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Title: Neurochemical characterization of a neuroprotective compound from *Parawixia bistriata* spider venom that inhibits synaptosomal uptake of GABA and glycine.

Authors: Renê Oliveira Beleboni; Renato Guizzo; Andréia Cristina Karklin Fontana; Andrea Baldocchi Pizzo; Ruither Oliveira Gomes Carolino; Leonardo Gobbo-Neto; Norberto Peporine Lopes; Joaquim Coutinho-Netto; Wagner Ferreira dos Santos[1].

Department of Biochemistry and Immunology, Ribeirão Preto School of Medicine, University of São Paulo (R.O.B.; R.O.G.C.; J.C.N.); Department of Biology, Ribeirão Preto Faculty of Philosophy, Sciences and Literature, University of São Paulo (R.G.; A.B.P.; W.F.S); Department of Physics and Chemistry, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (L.G.; N.P.L.); Department of Neurobiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA (A.C.K.F); Department of Biotecnology, University of Ribeirão Preto (R.O.B).

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Running title: Neuroprotective compound from spider venom.

Author for correspondence

Dr. Wagner F. dos Santos (PhD)

Biology Department, Faculty of Phylosophy, Sciences and Literature, University of São Paulo; Av. Bandeirantes, 3900 - Ribeirão Preto, São Paulo, Brazil, Zip code: 14040-901.

wagnerf@usp.br

Phone: 55 16 36023657

FAX: 5516 36331758

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Abbreviations: COSY, Correlation Spectroscopy; ESI-MS, Electrospray ionization mass spectrometry; FrPb, *Parawixia bistriata* fraction; GABA-T, γ-aminobutyrate: α-ketoglutarate aminotransferase - EC: 2.6.1.19; HMBC, Heteronuclear Multiple Bond Correlation; HMQC, Heteronuclear Multiple Quantum Coherence; LDH, lactic acid dehydrogenase; PbTx, *Parawixia bistriata* toxin.

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Abstract

The major contribution of this work is the isolation of a neuroprotective compound referred to as FrPbAII (2-amino-5-ureidopentanamide, M_r = 174), from *Parawixia bistriata* spider venom, and an investigation of its mode of action. FrPbAII inhibits synaptosomal GABA uptake in a dose-dependent manner and probably does not act on Na^+, K^+, Ca^{2+} channels, $GABA_B$ receptors or γ-aminobutyrate: α-ketoglutarate aminotransferase enzyme (GABA-T), therefore, it is not directly dependent on these structures for its action. Direct increase of GABA release and reverse transport are also ruled out as mechanisms of FrPbAII activities, as well as unspecific actions on pore membrane formation. Moreover, FrPbAII is selective for GABA and glycine transporters, having slight or no effects on monoamines or glutamate transporters. According to our experimental glaucoma data in rat retina, FrPbAII is able to cross the blood-retina barrier and promote effective protection of retinal layers submitted to ischemic conditions. These studies are of relevance by providing a better understanding of neurochemical mechanisms involved in brain function, and for possible development of new neuropharmacological and therapeutic tools.

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Introduction

γ-aminobutyric acid (GABA) is the most predominant inhibitory neurotransmitter in the mammalian central nervous system (Hendry et al., 1987). Also, it has been well established that a detailed understanding of the GABA pathways and new approaches to identify targets and drugs for the treatment of neural diseases are of relevance (Wong et al., 2003; Beleboni et al., 2004a).

Following endogenous synthesis, the build-up of GABA in synaptic vesicles is directed by a Na^+-independent proton-electrochemical gradient generated at the cost of a H^+-ATPase transporter (Christensen et al., 1991). Release of GABA occurs either by classical Ca^{2+}-dependent exocytosis or through a Ca^{2+}-independent mechanism probably involving reverse transport of the neurotransmitter (Agostinho et al., 1994). Once released, GABA acts through specific receptors localized in pre- and post-synaptic membranes, which may be classified as ionotropic ($GABA_A$ and $GABA_C$) or metabotropic ($GABA_B$) receptors (Bormann 2000; Bowery et al., 2002).

The physiological GABA concentrations in synaptic cleft are maintained by neuronal and glial membrane transporters. GABA transporters are proteins of the Na^+- and Cl^--dependent transporter family, composed of several sub-families like the choline, monoamine, taurine, glycine and betaine amino acid transporters (Worral and Willians, 1994). Up to now, the existence of four subtypes of GABA transporters has been postulated. The nomenclature of these proteins is not clear enough, varying according to the species from which they were cloned, that includes mice, rats and humans (Sarup et al., 2003).

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Spider venoms are useful sources of bioactive molecules and show a wide range of pharmacological effects on synaptic transmission. Neurotoxins from these venoms are evolutionary products that could function to immobilize prey or also in self-defense. Several of these molecules show high affinity for ion channels, receptors and transporters in invertebrates and vertebrates (for review, see Beleboni et al., 2004b). These molecules represent a rich source of useful probes for understanding of synaptic transmission events, for identifying insecticide targets and as aids for the designing of novel drugs for the treatment of neurological disorders (Harvey et al., 1998; Escoubas et al., 2000).

Parawixia bistriata is a social spider found in the South American “cerrados”. The injection of its venom produces irreversible and dose-dependent paralysis in termites (Fontana et al., 2000). Intracerebroventricular injection of the venom, as well as of its more purified fractions (including *P. bistriata* fraction AII; FrPbAII), abolishes convulsive tonic-clonic seizures induced by picrotoxin, bicuculline and pentylenetetrazole in rats (Cairrão et al., 2002). Also, a previous report has shown that a highly purified component of the venom (*P. bistriata* toxin 1.2.3; PbTx1.2.3) (M_r = 437) enhances glutamate uptake by a mechanism that seems to be independent from glutamate receptor activation. In addition, PbTx1.2.3 prevents neuronal death during retinal ischemia by enhancing glutamate clearance (Fontana et al., 2003).

Considering the recognized relevance of GABA neurotransmission, as well as of spider venoms as a rich source of bioactive substances, the major aims of this work were to identify a novel neuroprotective compound from *P. bistriata* spider venom, FrPbAII, and to investigate its mode of action in synaptosomes. According to our results,

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FrPbAII could serve as a basis for designing of therapeutic drugs that decrease GABA clearance hence decreasing the neuronal damage.

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Materials and Methods

Materials

Methanol, acetonitrile and trifluoroacetic acid (TFA) were of analytical grade, purchased from Merck (Germany), and deuterated methanol, from ACROS (Belgium). HPLC columns were from Shimadzu Techno-Research, Inc. (Kyoto, Japan), and Millipore filters (0.45 μm of porosity), from Millipore (Brazil).

4-Amino-n-[2,3-^{3}H]butyric acid ([^{3}H]GABA) (89 Ci/mmol), [^{3}H]Glycine (23 Ci/mmol), [^{3}H]Dopamine (10 Ci/mmol), [^{3}H]Serotonine (122 Ci/mmol), [^{3}H]Noradrenaline (15 Ci/mmol) and L-[G-^{3}H]glutamate (49 Ci/mmol) were obtained from Amersham Biosciences (UK). Reagents for Krebs-phosphate buffer, tetrodotoxin (TTX), α-ketoglutaric acid, and unlabelled neurotransmitters were from Sigma (St. Louis, MO, USA). Nipecotic acid, tetraethylammonium (TEA) and baclofen were from RBI (Massachusetts, USA).

3,5-Diaminobenzoic acid was from ACROS Organics (New Jersey, USA), and thiopental from Cristalia (Brazil). Scintillation cocktail ScintiVerse was obtained from Fisher Scientific (UK). Solutions for histological analyses and all other reagents were from Reagen, Vetec (Brazil) or Merck (Germany).

