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Gyroxin fails to modify in vitro release of labelled dopamine and acetylcholine from rat and mouse striatal tissue

M.A.P. Camillo[a,*], P.C. Arruda Paes[a], L.R.P. Troncone[b], J.R. Rogero[a]

[a]Instituto de Pesquisas Energéticas e Nucleares, IPEN/CNEN-SP, Travessa R, no. 400, Cidade Universitária, São Paulo, SP 05508-900, Brazil
[b]Instituto Butantan, Av. Vital Brazil, no. 1500, São Paulo, SP 05503-900, Brazil

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Abstract

Gyroxin fails to modify in vitro release of labelled dopamine and acetylcholine from rat and mouse striatal tissue. Gyroxin is a thrombin-like peptide with amidasic, esterasic and fibrinogenolitic activities, found in the venom of snakes like *Lachesis muta muta* and *Crotalus durissus terrificus*. Intravenous injections of small doses of gyroxin induce a typical barrel rotation behaviour that has been thought to be a neurotoxic effect. The aim of this study was to determine whether gyroxin-induced barrel rotation behaviour involves changes in neurotransmitter release. Gyroxin was isolated from crude venoms by gel filtration and affinity chromatography. Its properties were determined by assaying esterasic, amidasic and fibrinogenolitic enzymatic activities and tested for barrel rotation behaviour. Neurotransmitter release tests employed rat and mouse superfused brain striatal chopped tissue preloaded with [^{3}H]-dopamine, [^{3}H]-acetylcholine or in a double labelling procedure. They were stimulated by 20 mM K$^+$ in control conditions or in the presence of several concentrations of toxins. Crotoxin and crotamine were used as positive controls. Gyroxins failed at modifying both basal and stimulated neurotransmitter releases, suggesting a lack of direct neurotoxic effect. We therefore suggest that gyroxin may not be a neurotoxin but rather, induces this behavioural syndrome by other means possibly related to haemodynamic disturbance. The possible role of vasopressin is discussed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Crotalus durissus terrificus*; *Lachesis muta muta*; Gyroxin; Snake; Venom; Toxin; Dopamine; Acetylcholine; Release; Barrel rotation

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1. Introduction

Thrombin-like enzymes are of great interest due to their use as anticoagulants in thrombotic diseases (Reid and Chan, 1968; Stocker, 1990). These enzymes seem to be useful in the dissociation of clots in myocardial infarction, thrombosis in deep veins, trauma and lung clots (Markland, 1988). Some thrombin-like enzymes are commercially available like Batroxobin® and Reptilase® from *Bothrops atrox* venom, Defibrase® from *Bothrops moojeni* venom and Arvin® and Ancrod® from *Calloselasma rhodostoma* venom for treatment and as reagents for diagnosis (Latallo, 1983; Kornalik, 1990; Pirkle and Theodor, 1990).

The barrel rotation syndrome was observed for the first time by Barrio (1961) describing the effects of gyroxin from *Crotalus durissus terrificus* venom, however, only in 1975, Cohn and Cohn (1975) introduced this concept to neuroscience. They observed this behaviour after intracerebroventricular injection of high doses of somatostatin. Other peptides such as arginine–vasopressin (Kruse et al., 1977) and endothelin-3 (Kannan et al., 1994) also induced this effect. The mechanism underlying the barrel rotation syndrome has not yet been elucidated. The gyroxin-related syndrome could be attributed to labyrinthic lesions (Barrio, 1961; Seki et al., 1980) or other indirect effects on the central nervous system related to its enzymatic activity (Alexander et al., 1988; Da Silva et al., 1989). However, intra-hippocampal injection of gyroxin obtained from *Crotalus durissus terrificus* venom has been reported to induced epileptiform electrographic behavioural and

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* Corresponding author. Tel.: +55-11-3816-9229; fax: +55-11-3816-9232.
E-mail address: mcamillo@net.ipen.br (M.A.P. Camillo).

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neuropathologic alterations in a dose dependent manner (Moreira, 1993) suggesting a possible direct action on the nervous tissue.

The barrel rotation syndrome has been described as a sudden asymmetric increase in the stretch muscle tone, accompanied by opisthotonous, spastic distortions and rotation along the long axis of the body (Kruse et al., 1977). It may be clockwise or counter-clockwise; however, the same animal always rotates towards the same side, with 3% of exceptions (Wurpel et al., 1986a). The rolling behaviour is alternated with prostration periods. Tail lifting or tail pinching increases the rotation behaviour (Cohn and Cohn, 1975). Some rats die, apparently from breathing impairment, while others are asymptomatic for at least 1 h. A large proportion of animals die without presenting the barrel rotation behaviour but develop one or more symptoms of intoxication, such as immobility, stretching of posterior limbs and grooming behaviour. Sometimes it is preceded by a brief period of hyper-excitation with abrupt running or by paleness and tachypnea (Barrabin et al., 1978). The syndrome cannot be induced a second time by a new injection of toxin just after recovery from the first (Alexander et al., 1988). It shows an "all or none" pattern (Wurpel et al., 1986a) and is affected by prior exposure to the peptide (Wurpel et al., 1986b; Willcox et al., 1992).

Thus, this work aims to contribute to the understanding of this thrombin-like enzyme obtained from *Crotalus durissus terrificus* and *Lachesis muta muta* venoms, by testing possible direct action on brain tissue that could account for the neurotoxic activity and eventual barrel rotation behaviour. The striatum is a well-known subcortical structure involved in motor control. It bears several different cell figures like dopamine terminals, cholinergic, GABAergic and somatostatin intrinsic neurones, and glutamate cortical afferent and several other peptidergic neurones. It has been demonstrated that the release of acetylcholine and dopamine is under the influence of many of the transmitters (Angulo and McEwen, 1994; Wilson, 1998). Besides the expression of a variety of receptors, striatal neurones also express ion channels, therefore constituting a valuable target for neurotoxins. Previous work from our laboratory showed the sensitivity of this method (Troncone et al., 1995) and the results obtained with crotoxin and crotamine shown here further demonstrate its usefulness.

