MATHEUS DEROCO VELOSO DA SILVA

IDENTIFICAÇÃO DA REDE PERINEURONAL NA INERVAÇÃO ESPINAL DO CÓLON DISTAL DE CAMUNDONGOS COM RETOCOLITE ULCERATIVA

Londrina
2021

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Dissertação apresentada ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial para obtenção do título de Mestre

Orientador: Prof. Dr. Eduardo José de Almeida Araújo.

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Londrina, 19 de novembro de 2021.

Dedico este trabalho a todos que deram suas vidas a um bem maior e dedicaram-se de todo coração em prol da ciência.

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“The more I study science, the more I believe in God”

“Quanto mais me aprofundo na Ciência mais me aproximo de Deus”

Albert Einstein

SILVA, Matheus Deroco Veloso da. **Identificação da rede perineuronal na inervação espinal do cólon distal de camundongos com retocolite ulcerativa**. 2021. 81 f. Dissertação apresentada ao Programa de Pós-graduação em Patologia Experimental da UEL, como requisito parcial para obtenção do título de Mestre – Universidade Estadual de Londrina, Londrina, 2021.

RESUMO

As doenças inflamatórias intestinas (DII) estão entre os principais distúrbios que acometem o trato gastrointestinal, destacando-se a retocolite ulcerativa (RCU). O processo inflamatório na RCU é capaz de ativar fibras intestinais direcionadas aos gânglios de raíz dorsal (GRD), que transmite a informação ao sistema nervoso central (SNC) dando origem a dor. Em geral, no SNC, o espaço entre as células é ocupado por matriz extracelular amorfa e difusa, porém ao redor de populações neuronais específicas a matriz se torna altamente especializada, sendo chamada de Rede perineuronal (RPN). A RPN está relacionada como o desenvolvimento neuronal, sinaptogênese, plasticidade sináptica e neuroproteção.Os relatos da presença da RPN estão restritos ao SNC, não existindo estudos que avaliem sua relação com a inervação aferente extrínseca do cólon distal e com a inflamação presente na RCU. Por isso, objetivo deste estudo foi avaliar a presença da RPN na inervação espinal aferente do cólon distal, bem como a influência do processo inflamatório presente na RCU sobre esta rede. Foram utilizados 20 camundongos C57BL/6 machos distribuídos em 4 grupos (n=5): GCa: recebeu água autoclavada durante 7 dias; RCUa: receberam Dextran sulfato de sódio 3% (DSS 3%) durante 7 dias; GCc: água autoclavada durante 29 dias; RCUc: 3 ciclos de DSS 3% durante 5 dias, intercalados por 2 ciclos de 7 dias com água autoclavada. Durante o tratamento, os animais foram avaliados clinicamente. Ao fim, foram submetidos à eutanásia e coletou-se o cólon distal para análise histopatológica e os GRD de níveis espinais bilaterais L6-S1, seus respectivos nervos esplânicos, para avaliar a inervação espinal aferente do cólon distal. As secções de tecido nervoso foram submetidas à técnica de histoimunofluorescência, utilizando-se a lectina *Wisteria floribunda* aglutinina (WFA) e anticorpos contra o peptídeo relacionado ao gene da calcitonina (CGRP) ou contra periferina c- 19. Foram quantificados 500 corpos celulares e prolongamentos, além da mensuração dos pericários WFA+ e WFA-, além da co-marcação com CGRP nos GRD. Com auxílio de microscópio confocal, foram quantificados 30 neurônios por animal e identificados os subtipos de marcação de WFA, além do número de células gliais satélites ao seu redor. Em relação à marcação com WFA, foi possível observar a presença de RPN entorno de todas as estruturas analisadas. Também observamos que a RCU aumentou o número de neurônios WFA positivos bem como a área ocupada pelos seus corpos celulares no córtex de GRD ($p<0,05$). Observamos que a área ocupada pelos neurônios WFA+/CGRP+ era maior nos GRD de camundongos do grupo RCUc ($p<0,05$). Também observamos um aumento no número de células gliais satélites positivas para WFA nos GRD dos animais expostos ao DSS, o que contribuiu para a visualização de maior quantidade de marcação de WFA nos animais com RCU. Parte dos prolongamentos neuronais em nervos esplâncnicos também estava envolvida pela marcação com WFA, cuja proporção aumentou nos comundongos com RCUc ($P<0,05$). Neste trabalho verificamos evidências de que o ambiente inflamatório presente na RCU induziu um aumento na produção de

componentes de RPNs e, consequentemente, sua condensação. Sugere-se que, assim como no SNC, a RPN atue de modo a proteger os prolongamentos sensitivos do ambiente inflamatório e aumente a capacidade de condução da informação nociceptiva até o SNC.

Palavras-chave: inervação extrínseca; inflamação; matriz extracelular.

SILVA, Matheus Deroco Veloso da. **Identification of perineuronal net in spinal innervation of the distal colon of C57bl6 mice with ulcerative colitis.** 2021. 81 p. Dissertation submitted to the Graduate Program in Experimental Pathology at UEL, as partial requirement for obtaining the title of Master degree – Universidade Estadual de Londrina, Londrina, 2021.

ABSTRACT

Inflammatory bowel diseases (IBD) are among the main disorders that affect the gastrointestinal tract, with ulcerative colitis (UC) standing out. The inflammatory process in UC is capable of activating intestinal fibers directed to the dorsal root ganglia (DRG), which transmit information to the central nervous system (CNS) giving rise to pain. In general, in the CNS, the space between cells is occupied by a diffuse, amorphous extracellular matrix, but around specific neuronal populations the matrix becomes highly specialized, and is called the perineuronal network (PNN). The PNN is related as neuronal development, synaptogenesis, synaptic plasticity and neuroprotection.Reports of the presence of the RPN are restricted to the CNS, and there are no studies evaluating its relationship with the extrinsic afferent innervation of the distal colon and with the inflammation present in the UC. Therefore, the objective of this study was to evaluate the presence of the PNN in the spinal afferent innervation of the distal colon, as well as the influence of the inflammatory process present in the CU on this network. Twenty C57BL/6 male mice were distributed in 4 groups (n=5): CGa: received autoclavad water for 7 days; UCa: received Dextran sodium sulfate 3% (DSS3%) for 7 days; CGc: autoclavad water for 29 days; UCc: 3 cycles of DSS3% for 5 days, interspersed with 2 cycles of autocled water. After treatment, the animals were submitted to cardiac perfusion and we collected the DRG of bilateral spinal levels L6-S1, and their respective splanic nerves . The tissues were post-fixed and prepared for the histoimmunofluorescence technique. For this we used lectin Wisteria floribunda agglutinin (WFA), and antibodies against the calcitonin gene-related peptide (CGRP) and periferin c-19. We quantified 500 cell bodies and extensions, and measured the WFA+ and WFA- pericytes, as well as co-labeling with CGRP in DRGs. Using a confocal microscope, 30 neurons per animal were quantified and the subtypes of WFA labeling were identified, as well as the number of surrounding satellite glial cells In relation to the labeling with WFA it was possible to observe the presence of PNN around all the structures analyzed. We also observed that the number of positive WFA neurons as well as the area occupied in both models of UC induction by these in the ganglia was higher when compared to the control group. We infer that the area occupied by the WFA+/CGRP+ neurons was larger in the UCc group than in their respective control group (GCc). We also observed an increase in the number of satellite glial cells in animals exposed to DSS, in addition to the increase in the production of PNN components by these cells and their respective sensory aferent neurons. In this work we provide evidence that the inflammatory environment present in UC induces the increase in the production of PNNs and their condensation. And that, as in the CNS, the PNN act in order to protect the sensory prolongations of the inflammatory environment and increase the conduction capacity of the nociceptive information up to the CNS.

Key words: extrinsic innervation; inflammation; extracellular matrix.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

CGa	Grupo controle agudo
CGc	Grupo controle crônico
CGRP	Peptídeo relacionado ao gene da calcitonina
CSPG	Proteoglicanos de sulfato de condroitina
DC	Doença de Chron
DII	Doenças inflamatórias intestinais
DSS	Dextran sulfato de sódio
GRD	Gânglio de raíz dorsal
HAPLN	Ácido hialurônico
HAPLN-1	Ácido hialurônico do tipo 1
IFN	Interferon
IL-4	Interleucina 4
IL-5	Interleucina 5
IL-9	Interleucina 9
IL-10	Interleucina 10
IL-13	Interleucina 13
IL-33	Interleucina 33
MEC	Matriz extracelular
RCU	Retocolite ulcerativa
RPN	Rede perineuronal
RPNs	Redes perineuronais
SAH	sintetases do ácido hilurônico
SAH-2	sintetases do ácido hilurônico do tipo 2
SAH-3	Sintetases do ácido hilurônico do tipo 3
SN	Sistema nervoso
SNC	Sistema nervoso Central
SNE	Sistema nervoso entérico
SNP	Sistema nervoso periférico
TGI	Trato gastrointestinal
Th2	Resposta imune do tipo Th2
Th9	Resposta imune do tipo Th9
TN	Tenascina

TnC	Tenascina R
TNF-α	Fator de necrose tumoral-alfa
TnR	Tenascina R
TnW	Tenascina W
TnX	Tenascina X
UCa	Grupo retocolite ulcerativa aguda
UCc	Grupo retocolite ulcerativa crônica
WFA	*Wisteria Floribunda* aglutinin

SUMÁRIO

REVISÃO BIBLIOGRÁFICA

DOENÇAS INFLAMATÓRIAS INTESTINAIS

O trato gastrointestinal (TGI) apresenta uma grande interface entre o ambiente externo e o organismo. Diariamente este trato atua sobre moléculas que irão ser responsáveis pelo abastecimento do organismo (GUYTON et al., 2017), além dessas moléculas o TGI também entra em contato com diversos microorganismos, patogênicos ou não (MADINGAN et al., 2016). Assim como esta grande interface é essencial para manutenção da vida, ela também pode servir de porta de entrada para microorganismos. Os microorganismos ao ultrapassarem o lúmen intestinal, podem causar distúrbios que podem afetar severamente a vida de seus portadores (BROCK et al., 2016).

As doenças inflamatórias intestinais (DII) representam doenças capazes de gerar um intenso processo inflamatório intestinal e afetar o funcionamento do órgão (TORRES et al., 2017; UNGARO et al., 2017). As DII são divididas em duas principais doenças: a doença de Chron (DC) e a retocolite ulcerativa (RCU). Na doença de Chron o intenso processo inflamatório é presente de maneira focal podendo afetar todas as camadas da parede do TGI, assim como em qualquer região do intestino. Já na RCU, doença de estudo deste trabalho, a alteração tecidual causada pela inflamação é restrita a túnica mucosa do intestino grosso (DANESE et al., 2006).

A execução das funções necessárias para homeostasia corporal realizadas pelo intestino é acompanhada por um completo sistema de proteção (DOE, 1989). A primeira linha de defesa é realizada por uma camada de enterócitos que juntos irão formar uma barreira, impedindo com que agentes agressores ultrapassem o lúmen intestinal e adentrem a parede do TGI (BROCK et al., 2016). Junto a essa barreira também fazem parte do sistema de proteção: células imunes residentes, células imunes não residentes, células M, células de Paneth, células tuft e células caliciformes (BILLIPP et al., 2021; JUNQUEIRA, CARNEIRO, 2017).

A fisiopatologia das DII é iniciada pela resposta imunológica inata, seguido por uma resposta celular adaptativa a qual é o ponto chave que diferencia a patogênese de cada uma das doenças (BARBALHO et al., 2020). Enquanto a DC

possui um padrão Th1 bem definido (SAKURABA et al., 2009) a RCU é mediada por uma resposta linfocítica do tipo Th2 (GRAHAM et al., 2020; CAE et al., 2020).

RETOCOLITE ULCERATIVA

A RCU é uma doença idiopática e multifatorial, que apresenta grande representatividade na clínica médica, já que nos últimos anos o número de casos aumentou drasticamente (ZOU et al., 2021). Estimasse que na América do Norte e Europa a cada 200 pessoas, 1 possuí ou já desenvolveu a RCU em determinados momentos de sua vida (BARBALHO et al., 2018; NG et al., 2018; SHARARA et al., 2018; MAGRO et al., 2017; NG et al., 2013).

Essa doença é causada pela associação de fatores genéticos, ambientais e alimentares que resultam num processo inflamatório exacerbado, iniciado por células imunitárias inatas, principalmente neutrófilos que produzirão TNF-α, IL-6 e IL-8 (HEYLEN et al., 2014). Estas moléculas inflamatórias serão responsáveis pela ativação da resposta Th2 levando à cronificação da doença e um subsequente aumento de IL-4, IL-5, IL-9 e IL-13 (BAMIAS; COMINELLI, 2015). A IL-13 possuí um importante papel na destruição da barreira epitelial, já que essa interleucina é capaz de ativar células T citotóxicas que irão destruir as células epiteliais que formam o primeiro mecanismo de defesa do TGI, culminando na exposição das regiões mais internas da camada mucosa e na destruição da arquitetura tecidual (ANNUNZIATO et al., 2015; SEILLET et al., 2014).

Após a reposta Th2 e elevar os níveis de IL-4 e IL-9, essas interleucinas serão responsáveis por induzirem a transformação de linfócitos Th0 indiferenciados em linfócitos Th9 (HARUSATO et al., 2017). A resposta Th9, por sua vez, é capaz de induzir um efeito negativo sobre a proliferação e no reparo celular da barreira intestinal, levando a perpetuação da inflamação e impossibilitando a reconstrução fidedigna do tecido (Fig. 1) (SHOHAN et al., 2018).