Spider collection and preparation of venom extract

P. bistriata specimens were collected in the region of Ribeirão Preto, São Paulo State, Brazil. Upon arrival in the laboratory, spiders were frozen and stored at -20°C. The venom sacs were removed, crushed in Milli-Q water at 0-4°C, and the extract boiled for 10 min. After, this venom extract was cleared by centrifugation at 3,000 x *g* for 10 min, and the supernatant was lyophilized and weighted.

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Fractionation of the venom extract by reverse phase HPLC

HPLC procedures was performed on a Shimadzu LC-6A apparatus with a ultraviolet detector SPD-6AV coupled with an auto injector (SIL-10ADvp, Shimadzu) or on a Shimadzu LC-6AD apparatus with a Diode Array Detector (SPD-M10Avp, Shimadzu), associated with an auto injector (SIL-10AF, Shimadzu), both using the software CLASS-VP 6.14.

FrPbAII was obtained to homogeneity by two chromatographic steps carried out at room temperature. In the first, Milli Q water (solvent A) and acetonitrile (solvent B) were degassed prior to use. The lyophilized venom extract was dissolved in Milli-Q water (40 mg/ml), filtered on Millipore filters, and applied onto a reverse phase HPLC column (PREP-ODS 20 x 250 mm, 5 μm) previously equilibrated with 1% (v/v) of Solvent B. This sample was eluted by a linear gradient from 1-100% of Solvent B (v/v) with a hold of 5 min at 20% (v/v) of Solvent B. The flow rate was 8.0 ml/min, and elutes were continuously monitored at 215 nm. Seven fractions were collected in ice bath, lyophilized, dissolved in Krebs-phosphate buffer, and assayed for effects on synaptosomal GABA uptake, as described below.

In the second step, the active fraction referred to as to FrPbAI (*Parawixia bistriata fraction* AI; retention time of 7.25 min) (1.37 mg/ml) was chromatographed on a Shim-pack CLC-C8 (M) (4.6 x 250 mm, 5 μm) analytical column, coupled to a pre-column Shim-pack, CLC G-C8 (4 x 10 mm), following an isocratic profile of Milli-Q water/Metanol/TFA (99:1:0.1, v/v), for 8 min, at a flow rate of 1.0 ml/min. Again, eluates were continuously monitored at 215 nm. Five eluted fractions were collected in ice bath,

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lyophilized, dissolved in Krebs-phosphate buffer, and that able to inhibit synaptosomal GABA uptake referred to as FrPbAII (Retention time of 4.1 min).

Electrospray Mass Spectrometry

Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS) was performed on a Quattro-LC instrument (Micromass, UK). High resolution q-TOF ESI-MS spectrum was acquired on an UltrOTOF apparatus (Bruker Daltonics, Billerica, USA). Solutions were infused into the ESI source using a Harvard Apparatus model 1746 (Holliston, MA) syringe pump, at a flow rate of 10 μL/min. Collision-induced dissociation was performed on the isolated protonated molecule using argon as collision gas.

Nuclear Magnetic Ressonance

^{1}H and 2D Heteronuclear Multiple Quantum Coherence (HMQC), Heteronuclear Multiple Bond Correlation (HMBC), Correlation Spectroscopy (COSY ^{1}H-^{1}H) NMR spectra were recorded at 500MHz on a Bruker Avance DRX-500. Chemical shifts (δ) were referenced to TMS signal.

Preparation of synaptosomes

Male Wistar rats (200-250g) were bred at University of São Paulo (Ribeirão Preto/SP, Brazil). Animals were kept on a 12:12 h light:dark cycle, at room temperature, supplied with food and water ad libitum. Animals were decapitated without the use of anesthetics. All procedures followed the guidelines established by the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Every effort was made to avoid unnecessary stress and pain to the experimental animals.

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Cerebral cortex (for GABA, glutamate, glycine or noradrenaline uptake or release assays), retina (for GABA and glycine uptake assays), hippocampus or striatum (for serotonine and dopamine uptake assays, respectively) were used to prepare synaptosomes as previously described by Gray and Whittaker (1962). Synaptosomes were resuspended in Krebs-phosphate buffer (in mM: NaCl 124; KCl 5; KH_2PO_4 1.2; $CaCl_2$ 0.75; $MgSO_4$ 1.2; Na_2HPO_4 20; glucose 10; pH 7.4) and centrifuged for 20 min at 4°C. Protein content was determined according to Lowry et al. (1951), modified by Hartree (1972).

Neurotransmitters uptake assays

Synaptosomes were resuspended in Krebs-phosphate buffer and preincubated for 10 min at 25°C (for GABA uptake assays), for 10 min at 37°C (for glutamate, serotonine, dopamine, noradrenaline uptake assays) and for 15 min at 30°C (for glycine uptake assays) in the absence or presence of increasing concentrations of venom extract or fractions obtained following each chromatographic step. In the retina synaptosomes assays, $MgCl_2$ (1 mM, final concentration) was added to 0.32 M sucrose, during retina enucleating and homogenization, or in Krebs-phosphate buffer.

Uptake assays were initiated, in each case, by adding radio-labeled neurotransmitters ([^{3}H]GABA, 10 nM; [^{3}H]Glycine, 250 nM; [^{3}H]Dopamine, 5 nM; [^{3}H]Serotonine, 5 nM; [^{3}H]Noradrenaline, 50 nM or [^{3}H]L-glutamate, 100 nM; all at final concentration), to synaptosomal suspensions (100 µg of protein/ml, final concentration), and incubated for 3 min (for GABA and glutamate assays), 5 min (for noradrenaline assays), 10 min (for dopamine and serotonine assays), or 15 min (for glycine assays) at the above specified temperatures. The time and temperature of incubation were

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adjusted for 5 min at 25°C, for GABA or glycine uptake assays, when rat retina synaptosomes were used. The uptake rate measured under these conditions was found to be linear over time and amount of tissue used. For [^{3}H]Dopamine, [^{3}H]Serotonine and [^{3}H]Noradrenaline, ascorbic acid and pargyline (1.7 mM and 80 μM, final concentrations, respectively) were added to Krebs-phosphate buffer to avoid monoamines oxidation or degradation.

In order to evaluate FrPbAII effects on synaptosomal GABA uptake in the presence of agents that alter $GABA_B$ receptor and ion channels activities, synaptosomes from rat cerebral cortex (100 μg of protein/ml) were incubated for 3 min at 25°C, with 10 nM of [^{3}H]GABA in the presence or absence of FrPbAII (24 μg/ml); and at final concentrations of TTX (5 μM); $CdCl_2$ (1.0 mM); TEA (5.0 mM) or Baclofen (0.1mM).

Incubations were carried out in triplicate, and reactions were interrupted by centrifugation (3,000 x *g*, for 3 min at 4°C). Supernatants were discarded; pellets were washed twice with ice-cold distilled water, homogenized in 10% tricloroacetic acid (TCA) and centrifuged at 3.000 x *g*, for 3 min at 4°C. Aliquots of supernatants were transferred to scintillation vials containing 5 ml of the biodegradable scintillation cocktail ScintiVerse, and their radioactivity were quantified in a scintillation counter (Beckman, LS-6800) with a counting efficiency of 35-40% for [^{3}H]. Non-specific uptake was estimated in parallel probes with nipecotic acid (6 mM, final concentration) (for GABA assays), and using non-labeled neurotransmitters (1mM, final concentrations, for glutamate and glycine) or low temperature (0-4°C, for all other assays); values obtained were subtracted from those of the total uptake.

