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***CAMPUS* SÃO GABRIEL**

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CARLOS GABRIEL MOREIRA DE ALMEIDA

INVESTIGAÇÃO BIOQUÍMICA E ELETROFISIOLÓGICA DA ATIVIDADE NEUROTÓXICA DA UREASE DE *CANAVALIA ENSIFORMIS* SOBRE O SISTEMA NERVOSO DE MAMÍFEROS

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Orientador: Prof. Dr. Chárist on André Dal Belo

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“Ninguém é tão grande que não possa aprender, nem tão pequeno que não possa ensinar.”

Augusto Cury

RESUMO

A urease de *Canavalia ensiformis* (Jack Bean Urease, JBU) possui uma cadeia polipeptídica de 90.770 kDa, com 840 resíduos de aminoácidos. Do ponto de vista biotecnológico, já foram caracterizadas suas ações fungicida e inseticida. Quando administrada na corrente sanguínea de mamíferos, esta, induz convulsões que culminam em morte dos animais, um efeito cujo mecanismo de ação ainda carece de investigação. O objetivo desse trabalho foi investigar os padrões eletrofisiológicos da JBU no sistema nervoso central e periférico de roedores *in vivo* e *in vitro*, e as correlações bioquímicas em termos de viabilidade celular e exocitose do L-glutamato. Nos ensaios bioquímicos, verificou-se aumento na liberação de L-glutamato em sinaptossomas corticais de ratos na concentração de 100 nM. Os ensaios de viabilidade celular por MTT em fatias de hipocampo de camundongos não demonstraram alterações. Nos ensaios eletrofisiológicos, verificou-se redução significativa na amplitude do potencial de ação composto (CAP) de nervo ciático de camundongo, nas doses de 1 e 10 nM. Nos ensaios eletroencefalográficos em ratos a injeção da urease (10nM) no hipocampo induziu um traçado de espícula-onda. A redução na amplitude do CAP provavelmente está relacionada a uma inibição dos canais de sódio voltagem-dependentes, já que a administração posterior de tetrodotoxina, não aumentou o nível de bloqueio da condução. Nossos resultados demonstram um efeito excitatório da JBU do tipo crise de ausência sobre o sistema nervoso central de mamíferos. Este resultado sugere o envolvimento dos canais de Ca^{2+} do tipo T nos efeitos excitatórios da JBU. O bloqueio da condução do nervo ciático de camundongos sugere o envolvimento de canais de Na^{+} voltagem-dependentes, o qual corroboraria a atividade excitatória da urease sobre o sistema nervoso central de mamíferos.

Palavras-chave: Jack Bean Urease, bloqueio de canais de sódio voltagem-dependentes, liberação de L-Glu, crise de ausência.

ABSTRACT

The *Canavalia ensiformis* urease (Jack Bean Urease) has a 90,770 kDa polypeptide containing 840 aminoacid residues. JBU is known to exhibit insecticidal and fungicidal activities. When administered endovenously in mammalians, it induces tonic clonic convulsions culminating in the death of the animals. This mechanisms involved in the excitatory activity of JBU has not been elucidated so far. In this work, we sought to investigate the central and peripheral electrophysiological patterns of Jack Bean Urease in rodents, *in vivo* and *in vitro*, as well as the biochemical correlation of cell viability and glutamate release. In the biochemical assays, JBU induced increase in L-Glutamate release in rat cortical sinaptossomes, with no alteration of mice hippocampal cell viability. The electrophysiological assays, showed that JBU induce a significant decrease on mice sciatic nerve compound action potentials (CAP), and spike-wave discharges (SWD) similar to “petit mal” seizures when injected directly in the hippocampus (10 nM). The decrease in CAP amplitude is related to a blockage of voltage-gated sodium channels, since it was not affected by the concomitant application of tetrodotoxin. Our results show that JBU exerts an effect of spike wave discharges-like activity over the mammalian central nervous system. This later result suggests an involvement of T-type voltage gated calcium channels in the excitatory activity of JBU. The blockade of mouse sciatic nerve compound action potential conduction corroborates the excitatory activity of the urease upon the mammalian central nervous system.

Keywords: Jack Bean Urease, blockade of voltage-gated sodium channels, L-Glu release, absence crises.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

CA_1: *Cornu Ammonis* 1

Ca^{2+}: Íon de Cálcio bivalente

Cl^-: Íon de Cloro monovalente

CAP: *Compound Action Potential* – Potencial de Ação Composto

CEUA: Comitê de Ética no Uso de Animais

CNTX: Canatoxina

CONCEA: Conselho Nacional de Controle de Experimentação Animal

EDTA: Ácido etilenodiamino tetra-acético

EEG: Eletroencefalografia

GABA: Ácido-γ-amino-butírico

HEPES: 4-(2-hidroxietil)-1-piperazina etano ácido sulfônico

JBU: *Jack Bean Urease*

kDa: Quilodaltons

KRH: Krebs-Ringer-Hepes

L-Glu: L-Glutamato

MTT: Brometo tiazolil azul de tetrazólio

Na_V: Canais de sódio voltagem-dependentes

NMDA: N-metil-D-aspartato

LFP: *Local Field Potential* - Potencial de Campo Local

MDT: *Mediodorsal nucleus of the Thalamus* - Núcleo médio dorsal do Tálamo

mPFC: *Medial Pre-frontal Cortex* - Córtex Pré-frontal medial

SWD: *Spike-wave discharge* – Descarga espícula-onda

VMAT: *Vesicular Monoamine Transporter* – Transportador Vesicular de Monoaminas

APRESENTAÇÃO

No item INTRODUÇÃO, consta uma breve revisão da literatura sobre o tema abordado nesta dissertação.

A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados sob a forma de manuscrito, que se encontra no item MANUSCRITO. No mesmo constam as seções: Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas. O manuscrito será submetido ao periódico Journal of Neuroscience Research ISSN: 1097-4547.

O item CONCLUSÕES, encontrado no final desta dissertação, apresenta interpretações e comentários gerais sobre os resultados do manuscrito presentes neste trabalho.

As REFERÊNCIAS referem-se somente às citações que aparecem no item INTRODUÇÃO desta dissertação.

Os ANEXOS 1 E 2 referem-se a dois artigos científicos que serão submetidos esse ano e foram desenvolvidos pelo grupo ao longo desse mestrado.

No ANEXO 3 encontra-se o certificado do Comitê de Ética no Uso de Animais que autorizou a realização desse trabalho.

Nos ANEXOS 4,5 e 6 encontram-se os certificados dos principais congressos nos quais foram apresentados trabalhos com dados obtidos no decorrer do mestrado.

SUMÁRIO

INTRODUÇÃO

Revisão sobre ureases

Ureases são proteínas *moonlighting*, um termo cunhado por Jeffery (1999), para designar proteínas que desempenham diferentes atividades biológicas não relacionadas. Quer seja em função de sua atividade enzimática, que leva à formação do composto tóxico amônia, ou por suas várias outras propriedades biológicas não dependentes da liberação de amônia, as ureases e peptídeos derivados biologicamente ativos são considerados toxinas no mais amplo sentido da palavra, atuando em mecanismos de defesa e/ou como fatores de virulência dos organismos produtores. Em Carlini & Polacco (2008), encontra-se uma revisão sobre as propriedades tóxicas de ureases em diferentes contextos.

Na década de 1980, foi isolada da semente do feijão de porco (Jack bean), *Canavalia ensiformis*, uma proteína convulsivante, a qual foi denominada Canatoxina (Carlini et al., 1980; Carlini e Guimarães, 1981). Nesse estudo, demonstrou-se um efeito letal associado a uma atividade pró-convulsiva da Canatoxina após a sua administração por via intraperintoneal em camundongos e ratos. A observação do fenômeno de Straub, bem como o fato da reserpina, uma droga que inibe o transportador vesicular de monoaminas (VMAT), ter diminuído o limiar para o início das convulsões, levaram os autores a estabelecerem o sistema nervoso central, como o principal lócus de ação da neurotoxina para o desenvolvimento do estado convulsivo (Carlini et al., 1984). Por outro lado, foi observado também que a destruição da espinha dorsal na região torácica T5, levava a inibição das convulsões relacionadas aos membros

inferiores, mas que ainda permaneciam nos membros superiores. Além disso, observou-se um efeito bradicárdico desencadeado pela toxina, diferentemente do efeito hipertensor comumente observado com drogas pró-convulsivantes (Antonaccio e Taylor, 1977; Persson e Henning, 1980). Assim, esses achados demonstram a complexidade dos efeitos induzidos por essa toxina sobre o sistema nervoso de mamíferos, permanecendo ainda desconhecidos, o sítio celular e molecular envolvidos em sua inerente toxicidade.

Mecanismos relacionados aos processos convulsivos e epileptogênicos

Com mais de 10 bilhões de neurônios e uma quantidade estimada de 10^{14} conexões sinápticas, o cérebro humano ostenta uma complexidade elétrica sem paralelo. Diferentemente do tecido miocárdico, no qual os sinais elétricos propagam-se de modo sincrônico por um sincício de células, o funcionamento apropriado do cérebro requer o isolamento distinto de sinais elétricos e exige, portanto, um nível muito maior de regulação. O controle dessa complexa função começa em nível dos canais iônicos e é mantido por meio dos efeitos desses canais iônicos sobre a atividade de redes neuronais altamente organizadas. Qualquer anormalidade na função dos canais iônicos e das redes neurais pode resultar em rápida, sincrônica e descontrolada propagação da atividade elétrica, que constitui a base da convulsão (Cornes et. al., 2014). Uma convulsão isolada deve ser distinguida da epilepsia, que trata-se de uma desordem no sistema nervoso central em que o indivíduo é propenso a sofrer convulsões recorrentes.

Uma convulsão pode manifestar-se por meio de uma variedade de sintomas e ser proveniente de diversas causas. O cérebro humano normal na ausência de qualquer lesão ou anormalidade genética, é capaz de sofrer convulsão. A Tabela 1 resume os principais tipos de convulsões epilépticas conhecidas.

Se por um lado os peptídeos supracitados exibem uma inerente aplicabilidade biotecnológica como inseticidas naturais, também podem exibir uma importante atividade deletéria convulsivante, como demonstrado para a Canatoxina (Carlini e Guimarães, 1984). Por essa razão torna-se imprescindível, a determinação do mecanismo de neurotoxicidade central desses compostos em mamíferos, no sentido de contribuir para elucidação da relação estrutura-atividade e espécie seletividade. Por outro lado, se a determinação do mecanismo de neurotoxicidade tem a sua obvia importância clínica, também ressalta o potencial das neurotoxinas de origem natural como protótipos para o desenvolvimento de novos agentes terapêuticos (Koh et al., 2006).

Nesse aspecto, o funcionamento adequado do cérebro requer um isolamento distinto dos sinais elétricos demandando um alto nível de regulação. O controle dessa complexa função inicia-se no nível dos canais iônicos e é mantido posteriormente, pelo efeito desses canais iônicos sobre a atividade de redes neurais altamente organizadas (Cavalheiro et al., 1991). A função anormal dos canais iônicos e das redes neurais pode resultar em uma dispersão rápida, sincrônica e descontrolada dos potenciais de ação, os quais estão na base do processo de construção de uma convulsão (Griffin e Lowenstein, 2008). No sistema nervoso central de mamíferos, dois importantes elementos normalmente envolvidos no ajuste-fino do processo de sinalização celular também funcionam

para prevenir disparos repetidos e sincrônicos dos neurônios característicos das convulsões. Ao nível celular o período refratário induzido pela inativação dos canais de sódio e a hiperpolarização mediada pela abertura dos canais de potássio previnem o disparo repetitivo anormal nas células neuronais (Bezanilla, 2006). Nesse aspecto, os receptores NMDA são igualmente permeáveis ao sódio e ao potássio, influenciando diretamente o controle das respostas excitatórias no sistema nervoso central de mamíferos (Dingledine et al., 1999). Não obstante, esses receptores também controlam o influxo de Ca^{++}, sabidamente o principal acoplador das respostas elétricas às de sinalização bioquímicas (Brocard et al. 1993). Além disso, no controle do processo excitatório, respostas químicas associadas ao receptor do acido gama-aminobutírico (GABA-A), estão intimamente relacionas à hiperpolarização da membrana, pela indução de correntes de Cl^- em direção ao citoplasma celular (Pan et al., 2012). Dessa forma, várias neurotoxinas de origem vegetal já foram descritas na literatura, por modularem tanto os receptores NMDA (do glutamato) (Karangwa et al., 2007) como os de GABA (do ácido gama – amino- butírico) (Wang et al., 2006).

Tabela 1: Classificação das convulsões epilépticas.

Modificado de Cornes et al., 2014.

TIPO DE CONVULSÃO	SINTOMAS/MANIFESTAÇÕES ESSENCIAIS
Convulsões focais	
Sem alteração do estado mental	Os sintomas variam, dependendo da localização da atividade anormal do cérebro: movimento repetitivo involuntário (córtex motor), parestesias (córtex sensorial), luzes piscando (córtex visual), etc. A consciência é preservada. Propagações para regiões ipsilaterais no córtex (p. ex. “mancha jacksoniana”).
Com alteração do estado mental	Em geral, os sintomas resultam de atividade anormal em lobo temporal (corpo amigdaloide, hipocampo) ou lobo frontal. Alteração da consciência (cessação da atividade, perda de contato com a realidade). Frequentemente associada a “automatismos” involuntários, que incluem desde movimentos repetitivos simples (estalar os lábios, apertar a mão) até atividades que exigem alta habilidade (dirigir veículos, tocar instrumentos musicais). Memória comprometida na fase ictal. Classicamente precedida de aura.
Convulsão focal com generalização secundária	Manifesta-se inicialmente com sintomas de convulsão focal, com ou sem alteração do estado mental. Evolui para uma convulsão tônico-clônica, com contração sustentada (tônica)seguida de movimentos rítmicos (clônicos) de todos os membros. Perda da consciência. Precedida de aura.
Convulsões generalizadas primárias	
Crise de ausência (pequeno mal epiléptico)	Interrupção súbita e breve da consciência. Olhar parado. Sintomas motores ocasionais, como estalar os lábios, piscar rápido. Não precedida de aura.
Convulsão mioclônica	Contração muscular breve (de um segundo ou menos); os sintomas podem ocorrer em um músculo individual ou generalizar-se para todos os grupos musculares do corpo (podendo nesse último caso, resultar em queda). Associada a estados de doença sistêmica, como uremia, insuficiência hepática, afecções degenerativas hereditárias, doença de Creutzeldt-Jakob.
Convulsão tônico-clônica (grande mal epiléptico)	Sintomas conforme descrito anteriormente, entretanto, o início é abrupto e não precedido de sintomas de convulsão focal.

Crises de ausência (Pequeno mal epiléptico)

O pequeno mal epiléptico ou crise de ausência e sua correlação eletroencefalográfica (EEG), a generalizada, bilateral e sincrônica descarga espícula-onda de 3 a 4 Hz, diferencia-se de outros tipos de epilepsia em diversos aspectos importantes. Em termos de comportamento, as crises de ausência são caracterizadas por interrupções bruscas da atividade e responsividade, e estas por sua vez são retomadas ao final do traçado de EEG de espícula-onda sem nenhuma depressão pós-ictal (Loiseau, 1992). O pequeno mal epiléptico difere de outros tipos de epilepsia pela predominância da atividade inibitória ao passo que nos demais tipos de crises, o excesso de excitação prevalece.

De acordo com Loiseau 1992, crises de ausência são mais comuns na infância que na adolescência, começando normalmente na faixa de dois a doze anos de idade e acometendo principalmente meninos. O pico de ocorrência é entre seis e sete anos e as crianças afetadas podem ter de 20 a 200 crises por dia. Em 80% dos casos, as crises de ausência desaparecem espontaneamente na fase adulta. Nos demais casos são controladas facilmente com auxílio de medicamentos.

Dados obtidos de humano e outros animais são altamente sugestivos de que as crises de ausências generalizadas surgem a partir de ritmos de *feedback* oscilatório no eixo talamocortical (TC). Assim, as descargas de espícula-onda que caracterizam esse tipo de crise provavelmente estão associadas a mecanismos talamocorticais que mediam os fusos do sono e as respostas de recrutamento (Bruno-Neto, 2002; André ES, 2002). O papel do núcleo reticular talâmico e dos circuitos associados à geração do ritmo espícula-onda foi determinado (Avanzini et al., 1992) e associado à inbição dos receptores $GABA_b$ (Marescaux et. al., 1992 & Snead, 1992). No trabalho de França, 1998; Kwan et. al., 2000; Oliveira et. al., 2001, foi demonstrado que para ocorrer crises de ausência, são necessários potenciais excitatórios pós-sinápticos

mediados por receptores NMDA, seguidos de inibição mediada por receptores $GABA_a$ e $GABA_b$ que desencadeiam correntes de cálcio de baixo limiar de voltagem através de canais de cálcio voltagem-dependentes do tipo T em neurônios do núcleo reticular do tálamo (Rang et. al., 2012).

De acordo com Oliveira 2010, o mecanismo envolvido nas crises de ausência e que de forma geral é responsável pelas descargas bilaterais nas epilepsias generalizadas, envolve principalmente três populações de neurônios: os neurônios piramidais corticais, os neurônios relé do tálamo e os neurônios reticulares do tálamo. Os neurônios relé podem ativar os neurônios corticais de forma tônica ou fásica. A ativação tônica ocorre durante a vigília e no sono dessincronizado (sono REM). A ativação fásica ocorre no sono sincronizado. O modo de ativação do córtex (tônico ou fásico) é controlado por impulsos dos neurônios reticulares que podem hiperpolarizar os neurônios relés permitindo que eles disparem de forma fásica. Os neurônios reticulares podem ser inibidos por eles mesmos. Os neurônios corticais e os neurônios relé projetam para os neurônios reticulares fechando assim o circuito. No sono sincronizado normal, o córtex é ativado de forma fásica criando os elementos neurofisiológicos observados regularmente. Durante a vigília, os neurônios relé ativam o córtex de forma tônica permitindo a transferência de impulsos sensoriais de forma arrítmica. Uma falha nesse circuito ativará o córtex de forma fásica durante a vigília provocando dessa forma as descargas rítmicas observadas no EEG durante a crise de ausência.

O objetivo deste trabalho é contribuir para o entendimento de como as uréases interagem com o sistema nervoso de mamíferos. Assim, esses novos resultados, são a correlação entre a atividade inibitória induzida pela urease e o efeito contraditório eletrofisiológico excitatório. Como as ureases são similares em sua estrutura, as crises

de ausência induzidas pela JBU podem fornecer pistas para tentativas futuras de entender como as meningites bacterianas são capazes de produzir crises convulsivas em crianças.

OBJETIVOS

Objetivo Geral

Caracterizar a ação neurotóxica da urease de *Canavalia ensiformis* em preparações neurobiológicas de sistema nervoso central e periférico de mamíferos.

Objetivos Específicos

➔ Realizar ensaios de viabilidade neuronal de fatias hipocampais de camundongos expostas à JBU;

➔ Verificar os efeitos da JBU sobre a liberação de L-Glu em sinaptossomas corticais de ratos, para detectar se existe uma possível interação entre esse fenômeno e os processos excitatórios centrais.

➔ Realizar ensaios de EEG nas regiões CA_1, mDT e mPFC a fim de detectar alterações nos padrões de LFP decorrentes da atividade neurotóxica da JBU em microinjeções na via intrahipocampal;

➔ Verificar a amplitude do potencial de ação de composto de nervo ciático de camundongo sob efeito da JBU, a fim de detectar possíveis interações com canais de sódio e potássio voltagem-dependentes;

MANUSCRITO

Todos os resultados, bem como os itens Materiais e Métodos, Discussão e parte das Referências Bibliográficas que fazem parte desta dissertação estão apresentados sob a forma de manuscrito. Este manuscrito está disposto na forma na qual foi submetido ao periódico **Journal of Neuroscience Research** (ISSN: 1529-2401) intitulado como “Biochemical and Electrophysiological Investigation of the stimulating activity of *Canavalia ensiformis* urease in mammalian nervous system”.

Electrophysiological and Biochemical Investigation of the Stimulating Activity of *Canavalia ensiformis* Urease on Mammalian Nervous System

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Abstract

Canavalia ensiformis urease (Jack Bean Urease, JBU) is a homohexamer of 90,770 kDa polypeptide chains. JBU is known to exhibit insecticidal and fungicidal activities. When administered endovenously in rodents, it induces tonic clonic convulsions culminating in the death of the animals. The mechanisms involved in the excitatory activity of JBU have not been elucidated so far. In this work, we sought to investigate the central and peripheral electrophysiological effects of Jack Bean Urease in rodents, *in vivo* and *in vitro*, as well as on cell viability and glutamate release. In the biochemical assays, JBU increased L-Glutamate release in rat cortical synaptosomes, with no alteration of mice hippocampal cell viability. The electrophysiological assays showed that JBU induce a significant decrease of mice sciatic nerve compound action potentials (CAP), and spike-wave discharges (SWD) similar to "petit mal" seizures when injected directly into the rat hippocampus (10 nM). The decrease in CAP amplitude is related to a blockage of voltage-gated sodium channels, since it was not affected by the concomitant application of tetrodotoxin. Our results show that JBU exerts an effect of spike wave discharges-like activity in the mammalian central nervous system. This later result suggests an involvement of T-type voltage gated calcium channels in the excitatory activity of JBU. Together, the inhibitory actions of JBU over sodium channels may unveil a paradox between inhibition and excitation at the central levels.

