C-like systems in Cnidaria, as well as the involvement of calcium on the mechanisms regulating the activity of the hypostome during feeding in *Hydra* sp.

2. Materials and methods

2.1. Animals

Individuals of *Hydra* sp. were obtained from a colony originated from wild hydroids collected in Argentina. The individuals are maintained in dechlorinated water at 20 ± 2 °C with a 12:12 h light/dark period. Animals were fed with *Artemia salina* and the water was replaced every day.

2.2. Physiological assays

The specimens selected for experiments were placed in *Hydra* medium (HM) ($NaHCO_3$ 0.5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 0.1 mM, $MgSO_4$ 0.08 mM, KNO_3 0.03 mM) and starved during 48 h. The hydroids were then placed individually in HM and acclimated for 10 to 15 min. Once the hydroids were acclimated, the saline solution was replaced by fresh saline HM (control), and then by medium containing the different treatments assayed. Each experiment was performed on 6 to 7 individuals. Each specimen was kept isolated throughout the entire experiment. The same hydroid was used for both control (saline) and posterior treatment. The experimental specimens were examined individually under a binocular microscope, and their activity recorded with a digital video camera. For each treatment a time-lapse was recorded, taking a picture every 3 s during 15 min. The length of the hypostome was evaluated as the number of pixels measured at different times during the 15 min of exposition (i.e. 0.25, 0.50, 1, 3, 5, 8, 10, 12 and 15 min) by using the GNU Image Manipulation Program (GIMP) software (Alzugaray et al., 2016; Kulkarni and Galande, 2014).

2.3. Analysis of feeding behavior

Starved hydroids were maintained in HM, and the behavior of the hypostome was evaluated. Then the medium was replaced by fresh saline + eggs of *A. salina*.

2.4. Response of the hypostome to AT and AST-C

In previous studies we showed that individually assayed peptides induced a maximum response at a concentration of 10^{-6} M (Alzugaray et al., 2013, 2016). The same concentration was selected to analyze the response of the hypostome. After 15 min in saline the hydroids were treated with 10^{-6} M of *Aedes aegypti* AT (APFRNSEMMTARGF) or 10^{-6} M of *A. aegypti* AST-C (QIRYRQCYFNPISCF) in the presence of food (Alzugaray et al., 2013, 2016; Li et al., 2003, 2004).

2.5. Effect of simultaneous treatment with AST-C and AT on the hypostome extrusion in Hydra sp

To study the existence of an inhibiting effect of AST-C on the extrusion of the hypostome induced by AT, another group of hydroids was treated simultaneously with both neuropeptides using a concentration of 10^{-6} M for each peptide.

2.6. Effect of changes on the cytosolic calcium levels on the extrusion of the hypostome

To study how modulation of cytosolic calcium levels affects hypostome extrusion in the presence of food or the two allatoregulatory peptides we used two compounds: the inhibitor of the

sarco-endoplasmic calcium pump (SERCA) thapsigargin (TG) (Kijima et al., 1991; Lytton et al., 1991) and the blocker of L-type voltage sensitive calcium channel nifedipine (Cognard et al., 1990; Zsotér and Church, 1983). Groups of hydroids starved for 48 hs were treated with saline without (control) or with TG (10^{-6} M) or nifedipine (10 μM) (in the presence of food) (Sigma–Aldrich).

2.7. Analysis of the effect of AST-C and its relationship with changes in the cytosolic calcium levels

To test if the effect of AST-C is mediated by changes in the cytosolic calcium levels, we assayed the activity of the hypostome in hydroids treated with a solution containing AT (10^{-6} M) + AST-C (10^{-6} M) + TG (10^{-6} M).

2.8. Analysis of the involvement of the influx of extracellular calcium in the activity of AT

To test if the stimulatory effect of AT on the hypostome extrusion depends on the influx of calcium through voltage-sensitive calcium channels, we assayed the response of the hypostome in starved hydroids treated with AT in the presence of nifedipine (10 μM). As in previous experiments hydroids were first maintained in HM (control) and then exposed to the treatment

2.9. Statistics

Differences between treatments were analyzed by multifactorial ANOVA. Single post-hoc comparisons were tested by the least significant difference (LSD) method. Only differences of $p \leq 0.05$ were considered significant. Data are showed as mean ± SEM.