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Dose-response curves were fitted to the Hill equation in non-linear regression analyses using the GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, California, USA). Results were expressed as averaged percent control uptake values with their standard error of the mean (S.E.M.). Statistical significance was assessed using Student's *t*-test; $^{*}p<0.05$ values were considered significant.

Saturation curves were performed as described above, in the presence of increasing concentrations of unlabeled GABA (4.5 nM to 10 μM, final concentration). The kinetic values K_M and V_{max} for synaptosomal GABA uptake were obtained by means of Michaelis-Menten curves. All data are presented as means with their S.E.M. Briefly, high affinity GABA uptake assays were initiated by the [^{3}H]GABA (10 nM, final concentration) to synaptosomes (100 μg of protein/ml, final concentration) in triplicate. Statistical analyses of $V_{max.}$ and K_M values, obtained in each experiment in the presence or absence of FrPbAII (24 μg/ml, final concentration), were performed using Student's *t*-test for paired data ($^{*}p<0.05$).

GABA release assays

Synaptosomes (3 mg/ml) were pre-loaded with 0.5 μM [^{3}H]GABA in Krebs-phosphate buffer, for 20 min at 25°C. Samples were centrifuged for 3 min at 7.200 x *g* at 4°C, and pellets washed three times with cold buffer. To assess neurotransmitter release, the final pellet was resuspended in Krebs-phosphate buffer and incubated for 3 min at 25°C in the absence or presence of increasing concentrations of FrPbAII. These concentrations are representative of those used to access the FrPbAII activity in GABA uptake assays. Neurotransmitter release was also measured in the presence of 50 mM KCl and 5 μM TTX, to verify the functional properties of synaptosomal preparations.

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Reactions were stopped by centrifugation (3.000 x *g*, for 3 min at 4°C), and aliquots of supernatants and pellets were separately transferred to scintillation vials containing 5 ml of biodegradable scintillation cocktail; radioactivity was quantified in a scintillation counter. The amounts of released GABA were calculated as percent of control uptake average. Statistical analyses were performed using Student's *t*-test ($^{*}p<0.05$).

GABA-T (γ-aminobutyrate: α-ketoglutarate aminotransferase - EC: 2.6.1.19) activity in the presence of FrPbAII

To obtain whole brain homogenate preparations, rat brains were removed and homogenized in 5 ml of ice-cold water using a Potter-Elhvejen, Labo-Stirrer LS-50-Yamato homogenizer. Protein content was determined by the Lowry method (Lowry et al., 1951), as modified by Hartree (1972). GABA-T activity was measured based on the rate of succinic semialdehyde formation, according to Salvador and Albers (1959). The incubation medium contained α-ketoglutaric acid (10 mM), GABA (50 mM) and 0.2 ml of brain homogenate (150-250 μg of protein/ml), in the presence or absence of FrPbAII (24 μg/ml) in a final volume of 1 ml. Samples were incubated for 60 min at 38°C, cooled in an ice bath and centrifuged for 15 min at 3.000 x *g* at 4°C. Aliquots of 0.3 ml of supernatants were collected and added to 0.3 ml of a 3,5-diaminobenzoic acid solution (0.2 M; pH 6.0), and samples were heated for 60 min at 60°C. After these procedures, the samples were measured in Spectrofluorometer RS-540 (Shimadzu). The excitation and emission wavelength were set to 405 and 505 nm, respectively. For tissue blanks the incubation at 38°C was omitted.

Effects of FrPbAII in experimental glaucoma model

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Male Wistar rats (230-250g) were intraperitoneally anesthetized with thiopental (50 mg/kg), and their retinas submitted to ischemia according to Louzada-Jr. et al. (1992) and Fontana et al. (2003). Intraocular pressure was increased to 155 mmHg, by cannulating in the anterior chamber of the eye, with a sterile 27-gauge needle attached to a manometer/pump and an air reservoir. Ischemia was induced for 60 min, after which intraocular pressure was reduced to normal levels for 45 min (reperfusion period). The left retina of each animal was subjected to the experimental conditions, ischemia (n=5) and ischemia/reperfusion (n=5), while the right retinas served as a non-ischemic control (n=10). 25 μl of FrPbAII (6 mg/ml) were intravenously (i.v.) injected 15 min prior to ischemia (n=5) and ischemia/reperfusion (n=5) and the right retinas (contralateral retinas) served as a non-ischemic control (n=10). The animals were then sacrificed, the left and right eyes rapidly enucleated and fixed in Bouin's solution (75% picric acid, 25% formalin and 5% acetic acid) for 24 h. After fixation, cornea, aqueous humor, lens, vitreous humor were removed and the eyecups dehydrated in 70-100% ethanol and embedded in paraffin. Retinas were sectioned at 5 μm, approximately 1 mm from the emergency of the optic nerve, stained with hematoxilin-eosin and examined using a Zeiss Axiophot microscope. For each experimental group, five microscopic fields (160X or 636x474 pixels) of one sagital section at the superior retina were captured by light microscopy and digitalized with an analogic camera (JVC TK1270) connected to the microscope and a computer. A computer program KS 400 (Carl Zeiss Vision, Germany) was used to quantify the cell counts manually (means of cells ± SEM) in established areas (in mm^2), the outer nuclear layer (ONL, 47.16), inner nuclear layer (INL, 40.40) and ganglionar cell layer (GCL, 29.52). Significance of recorded differences between

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groups were determined using ANOVA ($^{*}p<0.05$). Qualitative analysis was performed to characterize histological damages as decreases of cell numbers, cytoplasm vacuolization, edema, disorganization and pyknotic nuclei.

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Results

Effects of the venom extract and purified venom fractions (FrPbAI and AII) on synaptosomal GABA uptake are dose-dependent

Preliminary experiments assessing the effects of *P. bistriata* boiled crude venom on GABA uptake into cortical synaptosomes, indicated that this venom extract inhibits this process (Fontana et al., 2003). To further explore these observations, we examined the dose-dependence of the venom extract on this system. Fig. 1a shows the dose-response curve of spider venom extract on GABA uptake. The maximum inhibition (~ 98.20%) was obtained in the presence of 13440 μg/ml; the IC_{50} was 1700 ± 130 μg /ml (final concentration).

Following these early results, the aqueous extract of venom (40 mg/ml) was applied to a C18 column, resulting in the purification of the first active fraction, referred to as FrPbAI (retention time of 7.25 min) (data not shown).

Fig. 1b shows the dose-response curve of FrPbAI on GABA uptake. Maximum inhibition (~ 98.30%) was obtained in the presence of 1344 μg/ml; the IC_{50} was 100 ± 12 μg/ml (final concentration). FrPbAI (1.37 mg/ml) was chromatographed on an analytical C8 column, producing the second active fraction referred to as FrPbAII (retention time, 4.1 min).

Fig. 1c shows the dose-response curve of FrPbAII on GABA uptake. Maximum inhibition (~ 96.20%) was obtained in the presence of 320 μg/ml; the IC_{50} was 24 ± 0.019 μg/ml (final concentration). Therefore, IC_{50} values from the venom extract to the FrPbAII followed a profile of increasing affinity.

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All other fractions obtained at each chromatographic step, which include both highly hydrophilic and hydrophobic components, were assayed for their ability to produce inhibition of GABA uptake, having slight or no effects on this system. Uptake assays were regularly accompanied in parallel, by lactic acid dehydrogenase (LDH) activity measurement or morphological examination by electron microscopy. No morphological damage or increase in LDH level in the supernatants were detected, indicating that the synaptosomes maintained their integrity in the presence of the venom extract or its active fractions (data not shown).