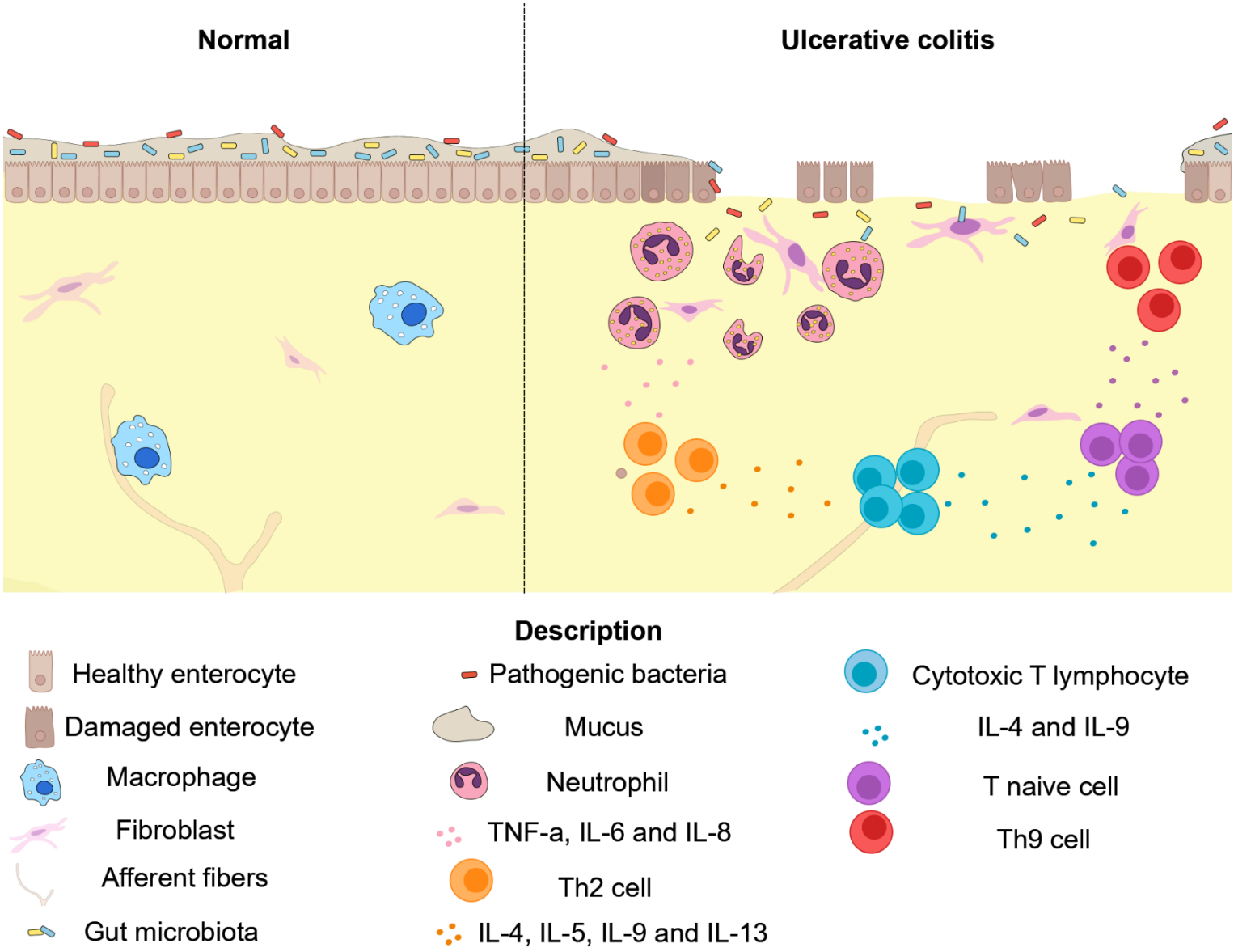

Figura 1 –Representação esquemática da parede intestinal do cólon (lâmina própria) representando a morfologia das células em condições normais à esquerda e no processo inflamatório da RCU.
Fonte: Elaborado pelo autor

As alterações histopatológicas são importantes indicativos da evolução da doença, em um primeiro momento quando o processo inflamatório está em seu início são observados dois fenômenos. O primeiro de caráter imunológico, é definido pelo intenso infiltrado inflamatório de células multinucleadas na camada mucosa, junto com a formação de granulomas e de macrófagos gigantes. Já no segundo de caráter morfológico, é observada a destruição da barreira epitelial junto a tentativa de reparo por fibroblastos (BONETTI et al., 2021).

Em situações em que o estímulo para a destruição de tecidual acontece repetidas vezes seguido por tentativas de recomposição da arquitetura tecidual, comumente é observado próximo ao lúmen o fenômeno de displasia (SU et al., 2020). Na displasia as células da camada mucosa se tornam menos diferenciadas, a formação de criptas de Lieberkühn se tornam raras, bem como a presença de células que remetam as células de um tecido sadio (BONETTI et al., 2021; SU et al., 2020). Além disso, o constante dano seguido pelas tentativas de reparo pode resultar em danos genéticos que por sua vez, pode levar ao surgimento de neoplasias no cólon, que são comumente encontradas na RCU (HIRSCH et al., 2020; SANDS et al., 2019; NEURATH et al., 2019).

INERVAÇÃO DO TRATOGASTROINTESTINAL

Os produtos gerados no processo inflamatório na RCU são capazes de interagir com a inervação presente no cólon (MOYNES et al., 2014). A inervação do TGI está envolvida na determinação dos padrões de movimentos intestinais, no controle da secreção de fluidos, na regulação do movimento dos fluidos no lúmen, na regulação do fluxo sanguíneo local e na liberação de hormônios intestinais (KANG et al., 2021).

O cólon, assim como em todo o TGI, é inervado pelo sistema nervoso entérico (SNE), o qual possue componentes intrínsecos (de origem na parede intestinal) e componentes extrínsecos (originados no sistema nervoso central e direcionados a parede intestinal) (FURNESS, 2006).

INERVAÇÃO INTRÍNSECA

A literatura tem como consenso que o SNE pode atuar de forma autônoma para controlar os reflexos de motilidade intestinais e realizar outras funções (GIBBINS et al., 1985; LANGLEY; MAGNUS, 1905; BAYLISS; STARLING, 1901) Já o sistema nervoso central (SNC) desenvolve um importante papel no controle da motilidade gástrica e esofágica (BERNE et al., 2009).

O SNE pode ser descrito como uma forma eficiente de deslocamento do controle visceral do SNC, através da eliminação do número de vias entre o SNC e

o tubo digestório, de modo a facilitar a troca de informações. Trata-se de um sistema muito complexo, cujo número total de neurônios que o constitui possuem a mesma magnitude do número de neurônios da medula espinal (FURNESS et al., 1987). Esse grande número de neurônios está localizado no TGI, principalmente entre os estratos musculares de sua parede (STERNINI, 1988) e na camada submucosa do intestino (Fig. 2) (GUYTON et al., 2017).

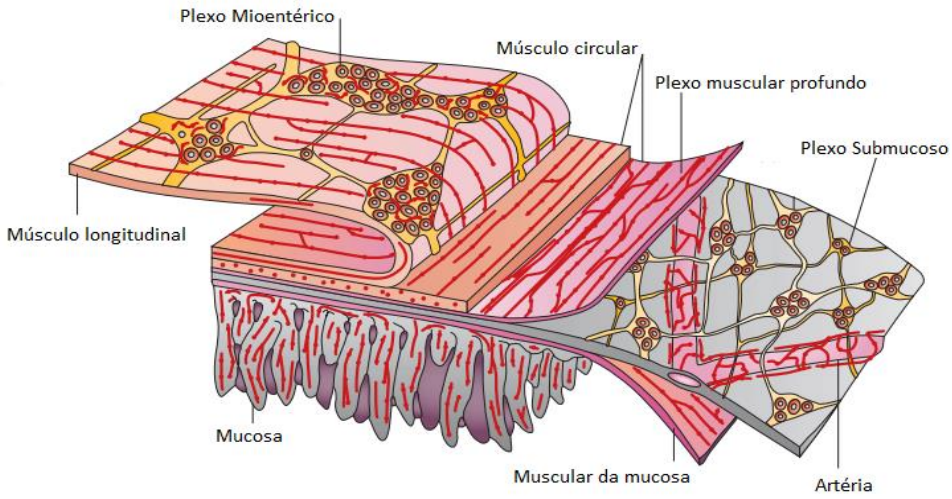

Figura 2 - Organização do SNE.
Fonte: Adaptado de Furness et al. (2012).

O SNE está relacionado ao controle das funções dos intestinos delgado e grosso, exercendo funcionamento mesmo quando separado do SNC. Por outro lado, mesmo atuando de maneira independente, o SNE é monitorado pelo SNC por intermédio dos gânglios extrínsecos ao TGI (BROOKES; DINNING; GLADMAN, 2009). Esses gânglios extrínsecos, por intermédio dos prolongamentos de seus nervos, participam da inervação extrínseca do TGI (FURNESS et al., 2014).

INERVAÇÃO EXTRÍNSECA

O cólon dos mamíferos é dividido anatomicamente em duas porções: proximal e distal, sendo a porção final a região com maior quantidade de fibras nervosas extrínsecas silenciosas do TGI (HARRINGTON et al., 2018). Essas fibras representam um conjunto de prolongamentos de neurônios presentes nos gânglios de raiz dorsal (GRD) e são ativadas principalmente por moléculas inflamatórias, como

citocinas e neurotransmissores pró-inflamatórios (BRIERLEY et al., 2018; BRIERLEY et al., 2004).

A informação captada pelos prolongamentos dos GRD é levada até o corno dorsal da medula espinal (substância cinzenta), em seguida viaja pelas vias espinotalâmicas ou espinorreticulares (LARSSON et al., 2012). A via espinotalâmica é responsável por conduzir a informação até o tálamo, onde será processada e transmitida até o córtex somato sensorial, córtex pré-frontal e córtex cingulado anterior (TILLISCH et al., 2011). De acordo com a intensidade e frequência do sinal captado, o córtex pode dar origem a diferentes sensações, como o desconforto e a dor (PUOPOLO et al., 2019; FRANCOIS et al., 2017; BANNISTER; DICKENSON, 2016; CHEN et al., 2017; OSSIPOV et al., 2010). Além disso, a via espinotalâmica realiza conecções com o núcleo vagal sensorial, que está ligado a centros superiores de processamento (COSS-ADAME et a., 2014; AMMONS et al.,1985) (Fig. 3).

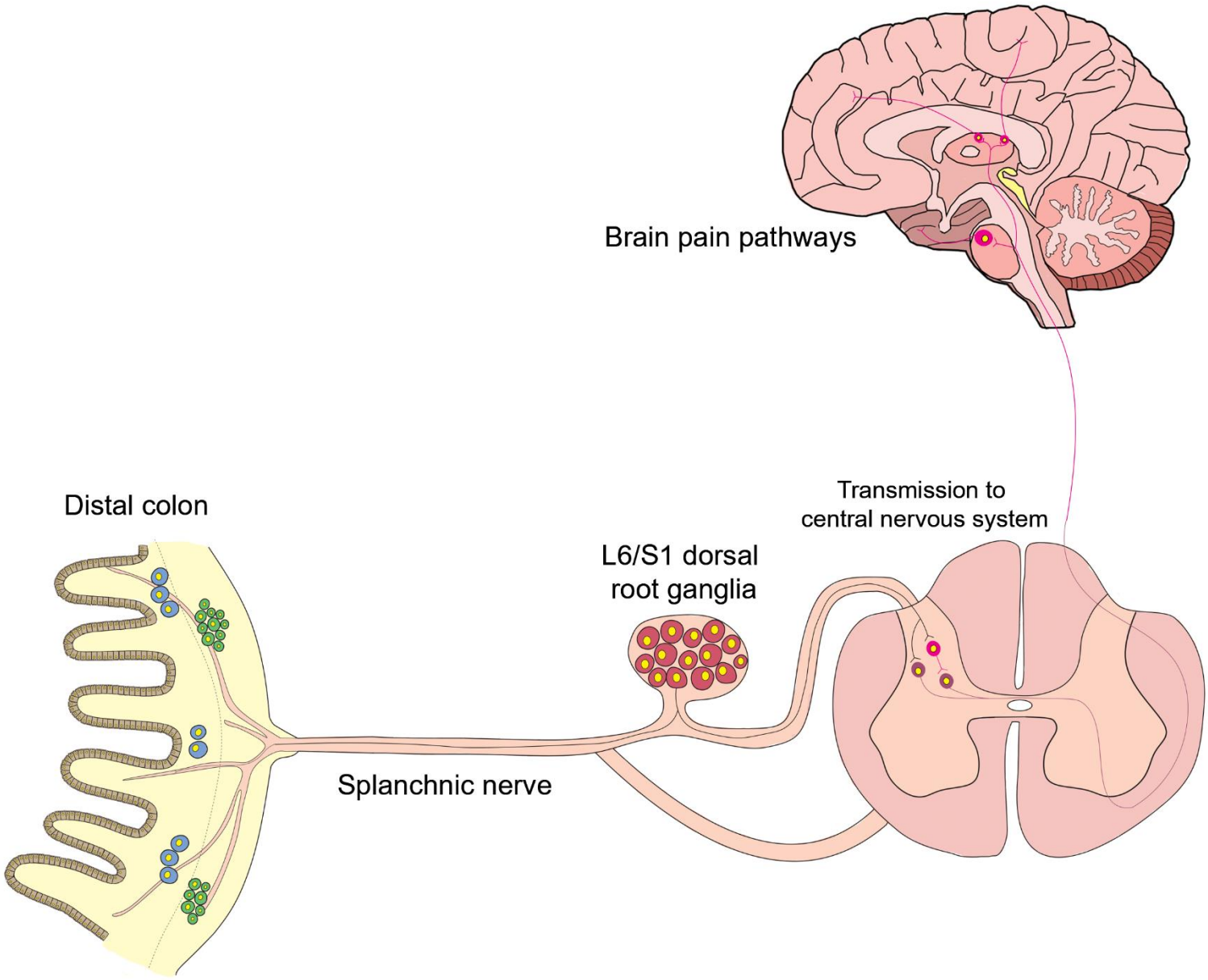

Figura 3. Representação esquemática do mecanismo de aferência realizada pelos neurônios presentes no DRG e a condução de informações até o SNC.

Fonte: Elaborado pelo próprio autor.

A importância da sinalização da inervação extrínseca aferente não se resume somente a inflamação, os neurônios presentes nos GRD também detectam a distenção da parede intestinal, mobilidade da camada mucosa, bem como a absorção de água e nutrientes (UESAKA et al., 2016; DEVROEDE; LAMARCHE, 1974). Esses sinais captados e transmitidos por gânglios sensitivos do TGI dão origem a uma variedade de sensações conscientes, como saciedade, náuseas, desconforto e dor

(HABERBERGER et al., 2019). Sendo que 90% da população neuronal destes gânglios possuem prolongamentos sensitivos (SPENCER et al., 2014). Os GRD representam o maior aglomerado de neurônios aferentes alocados fora do SNC (DEVOR, 1999), os quais são responsáveis por transmitirem informações sobre alterações de pressão, tato, ritmo de contração, secreção e alterações químicas no TGI, as quais são encaminhadas e interpretadas pelo SNC (KYLOH et al., 2011; LARSSON et al., 2003; NESS et al., 1990).

O conjunto de prolongamentos dos neurônios dos GRD que dão origem aos nervos pélvicos responsáveis pela inervação do cólon distal, transportam também informações aferentes de mecanorreceptores de baixo limiar. Esses prolongamentos são classificados como terminações nervosas laminares intra-ganglionares, semelhantes aos presentes no esôfago e no estômago. São acionados por sondagem direta ou pelo alongamento da parede do reto em uma ampla faixa, incluindo os níveis de dor (BROOKES; DINNING; GLADMAN, 2009).