2.10. In silico search of putative allatotropin/orexin receptors in Cnidaria

There are two highly conserved motifs described in AT/Ox GPCRs; one located in the second intracellular loop and another in the transmembrane 7 domain (TM7). Based on these conserved motifs, a protein BLAST search was performed in GenBank. Only those sequences that presented the complete transmembrane domains and the **E/D**RWYA motif in the second intracellular loop were included. Six sequences corresponding to non-bilaterian organisms were selected; including four sequences pertaining to phylum Cnidaria (i.e. *Nematostella vectensis*, *Acropora digitifera* and *H. vulgaris*) and two corresponding to Phylum Placozoa. Sequences representative of Ecdysozoa and Lophotrochozoa that presented the conserved domains were also included in the analysis (i.e. Arthropoda; Mollusca; Brachiopoda; Annelida) (Supplementary File 1).

The selected sequences were aligned using the Clustal Omega algorithm (http://www.ebi.ac.uk/Tools/msa/clustalo/) and further analyzed by the software JalView 2.8.2 (Waterhouse et al., 2009). The analysis of the probable evolutionary relationships between sequences was performed by the use of Mega 6.06 software (Tamura et al., 2013) based on the complete sequences (i.e. including amino and carboxyl-terminal sequences) (Alzugaray et al., 2016), using the Maximum likelihood method based on the Poisson correction model, including a 1000 replicates bootstrap analysis.

3. Results

3.1. Response of the hypostome to the presence of food

In a previous study we reported that individuals of *Hydra* sp. extrude the hypostome in the presence of food (Alzugaray et al., 2013). In the present study we used a new protocol to analyze the response of the hypostome, which consisted in the measurement of the hypostome length.

Fig. 1 shows that the average length of the hypostome of starved hydroids maintained in saline during 15 min was 24.7 ± 0.65 pixels, corresponding to the state of "hypostome no extruded". In the presence of food the hydroids increased significantly the length of the hypostome, reaching an average of 41.2 ± 1.35 pixels ($p \leq 0.05$) (Fig. 1A). The percentage of increment was 66%. The hypostome length throughout the experiment is shown in Fig. 1B. The extrusion in the control group is not evident, being the hypostome significantly longer in the presence of food during the experiment.

3.2. Effect of AT on the extrusion of the hypostome

We have previously reported that starved hydroids react to AT, extruding their hypostome with a maximum response at a concentration of 10^{-6} M. To further test the AT effect, we decided to evaluate the length of the hypostome. The results confirm that AT has a stimulatory effect, generating a statistically significant increment in the length (Fig. 1C). The average increment of the hypostome in hydroids undergoing AT treatment was 68% being similar to the response induced by the presence of food (Fig. 1A).

AT induced a significant increment along all the period analyzed being higher than controls in all time points analyzed (Fig. 1D).

3.3. Effect of AST-C on the hypostome extrusion induced by the presence of food

AST-C induced a slight increment in the length of the hypostome at a concentration of 10^{-6} M, although it retained the aspect of a non-extruded organ (data not shown). To further analyze the activity of this peptide we decided to assay the effect of AST-C in individuals stimulated by the presence of food. Fig. 2(A and B) shows that when hydroids were challenged with food in the presence of AST-C the extrusion of hypostome was inhibited, with no differences in the length compared with individuals maintained without food (control) (Fig. 2A). The analysis, showed that the length of the hypostome was similar to the control group during all the time registered (Fig. 2B).

3.4. Inhibitory effect of AST-C on the hypostome extrusion induced by AT

We also tested the effect of AST-C on the extrusion of the hypostome induced by AT. We evaluated the response of the hypostome in starved hydroids treated simultaneously with both peptides. The average length of the hypostome in treated hydroids was significantly higher than control (Fig. 2C). However, the average increment (29%) was lower than those obtained with AT treatment or in the presence of food (See Fig. 1A–D). When the time-response was analyzed, the length of the hypostome was similar to the control during the first 30 s of the treatment, showing later a gradual increment that was maintained during the rest of the experiment (Fig. 2D).