Keywords: Jack Bean Urease, blockade of voltage-gated sodium channels, L-Glu release, absence crises.

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1. Introduction

Ureases (EC 3.5.1.5 aminohydrolases) are nickel-dependent metalloenzymes that catalyses the hydrolysis of urea into two molecules of ammonia and one of carbon dioxide (Dixon et al., 1975). Ureases are synthesized by plants, bacteria and fungi, but not by animals (Mobley et al. 1995; Krajewska 2009). It is important to note, that although composed of different types of subunits, ureases from different sources extending from bacteria to plants and fungi exhibit high homology of amino acid sequences (Krajewska, 2009).

The leguminous plant *Canavalia ensiformis* produces at least three isoforms of urease (Carlini & Ligabue-Braun, 2016). The most abundant protein JBU, the first protein ever crystalized, has an 840-amino acid residues polypeptide chain, with molecular mass of 90,770 Da, and exists mainly as a homohexamer of 540,000 Da. Other urease isoforms in *C. ensiformis* are less abundant, including canatoxin (CNTX) and JBURE-II (Carlini & Ligabue-Braun, 2016). CNTX was first isolated from *C. ensiformis* seeds as a neurotoxic and convulsant protein (Carlini & Guimarães, 1981) and later characterized as an isoform of urease (Follmer et al., 2001). Both CNTX and JBU are known to exhibit insecticidal (Stanisçuaski & Carlini, 2012) and fungicidal (Becker-Ritt et al. 2007; Carlini and Polacco 2008) activities.

In mammalians, the toxic effect of *Canavalia ensiformis* crude extract has been assessed in rodents which showed dyspnea, ataxia, hypothermia, coma, and tonic convulsions preceding death (Carlini et al., 1984). The same effects are observed with JBU, although they appear only after endovenous injection of the protein while CNTX is also active by intraperitoneal route (Follmer et al., 2004b). The induction of Straub phenomenon and the ability of reserpine, a drug which inhibits the vesicular monoamines transporter (VMAT), to decrease the threshold of seizures, suggested the central nervous system as the target for CNTX convulsive activity (Carlini et al., 1984). On the other hand, it was also observed that the disruption of spinal column at T5 region inhibited the seizures related to the hind limbs (lower limbs) while non-affecting those of the front limbs.

Seizures are the clinical manifestation of an abnormal, excessive, hypersynchronous discharge of a population of cortical neurons (Broomfield et al., 2006). Experimental studies using animal epilepsy models have shown that NMDA, AMPA and kainate agonists induce seizure activity, whereas their antagonists are suppressive modulators (Broomfield et al., 2006). Thus, since glutamate is the main excitatory neurotransmitter at central the nervous system (CNS), it is the pivotal agent involved in seizures and other neurodegenerative disorders (Meldrum, 1994; Platt, 2007).

In this work, we sought to investigate the central and peripheral electrophysiological effects of Jack Bean Urease in rodents, *in vivo* and *in vitro*, as well as the biochemical correlation of cell viability and glutamate release. As far as our knowledge, this is the first work to present the correlation between the EEG activity of Jack Bean Urease with cell viability and the changes in glutamate release, therefore contributing to the understanding of the ureases convulsive-like activities.

2. Materials and Methods

2.1 Animals

Adult male Swiss white mice and adult male Wistar rats were obtained from PUCRS Animal House. The animals were housed in standard rodent cages in a colony room maintained at 24 ^{o}C under a 12 h light/12 h dark cycle with free access to food and water.

The studies presented on this paper have been done in accordance with the Brazilian Council for Animal Experimentation (CONCEA) guidelines and approved by the local animal care committee (UNIPAMPA-CEUA) under the authorized protocol number 043/2015. Experiments were designed to minimize the number of animals used and their suffering.

2.2 Reagents

All reagents used in these studies were of high purity, obtained from Sigma-Aldrich Co. Brazil.

2.2.1 JBU solutions

Highly purified crystalline urease of *Canavalia ensiformis* (type C3) was obtained from Sigma-Aldrich Brazil, hexamer molecular mass 545 kDa. The protein crystals were dissolved in phosphate buffered saline to give concentrations in the range of 1 mM. Protein solutions were dialysed against the same buffer and kept at 4 °C before de bioassays.

Biochemical assays

2.3.1 MTT Cell Viability Assay

2.3.1.1 Hippocampal Slices Preparation

Mice were decapitated, the brains removed immediately, and the hippocampus dissected on ice and humidified in cold HEPES-saline (124 mm NaCl, 4 mM KCl, 1.2 mM $MgSO_4$,12 mM glucose, 1 mM $CaCl_2$, and 25 mM HEPES pH 7.4) buffer gassed with O_2. Hippocampal slices were obtained according to Vinadé and Rodnight (1996), briefly: a McIlwain tissue chopper was used to obtain the slices (400 nm) that were separated and preincubated at 37 °C for 30 min in microwell plates filled with HEPES saline (200 μL/slice). Subsequently, fresh medium was replaced (200 μL/slice) for control condition and treatments with JBU (1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM and 1 pM), and incubated for 1 hour and 30 minutes (37 °C).

2.3.1.2 Hippocampal Slices Viability

Immediately after incubation with different treatments, slices were assayed for a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (0.05 % in HEPES-saline) for 30 min (37 °C). The MTT is converted into a purple formazan product after cleavage of the tetrazolium ring by mitochondrial dehydrogenases. Formazan was dissolved by the addition of 100% DMSO, resulting in a colored compound whose optical density (λ = 490 nm) was measured in an ELISA reader equipment. The MTT colorimetric assay was performed as described elsewhere (Dal Belo et al., 2013).

2.3.2 Synaptosomes preparation

Male adult Wistar rats (200–250 g) were sacrificed by decapitation. The brain cortex was removed and homogenized in 0.32 M sucrose solution containing dithiothreitol (0.25 mM) and EDTA (1 mM). Homogenates were then submitted to low-speed centrifugation (1000 g for 10 min) and the synaptosomes were purified from the supernatant by discontinuous Percoll density gradient centrifugation (39,000 g for 15 min) (Dunkley et al., 2008). The isolated nerve terminals were resuspended in Krebs–Ringer–HEPES (KRH) solution (124 mM NaCl, 4 mM KCl, 1.2 mM Mg2SO4, 10 mM glucose, 25 mM HEPES, pH 7.4) to a final concentration of approximately 1 mg/ml protein. Aliquots of 30 µL were pipetted/well and kept on ice until use.

2.3.3 Measurement of continuous glutamate release

Glutamate release was assessed by measuring the fluorescence increase due to the production of NADPH in the presence of glutamate dehydrogenase and $NADP^+$. This protocol was performed essentially as described (Lomeo et al., 2014). In short, the medium used for the reaction contained a mixture of rat cortical synaptosomes (± 30 µg of protein/well) and $NADP^+$

(1 mM) in KRH solution containing 2mM $CaCl_2$ transferred to Elisa microplates (300 µl/well) to be read in a spectrofluorimeter (Synergy 2, Winooski, USA). After 1 min, glutamate dehydrogenase (35 units per well) was added to the medium and the reading was restarted until the fluorescence reached a plateau (about 10 min). Subsequently, treatments with different concentrations of JBU were applied. Calibration curves were done in parallel by adding known amounts of glutamate to the reaction medium. The experimental data were expressed as nmol of glutamate released per mg of synaptosomal protein. The experiments were performed at 37 ºC for 45 min of continuous reading at 340 nm/440 nm excitation/emission wavelengths.

2.4 Electrophysiological recordings

2.4.1- In vitro recordings of compound action potentials from mouse sciatic nerve preparation

Sciatic nerves were obtained from adult male Swiss mice (28 – 35g). The extraction surgery was performed essentially as described by Bala et al. 2014. The mice sciatic nerves were mounted on a recording chamber according to Dal Belo et al. 2005. Standard extracellular recording techniques were used to record compound nerve action potentials. Pellet-type silver electrodes were dipped into each of the three compartments of the recording chambers, with stimulation occurring between the central and one of the external compartments. Recordings were obtained from the central compartment. A Grass S48 stimulator was used to supply supramaximal electrical impulses (0.4 Hz, 0.04 ms duration) via a model SIU 5A stimulus isolation unit (Grass Instrument Co.). The signals were amplified with a CED1902 transducer (Cambridge Electronic Design, Cambridge, England), digitized with a CED 1401 analogue-to-digital converter (Cambridge Electronic Design) and analyzed with custom built software

(Dempster, 1988). In each experiment, the amplitude, rise time, latency and threshold of the action potentials recorded were measured. Before adding the test compounds, the sciatic nerve preparations were incubated in physiological solution for 15 min under constant supramaximal stimulation to demonstrate viability of the preparation and consistency of the recordings.

2.4.2. In vivo rat electroencephalographic (EEG) Recordings

2.4.2.1. Implantation of animals for electroencephalographic recordings

Rats were anesthetized with ketamine 90 mg/kg and xylazine 13mg/kg. The anesthesia was maintained with extra doses of ketamine/xylazine (30% of initial dose), after checking tail pinch reflex and respiratory rate. Body temperature was maintained at 37 ± 0.5 ºC using a heating pad. For the EEG recordings, A-M SYSTEMS .0045" tungsten Teflon-insulated electrodes were employed. These electrodes were surgically implanted on mPFC (3.0 mm anterior to bregma, 0.4 mm lateral to midline and 3.2 mm ventral to dura mater); CA_1 (5.7 mm anterior to bregma, 4.6 mm lateral to midline and 2.5 mm ventral to dura mater) and TMD (-1.9 mm anterior to bregma, -0.4 mm to midline and -4.8 mm ventral to dura mater). The electrode implanting sites coordinates were followed according to Paxinos and Watson (2007). At the hippocampal CA1 area, a chemitrode (electrode + cannula) was implanted. The cannula was used for intrahippocampal injections of 10 nM JBU in a volume of 2 µl. The skull was exposed, and holes were drilled so that the recording electrodes and the chemitrode could be lowered at the mentioned stereotaxic coordinates. An additional hole was drilled over the parietal to cortex to implant a micro screw that served as a recording reference (ground). An additional electrode was used as miogram to detect the electrical activity of the animal's neck

muscles in order to detect REM sleep, noise caused by body movements or electrical oscilations.

2.4.2.2.Recording of Spike-and-Wave Discharges SWDs

EEG was recorded by an electroencephalograph (NIHONCOHDEN, Japan, Tokyo) attached to a data capture and analysis device (CED: Cambridge Electronic Design Ltd., UK, Cambridge; POWER 1401 mkII). Sampling rate was 2000 Hz whereas the bandwidth of the EEG recording was 0.3–150 Hz. Number of SWDs (frequency: 3–11 Hz; a train of asymmetric spikes and slow waves starting and ending with sharp spikes; the average amplitude at least twice as high as the basal EEG activity) (Kovács et al., 2006, 2015) and time of SWDs (average time and total time of SWDs) were measured between 30 and 270 min of post-injection time (from 4.00 PM to 8.00 PM). The software used to acquire the data was LabChat 7 from ADinstruments Inc., 2205 Colorado Springs CO 80906, USA.

EEG recordings were carried out in awake rats without anesthesia during 6h in a recording chamber containing food and water *ad libitum*. After 2h of basal recordings, the animals were injected with 2µl of JBU 10nM by using a Hamilton syringe connected to a 30G dental needle through a flexible polypropylene pipe. The needle was inserted into the cannula and stood 0,1mm above hippocampal CA_1. Every microliter of JBU solution was injected in one minute.

2.5. Statistical analysis

For the MTT cell viability assay, the results were expressed as the mean ±SEM and were compared statistically using ANOVA for repeated measures. Statistical analysis glutamate

release data was made by the method of two-way ANOVA followed by Bonferroni test. For compound action potentials recordings, the results were expressed as the mean ±SEM and were compared statistically, using Student's unpaired *t*-test.

3. Results

3.1 MTT Cell Viability Assay

On MTT Assay, no substantial cell loss could be verified according to our data (Fig. 1). Since MTT is based on mitochondrial dehydrogenases activity, our findings suggest no mitochondrial dysfunctions after exposure of the hippocampal slice to the highest dose of JBU (1 µM) for 90 minutes. A positive control with H_2O_2 (44 mM) was also carried out. The blank control was provided by comparing the absorbance of 100% DMSO.

3.2 L-Glutamate Release Assay

In brief, rat brain cortex synaptosomes are isolated nerve terminals which preserve the machinery related to neurotransmission and consist of a protocol for the study of the effects of compounds with potential activities upon the cortex on central nervous system (CNS). The analysis of L-Glu release in the presence of different concentrations of JBU (1 nM, 10 nM, 20 nM, 50 nM, and 100nM), showed a significative time-dependent increase in the release of L-GLU (n=6) Fig.2. Thus, 33.34±8.56% maximum increase of JBU-induced L-Glu release was observed with the concentration of 100nM in 45min readings ($p<0.05$) Table 1. Also, a positive control in depolarizing conditions (KCl) was assayed for comparison.

3.3 Compound action potential (CAP)

The effect of JBU on compound action potentials caused significant changes in the amplitude and rise time of the potentials. Fig. 3 displays JBU's effects in different doses. This result is summarized in Table 2. The most significant decrease (40% ???) in CAP amplitude was observed ($n = 6$, $p < 0.05$) for the lowest dose of JBU 0.001 μM. The "blocking" effect of JBU on the CAPs decreased for larger doses of the toxin, and almost imperceptible at 1 μM. Application of Tetrodoxin (1μM), added to the preparation either 120 min after or before JBU, did not increase or decrease the urease effect (Fig.4).

3.4 Electroencephalographic (EEG) Recordings

An intrahippocampal injection of JBU 10 nM, induced a 3 Hz generalized spike-wave discharge as shown in Fig. 5. This finding suggests a non-convulsive seizure, a "petit mal" seizure since the animals did not respond to external sound stimulation or show any tonic-clonic convulsion.

3.4.1 Effects of Jack Bean Urease on mean number of SWDs

The criteria for SWDs analysis was that it should last at least 3 seconds. Numbers of SWDs in all groups are presented in Fig.6. There was a remarkable number of SWDs in all areas, slightly higher but not statistically meaningful in CA_1.

3. Discussion

In this work we have shown that intrahippocampal injections of rats with Jack Bean Urease provoked spike-and-wave episodes related to absence crisis phenomena. Also, it was demonstrated that the induction of such electrophysiological deregulations is accompanied by

an increase of L-GLU release, without affecting the neuronal viability, as shown by the MTT assay in mice. The inability of TTX to induce a further inhibitory activity at the onset of JBU blockage of mouse sciatic nerve conduction, suggests that the urease displays an activity over voltage-gated sodium channels. The results will then be discussed in detail herein.

Spike-and-wave is the term that describes a particular pattern of the electroencephalogram (EEG) usually observed during epileptic seizures. The spike-and-wave discharge is a regular, symmetrical, generalized EEG pattern seen, related to absence epilepsy, also known as 'petit mal' epilepsy (Loiseau, 1992). The basic mechanisms underlying these patterns are complex and involve part of the cerebral cortex, the thalamocortical network, and intrinsic neuronal mechanisms (Snead, 1995 & Rang et. al., 2012). Some studies suggest that a thalamocortical (TC) loop is involved in the initiation of spike-and-wave oscillations (Snead, 1995; Akman et al., 2010).

Recent studies of thalamic brain slices suggest that inhibition of thalamic relay neurons by GABA-ergic interneurons hyperpolarizes the relay neuron, thus removing inactivation of the T-type Ca^{2+} channels (Mccormick and Contreras, 2001). This sequence of events leads to a rebound burst of action potentials after each inhibitory postsynaptic potentials (IPSP). The action potential stimulates the GABAergic neurons by a reciprocal excitatory connection. The action potentials in the relay neurons also excite cortical neurons and thus can be manifested in the EEG as a "spindle" (Mccormick and Contreras, 2001).

Block of $GABA_A$ channels enhances $GABA_B$ IPSPs in relay neurons, resulting in an increase in rebound bursts of action potentials. Thus, the T-type Ca^{2+} channel and $GABA_B$ receptors appear to play an important role in generating absence seizures activity in mammalians (Iftinca, 2011; Cain and Snutch, 2012; Chen et al., 2014).

In our experimental conditions, JBU was injected directly into the rat hippocampus, where the number of SWDs was slightly higher compared to other brain regions, although not

significative. This latter result suggests that JBU induces intense hypersynchronous bursts firing activity on CA1 area that turned the electrical activity to spread to other brain regions. Indeed, in a recent study, we have shown that JBU is increasing Ca^{2+} influx in primary hippocampal cultures (Piovesan et al., 2015). Although we have not addressed which type of calcium channel is involved in the JBU-increase of calcium influx in the hippocampus, there is a striking link between the involvement of T-type calcium in the CA1 area of the hippocampus and the development of SWDs (Iftinca, 2011).

In this regard, Bengtson et al. (2013) have shown that in a twelve-day hippocampal culture, neurons normally express markers for either glutamate (~ 90% of neurons) or GABA (~ 10% of neurons). As described above, the mechanisms involved in absence-like crises are complex. The specific cellular mechanisms by which absence seizures are produced are still a matter of debate, including the extent of involvement of GABA-ergic signaling (Cope et al., 2009). In this view, two main situations could be amenable to explain the pharmacological activity of JBU over mammalian nervous system in our laboratory conditions: the former and less reasonable, is that the blocking activity of JBU upon sodium channels, as demonstrated by the reduction of mouse CAP conductance, would involve specific GABA-ergic neurons, similarly to the central actions of local anesthetics. This pharmacological interaction with voltage-gated sodium channels would cause a relatively selective depression of inhibitory neurons, which ultimately would induce cerebral excitation, therefore provoking seizures (Hara et al., 1995). The second and more attractive possibility appears to be an increase of GABA-ergic transmission by the blockage of voltage-gated sodium channels in glutamatergic neurons, since these excitatory cells are more abundant in CNS. In this context, increased GABA-ergic transmission may predominantly represent a compensatory response of the brain in an attempt to decrease seizure propensity. However, enhanced GABA transmission has been reported paradoxically to promote seizures as well, at least in some specific types of epilepsy models

(Klaasen et al., 2006), such as absence epilepsy (Cope et al., 2009). On the other hand, how to explain the JBU-increase of L-Glu release in brain synaptosomes? The explanation may rely on the clinical observation that several antiepileptic drugs such as phenobarbital, benzodiazepines, phenytoin, carbamazepine, oxcarbazepine, valproate, ethosuximide among others can exacerbate seizures, in which glutamate content is positively altered (Otoom and Al-Hadidi, 2000). In addition to that, JBU is a pore-forming neurotoxin (Piovesan et al., 2014) which potentially is able to modulate positively or negatively the surrounding ion channels of a biological membrane, therefore increasing or decreasing neurotransmitter release (Schenning et al. 2006; Popoff and Poulain 2010). In this regard, the glutamate transporter GLT-1 is a sodium-dependent enzyme which is predominantly expressed in astrocytes (Nakagawa et al., 2008). The decrease of sodium conduction by a sodium channel blocker (e.g. JBU) could potentially promote the reversal of glutamate uptake (Jackson et al., 2014).

In addition to that, in the mouse sciatic nerve CAP experiments we have also observed a decrement of the pharmacological blockage with the increase of JBU concentration. This later phenomena was previously addressed by Follmer et al. 2004a, which demonstrated that in solution, JBU can assume different molecular architectures that are bigger than the original hexamer. Another possibility to explain the urease functional contradictory activity is a situation in which the urease is acting like an inverted agonist. In such a condition, the activity related to the active form of the receptor and higher efficacy would be annulated by the increase of the urease concentration and the consequent biding to an inactive form with higher affinity and less efficacy (Milligan, 2003).

Finally, the inability of JBU to decrease the neuronal cell viability may suggest that the amount of glutamate released during the onset of the toxin activity is not harmful to the cells. Indeed, a similar activity was observed by with pancreatic islets (Barja-Fidalgo et al., 1991) and platelets (Carlini et al., 1985). This later result permit to infer that mitochondrial

dysfunctions are not associated with the JBU-induced seizure activity (Henshall 2007; Zsurka and Kunz 2015).

