UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

ASPECTOS MOTORES E NEURAIS EM RATOS SUBMETIDOS À LESÃO DO SISTEMA NERVOSO CENTRAL E PERIFÉRICO E TRATAMENTO DE REABILITAÇÃO

ALINE DE SOUZA PAGNUSSAT

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ALINE DE SOUZA PAGNUSSAT

Orientador: Prof. Dr. Carlos Alexandre Netto
Co-orientadora: Profª. Drª. Stella Maris Michaelsen

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“Discovery consists not in seeking new landscapes but in having new eyes”

Marcel Proust

Para minha mãe Vilma de Souza Pagnussat (*In memoriam)*

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À UFRGS, ao Programa de Pós-Graduação em Neurociências e ao CNPq pela oportunidade e pela bolsa concedida durante o período.

Sumário

APRESENTAÇÃO

Esta tese está organizada em tópicos: *Introdução, Objetivos, Capítulos* (1 a 4 – referente a artigos científicos), *Discussão, Conclusões, Perspectivas* e *Bibliografia*.

A *Introdução* apresenta o embasamento teórico, que nos levou a formular a proposta de trabalho. O *objetivo geral* e os *objetivos específicos* estão dispostos no corpo da tese, e, especificamente dentro de cada capítulo. Os *capítulos* contêm os artigos científicos, os quais foram organizados como resposta aos objetivos propostos. Todos os experimentos foram desenvolvidos no departamento de Bioquímica-ICBS-UFRGS.

A seção *Discussão* contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Os tópicos seguintes, *Conclusões* e *Perspectivas*, abordam as conclusões gerais da tese, bem como, possibilidades de futuros trabalhos a partir dos resultados descritos.

O item *Bibliografia* lista as referências citadas na *Introdução* e *Discussão*. As referências utilizadas nos diferentes artigos estão listadas ao final de cada trabalho.

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LISTA DE ABREVIATURAS

ACM: Artéria Cerebral Média

AE: Ambiente Enriquecido

AMS: Área Motora Suplementar

APM: Área Pré-Motora

AVE: Acidente Vascular Encefálico

BDNF: Fator de Crescimento Derivado do Encéfalo

Ca^{+2}: Íon Cálcio

EROS: Espécies Reativas de Oxigênio

ET-1: Endotelina - 1

Fr: Isocórtex Frontal

GFAP: Proteína Glial Fibrilar Ácida

M1: Córtex Motor

NGF: Fator de Crescimento do Nervo

PT: Physical Therapy

SNC: Sistema Nervoso Central

SNP: Sistema Nervoso Periférico

trk: Tyrosine Kinase

RESUMO

O objetivo desta tese foi caracterizar o desempenho de ratos *Wistar* no teste do *Staircase;* avaliar se o treinamento de habilidade e de repetição poderia induzir diferente recuperação motora, adaptações morfológicas e plasticidade encefálica após lesão do plexo braquial e isquemia focal, além de analisar os efeitos da isquemia cerebral induzida por endotelina-1 na área média das fibras do músculo sóleo.

Nossos resultados mostraram que ratos *Wistar*, de forma semelhante ao observado em outras linhagens, apresentam bom desempenho na tarefa do *Staircase* em 2 semanas após iniciado o período de treinamento; todavia, o número de animais capazes de atingir o critério mínimo (alcance e consumo de 15 esferas comestíveis) aumenta em torno de 10% quando o tempo de treinamento é estendido por mais algumas semanas.

Após lesão do Sistema Nervoso Periférico (SNP), os animais submetidos à lesão do plexo braquial e treinamento de reabilitação por meio de tarefa de repetição apresentaram axônios em estágio mais avançado de recuperação, conforme evidenciado pela análise da espessura da bainha de mielina, diâmetro do axônio mielinizado e diâmetro total da fibra mielinizada. Em relação à avaliação comportamental, ambas as tarefas induziram aceleração da recuperação funcional, mas sem diferença entre os dois tratamentos propostos.

Após lesão do Sistema Nervoso Central (SNC), os animais submetidos à isquemia focal e treinamento de habilidade demonstraram níveis mais elevados de proteínas relacionadas à sinapse (Sinapsina-I e PSD-95) no córtex sensório-motor e hipocampo no hemisfério lesado. O conteúdo de GFAP no córtex aumentou em função da isquemia e não foi modificado por nenhuma das tarefas de reabilitação. No hipocampo, o treinamento de habilidade resultou em maior imunoconteúdo de proteína glial fibrilar ácida (GFAP). Em relação à avaliação comportamental, nenhum grau de recuperação, espontânea ou induzida pela reabilitação, foi observado ao final do experimento.

A análise das fibras do músculo sóleo não evidenciou atrofia no membro posterior contralateral ao hemisfério isquêmico. Por outro lado, a sobrecarga do membro não-parético (ipsilateral à lesão) foi suficiente para induzir hipertrofia muscular.

Concluímos que a lesão do plexo braquial por esmagamento e a isquemia focal induzida por endotelina – 1 (ET-1) causam prejuízo funcional, o qual pode ser avaliado por meio do teste do *Staircase* em ratos *Wistar*; que as atividades motoras de habilidade e de repetição induzem padrões diferentes de regeneração no SNP e no SNC; e que a isquemia focal, em longo prazo, pode induzir aumento da área média das fibras do músculo sóleo no membro posterior ipsilesional. Esses resultados ampliam os conhecimentos acerca da lesão no SNP e SNC, formas de avaliação comportamental e métodos para reabilitação funcional.

ABSTRACT

The aim of this study was to characterize the performance of *Wistar* rats in the *Staircase* test, to investigate whether skilled and unskilled training would induce different motor recovery, morphological improvement and brain plasticity after brachial plexus crush and focal brain ischemia and to analyze the effects of focal ischemia produced by Endothelin -1 on soleus muscle fiber cross-sectional area.

Our results showed that *Wistar* rats, similar to other strains, can display high performance on the *Staircase* test after 2 weeks of training; that the number of animals that attained the inclusion criterion increased by 10% with longer times of training; and that the stricter criterion of 15 pellets taken can be adopted as study inclusion criterion.

After Peripheral Nervous System (PNS) lesion, animals submitted to brachial plexus injury and unskilled training showed axons in more mature appearance, as evidenced by greater myelin thickness, myelinated fiber and axon diameter. With regard to functional evaluation, the rehabilitation resulted in no difference between treatments.

After Central Nervous System (CNS) lesion, animals submitted to focal brain ischemia and skilled training showed high levels of brain proteins related to synapse (synapsin-I and PSD-95) in sensorimotor cortex and hippocampus. The content of glial fibrillary acidic protein (GFAP) in ischemic sensorimotor cortex was augmented as result of focal brain ischemia and it was no modified by rehabilitation. In hippocampus skilled training resulted in higher GFAP levels when compared with ischemia and unskilled training. With regard to functional evaluation, animals remained permanently impaired at the end of evaluations.

The analyses of soleus muscle fibers showed no atrophic factor on hind limb contralateral to the ischemic hemisphere. Nevertheless, the overuse of non-paretic limb was sufficient to induce hypertrophy of soleus on limb ipsilateral to injured hemisphere.

We conclude that brachial plexus crushing and focal brain ischemia induce functional impairment, which can be evaluated by means of *Staircase* in *Wistar* rats; that skilled and unskilled motor activity can induce different pattern of regeneration in PNS and CNS; and that long term focal brain ischemia result in increase of transverse cross sectional area of soleus fibers in non-paretic limb. The findings extend the understanding about PNS and CNS lesion, evaluation and rehabilitation treatment.

1. INTRODUÇÃO

1.1 Controle Motor

A capacidade de gerar movimentos corporais coordenados depende da interconexão de uma série de estruturas envolvidas no controle motor. O primeiro passo necessário para a inicialização de um movimento voluntário é a ativação cortical. Em humanos, o córtex motor pode ser dividido em córtex motor primário (M1), área motora suplementar (AMS) e área pré-motora (APM). Enquanto a AMS e a APM estão envolvidas no planejamento da atividade motora, M1 é uma das principais origens do trato córtico-espinal lateral. M1 recebe densas aferências da AMS e APM e mantém-se organizado em agrupamentos neuronais altamente especializados no controle do movimento de cada segmento. A ativação coordenada dessa rede neuronal possibilita o controle multi-articular fino e independente, como o necessário para os movimentos complexos de alcance e preensão (KANDEL et al., 2003; ADKINS et al., 2006).

Em ratos, o córtex motor é denominado de isocórtex frontal (Fr). Trata-se de uma estrutura cuja marca característica é a heterogeneidade celular, e que pode ser dividida de acordo com suas características morfológicas, neuroquímicas e de padrões de conexão em 3 áreas: Fr1 (M1), Fr2 (M2) e Fr3 (Fig.1A-C). A área M1 corresponde ao córtex motor primário de primatas, a área M2 corresponde à APM e à AMS, enquanto Fr3 seria uma sub-região da representação somatotópica. Essas regiões são estruturadas em camadas de células, o que lhes confere um padrão de organização laminar. No córtex motor, as camadas celulares II e V são as mais proeminentes (PAXINOS, 2004; PAXINOS & WATSON, 2004).

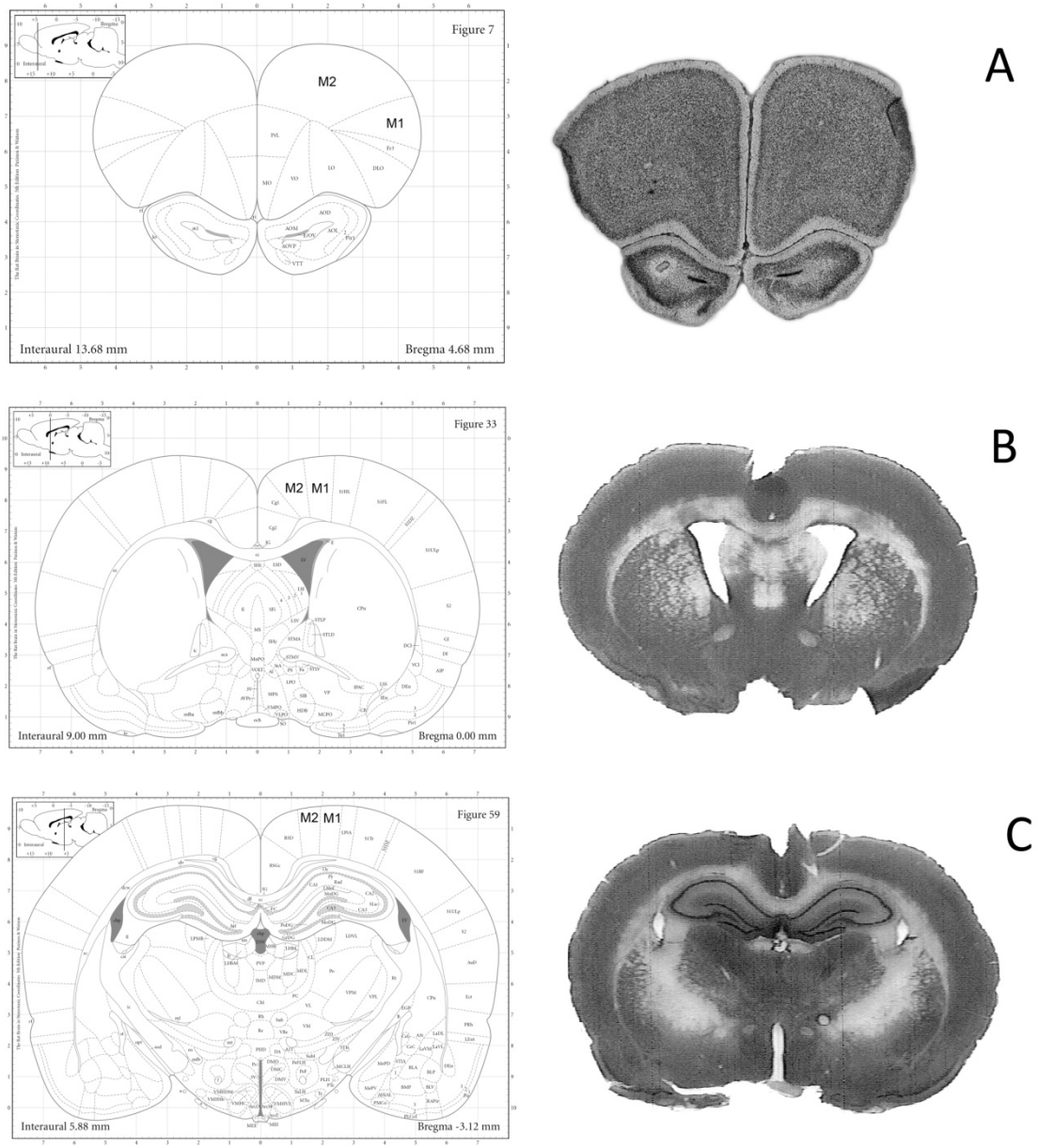

Figura 1. Cortes encefálicos transversais demonstrando as áreas motoras (adaptado de PAXINOS & WATSON, 2004)

O sistema de controle motor é constituído por um conjunto de 3 unidades: unidade de comando, de controle e de ordenação. O movimento é idealizado, planejado e comandado pelo córtex, sob supervisão da unidade de controle, a qual é constituída

pelos núcleos da base e pelo cerebelo. O sistema de controle do movimento funciona por meio de uma alça de retroalimentação, a qual leva informação acerca do movimento pretendido até as estruturas de controle, que corrigem, adéquam e aperfeiçoam o movimento e reenviam esta informação às estruturas corticais. Um modelo simplificado da circuitaria dos gânglios da base é demonstrado na Fig. 2 (para revisão veja DELONG & WICHMANN, 2007; HANDLEY et al., 2009).

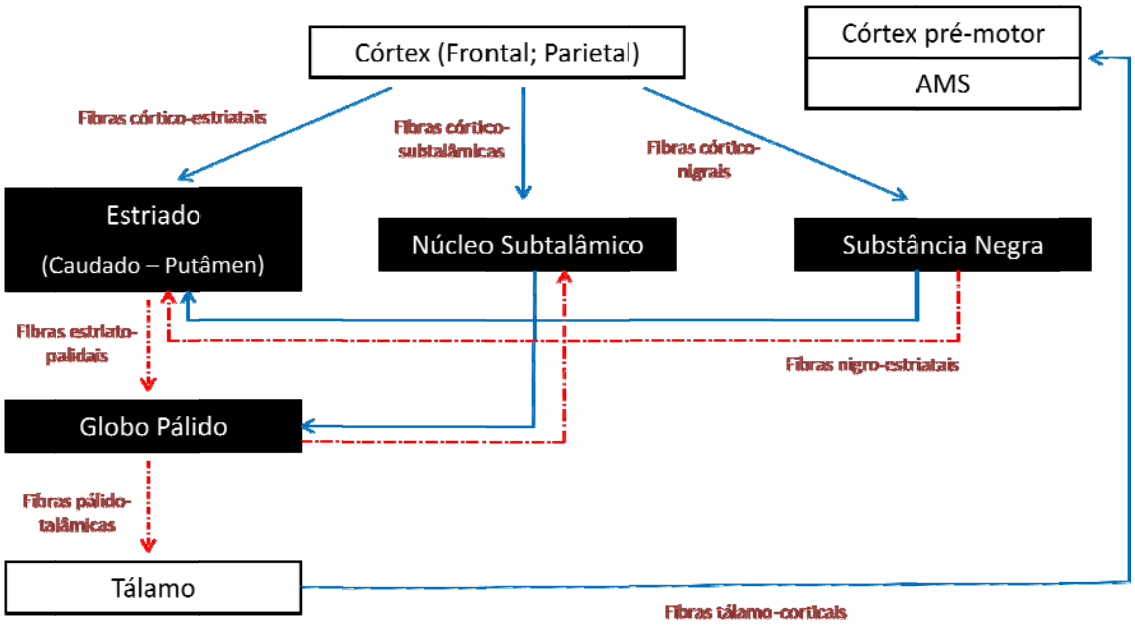

Figura 2. Diagrama esquemático simplificado das conexões dos gânglios da base. Vias excitatórias são mostradas pelas linhas contínuas (azuis) e as conexões inibitórias estão representadas pelas linhas pontilhadas (vermelhas); AMS: área motora suplementar (adaptado de HANDLEY et al., 2009).

Os sinais partem do córtex e são transmitidos por meio do corpo do estriado, globo pálido e tálamo de volta para o córtex (HANDLEY et al., 2009). O cerebelo, por sua vez, possui uma grande densidade de aferências, as quais provêem informações sobre sensibilidade somestésica, vestibular, visual e auditiva, além das aferências de

origem cortical (PROSKE & GANDEVIA, 2009). Do córtex, o cerebelo recebe as informações sensório-motoras por meio do trato córtico-pontino-cerebelar. A resposta de correção do movimento, por sua vez, parte dos núcleos cerebelares profundos para o núcleo centro-lateral do tálamo e deste para o córtex (HORNE & BUTLER, 1995; HOLSCHNEIDER et al., 2007). Após a adequação e correção do movimento a informação é transmitida até a unidade de ordenação. A unidade de ordenação é constituída pelos motoneurônios da medula e do tronco encefálico, os quais controlam a excitabilidade muscular por meio de potenciais de ação transmitidos pelos pares de nervos cranianos e espinais (KANDEL et al., 2003).

Lesões no sistema nervoso central e periférico podem afetar a capacidade de gerar e coordenar movimentos. A motricidade fina, um importante aspecto da preensão e funcionalidade manual, é freqüentemente prejudicada após lesão nervosa periférica (DUFF, 2005) e lesão do sistema nervoso central (FARR & WHISHAW, 2002).

1.2 Aspectos Fisiopatológicos da Lesão no Sistema Nervoso Periférico

Os nervos espinais são estruturas formadas por axônios associados a células de Schwann, e que são envoltos por três bainhas de tecido conjuntivo. Essas bainhas são constituídas por elementos distintos, dependendo de sua localização. A mais externa delas, envolve o nervo como um todo e é denominada epineuro. Envolvendo feixes de fibras nervosas e cada axônio individualmente, há o perineuro e o endoneuro,

respectivamente. O axônio é uma extensão longa e delgada do corpo celular, que possui uma estrutura arborescente em sua região distal, denominada terminação axonal. É por meio dela que os axônios realizam contatos sinápticos com os órgãos alvo. Em um nervo existem axônios mielinizados e não mielinizados. No primeiro tipo, as células de Schawnn se organizam ao redor do axônio formando estruturas tubulares denominadas de bainha de mielina, a qual é interrompida em intervalos regulares, em locais chamados de nodos de Ranvier. Os axônios amielínicos, embora não possuam bainha de mielina e nodos de Ranvier, também estão em contato íntimo com as células de Schwann (PETERS et al., 1976).

Após a lesão de um nervo periférico, inicia-se uma seqüência complexa e altamente regulada de eventos que visam a remoção do tecido lesado e o início do processo de reparo e regeneração. O entendimento acerca da regeneração nervosa periférica cresceu de forma significativa com os avanços na biologia celular e molecular. Hoje se sabe que as respostas frente ao dano periférico não envolvem somente reações locais, podendo incluir modificações proximais e distais ao segmento lesado, bem como alterações em nível do corpo neuronal, localizado na medula ou nos gânglios nervosos (CLARKE & RICHARDSON, 1994).

Diversos sistemas de graduação foram desenvolvidos a fim de permitir a correlação entre as alterações microscópicas e a sintomatologia clínica após lesão nervosa periférica. Dentre as mais amplamente aceitas, está a que divide as lesões em três categorias, de acordo com a severidade da lesão: neuropraxia, axonotmese e neurotmese (SEDDON, 1943).

As lesões classificadas em neuropraxia são do tipo mais leve, não envolvem perda da continuidade axonal e a perda da funcionalidade é transitória. Por outro lado, a

axonotmese ocorre quando há completa interrupção da continuidade de fibras axonais e prejuízo da camada de mielina circundante, mas com manutenção da integridade do tecido conjuntivo que envolve os feixes de fibras (perineuro) e o nervo (epineuro). Ocorre degeneração axonal e mielínica distais ao foco da lesão, causando completa denervação. O prognóstico de regeneração é favorável, uma vez que a preservação do tecido conjuntivo provê orientação para crescimento axonal e reinervação. Já a neurotmese é a forma mais grave, já que envolve a desconexão completa do nervo. Nesse tipo de lesão a perda funcional é acentuada e, em função da formação cicatricial e perda da continuidade de tecido conjuntivo, a recuperação sem reparo cirúrgico raramente ocorre (BURNETT & ZAGER, 2004).

