EDILENE SIQUEIRA SOARES

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Campinas, 2015

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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Orientadora: Dra. Maria Alice da Cruz-Höfling

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Estrutural do Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Mestra em Biologia Celular e Estrutural, na área de Biologia Tecidual.

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RESUMO

Cavéolas são estruturas em forma de vesículas presentes na membrana de diversas células, incluindo endoteliais, gliais e neurônios. As cavéolas situam-se em domínios da membrana ricos em colesterol, esfingolípides e têm como principal proteína estrutural a caveolina. As cavéolas atuam em diversas vias de sinalização incluindo as relacionadas ao tráfico de moléculas e neuroproteção. Nós investigamos se as cavéolas tomam parte na endocitose e aumento do tráfico vesicular trans-endotelial no cerebelo de ratos decorrente da quebra da barreira hematoencefálica (BHE) promovida pelo veneno da aranha *Phoneutria nigriventer* (PNV). Ratos Wista foram injetados com PNV e eutanaziados quando as manifestações tóxicas eram intensas (1 e 2 h), quando eram aparentes os sinais de melhora (5h) e na ausência de sinais de intoxicação (24 e 72 h). No endotélio, o envolvimento das cavéolas foi investigado através da expressão das proteínas caveolina-1 (Cav-1), Cav-1 fosforilada (pCav-1), dinamina-2 (Din2) e quinase da família Src (SKF) por Western Blotting (WB), imunofluorescência e microscopia eletrônica de transmissão. Possível mediação pela enzima eNOS foi avaliada por WB em baixa temperatura. O envolvimento dos astrócitos foi investigado pela expressão da Cav-3 e conexina-43 (Cx43) formadora de junções comunicantes. No cerebelo, o PNV aumentou a marcação anti-Cav-1 nos capilares das camadas granular e molecular e neurônios de Purkinje; aumentou a expressão Cav-1 e Din2, formadoras das cavéolas, ao mesmo tempo que diminuiu a expressão de SKF e pCav-1, envolvidas na internalização caveolar (1, 5 e 24 h). Inversamente, pCav-1 e SKF estavam superexpressas às 2 e 72 h, enquanto Cav-1 e Din2 estavam diminuídas. O PNV aumentou a metaloproteinase-9 da matriz, importante mediadora de quebra da BHE e aumentou a formação e o tráfego de vesículas. A análise de eNOS revelou desacoplamento (aumento de monômeros) nos períodos de envenenamento agudo (1-2 h) com progressivo re-acoplamento e super-expressão de dímeros às 72 h; ações essas correlacionadas a alterações nos níveis intracelulares de cálcio evidenciado pelo aumento na expressão de calmodulina e confimado pela localização de anti-calbindina-D28. Nos astrócitos, as cavéolas são formadas por Cav-3 e sua superexpressão é associada a doenças neurológicas. O PNV

aumentou Cav-3 em astrócitos GFAP positivos (astrogliose reativa) e Cx43 (1, 5 e 24 h) na vigência de edema citotóxico nos pés astrocitários e alterações nos contatos sinápticos axo-dendríticos e axo-somáticos. Em conjunto, os resultados revelam que: (a) a quebra da via transcelular da BHE pelo PNV é causa do aumento da endocitose via cavéolas; (b) no endotélio, os efeitos são mediados pelo sistema eNOS/NO; (c) a SKF ativa o sistema endocítico e aumenta transporte vesicular; (d) componentes da unidade neurovascular, como endotélio, astrócitos e neurônios estão intimamente envolvidos; (e) nos astrócitos, a dinâmica expressão de Cx43 e Cav-3 e o retorno aos níveis basais em paralelo com a ausência de sinais de intoxicação nos animais (72 h) dá evidências de que ambas as proteínas interagem na resposta astrocitária; (f) esses efeitos podem ser correlacionados aos sinais clínicos ao longo do envenenamento. Sugerimos que a ação em canais iônicos dos peptídeos neurotóxicos presentes no veneno de *Phoneutria nigriventer* estão no centro dos efeitos aqui relatados.

Palavras-chave: astrócitos, barreira hematoencefálica, cavéolas, óxido nítrico-sintase endotelial, veneno de aranha.

ABSTRACT

Caveolae are vesicles-shaped structures present in the membrane of different cells, including endothelial, glial and neurons cells. Caveolae are located in cholesterol and sphingolipids enriched membrane domains (*lipid-rafts* domains) and its main structural protein is the caveolin. The caveolae take part in several important cell signaling pathways, including those related with vesicle trafficking and neuroprotection. In this work, we investigated if caveolae mediate the endocytosis and the increase of the trans-endothelial vesicular trafficking in the rats' cerebellum following the blood-brain barrier breakdown (BBBb) induced by *Phoneutria nigriventer* spider venom (PNV). Wistar rats (*Rattus norvegicus*) were injected with PNV and euthanized when toxic manifestations were intense (1 e 2 h), when signals of recovery started (5 h), and in the absence of toxic signals (24 e 72 h). The involvement of caveolae in the BBB's endothelium was investigated through the expression of caveolin-1 (Cav-1), phosphorylated Cav-1 (pCav-1), dynamin-2 (Dyn2) and Src kinase family (SKF) proteins using Western Blotting (WB), immunofluorescence and transmission electron microscopy. A possible eNOS-mediation was evaluated using low-temperature WB. The involvement of astrocytes was investigated by measuring the expression of Cav-3 and Connexin-43 (Cx43), the latter the principal main component of gap-junctions. In the cerebellum, the PNV enhanced anti-Cav-1 labeling in capillaries of the granular and molecular layers and in Purkinje neurons; increased the expression of the caveolae-forming proteins, Cav-1 and Dyn2 (1, 5 e 24 h) at same time that reduced SKF and pCav-1 which have role in the caveolae internalization. By contrast, at 2 and 72, pCav-1 and SKF were up-regulated, whereas Cav-1 and Dyn2 were down-regulated. PNV increased matrix metalloproteinase-9, an important mediator of the BBBb, and increased formation and trafficking of vesicles. The eNOS analyses revealed enzyme uncoupling (increased monomers) in the periods of acute envenoming (1-2 h) with a progressive recoupling and overexpression of dimers at 72 h; those actions being correlated to intracellular calcium levels alterations evidenced by increased expression of calmodulin and confirmed by anti-calbidin-D28 localization. Astrocytes' caveolae are

formed by Cav-3 protein and its overexpression has been associated with neurological diseases. PNV increased both the Cav-3 expression in GFAP-positive astrocytes (reactive astrogliosis) and Cx43 (1, 5 and 24 h) and promoted astrocytes end-feet cytotoxic edema and alterations in axo-dendritic and axo-somatic synaptic contacts. Together the results revealed that: (a) the breakdown of the BBB's transcellular route is caused by an increase in endocytosis via caveolae; (b) in the endothelium those effects are mediated by eNOS/NO system; (c) the SKF activates endocytic system and enhances vesicular transport; (d) neurovascular unit components, such as endothelium, astrocytes and neurons are intimately involved; (e) in astrocytes, Cx43 and Cav-3 dynamic changes and their return to basal levels in parallel with absence of animals' toxic signals (72 h) provides evidence that both proteins interacts in the astrocyte response; (f) altogether the effects can be correlated with the progression of temporal signs of envenoming. The data allows suggesting that channel ions-acting neurotoxic peptides presented in *Phoneutria nigriventer* venom are in the center of the effects reported here.

Key-word: astrocytes, blood-brain barrier, caveolae, endothelium nitric oxide synthase, spider venom.

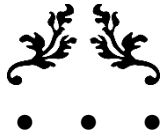
Sumário

"Ó mar salgado, quanto do teu sal
São lágrimas de Portugal!
Por te cruzarmos, quantas mães choraram,
Quantos filhos em vão rezaram!
Quantas noivas ficaram por casar
Para que fosses nosso, ó mar!

Valeu a pena? Tudo vale a pena
Se a alma não é pequena.
Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu."

Fernando Pessoa

Dedico este trabalho aos meus pais que do cais do porto tanto se sacrificaram para que eu tivesse um pedacinho do mar. Dedico também aos animais cujo sacrifício único nos move além do Bojador pois "navegar é preciso..."



"*The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka' but 'Hum, that's funny...*'" —Isaac Asimov (1920–1992)

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Lista de abreviações

Siglas em português

BHE barreira hematoencefálica
Cav-1 caveolina-1
Cav-3 caveolina-3
CaM calmodulina
Cx43 conexina-43
Din2 dinamina-2
ERO espécie reativa de oxigênio
JA junções de adesão
JO junções de oclusão
MB membrana basal
Phtx-1 phoneutria toxina 1
Phtx-2 phoneutria toxina 2
Phtx-3 phoneutria toxina 3
Phtx-4 phoneutria toxina 4
SNA sistema nervoso autônomo
SNC sistema nervoso central
SNP sistema nervoso periférico
Tx-3 toxina-3 de *Phoneutria nigriventer*

Siglas em inglês

BH4 tetrahydrobiopterin (Tetrahidrobiopterina)
eNOS endothelium nitric oxide synthase (Síntase de óxido nítrico endotelial)
FAD flavin adenine dinucleotide (Dinucleotídio de flavina-adenina)
FMN flavin mononucleotide (Mononucleotídio de flavina)
GJ gap-junctions (junções comunicantes)

IFN-γ interferon gama (Fator de interferência gama)

iNOS inducible nitric oxide synthase (Síntase induzível de óxido nítrico)

NADPH nicotinamide adenine dinucleotide phosphate (Fosfato dinucleotídio de nicotinamida-adenina)

nNOS neuronal nitric oxide synthase (Síntase de óxido nítrico neuronal)

NOS nitric oxide synthase (Síntase de óxido nítrico)

NO nitric oxide (Óxido nítrico)

NvU neurovascular unit (Unidade neurovascular)

pCav-1 phosphorylated caveolin-1 (Caveolina-1 fosforilada)

PNV *Phoneutria nigriventer* venom (Veneno de *Phoneutria nigriventer*)

TKR tyrosine kinase receptor (Receptor tirosina cinase)

SKF Src Family Kinase (Cinases da Família Src)

TGFβRI/II tumor growth factor beta receptor I/II (Receptor I/II para o fator de crescimento tumoral beta)

TNFα tumor necrotic factor alfa (Fator de necrose tumoral alfa)

TRPC1 transient receptor potential cation channel 1 (Receptor transiente potencial de canal catiônico 1)

TRPV4 transient receptor potential vanilloid channel 4 (Receptor transiente potencial de canal catiônico 4)

VEGF Vascular endothelial growth factor (Fator de crescimento endotelial de vaso)

I. INTRODUÇÃO

1. O cerebelo

Este órgão surge nos primeiros vertebrados como centro de controle da coordenação lateral do corpo de lampreias (os primeiros vertebrados marinhos com mandíbulas ósseas) (Butler and Hodo, 2005). Ao longo da evolução dos vertebrados, o cerebelo passou a participar do controle de diversas funções cerebrais, dentre elas temos o controle do movimento e da coordenação motora, a aprendizagem cognitiva, a memória, o comportamento e a linguagem (Broussard, 2014).

A organização tecidual do cerebelo permite dividi-lo em duas porções principais o 'corpo' cerebelar e o córtex cerebelar. O 'corpo' cerebelar é a região mais interna do órgão, sendo formado por fibras nervosas que constituem a substância branca. Já o córtex cerebelar é constituído pela substância cinzenta e pode ser dividido em três regiões de acordo com o tipo e organização celular que apresentam: camada granular, a camada de células de Pukinje e a camada molecular (Butler and Hodo, 2005).

A substância branca é rica em fibras nervosas aferentes e eferentes mielinizadas com nódulos de Ranvier (que facilitam a transmissão do impulso nervoso), essas fibras apresentam oligodendrócitos associados; nesta região encontramos ainda astrócitos do tipo fibroso que emitem prolongamentos longos e poucos ramificados que em sua porção terminal envolvem os nódulos de Ranvier (Wang & Bordey, 2008). Esta região constitui uma área para transmissão do impulso nervoso.

A substância cinzenta é rica em corpos celulares sendo a região responsável pelo processamento do impulso nervoso. As porções que compõe a substância cinzenta apresentam astrócitos protoplasmáticos, com prolongamentos curtos e muit ramificados. Tanto os astrócitos protoplasmáticos como os fibrosos envolvem e sustentam as sinapses e os vasos sanguíneos (Wang & Bordey, 2008).

A figura 1 é um esquema da organização celular neuronal nas diferentes camadas da substância cinzenta. A camada granular é a região mais interna do córtex cerebelar, em íntimo contato com a substância branca. Na camada granular estão situadas as fibras

musgosas oriundas do tronco (assim denominadas pelo formato de arbusto) e uma grande população de neuronios granulares, os menores neurônios do sistema nervoso central (SNC): as células granulares emitem axônios (fibras paralelas) para a camada molecular e ainda estabelecem sinapses com as fibras musgosas. Na camada granular encontram-se ainda as fibras trepadeiras e as células de Golgi (ou neurônios de Golgi), que estabelecem sinapses com as células de Purkinje. As células de Purkinje são os neurônios mais ramificados do cerebelo (Purves, 2010).

A camada molecular é a mais periférica do da substância cinzenta sendo recoberta pela pia mater. Ela é composta por uma quantidade menor de corpos de neurônios do que as outras camadas. Nela estão presentes a árvore dendrítica das células de Purkinje e as fibras paralelas das células granulares, além das células em cesto e estreladas. Os astrócitos nesta região são oriundos da glia radial e são denominados glia de Bergmann (Purves, 2010). A glia de Bergmann é essencial para a manutenção das células de Purkinje e das sinapses cerebelares sendo indispensável também durante a neurogênese deste órgão (Buffo e Rossi, 2013).

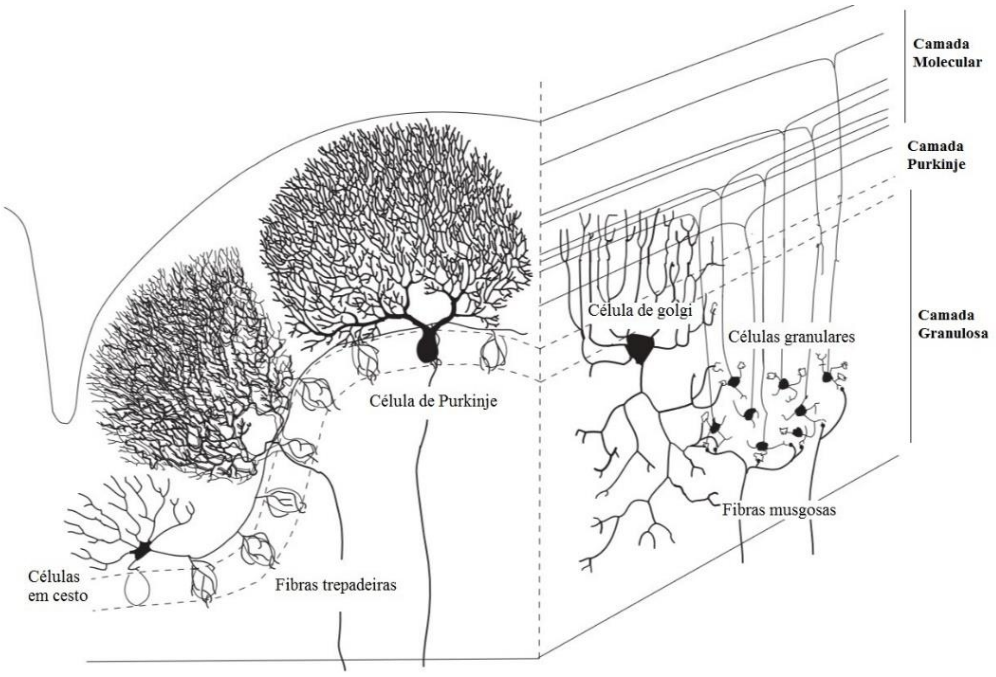

Figura 1: Córtex cerebelar humano. (Broussard, 2014). Camada molecular; com célula em formato de cesto. Camada de células de Purkinje (delimitada no tracejado). Camada granulosa: rica em diferentes tipos celulares e fibras: células ou neurônios granulares, fibras musgosas e trepadeiras, provenientes do tronco, e Células de Golgi.

2. A Barreira Hematoencefálica

Em condições normais, a barreira hematoencefálica (BHE) assegura a homeostase no ambiente neural, através do estabelecimento de uma barreira seletiva que permite o constante suprimento de oxigênio e glicose às células neurais e as protege de flutuações na composição do plasma sanguíneo ou da entrada de xenobióticos (Ballabh et al., 2004). A BHE é a estrutura responsável pelo rígido controle do tráfego bi-direcional de substâncias na interface sangue-cérebro; seus componentes, o endotélio, pericitos e astrócitos, em conjunto com neurónios e micrógia recebe a denominação de **unidade neurovascular** (Abbott, 2002).

2.1 A unidade-neurovascular

Composta pelos elementos da BHE (endotélio microvascular, a membrana basal, os pericitos e pelos pés astrocitários (Figura 2)) mais neurônios e micróglia, a unidade neurovascular (Wolburg et al., 2009) em concerto colabora na manutenção da homeostase no microambiente cerebral. O conjunto forma uma seletiva barreira contra substânicas indesejáveis ao parênquima cerebral, ajustando as flutuações no plasma sanguíneo e conectando assim a atividade nervosa e a função vascular (El Ali et al., 2014).

Os capilares sanguíneos contínuos e sem fenestrações formam a base estrutural da BHE. Além disso, as junções celulares entre as células endoteliais, denominadas de junções de oclusão e zônulas de adesão, são reforças de forma a impedir o tráfego paracelular de substâncias (Wolburg e Lippoldt, 2002); a via transcelular é altamente seletiva e regula o tráfego de vesículas e moléculas (Rubin e Staddon, 1999). Inúmeras moléculas são responsáveis pelo controle do tônus vascular e participam ativamente da manutenção de uma barreira endotelial seletiva.

A membrana basal (MB) é sintetizada pelas células endoteliais, pelos pericitos e pelos astrócitos. É uma estrutura de interface entre o endotélio e o tecido neural adjacente; a MB é formada por laminina, integrina, colágeno IV, fibronectina e ancora proteínas integrais de membrana das células endoteliais) (Persidsky et al., 2006). A MB auxilia na interface vascular e na função de barreira. Além disso, estudos recentes relacionam disfunções da membrana basal com patologias que acometem o insterstício do tecido neural, como doenças neurodegenerativas (Morris et al., 2014).

Os pericitos são as células menos estudadas da unidade neurovascular, seu papel na formação e manutenção da barreira hematoencefálica vem sendo aos poucos elucidado (Kamouchi et al., 2012). Essas células são capazes de responder a estímulos de outros componentes da BHE para diferenciação em novas células endoteliais e estão sendo relacionadas ao controle da sinalização de componentes do sistema imune ao nível da barreira (Hurtado-Alvarado et al., 2014).

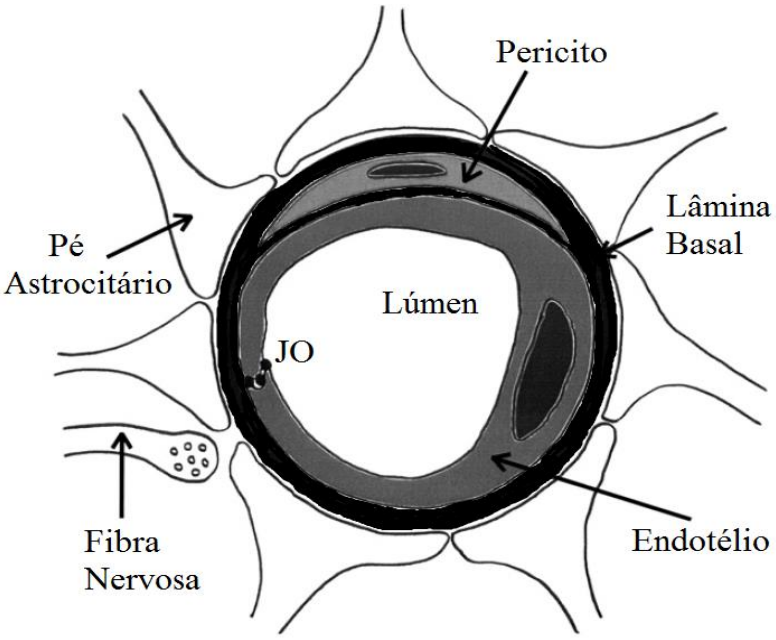

Figura 2: Imagem ilustrativa dos componentes da BHE que formam a unidade gliovascular: as células endoteliais com junções intercelulares reforçadas (junções de oclusão (JO)), circundadas pela membrana basal adjacente, os pericitos e os pés astrocitários, que medeiam a interação dos neurônios ou da fibra nervosa com os microvasos. Modificado de Stam, 2010.

Os astrócitos envolvem >99% da barreira endotelial (Hawkins e Davis, 2005); constituem uma região de interposição entre o parêquima cerebral e a circulação sanguínea. Essas células gliais são chamadas de astrócitos perivasculares e envolvem os capilares endoteliais através de expanções distais de seus prolongamentos denominadas pés-astrocitários. Além de terem papel na formação e manutenção da BHE, os astrócitos também são responsáveis pela nutrição e suporte estrutural dos neurônios (Abbott, 2006), pela manutenção e controle das sinapses neuronais (Perea et al., 2014; Covelo e Araque, 2015), além de diversas outras funções (Para revisão ver: Verkhratsky e Butt, 2007).

De modo geral, os astrócitos constituem uma população celular muito variada apresentando um formato e uma função característicos à região em que se encontram e o papel que desempenham. Destacam-se os atrócitos protoplasmáticos e fibrosos que ocorrem na substância cinzenta e branca, respectivamente e a glia de Bergmann um tipo especial de glia radial encontrada apenas no cerebelo. Devido a toda sua complexidade funcional compreendem cerca de metade de todas as células da glia encontradas no cérebro. Os astrócitos se intercomunicam e se comunicam com outras células da glia através de junções comunicantes ou "gap junctions" (Verkhratsky e Butt, 2007).

2.2. Vias de acesso ao SNC

A BHE se apresenta como uma barreira dinâmica capaz, em condições hígidas, de modular a interferência externa e manter o ambiente interno próprio para a função neural. A perda da integridade física ou fisiológica da BHE está relacionada a muitas patologias do SNC que comprometem a função neural exercida pelos neurônios e pelas células da glia (*e.g*. doenças neurodegenerativas) (Weiss et al., 2009). Muito embora a BHE seja essencial ao bom funcionamento do cérebro, ela cria dificuldades de acesso ao parênquima cerebral de substâncias terapêuticas para o tratamento de doenças e tumores. A permeabilização experimental da BHE, isto é, sua manipulação possibilita a elucidação de seus mecanismos intrínsecos.

Distinguem-se de modo geral duas vias de acesso à entrada de substâncias no SNC em condições patológicas: a primeira delas é através da **via paracelular,** com desestabilização de proteínas que constituem as junções celulares, a outra é através do aumento no tráfego de moléculas pela **via transcelular**, com participação de proteínas envolvidas na endocitose e no influxo de moléculas (Chen e Liu, 2012).

2.2.1 A via paracelular

No contato de duas células endoteliais adjacentes ocorre formação de junções intercelulares. As junções representam uma barreira ao fluxo de substâncias entre duas células e permitem a sinalização necessária para formação de nichos celulares que irão originar os diferentes tecidos e órgãos (Lecuit e Lenne, 2007).

Nas células endoteliais dos vasos que constituem a BHE, as junções intercelulares são modificadas para restringir de forma eficiente o fluxo de moléculas por essa via, a via paracelular. Duas classes de junções se destacam nesta função, as junções de oclusão (JO) e as junções de adesão (JA). As JO são formadas por proteínas integrais de membrana, como a ocludina e a claudina e por proteínas acessórias, como as proteínas da zônula de oclusão (ZO-1, ZO-2, ZO-3, entre outras) e a cingulina essas últimas citoplasmáticas. As JA apresentam proteínas denominadas caderinas conectadas ao citoesqueleto celular através de proteínas de ligação à actina, destacando-se nesta função as cateninas e as vinculinas. A ligação extracelular das moléculas de caderinas depende da ligação com os íons cálcio sendo uma junção cálcio-dependente. A Figura 3 resume a estrutura das junções nas regiões de contato entre duas células (Dejana et al., 2009; Wolburg e Lippoldt, 2002).

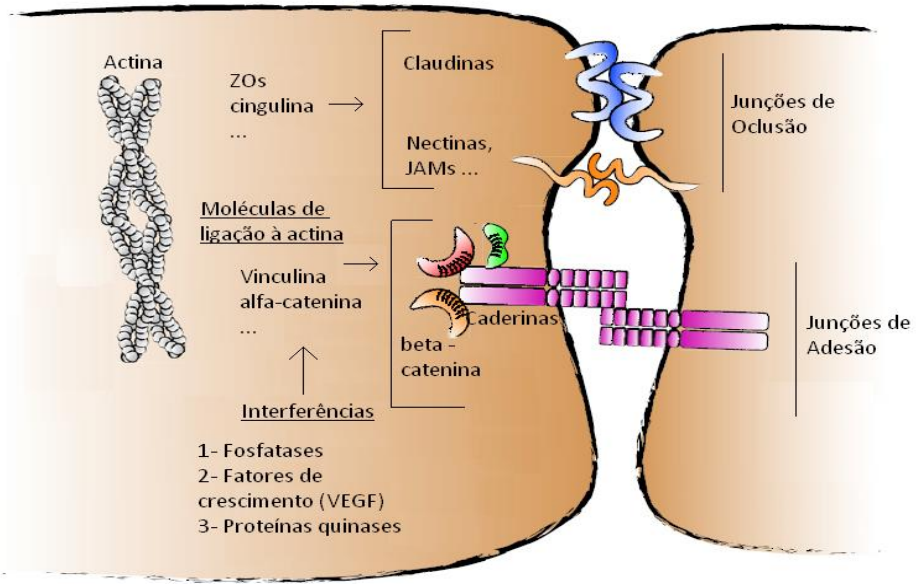

Figura 3: Imagem ilustrativa das junções de oclusão e adesão presente no endotélio dos vasos. Note a presença das proteínas citoplasmáticas de ligação ao citoesqueleto, esta ligação pode ser afetada pelos fatores listados (1-3). As junções intercelulares formam uma barreira ao tráfego de moléculas entre duas células vizinhas e são altamente especializadas no endotélio. Modificado de Dejana et al., 2009.

Outro tipo de junções celulares que tem papel chave na fisiologia do SNC são as junções comunicantes (*gap-junctions*, GJ). As GJ tem papel essencial na sinalização célula-célula e célula-matriz e na fisiologia do SNC (Cheung et al., 2014; Eugenin et al., 2012), sendo cruciais na integração entre neurônios e astrócitos (Rouach et al., 2002). Formadas

por proteínas hemi-canais denominadas conexinas, as GJ permitem a passagem de substâncias, solutos e moléculas sinalizadoras de até 1 kDa ao formar verdadeiros poros na membrana. As conexinas são as proteínasque sustentam e formam esses canais comunicantes intercelulares (Para Revisão ver: Oshima, 2014).

2.2.2. A via transcelular

No cérebro a via transcelular é altamente seletiva e seus mecanismos de transporte celular ocorrem de forma bidirecional, removendo substâncias (através de efluxo) ou internalizando moléculas de interesse (por influxo). Participam desta função diversas proteínas alvo, que tendo ou não afinidade pelo substrato a ser transportado medeiam seu transporte.

As proteínas responsáveis pelo estabelecimento de um fluxo pela célula podem estar relacionadas à transposição de nutrientes como a glicose (por influxo) (Persidsky et. al, 2006), ou com a remoção de moléculas que estejam no citoplasma endotelial ou em astrócitos, neste caso, destacam-se as proteínas de efluxo (como por exemplo, a poliglicoproteína (P-gp) (Higgins, 1992). O tráfego de vesículas pela célula é restrito sendo controlado por receptores de membrana e carreadores (Rubin e Staddon, 1999). Várias formas de transcitose podem ocorrer no endotélio, como a endocitose (*e.g*. para entrada de HRP), a endocitose adsortiva (*e.g.* para entrada de albumina catiônica) e a endocitose mediada por receptores (*e.g.* para entrada de insulina) (Broadwell et al., 1989). A Figura 4 resume os mecanismos principais de entrada de substâncias e células no parênquima cerebral em condições normais e patológicas (Abbott, 2013).

2.2.2.1. As cavéolas

As cavéolas constituem um tipo de estrutura engajada em processos de endocitose mediada por receptor. São de extrema importância para a fisiologia da BHE. Cavéola (do latim *caveolae*) significa pequena caverna, de fato o diâmetro aproximado das vesículas caveolares é pequeno e está em torno de 50-100 nm (Sandvig et al., 2008). Palade em 1953 descreveu e cunhou o termo *caveolae* ao observar por microscopia eletrônica de transmissão, pequenas invaginações em formato de balão na membrana de células endoteliais de capilares sanguíneos. Na realidade, hoje sabe-se que o formato e até a

composição das cavéolas varia dependendo de diferentes fatores, como: o tipo celular onde são encontradas, as proteínas estruturais e acessórias presentes nos seus domínios e também pela forma de processamento do material a ser analisado, neste contexto assumem formatos tubulares, de rosetas (com várias vesículas fundidas) ou mesmo o tradicional formato de balão (Razani et al., 2002; Lossinsky and Shivers 2004) (Figura 5).