2. Materials and methods

2.1. Venoms and gyroxin purification

Crystallised *Lachesis muta muta* (*Lmm*) venom, was obtained from the National Research Institute of Amazonas (INPA) and *Crotalus durissus terrificus* (*Cdt*) venom, lyophilised form, was obtained from Butantan Institute and were kept at −20°C. All reagents used were of analytical grade.

Purification of gyroxin was performed employing gel filtration and affinity chromatography as described elsewhere with minimal changes (Nakazone et al., 1984; Alexander et al., 1988; Silveira et al., 1989). Crotoxin and crotamine employed in this study were obtained by gel filtration. In some experiments gyroxin kindly supplied by Dr. I. Kaiser was used.

Briefly, samples of 100 mg of venom were dissolved in 1 ml of the respective buffer used in each gel filtration chromatography and centrifuged at 10,000*g* for 1 min. The soluble portion of the *Lmm* venom was applied to a Sephacryl® S.200 chromatographic column (2.6 × 90 cm) previously balanced with sodium bicarbonate 0.1 M pH 7.2. The similarly centrifuged *Cdt* venom was applied to a Sephadex G.100 column (2.6 × 90 cm) balanced with ammonium formiate buffer 0.1 M pH 3.0 and fractions of 2.1 ml/tube were collected by a constant flow of 15 ml/h. Fraction elution was monitored by absorbance at 280 nm. Further spectrophotometric measures were taken with a Pharmacia Ultrospec III spectrophotometer with an optical path of 1 cm. Peaks obtained were screened for enzymatic activities and behavioural tests. In order to calibrate the columns, prior to their use with venoms, some molecular weight markers were used to establish the elution volumes and calculate the partition coefficient (Kav): V_0 (Blue Dextran or Ferritin); V_t (Triptophan) and various V_e markers (bovine serum albumin; human albumin; chymotrysin; myoglobin and cytochrome *c*). Gel filtration and affinity chromatographies were performed at 2–4°C.

The active fractions from each venom, obtained in different gel filtration runs, were pooled and dialysed against Tris–HCl 0.05 M and NaCl 0.4 M pH 9.0 buffer for 18 h, with two buffer changes. The dialysed fractions were further purified using a Benzamidine Sepharose 6B affinity chromatography column (1.2 × 5 cm, with 2 ml of gel), with a flow of 12 ml/h. The column was then washed with 30 ml of the same Tris–HCl buffer. Elution of active peptides was carried out in sodium acetate 0.1 M pH 5.0 buffer in samples of 1 ml. The elution was monitored at 280 nm and by pH measures (Tornasol paper) and enzymatic assays as described below. Protein content of the samples was determined by Lowry's method as modified by Miller (1959) and allowed for quantification of toxin content in further assays. The active fractions were pooled and assayed by an in vivo test as described below.

2.2. Enzymatic assays and in vivo test

The active fractions were identified by enzymatic assays and an in vivo test. The enzymatic activities were tested according to the following methods: amidase assay, towards *N*α-benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA) was assayed according to the method of Erlanger et al., 1961; esterase assay, towards *p*-toluenosulfonyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-arginine ethyl ester (BAEE), performed as described by Barrabin et al., 1978;

coagulant activity, as described by Santoro and Sano-Martins (1993), with bovine fibrinogen (37.4% protein, 96.1% clottable) as substrate; phospholipase A_2 assay, as described by Araújo and Radvanyl (1987) using phenol red indicator; and platelet aggregation, as described by Born (1962), with activation of platelets in human plasma.

The in vivo test (barrel rotation test) was performed using Swiss male mice (maintained in cages with pine wood, with commercial food and water ad libitum, body weight 25–30 g), injected with test samples at 0.25 μg/g body weight by intravenous injection in the tail vein and observed for 30 min. Barrel rotation should appear spontaneously in this period or be triggered by mild stimulation, otherwise the fraction was considered inactive. A typical barrel rotation consisted of at least one full rotation over the long axis of the body, performed in a second or less. Usually a mouse would perform several of these rotations in response to a mild stimulation.

2.3. Polyacrylamide gel electrophoresis (SDS-PAGE)

Salt-free samples (10–40 μg) dried in a Speed-Vac were taken up in 100 μl of 0.048 M of Tris–HCl buffer pH 6.7, containing 1% (w/v) SDS, 0.05% (w/v) 2-β-mercaptoethanol, 0.005% bromophenol blue and 10% (v/v) glycerol. Samples were heated for 3 min at 100°C and applied on top of 12.5 or 15% polyacrilamide slab gel according to the method of Laemmli (1970). Protein bands were revealed by the Coomassie Brilliant Blue R-250 staining procedure. Molecular weight markers run in parallel were bovine serum albumin; ovalbumin; carbonic anydrase; trypsinogen and lyzosyme.

2.4. Analytical HPLC

Samples were dissolved in water or in the eluent (ammonium bicarbonate 0.025 M pH 7.2) for analytical HPLC (Waters Chromatographic System composed of two model 501 pumps, automatic controller, processor and a UV detector (Isco) at 280 nm). The samples were applied (volume of 20 μl, containing 6.5 to 65 μg of protein) to a Guardcolumn SW 7.5 mm ID × 7.5 cm TSK gel (Toso Haas) followed by a TSK G.2000SW 7.5 mm ID × 60 cm with 10 μm particles (Toso Haas); flow of 1 ml/min, at room temperature.