De forma geral os neurônios presentes nos GRD podem ser divididos em neurônios proprioceptivos e nociceptivos. Os neurônios nociceptivos podem ser: imunorreativos para a substância P e ao peptídeo relacionado ao gene da calcitonina (CGRP) (OHTORI et al., 2002); imunorreativos para a isolectina B4 (BRUMOVSKY et al., 2006); ou produtores de tirosina hidroxilase, enzima limitadora na taxa de síntese de catecolaminas (OHTORI et al., 2007).

Os neurônios nociceptivos presentes nos GRD podem secretar moléculas em resposta a detecção de sinais nocivos captados por seus prolongamentos, como é o caso da substância P e o CGRP (PRICE et al., 2007; CHRISTIANSON et al., 2006; ROBINSON et al., 2004). O CGRP é a principal neuropeptídeo envolvido no mecanismo de nocicepção e está distribuído tanto em vias nociceptivas no SNC como Sistema Nervoso Periférico (SNP) (STATON et al., 2007). Além de estimular a nocicepção, a secreção do CGRP também pode causar vasodilação no sistema vascular entérico (SMITH et al., 1992). Composto 37 aminoácidos, junto a adrenomedilina, amilina e calcitocina completa a família de peptídeos da calcitonina (BRAIN et al., 1996; AMARA et al., 1982). Em situações de normalidade sabe-se que até 50% dos neurônios presentes nos GRD de camundongos saudáveis expressam este neuropeptídeo (LEE et al., 1985; WIESENFELD et al., 1984). O aumento na produção de CGRP reduz o limiar

nociceptivo para a estimulação mecânica (OKU et al., 1987) e também leva a sensibilização de nociceptores (SCHOU et al., 2017).

INFLAMAÇÃO E COLITE

O ambiente inflamatório presente na RCU leva a alterações nas vias aferentes extrínsecas, alterações as quais irão ser interpretadas pelo SNC e darão origem a dor visceral (MOYNES et al., 2014).

A dor intestinal tem seu início na detecção de substâncias pronocipetivas, como a bradicinina e TNF-α, pelas terminações nervosas dos neurônios aferentes extrínsecos (PUOPOLO et al., 2019; MAKOWSKA et al., 2018; JUNIOR et al., 2017). No momento em que há o aumento da concentração destas moléculas na parede do cólon, os receptores destes neurônios presentes em suas terminações se tornam sensibilizados, iniciando o processo da dor (LA, GEBHART 2011; BEYAK et al. 2004; STEWART et al., 2003;). A detecção das moléculas prononiceptivas assim como a sensibilização dos receptores dos neurônios sensoriais aferentes extrínsecos é capaz de induzir a produção de CGRP, aumentando drasticamente a porcentagem de neurônios que expressam o neuropeptídeo (LAI et al., 2017; EYSSELEIN et al., 1992). Acredita-se que o papel do CGRP na colite atue em mão dupla: na parede do cólon este atue como um potente vasodilatador e como um importante agente anti-inflamatório (LI et al., 2013; MILLET et al., 2000). Assim como também pode ativar vias dopaminérgicas cerebrais responsáveis pela dor (PUOPOLO et al., 2019).

DOR

Os principais sintomas da RCU são a dor abdominal recorrente, diarreia, fezes com sangue e perda de peso contínua, sendo a dor o principal sintoma presente na RCU (YU; RODRIGUEZ 2017). De modo geral, a dor sempre esteve presente na rotina dos mamíferos, evolutivamente esta é associada a experiências prejudiciais, relacionadas a ações ou situações capazes de gerar danos (WLLIAMS et al., 2019). Acredita-se que seja um importante mecanismo inconsciente de defesa do SNC que visa valorizar a sobrevivência (STEARNS et al., 2016; ANDREWS et al., 2002).

É importante saber que nem tudo é dor, segundo a Associação

Internacional para o Estudo da Dor (IASP) a dor é uma experiência sensorial ou emocional desagradável associada a danos teciduais, reais ou potenciais, ou descritos em função da lesão. Diferente do sofrimento o qual inclui todos os tipos de experiências negativas, como fome, desconforto, cansaço ou privações (FORDYCE, 1998). Em suma, a dor se restringe a presença de possíveis danos teciduais que são captados pelas terminações nervosas aferentes extrínsecas, em sua maioria presente nos GRD (BRIERLEY et al., 2018).

As experiências dolorosas moldam a capacidade do indivíduo de responder ao ambiente por meio de complexos mecanismos de aprendizado, possibilitando que este tenha uma vida mais longa. Acredita-se também que parte desse aprendizado é passado pelo material genético, e que caso seja benéfico, se torna mais frequente em uma população (WALTERS, WILLIAMS 2019).

Por outro lado, essa sensação não é só benéfica, dependendo da intensidade e frequência, a dor se torna um elemento incapacitante, levando o indivíduo a abdicar-se de suas funções rotineiras, além de afetar sua qualidade de vida nos âmbitos emocionais, sociais e econômicos (BRUCE et al., 2009; SAASTAMOINEN et al., 2005).

DOR E COLITE

Como citado anteriormente a dor é o principal sintoma relatado por portadores das DII e RCU. Estudos indicam que a dor abdominal está presente em 50 a 70% dos pacientes com doenças inflamatórias intestinais, sendo este o principal motivo de procura aos consultórios médicos por RCU (BIELEFELDT et al., 2009).

A dor na RCU pode se tornar um elemento incapacitante, principalmente em condições crônicas, onde afeta diretamente a qualidade de vida de seus portadores (KIM; CHEON, 2017). Acredita-se que grande parte dos pacientes não procuram um consultório médico de imediato, principalmente pela dor ser um sintoma inespecífico. Este fato induz o uso da auto-medicação paliativa, o que dificulta o tratamento específico e aumenta os custos do tratamento (PARRAY et al., 2012; WHITTEN et al., 2005; CAMPOS et al., 2002).

MATRIX EXTRACELULAR

Em mamíferos o tecido conjuntivo é composto por células (imunitárias e fibroblastos) e pela MEC. A MEC é formada por fibras, glicoproteínas adesivas, proteoglicanos, ácido hialurônco e a substância fundamental amorfa sendo presente em todos os sistemas inclusive no sistema nervoso (BOSMAN, STAMENKOVIC 2003). No organismo como um todo, a MEC é uma substância amorfa e difusa que tem como principal função preencher o espaço entre as células e auxiliar em processos metabólicos e celulares (GARTNER, 2017).

Porém a composição dessa matriz não é homogênea em todos os sistemas, em algumas situações, a MEC pode adquirir uma composição específica, levando-a adquirir funções não convencionais (YURCHENCO, SCHITTNY 1990). A primeira especialização descoberta foi a membrana basal, que permite com que células epiteliais se ancorem no tecido conjuntivo, e que se mantenham firmes e unidas em situações onde essa função é de extrema valia, como acontece com as células epiteliais intestinais (JUNQUEIRA, CARNEIRO 2017; KELLEY, et al., 2014). Além disso, a membrana basal também auxilia no transito de moléculas que são absorvidos em direção ao sistema vascular (LEBLEU et al., 2007).

REDE PERINEURONAL

Ao final do século XIX, Ramón e Cajal (1880, 1887, 1898) publicaram estudos descrevendo a existência de uma “cobertura delicada” em torno dos neurônios, que percorria os corpos celulares e se estendia até seus dendritos. Em 1901, Arturo Donaggio descreveu que essas coberturas, agora chamadas de Rede perineuronais (RPNs), possuíam aberturas, pelas quais os terminais de neurônios vizinhos realizam sinapses (Fig. 4). As RPNs também foram estudadas por pesquisadores de renome como Golgi (1898) e Bethe (1899) (BODEGA et al., 1985).

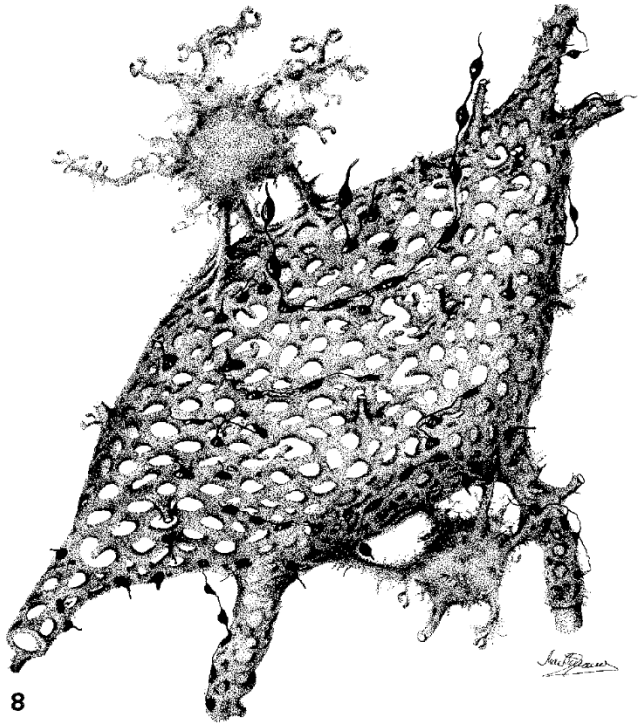

Figura 4 – Rede perineuronal.
Fonte: Adaptado de M. Lafarga; M. Berciano; M. Blanco. 2004

Com o avanço da microscopia e dos métodos proteomicos, foi possível verificar que a formação das RPN's era restrita somente a populações específicas do SNC. Já foram descritas no córtex cerebral (WEGNER et al., 2003), no cerebelo, no troco encefálico (BEKKU et al., 2012), no hipocampo, no hipotálamo (LENSJØ et al., 2017) e na medula espinal (MURAKAMI et al., 2003).

A especialização dessa matriz se deve a sua composição específica. A produção, secreção e deposição das moléculas constituintes da RPN é realizada pelos próprios neurônios e pelas células da glia (CELIO et al., 1994). Seus principais constituintes são: o ácido hialurônico, hialecticanas, proteínas de ligação e tenascinas (GALTREY et al., 2008; CARULLI et al., 2006; TOOLE et al., 2000).

O ácido hialurônico é um polímero linear de dissacarídeos de N-acetilglicosamina e ácido glucurônico unidos por ligações β1-4 e β1-3 (MEYER et al., 1951). Esta é a única glicosaminoglicana pertencente à MEC sem estar covalentemente ligada a um eixo proteico (TOOLE et al., 2000). Semelhante ao que ocorre em cartilagens, o ácido hialurônico interage com outras moléculas da MEC permitindo a formação de grandes agregados proteicos que envolvem neurônios e glia no SNC (KÖPPE et al., 1997). O ácido hialurônico é sintetizado por enzimas

(sintetases do ácido hilurônico - SAH) localizadas na superfície da membrana plasmática das células produtoras (WEIGEL et al., 2007). As três formas de SAH (1, 2 e 3) são codificadas por genes localizados em diferentes cromossomos. Com o uso de hibridização *in situ*, demonstrou-se que neurônios com RPN no cerebelo expressam SAH-2 e SAH-3, enquanto na medula espinal esses neurônios expressam SAH-1 e SAH-3 (GALTREY et al., 2008; CARULLI et al., 2006). Essas variações na expressão de SAH, bem como no padrão de síntese de ácido hialurônico, podem influenciar as propriedades da RPN e, portanto, o funcionamento de neurônios (FOWKE et al., 2017).

As proteoglicanas de sulfato de condroitina (CSPG) pertencem à família das hialectinas. As hialectinas são os principais componentes da RPN e podem ser divididas em quatro tipos: agrecanas, versicanas, neurocanas e bevicanas (ZIMMERMANN et al., 2008; YAMAGUCHI, 2000). Sendo que essas quatro glicoproteínas também estão presentes na RPN (CARULLI et al., 2006; HAGIHARA et al., 1999). As hialectinas partilham domínios estruturais, incluindo o domínio globular N-terminal, uma região longa e central que contém cadeias de sulfato de condroitina, ligadas covalentemente a um domínio globular C-terminal. Esses domínios se ligam ao ácido hialurônico e a Tenascina X (TnX), formando agregados macromoleculares no espaço extracelular (MIYATA et al., 2018). Enquanto as agrecanas estão presentes em todos os locais onde há RPN, as demais hialectinas são encontradas em parte das RPN, indicando que estão relacionadas a subpopulações de neurônios (GALTREY et al., 2008).

As proteínas de ligação constituem um grupo de proteínas que interagem tanto com o ácido hialurônico quanto com as glicosaminoglicanas das hialectinas e assim estabilizam os agregados formados por esses componentes. Elas pertencem a uma superfamília de módulos de ligação, que incluem as proteínas de ligação e as hialectanas. Com base em suas propriedades de ligação, considera-se que também pertençam à família de genes de proteínas de ligação que interagem com proteoglicanos e ácido hialurônico (HAPLN) (SPICER et al., 2003). Há quatro membros no grupo das HAPLN e todos compartilham uma estrutura similar. Três desses quatro membros têm sido encontrados no SNC (HAPLN-1, 2 e 4) (HIRAKAWA et al., 2000). HAPLN-1 e HAPLN-4 são os membros encontrados exclusivamente em RPN (GALTREY et al., 2008; BEKKU et al., 2003; CARULLI et al., 2006; RAUCH et al., 2004).

As tenascinas (Tn) fazem parte de uma família com cinco membros: X, C, R, W e Y. São glicoproteínas multiméricas da MEC importantes para a morfogênese e desenvolvimento neural, regeneração de nervos, migração celular e cicatrização de feridas (JONES et al., 2000). Enquanto TnC, TnX e TnW são expressas também fora do SNC, a expressão de TnR é mais restrita ao SNC (KIMURA et al., 2007; TUCKER et al., 2009) e estudos mostraram que sua localização é marcante em RPN (CARULLI et al., 2006; WEBER et al., 1999).

Acredita-se que o complexo formado por ácido hialurônico, hialectinas e tenascinas, quando ancorado no citoplasma neuronal, via receptores de CSPG (Fig. 5), funcione como uma barreira repulsiva a outros neurônios e dendritos (MIAO et al., 2014; BONNEH et al., 2009).

