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Distribution and characterization of Corazonin in the central nervous system of *Triatoma infestans* (Insecta: Heteroptera)

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ABSTRACT

The distribution of corazonin in the central nervous system of the heteropteran insect *Triatoma infestans* was studied by immunohistochemistry. The presence of corazonin isoforms was investigated using MALDI-TOF mass spectrometry in samples containing the brain, the subesophageal ganglion, the corpora cardiaca–corpus allatum complex and the anterior part of the aorta. Several groups of immunopositive perikarya were detected in the brain, the subesophageal ganglion and the thoracic ganglia. Regarding the brain, three clusters were observed in the protocerebrum. One of these clusters was formed by somata located near the entrance of the ocellar nerves whose fibers supplied the aorta and the corpora cardiaca. The remaining groups of the protocerebrum were located in the lateral soma cortex and at the boundary of the protocerebrum with the optic lobe. The optic lobe housed immunoreactive somata in the medial soma layer of the lobula and at the level of the first optic chiasma. The neuropils of the deutocerebrum and the tritocerebrum were immunostained, but no immunoreactive perikarya were detected. In the subesophageal ganglion, immunostained somata were found in the soma layers of the mandibular and labial neuromeres, whereas in the mesothoracic ganglionic mass, they were observed in the mesothoracic, metathoracic and abdominal neuromeres. Immunostained neurites were also found in the esophageal wall. The distribution pattern of corazonin like immunoreactivity in the central nervous system of this species suggests that corazonin may act as a neurohormone. Mass spectrometric analysis revealed that [Arg^7]-corazonin was the only isoform of the neuropeptide present in *T. infestans* tissue samples.

1. Introduction

Chagas' disease is endemic in Latin America. The prevalence of the infection caused by the parasite *Trypanosoma cruzi* is 16–18 million cases, and the World Health Organization [45] estimated that 120 million people are at risk of developing this disease. *Triatoma infestans*, a blood feeding heteropteran, is the main vector of Chagas' disease in Argentina and neighboring countries. Despite the epidemiological importance of this species, knowledge on the structure and function of its nervous system is still limited. Morphological studies on the central nervous system (CNS, [1,12,13]) were followed by descriptions of the distribution of a number of neuropeptides and neurotransmitters [26–30]. Strategies aimed at combatting this vector insect are challenged by the appearance of resistance to current chemical agents in certain populations [19,38]. Knowledge on the presence of neuropeptides and their receptors may contribute to the design of new drugs to control this disease-vector insect and related species, which cause infections to humans and cattle.

Neuropeptide corazonin (CRZ, [pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-amide]) was first isolated from the corpora cardiaca of the cockroach *Periplaneta americana* [39]. Corazonin production has been localized in lateral brain neurosecretory cells, which project to the corpora cardiaca–corpora allata complex, and in ventral nerve cord neurons [24,43]. Six CRZ isoforms are known to occur in insect species from different orders [2,22]. The [Arg^7]-CRZ isoform has been isolated from the cockroaches *P. americana* and *Nauphoeta cinerea*, the sphinx moth *Manduca sexta*, the silkworm *Bombyx mori* and the cricket *Gryllus bimacu-*

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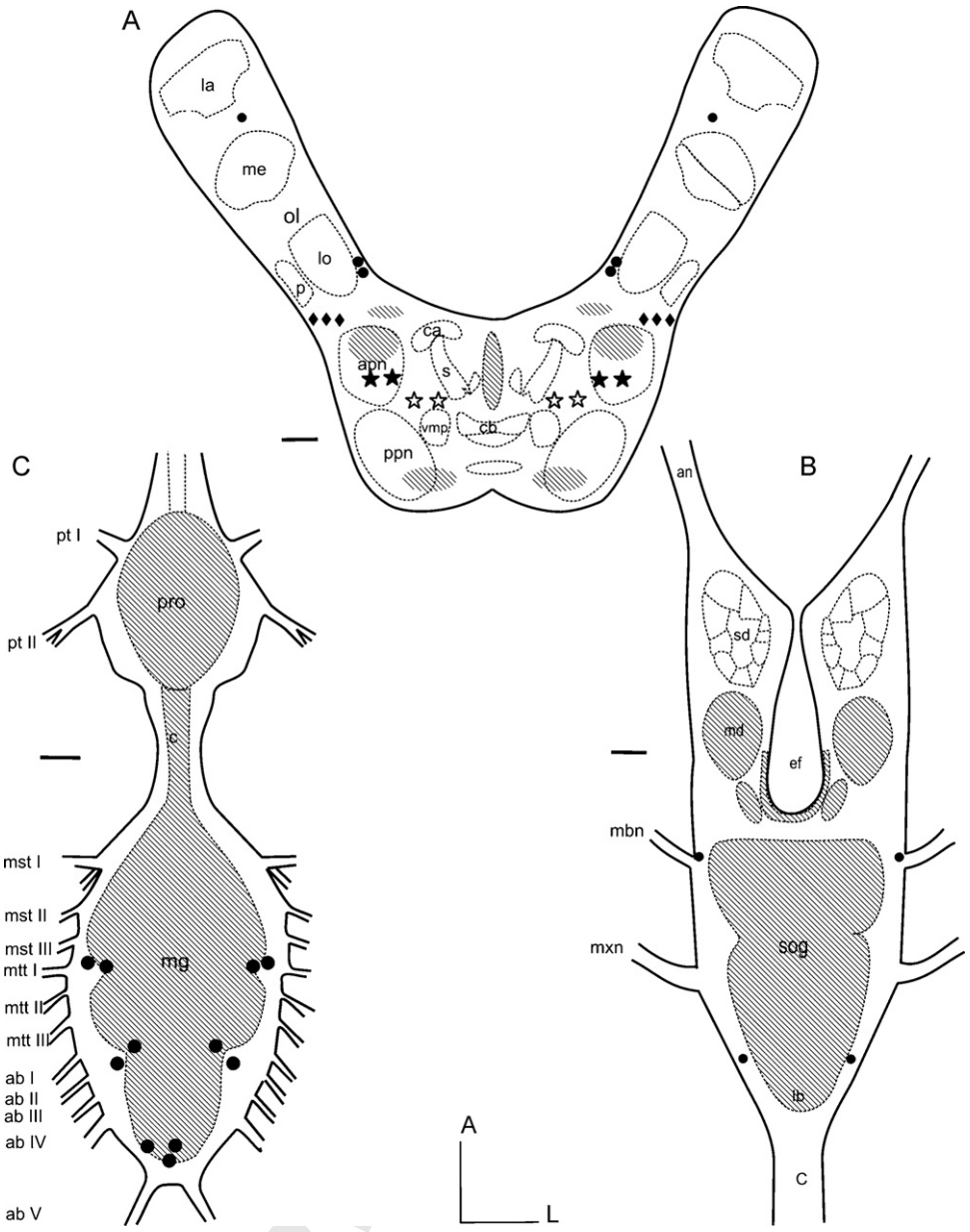

