UNIVERSIDADE ESTADUAL DE CAMPINAS

Catarina Rapôso Dias Carneiro

"AÇÃO CENTRAL DO VENENO DA ARANHA *Phoneutria nigriventer*: MODULAÇÃO AGUDA DA BARREIRA HEMATOENCEFÁLICA, ATIVAÇÃO NEURONAL E ENVOLVIMENTO DO ÓXIDO NÍTRICO"

Tese apresentada ao Instituto de Biologia, da Universidade Estadual de Campinas para obtenção do Título de Mestre em Biologia Celular e Estrutural na área de Histologia.

Orientadora: Profª Drª Maria Alice da Cruz-Höfling

Campinas/2006

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

C215a

Carneiro, Catarina Rapôso Dias
Ação central do veneno da aranha *Phoneutria nigriventer*: modulação aguda da barreira hematoencefálica, ativação neuronal e envolvimento do óxido nítrico / Catarina Rapôso Dias Carneiro. -- Campinas, SP: [s.n.], 2006.

Orientadora: Maria Alice da Cruz-Höfling.
Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Neurônios. 2. Cérebro. 3. Venenos de aranha. 4. Barreira hematoencefálica. 5. Cerebelo. I. Cruz-Höfling, Maria Alice da. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(scs/ib)

Título em inglês: Central action of *Phoneutria nigriventer* spider venom: acute modulation of the blood-brain barrier, neuronal activation and nitric oxide involvement.
Palavras-chave em inglês: Neurons; Brain; Spider venoms; Blood-brain barrier; Cerebellum.
Área de concentração: Histologia.
Titulação: Mestre em Biologia Celular e Estrutural.
Banca examinadora: Maria Alice da Cruz-Höfling, Alexandre Leite Rodrigues de Oliveira, Kátia Cristina Barbaro.
Data da defesa: 17/08/2006.
Programa de Pós-Graduação: Biologia Celular e Estrutural.

Campinas, 17 de Agosto de 2006

BANCA EXAMINADORA

Profª Drª Maria Alice da Cruz-Höfling (Orientadora) _____

Prof. Dr. Alexandre Leite Rodrigues de Oliveira _____

Profª Drª Kátia Cristina Barbaro _____

Profª Drª Elenice A. de Moraes Ferrari _____

Prof. Dr. Stephen Hyslop _____

Dedicatória

Ao esposo, Marcos André Lins de Melo,
pelo apoio e compreensão

Às filhas queridas, Raphaela e Maria Gabriela,
razão de tudo

À minha mãe, Maria do Socorro Brito Rapôso,
pelo esforço incondicional

Aos irmãos, Manuela e Thiago,
pela preciosa amizade

Ao Senhor Deus,
pois nada se realiza sem o Seu consentimento

Agradecimentos

À minha orientadora, Profª Drª Maria Alice da Cruz-Höfling, pelo profissionalismo e amizade e por me conduzir a desenvolver não apenas habilidades técnicas, mas a capacidade de solucionar problemas, interpretar resultados e tirar conclusões.

À técnica e amiga Marta Beatriz Leonardo, pelo precioso apoio.

Aos colegas de laboratório, pela sincera amizade e compreensão em todos os momentos.

À colega Gabriela Mariotoni Zago, pela rica parceria.

Ao professor Dr. Cláudio Toledo, pelo apoio e disponibilidade.

Aos Professores membros das bancas de análise prévia e de defesa, Prof. Dr. Alexandre Leite Rodrigues de Oliveira, Profª Drª Ione Salgado, Prof. Dr. Cláudio Toledo, Drª Kátia Cristina Barbaro, Prof. Dr. Stephen Hyslop, Profª Drª Elenice Ferrari, pelas valiosas sugestões e criteriosa correção da tese.

Aos Departamentos de Anatomia e Fisiologia, do Instituto de Biologia, UNICAMP, por disponibilizar sua infra-estrutura para a execução deste trabalho.

Ao Instituto Butantan, pela gentil doação do veneno.

Às agências de fomento, CAPES, CNPq, FAEP/UNICAMP e FAPESP, pelo suporte financeiro.

Resumo

Recentemente foi demonstrado em nosso laboratório que o veneno bruto da aranha *Phoneutria nigriventer* (PNV), Keyserling (1891) (Ctenidae, Araneomorphae), é capaz de alterar a permeabilidade da barreira hematoencefálica (BHE), quando injetado sistemicamente em ratos (observações feitas das 18 horas aos 9 dias). Afora a presença de edema vasogênico e de pés astrocitários edematosos, não foram detectadas alterações nos neurônios e células gliais, que indicassem a ação do veneno no interior do parênquima cerebral. O presente trabalho se propôs a avaliar se, em fases agudas do envenenamento, o PNV afeta a BHE e o tecido neural. Os parâmetros observados por microscopia eletrônica de transmissão (através do uso de traçador extracelular injetado na circulação e estudo quantitativo) e microscopia de luz (imunohistoquímica e estudo quantitativo) foram: alterações de permeabilidade vascular na microcirculação cerebral, alterações na expressão da laminina na membrana basal endotelial, ativação neuronal através da expressão da proteína c-FOS, modulação da síntese de óxido nítrico, através da expressão da óxido nítrico sintase neuronal (nNOS). Ratos Wistar machos, jovens (200-300 g) foram injetados pela veia da caudal com solução salina estéril (0.9%), ou com veneno (850 μg/Kg). Para análise da integridade da BHE, após 15 min, 1, 2 e 5 h, cerebelo e hipocampo foram coletados. Os resultados mostraram que o PNV provocou extravasamento significativo do traçador nitrato de lantano a partir de 1 h após a injeção (p.i.) e teve o seu maior pico em 2 h p.i. ($p< 0,05$). O cerebelo mostrou-se bem mais resistente ao aumento de permeabilidade vascular do que o hipocampo. Por outro lado, freqüentemente observou-se entumecimento dos pés-vasculares astrocitários no cerebelo, mesmo em vasos sem extravazamento do traçador, o que não ocorreu no hipocampo. Houve desaparecimento total da expressão da laminina dos capilares já aos 15 min p.i., seguida por recuperação gradativa, até que às 2 h p.i., sua expressão assemelha-se à mostrada pelos controles e 5 h p.i., a expressão dessa proteína estava acima da fisiológica, na membrana basal. A ativação de neurônios, avaliada às 2 h p.i. pela expressão da c-FOS, ocorreu em áreas relacionadas com atividade motora (substância cinzenta periaqueductal – partes ventral e dorso lateral, córtex frontal e parietal e núcleo talâmico periventricular) e em áreas envolvidas com stress agudo (córtex rinal e

núcleo septal lateral) ($p< 0,05$). Todas as áreas com significativo aumento de neurônios imunoreativos, também apresentaram neurônios positivos para nNOS, porém apenas a substância cinzenta periaqueductal parte dorsolateral, o córtex parietal e o núcleo talâmico periventricular apresentaram aumento no número de neurônios marcados para essa enzima. Nós concluímos que o veneno de *P. nigriventer* possui componentes neurotóxicos capazes de alterar a permeabilidade da BHE em tempos agudos após a intoxicação, cujo mecanismo precisa ser esclarecido. O veneno pode ser uma ferramenta importante para analisar as respostas dos componentes estruturais da BHE, bem como mapear as áreas cerebrais reativas, possibilitando esclarecer as vias do SNC envolvidas no envenenamento por *Phoneutria*. O conhecimento de vias relacionadas com neurotoxinas específicas do veneno pode ser útil para estudos relacionados com estrutura-função.

Abstract

It was recently demonstrated in our laboratory that the *Phoneutria nigriventer* spider venom (PNV), Keyserling (1891) (Ctenidae, Araneomorphae), is capable of altering the permeability of the blood brain barrier (BBB) when injected systemically in rats (observations done from 18 hours to 9 days). Apart from the presence of vasogenic edema and swollen atrocytic end-feet processes, no neuronal or glial cells alterations were detected, which would indicate the action of the venom within the cerebral parenchyma.The present work, intended to evaluate if at acute phases of the envenoming, the PNV affects the BBB. The parameters observed by transmission electron microscopy (by the use of electron opaque extracellular tracer injected in the circulation, and quantitative study) and by light microscopy (using immunohistochemistry and quantitative study) were: alterations of vascular permeability in the cerebral microcirculation, alterations of the expression of laminin at the endothelial basement membrane, neuronal activation seen through the expression of c-FOS protein, modulation of nitric oxide synthesis, trough the expression of the neuronal nitric oxide synthase (nNOS). Young male Wistar rats (200-300 g) were injected by the tail vein with sterile saline solution (0.9%), or with venom (850 μg/Kg). For BBB integrity analysis, after 15 min, 1, 2 and 5 h, cerebellum and hippocampus were collected. The results showed that the PNV provoked significant extravasation of $LaNO_3$ tracer at 1 h post injection (p.i.) which peaked at 2 h p.i. ($p< 0.05$). The cerebellum showed

to be more resistant to prevent vascular permeability increase than hippocampus. On the other hand, swollen vascular end-feet processes were more frequently seen in the cerebellum, even in vessels without vascular extravasation, what was not seen in hippocampus. There was complete vanishment of laminin immunolabeling of capillaries soon at 15 min p.i., followed by a gradual restoration to control levels at 2 h p.i. At 5 h p.i., the expression of the protein was upregulated in the basement membrane, compared to the physiologic control. The neuronal activation, evaluated through the expression of c-FOS at 2 h p.i., occurred at motor-related areas (periaqueductal gray matter pars ventral and dorsolateral, frontal and parietal cortex and paraventricular thalamic nuclei), and with acute stress-related areas (rhinal cortex and septal lateral nuclei) ($p < 0.05$). All cerebral areas with significant increase of c-FOS immunoreactive neurons, also exhibited nNOS-positive neurons, however only the pars ventral and dorsolateral of the periaqueductal gray matter, frontal and parietal cortex and paraventricular thalamic nuclei areas presented increase in the labeled-cells number. We conclude that *P. nigriventer* venom possesses neurotoxic components capable of altering the BBB permeability at acute stages of intoxication, whose mechanism needs clarification. The venom can be an important tool to analyse the responses of structural components of BBB, and to map the reactive cerebral areas, therefore shedding light to pathways involved with *Phoneutria* envenoming in CNS. The knowledge of the pathways related to specific venom containing neurotoxins may be useful for structure-function-related studies.

Sumário

II. INTRODUÇÃO

As aranhas pertencem à ordem Aranea, classe Arachnida. São animais essencialmente terrestres, com exceção de certas espécies que posteriormente adaptaram-se à vida aquática. Mais de 30.000 espécies foram identificadas no mundo. As aranhas variam de tamanho, de espécies muito pequenas, menores que 0,5 mm de comprimento até grandes tamanhos, como é o caso da caranguejeira, que atinge o tamanho de até 25-35 cm de comprimento, e da aranha armadeira, que em posição de ataque atinge o tamanho de 15 cm.

Geralmente, as aranhas possuem um importante papel como inimigos naturais de insetos considerados pestes. Como outros aracnídeos, as aranhas são predadoras e se alimentam em grande escala de insetos. Em geral, e felizmente para os seres humanos, os pares de presas de muitas aranhas são muito curtos ou fracos para penetrar na pele humana e, por isso, seu veneno pode ser quase inócuo. Entretanto, muitas aranhas podem produzir lesões de variável severidade no local da picada e, em uma série delas, podem causar sintomas generalizados, eventualmente levando à morte. Russel & Gertsch (1974) declararam que pelo menos 50 espécies de aranhas nos Estados Unidos estão implicadas nos acidentes com seres humanos. Ori (1977) também relatou casos de envenenamento por 10 diferentes espécies de aranhas no Japão.

No Brasil, os gêneros *Loxosceles* e *Phoneutria* são responsáveis pelos acidentes mais graves causados por aranhas venenosas. Os acidentes com aranhas do gênero *Phoneutria* são comuns na região de Campinas, embora os acidentes graves sejam raros, sendo observados em somente 0,5% do grupo estudado por Bucaretchi *et al.* (2000). Esses autores observaram ainda que 89,8% dos acidentes, naquela população, foram classificados como leves, 8,5% como moderados e 1,2% como assintomáticos, constituindo grupos de risco crianças com menos de 10 anos de idade e pacientes idosos (> 70 anos).

2.1. *Phoneutria nigriventer*

Aranhas que pertencem ao gênero *Phoneutria* fazem parte da rica biodiversidade da fauna que pode ser encontrada no Brasil. *Phoneutria nigriventer,* uma agressiva aranha sul americana, conhecida como aranha "armadeira" ou "errante", é responsável pela maioria dos acidentes com humanos por araneísmo, incluindo a morte de crianças, no Estado de São Paulo (Bucaretchi *et al.*, 2000).

Figura 1: *Phoneutria nigriventer* em posição de ataque. Por isso é conhecida como aranha "armadeira". www.washington.edu

O envenenamento por *P. nigriventer* é um importante problema de saúde pública, uma vez que é uma das principais aranhas responsáveis por acidentes por araneísmo no Brasil. Além disso, embora a maioria dos casos seja leve, dependendo da gravidade do acidente, as vítimas de picadas da aranha armadeira podem apresentar diversos sinais e sintomas clínicos, que incluem alterações cardiovasculares e neurológicas, hipertensão arterial, taquicardia, arritmia e, em casos considerados severos, distúrbios visuais e convulsões tônicas. Outras manifestações que geralmente ocorrem são dor e inchação locais, eritema, sudorese, náusea, vômitos, salivação, diarréia e priapismo. Em raros casos pode haver choque e edema pulmonar (Brazil & Vellard, 1925,1926; Bucaretchi *et al.,* 2000; Chávez- Olórtegui *et al.*, 2001).

2.2. Veneno de *P. nigriventer* (PNV)

2.2.1. Composição e neurotoxicidade

Os venenos dos aracnídeos pertencem a um grande grupo de substâncias tóxicas naturais, do qual os venenos de serpentes são os mais profundamente explorados. Só mais recentemente, a atenção de bioquímicos, farmacologistas, fisiologistas, patologistas, dentre outros, tem-se voltado ao aprofundamento do estudo dos venenos de artrópodos, incluindo as aranhas, por constatarem que, pelos sinais clínicos que produzem, podem ser potentes ferramentas de cunho investigativo. As substâncias naturais, de origem animal ou vegetal possuem considerável interesse farmacológico. Pelo fato de possuírem ações bastante específicas sobre as vítimas, ou em modelos experimentais, são úteis na compreensão dos mecanismos de várias funções fisiológicas ou alterações patofisiológicas do organismo. No caso das aranhas, como seu primeiro propósito é paralisar a presa, seu veneno possui uma variedade de toxinas com atividade neurotóxica (Rash & Hodgson, 2002).

O veneno de *P. nigriventer* (PNV) contém uma extensa variedade de proteínas e peptídeos, incluindo neurotoxinas que agem em canais iônicos e receptores químicos do sistema neuro-muscular de insetos e mamíferos. Esse veneno tem sido descrito como uma importante ferramenta para futuras descobertas e desenvolvimento de novas moléculas biologicamente ativas com potencial de aplicação em medicina e agricultura (Escoubas *et al.,* 2000; Gomez *et al.,* 2002; Rash & Hodgson, 2002).

Os primeiros estudos bioquímicos revelaram que o PNV possuía potentes neurotoxinas com ação excitatória, como salivação, lacrimação, priapismo, paralisia flácida e espástica dos membros anteriores e posteriores e morte, após injeção intracerebral em camundongos (Diniz, 1963; Schenberg & Pereira Lima, 1971; Entwhistle *et al.,* 1982). Subseqüentemente, três grupos de frações neurotóxicas (Phtx1, Phtx2 e Phtx3) e uma fração não tóxica, com atividade em músculo liso, foram purificados do veneno (Rezende *et al.,* 1991). Mais tarde, uma quarta fração (Phtx4) foi isolada, a qual foi extremamente tóxica em insetos da ordem Díptera e Dictióptera, mas com fracos efeitos tóxicos em camundongos (Figueiredo *et al.,* 1995).

Dessas frações, a seqüência completa de aminoácidos foi determinada para a toxina Tx1 (Diniz *et al.,* 1990), para quatro das toxinas Tx2 (Cordeiro *et al.,* 1992), para seis das toxinas Tx3 (Cordeiro *et al.,* 1993), para duas da fração não tóxica ativa em músculo liso (Cordeiro *et al.,* 1995) e para três das toxinas da fração inseticida Tx4 (Figueiredo *et al*., 1995, 2001; Oliveira *et al.,* 2003). A estrutura primária da maioria dessas moléculas foi posteriormente confirmada (Diniz *et al.,* 1993; Kalapothakis *et al.,* 1998 a, b; Kushmerick *et al.,* 1999; Penaforte *et al.,* 2000; Matavel *et al.,* 2002).

2.2.2. Ação conhecida do PNV no SNC

Estudos farmacológicos e eletrofisiológicos dos peptídeos purificados do veneno de *P. nigriventer* têm revelado que Tx1 age em canais de Ca^{2+} (Santos *et al.,* 1999), toxinas do tipo Tx2 afetam canais de Na^{+} (Araújo *et al*., 1993 a, b; Matavel *et al*., 2002), o grupo Tx3 age em canais de K^{+} ou Ca^{2+} (Troncone *et al*., 1995; Prado *et al*., 1996; Guatimosim *et al*., 1997; Leão *et al*., 2000; Miranda *et al*., 1998; Cassola *et al*., 1998; Kushmerick *et al*., 1999; Gomez *et al*., 2002; Santos *et al*., 2002; Vieira *et al*., 2003; Carneiro *et al*., 2003). A toxina inseticida Tx4 (6-1) estimula a liberação de glutamato das junções neuromusculares em barata (Figueiredo *et al*., 1997) e inativa a corrente de Na^{+} em SNC de insetos, porém não em canais de Na^{+} de mamíferos (De Lima *et al*., 2002). Yonamine *et al*. (2004) sugerem o envolvimento do óxido nítrico na intoxicação pela fração Tx2-5, em camundongos.

Apesar da sua aparente falta de toxicidade em mamíferos, a classe de toxinas inseticidas PhTx4 mostrou-se capaz de inibir a liberação de glutamato em sinapses cerebrais (Mafra *et al*., 1999). Igualmente, a fração Tx4 (5-5) foi capaz de inibir seletivamente e reversivelmente o subtipo de receptores de glutamato, o N-metil-D-aspartato (NMDA) em neurônios hipocampais de rato (Figueiredo *et al*., 2001).

A quantidade de neurotoxinas constituintes do veneno da aranha *P. nigriventer*, torna relevante, do ponto de vista médico, a investigação de sua ação central, bem como aponta o veneno como importante ferramenta para estudar a barreira sangue-cérebro.

2.3. Barreira Hematoencefálica (BHE)

2.3.1 Considerações gerais e importância

Todos os animais superiores que apresentam complexidade de organização do sistema nervoso central requerem uma barreira hemato-encefálica (BHE). A estrutura da BHE é, em primeira instância, proporcionada pela organização e propriedades das células endoteliais cerebrais, porém outros componentes estruturais são essenciais para a manutenção de sua integridade.

O cérebro é separado do contato direto com sangue e fluidos pela presença de duas barreiras: uma delas é a barreira entre o tecido nervoso e o fluido cerebroespinhal. Essa primeira barreira é composta pelo plexo coróide, células ependimárias, membrana aracnóide e órgãos circumventriculares. A função dessa barreira é proporcionada por zônulas de oclusão e de aderência presentes entre as células das estruturas mencionadas, as quais se encontram em contato direto com o fluido cerebroespinhal e impedem o transporte paracelular, direcionando o transporte entre tecido nervoso e ventrículos através da via transcelular, com controle da bomba de sódio, que ativamente mantém as diferenças na composição entre o tecido e o fluido cerebroespinhal. A segunda barreira é aquela entre o sangue e o tecido cerebral, denominada barreira hematoencefálica (BHE) (para revisão, ver Ballabh *et al.,* 2004) (Figura 2). A BHE permite a criação de um ambiente fluido extracelular único no SNC, cuja composição pode como conseqüência ser precisamente controlada e é diferente do ambiente de qualquer outra região do organismo (Consultar Begley, 2004, para revisão).

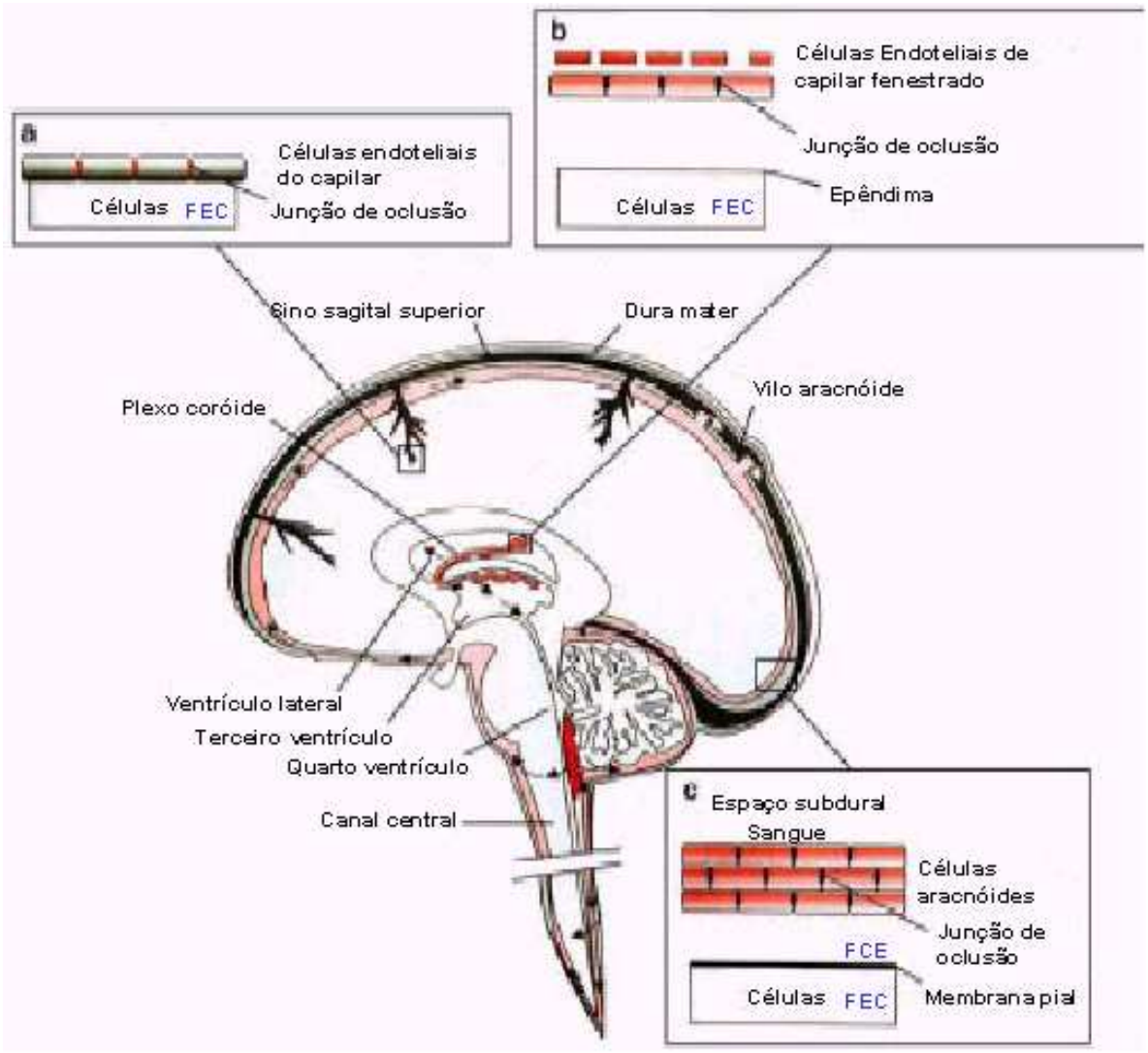

Figura 2. A BHE é formada pelas junções de oclusão entre as células endoteliais dos capilares cerebrais (**a**), células epiteliais dos plexo coróide (**b**) e células epiteliais da membrana aracnóide (**c**). **FEC** = fluido extracelular, **FCE** = fluido cerebroespinhal. Modificado de Ballabh *et al.* (2004).

A barreira hematoencefálica é uma barreira de difusão essencial para o funcionamento normal e manutenção da homeostase no SNC. Na BHE, as células endoteliais da microcirculação cerebral diferem daquelas do resto do corpo pela ausência de fenestrações e pela presença de zônulas de oclusão e adesão mais extensas e em maior número, aliado a um parcimonioso transporte vesicular pinocitótico. As zônulas de oclusão, com junções "tight" entre as células endoteliais limitam o fluxo paracelular de moléculas hidrofílicas na interface sangue-cérebro. Em contraste, pequenas substâncias lipofílicas como O_2 e CO_2 difundem-se livremente através das membranas plasmáticas a favor de um

gradiente de concentração (Grieb *et al.,* 1985). Nutrientes, incluindo glicose e aminoácidos entram no cérebro via transportadores, enquanto a endocitose mediada por receptor faz o aporte de grandes moléculas, incluindo insulina, leptina e transferrina (Pardridge *et al.,* 1985; Zhang & Pardridge, 2001). Adicionalmente às células endoteliais, a BHE é, em termos físicos, composta pela membrana basal dos capilares, pelos pés vasculares dos prolongamentos astrocitários, que envolvem os vasos, e pelos pericitos embutidos na membrana basal endotelial (Figura 3). Os pericitos são um componente celular pouco estudado da BHE, mas parecem exercer um papel chave na angiogênese, integridade estrutural e diferenciação de vasos e formação das junções de oclusão endoteliais (Allt & Lawrenson, 2001; Balabanov & Dore-Duffy, 1998; Bandopadhyay *et al.,* 2001)

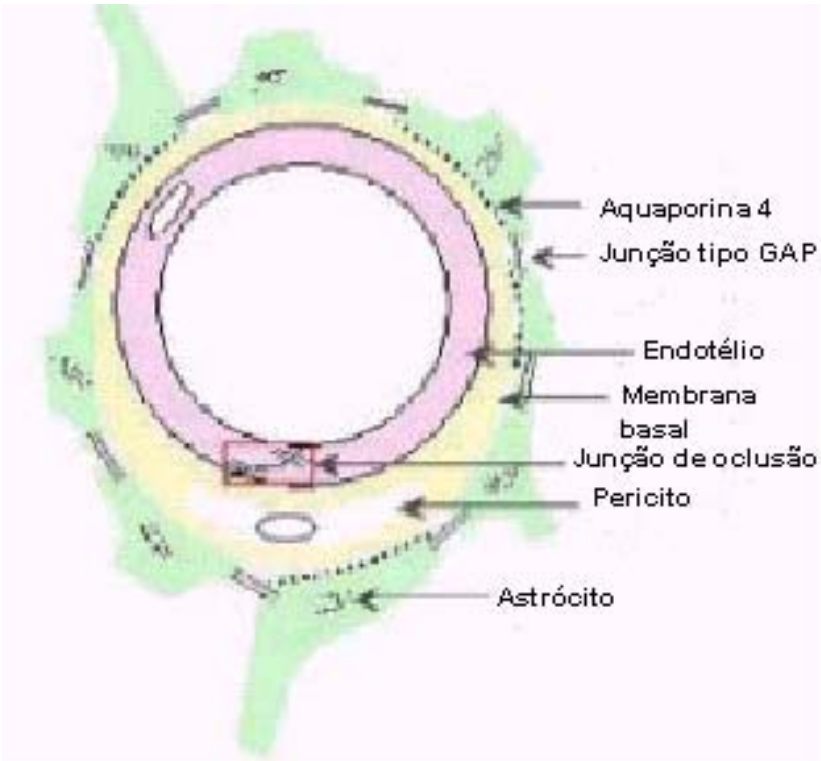

Figura 3. Esquema da BHE em secção transversa mostrando endotélio com junções de oclusão e junções comunicantes (tipo gap), membrana basal, pericitos e astrócitos. Modificado de Ballabh *et al.,* 2004.

Além da estrutura física, a BHE é também formada por vários sistemas de transportadores expressos na membrana das células endoteliais (consultar Ballabh *et al,* 2004; Luurtsema *et al,* 2004; Ohtsuki, 2004, para revisão). Acredita-se que todos os componentes da BHE são essenciais para a função normal e estabilidade da mesma.

O principal propósito da BHE é proteger, de forma dinâmica, o SNC de flutuações composicionais nas concentrações eletrolíticas e de metabólitos que ocorrem no sangue, mantendo a homeostase no SNC. A homeostasia cerebral é essencial para seu funcionamento e para o funcionamento do organismo como um todo. Outra importante função da BHE é proteger o SNC contra xenobióticos tóxicos. As moléculas precisam passar através das membranas das células endoteliais cerebrais para ter acesso ao tecido. As moléculas lipofílicas atravessam as membranas por difusão, no entanto as moléculas hidrofílicas precisam se ligar a proteínas transportadoras transmembrana específicas (via transcelular) (ver Luurtsema *et al,* 2004 para revisão).

Portanto, a BHE exerce essencialmente duas funções no SNC: a) possibilita a criação de um compartimento fluido extracelular intracerebral extremamente estável, cuja composição pode ser mantida distinta da do fluido extracelular somático. No interior desse compartimento protegido, a composição do fluido extracelular é regulada de forma precisa em termos de concentração de solutos. Essa estabilidade é essencial no SNC, por possibilitar uma correta transmissão e/ou inibição sináptica, bem como sua somatização espacial e temporal, necessárias para que haja uma complexa função integrativa. Em um ambiente que não esteja perfeitamente estável, uma perfeita transmissão sináptica e integração nervosa tornam-se impossíveis (Begley & Brightman, 2003). O fluido extracelular somático contém muitos neurotransmissores potenciais e outras substâncias neuroativas, cujas concentrações podem variar amplamente em curto período de tempo. O SNC não pode tolerar e continuar sua função diante de um ambiente com as significativas flutuações na concentração de substâncias neuroativas que ocorrem no fluido extracelular geral. Aminoácidos que estão presentes no sangue em altas concentrações (p.ex. glicina, ácido glutâmico e ácido aspártico) são potentes neurotransmissores excitatórios; então, sua concentração no fluido extracelular cerebral precisa ser mantida estável com níveis constantes (Begley, 2004**).** b) A BHE tem também uma função de neuroproteção. Em um tecido altamente complexo como o tecido nervoso, onde a divisão mitótica e proliferação das células neuronais são ausentes, qualquer aceleração na morte celular e no contato dos neurônios com substâncias tóxicas pode causar doenças degenerativas prematuras e patologias diversas. Muitas substâncias potencialmente neurotóxicas são continuamente ingeridas na dieta ou geradas pelo metabolismo. A BHE é, portanto, crucial em limitar o

acesso desses xenobióticos e metabólitos potencialmente prejudiciais ao SNC por bloquear sua entrada ou removê-los ativamente do cérebro via transportadores ABC (Begley & Brightman, 2003; Begley, 2004).

