MANUELLA BATISTA DE OLIVEIRA

Condições de lactação, exercício físico e envelhecimento na prole do rato albino: suas repercussões sobre parâmetros eletrofisiológicos cerebrais e comportamentais.

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Orientador: Prof$^{o.}$ Dr$^{o.}$ Rubem Carlos Araújo Guedes

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BANCA EXAMINADORA

Professor Cristovam Wanderley Picanço-Diniz
Universidade Federal do Pará – Instituto de Ciências Biológicas

Professora Luciana Maria Silva de Seixas Maia
Universidade Federal de Pernambuco – Departamento de Histologia e Embriologia

Professora Angela Amâncio dos Santos
Universidade Federal de Pernambuco – Departamento de Fisiologia e Farmacologia

Professora Juliana Maria Carrazzone Borba
Universidade Federal de Pernambuco – Departamento de Nutrição

Professora Angelica da Silva Tenório
Universidade Federal de Pernambuco – Departamento de Fisioterapia

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Aos meus pais, ao meu irmão, a Eric, meus amores, minha essência.

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RESUMO

Numerosas evidências têm descrito a influência do exercício físico e do estado nutricional sobre aspectos estruturais e funcionais do sistema nervoso durante o envelhecimento. Este trabalho investigou no rato albino como o exercício físico, as condições de lactação e a senescência modulam aspectos eletrofisiológicos e comportamentais do funcionamento cerebral. Ratos machos albinos *Wistar* foram amamentados em ninhadas com 12 (L12) ou 6 (L6) lactentes, constituindo dois grupos com condições diferentes de lactação. Esses grupos foram divididos em sedentários e exercitados em fases diferentes da vida (jovens, adultos e idosos). A propagação do fenômeno da "depressão alastrante cortical" (DAC) foi registrada em dois pontos da superfície do cérebro, em diferentes fases da vida, ou seja, no período pós-desmame (45-60 dias de vida nos grupos exercitados na lactação), na fase adulta (120-130 dias) e na senescência (600 a 700 dias). A condição desfavorável de lactação (L12) aumentou, e os fatores envelhecimento e exercício físico diminuíram a velocidade de propagação da DAC, com interação entre os fatores apenas nos grupos idosos. Nestes, o período no qual o exercício físico foi realizado influenciou significativamente a DAC. O estilo de vida sedentário prejudicou a memória espacial de ratos idosos e adultos independentemente das condições de lactação e o exercício reduziu estes efeitos em animais idosos de ninhadas pequenas, mas não daqueles criados em ninhadas grandes. Por outro lado, apenas animais idosos sedentários de ninhadas grandes e pequenas apresentaram memória de reconhecimento de objetos prejudicada e o exercício reduziu este efeito, independente das condições de lactação. Os resultados auxiliam na compreensão dos mecanismos subjacentes à influência do exercício físico e do envelhecimento sobre funções cerebrais, associados ou não a distintas condições de lactação, durante o desenvolvimento do cérebro.

Palavras-chave: Condições de lactação; Depressão alastrante cortical; Envelhecimento; Excitabilidade cerebral; Exercício físico; modulação cerebral dependente da nutrição;

ABSTRACT

Several evidence have presented the influence of physical exercise and nutritional status on structural and functional aspects of the nervous system during aging. Thus, we investigated how physical exercise, lactation conditions and aging interact and modulate brain function through neurophysiological and behavioral aspects. Wistar male rats were suckled in litters with 12 (L12) or 6 (L6) pups, performing two groups with different lactation conditions. These groups were divided in exercised and sedentary in different stages of life (young, adults and aged). The propagation of the phenomenon named cortical spreading depression (CSD) was recorded in two points of the brain surface, in distinct periods, for young groups (45 – 60 days old), during adulthood (120 – 130 days old) and during senescence (600 – 700 days old). The unfavorable lactation condition (L12) increased, while aging and physical exercise decreased the CSD velocity of propagation with an interaction between the factors seen just in the aged group. In another words, in the old groups, the exercise timing significantly influenced the CSD. We found that sedentary lifestyle impaired spatial memory of both mature and aged rats independent of the litter size, and that exercise reduced these effects in aged animals from small but not large litters. On the other hand, only sedentary aged animals both from large and small litters had impaired object recognition memory, and exercise reduced this effect regardless of litter size. The results contribute to the comprehension of the underlying mechanisms for the influence of exercise and aging on brain function, associated or not to distinct lactation condition during brain critical development period.

Keywords: Aging; Brain excitability; Lactation condition; Treadmill; Physical Exercise; Nutrition-dependent brain modulation;

SUMÁRIO

1. Apresentação

Um número crescente de evidências tem descrito os impactos fisiológicos negativos causados por alterações estruturais e funcionais observadas durante o envelhecimento. A manipulação do estado nutricional e do nível de atividade física, ao longo da vida, tem sido sugerida como abordagem preventiva e terapêutica, em contraposição aos danos à saúde ocasionados pelo envelhecimento. O presente trabalho desenvolvido no Laboratório de Fisiologia da Nutrição Naíde Teodósio estudou experimentalmente como o exercício físico, as condições de lactação e a senescência podem modular a função cerebral em relação a aspectos eletrofisiológicos e comportamentais. O estudo descreveu também como o exercício físico em diferentes fases da vida pode minimizar efeitos eletrofisiológicos indesejáveis decorrentes daquelas alterações nutricionais precoces, efeitos estes avaliados durante o processo de envelhecimento.

O fenômeno da depressão alastrante cortical (DAC) foi utilizado como parâmetro neurofisiológico e os testes de reconhecimento de objetos foram utilizados para a avaliação comportamental. Ratos machos *Wistar* recém-nascidos foram distribuídos aleatoriamente para constituir os grupos L6 e L12, formados por filhotes amamentados em ninhadas com 6 e 12 lactentes, respectivamente. Tanto os animais L6 quanto os L12 foram divididos em sedentários e exercitados, estudados em fases diferentes da vida (jovens, adultos e idosos).

Para o estudo de parâmetros comportamentais, testes de reconhecimento de objetos foram realizados após o término do período de exercício ou sedentarismo, em ratos adultos e idosos. O eletrocorticograma (ECoG) e a propagação da DAC foram registrados na juventude (45-60 dias), na fase adulta (120-130 dias) e na senescência (600 a 700 dias) em dois pontos da superfície cortical.

O trabalho possibilitou descrever alterações eletrofisiológicas e comportamentais associadas aos fatores estudados. Estes resultados auxiliam na compreensão dos mecanismos subjacentes à

influência do exercício físico e do envelhecimento sobre funções encefálicas, associados ou não a alterações nutricionais e aos cuidados maternos durante o desenvolvimento cerebral no início da vida.

Além dos resultados eletrofisiológicos apresentados, a oportunidade de duas colaborações com pesquisadores de outras universidades (Universidade Federal do Pará e Universidade de Harvard) trouxe dados complementares ao projeto de Tese. Graças à colaboração com o professor Cristovam Wanderley Picanço-Diniz e o seu grupo da Universidade Federal do Pará, o desempenho comportamental destes animais e as análises de células imunes inatas do sistema nervoso (microglias) foram realizadas em camadas do giro denteado de ratos. As análises da expressão microglial forneceram dados adicionais à compreensão da ação moduladora das condições de lactação e do exercício físico sobre a resposta inflamatória cerebral associada ao envelhecimento.

Por outro lado, a colaboração com o professor Felipe Fregni e o seu grupo da Universidade de Harvard ofereceu a oportunidade de acrescentar à formação da doutoranda, uma etapa de doutorado sanduíche naquela instituição. Nesse estágio-sanduíche, a aluna familiarizou-se com diferentes técnicas de estimulação elétrica e magnética transcranianas, utilizadas em seres humanos, relacionadas à neuromodulação e à avaliação da atividade cerebral. Em anexo, serão apresentados os frutos da colaboração com o estágio-sanduíche.

2.0 Objetivos

2.1 Objetivo geral

Avaliar, em ratos jovens, adultos e idosos submetidos a diferentes condições de aleitamento, os efeitos agudos e crônicos do exercício físico forçado em diferentes fases da vida sobre parâmetros comportamentais e eletrofisiológicos.

2.2 Objetivos específicos

- Acompanhar a evolução ponderal, como indicador do impacto das condições de lactação sobre o peso corporal;
- Averiguar os efeitos do exercício físico em diferentes momentos da vida, ou seja, em ratos jovens (entre 15-45 dias), adultos (90-120 dias) e idosos (530-600 dias), sobre a propagação da DAC;
- Investigar os efeitos do exercício físico sobre o reconhecimento de objetos, como indicador de alterações comportamentais;
- Avaliar se os efeitos, sobre os parâmetros eletrofisiológicos (registro da DAC) e comportamentais (reconhecimento de objetos), do exercício físico em fases distintas da vida, são influenciados pelas condições de lactação;

3.0 Revisão da literatura

3.1- Nutrição, envelhecimento e exercício físico: aspectos eletrofisiológicos

O envelhecimento é um processo fisiológico que envolve um conjunto multifatorial de mudanças deletérias no organismo. Dentre essas alterações, atenção especial tem sido dada àquelas decorrentes do estresse oxidativo, acumulado ao longo do tempo, que pode ser considerado "força propulsora" ("driving force") do envelhecimento celular (Kern e Behl, 2009). O aumento do contingente humano em fase de envelhecimento tem se tornado uma realidade crescente em todo o mundo. Infelizmente, muitas vezes o envelhecimento celular está associado a doenças crônicas não-transmissíveis (Lobo et al., 2000) que, além de representar um ônus econômico-social, ameaçam a saúde e a expectativa de uma vida ativa.

As pesquisas conduzidas nesse tema têm o intuito de melhor compreender os mecanismos envolvidos no processo de envelhecimento e assim contribuir para preservar a qualidade de vida da população idosa, mas ainda há muito por se esclarecer. Alguns estudos buscam alternativas para tentar reduzir os impactos fisiológicos negativos causados pelas alterações estruturais e funcionais observadas na senescência. Dentre estas alternativas, pode-se encontrar a manipulação do estado nutricional e o nível de atividade física, impostos ao longo da vida (Heilbronn et al., 2006; Fontana, 2009; Walford, 1985; Berchtold, et al. 2005; 2010; Chen et al., 2008; Cotman e Berchtold, 2002; Greenwood, et al. 2007).

Dados da literatura demonstram de forma marcante que para o sistema nervoso as fases iniciais da vida representam um período crítico. Isto se dá porque, nesse período, os processos de hiperplasia, hipertrofia, mielinização e migração neuronal, dentre outros, ocorrem com velocidades máximas, em relação a outras etapas da vida, o que torna o cérebro mais vulnerável às agressões do ambiente, inclusive as nutricionais (Dobbing, 1968; Morgane et al., 1993). Nos seres humanos o período crítico de desenvolvimento do sistema nervoso se inicia intra-útero, no terceiro trimestre de gestação, e se estende principalmente até os primeiros dois a quatro anos de vida. No rato albino, o mamífero mais usado para estudos experimentais sobre o tema, ele compreende as três primeiras

semanas de vida pós-natal, ou seja, o período de aleitamento (Smart e Dobbing, 1971). O fato de se reportar às fases iniciais da vida em estudos sobre envelhecimento se baseia em que, diferentemente dos demais sistemas, alguns danos ocasionados ao sistema nervoso durante o período crítico de desenvolvimento cerebral poderão não mais ser revertidos e persistir até a idade adulta (Morgane et al., 1993; Guedes et al., 1996; Guedes, 2011; Almeida et al., 2002), o que poderia talvez influenciar o processo de envelhecimento. Durante esses períodos de crescimento e maturação cerebral, há alguns momentos "vulneráveis", nos quais os efeitos deletérios da nutrição inadequada podem interferir criticamente. A nutrição inadequada pode influenciar os mecanismos reguladores do desenvolvimento, produzindo alterações estruturais e metabólicas do SN em desenvolvimento (Morgane et al., 1993; Grantham-McGregor, 1990; Guedes, 2011).

Apesar de estudos demonstrarem um declínio progressivo da incidência de desnutrição infantil nos países em desenvolvimento (Onis et al., 2000), a desnutrição em crianças ainda é considerado um sério problema de saúde pública nestes países, inclusive em algumas regiões do Brasil. Os efeitos da desnutrição sobre o desenvolvimento do sistema nervoso central (SNC) têm sido estudados, principalmente, devido à incidência da desnutrição infantil e às evidências consideráveis de seus efeitos neurais, alguns deles permanentes. Estes últimos estão geralmente associados com danos à função mental, inclusive *déficits* da inteligência (Grantham-McGregor, 1990 e Nahar et al., 2009).

Por outro lado, outros fatores que também parecem amenizar o impacto negativo do envelhecimento sobre as funções corporais, são o enriquecimento ambiental e o exercício físico. Dados da literatura têm evidenciado que a estimulação ambiental é capaz de minimizar seqüelas fisiológicas decorrentes de insulto nutricional precoce ocasionado pela desnutrição (Santos-Monteiro et al., 2000). Da mesma maneira, tem sido demonstrado que o enriquecimento ambiental promove neurogênese no hipocampo de ratos idosos (Segovia et al., 2006), a qual parece diminuir

com a idade tanto para ratos Bizon e Gallagher, 2003), quanto para camundongos (Kempermann, 1998).

Em relação ao exercício físico, tem sido mostrado que a manutenção, ao longo da vida, de certo nível desta prática são pré-requisitos para um envelhecimento bem-sucedido, embora respostas definitivas sobre os mecanismos fisiológicos responsáveis por este processo ainda não estejam bem estabelecidas (Godde et al., 2002). Foi demonstrado que o aumento das citocinas inflamatórias acelera o envelhecimento celular (Chen et al., 2008).

O envelhecimento, por sua vez, pode facilitar complicações comportamentais associadas a processos inflamatórios, provavelmente devido ao aumento da expressão dessas citocinas inflamatórias em áreas cerebrais responsáveis por mediar o processamento cognitivo. O exercício físico parece reduzir as respostas inflamatórias do tecido neural, ocasionadas naturalmente durante o envelhecimento, e dessa forma, retardar e/ou minimizar os fatores de risco periféricos responsáveis pelo declínio cognitivo e a neurodegeneração que acompanham este processo fisiológico (Berchtold et al, 2010). O exercício físico regular está relacionado a processos adaptativos que trazem efeitos benéficos ao funcionamento cerebral, incluindo aprendizagem, potenciação de longo prazo e memória (Praag et al., 1999; Radak et al., 2001; Ogonovszky et al., 2005). O exercício físico parece contribuir para manutenção da integridade cerebrovascular, aumentar o crescimento dos capilares, aumentar as conexões dendríticas (Pysh e Weiss, 1979; Ding et al., 2006; Lucas et al., 2012), bem como a eficiência do processamento de informações no sistema nervoso central (Dustman et al., 1990; Berchtold et al., 2010; Lucas et al., 2012).

Em nível comportamental, o exercício pode facilitar a aquisição e a retenção de informações em ratos jovens e idosos, em testes como o do labirinto aquático de Morris (Praag, et al., 2005; Mello et al., 2008) e o de reconhecimento de objetos (O'Callaghan, et al., 2007; Mello et al., 2008). Além dos efeitos sobre a citoarquitetura hipocampal e sobre propriedades eletrofisiológicas citados anteriormente, o exercício físico aumenta os níveis de proteínas sinápticas, como sinapsinas e sinaptofisinas (Vaynman, et al. 2006), receptores glutamatérgicos (Farmer, et al. 2004) e a

disponibilidade de fatores tróficos, incluindo BNDF (fator neurotrófico derivado do encéfalo) [Berchtold, et al. 2005] e o IGF-1 (fator trófico semelhante à insulina) [Trejo, et al. 2001]. Fatores tróficos estão implicados na sobrevivência e diferenciação celular, em alterações nas conexões sinápticas, memória e resistência aumentada ao estresse oxidativo (Leeds et al., 2005; Klumpp et al., 2006).

Embora evidências relevantes demonstrem que o exercício pode facilitar o aprendizado em humanos e outros animais, há uma lacuna no conhecimento sobre os tipos de aprendizado que são aprimorados pelo exercício. Essa lacuna recentemente começou a ser preenchida (Berchtold et al, 2010), incluindo os mecanismos subjacentes a essa influência do exercício físico sobre a estrutura e função encefálica nas diferentes fases da vida. A maior parte dos benefícios neurais do exercício físico parece ocorrer dependendo do número de sessões, ou seja, por período de maior duração (3 a 12 semanas, no rato) [Praag, et al., 2005 e O'Callaghan, et al, 2007], embora alguns autores tenham demonstrado, em camundongos, benefícios sobre a função sináptica mesmo após apenas três dias de exercício (Vaynman, et al. 2006).

Esses dados indicam que inúmeros fatores podem influenciar o processo de envelhecimento, mas é difícil precisar em que medida cada um deles participa, se os efeitos são generalizados a todos os sistemas orgânicos, bem como os mecanismos envolvidos. Dessa maneira, estudos experimentais relacionados ao processo fisiológico de envelhecimento e suas possíveis implicações na qualidade de vida, parecem ser de grande relevância, com impactos inclusive para a área social. Nesse cenário, este trabalho investigou se o estado nutricional nas fases iniciais da vida (no período de aleitamento) influencia a eletrofisiologia do sistema nervoso do rato idoso. Investigou-se também se o exercício físico poderia minimizar os possíveis efeitos indesejáveis decorrentes daquelas alterações nutricionais. A depressão alastrante cortical foi utilizada como parâmetro neurofisiológico (descrito no próximo tópico).

3.2 Depressão Alastrante Cortical (DAC)

A DAC foi descrita pela primeira vez como uma "onda" propagável, de depressão da atividade elétrica cortical espontânea (Leão, 1944). É uma resposta reversível do tecido cortical, provocada por estimulação elétrica, mecânica ou química, de um ponto desse tecido. Ela propaga-se de forma concêntrica por todo o córtex (com velocidade da ordem de 2 a 5 mm/min) e ao final de 10 a 15 min o tecido cortical acha-se recuperado. À medida que a DAC se propaga para regiões cada vez mais afastadas, a atividade elétrica começa a se recuperar a partir do ponto estimulado. Concomitante à depressão da atividade elétrica espontânea, foi descrita uma variação lenta de voltagem (VLV) na região cortical onde estava ocorrendo a DAC (Leão, 1947).

Desde a primeira descrição da DAC muitos estudos têm sido feitos para esclarecer os processos responsáveis por este fenômeno. O envolvimento de alguns íons (Guedes e Carmo, 1980), do sistema serotoninérgico (Amâncio-dos-Santos et al, 2006 e Guedes, 2002), do sistema gabaérgico (Guedes, 1992) e do colinérgico (Guedes e Vasconcelos, 2008) têm sido sugeridos. Em várias condições fisiopatológicas de importância clínica, como o envelhecimento (Guedes et al, 1996), a desnutrição (Guedes, 1987 e Rocha-de-Melo e Guedes, 1997), a estimulação ambiental (Santos-Monteiro et al., 2000) e a privação sensorial (Tenório et al., 2009) têm sido demonstradas alterações da propagação da DAC em modelos animais.

O uso do exercício físico ao longo da vida pode também representar estimulação multi-sensorial capaz de interferir na excitabilidade cerebral e na memória e/ou no aprendizado. Nesse contexto, os testes de reconhecimento de objetos em campo aberto foram utilizados, neste trabalho, para o estudo de parâmetros comportamentais relacionados à memória, como abordagem adicional ao estudo do fenômeno da DAC, este último considerado como modelo de estudo da excitabilidade cortical.

3.3 Nutrição, envelhecimento e exercício físico: aspectos comportamentais

O aumento na estimulação sensorial parece ser capaz de retardar e/ou minimizar os fatores de risco periféricos responsáveis pelo declínio cognitivo e a neurodegeneração que acompanham o processo de envelhecimento (Cotman et al., 2007). Dos estudos que têm investigado os efeitos do exercício sobre a memória, recentemente alguns têm utilizado para isto o teste de reconhecimento de objetos. Esse teste permite uma avaliação da capacidade do animal explorar e reconhecer objetos. Esse reconhecimento baseia-se na forma ou localização espacial destes objetos, podendo-se quantificar o tempo que o rato utiliza tocando um objeto pelo menos com o focinho com a finalidade de reconhecê-lo (O'Callaghan et al., 2007 e Kelly et al., 2003).

Os testes de reconhecimento de objetos perecem ser capazes de demonstrar aquisição e retenção de informações com o uso de aspectos relacionados à memória episódica. Estes testes auxiliam na avaliação da memória episódica para "o que", "onde" e o "quando" ao combinar versões diferentes para paradigmas de preferência da novidade (Dere et al., 2005). O tópico 4.3 descreve os métodos utilizados para avaliar o reconhecimento de objetos.

Neste projeto, pretendeu-se avaliar aspectos relacionados à memória para o reconhecimento de objetos pelo rato adulto jovem e idoso, amamentados em diferentes condições, a fim de investigar as mudanças observadas ao longo do envelhecimento. Investigou-se, de uma maneira pioneira, se tais mudanças seriam modificadas pelo exercício físico e condições de lactação.

