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EFEITOS DO EXERCÍCIO FÍSICO SOBRE A MORFOFISIOLOGIA DOS ASTRÓCITOS IMUNORREATIVOS PARA A PROTEÍNA GLIAL FIBRILAR ÁCIDA (GFAP) NO HIPOCAMPO DE RATOS WISTAR

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Efeitos do exercício físico sobre a morfofisiolgia dos astrócitos imunorreativos para a Proteína Glial Fibrilar Ácida (GFAP) no hipocampo de ratos Wistar

Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul, como requisito para obtenção de grau de Mestre.

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RESUMO

Diversos estudos demonstram que a prática de atividade física diária tem efeitos benéficos sobre a função cerebral, incluindo melhora da cognição e dos processos de aprendizagem e memória, além de apresentar efeitos neuroprotetores. Um dos mecanismos responsáveis por esses efeitos benéficos é a influência que o exercício físico exerce sobre a plasticidade neural. Neste sentido, o hipocampo é uma região encefálica especialmente importante nos processos de formação de novas memórias e caracterizado como um dos principais locais do encéfalo onde observamos neurogênese em fase adulta. Os astrócitos são importantes células da glia, fundamentais para o metabolismo energético neuronal, regulando a concentração de várias moléculas e assim como os neurônios, acredita-se que participem ativamente da função sináptica. Além disso, os astrócitos são células suscetíveis à plasticidade induzida por estímulos ambientais, como o exercício físico. O objetivo deste estudo foi investigar se o exercício físico é capaz de alterar a imunorreatividade para a Proteína Glial Fibrilar Ácida (GFAP), a densidade e a morfologia dos astrócitos GFAP positivos, do *stratum radiatum* da região CA1 do hipocampo de ratos Wistar. Treze ratos machos foram divididos em 2 grupos: Sedentário (n=6) e Exercício (n=7). Os animais do grupo exercício foram submetidos a 4 semanas de exercício físico diário em esteira durante 30 minutos por dia. A imunorreatividade para GFAP foi avaliada por densitometria óptica e a análise da ramificação astrocitária foi realizada por uma adaptação do método dos círculos concêntricos de Sholl. Os resultados obtidos demonstram que o exercício físico foi capaz de aumentar a densidade de astrócitos GFAP positivos, bem como a expressão regional e celular de GFAP. Além disso, os astrócitos alteraram sua morfologia em resposta ao exercício físico, o que foi demonstrado pelo aumento no grau de ramificação dos astrócitos nos quadrantes laterais e no comprimento dos processos astrocíticos nos quadrantes centrais. Estes achados demonstram importantes alterações astrocitárias após o exercício físico, corroborando a ideia de que estas células estão envolvidas na regulação da atividade neural e da plasticidade.

Palavras-chave: Exercício físico, Astrócitos, GFAP, Hipocampo, Sholl

ABSTRACT

A wide variety of studies have demonstrated that physical activity has beneficial effects on brain function, including the improvement of cognition, the enhancement of learning and memory processes, and also displays neuroprotective effects. One of the mechanisms responsible for these beneficial effects is the influence that exercise has on brain plasticity. In this sense, the hippocampus is a brain region particularly important in the formation of new memories and featured as one of the main structures of the brain where we observe neurogenesis in adulthood. The astrocytes are important glial cells, critical for neuronal energy metabolism by regulating the concentration of various molecules and as neurons, are believed to participate actively in the synaptic function. Furthermore, astrocytes are susceptible to plasticity induced by environmental stimuli such as physical exercise. The aim of this study was to investigate whether physical exercise can alter the immunoreactivity for Glial Fibrillary Acidic Protein (GFAP), density and morphology of GFAP positive astrocytes, in the stratum radiatum of the CA1 region of the rat hippocampus. Thirteen male rats were divided in two groups: Sedentary (n=6) and Exercise (n=7). The animals in the exercise group were submitted to a protocol of 30 minutes of daily physical exercise on a treadmill for 4 consecutive weeks. GFAP immunoreactivity was evaluated using optical densitometry and the analysis of the astrocyte ramification was done using an adaptation of Sholl's concentric circles method. The results show that physical exercise is capable of increasing the density of GFAP positive astrocytes as well as the regional and cellular GFAP expression. In addition, physical exercise altered astrocytic morphology as shown by the increased degree of ramification observed in the lateral quadrants and in the length of the longest astrocytic processes in the central quadrants. This data demonstrate important changes in astrocytes promoted by physical exercise, supporting the idea that these cells are involved in regulating neural activity and plasticity.

Keywords: Physical exercise, Astrocytes, GFAP, Hippocampus, Sholl

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CAPÍTULO 1

INTRODUÇÃO E OBJETIVOS

1 INTRODUÇÃO

1.1 GLIA E ASTRÓCITOS

Duas grandes classes de células são descritas no encéfalo – os neurônios e as células gliais (Verkhratsky e Butt, 2007). O conceito de "neuroglia" foi inicialmente introduzido em 1858 por Rudolf Ludwig Karl Virchow. Rudolf Virchow foi um dos patologistas mais influentes do século 19, e um dos criadores da teoria celular e da patologia celular (Verkhratsky e Butt, 2007). Para Virchow, a neuroglia era um material conjuntivo que mantinha as células nervosas unidas, apesar de admitir que este material também continha certo número de elementos celulares (Kettenmann e Verkhratsky, 2008).

Novas descobertas no campo da origem celular das células gliais resultaram dos esforços de muitos histologistas importantes, em particular Camillo Golgi (1843-1926), Santiago Ramón y Cajal (1852-1934) e Pio Del Rio Hortega (1882-1945) (Oberheim et al., 2012; Parpura et al., 2012; Verkhratsky e Butt, 2007). Camillo Golgi descreveu a enorme diversidade de células gliais do encéfalo, enquanto Ramon y Cajal estudou os astrócitos e a sua interação com outros elementos neurais e, por sua vez, Del Rio Hortega propôs os termos microglia e oligodendrócitos para caracterizar estas populações celulares distintas (Verkhratsky e Butt, 2007; Verkhratsky e Parpura, 2010). As células neurogliais do sistema nervoso central (SNC) incluem os astrócitos, oligodendrócitos, e microglia. Seus pares no sistema nervoso periférico (SNP) são as células de Schwann (Aschner et al., 1999).

Os astrócitos ("células estreladas") são as células gliais mais numerosas e diversificadas no SNC (Theodosis et al., 2008). Alguns astrócitos, de fato, tem uma aparência de estrela, com vários processos primários originando-se a partir do soma, mas eles também podem apresentar-se de formas distintas (Verkhratsky e Butt, 2007). A primeira sub-divisão dos astrócitos, realizada por Rudolf Albert von Kolliker e William Lloyd Andriezen, classificou essas células com base em sua localização e morfologia celular (Oberheim et al., 2012; Parpura et al., 2012; Verkhratsky e Parpura, 2010). Os astrócitos fibrosos preenchem a substância branca, normalmente têm contornos regulares e processos cilíndricos, obtendo-se o mais clássico exemplo de "aparência de estrela", além disso, estes astrócitos têm densos filamentos gliais que são imunorreativos para a Proteína Glial Fibrilar Ácida ("Glial Fibrilary Acidic Protein" – GFAP), proteína componente dos filamentos intermediários. Os astrócitos protoplasmáticos, por sua vez, preenchem a substância cinzenta e têm processos mais irregulares e poucos filamentos imunorreativos para GFAP (Kimelberg e Nedergaard, 2010; Molofsky et al., 2012; Oberheim et al., 2012, **Figura 1**).

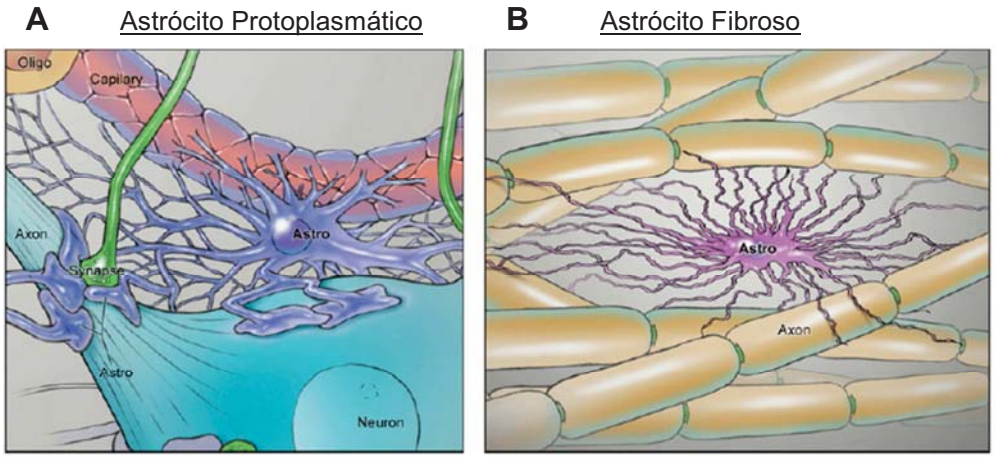

Figura 1: Desenho esquemático representando a morfologia astrocitária. A – Representação de um astrócito protoplasmático e suas respectivas interações com um neurônio e um capilar sanguíneo, formando a "unidade neurovascular", destacando o papel dos astrócitos na sinaptogênese e na manutenção da barreira hemato-encefálica. B – Representação de um astrócitos fibroso localizado na substância branca, onde este auxilia na indução do processo de mielinização. Figura adaptada de Molofsky et al. (2012)

Os astrócitos representam o principal elemento celular do sistema homeostático no SNC, sendo responsáveis pela maior parte dos aspectos do suporte metabólico, nutrição, regulação da concentração de íons e neurotransmissores no ambiente, manutenção da barreira hemato-encefálica e preservação da integridade dos tecidos após lesão (Catalani et al., 2002; Lo et al., 2003; Petty e Lo, 2002; Park et al., 2003; Theodosis et al., 2008). Evidências recentes também sugerem que os astrócitos influenciam vários aspectos da transmissão sináptica e, assim como os neurônios, participam ativamente do processamento da informação através das sinapses "tripartite" (Perea et al., 2009). A participação dos astrócitos nas sinapses ocorre de acordo com os seguintes processos neuroquímicos: 1 – Os processos astrocíticos contêm uma variedade de receptores de neurotransmissores, incluindo receptores de glutamato, ácido gama-aminobutírico ("γ-Aminobutyric acid" – GABA) e ATP, que permite a rápida percepção dos eventos de

transmissão sináptica; 2 – Os astrócitos respondem a liberação de neurotransmissores, durante a atividade sináptica, com a elevação da concentração intracelular de Ca^{2+}; 3 – Os astrócitos liberam gliotransmissores como glutamato, purinas (ATP, adenosina), GABA e D-serina, em resposta ao aumento na concentração de Ca^{2+} (Porter e McCarthy, 1996; Santello e Volterra, 2009; Verkhratsky e Steinhauser, 2000, **Figura 2**).