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Role of all authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: **C.A. Dal Belo, C. R. Carlini and A.P. Corrado.** Acquisition of data: **C.A. Dal Belo, R.S. Oliveira, C.N. da Silva, R.S. Floriano and C.G.M. de Almeida**. Analysis and interpretation of data: **C.A. Dal Belo, R.S. Oliveira, C.N. da Silva, C.G.M. de Almeida, and A.P. Corrado**. Drafting of the manuscript: **C.G.M. de Almeida and C.A. Dal Belo**. Critical revision of the manuscript for important intellectual content: **C. R. Carlini, M. E. de Lima, J. P. Leite, E.G. Rowan and L. Vinadé**. Statistical analysis: **C.G.M. de Almeida, C.A. Dal Belo, R.S. Oliveira, R. S. Floriano, C.N. da Silva**. Obtained funding: **C. R. Carlini, M. E. de Lima, J. P. Leite, E. G., Rowan, A. P. Corrado and C.A. Dal Belo**. Administrative, technical, and material support: **C. R. Carlini, M. E. de Lima, J. P. Leite, E. G., Rowan, A. P. Corrado and C.A. Dal Belo**. Study supervision: **M. E. de Lima, A.P. Corrado, E.G. Rowan**, **C. R. Carlini, C.A. Dal Belo.**

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5. Figure Legends

Figure 1. MTT cell viability assay results. The graphic displays the percentage of cell viability of mice hippocampal slices incubated with JBU in different concentrations for 90 min compared to control (HEPES). Our positive control was H2O2 44 mM and DMSO was the blank. Data are mean ± S.E.M., n= 6.

Figure 2. L-Glutamate release induced by JBU in rat synaptosomes. JBU induces synaptosomal glutamate release. A mixture containing synaptosomes (±30 mg of protein/well) and NADP+ (1 mM) in KRH (Krebs Ringer solution) was transferred to Elisa microplates (300 ml/well) coupled to a spectrofluorimeter (Synergy 2). After 1 min of reading, the enzyme glutamate dehydrogenase (35 units/well) was added and the reading was continued for 10 min. Subsequently, JBU (1, 10, 20, 50 and 100 nM) or KRH (control) or KCl (33 mM; positive control) was added and the reading continued for 30 min more (*$p < 0.05$).

Figure 3. Measurements of the compound action potential recorded in mouse sciatic nerve. A. The graphic displays the decrease in observed in CAP amplitude during incubation with JBU (0.001-1 μM, n=3). B. Representative record of a preparation treated with JBU 0.001 μM. In A, *$p<0.05$ compared to control preparations.

Figure 4. Effect of JBU and TTX on Compound Action Potential (CAP) Amplitude. The histogram displays the effects of JBU 1 nM; JBU 1 nM followed by TTX 1 µM 120 min after; TTX 1 µM followed by JBU 1 nM 120 min after, on CAP amplitude compared to control. The amplitudes are measured in percentage. All treatments were statistically different from control at $p < 0.01$, but not different from each other.*** significance at $p<0.01$ related to the control HEPES.

Figure 5. A representative spike-wave recording. Electroencephalographic recordings (EEG) showing an absence-like epileptical activity-induced by Jack Bean Urease (JBU), in vivo. The recordings show typical waveforms from the medial pre-frontal cortex (mPFC), medium dorsal nucleus of the thalamus (MDT), CA1 region of the hippocampus and an electromyogram (EMG), during the onset of urease's effect. Notice the spike-wave waveforms in all recordings. Note that the hypersyncronous low frequency spike-and-wave discharges signal was so intense and generalized that it also was recorded on the EMG.

Figure 6. Number of spike-wave discharges per region. The graphic displays the total amount of spike-wave discharges (SWDs) recorded in each electrode during three absence-like crisis. The electrodes were implanted in CA1 area (hippocampus), mPFC (medium prefrontal cortex) and TMD (Medial Dorsal Nucleus of the Thalamus). The amount of SWDs did not change significantly from one brain region to the others.

6. Tables legends

Table 1. Average percentage of L-glutamate (L-GLU) release of brain synaptosomes after the treatment with different concentrations of Jack Bean Urease (JBU). Note that after 45min incubation with JBU there was an increase in L-GLU release only with the highest concentration of JBU. * significance to $p<0.05$.

Table 2. Electrophysiological parameters recorded from mouse sciatic nerve compound action potentials showing the reduction of the amplitude by the application of Jack Bean Urease (JBU). *significance at $p<0.05$.

7. Figures

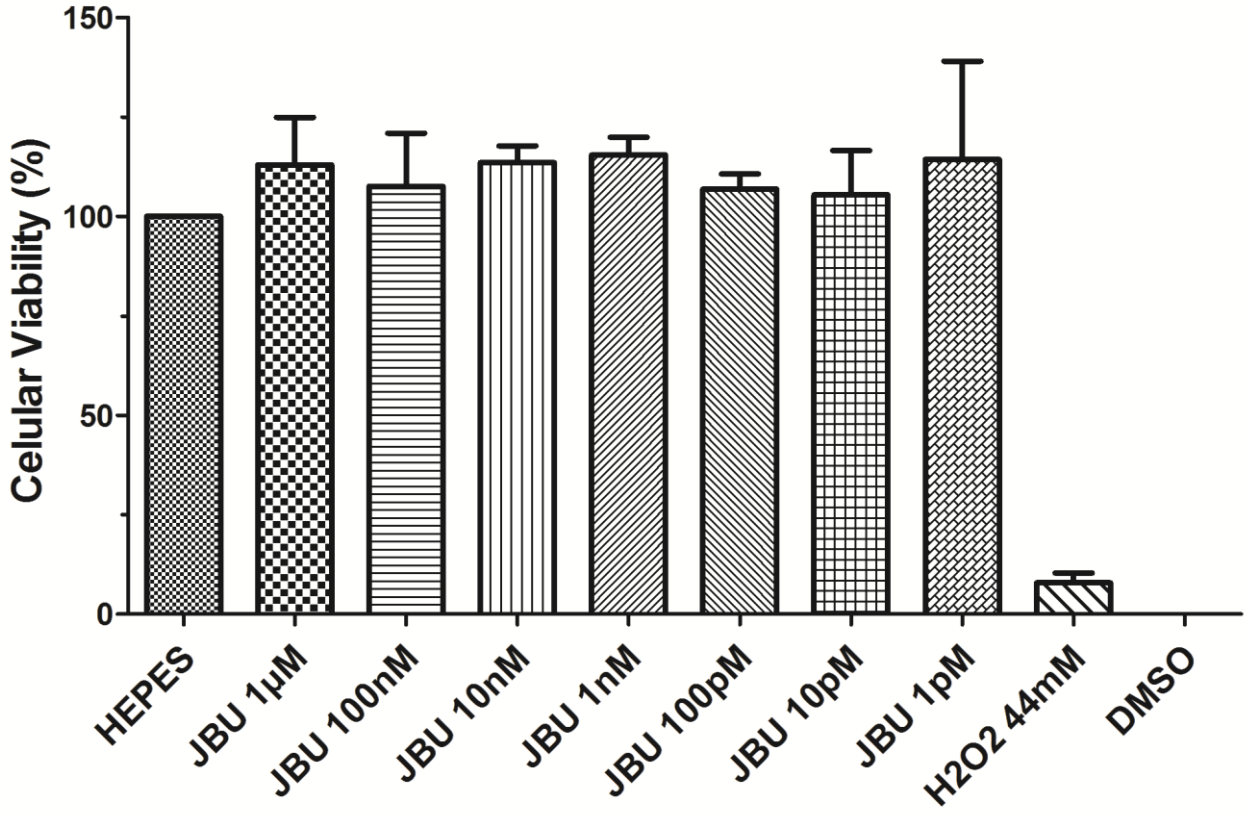


Figure 1- *MTT Cell Viability Assay*

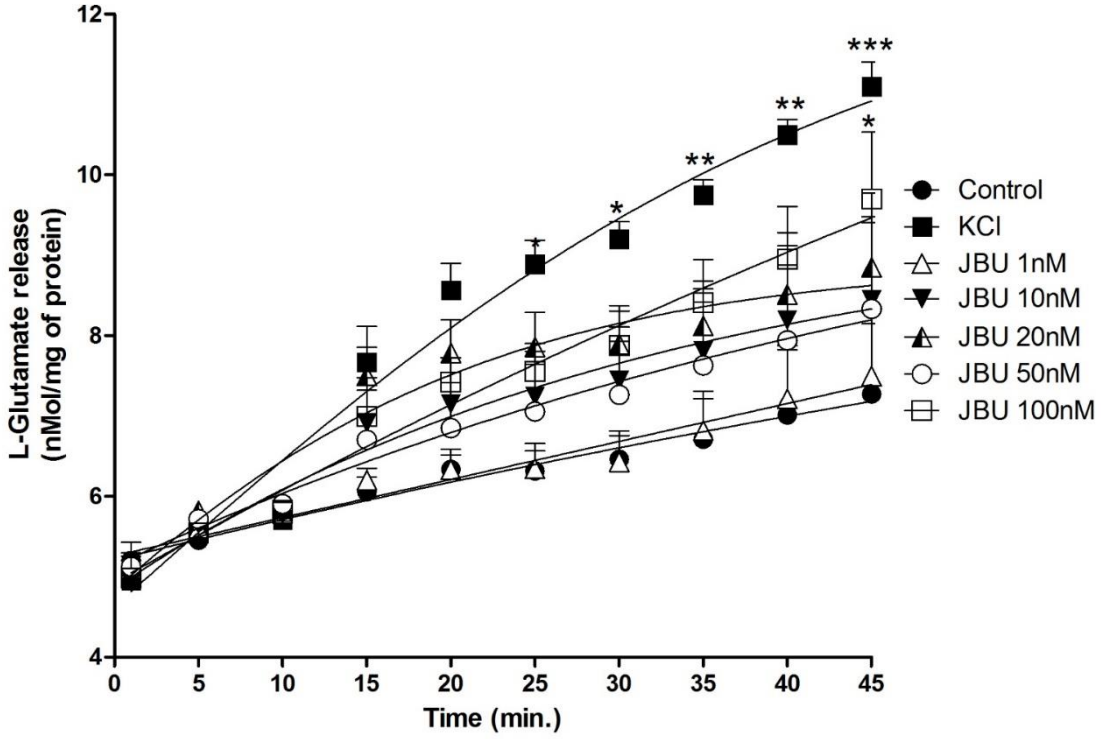
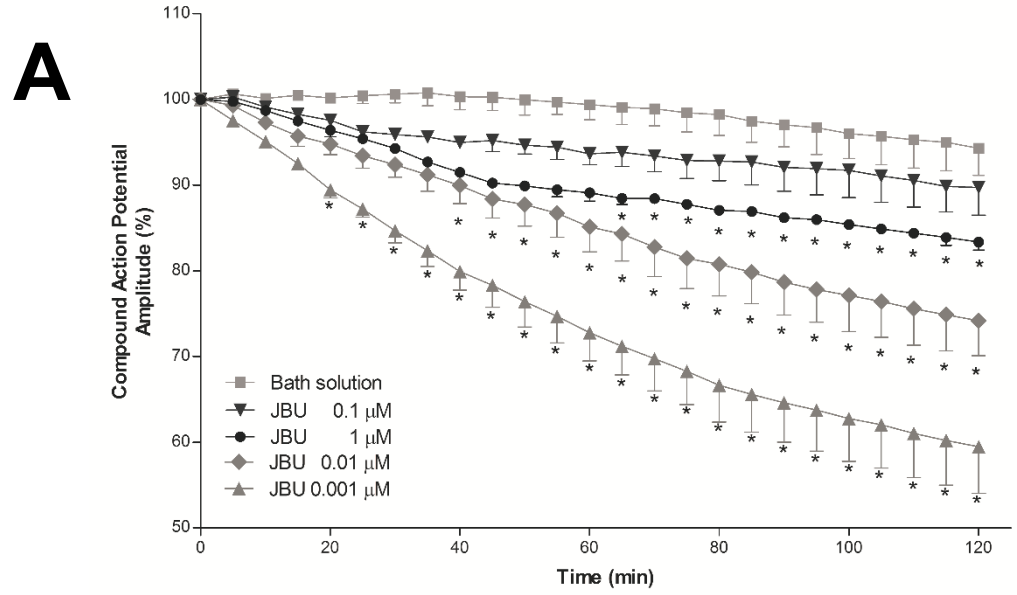


Figure 2 – *L-Glu release*



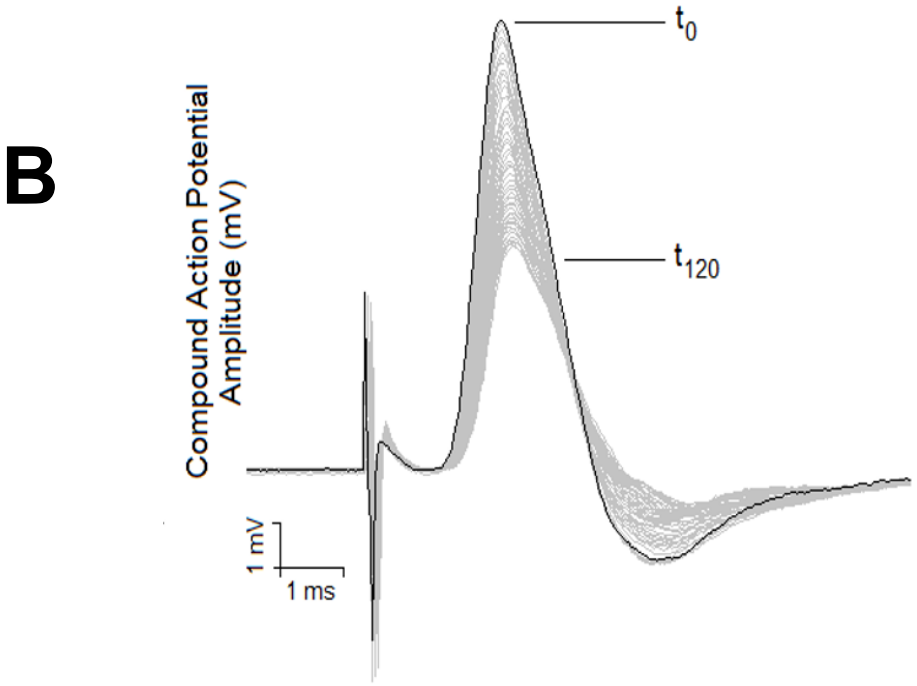


Figure 3 – *Compound Action Potential*

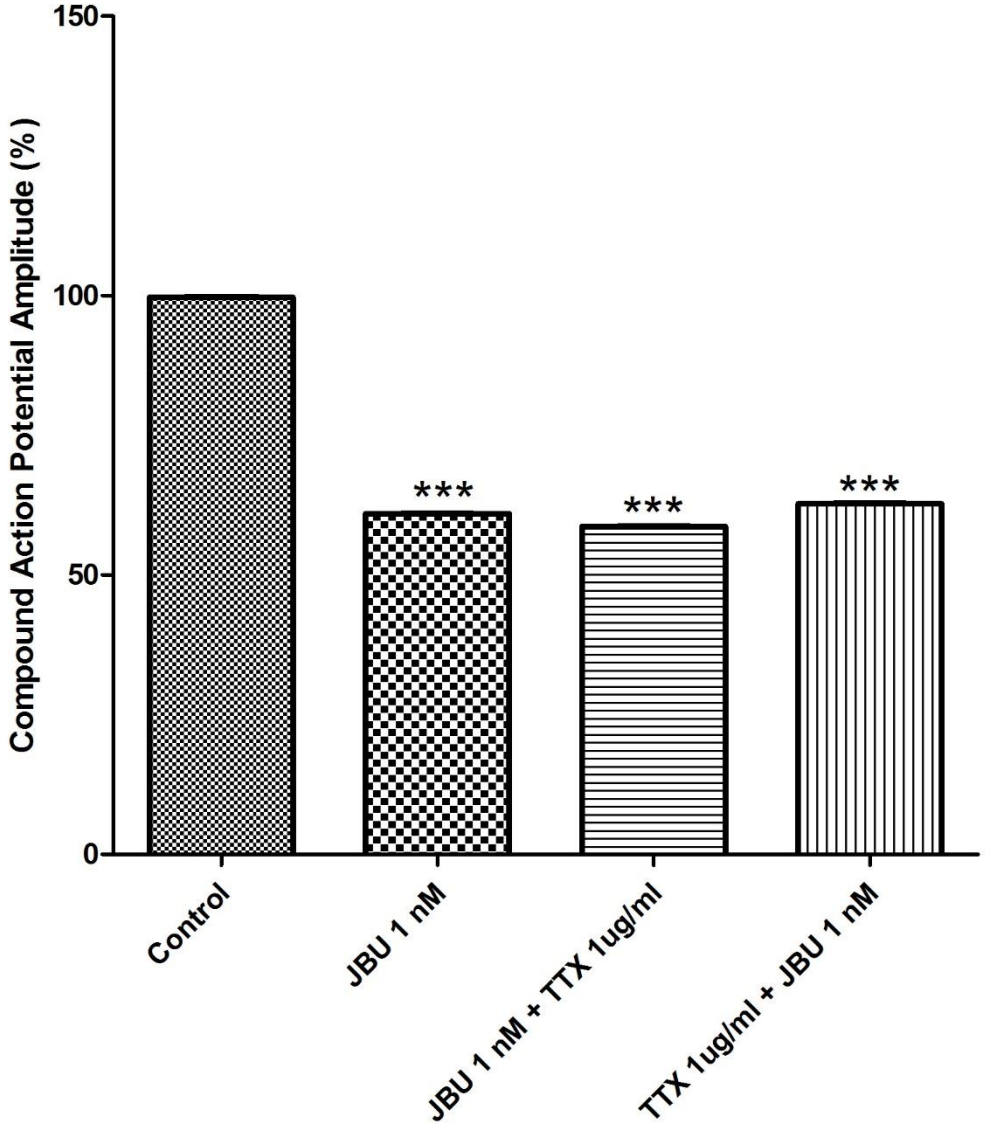


Figure 4 – *Compound Action Potential Average Amplitude.*

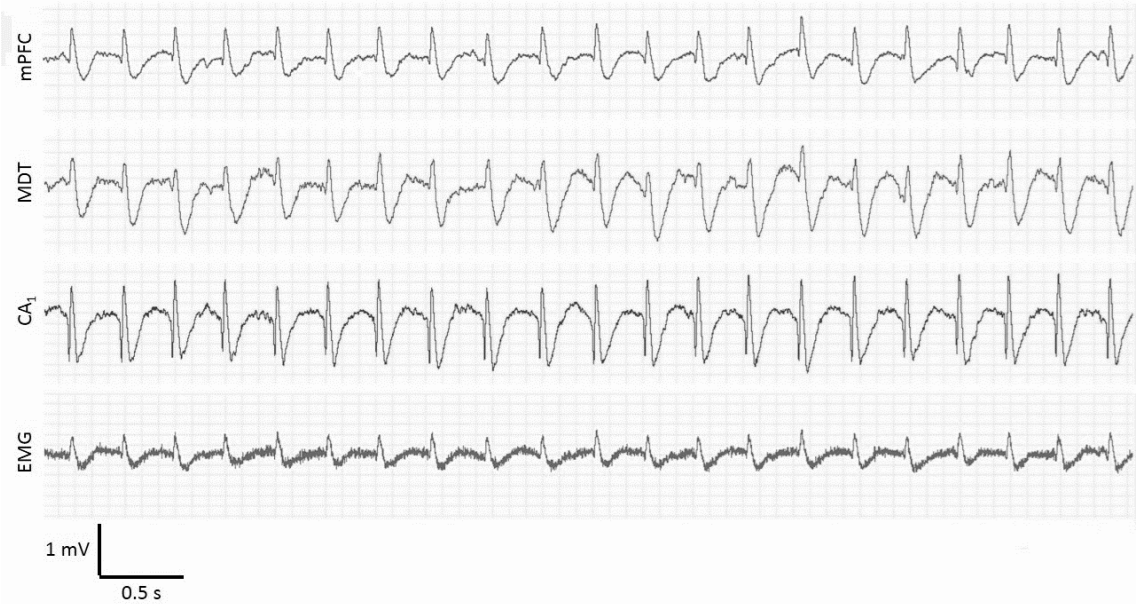


Figure 5 – *EEG Spike-wave discharges*

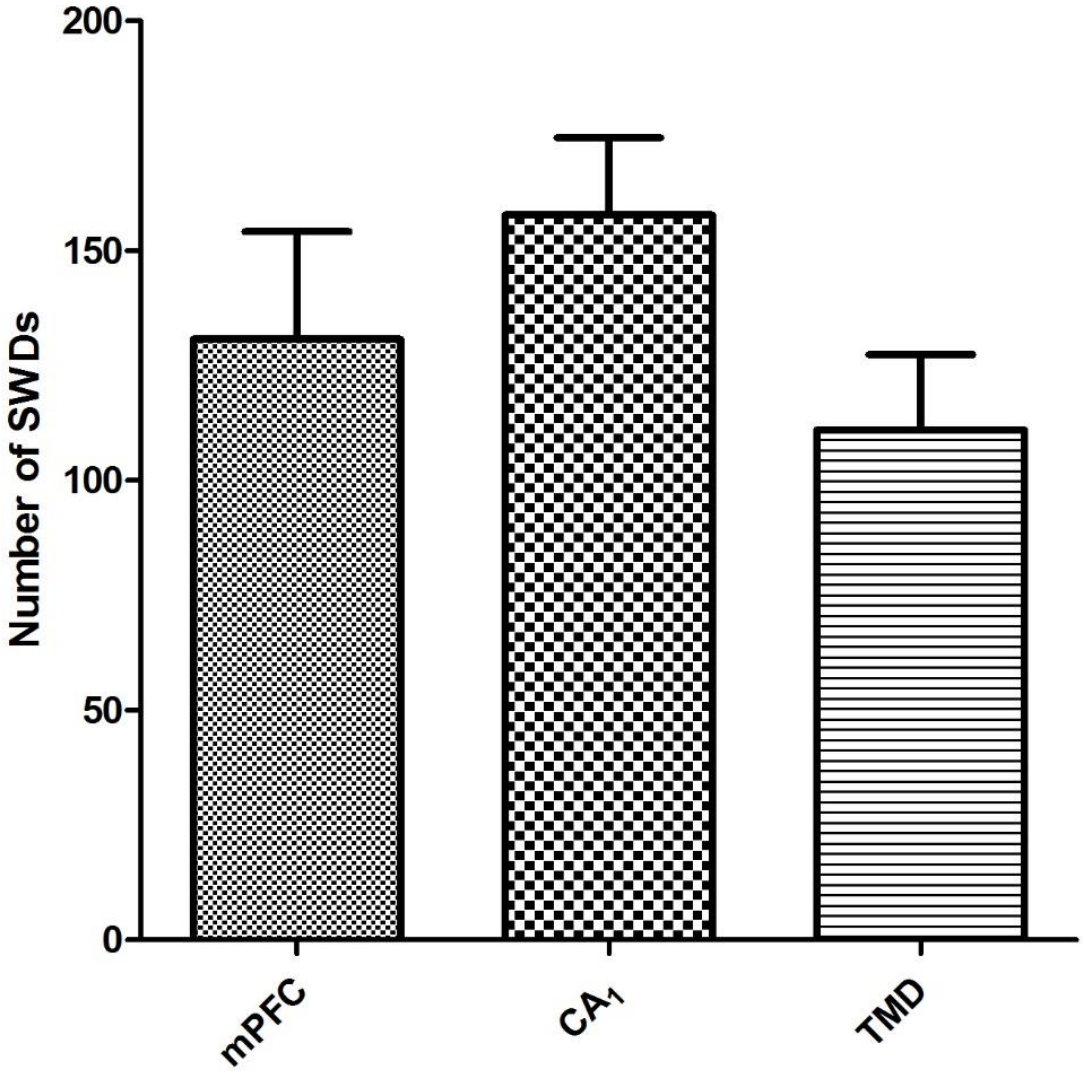


Figure 6 – *Number of SWDs*

8. Tables

Table 1

Average percentage of L-glutamate release increase of all treatments compared to control.