2.3.2. Via paracelular

a) Junções de oclusão (JO)

O complexo juncional das células endoteliais dos capilares cerebrais que compõe a BHE compreende as junções de oclusão e junções aderentes. As JOs, ultraestruturalmente parecem sítios de aparente fusão envolvendo os folhetos externos das membranas plasmáticas de células endoteliais adjacentes (Figuras 4 e 5). As JOs consistem em três proteínas integrais de membrana, denominadas claudina, ocludina e moléculas de adesão juncional e um número de proteínas acessórias citoplasmáticas, incluindo ZO-1, ZO-2, ZO-3, cingulina e outras. As proteínas citoplasmáticas conectam essas proteínas de membrana à actina, que é uma proteína do citoesqueleto, para manutenção da integridade estrutural e funcional do endotélio (ver Ballabh *et al.,* 2004, para revisão).

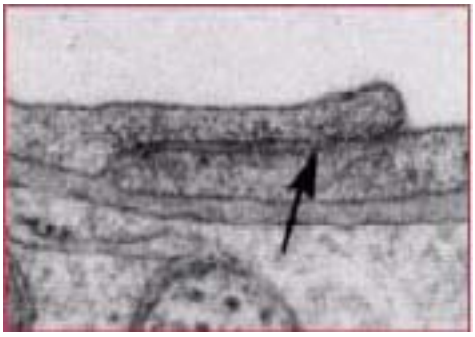

Figura 4. Micrografia eletrônica de BHE de mamífero, mostrando junção de oclusão (seta). Adaptado de: Biologia Celular e Molecular da Barreira Hematoencefálica, Pardridge W.M. (Ed.). Raven Press)

b) Junções aderentes (JA)

Essas junções possuem proteínas de membrana chamadas "caderinas", que se unem ao citoesqueleto via proteínas intermediárias, denominadas cateninas, para formar contatos adesivos entre as células. As JAs ligam-se via interações homofílicas entre os domínios extracelulares caderinas cálcio-dependentes, na superfície de células adjacentes. Os domínios citoplasmáticos das caderinas ligam-se, na placa submembranal, às proteínas beta ou gama-catenina, que fazem a conexão com o citoesqueleto de actina, via alfa-

catenina. Os componentes das JAs, incluindo caderinas, alfa-catenina e vinculina (homóloga à alfa-catenina), têm sido demonstrados em microvasos intactos da BHE de ratos (Figura 5). Os componentes das JOs e JAs parecem interagir, particularmente através das ZO-1 e cateninas, influenciando a formação das JOs (Matter & Balda, 2003).

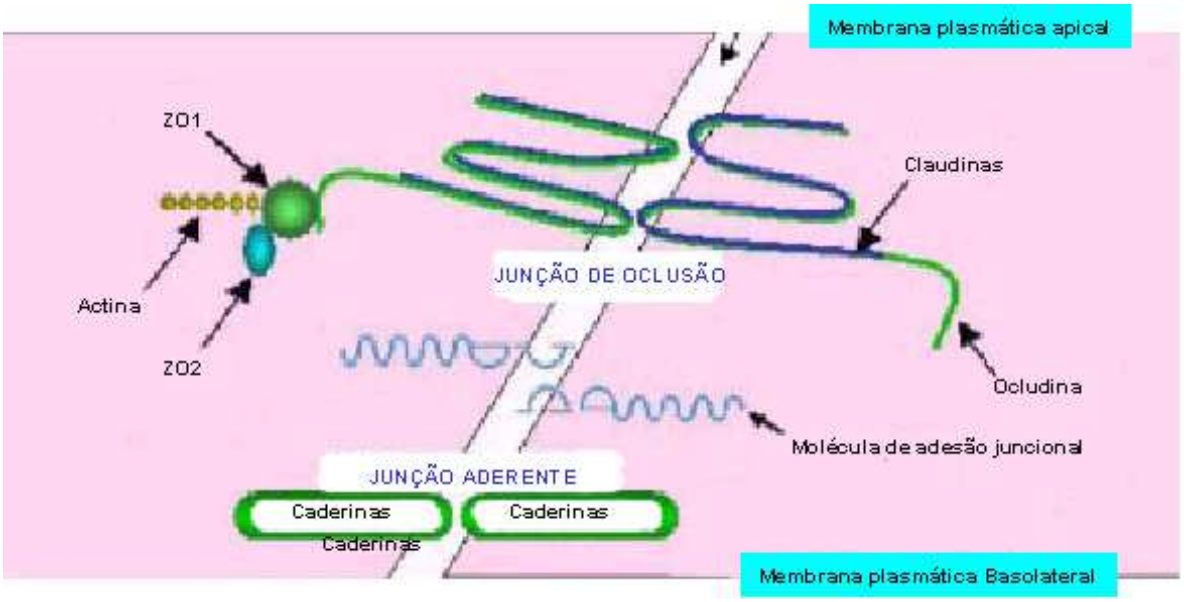

Figura 5. Representação esquemática da interação de proteínas associadas com as junções de oclusão da BHE. Claudina, ocludina e molécula de adesão juncional são proteínas transmembrana. ZO-1, ZO-2, ZO-3, cingulina e outras são proteínas citoplasmáticas. As claudinas são ligadas à actina através de proteínas citoplasmáticas Intermediárias. (Modificado de Ballabh *et al.* 2004)

2.3.3. Via transcelular

A BHE não é uma barreira estática, pois ela contém diferentes mecanismos de transporte. É uma interface dinâmica que possui várias proteínas dependentes de energia. Os transportadores de membrana podem agir no influxo ou no efluxo de substâncias, sendo que alguns trabalham bidirecionalmente. Os transportadores de influxo transportam, principalmente, nutrientes e componentes endógenos e exógenos do sangue para o cérebro. Os principais transportadores de influxo são: sistema de transporte de hexose, de aminoácidos, de ácidos monocarboxílicos, de amina e de nucleosídeos. Os transportadores de efluxo mais relevantes são: família de proteínas associadas à multi-resistência (e.g. P-gp, família MRP, BRCP), transportadores de ácidos monocarboxílicos e transportadores orgânicos de íons. O papel dos transportadores de efluxo na BHE é prevenir o acúmulo de componentes potencialmente tóxicos por ativação do bombeamento desses componentes

prejudiciais, do cérebro para a circulação periférica (Figura 6) (para revisão, ver Luurtsema *et al,* 2004).

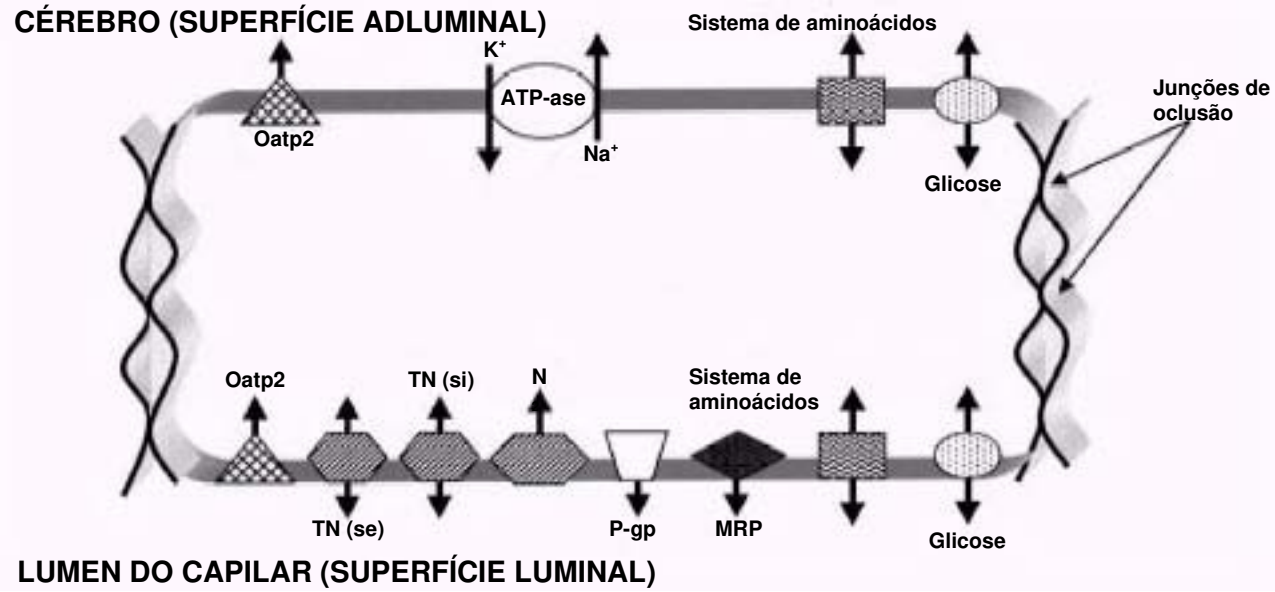

Figura 6. Diagrama esquemático dos transportadores de influxo e efluxo na BHE. Lee *et al* (2001) modificado

Os transportadores endoteliais cerebrais que abastecem o cérebro com nutrientes incluem o GLUT1 carreador de glicose, vários carreadores de aminoácidos (incluindo LAT1, sistema-L de grandes aminoácidos neutros) e transportadores de nucleosídeos, nucleobases e muitas outras substâncias (Begley & Brightman, 2003). Vários transportadores de ânions e cátions orgânicos identificados em outros tecidos e no plexo coróide são também expressos no endotélio cerebral.

Nos locais onde componentes precisam ser movidos contra um gradiente de concentração, a energia pode vir do ATP (como na família de transportadores ABC, incluindo P-glicoproteína - Pgp e proteínas relacionadas à resistência multidrogas-MRPs), ou um gradiente de Na^+ criado pela bomba ATPase Na^+/K^+ adluminal. Alguns transportadores (por exemplo, GLUT1 e LAT1) são bidirecionais, movendo o substrato sob um gradiente de conentração, e podem estar presentes tanto na membrana luminal quanto na adluminal, ou predominantemente em uma (para revisão ver Abbott *et al.,* 2006).

Entre os transportadores de efluxo, a Pgp está concentrada na membrana luminal (Schinkel, 1999), ao passo que transportadores dependentes de Na^+ são geralmente adluminais, especializados em movimentar solutos para fora do cérebro (Hawkins *et al.,*

2002; O'Kane & Hawkins, 2003). Eles incluem vários transportadores de glutamato dependentes de Na^+ (transportadores de aminoácidos excitatórios 1-3; EAAT1-3) (O'Kane *et al.,* 1999), que move o glutamato para fora do cérebro contra a grande oposição do gradiente de concentração. A clara polaridade apical-basal das células endoteliais notada acima é, portanto, refletida na sua polarizada função de transporte (Abbott *et al.,* 2006).

2.3.4. Membrana basal

A membrana basal é uma especialização da matriz extracelular, que está localizada em torno ou sob células endoteliais, epiteliais, nervos, células adiposas e musculares. Essa estrutura envolve a associação de proteínas da matriz e seus receptores da superfície celular: duas redes tridimensionais compostas por laminina ou colágeno tipo IV formando uma estrutura, na qual outras proteínas importantes da matriz extracelular, p.e., fibronectina e proteoglicanos, são intercaladas. As proteínas da membrana basal estão envolvidas em importantes processos do desenvolvimento e morfogenéticos, como diferenciação, proliferação, migração, adesão, crescimento axonal e regeneração (para revisão, consultar Paulsson 1992; Merker, 1994; Timpl, 1996; Schwarzbauer, 1999; Erickson & Couchman, 2000).

A laminina é composta por três diferentes cadeias polipeptídicas. Até a presente data, 12 diferentes lamininas foram identificadas em mamíferos (Colognato & Yurchenco, 2000). Ligações das células com a laminina ocorrem via uma variedade de integrinas e receptores não-integrínicos (Schwarzbauer, 1999; Belkin & Stepp, 2000), e participam em numerosos processos biológicos incluindo adesão, difusão, proliferação, migração e manutenção de fenótipos diferenciados (Timpl & Brown, 1994; Colognato & Yurchenco, 2000; Tunggal *et al.,* 2000). Além disso, a laminina pode se ligar a outras proteínas da membrana basal, contribuindo para a estruturação da lâmina basal e proporcionando as interações célula-matriz (Aumailley & Smyth, 1998).

Nos vasos sangüíneos cerebrais, os astrócitos estão separados das células endoteliais pela membrana basal (Figura 3). A quebra dessa membrana basal, como produzida experimentalmente pela injeção intracerebral de colagenase, resulta em um aumento da

permeabilidade da barreira hematoencefálica (Rosenberg *et al.,* 1992, 1993). Além disso, as isoformas de laminina endotelial -8 e -10 participam no recrutamento de células T através da barreira hematoencefálica durante lesões inflamatórias (Sixt *et al.,* 2001).

Morita *et al.* (2005) encontraram perda de função da barreira hematoencefálica relacionada com a idade. Essa perda foi devida, entre outros fatores, a uma diminuição na expressão de laminina em capilares e vênulas cerebrais.

2.3.5. Astrócitos e BHE

Tem sido esclarecido por estudos histológicos que os capilares cerebrais estão circundados por vários tipos celulares ou intimamente associados com elas. Essas células incluem os pés-vasculares dos astrócitos, pericitos, micróglia e processos neuronais (Figura 3). Essa associação fechada, célula-célula, particularmente entre astrócitos e capilares cerebrais, conduziu à sugestão de que essas células gliais podem mediar a indução de características específicas do fenótipo de barreira no endotélio dos capilares cerebrais (Davson *et al.,* 1967).

Astrócitos mostram um número de diferentes morfologias, dependendo da sua localização e associação com outros tipos celulares. De cerca de 11 fenótipos distintos que podem ser facilmente distinguidos, 8 envolvem interações específicas com vasos sangüíneos (Reichenbach & Wolburg, 2004). Evidências advindas particularmente de estudos com cultura de células, sugerem que os astrócitos podem regular muitas características da BHE, principalmente as firmes junções de oclusão (barreira física) (Dehouck *et al.,* 1990; Rubin *et al.,* 1991), a expressão e localização polarizada de transportadores, incluindo Pgp (Schinkel, 1999) e GLUT1 (barreira de transporte) (McAllister *et al.*, 2001) e sistemas de enzimas especializadas (barreira metabólica) (Hayashi, *et al,* 1997; Abbott, 2002; Haseloff *et al.,* 2005).

Além disso, tem sido mostrado que alguns dos outros tipos celulares presentes na BHE, incluindo pericitos, macrófagos perivasculares e neurônios, também contribuem para a indução da barreira (Duport *et al.,* 1998; Dohgu *et al.,* 2005). Tendo em vista a complexidade das propriedades da BHE, e a relação anatômica com outros tipos celulares, não é surpreendente que haja funções indutivas sinérgicas envolvendo mais de um tipo

celular. Por exemplo, os astrócitos são necessários para a correta associação de células endoteliais e pericitos, *in vitro* (Ramsauer *et al.,* 1998), sugerindo que a interação entre os três tipos celulares é também requerida para proporcionar a diferenciação dos capilares cerebrais *in vivo*. A indução inversa, ou seja, na qual o endotélio cerebral aumenta o crescimento e diferenciação de astrócitos, tem sido também descrita (Estrada *et al.,* 1990; Mi *et al.,* 2001).

2.3.6. Estruturas cerebrais que não apresentam BHE

A BHE está presente em todas as regiões cerebrais, exceto nos órgãos circumventriculares, incluindo área postrema, eminência mediana, neurohipófise, glândula pineal, órgão subfornical e lâmina terminalis (Figura 2). Os vasos sangüíneos dessas áreas do cérebro têm fenestrações que permitem a difusão de elementos do sangue, transportando bidirecionalmente moléculas através da parede vascular. Essas áreas, com barreira semipermeável, são áreas que regulam o sistema nervoso autonômo e as glândulas endócrinas (para revisão, ver Ballabh *et al.,* 2004).

2.3.7. Influência da BHE no acesso de agentes terapêuticos ao SNC: problemas e possibilidades

A presença da BHE representa um imenso obstáculo para o efetivo acesso de drogas terapêuticas ao SNC. Muitas drogas potenciais, que são efetivas nos seus sítios de ação, têm falhado e têm sido descartadas durante seu desenvolvimento para uso clínico devido à falha na sua distribuição em suficiente quantidade ao SNC. Em conseqüência, muitas doenças do SNC são subtratadas. Nos anos recentes, foi esclarecido que a BHE é formada não somente por barreiras anatômicas contra o livre movimento de solutos entre o sangue e o cérebro, mas também se constitui em uma barreira metabólica e de transporte (consultar Begley, 2004, para revisão).

Essas características da BHE são altamente restritivas ao tratamento de doenças do SNC, que requerem a entrada de drogas no cérebro em níveis terapêuticos. Algumas

pequenas drogas lipofílicas difundem-se através da BHE o suficiente para serem eficazes. No entanto, muitas drogas potencialmente úteis são excluídas (Chen *et al*., 2004).

A modulação da propriedade de barreira das junções existentes entre as células endoteliais, de modo a franquear a rota paracelular de acesso ao cérebro, seja parcialmente ou completamente, é uma estratégia que tem sido usada para permeabilizar transitoriamente a BHE às drogas, aumentando sua penetração num dado período de tempo (para revisão ver Begley, 2004).

Uma dessas estratégias é a "abertura osmótica" da barreira, uma técnica que tem sido aplicada com relativo sucesso há alguns anos no tratamento de tumores cerebrais em humanos (Neuwelt *et al.,* 1991; Rapoport, 2000). O agente osmótico usualmente empregado é o manitol hipertônico. Uma solução a 25% é introduzida na artéria carótida (com velocidade de 4-8 mL/seg em humanos) por um período de 30 s. Esse tratamento abre transitoriamente a barreira rapidamente por cerca de 30 min. O agente terapêutico é então administrado através da mesma cânula, enquanto a barreira está aberta, podendo difundir-se no SNC. A solução hipertônica age por puxar osmoticamente a água para fora da célula endotelial, causando redução celular, que pode causar desacoplamento dos domínios extracelulares das proteínas que formam as junções de oclusão, tornando essas junções mais permeáveis (Begley, 2004).

A prática de abertura osmótica, embora benéfica para o tratamento tumoral, pode trazer riscos adicionais, pois porções da via paracelular enquanto abertas, dão ensejo a que grandes partículas, incluindo partículas virais, albumina e neurotransmissores excitatórios, e outras substâncias potencialmente danosas ganhem acesso ao SNC, vindo a causar prejuízos adicionais ao paciente (Begley & Brightman, 200**3**).

A BHE pode ser permeabilizada pelo peptídeo bradicinina através de sua ação sobre a via de receptores B_2 expressos na membrana luminal do endotélio. Essa ação da bradicinina é feita pela modulação das junções de oclusão pela elevação intracelular dos níveis de cálcio livre. Esse cálcio livre ativa o sistema actina-miosina na célula, que encurtam e podem levar ao afastamento parcial das proteínas (ZO1, ZO2 e ZO3), acopladas ás proteínas juncionais ocludina e claudina da membrana celular endotelial, e então modificar as propriedades das junções de oclusão. Um análogo da bradicinina, o RMP7 ou Cereport, tem sido desenvolvido como um agente de abertura da BHE (Emerich *et al.,*

2001). De todo modo, a abertura da BHE por essa via, continua relativamente não-seletiva e pode admitir várias alterações, alguma perda de soluto plasmático como aminoácidos excitatórios, bem como da desejada dose terapêutica (Begley, 2004).

Foi mostrado que os alcilgliceróis são também moduladores da BHE (Lee *et al.,* 2002; Erdlenbruch *et al.,* 2003). Eles são administrados via artéria carótida, de modo similar ao manitol, induzindo a abertura osmótica. O acesso do methotrexato, durante terapia de tumores cerebrais, torna-se significantemente aumentado se co-injetado com um alcilglicerol (Erdlenbruch *et al.,* 2003). O monoacetil e o diacetil glicerol são efetivos, sendo o 1-O-hexildiglicerol o mais efetivo. A BHE parece ficar rapidamente aberta e retornar ao estado normal em 120 minutos. A toxicidade geral dos alcilgliceróis aparentemente é baixa. O mecanismo de modulação da BHE permanece não definido, mas tanto o cérebro normal, quanto a área de tumor são abertas, em contraste à abertura osmótica, que parece agir preferencialmente na BHE do tecido normal (Erdlenbruch *et al.,* 2003). A abertura da BHE torna-se, outra vez, presumivelmente não seletiva.

2.3.8. Ação do PNV sobre a BHE

Recentemente foi demonstrado em nosso laboratório que o veneno da aranha *Phoneutria nigriventer* provoca abertura da BHE no hipocampo de ratos Wistar, quando injetado endovenosamente (pela veia da cauda). Essa abertura parece ser transitória e os tipos de vasos da microcirculação afetados, variam no tempo. Dezoito horas após o envenenamento, algumas arteríolas e vênulas hipocampais encontravam-se permeabilizadas, mas não os capilares. Vinte e quatro horas após, grande número de vênulas e arteríolas estavam afetadas. Os capilares apresentaram alterações apenas após 9 dias da inoculação (Le Sueur *et al.*, 2003). Outros estudos realizados pelos autores com observações feitas 24 h e 9 dias após o envenenamento, revelaram que a quebra da BHE se dava através da alteração do transporte intravesicular microtúbulo-dependente (rota transendotelial) dos vasos hipocampais, permanecendo a rota paracelular aparentemente não afetada nesses períodos de observação (Le Sueur *et al.,* 2004, 2005).

No entanto, por ser essa rota a mais freqüentemente alterada em condições de modulação da BHE, e pelo fato de, em tempos mais precoces, quando da utilização de

traçador extracelular, este ter sido visto impregnando o local onde se localizam as "tight junctions", foi cogitado que essa via poderia ter sido transitoriamente aberta (Le Sueur *et al*., 2004). Nesse caso, poderia ocorrer alteração na estrutura das junções de oclusão e/ou adesão, as quais seriam primeiro restauradas, e só então haveria modificação da via transcelular, ou seja, as quebras das rotas trans- e para-celular seriam temporalmente diferentes. Desse modo, a alteração das junções (JOs e JAs) seriam coincidentes com os sinais clínicos do envenenamento, que são agudos e se resolvem em torno de 12 horas. A manifestação da permeabilização da BHE envolvendo a via transcelular, constatada por nossa equipe 24 h e 9 dias após o envenenamento, era entretanto sub-clínica, não mostrando sinais visíveis pelos animais que indicassem que nesses períodos a barreira estava rompida. Assim, no presente trabalho, períodos precoces foram estados de forma a avaliar os efeitos agudos do envenenamento sistêmico na permeabilização da BHE.

2.4. Proteína FOS

Muitos estímulos fisiológicos e farmacológicos, como estresse, inflamação, hipóxia e estimulação nervosa direta (Krukoff, 1993; Herrera & Robertson, 1996; Willoughby *et al,* 1997) induzem, rapidamente, aumento na expressão de genes primários, como o protooncogene *c-Fos*, no SNC. O mapeamento imunohistoquímico do produto protéico da expressão do gene *c-Fos*, isto é, a proteína FOS (uma fosfoproteína nuclear), tem sido usado como um marcador da ativação neuronal para identificar regiões específicas do cérebro, cuja atividade tenha sido alterada pelo estímulo aplicado (Harlan & Garcia, 1998).

O gene *c-Fos* é induzido minutos após o estímulo e a proteína FOS é expressa em 1-3 horas. A expressão de FOS no SNC é considerada um marcador da atividade neuronal que se segue a um estímulo apropriado e o sítio da expressão central de FOS em resposta a um estímulo tem sido usado para elucidar o curso de uma resposta (Shuai & Xie, 2004).

O padrão de expressão da proteína FOS pode ser influenciado por alguns fatores, como intensidade e natureza do estímulo, estado de consciência do animal (acordado ou anestesiado), infuência do biorritmo, e os métodos de detecção da sua expressão mais

usados são através de western blotting, imunohistoquímica, e hibridação *in situ* (Xavier, 1999).

O estudo da expressão de FOS em casos de envenenamento experimental já foi realizado com a toxina BmK I, do veneno do escorpião *Buthus martensi* (Bai *et al*., 2006). Nenhum estudo foi realizado com o veneno de escorpiões ou aranhas brasileiras e/ou suas toxinas, para analisar as vias acionadas no quadro tóxico, ou o grau de ativação das células neuronais.

2.5. Papel do NO e sua relação com a ativação neuronal

O óxido nítrico ("nitric oxide", ou NO) é um "neurotransmissor atípico" que é sintetizado a partir da L-arginina por enzimas denominadas óxido nítrico sintases ("nitric oxide synthase", NOS) (Forstermann *et al.,* 1991; Bredt & Snyder, 1990; Bredt, 1999). Essa enzima existe em várias isoformas (Forstermann *et al.,* 1991): a NOS neuronal é uma enzima constitutiva, citossólica, Ca^{2+}/calmodulina-dependente (Bredt *&* Snyder, 1990; Klatt *et al.,* 1992) que, no SNC, ocorre nos corpos celulares neuronais, dendritos e axônios (Bredt *et al.,* 1990) e apresenta discreta localização em estruturas cerebrais (Barjavel & Bhargava, 1995). No SNC, o NO é usado como um neurotransmissor (Bredt *et al.,* 1991; Brenman & Bredt, 1996). No sistema circulatório, o NO, gerado pela NOS endotelial (eNOS), funciona como vasodilatador (Hobbs & Ignarro, 1996), fator de angiogênese (Keifer *et al,* 2002) e fator de sobrevivência para as células endoteliais (Dimmeler & Zeiher, 1999).

O NO é um mensageiro intercelular no SNC. Foi primeiramente descoberto e descrito como um fator de relaxamento endotelial e, desde então, a sua atividade em um número de situações fisiológicas tem sido exaustivamente investigada (Marletta, 1993; McGeer *et al.*, 1993; Feelisch *et al.*, 1994; Hobbs & Ignarro, 1996; MacMicking *et al.*, 1997; Stamler *et al.,* 1997; Keifer *et al.*, 2002). O NO é um radical livre gasoso livremente difusível através das membranas, capaz de modificar muitas condições fisiológicas e patológicas.

O NO está envolvido na regulação da excitabilidade neuronal (para revisão, consultar Prast & Philippu, 2001), potenciação a longo prazo no hipocampo (Brenman &

Bredt, 1996**;** Son *et al*., 1996; Bohme *et al.,* 1991; O'Dell *et al.,* 1991), depressão a longo prazo no cerebelo (Shibuki & Okada, 1991; Linden & Connor, 1992), neurotoxicidade/neuroproteção (Buisson *et al*., 1993; Choi, 1993; Lipton *et al.,* 1993; Castagnoli *et al.,* 1999), nocicepção (Coderre, 1993), plasticidade sináptica (Bohme *et al.,* 1991; O'Dell *et al.,* 1991) e ansiedade (De Oliveira *et al.,* 2001; Guimarães *et al.,* 1994; Starr & Starr, 1995; Volke *et al.,* 1995). O NO pode também exercer um papel nas reações defensivas, uma vez que neurônios nNOS positivos estão localizados em numerosas regiões relacionadas com essa resposta, como a amigdala medial, núcleo pré-mamilar dorsal e paraventricular do hipotálamo e substância cinzenta periaquedutal (Guimarães *et al.,* 1994; Vincent & Kimura, 1992). A atividade da NADPH diaforase é utilizada como indicadora de atividade da NOS. Neurônios marcados para NADPH-d/NOS já foram detectados no córtex, putamen caudado, núcleo tegmental pedunculopontino e substânica negra compacta (Vincent & Kimura, 1992), regiões envolvidas no controle motor.

No metabolismo normal (fisiológico), o NO é necessário e útil para a célula, porém, em outras situações, como em injúrias e doenças, pode ser tóxico (patológico). Não está claro se isso ocorre pela quantidade de NO liberada pela célula, pela velocidade do fluxo, ou por causa do ambiente intracelular em que o NO é liberado. Como um gás altamente difusível e molécula de vida curta, o NO é, em geral, estudado indiretamente através de métodos de detecção das NOS (Shuai & Xie, 2004**;** Bishop & Anderson, 2005).

O NO é um neurotransmissor e/ou neuromodulador tanto no SNC, quanto no SNP, em ambos por mecanismos dependentes de cGMP (Lewko & Stepinski, 2002). O papel do NO no cérebro foi conhecido antes que a sua natureza química fosse revelada. Garthwaite *et al.* (1988) foram os primeiros a observar que a ativação dos receptores NMDA cerebrais resultavam na liberação de NO. Receptores NMDA induzem ativação da nNOS com um pico aos 5-15 min após ativação, retornando a níveis basais após 60 min, mais provavelmente por exaustão de substrato (Do *et al.,* 2002). Esse processo tem sido bem descrito em várias regiões cerebrais como hipocampo, striatum, hipotálamo e locus coeruleus (Fedele *et al.,* 2001; Maura, *et al.,* 2000; Trabace *et al.,* 2004).

Uma relação inversa entre NO e glutamato tem também sido observada. Estudos *in vivo* e *in vitro*, com doadores de NO, inibidores da NOS e antagonistas de receptores de glutamato, sugerem que em várias áreas do cérebro e medula espinhal, o NO aumenta a

liberação de glutamato (Prast *et al.,* 1998). Esse mecanismo retrógrado tem importância no processo de memória. Potenciação em longo prazo tem sido proposta como o principal mecanismo de estocar informações e consiste na ativação sináptica contínua em algumas partes do hipocampo. Para manutenção da ativação pós-sináptica, alguma comunicação retrógrada com o componente pré-sináptico deve existir. O NO tem sido sugerido como uma molécula retrógrada que ativa a liberação de glutamato em uma via dependente de cGMP (Nowicky & Bindman, 1993).

O efeito do NO na liberação de glutamato depende do nível de NO. Quando as concentrações de NO são baixas, ocorre diminuição da liberação de glutamato apesar da existência de elevados níveis de cGMP. Quando o NO aumenta os níveis de cGMP, o efeito inibitório na liberação de glutamato é revertido, sugerindo que cGMP exerce um efeito bifásico (Sequeira *et al.,* 1997).

É sabido que a ativação do receptor NMDA conduz à ativação da nNOS, que pode exercer um papel protetor através do bloqueio de caspases (Khaldi *et al.,* 2002). A S-nitrosilação do receptor NMDA parece ser um mecanismo inibitório pelo qual sua atividade é regulada para prevenir efeitos tóxicos. Mas o NO tem sido sugerido com um ativador neurotoxicidade-dependente do NMDA (Dawson *et al.,* 1991). Amônia é desintoxicada do cérebro pela glutamina sintetase. A ativação dos receptores NMDA aumentam a atividade da nNOS, produzindo NO capaz de inibir a glutamina sintetase por nitração ou nitrosilação (Kosenko *et al.,* 2003).

Estudos bioquímicos têm mostrado que o NO pode induzir a expressão de c-FOS em neurônios adjacentes, difundindo-se do corpo celular neuronal ou dos processos axonais/dendríticos dessas células para neurônios vizinhos. Esse neurotransmissor pode, portanto, agir nos neurônios Fos-positivos através das vias intracelulares, bem como extracelulares, para ativar a guanilil ciclase, que, por sua vez, ativa a via da c-GMP, a qual, por último, induz a expressão de c-FOS (Kayalioglu & Balkan, 2004).