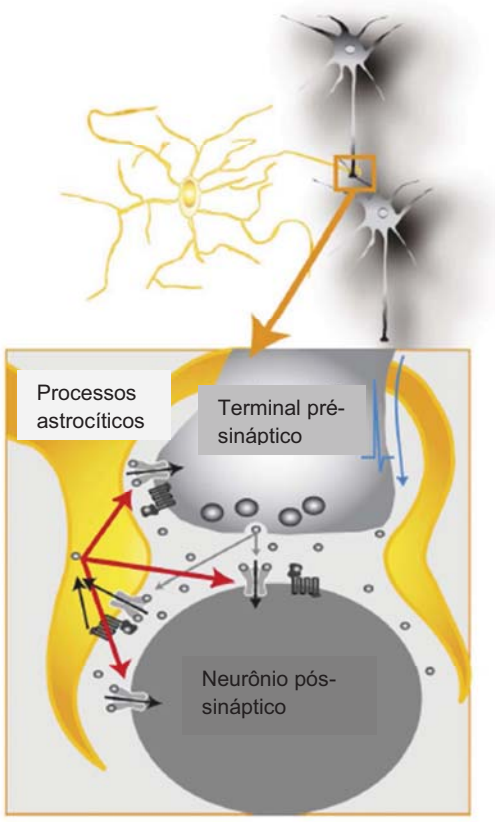

Figura 2: Os processos astrocíticos contribuem para a transmissão sináptica na sinapse "tripartite". O glutamato sinapticamente liberado (setas cinza) estimula receptores ionotrópicos e metabotrópicos nos astrócitos. Isso, então, ativa vias intracelulares astrocitárias (setas pretas), levando à liberação de gliotransmissores (setas vermelhas) que por sua vez agem sobre os receptores pré e pós-sinápticos dos neurônios. Figura adaptada de Theodosis et al. (2008)

Todavia, o papel dos astrócitos na transmissão sináptica ainda é um assunto em discussão, pois em um artigo recente, Sun e colaboradores (2013) demonstraram que, receptores metabotrópicos de glutamato tipo 5, não são expressos no hipocampo e no córtex de humanos e roedores depois de 3

semanas pós-natal e que, em idade adulta ao menos, os astrócitos não respondem a liberação de glutamato dos neurônios. Este dado sugere que a sinalização neuroglial no encéfalo adulto pode ocorrer de um modo essencialmente diferente da que é exibida em idade jovem, indo na contramão do modelo da sinapse tripartite (Sun et al., 2013).

Os astrócitos desempenham um papel na formação, manutenção e eliminação das sinapses durante o desenvolvimento (Ullian et al., 2001). Durante muitos anos, os processos de sinaptogênese, manutenção e eliminação dos contatos sinápticos foram considerados responsabilidade exclusiva dos neurônios. Recentemente, foi demonstrado que as células gliais (astrócitos no SNC e células de Schwann no SNP) controlam o nascimento, a vida e a morte de sinapses. Esses processos constituem a base da adaptação do encéfalo para as constantes mudanças no ambiente externo e são essenciais para processos como aprendizagem e memória (Verkhratsky e Butt, 2007).

Em regiões como o hipotálamo e o hipocampo, foi demonstrado que os astrócitos participam ativamente da plasticidade sináptica (Araque e Navarrete, 2010). Assim como os neurônios, os astrócitos são suscetíveis a plasticidade, e a remodelação dos processos astrocíticos está intimamente associada às alterações morfológicas em neurônios vizinhos (Theodosis et al., 2008). As principais alterações morfológicas observadas nos astrócitos ocorrem em seus processos, os quais podem sofrer mudanças em sua organização estrutural em resposta a estímulos ambientais (Haber et al., 2006). Atividade física e experiências de aprendizado provocam alterações na morfologia astrocitária. Como exemplo, temos o ambiente enriquecido, que altera a morfologia dos

astrócitos do hipocampo de ratos saudáveis aumentando a ramificação, a densidade e o comprimento dos processos astrocíticos (Viola et al., 2009); e o exercício físico, que aumenta a expressão de GFAP e o número de astrócitos GFAP positivos no córtex frontoparietal e no estriado (Li et al. 2005) e estimula a proliferação dos astrócitos na zona subgranular do hipocampo de roedores (Uda et al. 2006).

Um novo conceito da glia como fonte de novas células no encéfalo adulto vem se desenvolvendo gradualmente (Garcia et al., 2004; Pinto e Götz, 2007; Verkhratsky e Butt, 2007). Em regiões como a zona subventricular, a zona dos ventrículos laterais e a zona subgranular do giro denteado do hipocampo, foi demonstrado que as células gliais podem agir como precursores neurais pluripotentes produzindo todos os tipos de células nervosas: de neurônios a glia (Garcia et al., 2004; Pinto e Götz, 2007; Verkhratsky e Butt, 2007).

1.2 **GFAP**

A GFAP é o marcador imunoistoquímico mais confiável e amplamente utilizado para identificação de astrócitos (Catalani et al., 2002; Sofroniew e Vinters, 2010). A GFAP é um membro de uma família de proteínas dos filamentos intermediários, que incluem ainda a vimentina, a nestina e outros, que servem, em grande parte, para as funções cito-arquitetônicas, como a manutenção da integridade do citoesqueleto e nos processos de extensão e

retração das ramificações astrocitárias (Pekny e Pekna, 2004; Steffek et al., 2008).

A capacidade dos astrócitos de monitorar e responder às mudanças nas sinapses glutamatérgicas se torna possível, em parte, pelos seus extensos processos de ramificação (Allen e Barres, 2005). Os processos astrocíticos contêm GFAP, cuja função é modular a motilidade e a forma dos astrócitos, fornecendo estabilidade estrutural aos processos astrocíticos, além disso, a montagem e desmontagem do citoesqueleto proporcionam alterações na morfologia dos astrócitos (Rodnight et al., 1997). Experimentos *in vivo* e *in vitro* demonstraram que a ausência de GFAP por deleção ou supressão da expressão desta proteína, leva à diminuição da ramificação, ao funcionamento sináptico irregular, e ao comportamento anormal em roedores (Chen e Liem, 1994; McCall et al., 1996; Shibuki et al., 1996; Weinstein et al., 1991).

No SNC dos vertebrados superiores, após lesão traumática, patológica, ou por alteração genética gerada por insulto químico, os astrócitos tornam-se reativos aumentando a síntese de GFAP, o que resulta na hipertrofia das ramificações gliais em um processo conhecido como astrocitose reativa (Eng et al., 2000). As funções dos astrócitos reativos ainda não são bem compreendidas e tanto efeitos benéficos (por exemplo: formação de uma densa barreira física que protege as regiões do encéfalo intacto do tecido lesado, restringindo a inflamação e protegendo os neurônios e outras células, preservando assim a função do SNC) como prejudiciais (por exemplo: inibição da regeneração axonal) são atribuídos a esse processo (Sofroniew, 2005). Entretanto, o aumento na expressão de GFAP também pode se reflexo de um aumento substancial no metabolismo astroglial e na síntese de proteínas,

consistente com uma hipertrofia celular saudável em resposta ao aumento de exigências fisiológicas (Eddleston e Mucke, 1993).

1.3 **HIPOCAMPO**

A neuroanatomia hipocampal começou a ser desvendada com os estudos pioneiros de Camillo Golgi (1886), Sala Luigi (1891), Karl Schaffer (1892), Santiago Ramón y Cajal (1893) e Rafael Lorente de Nó (1934). Rafael Lorente de Nó dividiu o hipocampo nas áreas CA1, CA2 e CA3 (CA advém de *"cornu ammonis"*, latin para "corno de carneiro", uma comparação da estrutura em arco do hipocampo com um corno de um carneiro e também uma referência ao deus mitológico egípcio Amun Kneph, cujo símbolo era um carneiro), que representam o hipocampo propriamente dito e o giro denteado (Andersen et al., 2007, **Figura 3**).

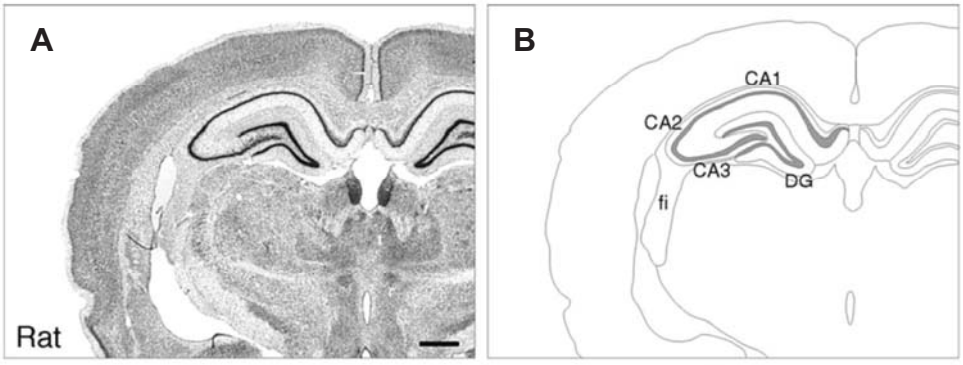

Figura 3: A – Imagem digitalizada da secção transversal de um encéfalo de rato corado com Nissl. B – Desenho esquemático da imagem A mostrando a localização das diferentes áreas do corno de Ammon. Figura adaptada de Andersen et al. (2007)

De acordo com a subdivisão descrita por Lorente de Nó, o hipocampo é estratificado em seis camadas distintas dispostas no sentido dorsal-ventral:

stratum alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunoso moleculare e stratum moleculare (**Figura 4**).

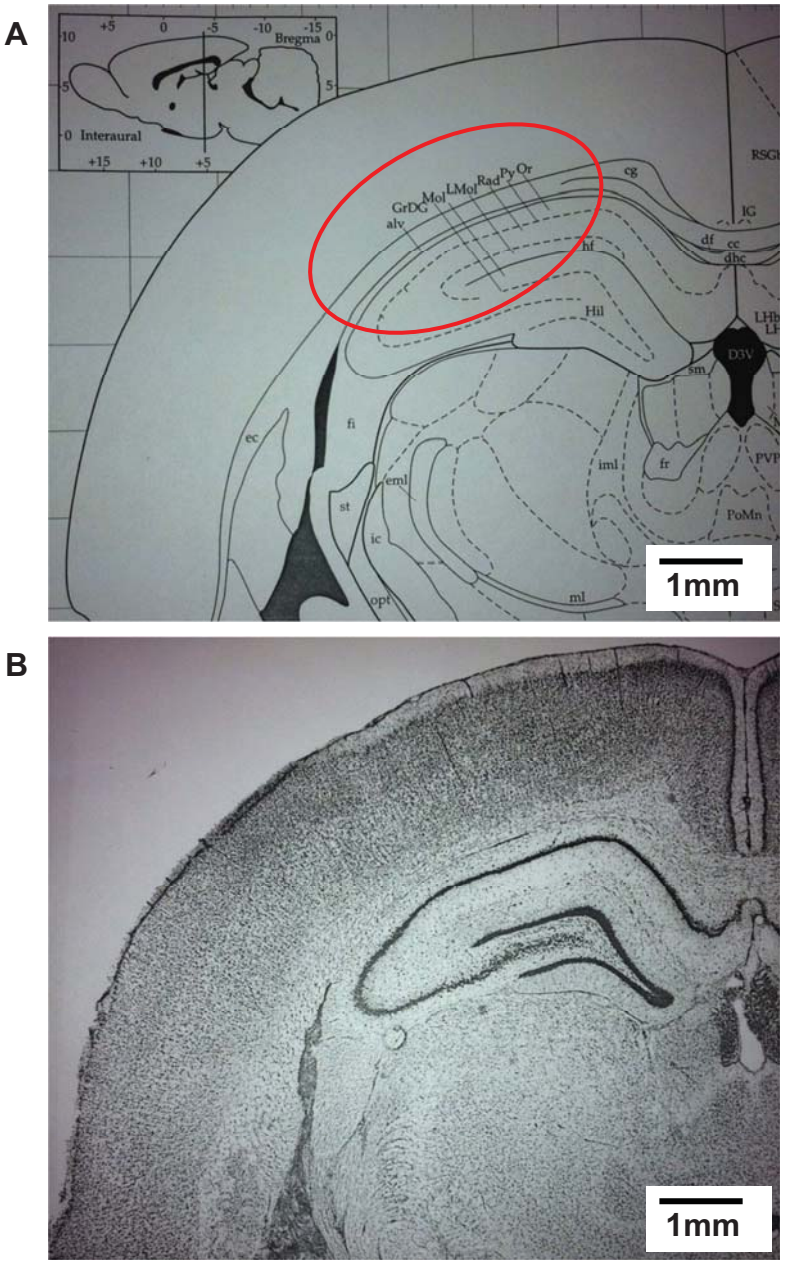

Figura 4: A – Desenho esquemático da secção transversal de um encéfalo de rato mostrando a localização das diferentes regiões descritas por Lorente de Nó. B – Imagem digitalizada de uma secção representativa da mesma região da figura A. Alv= *stratum alveus;* Or= *stratum oriens*; Py= *stratum pyramidale*; Rad= *stratum radiatum*; LMol= *stratum lacunoso moleculare*; Mol=*stratum moleculare* e GrDG=giro denteado. Figuras adaptadas de Paxinos e Watson (1998)

