GABRIELA MARIOTONI ZAGO

ALTERAÇÕES NA PERMEABILIDADE DA BARREIRA HEMATOENCEFÁLICA EM DIFERENTES REGIÕES CEREBRAIS, A RESPOSTA ASTROCITÁRIA E A PRODUÇÃO DE CITOCINAS PRÓ-INFLAMATÓRIAS NO SNC – MODELO EXPERIMENTAL DE PHONEUTRIISMO

Este exemplar corresponde à versão final da Dissertação de Mestrado, apresentada à Pós-Graduação da Faculdade de Ciências Médicas - UNICAMP, para obtenção do Título de Mestre em Farmacologia da Fisioterapeuta – Gabriela Mariotoni Zago.

Campinas, 30 de janeiro de 2007.

Profa. Dra. Maria Alice da Cruz-Höfling
- Orientadora -

CAMPINAS

2007

GABRIELA MARIOTONI ZAGO

ALTERAÇÕES NA PERMEABILIDADE DA BARREIRA HEMATOENCEFÁLICA EM DIFERENTES REGIÕES CEREBRAIS, A RESPOSTA ASTROCITÁRIA E A LIBERAÇÃO DE CITOCINAS PRÓ-INFLAMATÓRIAS NO SNC - MODELO EXPERIMENTAL DE PHONEUTRIISMO

Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Mestre em Farmacologia.

ORIENTADORA: *PROFA. DRA. MARIA ALICE DA CRUZ-HÖFLING*

CAMPINAS

2007

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS DA UNICAMP
Bibliotecário: Sandra Lúcia Pereira – CRB-8ª / 6044

Z13a

Zago, Gabriela Mariotoni
Alterações na permeabilidade da barreira hematoencefálica em diferentes regiões cerebrais, a resposta astrocitária e a produção de citocinas pró-inflamatórias no SNC – Modelo experimental de Phoneurtriismo / Gabriela Mariotoni Zago. Campinas, SP : [s.n.], 2007.

Orientador : Maria Alice da Cruz-Höfling
Dissertação (Mestrado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Cérebro. 2. Neurotoxinas. 3. Citocinas. 4. Astrócitos. I. Cruz-Höfling, Maria Alice da. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Título em inglês : REGIONAL DIFFERENCES IN THE PERMEABILITY OF DIFFERENT CEREBRAL REGIONS, THE ASTROGLIAL REACTION AND THE PRODUCTION OF CYTOKINES IN THE CNS – AN EXPERIMENTAL MODEL OF PHONEUTRIISM

Keywords:
- Brain
- Neurotoxins
- Cytokines
- Astrocytes

Titulação: Mestrado em Farmacologia

Banca examinadora: Profa. Dra. Maria Alice da Cruz-Höfling
Prof. Dr. Francesco Langone
Prof Dr. Osvaldo Augusto Brazil Esteves Sant' Anna

Data da defesa: 30-01-2007

Banca Examinadora da Dissertação de Mestrado

Orientador: Profa. Dra. Maria Alice da Cruz-Höfling

MEMBROS:

1- Profa. Dra. Maria Alice da Cruz-Höfling

2- Prof. Dr. Francesco Langone

3- Prof.Dr. Osvaldo Augusto Brazil Esteves Sant'Anna

Curso de pós-graduação em Farmacologia, da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 30/01/2007

DEDICO ESSE TRABALHO...

Ao meu pai, Cristóvão, que hoje já não está entre nós, mas que sempre me apoiou nos meus objetivos, por mais difíceis que eles parecessem.

À minha mãe, Cleusa, que foi persistentemente amiga e companheira, transmitindo muito amor e carinho.

Ao meu irmão, Marcelo, pelo incentivo.

Aos meus tios, Antônio e Lila, pela constante ajuda.

Em especial, à Profª. Maria Alice da Cruz-Höfling pela oportunidade, orientação, por seu exemplar profissionalismo acadêmico e científico e por suas valiosas críticas e sugestões.

À bióloga Marta Beatriz Leonardo, pela paciência e disponibilidade de tempo no ensino das técnicas laboratoriais.

À amiga Catarina Rapôso pelo companheirismo e auxílio na realização deste trabalho.

Aos amigos do laboratório: Ângela Ito, Juliana Godoy, Karina Fontana, Thalita Rocha, Luciano de Barros, Fernanda Barbosa, Rosana Andrade e Soraya.

À Cleusa Franco, pelo auxílio durante a fase de microtomia.

Aos bioteristas Marcos Silva e Miguel Silva, do Deptº de Farmacologia, e Baltazar de Paula, do Deptº de Histologia e Embriologia.

À Profª. Liana M. C. Verinaud, por compartilhar seu conhecimento.

Aos Deptos. de Histologia e Embriologia, Fisiologia e Biofísica, do Instituto de Biologia, e ao Deptº. de Anatomia Patológica, da Faculdade de Ciências Médicas, pela disponibilização da infra-estrutura, bem como do apoio profissional, necessários à execução desse trabalho.

Aos funcionários do Deptº. de Farmacologia da Faculdade de Ciências Médicas, especialmente ao Wanderlei C. Claro, pela prestatividade.

E a todos que, direta ou indiretamente, contribuíram para realização deste trabalho.

À CAPES pela bolsa de estudos concedida.

Esse trabalho teve o suporte financeiro da CAPES, CNPq e FAPESP.

Primeiro fruto da sabedoria é a paciência, que leva o homem a se controlar, esperando pelo momento oportuno.

(L. Sabedoria; 19-21)

BHE	Barreira Hematoencefálica
SNC	Sistema Nervoso Central
O_2	Oxigênio
CO_2	Gás Carbônico
JO	Junções de Oclusão
JÁ	Junções de Aderência
gp-P	Glicoproteína – P
SNP	Sistema Nervoso Periférico
GFAP	Proteína Acídica Fibrilar Glial
IL	Interleucina
IFN	Interferon
TNF	Fator de Necrose Tumoral
FGF	Fator de Crescimento Fibroblástico
HIV	Vírus da Imunodeficiência Humana
TTX	Tetrodoxina
PNV	Veneno *Phoneutria nigriventer*

RESUMO

A barreira hematoencefálica (BHE) é a principal estrutura controladora da manutenção da homeostase do SNC. A perda da integridade da BHE em respostas inflamatórias do SNC, desencadeada por agentes neurotóxicos, têm sido associadas ao desenvolvimento de sinais neurológicos. O veneno da aranha *Phoneutria nigriventer* (PNV) produz sinais e sintomas excitatórios em humanos e sua ação neurotóxica sugere habilidade potencial em alterar a permeabilidade da BHE. Nesse trabalho, o PNV foi utilizado como ferramenta para avaliar a susceptibilidade da BHE em diferentes regiões anatômicas cerebrais de ratos. Após injeção sistêmica do PNV (0.85 mg/Kg in 0.5 ml), os ratos anestesiados foram perfundidos 1, 2 e 5 h após a injeção, com solução fixadora à qual foi adicionado traçador extracelular eletron-opaco. Córtex motor fronto-parietal, substância cinzenta periaquedutal, núcleos da base e amigdala foram dissecados e processados para microscopia eletrônica de transmissão. O estado funcional da BHE foi avaliado considerando evidências do vedamento da barreira (edema vasogênico e extravasamento do traçador) e a resposta de elementos do tecido circunjacente (astrócitos, terminais sinápticos, populações de células). Além disso, foi investigada a expressão das proteínas GFAP, o principal filamento intermediário dos astrócitos, proteína S100, uma família de proteínas ligantes de cálcio e as citocinas pró-inflamatórias, IFN-γ e TNF-α, através de marcação imunohistoquímica, no hipocampo e cerebelo. Logo após a administração do PNV, os animais mostraram sinais indicativos de envolvimento do SNC, SNP e SNA. Nossos resultados mostraram que todas as regiões analisadas apresentaram sinais morfológicos de reação defensiva, tais como migração de micróglia perivascular reativa, pés-vasculares astrocitário edemaciados e macrófagos ativos circulantes. Entretanto, apenas o córtex motor fronto-parietal mostrou número significante de vasos afetados em relação aos controles e às outras áreas anatômicas (1 h p.i.). Nos grupos controle, uma expressão basal de GFAP e S100B foi mantida inalterada durante os períodos de observação, enquanto nenhuma produção fisiológica das proteínas TNFα e INFγ ocorreu. Por outro lado, a análise dos grupos tratados com PNV mostrou que, variavelmente, todas as proteínas investigadas aumentaram sua expressão no cerebelo e hipocampo ao longo do tempo após a injeção do veneno. O aumento da GFAP no cerebelo foi mais precoce e mais forte do que no hipocampo. Essa gliose mais evidente no cerebelo, provavelmente justifica estudos prévios, onde o extravasamento do traçador foi menor nessa região do que hipocampo, demonstrando

assim, uma maior resistência da BHE do cerebelo. Outros mecanismos moleculares envolvidos poderiam ser a expressão de citocinas pró-inflamatórias TNFα e INFγ, cuja modulação diferente em hipocampo e cerebelo de animais tratados com PNV, poderia também ter papel nas diferenças de permeabilidade da BHE vistas em ambas as áreas após PNV. Nosso trabalho dá suporte à hipótese de que os sinais e sintomas apresentados pelos animais durante o intervalo de tempo, após a injeção de PNV e o sacrifício, refletem alterações fisiológicas em curso, que por sua vez se revela ao nível histológico e ultraestrutural no desigual envolvimento da BHE nas diferente regiões cerebrais analisadas. Os vasos do córtex motor fronto parietal foram mais afetados pelo PNV, do que as demais regiões, confirmando a existência de diferenças regionais na capacidade do tecido local de mediar eventos requeridos para que ocorram as alterações da permeabilidade da BHE e para a invasão de populações celulares. O veneno de *Phoneutria nigriventer* representa uma importante substância natural, cuja complexa composição pode ser explorada em relação à ação de drogas que agem no SNC.

ABSTRACT

The blood–brain barrier (BBB) is of pivotal importance to maintain homeostasis of the CNS, as it closely regulates the composition of the interstitial fluid in the brain. The loss of BBB integrity in CNS inflammatory responses triggered by neurotoxic agents has been associated with the development of neurological signs. *Phoneutria nigriventer* armed spider venom (PNV) produces excitatory signals and symptoms in humans, and its recognized neurotoxic action suggests a potential ability to alter BBB permeability. In this work, the PNV was used as tool to analyzing the BBB susceptibility of different rat brain anatomic regions. After PNV systemic injection (0.85 mg/ Kg in 0.5 ml) the rats were perfused at 1, 2 and 5 h post-injection (p.i.) with fixative solution to which had been added an electro-opaque extracellular tracer. Frontal-Parietal Motor Cortex, Periaqueductal Gray Matter, Base Nucleus and Amygdala were dissected and processed for routine transmission electron microscopy. The functional state of the BBB was evaluated considering evidences of the tightness of the barrier (vasogenic edema and tracer extravasation) and the response of elements of circumjacent tissue (astrocytes, synaptic endings, cells population). Besides, it was investigated the expression of the GFAP, the major intermediate filament of astrocytes, S100 protein, a family of calcium-binding proteins, and IFN-γ and TNF-α pro-inflammatory cytokines, through imunohistochemistry labeling, in the hippocampus and cerebellum. Soon after PNV dministration the animals showed clinical signs indicative of peripheral, autonomic and central nervous system involvement. Our findings showed that all regions analyzed presented morphological signs of defensive reaction, such as migrating reactive perivascular microglia, swollen astrocytes end-feet and circulating active macrophages. However, only FPMC showed significant number of affected vessels in relation to controls and the other anatomic areas (1 h p.i.). A basal expression of GFAP and S100 was maintained unaltered along the periods of observation, whereas none physiologic production of TNFα and INFγ proteins has occurred in control groups. In contrast, analysis of the PNV-treated groups showed that all investigated proteins variably enhanced its expression along the time-course after venom injection in cerebellum and hippocampus. The increase of GFAP in cerebellum is more precocious and stronger than in hippocampus. A more prominent reactive gliosis by cerebellum over hippocampus would be supposedly one of the molecular events underlying the previous findings showing to be cerebellum BBB more resistant to leakage than hippocampus. Other possible molecular mechanism

involved would be the expression of proinflammatory TNFα and INFγ cytokines, whose different modulation in cerebellum and hippocampus of PNV-treated animals could be also involved in differences of permeation of BBB seen in both areas. Our study further support the idea that the symptomatic interval after systemic *P. nigriventer* spider venom injection is characterized by sequential physiologic changes that are reflected in histological and ultrastructural preparations and reveal that BBB impairment is unequal in different anatomical brain areas. The Fronto-Parietal Motor Cortex vessels are more targeted for PNV, confirming the existence of regional differences in the capacity of the local tissue of mediating the events required for the changes of BBB permeability and for cell invasion. *Phoneutria nigriventer* venom represents an important natural substance, whose complex composition should be explored in terms of CNS acting drugs.

1- INTRODUÇÃO

Foram estudadas as alterações na permeabilidade da Barreira Hematoencefálica (BHE) em diferentes regiões cerebrais. Para atingir esse propósito, foi utilizado um modelo experimental para mimetizar acidente com a aranha armadeira *Phoneutria nigriventer,* que em caso de acidentes graves induz na vítima uma série de sinais e sintomas que sugerem o envolvimento do sistema nervoso periférico, autônomo e central. Ratos adultos receberam injeção endovenosa do veneno bruto da aranha *Phoneutria nigriventer* e o curso das alterações da permeabilidade foram avaliadas em diferentes períodos de tempo através de microscopia eletrônica de transmissão e imunohistoquímica; sendo os dados analisados qualitativamente e quantitativamente.

1.1- Origem e estrutura básica do sistema nervoso

O primeiro indício da formação do sistema nervoso ocorre na fase de gástrula e se apresenta como um espessamento do ectoderma, chamado placa neural, que cresce progressivamente tornando-se mais espessa, formando as pregas neurais, concomitante à formação de um sulco longitudinal denominado sulco neural, que se aprofunda para a goteira neural (Figura 1). Os lábios da goteira, isto é, as pregas neurais se fundem para formar o tubo neural. Ocorre proliferação de células de cada lado do tubo dando origem à crista neural, que por sua vez, dará origem a uma variedade de neurônios que vão migrar e se localizar fora do SNC (gânglios simpáticos e neurônios dos gânglios da raiz dorsal). O tubo neural irá originar neuroblastos e espongioblastos, que se diferenciarão em neurônios e células da glia, componentes do sistema nervoso central (Moore, 1984).

A estrutura do tecido nervoso do SNC é constituída por neurônios e células gliais, provenientes de uma camada simples de neuroeptélio proliferativo ou células do tubo neural primitivo (para revisão ver Watson, 1976; Pereira e Graça, 1990), e matriz extracelular não colagênica (Bertolotto et al., 1990).

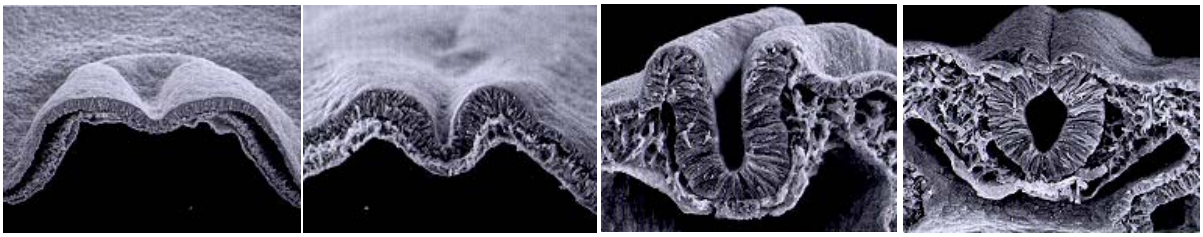

Figura 1- Micrografias de Microscopia Eletrônica de Varredura. Desenvolvimento do sistema nervoso. Imagens gentilmente cedidas pelo Prof. Dr. Sérgio Eduardo de Andrade Perez, Universidade Federal de São Carlos.

1.2- Barreiras do SNC

O sistema nervoso central (SNC) é o sistema mais crítico e sensível em todos os mamíferos; por isso, foi necessário o desenvolvimento de estruturas especializadas para isolar suas células principais, os neurônios, do sangue ou do fluido cérebroespinhal, de forma a que funcionassem em um ambiente iônico estável e assim assegurar que exercessem suas atividades de forma adequada.

A interface entre SNC e o sistema circulatório (sangue ou líquido cefaloraquidiano) é representada por uma barreira que regula o equilíbrio iônico, facilita o transporte de nutrientes e bloqueia moléculas potencialmente nocivas. As células endoteliais dos capilares e o fluido extracelular entre os neurônios e células gliais do SNC promovem esse ambiente.

Há três barreiras principais no SNC: o endotélio dos capilares cerebrais formando a barreira hematoencefálica (BHE), no epitélio aracnóide, e no epitélio do plexo coróide, formando a barreira entre o tecido nervoso e o fluído cerebroespinhal (Abott, 2005). Essas barreiras diferem em localização, tamanho, morfologia e função. A barreira entre o tecido nervoso e o fluido cerebroespinhal é composta pelo epitélio do plexo coróide, células ependimárias, membrana aracnóide e órgãos circumventriculares. A função de bloqueio é proporcionada pela presença de junções de oclusão (“tight junctions”) e de aderência presentes entre as células epiteliais das estruturas mencionadas, que se encontram em contato direto com o fluido cérebroespinhal e impedem o transporte paracelular,

direcionando o transporte entre tecido nervoso e ventrículos através da via transcelular. A existência de uma bomba de sódio controla ativamente esse transporte e mantém as diferenças na composição entre o tecido e o fluido cerebroespinhal (Abbott et al., 2006), entretanto, os capilares sangüíneos dessas regiões são fenestrados. Já a BHE é uma barreira endotelial, onde as "tight junctions" entre as células endoteliais, isolam a luz capilar direcionando o transporte transcelular através de um fluxo altamente seletivo entre o sangue e o cérebro.

1.2.1- Barreira Hematoencefálica (BHE) – Aspectos gerais e localização

A barreira hemato-encefálica (BHE) é uma barreira de difusão com propriedades essenciais para o funcionamento normal e homeostasia do SNC (Ballabh et al., 2004); sendo a principal interface controladora de trocas bidirecionais de nutrientes, substratos metabólicos e drogas, entre o sangue e o cérebro (Dermietzel e Krause, 1991; Gloor et al., 2001).

A existência física da BHE foi identificada primeiramente em 1885, quando Ehrlich observou que um corante introduzido por via intravenosa impregnava os tecidos de todo o organismo, com exceção do encéfalo, sugerindo que os capilares do SNC, diferentemente dos capilares de outras regiões, eram impermeáveis a este agente químico. Goldmann (1913), através de experimentos com azul de tripan e vários corantes vitais derivados da anilina, constatou após a introdução pela corrente sangüínea, que impregnavam o corpo inteiro do animal, com exceção do neuro-eixo. Entretanto, se o corante fosse introduzido por via subaracnóidea, o tecido nervoso ficava fortemente marcado, não atingindo a circulação periférica, demonstrando que há impedimento bi-direcional à livre passagem de substâncias para o encéfalo.

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. Os vasos sangüíneos dessas áreas do cérebro contêm fenestrações que permitem a passagem bi-direcional de substâncias entre sangue-cérebro sem passar pelo citoplama das células endoteliais. Essas áreas, com barreira

semipermeável regulam o sistema nervoso autonômo e as glândulas endócrinas (para revisão, ver Ballabh et al., 2004). Diferentemente, vasos com endotélio contínuo, não fenestrado, constituem as regiões cerebrais onde há BHE.

1.2.2- Componentes estruturais da BHE

A BHE é uma barreira de difusão seletiva caracterizada por "tight junctions" entre as células endoteliais, ausência de fenestrações no endotélio e transportadores específicos. Três elementos da microvasculatura cerebral compõem a BHE – células endoteliais dos capilares sangüíneos, pés-astrocitários (pés terminais vasculares dos prolongamentos dos astrócitos) e pericitos; as células endoteliais e os pericitos estão envolvidos pela membrana basal, composta de colágeno tipo IV, laminina, fibronectina e proteoglicanos (Farkas e Luiten, 2001). Além desses constituintes, a presença de terminais nervosos próximos aos capilares, micróglias, mastócitos e neurônios também contribuem para manutenção, regulação da permeabilidade e reparo da BHE (Risau e Wolburg, 1990; Janzer, 1993; Abbott, 2002; Dohgu et al., 2005).

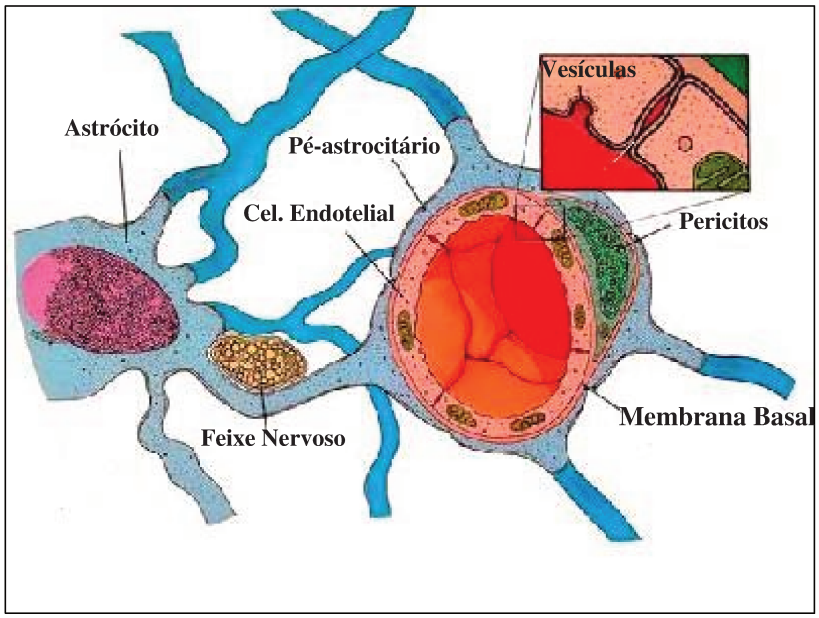

Figura 2- Constituintes da barreira Hematoencefálica – BHE. Modificado de Hawkins et al., 2006.

Embora o conceito de Barreira Hematoencefálica ter sido estabelecido há aproximadamente 100 anos atrás, o papel das células endoteliais nesta barreira só foi aceito posteriormente. A estrutura básica da BHE foi estabelecida nos anos 60 (Reese e Karnovsky, 1967; Brightman e Reese, 1969). As células endoteliais da BHE diferem de outras pelo seu metabolismo ativo, um número muito maior de mitocondrias (Oldendorf et al., 1977) e baixo número de vesículas pinocíticas (Sedlakova et al., 1999). Além disso, essas células diferem das células endoteliais do resto do corpo, pela ausência de fenestrações, e pela presença de junções de oclusão (JO) com alta resistência elétrica, e junções de adesão (JA) mais extensas e em maior número, apresentando baixa taxa de transporte vesicular pinocitótico. (Rubin e Staddon, 1999), sendo que essas JO limitam o fluxo paracelular de moléculas hidrofílicas, através da BHE. Em contraste, pequenas moléculas gasosas como O_2 e CO_2 difundem-se livremente através da membrana plasmática, podendo ser também uma rota de entrada para pequenos agentes lipofílicos, incluindo drogas como os barbitúricos e etanol (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 e Pardridge, 2001).

As células endoteliais apresentam propriedades responsáveis por características funcionais da BHE e manutenção da homeostase cerebral, uma vez que, fisicamente e fisiologicamente a via paracelular é vedada à difusão de íons, peptídeos e células imunes do sangue para o SNC (Huber et al., 2001), enquanto a via transcelular é altamente seletiva (Ballabh et al., 2004). Essas rotas de transporte serão discutidas a seguir.

1.2.3- Rotas de acesso a BHE

O complexo juncional das células endoteliais dos capilares cerebrais que compõe a BHE compreende as junções de oclusão (JO) e as aderentes (JA), ambas especializações intercelulares altamente restritivas ao transporte pela via paracelular.

As JOs, ultraestruturalmente aparecem como sítios de fusão envolvendo os folhetos externos das membranas plasmáticas de células endoteliais adjacentes.

As JOs são as estruturas chave na função da BHE. Elas 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. Essas proteínas acessórias conectam as proteínas de membrana à actina, que é uma proteína do citoesqueleto, e colaboram para a manutenção da integridade estrutural e funcional do endotélio (ver Ballabh et al., 2004, para revisão).

As JAs são junções que 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. Os componentes das JOs e JAs parecem interagir, particularmente através das ZO-1 e cateninas, influenciando a formação das JOs (Matter e Balda, 2003).

Outra rota presente na BHE é a via transcelular, como dito anteriormente, trata-se de uma rota altamente seletiva, onde suas proteínas são 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 (p. ex., a glicoproteína P, ou P-gp), 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 (para revisão, ver Luurtsema et al, 2004).