Treatment	Increase in L-Glutamate release (%)
Control	0
KCl	52.55 ± 2.7*
JBU 1 nM	3.08 ± 10.4
JBU 10 nM	16.08 ± 12.25
JBU 20 nM	21.65 ± 10.43
JBU 50 nM	14.56 ± 12.9
JBU 100 nM	33.34 ± 8.56*

*$p<0.05$ compared to t_0 values in all cases.

Table 2

Electrophysiological parameters recorded from the mouse sciatic nerve pre-incubated with different concentrations of JBU.

Treatment	Amplitude (mV)		Latency (ms)		Rise Time (ms)	
	Time 0 (absolute values)	End of incubation (% time 0)	Time 0 (absolute values)	End of incubation (% time 0)	Time 0 (absolute values)	End of incubation (% time 0)
Bath solution	8.0±0.6	94.3±3.2	0.26±0.06	122±8.8	0.25±0.03	90.1±2.9
JBU 1 µM	8.9±2.5	83.4±0.9*	0.35±0.10	114±1.8	0.25±0.02	93.7±4.3
JBU 100 nM	10.8±2.9	74.2±4.1*	0.44±0.05	106±2.4	0.26±0.03	89.3±8.8
JBU 10 nM	12.9±2.9	89.7±2.9	0.59±0.11	105±0.9	0.33±0.08	94.5±5.8
JBU 1 nM	7.7±3.7	59.5±5.4*	0.55±0.06	107±3.6	0.28±0.01	91.5±3.9

*$p<0.05$ compared to t_0 values in all cases.

CONSIDERAÇÕES FINAIS

Em conjunto os resultados obtidos nesse trabalho nos permitem estabelecer as seguintes considerações:

1-A urease de Canavalia ensiformis induz alterações exitatórias sobre o sistema nervoso central de mamíferos, quando injetada diretamente sobre o hipocampo;

2-Essas alterações do tipo crise de ausência sugerem a ativação de canais de cálcio do tipo T no hipocampo;

3-O bloqueio dos canais de sódio voltagem-dependentes no sistema nervos central induz uma exacerbação da neurotransmissão gabaérgica induzindo uma excitação paradoxal;

4-O efeito excitatório paradoxal é acompanhado por uma aumento da liberação de glutamato no sistema nervoso central;

5- A atividade excitatória da urease de *Canavalia ensiformis* sobre o sistema nervoso central de murinos não induz um processo de excitotoxicidade.

6- O bloqueio da condução do nervo ciático de camundongos demonstra uma atividade inibitória sobre os canais de sódio voltagem dependentes;

PERSPECTIVAS

Considerando o andamento do trabalho até o momento, temos como perspectivas:

➔ Realizar estudos comparativos entre os efeitos da JBU, sem e com diferentes instrumentos farmacológicos clássicos usados para o tratamento de pequeno mal epiléptico, tais como: etoxisumida, trimetadiona e ácido valpróico;

➔ Realizar ensaios com drogas utilizadas no tratamento do grande mal epilético, tais como: Carbamazepina e fenitoína, esperando-se um agravamento das crises de ausência;

➔ Realizar ensaios *in vitro* usando-se fatias de cérebro de ratos e camundongos para investigar a influência de moduladores de canais de cálcio sobre as respostas induzidas pela JBU em registros eletrofisiológicos intra e extracelulares.

➔ Identificar, por meio de ensaios de imunohistoquímica e Western Blot com anticorpos específicos, alvos moleculares relacionados a processos neurofisiológicos que envolvam o cálcio, como a calpaína, a cálcio camodulina cinase do tipo II e parvalbumina. E a relação entre os níveis dessas proteínas e as alterações eletrofisiológicas induzidas pela JBU no hipocampo.

➔ Investigar a influência do óxido nítrico no processo convulsivante induzido pela urease, por meio de ensaios de fluorescência com o marcador de óxido nítrico DAF-FM em cultura de células de hipocampo de ratos.

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ANEXO 1

Artigo científico a ser submetido para a revista BBA General Subjects.

Central and Peripheral Neurotoxicity Induced by Jack Bean Urease (JBU) in *Nauphoeta cinerea* cockroaches: interplay among cholinergic, gabaergic and octopaminergic neurotransmission

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Abstract

Ureases of *Canavalia ensiformis* plant have been studied for their potent insecticide activity. However the mode of action of their entomotoxic activity is still elusive. In this work we sought to investigate the mechanisms involved in the neurobiological alterations induced by the main Jack Bean Urease (JBU) in cockroaches *Nauphoeta cinerea*. The injection of insects with different doses of JBU was not lethal in doses of 0.75 to 6 µg/g animal within 24h. The of AChE activity of brain homogenate of JBU-injected insects was significantly inhibited in a dose-dependent manner with maximal inhibition (73.5±5nmol TNB/min/mg protein versus; n=3;

p<0.05) observed for the highest JBU dose (6 µg/g). The addition of JBU (1.5 µg/200µL), ACh (5 µM) or neostigmine (5 µM), to the cockroach semi-isolated heart preparation induced a maximum increase of heart rate of 24 ± 6, 26 ± 5 and 28 ± 6 beats/min, respectively (n=9, p<0.05). JBU (1.5, 3 and 6 µg/g) or octopamine (15 µg/g) also induced a pronounced behavioral alterations by causing a significative modulation of grooming activity that, in the case of JBU, was maximum for the higher dose (253 ± 33 s/30min; n=29; *p*<0.05). In this set of experiments the pre-treatment with phentolamine also inhibited the JBU or octopmine-induced positive modulation of grooming activity (p<0.05). The recordings of the insect neuromuscular function showed that JBU induced a maximum decrease in the muscle twitches of 65 ± 9%, this decrease was counteracted by the previous treatment of the cockroaches with bicuculline 5 µg/g (p<0.05, n=6, respectively). The acquisition of SNCAPs in presence of JBU (6µg/g) showed a decrease in the mean frequency whilst increased the amplitude of the potentials, when compared with the control saline (1425 ± 52.60min^{-1} and 1.102 ± 0.032mV, n=6, p<0.05 respectively). Taken together the results indicate that jack bean urease induce robust behavioral alterations at *Nauphoeta cinerea* cockroaches. These pharmacological activities involves, at CNS, the manipulation of grooming activity and the blockade of neuromuscular junctions at PNS. The mimetic activity of those responses induced by both octopamine and acetylcholine suggests the involvement of these neurotransmitters in the effects inducedby JBU in cockroaches.

Key words: Plant ureases, *Nauphoeta cinerea*, neurotoxicity, insect behavior, neuromuscular junctions.

1. Introduction

Throughout the last century, the majority of natural insecticides were replaced by their synthetic counterparts. Amongst the main reasons for the popularization of synthetic insecticides were their low cost, broad spectrum for use upon different insect species, and longevity in the field. However, although these characteristics appeared to be initially sound, they revealed catastrophic effects for the environment, since these chemical insecticides do not discriminate between insects and other species, nor against beneficial insects. In addition, the simplicity of their chemical structures favors insect resistance [1,2,3], which to overcome normally requires increasing both dosage and frequency of administration further endangering the environment. For these reasons, the search for novel natural molecules with insecticide potential has become a necessity more than an alternative. The growing knowledge on the non-

enzymatic properties of ureases, plant proteins crucial for the conversion of urea to ammonia, has unveiled their potential as biological insecticides**.**

Ureases (EC 3.5.1.5, urea amidohydrolase) are nickel-dependent metalloenzymes that catalyze the conversion of urea to two molecules of ammonia and one of carbon dioxide [4], enhancing the rate of the uncatalyzed hydrolysis by a factor of 8×10^{17} [5]. Ureases are widespread in plants, fungi, and bacteria but are not synthesized by animals [6]. *Canavalia ensiformis* displays several urease isoforms: the most abundant jackbean urease (JBU) [7], canatoxin (CNTX) [8,9] and JBURE-II [10,11]. The entomotoxic potential of *C. ensiformis* ureases has been proven in different insect models [12]. Indeed, a recombinant peptide named Jaburetox derived from the JBURE-II urease of *C. ensiformis* produced in *Escherichia coli* and its direct paralyzing effect on the cockroach neuromuscular junctions was characterized [13]. However, the specific mechanisms enrolled on this biocide activity are still not fully elucidated.

Cockroaches are quite diverse representing more than 4000 species comprising the suborder Dictyoptera. *Nauphoeta cinerea* is an ovoviviparous cockroach of the Blaberidae family. This specie is frequently used in biomedical research not only because the easiness in terms of breeding and maintenance but also because their relative simplicity and applicability for certain experimental procedures [14]. Therefore, despite their diversity, there are many similarities between the cockroach nervous system and other insect species [14]. In addition their amenability to experimental manipulation and biophysical parallelism with vertebrates makes then suitable for a number of neurophysiological approaches [15].

In this work we sought to investigate the alterations induced by jack bean urease in the insect central and peripheral nervous system. To accomplish this we carried out electromyographic, electrophysiological and behavior experiments using the cockroach *Nauphoeta cinerea.*

2. Materials and Methods

2.1. Experimental Animals

All experiments were performed on adult male *Nauphoeta cinerea* cockroaches (3-4 month after adult molt). The animals were reared at laboratory conditions with controlled temperature (22-25°C) on a 12h:12h L:D cycle. All cockroaches were provided with water and dog chow *ad libitum*.

2.2. Jack Bean Urease

Highly purified crystalline urease of *Canavalia ensiformis* (type C3) was obtained from Sigma-Aldrich Brazil, hexamer molecular mass 545 kDa. The protein crystals were dissolved in phosphate buffered saline to give concentrations in the range of 1mM. Protein solutions were dialysed against the same buffer and kept at 4°C before de bioassays.

2.3.Reagents and solutions

All chemicals and reagents used were of the highest purity available and were obtained from Sigma-Aldrich, Merck, Roche, Life Technologies or BioRad. Test-solutions were prepared daily by dilution in insect saline (Sturmer et al., 2014) immediately before use. The insect saline is a carbonate-buffered solution prepared with the following composition in mM: were freshly prepared in HEPES saline of the following composition (in mMol l^{-1}): NaCl, 214; KCl, 3.1; $CaCl_2$, 9; sucrose, 50; HEPES buffer, 5 and pH 7.2. All drugs were injected into the third abdominal hemocoel segment, at a final volume of 20µl, by means of a Hamilton syringe.

2.4. Lethality Assay

The insecticidal assay against adult *Nauphoeta cinerea*, was carried out essentially as described by Kagabu [16]. Various concentrations of JBU dissolved in 10µl insect saline, were injected between the third and the fourth abdominal segments of *N. cinerea*. All the experiments

were made in triplicate. Ten insects were used to test each dose and were kept at 22-25°C. Survival rate was registered 24 h after injection.

2.5. Assay for acetylcholinesterase activity

The *in vitro* inhibition of AChE was evaluated essentially as described by Stürmer [17]. The whole amount of protein was measured according to Bradford [18]. In brief, three cockroaches were injected with JBU (0.75, 1.5 and 3µg/g animal weight), or with trichlorfon (40 µg/g), an inhibitor of acetylcholinesterase, thirty minutes before the analysis. The animals were previously anesthetized by chilling at 5-7min at -20°C and their brains collected after cuticle removal. The material was mixed with 750µL of potassium phosphate-KPi buffer pH 7.0 (500 rpm/5 min/4º C) and 400 µl of supernatant were collected. From this sample, 50 µl was added to 50 µl of 50 mM (5,5'-dithiobis-(2-nitrobenzoic acid)-DTNB, 500 µl Kpi (pH 8.0) and 2.5 µl acetylthiocholine. The reaction was measured during 60 seconds (s) at 412 nm using a UV- Visible Spectrophotometer (model Evolution 60S, Thermoscientific, New Hampshire, USA) and analyzed by the software VISION lite (Thermoscientific).

2.6. Semi-isolated cockroach heart preparation

A semi-isolated cockroach heart bioassay was mounted essentially as previously described by Rodrigues [19]. Briefly adult male cockroaches were anesthetized by chilling (5-7min) until immobile and placed ventral side up. The lateral margins of the abdomen were cut along each side, and the ventral abdominal body wall was peeled up to expose the viscera. The viscera were carefully moved aside to expose the heart, which was still contracting while attached to the dorsal body wall. The heart preparations were washed by bathing them in drops of insect saline solution at room temperature (21-24ºC). After 5 min of heart beat stabilization, 200 µL of insect saline were added to the exposed heart. The treatments were delivered by

exchanging the bathing solution. The mean beats.min^{-1} in the first 5 min was taken as a reference. Heartbeat frequency was monitored for 30 min under a stereoscopic microscope. Nine cockroaches were used for each group. In the control group, only saline solution was added.

2.7. Behavioral assays

For general behavioral study, animals were placed in a demarked open-field arena with a video camera mounted overhead as previously described by Stürmer [18].

For each specific biological assay, the activities were recorded during 30 min by using a video-camera (Panasonic coupled to a 50mm Karl-Zeiz lens) connected or not to an eyepiece of micro-scopy (Olympus, model SZ51, Germany).The camera had a frame-by frame(60/ s) and was connected to a PC (Infoway, ItauTec, Brazil).Video movies were later analyzed using a HD Writer AE 2.6 T system (Panasonic) with variable speed control.

2.7.1. Grooming activity

The grooming behavior of cockroaches was monitored in an opaque plastic box (29 cm x 18 cm x 13 cm) with a clear plastic cover and was recorded with a camera for later analysis of motion time.The time of continuous groomingin seconds was measured for a 30min period immediately follow ingtreatment. Animals had never been placed in the testing box previously,and it was therefore a novel environmentinall cases. Thetemperature in the testing room was maintained at 22–30 °C. Testing was performed 2–8 h after the beginning of the light cycle. Control cockroaches were injected only with insect saline.

2.8. Electromyographic recordings

2.8.1.In vivo Cockroach Metathoracic Coxal-Adductor Nerve-Muscle Preparation

To analyze the effect induced by JBU at insect neuromuscular junctions we used the *in vivo* cockroach metathoracic-coxal adductor muscle preparation that was mounted essentially as described in Martinelli [13]. Briefly, animals were immobilized by chilling and mounted, ventral side up, in a Lucite holder covered with 1cm soft rubber that restrained the body and provided a platform to which the metathoracic thigh could be firmly attached using entomologic needles. The left leg was then tied at the medial joint with a dentistry suture line connected to a 1g force transducer (AVS Instruments, São Carlos, SP, Brazil). The transducer was mounted in a manipulator which allowed adjustment of muscle length. The exoskeleton was removed from over the appropriated thoracic ganglion. Nerve 5, which includes the motor axon to the muscle, was exposed and a bipolar electrode inserted to provide electrical stimulation. The nerve was stimulated at 0.5Hz/5ms, with twice the threshold during 120mim. After the insertion of the electrodes, the opening in the exoskeleton was covered with mineral oil to prevent dryness. Twitch tensions were recorded, digitalized and retrieved using a computer based software AQCAD (AVS Instruments, São Carlos, SP, Brazil). Data were further analyzed using the software ANCAD (AVS Instruments, São Carlos, SP, Brazil).

2.9. Electrophysiological recordings

2.9.1. In vitro extracellular recordings of spontaneous neural compound action potentials (SNCAP) of cockroach leg

For the recordings of SNCAP male cockroaches were anesthetized by chilling during 5-7min and the metathoracic leg was removed by cutting as closely as possible to the body ensuring that the thigh, femur, tibia, and tarsus remained intact. The leg was then carefully fixed by means of three Ag/AgCl needle electrodes in a petri dish filled with 10mm Sylgard® layer. One of these electrodes was connected to the ground connector of the amplifier (Axoclamp 2B,

Molecular Devices, USA) and the other to the indifferent (-) connector of the amplifier. The third electrode was placed in the femur and was the active recording electrode (+). The signals were recorded at a sampling rate of 1kHz and digitalized using a digitizer Digidata1320A (Molecular Devices, USA). The action potentials were visualized, recorded and retrieved for later analysis in a computer-based software Clampex (Molecular Devices, USA). The posterior analysis of the potentials was done using the software WinWCP (John Dempster, University of Strathclyde). Using this configuration the preparation could be used for at least one hour without changing the physiological characteristics of the potentials. The treatments were injected in the leg by using a Hamilton serynge and the doses calculated in µg/g based in body weight of the animal. Thus, the final volume to be adminstred was calculated based on the weight of an isolated leg.

2.10. Statistical Analysis

The results were expressed as the mean ± SEM and were analyzed by means of analysis of variance ANOVA/MANOVA or Student "t" test when convenient. A *p*-value ≤ 0.05 indicated significance. Statistics and graphs were made using the Software OriginPro 8.6 (OriginLab Corporation, MA, USA).

3. Results

3.1. Entomotoxic activity o Jack Bean Urease (JBU)

To determine the insecticidal activity of JBU, four doses were assayed (0.75, 1.5, 3 and 6µg/gAfter 24h no lethality was observed (data not shown). Although not lethal, the animals displayed a notorious grooming activity starting soon after the injection of urease and lethargy at the end of the 24h observation.

3.2. Assay for Acetylcholinesterase activity on cockroach brain homogenates

The analysis of AChE activity on cockroach brain homogenates of animals injected with different concentrations of JBU (1.5, 3 and 6µg/g animal weight) or Trichlorfon (40µg/g) revealed a dose-dependent enzyme inhibition, compared to the control (insects injected with saline only). Thus, the control values of AChE activity with saline was 186±3nmol NADPH/min/mg protein. When JBU was incubated (1.5 µg/g animal weight) the AChE activity decreased 33±6% (n=3; $p>0.05$). When JBU (3µg/g animal weight) was incubated there was the same decrease of AChE activity (33±3%; n=3; $p<0.05$) compared to control saline. On the other hand, a significative increase in AChE inhibition was also seen when JBU (6µg/g animal weight) was incubated (60±5%; n=3; $p<0.05$). Trichlorfon (1µM) administration, resulted in an AChE inhibition (77±6%; n=3; $p<0.05$). Results shown in Fig.1. indicated that 30 minutes after injection of JBU, the AChE activity in the cockroaches' brain was reduced significantly as compared to control values, with 60% inhibition for the largest tested JBU dose. At this dose, the effect of JBU was comparable to that observed in brain homogenates of insects injected with trichlorphon, a well known AChE inhibitor.