O termo formação hipocampal é diferente do termo hipocampo, o qual compreende as regiões CA e o giro denteado. A formação hipocampal abrange o hipocampo propriamente dito mais o subiculum, presubiculum, parasubiculum (que juntos são chamados de complexo subicular) e o córtex entorrinal. A principal justificativa para a inclusão das regiões citadas acima sob o termo "formação hipocampal" é que elas estão ligadas por um circuito de neurônios conhecido como via tri-sináptica. Essa via conecta uma região a outra de forma sequencial e unidirecional (Andersen et al., 2007). As células nas camadas superficiais do córtex entorrinal dão origem a axônios que se projetam, entre outros destinos, para o giro denteado. As projeções do córtex entorrinal para o giro denteado formam a via perfurante. Da mesma forma, as células granulares do giro denteado, dão origem a axônios, chamados de fibras musgosas, que se ligam às células piramidais da região CA3. As células piramidais de CA3, por sua vez, são a principal fonte de entrada de informação para a região CA1 (os axônios colaterais de Schaffer). A região CA1, em seguida, projeta para o subiculum e para o córtex entorrinal. Além disso, o subiculum se projeta para o presubiculum, parasubiculum e também para o córtex entorrinal (Langston et al., 2010; van Striem et al., 2009, **Figura 5**).

Através dessas conexões, tanto CA1 como o subiculum fecham o ciclo de processamento do hipocampo, que começa nas camadas superficiais do córtex entorrinal e termina em suas camadas mais profundas.

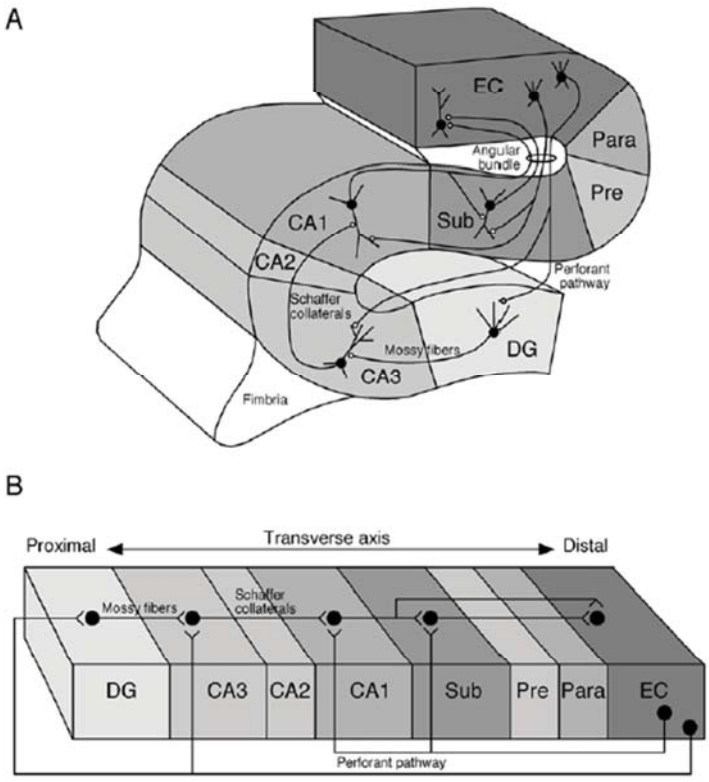

Figura 5: Representação esquemática da formação hipocampal de ratos. A – Os neurônios na camada II do córtex entorrinal projetam-se para o giro denteado, e para a região CA3 através da via perfurante. Neurônios na camada III do córtex entorrinal projetam para a região CA1 e para o subiculum. As células granulares do giro dentado projetam para a região CA3 através das fibras musgosas. Os neurônios piramidais de CA3 projetam para CA1 via colaterais de Schaffer. As células piramidais de CA1 projetam para o subiculum e tanto CA1 como o subiculum projetam de volta para as camadas mais profundas do córtex entorrinal. B – Projeções ao longo do eixo transversal da formação hipocampal. EC=córtex entorrinal; Para=parasubiculum; Pre=presubiculum; Sub= subiculum e DG=giro denteado. Figura adaptada de Andersen et al. (2007)

O *stratum radiatum* de CA1, região que abordaremos nesse estudo, é definido como a região que recebe projeções de CA3 (via colaterais de Schaffer). Essa região está localizada imediatamente abaixo das células piramidais. Duas árvores dendríticas emergem do soma dos neurônios piramidais de CA1: os dendritos basais ocupam o *stratum oriens*, e os dendritos apicais ocupam o *stratum radiatum* e o *stratum lacunoso moleculare*.

No entanto o *stratum radiatum* é o local de maior densidade de espinhos dendríticos e sinapses dos neurônios piramidais de CA1 (Andersen et al., 2007, **Figura 6**).

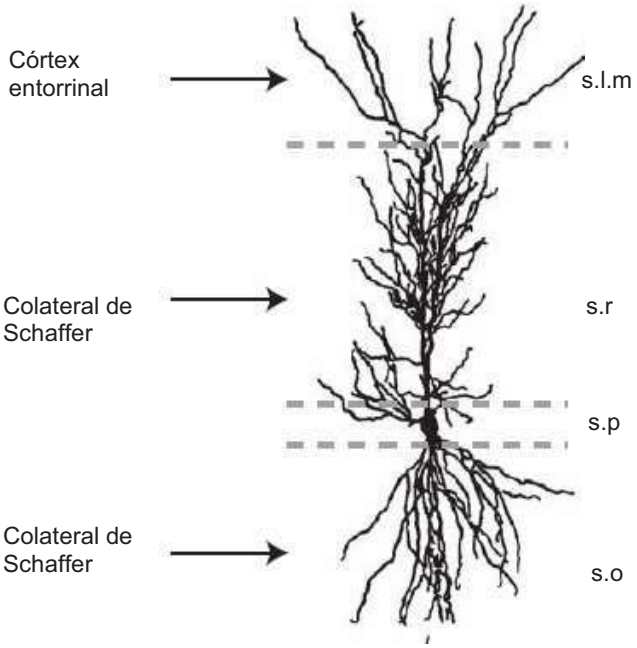

Figura 6: Morfologia e conexões dos dendritos dos neurônios piramidais da região CA1. Observe que os dendritos desse neurônio ocupam toda a extensão do *stratum radiatum* e as projeções dos axônios de CA3 chegando ao *stratum radiatum* via colaterais de Schaffer. S.l.m= *stratum lacunoso moleculare*, s.r=*stratum radiatum*, s.p=*stratum pyramidale* e s.o=*stratum oriens*. Figura adaptada de Andersen et al. (2007)

A maioria dos astrócitos no *stratum radiatum* tem um formato fusiforme, quase perpendicular ao *stratum pyramidale* com o seu eixo longo orientado paralelamente aos dendritos apicais das células piramidas de CA1 (Nixdorf-Bergweiler et al., 1994; Bushong et al., 2002) (Figura 7).

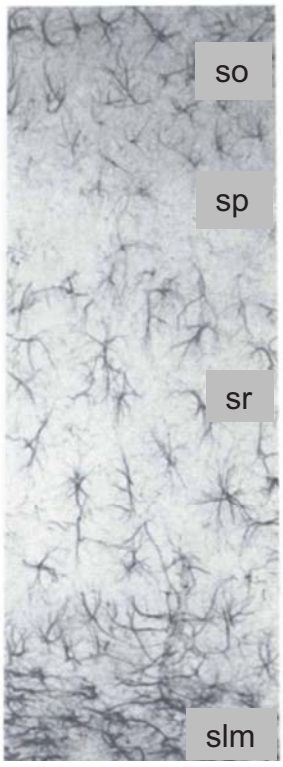

Figura 7: Imagem digitalizada dos astrócitos da região CA1 do hipocampo de rato adulto. Observe a orientação perpendicular dos prolongamentos dos astrócitos do *stratum radiatum* em relação ao *stratum pyramidale*. So=*stratum oriens,* sp=*stratum pyramidale,* sr=*stratum radiatum e* sm= *stratum lacunoso moleculare*. Figura adaptada de Nixdorf-Bergweiler et al. (1994)

O hipocampo é parte de um sistema que desempenha um papel crítico na formação e evocação da memória de longo prazo de fatos e eventos, a chamada memória declarativa. A formação de uma nova memória declarativa é um processo sequencial que inclui a aquisição de novos conhecimentos (codificação), retenção da informação (armazenamento), e rememoração (evocação) (Sharma et al., 2010). Além disso, o hipocampo é um alvo precoce de mudanças estruturais e fisiológicas relacionadas à idade, e danos no hipocampo resultam em déficits cognitivos semelhantes aos vivenciados por pessoas idosas, tais como prejuízos na aquisição/formação de novas memórias declarativas (Sharma et al., 2010).

1.4 EXERCÍCIO FÍSICO

O exercício físico pode ser dividido em isométrico e isotônico dependendo do tipo de atividade muscular realizada. No exercício isométrico há um aumento da tensão muscular sem movimento, ou seja, é um exercício estático. Esse tipo de exercício resulta em um aumento da massa e da força muscular. Em contraste, o exercício isotônico é repetitivo, com movimentos rítmicos e que envolve grandes massas musculares, são conhecidos também como exercício aeróbico ou dinâmico. Embora a maioria das atividades envolva exercício isotônico e isométrico, a corrida é uma atividade física predominantemente isotônica (Hammond e Froelicher, 1985).

O exercício físico pode ser realizado em diferentes intensidades: leve, moderada e alta. Em ratos, para a determinação da intensidade de exercício, os animais são submetidos ao Teste de Esforço Máximo (TEM), que consiste em exercício gradual na esteira, partindo-se de uma velocidade de 5m/min, a qual é aumentada, em um protocolo em degrau, continuamente em mais 5m/min a cada 3 minutos, até que seja atingida a velocidade máxima (VM) de cada animal – definida pelo momento em que o animal para de realizar o teste por exaustão (Ilha et al., 2008; Melo et al., 2003). Em ratos jovens, um exercício de intensidade leve a moderada corresponde a cerca de 30 a 60% da velocidade máxima atingida no TEM (aproximadamente de 5 a 11m/min) (Ilha et al., 2008; Lou et al., 2008; Nico et al., 1997).