Structural elucidation of FrPbAII

FrPbAII was chemically identified as 2-amino-5-ureidopentanamide (Fig. 2) by NMR and ESI-MS analyses. Two multiplets (2 H each) at δ = 1.57 and 1.78ppm, two triplets at δ = 3.09 (2 H, J = 6.8Hz) and 3.70ppm (1 H, J = 6.0Hz) were observed in ^{1}H NMR. Taken together with COSY ^{1}H-^{1}H data, these results indicate the saturated carbon chain. HMBC and HMQC confirmed this part of the molecule and provided ^{13}C chemical shift data suggesting the presence of an amide carbonil (δ = 175.5 ppm) in one edge of the molecule. The other functional groups linked to the carbon chain were proposed based in chemical shifts of ^{1}H and ^{13}C provided by NMR spectra.

Positive ESI-MS scan of FrPbAII showed the most intense signal at *m/z* 175. ESI-MS/MS experiments undertaken with this peak confirmed that all other peaks present in ESI-MS spectrum were its daughters, formed by in source dissociation. Therefore, $[M + H]^{+}$ 175 give us the M_r 174 to FrPbAII. In ESI-MS/MS experiments it could be observed the following three competitive losses, which confirmed all the functional groups linked to the carbon chain: loss of NH_3 (17 m.u.), affording *m/z* 158; or a loss of $CONH_3$ (45

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m.u.) giving *m/z* 130; or a loss of $NHCONH_2$ (59 m.u.) yielding the more stable ion at *m/z* 116. High Resolution q-TOF ESI-MS analyses (Supplemental Data) afforded *m/z* 175.1182 for the protonated parent ion $[M + H]^+$. Taken together with NMR and MS/MS fragmentation data, this information confirmed the molecular formula $C_6H_{15}N_4O_2^+$ (4.6 ppm error) for $[PbFrAII + H]^+$, since no other reasonable molecular formula is possible within a 50 ppm error.

FrPbAII does not affect maximum transport velocity but does alter apparent transport affinities

By measuring the synaptosomal GABA uptake over a range of substrate concentrations, the transport activity was shown to possess both saturability and high affinity. Fig. 3 shows the alteration of apparent transport affinities evoked by 24 μg/ml of FrPbAII (IC_{50}) (final concentration); it also shows that the V_{max} of GABA transport was not modified. The control values obtained for K_M and V_{max} were 1.17 $\pm$ 0.2 μM and 8.19 $\pm$ 0.3 pmol/min/mg, respectively. In the presence of FrPbAII, the K_M value was increased to 2.22 $\pm$ 0.2 μM and V_{max} was 8.15 $\pm$ 0.2 pmol/min/mg, a value quite similar to that obtained in control. Therefore, K_M was increased whereas V_{max} was not significantly changed, suggesting a competitive inhibition.

Uptake inhibition is not caused by alterations in GABA release or GABA-T activity

Synaptosomes can also mediate GABA release in a Ca^{+2}-dependent manner, or by means of reverse transport, especially when GABA-T is inhibited (Bernath and Zigmond, 1988). In order to confirm that GABA release was not directly affected in our experiments, we measured the neurotransmitter release in the presence or absence of FrPbAII, at a representative range of concentrations used in the GABA uptake assay.

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Fig. 4 shows the effect of FrPbAII (20-320 μg/ml, final concentration) on GABA basal release. Spontaneous release of GABA was not altered by FrPbAII. Control samples were incubated in the presence of KCl (50 mM) or TTX (5 μM), in order to verify the functional integrity of preparation. As expected, basal GABA release as increased by KCl, and TTX did not alter the process (data not shown). In addition, our experiments measuring GABA-T activity in the presence of FrPbAII (24 μg/ml, final concentration) did not showed significant alterations (data not shown).

FrPbAII effects are maintained even in the presence of ion channel inhibitors or a $GABA_B$ receptor agonist

Fig. 5 shows that the inhibition of synaptosomal GABA uptake caused by 24 μg/ml of FrPbAII (approximately 50%) is maintained even when the experiment was performed using ion channels inhibitors (TTX, 5 μM; $CdCl_2$, 1.0 mM; and TEA, 5.0 mM, final concentrations) or a $GABA_B$ receptor agonist (Baclofen, 0,1 mM, final concentration).

FrPbAII is selective for GABA and glycine systems

Monoamines and glycine transporters are homologous with GABA transporters; glutamate transporters are only similar, but also involved in ischemic damage. To verify the selectivity of the action of FrPbAII, synaptosomal uptake of [^{3}H]Glycine, [^{3}H]Serotonine, [^{3}H]Dopamine, [^{3}H]Noradrenaline and [^{3}H]L-glutamate was studied in the presence or absence of increasing concentrations of FrPbAII (20–320 μg/ml), at same range used in the [^{3}H]GABA uptake in this assay. Fig. 6 shows that FrPbAII is a selective inhibitor of [^{3}H]GABA and [^{3}H]Glycine uptake, having slight or no effect on other homologous or glutamate transporters.

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Neuroprotective effects of FrPbAII in experimental glaucoma

To investigate the effect of FrPbAII *in vivo*, rat retinas were submitted to experimental glaucoma. In these experiments, rats received i.v. injections of 25 μL FrPbAII (6mg/ml) or saline 15 min prior to ischemic treatment. Fig. 7 shows micrographs of non-ischemic control retinas, subjected to ischemia or to ischemia followed by reperfusion. Marked histological alterations could be observed when comparing non-ischemic control (Fig. 7a), ischemic (Fig. 7b) and ischemic/reperfused retinas (Fig. 7c).

Compared to non-ischemic control retinas, ischemic retinas (Fig. 7b), showed cytoplasm vacuolization, pyknotic nuclei and a decrease in cell number in the GCL. The INL displayed greater edema, pyknotic nuclei and cellular disorganization. The ONL exhibited decreased cell number, compared to control retinas. Edema was also observed in the inner plexiform layer (IPL).

In the ischemia/reperfusion retinas (Fig. 7c) the GCL showed lower cell density, and increased vacuolization and number of pyknotic nuclei as well as cellular disorganization. The INL also had fewer cells and those remaining had a greater amount of pyknotic nuclei and cytoplasm vacuolization, as well as, enhanced edema and cellular disorganization. There were also fewer cells in the ONL.

Compared to ischemic and ischemic/reperfused retinas (Fig. 7b and 7c), retinas of animals treated with FrPbAII (Fig. 7d, 7e and 7f) displayed normal cellular morphology and a remarkable reduction in cell death in all layers. Fig. 7d represents another control, a non-ischemic retina of animal treated with FrPbAII that showed no signs of cell damage. Fig. 7e shows ischemia-induced retinas treated with FrPbAII. No degeneration, pyknotic nuclei or disorganization of the cells, but some cell loss were

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observed. Fig. 7f shows ischemic/reperfused retinas treated with FrPbAII, where protection was observed in all layers and there was a decreased in cell loss.

The number of cells in control, ischemic and ischemic/reperfused retinas with or without treatment with FrPbAII are presented in Table 1. There was a decrease in number of cells in all layers following ischemia and in those that were reperfused following ischemia. The cell numbers were significantly decreased by 38% in the ONL, 54% in the INL and 51% in the GCL after ischemia and were decreased by 40% in the ONL, by 63% in the INL, and 58% in the GCL in the retinas subjected to ischemia/reperfusion compared to the non-ischemic control retinas ($^{*}p<0.05$). In contrast, retinas of animals treated with FrPbAII, showed significant protection of 27% in ONL; 57% in INL and 50% in GCL after ischemia and of 20% in ONL; 81% in INL and 44% in GCL in ischemia/reperfusion compared to ischemic and ischemic/reperfusion conditions ($^{*}p<0.05$), respectively (Table 1).