4.0 Materiais e métodos

Ratos machos neonatos da linhagem *Wistar,* da colônia do Departamento de Nutrição da Universidade Federal de Pernambuco foram distribuídos aleatoriamente 24h após o nascimento de acordo com duas condições de lactação (denominadas **L6** e **L12**), segundo descrito no tópico adiante. Em ambas as condições de lactação, os animais foram subdivididos em dois grupos, denominados de **exercitados** e **sedentários**, de acordo com o exercício físico. Finalmente, os animais foram ainda subdivididos em distintos grupos etários (**jovens**, **adultos** e **idosos**), segundo a idade em que foram estudados. No total, foram analisados dezoito grupos, conforme a divisão apresentada na Tabela 1.

Tabela 1. Os grupos experimentais estão descritos de acordo com as condições de lactação, realização do exercício ou sedentarismo e o fator idade. O número de ratos por grupo está apresentado entre parênteses.

Grupo	Condição de lactação	Condição de exercício	Grupo por idade	Período de experimentação
1	[+] L6 (N=81)	[++] Ex (N=52)	**J**ovem (n=10)	E1 (15 - 45d)
2			**Ad**ulto (n=14)	E1 (15 - 45d)
3				E2 (90 – 120d)
4			**Id**oso (n=28)	E1 (15 - 45d)
5				E2 (90 – 120d)
6				E3 (530 – 600d)
7		Sed (N=29)	**J** (n=8)	15 – 45d
8			**Ad** (n=10)	90 - 120d
9			**Id** (n=11)	530 – 600d
10	L12 (N=80)	Ex (N=44)	**J** (n=9)	E1 (15 - 45d)
11			**Ad** (n=14)	E1 (15 - 45d)
12				E2 (90 - 120d)
13			**Id** (n=21)	E1 (15 - 45d)
14				E2 (90 - 120d)
15				E3 (530 – 600d)
16		Sed (N=36)	**J** (n=10)	(15 - 45d)
17			**Ad** (n=13)	(90 - 120d)
18			**Id** (n=13)	(530 – 600d)

[+] As ninhadas eram formadas por 6 (L6) ou 12 filhotes (L12). [++] Sed para sedentários e Ex para ratos exercitados.

E1, E2 e E3 indicam grupos exercitados em idades diferentes: respectivamente aos 15-45 dias, 90-120 dias e 530-600 dias de vida.

4.1 Manipulação Nutricional

A manipulação do estado nutricional foi realizada através da modificação do número de filhotes em cada ninhada, conforme descrito por Rocha-de-Melo et al (2004; 2006). Assim, utilizamos grupos formados por filhotes amamentados em condições de lactação diferentes, em ninhadas contendo 12 e 6 lactentes; esses dois grupos nutricionalmente distintos foram chamados: L12 e L6, respectivamente. Após o desmame, aos 21 dias, todos os filhotes passaram a receber a dieta de manutenção do biotério ("Labina", com 23% de proteína).

4.2 Exercício físico nas diferentes fases da vida

Os ratos foram subdivididos em grupos por faixas etárias diferentes; alguns iniciaram o exercício físico aos 15 dias de idade, outros aos 90 dias e os demais aos 530 dias, perfazendo respectivamente o grupo de jovens, adultos e idosos exercitados, os quais foram comparados aos grupos sedentários correspondentes.

4.2.1 Procedimentos gerais para o exercício físico

Os animais foram submetidos ao exercício físico forçado, representado pela corrida em esteira motorizada (Insight EP-131, 0º inclinação), em diferentes fases da vida, conforme adaptação de parâmetros de exercício moderado descritos na literatura (Scopel et al., 2006 e Gomes-da-Silva et al., 2010). O exercício físico teve a duração de cinco semanas. Nas três primeiras semanas, os animais foram submetidos a cinco sessões por semana (uma sessão por dia, de segunda a sexta-feira), com duração de 30 minutos por sessão.

A velocidade da corrida na esteira aumentou gradualmente de 5 para 10 e depois para 15 m/min, na primeira, segunda e terceira semanas, respectivamente. Na quarta e quinta semanas de

atividade física, foram realizadas, respectivamente, três e duas sessões de 45 min em dias alternados a uma velocidade de 25m/min. A tabela 2.0 ilustra estes parâmetros.

No grupo sedentário, os animais passaram pelos mesmos procedimentos descritos acima, sendo colocados na esteira pelo mesmo tempo, porém a esteira permaneceu desligada.

Tabela 2.0 Parâmetros de realização do exercício físico dos ratos L6 ou L12, aos 15, 90 ou 530 dias de idade, perfazendo respectivamente o grupo de jovens, adultos e idosos exercitados.

Semanas de treino	**1ª semana**	**2ª semana**	**3ª semana**	**4ª semana**	**5ª semana**
Duração de cada sessão diária	30 min	30 min	30 min	45 min	45 min
Número de sessões de treino	5	5	5	3	2
Velocidade percorrida	5 m/min	10 m/min	15 m/min	25 m/min	25 m/min

4.3 Avaliação comportamental

4.3.1 Tarefa de reconhecimento de objetos

O aparato consiste em uma arena (campo aberto) localizada em um ambiente com iluminação reduzida. Logo após a última sessão de exercício físico, os ratos foram colocados, por 5 minutos na arena, para se adaptarem ao ambiente e a sessão de teste foi realizada no dia seguinte à adaptação. Nestes testes, foram avaliadas as diferenças, entre os grupos, na capacidade de identificação de objetos com base na sua forma e localização no campo aberto.

Em cada uma dessas três tarefas, os animais, em uma primeira sessão, exploraram por 5 minutos o ambiente, enquanto dois examinadores (de modo "cego") treinados previamente, utilizando cronômetros, registraram o tempo gasto pelo animal para explorar cada objeto (ver abaixo). Numa segunda sessão, após 50 minutos, foram avaliados o reconhecimento das características de forma e localização espacial dos objetos, como descrito adiante. Se, nessa segunda análise, diante de dois objetos, um conhecido e outro desconhecido, o rato reconheceu o objeto apresentado na primeira análise, ele, então, passaria mais tempo explorando o objeto desconhecido, demonstrando assim reconhecimento do objeto previamente apresentado. Entre as sessões, os objetos, bem como o campo aberto, foram adequadamente limpos com álcool a 70%, para eliminar pistas olfativas que pudessem influenciar o ensaio seguinte.

O critério para definir exploração foi baseado na "exploração ativa", ou seja, quando o animal está tocando os objetos pelo menos com o focinho (O'Callaghan, et al., 2007; Mello et al., 2008; Dere et al., 2005). Ennaceur and Delacour (1988) (apud Dere et al., 2005) demonstra esses métodos utilizados para os testes de reconhecimento de objetos, brevemente descritos a seguir:

(1) Na **discriminação das formas**: dois objetos idênticos (A e B) foram posicionados na arena para a primeira análise. Após 50 minutos, os animais foram recolocados no campo aberto (segunda sessão) com o mesmo objeto A (conhecido), porém, o objeto B foi substituído por outro, C (desconhecido), da mesma cor, tamanho e cheiro do objeto A, mas com uma forma diferente. O

animal demonstra que pode diferenciar as formas quando, nessa segunda sessão, passa mais tempo explorando o objeto com a forma desconhecida.

(2) Para avaliar a **distinção de localização espacial**: dois objetos idênticos (A e B) foram colocados em determinadas posições no campo aberto. Passados 50 minutos, os animais foram novamente colocados no campo aberto (segunda sessão) na presença dos mesmos objetos (A e B), todavia, neste segundo momento a posição de A se mantém (posição conhecida), já a localização de B modifica-se. Se o animal distingue uma posição desconhecida, ele gasta mais tempo explorando o objeto nessa posição.

4.4 Determinações Ponderais

Para acompanhar a evolução ponderal, o peso corporal foi obtido aos 7, 14, 21, 60, 90, 600 dias de vida e no dia do registro eletrofisiológico da DAC. Os ratos foram pesados em balança Marte (modelo 1001).

Os pesos corporais foram comparados entre os grupos nutricionais (L6 e L12) e de exercício físico (sedentários e exercitados) e analisados estatisticamente com a ANOVA, seguida do teste "post hoc" (Tukey), quando indicado. As diferenças em que $p \leq 0,05$ foram consideradas significantes.

4.5 Procedimentos cirúrgicos para os registros eletrofisiológicos

Nos grupos jovens, adultos e idosos, os registros eletrofisiológicos foram realizados, respectivamente aos 45-60 dias, aos 120-130 dias, e aos 600-700 dias. Nessas idades, os animais foram anestesiados com uma solução de uretana 10% + cloralose 0,4%, à dose de 1000 mg/kg de uretana + 40 mg/kg de cloralose, via intra-peritoneal. O animal permaneceu respirando espontaneamente e foi colocado em decúbito ventral sobre um aquecedor elétrico de temperatura

regulável, para manutenção da sua temperatura retal em 37,5 ± 1°C, que foi verificada continuamente por um termômetro.

Em seguida, a cabeça do animal foi fixada à base de um aparelho estereotáxico (marca "David - Kopf" USA, modelo 900), de modo que permitiu a incisão da pele e a remoção do periósteo para exposição do crânio. Por meio de trepanação, foram feitos 3 orifícios, de cerca de 2 a 4 mm de diâmetro cada, em um dos lados do crânio, alinhados no sentido antero-posterior e paralelamente à linha média.

4.6 Registro eletrofisiológico

Os registros eletrofisiológicos foram feitos com eletrodos do tipo "Ag-AgCl", confeccionados no próprio laboratório (ver Guedes et. al.,1992), conectados a um polígrafo modelo 7D (Grass Medical Instruments). Os registros da variação lenta de voltagem (VLV) que acompanha a DAC foram feitos durante 4 horas, por 1 par de eletrodos "registradores", localizados em um dos hemisfério na área parietal. Um terceiro eletrodo do mesmo tipo foi colocado sobre os ossos nasais e serviu de referência comum ("eletrodo de referência") aos 2 eletrodos registradores.

A DAC foi provocada a cada 20 minutos, por meio de estimulação química, com uma pelota de algodão de 1 a 2 mm de diâmetro, embebida em uma solução de cloreto de potássio (KCl) a 2%, colocada durante 1 minuto sobre um ponto da superfície cortical através do orifício de estimulação, na região frontal. A propagação da DAC foi observada através do registro eletrofisiológico em dois pontos da região parietal, denominados pontos 1 e 2, respectivamente.

A velocidade de propagação da DAC foi calculada com base na distância entre os eletrodos registradores e no tempo gasto pela DAC para percorrer esta distância. Diferenças inter-grupos, dessas velocidades, foram analisadas estatisticamente com a ANOVA, seguida de teste "post hoc" (Tukey), quando indicado, tendo-se como fatores as condições de lactação (L6 e L12), o exercício

físico (sedentário e exercitado) e a idade (jovem, adulto e idoso), sendo consideradas significantes as diferenças em que $p \leq 0,05$. Além do cálculo da velocidade de propagação da DAC, as amplitudes das ondas de variação lenta de voltagem que acompanham a DAC também foram avaliadas.

5.0 Resultados e discussão – artigos originais

5.1 ARTIGO 01

TITLE: Aging-dependent brain electrophysiological effects in rats after distinct lactation conditions, and treadmill exercise: A spreading depression analysis

AUTHORS: Manuella Batista-de-Oliveira, Andréia Albuquerque Cunha Lopes, Rosangela Figueiredo Mendes-da-Silva, Rubem Carlos Araujo Guedes

AFFILIATION: Laboratory of Nutrition Physiology, Department of Nutrition, Universidade Federal de Pernambuco, 50670-901 Recife, PE, Brazil

Corresponding Author: Rubem C. A. Guedes, MD, PhD

Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE - CEP: 50670-901 |

Phone: +55 81 2126-8936

Email: guedes.rca@gmail.com or rguedes@ufpe.br

Abstract

Aging-related neurophysiological alterations are a matter of growing concern in gerontology. Physical exercise has been therapeutically employed to ameliorate aging-associated deleterious neurological changes. The aging process, as well as the effects of treadmill exercise on brain excitability, can be influenced by nutritional demands during lactation. In this study we investigated whether physical exercise, lactation conditions, and aging interact and modulate brain electrophysiology as indexed by the excitability-related phenomenon known as cortical spreading depression (CSD). Wistar male rats were suckled in litters of 12 or 6 pups (constituting two groups named L12 and L6), with different lactation conditions. Each group was subdivided into exercised (treadmill) and sedentary. CSD was recorded immediately after the exercise period for young, adult, and aged groups (respectively 45–60, 120–130, and 600–700 days old). In L6 groups, the mean CSD velocity (in mm/min) ranged from 2.57±0.24 in aged rats to 3.67±0.13 in young rats, indicating an aging-related CSD deceleration. The L12 condition accelerated CSD (velocities ranging from 3.11±0.21 to 4.35±0.16 in aged and young rats, respectively) while treadmill exercise decelerated it in both L6 groups (range: 3.02±0.19 to 2.57±0.24) and L12 groups (3.32±0.16 to 3.11±0.21), with an observed interaction between factors in the aged group. Furthermore, aging led to a significant failure of CSD propagation. These results contribute to the understanding of underlying mechanisms by which exercise and aging influence brain electrophysiological functioning, previously associated with distinct lactation conditions during the period of brain development.

Keywords: Aging; Brain excitability; Lactation condition; Treadmill exercise; Aging-dependent brain modulation

1. Introduction

The increasing population of elderly facing the physiological disturbance of aging and related neurodegenerative diseases has become a worldwide concern. Aging consists of a natural process associated with deleterious changes in the different organic systems. In addition to representing a social and economic problem, cellular aging has been associated with degenerative diseases that threaten the expectations of a healthy, long-lasting life. Nutritional support and physical exercise have been considered a valuable therapeutic approach to reduce aging's negative impacts on the organism (Fontana, 2009; Berchtold et al., 2010; Chen et al., 2008). The optimal time of life stage to better prevent aging-related deleterious changes is still unknown. However, translational investigations have focused on one unique sensitive phase of life, the perinatal period, when early-life experiences can confer enduring effects on brain structure and function (Korosi et al., 2012).

In the human nervous system, the critical development period begins in the third trimester of pregnancy and lasts until two to four years of age. In the rat, which is the most commonly used mammal in experimental neurophysiological studies, the critical phase of brain development includes both the gestation and lactation periods (Morgane et al., 1993). Physiological brain events during this phase occur in a similar sequence in the rat and in human beings, albeit on a different time scale (days versus months for rats and humans, respectively) (Smart and Dobbing, 1971). Physiological processes such as hyperplasia, hypertrophy, myelination, and neuronal migration occur with maximal velocity during this critical period, in comparison with other phases of life. In this case, the brain becomes more vulnerable to environmental demands, including the conditions under which lactation is carried out (Dobbing, 1968; Morgane et al 1993; Zippel et al., 2003). The deleterious effects of inadequate lactation can influence the regulatory mechanisms of the developmental process, leading to metabolic and structural alterations in the developing nervous system (Morgane et al., 1993; Guedes, 2011). Therefore, there is a growing interest in studying the

effects of early-life environmental demands on the aging process, since damages to the nervous system that occur during the critical brain development period can persist until adulthood, and may be irreversible (Morgane et al., 1993; Guedes, 2011). Therefore, early developmental events may influence brain function during aging.

Because of the great incidence of childhood undernourishment (Fanzo and Pronyk, 2011) and related neural effects (Frazão et al., 2008; De Frías et al., 2010), the effects of unfavorable lactation conditions on nervous system development have been widely studied (Tenório et al., 2009; Rocha-de-Melo et al., 2006; Zippel et al., 2003). Some neural effects are long-lasting and are followed by damage to mental function, leading to cognitive deficits (Nahar et al., 2009). Currently, factors such as environmental enrichment and physical exercise are considered capable of attenuating negative effects on the brain that are caused by physiological changes associated with aging (Kobilo et al., 2011; O'Callaghan et al., 2007).

In the rat brain, environmental stimulation can reduce the electrophysiological consequences of nutritional aggression generated by undernourishment during suckling (Santos-Monteiro et al., 2000). Environmental enrichment promotes neurogenesis in the hippocampus of young and aged rats (Segovia et al., 2006), and a lifestyle involving prolonged and maintained physical activity has been associated with "successful aging" (Godde et al., 2002). However little information is available concerning the long-lasting electrophysiological effects (observed at adulthood and in the elderly) of physical exercise episodes performed at earlier stages of life (during youth or adulthood, respectively). Therefore, experimental studies related to the process of aging and its effects on life quality appear to be of great relevance, with an important impact on society. We used a phenomenon known as cortical spreading depression (CSD) to investigate these points electrophysiologically.

CSD was first described as a slowly propagating wave of depression of spontaneous cortical electrical activity (Leão, 1944). The phenomenon is fully reversible and can be elicited by

chemical, mechanical, or electrical stimulation of one point in the cortical tissue. Cortical electrical activity has been completely recovered ten to 15 min after CSD elicitation, and this recovery process begins from the first stimulated point. Simultaneous to the depression of spontaneous electrical activity, a slow direct-current potential change (SPC) appears in the cortical region where the CSD is observed, and this "all or none" signal constitutes the hallmark of the phenomenon (Leão, 1947). The neural tissue usually presents some resistance to CSD, and its propagation velocity is inversely related to that resistance (Amaral et al., 2009). As presently demonstrated, the brain's susceptibility to CSD can be easily estimated by determining the velocity of the CSD along the cortical tissue. Furthermore, by characterizing changes in the brain's inherent capability to propagate CSD, as a consequence of experimental conditions like those detailed in the present work, we are able to provide knowledge about CSD-related diseases, such as migraine (Lehmenkühler et al., 1993) and epilepsy (Guedes and Cavalheiro, 1997; Guedes et al., 2009). In this context, the present work addressed three issues: (1) Would lactation conditions influence the brain CSD during aging? (2) Could the adoption of treadmill exercise during different life stages (youth, adulthood, and senescence) counteract the deleterious brain CSD effects provoked by early nutritional imbalance? (3) Would the interaction between the effects of lactation conditions and treadmill exercise on CSD depend on the degree of aging? An abstract that discusses part of these results has been presented previously (Batista-de-Oliveira et al., 2010).

2. Methods

2.1. Animals and lactation conditions

Newborn male Wistar rat pups born from distinct dams were randomly distributed to be suckled in litters of either 6 pups (group L6; n=81) or 12 pups (group L12; n=80) to represent two distinct lactation conditions that differentially affect the pups' nutritional status, as previously described (Rocha-de-Melo et al., 2006; Frazão et al., 2008; Tenório et al., 2009). All experiments

were carried out at the Universidade Federal de Pernambuco in accordance with the guidelines of the Institutional Ethics Committee for Animal Research, which comply with the "Principles of Laboratory Animal Care" (National Institutes of Health, Bethesda, USA). Animals were raised from birth until the day of the electrophysiological recording in a room with a temperature of 23±1°C and a 12-h light/dark cycle (lights on from 7:00 am to 7:00 pm), with free access to food and water. After weaning, all pups were housed in groups of 3–4 per cage (51 × 35.5 × 18.5 cm), and maintained on a commercial laboratory chow diet (Purina do Brazil Ltd., Paulinia, São Paulo, Brazil) with 23% protein. Body weights were measured at postnatal days 7, 14, 21, 60, 90, and 600.

2.2. Treadmill exercise

Both nutritional groups were subdivided into sedentary and exercised. The exercised groups were subjected to treadmill running at three different ages: from 15 to 45 days, from 90 to 120 days, and from 530 to 600 days of life (young, adult, and aged animals, respectively).

All rats exercised in a treadmill apparatus (Insight EP-131, 0° inclination) following the parameters of moderate exercise, as previously described (Scopel et al., 2006; Gomes-da-Silva et al., 2010). Periods of treadmill exercise lasted 5 weeks. During the first 3 weeks, the animals were subjected to the treadmill for 30 min/day (from Monday to Friday). The treadmill running velocity was increased from 5 m/min during the first week to 10 m/min during the second week, and increased again to 15 m/min during the third week. During the fourth and fifth weeks, the treadmill running velocity was increased to 25 m/min, and rats were subjected to three and two 45-min sessions, respectively, on alternate days. Rats from the sedentary groups were placed in the treadmill for the same period as the exercised animals, but the treadmill was not turned on. Table 1 summarizes the group distribution.

2.3. Recording cortical spreading depression

In the three age groups, CSD was recorded at 45-60, 120-130, and 600-700 days of life. Three trephine holes (2–4 mm diameter) were drilled in the right hemisphere of each rat under anesthesia with a mixture of 1g/kg urethane plus 40 mg/kg chloralose (both from Sigma Co., USA). Only the right hemisphere was used for CSD elicitation and recording. The holes were aligned in the anteroposterior direction and parallel to the midline (the dura mater was left intact). During surgery and CSD recording, animals breathed spontaneously and rectal temperature was continuously monitored and maintained at 37±1°C with a heating pad placed underneath the animal. As a rule, after the topical application of 2% KCl (approximately 270 mM) for 1 min at the exposed cortical surface, a single CSD wave was elicited in the frontal area. KCl application was repeated every 20 min for a total of 4 h. This CSD wave was recorded using Ag–AgCl, agar-Ringer electrodes located more posterior in the stimulated hemisphere. A third electrode of the same type was placed on the nasal bones and served as a common reference for the other two recording electrodes (see Fig. 3 for electrode placement). The velocity of CSD wave propagation was calculated from the time required for a CSD wave to cross the distance between the two cortical recording points. This time was measured using the beginning of the rising phase of the negative SPC as the initial point, as previously reported (Abadie-Guedes et al., 2008). In addition to the CSD velocity of propagation, for each rat we evaluated the incidence of CSD propagation failure and the amplitude of the negative, CSD-related SPC.