Estudos em humanos e animais demonstram que o exercício físico isotônico apresenta efeitos benéficos sobre o funcionamento do encéfalo, incluindo a promoção da plasticidade e melhora nos processos de

aprendizagem e memória (Albeck et al., 2006; Alaei et al., 2008; de Senna et al., 2011; Ding et al., 2006; Hillman et al., 2008; Kashihara et al., 2009; Kramer et al., 2006). A prática de atividade física diária tem sido associada à redução do risco de déficits cognitivos e demência com o avanço da idade (Laurin et al., 2001). Um estilo de vida ativo, incluindo exercício físico regular, previne a ansiedade, depressão, obesidade e o risco de desenvolvimento de doenças cardiovasculares (Martinsen, 2008; Van der Borght et al., 2009). Além disso, o exercício também tem efeitos protetores contra várias doenças neurológicas, incluindo a doença de Parkinson (Smith e Zigmond, 2003), a doença de Alzheimer (Mirochnic et al., 2009) e acidente vascular cerebral isquêmico (Stummer et al., 1994).

O exercício afeta especialmente o hipocampo, aumentando a sua atividade, por meio do aumento do fluxo sanguíneo local (Holschneider et al., 2007) e induzindo a plasticidade sináptica hipocampal, principalmente através do aumento da eficácia sináptica e da expressão de moléculas envolvidas na aprendizagem e memória (Farmer et al., 2004; Vaynman et al., 2003). O exercício é capaz de induzir o aumento de fatores neurotróficos, tais como o fator neurotrófico derivado do encéfalo (BDNF), o fator de crescimento neural (NGF), o fator de crescimento de fibroblastos (FGF), bem como os seus mRNAs (Berchtold et al., 2010; Gomez-Pinilla et al., 1997; Neeper et al., 1996). Componentes da vesícula sináptica, como sinapsina I e sinaptofisina também têm seus níveis aumentados após atividade física (Vaynman et al., 2006). Além disso, o exercício é capaz de induzir potenciação de longa duração (“Long-Term Potentiation” - LTP) (van Praag et al., 1999a) e angiogênese (Black et al., 1990; van der Borght et al. 2009).

O exercício físico está associado também ao aumento da proliferação e da sobrevivência celular. Vários estudos relatam o aumento da neurogênese e da densidade e complexidade dos espinhos dendríticos no hipocampo de roedores quando submetidos a protocolo de atividade física (Eadie et al., 2005; Kim et al., 2003; van Praag et al., 1999a; van Praag et al., 1999b; van Praag et al., 2005). A proliferação celular hipocampal pode ocorrer em vários estágios do desenvolvimento, incluindo adultos (van Praag et al., 1999a) e idosos (van Praag et al., 2005). Filhotes recém-nascidos com mães que haviam realizado exercícios aeróbicos durante o período gestacional da gravidez também apresentaram um maior número de células no hipocampo do que os filhotes nascidos de mães sedentárias (Kim et al., 2007; Lee et al., 2006). Mais interessante ainda é que o aumento na proliferação celular está intimamente relacionado a melhoras no desempenho em testes comportamentais, sugerindo que as células recém-formadas podem contribuir nos processos de aprendizagem e memória. A proliferação de novos neurônios no encéfalo é acompanhada por um aumento da necessidade de nutrientes (Hillman et al., 2008). Esta necessidade é suprida pela estimulação do crescimento de novos vasos sanguíneos em regiões encefálicas como o córtex cerebral (Kleim et al., 2002; Swain et al., 2003), o cerebelo (Black et al., 1990; Isaacs et al., 1992), o estriado (Ding et al., 2004) e o hipocampo (Lopez-Lopez et al., 2004).

Os estudos sobre os efeitos da atividade física nas células astrogliais no SNC são escassos e tampouco analisam os efeitos do exercício físico sobre a morfologia dos processos astrocíticos, componente que, provavelmente, apresenta significativa alteração devido a sua íntima associação com os neurônios.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Analisar as possíveis alterações nos astrócitos GFAP positivos do *stratum radiatum* da região CA1 do hipocampo de ratos Wistar, submetidos a exercício físico isotônico de intensidade leve.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar, através da técnica imunoistoquímica para GFAP associada a técnica de morfometria planar, a densidade de astrócitos GFAP positivos no *stratum radiatum* da região CA1 do hipocampo;

- Avaliar, através da técnica imunoistoquímica para GFAP associada a medida densitométrica, a imunorreatividade para esta proteína nos astrócitos do *stratum radiatum* da região CA1 do hipocampo;

- Avaliar através da técnica dos círculos concêntricos de Sholl, as possíveis alterações morfológicas nos astrócitos GFAP positivos do *stratum radiatum* da região CA1 do hipocampo.

CAPÍTULO 2

ARTIGO CIENTÍFICO

Os materiais e métodos utilizados, os resultados e a discussão serão apresentados na forma de artigo científico publicado no periódico:

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ORIGINAL ARTICLE

Physical exercise increases GFAP expression and induces morphological changes in hippocampal astrocytes

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Abstract Physical exercise has an important influence on brain plasticity, which affects the neuron–glia interaction. Astrocytes are susceptible to plasticity, and induce and stabilize synapses, regulate the concentration of various molecules, and support neuronal energy metabolism. The aim of our study was to investigate whether physical exercise is capable of altering the morphology, density and expression of glial fibrillary acidic protein (GFAP) in astrocytes from the CA1 region of rat hippocampus. Thirteen male rats were divided in two groups: sedentary ($n = 6$) and exercise ($n = 7$). The animals in the exercise group were submitted to a protocol of daily physical exercise on a treadmill for four consecutive weeks. GFAP immunoreactivity was evaluated using optical densitometry and the morphological analyses were an adaptation of Sholl's concentric circles method. Our results show that physical exercise is capable of increasing the density of GFAP-positive astrocytes as well as the regional and cellular GFAP expression. In addition, physical exercise altered astrocytic morphology as shown by the increase observed in the degree of ramification in the lateral quadrants and in the length of the longest astrocytic processes in the central quadrants. Our data demonstrate important changes in astrocytes promoted by physical exercise, supporting the idea that these cells are involved in regulating neural activity and plasticity.

Keywords Physical exercise · Astrocytes · GFAP · Hippocampus · Sholl

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Introduction

There is a considerable evidence to show that physical exercise has a positive effect on brain function in both humans (Hillman et al. 2008; Laurin et al. 2001) and animals (Albeck et al. 2006; Farmer et al. 2004; Stranahan et al. 2010). Physical exercise improves cognitive functions (Kashihara et al. 2009; Kramer et al. 2006) and memory (Alaei et al. 2008; de Senna et al. 2011), reduces anxiety and depression (Martinsen 2008), and has protective properties on a wide variety of neurological diseases, such as Parkinson's disease (Smith and Zigmond 2003), Alzheimer's disease (Mirochnic et al. 2009) and ischemic stroke (Stummer et al. 1994).

Studies designed to shed light on the neurobiological bases of these benefits have demonstrated that physical exercise is involved in cerebral plasticity. Exercise can induce neurogenesis (Kim et al. 2003; van Praag et al. 1999a, b, 2005) and increase the release of neurotrophic factors such as: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), fibroblast growth factor (FGF) and their mRNAs (Berchtold et al. 2010; Gómez-Pinilla et al. 1997; Neeper et al. 1996). Physical exercise can also induce long-term potentiation (LTP) (van Praag et al. 1999a)

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and angiogenesis (van der Borght et al. 2009). In addition, physical activity can increase the most common markers of hippocampal synaptic and structural plasticity, such as synapsin I, neurofilaments, microtubule-associated protein 2 (Ferreira et al. 2011).

Nevertheless, there are only a few studies that have investigated the effects of exercise in astrocytes in animals and humans. Some of these studies report that physical exercise was able to increase the glial fibrillary acidic protein (GFAP) expression as well as the number of GFAP-positive astrocytes in the frontoparietal cortex and striatum (Li et al. 2005), and stimulate the proliferation of the astrocytes in the subgranular zone of the hippocampus of rodents (Uda et al. 2006).

Thus, the goal of our study was to analyze the effects of physical exercise in the morphology of GFAP-positive hippocampal astrocytes, more specifically, in the *stratum radiatum* within the CA1 (Interaural 6.70 mm/Bregma −2.30 mm to Interaural 4.70 mm/Bregma −4.30 mm), a region that contains numerous astrocytes and is involved in important functions including learning and memory (Catalani et al. 2002; Squire et al. 2004).

Materials and methods

Animals

For this study, 13 male Wistar rats, aged approximately 3 months and weighing about 200–300 g were obtained from the Instituto de Ciências Básicas da Saúde (ICBS), UFRGS. They were maintained in a controlled environment with food and water ad libitum, at a 12:12 h dark:-light schedule. The animals were divided into two groups: 1-Sedentary (Sed; 6 animals), 2-Exercise (Exe; 7 animals). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA). All efforts were made to minimize animal suffering and reduce the number of animals needed.

Exercise program

Considering that for humans the guidelines recommend practicing physical activity for at least 30 min most days of the week (Hillman et al. 2008), our investigation adopted a protocol of light intensity exercise adapted from a previous study (Yoon et al. 2007). In summary, the animals in the exercise group walked on an adapted motorized treadmill for 30 min, 5 days a week for four consecutive weeks. In all the exercise sessions, the treadmill was maintained at a speed of 4 m/min for the first 5 min, and then 6 m/min for the remaining 25 min.

GFAP immunohistochemistry

For the immunohistochemical study, all rats were deeply anesthetized with ketamine (90 mg/kg) and xylazine (15 mg/kg) (i.p.) and injected with heparin (1,000 IU; Cristalia, Brazil). Using a peristaltic pump (Milan, Brazil, 50 mL/min), the animals were perfused through the left cardiac ventricle with 200 mL of saline solution followed by 200 mL of fixative solution of 4 % paraformaldehyde (Reagen, Brazil) diluted in 0.1 M phosphate buffer (PB), pH 7.4. Brains were extracted from the skull, post-fixed for 4 h in the same fixative solution at room temperature, cryoprotected in 15 % sucrose solution in PB at 4 °C until they sank (about 24 h) and then transferred to a solution of 30 % sucrose (Synth, Brazil) in PB at 4 °C until they sank (also about 24 h), and then frozen in liquid nitrogen (Nitrovet, Brazil). After these procedures, the brains were kept in a freezer (−70 °C) for further analyses. Coronal brain sections (50 µm) were obtained using a cryostat (Leica, Germany) and one in every five sections was collected for analysis. Brain sections were collected in phosphate-buffered saline (PBS) and processed for GFAP immunohistochemistry (Dutra et al. 2012). Free floating sections were washed and blocked with 2 % bovine serum albumin (BSA) in PBS containing 0.4 % Triton X-100 (PBS-Tx, Sigma Chemical Co., USA) for 30 min. They were then incubated with polyclonal GFAP antiserum raised in rabbit (Dako, UK) diluted 1:500 in 0.3 % of PBS-Tx for 48 h at 4 °C. After being washed with PBS-Tx twice, sections were incubated in anti-rabbit IgG whole molecule peroxidase-conjugated antibody produced in goat (Sigma, USA) diluted 1:150 in PBS-Tx at room temperature for 2 h. The reaction was developed by incubating the sections in a medium containing 0.06 % 3,3′-diaminobenzidine (DAB, Sigma-Chemical Co., USA) dissolved in PBS for 10 min and in the same solution containing 1 µL of 3 % H_2O_2 per mL of DAB medium for an additional 10 min. Immediately after the DAB + H_2O_2 revelation, the sections were rinsed in PBS, dehydrated in series of increasing ethanol concentrations (70, 90 and 100 %, 2 min each) cleared with xylene and covered with Permount and coverslips. As a control to rule out unspecific binding, in a few sections the primary antibody was omitted and replaced by PBS. In order to minimize differences in the staining of astrocytes and in background levels, the brains in both experimental groups were fixed and post-fixed in identical solutions for the same length of time, processed at the same time and incubated in the same immunostaining medium for the same period of time.

