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Effect of the urease-derived peptide Jaburetox on the central nervous system of *Triatoma infestans* (Insecta: Heteroptera)

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ABSTRACT

Background: Triatoma infestans is the main vector of Chagas'disease in Southern Cone countries. In triatomines, symptoms suggesting neurotoxicity were observed after treatment with Jaburetox (Jbtx), the entomotoxic peptide obtained from jackbean urease. Here, we study its effect in the central nervous system (CNS) of this species.
Methods: Immunohistochemistry, Western blots, immunoprecipitation, two-dimensional electrophoresis, tandem mass spectrometry and enzymatic assays were performed.
Results: Anti-Jbtx antibody labeled somata of the antennal lobe only in Jbtx-treated insects. Western blot assays of nervous tissue using the same antibody reacted with a 61 kDa protein band only in peptide-injected insects. Combination of immunoprecipitation, two-dimensional electrophoresis and tandem mass spectrometry identified UDP-*N*-acetylglucosamine pyrophosphorylase (UDP-GlcNAcP) as a molecular target for Jbtx. The activity of UDP-GlcNAcP increased significantly in the CNS of Jbtx-treated insects. The effect of Jbtx on the activity of nitric oxide synthase (NOS) and NO production was investigated as NO is a recognized messenger molecule in the CNS of *T. infestans*. NOS activity and NO levels decreased significantly in CNS homogenates of Jbtx-treated insects.
Conclusions: UDP-GlcNAcP is a molecular target of Jbtx. Jbtx impaired the activity of *T. infestans* nitrergic system, which may be related with early behavioral effects.
General Significance: We report that the CNS of *Triatoma infestans* is a target for the entomotoxic peptide and propose that a specific area of the brain is involved. Besides potentially providing tools for control strategies of Chagas' disease vectors our data may be relevant in various fields of research as insect physiology, neurobiology and protein function.

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1. Introduction

Isoforms of urease from *Canavalia ensiformis* seeds possess fungitoxic and insecticidal properties [3,7,9,35]. This entomotoxicityis mostly due to the release of an internal peptide by the hydrolytic activity of insect cathepsin-like enzymes [8,12,25,27]. *Rhodnius prolixus* and *Triatoma infestans*, two of the triatomine vectors of Chagas' disease, belong to the group of insects in which digestion is based on cathepsin-like enzymes. An entomotoxic peptide derived from jackbean urease (JBU) with 93 amino acids was obtained by heterologous expression in *Escherichia coli* and named Jaburetox [39,22,26]. Two recombinant His-tagged versions of Jaburetox (Jbtx) were developed, Jbtx-2Ec [22] and Jbtx [26], differing from each other only by the presence of a V5

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viral epitope in the first one, both displaying potent and equivalent insecticidal activity [20].

A combination of molecular modeling, leakage experiments in large unilamelar vesicles and electrophysiological studies using planar lipid bilayers revealed that both, JBU and Jbtx, disrupt acidic lipid bilayers producing membrane permeabilization and display ion channel activity [2,20,24]. This mechanism may be relevant for the in vivo action of these molecules as entomotoxic agents [6].

The effects of JBU and Jbtx were tested in several insect species and their mechanism of action has been characterized in Malpighian tubules [36] and in the crop [37] of *R. prolixus*. In triatomines, several symptoms suggestive of neurotoxicity were observed after a meal of urease or Jbtx, which may indicate that the central nervous system (CNS) of the insect is a target of the entomotoxic molecules. Furthermore, RNAi experiments revealed that the urease toxicity is linked to a phospholipase A_2 XII gene, which is abundantly expressed in the *R. prolixus* CNS [10].

T. infestans, a blood feeding heteropteran, is the main vector of Chagas' disease in Argentina and neighboring countries. Morphological, biochemical and molecular biology approaches have been employed to study the CNS of this triatomine [30,31], making this species a suitable model for the investigation of neurotoxicity of entomotoxic molecules [29]. In this study, we investigated the effect of Jbtx in the CNS of *T. infestans*. The impairment of the nitrergic system of the insect and the role of UDP-*N*-acetylglucosamine pyrophosphorylase (UDP-GlcNAcP), a putative Jbtx binding protein in *T. infestans* CNS, are also discussed.

2. Materials and methods

2.1. Chemicals

ABC reagent (Vector Laboratories Inc., Burlingame, CA, USA), rabbit polyclonal universal nitric oxide synthase (uNOS) antibody (Thermo Scientific - Pierce, IL, USA) and biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Inc) were purchased from indicated commercial sources. ECL Advance Western Blotting Detection Kit; horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies; Hybond-P PVDF membranes; protein A-Sepharose and IPG strips were acquired from GE Healthcare (Buckinghamshire, UK). Bacitracin; 3,3′-diaminobenzidine tetrahydrochloride; Permount medium; protease inhibitor cocktail; vanadium chloride; mouse monoclonal anti-β-tubulin antibody and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Insects

Male adults of *T. infestans* were used in this study. The insects, free of *Trypanosoma cruzi* and *Blastocrithidia triatomae*, were supplied by the National Center for the Control of Chagas' disease (Santa María de Punilla, 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%). Fifth-instar insects of the phytophagous hemipteran *Dysdercus peruvianus* (cotton-stainer bug) from a colony established in our laboratory were used in this study. The colony was kept under controlled temperature (24 ± 2 °C) and humidity (70 ± 5%) and in a 14/10 h photoperiod (light/dark). The insects were fed on cotton (*Gossypium hirsutum*) seeds and had free access to water.