2.5. [^{3}H]-neurotransmitter release from striatal chopped tissue

Neurotransmitter release was assessed as described by Marien et al., 1983 and Troncone et al., 1995. Briefly, adult male Wistar rats were killed by decapitation and the striatum was dissected and placed in ice-cold Krebs Ringer bicarbonate buffer (KRB) with the following mM composition: NaCl 118, $NaHCO_3$ 25, KCl 4.8, $CaCl_2$ 1.2, KH_2PO_4 1.2, $MgSO_4$ 1.2 and Glucose 10 pH 7.3; constant gassing with CO_2/O_2 was maintained. The striatal tissue was then chopped twice in a McIlwain tissue chopper set to cut at 250 μm to form prisms. The chopped tissue was dispersed with a pipette and washed twice in 20 ml ice cold KRB and transferred to a beaker containing 3 ml KRB with 0.135 μM of [^{3}H]-dopamine (New England Nuclear, specific activity 1.4 TBq/mmol) and 10 μM pargyline to prevent dopamine degradation, or 0.055 μM of methyl-[^{3}H]-choline (New England Nuclear, specific activity 3.3 TBq/mmol) and maintained at 37°C for 20 min to allow uptake. The tissue was then filtered and washed twice with normal ice cold KRB and distributed in 10 superfusion chambers with internal volume of 0.25 ml. Superfusion was performed at a rate of 0.25 ml/min with a 10 channel peristaltic pump, for 60 min in order to achieve a stable baseline of [^{3}H]-transmitter release. After this time, three successive baseline samples were collected with an adapted home-made sample collector, at three-minute intervals. Superfusion with stimulating agent followed in the fourth interval and lasted two minutes. A 20 mM K^+ KRB (NaCl was reduced to maintain isoosmolarity) was used to depolarise the striatal tissue and induce release. A second identical stimulus was performed in the 11th sample and six more samples were afterwards collected. In the 18th interval, the tissue was perfused with 0.1 N HCl for two periods of three minutes to induce release of all the [^{3}H]-dopamine or [^{3}H]-acetylcholine still present in the tissue.

Superfusion with toxins was started in the 8th sample and was maintained until the 14th sample in the experimental samples. Crotoxin 1.3 μM and crotamine 5 μM were used as positive controls. The *Lmm* and *Cdt* gyroxins were assayed in 0.02; 0.20 and 1.00 μM. Control samples were perfused with normal KRB.

Results are expressed as fractional release, i.e. percent of [^{3}H]-neurotransmitter released over the total contained in the tissue at the time of release. The effects of toxins were evaluated initially by the fractional release profile compared to the control sample profile and afterwards by comparing the values of B_2/B_1 and S_2/S_1. S_1 is the release obtained by stimulation in control conditions and S_2 is the stimulated release obtained in the presence of toxin. Basal releases in the presence of toxin (B_2) and control basal release (B_1) were used to evaluate the effects of toxin on basal release using the B_2/B_1 ratio. $S(n)$ is calculated subtracting $B(n)$ from the total release obtained under stimulation. B_1 corresponds to the mean of the three samples that preceded stimulation and the last fractional release before S_2 was considered as B_2. All radioactivity counts were obtained in dpm, as corrected by external standard counting performed in a Wallac Scintillation counter.

Considering that the barrel rotation syndrome has been described in mice, our study approached both species but rats have been used in most of the experiments because of the size of the striatum and ease of dissection and handling. Also, three rats yielded enough materials for one experiment while eight mice were necessary for a single experiment. When mouse striatal tissue was assayed, a double labelling procedure was employed. 121 μM methyl-[^{14}C]-choline

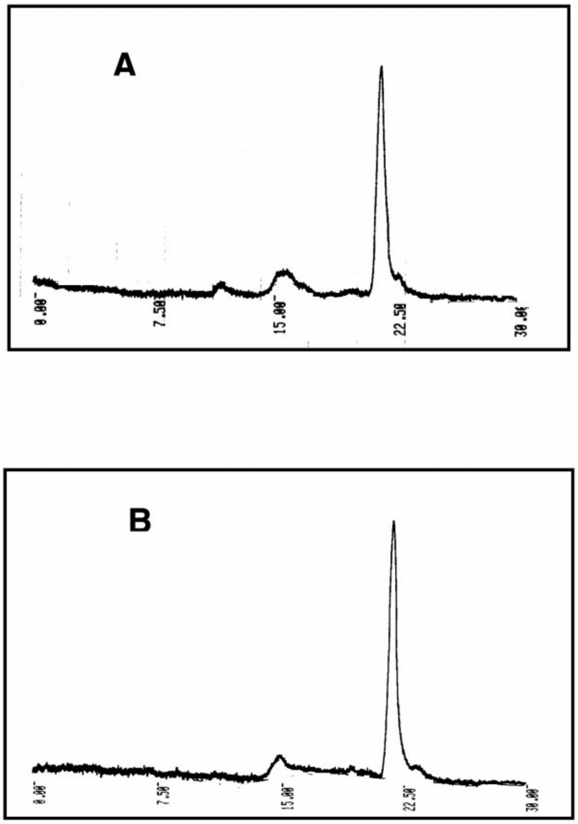

Fig. 1. HPLC elution profile obtained from *Lachesis muta muta* (A) and *Crotalus durissus terrificus* (B) gyroxin, using a TSK G2000 column and 25 mM ammonium bicarbonate buffer pH 7.2; flow at 1 ml/min and detection by UV absorbance at 280 nm. Retention times were very similar at 21.7 min for *Lachesis* and 21.8 min for *Crotalus* gyroxin.