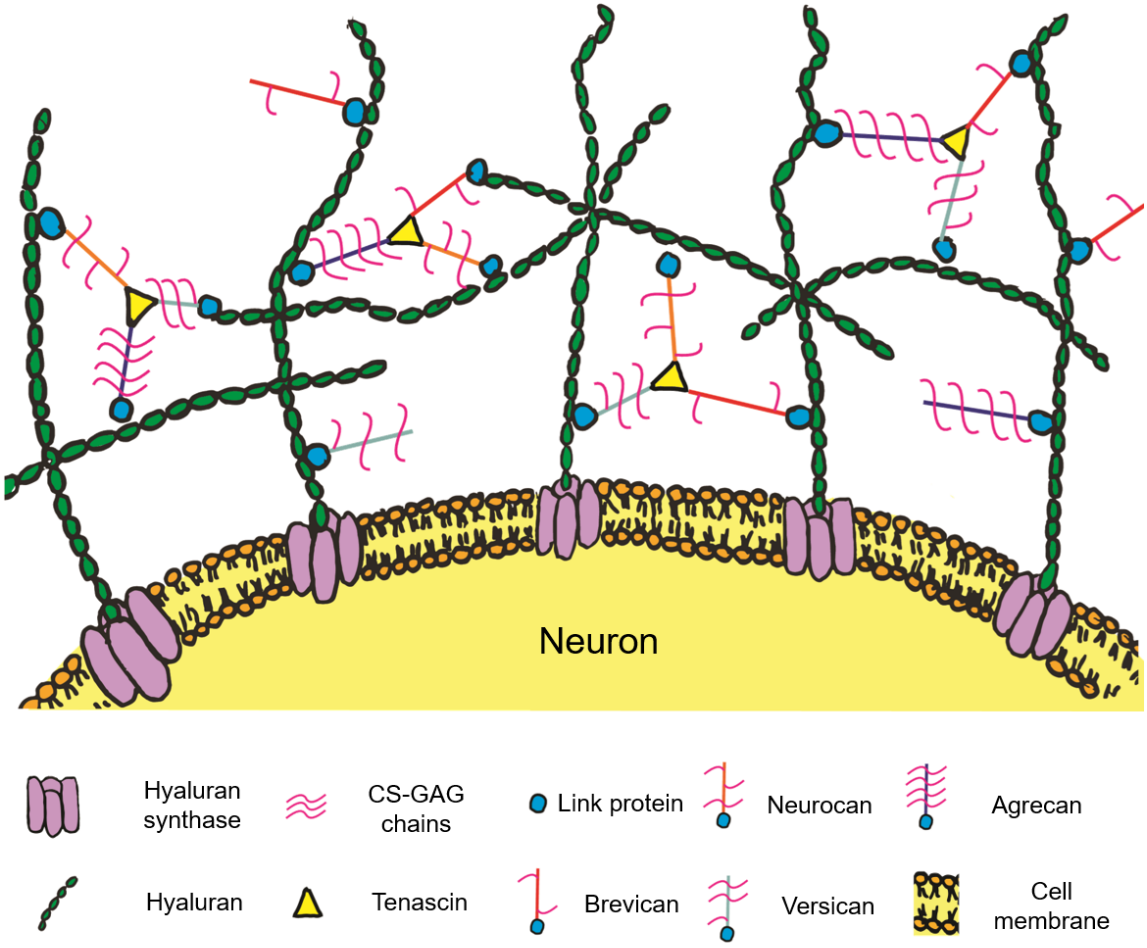

Figura 5 - Representação dos componentes da RPN.

Fonte: Elaborado pelo próprio autor.

Novas descobertas nos últimos anos demonstraram que a RPN é fundamental para a manutenção de funções essenciais tanto em condições normais quanto nos distúrbios e doenças (REICHELT et al., 2019). Sabe-se que RPN está envolvida no funcionamento de tipos específicos de neurônios e na plasticidade de neurônios de indivíduos adultos (KWOK et al., 2014; KWOK et al., 2011; GALTREY et al., 2007; PIZZORUSSO et al., 2006), bem como na estabilização de sinapses após o nascimento (DITYATEV et al., 2003; BRUCKNER et al., 2000).

A RPN está relacionada com processos fisiológicos, como o desenvolvimento do aprendizado e memória (YANG et al., 2015; XUE et al., 2014; GOGOLLA et al, 2009). Em distúrbios patológicos, observou-se envolvimento da RPN na recuperação em casos de danos no SNC (FAWCETT, 2009; GALTREY et al., 2007), esquizofrenia (MAUNEY et al., 2013), doença de Alzheimer (ITTNER et al., 2011), derrame, epilepsia (YUTSUDO et al., 2015), traumas psicológicos, espectro de autismo (ABDALLAH et al., 2012), depressão (RYBAKOWSKI et al., 2013) e dependência de drogas (SLAKER et al., 2015).

Foi demonstrado no SNC, que a RPN representa um freio molecular, capaz de limitar a plasticidade neuronal na idade adulta e que sua degradação pode ocorrer para reestabelecer à plasticidade (ROMBERG et al., 2013; GOGOLLA et al., 2009). Como resultado de sua perda, há brotamento de axônios e, em caso de lesões, a RPN auxilia na regeneração de neurônios danificados em várias regiões do cérebro (HAPPEL et al., 2014; XUE et al., 2014; PIZZORUSSO et al., 2002). Também foi possível identificar que a RPN possui papel chave no desenvolvimento neuronal, na sinaptogênese (MCRAE et al., 2012), na neuroproteção (CELIO et al., 1998) e na plasticidade sináptica (SUTTKUS et al., 2016; DITYATEV et al., 2003).

Köppe et al. (1997) identificaram que a presença de RPN somente é expressa em períodos pós-natais, sendo caracterizada como contínua e não transitória. Também relataram que sua presença parece estar relacionada com a maturação neuronal. Ou seja, em indivíduos mais jovens é necessário que essa rede seja menos expressa para que mecanismos de plasticidade neuronal ocorram com mais frequência, diferente de indivíduos adultos que necessitam de maior estabilidade dos circuitos neuronais já formados.

Já é definido que em locais onde já exista a capacitação genética dos neurônios e células da glia visando a produção da RPN, inicia-se o processo de transcrição gênica visando a secreção dos constituintes da RPN frente a um possível

dano neuronal (DZYUBENKO et al., 2018). A detecção do dano neuronal é feita pelo reconhecimento de citocinas, moléculas inflamatórias ou pelo aumento do estresse oxidativo, comumente ligado a focos inflamatórios (MORAWSKI et al., 2004). Subsequentemente os componentes da RPN são secretados e organizados ao redor dos neurônios com o objetivo de protegê-lo, bem como preservar a função a qual esse exerce ou as informações contidas nele (CABUNGCAL et al., 2013).

Na doença de Alzheimer, experimentos utilizando diferentes modelos animais demonstraram alterações na RPN nos diferentes estágios da doença, consequentemente defasagem na funcionalidade da RPN, incluindo o mecanismo de proteção neuronal (ITTNER et al., 2011; CREWS et al., 2010). Após marcação com a aglutinina *Wisteria Floribunda* (WFA), foi possível observar uma perda significativa de RPN no córtex cerebral (BAIG et al., 2005; ARNOLD et al., 1991; BEACH et al., 1988). Outro estudo, utilizando tecido cerebral humano, demonstrou que o ácido hialurônico é altamente expresso nas placas amiloides. Já as RPN contendo N-acetil-D-galactosamina, resistem à decomposição e são degradadas somente após a degeneração das estruturas neuronais. Isto sugere que a RPN ao aumentar a expressão de ácido hialurônico, possibilita a penetração das células da glia nas regiões afetadas pela doença de Alzheimer e que a RPN exerce um papel protetor sob as estruturas neuronais (MORAWSKI et al., 2012).

Abdallah et al. (2012), sugeriram que no espectro austista, a hiperplasticidade neuronal é causada pelo aumento na expressão de metaloproteinases, com função de degradar a RPN. Essa hiperplasticidade influenciará na expressão dos sintomas e evolução do distúrbio, por meio da exposição neuronal, ao degradar um dos seus mecanismos de defesa: a RPN.

REDE PERINEURONAL E A INERVAÇÃO DO TGI

A literatura tem como consenso que a barreira hematoencefálica é responsável por grande parte da proteção do SNC (BALLABH et al., 2004), assim como a barreira formada pelo sistema sanguíneo (SHIMIZU et al., 2011) é responsável por proteger as estruturas constituintes do SNP. Os GDR possuem característica única, uma vez que são desprovidos de barreiras físicas projetadas exclusivamente para sua proteção, a qual fica sob responsabilidade das células gliais que estão em

contato íntimo com os corpos celulares dos neurônios presentes nos gânglios. Por não possuir nenhuma barreira física, os neurônios de GRD ficam expostos a moléculas, patógenos e outros agentes (HU et al., 2002).

Sabe-se que ao redor de populações neuronais específicas no SNC, a existência e a composição da RPN são bem descritas. Sabe-se também que essa estrutura é essencial para o desenvolvimento e funcionamento desses grupos de neurônios, seja por meio dos mecanismos de neuroproteção (CELIO et al., 1994), controle da plasticidade (DITYATEV et al., 2003) ou estabilização sináptica (SUTTKUS et al., 2016). Por outro lado, poucos são os relatos investigando a existência de RPN no SNP e nenhum estudo avaliando a presença desta rede nos neurônios de gânglios sensitivos do intestino. Recentes estudos, publicados por nosso grupo de pesquisa, revelaram que pacientes com deficiência de TnX, importante constituinte da RPN no SNC, apresentavam dor abdominal e constipação. Demonstramos também que pacientes e camundongos deficientes de TnX apresentavam alterações estruturais em neurônios intrínsecos do intestino grosso, quando comparados ao grupo controle (AKTAR et al., 2018). Identificarmos também que a TnX exerce um papel fundamental na inervação aferente do estômago, funcionando como uma “ancoradoura” das terminações nervosas em seus ligantes (AKTAR et al., 2019).

Dada a importância dos GRD para a homeostase do tubo digestório e sua influência em processos patológicos, combinada com a ausência de estudos que descrevam a presença da RPN nesses gânglios, faz-se necessário explorar os neurônios constituintes dos GRD e seus prolongamentos, em camundongos no intuito de investigar a presença de RPN, bem como identificar sua função, sobretudo sua relação com o mecanismo de inflamação e dor presente na RCU.

Nossa hipótese é de que assim como no SNC, a RPN esteja presente nos GRD e em seus prolongamentos presentes no nervo splânico. Produzida pelos neurônios e células auxiliares, a RPN possivelmente sofre alterações quando o metabolismo destas células é alterado, como acontece na RCU. Sendo assim acreditamos que o ambiente inflamatório presente na RCU seja capaz de alterar a organização da RPN ao entorno de neurônios presentes nos GRD e em seus prolongamentos.

OBJETIVOS

OBJETIVO GERAL

- Descrever a presença de RPN e suas alterações na inervação lombossacral aferente do cólon distal de camundongos com RCU.

OBJETIVOS ESPECÍFICOS

- Investigar se há a presença de RPN em gânglios de raiz dorsal, e em seus respectivos nervos esplânicos de níveis L6 e S1 de camundongos C57Bl/6.
- Quantificar o número e a área de corpos celulares de neurônios envoltos por RPN em gânglios de raiz dorsal e de seus respectivos nervos splanicos de níveis L6 e S1 durante o processo inflamatório presente na RCU aguda e crônica.
- Investigar o papel das células gliais satélites na produção da RPN.

MATHEUS DEROCO VELOSO DA SILVA

PRESENCE OF THE PERINEURONAL NET IN THE EXTRINSIC INNERVATION OF THE DISTAL COLON OF MICE AND YOUR REMODELING IN ULCERATIVE COLITIS

Artigo de dissertação apresentado ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial para obtenção do título de Mestre

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PRESENCE OF THE PERINEURONAL NET IN THE EXTRINSIC INNERVATION OF THE DISTAL COLON OF MICE AND YOUR REMODELING IN ULCERATIVE COLITIS

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ABSTRACT

Perineuronal net (PNN) is a highly specialized extracellular matrix well described in the Central Nervous System. There are no reports of its presence neither its relationship with patological processes in the peripheral nervous system. This study described the presence of PNN in the spinal afferent innervation of the distal colon of mice and characterized morphological alterations on this structure induced by experimental ulcerative colitis (UC). Acute and chronic UC was induced by exposing C57Bl/6 mice to 3% dextran sulfate sodium (DSS). L6/S1 dorsal root ganglia (DRG) and their splanic nerves were collected. PNN was labeled using fluorescein-conjugated Wisteria floribunda (WFA) lectin and neurons were labeled by immunofluorescency using antibodies anti-calcitonin gene-related peptide (CGRP) or anti-periferin c-19. Our results demonstrated the presence of a PNN-like structure (WFA+) surrounding most of DRG neuronal cell bodies and also their extensions through the nerves. Confocal microscopy images showed presence of WFA+ labelling in the cytoplasm and on the pericellular surface of neurons and satellite cells. Both acute and chronic UC increased the number of WFA+ neuronal cell bodies, however the number of WFA+ satellite cells increased only in acute UC. The PNN-like structure around cell bodies was more dense in UC mice. Only chronic UC increased the number of WFA+ neuronal extensions in the splanic nerves. In conclusion, we found a PNN-like structure around DRG neuronal cell bodies and their extension in splanic nerves. UC changed the quantity and morphology of this structure.

Key words: Inflammation; Extrinsic innervation; Large intestine; Extracellular matrix.

INTRODUCTION

Inflammatory bowel diseases are among the main disorders that affects the gastrointestinal tract [1,2]. Crohn's disease and ulcerative colitis (UC) represent the main groups of the inflammatory bowel diseases. In UC, the inflammation is restricted to the mucosa layer of the large intestine [3,4]. Its etiology involves the association of genetic and environmental factors that result in an exacerbated inflammatory process, involving neutrophils that will secrete TNF-a, IL-6 and IL-8 [5]. These inflammatory molecules will be responsible by activating the Th2 response, along with the increase of IL-4, IL-5, IL-9 and IL-13 [6].

The inflammation caused by UC may interact with colon innervation [7] , as the distal colon of mammals has the largest amount of silent extrinsic nerve fibers of the gastrointestinal tract [8]. These fibers represent a set of neuronal extensions present in the dorsal root ganglia (DRG) [8,9]. DRG neurons have a peripheral extension towards to the intestinal wall and a central extension towards to spinal cord neurons, which connectes the intestine to the pain pathway in the Central Nervous System (CNS) [10–14].

The nociceptive aferential neurons of the DRG secrete molecules in response to stimuli of harmful agents perceived by their extensions located in the intestinal wall, such as the substance P and the Peptide related to the Calcitonin Gene (CGRP) [15–17]. CGRP is the main neuropeptide involved in the nociception mechanism and it is distributed in both nociceptive pathways in the CNS and in the peripheral nervous system[18]. In addition to nociception stimulation, the CGRP secretion can cause vasodilation in the enteric vascular system [19]. Increased CGRP production reduces the nociceptive threshold for mechanical stimulation [20] leading to sensitization of nociceptors [21]. The UC inflammatory environment drives to alterations in the extrinsic afferents which are interpreted by the CNS causing visceral pain [7].

In the CNS, the space between neurons is occupy by an extracellular matrix (ECM) [22] . Around specific CNS neurons, the composition of the ECM is modified, becoming highly specific, which are called perineuronal network (PNN) [23]. The production, secretion, and deposition of the constituent molecules of the PNN is carried out by neurons and glial cells [24]. The main constituents are: hyaluronic acid, hyalectins, binding proteins and tenascins [25,26].