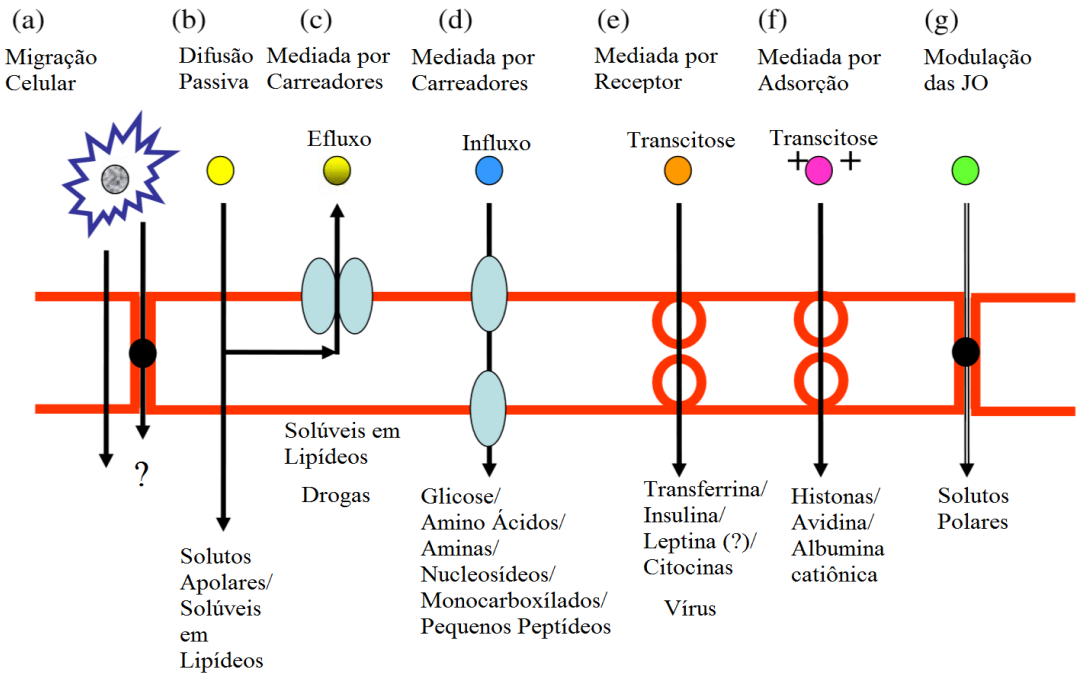

Figura 4: Possíveis vias de acesso ao SNC. **(a)** Representa a entrada pela via paracelular de células através da migração celular. **(b)** A Difusão passiva de substâncias ocorre através da membrana e permite a passagem de substâncias pequenas apolares ou solúveis em lipídeos. A entrada mediada por carreadores pode feita por duas vias: efluxo **(c)** com bombas que removem substâncias da célula: como drogas, e influxo **(d)** com carreadores que permitem a passagem de moléculas de interesse como: glicose e pequenos peptídeos. **(e)** A transcitose mediada por receptor permite a entrada e/ou sinalização de diversas moléculas, como insulina e até mesmo vírus. **(f)** A transcitose mediada por adsorção é a entrada de moléculas induzida pela diferença de carga. **(g)** Passagem de moléculas mediada pela modulação por junções de oclusão (JO). Modificado de Abbott, 2013.

As cavéolas formam invaginações na membrana plasmática em regiões ricas em esfingolipídios e colesterol (denominadas *lipid rafts*) e apresentam proteínas estruturais de suporte. Múltiplas funções lhes são atribuídas, dentre elas, sua contribuição para a homeostase de lipídios (Fielding e Fielding, 2001), participação na sinalização intracelular

(Frank et al., 2003; Williams e Lisanti, 2004a; Luoma et al., 2008; Stern et al., 2010; Sowa et al., 2012), no tráfego de vesículas pela célula (Parton e Richards, 2003; Frank et al., 2009) e, mais recentemente, na sinalização e controle do estresse mecânico de membrana (Nassoy e Lamaze, 2012). As cavéolas desempenham papel chave no tráfego vesicular por endocitose, transcitose e potocitose (Pelkmans e Helenius, 2002; Williams e Lisanti, 2004b). Essas regiões da membrana celular são bastante estáveis devido a abundância em moléculas de colesterol e esfingolipídios. O arcabouço das cavéolas é formado por duas famílias de proteínas integrais transmembranas denominadas caveolinas e mais recentemente descobertas as *cavin* ou cavinas (tradução livre do autor) (Hansen e Nichols, 2010). As caveolinas e as cavinas formam em conjunto o complexo caveolar, sendo as cavéolas compostas por repetições desses complexos (Ludwig et al., 2013).

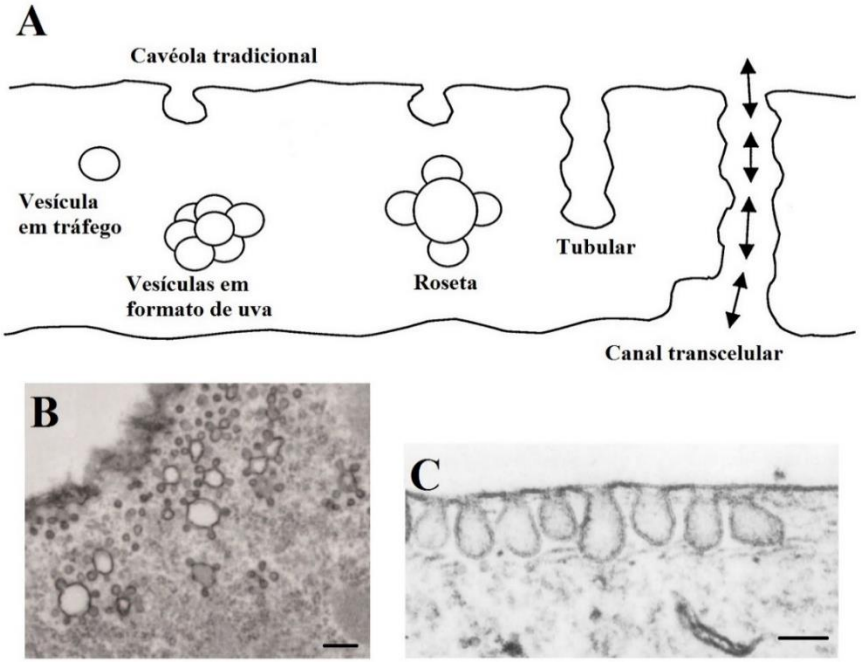

Figura 5: Representação dos diferentes formatos das cavéolas. (**A**) Representação da morfologia das cavéolas: vesículas em tráfego, vesículas em formato de uva, formato de roseta, formato tubular e fomação de canal transcelular. (**B**) Imagem de cavéolas em formato de roseta arranjo encontrado em adipócitos. (**C**) Imagem de cavéolas tradicionais na membrana de células endoteliais. Barra de calibração de 100 µm. NB: As vesículas revestidas por proteínas caveolina são menos elétron-densas do que vesículas revestidas por clatrina (via clássica para endocitose). Modificado de Razani et al., 2002; Parton e Simons, 2007; Rothberg et al., 1992.

As primeiras proteínas estruturais das cavéolas a serem descritas foram as caveolinas (Rothberg et al., 1992), e de fato, as caveolinas são as moléculas estruturais mais importantes das cavéolas. Seus domínios terminais são voltados para o citoplasma celular e sua forma ativa na membrana só ocorre através da união de duas moléculas de caveolina pela porção amino-terminal conectada à membrana plasmática (Williams e Lisanti, 2004b). A inserção das caveolinas na membrana celular se dá como ilustra a figura 6A. Essas proteínas são formadas por um domínio terminal carboxila livre, seguido de uma porção terminal carboxila ligada à membrana plasmática, um domínio transmembrana, seguido de uma porção amino-terminal ligada à membrana onde se encontra o sítio de polimerização e por fim uma porção amino terminal livre (Figura 6B).

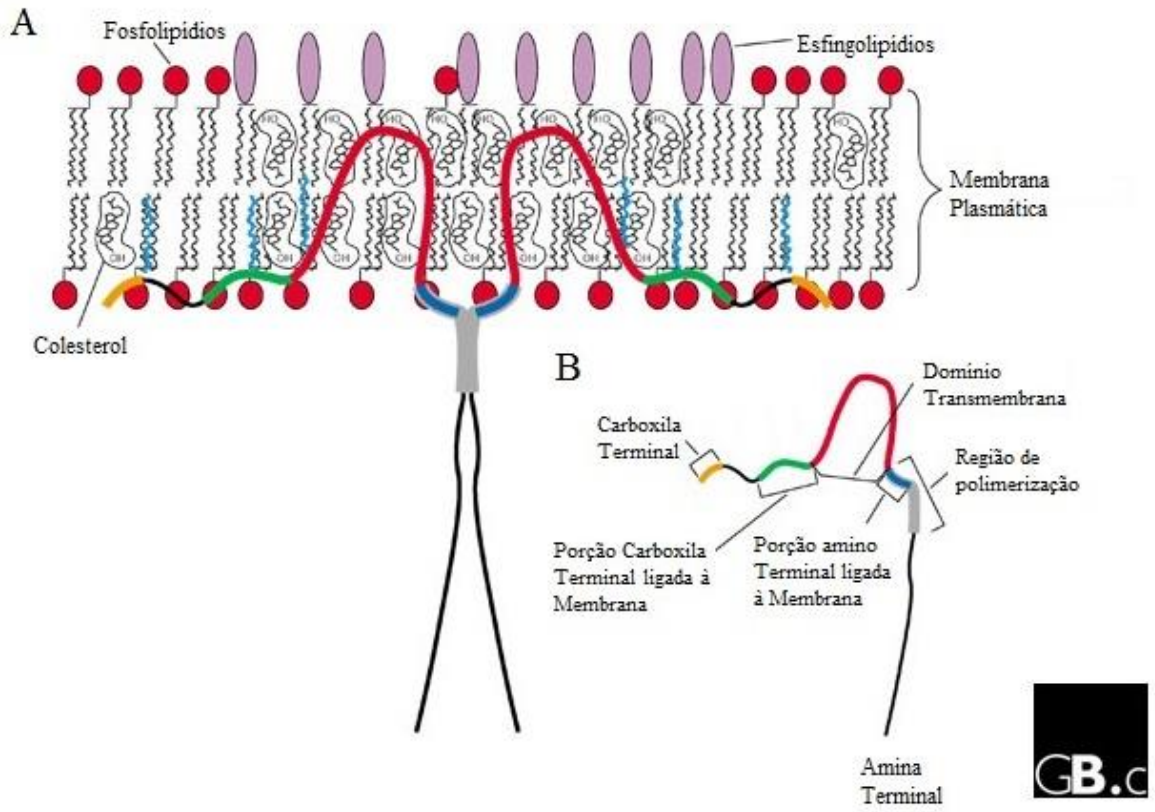

Figura 6: (A) Imagem ilustrativa da inserção das proteínas caveolinas na membrana celular, note que estas proteínas ocorrem em pares na membrana e suas porções terminais são voltadas para o citoplasma da célula. **(B)** Imagem ilustrativa da estrutura plana da caveolina isolada, formada por cinco domínios: dois terminais, dois ligados à membrana e um domínio transmenbrana. Modificado de Williams and Lisanti, 2004b.

As proteínas da família das caveolinas são formadas por três membros: caveolina-1 (Cav-1), caveolina-2 (Cav-2) e caveolina-3 (Cav-3). O que caracteriza essas proteínas como pertencentes à mesma família é uma sequência conservada de aminoácidos "FEDVIAEP" (na ordem temos: Fen/Glu/Asp/Val/Iso/Ala/Glu/Pro) referida como a assinatura motivo das caveolinas (Razani et al., 2002). No cérebro encontramos em abundância a Cav-1 em células endoteliais e nos pericitos dos microvasos (Virgintino et al., 2002) e a Cav-3 nos pés astrocitários (Ikezu et al., 1998); Cav-2 aparece complexada com ambas Cav-1 e Cav-3 (Cameron et al., 1997). Do que se sabe até o momento, das três proteínas apenas caveolina-1 e caveolina-3 são essenciais para a formação caveolar na membrana (Stan, 2005).

A proteína Cav-1 é a proteína integral da membrana caveolar mais abundante de sua família e tem sido descrita como um marcador seletivo para as regiões das cavéolas (Para revisão: Razani et al., 2002). Cav-1 apresenta duas isoformas (α e β) estruturalmente distintas (Scherer et al., 1995) sendo que a isoforma alpha representa cerca de 90% da Cav-1 expressa no cérebro (Ikezu et al., 1998). Cav-1 é encontrada em diferentes tipos celulares inclusive no endotélio cerebral, aparecendo como elemento chave da permeabilidade da BHE (Nag et al., 2007, 2011) através por exemplo da indução da expressão de metaloproteinases de matriz (MMP). No cérebro, a MMP9 é mobilidada durante episódios de injúria sendo responsável pela degradação da matriz extracelular contribuindo para a permeabilidade da BHE (Muradashvili et al., 2014).

A proteína Cav-2 tem distribuição similar à Cav-1 ocorrendo juntas na membrana celular, apesar de consideradas marcadoras de cavéolas e fazerem parte de seu arcabouço estrutural, estudos demonstraram que Cav-2 não é essencial para a formação caveolar no endotélio (Stan, 2005). Por outro lado, animais *knock-out* para Cav-2 apresentam significativas alterações pulmonares, sendo que o papel de Cav-2 nos pneumócitos vem sendo abordado, além disso, Cav-2 também tem presença marcante em cavéolas de adipócitos (Zhu et al., 2010).

A Cav-3 é expressa preferencialmente no tecido muscular (Tang et al., 1996). No cérebro foi encontrada em astrócitos (Cameron et al., 1997; Ikezu et al., 1998) participando da formação de plataformas de sinalização na membrana celular astrocitária, sendo que sua regulação negativa foi relacionada a alterações patológicas na expressão de proteínas das junções comunicantes (*e.g.* conexina-43) (Liao et al., 2010). Em contraste, o aumento na

expressão de Cav-3 é associado com agravamento de doenças neurodegenerativas sendo considerado um mediador patológico nessas doenças (Nishiyama et al., 1999). As poucas funções atribuídas à Cav-3 nos astrócitos se deve ao pouco conhecimento que se tem de seu papel neste tipo celular.

Já foram descritos até o momento 4 membros para a família das cavinas, todos como tendo função auxiliar ou regulatória das cavéolas. De fato, as 'cavinas' são consideradas proteínas adaptadoras das cavéolas (Sowa, 2012). Cavina-1 é um fator de transcrição essencial para a formação das proteínas caveolinas. Cavina-2 pode estar relacionada ao formato e curvatura das vesículas e tem papel importante na sua formação. Cavina-3 pode estar relacionada à função endocítica das cavéolas sendo mobilizada durante o fenômeno de desprendimento das vesículas da membrana celular. Por último, Cavina-4 tem papel importante nas cavéolas presentes no tecido muscular cardíaco e pode estar relacionada ao controle e à mobilizacão do cálcio presente no retículo endoplasmático (Hansen e Nichols, 2010; Briand et al., 2011).

2.2.2.2. Endocitose

As cavéolas foram inicialmente descritas com uma possível rota alternativa para a endocitose celular (Palade, 1953; Yamada, 1955; Sandvig et al., 2008). A Cav-1 é um substrato das proteínas tirosina quinase da família Src (*Src kinase family*, SKF) e sua fosforilação no resíduo 14 de tirosina (pCav-1) dá início à internalização das invaginações vesiculares que constituem as cavéolas (Fagerholm et al., 2009). As SKF participam ainda da permeabilidade vascular através do controle que exercem sobre os componentes das vias paracelular e transcelular (Hu et al., 2008).

Além da fosforilação da Cav-1 por proteínas SKFs, outra proteína é necessária para a formação/desprendimento das vesículas revestidas por caveolinas: a dinamina. As proteínas dinaminas constituem uma família multigênica de GTPases já descritas como constituintes das vesículas revestidas por clatrina (via clássica de endocitose). Essas proteínas são responsáveis pela formação do gargalo das cavéolas e estão diretamente relacionadas ao desprendimento das vesículas da membrana. Seu papel na internalização das cavéolas vem sendo descrito e nesta função destaca-se a dinamina-2 (Din2), cuja fosforilação é necessária para a internalização caveolar (Henley et al., 1998).

Os mecanismos iniciais que promovem a ativação da via para internalização das cavéolas não estão totalmente compreendidos. Um dos fatores determinantes para a formação das vesículas nas células do endotélio pode estar relacionado à função de plataforma de sinalização desempenhada pelas cavéolas. As cavéolas oferecem uma região estável para a inserção de diversos receptores celulares, sendo a própria Cav-1 relatada como responsável por interagir e regular tais receptores (Figura 7). Vários receptores estão presentes nas cavéolas: o receptor 2 (R2) para o fator de crescimento endotelial de vaso (*vascular endothelium growth factor*, VEGF), também denomin Flk-1, canais de cálcio (TRPC1 e TRPV4, ambas siglas em inglês) e a enzima sintase endotelial para óxido nítrico (*endothelial nitric oxide synthase*, eNOS) (Sowa, 2012).

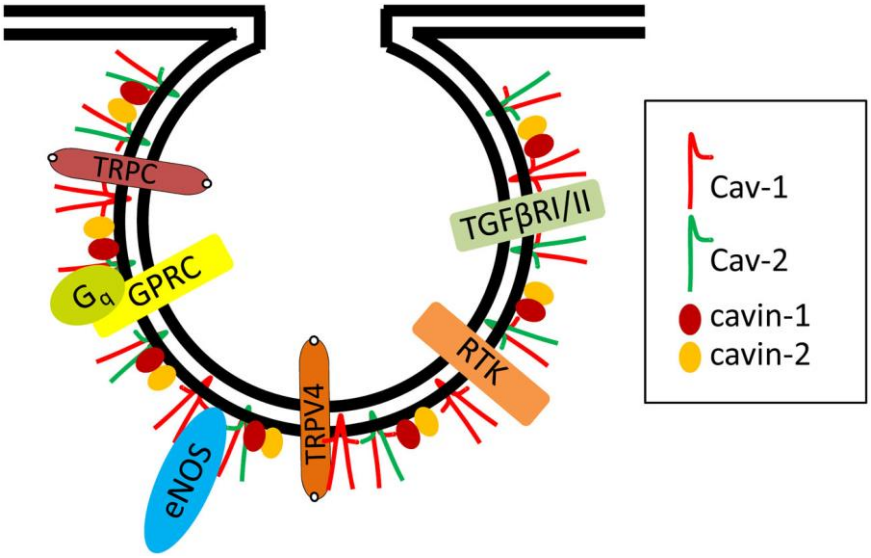

Figura 7: Imagem ilustrativa de uma região de *lipid rafts*, região especializada da membrana celular onde há uma maior ocorrência de colesterol e esfingolipídios o que confere uma maior estabilidade à região. Nesta figura revestindo a vesícula estão Cav-1 e Cav-2 e as proteínas acessórias cavina-1 e -2. Na figura temos seis receptores de membrana compartimentalizados nas regiões das cavéolas, TRPC1 e TRPV4, ambos são receptores ou canais de cálcio; receptores GPRC (*e.g.* receptor de bradicinina, receptor muscarínico ou receptor endotelial); eNOS, enzima formadora de NO endotelial; RTK, receptor 2 do VEGF; TGFβRI/II, receptores 1 e 2 do TGFβ (transforming growth factor beta) (Extraído de Sowa et al., 2012).

2.2.2.3. Controle da sinalização celular

Dentre os receptores intracelulares compartimentalizados nas cavéolas destaca-se a enzima eNOS. A eNOS faz parte de uma família de enzimas de síntese do óxido nítrico

(*nitric oxide syntase*, NOS) presente em diversas células após diferentes estímulos. As três isoformas de NOS em mamíferos são: NOS I ou nNOS preferencialmente expressa em neurônios, NOS II ou iNOS é a forma induzível de NOS expressa em diferentes tipos celulares e que participa ativamente da ativação de respostas pró-inflamatorias e a NOS III ou eNOS que é expressa principalmente no endotélio (Förstermann e Li, 2011).

O NO é um importante sinalizador intracelular e neurotransmissor. No endotélio o NO é uma molécula bioativa que atua no controle da vasodilatação, na resposta ao estresse mecânico (Rath et al., 2009), na sinalização celular, na adesão de leucócitos e na inibição da agregação plaquetária (Qian e Fulton, 2013). O NO controla também a pressão arterial através da promoção da vasodilatação e da remodelação vascular com participação na angiogênese (Minshall et al., 2003). No endotélio cerebral o NO é essencial para a manutenção da homeostase do parênquima cerebral ao controlar a função vascular (Gulati et al., 2014).

A produção de NO pelas NOS ocorre através da oxidação de L-arginina resultando em NO e L-citrulina (Michel e Vanhoutte, 2010). A síntese de NO se dá através do estabelecimento de um fluxo de elétrons entre o domínio oxigenase de um monômero da enzima até o domínio redutase do outro monômero; assim a enzima está completamente ativada quando ocorre na forma de dímeros (Panda et al., 2002).

No caso da eNOS, a ativação requer que a enzima esteja ligada a vários mediadores celulares (Figura 8), com detaque para a proteína calmodulina (CaM). A CaM é uma proteína ligante de cálcio, essencial para a função da enzima eNOS, pois responde à alterações nos níveis de cálcio citoplasmático e se liga a eNOS permitindo sua dimerização e estabilização (Persechini et al., 2013), além de impedir a ligação de eNOS com Cav-1 (ligação inibitória) (Michel e Vanhoutte, 2010).

Em condições normais, eNOS produz NO através do fluxo de elétrons entre seus monômeros ligados, porém diversas condições patológicas podem induzir alterações estruturais na enzima e promover um fenômeno denominado desacoplamento de eNOS, no qual a proporção de monômeros ultrapassa a formação de dímeros e o conjunto de enzimas fica inativado (Masano et al., 2008; Yang et al., 2009; Sabri et al., 2010). Os monômeros ainda realizam um gradiente de elétrons que flui do domínio oxigenasse para o domínio redutase, porém como a enzima está monomérica e não ligada a outro monômero, o

substrato da oxidação deixa de ser a L-arginina e passa a ser o NADPH sozinho (Rafikov et al., 2011). O desacoplamento de eNOS tem como consequência direta a produção de superóxido (um tipo de espécie reativa de oxigênio, ERO) em detrimento da produção de NO. Portanto, o desacoplamento é uma disfunção da enzima eNOS (Förstermann e Münzel, 2006).

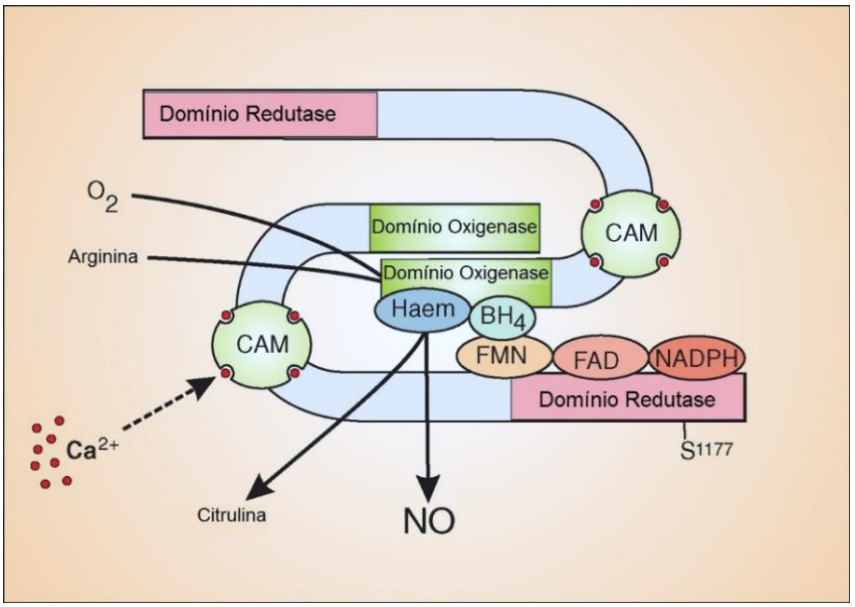

Figura 8: Esquema representativo da forma ativada (dimérica) da enzima eNOS complexada com seus principais ativadores: CaM ou CAM (Calmodulina), motivo haem, FMN (monucleotídio de flavina), FAD (dinucleótido de flavina-adenina), NADPH (fosfato dinucleótido de nicotinamida-adenina) e BH4 (Tetrahidrobiopterina) (todas siglas em inglês traduzidas). Note que ambos os dímeros são formados por uma região denominada domínio oxigenase e outra região denominada domínio redutase ambas essenciais para a formação de NO. Note ainda que o cálcio intracelular ativa CaM e permite que na presença de oxigênio (O_2) e Arginina haja formação de citrulina e NO. Modificado de Berridge, 2012.

3. O envenenamento acidental

Os animais com peçonha são aqueles que produzem toxinas (misturas de moléculas de baixo peso molecular, peptídeos e proteínas) que afetam os tecidos biológicos. Esses animais geralmente possuem estruturas próprias para a inoculação destas substâncias em outros organismos e este comportamento está relacionado à captura de alimento através da imobilização de presas, ou mesmo como recurso para defesa quando se sentem ameaçados (Escoubas et al., 2008).

Anualmente são relatados diversos incidentes envolvendo o contato acidental de humanos com animais peçonhentos. A gravidade do acidente depende de diversos fatores, como a espécie responsável pelo acidente, as condições de saúde do paciente, o local de inoculação do veneno, dentre outras. Nos casos mais graves, além do desconforto no local da picada, a vítima pode apresentar alterações decorrentes da ação sistêmica da peçonha e em alguns casos o quadro clínico pode evoluir para óbito. Neste sentido, o envenenamento acidental ganhou destaque na comunidade médica e culminou com estudos envolvendo o tratamento das vítimas e o desenvolvimento de soroterapia. Acidentes com animais peçonhentos predominam em países tropicais, e as condições clínicas a que ficam sujeitas as vítimas são considerados pela Organização Munidal de Saúde como negligenciadas.

Vital-Brazil iniciou já no fim do século XIX, com a criação do Instituto Butantã, a produção de soros no Brasil; a partir do início do século XX o Instituto foi declarado instituição autônoma e nesta mesma época a produção de soro antiofídico foi iniciada (Instituto Butantan). Porém, para atender a demanda nacional era necessário o aumento na produção de soros, assim no início da década de 80 casos de óbito por falta de assistência envolvendo acidentes ofídicos levaram a mobilização do Ministério da Saúde que se tornou órgão regulador da produção e da distribuição de soros. Antes disso, o abastecimento das instalações médicas era feito através da parceria com indústrias privadas (Cardoso et al., 2003). Segundo o Ministério da Saúde, os acidentes envolvendo animais peçonhentos são mais comuns no verão, de modo que ocorre neste período um alerta das unidades de saúde em prol da prevenção e mesmo tratamento dos acidentes (Brasil, 2011). Outro fator que pode desencadear um surto de acidentes é a interferência humana no habitat natural dos animais peçonhentos ou mesmo a própria irradiação destes para ambientes domésticos.

Nem todos os casos de exposição ao animal peçonhento requerem soroterapia, entretanto em casos de envenenamento acidental grave o tratamento é essencial para assegurar o bem-estar do paciente. A identificação do animal por parte das vítimas ou mesmo a coleta do animal e encaminhamento com o paciente a unidade de saúde facilitam a escolha do antissoro. Porém, nem sempre é possível ao paciente ou aos acompanhantes identificarem o animal ou mesmo levá-lo às unidades de saúde. Na perspectiva de vigilância epidemiológica, os sintomas característicos de cada envenenamento foram sistematizados a partir da observação clínica das vítimas com o intuito de facilitar a identificação do gênero causador do acidente e a possível evolução dos sintomas. Dessa forma, as alterações fisiológicas e celulares desencadeadas pelas peçonhas dos diferentes animais começaram a ser estudadas com grande interesse médico-terapêutico.

Ou seja, além da preocupação com a Saúde Pública, as peçonhas se mostraram potenciais ferramentas para desvendar processos fiológicos e fisiopatológicos, uma vez que podem mimeticar eventos celulares. A identificação da estrutura-função das toxinas componentes dessas peçonhas permitiram o avanço no conhecimento de fenômenos morfofisiológicas ou celulares de interesse da comunidade médico-científica. Atualmente, investiga-se o potencial farmacológico e terapêutico para o tratamento de doenças das moléculas biologicamente ativas presentes em diferentes peçonhas de origem animal (para revisão ver: Lewis e Garcia, 2003).

No Brasil, destacam-se as pesquisas envolvendo a peçonha de serpentes (principalmente família Viperidae), escorpiões, himenópteros (abelhas e formigas) e **aranhas.**

3.1. O caso das aranhas

As aranhas estão entre os invertebrados mais bem-sucedidos do mundo atual com cerca de 40 000 espécies descritas (Ruppert et al., 2005). Esses animais se alimentam basicamente de insetos ou outros artrópodes, sendo a captura da presa feita através da caça (caçadoras ou errantes) ou através de teias de seda que as próprias aranhas tecem (construtoras de teia). Após a captura, a presa é imobilizada com o uso da peçonha produzida pela aranha, sendo que todas as aranhas venenosas apresentam glândulas de veneno (Rash e Hodson, 2002). Em geral estas glândulas estão associadas às quelíceras

para inoculação imediata da peçonha após a captura. Em geral são substâncias neurotóxicas que imobilizam ou matam a presa.

Os venenos das aranhas são compostos de moléculas bioativas que passaram por uma longa seleção evolutiva apresentando grande diversidade de componentes com ação sobre os tecidos biológicos (Vassilevski et al., 2009). Apesar da eficiência da peçonha, poucos gêneros de aranhas representam uma ameaça aos humanos, isso porque, esses animais produzem venenos em quantidades pequenas, que ao serem inoculados, não acarretam alterações significativas à saúde ou até devido ao tamanho reduzido da própria aranha, sendo a pequena quelícera incapaz de penetrar a camada espessa da pele. Ainda em raras exceções a aranha pode não apresentar a glândula de veneno próximo à quelícera o que torna a peçonha inócua (Brasil, 2001; Lucas et al., 2003).

Araneísmo é o nome dado aos acidentes causados por aranhas. Segundo a Organização Mundial de Saúde (OMS) três gêneros e uma família de aranhas destacam-se como importantes em termos de Saúde Pública por causarem acidentes que tem morbidade ou letalidade. São aranhas pertencentes à Ordem Araneae, a saber, *Loxosceles*, *Phoneutria*, *Latrodectus* e a família *Theraphosidae*, popularmente conhecidas como aranha-marrom, armadeira, viúva-negra e tarântula, respectivamente. No Brasil, apenas os três primeiros gêneros representam ameaças à Saúde Pública (Lucas, 2003).

Os acidentes causados por *Loxosceles* e *Latrodectus* envolvem o contato acidental com essas aranhas, sendo frequente o acidente doméstico em que a vítima espreme a aranha contra o corpo; esses gêneros não costumam apresentar indivíduos agressivos e só picam os humanos em casos de contato como o acima citado (Lucas, 2003).

Em contraste, o gênero *Phoneutria* (Araneae, Ctenidae) apresenta indivíduos bastante agressivos que ao se sentirem ameaçados atacam o seu opressor. Nessas ocasiões, colocam-se em posição de ataque bastante característica: se posicionam sobre as patas posteriores e erguem as patas dianteiras, em seguida expõem os palpos e as quelíceras deixando visíveis os ferrões de inoculação do veneno, esta posição é característica para o ataque (Figura 9) sendo responsável pelo nome popular de armadeira que essa aranha recebe (Brasil, 2001).

