UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

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PLASTICIDADE SINÁPTICA EM MOTONEURÔNIOS ALFA MEDULARES DE ANIMAIS SUBMETIDOS À ENCEFALOMIELITE AUTOIMUNE EXPERIMENTAL

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"I know someday you'll have a beautiful life.
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SUMÁRIO

Capítulo 1

RESUMO

Durante o curso da encefalomielite autoimmune experimental ocorre uma grave redução das funções motoras e sensitivas. Esses eventos têm sido classicamente atribuídos ao processo desmielinizante da doença. Em ratos, os sinais clínicos da doença desaparecem 5 dias após completa tetraplegia, indicando que o processo desmielinizante não é a única causa da rápida evolução da doença. Assim sendo, investigamos as alterações sinaptológicas e o processo inflamatório induzidos pela encefalomielite autoimune experimental (EAE) em motoneurônios medulares e sua relação com o surto e remissão da doença. Para esse estudo, foram utilizados ratos Lewis, fêmeas de 7 semanas. Os animais foram induzidos à EAE por meio de dose única de proteína básica de mielina emulsificada com adjuvante completo de Freund e sacrificados no 13º dia após indução (surto grau 3) e no 26º dia (remissão da doença). Também, para investigar a possibilidade de que o tratamento com acetato de glatirâmer, uma droga imunomoduladora baseada na estrutura de aminoácidos da proteína básica de mielina, interfira no processo de plasticidade sináptica, os animais foram induzidos à EAE, tratados com AG diariamente e sacrificados após 2 semanas. Os grupos experimentais foram divididos em: estudo da aposição sináptica durante surto e remissão da doença e tratamento dos animais induzidos à EAE com AG. Assim, os espécimes foram processados para análise através de imunohistoquímica e microscopia eletrônica de transmissão.

Nossos resultados indicaram que os componentes gliais (astrócitos e microglia), estimulados pela inflamação, desempenham papel ativo no processo de retração

sináptica em motoneurônios alfa. Apresentamos evidências de que a eliminação de terminais sinápticos contribui para a perda da função motora observada no curso da doença e que o imunomodulador AG não só possui efeito antiinflamatório, mas também influencia diretamente na plasticidade de elementos neurais no microambiente medular. Reforçam, também, que um processo agudo de inflamação pode colaborar diretamente para a recuperação e sobrevivência neuronal, uma vez que as células inflamatórias produzem citocinas e fatores neurotróficos no microambiente medular.

ABSTRACT

During the course of experimental autoimmune encephalomyelitis, a massive loss of motor and sensitive function occurs, which has been classically attributed to the demyelination process. In rats, the clinical signs disappear within 5 days following complete tetraplegia, indicating that demyelination might not be the only cause for the rapid evolution of the disease. The immunomodulador glatiramer acetate (GA) has been shown significantly reduce the seriousness of the symptoms during the exacerbation of the disease. However, little is known about its effects on the spinal motoneurons and on their afferents. The present work investigated the occurrence of experimental autoimmune encephalomyelitis-induced changes of the synaptic covering of spinal motoneurons during exacerbation and after remission and investigated whether GA has a direct influence on synapse plasticity and on the deafferentiation of motoneurons during the course of EAE in rats. Lewis rats were subjected to EAE associated with GA or placebo treatment. The animals were sacrificed after fifteen days of treatment. For the both cases the spinal cords was processed for immunohistochemical analysis (IH) and electron transmission microscopy.

The terminals were typed with transmission electron microscopy as C-, F- and S-type. Immunohistochemical analysis of synaptophysin, glial fibrillary acidic protein and the microglia/macrophage marker F4/80 were also used in order to draw a correlation between the synaptic changes and the glial reaction. The ultrastructural analysis showed that, during exacerbation, there was a strong retraction of both F- and S-type terminals. In this sense, both the covering as well as the length of the remaining

terminals suffered great reductions. However, the retracted terminals rapidly returned to apposition, although the mean length remained shorter. A certain level of sprouting may have occurred as, after remission, the number of F-terminals was greater than in the control group. The immunohistochemical analysis showed that the peak of synaptic loss was coincident with an increased macro- and microglial reaction. Interestingly, although the GA treatment preserved synaptophysin labelling, it did not significantly reduce the glial reaction, indicating that inflammatory activity was still present.

Our results suggest that the major changes occurring in the spinal cord network during the time course of the disease may contribute significantly to the origin of the clinical signs as well as help to explain their rapid recovery and that the immunomodulator GA has a direct influence on the stability of nerve terminals in the spinal cord, which in turn may contribute to its neuroprotective effects during the course of multiple sclerosis.

Lista de Abreviaturas:

EAE – encefalomielite autoimune experimental

EM – esclerose múltipla

AG – acetato de glatiramer

BDNF – *brain derivated neural factor*

NGF – *neural growth factor*

SNC – Sistema Nervoso Central

MBP – proteína básica de mielina

PLP – proteolipoproteína

MAG – glicoproteína associada à mielina

MOG – glicoproteína da mielina de oligodendrócitos

Células NK – células *natural killer*

RNM – ressonância magnética nuclear

MHC – complexo principal de histocompatibilidade

MT – *Mycobacterium tuberculosis*

CFA – completo adjuvante de Freund

MAP-2 - *microtubule associated protein –2*

INF-ß – Interferon beta

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ESTRUTURA DA TESE

A presente Tese de Doutorado contém 4 capítulos. No capítulo 1 é apresentada a Introdução ao trabalho. Os resultados estão organizados na forma de artigos científicos, apresentados no capítulo 2. O capítulo 3 apresenta as conclusões gerais do trabalho e o capítulo 4, as referências bibliográficas referentes ao capítulo 1 (Introdução).

O primeiro artigo (Artigo I – Capítulo 2) relata que apesar dos eventos de importantes perdas das funções motoras e sensitivas serem atribuídos ao processo desmielinizante decorrente da Esclerose Múltipla, há evidências de que a retração sináptica decorrente da inflamação local também contribua para os sinais clínicos observados. Assim sendo, investigamos as alterações sinaptológicas e o processo inflamatório induzidos pela encefalomielite autoimune experimental em motoneurônios medulares e sua relação com o surto e remissão da doença. Esse estudo recebeu o prêmio de Honra ao Mérito na XX Reunião Anual da FeSBE- 2005. O artigo completo encontra-se publicado no periódico internacional: Marques, K.B.; Santos, L.M.B.; Oliveira, A.L.R. (2006) Spinal motoneuron synaptic plasticity during the course of an animal model of multiple sclerosis. *European Journal of Neuroscience*, 24 (11): 3053-62.

No segundo artigo (Artigo II – Capítulo 2) foi investigada a relação do acetato de glatirâmer, uma droga imunomoduladora baseada na estrutura de aminoácidos da proteína básica de mielina, com a retração sináptica dos motoneurônios alfa medulares decorrente da esclerose múltipla. O acetato de glatirâmer tem-se mostrado eficiente

para o tratamento da esclerose múltipla. Tendo-se em vista as propriedades antiinflamatórias do medicamento, investigamos no presente estudo, a possibilidade de que este interfira no processo de plasticidade sináptica induzido a cada surto da doença. Este trabalho foi submetido ao periódico internacional *Neuroscience.*

CAPÍTULO 1

I – INTRODUÇÃO

1. Medula Espinhal e Motoneurônios Medulares

A medula é uma porção do Sistema Nervoso Central (SNC) situada no interior do canal vertebral e possui forma cilíndrica. Apresenta as intumescências cervical e lombar, de onde partem os nervos espinhais que compõem os plexos braquial e lombossacral, responsáveis pela inervação dos membros superiores e inferiores, respectivamente. A medula espinhal recebe impulsos sensoriais de receptores periféricos e envia impulsos motores a efetuadores tanto somáticos quanto viscerais.

Cada segmento da medula compreende quatro raízes nervosas, ou seja, um par ventral (direito e esquerdo) e um par dorsal (direito e esquerdo). Na raiz dorsal de um nervo espinhal típico, próximo à junção com a raiz ventral, localiza-se o seu gânglio, que contém corpos de neurônios sensitivos.

As raízes ventrais contêm axônios de neurônios motores alfa de grande diâmetro para as fibras musculares estriadas esqueléticas; os axônios dos neurônios motores gama, mais delgados, inervam as fibras intrafusais. As fibras autonômicas pré-ganglionares nos níveis torácico, lombar superior e mediossacro (S2- S4) transmitem informações motoras e sensitivas das vísceras torácicas e abdominais. Na medula espinhal, a interação entre os motoneurônios e o microambiente medular, desempenha uma função crucial para a sobrevivência, regulação do estado funcional e conectividade sináptica dos mesmos (Hamburger, 1958; Levi-Montalcini, 1987; Oppenheim, 1991; Huh *et al.*, 2000; Oliveira *et al.*, 2001).

2. Células não neurais do Sistema Nervoso Central

Na embriogênese do SNC, as células precursoras da glia (glioblastos ou espongioblastos) originam a macróglia, formada de oligodendrócitos e astrócitos. Os oligodendrócitos são formados a partir da camada marginal e, portanto, estão presentes na substância branca e são responsáveis pela mielinização dos axônios do SNC. Os astrócitos constituem o principal componente celular do SNC, que participam do reparo do tecido nervoso lesionado, através da remoção de restos celulares e pela extensão de seus processos citoplasmáticos nos espaços extracelulares. Os astroblastos oriundos dos glioblastos e precursores dos astrócitos, conforme são formados na zona intermediária ou na zona marginal, se diferenciam em astrócitos protoplasmáticos (presentes na substância cinzenta) ou fibrosos (presentes na substância branca). Os microgliócitos são células que constituem a microglia e que são derivadas dos monócitos.

3. Integração Neurônio – Glia

As células da Glia, até recentemente, eram consideradas apenas como provedoras de suporte estrutural e metabólico para o neurônio executar a transmissão sináptica. Posteriormente, foram descritos efeitos relacionados à modulação da fisiologia sináptica por meio de sua conhecida capacidade de regular o meio iônico extracelular e de metabolizar neurotransmissores. Estas células respondem a

neurotransmissores liberados em terminais sinápticos ativos sob o controle dinâmico da atividade neuronal.

Em resposta a esta atividade neuronal, os astrócitos podem, por meio de oscilações nos níveis citosólicos de cálcio, enviar retrogradamente ao neurônio, uma mensagem estimulando a liberação de vários neurotransmissores, como por exemplo, o neurotransmissor excitatório glutamato (Glowinski *et al.*, 1994; Griffith e Sutin, 1996; Hu *et al.*, 1997; Kotter *et al.*, 1999; Krushel *et al.*, 1995 Krushel *et al.*, 1998). O glutamato despolariza o astrócito, induzindo um aumento nos níveis de cálcio intracelular, que, por sua vez, induz a liberação de neurotransmissores pelos neurônios (Laming *et al.*, 2000).

Além desse efeito neuromodulatório e do provimento de substratos glutamatérgicos para os neurônios, as funções gliais decorrentes da integração com o neurônio incluem a regulação neuronal dos níveis extracelulares de potássio, e esta regulação está associada à atividade sensorial, ao aprendizado e aos estados motivacionais (Sykova *et al.*, 1990). Além do glutamato, a glia também modula diretamente a neurotransmissão serotoninérgica e noradrenérgica, via receptores pós-sinápticos gliais (Griffith e Sutin, 1996; Rajkowska, 2000).

Nos astrócitos de ratos, a noradrenalina ativa a glicogenólise e o metabolismo oxidativo (Aoke e Pickel, 1992), enquanto a serotonina induz a despolarização astrocítica (Walz e Schlue, 1982).

Também têm sido descritas alterações, em ratos, na plasticidade astrocítica no córtex e no cerebelo durante o aprendizado (Black *et al.*, 1990), modificações que até recentemente estavam relacionadas somente ao metabolismo neuronal.

A produção de fatores neurotróficos por células gliais, como, por exemplo, o fator neurotrófico derivado do encéfalo (BDNF), parece ser fundamental para a sobrevivência e a plasticidade de neurônios corticais (Ohgoh *et al*., 1998). Estas novas descobertas propõem o conceito de que a plasticidade estrutural neuronal e os diversos efeitos tróficos no SNC adulto apresentam-se modulados ou influenciados pelos astrócitos (Black *et al*., 1990).

Um peptídeo denominado de fator de crescimento neural (NGF - neural growth factor) foi o primeiro fator trófico a ser identificado em 1940 pela bióloga italiana Rita Levi-Montalcini. O NGF é produzido pelos alvos dos axônios na divisão simpática do sistema nervoso vegetativo. Levi-Montalcini (1987) observou que a injeção de anticorpos contra o NGF em camundongos recém-nascidos resultava em total degeneração dos gânglios simpáticos. O NGF, produzido e liberado pelos tecidos-alvo, é captado pelos axônios e transportado retrogradamente, onde age promovendo a sobrevivência neuronal. Na realidade, se o transporte axoplasmático é interrompido, os neurônios degeneram, apesar da liberação de NGF pelos tecidos-alvo.

O NGF é um dos membros da família de proteínas tróficas coletivamente chamadas de neurotrofinas. Membros desta família incluem as proteínas NT-3, NT-4 e BDNF, que são importantes para a sobrevivência de neurônios, como por exemplo, do córtex visual. As neurotrofinas agem em receptores específicos da superfície celular. A maioria desses receptores é proteína cinase ativada por neurotrofinas, chamadas de receptores trk (tyrosine kinase, ou tirosina cinase), que fosforilam resíduos de tirosina nos seus substratos protéicos. Esta reação de fosforilação estimula uma cascata de segundos mensageiros que altera a expressão gênica no núcleo da célula podendo

assegurar a sobrevivência neuronal. Dessa forma, Hammarberg *et al.* (2000) demonstraram que uma axotomia proximal, quando realizada durante a fase de surto do modelo experimental da esclerose múltipla, induz a morte de um número significativamente menor de motoneurônios medulares. Tal neuroproteção foi atribuída a uma possível produção local de fatores neurotróficos tais como BDNF e NT-3.

4. Morte Celular e Neurodegeneração

Degeneração nervosa é um processo onde estruturas e funções são progressivamente afetadas, podendo ser perdidas parcial ou totalmente. A neurodegeneração envolve alterações estruturais complexas, modificando a atividade das células nervosas, podendo resultar em eventual morte celular. Degeneração progressiva de regiões específicas do SNC é um acontecimento bem estabelecido em doenças neurodegenerativas tais como o Alzheimer, Parkinson, Huntington e Esclerose Múltipla (Przedborski *et al.*, 2003).

Existem evidências da ocorrência de processos de apoptose ou necrose celular em doenças neurológicas (Majno e Joris, 1995). Essa progressão pode ocorrer via alteração do processo constante, em organismos adultos, de regulação e rearranjo das sinapses, durante o curso de algumas doenças. Tais alterações podem progredir acarretando, assim, morte celular.