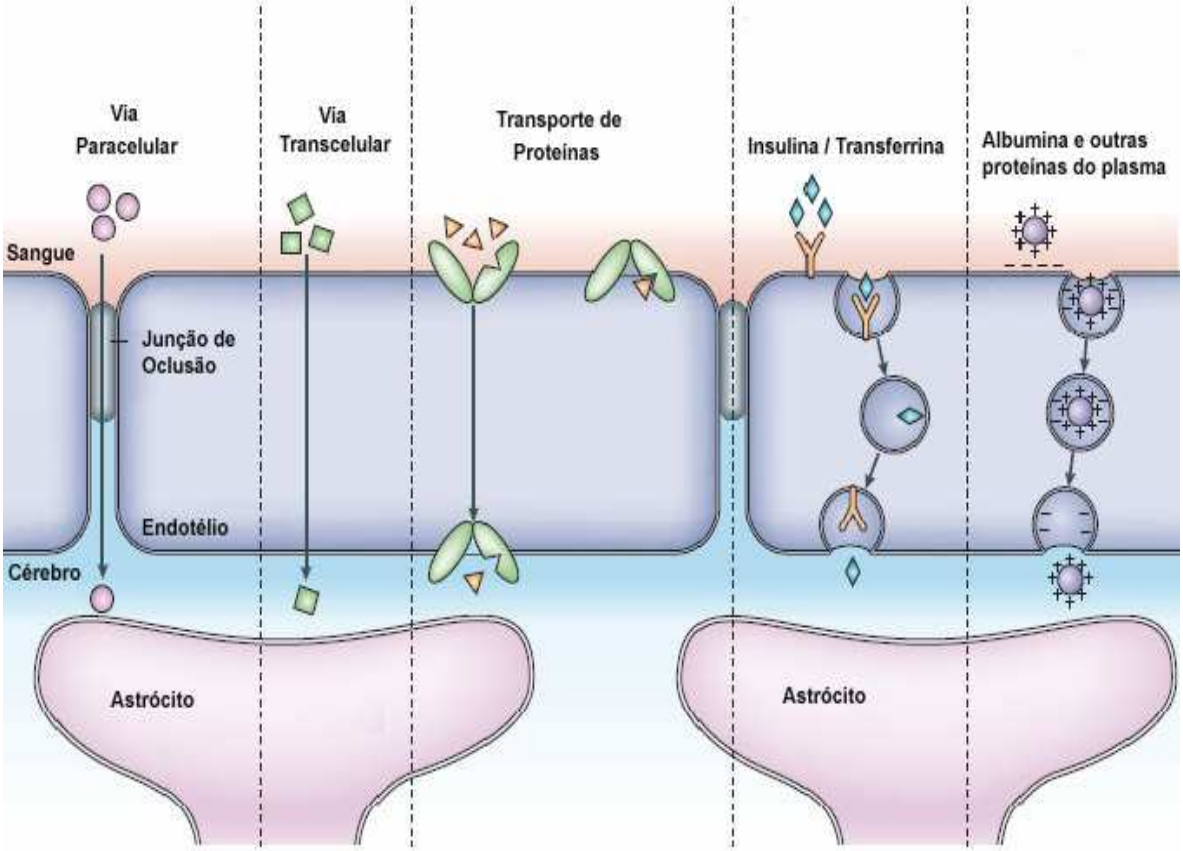

Figura 3- Vias de acesso à BHE. Note as células endoteliais que formam a BHE e sua associação com os pés astrocitários. Modificado de www.nature.com/reviews/neuro

1.2.4- Funções da BHE

A BHE tem várias funções (Abbott e Romero, 1996; Abbott, 2005; Begley e Brightman, 2006). Ela supre o cérebro com nutrientes essenciais e controla o efluxo de produtos indesejáveis. Restringe o movimento de fluídos entre o sangue e o cérebro, permitindo transporte iônico específico e, portanto, regulando o tráfico iônico. Produz o fluído intersticial do cérebro, o qual é responsável pelas funções neuronais.

Além disso, a BHE protege, de forma dinâmica, o SNC de flutuações na composição iônica, que ocorrem no sangue, após uma refeição ou exercício, a qual perturbaria a sinalização sináptica e axonal (Cserr e Bundgaard, 1984). O SNC não pode tolerar e continuar sua função diante de um ambiente com 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 e com níveis constantes (Begley, 2004)**.**

Desta forma, concluímos que a BHE age primordialmente na homeostase do SNC. A homeostasia cerebral é essencial para seu funcionamento e para o funcionamento do organismo como um todo. Outra importante função da BHE é de neuroproteção do SNC contra xenobióticos tóxicos. Em um tecido altamente complexo como o tecido nervoso, onde a divisão mitótica e proliferação das células neuronais são restritas, 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 e Brightman, 2003; Begley, 2004). Estudos mostraram que a proteína P-gp impede a ação dos xenobióticos (Gaillard et al., 2000).

A BHE também tem papel na manutenção dos neurotransmissores e agentes neuroativos agem centralmente (SNC) e perifericamente (SNP) separados (Abbott et al., 2006).

1.3- Astrócitos e sua relação com a BHE

No cérebro há 10 vezes mais células gliais do que neurônios, e dentre as células da glia, os astrócitos são os mais numerosos (Kimelberg e Norenberg, 1989; Benveniste, 1992). Classicamente, os astrócitos foram divididos em protoplasmáticos, encontrados predominantemente na substância cinzenta, e fibrosos, achados ao longo de tractos mielinizados e dotados de mais fibrilas gliais (GFAP), que os anteriores (Wilkin et al., 1990). Entretanto, os astrócitos mostram um número de diferentes morfologias, dependendo da sua localização e associação com outros tipos celulares. Aproximadamente 11 fenótipos distintos podem ser facilmente distinguidos, 8 envolvem interações específicas com vasos sangüíneos (Reichenbach e Wolburg, 2004). Os astrócitos foram considerados a "cola do SNC", com o papel de suporte estrutural para o desenvolvimento e as interações neuronais.

Evidências recentes sugerem que os astrócitos desempenham uma ampla cadeia de funções no sistema nervoso de mamíferos, incluindo regulação da densidade sináptica (Meshul et al., 1987), síntese e secreção de fatores tróficos (Liesie e Silver, 1988), reparação e regeneração de injúrias (Rudge et al., 1989), fagocitose de restos celulares e funções imunes, tais como secreção de citocinas (IL-1, IL-3, IL-6, IFN-γ e β, TNF-α) (Barres et al., 1991; Chen e Swanson, 2003), remoção de íons K^+ e Na^+ excedente da neurotransmissão, neurogênese e sinaptogênese e liberação de neurotransmissor (Diamond e Jahr, 1997). Devido a suas várias funções, os astrócitos tornam-se uma importante ferramenta quando investigamnos mecanismos que agem no SNC. Proteína Ácida Fibrilar Glial (GFAP), Síntese de Glutamina e S100 são os três principais marcadores de astrócitos do SNC (Walz e Lang, 1998; Savchenko et al., 2000).

Evidências advindas particularmente de estudos com cultura de células, sugerem que os astrócitos podem regular muitas características da BHE, principalmente o tônus vascular e 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) (Hayagashi, et al., 1997; Abbott, 2002; Haseloff et al., 2005).

Os astrócitos ou os fatores astrocíticos induzem a expressão de proteínas oxidativas (p. ex. superóxido dismutase manganês-dependente) nas células endoteliais, protegendo o cérebro de insultos oxidativos induzidos por radicais livres (Schroeter et al., 2001). Além dessas funções, os astrócitos também regulam a liberação de glutamato, transporte de água, e produção de óxido nítrico (Chen e Swanson, 2003).

Em resposta à injúria cerebral, o reparo do tecido é sempre realizado por participação astrocítária (Eddeleston, 1993). Nesses casos, os astrócitos sofrem alteração em suas características: proliferação e/ou hipertrofia de seus processos celulares, fenômeno como gliose astrocitária, astrocitose, astrogliose, cicatriz glial, ou simplesmente gliose. Outra reação dessas células diante dessa situação é aumentar a produção de filamentos intermediários, e também, da expressão da GFAP, mas outras proteínas filamentosas como a vimentina e a nestina também são expressas em astrócitos imaturos (Montgomery, 1994). Desta forma, concluímos que a reatividade dos astrócitos associada à ativação de microglias são potentes marcadores de injúria cerebral, ou pelo menos é um sinal de reatividade do SNC à modificação do microambiente, ou a quebra da homeostasia cerebral.

Essas células, em caso de injúria, são afetadas principalmente por tumores cerebrais (Collins, 2002) e áreas com alto stress oxidativo (Abdul-Khaliq et al., 2000).

A tentativa de depauperar astrócitos de GFAP e outras proteínas filamentosas tem sido um caminho para aprender mais sobre as funções fisiológicas e patológicas dessas células em geral e das proteínas em particular (Pekny, 2001), uma vez que o conhecimento sobre suas funções na saúde e na doença é ainda limitado (Penky e Pekna, 2004).

As principais características da BHE, isto é, a presença de endotélio não fenestrado, com junções intercelulares altamente oclusivas e impermeáveis, o transporte restritivo e seletivo, dentre outras, tem sido associados à presença dos astrócitos e há inequívocos sinais da estreita associação entre os prolongamentos terminais dos pés astrocitários e as células endoteliais (Orte et al., 1999).

Os astrócitos envolvem com seus "pés terminais" os pericitos (Kacem et al., 1998) e, além disso, interconectam as células endoteliais com os neurônios circunjacentes. À vista da associação anatômica com as células endoteliais, é de se supor que os astrócitos desempenham um papel proeminente no desenvolvimento, manutenção e quebra da BHE (Janzer e Raff, 1997). Pesquisas com várias linhagens de células endoteliais, inclusive de células endoteliais fora do SNC, têm mostrado que a presença dos astrócitos é essencial para o desenvolvimento do fenótipo de BHE, inclusive induzindo a formação de "tight junctions" e o aumento da expressão de reguladores da BHE, (Hurst e Fritz, 1996; Kuchler-Bopp et al., 1999).

1.3.1- GFAP - Proteína Ácida Fibrilar Glial

Apesar da heterogeneidade morfológica, bioquímica e funcional, com poucas exceções, todos os astrócitos caracterizam-se pela presença de prolongamentos citoplasmáticos contendo filamentos intermediários (fibrilas gliais) cujo componente principal, a proteína GFAP, serve como marcador desse tipo celular no SNC (Montgomery, 1994). O filamento intermediário mede cerca de 8-9 nm em astrócitos maduros, sendo encontrada em células homólogas a astrócitos, no que tange à sua situação perivascular como fator influenciador da permeabilidade a macromoléculas, peptídeos e íons no transporte transendotelial. Têm sido acumuladas evidências indicando a presença de GFAP nas células perivasculares: astrócitos (Bock et al., 1977), células perisinusoidais do fígado – armazenadora de vitamina A (Gard et al., 1985; Buniantian et al., 1994; Niki et al., 1996), células de Leydig, produtoras de testosterona, nos testículos (Holash et al., 1993), em condrócitos de cartilagem elástica (Kepes e Perentes, 1988) e nas células mesangiais e podócitos do rim (Buniantian et al., 1994). Mutações do gene para a GFAP têm sido encontradas na doença de Alexander, uma condição neurodegenerativa fatal em humanos (Pekny e Pekna, 2004).

O aumento da expressão de GFAP tem sido bem documentado com doenças relacionadas à idade (Kushner et al., 1991; Schipper et al., 1998), apresentando uma forte expressão na doença de Alzheimer, quando há presença de placas senis (Nagele et al., 2004).

Em casos de injúria cerebral e alterações hormonais, também ocorre aumento dessa proteína (Ke e Gibson, 2004). GFAP está associada com processos iniciais do desenvolvimento do sistema nervoso, provavelmente através da reestruturação dos componentes do citoesqueleto envolvidos com as alterações celulares associadas à diferenciação glial (Weier et al., 1984; DeArmond et al., 1986), ou em processos de reparo visando aspectos regenerativos do tecido cerebral adulto após lesões traumáticas ou químicas (Kindy, 1992).

1.3.2- S100

Pertence a um grupo de proteínas ácidas intimamente ligadas ao cálcio, com massa molecular de 21kDa, consistindo de 2 sub-unidades – alpha e beta: S100a0, S100a e S100b, as quais são alpha-alpha, alpha-beta e beta-beta, respectivamente. A S100 é encontrada abundantemente no cérebro, como uma mistura de S100a e S100b (nomeada S100–beta). Tipos celulares de origem neuroectodérmica e não – neuroectodérmica podem expressar essa família de proteínas (Donato, 1986; Beaudeux et al., 1999). Apesar da sua abundância no SNC de vertebrados, ela é inexistente nos invertebrados.

Nos cérebros de mamíferos, a S100a e S100b são confinadas a células gliais, enquanto S100a0 à neurônios. Alterações na estrutura das células gliais provocam extravasamento das proteínas S100 dentro do compartimento extracelular e do fluído cérebro espinhal, podendo alcançar o fluxo sangüíneo. A proteína S100 não tem atividade enzimática, mas está envolvida em uma variedade de processos celulares, tais como: regulação do ciclo celular, crescimento, diferenciação e motilidade celular (Marenholz e Heizmann 2004). Atua também em outras atividades, incluindo processos de memória, regulação da difusão de cátions monovalentes através das membranas, modulação do estado físico das membranas, regulação da fosforilação de muitas proteínas, controle da união e desunião de microtúbulos (Donato, 1986).

Além das funções intracelulares, muitas proteínas S100 como, S100B, S100A4, S100A8, S100A9, S100A12, e S100A13, são secretadas e agem como citocinas. Por exemplo, as S100A8/A9 atuam como moléculas quimiotáxicas na inflamação (Newton e Hogg, 1998). As S100B exibem a atividade neurotrófica (Huttunen et al., 2000),

S100A4 tem efeito angiogênico (Ambartsumian et al., 2001), a S100A12 está envolvida na resposta parasita-hospedeiro e a S100A13 é um componente do complexo secretado contendo fator de crescimento fibroblástico (FGF-1).

A proteína S100 beta é primariamente sintetizada no cérebro pelos pés–vasculares dos astrócitos e rapidamente liberada do cérebro para o sangue, quando a BHE é insultada (Donato, 1986; Mercier e Hatton, 2000; Kapural et al., 2002; Marchi et al., 2003). A S100 beta também tem sido encontrada em outros tecidos, mas em baixas concentrações (Jonson et al., 1999; Rickmann e Wolff, 1995).

Segundo Marchi et al. (2004), a S100 beta é um marcador, potencialmente usado para permeabilidade da BHE, uma vez que está diretamente relacionada com a integridade dessa barreira (Marchi et al., 2003). Doenças associadas com alterações nos níveis de expressão das proteínas S100 podem ser classificadas em 4 categorias: doenças cardíacas, doenças do SNC, desordens inflamatórias e câncer (ver tabela 1).

Tabela 1- Doenças humanas associadas com alterações nos níveis da proteína S100. Modificado de Marenholz et al., 2004

Proteínas	***Doenças***
S100A1	Cardiopatias
S100A2	Câncer
S100A3	
S100A4	
S100A5	
S100A6	
S100A10	
S100P	
S100B	
S100A8	Inflamatórias
S100A9	
S100A12	
S100B	Neurológicas

Dentre as patologias cerebrais, que apresentam alteração na expressão da S100, podemos destacar: Alzheimer, síndrome de Down (Mrak e Griffin, 2004) e esclerose múltipla. Em processos cancerígenos é encontrado alto nível dessa proteína em metástase de melanoma (Brochez e Naeyaert, 2000).

1.4- Pericito e Micróglia

Os pericitos são células que se originam da crista neural. Durante a angiogênise, que ocorre no curso do desenvolvimento do SNC os pericitos migram para uma camada de células neuroepiteliais. Essas células ficam em estreita proximidade às células endoteliais, delas separadas apenas pela membrana basal, que, aliás, partilham. Entre ambas as células se estabelecem interações que são mediadas pela endotelina-1 (Dehouck et al., 1997).

Pouco se tem explorado o papel dos pericitos na BHE. Os pericitos possuem proteínas contráteis propiciando a essas células contrair o diâmetro dos capilares cerebrais, dessa forma regulando o fluxo sangüíneo (Bandopadhyay et al., 2001). Essas células parecem induzir ou ter papel na manutenção do fenótipo da BHE apresentado pelas células endoteliais da microcirculação cerebral, à maneira como migram os astrócitos (Hori et al., 2004). Entretanto, os pericitos parecem migrar separando-se das células endoteliais em situações de hipóxia e trauma (Dore-Duffy et al., 2000).

As microglias são células gliais do SNC que se originam de células do sangue, os monócitos e correspondem aos macrófagos do parênquima neural. Em seu destino para o SNC, as microglias migram juntamente com as células endoteliais, sendo estas últimas olhadas como células imunes endógenas do SNC. Embora funções como fagocitose de materiais estranhos ao tecido nervoso (Xu e Ling, 1994), ou função de guia para monócitos atravessarem as células endoteliais (Peridsky et al., 1999) sejam sugeridas, o real papel das microglias na estrutura da BHE permanece a ser elucidado.

1.5- O SNC e a resposta imune

O SNC tem sido muitas vezes considerado como um sítio imunologicamente privilegiado devido à presença da BHE (Fabry et al., 1994). Condições patológicas do SNC freqüentemente resultam em ruptura da BHE, induzindo a produção de uma sucessão de citocinas e permitindo o aparecimento de abundantes infiltrados inflamatórios compostos de células T ativadas, linfócitos B e macrófagos. Esses fatores solúveis das células linfóides, por sua vez, mostram-se capazes de modular o crescimento e função das células gliais.

Por outro lado, essas células são capazes de secretar inúmeras moléculas imunorregulatórias como interleucina (IL-1, IL-3, IL-6), INT-γ e β e TNF-α, que, em contrapartida, influenciam o sistema imune, estabelecendo-se assim uma aparente comunicação bidirecional mediada entre células linfóides e gliais (Benveniste, 1992; Montgomery, 1994), criando uma cadeia de interação entre derivados do sangue e células nervosas.

As citocinas pertencem a um grande grupo de polipeptídios produzidos por uma ampla variedade de células. Os linfócitos T são os maiores produtores de citocinas (Munoz-Fernandez e Fresno, 1998). A ação regulatória das citocinas vai evocar uma seqüência altamente ordenada de eventos, levando ao reparo da lesão e à formação cicatricial (Pawlinski e Janeezko, 1997). Nesses casos há transformação morfológica, isto é, os astrócitos exibem inúmeros fenótipos, além de haver, proliferação dos mesmos (Yong et al., 1996).

Estudos mostraram que o IFN-γ pode ser um fator de forte atividade biológica iniciando e controlando alterações inflamatórias no tecido cerebral (Simmons e Willenbourg,1990; Sethna e Lmpson, 1991). Em situações em que a BHE é alterada, os linfócitos penetram na área injuriada, tornando-se a principal fonte de IFN-γ.

O TNF-α é produzido por astrócitos, microglia/macrófago, e agem na inflamação promovendo extravasamento de neutrófilos, linfócitos e monócitos e favorecendo a adesão local para células endoteliais. Ele também controla a resposta imune

pela modulação da ativação das células T e induz a síntese de citocinas (Fernandez et al; 2004). Segundo Renno et al. (1995) altas concentrações dessa citocina aparecem no local do dano cerebral, e no fluído cerebroespinhal.

Estudos relataram que injeções intracranianas de TNF-α, em roedores, resultaram em uma forte resposta astrogliótica e ativação microglial (Kahn et al., 1997).

As células endoteliais do SNC são resistentes à inflamação e limitam a resposta inflamatória. Apesar disso, nas doenças inflamatórias do SNC, a quebra da BHE é acompanhada pelo rompimento das "tight junctions" (Petty e Lo, 2002). Vários mediadores inflamatórios mediam a permeabilidade da BHE (Abbott, 2000). Por exemplo, embora a bradicinina aja diretamente sobre as células endoteliais, ela também pode ativar o NF-kB nos astrócitos para que liberem interleucina-6 (IL-6) (Schwaninger et al., 1999). O TNF-α produz endotelina-1 endotelial e leva à liberação de interleucina 1b dos astrócitos (Deli et al., 1995).

As citocinas secretadas nos processos inflamatórios periféricos geralmente não comprometem a BHE, a menos que seja acompanhada por patologia do SNC. É interessante notar que algumas inflamações periféricas como a depressão cortical (Gursoy-Ozdemir et al., 2004) e o "stress" por dor cirúrgica aguda (Oztas et al., 2004) tem efeito sobre as proteínas relacionadas às "tight junctions" das células cerebrais.

1.6- Neurônios

Os vasos sanguíneos cerebrais estão estreitamente ligados à atividade neuronal. É sabido que o fluxo sanguíneo aumenta em resposta à ativação neuronal local (Iadecola, 1993). Porém, os mecanismos celulares deste processo não são ainda bem entendidos (Leybaert, 2005). A quebra da BHE é frequentemente seguida por alterações patológicas do fluxo sanguíneo e da pressão de perfusão, o que seria mais um mecanismo compensatório do que um simples rompimento anatômico (Lee et al., 1999). No SNC adulto, os neurônios não se contactam diretamente com as células endoteliais, pois entre eles estão dispostos os astrócitos. São estas células que mediam a conexão neurovascular.

Entretanto, durante o desenvolvimento, os neurônios indiferenciados e em diferenciação podem contactar-se com as células endoteliais.

Por isso, a relação que se estabelece precocemente entre neurônios imaturos e células endoteliais, que ocorre no decorrer do desenvolvimento e formação do SNC, pode influenciar a indução da BHE nas células endoteliais (Pardridge, 1999).

1.7- Relação da BHE com doenças e injúrias neurológicas

Progresso substancial tem sido feito pelo entendimento dos mecanismos patofisiológicos envolvidos na permeabilidade da BHE. Em muitas patologias que acometem o SNC, o endotélio cerebral, tem um papel importante nos processos dessas doenças, pois ocorre ruptura ou modificações na estrutura da BHE, aumentando a permeabilidade vascular (Marchi et al, 2003).

Essa disfunção na integridade estrutural e funcional da BHE pode ser a causa de vários processos neurológicos (Greenwood, 1991, Tanobe et al., 2003), podendo provocar sua ruptura em humanos em condições de hipertensão aguda (Nag e Harik, 1987) isquemia (Belayev et al., 1996), o uso de drogas convulsivantes (Nitsch e Klatzo, 1993), choque osmótico (Kajiwara et al., 1990), epilepsia (Nitsch et al., 1986), trauma medular espinhal (Lossinsky et al., 1979), esclerore múltipla (Cláudio et al., 1995), malária severa (Adams et al., 2002), encefalopatia e tumores cerebrais (Davies, 2002), infecção por HIV-1 (Toborek et al., 2005) e desordens humorais (Rubin e Staddon, 1999). Em animais de laboratórios quando submetidos ao envenenamento pela aranha *Phoneutria nigriventer* (Le Sueur et al., 2003; Raposo et al., submetido) e pela lagarta *Lonomia obliqua* (Sílva et al., 2004).

O efeito dessas doenças na BHE pode secundariamente afetar o fluxo sanguíneo cerebral e o tônus vascular, os quais adicionalmente influenciam o transporte através da membrana (Marchi et al, 2003). (Ver tabela 2).

A medida da função da BHE pode ser uma importante ferramenta no diagnóstico de doenças progressivas. Atualmente somente técnicas invasivas e caras, como a ressonância magnética, a tomografia computadorizada e a punção lombar, são disponíveis para avaliar clinicamente a integridade da BHE. Uma abordagem alternativa tem sido proposta, consistindo de detectar alterações na composição sangüínea que indiquem ruptura da BHE (Kapural et al., 2002).

Tabela 2- Desordens do SNC associadas com disfunção da BHE. Modificado de Kim et al., 2006.

Mecanismos	**Doenças do SNC**
Neoplasias	Tumores benignos e malignos do SNC
Vasculares	Hemorragia, isquemia, hipertensão, mal formação vascular
Metabólicas	Diabetes, neurointoxicação (metais pesados e químicos)
Inflamatórios	Esclerose múltipla, meningite ou encefalite (viral, bacteriana, fungos e alérgica)
Traumáticos	Dano térmico, mecânico ou químico, irradiação
Epilepsias	Diversos tipos

1.8- Acesso de drogas terapêuticas no SNC - através da BHE

Enquanto a diminuição da função da BHE é um componente etiológico de várias doenças cerebrais, a integridade dessa barreira pode restringir a entrada de substâncias terapêuticas no cérebro, restringindo o possível tratamento dessas doenças. Segundo Chen et al., (2004) pequenas drogas lipofílicas difundem-se através da BHE o suficiente para serem eficazes, no entanto, muitas drogas potencialmente úteis são excluídas.

Uma técnica de modulação da BHE, através da via paracelular 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, 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 2 5% 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 (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 e Brightman, 2003).

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.

1.9- Neurotoxinas: seu uso no estudo das funções fisiológicas e patológicas

Em um passado de 15 a 20 anos, houve um dramático aumento nos estudos dos venenos de muitos animais, particularmente escorpiões e aranhas. Especialistas, bioquímicos, farmacologistas, fisiologistas, patologistas, dentre outros, tem-se voltado ao estudo detalhado dos venenos de artrópodos, especialmente as aranhas, pois representam uma inacreditável fonte de substâncias biologicamente ativas, agindo em várias funções fisiológicas vitais, em mamíferos e insetos. Especialistas constataram que os sinais clínicos produzidos nas vítimas de aracneismo podem ser potentes ferramentas de caráter investigativo. As substâncias naturais, de origem animal ou vegetal possuem considerável interesse farmacológico, uma vez que, em modelos experimentais, torna-se possível a compreensão dos mecanismos fisiológicos ou patológicos que ocorrem no ser humano (Rash e Hodgson, 2002).

A disponibilidade de diversas neurotoxinas de ocorrência natural pode ser usada como meio específicos para a marcação de moléculas de canais iônicos, uma vez que, essas neurotoxinas se ligam fortemente a esses canais, alterando suas funções (Cruz-Höfling et al., 1985; Love et al., 1986; Massacrier et al., 1990; Prado et al., 1996). Dentre as neurotoxinas destacamos a tetrodoxina (TTX), tetraetilamônio, a saxitoxina extraída de um dinoflagelado que infecta moluscos, a batracotoxina de rãs venenosas da América do Sul, além de venenos de escorpiões e aranhas.

Estudos mostraram que as moléculas de TTX, que são acrescentadas à solução que banha a célula bloqueiam os canais vontagem-dependentes de Na^+, diminuindo gradativamente as amplitudes do potencial de ação pré e pós-sináptico, e o uso do tetraetilamônio, bloqueia os canais voltagem-dependentes de K^+ (Kandel et al., 2000).

1.10- Aranhas – aspectos gerais

Constituem as aranhas uma ordem Aranea de artrópodes da classse dos aracnídeos, cujos representantes se distribuem por três subordens: mesotelas (Mesothelae), caranguejeiras (Orthognatha) e aranhas verdadeiras (Araneomorphae).

Há cerca de 33 mil espécies descritas, a maioria de hábitos terrestres. Habitam todos os continentes e climas, desde o tropical até o frio. Apenas na Antártica ainda não se encontram aranhas. Seu tamanho pode variar de 0.5 mm até mais de 20 cm. Todas são predadoras e alimentam-se principalmente de insetos. 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 acima de 70 anos.

Venenos de aranhas representam uma inestimável fonte de substâncias biologicamente ativas, agindo em várias funções fisiológicas vitais, em mamíferos e insetos. Muitas toxinas isoladas de venenos de aranhas e escorpiões têm sido fundamentais na determinação do papel de diversos canais iônicos neurais e o processo de exocitose de neurotransmissores.

1.10.1- A aranha *Phoneutria nigriventer*

Phoneutria nigriventer é conhecida como aranha "armadeira", devido à posição de ataque que assumem, quando se sentem ameaçadas. Trata-se de uma aranha sul americana, extremamente agressiva, com hábitos noturnos, que se alimenta de uma vasta variedade de animais, incluindo muitas espécies de insetos, outras aranhas e pequenos roedores. Essa espécie não constrói teias, e seu sucesso como predador é explicado pela alta toxicidade de seu veneno (Brazil e Vellard, 1925; Schenberg e Lima, 1966; Lucas, 1988).