2.3. Jaburetox

The recombinant peptide used in this study, previously named Jbtx-2Ec [22], contained a 93 amino acid sequence derived from jackbean urease plus a V5 viral epitope and was produced according to Mulinari et al. [22]. We have shown that the presence of the V5 antigen did not contribute nor interfere with the insecticidal activity of the urease-derived peptide [20]. Thus here, with the purpose of simplification, we called Jbtx-2Ec simply Jbtx. The rabbit polyclonal anti-Jbtx antibody employed in the experimental approaches was obtained as reported previously [22].

2.4. Injections

The insects were weighed and placed ventral side up under a dissecting microscope. Injections of Jbtx (dose of 0.1 μg/mg body weight) in 20 mM sodium phosphate buffer (PB, pH 7.1) were performed with a Hamilton syringe attached to a stereotaxic instrument (David Kopf Instruments, CA, USA). Control insects were injected with an equivalent volume of PB. Jbtx or vehicle was injected between the third tergite and the first sternite, at the midline [39]. After the injection, control and treated insects were placed in individual containers where they were allowed to recover. All the experiments were performed 3 h after injections.

2.5. Immunohistochemistry

Jbtx- and vehicle-injected insects were processed for immunohistochemistry. They were placed under a dissecting microscope and the dorsal cuticle of the head and thorax was quickly removed. The head and thorax of 5 insects for each treatment were flushed with ice-cold fixative (4% paraformaldehyde and 0.4% picric acid in 0.16 M PB, pH 6.9) [32]. Dissection of the brain and ganglia proceeded with the tissues bathed in the cold fixative. Samples were pooled separately and remained in the same fixative overnight, at 4 °C. After that, they were rinsed several times in phosphate buffered saline (PBS, 6.6 mM Na2HPO4/KH2PO4, 150 mM NaCl, pH 7.4), passed through increasing concentrations (5%–30%) of sucrose in PBS plus 0.02% bacitracin and 0.01% sodium azide for at least 48 h at 4 °C until processed for immunohistochemistry [33]. The pools of brains and CNS ganglia from Jbtx- and vehicle-injected insect groups were serially sectioned with a cryostat (Microm, Waldorf, Germany) and mounted onto gelatin coated glass slides. After that, sections were processed according to the avidin–biotin immunoperoxidase protocol [16]. The sections (16 μm) were incubated overnight with the rabbit anti-Jbtx antibody (1:5000) [22]. After that, the slides were rinsed in PBS, incubated at room temperature for 30 min in biotinylated goat anti-rabbit secondary antibodies (1:100), rinsed again in PBS and further incubated for 1 h in ABC reagent. Peroxidase activity was revealed by reaction with 3,3′-diaminobenzidine tetrahydrochloride using hydrogen peroxide and nickel salts for enhancement of the reaction product [34]. The sections were dehydrated in graded ethanol series of 20, 40, 60, 90% (v/v) to absolute ethanol and mounted with Permount medium. Photographs were taken with a Nikon E800 microscope equipped with a Nikon digital sight DS-5Mc camera. Images were modified only to enhance contrast (Adobe Photoshop, Adobe Systems Inc). The experiment was repeated three times.

The specificity of the immunohistochemistry protocol was tested by overnight pre-incubation of the anti-Jbtx antibody diluted 1:5000 with 5 μM Jbtx. This pre-absorbed antibody was used for incubating sections which were further processed following the ABC method as stated above. Other controls were performed by incubating sections without either the first or the second antibodies

2.6. Western blot

For western blot assays, three groups containing 10 brains with the attached CNS ganglia from Jbtx-injected or control insects were processed. Tissue samples were obtained by dissecting the insects with the head and thorax constantly bathed in ice-cold sterile saline solution. Samples of each group were stored at −70 °C in 0.01 M PBS plus a protease inhibitor cocktail until assayed.

To confirm that the injected Jbtx had reached the CNS, tissue samples were homogenized, centrifuged at 10,000 *g* (10 min at 4 °C)

and the protein concentration in the supernatants was determined [4]. Twenty-five μg protein samples were fractionated by 13% SDS-PAGE [18] in the absence of reducing agent. Proteins were transferred to PVDF membranes and thereafter, they were incubated with rabbit polyclonal anti-Jbtx antibody (1:5000) followed by an anti-rabbit HRP-conjugated antibody (1:10,000). The bands were detected by enhanced chemiluminescence (ECL) using a commercial kit. To have a Western blot loading control, membranes were stripped in a buffer containing 0.2 M glycine, 0.1% sodium dodecyl sulfate, 1% Tween 20, pH 2.2 (room temperature, 2–10 min incubations) and washed. They were re-blotted with mouse monoclonal anti-β-tubulin antibody (1:3000) followed by incubation with an anti-mouse HRP-conjugated antibody (1:12,000) and further examined as previously described.

To evaluate the levels of nitric oxide synthase (NOS) protein in treated and control insects, tissues were processed as described above, and samples of 25 μg protein were subjected to 10% SDS-PAGE electrophoresis and transferred to Hybond-P PVDF membranes. Blots were probed with rabbit polyclonal uNOS antibody (1:500). Membranes were washed and incubated with an anti-rabbit HRP-conjugated antibody (1:10,000). Loading controls were performed using the anti-β-tubulin antibody as described above. The X-ray films were scanned and densitometric analyses were performed under conditions that yielded a linear response, with gray color scale used as standard (Scion Image software).