(Amersham Pharmacia, specific activity 2.04 GBq/mmol) was added to 0.135 μM [^{3}H]-dopamine plus pargiline in 3 ml KRB and all following steps were performed in the same way as for the single labelling experiments described above. *Lmm* and *Cdt* gyroxin were assayed in 0.2 and 2.2 μM. Crotoxin and crotamine were employed in the same conditions described above to illustrate how mouse tissue responds to known neurotoxins. Scintillation counting was performed in a Packard Tricarb counter and output given in dpm for [^{3}H] and [^{14}C] by internal programming.

2.6. Statistics

To compare the control values obtained with rats and mice striatal tissue statistical analysis ANOVA and unpaired *t*-test were employed. One-Way ANOVA followed by Dunnet's test was applied to compare toxin groups with a control group. A value of $p < 0.05$ was assumed for the significance limit. All analysis was performed with the software GraphPad Prism.

3. Results

The yield of our purification procedure was similar to those obtained by others (Seki et al., 1980; Campos et al., 1988; Alexander et al., 1988). The gel filtration conditions proved to be ideal for the fractionation of *Crotalus durissus terrificus* venom. Crotoxin and crotamine were used in the release experiments without further purification and showed 81.4 and 80.8% purity, estimated by HPLC.

Based on gel filtration data (partition coefficient (Kav) and molecular weight markers), the purified toxins showed molecular weights of 48 kDa for *Lmm* gyroxin and 47 kDa for *Cdt* gyroxin. These close molecular radii were also observed in HPLC analysis. Fig. 1A and B shows HPLC elution profiles and retention times of 21.7 (*L. m. muta*) and 21.8 (*C. d. terrificus*) min. The toxin peaks correspond, respectively to 70 and 80% of the total amount of protein applied to the column and the contaminants may well be dimers as suggested by Barrabin et al., 1978. In the electrophoretic analysis the dimer forms could have dissociated during treatment of the samples before electrophoresis. This observation suggests that the involved links are weak and the addition of urea and β-mercaptoethanol confirmed that it was a single protein.

SDS-PAGE attributed a value of 29 kDa for the *Lmm* toxin obtained in gel filtration and 28 kDa after affinity chromatography. For *Cdt* toxin these values stood at 30–33 and 36 kDa, respectively. Results are presented in Fig. 2A and B for gel filtration and Fig 3A and B for affinity.

Electrophoresis and gel filtration data were in disagreement regarding the estimation of the molecular weight (MW) of these two samples of toxins. Nevertheless, these values are in the same range of data from other laboratories (Seki et al., 1980; Da Silva et al., 1989; Silveira et al., 1989; Yarlequé et al., 1989; Orejuela et al., 1991). Since gyroxin is a glycoprotein, different degrees of glycosilation could account for the different MW obtained by these methods. Based on the amino acid sequence of *Lmm* gyroxin the calculated MW should be 25.656 Da (Magalhães et al., 1993).

The results of the enzymatic assays are summarised in Table 1. Both *Cdt* and *Lmm* gyroxins presented similar profiles, being positive for esterasic, amidasic and coagulant activities, and negative for phospholipase A_2 and platelet aggregation assays. The induction of barrel rotation was confirmed for both, in agreement with other studies (Alexander et al., 1988; Aguiar et al., 1996).

Neurotransmitter release experiments performed in rat and mouse striatal tissue yielded similar results. Control values of basal and stimulated release of dopamine in rat and mouse striatal tissue (B_2/B_1 0.85 ± 0.07 for the rat tissue and 1.00 ± 0.04 for mouse tissue; S_2/S_1 0.79 ± 0.10 for rat and 0.68 ± 0.09 for mouse) and for acetylcholine (B_2/B_1 0.80 ± 0.08 for rat and 0.68 ± 0.01 for mouse; S_2/S_1 0.88 ± 0.04 for rat and 0.81 ± 0.01 for mouse), showed no significant difference.

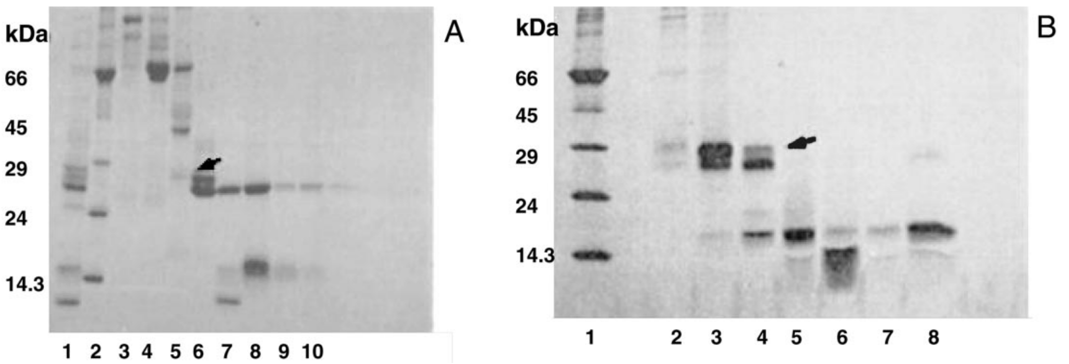

Fig. 2. Electrophoretic profiles obtained after gel filtration chromatography. Arrows point to gyroxin bands: A — SDS polyacrilamide 12.5% gel electrophoresis of *Lachesis muta muta* crude venom (lane 1), molecular weight markers (lane 2), samples separated by chromatography in Sephacryl S 200 (lanes 3–10). B — SDS polyacrilamide 15% gel electrophoresis of *Crotalus durissus terrificus* crude venom (lane 8), molecular weight markers (lane 1), samples separated by chromatography in Sephadex G100 (lanes 2–7).