Diversos estímulos levam à expressão de c-FOS e os neurônios ativados podem ser relacionados com a presença da enzima óxido nítrico sintase. Pardutz *et al.* (2000) mostraram que a injeção sistêmica de nitroglicerina aumenta os níveis de nNOS e de FOS em neurônios do núcleo trigeminal caudado de rato. Após estimulação das fibras cardíacas simpáticas aferentes, através da aplicação tópica de bradicinina na superfície anterior do

ventrículo esquerdo, os neurônios expressam tanto FOS como NOS no núcleo do hipotálamo (Guo & Moazzami, 2004). Injeção de formalina 5% no dorso da pata traseira de ratos revelou que neurônios do núcleo pedunculopontino tegmental contêm Fos e/ou NADPH-d (Kayalioglu & Balkan, 2004). Neurônios revelados por histoquímica para NADPH-d coincidem com aqueles marcados imunohistoquimicamente para NOS. Esses trabalhos também revelam haver uma relação anatômica de neurônios que co-expressam FOS e NOS, sugerindo que o NO pode ter um importante papel em vários processos que ativam a expressão da FOS.

2.6. Influência do NO na intoxicação por toxinas do veneno de *P. nigriventer*

Yonamine *et al.* (2004) investigaram o mecanismo de ação da toxina Tx2-5, do veneno de *P. nigriventer*, e relataram que a injeção intraperitoneal dessa toxina induziu uma síndrome tóxica que incluiu ereção peniana (priapismo), hipersalivação e morte por angústia respiratória e, provavelmente, edema pulmonar em ratos. Afirmaram que o pré-tratamento com inibidor não seletivo da NOS (L-NAME) reduziu a ereção peniana e, parcialmente, protegeu dos efeitos letais de Tx2-5, enquanto o pré-tratamento com o inibidor seletivo da nNOS (7-NI) aboliu completamente todos os efeitos tóxicos de Tx2-5, incluindo ereção peniana e morte, sugerindo que a nNOS exerce um importante papel nessa intoxicação.

III. REFERÊNCIAS BIBLIOGRÁFICAS

Abbott, N.J. Astrocyte–endothelial interactions and blood–brain barrier permeability. *J. Anat*. (2002) **200**:629–638.

Abbott, N.J.; Ronnback, L.; Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* (2006) **7**:41-53

Allt, G.; Lawrenson, J.G. Pericytes: cell biology and pathology. *Cells Tissues Organs* (2001) **169**:1-11.

Araujo, D.A.M.; Cordeiro, M.N.; Richardson, M.; Diniz, C.R.; Beirao, P.S.L. A novel class of polypeptide toxin modifies sodium current inactivation and activation in isolated frog skeletal muscle. *J. Physio.* (1993a) **467:**365.

Araujo, D.A.M.; Cordeiro, M.N.; Diniz, C.R.; Beirão, P.S.L. Effects of a toxic fraction, PhTx2 from the spider *Phoneutria nigriventer* on the sodium current. *Naunyn-Schmiedeberg's Arch. Pharmacol.* (1993b) **347**:205-208.

Aumailley, M.; Smyth, N. The role of laminins in basement membrane function. *J. Anat.* (1998) **193**:1–21

Bai, Z.T.; Zhao, R.; Zhang, X.Y.; Chen, J.; Liu, T.; Ji, Y.H. The epileptic seizures induced by BmK I, a modulator of sodium channels. *Exp. Neurol.* (2006) **197**:167-176.

Balabanov, R.; Dore-Duffy, P. Role of the CNS microvascular pericyte in the blood-brain barrier. *J. Neurosci. Res.* (1998) **53**:637-644.

Ballabh, P.; Braun, A.; Nedergaard, M. The blood-brain barrier: an overview structure, regulation, and clinical implications. *Neurobiol. Dis.* (2004) **16:**1 -13.

Bandopadhyay, R.; Orte, C.; Lawrenson, J.G.; Reid, A.R.; De Silva, S.; Allt, G. Contractile proteins in pericytes at the blood-brain and blood-retinal barriers. *J. Neurocytol.* (2001) **30**:35-44.

Barjavel, M.J.; Bhargava, H.N. Nitric oxide synthase activity in brain regions and spinal cord of mice and rats: kinetic analysis. *Pharmacology* (1995) **50**:168-174

Begley, D.J.; Brightman, M.W. Structural and functional aspects of the blood–brain barrier. *Prog. Drug Res.* (2003) **61:**40–78.

Begley, D.J. Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. *Pharmacology & Therapeutics* (2004) **104:**29-45.

Belkin, A.M., Stepp, M.A. Integrins as receptors for laminins. *Microsc. Res. Tech.*. (2000) **51**:280–301.

Bishop, A.; Anderson, J.E. NO signaling in the CNS: from the physiological to the pathological. *Toxicology* (2005) **208:**193-205.

Bohme, G.A.; Bon, C.; Stutzmann, J.M.; Doble, A.; Blanchard, J.C. Possible involvement of nitric oxide in long-term potentiation. *Eur. J. Pharmacol.* (1991) **199**:379-381.

Brazil, V.; Vellard, J. Contribuição ao estudo do veneno das aranhas. *Mem. Inst. Butantan* (1925) **2:** 5-77.

Brazil, V.; Vellard, J. Contribuição ao estudo do veneno das aranhas. *Mem. Inst. Butantan* (1926) **3**:3-77.

Bredt D.S.; Snyder, S.H. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci.* (1990) **87**:682-685.

Bredt, D.S.; Hwang, P.M.; Glatt, C.E.; Lowenstein, C.; Reed, R.R.; Snyder, S.H. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* (1991) **351:**714-718.

Bredt, D.S.; Hwang, P.M.; Snyder, S.H. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* (1990) **347**:768-770.

Bredt, D.S. Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radic Res.* (1999) **31**:577-596.

Brenman, J.E., Bredt, D.S. Nitric oxide signaling in the nervous system. *Meth. Enzymol.* (1996) **269:**119-129.

Bucaretchi, F.; Deus Reinaldo, C.R.; Hyslop, S.; Madureira, P.R.; De Capitani, E.M.; Vieira, R.J. A clinico-epidemiological study of bites by spiders of the genus *Phoneutria.* Rev. Inst. Méd. Trop. São Paulo (2000) **42**:17-21.

Buisson, A.; Margail, I.; Callebert, J.; Plotkine, M.; Boulu, R.G. Mechanisms involved in the neuroprotective activity of a nitric oxide synthase inhibitor during focal cerebral ischemia. *J. Neurochem.* (1993) **61**:690-696.

Carneiro, A.M.; Kushmerick, C.; Koenen, J.; Arndt, M.H.; Cordeiro, M.N., Chavez-Olortegui, C.; Diniz, C.R.; Gomez, M.V.; Kalapothakis, E.; Prado, M.V.; Prado, V.F. Expression of a functional recombinant *Phoneutria nigriventer* toxin active on K+ channels. *Toxicon* (2003) **41**:305-313.

Cassola, A.C.; Jaffe, H.; Fales, H.M.; Castro, A.S.; Magnoli, F.; Cipolla-Neto, J. Omega-phonetoxin-IIA: a calcium channel blocker from the spider *Phoneutria nigriventer, Pflugers Arch.* (1998) **436**:545-552.

Castagnoli, K.; Palmer, S.; Castagnoli, N. Jr. Neuroprotection by ®-deprenyl and 7-nitroindazole in the MPTP C57BL/6 mouse model of neurotoxicity. *Neurobiology* (1999) **7**:135-149.

Chavez-Olortégui, C.; Bohorquez, K.; Alvarenga, L.M.; Atapothakis, E.; Campolina, D.; Maria, W.S.; Diniz, C.R. Sandwich-ELISA detection of venom antigens in envenoming by *Phoneutria nigriventer* spider. *Toxicon* (2001) **39**:909-911.

Chen, Y.; Dawaldi, G.; Benson, H.A. Drug delivery across the blood-brain barrier. *Curr. Drug. Deliv*. (2004) **1:**361-376.

Choi, D.W. Nitric oxide: foe or friend to the injured brain? *Proc. Natl. Acad. Sci. USA*. (1993) **90**:9741-9743.

Coderre, T.J. The role of excitatory amino acid receptors and intracellular messengers in persistent nociception after tissue injury in rats. *Mol. Neurobiol.* (1993) **7**:229-246.

Colognato, H.; Yurchenco, P.D. Form and function: the laminin family of heterotrimers. *Dev. Dyn.* (2000) **218**:213–234.

Cordeiro, M.N.; Diniz, C.R.; Valentim, A.C.; Von Eickstedt, V.R.D.; Gilroy, J.; Richardson, M. The purification and amino acid sequences of four Tx2 neurotoxins from the venom of the Brazilian 'armed' spider *Phoneutria nigriventer*. *FEBS Lett.* (1992) **310:**153-156.

Cordeiro, M.N.; Figueiredo, S.G.; Valentim, A.C.; Diniz, C.R.; Von Eickstedt, V.R.D.; Gilroy, J.; Richardson, M. Purification and amino acid sequences of six Tx3 type neurotoxins from the venom of the Brazilian 'armed' *Phoneutria nigriventer. Toxicon* (1993) **31**:35-42.

Cordeiro, M.N.; Richardsom, M.; Gilroy, J.; Figueiredo, S.G.; Beirao, P.S.L.; Diniz, C.R. Properties of the venom from the South American Armed spider *Phoneutria nigriventer. J. Toxicol. Toxin Rev.* (1995) **14** :309-326.

Davson, H.; Oldendorf, W. H. Transport in the central nervous system. *Proc. R. Soc. Med.* (1967) **60:**326–328.

Dawson, V.L.; Dawson, T.M.; London, E.D.; Bredt, D.S.; Snyder, S.H. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A*. (1991) **88**:6368–6371.

De Lima, M.E.; Stankiewicz, M.; Hamon, A.; Figueiredo, S.G.; Cordeiro, M.N.; Diniz, C.R.; Martin-Eauclaire, M.F.; Pelhate, M. The toxin Tx4(6-1) from the spider *Phoneutria nigriventer* slows down Na+ current inactivation in insect CNS via binding to receptor site 3. *J. Insect Physiol.* (2002) **48:**53-61.

De Oliveira, R.M.; Del Bel, E.A.; Guimaraes, F.S. Effects of excitatory amino acids and nitric oxide on flight behavior elicited from the dorsolateral periaqueductal gray. *Neurosci. Biobehav. Rev.* (2001) **25**:679-685.

Dehouck, M.P.; Meresse, S.; Delorme, P.; Fruchart, J. C.; Cecchelli, R. An easier, reproducible, and mass-production method to study the blood–brain barrier *in vitro*. *J. Neurochem.* (1990) **54**:1798–1801.

Dimmeler, S.; Zeiher, A.M. Nitric oxide - an endothelial cell survival factor. *Cell Death Differ.* (1999) **6:**964-968.

Diniz, C.R. Separation of proteins and characterization of active substances in the venom of Brazilian spiders. *Anais da Acad. Bras. Cien.* (1963) **35:**283-291.

Diniz, C.R.; Cordeiro, M.N.; Rezende Jr, L.; Kelly, P., Fischer, S.; Reimann, F.; Oliveira, E.B.; Richardson, M. The purification and amino acid sequence of the lethal neurotoxin Tx1 from the venom of the Brazilian 'armed' spider *Phoneutria nigriventer. FEBS Lett.* (1990) **263:**251-253.

Diniz, M.R.; Paine, M.J.; Diniz, C.R.; Theakston, R.D.; Crampton, J.M. Sequence of the cDNA coding for the lethal neurotoxin Tx1 from Brazilian "armed" spider *Phoneutria nigriventer* predicts the synthesis and processing of a preprotoxin. *J. Biol. Chem.* (1993) **268**:15340-15342.

Do, K.Q.; Grima, G.; Benz, B.; Salt, T.E. Glial-neuronal transfer of arginine and S-nitrosothiols in nitric oxide transmission. *Ann. N.Y. Acad. Sci.* (2002) **962**:81-92.

Dohgu, S; Takata, F.; Yamauchi, A.; Nakagawa, S.; Egawa, T.; Naito, M.; Tsuruo, T.; Sawada, Y.; Niwa, M.; Kataoka, Y. Brain pericytes contribute to the induction and up-regulation of blood–brain barrier functions through transforming growth factor-β production. *Brain Res.*(2005) **1038:**208–215.

Duport, S.; Robert, F.; Muller, D.; Grau, G.; Parisi, L.; Stoppini, L. An *in vitro* blood–brain barrier model: cocultures between endothelial cells and organotypic brain slice cultures. Proc. Natl. Acad. Sci. USA (1998) **17:**1840–1845.

Emerich, D.F.; Dean, R.L.; Osbom, C.; Bartus, R.T. The development of the bradykinin agonist labradamil as a means to increase the permeability of the blood-brain barrier: from concept to clinical evaluation. *Clin. Pharmacokinet.* (2001) **40:**105-123.

Entwhistle, I.D.; Johnstone, R.A.W.; Medziradski, D.; May, T.E. Isolation of a pure toxic peptide from the venom of the spider *Phoneutria nigriventer* and its neurophysical activity on an insect femur preparation. *Toxicon* (1982) **20**:1059-1067.

Erdlenbruch, B.; Schinkhof, C.; Kugler, W.; Heinemann, D.E.H.; Herms, J.; Eibl, H.; Lakomec, M. Intracarotid administration of short-chain alkylglycerols for increased delivery of methotrexate to the rat brain. *Br. J. Pharmacol.* (2003) **139:**685-694.

Erickson, A.C.; Couchman, J.R. Still more complexity in mammalian basement membranes. *J Histochem Cytochem* (2000) **48**:1291–1306.

Escoubas, P.; Diochot, S.; Corzo, G. Structure and pharmacology of spider venom neurotocins. *Biochemie* (2000) **82:**893-907.

Estrada, C.; Bready, J.V.; Berliner, J.A.; Pardridge, W.M.; Cancilla, P.A. Astrocyte growth stimulation by a soluble factor produced by cerebral endothelial cells *in vitro*. *J. Neuropathol. Exp. Neurol*. (1990) **49:**539–549.

Fedele, E.; Marchi, M.; Raiteri, M. In vivo NO/cGMP signalling in the hippocampus. *Neurochem. Res.* (2001) **26**:1069–1078.

Feelisch, M.; te Poel, M.; Zamora, R.; Deussen, A.; Moncada, S. Understanding the controversy over the identity of EDRF. *Nature* (1994) **368:**62-65.

Figueiredo, S.G.; Lima-Perez Garcia, M.E.; Valentim, A.C.; Cordeiro, M.N.; Diniz, C.R.; Richardson, M. Purification and amino acid sequence of the insecticidal neurotoxin Tx4(6-1) from the venom of the 'armed' spider *Phoneutria nigriventer. Toxicon* (1995) **33:**83-93.

Figueiredo, S.G.; Mafra, R.A.; Pimenta, A.M.C.; Cordeiro, M.N.; Diniz, C.R.; De Lima, M.E. Purification and pharmacological activity of two potent insecticidal neurotoxins from *Phoneutria nigriventer* spider venom. *J. Venom. Anim. Toxins* (1997) **3:**84.

Figueiredo, S.G.; Perez-Garcia, M.E.L.; Cordeiro, M.N.; Diniz, C.R.; Patten, D.; Halliwell, R.F.; Gilroy, J.; Richardson, M. Purification and amino acid sequence of a highly insecticidal toxin from the venom of the Brazilian spider *Phoneutria nigriventer* which inhibits NMDA-evoked currents in rat hippocampal neurons. *Toxicon* (2001) **39:**309-317.

Forstermann, U.; Closs, E.I.; Pollock, J.S.; Nakane, M.; Schwarz, P.; Gath, I.; Kleinert, H. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* (1991) **23**:1121–31.

Garthwaite, J.; Charles, S.L.; Chess-Williams, R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* (1988) **336**:385-388.

Gomez, M.V.; Kalapothakis, E.; Guatimosim, C.; Prado, M.A.M. *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels. *Cell. Mol. Neurobiol.* (2002) **22:**579-588.

Grieb, P.; Forster, R.E.; Strome, D.; Goodwin, C.W.; Pape, P.C. O2 exchange between blood and brain tissues studied with 18O2 indicator-dilution technique. *J. Appl. Physiol.* (1985) **58**:1929-1941.

Guatimosim, C.; Romano-Silva, M.A.; Cruz, J.S., Beirão, P.S.L. Kalapothakis, E.; Moraes-Santos, T.; Cordeiro, M.N.; Diniz, C.R.; Gomez, M.V.; Prado, M.A.M. A toxin from the spider *Phoneutria nigriventer* that blocks calcium channels coupled to exocytosis. *Br. J. Pharmacol.* (1997) **122:**591-597.

Guimarães, F.S.; de Aguiar, J.C.; Del Bel, E.A.; Ballejo, G. Anxiolytic effect of nitric oxide synthase inhibitors microinjected into the dorsal central grey. *Neuroreport* (1994) **5**:1929-1932.

Guo, Z.L.; Moazzami, A.R. Involvement of nuclei in the hypothalamus in cardiac sympathoexcitatory reflexes in cats. *Brain Res.* (2004) **1006:**36-48.

Harlan, R.E.; Garcia, M.M. Drugs of abuse and immediate-early genes in the forebrain. *Mol. Neurobiol.* (1998) **16**:221-267.

Haseloff, R.F.; Blasig, I.E.; Bauer, H.C.; Bauer, H. In search of the astrocytic factor(s) modulating blood-brain barrier functions in brain capillary endothelial cells *in vitro. Cell Mol. Neurobiol.* (2005) **25**:25-39.

Hawkins, R.A.; Peterson, D.R.; Vina, J.R. The complementary membranes forming the blood–brain barrier. *IUBMB Life* (2002) **54**:101–107.

Hayashi, Y.; Nomura, M.; Yamagishi, S.; Harada, S.; Yamashita, J.; Yamamoto, H. Induction of various blood–brain barrier properties in non-neural endothelial cells by close apposition to co-cultured astrocytes. *Glia* (1997) **19**:13–26.

Herrera, D.G.; Robertson, H.A. Activation of c-Fos in the brain. *Prog. Neurobiol.*(1996) **50:**83-107.

Hobbs, A.J.; Ignarro, L.J. Nitric oxide-cyclic GMP signal transduction system. *Meth. Enzymol.* (1996) **269:**134-148.

Kalapothakis, E.; Penaforte, C.L.; Leão, R.M.; Cruz, J.S.; Prado, V.F.; Cordeiro, M.N.; Diniz, C.R.; Romano-Silva, M.A.; Prado, M.A.; Gomez, M.V.; Beirao, P.S. Cloning, cDNA sequence analysis and patch clamp studies of a toxin the venom of the armed spider (*Phoneutria nigriventer*). *Toxicon* (1998a) **36**:1971-1980.

Kalapothakis, E.; Penaforte, C.L.; Beirao, P.S.; Romano-Silva, M.A.; Cruz, J.S.; Prado, M.A.; Guimaraes, P.E.; Gomez, M.V.; Prado, V.F. Cloning of cDNAS encoding neurotixic peptides from spider *Phoneutria nigriventer. Toxicon* (1998b) **36**:1843-1850.

Kayalioglu, G.; Balkan, B. Expression of c-Fos and NADPH-d after peripheral noxious stimulation in the pedunculopontine tegmental nucleus. *NeuroReport* (2004) **15:**421-423.

Keifer, F.N.; Mistell, H.; Kalak, N.; Tshudin, N.K.; Fingerle, J.; Van der Kooij, M.; Stumm, M.; Sumanovski, L.T.; Sieber, C.C.; Battegay, E.J. Inhibition of NO biosynthesis, but not elevated blood pressure, reduces angiogenesis in rat models of secondary hypertension. *Blood Press.* (2002) **11:**116-124.

Khaldi, A.; Chiueh, C.C.; Bullock, M.R.; Woodward, J.J. The significance of nitric oxide production in the brain after injury. *Ann. N.Y. Acad. Sci*. (2002) **962**:53–59.

Klatt, P.; Heinzel, B.; John, M.; Kastner, M.; Bohme, E.; Mayer, B. Ca2+/calmodulin-dependent cytocrome c redutase activity of brain nitric oxide synthase. *J. Biol. Chem.* (1992) **267**:11374-11378.

Kosenko, E.; Llansola, M.; Montoliu, C.; Monfort, P.; Rodrigo, R.; Hernandez- Viadel, M.; Erceg, S.; Sanchez-Perez, A.M.; Felipo, V. Glutamine synthetase activity and glutamine content in brain: modulation by NMDA receptors and nitric oxide. *Neurochem. Int.* (2003) **43**:493–499.

Krukoff, T.L. Expression of c-fos in studies of central autonomic and sensory systems. *Mol. Neurobiol*. (1993) *Fall-Winter* **7:**247-263.

Kushmerick, C.; Kalapothakis, E.; Beirão, P.S.L.; Penaforte, S.L.; Prado, V.F.; Cruz, J.S.; Diniz, C.R.; Cordeiro, M.N. Gomes, M.V.; Romano-Silva, M.A.; Prado, M.A.M. *Phoneutria nigriventer* toxin Tx3-1 blocks A-type K+ currents controlling Ca++ oscillation frequency in GH3 cells. *J. Neurochem.* (1999) **72:**1472-1481.

Le Sueur, L.; Collares-Buzato, CB.; Kalapothakis, E.; Cruz-Höfling, M.A. In vitro effect of the *Phoneutria nigriventer* spider venom on cell viability paracellular barrier function and transcellular transport in cultured cell lines. *Toxicon* (2005) **46**:130-141.

Le Sueur, L.P.; Collares-Buzato, C.B.; Cruz-Höfliing, M.A. Mechanisms involved in the blood-brain barrier increased permeability induced by *Phoneutria nigriventer* spider venom in rats. *Brain. Res.* (2004) **1027**:38-47.

Le Sueur, L.; Kalapothakis, E.; Cruz-Höfling, M.A. Breakdown of the blood-brain barrier and neuropathological changes induced by *Phoneutria nigriventer* spider venom. *Acta neuropathol. (Berl).* (2003) **105**:125-134.

Leão, R.M.; Cruz, J.S.; Diniz, C.R.; Cordeiro, M.N.; Beirão, P.S.L. Inhibition of neuronal high-voltage activated calcium channels by the *Phoneutria nigriventer* Tx3-3 peptide toxin. *Neuropharmacology* (2000) **39:**1756-1767.

Lee, G.; Dallas, S.; Hong, M.; Bendayan, R. Drug transporters in the central nervous system: brain barriers and brain parenchyma considerations. *Pharmacol. Rev.* (2001) 53:569-596.

Lee, H.J.; Zhang, Y.; Pardridge, W.M. Blood-brain barrier disruption following the internal carotid arterial perfusion of alkyl glycerols. *J. Drug Targ.* (2002) **10:**463-467.

Lewko, B.; Stepinski, J. Cyclic GMP signaling in podocytes. *Microsc. Res. Tech.* (2002) **57**:232-235.

Linden, D.J.; Connor, J.A. Long-term depression of glutamate currents in cultured cerebellar Purkinje neurons does not require nitric oxide signaling. *Eur. J. Neurosci.* (1992) **4**:10-15.

Lipton, S.A.; Choi, Y.B.; Pan, Z.H.; Lei, S.Z.; Chen, H.S.; Sucher, N.J.; Loscalzo, J.; Singel, D.J.; Stamler, J.S. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* (1993) **64**:626-632.

Luurtsema, G.; Lange, E.C.M.; Lammertsma, A.A.; Franssen, A.J.F. Transport across the blood-brain barrier: stereoselective and PET-tracers. *Mol. Imag. Biol.* (2004) **6:**306-318.

MacMicking, J.; Xie, Q.W.; Nathan, C. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* (1997) **15:**323-350.

Mafra, R.A.; Figueiredo, S.G.; Diniz, C.R.; Cordeiro, M.N.; Cruz, J.D.; De Lima, M.E. PhTx4, a new class of toxins from *Phoneutria nigriventer* spider venom, inhibits the glutamate uptake in rat brain synaptosomes. *Brain Res.* (1999) **831**:297-300.

Marletta, M.A. Nitric oxide synthase: function and mechanism. *Adv. Exp. Med. Biol.* (1993) **338:**281-284.

Matavel, A.; Cruz, J.S.; Penaforte, C.L., Araujo, D.A.M.; Kalapothakis, E.; Prado, V.F.; Diniz, C.R.; Cordeiro, M.N.; Beirão, P.S.L. Electrophysiological characterization and molecular identification o the *Phoneutria nigriventer* peptide toxin PnTx2-6. *FEBS Lett.* (2002) **523** :219-223.

Matter, K.; Balda, M.S. Holey barrier: claudins and the regulation of brain endothelial permeability. *J. Cell Biol.* (2003) **161**:653-660.

Maura, G., Marcoli, M., Pepicelli, O., Rosu, C., Viola, C., Raiteri, M. Serotonin inhibition of the NMDA receptor/nitric oxide/cyclic GMP pathway in human neocortex slices: involvement of 5-HT(2C) and 5-HT(1A) receptors. *Br. J. Pharmacol*. (2000) **130:**1853–1858.

McAllister, M.S.; Krizanac-Bengez, L.; Macchia, F.; Naftalin, R.J.; Pedley, K.C.; Mayberg, M.R.; Marroni, M.; Leaman, S.; Stanness, K.A.; Janigro, D. Mechanisms of glucose transport at the blood–brain barrier: an *in vitro* study. *Brain Res.* (2001) **409:**20–30.

McGeer, P.L.; Kawamata, T.; Walder, D.G.; Akiyama, H.; Tooyama, I.; McGeer, E.G. Microglia in degenerative neurological disease. *Glia* (1993) **7:**84-92.

Merker, H.J. Morphology of the basement membrane. *Microsc. Res. Tech.* (1994) **28**:95–124

Mi, H.; Haeberle, H.; Barres, B.A. Induction of astrocyte differentiation by endothelial cells. *J. Neurosci.* (2001) **21:**1538–1547.

Miranda, D.M.; Romano-Silva, M.A.; Kalapothakis, E.; Diniz, C.R.; Cordeiro, M.N.; Moraes-Santos, T.; Prado, M.A.; Gomez, M.V. *Phoneutria nigriventer* toxins block tityustoxin-induced calcium influx in sunaptosomes. *Neuroreport.* (1998) **9**:371-1373.

Morita, T.; Mizutani, Y.; Sawada, M.; Shimada, A. Immunohistochemical and ultrastructural findings related to the blood-brain barrier in the blood vessels of the cerebral white matter in aged dogs. *J. Comp. Pathol.* (2005) **133**:14-22.

Neuwelt, E.A.; Goldman, D.L.; Dahlborg, S.A.; Crosen, J.; Ramsey, F.; Roman-Goldstein, S.; Brazoel, R.; Dana, B. Primary CNS lymphoma trated with osmotic blood-brain barrier disruption; prolonged survival and preservation of cognitive function. *J. Clin. Oncol.* (1991) **9:**1580-1590.

Nowicky, A.V.; Bindman, L.J. The nitric oxide synthase inhibitor, Nmonomethyl-L-arginine blocks induction of a long-term potentiationlike phenomenon in rat medial frontal cortical neurons in vitro. *J. Neurophysiol*. (1993) **70**:1255–1259.

O'Dell, T.J.; Hawkins, R.D.; Kandel, E.R.; Arancio, O. Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl., Acad. Sci.* USA (1991) **88**:11285-11289.

O'Kane, R.; Hawkins, R.A. Na+-dependent transport of large neutral amino acids occurs at the abluminal membrane of the blood–brain barrier. *Am.J. Physiol. Endocrinol. Metab.* (2003) **285**: E1167–E1173.

O'Kane, R.L.; Martinez-Lopez, I.; DeJoseph, M.R.; Vina, J.R.; Hawkins, R.A. Na+-dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) of the blood–brain barrier. *J. Biol. Chem.* (1999) **274**:31891–31895.

Ohtsuki, S. Physiological function of blood-brain barrier transporters as the CNS supporting and protecting system. *Yakugaku Zasshi* (2004) **124:**791-802.

Oliveira, L.C.; De Lima, M.E.; Pimenta, A.M.; Mansuelle, P.; Rochat, H.; Cordeiro, M.N.; Richardson, M.; Figueiredo, S.G. PnTx4-3, a new insect toxin from *Phoneutria nigriventer* venom elicits the glutamate uptake inhibition exhibited by PhTx4 toxic fraction. *Toxicon* (2003) **42**:793-800.

Ori, M. Aranea. In: *Animals of Medically Importance in Nansei Islands in Japan* (Sasa, M., Tanaka, H., Kano, R., Tanaka, H., Eds). (1977) Pp.410 Shinjuku Shobo: Japan.

Pardridge, W.M.; Eisenberg, J; Yang, J. Human blood-brain barrier insulin receptor. *J. Neurochem.* (1985) **44**:1771-1778.

Pardutz, A.; Krizbal, I.; Multon, S.; Vecsei, L.; Schoenen, J. Systemic nitroglycerin increases nNOS levels in rat trigeminal nucleus caudalis. *NeuroReport* (2000) **11:**3071-3075.

Paulsson, M. Basement membrane proteins: structure, assembly, and cellular interactions. Crit *Rev. Biochem. Mol. Biol.* (1992) **27**:93–127.

Penaforte, C.L.; Prado, V.F.; Prado, M.A.M.; Romano-Silva, M.A.; Guimarães, P.E.M.; De Marco, L.; Gomez, M.V.; Kalapothakis, E. Molecular cloning of cDNAs encoding neurotoxic peptodes from the spider *Phoneutria nigriventer. Toxicon* (2000) **38:**1443-1449.

Prado, M.A.M.; Guatimosin, C.; Gomez, M.V.; Diniz, C.R.; Cordeiro, M.N.; Romano-Silva, M.A. A novel tool for the investigation of glutamate release from rat cerebrocortical synaptosomes: the toxin Tx3-3 from the venom of the spider *Phoneutria nigriventer. Biochem. J.* (1996) **314:**145-150.

Prast, H.; Tran, M.H.; Fischer, H.; Philippu, A. Nitric oxide-induced release of acetylcholine in the nucleus accumbens: role of cyclic GMP, glutamate, and GABA. *J. Neurochem*. (1998) **71**:266–273.

Prast, H.; Philippu, A. Nitric oxide as modulator of neuronal function. *Prog. Neurobiol.* (2001) **64**:51-68.

Ramsauer, M.; Krause, D.; Dermietzel, R. Angiogenesis of the blood–brain barrier *in vitro* and organotypic brain slice cultures. *Proc. Natl Acad. Sci.* (1998) **95**:1840–1845.

Rapoport, S.I. Osmotic opening of the blood-brain barrier: principles mechanism and therapeutic applications. *Cell. Mol. Neurobiol.* (2000) **20:**217-230.

Rash, L.D.; Hodgson, W.C. Pharmacology and biochemistry of spider venoms. *Toxicon* (2002) **40:**225-254.

Reichenbach, A.; Wolburg, H. in *Neuroglia* 2nd edn (eds Kettemann, H. & Ransom, B. R.) 19–35 (Oxford Univ. Press, New York, 2004).

Rezende Jr, L.; Cordeiro, M.N.; Oliveira, E.B., Diniz, C.R. Isolation of neurotoxic peptides from the venom of the 'armed' spider *Phoneutria nigriventer. Toxicon* (1991) **29:**1225-1233.

Rosenberg, G.A.; Estrada, E.; Kelley, R.O.; Kornfeld, M. Bacterial collagenase disrupts extracellular matrix and opens bloodbrain barrier in rat. *Neurosci. Lett.* (1993) **160**:117–119.

Rosenberg, G.A.; Kornfeld, M.; Estrada, E.; Kelley, R.O.; Liotta, L.A.; Stetler-Stevenson, W.G. TIMP-2 reduces proteolytic opening of blood-brain barrier by type IV collagenase. *Brain Res.* (1992) **576**:203–207.