O envenenamento por *P. nigriventer* é um importante problema de saúde pública, uma vez que, os acidentes causados pelas aranhas dessa espécie constituem cerca de 42% dos casos registrados no Estado de São Paulo (Boletim do Ministério da Saúde, 1989; Barraviera, 1992; Bucaretchi et al., 2000).

As manifestações clínicas apresentadas pelos indivíduos picados por essas aranhas, são classificadas em leve, moderada e grave. Nas manifestações leves observamos dor irradiada no sítio da picada, edema local, parestesia e sudorese generalizada. As manifestações moderadas são caracterizadas por tremores, paralisia espástica, sialorréia, taquicardia, hipertensão arterial, agitação psicomotora e distúrbios visuais (Brazil e Vellard, 1925; Lucas, 1988). Nas manifestações graves, observam-se vômitos freqüentes, hipotensão arterial, bradicardia, dispinéia, insuficiência e arritmias cardíacas, convulsões, choque, coma, priapismo, edema pulmonar agudo e parada cardiorespiratória (Schenberg e Pereira Lima, 1971; Bucaretchi, 2000). Todavia a maioria das manifestações clínica é do tipo leve ou moderada, sendo raro casos graves.

Figura 4- Aranha *Phoneutria nigriventer,* foto cedida pelo Agrônomo Antônio de Pádua Rosolino.

1.10.2- Composição, neurotoxicidade e farmacologia do veneno

A composição do veneno dessa espécie é uma mistura de polipeptídeos e moléculas biologicamente ativas, a maioria delas neurotóxicas (Brasil e Vellard, 1925; Diniz, 1963;Shenberg e Pereira Lima, 1971; Fontana e Vital Brazil, 1985). Por esse motivo, é freqüente a observação de efeitos nocivos em animais experimentais e em vítimas de phoneutriismo (Chavez-Olortegui et al, 2001).

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. Os primeiros estudos bioquímicos revelaram que o PNV possuía potentes neurotoxinas com ação excitatória, induzindo salivação, lacrimação, priapismo, paralisia flácida e espástica dos membros anteriores e posteriores e morte, após injeção intracerebral em camundongos (Diniz, 1963; Schenberg e Pereira Lima, 1971; Entwhistle et al., 1982).

O PNV causa uma variedade de efeitos farmacológicos, como a ativação dos canais de sódio voltagem-dependente levando à despolarização repetitiva e finalmente ao bloqueio neuromuscular em preparações do nervo frênico do diafragma de camundongos (Fontana e Vital Brazil, 1985), e ou atraso na inativação dos canais de sódio *in vivo*

(Cruz-Hofling et al., 1985). Em terminações nervosas autônomas de aurículas de porquinhos da Índia, foi observada a liberação de acetilcolina e norepinefrina (Vital-Brazil et al., 1988). Em ratos produziu a contração do músculo liso de vasos (Antunes et al., 1992; Marangone et al., 1993; Bento et al., 1995), o relaxamento do corpo cavernoso (Rego; 1996), uma resposta bifásica de pressão arterial sanguínea no sistema cardiovascular, caracterizada por hipotensão de curta duração, seguida por hipertensão prolongada e sustentada (Costa et al., 1996). Em coelhos o veneno de *Phoneutria* nigriventer também produzia resposta hipertensora prolongada, sugerindo alterações hemodinâmicas de origem periférica e central (Estato et al., 2000).

O veneno provoca a ativação dos receptores de histamina e serotonina, que parcialmente produzem a formação de edema, devido ao aumento da permeabilidade vascular na pele de rato e camundongos (Antunes et al., 1992), também observado na pele dorsal de coelhos (Marangoni et al., 1993).

No SNC, particularmente na região do hipocampo e cerebelo, a injeção sistêmica do veneno provoca alterações na modulação da BHE, em tempos tardios (Le Sueur et al, 2003;2004) e agudos (Raposo et al., submetido).

Várias frações desse veneno têm sido isoladas. Verificou-se a existência de 3 frações neurotóxicas – letais em camundongos denominadas Phtx1, Phtx2 e Phtx3 e uma fração não tóxica, com atividade em músculo liso (Rezende et al., 1991). Posteriormen**e,** uma fração de ação inseticida, denominada Phtx4 a qual foi extremamente tóxica em insetos da ordem Díptera e Dictióptera, mas com fracos efeitos tóxicos em camundongos (Entwistle et al., 1982; Diniz et al., 1990; Rezende et al., 1991; Cordeiro et al., 1992; 1993; Figueiredo et al., 1995; Romano-Silva et al., 1996). As frações diferem entre si pela massa molecular, composição de aminoácidos, sequencia do N-terminal e ação biológica.

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 (Escoubas et al., 2000; Gomez et al., 2002; Rash e Hodgson, 2002).

2- OBJETIVOS

2.1- Geral

Avaliar a permeabilidade da BHE em diferentes regiões cerebrais, em tempos agudos após a injeção intravenosa do veneno bruto da aranha *Phoneutria nigriventer*.

2.2- Específicos

Investigar possíveis alterações histopatológicas relacionadas às alterações de permeabilidade da BHE através da microscopia de luz (ML) e da microscopia eletrônica de transmissão (MET).

Analisar a resposta astrocitária, através das proteínas GFAP e S100, como indicativo de injúria do SNC - Imunohistoquímica.

Analisar possível processo inflamatório, através da expressão de citocinas pró-inflamatórias, INF-γ e TNF-α, através de imunohistoquímica.

3- CAPÍTULOS

Esta dissertação está baseada na informação CCPG/001/98 UNICAMP que regulamenta o formato alternativo para a dissertação de mestrado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato (Anexo 1).

Desta forma, esta dissertação é composta de dois artigos, os quais foram submetidos para publicação, conforme descrito abaixo:

3.1- Artigo 1 - Acute Blood-Brain Barrier Modulation by Neurotoxic venom, submetido a Neuroscience Research.

3.2- Artigo 2 - Astroglial Reaction during acute injury of the CNS against systemic neurotoxins-containing spider venom, submetido a Cellular and Molecular Neurobiology.

CAPÍTULO I

[*]Maria Alice da **Cruz-Höfling**
Departamento de Histologia e Embriologia
Instituto de Biologia, C.P. 6109
Universidade Estadual de Campinas (UNICAMP)
13 087-130, Campinas – SP – BRASIL
E-mail: hofling@unicamp.br
Tel/Fax (55)(19) 3521 6247; Fax. (55)(19) 3521 3124

ACUTE BLOOD-BRAIN BARRIER MODULATION BY NEUROTOXIC VENOM

[*]Gabriela Mariotoni Zago[1,2]; [*]Catarina Rapôso[1]; Gustavo Henrique da Silva[3];
Marta Beatriz Leonardo[1]; Maria Alice da Cruz-Höfling[1,2]

[1]Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas – Universidade Estadual de Campinas, [2]Departmento de Farmacologia and [3]Departamento de Anatomia Patológica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas. Campinas, SP 13083-970, Brazil.

*****These authors contributed equally to the development of this work.

Abstract

Several stimuli provoke changes in vascular permeability in CNS and threat the homeostasis of the CNS. *Phoneutria nigriventer,* the most aggressive spider from South America, causes neurological disturbances in the victims in accidents graded as severe. The venom (PNV) contains neurotoxins and was shown to alter BBB permeability of hippocampus. In this work, PNV was used for analyzing regional BBB susceptibility in the Frontal-Parietal Motor Cortex (FPMC), Periaqueductal Gray Matter (PAG), Base Nucleus (BN) and Amygdala (AM) of Wistar rats. After PNV systemic injection (0.85 mg/ Kg, in 0.5 ml saline) the rats were perfused with fixative to which had been added an electro-opaque extracellular tracer The functional state of the BBB was evaluated considering the tightness of the barrier (vasogenic edema and tracer extravasation) and the response of tissue circumjacent (astrocytes, synaptic endings, cells population) to the microvessels at 1, 2 and 5 h post-injection. All regions showed morphological signs of defensive reaction, such as migrating reactive perivascular microglia, swollen astrocytes end-feet and circulating active macrophages. However, only FPMC showed significant number of affected vessels in relation to controls and the other anatomic areas (1 h p.i.). This work reinforces the view of regional differences in the selectivity of BBB. *Phoneutria nigriventer* venom represents an important natural substance, whose complex composition should be explored in terms of CNS acting drugs.

Key works: Central nervous system; Venom; *Phoneutria nigriventer;* BBB; Toxic peptides

Introduction

The interface between blood and brain is a physical three-layered barrier composed of the cell bodies of endothelial cells and pericytes and the surrounding end-feet processes of astrocytes. Interspersed among the cell entities a basal lamina is found. Altogether they constitute a diffusion barrier known as blood-brain barrier (BBB). It hampers influx of most compounds from blood to brain. Two general mechanisms underlie the function of the BBB. Firstly, a high electrical resistance and extensive tight junctions seal the interface of juxtaposed endothelial cells to prevent most blood-borne substances from enter into the brain without passing across the cell membrane and cytoplasm (Rubin and Staddon, 1999). Secondly, the endothelial cells (EC) themselves exhibit a low rate of endocytosis ought to the presence of numerous highly specific membrane receptors and cytosol transporters. Specific transporters working at the BBB enables nutrients to enter. Drug-efflux transporters highly concentrated in the luminal endothelium membrane prevent and actively remove a broad range of drug molecules from the endothelial cell cytoplasm before they cross into the brain parenchyma (Tamai and Tsuji, 2000). These attributes entitle EC to exert selective trancytosis of macromolecules and solutes from blood to brain (Miller, 2003), which is also highly based on lipid solubility, molecular size, and charge (Habgood et al., 2000). Finally, but not less important, astrocytic end-feet tightly enwrap the vessel wall what seems to be critical for the induction and maintenance of the BBB tightness (Ballabh et al., 2004).

As result of all these physical, chemical and metabolic barriers, a stable composition of the extra-cellular fluid can be precisely regulated in terms of solute concentrations. This permit the CNS relies on accurate synaptic transmission and inhibition, and spatial and temporal summation, to perform its complex integrative functions (Begley and Brightman, 2003). If by one side a highly controlled system is destined to maintain a stable microenvironment crucial for CNS well-functioning, by other it constitutes the main obstacle for neurotherapeutics. In this context, researches aimed to show changes in the BBB tightness in different anatomic regions of the CNS by the use of pharmacologically active substances can be helpful in brain therapy managements.

We recently demonstrate, through transmission electron microscopy using lanthanum nitrate as an extracellular tracer, that *Phoneutria nigriventer* spider venom (PNV) infused in the circulation causes modulation of the BBB permeability in hippocampus of adult rats at chronic periods after envenoming (18 h to 9 days) (Le Sueur et al., 2003). A subsequent study where observations were done on days one and 9 after envenoming showed that the BBB permeation occurred by increased transcellular vesicular transport by a mechanism microtubule-dependent, since it was abolished in animals pre-treated with colchicine. No visible involvement of the paracellular route at these very same periods was observed (Le Sueur et al., 2004). A further study, now seeking for BBB impairment at acute stages of envenoming showed that the microvessels in cerebellum and hippocampus were affected and that cerebellum's microvessels were more resistant to the venom. Interestingly the changes observed were transient. After peaking between 1 to 2 h post-PNV, a tendency to normality gradually was established (manuscript submitted).

The co-existence of an "enzymatic barrier" together with a physical one provides to BBB the conditions for rapid metabolizing peptide drugs and nutrients (Vorbrodt, 1988; Minn et al., 1991; Brownlees and Williams, 1993; El-Bacha and Minn, 1999). As amino acids and their associated linkage are highly susceptible to enzymatic degradation, the nature and concentration of specific enzymes at the BBB environment can greatly affect the efficacy of a given peptide-based drug (Pardridge, 1991; Witt et al., 2001).

The *P. nigriventer* spider venom is basically a mixture of relatively low molecular mass basic polypeptides (5,000 – 6,000 Da) (Schenberg and Pereira-Lima, 1966), most of which neurotoxic (Brazil and Veillard, 1925). In the last decade, the venom has been studied in great biochemical detail. Among others, it was shown that it represents a cocktail of biologically active ion channels-acting toxins (Gomez et al., 2002).

The controlled transient opening of the BBB can be a useful strategy for the entry of therapeutic drugs into the CNS, and also of great significance in regard to venom peptide viability. In the current study, we screened four areas of the CNS aiming at

mapping the behavior of the BBB microvessels after systemic injection of a sub-lethal single intravenous injection of *P. nigriventer* venom.

Materials and Methods

Animals and venom

Male Wistar 8-10-week-old rats (250-350 g) obtained from an established colony from Centro Multi-Institucional de Bioterismo (CEMIB) at State University of Campinas (UNICAMP) were used. Animal were housed in a temperature-controlled room (25-28ºC) on a 12 h light/dark cycle with lights on at 6 a.m. and were fed standard Purina chow and water *ad libitum*. One lot of lyophilized *P. nigriventer* crude venom (PNV) was kindly donated by Instituto Butantan (São Paulo, SP, Brazil). The venom was stored at –20ºC and dissolved in 0.9% sterile saline solution immediately before use. All procedures were carried out in accordance with the guidelines proposed by the Brazilian Council on Animal Care (COBEA) and approved by the animal care committee of UNICAMP.

PNV envenoming

A single intravenous (i.v.) injection of PNV at a selected concentration of 850 μg/Kg, in 0.5 ml of 0.9% sterile saline was given to rats in the tail vein (Le Sueur *et al.,* 2003). To another group was given the same volume of the vehicle by the same via (sham controls). Animals were monitored in the periods comprised between saline or venom injections and sacrifice to follow the signs caused by the procedures done. After 1, 2, and 5 h post-injection (n=4/period), animals of each group were anesthetized by intra peritoneal injection of ketamine (100 mg/Kg) and xylasine (10 mg/Kg) and then perfused through the left ventricle with 150 ml of a 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). The pressure of perfusion of both solutions was controlled up to 70 mm Hg using a mercury manometer. Lanthanum nitrate has been used as an anionic extracellular marker of small size and electron opacity to electrons.

Transmission electron microscopy

After perfusion, the rat corpses were maintained at 4ºC overnight (18 h) before dissection. This procedure prevents artifact damage of the brains. Periaqueductal gray matter, fronto-parietal motor cortex, amygdala and base nucleus (striatum) were dissected and 1-2 mm samples were kept in the same fixative without lanthanum nitrate for one hour.

Then they 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 resin. Semithin sections (1 μm thick) were cut on an ultramicrotome (Reichert S Ultra-Cut, Leica), stained with 1% toluidine blue and examined by light microscopy. Ultrathin sections (60 nm thick) obtained from selected areas were mounted on copper grids (200 mesh) and double-contrasted with uranyl acetate and lead citrate for examination in a LEO 906 (Zeiss) transmission electron microscope operated at 60 kV.

Quantitative analysis

Coordinates of transversal planes and limits of the brain regions analyzed were selected according Palkovits and Brownstein atlas of rat brain anatomy (1988). The stereotaxics coordinates and regions examined are indicated in Figure 1.

The blood vessels of periaqueductal gray matter, fronto-parietal motor cortex, amygdala and base nucleus (striatum) affected by the i.v. injection of *P. nigriventer* spider venom or saline solution were counted in semithin toluidine blue section by light microscopy (LM, 20x objective) and in ultrathin sections by transmission electron microscopy (TEM). For LM, the criterium used was the presence of perivascular edema, characterized by spaces in the periphery of the vessels wall. A total of eight sections (1 mm^2 each) per period were examined in each anatomic region (2 sections x 4 animals=8 sections/per time interval post-injection in each of the brain region studied). The percentage of vessels affected in each region per period was calculated by dividing the number of vessels with vasogenic edema by the total number of vessels contained in the whole sectional area of each of the eight sections examined. The results were compared with

paired sham controls. Comparison among the brain regions at each time interval was done in PNV-treated animals.

For TEM, the criteria used were two: presence of swollen perivascular astrocytes end-feet around the vessels and the existence of blood vessels with lanthanum tracer extravasation out of the lumen. The tracer could have invaded the interendothelial cleft, achieved the underlying basal lamina or deeper and/or be filling endocytotic or pinocytotic vesicles of the endothelial cell cytoplasm. Only vessels with more than two pinocytotic vesicles were considered affected. At each region, at least 10 vessels were counted in one ultrathin section (~1000 μm^2), meaning that four sections were examined per period (since n=4 rats/period) totaling 40 vessels counted/per period. The percentage of blood vessels (arterioles, venules and capillaries) affected was calculated by dividing the number of vessels presenting swollen perivascular astrocyte end-feet and/or leakage of the tracer, by the total number of vessels (40 vessels) in the sections observed. The figures obtained at each region per period of time in the PNV-injected group were compared with those of saline-injected ones (sham control). Comparison among the regions along the time-course of envenoming was also done.

Besides vessels quantification, the qualitative morphological characteristics of the surrounding parenchyma of PNV-injected and controls were analyzed and compared.

The results were expressed as means ± S.E.M. using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer Multiple Comparisons Test to compare regions of controls versus matched of PNV-treated rats. Inter-regions comparison was also done in PNV group, at each time point after envenoming. A value of $P < 0.05$ indicated statistical significance.

Results

While saline-injected animals were normal in their activities inside the cage, the envenomed animals presented various excitatory and inhibitory clinical signs of intoxication. The envenoming probably resulted in over-stimulation of muscarinic and

nicotinic receptors in the autonomic and central nervous system and at the neuromuscular junction. Signs indicating increased parasympathetic stimulation were sialorrhea, bronchospasm and tremors (11 rats). Effects at the neuromuscular junction were inferred by weakness, fasciculations, and eventually flaccid paralysis followed by spastic paralysis (10 rats) of posterior legs in the rats. Central effects included altered behaviour, apnea and seizures (9 rats). Lethality was surprisingly low (8.3%), caused by respiratory failure. This animal was discarded and has to be replaced. The necropsy showed lung edema. The intensity of the signs varied among the animals indicating individual susceptibility to the spider venom neurotoxic effect.

The observation of 1 μm thick toluidine blue-stained sections showed that a consistent vascular damage in PNV-envenomed rats, although variable in number, was the presence of wide spaces around the microvessels of the regions examined. The changes were interpreted as vasogenic edema. Frontal parietal motor cortex (FPMC), periaqueductal gray matter (PAG), base nucleus (BN, mainly striatum) and amygdala (AM) showed to have different interactions with the venom, as inferred by the variability in the number of vessels with vasogenic edema. Probably such variability reflects the variable intensity of the clinical signs exhibited by the animals.

The quantitative analysis of the toluidine blue stained sections showed that the impairment of the BBB was significant only in the FPMC region. This was seen by counting the vessels with perivascular edema, characterized by wide spaces around the microvessels (Fig. 2A,B,C,D). In this region, in comparison to controls, the peak of BBB modulation occurred one hour after venom injection ($P<0.001$; Fig. 2B), after which a clear significant recovery was seen at 2 and 5 h after PNV administration ($P<0.01$). In contrast, no significant number of vessels with vasogenic edema was seen in AM, PAG and BN in any of the time points after envenoming, in comparison with the sham group (Fig.2 A,C,D). The comparison among the four anatomic regions examined at the same period after envenoming showed that FPMC has significantly higher percentage of vessels with vasogenic edema in relation to all other regions at one hour p.i. ($P<0.01$, except for FPMC versus NB where $P<0.05$).

The counting of vessels showing leakage of the electron opaque tracer and/or swelling of the perivascular astrocytes end-feet (TEM examination) reproduced the results obtained by LM quantification (Fig. 3A,B,C,D). In comparison to matched controls, BBB increased permeability was significant only in FPMC region one hour post-PNV ($P<0.05$; Fig. 3B). Inter-region comparisons at the time points scheduled showed that the variation in the number of affected vessels by circulating PNV was not significant ($P<0.05$).

FPMC of saline controls examined by TEM showed the tracer confined into the vessel's lumen (Fig. 4A). In envenomed animals, leakage of $LaNO_3$ occurred mostly by appearance of increased number of tracer containing-pinocytotic vesicles from lumen to the endothelial cytoplasm. In the vessels affected (peak at one hour p.i.), the tracer could reach the intercellular spaces underneath endothelium (Fig. 4B), or cause strong vasogenic edema concomitant with alterations of the neural structures around (Figs. 4B,C,D). Figure 4E shows the pinocytotic vesicle filled with tracer. Changes in the mitochondrial population and in synaptic endings are a common finding In FPMC. In the PAG region, tracer extravasation was seen impregnating the interendothelial cleft, but not the underlying basal lamina or elements underneath in all periods observed (Fig. 5). In spite of being the region more resistant to the venom effect in comparison to the others examined (Figs. 2 and 3), PAG was the region where perivascular tissue damage was more intense (Figs. 5B,C,D). There the swelling of the perivascular end-feet processes of astrocytes was more evident than in any of the other regions. In addition, in the aqueduct some vessels with marked tracer extravasation also contained macrophages with phagosomes that had phagocytized the tracer (Figs. 5C,D). AM and BN were the regions where the morphological changes of the surrounding tissue were less evident, indicating resistance against the circulating venom (Fig. 6). In these areas the tracer never attained the endothelial basement membrane, remaining restrict to pinocytotic vesicles or at least reaching only the luminal aspect of the cleft, just until part of the endothelial junctional complex. Besides the presence of reactive swollen astrocyte end-feet, common to all regions examined, other signs of strong defensive response were represented by reactive microglia (FPMC, AM and BN), multinuclear macrophages (PAG - aqueduct) and monocyte in margination (AM, Fig. 6C).

Discussion

Our results showed that the *P. nigriventer* spider venom when injected systemically in rats induces a series of clinical signs that prove the neurotoxic action of the venom on the central, autonomic and peripheral nervous system. In the current study, the signs persisted until the sacrifice of the animals (at 1, 2 and 5 h after PNV). However, previous studies of longer term, using the same dose and concentration of venom reported that 12 h after envenoming all the clinical signs are clinically resolved (Le Sueur et al., 2003). The loss of BBB integrity in CNS injuries has generally been associated with the development of neurological signs (Phares *et al.,* 2006). Disruption of the BBB seen in this study is an unquestionable feature of injury to the central nervous system caused by venom. The venom caused appearance of a significant number of blood vessels with vasogenic edema in the fronto-parietal motor cortex (FPMC), but the effects in the vessels of the periaqueductal gray matter (PAG), amygdala (AM) and base nucleus (BN, striatum) were not numerically significant when compared to saline-injected controls ($P<0.05$). In general, the data on the affected vessels seen by LM examination coincided with the observations done by TEM, the peak of damage was at one hour post-injection, after which a gradual tendency to normalizing occurs. If we analyzed the figures obtained in each region it became clear that the percentage of affected vessels was higher when the counting was done by TEM, except for FPMC. A plausible explanation for that could be the lower resolution of the LM, which in comparison to TEM permits detection of changes at a sub-cellular level, therefore enabling a finer exploration of changes. On the other hand, the subtle lower number of affected vessels seen by TEM in FPMC could be ascribed to the fact that LM lower resolution favors a more panoramic view in contrast to TEM's restrict field of vision due to the high resolution of the electron microscope. The fact that LM and TEM quantification were unanimous in showing significant higher percentage of affected vessels in FPMC, than in the other brain areas and in relation to control gives additional reliability to the findings. Our observations permitted to see that regions with higher percentage of affected vessels are regions in which gray matter surpasses white matter (FPMC). We also observed that white matter is less vascularized than gray matter. In support to our observations, Hagen et al. (1999) and Hendrikse et al. (2004) demonstrated in different anatomic areas that the brain blood flow is higher in gray (48.5 ml/100 g/min)

than in white matter (22.6 ml/100 g/min). This could explain why amygdala and base nucleus regions presented relatively low percentage of affected vessels, since in these regions there is predominance of white matter. On the other hand, periaqueductal gray matter in which predominates gray matter, although not having significant increase of affected vessels, was the region where the changes in the surrounding neural tissue were quite severe. In this work, the analysis of FPMC, BN, AM and PAG vessels, including the aqueductal ones revealed a difference in resistance of BBB against the systemic injection of PNV, since they did not respond equally to the action of neurotoxins contained in venom.

Regional differences have already been seen in hippocampus and cerebellum of rats at acute stages of *P. nigriventer* envenoming. The study showed that BBB in cerebellum exhibited higher resistance for damage than in hippocampus. In addition of being cerebellar microvessels least permeable to tracer passage, they also exhibit faster recovery of laminin expression after their drastic down-regulation caused by PNV, than the vessels of hippocampus (Raposo et al., submitted).

Regional differences in the tightness of the BBB have been reported elsewhere. For instance, the clearance of the attenuated rabies virus CVS-F3 in CNS provoked up-regulation chemokines and loss of BBB integrity more extensive in the cerebellum than in the cerebral cortex (Phares *et al*., 2006). Differences in the tightness of BBB would be also responsible for the higher uptake of amylin over insulin by whole brain, midbrain, frontal and occipital cortex and cerebellum (Banks and Kastin, 1998). Yet, it has been shown that a single dose of pentylenetetrazole administered to rats resulted in 100% bilateral BBB impairment in the preoptic area, caudate nucleus, putamen, thalamus, hypothalamus, midbrain, and superior colliculus, whereas repeated treatment induced BBB impairment only in hypothalamus, caudate nucleus, cerebellum, thalamus, and pons in a few animals (Sahin *et al.,* 2003). Although the mechanisms behind these differences are unclear, it is not unrealistic suppose that the quality and intensity of the stimulus can modulate inversely a same brain region. On the other hand, because a same toxicant agent modulates differently different brain regions it could be inferred that the molecular structure of the BBB varies depending on the microenvironment. In such circumstances the physical, metabolic and "enzymatic" components of the BBB actually should be anatomical

area-specific. However, being the progression and so the regression of BBB impairment a dynamic process, interaction with neurotoxic drugs may induce changes in the local BBB phenotype, hence on their physiology.

Here, the interaction of circulating PNV with components of the BBB was used to target the vasculature of different regions of the CNS. The study can also open possibilities of using physiologic concentrations of *P. nigriventer* toxins to provoke temporary openings of the BBB, a feature already seen be typical of the PNV on peripheral (Cruz-Höfling et al., 1985; Love and Cruz-Höfling, 1986) and in central nervous system (Le Sueur et al. 2003, Raposo et al., submitted). Despite the brain is an organ with high systemic nutrients requirements, the BBB exerts a rigid control of the most of the soluble molecules in the blood. This control is collaborative since involves specialized endothelium, pericytes, basement membrane and astrocyte cell processes and eventually neurons. Putatively, an extensive repertoire of messages should be changed among all the BBB entities. From all the characters played by the components of the BBB, is far the endothelium traditionally the first protagonist. The unusual impermeability of brain vessels, particularly the capillaries, is attributed to the highly organized tight junctions sealing contacts between outer leaflets of endothelial cells plasma membrane. Junctional proteins, such as occluding, junctional adhesion molecule, claudins, zonula occludens family (ZO-1, ZO-2 and ZO-3), and cingulin, all present at the plasma membrane contact constitute the fundament which explain the high electrical resistance of the capillary endothelium responsible for the repressive paracellular transport of solutes. In addition, an extremely tutored vesicular transport is responsible for the restrictive transcellular transport at BBB (Rubin and Staddon, 1999).