2.7. Immunoprecipitation, two-dimensional electrophoresis and tandem mass spectrometry

The dissected brains and CNS ganglia from 17 to 18 vehicle or Jbtx-injected insects obtained as stated in Section 2.6 were pooled, placed in sterile tubes containing 100 μl of a protease inhibitor cocktail and stored at −70 °C until assayed. Samples were processed in glass homogeneizers at a final volume of 300 μl containing the following protease inhibitors: 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 1 mM N-α-p-tosyl-1-lysine chloromethylketone (TLCK), 0.3 μM aprotinin and 1 μM pepstatin. Twelve μl of rabbit anti-Jbtx antibody was added to control and treated samples which contained a total amount of 250 μg and 280 μg protein (vehicle and Jbtx-injected insects, respectively). Incubation with the antiserum was performed under gentle agitation for 5 h. After that, 50 μl of protein A-Sepharose was added to each of the samples and left overnight under gentle agitation. Two-dimensional (2D) electrophoresis was carried out using Ettan IPGphor equipment (GE Healthcare) and 7 cm long, pH 3–10, IPG strips for both samples. Spots of interest were excised from the gels, dehydrated by lyophilization and the gels were digested with trypsin before analysis by liquid chromatography–electrospray ionization–quadrupole-time of flight (LC–ESI–QTOF). Briefly, 5 μl was applied on a Q-TOF Micro instrument (Waters Corporation) with a nanoflow electrospray probe, capillary voltage at 3500 V and a cone voltage of 30 V, calibrated in the m/z region region 50–20,000 using phosphoric acid. Chromatographic separation by LC–MS was performed in a Nano Acquity Ultra Performance LC, equipped with columns Symmetry C18 and BEH 130-C18 (Waters Corporation) in a linear gradient of 0–90% acetonitrile in 0.1% formic acid. The mass spectrometric data were collected in a full scan mode from m/z 50 to 1400 in positive mode. Data were processed and analyzed using Mass Lynx™ v 4.1 software. Three independent immunoprecipitation experiments were performed.

2.8. Measurement of UDP-GlcNAcP activity

Brains and CNS ganglia from vehicle or Jbtx-injected insects (8 insects in each group) were dissected out from surrounding tissues. Nervous tissue was then rinsed in a 10% solution of protease inhibitors in sterile saline (0.13 M NaCl). Samples were pooled separately and homogenized in 50 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$ and 1 mM DTT. The homogenates were then centrifuged at 10,000 *g* (10 min, 4 °C); protein concentration was determined in the CNS supernatants [4].

UDP-GlcNAcP activity was determined as described by Mio et al. [21] with modifications. The assay was performed in a 96-well plate at 37 °C with each well containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 25 μM UTP, 20 μM *N*-acetyl-α-D-glucosamine 1-phosphate, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.4 units/ml pyrophosphatase, and 0.01–0.05 mg of protein from CNS supernatants. After incubation for 15 min, an equal volume of the color reagent containing 0.03% (w/v) malachite green, 0.2% (w/v) ammonium molybdate, and 0.05% (v/v) Triton X-100 in 0.7 M HCl was added to the reaction mixture, and further incubated at 37 °C for 10 min. Inorganic phosphate derived from the pyrophosphate formed by the enzyme was quantified at 655 nm therefore representing UDP-GlcNAcP activity. Specific activity was expressed as the absorbance (A_{655}) per mg of protein, measured after 15 min.

2.9. In vitro effect of Jbtx on UDP-GlcNAcP activity

The in vitro effect of Jbtx on the activity of UDP-GlcNAcP was tested on brain homogenates of the cotton-stainer bug, *D. peruvianus*. Brains and CNS ganglia from insects not treated with Jbtx were dissected out from surrounding tissues in cold sterile saline. Samples from 10 insects were pooled and homogenized in 50 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). The homogenates were then centrifuged at 10,000 *g* (10 min, 4 °C); protein concentration was determined in the CNS supernatants [4]. Different concentrations of Jbtx were added to CNS supernatants and incubated for 1 h in an ice bath. Aliquots of CNS supernatants were incubated in the same conditions in the presence of buffer alone, as controls of the enzymatic reaction. Thereafter, the UDP-GlcNAcP activity was determined as described in Section 2.8.

2.10. Measurements of NOS activity and NO levels

Brains and CNS ganglia from Jbtx- and vehicle injected insects (8 insects in each group) were dissected out from surrounding tissues. After that, nervous tissue was rinsed in a 10% solution of protease inhibitors in sterile saline (0.13 M NaCl). Samples were homogenized in 20 mM Tris–HCl (pH 7.4) containing 0.32 M sucrose, 2 mM Na_2EDTA, 2 mM dithiothreitol (DTT) and 10% protease inhibitors. The homogenates were then centrifuged at 10,000 *g* (10 min, 4 °C); protein concentration was determined in the CNS supernatants [4] which were later stored at −20 °C until use.