A secção de um nervo periférico produz alterações morfofuncionais observáveis na região proximal e distal à lesão. Na porção distal essas alterações ocorrem de maneira dependente de Ca^{+2} e constituem o processo denominado degeneração Walleriana (ou anterógrada). As adaptações morfológicas primárias da degeneração envolvem edema e fragmentação axonal, além da desorganização de neurotúbulos e neurofilamentos. Concomitantemente, o contato entre axônios e células de Schwann é perdido, ocorrendo fragmentação e degeneração da bainha de mielina. Durante este processo, macrófagos são intensamente recrutados da circulação periférica para o lócus da lesão, onde fagocitam e degradam os resíduos celulares (SHEN et al., 2000).

As células de Schwann desempenham um papel fundamental no processo de degeneração/regeneração na lesão nervosa periférica. Essas células tornam-se ativas dentro das primeiras 24 h após a lesão, exibindo aumento nuclear e citoplasmático, bem como aumento da taxa mitótica. A rápida divisão celular é responsável pela supraregulação da expressão de uma série de moléculas que auxiliam no processo de

orientação e estimulação do crescimento dos neuritos em regeneração (FAWCETT & KEYNES, 1990).

Os macrófagos e as células de Schwann mantêm uma íntima interatividade após a lesão nervosa periférica. Ao mesmo tempo em que as células de Schwann auxiliam os macrófagos na remoção do axônio degenerado e dos resíduos de mielina, os macrófagos estão envolvidos na produção de fatores que estimulam a mitose das células de Schawnn (BAICHWAL et al., 1988) e a regulação da síntese de fatores de crescimento por essas células (LINDHOLM et al., 1987). A presença de moléculas tróficas no microambiente neural periférico, como o fator de crescimento do nervo (NGF) e o fator de crescimento derivado do encéfalo (BDNF), é um dos fatores responsáveis pela maior capacidade de regeneração em lesões do SNP quando comparado a lesões no SNC (DAVID & AGUAYO, 1981, YAN et al., 1992; YIN et al., 1998; BURNETT & ZAGER, 2004).

As modificações no corpo neuronal e no segmento proximal das fibras dependem da severidade da lesão, assim como da proximidade entre o segmento lesado e o corpo do neurônio (CULLHEIM et al., 2002). As células de Schwann inevitavelmente degradam no segmento próximo à lesão e os axônios e a mielina tornam-se visivelmente reduzidos em diâmetro. Essa degradação proximal pode ser mínima (até o nodo de Ranvier mais próximo) ou estender-se até o corpo do neurônio. Se o corpo do neurônio degenera, o que ocorre em casos de trauma moderado a severo, todo o segmento proximal sofre degeneração e é fagocitado (LUNDBORG, 2000).

Mesmo em lesões brandas, o corpo neuronal passa por modificações visíveis após a lesão. O núcleo migra para a periferia da célula e ocorre desmembramento dos corpúsculos de Nissl e do retículo endoplasmático rugoso, processo denominado de

cromatólise. Simultaneamente, ocorre rápida resposta proliferativa das células gliais, de certa forma sinalizada pela cromatólise, que se estendem pelo neurônio afetado e interrompem as conexões sinápticas, possivelmente para isolar o neurônio durante a fase de recuperação (BURNETT & ZAGER, 2004).

Em humanos, a maioria das lesões em nervos periféricos afeta a extremidade superior e é responsável por perda ou restrição da capacidade funcional de alcance, preensão e manipulação de objetos (DUFF, 2005). Em função da perda evidente na qualidade de movimento após lesões nervosas periféricas, a potencialização da regeneração nervosa e a recuperação da função têm sido alvo de diversos estudos (VAN MEETEREN et al., 1998; BONTIOTI et al., 2003; GORDON et al., 2003; BONTIOTI et al., 2005; ILHA et al., 2008; SEBATIER et al., 2008).

Conforme anteriormente descrito, o processo de reinervação depende da expressão de fatores de crescimento e outras moléculas de adesão envolvidas no reparo e comunicação inter-celular (ZHANG et al., 2000; MAKWANA & RAIVICH, 2005; VÖGELIN et al., 2006). Todavia, os mecanismos que regulam a expressão de neurotrofinas ainda não estão completamente elucidados. Existe uma série de evidências de que a expressão de alguns membros da família de fatores tróficos está correlacionada com o grau de atividade neuro-muscular após a lesão (ILHA et al., 2008; SABATIER et al., 2008). Entretanto, ainda não existe consenso acerca do tipo e intensidade de atividade neuromuscular ideal para o favorecimento do reparo e reinervação (CUP et al., 2007).

1.3 Aspectos Fisiopatológicos da Lesão no Sistema Nervoso Central

No Brasil, a doença cerebrovascular é uma das principais causas de óbito e dependência, observando-se uma incidência anual de 156 casos a cada 100.000 habitantes (RADANOVIC, 2000). O Acidente Vascular Encefálico (AVE) pode ser definido como uma perturbação focal ou global da função cerebral, de rápido desenvolvimento, supostamente de origem vascular, e que resulta em sinais clínicos duradouros ou morte sem outra causa aparente, a não ser de origem vascular (WHO, 1988). Pode tratar-se de uma interrupção do suprimento sangüíneo ou de uma hemorragia encefálica, que, normalmente, envolve prejuízo das vias sensoriais e motoras. Aproximadamente 80% dos AVEs são decorrência da oclusão vascular, sendo que a artéria mais comumente ocluída é a artéria cerebral média (ACM) ou suas ramificações profundas (STOKES, 2004).

O encéfalo possui alta taxa metabólica e ausência de significativa reserva energética. Isso faz com que esta estrutura seja particularmente sensível à isquemia. A redução da taxa de fluxo sangüíneo e/ou conteúdo arterial de oxigênio pode afetar gravemente a função cerebral, ocasionar alterações bioquímicas e moleculares, e manifestar-se como seqüela neurológica (RODRIGO et al., 2005).

O tecido encefálico, submetido à isquemia, passa por uma série de eventos complexos e intrincados, os quais conjuntamente podem ser denominados de ‘cascata isquêmica’. Em poucos minutos de oclusão vascular, uma seqüência complexa de eventos fisiopatológicos espaciais e temporais acontece em certa ordem, apresentando importantes inter-relações entre si, e perdurando por várias horas ou dias (DURUKAN

& TATLISUMAK, 2007). Decorrente da falha energética, ocorre despolarização neuronal, excessiva liberação e falha na recaptação do neurotransmissor glutamato, aumento dos níveis intracelulares de Ca^{+2}, produção excessiva de espécies reativas de oxigênio (EROS), depleção dos níveis de enzimas anti-oxidantes, produção de ácido araquidônico e mediadores inflamatórios, além da ativação de segundos-mensageiros envolvidos na sinalização da morte celular programada. Em função de todas essas modificações e da ativação de enzimas que danificam a estrutura das membranas celulares, ocorre perda da compartimentalização, abalo da homeostase celular e, finalmente, morte neuronal. Acompanhando as adaptações que acontecem nas células neuronais, também ocorre ativação das células da microglia, astrogliose reativa e rompimento da barreira hematoencefálica (HARUKUNI & BHARDWAJ, 2006; MEHTA et al., 2007).

Durante a isquemia, ocorre redução gradativa do dano tecidual do centro para a periferia, de forma que o dano máximo ocorre na área central do infarto. A região em torno do foco da lesão é denominada de 'penumbra isquêmica' e normalmente é suprida por vasos colaterais à artéria inicialmente ocluída (MEHTA et al., 2007). Caso o fluxo sangüíneo na região da penumbra não seja restaurado em poucas horas, a região de penumbra torna-se parte da região central afetada pela isquemia (GREEN et al., 2003). Os processos de morte celular são consideravelmente diferentes nestas duas regiões (SMITH, 2004). Dentre os mecanismos característicos da morte celular por isquemia, a necrose e apoptose parecem atuar de modo importante. O pensamento de que o infarto cerebral era exemplo clássico de necrose foi substituído por outro, em que necrose e apoptose contracenam em um processo contínuo de morte celular (UNAL-CEVIK et al., 2004; PAGNUSSAT et al., 2007). Enquanto a necrose é mais evidente na região central,

nas células da penumbra tanto necrose quanto apoptose ocorrem, com predominância de apoptose (SMITH, 2004).

Uma vez que cerca de 80% das pessoas sobrevive ao AVE, essa é uma das principais causas de incapacidade no adulto (CARMICHAEL, 2005). Dentre os prejuízos motores decorrentes estão: a hemiparesia, a incoordenação motora e a hipertonia espástica do membro superior e inferior contralaterais à lesão. A fraqueza é reportada de forma mais acentuada no hemicorpo contralateral ao hemisfério lesado, todavia, pode estar presente também no hemicorpo localizado ipsilateralmente à lesão (BOHANNON, 1990; BOHANNON, 1995; ANDREWS & BOHANNON, 2000; SCHAECHTER, 2004). Sabe-se que esses pacientes apresentam alterações morfológicas musculares e neurais, as quais sustentam a deficiência de força muscular. Existe uma expressiva diminuição no número total de unidades motoras (SEGURA & SAHGAL, 1981), baixas taxas de disparo e padrões anormais de despolarização muscular no lado acometido (HAMMOND et al., 1988).

A severidade da isquemia e a intensidade do prejuízo sensório-motor dependem de uma série de fatores, incluindo o local e a extensão da lesão encefálica. A lesão dos corpos neuronais, das vias de projeção envolvidas no controle motor e o desenvolvimento do ‘desuso aprendido’ dos segmentos corporais acometidos são as principais causas da perda da funcionalidade após o AVE (JORGENSEN et al, 1995).

A recuperação funcional pode ser influenciada por uma série de fatores biológicos e ambientais, sendo que o perfil de reabilitação motora é caracterizado por uma grande variabilidade interindividual. Todavia, existem fortes evidências de que os melhores resultados terapêuticos dependem da escolha e execução adequada da atividade motora (MICHAELSEN et al., 2006; THIELMAN et al., 2004), intensidade e

freqüência do tratamento, assim como, do início precoce da reabilitação (HUANG et al., 2009).

Sabe-se que a experiência é capaz de produzir mudanças plásticas em encéfalos de animais adultos saudáveis e após lesão (KLEIM et al., 1998; KLEIM et al., 2003). A reabilitação após pequenas lesões isquêmicas provoca reorganização no tecido cortical adjacente e alteração dos mapas corticais (NUDO et al., 1996), expressão de proteínas e modificações na morfologia dendrítica (GONZALEZ et al., 2003). Essa tendência das sinapses e dos circuitos neuronais se modificarem devido à atividade está diretamente relacionada à recuperação motora das funções perdidas e é denominada 'neuroplasticidade' (CAURAUGH & SUMMERS, 2005).

A plasticidade pode ocorrer em curto e longo prazo. As sinapses geralmente apresentam uma capacidade notável para mudanças fisiológicas em curto prazo (horas), que aumentam ou diminuem a eficiência da sinapse. Mudanças em longo prazo (dias ou semanas) podem gerar modificações fisiológicas adicionais, que levam a alterações anatômicas, incluindo a remoção de conexões pré-existentes e o crescimento de novas conexões (KANDEL et al., 2003). De fato, dados experimentais demonstram que o aumento da síntese protéica, a sinaptogênese e a reorganização dos mapas corticais ocorrem de forma coordenada ao longo do tempo, objetivando a aquisição e aperfeiçoamento motor (Fig. 3) (ADKINS et al., 2006).

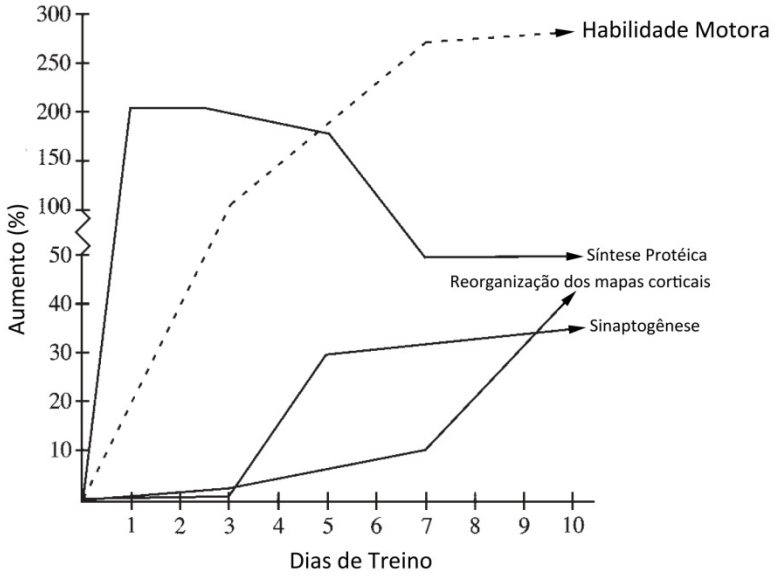

Figura 3. Curso da plasticidade molecular, anatômica e fisiológica no córtex motor de roedores durante aprendizado de tarefa motora de habilidade. As medidas são expressas como porcentagem dos níveis pré-treino obtidos de animais intactos. O desempenho motor ascende rapidamente dentro dos primeiros dias de treino A fase inicial de aprendizado é caracterizado por aumento na síntese de várias proteínas. A fase tardia da aquisição motora é acompanhada por aumento significativo no número de sinapses e pela reorganização dos mapas corticais (Adaptado de ADKINS et al., 2006).

Existem evidências de que o retorno da função em indivíduos hemiparéticos seja devido à plasticidade adaptativa nas regiões motoras encefálicas, corticais e subcorticais, as quais permaneceram ilesas (LIEPERT et al., 2000). Por meio do treinamento de uma atividade motora, observa-se que a topografia das representações corticais é alterada (KARNI et al., 1998). Os segmentos envolvidos nos movimentos realizados na tarefa aprendida são representados com maior extensão na região cortical. Além disso, o aprendizado de novas tarefas também é acompanhado por aumento na

densidade sináptica nas camadas II/III do córtex motor (NUDO et al., 2001; NUDO, 1999; NUDO & FRIEL, 1999).

Imediatamente após a isquemia os prejuízos motores são bastante pronunciados, entretanto, com o decorrer do tempo, os animais apresentam algum grau de recuperação do membro anterior, independente de receberem ou não tratamento (WHISHAW, 2000; GONZALEZ & KOLB, 2003; REGLODI et al., 2003). Essa melhora "espontânea", no entanto, é reduzida quando há lesão concomitante do córtex e estriado (BIERNASKIE & CORBETT, 2001), uma vez que o estriado dorsolateral contribui tanto para a iniciação quanto para a execução da habilidade de alcance (WHISHAW et al*.,* 1986).

Em humanos, a recuperação motora parece ser mais rápida durante o primeiro mês após a lesão, decrescendo gradativamente durante os meses seguintes (HENDRICKS et al, 2002). Também ocorre certo grau de recuperação espontânea, principalmente durante os 3 primeiros meses após o AVE. Com relação aos processos relacionados à recuperação motora espontânea, três podem ser citados: (a) mudanças na organização funcional do tecido cortical que circunda a área lesada; (b) ativação de áreas motoras e fibras corticoespinhas no hemisfério não afetado e (c) aumento na ativação de áreas 'primariamente' não motoras, como AMS, córtex parietal inferior, cingulado e ínsula (CAURAUGH & SUMMERS, 2005).

A capacidade de recuperação das assimetrias motoras depende, em parte, do grau de supressão do uso do segmento corporal acometido (NUDO et al*,* 1996). Sabe-se que a experiência comportamental específica (treinamento em movimentos orientados à tarefa, como alcance e preensão de objetos) posterior à lesão encefálica experimental (NUDO et al., 1996) e a terapia induzida por constrição (LIEPERT et al., 2000) resultam na modificação dos mapas corticais e promovem a recuperação funcional

(NUDO, 2003; WARD et al., 2003). Isto indica que esses tipos de tratamento poderiam provocar adaptações na circuitaria neuronal adjacente à lesão, e que tal reorganização contribuiria para a recuperação de funções motoras comprometidas (BIERNASKIE & CORBETT, 2001). O tratamento por meio de atividade física voluntária (GRIESBACH et al., 2004) e a exposição a ambiente enriquecido (AE) (BIERNASKIE & CORBETT, 2001) também promovem melhora funcional após isquemia experimental. Em relação ao AE, animais com pequenas áreas de infarto são capazes de recuperar o desempenho motor pré-isquêmico, enquanto que, animais com grandes áreas de infarto são mais resistentes a este tipo de terapia (BIERNASKIE et al., 2005).

A aquisição e o refinamento de seqüências de movimento envolvidos em tarefas de habilidade envolvem modificações na conectividade da rede neuronal (ADKINS et al., 2006; GRAZIANO, et al., 2006). Essas adaptações incluem ajustes na síntese de proteínas (HERNANDEZ et al., 2006), no número e efetividade das sinapses (KLEIM et al., 1997; KLEIM et al., 1998), além de alterações na representatividade cortical dos segmentos treinados (MONFILS, et al., 2005).

Sabe-se que em primatas e roedores intactos, ocorre aumento da área de representatividade cortical dos segmentos envolvidos na tarefa (VAZQUEZ et al., 2004), e que essa plasticidade adaptativa é proporcional ao tempo e continuidade do treino (MONFILS, et al., 2005). A reorganização dos mapas corticais é específica para os grupos musculares solicitados durante a execução da tarefa treinada, e, não ocorre em mesma proporção pelo simples aumento do uso muscular, como, por exemplo, na execução de atividade repetitiva (MONFILS, et al., 2005; GRAZIANO, et al., 2006).

O treinamento em tarefas de habilidade em animais, ou a terapia orientada à tarefa em humanos, baseia-se no re-treinamento de atividades funcionais por meio da

utilização e inter-relação de múltiplos sistemas, incluindo o músculo-esquelético, perceptivo e cognitivo. Estudos de neuroimagem sugerem que os ganhos funcionais obtidos por meio desse tipo de treinamento, possivelmente sejam devido ao restabelecimento do controle exercido pelo córtex sensório-motor ipsilateral à lesão, aumento na atividade no córtex sensório-motor primário ipsilesional e redistribuição da atividade em diversas áreas da rede sensório-motora (CONNER et al., 2003; SCHAECHTER, 2004; ADKINS et al., 2006).

2. OBJETIVO

2.1 Objetivo Geral

O objetivo geral deste estudo foi verificar os efeitos terapêuticos de atividades motoras de habilidade e de repetição na recuperação motora, funcional e tecidual de animais expostos à lesão do sistema nervoso central e periférico.

2.2 Objetivos Específicos

(1) Caracterizar a aquisição e o desempenho motor de ratos *Wistar* adultos no teste de habilidade motora fina: Teste do *Staircase* – Capítulo 1.

(2) Investigar os efeitos da tarefa de habilidade e da tarefa de repetição na recuperação motora e na regeneração nervosa em ratos adultos submetidos à lesão dos nervos mediano e ulnar – Capítulo 2;

(3) Analisar os efeitos da tarefa de habilidade e da tarefa de repetição na recuperação motora e na plasticidade encefálica em ratos adultos submetidos à isquemia focal induzida pela injeção de Endotelina-1 (ET-1) no córtex sensório-motor e estriado – Capítulo 3;

(4) Avaliar as características morfológicas do músculo sóleo de ambos os membros posteriores de ratos adultos 6 semanas após a indução de isquemia focal por meio da injeção de ET-1 no córtex sensório-motor e estriado – Capítulo 4.