2.4. Statistical analysis

Intergroup differences were compared using analysis of variance (ANOVA) including nutritional status (L6 and L12), exercise condition (sedentary and exercised), and age (young, adult and aged) as factors and followed by a post-hoc test (Tukey) when indicated. Intragroup differences (exercised rats versus sedentary rats, in the same lactation and age conditions) were analyzed using

the unpaired *t*-test. Differences in the incidence of CSD propagation failure were analyzed using Fisher's exact probability test. Differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Body weights

Body weights were significantly lower among L12 rats than corresponding L6 rats at the 7th, 14th, 21st, 60th, and 90th postnatal days ($p \leq 0.05$), confirming that litter size manipulation can effectively alter body weight early in life. When the pups reached the elderly stage (600 days old), body weight differences between L12 and L6 rats were no longer observed. ANOVA showed that the main effect of the lactation condition was significant for body weight at postnatal days 7 ($F[1,46]=253,37$; $p<0.001$), 14 ($F[1,44]=296.57$ $p<0.001$), 21 ($F[1,46]=482.49$; $p<0.001$), 60 ($F[1,43]=29.46$; $p<0.001$) and 90 ($F[1,24]=16.24$; $p<0.001$). Physical exercise did not significantly affect the evolution of body weight in pups subjected to either lactation condition (Fig. 1).

3.2. Cortical spreading depression

Figure 2 presents examples of electrophysiological recordings documenting KCl-elicited CSD in L6 and L12 groups of young, adult, and aged rats (exercised and sedentary groups). The topical application of 2% KCl for 1 min in one point of the frontal cortex in the right hemisphere usually elicited a single CSD wave that propagated and was sequentially recorded by two epidural electrodes gently placed over the parietal region of the same hemisphere (see Fig. 2 central inset for electrode placement). The recordings demonstrate the electrocorticographic depression and the SPC that accompany CSD. Aside from the elicitation of the CSD wave, its propagation velocity was also evaluated as described below. In a few cases in the adult exercised (E2) group, and in a greater

number of cases in the aged groups from both lactation conditions, the KCl-elicited CSD failed to propagate to the more remote recording point after propagating to the recording point nearest to the stimulation site. Data about this propagation failure, including statistical significances, are presented in Table 2, and examples of CSD propagation failure are documented in Figure 3.

Regarding CSD propagation velocity, three-way ANOVA revealed that lactation condition ($F[1,100]=352.91$; $p<0.001$); age ($F[2,100]=119.74$; $p<0.001$), and physical exercise ($F[1,100]=181.93$; $p<0.001$) have significant main effects on CSD propagation. Post-hoc comparisons demonstrated that the unfavorable lactation condition (L12 groups) accelerated the CSD velocity of propagation, which ranged in the L12 sedentary groups from 3.40±0.23 to 4.35±0.16 mm/min; by comparison, CSD velocity in the L6 condition ranged from 2.92±0.17 to 3.67±0.13 mm/min. Exercise and aging decelerated CSD compared with the corresponding controls (sedentary and young groups, respectively). In the L6 exercised groups, the mean CSD velocities ranged from 2.57±0.24 to 3.02±0.19 mm/min. In the different L6 sedentary age groups, the CSD velocities were 2.92±0.17 mm/min (elderly), 3.29±0.08 mm/min (adult), and 3.67±0.13 mm/min (young). In the L12 exercised groups, CSD velocities ranged from 3.11±0.21 to 3.32±0.16 mm/min. These findings are illustrated in Figure 4, where interaction effects (aging/lactation condition interaction and aging/exercise-timing interaction) can also be observed. The SPC amplitudes of the CSD are presented in Table 3. No significant intergroup differences in CSD amplitude were observed.

4. Discussion

In the present study, we have demonstrated interactions between aging, exercise, and lactation conditions that affect the brain propagation features of the excitability-related CSD phenomenon. Data clearly show that aging and exercise decelerate CSD, while unfavorable

lactation conditions (L12 group) accelerate it. These three factors had not yet been combined in a single study involving CSD. The novel aging-related electrophysiological findings may be considered an interesting contribution to the understanding of effects of aging, exercise, and lactation on the brain.

Brain development largely occurs early in life, during the perinatal period. In the rat, the perinatal period includes both gestation and lactation phases, and is very sensitive to adverse environmental and nutritional conditions (Morgane et al, 1993; Smart and Dobbing, 1971). Distinct lactation experiences can induce robust neural changes that may modify brain function in a long-lasting manner (Guedes, 2011). Regarding the influence of lactation conditions on brain function, in the present work, suckling in 12-pup litters (L12 groups) also led to permanent or at least long-lasting effects on brain excitability, as indexed by accelerated CSD compared with rats suckled in 6-pup litters (L6 groups). The present results confirmed previous findings regarding the enduring effects of unfavorable lactation conditions on brain CSD features in adult rats (Rocha-de-Melo et al., 2006; Frazão et al., 2008), and also demonstrated that such effects can persist into old age. These robust effects of unfavorable lactation conditions on CSD may be of interest to neurophysiologists, gerontologists, and other specialists because of the possibility that this early-life negative experience generates long-lasting neuropathological impact (for a review, see Korosi et al., 2012). Furthermore, CSD has been implicated in important neurological human disorders, such as migraine (Lehmenkühler et al, 1993). The global importance of this concern can clearly be perceived if one considers the persistently high prevalence of nutritional deficiency among children. Recently, evaluations in 36 low- and middle-income countries estimated that prevalence at approximately 125 million underweight and 195 million stunted children younger than five years of age (for a review, see Fanzo and Pronyk, 2011).

Despite the growing population of elderly individuals, the framework of aging-related deleterious neurological changes remains unclear. This framework includes not only structural and

functional changes per se, but also the timing of the alterations and the consequences of that timing on brain function. Studies have been targeted to provide insights about useful therapeutic approaches and better timing for their application to prevent or modulate age-related negative changes in brain function. In this context, treadmill exercise has been considered a valuable therapeutic intervention to attenuate the effects of physiological disturbances on the nervous system during the aging process (Kobilo et al., 2011). However, lack of evidence persists concerning the optimal timing and parameters of treadmill exercise to track enduring beneficial effects on brain excitability.

Our CSD findings can be explained by different mechanisms. We hypothesize that two mechanisms are most likely involved, and deserve comment: oxidative stress and age-related impairment of cerebral blood flow. Aging is associated with a multifactorial set of deleterious changes. Among these alterations, special attention is devoted to those provoked by oxidative stress. Oxidative stress alterations that have been accumulated throughout life are considered a driving force for cellular aging (Kern and Behl, 2009; Poon et al., 2004). During aging, the oxidation of DNA, proteins, and lipids by reactive oxygen species (ROS) can functionally affect the brain (Poon et al., 2004). The beneficial effects of exercise on the brain appear to depend on counteracting lifelong-accumulated oxidative stress. Aerobic physical exercise protects neural tissue against degeneration, and consequently ameliorates brain function, by improving redox homeostasis (García-Mesa et al., 2011). In this work, treadmill exercise led to CSD deceleration, in agreement with previous CSD findings in rats treated with carotenoid antioxidants, which exhibited protective action against the CSD-accelerating effects of chronic ethanol consumption (Bezerra et al., 2005; Abadie-Guedes et al., 2008). The deleterious effects of ethanol on the brain are thought to occur through the production of ROS (Rashba-Step et al., 1993). Notwithstanding these pieces of evidence, we believe that further studies are necessary to confirm this hypothesis. Because CSD is an excitability-related brain phenomenon, it is also pertinent that the beneficial effects of regular

physical exercise include the improvement of processes like learning, long-term potentiation, and memory (Radak et al., 2001; Ogonovszky et al., 2005), which are intrinsically linked to changes in brain excitability (Passecker et al., 2011). Notably, changes in brain excitability also influence CSD propagation, lending support to the idea that CSD is a useful index of brain excitability (Guedes et al., 2009; Souza et al., 2011).

Physical exercise preconditioning is known to help preserve cerebrovascular integrity and attenuate the age-related impairment of cerebral blood flow (Ding et al., 2006; Lucas et al., 2012). Similarly, alterations in the cerebral blood flow strongly influence CSD (Sun et al., 2011). CSD is therefore clinically relevant, because the pathological framework of migraine includes important alterations in the brain circulation, and CSD is postulated to be involved in migraine vascular disorders (Lehmenkühler et al, 1993). In addition, serotoninergic antimigraine/antidepressant drugs are also capable of blocking CSD (Barkley et al., 1992; Cabral-Filho et al., 1995; Guedes et al., 2002). We conclude that it is reasonable to consider the involvement of cerebrovascular changes in the effects of treadmill exercise on CSD propagation.

Forced exercise, rather than voluntary exercise, provides neuroprotection by increasing cerebral glycolysis and metabolism (Kinni et al., 2011). Costa-Cruz and Guedes (2001) observed an inverse relationship between glycemic changes and CSD propagation, with hyperglycemia significantly decelerating CSD. Therefore, these findings allow us to speculate whether exercise-induced cerebral metabolism increase would help to decelerate CSD.

The deceleration of CSD propagation velocity might also be due to either a larger extracellular space or a more hindered diffusion caused by more cellular elements (Richter et al., 2003). Additional specific studies shall deeper investigate this possibility, as well as the “oxidative stress” and the “cerebrovascular” hypotheses.

Previous studies have consistently confirmed that early malnutrition accelerates CSD (see Guedes (2011) for a review). In comparison to the well-nourished control, the early-malnourished rat has a smaller and lighter brain with reduced myelin content and smaller cells packed in a denser manner, resulting in reduced volume of extracellular space. All of these conditions are thought to favor CSD propagation (Guedes et al., 2002; Merkler et al., 2009).

In conclusion, the present in vivo study describes novel and enduring electrophysiological CSD effects in aged rats previously preconditioned by early-life experiences, such as lactation conditions and forced (treadmill) exercise. The results allow us to draw three conclusions. First, after unfavorable lactation and exercise, CSD propagation accelerates and decelerates, respectively, in a long-lasting manner. Second, the aging process interacts with the two factors (lactation and exercise), modulating their effects on CSD. Third, the conditions in the aged brain favor the failure of CSD propagation, reinforcing the inverse relationship between age and CSD velocity. The present data might advance understanding of the CSD/brain excitability/nutrition/exercise relationship in the aged brain.

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Figure Legends

Fig. 1. Body weights (mean ± SD) of male Wistar rats suckled in litters of 6 pups (L6) or 12 pups (L12). These animals were also subdivided into exercised groups (n=10 per group) and sedentary groups (n=12 per group). Body weights were measured on postnatal days 7, 14, 21, 60, 90, and 600. Asterisks indicate L12 values that differ significantly from the corresponding L6 rats ($p<0.05$; ANOVA plus Tukey test). Exercise did not influence body weight.

Fig. 2. Representative recordings of spontaneous cortical activity (electrocorticogram; E) and slow potential change (P) of KCl-elicited cortical spreading depression (CSD) in 12 rats (six L6 rats and six L12 rats, suckled in litters of 6 and 12 pups, respectively), as follows: four young, four adult and four aged rats (two L6 and two L12 from each age group). In each age group, two recordings were from exercised (Ex), and two from sedentary (S) rats (one L6 and one L12 from each exercised/sedentary group). The vertical bars indicate 10 mV for P and 1 mV for E (negative upwards). CSD was elicited by 2% KCl applied epidurally for 1 min, as indicated by the horizontal bars over P1 traces. The CSD was recorded by the two cortical electrodes located posterior to the area of stimulation (at points 1 and 2, as shown in the central inset). A third electrode of the same type was placed on the nasal bones and served as a common reference (R) for the recording electrodes.

Fig. 3. Recordings of CSD from one sedentary aged rat previously suckled in favorable lactation condition (L6), documenting the occurrence of CSD propagation failure. (A) KCl stimulation for 1 min (horizontal bar) in the frontal cortex elicited CSD that propagated and was recorded at points 1 and 2, in the parietal region. (B) Another KCl-elicited CSD episode propagated to point 1, but failed to reach point 2. (C) KCl stimulus was applied posterior to point 2 (where it was recorded), but failed to propagate to point 1. In the top-right inset, R, KCl, 1, and 2 indicate the site of the reference electrode, of the KCl stimulation, and the recording points 1 and 2, respectively. The vertical bars indicate 10 mV for slow potential change (P) and 1 mV for spontaneous cortical activity (electrocorticogram; E) (negative upwards). Recordings in B and C were taken at the following time intervals (t) after the recording A, which was defined as t=0 min: B, t=21 min; C, t=197 min.

Fig. 4. CSD velocity of propagation (in mm/min) in young, adult, and elderly rats previously suckled in litters of either 6 pups (L6 group) or 12 pups (L12 group). Each group was subdivided into sedentary (S) and exercised animals. Exercise occurred from postnatal days 15 to 45 (groups E1), 90 to 120 (E2) or 530 to 600 (E3). Values are presented as mean ± SD. Asterisks indicate the L12 values that differ significantly from the corresponding L6 values. The symbol (#) indicates exercise *versus* sedentary difference, and the white ellipse indicates lack of difference between distinct exercise groups (E1, E2, and E3), within the same age and lactation condition. The symbol @ denotes age-related difference within the sedentary groups. The symbol (+) indicates adult *versus* aged difference regarding the E2 group, within the L12 condition ($P<0.05$; ANOVA plus Tukey test).

Table 1. Experimental groups of this study, described according to the lactation conditions, exercise conditions and ages. The number of rats per group is presented in parentheses.

Group	Lactation condition	Exercise condition	Age-group	Age of exercising
1	L6* (N=81)	Ex** (N=52)	**Young** (n=10)	E1 (15-45d)+
2			**Ad**ult (n=14)	E1 (15-45d)
3				E2 (90-120d)
4			**Ag**ed (n=28)	E1 (15-45d)
5				E2 (90-120d)
6				E3 (530-600d)
7		Sed (N=29)	**Y** (n=8)	15-45d
8			**Ad** (n=10)	90-120d
9			**Ag** (n=11)	530-600d
10	L12 (N=80)	Ex (N=44)	**Y** (n=9)	E1 (15-45d)
11			**Ad** (n=14)	E1 (15-45d)
12				E2 (90-120d)
13			**Ag** (n=21)	E1 (15-45d)
14				E2 (90-120d)
15				E3 (530-600d)
16		Sed (N=36)	**Y** (n=10)	(15-45d)
17			**Ad** (n=13)	(90-120d)
18			**Ag** (n=13)	(530-600d)

*L6 and L12 represent the groups of rats suckled in litters formed by either 6 or 12 pups, respectively.

**Ex and Sed are exercised and sedentary groups, respectively.

+E1, E2 and E3 indicate groups exercised in different ages: respectively at 15-45days, 90-120days and 530-600days of life.

Table 2

CSD propagation failure in rats as a function of age, lactation condition, and physical exercise. The number of rats per group is in parentheses. E1, E2, and E3 indicate groups subjected to exercise at different ages: at postnatal days 15-45 (during the lactation period), 90-120 (during adulthood), or 530-600 (during elderhood), respectively. S: sedentary rats (not subjected to exercise); L6: rats suckled in 6-pup litters; L12: rats suckled in 12-pup litters. Values marked with lower-case letters differ significantly from the corresponding values of the groups marked with the same letters in the left column (Fisher's test).

Age group	**Exercise group**	**Lactation Condition**			
		L6		**L12**	
		No. of KCl applications	**No. of propagation failures (%)**	**No. of KCl applications**	**No. of propagation failures (%)**
Young	**E1 (a)**	101 (10)	0	108 (9)	0
	S (b)	116 (8)	0	120 (10)	0
Adult	**E1 (c)**	96 (8)	0	59 (5)	0
	E2 (d)	70 (6)	5[c, e] (7.14)	106 (9)	5[e] (4.72)
	S (e)	122 (10)	0	156 (13)	0
Aged	**E1 (f)**	90 (9)	24[a, c, h] (26.67)	68 (5)	14[a, c] (20.59)
	E2 (g)	108 (9)	21[d] (19.44)	71 (6)	15[d] (21.13)
	E3 (h)	116 (10)	15[i] (12.93)	115 (10)	15 (13.04)
	S (i)	143 (11)	40[b, e] (27.97)	153 (13)	17[b, e] (11.11)

Table 3

Amplitudes of the cortical spreading depression (CSD) slow potential shifts in 18 groups of rats (9 groups per lactation condition), according to age and exercise condition. Data are expressed as mean ± standard deviation. The number of rats per group is in parentheses. No significant differences were observed.

		Amplitudes of CSD slow potential shifts (mV)	
Age group	**Exercise group**	**Lactation condition**	
		L6	**L12**
Young	**E1**	9.27 ± 2.08 (5)	8.65 ± 2.38 (8)
	S	9.58 ± 2.30 (5)	11.69 ± 3.30 (8)
Adults	**E1**	10.12 ± 3.89 (6)	13.50 ± 6.69 (5)
	E2	12.47 ± 5.25 (6)	12.49 ± 3.83 (8)
	S	9.49 ± 2.98 (6)	11.02 ± 3.79 (5)
Aged	**E1**	11.49 ± 3.04 (7)	13.60 ± 5.33 (5)
	E2	12.60 ± 3.90 (6)	10.54 ± 3.04 (6)
	E3	9.41 ± 2.02 (7)	12.73 ± 4.92 (10)
	S	9.20 ± 4.33 (7)	9.24 ± 2.44 (10)

E1, E2, and E3 indicate groups subjected to exercise at different ages: at postnatal days 15-45 (during the lactation period), 90-120 (during adulthood), or 530-600 (during elderhood), respectively. S: sedentary rats (not subjected to exercise); L6: rats suckled in 6-pup litters; L12: rats suckled in 12-pup litters.