3.3. Effects of JBU on cockroach heart rate

The semi-isolated heart preparation of *N. cinerea* in the presence of saline had a control value of 94±5 beats/min (n = 9) (Fig. 2A). The addition of JBU (0.75, 1.5 and 3µg/200µL) to the preparation revealed a time-dependent and U-shaped dose-dependent effect. At the lowest dose of JBU (0.75µg/200µL) there was a tendency of increasing the chronotropic response of the heart. Starting immediately after addition of 1.5µg/200µL JBU, there was a significative increase of chronotropic responses peaking at 5 min with 24±6 beats/min ($p<0.05$ compared to the control saline, n = 9) and lasting until de end of 30 min of recording (Fig.2A). In contrast, when the highest concentration of JBU 3µg/200µL was assayed, there was a negative

chronotropic effect compared to the control saline (n = 9, $p > 0.05$) Fig.2A. For all JBU treatments, the washout of the preparation with insect saline, partially reversed the effects induced by urease (Fig. 2A). When ACh (5µM) or neostigmine (5µM) were assayed in the same preparation there was a similar positive modulation of heart beats of 26±5 and 28±6, respectively in 30 min recordings (n=6). The washout of the preparations partially reversed the choronotropic effect (Fig. 2).

3.4. Effects of JBU on cockroach grooming activity

In saline-injected cockroaches the mean time of continuous grooming was 153 ± 8s/30min for the legs and 70 ± 6s/30min for the antennae (n=32, respectively). The manipulation of the insects or the introduction of the syringe needle alone did not interfere with normal behavior (158.5 ± 12s/30min; n=28; $p>0.05$, Fig.3 A).

Injection of JBU (1.5, 3 and 6µg/g of animal weight) produced a significative dose-dependent increase in the grooming activity of the leg but this effect was not noticeable for the antennae. Thus, JBU 1.5µg/g induced an increase in leg grooming activity of (16±2%; n=40; $p<0.05$ compared to control saline) Fig. 3, Table 1. When the animals were treated with a dose of (3µg/g) there was a further increase in the grooming parameters (65±8%; n=29; $p<0.05$) Fig.3. When the highest dose 6µg/g was administered, there was also a further increase in the time spending leg grooming, above to the former and the second concentrations (137±7%; n=30; $p<0.05$) (Fig. 3).The percentage values for antennae grooming were (68±7%, 81±9% and 115±11%) respectively for 1.5, 3 and 6µg/g of animal weight JBU ($p>0.05$, Fig. 3).

3.5. Effects of different pharmacological treatments on JBU-induced grooming activity

Leg grooming activity is thought to be modulated mainly by the neurotransmitter octopamine in insects [20]. Thus, we tested the effect of the neurotransmitter octopamine and

of phentolamine, the selective octopamine receptor blocker, in modulating the behavioral changes induced by JBU. When octopamine (15µg/g) was injected in the cockroaches there was an increase in the time spending leg grooming was of (134±10%; n=40; $p<0.05$ compared to control saline) Fig.4, Table 1. The previously administration of phentolamine (0.1µg/g) 15min prior the treatment with octopamine inhibited the octopamine-induced increase of leg groomings to a number slightly below to the control saline (52±8%; n=40; $p<0.05$ compared to the control octopamine) Fig. 4. When phentolamine (0.1µg/g) was previously administered 15min before the treatment with JBU (6µg/g) there was a decrease in the time spending leg groomings by JBU to (37±5%; n=40; $p<0.05$ compared to the control urease) Fig.4, Table 1.

3.6. Neuromuscular blockade induced by JBU in vivo on a cockroach nerve-muscle preparation

To further analyze the effect of JBU on cockroach nervous system, we used the i*n vivo* metathoracic coxal-adductor nerve-muscle preparation. The administration of insect saline alone did not interfere with neuromuscular responses during 120min recordings (n=6) (Fig. 5A). The injection of JBU (1.5, 3 and 6 µg/g of animal weight) induced a time-dependent neuromuscular blockade in 120 min recordings. When JBU (1.5µg/g) was assayed there was 31±3% blockade of twitch tension in 120min (n=6, $p<0.05$) (Fig 5A). The injection of the highest concentration of JBU (6µg/g) induced an inhibitory effect noticeable after 30 min, increasing steadly for about 1 hour, then reaching a maximal of 65±9% (n=6, $p<0.05$) after 120 min (Fig.5A). However, the administration of the intermediate dose (3 µg/g of animal weight) induced only 16±4% inhibition (n=6) of the twitches ($p>0.05$ compared to the control, Fig. 5A). In the same set of experiments, the treatment of the animals with bicuculline (5µg/g), a selective blocker of gama-amino-butyric acid (GABA) receptor induced 27±6% inhibition of the twitches ($p>0.05$, n=6) (Fig. 6A). When bicuculline(5µg/g) was previously injected in the insect

15min before JBU (6 µg/g) there was ~40% prevention of JBU-induced neuromuscular blockade ($p<0.05$ compared to the JBU alone) (n=6, Fig 6A).

3.7. Effect of JBU on spontaneous activity of cockroach leg nerve-compound action potentials (SNCAP)

The cockroach leg nerve-compound action potential has a relative high rate of rise when compared to other neuronal models (Table 1). Injection of JBU (6µg/g) into the cockroach leg caused a significative decrease in the frequency (1425 ±5 2.60min^{-1}) but an increase in the mean amplitude (1.102 ± 0.032mV) of SNCAP (n=6, $p<0.05$ respectively) in the 60min period of observation (Fig.7A, C). The rise time decreased (0.64±0.044 ms) together with the decay time (3.72±2.121ms, $p<0.05$ respectively). The treatment with JBU 9 % decrease of the duration of the potentials (Table 1), a result that agreed with the increase of the mean area of the potentials (data not shown).

4. Discussion

In this work we have characterized the neurotoxic activity induced by the main urease isoform of *Canavalia ensiformis* plant, the so-called jack bean urease, in the cockroach *Nauphoeta cinerea*. Aspects related to the cellular and biochemical mechanisms involved in the neuromodulation of the insect central and peripheral nervous system by JBU were elucidated in this study.

Although JBU has been proven to be entomotoxic to different insects [12], it displayed no lethality in *Nauphoeta cinerea* cockroaches, at least not by intrabdominal administration. In previous studies, we reported that the lethality induced by the Jack Bean ureases given orally to insects depends on characteristics of their digestive system (Stanisçuaski and Carlini, 2012). At least part of the entomotoxic effect is caused by toxic peptides derived from ureases upon

cleavage by insect's digestive enzymes. Thus, only insects relying on cathepsin-like enzymes (cysteine and aspartic proteases) e.g. *Callosobruchus maculatus* and *Rhodnius prolixus*, died upon ingestion of ureases, while insects with digestion based on trypsin-like enzymes (serine proteases), such as *Manduca sexta*, *Schistocerca americana*, *Drosophila melanogaster* and *Aedes aegypti*, were insensitive. Elpidina and cols [20] showed that the digestion in *N. cinerea* midgut is related to three types of serine proteinases and one cysteine proteinase activities, that have demonstrated an optimal activity under pH 11.5 and what would explain the absence of lethality of JBU in our experimental insect model. On the other hand, it is already known that urease-derived peptides do not account for all entomotoxic properties of these proteins. The whole protein can be found circulating in the hemolymph of insects after feeding (Stanisçuaski et al., 2010). Urease itself was shown to produce entomotoxic effects in the hemipteran Rhodnius prolixus that were independent of enzymatic cleavage, such as impairment of diuresis in isolated Malpighiam tubules (Stanisçuaski et al., 2009) or increase in the frequency of crop contraction (Stanisçuaski et al., 2010). Here we have shown that, even in an insect model probably unable to cleave JBU to release its insecticidal peptides, thus not causing lethality, the protein itself induces profound alterations in the cockroach physiology impacting its central and peripheric nervous system.

JBU showed effects consistent with inhibition of acetylcholinesterase (AChE) activity in brain homogenates of treated insects. Although not lethal to the cockroach, the anti-acetylcholinesterase activity of JBU could explain the behavioral alterations induced by the protein in the treated insects [17]. Therefore, the increase in the leg rather than the antennal grooming and its antagonism by phentolamine suggests the involvement of the neurotransmitter octopamine in the modulation of the cockroach behavior triggered by JBU [21].

The anti-AChE activity of JBU can also account for the increase in the heart rate in our experimental model. In *Periplaneta americana*, the rate of heart beats is determined by a

neurogenic pace-maker with cholinergic properties [22]. Thus, this system is stimulated by acetylcholine and by substances that inhibit the breakdown of acetylcholine by AChE. Indeed, our group has demonstrated in a previous study the increase of heart rate by natural inhibitors of acetylcholinesterase in *Leurolestes circunvagans* cockroaches [19]. The molecular mechanisms by which JBU can act as an inhibitor of AChE was not investigated in the present work.

The electromyographic recordings of cockroaches injected with JBU unveiled the neuromuscular blocking activity of the protein itself. This effect was previously reported for jaburetox, a urease-derived recombinant peptide (Martinelli et al., 2014). Several biological properties, but not all of them, are shared between the whole urease molecule and Jaburetox. Particularly relevant to the present data, both JBU and Jaburetox in nanomolar concentrations were shown to insert themselves into artificial lipid bilayers creating cation-selective channels. Since the region of the JBU molecule comprising this peptide sequence is mostly exposed at the protein's surface, it probably mediates most of the urease's interactions with its targets (Piovesan et al., 2014).

There is a considerable amount of work showing that insect neuromuscular junctions use glutamate as the main excitatory neurotransmitter and gama-aminobutiric acid (GABA) as the main inhibitory one [23,14,24]. In insects the release of glutamate or GABA at the synaptic cleft induces both increase or decrease of strength of muscle contraction [25]. Thus, JBU could primarily induce neuromuscular blockade in *N. cinerea* by increasing gabaergic neurotransmission or inhibiting the glutamatergic counterparts. Accordingly, pretreatment of the animals with bicucculine decreased the level of neuromuscular blockade induced by JBU, suggesting that GABA may be involved in the peripheral inhibitory activity of the protein [26].

In our experimental model, the neuromuscular twitches were obtained by stimulating the nerve 5. In cockroaches, the axon of the slow depressor coxal motor neuron (Ds) leaves the

ganglion via nerve 5 and innervates the coxal depressor muscle (muscle 177D) [27]. Immunostaining of metathoracic region in *Locusta migratoria* has revealed that at least two branchs of inhibitory neurons (gabaergic neurons) departs from nerve 5 at the metathoracic ganglion. Along the nerve 5 the activity is conducted centrally (coming from afferent signals of sensila) or peripherally, toward the methatoracic ganglion through monosynapticaly connected motorneurons via cholinergic synapses [27]. Thus, we suggest that at least in part, the neuromuscular blockade induced by JBU in *N. cinerea* consequent to an interplay between cholinergic and gabaergic interneurons.

JBU induced a decrease in the frequency of SNCAP concomitant with an increase in the amplitude. The hair plate neurons located in the animal leg are connected to the ganglion via of nerve 5, thus the anti-AChE activity of JBU could also account for the increase in the amplitude of the spikes. In the walking animal, deflection of the hair plate sensilla produces afferent spikes which modulate tonic Ds activity, thereby coordinating the animal stepping [27]. Electrically charged ions move inward or outward to the membrane of dendrites through stretch-activated ion channels [28]. In the crayfish, stretch receptors have been associated to changes in the potassium conductance of the membrane or in the activity of the sodium-potassium pump, which could directly affect the amplitude of the receptor potential [29, 30,31,32]. Thus, it is possible that somehow JBU is inducing hyperpolarization of nerve 5 either by increasing gaba-induced activity or by a direct membrane interaction, resulting in an improved potassium conductance [33]. Such an effect would create a situation where only superimposed spikes with amplitudes above the normal tonic firing pattern of Ds would transpass the resting membrane threshold during the onset of urease activity.

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Conflict of interests: The authors declare that there is no conflict of interests regarding this work.

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Legends

Figure 1. Graph of acetylcholinesterase inhibition in total cockroach brain homogenate (n=3 for each dose) after 6 hours exposure to JBU (1.5, 3.0 and 6µg/g) and trichlorfon (1µM). Data were expressed as mU/mg protein. $p<0.05$ in comparison to control saline.

Figure 2. Effect of different concentrations of Jack Bean Urease (JBU) on Nauphoetacinerea heart rate. In the graph each point corresponds to the mean ± S.E.M. of the percentage of the insect heart beats in 30min. Note that JBU (1.5µg/200µl) induced a cardio acceleratory activity similarly to those induced by the application of acetylcholine or neostigmine (5µM, respectively). * $p<0.05$ in comparison to the control saline.

Figure 3. Increase of grooming behavior induce by different doses of JBU (1.5, 3.0 and 6µg/g) in *Nauphoeta cinerea* cokroaches. The grooming activity was recorded during 30 min and the results expressed as the total time of grooms in seconds. In the graph, each bar represent the mean ±S.E.M. of the number of grooms.30min-1. Note that only the leg grooming behavior was significantly modified by the urease. **$p<0.05$ in comparison to the controlsaline.

Figure 4. Effect of different octopaminergic modulators on JBU-induced grooming increase in cockroaches. The grooming activity was recorded during 30 min and the results expressed as the total time of grooms in seconds. In the graph, each bar represent the mean ± S.E.M. of the number of grooms.30min-1. In the case of concomitant treatment with JBU (6µg/g) or octopamine (15µg/g), phentolamine (0.1µg/g) was injected in the third abdominal segment 10 min before. *$p<0.05$ in comparison to control saline; **$p<0.05$ in comparison to the control octopamine; **$p<0.01$ in related to the control JBU.

Figure 5. Neromuscular blockade induced by Jack Bean Urease (JBU) in *Nauphoeta cinerea* cockroaches. In the graph each point represents the mean ± S.E.M. of the percentage of the twitch tension related to before treatment. Panel B shows a representative recording of a control saline experiment. On C, a representative trace of the JBU-induced neuromusucular paralysis. *$p<0.05$ related to the control saline.

Figure 6. Neuromuscular blocking activities induced by Jack Bean Urease, octopamine and acetylcholine and its prevention by bicuculline using *in vivo* essays with *Nauphoeta cinerea* cockroaches. In the graphs each point correspond to the mean ± S.E.M. the percentage of the twitch tension related to before treatment. On A, B and C, note the similarity among the neuromuscular inhibitory activity of jack bean urease, acetylcholine and octopamine. In all cases, the administration of bicuculline (5µg/g) was able to counteract the inherent neuromuscular blockade. *$p<0.05$ related to the control saline.

Figure 7. Effect of Jack Bean Urease (JBU) on cockroach leg nerve-compound action potentials (SNCAP) kinetics. Panel A shows representative traces of the SNCAP under control saline and JBU-treated preparations. Noticed the decrease in the frequency of the potentials. On

B, comparative histograms of control saline and JBU-treated preparations. Notice the increase in the frequency of higher amplitudes at JBU-treated situations.

Figure 1

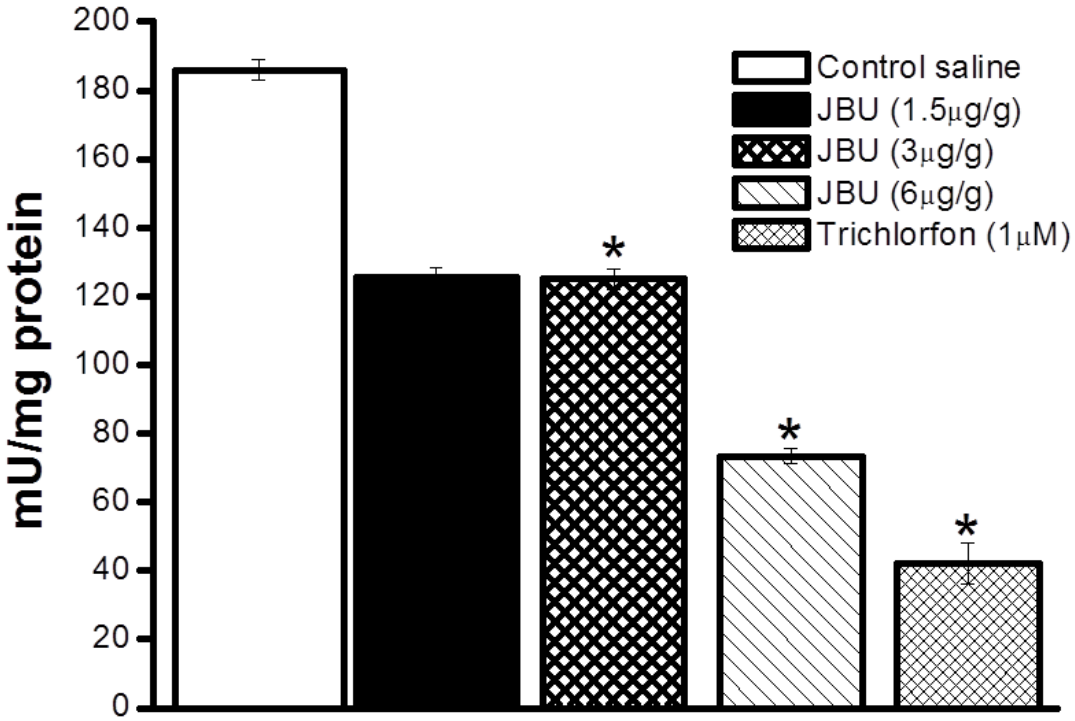

Figure 2

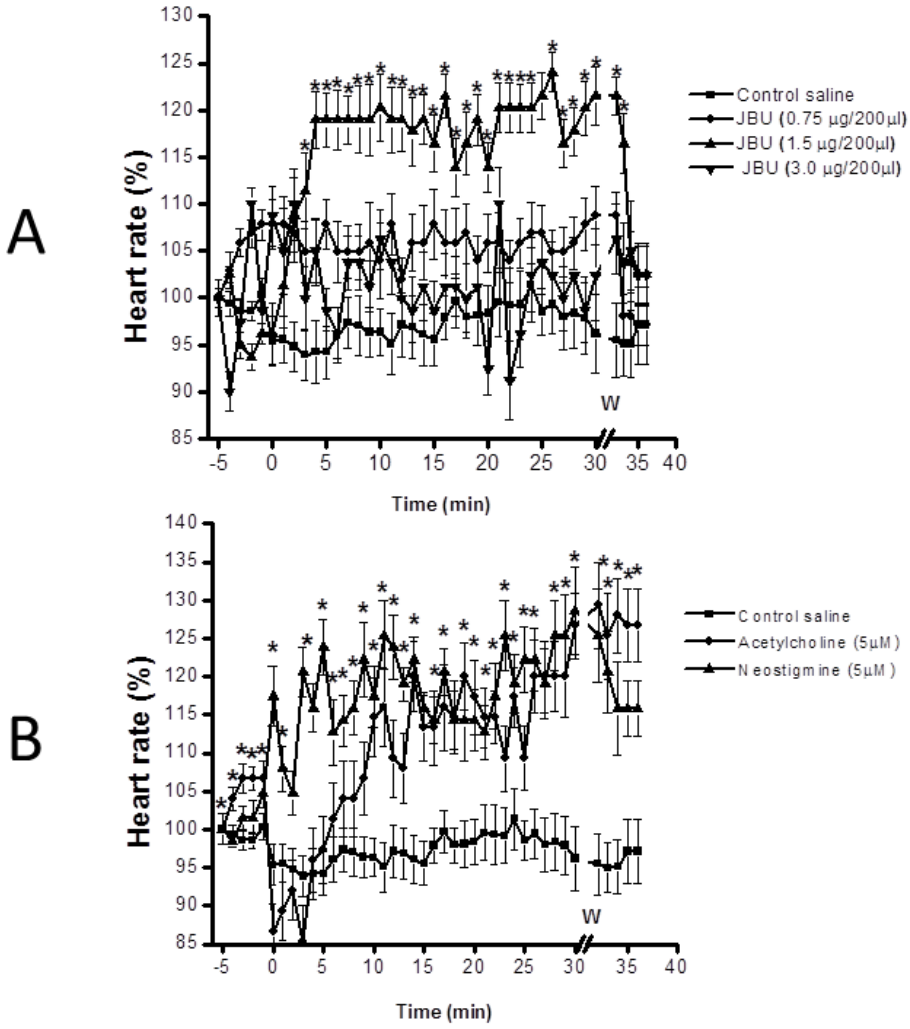
A
Heart rate (%)
Time (min)
Control saline
JBU (0.75 μg/200μl)
JBU (1.5 μg/200μl)
JBU (3.0 μg/200μl)
W
B
Heart rate (%)
Time (min)
Control saline
Acetylcholine (5μM)
Neostigmine (5μM)
W