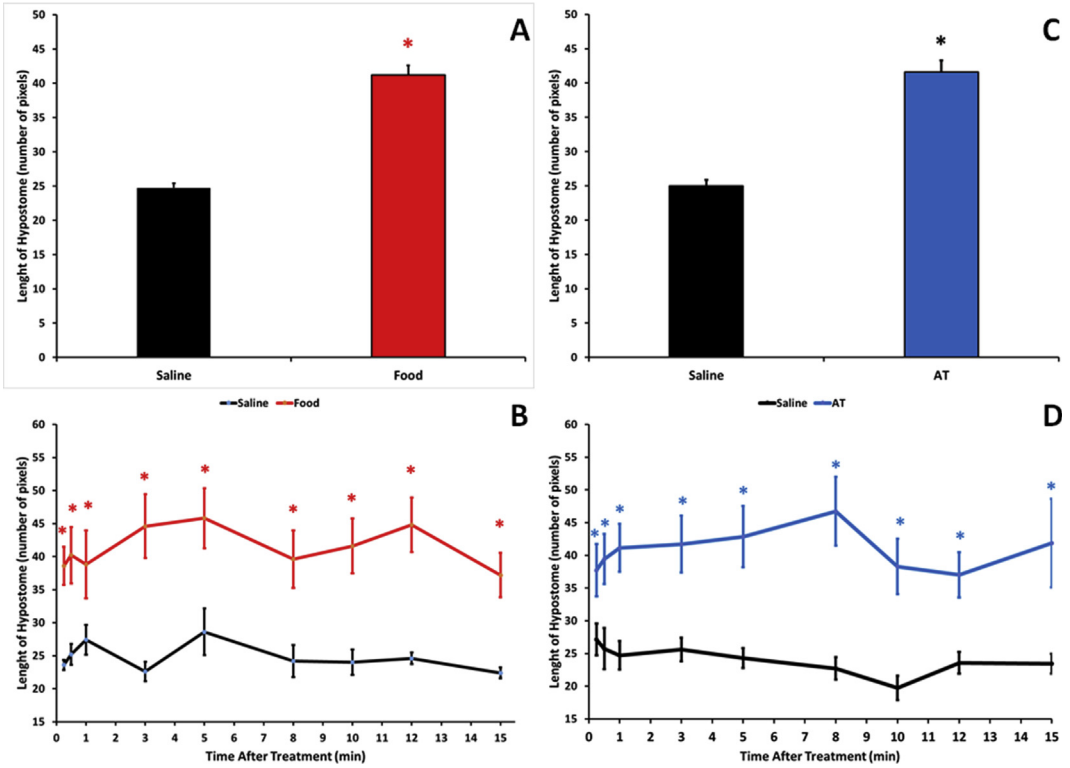

Fig. 1. Effect of the presence of food and allatotropin on the length of the hypostome in *Hydra* sp. (A) Average length of the hypostome in starved control hydroids (saline) and hydroids exposed to food for 15 min. (B) Changes in the length of the hypostome induced by food during the experiment. (C) Average length of the hypostome in starved control hydroids (saline) and hydroids exposed to AT 10^{-6} M for 15 min. (D) Changes in the length of the hypostome induced by AT during the experiment. Each point represents mean ± SEM (n = 7). Asterisks represent significant differences between the treatment and control.