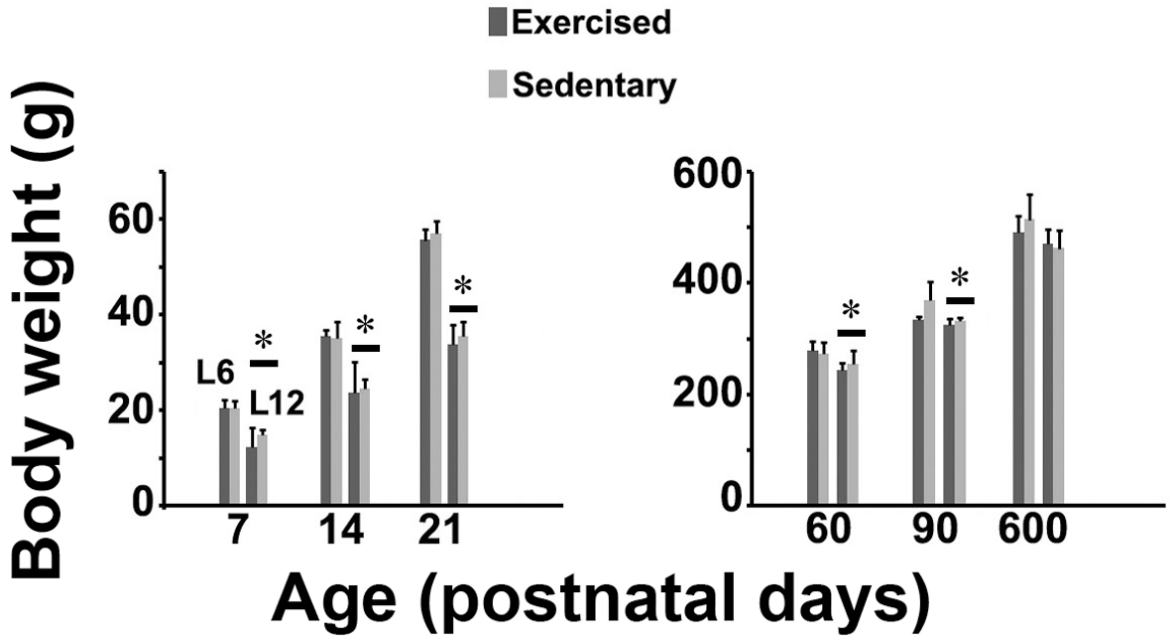
Exercised
Sedentary
Body weight (g)
60
40
20
0
L6
L12
*
*
*
7
14
21
600
400
200
0
*
*
60
90
600
Age (postnatal days)

Figure 1

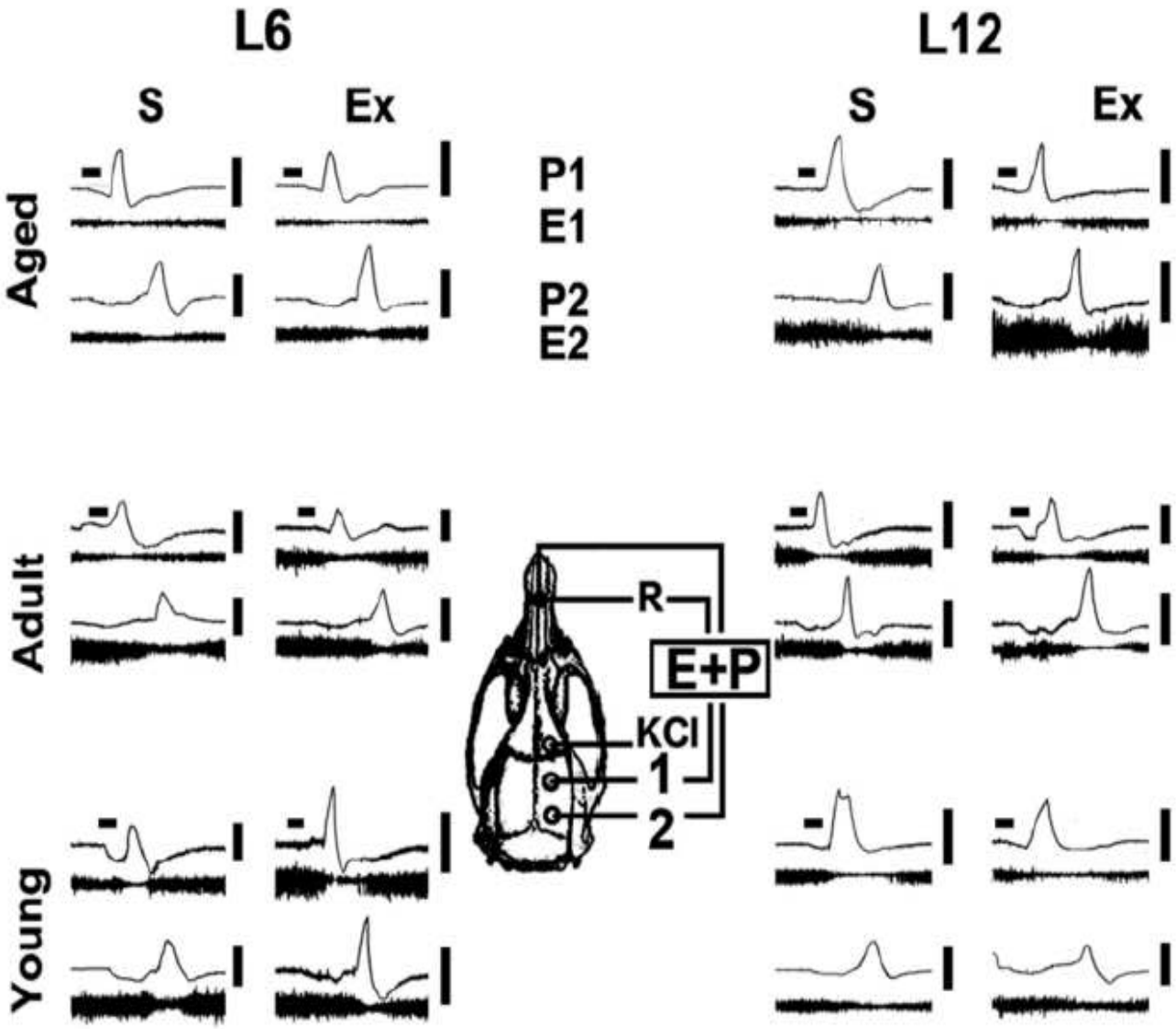

Figure 2

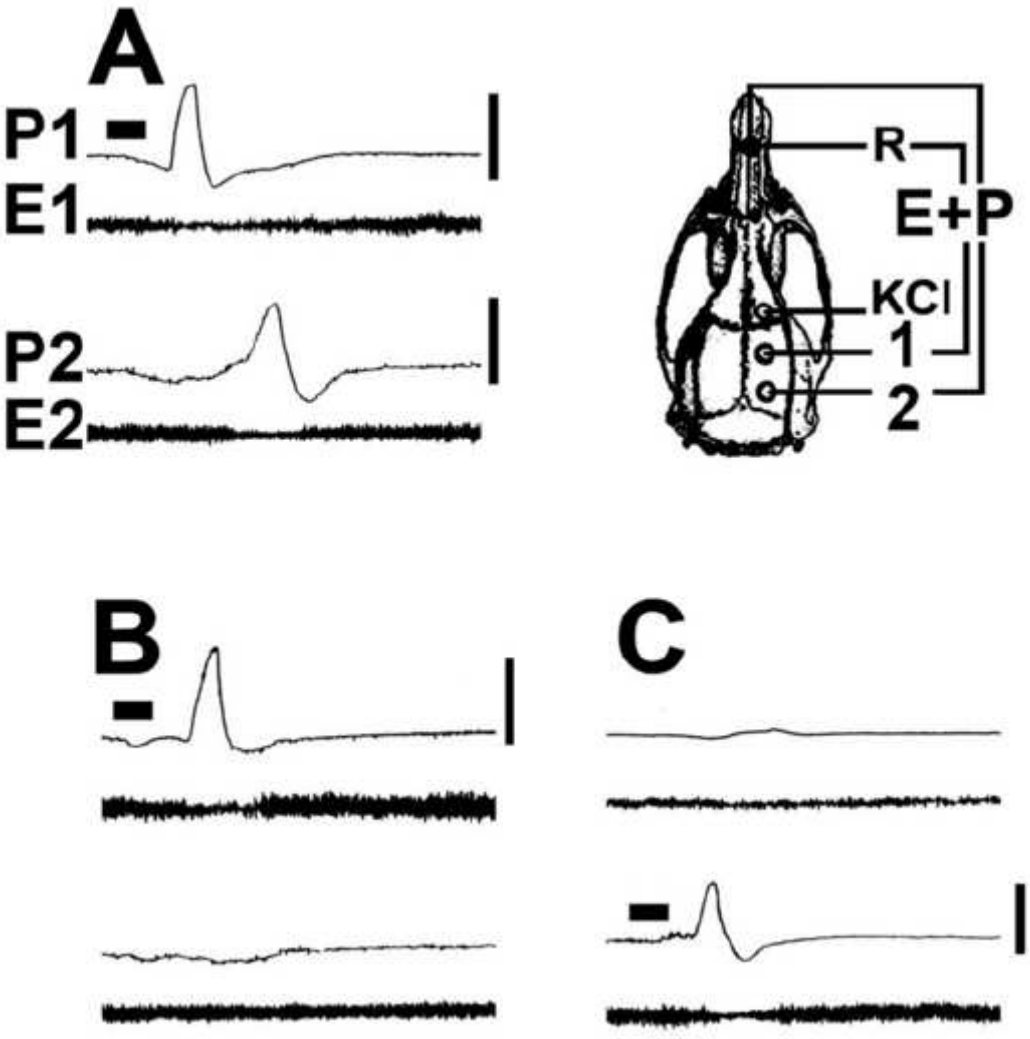

Figure 3

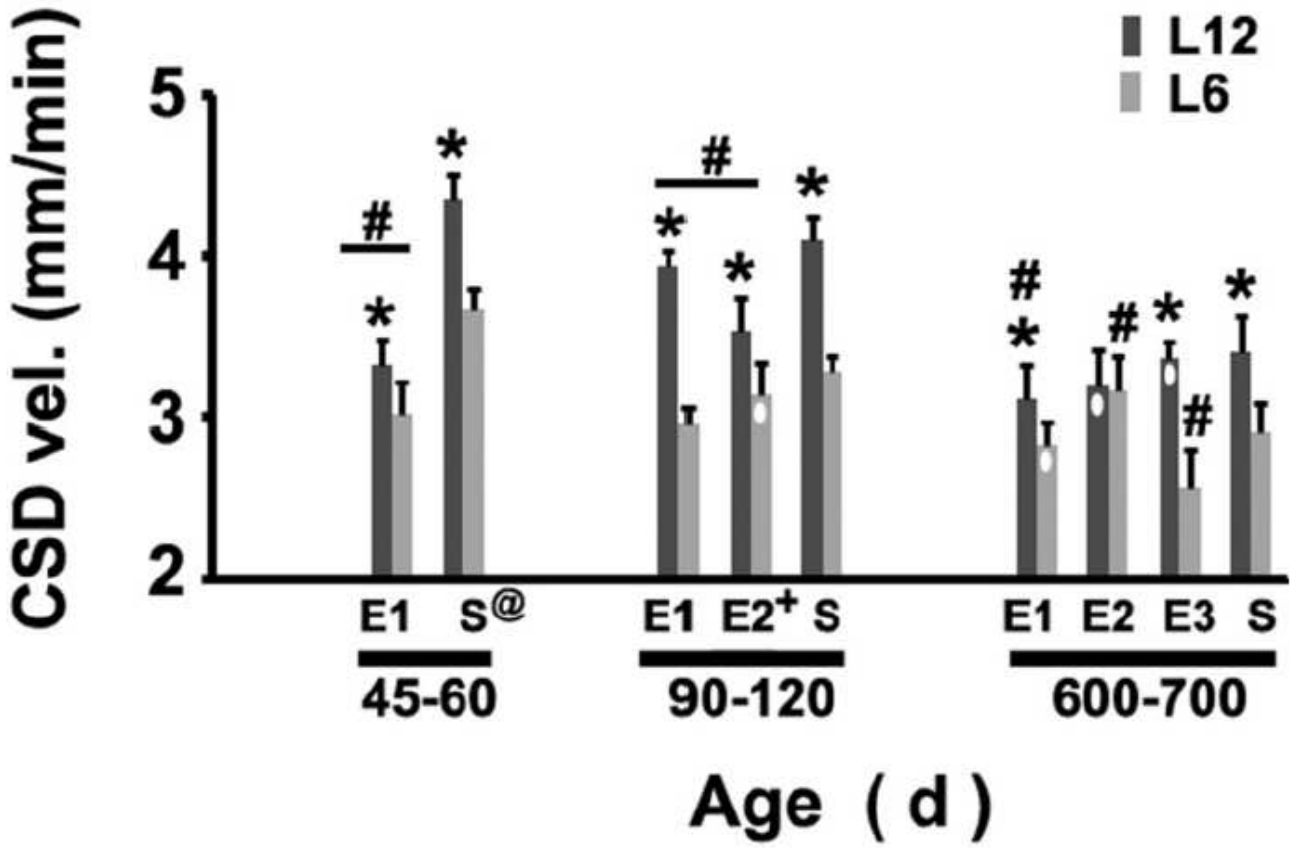
L12
L6
CSD vel. (mm/min)
5
4
3
2
E1
S@
E1
E2+
S
E1
E2
E3
S
45-60
90-120
600-700
Age (d)

Figure 4

5.2 ARTIGO 02

Title: Litter size and sedentary lifestyle affect aging cognitive decline and microglial number in the rat dentate gyrus.

Lane C. Viana1, Camila M. Lima1, César A. R. Fôro1; Marcus A. Oliveira1, Ronaldo P. Borges1, Tatiana T. Cardoso1, Izabela N. F. Almeida1, Daniel G. Diniz1, João Bento-Torres1, Antonio Pereira1, Manuella Batista-de-Oliveira2, Andreia A. C. Lopes, Rosângela F. M. Silva, Ricardo Abadie-Guedes2, Angela Amâncio dos Santos2, Denise S. Lima2, Pedro Fernando C. Vasconcelos3, Colm Cunningham5, *Rubem C. A. Guedes2, *CristovamW. Picanço-Diniz 1

Affiliations:

1Laboratório de Investigações em Neurodegeneração e Infecção, Hospital João de Barros Barreto, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, Pará, Brasil

2Laboratório de Fisiologia da Nutrição Naíde Teodósio, Departamento de Nutrição, Universidade Federal de Pernambuco, 50670901, Recife, Pernambuco, Brazil

3Instituto Evandro Chagas, Departamento de Arbovirologia e Febres Hemorrágicas. Ananindeua, Pará, Brazil

Corresponding author: Cristovam Wanderley Picanço Diniz, MD, PhD

Address: Laboratório de Investigações em Neurodegeneração e Infecção, Hospital João de Barros Barreto, Instituto de Ciências Biológicas, Universidade Federal do Pará,

Rua Mundurucus, 4487, Bairro do Guamá, Zip Code: 66073-000, Belém, Pará, Brazil

ABSTRACT

Background

It has been proposed that aging increase the responsiveness of innate immune response but is not known whether microglial changes induced by aging are affected by early in life influences of litter size and sedentary life style.

Methods

To address this question, rats suckled in litters of 6 or 12pups/mother were raised sedentarily in groups of 2–3 from the 21stpostnatal day onwards. At 4 (mature adult) or 23 (aged) months of age, half of the sedentary rat group underwent progressive daily treadmill exercise for five weeks, while the others remained sedentary. After spatial memory and object recognition tests, animals were sacrificed and their brains processed for microglia immunolabeling.The number and laminar distributions of IBA-1–immunolabeled cells in the dentate gyrus were estimated using the optical fractionator stereological method.

Results

We found that sedentary lifestyle impaired spatial memory of both mature and aged rats independent of the litter size, and that exercise reduced these effects in aged animals from small but not large litters. On the other hand, only sedentary aged animals both from large and small litters had impaired object recognition memory, and exercise reduced this effect regardless of litter size. Of interest, compared with age-matched rats from small litters, aged animals from larger litters showed an increased microglia number in all layers of the dentate gyrus, and exercise reduced this effect. Although mature adult sedentary rats from larger litters showed a similar effect in all dentate gyrus layers, exercise reduced the effect in the granular but not the molecular and polymorphic layers.

Conclusion

Taken together, the results demonstrate that litter size and early changes in maternal care, may affect immune cells of the central nervous system in both mature and aged brains and that exercise reduces microglial numbers and memory impairments.

Key words: litter size, aging, microglial response, exercise, dentate gyrus, stereology.

Running Title: Litter size and exercise affect CNS immune cells

BACKGROUND

The early postnatal environment in rodents has long-term physiological and behavioral consequences late in life[1-5]; for a recent review see [6]. Indeed, a series of experiments described experiments designed to measure the impact of reduced maternal care have demonstrated important detrimental effects on rat central nervous system (CNS) both development and adult brain function [7-11] and has shown that epigenetic changes in DNA methylation alter glucocorticoid receptor expression, changing stress responses in the offspring[12]. It has also been demonstrated that partial reversal of these effects of reduced maternal care on cognitive function can be achieved through environmental enrichment [13, 14].A number of studies have shown that extreme litter size changes such as three or four pups per litter [15-17] or 16 to 18 pups per litter [18, 19] may induce permanent changes in metabolic profile and this is associated with epigenetic changes by acquired alterations of the DNA methylation pattern [20].

Another series of experiments in a variety of small mammals has demonstrated that litter size is a potential threat in early postnatal life because sibling competition within litters depends on the offspring's number competing for access to milk from each nipple [21, 22]. In extreme cases when the number of pups exceeds the number of nipples, the consequences of this competition may be fatal[23]. However, even nonlethal consequences of this natural competition may result in important differences in individual's postnatal life. For example, growth rates favoring animals with more access to maternal milk, possibly resulting in higher reproductive success and longer life, may represent evolutionary advantages [22, 24-27]. In Furthermore, it has been demonstrated that spontaneous litter size changes may affect emotionality in adulthood, and that these changes canot be explained by concomitant changes in maternal care [28].

However, very little is known about the possible long-term consequences of litter size on the immune system[29],particularly on the resident immune cells of the brain, the microglia, of adult and aged animals[30]. Recent evidence indicates that litter size affects corticosterone levels at

the early postnatal stages [6, 31, 32],but very little is known about its long-term consequences on microglia populations or responsiveness [33]. Although it has been demonstrated that voluntary exercise attenuates microglia proliferation i n the hippocampus of aged mice, increasing a proneurogenic phenotype [30], and moderate physical training attenuates the effects of perinatal undernutrition on the peripheral immune system[34] it is not known whether physical training influences the impact of litter size on microglial numbers in aged and young rats.

A previous report described the long-term effects of early undernutrition and environmental stimulation on learning performance in mature rats (11 weeks old) maintained in litters of 18 pups/dam during the weaning period and compared with age-matched control rats, from litters of 6pups/dam [35].The authors found that the induced undernourishment did not impair Hebb-Williams maze test performance and that environmental stimulation improved the learning performance both in control and previously undernourished groups.

In a previous report we have demonstrated that the dentate gyrus is particularly sensitive to aging and impoverished environment and that environmental enrichment may contribute to partially recover memory impairments and associated astroglial changes[36]. We investigated the long-term effects of two different litter sizes (6 and 12 pups/dam) on the number and laminar distribution of microglias in the dentate gyrus and on the object recognition and spatial memories of adult mature (6 months old) and aged (24 months old) rats that were either sedentary throughout their lives or exercised for 5 weeks later in life. We estimated the number of microglia in the dentate gyrus by optical fractionator to test the hypothesis that litter size and sedentary lifestyle alter the number and laminar distribution of microglia. We also investigated whether or not these alterations may be associated with object recognition and spatial memory impairments and whether or not both effects (microglial number and cognitive decline) are affected by five weeks of exercise later in life.

METHODS

All procedures in this investigation were submitted to and approved by the institutional animal care committee of the Federal University of Pernambuco, Brazil, and handled in accordance with the "Principles of Laboratory Animal Care" (NIH).

Experimental groups

The experiments were performed using the offspring of an outbred colony strain of Wistar rats obtained from the Department of Nutrition of the Federal University of Pernambuco. Wistar female rats fed ad libitum with a rodent laboratory chow diet (Purina do Brazil Ltd) with 23% protein were maintained in groups of 2 or 3. After mating and gestation, Wistar female pregnant rats delivered 7 to 12 pups per litter. To manipulate maternal care and the level of competition for suckling, a pup-to-dam ratio of either 6:1 (small litter size, N = 20) or 12:1 (larger litter size, N = 20) was established 48h after birth. In our model, pups from different dams were pooled and then divided among the dams to yield varying ratios of pups per dam. The assumption was that with many pups and only one dam, competition for milk access and maternal care differ significantly. Our choice assumed, as previously demonstrated, that under these two litter sizes does not induce undernourishment [37-39] but that each pup from the 12:1 condition received less licking, grooming and is under a higher level of competition for nipples as compared to pups from the 6:1 litter.

Body weights were measured at different time windows to follow its evolution in the different experimental conditions. Figure 1 shows the timeline of the experimental procedures with pre- and postweaned Wistar rats.

Environment, exercise, and sedentary conditions

After the suckling period, all experimental groups were fed ad libitum with the same rodent laboratory chow diet (Purina do Brazil Ltd) with 23% protein and maintained in groups of 2 or 3 animals polypropylene cages (51 × 35.5 × 18.5 cm) in a room with a light–dark cycle (12/12h; lights on at 6 a.m.) and room temperature (23 ± 1°C), similar to standard housing conditions in most laboratories. All animals were housed under these standard conditions after the weaning period until the day of sacrifice. After 4 or 17 months, half of each experimental group was submitted every morning to 5 weeks of progressive exercise on a treadmill (n=10) as described in Table 1, and the other half left sedentary (n=10). We used a treadmill (Insight Equipamentos Ltda, Ribeirão Preto, São Paulo, Brazil) where time and speed of the moving platform was under control. Sedentary animals were also transferred every morning to a switched-off treadmill for an equal amount of time, as a control procedure.

Behavioral tests

After the exercise period, all mature (4-month-old) and aged (23-month-old) rats no matter whether sedentary or exercised, from all experimental groups, were submitted to spatial memory and object recognition tests. Figure 2 is a schematic diagram of the object recognition and object placement apparatus and test procedure. In the present work, we used single trial tests to assess object identity and object placement recognition memories.

The apparatus for the single trial object recognition and spatial memory tests consisted of an open circular container (1m diameter) made of painted, varnished wood. The floor was painted with lines to distinguish four quadrants, and the luminance at the center of the circular box floor was 2.4 cd/m2. Detailed protocols and reasons for test choices provided are discussed elsewhere [40-42]. In brief, behavioral essays were performed over 5 days: 1 day for open field habituation, 2 days for object habituation, and 2 days for testing;1 day for each test.

To minimize the influence of natural preferences for particular objects or materials, we chose objects of the same material but different geometries that could be easily discriminated and had similar possibilities for interaction [43]. All objects were plastic with different shapes, heights, and colors. Before each rat entered the arena, the arena and objects were cleaned with 75% ethanol to minimize distinguishing olfactory cues. The testing procedures were as follows. For open field habituation, each animal was placed in the arena, free of objects, for 5 min to explore the open field; for object habituation, each animal was exposed to two identical objects (not used on test days) placed at the same quadrants of the arena for 5 min, three times, with 50 min in between. For the testing one-trial recognition tests were administered on 2 consecutive days. One was the object identity test, a 5-min sample trial, during which animals explored two identical objects in a familiar arena, followed by a 50-min intermission and then a second 5-mintest trial, in which a "novel" object was presented together with one "familiar" object already explored during the sample trial. Objects differed in form, dimensions, color, and texture and had no ethological significance for rats. It was expected that rats would spend more time with the "novel" object than with the "familiar" one. The second test was a one-trial object identity recognition, which followed the same procedure as above, except in the test trial, one of the two identical objects was shifted to a novel location ("displaced" object). It was expected that rats would spend more time with the "displaced" object than with the "stationary" one.