Fig. 1. Schematic drawings of dorsal views of *Triatoma infestans* protocerebrum with optic lobes (A), deutocerebrum (upper part of B), subesophageal ganglion (lower part of B) and thoracic ganglia (C) showing CLI. In A, immunoreactive somata of the A1, A2 and A3 groups are marked by,☆, ⋆, ♦ respectively. Circles represent immunoreactive somata other than those of the A1–A3 clusters; shadowings in neuropils indicate the location of immunolabeled processes. *Abbreviations*: ab I–V (abdominal nerves I–V), an (antennal nerve), apn (anterolateral protocerebral neuropil), ca (calyx of mushroom body), c (connectives), cb (central body), ef (esophageal foramen), la (lamina ganglionaris), lb (labial neuromere), lo (lobula complex), md (mechanosensory and motor deutocerebrum), mdn–mxn (mandibular, maxillary nerves), me (medulla), mst I–III (mesothoracic nerves I–III), mtta I–III (metathoracic nerves I–III), ol (optic lobe), p (lobula plate), ppn (posterolateral protocerebral neuropil), pro (prothoracic ganglion), mg (mesothoracic ganglionic mass), pt I–II (prothoracic nerves I–II), sd (sensory deutocerebral glomeruli), sog (subesophageal ganglion), s (stalk), vmp (lateromedial protocerebral neuropil). Anterior (A), lateral (L). Bars: 100 μm.

latus [11,39,40]. Moreover, in the fruitfly *Drosophila melanogaster* and in the waxmoth *Galleria mellonella* [Arg^7]-CRZ pre-prohormone has been cloned [8,41]. The widespread distribution of this isoform in insects, suggested that it appeared early during the evolution of insects [22].

[His^7]-CRZ has been found in the corpora cardiaca of the locusts *Schistocerca americana* [39], *S. gregaria* and *Locusta migratoria* [37] as well as in the retrocerebral complex of the stick insect *Carausius morosus* [22]. It was also found that in *Apis mellifera* [His^7]-CRZ, the glutamine^4 residue was substituted by a threonine, a fact which suggested the possible existence of further variations in the sequence of this neuropeptide [44]. Indeed, other sequence modifications of CRZ were recently reported in species of the insect orders Diptera, Hymenoptera and Mantophasmatodea [22].

Physiological experiments have shown different roles for CRZ. In *P. americana*, application of the neuropeptide increased the heart beat rate [20,32,39] and elicited the contractions of the hyperneural muscle and the antennal heart [20]. It was also found that in locusts, CRZ produced changes in body coloration [35,37]. Other functions ascribed to CRZ were the reduction of the rate of silk spinning in the larval–pupal transition of *B. mori* [36] and the induction of the release of pre-ecdysis triggering hormones in *M. sexta* [14]. From the available experimental data, it appears that no common function may be attributed to CRZ [2,42].