Astrocytic density estimation

The number of GFAP-immunoreactive astrocytes per mm^2 in the *stratum radiatum* of the CA1 was estimated using an

Olympus BX 50 microscope coupled to a Motic Images Plus 2.0 camera and Image Pro Plus (Image Pro-Plus 6.1, Media Cybernetics, Silver Spring, EUA) software.

For this analysis, three digitized images (20×) from selected areas were obtained from each section. Altogether, five sections from each animal were analyzed. Thus, 15 images were analyzed per animal. Three randomized squares measuring 5,828 μm^2 and named areas of interest (AOIs) were overlaid on each image. The astrocytes located inside this square or intersected by the upper and/or right edges of the square were counted. Astrocytes intersected by the lower and/or left edges of the square were not counted.

GFAP immunoreactivity evaluation

The intensity of GFAP immunoreactivity was measured using semi-quantitative densitometric analysis (Ferraz et al. 2003; Xavier et al. 2005; Martinez et al. 2006) with the same software employed to estimate the astrocytic density. The same images used to estimate astrocytic density were used in the analysis of regional optical density (OD). The images were converted to an 8-bit gray scale (256 gray levels) and three AOIs (5,828 μm^2) were overlaid on each image.

For the analysis of cellular OD, three digitized images (40×) were obtained from each section. Altogether, five sections from each animal were analyzed. Thus, fifteen images were analyzed in each animal. The images were converted to gray scale and one AOI measuring 10.37 μm^2 was placed over the astrocytic soma in each image. Cellular GFAP expression was only measured in the glial soma, immunoreactivity in the processes was not measured.

All lighting conditions and magnifications were kept constant during the process of capturing the images. Blood vessels and other artifacts were avoided and the background correction was performed according to the formula previously described in Xavier et al. (2005).

Morphological analysis of astrocytes

The morphological analysis was done using the same images employed to measure cellular optical density. For the analysis of astrocytic ramification, an adaptation of Sholl's concentric circles technique was used (Sholl 1953; Dall'Oglio et al. 2008). Briefly, seven virtual circles with 3.91 μm intervals were drawn around each astrocyte.

The degree of ramification of the astrocytes was measured by counting the number of times the astrocytic processes intersected with each virtual circle in both the lateral (i.e. right/left) and central (i.e. superior/inferior) quadrants around the astrocytes.

Primary process quantification was performed by counting the processes extending directly from the soma in both the lateral and central quadrants of astrocytes in the same sections.

The longest primary process in each quadrant was measured by tracing the process with a manual measurement tool found in the Image Pro Plus software.

Statistical analysis

An unpaired (Student's) t test was used to compare the groups ($p < 0.05$), using Graph Pad 4.0 software. Data are expressed as mean ± standard deviation.

Results

In our study, astrocytes from the *stratum radiatum* within the CA1 region of the hippocampus were analyzed (Fig. 1). In both groups, it was possible to observe the soma and processes of GFAP-positive astrocytes. Some of these processes were long and thin, extending from the soma (primary process) and giving rise to many fine ramifications (Fig. 1). These astrocytes were seen to connect to neighboring astrocytes by slightly touching their distal processes (Fig. 1).

In the qualitative analysis, we observed an increased number of GFAP-positive astrocytes, and a stronger immunoreaction in the exercise group when compared to sedentary animals (Fig. 1).

In order to confirm our qualitative morphological findings, quantitative and semi-quantitative evaluations were performed, respectively, involving an estimation of astrocytic density and measurements of regional and cellular optical density.

Astrocytic density, regional and cellular optical density

Daily physical exercise was able to increase astrocytic density (Fig. 2a; $p < 0.001$). Increases in regional (Fig. 2b; $p < 0.05$) and cellular (Fig. 2c; $p < 0.01$) GFAP immunoreactions were also observed in the exercise group.

Analysis of astrocytic ramification

As shown in Fig. 3b, physical exercise induced an increase in the number of total ramifications ($p < 0.05$). This increase in the total number of intersections is due to the fact that the number of intersections increased in the lateral quadrants in the exercise group (Fig. 3d; $p < 0.05$). No difference was observed in the number of intersections counted in the central quadrants (Fig. 3c).

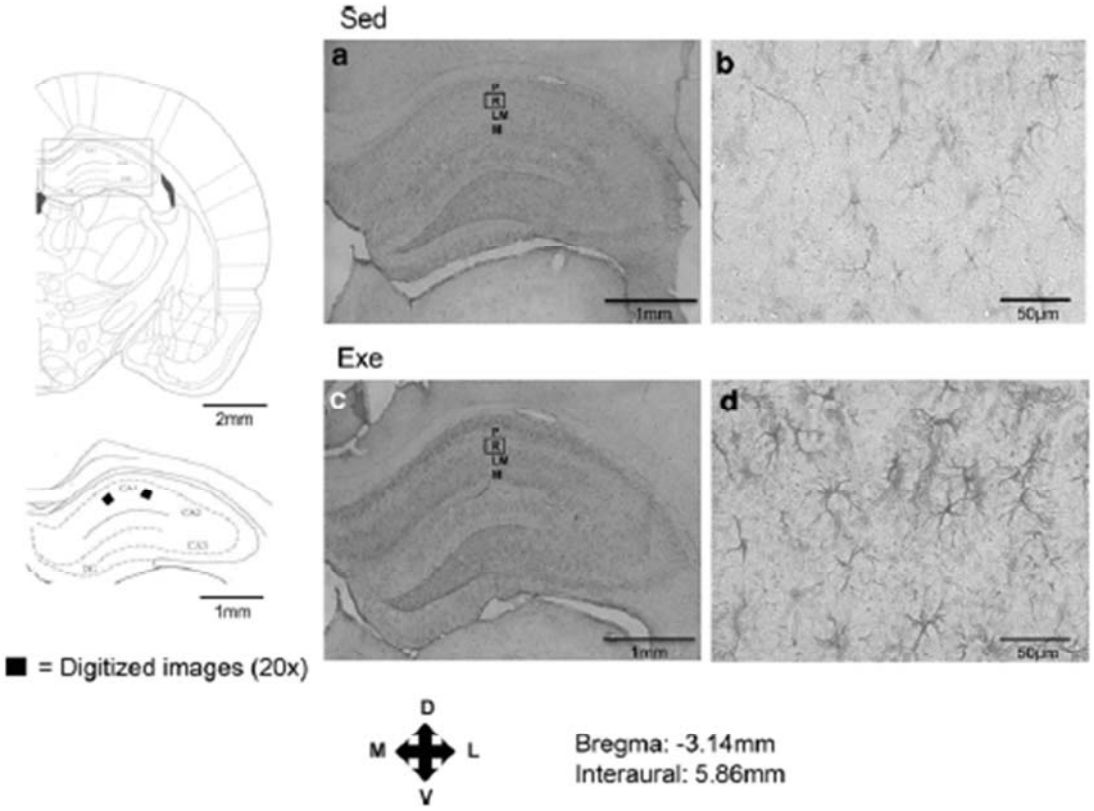

Fig. 1 Digitized images of the hippocampus after GFAP immunohistochemistry showing the CA1 region. **a**, **b** sedentary, **c**, **d** exercise. Note the astrocytic soma and processes stained for GFAP and the increase in astrocytic density and GFAP expression in the exercise group. *Filled square* areas of capture at 20×. *P* stratum pyramidale, *R* stratum radiatum, *LM* stratum lacunosum moleculare and *M* stratum moleculare. Adapted from Paxinos and Watson (1998)

Analysis of the primary processes

There were no significant differences in the number of central, lateral and total primary processes (Fig. 4a–c).

Length of the primary processes

The analysis of the length of the longest primary central and lateral processes demonstrated that physical exercise was able to increase the length of the astrocytic processes in the central quadrant (Fig. 5a; $p < 0.05$) when compared to the sedentary group. No differences were observed in the astrocytic process length in the lateral quadrant (Fig. 5b).

Discussion

In our study, we used GFAP as an astrocytic marker. Other markers including, S100 protein, vimentin and glutamine synthetase are used to analyze alteration in the proprieties of glial cells (Catalani et al. 2002). However, our choice to use GFAP is based on the fact that other markers present some serious disadvantages when compared to GFAP.

For example, in the study by Wu et al. (2005), while antibodies for glutamine synthetase and S100β were found to clearly stain the nuclei of astrocytes, the cytoplasm and processes were only poorly stained. It has also been noted that with S100 immunohistochemistry the astrocytic processes appear to be smaller when compared to GFAP immunostained astrocytes (Björklund et al. 1983). Moreover, although glutamine synthetase was first thought to be specific for astrocytes, later studies revealed that this enzyme is also detectable in oligodendrocytes (Tansey et al. 1991).

Like GFAP, vimentin is also a good marker of astrocytic morphology, but it is predominantly expressed in immature glial cells (Dahl et al. 1981; Pixley and Vellis 1984), and in our study, we only focused on the effects of exercise on the structure and function of fully developed astrocytes.

Other glial markers that could have been used are members of the glutamate transporters family. Some studies have used immunoreaction to detect these transporters (i.e. GLAST and GLT). However, these markers are

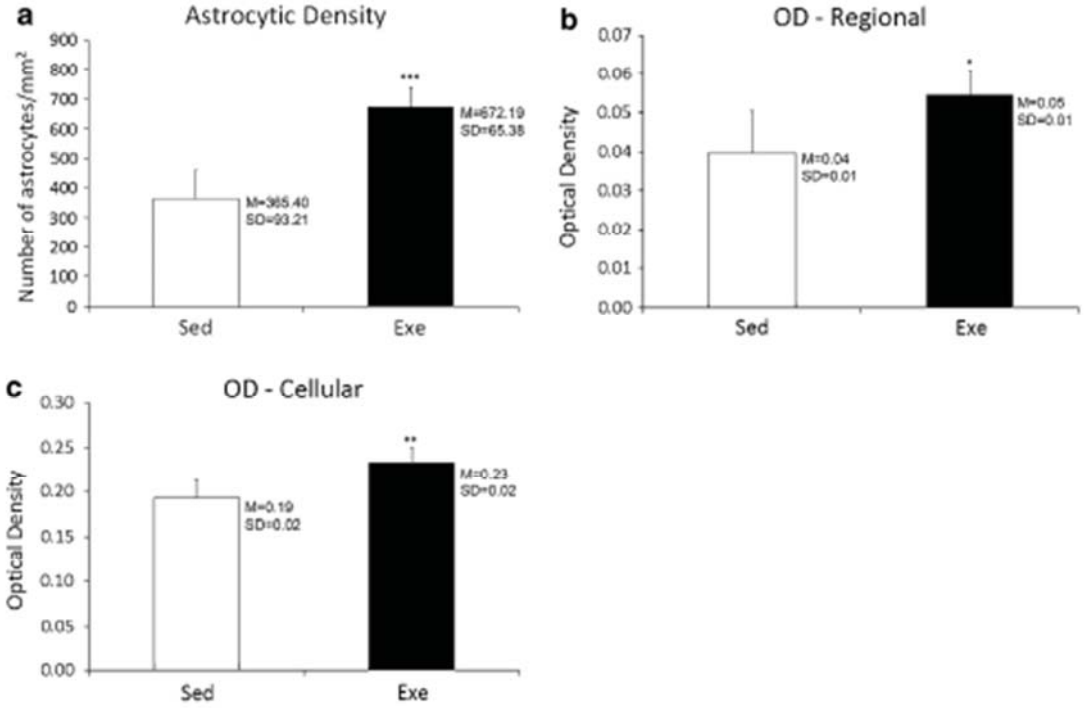

Fig. 2 Effects of physical exercise on the astrocytic density and GFAP expression. **a** Astrocytic density, **b** regional optical density ($p = 0.015$) and **c** cellular optical density ($p = 0.007$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (mean ± SD). *Sed* sedentary, *Exe* exercise, *OD* optical density

not good tracers for morphological analyses, because they usually produce unclear images (Coleman et al. 2004; Zhang et al. 2011).