The basic measure was the time a rat spent exploring each object during the test trial, and scores were determined for object recognition (novel vs familiar) and placement (displaced vs stationary) memories. In these tests, the exploration of an object was assumed when a rat approached an object, the head was directed towards it, and the head was placed within 0–3cm from the object. This definition required that each object be fixed to the apparatus floor; thus, we chose heavy objects for interaction.

Behavioral data were analyzed using parametric statistics, and the two-tailed t-test for dependent groups was used to detect significant differences between the periods of time a rat spent during the test trial on each object as compared to the total time of exploration. The performance was the time of exploration for each object expressed as a proportion (percentage) of the total time of exploration, and possible significant differences were detected with the two-tailed t-test for dependent groups [44]. In addition, differences were considered significant in the time of exploration only if the average time with one of the objects was 60% or higher than with the other. In all statistical tests, the threshold for significance was set at $p<0.05$.

Immunohistochemistry

After behavioral tests, all rats were weighed and anesthetized with intraperitoneal 2,2,2-tribromoethanol (0.04 ml/g of body weight) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4). Alternate series of sections (70 μm thickness) obtained with a Vibratome (Micron) were immunolabeled with a polyclonal antibody against ionized calcium-binding adapter molecule 1(IBA-1) to detect microglia and/or macrophages (anti-Iba1, #019-19741; Wako Pure Chemical Industries Ltd., Osaka, Japan). All chemicals used in this investigation were supplied by Sigma-Aldrich (Poole, UK) or Vector Labs (Burlingame, CA, USA).

For immunolabeling, free-floating sections were pre-treated with 0.2 M boric acid (pH 9) at 65–70 °C for 60 min to improve antigen retrieval, washed in 5% phosphate-buffered saline (PBS), immersed for 20 min in 10% normal goat serum (IBA-1 immunolabeling)(Vector Laboratories), and then incubated with anti-Iba1 (2 μg/ml in PBS) diluted in 0.1 M PBS (pH 7.2–7.4) for three days at 4 °C with gentle and continuous agitation. Washed sections were then incubated overnight with biotinylated secondary antibody (goat anti-rabbit for IBA-1, 1:250 in PBS, Vector

Laboratories). We inactivated endogenous peroxidases by immersing the sections in 3% H2O2 in PBS, washed the sections in PBS, and transferred them to a solution of avidin–biotin–peroxidase complex (VECTASTAIN ABC kit; Vector Laboratories) for 1 h. The sections were washed again before incubation in 0.1 M acetate buffer (pH 6.0) for 3 min and developed in a solution of 0.6 mg/ml diaminobenzidine, 2.5 mg/ml ammonium nickel chloride, and 0.1 mg/ml glucose oxidase [45]. We confirmed the specificity of the immunohistochemical pattern by omitting the primary antibody [46]. The negative control resulted in the absence of immunoreactivity in all structures.

Microscopy and the optical fractionator

The optical fractionator is an accurate stereological method of quantification that combines the properties of an optical dissector and a fractionator; it has been used in a variety of studies to determine cell numbers in multiple brain regions [47-49]. The optical fractionator is unaffected by histological changes, shrinkage, or damage-induced expansion with injury (West et al. 1991). We tested the hypothesis that aging and sedentary lifestyle would aggravate microglial changes associated with litter size changes. At all levels in the histological sections, we delineated the layers of the region of interest(dentate gyrus) by placing counting probes and digitizing directly from sections with a low-resolution, 4× objective on a NIKON, Eclipse 80i microscope (Nikon, Japan) equipped with a motorized stage (MAC200, Ludl Electronic Products, Hawthorne, NY, USA).This system was coupled to a computer that ran the StereoInvestigator software (MicroBrightField, Williston, VT, USA) to store and analyze the x, y, and z coordinates of the digitized points. To unambiguously detect and count microglia with the dissector probe, the low-resolution objective was replaced with a high-resolution, 100× oil immersion plan fluoride objective (Nikon, NA 1.3, DF = 0.19μm).

The thickness of the section was carefully assessed at each counting site with the high-resolution objective, and the fine focus of the microscope was used to define the immediate layers at the top and bottom of the section. Because the thickness and the distribution of cells in the section were variable, the total number of objects of interest was weighted with the section thickness. All microglial cell bodies that came into focus inside the counting frame were counted and added to the total number of markers, provided they were entirely within the counting frame or intersected the acceptance line without touching the rejection line [50]. The counting boxes were randomly, systematically placed within a grid.

Planimetric estimations of dentate gyrus volumes

The Stereo Investigator software was also used to estimate the volumes of unilateral dentate gyrus in all experimental conditions. Optical fractionator bases the volume calculation in planimetric data [51]. The distance between sections is constant throughout the sequence. The area estimates of multiple sections can be combined to give a total volume estimate and the coefficient of error (CE). We have used this approach to estimate the volumes of the actual thickness of the sections after histological preparation. Because the shrinkage induced by these processes is nonlinear and the z-axis is more affected than the x and y axes by dehydration, all volumes were estimated with no corrections for shrinkage.

Photomicrographic documentation and processing

To obtain digital photomicrographs, we used a digital camera (MicroFire, Optronics, Goleta, CA, USA) coupled to a NIKON, Eclipse 80i microscope. Digital photomicrographs were processed with Adobe Photoshop software; scaling and adjustment of the brightness and contrast levels were

applied to the whole image. The selected micrographs display representative sections from each experimental group in which the microglia number in the region of interest was closest to the mean value.

Statistical analyses

Data are reported as the mean ± the standard error of the mean. Tables S2–S6 presented as supplementary material show the experimental parameters and average counting results from the optical fractionator. The grid size was adapted to achieve an acceptable CE. For the CE of the total microglial counts for each rat, we adopted the one-stage systematic sampling procedure (Scheaffer CE) used previously[52].

The level of acceptable CE for stereological estimations was defined as the ratio of the intrinsic error introduced by the methodology and the coefficient of variation (CV) [52]. The CE expresses the accuracy of the cell number estimates; a CE ≤ 0.05 was deemed appropriate for the present study because the variance introduced by the estimation procedure contributed little to the observed group variance [53]. The ratio between CE2/CV2 should not be higher than 0.5, although there are exceptions, and strict adherence to this rule is not advised [53]. We detected these exceptions in this investigation, and CE2/CV2 values were higher than the rule recommends, but in these cases, the biological variance and CE introduced by the methodology was very low, and application of the rule was neither meaningful nor practical [53].

Stereological estimations of all groups were compared by multifactorial ANOVA using ezANOVA free statistical software applied as Design 3 Between Subject Factors, and pairwise comparisons with Tukey's honestly significant difference test (HSD) that attempts to control for multiple comparisons expressing a standardized Q score. Significant levels were set at $p<0.05$.

RESULTS

Body weights and dentate gyrus volumes

Figure 3 shows a comparative survey of the mean values and respective standard errors of body weight after 7, 14, 21, 30, 90, and 600 postnatal days. A progressive and significant body weight gain independent of litter size was observed in all time windows. As compared with animals from small litters, the mean values of body weights from animals of larger litters were significant smaller from the 7th to 30th postnatal days. After that period, the mean body weight values of rats from large and small litters were indistinguishable from each other in the 90th post-natal day. However at the 600thpostnatal day, animals from small litters showed higher body weights than rats from larger litters. Larger body weight mean differences (indicated by dotted line) were found between 14 and 21st days.

Although three-way ANOVA indicated that aging but not litter size and exercise, influenced dentate gyrus volumes, only aged rats from small litters raised sedentarily were different from age-matched exercised animals raised in similar-sized litters ($F_{1,32} = 6.16$; $p<0.018$,three-way ANOVA, pairwise comparisons [Q=Tukey HSD:$t(8)=2.40$ $p <0.0434$]. See table 2.

Behavioral assays

Figure 4 shows the results of the object recognition and spatial memory tests. Taken together, the results show that a sedentary lifestyle impaired spatial memory (placement object recognition) of both young and aged rats no matter the litter size and that exercise reduced these effects in the aged animals from small but not from large litters. On the other hand, only sedentary aged animals from

both large and small litters had their identity object recognition memory impaired, and exercise reduced this effect regardless of litter size.

One-trial object identity recognition

Mature adult and aged exercised rats, independent of litter size, could distinguish familiar from novel objects. In contrast, aged sedentary animals regardless of litter size could not make this distinction. Mature sedentary rats from small but not from large litters could distinguish the two objects (Figure 4A). Two tail t-tests: (Small litter size: M-Sed, $t = -2.58$, $p = 0.042$; M-Ex, $t = -4.03$, $p = 0.002$; A-Ex, $t = 2.91$, $p = 0.023$; Larger litter size: M-Ex, $t = -3.14$, $p = 0.01$; A-Ex, $t = -2.76$, $p = 0.033$)

One-trial object placement recognition

All sedentary rats regardless of litter size and age were unable to distinguish stationary from displaced objects. In contrast, exercised rats from the mature and aged groups from small litters and exercised mature but not aged animals from larger litters could distinguish stationary from displaced objects (Figure 4B). Two tail t-tests: (Small litter size: M-Ex, $t = -2.72$, $p = 0.021$; A-Ex, $t = -3.79$, $p = 0.006$; Larger litter size: M-Ex, $t = -2.28$, $p = 0.047$).

Microglial numbers in the dentate gyrus

Figure 5A illustrates a series of photomicrographs from IBA-1–immunolabeled sections of the dentate gyrus from animals close to the mean value of each experimental group. Curved lines in the photomicrographs are used to indicate the granular layer located between the molecular and

polymorphic layers of the dentate gyrus. Figure 5B-D shows the mean values, standard errors, and significant differences. Three-way ANOVA indicated that exercise, litter size and age influenced the results but interactions between variables is laminar-dependent. Molecular layer: subjects from larger litters present higher significant number of microglias than animals from small litters no matter age or physical condition. Aged sedentary animals present a higher a significant number of microglias than sedentary adult mature animals from similar litter size and exercise reduced these effects. Litter size ($F(1,32) = 66.4$ $p<0.000001$), age ($F(1,32) = 10.5$ $p<0.0027$) and exercise ($F(1,32) = 4.71$ $p<0.0374$) affected the number of molecular layer microglias. In this layer it were detected interactions between litter size vs exercise ($F(1,32) = 8.82$ $p<0.0056$) and age vs exercise ($F(1,32) = 8.81$ $p<0.0056$). Granular layer: Different from molecular layer, exercise seems to protect adult mature animals from large litters. In addition, aged exercised showed a higher number of microglias than mature adult exercised animals. Litter size ($F(1,32) = 50.0$ $p<0.000001$), age ($F(1,32) = 34.8$ $p<0.000001$) and exercise ($F(1,32) = 7.48$ $p<0.01$) affected the number of microglias in granular layer. Litter size vs exercise ($F(1,32) = 8.25$ $p<0.0071$) and litter size vs age ($F(1,32) = 4.75$ $p<0.0367$) showed interactions. Polymorphic layer: Sedentary rats from larger litters, independent of age, showed a greater number of microglias than age-matched animals from small litters, and exercise reduced these differences in the aged groups. In this layer aged animals from small litters, independent of the physical condition show a higher number of microglias than adult mature groups. Litter size ($F(1,32) = 104$ $p<0.000001$), age ($F(1,32) = 57.3$ $p<0.000001$) and exercise ($F(1,32) = 4.91$ $p<0.0338$). Interactions were observed between all variables in this layer (litter size vs age vs exercise: $F(1,32) = 12.3$ $p<0.0013$). Detailed stereological data are presented as supplementary material (Tables S1–S6).

Figure 6A illustrates the influence of litter size, aging, and exercise on the laminar distribution of microglia in the dentate gyrus of adult mature and aged rats. The laminar distribution of microglias in the molecular and polymorphic layers seems to be influenced by aging in opposite

ways: the number of cells increase in the polymorphic and equivalently decreases in the molecular layer (Figure 6B). However sedentary animals from small litters did not change laminar distribution after aging whereas exercised animals from larger litters increase the percentage of microglias in the granular and reduces in the molecular layer. Finally aged exercised animals from small litters showed an increase in the polymorphic with equivalent decrease in the molecular. Note the occurrence of laminar redistribution of microglia, an effect that seems to be mainly associated with litter size and aging, and that exercise reduced these laminar changes. Aging influences on the laminar distribution of microglia in the polymorphic $F(1,32) = 11.9$ $p<0.0015$ and molecular $F(1,32) = 17.4$ $p<0.0002$ layers were detected by three-way ANOVA with significant interactions between all variables only in the polymorphic layer (litter size vs age vs physical status $F(1,32) = 5.13$ $p<0.0303$). Litter size and aging ($F(1,32) = 6.72$ $p<0.0142$) affect the granular layer with significant interaction between them ($F(1,32) = 6.72$ $p<0.0142$).

No simple correlations between the number of microglias and behavioral performances were detected.

DISCUSSION

We investigated the influences of litter size and treadmill exercise late in life on the laminar distribution of microglia in the dentate gyrus of mature and aged rats. Litter size affected the number of microglia, and aging induced re-distribution of these cells in a laminar-dependent fashion. Larger litters and aging were associated with object identity and placement recognition and exercise reduced these effects.

Litter size, growth, and somatic maturation

Our experimental manipulation was based on the protocol of Celedon et al. [35] with an adaptation to reduce the number of pups of the larger litter size to 12 pups per dam to avoid undernourishment. In the present report, the large litter group had a pup:dam ratio of 12:1, and the small litter group had a pup:dam ratio of 6:1. The assumption is that on average, a higher ratio of pups-to-dam results in a lower number of sucklings per pup, as compared to the group with a lower ratio of pups per dam. This assumption seems to be reasonable and was in line with body-weight curve in which significant differences in body weight were found until 30th day but not at 90th postnatal day, when age-matched animals from small and large litters were indistinguishable from each other. Indeed, a previous report [54]showed three classes of natural litter sizes in Wistar rats in which only minor differences regarding pup growth and somatic maturation could be detected: Class 1,litters with 6, 7,and 8pups (representing 27% of all pups); Class 2,litters with 9 and 10 pups (42% of all pups); and Class 3, litters with 11 and 12 pups (23% of all pups). Our experimental design encompasses classes 1 (six pups/dam) and 3 (12 pups/dam). In addition, a series of previous reports has demonstrated that normal litter size for Wistar rats may vary from 1 to 13 pups but that no undernutrition is produced with a ratio of6 or 12 pups/dam during the weaning period [54, 55].

Litter size and microglial response

Aside from nutritional differences a considerable literature has explored the epigenetic effects of maternal behavior (licking and grooming) on pups. Much of this work was pioneered by Meaney's group[12], who showed that maternal behavior in the first weeks of life has profound epigenetic effects on genes regulating the hypothalamic–pituitary–adrenal axis. Because glucocorticoids have important influences on microglia throughout life[56, 57] and litter size seems to affect corticosterone levels [27], it is reasonable to expect microglial changes as a function of litter size.

In line with this expectation, optical density analysis of IBA-1 immunoreactivity in the dentate gyrus of adult and aged groups after chronic restraint stress as compared to control groups revealed significant differences, and these differences were associated with higher levels of corticosterone in both adult and aged stressed groups [58]. Gi The present study manipulated the ratio of pups to dam and that minor differences in nutritional status were detected, the differences in maternal behavior induced by the different number of pups/mother may be relevant to the results. Indeed, it is highly likely that pups in the high ratio group were exposed to less maternal licking and grooming compared to the low ratio group. Therefore, a reduction in maternal care can interfere with the innate immune response in adulthood, imprinting permanent alterations on the offspring's immune system [59, 60].

Alternatively litter size may affect the early life development of social interactions between offspring that may lead in adulthood to a variety of permanent changes in anxiety, exploration of novelty, and adaptation to stressful situations and that cannot be directly explained by differences in maternal care[28]. How large the contribution of reduced maternal care and differential infant–infant interactions and whether or not these variables interact to affect cognition and emotion later in life.

Thus far, however, no information is available regarding the impact of litter size on microglia numbers later in life. We have demonstrated a vigorous long-term effect on microglial numbers in the dentate gyrus of young and aged rats from larger litters during the suckling period. It seems that in larger litters, brain development is on average associated with permanent changes in the innate immune system in the brain, with a significant impact on the microglial homeostasis of aged rats. Whether or not there is a direct correlation between litter size, age-related dentate gyrus plasticity, and changes in the microglia numbers remains to be established.

Litter size, aging, and cognitive decline

Under homeostatic conditions, microglia across different regions of the CNS exhibit a typical branching and ramified morphology that distinguishes them from tissue macrophages [61]. However, even in the absence of neurological disease, more-reactive phenotypes of astrocytes and microglia are expressed during aging as part of an increased and maintained pro-inflammatory profile [62, 63]. Age-related physiological changes in microglia include cytokine production [64, 65], altered expression of activation markers [66, 67], and dystrophic morphologies [68]. In addition, neuron and microglia crosstalk during aging is dysregulated, with concomitant loss of neuronal-derived factors that control microglial activation [69]. Because a marked, aged-related induction of pro-inflammatory microglial profiles in the hippocampus and dentate gyrus are not necessarily associated with cognitive impairment [70]and a higher number of these profiles is found in the dentate gyrus of sedentary animals [30], it is difficult to directly associate microglial changes with memory impairments. However, exercise attenuates microglia proliferation and increases expression of a proneurogenic phenotype in the hippocampus and dentate gyrus[30].In line with these findings, we found here no cognitive dysfunction in the exercised group except in the aged animals from larger litters in which exercise did not ameliorate spatial memory decline: a higher number of microglia was found in all layers of the dentate gyrus in this group as compared with age-matched exercised rats. Because we are not assessing any inflammatory mediators or cell surface markers of microglia to distinguish phenotypes, it remains to be investigated whether or not there may be two microglial phenotypes (proneurogenic and pro-inflammatory) in the dentate gyrus of Wistar rats as previously described in mice [30], and if exercised rats show the dominant effects of the proneurogenic phenotype.

Two key molecules are involved in microglia survival: granulocyte colony-stimulating factor (GCSF) and macrophage colony-stimulating factor-1 (MCSF-1). Mice lackingMCSF-1,the so-called osteopetrotic mouse (op/op),have 24% fewer microglia than wild-type controls in the

cerebral cortex, but the morphology is relatively normal[71]. After an injury, microgliosis in MCSF-1–deficient mice is significantly smaller than in wild-type control mice, and morphologies of both mutant and control mice change to an activated morphology profile [71]. Recently, another ligand for the MCSF-receptor, designated as IL-34, was identified and this ligand is highly expressed in the brain at the mRNA level and to a lesser extent in other tissues; see [72] for review. Another factor that controls the production of circulating blood cells by bone marrow the granulocyte stimulating factor (GCSF), has also been implicated in modulation of systemic immune responses by inhibiting pro-inflammatory cytokines[73]. Of interest, lower plasma levels of GCSF are associated with cognitive dysfunction in transgenic Alzheimer's mice [74], and a lower level in human plasma is predictive regarding the conversion of mild cognitive decline to dementia of Alzheimer's type [75].

Whether or notMCSF-1or IL-34 is increased after changes in litter size or whether GCSF is reduced in mature and aged sedentary rats from larger litters are questions requiring further investigation.

Technical limitations

Estimation of the number of objects in histological sections using stereological methods may vary from study to study as a result of different estimation methods, animal lineages, histological procedures, stereological protocols, and ambiguities in the definition of the objects and areas of interest [76]. To reduce these possible sources of error when comparing animal groups, we processed all samples with the same protocols, and all data were collected and analyzed with the same stereological method, software, and hardware. To detect possible variations in the criteria for identifying the objects of interest, we performed checking procedures of the objects of interest by having different investigators count the same regions using the same anti-Iba1 antibody as a

microglial marker. As a result, we reduced possible variations associated with non-biological sources to acceptable levels.

Finally, microglial plasticity may be affected by corticosteroids that inhibit microglial activation [77]. In the present report, manipulation-induced stress during treadmill exercise could have altered plasma corticosteroid levels and thus affected microglial numbers. We did not measure plasma corticosteroid levels after exercise; therefore, we cannot exclude the possibility that different levels of corticosteroids may underlie our observations.

CONCLUSIONS

We have shown that litter size changes during the suckling period followed by ad libitum access to a conventional laboratory diet in conventional sedentary laboratory conditions may have significant effects on the microglia population in aged rats. A relatively brief period of exercise later in life further influences these effects on the microglia. Because exercise reduces the cognitive dysfunction associated with large litter size and exercise attenuates microglial proliferation and increases a proneurogenic phenotype in the dentate gyrus [30],we suggest that at least part of the cognitive dysfunction found in the sedentary animals may be related to changes in the microglial profile. The cellular and molecular factors that contribute to the number and phenotype of microglia and their influence on the development of memory remain to be established.