3. CAPÍTULO 1

Artigo: Skilled forelimb reaching in *Wistar* rats: Evaluation by means of Montoya Staircase test – Publicado no Journal of Neuroscience Methods

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Skilled forelimb reaching in Wistar rats: Evaluation by means of Montoya staircase test

Aline de Souza Pagnussat[a,c], Stella Maris Michaelsen[b], Matilde Achaval[c], Carlos Alexandre Netto[d,*]

[a] Programa de Pós-graduação em Neurociências, ICBS, Universidade Federal do Rio Grande do Sul (UFRGS), Brazil
[b] Departamento de Fisioterapia, Universidade do Estado de Santa Catarina, UDESC, Brazil
[c] Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde (ICBS), UFRGS, Brazil
[d] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde (ICBS), UFRGS, Brazil

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ABSTRACT

Experimental animals have been used as models for several neurological disorders; their performance in behavioral tests is useful in determining the success of lesion repair procedures and assessing functional recovery. The staircase test is a behavioral test that consists in reaching for food inside a special box and allows for a sensitive measure of skilled reaching by each limb in an independent manner. In most laboratories in the south of Brazil, Wistar rats are used for the study of experimental stroke, hypoxia and peripheral neuropathy, but most studies with the staircase test have used other strains such as Sprague–Dawley and Long–Evans. Because skilled reaching, grasping and performance can differ among strains, the purpose of the present study was to characterize the performance of Wistar rats in the staircase test and determine the effect of median and ulnar nerve crush. Our results with Wistar rats on the staircase test showed that: similar to other strains, Wistar animals can display high performance after 2 weeks of training; the number of animals that attained the inclusion criterion increased by 10% with longer times of training; the stricter criterion of 15 pellets taken can be adopted as study inclusion criterion; the test has an unquestionable value in assessing lateralized deficits, as evidenced by the lack of performance deficit of the non-manipulated forelimb at any time point. These results extend the understanding about the performance of Wistar rats in the staircase test, which will be used for the best training and research using this strain.

1. Introduction

Rodents, including rats and mice, have been used as models for several neurological diseases. Their performance in behavioral tests are widely used to evaluate the success of surgical procedures that cause impairments to motor control, as well as to assess the pattern of functional recovery after different types of treatment (Biernaskie and Corbett, 2001; Windle et al., 2006).

The need to assess goal-directed movement abilities in animal models has led to the development of a variety of tests. The Montoya staircase test was developed to evaluate lateralized forepaw ability (the animal must reach with the left or right forepaw independently), and it allows a sensitive and quantitative measure of skilled reaching and grasping in rats and mice (Montoya et al., 1991; Whishaw et al., 1997; Baird et al., 2001; Clarke et al., 2007). This skilled forelimb task is largely used to evaluate lesion-induced impairments and recovery after nervous system lesion in different strains. The staircase test has been mostly applied in Sprague–Dawley rats for assessment of recovery from brain ischemia after training, or in Long–Evans rats to evaluate either toxicological effects of drugs (Samsam et al., 2004) or motor cortical electrical stimulation (Adkins-Muir and Jones, 2003). Besides its use for the study of central nervous injury, the staircase test has been recently employed to evaluate recovery after peripheral nerve injury in Lister–Hooded rats (Galtrey and Fawcett, 2007).

Although Wistar rats have been rarely used in studies of skilled forelimb reaching, compared to Sprague–Dawley and Long–Evans rats, they have been widely used in the south of Brazil in most research laboratories studying experimental stroke, hypoxia and ischemia (Valentim et al., 1999; Pagnussat et al., 2007; Pereira et al., 2007) and peripheral neuropathy (Rodrigues-Filho et al., 2003). Skilled reaching, grasping acquisition and performance can, however, vary with rat strain (Nikkhah et al., 1998). Galtrey and Fawcett (2007) reported a poor performance of Lewis rats in the staircase test. Differences in motor behavior have been described in a task

* Corresponding author at: Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde - ICBS, Universidade Federal do Rio Grande do Sul - UFRGS, Rua Ramiro Barcellos 2600, 90035-003/Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5531; fax: +55 51 3308 5540.
E-mail address: alex@prograd.ufrgs.br (C.A. Netto).

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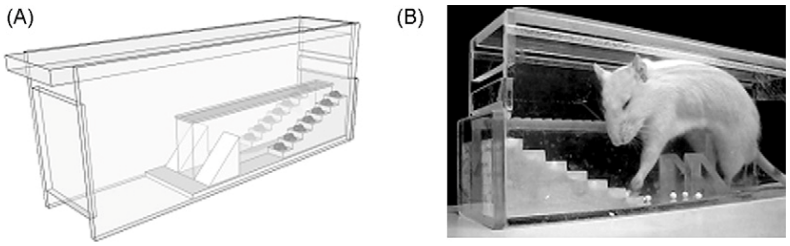

Fig. 1. (A) Illustration of the apparatus used for the staircase test. (B) Representation of animal inside staircase apparatus during the test.

of reaching for food from a shelf (single pellet reaching) either between Long–Evans and Fischer-344 rats (VandenBerg et al., 2002) or between Long–Evans and Sprague–Dawley rats (Whishaw et al., 2003). Little has been reported about Wistar rats' reaching performance (Metz et al., 2005), particularly using the staircase test.

Furthermore, we can find in the existing literature several differences in training protocols (number of trials, number of steps, number of pellets in well, time of test and inclusion criteria). Most studies use three pellets in each step, 2 weeks of training and 15 min for the test (Biernaskie and Corbett, 2001; Clarke et al., 2007). For a total of 21 pellets per side, Hewlett and Corbett (2006) adopted a criterion of a minimum of 12 pellets reached on the last eight trials, with a standard deviation less than ±2 pellets. However, because rats could access the food pellets on each bottom stair with their tongue (Hoane et al., 2004) a stricter criterion can be adopted.

To be confident in assessing the efficacy of a treatment approach in Wistar rats, we must know if skilled forelimb use, in terms of scores of pellets taken and performance at the end of training, is similar to those described for other rats. The brachial plexus lesion provides a good experimental model to assess the control of muscle function, examine the mechanisms underlying functional recovery, and test the effects of treatments to enhance recovery (Bertelli et al., 2005).

Therefore, the aims of this study were: (1) to characterize acquisition of the staircase test for inbred albino Wistar rats in terms of pellets taken; (2) to compare final performance of inbred albino Wistar rats after two different periods of training (completing 20 and 50 trials); (3) to evaluate performance before and after brachial plexus avulsion.

2. Materials and methods

2.1. Animals

Adult Wistar rats (obtained from a local breeder-Central Animal House of the Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul), weighing approximately 300 g at the beginning of the experiment, were used. Animals were housed in groups of four or five in Plexiglas cages under standard conditions with a 12 h light/dark cycle (lights off at 7:30 p.m.) and controlled temperature (22 ± 2 °C).

Water and standard laboratory chow were provided *ad libitum* except during behavioral testing periods. On the day before the start of the testing, animals were not furnished with any food (completely food deprived). From then on, and after each training session, animals were provided with a measured amount of food each day (10–15 g of standard laboratory chow at the end of day) to maintain body weight at approximately 80–90% of free feeding level. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted from the National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology.

For the first goal of the study, a group of 52 animals were trained for a period of 2 weeks. For the second goal a new group of 38 animals was added, and these were trained for 5 weeks. Finally, to test the sensitivity of the staircase test to both peripheral lesion and spontaneous recovery, 24 animals belonging to the group trained for 2 weeks were randomly assigned to sham, control or surgery groups, where the latter surgical procedure was aimed at the brachial plexus injury. For ethical purposes, surplus animals (those that were subjected to only the training phase) were used for other on-going experiments in our laboratory.

2.2. Behavioral assessment by the Montoya staircase test

This behavioral test provides a sensitive measurement of skilled reaching and was initially described by Montoya et al. (1991) to assess reaching performance in rats and mice (Whishaw et al., 1997; Baird et al., 2001). The test apparatus was made of clear Plexiglas and is illustrated in Fig. 1. Briefly, the boxes consist of a chamber with a central platform, which allows the animal to climb onto it; a set of seven steps is located on each side, and three small food pellets can be placed on every step (totaling 21 pellets on each side). This stair arrangement and the narrowness of the corridor are such that the rat cannot turn around and/or retrieve dropped pellets and just allow the animal to reach from the right steps using the right forepaw and from the left steps only with the left forepaw (for details see Montoya et al., 1991). For the realization of the test, small food pellets were used (white sucrose spheres with a sweet flavor and smell—4.6 mm) weighting 65 mg ± 10% (Glóbulos Inertes n° 7 – Farmacopéia Brasileira Homeopática – Brazil). Animals were first familiarized with these pellets by placing some in each home cage on three consecutive days prior to the beginning of training.

2.3. Training procedure

2.3.1. Training procedure (acquisition) and establishment of best criteria

The animals were trained 5 days per week, 2 trials per day, for 2 weeks, totaling 20 trials. Rats remained in the staircase boxes for 15 min and the total numbers of pellets eaten, dropped and remaining on the steps on each side were recorded.

As pointed out before, pellets located in the first one or two steps (3 in each step) could be obtained with the tongue or mouth. Thus, the fact that some authors affirm that the minimum reaching performance for the animal to be included in the sample is to retrieve 12 pellets, is dubious if they do not take into account the 3 or 6 pellets from the upper steps of staircase boxes. Instead of only using as test inclusion criterion the reaching of at least 12 pellets, we also looked for how many animals could reach at least 15 and 18 pellets at the end of training, and for those, how many days they took to attain this criterion.

Because some studies have identified limb preference in pellets retrieved in a skilled reaching task (single pellet; VandenBerg et

al., 2002), we also compared the average number of pellets taken in each trial between right and left side. We identified individually what side had the best performance (greater number of pellets taken), and this side was called the preferred side. We then calculated the average number of pellets taken with the preferred side.

2.3.2. Testing procedure (final performance)

To test if training duration can influence the final performance, we compared a set of additional animals that were trained in the same procedures as described before, but for a longer period (5 weeks). After 2 or 5 weeks, animals were tested on two different days, two trials per day, and the performance in reaching was based on the average of these four trials, for right and left side. The preferred paw was then determined and the final performance for the preferred paw was determined.

2.3.3. Lesion effect and spontaneous recovery

For the last goal of this study, animals submitted to surgery, were again given the staircase test for another five times (two trials per day): 24/48 h (Post 1) and 1, 2, 3 and 4 weeks after the surgery (Post 2, 3, 4, and 5, respectively).

2.4. Surgical procedure

After the two training weeks and before the surgical procedure, 24 animals were randomly divided into three groups: (1) control group (unoperated – $n = 8$); (2) sham group (submitted to all procedures, except to brachial plexus crush – $n = 8$); (3) crush group – $n = 8$).

For the surgery procedure, all rats except the control group were deeply anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. The animals were then immobilized on a wood surface, and a horizontal incision was made parallel to the clavicle, running from the sternum to the axillary region; the forepaw that showed better practice in the staircase test was chosen. The brachial plexus was approached through this opening and the median and ulnar nerves were crushed together with 1-mm hemostat for 30 s (adapted from Bridge et al., 1994; Galtrey and Fawcett, 2007). Given that the purpose of study was to evaluate reaching performance, the crush was done in both, median and ulnar nerves. In rats, the median nerve is responsible for finger flexion and ulnar nerve injury affects the ability of grasping (Bertelli and Mira, 1995; Bontioti et al., 2003; Papalia et al., 2003).

2.5. Histological technique

After 4 weeks, animals from control, sham and lesion effect with spontaneous recovery groups were anesthetized with chloral hydrate (30%, 10 ml/kg, i.p.) and injected with 1000 IU heparin (Cristália, Brazil). Afterward, they were transcardially perfused through the left ventricle, using a peristaltic pump (Control Company, São Paulo, Brazil) with 200 ml of saline solution followed by 100 ml of fixative solution composed of 2.5% glutaraldehyde (Sigma, St. Louis, MO, USA) and 2% paraformaldehyde (Reagen, Rio de Janeiro, Brazil) in 0.1 M phosphate buffer (PB), pH 7.4, at room temperature. For nerve analysis, one short segment (~3 mm) of ulnar and median nerves was excised from 5 mm after the crush injury site (distal portion). The specimens were fixed by immersion in the same fixative solution for at least 1 h. Next, they were post-fixed in 1% OsO_4 (Sigma, St. Louis, MO, USA) in PB, dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, Hatfield, PA, USA), embedded in araldite (Durcupan ACM, Fluka, Buchs, Switzerland) and polymerized for 48 h at 60 °C. Semithin cross-sections (1 μm) were obtained using an ultramicrotome (MT 6000-XL, RMC, Tucson, USA) and stained with 1% toluidine blue (Merck, Darmstadt, Germany) in 1% sodium tetraborate (Ecibra, São Paulo, Brazil). Afterward, images of the nerves were digitized using a Nikon Eclipse E-600 microscope (Japan) coupled to a Pro-Series High Performance CCD camera. The purpose of this evaluation was to observe qualitatively the morphological characteristics of nerves 4 weeks following crush.

2.6. Statistical analysis

Test performance was evaluated by the following parameters: (a) the training time (number of trials) necessary to learn the staircase test adopting the criterion to reach at least 12, 15 and 18 pellets; (b) reaching preference (as defined before) evaluated by Student's *t*-test; (c) the influence of the surgical procedure on absolute performance and the recovery period, evaluated by two-way ANOVA having as factors *time* (pre; 24 h post-surgery; and 7, 21 and 28 days post-surgery) and *group* (control, sham and crush), followed by Bonferroni's post hoc test as indicated. The level of statistical significance was set at $p < 0.05$ in all tests, which were performed using the SPSS 11.5 software for Windows®.

3. Results

All animals learned to collect pellets from the staircases in the first two days of training. The success score in reaching progressively improved over the days, as evidenced by the number of pellets eaten. The learning curve of pellets taken and eaten with the right and left forepaw is illustrated in Fig. 2. At the end of the training period (2 weeks), Wistar rats could take on average 15.3 ± 0.5 pellets with the right and 14.2 ± 0.6 with the left paw (mean ± S.E.M.).

3.1. Time to learn the staircase test

The number of trials necessary to perform the minimum criteria of 12, 15 or 18 pellets with right, left and preferred paw, as well as the percentage of animals that attained these criteria when trained for 2 weeks is presented in Table 1 (mean ± S.E.M.). After 6 trials (3 days of training), the majority of the animals were able to take at least 12 pellets with their right or preferred paw. At the end of the first week (10 trials), 23.1, 30.8 and 17.4% of the animals were unable to take 15 pellets with right, left and preferred paw, respectively. At the same time, the percentage of animals that could not take 18 pellets with their right, left and preferred paw was, respectively 40.4, 48.1 and 36.3%.

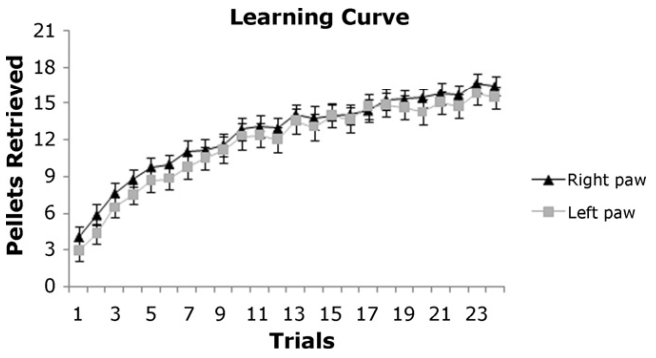

Fig. 2. Overview of the right and the left forelimb practice in the staircase test during 20 trials of training (2 weeks; $n = 52$). Results are expressed as the mean number of pellets taken and eaten ± S.E.M., and demonstrate the continuous learning during this period.

Table 1
Necessary trials to learn and to take at least 12, 15 or 18 pellets.

	12 pellets		15 pellets		18 pellets	
	Attain	Trials	Attain	Trials	Attain	Trials
right paw	94.2%	6.4 ± 0.5	76.9%	10.3 ± 0.7	59.6%	11.8 ± 0.8
Left paw	92.3%	9.3 ± 0.7	69.2%	9.5 ± 0.7	51.9%	11.5 ± 0:8
Preferred paw	96.1%	6.8 ± 0.5	82.6%	9.5 ± 0.7	63.4%	10.9 ± 0:8

3.2. Preference for reaching with right or left forepaw

Paw preference was determined on the basis of the best forelimb performance in the last four trials, in animals trained for 2 and 5 weeks. Animals trained in the staircase test for 2 weeks had a preference for the right paw most of the times (59.6%), for the left paw 36.5% of times, and no preference in 3.8% of cases. Animals trained for 5 weeks showed a similar proportion between preference for the right (57.8%) or the left (39.4%) and both (2.6%) forepaws.

The average number of pellets reached with the preferred paw in the testing trials was significantly higher (16.9 ± 0.4) when compared with the non-preferred (14.1 ± 0.5) (paired Student's t-test; $t_{(49)} = 3.90$, $p < 0.01$). After 5 weeks of training, animals retrieved 17.2 ± 0.4 with preferred and 14.8 ± 0.5 with non-preferred paw (mean ± S.E.M.; paired Student's t-test; $t_{(37)} = 3.51$, $p = 0.001$) (Fig. 3). Because we found this difference between preferred and non-preferred forepaw, for the next results we used the preferred forepaw.

3.3. Comparison between 2 and 5 weeks of training

The mean performance (number of pellets taken) did not differ after 2 and 5 weeks. Adding 3 weeks (30 trials) of training, the number of animals that could take 12 and 15 pellets with the preferred paw was increased by ~10%, but the same did not occur with those that could take 18 pellets (Fig. 4).

3.4. The influence of the surgical procedure on absolute performance reaching

There were no differences between groups during pre-operative training. Nevertheless, immediately after the surgery (Post 1), the number of pellets retrieved with the damaged forepaw over time was significantly lower than that retrieved using the non-damaged side only in the crush group (5.47 ± 0.1 for damaged side; 13.95 ± 0.7 for non-damaged side; $p < 0.05$). As expected, after the surgery, the reaching rate of control and sham (non-manipulated and manipulated) and non-damaged paw (surgery group) did not show differences over the periods analyzed ($p > 0.05$).

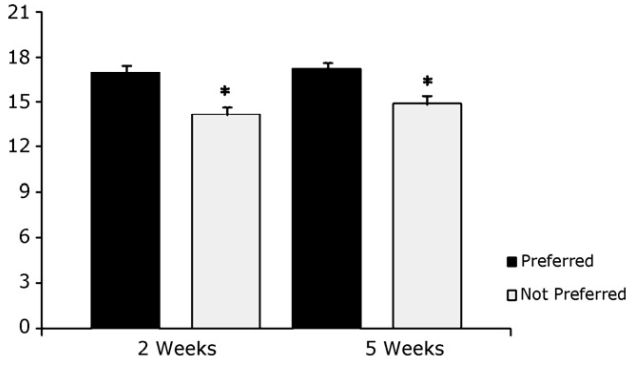

Fig. 3. Number of pellets retrieved with preferred and non-preferred forepaw during the 2 weeks ($n = 52$) and 5 weeks ($n = 38$) of training, showing higher scores obtained with the preferred paw. Values are means ± S.E.M. (*$p = 0.0006$).

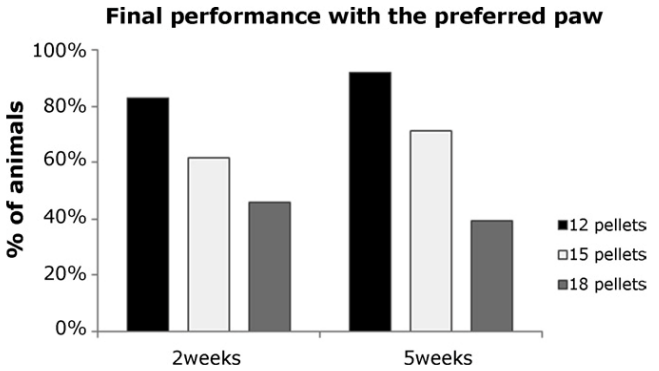

Fig. 4. Final performance displayed with preferred forepaw after 2 ($n = 52$) and 5 weeks ($n = 38$) of training. Values presented as percentage of animals that could reach 12, 15 and 18 pellets at end of training periods described above.

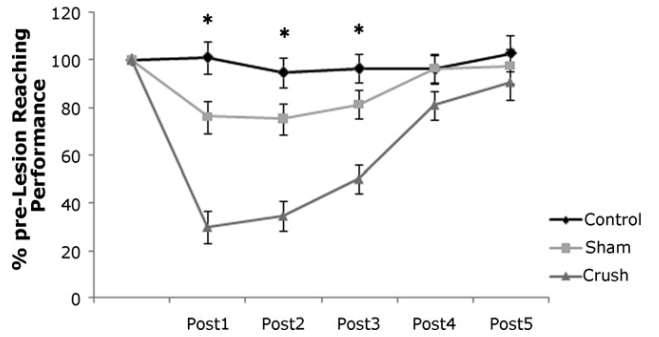

Fig. 5. Staircase test evaluation at 24 h (Post 1), day 7 (Post 2), day 14 (Post 3), day 21 (Post 4) and day 28 (Post 5) after median and ulnar nerves crushing. Impaired forepaw scores are presented as percentage of pre-surgery performance. Control and sham animals performed better than crush animals on Post 1–3 ($p < 0.01$). Values are means ± S.E.M.