Rubin, L. L.; Hall, D.E.; Porter, S.; Barbu, K.; Cannon, C.; Homer, H.C.; Janatpour, M.; Liaw, C.W.; Manning, K.; Morales, J.; Tanner, L.; Tbmaselli, K.J.; Bard, F. A cell culture model of the blood–brain barrier. *J. Cell Biol*. (1991) **115**:1725–1735.

Russel, F.E.; Gertsch, W.J. Bites os spiders and other arthropods.In: *Current t Therapy*. (Conn, H.F., Eds.) (1974) pp.856-867, W.B. Saunders: Philadelphia.

Santos, R.G.D.; Diniz, C.R.; Cordeiro, M.N.; De Lima, M.E. Binding sites and acrions of Tx1, a neurotoxin from the venom of the spider *Phoneutria nigriventer,* in guinea pig ileum. *Braz. J. Med. Biol. Res.* (1999) **32**:1565-1569.

Santos, R.G.D.; Van Renterghem, C.; Martín-Moutot, N.; Mansuelle, P.; Cordeiro, M.N.; Diniz, C.R.; Mori, Y.; De Lima, M.E.; Seagar, M. *Phoneutria nigrivnter* Omega-phonetoxin IIA blocks the Cav2 family of calcium channels and interacts with Omega-conotoxin-binding sites. *J. Biol. Chem.* (2002) **277:**13856-13862.

Schenberg, S.; Pereira Lima, F.A. *Phoneutria nigriventer* venom In: Bucherl, W., Buckley, E.E. (Eds.) Venomous Animals and their Venom, Academic Press, New York, (1971) **3:**279-285.

Schinkel, A.H. P-glycoprotein, a gatekeeper in the blood–brain barrier. *Adv. Drug Deliv. Rev*. (1999) **36**:179–194.

Schwarzbauer, J. Basement membranes: putting up the barriers. *Curr. Biol.* (1999) **9**:R242-R244.

Sequeira, S.M.; Ambrosio, A.F.; Malva, J.O.; Carvalho, A.P.; Carvalho, C.M. Modulation of glutamate release from rat hippocampal synaptosomes by nitric oxide. *Nitric Oxide* (1997) **1**:315–329.

Shibuki, K.; Okada, D. Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature* (1991) **349**:326-328.

Shuai, X.W.; Xie, P.Y. Expression and localization of c-Fos and NOS in the central nerve system following esophagitis stimulation in rats. *World Gastroenterol.* (2004) **10:**2287-2291.

Sixt, M.; Engelhardt, B.; Pausch, F.; Hallmann, R.; Wendler, O.; Sorokin, L.M. Endothelial cell laminin isoforms, laminins-8 and -10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. *J. Cell. Biol.* (2001) **153**:933–946.

Son, H.; Hawkins, R.D.; Martin, K.; Kiebler, M.; Huang, P.L.; Fishman, M.C.; Kandel, E.R. Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell* (1996) **87:**1015-1023.

Stamler, J.S.; Jia, L.; Eu, J.P.; McMahon, T.J.; Demchenko, I.T.; Bonaventura, J.; Genert, K.; Piantadose, C.A. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* (1997) **276:**2034-2037.

Starr, M.S.; Starr, B.S. Do NMDA receptor-mediated changes in motor behaviour involve nitric oxide. *Eur. J. Pharmacol.* (1995) **272**:211-217.

Timpl, R. Macromolecular organization of basement membranes. *Curr. Opin. Cell Biol.* (1996) **8**:618–624

Timpl, R.; Brown, J.C. The laminins. *Matrix. Biol.* (1994) **14**:275–281

Trabace, L.; Cassano, T.; Tucci, P.; Steardo, L.; Kendrick, K.M.; Cuomo, V. The effects of nitric oxide on striatal serotoninergic transmission involve multiple targets: an in vivo microdialysis study in the awake rat. *Brain. Res*. (2004) **1008**:293–298.

Troncone, L.R.P.; Lebrun, I.; Magnoli, F.; Yamane, T. Biochemical and pharmacological studies on a lethal neruotoxic polypeptide from *Phoneutria nigriventer* spider venom. *Neurochem. Res.* (1995) **20**:879-883.

Tunggal, P.; Smyth, N.; Paulsson, M.; Ott, M.C. Laminins: structure and genetic regulation. *Microsc. Res. Tech.* (2000) **51**:214–227

Vieira, L.B.; Kushmerick, C.; Reis, H.J. Diniz, C.R.; Cordeiro M.N.; Prado, M.A.M.; Kalapothakis, E.; Romano-Silva, M.A.; Gomez, M.V. PnTx3-6 a spider neurotoxin inhibits K+ -evoked increase in [Ca2+]i and Ca2+ -dependent glutamate release in synaptosomes. *Neurochem. Int.* (2003) **1267:**1-6.

Vincent, S.R.; Kimura, H. Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience* (1992) **46**:755-784.

Volke, V.; Koks, S.; Vasar, E.; Bourin, M.; Bradwejin, J.; Mannisto, P.T. Inhibition of nitric oxide synthase causes anxiolytic-like behaviour in an elevated plus-maze. *Neuroreport* (1995) **6**:1413-1416.

Willoughby, J.O.; Mackenzie, L.; Medvedev, A.; Hiscock, J.J. FOS induction following systemic kainic acid: early expression in hippocampus and later widespread expression correlated with seizure. *Neuroscience* (1997) **77** :379-392.

Xavier, G.F. Técnicas para o estudo do sistema nervoso. (1999) Editora Plêiade, São Paulo, SP.

Yonamine, C.M.; Troncone, L.R.P.; Camilloa, M.A.P. Blockade of neuronal nitric oxide synthase abolishes the toxic effects of Tx2-5, a lethal *Phoneutria nigriventer* spider toxin. *Toxicon* (2004) **44:**169-172.

Zhang, Y.; Pardridge, W.M. Rapid transferrin efflux from brain to blood across the blood-brain barrier. *J. Neurochem.* (2001) **76**:1597-1600.

IV – OBJETIVOS

Geral

Avançar no entendimento de possíveis mecanismos que estariam envolvidos nas alterações provocadas pelo veneno da aranha *Phoneutria nigriventer* no Sistema Nervoso Central.

Específicos

O presente trabalho teve como propósito examinar a quebra aguda da BHE, nos períodos de 15 minutos, 1, 2 e 5 horas após a administração endovenosa do veneno da aranha *Phoneutria nigriventer*, analisando alguns componentes físicos da barreira (endotélio e membrana basal dos microvasos cerebrais), bem como avaliar a ação do veneno no tecido neural, através da observação da resposta neuronal central (expressão de c-FOS e n-NOS pelos neurônios centrais).

V. RESULTADOS

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Acute blood–brain barrier permeabilization in rats after systemic *Phoneutria nigriventer* venom

Catarina Rapôso[a,1], *Gabriela Mariotoni Zago*[a,b,1], *Gustavo Henrique da Silva*[c], *Maria Alice da Cruz Höfling*[a,*]

[a]*Departamento de Histologia e Embriologia, Instituto de Biologia, C.P. 6109, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, 13083-970, Brazil*
[b]*Departmento de Farmacologia, Faculdade de Ciências Médicas, UNICAMP, Campinas, SP, 13083-970, Brazil*
[c]*Departamento de Patologia Clínica, Faculdade de Ciências Médicas, UNICAMP, Campinas, SP, 13083-970, Brazil*

ARTICLE INFO

Article history:
Accepted 20 February 2007
Available online 7 March 2007

Keywords:
Basal membrane
BBB
Laminin
MET
Lanthanum nitrate
Phoneutria nigriventer

ABSTRACT

A highly controlled transport of substances at the interface between blood and brain characterizes the blood–brain barrier (BBB), fundamental for maintenance of the homeostasis of the cerebral milieu. In this study, we investigated the time course (15 min, 1, 2, and 5 h) of BBB opening induced by intravenous (i.v.) injection of *Phoneutria nigriventer* spider venom (PNV) using quantitative and morphological approaches on cerebellum and hippocampus vessels for assessment of BBB permeability. The results showed vasogenic edema and tracer extravasation faster and severalfold higher in hippocampus than in cerebellum. Reactive astrocytes with swollen perivascular end-feet processes were found only in cerebellum. An immediate and total degradation of laminin in capillaries occurred resulting in the disappearance of the basement membrane. In medium-sized vessels, this effect was less prominent. The changes were transient, with cerebellum in general presenting a faster recovery. However, at 5 h laminin was overexpressed, principally in hippocampus. The rapid and abrupt shift of laminin expression in capillaries (at 15 min) coincided with the immediate and severe signs of intoxication shown by the animals, but not with the peak of leakage of vessels and vasogenic edema, which occurred later (1–2 h). The findings suggest a complex regulatory mechanism, since the extension of BBB impairment caused by PNV depends on the region of the SNC, and on the vessels types. It is suggested that the components of the BBB (gliovascular unit) have a critical role in these differences. *P. nigriventer* venom can be a useful tool to explore the mechanisms of BBB.

1. Introduction

Blood–brain barrier (BBB) is a diffusion barrier essential for the normal functioning of the CNS. Endothelial walls from brain blood vessels differ from peripheral ones by being continuous, fenestration-free and possessing an extensive highly resistant tight junction (TJ) occluding the intercellular pathway as a route for transit of molecules. In addition, a very selective transcellular transport largely controlled by membrane receptors, carriers and metabolic barriers acting-enzymes expressed in the

* *Corresponding author*. Fax: +55 19 3289 3124.
E-mail address: hofling@unicamp.br (M.A. da Cruz Höfling).
[1] Contributed equally to the development of this work.

0006-8993/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2007.02.086

endothelial plasma membrane limits the entrance of substances into brain. Nutrients such as glucose and amino acids enter the brain via cell transporters. Membrane receptor-mediated endocytosis controls the uptake of larger molecules such as insulin, leptin, and iron transferring (Zhang and Pardridge, 2001). In contrast, small lipophilic substances, such as O_2 and CO_2, diffuse freely across plasma membranes to tissue according concentration gradient (Grieb et al., 1985). In addition, outer coverings represented by capillary basement membrane (BM), perivascular astroglial end-feet, pericytes, perivascular microglia and neurons are also important part of the BBB complex (Ramsauer et al., 2002). These cells contribute to the synthesis of proteins of the extracellular matrix which in turn influences behavior and differentiation of the cells. The BBB complex has been referred to as neurovascular unit (Abbott, 2002).

The BBB can be altered in several brain diseases. A variety of pathological conditions may either weaken the barrier efficiency or contribute to the development of the disease processes (Neuwelt, 2004). Examples of BBB dysfunction were demonstrated in Alzheimer's disease (Zlokovic, 2004), neuroinflammatory diseases, such as multiple sclerosis (Plumb et al., 2002; Werring et al., 2000), HIV encephalitis (Toborek et al., 2003), and brain tumors (Schlageter et al., 1999). Natural toxins contained in the venom of caterpillars of the saturniid moth *Lonomia obliqua* (Silva et al., 2004), and in the venom of the armed spider *Phoneutria nigriventer* (Le Sueur et al., 2003, 2004) are among a large list of xenobiotics which can disturb the tight-controlled bi-directional transport blood–nervous tissue.

P. nigriventer venom has long been investigated by Brazilian researchers because the majority of accidents caused by venomous spiders, in São Paulo State, Brazil, were due to this species (Bucaretchi et al., 2000). The victims of bite complain of intense local pain (92.1%) and edema (33.1%). The accidents are classified as mild (89.8%), moderate (8.5%) and severe (0.5%) (Bucaretchi et al., 2000). Severe accidents occur mainly in children and elderly people who may develop acute pulmonary edema and eventually death. Experimental animals injected systemically with the whole venom show excitatory signs including salivation, flaccid followed by spastic paralysis of hindlimbs, tremors, spasms and tonic convulsions, which are indicate that venom toxins affect autonomic (Antunes et al., 1993; Gomez et al., 1995), peripheral (Fontana and Vital-Brazil, 1985; Cruz-Höfling et al., 1985; Love and Cruz-Höfling, 1986; Love et al., 1986) and central nervous system (Le Sueur et al., 2003, 2004; Zanchet et al., 2004).

Experimental studies have shown that the venom affects the excitability of nerve fibers by producing conformational changes in Na^+ channels that are highly concentrated at nodes of Ranvier, to allow inward current during depolarization to sustain saltatory conduction. Electrophysiological studies in sciatic nerve–soleus preparations of mice and on dorsal and ventral nerve roots of rats indicate that the venom delays inactivation of sodium channels (Baker et al., 1985; Cruz-Höfling et al., 1985). The electrophysiological disturbances were associated with striking changes in the appearance of myelinated fibers, which were prevented if pretreatment with tetrodotoxin, a sodium channel blocker, was applied (Cruz-Höfling et al., 1985; Love et al., 1986; Love and Cruz-Höfling, 1986). More recently, it was shown that the venom of *P. nigriventer* increases the microtubule-mediated transendothelial transport at the brain microvasculature in rats (Le Sueur et al., 2004). The paracellular transport at the periods then observed (1 and 9 days after envenoming) seemed unaffected, although the interendothelial cleft was seen filled with an intravenous (i.v.)-injected extracellular tracer (Le Sueur et al., 2003).

In this work, we investigate the permeation of the BBB of cerebellum and hippocampus to the extracellular tracer lanthanum nitrate in rats provoked by the intravenous injection of PNV at the first stages of the systemic envenoming (15 min, 1, 2 and 5 h). In addition, the integrity of the basement membrane (BM) of the microvessels was evaluated by the expression of its major protein, laminin through immunofluorescence. This study is part of a series aimed at characterizing the time course of the alterations involving impairment of the blood–brain barrier caused by the *P. nigriventer* spider venom.

2. Results

To investigate the time-course of BBB breakdown and the participation of basement membrane on it caused by PNV in hippocampus and cerebellum at acute stages of envenoming, 32 rats were divided into four treated groups and four control groups ($n=4$ rats/period after venom or saline i.v. injection, respectively).

Clinically, the animals presented immediate signs of intoxication after PNV injection, such as hyperemia, tremors, eye paleness, salivation and motionless. Whereas hyperemia and eye paleness disappeared almost as quickly as they appeared, the other signs persisted and some minutes after the animals also showed flaccidity followed by spastic paralysis of hindlimbs. These signs persisted until their sacrifice. Of the four rats used in each period of envenoming, one or two showed temporary tonic convulsion. Some animals that died by cardiac–respiratory arrest were discarded and have to be replaced. Necropsy of these animals revealed lung edema. Saline-injected animals were clinically normal in appearance.

LM histological observations of 1-μm-thick TB-stained sections showed that the neural parenchyma looked normal, and so neuronal and glial cells, both in saline- and PNV-injected rats. However, whereas all segments of the microvasculature of the cerebellum and hippocampus showed no abnormality in control groups, blood vessels with wide spaces around, characterizing perivascular edema were present in both regions of PNV-treated rats (Fig. 1).

Quantification of the affected vessels aimed at evaluating the extension of barrier impairment permitted estimation of the time-course of the alterations from 15 min to 5 h post-injection (p.i.). It also permitted comparison between hippocampus and cerebellum. At the hippocampus, a marked 7.4-fold increase of vessels with vasogenic edema was seen 1 h after PNV injection in comparison to controls ($P<0.01$). In the succeeding periods examined (2 and 5 h), there was reduction in number of hippocampal vessels with perivascular edema (Fig. 2A). In cerebellum, the perivascular edema was not so marked as for hippocampus, nor was so high as the percentage of affected vessels. Probably, as a consequence of this, the highest number of affected vessels in cerebellum was achieved later than in hippocampus, i.e., it occurred at 2 h p.i., after which there was reduction, being all changes not significant in

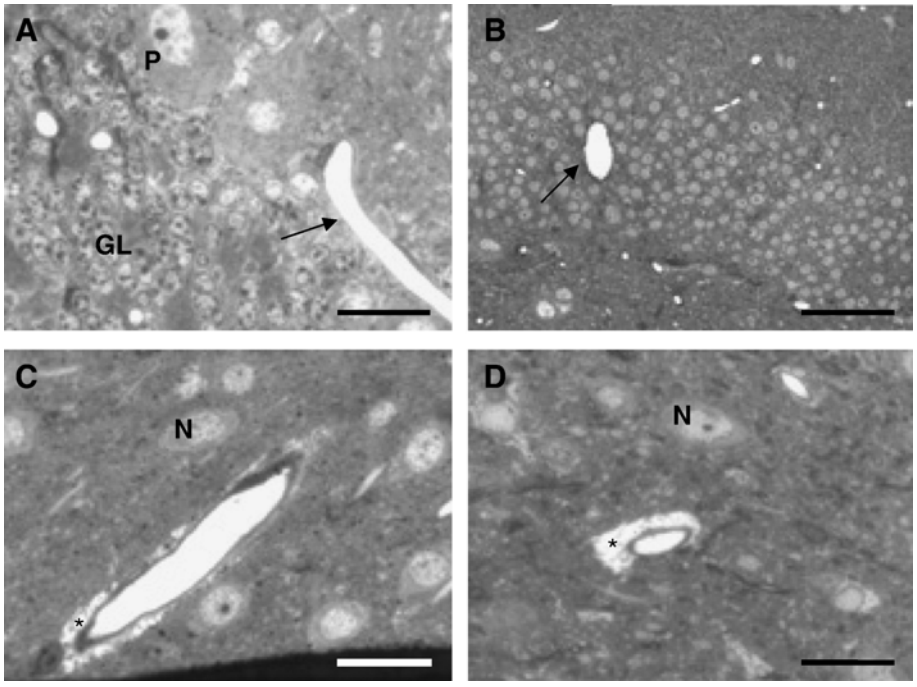

Fig. 1 – Light micrographs of toluidine blue-stained sections. (A, B) Cerebellum and hippocampus, respectively, injected with saline solution: no changes were observed around the blood vessels (arrows). (C) Cerebellum 1 h after PNV injection. (D) Hippocampus 2 h after PNV injection. Note a noticeable perivascular edema (asterisks), which probably represents fluid extravasation and hence breakdown of the blood–brain barrier. P – Purkinje cells; GL – granular layer; N – neuron. Bars: A, C and D = 20 μm; B = 50 μm.

relation to control (Fig. 2B) ($P<0.05$). See panels A and B of Fig. 2 to compare the curves of affected vessels in hippocampus and cerebellum. Comparison of both regions per time interval showed that difference in the percentage of vessels with vasogenic edema was significant only at 1 h p.i., being the hippocampus 2.6-fold higher than cerebellum ($P<0.05$).

Observations done by TEM revealed that in hippocampus a significant increase of vessels with tracer extravasation (17.4-fold higher) occurred after 2 h p.i. in comparison to matched controls ($P<0.001$). At 5 h p.i., the number of affected vessels continued significantly higher (9.8-fold) compared to controls ($P<0.05$), but significantly reduced at 2 h p.i. ($P<0.05$, Fig. 3A). Cerebellum showed significant gradual increase of vessels with tracer extravasation achieving 3.5- and 3.8-fold higher than controls at 1 and 2 h post-PNV, respectively ($P<0.05$) (Fig. 3B). On the other hand, although the number of affected vessels in cerebellum was in general smaller than in hippocampus at each of the periods studied, this difference was not significant.

Ultrastructural observations by TEM done in the hippocampus of animals injected with saline solution (sham group) showed the tracer confined into the vessels lumen and normality in the perivascular tissue (Figs. 4A–C). However, in vessels of PNV-injected animals, the tracer reached the basal lamina (referred to as basement membrane by LM) of endothelium and muscular layer (Figs. 4D–G), or was filling the endocytotic vesicles of endothelial cells (Figs. 4H, I). As for hippocampus, cerebellum of sham animals presented no leakage of the extracellular tracer from the lumen of vessels (Figs. 5A, B). In contrast, whereas in the cerebellum astrocyte end-feet swelling were found both in vessels without or with tracer extravasation (Figs. 5C, D), in hippocampus swollen astrocyte end-feet was never seen even in lanthanum-leaked vessels (Figs. 4D–G).

Laminin immunolabeling was obviously seen in places where BM was present. All the segments of microvasculature were well-labeled (see control groups in Figs. 6A, B for hippocampus, and Fig. 7A for cerebellum). As soon as 15 min PNV p.i., the laminin labeling decreased drastically in small vessels becoming practically negative in the capillaries of both regions, when compared with control groups. On the other hand, whereas medium-sized vessels of hippocampus maintained practically unaltered the expression of laminin at the BM (Figs. 6C, D), in the cerebellum a strong reduction was seen (Fig. 7B). Two hours p.i., recovery of the labeling in capillaries was better in cerebellum (Fig. 7C), than in hippocampus (Fig. 6E). At this very same period post-PNV, in comparison to controls, a slight recovery of laminin expression was seen in small hippocampal vessels (compare Figs. 6A and E), whereas it was outshining in cerebellum (compare Figs. 7A and C). After 5 h p.i., the laminin was overexpressed in small diameter vessels including capillaries with predominance in hippocampus over cerebellum (Figs. 6F, G, and 7D).

3. Discussion

The existence of a restrictive receptor- and transporter-mediated BBB is essential to provide a homeostatic chemical environment for the proper brain functioning. The main BBB component entitled to have these properties is the endothelial

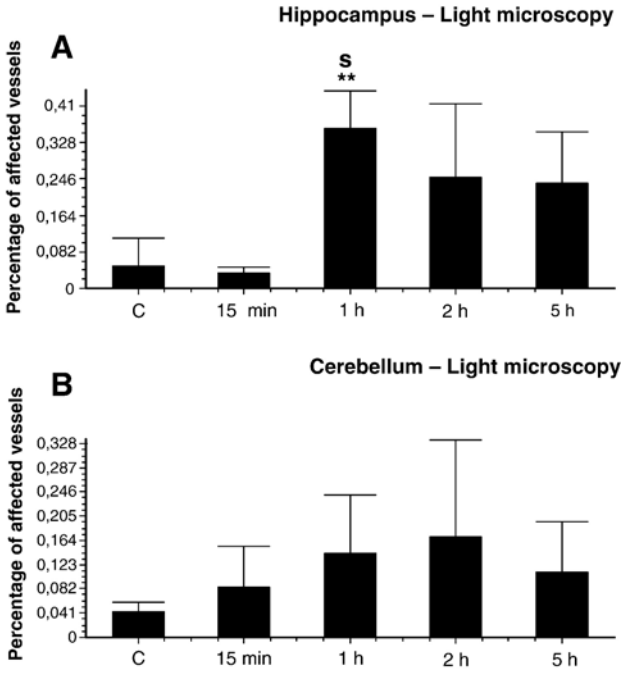

Fig. 2 – Percentage of hippocampal (A) and cerebellar (B) affected vessels (vasogenic edema) after PNV injection, in comparison to control. The counting was done in toluidine blue-stained sections (1 μm thick). Each bar represents the mean number of affected vessels per period (±SD) divided by the total number of vessels counted in twelve sections of ~1 mm^2 each, representing different sub-regions of hippocampus and cerebellum taken at random (n=4 rats×3 sections per rat; total=12 sections/time interval). Hippocampus – $^{**}P<0.01$ compared with control group; cerebellum – no significant difference was seen in relation to control ($P>0.05$). Comparison between cerebellum and hippocampus showed significant difference at 1 h ($^{\$}P<0.05$).

cells (ECs) of capillaries (followed by ECs of venules and arterioles). ECs preclude passage of macromolecules through a tight, belt-like occluding junctional complex located at adjacent EC membranes (Brightman and Reese, 1969; Lossinsky and Shivers, 2004), and permit a parsimonious vesicular transport across the EC cytoplasm (Reese and Karnovsky, 1966; Brightman and Reese, 1969). In addition, vicinal astrocytes, pericytes, neurons and the extracellular matrix of the BM work in concert with the cerebral microvascular endothelium to maintain the properties of the BBB.

The knowledge of how neurotoxic agents affect the BBB components and the existence of regional anatomic differences in this response is of primary importance to advance in the understanding of events involved in changes of permeability under a variety of conditions. Animal venoms are rich sources of bioactive substances. The identification of their targeted BBB components can open possibility to a large line of investigations. Yet, knowledge of BBB-acting natural substances and their pharmacological properties could be meaningful therapeutically.

P. nigriventer crude venom (PNV) is a cocktail of ion channels acting toxins (Gomez et al., 2002), the majority of which low molecular mass basic polypeptides (3500–9000 Da) (Schenberg and Pereira-Lima, 1966), described as activators or delayers of inactivation of sodium channels (Fontana and Vital-Brazil, 1985; Cruz-Höfling et al., 1985; Baker et al., 1985; Araújo et al., 1993), and blockers of calcium and potassium channels (Guatimosim et al., 1997; Cassola et al., 1998; Carneiro et al., 2003; Vieira et al., 2005), most of them excitatory and neurotoxic (Brazil and Vellard, 1925; Diniz et al., 1990; Rezende et al., 1991). In addition, a small parcel of histamine (0.06–1%) and serotonin (0.03–0.25%) (Diniz, 1963; Schenberg and Pereira-Lima, 1978) are also components of the venom. Some of the toxins abolished Ca^{2+}-dependent glutamate release, but did not alter the Ca^{2+}-independent secretion of glutamate when brain cortical synaptosomes were depolarized by KCl, an effect attributed to interference on voltage activated calcium channels (Vieira et al., 2005).

In this study, we investigated the time course (15 min, 1, 2 and 5 h) of BBB opening induced by intravenous injection of PNV using quantitative and morphological approaches on brain blood vessels for assessment of BBB permeability. In addition, the BM integrity of the brain vasculature was examined by immunodetection of laminin, its major component. Comparison of the time course of alterations in microvasculature was done in hippocampus and cerebellum. The results showed that the venom produced vasogenic edema

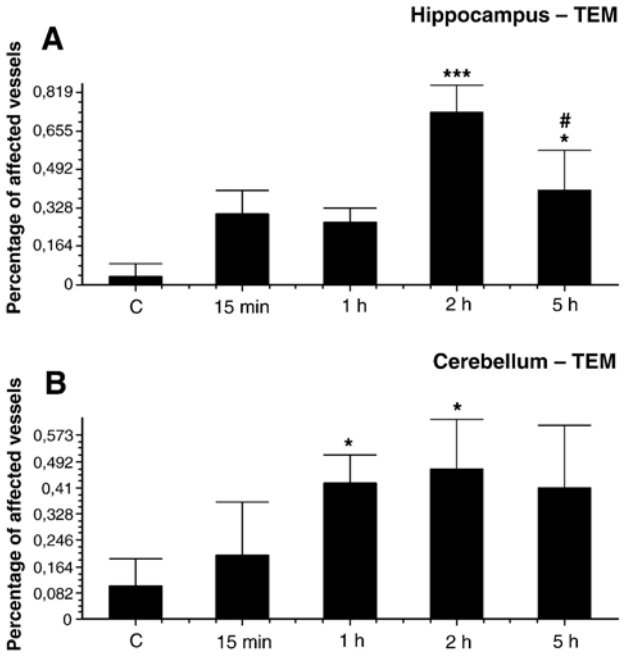

Fig. 3 – Percentage of hippocampal (A) and cerebellar (B) vessels presenting lanthanum nitrate leakage after PNV injection in comparison to control. The counting was done in double contrasted ultra-thin sections seen by transmission electron microscopy (TEM). Each bar represents the mean number of affected vessels per period (±SD) divided by the total number of vessels counted in one section (~1 mm^2) per rat at each time interval (n=4 rats×10 vessels per rat=40 vessels/time interval). Hippocampus – $^{*}P<0.05$; $^{***}P<0.001$ (compared with control group); $^{\#}P<0.05$ comparing 2 and 5 h. Cerebellum – $^{*}P<0.05$ compared with control group.

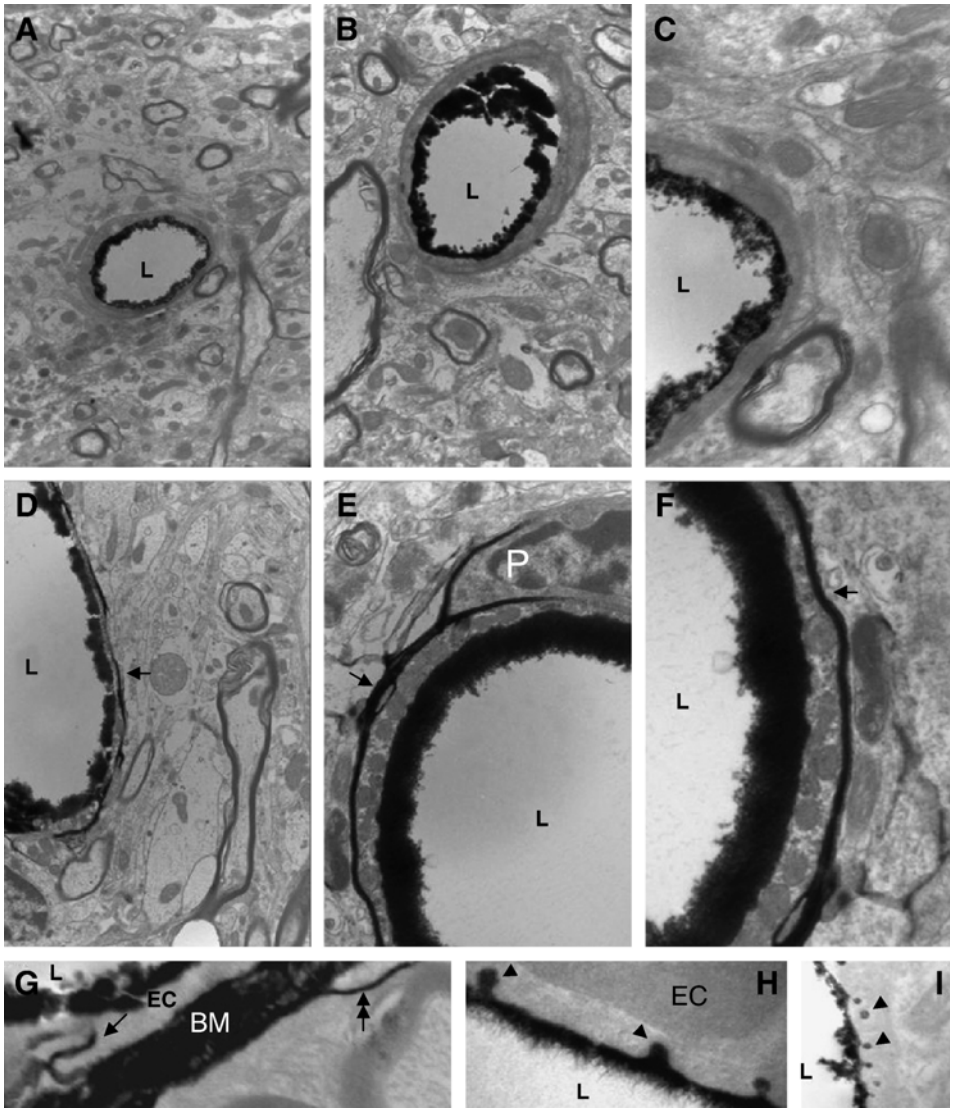

Fig. 4 – (A–C) Three views of hippocampal vessels and parenchyma of saline-injected rats (sham) showing lanthanum nitrate circumscribed at the luminal (L) space. The surrounding neural tissue appears normal. (D–G) Images at 2 h post-PNV injection. The extracellular tracer appears in basement membranes (BM) of endothelial cell (EC) (arrows in panels D–F, and BM in panel G), involving pericytes (P, in panel E), in the basement membrane of muscular layer (double-headed arrow in panel G) and into the inter-endothelial cleft (arrow in panel G). Arrowheads point the uptake of the luminal tracer by endocytosis and fluid-phase pinocytotic vesicle pinching off from the endothelial cell surface. Note that the circumjacent tissue around affected vessels is apparently normal in all panels. Also note that any of the affected vessels is a capillary. The same findings are seen 5 h after i.v. injection of PNV (not shown). Bars: A=1 μm; B–D=0.5 μm; E–I=0.25 μm.

and tracer extravasation faster and severalfold higher in hippocampal's than in cerebellar microvessels, that appearance of reactive astrocytes with swollen perivascular end-feet processes (present even in vessels without visible tracer extravasation) was exclusive for cerebellum, and that an immediate (15 min p.i.) and total degradation of laminin occurred in the capillary network, followed to a moderate extent in medium-sized blood branches. At the subsequent periods there was a gradual remodeling of the BM. Laminin immunolabeling recovered faster in capillaries of cerebellum than in hippocampus', a condition that was inverted at 5 h p.i., when hippocampus surpassed cerebellum and visually presented an overexpression of laminin in all post-capillaries segments, including in relation to the basal expression of control saline-injected samples (sham animals). Our present data on the number of vessels with perivascular edema, and BM laminin expression, seen by LM, showed that changes are transient. These short-lived changes were comparable to the striking alterations of myelinated fibers seen 15 min after intrasciatic nerve injection of PNV, and which were greatly resolved at 24 h p.i. (Cruz-Höfling et al., 1985). The authors attributed the changes to osmotic disturbance due to excess of Na^+ influx, followed by fluid, ought to the activation of axolemmal voltage-gated sodium channels by PNV.