FrPbAII is also able to inhibit GABA and glycine uptake in synaptosomes from rat retinas

Synaptosomes from rat retina were used in order to verify the direct correlation between neuroprotection and inhibition of GABA and glycine uptake caused by FrPbAII. In these experiments, FrPbAII also caused a dose-dependent inhibition of neurotransmitter uptake (Fig. 8), as it was demonstrated by our assays using synaptosomes from rat cerebral cortex (Fig. 1c). IC_{50} values for FrPbAII in GABA and glycine uptake were 13.80 and 13.10 μg/ml, respectively.

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Discussion

In this work, we show the novel neuroprotective compound from *P. bistriata* venom extract (FrPbAII), which inhibits synaptosomal GABA uptake in a dose-dependent manner (Fig. 1c) and probably by a competitive antagonism (Fig. 3). In addition to this neuroprotective property, FrPbAII has previously been reported as a significant anticonvulsant against seizures induced in rats (Cairrão et al., 2002).

Synaptosomes are recognized as a useful model to neurochemical studies since they retain all machinery for the uptake, storage, release of neurotransmitters and ionic conductance (Gray and Whittaker, 1962, Bicalho et al., 2002, Wang and Sihra, 2003). In our studies, morphological examination of the synaptosomal preparations by electron microscopy demonstrated that membrane integrity was maintained, and levels of LDH were not changed when synaptosomes were incubated with either the venom extract, FrPbAI or FrPbAII (results not shown). These results indicate that the observed uptake inhibition was not due to plasma membrane disruption or pore formation.

Synaptosomes mediate residual GABA release during the uptake process. If FrPbAII was elevating basal release, it could enhance the unlabeled/labeled GABA ratio in the assay buffer producing an apparent and unreal decrease of neurotransmitter transport. Therefore, we also examined the effect of FrPbAII on GABA efflux. The synaptosomal release of neurotransmitter was stimulated by K^+ and was not altered by TTX, confirming the functional integrity of the preparation (data not shown). FrPbAII did not affect the levels of basal GABA release (Fig. 4), an indication that it does not act indirectly by altering tonic or depolarization-dependent GABA release or even via membrane pore formation.

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$GABA_B$ receptors are present on neural terminals throughout the CNS, acting as autoreceptors when localized on the pre-synaptic membrane. In this case, their activation inhibits release of further synaptic vesicles through the suppression of high-threshold Ca^{2+} channels (Harayama et al., 1998).

Upon the uptake process, GABA can be catabolized by the action of GABA-T. The enzyme inhibition increases GABA concentration in the brain, especially by reverse transport, decreasing susceptibility to convulsions and epileptic conditions (Sherif and Ahmed 1995). This process is distinguished from exocytotic release by being non-vesicular and independent of Ca^{2+} influx via selective voltage-dependent channels (Agostinho et al., 1994; Beleboni et al., 2004a).

If FrPbAII is able to inhibit GABA-T, K^+ channel activity and $GABA_B$ receptors function, or to enhance Na^+ or Ca^{2+} channels activity, these effects also could be responsible for an apparent and unreal decrease of neurotransmitter uptake, ruling out the possibility of a direct effect of FrPbAII on the GABA transporter.

In order to evaluate whether inhibition of GABA uptake by FrPbAII is mediated by an indirect mechanism through $GABA_B$ receptors or ion channels, we investigated whether channel and receptor agonists or antagonists could modify its effects on GABA uptake. These agents included TTX a Na^+ channel blocker, cadmium chloride a Ca^{2+} channel blocker, TEA a K^+ channel blocker, and baclofen a $GABA_B$ receptor agonist.

FrPbAII-inhibited GABA uptake was unchanged by the blockade of voltage-dependent Na^+ channels, Ca^{2+} or K^+ channels, when these blockers were tested concentrations known to produce effective inhibition of channel activity (Fig. 5). Despite of its potential effects on neuronal GABA concentration, baclofen did not alter the ability of FrPbAII to inhibit GABA uptake into synaptosomes, suggesting that those effects are

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not dependent on $GABA_B$ receptor inhibition (Fig. 5). Moreover, GABA-T activity is maintained in the presence of FrPbAII, indicating that reverse transport is not involved in FrPbAII effects (data not shown). Therefore, FrPbAII effect can not be directly dependent on Na^+, K^+, Ca^{2+} channels, $GABA_B$ receptors or GABA-T for its action. These results associated to those demonstrated on Fig. 4 rule out the possibility that FrPbAII could act by inhibition or activation of these structures, indicating its selectivity of action.

As demonstrated on Fig. 6, FrPbAII is highly selective for GABA and glycine transporters, having slight or no effects on homologous (serotonine, dopamine, noradrenaline) or glutamate transporters. These results reinforce the concept of a remarkable selectivity of FrPbAII, a desirable attribute for future development of new therapeutic drug or pharmacological tools.

GABA and glycine are relevant inhibitory transmitters in retinal synapses, as it was well established in the CNS, and many reports suggest a heterogeneous distribution of GABA/glycine receptors and transporters in the vertebrate retina (Honda et al., 1995; Wässle et al., 1998; Gadea et al., 1999). The organization and accessibility of the retina has made it the best-characterized system for examining the physiology and function of amino acid transporters and an excellent model to study the effects of drugs on ischemia/reperfusion in the CNS (Louzada Jr et al., 1992; Eliasof et al., 1998).

It is well-established that drugs acting on GABA and glycine uptake may provide an effective means for protecting the brain against neuronal injury and for treating epilepsy, as illustrated by the clinical use of tiagabine (Meldrum 1997; Fisher and Bogousslavsky 1998). Ichinose and Lukasiewicz (2002) using the selective GAT-1 blocker NO-711 showed that blockade of GABA uptake resulted in increased activation of $GABA_C$ receptors. This compound has proved to be effective as an anticonvulsant in

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animal models and as a neuroprotector against ischemia of CA1 pyramidal neurons in gerbil (O'Connell et al., 2001). Thus, to obtain insight into the possible neuroprotective actions, consequent to inhibition of GABA and glycine uptake, we examined FrPbAII effects during retinal ischemia and ischemia followed by reperfusion, using the experimental glaucoma model. In our experiments, FrPbAII was found to protect neurons from injury in all retinal layers, particularly in the INL (Fig. 7 and Table 1).

Thus, inhibition of GABA and glycine uptake by FrPbAII can increase GABA and glycine levels on the synaptic cleft promoting an activation of their receptors. These processes hyperpolarize neurons leading to a reduced transmitter release and/or action potential firing, effects that can act in the ischemic cascade promoting a neuroprotective effect of the retina in the experimental glaucoma model. Similar conclusions could be taken to explain our previous results, indicating a marked anticonvulsant activity presented by FrPbAII (Cairrão et al., 2002).

The neuroprotective effects of FrPbAII are observed in both ischemic and ischemic/reperfused retinas. Louzada Jr. et al. (1992) have shown that there is a higher percentage of glutamate release, and subsequent neuronal death during the reperfusion period than during ischemia; nevertheless, we still observed protection when FrPbAII was administered in this condition.

FrPbAII is also able to inhibit GABA and glycine uptake in synaptosomes prepared from rat retina (Fig. 8). This result confirms the view that the neuroprotective action of FrPbAII is due to inhibition of GABA and glycine uptake. Moreover, an enhancement of glutamate uptake could be discarded as a mechanism by which FrPbAII could act as a neuroprotective compound, since no effect on glutamate transport is observed when synaptosomes were incubated with FrPbAII (Fig. 6). A direct

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FrPbAII interaction with $GABA_A$/$GABA_C$ receptors on GABA binding site is also ruled out as a neuroprotective mechanism, since only a very high concentration of FrPbAII is able to induce [^{3}H]-GABA displacement on binding assays (Zukin et a., 1974; data not shown).