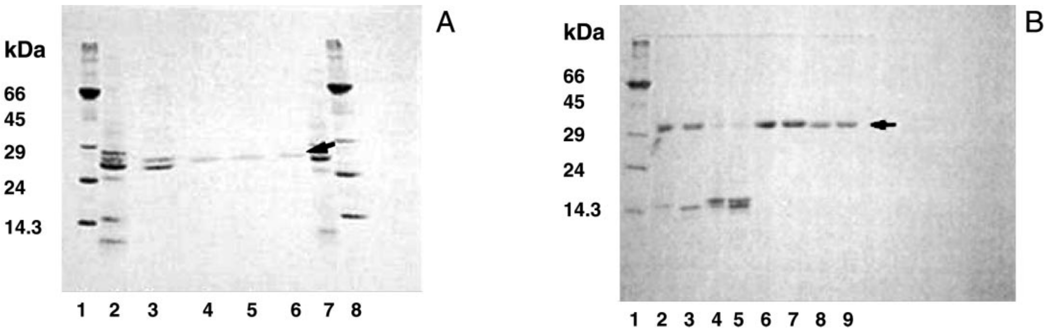

Fig. 3. Electrophoretic profiles obtained after affinity chromatography. Arrows point to gyroxin bands: A — SDS polyacrilamide 12.5% gel electrophoresis of *Lachesis muta muta*. Molecular weight markers (lanes 1 and 8), crude venom (lanes 2 and 7), samples purified by affinity chromatography as described in Section 2 (lanes 3–7). B — SDS polyacrilamide 15% gel electrophoresis of *Crotalus durissus terrificus*. Molecular weight markers (lane 1), semi-purified gyroxin, before affinity column (lanes 2 and 3), compounds not retained (lanes 4 and 5), purified gyroxin (lanes 6–9) being lanes 8 and 9 after treatment with β-mercaptoethanol and urea.

Table 1
Test of biological activities of *Lachesis* and *Crotalus* gyroxin

Activities	*Lachesis* gyroxin	*Crotalus* gyroxin
Esterasic (BAEE, TAME)	+	+
Amidasic (BAPNA)	+	+
Coagulant (bovine fibrinogen)	+	+
Phospholipasic	−	−
Platelet aggregation	−	−
Barrel rotation	+	+

Crotoxin as well as crotamine, produced a clear increase in basal release of acetylcholine in rat and mouse striatal tissue. However, crotoxin was more effective in eliciting release in mouse tissue and crotamine was more effective on rat striatal tissue. Fig. 4 represents the release profiles obtained in these experiments.

Both *Lmm* and *Cdt* gyroxins failed to modify the release of [^{3}H]-dopamine, [^{3}H]-acetylcholine or [^{14}C]-acetylcholine from rat and mouse striatal tissue. Basal release in the absence and presence of toxin was similar to the control as well as the release obtained by stimulation with 20 mM K$^+$, as can be observed in Fig. 5, showing representative