Figure 3

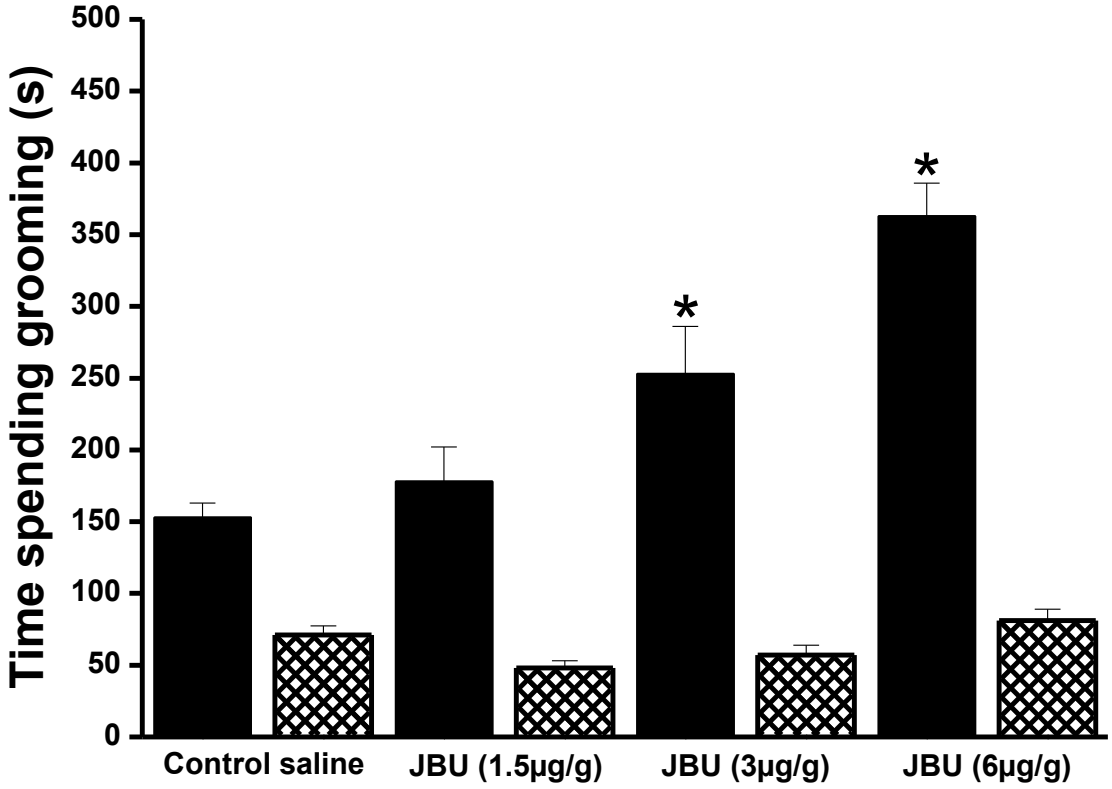
Time spending grooming (s)
500
450
400
350
300
250
200
150
100
50
0
*
*
Control saline
JBU (1.5μg/g)
JBU (3μg/g)
JBU (6μg/g)

Figure 4

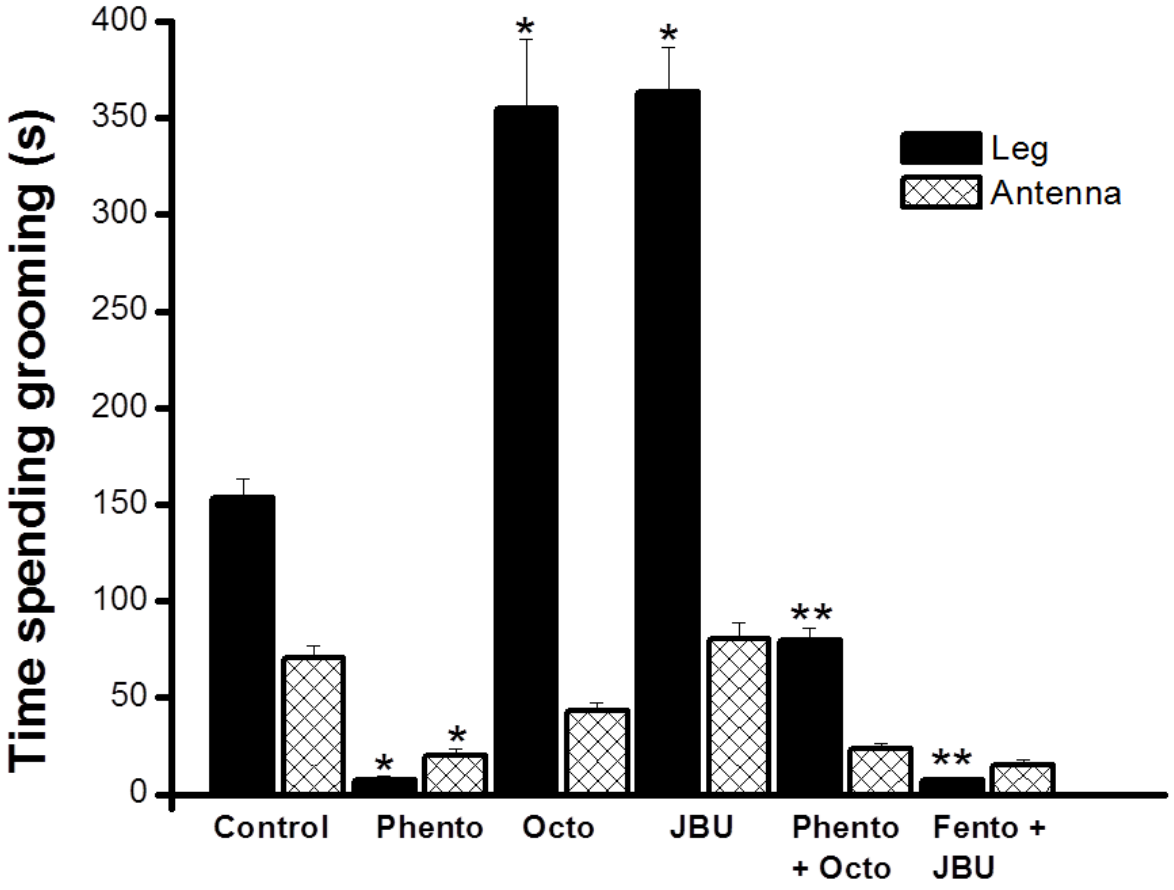
Time spending grooming (s)
400
350
300
250
200
150
100
50
0
Leg
Antenna
*
*
*
*
**
**
Control
Phento
Octo
JBU
Phento + Octo
Fento + JBU

Figure 5

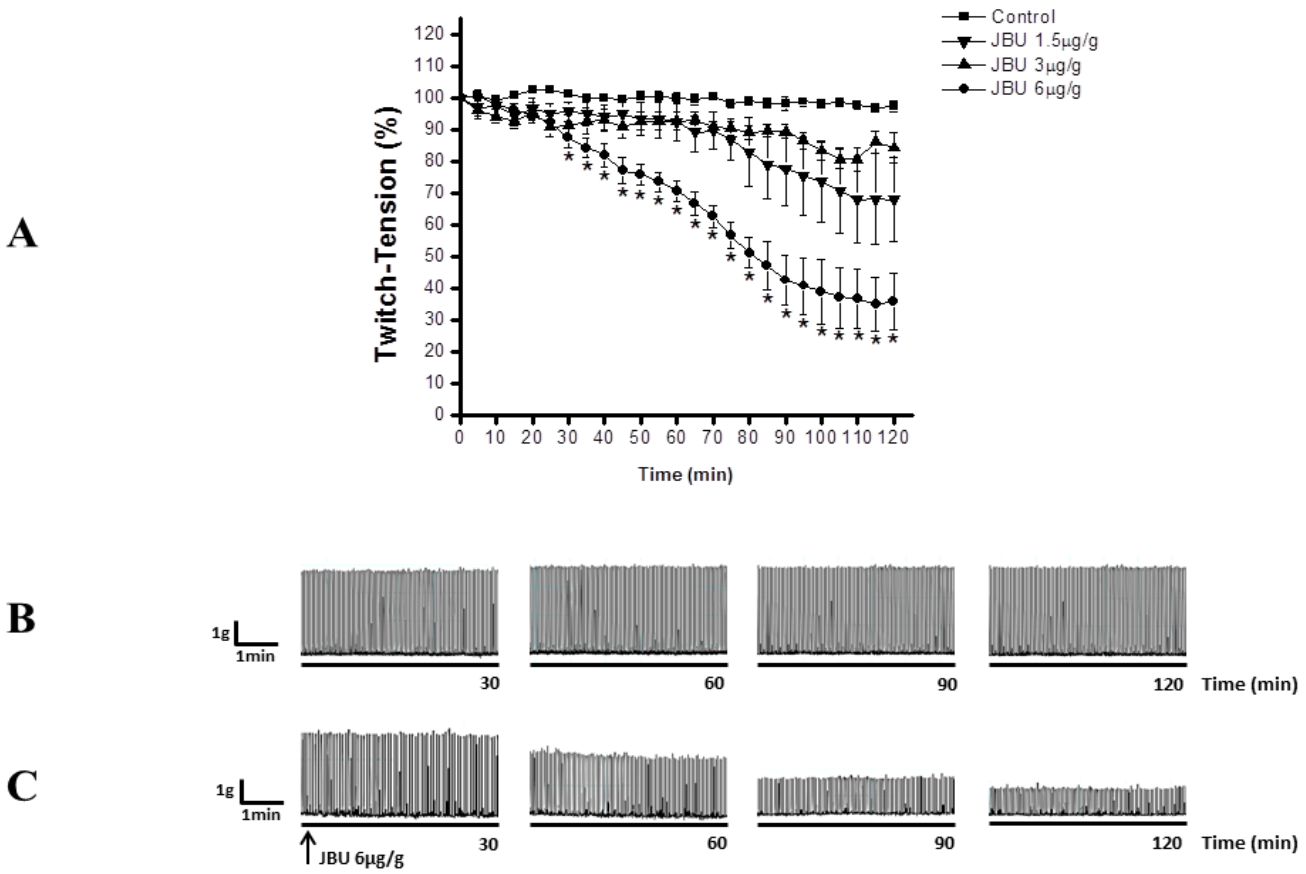
A
Control
JBU 1.5μg/g
JBU 3μg/g
JBU 6μg/g
Twitch-Tension (%)
Time (min)
B
1g
1min
30
60
90
120
Time (min)
C
1g
1min
JBU 6μg/g
30
60
90
120
Time (min)

Figure 6

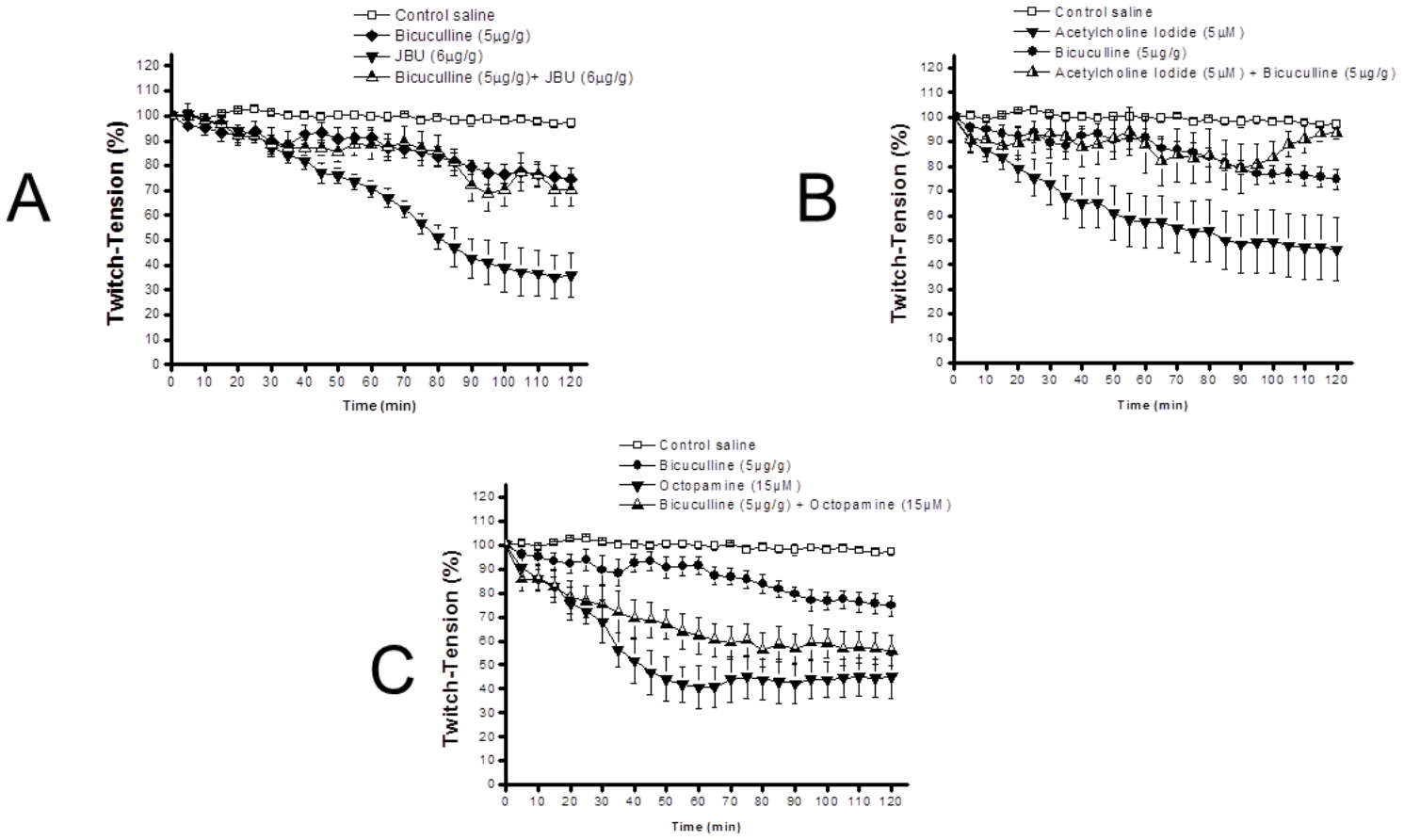
A
Control saline
Bicuculline (5µg/g)
JBU (6µg/g)
Bicuculline (5µg/g)+ JBU (6µg/g)
Twitch-Tension (%)
Time (min)
B
Control saline
Acetylcholine Iodide (5µM)
Bicuculline (5µg/g)
Acetylcholine Iodide (5µM) + Bicuculline (5µg/g)
Twitch-Tension (%)
Time (min)
C
Control saline
Bicuculline (5µg/g)
Octopamine (15µM)
Bicuculline (5µg/g) + Octopamine (15µM)
Twitch-Tension (%)
Time (min)

Figure 7

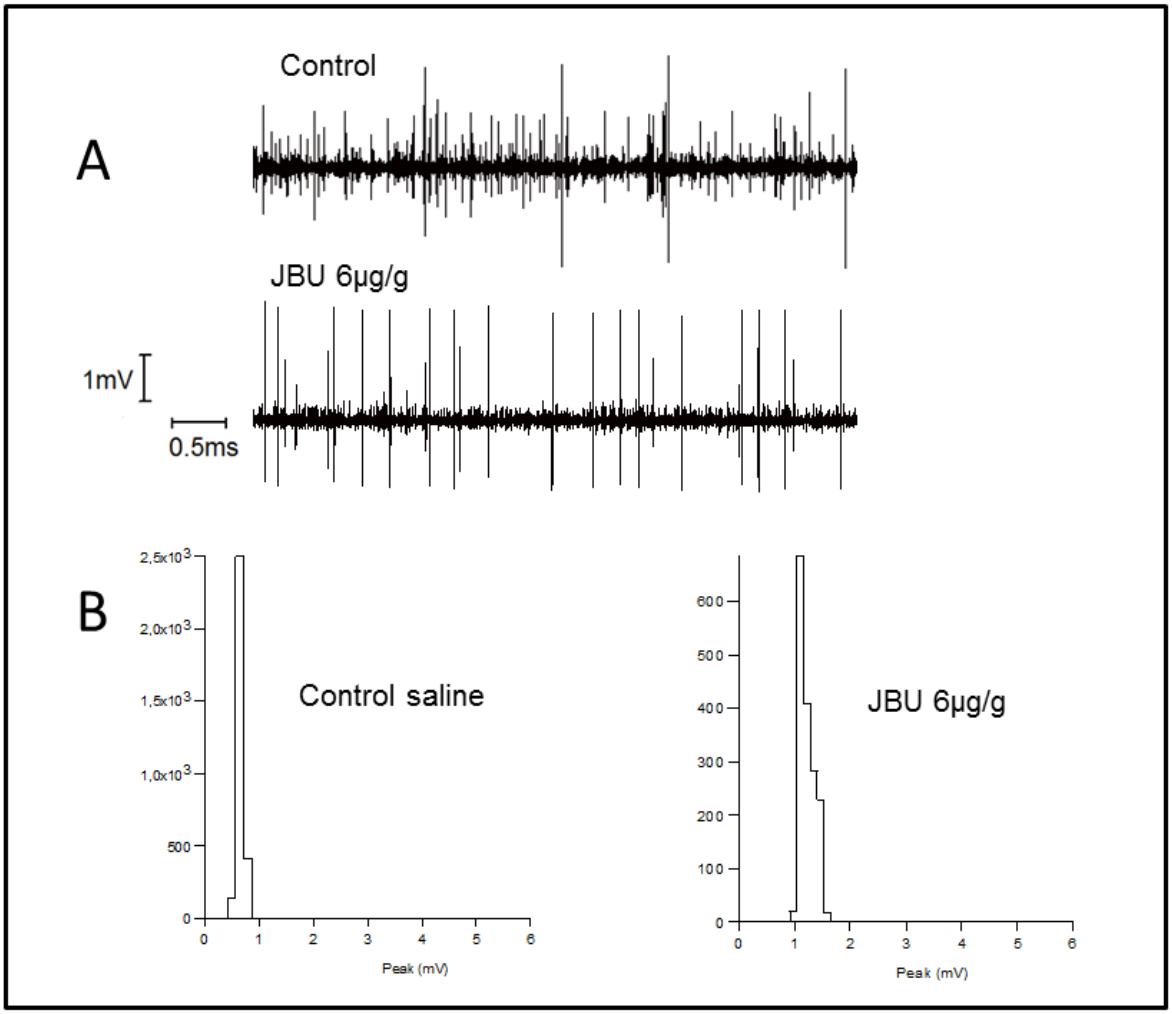
A
Control
JBU 6µg/g
1mV
0.5ms
B
Control saline
JBU 6µg/g
Peak (mV)
Peak (mV)

Table 1

Gromming treatments	Leg (time of continuous) Grooming	Antennae (time of continuous) Grooming
Control saline	153 ± 8s/30min	70 ± 6s/30min
JBU 1.5µg/g	177±23s/30min	47.6±5.2s/30min
JBU 3µg/g	253±33s/30min	57.2±7s/30min
JBU 6µg/g	363±23s/30min	80.7±8s/30min
Octopamine 15µg/g	358 ± 27s/30min	
Phentolamine 0.1µg/g	79±13s/30min	
Phentolamine 0.1µg/g -15min	56±7s/30min	

***-15min means that the drug was applied fifteen minutes before Jack Bean Urease**

Table 2

	Frequency (Events.60min^{-1})	Amplitude Average (mV)	Rise time (ms)	Decay time (ms)	Area (mV.ms)
Control saline	3685 ±273	0.0599±0.0.19	3.72±0,264	13.866±8.03	0.939 ±0.1339
JBU 6µg/g	1425±52.19*	1.102± 0.06*	0.64±0.04*	3.72±2.121*	0.724±0.099

ANEXO 2

Artigo científico a ser submetido para revista Neuropharmacology.

Neurobiology of Jack Bean Urease at mammal central nervous system: Investigation of its convulsive-like activity

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Abstract

Ureases are convulsive-like enzymes whose mechanism of action is still a matter of interest. In this work we have demonstrated that the urease of *Canavalia ensiformis* induced an increase of calcium influx at primary hippocampal cultures, with the risein action potential firing rates at pyramidal cells of both rat hippocampal slices and cultures. These excitatory effects are accompanied by the activation of sodium currents and by an increase of the frequency of EPSPs in hippocampal tissue cultures. However the pro-convulsive activity of JBU induced no excitotoxic effects in hippocampal cells as demonstrated by the MTT assay in mice brain slices. In addition, this toxin showed an inhibitory action over the LTP induction of rat hippocampal slices, similarly to those observed with anesthetic-like drugs such as lidocaine.

1-Introduction

Ureases are nickel-dependent metalloenzymes, synthesized by plants, some bacteria and fungi but not by animals (Mobley and Hausinger, 1989). It catalyzes the hydrolysis of urea into ammonia and carbon dioxide. Although the amino acid sequences of plant and bacterial ureases are closely related, some biological activities differ significantly. In 1926, Sumner isolated from jack beans (*Canavalia ensiformis*) the first crystalline enzyme, urease, and proposed that enzymes could be proteins devoid of organiccoenzymes and metal ions. The basic structural unity of this urease is a single polypeptide chain with 840 amino acid residues with a molecular weight of 90.770 Da(Riddles et al., 1991). Therefore, *C. ensiformis* displays several urease isoforms: jackbean urease (JBURE-I) (Sumner, 1926), canatoxin, (CNTX) (Carlini and Guimarães, 1981 and Follmer et al., 2001) and JBURE-II (Pires-Alves et al., 2003 and Mullinari

et al., 2011).The widespread presence of “CNTX-like” proteins in other leguminous seeds (Carlini et al., 1988, Vasconcelos et al., 1994), as well as thetoxin accumulation pattern during seed maturation (Barcellos et al., 1993) strongly suggested that this protein might playan important physiological role, perhaps related to the plant defense. Beyond roles related to ureolytic activity, our group have discovered unique toxicityof these ureases toward some insects and fungi toxicity independent of enzyme activity.Indeed, Jaburetox-2Ec (Jbtx-2Ec), a recombinant peptide from Jack Bean Urease, exhibited a potent insecticidal effect in important economical insect crop pests, reinforcing the defense roles of ureases in plants (Mullinari et al., 2007; Staniçuaski et al., 2005).However, canatoxin exhibits toxic effects on mammals,in addition to fungitoxic and insecticidal activities. CNTX was shown to induce convulsions and death in mice and rats when injectedintraperitoneally(Carlini et al., 1984), but it was ineffective if given orally to the animals (Carlini and Guimarães, 1991).Therefore, the similarities among the ureolitic activity of ureases from different sources, including those with clinical interest, raised the wish to exploit the mechanisms of convulsive-like activity of Jack Bean Urease. In this work we sought to investigate the electrophysiological and molecular interactions of Jack Bean Urease at murine central nervous system.