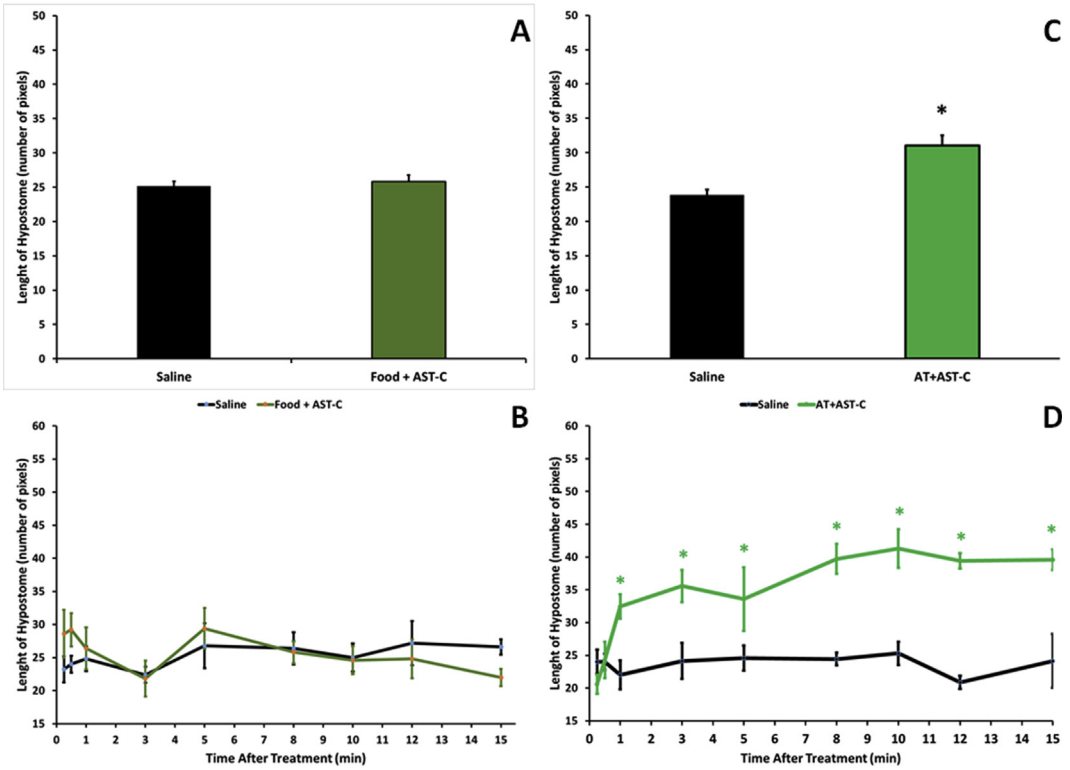

Fig. 2. Effect of AST-C on the hypostome extrusion induced by food and by AT. (A) Average length of the hypostome in starved hydroids (saline) and hydroids exposed to food treated with AST-C 10^{-6} M for 15 min. (B) Length of the hypostome in hydroids undergoing AST-C treatment during the experiment. (C) Average length of the hypostome for 15 min in control (saline) and in hydroids treated with a solution containing AT (10^{-6} M) plus AST-C (10^{-6} M). (D) Changes in the length of the hypostome induced by treatment with both peptides during the experiment. Each point represents mean ± SE (n = 7). Asterisks in C and D represent significant differences between treated and control hydroids.

3.5. Effect of changes on the cytosolic calcium levels on the hypostome extrusion

As a first approach to understand the involvement of calcium in the myoregulatory mechanisms caused by the presence of food and peptides, we decided to assay the effect of TG, a compound that increases the cytosolic calcium levels by blocking the ability of the cell to pump it back into the sarcoplasmic reticulum.

Fig. 3A shows that the incubation of hydroids with TG (10^{-6} M) causes a significant increment on the length of the hypostome, suggesting that an increment of the cytosolic level of calcium is involved in the extrusion of this structure. The time-response analysis showed that the stimulatory effect was already evident one minute after the beginning of the experiment and sustained along the rest of the period assayed, with a maximum response at 3 min (Fig. 3B).

To further analyze the involvement of Ca^{2+}, we decide to test the effect of nifedipine, a compound that decreases cytosolic calcium levels by blocking L-type voltage sensitive calcium channels in the plasma membrane. When starved hydroids were treated with nifedipine (10 μM) no changes on the length of the hypostome were observed (data not shown). On the other hand, when nifedipine was applied to starved hydroids exposed to food, the length of the hypostome was significantly lower than in the control group (i.e. saline + food), suggesting that the influx of extracellular Ca^{2+} might be involved in the mechanism of extrusion of the hypostome induced by the presence of food (Fig. 3C). The time-response analysis showed that, similarly to that observed with TG, changes did not occur from the beginning of the treatment. Indeed, statistical differences appeared only after 3 min of treatment, and tended to revert after 10 min (Fig. 3D).

3.6. Is the inhibitory effect of AST-C mediated by changes in cytosolic calcium levels?

To further analyze the mechanism involved in the antagonistic effects of AST-C and AT, we tested the response of the hypostome to the simultaneous treatment with both peptides in the presence of TG.

Fig. 4(A and B) shows that the hypostome of starved hydroids was extruded when treated with a solution containing AT (10^{-6} M) plus AST-C (10^{-6} M) and TG (10^{-6} M) eliminating the inhibitory effect of AST-C. The length of the hypostome shows an average increment of 75 %, reaching values similar to those of hydroids challenged with food or treated with AT alone.

Taking into account that hydroids exposed to food undergoing Ca^{2+} entry blockade showed a decrease on the extrusion of the hypostome, and that the effect of AT seems to be mediated by Ca^{2+}, we tested the effect of nifedipine on the extrusion of the hypostome induced by AT. The length of the hypostome in hydroids treated with a solution containing AT plus nifedipine was similar to the control group during the experiment, suggesting that the extrusion induced by AT depends on the extracellular Ca^{2+} (Fig. 4C and D).

3.7. Phylogenetic relationships of cnidarian allatotropin receptors

We identified several sequences that presented the conserved **E/D**RWYA motif. Four of them from Cnidaria: one in *H. vulgaris* (Hydrozoa) and three in two species of Anthozoa (i.e. *N. vectensis* and *A. digitifera*). Two additional sequences were identified in Placozoa (*Trichoplax adhaerens*). The sequence from *H. vulgaris* showed a high degree of identity in two different regions that are con-