3.1.1. A aranha armadeira

No Brasil, até o momento são descritas cinco espécies principais para o gênero *Phoneutria*, dentre elas três são destaque por ocasionarem acidentes em humanos, *Phoneutria fera, P. reidgyi* e *P. nigriventer* (Simo e Brescovit, 2001). As espécies *P. fera* e *P. reidgyi*, são encontradas na região amazônica, enquanto a espécie *P. nigriventer* tem ampla distribuição geográfica, sendo encontrada em pelo menos oito estados brasileiros, com preferência de ocorrência na região Sul (Santa Catarina, Rio Grande do Sul e Paraná) e Sudeste (Minas Gerais, Rio de Janeiro, São Paulo) e com acidentes relatados em Goiás e no Mato Grosso do Sul (Lucas, 2003).

Figura 9: Foto da posição de ataque assumida pela aranha *Phoneutria nigriventer*. Note que a aranha ergue as patas dianteiras enquanto se equilibra nas patas traseiras assim expõe as quelíceras (o par de estruturas em vermelho). Foto: *site* do Museu Escola: Animais Sinantrópicos (UNESP).

A espécie *Phoneutria nigriventer*, Keyserling 1891, é também conhecida como aranha macaco ou aranha-das-bananeiras por sua ocorrência em bananeiras. Além disso, podem habitar locais abrigados da luz, como arbustos e até mesmo entulho. Adultos e filhotes apresentam diferenças quanto ao hábito de vida, sendo que os filhotes frequentemente se expõe mais facilmente se afastando dos abrigos (Almeida et al., 2000; Ramos et al., 1998). Em razão de seu comportamento bastante agressivo e sua ampla distribuição geográfica, detêm o segundo lugar no rol das aranhas responsáveis por

acidentes de importância médica no Brasil envolvendo o envenenamento acidental por artrópodes (Brasil, 2001). Os acidentes aumentam nos períodos de acasalamento da aranha que ocorre nos meses de março a junho.

O envenenamento por *P. nigriventer* é classificado quanto à gravidade dos sinais clínicos: em leve, tratado com analgésicos para alívio da dor e edema (inflamação) locais e observação do paciente; moderado, com administração de soro e internação hospitalar, nestes casos os sintomas são sistêmicos com possíveis vômitos, sudorese e aumento da pressão arterial e por último, os envenenamentos graves que são caracterizados por sua intensidade. Vítimas de acidentes graves apresentam, além da dor e edema locais, tremores, intensa sudorese, convulsões, arritmia cárdio-respiratórias, priapismo, diarréia, bradicardia, hipotensão, edema pulmonar, choque e, em raras ocasiões, seu quadro clínico evolui para óbito (Antunes e Málaque, 2003; Brazil and Vellard, 1925). Ao conjunto dos sintomas, denomina-se síndrome do foneutrismo (Antunes e Málaque, 2003). Ainda, essas alterações aparentam ser mais severas em crianças abaixo de 10 anos e idosos acima de 70 anos (Bucaretchi et al., 2000; 2008; Cupo et al., 2013).

O veneno da aranha *P. nigriventer* (PNV) tem ação sobre o sistema nervoso autônomo (SNA), central (SNC) e periférico (SNP) de animais (vertebrados e invertebrados). O PNV é uma mistura de 17 peptídeos simples ativos (de peso molecular entre 3000 a 9000 Da), além de serotonina e histamina (Diniz, 1963). O PNV altera a fisiologia dos canais iônicos (Na^+, K^+ e Ca^{2+}) e receptores de membranas excitáveis, o que justifica sua potente ação neurotóxica e neuroexcitatória (para revisão ver Gomez et al, 2002).

Várias frações já foram isoladas do veneno bruto. Inicialmente foram isoladas por filtração em gel e cromatografia de fase líquida, três principais toxinas derivadas do PNV: PhTx-1, PhTx-2, PhTx-3, todas foram testadas em camundongos e demonstraram ação sobre o sistema nervoso desses animais (Rezende Jr et al., 1991).

Nos estudos preliminares envolvendo o envenenamento experimental dos camundongos, a toxina Phtx-1 induziu paralisia espástica do membro posterior, excitação e elevação da cauda, com sua ação principal sobre canais de Ca^{2+} (Santos et al., 1999). Phtx-2 induziu salivação, lacrimação, priapismo, convulsão e paralisia espástica, atrasando a inativação dos canais de Na^+ (Araújo et al., 1993). Phtx-3 induziu paralisia flácida das patas

traseiras por até 24 horas após administração sistêmica, afetando dois diferentes canais, os canais iônicos de K^+ e/ou Ca^{2+} (Troncone et al., 1995; Prado et al., 1996; Guatimosin et al., 1997). A toxina PhTx3-3 promoveu inibição da liberação de glutamato dependente de Ca^{2+} nas sinapses de camundongos (Prado et al., 1996); PhTx3-4 inibiu canais de Ca^{2+} e liberação de glutamato nas sinapses cerebrais (Miranda et al., 1998); PhTx3-4 (5-5) inibiu seletivamente e reversivelmente o subtipo de receptores de glutamato N-metil-D-aspartato (NMDA) em neurônios hipocampais de rato (Figueiredo et al., 2001) de modo geral, a fração Tx3 e suas derivadas tem ação mais potente sobre o SNC (Antunes e Málaque, 2003).

4. Interação PNV/BHE

Em ratos e camundongos, o veneno de *P. nigriventer* tem ação neurotóxica e excitatória sobre SNC, SNA e SNP, desencadeando os seguintes sinais clínicos: salivação, paralisia flácida e espática dos membros, vermelhidão das orelhas, piloereção, priapismo, convulsão e, em caso de morte, constata-se que houve edema pulmonar (Diniz, 1963; Schenberg e Pereira Lima, 1971; Mendonça et al., 2012). Estudos mostram que o PNV desencadeia aumento na permeabilidade vascular da pele (Antunes et al., 1992) e alterações na pressão sangüínea arterial (Costa et al., 1996). A figura 10 resume as alterações morfológicas e moleculares mais importantes da BHE em uma escala de tempo de 15 minutos até 3 dias após o envenenamento.

No SNC, a ação do PNV sobre os componentes da BHE tem grande destaque. O cerebelo e hipocampo são as regiões do SNC aonde a BHE é mais afetadas pela peçonha de *P. nigriventer* tanto em ratos. Estudos com microscopia eletrônica de transmissão (MET) demonstraram edema dos pés astrocitários principalmente no cerebelo e extravasamento de nitrato de lantânio (marcador extracelular) para o intertício. Em paralelo observou-se aumento na quantidade e no tráfego de vesículas na membrana do endotélio em vasos hipocampais (Le Sueur et al., 2003, 2004; Rapôso et al., 2007). Em conjunto esses dados demonstram uma permeabilidade temporária da BHE após envenenamento sistêmico dos ratos.

Estudos posteriores envolvendo a administração sistêmica do corante azul de Evans comprovaram a habilidade do PNV em permeabilizar temporariamente a BHE. Em condições normais após administração intravascular do corante todos os órgãos do animal adquirem a cor azul, exceto a medula espinhal e o cérebro, pois estes últimos apresentam a seletiva BHE; quando ocorre um prejuízo dos componentes da BHE, há extravasamento de corante no SNC; o que foi relatado em períodos iniciais ao envenenamento (entre 1 h e 2 h após administração do PNV) comprovando a desestabilização dos compontes da BHE e consequente perda da barreira seletiva (Mendonça et al., 2014).

A ação do PNV sobre a BHE envolve dois caminhos: a via paracelular e a via transcelular. A primeira via a ser afetada após o envenenamento foi a via paracelular, com diminuição da expressão das proteínas que constituem as junções celulares: ocludina, ZO-1, claudina-5 pertencentes as juções de oclusão; e, β-catenina, pertencente a junção de adesão

(Rapôso et al., 2012; Mendonça et al., 2014). As junções comunicantes também são afetadas, com aumento de sua principal proteína, a Cx43 (Râposo et al., 2014). A lâmina basal também é afetada e há prejuízo de sua principal proteína componente: a laminina (Rapôso et al., 2007).

As alterações das proteínas que compoem as junções celulares e a lâmina basal são dependentes do tempo após exposição ao PNV, e em geral, tem sua expressão reduzida nos períodos inicias de envenenamento entre 15 minutos e 2 horas; após este período essas proteínas retornam aos níveis basais ou até mesmo se encontram super expressas (Rapôso et al., 2007; 2012; Mendonça et al., 2014). Este fenômeno é corroborado por dados morfológicos de MET que demonstraram a passagem de marcadores extracelulares (*e.g.* nitrato de lantânio) em vasos em períodos de até 2 h após PNV (Rapôso et al., 2007). Em contraposição, nestes nos períodos inicias após o envenenamento ocorre aumento da forma inativada e da forma ativada de P-gp (do inglês *poly-glycoprotein*) responsável pela formação de bombas de efluxo nas células endoteliais e astrócitos (Rapôso et al., 2012) o que sugere um mecanismo compensatório face à perda da integridade das junções celulares, de forma a remover substâncias que transpassaram para o interior das células.

O endotélio cerebral é afetado inicialmente pela desestabilização de suas junções celulares como acima descrito e posteriormente pelo aumento na via endocítica em períodos tardios ao envenenamento (às 24 h) tanto em modelos *in vitro* quanto *in vivo* (Le Sueur et al., 2004, 2005). O aumento na endocitose ocorre através do aumento no tráfego de vesículas em trânsito pelo endotélio de vênulas e arteríolas (primeiro a serem afetadas) e posteriormente de capilares, este fenômeno é mediado por microtúbulos do citoesqueleto celular (Le Sueur et al., 2003, 2004). Ainda, a proteína transportadora de glicose (GLUT-1) é super expressa no endotélio em períodos intermediários aos do envenenamento (5 h), possivelmente contribuindo para o aumento no aporte de glicose necessário ao reparo tecidual após as alterações agudas desencadeadas pelo PNV (Rapôso et al., 2014).

Em neurônios, o PNV induziu aumento imediato do gene c-Fos e indução de sua proteína FOS em paralelo ao aumento na expressão de nNOS em regiões envolvidas na motricidade e estresse agudo do córtex cerebral (Cruz-Höfling et al., 2007). A inibição da nNO pelo inibidor seletivo dessa isoforma, o 7-nitroindazol, demonstrou a participação mais clara do NO e da nNOS na promoção de fenótipos pro-inflamatórios após

envenenamento por PNV (Rapôso et al., 2014); de fato há aumento em citocinas pro-inflamatórias (IFN-γ e TNF-α) após o envenenamento no cerebelo e no hipocampo de ratos (Cruz-Hofling et al., 2009).

Os astrócitos em curtos períodos após o envenenamtno (envenenamento agudo) apresentaram edema citotóxico (inchaço dos pés astrocitários perivasculares) e vasogênico (edema interticial) (Le Sueur et al., 2003; Rapôso et al., 2007). Tanto a resolução e também indução do edema observada após a administração do PNV foi relacionada ao aumento na proteína envolvida com a formação dos canais de água, as aquaporinas, neste caso em particular a proteína aquaporina-4 (Stávale et al., 2013). A aquaporina-4 é considerada um marcador de astrócitos e tem papel chave no edema cerebral (Nielsen et al., 1997). Em resposta ao estímulo neurotóxico causado pelo PNV, ocorreu astrogliose reativa, mediada pelo aumento da proteína do citoesqueleto astrocitário denominada proteína fibrilar ácida glial (*glial fibrillary acidic protein*, GFAP) (Cruz-Höfling et al., 2009); a proteína Cx43, principal formadora das junções comunicantes e envolvida em mecanismos pro-inflamátorios é superexpressa após envenenamento (Rapôso et al., 2014); ainda S-100, proteína associada ao metabolismo de cálcio em astrócitos, considerada um marcador de injúria cerebral também foi intensamente marcada após envenenamento no cerebelo e hipocampo de ratos (Cruz-Höfling et al., 2009).

A administração intraperitoneal do PNV causa alteração na expressão do VEGF e seus receptores, com aumento na expressão do receptor Flt-1 (Mendonça et al., 2012), receptor Flk-1 e do próprio VEGF no hipocampo e cerebelo de ratos (Mendonça et al., 2013, 2014). Dado que o VEGF tem tanto um papel na permeabilização da BHE como pode mostrar um caráter protetor do VEGF em modelos de injúria cerebral, sua alteração foi relacionada aos mecanismos de reparo mobilizados pelas células cerebrais estimuladas pelos componentes neurotóxicos do veneno. Entretanto, estudos estão em andamento para determinar qual o real significado das alterações promovidas pelo PNV no sistema VEGF/Flt-1/Flk-1.

Em cultura, o PNV não se mostrou letal para linhagens de células epidteliais ((*Madin-Darby canine cell kidney*, MDCK) ou de cultura primária afetando apenas o crescimento de linhagens de células de glioma (C6) (Le Sueur et al., 2005; Rapôso et al., 2012, 2014). Em células endoteliais de cordão umbilical (HUVEC, *human umbilical vein*

endothelial cells) e em cultura de astrócitos corticais, a incubação com PNV promoveu aumento na atividade bombeadora da proteína de efluxo MRP-1 (*Multidrug-resistance protein-1*) responsável pela retirada de substâncias indesejáveis ao SNC. Células MDCK incubadas com PNV mostraram grande endocitose apical com consequente aumento no fluxo de vesículas em tráfego pelo citoplasma da célula (Le Sueur et al., 2005). Os experimentos em cultura corroboraram os ensaios *in vivo*.

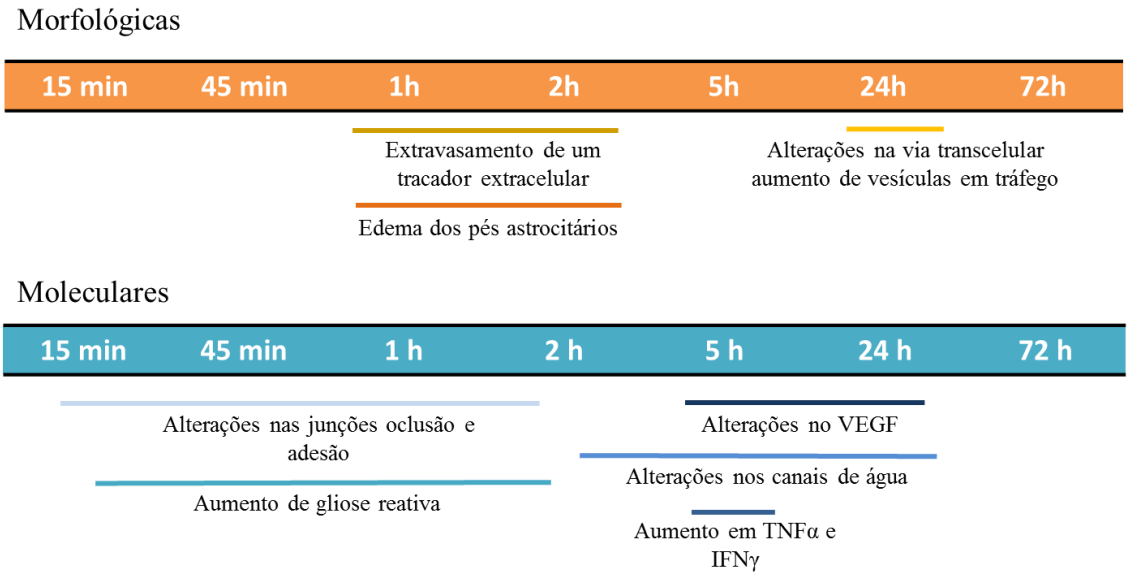

Figura 10: Esquema temporal das alterações morfológicas e moleculares apresentadas pelos componentes da BHE após o envenenamento sistêmico por PNV em ratos, a partir de 15 minutos após administração do veneno até 3 dias.

Por último, um dos pontos relevantes no estudo da ação do PNV sobre componentes celulares da BHE foi a elucidação da resposta celular de ratos neonatos comparados aos ratos adultos após o envenenamento. De modo geral, os sinais neurotóxicos apresentados por animais neonatos são maiores do que os sinais dos animais adultos (mesmo ~~a~~ sendo a dose administrada peso-dependente – 1,7 mg/Kg). As alterações celulares ao nível da BHE também foram mais presentes em neonatos (Mendonça et al., 2012, 2013; 2014; Stávale et al., 2013). Em conjunto, a maior fragilidade dos neonatos em comparação aos adultos pode ser reflexo de uma resposta mais eficiente dos ratos adultos uma vez que nestes a BHE está madura e portanto com mais eficiência na regulação do transporte na interface sangue-cérebro. Além disso, pode refletir o processo de desenvolvimento do cerebelo que ainda

está em curso em neonatos o que contribue para a menor eficiência dos neonatos frente ao envenenamento (Altman e Bayer, 1997).

II. OBJETIVOS

2.1. Objetivos Gerais

O trabalho teve como objetivo avançar na compreensão dos mecanismos envolvidos na permeabilidade temporária da BHE no cerebelo após administração da peçonha de *P. nigriventer* (PNV) atráves do estudo da via transcelular com enfoque nas cavéolas. A hipótese foi verificar se o aumento de vesículas presentes na membrana adluminal do endotélio vascular no cérebro de ratos que receberam PNV refletiria processo endocítico mediado por "cavéolas não revestidas por clatrina". Além disso, verificar se o aumento das vesículas na membrana abluminal desse endotélio refletiria tráfego aumentado de vesículas via caveolina fosforilada e participação de quinase da família Src.

2.2. Objetivos específicos

Foram objetivos específicos deste trabalho:

1. Investigar a expressão total (através de western blotting) e localização tecidual (através de imunohistoquímica) da proteína e expressão do gene (através de PCR real time) da caveolina-1 no cerebelo de ratos neonatos (14 dias) e de ratos adultos (8 a 10 semanas) em diferentes tempos (2 h, 5 h e 24 h) após exposição intraperitoneal única ao PNV.
2. Investigar alterações envolvendo a expressão (através de western blotting) e localização (através de imunofluorescência) de proteínas que formam as cavéolas e que estão envolvidas em sua sinalização no endotélio vascular cerebral e nas células gliais (principalmente astrócitos) do cerebelo de ratos, após exposição intravenosa única ao PNV nos períodos de 1 h, 2 h, 5 h, 24 h e 72 h.
3. Investigar alterações morfológicas no parênquima cerebral, com ênfase no endotélio de vasos cerebrais e nos pés astrocitários que os circundam através de microscopia

eletrônica de transmissão após exposição intravenosa única ao PNV nos períodos de 1 h, 2 h, 5 h, 24 h e 72 h.

4. Investigar a atividade da enzima eNOS após é desacoplada, passando à forma monomérica inativa por através de western blotting de baixa temperatura após exposição intravenosa única ao PNV nos períodos de 1 h, 2 h, 5 h, 24 h e 72 h.

III. 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 coautoria do candidato (**Anexo 1**). Desta forma, esta dissertação é composta de quatro artigos (apresentados em capítulos), os quais foram e serão submetidos para publicação, conforme descrito abaixo:

3.1. CAPÍTULO 1: Evidences of endocytosis via caveolae following blood-brain barrier breakdown by *Phoneutria nigriventer* spider venom.

3.2. CAPÍTULO 2: Caveolae, a novel *Phoneutria nigriventer* spider venom target.

3.3. CAPÍTULO 3: eNOS uncoupling in the cerebellum after BBB disruption by exposure to *Phonuetria nigriventer* venom.

3.4. CAPÍTULO 4: Are synchronized changes in Cx43 and Cav-3 a bystander effect in a *Phoneutria nigriventer* venom model of blood-brain barrier breakdown?

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Evidences of endocytosis via caveolae following blood–brain barrier breakdown by *Phoneutria nigriventer* spider venom

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HIGHLIGHTS

- *Phoneutria nigriventer* venom (PNV), known to induce BBB breakdown, increases cav-1 expression.
- The cav-1-labeled capillaries number is higher in PNV-treated P14 rats than in adult rats.
- The number of Purkinje cells expressing cav-1 increases after PNV envenoming.
- The variable age affects cav-1 induction by PNV in the white matter and granular layer.

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ABSTRACT

Spider venoms contain neurotoxic peptides aimed at paralyzing prey or for defense against predators; that is why they represent valuable tools for studies in neuroscience field. The present study aimed at identifying the process of internalization that occurs during the increased trafficking of vesicles caused by *Phoneutria nigriventer* spider venom (PNV)-induced blood–brain barrier (BBB) breakdown. Herein, we found that caveolin-1α is up-regulated in the cerebellar capillaries and Purkinje neurons of PNV-administered P14 (neonate) and 8- to 10-week-old (adult) rats. The white matter and granular layers were regions where caveolin-1α showed major upregulation. The variable age played a role in this effect. Caveolin-1 is the central protein that controls caveolae formation. Caveolar-specialized cholesterol- and sphingolipid-rich membrane sub-domains are involved in endocytosis, transcytosis, mechano-sensing, synapse formation and stabilization, signal transduction, intercellular communication, apoptosis, and various signaling events, including those related to calcium handling. PNV is extremely rich in neurotoxic peptides that affect glutamate handling and interferes with ion channels physiology. We suggest that the PNV-induced BBB opening is associated with a high expression of caveolae frame-forming caveolin-1α, and therefore in the process of internalization and enhanced transcytosis. Caveolin-1α up-regulation in Purkinje neurons could be related to a way of neurons to preserve, restore, and enhance function following PNV-induced excitotoxicity. The findings disclose interesting perspectives for further molecular studies of the interaction between PNV and caveolar specialized membrane domains. It proves PNV to be excellent tool for studies of transcytosis, the most common form of BBB-enhanced permeability.

1. Introduction

The blood–brain barrier (BBB) steady-state is crucial for preserving the homeostasis of the central nervous system (CNS) and normal neuronal and glial activity (Bradbury, 1993; Abbott

Abbreviations: BBB, blood–brain barrier; Cav-1, caveolin-1; CNS, central nervous system; GL, granular layer; ML, molecular layer; PC, Purkinje cells; PL, Purkinje layer; PNV, *Phoneutria nigriventer* venom.

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et al., 2010). BBB disruption results from disarray of tight and adhesion endothelial cell proteins that prevent bi-directional paracellular diffusion and/or disturbance of the transport proteins and enzymes that restrict trans-cellular movement of solutes across the blood–brain border.

The armed-spider *Phoneutria nigriventer* (*Araneae*, *Ctenidae*), also known as the wandering spider or armed-spider, is responsible for a great number of accidents in the Southeast of Brazil. The majority of accidents only cause local pain and edema (89.8%). Less than 0.5% of cases are considered severe, involving systemic neurotoxic manifestations such as tremors, convulsions, spastic paralysis, priapism, cardiovascular arrhythmia, intense sudoresis and visual disturbances (Brazil and Vellard, 1925; Bucaretchi et al., 2008). These symptoms appear to be more severe in children (Bucaretchi et al., 2000). *P. nigriventer* venom (PNV) contains several types of simple peptides, capable of blocking Ca^{2+} and K^{+} channels and delaying inactivation of Na^{+} channels, consequently affecting neurotransmitter handling (Gomez et al., 2002; Love and Cruz-Höfling, 1986).

In the brain of rats, data have shown that increased trans-endothelial vesicle trafficking is one of the pathways affected after PNV-enhanced BBB breakdown (Le Sueur et al., 2004). Rows of omega-shaped indentations are formed in the adluminal and abluminal aspect of the endothelial plasma membrane followed by extravasation of the electron-opaque extracellular tracer lanthanum nitrate into the neuropil interstitial space. In addition, swollen peri-vascular end-feet processes of astrocytes and edematous synaptic contacts are observed (Le Sueur et al., 2003). However, it remains to be seen how transcytosis in PNV-induced BBB permeabilized vessels is intensified.

The rise of vesicles through the brain microvascular endothelium in cultures has been associated with a high expression of caveolae frame-forming proteins (Xia et al., 2009). Caveolae are flask-shaped invaginations of the plasma membrane described as playing important roles in endocytosis (Lajoie and Nabi, 2010), trans-cellular trafficking (Hansen and Nichols, 2010; Pelkmans and Helenius, 2002), compartmentalization of signaling molecules (Quest et al., 2004), regulation of the endothelium function during blood–brain barrier breakdown (Nag et al., 2009; Predescu et al., 2007), management of lipid homeostasis (Fielding and Fielding, 2001) and intracellular signaling (Stern and Mermelstein, 2010; Sowa, 2012; Luoma et al., 2008). Located in the endothelial cells and pericytes of all of the cortex micro-vessels (Virgintino et al., 2002), caveolin-1 (cav-1) appears to be the most abundant protein of its family and has been described as a selective marker for caveolae (see Razani et al., 2002 for review) in the BBB endothelium (see Zhao et al., 2014 for review). Cav-1 is formed by two structurally distinct isoforms (α and β) (Scherer et al., 1995), of which the alpha isoform is the most highly expressed in the brain (Ikezu et al., 1998).

The aim of the present study was to verify whether the increased trans-cellular trafficking of vesicles found following *P. nigriventer*-induced BBB breakdown involves caveolin-1α. The hypothesis is that omega-shaped indentations present in the capillary and venule endothelia of PNV-administered rats result from caveolae formation and subsequently the endocytotic mechanism used in enhanced transcytosis. In addition, a possible age-related difference in the expression of caveolin-1α was assessed in the white matter, granular layer and molecular layer of the cerebellum of neonate and adult rats at early (2 h), intermediate (5 h) and late (24 h) time periods after PNV envenoming. These periods were associated with severe intoxication, the onset of recovery and the absence of signs of intoxication, respectively. The present study contributes to an understanding of the molecular changes that accompany the PNV-induced BBB permeability and the toxic manifestations seen in victims of accidents involving the armed-spider. Understanding the functional endothelial alterations is likely to provide innovative ways to target the BBB in pathological conditions.

2. Materials and methods

2.1. Animals and venom

Male Wistar rats, aged 14 days (P14 - neonate group) and 8–10 weeks (adult group) were obtained from the Multidisciplinary Center for Biological Research (CEMIB) of the UNICAMP. The adult rats were maintained in a temperature-controlled room (25–28 °C), a 12–12 h light–dark cycle with ad libitum access to food and water for acclimatization. One batch of lyophilized *P. nigriventer* spider venom (PNV) was supplied by the Butantan Institute (São Paulo, SP, Brazil) and stored at −20 °C. It was dissolved in 0.9% sterile saline solution (0.5 mg PNV/ml in 0.9% sterile saline) immediately before use.

2.2. Exposure to P. nigriventer *spider venom*

The rats ($n = 45$ per age) were given an i.p. sub-lethal injection of PNV (170 μg/kg) while rats in the control group ($n = 45$ per age) received the same volume of 0.9% sterile solution. PNV dose was selected based on previous dose-response assessment (Mendonça et al., 2012). After 2 h (period of intense intoxication signs), 5 h (signs that recovery from intoxication is underway) and 24 h (no sign of intoxication is apparent) ($n = 5$ per time period), the animals were anesthetized with a lethal dose of a mixture (3:1) of ketamine chloride (Dopalen®, 100 mg/kg) and xylazine chloride (Anasedan®, 10 mg/kg) and the cerebellum was excised for the cav-1 immunohistochemistry assay. For qPCR and western blotting assay, the animals were euthanized by decapitation after CO_2 inhalation at 2 h, 5 h and 24 h after PNV injection ($n = 5$ per time period and per technique).

2.3. Imunohistochemistry (IHC)

After anesthesia and thoracotomy, the animals were perfused through the left ventricle with 0.9% sterile saline (150 ml for 8–10 weeks and 20 ml for neonates), followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), with a pH of 7.4 (250 ml for 8- to 10-week-old and 50 ml for neonate rats). The cerebellum was immediately removed and post-fixed in the same fixative overnight. Then, the organ was dehydrated in ethanol series, cleared in xylene and embedded in paraffin (Paraplast®, Sigma–Aldrich, St. Louis, MO, USA). Cav-1 immunohistochemistry was performed in 5 μm thick sections (2035 RM microtome, Reichert S, Leica), as described by Mendonça et al. (2012) using the rabbit polyclonal primary antibody for caveolin-1: isoform α (Santa Cruz, CA, USA – code: SC-894; dilution, 1:500). Negative control was performed with 1% PBS-bovine serum albumin (BSA) but without the primary antibody. The intensity of the cav-1 labeling was analyzed in the white matter and the granular and molecular layers of the cerebellum through the density of pixels obtained in images captured by an Olympus BX51 photomicroscope (Japan), equipped with Image-Pro Plus image analyzer software (Silver Spring, MD, USA). Ten pictures per period/treatment of each cerebellar region were taken using an objective of 40× and fixed illumination parameters. The expression of the protein was assessed using the free access program GIMP 2.6.4 software (GNU Image Manipulation Program, CNE, Free Software Foundation, Boston, MA, USA), which segments the immunochemical reaction by color (Solomon, 2009). Also see Mendonça et al. (2012) and Stávale et al. (2013).

2.4. Number of vessels and Purkinje cells expressing cav-1

2.4.1. Vessels

The number of vessels expressing cav-1α was counted in 10 random fields per period and treatment (2 pictures per layer, $n = 5$ animals) in the white matter, as well as the granular and molecular layers using an objective of 40× (Olympus BX51, Japan).

2.4.2. Purkinje cells

Due to the reduced area of the Purkinje layer, it was difficult to count the vessels within the layer. On the other hand, a number of Purkinje cells appeared to express cav-1, which led us to investigate if this labeling differed significantly in the envenomed group relative to the control group. For that matter, 50 cells per animal were counted (totaling 250 cells per time). Vessels and Purkinje neurons were counted with the assistance of the UTHSCSA ImageTool Program (by C. Donald Wilcox and colleagues at UTHSCSA Dental Diagnostic Science, San Antonio, TX, USA).

2.5. Western blotting (WB)

After euthanasia of the animals (from the control and PNV groups), the cerebella were removed and homogenized with a protein extraction cocktail (10 mM EDTA, 2 mM PMSF, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 0.1 mg of aprotinin/ml and 100 mM tris, pH 7.4). Cellular proteins were quantified by the Bradford assay (Bio-Rad Hercules, CA, USA). Next, 40 μg of the cleared lysates were separated in 12% SDS-PAGE and electro-transferred onto the nitrocellulose membrane (Bio-Rad Hercules, CA, USA). As previously described, the total cell lysates were prepared and analyzed by western blotting (Rapôso et al., 2012). The primary antibody used was specific for rabbit polyclonal antibodies against caveolin-1 isoform α (1:1000). The mouse polyclonal antibody against β-actin (1:1000, Sigma–Aldrich, St. Louis, MO, USA) was used as an endogenous control. The bands were visualized through chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA) and the density of pixels in each band was determined by Image J 1.45s software (Wayne Rasband, NIH, Bethesda, MD, USA). The experiments were done in triplicate. The quantification was normalized with the corresponding value of β-actin expression.