Overall the results indicate increased permeability of BBB with substantial difference in timing and extent of these

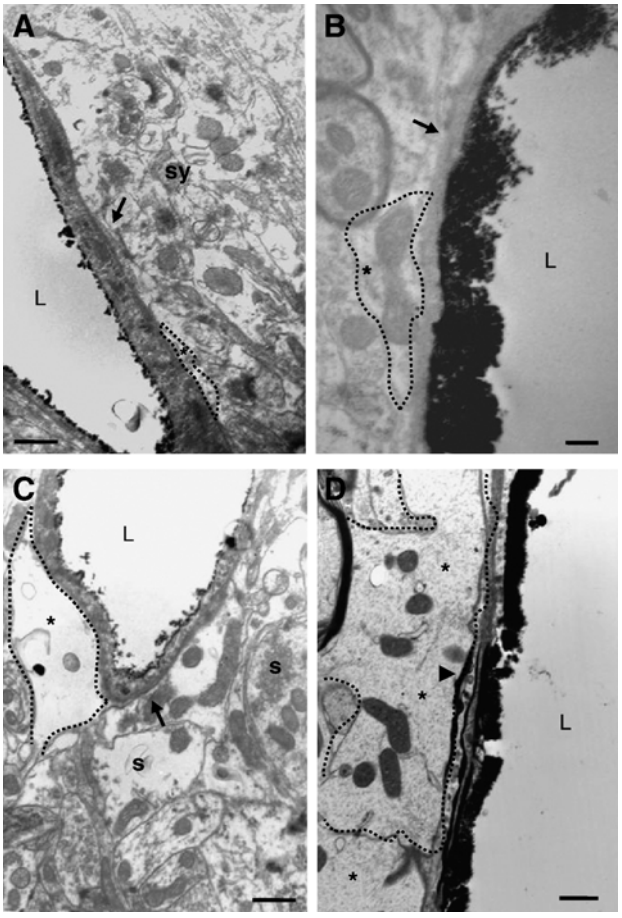

Fig. 5 – (A, B) Electron micrographs of sections of cerebellum tissue of saline-injected rats (sham) showing the neural tissue looking normal (asterisk) and the tracer confinement at the vessels' lumen (L). No lanthanum tracer is reaching the basal lamina (arrow). Axon ending synapse (sy) is normal in appearance. (C, D) At 2 h p.i.: perivascular edema of astrocytes end-feet (asterisks) and presence of tracer impregnating the basal lamina (arrowhead). Note that in panel C (envenomed rat) despite the absence of the tracer out of the vessel lumen there is swelling of the glial ending processes (asterisk) as well as of axon ending synapse (s). Dotted areas in panels C and D outline the edematous end-feet processes of envenomed specimens in comparison to the correspondent normal looking ones seen in control depicted in panels A and B. Bars: A, C and D=0.5 μm; B=0.25 μm.

permeability changes in cerebellum and hippocampus. However, varying susceptibility of individual animals to the PNV treatment was found. This was expressed by differences in the intensity and extent of vessels leakage and vasogenic edema of animals.

BM, a specialized type of extracellular matrix (ECM), completely underlies the endothelial cells of cerebral microvessels, surrounds the pericytes, and is ~99% invested over by the end-feet processes of astrocytes. By their turn astrocytes have their own BM, referred to as parenchymal BM (pBM) (Sixt et al., 2001). Together with endothelium and astrocytes, both BMs represent critical physical barriers to free movement of molecules and cells at the blood–brain interface. pBM is produced by astrocytes and associated leptomeningeal cells and is present in post-capillary venules (Reese and Karnovsky, 1966), therefore being these vessels ensheathed by two types of BMs. Both BMs involves self-association of matrix proteins and their binding to specific endothelial or astrocytic cell surface receptors, respectively, and so bridging their selves with cell cytoskeleton. Interactive roles of all these closely apposed components in the BBB physiology are predictable. In this sense, matrix proteins can influence the expression of cell-to-cell tight and adherens junction proteins located at adjacent ECs membranes (Tilling et al., 1998; Savettieri et al., 2000). However, endothelial and parenchymal BMs are biochemically distinct and hence potentially feasible of being distinctly influenced by a given effector. Endothelial BM expresses laminin isoforms 8 and/or 10, whereas parenchymal (astrocytic) BM expresses laminin isoforms 1 and 2 (Sixt et al., 2001). Contact endothelium-to-matrix is established via laminin to integrin receptors (Hynes, 1992). Conversely, the contact astrocyte end-feet-to-matrix is established via laminin, perlecan and agrin to dystroglycan extracellular (α-) or transmembrane (β-) receptors (Sixt et al., 2001; Agrawal et al., 2006). Molecular heterogeneity of matrix proteins and cell receptors proteins in both BMs presupposes, and was proven to evoke differential interactions with T-cell lines emigrating into the CNS (Sixt et al., 2001), with pro-inflammatory cytokines, angiostatic agents (Agrawal et al., 2006), and/or either endogenously, through an unbalanced matrix metalloproteinases (MMP) activity (Lukes et al., 1999). Likely, each of the BMs could react differently to xenobiotics in the circulation, such as PNV. In light of these discoveries, we conjecture if the difference of action of *P. nigriventer* venom on the expression of laminin isoforms in capillaries and post-capillaries segments could be ought, at least partially, to differences in BM characteristics in these types of vessels.

Although very restrict into the CNS, and typically noticeable at the BM, vascular ECM disruption has been strongly associated to increased permeability of the BBB in several pathological states (see Hawkins and Davis, 2005), predicting so its role in the blood–brain interface. Whether the differences in BBB tightness between cerebellum and hippocampus, seen in the current study, could be ascribed to qualitative and quantitative differences in the characteristics of endothelial and parenchymal BMs is elusive. Whether the fact of astrocytic end-feet swelling be only a manifestation of cerebellar vessels, even in those vessels with no visible tracer leakage, has to do with these BM dissimilarities is also unknown.

Astrocytes have been suggested to play several roles in the complex control of brain microenvironment (see Ballabh et al., 2004). Astrocytes express several membrane proteins and enzymes that are critical for calcium-mediated uptake of glutamate at the synapses, ammonia detoxification, buffering of extracellular K^+, and volume regulation (reviewed by Benarroch, 2005). Disruption of ionic homeostasis (as can be brought about with circulating *P. nigriventer* venom containing ion channel-acting toxins) is one of the factors leading to water influx into astrocytes and as consequence the edema formation (Panickar and Norenberg, 2005). Recent studies have

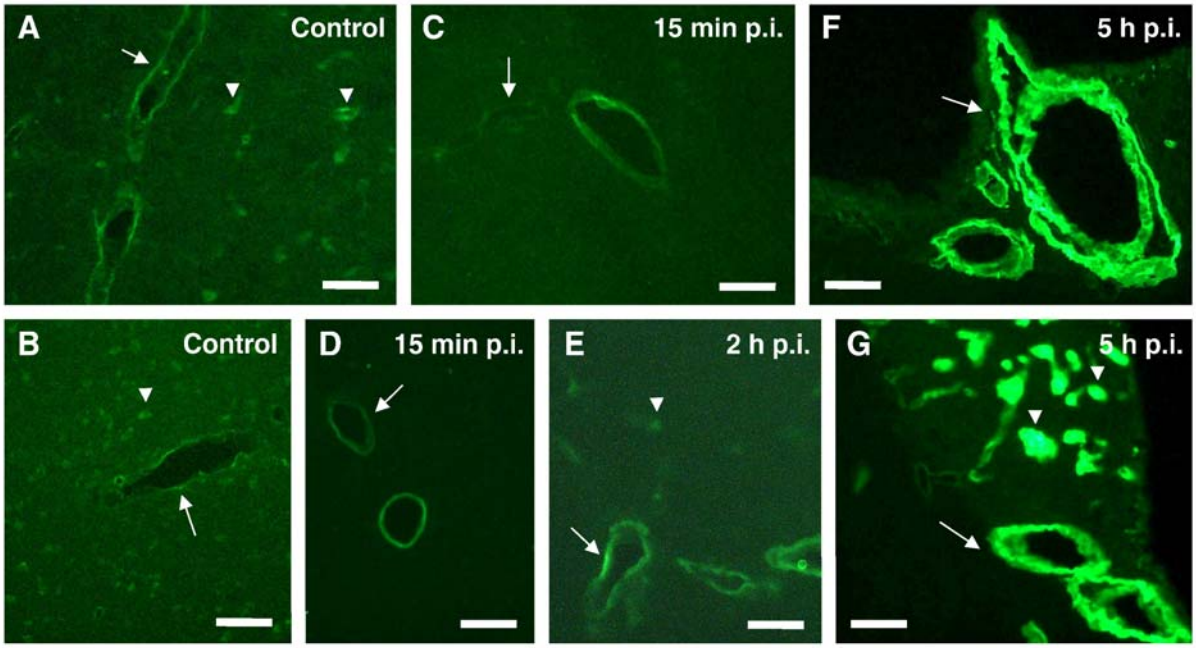

Fig. 6 – Laminin expression in hippocampal vessels. (A, B) Saline-injected (sham): this component of the basement membrane is being expressed in all sort of vessels of the microvasculature (arrows and arrowheads). (C, D) At 15 min p.i. of venom, there was a complete disappearance of laminin expression in the capillaries and pre- and post-branches of the capillary network. (E) At 2 h after PNV, laminin labeling recovered incipiently in smaller segments and was modestly increased in higher caliber ones. (F, G) At 5 h after PNV injection, laminin was overexpressed both in capillaries (arrowheads) and in major vessels (arrows) in comparison to controls. Bars: A–C = 80 μm; D–G = 40 μm.

suggested that aquaporin-4 (AQ4), a water-transporting protein, is the primary route by which water moves in and out of astrocytes and contributes to astrocytic swelling. AQ4 is upregulated in astrocytes in hypoxia and increases vascular permeability in the rat cerebellum suggesting these glial cells are involved in edema formation (Kaur et al., 2006). The homeostasy-related predicates of astrocytes imply their close involvement in rapid and extensive intracellular signaling pathways by second messengers. Immunolabeling studies detected proteins required for calcium and purinergic signaling in their vascular end-foot processes, besides to have demonstrated that ATP mobilizes cytosolic calcium in astrocytic end-feet. The study pointed astrocytes as a center for purinergic signaling and speculated "that calcium signaling may play a role in astrocytic functions related to the blood–brain barrier, including blood flow regulation, metabolic trafficking, and water homeostasis" (Simard et al., 2003). Purinoceptors were shown to be involved on neurogenic plasma extravasation evoked by *P. nigriventer* venom in rat dorsal skin (Costa et al., 2000). Experimentally, PNV causes sustained hypertension in anesthetized rats, after a short-lasting initial hypotension, the latter being partially attributed to ATP-dependent K^+ channel activation (Costa et al., 1996). The existence of astrocytes populations with different tuning in their function of ion buffers in cerebellum and hippocampus is yet undetermined. However, it is possible that these astrocytic signalization pathway and second messengers are responsible for the modulation in the responses of the cerebellum and hippocampus to PNV.

Our results also showed that despite the fact that vessels of small and medium calibers have modest changes in laminin expression, they were those which more exhibited vasogenic edema, as seen by LM. In such type of vessels, smooth muscle cells are interposed between astrocytes and ECs, as well as by both endothelial and parenchymal BMs (astrocytic) and as so submitted to their own intrinsic properties. It is likely that the laminin isoforms 1 and 2 from pBM could be more resistant to PNV-degrading effects than the laminin isoforms 8 and 10 from capillary BM. If so, it is an attractive hypothesis to think that laminin's higher resistance of medium-caliber vessels could be a compensatory mechanism developed to neutralize the less-efficient paracellular junctional apparatus between adjacent ECs of microvessel branches other than capillaries. Corroborating this view, previous studies with a similar experimental design, in which 18 h post-envenoming was the earliest period studied venules and arterioles were the former to be affected by PNV and this condition remained up to day 5. Only on day 9 post-PNV that capillaries showed tracer leakage. Yet, only vessels of hippocampus were vulnerable in the periods studied, whereas vessels of cerebral cortex, thalamus, hypothalamus and cerebellum were unaffected even at the earliest period (18 h p.i.) of the experiment (Le Sueur et al., 2003).

Our present findings show that laminin was overexpressed at the time point 5 h after PNV injection but edema and lanthanum extravasations were still relatively high after 5 h. Nonetheless, laminin was reduced as soon as 15 min after injection but edema and contrast extravasations appeared

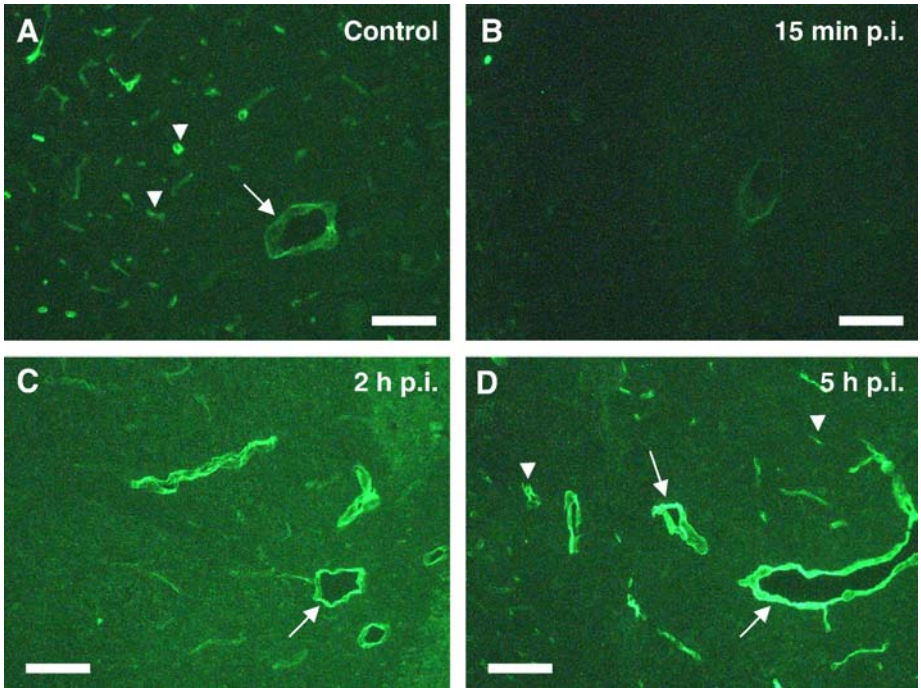

Fig. 7 – Light micrographs showing laminin expression in cerebellum vessels. (A) Sham control: observe the indiscriminate physiologic expression in basement membrane of major vessels (arrows) and capillaries (arrowheads). (B) PNV-treated rat (15 min p.i.): strong decrease of expression of this protein occurred in capillaries and other vessels of the microcirculation. (C) PNV-treated rat (2 h p.i.): there was a noticeable increase of laminin expression, in comparison to control and to 15 min PNV-treated rats. (D) At 5 h after PNV injection: note the overexpression of this protein in the basement membrane of vessels of the microvasculature (arrows and arrowheads). Bars: 80 μm.

only 1 h later. Although these results seem to be conflicting, actually the strong reduction of laminin expression at 15 min after PNV coincided with the immediate neurological signs of intoxication. Laminin degradation could be seen as the earliest molecular event of envenoming and BM disappearance one of the first visible anatomic target of the venom. In sequence, the aggravation of neurological signs will correlate to the peak at 1 h and 2 h of edema and tracer extravasation. Five hours post-PNV injection when animals start exhibiting signs of clinical recovery, laminin was overexpressed pointing for a BM supra-remodeling. This time point matched with the process of restoration of vascular integrity, as interpreted by the significant reduction of tracer leakage ($P<0.05$ in comparison to 2 h and $P<0.0001$ in comparison to controls). A recent study on biodistribution of TX2-6, a toxin from PNV (Cordeiro et al., 1992) in rats after intraperitoneal administration and using radiotracer (^{125}I-PnTX2-6) has shown that its maximum concentration in brain occurred between 5 min (0.623± 0.396%) and 15 min (0.507±0.130%), after which a gradual clearance occurred leading to a remains of 0.027±0.015% at 24 h. On the other hand, in thyroid the toxin concentration was ~0.607% at 5 min and increased to ~18.387% at 24 h p.i. (Yonamine et al., 2005). The findings indicate that the toxin lasts long in the circulating blood from which it was uptaken by gland. It also indicates that toxin rapid and immediate passage into brain is not obstructed by BBB, although soon after the access of the toxin into the brain seems to be impeded. These data corroborate with our findings related to laminin degradation and recovery and MB remodeling, as well with the transient effects on lanthanum leakage and vasogenic edema.

In contrast with findings of Le Sueur et al. (2003), which showed unaltered cerebellar vessels in periods ranging from 18 h to 9 days, in the present study the vessels of the cerebellum showed tracer leakage meaning that the process of BBB opening changes over time. The results of the current study, obtained from 15 min to 5 h post-PNV confirmed that the BBB of cerebellum was significantly more resistant than hippocampal's (~13% for cerebellum *versus* 35% for hippocampus of vessels with vasogenic edema at 1 h). At 2 and 5 h, the changes were not significant, although cerebellum continued less affected than hippocampus. On the other hand, compiling the data of the current study and those of Le Sueur et al. (2003), it was seen that the percentage of vessels with vasogenic edema in hippocampus obtained by LM counting was ~3% (15 min), 35% (1 h), 25% (2 h), 23% (5 h), and 10% (18 h), 17% (24 h), 60% (3 days), 22% (5 days), and 11% (9 days), whereas in cerebellum it was ~8% (15 min), 13% (1 h), 17% (2 h) and 10 % (5 h). As aforementioned in the time elapsed between 18 h and 9 days p.i., vasogenic edema in vessels of cerebellum was not detected by LM. In summary, the results suggest that the increased vascular permeability is more modest and also a shorter-lasting event in cerebellum, since it apparently has been vanished in the interval between 5 and 18 h p.i., whereas it is more prominent and long-lasting in hippocampus (present as early as 15 min p.i. and prolonged until day 9 p.i.). The time course of changes obtained from TEM observations paralleled to those obtained from LM observations. Taken together the present

findings and comparing with those of Le Sueur et al. (2003) one can see that the percentage of vessels with tracer extravasation in hippocampus was about 29% (15 min), 25% (1 h), 72% (2 h), 40% (5 h), and 31% (18 h), 42% (24 h), 22% (3 days) 21% (5 days) and 39% (9 days). Seemingly, the percentage of vessels with tracer extravasation in cerebellum was about 19.5% (15 min), 43% (1 h), 47% (2 h), 40% (5 h) and was no longer seen from 18 h onwards. In brief, the results indicate fluctuations of the BBB permeability along time in hippocampus, whereas in cerebellum it is likely a self-limited permeation event. The fact percentage of affected vessels be in general higher when calculated with TEM than with LM could be attributed to vessels with tracer leakage be only detected by TEM resolution. Exception was given in PNV-1 h group, which showed in hippocampus a percentage of affected vessels higher when counted by LM than by TEM. A possible explanation could be that LM quantification included all the vessels present in 12 sections per time interval in each animal group, whereas in TEM quantification we counted always 40 vessels (taken at random) at each time point per group. It is likely that tracer leakage was unapparent at this very initial step of envenoming, despite unnoticed changes could be already in course.

In the brain, the first physical contact of the circulating venom was with the luminal surface of endothelial cells (ECs) of vessels of all calibers. Vascular permeability is controlled primarily by tight junction proteins which seal passage between adjacent ECs (Gloor et al., 2001; Stevenson and Keaon, 1998). In addition, adherens junctional proteins and proteins which establish contact between adjacent ECs, ECs and BM and the cytoskeleton stress fibers complete the apparatus responsible for closure of the paracellular route (Yap et al., 1998; Lum and Malik, 1994; Huber et al., 2001a,b). Conformational changes or disassembly of cell–cell or cell–matrix proteins can unseal this passage. Also disturbances of receptors and transporters involved in the restrictive transcellular transport alter permeability. A number of pathological conditions can impair the selective transcellular transport (Kirk et al., 2003), and/or unfasten paracellular junctional complex and alter brain–blood exchanges.

In a subsequent study of our group (Le Sueur et al., 2004), we investigated the mechanisms involved in BBB breakdown (BBBb) in hippocampus at 1 and 9 days after PNV injection. At these time intervals it was shown that the vascular permeability occurred by the BBBb of the microtubule-mediated transcellular transport, since the treatment with colchicine prior to PNV significantly prevented the leakage of vessels to tracer. On the other hand, investigation on the expression and phosphorylation of some tight- (ZO-1 and occludin) and adherens junction-associated (β-catenin) proteins in hippocampal homogenate and hippocampal microvessels homogenate to assess the involvement of the paracellular barrier did not show significant changes, then precluding participation of this route in the BBBb caused by PNV, at least in these time intervals.

In spite of this finding, the way(s) in which lanthanum nitrate permeates the BBB after systemic presence of PNV have not yet been established unequivocally in our model. The disturbances, either on transcellular or paracellular pathway of the BBB can be transient if the noxious stimulus is suppressed, or the organism reaction was able to neutralize it by restoring the original molecular status. It was this latter hypothesis that seems to happen in our model since venom apparently continues in circulation for an appreciable time. It was seen that in hippocampus the impairment of the BBB had alternating periods of worsening and recovery, and periods examined could coincide with moments of relapse or not.

On top of it, phosphorylation of junctional proteins pointed out as an important regulatory mechanism controlling junctional permeability is an extremely fast process, turning the actual scenario which excludes tight and adherens junctions as target for PNV quite questionable. Nonetheless, *in vitro* studies on the direct effect of PNV in MDCK-cultured cells showed increased endocytosis of horseradish peroxidase, and at same time increased the transendothelial electric resistance of the monolayer, suggesting that the more permeable transcellular route, the more tight-resistant the paracellular one (Le Sueur et al., 2005).

Since the disturbances of BBB permeability here saw were an uneven event, as occurred in hippocampus, it is not unrealistic to suggest that at the molecular point of view the mechanisms that regulates BBB (involved in controlling the transcellular and paracellular traffic of solutes) could be alternatively up- and downregulated after exposure of endothelium to venom. In this sense, this mechanism could be distinctly modulated in cerebellum and hippocampus. Yet, as for cerebellum, these molecular changes could be transient, if the molecular recovery prevails over the noxious stimulus or either if the toxicant agent is suppressed, or both. Yet, we cannot discard that proteins of the TJ could be disturbed temporally in an intermittent manner by the action of PNV.

Since circulating PNV is supposed to reach all encephalic anatomic areas, the differences here seen could probably be ascribed to subtle differences in the regional molecular composition and constitution of the BBB components.

Taken together all these points reflect the complex interactions established by the various regulatory components of the BBB. It reflects also that subjacent alterations can be in course without a paralleled morphological (and/or clinical) visibility and vice-versa.

4. Experimental procedures

4.1. *Animals and venom*

Male Wistar rats (200–300 g) obtained from an established colony maintained by the Central Animal House Service at UNICAMP were housed in a temperature-controlled room (25–28 °C) on a 12-h light/dark cycle with lights on at 6 a.m. and fed standard Purina chow with free access to water. One lot of lyophilized *P. nigriventer* crude venom (PNV) was donated by Instituto Butantan (São Paulo, SP, Brazil). The venom stored at −20 °C was dissolved in 0.9% sterile saline solution immediately before use.

4.2. *PNV envenoming*

Male Wistar 7- to 10-week-old rats were divided in two major groups for light and electron microscopy. A group received a single intravenous (i.v.) injection of PNV (850 μg/kg in 0.5 ml

of 0.9% sterile saline) in the tail vein, while the control group was given the same volume of vehicle. The PNV concentration was selected according to the study of Le Sueur et al. (2003, 2004).

The experimental protocol was approved by the University's Committee for Ethics in Animal Experimentation (Proc. 702-1) and followed the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

4.3. *Morphological studies*

To investigate if PNV induced BBB breakdown in early stages, rats were anaesthetized at 15 min, 1, 2 and 5 h (n=4 rats/time interval) after venom systemic injection and immediately sacrificed by transcardiac perfusion with 150 ml of prefixative solution (100 mM Tris, pH 7.2, 150 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 3.7 mM glucose and 3.6 mM procaine) followed by 250 ml of fixative [2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 containing 2% lanthanum nitrate ($LaNO_3$)] as an extracellular marker using a peristaltic pump. Rats of control group, injected with sterile saline, were anesthetized and received the same pre-fixative and fixative solution, at the same time intervals (n=4 rats/period). The perfusion pressure was monitored with a mercury manometer to never exceed 70 mm Hg.

After perfusion, the rats were maintained at 4 °C overnight (18 h) before dissecting the brains. Hippocampus and cerebellum were excised and 1 to 2 mm samples were kept in the same fixative without lanthanum nitrate for 1 h. The samples of cerebellum comprised both white and grey matter, and the hippocampus comprised all sub-regions, taken at random. After, the samples were rinsed in washing solution (0.15 M NaCl plus 0.2 M sucrose), post-fixed in 1% OsO_4 diluted in the same solution, dehydrated in a graded acetone series and embedded in Epon 812. For light microscopic (LM) examination, semithin sections (1 μm thick) were cut on an ultramicrotome (Reichert S Ultra-Cut, Leica) and stained with 1% toluidine blue (TB). For transmission electron microscopy (TEM) studies, ultrathin sections (60 nm thick) were obtained of selected blocs of hippocampus and cerebellum regions after histological examination of TB sections. The ultrathin sections were mounted on copper grids (200 mesh) and double-contrasted with uranyl acetate and lead citrate for examination in a LEO 906 (Zeiss, Oberkochen, Germany) transmission electron microscope operated at 60 kV.

4.4. *Immunofluorescence studies (laminin–BM)*

PNV- and saline-injected animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) at 15 min, 1, 2 and 5 h (n=4 rats/time interval) post-injection (p.i.) of venom and then transcardially perfused with 150 ml physiological saline followed by 250 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS). Then, the brain of each animal was removed, post-fixed with the same fixative for 2 h and cryoprotected with 30% sucrose overnight, after which the brain was frozen in liquid nitrogen, followed by n-hexane and maintained at −80 °C until use. Cryostat sections (10 μm thick) from cerebellum and hippocampus were serially collected using a cryostat (Micron HM 505 E, USA). The sections were maintained in 0.01 M PBS with 1% bovine serum albumin (BSA) for 1 h. The brain sections were kept overnight at 4 °C in rabbit polyclonal laminin antibody, at a dilution of 1:100 (Chemicon, USA). The secondary antibody utilized was anti-Rabbit IgG FITC conjugated (1:100; Sigma, USA). Specificity of the immune reactions was controlled by omitting the primary antiserum. The sections were mounted in glycerol gelatin and the intensity of laminin labeling was analyzed by fluorescence microscopy. The images of all periods studied (saline- and PNV-treated) were captured using a Nikon Eclipse E800 light microscope (Japan) equipped with Image-Pro Plus image analyzer software (USA).

4.5. *Quantitative analysis*

Both affected and unaffected blood vessels were counted by LM using semithin sections, and TEM using ultrathin sections. The criteria used for considering blood vessels as affected was existence of perivascular edema (seen by LM), or presence of $LaNO_3$ impregnating the interendothelial cleft and/or filling three or more pinocytotic vesicles inside the endothelial cell cytoplasm (seen by TEM). Vessels with $LaNO_3$ reaching the underlying basal lamina (corresponding to BM when by LM) or deeper, were also considered affected. Vessels with swollen astrocyte end-feet when not accompanied by $LaNO_3$ extravasation were not considered leaked.

The percentage of vessels with vasogenic edema was calculated in three semi-thin sections (LM examination) of hippocampus and cerebellum, where their regions (Ca1, Ca2, Ca3 and grey and white matter, respectively) were randomly present. The percentage was calculated by dividing the number of affected vessels by the total number of vessels per section (~1 mm^2) per animal. A total of 12 sections was examined in PNV- and saline-treated groups (3 sections×4 animals=12 sections/~1 mm^2 each of the hippocampus and cerebellum regions) per time interval. The quantification was done by two observers.

For TEM, the evaluation of the tracer leakage was done by counting at least 10 randomly selected microvessels (arterioles, venules and capillaries) in one ultrathin section (~1 mm^2) of cerebellum and hippocampus per animal (40 vessels per time interval after PNV or saline injection). The percentage of vascular leakage was calculated by dividing the number of affected vessels by the total number of vessels examined in that section. Besides vessels quantification, the morphological characteristics of the surrounding parenchyma of PNV-injected and controls were compared.

The percentages of blood vessels affected were compared between PNV-injected and saline-injected (control) groups at each time interval, both in the hippocampus and cerebellum regions. The percentage of affected vessels/per period was also compared between hippocampus and cerebellum to evaluate susceptibility of each region to venom at each time point.

The results were expressed as means±SD using one-way analysis of variance (ANOVA) followed by Tukey–Kramer Multiple Comparisons Test to compare treated and control groups. A P value of <0.05 indicated statistical significance.

Acknowledgments

This work has been funded by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP # 98/01999-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq # 522131/95-6). We gratefully acknowledge the Butantan Institute for venom donation and Ms. Marta B. Leonardo MSc for excellent technical assistance. C.R. and G. M.Z. are recipients of scholarships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

Abbott, N.J., 2002. Astrocyte–endothelial interactions and blood–brain barrier permeability. J. Anat. 200, 629–638.

Agrawal, S., Anderson, P., Durbeej, M., van Rooijen, N., Ivars, F., Opdenakker, G., Sorokin, L.M., 2006. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. J. Exp. Med. 203, 1007–1019.