Therefore, GFAP immunolabeling is still generally considered a reliable means of identifying astrocytes (Theodosis et al. 2008). GFAP has been used as the main marker for astrocytical reactivity in several different areas such as: obesity (Buckman et al. 2012), schizophrenia (Williams et al. 2012), neuroinflammation (Schäfer et al. 2012), the effects of vitamin C (Hashem et al. 2012), and the effects of resveratrol (Yuan et al. 2012). Furthermore, GFAP is a cytoskeletal protein required for the formation of stable astrocytic processes (Weinstein et al. 1991), which is ideal for morphological analyses.

In our study, we have demonstrated that physical exercise can morphologically alter GFAP-positive astrocytes in the *stratum radiatum* within the CA1 region of the hippocampus in healthy rats. We found an increase in the density of astrocytes in the exercise group (Fig. 2a). This result is supported by previous studies which demonstrated an increase in astrocytic density (Uda et al. 2006) and in the proliferation of GFAP-positive cells, in the subgranular zone of the hippocampus, in exercised rats (Komitova et al. 2005). One explanation for this increase in astrocytic density is that physical exercise is capable of increasing FGF and NGF (Neeper et al. 1996; Gómez-Pinilla et al. 1997), since previous studies have demonstrated that FGF and NGF are able to induce astrocytic proliferation (Gómez-Pinilla et al. 1995; Lewis et al. 1992; Yokoyama et al. 1993).

We observed an increase in regional GFAP expression brought on by physical exercise (Fig. 2b). Our finding is in accordance with previous studies which have demonstrated similar alterations in the hippocampus (Ferreira et al. 2011; Rodrigues et al. 2010). Another study also found exercise evoked an increase in regional GFAP expression in different brain regions (Li et al. 2005). However, using a more intense exercise protocol, de Senna et al. (2011) reported no changes in GFAP regional optical density, in contrast to our study. This difference is probably a result of the different exercise protocols used, indicating that regional optical density generated by GFAP could differ according to the protocol used. Unfortunately, the study by de Senna et al. (2011) did not include an analysis using Sholl method, which could have provided some very interesting information.

The increase in GFAP expression has been widely described in neurologic dysfunctions such as depression (Kraig et al. 1991), electrically induced seizures (Steward et al. 1991, 1997) and augmented cerebral activity brought on by an increase in extracellular potassium concentration (Canady et al. 1990). But in many instances, these alterations observed in astrocytes may reflect a substantial increase in astroglial metabolism and protein synthesis,

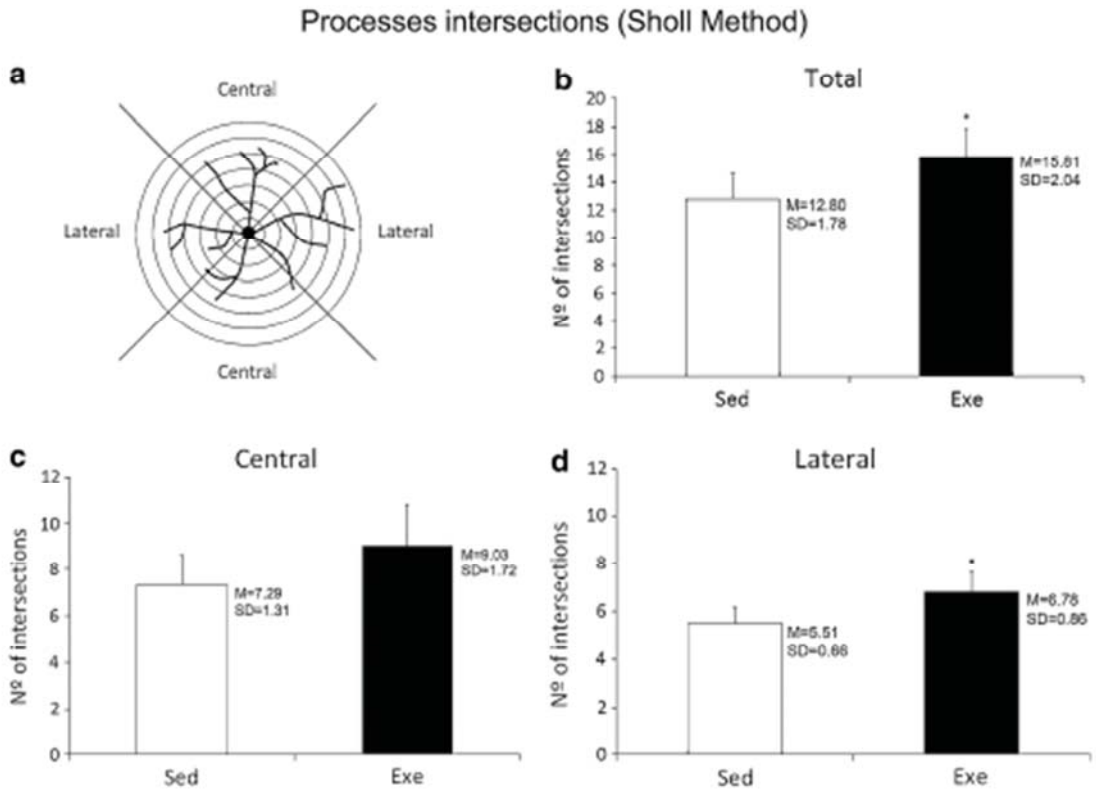

Fig. 3 Effects of physical exercise on the ramification in GFAP-positive astrocytes. **a** A schematic representation of Sholl's concentric circles method, **b** total intersections of the processes with the *circles* ($p = 0.026$), **c** number of intersections of astrocytic processes in the central quadrants ($p = 0.089$) and **d** number of intersections in the lateral quadrants ($p = 0.020$). *$p < 0.05$ (mean ± SD). *Sed* sedentary, *Exe* exercise, *Central* central quadrants, *Lateral* lateral quadrants

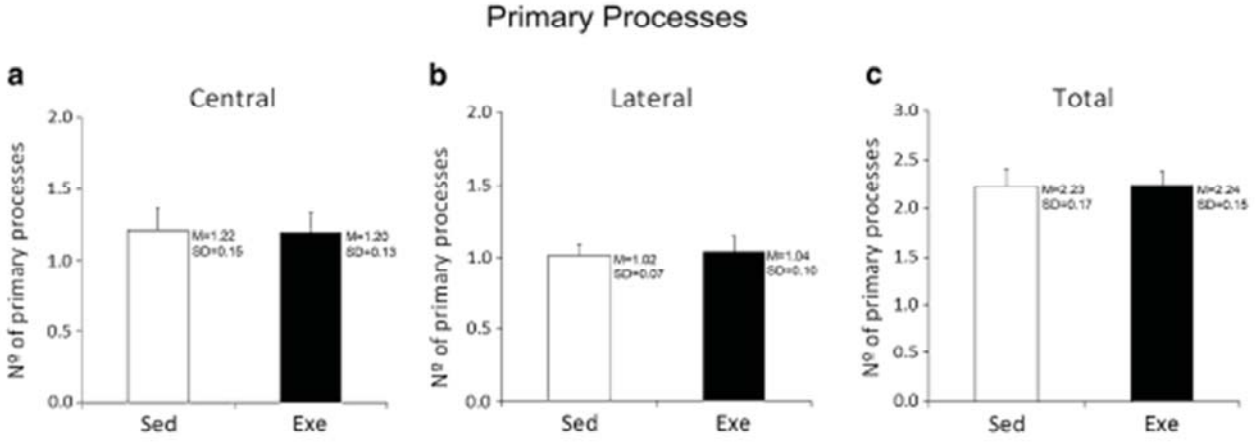

Fig. 4 Effects of physical exercise on the number of primary processes in GFAP-positive astrocytes. **a** Primary processes in the central quadrants ($p = 0.711$). **b** Primary processes in the lateral quadrants ($p = 0.640$) and **c** total primary processes, $p = 0.925$ (mean ± SD). *Sed* sedentary, *Exe* exercise

consistent with a healthy cellular hypertrophy in response to increased physiologic demands (Eddleston and Mucke 1993).

Furthermore, the astrocytes activation can play an important role in neural plasticity in healthy animals as shown by the increase in the density and expression of GFAP immunoreactive astrocytes following behavioral and environmental manipulations (Jones et al. 1996; Sirevaag and Greenough 1991; Matsutani and Leon 1993; Gómez-Pinilla et al. 1998). In addition, some studies have observed

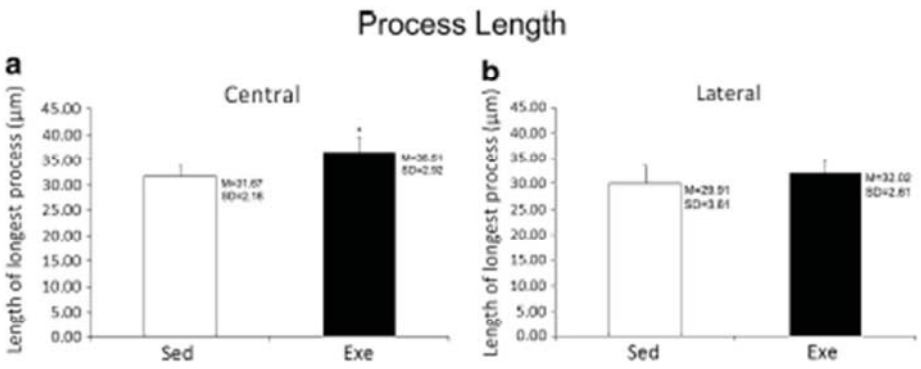

Fig. 5 Effects of physical exercise on the length of the longest primary processes in GFAP-positive astrocytes. **a** Length of the longest primary process in the central quadrants, $p = 0.011$ and **b** length of the longest primary process in the lateral quadrants ($p = 0.287$). $^{*}p < 0.05$ (mean ± SD). *Sed* sedentary, *Exe* exercise

that the decrease in GFAP expression leads to abnormal astrocyte–neuronal interaction, neuronal physiology and abnormal behavior in rodents (McCall et al. 1996; Shibuki et al. 1996).

Another finding of our study is the increase in cellular GFAP expression (Fig. 2c). In a very similar study, in which the researchers analyzed the effects of environmental enrichment (EE) on astrocytes, no alteration in this parameter was observed (Viola et al. 2009). EE is a housing condition that facilitates enhanced sensory, cognitive and also motor stimulation by placing various objects/tasks inside the home cages in which the animal is free to interact at will (Nithianantharajah and Hannan 2006). The differences between the results of the present study and that of Viola et al. (2009) might be related to the motor stimulus employed. EE is very broad, whereas physical exercise is specific and uniform. Thus, the increase in the cellular GFAP expression found in the present study could be associated to the regular motor stimulus provided by our protocol.