Doenças neodegenerativas são caracterizadas por uma eventual perda neuronal em certas regiões do SNC, muitas vezes por mecanismos desconhecidos. Alguns destes envolvem a exitotoxicidade por glutamato, radicais livres, desregulação da

síntese de proteínas envolvidas no processo de morte celular, bem como acúmulo de agregados protéicos (ex.: emaranhado de neurofibrilas e peptídeos-Aβ) (Honig e Rosenberg, 2000; Kanazawa, 2001; Sherman e Goldberg, 2001; Przedborski *et al*., 2003; Vila e Przedborski, 2003).

4.1. Plasticidade Sináptica

O estabelecimento e a manutenção dos circuitos neuronais no SNC constitui-se num processo complexo e pouco conhecido, o qual envolve milhares de neurônios interconectados precisamente através das sinapses (Huh *et al*., 2000; Boulanger *et al*., 2001). Na medula espinhal, acredita-se que a interação entre os motoneurônios e o microambiente medular, bem como com o órgão alvo, desempenhe uma função crucial para a sua sobrevivência, regulação de seu estado funcional e conectividade sináptica (Hamburger, 1958; Levi-Montalcini, 1987; Oppenheim, 1991; Huh *et al*., 2000; Oliveira *et al*., 2001). Após uma lesão que resulte na interrupção do contato entre o motoneurônio e as fibras musculares alvo, uma série de alterações ocorre no corpo celular do neurônio, sendo, em conjunto, denominadas cromatólise (Romanes, 1946; Lieberman, 1971; Kreutzberg, 1981; Aldskogius and Svensson, 1993). Associado a isso, há uma retração significativa das terminações nervosas em contato com o corpo celular desses motoneurônios, sendo as excitatórias do tipo glutamatérgicas as mais afetadas (Choi & Rothman, 1990; Lindå *et al*., 2000). Existem evidências de que um processo semelhante, envolvendo mecanismos relacionados a excitotoxicidade por glutamato ocorra no desenvolvimento da esclerose amiotrópica lateral (ALS) e durante

os episódios da doença neurodegenerativa denominada esclerose múltipla (EM) (Lindå *et al*., 2000, Zhu *et al*., 2003).

4.2. Esclerose Múltipla (EM)

A EM é uma doença autoimune de caráter progressivo caracterizada pela destruição da bainha de mielina, com perda eventual de oligodendrócitos (Waksman, 1985). A desmielinização da substância branca do sistema nervoso central desencadeia vários sintomas neurológicos (Kornek *et al*., 2000; Olson *et al*., 2000; Swanborg, 2001; Pedotti *et al*., 2003). As manifestações clínicas são muito variáveis, não existindo nenhum sintoma ou sinal específico da doença.

Sua ocorrência é em adultos jovens, entre 20 e 45 anos, manifestando-se raramente antes dos 15 ou após os 50 anos. A evolução da EM é extremamente variável e imprevisível. Identificam-se dois cursos bem distintos da EM: o primeiro se denomina curso remitente / recorrente, mais comum no adulto jovem, onde os sintomas e sinais neurológicos são transitórios, e o segundo, denominado curso progressivo, no qual os sinais e sintomas neurológicos instalados se intensificam, sem que ocorram remissões, sendo o quadro neurológico mais agudo, geralmente com comprometimento motor (sistema piramidal e/ou cerebelar) e manifesta-se mais freqüente após os 40 anos.

A mortalidade em pacientes com EM não é muito diferente da observada em indivíduos sem a doença. Porém, a progressão dos déficits neurológicos ocorre em todos os portadores da doença. Sabe-se que 15 anos após o início da doença, cerca

de 50% dos pacientes necessitam de auxílio para deambular e, após 25 anos, a maioria está incapacitada para tal. Estas incapacidades parecem relacionar-se com o número e a gravidade dos surtos nos primeiros anos da doença.

A maioria dos pacientes inicia seu quadro clínico com surtos que se alternam com remissões. Considera-se surto a ocorrência de sintomas de disfunção neurológica, com mais de 24 horas de duração, na ausência de febre ou infecção e com comprometimento de funções motoras e/ou sensitivas.

O exame neurológico, durante o surto, demonstra piora, principalmente por aumento de pelo menos um ponto em um dos escores da escala de incapacidade funcional. O índice anual de surtos e os intervalos entre eles (remissões) variam entre pacientes, sendo estimado em 0,85 surtos/ano. Durante a remissão o paciente permanece estável.

4.3. Encefalomielite Autoimune Experimental (EAE)

Um modelo experimental para o estudo da EM é denominado encefalomielite autoimune experimental (EAE), cuja investigação tem sido de grande utilidade para o melhor entendimento da EM (Olson *et al.*, 2000; Kornek *et al.*, 2000).

A EAE começou a ser estudada após a descoberta da vacina contra a raiva por Pasteur, em 1875. Esse tratamento anti-rábico consistia em injetar, na pessoa infectada pelo vírus, medula de coelho raivoso, dissecada e triturada com solução salina. A vacina permitiu a cura de muitos indivíduos. No entanto, observou-se que um

número significativo de pacientes desenvolveu uma paralisia, muitas vezes fatal (Encefalomielite pós-vacinal).

Estudos, realizados em 1946, mostraram que a injeção repetida do extrato de mielina do sistema nervoso central provocava uma encefalomielite no macaco (Kabat et al., 1946). A EAE passou então a ser considerada modelo experimental para o estudo dos mecanismos imunopatológicos nas doenças inflamatórias desmielinizantes.

A doença pode ser induzida em animais geneticamente susceptíveis, pela imunização com mielina e seus componentes, como a proteína básica de mielina (MBP), proteolipoproteína (PLP), glicoproteína associada à mielina (MAG), glicoproteína de mielina do oligodendrócito (MOG) e peptídeos encefalitogênicos derivados desses antígenos, ou ser transferida para animais normais por clones de linfócitos sensibilizados a estes componentes. (Ben-Nun & Cohen, 1982; Holoshitz *et al.*, 1983).

Dependendo do animal utilizado, a doença se apresenta de forma aguda monofásica ou crônica com surtos e remissões. Os ratos Lewis – imunizados com MBP - desenvolvem a forma aguda e monofásica da doença, enquanto os camundongos – imunizados com MOG - desenvolvem preferencialmente a forma crônica.

Como resultado da imunização ocorre a expansão e ativação de células T encefalitogênicas que invadem o SNC. Subseqüentemente, a produção de quimiocinas desencadeia um afluxo de monócitos e células NK. Secundariamente a esses eventos, aumenta a produção e liberação de substâncias neurotóxicas tais como óxido nítrico e agonistas de glutamato, tanto por parte da microglia residente quanto por parte dos macrófagos derivados de monócitos (Hammarberg *et al.*, 2000). Tais eventos podem

contribuir diretamente para o déficit funcional observado durante as crises da doença, afetando diretamente os terminais nervosos em aposição ao corpo celular dos motoneurônios medulares.

Depois da axotomia do funículo ventral da medula espinhal de gatos, por exemplo, aproximadamente metade da população de motoneurônios lesionados se degenera. Os terminais tipo S (excitatórios) são mais afetados do que os do tipo F (inibitórios) (Huh *et al*., 2000).

Através da análise destas estruturas, em modelos agudos e crônicos do modelo experimental da esclerose múltipla, foram observadas alterações significativas da expressão da proteína MAP-2 (microtubule associated protein -2), na extensão dos dendritos presentes na medula espinhal, durante a exacerbação e remissão da doença. Marcações retrógradas confirmaram que a maioria dos dendritos dos motoneurônios são desmielinizados na medula espinhal durante o surto da EAE e mostraram-se em processo de remielinização durante a fase de remissão (Zhu *et al*., 2003).

5. Os Imunomoduladores

A partir de abril de 1993, os imunomoduladores foram incorporados como alternativa para o tratamento de pacientes portadores da forma remitente/recorrente da EM, oferecendo eficientes resultados quanto à possibilidade de modificação da progressão da doença. Os primeiros resultados publicados com o uso do interferon beta 1-a em mais de 350 pacientes, demonstraram redução do número e gravidade dos surtos, prolongando o interstício entre eles. Adicionalmente, este tratamento reduziu o

número e volume de lesões analisadas sob ressonância nuclear magnética (RNM) (Paty e Li, 1993). Nos últimos anos, outros imunomoduladores têm sido empregados no tratamento da EM (Jacobs *et al.*, 1996; van Oosten, *et al.*, 1995). Os atualmente utilizados são: o interferon beta 1-a, o interferon beta 1-b, e o acetato de glatirâmer.

- Interferon beta 1-a - preparação recombinante glicolisada que tem a cadeia de aminoácidos idêntica a do interferon beta natural humano.
- Interferon beta 1-b - recombinante, porém não glicolisado, apresentando na sua estrutura de aminoácidos a cisteína na posição 17.
- Acetato de glatirâmer (Copaxone®) uma mistura de polímeros de quatro aminoácidos. Uma das seqüências é o fragmento peptídico não encefalitogênico da MBP. Foi demonstrado ter efeitos semelhantes aos do INF-ß com relação ao controle da exacerbação, dos sintomas e da gravidade dos episódios. O acetato de glatirâmer (AG) é uma droga aprovada para o tratamento da esclerose múltipla, diminuindo a intensidade e frequência dos episódios (Aharoni *et al.*, 2003; Gilgun-Sherki *et al.*, 2003). Seu mecanismo de ação não é completamente conhecido. Sabe-se que inclui a competição com a proteína básica da mielina, através do complexo principal de histocompatibilidade classe II (MHC II) (Racke *et al.*, 1992; Teitelbaum *et al.*, 1992; Fridkis-Hareli *et al.*, 1994). Também, a habilidade em estimular a proliferação de células Th2 como a supressão da atividade de células T (Teitelbaum *et al.*, 1997). Porém, há a possibilidade de que o AG atue diretamente nos neurônios e axônios e células gliais. Essa hipótese tem sido explorada em alguns poucos estudos com lesões no nervo

óptico, toxicidade por glutamato e modelos de doenças nos motoneurônios (Kipnis *et al.*, 2000; Schori *et al.*, 2001; Angelov *et al.*, 2003).

Nunca o impacto do AG no tecido nervosa, especialmente em neurônios durante o curso da esclerose múltipla, foi avaliado. Baseado no fato que o AG influencia a proliferação celular como a síntese de fatores neurotróficos (Ziemssen *et al.*, 2002), é possível que ele atue no controle de eliminação/retração sináptica.

II- JUSTIFICATIVA

Os conhecimentos baseados no processo de retração e remodelação dos circuitos medulares prestam sustentação à ocorrência de alterações sinaptológicas medulares, durante surtos da doença no modelo experimental da esclerose múltipla.

Esse trabalho visou a investigação dessas possíveis alterações sinaptológicas durante o surto e após a remissão da EAE com ou sem tratamento com acetato de glatirâmer.

O Departamento de Neurologia da UNICAMP dispõe hoje de aproximadamente 250 pacientes com diagnóstico definitivo da Esclerose Múltipla e recentemente a Universidade foi considerada como referência para o estudo da doença em nossa região. Dessa forma, associando a pesquisa clínica à experimental contribuímos para o melhor entendimento dos mecanismos associados à evolução da doença, os quais poderão, futuramente, ser aplicados para o ser humano.

III. OBJETIVOS

Através de técnicas histológicas de Microscopia Eletrônica de Transmissão e Imunohistoquímica:

- Realizar o estudo da dinâmica das sinapses em aposição ao corpo celular e dendritos proximais de motoneurônios alfa medulares durante o surto e após a remissão da encefalomielite autoimune experimental (EAE).
- Investigar a interferência da droga imunomoduladora acetato de glatirâmer no processo de plasticidade sináptica induzido a cada surto da doença.

IV – MATERIAIS e MÉTODOS

1. Comitê de Ética

Para a experimentação animal, obteve-se a autorização do Comitê de Ética – Protocolo nº 794-2.

2. Indução da EAE

Foram realizadas as induções da EAE em ratos Lewis fêmeas de 7 semanas, adquiridos no CEMIB, através da microinjeção de 100µl de proteína básica de mielina (MBP) e *Mycobacterium tuberculosis* (MT) emulsificadas em adjuvante completo de Freund, nas plantas das patas traseiras dos animais. Após a indução, os animais foram mantidos no biotério do Depto. de Anatomia recebendo ração e água *ad libitum*.

Diariamente, foi realizada a análise e classificação dos sinais e sintomas da doença, com o propósito de identificar o período de surto (que ocorre entre o dia 9 e 14 após a indução). Os primeiros sinais da doença são evidenciados com a flacidez da cauda do animal (que ocorre ~no 9º dia após indução - grau 1), depois ocorre a paralisia parcial do membro posterior (~11º dia após indução – grau 2), tetraplegia (~13º dia após indução – grau 3) e paraplegia (~14º dia após indução – grau 4). Após a exacerbação da doença, o animal começa a se recuperar e não apresenta mais nenhum sintoma da EAE (remissão ~20ºdia).

Para o estudo da dinâmica das sinápses, foram sacrificados dez animais que apresentavam o grau 3 da doença – 13º dia e dez animais na fase de remissão – 26º dia após indução. Na fase de remissão tivemos maiores dificuldades, perdendo alguns

animais, uma vez que estes precisaram passar pelo grau 4 da doença, onde apresentaram o grau de paraplegia, tendo dificuldades para beber e se alimentar.

Para o grupo controle, dez ratos Lewis fêmeas, também de 7 semanas, foram sacrificados.

3. Tratamento com Acetato de Glatirâmer (Copaxone®)

O tratamento foi iniciado trinta minutos após os procedimentos de indução da EAE. Dez animais receberam diariamente, uma dose subcutânea 0,5mg/animal 280g/dia de uma solução contendo o acetato de glatirâmer (Copaxone®) em solução PBS, sendo sacrificados 2 semanas após o início do tratamento.

Os grupos controle foram constituídos de dez animais tratados de forma idêntica aos dos grupos experimentais, exceto pela aplicação do acetato de glatirâmer que fora substituído por solução placebo (mesma dose de solução PBS não contendo o acetato de glatirâmer).

4. Grupos experimentais

Grupo 1: Induzidos à EAE e sacrificados no 13º (surto #3) e 26º dia (remissão) pós-indução.

Grupo 2: Induzidos à EAE e tratados com acetato de glatiramer e com solução placebo, e sacrificados 15 dia pós-indução.

Todos os espécimes foram processados para análise de microscopia eletrônica de transmissão, imunohistoquímica, microscopia de luz (coloração de Sudan Black e azul de Toluidina).

5. Processamento e análise dos espécimes para Imunohistoquímica

Após os períodos de sobrevida pré-determinados os animais foram submetidos à toracotomia e a seguir perfundidos transcardiacamente com tampão fosfato de sódio (PBS pH 7,4 – 0,1M) e formalina 10% em PBS pH 7,4 – 0,1M.