Previous studies focusing on the alterations of the junctional proteins of hippocampus microvessels induced by PNV i.v. injection showed that on day one and 9 the BBB remains impaired despite signs of envenoming were sub-clinical (Le Sueur et al., 2004). The authors found that at these late periods of PNV envenoming, the BBB impairment occurred by vesicular transport disarrangement, while the junctional proteins expression were unchanged. In the present study, BBB disruption and vascular damage undoubtedly implicate endothelial cells as targets of PNV. However, swelling of astrocytes

end-feet suggests the importance of astrocytes in the BBB functioning and in addition points these glial cells as another candidate for the venom action. We saw that in addition to the different susceptibility of FPMC, PAG, AM and BN in relation to the systemic presence of PNV, there was recruitment of different cell types to the BBB environment. Reactive microglia was seen in the white matter of AM and BN, reactive macrophages were seen in PAG, swollen astrocytes were found everywhere.

Although the regional differences seen in this study could be partially attributable to the involvement of different cell populations, as well as to the anatomic characteristic of each region, one cannot neglect the cross talk among the various intrinsic components of the barrier able to interact with the toxicant agent. Studies in course suggest the involvement of pro-inflammatory cytokines TNF-α and IFN-γ in the response of hippocampus and cerebellum to PNV, and a stronger reactive gliosis in the cerebellum over hippocampus, revealed by a marked production of the cytoskeletal GFAP and metabolic S100 proteins.

Our study further supports the idea that during the symptomatic interval after systemic *P. nigriventer* spider venom injection a row of sequential physiologic changes should be coursing with. These changes were reflected in histological and ultrastructural preparations and revealed that BBB impairment is unequal in different anatomical brain areas. A systematic study aimed to identify if the extent of gray matter in a particular anatomic area could represent a key point in these differences is welcome. This measurable increase in microvessels permeability is the final product of a multi-protagonist piece of events in which many cells and molecular targets are involved. Our study also further support the hypothesis that *P. nigriventer* neurotoxins, some of which ion channels acting-toxins can be promising tools either for testing regional selectivity of BBB a pre-requisite in the search for neurotherapeutic drugs, and/or for themselves be tested as transient openers of the BBB.

Acknowledgments

The authors thank Instituto Butantan (São Paulo, SP, Brazil) for venom donation, Mrs. Marta Beatriz Leonardo (BSc) for excellent technical assistance, and Mr. Miguel B. Silva and Marcos A. Silva for animal care. This study was supported by grants of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP # 98/00341-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq # 522131/95-6). G.M.Z. and C.R. are recipients of scholarships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

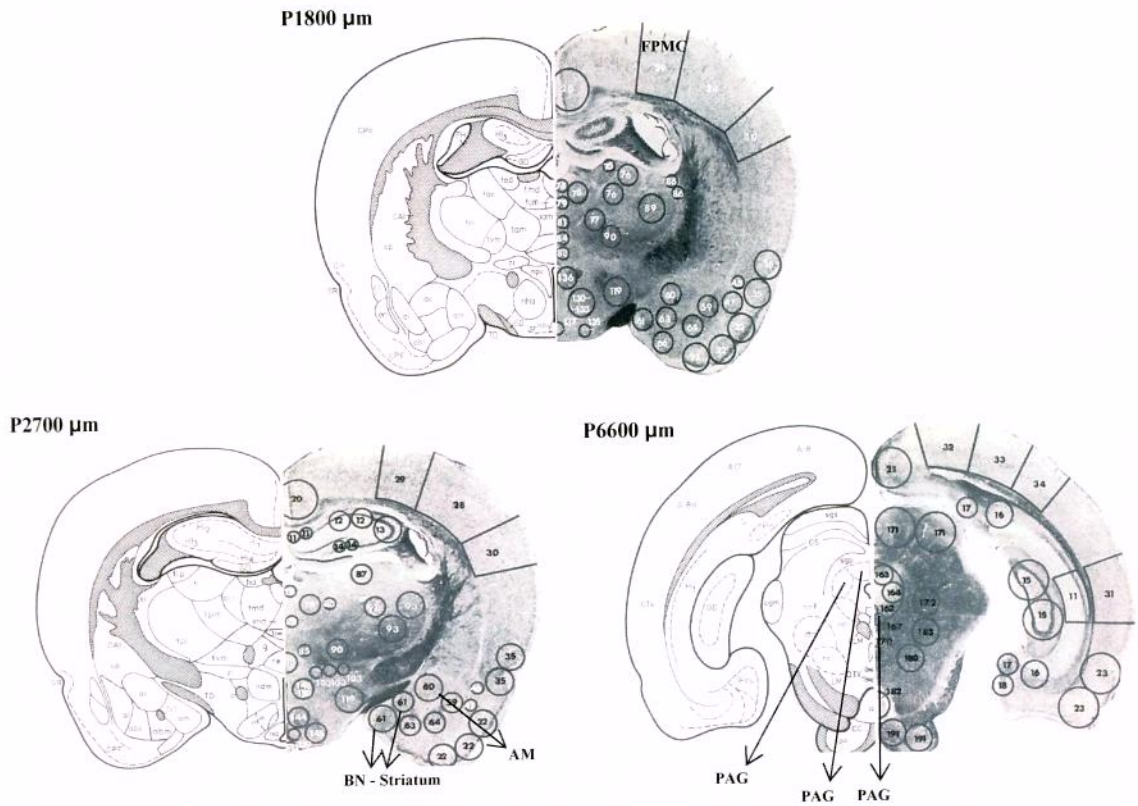

Figure 1: Schematic representation of cross sectional sections of the brain illustrating the location of areas whose BBB integrity was valued after *P. nigriventer* spider venom injected intravenously. Brain areas were selected according Palkovits and Brownstein [28]. FPMC - Frontal-Parietal Motor Cortex; PAG - Periaqueductal Gray Matter; BN - Base Nucleus (Striatum) and AM – Amygdala.

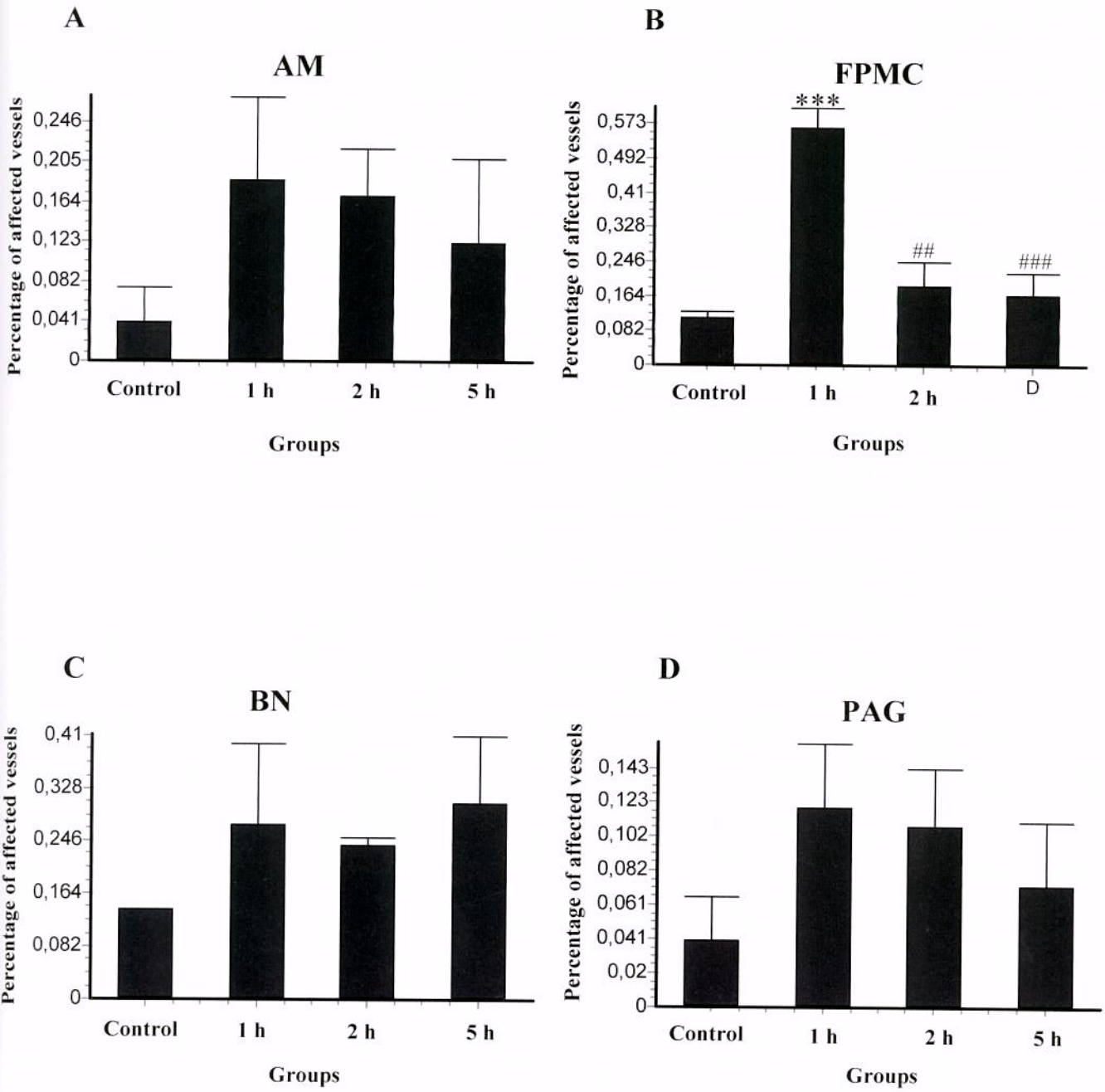

Figure 2: Quantitative analysis of amygdala (A), fronto-parietal motor cortex (B), base nucleus (striatum) (C), periaqueductal gray matter (D) affected vessels 1, 2, 5 h after PNV injection. The counting was made by light microscopy. Each bar represents the number of vessels with vasogenic edema per period divided by the total number of vessels counted in eight sections of ~1 mm2 each, (n= 4 rats x 2 sections per rat. Total = 8 sections/time interval). Only FPMC presented significant higher number of vessels with vasogenic edema at one hour after PNV, compared to sham group (*P<0.001). This alteration was transient, after 2 (##P<0.01) and 5 h (###P<0.001), a significant decrease of the vasogenic edema occurred in FPMC. A relative individual variability has been perceived and probably responds by the lack of difference among regions and groups. Inter-regions comparison showed a significant difference at one hour post-PNV between FPMC and other regions (P<0.01), except in relation to NB (P<0.05). Mean ± SEM.; One-way variance analysis (ANOVA) and Tukey-Kramer Multiple Comparison Test.**

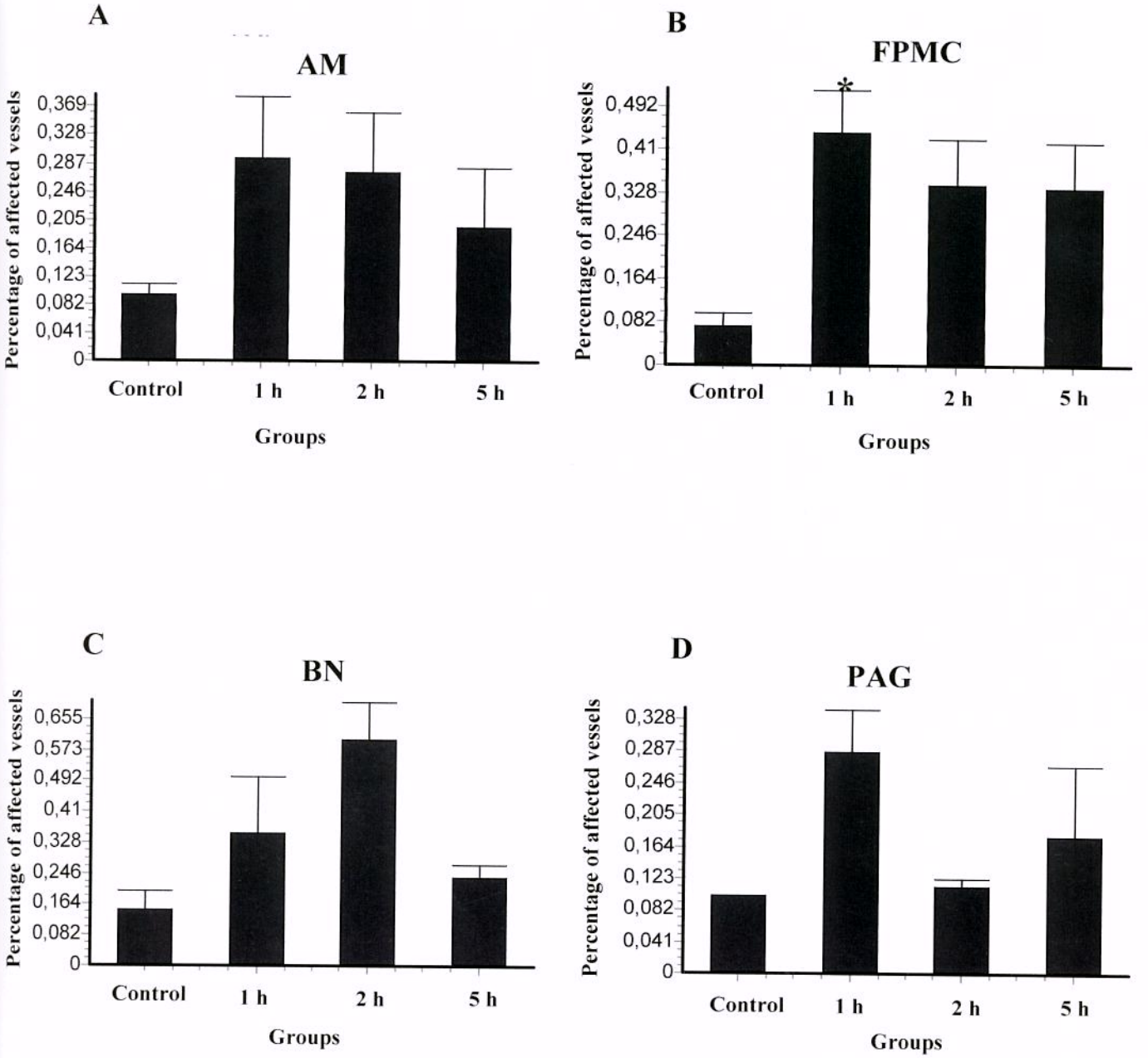

Figure 3: Percentage of vessels of amygdala (A), fronto-parietal motor cortex (B), base nucleus (striatum) (C) and periaqueductal gray matter (D) which presented lanthanum nitrate leakage after 1, 2 and 5 h after PNV injection (i.v.) in comparison to control, and in comparison among regions at matched time point. The counting was done in double-contrasted ultra-thin sections seen by transmission electron microscopy. Each bar represents the mean number of affected vessels per period (± SD) divided by the total number of vessels counted in one section (~1 mm2) per rat at each time interval (n= 4 rats x 10 vessels per rat = 40 vessels/time interval). FPMC, one hour after PNV presented increased percentage of affected vessels- *$P < 0.05$ *(compared with control group).*

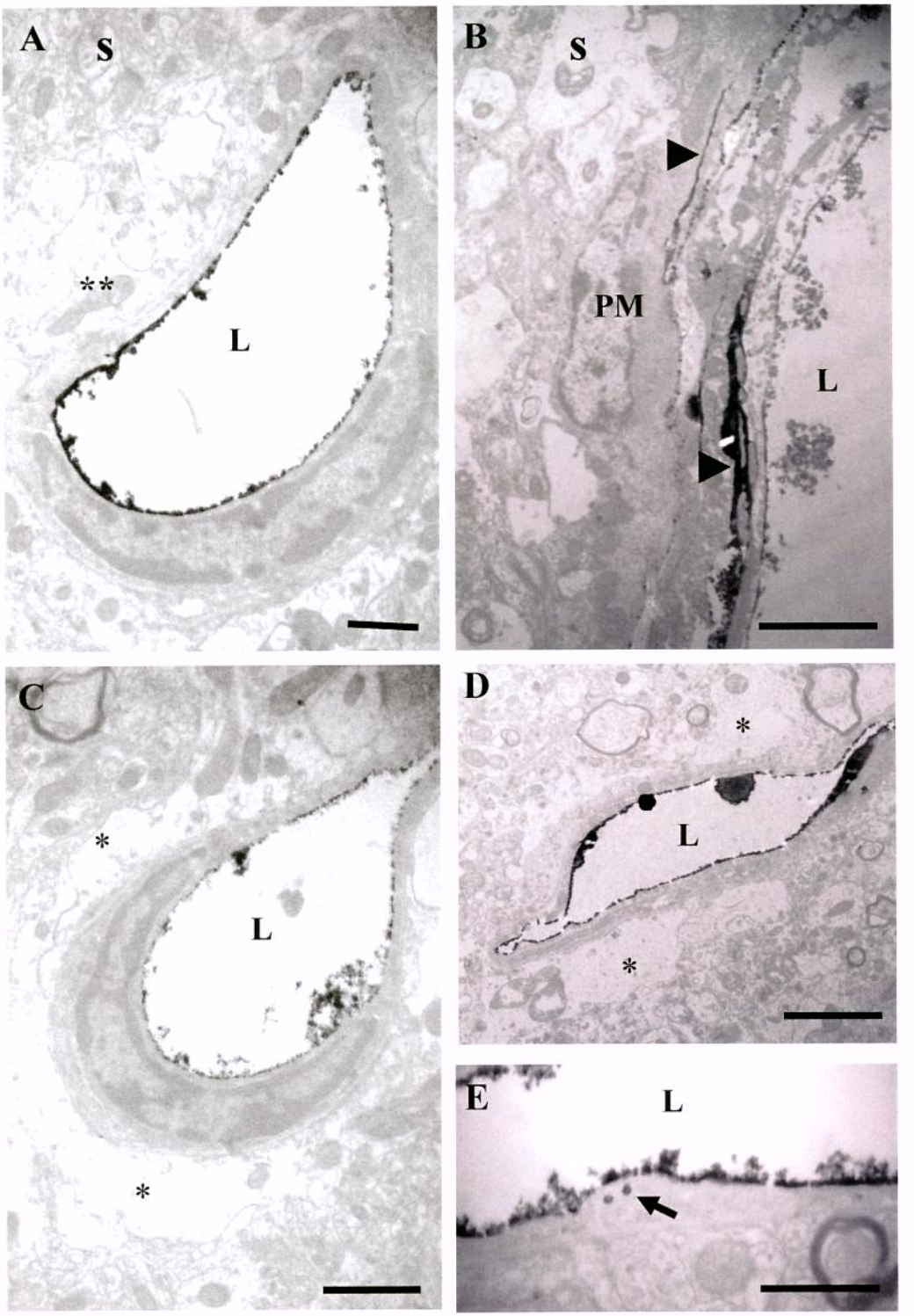

Figure 4: Electronmicrographs of Fronto-Parietal Motor Cortex: In control group (A), note the lanthanum tracer confined into the vascular lumen (L): endothelial cell and neural parenchyma did not show damage. In envenomed group, at 1 h p.i., corresponding to the peak of vessels with altered permeability (B – E): observe in panel B the electrondense tracer permeating the nervous tissue (arrowheads), the presence of reactive perivascular microglia (PM), and the dilated synapses (s) devoid of synaptic vesicles and with abnormal mitochondria, compared to control (s). The panels C and D show the swollen astrocyte end-feet (asterisk), compared to normal ones in control (**). In E, note the containing tracer-pinocytotc vesicles (arrow). Bars: A - C = 0.5 µm; D = 1 µm; E = 0.25 µm.

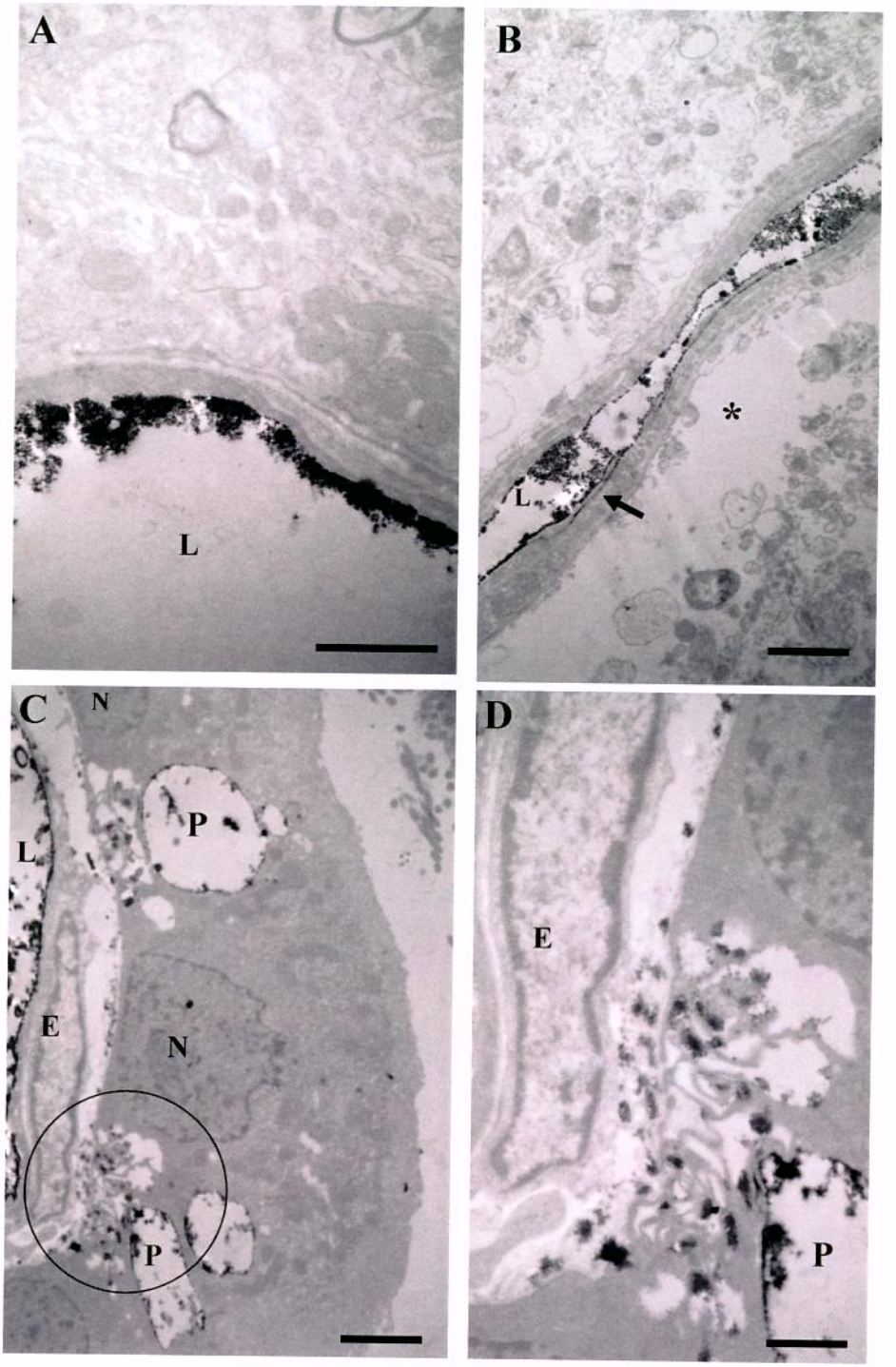

Figure 5: Electronmicrographs of Periaqueductal Gray Matter (one hour post-PNV or pot-saline injections). Sham (A): showing part of the endothelial wall and parenchyma without morphological changes. In envenomed animals (B – D), tissue damage and a strong defensive reaction was seen. Note in panel B, the marked perivascular edema (asterisk) and lanthanum tracer into the interendothelial junction (arrow). See that the structures at the vessel periphery are altered. Panel C shows an activated macrophage adhered to the vessel wall in the aqueduct. Note the tracer being phagocytized and incorporated by phagosomes (P). In D, a detail of the region encircled in C is shown. Macrophages are engulfing electron-dense particles of lanthanum nitrate which are being accumulated into phagosomes (P). L - vascular lumen; N - macrophage nucleus; E - endothelial cell. Bars: A = 0.5 µm; B and C = 1 µm; D = 0.25 µm.

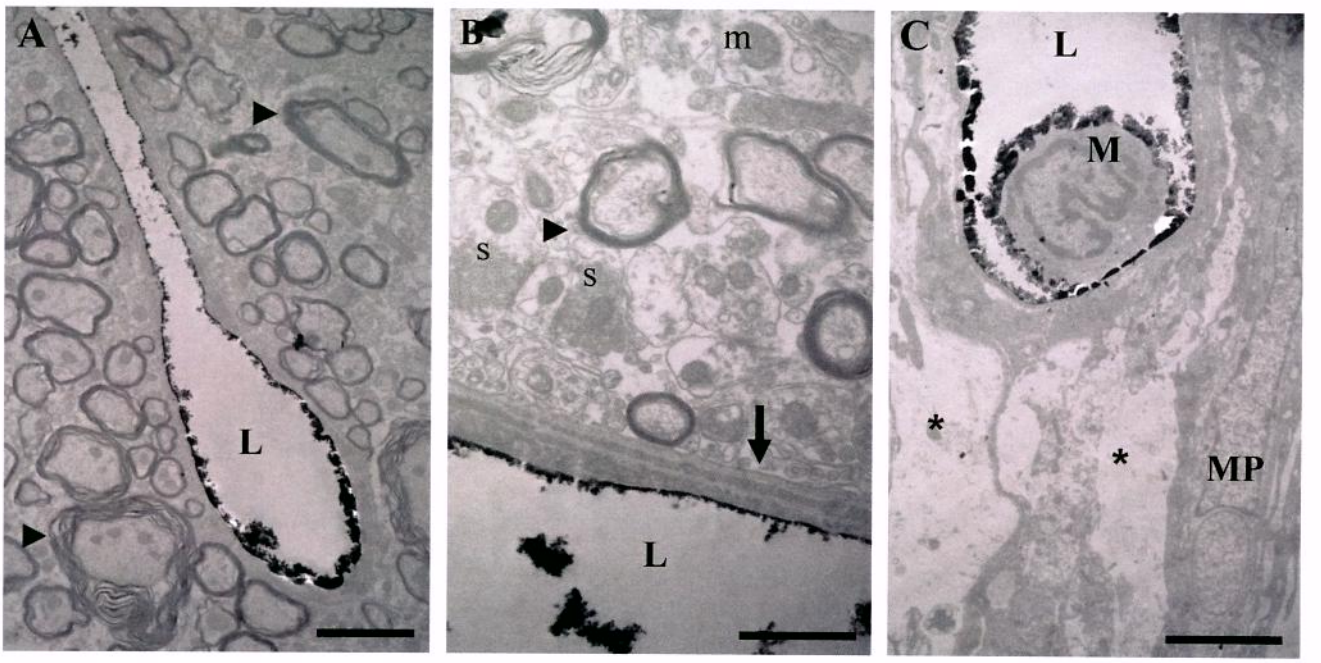

Figure 6: Eletronmicrographs of Amygdala of rat one hour after PNV i.v. or saline i.v. injection. Sham (A and B): showing rows of synapses (s) with normal neurotransmitter vesicles, and mitochondria (m); the myelinated axons (arrowheads) and endothelial cell (arrow) are normal looking. In envenomed group, one hour p.i., (C) any perceptible change was seen at the vessel wall however there was strong reactive response by the neural tissue. Note swollen astrocytes end-feet (asterisks), reactive perivascular microglia (PM) and monocyte in margination (M). These findings suggest a defensive reaction of tissue. L - vascular lumen. Bars: A = 1 μm; B and C = 0.5 μm.