Although a number of classical GABA analogues are useful as pharmacological tools in research epilepsy and ischemic cerebral damage, they were shown to be inefficient in therapy due to their low permeability of the blood-brain barrier (Krogsgaard-Larsen et al., 1998). FrPbAII protects retinas against neuronal damage when administered intravenously, suggesting that this compound is able to cross the blood-retina barrier, which is structurally and functionally similar to the blood-brain barrier (Steuer et al., 2005).

To summarize, our results taken together provide an insight into the effects of a new neuroprotective compound from *P. bistriata* spider venom that acts primarily and directly on GABA and glycine transporters. In fact, the significant inhibition of GABA and glycine uptake caused by FrPbAII associated with its selectivity and blood-retina barrier permeability strongly suggest its high potential value in studies of transport mechanisms and the role of uptake during inhibitory neurotransmission. Moreover, an understanding of the structure and activity of active compounds from arthropod venoms may provide "proof of principle" for a new class of neuroprotective and anticonvulsant drugs that act by inhibiting GABA and glycine clearance.

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Legends for figures

Fig. 1 Dose response curves for the inhibitory effects of increasing concentrations of *P. bistriata* venom extract (a), FrPbAI (b) or FrPbAII (c), on [^{3}H]GABA uptake in synaptosomes from rat cerebral cortex. Uptake assays were initiated by adding [^{3}H]GABA (10 nM, final concentration). Synaptosomes were pre-incubated in the presence or absence of venom extract, FrPbAI and FrPbAII, at final concentrations of 210-13440 μg/ml, 21-1344 μg/ml, 2.5–320 μg/ml, respectively, for 3 min at 25°C. Concentrations of venom extract and fractions are plotted on a logarithmic scale. Higher concentrations than those shown also inhibit GABA uptake, but were not included in the Figure. Data from four-six independent experiments, performed in triplicate, generated an IC_{50} of 1700 $\pm$ 130 μg/ml, 100 $\pm$ 12 μg/ml and 24 $\pm$ 0.019 μg/ml, for respectively, venom extract, FrPbAI and FrPbAII in the GABA uptake assays.

Fig. 2 Chemical structure of FrPbAII.

Fig. 3 Kinetic analysis of high affinity GABA uptake by synaptosomes pre-incubated in absence (■) or presence (▼) of IC_{50} of FrPbAII (24 μg/ml) (final concentration). Uptake was measured in the presence of increasing concentrations of unlabeled neurotransmitter (from 4.5 nM to 30 μM, final concentration) and [^{3}H]GABA (10 nM, also at final concentration). Data are means ± S.E.M. of four independent experiments, performed in triplicate. Inset, Eadie-Hofstee plot.

Fig. 4 Effects of final increasing concentrations of FrPbAII on [^{3}H]GABA release. Synaptosomes were preloaded with [^{3}H]GABA (0.5 μM) for 20 min at 25°C. Release, expressed as % of neurotransmitter released over the control, was initiated by the addition of FrPbAII from 20 to 320 μg/ml. No significant difference was observed

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between control and experimental treatments ($^{*}p<0.05$). Each bar is a mean ± S.E.M of three independent experiments, performed in triplicate.

Fig. 5 GABA uptake inhibition caused by 24 μg/ml of FrPbAII (approximately of 50%) is maintained even in presence of Na^{+} (TTX, 5 μM), Ca^{2+} ($CdCl_2$, 1 mM), K^{+} (TEA, 5 mM) channels inhibitors or the $GABA_B$ receptor agonist (Baclofen, 0.1 mM) at final concentration. Each bar is a mean ± S.E.M of five independent experiments, performed in triplicate. Baclo= baclofen; AII= FrPbAII.

Fig. 6 Dose response curves for the effects of increasing concentrations of FrPbAII (20-320 μg/ml) on neurotransmitters uptake. (a) [^{3}H]GABA; (b) [^{3}H]glycine; (c) [^{3}H]glutamate; (d) [^{3}H]serotonine; (e) [^{3}H]dopamine; and [^{3}H]noradrenaline uptake. Data are means ± S.E.M. of three independent experiments, performed in triplicate.

Fig. 7 Effects of the intravenous injection of 25 μl FrPbAII (6mg/ml) in ischemic and ischemic/reperfused rat retinas. Retinal sections were stained with Hematoxilin-Eosin. Arrowheads denote areas of vacuolization and arrows, pyknotic nuclei. (a) control; (b) ischemic; (c) ischemic/reperfused; (d) control retina of animal pre-treated with FrPbAII (e) ischemic pre-treated with FrPbAII, (f), ischemic/reperfused pre-treated with FrPbAII. ONL, outer nuclear layer; OPL, outer plexiform layer, INL, inner nuclear layer, IPL, inner plexiform layer; GCL, ganglion cell layer. Bars= 50 μm.

Fig. 8 Dose response curves for the inhibitory effects of increasing concentrations of FrPbAII on [^{3}H]GABA (a) or [^{3}H]glycine (b) uptake in synaptosomes from rat retinas. Uptake assays were initiated by adding [^{3}H]GABA or [^{3}H]glycine (at respectively, 10 nM and 250 nM final concentrations). Synaptosomes were pre-incubated for 3 min at 25°C, in the presence or absence of FrPbAII at final concentrations of 2.5–320 μg/ml, the

same range used in assays with synaptosomes from cerebral cortex. Concentrations are plotted on a logarithmic scale. Data from four independent experiments, performed in triplicate, generated an IC_{50} of 13.80 and 13.10 mg/ml respectively, for FrPbAII in the GABA and glycine uptake assays.

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Table 1. Number of cells in retinal layers

	ONL	INL	GCL
Control	953 ± 43	259 ± 9	37 ± 4
Ischemia	590 ± 15 (38%)*	119 ± 6 (54%)*	18 ± 2 (51%)*
Ischemia/reperfusion	574 ± 30 (40%)*	96 ± 13 (63%)*	16 ± 1 (58%)*
FrPbAII/Ischemia	748 ± 28 (27%)♦	186 ± 10 (57%)♦	27 ± 2 (50%)♦
FrPbAII/Ischemia/reperfusion	688 ± 23 (20%)•	174 ± 9 (81%)•	23 ± 1 (44%)•

ONL, outer nuclear layer; INL, inner nuclear layer, GCL, ganglion cell layer.

*Percentage of cell losses compared to control retinas in non ischemic conditions;

♦Percentage of decrease in cell losses with FrPbAII treatment compared to ischemic conditions (Protection percentage);

•Percentage of decrease in cell losses with FrPbAII treatment compared to ischemic/reperfusion conditions (Protection percentage).