When staircase test performance was evaluated for longer periods, it was possible to observe an effect of time ($F_{1,21} = 24.27$) and group ($F_{2,21} = 21.10$) and time-group interaction ($F_{2,21} = 8.81$) ($p < 0.01$ in all). The percentage of pre-lesion performance with preferred paw (for control group), manipulated paw (for sham group) and damaged forepaw (for crush group) in the staircase test evaluated on Post 1–5 is shown in Fig. 5.

3.5. Histological observation

Distal portions of median and ulnar nerves of control, sham and crush groups are shown in Fig. 6. Control and sham images, of median and ulnar nerves (C and S), exhibited normal appearance and regular distribution of fibers. In the distal portion of the crush group, there was the presence of degeneration debris, fibers with reduced diameter and thinner myelin sheath and enlarged endoneurial connective tissue between fibers.

4. Discussion

Since its development, the staircase test has been widely used to evaluate the performance of forelimb reach in a variety of studies (Montoya et al., 1990; Whishaw et al., 1997; Kloth et al., 2006; Milman et al., 2006; Ago et al., 2007; Clarke et al., 2007). This is a standardized method to assess the functional integrity of forelimbs by means of reaching and grasping movements in rodents (Montoya et al., 1991; Baird et al., 2001). To obtain the food, the animal must

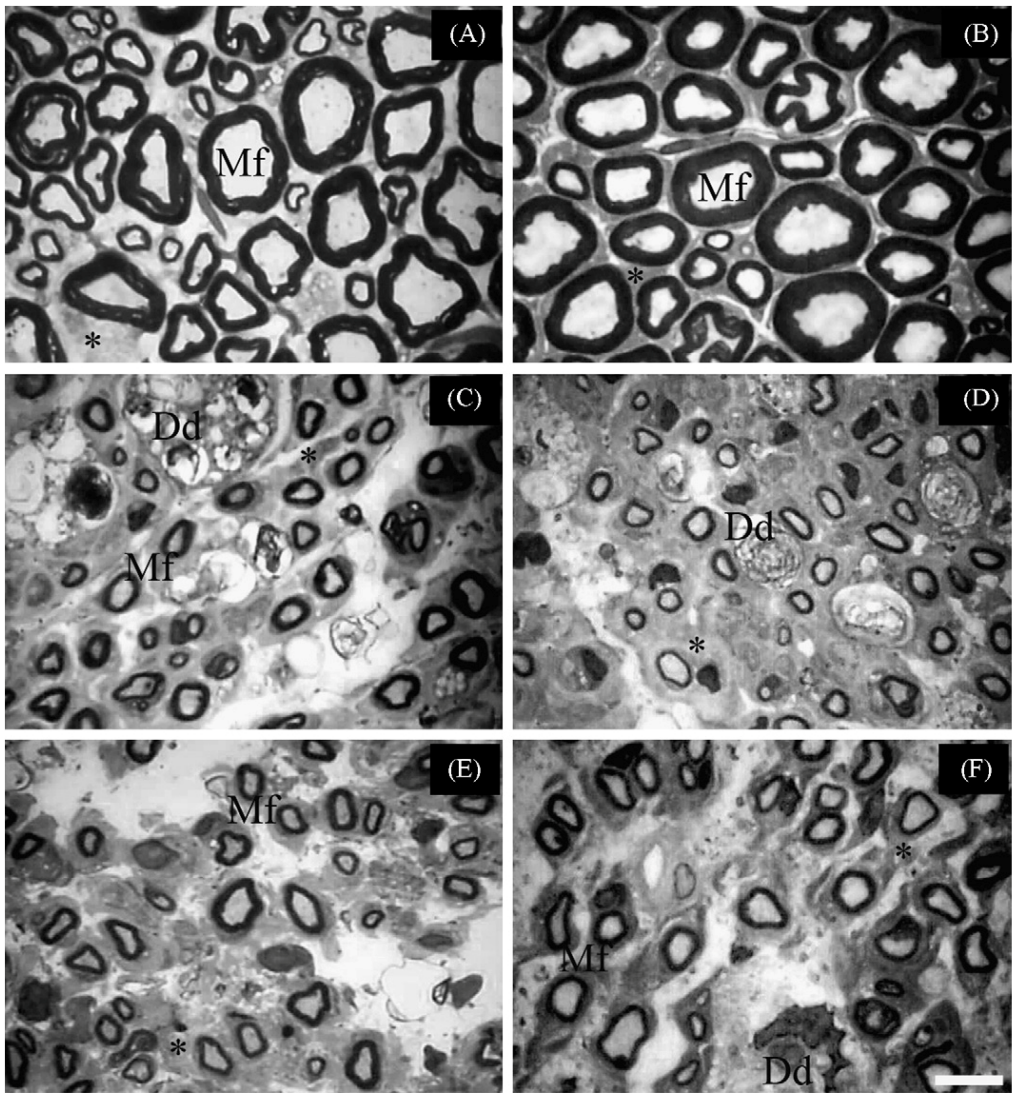

Fig. 6. Images of semithin cross-sections (1 μm) obtained from median and ulnar nerves 4 weeks after crushing. (A) and (B) distal portions of median nerve of Control and ulnar nerve of Sham, respectively. Note appearance of myelinated fibers and restricted endoneurial connective tissue between fibers; (C) and (D) distal portions of median nerve of crush group; (E) and (F) distal portions of ulnar nerve of crush group; Images from lesioned group displayed a predominance of fibers with thinner myelin sheath, reduced diameter and increased surrounding connective tissue. Mf indicates myelinated nerve fiber; * (asterisk), endoneurial connective tissue; Dd, degeneration debris. Semithin sections were stained with toluidine blue. Scale bar = 20 μm.

reach for, grasp, and retrieve the pellets with its forepaws, requiring finely controlled sensorimotor function of the forelimbs (Samsam et al., 2004).

The number of steps, number of pellets supplied, training time and daily frequency in the staircase test vary greatly among studies (Whishaw et al., 1997; Baird et al., 2001; Döbrössy and Dunnett, 2004; Klein et al., 2007). This could be due to the fact that different strains can learn and attain the plateau of pellets retrieved faster than others (Nikkhah et al., 1998). In a period of 10 days, VandenBerg et al. (2002) showed that Fischer-344 rats improved much more slowly and had inferior performance in a single pellet-reaching task when compared with Long–Evans rats. Similarly, Galtrey and Fawcett (2007) showed that Lewis rats had the poorest participation and the lowest performance success on the staircase when compared with Long–Evans and Lister–Hooded rats; between these two last strains, the Lister–Hooded rats have shown the highest scores. Kloth et al. (2006) demonstrated that Sprague–Dawley rats can easily learn and achieve their maximal performance level just after 3 days of training. Our results demonstrate that Wistar animals can easily learn the task and display high scores just after 1 week of training.

Moreover, different animals may require different times to complete the task. Whereas rats retrieve and eat several pellets in rapid succession, mice normally retrieve one pellet at a time, and back out of the corridor to eat it at the start of the chamber (Baird et al., 2001). Therefore, to monitor the performance capabilities it is necessary to select an appropriate session time, sessions of 30 min for mice and sessions of 15 min for rats (Montoya et al., 1990; Baird et al., 2001; Kloth et al., 2006). We found few studies that used the staircase test with Wistar rats. Nonetheless, in the other strains, the time adopted by the majority of authors to complete the task is 15 min. This time was used in the few studies that we found using Wistar rats, except that by Wakayama et al. (2007) which used a 20-min session (Bayona et al., 2004; Whitehead et al., 2005). These authors did not give an explanation for this longer time. Contrarily, Samsam et al. (2004) showed that for Long–Evans rats the maximum number of pellets was collected within 10 min, suggesting that longer times do not result in higher scores.

Normally, a condition to be included in the samples is to retrieve a minimum of 55–57% of furnished pellets and to have a stable baseline (Whishaw et al., 1997; Biernaskie et al., 2004; Galtrey and Fawcett, 2007). Despite, a strain-dependent variation in skilled forelimb use in Sprague–Dawley rats described in the literature (Nikkhah et al., 1998), our results show that the criterion of taking at least 12 pellets in four consecutive trials can also be used for Wistar rats. However, since animals can use their tongue to get the food from the first couple of stairs and just use their paws to retrieve food further away, the criterion of reaching at least 15 pellets can be adopted for more reliability.

Similar to that previously described by Nikkhah et al. (2001) for other strains, Wistar rats showed the ability to retrieve pellets with both forelimbs. Although there was no difference in the mean number of pellets retrieved with the right versus left paw, when each paw was individually analyzed it was possible to identify a difference between preferred and non-preferred forepaw. In this analysis, the amount of food reached with the preferred paw was significantly higher than that reached with the non-preferred. Thus, since the criterion is attained, both limbs can be selected; but the best choice is the preferred paw.

One purpose of this study was to evaluate and characterize the staircase test for the Wistar rat strain after different periods of training. Our results demonstrate that Wistar animals are able to continuously improve their success rate over the first 20 trials of training. This was evidenced by the number of collected pellets at the end of this period, which was as high as 16.9 ± 0.4 (mean ± S.E.M.) with the preferred paw. When trained for a longer period (5 weeks), in order to verify if this performance could be improved, we found no difference in the number of pellets taken (17.2 ± 0.4; mean ± S.E.M.), but the number of animals that was able to reach 12 and 15 pellets with the preferred paw increased about 10%. Since most animals were able to reach 12 and 15 pellets with the preferred paw after longer training time (as depicted in Fig. 4), the period designated for acquisition of the staircase test could exceed 2 weeks. This will reduce the number of animals that should be excluded for not attaining the inclusion criterion and minimize the total number of animals employed.

Recently, it was demonstrated that one of the tests that most clearly reveals the effects of axon misdirection on function is the staircase test (Galtrey and Fawcett, 2007). It has become evident that the staircase test has an unquestionable value in assessing lateralized deficits since our results demonstrated no "statistical difference" in reaching performance with the non-manipulated paw at any time point. Perhaps, after the surgery possibly not the skilled movements involved in reach are harmfully altered, but the number of attempted reaches may be fewer than prior to the procedure. Impaired animals might have learned through experience that attempting to reach with damaged forepaw results in limited success, developing a "learned non-use" (Whishaw et al., 1997; Clarke et al., 2007).

The staircase test would be useful in examining the control of skilled limb movements (measured by counting the number of pellets picked up and eaten) and in measuring gross limb movement (provided by measuring maximum distance reached) (Döbrössy and Dunnett, 2004; Van Kampen and Eckman, 2006; Galtrey and Fawcett, 2007). However, in view of the analyses of the maximum distance reached, it is not possible to assert that pellets remaining on steps are those originally present or those that were dropped from higher stairs. Thus, even though the staircase test is a simple, good and trustworthy method, in its original form, it is not possible to assess the "effectiveness" of the reaching movement. This is because the grasping of pellets situated at lower levels represents an activity more complex than reaching for food located on higher steps (Montoya et al., 1991). In order to increase the power of the quantitative data obtained by this test, use can be made of a color-coded pellet system that allows the discrimination of the different levels of reaching and grasping difficulties (Kloth et al., 2006) or of slow motion video-recording analysis (Clarke et al., 2007).

The muscle synergy patterns required to execute the components of reaching in the staircase apparatus (for details see Clarke et al., 2007) were successfully impaired by brachial plexus crush, as demonstrated by the number of pellets eaten after the surgery. Previous studies from our laboratory demonstrated that the distal portion of nerves injured by means of crushing shows a predominance of small, thin myelinated fibers, enlarged endoneurial connective tissue space between the fibers and degeneration debris (Ilha et al., 2008). Similar results were obtained when semithin sections of ulnar and median nerves were analyzed after 4 weeks of lesion. Morphological characteristics remained markedly different from control and sham, even when there was motor or functional recovery, as demonstrated by staircase test performance at Post 4–5 (as depicted in Fig. 5).

The staircase test apparatus is simple and inexpensive; moreover, animals can easily learn and run it with minimal supervision. Nevertheless, the strain variations should be viewed as a potential influence, which should be considered at the moment of choosing the strain for the skilled reaching study (Galtrey and Fawcett, 2007). We conclude that Wistar animals can display higher performance after 2 weeks of training; but with longer period of training most animals were able to reach a minimum of 12 and 15 pellets. Furthermore, either forelimb can be chosen for lesions, but it is always more convenient to select the paw with best performance in the task. These data extend the understanding about skilled forelimb use in Wistar rats and can be used as an aid in research using this rat strain.

Acknowledgments

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4. CAPÍTULO 2

Artigo: Effects of skilled and unskilled training on nerve regeneration and functional recovery after brachial plexus crush – Submetido ao Neurorehabilitation and Neural Repair

Neurorehabilitation and Neural Repair

Neurorehabilitation
& Neural Repair

Effects of skilled and unskilled training on nerve regeneration and functional recovery after brachial plexus crush

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Complete List of Authors:	Pagnussat, Aline; Federal University of Rio Grande do Sul, Biochemistry Department Michaelsen, Stella; Universidade do Estado de Santa Catarina, Fisioterapia Achaval, Matilde; Universidade Federal do Rio Grande do Sul, Departamento de Ciências Morfológicas Ilha, Jocemar; Universidade Federal do Rio Grande do Sul, Departamento de Ciências Morfológicas Hermel, Erica; Universidade Federal do Rio Grande do Sul, Ciências Morfológicas Back, Franklin; Universidade Federal do Rio Grande do Sul, Bioquímica; Universidade Federal do Rio Grande do Sul, Ciências Morfológicas Netto, Carlos; Universidade Federal do Rio Grande do Sul, Bioquímica
Keyword:	Peripheral nerve injury, Functional recovery, Median nerve, Ulnar nerve, Nerve morphometry

Abstract

The majority of human peripheral nerve injuries affects the upper limbs and the most disabling aspect of this injury is the loss of skilled hand movements. There are evidences that the type and intensity of activity induces morphological and electrophysiological remodeling of neuromuscular junction and this could exert influence in nerve repair. Then, we decided to investigate the effects of two types of treatment on functional and morphological recovery after a brachial plexus crush in adult male rats. After one week of nerve crush, animals were submitted to skilled (reaching for small food pellets) and unskilled (walking on a motorized treadmill) training for 3 weeks. Over this period, functional recovery was monitored weekly using the Staircase and the Cylinder Tests. Histological and morphometric nerve analyses were used to assess nerve regeneration at the end of the treatment. The functional evaluation evidenced benefits of both tasks but no difference between them. However, the unskilled training induced a greater degree of nerve regeneration as evidenced by histological measurement. These data provide evidence that both tasks used in this study for forelimb training after brachial plexus injury can accelerate the functional recovery, but that unskilled training induce a slightly greater degree of peripheral nerve regeneration than skilled treatment.

Keywords:

Peripheral nerve injury, Functional recovery, Median nerve, Ulnar nerve, Nerve morphometry.

Introduction

Although the majority of human peripheral nerve injuries affects the upper extremity, the sciatic nerve injury is the most widespread experimental model used for study of the nerve repair [1]. Recently, experimental nerve injury of the brachial plexus in studies of functional recovery has been defended by several authors [2, 3, 4, 5].

There are several advantages in the use of the brachial plexus injury as an experimental model. The brachial plexus of rats and humans exhibit various similarities with regard to its components and branches[6, 7]; furthermore, in upper extremity of rats the distance between nerves and targets is shorter than in lower extremity which could reduce the time required for studies of functional recovery[8] and, generally, no autotomy and contractures are observed in upper extremity lesions[5, 9, 10].

The neuronal response after injury is associated with the expression of growth factors and other secreted molecules involved in cell-to-cell communication[11, 12 13]. The mechanisms which regulate expression of the different neurotrophins are not completely known. In the central nervous system, the expression of some members of the family of neurotrophic factors could be correlated with the degree of neuronal activity[14, 15, 16]. In peripheral nervous system, the correlation between depolarizing signals and neurotrophin expression is not well established[17]. However, there are evidences that the type and intensity of activity induces morphological and electrophysiological remodeling of neuromuscular junction. Although adults' synapses can be stable structures after they have been fully established, the motor nerve endings are continuously changing and can suffer influence of modifications in functional demands[18, 19, 20]. These adaptations of synapses can exert influence in nerve orientation and repair since one of the mechanisms governing the regeneration is the secretion of growth factors by target muscles during the period of denervation [20, 21].

There are a variety of neuromuscular diseases with great diversity in referral to the physical therapy (PT). Despite the advances in surgical techniques and the knowledge about nerve regeneration, functional recovery often remains unsatisfactory[22]; besides that, there is no consensus regarding the type and intensity of PT[23]. Thus, it is important to know how the nervous system recovery is influenced by the type of exercise[24].

Rats are adept at reaching for, grasping and bringing food to the mouth with a single paw (behavior termed skilled reaching)[25]. As the appropriated strategy for rehabilitation after peripheral nerve injury has unquestionable clinical relevance, we decided to investigate the effects of two types of treatment: a skilled and an unskilled task on functional recovery and morphological differentiation of regenerating nerve after a brachial plexus crush lesion in adult rats.

Methods

Experimental procedures

All procedures were in accordance with Brazilian laws and the recommendations of the Brazilian Society for Neurosciences and are in compliance with the National Institute of Health's Guidelines for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985).

Animals

Subjects were male *Wistar* rats (n=42) weighing between 280 and 330 g at time of surgery, housed in groups of four or five in plexiglass cages under standard conditions (12-h light/dark, 22 ± 2 °C). Water and standard laboratory chow were provided *ad libitum* except during all behavioral training and testing periods. On the day before the start of training, animals were not furnished with food. From then on, and after each training session, all rats were provided with a measured amount of standard laboratory chow each day (12-15 g) to keep their body weight at ~ 80 – 90% of free feeding level. The training period was finished two days prior the surgery and animals returned to the normal feed.

Experimental design

Three weeks before the surgery animals were randomly assigned to one of 5 groups described in table 1.

Table 1 about here

During three weeks (5 days/week) before the surgical procedure, animals were habituated in all tasks. The habituation phase consisted of three weeks of training in Single Pellet task, two weeks of training in *Staircase* test and one week of habituation in electrical treadmill. A time line of experimental events is presented in Fig. 1.

Figure 1 about here

Surgical procedures

For the surgical procedure all animals, except those of Control group, were deeply anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Then, rats were immobilized on a wood surface and a horizontal incision parallel to the clavicle, running from the sternum to the axillary region was done (the forepaw which showed higher performance on *Staircase* test prior surgery was chosen). The brachial plexus was approached through this opening and the ulnar and median nerve were crushed together (1 cm distal to the sternum) with a 1 mm haemostatic forceps for 30 seconds (adapted from[24, 26]). Inasmuch as median and ulnar nerve injury affects the capacity of finger flexion and grasping[8, 9, 27], the crush was done in these two nerves. This procedure induces axonal interruption preserving the connective sheaths (axonotmesis)[28].

Rehabilitation protocols

After one week of brachial plexus crush animals received one of the following treatments. Both protocols were performed along 3 weeks, 5 days per week.

Skilled task

This task consisted of skilled reaching for food inside reaching boxes, made of clear Plexiglass (20 cm x 25 cm x 40 cm high)[29]. In the middle of the front wall, a 1.1 cm wide vertical slot

allowed animals to reach for food pellets placed on a shelf situated 4 cm above the floor. Two small indentations (0.5 cm in diameter, 0.15 cm deep) on the upper side of this shelf, each aligned with one side of the slit, served to stabilize the food pellets (sucrose spheres of sweet flavor – 4.6 mm; 65 mg ± 10% – Brazilian Homeopathic Pharmacopoeia –Brazil). The distance of the indentations to the front wall was 1.5 cm.

Animals received an individual preoperative training. Once animals began to reach, the food pellets were placed in the indentation contralateral to their preferred limb to provide easier access to the food and prevent simultaneous use of the non-preferred limb. In the training period (before the surgery) rats were motivated to reach during 20 min. The treatment was performed similarly to training period. Reaching with the impaired forelimb was effectively enforced by the insertion of an inner chamber wall ipsilateral to this limb and the placement of pellets, one at a time, in the well opposite this limb. Since animals need to cross the midline to reach, this prevents the use of the unaffected forelimb.