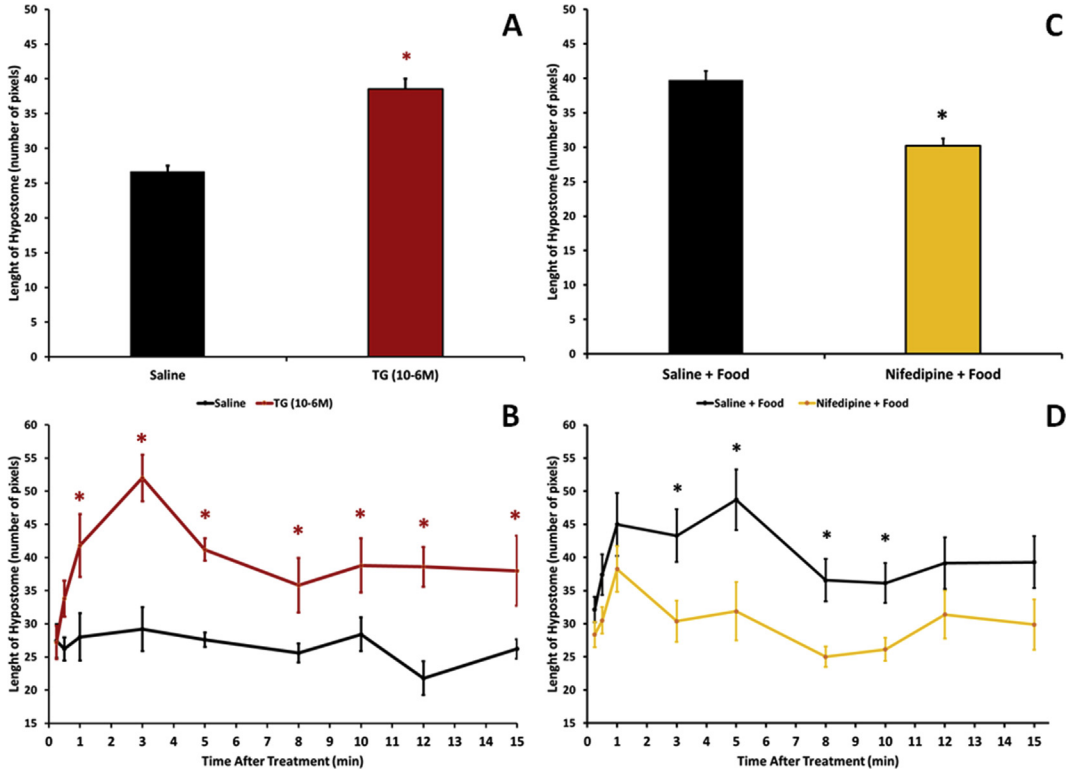

Fig. 3. Effect of modulation of the cytosolic Ca^{2+} levels on the length of the hypostome caused by TG and nifedipine in *Hydra* sp. (A) Average length of the hypostome in starved hydroids treated with the inhibitor of the sarcoplasmic calcium pump TG (10^{-6} M) for 15 min. (B) Changes in the length of the hypostome induced by TG treatment during the experiment. (C) Average length of the hypostome in starved hydroids exposed to food, and starved hydroids exposed to food in the presence of nifedipine (10 μM) for 15 min. (D) Changes in the length of the hypostome induced by treatment with food plus nifedipine during the experiment. Each point represents mean ± SE (n = 7). Asterisks represent significant differences between treated and control hydroids.