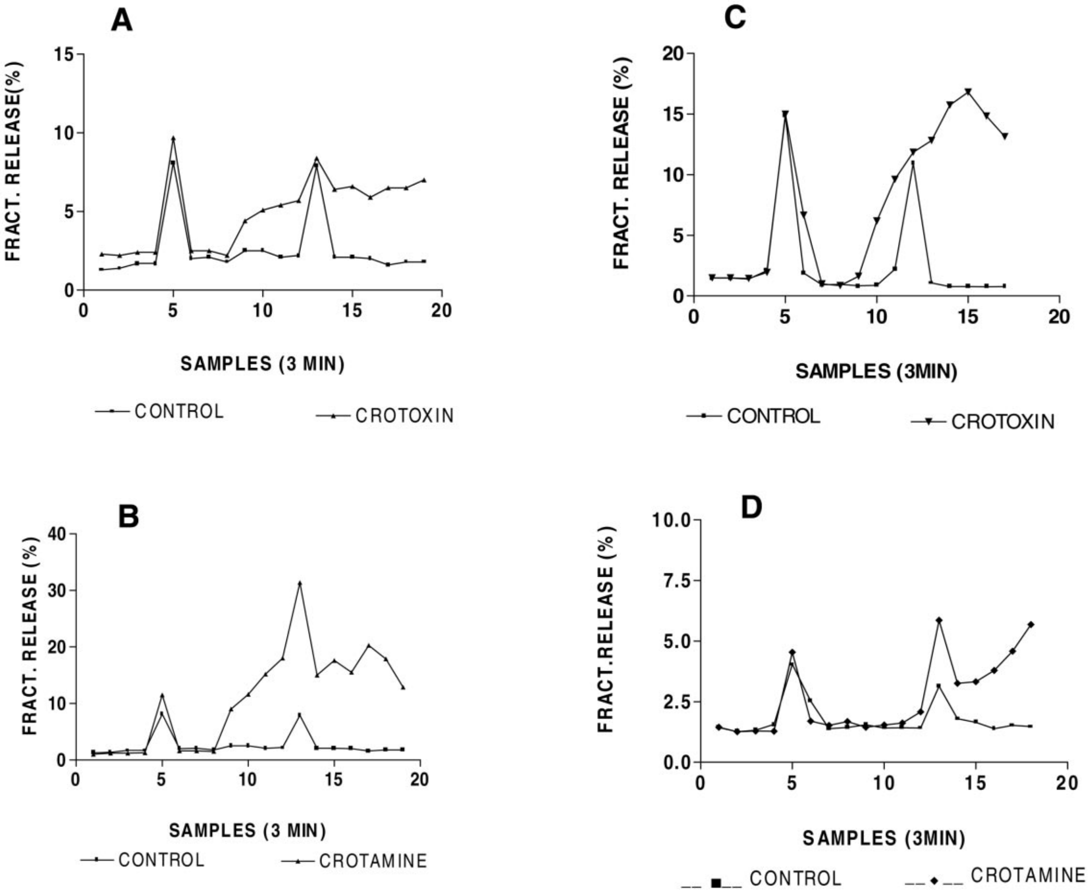

Fig. 4. Results of release experiments using neurotoxins as positive controls. (A) Crotoxin 1.3 μM on acetylcholine release from rat striatal tissue ($B_2/B_1 = 3.06 \pm 0.35^*$; $S_2/S_1 = 0.3 \pm 0.13^*$); (B) Crotamine 5 μM on acetylcholine release from rat striatal tissue ($B_2/B_1 = 10.3 \pm 1.37^*$; $S_2/S_1 = 2.17 \pm 0.30^*$); (C) Crotoxin 1.3 μM on acetylcholine release from mouse striatal tissue (it was impossible to calculate the basal and stimulated releases with toxin given to the shape of the profile); (D) Crotamine 5 μM on dopamine release from mouse striatal tissue ($B_2/B_1 = 1.26 \pm 0.05^*$; $S_2/S_1 = 1.24 \pm 0.06$). Significant difference $^*p < 0.05$.

release profiles obtained with the highest doses of toxin tested in rat striatum. Several doses of both toxins were tested and the normalised results (B_2/B_1 and S_2/S_1 with control values taken as absolute value of one) are displayed in Fig. 6 for rat striatum and Fig. 7 for mouse striatum. None of the doses modified significantly these release parameters.

4. Discussion

The lack of effect of both gyroxin preparations on [^{3}H]-dopamine, [^{3}H]-acetylcholine and [^{14}C]-acetylcholine release in rat and mouse striatum argues against a direct neurotoxic activity since in this case there is no blood–brain barrier to limit the access of the toxins to the eventual receptor/target in brain tissue. The neurotransmitter release assay employed here proved to be sensitive to a variety of different toxins as showed with crotoxin and crotamine and from our previous observations (Troncone et al., 1995). Crotoxin produced similar results elsewhere (Delot and Bon, 1992).

Results by Moreira et al., (1992a,b) and Moreira (1993) report convulsive activity for gyroxin. These authors failed, though, to report that the toxins used in their studies were indeed pure since all the results described for gyroxin, crotoxin and crotamine were very similar, suggesting that a considerable degree of contamination could account for their results. In order to ensure that our purified gyroxin from *Cdt* venom was free of potential contaminants like crotoxin, convulxin and crotamine, powerful toxins that could lead to false results, assays were performed in this study and proved that these fractions were free of PLA_2 activity and platelet aggregating factors, as can be seen in Table 1.

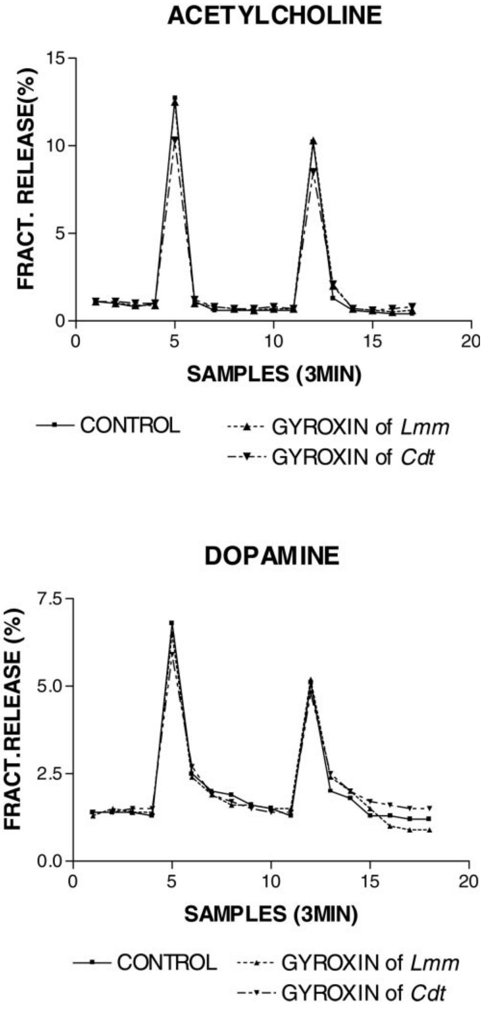

Fig. 5. Results of superfusion experiment in rat striatal tissue showing the effect of gyroxins on the release of acetylcholine. (A) *Lmm* 1 μM (B_2/B_1 0.75 ± 0.03; S_2/S_1 0.85 ± 0.02) and *Cdt* 1 μM (B_2/B_1 0.73 ± 0.03; S_2/S_1 1.04 ± 0.19) and dopamine (B) *Lmm* 1 μM (B_2/B_1 0.73 ± 0.03; S_2/S_1 0.87 ± 0.07) and *Cdt* 1 μM (B_2/B_1 0.73 ± 0.03; S_2/S_1 0.87 ± 0.04).