In this context, studies on the distribution of CRZ in insect species with different behaviors become relevant. In view of the fact that the presence of CRZ is unknown in *T. infestans*, as well as in other hematophagous insect vectors, we investi-

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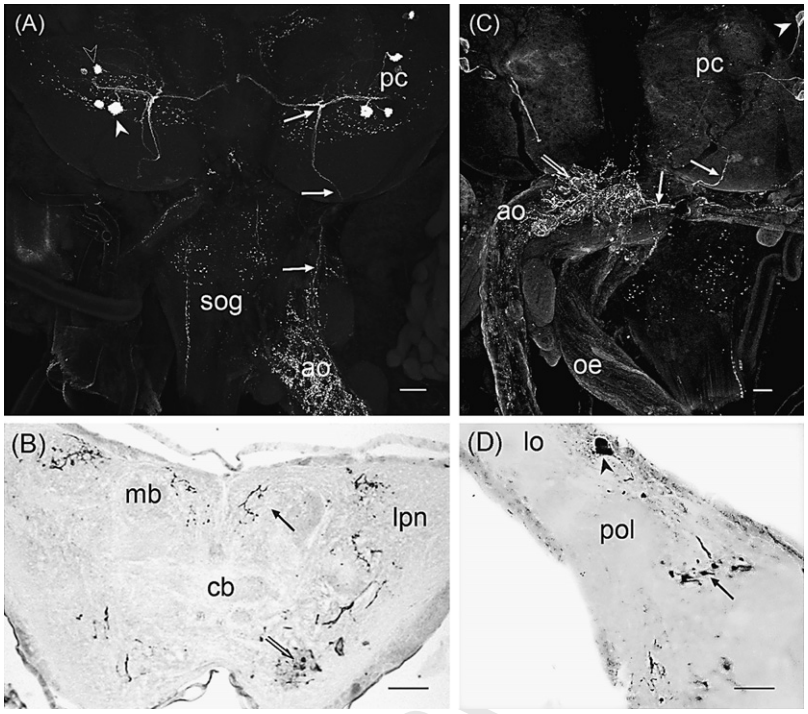

Fig. 2. Confocal immunofluorescence (A and C) and bright field micrographs showing CLI in the protocerebrum (b) and optic lobe (d). (A) Immunoreactive perikarya (arrowheads) and neurites in the protocerebral (pc) lobes. Filled arrowhead marks a perikaryon of the A1 cluster while open arrowhead points to a soma of the A2 group. Arrows point to fibers with origin in pc somata traveling to the aorta (ao). sog, subesophageal ganglion. (B) Immunostained neurites in the protocerebrum. Arrow points to positive neurites close to the median line while open arrow marks neurites in the posterior protocerebrum. cb, central body; lpn, lateral protocerebral neuropil; mb, mushroom bodies. (C) CLI in the protocerebrum and retrocerebral complex (lateral view). Arrowhead points to an immunostained perikaryon of the A2 cluster. Filled arrows mark the course of an immunostained axon from the protocerebral (pc) lobes to the corpora cardiaca–corpus allatum complex. An open arrow points to neurites in the aorta (ao). oe, esophagus. (D) An immunopositive soma (arrowhead) and its emerging neurite in the medial soma rind of the optic lobe. Arrow marks positive neurites in the proximal optic lobe (pol). lo, optic lobula. Anterior is to the top of each figure. Bars: 50 μm.

gated the distribution of CRZ-like immunoreactivity (CLI) with immunohistochemistry. Recent advances in mass spectrometry allow the identification of neuropeptides in the insect nervous system, providing information for physiological investigations and phylogenetic analyzes [6,21,23]. With this tool, several neuropeptides have been characterized in species of the order Heteroptera [17,23]. Information about the distribution pattern and amino-acid sequence is required to advance into the study of the function of CRZ in triatomine bugs.

In the present study, we describe the distribution pattern of CLI in the CNS of *T. infestans* and identify [Arg^7]-CRZ as the isoform of the neuropeptide present in this species.

2. Materials and methods

2.1. Insects

Male adults of *T. infestans* were used in this study. The insects, free of *T. cruzi* and *Blastocrithidia triatomae*, were provided by the Center for the Control of Chagasĩ disease (Santa María, Córdoba, Argentina). They were housed under controlled conditions of light (L:D = 12:12, lights on at 6.00 a.m.), temperature (27 ± 1 °C) and relative humidity (70–80%) [25].

2.2. Dissection and fixation

The insects (n = 70) were processed for immunohistochemistry two weeks after last feeding. The dorsal cuticle of the head and thorax was quickly removed and the tissues were immediately flushed with ice-cold fixative, a mixture of formalin and picric acid (4% paraformaldehyde and 0.4% picric acid; [46]) in 0.16 M sodium phosphate buffer (PB), pH 6.9. Dissection of the brain and ganglia proceeded with the tissues bathed in the cold fixative. Samples remained in the same fixative overnight, at 4 °C. After that, they were rinsed several times in 0.01 M PB saline (0.13 M NaCl in PB at pH 7.4, PBS).

2.3. Immunohistochemistry

Whole-mount preparations (n = 10) of the brain, subesophageal (SOG) and thoracic ganglia were transferred to PBS containing 1% Triton X100 (PBST) for at least 48 h. After that, they were immersed in PBS containing 10% normal donkey serum for 1 h in order to diminish non-specific binding. The same solution was also used to dilute the primary and secondary antisera. The samples were incubated overnight at room temperature (RT) with anti-CRZ antibodies (a gift from Dr. J Veenstra, Université Bordeaux I, Talence, France) diluted 1:1500. The polyclonal antiserum was obtained by injecting rabbits with a conjugate prepared by coupling corazonin via its tyrosine residue to bovine serum albumin with 1,5-difluoro-2,4-dinitrobenzene [43].