AUTHORS' CONTRIBUTIONS

CWPD, RCAG, and AP designed the study; LCV, CML, CRF, MAO, RPB, TTC, INFA, DGD, JBT, MBO, AACL, RFMS, RAG, AAS, and DSL carried out most of the lab work and analyzed the data. LCV was responsible for the stereological measurements.JBT carried out statistical

analysis and prepared the figures. CWPD, RCAG, VHP, CC, and PFCV participated in writing the manuscript.All authors read and approved the final manuscript.

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Figure Legends

Figure 1. Experimental timeline. Time schedule conducted with pre- and postweaned Wistar rats from small (6pups/dam) and large litters (12/dam).

Figure 2. Diagram of the experimental designs for object recognition tests. A. Object identity recognition: It is expected that rats spend more time with the "novel" object than with the "familiar" one. B. Object placement recognition: It is expected that rats spend more time with the "displaced" object than with the "stationary" one. Modified from[42].

Figure 3. Body weight evolution. Mean body weight (mean ± standard error of the mean) of rats reared in litters of 6(small litters) and 12 (large litters) as a function of age. (*) indicates significant differences after two-tailed t-test (7^{th} to 90^{th}postnatal day, PND)or after three-way ANOVA at 600^{th} PND.

Figure 4. Object identity and placement recognition. Graphic representation of object identity recognition (A) and object placement recognition results (B).M-Sed = mature sedentary rats; M-Ex = mature exercised rats; A-Sed =aged sedentary rats; A-Ex = aged exercised rats. (*)$p<0.05$ and (**)$p<0.01$ indicate different levels of statistical significance in two-tailed t-tests for related events.

*Figure 5.*Laminar distribution of microglia in dentate gyrus of Wistar rats.

A. Photomicrographs of immunolabeled sections from mature (M) and aged (A) rats raised in reduced (6pups/dam) or larger litters (12 pups/dam) submitted to a short period of exercise (Ex) later in life or raised sedentarily (Sed). Pictures were selected to illustrate rats with microglia counts close to the mean values of different experimental groups. Curved lines indicate limits of the granular layer (GR) located between the molecular (MOL) and polymorphic (POL) layers of the dentate gyrus. Graphic representation of the mean values and standard error bars of unilateral dentate gyrus microglial counts in the molecular (B), granular (C), and polymorphic (D) layers. (*) indicates $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, different levels of statistical significance in 3-way ANOVA. (#) indicates significant differences between ages.

Figure 6. Laminar redistribution of microglia in rat dentate gyrus after litter size changes early in life and later exercise. A. Percent distribution of microglial counts in the polymorphic (POL), granular (GR), and molecular (MOL) layers of the dentate gyrus under each experimental condition. B. Equivalent differences expressed by absolute numbers of microglia in the polymorphic (top), granular (middle), and molecular (bottom) layers. M = mature adult rats; A = aged rats; Sed-L = sedentary rats from large litters; Sed-S = sedentary rats from small litters; Ex-L = exercised rats from large litters; Ex-S = exercised rats from small litters. (*) indicates $p<0.05$ and (**) $p<0.01$, different levels of statistical significance in 3-way ANOVA.

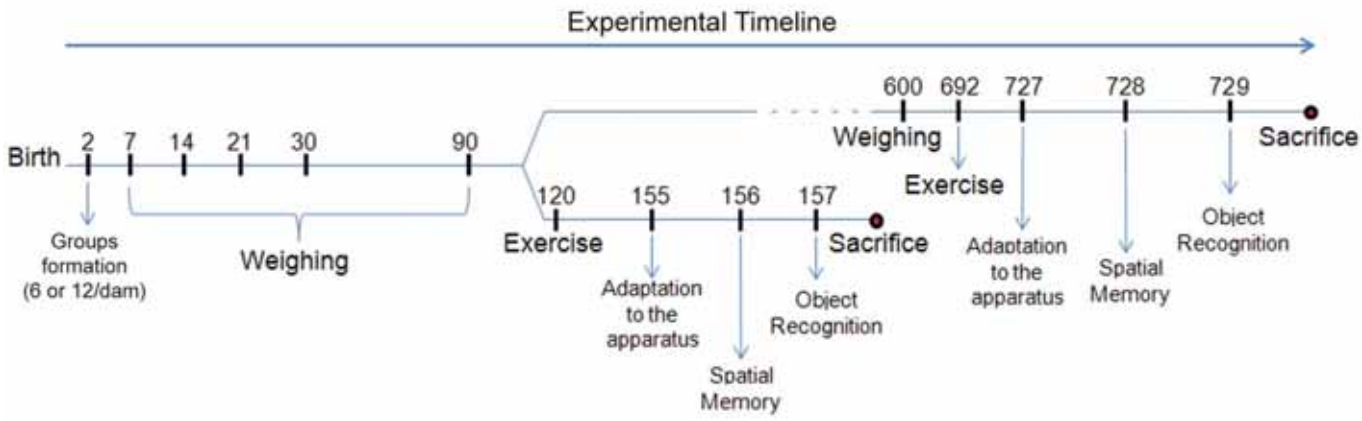

Figure 1

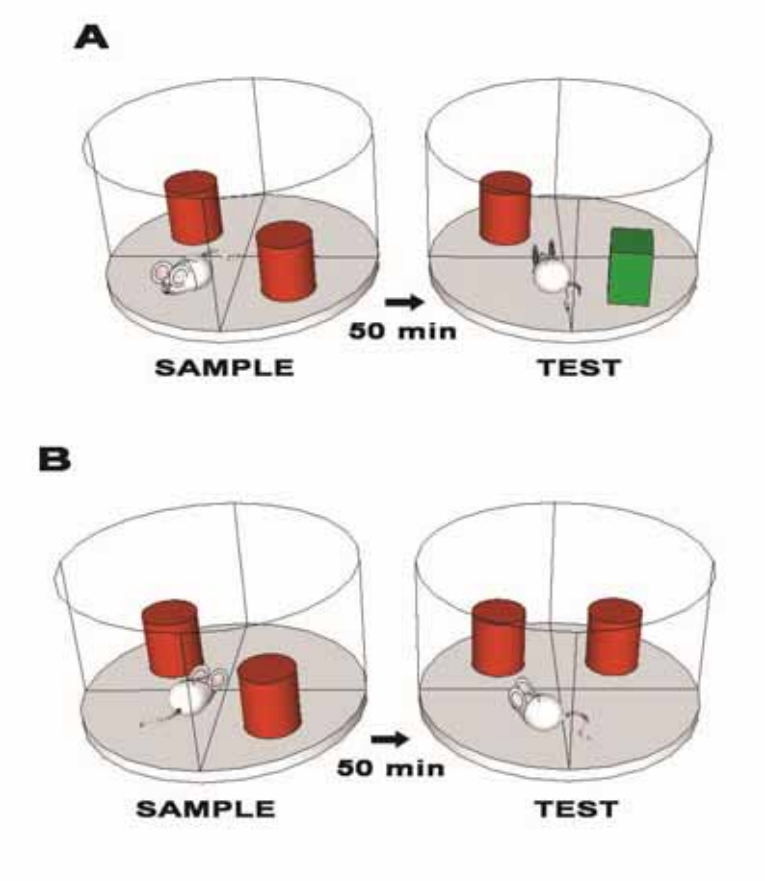

Figure 2

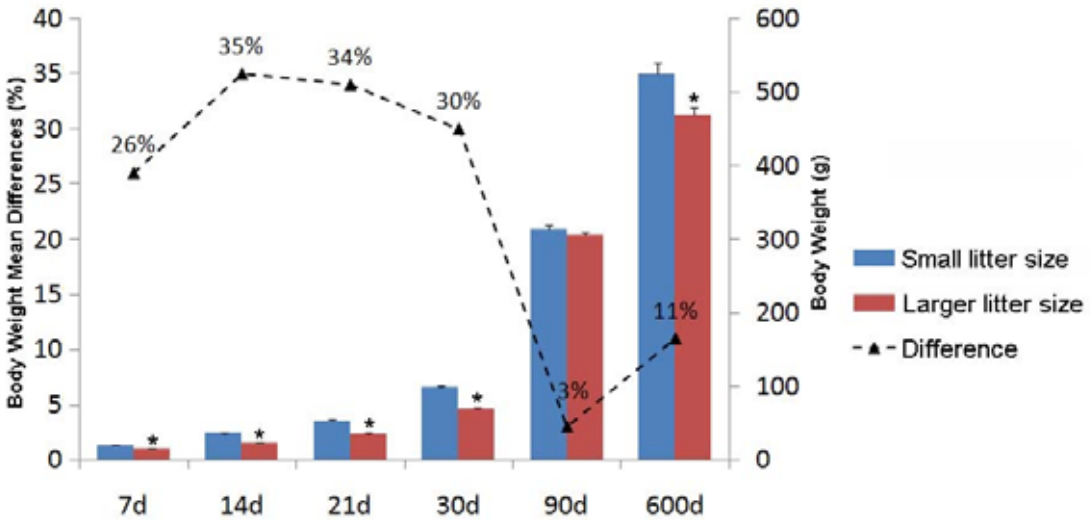

Figure 3

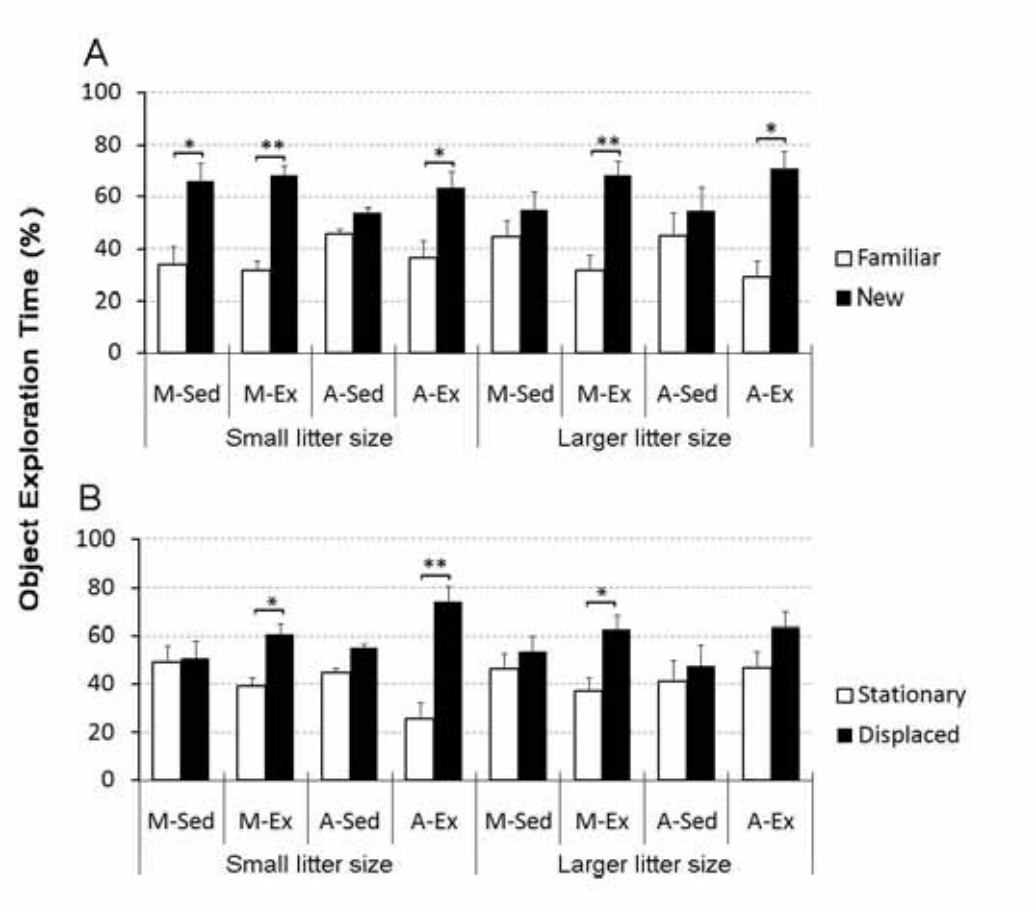

Figure 4

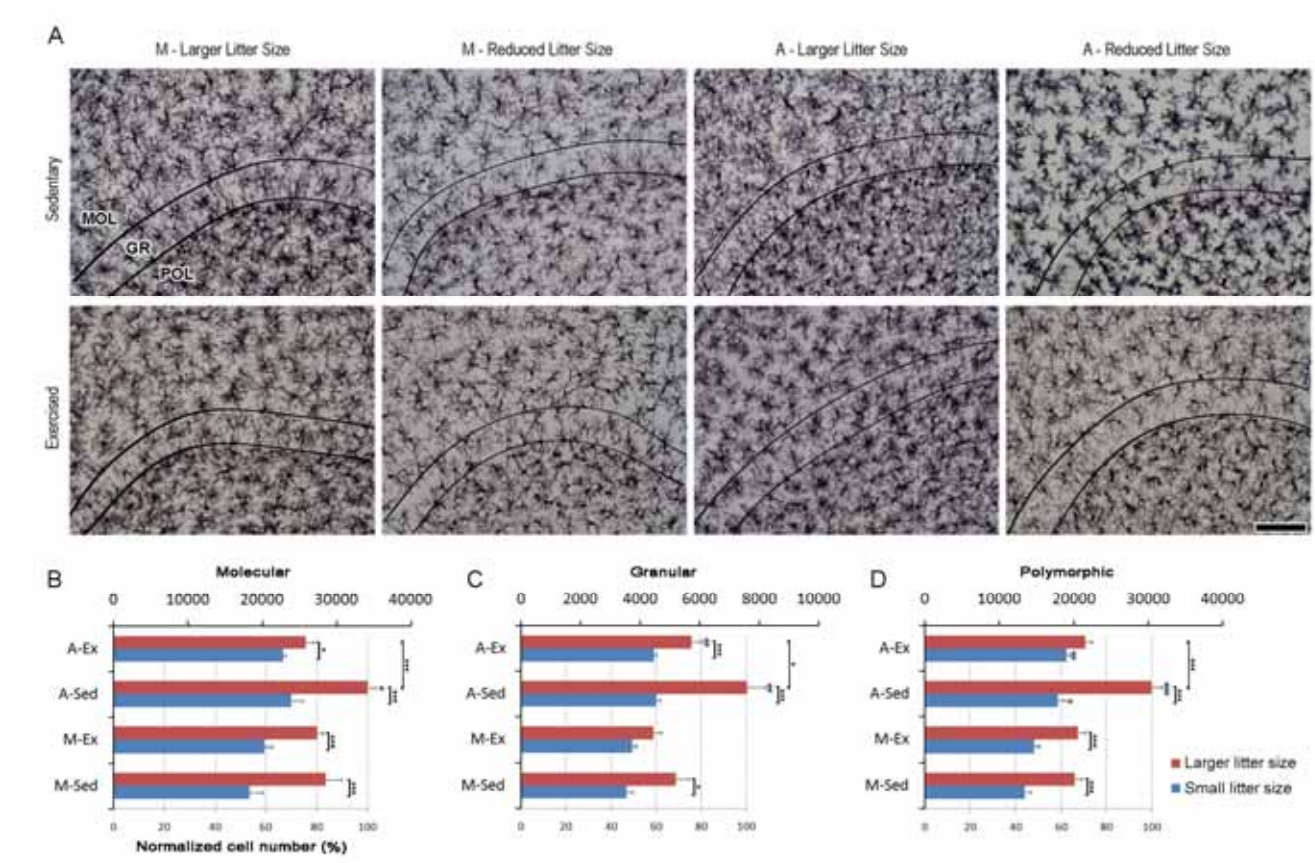

Figure 5

Figure 6

Table 1- Parameters of the Physical Exercise (running in the treadmill).

	1st week	2nd week	3rd week	4th week	5th week
Duration of the daily sessions	30 min	30 min	30 min	45 min	45 min
Number of sessions per week	5	5	5	3	2
Running speed	5 m/min	10 m/min	15 m/min	25 m/min	25 m/min

Table 2- Dentate Gyrus Volumes

	A-Sed	A-Ex	M-Sed	M-Ex
Small litter size	2.707 ± 0.086	2.946 ± 0.051(*)	2.979 ± 0.141	3.019 ± 0.040
Larger litter size	2.817 ± 0.033	2.871 ± 0.070	2.919 ± 0.066	2.963 ± 0.074

Aging impact on the dentate gyrus volumes F(1, 32) = 6.16; p , 0.018 Three-way ANOVA

(*) indicates significant differences between A-Sed *vs* A-Ex from small litters, pairwise comparisons [Q=TukeyHSD: t(8)=2.40 p< 0.0434]

Table S1. Microglial Granular Layer Estimates for Aged, Exercised and Sedentary Rats Raised in Large and Small Litters. Experimental Parameters, Optical Fractionator Counting Results and Individual Unilateral Microglial Numbers (N) and Mean Groups with the Coefficient of Error (CE).

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Aged Sedentary from Large Litters						
SMG20 EX62	31.5 ± 7.55	7354.49	0.070	0.258± 0.037	198	126
VIE G21 EX66	23.5 ± 0.22	8337.7	0.059	0.298 ± 0.003	206	184
VSDE G21 EX64	32.7 ± 5.33	9083.46	0.0615	0.236 ± 0.034	199	146
VSDE G29EX119	26.7 ± 0.39	7679.83	0.068	0.263± 0.004	201	150
VSDEG29EX120	32.2 ± 3.10	5493.52	0.085	0.224 ± 0.017	200	90
Mean	29.3± 1.80	7589.8	0.069			
SD		1346.12784				
$CV^2=(SD/Mean)^2$		0.031				
CE^2		0.005				
CE^2/CV^2		0.1507				
CVB^2		0.027				
CVB^2 (% of CV^2)		85				
Aged Sedentary from Small Litters						
DOR EXP 122	27.6 ± 4.06	4501.15	0.077	0.284± 0.049	155	88
SM G01B	18.1 ± 0.06	4644.68	0.071	0.388 ± 0.001	171	134
VME G04B	19.2 ± 0.40	4642.5	0.071	0.366 ± 0.007	194	125
VSD G04B	21.5 ± 0.69	4862.41	0.068	0.327 ± 0.011	173	118
VSE G01	20.2 ± 0.34	4112.38	0.074	0.348 ± 0.006	190	106
Mean	21.3 ± 1.66	4552.62	0.072			
S.D.		277.93				
$CV^2=(D.P./Mean)^2$		0.004				
CE^2		0.005				
CE^2/CV^2		1.3958				
CVB^2		-0.001				
CVB^2 (% of CV^2)		-40				

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Aged Exercised from Large Litters						
PAE G13	17.5 ± 0.14	5088.23	0.063	0.400 ± 0.003	220	151
SM G13	40.2 ± 0.81	6841.19	0.083	0.175 ± 0.003	213	89
SM G32	30.5 ± 3.11	6241.22	0.081	0.241 ± 0.025	184	108
VIE G32 A	24.9 ± 1.14	5440.69	0.073	0.284 ± 0.012	203	114
VSE G32 A	27.5 ± 1.44	4986.48	0.084	0.257 ± 0.013	210	94
Mean	28.1 ± 3.7	5719.6	0.077			
SD		797.4859				
$CV^2=(SD/Mean)^2$		0.019				
CE^2		0.006				
CE^2/CV^2		0.3032				
CVB^2		0.014				
CVB^2 (% of CV^2)		70				
Aged Exercised from Small Litters						
SMG23EX56	22.2±0.71	4345.33	0.075	0.317 ± 0.01	203	100
VIEG23EX58	21.4±1.21	4493.32	0.072	0.333 ± 0.019	195	110
VSDG01A	18.7±0.62	4277.57	0.076	0.376 ± 0.011	196	117
VSEG23EX59	24.4±1.04	4738.17	0.080	0.289 ± 0.012	196	99
VSEG25	22.1±0.76	4506.42	0.079	0.319 ± 0.011	223	105
Mean	21.8±0.91	4472.16	0.076			
S.D.		177.72				
$CV^2=(D.P./Mean)^2$		0.002				
CE^2		0.006				
CE^2/CV^2		3.668				
CVB^2		-0.004				
CVB^2 (% of CV^2)		-267				

[a]All evaluations were performed using a 100X objective lens (Nikon, NA 1.3, DF = 0.19µm). a(frame)' area of the optical dissector counting frame = 60 x 60 μm^2; A(x,y step), x and y step sizes = 90 x 90; asf, area sampling fraction [a(frame)/A(x,y step)] = 0.44; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7µm divided by section thickness, h/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; $\sum Q^-$, counted microglial markers.

Table S2. Microglial Granular Layer Estimates for Mature, Exercised and Sedentary Rats Raised in Large and Small Litters. Experimental Parameters, Optical Fractionator Counting Results and Individual Unilateral Microglial Numbers (N) and Mean Groups with the Coefficient of Error (CE).