References

Alexander, B., Li, X., Benjamin, I.S., Segal, M.B., Sherwood, R., Preston, J.E., 2000. A quantitative evaluation of the permeability of the blood brain barrier of portacaval shunted rats. Metab Brain Dis.15, 93-103.

Alvarez, J.I., Teale, J.M., 2006. Breakdown of the blood brain barrier and blood-cerebrospinal fluid barrier is associated with differential leukocyte migration in distinct compartments of the CNS during the course of murine NCC. J Neuroimmunol. 173, 45-55.

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

Banks, W.A., Kastin, A.J., 1998. Differential permeability of the blood brain barrier to two pancreatic peptides: insulin and amylin. Peptides. 19, 883-889.

Begley, D.J., 2004. Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol Ther. 104, 29-45.

Begley, D., Brightman, M.W., 2003. Structural and functional aspects of the blood-brain barrier. In: Prokai, L., Prokai-Tatrai, K., Peptide Transport and Delivery into the Central Nervous System. Progress in Drug Research. 61, 39-78.

Benoit, J.P., Faisant, N., Venier-Julienne, M.C., Menei, P., 2000. Development of microspheres for neurological disorders: from basics to clinical applications. J Control Release. 65, 285-296.

Brazil, V., Veillard, J.,1925. Contribuição ao estudo do veneno das aranhas. Mem Inst Butantan. 2: 5-77.

Brownlees, J., Williams, C.H., 1993. Peptidases, peptides, and the mammalian blood-brain barrier. J Neurochem. 60, 793-803.

Crone, C., 1971. The blood brain barrier facts and questions. In: Siesjo, B., Sorensen, S., Ion Homeostasis of the Brain. Munksgaard. Pp. 52-62.

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

El-Bacha, R.S., Minn, A., 1999. Drug metabolizing enzymes in cerebrovascular endothelial cells afford a metabolic protection to the brain. Cell Mol Biol. 45, 15-23.

Emerich, D.F., Tracy, M.A., Ward, K.L., 1999. Biocompatibility of poly-(DL-lactide-co-glycolide) microspheres implanted into the brain. Cell Transplant. 8, 47-58.

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

Habgood, M.D., Begley, D.J., Abbott, N.J., 2000. Determinants of passive drug entry into the central nervous system. Cell Mol Neurobiol. 20, 231-253.

Hagen, T., Bartylla, K., Piepgras, U., 1999. Correlation of regional cerebral blood flow measures by stable xenon CT and perfusion MRI. J Comput Assist Tomogr. 23, 257-264.

Hendrikse, J., van der Grond, J., Lu, H., van Zijl, P.C., Golay, X., 2004. Flow territory mapping of the cerebral arteries with regional perfusion MRI. Stroke. 35, 882-887.

Kroll, R.A., Neuwelt, E.A., 1998. Outwitting the blood-brain barrier for therapeutic purposes: osmotic opening and other means. Neurosurgery. 42, 1083-1099.

Le Sueur, L.P., 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. 19, 38-47.

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.

Miller, D.S., 2003. Confocal imaging of xenobiotic transport across the blood-brain barrier. J. Exp Zool Part A Comp Exp Biol. 300, 84-90.

Minn, A., Ghersi-Egea, J.F., Perrin, R., Leininger, B., Siest, G., 1991. Drug metabolizing enzymes in the brain and cerebral microvessels. Brain Res. 16, 65-82.

Pardridge, W.M., 1991. Peptide Drug Delivery to the Brain. New York: Raven Press Ltd.

Phares, T.W., Kean, R.B., Milheeva, T., Hooper, D.C., 2006. Regional differences in blood-brain barrier permeability changes and inflammation in the apathogenic clearance of virus from the central nervous system. J Immunol. 176, 7666-7675.

Palkovits, M., Brownstein, M., 1988. Maps and Guide to Microdissection of the Rat rain, Elsevier, New York.

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

Rubin, L.L., Staddon, J.M., 1999. The cell biology of the blood-brain barrier. Annu Rev Neurosci. 22, 11-28.

Sahin, D., Ilbay, G., Ates, N., 2003. Changes in the blood-brain barrier permeability and in the tissue trace element concentrations after single and repeated pentylenetetrazole-induced seizures in rats. Pharmacol Res. 48, 69-73.

Schenberg, S., Pereira-Lima, F.A., 1966. Pharmacology of the polypeptides from the venom of the spider *Phoneutria nigriventer*. Mem Inst Butantan. 33, 627-638.

Silva, G.H., Hyslop, S., Cruz-Höfling, M.A., 2004. *Lonomia obliqua* caterpillar venom increase permeability of the blood-brain barrier in rats. Toxicon. 44, 625-634.

Tamai, I., Tsuji, A., 2000. Transporter-mediated permeation of drugs across the blood-brain barrier. J Pharm Sci. 89, 1371-1388.

Vorbrodt, A.W., 1988. Ultrastructural cytochemistry of blood-brain barrier endothelia. Prog Histochem Cytochem. 18, 1-99.

Witt, K.A., Gillespie, T.J., Huber, J.D., Egleton, R.D., Davis, T.P., 2001. Peptide drug modifications to enhance bioavailability and blood-brain barrier permeability. Peptides. 22, 2329-2343.

CAPÍTULO II

[*]Maria Alice da Cruz-Höfling

Departamento de Histologia e Embriologia

Instituto de Biologia, C.P. 6109

Universidade Estadual de Campinas (UNICAMP)

CEP13 087-130, Campinas – SP – BRASIL

E-mail: hofling@unicamp.br

Tel/Fax (55)(19) 3521 6247; Fax. (55)(19) 3289 3124

Astroglial reaction during acute injury of the CNS against systemic neurotoxins-containing spider venom

Maria Alice da Cruz Höfling[1*];
Catarina Rapôso[1]; Liana Verinaud[2], Gabriela Mariotoni Zago[1,3]

[1]Departamento de Histologia e Embriologia and [2]Departamento de Microbiologia e Imunologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP); [3]Departamento de Farmacologia, Faculdade de Ciências Médicas, UNICAMP, Campinas, SP, CEP 13083-970, Brazil.

Abstract

Phoneutria nigriventer spider venom (PNV), a rich mixture of toxins that affect ion channels of excitable membranes, neurotransmitter release, and impairs BBB permeability was injected intravenously (i.v.) to investigate the astrocyte reaction and involvement of pro-inflammatory cytokines in cerebellum and hippocampus of rats. We investigate by imunohistochemistry the expression of GFAP, the major intermediate filament of astrocytes, S100 protein a family of calcium-binding proteins, IFN-γ and TNF-α, 1, 2 and 5 h after i.v. injection of PNV (0.85 mg/Kg). Immediate signs of intoxication revealed central, peripheral and autonomic nervous system as target for venom action. All proteins enhanced variably its expression along the time-course analyzed. GFAP and S100 showed gradual increase with cerebellum being more reactive than hippocampus. The expression of TNF-α and IFN-γ along the time-points was the same. However, whereas expression started earlier in cerebellum (1 h) than hippocampus (2 h), they rendered more expressed in hippocampus than in cerebellum at 5 h post-PNV. We suggest that TNFα and INFγ could be another possible mechanism involved in BBB permeation seen after PNV i.v. injection. A more prominent reactive gliosis of cerebellum over hippocampus could be supposedly one of the molecular events underlying previous findings showing cerebellum BBB as less permeable under the PNV effects than hippocampus. We suggest the existence of a mechanism of defense in the CNS apt to promptly trigger machinery for synthesis of proteins, such as GFAP, S100, and proinflammatory cytokines in response to systemic *P. nigriventer* venom.

Key words: brain, astrocytes, GFAP, S100, TNF-α, IFN-γ, *Phoneutria nigriventer*

Introduction

Astrocytes, the most abundant cell type in the CNS, carry out numerous functions critical to the development and maintenance of the central nervous tissue. They are suspected of being involved in a wide range of CNS pathologies, including trauma, ischemia, and neurodegeneration (Eng and Ghirnikar, 1994, Ransom et al., 2003). The sheer number and functions of astrocytes make them an important study target when investigating the mechanisms of different sort of insults against the central nervous system (CNS). Glial fibrillary acidic protein (GFAP), glutamine synthase, and S100 beta are three major markers of astrocytes in CNS (Walz and Lang, 1998; Savchenko et al., 2000), and have been used to monitor astrocyte behavior. Astrocytes respond to neuronal injury and BBB damage by hypertrophy and some limited proliferation, although this latter response varies. In both cases, an upregulated synthesis of intermediary filaments occurs, turning this glial cell intensely GFAP positive (Eng et al., 2000). S100 represents a family of protein isoforms expressed abundantly in astrocytic cells population. Particularly the extracellular S100ß has been used as parameter of glial activation or death in several situations of brain injury (Rothermundt et al., 2003). Mrak et al. (1996) found that the number of activated astrocytes overexpressing S100ß is highly correlated with the amount of dystrophic neurites present in plaque-rich brain regions, as well as per plaque in Alzheimer's disease. GFAP and S-100 immunohistochemistry are shown to be increased in astrocytes in cerebellum and hippocampus of vanadium toxic metal-exposed animals (Garcia et al., 2005).

The blood-brain barrier (BBB) is of pivotal importance to maintain homeostasis of the CNS given its property of closely regulating the composition of the interstitial fluid aimed to providing an optimal chemical environment for cerebral functioning (see Boer and Gaillard, 2006 for review). To perform this task, the barrier interposes between the brain and blood an intricate system of selective transcellular exchange through the capillaries endothelial cells which involves cell receptors and specific transcellular carriers (Zhang and Pardridge, 2001). In addition, the junctional complex situated at the interendothelial cleft possesses a special type of tight junction whose tightness prevents this via to be route of solutes between the blood and brain tissue (see Engelhardt, 2003 for review). As part of the

BBB, a basement membrane consisting of type IV collagen, fibronectin and laminin, completely covers the endothelium and pericytes, another cellular component of the barrier.

Astrocytes, the third cell type of the BBB, emit end-feet processes which completely cover all the former components, and among which is established a cross-talk for the good of the barrier. Each of these layers could, potentially, restrict the movement of solutes (Grieb et al., 1985; Hawkins et al., 2006).

Failure of the BBB following brain injury induces a succession of pro-inflammatory mediators (cytokines) and other factors creating a chain of interactions between blood derived-cells and glial cells (Wong et al., 2004). Inflammatory processes play a critical role in the pathogenesis of human diseases including neurological disorders, like multiple sclerosis. The hallmark of neuroinflammation is the activation of resident glial cells, and recruitment of peripheral immune cells, as well as production of cytokines and free radicals (Lovell et al., 1998). Among the cytokines, Interferon-gama (INFγ) and Tumor Necrosis Factor-alpha (TNFα) are the main mediators in the pathogenesis of inflammatory lesion leading to central nervous system autoimmune disease (Minghetti and Levi, 1998; Hirsch, 2000; Streit, 2000; Neumann, 2001; Liu et al., 2002). TNFα mediates cytotoxic damage to glia cells and possibly neurons too, while INFγ seems to act by inducing cell surface molecules required for interactions between immune and brain cells (Imai et al., 2006). Observations mainly in tissue culture suggest that both INFγ and TNFα are able to affect the differentiation of neurons (Mehler et al., 1993) and their electric function (Calvet and Gresser, 1979). In the adult brain, proinflammatory cytokines are known to profoundly influence behavioral functions like sleep, feeding, and temperature regulation (Dinarello et al., 1986).

These reactions of neural tissue are complexes and may be unchained by different stimuli. Accidents caused by venomous arthropods, such as caterpillars, scorpions and spiders, may elicit clinical signs and symptoms indicative of involvement of central, peripheral and autonomic nervous system. In this sense, the venoms produced by venomous animals can be useful tools for understanding CNS function in health and disease. *Phoneutria nigriventer* spider venom (PNV) is rich in toxins that affect ion channels and neurotransmitter release. Voltage-gated sodium, calcium, and potassium channels have

been described as the main targets of some of these toxins. The study of the effects of the venom of *P. nigriventer* (popularly known as armed-spider) started on the 80's (Cruz-Höfling et al., 1985; Fontana and Vital Brazil, 1985) and since a bulk of information has been accumulated on its toxin composition and some of their biological actions (Rezende et al., 1991; Prado et al., 1996; Vieira et al., 2005). Nevertheless, we still lack a clear understanding of the mechanism of action of the effects caused by each of the venom toxins, or the whole venom, and even their specific target in different biological systems. Lately, our laboratory has been involved in researches investigating the action of the PNV in the CNS. This line of investigation has emerged from convulsions presented by the victims of severe envenoming by *P. nigriventer*.

It has been shown that in acute stage of envenoming, circulating PNV modulates the BBB of several regions, apparently is innocuous for others, downregulates the expression of laminin, and activates neurons at a number of motor- and stress-related areas of the CNS (Raposo et al., *submitted;* Cruz-Höfling et al., *submitted*). The time course of envenoming investigated at longer periods not only showed disruption of the BBB in hippocampus, but that permeation was transient, gradually being restored at later stages, but that other areas investigated were more resistant to undergo rupture (Le Sueur et al., 2003). It was also proved that the mechanism of BBB enhanced-permeability in hippocampus involved the transcellular pathway and was microtubule-dependent whereas the intercellular (paracellular) route was unaffected at these late stages of envenoming (Le Sueur et al., 2004).

In this work, we investigate the expression of the GFAP, S100, IFN-γ and TNF-α in the hippocampus and cerebellum of rats one, two and five hours after intravenous injection of PNV, corresponding to acute stages of envenoming. This study is part of a series trying to characterize the time course of the reactions of components of the neural tissue which run in parallel with the blood brain barrier breakdown caused by *P. nigriventer* spider venom.

Materials and Methods

Venom and animals

Lyophilized *P. nigriventer* crude venom (PNV) was supplied by Instituto Butantan (São Paulo, SP, Brazil) and stored at –20ºC. The venom was dissolved in sterile physiological saline [0.9% (w/v) NaCl solution] at the moment of use and administered by the intravenous (i.v.) route.

Male Wistar rats (3-week-old) were obtained from an established colony maintained by the Central Animal House Service at UNICAMP and kept under standard animal colony conditions including 12 h-12 h light-dark cycle, 22 ± 2°C temperature with free access to food and water until reaching 8–10-week-old (250-300 g).

At least 24 h before the experiment, animals were transported in their home cages from the animal colony to the laboratory and allowed to habituate. Acoustic, visual, and olfactory stimuli in the experimental room were kept to a minimum. All experiments were conducted between 9:00 a.m. and 1:00 p.m. in order to reduce circadian influences.

Envenoming procedure

Fifteen male rats were given a single intravenous (i.v.) injection of PNV (850 μg/Kg in 0.5 ml saline) in the tail vein. The PNV concentration was selected based on previous studies in our laboratory (Le Sueur *et al.,* 2003, 2004). One, 2 and 5 h of PNV injection (n=5/time interval), the animals were anesthetized with an intramuscular injection of 100 mg kg^{-1} Ketamine and 10 mg kg^{-1} Xylasine, and transcardially perfused with physiological saline (150 ml) followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.2. To control group (n = 2-3/time interval) was given the same volume of 0.9% sterile saline solution. All procedures were carried out in accordance with the guidelines proposed by the "Principles of Laboratory Animal Care" (NIH publication n^{o}. 85-23, revised 1985) and approved by the Animal Care and Use Committee of the State University of Campinas (UNICAMP).

Immunohistochemistry

After anesthesia and perfusion fixation, the brain was immediately removed, post-fixed with the same fixative overnight. Fragments of hippocampus and cerebellum regions taken at random were dehydrated in a graded ethanol series, cleared in xylol, and embedded in Histosec (Merck, Rio de Janeiro, RJ, Brazil). Sections 5 μm thick were cut on an RM 2035 microtome (Reichert S, Leica), washed several times in 0.05 M PBS, and later incubated in this buffer with 1% bovine serum albumin (BSA) for one hour for immunohistochemical detection of GFAP, S100, INFγ and TNFα. Endogenous peroxidase was blocked by incubation for 30 min in PBS containing 3% hydrogen peroxide.

Nonspecific binding sites were blocked by incubating slides with normal rabbit serum for 15 min at room temperature. Before incubation with TNFα and INFγ primary antibodies antigen retrieval was performed by microwave pre-treatment (20 mM citrate buffer, pH 6.0) for 12 min. Four sections per animal were incubated with the following rabbit polyclonal primary antibodies: anti-GFAP and anti-S100 (both from DakoCytomation, CA.,USA) used at a dilution of 1/100 and 1/400, respectively, anti-IFNγ and anti-TNF-α (Peprotech-Rocky Hills, NJ, USA) both used at a dilution of 1/500, for 30 min at room temperature and then overnight at 4ºC. After washing, sections were overlaid for 1 h with a secondary antibody biotin-conjugated. The immunohistochemical reaction was amplified using the kit Rabbit Immunocruz Staining System (Santa Cruz, CA, USA), and visualized with 3`3-Diaminobenzidine (DAB) as chromogen. All incubations were carried out in a darkened, humidified chamber at room temperature. The reactions were stopped by washing the slides in distilled water. The slides were then weakly counter-stained with Harris' hematoxylin, dehydrated in ethanol and mounted in Canada balsam.

Statistic Data Analysis

The clinical signs of the envenoming presented by each animal were monitored by two observers and carefully registered.

The stereotaxics coordinates of transversal planes and limits of the regions analyzed were selected according to Palkovits and Brownstein's atlas of rat brain anatomy (1988). Preliminary observations were done at light microscope with x10 objective (Leica,

Germany) to identify hippocampus and cerebellum regions. Then, using x20 and x40 objectives, a qualitative evaluation was done comparing the labeling intensity of control group at each period of envenoming.

The qualitative analysis of GFAP, S100, IFN-γ and TNF-β expression was scored as (+++), (++), (+) and (-), indicating strong, moderate, weak and negative labeling, respectively. Whereas GFAP immunoreactivity was observed in the astrocytes cell bodies and processes among and around the vessels, S100 labeling was seen mainly in the cell body. IFN-γ and TNF-α were detected in neuronal and astrocytic cellular bodies. The analyses were done by two different researchers working blind.

The images of all envenoming periods were captured using a Nikon Eclipse E800 light microscope (Japan) equipped with Image-Pro Plus image analyzer software (USA).

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

Results

Clinical observations

The observation of clinical signs of envenoming showed immediate signals of intoxication, including hyperemia, tremors, paleness, salivation followed within few minutes by reduced locomotor's activity and some difficulty in breathing. Practically all animals presented flaccidity followed by spastic paralysis of posterior legs. Off the five rats used in each period of envenoming, at least two showed tonic convulsion. Three animals died after a while of venom injection, probably by respiratory arrest, since necropsy showed lung edema, being so discarded. Rats injected with sterile saline alone showed none of the alterations described above.

Immunohistochemistry

In control groups a basal expression of GFAP and S100 was maintained still along the periods of observation, whereas none physiologic production of TNFα and INFγ proteins has occurred. In contrast, the analysis of immunohistochemistry of the proteins investigated showed that all of them variably enhanced its expression along the time-course after venom injection. There was also variability in phenotypic labeling of astrocytes if belonging to hippocampus or cerebellum.

GFAP

After PNV injection, the GFAP expression was quite the same for all layers of hippocampus. One hour after, it was graded as weak (+) therefore not much different from the basal expression seen in controls, however, it increased in cerebellum where was graded as moderate (++). In the 2 h-treated group, the labeling also enhanced to moderate (++) in hippocampus and strong (+++) in cerebellum. After 5 h, the labeling was strong (+++) in hippocampus and was kept intense in cerebellum (+++) (Table 1). In cerebellum, the pattern of labeling of astrocytes processes changed radically, mainly in the molecular layer in comparison to controls (Figures 1A,B versus 1E,G). There they appeared arranged in palisade, running in parallel to each other and perpendicular to the pia-mater (Fig. 1E,G). Although there were no changes in the spacial arrangement of the astrocytic processes in the molecular layer between controls (Fig. 1A) and PNV-treated samples (Figs. 1G), the intensity of labeling, length and thickness of the processes differed markedly, indicating that the processes underwent hypertrophy under the venom effect.

At the peak of GFAP expression which was 5 h for hippocampus, astrocytes showed an enlarged cell body with more ramified and tortuous cytoplasmic processes, a phenotype clearly visible as a more intense immunostaining compared to the astrocytes from controls (compare Fig. 1C,D from controls to 1F,H from envenomed). GFAP at the astrocytes end-feet was heavier labeled around vessels than in the astrocytic processes away from vessels. Because stained tiny astroglial ramifications are widespread all over the hippocampal parenchyma, the tissue background appeared shadowed in brown whereas in controls it appeared bluish (Figs. 1C,D versus 1F,H). This feature appeared at 2 and 5 h

post-PNV. It is worth mention the appearance of vasogenic edema in the PNV-treated animals (Fig. 1F,H).

S100

Intracellular S100 was observed in the astrocytic cell bodies in cerebellum of control animals (Fig. 2A,B), and was scored (+), however differed from GFAP, since the major labeling of this protein was in the granular layer instead of the molecular as for GFAP (Fig 1E,G). From 1 to 2 h after envenoming, the labeling increased faster (faster upregulation) in cerebellum (+++) than in hippocampus (++) (Table 1). A concomitant staining of the background took place as result of labeling the delicate branching of the astrocytic ending processes. A strong labeling impregnated astrocytes of the Purkinje layer (Fig. 2E,G).

The localization and expression of S100 protein in the hippocampus of controls was in the perikaryon of astrocytes (Fig. 2C,D). The immunostaining was then graded as weak (+). In the PNV-treated animals, a gradual increase was seen in the intensity and extent of labeling (Table 1), with the staining progressively reaching the cell processes, visually inferred by the increase of the brownish shade of the tissue background in all periods. Figures 2F,H illustrate the labeling pattern seen at 5 h p.i.

Cytokines: TNFα and INFγ

Controls were negative for both cytokines in all periods observed both in cerebellum (Fig. 3A,B for TNFα and Fig. 4A,B for INFγ) and hippocampus (Fig. 3C,D for TNFα and 4C,D for INFγ) After one hour of PNV injection the animals presented no cytokines expression in the hippocampus (-) and weak (+) expression in cerebellum (Table 1). In the 2 h-treated group, as for cerebellum, hippocampus TNFα and INFγ labeling was weak (+). However, TNFα achieved its peak (+++) at 5 h becoming strongly labeled for hippocampus, whereas was moderately labeled (++) for cerebellum. On the other hand, whereas INFγ remained with a score of (++) in cerebellum, in hippocampus it achieved the score of (+++). Figures 3E,H,I, for cerebellum and Figure 3F,G,J, for hippocampus illustrate immunohistochemistry of TNFα 5 h after venom administration via

the tail vein of rats. Figures 4E,G, for cerebellum and 4F,H, for hippocampus illustrate immunolabeling for INFγ 5 h after PNV systemic administration. It is worth mention that the labeling was found primarily in the cytoplasm of neurons and astrocytes, in both regions studied. In the cerebellum, staining was more frequently found in Purkinje cells (strong labeling) and astrocytes (weak labeling, see Figs. 3E and 4E) from the Purkinje layer, as for S100 protein. Interestingly, while in hippocampus of control groups, cytokine-negative-astrocytes and -neurons were separated apart from each other, in PNV-treated groups these cells were closely-applied, maintaining tight contact among them (compare Fig. 3D with 3G,J and Fig. 4D with 4F,H).

Discussion

Venoms and toxins are important tools for analysis of different tissue responses, since they are pharmacologically active substances, binding at specific sites of the targeted-cells or -tissues. These substances, if neurotoxic, can be used in the investigation of a wide range of nervous tissue related-phenomena, among which astrogliosis and neuroinflammation.

In this work, the clinical observations done at acute stages (ranging from 1 h to 5 h) after *P. nigriventer* envenoming showed the rats with excitatory signs including tonic convulsion, which unequivocally point to CNS involvement. Yet, the histological observations showed that not only the cytoskeletal GFAP and metabolic S100 proteins time-dependently were overexpressed, peaking at 5 h after envenoming, but that hypertrophy of the astrocytes processes branching has occurred. In addition, the venom provoked vasogenic edema characterized by spaces at the periphery of the endothelium of affected vessels. Association of increased astrocytic GFAP expression, vasogenic edema and increased permeability of BBB in hippocampus and cerebellum of rats has been reported by our research group after systemic injection of the *Lonomia obliqua* caterpillar venom (Silva et al., 2004). In this study, the astrocyte reactivity was correlated with the neurotoxic effects of the circulating venom in the brain vasculature despite the venom itself

has not been immunodetected into the tissue in the periods studied (6, 18, 24, and 72 h after injection).

Enhanced expression of GFAP is one of the manifestations of astrogliosis and has been generally accepted as a marker of neurotoxicity (O'Callaghan, 1988). The ubiquity of gliosis phenomenon given in response to a wide range of nervous tissue insults, such as stroke, trauma, tumor growth, neurodegenerative disease or neurotoxic agents, gives the dimension of astrocytes importance in the CNS.