Fig. 1

Top

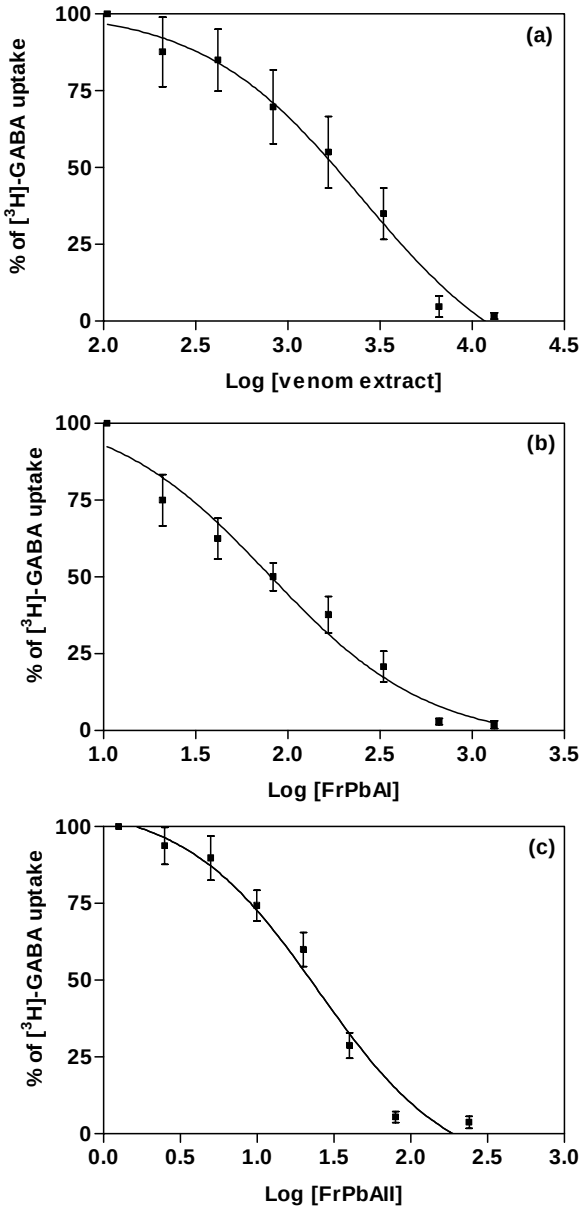

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Fig. 2

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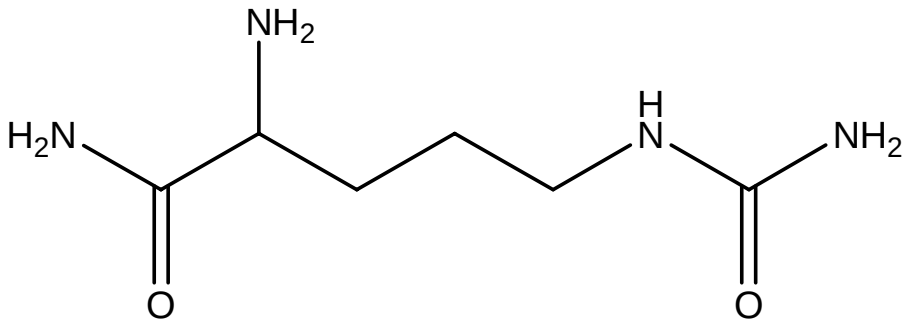

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Fig. 3

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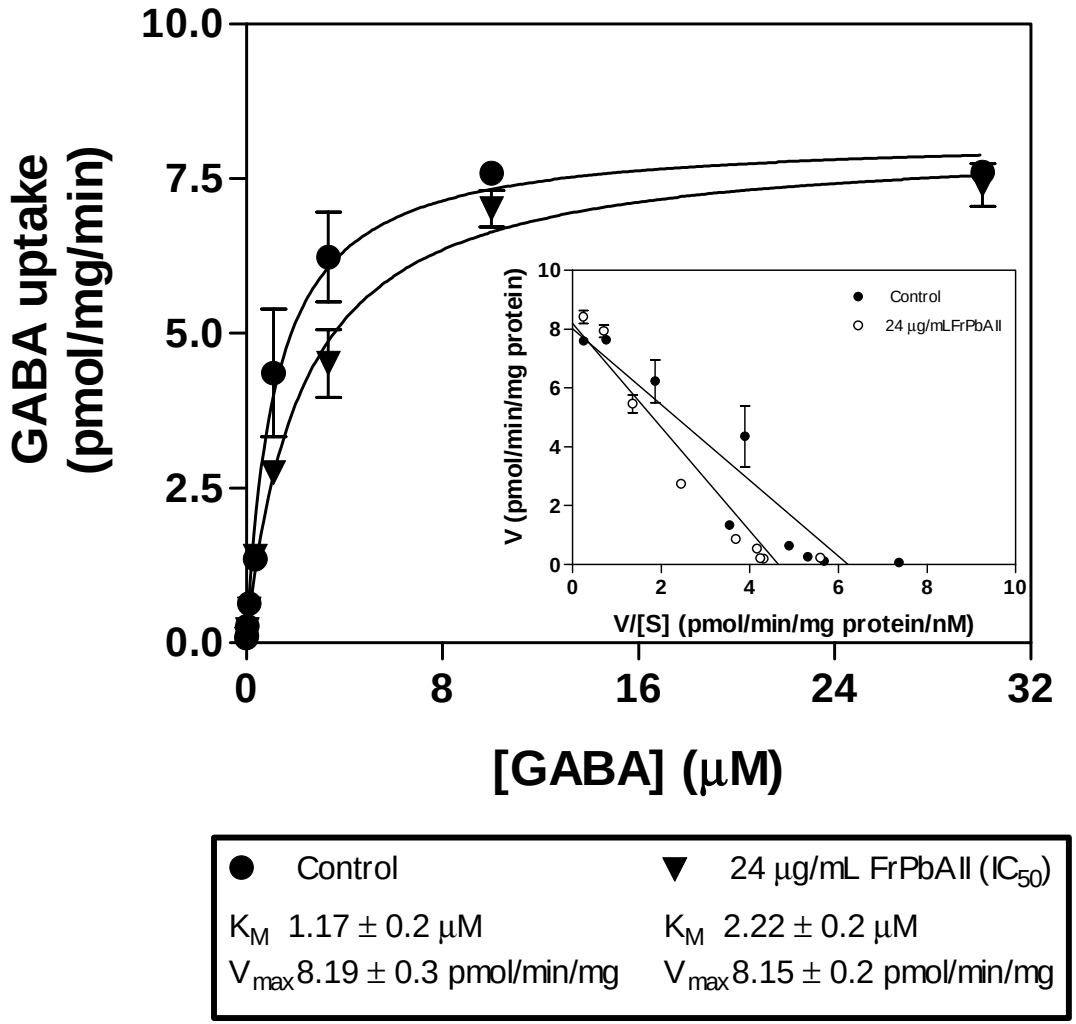

● Control	▼ 24 μg/mL FrPbAII (IC_{50})
K_M 1.17 ± 0.2 μM	K_M 2.22 ± 0.2 μM
V_{max} 8.19 ± 0.3 pmol/min/mg	V_{max} 8.15 ± 0.2 pmol/min/mg

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Fig. 4

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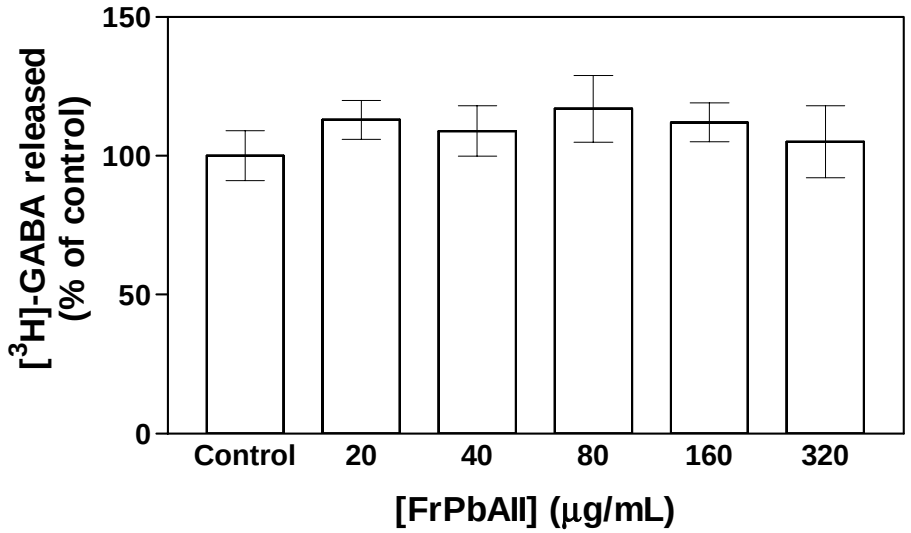