After the incubation of the whole mounts with the primary antiserum, they were rinsed in PBST and subsequently incubated overnight at RT with tetramethyl-rhodamine isothiocyanate (TRICT)-conjugated goat anti-rabbit secondary antibodies (1:200, Jackson ImmunoResearch Labs, PA, USA). Brains and ganglia were passed through solutions from 20% to 80% glycerol in 0.05 M sodium carbonate–bicarbonate buffer, pH 7.0. The samples were observed with a Nikon PCM 2000 laser-scanning confocal microscope, mounted on an E800 microscope equipped with green He/Ne (543 nm), red He/Ne (633 nm) and argon (488 nm) lasers and the appropriate filters. Stacks of digitized images were merged and

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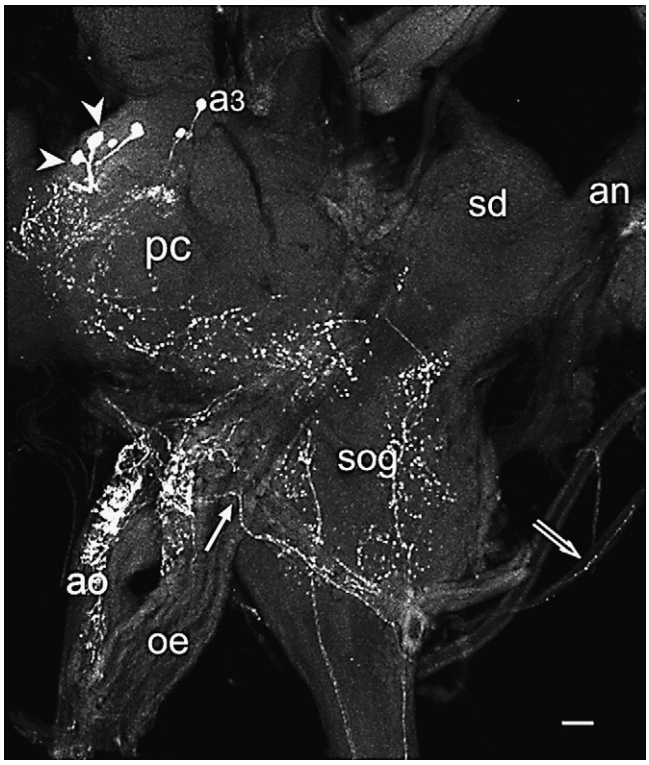

Fig. 3. Confocal immunofluorescence micrograph showing CLI in the protocerebrum (pc), deutocerebrum and subesophageal ganglion (sog). Note the location of the A3 (a3) cell-body cluster in this lateral view of a pc hemisphere. Arrowheads point to somata belonging to the A1 group. A filled arrow marks the course of an immunopositive neurite which after traversing the esophagus (oe), enters the subesophageal ganglion (sog). The antennal lobe (sd) and the antennal nerve (an) show no CLI. Note the high density of immunoreactive neurites in the aorta (ao). An open arrow marks the peripheral course of a positive neurite. Bar: 50 μm.

processed using Simple PCI 3.2 software (Compix Inc., Cranberry Township, PA, USA) as image acquisition software. If required, images were modified only to enhance contrast (Adobe Photoshop, Adobe Systems Inc., San José, CA, USA).

Serial horizontal cryostat sections (Microm, Waldorf, Germany) from 60 insects were processed according to the avidin–biotin immunoperoxidase protocol (ABC, [10]). Before sectioning, the tissues were passed through increasing concentrations of sucrose (5–30%) in PBS containing 0.02% bacitracin (Sigma–Aldrich, St. Louis, MO, USA) and 0.01% sodium azide (Merck, Darmstad, Germany) [26] for cryoprotection. The sections (16 μm) were incubated overnight with CRZ antiserum diluted 1:2000. After that, the slides were rinsed in PBS, incubated at RT for 30 min in biotinylated goat anti-rabbit secondary antibodies (1:100, Vector Laboratories, Burlingame, CA, USA), rinsed again in PBS and further incubated for 1 h in ABC reagent (Vectastain Elite kit, Vector Laboratories). Peroxidase activity was revealed by reaction with 3,3′-diaminobenzidine tetrahydrochloride (Sigma–Aldrich) using hydrogen peroxide (0.005%) and nickel salts for enhancement of the reaction product [31]. The sections were dehydrated and mounted with Permount medium (Sigma–Aldrich) and photographed using a Nikon E800 microscope equipped with a Nikon DN100 digital-net camera. Images were modified only to enhance contrast (Adobe Photoshop, Adobe Systems Inc.).

2.4. Controls

The specificity of the CRZ antiserum was tested by overnight preincubation of synthetic corazonin (pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-NH_2, Sigma–Aldrich) at a concentration of 10 μg/ml in the antiserum diluted 1:2000. After the incubation of the sections with the preabsorbed antiserum, they were processed following the ABC method as stated above. Other controls were performed by incubating the sections without either the primary or the secondary antisera.

2.5. Mass spectrometry

2.5.1. Sample preparation

The brains and the SOGs of adult insects ($n = 30$) were quickly dissected out from surrounding tissues. Groups of 5 brains together with SOGs were placed in sterile tubes containing 50 μl of 0.01 M PBS plus a protease inhibitor cocktail (Sigma–Aldrich, P2714). Samples were stored at −70 °C until assayed. Special care was taken to include in each of the samples the corpora cardiaca–corpus allatum complex (CC–CA) together with the adjoining cephalic portion of the aorta.