Assuming that gyroxin is devoid of direct neurotoxic action, alternative hypotheses could be proposed to explain the barrel rotation syndrome:

(a) Initially, the release of active products of enzymatic activity upon the inactive endogenous substrate could account for the syndrome. Alexander et al. (1988) verified that the crotalic gyroxin purified by different methods as well as thrombin-like enzymes available on the market (Crotalase® from *Crotalus adamanteus* venom and Ancrod® from *Calloselasma rhodostoma*) induced barrel rotation and fibrinogen coagulation, and had amidasic and esterasic enzymatic activities. According to these authors the capacity to induce barrel rotation would be associated with the enzymatic activities, correlating better with the coagulant activity than with the esterasic one. In addition, a second IV injection of gyroxin failed to induce barrel rotation. Inhibition of enzymatic activity by heat, 2-mercaptoethanol or dithiothreitol as well as the addition of specific inhibitors like PMSF (*p*-toluene sulphonyl fluoride); DFP (fluorphosphate diisopropyl) and TACK (tosil-arginine-chlorometyl-ketone) eliminated the barrel rotation syndrome (Barrabin et al., 1978; Alexander et al., 1988). Also, the long delay in the onset of barrel rotation agrees with the hypothesis of accumulation of a product of enzymatic activity.

Challenging this hypothesis, Wurpel et al. (1986a) showed that under continuous intracerebroventricular infusion of arginine–vasopressin for 60 min, the behavioural syndrome continued to show the same delay and some animals failed to present the behaviour. Later, these authors proposed that barrel rotation would demand that several stages and/or several active sites should be activated to elicit the syndrome (Wurpel et al., 1986a). Moreira (1993) suggested the involvement of intracellular processes (production of second and third messengers) that would be slower than those linked to synaptic facilitation or block.

(b) Alternatively, as stated above, some neuropeptides like somatostatin, arginine–vasopressin and endothelin-3 have been shown to induce barrel rotation when injected intracerebroventricularly. It seems possible that one or more of these peptides could be released in the brain as a physiological response to variations in blood pressure produced by gyroxins, and therefore, these peptides would be responsible for the behavioural syndrome (Yoshizawa et al., 1990; Willcox et al., 1992; De Wied et al., 1993; Diamant et al., 1994; Kannan et al., 1994). In this way, some thrombin-like enzymes from crotalic venom (Alexander et al., 1988; Markland et al., 1982) cause a pronounced decrease in blood pressure that could lead to release of vasopressin. Again, intracerebroventricular injection of endothelin-3 produced a blood pressure response increasing vasopressin release and could trigger barrel rotation behaviour (Yoshizawa et al., 1990; Kannan et al., 1994). The involvement of vasopressinergic V1 receptors was proven since pre-treatment with specific antagonists of this receptor prevented barrel rotation induced by high doses of intracerebroventricular arginine–vasopressin (Diamant et al., 1994). An association of barrel rotation behaviour with hypothermia was proposed since intracerebroventricular arginine–vasopressin induced the behaviour at the same time as when it produced a decrease in body temperature (Kasting et al., 1980; Diamant et al., 1994). Local vasoconstriction was proposed to generate barrel rotation; however, this relationship was questioned by several authors because angiotensin II is more potent than arginine–vasopressin as a vasoconstrictor and fails to increase motor activity in the rat, and lisine–vasopressin, which induces higher motor activation than arginine–vasopressin, has less vasoactive effect (Kruse et al., 1977; Willcox et al., 1992). Angiotensin-II

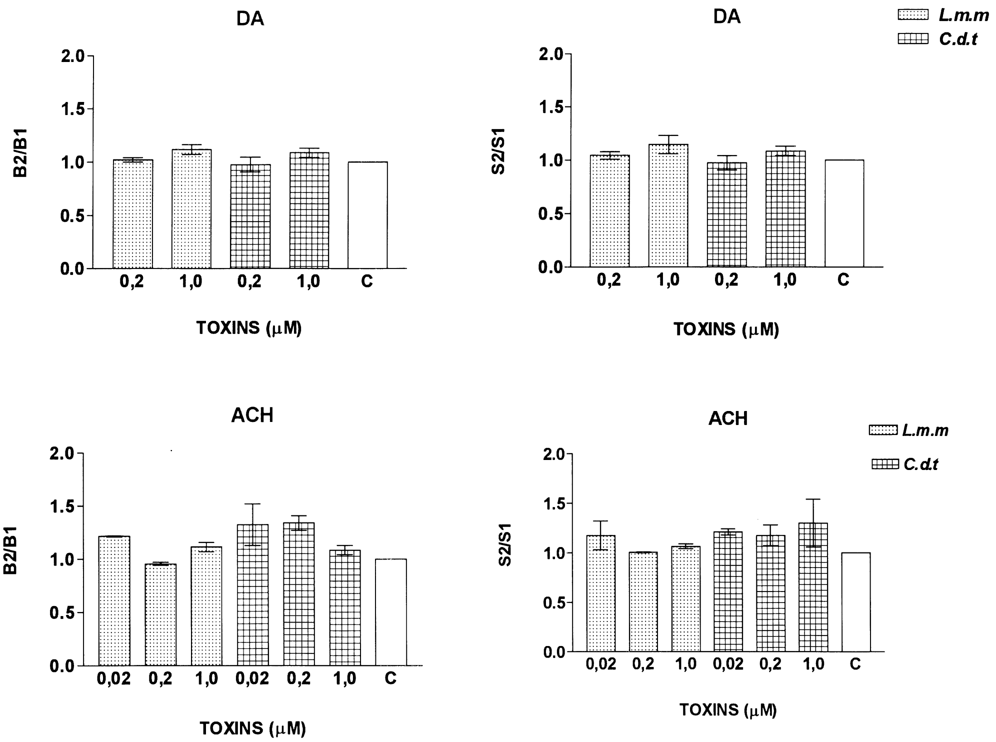

Fig. 6. Effect of several concentrations of gyroxins on basal and stimulated release of acetylcholine (ACH) and dopamine (DA) from rat striatal tissues. B_2/B_1 and S_2/S_1 were calculated as described in Section 2 and normalised considering control samples as numeric value of one. Each bar represents the average of release with S.E.M to different concentration samples. There was no significant difference between control and toxins groups.