2.6. RNA isolation and qPCR (quantitative real-time reverse transcription-PCR)

Using Trizol reagent (Life Technologies, Gaithersburg, MD, USA), the total RNA was extracted from the cerebellum of the control and PNV-treated groups. As described, standard reverse-transcription PCR was performed using total RNA (Hirota and Moro, 2006). Quantitative real-time PCR was performed in an ABI Prism7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA), using TaqMan® Universal Master Mix. GAPDH was used as an endogenous control for normalizing the RNA levels. The primer utilized was caveolin-1α and its assay identification number in the Applied Biosystem catalogue was Rn00755834_m1. The optimal concentrations of cDNA and primer, as well as the maximum efficiency of amplification, were obtained through five-point, two-fold dilution curve analysis for the gene. Each PCR contained 3.0 ng of reverse-transcribed RNA, 200 nM of each specific primer, SYBR SAFE PCR master mix and RNAse free of water to a final volume of 20 μl. Real-time data were analyzed using the Sequence Detector System 1.7 (Applied Biosystems, Carlsbad, CA, USA).

2.7. Statistics

The student's *t*-test was used to determine significant differences between data of the control and PNV-treated samples. Three-way ANOVA was conducted to assess the impact of the animal's age, the treatment and the time of venom/saline exposure on the density of pixels (immunostaining) in the white matter, granular layer and molecular layer of the cerebellum. Data were expressed as mean ± SEM. The significance level was set at $^{*}p \leq 0.05$.

3. Results

3.1. Immunohistochemistry

Cav-1α was expressed in the endothelium of vessels in P14 (Fig. 1A) and adult controls (Fig. 1E) and respective PNV-treated rats (Fig. 1B and F) in the white matter (WM), granular layer (GL) and molecular layer (ML). Digitized image processing following immunostaining color segmentation (Solomon, 2009) showed that the expression of cav-1α was higher in the cerebellum of envenomed rats (Fig. 1D and H) than in the control (Fig. 1C and G) rats. However, there were region- and age-related differential labeling dynamics after different venom administration times (Fig. 2A–C). Given that the Purkinje layer (PL) comprises a single cell layer formed by rows of Purkinje neurons, it was difficult to detect and count the vessels therein.

3.1.1. White matter

PNV induced significant increases of cav-1α expression in the WM of neonate (P14) rats over time. The increases recorded were 29% (2 h), 72% (5 h) and 95% (24 h), relative to baseline level. In PNV-treated adult rats (8–10 weeks), cav-1α expression increased by 68% after 2 h, 83% after 5 h and did not differ from baseline after 24 h. Three-way ANOVA revealed that the variable age influenced the higher level of cav-1α in the WM of PNV-treated neonates after 24 h compared with adult rats (Fig. 2A).

3.1.2. Granular layer

The expression of cav-1α increased by 82% (2 h), 94% (5 h) and 44% (24 h) in the vessels of the GL of PNV-administered P14 rats, whereas it increased by 132% (2 h), 102% (5 h) and 140% (24 h) in adult rats. Three-way ANOVA revealed that the variable age influenced the higher level of cav-1α in the GL of PNV-treated adults after 24 h compared with P14 rats (Fig. 2B).

3.1.3. Molecular layer

The expression of cav-1α increased by 95%, 97% and 67% after 2, 5 and 24 h, respectively in P14 rats exposed to PNV, whereas for adult rats, the increases were only significant (108%) after 5 h. ML did not exhibit age-related differences in the level of caveolin-1 after envenoming (Fig. 2C).

3.2. Number of caveolin-1-labeled capillaries

In the WM of PNV-treated P14 rats, the number of capillaries cav-1α-labeled at 2, 5 and 24 h was 33%, 42% and 53% higher than the controls, respectively. The corresponding number in adults was 24% at 2 h and 36% at 5 h, relative to the controls (Fig. 2D).

In the GL of P14 and adult rats exposed to PNV, the number of cav-1α-labeled capillaries increased by 26% and 16% after 2 h, by 53% and 27% after 5 h and by 26% and 12% after 24 h, respectively, relative to the P14 and adult controls (Fig. 2E).

In the ML, significant increases in the number of cav-1α-labeled capillaries occurred after 24 h for P14 (34%) and after 5 h for adult rats (18%) (Fig. 2F).

3.3. Number of Purkinje cells labeled for cav-1

Fig. 3 shows that the number of Purkinje neurons expressing cav-1α in PNV-treated P14 rats increased by 66% and 53% after 2 h

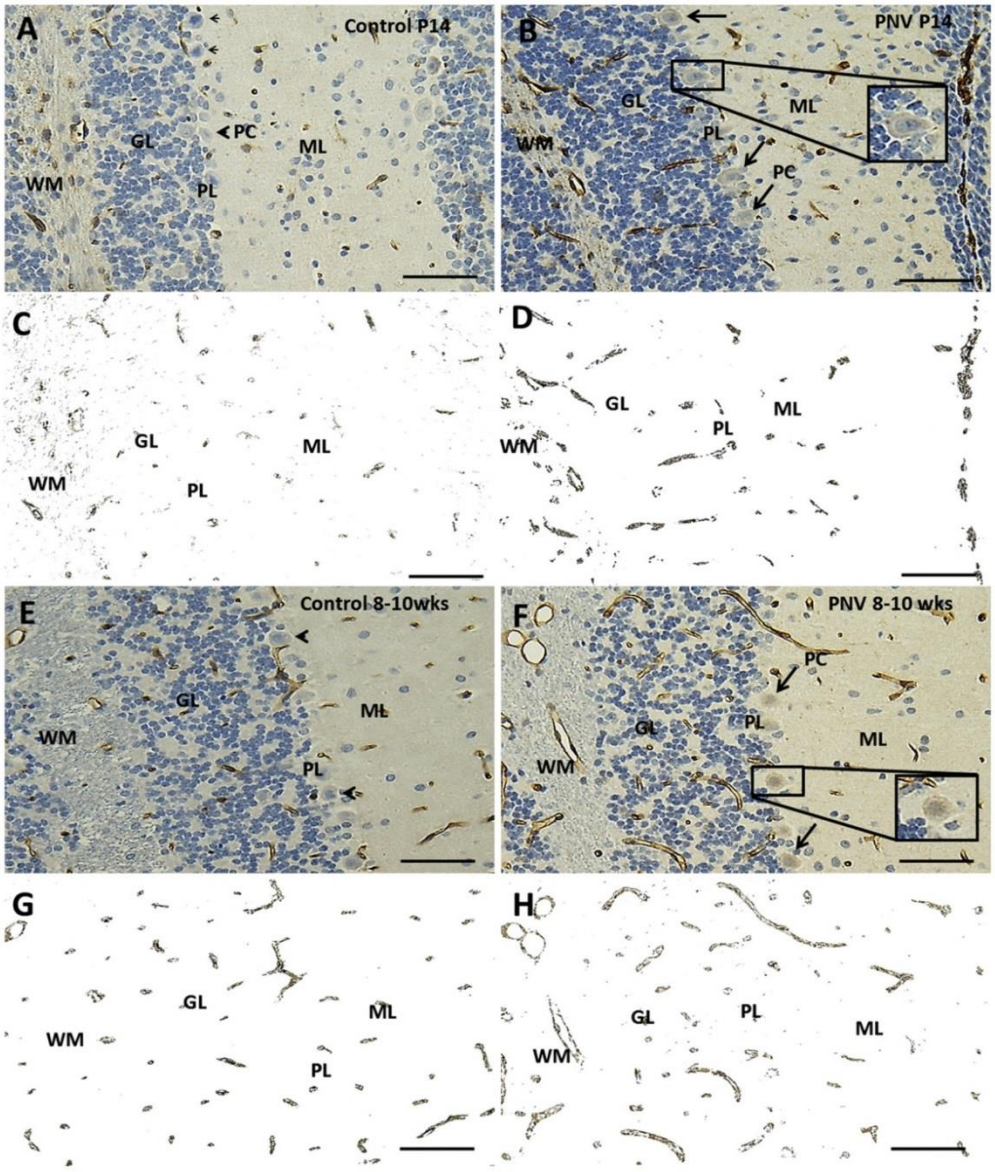

Fig. 1. Immunostaining for cav-1 (A, B, E, F) and selection by color performed by GIMP program (C, D, G, H) for both P14 and 8–10 weeks animals. WM = white matter; GL = granular layer; PL = Purkinje layer; ML = molecular layer; PC = Purkinje cells; arrows = Purkinje cells intensely positive for cav-1α; arrowheads = Purkinje cells cav-1 negative. Zoom square confirmed a cav-1 positive Purkinje cell (mainly close to membrane). Bars: 100 μm.

and 5 h, respectively, while there was no difference from the control after 24 h. Adult rats exhibited 103%, 73% and 57% increases in the number of cav-1α positive Purkinje neurons after 2 h, 5 h and 24 h, respectively. Fig. 1A–E are illustrative of the Purkinje cells that were immunostained for cav-1α in P14 and adult rats treated with saline and PNV.

3.4. qPCR and western blotting (WB)

Analysis of cav-1α mRNA expression showed no change in P14 animals after PNV administration. In contrast, 8 to 10-week rats exhibited a 20% increase after 24 h post PNV (Fig. 4A).

WB analysis of cerebella homogenates revealed a punctual and transitory increase (after 5 h) of 18% and 15% in the total cav-1α level in neonate and adult envenomed rats, respectively (Fig. 4B).

4. Discussion

Previous studies have shown that PNV affects the BBB steady-state by disturbing members of the neurovascular unit, such as the endothelium, astrocytes and neurons (Le Sueur et al., 2003). This event, also detected in smooth muscle cells of small venules and arterioles, is accompanied by important synaptic contact remodeling, perivascular astrocytes end-feet cytotoxic edema (Le Sueur et al., 2003) and vasogenic (interstitial) edema (Rapôso et al., 2007; Mendonça et al., 2012). Previous treatment with colchicine eradicates such effects indicating that PNV-increased vesicle formation and trans-cellular trafficking is microtubule-mediated (Le Sueur et al., 2004). In astrocytes, PNV promotes cytoskeleton remodeling (GFAP up-regulation), expressional increases of pro-inflammatory cytokines, S-100 protein (Cruz-Höfling et al., 2009),

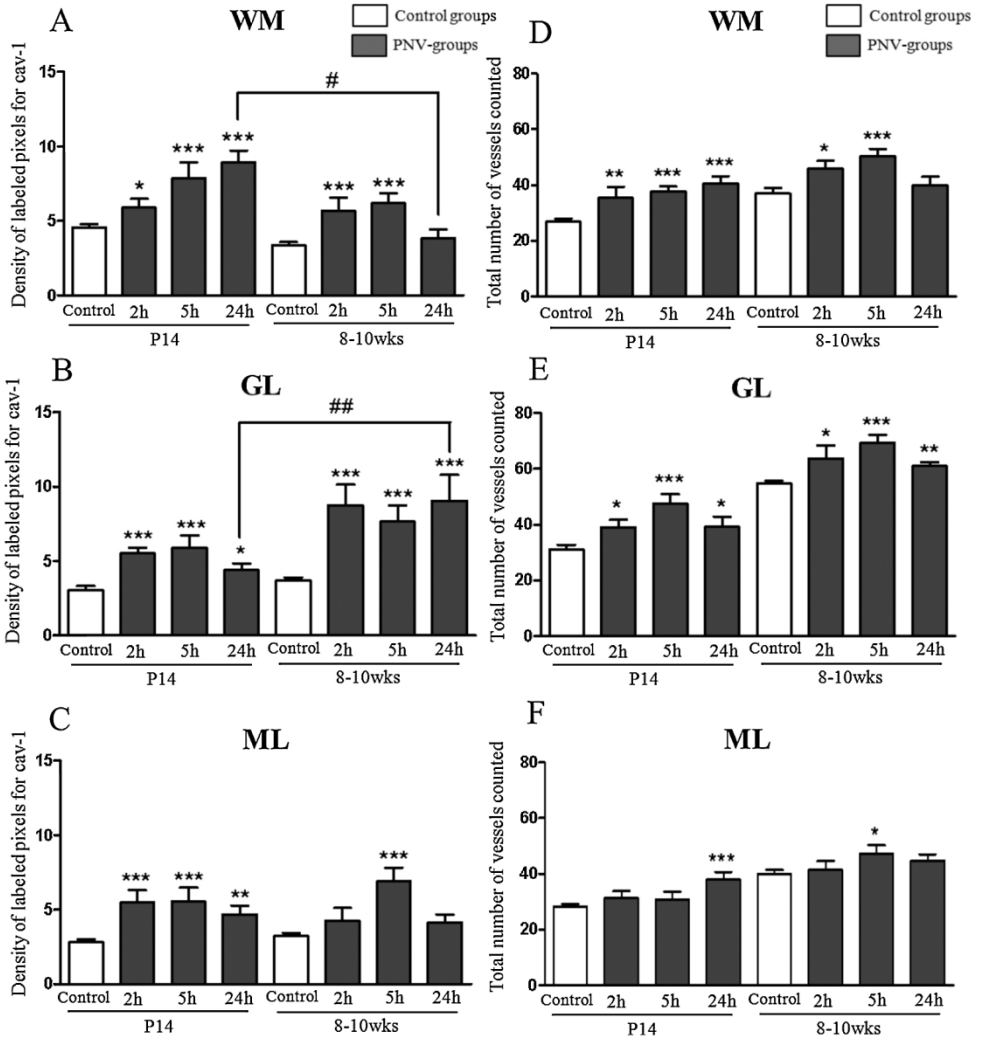

Fig. 2. Graphic representation of cav-1α IHC analyses (panels A–C) and number of vessels cav-1 immunostained (D–F) in the cerebellum of control ($n = 15$) and PNV-treated neonate (P14) and adult (8–10 weeks) rats ($n = 5$ per time). WM = white matter; GL = granular layer; ML = molecular layer. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ relative to respective pooled-control. Data were expressed as mean ± SEM, student *t*-test. $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$: three-way ANOVA indicated interference of the variable age in cav-1 labeling at 24 h for WM and GL.

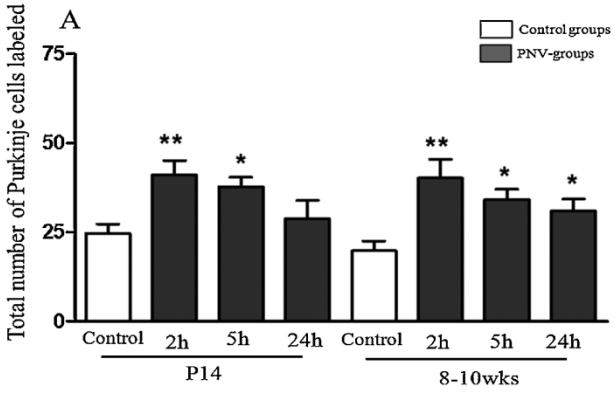

Fig. 3. Number of Purkinje cells immunostained for cav-1α in PNV ($n = 5$/time) and saline (control) groups ($n = 10$) for P14 and 8–10 weeks animals. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ relative to pooled-control. Data were expressed as mean ± SEM, student *t*-test.

aquaporin-4, a water channel-forming protein involved in edema formation and resolution (Stávale et al., 2013) and inhibition of P-gp efflux protein (Rapôso et al., 2012). In neurons, PNV causes c-Fos induction in several motor- and stress-related brain regions (Cruz-Höfling et al., 2007).

The results of the present study include an interesting and new finding related to the neurotoxic effects caused by PNV at the BBB. The findings reveal that PNV acts on membrane domains containing the main protein of the caveolae frame (Rothberg et al., 1992). The expressional increases of endothelial cav-1α indicate that the omega-shaped indentations at the luminal aspect of the plasma membrane of capillaries following PNV-enhanced BBB breakdown (Le Sueur et al., 2003) may represent endocytosis via caveolae formation (Pelkmans and Helenius, 2002). Additionally, the omega-shaped indentations at the ab-luminal aspect of the endothelium may represent ongoing increased trans-cellular trafficking. Cav-1α is a selective marker for caveolae in the brain endothelium. Its isoform α is the major representative in brain vessels (Ikezu et al., 1998). Studies have shown the participation of cav-1 in BBB fence weakening. Brain injury and the consequent

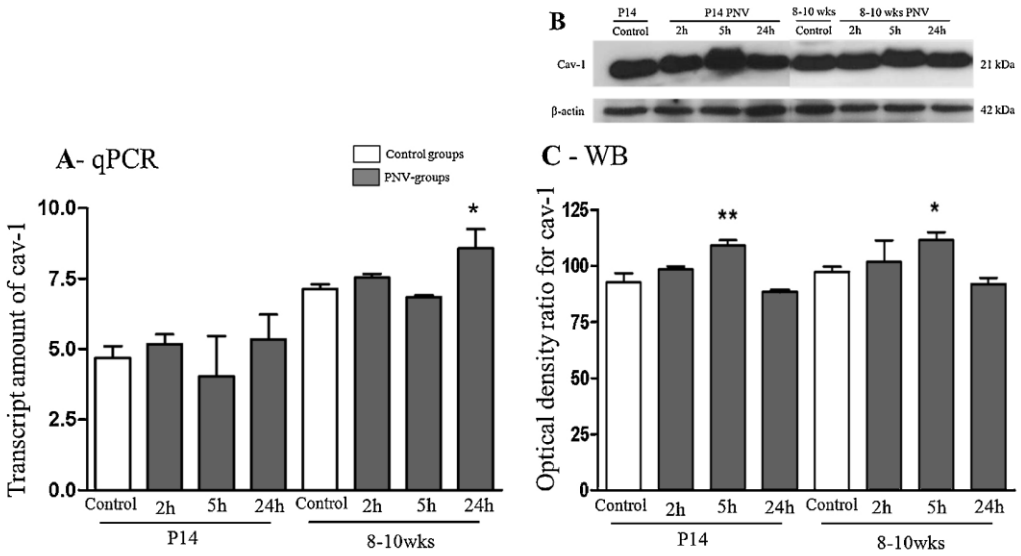

Fig. 4. Caveolin-1α (Cav-1α) mRNA analysis (panel A); immunoblots of cav-1α (Panel B) and graph representations of total cav-1α protein level (panel C) of PNV ($n = 5$/time) and control groups ($n = 10$); P14 = neonate; 8–10 weeks = adult rats. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ relative to control. Data were expressed as mean ± SEM, student t-test.

destabilization of the BBB have been associated with the up-regulation of cav-1 while its over-expression in the endothelium promotes trans-cellular trafficking of plasma proteins and contributes to vasogenic edema in BBB breakdown models (Nag et al., 2009, 2011). In contrast, cav-1 down-expression can prevent the formation of edema and neuronal death (Chang et al., 2011). The data in the present study correlate the up-regulation of the cav-1α with putative greater caveolae-mediated microvascular endocytosis and the trafficking and leakage of certain plasma solutes into the neuropil. This explains the extravascular diffusion of lanthanum nitrate into the interstitial space of neuropil in the course of BBB breakdown caused by PNV (Le Sueur et al., 2003, 2004). This hypothesis is not unlikely since recent studies have shown that PNV inhibits P-gp efflux activity in cultured astrocytes, thus allowing the entrance of toxicants into the CNS (Rapôso et al., 2012). Furthermore, the resulting endocytotic activity from circulating solutes and their transcellular transport explains the edema of perivascular astrocytes end-feet and extravasation into interstitial neuropil spaces (Le Sueur et al., 2003; Rapôso et al., 2007). This is consistent with the PNV-induced aquaporin-4 increases (Stávale et al., 2013), an integral transmembrane protein with a key-role in the brain water balance and K^+ buffering after neuron excitability (Nico et al., 2002; Verkman et al., 2006).

It is interesting to note that the increase in cav-1α immunostained vessels herein demonstrated could also be related to a new role attributed to caveolae as a mechanical sensor of shear stress at the membrane (Rizzo et al., 2003). Studies have reported that cav-1α redistribution at the cell surface, which results in molecular changes derived from caveolae assembly/disassembly alternate cycles, induces mechanical stress due to tension variation in the plasma membrane (Yu et al., 2006; Nassoy and Lamaze, 2012). In turn, membrane tension contributes to signaling mechanisms involved in the vessel's response to shear stress (Boyd et al., 2003). In fact, it makes sense that the increased density of omega-shaped indentations (caveolae-like) at the plasma membrane (Le Sueur et al., 2003) would reflect redistribution of cav-1 in the brain microvasculature thereby leading to shear stress and alteration of membrane tension status. In line with this, caveolae would function as a mechanotransducer, transforming hemodynamic changes into biochemical signals for vascular function regulation (Yu et al., 2006).

The present IHC-based data showed up-regulation of cav-1α at practically every post-envenoming time interval, whereas WB-based data showed that total cerebellar cav-1α content only increased after 5 h in animals of both ages. Messenger RNA levels (qRT-PCR) only increased in adult rats after 24 h. These differences can be attributed to technique specificity. While IHC reveals sensible and regional alterations in cav-1α expression in the cerebellum, WB data obtained through the maceration of the whole cerebellum dissipate delicate local regulations of the protein. Moreover, intrinsic regulatory mechanisms at transcriptional and translational levels can lead to an abundance of protein and low gene expression and vice-versa (Schwanhäusser et al., 2011). Moreover, it has been reported that cav-1α has a very low turnover (great stability) and that very little variation in its mRNA would be sufficient to increase protein expression (Parat and Fox, 2001). Boyd et al. (2003) described the rise in cav-1α redistribution in vessels submitted to mechanical tension without a corresponding increase in total protein expression or it's mRNA. This could also suggest that cav-1α increases during the post-envenoming period might not occur by neosynthesis but rather through increased trafficking from the Golgi complex to the plasma membrane (Boyd et al., 2003).

The venom of *P. nigriventer* contains a plethora of Na^+-, K^+- and Ca^{2+} channels-acting excitotoxic neuropeptides that interfere in the handling (both release and uptake) of glutamate (Gomez et al., 2002; Pinheiro et al., 2006). Neuron excitotoxicity induces glutamate release by astrocytes and astrocytic glutamate in turn activates neuronal glutamate receptors (Bergensen and Gundersen, 2009; Nedergaard, 1994). Previous studies have shown that PNV activates neuron cells (cFos induction) in motor- and stress-related regions of the CNS (Cruz-Höfling et al., 2007), and disturbs synaptic contacts structure (Le Sueur et al., 2003). We suggest that these effects that can be associated with PNV-induced glutamate and ion channels unbalanced state. We also suggest that the presence of membrane micro-domains expressing the main protein of the caveolae frame in Purkinje neurons of rats intoxicated with PNV could be associated with glutamate and ion channel disturbances. Increasing evidence shows that lipid rafts, caveolar flask-shaped invaginations of plasma membrane enriched with cholesterol, sphingolipids and caveolin-1 scaffolding proteins are essential for synapse formation, stabilization and maintenance (Willmann et al., 2006). These membrane sub-

domains have been reported to promote signaling pathways to stimulate neuronal survival and growth (Head et al., 2011). The authors believe that neuronal increases in cav-1α expression could be a new way to preserve, restore and enhance neuronal function following injury. If this is the case, age-associated differences in the cav-1α positive Purkinje cells (see Section 3.3 and Fig. 3) suggest that adult rats could counteract the PNV toxic effects longer than P14 rats, which could configure a better instrumental in adulthood for preserving neuron function than among neonates. Moreover, cerebellum development is only completely achieved after several months of post-natal life (Altman and Bayer, 1997) and during this period newborn rats exhibit less restrictive transport regulation at the BBB (Risau et al., 1998), which could affect the metabolism of venom components thus signaling neuron viability.

5. Conclusion

Considering the role of the caveolae as mechanical sensors and vascular hemodynamics regulator, and the ability of the PNV to promote caveolae formation, vasogenic edema and BBB breakdown, the data suggest that the up-regulation of cav-1α and the increase of vessels immunostained with the principal caveolae-forming protein would reflect changes in the biophysical properties of cell membranes at the BBB level, associated with increased endocytosis and trans-cellular trafficking. On the other hand, the increased expression of caveolin-1 in Purkinje cells could be evidence of neuronal protection against the excitotoxicity promoted by PNV. The factors that underlie the up-regulation of cav-1α by PNV remain to be explored. The present study proves this spider venom is an excellent tool for studying transcytosis, which is considered the most common but least studied route, during BBB-enhanced permeability. The findings disclose interesting perspectives for further studies on drug delivery by investigating the interactions between *P. nigriventer* venom and caveolar specialized membrane domains. Understanding the mechanisms that cause functional endothelial alterations is likely to provide innovative ways to target BBB in pathological conditions.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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CAPÍTULO 2

Caveolae, a novel *Phoneutria nigriventer* spider venom target

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Abstract

Neuropeptides of *Phoneutria nigriventer* spider venom (PNV) cause neuroinflammation and induce blood-brain barrier breakdown (BBBb). Caveolae are specialized flask-shaped invaginations of the endothelial plasma membrane that are involved in endocytosis/transcytosis. The structural protein of caveolae is caveolin 1 (Cav-1). In the brain, Cav-1 plays a key role in microenvironment homeostasis by regulating cell signaling. The present study investigated whether caveolae mediate the effects of PNV in the cerebellum at critical periods following envenomation. Cav-1 and dynamin-2 (Dyn2), which are essential molecules for caveolae formation, were upregulated in the early (1 h), intermediate (5 h) and late (72 h) periods. Inversely, phosphorylated Cav-1 and Src tyrosine kinase family (SKF), which are essential molecules for internalization and transport, decreased during the periods when Cav-1 and Dyn2 increased. MMP9 was upregulated in the same periods as Cav-1 and Dyn2. Overall, the data indicates that periods of vesicles trafficking across the endothelium (high pCav/SKF levels) coincided with lower numbers of caveolae (Cav-1/Dyn2 dowregulation) and lower MMP9 expression. It can be concluded that internalization and trafficking activities are responsible for the flattening of plasma

membranes, revealed by a decrease in caveolae. The findings reveal the ability of PNV to induce MMP9 activity and interfere in caveolae specialized membrane domains. Therefore, caveolae is a main PNV target, and contributes to major alterations in BBBb following systemic envenomation.

Keywords: caveolae, spider venom, vesicle trafficking, blood-brain barrier breakdown.

Abbreviations: BBB, blood-brain barrier; BBBb, blood brain-barrier breakdown; Cav-1, caveolin-1; Cav-3, caveolin-3; CNS, central nervous system; Dyn2, Dynamin-2; IF, immunofluorescence; MMP9, metalloproteinase-9; pCav-1, phosphorylated caveolin-1; PNV, *Phoneutria nigriventer* venom; SKF, Src tyrosine kinase family; TEM, transmission electron microcopy; WB, western blotting.

1. Introduction

Under physiological conditions, the blood brain barrier (BBB) maintains a suitable and stable microenvironment within the central nervous system (CNS), a prerequisite for neuron and glia cells to function in brain homeostasis. Homeostasis of the CNS can be disrupted by many neurological factors, including but not limited to endogenous neurodegenerative diseases and exogenous neurointoxication caused by accidents with venomous animals. Brazil is home to a medically important spider, the *Phoneutria nigriventer* armed-spider, whose venom (PNV) contains a variety of pharmacologically-active neuropeptides. Some of these activate or delay inactivation of tetrodotroxin (TTX)-sensitive Na^+ channels, resulting in excitable membrane depolarization, thought to increase Na^+ influx and intracellular Ca^{2+} stores. PNV also blocks K^+ channels and different types of Ca^{2+} channels (Fontana and Vital Brazil, 1985; Love and Cruz-Höfling 1986; Gomez et al., 2002). The interference on ion channel physiology by these PNV neuropeptides affects neurotransmission, as shown by interference in both glutamate release and glutamate uptake (see Gomez et al., 2002). The clinical profile of severe Phoneutrism accidents include muscle weakness, arterial hypertension, cardiac arrhythmias, respiratory distress, pain, intense sudoresis, agitation, blurred vision and sometimes convulsions (Bucaretchi et al., 2000).

Experimental probes indicate that PNV, along with neurotoxic manifestations, activates pro-inflammatory cytokine. It also disrupts the BBB and alters the expression of proteins related directly or indirectly to the barrier (Cruz-Höfling et al., 2009; Le Sueur et al, 2003; 2004, 2005; Mendonça et al., 2012, 2013, 2014; Rapôso et al., 2007, 2012, 2014; Stávale et al., 2013). It has been shown that PNV-induced BBB opening occurs through a marked increase in transcellular vesicle transport (Le Sueur et al., 2004; 2005) and redistribution of endothelium junction proteins (Rapôso et al., 2012; Mendonça et al., 2013). Augmented endothelial transcytosis in the brain microvessels of rats administered with PNV was manifested by rows of flask-shaped indentations at the ad- and abluminal aspects of the endothelium and smooth muscle cells of venules and arterioles, indicating acceleration of vesicle trafficking across the BBB (Le Sueur et al., 2003, 2004). The authors also found vesicle content delivered into the interstitial spaces of the neuropile, and the presence of cytotoxic and vascular edema. These flask-shaped indentations, known as caveolae, are cholesterol- and glycosphingolipid-rich membrane microdomains that gather together and compartmentalize many molecules, including caveolin, endothelial nitric oxide synthase, G proteins, and many phosphatases, to cite just a few (Pattel et al., 2008).

Endothelium of the brain microcirculation possess few caveolae, as high electrical resistance and highly receptor- and carrier-regulated transcytosis restrict transport at the blood-brain border. Therefore, increases in the number of caveolae following PNV exposure implies BBB permeabilization through impairment of the transcellular pathway (Le Sueur et al., 2003). The authors of the present study recently showed that PNV upregulates the expression of caveolin-1α (Cav-1), the main caveolae scaffolding protein, and increases both the number of vessels labeled with Cav-1, and Cav-1+ Purkinje neurons in the cerebellum of rats (Soares et al., 2014a). The findings suggest a role for Cav-1 in the increased endocytosis and transcytosis shown earlier in pioneer papers describing PNV-induced BBB breakdown (Le Sueur et al., 2003; 2004), and probable neuronal signaling, as has already been described (Stern and Melmerstein, 2010).

In fact, caveolin coated-caveolae contribute to regulate cell membrane dynamics (Nassoy and Lamaze, 2012). Caveolae mediate endocytosis and trafficking of substances by sorting transporting vesicles into cell cytoplasm (Pelkmans and Helenius, 2002; Shajahan et al., 2004a). Increased transcytosis of plasma protein by endothelial caveolae

has also been linked to BBB breakdown and cerebral edema (Nag et al., 2009). Caveolae-derived vesicle trafficking requires caveolin phosphorylation (pCav-1) by activation of the Src tyrosine kinase family (SKF) (Shajahan et al., 2004a; Nag et al., 2009) and dynamin-2 (Dyn2), a caveolae neck forming protein with a role in caveolae scission and internalization (Yao et al., 2005), resulting in plasma membrane flattening (Henley et al., 1998).