NOS activity was determined in the CNS supernatants by following spectrophotometrically the oxidation of oxyhemoglobin to methemoglobin as described previously [11,30]. Briefly, each sample (1 mg protein/ml) was incubated at 37 °C in a reaction medium with 50 mM PB pH 7.0, containing 1 mM $CaCl_2$, 1 mM L-arginine, 100 μM NADPH, 10 μM DTT, 0.1 μM catalase, 4 μM superoxide dismutase (SOD) and 5 μM oxyhemoglobin. The formation of methemoglobin was followed at 401 nm ($\varepsilon = 38\ mM^{-1}\ cm^{-1}$). Specific NOS activity was expressed as nmol of NO produced/min mg protein. Catalase and SOD were added to the assays to prevent the interference of superoxide and hydrogen peroxide, respectively. Parallel assays were run adding NG-methyl-L-arginine (L-NMMA, 1 mM) to confirm that hemoglobin oxidation was occurring due to NO production only.

The levels of NO in the CNS supernatants were evaluated by measuring its stable degradation products, nitrite and nitrate (NOx), using the NO Analyser 280 (Sievers Instruments Inc., Boulder, CO, USA). NOx present in the samples were reduced with vanadium chloride in HCl at 90 °C to form NO [5]. NO products reacted with ozone in a gas-phase chemiluminescent reaction generating nitrogen dioxide, whose emissions in the red and near-infrared region of the spectrum were detected by a photomultiplier tube. The analyzer was calibrated with 1–100 μM $NaNO_3$ solution.

2.11. In vitro effect of Jbtx on NOS activity

The CNS supernatants from 10 insects not treated with Jbtx were obtained as indicated in Section 2.10 and divided into two aliquots. One of these aliquots was incubated with 1 nM Jbtx while the other one was incubated with the same volume of vehicle, for 40 min in an ice bath. Thereafter, NOS activity was determined in both samples following the procedure as stated in Section 2.10.

2.12. Statistical analyses

For measurements of NOS and UDP-GlcNAcP activities as well as NO levels, control or treated groups of 8–10 insects were processed as stated in each section. Four to six independent experiments were performed and data were expressed as mean ± Standard Error of the Mean (SEM). Graphs and statistical tests were performed using GraphPad Prism and GraphPad Instat 3.0 (GraphPad Software, San Diego, CA, USA). Results from activation of UDP-GlcNAcP by Jbtx in *D. peruvianus* were analyzed by One-way parametric ANOVA for comparisons between means and employing the Student–Newman–Keuls as post-test. All other comparisons were conducted using the Student's *t*-test. A P value <0.05 was considered statistically significant. The degrees of freedom (*df*) are informed in the legends to figures.

3. Results

3.1. Effects of Jbtx injection on insect behavior

Insects injected with Jbtx at a dose of 0.1 μg/mg body weight had 100% mortality within 18 ± 1 h after the injection as reported [39]. After the injection of either the peptide or the vehicle, insects were placed in individual jars with the floor covered with a disk of blotting paper. To provide the insects with shelter, a folded sheet of the same material stood from the base to the top of the jar.

During the first hour after the treatment, Jbtx-treated insects displayed signs of neurotoxicity as paralysis of the legs, proboscis extension and abnormal movements of the antennae [40]. It was observed that the antennae of peptide-injected insects were bent upwards and while in this position they executed irregular movements around the vertical plane. All these signs were transient, one hour after the injection treated insects behaved apparently normal and were observed standing vertically inside the folds of the paper as their control counterparts.

None of the signs mentioned above were observed in vehicle treated insects, which survived for at least 1 week after the injection.

3.2. Jbtx in the CNS

To investigate the effect of Jbtx in the CNS, insects were either injected with 0.1 μg/mg body weight Jbtx or the corresponding volume of 20 mM PB. The insects were killed, their CNS ganglia were dissected out and processed for immunohistochemistry. Sections of the brain and ganglia were incubated with the anti-Jbtx antibody. We observed immunolabeled somata only in sections from Jbtx-treated insects. Thus, several medium-sized immunostained somata (10–15 μm) were observed in the lateral cell body layer of the antennal lobe (Fig. 1A). Fig. 1C depicts the position of immunostained somata of treated insects, which were detected in all the processed samples. The antennal glomeruli were unstained as well as other neuropil areas, indicating that the immunoreactive product was present only in perikarya. A few scattered immunopositive cells were also found in the suboesophageal ganglion (Fig. 1C). In vehicle-injected insects, no immunoreactive somata or neurites were seen (Fig. 1B). Also, no immunostained perikarya or neurites were found when the sections were incubated with anti-Jbtx antibody previously pre-absorbed with the peptide. Immunostaining was also negative when the primary or the secondary antibodies were omitted in the corresponding solutions.

Western blots of nervous tissue homogenates from peptide- or vehicle-injected insects were incubated with the anti-Jbtx antibody.