Unskilled task

This task consisted in walking to an adapted motorized rodent treadmill (INBRAMED TK 01, Porto Alegre, Brazil) during 20 min (0.03 m/s - 3 initial min; 0.05 m/s - 14 min; 0.03 m/s - last 3 min). The grade of the treadmill remained at 0% and no aversive stimulus was used. This velocity was chosen in order to avoid possible effects of aerobic treadmill exercise.

Behavioral assessment

The behavioral assessment was performed using a skilled reaching test (Staircase test) and an asymmetrical forelimb use test (Cylinder test).

Skilled reaching test

The fine motor ability was assessed by means of *Staircase* test, which provides a sensitive measure of skilled reaching of both forepaws independently[30]. This test has been recently demonstrated as one of the tests that most clearly reveals the effects of axon misdirection on forelimb function[2]. The boxes were made of clear Plexiglas and consist of a chamber with a central platform. Stairs were positioned on each side of the animal and a set of seven steps placed on each side allows placing three small food pellets (Brazilian Homeopathic Pharmacopoeia –Brazil). Animals were trained during two weeks before the surgical procedure (2 trials/day). The rats remained in the *Staircase* for 15 min and the total number of pellets eaten on each side was recorded. Animals were tested before the surgery in two different days (2 trials/day) and the performance in reaching was made by the average of these trials. Animals were submitted to *Staircase* test 2 and 7 days after the surgery and weekly along the 3 weeks of treatment.

Forelimb asymmetry test

To examine the effect of brachial plexus crush and treatment on spontaneous forelimb use during exploratory activity movements, animals were individually placed into a transparent cylinder (20 cm diameter and 40 cm high) on a glass tabletop and video recorded from below through an angled mirror for 4 min during each test session. The cylindrical shape encouraged rearing and vertical exploration of the walls with the forelimbs. The number of forepaws wall contacts used for postural support was counted and the percentage of asymmetry of single-limb wall contacts [(contralateral/contralateral + ipsilateral) x 100] was calculated. A single cylinder test session was performed on day -2 before the surgery, at 2 days after the surgery and weekly after the start of treatment.

Morphometric analyses

Two days after the end of treatment period animals were anesthetized with chloride hydrate (30%, 10 mL/kg, i.p.) and injected with 1000 UI heparin (Cristália, Brazil). After that, they were transcardially perfused through the left ventricle, using a peristaltic pump (Control Company, São Paulo, Brazil) with 100 mL of saline solution followed by 200 mL of fixative solution composed of 0.5% glutaraldehyde (Sigma, St Louis, MO, USA) and 4% paraformaldehyde (Reagen, Rio de Janeiro, Brazil) in 0.1 M phosphate buffer (PB) pH 7.4 at room temperature. For nerve regeneration analysis, one short segment (~ 3 mm) of ulnar and median nerves was rapidly excised 5 mm after the crush injury site. The specimens were fixed by immersion in the same fixative solution for 1 h. They were then post-fixed in 1% OsO4 (Sigma, St Louis, MO, USA) in PB, dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, Hatfield, PA, USA), embedded in araldite (Durcupan ACM, Fluka, Buchs, Switzerland) and polymerized for 48 h at 60°C.

Transverse-semithin sections (1 μm) were obtained using an ultramicrotome (MT 6000-XL, RMC, Tucson, USA) and stained with 1% toluidine blue (Merck, Darmstadt, Germany) in 1% sodium tetraborate (Ecibra, São Paulo, Brazil). Afterwards, images of the distal portions of the nerves were digitized (initially 100x and further amplified 200x for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a Pro-Series High Performance CCD camera and Image Pro Plus Software 6.0 (Media Cybernetics, USA). For morphological evaluation, a set of 6 images was obtained from each nerve portion, 3 random images from the periphery and 3 random images from the center of the nerve, in order to obtain a representative area per nerve segment (0.010 mm^2; 100% of the area analyzed per segment)[24,31]. The morphometric measurements

used to assess the differentiation of regenerating nerves include: (1) myelinated fiber density (number of fibers/mm^2); (2) average myelinated fiber area (μm^2); (3) average myelinated fiber diameter (μm); (4) average axon diameter (μm) of the myelinated fiber; (5) average myelin sheath thickness (μm); (6) *g* ratio (the quotient axon diameter/fiber diameter, a measurement of the degree of myelination). Individual myelinated fibers were counted and the myelinated fiber density was determined by examining the ratio of the myelinated fibers/total area analyzed. The average myelin sheath thickness was estimated using the measurement tools of the Image Pro Plus software. The measurements of areas were estimated with a point-counting technique[32, 33] using grids with point density of one point per 1.87 μm^2 and the following equation:

$\hat{A}=\Sigma p.a/p$

Where $\hat{A}$ is the area, Σp the sum of points and a/p the area/point value (1.87 μm^2). In order to estimate the axon and fiber diameters, the area of each individual fiber was measured and the value obtained was converted to the diameter of a circle having an equivalent area. In each nerve segment 10.479 μm^2 was 100% of the analyzed area.

Statistical analysis

SPSS® 16.0 (Statistical Package for the Social Sciences, Inc., Chicago, USA) was used for data analyses. To determine differences between groups in behavioral evaluation repeated measures analyses of variance (ANOVA) was utilized. Morphological measurements were analyzed by one way ANOVA and Duncan multiple range test was used when appropriated. Significance was set at $p < 0.05$ for all analyses and results are presented as means ± standard error of the mean (SEM).

Results

Staircase test

Repeated measures ANOVA of Staircase test showed time ($F_{(1, 37)}$= 12.02, p=0.01) and group effects ($F_{(4,37)}$= 7.24, p<0.01); but no significant interaction between time and group ($F_{(4, 37)}$=1.71, p=0.16). As shown in Fig. 2, Duncan's *post hoc* analysis revealed that nerve lesion resulted in a significant decrease in performance of the crushed groups when compared to Sham and Control groups at Post (evaluation performed at 24 hours after crush) and day 7 (p<0.05). At day 14, just Crush group persisted impaired (p<0.05); at this point no difference between treated groups, Sham and Control were observed. At days 21 and 28 no difference between groups were observed (data not shown).

Figure 2 about here

Cylinder test

For the forelimb asymmetry task, repeated measures ANOVA showed an effect of time ($F_{(1, 37)}$= 4.21, p<0.05), group ($F_{(4, 37)}$= 15.50, p<0.01) and time-group interaction ($F_{(4, 37)}$= 7.61, p<0.01). As demonstrated in Fig. 3, all injured groups were significantly impaired immediately following brachial plexus crush (p<0.05) and used the damaged limb for support less frequently than Sham and Control group. At 14 days both treated groups improved at Sham and Control levels. Animals from crush group persisted impaired (p<0.05) at 14 days and showed spontaneous recovery at day 21. There were no differences between groups at days 21 and 28 (data not shown).

Figure 3 about here

Histological analysis

The histological characteristics of distal portions of median and ulnar nerves (Fig. 4) evidenced pathological features in the Crush group (Fig. 4C,H) compared with the Control (Fig. 4A,F) and Sham (Fig. 4B,G) groups, which comprise enlargement of endoneurial connective tissue between the nerve fibers, reduction of myelinated fiber diameter and myelin sheath thickness and presence of degeneration debris. In injured treated groups (Fig. 4D,E; 4I,J) these pathological features were apparently reduced and less endoneurial connective tissue and tissue debris were observed. In a qualitative view, unskilled task accelerated axonal regeneration (as demonstrated by axons in more mature appearance) when compared to skill training with regard to the myelin sheath thickness and axon diameter (Fig. 4D,E; 4I,J).

Figure 4 about here

Morphometric analysis of median nerve

For median nerve, one way ANOVA analyses evidenced effect of lesion for Myelinated fiber area ($F_{(4,18)}$=54.16, p<0.01); Myelin sheath thickness ($F_{(4,18)}$= 67.06, p<0.01); Myelinated fiber diameter ($F_{(4,18)}$= 54.60, p<0.01); Axon diameter ($F_{(4,18)}$=24.80, p<0.01) and g ratio ($F_{(4,18)}$=11.36, p<0.01), but not for Myelinated fiber density ($F_{(4,18)}$=1.23, p=0.33). At 3 weeks of rehabilitation Duncan *post-hoc* points differences between injured (Crush, Skilled task and Unskilled) and uninjured groups (Sham and Control) in Myelinated fiber area, Axon diameter and g ratio (Fig. 5 A, D e E, respectively). For Myelin sheath thickness (Fig. 5 B) differences were found between injured and uninjured groups and between Crush and Unskilled groups; and for

Myelinated fiber diameter (Fig. 5 C) there were differences between injured and uninjured groups and between injured treated (Skilled and Unskilled task) and injured group (Crush), but no difference between two types of treatment. No difference was observed in Myelinated fiber density between groups (Fig. 5 F).

Figure 5 about here

Morphometric analysis of ulnar nerve

As for median nerve, for ulnar nerve one way ANOVA analyses evidenced effect of lesion for Myelinated fiber area ($F_{(4,18)}$=17.17, p<0.01); Myelin sheath thickness ($F_{(4,18)}$=19.47, p<0.01); Myelinated fiber diameter ($F_{(4,18)}$=15.53, p<0.01); Axon diameter ($F_{(4,18)}$=11.15, p<0.01) and g ratio ($F_{(4,18)}$=11.58, p<0.01), but not for Myelinated fiber density ($F_{(4,18)}$=0.48, p=0.74).

At 3 weeks of rehabilitation Duncan post-hoc points differences between injured (Crush, Skilled and Unskilled task) and uninjured groups (Sham and Control) in Myelinated fiber area, Myelin sheath thickness and g ratio (Fig. 6 A, B e E, respectively). For Myelinated fiber diameter (Fig. 6 C) differences were found between injured and uninjured groups and between Crush and Unskilled groups; and for Axon diameter (Fig. 6 D) there were difference between Crush, Control and Sham, but no difference between Control and Unskilled groups and between both of treated groups (Skilled and Unskilled task). No differences were observed in Myelinated fiber density between groups (Fig. 6 F).

Figure 6 about here

Discussion

The most disabling aspect of upper extremity peripheral nerve injury in humans is the loss of skilled hand movements[34]. Rats have been shown to be capable of performing skilled reaching and grasping movements with their forepaws. This ability can be used for rehabilitative treatment[35] as well as for the assessment of post-injury performance[29]. However, previous studies have neither used skill training as treatment for brachial plexus injury nor tests of skilled forelimb function to assess recovery after peripheral nerve injury and repair, except that of Galtrey and Fawcett[2].

Although small physiological variations (as increase and decrease in locomotor activity for a short period) can modify the ultrastructure of nerve terminal[19,21,36] the differences obtained by the use of different types of physical exercises are not frequently examined in brachial plexus injury model.

In the present study we investigated the hypothesis that skilled and unskilled training, started 1 week after the crush, would produce different effects on functional recovery and morphological changes in regenerating nerve. This schedule was chosen because the analysis of functional activity of the forepaws using the grasping strength test revealed that brachial plexus crush lead to a marked reduction in grasping on the 5th day after the nerve damage, and that this impairment was gradually reduced about 20 days after the lesion[10].

Our results demonstrate that both types of treatment promoted the normalization of skill and gross forelimb motor function after 1 week of treatment, as evaluated by means of Staircase and Cylinder test (Fig. 2 and 3). However, the unskilled training accelerated axonal regeneration as demonstrated by the increased fiber myelination and tissue recovery (Fig. 4). For the median nerve, greater myelin thickness and myelinated fiber

diameter was found in those animals trained in unskilled task compared to animals submitted to crush and no treatment (Fig. 5). Equally, for the ulnar nerve, morphological parameters indicated axons in more mature appearance in animals from unskilled training in comparison with crush group (as evidenced by analysis of myelinated fiber diameter and axon diameter of the myelinated fiber) (Fig. 6).

As visualized in figure 6 sham animals showed a lower density of myelinated fibers when compared to control animals. This variability has seen in both, ulnar and median evaluation, and no statistical difference was observed between these two groups. Probably this difference is due a biological variability.

In humans, the major aim of physiotherapy after nervous system injury is to restore the patient's autonomy in activities of daily living and to avoid permanent disability. In spite of the evident relevance in asses the functional performance, experimental studies demonstrated that recovery of function does not necessarily correlate with histological and electrophysiological evidence of regeneration[7,37], as confirmed in the present study.

Previous studies have evaluated the role of treadmill exercise in peripheral nerves following injury[38, 39]. Regeneration of neural processes is likely an activity-dependent process[34] and in some cases is more directly influenced by the quantity of exercise than by the type of exercise[40]. Sabatier et al. [40], demonstrated that training for large amounts of time at slower speeds and training for short amounts of time (even as little as 4 minutes) at higher speeds increased axon profile lengths at two weeks after nerve transection and repair.

Physiological activities, as these utilized in this study, could induce greater regeneration than intensive training. Several studies have reported that muscle overwork was prejudicial to muscle reinnervation since overexerting muscles might accelerate disease

progression[24,38,41,42]. However, it is known that for the improvement of the nerve regeneration the intervention should be of enough intensity to provide a training stimulus[23] and maybe this goal has not been reached in those animals submitted to skill training.

Although previous studies have shown that voluntary physical activity can prime for enhanced axonal regeneration after subsequent axotomy[43], in our study this bias was avoided since all animals was submitted to the same pretreatment protocol.

Even though fine dexterity is often lost or diminished after nerve injury and goal-direct activity may promote greater use of the hand and limb[34], morphological evaluation of animals submitted to unskilled training showed characteristics of superior nerve regeneration than skill training. The discharge of weight on a treadmill (used as unskilled training) demands more intense and global muscular activity than skilled training. It is possible that the pattern of peripheral regulation of growth factors may differ given the type of training[24]. Thus, unskilled training, as the treadmill training employed in our study, could induce greater release and expression of substances and their respective receptors involved in nerve regeneration, as brain-derived neurotrophic factor (BDNF), neurotrophins (NT 3, 4 and 5) and specific members of the tyrosine kinase gene family (trk) (17 and 42 for review).

A limitation of our study is that we used a technical that just allows visualizing the final nerve regeneration. Further studies are necessary to determine the rate of regeneration or the time of target reinnervation in animals submitted to both rehabilitation protocols.

In conclusion the present study showed that both tasks used for forelimb training (skilled and unskilled training) can accelerate the motor and functional recovery after brachial plexus injury. However, the morphological analyses showed that unskilled training induced a greater degree of tissue recovery. The need for effectiveness in

recovery of hand and forelimb movements requires that further studies are needed to verify the success of different methods of treatment in promoting neuronal reorganization and functional improvement after peripheral nervous system injury.

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Table and Legend

Group Nº.	Group Description	Designation (Abbreviation)	Nº. of animals	Behavioral evaluation	Morphometric analysis
1.	Control animals (untouched - unoperated)	Control (C)	8	8	4
2.	Animals submitted to all procedures (anesthesia, incision and suture), except to brachial plexus crush	Sham (S)	8	8	4
3.	Animals submitted to median and ulnar nerve crush and not treated.	Crush (CC)	8	8	5
4.	Animals submitted to median and ulnar nerve crush and treated with motor skilled task.	Skilled (Sk)	10	10	5
5.	Animals submitted to median and ulnar nerve crush and treated with motor repetitive movement (unskilled task)	Unskilled (Usk)	8	8	5

Table 1. Experimental groups and number of animals used for each analyses.

Figures and Legends

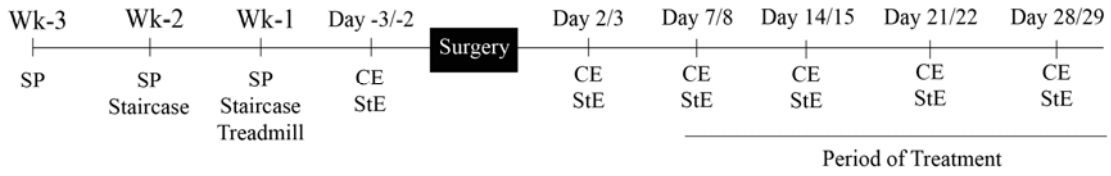

Figure 1: Time line of experimental procedures: SP: Single Pellet training; *Staircase*: *Staircase* Test training; Treadmill: Electrical Treadmill habituation; CE: Cylinder evaluation; StE: Staircase Test evaluation.

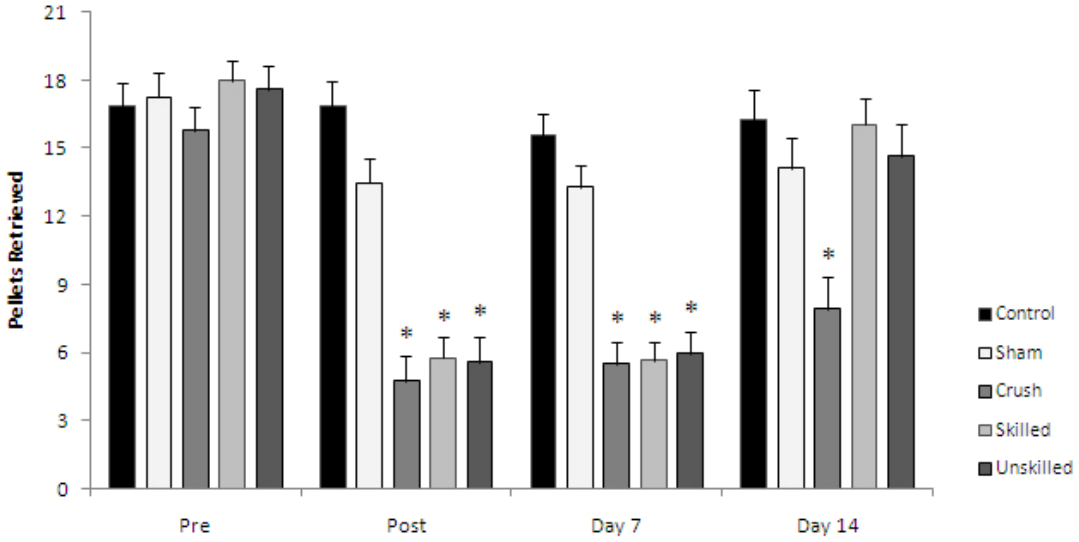

Figure 2: Staircase Test of skilled forelimb reaching: All damaged groups were significantly impaired compared to Control and Sham animals at Post (24h) and day 7. At day 14 animals from crush group remain impaired and animals from both treated groups improved but without difference between them. Values presented are mean ± SEM (* indicates a significant difference from Control and Sham group, $p<0.05$).

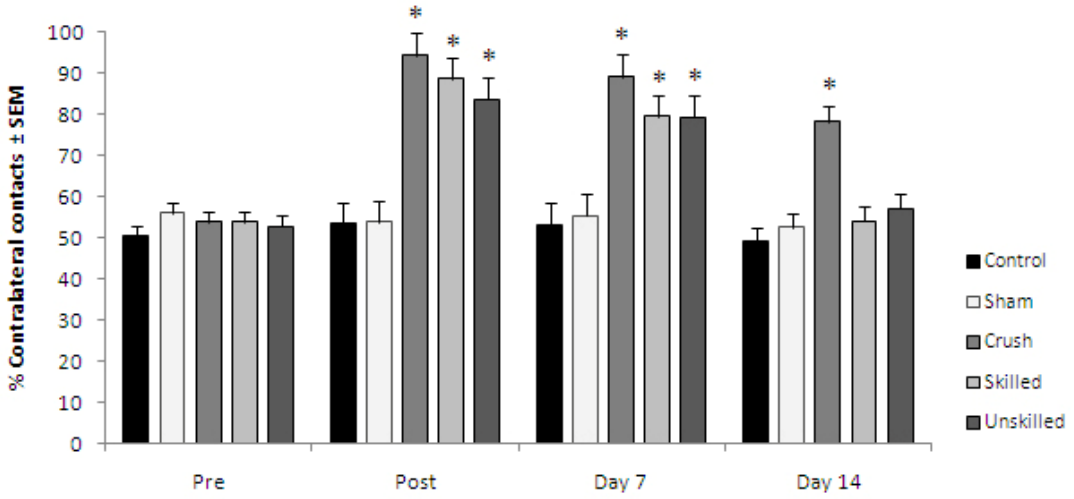

Figure 3: Forelimb asymmetry task: Forelimb asymmetry task measured the number of contralateral forelimb contacts compared to ipsilateral while the animal reared in a cylinder. All damaged groups showed an increase in contralateral forelimb use after brachial plexus crush at Post (24h) and day 7. At day 14 both treated groups improved with no difference between treatments. At this time just animals from crush group remain impaired. Values presented are mean ± SEM (* indicates a significant difference from Control and Sham group, $p<0.05$).