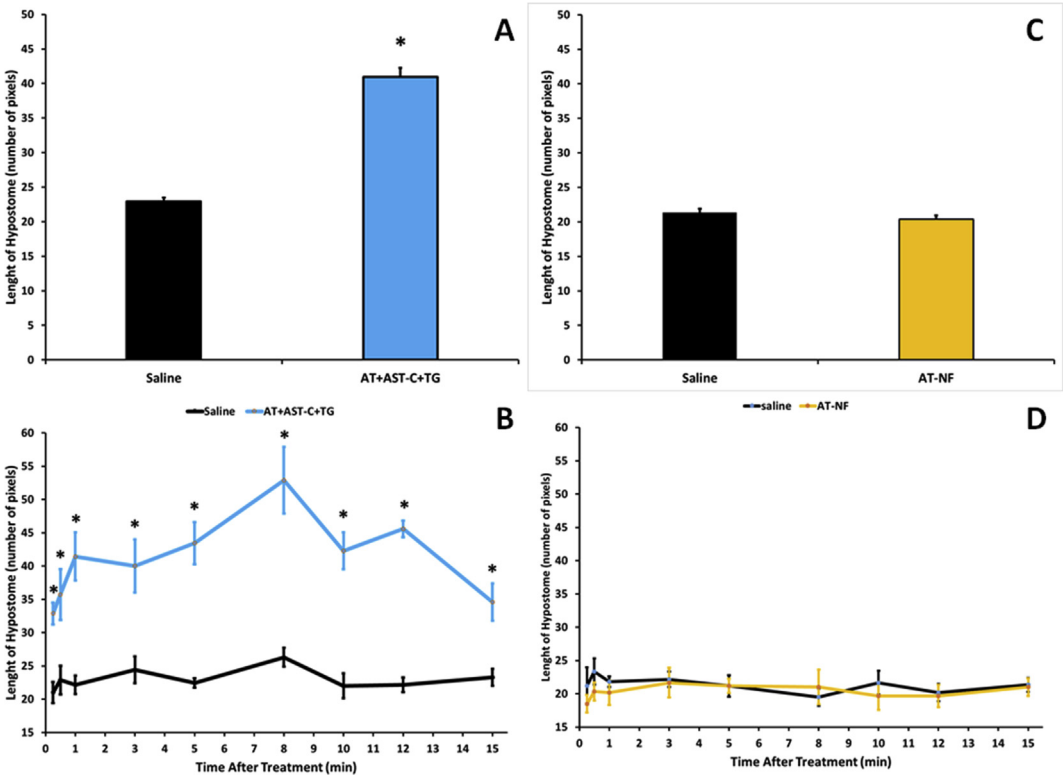

Fig. 4. Effect of the use of modulators of the cytosolic calcium levels on the activity of AT and AST-C. (A) Average length of the hypostome in starved hydroids induced by simultaneous treatment with both peptides in the presence of TG for 15 min. (B) Changes in the length of the hypostome induced by the simultaneous treatment with the peptides and TG during the experiment. Each point represents mean ± SE (n = 7). Asterisks represent significant differences between treatment and control. (C) Average length of the hypostome of starved hydroids exposed to AT plus nifedipine (10 μM) during 15 min. (D) Length of the hypostome in hydroids undergoing AT plus nifedipine (10 μM) treatment during the experiment. Each point represents mean ± SE (n = 6).

served in members of the AT/Ox receptor family. The first region consists in a segment of 10 amino acids located in the second intracellular loop. When compared with the insect consensus sequence, the *H. vulgaris* sequence showed percentages of identity and similarity of 80 and 90% respectively (Table 1). Interestingly, this sequence is the only one analyzed that presents an asparagine residue (N) instead of a tyrosine residue (Y). The other portion of the protein conserved is a 13 amino acids sequence that is part of the TM7 domain. This region showed a percentage of identity and similarity with the insect consensus of 61.5 and 76.9% respectively (Table 1).

An alignment of the sequences from non-bilaterian organisms, together with sequences of several species of protostomes is shown in the Supplementary File 2. A phylogenetic analysis of all the selected sequences revealed that *H. vulgaris* AT putative receptor clusters together with the all bilaterian sequences, while the other three cnidarian sequences cluster together with the sequences of the placozoan *T. adhaerens* (Fig. 5).

4. Discussion

Although AT and AST-C were originally characterized in insects, the existence of genes codifying for homologous peptides in species pertaining to other phyla of invertebrates was proposed (Veenstra, 2010, 2011). In addition, the presence of AT-like peptides has been described in neurons associated to muscle tissue in Platyhelminthes (Adami et al., 2011, 2012). AT induced contractions of the muscle fibers associated with the mouth and pharynx, revealing that AT-like systems are present in other groups of Protostomia (Adami et al., 2012).

Looking for the ancestral origin of allatoregulatory peptides, we have previously analyzed the presence of these systems in *Hydra* sp. In fact, by the use of QDot-conjugates we have shown that AT and AST-C are specifically recognized by different myoepithelial cells in several regions of the *Hydra* (Alzugaray et al., 2013, 2016). Using several types of assays, we showed that both peptides are able to elicit time and dose dependent myoregulatory effects, suggesting a physiological role of these peptides (Alzugaray et al.,

Table 1
Insect allatotropin receptors and putative orthologues identified in Cnidaria. The table includes the percentage of identity and similarity of the sequences considered as signatures for the AT/Ox receptor family and accession numbers. Red letters show amino acids residue identity. Blue letters show conservative and semiconservative changes.