Antunes, E., Marangoni, R.A., Borges, N.C.C., Hyslop, S., Fontana, M.D., De Nucci, G., 1993. Effects of *Phoneutria nigriventer* venom on rabbit vascular smooth muscle. Braz. J. Med. Biol. Res. 26, 81–91.

Araújo, D.A., Cordeiro, M.N., Diniz, C.R., Beirão, P.S., 1993. Effects of a toxic fraction, PhTx2, from the spider *Phoneutria nigriventer* on the sodium current. Naunyn-Schmiedebergs Arch. Pharmacol. 347, 205–208.

Baker, M., Bostock, H., Brook, G.A., Love, S., 1985. *Phoneutria* and *Leiurus* venoms induce spontaneous compound action potentials in rat spinal roots. In Proceedings of the Physiological Society, J. Physiol. pp. 366, 363.

Ballabh, P., Braun, A., Nedergaard, M., 2004. The blood–brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol. Dis. 16, 1–13.

Benarroch, E.E., 2005. Neuron–astrocyte interactions: partnership for normal function and disease in the central nervous system. Mayo Clin. Proc. 80, 1326–1338.

Brazil, V., Vellard, J., 1925. Contribuição ao estudo do veneno das aranhas. Mem. Inst. Butantan 2, 5–77.

Brightman, M.W., Reese, T.S., 1969. Junctions between intimately-apposed cell membranes in the vertebrate brain. J. Cell Biol. 40, 648–677.

Bucaretchi, F., Deus Reinaldo, C.R., Hyslop, S., Madureira, P.R., De Capitani, E.M., Vieira, R.J., 2000. A clinico-epidemiological study of bites by spiders of the genus *Phoneutria*. Rev. Inst. Med. Trop. 42, 17–21.

Carneiro, A.M.D., Kushmerick, C., Koenen, J., Arndt, M.H.L., Cordeiro, M.N., Chavez-Olortegui, C., Diniz, C.R., Gomez, M.V., Kalapothakis, E., Prado, M.A.M., Prado, V.F., 2003. Expression of a functional recombinant *Phoneutria nigriventer* toxin active on K^+ channels. Toxicon 41, 305–313.

Cordeiro, M.N., Diniz, C.R., Valentim, A.C., von Eickstedt, V.R., Gilroy, J., Richardson, M., 1992. The purification and amino acid sequences of four Tx2 neurotoxins from the venom of the Brazilian 'armed' spider *Phoneutria nigriventer*. FEBS Lett. 310, 153–156.

Cassola, A.C., Jaffe, H., Fales, H.M., Castro Afeche, S., Magnoli, F., Cipolla-Neto, J., 1998. Omega-phonetoxin-IIA: a calcium channel blocker from the spider *Phoneutria nigriventer*. Pflugers Arch. 436, 545–552.

Costa, S.K., Moreno Jr., H., Brain, S.D., De Nucci, G., Antunes, E., 1996. The effect of *Phoneutria nigriventer* (armed spider) venom on arterial blood pressure of anaesthetised rats. Eur. J. Pharmacol. 298, 113–120.

Costa, S.K., De Nucci, G., Antunes, E., Brain, S.D., 2000. Involvement of vanilloid receptors and purinoceptors in the *Phoneutria nigriventer* spider venom-induced plasma extravasation in rat skin. Eur. J. Pharmacol. 391, 305–315.

Cruz-Höfling, M.A., Love, S., Brook, G., Duchen, L.W., 1985. Effects of *Phoneutria nigriventer* spider venom on mouse peripheral nerve. Q. J. Exp. Physiol. 70, 623–640.

Diniz, C.R., 1963. Separação de proteínas e caracterização de substâncias ativas em venenos de aranhas do Brasil. An. Acad. Bras. Cienc. 35, 283–291.

Diniz, C.R., Cordeiro, M.N., Junor, L.R., Kelly, P., Fischer, S., Reimann, F., Oliveira, E.B., Richardson, M., 1990. The purification and amino acid sequence of the lethal neurotoxin Tx1 from the venom of the Brazilian "armed" spider *Phoneutria nigriventer*. FEBS Lett. 263, 251–253.

Fontana, M.D., Vital-Brazil, O., 1985. Mode of action of *Phoneutria nigriventer* spider venom at the isolated phrenic nerve-diaphragm of the rat. Braz. J. Med. Biol. Res. 18, 557–565.

Gloor, S.M., Wachtel, M., Bolliger, M.F., Ishiaha, H., Landmann, R., Frei, K., 2001. Molecular and cellular permeability control at the blood–brain barrier. Brain Res. Brain Res. Rev. 36, 258–264.

Gomez, R.S., Casali, T.A., Romano-Silva, M.A., Cordeiro, M.N., Diniz, C.R., Moraes-Santos, T., Prado, M.A., Gomez, M.V., 1995. The effect of PhTx3 on the release of ^{3}H-acetylcholine induced by tityustoxin and potassium in brain cortical slices and myenteric plexus. Neurosci. Lett. 196, 131–133.

Gomez, M.V., Kalapothakis, E., Guatimosim, C., Prado, M.A., 2002. *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels. Cell. Mol. Neurobiol. 22, 579–588.

Grieb, P., Forster, R.E., Strome, D., Goodwin, C.W., Pape, P.C., 1985. O_2 exchange between blood and brain tissues studied with $18O_2$ indicator dilution technique. J. Appl. Physiol. 58, 1929–1941.

Guatimosim, C., Romano-Silva, M.A., Cruz, J.S., Beirão, P.S., Kalapothakis, E., Moraes-Santos, T., Cordeiro, M.N., Diniz, C.R., Gomez, M.V., Prado, M.A., 1997. A toxin from the spider *Phoneutria nigriventer* that blocks calcium channels coupled to exocytosis. Br. J. Pharmacol. 122, 591–597.

Hawkins, B.T., Davis, T.P., 2005. The blood–brain barrier/ neurovascular unit in health and disease. Pharmacol. Rev. 57, 153–185.

Huber, J.D., Egleton, R.D., Davis, T.P., 2001a. Molecular physiology and pathophysiology of tight junctions in the blood–brain barrier. Trends Neurosci. 24, 719–725.

Huber, J.D., Witt, K.A., Hom, S., Egleton, R.D., Mark, K.S., Davis, T.P., 2001b. Inflammatory pain alters blood–brain barrier permeability and tight junctional protein expression. Am. J. Physiol. Heart. Circ. Physiol. 280, 1241–1248.

Hynes, R.O., 1992. Integrins: versatility, modulations, and signaling in cell adhesion. Cell 69, 11–25.

Kaur, C., Sivakumar, V., Zhang, Y., Ling, E.A., 2006. Hypoxia-induced astrocytic reaction and increased vascular permeability in the rat cerebellum. Glia 54, 826–839.

Kirk, J., Plumb, J., Mirakhur, M., McQuaid, S., 2003. Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood–brain barrier leakage and active demyelination. J. Pathol. 201, 319–327.

Le Sueur, L., Collares-Buzato, C.B., Kalapothakis, E., Cruz-Höfling, M.A., 2005. In vitro effect of the *Phoneutria nigriventer* spider venom on cell viability paracellular barrier function and transcellular transport in cultured cell lines. Toxicon 46, 130–141.

Le Sueur, L., Kalapothakis, E., Cruz-Höfling, M.A., 2003. Breakdown of the blood–brain barrier and neuropathological changes induced by *Phoneutria nigriventer* spider venom. Acta Neuropathol. 105, 125–134.

Le Sueur, L.P., Collares-Buzato, C.B., Cruz-Höfling, M.A., 2004. Mechanisms involved in the blood–brain barrier increased permeability induced by *Phoneutria nigriventer* spider venom in rats. Brain Res. 1027, 38–47.

Lossinsky, A.S., Shivers, R.R., 2004. Structural pathways for macromolecular and cellular transport across the blood–brain barrier during inflammatory conditions. Histol. Histopathol. 19, 535–564.
Love, S., Cruz-Höfling, M.A., 1986. Acute swelling of nodes of Ranvier caused by venoms which slow inactivation of sodium channels. Acta Neuropathol. 70, 1–9.
Love, S., Cruz-Höfling, M.A., Duchen, L.W., 1986. Morphological abnormalities in myelinated nerve fibres caused by *Leiurus*, *Centruroides* and *Phoneutria* venoms and their prevention by tetrodotoxin. Q. J. Exp. Physiol. 71, 115–122.
Lukes, A., Mun-Bryce, S., Lukes, M., Rosenberg, G.A., 1999. Extracellular matrix degradation by metalloproteinases and central nervous system diseases. Mol. Neurobiol. 19, 267–284.
Lum, H., Malik, A.B., 1994. Regulation of vascular endothelial barrier function. Am. J. Physiol. 267, 223–241.
Neuwelt, E.A., 2004. Mechanisms of disease: the blood–brain barrier. Neurosurgery 54, 131–140.
Panickar, K.S., Norenberg, M.D., 2005. Astrocytes in cerebral ischemic injury: morphological and general considerations. Glia 50, 287–298.
Plumb, J., McQuaid, S., Mirakhur, M., Kirk, J., 2002. Abnormal endothelial tight junction in active lesions and normal-appearing white matter in multiple sclerosis. Brain Pathol. 12, 154–169.
Ramsauer, M., Krause, D., Dermietzer, R., 2002. Angiogenesis of the blood–brain barrier *in vitro* and the function of cerebral pericytes. FASEB J. 16, 1274–1276.
Reese, T.S., Karnovsky, M.J., 1966. Fine structural localization of a blood–brain barrier to exogenous peroxidase. J. Cell Biol. 34, 207–217.
Rezende, L.J, Cordeiro, M.N., Oliveira, E.B., Diniz, C.R., 1991. Isolation of neurotoxic peptides from the venom of the "armed" spider *Phoneutria nigriventer*. Toxicon 29, 1225–1233.
Savettieri, G., Di Liegro, I., Catania, C., Licata, L., Pitarresi, G.L., D'agostino, S., Schiera, G., De Caro, V., Giandalia, G., Giannola, L.I., Cestelli, A., 2000. Neurons and ECM regulate occludin localization in brain endothelial cells. Mol. Neurosci. 11, 1081–1084.
Schenberg, S., Pereira-Lima, F.A., 1966. Pharmacology of the polypeptides from the venom of *Phoneutria nigriventer*. Mem. Inst. Butantan 33, 627–638.
Schenberg, S., Pereira-Lima, F.A., 1978. Venom of the Ctenidae. Handbook of Experimental Pharmacology, vol. 48. Springer, pp. 217–244.
Schlageter, K.E., Molnar, P., Lapin, G.D., Groothuis, D.R., 1999. Microvessel organization and structure in experimental brain tumors: microvessel populations with distinctive structural and functional properties. Microvasc. Res. 58, 312–328.
Silva, G.H., Hyslop, S., Cruz-Höfling, M.A., 2004. *Lonomia obliqua* caterpillar venom increases permeability of the blood–brain barrier in rats. Toxicon 44, 625–634.
Simard, M., Arcuino, G., Takano, T., Liu, Q.S., Nedergaard, M., 2003. Signaling at the gliovascular interface. J. Neurosci. 23, 9254–9262.
Sixt, M., Engelhardt, B., Pausch, F., Hallmann, R., Wendler, O., Sorokin, L.M., 2001. Endothelial cell laminin isoforms, laminin 8 and 10, play decisive roles in T-cell recruitment across the blood–brain barrier in an EAE model. J. Cell Biol. 153, 933–945.
Stevenson, B.R., Keaon, B.H., 1998. The tight junction: morphology to molecules. Annu. Rev. Cell Dev. Biol. 14, 89–109.
Tilling, T., Korte, D., Hoheisel, D., Galla, H.J., 1998. Basement membrane proteins influence brain capillary endothelial barrier function *in vitro*. J. Neurochem. 71, 1151–1157.
Toborek, M., Lee, Y.W., Pu, H., Malecki, A., Flora, G., Garrido, R., Hennig, B., Bauer, H.C., Nath, A., 2003. HIV – Tat protein induces oxidative and inflammatory pathways in brain endothelium. J. Neurochem. 84, 169–179.
Vieira, L.B., Kushmerick, C., Hildebrand, M.E., Garcia, E., Stea, A., Cordeiro, M.N., Richardson, M., Gomez, M.V., Snutch, T.P., 2005. Inhibition of high voltage-activated calcium channels by spider toxin PnTx3-6. J. Pharmacol. Exp. Ther. 314, 1370–1377.
Werring, D.J., Brassat, D., Droogan, A.G., Clark, C.A., Symms, M.R., Barker, G.J., MacManus, D.G., Thompson, A.J., Miller, D.H., 2000. The pathogenesis of lesions and normal-appearing white matter changes in multiple sclerosis: a serial diffusion MRI study. Brain 123, 1667–1676.
Yap, A.S., Mullin, J.M., Stevenson, B.R., 1998. Molecular analyses of tight junction physiology: insights and paradoxes. J. Membr. Biol. 163, 159–167.
Yonamine, C.M., Costa, H., Silva, J.A.A.A., Muramoto, E., Rogero, J.R., Troncone, L.R.P., Camillo, M.A.P., 2005. Biodistribution studies of bee venom and spider toxins using radiotracers. J. Venom. Anim. Toxins Incl. Trop. Dis. 11, 39–50.
Zanchet, E.M., Longo, I., Cury, Y., 2004. Involvement of spinal neurokinins, excitatory amino acids, proinflammatory cytokines, nitric oxide and prostanoids in pain facilitation induced by *Phoneutria nigriventer* spider venom. Brain Res. 1021, 101–111.
Zhang, Y., Pardridge, W.M., 2001. Rapid transferring efflux from brain to blood across the blood–brain barrier. J. Neurochem. 76, 1597–1600.
Zlokovic, B.V., 2004. Clearing amyloid through the blood–brain barrier. J. Neurochem. 89, 807–811.

ELSEVIER

Brain Research Bulletin 73 (2007) 114–126

BRAIN RESEARCH BULLETIN

www.elsevier.com/locate/brainresbull

c-FOS and n-NOS reactive neurons in response to circulating *Phoneutria nigriventer* spider venom

Maria Alice da Cruz-Höfling [a,*], Gabriela Mariotoni Zago [a,b], Liana Lins Melo [c], Catarina Rapôso [a]

[a] *Departamento de Histologia e Embriologia, Instituto de Biologia, C.P. 6109, Universidade Estadual de Campinas (UNICAMP), Campinas, SP 13083-970, Brazil*

[b] *Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), Campinas, SP 13083-970, Brazil*

[c] *Laboratório de Neuropsicofarmacologia (NAAC2), Universidade São Francisco, Bragança Paulista, SP 12910-900, Brazil*

Received 11 July 2006; received in revised form 22 December 2006; accepted 24 January 2007
Available online 22 March 2007

Abstract

Drugs and neurotoxins activate specific neural circuits by increasing or decreasing the formation and release of neurotransmitters, such as nitric oxide (NO), and by inducing immediate early genes, such as FOS. We have previously shown that *Phoneutria nigriventer* spider venom (PNV) impairs the microtubule-dependent transcellular barrier of the blood–brain interface and causes structural alterations in perivascular astrocytic end-feet without producing morphological changes in central neuronal cells. In the present study, we used FOS and neuronal nitric oxide synthase (n-NOS) immunolabeling to investigate the ability of PNV to activate the central nervous system. Three groups of rats were used: the first group received a sublethal dose of PNV (850 μg/kg, via a tail vein), the second received an equal volume of 0.9% saline (sham group) and the third group received no injection. Envenomed rats showed salivation, lachrymation, tremors and flaccidity followed by spastic paralysis of the hind limbs and convulsions. Cryosections (30 μm thick) were serially collected at 600 μm intervals for free-floating immunohistochemical analysis. FOS-like positive neurons predominated in motor-related areas such as dorsolateral (dlPAG) and ventral periaqueductal gray matter (vPAG), frontal (FCM) and parietal motor cortex (PCM), and periventricular thalamic nucleus (PTN) and in acute stress-related areas (rhinal cortex and lateral septal nuclei). The greatest relative increases in FOS-like positive neurons occurred in the vPAG, PCM and PTN motor-related areas. n-NOS-positive neurons predominated in the periventricular thalamic nuclei, followed by the dorsolateral periaqueductal gray matter and parietal cortex motor area. The marked activation of motor areas and, to a lesser extent, of acute stress-related areas suggested the involvement of neuronal pathways in these regions in the response to envenoming by PNV. In addition, the occurrence of n-NOS immunolabeling in some anatomical regions with FOS-like positive neurons suggests that NO may modulate the response to PNV in these regions.

Keywords: Acute stress-related area; Brain; Convulsion; Motor-related area; Neuronal activation; Nitric oxide

1. Introduction

The induction of c-FOS immediate early genes in response to toxic stimuli in the central nervous system (CNS) can be used to indirectly monitor intercellular communication in central nervous tissue. FOS protein is a useful marker for monitoring neural activity in the CNS because its expression can be rapidly elicited by various stimuli [1,13,27]. Nitric oxide (NO) is an important messenger molecule in signal transduction pathways in the CNS [23]. Of the three isoforms of nitric oxide synthase (NOS), neuronal NOS (n-NOS) is the most abundant and is located mainly in neurons [15]. n-NOS and endothelial NOS (eNOS) are constitutively expressed in the CNS, where they play an essential role in several processes, including intracellular signaling, neurotransmission and vasoregulation [7,38]. Although increased NO formation has been associated with upregulation of inducible NOS (iNOS) during inflammatory/neurodegenerative diseases in the CNS, n-NOS and eNOS may also be involved. Despite n-NOS and eNOS are generally considered to be constitutive, under some circumstances, these enzymes are also inducible

* Corresponding author. Tel.: +55 19 3521 6247; fax: +55 19 3289 3124.
E-mail address: hofling@unicamp.br (M.A. da Cruz-Höfling).

0361-9230/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2007.01.017

[35,38]. The immunodetection of n-NOS and c-FOS has been extensively used as a marker of brain activation in numerous pathological and experimental conditions.

Phoneutria nigriventer (Keyserling, 1891) (Ctenidae, Araneomorphae), a wandering spider found in South America, is responsible for most bites by venomous spiders in São Paulo State, Brazil [8,24]. Bites by this species are classified as mild, moderate or severe based on the accompanying clinical manifestations. Mild bites are characterized by intense, radiating local pain that is generally followed by tremors, local swelling and erythema. Severe accidents, although rarer, additionally involve excitatory cardiovascular and neurological effects, such as arterial hypertension, tachycardia, arrhythmia, visual disturbances, priapism and tonic convulsions [6,50]. *P. nigriventer* venom (PNV) and its toxins have been extensively studied in the last two decades, with most interest focusing on its neurotoxic components [47]. The main neurotoxic action of the venom involves the activation or delayed inactivation of voltage-gated Na^+ channels, which can induce iterative action potential discharges in nerve and muscle fiber membranes and increase the frequency of miniature endplate membrane potentials [4,12,22]. In mice, the intra-muscular or intra-sciatic nerve injection of PNV causes spastic paralysis of the hind limbs and morphological alterations in the nodes of Ranvier of myelinic axons that are compatible with an increased influx of Na^+ that is followed passively by fluid [12,33,34]. These physiological and morphological alterations are abolished by pre-treatment with TTX, a Na^+ channel blocker. In experimental animals, the use of sublethal venom concentrations has shown dysfunction of the peripheral rather than the central nervous system.

We have recently shown that the intravenous injection of PNV causes vasogenic edema, swelling of the astrocytic end-feet processes around microvessels, and blood–brain barrier (BBB) breakdown in adult rats, particularly in the hippocampus [32]. The BBB impairment involved a microtubule-dependent increase in transendothelial vesicular transport, whereas paracellular transport was apparently unaffected in the intervals studied (24 h and 9 days post-injection) [30–32]. *P. nigriventer* venom also impairs the BBB in the cerebellum and hippocampus during the acute stages of envenoming (15 min, 1, 2 and 5 h) [44]. In the cerebellum, the number of vessels with edematous perivascular astrocytic end-feet processes is greater than in the hippocampus, whereas the number of vessels showing extravasation of an extracellular tracer is greater in the hippocampus than in the cerebellum.

Despite the evidence for a neurogenic origin of the clinical symptoms and effects induced by PNV in the BBB, no visible damage to neuronal bodies has been reported so far. Astrocytes play an important role in communication among many neuronal cells. Many of the functions of astrocytes in the CNS are related to their structural organization around the BBB [18]. These cells are essential for the development and maintenance of the BBB [2], with their role in neuronal homeostasis being essential for adequate synaptic transmission [40]. The expression of proteins that are essential for intercellular calcium signaling in the perivascular astrocytic end-feet facing the vessel wall, such as purinergic receptors and gap junctions, is further evidence that astrocytes are involved in cell signaling in the CNS [51].

In the present study, we used immunohistochemistry to investigate the ability of intravenously (i.v.) administered PNV to alter c-FOS and n-NOS expression in rat cortical and subcortical neurons.

2. Materials and methods

2.1. Animals and venom

Male Wistar rats were obtained from an established colony maintained by the university's Central Animal House Service and housed in a temperature-controlled room (25–28 °C) on a 12 h light/dark cycle with lights on at 6 a.m. and free access to standard Purina® chow and water. The experimental protocols were approved by the university's Committee for Ethics in Animal Experimentation (protocol no. 785-1) and were done in accordance with the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

Lyophilized *P. nigriventer* venom obtained by electrical stimulation of adult spiders of both sexes was supplied by the Instituto Butantan (São Paulo, SP, Brazil). The venom was stored at −20 °C and dissolved in sterile 0.9% saline solution immediately before use.

2.2. Experimental groups

Male Wistar rats (7–10 weeks old; 200–300 g) were divided into three groups (5 rats/group): (a) the first group received an i.v. injection of PNV via a tail vein (850 μg/kg in 0.5 ml), (b) the second group received the same volume of sterile 0.9% saline solution (sham group) and (c) the third group consisted of non-envenomed rats that were housed in a silent environment until sacrifice. The dose of PNV used was sublethal but produced severe intoxication in the rats and was based on previous studies in our laboratory [30,32]. At least 24 h before the experiment, the rats were transported in their cages from the animal house to the laboratory and allowed to acclimatize. Acoustic, visual and olfactory stimuli in the experimental room were kept to a minimum. All experiments were done between 9:00 a.m. and 1:00 p.m. in order to reduce circadian influences. Two hours after venom injection, the rats were anesthetized with ketamine–xylazine (100 and 10 mg/kg, respectively, i.p.) and perfused transcardially with 150 ml of physiological saline followed by 250 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS).

2.3. Immunohistochemistry

After anesthesia and perfusion fixation, the brain was removed, post-fixed with same fixative for 2 h and cryoprotected with 30% sucrose overnight. The brains were then frozen in liquid nitrogen and embedded in tissue freezing medium. Serial cryostat sections (30 μm thick) were collected in 16 wells containing cold PBS (free-floating sections). Free-floating sections were used because their thickness was almost the diameter of a neuron and this allowed the detection of a higher number of cells, which was advantageous for counting and subsequent comparisons. Consecutive wells received sections cut 600 μm apart. Immunohistochemical detection was done essentially as described by Pardutz et al. [41]. After pretreatment with 0.3% H_2O_2 to inhibit endogenous peroxidase, the free-floating sections were kept in 0.01 M PBS containing 1% Triton X-100 and 1% bovine serum albumin (BSA) and incubated for two nights at 4 °C with rabbit polyclonal n-NOS antibody (diluted 1:500). Other sections from the same areas (also collected serially) were incubated with rabbit polyclonal FOS antibody (diluted 1:2000) for three nights at 4 °C. The immunohistochemical reaction was visualized using an avidin-biotin kit (ABC - Staining System) and staining with 3,3′-diaminobenzidine. The specificity of the immune reactions was assessed by omitting the primary antiserum. All of the antibodies and the ABC kit were acquired from Santa-Cruz Biotechnology (Santa Cruz, CA, USA).

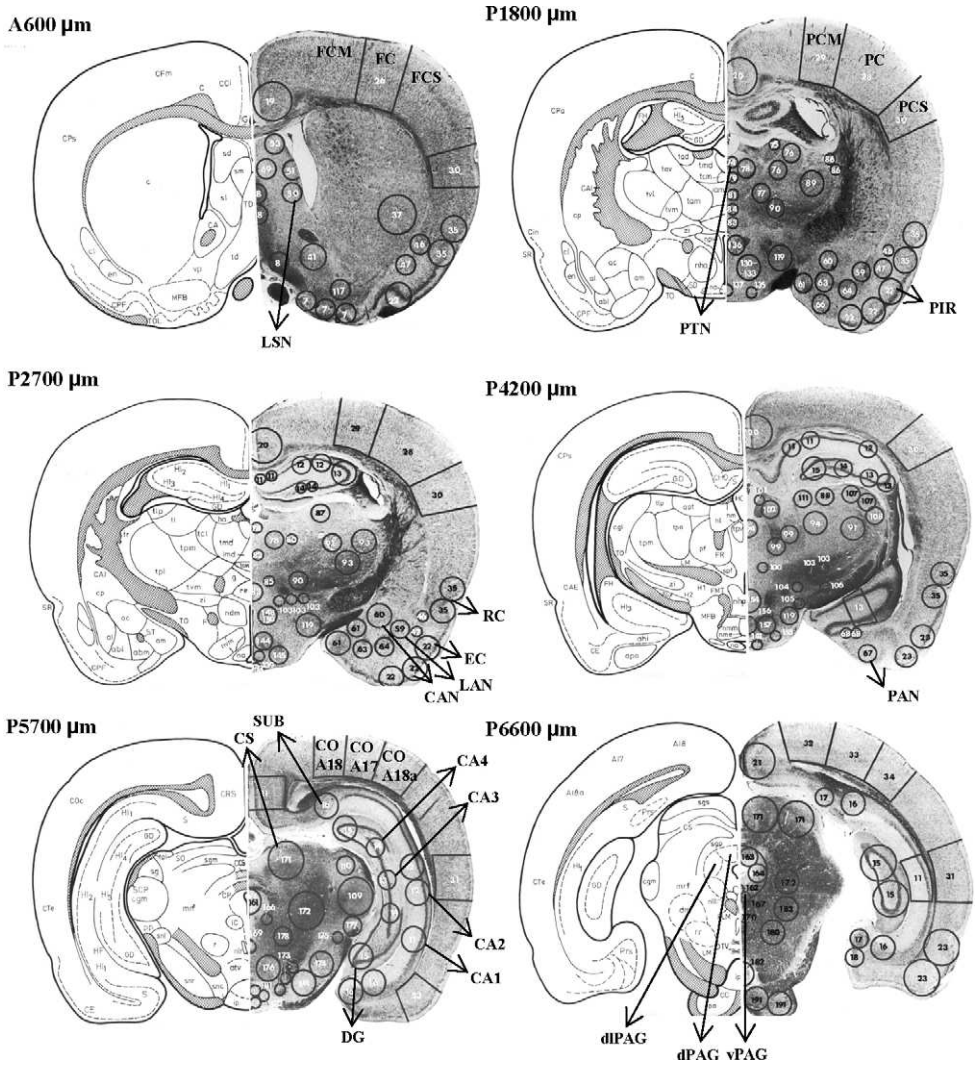

Fig. 1. Schematic representation of cross-sections of the brain showing the location of the areas in which FOS immunoreactivity was quantified. Brain areas were selected according to Palkovits and Brownstein [39]. Abbreviations are defined in Table 1.

2.4. Data analysis

The coordinates of the transversal planes and limits of the brain regions analyzed were selected using the stereotaxic atlas of Palkovits and Brownstein for rat brain [39]. The stereotaxic coordinates and regions examined are indicated in Fig. 1. Preliminary observations were done by light microscopy using a 10× objective (Leica, Germany) to identify the brain regions and the pattern of protein labeling. FOS reactivity was detected in neurons nuclei of the brain sections and appeared as brownish round nuclear-like dots whereas n-NOS was detected in cell bodies and neuronal processes. Both FOS-like and n-NOS-positive neurons were counted using 20× objective. The total number of rats used for counting c-FOS and n-NOS-positive neurons and for clinical observations was 15 ($n = 5$/group), i.e., all of the rats used were evaluated. For each rat, six sections (30 µm thick) from different stereotaxic coordinates were collected to ensure that all of the areas of interest were included (see Fig. 1). Each anatomical region was counted in only one section, i.e., in the section where it was more representative per rat. For example, PAG given its dimension appeared in two sections, but only the one where it was more complete was chosen. In general, a given area was seen in only two to three of the six sections examined.

For all of the brain regions examined, the total number of neurons positive for the proteins studied was obtained by counting them bilaterally in each of the six sections per rat ($n = 5$). For each anatomical region, the number of n-NOS and FOS-like positive cells was counted in an area of fixed size (0.35 mm^2) using a computer-aided image analysis system (Image ProPlus). The images were captured with an Olympus BX51 microscope coupled to a digital camera. The counting was done manually by one observer who was unaware of the group treatments. The results were expressed as the mean ± S.E.M. using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test to compare the treated and control groups. A P value less than 0.05 was considered significant.

To determine whether one area was more activated than another, the relative increase in FOS-like positive neurons was calculated among areas. Only areas that showed a significant increase in labeled cells in the previous analysis were considered. For each rat, the relative increase was calculated using the equation:

$$\mathrm{RI} = \frac{R_{\mathrm{PNV}} - M_{\mathrm{C}}}{M_{\mathrm{C}}}$$

where RI is the relative increase in FOS-like positive neurons in a given area in PNV-treated rats, R_{PNV} the number of FOS-like positive neurons in a given area of PNV-treated rats, and M_{C} is the mean number of labeled neurons in the corresponding area of the control group (intact or sham). To assess whether the relative increase was significant, the values for each area were compared by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test. The results were expressed as the mean ± S.E.M. and a value of $P < 0.05$ indicated significance.

3. Results

3.1. Clinical observations

Immediately after PNV injection, the rats were hyperemic, motionless, and showed salivation, lachrymation, tremors and flaccidity followed by spastic paralysis of the hindlimbs. Of the nine rats envenomed, six developed tonic–clonic convulsions, four of which died and were excluded from the study; the remaining five were used for immunohistochemical analysis. The six rats that developed convulsions showed all of the foregoing signs and symptoms within 15 min after envenoming and, in the two that survived these manifestations lasted until the rats were killed 2 h after venom injection. The tonic–clonic convulsions were the main sign of the CNS toxicity of PNV, and the seizures were characterized by spastic tonus followed by generalized clonic spasms involving the limbs, head spasms and loss of righting ability. The convulsions lasted for approximately 5 min.