Secções transversais da intumescência lombar foram realizadas (12µm de espessura) em criostato (Microm) e posteriormente incubadas com os seguintes anticorpos primários: anti-sinaptofisina humana (Dako, 1 : 100), *goat antiglial fibrillary acidic protein* ((GFAP) Santa Cruz, 1:200) e anti-F4⁄80 (Península, 1:1000). Adicionalmente, secções do nível frontal cerebral foram incubadas com anti-sinaptofisina. As secções foram mantidas 24h a 4ºC. Os anticorpos primários foram diluídos em solução contendo albumina bovina e Triton X em tampão fosfato de sódio (0,01M).

Em seqüência à primeira incubação, as lâminas foram lavadas em PBS pH 7,4 - 0,01M e incubadas com os anticorpos secundários CY-2 e/ou CY-3 (Jackson Lab., USA) por 45 minutos. As laminas foram lavadas em PBS, montadas em uma mistura de glycerol/PBS (3:1) e observadas através de microscopia confocal de varredura a laser (Bio-Rad).

O comprimento de onda era de 488 nanômetros para CY2 e 568 nanômetro para o CY3. Para medidas quantitativas da imunorreatividade da sinaptofisina, GFAP e F4/80, imagens alternas do corno ventral L5 foram capturadas em uma ampliação final de 40x. A quantificação foi executada através da análise do contraste e a densidade realçada através do software Image Tool (versão 1.55; NIH, Bethesda, MD, EUA). A

densidade integrada de seis áreas (0.25 pixels quadrados cada um) foi medida em três seções alternas de cada espécime. A média e desvio padrão foram calculados para cada grupo e análise estatística foi executada.

6. Processamento e análise dos espécimes para microscopia eletrônica de transmissão (MET)

Após a fixação e inclusão em resina (Durcupan), seguindo protocolo para MET, foi padronizada a técnica para identificação dos motoneurônios alfa: neurônios com grandes corpos celulares (>35µm em diâmetro) encontrados no grupo dorso-lateral da coluna anterior da medula, seccionados ao nível do plano nuclear e identificados pela presença de terminais nervosos do tipo C.

A superfície celular foi, então, digitalizada sequencialmente em uma ampliação de 10.000x com uma câmera de vídeo conectada a um sistema computarizado, usando a característica de aquisição do software Kontron KS300 (Zeiss). As imagens foram montadas utilizando o software da Adobe - Photoshop e o perímetro total dos neurônios medidos.

O soma dos motoneurônios foram identificados e seus terminais sinápticos em aposição a membrana celular foram medidas pelo comprimento da justaposição.

Como ferramenta da medida utilizou-se software Image Tool versão 3.0, da Universidade do centro da saúde de Texas em Santo Antonio, TX, EUA. Os terminais foram ampliados (pelo menos 27.500x) e identificados como F (com as vesículas achatadas), S (com vesículas esféricas) e C (com uma cisterna sub-sináptica), de acordo com a nomenclatura de Conradi & de Skoglund (1969). Os terminais M-

glutamatérgicos são relativamente infrequëntes, contendo vesículas sinápticas esféricas originadas das fibras aferentes preliminares, assim foram classificados como terminais S.

É importante enfatizar que, durante o surto da doença quando os terminais indicaram alterações morfológicas, a forma das vesículas poderia ainda ser identificada. A distância entre os terminais nervosos que cobrem os motoneurônios foi determinada também. Um total de 50 motoneurônios foi estudado (dois neurônios por animal).

7. Indução de desmielinização não-imune

Dez ratos Lewis fêmeas, adultos jovem de sete semanas (pesando ~250g), foram anestesiados com uma combinação de Kensol (xylasin, 10mg/kg, Koning, Argentina) e Vetaset (ketamin, 50mg/kg, Fort Dodge, USA), tiveram suas vértebras laminectomizadas e a dura-máter aberta com uma incisão longitudinal. As raízes dorsais foram movidas cuidadosamente para expor a região do funículo lateral. Um volume de aproximadamente 5µl de 1% lisolecitina em salina 0.9% foi injetado lentamente no funículo lateral, de acordo com a técnica descrita por Jeffery e Blakemore (1995). Os músculos e pele foram suturados usando fio de sutura 4.0. Os animais foram mantidos individualmente por um período de 7 dias depois da cirurgia com ração e água *ad libitum*.

8. Análise da inflamação e desmielinização durante o curso da EAE

Técnicas de coloração com Sudan Black foram utilizadas para demonstrar o processo de desmielinização durante o desenvolvimento da doença. Para isso, secções congeladas foram mantidas em temperatura ambiente, lavadas em PBS 0,1M e coradas com Sudan Black 70% em solução de etanol por 30min. Os espécimes foram diferenciados em etanol 50%, lavados em água destilada e montados com uma mistura de glicerol/PBS (3:1).

Para o estudo do processo inflamatório através do influxo de linfócitos na região dos motoneurônios medulares, foram coradas lâminas contendo cortes semifinos (500nm) com azul de toluidina.

As análises foram realizadas através de microscopia de luz em microscópio Axiovert 135 (Zeiss, Germany).

9. Análise estatística

Os dados foram analisados e aplicados análise de variância (ANOVA) complementados com Test t ($p<0,05$(*) e $p<0,01$(**)).

CAPÍTULO 2

Artigo I: Spinal motoneuron synaptic plasticity during the course of an animal model of multiple sclerosis. European Journal of Neuroscience. 24: 3053-3062, 2006.

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Spinal motoneuron synaptic plasticity during the course of an animal model of multiple sclerosis

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Abstract

During the course of experimental autoimmune encephalomyelitis, a massive loss of motor and sensitive function occurs, which has been classically attributed to the demyelination process. In rats, the clinical signs disappear within 5 days following complete tetraplegia, indicating that demyelination might not be the only cause for the rapid evolution of the disease. The present work investigated the occurrence of experimental autoimmune encephalomyelitis-induced changes of the synaptic covering of spinal motoneurons during exacerbation and after remission. The terminals were typed with transmission electron microscopy as C-, F- and S-type. Immunohistochemical analysis of synaptophysin, glial fibrillary acidic protein and the microglia/macrophage marker F4/80 were also used in order to draw a correlation between the synaptic changes and the glial reaction. The ultrastructural analysis showed that, during exacerbation, there was a strong retraction of both F- and S-type terminals. In this sense, both the covering as well as the length of the remaining terminals suffered great reductions. However, the retracted terminals rapidly returned to apposition, although the mean length remained shorter. A certain level of sprouting may have occurred as, after remission, the number of F-terminals was greater than in the control group. The immunohistochemical analysis showed that the peak of synaptic loss was coincident with an increased macro- and microglial reaction. Our results suggest that the major changes occurring in the spinal cord network during the time course of the disease may contribute significantly to the origin of the clinical signs as well as help to explain their rapid recovery.

Introduction

The establishment and maintenance of the central nervous system neuronal circuits are complex and still poorly understood processes that involve millions of neurons precisely interconnected by the synapses (Cullheim *et al.*, 2002). Following axonal damage (e.g. sciatic nerve transection), spinal motoneurons undergo chromatolysis, which is followed by a significant retraction of the nerve terminals in contact with the cell surface, directly affecting excitability and synaptic transmission (Kuno & Llinás, 1970a,b). As shown by Linda *et al.* (2000), there is a preferential elimination of glutamatergic boutons, which has been interpreted as a way to avoid glutamate excitotoxicity. Interestingly, there is evidence that excitotoxicity with glutamate may also occur during the development of amyotrophic lateral sclerosis and during the episodes of multiple sclerosis (MS) (Zhu *et al.*, 2003).

However, the data, as they relate to demyelinating diseases, have been focused on the perikarya of demyelinated axons rather than the synaptic terminals. In this sense, the impact of demyelination on the spinal cord network is still poorly understood and underestimated (Gehrmann *et al.*, 1993). Although there is no significant neuronal death during MS exacerbation, it is clear that the influx of lymphocytes to the central nervous system territory induces dramatic changes in homeostasis, partly as a result of a local production of cytokines and neurotrophic factors (Hammarberg *et al.*, 2000; Novikova *et al.*, 2000). Also, inflammation induces proliferation of microglial cells and reactive astrogliosis, which may in turn induce changes in the spinal cord network during the episodes of the experimental autoimmune encephalomyelitis (EAE). It is important to emphasize that, under normal circumstances, nerve terminals, also referred to as boutons, have a degree of surface contact with the motoneuron cell membrane. Their ultrastructure has been thoroughly described and, according to the subsynaptic specializations and shape of the vesicles within the terminal, one can determine if it is excitatory or inhibitory. Certainly the interaction between the motoneurons and the surrounding environment, including the extracellular components as well as non-neuronal cells, is of crucial importance for the stability of synaptic connectivity and homeostasis (Hamburger, 1958; Levi-Montalcini, 1987; Oppenheim, 1991; Huh *et al.*, 2000; Boulanger *et al.*, 2001; Oliveira *et al.*, 2001, 2004). Such changes may occur in a very brief period of time and may well account for many of the motor problems observed during episodes of MS. Most of these changes have been attributed to the demyelination process itself although, at least in the EAE model, the animal's recovery from tetraplegy to normal occurs within 3–4 days, whereas the myelination process is far slower.

Based on the above, the present work investigated the occurrence of EAE-induced changes in the synaptic covering of spinal alpha motoneurons during exacerbation and after remission of the disease. Our results suggest that the major changes occurring in the spinal cord network during the time course of the disease may significantly

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contribute to the origin of the clinical signs as well as help to explain their rapid recovery.

Materials and methods

Animals

Thirty adult female Lewis rats (7 weeks old, ~250 g body weight) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP) and housed using a 12-h light/-dark cycle with free access to food and water. The study was approved by the Institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, proc. 794-2) and the experiments were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation. The rats were allocated to one of three groups. The first group of rats ($n = 10$) was submitted to EAE and killed when presenting paraplegia (grade 3, see below), whereas the second group of rats ($n = 10$) was killed 2 weeks after the peak of the EAE symptoms. The third group of rats ($n = 10$) was used as normal control. In all cases, the spinal cords were processed for immunohistochemical ($n = 5$ in each group) and ultrastructural ($n = 5$ in each group) analysis.

Induction of experimental autoimmune encephalomyelitis

All reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) except those specifically mentioned. The rats were immunized with a single injection of 100 μL of guinea pig myelin basic protein (25 μg) associated with heat-inactivated *Mycobacterium tuberculosis* H37RA (2.0 mg/mL; Difco Laboratories, Detroit, MI, USA) and complete Freund's adjuvant emulsion. This solution was injected subcutaneously in the footpad of the animals' hind legs. Control rats received the complete Freund's adjuvant alone (containing *M. tuberculosis*). A daily analysis and scoring of the signs and symptoms of the severity of EAE were carried out as follows: grade 0, no clinical signs; grade 1, tail weakness or paralysis; grade 2, hind limb paraparesis; grade 3, hind limb paralysis; grade 4, complete paralysis (tetraplegy).

Induction of non-immune demyelination

Ten adult female Lewis rats (7 weeks old, ~250 g body weight) were anesthetized with a combination of Kensol (xylasin, 10 mg/kg, Köning, Argentina) and Vetaset (ketamin, 50 mg/kg, Fort Dodge, USA). A half-sided laminectomy was performed on the L3 vertebrae and the dural sac was opened with a longitudinal incision. The dorsal roots were carefully moved upwards in order to expose the region of the lateral funiculus. A volume of approximately 5 μL of 1% lysolecithin (L-alpha lysophosphatadyl choline type I, Sigma) in 0.9% saline was slowly injected into the lateral funiculus, according to the technique described by Jeffery & Blakemore (1995). The epiaxial muscles and skin were closed separately using 4/0 monofilament nylon. The animals were housed individually for a period of 7 days after surgery with free access to food and water.

Specimen preparation

Following the pre-determined survival periods, the animals were anesthetized with a mixture of Kensol (xylasin, 10 mg/kg, Köning) and Vetaset (ketamin, 50 mg/kg, Fort Dodge) and the vascular system was rinsed by transcardial perfusion with phosphate buffer (pH 7.4). For the immunohistochemical detection of synaptophysin, glial fibrillary acidic protein and F4/80, the rats were fixed by vascular perfusion with 4% paraformaldehyde in phosphate buffer (pH 7.4). Thereafter, the brain and lumbar intumescence were dissected out, post-fixed overnight, washed in phosphate buffer and stored in sucrose (20%) for 8 h before freezing. Cryostat transverse sections (12 μm thick) of the spinal cords and frontal sections from the brains were obtained and transferred to gelatin-coated slides, dried at room temperature (~21 °C) for 30 min and stored at −20 °C until utilization. For electron microscopy, 100 mL of a fixative containing 2.5% glutaraldehyde and 0.5% paraformaldehyde in phosphate buffer (pH 7.4) was perfused through the ascending aorta. The lumbar spinal cords were removed and stored overnight in the same fixative at 4 °C. The specimens were then trimmed and osmicated, dehydrated and embedded in Durcupan (Fluka, Steinheim, Switzerland). Ultra-thin sections from the L4–L6 segments were collected on formvar-coated copper grids, contrasted with uranyl acetate and lead citrate, and examined under a Leo906 transmission electron microscope operated at 60 kV.

Immunohistochemistry

Transverse sections of the spinal cord (12 μm thick) were cut in a cryostat (Microm) and incubated with the following primary antibodies: human anti-synaptophysin (Dako, 1 : 100), goat anti-glial fibrillary acidic protein (Santa Cruz, 1 : 200) and rat anti-F4/80 (Peninsula, 1 : 1000). Additionally, frontal brain sections at the level of the motor cortex and basal ganglia were incubated with anti-synaptophysin antibodies. Sections were incubated overnight in a moist chamber at 4 °C. The primary antiserum was diluted in a solution containing bovine serum albumin and Triton X in 0.01 M phosphate-buffered saline (PBS). After rinsing, the secondary antibodies were applied and incubated for 45 min, according to the host of the primary antibodies (CY-2 and CY-3, Jackson Immunoresearch, 1 : 250). The sections were then rinsed in PBS, mounted in a mixture of glycerol/PBS (3 : 1) and observed with a laser scanning confocal system (Bio-Rad) mounted on an Axiovert 135 inverted microscope (Zeiss, Germany). The excitation wavelength was 488 nm for CY2 and 568 nm for CY3 fluorescence. For quantitative measurements of synaptophysin, glial fibrillary acidic protein and F4/80 immunoreactivity, alternate images of the L5 ventral horn were captured at a final magnification of 400 ×, with the microscope settings being kept the same for all slides. Quantification was performed with the enhance contrast and density slicing feature of IMAGE software (version 1.55; NIH, Bethesda, MD, USA). The integrated density of six areas (0.25 square pixels each) was measured in three alternate sections of each specimen. The mean and SE were calculated for each group and a statistical analysis was performed.