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Fig. 5

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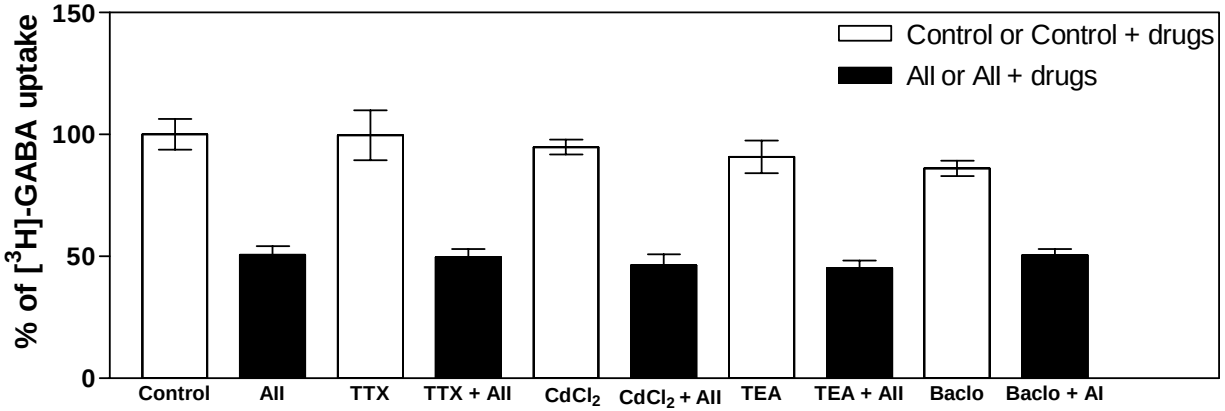

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Fig. 6

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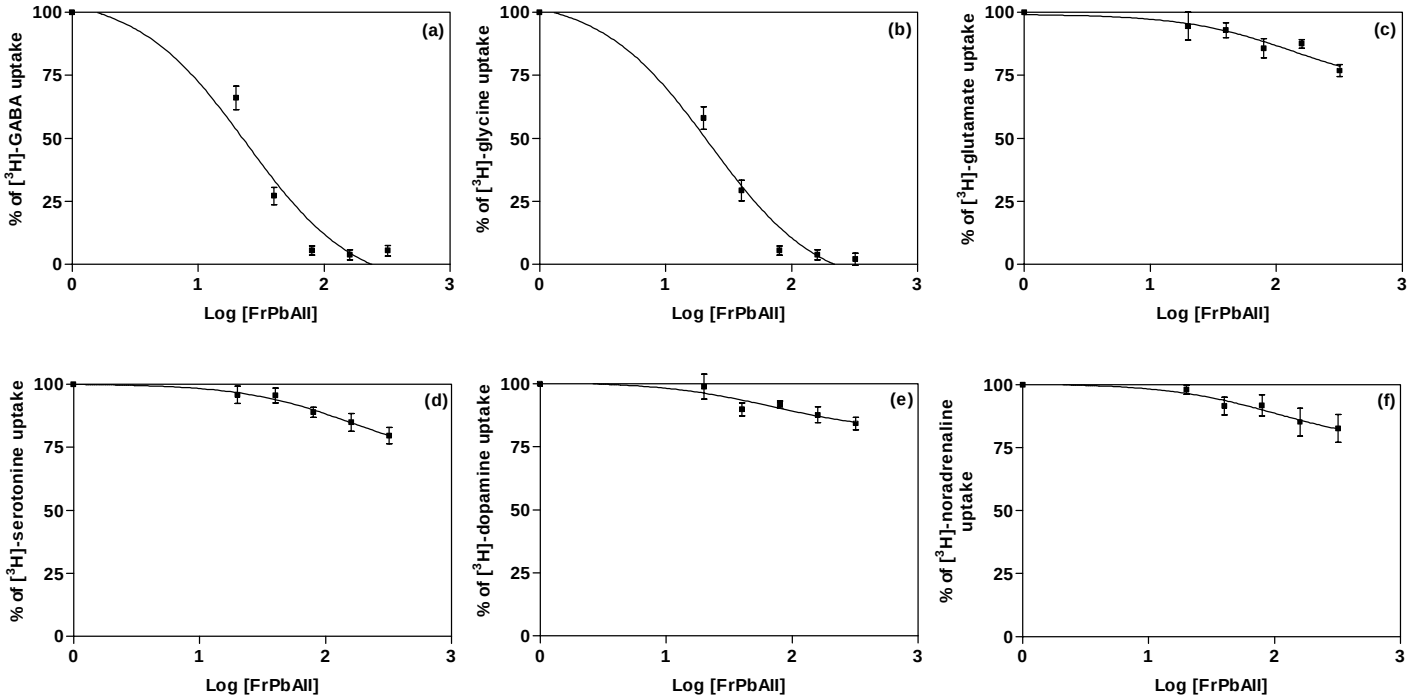

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Fig. 7

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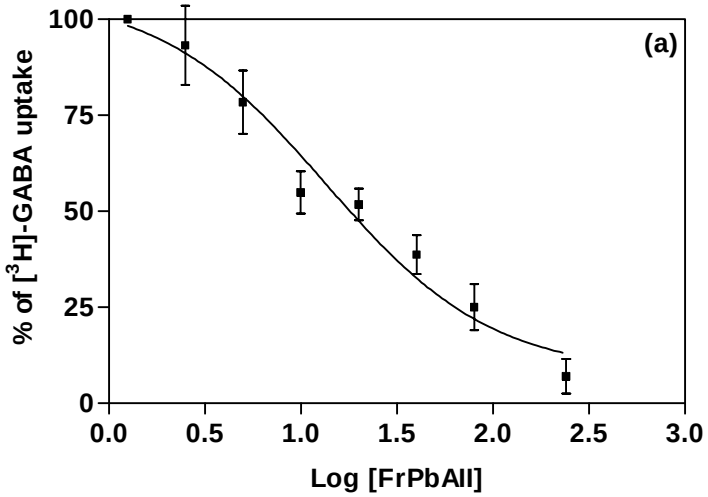
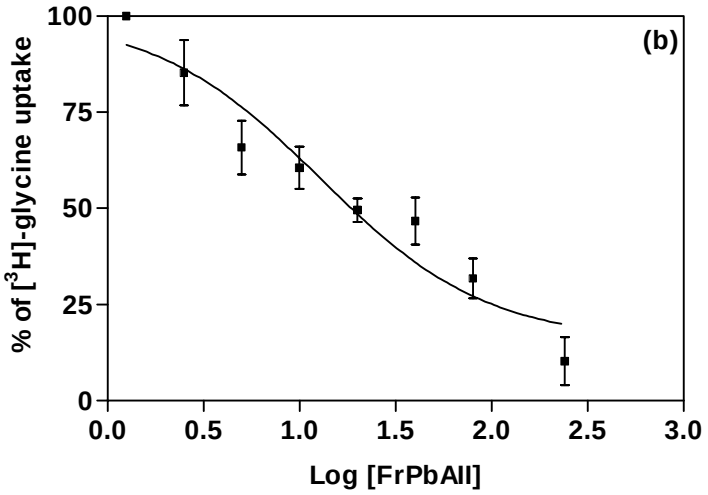
Fig. 8

Top





Bottom