In this study, we also observed morphological alterations analyzed by the Sholl method in GFAP-positive astrocytes. Most astrocytes in the *stratum radiatum* region have a fusiform shape almost perpendicular to *stratum pyramidale* with the long axis oriented parallel to the descending apical dendrites of the CA1 pyramidal cells (Nixdorf-Bergweiler et al. 1994; Bushong et al. 2002). The longitudinal arrangement of astrocytes in relation to apical CA1 dendrites suggests that a structural arrangement between astrocytes and neurons exists in this region (Bushong et al. 2002). Our results show an increase in the degree of ramification of astrocytes in lateral quadrants, but not in central quadrants in the exercised group (Fig. 3d). Therefore, the increase observed in the ramification indicates that physical exercise induced a slight change in the morphology of the astrocytes in this region, with them adopting more stellate shape. Similar data have been described by Viola et al. (2009), in which they demonstrated that housing in an enriched environment was capable of changing the same parameter in the *stratum radiatum*.

We also observed an increase in the length of the longest primary process in the central quadrant in the exercised animals (Fig. 5a). This is in accordance with a recent study that reported that reaching skills training can induce an increase in the length of astrocytic processes, which was observed in association with enhanced sensorimotor recovery after intracerebral hemorrhage (Mestriner et al. 2011). Astrocyte processes contain cytoskeletal GFAP molecule, in which the assembly of the cytoskeleton facilitates such changes in astrocyte morphology (Rodnight et al. 1997) and some studies have observed that decreased GFAP expression leads to a reduced capacity to form stable astrocytic processes in rodents (Chen and Liem 1994; Weinstein et al. 1991).

As previously mentioned, physical exercise is capable of increasing BDNF expression (Neeper et al. 1996) and a related study demonstrated that the astrocytes from layer I within the motor cortex treated with BDNF presented similar alterations in astrocytic morphology to those observed in our study (Ohira et al. 2007). Thus, BDNF could be one of the molecular mechanisms responsible for the alterations that we observed in the astrocytes from exercised animals in our study. Moreover, physical exercise is capable of inducing LTP (van Praag et al. 1999a), which has been shown to produce alterations in the morphology of astrocytes around potentiated synapses to accompany neuronal plasticity in the *dentate gyrus* (Wenzel et al. 1991).

In the same way, the dendritic spines are adaptable and respond to changes in activity by altering their structure, astrocytic processes dynamically alter their morphology and interact with synapses in response to environmental cues (Allen and Barres 2005). Physical exercise is able to increase the length, density and complexity of the dendritic spines in the hippocampus (Dietrich et al. 2008; Eadie et al. 2005; Lin et al. 2012) and changes in the dendritic spines are typically coordinated with changes in the astrocytic processes (Haber et al. 2006).

Evidence clearly shows that remodeling of astrocytic processes is closely linked to neuronal activity and often

occurs in synchrony with morphological changes in neighboring neurons and synaptic inputs, a kind of astrocytic-neuronal plasticity that highlights the brain's capacity for activity-dependent modulation (Theodosis et al. 2008). The "tripartite" synapse, in which information flows not only between the traditional pre- and postsynaptic neuronal partners but, in addition, between astrocytic processes (Perea et al. 2009), suggests astrocytic processes directly influence synaptic activity, which reflects the cooperation between neurons and astrocytes. Therefore, the alterations seen in the astrocytes in our study could reflect neuronal and synaptic changes in response to physical exercise, suggesting that structural changes in both neurons and glia contribute to synaptic plasticity in the hippocampus.

An interesting hypothesis is that astrocytes could present some degree of polarization, as found in neurons and epithelial cells (Alberts et al. 2008). Thus, the "apical", "basal" and "lateral" portions of astrocytes could present different types of proteins, receptors, etc. The concept of polarity in astrocytes hypothesized in our study has been previously suggested in other studies (Nixdorf-Bergweiler et al. 1994; Derouiche et al. 2012).

The polarization of astrocytes is an exciting idea because, in the *stratum radiatum* region of the CA1, the apical dendrites of the pyramidal cells are heavily innervated by Schaffer collaterals that produce an excitatory glutamatergic input at a distal site and also by interneurons that generate inhibitory GABAergic inputs, at a proximal shaft (Andersen et al. 2007; Freund and Buzsáki 1996; Verkhratsky and Butt 2007). These differences in the synaptic configurations could explain the anisotropic nature found using Sholl analysis.

The involvement of astrocytes in angiogenesis has long been recognized (Penfold et al. 1990; Suárez et al. 1994), suggesting astrocytes have a functional role in the vascularization of neural tissue. These studies also indicate that astrocytes might participate directly and actively in the regulation of capillary formation. Another study also showed that exercise induces astroglial proliferation in the same areas that exhibit angiogenesis (Li et al. 2005). This association of angiogenesis and astroglial proliferation during exercise suggests that both astrocytes and endothelial cells could participate in the formation of new blood vessels in the brain.

Various studies have reported important neuronal and molecular alterations related to physical exercise, however, there is little is clear evidence about the relation between physical exercise and astrocytes. Thus, the main contribution of our study was to demonstrate that physical exercise is able to increase regional and cellular GFAP, as well as the number of GFAP-positive astrocytes in the hippocampus. Furthermore, these alterations were accompanied by important morphological changes in the degree of ramification and the length of the astrocytic processes.

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Conflict of interest The authors declare that they have no conflicts of interest.

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CAPÍTULO 3

CONSIDERAÇÕES FINAIS

3 CONSIDERAÇÕES FINAIS

Os resultados deste trabalho demonstram que o exercício físico é capaz de alterar a densidade de astrócitos GFAP positivos, a expressão de GFAP e a morfologia dos astrócitos do *stratum radiatum* da região CA1 do hipocampo de ratos saudáveis.

Neste estudo, foi utilizado GFAP como marcador astrocitário. Outros marcadores como a proteína S100, vimentina e glutamina sintetase também são utilizados para analisar alteração nas propriedades de células gliais (Catalani et al. 2002). Entretanto nossa escolha pela GFAP baseia-se no fato dessa proteína ser um marcador confiável para identificação de astrócitos maduros, além de ser uma proteína de citoesqueleto necessária para a formação de processos astrocíticos estáveis (Weinstein et al. 1991), portanto, ideal para análises morfológicas.

Observamos que nos animais exercitados houve um aumento na densidade de astrócitos GFAP positivos e esse aumento é seguido tanto pelo aumento na expressão regional como celular de GFAP. Outros estudos também demonstraram que o exercício físico é capaz de produzir efeitos semelhantes, mas em outras regiões encefálicas (Komitova et al., 2005; Li et al. 2005; Uda et al., 2006). O aumento na densidade de astrócitos e na expressão de GFAP pode ser resultado do aumento do metabolismo astroglial e da síntese de proteínas, em resposta ao acréscimo na demanda fisiológica gerada pela prática de atividade física (Eddleston e Mucke 1993). Outros estudos já documentaram aumento na expressão de GFAP após manipulações

ambientais e comportamentais (Gómez-Pinilla et al., 1998; Jones et al., 1996; Matsutani e Leon, 1993; Sirevaag e Greenough, 1991).

Em relação as alterações morfológicas, observamos que nos animais exercitados houve um aumento no grau de ramificação dos astrócitos nos quadrantes laterais e um aumento no comprimento do maior processo astrocítico nos quadrantes centrais. Da mesma maneira que os espinhos dendríticos são adaptáveis e respondem a mudanças na atividade neuronal alterando a sua estrutura, os processos astrocíticos são capazes de alterar a sua morfologia em resposta aos estímulos ambientais (Allen e Barres, 2005). Estima-se que cada astrócito faz contato com aproximadamente 140.000 sinapses, sugerindo que um único astrócitos pode integrar os sinais de múltiplas sinapses (Bushong et al., 2002).

A atividade astrocitária é intimamente relacionada a atividade neuronal, consequentemente, o remodelamento astrocítico é intimamente relacionado as mudanças nos neurônios vizinhos, constituindo um tipo de plasticidade neurônio-glia (Theodosis et al., 2008). Além disso, os astrócitos localizados próximos às sinapses potenciadas desenvolvem um maior grau de ramificação e um aumento da sua superfície (Wenzel et al., 1991). Os processos astrocíticos estabelecem um contato mais próximo com essas sinapses potenciadas e tendem a cobrir toda a superfície sináptica, bem como limitar a fenda sináptica lateralmente (Wenzel et al., 1991). Portanto, a remodelação dos processos astrocíticos deve ser considerada um mecanismo importante quando se examina os fatores que podem modificar a estrutura sináptica.

Os resultados deste estudo corroboram com a hipótese de que os astrócitos devem apresentar algum grau de polarização, como o encontrado

em neurônios e células epiteliais (Alberts et al. 2008). A maioria dos astrócitos no *stratum radiatum* tem uma forma fusiforme, quase perpendicular ao *stratum pyramidale* (Nixdorf-Bergweiler et al, 1994;. Bushong et al 2002.). Foi observado um aumento no grau de ramificação dos astrócitos nos quadrantes laterais, portanto, estes resultados indicam que os astrócitos nessa região alteraram a sua morfologia para uma forma mais estrelada. Estes achados sugerem que as porções “basais”, “apicais” e “laterais” podem apresentar diferentes tipos de proteínas e receptores. Contudo, estudos futuros utilizando microscopia confocal e microscopia eletrônica, são fundamentais para a consolidação desta hipótese.

A principal contribuição desse estudo foi demonstrar que o exercício físico é capaz de aumentar a expressão regional e celular de GFAP, bem como o número de astrócitos GFAP positivos e que essas alterações são acompanhadas por mudanças morfológicas no grau de ramificação e no comprimento dos processos astrocíticos, sugerindo que os astrócitos participam ativamente da plasticidade hipocampal promovida pelo exercício físico.

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ANEXOS

COMPROVANTE DE APROVAÇÃO DO PROTOCOLO PELO COMITÊ DE ÉTICA PARA USO DE ANIMAIS (CEUA)

PRODUÇÃO CIENTÍFICA DURANTE O CURSO DE MESTRADO

Este trabalho faz parte de um projeto “guarda-chuva”, analisado e aprovado pelo CEUA-PUCRS com registro 10/00147.

PUCRS CEUA Comitê de Ética para o Uso de Animais

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 063/10 – CEUA

Porto Alegre, 22 de abril de 2010.

Senhor Pesquisador:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 10/00147, intitulado: **"Efeitos do exercício físico sobre a morfofisiologia estrital e marcha de ratos wistar com parkinsonismo induzido por bloqueio farmacológico de receptores D2".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora do CEUA – PUCRS

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Outros artigos científicos produzidos ao longo do mestrado:

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2. Pedro Porto Alegre Baptista, Priscylla N de Senna, Mariana F Paim, **Lisiani Saur**, Martina Blank, Patricia do Nascimento, Jocemar Ilha, Monica R Vianna, Regis G Mestriner, Matilde Achaval, Leder L Xavier. Physical exercise down-regulated locomotor side effects induced by haloperidol treatment in Wistar rats. Pharmacology, Biochemistry and Behavior 104:113-118. 2013

3. Deivis de Campos, Joel Henrique Ellwanger, Patrícia Severo do Nascimento, Helen Tais da Rosa, **Lisiani Saur**, Geraldo Pereira Jotz, Léder Leal Xavier. Sexual dimorphism in the human vocal fold innervation. Journal of Voice. (No Prelo) (2013)

4. Régis Gemerasca Mestriner, Patrícia Maidana Miguel, Pamela Brambilla Bagatini, **Lisiani Saur**, Lígia Simões Braga Boisserand, Pedro Porto Alegre Baptista, Léder Leal Xavier, Carlos Alexandre Netto. Behavior outcome after ischemic and hemorrhagic stroke, with similar brain damage, in rats. Behavioural Brain Research. (No Prelo) (2013)

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ORIGINAL PAPER

The role of calcium channel blockers and resveratrol in the prevention of paraquat-induced parkinsonism in *Drosophila melanogaster*: a locomotor analysis

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Abstract Studies have suggested that neuronal loss in Parkinson's disease (PD) could be related to the pacemaker activity of the substantia *nigra pars* compacta generated by L-type Ca_V 1.3 calcium channels, which progressively substitute voltage-dependent sodium channels in this region during aging. Besides this mechanism, which leads to increases in intracellular calcium, other factors are also known to play a role in dopaminergic cell death due to overproduction of reactive oxygen species. Thus, dihydropyridines, a class of calcium channel blockers, and resveratrol, a polyphenol that presents antioxidant properties, may represent therapeutic alternatives for the prevention of PD. In the present study, we tested the effects of the dihydropyridines, isradipine, nifedipine, and nimodipine and of resveratrol upon locomotor behavior in *Drosophila melanogaster*. As previously described, paraquat induced parkinsonian-like motor deficits. Moreover, none of the drugs tested were able to prevent the motor deficits produced by paraquat. Additionally, isradipine, nifedipine, resveratrol, and ethanol (vehicle), when used in isolation, induced motor deficits in flies. This study is the first demonstration that dyhidropyridines and resveratrol are unable to reverse the locomotor impairments induced by paraquat in *Drosophila melanogaster*.