Analysis of the inflammation and demyelination during the course of experimental autoimmune encephalomyelitis

The Sudan black technique was used to demonstrate the demyelination process during the development of the disease. For this purpose, frozen sections were left at room temperature, rinsed with 0.01 M PBS and stained with Sudan black in 70% ethanol solution for 30 min. The specimens were differentiated in 50% ethanol, rinsed with distilled water and mounted in a mixture of glycerol/PBS (3 : 1).

The influx of lymphocytes close to the spinal motoneurons was studied on semithin toluidine blue-stained sections, obtained previously to the ultra-thin sections.

Analysis of the ultra-thin sections

Neurons with large cell bodies (> 35 μm in diameter) found in the sciatic motoneuron pool and cut in the nuclear plane were identified as alpha motoneurons by the presence of C-type nerve terminals. The surface of the cells was then sequentially digitalized at a magnification of 10 000 × with a video camera connected to a computerized system, using the acquisition feature of the KONTRON KS300 software (Zeiss). The images were then mounted together using the Adobe PHOTOSHOP software and the total perimeter of the neurons (in μm) was measured. Synaptic terminals apposing the motoneuron somata were identified and their numbers per 100 μm of cell membrane and the length of apposition in percent of membrane length were calculated using the measurement tool of the IMAGE TOOL software (version 3.0, The University of Texas Health Center in Santo Antonio, TX, USA). The terminals were typed under high magnification (at least 25 000 ×) as F (with flattened synaptic vesicles), S (with spherical synaptic vesicles) and C (with a subsynaptic cistern), according to the nomenclature of Conradi & Skoglund (1969). The relatively infrequent glutamatergic M-boutons, containing spherical synaptic vesicles and originating from Ia primary afferent fibers, were typed as S-boutons.

It is important to emphasize that, even during the exacerbation of the disease when the terminals displayed morphological alterations, the shape of the vesicles could still be identified. The distance between consecutive nerve terminals covering the motoneurons was also determined. A total of 30 sciatic motoneurons (two neurons per animal) in three groups of five animals ($n = 10$ neurons per group, in three groups of five animals: control, degree 3 of EAE and remission) were studied in this way.

Statistical analysis

The data were analysed with ANOVA and the two-tailed Student's *t*-test at $P < 0.05$ and $P < 0.01$.

Results

Loss of synaptophysin immunoreactivity and increase of glial reaction in experimental autoimmune encephalomyelitis-affected motoneurons

In order to assess the changes in synaptic activity during the course of EAE, qualitative and quantitative analyses of the immunoreactivity of the synaptic protein synaptophysin were performed in the spinal cord motor nuclei in control animals as well as in EAE-induced rats at the grade 3 and remission stages. The immunolabeling results are presented in Fig. 1A–C and the quantitative analysis revealed a

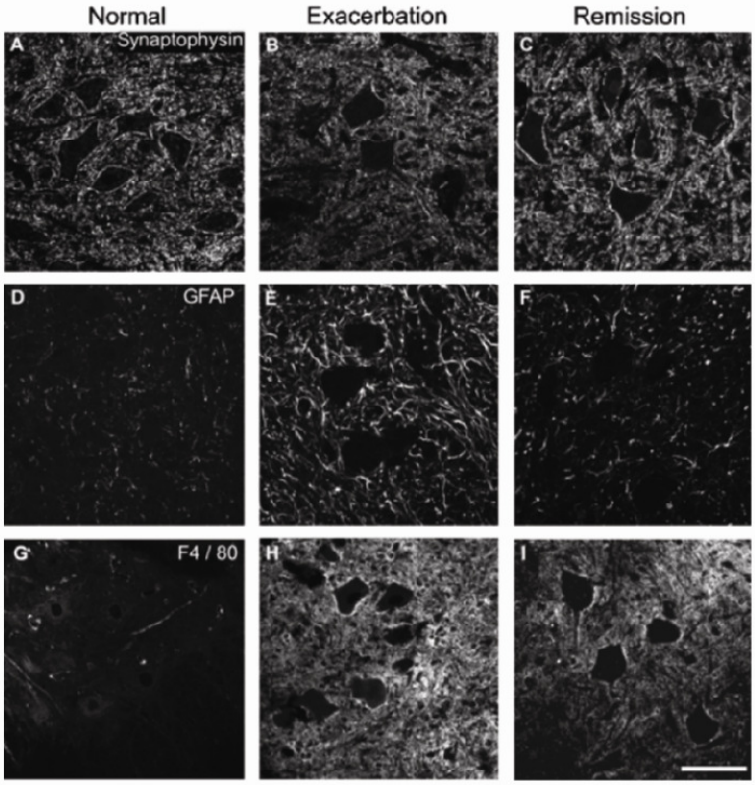

FIG. 1. The reduction of synaptophysin and increase in glial fibrillary acidic protein (GFAP) and F4/80 during the peak of experimental autoimmune encephalomyelitis (EAE) followed by synaptic covering recovery as well as a reduction in glial reactivity after remission. Immunolabeling against synaptophysin in the normal (A), exacerbation (degree 3) of EAE (B) and remission (C) stages. Immunofluorescence micrograph showing astrocyte reactivity in the normal (D), degree 3 of EAE (E) and remission (F) stages. Immunofluorescence micrograph showing microglial reactivity in the normal (G), degree 3 of EAE (H) and remission (I) stages. Scale bar, 50 μm.

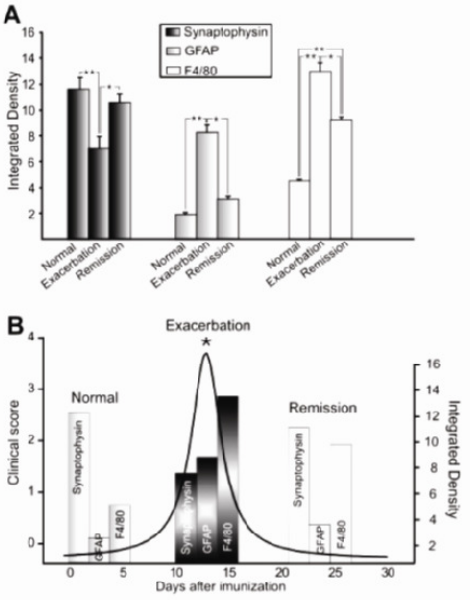

FIG. 2. The graph in A represents a quantitative evaluation of the levels of immunoreactivity during the course of the disease, depicting a decrease in synaptic activity as well as an increase in glial response in the lumbar spinal cord, coincident with the period of paraplegia. The graph in B represents the time course of the experimental autoimmune encephalomyelitis and the correlation between the clinical scores and immunohistochemical findings. The peak of the disease (*) occurred around 14 days after immunization. GFAP, glial fibrillary acidic protein. In A, $*P < 0.05$; $**P < 0.01$; in B, *represents the peak of the disease.

statistically significant twofold decrease in the labeling at exacerbation/grade 3, corresponding to the period of paraplegia (control, 11.5 ± 1.0 mean ± SEM; exacerbation, 6.9 ± 1.0; remission, 10.5 ± 0.8, $n = 5$ for all groups; Fig. 2A). In the remission phase, immunolabeling recovered to a level similar to that of the control group. A qualitative analysis of the brain sections, stained with anti-synaptophysin, revealed a general decrease of immunoreactivity during the peak of the disease (not shown).

Immunoreactivity to glial fibrillary acidic protein and F4/80 was used in order to assess the degree of glial reactivity during the course of the disease. In this sense, statistically significant increases in both astrocyte (Fig. 1D–F, control, 1.9 ± 0.2 mean ± SEM; exacerbation grade 3, 8.2 ± 0.6; remission, 3.1 ± 0.2, $n = 5$ for all groups; Fig. 2A) and microglial reactivity (Fig. 1G–I, control, 4.5 ± 0.1 mean ± SEM; exacerbation grade 3, 13.0 ± 0.7; remission, 9.3 ± 0.2, $n = 5$ for all groups; Fig. 2A) were observed, especially in the motoneuron pool area. In both cases the increase in labeling was about three- to fourfold, remaining elevated after remission. Figure 2B represents the correlation between the immunolabeling and the clinical scores obtained during the course of the disease. In this sense, at the exacerbation phase that occurred around the 14th day after immunization, the maximum degree of synaptic elimination was coincident with the intense glial reaction.

Presence of demyelinated fibers in the spinal cord after motor recovery (remission phase)

Sudan black-stained myelinated axons in the spinal cord are shown in Fig. 3A–C. The degree of staining was greatly reduced during the exacerbation of the disease (Fig. 3B) and it was at a minimum at the stage when the animals were paralysed (Fig. 3G). Also, a clear degree of demyelination was still present in the remission phase (Fig. 3C), at the stage in which the rats had already recovered motor function. The demyelination process was coincident with the influx of immune cells to the nervous tissue, as seen by the toluidine blue-stained material. In this sense, the peak of the inflammatory response was also at the exacerbation of the disease (Fig. 3E). Representative pictures from normal and remission phase material are shown in Fig. 3D and F, respectively.

Non-autoimmune inflammation and non-immune demyelination do not induce synaptic stripping

The impact of a non-immune inflammation triggered after complete Freund's adjuvant was analysed with anti-synaptophysin and anti-F4/80. Hind limb mobility was monitored during 2 weeks after complete Freund's adjuvant injection. The immunolabeling revealed no visible synaptological changes in the ventral horn of the spinal cord (not shown) and the motor function was not affected.

The results regarding the demyelination induced after lysolecithin injection are presented in Fig. 4A–F. Sudan black staining revealed the presence of demyelinated axons in the white matter, close to the interface with the gray matter. In this sense, a certain amount of affected fibers within the motoneuron area (lamina IX) was also observed (Fig. 4C). These results contrasted with the morphology seen in the contralateral side of the spinal cord (Fig. 4D). The impact of such non-immunological demyelination on the synaptic covering of the motoneurons is shown in Fig. 4E. Although a general decrease of synaptophysin labeling could be observed, the density of stained terminals apposed to the alpha motoneurons remained similar to the contralateral (unlesioned) side (Fig. 4F).

Increased synaptic elimination during the exacerbation of experimental autoimmune encephalomyelitis

A series of synaptic changes have been identified at the ultrastructural level during the course of the disease. Regarding the alpha motoneurons in the spinal cord, it was possible to observe an axotomy-like morphology that included chromatolysis, displacement of the cell nucleus, alterations on the surface of the plasma membrane (membrane bubbling) and elimination of synapses from the cell body (Aldskogius & Svensson, 1999). Nevertheless, no significant changes regarding the cell body diameter were found (Fig. 5C). The location of the neurons sampled from the spinal cord as well as the ultrastructural differences in the control, degree 3 and remission are represented in Fig. 5. A total of 30 sciatic alpha motoneurons ($n = 10$ neurons per group, two per animal in three groups of five animals, control, degree 3 of EAE and remission) were analysed in detail with respect to their synaptic covering, as well as the number and type of synapses in apposition to the cell body. The total covering of F-, S- and C-terminals is presented in Fig. 6A. A statistically significant reduction in both the F-terminals (representing inhibitory inputs: normal group, 43.9 ± 0.9%; exacerbation, 22.6 ± 0.8%; i.e. a ~50% reduction) and S-terminals (representing excitatory inputs other than except C-boutons: normal, 21.9 ± 0.5%; exacerbation, 9.7 ± 0.8%; i.e. ~70% reduction) occurred during the peak of the disease, resulting in a 50% decrease in the overall synaptic covering. This loss of inputs was fully recovered at the remission stage. However, as seen in Fig. 6B and C, the number of F- and S-terminals after remission increased in comparison with the control neurons, indicating a

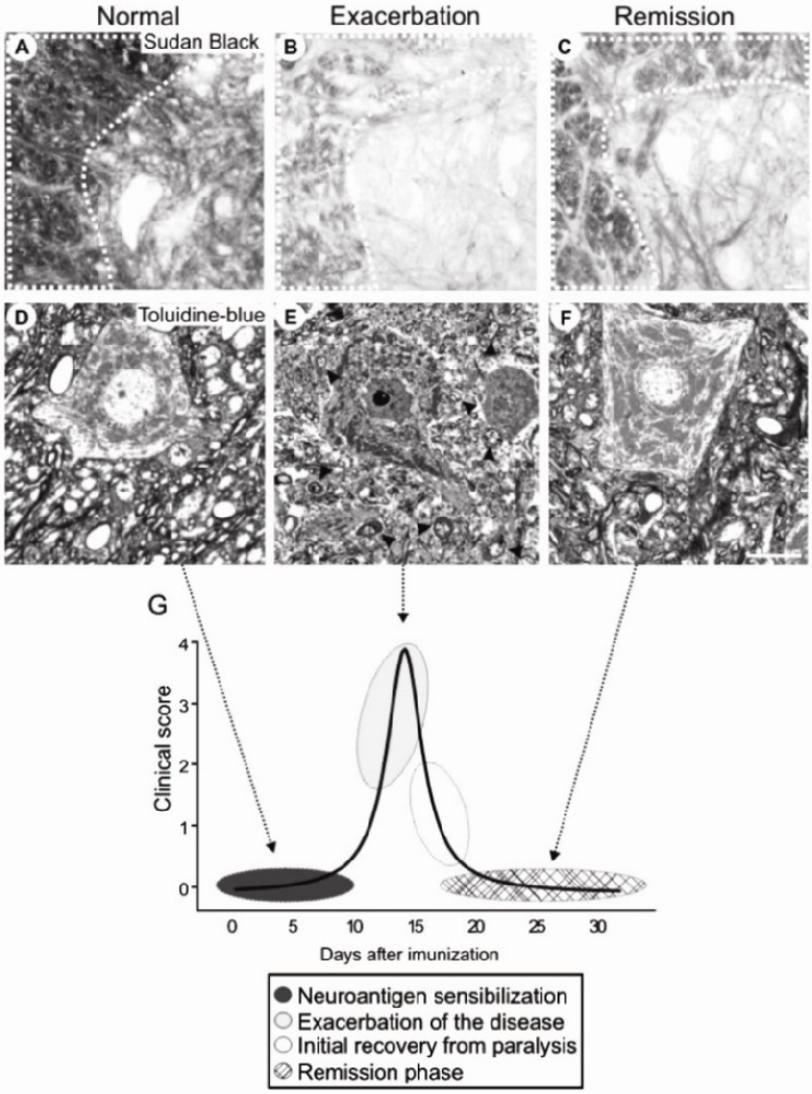

FIG. 3. Correlation between demyelination/inflammation and the clinical scores of experimental autoimmune encephalomyelitis (EAE). Sudan black staining at the ventral horn of the spinal cord, showing the degree of myelination in normal (A), paralysed [degree 3 (B)] and recovered [remission (C)] animals. Note that, after remission, axons in the white matter are not fully remyelinated. Scale bar, 20 μm. Toluidine blue-stained semithin sections at the different stages of the disease. Note the influx of immune cells in the vicinity of the motoneurons (arrowheads, E). Normal (D) and remission (F) material are also shown. Scale bar, 15 μm. (G) Time course of the EAE and its correlation with the degree of demyelination and inflammation in the spinal cord.

possible sprouting of both glutamatergic and glycin/GABAergic terminals after the exacerbation of the disease. Although retraction of F-terminals was evident during degree 3 of EAE, no statistically significant difference regarding the number of S-boutons was observed. This fact indicates the occurrence of a partial detachment of such excitatory terminals, which is clearly indicated by the reduction in bouton length shown in Fig. 6D ($P < 0.01$). This reduction was still present for both F- and S-boutons, to a smaller degree, after remission.