2.5.2. MALDI-TOF mass spectrometry analysis

Samples were extracted in the solution they were stored using a glass microhomogenizer and the protein concentration was determined [3]. 1 μl protein sample was mixed with 1.0 μl of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 0.1% TFA in 1:1 acetonitrile/methanol), delivered to the target plate and dried at room temperature. MALDI-TOF mass spectra were acquired in a positive-ion mode first on a MALDI micro MX (Waters, Milford, USA), equipped with a pulsed nitrogen laser emitting at 337 nm. Samples were analyzed in the reflectron mode using a delayed extraction time of 150 ns, 75% grid voltage, 0.06% guide wire voltage and an accelerating voltage of 20 kV. Mass spectrometry analysis was performed at the UNIPROTE-MS facility, Center of Biotechnology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

In order to compare to the native CRZ present in the samples of adult insects, standard synthetic CRZ (pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-NH_2, MW 1369.44, Sigma–Aldrich) was analyzed by mass spectrometry under the same conditions. For the external calibration, a polyethylene glycol mix (PEG, 400, 1000 and 2000) was used. Due to the nature of the samples, all acquisitions were taken in manual mode and each spectrum was produced by accumulating data from 100 consecutive laser shots.

3. Results

The central nervous system of *T. infestans* is composed of the brain, the subesophageal ganglion (SOG) and two thoracic ganglia. The head of this triatomine insect is tubular and compressed laterally. Due to the huge development of the pharyngeal pump muscles, the brain and the SOG lie posteriorly in the postocular region of the cephalic capsule. Upon removal of the cuticle of the frons, the protocerebral hemispheres and the optic lobes can be observed (Fig. 1A). Each optic lobe extends anterolaterally, beyond the anterior margin of the protocerebrum (PC), to reach the ipsilateral compound eye. In this dorsal view, the deutocerebrum (DC, Fig. 1B), the tritocerebrum (TC) and the anterior third of the SOG are not visible, as they are concealed by the dome-shaped protocerebral lobes.

The thoracic ganglia comprise the prothoracic ganglion (PRO) and the mesothoracic ganglionic mass (MG), situated near the prosternum and the mesosternum respectively (Fig. 1C). The MG represents the fused mesothoracic, metathoracic and abdominal ganglia.

3.1. Corazonin-like immunoreactivity in the brain

The distribution of CLI in cell bodies and fibers is depicted in Fig. 1A and B. Few immunoreactive somata were detected in

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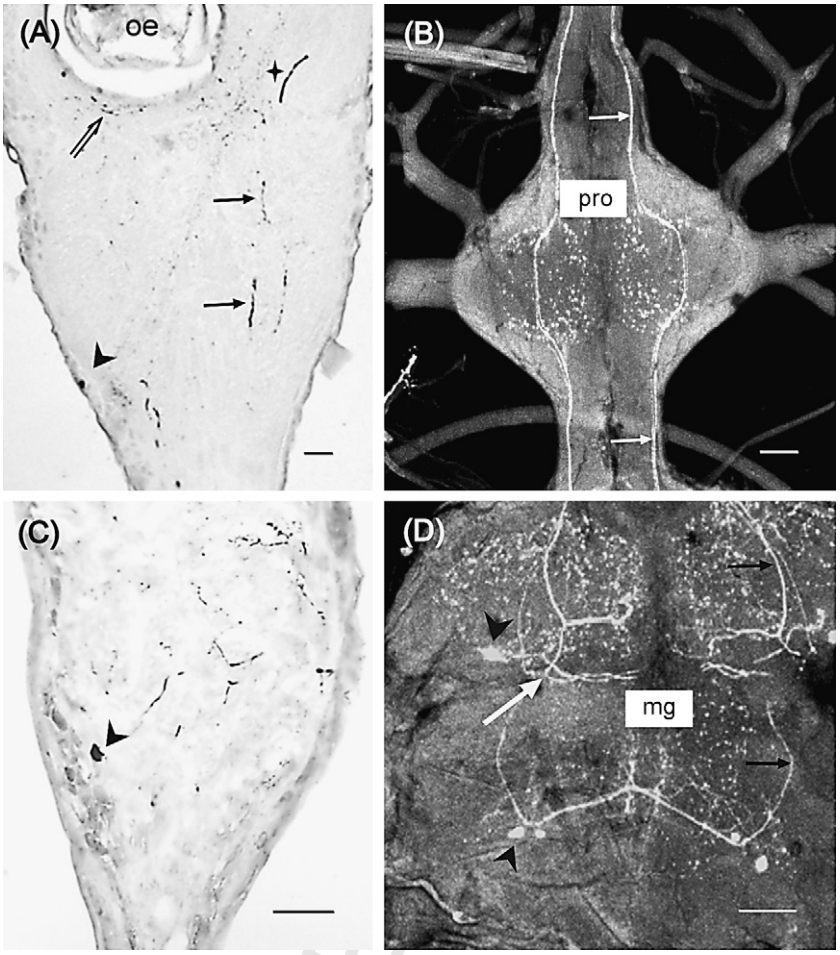