3.2. Immunohistochemistry

3.2.1. FOS

Rats that were maintained intact or injected with saline (control groups) showed a basal FOS expression in the areas of the forebrain and brainstem examined, with no alteration in the density of labeled cells ($P > 0.05$). However, the density of labeled cells increased after PNV injection in the frontal cortex motor area (FCM), parietal cortex motor area (PCM), ventral and dorsolateral periaqueductal gray matter (vPAG and dlPAG), periventricular thalamic nuclei (PTN), and rhinal cortex (RC) when compared to the sham and intact control groups ($P < 0.05$). In contrast, in the lateral septal nuclei (LSN), there was significantly greater labeling when compared only to the sham group. Similarly, the density of FOS-positive cells in the periaqueductal gray matter pars dorsal (dPAG) was greater than in the intact control group but not significantly different from that of sham rats. Table 1 summarizes the results of the quantitative analyses of FOS-like labeled neurons in all of the regions

Table 1
Number of FOS and n-NOS-positive neurons in anatomical regions of the brain in PNV-treated rats compared to intact controls and sham controls

	Intact control		Sham control		PNV-treated	
	FOS	n-NOS	FOS	n-NOS	FOS	n-NOS
Brain area cortex (C)						
Frontal motor cortex (FCM)	12.4 ± 5.06	16.5 ± 2.88	9.2 ± 2.83	10.6 ± 2.88	63 ± 17.95*(##)	14 ± 5.2
Frontal cortex (FC)	9.8 ± 1.39	17 ± 1	3.8 ± 1.62	12 ± 2.94	20 ± 10.40	9.2 ± 3.27(**)
Frontal somatosensory cortex (FCS)	10.6 ± 4.54	11.2 ± 2.38	3.2 ± 2.08	16 ± 3.74	27.8 ± 16.56	8 ± 3.74(#)
Parietal cortex (PC)	8.2 ± 5.04	9.6 ± 2.07	6.2 ± 3.17	8.2 ± 2.36	41.2 ± 18.26	9.8 ± 1.48
Parietal motor cortex (PCM)	11.2 ± 6.79	10.6 ± 2.07	18.4 ± 7.33	12 ± 1.58	114 ± 29.5**(##)	17.8 ± 4.49**(#)
Parietal somatosensory cortex (PCS)	26.8 ± 16.77	9 ± 3.16	11.8 ± 3.27	11 ± 2.82	91.4 ± 43.93	11.6 ± 1.81
Temporal cortex (TC)	52.8 ± 15.41	14.2 ± 2.87	45.6 ± 6.51	11.4 ± 3.05	81.6 ± 14.17	11.57 ± 3.04
Occipital cortex-A18 (OC18)	44.4 ± 32.03	10 ± 2.7	49.8 ± 15.44	7.7 ± 2.06	62.6 ± 19.70	8.8 ± 4.01
Occipital cortex-A17 (OC17)	34.8 ± 19.58	7.7 ± 1.25	33.2 ± 13.57	7.2 ± 1.5	51.2 ± 15.49	8.5 ± 2.58
Occipital cortex-A18a (OC18a)	46 ± 19.04	9.7 ± 1.7	30.4 ± 13.09	8.5 ± 1.73	66.8 ± 22.6	10.3 ± 1.49
Entorhinal cortex (EC)	28.4 ± 10.11	8.2 ± 2	49 ± 4.45	10.2 ± 1.3	41.2 ± 9.03	10.8 ± 3.76
Piriform cortex (PIR)	188.2 ± 69.04	13 ± 8.3	146.8 ± 30.41	16.2 ± 2.5	128.4 ± 18.58	10.6 ± 5.12
Rhinal cortex (RC)	7.6 ± 3.18	3.8 ± 3.56	31.8 ± 2.97	6.4 ± 2.6	64.8 ± 11.3***(#)	5 ± 1.87
Periaqueductal gray matter (PAG)						
P. gray matter dorsolateral (dlPAG)	10.4 ± 3.21	99.8 ± 35.27	23.2 ± 8.15	112.8 ± 17.75	90 ± 14.4***(##)	139.7 ± 58.95
P. gray matter dorsal (dPAG)	7.8 ± 2.29	40.2 ± 15.6	16.6 ± 6.23	29.7 ± 6.39	33.2 ± 7.1**	41.14 ± 8.68
P. gray matter ventral (vPAG)	8.8 ± 3.35	67.8 ± 39.86	6 ± 2.34	34 ± 30.79	60 ± 12.38**(##)	44.5 ± 33.24
Hippocampus (HPC)						
CA1	1.8 ± 1.78	0.7 ± 0.95	0.4 ± 0.4	3 ± 2.16	1.2 ± 0.58	3.33 ± 2.06
CA2	3.6 ± 3.35	3 ± 3.46	0.8 ± 0.8	3.7 ± 4.5	0.8 ± 0.8	3.5 ± 2.95
CA3	0.6 ± 0.6	4.2 ± 4.03	2.6 ± 1.12	2.7 ± 2.06	2.2 ± 0.73	4.8 ± 3.65
CA4	0.8 ± 0.8	3.7 ± 3.86	0.8 ± 0.8	2.5 ± 2.08	2.4 ± 1.03	4.2 ± 3.48
Subicculum (SUB)	19.4 ± 15.17	12 ± 3.5	22.8 ± 4.13	4.7 ± 5.73	45.8 ± 15.66	11.3 ± 9.79
Amigdala (A)						
Posterior amygdaloid nucleus (PAN)	33.8 ± 13.94	2.2 ± 1.5	37.8 ± 10.85	5 ± 2.44	26.6 ± 6.03	2.2 ± 1.72
Central amygdaloid nucleus (CAN)	7 ± 3.33	36 ± 15.38	18.6 ± 6.67	41.5 ± 16.27	47.4 ± 23.76	31.1 ± 6.76
Lateral amygdaloid nucleus (LAN)	19.4 ± 10.43	19.2 ± 6.18	51.2 ± 13.25	14.5 ± 4.12	46.4 ± 3.77	9.5 ± 3.72
Other areas						
Dentate gyrus (DG)	11.2 ± 9.95	18 ± 5.47	14.2 ± 5.83	13.2 ± 1.25	4 ± 1.95	20.6 ± 7.73
Superior colliculus (SC)	0.2 ± 0.2	10.2 ± 5.56	6.4 ± 4	8 ± 3.74	1.8 ± 0.66	10.4 ± 5.44
Lateral septal nuclei (LSN)	40.8 ± 16.85	9.6 ± 6.53	12.6 ± 5.57	6.2 ± 4.65	76.6 ± 19.87(#)	4.6 ± 3.84
Periventricular thalamic nuclei (PTN)	11.4 ± 7.09	21.6 ± 7.12	45 ± 10.4	27.6 ± 24.9	153 ± 24***(##)	98 ± 32.3***(##)

The results are expressed as the mean ± S.E. per region (5 rats/group). For each rat, six 30 μm-thick sections were collected to ensure that all areas of interest were sampled (see stereotaxic coordinates in Fig. 1). Each anatomical region was counted in only one section per rat. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for PNV-treated rats vs. intact rats and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ for PNV-treated rats vs. sham rats (ANOVA and Tukey–Kramer multiple comparisons test).

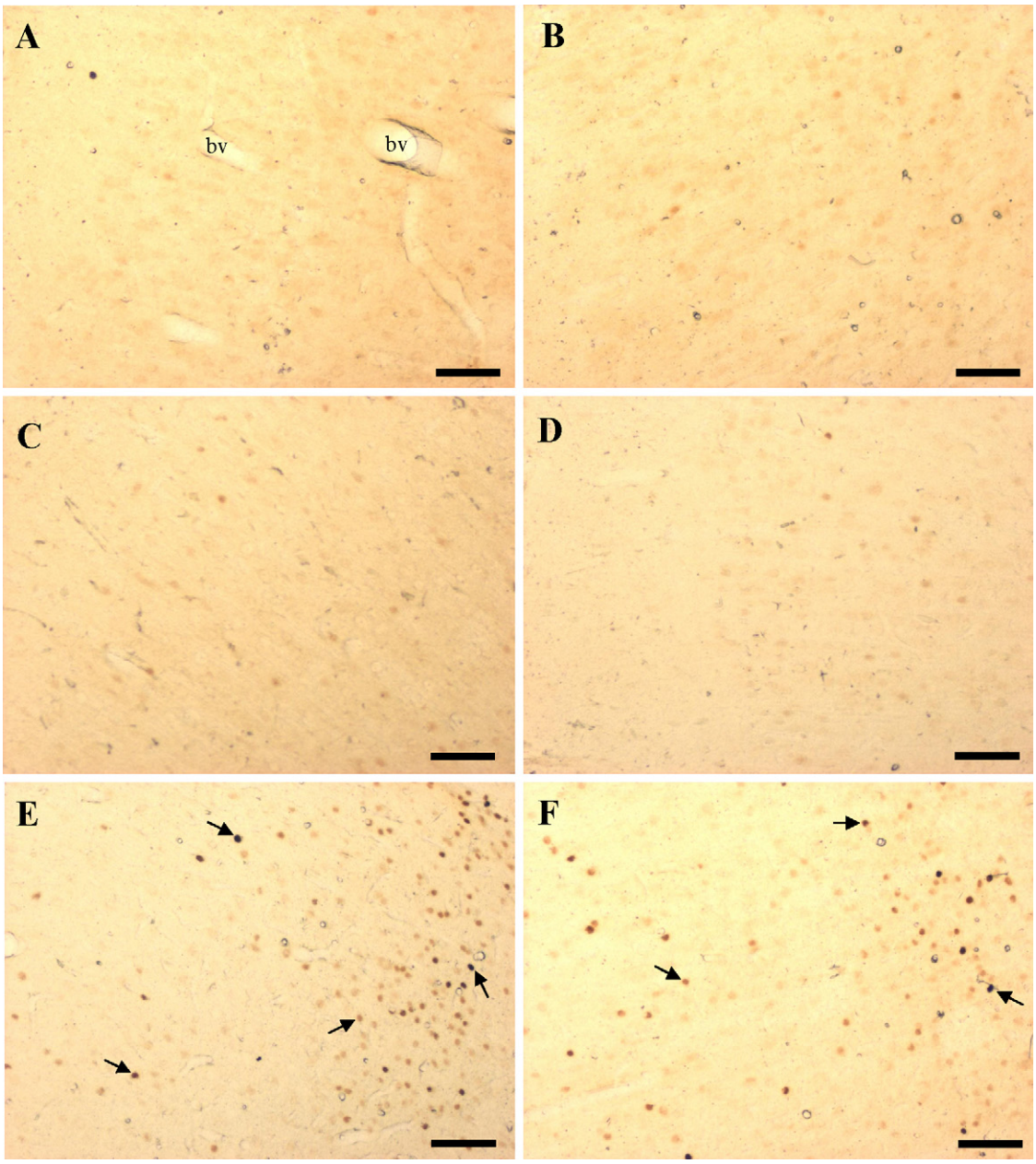

Fig. 2. Light micrographs of FOS-immunoreactive neurons in the frontal motor cortex (A, C and E) and parietal motor cortex (B, D and F). (A and B) Refer to intact controls, (C and D) to sham (saline-injected) controls and (E and F) to PNV-injected rats. Note the intense labeling of neuronal nuclei in (E and F) and the absence of labeling in the controls (intact and sham rats). Bv, blood vessels; arrows, FOS-like positive nuclei. Bars: 70 μm.

studied. Figs. 2–6 show the immunolabeling in selected regions and Fig. 7 shows the regions with significant changes in FOS expression.

Comparison of the areas that showed significant activation in PNV-treated rats with the corresponding regions in sham control group indicated that the relative increase in FOS-like labeled neurons was more prominent in the vPAG than in the dPAG, dlPAG, RC and PTN. A similar comparison between PNV-treated and intact control rats showed that the PTN had a higher relative increase in FOS-like positive neurons than the dPAG, FCM and SLN. The relative increase in reactive neurons in the PCM area was also higher than in the SLN (see Fig. 8).

3.2.2. n-NOS

All of the structures and areas analyzed in the control groups (intact and sham rats) had a basal expression of n-NOS, with the pattern of labeling being the same in both groups. In PNV-treated rats, the greatest changes in expression occurred in the periventricular thalamic nuclei (PTN) and parietal cortex motor area (PCM), in which there was a significant increase in the density of n-NOS-positive neurons compared to both control groups ($P < 0.05$) (Figs. 3 and 6). In most of the other areas, PVN did not significantly alter with the density and distribution of n-NOS-labeled neurons ($P > 0.05$) (Table 1), although the neuronal processes in the dlPAG had enhanced labeling, as indicated by the tiny-labeled ramifications throughout the neural

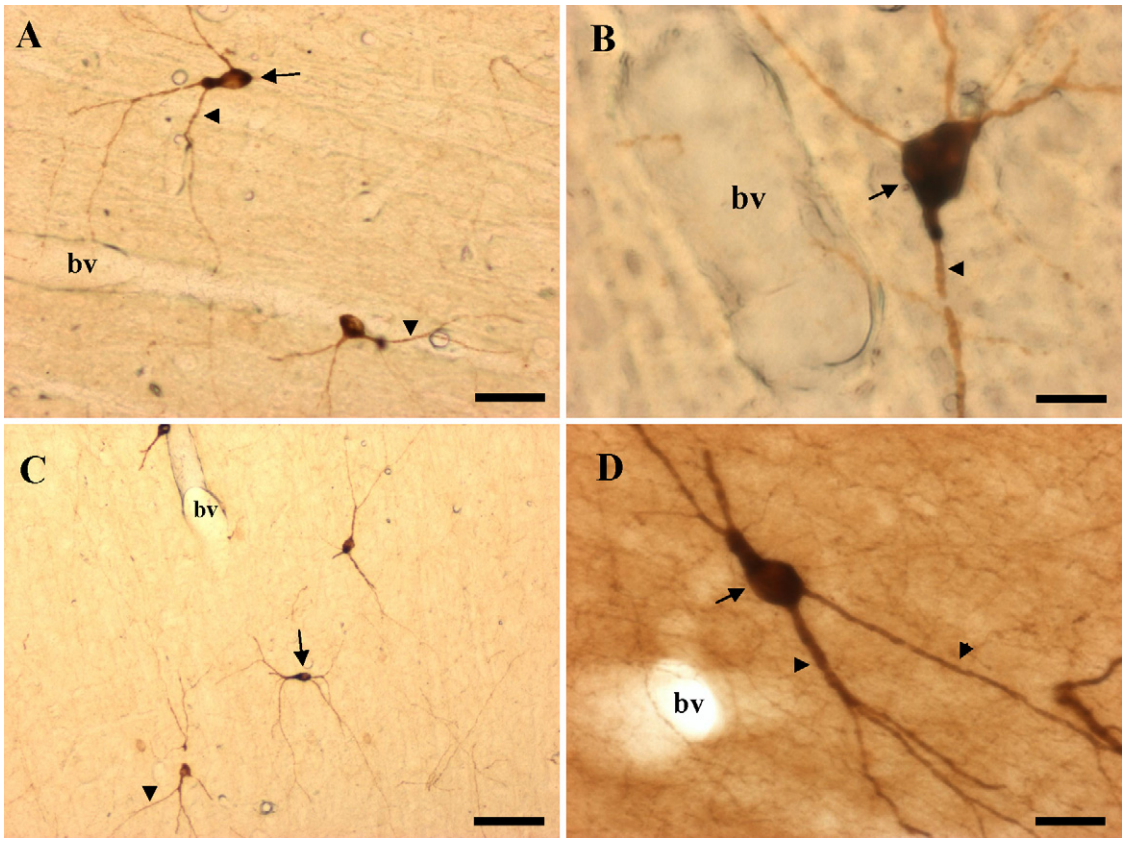

Fig. 3. Light micrographs of n-NOS-immunoreactive neurons in the parietal motor cortex of intact controls (A and B) and in PNV-injected rats (C and D). Compared to rats that did not receive venom, PNV-treated rats showed a stronger reaction in processes arising from the cell body and in the cell body itself; there was also widespread immunoreactivity throughout the neuronal parenchyma. Bv, blood vessels; arrows, cellular body; arrowheads, neuronal processes. Bars: (A) 35 μm, (B) 12 μm, (C) 70 μm and (D) 20 μm.

parenchyma (compare panels B and F of Fig. 5). Since we only counted neuronal bodies, the possible PNV-induced increase in n-NOS expression in this region was not quantified and may have been underestimated. In contrast, there was a significant decrease in the number and density of n-NOS reactive neurons in the frontal cortex (FC) and frontal cortex somatosensory area (FCS) in envenomed rats.

4. Discussion

Spider and scorpion toxins are valuable molecular tools for understanding the mechanisms of neuronal excitatory and/or inhibitory responses [3,28,37,45,49]. These toxins may affect different neuronal populations, thereby triggering the release or inhibition of several types of neurotransmitters.

Drugs and neurotoxins activate specific neural circuits, as indicated by changes in neurotransmitter release, including NO [29,55], and by the induction of immediate early genes such as FOS [36,52]. The inducibility of FOS expression has been regarded as a functional marker for mapping the hyperexcitability of neuronal populations in the brain under a variety of stimuli [13]. In the present study, several structures and regions representing different neuronal populations in the CNS were screened for their expression of FOS and n-NOS in order to identify the brain regions that are modulated by PNV. Based on the changes in FOS expression, PNV upregulated neuronal activity in areas associated with motor activities, e.g., dlPAG, vPAG, FMC, PCM and PTN, and with acute stress (RC and LSN) when compared with sham rats.

The dlPAG and vPAG, but not the dPAG, showed an increased density of FOS-positive cells. In rats, a gradual increase in the intensity of electrical stimulation of the periaqueductal gray matter (PAG) results in the progressive development of characteristic aversive reactions such as arousal, freezing and escape behavior [25,26] accompanied by autonomic responses [10,11,19]. The brain and brainstem networks involved in the organization of this fear-induced behavior are formed by the medial hypothalamus, amygdaloid complex and PAG (as a mesencephalic output). However, distinct regions of the PAG appear to play different roles in various defensive behaviors [5,9,14,15,20,21,46]. The ventral portion of the PAG is apparently involved exclusively in the freezing response that occurs when an animal is exposed to innate or learned aversive stimuli, whereas the dorsal portion of the PAG mediates active and inhibitory behavioral patterns of defensive responses. For example, lesions of the dPAG enhance conditioned freezing [16] and reduce escape reactions to electrical footshock [20]. Since PNV enhanced FOS expression in the dPAG, it would be interesting to determine whether this venom also provokes pro-aversive reactions in rats.

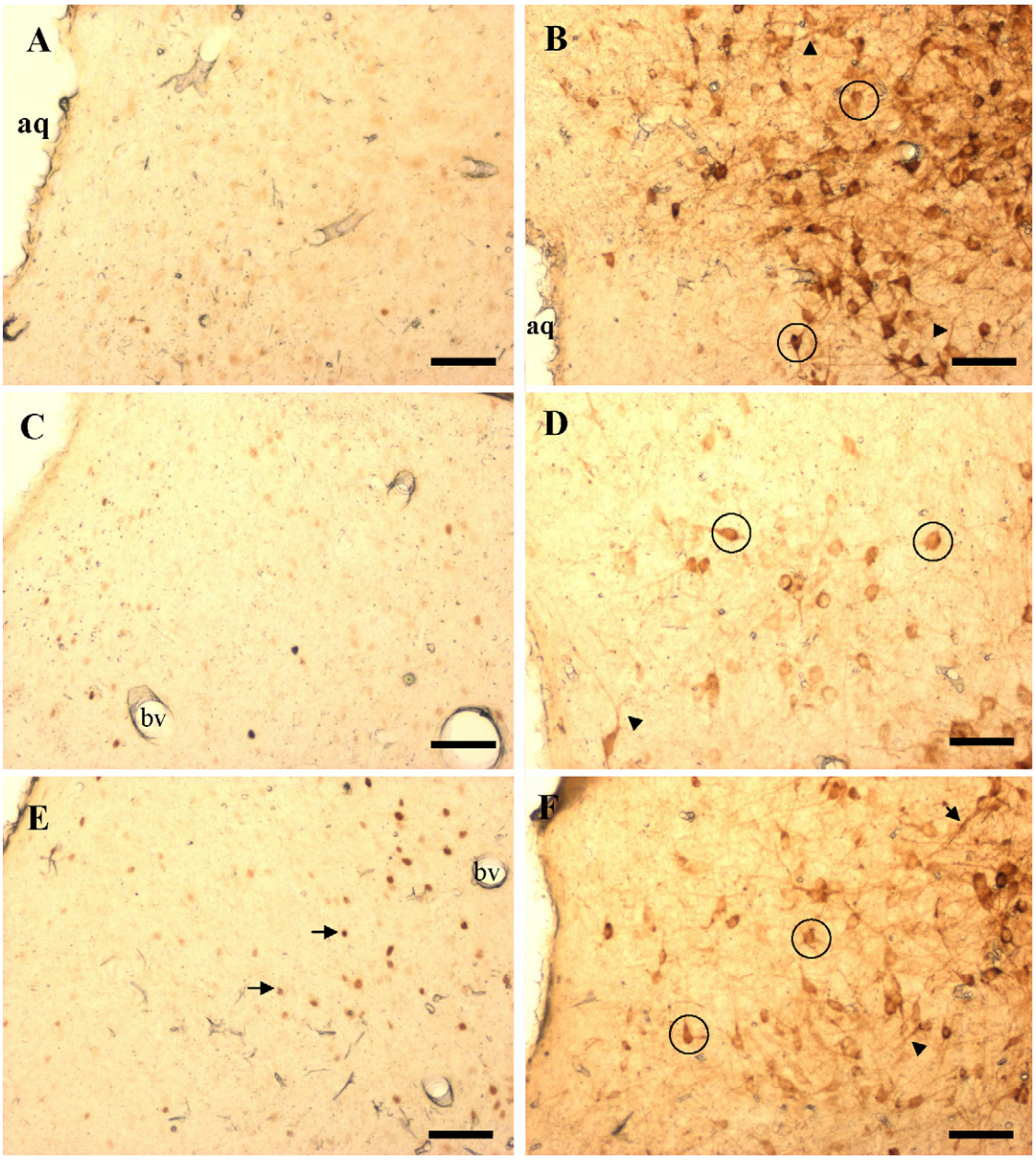

Fig. 4. Light micrographs of the periaqueductal gray matter pars ventral. (A, C and E) FOS-positive nuclei; (B, D and F) n-NOS-positive neurons. (A and B) Intact controls, (C and D) sham controls and (E and F) PNV-injected rats. There was a significant increase in the number of FOS-labeled nuclei in venom-treated rats. In contrast, n-NOS expression was unchanged compared to the controls. Aq, aqueduct; arrows, FOS-labeled nuclei; arrowheads, n-NOS-labeling of neuronal processes; circles, n-NOS-labeled neuronal bodies; bv, blood vessels. Bars: 70 μm.

A number of studies have shown that the systemic or local microinjection of drugs into the PAG also induces motor reactions. Drugs injected directly into this region can induce seizures or a flight reaction, indicating that local neurons could be involved in motor activities [42]. Peterson et al. [42] demonstrated that the PAG is a component of the neural network through which brainstem seizures elicit forebrain seizures. These authors showed that bilateral microinjection of the cholinergic agonist carbamylcholine chloride (carbachol) into the PAG region of rats induced arrested, staring behavior that was accompanied by epileptiform electrocorticograms based on recordings from the parietal cortex.

The motor areas activated by PNV are involved in the action of drugs that cause convulsions and motor reactions. Vergnes et al. [53,54] showed that picrotoxin-elicited convulsions were accompanied by well-defined and consistent FOS expression in the fronto-parietal, piriform and perirhinal cortex, with low expression in the amygdala. Willoughby et al. [56] correlated the distribution of FOS expression and the development and type of seizures with at least two patterns of neuronal involvement. The cortex, part of the caudate-putamen, amygdala and thalamus were involved in restricted seizures whereas the hippocampus, cortex and thalamus were involved in generalized seizures. In the present study, PNV produced tremors, spasms, spastic paralysis

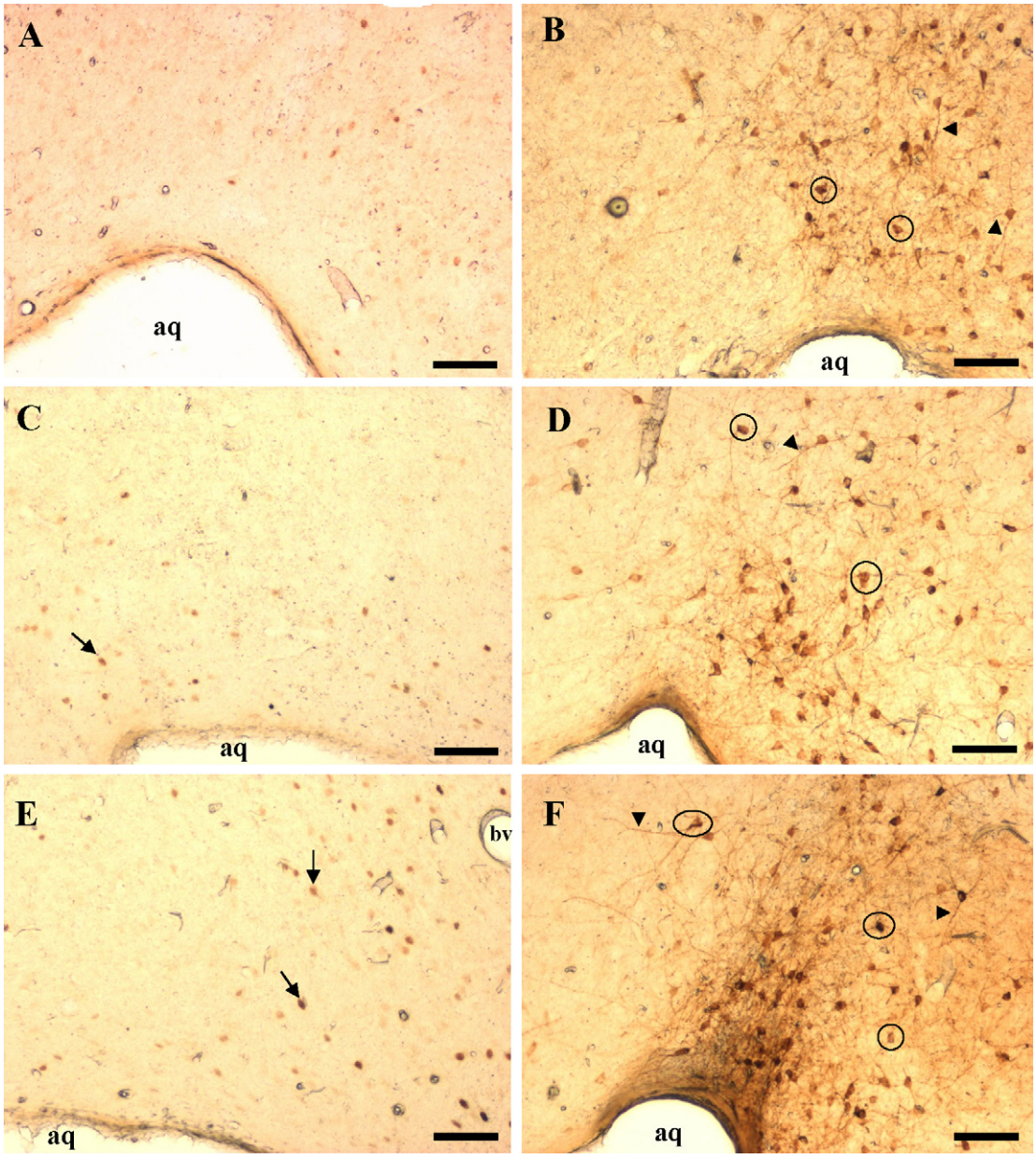

Fig. 5. Periaqueductal gray matter pars dorsolateral. (A, C and E) FOS immunolabeling; (B, D and F) n-NOS immunolabeling. (A and B) Intact controls, (C and D) sham controls and (E and F) PNV-treated rats. Compared to the controls, there was a marked increase in FOS expression in envenomed rats (E), whereas n-NOS expression was unaltered (F). aq, aqueduct; arrows, Fos-positive nuclei; circles, n-NOS-positive neuronal bodies; arrowheads, n-NOS-labeling in neuronal branchings; bv, blood vessels. Bars: 70 μm.

and, in several cases, generalized convulsions, all of which could have been triggered by FOS-reactive motor pathways since in some of these areas (PCM, FCM, PTN) there was a significant increase in this protein.

Among the regions that showed significant FOS labeling, the relative increase in FOS-like positive neurons was most prominent in the vPAG of envenomed rats when compared to sham controls. In contrast, when compared to intact controls, the PTN and PCM of envenomed rats showed the highest relative increase in FOS-like positive neurons. The finding that motor-related areas were the most activated in this study suggests that PNV stimulate preponderantly motor-related structures of the CNS and to a lesser extent acute stress-related areas.

The lack of a significant increase in FOS-like reactive neurons in the hippocampus agrees to a certain extent with our findings [44] that in the acute stages of envenoming there is a decrease in the percentage of vessels with swollen perivascular astrocytic end-feet processes in the hippocampus compared to the cerebellum. Similarly, during the acute stages of envenoming, circulating venom may induce a "defensive response" that is translated in the hippocampus as a non-reactivity of the neurons populations to induce immediate early genes and express FOS protein.

These observations support the view that PNV acts selectively in certain regions of the CNS since the number of FOS-like positive neurons increased mainly in areas that rats use to defend

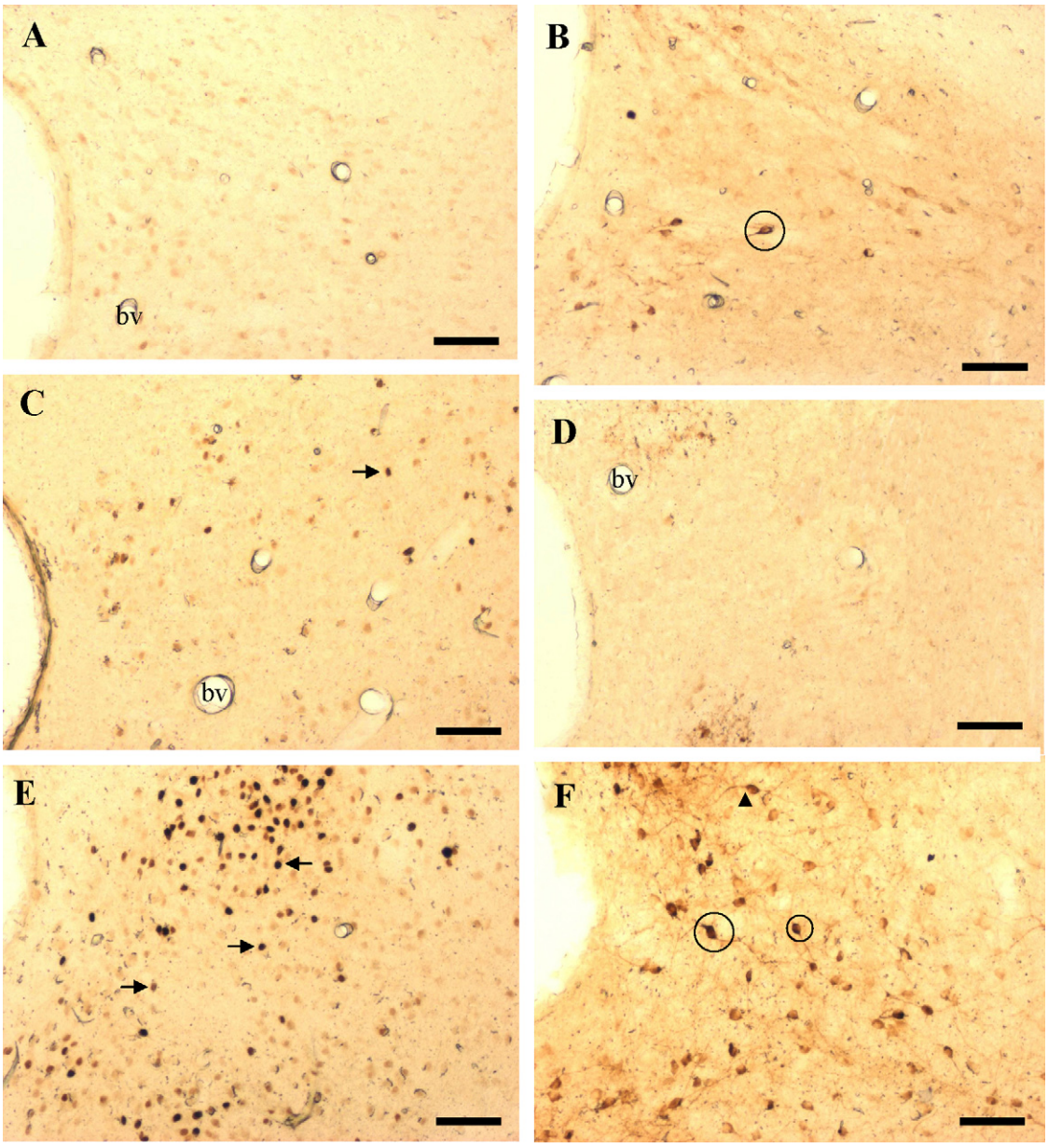

Fig. 6. Periaqueductal thalamic nucleus. (A, C and E) Fos-positive nuclei; (B, D and F) n-NOS-positive neurons. (A and B) Intact controls, (C and D) sham controls and (E and F) PNV-treated rats. Note the marked increase in the number of FOS-labeled neurons in venom-treated rats (E) compared to the controls (A and C). A similar marked increase was observed in n-NOS in PNV-treated rats (F) compared to the controls (B and D). Arrows, FOS-labeling in nuclei; arrowheads, n-NOS-labeling in neuronal processes; circles, n-NOS-labeling in neuronal bodies. Bars: 70 μm.

themselves. On the other hand, we have previously shown that in later stages of envenoming (1 and 9 days), PNV disrupts the microtubule-mediated transendothelial pathway of the BBB in the hippocampus [30]. At this stage, different signaling pathways could be involved since 12 h after the i.v. injection of venom the signs and symptoms normally associated with the early stages of envenomation are no longer present [32].