Pattern of elimination of the boutons induced by experimental autoimmune encephalomyelitis inflammation

A particular feature of the synaptic elimination process is that the retraction of boutons is not by chance but rather a finely orchestrated event. In this sense, synaptic removal after lesion tends to preserve clusters of terminals at specific points along the cell membrane of injured neurons. This is demonstrated by the fact that, even during the peak of the disease, about 26% of the gaps between terminals were

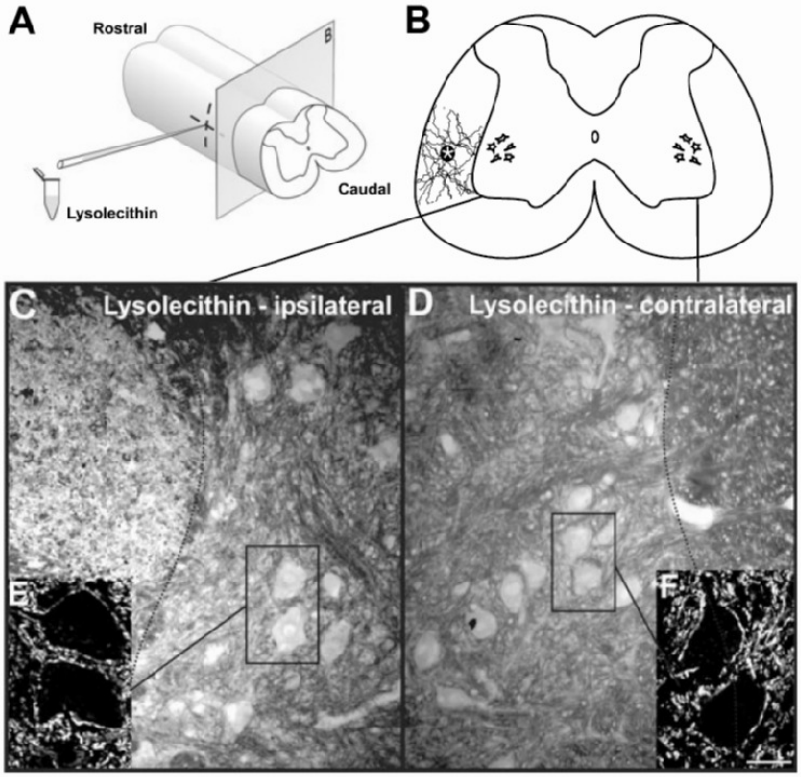

FIG. 4. (A) Schematic drawing of the rat spinal cord at the lumbar level, showing the site of lysolecithin injection (non-immunological demyelination experiment). (B) Transverse section, caudally to the injection site, showing the area of demyelination in the white matter (*). Sudan black staining at the ventral horn ipsilateral (C) and contralateral (D) to the lesion. Synaptophysin immunoreactivity on the surface of spinal motoneurons ipsilateral (E) and contralateral (F) to the lesion (adjacent sections to C and D). Note that the immunolabeling is not affected by the lysolecithin-induced demyelination. Scale bar, 60 μm (C and D) and 20 μm (E and F).

<2 μm (~42% in the normal and ~50% after remission), so that a number of boutons remained close together. Figure 7 represents the pattern of terminal distribution on motoneurons [normal (Fig. 7A), exacerbation (Fig. 7B) and after remission (Fig. 7C)]. In normal neurons the spacing of the terminals was restricted to a shorter soma membrane length, typically from 1 to 4 μm. Such a distribution resulted in many bouton clusters. However, during the peak of the disease, due to terminal retraction, the size of the interval increased up to 10 μm.

Taken together, the morphological data presented here indicate that the elimination of inputs during the course of EAE is strikingly similar to that observed after peripheral axotomy (Oliveira *et al.*, 2004).

Discussion

The present study has provided evidence for the occurrence of intense synaptic plasticity in the spinal cord during the course of EAE, an animal model for MS. Such changes directly affect the spinal motoneurons, profoundly modifying the number, length and balance of the inputs. In particular, an axotomy-like reaction in the gray matter was described, triggered by the inflammatory process together with the activated glia. In this way, by the use of ultrastructural techniques, it was possible to assess the process of synaptic retraction that takes place during the exacerbation of the disease and the recovery of the pre-synaptic contacts that occurred in the remission phase. The great ultrastructural alterations observed were in agreement with the immunohistochemical data herein, indicating a decrease in synaptic activity (in both the spinal cord and telencephalic structures) as well as an increase in glial reactivity during the course of the disease. In this way, the present results suggest that the several input changes to the alpha motoneurons in the spinal cord could account, at least in part, for the paralysis observed during the EAE peak.

Due to the great demyelination of the spinal tracts already described in the literature (Kornek *et al.*, 2000; Wujek *et al.*, 2002; Zhu *et al.*, 2003; Kerschensteiner *et al.*, 2004; Heppner *et al.*, 2005), it is possible that myelinated axonal recovery takes much longer to occur than the disease remission phase. This is also supported by the present results with Sudan black staining, showing that recovered animals still presented extensive demyelination in the spinal cord. However, pre-synaptic input retraction and reapposition are fast phenomena, usually occurring within hours, as described in several reports in the literature (for a review, see Luo & O'Leary, 2005). Recently, original studies have provided evidence for corticomotor reorganization in MS,

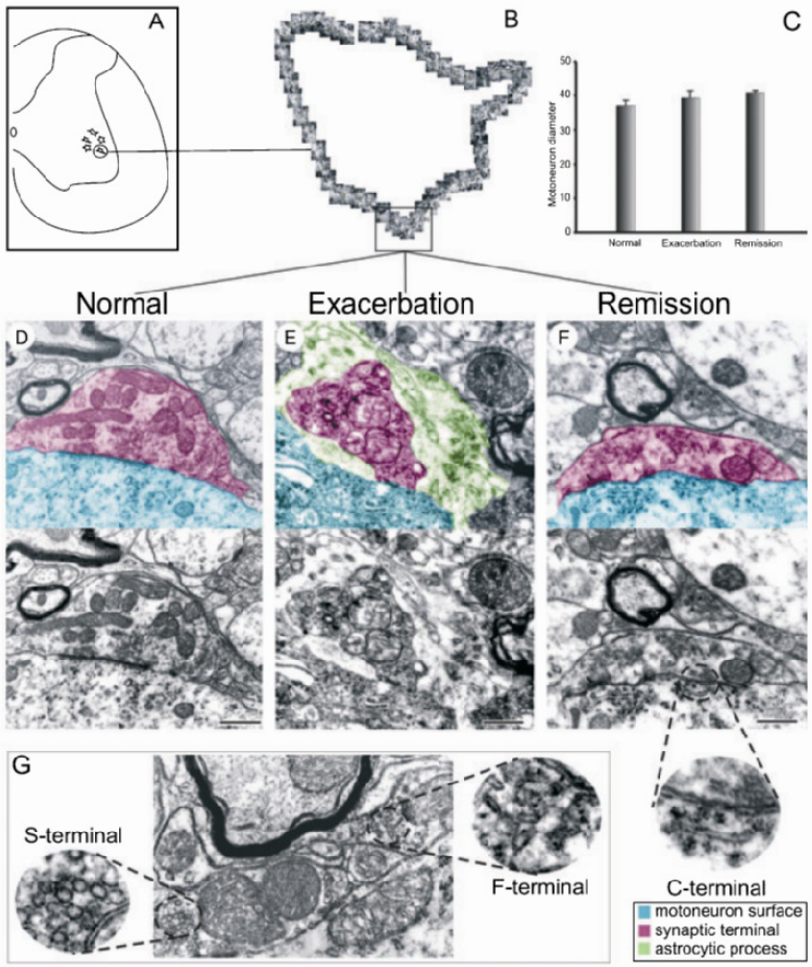

FIG. 5. (A) Schematic drawing of a transverse section of the rat spinal cord at the lumbar level, showing the dorsolateral motoneuron pool that was used for the ultrastructural analysis. (B) Ultrastructurally reconstructed alpha motoneuron. The entire cell surface was obtained at a magnification of 10 000 × by sequentially photographing the neuron. (C) Diameter of motoneuron cell bodies in the different experimental conditions. Electron micrographs from the motoneuron surface, showing the apposition of a pre-synaptic terminal to the plasma membrane. (D) Normal covering in a control section. (E) Example of a degree 3 experimental autoimmune encephalomyelitis specimen. Note the retraction of the nerve terminal from the surface of the motoneuron. (F) Example of the remission phase, showing the reapposition of a bouton. Scale bar, 1.0 μm. (G) Different morphology of the synaptic terminals, illustrating S-, F- and C-boutons.

indicating that demyelination as well as axonal microenvironment inflammation trigger synaptic plasticity in the brain, altering the motor representation (Thickbroom *et al.*, 2005). In this respect, evidence of neural plasticity has been provided mostly by neuroimaging, where the level of activation correlates with the diffusion weighted imaging (Lee *et al.*, 2000; Boniface, 2001; Filippi, 2001; Cifelli & Matthews, 2002; Pantano *et al.*, 2002; Rocca *et al.*, 2002, 2003; Filippi & Rocca, 2003). In this sense, such changes have been shown in most MS studies, including patients in the earliest clinical stages of the disease and still with subclinical alterations.

Kerschensteiner *et al.* (2004) demonstrated that the EAE lesion induces axonal remodeling at many levels in the circuitry surrounding the lesion, as well as in descending motor tracts and at the cortical level itself. These authors used a targeted EAE lesion, induced in the dorsal column of the midthoracic spinal cord by injection of proinflamatory cytokines in rats immunized with oligodendrocyte glycoprotein peptide. Using this approach, associated with neuroimaging techniques, it was possible to show axonal branching as well as the formation of new axon collaterals. Although they assessed the occurrence of synaptic plasticity to a certain degree, no ultrastructural analysis has been performed. Taking this into account, the ultrastructural morphology of the synapses was used in the present study in order to investigate the input changes to spinal alpha motoneurons during the development of EAE.

We also provide evidence for the occurrence of retraction of both the inhibitory and excitatory inputs during the exacerbation of the disease. In this sense, the total synaptic covering was drastically reduced, similar to that occurring after ventral root avulsion or an

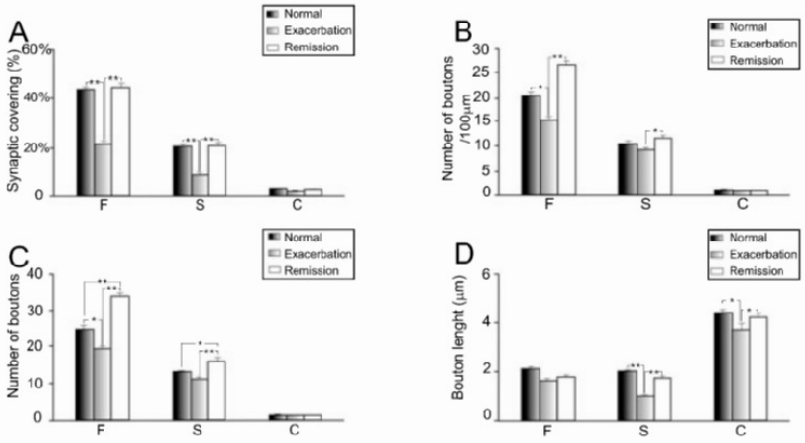

FIG. 6. Detailed analysis of the synaptic covering and number of synaptic boutons during the course of experimental autoimmune encephalomyelitis (EAE) revealing that, during the peak of the disease, there is a significant reduction in covering, similar to the response occurring after a peripheral lesion. (A) Graph showing the overall reduction of both excitatory and inhibitory inputs followed by recovery, during the remission phase. (B and C) Reduction in the inhibitory terminals during the peak of EAE both in absolute and relative (number of boutons/100 µm of membrane) numbers. After remission, note the increase in the number of F-terminals, indicating the occurrence of input sprouting. (D) The graph shows that the reduction in S covering was mainly due to partial retraction of the terminals. Inhibitory terminal partial detachment was less prominent, indicating that the majority of the F-terminals were fully retracted during the peak of the disease. $*P < 0.05$; $**P < 0.01$.

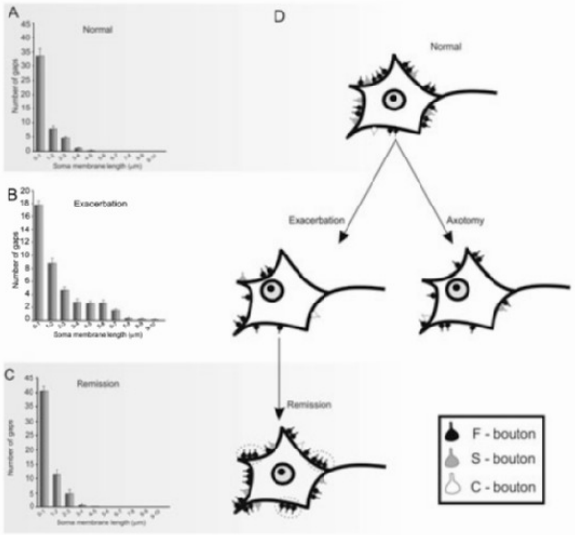

FIG. 7. The distribution of the gap length between clusters of terminals apposing the cell soma membrane was affected during the course of experimental autoimmune encephalomyelitis (EAE). (A) Normal distribution of intervals between nerve terminals showing that the boutons are organized in clusters of inputs. (B) The distribution of terminals during the EAE peak was altered by retraction of the F- and S-terminals. Note that the gaps between the terminals increased but the overall pattern did not change, similar to what has been observed after axonal lesion. (C) Recovery of retracted terminals restoring the original distribution of clusters along the motoneuron membranes. Note that, due to fiber sprouting, the gap size tended to be shorter than in the normal situation. (D) Schematic representation of the changes in the synaptic covering during the course of EAE. Note the similarity of the synaptic alterations occurring during the exacerbation of EAE with those of a peripheral axotomy. In the remission phase, the size of the inhibitory clusters increased, probably due to pre-synaptic input sprouting (dashed circles).

intramedullary axotomy, as described ultrastructurally by Lindå *et al.* (2000). In the case of an intramedullary lesion, only about 12–25% of the terminals remained in apposition to the cell somata 3 weeks after lesion. Additionally, about 90% of the excitatory (glutamatergic) terminals were affected, whereas inhibitory boutons (glycine and GABA) were less affected (70%). The present results show a decrease

of about 70% in the excitatory and about 50% in the inhibitory input at the stage of the disease where the rats were paraplegic. Although these percentages are smaller than after intramedullary section, they may be sufficient to cause a significant imbalance in terms of excitability of the affected motoneurons.