The PNN is related to physiological processes, such as the development of learning and memory [27–29]. It also plays a key role in neuronal development, synaptogenesis [30], neuroprotection [31] and synaptic plasticity [32,33]. In pathological disorders, the PNN was involved in recovery cases of CNS damage [34,35] , schizophrenia [36] , Alzheimer's disease [37], stroke, epilepsy [38], psychological trauma, autism spectrum [39] , depression [40] and drug dependence [41].

It is known that the existence and composition of the PNN are well described around specific neuronal populations in the CNS [24,32,33]. However, there no reports investigating the existence of PNN in the peripheral nervous system and studies that evaluated the presence of this network around neurons of the extrinsic bowel innervation. Our research group recently demonstrated that patients and mice with Tenascin X (TnX) deficiency, an important constituent of PNN in the CNS, had abdominal pain, constipation and hyperresponsiveness to colon distension when compared to the control group [42].

Given the importance of intestinal extrinsic innervation for the homeostasis of the digestive tube and its influence on pathological processes, combined with the absence of studies describing the presence of PNN in this structure, it is necessary to explore the constituent neurons of DRG and its prolongations. In order to investigate the presence of PNN, especially the relationship with the mechanism of inflammation and abdominal pain in UC. Thus, the aim of this study was to evaluate the presence of PNN in the spinal afferent innervation of the distal colon of C57Bl/6 mice. In addition, we evaluated if the inflammatory process observed in UC was capable of modulate the structure of PNN around cell bodies of DRG neurons and their extensions.

Our hypothesis is that, as in the CNS, PNN is present around the neurons present in DRG and in their extensions in the splenic nerve. We believe that the detection of inflammatory molecules present in the UC, induces the condensation of PNN and the activation of glial stellate cells, generating a denser PNN when compared to control groups.

METHODOLOGY

Animals

The procedure protocol was previously approved by the Ethics Committee on Animal Experimentation (CEUA) of the University of Londrina (UEL) (OF CIRC No. 032/2015) (Appendix A).

In this study, twenty C57BL/6 mice, aged 8 to 12 weeks old, were allocated in polyethylene boxes, with a maximum capacity of 5 animals per box. During the experiment, the boxes were kept in the sectorial bioterium of the Histology Department of the Universidade Estadual de Londrina (UEL), with a controlled temperature (21 to 24ºC) and cycle of 12 hours between light/dark.

Experimental design

The animals were assigned into four groups (n=5) as follow: (i) Control group for the acute UC experiment (CGa): mice that ingested clean (DSS-free) water for 7 days; (ii) Acute UC group (UCa): mice exposed to 3% sodium dextran sulfate (DSS) 40 kDa dissolved in the drinking water for 7 days (FIG. 1A); (iii) Control group for the chronic UC experiment (CGc): mice that ingested clean (DSS-free) water for 29 days; and (iv) Chronic UC group (UCc): animals exposed to 3 cycles of 3% DSS 40 kDa diluted in autoclavated water (5 days/cycle). These cycles were alternated with 2 cycles when mice ingested DSS-free water (7 days/cycle), totalizing 29 days for the chronic UC experiment (FIG. 1B).

Disease activity index (DAI)

In order to observe the development of UC, the animals were monitored daily along the experiment to determine the Disease Activity Index (DAI), considering the criteria suggesty by Bento et al. (2011)[43]:

1. Body mass loss: when it was equal to 5% received a score of 1, from 5% - 10% a score of 2, from 10%-15% a score of 3, and weight loss above 15% received a score of 4.
2. Consistency of the feces: 0 corresponded to absence of diarrhea, 2 to loose feces that do not stick to the rehest and 4 for liquid feces that did not adhere to the anus.
3. Presence of blood in feces: 0 correspond to no bleeding, 2 to moderate bleeding and 4 to coarse bleeding. A test for occult blood detection was used to increase the sensitivity of this evaluation, when there was no visual sign of bleeding [44].

Collection and Processing

The mice were anesthetized using halothane vapor (1%), oxygen (14%) nitrogen (86%). Then, cardiac perfusion was performed with saline solution at 24 ºC and, then, buffered 4% paraformaldehyde (PFA) at 24ºC.

The total colon of each mice was removed, measured according its length and circumference, weighed, post-fixed in PFA for three hours, and then submitted to routine processing for histopathological analysis. Cross sections (7 µm thickness) were stained with hematoxylin and eosin.

Dorsal Root Ganglia (DRG) at levels L6 and S1 and their respective splanic nerves were removed. Then the fragments were post-fixed in PFA and then submitted to routine processing to freeze biological material in OCT. Semi-serial sections (10 µm thickness) were submitted to histofluorence technique for labelling perineuronal net and immunofluorescence to labell neuronal strucutures.

Histofluorecence e Imunofluorescence

Frozen DRG and their splanic nerve sections were double labelled using Wisteria floribunda Lectin - WFA (histofluorescence) and antibody anti-Calcitonin Gene Related Peptide - CGRP (in DRG sections) or antibody anti-peripherin c-19 (in splanic nerve sections). First of all, the sections were submitted to antigenic recovery using sodium citrate buffer pH 6.0 heated in microwave until reaching 95ºC; 3x 5' wash in PBS 0.1M; antigenic blockade using 2% bovine serum albumin, 10% donkey serum, 0.1% Triton X-100 in PBS 0.1 M pH 7.4; 3x 5' wash in PBS 0.1 M; incubation with WFA lectin conjugated with fluorescein (VECTOR LAB, ZB0107, 1:500) and with antibody goat anti-CGRP (SANTA CRUZ, SC8857, 1:300) in DRG, or antibody goat anti-peripherin c19 (SANTA CRUZ BIOTECNOLOGY, SC7604, 1:200) in splanic nerve sections, for 24 hours; 3x 5' wash in PBS 0.1 M; incubation with Alexa Fluor 568 donkey anti-goat IgG antibody (MOLECULAR PROBES, A11057, 1:500) for 2 hours; 3x 5' wash in PBS 0.1 M; the slides were covered using slip and DAPI mounting medium (INVITROGEN, P36931).

Perineuronal net and nociceptive neurons analysis

A number of 500 cell bodies and prolongaments were analyzed per animal using the ImageJ software. Cell bodies were quantified and their area was measured, distinguishing WFA positive (WFA^+) and negative (WFA-) labelling, as well as CGRP+ and CGRP-. For the neuronal extensions in the nerves, we quantified only WFA+ and WFA- labeling.

Confocal microscope analysis

Using confocal microscopy, we performed: 1) morphological characterization of the different WFA labelling patterns; 2) quantification of different WFA labeling patterns in 30 general neurons/animal; 3) quantification of different WFA labeling patterns in 30 CGRP+ neurons/animal; 4) quantification of WFA+ satellite cells associated with 30 general and CGRP neurons/animal .

In addition, three WFA+ neurons per animal were randomly chosen for analysis of pericytoplasmic PNN density using Z stack images captured by a Confocal microscopy (Leica TCS SP8) and the LeicaX software version 3.7. On 10 µm thick DRG sections, images of 10 sections (Z stack), 1µm apart, of the three neurons were captured. Over the PNN (WFA+ pericytoplasmic staining of neuron cell bodies), three perpendicular lines of 10 µm length were drawn, where the WFA staining was converted into pixels and the pixels present in the line length were analyzed following the parameters in ImageJ Fiji Version Software: 1) Distance between each other; 2) intensity (size); and 3) number. The background for each section was defined and its value was subtracted from the values analyzed in the PNN. Then the values were averaged per neuron, then per animal and group.

Statistical analysis

The analyses were performed using the Software GraphPad Prism 9. Data distribution was verified using the Shapiro-Wilk test, then the Student's T test was applied. For all statistical analysis a $P<0.05$ was considered as significance level.

RESULTS

Development of DSS-induced ulcerative colitis

Ulcerative colitis was confirmed in DSS-exposed mice considering the results for disease activity index (DAI) and histopathological analysis of the distal colon.

The DAI increased progressively in mice of the UCa group during DSS exposure, becaming statistically different in relation to the control group after the second day of DSS exposition (Fig. 2A). Loss of body mass was observed after the fourth day of exposition to DSS (Fig.2B), although the loss of consistency of the feces was the most component of the DAI score increasing (Fig. 2C). After euthanasia, it was observed that the colonic length was shorted (Fig. 2D).

DAI also increased progressively in mice of the UCc group during each cycle of DSS exposure. Aalthough the score decreased during the intervals in which the animals ingested DSS-free water, it did not return to the basal state (Fig. 2E). Mice body mass decrased progressively during the first week of DSS exposure, remaining relatively stable during the other two cycles of exposure (Fig. 2F). In the first week DSS exposure, the loss of body mass stood out among the components of the DAI score, but the loss of feces consistency was again more determinant during the other cycles (Fig. 2G). The animals colon length from the UCc group were shorter than the control group (Fig.2H).

Regarding the histopathological analysis, as a reference of normality, it was observed that the distal colon of the control groups mice had natural architecture of the histological layers (Fig. 3A), as well as the maintenance of the epithelial barrier (Fig. 3B) and muscle layers preservation (Fig. 3C). In UCa mice, large number of multinucleated cell was observed in the mucosal and submucosal layers (Fig. 3D); besides, the epithelial barrier was ruptured and the *lamina propria* presented an intense inflammatory process (Fig. 3E), but no changes was observed in the muscle layer structure (Fig. 3F).

In the UCc group, typical crypts were absent due to the presence of several inflammatory cells in the *lamina propria* and, in addition, the submucosal layer was swollen (Fig. 3G). Dysplasia in the mucosa layer, presence of undifferentiated cells and the absence of the intact epithelial barrier were also observed in these animals (Fig. 3H). Besides, it was visualized the presence of mononucleated immunological cells, fibroblasts and dysplastic cells in the *lamina propria* (Fig. 3H). The muscle layer remained intact even after the three cycles of DSS exposure (Fig. 3I).

There are different types of WFA labelling in DRG neurons of mice.

Considering the WFA labeling images, our results indicate the existence of a PNN-like structure in neurons (cell body and extensions) of DRG. WFA labeling occurred in the cytoplasm and pericytoplasmic surface heterogeneously among neurons. These results indicate that part of the DRG neurons could produce PNN components, but was not enveloped by this network (WFA labeling only in the cytoplasm; Fig. 4A); another part was enveloped by the PNN, but possibly did not produce its components (WFA labeling only in the pericellular region; Fig. 4B); and other group neurons appeared to be able to produce and were enveloped by the PNN (WFA labeling in the cytoplasm and pericellular region; Fig. 4C). Part of these neurons was positive for CGRP labeling (Fig. 4D and 4E). We also found neurons that did not show WFA labeling but were CGRP-positive (Fig. 4F), and neurons negative for both labelings (Fig. 4G). Additionally, we also observed WFA-labeled satellite cells in the pericyte region (Fig. 4F), but part of them did not show intracellular WFA labeling (Fig. 4H).

UC enhanced WFA labeling in spinal afferent neurons of the distal colon.

Both acute and chronic UC modified the WFA labelling in the DRG. Regarding the acute UC, the number and the proportion of WFA+ neuronal cell body area/total DRG cortical area were increased in 7.5 and 4.3% in relation to control, respectively ($P<0.05$; Fig. 5 A and B), especially those presented WFA+ labelling in both cytoplasm and pericellular region (↑123.1% vs CG; $P<0.05$; Fig. 5C). Obviously, the number of neurons with no labelling for WFA was reduced (↓79% vs CG; $P<0.05$; Fig. 5C). Intriguingly, the number and area of WFA+ and CGRP+ neurons were not altered by the intestinal acute inflammation (Fig. 5A and B), independently the localization of WFA labelling in the neurons (Fig. 5D - F).

Chronic UC also led to a significative increase in both number (7.5%) and area (5.3%) of neurons surrounded by PNN-like structure ($P<0.05$; Fig. 5 G and H), which was WFA+. Similar to the acute inflammation, the number of WFA+ neurons labelled in both cytoplasm and pericellular region has increased (↑80% vs CG; $P<0.05$; Fig. 5I) and the number of neurons negative labelled for WFA was reduced (↓68% vs CG; $P<0.05$; Fig. 5I). Regarding the specific group of CGRP+ neurons, the number of neurons WFA+ labelled only in the pericellular region was 100% higher in the chronic UC mice ($P<0.05$; Fig. 5J - L).

UC caused hyperplasia of WFA labelled satellite cells in DRG.

The intestinal inflammation also caused changes in the number of satellite

glial cells labelled with WFA. There was an increase in the number of WFA+ satellite glial cells/total neuron in 25.7% and 70% in the acute and chronic UC, respectively ($P<0.05$; Fig. 6A). In relation to CGRP neurons, the number of WFA+ satellite cells was augmented (30%) only in acute UC mice ($P<0.05$; Fig. 6B). Especificaly, acute UC caused intense hyperplasia of WFA+ satellite cells which were surrouding general (↑294% vs CG; $P<0.05$; Fig. 6C) and CGRP (↑225% vs CG; $P<0.05$; Fig. 6D) neurons that presented labelling for this lectin in both cytoplasm and pericellular region. Chronic UC also caused hyperplasia (↑110%) of WFA+ satellite cells which were around gernal (↑110% vs CG; $P<0.05$; Fig. 6E) and CGRP (↑206% vs CG; $P<0.05$; Fig. 6F) neurons presented WFA+ labelling only the pericellular region (Fig. 6 G – J).

UC provoked a rearrangement of the PNN in DRG neurons of mice.

Using a 3D graphic construction from the Z-stack images captured on the confocal microscopy (Fig. 7A – Q), it was observed that the WFA labeling around neurons in DRG from the acute UC mice had pixels presenting greater number, greater intensity and less distant from each other when compared to control ($P<0.05$; Fig. 7R - T). On the other hand, the WFA labeling around neurons from the chronic UC mice was altered only in relation to the intensity of the pixels, which was higher in relation to the CGc ($P<0.05$; Fig. 7U - W).

Only chronic UC modified the PNN-like in neuronal extensions in the splanic nerve

WFA+ labelling (PNN-like) was also observed surrounding some neuronal extensions in the splanic nerve of control mice (Figs. A and C). We observed that only chronic inflammation was able to increase the number of neuronal extensions involved by PNN (↑13% vs CG; $p<0.05$; Fig. 8 D and E).

DISCUSSION

The description of PNN has been limited to the CNS only, since there are no reports of the presence and function of PNN in peripheral nervous system structures, including the extrinsic innervation of the colon. In order to contribute to filling this gap, this work demonstrated the presence of PNN-like components of the CNS surrounding extrinsic spinal afferent neurons innervating the distal colon of C57BL6 mice. Furthermore, we described the modifications that this PNN-like structure undergoes during the inflammatory process (acute and chronic phase) that occurs in the colon wall exposed to DSS, which is one of the most used experimental models of ulcerative colitis (UC).