All of the areas with increased FOS expression after the injection of PNV, including the frontal motor cortex (FCM), parietal motor cortex (PCM), periaqueductal gray dorsolateral (dlPAG), periaqueductal gray ventral (vPAG) and lateral septal nuclei (LSN), also had n-NOS-positive neurons, with the PCM and PTN showing the most significant increases in n-NOS expression. Even areas in which n-NOS expression was not enhanced after PNV injection had a higher basal expression of n-NOS-positive neurons. The periventricular thalamic nuclei (PTN), which had the lowest basal expression of n-NOS, showed the greatest labeling of n-NOS and FOS after venom administration ($P < 0.05$). This immunohistochemical evidence for a close association between n-NOS and FOS agrees with the observation that NO can induce FOS expression in adjacent neurons (paracrine stimulus) by diffusing from neuronal cell bodies or dendritic/axonal processes to adjacent neurons [48]. Salter et al. [48] suggested that NO acts on FOS-positive neurons to acti-

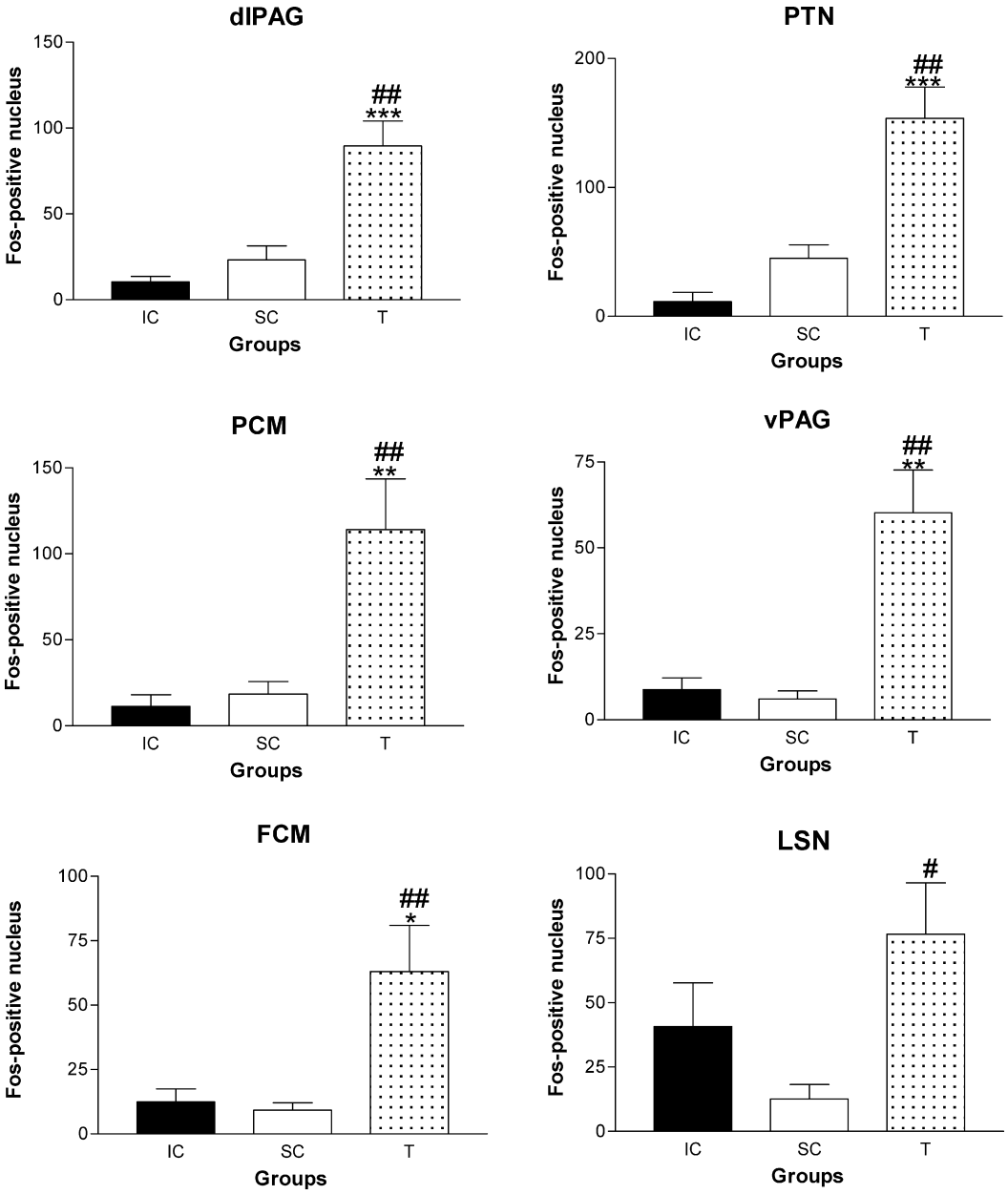

Fig. 7. Histogram showing the anatomical regions in which there were significant differences in the number of FOS and n-NOS immunoreactive neurons per 0.35 mm^2 between PNV-treated rats (T) and intact controls (IC) and/or sham controls (SC). The results are expressed as the mean ± S.E. $^{*}P<0.05$, $^{**}P<0.02$, $^{***}P<0.001$, $^{\#}P<0.05$ and $^{\#\#}P<0.02$ (ANOVA followed by the Tukey–Kramer multiple comparison test). dlPAG, dorsolateral periaqueductal gray matter; PTN, periventricular thalamic nucleus; PCM, parietal cortex motor area; vPAG, ventral periaqueductal gray matter; FCM, frontal cortex motor area; LSN, lateral septal nucleus.

vate guanylate cyclase and produce cGMP that induces FOS expression.

There was a significant decrease in n-NOS-labeled neurons in the frontal cortex (FC) and frontal cortex somatosensory area (FCS) in envenomed rats when compared with the intact and sham controls. The mechanism behind this effect is unknown, as is the reason for the lack of activation or inhibition of n-NOS expression in most of the areas investigated here.

The injection of NO donors into the dlPAG induces a motor reaction [17] and, as shown here, there was a significantly higher expression of n-NOS and FOS in the dlPAG neurons of PNV-treated rats. These findings suggest that NO may be an important modulator during envenoming.

The modulatory role of NO following intoxication by different agents has been investigated, and the generation of this messenger through the activation of n-NOS is the best characterized signal transduction pathway implicated in neuronal excitotoxicity [29]. The influence of NO in the toxicity of PNV has been studied by using toxins isolated from this venom. Yonamine et al. [57] reported that pretreatment with 7-nitroindazole (7-NI), a selective inhibitor of n-NOS, abolished all of the signs of intoxication associated with the intraperitoneal injection of the toxin Tx2-5, including priapism, salivation and lung edema. Similarly, pretreating mice with the non-selective NOS inhibitor N^{ω}-nitro-L-arginine-methyl-ester (L-NAME) reduced the penile erection and partially protected the animals from the

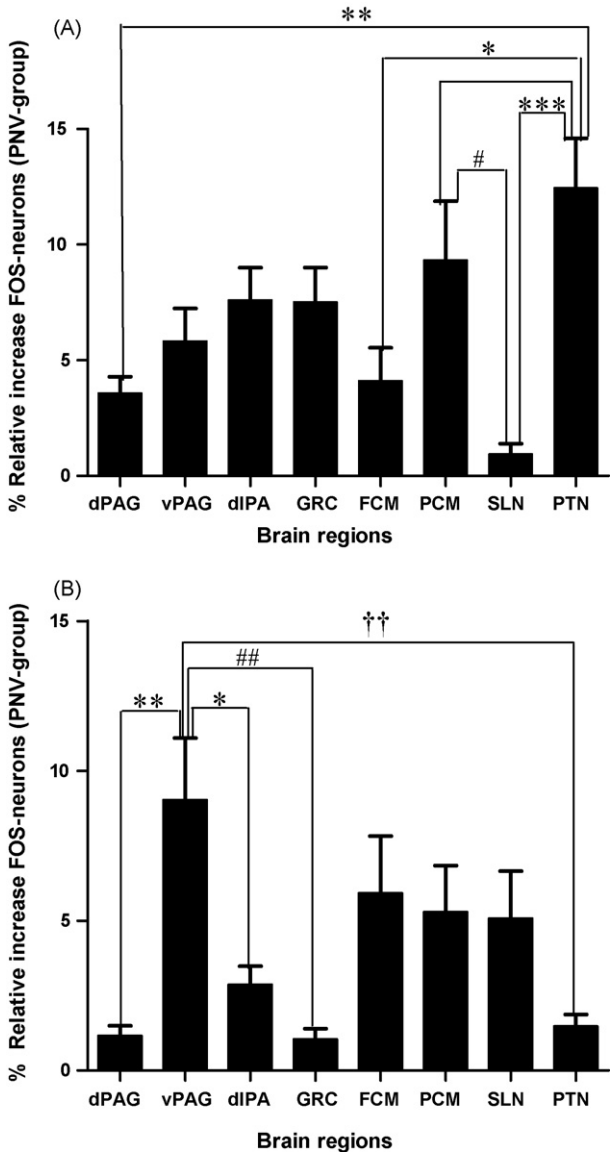

Fig. 8. Comparison of the relative increase in FOS-like positive neurons among different areas of the CNS in PNV-treated rats vs. intact control (A) and vs. sham control (B) rats. The data were analyzed by one-way ANOVA followed by the Tukey–Kramer multiple comparisons test. (A) $^{*}P<0.05$ for PTN vs. FCM, $^{\#}P<0.05$ for SLN vs. PCM, $^{**}P<0.01$ for PTN vs. dPAG and $^{***}P<0.001$ for PTN vs. SLN. (B) $^{*}P<0.05$ for vPAG vs. dlPAG, $^{**}P<0.01$ for vPAG vs. dPAG, $^{\#\#}P<0.01$ for vPAG vs. RC and $^{\dagger\dagger}P<0.01$ for vPAG vs. PTN.

lethal effects of Tx2-5. The authors concluded that NO was the major mediator involved in the responses to this toxin.

PhTx3, another toxin from PNV, is a broad-spectrum Ca^{2+} channel blocker that inhibits Ca^{2+}-dependent calcium uptake and glutamate release and uptake in synaptosomes, and provokes flaccid paralysis when injected intracerebroventricularly [43]. Since PhTx3 blocks voltage-gated Ca^{2+} channels, this toxin could also inhibit n-NOS-Ca^{2+}/calmodulin-dependent activity, although this action would be contrary to the enhanced expression of n-NOS seen here with the venom. Overall, our findings do not allow us to determine whether an increase in NO production in some of the motor-related-areas (seen indirectly as an increase in n-NOS expression) enhances the toxicity of PNV.

In conclusion, the intravenous injection of PNV results in a central action that involves the neuronal networks of motor and stress-related areas of the CNS, with a predominance of the former. The extent to which circulating venom can trigger endothelial and astrocytic responses that are then transmitted to different neuronal populations in the CNS remains to be determined. Since our results show that neuronal populations react differently to PNV, it would be interesting to identify the toxins that directly activate specific areas, as well as the pathway(s) and neurotransmitter(s) involved in this activation.

Acknowledgements

We thank the Butantan Institute for donating the venom, Marta Beatriz Leonardo (B.Sc.) for excellent technical assistance, and Miguel B. Silva and Marcos A. Silva for caring for the rats. This work was partially supported by CNPq, FAPESP and FAEP/UNICAMP. CR and GMZ are supported by scholarships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). MACH is supported by research fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We also thank the anonymous referees whose comments and criticisms improved the clarity of the manuscript, and Dr. Stephen Hyslop for reviewing the English.

References

[1] C. Abbadie, B.K. Taylor, M.A. Petern, A.I. Basbaum, Differential contribution of the two phases of the formalin test to the pattern of c-Fos expression in spinal cord: studies with remifentanil and lidocaine, Pain 69 (1992) 101–110.
[2] N.J. Abbott, Astrocyte-endothelial interactions and blood–brain barrier permeability, J. Anat. 200 (2002) 523–534.
[3] Z.T. Bai, R. Zhao, X.Y. Zhang, J. Chen, T. Liu, Y.H. Ji, The epileptic induced by BmK I, a modulator of sodium channels, Exp. Neurol. 197 (2006) 167–176.
[4] M. Baker, H. Bostock, G.A. Brook, S. Love, *Phoneutria* and *Leiurus* venoms induce spontaneous compound action potentials in rat spinal roots, J. Physiol. 366 (1985) 63.
[5] R. Bandler, M.T. Shipley, Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? Trends Neurosci. 17 (1994) 379–389.
[6] V. Brazil, J. Vellard, Contribuição ao estudo do veneno das aranhas, Mem. Inst. Butantan 2 (1925) 5–77.
[7] D.S. Bredt, S.H. Snyder, Nitric oxide, a novel neuronal messenger, Neuron 8 (1992) 3–11.
[8] F. Bucaretchi, C.R.D. Reinaldo, S. Hyslop, P.R. Madureira, E.M. de Capitani, R.J. Vieira, A clinico-epidemiological study of bites by spiders of the genus *Phoneutria*, Rev. Inst. Med. Trop. 42 (2000) 17–21.
[9] P. Carrive, The periaqueductal gray and defensive behavior: functional representation and neuronal organization, Behav. Brain Res. 58 (1993) 27–47.
[10] N.C. Coimbra, M.L. Brandão, GABAergic nigro-collicular pathways modulate the defensive behavior elicited by midbrain tectum stimulation, Behav. Brain Res. 59 (1993) 131–139.
[11] N.C. Coimbra, M.L. Brandão, Effects of 5-HT_2 receptors blockade on fear induced analgesia elicited by electrical stimulation of the deep layers of the superior colliculus and dorsal periaqueductal gray, Behav. Brain Res. 87 (1997) 97–103.
[12] M.A. da Cruz-Höfling, S. Love, G. Brook, L.W. Duchen, Effects of *Phoneutria nigriventer* spider venom on mouse peripheral nerve, Q. J. Exp. Physiol. 70 (1985) 623–640.

[13] G.H. Daniel, A.R. Harold, Activation of c-Fos in the brain, Prog. Neurobiol. 50 (1996) 83–107.
[14] M. Davis, The role of the amygdala in conditioned fear, in: J.P. Aggleton (Ed.), The Amygdala: Neurobiological Aspects of Emotion, Memory and Mental Dysfunction, Plenum Press, New York, 1992, pp. 255–305.
[15] T.M. Dawson, S.H. Snyder, Gases as biological messengers: nitric oxide and carbon monoxide in the brain, J. Neurosci. 14 (1994) 5147–5159.
[16] B.M. de Oca, J.P. de Cola, S. Maren, M.S. Fanselow, Distinct regions of the periaqueductal gray are involved in the acquisition and expression of defensive response, J. Neurosci. 18 (1998) 3426–3432.
[17] R.W. de Oliveira, E.A. del Bel, F.S. Guimarães, Behavioral and c-Fos expression changes induced by nitric oxide donors microinjected into the dorsal periaqueductal gray, Brain Res. Bull. 51 (2000) 457–464.
[18] G.J. del Zoppo, J.M. Hallenbeck, Advances in the vascular pathophysiology of ischemic stroke, Thromb. Res. 98 (2000) 73–81.
[19] G.C.D. Eichenberger, S.J. Ribeiro, M.Y. Osaki, R.Y. Maruoka, G.C.C. Resende, S.A. Castellan-Baldan Correa, L.A. da Silva, N.C. Coimbra, Neuroanatomical and psychopharmacological evidence for interaction between opioid and GABAergic neural pathways in the modulation of fear and defense elicited by electrical and chemical stimulation of the deep layers of the superior colliculus and dorsal periaqueductal gray matter, Neuropharmacology 42 (2002) 48–59.
[20] M.S. Fanselow, The midbrain periaqueductal gray as a coordinator of action in responses to fear and anxiety, in: A. DePaulis, R. Bandler (Eds.), The Midbrain Periaqueductal Grey Matter: Functional, Anatomical and Immunohistochemical Organization, Plenum Publishing Corpo, New York, 1991, pp. 151–173.
[21] M.S. Fanselow, J.P. de Cola, B.M. de Oca, J. Landeira-Fernandez, Ventral and dorsolateral regions of the midbrain periaqueductal gray PAG control different stages of defensive behavior, Aggress. Behav. 21 (1995) 63–77.
[22] M.D. Fontana, O. Vital-Brazil, Mode of action of *Phoneutria nigriventer* spider venom at the isolated phrenic nerve-diaphragm of the rat, Braz. J. Med. Res. 18 (1985) 557–565.
[23] J. Garthwaite, S.L. Charles, R. Chess-Williams, Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intracellular messenger in the brain, Nature 326 (1988) 385–387.
[24] M.V. Gomez, E. Kalapothakis, C. Guatimosim, M.A.M. Prado, *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels, Cell. Mol. Neurobiol. 22 (2002) 579–588.
[25] F.G. Graeff, Brain defense systems and anxiety, in: M. Roth, G.D. Burrows, R. Noyes (Eds.), Handbook of Anxiety, vol. 3, Elsevier, Amsterdam, 1990, pp. 307–354.
[26] F.G. Graeff, F.S. Guimarães, T.G.C.S. de Andrade, J.F.W. Deakin, Role of 5-HT in stress, anxiety and depression, Pharmacol. Biochem. Behav. 54 (1996) 129–141.
[27] S.P. Hunt, A. Pini, G. Evan, Induction of c-Fos-like protein in spinal cord neurons followed by sensory stimulation, Nature 328 (1987) 632–634.
[28] M. Khvotchev, G. Lonart, T.C. Sudhof, Role of calcium in neurotransmitter release evoked by α-latrotoxin or hypertonic sucrose, Neuroscience 101 (2000) 793–802.
[29] J.S. Kim, H.J. Lee, J. Kim, C. Jin, J.H. Ryu, Luteolin inhibits LPS-stimulated inducible nitric oxide synthase expression in BV-2 microglial cells, Planta Med. 72 (2006) 65–68.
[30] L.P. Le Sueur, C.B. Collares-Buzato, M.A. da Cruz-Höfling, Mechanisms involved in the blood–brain barrier increased permeability induced by *Phoneutria nigriventer* spider venom in rats, Brain Res. 1027 (2004) 38–47.
[31] L. Le Sueur, C.B. Collares-Buzato, E. Kalapothakis, M.A. da Cruz-Höfling, *In vitro* effect of the *Phoneutria nigriventer* spider venom on cell viability, paracellular barrier function and transcellular transport in cultured cell lines, Toxicon 46 (2005) 130–141.
[32] L. Le Sueur, E. Kalapothakis, M.A. da Cruz-Höfling, Breakdown of the blood–brain barrier and neuropathological changes induced by *Phoneutria nigriventer* spider venom, Acta Neuropathol. 105 (2003) 125–134.
[33] S. Love, M.A. da Cruz-Höfling, Acute swelling of nodes of Ranvier caused by venoms which slow inactivation of sodium channels, Acta Neuropathol. 70 (1986) 1–9.
[34] S. Love, M.A. da Cruz-Höfling, L.W. Duchen, Morphological abnormalities in myelinated nerve fibres caused by *Leiurus*, *Centruroides* and *Phoneutria* venoms and their prevention by tetrodotoxin, Q. J. Exp. Physiol. 71 (1986) 115–122.
[35] L. Ma, I. Morita, S. Murota, Presence of constitutive type nitric oxide synthase in cultured astrocytes isolated from rat cerebra, Neurosci. Lett. 174 (1994) 123–126.
[36] V.A. Maisky, N.N. Oleshko, O.V. Bazilyuk, S.A. Talanov, V.F. Sagach, O. Appenzeller, Fos and nitric oxide synthase in rat brain with chronic mesostriatal dopamine deficiency: effects of nitroglycerin and hypoxia, Parkinsonism Relat. Disord. 8 (2002) 261–270.
[37] J.R. Moura, M.A.M. Prado, M.V. Gomez, E. Kalapothakis, C.R. Diniz, M.N. Cordeiro, M.A. Romano-Silva, Investigation of the effect of PhTx2, from the venom of the spider *Phoneutria nigriventer*, on the release of [^{3}H]-acetylcholine from rat cerebrocortical synaptosomes, Toxicon 36 (1998) 1189–1192.
[38] S. Murphy, M.L. Simmons, L. Agullo, A. Garcia, D.L. Feinstein, E. Galea, D.J. Reis, D. Minc-Golomb, J.P. Schwartz, Synthesis of nitric oxide in CNS glial cells, Trends Neurosci. 16 (1993) 323–328.
[39] M. Palkovits, M. Brownstein, Maps and Guide to Microdissection of the Rat Brain, Elsevier, New York, 1988.
[40] W.M. Pardridge, Blood–brain barrier biology and methodology, J. Neurovirol. 5 (1999) 556–569.
[41] A. Pardutz, I. Krizbai, S. Multon, L. Vecsei, I.J. Schoenen, Systemic nitroglycerin increases n-NOS levels in rat trigeminal nucleus caudalis, NeuroReport 11 (2000) 3071–3075.
[42] S.L. Peterson, J.J. Armstrong, M.K. Walker, Focal microinjection of carbachol into the periaqueductal gray induces seizures in the forebrain of the rat, Epilepsy Res. 42 (2000) 169–181.
[43] M.A.M. Prado, C. Guatimosim, M.V. Gomez, C.R. Diniz, M.N. Cordeiro, M.A. Romano-Silva, A novel tool for the investigation of glutamate release from rat cerebrocortical synaptosomes: the toxin Tx3-3 from the venom of the spider *Phoneutria nigriventer*, Biochem. J. 314 (1996) 145–150.
[44] C. Rapôso, G.M. Zago, G.H. da Silva, M.A. da Cruz-Höfling, Acute blood-brain barrier permeabilization in rats after systemic Phoneutria nigriventer venom, Brain Res. 1149 (2007) 18–29.
[45] H.J. Reis, M.A. Prado, E. Kalapothakis, M.N. Cordeiro, C.R. Diniz, L.A. de Marco, M.V. Gomez, M.A. Romano-Silva, Inhibition of glutamate uptake by a polypeptide toxin (phoneutriatoxin 3-4) from the spider *Phoneutria nigriventer*, Biochem. J. 343 (1999) 413–418.
[46] T.A. Rizvi, M. Ennis, M.M. Behbehani, M.T. Shipley, Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: topography and reciprocity, J. Comp. Neurol. 303 (1991) 121–131.
[47] M.A. Romano-Silva, M.V. Gomez, C.R. Diniz, M.N. Cordeiro, A.M. Ribeiro, Acetylcholine release from rat brain cortical slices evoked by the fraction P4 of the venom of the spider *Phoneutria nigriventer* has Ca^{2+} and temperature independent components, Neurosci. Lett. 219 (1996) 159–162.
[48] M. Salter, P.J. Strijbos, S. Neale, C. Duffy, R.L. Follenfant, J. Garthwaite, The nitric oxide-cyclic GMP pathway is required for nociceptive signaling at specific loci within the somatosensory pathway, Neuroscience 73 (1996) 649–655.
[49] S.V. Sampaio, J. Coutinho-Netto, E.C. Arantes, M.H. Toyama, J.C. Novello, J.R. Giglio, TsTX-VII, a new toxin from *Tityus serrulatus* scorpion venom able to induce the release of neurotransmitters from rat brain synaptosomes not blocked by tetrodotoxin, Biochem. Mol. Biol. Int. 41 (1997) 1255–1263.
[50] S. Schenberg, F.A. Pereira Lima, *Phoneutria nigriventer* venom, in: W. Bücherl, E.E. Buckley (Eds.), Venomous Animals and their Venom, vol. 3, Academic Press, New York, 1966, pp. 279–285.
[51] M. Simard, G. Arcuino, T. Takano, Q.S. Liu, M. Nedergaard, Signaling at the gliovascular interface, J. Neurosci. 23 (2003) 9254–9262.
[52] E. Taracha, M. Lehner, A. Wislowska-Staned, M. Zienowicz, P. Maciejak, A. Bidzinski, A. Skorzewska, D. Turzynska, A. Plaznik, Effects of methadone and morphine on c-Fos expression in the rat brain: similarities and differences, Pharmacol. Rep. 58 (2006) 120–124.
[53] M. Vergnes, A. Boehrer, A. Nehlig, Developmental characteristics of picrotoxin-induced convulsions in rats with genetic absence epilepsy, Exp. Neurol. 184 (2003) 549–551.

[54] M. Vergnes, A. Boehrer, S. Reibel, S. Simler, C. Marescaux, Selective susceptibility to inhibitors of GABA synthesis and antagonists of GABA(A) receptor in rats with genetic absence epilepsy, Exp. Neurol. 161 (2000) 714–723.

[55] H. Wang, Y.B. Wu, X.H. Du, Effect of dexamethasone on nitric oxide synthase and caspase-3 gene expressions in endotoxemia in neonate rat brain, Biomed. Environ. Sci. 18 (2005) 181–186.

[56] J.O. Willoughby, L. Mackenzie, A. Medvedev, J.J. Hiscock, Distribution of Fos-positive neurons in cortical and subcortical structures after picrotoxin-induced convulsions varies with seizure type, Brain Res. 683 (1995) 73–87.

[57] C.M. Yonamine, L.R.P. Troncone, M.A.P. Camillo, Blockade of neuronal nitric oxide synthase abolishes the toxic effects of Tx2-5, a lethal *Phoneutria nigriventer* spider toxin, Toxicon 44 (2004) 169–172.

VI. RESUMO DOS RESULTADOS

1) A análise clínica da intoxicação de ratos pelo veneno bruto de *P. nigriventer* sugere que há quebra da barreira hematoencefálica (BHE) em tempos precoces do envenenamento, uma vez que ocorrem sinais da ação central do veneno em poucos minutos da sua injeção sistêmica.

2) A observação ao microscópio de luz mostrou a presença de edema perivascular em todos os períodos investigados (15 min, 1, 2 e 5 horas após o envenenamento), o que veio a fortalecer a hipótese acima. Ao microscópio eletrônico, vasos com alteração de permeabilidade foram vistos, uma vez que o traçador eletrondenso estava presente tanto em vesículas de pinocitose, como nas áreas de junção intercelular, bem como se infiltrando para a membrana basal e para os espaços intercelulares entre os pés vasculares astrogliais, pericitos e células musculares lisas das arteríolas e vênulas, no cerebelo e hipocampo.

3) O extravasamento do traçador foi visto mais freqüentemente no hipocampo do que no cerebelo. Neste último houve pronunciado entumecimento dos pés-astrocitários, o que foi menos evidente no tecido hipocampal. O edema astrocitário visto nos vasos hipocampais estava presente mesmo em vasos aparentemente íntegros, isto é, sem extravasamento do traçador.

4) A expressão da laminina na membrana basal de vasos cerebrais e cerebelares diminuiu após 15 minutos de envenenamento, sendo posteriormente aumentada, principalmente após 5 h do envenenamento, quando a expressão dessa proteína foi visivelmente maior do que a fisiológica vista nos animais controles.

5) Houve envolvimento das áreas corticais frontal e parietal, substância cinzenta periaqueductal, núcleo talâmico periventricular, além do núcleo septal lateral no envenenamento, tendo em vista que neurônios ativados cFOS-positivos foram detectados nesses locais.

6) A expressão da óxido nítrico sintase (nNOS) aumentou após 2 h do envenenamento, no núcleo talâmico periventricular e no córtex parietal. Além disso, essa enzima foi detectada nas mesmas áreas da c-FOS, sugerindo um papel do NO na modulação do mecanismo da intoxicação.

VII. *CONCLUSÕES*

Nós concluímos que o veneno da aranha Phoneutria nigriventer é capaz de ativar diferentes áreas do SNC, relacionadas com atividade motora e stress. A localização coincidente da nNOS nas regiões que foram reativas para c-FOS, sugere que o NO pode estar envolvido na modulação da intoxicação. O fato de diferentes populações de neurônios reagirem diferentemente à toxicidade do veneno, levanta a possibilidade de neurotoxinas expecíficas do veneno podem ter como alvo áreas específicas. A modulação funcional de diferentes populações neuronais por uma toxina particular é importante para lançar luz no entendimento da relação estrutura-função. Será interessante correlacionar as diferentes toxinas do veneno com essas áreas. Os presentes achados suportam observações prévias na ação do veneno de Phoneutria no SNC e pode esclarecer, ao menos em parte, o dano à BHE.

O fato de a membrana basal endotelial ter sido afetada logo aos 15 min p.i., num momento em que os vasos sangüíneos não mostraram extravazamento do traçador, não havia entumecimento dos pés-astrocitários, ou vacúolos perivasculares, sugere que a quebra da BHE possa ser subestimada, uma vez que nem sempre os eventos morfológicos e fisiológicos podem ser sincrônicos. Os sinais clínicos observados imediatamente após o envenenamento suportam essa suposição. Os achados apontam para a existência de diferenças no vedamento da BHE, em diferentes regiões do cérebro e/ou diferentes sensibilidades de cerebelo e hipocampo ao veneno.

O veneno pode ser uma importante ferramenta para analisar a resposta de componentes estruturais da BHE, e para mapear as áreas cerebrais reativas, lançando luz, portanto, às vias envolvidas com o envenenamento por Phoneutria nigriventer no SNC. O conhecimento das vias relacionadas com neuropeptídeos específicos contidos no veneno pode ser útil para estudos relacionados com estrutura-função.

O fato de os efeitos do veneno de Phoneutria nigriventer no SNC serem reversíveis, pode fazer do mesmo uma interessante ferramenta para promoção de aberturas transitórias da BHE relevante para fins terapêuticos.