In contrast to the proximal or intramedullary axotomy, the EAE-induced changes regarding the input covering seemed to recover rapidly during the remission phase. This strategy, as used by the motoneurons, has been suggested as a way to avoid excitotoxicity, mainly due to an influx of glutamate, and to focus the cell resources on survival and axonal recovery. From our point of view, this would contribute to the explanation of the fast recovery of EAE during remission, regardless of the demyelination process and its consequences.

Another key point during the development of EAE is the glial reaction which involves, in the spinal cord, both astrocytes and microglia (Ullian *et al.*, 2001; Pedotti *et al.*, 2003; Heppner *et al.*, 2005; Pannu *et al.*, 2005). Such cell lineages have been implicated in the process of synaptic elimination (Blinzinger & Kreutzberg, 1968; Conradi, 1969; Gehrmann *et al.*, 1993; Brännström & Kellerth, 1998; Aldskogius & Svensson, 1999), as well as the production of neurotoxic and pro-inflammatory molecules (Ulvestad *et al.*, 1994; Becher *et al.*, 2000; Carson, 2002; Ambrosini & Aloisi, 2004). Thus, the role of glia in the neuronal response to injury or inflammation has to be taken into account and might be an important issue during the course of EAE and MS. The present immunohistochemical results show that both astrocytes and microglia were reactive in the surroundings of the motoneurons. Such reactivity and location were confirmed by electron microscopy, where it was also possible to identify a large number of astrocytic processes filling the gaps between synaptic boutons. An important point is that both astroglial and microglial thin processes are very plastic and can change in shape and size within a short period of time (Schneider *et al.*, 1992; Shao *et al.*, 1994). In this regard, in a review by Gehrmann *et al.* (1993), the possibility that microglial reactivity could be related to synaptic reorganization around the spinal motoneurons has been raised, strengthening the data presented herein.

We suggest that the fast recovery of the terminal covering seen during the remission phase, which included a possible sprouting of pre-synaptic terminals, is optimized by the fast reshaping of glial processes, allowing the return of boutons to apposition, contributing to the quick motor recovery. In this sense, the gaps between the fully, as well as the partially, retracted terminals were filled with lamellar glial processes. An important finding in the present work was that the surplus removal of synaptic terminals was preferentially directed towards leaving a few clusters of terminals. It is possible that these remaining clusters helped with the maintenance of the original spinal cord circuits that were re-established during the remission phase.

An important point is whether demyelination alone is able to induce significant disability and synaptic stripping. This matter was addressed by injecting lysolecithin in the white matter of the spinal cord, adjacent to lumbar motoneurons. Under these circumstances, no visible motor impairment was observed and the labeling with anti-synaptophysin showed a rather conserved density of inputs on the surface of the alpha motoneurons. Although, in this particular experiment, the demyelination was not as extensive as during an EAE exacerbation, the results reinforce the hypothesis that inflammation in the spinal cord microenvironment may be the most important factor for inducing network changes during the course of EAE. However, the observation that rats immunized with non-central nervous system immunogen did not display any sign of synaptic retraction or clinical signs of motor impairment indicates that the autoimmune characteristics of EAE are pivotal for the occurrence of transient plastic phenomena in the central nervous system during the course of the disease.

In summary, the data presented in this study provide evidence that synaptic retraction and reapposition, together with the reactive glia, play important roles in the motor deficit and recovery during the course of EAE and also that the strategy used by the motoneurons during the peak of the disease and after recovery resembles that after very proximal lesions or when the motoneuron-terminal signaling pathway is impaired.

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Abbreviations

EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; PBS, phosphate-buffered saline.

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Artigo II: The immunomodulator glatiramer acetate directly influences spinal motoneuron plasticity during the course of an animal model of multiple sclerosis. Em preparação.

The immunomodulator glatiramer acetate directly influences spinal motoneuron plasticity during the course of an animal model of multiple sclerosis

Abbreviated title: Glatiramer acetate increases synaptic stability in the spinal cord

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Abstract

Most of the motor disabilities occurring in multiple sclerosis and in its animal model - experimental autoimmune encephalomyelitis (EAE), have been classically attributed to the demyelination process. However, the occurrence of major changes in the spinal cord network during the time course of the disease also trigger the clinical signs, as well as contributing to a rapid sensitive/motor recovery. The immunomodulador glatiramer acetate (GA) has been shown significantly reduce the seriousness of the symptoms during the exacerbation of the disease. However, little is known about its effects on the spinal motoneurons and on their afferents. In the present study, we investigated whether GA has a direct influence on synapse plasticity and on the deafferentiation of motoneurons during the course of EAE in rats. Lewis rats were subjected to EAE associated with GA or placebo treatment. The animals were sacrificed after fifteen days of treatment and the spinal cords processed for immunohistochemical analysis (IH) and electron transmission microscopy. A correlation between the synaptic changes and glial activation was obtained by performing labelling of synaptophysin, GFAP and F4/80 using IH. Also an ultrastructural analysis of the terminals apposed to alpha motoneurons was performed by electron transmission microscopy. Interestingly, although the GA treatment preserved synaptophysin labelling, it did not significantly reduce the glial reaction, indicating that inflammatory activity was still present. Also, the ultrastructural analysis showed that GA treatment significantly prevented retraction of both F and S type terminals in comparison with the placebo. The present results indicate that the immunomodulator GA has a direct influence on the stability of nerve terminals in the

spinal cord, which in turn may contribute to its neuroprotective effects during the course of multiple sclerosis.

Keywords: EAE, spinal cord, synapse elimination, motoneuron, immunomodulator, glatiramer acetate.

Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) of which the aetiology is unknown, although, once initiated, it actively involves the immune system. Demyelination, similarly to that seen during the course of MS, may be induced in animals, in a model called experimental autoimmune encephalomyelitis (EAE) (Trapp et al., 1999; Kornek et al., 2000; Kerschensteiner et al., 2004; Gold et al., 2006; Marques et al., 2006). During the course of EAE, damage to the axons as well as the occurrence of synaptic plasticity phenomena in the brain and spinal cord, contribute to the clinical signs of the disease (Lassmann et al., 2001; Hemmer et al., 2002; Kerschensteiner et al., 2004). Interestingly, synaptic plasticity in the spinal cord may explain, at least in part, the rapid sensitive/motor recovery of the animals soon after tetraplegia (Kerschensteiner et al., 2004; Marques et al., 2006).

Taking into account the importance of a better understanding of MS as well as its experimental models, major advances in understanding how cellular and humoral immune responses contribute to the pathogenesis of these diseases have been made in recent years (Cross and Stark, 2005; Schmidt et al., 2005; Ziemssen and Ziemssen, 2005; Wegner, 2005; Merkler et al, 2006). Thus targeting key elements of the immunological cascade that culminate in neural and glial tissue damage, may offer a number of advantages over currently available treatment strategies (Kust et al., 2006; Li and Schluescener, 2006; Li et al., 2006; Muthian et al., 2006; Vogler et al., 2006).

Glatiramer acetate (GA), an approved drug for the treatment of MS, displays a variety of immunomodulatory effects, decreasing the intensity and frequency of the

episodes (Aharoni et al., 2003; Gilgun-Sherki et al., 2003). Although its mechanism of action is not completely known, the inhibition of CNS myelin antigens by competing as a MHC ligant may be of importance (Racke et al., 1992; Teitelbaum et al., 1992; Fridkis-Hareli et al., 1994). Also, the ability to stimulate proliferation of Th2 over Th1 cells, suppressing T-cell activity is of relevance as well (Teitelbaum et al., 1997). However, it is possible that GA may act directly on neurons and axons and glial cells. Such hypothesis has been explored in just a few studies dealing with optic nerve injury, glutamate toxicity and in motoneuron desease models (Kipnis et al., 2000; Schori et al., 2001; Angelov et al., 2003)

Nevertheless, the impact of GA on the nervous tissue, especially on neurons during the course of the disease, is virtually unknown. Based on the fact that GA influences cell proliferation as well as neurotrophic factor synthesis (Ziemssen et al., 2002), it is possible that it might have a role in synaptic elimination/retraction, changing and reducing the seriousness of the course of the disease.

Taking into account the possible influence of GA on plastic events in the spinal cord during the exacerbation of EAE, the present work investigated the synaptic elimination process as well as the glial reaction in the spinal cord during the different stages of the disease, using treatment on a daily basis with the drug.

Material and Methods

Animals

Forty adult female Lewis rats, seven weeks old (~250g of body weight), were obtained from the Multidisciplinary Centre for Biological Investigation (CEMIB/UNICAMP) and were housed using a 12h light/dark cycle with free access to food and water. The Institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, proc. 794-2) approved the study, and the experiments were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Induction of EAE

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) except those specifically mentioned. The rats were immunized with a single injection of 100µl of guinea pig myelin basic protein (MBP, 25µg) associated with heat-inactivated *Mycobacterium tuberculosis* H37RA (2.0 mg/ml; Difco Laboratories, Detroit, MI) and complete Freund's adjuvant emulsion. This solution was injected subcutaneously in the footpad of the animals' hind legs. A daily analysis and scoring of the signs and symptoms of the severity of EAE was carried out as follows: grade 0, no clinical signs; grade 1, tail weakness or paralysis; grade 2, hind leg paraparesis; grade 3, hind leg paralysis; grade 4, complete paralysis (tetraplegy).

Glatiramer acetate treatment

GA from batch 334190 was used throughout the study. GA treatment was performed daily by subcutaneous injections (0.5mg/animal/day) in phosphate buffer) for 2 weeks. GA treatment (n=10) was started thirty minutes after the procedures for EAE induction, being repeated daily by subcutaneous injections containing: GA solution (treated group) and placebo (placebo group). The animals were sacrificed 2 weeks after the beginning of treatment, and processed for analysis by immunohistochemistry and electron microscopy. The alpha motoneurons present in the spinal cord lumbar intumescence were used for the synaptic covering investigation. The results were compared to those from placebo treated (n=10), non-treated EAE induced (n=10) and normal animals (n=10). In all cases, the spinal cords were processed for immunohistochemical (n=5 for each group) and ultrastructural analyses (n=5 for each group).

Specimen Preparation

Following the pre-determined survival periods, the animals were anaesthetized with a mixture of Kensol (xylasine, Köning, Argentina, 10 mg / Kg) and Vetaset (Cetamine, Fort Dodge, USA, 50 mg / Kg) and the vascular system was rinsed by transcardial perfusion with phosphate buffer (pH 7.4). For the immunohistochemical detection of synaptophysin, glial fibrillary acidic protein (GFAP) and F4/80, the rats were fixed by vascular perfusion with 4% paraformaldehyde in phosphate buffer (pH 7.4). Thereafter, the lumbar intumescence was dissected out, post-fixed overnight and then frozen. Cryostat transverse sections of the spinal cords was obtained and transferred to

gelatine-coated slides, dried at room temperature for 30 min and stored at -20ºC until analysed. For electron microscopy, 100 ml of a fixative containing 2.5% glutaraldehyde and 0.5% paraformaldehyde in phosphate buffer (pH 7.4) were perfused through the ascending aorta. The lumbar spinal cords were removed and stored overnight in the same fixative at 4°C. The specimens were then trimmed, osmicated, dehydrated and embedded in Durcupan (Fluka, Steinheim, Switzerland). Ultra-thin sections from the L4-L6 segments were collected on formvar-coated copper grids, contrasted with uranyl acetate and lead citrate, and examined under a Leo906 transmission electron microscope operated at 60 KV.

Immunohistochemistry

Transverse sections of the spinal cord (12µm-thick) were cut in a cryostat (Micron) and incubated with the following primary antibodies: human anti-synaptophysin (Dako, 1:100), goat anti-GFAP (Santa Cruz, 1:200) and rat-anti-F4/80 (Peninsula, 1:1000). Sections were incubated overnight in a moist chamber at 4°C. The primary antiserum was diluted in a solution containing BSA and Triton X in 0.01M PBS. After rinsing, the secondary antibodies were applied and incubated for 45 minutes, according to the primary antibody hosts (CY-2 and CY-3, Jackson Immunoresearch; 1:250). The sections were then rinsed in PBS, mounted in a mixture of glycerol/PBS (3:1) and observed with a laser scanning confocal microscope (Bio-Rad), mounted on an Axiovert 135 inverted microscope (Zeiss, Germany). The excitation wavelength was 488nm for CY2 fluorescence and 568nm for CY3. For quantitative measurements of synaptophysin and GFAP and F4/80 immunoreactivity, alternate images of the L5 ventral horn were

captured at a final magnification of (x 400), with the microscope settings being kept the same for all slides. Quantification was performed with the enhance contrast and density slicing feature of the NIH Image Software (Version 1.55; NIH, Bethesda, MD, USA). The integrated density of six areas was measured in three alternate sections of each specimen according to the method described by Emirandetti et al. (2006). The mean and standard deviation were calculated for each group and a statistical analysis was performed.

Analysis of inflammation and demyelination during the course of EAE

The Sudan Black technique was used to demonstrate the demyelination process during disease development. For this purpose, frozen sections were left at room temperature, rinsed with 0.01M PBS and stained with Sudan Black in 70% ethanol solution for 30 min. The specimens were differentiated in 50% ethanol, rinsed with distilled water and mounted in a mixture of glycerol/PBS (3:1).

The influx of lymphocytes close to the spinal motoneurons was studied on semi-thin toluidine blue stained sections, obtained prior to the ultra-thin sections.

Analysis of the Ultra-thin Sections

Neurons with large cell bodies (average of 25 μm in diameter) found in the sciatic motoneuron pool and cut in the nuclear plane, were identified as alpha motoneurons from the presence of C-type nerve terminals. The surface of the cells was then sequentially digitalized at a magnification of (x 10,000) using a video camera connected to a computerized system and the acquisition feature of the Kontron KS300 software (Zeiss). The images were then mounted together using the a vectorial software. Synaptic terminals apposing the motoneuron somata were identified and their numbers per 100μm of cell membrane and the membrane covering of all terminals in percent of membrane length, calculated using the measurement tool of the Image Tool software (Version 3.0, The University of Texas Health Center in Santo Antonio, USA). The terminals were classified as type F (with flattened synaptic vesicles), type S (with spherical synaptic vesicles) and type C (with a subsynaptic cistern), according to the nomenclature by Conradi and Skoglund (1969). The distance between consecutive nerve terminals covering the motoneurons was also determined. A total of 40 sciatic motoneurons (2 neurons/animal in four groups of 5 animals; normal, EAE induced - degree 3 / exacerbation, EAE induced and GA treated and EAE induced and placebo treated) were studied in this way. Only large neurons present in the sciatic motor pool and presenting at least one C-terminal were selected for the ultrastructural analysis

Statistical analysis

The data were analysed by ANOVA and the two-tailed Student t test at $p<0.05$ (*) and $p<0.01$ (**).