Fig. 4. Bright field (A and C) and confocal immunofluorescence micrographs (B and D) showing CLI in the subesophageal ganglion (sog, A) and thoracic ganglia (B–D). (A) Immunoreactive neurites bordering the esophageal foramen (open arrow) and in the neuropil of the sog (arrows). An arrowhead points an immunostained soma in the lateral soma rind of the labial neuromere. A star marks an immunoreactive neurite in the deutocerebral neuropil. (B) Immunostained neurites in the neuropil of the prothoracic ganglion (pro) and fiber tracts connecting the cephalic and posterior connectives (arrows). (C) Parasagital section of the mesothoracic ganglionic mass showing an immunoreactive soma in the dorsal soma rind (arrowhead) and positive neurites in the neuropil. (D) Immunostained somata (black arrowheads) in the mesothoracic and metathoracic neuromers of the mesothoracic ganglionic mass (mg). Black arrows mark the course of a bilateral immunopositive neurite while a white arrow points to an immunostained commissure. Anterior is to the top of figures. Dorsal side is to the left of plate. (C) Bars: 50 μm.

the brain. Three groups of somata showing CLI were observed in the protocerebrum which were termed A1, A2 and A3. The A1 cluster was formed by two intensively immunostained perikarya (Figs. 1A and 2A), which were located in a dorsomedial position near the entrance of the ocellar nerves. One soma of the A1 cluster was large (about 25 μm) and showed granular deposits of the immunoreactive material. Perikarya of this cluster originated two branches. One of them was observed running dorsally to the central body toward the median line where it ended close to the contralateral partner. The other branch was seen traveling posteriorly, and after leaving the brain it ramified in the cephalic aorta and in the corpora cardiaca (Fig. 2A and C).

The A1 cluster gave also origin to an immunopositive branch which spread into varicose fibers in the lateral protocerebrum and in the SOG (Figs. 2A, C and 3). The neuropils of both the mechanosensory-motor deutocerebrum and the tritocerebrum housed immunostained neurites (Figs. 3 and 4A).

The A2 cluster was formed by two immunolabeled somata located in an anterolateral position to the A1 group (Figs. 1A and 2A, C). Their neurites arborized in the lateromedial protocerebral neuropil. An immunopositive fiber originated in one of these cell bodies was observed running toward the A1 cluster (Fig. 2A). The A3 group was formed by three medium-sized immunostained somata (10–15 μm). They were detected in the dorsolateral cell-body layer, at the boundary of the protocerebrum with the optic lobe (Figs. 1A and 3).

The optic lobe housed three immunoreactive cell bodies (Fig. 1A). One of them was found at the level of the first optic chiasma. The remaining perikarya were observed in the medial cell-body layer close to the base of the lobula. An immunopositive fiber from one of these somata was seen running toward the PC neuropil (Fig. 2D).

Immunostained varicosities were observed along the median line of the protocerebrum (Fig. 2A and B), the anterolateral and posterolateral protocerebral neuropils and in the proximal optic lobe (Figs. 1A and 2B, D). The central body complex and the mushroom bodies showed no immunostaining (Fig. 2B).

No immunopositive perikarya were observed in cortical regions of the deuto- and tritocerebrum (Figs. 1B and 3A).

3.2. *Corazonin-like immunoreactivity in the subesophageal and thoracic ganglia*

In the SOG, immunoreactive somata were observed in the lateral soma cortices of the mandibular and labial neuromeres (Figs. 1B and 4A). Immunolabeled fibers were seen bordering the esophageal foramen (Figs. 3 and 4A).

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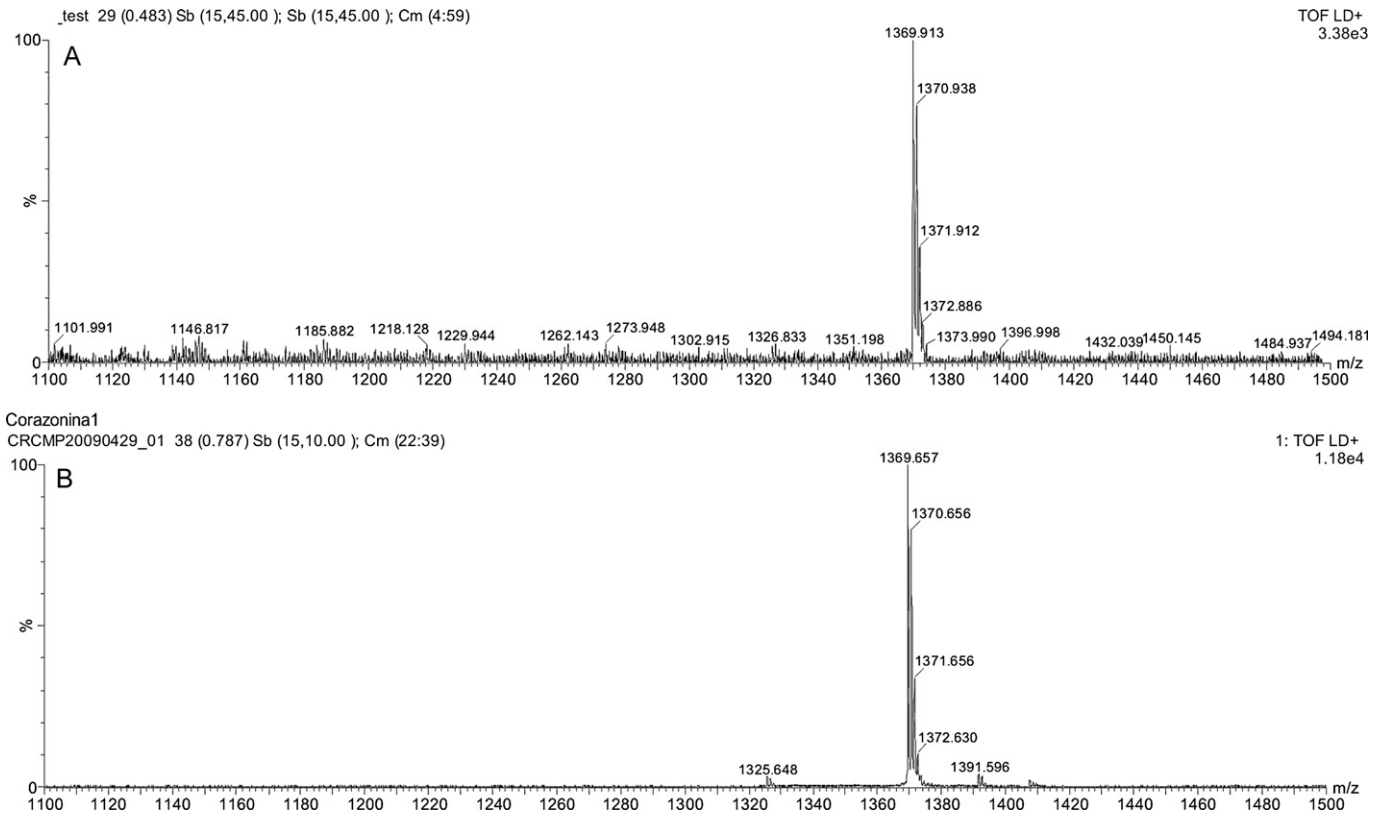