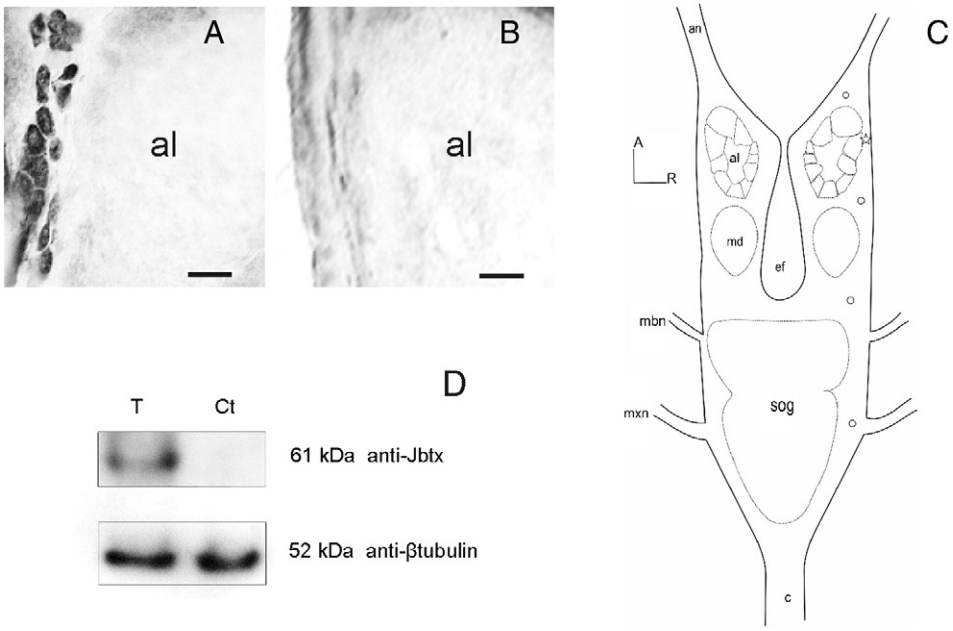

Fig. 1. *Immunohistochemistry and Western blots of T. infestans CNS samples.* Bright-field micrographs of antennal lobe sections from insects injected either with Jbtx (A) or vehicle (B). Both sections were incubated with anti-Jbtx antibody and processed with the ABC protocol. Immunopositive somata were only detected in sections obtained from Jbtx-injected insects. Bars = 25 μm. Similar results were obtained in three separated experiments. (C) Schematic drawing of deutocerebrum and subesophageal ganglion showing immunoreactivity to anti-Jbtx antibody. Symbols represent the number of immunoreactive somata. (D) Western blot of CNS homogenates from Jbtx- (T) and vehicle-injected insects (Ct). Blots were incubated with anti-Jbtx antibody (top panel) and an immunoreactive band at ~61 kDa was observed only in treated insects. Blots were also incubated with an anti-β-tubulin antibody to assess loading control (bottom panel), with immunopositive bands seen in both samples at the expected size of 52 kDa. The Figure shows a representative experiment of three independent assays. Abbreviations: an (antennal nerve), c (connectives), ef (oesophageal foramen), md (mechanosensory and motor deutocerebrum), al (antennal lobe), sog (suboesophageal ganglion). (○ 1–5 immunoreactive somata; ☆, 11–15 immunopositive somata).

An immunoreactive band of ~61 kDa was observed in Jbtx-injected insects (Fig. 1D, upper panel). This band was not detected in vehicle-injected animals. The expression of β-tubulin, used as loading control, was similar in both groups of insects (Fig. 1D, lower panel).

3.3. Characterization of Jbtx binding protein(s)

The presence of cells displaying immunoreactivity in Jbtx-treated insects as well as the presence of an immunoreactive band in Western blots of treated insects indicated that the peptide, after injection into the hemocele, reached the CNS and localized in specific subsets of somata. To identify putative Jbtx-binding protein(s) in those groups of cells, we performed immunoprecipitation of nervous tissue homogenates of peptide-treated insects with the anti-Jbtx antibody. Two-dimensional electrophoresis revealed the presence of a specific spot in Jbtx-treated insects (Fig. 2A), which was absent in vehicle injected animals (Fig. 2B). The protein present in the spot displayed a molecular weight (MW) of ~59 kDa and an isoelectric point (pI) of ~3.8. The spot was further excised from the gel and digested with trypsin. The resulting peptides were submitted to liquid chromatography–mass spectrometry for its identification using the NCBI (Insecta) database (Table 1). UDP-*N*-acetylglucosamine pyrophosphorylase (UDP-GlcNAcP) was the protein identified in the spot.

3.4. Effect of Jbtx on the activity of UDP-GlcNAcP

To investigate a possible interaction between Jbtx and UDP-GlcNAcP, as suggested by the immunoproteomic approach (see Section 3.3), the activity of this enzyme in CNS homogenates of Jbtx-injected or vehicle-injected bugs (control) was assessed. Fig. 3A shows that UDP-GlcNAcP activity increased significantly in Jbtx-injected insects as compared to vehicle-injected ones (13.06 ± 1.76 vs. 7.48 ± 0.98 A_{655} nm/mg protein, $P < 0.05$). Activation of UDP-GlcNAcP by Jbtx also occurs in vitro, and a significant increase of the enzyme activity was found upon incubation of CNS homogenates of the hemipteran *D. peruvianus* with 50 nM of the peptide (8.44 ± 2.2 A_{655} nm/mg protein) in comparison with the activity detected in the controls (4.43 ± 0.37 A_{655} nm/mg protein) ($P < 0.05$; Fig. 3B).

3.5. Effect of Jbtx on NOS activity and levels of NO

As shown in Fig. 1A, the anti-Jbtx antibody labeled somata in the antennal lobe, which are known to produce NOS in *T. infestans*. Also, consistent with the observation of abnormal movements of the antennae in Jbtx-treated insects, a possible effect of the peptide on the nitrergic system was evaluated. It was found that NOS activity decreased significantly in CNS homogenates of insects injected with the peptide, from a value of 7.29 (control) to 1.82 nmol/mg.min (Jbtx-treated) ($P < 0.001$; Fig. 4A). Preincubation of CNS homogenates of non-injected insects in vitro, with 1 nM Jbtx also inhibited the enzyme activity from 7.42 nmol/mg.min (control) to 2.23 nmol/mg.min ($P < 0.001$; Fig. 4B), suggesting a direct effect of the peptide on NOS itself or indirectly, acting on the nervous tissue in a way that affected the enzyme activity.