has been shown to induce dopamine release from striatal tissue in similar experiments to those described here, but since gyroxin failed to modify the release of this transmitter, it is likely that gyroxin exerts an indirect effect on release, or a distinct brain structure may be involved in this behaviour (Simonnet and Giorguieff-Chesselet, 1979). Endothelin-3, also a potent vasoconstrictor, did not induce barrel rotation after intravenous injections (Kannan et al., 1994), but only after intracerebroventricular injection. Therefore, taken together, the mass of evidence suggests that vasopressin may be a necessary intermediate step in the induction of the barrel rotation syndrome. The precise mechanism of action and complete sequence of events that lead to the behaviour described have yet to be elucidated.

Finally, a high degree of homology is found in the amino acid sequences of the catalytic site of several thrombin-like toxins, serine-proteases as trypsin, thrombin and kallikrein. However, these enzymes do not induce barrel rotation or the cleavage of fibrinogen by thrombin in doses up to 10 times that of gyroxin (Alexander et al., 1988). These lead us to think that the enzymatic activity of gyroxin is essential to induce rotation and that the enzymatic sites are highly specific. A remarkable feature was indeed described by Massova et al. (1997), by analysing the three-dimensional structure of crotalase after modelling. They observed that “the topology of the molecular surface surrounding the active-site cavity of crotalase is unique and different from other serine-proteases”. These authors described two recognition exosites in crotalase, in contrast to all other serine-proteases, which have only one. This additional site identified in crotalase could be somehow related to barrel rotation because it is exclusive of the thrombin-like enzymes, not having been described in other serine-proteases.

In conclusion, a direct effect of gyroxins on neurotransmitter release in striatal tissue is rather unlikely, as demonstrated in this study, and possibly does not occur at all since more consistent data give support to the role of vasopressin in this behaviour, which indeed, plays an important role in blood pressure control modified by gyroxin.

Gyroxin seems to be the most active toxin in the venom of *Lachesis muta muta* (Da Silva et al., 1989) and the hypotension induced by the haemorrhagic kinin-releasing enzymes (Diniz and Oliveira, 1992) as well as the haemorrhage caused by other toxins (Sanchez et al., 1987; Sanchez et al., 1991) increase its action (Burnard et al., 1983), demonstrating once more the synergistic effect between components of venoms. In the *Crotalus durissus terrificus* venom its importance is overshadowed by crotoxin. This also gives

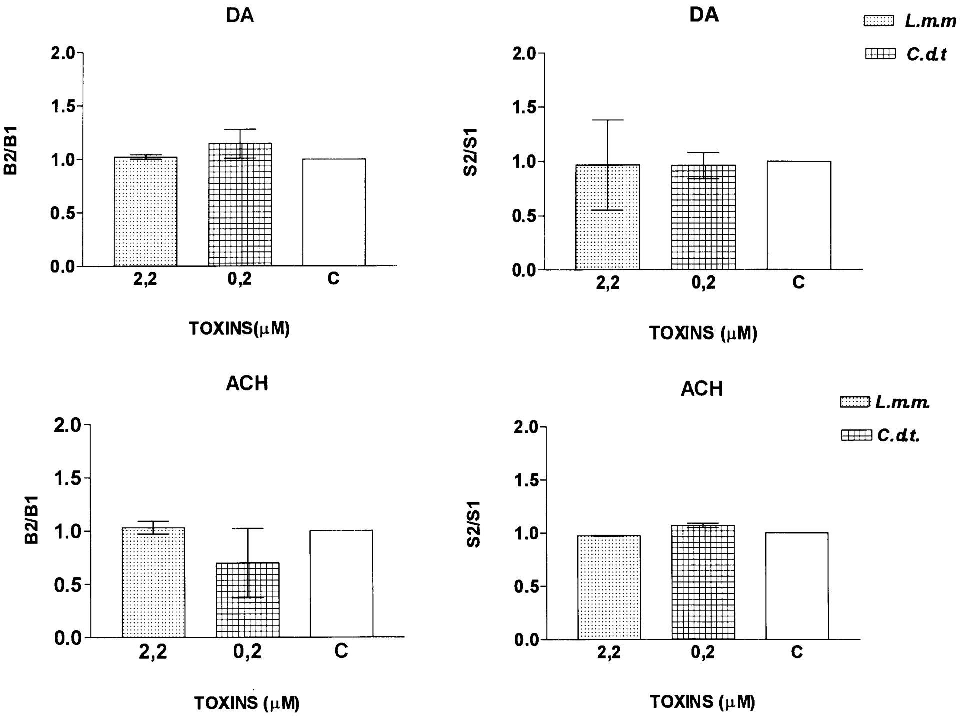

Fig. 7. Effect of several concentrations of gyroxins on basal and stimulated release of acetylcholine (ACH) and dopamine (DA) from mouse striatal tissues. B_2/B_1 and S_2/S_1 were calculated as described in Section 2 and normalised considering control samples as numeric value of one. Each bar represents the average of release with SEM to different concentration samples. There was no significant difference between control and toxins groups.

support to the position of the two snakes in the evolutionary process (Brattstrom, 1964).

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