In the present study, two proteins that have pivotal importance in the structure and function of caveolae were investigated, and part of the mechanistic pathway triggered in response to the presence of a BBB disrupting spider venom in the circulation of rats was assessed. The study was followed up during the intense toxic manifestation of the rats (1 to 2 h), when recovery was in process (5 h) and when clinical manifestations were not observe and the animals presented normal behavior (24 and 72 h).

Materials and Methods

Phoneutria nigriventer venom (PNV)

The lyophilized venom of the spider *Phoneutria nigriventer* (PNV) was donated by Prof. Dr. Evanguedes Kalapothakis (UFMG, Belo Horizonte, MG, Brazil) and stored at -20ºC. At the moment of use the venom was dissolved in 0.9% sterile saline solution (dilution: 0.5 mg PNV/ml sterile saline).

Animals and envenoming procedure

Male Wistar rats (*Rattus norvegicus*), 6 to 7 weeks old were obtained from the Multidisciplinary Center for Animal Investigation (CEMIB) at UNICAMP (Campinas, SP, Brazil). They were maintained at 12 h light-dark cycle (25-28ºC) with free access to food and drinking water until the experimental procedure was performed. A single intra-vascular injection (i.v.) of 0.5 mg/Kg sub-lethal dose of PNV was administered to rats (n = 55); animals were euthanized 1, 2, 5, 24 and 72 h (n = 11/time, i.e., n = 5 for western blotting and n = 3 for immunofluorescence and transmission electron microscopy assays per time) after PNV injection. A single control group received the same of volume of vehicle and was euthanized 5 h later (n = 11, i.e., n = 5 for western blotting and n = 3 for immunofluorescence and transmission electron microscopy assays). The experiments were

approved by the institution's Committee for Ethics in Animal Use (CEUA-Unicamp, protocol nº 3609-1) which follows the Brazilian Society for Laboratory Animal Science (SBCAL) guidelines.

Western blotting (*WB*)

After venom or saline treatment, the animals were euthanized in a CO_2 chamber and their brains removed and immediately frozen in liquid nitrogen. Then the proteins were separated in an extraction cocktail and submitted to a 12% SDS-PAGE as describing by Rapôso et al. (2012). The membrane was blocked overnight with 5% non-fatty milk, and incubated for four hours with primary antibody against caveolin-1 (1:1000), dynamin-2 (1:500), both from Santa Cruz Biotechnology, Santa Cruz, CA, USA, Src tyrosine kinase family (SKF) (1:1000), p-caveolin-1 (1:1000), both from Cell Signaling Technology, Danvers, MA, USA, β-actin (1:1000; Sigma Aldrich, St. Louis, MO, USA). After, the membranes were incubated with secondary antibodies: anti-rabbit (1:1000) for caveolin-1, SKF and p-caveolin-1, anti-goat (1:1000) for dynamin-2 and anti-mouse for β-actin (1:40000) all from Sigma Aldrich). Proteins bands were visualized by chemiluminescence (Thermo Scientific, Waltham, MA, USA) and proteins content were quantified by measuring the density of pixels of each band using Image J 1.45s program (Wayne Rasband, NIH, Bethesda, MD, USA). The quantification was normalized with the corresponding value of endogenous β -actin expression.

Immunofluorescence (*IF*)

After 1, 2, 5, 24 and 72 h post envenoming or saline administration the animals were euthanized under anesthesia with an over dose of a 3:1 mixture of ketamine chloride (Dopalen®, 100 mg/Kg) and xylazine chloride (Anasedan®, 10 mg/Kg) and immediately perfused with 0.9% saline solution (100 ml) via the left ventricle followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (200 ml) fixative. The cerebella were excised and immersed in 15% sucrose and further in 30% sucrose for cryoprotection (24 h each). Cerebella samples were immersed in OCT-Tissue Tek (Sakura Finetek, Torrance, CA, USA) and frozen in n-hexane in liquid nitrogen (-70ºC). Frozen sections 5 µm thick were mounted in silanized glass slides and after rinsing with Trizma® buffer pH 7.4 the

slides were permeabilized with 0.1% Triton X-100 for 10 min followed by phosphate buffered 0.1% Tween 20 with 1% non-fat milk at room temperature for blockade of non-specific antigens. The slides were incubated overnight with primary antibodies diluted in the blocked solution (phosphate buffered 0.1% Tween 20 and 1% non-fat milk): anti-caveolin-1 (1:100), anti-dynamin-2 (1:50), anti-Src tyrosine kinase family (1:100). After rinsing with phosphate buffer, slides were incubated with respective secondary antibodies conjugated with anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 45 minutes in the dark. The slides were sealed using commercial vehicle (Vectashield, Vetor Labs, Burlingame, CA, USA) and examined under fluorescence in Olympus BX51 photomicroscope (Olympus Corporation, Tokyo, Japan).

Transmission electron microscopy (TEM)

After anesthesia, the animals were cut open in the chest and the left ventricle exposed for animals to be first perfused with 0.9% saline solution (80 ml) in order to remove the circulating blood, followed by 250 ml of a fixative containing 2.5% paraformaldehyde, 2.5% glutaraldehyde and 2% lanthanum nitrate in 0.1 M sodium cacodylate buffer, pH 7.2), which was filtrated before perfusion. After perfusion, the animals were maintained at 4ºC for 12 to 18 h to avoid artifactual changes to tissue. Then, fragments of cerebella (0.5 - 1 mm) were immersed in the same fixative for 2 h at room temperature (RT). The samples were washed with a glucose solution (0.1 M sodium chloride plus 0.2 M sucrose) and post-fixed in 1% osmium tetroxide plus1% potassium ferrocyanide in glucose solution during 1 h in the ice. After rinsing with glucose solution, the samples were dehydrated in an ethanol crescents series until 1:1 ethanol/acetone (v/v) and three pure acetone baths for 30 minutes each. Then, they were embedded in 1:1 acetone/resin for 4 h and pure resin for 24 h under rotation, followed by pure resin at 60ºC for 72 h for polymerization. Semi-thin 0.5 µm thick sections obtained with glass knives taken with Supernova LKB ultra-microtome (Bromma, Suécia) were stained with 0.5% Toluidine Blue for light microscope selection of cerebella regions. Ultrathin 60 to 70 nm thick sections obtained with diamond knife in an Ultra-cut Reichert S, Leica ultra-microtome (Wetzlar, Germany) and collected on 200 mesh copper grids and contrasted with 2% uranyl acetate methanolic solution and 0.5% lead citrate aqueous solution.

Ultrastructure analyses were done with transmission electron microscopy (Zeiss LEO 906 operated at 60 kV).

Statistics

The data were analyzed using the Software Graph Pad Prism (San Diego, CA, USA) and were shown as mean ± standard error. Controls and PNV-groups were submitted to Student's *t*-test to address differences. A value of $p \leq 0.05$ was considered significant. ANOVA two-way followed by Bonferroni's test was conducted to assess the impact of treatment and the time elapsed from envenoming to euthanasia.

3. Results and Discussion

The venomous spider species, *Phoneutria nigriventer* (Lucas, 1988), represents a significant public health problem in Brazil. Clinically, the venom of the spider (PNV) causes neurotoxic manifestations evidencing damage to the peripheral and central nervous system; severe accidents can lead to convulsion and, while rarely, death (Bucaretchi et al., 2000, 2008). Animal venoms are sources of neurotoxic substances aimed at paralyzing and/or killing prey, or as a defense against predators (Lewis & Garcia, 2003). PNV contains a vast range of pharmacologically important neuropeptides affecting the physiology of the Na^+, K^+ and Ca^{2+} channels and causing neurotransmission disturbances (For review see Gomez et al., 2002).

Experiments in rats have largely mirrored the toxic signs observed in humans; cellular and molecular assessment has also shown that systemic venom causes BBB breakdown, principally in the hippocampus and cerebellum, although regionally and temporally heterogeneous in magnitude in the same brain region (Le Sueur et al., 2003; 2004; Rapôso et al., 2007). One of the criteria for indicating that the BBB has broken is an increase in solute and macromolecule traffic across the endothelium of brain microvessels. In fact, BBB breakdown from an increase of permeability is very often not a result of disruption of the integrity of the tight junction at the interendothelial cleft. Instead, the majority of studies have evidenced that failure of the transcellular transport system regulation is the initial mechanism responsible for the majority cases of increased

permeability at the BBB (Lossinsky and Shivers, 2004; Le Sueur et al., 2004; 2005; Soares et al., 2014).

Damage to the stability of BBB structure and physiology induces pathological conditions, which affect together or separately, in concert or otherwise, with the BBB transporters, basement membrane matrix and/or tight-adhesion junction interendothelial apparatus. A significant transcellular transport system mechanism at the BBB involves a special and dynamic delta-shaped domain of the endothelial plasma membrane enriched in cholesterol, glycosphingolipids and the scaffolding protein Cav-1. These lipid raft domains are named caveolae and their main marker is the caveolin-1 protein (Rothberg et al., 1992; Pelkmans and Helenius, 2002; Shajahan et al., 2004a).

In the present study, IF and WB assays were used to find that PNV either induced upregulation or downregulation of Cav-1 expression, independently of being elicited in the initial or late periods of envenoming. In the cerebella of control rats, Cav-1 labeling was identified in 8-12 μm width capillaries; the venules and arterioles were Cav-1 negative (Figure 1A); PNV altered time-dependently expression of the protein, but the only capillaries remained Cav-1+; the labeling peak was 1, 5 and 24 h after envenoming (Figure 1 B–F). WB data endorsed the dynamic alteration of Cav-1 expression, observed by IF. Significant increases were found at 1, 5 and 24 h, while decreases were found at 2 and 72 h (Figure 1 G–H). Two-way ANOVA revealed interactions between the variables time *vs.* treatment, meaning that the time elapsed after PNV exposure influenced venom effect ($p<0.05$).

Caveolin 1, the main molecular marker of the caveolae, is indispensable for the formation of caveolae and non clathrin-coated endocytosis; deletion of Cav-1 impedes caveolae formation (Razani et al., 2002) and interaction with other proteins for signal transduction (see, Quest et al., 2004), and accelerates neurodegeneration (Head et al., 2010). Both the downregulation and upregulation of Cav-1 expressed in the capillaries of PNV- treated rats implied increased and decreased density of caveolae on the surface of endothelial cells and interference in plasma membrane dynamics. In fact, transmission electron microscopy observation showed an increase in vesicle attachment to both the ad-luminal and ab-luminal membrane surface, thus evidencing vesicle trafficking across the cerebellar endothelium of PNV administered rats (Figure 2). Ultrastructural findings

corroborate IF and WB data, thus suggesting that increases in Cav-1 expression implie caveolae neoformation, whilst Cav-1 reduction implies caveolae flattening. Alternatively, up and down regulations of Cav-1 indicate the existence of reassembly/disassembly cycles throughout envenomation periods.

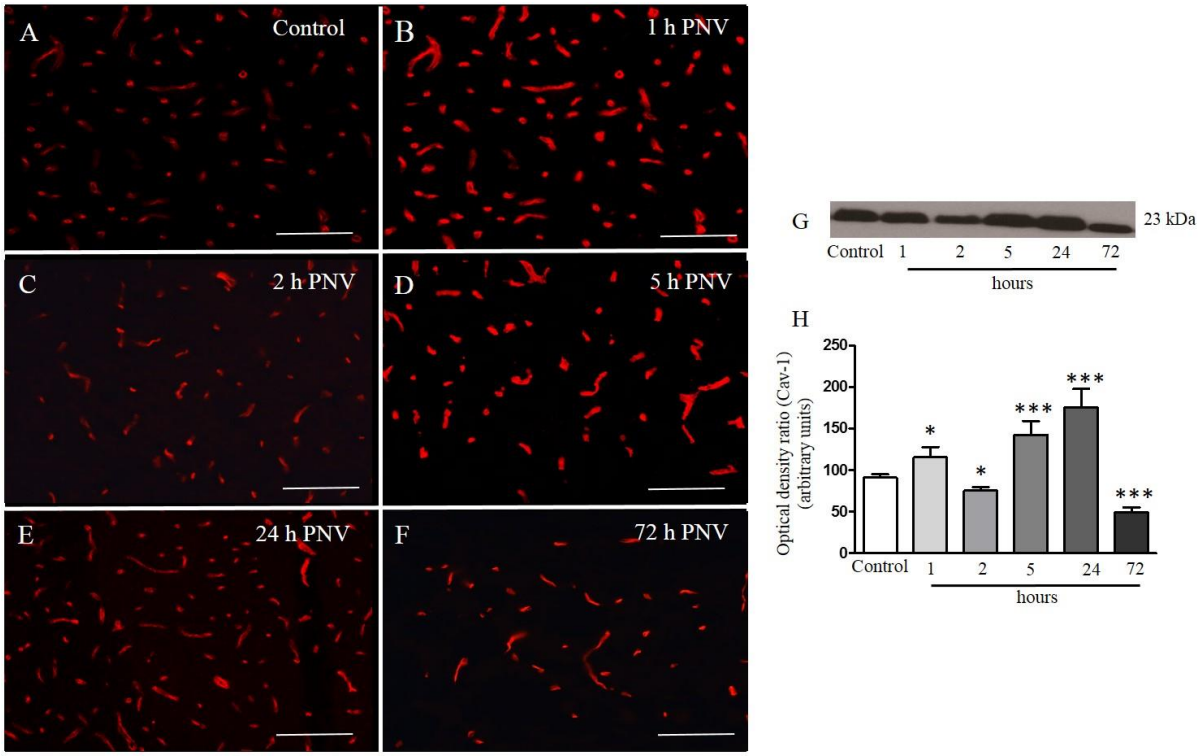

Figure 1: Immunolabeling of caveolin-1 (Cav-1) in the cerebellum of rats injected i.v. with saline (**A**) or with PNV (**B – F**); Cav-1 blots and normalized (β-actin) band histogram (**G – H**) in cerebella homogenate**.** Data is mean ± SE; Student *t*-test; * p≤0.05; ** p≤0.01; *** p≤0.001 relative to control. Bars: 40 µm.

Such caveolae dynamics imply tensional changes and mechanical stressing of the endothelial membrane (Nassoy and Lamaze. 2012). Mechanical stress of the endothelial plasma membrane of brain capillaries is an important stimulus for triggering signaling mechanisms (Boyd et al., 2003), as it is supposed occurred in the present experimental model.

Caveolae-derived vesicles and internalization of caveolae content requires phosphorylation of caveolin-1 (pCav-1) and NH_2-terminal tyrosine residue by activation of the Src tyrosine kinase family (SKF) (Quest, 2004; Shajahan et al., 2004a). This also requires interaction with dynamin-2 (Dyn2), a protein that forms the caveolae neck and participates in vesicle detachment and internalization (Henley et al., 1998; Yao et al.,

2005). In this context, it was tested if PNV induces phosphorylation of Cav-1 (pCav-1) and alters SKF expression.

SKF cytoplasmic immunolabeling was detected in cells of the granular and molecular layers; relative to controls, PNV-administered rats showed upregulation of SKF mainly at 2, 5 and 72 h; while some venules were anti-SKF + (Figure 3 A–F). WB of total SKF and pCav-1 in cerebella homogenate revealed increases at 2 and 72 h after envenoming and decreases at 1, 5 and 24 h after PNV (Figure 3 G–J**)**. Two-way ANOVA revealed that expression of SKF and pCav-1 expression is affected by the variable time ($p \leq 0.05$).

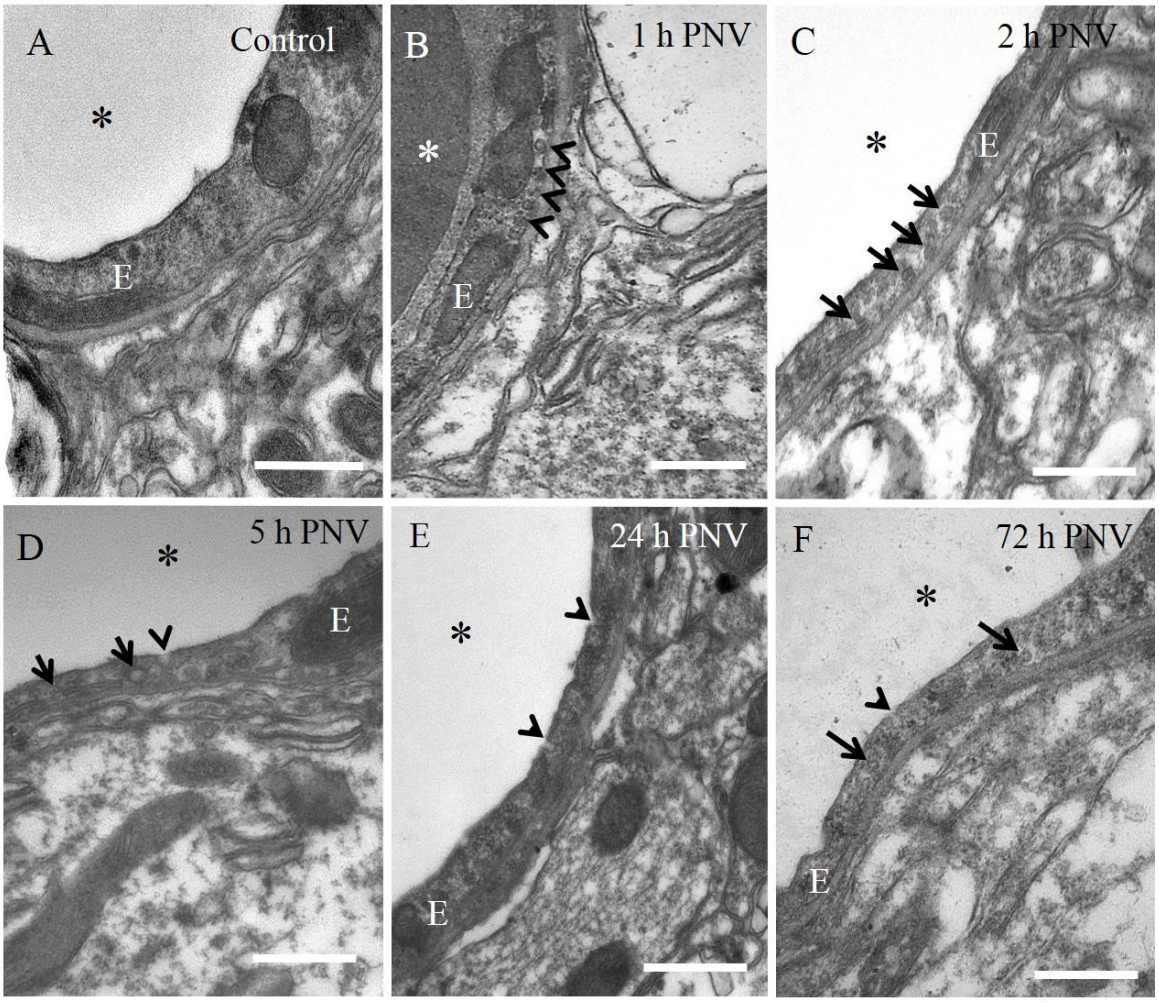

Figure 2: Transmission electron microscopy images of the endothelium in the cerebellum of control animals **(A)** and at different time points following PNV **(B-F)**. Asterisks = endothelial lumen; *arrows* show vesicles attached to ad-luminal and ab-luminal membrane aspects; *arrowheads* show vesicles influx; E: endothelium. Bar: 500 nm.

The findings of the present study showed that PNV-induced alternate phosphorylation/dephosphorylation of Cav-1 coupled to SKF upregulation/downregulation. This data suggests the triggering of a pathway to vesicular trafficking across the endothelium; the findings suggest discontinuous vesicle shortage and caveolae internalization over time.

SKF activation also has a role in Dyn2 participation in the disassembly/reassembly of the caveolae by flattening (detachment from plasma membrane) and neck reestablishment of the membrane vesicle (Shajahan et al., 2004b). Dyn2 was found to interact directly with Cav-1 and interfere in the process of caveolae internalization (Yao et al., 2005).

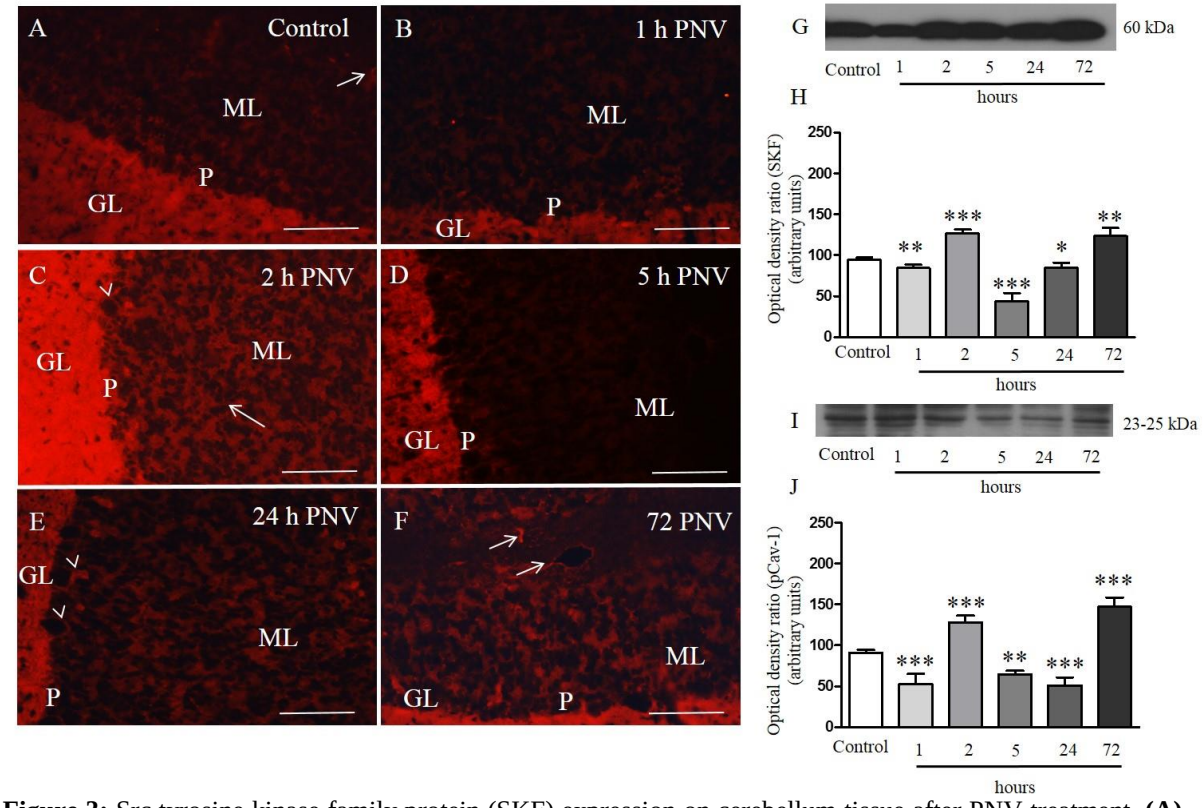

Figure 3: Src tyrosine kinase family protein (SKF) expression on cerebellum tissue after PNV treatment. **(A)** Control group; and **(B-F**) PNV groups; **(G** and **I**) SKF and phosphorylated caveolin-1 (pCav-1) blots and **(H and J**) respective histogram depicting the density level of both proteins after normalization. Data is mean ± SE; Student *t*-test; *p≤0.05; **p≤0.001; ***p≤0.001 relative to control. **ML**, molecular layer; **GL**, granular layer; **P**, Purkinje layer; *Arrows*: endothelial labeled. *Arrows head*: Purkinje cells labeling. Bars: 40 µm.

WB data of the total protein expression of Dyn2 increased at 5 and 24 h after PNV, and decreased at 2 and 72 h. Dyn2 immunofluorescence appeared discontinuous on the

cerebellum vessels (Figure 4 A– H). Two-way ANOVA revealed that the time since PNV injection affects Dyn2 expression ($p \leq 0.05$). Curiously, the histogram profile which depicts the changes of Cav-1 over time mirrored that of Dyn2 exactly (compare Figure 1 H with 4 H).

Dyn2 is ubiquitously expressed in a variety of vesicle structures, including synaptic, endocytic, phagocytic, and vesicles budding from Golgi lamellae and forming the neck of caveolae (González-Jamett et al., 2014). Such strategic locations have a direct role in membrane severing for the release of vesicles (Henley et al., 1998). In the CNS, besides having a role in caveolae budding (Oh et al., 1998) at the BBB, Dyn2 participates in synaptic vesicle recycling, post-synaptic receptor internalization, neurosecretion, and preservation of synaptic transmission (González-Jamett et al., 2014, see for review; Yao et al., 2005). Dyn2 is a large mechanochemical GTPase which is found to interact with multiple cellular proteins, including cytoskeleton proteins (Yao et al., 2005). It is suggested that alternate increases-decreases of caveolae numbers in the endothelium of the cerebella capillaries remodels the lipid raft membranes microdomain containing Cav-1, and creates oscillatory frictional forces at the capillary wall, triggering or inhibiting cell signal mechanisms. In fact, a number of studies report that caveolae is a mechanosensor of shear stress, and describe a direct relation between their luminal location and role in endothelial mechanosensing (Boyd et al., 2003; Nassoy and Lamaze, 2012; Parton and del Pozo, 2013; Rizzo et al., 2003). Shear stress also affects the activation pattern of ERK and AKT, two well-known shear-detecting signaling molecules (Boyd et al., 2003).

In the present study, the matched upregulation/downregulation of Dyn2 and Cav-1 over time suggests that the proteins that form both parts of caveolae, *i.e.* the neck and vesicle body, have a synchronous pace in response to PNV. Intriguingly, Dyn2 (and thus Cav-1) upregulation/downregulation was asynchronous to the SKF and pCav-1 counterparts (compare the histogram of Figures 3 H and 3 J with the histogram of Figure 4 H). It is suggested that by caveolae disassembling (SKF/pCav-1 activation) and caveolae reassembling (increased expression of Cav-1 and Dyn2) the hemodynamics are altered, thus provoking mechano-transduction of signals that affect the regulation of BBB permeability.

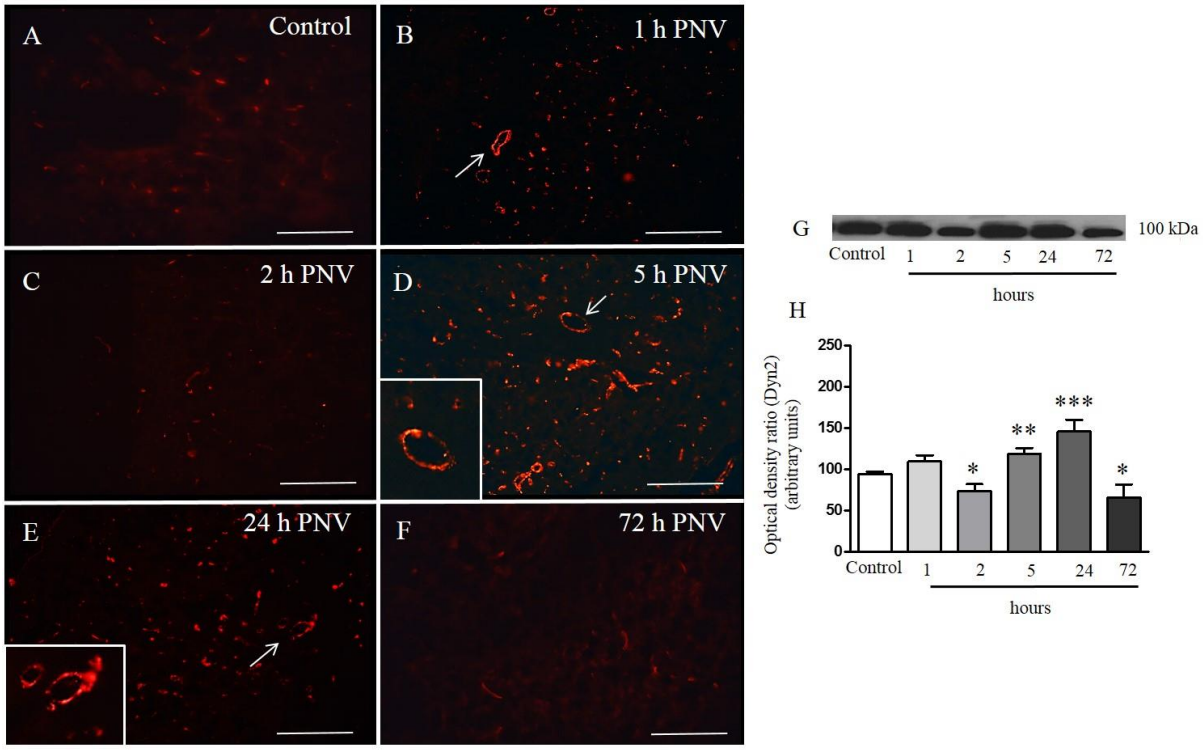

Figure 4: Dyn2 protein expression on cerebellum tissue after PNV treatment. Dyn2 labeled in control groups **(A)** and in PNV **(B-F).** (**G-H**) Din2 blots and respective histogram depicting the density level of both proteins after normalization. Data is mean ± SE; Student *t*-test; *p≤0.05; **p≤0.001; ***p≤0.001 relative to control. Bars: 40 µm.

Shear stress resulting from changes in hemodynamic parameters also affects endothelial nitric oxide synthase (eNOS). Interestingly, eNOS is also a signaling molecule compartmentalized inside the caveolae microdomain (Rizzo et al., 1998), a tactical location for mechanosensing and signal transduction. Research by the authors' group in a similar rat model and using the same experimental design has shown that eNOS is inactivated at periods of intense PNV toxic manifestation because PNV disrupts its active dimeric form, concurrently with increases of intracellular calcium indirectly inferred by a PNV-induced rise in calmodulin (Soares et al., 2014b). eNOS was shown to be negatively regulated by Cav-1 (García-Cardeñas et al., 1997), but eNOS inhibition is blocked by a rise in intracellular calcium levels. Taken together, it can be supposed that at periods of signs of serious envenomation in animals, the lesser availability of nitric oxide on one side and the positive regulation of endocytosis and transcytosis on the other side, contributes to BBB enhanced permeability and vascular dysfunction in response to PNV. Also, the labeling of

neurons of the Purkinje, granular and molecular layers in the cerebellum of PNV-treated rats, as shown earlier (Soares et al., 2014a) reinforces the roles of Cav-1 and Dyn2 in numerous steps related to the neuronal intracellular signaling pathway (Francesconi et al., 2009; García-Cardeñas et al., 1997; Gaudreault et al., 2005; Head et al., 2011; Stern and Melmerstein, 2010).