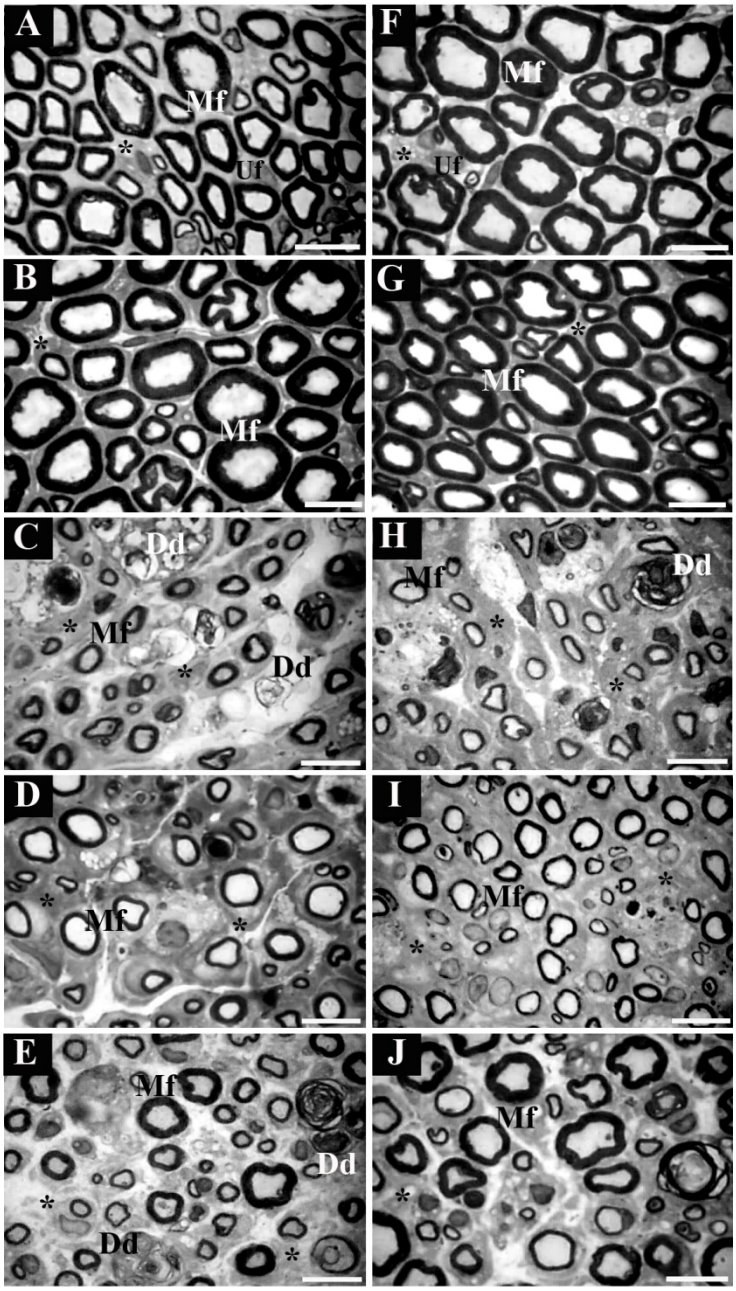

Figure 4: Morphological images from median and ulnar nerves: Digitalized images of transverse-semithin sections (1 μm) obtained from regenerating median and ulnar nerves after 3 weeks of skilled and unskilled training. The left column represents images from distal portions of median nerve and right column represents images from distal portions of ulnar nerve. Letters indicate distal portions from nerves of: (A) and (F) Control group; (B) and (G) Sham group; (C) and (H) Crush group; (D) and (I) Skilled training group; (E) and (J) Unskilled training group. Mf indicates myelinated nerve fiber; Uf, unmyelinated nerve fiber; Dd, degeneration debris; *(asterisk) endoneurial connective tissue. Semithin sections were stained with toluidine blue. Scale bar = 10 μm.

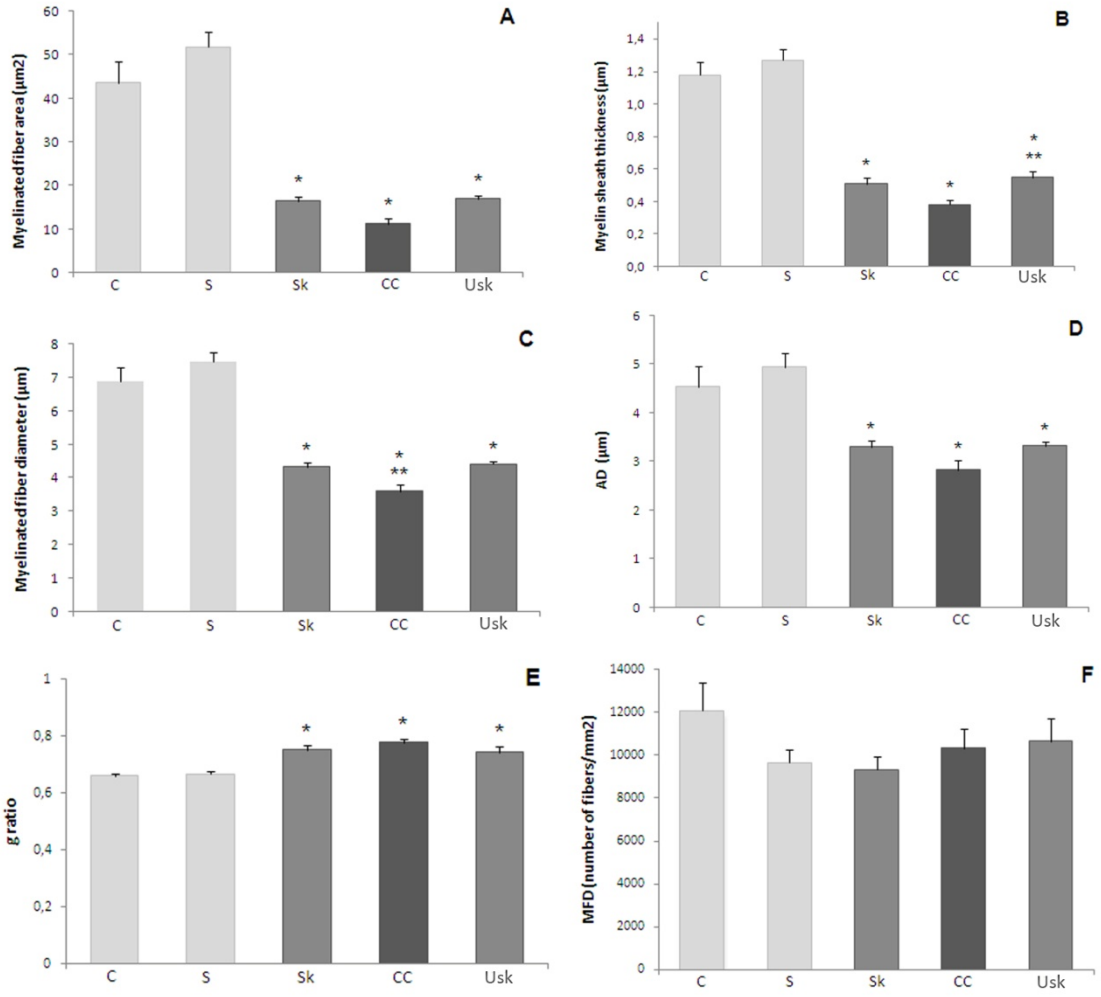

Figure 5: Effects of treatment on the morphometric parameters of regenerating median nerve fibers: Graphics show the mean ± SEM of (A) Myelinated fiber area; (B) Myelin sheath thickness; (C) Myelinated fiber diameter; (D) Axon diameter; (E) g ratio and (F) Myelinated fiber density; * (asterisk) correspond to significant difference from Control and Sham group ($p<0.05$); In (B) ** (double asterisk) indicates significant difference between Unskilled training and Crush group ($p<0.05$); In (C) ** (double asterisk) indicates significant difference between injured treated (Skill and Unskilled training) and injured group (Crush) ($p<0.05$). In D: AD: Axon diameter of the myelinated fiber; F: MFD: Myelinated fiber density; Groups: C: Control; S: Sham; Sk: Skill training; CC: Crush; Usk: Unskilled training.

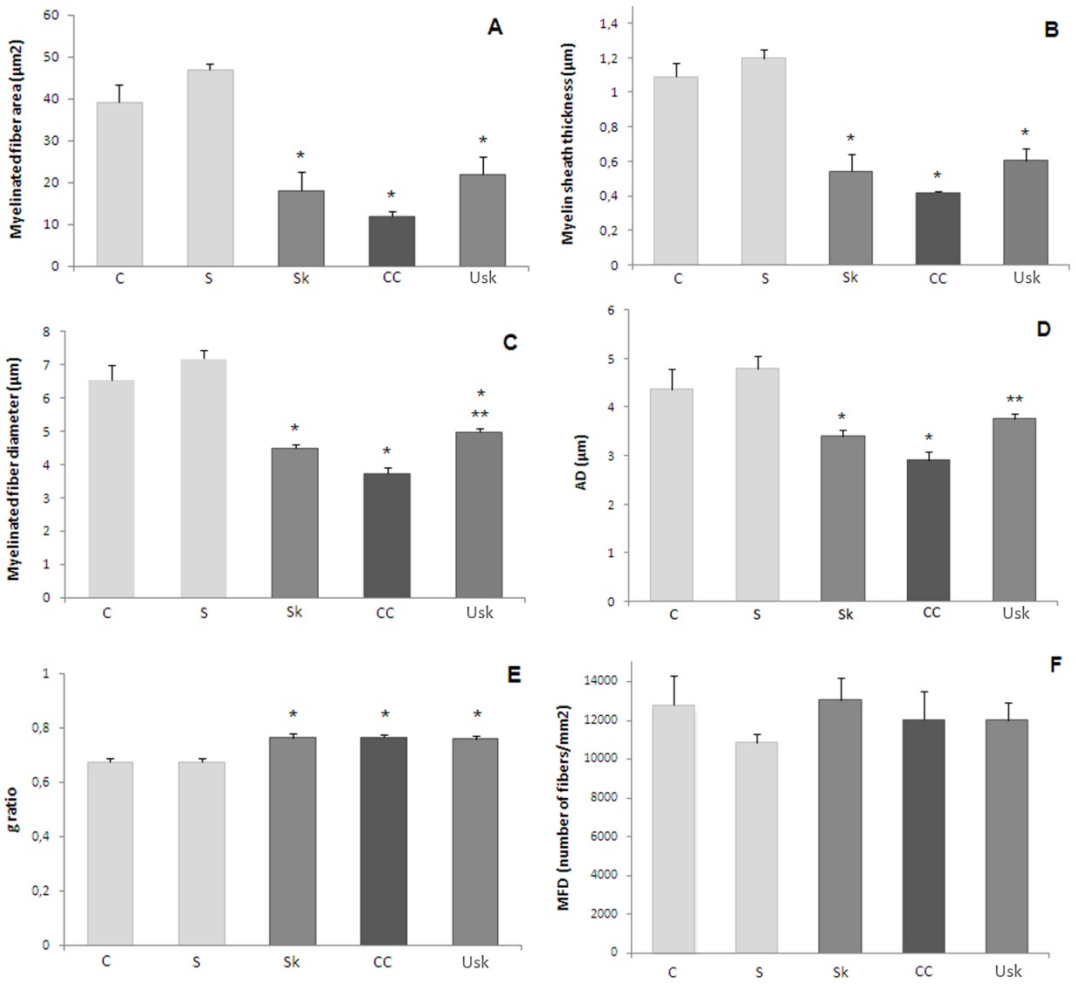

Figure 6. Effects of treatment on the morphometric parameters of regenerating ulnar nerve fibers. Graphics show the mean ± SEM of (A) Myelinated fiber area; (B) Myelin sheath thickness; (C) Myelinated fiber diameter; (D) Axon diameter; (E) g ratio and (F) Myelinated fiber density; * (asterisk) correspond to significant difference from Control and Sham group ($p<0.05$); In (C) and (D) ** (double asterisk) indicates significant difference between Unskilled training and Crush group. In D: AD: Axon diameter of the myelinated fiber; F: MFD: Myelinated fiber density; Groups: C: Control; S: Sham; Sk: Skill training; CC: Crush; Usk: Unskilled training.

5. CAPÍTULO 3

Artigo: Effects of skilled and unskilled training on functional recovery and brain plasticity after focal ischemia in adult rats – a ser submetido à Brain Research.

Effects of skilled and unskilled training on functional recovery and brain plasticity after focal ischemia in adult rats

Pagnussat AS[1,2,3]; Anastacio J[2]; Simao F[2]; Mestriner RG[2]; Salbego, C[2]; Michaelsen SM[4]; Netto CA[1,2].

[1]Programa de Pós-graduação em Neurociências, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Brazil.
[2]Departamento de Bioquímica, ICBS, UFRGS, Brazil.
[3]Departamento de Fisioterapia, Universidade Federal do Pampa (UNIPAMPA), Brazil.
[4]Departamento de Fisioterapia, Universidade do Estado de Santa Catarina (UDESC) Brazil.

Corresponding author:
Aline de Souza Pagnussat
Departamento de Bioquímica
Instituto de Ciências Básicas da Saúde - ICBS
Universidade Federal do Rio Grande do Sul - UFRGS
Rua Ramiro Barcelos, 2600.
90035-003 / Porto Alegre, RS, Brazil.
Phone: + 55-51-3308 5530 / Fax: +55 51 3308 5540
Email: alinespagnussat@gmail.com

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Abstract

Stroke is a leading cause of morbidity and mortality worldwide. After a stroke the recovery of motor functions can be modified by post-injury experience, but the majority of surviving patients exhibit a persisting motor disorder even after rehabilitative therapy. The aim of the present study was to investigate whether skilled and unskilled training would induce different motor recovery and brain plasticity after focal ischemia. We tested this hypothesis by evaluating of the motor skill relearning and the immunocontent of Synapsin-I, PSD-95 and GFAP (pre, post-synaptic elements and surrounding astroglia) in sensorimotor cortex and hippocampus of both hemispheres six weeks after endothelin-1–induced focal brain ischemia in rats. Synapsin-I and PSD-95 levels were increased by skilled training in ischemic sensorimotor cortex and hippocampus. The content of GFAP in ischemic sensorimotor cortex was augmented as result of focal brain ischemia and it was no modified by rehabilitation. In hippocampus skilled training resulted in higher GFAP levels when compared with ischemia and unskilled training. Animals remained permanently impaired at the end of motor/functional evaluations. No significant modification in protein expression was observed in undamaged sensorimotor cortex and hippocampus. We conclude that skilled motor activity can induce higher brain plasticity than unskilled training after brain ischemia in spite of no functional improvement.

Keywords: Focal ischemia, Stroke rehabilitation, Neuroplasticity, Synapsin-I, PSD-95, GFAP

Introduction

Stroke is a leading cause of morbidity and mortality worldwide (Murray & Lopez, 1997). Ischemic stroke accounts for approximately 80% of all strokes and generally results from an occlusion of middle cerebral artery or its branches. Experimental focal cerebral ischemia models have been developed to mimic human stroke and serve as an important indispensable tool in the stroke research field (Durukan & Tatlisumak, 2007). Endothelin – 1 (ET-1) is a potent vasoconstrictor that reduces blood flow to ischemic levels when administered directly into the brain parenchyma (Sharkey & Butcher, 1995). Intracerebral injection of ET-1 involves simple stereotaxic surgery, is associated with minimal morbidity (Biernaskie et al., 2001) and induces long-term functional and motor deficits (Windle et al., 2006).

The damage of cortical networks for movement, their projections and disuse-related mechanisms are considered a major cause of disability after stroke. Up to two thirds of stroke survivors experience impaired function of the upper limb (Jorgensen et al, 1995) and the majority of surviving patients exhibit a persisting motor disorder even after rehabilitative therapy (Hendricks et al, 2002). Functional recovery is influenced by a variety of biologic and environmental factors and recovery profiles are characterized by a high interindividual variability. However, it is well known that there is a 'dose-dependent' effect of rehabilitation and that earlier delivery of rehabilitation has lasting effects on the functional recovery of stroke patients (Huang et al., 2009).

Recovery of motor functions after a stroke the can be modified by post-injury experience. Several studies report activity-dependent brain plasticity proportional to the complexity of motor learning task (Maldonado et al., 2008; Adkins et al., 2006; Kleim et al., 2004). The primary motor cortex (M1) is organized into highly interconnected groups of neurons, whose coordinated activation encodes complex and multijoint

movements (Adkins et al., 2006; Graziano, 2006). Noninvasive functional imaging studies have indicated that M1 is involved in the process of motor learning and that learning-associated activity in M1 can be greater in magnitude and areal extent than the activity associated with simple motor use either in healthy or in lesion conditions (Kleim et al., 2002; Plautz et al., 2000; Kleim et al., 1998).

The cellular mechanisms of learning-dependent plasticity and motor recovery after stroke involve coordinated neuronal changes within motor cortex that include up regulation of trophic factors, like BDNF, increase in protein synthesis, synaptogenesis, and map reorganization (Adkins et al., 2006). Synapses consist of three components including the pre- and post-synaptic elements and the surrounding astroglial ensheathment (Volterra et al., 2002). Synapsin-I is a protein implicated in neuroplasticity that tethers synaptic vesicles to the actin cytoskeleton and regulates the proportion of vesicles available for release in the presynaptic terminal (Jovanovic et al., 2000). PSD-95 is highly abundant in the postsynaptic density (PSD) and has been proposed to regulate many aspects of synaptic transmission (Steiner et al., 2008). On the other hand, glial fibrillary acidic protein (GFAP) is the principal intermediate filament in mature astrocytes, is important for astrocyte-neuronal interactions and plays a vital role in modulating synaptic efficacy in the central nervous system (CNS) (Eng et al., 2000).

In this study we investigated whether skilled and unskilled training would induce different motor recovery and brain plasticity after focal ischemia. We hypothesized that skilled rehabilitation would result in superior performance on behavioral evaluation as well as in a greater expression of proteins related to synaptogenesis on sensoriomotor cortex and hippocampus in both brain hemispheres.

Results

Skilled Reaching Performance

The Staircase test showed a significant effect of time ($F_{(1,39)}$ = 16.34; $p<0.001$) and an interaction of time and group ($F_{(3,39)}$ = 16.04; $p<0.001$). Ischemic injury resulted in a significant reduction in pellets retrieved compared with sham ($p<0.01$), but there was no difference between ischemic and treated groups along weeks ($p>0.05$), as depicted in Fig 1.

Figure 1 about here

Forelimb Asymmetry Use

Animals did not exhibit asymmetrical use of the forelimbs during postural support before ischemic injury. After surgery all ischemic animals developed a reliance on the ipsilateral limb for postural support that persisted during the six weeks of the experiment. Repeated-measures ANOVA evidenced a main effect of group ($F_{(1,39)}$ = 27.27; $p<0.01$) and interaction between time and group ($F_{(3,39)}$ = 5.46; $p<0.01$). Animals in all ischemic groups maintained the impairment with the contralateral forelimb over the course of the experiment. As showed in Fig 2 there was no difference between ischemic and treated groups at the end of six weeks of rehabilitation ($p>0.05$).