Our pioneer study which investigated the neurotoxic effects induced by PNV at the BBB breakdown in different regions (cerebellum not included) of the brain used longer periods after envenoming (ranging from 18 h to 9 days) and showed hippocampus as the main target. Although the signs were clinically resolved 12 h after envenoming, at the biological level the effects of the venom still persisted over days (Le Sueur et al., 2003). In this particular study, using an extracellular tracer, we showed that the venom modulated differentially the permeability of the BBB. Changes were first seen in pre-capillary arterioles and post-capillary venules (from 18 h to 5 days post-PNV) and later in capillaries (9 days post-PNV). Changes included the extracellular tracer permeating the interendothelial cleft (BBB paracellular pathway) and filling an increased number of pinocytotic vesicles (BBB transcellular pathway). A further study proved that the mechanism of BBB enhanced-permeability in hippocampus occurred by the increase of microtubule-mediated transcellular vesicular transport, since it was abolished in colchicine pretreated animals whereas the intercellular (paracellular) route was unaffected at these late stages of envenoming (Le Sueur et al., 2004). Since astrocytes are involved in brain capillaries phenotype for BBB, we now raise the hypothesis that the higher susceptibility of precapillary arterioles and postcapillary venules could be associated to the inexistence of close apposition of the endothelium with the astrocyte end-feet, typical of the capillary network, hence impeding a finer tuned communication between the two cells.

In another study, now comparing the PNV effects in BBB of cerebellum and hippocampus at acute stages after envenoming (1 to 5 h) we showed that the hippocampus was significantly more severely affected than cerebellum, since the latter presented lesser percentage of vessels with extracellular tracer extravasation. Nevertheless, swollen

perivascular astrocytes end-feet have been frequently observed in the cerebellum indicating that the perivascular components of the nervous tissue (namely astrocytes) reacted to the luminal circulating venom supposedly in response to messages interchanged between the endothelial and astrocytic cells (Raposo et al., *submitted*). Interestingly, we saw in the current study that cerebellum showed more intense gliosis than hippocampus, corroborating the former findings. These findings suggest that the reactive gliosis protected the cerebellar BBB structure against permeation as confirmed by the smaller number of vessels with tracer leakage in cerebellum (Rapôso et al, *submitted*).

There is now strong evidence, particularly from studies in cell culture indicating that astrocytes can upregulate many BBB features, mainly those viewing the tightness of the physical barrier between blood and brain (Dehouck et al., 1990; Rubin et al., 1991). A growing body of evidences has given astrocytes a number of different morphologies, depending on their location and association with other cell types. However, the specific structural and functional characteristics of the process of astrocyte activation remain rather enigmatic. Whether the morphology of cerebellar astrocytes (disposed in palisade along the molecular layer and perpendicularly to pia mater) has to do with the strength of the cerebellum' BBB over hippocampus's or not, as seen in this work, is an attractive possibility, given the concept that morphology of an structure is always associated with the function exerted.

Altogether, the findings of the current study also suggest a better transcriptional control of GFAP genes sensitive to *Phoneutria nigriventer* venom in the cerebellum, with the envenoming probably acting as a regulatory gene event for intermediate filament protein synthesis in situations which require alert of the perivascular astrocytes to insults against vessels of a given area of the encephalon. This was validated by the stronger labeling seen around the vessels, mainly in the vasogenic ones.

PNV also induced increase in S100 expression in hippocampus and cerebellum, being the latter region more responsive than the former. S100 proteins belong to a family of 21 members of calcium binding proteins that have shown to be implicated in a wide range of intracellular (such as regulation of enzyme activities, dynamics of cytoskeleton constituents, growth and differentiation and calcium homeostasis), and extracellular

activities (such as regulatory effects on inflammatory cells, neurons, astrocytes, microglia and endothelial cells) (see Donato, 2003). Signaling pathways between astrocytes and astrocytes:endothelial cells involve intercellular Ca^{2+} signals (Paemeleire, 2002). Studies of astrocyte-neuron interactions have shown that calcium signaling is a potent modulator of the strength of both excitatory and inhibitory synapses (Simard et al., 2003). Garcia et al. (2005) in a model of vanadium-induced neurotoxicity in the rat CNS reported that NADPH-diaphorase expression was higher in cerebellum than in hippocampus, whereas heat shock protein (hsp) 70 showed reactive neurons only in cerebellum. The authors also reported GFAP and S-100 proteins immunolabeling in enlarged astrocytes of cerebellum and hippocampus.

S100ß, which is found only in astrocytes, directly interacts with different cytoskeletal proteins, including GFAP, and inhibits their phosphorylation, thus inducing a cytoskeletal stabilization that promotes neuritis outgrowth (Ziegler et al., 1998). In this sense, the overexpression of S100 seen in our experimental model agrees with the increased labeling of GFAP. The fact that astrocytes function as a syncytium of interconnected cells in both health and disease, rather than as individual cells (Pekny and Nilsson, 2005), implies in cross-talks transmitted by an extensive network of communication and adds yet another dimension to the process of reactive gliosis. Our findings find support in a presumed cross talk between the mechanisms controlling the synthesis of both GFAP and S-100. The fact that the synthesis of both proteins was more clearly upregulated in the cerebellum than in hippocampus raises the possibility on the existence of a differential regulatory role of the venom in function of local molecular phenotype. As for GFAP, the envenoming can act as regulatory gene event which resulted in the increased expression of S100.

On the other hand, S100ß release from astrocytes has been shown to be mediated by serotonin, acting through 5-HT_{1A} receptors (Whitaker-Azmitia et al., 1990; Ramos et al., 2000). It has been shown that PNV provokes vascular permeability in rat and rabbit skin that, at least partially, is caused by the activation of histamine and serotonin receptors by venom (Antunes et al., 1992), and activates type 5-HT_4 receptors in vagus nerve preparations, a mechanism probably involved in the generation of pain and

inflammation caused by the venom (Costa el al., 2003). Studies aimed to investigate the role of serotonin receptors in the upregulation of S100 synthesis and in the enhanced permeability of BBB can contribute for shedding light in molecular mechanisms of the *P. nigriventer* action at the CNS in general and in BBB in particular.

The brain lacks elements of lymphoid tissue but contains instead two resident cells, astrocytes and microglia that form an innate immune system in CNS with potential to initiate responses to exogenous antigens, like toxic CNS molecules, or endogenous molecules born from degenerative process (Aloisi, 1999; Nelson et al., 2002).

It has been previously shown that brain immune cells respond to various stimulations, such as lipopolysaccharide, or viral infection *in vitro* by the induction of inflammatory cytokines (Lieberman et al., 1989; Aranguez et al., 1995; Olson et al., 2001) and that astrocytes play an important role in the fine tuning of brain inflammation (Dietrich et al., 2003).

Since astrocytes and microglia exhibit a reactive phenotype in response to neurotoxic insults, we have investigated inflammatory cytokines (TNFα and INFγ) since they are thought to contribute to the glia activation process (Woodroofe et al., 1991; Balasingam et al., 1994; Balasingam and Yong, 1996; Yong, 1996; Rostworowski et al., 1997). Among these inflammatory cytokines, TNFα has been implicated as playing a proactive role (Benveniste, 1995). High levels of these cytokines are known to appear at the injury site and/or in the cerebrospinal fluid (Renno et al., 1995). TNFα is produced by astrocytes, microglia/macrophages, and infiltrating immune cells in injured brain (Hofman et al., 1989; Medana et al., 1997) and may play a role in regulating astrocyte and microglia reactivity and proliferation (Selmaj et al., 1990; Ganter et al., 1992).

In this paper we have shown that PVN injection provoked vigorous inflammatory process in neural tissue, inferred by enhanced TNFα and INFγ expression at 5 h p.i.. In cerebellum cytokines expression was more precocious ((scored as (**+**)) 1 h post-PNV inoculation) than hippocampus which achieved the score (+) at 2 h after envenoming. At 5 h p.i., TNFα and INFγ achieved their maximal expression in both cerebellum (++) and hippocampus (+++). Although not measurable to infer whether the

cytokines scores could be considered as having a proactive role in the first two hours after envenoming and a cytotoxic one after 5 h, mainly seen for hippocampus, the hypothesis is attractive. The apparent region-selective and time-selective responses suggest that proinflammatory cytokines can be cytotoxic to some CNS regions and neurotrophic to others having the period of exposure to the toxicant a role in the modulatory action.

Evidences suggest that inflammatory mediators such as cytokines produced at the inflammatory site are at least partly responsible for changes in vascular permeability. It has been observed that intracranial injections of TNFα and interleukin 1 beta (IL-1β) increase BBB permeability in rats, while IFNγ, TNFα, and lipopolysaccharide (LPS) can increase the permeability of *in vitro* models of BBB (Abbott and Revest, 1991; Burke-Gaffney and Keenan, 1993; Yuan, 2000; Mayhan, 2001).

Until now, we do not know if these cytokines are locally released by autochthonous brain cells or secreted from cells invading the CNS tissue from blood stream. Additionally, as TNFα increases vascular-brain permeability, systemic TNFα may gain access to the brain through a specific transport system (Gutiérrez et al., 1993). This question remains to be solved but experiments with purified cultures of astrocytes and microglia from envenomed animals for evaluation of inflammatory cytokine gene expression by reverse transcriptase PCR and protein expression by western blotting are being planned in our laboratory.

Diffuse cytoplasmic staining for TNFα and IFNγ was also evident in neurons and this fact led us to assume that these neurons express functional cytokine receptors. Two types of TNF-α receptors (TNFR) have been identified: P75 TNFR and P55 TNFR (Carlson et al., 1998; Haviv and Stein, 1998). Although the neuronal distributions of these receptors have not yet been characterized (Pan et al., 1997), the highest density of TNF-α-binding sites is in the medullary brain stem (Kinouchi et al., 1991). Neumann et al. (1997) have demonstrated that functionally mature brain neurons derived from cultures of dissociated rat hippocampal tissue transcribed the α-chain of the interferon-type II receptor (binding IFN-γ) along with the p55 receptor for TNF-α. Interestingly, we found evidences of "physical contact" between TNF-α-positive neurons and TNF-α-positive astrocytes in

the PNV-group, while in controls these cells, which were negative for TNF-α, were separated apart from each other (compare Fig. 3D with 3G,J and Fig. 4D with 4F,H). Certainly, this assumption in our experimental model needs to be confirmed by further investigations.

Immune responses protect the CNS against pathogens, but the fact that there is little dispensable tissue in the brain, a vigorous regulation is necessary to avoid disastrous immune-mediated damage. So, neuroinflammation can be seen as a double-sided sword since it can cause neuronal damage but it also has neuroprotective and neurotrophic effects at some stages.

Our results suggest that the expression of proinflammatory cytokines in astrocytes and neurons is part of the immune response against the neurotoxic injury caused by *P. nigriventer* venom and, at least partially, could also be involved in the transient characteristics of the changes of permeability of the BBB at the cerebellum and hippocampus.

Besides, the results suggest the existence of a mechanism of defense in the CNS apt to promptly trigger machinery for synthesis of proteins, such as GFAP and S100, and to induce the expression of proinflammatory cytokines, all of them in response to circulating *Phoneutria nigriventer* venom. Whether the bulk of effects hitherto known be provoked by systemic PNV in different areas of the CNS result from a direct action of the venom, and/or from secondary messengers (such as pro-inflammatory cytokines) elicited during envenoming is a matter to be investigated. The richness of neurotoxins in the *P. nigriventer* armed-spider venom elects it as a powerful tool for investigating physiopathological processes of the CNS, including inflammatory ones.

Acknowledgements

The authors thank Instituto Butantan (São Paulo, SP, BR) for venom donation. The authors also thank Ms. Marta Beatriz Leonardo (BSc) for excellent technical assistance, and Mr. Miguel Silva and Mr. Silva for animal care. This work has been funded by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP # 98/00341-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq # 522131/95-6). G.M.Z. and C.R. are recipients of scholarships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

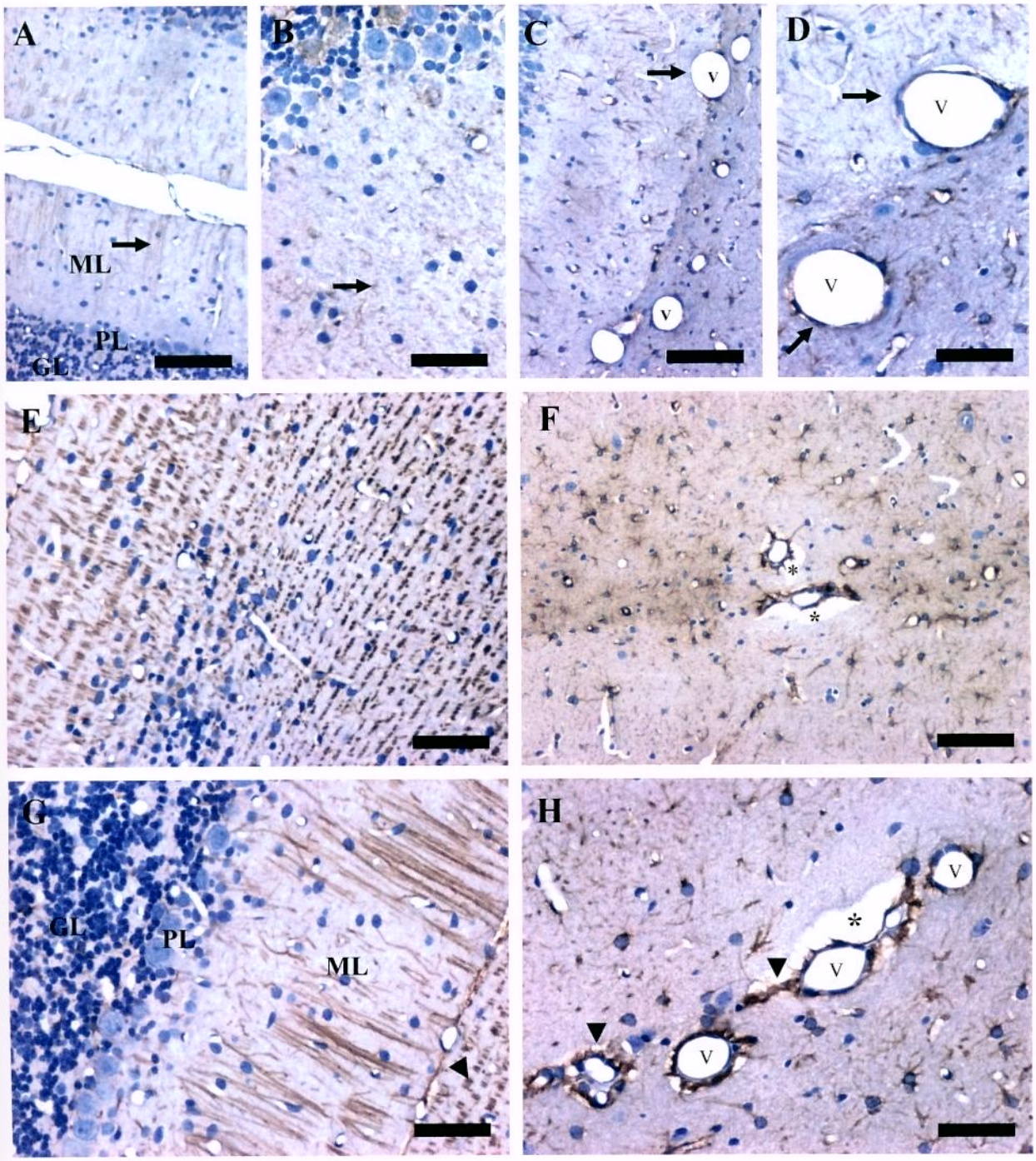

Figure 1: Glial fibrillary acidic protein (GFAP) immunohistochemistry in the cerebellum and hippocampus. A, B – Cerebellum; C, D – Hippocampus: Micrographs of astrocytes from control rats showing the physiologic expression of this protein (arrows). E and G – Cerebellum (envenomed group – 5 h p.i.). The GFAP expression was strongly enhanced. Observe the cross (E) and longitudinal (G) sectional profiles of the astrocytic processes. F and H – Hippocampus (treated group – 5 h p.i.) In this region occurred increased expression of GFAP, suggesting reactive gliosis mainly around vessels (arrowhead). Observe vasogenic edema around the affected vessels (asterisks). v: vessels; PL = Purkinge layer; ML = Molecular layer; GL = Granular layer. Bars: A, C = 80 µm B, D - H = 40 µm.

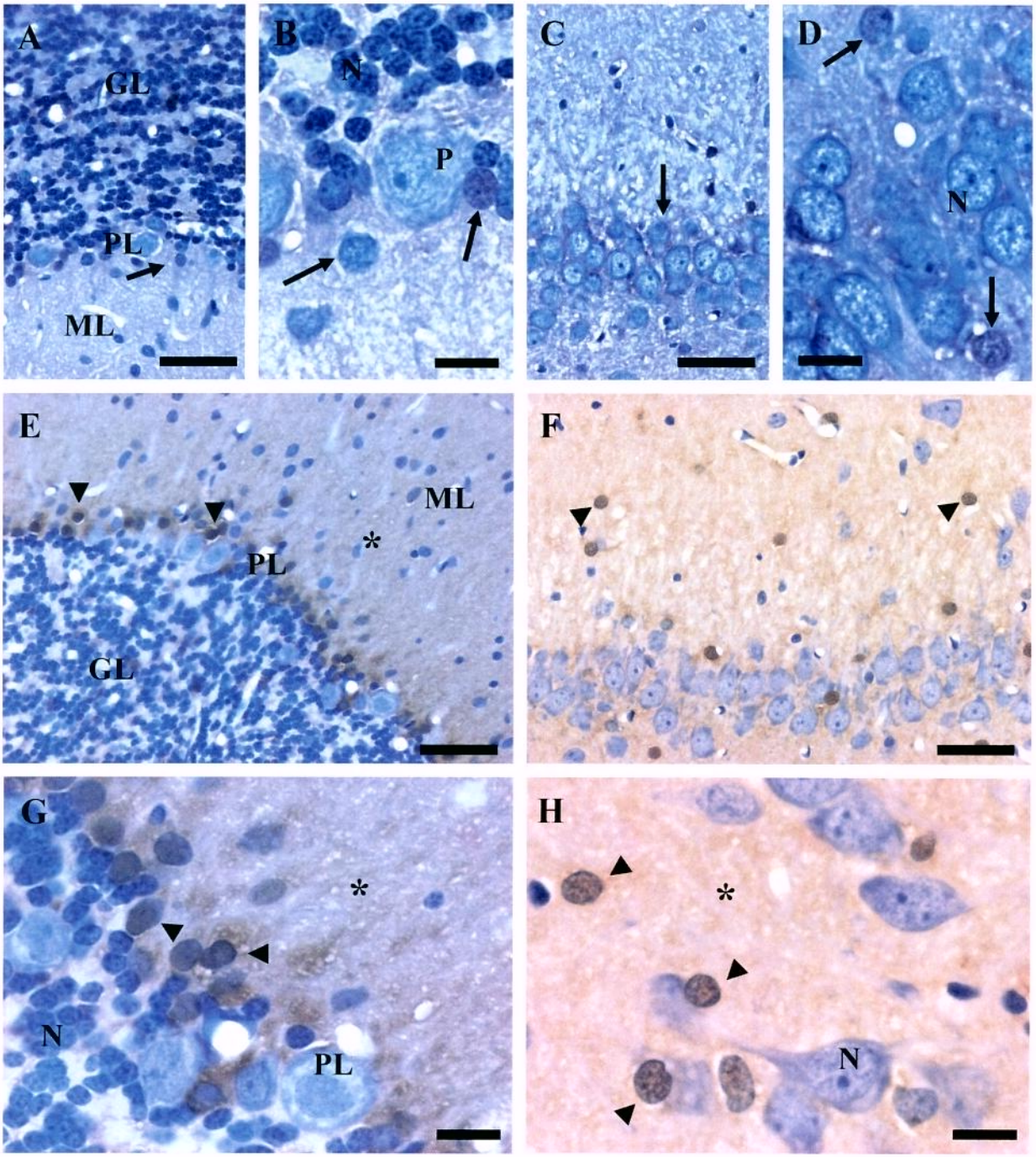

Figure 2: S100 labeling in cerebellar and hippocampal cells. A, B – Cerebellum; C, D – Hippocampus: Note the very low expression of S100 in astrocytes of both regions from control group (arrows). E, G – Cerebellum of PNV-group (5 h p.i.). S100 expression enhanced strongly in astrocytes localized mainly in Purkinje Layer (PL), and in astrocytic processes throughout the parenchyma (asterisks), giving a brownish shadow to the background of tissue. F, H – Hippocampus of PNV-group (5 h p.i.). See that both astrocyte bodies (arrowheads) and processes (asterisk – brownish ground) exhibited S100 overexpression. GL = Granular layer, ML = Molecular layer, N = Neurom Bars: A, C, E, F = 40 μm; B, D, G, H = 12 μm.

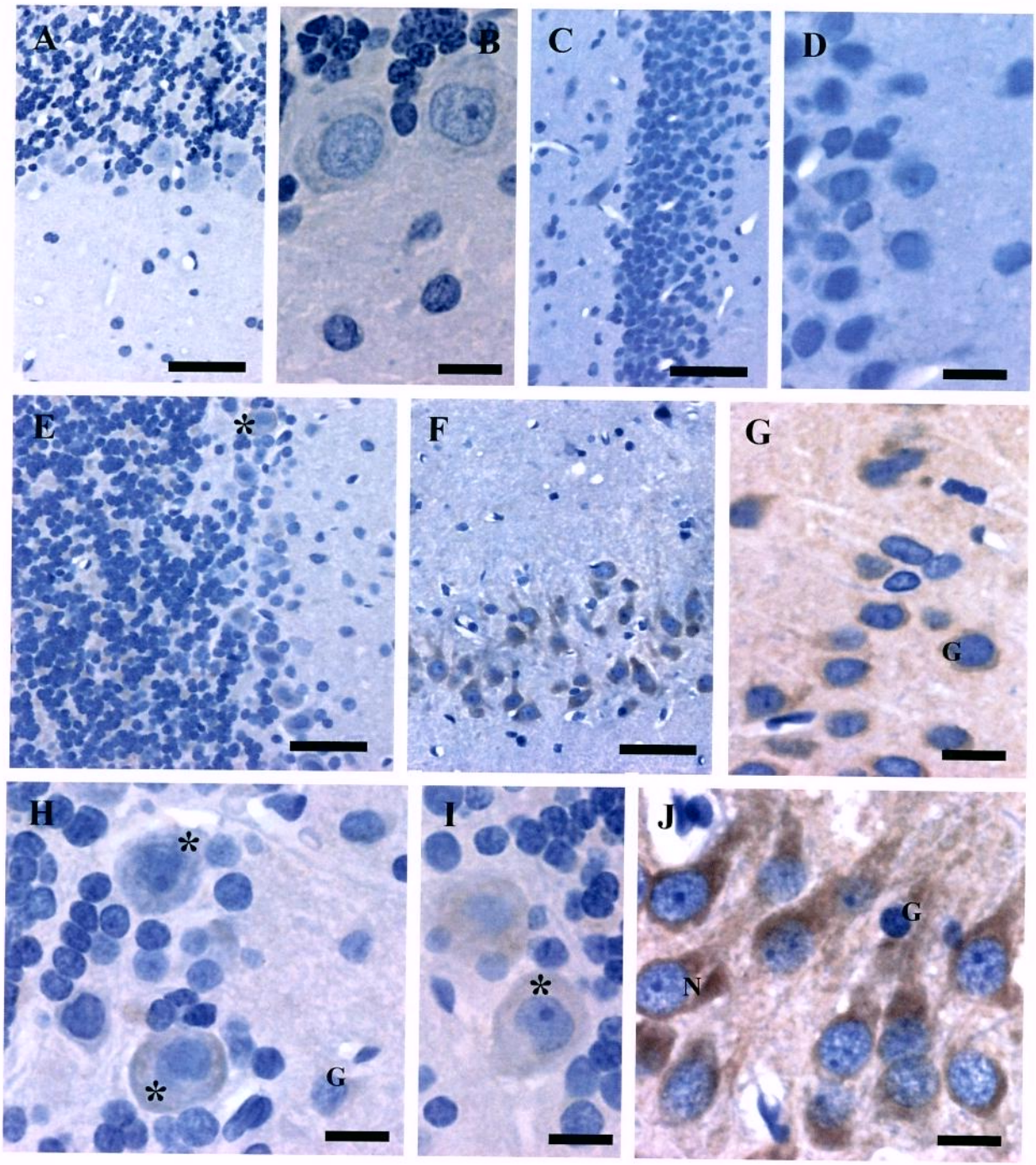

Figure 3: Light micrographs of TNF-α immunolabeling in cerebellum A, B and hippocampus C, D of control rats. Observe that any expression of the inflammatory factor is noted after saline solution injection. E, H, I – Cerebellum of PNV-treated animals (5 h p.i): The cells showed a relatively moderate cytoplasmatic expression of this cytokine (asterisks). F, G, J – Hippocampus of envenomed group (5 h p.i.). Note the strong labeling in neurons (N) and glial cells (G) and that cells are closely together in envenomed animals (panels G,J) in contrast to controls where they are more dispersed (panel D). Bars: A, C, E, F = 40 µm; B, D, G - J = 12 µm.