Name	Second Intracellular Loop	% identity	% similarity	Accession
Insect Consensus	FISIDRWYAI	80.0	90	XP_012554847
Hydra vulgaris	||||:||:||			
	FISIERWNAI			
Name	TM7	% identity	% similarity	Accession
Insect Consensus	NSAVNPLIYNFMS	61.5	76.9	XP_012554847
Hydra vulgaris	||.:||||:.|.|			
	NSFINPLIFCFES			

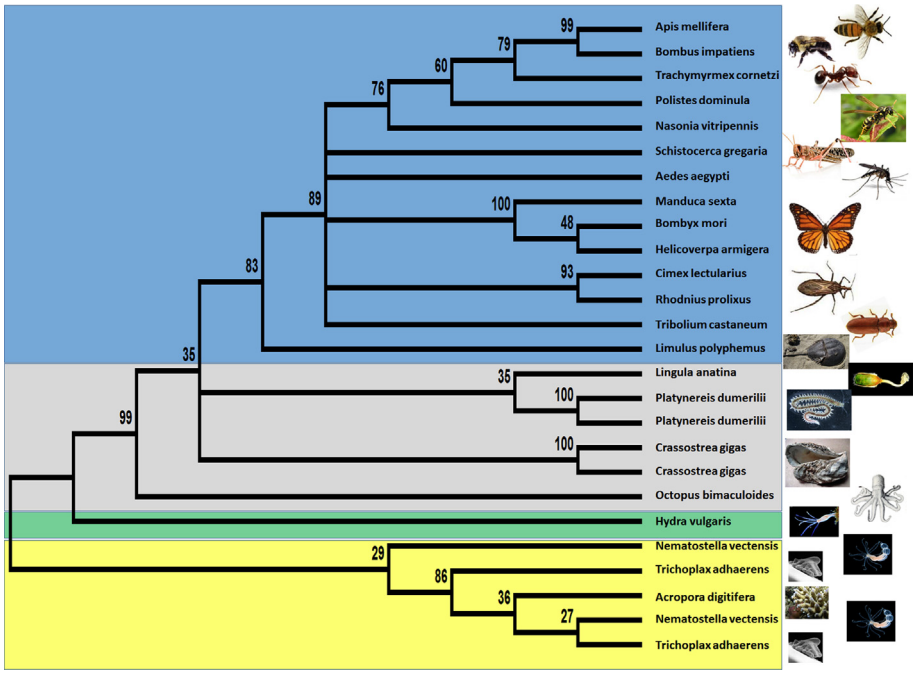

Fig. 5. Phylogenetic analysis of AT/Orexin receptors and probable orthologues in Cnidaria. The analysis was performed by Maximum likelihood methodology based on the Poisson correction model. The phylogram shows the relationships between different species of representative phyla of Metazoa including Arthropoda, Annelida, Brachiopoda, Mollusca, Cnidaria and Placozoa corresponding to the highest log likelihood (−8095.5722). Note that *H. vulgaris* clusters together with bilaterian groups suggesting a close relationship between sequences. Numbers on the branches indicate the percentage of trees in which the associated taxa clustered together.

2013, 2016). Altogether, these results suggest that AT and AST-C systems might be an ancestral trait, associated to prey capture and feeding behavior (Alzugaray et al., 2013, 2016; Elekonich and Horodysky, 2003).

As mentioned above, the populations of myoepithelial cells that recognize AT and AST-C are different, as well as the responses caused by the peptides. While AST-C induced changes in the total length of the body, tentacles and gastrovascular cavity, AT was mainly responsible for the stimulation of the extrusion of the hypostome, mimicking the reaction of the hydroids in the presence of food (Alzugaray et al., 2013, 2016). Furthermore, the response induced by food was prevented by treatment with an AT-antiserum, supporting the involvement of an AT-like system in this process (Alzugaray et al., 2013).

Proteins sharing high levels of identity and similarity with the AT/Ox and AST-C/SST families of GPCRs have been predicted in Cnidaria, suggesting the existence of this type of receptors in non-bilaterian groups (Alzugaray et al., 2013, 2016). In the present study we described a new sequence from *Hydra* that shares a high level of identity and similarity in two critical conserved domains that might be considered as signatures for this family of GPCRs (Ronderos et al., 2016; Verlinden et al., 2015). Interestingly, this sequence is the only one analyzed that presents an asparagine residue (N) instead of a tyrosine residue (Y). We would like to propose that this change is a sequencing artifact. In fact, when the codons for asparagine and tyrosine are compared, differences are present only in the first nucleotide of the codon. When a phylogenetic analysis was performed, the tree with the highest log likelihood clustered this sequence together with those of bilaterian groups, sharing a common ancestor with another cluster that includes other species of the phylum Cnidaria and Placozoa.

In the current study we analyzed the responses of the hypostome to treatments with AT and AST-C, as well as with different combinations of both peptides under different physiological conditions. Our assays confirmed that the presence of food has a stimulatory effect on the extrusion of the hypostome, inducing a significant increment in the length of this structure. Similarly, the treatment with AT also induced an increment in the length of the hypostome that was sustained along the entire period assayed, clearly mimicking the behavior induced by the presence of food, and suggesting that an AT-like system is involved in the regulatory mechanisms associated with feeding.