Fig. 5. (A) MALDI-TOF mass spectrum from a preparation containing tissues from the brain, subesophageal ganglion, corpora cardiaca–corpus allatum complex and adjoining cephalic portion of the aorta. A single peak signal at *m/z* 1369.913 nearly identical to the mass of [Arg^7]-CRZ is shown in (B). (B) Pattern obtained for standard [Arg^7]-CRZ analyzed under the same conditions, showing a peak signal at 1369.657.

Bilateral immunostained fibers were observed along the SOG, they gave rise to varicose branches in each hemineuromere (Figs. 2A, C and 3). After traveling through the cephalic connectives they entered the PRO. These bilateral tracts could be traced backwards to the A1 cluster (Fig. 3).

No immunolabeled somata were detected in the PRO. Bilateral fibers arborized in the neuropil before entering the posterior connectives (Figs. 1C and 4B). Paired somata showing CLI were observed in the infoldings of soma layer between the neuromeres of the MG (Figs. 1C and 4C, D). Unpaired immunostained perikarya were only present in the abdominal neuromeres. The neuropil of this ganglionic mass contained a high density of immunoreactive varicose neurites. Bilateral immunopositive axons were observed running along the entire MG while commissures connected the mesothoracic and metathoracic hemineuromeres (Fig. 4D).

3.3. Other corazonin-like immunoreactive elements

The esophageal wall received immunoreactive axons from somata of the A1 group. Immunostained neurites were traced from the esophagus to supply both the dorsal and the ventral esophagus dilator muscles. The wall of the anterior third of the aorta housed a high density of varicose neurites (Fig. 3). Also, immunopositive fibers linked the aorta and the esophagus (Fig. 3).

3.4. Controls

No immunostained perikarya or neurites were found when the sections were incubated with the CRZ antiserum preabsorbed with the synthetic neuropeptide. Immunostaining of the tissues was abolished when the primary or the secondary antibodies were omitted in the corresponding solutions.

3.5. Mass spectrometry analysis of CRZ isoforms

Fig. 5A shows the MALDI-TOF spectra of sample homogenates containing the brain, SOG, CC–CA complex and the adjoining portion of the aorta. Fig. 5B displays the pattern obtained for standard [Arg^7]-CRZ (MW 1369.44) analyzed under the same conditions. Comparison of both spectra indicates a positive identification of [Arg^7]-CRZ in the sample. The four peaks shown in Fig. 5A represent the isotopic pattern of [Arg^7]-CRZ.

4. Discussion

Since the first description of CLI in *P. americana* by Veenstra and Davis [43], various studies have contributed to increase our knowledge on the distribution pattern of this neuropeptide in insects [4,7,24,40,47].

In the disease vector insect *T. infestans*, CLI is present in the retrocerebral neuroendocrine system and in interneurons. In these triatomine bugs, the finding of immunoreactive somata of the lateral PC with ipsilateral projections to the retrocerebral complex is in agreement with previous reports in species from five insect orders [24]. It also provides additional evidence for the hypothesis that the pattern of CLI in the retrocerebral complex is highly conserved in insects.

Instead, in other regions of the brain and in the mesothoracic ganglionic mass differences in the pattern of CLI were found between the phytophagous bug *Pyrrhocoris apterus* and *T. infestans*. These differences suggest the possibility of a species-specific pattern of CLI in the aforementioned regions within the heteropterans. However, as different immunohistochemistry protocols have been used in our study and in that by Roller et al. [24], it is possible that at least some differences in the immunostainings may be the result of employing different methodologies.