As one could expect from the inhibition of NOS activity, Jbtx also promoted a reduction in the levels of NO in CNS homogenates of treated insects. Thus, the amount of NOx decreased significantly from 5.91 ± 0.39 pmol/μg protein (control) to 2.98 ± 0.30 pmol/μg protein (Jbtx-injected, $P < 0.001$; Fig. 5).

To evaluate if the treatment with the peptide affected the expression of the NOS protein, the same type of samples were submitted to SDS-PAGE and Western blot. Membranes were incubated with the anti-uNOS antibody. Immunoreactive bands were found at the expected size of 132 kDa in both groups of animals (Fig. 6A). The ratio between NOS/β-tubulin (Fig. 6B) was similar in control and treated animals (Fig. 6C), suggesting that Jbtx treatment did not reduce the levels of NOS expression in the CNS of the insects.

4. Discussion

Our results clearly show that *T. infestans* CNS is a target for the entomotoxic peptide Jbtx. The behavioral effects of the peptide on the insects were observable soon after injection. Treated insects displayed abnormal movements of the antennae, uncoordinated movements of the legs and did not show escape reaction to an attempt in handling. Immunohistochemistry experiments revealed that anti-Jbtx antibody marked somata of lateral layer of the antennal lobe in treated insects. It has been reported that in other insect species both uni- and multi-glomerular projection neurons and inhibitory multi-glomerular interneurons are located in the lateral layer of the antennal lobe [15]. To determine the identity of these immunolabeled cells would require employing immunocytochemistry together with neuronal tracing experiments, which will be matter of future work. No labeling was observed in any layer of the antennal glomeruli, or in fibers. The anti-Jbtx antibody staining was restricted to the soma of the reactive cells; this fact prevented us to relate the behavioral changes observed in treated bugs with the localization of Jbtx in a specific brain area.

The labeling of brain somata with the anti-Jbtx antibody suggested that the peptide was internalized by neural cell bodies. The CNS of insects is surrounded by a neural sheath formed by a layer of large glial cells, the subperineurial glia, which insulates the nervous tissue from the surrounding environment [23,38]. Previously, it has been shown that Jbtx interacts with acidic lipid bilayers, causing membrane permeabilization [2,20] and forming ion channels [25]. Thus, these properties of Jbtx could allow the entomotoxic peptide, upon injection into the body cavity, to cross the neural sheath and localize within a cell body of a specific area of the insect brain.

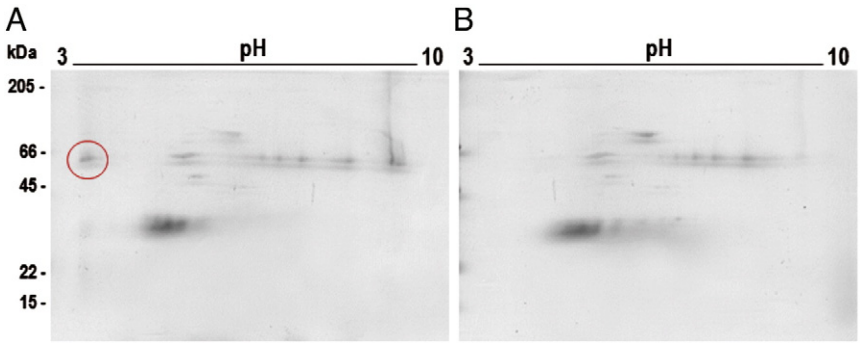

Fig. 2. *Characterization of Jbtx binding protein(s) in the CNS of T. infestans.* CNS homogenates from Jbtx- (A) or vehicle-injected insects (B) were subjected to immunoprecipitation with anti-Jbtx antibody followed by 2D electrophoresis. One spot of ~59 kDa with a pI of ~3.8 was present only in Jbtx-treated insects (red circle). The Figure shows a representative experiment of three independent assays.

Table 1
Identification of the Jbtx interacting protein after LC–MS/MS of the spot excised from the gel depicted in Fig. 2. The spot which was present only in the samples from Jbtx-treated insects was excised from the gel, digested with trypsin and the resulting peptides were submitted to liquid chromatography–mass spectrometry for its identification with the NCBI (Insecta) database.

Accesion	Annotation	Organism	Observed pI/MW	Identified peptides
AEL88647	UDP-*N*-acetylglucosamine pyrophosphorylase	*Nilaparvata lugens*	3.8/59 kDa	KSLYQIQAERI SLYQIQAER

The presence of Jbtx in the CNS was confirmed by western blot, after protein fractionation in the absence of reducing agent. Under this condition, the assays showed an immunoreactive band of ~61 kDa only in insects injected with the peptide. The fact that the molecular weight of this band is higher than the expected for the recombinant peptide alone is an indication that Jbtx is interacting with one or more proteins of the CNS. In an attempt to characterize the binding of Jbtx to brain protein(s) we performed immunoprecipitation of brain tissue homogenates with the anti-Jbtx antibody followed by 2D electrophoresis. One protein spot (~59 kDa, pI ~3.8) found only in samples of Jbtx-treated insects was subjected to identification by tandem mass spectrometry. The results identified UDP-N-acetylglucosamine pyrophosphorylase (UDP-GlcNAcP) as a putative Jbtx-binding protein in *T. infestans* CNS. In fact, the activity of UDP-GlcNAcP significantly increased in CNS homogenates of *T. infestans* injected with Jbtx and killed 3 h later the injection. The property of Jbtx to activate UDP-GlcNAcP was seen also upon in vitro incubation with CNS homogenates from *D. peruvianus*, another insect model well known for its susceptibility to urease and Jbtx [13,35], suggesting that the ability of Jbtx to interact with UDP-GlcNAcP is not restricted to *T. infestans* nervous tissue.