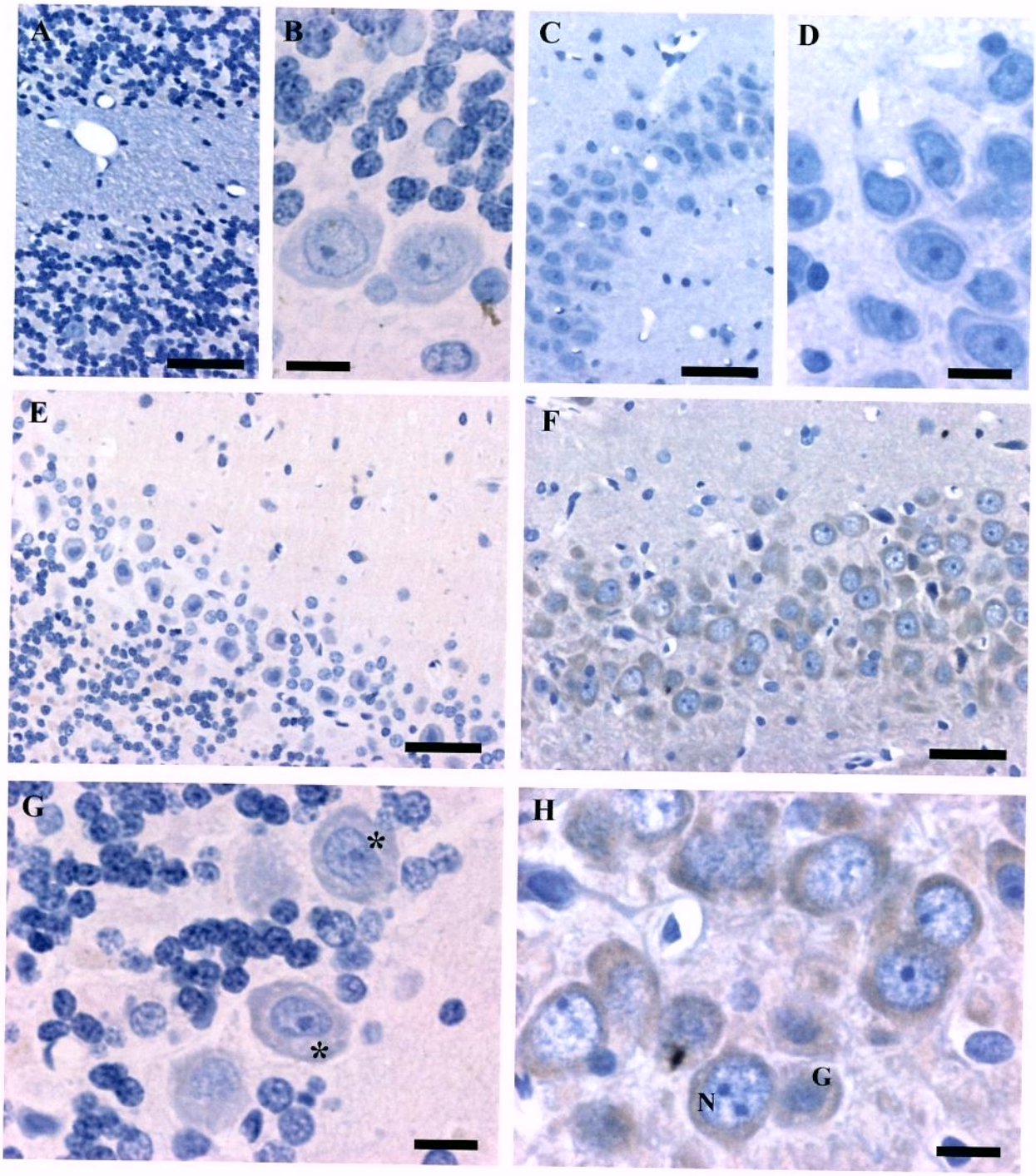

Figure 4: Light micrographs of INF-γ immunolabeling in cerebellum A, B and hippocampus C, D of control rats: Note that any expression of this pro-inflammatory cytokine was seen after saline solution injection. E, G – Cerebellum of envenomed animals: After 5 h of systemic PNV injection, a moderate cytoplasmatic labeling was observed (asterisks). F, H – Hippocampus of treated rats: Observe that 5 h p.i. a very strong expression of this inflammatory cytokine was seen both in neurons (N) and glial cells (G). See also the remarkable proximity assumed by cells (panel H), an aspect not observed in controls (panel D). Bars: A, C, E, F = 40 µm; B, D, G, H = 12 µm.

Markers / Groups	GFAP		S100		TNF α		INF γ	
	hip	cer	hip	cer	hip	cer	hip	cer
Control (1 to 5 h)	+	+	+	+	-	-	-	-
Treated 1 h	+	++	++	++	-	+	-	+
Treated 2 h	++	+++	++	+++	+	+	+	+
Treated 5 h	+++	+++	+++	+++	+++	++	+++	++

Table 1: Qualitative analysis of relative expression of GFAP, S100, TNFα and IFNγ in hippocampus (hip) and cerebellum (cer) of rats. Note a gradual increase of proteins expression from 1 to 5 h after PNV injection, when compared to control group.

References

Abbott, N.J., Revest, P.A. (1991). Control of brain endothelial permeability. Cerebrovasc. Brain Metab. Rev. 3: 39-72.

Aloisi, F. (1999). The role of microglia and astrocytes in CNS immune surveillance and immunopathology. Adv. Exp. Med. Biol. 468:123-133.

Antunes, E., Marangoni, R.A., Brain, S.D., De Nucci, G. (1992). *Phoneutria nigriventer* (armed spider) venom induces increased vascular permeability in rat and rabbit skin *in vivo*. Toxicon. 30: 1011-1016.

Aranguez, I., Torres C., Rubio N. (1995). The receptor for tumor-necrosis-factor on murine astrocytes-characterization, intracellular degradation, and regulation by cytokines and Theiler's murine encephalomyelitis virus. Glia 13: 185-194.

Balasingam, V., Tejada-Berges, T., Wright, E., Bouckova, R., Yong, V.W. (1994). Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines. J. Neurosci. 14: 846-856.

Balasingam, V., Yong, V.W. (1996). Attenuation of astroglial reactivity by interleukin-10. J. Neurosci. 16: 2945-2955.

Benveniste, Y. (1995). Magnetoelectric effect in fibrous composites with piezoelectric and piezomagnetic phases. Phys. Rev. B. Condens. Matter. 51: 16424-16427.

Boer, A.G., Gaillard, P.J. (2006). Blood-brain barrier dysfunction and recovery. J. Neural. Transm. 113: 455-462.

Burke-Gaffney, A., Keenan, A.K. (1993). Modulation of human endothelial cell permeability by combinations of the cytokines interleukin-1 alpha/ beta, tumor necrosis factor-alpha and interferon-gamma. Immunopharmacology 25: 1-9.

Calvet, M.C., Gresser, I. (1979). Interferon increases the excitability of cultured neurons. Nature (Lond.). 278: 558-560.

Carlson, N.G., Bacchi, A., Rogers, S.W., Gahring, L.C. (1998). Nicotine blocks TNF-alpha-mediated neuroprotection to NMDA by an alpha-bungarotoxin-sensitive pathway. J Neurobiol 35: 29-36.

Costa, S.K., Brain, S.D. Antunes, E., De Nucci, G., Docherty, R.J. (2003). *Phoneutria nigriventer* spider venom activates 5-HT_4 receptors in rat-isolated vagus nerve. Br. J. Pharmacol. 139: 59-64.

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

Cruz-Höfling, M.A., Zago, G.M., Melo, L.L., Raposo, C. (2006) c-FOS and n-NOS reactive neurons in response to circulating *Phoneutria nigriventer* spider venom (submitted)

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

Dietrich, P. Y., Walker, P. R., Saas, P. (2003). Death receptors on reactive astrocytes: a key role in the fine tuning of brain inflammation? Neurology. 60: 548-55.

Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B.A., Cerami, M.A., O'Connor, J.V. (1986). Tumor necrosis factor (cachectin) is an endogenous pyrogen and produces production of interleukin-1. J. Exp. Med. 163: 1433-1450.

Donato, R. (2003). Intracellular and extracellular roles of S100 proteins. Microc. Res. Tech. 60: 540-551.

Eng, L., Ghirnikar, F. (1994). GFAP and astrogliosis. Brain Pathol., 4: 229-237.

Eng, L. F., Ghirnikar, R. S., Lee, Y. L. (2000). Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem. Res. 25: 1439-1451.

Engelhardt, B. (2003). Development of the blood-brain barrier. Cell. Tissue. Res. 314: 119-129.

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

Ganter, S., Northoff, H., Mannel, D., Gebicke-Harter, P.J. (1992). Growth control of cultured microglia. J. Neurosci. Res. 33: 218-230.

Garcia, G.B., Biancardi, M.E., Quiroga, A.D. (2005). Vanadium (V)-induced neurotoxicity in the rat central nervous system: a histoimmunohistochemical study. Drug. Chem. Toxicol. 28: 329-344.

Grieb, P., Forster, R.E., Strome, D., Goodwin, C.W., Pape, P.C. (1985). O_2 exchange between blood and brain tissues studied with 18O2 indicatordilution technique. J. Appl. Physiol. 58, 1929-1941.

Gutierrez, E.G., Banks, W.A., Kastin, A.J. (1993). Murine tumor necrosis factor alpha is transported from blood to brain in the mouse. J Neuroimmunol 47: 169-176.

Haviv, R., Stein, R. (1998). The intracellular domain of P55 tumor necrosis factor receptor induces apoptosis which requires different caspases in naïve and neuronal PC12 cells. J Neurosci Res 52: 380-389.

Hawkins, R.A., O´Kane, R.L., Simpson, I.A., Vina, J.R. (2006). Structure of the blood-brain barrier and its role in the transport of amino acids. J. Nutr. 136: 218-226.

Hirsch, E., C. (2000). Glial cells and Parkinson´s disease. J. Neurol. 247: 1158-1162.

Hofman, F. M., Hinton, D. R., Johnson, K., Merril, J. E. (1989). Tumor necrosis factor identified in multiple sclerosis brain. J. Exp. Med. 170: 607-612.

Imai, F., Suzuki, H., Oda, J., Ninomiva, T., Ono, K., Sano, H., Sawada, M. (2006). Neuroprotective effect of exogenous microglia in global brain ischemia. J. Cereb. Blood Flow. Metab. [Epub ahead of print].

Kinouchi, K., Brown, G., Pasternak, G., Donner, D.B. (1991). Identification and characterization of receptors for tumor necrosis factor-α in the brain. Biochem. Biophys. Res. Commun. 181: 1532-1538.

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

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. (Berl). 105: 125-134.

Lieberman, A.P., Pitha, P.M., Shin, H.S., Shin, M.L. (1989). Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. Proc. Natl. Acad. Sci. USA 86: 6348-6352.

Liu, B. Gao, H.M., Wang, J.Y., Jeohn, G.H., Cooper, C.L., Hong, J.S. (2002). Role of nitric oxide in inflammation-mediated neurodegeneration. Ann. N. Y. Acad. Sci. 962: 318-331.

Lovell, M.A., Robertson, J.D., Teesdale, W.J., Campbell, J.L., Markesbery, W.R. (1998). Copper, iron and zinc in Alzheimer´s disease senile plaques. J. Neurol. Sci. 158: 47-52.

Mayhan, W.G. (2001). Regulation of blood–brain barrier permeability. Microcirculation. 8: 89–104.

Medana, I.M., Hunt, N.H., Chaudhri, G. (1997). Tumor necrosis factor-alpha expression in the brain during fatal murine cerebral malaria: evidence for production by microglia and astrocytes. Am. J. Pathol. 150: 1473-1486.

Mehler, M.F., Rozental, R., Dougherty, R., Spray, D.C., Kessler, J.A. (1993). Cytokine regulation of neuronal differentiation of hippocampal progenitor cells. Nature (Lond.). 362: 62-65.

Minghetti, L., Levi, G. (1998). Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. Prog. Neurobiol. 54: 99-125.

Mrak, R.E., Sheng, J.G., Griffin, W.S. (1996). Correlation of astrocytic S100 beta expression with dystrophic neuritis in amyloid plaques of Alzheimer's disease. J. Neurophatol. Exp. Neurol. 55: 273-279.

Nelson, P.T., Soma, L.A., Lavi, E. (2002). Microglia in diseases of the central nervous system. Ann. Med. 34**:** 491-500.

Neumann, H., Schmidt, H., Cavalié, A., Jenne, D., Wekerle, H. (1997). Major Histocompatibility Complex (MHC) Class I Gene Expression in Single Neurons of the Central Nervous System: Differential Regulation by Interferon (IFN)-γ and Tumor Necrosis Factor (TNF)-α**.** J. Exp. Med. 185: 305.

Neumann, H. (2001). Control of glial immune function by neurons. Glia. 36: 191-199.

O'Callaghan J.P. (1988). Neurotypic and gliotypic proteins as biochemical markers of neurotoxicity. Neurotoxicol. Teratol. 10: 445-452.

Olson, J.K., Girvin, A.M., Miller, S.D. (2001). Direct activation of innate and antigen-presenting functions of microglia following infection with Theiler's virus. J. Virol. 75: 9780-9789.

Paemeleire, K. (2002). Calcium signaling in and between brain astrocytes and endothelial cells. Acta Neurol. Belg. 102: 137-140.

Palkovits, M., Brownstein, M. (1988). Atlas of Rat Brain Anatomy. Elsevier, New York, USA.

Pan, W., Zadina, J.E., Harlan, R.E., Weber, J.T., Banks, W.A., Kastin, A.J. (1997). Tumor necrosis factor-alpha: a neuromodulator in the CNS. Neurosci Biobehav Rev 21: 603-613.

Pekny, M., Nilsson, M. (2005). Astrocyte activation and reactive gliosis. Glia. 50: 427-434.

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

Ramos, A.J., Tagliaferro, P., Lopez, E.M., Pecci Saavedra, J., Brusco, A. (2000). Neuroglial interactions in a model of para-chlorophenylalanine-induced serotonin depletion. Brain Res. 883:1-14.

Ransom, B., Behar, T., Nedergaard, M. (2003). New roles for astrocytes (stars at last). Trends Neurosci. 26: 520-522.

Raposo, C., Zago, G.M., Silva, G.H., Cruz-Höfling, M.A. (2006). Acute blood brain barrier permeabilization in rats after systemic *Phoneutria nigriventer* venom (submitted).

Renno, T., Krakowski, M., Piccirillo, C., Lin, J.Y., Owens, T. (1995). TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis regulation by Th1 cytokines. J. Immunol. 154: 944-953.

Rezende, J.L., 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.

Rostworowski, M., Balasingam, V., Chabot, S., Owens, T., Yong, V.W. (1997). Astrogliosis in the neonatal and adult murine brain post-trauma: elevation of inflammatory cytokines and the lack of requirement for endogenous interferon-gamma. J. Neurosci. 17: 3664-3674.

Rothermundt, M., Peters, M., Prehn, J.H., Arolt, V. (2003). S100B in brain damage and neurodegeneration. Microsc. Res. Tech. 60: 614-632.

Rubin, L.L., Hall, D.E., Porter, S., Barbu, K., Cannon C., Horner, H.C., Janatpour, M., Liaw, C.W., Manning, K., Morales, J. (1991). A cell culture model of the blood-brain barrier. J. Cell. Biol. 115: 1725-1735.

Savchenko, V.L., McKanna, J.A., Nikonenko, I.R., Skibo, G.G. (2000). Microglia and astrocytes in the adult rat brain: comparative immunocytochemical analysis demonstrates the efficacy of lipocortin 1 immunoreactivity. Neuroscience. 96: 195-203.

Selmaj, K.W., Faroog, M., Norton, W.T., Raise, C.S., Brosnan, C.F. (1990). Proliferation of astrocytes in vitro in response to cytokines. A primary role for tumor necrosis factor. J. Immunol. 144: 129-135.

Silva, G.H., Panuto, P.C., Hyslop, S., Cruz-Höfling, M.A. (2004). Immunochemical detection of *Lonomia obliqua* caterpillar venom in rats. Microsc. Res. Tech. 65: 276-281.

Simard, M., Arcuino, G., Takano, T, Liu, Q.S., Nedergaard, M. (2003). Signalling at the gliovascular interface. J. Neurosci. 23: 9254-9262.

Streit, W.J. (2000). Microglial response to brain injury: a brief synopsis. Toxicol. Pathol. 28: 28-30.

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.

Walz, W., Lang, M.K. (1998). Immunocytochemical evidence for a distint GFAP-negative subpopulation of astrocytes in the adult rat hippocampus. Neurosci. Lett. 257: 127-130.

Whitaker-Azmitia, P.M., Murphy, R., Azmitia, E.C. (1990). Stimulation of astroglial 5-HT1A receptors releases the serotonergic growth factor, protein S-100, and alters astroglial morphology. Brain Res. 528: 155-158.

Wong, D., Dorovini-Zis, K., Vincent, S.R. (2004). Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood-brain barrier. Exp. Neurol. 190: 446-455.

Woodroofe, M.N., Sarna, G.S., Wadhwa, M., Hayes, G.M., Loughlin, A.J., Tinker, A., Cuzner, M.L. (1991). Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. J. Neuroimmunol. 33: 227-236.

Yong, K.L. (1996). Granulocyte colony-stimulating factor (G-CSF) increase neutrophil migration across vascular endothelium independent of an effect on adhesion: comparison with granulocyte-macrophage colony-stimulating factor (GM-CSF). Br. J. Haematol. 94: 40-47.

Yuan, S.Y. (2000). Signal transduction pathways in enhanced microvascular permeability. Microcirculation 7: 395–403.

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

Ziegler, D.R., Innocente, C.E., Leal, R.B., Rodnight, R., Goncalves, C.A. (1998). The S100B protein inhibits phosphorylation of GFAP and vimentin in a cytoskeletal fraction from immature rat hippocampus. Neurochem. Res. 23: 1259-1263.

4- CONCLUSÃO

Com base nos resultados obtidos neste trabalho, confrontados com resultados anteriormente obtidos e com a literatura é possível sugerir que:

- *Os sinais clínicos apresentados pelos animais que receberam injeção i.v. do veneno de P. nigriventer confirmam sua neurotoxicidade e ação no SNC, além do SNP e SNA..*

- *Há diferenças regionais nas características da BHE, as regiões onde a quantidade de substância cinzenta é maior do que as áreas de substância branca parecem ser as mais susceptíveis à ação tóxica do veneno de Phoneutria nigriventer. Uma explicação imediata seria o fato de as regiões de substância cinzenta mostrarem-se significativamente mais vascularizadas que as áreas de substância branca. É possível que a maior vascularização esteja associada ao fato de ser a substância cinzenta o local onde localizam-se os corpos dos neurônios e os astrócitos que estão envolvidos nas várias funções de assessoramento da função neuronal e neural de maneira geral.*

- *Parece haver maior fragilidade da via transcelular da BHE, o que sugere que as neurotoxinas do veneno apresentam um grau de especificidade, tornando-as portanto excelentes ferramentas para o estudo dos fenômenos fisiopatológicos.*

- *As áreas estudadas embora possuam mecanismos defensivos em comum, devem ter variações nos componentes moleculares metabólicos,e enzimáticos que constituem a BHE, que ao interagir com as toxinas do veneno mostram as diferenças regionais no vedamento da interface sangue-cérebro.*

- *O contato dos componentes do veneno com o endotélio dos microvasos cerebrais parece acionar o recrutamento de diferentes populações de células de defesa, dependendo da área anatômica considerada, exceção feitas as astrócitos que mostram-se reativos em tôdas as áreas examinadas, quer pela expressão alterada das proteínas GFAP e S-100, como também pela presença de alterações de sua permeabilidade (edema).*

- *Os mediadores inflamatórios imunomarcados nos astrócitos e neurônios do cerebelo e hipocampo, são parte da resposta imune à injúria neurotóxica causada pelo veneno circulante, e podem estar, pelo menos parcialmente, envolvidas na transitoriedade das alterações da permeabilidade vascular na interface sangue-cérebro. Podem ainda atuar como segundo mensageiros no estímulo toxicante. O fato de os neurônios e astrócitos IFN-γ and TNF-α positivos do hipocampo estabelecerem graus de proximidade não observados nos animais contrôle sugere que pode ocorrer trocas de mensagens entre as duas células sob o efeito tóxico do veneno. A aparente seletividade de resposta regional pode estar associada a um possível papel diferencial dessas citocinas pró-inflamatórias que poderia ser citotóxica para algumas regiões do cérebro e neurotróficas para outras.*

- *Concluímos que o entendimento das características regionais da BHE é importante para desenvolver uma base de conhecimento fundamental nas propriedades de transporte sangue-cérebro, com vistas para o controle de acesso de drogas terapêuticas em áreas cerebrais de interesse.*

5- REFERÊNCIAS BIBLIOGRÁFICAS

Abbott NJ. Dynamics of CNS barriers: evolution, differentiation, and modulation. Cell Mol Neurobiol 2005; 25(1): 5-23.

Abbott NJ. Inflammatory mediators and modulation of blood-brain barrier permeability. Cell Mol Neurobiol 2002; 20: 131-47.

Abbott NJ. Romero IA. Transporting therapeutics across the blood-brain barrier. Mol Med Today 1996; 2(3): 106-13.

Abbott NJ, Rönnbäck L, Hanson E. Astrocyte-endothelial interations at the blood-brain barrier. Neuroscience 2006; 7: 41-53.

Abdul-Khaliq H, Schubert S, Stoltenburg-Didinger G, Troitzsch D, Bottcher W, Hubler M, et al. Protein S100 beta in brain an dserum after deep hypothermic circulatory arrest in rabbits: Relationship to perivascular astrocytic swelling. Clin Chem Lab Med 2000; 38: 1169-72.

Adams S, Brown H, Turner G. Breaking down the blod-brain barrier: signaling a path to cerebral malaria. Trends Parasitol 2002; 18: 360-66.

Ambartsumian N, Klingelhofer J, Grigorian M, Christensen C, Kriajevska M, Tulchinsky E, et al. The metastasis-associated Mts 1(S100A4) protein could act as an angiogenic factor. Oncogene 2001; 20(3-4): 4685-95.

Antunes E, Marangoni RA, Brain SD, De Nucci G. *Phoneutria nigriventer* (armed spider) venom induces increased vascular permeability in rat and rabbit skin in vivo. Toxicon 1992; 30: 1011-16.

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

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

Barres BA. New roles for glia. J Neurosc 1991; 11: 3685-94.

Barraviera B. Teaching about mishaps with venomous animals in the medical schools of Brazil. Rev Soc Bras Med Trop 1992; 25(3): 203-4.

Beaudeux J, Dequen L, Foglietti M. Pathophysiologic aspects of S100 beta protein: a new biological marker of brain pathology. Ann Biol Cli 1999; 57(3): 261-72.

Begley DJ. Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol Ther 2004; 104: 29-45.

Begley D, Brightman MW. Structural and functional aspects of the blood-brain barrier. In Prokai L, Prokai-Tatrai K. Peptide Transport and Delivery into the Central Nervous System. Progress in Drug Research 2006; 61: 39–78.

Belayev L, Busto R, Zhao W, Ginsberg MD. Quantitative evaluation of blood-brain barrier permeability following middle cerebral artery occlusion in rats. Brain Res 1996; 739: 88-96.

Bento AC, Rego E, Pedroso-Mariani SR, Mancuso LC, Marangoni S, Caracelli I, et al. Isolation of a polypeptide from *Phoneutria nigriventer* spider venom responsible for the increased vascular permeability in rabbit skin. Toxicon 1995; 33: 171–8.

Benvenist EN. Citokynes: influence on glial cell gene expression and function. In: Blalock JE. Neuro Immuno Endocrinology 1992; 2: 106-43.

Bertolotto A, Rocca G, Schiffer D. Chondroitin 4-sulfate proteoglycan forms an extracellular network in human and rat central nervous system. J Neurol Scien 1990; 100: 113-23.

Bock E, Moller M, Nissen C, Sensenbrenner M. Glial fibrillary acidic protein in primary astroglial cell culture derived from newborm rat brain. Febs Lett 1977; 83: 207-211.

Boletim do Ministério da Saúde. Acidentes Araneídeos: contribuição ao estudo da morbidade, 1989, bol 22. Brasília, Brasil.

Brightman MW, Reese TS. Junctions between brain cappilary endothelial cells and glial cells increase the transcellular permeability of the blood-brain barrier during ischemia. J Neurochem 1969; 83: 807-17.

Buniantian G, Gebhardt R, Hamprecht B. The astroglial marker the GFAP expressed in cultured cell populations containing stellate cell. J Neurochem 1994; 63: 521.

Bucaretchi F, Deus Reinaldo CR, Hyslop S, Madureira PR, De Capitani EM, Vieira RJ. A clinico-epidemological study of bites by spiders of the genus *Phoneutria*. Rev Inst Med Trop S Paulo 2000; 42: 17-21.

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

Brochez L, Naeyaert JM. Serological markers for melanoma. Br J Dermatol 2000; 143: 256-68.

Claudio L, Raine CS, Brosnan CF. Evidence of persistent blood-brain barrier abnormalities in chronic-progressive multiple sclerosis. Acta Neuropathol 1995; 90: 228-38.

Chavez-Olortegui C, Bohorquez K, Alvarenga LM, Kalapothakis E, Campolina D, Maria WS, et al. Sandwich-Elisa detection of venom antigens in evenoming by *Phoneutria nigriventer* spider. Toxicon 2001; 39(6): 909-11.

Chen Y, Dalwadi G, Benson HA. Drug delivery across the blood-brain barrier. Curr Drug Deliv 2004; 4: 361-76.

Chen Y, Swason RA. Astrocytes and brain injure. J Cereb Blood Flow Metab 2003; 23:137-49.

Collins VP. Cellular Mechanisms targeted during astrocytoma progression. Cancer Lett 2002; 188:1-7.

Cordeiro MN, Diniz CR, Valentim AC, Von Eicksted VRD, Gilroy J, Richardson M. The purification and amino acid sequence of four Tx2 neurotoxins from the venom of the Brazilian "armed" spider *Phoneutria nigriventer*. FEBS Lett 1992; 310: 153-6.

Cordeiro MN, Figueiredo SG, Valentin AC, Diniz CR, Von Eicksted VRD, Gilroy J, Richardson M. Purification and amino acid sequence of six Tx3 type neurotoxins from the venom of the Brazilian "armed" spider *Phoneutria nigriventer.* Toxicon 1993; 11: 35-42.

Costa SKP, Moreno H Jr, Brain SD, de Nucci G, Antunes E. The effect of *Phoneutria nigriventer* (armed spider) venom on arterial blood pressure of anesthetised rats. Eur J Pharmacol 1996; 298: 113-20.

Cserr HF, Bundgaard M. Blood-brain interfaces in vertebrates: a comparative approach. Am J Physiol 1984; 246: 277-88.

Cruz-Höfling MA, Love S, Brook G, Duchen LW. Effects of *Phoneutria nigriventer* spider venom on mouse peripheral nerve. Quartl J Exp Physiol 1985; 70: 623-40.

Davies DC. Blood-brain barrier breakdown in septic ancephalopathy and brain tumours. J Anat 2002; 200: 639-46.

DeArmond SJ, Lee YL, Kretzchmar HA, Eng LF. Turnover of glial filaments in mouse spinal cord. JNeurochem 1986; 47(6): 1749-53.

Dehouck MP, Meresse S, Delorme P, Fruchart JC, Cecchelli R. An easier, reproducible, and mass-production method to study the blood-brain barrier in vitro. J Neurochem 1990; 54(5):1798-801.

Dehouck MP, Vigne P, Torpier G, Breittmayer JP, Cecchelli R, Frelin C. Endothelin-1 as a mediator of endothelial cell-pericyte interactions in bovine brain capillaries. J Cereb Blood Flow Met 1997; 17: 464-69.

Deli MA, Descamps L, Dehouck MP, Cechelli R, Joo F, Alraham CS, et al. Exposure of tumornecrosis factor-a to terminal membrane of bovine capillary endothelial cells cocultured wih astrocytes induces a delayed increase of permeability and cytoplasmic stress formation of actin. J Neurosci Res 1995; 41: 717-26.