Figure 2 about here

The effects of rehabilitation on expression of Synapsin-I, PSD-95 and GFAP

In order to assess pre - and post-synaptic elements as well as the surrounding astroglial ensheathment we performed Western blotting assays using antibodies against Synapsin-I, PSD-95 and GFAP in the sensorimotor cortex and hippocampus in both hemispheres. Synapsin-I levels was modified in sensorimotor cortex ($F_{(3,19)}$ = 6.66, $p<0.01$) and hippocampus ($F_{(3,19)}$ = 21.3, $p<0.01$) in ischemic hemisphere (ipsilateral). Similarly, ANOVA evidenced differences in quantification of PSD-95 content in sensorimotor cortex ($F_{(3,19)}$ = 5.23, $p\leq0.01$) and hippocampus ($F_{(3,20)}$ = 8.12, $p<0.01$) and in quantification of GFAP in sensorimotor cortex ($F_{(3,19)}$ = 5.54, $p<0.01$) and hippocampus ($F_{(3,22)}$ = 4.50, $p\leq0.01$) only in the ipsilateral hemisphere. As displayed in Fig. 3, Synapsin-I and PSD-95 levels were increased by skilled training in sensorimotor cortex and hippocampus. The content of GFAP was augmented as result of focal brain ischemia (ischemia versus sham $p<0.05$; skilled and unskilled versus sham $p<0.01$). In hippocampus skilled training resulted in higher GFAP levels when compared with ischemia and unskilled groups ($p<0.01$).

Figure 3 about here

Discussion

The majority of the stroke survivors experience hemiparesis, resulting in long lasting impairment of one upper extremity after stroke (Carod-Artal et al., 2000). Upper extremity sensorimotor recovery after stroke may be slower (Levin et al., 2009) or more complex than that of the lower limb (Duncan, 1994). The partial recovery of upper limb movements does not usually translate into functional use because the upper extremity control involving a wider inventory of coordinated trunk and multi–joint movements (Duncan, 1994; Duncan et al., 1994). Since the most rapid recovery occurs within the first months after stroke (Duncan et al, 1994) choosing the appropriate approach is determinant for a successful rehabilitation, recovery of arm and hand function and social reintegration of the surviving stroke victims.

Rodent models of skilled reaching have been used extensively to study the neural bases of movement (Whishaw et al., 1993), recovery from brain damage (Ploughman et al., 2007) and anatomical or functional organization of the sensorimotor cortex after motor skill learning (Kleim et al., 1998; Anderson et al., 2002). It has been proposed that the acquisition and refinement of novel movement sequences involve sensorimotor cortex alterations in synapse number (Kleim et al., 1996), synaptic strength, dendritic growth (Biernaskie and Corbett, 2001) and can modify the topography representation of segments involved (Kleim et al., 1998; see Adkins et al., 2006 for review). Behavioral training would also result in similar changes in the hippocampus (Briones et al., 2000; Briones et al., 2004).

These structural changes should require the synthesis and/or redistribution of various neuronal proteins necessary for motor learning. Then, we decided to evaluate the functional recovery and the expression of proteins related to synaptogenesis after focal brain ischemia and rehabilitation using skilled and unskilled movements. We had

hypothesized that skilled task would result in superior functional recovery and expression of these proteins in both brain hemispheres. Our hypothesis was partially confirmed. Animals remained permanently impaired at the end of evaluations. No difference was observed in ischemic animals in spite of treatment on recovery, neither in fine motor control necessary for reaching in Staircase task, nor in recuperation of symmetrical forelimb use for postural support, as evaluated by cylinder test.

After stroke animals can develop compensatory mechanisms in order to improve the performance on functional tasks by the introduction of additional degrees of freedom in movement (Metz et al., 2005), including rotatory movements of the trunk and the assistance of the non-reaching paw (Farr & Whishaw, 2002). In humans the use of the trunk becomes part of the general reaching strategy (Michaelsen et al., 2001) as a compensatory mechanism by which the CNS may extend the reach of the arm when the control of the active range of arm joints is limited (Levin et al., 2002). In our experiment ischemic animals could have adopted undesirable compensatory strategies for reaching in single pellet task which prevented the satisfactory performance when trunk was in a more 'restricted' position in *Staircase* test.

The ET-1 model used in our study induces a consistent injury and ensuing behavioral deficits what makes it attractive for recovery studies. Nevertheless, it could cause an excessive brain damage and severe functional impairments resistant to physical therapy, what is evidenced by large infarct volume (Windle et al., 2006) and the lack of spontaneous recovery in tasks here used (Fig. 1 and 2).

Brain plasticity after stroke rehabilitation was assessed by means of evaluation of proteins involved with synaptic contacts: Sinapsin-I, PSD-95 and GFAP. Synapsins represent one of the most abundant families of synaptic proteins. They are expressed only in neurons and are specifically localized in the presynaptic compartment of the

synapses (Ferreira & Rapoport, 2002). Synapsin-I is implicated in neurotransmitter release, axonal elongation and maintenance of synaptic contacts (Wang et al. 1995). Our results demonstrated that skilled training increased the expression of Synapsin-I in both ipsilateral structures evaluated. Synapsin-I reflects brain plasticity since it is a downstream mediator of BDNF's effects (Cotman & Berchtold, 2002; Jovanovic et al., 2000) which is expressed in a use-dependent fashion (Gómez-Pinilla et al., 2002). This neurotrophin influences synaptic efficacy, neuronal connectivity and use-dependent plasticity (Schinder & Poo, 2000) as a result of binding to its tyrosine kinase receptor and subsequent activation of several signal transduction pathways (Waterhouse & Xu, 2009).

BDNF and Synapsin-I are upregulated by neuromuscular activity, including endurance exercise and functional rehabilitation, in normal (Vaynman et al., 2004; Klintsova et al., 2004) and ischemic animals and contribute to improved performance in cognitive and motor tasks (Kleim et al., 2003; Ploughman et al., 2005; Ploughman et al., 2007; Ploughman et al., 2009). In our study skilled training induced a long term increase of Sinapsin-I when compared with unskilled training in sensorimotor cortex and hippocampus (Fig. 3A e 3B). This is not a surprising since skilled training is a rehabilitation task that associates elaborated motor activity and higher cognitive function.

PSD-95 is a scaffolding molecule enriched at post-synaptic excitatory synapses (Keith & Husseini, 2008). It is involved in the anchoring of NMDA receptor (NMDAR) subunits (Kornau et al., 1995) and is responsible by maintaining the normal architecture and functionality of the post-synaptic density (Niethammer et al., 1996). In the present study we found that the immunocontent of PSD-95 was markedly increased in both sensorimotor cortex and hippocampus after focal ischemia and skilled training.

Previous studies support the importance of PSD-95 proteins in synaptic plasticity and in the control of synaptic strength (Abe et al., 2004). Behavioral training can increase the PSD-95 expression (Dietrich et al., 2005; Xu & Ruan, 2009). One the other hand, the PSD-95 content can be diminished after brain lesion (Hu et al., 1998). The truncation of the C-terminal domain of NMDAR subunits by calpain could result in the loss of the ability of the subunits to bind to PSD-95 in hypoxic-ischemic conditions (Chen et al., 2007). Maybe this decrease in PSD-95 expression is related to thickness of PSD instead of reduction in synaptic numeric density (Xu & Ruan, 2009). PSD is a dynamic structure whose morphology and composition change with experience and motor activity (Kennedy, 2000). In our study the skilled training could have induced an increase in synaptic effectiveness in both sensorimotor cortex and hippocampus when compared with unskilled training, as evidenced by enhanced levels of PSD-95 (Fig 3C e 3D).

Astrocytes provide energy substrates to neurons and play a crucial role in the maintenance of the extracellular environment. The astrocytic process is in intimate contact with both the pre- and postsynaptic terminal (Fellin et al., 2006) and are intricate in a bi-directional communication with neurons (Araque, 2008) influencing the neurotransmission (Anderson et al., 2002). GFAP is the major constituent of astrocytic filaments and a marker for reactive astrocytes (Ridet et al., 1997).

In our study we found that the immunocontent of GFAP was increased in ipsilateral sensorimotor cortex in all ischemic animals. The rehabilitation treatment did not alter the GFAP expression (Fig 3E). Strong evidence exists that injury to the CNS resulted in an increase in the expression of GFAP protein (Ridet et al., 1997). This reactive astrocytic response after injury could form glial scar tissue and sometimes hindered axonal growth (Alonso et al., 1993).

However, our results shown that the content of GFAP in ipsilateral hippocampus was increased by skilled training in ischemic animals (Fig 3F). Previous studies demonstrated that reactive astrocytes can play a role in neural plasticity in intact animals (Sirevaag et al., 1991). One the other hand, either cerebral ischemia or rehabilitation training can increase expression of reactive astrocytes in hippocampus and this enhanced astrocytic response might contribute to the amelioration of functional impairment (Briones et al., 2006).

Our data show that skilled training can increase brain plasticity in ischemic hemisphere when compared with unskilled training. The increased limb use in the absence of skill acquisition (as unskilled task) has been shown to result in no net change in movement topography within the primate motor cortex (Nudo et al. 1997) or synapse number within the rat motor cortex (Kleim et al. 1996). Although neuroplastic changes in the cortex of the undamaged side may contribute to functional recovery after stroke (Biernaskie and Corbett, 2001) we observed no significant modification in protein expression in undamaged sensorimotor cortex and hippocampus.

Since neuronal connections can be remodeled by physical and cognitive experience, our results evidenced that the skilled motor activity can induce higher brain plasticity than unskilled training after brain ischemia in spite of no functional improvement. The optimal rehabilitation depends of several factors, including the stroke severity and type/intensity/duration of treatment. Skilled rehabilitation would be a good choice even after a severe stroke and the restriction of compensatory mechanisms during rehabilitation would be important for functional improvement.

Experimental Procedures

Animals

Forty three male *Wistar* rats from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul, Brazil) weighing approximately 300 g at the time of surgery were housed in standard plexiglass boxes, under 12:12 h light/dark cycle, in a temperature-controlled environment (20±1 °C), with food and water available *ad libitum,* except during all behavioral training and testing periods. All experimental protocols were in accordance with Brazilian law and were approved by the Ethical Committee at the Federal University of Rio Grande do Sul.

Food deprivation

Four weeks before the surgery all rats were placed on a food deprivation schedule. On the day before the start of training, animals were not furnished with food. From then on, and after each training session, all rats were provided with a measured amount of standard laboratory chow each day (12-15 g) to keep their body weight at ~ 80 – 90% of free feeding level. The training period was finished two days prior the surgery and animals returned to the normal feed. Body weights were monitored throughout the study, and, within ages, there were no group differences in weight change over the course of the experiment.

Experimental design and groups

Four weeks before the surgery rats were familiarized with the apparatus and the tasks used in post surgery period. The time line is of experimental design is presented in Fig. 4. After this period, animals were randomly assigned to four different groups: sham [S]

(n=10), focal ischemia [FI] (n=11), Skilled training [Sk] (n=12) and Unskilled training [Usk] (n=10).

Figure 4 about here

Surgical procedures

Anesthesia was induced using a mixture of 2% halothane in 30% oxygen and 70% nitrous oxide and was maintained with 0.5 - 0.7% halothane. Temperature was maintained between 36.5°C and 37.5°C throughout the surgery using a self-regulating heating blanket (Letica, Spain). Animals were placed in a stereotaxic apparatus, a midline incision was made and small burr holes were drilled at the following coordinates: (1) anterioposterior (AP) 0.0 mm, mediolateral (ML) ±2.0 mm and dorsoventral (DV) -2.0 mm; (2) AP +2.3 mm, ML ±2.0 mm, DV -2.0 mm; (3) AP +0.7 mm, ML ± 3.8 mm, DV -6.0 mm. Using a 10 μL Hamilton Syringe, ET-1 (800 pmol - human and porcine, Calbiochem) was injected into the motor cortex (2 μL) and into the dorsolateral striatum (2 μL) (Windle et al., 2006)

ET-1 was injected in left or right hemisphere (the contralateral hemisphere to the preferred limb used for reaching) at a rate of 1.0 μL/2 min with a 1-min pause between each μL. After infusion, the needle was kept in position for an additional 3 minutes to minimize backflow. In sham surgeries ET-1 was replaced with sterile saline. The wound was sutured closed and topical anesthetic were applied.

Rehabilitation protocols

Five days after surgery animals received one of the following treatments. Both protocols were performed along 20 min/day, 5 days/week, during 6 weeks.

Skilled training consisted of skilled reaching for food inside reaching boxes, made of clear Plexiglass (20 cm x 25 cm x 40 cm high) (Whishaw & Pellis, 1990). Five centimeters from the side of each front wall was a 1 cm wide slit that extended from the floor to a height of 15 cm. On the outside of the wall, in front of the slit, mounted 4 cm above the floor was a 2 cm wide by 4 cm long shelf. Two small indentations (0.5 cm in diameter, 0.15 cm deep) on the upper side of this shelf, each aligned with one side of the slit, served to stabilize the food pellets (sucrose spheres of sweet flavor – 4,6 mm; 65 mg ± 10% – Brazilian Homeopathic Pharmacopoeia –Brazil). The distance of the indentations to the front wall was 1.5 cm. For each rat, food was placed in the indentation contralateral to the limb with which the rat reached. Animals received an individual preoperative training for 4 weeks. Training was administered in such a way that when a rat made a successful reach, a short pause preceded presentation of the next food pellet. After focal ischemia, reaching with the impaired forelimb was enforced by the insertion of an inner chamber wall ipsilateral to this limb and the placement of pellets in the well opposite to this limb. Since animals need to cross the midline to reach, this prevents the use of the unaffected forelimb.

Unskilled training consisted in walking to an adapted motorized rodent treadmill (INBRAMED TK 01, Porto Alegre, Brazil) at 0.03 m/s. Subjects did not require shock or other stimulus to walk. This velocity was chosen in order to allow slow walk and to avoid possible effects of aerobic treadmill exercise.

Behavioral outcome measures

Behavioral evaluation was performed before and after the stroke and weekly over the 6 weeks of treatment, as depicted in Fig 4.

Skilled reaching test

Animals were trained to reach for food pellets in the *Staircase* test of independent forelimb reaching ability over 4 weeks (2 trials/day; 15 min/trial) (Pagnussat et al., 2009). The boxes were made of clear Plexiglas and consist of a chamber with a central platform. Stairs were positioned on each side of the animal and a set of seven steps placed on each side was baited with 3 food pellets (Brazilian Homeopathic Pharmacopoeia –Brazil). The stair arrangement and the narrowness of the corridor are such that the rat cannot turn around and/or retrieve dropped pellets and just allow the animal to reach from the right steps using the right forepaw and from the left steps only with the left forepaw (Montoya et al., 1991).

Forelimb asymmetry test

Animals were individually placed into a transparent cylinder (20 cm diameter and 40 cm high) on a glass tabletop and videorecorded from below through an angled mirror for 4 min during each test session. The number of forepaws wall contacts used for postural support was counted and the percentage of asymmetry of single-limb wall contacts [(contralateral/contralateral + ipsilateral) x 100] was calculated (Schallert et al., 2000).

Western Blotting Assay

For immunoblotting studies, rats were decapitated, their brains removed and cortex and hippocampus were dissected out and homogenized in lysis buffer (4% SDS, 2.1 mM EDTA, 50 mM Tris). Brains from control rats were processed similarly. Aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5%. Protein samples (60 μg) were separated by 10% sodium dodecyl

sulfate–polyacrylamide gel electrophoresis following transfer to nitrocellulose membranes (Amersham, GE Healthcare). After 1 h incubation in blocking solution containing 5% skim milk powder and 0.1% Tween-20 in Tris-buffered saline (TBS; 50 mM Tris–HCl, 1.5% NaCl, pH 7.4), membranes were incubated overnight at 4 °C with the appropriate primary antibody diluted in TBS containing 5% skim milk powder and 0.1% Tween-20. Primary antibodies against the following proteins were used: anti-GFAP (1:2000 dilution, rabbit polyclonal, sigma); anti-PSD-95 (1:2000 dilution, rabbit polyclonal, cell signalling) and anti-synapsin (1:3000, rabbit polyclonal, millipore). After washes in TBS containing 0.1% Tween-20, membranes were incubated with horseradish peroxidase-coupled secondary antibodies recognizing antigens from the same host as the corresponding primary antibody (rabbit IgG from Amersham, GE Healthcare; 1:1000 dilution in blocking solution). The chemiluminescence (ECL, Amersham, GE Healthcare) was detected using X-ray films (Kodak X-Omat). The films were scanned and the percentage of band intensity was analyzed using OptiQuant software. All blots were re-probed with β-actin antibody from Sigma (1:2000 dilution, mouse monoclonal) as an internal control.

Statistical Analyses

SPSS® 16.0 (Statistical Package for the Social Sciences, Inc., Chicago, USA) was used for data analyses. To determine differences between groups in behavioral evaluation repeated measures analyses of variance (ANOVA) was utilized. Western blot was analyzed by one way ANOVA. Tukey's post-hoc test was used when appropriated. Significance was set at $p < 0.05$ for all analyses and results are presented as means ± standard error of the mean (S.E.M.).

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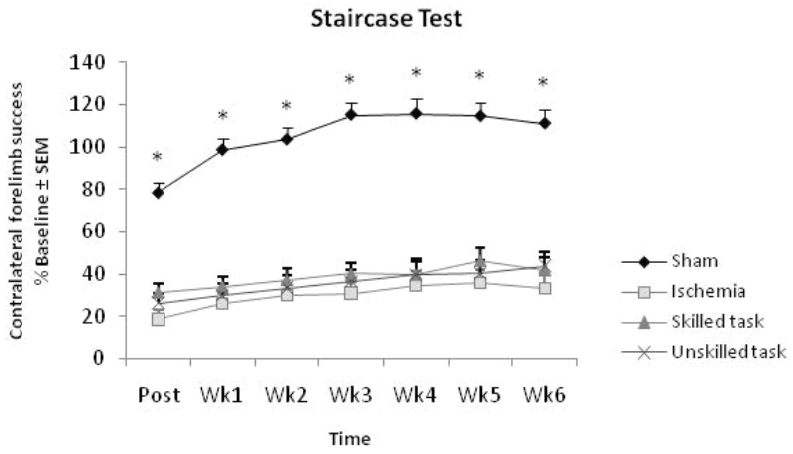


Fig 1: Skilled reaching ability in the *Staircase* test expressed as % of baseline performance (**p*<0.01 sham versus ischemia, skilled and unskilled; n=10 for sham and unskilled; n=11 for ischemia; n=12 for skilled). Values are mean ± S.E.M. Repeated measures ANOVA, Tukey test.