Eukaryotic UDP-GlcNAcP is ubiquitously expressed and highly conserved [21]. The enzyme plays a central role in glycosylation pathways, providing the activated precursor, UDP-N-acetylglucosamine, for the synthesis of chitin, glycoproteins and glycosylphosphoinositide (GPI) anchors of some membrane proteins attached to the exterior leaflet of the cell membrane [21]. In *R. prolixus*, a putative UDP-GlcNAcP was identified using the Blast tool genome assembly (Gene RPRC015300, VectorBasehttp://www.vectorbase.org). The deduced protein has a predicted MW of ~54.5 kDa and a pI of ~6.1, considering its amino acid sequence and disregarding possible post-translational modifications. Post-translational modifications, besides binding to Jbtx itself, can possibly explain the differences in MW and pI found for the putative UDP-GlcNAcPfrom *R. prolixus* and the corresponding protein from *T. infestans*. However, since most proteins differ widely in their buffering capacity, it is very difficult to speculate if a specific post-translational modification would lead to a particular difference in pI.

RNAi experiments performed in the beetle *Tribolium castaneum* [1] or in the locust *Locusta migratoria* [19] against one of the isoforms of UDP-GlcNAcP resulted in lethality probably caused by defective glycosylation in molecules with critical functions in the insects. *Drosophila melanogaster* with mutations in the gene encoding this enzyme displayed defects in the development of CNS and peripheral nervous system among other alterations [28,17].

The brain of *T. infestans* contains numerous NOS-immunoreactive somata and a fully active enzyme [30]. The entomotoxic peptide Jbtx had inhibitory effects upon NOS activity both in vivo and in vitro, producing a 50% decrease in the NOx CNS levels of treated insects respect to those of controls. This inhibition of NOS activity could be the result of a direct action of Jbtx on the enzyme as shown by the fact that in vitro incubation of the entomotoxic peptide with the CNS homogenates also decreased the activity of the enzyme. Alternatively, Jbtx could be affecting NOS activity indirectly, for instance, by altering membrane permeability and diffusion of NO, or interfering with a number of adaptor proteins that modulate/regulate the enzyme [41]. On the other hand, Jbtx treatment of the bugs did not alter the protein levels of NOS

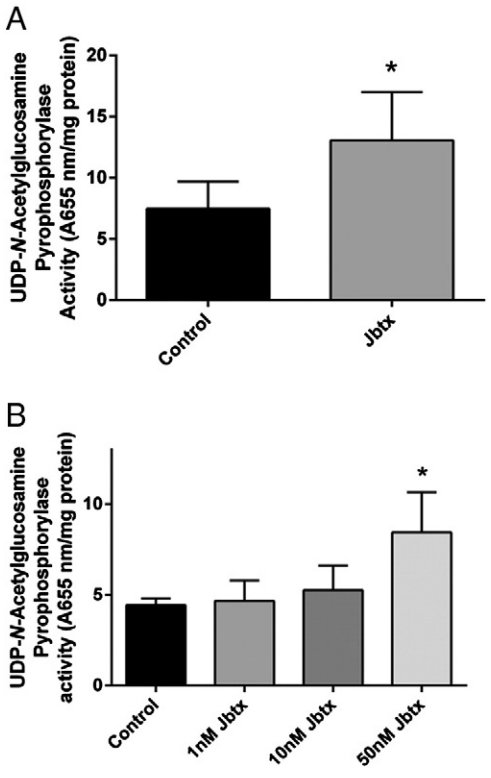

Fig. 3. *Activation of insect UDP-N-acetylglucosamine pyrophosphorylase (UDP-GlcNAcP) by Jbtx.* (A) CNS homogenates of *T. infestans* injected with Jbtx or vehicle solution (control) were assayed for UDP-GlcNAcP activity as described in Section 2. Results are expressed as specific activity (A_{655} nm/mg protein) and are means ± SEM (n = 6). $^{*}P < 0.05$, $df = 10$, Student t test. (B) UDP-GlcNAcP activity was measured in CNS homogenates of non-injected *D. peruvianus* incubated in vitro with different concentrations of Jbtx. Controls were performed without addition of Jbtx. Results are means ± SEM (n = 6). $^{*}P < 0.05$, $df = 23$, One-way parametric ANOVA.