Dermietzel R, Krause D. Molecular anatomy of the blood-brain barrier as defined by immunocytochemistry. Int Rev Cytol 1991; 127: 57-109.

Diamond JS, Jahr CE. Transporter buffer synaptically released glutamate on submillisecond time scale. J Neuroaci 1997; 17: 4672-87.

Diniz CR. Separação de proteínas e caracterização de substâncias ativas em venenos de aranhas do Brasil. An Acad Bras Ciênc 1963; 35: 283-91.

Diniz CR, Cordeiro MN, Resende Jr, Kelly P, Fisjer S, Reimam F, et al. The purification and amino acid sequence of letal neurotoxin Tx1 type neurotoxins from the venom of the Brazilian “armed” spider *Phoneutria nigriventer*. FEBS Lett 1990; 263: 251-53.

Dohgu S, Takata F, Yamauchi A, Nakagawa S, Egawa T, Naito M, et al. Brain pericytes contribute to the induction and up-regulation of blood-brain barrier functions through transforming growth factor-beta production. Brain Res 2005; 1038(2): 208-15.

Donato R. S-100 protein. Cell Calcium 1986; 7(3): 123-45.

Dore-Duffy P, Owen C, Balanov R, Murphy S, Beaumont T, Rafols JA. Pericyte migration from the vascular wall in response to traumatic brain injury. Microvasc Res 2000; 60(1): 55-69.

Eddeleston M, Mucke L. Molecular profile of reactive astrocytes – implications for their role in neurologic disease. Neuroscience 1993; 54:15-36.

Entwhistle ID, Johnstone RAW, Medziradski D, May TE. 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: 1059-67.

Erdlenbruch B, Alipour M, Fricker G, Miller DS, Kugle W, Eibl H, et al. Alkyl glycerol opening of the blood-brain barrier to small and large fluorescence markers in normal and C6 glioma-bearing rats and isolated rat brain capillaries. Br J Pharmacol 2003; 140(7): 1201-10.

Erlish P. "Das sauerstoffbedürfnis des organismus". Eine farbanalitische studie. 1885; Berlim; Hirschwald.

Escoubas P, Diochot S,Corzo G. Structure and pharmacology of spider venom neurotoxins. Biochimie 2000; 82: 893-07.

Estato V, Antunes E, Machado B, De Nucci G, Tibiriçá. Investigation of the haemodynamic effects of Phoneutria nigriventer venom in anaesthetised rabbits. Toxicon 2000; 38: 841-53.

Fabry Z, Raine CS, Hart MN. Nervous tissue as an immune compartment: the dialect of the immune response in the CNS. Immunology Today 1994; 15: 218-24.

Farkas E, Luiten PG. Cerebral microvascular pathology in aging and Alzheimer's disease. Prog Neurobiol 2001; 64: 575-11.

Fernandez C, Buyse M, Gimenez F. Influence of the pro-inflammatory cytokines on P-glycoprotein expression and functionality. J Pharm Pharmaceut Sci 2004; 7(3): 359-71.

Figueiredo SG, Garcia MELP, Valentim AC, Cordeiro MN, Diniz CR, Richardson MN. Purification and amino acid sequence of the inseticidal neurotoxin Tx4 (6-1) from the venom of the spider *Phoneutria nigriventer.* Toxicon 1995; 33: 83-93.

Fontana MD, Vital Brazil O. Mode of action of *Phoneutria nigriventer* spider venom at the isolated phrenic nerve-diaphragm of the rat. Braz J Med Biol Res 1985; 18: 557-65.

Gaillard PJ, Van Der Sandt IC, Voorwinden LH, Vu D, Nielsen JL, de Boer AG, et al. Astrocytes increase the functional expression of P-glycoprotein in an in vitro model of the blood-brain barrier. Pharm Res 2000; 11(10): 1198-205.

Gard AL, White FE, Dutton GR. Extraneural glial fibrillary acid protein (GFAP) immunoreactivity in perisinusoidal stellate cells os rat liver. J Neuroimmunol 1985; 8: 359-75.

Goldmann EE. Vitalfärbugen am zentralnervensystem. Beitrag zur physiologie des plexus choroideus und der hirnhãute. 1913; Berlim: Hirschfeld.

Gloor SM, Wachtel M, Bolliger MF, Ishihara H, Landmann R, Frei K. Molecular and cellular permeability control at theb blood-brain barrier. Brain Res Rev 2001; 36(2-3): 258-64.

Gomez MV, Kalapothakis E, Guatimosim C, Prado MA. *Phoneutria nigriventer* venom: a cocktail of toxins that affect íon channels. Cell Mol Neurobiol 2002; 22: 579-88.

Greenwood J. Mechanisms of blood-brain barrier breakdown. Neuroradiology 1991; 33: 95-100.

Grieb P, Forster RE, Strome D, Goodwin CW, Pape PC. O2 exchange between blood and brain tissues studied with 18O2 indicator-dilution technique. J Appl Physiol 1985; 58(6): 1929-41.

Gursoy-Ozdemir Y, Qire J, Matsuoka N, Bolay H, Bermpohl D, Jin H, et al. Cortical spreading depression activates and repregulates MMP-9. J Clin Investig 2004; 113: 1447-55.

Haseloff RF, Blasing IE, Bauer HC, Bahuer H. In search of the astrocytic factor(s) modulation blood-brain barrier functions n brain capillary endothelial cells in vitro. Cell Mol Neurobiol 2005; 25(1):25-39.

Hayagashi Y, Nomura M, Yamagishi S, Harada S, Yamashita J, Yamamoto H. Introduction of various blood-brain barrier properties in nn-neural endothelial cells by close apposition to co-cultured astrocytes. Glia 1997; 19:13-26.

Hawkins RA, O' Kane RL, Simpson IA, Vina JR. Structure of the blood-brain barrier and its role in the transport of amino acids. J Nutr 2006; 136: 218-26.

Holash JA, Harik SI, Perry G, Stewart PA. Barrier properits of testis microvessels. Proc Nat Acad Sci 1993; 90: 11069-73.

Hori S, Ohtsuki S, Hosoya K, Nakashima E, Terasaki T. A pericyte-derived angiopoietin-1 multimeric complex induces ocluding gene expression in brain cappilary endothelial cells through Tié-2 activation in vitro. J Neurochem 2004; 89: 503-13.

Huber JD, Egleston RD, Davis TP. Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. Trends Neurosci 2001; 24: 719-25.

Hurst RD, Fritz JB. Properties of an immortalised vascular endothelial/glioma cell co-culture model of the blood-brain barrier. J Cell Physiol 1996; 167(1): 81-8.

Huttunen HJ, Kuja-Panula J, Sorci G, Agneletti AL, Donato R, Rauvala H. Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation and products (RAGE) activation. J Biol Chem 2000; 275(51): 40096-105.

Iadecola C. Regulation of the cerebral microcirculation during neural activity: in nitric oxide the missing link?. Trends Neurosci 1993; 16: 206-14.

Jonsson H, Johnson P, Alling C, Backstrom M, Bergh C, Blomquist S. S100B after coronary artery surgery: release pattern, source of contamination, and relation to neuropsychological outcome. Ann Thorac Surg 1999; 68: 2202-8.

Janzer RC, The blood-brain barrier: cellular basis. J Inherit Metab Dis 1993; 16: 639-49.

Janzer RC, Raff MC. Astrocytes induce blod-brain barrier properties in endothelial cells. Nature 1997; 325: 253-57.

Kacem K, Lacombe P, Seylaz J, Bonvento G. Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: a confocal microscopy study. Glia 1998; 23: 1-10.

Kahn MA, Ellison JA, Chang RP, Speight GJ, de Vellis J. CNTF induces GFAP in a S100 alpha brain cell population: the pattern of CNTF-alpha R suggests an indirect mode of action. Dev Brain Res 1997; 98: 221-23.

Kajiwara K, Ito H, Fukumoto T. Lymphocyte infiltration into normal rat brain following hyperosmotic blood-brain barrier opening. J Neuroimmunol 1990; 25: 133-40.

Kapural M, Krizanac-Bengez LJ, Barnett G, Perl J, Masaryk T, Apollo D, et al. Serum S-100beta as a possible marker of blood-brain barrier disruption. Brain Res 2002; 940(1-2): 102-4.

Ke ZJ, Gibson GE. Selective response of various brain cell types during neurodegeneration induced by mild impairment of oxidative metabolism. Neurochem Int 2004; 45(2-3): 361-9.

Kepes JJ, Perenres E. Glial fibrillary acid protein in chondrocytes of elastic cartilage in the human epiglottis. An immuno histochemical study with polyvalent and monoclonal antibodies. Anat Rec 1988; 220: 296-99.

Kim JH, Kim JH, Park JA, Lee SW, Kim WJ, Yu YS, et al., Blood-neural Barrier: Intercellular Communication at Glio-Vascular Interface. J Biochem Mol Biol 2006;39: 339-45.

Kimelberg HK, Norenberg MD. Astrocytes. Sci Am 1989; 260(4): 72-6.

Kindy MS, Bhat AN, Bhat NR. Transient ischemia stimulati glial fibrillary acid protein and vimentin gene expression in gerbil neocortex, striatum and hippocampus. Mol Brain Res 1992; 13: 199-206.

Kuchler-Bopp S, Delanoy JP, Antault JC, Zaepfel M, Dietrich JR. Astrocytes induce several blood-brain barrier properties in non-neuronal endothelial cells. Neuro Report 1999; 10: 1347-53.

Kushner PD, Stephenson DT, Wright S. Reactive astrogliosis is widespread in the subcortical white matter of amyotrophic lateral sclerosis brain. J Neuropathol Exp Neurol 1991; 50(3): 263-77.

Lee EJ, Hung YC, Lee MY. Early alterations in cerebral hemodynamics, brain metabolism and blood-brain barrier permeability in experimental intracerebral hemorrhage. J Neurosurg 1999; 91: 1013-19.

Lee HJ, Zhang Y, Pardridge WM. Blood-brain barrier disruption following the internal carotid arterial perfusion of alkyl glycerols. J Drug Target 2002; 10(6): 463-7.

Le Sueur LP, Kalapothakis E, Cruz-Höfling MA. Breakdown of the blood-brain barrier and neuropathological changes by *Phoneutria nigriventer* spider venom. Acta Neuropathol 2003; 105: 125-34.

Le Sueur LP, Collares-Buzato CB, Cruz-Höfliing MA. Mechanisms involved in the blood-brain barrier increased permeability induced by *Phoneutria nigriventer* spider venom in rats. Brain Res 2004; 1027: 38-47.

Leybaert L. Neurobarrier coupling in the brain: a partner of neurovascular and neurometabolic coupling? J Cereb Blood Flow Metab 2005; 25: 2-16.

Liesi P, Silver J. Is astrocytes laminin involved in axon guidance in the mammalian CNS? Dev Biol 1988; 130: 774-85.

Lossinsky AS, Garcia JH, Iwanowski L, Lightfoote WE. New ultrastructural evidence for a protein transport system in endothelial cells of gerbil brains. Acta Neuropathol 1979; 47: 105-10.

Love S, Cruz-Höfling MA, Duchen LM. Morphological abnormalities in myelinated nerve fibres caused by *Leiurus, Centruroides* and *Phoneutria* venoms and their prevention by tetrodoxin. Quartl J Exp Physiol 1986; 71: 115-22.

Lucas S. Spiders in Brazil. Toxicon 1988; 26: 759-72.

Luurtsema G, De Lange EC, Lammertsma AA, Franssen EJ. Transport across the blood-brain barrier: stereoselectivity and PET-tracers. Mol Imaging Biol 2004; 6(5): 306-18.

McAllister MS, Krizac-Bengez L, Macchia F, Naftalin RJ, Pedley KC, Marroni M, et al. Mecanism of glucose transport at the blood-brain barrier: an in vitro study. Brain Res 2001; 904(1): 20-30.

Marenholz I, Heizmann CW. Fritz G. S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun 2004; 322: 1111-22.

Marenholz I, Heizmann CW. S100A16, a ubiquitously expressed EF-hand protein which is up-regulated in tumors. Biochem Biophys Res Commun 2004; (313) 2: 237-44.

Marangoni RA, Antunes E, Brain SD, De Nucci G. Activation by *Phoneutria nigriventer* (armed spider) venom of tissue Kallikrein-Kininogen-Kininsystem in rabbit skin in vivo. Br J Pharmacol 1993; 109: 539-43.

Marchi N, Rasmussen P, Kapural M, Fazio V, Kight K, Mayberg MR, et al. Peripheral markers of brain damage and blood-brain barrier dysfunction. Restor Neurol Neurosci 2003; 21(3-4): 109-21.

Marchi N, Cavaglia M, Fazio V, Bhudia S, Hallene K, Janigro D. Peripheral markers of blood-brain barrier damage. Clin Chim Acta 2004; 342: 1-12.

Mrak RE, Griffin WS. Trisomy 21 and the brain. J Neuropathol Exp Neurol 2004; 63(7): 679-85.

Massacrier A, Couraud F, Cau P. Voltage-sensitive Na+ channels peripheral nerves detected using scorpion toxins. J Neurocytol 1990; 19(6): 850-72.

Matter K, Balda MS. Signalling to and from tight junction. Nat Rev Mol Cell Biol 2003; 4(3): 225-36.

Mercier F, Hatton GI. Immunocytochemical basis for a meningeo-glial network. J Comp Neurol 2000; 420(4): 445-65.

Meshul CK, Seil FJ, Herndon RM. Astrocytes play a role in regulation of synaptic density. Brain Res 1987; 402(1): 139-45.

Montgomery DI. Astrocytes: form, functions, and roles in disease. Veterinary Pathol 1994; 31:145-67.

Moore K. Embriologia Clínica. 3 ed. Rio de Janeiro: Interamericana; 1984. p. 354-88.

Munoz-Fernandez MA, Fresno M. The role of tumour necrosis factor, interleukin 6, interferon gamma and inducible nitric oxide synthase in the development and pathology of the nervous system. Progress Neurobiol 1998; 56: 307-40.

Nag S, Harik SI. Cerebrovascular permeability to horseradish peroxidase in hypertensive rats: effects of unilateral locus cereleus lesion. Acta Neuropathol 1987; 247-53.

Nagele RG, Wegie J, Venkataraman V, Imaki H, Wang KC, Wegiel J. Contribution of glial cells to the development of amyloid plaques in Alzheimer`s disease. Neurobiol Aging 2004; 25(5): 663-74.

Newton RA, Hogg N. The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. J Immunol 1998; 160(3): 1427-35.

Neuwelt EA, Goldman DL, Dahlborg SA, Crossen J, Ramsey F, Roman-Goldstein S, et al. Primary CNS lymphoma treated with osmotic blood-brain barrier disruption: prolonged survival and preservation of cognitive function. J Clin Oncol 1991; 9(9): 1580-90.

Niki T, De Bleser PI, Xu G, Van Den Berg K, Wisse E, Gerts A. A comparasion of glial fibrillary acidic protein and desmin staining in normal and CC14 induced fibrotic rat livers. Hepatology 1996; 23: 1538-45.

Nitsch C, Klatzo I. regional patterns of blood-brain barrier breakdown during epileptiform seizures induced by various convulsive agents. J Neurol 1993; 59: 305-22.

Nitsch C, Goping G, Laursen H, Klatzo I. The blood-brain barrier to horseradish peroxidase at onset of bicuculline-induced seizures in hypothalamus, palladium, hippocampus, and other selected regions of the rabbit. Acta Neuropathol 1986; 69: 1-16.

Oldendorf WH, Conford ME, Brown WJ. The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of cappilary endothelial cells in brain and others tissues of the rat. Ann Neurol 1977; 1: 409-17.

Orte C, Lawrenson JG, Finn TM, Reid AR, Alit GA. Comparison of blood-brain barrier and blood-nerve barrier endothelial cell markers. Anat Embryol 1999; 199: 509-17.

Oztar B, Akgul S, Arslan FB. Influence of surgical pain stress on the blood-brain barrier permeability in rats. Life Sci 2004; 74: 1973-79.

Pardridge Wm, Eisenberg J, Yang J. Human blod-brain barrier insulin receptor. J Neurochem 1985; 44(6): 1771-8.

Peridsky Y, Ghorpade A, Rasmussen J, Limoges J, Liu XJ, Stins M, et al. Microglial and astrocytes chemokines regulate monocyte migration through the blood-brain barrier in human immunodeficiency virus-1 encephalitis. Am J Pathol 1999; 15: 1599-11.

Prado MA, Guatimosim C, Gomez MV, Diniz CR, Cordeiro MN, Romano-Silva MA. A novel tool for the investigation oof glutamate release from rat cerebrocortical synaptosomes: the toxin Tx3-3 from the venom of the spider *Phoneutria nigriventer*. Biochem J 1996; 15(314): 145-50.

Pawlinski R, Janeczko k. Intracerebral injection of interferon-gamma inhibits the astrocytes proliferation following the brain injury in the 6-day-old rat. J Neurosci Res 1997; 50(6): 1018-22.

Pekny M. Astrocytic intermediate filaments: lesons fraom GFAP and vimentim Knock-out mice. Program Brain Res 2001; 132: 23-30.

Pekny M, Pekna M. Astrocyte intermediate filaments in CNS pathologies and regeneration. J Pathology 2004; 204: 428-37.

Pereira LAV, Graça DL. Biologia da Neuroglia. Anais da XLII Reunião da Sociedade Brasileira para o Progresso da Ciência; 1990. p. 427-9.

Rapoport J. Osmotic opening of the blood-brain barrier: principles mechanism and therapeutic applications. Cell Mol Neurobiol 2000; 20: 217-30.

Rash LD, Hodgson WC. Pharmacology and biochemistry of spider venoms. Toxicon 2002; 40: 225-54.

Reese TS, Karnorsky MJ. Fine structural localization of a blood-brain barrier to exogenous peroxidase. J Cell Biol 1967; 34: 207-17.

Rego E, Bento AC, Lopes-Martins RAB, Antunes E, Novello JC, Marangoni S, et al. Isolation and partial characterization of a polypeptide from *Phoneutria nigriventer* spider venom that relaxes rabbit corpus cavernosum in vitro. Toxicon 1996; 34: 1141-47.

Reichenbach A, Wolburg H. In neuroglia. 2 ed. Oxford, 2004. p. 19-35.

Renno T, Krakowiski M, Piccirilio C, Lin JY, Owens T. TNF alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis: regulation by TH1 cytokines. J Immunol 1995; 154: 944-53.

Rickmann M, Wolff JR. S100 immunoreactivity in a subpopulation of oligodendrocytes and Ranvier´s nodes of adult rat brain. Neurosci Lett 1995; 186(1): 13-6.

Risau W, Wolburg H. Development of blood-brain barrier. Trends Neurosci 1990; 13: 174-8.

Rezende JrL, Cordeiro MN, Oliveira EB, Diniz CR. Isolation of Neurotoxic peptides from the venom of the "armed" spider *Phoneutria nigriventer*. Toxicon 1991; 29: 1225-33.

Romano-Silva MA, Gomes MV, Diniz CR, Cordeiro MN, Ribeiro AM. Acetylcholic 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. Neurots Lett 1996; 219: 159-62.

Rubin LL, Staddon JM. The cell biology of the blood-brain barrier. Annu Rev Neurosci 1999; 22: 11-28.

Rubin LL, Barbu K, Bard F, Cannon C, Hall DE, Horner H, et al. Differentiation of brain endothelial cells in cell culture. Ann N Y Acad Sci 1991; 633: 420-5.

Rudge JS, Smith GM, Silver J. An in vitro model of wound healing in the CNS: analysis of cell reaction and interaction at different ages. Exp Neurol 1989; 103: 1-16.

Savchenko VL, Mackanna JA, Skibo GG. Microglia and astrocytes in the adult rat brain: comparative immuno-cytochemical analysis demonstrates the efficacy of lipocortin 1 immuno-noreactivity. Neuroscience 2000; 96: 195-203.

Schenberg S, Lima FA. Pharmacology of the polypeptides from the venom of the spider *Phoneutria nigriventer.* Mem Inst Butantan 1966; 33(2): 627-38.

Schenberg S, Pereira Lima FA. *Phoneutria nigriventer* venom In: Bucherl W. Buckley EE. (Eds.). Venomous Animals and their Venom, Academic Press New York 1971; 3: 279-85.

Schinkel AH. P-glycoprotein, a gatekeeper in the blood–brain barrier. Adv Drug Deliv Rev 1999; 36: 179–94.

Schipper HM, Vininsky R, Brull R, Small L, Brawer JR. Astrocyte mitochondria: a substrate for iron deposition in the aging rat substantia nigra. Exp Neurol 1998; 152(2): 188-96.

Schroeter ML, Muller S, Lindenau J, Wiesner B, Hanisch UK, Wolf G, et al. Astrocytes induce manganese superoxide dismutase in brain capillary endothelial cells. Neuroreport 2001; 11:2513-7.

Schwaninger M, Sallmann S, Petersen N, Schneider A, Prinz S, Libermann TA, et al. Bradykinin induces interleukin-6 expression in astrocytes through activation of nuclear factor-kappaB. J Neurochem 1999; 4:1461-6.

Sedlakova R, Shivers RR, Del Maestro RF. Ultrastructure of the blood-brain barrier in the rabbit. J Submicrosc Cytol Pathol 1999; 31(1): 149-61.

Sethna MP, Lampson LA. Immune modulation within the brain: Recruitment of inflammatory cells and increases major histocompatibility antigen expression following intracerebral injection of interferon-gamma. J Neuroimmunol 1991; 34: 121-32.

Silva GH, Hyslop S, Cruz-Höfling MA. *Lonomia oblique* caterpillar venom increases permeability of the blood-brain barrier in rats. Toxicon 2004; 44(6): 625-34.

Simmons RD, Willenbourg DO. Direct injection of cytockines into the spinal cord causes autoimmune encephalomyelitis-like inflammation. J Neurol Sci 1990; 100: 37-42.

Tanobe K, Nishikawa K, Hinohara H, Okanmoto T, Saito S, Goto F. Blood-brain barrier and general anestheties. Massui 2003; 52(8): 840-5.

Toborek M, Lee YW, Flora G, Pu H, András IE, Wylegala E, et al. Mechanisms of the blood-brain barrier disruption in HIV-1 infection. Cell Mol Neurobiol 2005; 25: 181-99.

Vital-Brasil O, Leite GB, Fontana MD. Modo de ação da peçonha da aranha *Phoneutria nigriventer*, nas aurículas isoladas de cobaia. Ciência e Cultura 1988; 40: 181-85.

Walz W, Lang, MK. Immunocytochemical evidence for a distinct GFAP-negative subpopulation of astrocytes in the adult rat hippocampus. Neurosci Lett 1998; 257: 127-30

Watson WE. Cell Biology of the Brain. London: Chapman and Hall; 1976.

Weier MD, Patel AJ, Hunt A, Thomas DGT. Development changes in the amount of glial fibrillary acid protein in three regions of the rat brain. Dev Brain Res 1984; 15:147-54.

Wilkin GP, Marriott DR, Cholewinski AJ. Astrocyte heterogeneity. Trends Neurosci 1990; 13(2): 43-6.

Yong VW, Moundjian R, Yong FP, Ruijs TC, Freedman MS, Cashman N, et al. Gamma-interferon promotes proliferation and down-regulation of intercellular adhesion molecule-1 (ICAM-1) are interrupted by interferon gamma. Clin Exp immunol 1996; 103: 475-81.

Xu J, Ling EA. Upregulation and induction of major histocompatibility complex class I and II antigens on microglial cells in early postnatal rat brain following intraperitoneal injections of recombinant interferon-gamma. Neuroscience 1994; 4: 959-67.

Zhang Y, Pardridge WM. Rapid transferrin efflux from brain to blood across tha blood-brain barrier. J Neurochem 2001; 76(5): 1597-600.

6- ANEXOS

Anexo I- Resolução do formato alternativo para defesa da dissertação de mestrado

DELIBERAÇÃO CCPG – 001/98

Dispõe a respeito do formato das teses de Mestrado
e de Doutourada aprovadas pela UNICAMP

Tendo em vista a possibilidade, segundo parecer PG N^{o} 1985-96, das teses de mestrado e de doutourado terem um formato alternativo àquele já bem estabelecido, a CCPG resolve:

Artigo 1: Todas as teses (alternativas) de mestrado e de doutorado da UNICAMP terão, a partir de janeiro de 1999, o seguinte formato padrão:

I) Capa com formato único, dando visibilidade ao nível (mestrado ou doutorado), e à Universidade

II) Primeira folha interna dando visibilidade ao nível (mestrado ou doutorado) à Universidade, à Unidade em que foi defendida e à banca examinadora, ressaltando o nome do orientador e co-orientador. No seu verso deve constar a ficha catalográfica.

III) Segunda folha interna onde conste o Resumo em português eo Abstract em inglês.

IV) Introdução Geral

V) Capítulos

VI) Conclusão Geral

VII) Referências Bibliográficas

VIII) Apêndices (se necessário)

Artigo 2: A critério do orientador, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

Parágrafo único: Os veículos de divulgação deverão ser expressamente indicados.

Artigo 3: A PRPG providenciará o projeto gráfico das capas bem como a impressão de um número de exemplares, definido e pago pelo canditato, da versão final da tese a ser homologada.

Artigo 4: Fica revogada a resolução CCPG 17/97.

Anexo II- Certificado do Comitê de Ética em Pesquisa Animal

Universidade Estadual de Campinas
Instituto de Biologia

UNICAMP

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

C E R T I F I C A D O

Certificamos que o Protocolo nº **702-1**, sobre **"ENVOLVIMENTO DA VIA PARACELULAR NA QUEBRA DA BARREIRA HEMATOENCEFÁLICA PELO VENENO DA ARANHA PHONEUTRIA NIGRIVENTER (CTENIDAE, LABIDOGNATHA"** sob a responsabilidade de **Profa. Dra. Maria Alice da Cruz-Höfling/Gabriela Mariotoni Zago** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 05 de Agosto de 2004.

C E R T I F I C A T E

We certify that the protocol nº **702-1**, entitled **"INVOLVEMENT OF THE PARACELLULAR TRANSPORT ROUTE IN THE BLOOD-BRAIN BARRIER BREAKDOWN INDUCED BY PHONEUTRIA NIGRIVENTER SPIDER VENOM"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on August 5, 2004.

Campinas, 05 e Agosto de 2004

Profa. Dra. Liana Verinaud
Presidente

Fátima Alonso
Secretária

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

TELEFONE 55 19 3788-6359
FAX 55 19 32893124