Research from the laboratory of the authors showed that together with BBB breakdown, PNV decreases laminin from the endothelial basement membrane (Rapôso et al., 2007; Mendonça et al., 2013) and increases the expression of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) in the cerebellum and hippocampus (Cruz-Höfling et al., 2009). During episodes of brain injury, activation of matrix metalloproteinases (MMPs) degrades the extracellular matrix covering the blood microvessels and induces permeabilization of BBB (Gu et al., 2011). A recent study established a link between MMP9 activity and caveolar transcytosis and BBB permeation (Muradashvili et al., 2014). The authors reported a direct relation of caveolae formation, assessed by increased Cav-1 expression and activation of MMP9, and in contrast, the preservation of BBB integrity by inhibiting MMP9 activity. In the current study, the PNV effects on MMP9 expression were assessed to confront the expression of Cav-1, pCav-1, Dyn2 and SKF in the cerebellum of PNV-treated rats.

MMP9 protein expression increased at 1, 5 and 24 h after PNV exposure, was unaltered at 2 h and decreased at 72 h (Figure 5 G–H). The labeling of MMP9 was unspecific due to the matrix protein location; PNV intensified anti-MMP9 detection, which peaked at 24 h and substantially declined at 72 h (Figure 5 A–F). Two-way ANOVA revealed interactions between the variables times *vs.* treatment in the expression of MMP9, meaning that time elapsed after PNV exposure influenced the venom effect ($p \leq 0.05$).

The findings showed that MMP9, Dyn2 and Cav-1 expression was synchronously up- and downregulated in response to PNV, and was asynchronous to SKF and pCav-1 expression. The data showed a direct relation between the peaks of MMP9 expression and those of caveolae formation, assessed by peaks of Cav-1 and Dyn2 expression. The peaks of MMP9, Cav-1 and Dyn2 coincided with minor expressions of pCav-1 and SKF. The data suggested that PNV induces a pro-inflammatory phenotype and triggers Cav-1 signaling,

for a process of endocytosis and trancytosis and increased permeability at the blood-brain boundary.

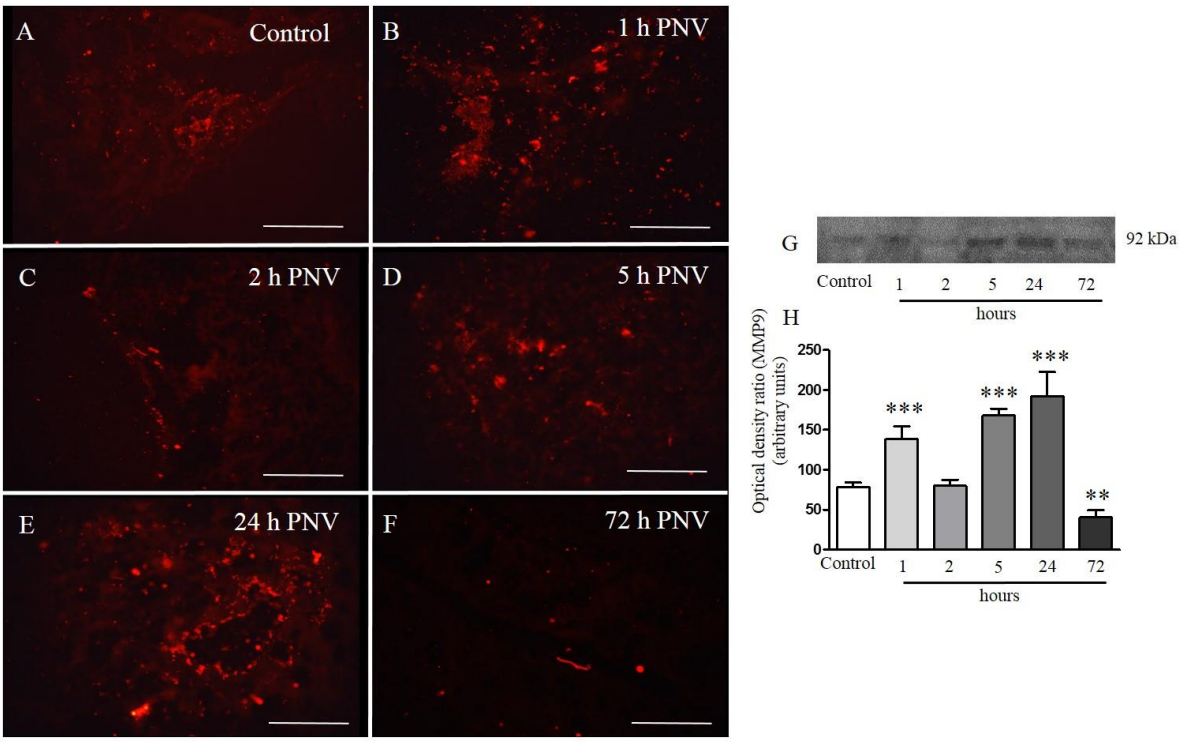

Figure 5: MMP9 protein expression on cerebellum tissue after PNV treatment. MMP9 expression in control group **(A)** and in PNV **(B-F). (G-H)** MMP9 blots and respective histogram depicting the density level of both proteins after normalization. Data is mean ± SE; Student *t*-test; *p≤0.05; **p≤0.001; ***p≤0.001 relative to control. Bar: 40 µm.

In summary, the present study provides evidence of how the venom of *Phoneutria nigriventer* can induce a pro-inflammatory phenotype and triggers signals for caveolae formation and enhancement of transport across the BBB. It is suggested that by caveolae disassembling (SKF/pCav-1 activation) and caveolae reassembling (increased expression of Cav-1 and Dyn2) the hemodynamics are altered, thus provoking the mechano-transduction of signals that affect BBB permeability. Caveolae dynamics have a role in shear stress and BBBb regulatory mechanisms, and can be a relevant target for therapeutic purposes following armed-spider envenomation.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

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CAPÍTULO 3

eNOS uncoupling in the cerebellum after BBB disruption by exposure to *Phonuetria nigriventer* venom.

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Abstract

Numerous studies have shown that the venom of *Phoneutria nigriventer* (PNV) armed-spider causes excitotoxic signals and blood-brain barrier breakdown (BBBb) in rats. Nitric oxide (NO) is a powerful signaling molecule which has a role in endothelium homeostasis and affects vascular health. The present study investigated the pathophysiological relevance of endothelial NO synthase (eNOS) uncoupling to clinical neurotoxic evolution induced by PNV. eNOS immunoblotting of cerebellum lysates processed through low-temperature SDS-PAGE revealed significantly increased monomerization of the enzyme, at critical periods of severe envenoming (1-2 hours), whereas eNOS dimerization reversal paralleled amelioration of animals condition (5-72 hours). Moreover, eNOS uncoupling was accompanied by increased expression in calcium-sensing calmodulin protein and calcium-binding calbindin-D28 protein in cerebellar neurons. It is known that greater eNOS monomerization than dimerization implies the inability of eNOS to produce NO leading to superoxide production and endothelial/vascular barrier dysfunction. It is suggested that detrimental transient eNOS deactivation and disturbances in calcium handling contributed to endothelial cell injury in the cerebellum vasculature of PNV-administered rats. We

suggest that eNOS uncoupling compromises the enzyme capacity to respond properly to shear stress contributing to perivascular edema and it is one of the mechanisms involved in the transient BBBb promoted by PNV.

Keywords: blood-brain barrier, calmodulin, calbindin-D28, cerebral microvessels, eNOS dysfunction, spider venom

Abbreviations: CaM, calmodulin; CaB, calbidin-D28; Cav-1, type 1 caveolin; eNOS, endothelium nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NVu, neurovascular unit; PNV, *Phoneutria nigriventer* venom; WB, western blotting.

1. Introduction

Accidents caused by *Phoneutria nigriventer* (armed-spider), are the second most important cause of envenoming in the south east of Brazil. Accidents graded as severe cause excruciating pain, intense sudoresis, blurred vison, excitotoxic manifestations, priapism, cardiovascular and respiratory distress (Bucaretchi et al., 2000, 2008). The venom of *P. nigriventer* (PNV) contains neuropeptides acting on Ca^{2+}, K^{+} and Na^{+} channels and which affects acetylcholine and glutamate release and uptake (Fontana and Vital-Brasil, 1985; Love and Cruz-Höfling, 1986; Gomez et al., 2002). Experimentally, it has been shown that PNV transiently alters endothelial cell-cell and cell-matrix interactions and increases vesicular transendothelial transport causing dysfunction to the blood-brain barrier (BBB) and neurovascular unit (NVu) (Le Sueur et al., 2003, 2004, 2005; Rapôso et al., 2007, 2012, 2014).

The unique characteristics of the BBB provide a safe and controlled environment for neuronal and glial function. BBB endothelial cells tightly adhere to one another by a range of proteins and an entourage of astrocytic end-feet processes and pericytes that confer the brain microvessels with a high electric resistance and highly restrict vesicular transport. The BBB complex is controlled by a number of regulatory molecules that maintain endothelium homeostasis by responding to different stimuli (see Abbott, 2002 for review).

Despite the existence of a substantial body of knowledge related to the effects of PNV on BBB cell biology (Le Sueur et al., 2003, 2004, 2005; Cruz-Höfling et al., 2009, 2007; Rapôso et al., 2007, 2012, 2014; Mendonça et al., 2011, 2012, 2013; Stávale et al., 2013; Soares et al., 2014), it has not yet been confirmed which signaling molecule is involved in the mediation of the transitory opening of the BBB. Nitric oxide (NO) is a ubiquitous messenger with pleiotropic effects in different tissues. It was hypothesized that endothelial nitric oxide enzyme (eNOS) is a potential target for PNV, and that rates of the monomer and dimer forms of the enzyme could run parallel to the stages of intoxication/amelioration following envenoming.

NO produced by eNOS plays a key role in hemodynamics by regulating blood flow and blood pressure through the control of vasodilation and vascular resistance, platelet adhesion and aggregation, and leukocyte-to-endothelium interaction. A number of pathological settings disturb such nitrergic control, resulting in endothelial and vascular dysfunction (Iadecola, 1997; see Atochin and Huang, 2010). In the brain, the proper functioning of the eNOS/NO system accounts for the microenvironment homeostasis needed for normal operative neuron and glial cells. Several studies correlate incapacity of normal synthesis of eNOS derived-NO with enhanced production of reactive oxygen species, disruption of endogenous control of eNOs inhibitors and worsened pathological conditions (Toda et al., 2010). Defective eNOS expression and activity and/or NO reduction impairs endothelial and vascular homeostasis, with a corresponding effect on BBB and NVu functioning (Förstermann and Münzel, 2006; Beauchesne et al., 2009; Förstermann and Li, 2011; Santhanam et al., 2012).

The hypothesis that eNOS uncoupling is associated with the critical neurotoxic manifestations of envenomed rats and that recovery from a toxic condition is associated with a resumption of eNOS dimerization, which is mandatory for its catalytic activity (Persechini et al., 2013), was tested. To this end, eNOS monomer/dimer ratio and the expression of eNOS were accessed along with if changes in the expression of calcium regulatory-related proteins were concurrent to eNOS alterations, expressional alteration of calcium-calmodulin and calbindinD-28 was also assessed. Such findings were assessed at critical times in the clinical evolution of animals: early (1-2 h), intermediate (5 h) and late (24 and 72 h) periods of severe envenoming, onset of recovery and no clinical

manifestation of envenoming, respectively. The focus on the cerebellum is a result of well-established venom disruption on both the paracellular and transcellular pathway of the BBB, and the effect on components of NVu in this region of the brain (Cruz-Höfling et al., 2009; Rapôso et al., 2007; 2012, 2014; Mendonça et al., 2014; Stávale et al., 2013; Soares et al., 2014).

2. Materials and Methods

2.1. Animal, spider venom and experimental envenomation

Male Wistar rats (*Rattus norvegicus*) aged 6-7 weeks were obtained from the Central Animal Facility (CEMIB) of UNICAMP (Campinas, SP, Brazil). The lyophilized venom of the *Phoneutria nigriventer* (PNV) spider was stored at -20ºC and dissolved in sterile saline (dilution: 0.5 mg PNV/ml of 0.9% of sterile saline solution) at the moment of use. A single intra-vascular (i.v.) sub-lethal dose (0.5 mg PNV/Kg) of PNV or sterile saline solution was administered to male rats. The experiment featured six groups: one control euthanized 5 h after i.v. saline administration (n = 11, of which n = 8 were used for western blotting (WB), and n = 3 were used for immunofluorescence (IF)); and 5 groups (n = 40 rats) which received PNV and were euthanized at different times after envenomation (at 1, 2, 5, 24 and 72 hours) (n = 5 per time for WB; n = 3 per time for IF). The experiments were approved by the Unicamp Committee for Ethics in Animal Use (CEUA-Unicamp, protocol nº 3609-1) which follows the guidelines of the Brazilian Society for Laboratory Animal Science (SBCAL).

2.2. Western blotting (WB)

Animals were euthanized in a CO_2 chamber and had their heads cut off. The cerebellum of each animal was immediately removed and frozen in liquid nitrogen. Proteins were separated with an extraction cocktail and submitted to 6-8% SDS-PAGE (Rapôso et al., 2012). Nitrocellulose membranes were kept overnight in 5% bovine serum albumin for the blocking of unspecific epitopes. The primary antibodies used were rabbit polyclonal antibody against eNOS (1:400; BD Transduction Laboratories™, Franklin Lakes, NJ, USA), Calmodulin (1:500; Santa Cruz Biotechnology, CA, USA), and β-actin

endogenous control (1:1000; Sigma Aldrich, St. Louis, MO, USA). Corresponding secondary antibodies were used: anti-rabbit (1:1000; Sigma Aldrich) for eNOS and Calmodulin; and anti-mouse for β-actin (1:40000; Sigma Aldrich). The bands were visualized using a chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA). Differences were quantified by the density of pixels of each band using Image J 1.45s program (by Wayne Rasband, NIH, Bethesda, MD, USA). The corresponding β-actin expression value normalized the pixel density value of proteins.

2.3. Low-temperature WB

In order to determine the concentration of the active (dimer) and inactive (monomer) forms of the eNOS enzyme SDS-PAGE was performed at a low temperature (LT-PAGE), in accordance with the procedure described by Yang et al. (2009). Tissue preparation and protein extraction followed the WB technique described above; then sample preparation was performed with routine Laemmli buffer without β-mercaptoethanol in a water bath (at 37ºC) for 5 minutes. Next, the prepared samples were submitted to 6-8% gel for LT-PAGE. LT-PAGE was performed at 4ºC and the electrophoresis chamber was maintained in an iced bath the temperature of which did not exceed 15°C. After LT-PAGE, proteins were transfered to nitrocellulose membrane and incubation were performed as per typical WB of a primary eNOS antibody (for details see Western Blotting section, Rapôso et al., 2012).

2.4. Immunofluorescence (IF)

The animals were euthanized under deep anesthesia and perfusion was performed through the left ventricle, with a needle clipping the aorta; first with 0.9% saline (50 ml) and then by 200 ml of 4% paraformaldehyde fixative in phosphate buffer (0.1 M; pH 7.4). The cerebellum was removed and kept in the same fixative for 2 h. It was then kept in 15% sucrose overnight and in 30% sucrose the next day. The material was immersed in OCT-Tissue Tek (Sakura Finetek, Torrance, CA, USA) and frozen in n-hexane in liquid nitrogen. The tissue was cryo-sectioned (5 μm thick) and sections were permeabilized with 0.1 % triton X-100 for 8 minutes. Unspecific reactions were blocked with 1% dry milk with 0.1% Tween 20 in Trizma® (Sigma Aldrich) buffer (0.05 M; pH 7.4) at room temperature (RT)

for 1 h. Sections were kept at 4ºC overnight in primary antibodies diluted in blocking solution: anti-Calbidin-D-28 (Sigma Aldrich), (see Western Blotting section for CaM and eNOS antibodies details). Respective anti-rabbit secondary antibody conjugated with anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), also diluted in blocking solution, was used for 45 minutes at RT. All reactions were interrupted and washed in Trizma® buffer. The slides were prepared with commercial media (Vectashield, Vector Labs, Burlingame, CA, USA) and examined using a fluorescence microscope (Olympus BX51 photomicroscope, Tokyo, Japan) using DAPI for nuclei labeling (not shown).

2.5. Statistics

All data were analyzed with the Graph Pad Prism software (San Diego, CA, USA) and represented as mean ± SEM. Groups (controls *vs*. PNV) were compared using the Student's *t*-test. Values of $p \leq 0.05$ were considered significant.

3. Results and Discussion

By using the same venom batch used in the experimental model of PNV-induced BBB breakdown in the cerebellum (Rapôso et al., 2007, 2012, 2014; Stávale et al., 2013) it was possible to demonstrate that the venom induced a transient eNOS uncoupling and alterations to eNOS, calmodulin and calbindin-D28 expression. The PNV used was from the venom batch that induced neuronal activation and astrocyte reaction (Cruz-Höfling et al., 2007; 2009; Rapôso et al., 2007) and enhanced BBB permeability (Raposo et al, 2012, 2014).

3.1. eNOS uncoupling

To assess whether eNOS could be uncoupled after the administration of PNV the eNOS monomer/dimer ratio was evaluated. As shown in Figure 1A,B,C,D, low temperature SDS-PAGE revealed a significant increase in the eNOS monomers in comparison with eNOS dimers in the cerebellum of PNV-treated rats. By dividing eNOS monomer content (Figure 1B) from that of eNOS dimers (Figure 1A), which is represented by the bands on panel 1C, it was found that the eNOS monomer/dimer ratio varied from 1 to 72 h in the

cerebellum lysates of PNV-treated rats. Panel 1D shows that the growing proportion of eNOS monomers significantly exceeded eNOS dimers in periods of severe intoxication (by 24% at 1 h and 42% at 2 h); the eNOS monomer to dimer ratio was equitative both with each other and with control when signs that recovery was underway (5 h), and the eNOS dimer level significantly surpassed its monomer counterparts (by 55% at 24 h and 51% at 72 h) in periods where there was no visible clinical neurotoxic condition.

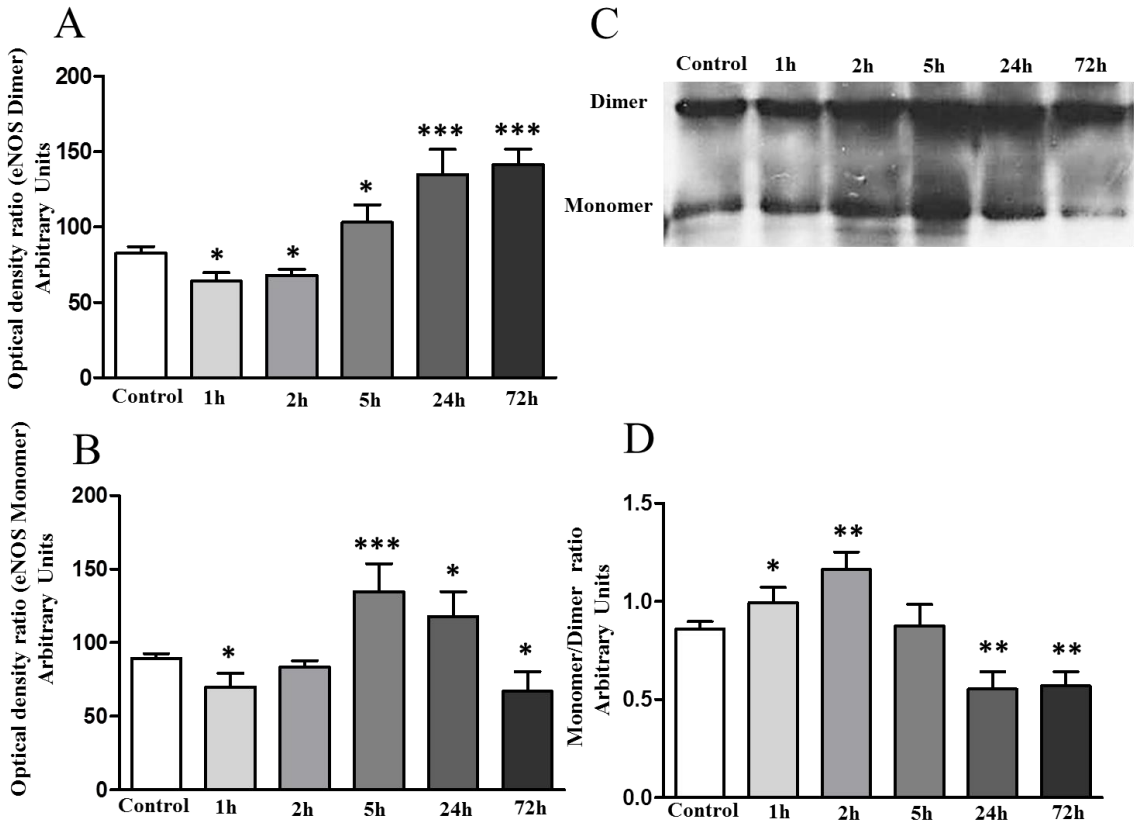

Figure 1: Representation of low-temperature western blotting for eNOS monomer and dimer expression at the time-points studied (1, 2, 5, 24 and 72 h). **(A** and **B) -** Representative graph of eNOS dimer and eNOS monomer expression, respectively. **(C)** - eNOS dimer (280 kDa) and eNOS monomer (140 kDa) bands obtained at low-temperature SDS-PAGE. **(D)** - proportion between monomer/dimer reveals eNOS uncoupling at 1 and 2 h; onset of eNOS recoupling at 5 h and higher rate of eNOS dimer at 24 and 72 h. Significance set at *p≤0.05; **p≤0.01; ***p≤0.001; Data is mean ± SEM, unpaired Student *t*-test.

NO produced by eNOS is a regulatory signaling molecule produced in abundance by endothelium and vascular smooth muscle cells, and has a central role in the control of vascular tone. Such function deteriorates through eNOS uncoupling, and is characterized by increased monomerization, leading to a decline in NO bioavailability and enhanced

production of superoxide (Domenico, 2004; Masano et al., 2008; Münzel et al., 2005; Sabri et al., 2011; Yang et al., 2009). Dysfunctional eNOS impairs endothelium-mediated relaxation and promotes vascular dysfunction. In addition, eNOS uncoupling causes instability of the BBB steady-state by generating oxidative stress (Beauchesne et al., 2009; Förstermann and Münzel, 2006; Förstermann and Li, 2011; Santhanam et al., 2012). The present data revealed transient eNOS uncoupling evidenced by a high rate of eNOS monomers occurring during early stages of severe PNV intoxication. Interestingly, the resume of the functionality of enzymes shown by increasing rates of eNOS dimers, in an active form, parallels periods of amelioration caused by a toxic condition in animals. It is suggested that eNOS uncoupling after PNV reduced NO bioavailability, and in its place enhanced the production of superoxide, contributing to the severe intoxication seen during the acute stages of PNV envenoming.

Phoneutria nigriventer venom contains a complex mixture of substances possessing either synergistic or antagonistic effects; some PNV components and PNV effects are capable of activating eNOS. For example, excitotoxic neuropeptides contained in venom are ion channel-acting neurotoxins, the pharmacological activity of which blocks K^+ and Ca^{2+} channels and activates and/or delays inactivation of the Na^+ channels (Fontana and Vital Brazil, 1985; Cruz-Höfling et al., 1985; Gomez et al., 2002) either resulting in membrane depolarization and increase of intracellular Ca^{2+} and glutamate release (PhTX2 toxins from PNV) (Romano-Silva et al., 1993), or in the blocking of Ca^{2+} channels and defective Ca^{2+} and glutamate uptake (Tx-3-4 toxins from PNV) (Mafra et al., 1999; Reis et al., 2000; see Gomez et al., 2002 for review). Disturbances of calcium management affect constitutive calcium-dependent eNOS function. Similarly, glutamate handling disturbances affect NO production by constitutive NOS isoforms (Garthwaite et al. 1988; Dawson et al., 1991).

Moreover, PNV induces edema formation and vessel permeability in rabbit skin by potent activation of the tissue kallikrein-kinin system (Marangoni et al., 1993). Also, hyperalgesia caused by intraplantar PNV injection in rats is mediated through a spinal mechanism involving induction of central neurokinins and excitatory amino acids (Zanchet et al., 2004). In the brain, PNV causes neuroinflammation (Cruz-Höfling et al., 2009). Kinins produced in endothelial compartments are known to be a strong eNOS activator and

inducer of endothelium permeability (see Domenico, 2004). In conjunction, the findings of the present study support the hypothesis that the pharmacological attributes of PNV tend to promote disarray in the physiologic production of NO derived from eNOS, thus contributing to the instability of BBB function in the cerebellum (Rapôso et al., 2007, 2012, 2014), resulting in neuroexcitotoxic manifestations.

Contrastingly, increased intracellular calcium as induced by some PNV toxins (Romano-Silva et al., 1993), promotes the binding of calmodulin to eNOS enzyme resulting in catalytic activity (Förstermann and Sessa, 2012). To investigate whether eNOS dysfunction was accompanied by an imbalance in calcium handling, expression of eNOS and calmodulin (CaM) was evaluated by western blotting.

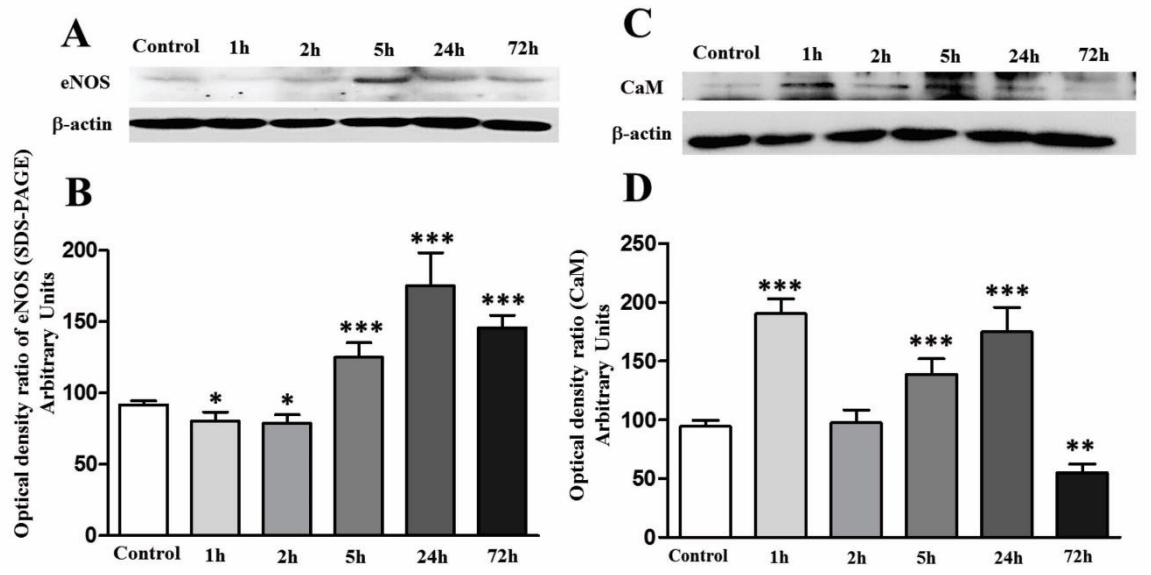

Figure 2: *eNOS and CaM alterations addressed through routine SDS-PAGE western blotting assayed at different time-points after envenoming.* (**A**) Blots of eNOS (140 kDa) and β-actin endogenous control (42 kDa). (**B**) Graphic representation of eNOS resulting from densitometric measurement expressed in arbitrary units. (**C**) Bands of CaM (17 kDa) and β-actin endogenous control. (**D**) Graph resulting from CaM blots measurements expressed in arbitrary units. *p≤0.05; **p≤0.01; ***p≤0.001 relative to control baseline; Student *t*-test; the data represents means ± SEM.

3.2. eNOS and Ca^{2+}/Calmodulin expression

The same eNOS antibody and cerebellum lysates used for examining eNOS uncoupling were employed for assessing eNOS and CaM expression. PNV induced a moderate decrease of eNOS at early stages of envenoming (nearly 20% at 1 and 2 h), which

was concurrent with a transient 100% marked increase in the level of CaM at 1 h, and subsequently a return to baseline at 2 h. Animals showed intense neurotoxic manifestation at this early period of envenoming. During intermediate envenoming periods (5 h), when animals showed signs of recovery, eNOS and CaM increased by 36% and 47%, respectively. During late envenoming (24 and 72 h), the animals were alive and no longer showed signs of envenoming; at the same time there was a 90% and 60% increase in eNOS level and an 85% increase followed by a 42% decrease in CaM level, at 24 and 72 h respectively. The data obtained from measuring eNOS and CaM protein expression is summarized in Figure 2A, B, C, D; the clinical manifestation was previously described (see also Le Sueur et al., 2004; Rapôso et al., 2012; Mendonça et al., 2013).

Endothelial cells and eNOS regulate vessel tone through a complex and not yet completely understood signaling mechanism that is dependent on the intracellular calcium/calmodulin interaction (Michel and Vanhoutte, 2010) and other regulators, including caveolin-1 (García-Cardeña, et al., 1997). CaM is a calcium binding protein that is essential to eNOS dimerization and the proper functioning of enzyme (Persechini et al., 2013), thereby contributing to balanced BBB and brain homeostasis (Bock et al., 2013). eNOS activation and NO production requires increases in intracellular calcium levels or Akt signaling, thus promoting the interaction of eNOS to a series of molecules such as calmodulin (CaM), a calcium sensing protein. On the other hand, low extracellular calcium bioavailability induces brain endothelium permeability (Bock et al., 2012).

The findings of the present study showed that eNOS and CaM were physiologically at the same level of abundance in the cerebellum of control rats. PNV disrupted the physiologic homeostasis of calcium and vascular endothelium; at periods of severe PNV intoxication and eNOS uncoupling, there was a burst of CaM expression (1 h) and a decrease of about 20% in eNOS expression; subsequently, when eNOS functionatity was resumed (increasing eNOS dimerization levels) eNOS/CaM expressions increased (5 and 24 h) and then further decreased (72 h) towards physiological levels. The findings imply that local regulation of cerebellar circulation can be placed under stress in response to PNV exposure. It is suggested that such alterations could be implicated in BBB permeability during oxidative stress episodes, as has already been observed (Fischer et al., 2005; Förstermann and Münzel, 2006; Beauchesne et al., 2009; Förstermann and Li, 2011;

Santhanam et al., 2012). This subsequently reflects in the clinical evolution of PNV envenomation.

To substantiate the data obtained from WB analysis, anti-calmodulin, anti-calbindin-D28 and anti-eNOS labeling was assessed by immunofluorescence in the cerebellar tissue.