Results

Preserved synaptophysin immunolabelling and increased glial reaction after GA treatment

Fig. 1 shows the synaptophysin and GFAP and F4/80 labelling. Quantitative measurements were made in the spinal cord motor nuclei of all experimental groups and are presented in Fig. 2. The basal immunolabelling was obtained from normal specimens, showing relatively intense synaptophysin staining (11.54±0.98; mean integrated density of pixels) and rather low levels of GFAP (1.87±0.18) and F4/80 (4.45±0.14). The exacerbation of EAE led to a significant decrease in synaptophysin expression in both untreated (2.77±0.10) and placebo-treated animals (2.08±0.29). Nevertheless, the GA treatment resulted in a marked preservation of synaptophysin (6.44±0.45), especially in the motoneuron surroundings, indicating a decreased loss of afferents, this observation being coupled with the fact that GA treated animals displayed a milder form of the disease (Fig. 3G).

Surprisingly, the decrease in input elimination after GA treatment was not paralleled with a significant reduction in macro and microglial reaction. In this sense, the GA treatment did not reduce GFAP (9.81±1.06) and F4/80 (11.69±0.96) labelling, which displayed similar levels to that of the placebo (GFAP – 10.01±0.80; F4/80 – 11.77±0.70) and non-treated EAE induced specimens (GFAP – 8.24±0.61; F4/80 – 12.98±0.71). The basal levels of GFAP (1.87±0.18) and F4/80 (4.45±0.14) were statistically lower than those of the experimental groups.

Treatment with GA results in a milder form of EAE

The degree of myelination of the white matter in the spinal cord was studied in Sudan Black stained sections of lumbar spinal cord in normal, placebo and GA treated animals, at the exacerbation stage of the disease (Fig. 3). The degree of demyelination was minimum in placebo treated animals at the stage of paraplegia. GA treated rats presented lower scores and milder clinical signs of the disease and did not reach paraplegia. Also, axonal myelination, although reduced in comparison to normal material, was more preserved than in the placebo group.

Interestingly, GA treated specimens displayed an influx of immune cells, as seen in the toluidine blue stained material. Nevertheless, inflammation in placebo treated rats as well as in untreated rats subjected to EAE, was significantly more prominent. Representative pictures from normal, placebo and GA treated sections are shown in Fig. 3.

GA treatment preserves the synaptic terminals during the course of EAE

Several synaptic changes have been identified at the ultrastructural level during the course of the disease in positive control rats. With respect to the alpha motoneurons in the spinal cord, it was possible to observe chromatolysis, cell nucleus displacement and retraction of synapses from the cell body. GA treatment reduced such signs, and a greater number of terminals in apposition to the motoneuron cell surface were evident. Representative examples showing the morphology of motoneurons and pre-synaptic terminals at the dorsal lamina IX can be seen in Fig. 4 A-C.

In addition to qualitative observations, a detailed analysis of the afferents of these neurons was carried out and the synaptic covering as well as the number and type of boutons studied. The total covering of the F, S and C terminals is presented in Fig. 4D. A statistically significant preservation of inputs was observed after GA treatment, parallel to a reduction in the maximum score reached after immunization. It is important to emphasize, however, that after treatment the covering remained lower than in the normal situation. A relevant finding is the positive effect of the treatment for both excitatory as well as inhibitory terminals (Fig. 4 D-G). With respect to this, GA administration preserved the mean length of apposition as well as the number of excitatory and inhibitory inputs (Fig. 4 E-G).

Pattern of button elimination induced by EAE inflammation

To investigate whether there was a quantitative change in the pattern of synaptic elimination, an analysis of the frequency distribution of terminals apposed to the motoneuron membrane was carried out. A particular feature of the synaptic elimination process is that the retraction of boutons is not by chance, but rather a finely organized event. In this sense, synaptic removal after lesion tends to preserve clusters of terminals at specific points along the cell membrane of injured neurons. This feature is evidenced by the fact that in a normal situation, a number of boutons can be found close together. After injury, however, there is a tendency to increase the distance between groups of terminals. This occurrence may be studied by observing the frequency distribution of the gaps between boutons. Considering this concept, during exacerbation of the disease, the placebo treated animals displayed an increase in larger

intervals, greater than 4µm, exposing, on average, an additional 35% of motoneuron membrane. Interestingly, the GA treatment that preserved more terminals in apposition to the motoneurons, resulted in a distribution of gaps strikingly similar to that of normal animals (Fig. 5).

Discussion

The present study strengthens the concept that maintenance of the afferents of spinal motoneurons may positively influence their functionality during the course of EAE. This hypothesis is based on the observation that, during the course of the disease, a series of plastic phenomena involving alpha motoneuron afferents correlate with a worsening of the symptoms of the disease (Marques et al., 2006). Also a highly plastic response of the motor system after neuroinflammatory lesion was reported by Kerschensteiner, et al 2004. In this sense, axonal remodelling at multiple levels can´t be observed, including the surrounding area to the lesion, and above as well as in the motor cortex (Kerschensteiner, et al 2004).

At present, the treatment of the remittent/recurrent form of multiple sclerosis is based on the administration of glatiramer acetate and beta interferon (Dhib-Jalbut et al., 1998; Polman and Uitdehaag, 2003; Gold et al., 2006). Although both are approved drugs for MS treatment, GA has the unique characteristic of being a synthetic polymer composed of L-alanine, L-glutamine, l-lysine and l-tyrosine, exerting a marked suppressive and protective effect on EAE in various animal species (Dhib-Jalbut et al., 1998). Interestingly, although GA is not encephalitogenic, its therapeutic effect is related to the induction of both cellular and humoral immune responses in experimental models and in humans. Currently, its therapeutic effects are attributed to the induction of specific Th2 suppressor cells that are able to release, upon an encephalitogenic stimulus, anti-inflammatory cytokines that minimize the progression of the disease (Dhib-Jalbut et al., 1998). However, apart from such an immunological role, it is possible that GA acts directly on the nervous tissue, stimulating the release of trophic and

neurotrophic factors by the glial cells, influencing the response to the inflammatory injuries caused by the autoimmune response. In this sense, it has been reported that GA induced an elevation of BDNF, TGFβ, IL-4 and 10 as well as suppressing TNFα and INFγ (Aharoni et al., 2003; Hammarberg et al., 2000; Ziemssen et al., 2002).

The above-mentioned effects of GA may directly influence the synaptic stability of the spinal cord circuits, which are perturbed during exacerbation of the disease. An analysis of the results revealed by immunohistochemistry in the present study, showed that the GA treatment significantly preserved synaptophysin labelling in the spinal cord during the period in which the placebo treated animals presented a great reduction in such immunolabelling. This observation, coupled with the decrease in clinical scores in the GA treated animals, indicated a correlation between synaptic activity and the degree of seriousness of the disease. Strikingly, the treatment with GA did not reduce the glial reaction in the surroundings of the alpha motoneurons, as seen by the GFAP and F4/80 immunolabelling, as well as at the ultra-structural level. This fact strengthens the idea that a certain degree of inflammation was still present, and was reinforced by the elevated number, although significantly smaller than that in the placebo group, of immune cells, presumably mononuclear cells, both in the white and grey matter.

Another relevant observation was obtained with the Sudan Black staining that revealed the presence of demyelination in the GA treated animals. This indicates that the disease was still progressing, although in a milder way than in the placebo treated animals. In this sense, it is acceptable that part of the beneficial effects of the treatment is a result of the direct stabilization of the inputs in the spinal cord microenvironment.

The results discussed above are also reinforced by the ultrastructural analysis of the afferents in contact with the alpha motoneurons during the course of the disease. Such data indicated that the GA treatment decreased retraction of both excitatory and inhibitory synapses to a level close to normality. In this sense, the total synaptic covering seems to have been preserved, apart from the relatively high reactive gliosis, indicating a direct influence of GA on the synaptic elimination process. The administration of GA also positively influenced the clustering pattern of the terminals, which was preserved, in contrast to the placebo treated condition (Cullheim et al., 2002). Taking into account that a motoneuron receives up to 100,000 inputs from various sources, the maintenance of active synapses is of crucial importance to the correct functionality of the motor pathway.

In summary, the results presented in this study demonstrated that the immunomodulator GA not only possesses anti-inflammatory effects, but also directly influences the plasticity of neural elements in the spinal cord. An interpretation of this fact is the possible GA induced expression of neurotrophic factors, such as BDNF, NT-3, and NT-4 (Aharoni et al., 2003). This hypothesis may be of relevance in the development of new strategies for the treatment of MS, using or combining drugs capable of acting directly on the synapses, increasing the stability of the neural circuits, which, in turn, may significantly reduce the clinical signs of neurodegenerative diseases.

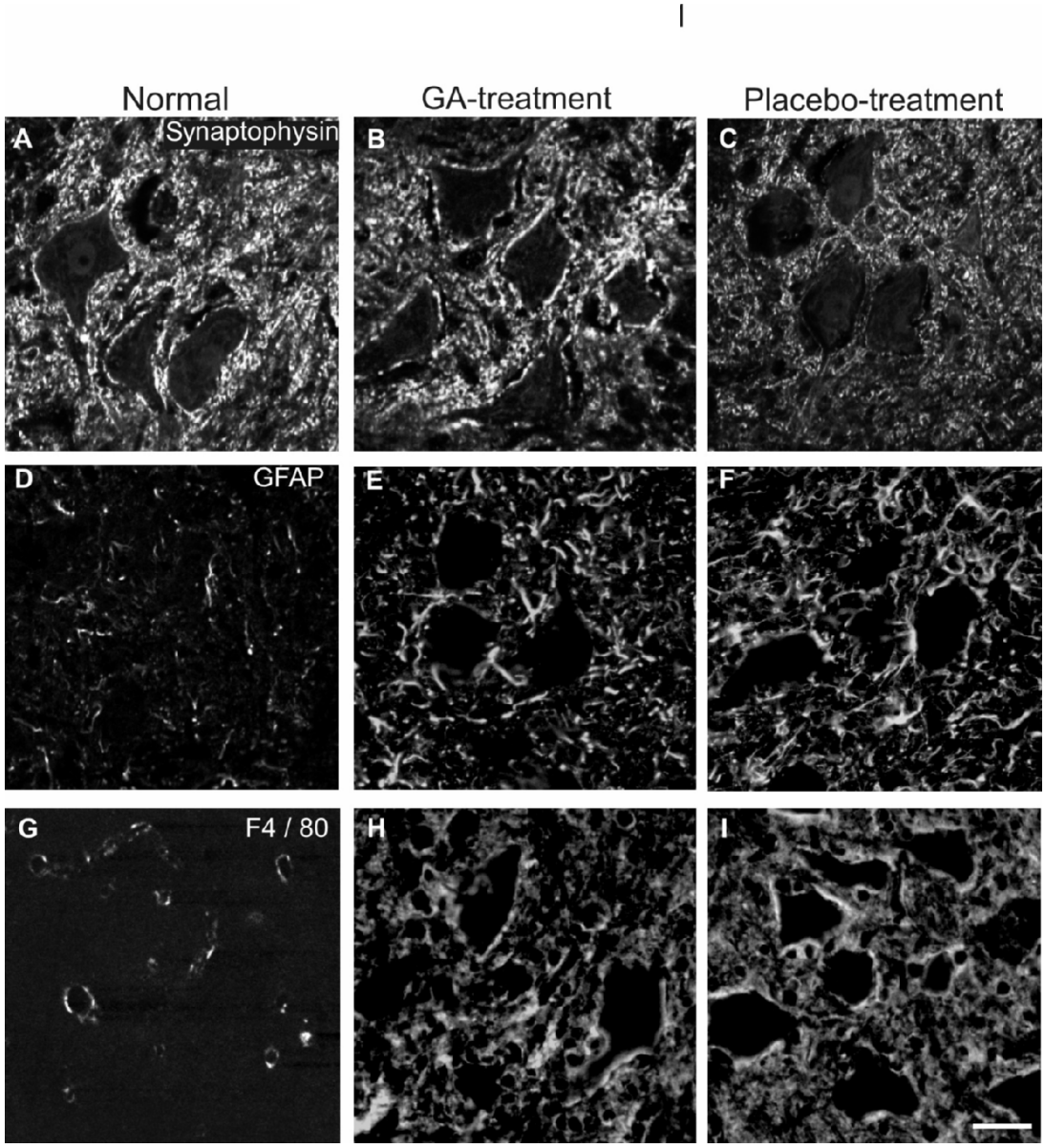

Figure 1 - The preservation of synaptophysin and increase in GFAP and F4/80 during the EAE peak, followed by glatiramer acetate treatment. A - C - immunolabelling against synaptophysin in the normal (A), EAE + GA (B) and placebo treated (C) animals. D - F - immunofluorescence micrographs showing astrocyte reactivity in the normal (D), EAE +GA (E) and placebo treated (F) animals. G - I - immunofluorescence micrographs showing microglial reactivity in the normal (G), EAE + GA (H) and placebo treated (I) groups. Scale bar = 35 μm.