Our results indicate that mice exposed to DSS indeed developed UC, as they showed DAI similar to studies that also used the DSS exposure to induce UC[43,45,46]. The development of UC was also confirmed by the histological changes observed in the colonic mucosa, and the shortening of the colon, which are typical features of DSS-induced UC in mice [47,48].

In general, inflammatory environments can reduce the threshold for neuronal action potential firing from pain-provoking stimuli, such as the cytokines IL-1, IL-6, IL-10, IFN, and TNF-α. [49–51]. Specifically, in experimental models of UC using DSS, there is a significant increase in IL-6, IFN-γ, IL-4, and IL-10 in the colon wall[52]. These cytokines can decrease the action potential threshold of nociceptive neurons present in DRG, which perceives stimuli in the intestinal wall that originate pain sensation [49].

This process also occurred in extrinsic afferent neurons, whose cell bodies are allocated in ganglia at the L6 and S1 spinal level in C57Bl/6 mice. These neurons are pseudo-unipolar in that they have a single extension that splits into two that one towards to the CNS (spinal cord) and the other towards to the distal colon wall[53]. This second prolongation is mainly responsible for realizing strength and rhythm of contraction perfomed by the muscle cells[54] and intraluminal pressure[55]. This information is conducted to the CNS and can be interpreted as pain, swelling, and discomfort [10–14], as happens in UC [56]. We observed in this work that neurons whose cell bodies are found in DRGs were surrounded by PNN and also had intracytoplasmic labeling for WFA, suggesting that they are capable of producing the network components.

We also demonstrated that both models of UC increased the proportion of neurons surrounded by PNN, as well as the area occupied by them. Interestingly, only in the chronic UC model did an increase in the area occupied by neurons double-labeled by CGRP and WFA. When analyzing the pattern of WFA labeling, it was found that the increase of WFA

positive neurons in acute UC was concentrated in producer neurons and surrounded by PNN, whereas in chronic UC the increase was concentrated in nociceptive neurons, which were surrounded by PNN.

In similar situations in the CNS, neurons and glial cells upon detecting pro-inflammatory molecules may produce and accumulate PNN components, to protect them against possible damage from the inflammatory process[57,58]. In addition, we suggest that neurons whose cell bodies are present in the DRG, upon detecting inflammatory molecules present in the intestinal wall, originating from the inflammatory process of UC, trigger the remodeling process of the ECM into PNN. PNN in DRG neurons, as well as in the CNS, probably act to potentiate neuronal electrical activity as well as protect the information conducted by these neurons[59], especially nociceptive information in the chronic DSS exposure model.

We also observed in this study that glial satellite cells had intracellular WFA labeling, they are possibly able to produce PNN components, similar to the mechanism described in the CNS[60,61]. We visualized that the inflammatory process present in both models of DSS exposure was able to elevate the number of WFA-producing satellite cells surrounding the neurons present in the DRG. We suggest that these gilal cells were concentrated around PNN-producing and network-wrapped neurons in the acute model of the disease, whereas in the chronic model they were present mainly around neurons wrapped by the network. This mechanism was observed both around the general neuronal population and in nociceptive neurons. It is known that glial satellite cells besides playing an immunoprotective role, can control the neuronal homeostasis of DRG and act on neurotransmission in neurons present in these ganglia[62]. When there is the detection of inflammatory molecules by the nerve extensions, satellite cells are also able to secrete neurotropic cytokines that lead to activation of the nociception mechanism [63]. Specifically, when we detect the increase of glial satellite cells around the neuronal populations mentioned above, we believe that these auxiliary cells also contribute to the process of conduction of information to the CNS, whether through the secretion of cytokines and the increase of nociception, as well as through the production of PNN compounds. Placing all together, these facts suggest that in the acute UC most of the production of the molecules present in the PNN is performed by the neurons, and after the chronicification of the disease, the consolidation and maintenance of the network around the neurons is probably destined to the glial satellite cells.

The density analysis of PNN indicated that the PNN-like structure present in the DRG neurons of the UCa group had more intense, numerous and closer pixels when

compared to their control, while in the neurons of the UCc group the pixels were only more intense when compared to GCc. These results suggest that acute exposure to DSS induces condensation and compaction of PNN, which is not maintained in chronic exposure, as it was only denser compared to its control. The condensation of PNN around neurons has already been described in the CNS in some disorders such as cerebral ischemia [64], stroke[65], and Alzheimer's disease [66]. It is worth noting that the cell bodies of neurons present in DRG are anatomically far from the colon wall [67], yet the neuronal extensions in contact with the inflammatory process induced ECM modifications within the ganglia. Considering what occurs in the CNS, perhaps this occurs to stabilize the neuronal electrical gradient of neurons [68], which possibly conducted more electrical impulses from the intestinal wall on the CNS. It is possible to infer that intracellular pathways activated by inflammatory mediators cause a hyperproduction of glycosaminoglycans around neurons to potentiate the neuronal activity, similar to what Raghunathan et al. (2020) [69] described in Parkinson's disease.

Furthermore, the neuronal extensions present in the splanchnic nerve of the control group mice were surrounded by the PNN. And that only the chronic DSS exposure model could increase the number of structures surrounded by the network. This result added to those described in the GRD corroborates once again the hypothesis that PNN act "protecting" the conduction of information captured in the distal colon toward the CNS.

We conclude that the data presented in this paper demonstrate the first report of the presence of PNN outside the CNS. The evidence provided here indicates the existence of PNN in the spinal afferent innervation of the distal colon of C57Bl/6 mice. Furthermore, the inflammatory process present in the colon observed in the UC groups can induce increased production of PNN components present in DRG neurons and glia cells, resulting in a denser network relative to control. Future studies may demonstrate the importance of this structural modification for optimizing the process of conduction of electrical impulses from the colon to the spinal cord and thus become a pharmacological target for reducing the abdominal pain threshold in patients with UC.

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ARTICLE ABBREVIATIONS

CEUA - Ethics Committee on Animal Experimentation
CG - Control group
CGa - Control group Acute
CGc - Control group chronic
CGRP - Calcitonin Gene Related Pepitide
CNS - Central nervous system
DAI - Disease Activity Index
DRG - Dorsal root ganglia
DSS - Sodium Dextran Sulfate
ECM - Extracelular matriz
IL-4 – Interleukin 4
IL-5 - Interleukin 5
IL-6 - Interleukin 6
IL-8 – Interleukin 8
IL-9 – Interleukin 9
IL-13 - Interleukin 13
kDa - Kilodaltons
OCT - Optimal Cutting Temperature
PBS - Phosphate Buffered Saline
PNN - Perineuronal net
PNNs - Perineuronal nets
Th2 – Type 2 immune response
TNF-α - Tumor Necrosis Factor-α
TnX - Tenascin X
UC - Ulcerative colitis
UEL - State University of Londrina
WFA - Wisteria Floribunda Aglutinin

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FIGURES

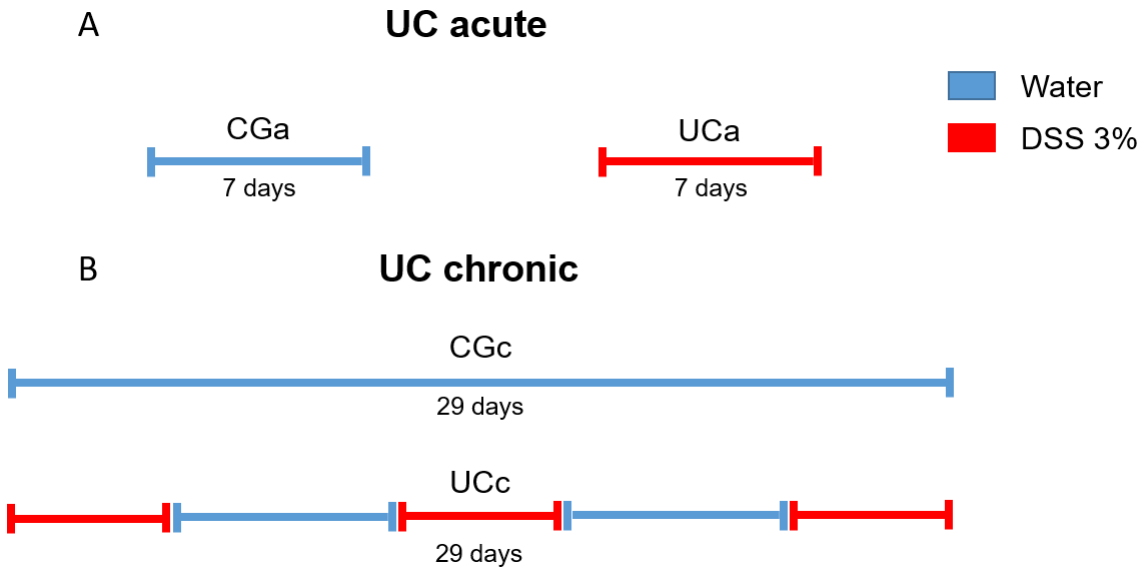

Figure 1. Scheme of the model of induction of ulcerative rectocolitis in C57/BL6 mice with Dextran Sulfate Sodium.

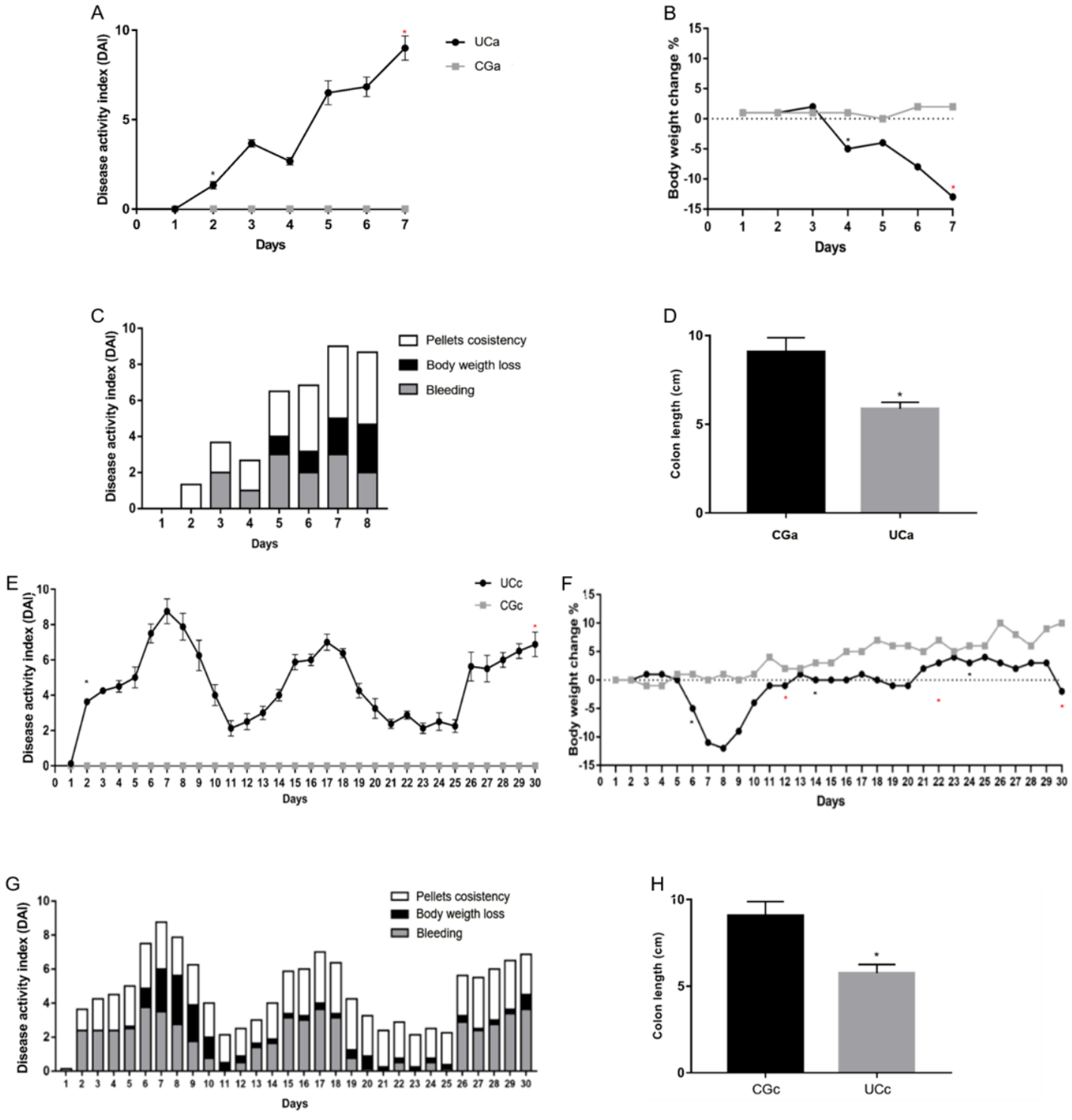

Figure 2 – Evaluation of the development of UC in mice exposed to DSS 3%. A-D correspond to acute UC and E-H correspond to chronic UC. A and E) Disease activity index (DAI). B and F) Body mass during DSS exposure. C and G) Evaluation of parameters related to the DAI calculation: consistency of feces, loss of body mass and presence of blood in the feces. D and H) analysis of colon length. The asterisk corresponds to $P<0.05$ found in student's T test.