3.3. Immunofluorescence (IF) of calmodulin, calbindin-D28 and eNOS

The immunolabeling and distribution of these proteins was compared in saline-(control) and PNV-treated groups (Figure 3). The IF findings revealed CaM labeling, primarily in the Purkinje and molecular layers of rats treated with saline (panel A). The variations in CaM labeling matched with those of the western blotting data, weak labeling relative to the control during the early stages of envenoming, followed by an increase (5 and 24 h) and subsequent decrease (72 h) (Figures 3B-F). Another calcium sensing protein, calbindinD-28 (CaB), confirmed that the molecular and Purkinje layers retained the most labeling intensity. Anti-CaB labeling was prominent in the Purkinje neurons (panel G). PNV induced a marked increase in anti-CaB labeling until 24 h, and thereafter labeling decreased (Figure 3 H-L). Anti-eNOS labeling was restricted to the endothelium vessels; PNV transiently increased eNOS labeling intensity which remained upregulated in relation to control (Figure 3M-R).

The enzyme eNOS is compartmentalized in caveolae (Rath et al., 2009) special regions of the cell plasma membrane, that are rich in cholesterol and sphingolipids and a coated protein, caveolin-1 (Rothberg et al., 1992). The caveolin/eNOS interaction is inhibitory (García-Cardeña, et al., 1997). The blood vessels of Cav-1 deficient mice show dysfunctional caveolae and impaired endothelium-dependent relaxation (Drab et al., 2001). Calmodulin binding in response to increased intracellular calcium interrupts caveolin inhibition, allowing electrons to flow between the oxygenized domain of one monomer to the reductase domain of another monomer, which fully activates eNOS and leads to NO synthesis (Panda et al., 2002). Cav-1 is a target for PNV in the cerebellum after envenomation (Soares et al., 2014). PNV increased vessels labeled with Cav-1 and promoted upregulation of the protein in the entire cerebellar tissue. Interestingly, the peak of Cav-1 expression was at 5 h, which coincides with the highest levels of eNOS monomer

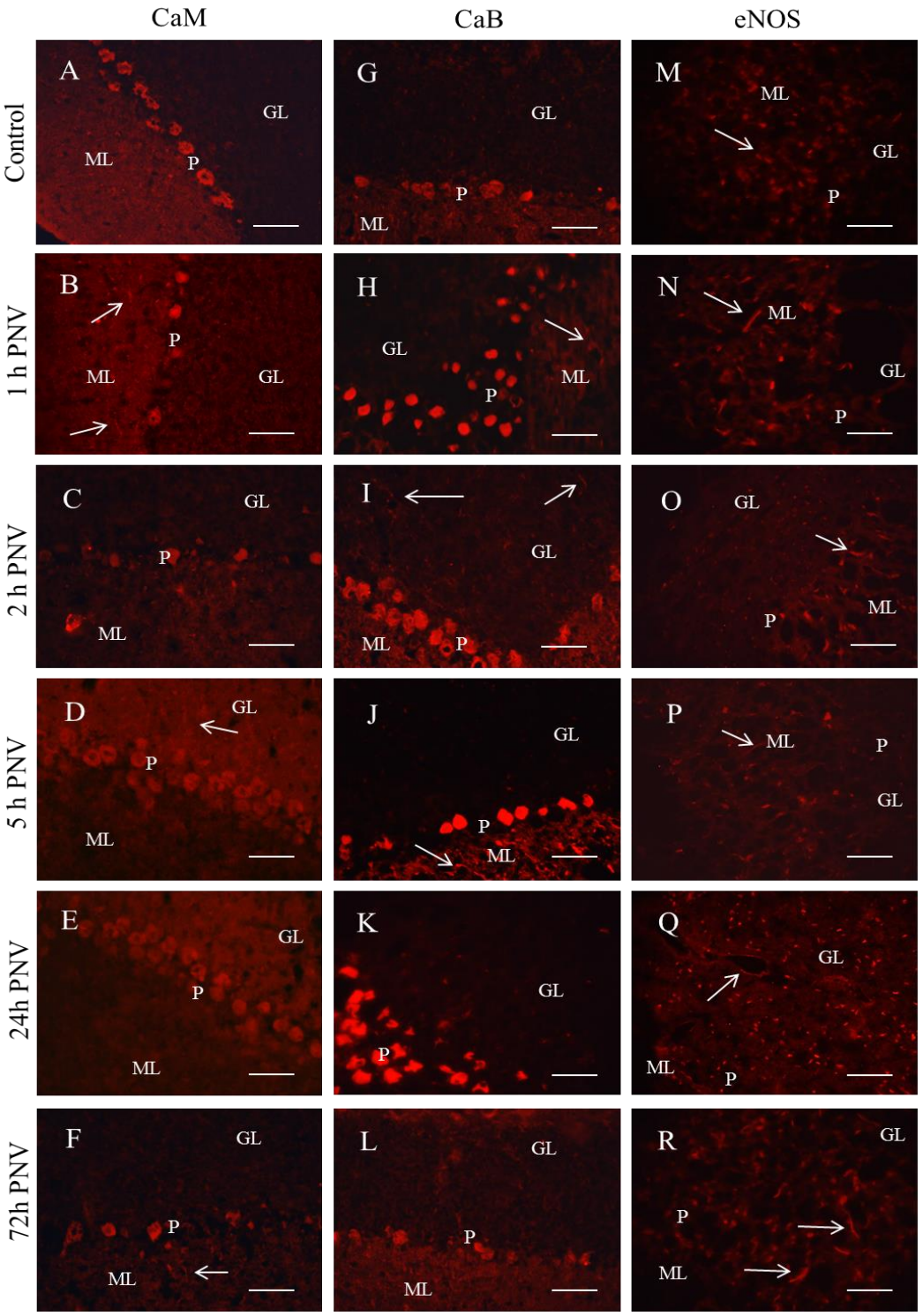

Figure 3: *CaM, CaB and eNOS immunofluorescence in the cerebellum of rats 5 h following sterile saline exposure and at 1, 2, 5, 24 and 72 h after P. nigriventer venom exposure*. CaM (panels **A** to **F**) and CaB (panels **G** to **L**) labeling occurs in neurons of the granular, molecular and Purkinje layers. CaB location is similar to the CaM location but differs in the levels of expression at the time points examine d. eNOS labeling occurs in the endothelium wall in all layers of the cerebellum (panels **M** to **R).** *Arrows*: endothelium labeled for CaM, CaB and eNOS. Scale bar: 40 µm.

(Figure 1B), whereas Cav-1 expression declines at 24 h, a period which has the highest levels of eNOS dimer (Figure 1A). The data could represent a balance between calmodulin stimulatory effects *versus* caveolin inhibitory effects on the functionality of eNOS in the cerebellum of rats administered with *P. nigriventer* venom.

4. Conclusion

In conclusion, *Phoneutria nigriventer* venom (PNV) induced transient eNOS uncoupling and alterations in the proteins involved in calcium handling. Uncoupling of eNOS occurred in parallel to the severe toxic manifestation of rats administered with PNV. The rise in eNOS monomers implies endothelium and vascular dysfunction. Recoupling of eNOS was concurrent with amelioration of the toxic condition and eventually with absence of signs of envenoming in animals. This implies a capacity to resume NO production, and thus the potential to maintain vascular health. PNV is a rich source of bioactive neuropeptides affecting the physiology of Ca^{2+}, K^{+} and Na^{+} channels and acetylcholine and glutamate release and uptake. Experimentally, PNV impairs the controlled BBB trancellular and paracellular trafficking of substances at the blood-brain interface. It is suggested that transient eNOS uncoupling may contribute to the toxic condition of animals due to a decline in NO production and thus the inability to maintain homeostasis of endothelium and vascular function. It is suggested that eNOS monomer-dimer fluctuation and alterations in calcium handling in the course of envenoming has an implication on endothelial dysfunction with a likely repercussion in venom induced BBB breakdown. Such time-related effects may partially explain the self-limiting toxic manifestations exhibited by rats, which evolve from intense intoxication (1-2 h) to complete absence of clinically identified toxicity (24-72 h). It is proposed that eNOS-derived NO is a neuromodulator that participates in BBB control in the cerebellum of PNV-treated rats. This is the first evidence of eNOS uncoupling in the cerebellum of PNV-exposed rats.

Conflict of interest

The authors declare no conflict of interest exists.

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CAPÍTULO 4

Are synchronized changes in Cx43 and Cav-3 a bystander effect in a *Phoneutria nigriventer* venom model of blood-brain barrier breakdown?

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Abstract

Caveolin-3 (Cav-3) is the main structural protein of the caveolar lipid raft membrane domain in astrocytes, an organelle involved in critical signaling pathways. However, few studies have examined the regulation of Cav-3 expression in glial cells, although Cav-3 up-regulation in astrocytes has been associated with worsening of neurological disorders. Similarly, few studies have investigated the changes in Cav-3 and Cx43 expression and activity after brain injury. In this work, we used *Phoneutria nigriventer* spider venom (PNV) to induce blood-brain barrier breakdown (BBBb) in rats in order to investigate Cav-3 and Cx43 expression in the cerebellum over critical periods of envenomation. Immunofluorescence (IF), western blotting (WB) and transmission electron microscopy (TEM) were used to assess changes at 1, 2, 5, 24 and 72 h post-venom. WB showed immediate increases in Cx43 and Cav-3 at 1 h (interval of greatest manifestations of envenomation) that persisted at 5 h (when there were signs of recovery) and peaked at 24 h

when no signs of envenomation were detectable. At 2 h and 72 h, Cav-3 was down-regulated and Cx43 had returned to baseline. IF closely mirrored the changes seen in WB; Cx43 and Cav-3 were poorly expressed in astrocytes of control rats and Cx43 was also weakly expressed in Purkinje neurons and granule neurons. PNV markedly intensified Cx43 and Cav-3 immunoreactivity. Cav-3 co-localization to GFAP increased markedly in PNV-induced reactive astrogliosis, suggesting increased glial caveolae. TEM showed swelling of perivascular astrocytic end-feet and alterations in synaptic contacts that had generally resolved by 72 h. Based on these findings, we postulated that the down-regulation of Cav-3 at 72 h post-venom coupled to the return of Cx43 to baseline indicated a return to normal homeostasis. The results of this investigation indicate that in rats PNV causes important changes in the expression of Cx43 and Cav-3 that apparently contribute to changes in BBB permeability.

Keywords: astrocytes, caveolae, gap-junctions, *Phoneutria nigriventer*, ultrastructural changes.

Abbreviations: AD – Alzheimer's disease, AQP4 – aquaporin-4, BBB – blood brain barrier, BBBb – blood brain barrier breakdown, Cav-1 – caveolin-1, Cav-3 – caveolin-3, CNS – central nervous system, Cxs – connexins, Cx43 – connexin-43, eNOS – endothelium nitric oxide synthase, EAE – experimental autoimmune encephalomyelitis, GFAP – glial fibrillary acid protein, GJ – gap-junctions, IF – immunofluorescence, nNOS – neuronal nitric oxide synthase, NO – nitric oxide, NvU – neurovascular unit, pERK – phosphorylated extracellular signal-regulated kinase, PNV – *Phoneutria nigriventer* venom, TEM – transmission electron microscopy, WB – western blotting.

Introduction

Bites by the wandering spider, *Phoneutria nigriventer*, are a public health problem in southeastern Brazil (Bucaretchi et al., 2000, 2008; Bucaretchi and Hyslop, 2009). Peptides in *P. nigriventer* spider venom (PNV) interfere with Na^+, K^+ and Ca^{2+} channels (Fontana et al., 1985; Cruz-Höfling et al., 1986; Gomez et al., 2002) and can adversely affect excitable tissues in the central nervous system (CNS) that depend on ion channels for proper functioning. We have shown that PNV alters the expression and/or activity of proteins associated with the blood-brain barrier (BBB) and cells of the neurovascular unit (NVu), i.e., neurons, endothelial and astrocytes.

In the endothelium, PNV remodels tight and adhesion junction proteins involved in paracellular pathway regulation, leading to enhanced BBB permeabilization (Rapôso et al., 2007, 2012, 2014). PNV also induces microtubule-mediated activation of the transcellular trafficking of vesicles (Le Sueur et al., 2003, 2004) and increases the formation of endothelial caveolae and expression of its structural protein caveolin-1 (Cav-1) (Soares et al., 2014). Enhanced transcytosis is associated with the activation of Src kinase family proteins and post-translational modification of Cav-1 by phosphorylation (Soares et al. *a*, *submitted*). The uncoupling of endothelial nitric oxide synthase (eNOS) could contribute to these neurovascular effects (Soares et al.*b, submitted*).

In neurons, PNV induces the immediate-early-gene c-fos with its protein product Fos in diverse brain regions, apparently mediated by neuronal nitric oxide synthase (nNOS) activation (Cruz-Höfling et al., 2007). PNV also increases TNF-α and affects the expression of vascular endothelial growth factor and its receptors (Cruz-Höfling et al., 2009; Mendonça et al., 2013). In astrocytes, PNV increases the expression of GFAP and S100 proteins (Cruz-Höfling et al., 2009) and aquaporin-4 (AQP4) (Stávale et al., 2013). Connexin-43 and the multidrug resistance protein-1 (MRP1) may mediate transient BBB breakdown by PNV *in vivo* and *in vitro*, possibly through enhanced production of nitric oxide (NO) by nNOS (Rapôso et al., 2014).

Astrocytes have a central role in key aspects of brain development and functioning through their ability to modulate CNS homeostasis (Reviewed in Verkhratsky and Nedergaard, 2014, and Ota et al. 2013). These cells respond to changes in neuron activity and in the CNS microenvironment. The term “tripartite synapsis” describes the

participation of astrocytes in the regulation of synaptic activity and bi-directional exchange of information with neuronal cells (Araque et al., 1999; Perea et al., 2009). The signaling and metabolic events coordinated by astrocytes are critically dependent on the key role played by gap junctions (GJs) that couple cells via their plasmalemma (Kielian and Esen, 2004; Eugenin, 2012). GJ channels are composed of connexins (Cxs), of which connexin-43 (Cx43) is predominant in astrocytes (Dermietzel et al., 1989; Rouach et al., 2004). Changes in connexin expression and function are relevant in a number of brain lesions and pathologies (Rouach et al., 2002) because they contribute to disease dissemination and/or the accumulation of toxic molecules in the absence of functional GJ channels (Wallraff et al., 2006).

Caveolins are structural proteins of caveolae, non-clathrin coated lipid raft domains of cells membrane that bring together several molecules, primarily caveolin 1 (Cav-1), but also caveolins 2 (Cav-2) and 3 (Cav-3), cavins and eNOS, in addition to associating with dynamin 2 and c-Src (Ikezu et al., 1998; Sowa, 2012). Cav-1 and Cav-2 are expressed in endothelial cells, whereas Cav-3 is expressed in astrocyte caveolae (Ikezu et al., 1998; Virgintino et al., 2002). Although caveolae/Cav-1 have important roles in endothelial endocytosis, transcytosis, exocytosis and vesicle trafficking (Pelkmans and Helenius, 2002), neuronal signaling (Stern and Melmerstein, 2010), BBB enhanced permeation (Nag et al., 2009), mechanosensing and hemodynamics changes (Nassoy e Lamaze, 2012), significantly less is known about Cav-3, especially with regard to its role in astrocytes (Ikezu et al., 1998; Badaut et al., 2015).

Since astrocytes are hallmark protagonists in BBB structure/function and major players in neuronal activities (Verkhratsky and Nedergaard, 2014), and since the MRP-1 efflux protein is active in astrocytes incubated with PNV (Rapôso et al., 2014), we hypothesized that astrocytic caveolae and its main protein could have a role in the effects of PNV on BBB permeability. To test this hypothesis, we investigated the expressional changes in Cav-3 and Cx43 in the cerebellum of rats at different time points following i.v. injection of PNV.

Materials and Methods

Venom and envenoming procedure

Venom was obtained by electrical stimulation of adult *P. nigriventer* of both sexes, lyophilized and stored at -20 ºC until used. Immediately before the experiment, venom was dissolved in 0.9% sterile saline solution (dilution: 0.5 mg PNV/ml). Male rats (*Rattus norvegicus*) 6-7 weeks old were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP). Venom-treated rats received a sub-lethal dose of PNV (0.5 mg/kg) administered intravenously via a tail vein, while the control group received the same of volume of 0.9% sterile saline solution. The rats were killed 1 h, 2 h, 5 h, 24 h and 72 h post-venom and after 5 h for the control group (n=5 per group for western blotting (WB) and n=3 for immunofluorescence (IF) and transmission electron microscopy (MET) per time interval). For WB, the rats were sacrificed in a CO_2 chamber, while those for IF and MET received a lethal dose of a 3:1 mixture of ketamine chloride (Dopalen®, 100 mg/kg) and xylazine chloride (Anasedan®, 10 mg/kg). The animal experiments were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol no. 3609-1).

Western blotting (WB)

After sacrifice, the cerebella were removed and immediately frozen in liquid nitrogen. The cerebella were subsequently lysed with a protein extraction cocktail (10 mM EDTA (Amresco, Solon, Ohio, USA), 2 mM phenylmethane sulfonyl-fluoride (PMSF), 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 10 mg of aprotinin/ml and 100 mM Tris, pH 7.4, all from Sigma, St. Louis, MO, USA). Samples were subjected to SDS-PAGE on 12% gels, as described elsewhere (Rapôso et al., 2012). The primary antibodies used were caveolin-3 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), connexin-43 (1:1,000; Santa Cruz Biotechnology) and β-actin (1:1,000; Sigma-Aldrich). The secondary antibodies were anti-mouse for β-actin and caveolin-3 (1:40,000 and 1:1,000; Sigma-Aldrich) and anti-rabbit for connexin-43 (1:1,000; Sigma-Aldrich). Protein bands were visualized with chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA). Band densities were quantified (in pixels) using Image J 1.45s software (developed by Wayne Rasband, NIH, Bethesda, MD, USA). The quantification was normalized relative to the expression of β-actin.

Immunofluorescence (IF) and co-localization

Rats were perfused transcardiacally with 0.9% saline solution (100 ml) and then 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) (200 ml). The cerebella were removed and cryoprotected in 15% and 30% sucrose (24 h each). Samples were covered in OCT-Tissue Tek (Sakura Finetek, Torrance, CA, USA) and frozen in n-hexane with liquid nitrogen (-70 ºC). Frozen sections (5 µm thick) were cut and collected on glass slides. Immunofluorescence was done as described by Rapôso et al. (2014). The primary antibodies used were caveolin-3 (1:100), connexin-43 (1:50) and GFAP (1:100, Dako Cytomation, Carpinteria CA, USA). The corresponding secondary antibodies were anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and anti-mouse FITC conjugate (Sigma Aldrich) for co-localization. The slides were prepared with Vectashield (Vector Labs, Burlingame, CA, USA) and visualized using a Zeiss LSM 780-NLO confocal microscope and Axio Observer Z.1 microscope (Carl Zeiss AG, Germany) in the laboratory of the National Institute of Science and Technology on Photonics Applied to Cell Biology (INFABIC) at the UNICAMP.

Transmission electron microscopy (TEM)

Rats were perfused transcardiacally with 0.9% saline solution (80 ml) followed by fixative solution (2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2; 250 ml) at a perfusion pressure <80 mmHg. The rats were subsequently held at 4 ºC for 12-18 h before removing the cerebellum. Fragmented cerebella (0.5-1 mm fragments) were immersed in fixative solution for 2 h after which the samples were rinsed in washing solution (0.15 M NaCl plus 0.2 M sucrose) and placed in 1% osmium tetroxide with 1% potassium ferrocyanide (1 h) on ice. The tissues were subsequently dehydrated in a graded ethanol/acetone series and embedded in Epon 212X. Glass blades were used to obtain semi-thin (0.5 µm) sections on an ultra-microtome (Ultra-cut Reichert S, Leica, Wetzlar, GE); the sections were subsequently stained with 0.5% toluidine blue in borax and examined by light microscopy. Ultrathin (60 nm thick) sections were cut, collected on copper grids (200 mesh) and double-contrasted with methanolic 2% uranyl acetate and an aqueous solution of 0.5% lead citrate for transmission electron microscope. The samples were examined with a Zeiss LEO 906 transmission electron microscope operated at 60 kV.

Statistical analyses

The results were expressed as the mean ± SEM and were analyzed using Prism software (GraphPad Inc., San Diego, CA, USA). The significance of differences between the control and PNV-treated groups was assessed using Student's *t*-test, whereas two-way ANOVA followed by the Bonferroni post-hoc test was used to assess time-dependent alterations within PNV groups. A value of $p \leq 0.05$ indicated significance.

Results and Discussion

Experimental and accidental envenoming by *P. nigriventer* induces neurotoxic manifestations indicative of the involvement of peripheral, autonomic and central nervous systems (Bucaretchi et al., 2000, 2008; Rapôso et al., 2012; Mendonça et al., 2013). We have previously shown that PNV disrupts the BBB and affects endothelial, astrocyte and neuronal cells. These effects have been attributed to the rich diversity of ion channel-acting toxins in PNV and their ability to disturb glutamate and acetylcholine neurotransmission (Gomez et al., 2002).

In this work, we examined the effect of PNV on two astrocyte proteins (Cx43 and Cav-3) intimately associated with cellular signaling in health and disease. A major finding of this investigation was that Cx43 and Cav-3 were significantly up-regulated in the cerebellum of rats exposed to PNV. In addition, there was a remarkable up-regulation of Cx43 in Purkinje neurons and possibly in granule neurons of the granular layer. A third, not less important finding was that Cav-3 was significantly expressed in reactive astrocytes overexpressing GFAP. Finally, we observed a close parallel between the changes in Cx43 and Cav3 expression over time in response to PNV. We suggest that the return of Cx43 to basal expression levels at 72 h post-venom, coupled to the down-regulation of Cav-3, was indicative of a return to the normal, pre-venom homeostatic state.

Increased expression of Cx43 in PNV-affected astrocytes at the time of greatest intoxication and when rats were clinically normal

Compared to the control basal level, Cx43 in cerebella of PNV-treated rats underwent cyclic changes in expression (periods of increase followed by a return to

baseline) that were apparently unrelated to the severity of envenoming exhibited by the rats. Significant increases were observed when the signs of envenoming were intense (78% increase at 1 h), when the manifestations of envenoming began to decrease (24% at 5 h) and were highest at 24 h (115%) when rats showed no signs of intoxication (Figure 1A,B). Immunofluorescence for Cx43 showed a similar pattern of changes as seen with WB (Figure 1C-H). Relative to control rats, PNV induced intermittent high/low anti-Cx43 immunoreactivity: basal labeling was detected in astrocyte populations in the molecular and granular layers and notably in the Purkinje cells perikaryon.

Gap junction (GJ)-based networks coordinate extracellular homeostasis by regulating pH, K^+ and glutamate release resulting from synaptic work (Zahs, 1998; Kielian, 2008). In basal conditions, GJ functioning is minimal, but can be accelerated by changes in Ca^{2+} handling, kinase activity, mechanical stress and peri-synaptic K^+ buffering during membrane depolarization (Stout et al., 2004). Astrocytes respond to the activation of neurons by irritating stimuli by emitting Ca^{2+} signals and triggering the release of gliotransmitters that in turn show feed-back regulation of synaptic activity (reviewed in Perea et al., 2014). The up/down regulation of Cx43 expression seen here based on immunoreactivity may reflect a bi-directional exchange of signaling factors between reactive astrocytes and neurons (Purkinje and granular), the nature of which is unknown but may likely represent mutual glia-neuronal regulations.

We have observed increases in endothelial phosphorylated Cav-1, a phenomenon that implies the activation of Src kinase family proteins, of which c-Src is part (Soares et al., *submitted* a). Cx43 membrane lipid raft domains can be regulated by c-Src in reactive astrocytes after excitotoxic insult and downregulation of Cx43 has been correlated with c-Src activation (Gangoso et al., 2012). We suggest that the ability of astrocytes to sense alterations in brain homeostasis (Zorec et al., 2012) promoted by the pharmacological effects of PNV on ion channels and neurotransmitter (glutamate and acetylcholine) handling (Gomez et al., 2002) could have led to the modulation of Cx43 expression and GJs activity by Src kinase family proteins; Cx43 expression could also be enhanced by the propagation of PNV-generated noxious stimuli by GJ channels. On the other hand, Cx43 down-regulation may imply a counter-protective mechanism mediated by inoperative GJs channels to avoid the propagation of harmful stimuli. However, cellular uncoupling could

lead to the accumulation of toxic substances and a new cycle of Cx43 up-regulation until its return to baseline at 72 h post-venom, a time-point with no clinical manifestations of intoxication in rats. The high density of Cx43 seen in the perivascular and perisynaptic end-feet processes of astrocytes (Danesh-Meyer and Green, 2008), and the role of this protein in pathophysiological conditions such as edema (Scemes and Spray, 1998) and neuroinflammation (Bennett et al., 2012; Chew et al., 2010), are in line with our findings (Le Sueur et al., 2003, 2004; Rapôso et al., 2007; Cruz-Höfling et al., 2009; Soares et al., *submitted* a).

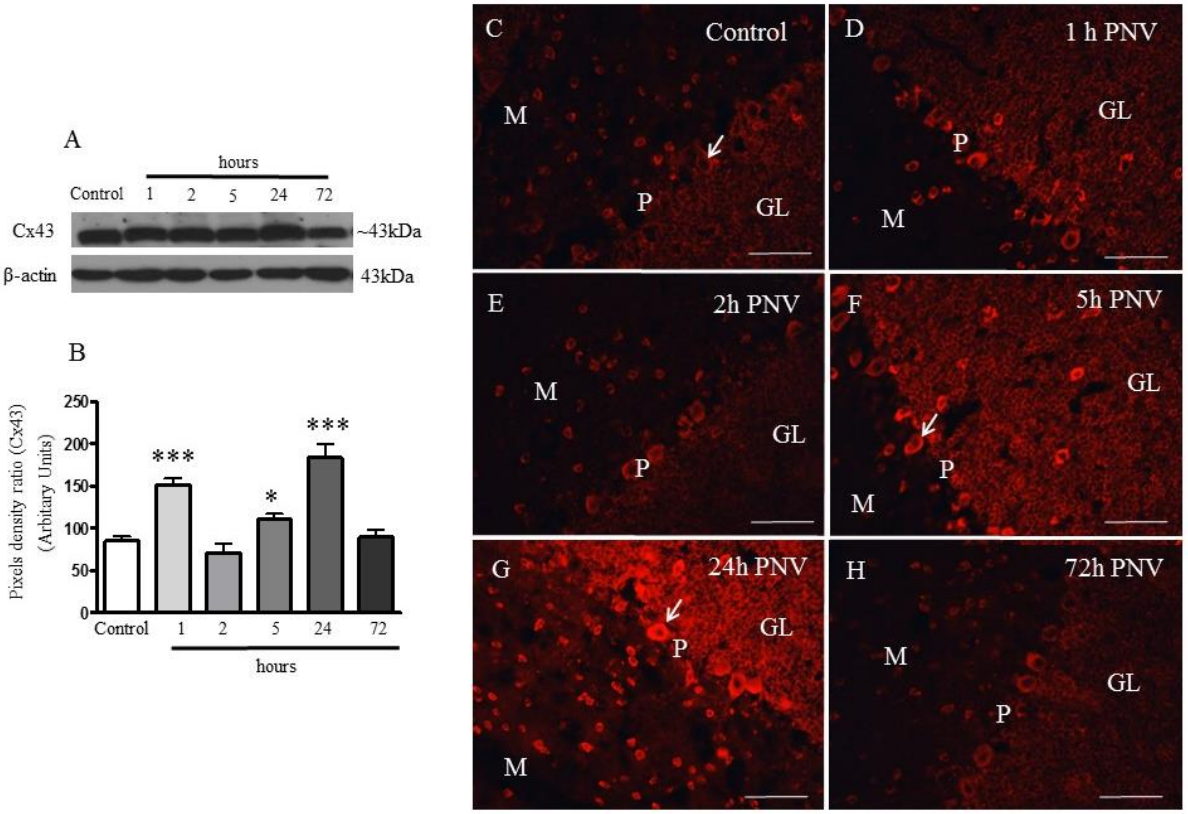

Figure 1: *Cx43 expression in rat cerebellar cortex.* (A) Representative western blot for Cx43 in saline- (control) and PNV-treated rats. (B) Densitometric quantification of Cx43 (~43 kDa) expression in PNV-treated rats compared to saline-treated controls. There were significant increases (1, 5 and 24 h post-venom) or no change (2 and 72 h) in cerebellar Cx43 expression. Band densities were normalized to endogenous β-actin (43 kDa). (C-H) The changes in anti-Cx43 immunoreactivity paralleled those observed in western blotting. GL, granular layer; M, molecular layer; P, Purkinje layer. *Arrows* indicate peri-nuclear labeling of Purkinje cells: astrocytes scattered in the molecular and granular layers were labeled. Bars: 40 µm. The columns in panel B represent the mean ± SEM. *p≤0.05 and ***p≤0.001 relative to saline control group (Student´s *t*-test).

Cav-3 upregulation increases co-localization of the protein with GFAP in reactive astrogliosis promoted by PNV

We also examined whether PNV alters the expression of Cav-3. However, to be sure that the cells expressing Cav-3 were astrocytes, we initially used immunofluorescence to assess the co-localization of Cav-3 and GFAP (glial fibrillary acidic protein) in serial cryo-sections.

Figure 2 shows the baseline expression and distribution of Cav-3 and GFAP immunoreactivity in separate images and after merging (panels A, B and C, respectively). In control preparations, there was partial co-localization of the two proteins and a weak presence of Cav-3 in the processes of Bergmann glia directed to the pial border, as reported elsewhere (Badaut et al., 2015; Ikezu et al., 1998; Jin et al., 2007; Nishiyama et al., 1999). Similarly, panels D, E and F display the changes in labeling intensity in the cerebellar cortex of envenomed rats. Interestingly, while Cav-3 in PNV-treated rats showed increased co-localization with GFAP in reactive astrocytes (panel F), much less co-localization was found in non-reactive ones (panel C). This finding suggests coupled astrogliosis and increased caveolae in astrocytes after PNV-induced injury. In addition to Bergmann glia astrogliosis and Cav-3 upregulation, PNV also altered the morphological pattern of cytoskeletal organization.