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Mature Sedentary from Large Litters						
SM G39 EXP 96	20.4 ± 1.07	6845.66	0.063	0.349 ± 0.02	204	174
VIDE G38 EXP 86	19.2 ± 1.09	4337.82	0.073	0.372 ± 0.02	193	117
VIE G39 EXP 94	21.8 ± 0.96	4777.27	0.074	0.325 ± 0.02	183	114
VSD G38 EXP 89	19.7 ± 0.87	5219.27	0.064	0.359 ± 0.01	189	137
VSE+VID G39 EXP 92	19.9 ± 1.75	4554.58	0.072	0.362 ± 0.03	197	126
Mean	20.2 ± 0.44	**5146.92**	0.069			
SD		1004.129				
$CV^2=(SD/Mean)^2$		0.038				
CE^2		0.005				
CE^2/CV^2		**0.1257**				
CVB^2		0.033				
CVB^2 (% of CV^2)		87%				
Aged Sedentary from Small Litters						
PAD G52 EXP 136	15.6 ± 0.35	3193.78	0.075834	0.450 ± 0.010	181	106
PPE G52 EXP 135	13.9 ± 0.90	3322.62	0.076046	0.515 ± 0.034	200	123
SM G32 EXP 148	15.5 ± 0.37	3090.66	0.079999	0.453 ± 0.011	186	103
SM G52 EXP 134	18.9 ± 0.82	4153.19	0.1	0.375 ± 0.015	206	113
VSDE G37 EXP 71	14.8 ± 0.68	3928.22	0.067753	0.479 ± 0.021	187	137
Mean	15.7 ± 0.84	**3537.694**	0.079926			
S.D.		473.21				
$CV^2=(D.P./Mean)^2$		0.018				
CE^2		0.006				
CE^2/CV^2		0.3570				
CVB^2		0.011				
CVB^2 (% of CV^2)		64.3%				

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Mature Exercised from Large Litters						
CAB G56 EXP 143	17.9 ± 0.39	3683.76	0.075	0.392 ± 0.008	195	107
DOR G56 EXP 142	18.8 ± 0.59	4990.08	0.063	0.374 ± 0.011	185	138
PPE G56 EXP 144	20.1 ± 0.72	3948.6	0.082	0.350 ± 0.013	188	102
VIDE G41 EXP 105	24.1 ± 0.46	4425.97	0.078	0.291 ± 0.006	183	96
VME G47 EXP 106	21.3 ± 1.38	5058.2	0.070	0.335 ± 0.023	191	124
Mean		**4421.32**	0.074			
SD		611.7				
$CV^2=(SD/Mean)^2$		0.019				
CE^2		0.005				
CE^2/CV^2		0.2826				
CVB^2		0.014				
CVB^2 (% of CV^2)		72				
Mature Exercised from Small Litters						
DOR G51 EXP 126	22.9 ± 0.98	3861.76	0.086	0.301 ± 0.014	214	87
CAB G32 EXP 124	23.2 ± 0.31	3417.89	0.095	0.303 ± 0.004	195	76
VID G37 EXP 70	18.5 ± 1.15	3875.78	0.078	0.386 ± 0.027	185	108
VMD EXP 52	19.0 ± 0.89	3348.55	0.08	0.374 ± 0.018	193	93
VME G36 EXP 67	19.3 ± 1.36	4112.68	0.074	0.369 ± 0.022	193	112
Mean	20.6 ± 1.01	**3723.332**	0.082			
S.D.		327.01				
$CV^2=(D.P./Mean)^2$		0.002				
CE^2		0.007				
CE^2/CV^2		0.8812				
CVB^2		0.001				
CVB^2 (% of CV^2)		12				

[a]All evaluations were performed using a 100X objective lens (Nikon, NA 1.3, DF = 0.19µm). a(frame)' area of the optical dissector counting frame = 60 x 60 μm^2; A(x,y step), x and y step sizes = 90 x 90; asf, area sampling fraction [a(frame)/A(x,y step)] = 0.44; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7µm divided by section thickness, h/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; $\sum Q^-$, counted microglial markers.

Table S3. Microglial Molecular Layer Estimates for Aged, Exercised and Sedentary Subjects Raised in Large and Small Litters. Experimental Parameters, Optical Fractionator Counting Results and Individual Unilateral Microglial Numbers (N) and Mean Groups with the Coefficient of Error (CE).

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Aged Sedentary from Large Litters						
SMG20 EX62	31.8 ± 7.43	31315.52	0.047	0.253 ± 0.036	199	305
VIE G21 EX66	23.4 ± 0.24	35491.54	0.040	0.299 ± 0.003	213	439
VSDE G21 EX64	33.1 ± 5.21	36923.36	0.044	0.233 ± 0.034	203	338
VSDE G29EX119	26.7 ± 0.41	32348.03	0.045	0.262 ± 0.004	203	351
VSDEG29EX120	32.4 ± 1.80	34783.94	0.045	0.219 ± 0.011	216	311
Mean	29.5± 1.89	**34172.48**	0.044			
SD		2300.65108				
$CV^2=(SD/Mean)^2$		0.005				
CE^2		0.002				
CE^2/CV^2		**0.4288**				
CVB^2		**0.003**				
CVB^2 (% of CV^2)		57				
Aged Exercised from Large Litters						
PAE G13	17.7 ± 0.38	26842.82	0.038	0.396 ± 0.0083	224	443
SM G13	40.2 ± 0.65	28099	0.052	0.175 ± 0.0028	212	203
SM G32	30.5 ± 3.55	27355.25	0.050	0.244 ± 0.0281	210	266
VIE G32 A	25.8 ± 1.64	22590.08	0.047	0.277 ± 0.0163	198	258
VSE G32 A	28.1 ± 1.47	24445.39	0.049	0.252 ± 0.0132	211	255
Mean	25866.51	**25866.51**	0.047			
SD		2286.27				
$CV^2=(SD/Mean)^2$		0.008				
CE^2		0.002				
CE^2/CV^2		0.2865				
CVB^2		0.006				
CVB^2 (% of CV^2)		71				
Aged Sedentary from Small Litters						
DOR EXP 122	29.5 ± 3.89	29682.22	0.043	0.262 ± 0.047	197	304
SM G01B	18.6 ± 0.31	24145.84	0.039	0.378 ± 0.006	198	380
VME G04B	18.9 ± 0.29	21524.84	0.044	0.370 ± 0.006	207	330
VSD G04B	21.6 ± 0.64	22572.45	0.046	0.325 ± 0.010	212	304
VSE G01	19.8 ± 0.58	21415.26	0.045	0.356 ± 0.010	211	316
Mean	21.7 ± 2.02	**23868.122**	0.044			
S.D.		3430.27445				
$CV^2=(D.P./Mean)^2$		0.021				
CE^2		0.002				
CE^2/CV^2		**0.0921**				
CVB^2		**0.019**				
CVB^2 (% of CV^2)		91				
Aged Exercised from Small Litters						
SMG23EX56	22.7 ± 0.26	22578.74	0.045	0.309 ± 0.036	211	289
VIEG23EX58	22.1 ± 1.50	21724.54	0.048	0.323 ± 0.021	210	293
VSDG01A	18.4 ± 0.61	23877.26	0.040	0.382 ± 0.012	215	378
VSEG23EX59	24.8 ± 1.17	22193.94	0.051	0.285 ± 0.013	209	261
VSEG25	22.4 ± 0.59	23503.34	0.043	0.314 ± 0.008	228	305
Mean		**22775.56**	0.045			
S.D.		897.9239				
$CV^2=(D.P./Mean)^2$		0.002				
CE^2		0.002				
CE^2/CV^2		**1.3170**				
CVB^2		**0.000**				
CVB^2 (% of CV^2)		-32				

[a]All evaluations were performed using a 100X objective lens (Nikon, NA 1.3, DF = 0.19µm). a(frame)⁻ area of the optical dissector counting frame = 60 x 60 μm^2; A(x,y step), x and y step sizes = 90 x 90; asf, area sampling fraction [a(frame)/A(x,y step)] = 0.44; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7µm divided by section thickness, h/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; ∑Q⁻, counted microglial markers.

Table S4. Microglial MolecularLayer Estimates for Mature, Exercised and Sedentary Rats Raised in Large and Small Litters. Experimental Parameters, Optical Fractionator Counting Results and Individual Unilateral Microglial Numbers (N) and Mean Groups with the Coefficient of Error (CE).

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Mature Sedentary from Large Litters						
SM G39 EXP 96	21.2 ± 1.55	30981	0.05	0.341 ± 0.031	241	426
VIDE G38 EXP 86	19.7 ± 1.09	21664.67	0.05	0.363 ± 0.019	208	322
VIE G39 EXP 94	22.5 ± 1.16	27878.07	0.04	0.316 ± 0.017	211	368
VSD G38 EXP 89	19.6 ± 0.69	34254.02	0.03	0.360 ± 0.013	228	509
VID G39 EXP 92	20.3 ± 1.23	26981.75	0.04	0.342 ± 0.020	205	395
Mean	20.7 ± 0.54	**28351.9**	0.04			
SD		4705.53				
$CV^2=(SD/Mean)^2$		0.028				
CE^2		0.002				
CE^2/CV^2		0.0703				
CVB^2		0.026				
CVB^2 (% of CV^2)		93%				
MatureSedentary from Small Litters						
PAD G52 EXP 136	16.0 ± 0.44	16082.51	0.045	0.441 ± 0.013	213	291
PPE G52 EXP 135	14.1 ± 0.92	16272.66	0.051	0.506 ± 0.033	226	327
SM G32 EXP 148	15.9 ± 0.19	15834.46	0.049	0.442 ± 0.005	201	292
SM G52 EXP 134	19.4 ± 0.98	24809.92	0.038	0.365 ± 0.018	268	373
VSDE G37 EXP 71	15.8 ± 0.92	18657.07	0.048	0.452 ± 0.026	216	340
Mean	16.2 ± 0.86	**18331.32**	0.046			
S.D.		3794.99				
$CV^2=(D.P./Mean)^2$		0.043				
CE^2		0.002				
CE^2/CV^2		0.0499				
CVB^2		0.041				
CVB^2 (% of CV^2)		95%				

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Mature Exercised from Large Litters						
CAB G56 EXP 143	19.1 ± 0.58	25352.66	0.04	0.36946 ± 0.0109	247	388
DOR G56 EXP 142	19.6 ± 0.89	26975.39	0.04	0.36086 ± 0.0158	223	399
PPE G56 EXP 144	21.2 ± 0.94	28779.58	0.04	0.33401 ± 0.0159	215	399
VIDE G41 EXP 105	24.4 ± 0.42	26054.13	0.04	0.28773 ± 0.0049	204	310
VME G47 EXP 106	21.9 ± 1.66	29130.15	0.04	0.32774 ± 0.0258	225	389
Mean	21.2± 0.94	**27258.4**	0.04			
SD		1656.78				
$CV^2=(SD/Mean)^2$		0.004				
CE^2		0.002				
CE^2/CV^2		0.5060				
CVB^2		0.002				
CVB^2 (% of CV^2)		49%				
Mature Exercised from Small Litters						
DOR G51 EXP 126	23.4 ± 1.01	21531.2	0.05	0.304 ± 0.014	236	268
CAB G32 EXP 124	23.9 ± 0.28	20991.13	0.05	0.297 ± 0.004	234	255
VID G37 EXP 70	19.4 ± 1.43	21096.16	0.05	0.370 ± 0.031	224	320
VMD EXP 52	19.8 ± 0.77	17486.28	0.05	0.359 ± 0.014	224	254
VME G36 EXP 67	20.0 ± 1.49	23311.8	0.05	0.358 ± 0.023	217	344
Mean	21.3 ± 0.96	**20883.3**	0.05			
S.D.		2116.25				
$CV^2=(D.P./Mean)^2$		0.010				
CE^2		0.002				
CE^2/CV^2		0.2387				
CVB^2		0.008				
CVB^2 (% of CV^2)		76%				

[a]All evaluations were performed using a 100X objective lens (Nikon, NA 1.3, DF = 0.19µm). a(frame)' area of the optical dissector counting frame = 60 x 60 µm^2; A(x,y step), x and y step sizes = 90 x 90; asf, area sampling fraction [a(frame)/A(x,y step)] = 0.44; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7µm divided by section thickness, h/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; $\sum Q^-$, counted microglial markers.

Table S5. Microglial Polymorphic Layer Estimates for Aged, Exercised and Sedentary Rats Raised in Large and Small Litters. Experimental Parameters, Optical Fractionator Counting Results and Individual Unilateral Microglial Numbers (N) and Mean Groups with the Coefficient of Error (CE).

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ˙
Aged Sedentary from Large Litters						
SMG20 EX62	32.4 ± 7.69	27768.73	0.045	0.249 ± 0.035	208	265
VIE G21 EX66	24.4 ± 0.30	30496.62	0.041	0.288 ± 0.003	200	363
VSDE G21 EX64	33.9 ± 5.18	33614.91	0.042	0.227 ± 0.032	200	302
VSDE G29EX119	27.1 ± 0.28	33714.75	0.048	0.258 ± 0.002	214	362
VSDEG29EX120	33.4 ± 2.57	26736.54	0.052	0.214 ± 0.014	215	234
Mean	30.2± 1.89	30466.31	0.046			
SD		3227.03754				
$CV^2=(SD/Mean)^2$		0.011				
CE^2		0.002				
CE^2/CV^2		0.1861				
CVB^2		0.009				
CVB^2 (% of CV^2)		81%				
Aged Sedentary from Small Litters						
DOR EXP 122	30.4 ± 3.91	18690.85	0.053	0.257 ± 0.049	161	187
SM G01B	19.3 ± 0.20	15951.26	0.055	0.364 ± 0.004	165	239
VME G04B	19.4 ± 0.52	16177.82	0.050	0.362 ± 0.009	191	243
VSD G04B	22.2 ± 0.72	17137.98	0.047	0.317 ± 0.011	212	227
VSE G01	21.2 ± 0.33	21281.11	0.042	0.331 ± 0.005	219	290
Mean	22.5 ± 2.04	17847.8	0.049			
S.D.		2201.58423				
$CV^2=(D.P./Mean)^2$		0.015				
CE^2		0.002				
CE^2/CV^2		0.1593				
CVB^2		0.013				
CVB^2 (% of CV^2)		84%				

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ˙
Aged Exercised from Large Litters						
PAE G13	18.2 ± 0.29	21137.91	0.044	0.386 ± 0.006	216	338
SM G13	41.1 ± 0.60	23926.4	0.053	0.170 ± 0.003	211	168
SM G32	33.5 ± 3.12	23285.19	0.063	0.222 ± 0.023	205	205
VIE G32 A	26.2 ± 1.21	18720.87	0.052	0.269 ± 0.012	205	208
VSE G32 A	29.3 ± 1.30	20622.89	0.051	0.241 ± 0.010	204	206
Mean	29.7 ± 3.80	21538.65	0.053			
SD		2103.024				
$CV^2=(SD/Mean)^2$		0.010				
CE^2		0.003				
CE^2/CV^2		0.2918				
CVB^2		0.007				
CVB^2 (% of CV^2)		71				
Aged Exercised from Small Litters						
SMG23EX56	22.2±0.71	19418.85	0.048	0.310 ± 0.007	206	254
VIEG23EX58	21.4±1.21	19125.14	0.046	0.309 ± 0.012	223	248
VSDG01A	18.7±0.62	18840.02	0.044	0.378 ± 0.005	214	294
VSEG23EX59	24.4±1.04	20141.05	0.046	0.275 ± 0.007	207	229
VSEG25	22.1±0.76	17835.57	0.045	0.307 ± 0.015	213	226
Mean	22.5 ± 1.11	19072.13	0.046			
S.D.		843.88				
$CV^2=(D.P./Mean)^2$		0.002				
CE^2		0.0021				
CE^2/CV^2		1.0675				
CVB^2		-0.0001				
CVB^2 (% of CV^2)		-6.7453				

[a]All evaluations were performed using a 100X objective lens (Nikon, NA 1.3, DF = 0.19µm). a(frame)' area of the optical dissector counting frame = 60 x 60 μm^2; A(x,y step), x and y step sizes = 120 x 120; asf, area sampling fraction [a(frame)/A(x,y step)] = 0.25; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7µm divided by section thickness, h/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; $\sum Q^-$, counted microglial markers.

Table S6. Microglial Polymorphic Layer Estimates for Mature, Exercised and Sedentary Rats Raised in Large and Small Litters. Experimental Parameters, Optical Fractionator Counting Results and Individual Unilateral Microglial Numbers (N) and Mean Groups with the Coefficient of Error (CE).

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Mature Sedentary from Large Litters						
SM G39 EXP 96	21.0 ± 0.95	22123.43	0.044	0.338 ± 0.017	207	305
VIDE G38 EXP 86	19.6 ± 1.06	16861.75	0.047	0.363 ± 0.018	198	253
VIE G39 EXP 94	22.2 ± 0.96	20119.39	0.050	0.318 ± 0.033	207	263
VSD G38 EXP 89	19.0 ± 0.57	21918.56	0.043	0.370 ± 0.011	209	336
VID G39 EXP 92	20.8 ± 1.79	18787.87	0.048	0.347 ± 0.028	203	274
Mean	20.5 ± 0.56	**19962.2**	0.046			
SD		2208.778				
$CV^2=(SD/Mean)^2$		0.012				
CE^2		0.002				
CE^2/CV^2		0.1739				
CVB^2		0.010				
CVB^2 (% of CV^2)		83				
MatureSedentary from Small Litters						
PAD G52 EXP 136	16.3 ± 0.30	11971.18	0.054	0.441 ± 0.013	202	214
PPE G52 EXP 135	14.5 ± 0.91	13099.32	0.047	0.506 ± 0.033	200	259
SM G32 EXP 148	15.3 ± 0.29	11548.52	0.050	0.442 ± 0.005	201	220
SM G52 EXP 134	18.9 ± 0.92	15869.08	0.046	0.365 ± 0.018	222	243
VSDE G37 EXP 71	15.6 ± 0.75	14771.88	0.047	0.452 ± 0.026	212	275
Mean	16.1 ± 0.75	**13452**	0.049			
S.D.		1838.804				
$CV^2=(D.P./Mean)^2$		0.019				
CE^2		0.002				
CE^2/CV^2		0.1268				
CVB^2		0.016				
CVB^2 (% of CV^2)		87				

Subjects	Section thickness (µm)	N	CE	tsf	No. of counting frames	ΣQ⁻
Mature Exercised from Large Litters						
CAB G56 EXP 143	19.0 ± 0.64	16844.02	0.048	0.371 ± 0.012	211	259
DOR G56 EXP 142	19.5 ± 0.56	21365.45	0.042	0.361 ± 0.010	211	320
PPE G56 EXP 144	20.4 ± 0.68	23465.51	0.042	0.345 ± 0.011	202	328
VIDE G41 EXP 105	24.5 ± 0.32	20521.48	0.047	0.286 ± 0.003	218	242
VME G47 EXP 106	21.5 ± 1.45	20123.41	0.048	0.332 ± 0.023	209	278
Mean	20463.97	**20463.97**	0.045			
SD		2400.273				
$CV^2=(SD/Mean)^2$		0.014				
CE^2		0.002				
CE^2/CV^2		0.1497				
CVB^2		0.012				
CVB^2 (% of CV^2)		85				
Mature Exercised from Small Litters						
DOR G51 EXP 126	24.0 ± 1.11	12888.41	0.065	0.296 ± 0.015	216	156
CAB G32 EXP 124	24.4 ± 0.35	14652.8	0.059	0.289 ± 0.004	210	174
VID G37 EXP 70	19.4 ± 0.97	16278.53	0.049	0.367 ± 0.019	212	248
VMD EXP 52	20.1 ± 0.91	14034.62	0.055	0.354 ± 0.017	208	204
VME G36 EXP 67	19.5 ± 1.26	15602.15	0.049	0.366 ± 0.022	210	235
Mean	21.5 ± 1.11	**14691.3**	0.055			
S.D.		1325.903				
$CV^2=(D.P./Mean)^2$		0.010				
CE^2		0.003				
CE^2/CV^2		0.3768				
CVB^2		0.005				
CVB^2 (% of CV^2)		62				

*All evaluations were performed using a 100X objective lens (Nikon, NA 1.3, DF = 0.19µm). a(frame)' area of the optical dissector counting frame = 60 x 60 µm^2; A(x,y step), x and y step sizes = 120 x 120; asf, area sampling fraction [a(frame)/A(x,y step)] = 0.25; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7µm divided by section thickness, h/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; ∑Q⁻, counted microglial markers.