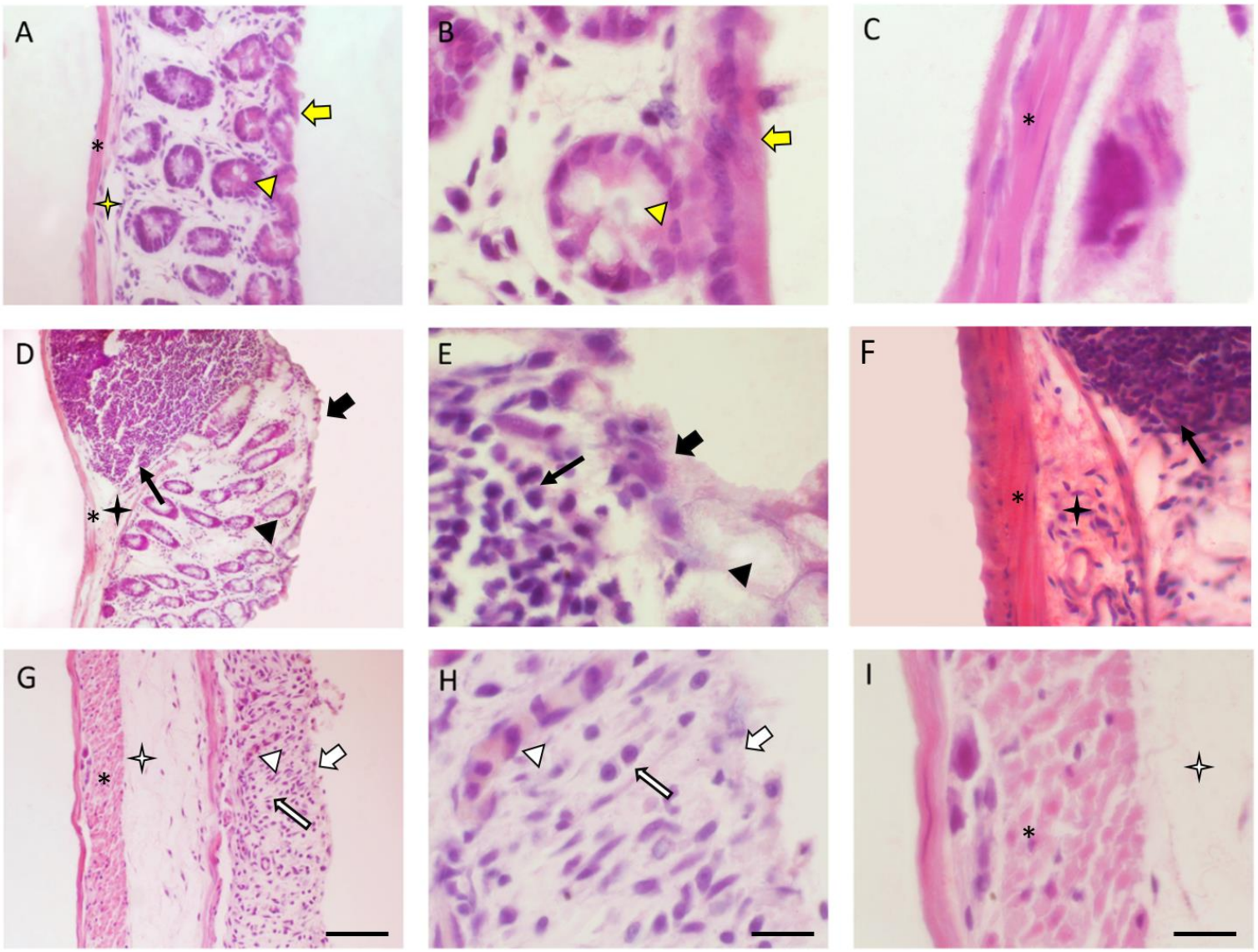

Figure 3. Photomicrographs of histological sections of the distal colon of mice exposed to 7 days of DSS (UCa) or three cycles of DSS (UCc). A-C are images of the control group. The loss of intestinal architecture in both groups exposed to DSS was observed, but in the UCa group (D-F) the presence of intact intestinal crypts was maintained and in the UCc group (G-I) the mucosa was full of inflammatory cells. Asterisk: muscle layers with integrative morphology; Yellow star: submucosal layer integrates; Black star: slightly swollen submucosal layer with the presence of leukocytes**;** White star: swollen submucosal layer with slight fibroblast increase; Yellow arrow head: normal morphology of liberkuhn crypts; Black arrowhead: normal morphology of liberkuhn crypts; White arrow head: change in the morphology of liberkuhn crypts (undifferentiated cells) Yellow arrow: normal morphology of enterocytes that form the intestinal barrier; Black arrow: replacement of enterocytes by fibroblasts; White arrow: replacement of enterocytes by undifferentiated filling tissue; Black thin arrow: inflammatory infiltrate of multinucleated cells; White thin arrow: inflammatory infiltrate of mononucleated cells. For figures A, D and G the bar corresponds to 50 µm. For B, E and H 25 µm. For C, F and I 12,5 µm.

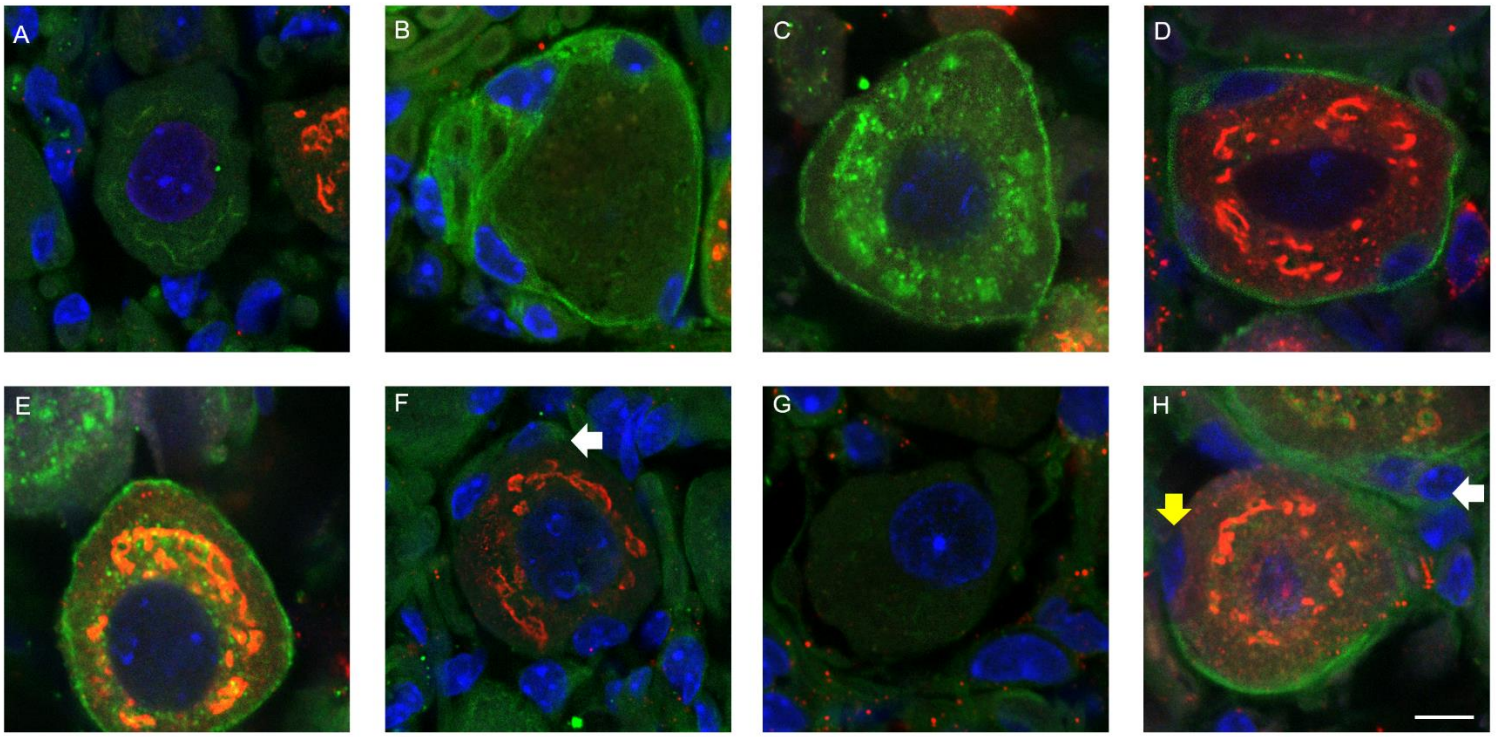

Figure 4. photomicrographs of neuron cell bodies present in dorsal root ganglion (DRG) of C57BL6 mice showing different WFA labeling patterns. A) WFA labeling in the perinuclear region; B) WFA labeling on the pericotoplasmic surface; C) WFA labeling in the perinuclear and pericytoplasmic regions. D) Neuronal cell body labeled for WFA (green) and CGRP (red). E) Neuronal cell body producing and surrounded by PNN (WFA+) also positive for CGRP. F) Neuronal cell body unlabeled for WFA but positive for CGRP and surrounded by WFA-labeled satellite cells inside (arrow); G) WFA and CGRP negative neuronal cell body; H) CGRP positive neuronal cell body and surrounded by PNN and PNN-producing (white arrow) and non-producing (yellow arrow) satellite cell. Nuclei in blue (DAPI staining). Bar corresponds to 3 μm.

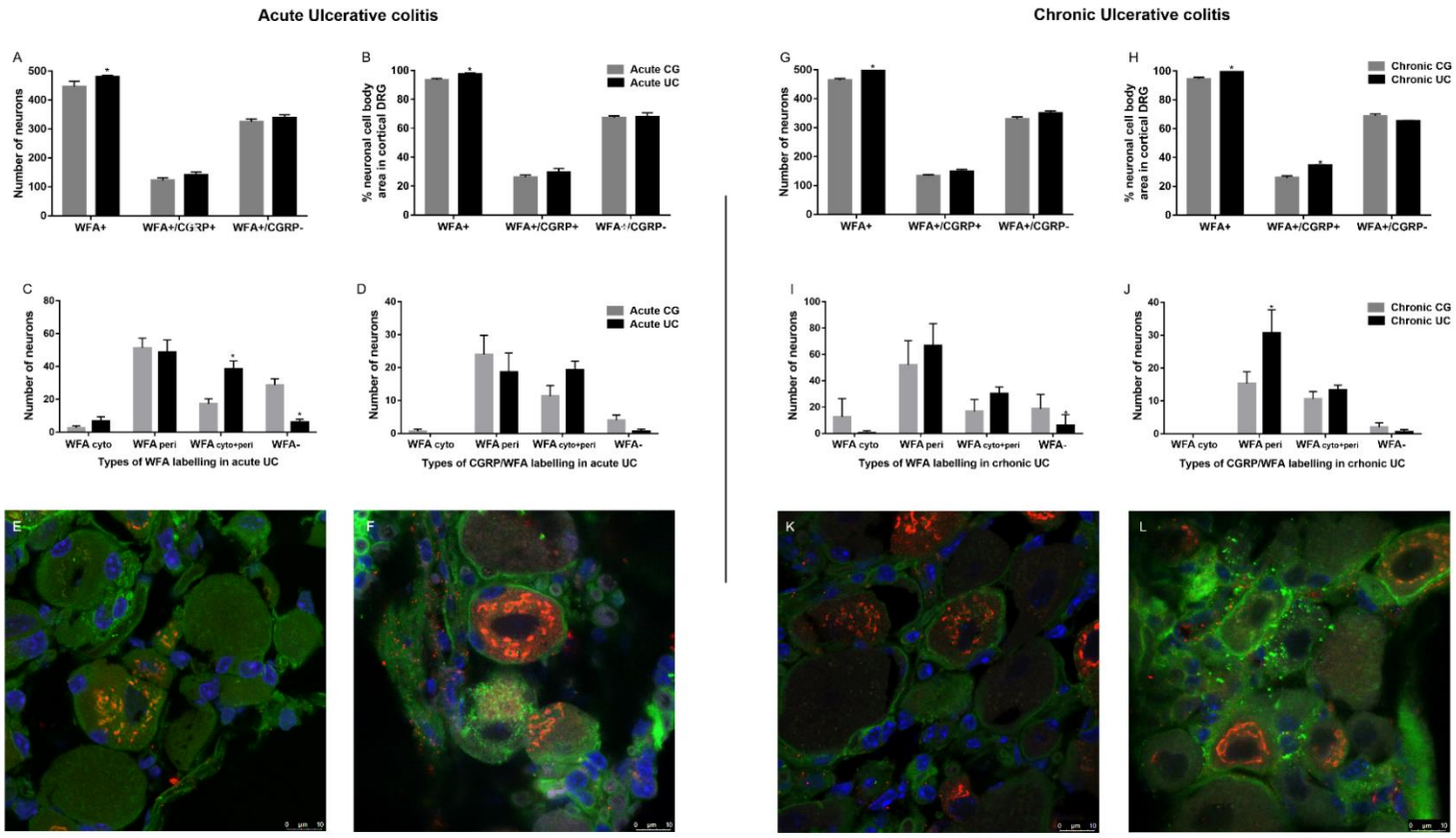

Figure 5. Number and area of L6 and S1 dorsal root ganglion neurons (aference of distal colon) of mice exposed to DSS. A and G) Number of positive neurons for labelling of WFA and/or CGRP in a total of 500 neurons. B and H) Area of cell body neurons positive for WFA and/or CGRP in proportion (%) to the total DRG cortical area. C and I) WFA labeling patterns in the DRG neurons . D and J) WFA labeling patterns in CGRP positive neurons. For photomicrographs of E, F, K and L: Wfa (PNN+), in green; CGRP, in red; DAPI, in blue.The images corresponds to: E) CGa; F) UCa; K) CGc and L) UCc. Abbreviations correspond to: WFA cyto: intracytoplasmic labeling of WFA; Peri WFA: pericellular WFA+ labeling; WFA cyto+peri: intracytoplasmic and pericellular WFA+ labeling; WFA-: negative labeling for WFA.Asterisk corresponds to $P<0.05$ found in student's T test.

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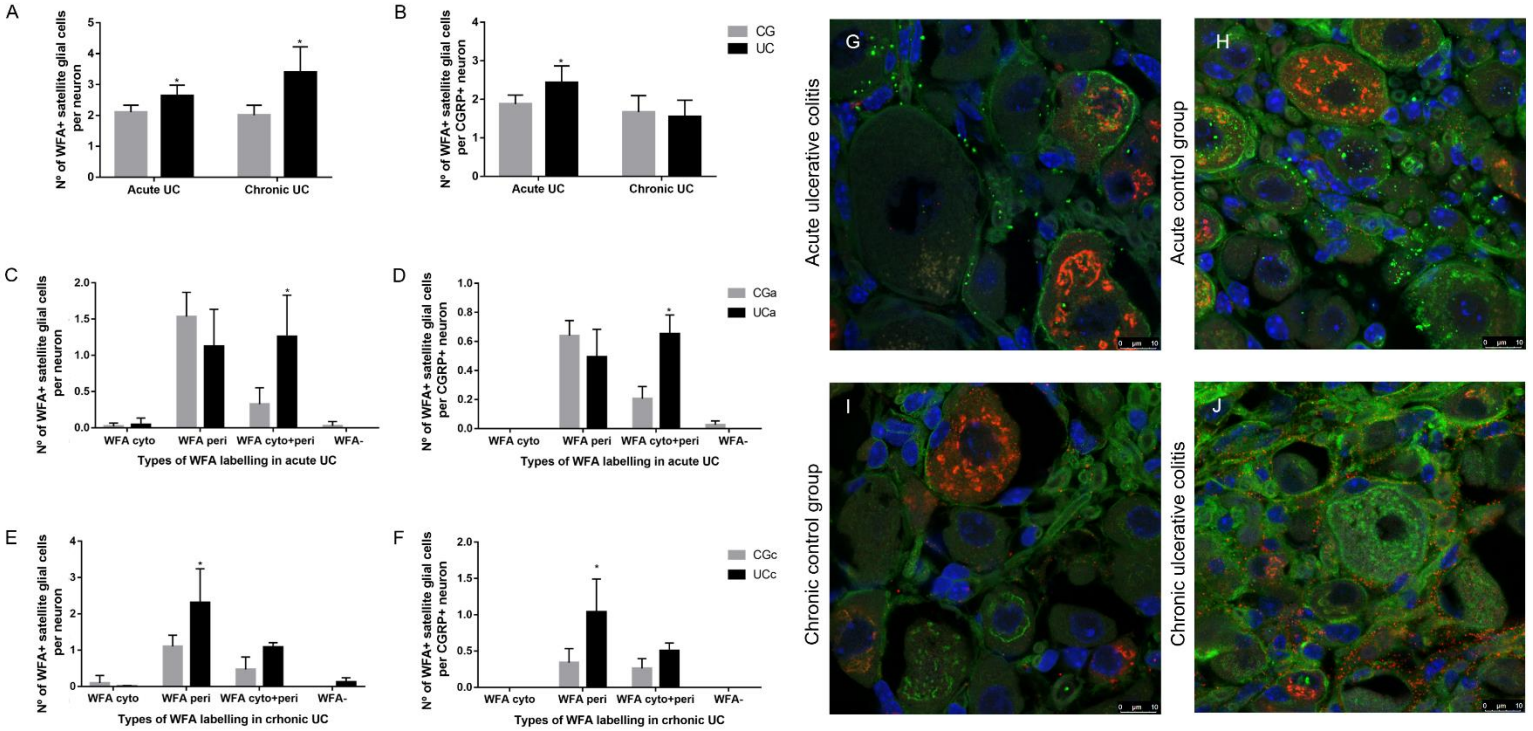