PNV-induced changes in total Cav-3 expression overlap those of Cx43

To substantiate the findings described above, we next examined the total level of Cav-3 expression in basal conditions and after PNV administration (Figure 3). Western blotting (WB) of cerebellar lysates in PNV-treated rats also showed cyclic periods of significant up-regulation (60% at 1 h, 282% at 5 h and 74% at 24 h) and down-regulation (40% at 2 h and 110% at 72 h) compared to control rats (Figure 3A,B). Two-way ANOVA revealed that the interaction between the variables treatment *vs*. time affected the expression of Cx-43 and Cav-3. Anti-Cav-3 immunofluorescence revealed astrocytes in the molecular and granular layers and faintly immunoreactive body cell and processes in the controls (Figure 2A); in PNV-treated rats, the labeling was stronger at 1, 5 and 24 h, thus corroborating the WB data (Figure 2B-F).

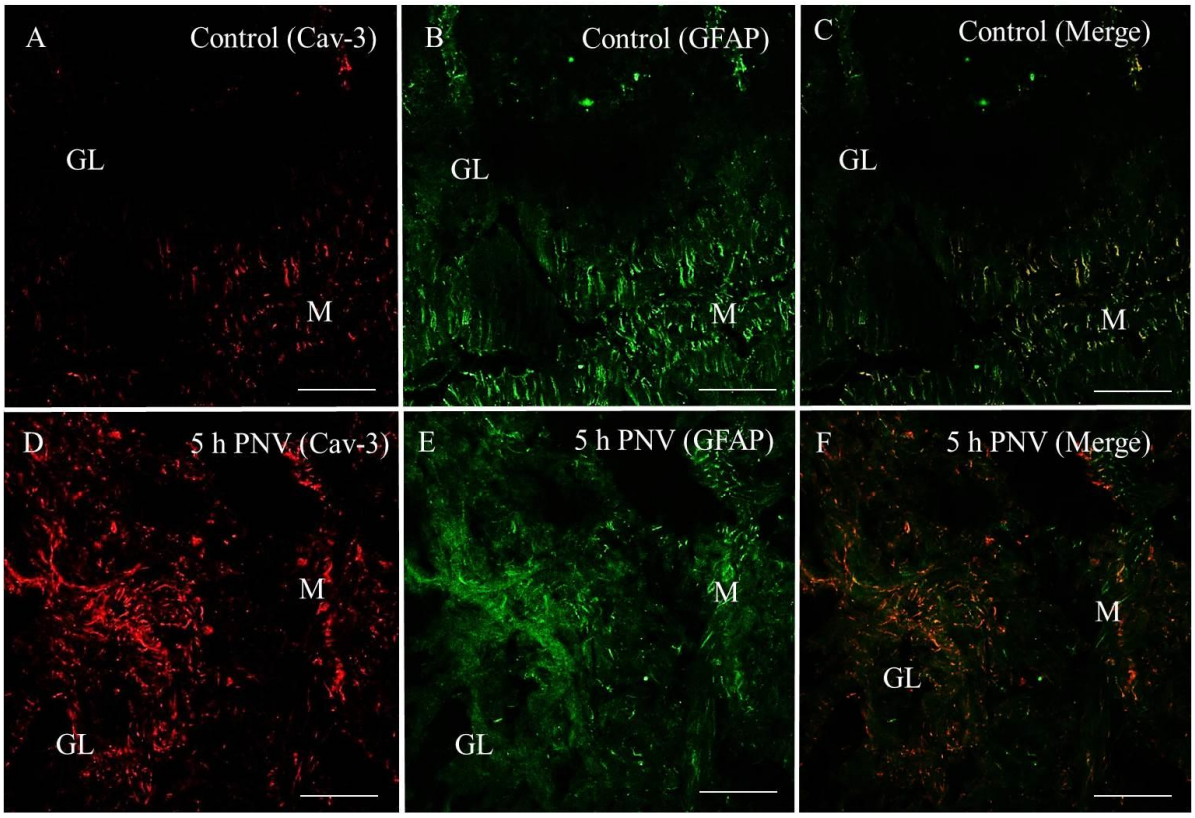

Figure 2: *Co-localization of Cav-3 and GFAP in rat cerebellar astrocytes.* (A-B) Weak expression of Cav-3 and GFAP in control rats, (C) Merged image of A and B showing increased co-localization of Cav-3 and GFAP, (D-E) Illustrative images of Cav-3 and GFAP labeling showing marked increase at 5 h after PNV administration, and (F) Merged image of D and E showing that the reactive gliosis was accompanied by overexpression of Cav-3 and that regions of GFAP/Cav-3 overlap exceeded those observed in the merged image of control rats. GL, granular layer; M, molecular layer. Scale bars: 60 µm.

The caveolar membrane domain is a dynamic structure that cycles between an open endocytic-like state and a closed flat-like state resulting from vesicle detachment and internalization. Cav-3 and Cav-1 show a high degree of sequence homology (65% identity and 85% similarity) and have a highly conserved sequence of eight amino acids (Tang et al., 1996). Both are structural proteins of the caveolar frame, a cell organelle with a key role in cell signaling and transduction (Razani et al., 2002). Whereas Cav-1 is localized predominantly in endothelial caveolae, in the brain Cav-3 is the scaffolding protein of caveolar membrane domains of the astrocyte plasmalemma (Ikezu et al., 1998) but is also present in human brain microvessels (Virgintino et al., 2002). Cav-3 is canonically expressed in skeletal, cardiac and smooth muscle sarcolemma, where caveolae have a key

role in muscle function (Tang et al., 1996). Caveolinopathies resulting from Cav-3 gene mutation or deletion can disrupt various signaling pathways, leading to pathogenetic dystrophic mechanisms that result in skeletal muscle diseases and cardiomyopathies (Gazzero et al., 2010).

The role of Cav-3 in astrocyte physiology is still poorly understood, although overexpression of Cav-3 results in dysfunctions related to neurological disorders. For instance, prominent down-regulation of Cav-3 during C6 glioma cell differentiation into an astrocyte-like phenotype can have a role in homeostasis (Silva et al., 2005); this agrees with the minimal expression of Cav-3 in healthy human brain (Nishiyama et al., 1999) and normal spinal cord of rats (Jin et al., 2007), as well as with our results for saline-treated rats. In contrast, Cav-3 up-regulation has been associated with diseases related to BBB disruption, such as Alzheimer's disease (AD) and experimental autoimmune encephalomyelitis (EAE). Specifically, Cav-3 overexpression has been associated with the processing of amyloid precursor protein by senile plaques in reactive astrocytes in AD (Nishiyama et al., 1999), while in EAE Cav-3 up-regulation was highest in the peak stage and decreased in the recovery stage (Shin et al., 2005; Jin et al., 2007).

These findings for Cav-3 share considerable similarity with our results for PNV-induced disruption of BBB model in rats. In rats injected i.v. with saline, Cav-3 was barely detected, while in rats treated with PNV, Cav-3 was up-regulated (1 h), down-regulated (2 h), dramatically up-regulated (5 h) and gradually decreased to half of the baseline value at 72 h, when the rats showed no signs of envenomation. These changes in Cav-3 expression suggested a differential pattern of Cav-3 gene expression or interference with RNA translation during envenoming with PNV. Silva et al. (2005) considered that understanding the experimental down-regulation of Cav-3 may be relevant in promoting neuroprotection in CNS diseases. As an extension of their reasoning, the PNV model described here could be useful for studying the mechanisms involved in neurological disorders associated with Cav-3 overexpression. Cav-3 may also be an important therapeutic target for managing PNV-induced BBB opening.

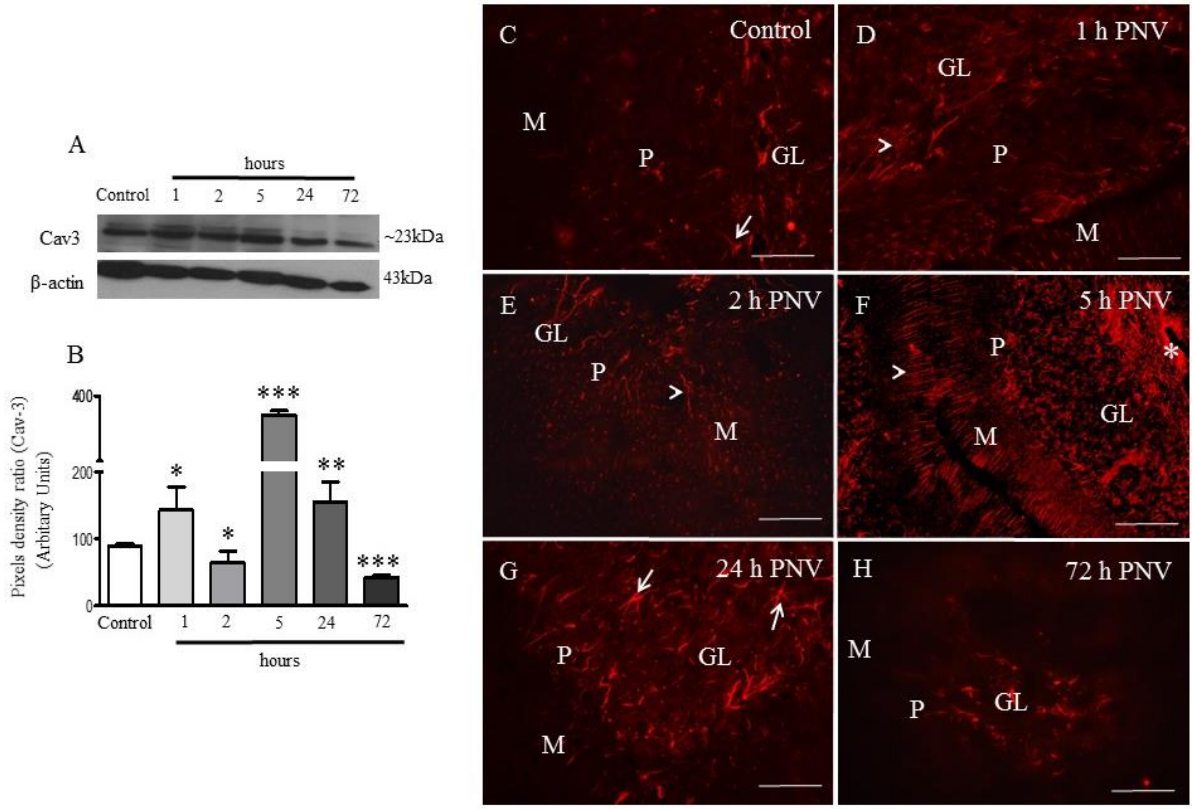

Figure 3: *Cav-3 expression in the cerebellum*. (A) Western blot for Cav-3 in a control rat and during systemic envenoming by PNV (time intervals are post-venom). (B) Quantification of Cav-3 bands (~23 kDa) normalized to endogenous β-actin (43 kDa) showed significant increases (1, 5 and 24 h post-venom) that alternated with decreases (2 and 72 h post-venom) in cerebellar lysates of PNV-treated rats compared to rats treated with saline (control). *p<0.05, **p<0.01 and ***p<0.001 compared to control (Student´s *t*-test). (C-H) Changes in the intensity of anti-Cav-3 immunolabeling that generally followed the pattern seen in western blotting. *Arrows* indicate Cav-3-labeled astrocyte cell body; *Arrowheads* indicate Bergmann glial processes arranged perpendicularly to the pial border and perivascular end-feet processes (*asterisk*) with Cav-3 positivity. GL, granular layer; M, molecular layer; P, Purkinje layer. Scale bars: 40 µm.

Interestingly, the changes in Cav-3 levels over time perfectly paralleled the pattern of changes in total Cx43 (compare Figure 1H with Figure 3H). As for Cx43, Cav-3 is involved in neuroinflammatory processes, with both proteins being expressed in microglia and participating in the modulation of injury-induced inflammation (Rouach et al., 2002; Niesman et al., 2014).

Cx43 has been shown to interact with Cav-3 (Liao et al., 2010). These authors found that Cx43 expression was regulated by Cav-3, with the participation of inducible NOS (iNOS) in lipolysaccharide (LPS)-treated primary cultured astrocytes. Treatment with LPS

inhibited Cx43 and significantly increased the level of phosphorylated extracellular signal-regulated kinase (pERK); this increase was concomitant with the down-regulation and weaker immunolabeling of Cav-3. These effects were prevented by inhibition of iNOS but not of ERK. In addition, the deletion of Cav-3 by siRNA induced down-regulation of Cx43 and inhibited communication through GJ channels. The downregulation of Cav-3 in this model was suggested to occur via a TLR4-mediated signaling pathway (Liao et al., 2010). To our knowledge, these authors were the first to identify a role for Cav-3 in the regulation of Cx43 in astrocytes incubated with pro-inflammatory stimuli. The participation of Cav-3 in Cx43 expression and in the functional regulation of GJs has previously been shown for heart (Liu et al., 2010).

Connexins, caveolins, water channels, potassium channels and glutamate receptors are located primarily in the astrocyte end-feet processes surrounding brain microvessels. Since PNV causes excitotoxic manifestations in experimental animals and humans, disrupts the BBB and causes perivascular edema (Le Sueur et al., 2003, 2004; Rapôso et al., 2007; Mendonça et al., 2013), and in view of the results described here, we extended our investigation to assess whether variations in the expression of astrocyte Cav-3 and Cx43 were followed by submicroscopic changes.

Transmission electron microscopy (TEM) reveals transient swelling of astrocyte end-feet and alterations in synaptic boutons in PNV-treated rats

The most noticeable changes in cerebellar ultrastructure included swelling of the perivascular end-feet processes and alterations in the synaptic contacts (Figure 4A-D). Panels A and B show prominent changes 1 h and 2 h after envenoming; swollen astrocyte end-feet processes were seen next to capillary walls. In addition, dendritic branches of Purkinje cells showed distortion and axon terminal detached from synaptic contacts (panel C) or synaptic vesicles clumped together without contact with synaptic densities (panels A, C and D).

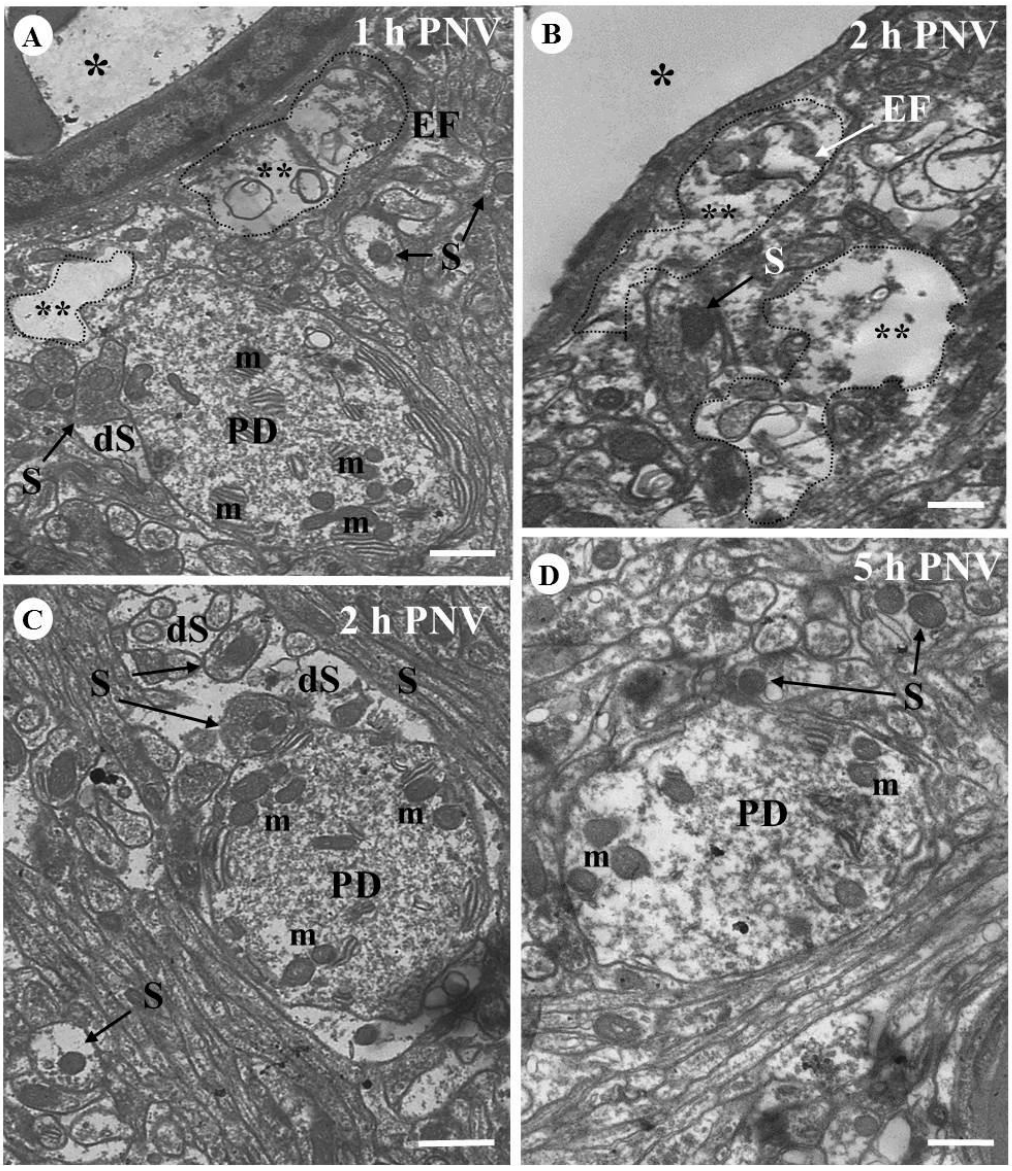

Figure 4: Electron micrographs of cerebellar gray matter of rats at different times after i.v. injection of *P. nigriventer* venom (PNV). The main ultrastructural changes occurred at 1 h and 2 h post-venom and included striking enlargement (cytotoxic edema, 3-4 µm width) of astrocyte end-feet (**EF**) and marked disorganization of synaptic contacts (**S**). Note in panels **A**, **B, C** and **D** the morphological abnormalities of Purkinje dendrites (**PD**) with the detachment between dendrites and axon (**Ax**) contacts. ***Double asterisks*** and dotted lines in panels **A** and **B**: probable evidence of vasogenic edema (when edema is interstitial). ***Dashed line*** delineates regions of cytotoxic edema in perivascular astrocytes and vasogenic edema. dS, disassembled synapses; m, mitochondria. Scale bars: 2 µm.

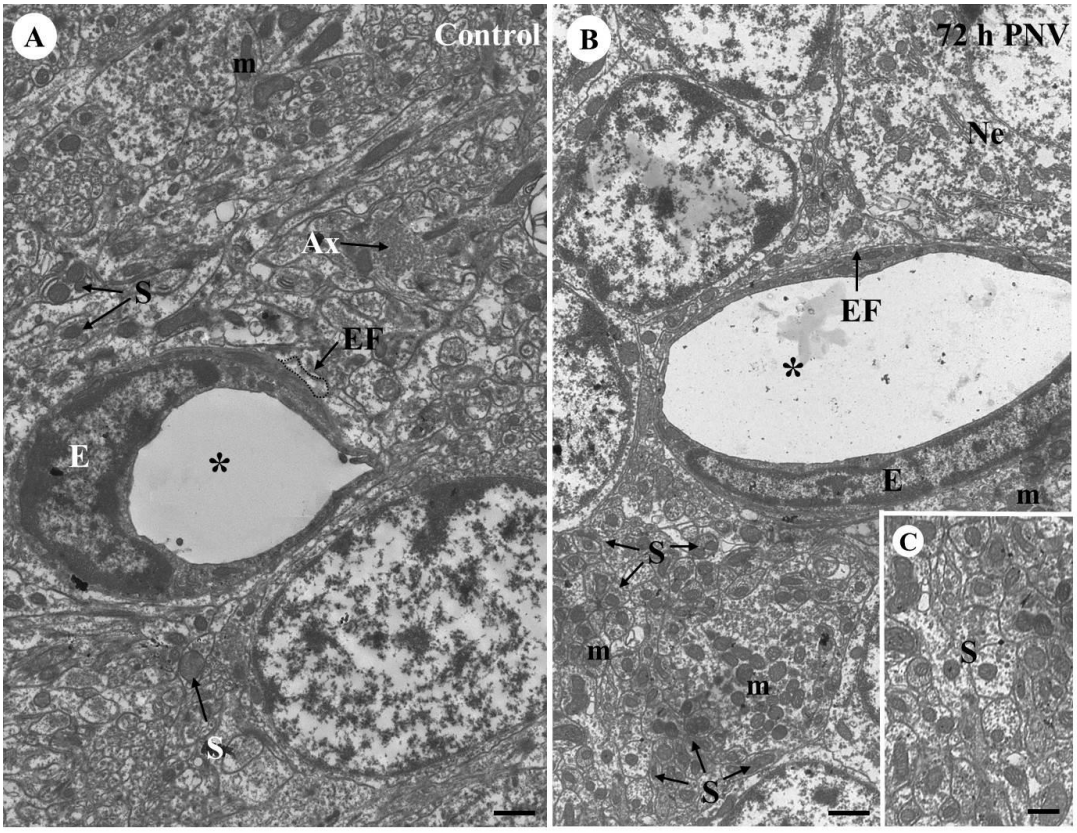

Figure 5: Representative electron micrographs of rat cerebellar gray matter 5 h after i.v. injection of sterile saline (**A**) or 72 h after the i.v. injection of *P. nigriventer* venom (PNV) (**B**). Note that in control and PNV-treated rats the ultrastructure of the cerebellar cortex parenchyma is normal in appearance. There was no visible swelling of perivascular astrocytic end-feet **(EF)** and the synaptic contacts **(S)** are well-structured. Ax, axon; E, endothelial cell; m, mitochondria; Ne, neuron; (*****) capillary; ***Asterisks*** indicate endothelium lumen. Scale bars: 2 µm.

Electron micrographs of control cerebellum revealed normal ultrastructure and organization of the gray matter parenchyma, i.e., a capillary surrounded by normal end-feet processes and a profusion of axo-somatic and axo-dendritic synaptic contacts (Figure 5A). In PNV-treated rats, the parenchyma of the cerebellar gray matter 72 h post-venom was similar to that of control rats, indicating that any venom-induced changes were reversible. At this time interval, the end-feet appeared normal since there was no more swelling (Figure 5B). Perivascular end-feet swelling results from osmotic changes generally caused by dysregulation of endothelial permeability through transcellular transport pathways. Typically, the extravasation of albumin and other plasma proteins from the peripheral circulation is the major cause of isosmotic disturbances. In the BBB, the transport of

macromolecules such as albumin involves receptor-mediated endothelial vesicle trafficking (Ghitescu et al., 1986). The increased uptake of albumin by perivascular astrocytic end-feet leads to increased fluid influx, resulting in cytotoxic edema (Klatzo, 1987). We have recently shown that aquaporin-4 (AQP4) is upregulated in cerebellar astrocytes (mainly Bergman glia type) of PNV-treated rats (Stávale et al., 2013). AQPs are integral proteins that form water channels and are related to edema formation and resolution (Nielsen et al., 1997; Nicchia et al., 2004). Our TEM observations showing perivascular swelling of astrocytic end-feet agree with AQP4 up-regulation during PNV-induced BBB breakdown in rats.

Conclusions

In summary, PNV upregulated the expression of Cx43 and Cav-3 in rat cerebellar astrocytes. This altered expression paralleled the reactive astrogliosis and ultrastructural alterations in perivascular glial end-feet that were swollen relative to the controls. Interestingly, the temporal changes in the levels of these two proteins were nearly identical, perhaps indicative of some form of synchronism in their responses. These parallel changes raise the possibility of cross-talk between Cx43 and Cav-3 in the astrocytic response to PNV. The findings that after marked up-regulation (24 h) the Cav-3 level was significantly below baseline, Cx43 expression had returned to basal level and end-feet swelling had reverted (72 h) suggested that intercellular communication via GJs had returned to its resting state. The significant down-regulation of Cav-3 may be indicative of a protective reaction triggered in the absence of neurotoxic manifestations, and could underlie the reduction in Cx43. Further studies are needed to define whether there was interaction between the two proteins or whether the parallel changes in expression simply represented a bystander effect.

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Conflict of interest statement

The authors have not conflict of interest related to the publication of this work.

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VI. CONCLUSÕES

Com base nos resultados apresentados neste trabalho e confrontados com dados da literatura pertinente é possível sugerir que:

- ✓ O veneno bruto da aranha *Phoneutria nigriventer* administrado via intravenosa ou intraperitoneal (dose de 0,5 mg/Kg ou 1,7 mg/Kg, respectivamente) induz um quadro de envenenamento severo em ratos neonatos e adultos.
- ✓ Considerando a janela de observação do presente estudo (1, 2, 5, 24 e 72 h) é possível inferir: os sinais de intoxicação pelo PNV se iniciam segundos após sua adminitração permanecendo intensos até às 2 horas após envenenamento; às 5 horas os ratos apresentam sinais de melhora; às 24 h e até as 72 h os animais tem aparência normal e comportamento idêntico aos dos animais do grupo controle. Entretanto, a melhora clínica não corre paralela às alterações celulares e moleculares que continuam subjacentes ainda às 72 h após envenenamento. Ainda, os animais neonatos são mais suceptíveis aos efeitos neutóxicos do PNV.
- ✓ No cerebelo, o PNV é capaz de induzir alterações morfológicas nos elementos da unidade neurovascular que compõem a BHE: aumentam o número de vesículas no endotélio vascular cerebral; há astrogliose reativa, inchaço dos pés-astrocitários; aumento de Cav-1 em células de Purkinje e desestabilização das sinapses neuronais.
- ✓ As alterações intermitentes apresentadas pelas proteínas envolvidas na sinalização para fomação/internalização cíclica das cavéolas (Cav-1/pCav-1/Din2/SKF) implicam em aumento das tensões na membrana endotelial e na hemodinâmica após o envenenamento e contribuiriam para ativação de vias de sinalização e permeabilização temporária da BHE. O edema nos pés astrocitários perivasculares poderia ter nessa dinâmica uma de suas causas.
- ✓ O endotélio se mostrou muito reativo ao PNV tanto pelas vesículas em tráfego quanto pelas alterações no ambiente extracelular com aumentos intermitentes de metaloproteinase-9 (MMP9) em consequência do aumento de Cav-1 o que em conjunto agrava o quadro de neuroinflammação e permeabilidade da BHE.

- ✓ A super expressão de conexina-43 colabora com a neuroinflamação induzida pelo PNV ao disseminar o estímulo tóxico e influencia o aumento da expressão de Caveolina-3 em astrócitos positivos para GFAP (aumento de gliose reativa) potencializando o quadro de dano e ativação astrocitária. As alterações pareadas entre Cav-3/Cx43 podem indicar um *cross-talk* entre as duas proteínas em resposta ao envenenamento. Em períodos tardios a redução de Cav-3 e o retorno de Cx43 aos níveis basais refletem os mecanismos de reparo após injúria.
- ✓ A inativação ou desacoplamento de eNOS nos períodos de envenenamento agudo e a consequente disfunção vascular, ajudam a explicar a intoxicação severa dos animais nos perídos iniciais ao envenenamento. Da mesma forma, nos períodos de recuperação e ausência de sinais clínicos a enzima está ativa e os dímeros (reflexo de ativação) super expressos.
- ✓ Os níveis intracelulares do íon cálcio, conhecido alvo do PNV, são alterados após o envenenamento interferindo na expressão de Calmodulina, proteína ativadora de eNOS e Calbidina-D28, ambas proteínas sensíveis ao cálcio intracelular; ainda, essas alterações estão envolvidas na disfunção e ativação da enzima eNOS após o envenenamento.
- ✓ O conjunto de ações do PNV sobre o endotélio, com aumento das tensões de membrana decorrentes dos ciclos de formação e desprendimento de cavéolas, a produção intermitente de metaloproteinases-9 e o prejuízo na produção de óxido nítrico pelo desacoplamento da eNOS, importante molécula reguladora do tônus vascular, apontam para importante ação do PNV sobre a homeostase do endotélio e da própria BHE, reflexo da sua potente ação neurotóxica.
- ✓ Por fim, a quebra da BHE pelo aumento de transcitose de vesículas é mediada por cavéolas revestidas por caveolina. Portanto, as cavéolas são importantes alvos do PNV após administração sistêmica do veneno colaborando com a permeabilidade da BHE tanto pelo aumento de vesículas no endotélio como pela influência que exercem sobre a homeostase de astrócitos e no controle de moléculas como as metaloproteinases e conexinas.

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IX. ANEXOS

9.1. 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 Doutorado aprovadas pela UNICAMP

Tendo em vista a possibilidade, segundo parecer PG No 1985-96, das teses de mestrado e de doutorado 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 coautoria 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 candidato, da versão final da tese a ser homologada.

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

9.2. Anexo II

Certificado do Comitê de Ética em Pesquisa Animal 1

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto **"Efeito do veneno de expressão de caveolina-1 (CAV-1) no hipocampo e cerebelo no envenenamento por *Phoneutria nigriventer* na barreira hematoencefálica"** (protocolo nº **2411-1**), sob a responsabilidade de **Profa. Dra. Maria Alice da Cruz Höfling / Edilene Siqueira Soares**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **02 de maio de 2011**.

Campinas, 02 de maio de 2011.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

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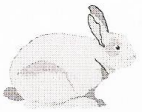
9.3. Anexo III

Certificado do Comitê de Ética em Pesquisa Animal 2

UNICAMP



CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto **"Envolvimento das cavéolas na permeabilidade da barreira hematoencefálica após envenenamento por *Phoneutria nigriventer* em ratos wistar (*Rattus norvegicus*)"** (protocolo nº **3609-1**), sob a responsabilidade de **Profa. Dra. Maria Alice da Cruz-Höfling / Edilene Siqueira Soares**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **03 de novembro de 2014**.

Campinas, 03 de novembro de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

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9.4. Anexo IV

ELSEVIER LICENSE
TERMS AND CONDITIONS

Mar 10, 2015

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9.4. Anexo V

Declaração de autorização de Comites em ética em Pesquisa.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Dissertação de Mestrado** intitulada "Envolvimento das cavéolas na permeabilidade da barreira hematoencefálica após envenenamento por *Phoneutria nigriventer* em ratos":

( ) não se enquadra no § 4º do Artigo 1º da Informação CCPG 002/13, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio – Comissão Interna de Biossegurança , projeto No. _____, Instituição: _____.

(X) CEUA – Comissão de Ética no Uso de Animais , projeto No. 3609-1, Instituição: Universidade Estadual de Campinas.

( ) CEP - Comissão de Ética em Pesquisa, protocolo No. _____, Instituição: _____

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Edilene Siqueira Soares

Aluna: Edilene Siqueira Soares

Orientadora: Maria Alice da Cruz-Höfling

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Prof. Dr. ALEXANDRE LEITE RODRIGUES DE OLIVEIRA
Presidente da Comissão de Ética no Uso de Animais CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

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