6.0 Considerações finais

Do ponto de vista eletrofisiológico, eventos vivenciados pelo organismo durante o seu desenvolvimento, tais como aqueles representados por distintas condições de lactação e pelo exercício forçado em esteira modularam a atividade cortical espontânea de uma maneira duradoura a julgar pelos efeitos sobre a velocidade de propagação da DAC. Dentre tais alterações, observamos que a lactação em ninhadas de tamanho maior (L12) e o exercício influenciam a propagação da DAC de forma oposta: aquela facilita, enquanto o exercício dificulta a DAC. O processo de envelhecimento, por sua vez, interage com esses dois fatores, modulando os seus efeitos sobre a propagação da DAC. Além desta interação, o envelhecimento favorece a falha na propagação da DAC, ratificando a relação inversa entre idade e susceptibilidade cortical à DAC. Por outro lado, sobre o ponto de vista comportamental, ratos adultos e idosos sedentários apresentaram memória espacial prejudicada de forma independente das condições de lactação. O exercício em esteira reduziu este prejuízo na memória espacial de animais idosos criados em ninhadas pequenas, mas não daqueles amamentados em ninhadas grandes. Com relação à memória de reconhecimento de objetos, apenas animais idosos sedentários de ambas as condições de lactação apresentaram prejuízo para o reconhecimento de objetos. Assim como para memória espacial, o exercício também reduziu este prejuízo na memória de reconhecimento de objetos, entretanto, desta vez, este efeito foi independente das condições de lactação. Estas conclusões nos permitem inferir que os dados eletrofisiológicos e comportamentais apresentados representam importante avanço na compreensão da relação entre a excitabilidade cerebral, o comportamento, a nutrição e o exercício, no encéfalo em processo de envelhecimento.

7.0 Perspectivas

Algumas perspectivas são sugeridas para a continuidade do presente estudo:

- Comparar os efeitos eletrofisiológicos de paradigmas diferentes de exercício físico, forçado ou voluntário, assim como um programa com ambos, a julgar pelas alterações na propagação da DAC;
- Estudar a associação entre os efeitos do exercício voluntário e o estado nutricional imposto no início da vida sobre a propagação da DAC;
- Avaliar a possível relação dos efeitos da combinação do exercício voluntário e forçado, com as condições de lactação, sobre a excitabilidade cortical, através da análise da propagação da DAC;
- Analisar os efeitos do exercício voluntário e forçado sobre a memória episódica de ratos;
- Caracterizar os efeitos de uma "curva freqüência-resposta" de exercício sobre parâmetros eletrofisiológicos e comportamentais;
- Avaliar os efeitos do exercício físico sobre a DAC utilizando fármacos, para testar o envolvimento dos sistemas glutamatérgico, GABAérgico, dopaminérgico, de opióides e serotoninérgico, nos mecanismos neuroquímicos associados a esse efeito;

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ANEXOS

Anexo 01: comprovante de submissão do artigo 01 após correções solicitadas pela "Experimental Gerontology.

---------- Mensagem encaminhada ----------
De: **Experimental Gerontology** <exg@elsevier.com>
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Para: guedes.rca@gmail.com

Ms. Ref. No.: EXG-12-10R1
Title: Aging-dependent brain electrophysiological effects in rats after distinct lactation conditions, and treadmill exercise: A spreading depression analysis
Experimental Gerontology

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Anexo 02: Comprovante de submissão do artigo 02

Article title: Litter size and sedentary lifestyle affect aging cognitive decline and microglial number in the rat dentate gyrus.
MS ID : 2032584024674832
Authors : Lane C. Viana, Camila M. Lima, César A. R. Fôro, Marcus A. Oliveira, Tatiana T. Cardoso, Izabela N. F. Almeida, Daniel G. Diniz, João B. Torres, Antonio Pereira, Manuella B. Oliveira, Andreia A. C. Lopes, Rosângela F. M. Silva, Ricardo A. Guedes, Angela A. Santos, Denise S. Lima, Pedro F. C. Vasconcelos, Colm Cunningham, Rubem C. A. Guedes, Ronaldo P. Borges and Cristovam W. Picanço Diniz
Journal : Journal of Neuroinflammation

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Best wishes,

The JNI Editorial Team

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Anexo 03: capítulo de livro publicado.

Batista-de-Oliveira M. ; Fregni, F. **Estimulação transcraniana por corrente alternada**. In: Felipe Fregni; Paulo Sérgio Boggio; André Russowsky Brunoni.. (Org.). Neuromodulação Terapêutica. Princípios e Avanços da Estimulação Cerebral Não Invasiva em Neurologia, Reabilitação, Psiquiatria e Neuropsicologia. 1ª ed. São Paulo: Sarvier, 2012, p. 570.

Anexo 04: matéria publicada na Revista Psique

dossiê

Estimulação TRANSCRANIANA magnética ou com corrente contínua

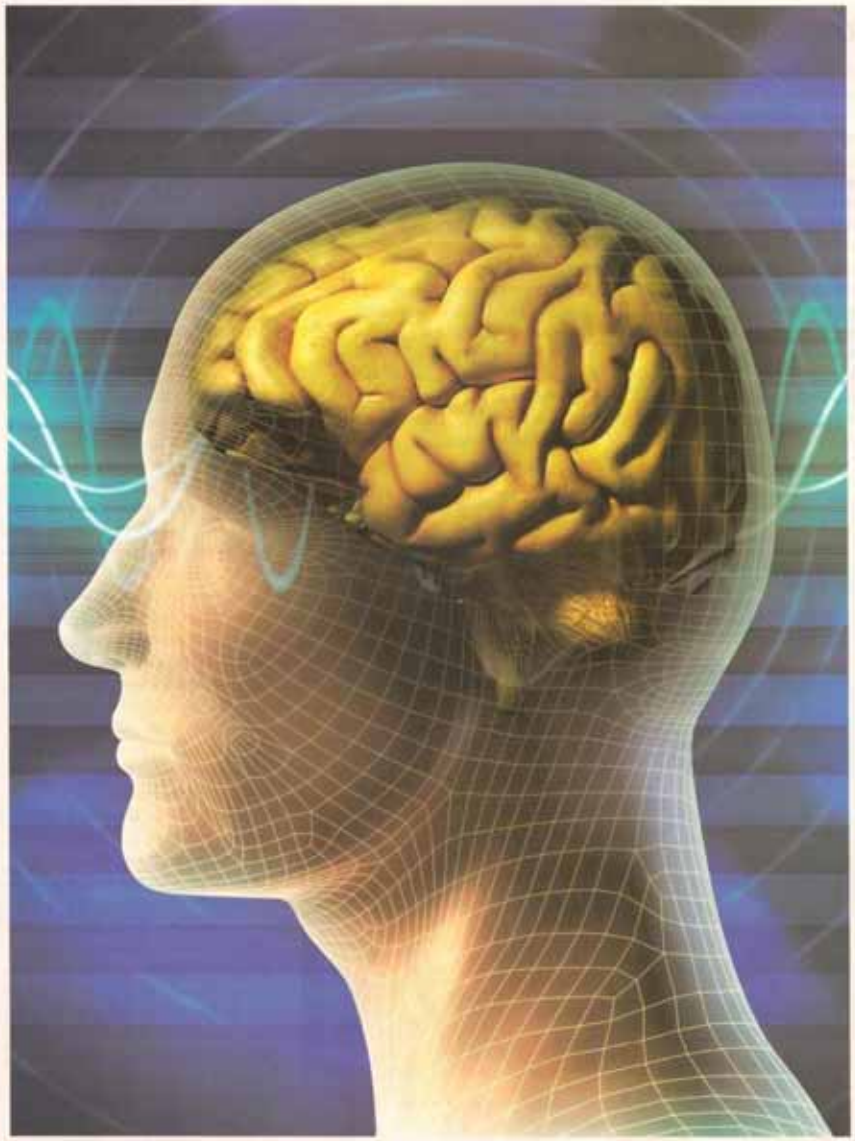
Considerados métodos de estimulação cerebral não invasivos úteis ao tratamento da depressão. Estudos preliminares recentes demonstram bons resultados com o uso da EMTr e da ETCC para alívio dos sintomas da doença

Por Manuella Batista de Oliveira e Felipe Fregni

A depressão pode ser definida como humor deprimido e/ou perda de interesse ou prazer por quase todas as atividades em um período mínimo de duas semanas. Para o diagnóstico da depressão, pelo menos quatro dos seguintes sintomas devem estar presentes: alterações de apetite, distúrbio no sono, agitação ou retardo psicomotor, fadiga ou perda de energia, sentimento de culpa excessiva ou inutilidade, dificuldade de concentração ou pensamentos recorrentes relacionados à morte. A ocorrência destes sinais e sintomas deve ser observada na maior parte do dia e acarretar em sofrimentos

Manuella Batista de Oliveira é nutricionista. Doutoranda (programa de doutorado sanduíche) do Laboratório de Neuromodulação. Spaulding Rehabilitation Hospital. Harvard Medical School. Boston-MA. USA. Doutoranda do Laboratório de Fisiologia da Nutrição Nalde Teodosio. Departamento de Nutrição. Universidade Federal de Pernambuco. Recife-PE. Brasil MSc.

Felipe Fregni é médico e professor associado da Harvard Medical School. Diretor do Laboratório de Neuromodulação. Spaulding Rehabilitation Hospital. Harvard Medical School. Boston-MA. USA. Diretor do Curso de Princípios e Práticas em Pesquisa Clínica. Harvard Medical School.

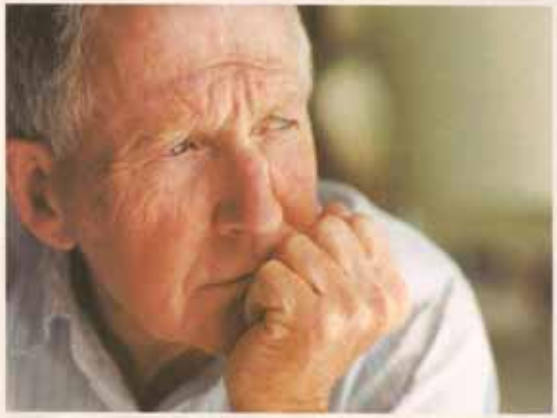


Perspectivas futuras

A incidência crescente dos sintomas depressivos e dos casos refratários aos tratamentos tradicionais tem aumentado a procura por novas abordagens terapêuticas. Estas novas terapias buscam identificar prováveis pontos falhos das técnicas anteriormente empregadas, atuar por mecanismos alternativos e ajudar pacientes portadores de distúrbios neuropsiquiátricos (por exemplo, depressão), melhorando o tratamento destes distúrbios e, dessa forma, melhorando a interação social e profissional.

Tanto a EMTr como a ETCC apresentam resultados promissores para o estudo dos mecanismos neurais associados à ocorrência dos casos depressivos, assim como para o tratamento dos sintomas depressivos. No Brasil, alguns centros oferecem o tratamento com o uso destas técnicas, como o Centro de Dor e Neurocirurgia Funcional do Hospital 9 de Julho. Mais estudos são necessários para elucidar, por exemplo: (1) os melhores parâmetros empregados ao tratamento da depressão, como duração e intensidade da estimulação, montagem e tamanho dos eletrodos e melhor área cortical a ser estimulada; (2) o melhor momento para a intervenção, ou seja, ponto crítico para se utilizar a ETCC durante o curso do distúrbio depressivo.

A ocorrência do episódio depressivo varia de acordo com a população estudada e com os instrumentos utilizados para diagnóstico



É cada vez maior o interesse da comunidade clínica e científica na busca de abordagens terapêuticas alternativas para pacientes com depressão

ou prejuízos clinicamente significativos no convívio social e profissional. Essas etapas e sintomas para o diagnóstico da depressão podem ser encontrados no Manual Estatístico e Diagnóstico para Distúrbios Mentais (DSM-IV). Atualmente, a Associação Americana de Psiquiatria (APA – *American Psychiatric Association*) está trabalhando no novo DSM-5 que será publicado oficialmente em maio de 2013.

A ocorrência do episódio depressivo, por sua vez, varia de acordo com a população estudada e com os instrumentos utilizados para diagnóstico. Evidências têm demonstrado que a prevalência da depressão pode oscilar entre 3% (São Paulo) e 10% (Porto Alegre). O aumento crescente na incidência de episódios depressivos tem gerado grande interesse da comunidade clínica e científica na busca de abordagens terapêuticas alternativas para estes pacientes, especialmente para aqueles refratários ao tratamento medicamentoso usual.

Algumas técnicas de estimulação cerebral têm sido utilizadas para o tratamento de depressão, tais como: (1) terapia eletroconvulsiva; (2) estimulação do nervo vago; (3) estimulação cerebral profunda; (4) estimulação magnética transcraniana repetitiva (EMTr); e (5) estimulação transcraniana com corrente contínua (ETCC) de baixa intensidade.

Dentre estas técnicas, a terapia eletroconvulsiva é a mais antiga. Apesar dos mecanismos ainda não estarem bem esclarecidos, esta técnica é capaz de causar melhoras no quadro depressivo refratário a outros tratamentos, como o medicamentoso. Por outro lado, estudos preliminares recentes demonstram bons resultados com o uso da EMTr e da ETCC para alívio dos sintomas depressivos. A EMTr e a ETCC são métodos de estimulação cerebral não invasivos que podem ser bastante úteis ao tratamento da depressão. A intensidade de corrente

Para o tratamento de depressão, algumas técnicas de estimulação cerebral têm sido utilizadas, tais como a estimulação magnética transcraniana repetitiva (EMTr)

Atualmente, o melhor ganho clínico tem sido observado com o uso da EMTr para o tratamento da depressão maior

apropriada, o tamanho dos eletrodos, assim como as montagens dos eletrodos são cruciais para a aplicação dessas técnicas. O artigo recentemente publicado no *Journal of Visualized Experiments* – JOVE apresenta os parâmetros para aplicação da ETCC (segue o link para o periódico *on-line* http://www.jove.com/details.php?id=2744 ou vide referência abaixo).

Como funcionaria a EMTr para o tratamento de depressão? Quais as vantagens desta técnica? Em seguida, e a ETCC, qual a diferença em relação à EMTr? E, por fim, as perspectivas no campo clínico e experimental para o emprego destas técnicas no tratamento da depressão.

• O uso da estimulação elétrica cerebral em busca de neuromodulação •

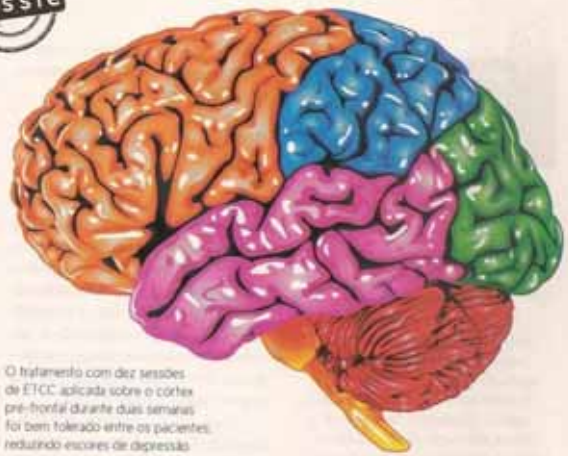
Quando falamos em estimulação elétrica cerebral, ou treinamento cerebral, ou neuromodulação, frequentemente escutamos as pessoas dizerem que é um absurdo, provavelmente não causa efeitos significativos e alguns até duvidam da credibilidade destas técnicas. Estas opiniões geralmente são devido à falta de informação. De fato, o uso da estimulação elétrica para investigação e/ou treinamento da atividade cerebral é relativamente recente do ponto de vista técnico-científico, por exemplo. A estimulação magnética transcraniana foi introduzida em 1985 e o uso desta técnica para o tratamento de depressão tem menos de 20 anos. Entretanto, valiosos resultados já foram encontrados e publicados na literatura atual, alguns deles descritos neste artigo. Desta forma, há atualmente um interesse crescente pelo entendimento dos mecanismos destas técnicas, dos efeitos da neuromodulação, bem como o aperfeiçoamento de seus resultados para o tratamento de distúrbios psiquiátricos. A neuromodulação pode ser definida como alterações químicas e/ou físicas observadas nos neurônios após a aplicação de estímulos elétricos no tecido cerebral. O que pode ocasionar facilitação ou inibição de uma determinada ação fisiológica ou resposta comportamental, dependendo do local de aplicação do estímulo, da sua duração, dentre outros parâmetros descritos ao longo deste artigo. Ao final, o leitor será capaz de formar sua própria opinião. Seria a neuromodulação através da estimulação elétrica o caminho para o tratamento da depressão? Ou seria esta pergunta uma superestimação destes efeitos? Ou apenas o início de tudo?

A estimulação magnética transcraniana repetitiva (EMTr)

Barker e colaboradores (1985) desenvolveram o primeiro equipamento capaz de produzir a estimulação cerebral não invasiva, introduzindo a estimulação magnética transcraniana. A EMTr pode influenciar a excitabilidade cortical, ora aumentando-a, ora reduzindo-a, dependendo dos parâmetros de estimulação utilizados e de uma maneira mais focal que a ETCC.

Logo após o surgimento da EMTr e descrição da sua capacidade em influenciar a excitabilidade cortical, grupos de pesquisadores começaram a investigar o uso da EMTr para o tratamento de distúrbios psiquiátricos. Neste contexto, cabe ressaltar que, atualmente, o melhor ganho clínico tem sido observado com o uso da EMTr para o tratamento da depressão maior.

A incidência crescente dos sintomas depressivos e dos casos refratários aos tratamentos tradicionais tem aumentado a procura por novas abordagens terapêuticas.



O tratamento com dez sessões de ETCC aplicada sobre o córtex pré-frontal durante duas semanas foi bem tolerado entre os pacientes, reduzindo escores de depressão

A EMTr pode influenciar a excitabilidade cortical, ora aumentando-a, ora reduzindo-a, dependendo dos parâmetros de estimulação utilizados

A literatura atual apresenta revisões sistemáticas, metanálises e inúmeros ensaios clínicos avaliando os efeitos da EMTr para o tratamento da depressão (Gross et al., 2007). Embora a maior parte destes achados tenha demonstrado resultados favorecendo o uso da EMTr, quando comparado ao tratamento controle (placebo), os efeitos da EMTr podem ser heterogêneos. Alguns dos ensaios clínicos são de baixa qualidade, por exemplo, com um número reduzido de pacientes, em alguns casos menos de dez pacientes e, em outros, os parâmetros de estimulação podem ter sido utilizados de maneira inadequada.

Mais de 15 anos se passaram desde o primeiro ensaio clínico publicado sobre os efeitos da EMTr para o tratamento da depressão. Portanto, tanto no ambiente experimental como no clínico, há um crescimento contínuo destes profissionais para tentar descrever os melhores parâmetros empregados ao tratamento da depressão com a EMTr, bem como os mecanismos relacionados a tais efeitos sobre os sintomas deste distúrbio.

A estimulação com corrente contínua (ETCC)

Dentre os métodos de estimulação cerebral não invasiva, além da EMTr, a estimulação transcraniana com corrente contínua (ETCC) tem sido empregada para o tratamento de alguns sintomas depressivos. A ETCC é empregada com a aplicação de correntes contínuas de baixa intensidade através de eletrodos posicionados sobre o couro cabeludo. Embora apenas uma parte desta corrente alcance o tecido cerebral, esta quantidade é suficiente para modular a atividade cerebral. Esta modulação, ou seja, aumento ou redução da excitabilidade cortical, depende dos parâmetros de estimulação empregados, como montagem e tamanho dos eletrodos, intensidade de corrente e duração do período de estimulação.

Estudos recentes têm apresentado a ETCC como uma técnica eficiente de neuromodulação. Em outras palavras, a aplicação da ETCC leva à modulação da atividade espontânea do tecido nervoso. De uma maneira diferente da EMTr, a ETCC não causa a geração de potenciais de ação, disparo neuronal propriamente dito, mas, sim, provoca uma facilitação ou inibição da ocorrência destes potenciais de ação, devido à manipulação da permeabilidade iônica através da membrana neuronal.

No início deste artigo, além da ETCC, outras técnicas de estimulação cerebral foram citadas. Quando comparadas àquelas técnicas, a ETCC apresenta certas vantagens. Por exemplo, a ETCC é de fácil aplicação, sendo simples e de baixo custo, e oferece um modelo de estimulação placebo (controle) que é eficaz. Além disso, tanto a EMTr como a ETCC não requerem nenhum procedimento cirúrgico, já que são aplicadas sobre o couro cabeludo.

Uma desvantagem do uso da ETCC é a baixa focalidade. Apesar disso, a aplicação da ETCC para o tratamento de episódios depressivos tem proporcionado resultados interessantes. Por exemplo, o tratamento com dez sessões de ETCC aplicada sobre o córtex pré-frontal durante um período de duas semanas foi bem tolerado entre os pacientes. Neste ensaio clínico, encontramos uma redução significante nos escores de depressão, quando comparados aos grupos controles. Os efeitos benéficos da aplicação da ETCC sobre o córtex pré-frontal persistiram por um mês após o término do tratamento (Boggio et al., 2008). Desta forma, estes achados, dentre outros apresentados na literatura atual, apoiam a investigação dos efeitos desta nova abordagem terapêutica para o tratamento da depressão.

Anexo 05:

----- Original Message -----
From: "Life Sciences" lifesci@elsevier.com
To: rc.guedes@terra.com.br, rguedes@ufpe.br
Cc:
Sent: Qui 09/02/12 23:03
Subject: Fwd: Submission Confirmation

Dear Prof. Guedes,

Your submission entitled "Favorable and unfavorable lactation modulate the effects of electrical stimulation on brain excitability: a spreading depression study in adult rats" has been received by Life Sciences

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