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Corazonin immunoreactive somata of the blowfly pars laterals have been classified according to their projection fields and colocalizations with other neuropeptides [7]. The pattern of projections shown by cells of the A1 cluster in *T. infestans*, resembles that of the blowfly PL-i cells. Comparison of the morphology and immunoreactivities of the A1 cells in both species, renders unlikely that in *T. infestans* these cells co-express CRZ-, <beta>-pigment-dispersing hormone and cholecystokinin-like immunoreactivities [29,30] as it occurs in the blowfly.

In the optic lobe of *T. infestans*, CLI was represented by somata at the level of the first optic chiasma and at the base of the lobula. These immunoreactive cell bodies bear no resemblance to those in which circadian-clock related proteins have been immunolocalized [29]. Optic lobe neuropils were devoid of immunostaining, only immunoreactive fibers from lobula perikarya were seen running toward the PC lobes. In other insect species, immunoreactive fibers were detected in relationship to the accessory medulla [18], suggesting that lobula somata showing CLI might play a role in the regulation of circadian rhythmicity. Although our observations do not provide support to a role of CRZ in the circadian periodicity, the possible coexistence of CLI with known clock proteins is an important matter to be investigated in *T. infestans*, particularly because some activity patterns have been well documented [15,25].

The localization by immunocytochemistry of a neuropeptide can give clues about its function. The mushroom bodies and the central complex of this triatomine species displayed no CLI. Neuroanatomical studies in insects have shown that these neuropils are related to the integration of information from different sensory modalities and the subsequent elaboration of the appropriate behavioral responses [9,16,33,34]. The antennal lobe of *T. infestans* lacked immunolabeled perikarya and only a few cell bodies were present in the optic lobe, which also showed unstained neuropils. Thus, it appears that CRZ is not involved in the processing of visual and olfactory information by integration centers of the protocerebrum. On the other hand, projections from somata of the A1 cluster supplied the cephalic aorta with numerous branches. The aorta is considered an important site of release of brain products. It is possible that in this region, CRZ is released into the hemolymph and may act as a neurohormone.

It was found that branches from a dorsal unpaired median neuron in the SOG are the source of CLI the antennal lobe and the tritocerebrum [43]. The antennal lobe of *T. infestans* was devoid of immunoreactive perikarya and fibers. Instead, the neuropils of both the antennal mechanosensory-motor center and the tritocerebrum contained immunolabeled fibers. Due to the fact that a similar immunoreactive SOG neuron was not detected in *T. infestans*, it appears that the A1 cluster is the only source of CLI in the deutocerebral and tritocerebral neuropils.

The presence of CRZ immunoreactive somata in the SOG has been reported in *P. americana* [43], *Ctenolepisma lineata*, *P. apterus* [24] and some termites [47]. The detection of immunostained fibers extending from the SOG to the tritocerebrum served to propose a link between both neural areas in *Neotermes castaneus* [47] and *P. americana* [43]. In our triatomine bugs, the SOG and the tritocerebrum receive immunoreactive neurites from somata of the A1 cluster. A local link between both neural regions seems unlikely as ascending immunolabeled neurites from the SOG to the tritocerebrum were not detected.

Bilateral CRZ immunoreactive longitudinal tracts and bilateral serially distributed interneurons have been reported in the ventral nerve cords of different insect species [4,5,43]. In *T. infestans*, bilateral axons having CLI were observed along the neuropils of both thoracic ganglia; immunoreactive perikarya were found only in the MG. CRZ immunoreactive somata of the ventral nerve cord disappear during the metamorphosis of blowflies and fruitflies [4,5]. Instead, these ventral nerve cord perikarya persist in most of the reported species, a fact which suggests the conservation of these immunostainings among different insect orders. Regarding the immunolabelings in neuropils and tracts, CRZ immunoreactive ascending projections from ventral cord neurons have been considered as a way of interaction between the latter and the protocerebral neurons. Conversely, in our specimens, ascending neurites were observed only in somata of the metathoracic neuromere and they could not be traced to the brain.

Structural conservation of CRZ in various insects suggests that this neuropeptide may be also present in hematophagous heteropterans. Here, MALDI-TOF mass spectrometric analysis from samples of adult *T. infestans* which included the brain, SOG, corpora cardiaca–corpus allatum complex and the cephalic portion of the aorta revealed (by mass-match) the presence of the [Arg^7]-CRZ isoform in this species. Mass spectrometric analysis of *Rhodnius prolixus* nymphal brains, without the corpora cardiaca–corpus allatum complexes, provided the sequence pQTFQYSRGWTN-NH_2 (m/z 1369.64) which also corresponds to [Arg^7]-CRZ [17]. Since the antiserum used in this study [43] was raised against the most abundant peptide of the corpora cardiaca of *P. americana* [Arg^7]-CRZ, the pattern of CLI observed in the brain, SOG and retrocerebral complex of *T. infestans* correlates with the presence of the isoform detected by mass spectrometry. [Arg^7]-CRZ isoform is present in species from different genera of the subfamily Triatominae as well as in four members of the Pentatomidae [23]. Thus, these species may be appropriate experimental models to develop functional studies.

In conclusion, the presence of CLI in specific areas of the CNS of *T. infestans*, together with the pattern of some projections of immunoreactive somata suggest mainly a neurohormonal role for [Arg^7]-CRZ.

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