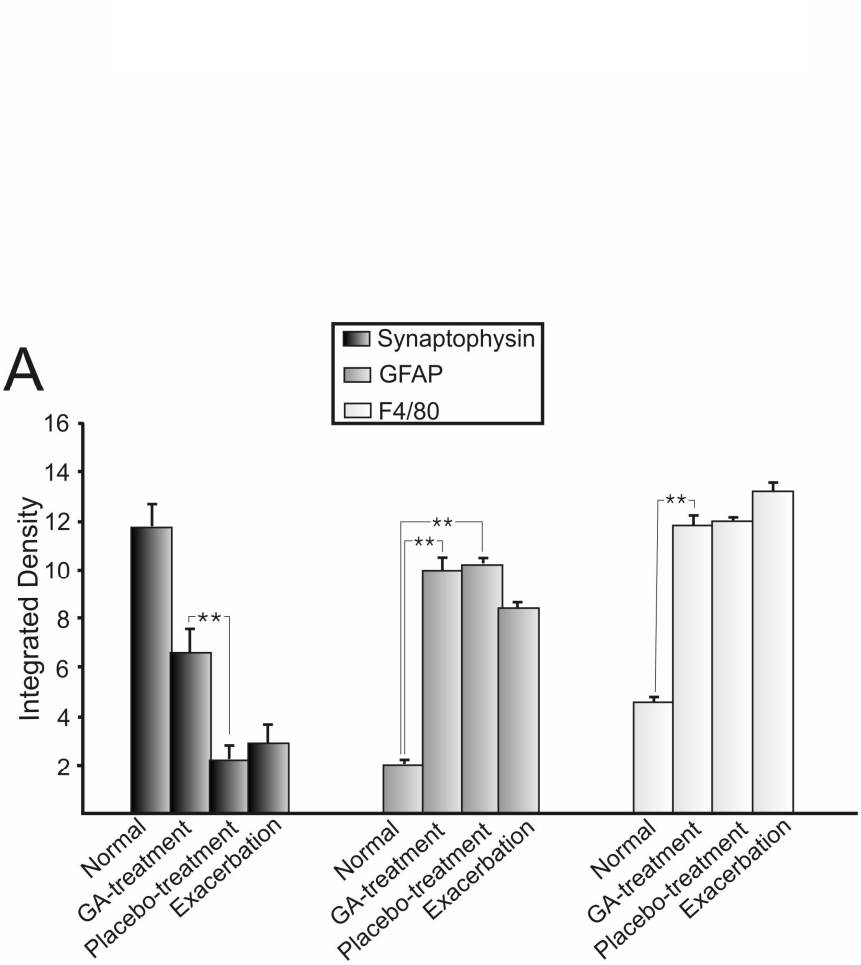

Figure 2 - The quantitative evaluation of the levels of immunoreactivity under the different experimental conditions, depicting the maintenance of synaptic activity after GA treatment. However, a significant increase in glial response in the lumbar spinal cord was still present during treatment.

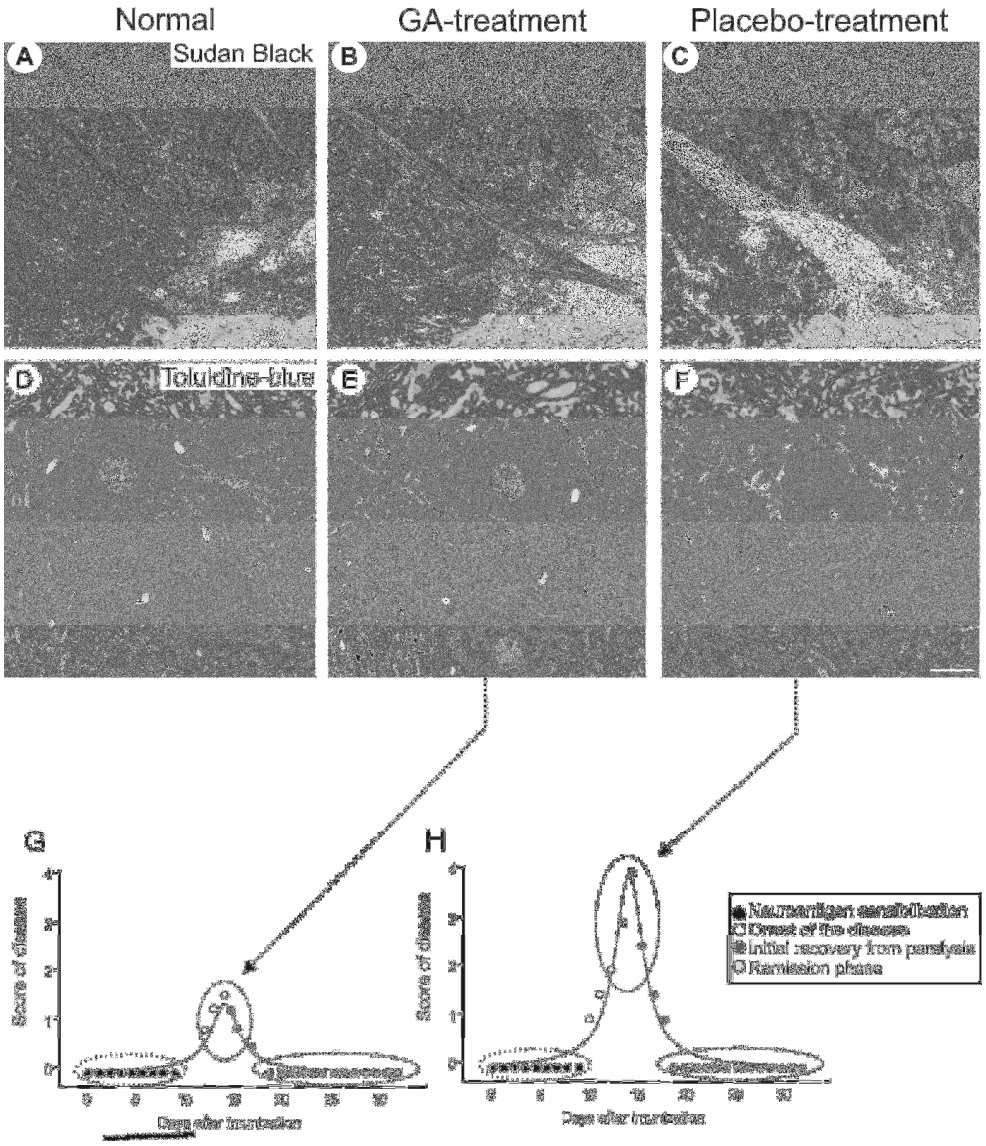

Figure 3 - Correlation between demyelination/inflammation and the clinical scores for EAE. A-C - Sudan Black staining at the ventral horn of the spinal cord showing the degree of myelination in normal (A), GA-treated (degree 1, B) and placebo-treated (C) animals. Note that GA preserved a significant degree of myelination. The placebo-treated material showed evident signs of demyelination. D-F - Toluidine blue stained semi-thin sections obtained from the different experimental situations. Observe the influx of immune cells in the vicinity of the motoneurons (F). Normal (D) and GA-treated specimens (E) are also shown. Scale bar A-C = 35µm, D-F = 15µm. G. The graphs in G and H represent schematic illustrations of the time course of the experimental autoimmune encephalomyelitis and the correlation between the clinical scores after GA and placebo treatment. The peak of the disease occurred around 14 days after immunization.

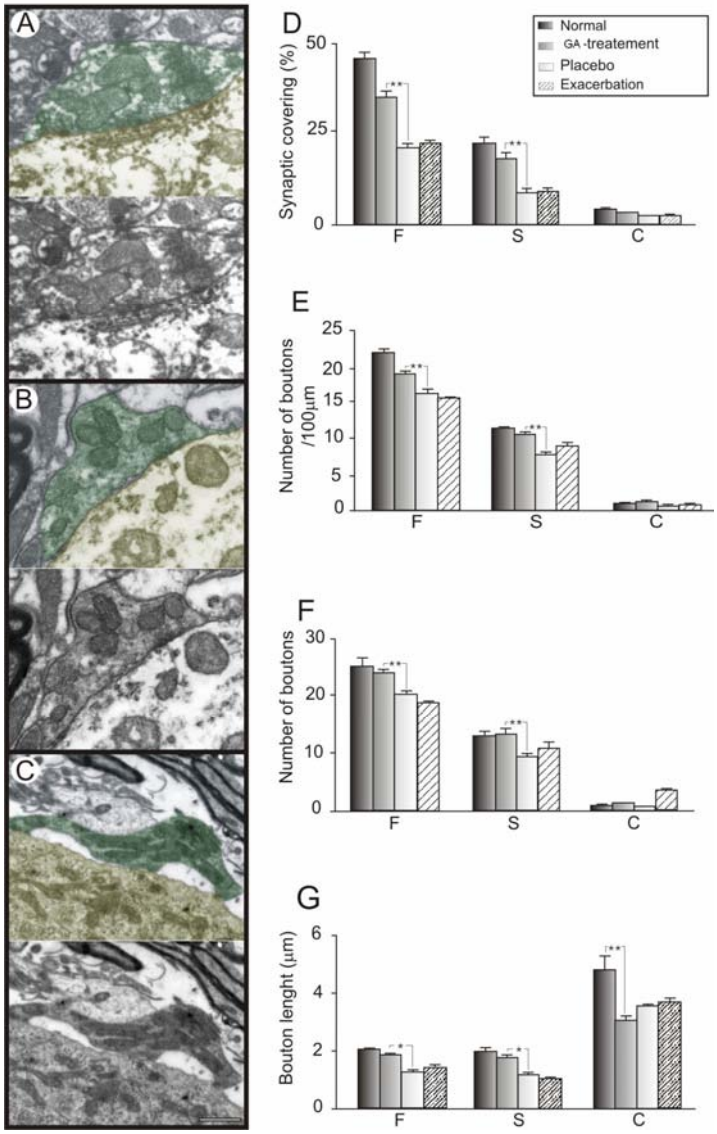

Figure 4 – A-C - Electron micrographs from the surface of a motoneuron, showing the nerve terminals in apposition to the plasma membrane. A represents the normal covering of a control rat. B is an example of a GA-treated specimen - note the normal apposition of the nerve terminal. C - Micrograph of a motoneuron in the placebo-treated situation, showing an almost fully retracted terminal. Scale bar = 0.5 µm. D**-**G **-** Detailed analysis of the synaptic covering and of the number of synaptic boutons after GA treatment, revealing the positive effects of GA in comparison to the placebo group. D - graph showing the preservation of both F- and S-boutons after GA treatment. E and F – Preservation of both inhibitory and excitatory terminals by GA-treatment in both absolute and relative (number of boutons / 100 µm of membrane) numbers. G. The button length of the F-, S- and C-terminals under the different experimental circumstances.

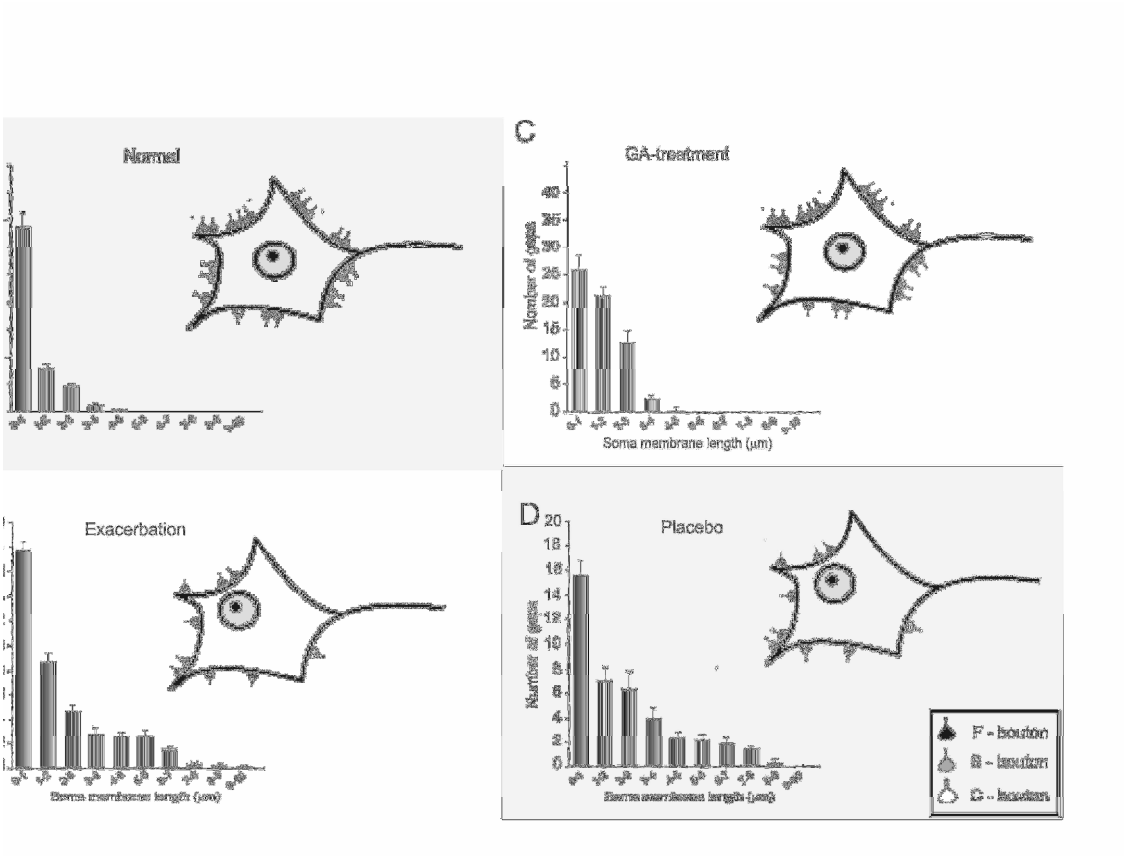

Figure 5 - The distribution of the gap length between clusters of terminals apposing the cell soma membrane was close to normality after GA treatment. A - Normal distribution of intervals between nerve terminals showing that the boutons were organized in clusters of inputs. B - The distribution of terminals during the exacerbation of the disease (degree 3). C - Treatment with GA keeps the clusters of terminals stable regardless of the increased surrounding glial reaction. D – Placebo treatment, which was similar to the positive control (degree 3 of the disease).

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CAPÍTULO 3

CONCLUSÕES GERAIS

- Os dados apresentados neste estudo indicam que ocorre intensa plasticidade sináptica na medula espinhal durante o curso da EAE – modelo experimental da esclerose múltipla.
- Evidenciou-se que a reaposição sináptica juntamente com a reatividade glial desempenhe papel importante no déficit motor e na recuperação após o surto da EAE.
- Os eventos de plasticidade sináptica apresentados pelos motoneurônios durante o curso da doença são semelhantes aos observados após axotomia de um nervo periférico.
- O tratamento com acetato de glatirâmer diminui a retração de sinapses excitatórias e inibitórias a um nível próximo ao da normalidade. Assim, a cobertura sináptica total dos motoneurônios é preservada.
- Os resultados apresentados neste estudo demonstram que o imunomodulador acetato de glatirâmer possui não somente efeitos anti-inflamatórios, mas também influencia diretamente na plasticidade de elementos neurais da medula espinhal.

CAPÍTULO 4

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CAPÍTULO 5

ANEXOS

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha tese de doutorado intitulada Plasticidade sináptica em motoneurônios alfa medulares de animais submetidos à encefalomielite autoimune experimental:

( ) não se enquadra no Artigo 1º, § 3º da Informação CCPG 002/06, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo nº_____), intitulado _____

(x) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo nº 794-2).

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (?) (Protocolo nº_____).

Aluna: Karina de Brito Marques

Orientador: Prof. Dr. Alexandre Leite Rodrigues de Oliveira

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Nome: Profa. Dra. ANA MARIA A. GUARALDO
Função: Presidente
Comissão de Ética na Experimentação Animal
CEEA/IB - UNICAMP