We recently reported that AST-C induces dose and time-dependent changes in several regions of the body of starved hydroids (Alzugaray et al., 2016). Interestingly this peptide did not show any relevant effect on the hypostome of the starved hydroids, causing only a slight increment in the length with the maximum concentration assayed (data not shown). When AST-C (10^{-6} M) was applied on hydroids stimulated with food, the peptide prevented the hypostome extrusion, suggesting that the peptide is also modulating feeding behavior.

Allatotropin and allatostatins were originally characterized by their opposite effects on the activity of the CA. In fact, the two peptides used in these experiments are involved in the regulation of the JH synthesis in mosquitoes (Li et al., 2003, 2004, 2006). Furthermore, it was proposed that the increment of the cytosolic Ca^{2+} levels is involved in the activity of AT on the JH synthesis in *Heliothis virescens* (Rachinsky et al., 2003).

Regarding the myoregulatory activity of both peptides, it has recently been shown the existence of an antagonistic effect between AST-C and AT in insects. In fact, AT has myostimulatory effects in two species of kissing bugs (Sterkel et al., 2010; Villalobos-Sambucaro et al., 2015), and AST-C decreases the frequency of contractions in *R. prolixus* that was counteracted by treatment with TG (Villalobos-Sambucaro et al., 2016). This fact suggests that the effect of this peptide involves changes in the cytosolic Ca^{2+} levels. The present studies suggest that AT and AST-C also have opposite myoregulatory effects on the hypostome extrusion in Cnidaria. When starved hydroids were treated with both peptides simultaneously, the response of the hypostome was completely inhibited at the beginning of the experiment, increasing gradually, showing that AST-C transiently inhibits the

effect caused by AT. The reversible nature of AST-C action suggests a physiological role for the peptide.

Although the involvement of Ca^{2+} in the mechanisms of contraction of myoepithelial cells in Cnidaria is not fully understood, Lin and Spencer (2001a,b) proposed that in the striated muscle of the jellyfish *Polyorchis penicillatus* (Cnidaria: Hydrozoa) both, intracellular stores and the influx from the extracellular space mediated by voltage-dependent Ca^{2+} channels are involved. To understand the involvement of this ion in the extrusion of the hypostome, we analyzed it behavior in the presence of TG, a compound that inhibits the reuptake of Ca^{2+} into the sarcoplasmic reticulum, inducing an increment of its cytosolic levels (Kijima et al., 1991; Lytton et al., 1991), and by the use of nifedipine, a blocker of voltage-dependent Ca^{2+} channels that prevent the influx of this ion into the cell (Cognard et al., 1990; Zsotér and Church, 1983). While, similarly to that observed in those individuals challenged to food, the addition of TG to starved hydroids caused an increment in the length of the hypostome, nifedipine applied to hydroids exposed to food prevented this process, showing that both mechanisms are relevant in the extrusion of the hypostome in *Hydra* (i.e. release from intracellular stores and extracellular influx).

The inhibitory effect of AST-C on the extrusion of the hypostome induced by AT, was analyzed on hydroids treated simultaneously with both peptides plus TG. The presence of TG clearly eliminated the effect of AST-C, suggesting that this peptide causes a reduction in the levels of cytosolic Ca^{2+}. Although there is no information about the *in vivo* mechanism of action of AST-C, it is known that its receptor is an orthologue of the SST receptors in vertebrates. Somatostatin, originally characterized by its inhibitory effect on the secretion of growth hormone, acts mainly through a reduction of cytosolic Ca^{2+} levels (Barbieri et al., 2013; Farrell et al., 2014; Patel, 1999). On the other hand, it was proposed that the action of *Manduca sexta* AT on the CA is mediated by the increment of cytosolic Ca^{2+} levels (Rachinsky et al., 2003). Our studies revealed that nifedipine abolished the stimulatory effect of AT on hypostome extrusion, suggesting that the peptide is facilitating the influx of the ion from the extracellular space.

In spite of the presence of these peptides in *Hydra* sp. could not be yet proved, due to the difficulties of analyzing highly diverse pro-peptides, the relationships among different families of peptides is supported by the orthology of their receptors and similar roles (Jékely, 2013). In summary, our studies strongly suggests that AT/Ox and AST-C/SST like systems were conserved during the evolution of Metazoa, and are present in a non-bilaterian phylum like Cnidaria, regulating the behavior of feeding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2017.07.020.

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