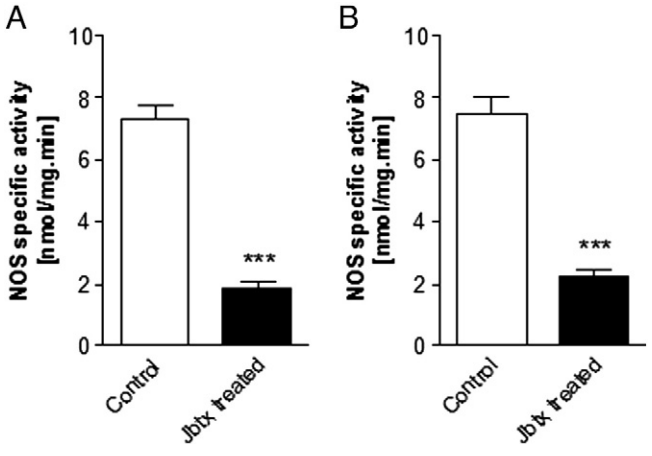

Fig. 4. *Effect of Jbtx on NOS activity in T. infestans CNS.* (A) Insects were injected with vehicle (control) or with the peptide (Jbtx treated, 0.1 μg/mg body weight). For each treatment, the CNS of the insects were dissected, pooled and processed for determination of the enzymatic activity as described in Section 2. Results are expressed as nmol of NO produced/mg.min and are means ± SEM (n = 4–6). $^{***}P < 0.001$, $df = 8$, Student t test. (B) For in vitro experiments, CNS homogenates were incubated with vehicle (control) or with 1 nM Jbtx (Jbtx treated), followed by NOS activity determination. Results are means ± SEM (n = 4). $^{***}P < 0.001$, $df = 6$, Student t test.

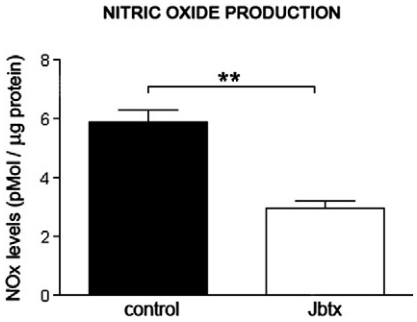

Fig. 5. *Effect of Jbtx on NO levels of T. infestans CNS.* Insects were injected with vehicle (control) or with Jbtx (0.1 µg/mg body weight). The CNS of the insects were dissected, pooled and processed for the determination of NO levels as described in Section 2. Results are expressed as pmol of NOx/µg protein and are means ± SEM (n = 4–5). $^{**}P < 0.01$, $df = 7$, Student t test.

in CNS homogenates, a fact more consistent with the hypothesis of an indirect effect of Jbtx on NOS activity. Glycosylinositolphospholipids, which derive indirectly from the activity of UDP-GlcNAcP produced by trypasonomatids, downregulate NO synthesis in the salivary glands of *R. prolixus*, without greatly affecting NOS mRNA levels [14]. It is thus tempting to speculate that the binding of Jbtx to *T. infestans* UDP-GlcNAcP, as detected here by a proteomic approach, which leads to concomitant activation and increased production of glycosylinositolphospholipids, is the cause of NOS inhibition in the CNS of Jbtx-treated bugs.

Altogether, our data indicate that the normal activity of nitrergic pathway in the CNS of *T. infestans* is impaired by the entomotoxic urease-derived peptide, Jbtx. UDP-GlcNAcP was identified as a Jbtx-binding protein in the CNS of this species, leading to enzyme activation probably by an allosteric mechanism. Besides its membrane-disturbing and ion channel activity, binding of Jbtx to UDP-GlcNAcP may result in

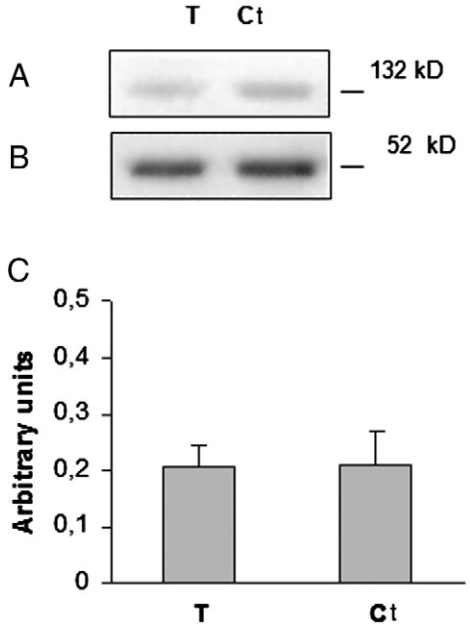

Fig. 6. *Western blot for anti-NOS. T. infestans* CNS homogenates from Jbtx- (T) and vehicle-injected insects (Ct) were subjected by SDS-PAGE and Western blot was developed with anti-universal NOS antibody as described in Section 2. An immunoreactive band was observed at the expected size of 132 kDa in both, Jbtx-treated and control insects (A). Afterwards, the blots were also incubated with an anti-β-tubulin antibody for loading control purposes (B). Immunopositive bands were seen in both samples at the expected size of 52 kDa. A and B show a representative experiment of three independent assays. The ratios between NOS/β-tubulin in control (Ct) and treated (T) insects expressed as arbitrary units are shown in (C) as mean ± SEM (n = 3).

alterations of glycosylphospholipds structure and levels, affecting NOS activity as well as the structure of neuronal membranes, disturbing cell to cell communication in the early steps of lethality. Further research is needed to address the biological functions of UDP-GlcNAcP in the CNS of this triatomine species.

5. Conclusions

Administration of Jbtx to adults of *T. infestans*, at the dose employed in this study caused death of all the insects in less than 24 h. It was found that Jbtx was internalized by CNS somata. UDP-*N*-acetylglucosamine pyrophosphorylase was identified as a putative Jbtx-binding protein; alteration of its normal activity in Jbtx-treated insects suggests that lethality may be caused by changes in glycosylation of molecules with critical functions in insect survival.

Treatment with the entomotoxic molecule also impaired the normal activity of the nitrergic system. Some of the early behavioral effects observed in treated animals may be related with alterations in the NO pathway.

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