Figure 6. Analysis of glial satellite cells in the DRG. A) comparison of the number of PNN-producing satellite glial cells in the general population; B) comparison of the number of PNN-producing satellite glial cells around CGRP positive neurons; Graphs C - F show the comparison of the number of glial satellite cells around the different WFA positive neuronal populations. C) CGa vs UCa in WFA positive neurons; D) CGa vs UCa in CGRP and WFA positive neurons; E) CGc vs UCc in WFA positive neurons; F) CGc vs UCc in CGRP and WFA positive neurons. For photomicrographs G - J: WFA (PNN+), in green; CGRP, in red; DAPI, in blue.The images corresponds to: G) CGa; H) UCa; I) CGc and J) UCc. Asterisk corresponds to $P<0.05$ found in student's T test. Abbreviations correspond to: WFA cyto: intracytoplasmic labeling of WFA; Peri WFA: pericellular WFA+ labeling; WFA cyto+peri: intracytoplasmic and pericellular WFA+ labeling; WFA-: negative labeling for WFA. Asterisk corresponds to $P<0.05$ found in student's T test.

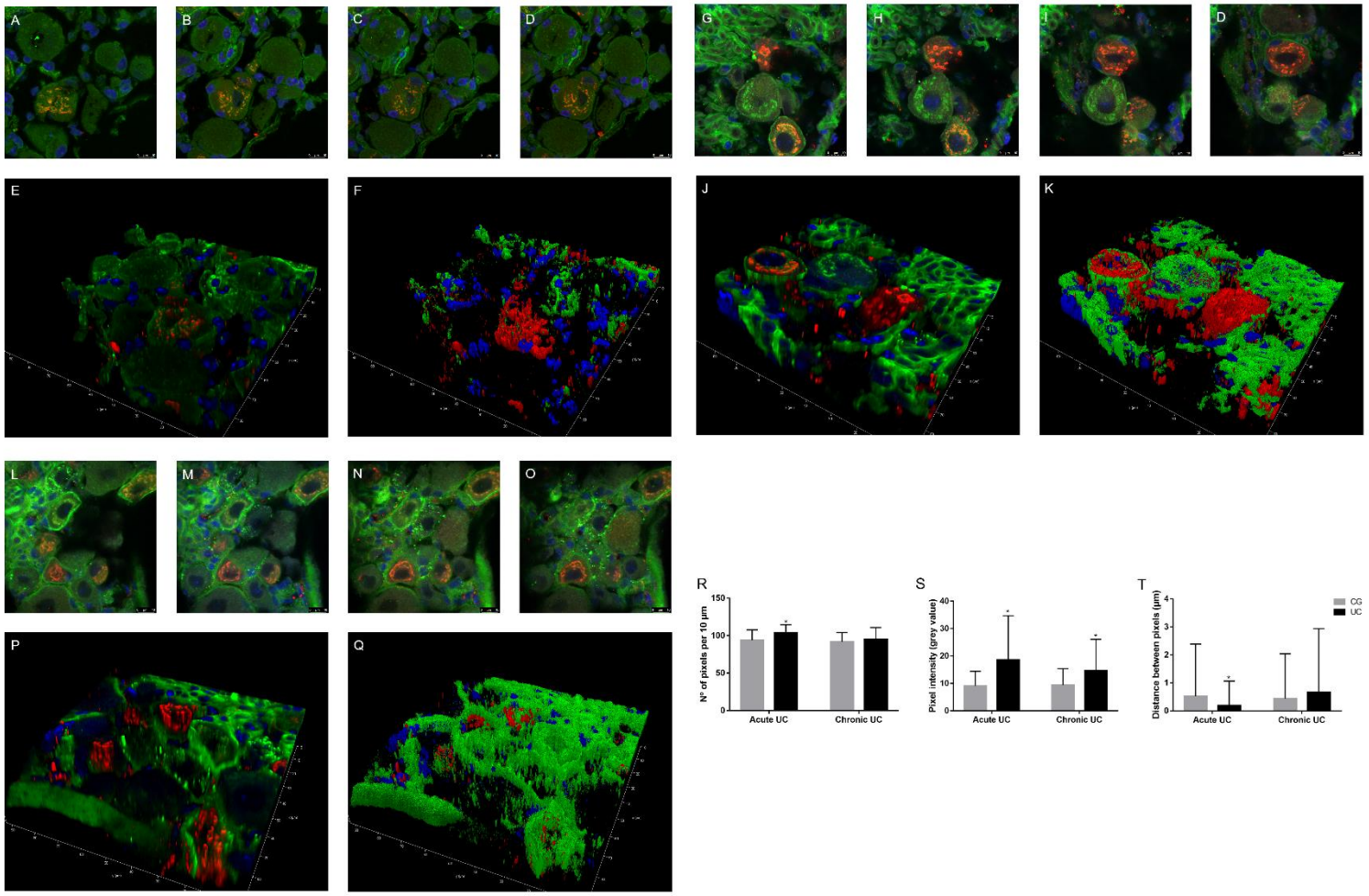

Figure 7. Morphological appearance of PNN in neurons present in the DRG of L6/S1 levels of C57BL6 mice. The images represent sections submitted to histoimmunofluorescence technique using WFA (in green), anti CGRP antibodies (in red) and DAPI (in blue), were captured by performing the Z-stack technique with the aid of a confocal microscope. Images E, J and P represent the graphic union of the images performed by LasX software. of the sections presented from A-D (for E), G-D (for J), L - O (for P). In image F the same LasX construction technique was used, but highlighting the pixels labelled by each antibody in the sections. R) Average number of pixels for each 10 µm analyzed; S) Average pixel intensity; T) Average distance between pixels. The samples were compared by Student T test.

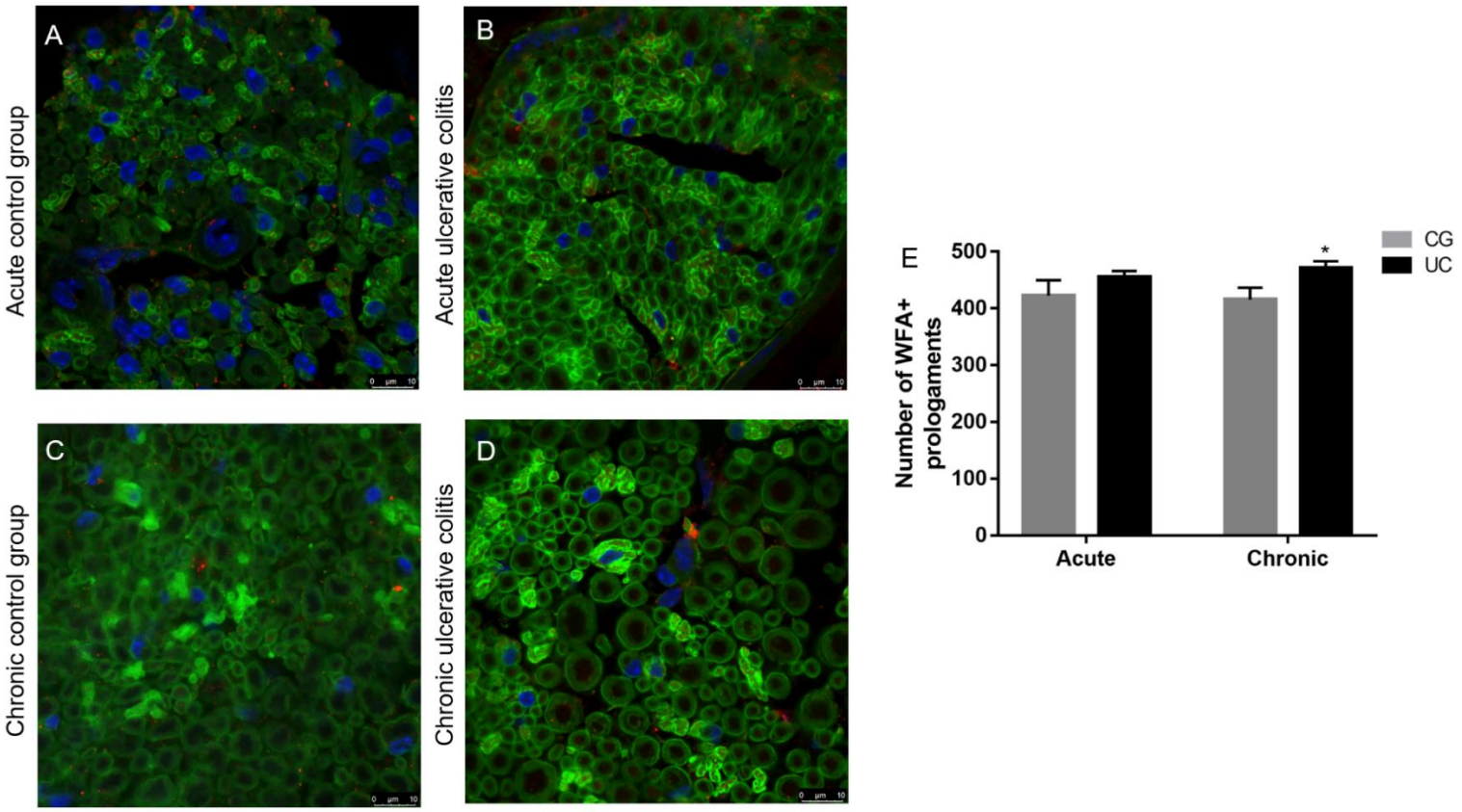

Figure 8. Presence of PNN in the splanic nerve. Number of WFA+ prolongaments in splanic nerve (L6 and S1 aference of distal colon) of mice exposed to DSS for 7 days (acute ulcerative colitis). Histoimmunofluorescence of splanic nerve section, labeled with peripherin (in red) and WFA (in green) of: A) CGa; B) UCa; C) CGc; D) UCc. E) Comparison graph between the number of positive WFA extensions. For image E the asterisk corresponds to $P<0.05$ found in student's T test.

CONSIDERAÇÕES FINAIS DA DISSERTAÇÃO

Este é o primeiro trabalho a descrever a presença da RPN na inervação espinal aferente do cólon distal de camundongos C57Bl/6. Também fornecemos evidências de que a produção de componentes desta rede é feita tanto por neurônios como células gliais satélites presentes nos GRD em nível L6/S1. Inferimos que mediadores inflamatórios secretados na parede do cólon de camundongos com RCU provavelmente ativam vias intracelulares de neurônios aferentes de GRD que modificam a expressão de componentes da RPN, tornando-a mais condensada e densa ao redor dessas células. Os resultados apresentados abrem porta para futuros estudos que poderão elucidar a participação da RPN na modulação do limiar da resposta da dor, assim como quais moléculas inflamatórias presentes na RCU aguda e crônica são responsáveis pela condensação da rede.

Ao descrever a presença da RPN na inervação extrínseca do cólon distal na RCU, esperamos também que sejam realizados novos estudos que possam caracterizar a presença da RPN na inervação intrínseca do cólon distal e compará-la durante o desenvolvimento da doença. Juntos, esses resultados poderão contribuir para novos estudos que tenham a RPN como alvo farmacológico e analgésico.

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APPENDIX

APPENDIX A

Protocol of Approval CEUA-UEL.

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 032/2015 **Londrina, 01 de Abril de 2015**

Prezado Pesquisador,

A CEUA/UEL reunida em 10 de Março de 2015 avaliou o projeto de pesquisa intitulado **"O papel da rede perineural sobre o sistema nervoso motor e sensorial de humanos e camundongos"** registrado sob processo CEUA **nº1454.2015.56**, pesquisa do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade, julgando-o **aprovado** para execução entendendo-se que os princípios éticos postulados pelo Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Serão utilizados 450 camundongos sendo 150 camundongos C57BL/6, machos/fêmeas, com 50 dias de idade, com peso entre 20-25g e provenientes do Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório (CEMIB) da Universidade Estadual de Campinas (UNICAMP) e 300 camundongos *Knockout* de linhagem a definir segundo experimentos inclusos neste projeto, com 50 dias de idade, peso de 20 a 25g e adquiridos comercialmente. As matrizes dos camundongos *Knockout* serão importadas do Reino Unido e o coordenador do projeto é responsável pela documentação necessária. O projeto tem como objetivo avaliar o papel da rede perineuronal (RPN) para o funcionamento de neurônios sensoriais e motores utilizando o sistema nervoso entérico com modelo. Para tanto, os animais serão anestesiados e submetidos à inalação, em câmara, de vapor de halotano (1%), oxigênio (14%) e nitrogênio (86%) para a coleta do material biológico visando análise por PCR e haverá aprofundamento anestésico (halotano 5%) até observação de parada cardiorrespiratória. Após a comprovação da ausência de reflexos, será feita perfusão cardíaca com solução salina a 32°C até completa remoção do sangue presente no interior dos vasos, a perfusão de paraformaldeido tamponado a 4% (70mL/animal) e os animais serão submetidos a procedimentos para a remoção dos órgãos que posteriormente serão avaliados. Os protocolos experimentais estão aprovados para execução em 36 meses.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente,

Waldiceu Ap.º Verri Junior

Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador da CEUA/UEL

Ilmo. Sr.
Prof. Dr. Eduardo José de Almeida Araújo
Coordenador do Projeto
Departamento de Histologia
Centro de Ciências Biológicas

08 ABR 2015

Com cópia para Sra. Edilamar dos Anjos (Chefe da DCA/PROPPG), Chefe do Departamento de Histologia e Diretor(a) do Centro de Ciências Biológicas.