2-**Methods**

2.1. Reagents and Jack Bean Urease

All chemicals and reagents usedwere of the highest purity and were obtained from Sigma,Aldrich,Merck or BioRad. The urease from *Canavaliaensiformis* was obtained from Sigma-Aldrich Brazil,hexamer molecular weight 545 kDa.

2.2. Animals

Adult Swiss white mice (28–35g) were supplied by the animal facility from Universidade Federal de Santa Maria(UFSM). Juvenile Wistar rats were obtained from Centre for Biological Experimental Models (CeMBE) of PUCRS. Newborn Sprague Dawley rats were supplied by the University of Strathclyde Animal House.The animals were housed at 25 ºC with access *ad libitum* to food and water. These studies have been done in accordance with the guidelines of the Brazilian College forAnimal Experimentation (COBEA) and with UK HomeOffice guidelines.

2.3. Cell culture

Primaryhippocampal cultures were prepared as describedpreviously (Greenwood et al., 2007). Briefly, 1- to2-day-old Sprague Dawley rats were sacrificed by cervical dislocation and decapitated. Once hippocampi were removed and triturated, cells were plated at adensity of 3 x 10^5 cells·mL^{-1} onto poly-L-lysine coatedcovers lips. Cultures were incubated in a medium consisting of Neurobasal-A Medium (Invitrogen, Paisley, UK) supplemented with 2% (v/v) B-27 (Invitrogen) and 2 mM L-glutamine and maintained in ahumidified atmosphere at 37°C/5% CO_2 for 10–14days *in vitro* (DIV). After 5DIV, cytosine-D-arabinofuranoside (10 μM) was added to inhibit glialcell proliferation. All experiments were performed on cells taken from at least three separate cultures obtained from different rats.

2.4. Mice Hippocampal Slices Preparation

Mice were decapitated, the brains removed immediately and the hippocampus dissected outon ice and humidified in cold HEPES-saline buffergassed with O_2 (124mm NaCl, 4mM KCl, 1.2mM $MgSO_4$,12mM glucose, 1mM $CaCl_2$, and 25mM HEPES pH 7.4).Hippocampal slices were obtained according to Vinadé and Rodnight, (1996), briefly: a Mcilwain tissue chopper was used to obtain the slices (0.4mm) that were separated and pre-incubated at 37∘C for 30min

in microwell plates filled with HEPES saline (200 μL/slice). Subsequently, fresh medium was replaced (200 μL/slice) to control conditions or treatments with JBU (0.01, 0.1 and 1μM) and incubated for additional 1 hour at 37°C.

2.5. Rat hippocampal slice preparation

Juvenile Wistar rats were sacrificed and its brains were rapidly removed and placed in ice-cold (0–3°C) and oxygenated (95% O_2/5% CO_2) cutting solution(pH?)containing(in mM): $NaHCO_3$2, sucrose 26,$NaH2PO_4$2, $MgSO_4$3, KCl 2, $CaCl_2$?and D-glucose 10. Eu prefiro padronizar a linguagem do artigo – descrever assoluções, as unidades de medida, as nomenclaturas, etc, sempre da mesma forma, usar!!. Parasagittal whole brain slices(400 µm thick) were cut using a vibratome and placedinto oxygenated artificial cerebrospinal fluid (aCSF)with the same composition as the cutting solution,but with NaCl (124 mM) replacing the sucrose. Hippocampalregions were then dissected free andplaced in a submerged holding chamber containing a CSF continuously bubbled with 95% O_2/5% CO_2.Slices were allowed to equilibrate for a minimum of1 h at room temperature prior to use.

2.6. Mice hippocampal slices viability assay

Immediately after treatment with JBU and control conditions, mice hippocampal slices were assayed for a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test according to Dal Belo et al., 2013. Briefly, slices were incubated in diluted MTT(0.05% in HEPES-saline) for 30 min at 37°C and the resulting purple formazan product was dissolved in DMSO. The subsequent colored compound was measured in an ELISA reader equipment atλ = 550 nm.

2.7. Ca^{2+} imaging

All imaging experiments were performed on a digital epifluorescenceimaging system (WinFluor, J. Dempster, Universityof Strathclyde) mounted on a Nikon TE300 microscope usinga 20x objective. Hippocampal cultures (DIV 10–14) wereloaded with FLUO-4 AM (5

μM, 45–60 min, room temperature)prior to experiments. Images (390/510 nm) wereobtained continually during 20min. Experimentswere performed on cultures continually perfused(1–2 $mL \cdot min^{-1}$) with HEPES-buffered saline (HBS) containing(in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2,HEPES 10, D-glucose 10, pH was adjusted to 7.4 andosmolarity adjusted to 310 mOsm with sucrose ifrequiredHBS, at room temperature. The urease was added via the perfusate. Cells wereidentified as astrocytes based on their morphological characteristicsand their lack of response to high extracellular potassium(5mM). Data were calculated as changes influorescence ratio and expressed as $\Delta F/F_0$.

2.8. Electrophysiology

Extracellular field excitatory postsynaptic potentials(fEPSP)

For extracellular electrophyisiological recordings, individual hippocampal slices from control rats or JBU-treated individuals were transferred to a submersion-type recording chamber and submerged beneath the continuously perfusion gas (95%, O_2, 5% CO_2)-saturated ACSF at a rate of 3.0 ml/min. Field excitatory postsynaptic potentials (fEPSP) from Schaffer collateral/commissural-CA1 slices were evoked (0.2 ms constant-current pulses every 20 s) using a differential alternating current (IsoflexM.P.I., Israel). The stimulation electrode consisted of a twisted bipolar pair of 75 μm platinum–iridium wires (A-M Systems, Carlsborg, WA, USA). The synapses were recorded by stimulating the CA1 stratum radiatum with bipolar electrodes and placing a recording glass electrode (1–10 MΩ) filled with ACSF.

The fEPSPs were recorded using an Axoclamp 2-B amplifier (Axon Instruments, Foster City, CA, USA) connected to a computer. At the beginning of each record, an input–output (I/O) curve for the fEPSP amplitude relative to the stimulus intensity (increased in 50 μA in a stepwise manner ranging from 50 to 250 μA) was recorded until the saturation of the fEPSP amplitudes. This current intensity was adjusted to evoke a baselinefEPSP amplitude that was 50–60% of the maximal fEPSP amplitude obtained in the I/O curve. Baseline responses to 0.05

Hz paired-pulse stimuli (0.2 ms) were recorded for 20 min before long-term potentiation (LTP) induction. After the observation of a stable baseline-evoked response, the high-frequency stimulation (HFS) protocol was applied (four trains of 1 s duration at 100 Hz, pulse duration of 0.2 ms, with an intertrain interval of 20 s). Field potentials were monitored for at least 60min after the tetanic stimulus. Evoked fEPSPs were amplified and low-pass filtered at 600 Hz (Cyber Amp 320, Axon Instruments), digitized (Digidata 1322A, Axon Instruments) and recorded (Axoscope 9.2, Axon Instruments). The amplitude of the evoked synaptic response was measured by Clampfit 9.2 device (Axon Instruments). The recorded values were normalized on a per recording basis and then plotted as the mean of 2 min (6fEPSPS) ± S.E.M. of one to three slices each rat in five rats per group.

After the recording of a stable baseline during 10 min, the slices were than perfused with MP-ACSF (3, 30 or 300µM) at 2 ml/min for 10min. After the 10 min perfusion the LTP was induced by repeated HFS (4 x 100 Hz for 1 s) in the hippocampus and the responses were recorded subsequently for 60 min.

Whole cell patch clamp recordings

Primary hippocampal neurons

Cells were perfused at2 $mL \cdot min^{-1}$ with a (HBS), pH was adjusted to 7.4 andosmolarity adjusted to 310 mOsm with sucrose ifrequired. Current clamp recordings were madeusing whole cell patch clamp in current clamp modewith glass pipettes (4–6 MW) filled with a internalsolution containing (in mM): KCl 150, $MgCl_2$ 1,$CaCl_2$ 1, HEPES 10, EGTA 0.5, Mg-ATP 3, GTP 0.3,pH was adjusted to 7.2 and osmolarity was adjustedto 290 mOsm using sucrose if required. Data wereacquired with an Axopatch-200B amplifier (MolecularDevices, Sunnyvale, CA, USA) and usingClampex software (MolecularDevices, Sunnyvale, CA, USA).

No capacitance and series resistancecompensation were applied. JBU wasadded via the perfusate, for 5 min followinga 5 min steady baseline period and then washed out.

Acute rat hippocampal slices

Slices were transferredto a submerged recording chamber continually perfused Withthe same HBS used for cells oxygenated at room temperature. Data were acquired with an EPC 8 amplifier (HEKA Elektronik, Lambrecht, GE) and recorded and analyzed using Patch Master software (HEKA Elektronik, Lambrecht, GE). No capacitance and series resistance compensation were applied. JBU was added via the perfusate, for 5 min following a 5 min steady baseline period and then washed out.

Statistical analysis

All data are expressed as mean ±SEM. Data werecompared by paired or unpaired Student's t-tests, orone-way analysis of variance with Tukey's comparisonas appropriate with $P< 0.05$ consideredsignificant.

3. Results

3.1. Increase of calcium influx by Jack Bean Urease (JBU) at rat primary hippocampal cultures

The perfusion of hippocampal cultures with different concentrations of JBU (0.001-1µM) induced an inverted pharmacological rise in the $[Ca^{2+}]_i$. Thus, withJBU (1µM) there was an increase in the $[Ca^{2+}]_i$of 350±20% (n=90 cells, $p<0.05$)Fig. 1A. With the lowest concentration of JBU, there was a maximum increase in the $[Ca^{2+}]_i$650±50% (n=90 cells, $p<0.05$)Fig. 1A. In this set of experiments, the individual analysis of the cells showed that only

some specific neurons were activated, as indicated by the KCl (5mM) perfusion Fig. 2A-D and Fig3.

3.2. Jack Bean Urease does not affect mice hippocampal cell viability

The effect of different concentrations of JBU (0.001-1µM) were evaluated on mice hippocampalslices by theMTT cell viability assay.Inthissetofexperimentsthe incubation of the tissues with $H_2O_2$44mM reduced in 80% de cell viability n=6, $p<0.05$). However, none of the concentrations assayed of JBU were able to alter significantly the cell viability of hippocampus when compared to the control hepes-saline (Fig.4).

3.3. Electrophysiology

3.3.1. Effect of JBU on extracellular field excitatory postsynaptic potentials (fEPSP)

In the next set of experiments we investigated the effect of two concentrations of JBU on LTP evoked by electrical stimulation (Fig. 5). Normalized field responses in the *stratum radiatum* were likewise increased after high frequency stimulation but differently for each concentration of JBU. In presence of a very low concentration (0.1 µM) JBU LTP was significantly impaired (Fig. 5A). When(1 µM) JBU was perfused the inhibitory effect of the drug on LTP, although rather small, was already visible in the facilitation of the fEPSP response followed by their gradual increased after 40 min the high-frequency stimulus train (Fig. 5A, open black circle). Also, between 42-54 minutes after posttetanic response we found significant difference in the magnitude of LTP in the JBU groups (JBU 1 µM: 162.7 ± 0.45 mV; JBU 0.1 µM: 159.8 ± 0.50; $p<0.01$) compared to controls (185.4 ± 0.34 mV)n = 6 respectively. There was also a significant difference in LTP magnitude between JBU groups ($p<0.05$; Fig.5B).

3.3.2. Patch clamp recordings

Increase of action potential (APs) and excitatory postsynaptic currents (EPSCs) firing at hippocampal cultured neuronsand slices

At cultured hippocampal cells the control parameters of spontaneous frequency of action potentials (APs) and excitatory postsynaptic currents (EPSCs) for a holding potential of -65mV were 100±15/min and 100±25/min, respectively in 5min (n=9). The perfusion of JBU (0.1µM)on hippocampal cells for 5min induced a maximum increase in the frequency of both APsand EPSCs to 300±100/min and 150±50/min, respectively (n=9, $p<0.05$ each) Fig.6A, B and C. During the onset of JBU activity there was the appearance of spontaneous action potentials in the EPSCs recordings. The washout of the preparations reduced the frequency of both APs and EPSCs, but that remained above to the control values (Fig.6.). The evoke of Hippo/IV curves of hippocampal cells by applying -100 to +30mV voltage-steps showed that during the onset of JBU (0.1µM)activity there was a significative increase in amplitude of the sodium currents (Fig.3B). In this set of protocols the values for control parameters were 1000±20pA and 1300±50pA for JBU-treated cells (n=9, $p<0.05$) Figure 6. There was no change in the potassium current amplitude.

Wenextexaminedtheelectrophysiologicalpropertiesof JBU on rat hippocampal slices. The recordings were carried out at CA1 area with a holding potential of -65mV. At control conditions the neurons exhibited an AP frequency of 67±13.min^{-1} (n=4) Fig.7. During the onset of the 5min JBU (0.1µM) perfusion there was a maximum increase in thefiring of APs to 127±13.min^{-1} (n=4, $P<0.05$) Figure 7A. The 10min washout of the preparation did not reverse completely the increase of frequency.

4. Discussion

In this work we have shown that the application of the urease of *Canavaliaensiformis* at hippocampal cell cultures induce excitatory activities characterized by an increase of both calcium influx and action potential firing rates. We have demonstrated that this calcium-

dependent activity must be associated to an activation of sodium currents at hippocampal neurons. In order to explore in further detail the effects of JBU at pre- and post-synaptic levels, we performed a series ofexperiments using calcium images, extracellular electrophysiology and patch clamp recordings at rat primaryhippocampal cultures and brain slices. The results related to this investigation will be discussed therein.

The voltage-dependentactivation of Na^+channels which leads togeneration of action potentials is due to cell depolarizationphysiologically produced by the EPSP. The depolarizing effectsof EPSPs leading to generation of action potentials are alsomimicked by injecting positive current steps through therecording electrode. Low concentrations of JBU (0.1 µM) increased the probability of firing, showed by the increase of EPSP frequency. Furthermore, EPSPs of a larger amplitude were seen in the presence of JBU.Consistent with these findings, JBUdecreased the thresholdand the latency of firing action potentials when theneurones were treated with this toxin. Together with the depolarization observed during the recordings, this later observation suggests that JBU affects the mechanism(s) of action potential generation andpoints to the voltage-operated Na^+ channels as one of its molecular targets.

Previous experiments with the use of tetrodotoxin (Hu etal., 1992) andfelbamate (Pugliese et al., 1996) havesuggested that CA1 pyramidal cells express Na^+channels which aredifferentially able to respond to drugs. Both compoundsdecreased synaptically-driven cell firing without changing theaction potential elicited by direct neuronal activation,suggesting that they were affecting subpopulations of Na^+channels.

Our prelimarydata agreed with those obtained in hippocampal cell cultures treated with veratridine. Propagation of action potentials in axons and dendritesincreases intracellular Na$[Na^+]_i$and Ca^{2+} concentrations$[Ca^{2+}]$i. Veratridine also increase glutamate quantal release, what in its turn, also agreed with the appearance of giant EPSCs observed in our experimental conditions (Bouron and Reuter, 1996).

Acknowledgements

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Figures Legends

Figure 1. Increase ofcalcium influx induced by JBU at hippocampal cell cultures. In the graph each bar represents the mean ± S.E.M. of the maximum $[Ca^{2+}]_i$during theonset of JBU activity (n=90 cells). Note that JBU increases cellular $[Ca^{2+}]_i$in an inverted manner (*p<0.01, n=90 cells). *significance at P<0.01.

Figure 2. Increase of $[Ca^{2+}]_i$ following application of jack bean urease (JBU). (A) Representative pictureof control HEPESat resting $[Ca^{2+}]_i$conditions. (B) Representative image of the onset activity of JBU (0.001µM) eliciting spike increases in $[Ca^{2+}]_i$. The arrows indicate cells activated by JBU. (C) Responses of calcium riseduring perfusion of HEPES-Saline buffer with extraKCl (5 mM). The arrows indicate the activated cells. (D) The graph illustrates the kinetics of $[Ca^{2+}]_i$before and after perfusion of JBU (0.001µM). Note that the application of JBU elicits transitory spikes of calcium influx.

Figure 3. Increase of calcium influx in hippocampal neural cells loaded with FLUO-4 AM after treatment with Jack Bean Urease (JBU). The panels shows representative pseudocolour images of calculated calcium influx at resting state of control HEPES and during the onset of JBU (0.1µM) and HEPES-Saline with extra K^+ (5mM). Note that JBU induces a significative and localized increase in calcium influx, when compared to high K^+.

Figure 4. Absence ofactivity of Jack Bean Urease (JBU) on cell viability of mice hippocampal slices. The tissues were incubated with different concentrations of JBU during 90 min.

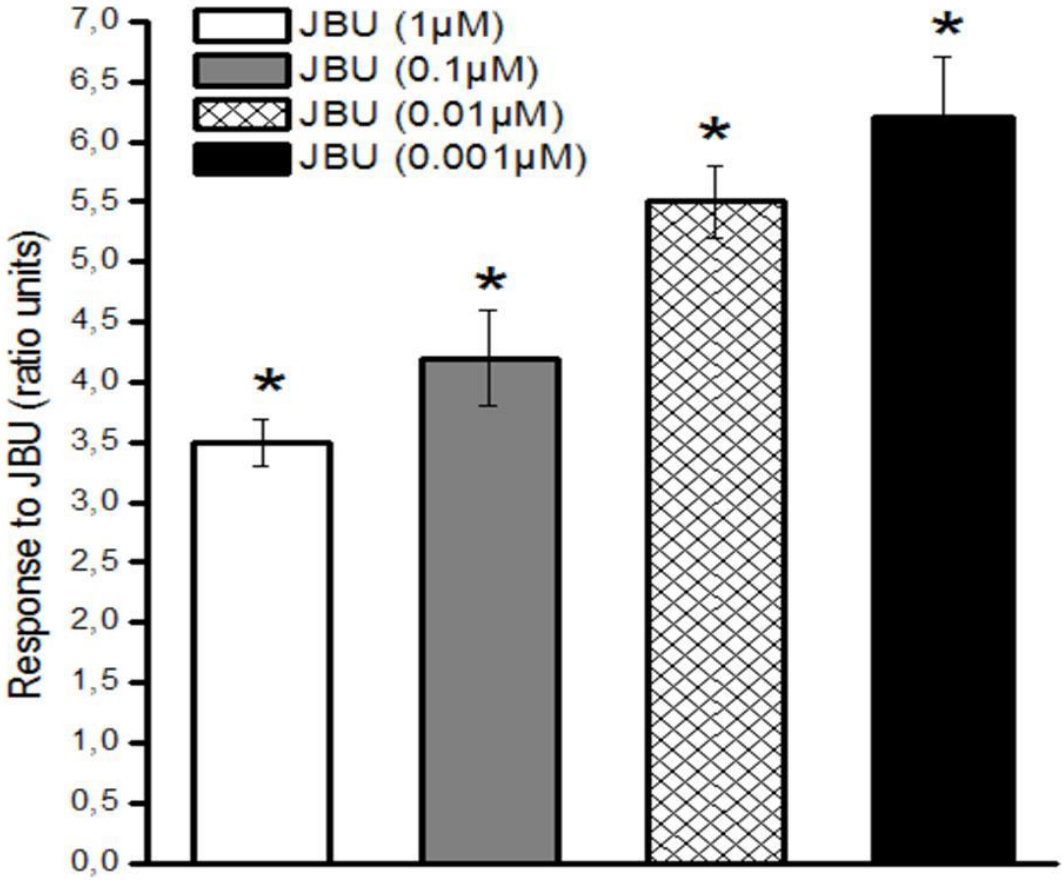
Figure5. Effect of JBU activity on the long-term potentiation in hippocampal CA3-to-CA1 network induced by 4x100 Hz stimulus train. (A) A high-frequency stimulation (HFS) protocol was applied at 0 min (arrow) to hippocampal slice from control (solid black circle), JBU 1 µM (squares symbols) or JBU 0.1 µM treated slices (open circles symbols) (n = 6 each). (B) Each bar represent the mean ± S.E.M. of the percentage of normalized amplitude of fPSPs before (Control) and after 54min JBU 0.1µM perfusion. The graphic shows that variation of amplitude fEPSP significantly decreases42-54min after HFS in slices treated with JBU. All values are represented as mean ± SEM. (***$p < 0.01$); # $p<0.05$ related to JBU 1µM.