Keywords Dihydropyridines · Resveratrol · *Drosophila melanogaster* · Paraquat · Parkinson's disease · Voltage-dependent calcium channels

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Introduction

Parkinson's disease (PD) is primarily characterized by motor symptoms including bradikinesia, akinesia, and tremor. PD patients present neuronal loss in different brain areas, mainly in the substantia *nigra pars* compacta (SNpc) and, to a lesser degree, in the ventral tegmental area (VTA) and retrorubral field (Halliday et al. 1996; Dauer and Przedborski 2003).

Recently, it has been suggested that the neuronal loss related to PD could be associated with SNpc neurons' pacemaker activity generated mainly by L-type voltage-dependent calcium channels (VDCC), which substitute voltage-dependent sodium channels (VDSC), in this region, during aging (Chan et al. 2007). In the VTA, a dopaminergic brain region damaged to a lesser extent in PD (Damier et al. 1999), the pacemaker activity is continuously generated by sodium channels, suggesting VDCC

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Physical exercise down-regulated locomotor side effects induced by haloperidol treatment in Wistar rats

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ABSTRACT

Extra-pyramidal symptoms (EPS) such as akinesia, dystonia, gait alteration and tremors are observed when dopamine D2-receptors are blocked by pharmacological agents such as haloperidol. These alterations produce a Parkinson disease-like state (PLS). Physical exercise has been proven to improve gait and locomotor symptoms in Parkinson's disease; we sought to elucidate the effects of physical exercise on PLS induced by chronic administration of haloperidol in rats. We used 48 rats distributed into four groups: Control, Exercise, Haloperidol, and Hal + Exe. All the animals received a daily injection of saline or haloperidol for 30 days, and the exercise groups underwent a daily 30-minute exercise protocol for 20 days. The animals were subjected to the ink–paw test, bar test and open-field test throughout the training period. The haloperidol-induced akinesia increased throughout the days of injections, but exercise was shown to alleviate it. The assessment showed shortened stride length and increased stance width with the use of haloperidol, which were significantly alleviated by exercise. These results indicate that exercise could be an interesting approach towards reducing unwanted EPS caused by haloperidol.

1. Introduction

Extrapyramidal symptoms (EPS) are a collection of motor side-effects that can arise with the use of dopamine D2-receptor blockers. Drugs of this nature are widely used in the treatment of psychotic illnesses such as schizophrenia and bipolarity (Inada et al., 2002). Haloperidol is an example of a dopamine antagonist and, although it belongs to the first generation of antipsychotics drugs (APD), it is still the reference treatment for schizophrenia (McCue et al., 2006). EPS presents a very specific set of motor deficits such as tremors, akinesia, dystonia and gait alterations (Lieberman et al., 2005; Miyamoto et al., 2005), which greatly resemble the motor characteristics observed in Parkinson's Disease (PD) patients and animal models (Amende et al., 2005; Guillot et al., 2008; Kurz et al., 2007). For this reason, APD is said to cause Parkinsonism (Peluso et al., 2012) or, as it will be referred to in this study, a Parkinson's-like state (PLS).

Physical exercise is widely prescribed to PD patients in an attempt to improve motor control and enhance life quality (Uitti, 2012). Treadmill training, in particular, has been shown to greatly improve the gait quality of PD patients (Herman et al., 2008) and in PD animal models (Pothakos et al., 2009). On the other hand, very little has been written about gait alterations in PLS induced by APD, with some studies merely mentioning the presence of a gait deficit in this state (Hansen et al., 1997; Lieberman et al., 2005). Additionally, previous studies have shown that physical exercise has some beneficial effects on EPS induced by haloperidol in rats (Teixeira et al., 2011).

Given that APD induces PLS, generating important gait alterations that are not completely understood, and that physical exercise has a beneficial effect on EPS (Herman et al., 2008; Uitti, 2012), the main goals of this study were to improve the knowledge about the motor gait deficit induced by D2 blockers and to investigate the effects of physical exercise in PLS induced by haloperidol.

2. Materials and methods

2.1. Animals

For this study, 48 male Wistar rats, three months old and weighing 200–300 g were obtained from the Institute of Basic Health Sciences (ICBS) — UFRGS. They were maintained in a controlled environment and housed in groups of five with food and water ad libitum, in a 12:12 h dark:light schedule. The animals were allocated into four groups (twelve each): 1 — Saline and Sedentary (Control), 2 — Saline and

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Research report

Behavior outcome after ischemic and hemorrhagic stroke, with similar brain damage, in rats

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HIGHLIGHTS

- ET-1 and collagenase could produce comparable ischemic and hemorrhagic injuries.
- Ischemic and hemorrhagic rats showed similar spontaneous locomotor activity.
- Ischemic and hemorrhagic rats showed similar forelimb asymmetry profile.
- Hemorrhagic stroke showed better performance in skilled walking then ischemic form.

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Anymaze

ABSTRACT

Stroke causes disability and mortality worldwide and is divided into ischemic and hemorrhagic subtypes. Although clinical trials suggest distinct recovery profiles for ischemic and hemorrhagic events, this is not conclusive due to stroke heterogeneity. The aim of this study was to produce similar brain damage, using experimental models of ischemic (IS) and hemorrhagic (HS) stroke and evaluate the motor spontaneous recovery profile. We used 31 Wistar rats divided into the following groups: Sham ($n = 7$), ischemic (IS) ($n = 12$) or hemorrhagic (HS) ($n = 12$). Brain ischemia or hemorrhage was induced by endotelin-1 (ET-1) and collagenase type IV-S (collagenase) microinjections, respectively. All groups were evaluated in the open field, cylinder and ladder walk behavioral tests at distinct time points as from baseline to 30 days post-surgery (30 PS). Histological and morphometric analyses were used to assess the volume of lost tissue and lesion length. Present results reveal that both forms of experimental stroke had a comparable long-term pattern of damage, since no differences were found in volume of tissue lost or lesion size 30 days after surgery. However, behavioral data showed that hemorrhagic rats were less impaired at skilled walking than ischemic ones at 15 and 30 days post-surgery. We suggest that experimentally comparable stroke design is useful because it reduces heterogeneity and facilitates the assessment of neurobiological differences related to stroke subtypes; and that spontaneous skilled walking recovery differs between experimental ischemic and hemorrhagic insults.

1. Introduction

Stroke is an important public health problem and is broadly subdivided into ischemic and hemorrhagic subtypes [1]. Although considerable development has been made in acute stroke care, current data on functional recovery according to stroke subtypes are not conclusive [2]. Clinical observations have shown that the hemorrhagic form presents greater functional impairment than ischemic stroke at hospital admission, but shows greater functional improvement at discharge [3]. On the other hand, ischemic

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Sexual Dimorphism in the Human Vocal Fold Innervation

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Summary: This study investigated the sexual dimorphism in the recurrent laryngeal nerve (RLN) and thyroarytenoid (TA) muscle, which control the vocal fold. The RLN and TA were bilaterally studied in human specimens obtained from necropsies (seven men and seven women). Analysis of the morphometric parameters showed that the RLN of the men were significantly larger, as shown by the intraperineural area (42.5%) ($P = 0.006$), total number of fibers (38.0%) ($P = 0.0002$), axonal area (34.3%) ($P = 0.0001$), axonal diameter (19.0%) ($P = 0.0001$), and the area of the nerve occupied by myelinated fibers (34.9%) ($P = 0.001$). By contrast, in women, our results showed that the area of the RLN occupied by endoneurial connective tissue was larger (5.7%) ($P = 0.001$). Estimation of the fiber area and shape coefficient showed that the histologic organization of TA is similar in men and women. These results may contribute toward enhancing our understanding about the voice neurobiology.

Key Words: Sexual dimorphism–Recurrent laryngeal nerve–Thyroarytenoid muscle–Voice.

INTRODUCTION

Several studies have demonstrated the presence of sexual dimorphism in the organization of the nervous system in different groups of vertebrates, such as amphibians,[1] reptiles,[2,3] birds,[4] and mammals.[5–9] Similarly, numerous studies with animals have shown sexual dimorphism in different regions of the nervous system involved in vocalization/vocal control.[4,10,11] However, little is known about this aspect in humans.

Although some authors have reported the existence of sexual dimorphism in the neural structures involved in vocal control at the level of the central nervous system in humans,[12] to our knowledge, there is no study in the current literature that shows the presence or absence of sexual dimorphism in structures related to the peripheral nervous system and muscles related to vocalization, especially in the recurrent laryngeal nerve (RLN) and thyroarytenoid (TA) muscle.

In addition, classically, the variability between the voices of men and women has been explained by the differences in the mass of the vocal folds.[13] This sexual dimorphism is attributable to increased testosterone at puberty in males, which stimulates growth in the laryngeal cartilages.[14] In the 20th century, the dominant model of sexual differentiation stated that genetic sex (*XX* vs *XY*) causes differentiation of the gonads, which then secrete gonadal hormones that act directly on tissues to induce sex differences in function. This serial model of sexual differentiation was simple, unifying, and seductive. Recent evidence, however, indicates that the linear model is incorrect and that sex differences arise in response to diverse sex-specific signals originating from inherent differences in the genome and involves cellular mechanisms that are specific to individual tissues or brain regions.[15]

Likewise, studies of songbirds and rodents suggest that male and female brain cells are also intrinsically different because of the sex differences in the expression of sex chromosome genes within the cells.[16] As those differences in gene expression and alterations in brain structures are often responsible for important changes in different body characteristics, such as innervation and morphology, our hypothesis is that sexual dimorphism may be present in the RLN and TA of subjects of different genders. This study aims to investigate this matter.

MATERIALS AND METHODS

Specimens

All the nerves and muscles analyzed were obtained from necropsies of 14 Caucasian subjects who had died suddenly (seven men [age = 71.14 ± 8.07 years] and seven women [age = 75.71 ± 7.83 years]) (mean ± standard deviation), from the Department of Forensic Medicine. It should be pointed out that although our analysis was performed in older individuals because of the difficulty in obtaining younger specimens, this limitation was also found in a previous study, in which the subjects with average age of 70 years for men and 75 years for women were analyzed.[17] Furthermore, in the present study, the men and women were in the same age group. This study was approved by the Ethics Committee of the *Universidade Federal do Rio Grande do Sul*, Rio Grande do Sul, Brazil.

Dissection

The dissection of the RLN was performed according to Jotz et al.[18] The TA muscle was bilaterally removed (~10 mm) from the larynx, and the middle region of the TA was chosen for our study. This choice was based on a previous study,[19] which showed that this region presents more and better defined

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