Figure 6. Excitatory activity induced by JBU on rat hippocampal cells. Panel (A) shows the increase in firing of action potentials during JBU (0.1µM) perfusion of hipocampal cells (n=8, $p<0.05$). The black bar indicates the time of JBU perfusion on cells. Panel (B) shows the increase of sodium currents recorded during the perfusion with JBU (0.1 µM) ($p<0.05$, n=8). Panel (C) and (D) show representative recordings of spontaneous action potentials and excitatory postsynaptic currents under -65 mV holding potentials, respectively. •controlHEPES conditions ; ♦ JBU-treated preparation. Note the increase of frequency of both Aps and EPSCs in presence of JBU (0.1 µM). * significance at $p<0.05$.

Figure 7. Increase of action potentials (APs) firing in rat brain slices by perfusion of Jack Bean Urease (JBU). Panel A, shows the mean frequency of APs of control HEPES and during the onset of JBU. In the graph each bar represents the mean ± S.E.M. of 4 experiments. Note that JBU induces a significative ($p<0.05$) increase of AP frequency after 2 min perfusion. Panel B show a representative image of the brain slices under whole cell recordings.

Figures

Figure 1.



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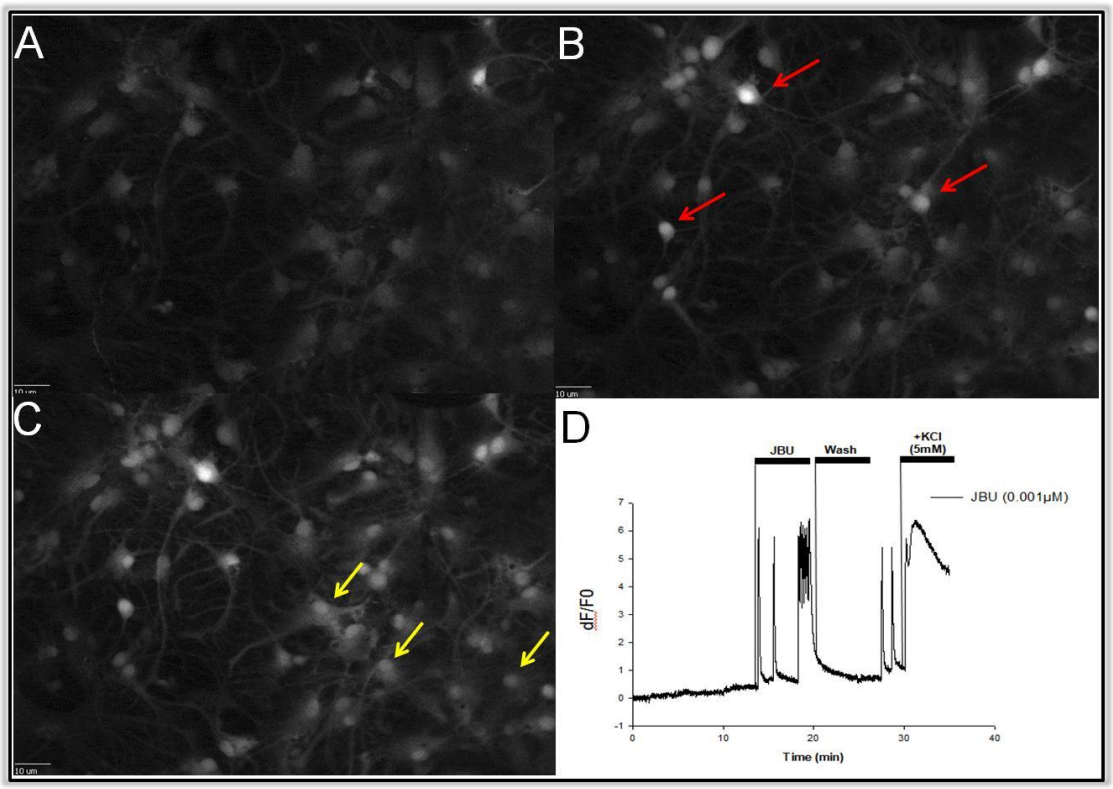

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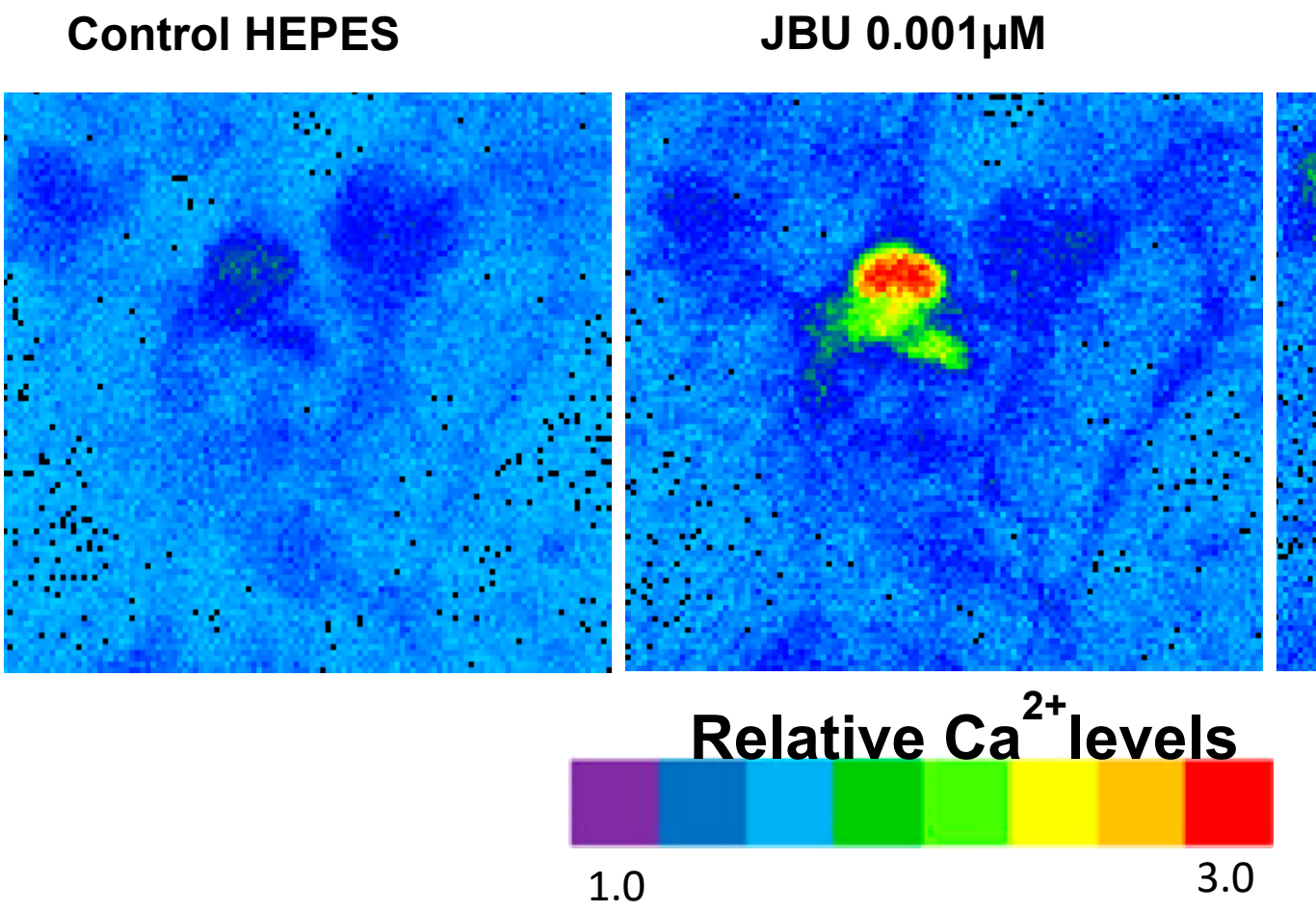

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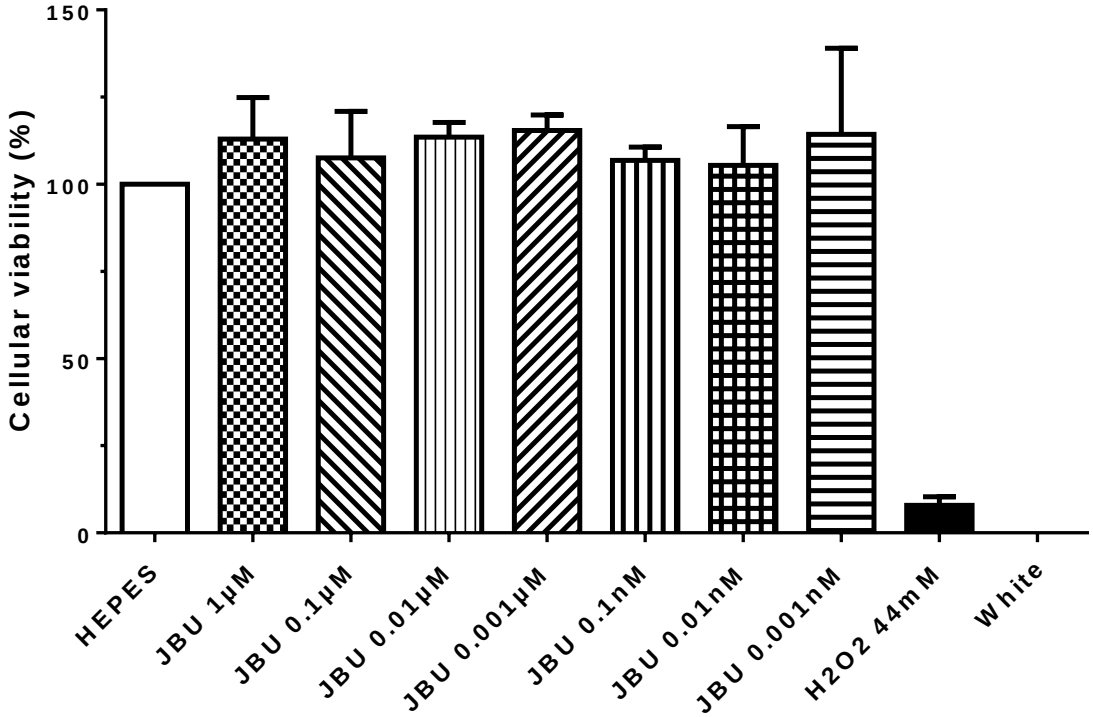
Cellular viability (%)
150
100
50
0
HEPES
JBU 1µM
JBU 0.1µM
JBU 0.01µM
JBU 0.001µM
JBU 0.1nM
JBU 0.01nM
JBU 0.001nM
H2O2 44mM
White

Figure 5.

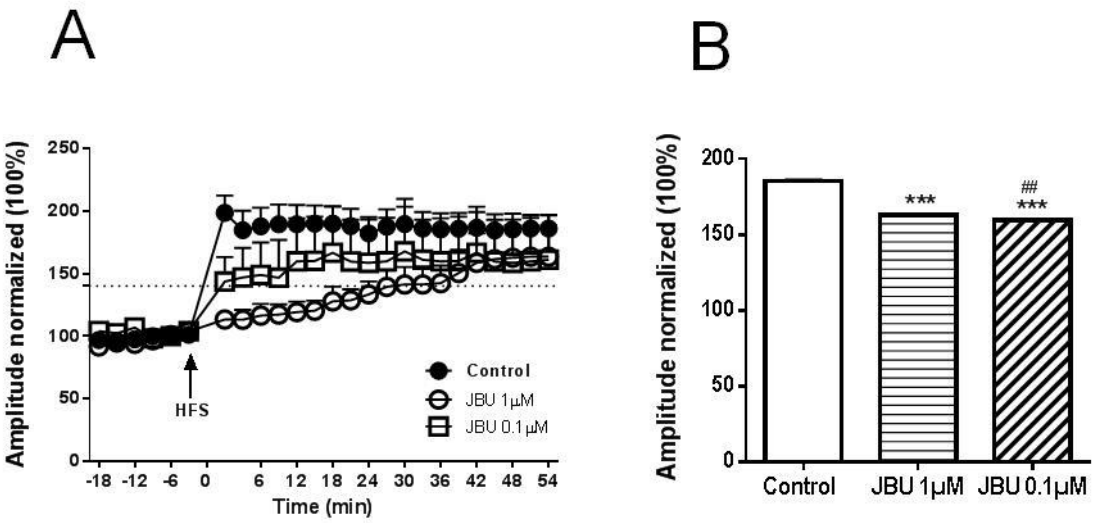
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Figure 6.

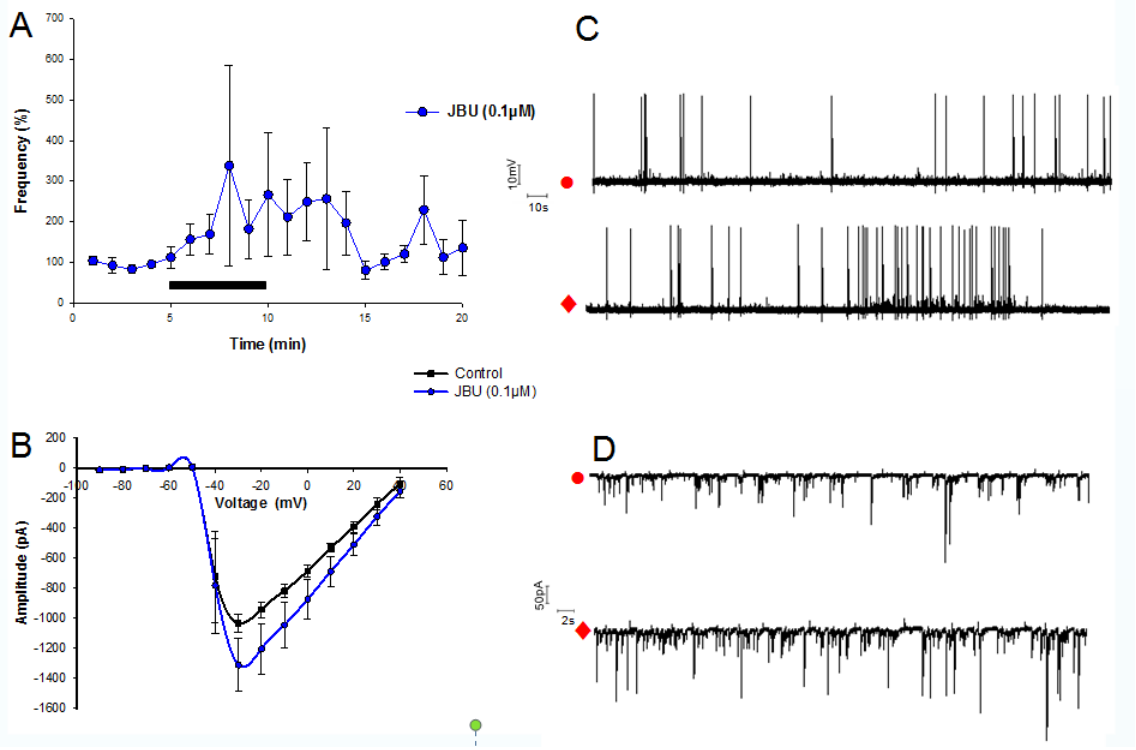
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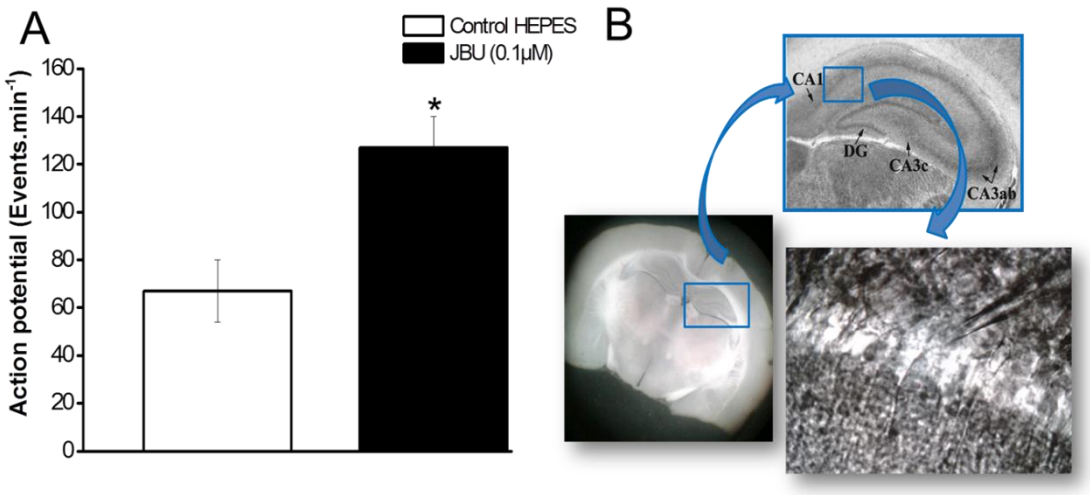
Figure 7.



ANEXO 3

Formulário do Comitê de Ética no Uso de Animais autorizando a realização dos experimentos desse trabalho.

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **043/2015**

Título: Ureases como proteínas multifuncionais: Investigação eletrofisiológica do mecanismo de ação sobre o sistema nervoso central e periférico de murinos

Data da aprovação: **18/01/2016**

Período de vigência do projeto: De: **01/2016** Até: **01/2019**

Pesquisador: Chárist on André Dal Belo

Campus: **Uruguaiana**

Telefone: (55) 99627034

E-mail: charistonbelo@unipampa.edu.br

Marcelo Dal Pozzo
Médico Veterinário
Coordenadora Pro Tempore da CEUA/UNIPAMPA

ANEXO 4

Certificado de apresentação de trabalho no 23º SBBq.

SBBq | IUBMB 2015

We hereby certify that the abstract entitled

ANALYSIS OF THE NEUROTOXICITY OF JACK BEAN UREASE (JBU) IN RODENT MODELS

by authors: CARLOS GABRIEL MOREIRA DE ALMEIDA (UNIPAMPA,PUC-RS), RAQUEL SOARES OLIVEIRA (UNIPAMPA), LÚCIA HELENA DO CANTO VINADÉ (UNIPAMPA), DOUGLAS SILVA SANTOS (UNIPAMPA,UFSM), CÉLIA REGINA RIBEIRO DA SILVA CARLINI (PUC-RS,UFRGS), CHÁRISTON ANDRÉ DAL BELO (UNIPAMPA,PUC-RS,UFSM)

was presented during the 23rd International Congress of the IUBMB and 44th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (SBBq) in Foz do Iguaçu, Paraná, Brazil, on August 24th to 28th, 2015.

Glaucius Oliva
Chair of the 23rd IUBMB Congress

Jerson Lima Silva
President of SBBq

ANEXO 5

Trabalho apresentado no IST, em 2015.

INTERNATIONAL SOCIETY ON TOXINOLOGY

18th World Congress IST 2015 25-30 September OXFORD United Kingdom

Sfet

GLOBAL SNAKEBITE INITIATIVE

CERTIFICATE

The 18th World Congress of the International Society on Toxinology

25-30 September 2015, Oxford, UK

The scientific work entitled "**Neurotoxicity of Jack Bean Urease: Investigation of its convulsive-like activity**" by Chárіston A. Dal Belo, Carlos G. Moreira de Almeida, Douglas S. Santos, Louise Wilson, Paulo M. Pinto, Lúcia Vinadé, Jaderson C. DaCosta, Célia R. Carlini and Trevor J. Bushell was presented as **Poster** in this congress.

Managing Director
LibPubMedia Ltd
Oxford, United Kingdom

Dr M Sohail
Congress Administrator

Biochemistry Lecturer, St Hilda's College, Oxford & Managing Director, LibPubMedia Ltd, Oxford, United Kingdom

ANEXO 6

Trabalho apresentado no IBRO, em 2015.


IBRO 2015
9th WORLD CONGRESS
International Brain Research Organization
IBRO
July 7 - 11, 2015
Rio de Janeiro • Brazil
Certificate
We hereby certify that the scientific work entitled
NEUROTOXICITY INDUCED BY JACK BEAN UREASE (JBU) ON MAMMALIAN NERVOUS SYSTEM
with the authors
CHÁRISTON ANDRÉ DAL BELO; CARLOS GABRIEL MOREIRA DE ALMEIDA; RAFAEL STUANI FLORIANO; CLEITON LOPES AGUIAR; LUCIA HELENA VINADÉ; JOÃO PEREIRA LEITE; ALEXANDRE PINTO CORRADO; EDWARD G ROWAN; CÉLIA REGINA CARLINI
was presented as POSTER
in the 9th IBRO World Congress on Neuroscience.
Rio de Janeiro, July 11 , 2015
Roberto Lent
Local Organizing Committee Chair