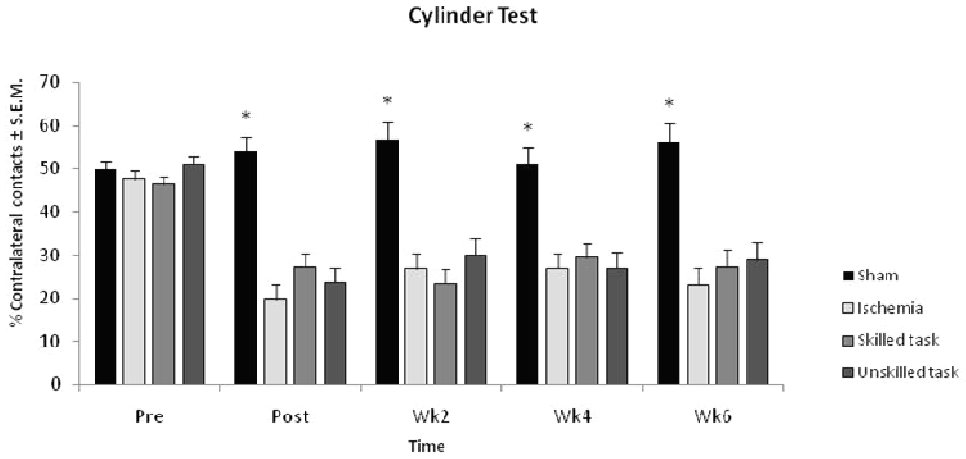
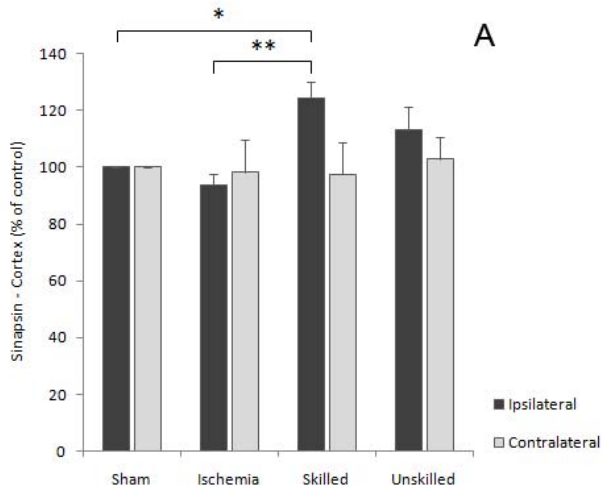


Fig 2: Contralateral limb use in the cylinder test. All ischemic groups used their contralateral forelimb significantly less after ischemia. This impairment persisted for the duration of the experiment in ischemic and ischemic treated groups (*p<0.01 sham versus ischemia, skilled and unskilled; n=10 for sham and unskilled; n=11 for ischemia; n=12 for skilled). Values are mean ± S.E.M. Repeated measures ANOVA, Tukey test.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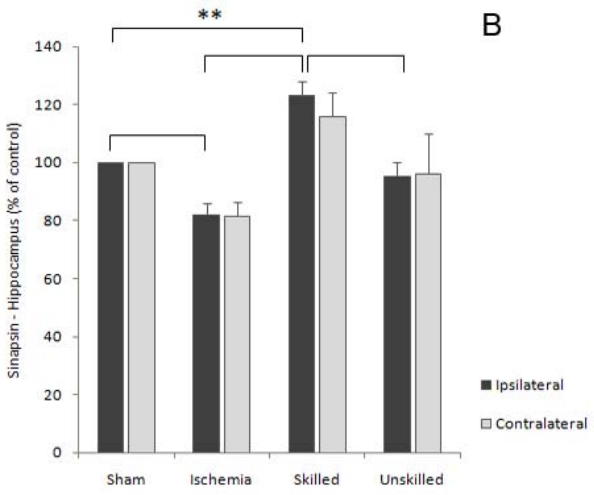
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Sinapsin - Hippocampus (% of control)
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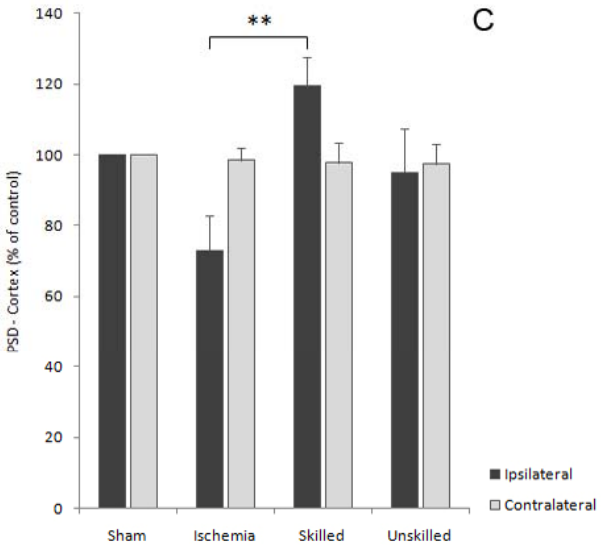
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PSD - Cortex (% of control)
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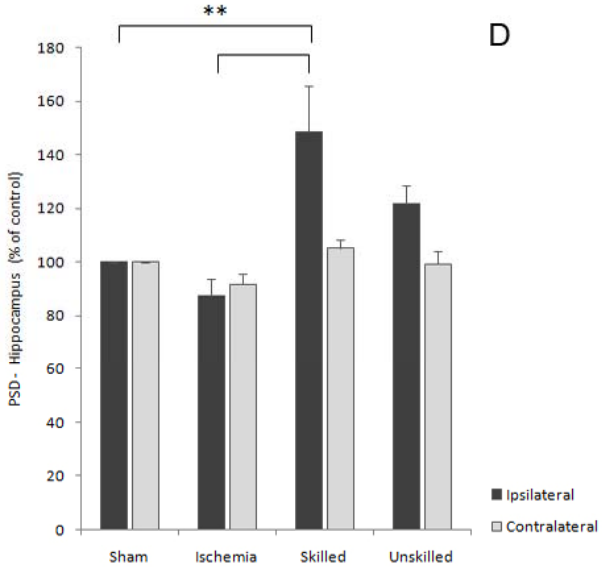
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PSD - Hippocampus (% of control)
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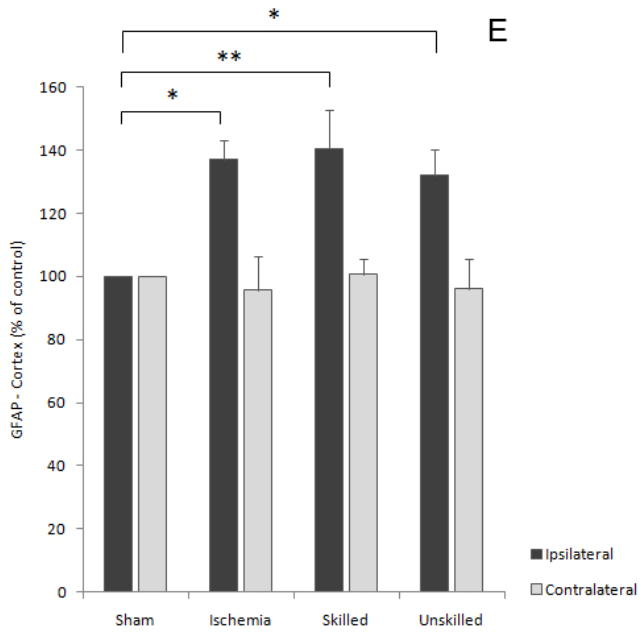
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GFAP - Cortex (% of control)
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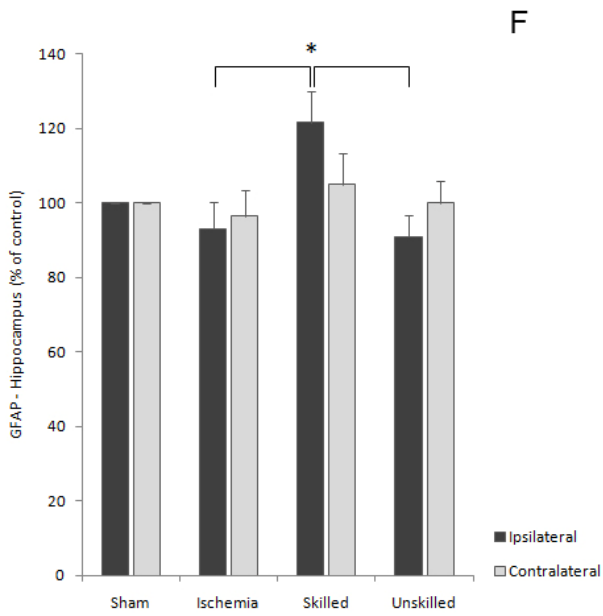
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GFAP - Hippocampus (% of control)
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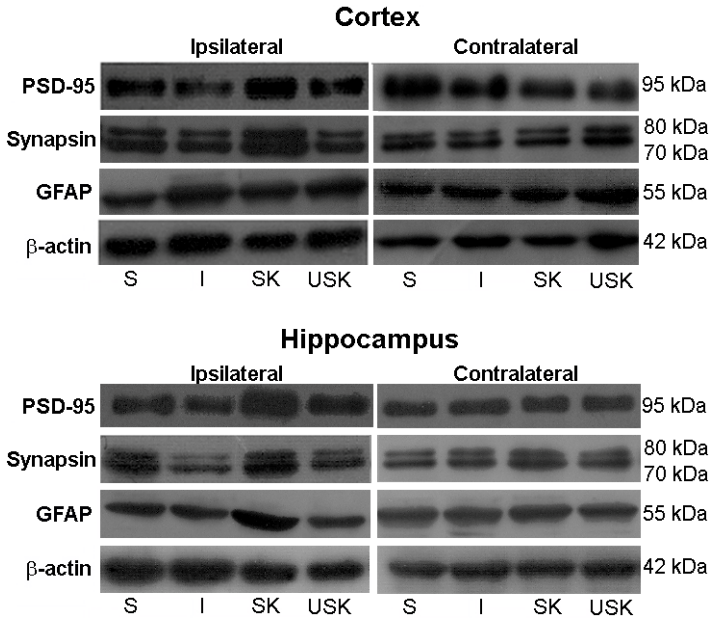

Fig 3: Synapsin-I, PSD-95 and GFAP levels measured in the sensorimotor cortex and hippocampus from ischemic (ipsilateral) and intact (contralateral) hemispheres. A: Synapsin-I in the sensorimotor cortex (*p<0.05 skilled versus sham; **p<0.01 skilled versus ischemia; n=5). B: Synapsin-I in the hippocampus (**p<0.01 ischemia versus sham; skilled versus sham, ischemia and unskilled; n=5). C: PSD-95 in the sensorimotor cortex (**p<0.01 skilled versus ischemia; n=5-6). D: PSD-95 in the hippocampus (**p≤0.01 skilled versus sham and ischemia; n=5-6). E: GFAP in the sensorimotor cortex (*p<0.05 ischemia versus sham; **p<0.01 skilled versus sham, unskilled versus sham; n=5). F: GFAP in the hippocampus (*p<0.01 skilled versus ischemia and unskilled; n=5-7). G: Representative Western blots images from each of the groups. Uniformity of gel loading was confirmed with β-actin as the standard. Values are expressed as a percentage of sham levels. Bars represent the mean ± S.E.M. One way ANOVA, Tukey test.

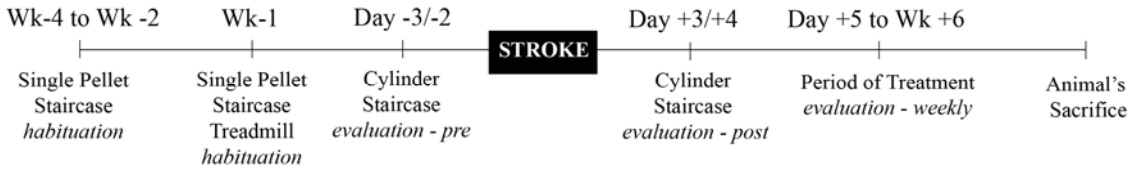

Fig 4: Time line of experimental events.

6. CAPÍTULO 4

Artigo: Effects of focal brain ischemia induced by Endotelin - I on skeletal muscle morphology - a ser submetido a Neuroscience Letters.

Effects of focal brain ischemia induced by Endotelin - I on skeletal muscle morphology

Aline S Pagnussat[1,2,3], Anna R S Gomes[4], Ana P Loureiro[5], Régis G Mestriner[2,6], Carlos A Netto[2]

[1]Programa de Pós-graduação em Neurociências, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Brazil.
[2]Departamento de Bioquímica, ICBS, UFRGS, Brazil.
[3]Departamento de Fisioterapia, Universidade Federal do Pampa (UNIPAMPA), Brazil.
[4]Departamento de Fisioterapia, Universidade Federal do Paraná (UFPR – Setor Litoral), Brazil.
[5]Departamento de Fisioterapia, Pontifícia Universidade Católica do Paraná (PUCPR), Brazil.
[6]Programa de Pós-graduação em Fisiologia, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Brazil.

Corresponding author:
Carlos Alexandre Netto
Departamento de Bioquímica
Instituto de Ciências Básicas da Saúde - ICBS
Universidade Federal do Rio Grande do Sul - UFRGS
Rua Ramiro Barcelos, 2600.
90035-003 / Porto Alegre, RS, Brazil.
Phone: + 55-51-3308 5530 / Fax: +55 51 3308 5540
Email: netto@gabinete.ufrgs.br

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Keywords: Focal brain ischemia, Soleus muscle, Muscle fiber area.

Abstract

Stroke is an important cause of physical disability in adults. Neurological disorders as stroke are frequently accompanied by impairments in motor units, skeletal muscle atrophy and weakness. Muscle weakness in the post-stroke population often result in decreased functional ability. Thus, the understanding about effects of focal brain ischemia on skeletal muscle is needed for definition of the best strategy for rehabilitation and to optimize results with therapy. The present study investigated the effects of focal ischemia produced by Endothelin -1 on body weight, muscle weight and soleus muscle fiber cross-sectional area of both paretic and non-paretic limbs six weeks after the brain injury. No difference was observed on body and muscle weights six weeks after the ischemia. Muscle fiber cross sectional area was significant augmented in non-paretic limb of ischemic animals when compared to paretic limb ($p=0.01$) and to muscle from sham animals ($p=0.03$). These results suggest that focal ischemia induced by ET-1 does not have long term effects on body or muscle weight but the overuse of non-paretic limb was sufficient to induce hypertrophy of soleus muscle ipsilateral to injured hemisphere. Further studies are necessary to determine if this experimental model of ischemia can be used in studies of strengthening after stroke.

Introduction

Stroke is an important cause of death and physical disability in adults [14]. It is characterized as an interruption of the blood supply to the brain or hemorrhage into the brain tissue that involves a disruption in the motor and sensory pathways [27]. Following stroke, several patients lead their lives with persistent limb weakness which is correlated with loss of dexterity and reduced performance on functional tasks [6].

Muscle weakness is not always the major problem associated with a neurological diagnosis but it can accompany other motor problems and is recognized as a limiting factor for rehabilitation [6]. Stroke patients have been shown to be weak on the apparently non-paretic side (ipsilateral to the brain lesion), as well as on the obviously paretic side (contralateral to the brain lesion) [5]. The impairment of voluntary strength is associated with loss of motor units, deficient motor unit recruitment and firing frequencies inadequate to sustain muscle contraction and muscle fiber atrophy [9, 15, 19]. Optimizing patient outcomes after stroke necessitates understanding about the effects of cerebral ischemia on skeletal muscle which may have an impact on the patient's rehabilitation potential [12].

Endothelin-1 (ET-1) is a potent vasoconstrictor that induces ischemic injury when injected directly into brain tissue. This model is very attractive because it produces long-lasting functional impairments and has greater similarity to clinical stroke [29], and maybe can be useful to study weakness associated with atrophy after stroke. Thus, the present study investigated the effects of focal ischemia produced by ET-1on body and muscle weights and muscle fiber cross-sectional area of both paretic and non-paretic soleus muscle six weeks after the brain injury. It was hypothesized that this experimental model of ischemia would induce reductions in body and muscle weight

and muscle fiber cross-sectional area of paretic limb and could be useful for studies of strengthening after stroke.

Material and Methods

Ten male *Wistar* rats weighing 300–350 g at the time of surgery were housed in standard plexiglass boxes, under 12:12 h light/dark cycle, in a temperature controlled environment (20±1 °C), with food and water available *ad libitum*. All the procedures were approved by the Ethical Committee at the Federal University of Rio Grande do Sul. Attempts were made to minimize suffering and reduce the numbers of animals used.

Rats were randomly assigned to two different groups: sham [S] (n=5) and focal ischemia [FI] (n=5). Animals were anesthetized initially with 2% halothane in 30% oxygen and 70% nitrous oxide and were maintained with 1% halothane. Temperature was maintained between 36.5°C and 37.5°C throughout the surgery using a self-regulating heating blanket (Letica, Spain). Animals were placed in a stereotaxic apparatus, a midline incision was made and small burr holes were drilled at the following coordinates: (1) anterioposterior (AP) 0.0 mm, mediolateral (ML) ±2.0 mm and dorsoventral (DV) -2.0 mm; (2) AP +2.3 mm, ML ±2.0 mm, DV -2.0 mm; (3) AP +0.7 mm, ML ± 3.8 mm, DV -6.0 mm. Using a 10 μL Hamilton Syringe, ET-1 (800 pmol - human and porcine, Calbiochem) was injected into the motor cortex (2 μL) and into the dorsolateral striatum (2 μL). ET-1 was injected in left hemisphere at a rate of 1.0 μL/2 min with a 1-min pause between each μL and a 3-min delay before syringe withdrawal. In sham surgeries ET-1 was replaced with sterile saline. Six weeks after the surgery animals were anesthetized with chloride hydrate (30%, 10 mL/kg, i.p.) and injected with 1000 UI heparin (Cristália, Brazil). After that, they were transcardially

perfused with 100 mL of saline solution followed by 200 mL of fixative solution composed of 0.5% glutaraldehyde (Sigma, USA) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB) pH 7.4 at room temperature. After cryoprotection brains were sectioned using a cryostat (Leitz, Germany) at 30 μm to identify the area of cerebral infarction. The slices were mounted and stained with Cresyl Violet, as visualized in Fig 1.

Figure 1 about here

Right and left soleus muscles were carefully dissected free from surrounding tissue. The weights of dissected individual muscles were measured using a microbalance, whose precision was ±0.0001 g (Sartorius BP 210s, USA). Small samples (2×1 mm) of the belly soleus muscle were selected and postfixed in the same fixative solution by 24 h. Then, samples were washed in PB and postfixed in 1% OsO4 (Sigma, USA) in PB for 1 h, washed with PB and dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, USA), embedded in resin blocks (Durcupan, ACM-Fluka, Switzerland), maintained in vacuum for 24 h, and, afterwards, polymerized for 48 h at 60 °C. Transverse-semithin sections (1 μm) were obtained using an ultramicrotome (MT 6000-XL, RMC, Tucson, USA) and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil). Afterwards, images of the soleus muscles were captured and digitalized (initially 20x and further amplified 200% for analysis) using a Nikon Eclipse E-600microscope (Japan) coupled to a Pro-Series High Performance CCD camera and Image Pro Plus Software 6.0 (Media Cybernetics, USA).

The cross-sectional area of 100 muscle fibers randomly chosen from six slices were estimated with a point-counting technique [18], using grids with point density of one point per 46.81 μm^2 and the equation: $\hat{A}=\Sigma p.a/p$. Where $\hat{A}$ is area, Σp is the total of counted areas/point and a/p is the area/point value (46.81 μm^2).

Body weight was analyzed by the Paired Student's *t*-test. Muscle weight and cross sectional area were submitted to one and two-way ANOVA, considering the factors: lesion and limb. Significance was set at $p < 0.05$ for all analyses and results are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS® 16.0 (Statistical Package for the Social Sciences, Inc., Chicago, USA).

Results

There were no differences in body and muscle weights between sham and focal ischemia groups, as depicted in the Fig 2.

Figure 2A and B about here

Morphological Analysis

Soleus muscle fibers were regular and polygonal in shape, with minimal variations in size, in sham group as well as in affected of focal ischemia group. the In the unaffected limb of focal ischemia the fibers were found more variable in size, sometimes with nuclei was found in a central position and splint fibers was observed as presented in Fig 3.

Figure 3 about here

Pearson correlation coefficient evidenced no correspondence between muscle weight and mean fiber area, so we decided to use the absolute mean area for the following analysis.

The soleus muscle fiber cross-sectional area is shown in Fig 4. Two way ANOVA indicated no effects of lesion ($F_{1,16} = 2.31$, $p=0.147$) and limb ($F_{1,16} = 3.77$, $p=0.07$) but an interaction between lesion and limb ($F_{1,16} = 8.76$, $p=0.009$) on mean muscle fiber area.

With regard to cross sectional mean area it was compared the right limb (referred as affected limb for ischemic animals) and left limb (referred as unaffected for ischemic animals). One way ANOVA indicated effect of group ($F_{3,19}=4.95$, $p=0.01$) and Bonferroni post hoc evidenced significant differences between affected and unaffected limbs in [FI] group ($p=0.01$) and between unaffected limbs from [S] and [FI] groups ($p=0.03$).

Figure 4 about here

Discussion

The present study examined the long term effects of focal ischemia induced by ET-1 on body weight, muscle weight and soleus muscle morphology. Animal studies provide evidence of muscle weight reduction on both affected and unaffected sides during the immediate period (7 days) following stroke [2]. In our study we did not identify differences in body or muscle weight between groups along time. Similarly, Ansved et al., 1996 [3] showed no difference on body weight of ischemic and control

animals and on muscle weight between paretic and non-paretic side of ischemic animals 14 weeks after surgery.

The most common surgery used to induce focal ischemia is the occlusion of the middle cerebral artery by insertion of an intraluminal suture. This surgery is invasive and often results in feeding difficulties [29]. Maybe our results about weight were due the fact of evaluation has been conducted on long term. In addition we used an ET-1 model, which induces long-lasting impairments and is less invasive compared to methods that expose the artery or introduce a suture into the lumen [24, 29].

We worked with the hypothesis that stroke would reduce the cross sectional area of affected limb. This hypothesis was not confirmed. Significant increase was found in the area of unaffected limb of [FI] group as compared to affected limb of [FI] and unaffected limb of [S] group. Experimental studies demonstrated no significant differences in morphological, morphometrical or enzyme-histochemical muscle characteristics between paretic and non-paretic or between ischemic and control rats, in spite of marked contralateral motor dysfunction, long periods after brain ischemia [3]. However, short term after stroke, Choe et al., 2004 [12] demonstrated significant decreases on type I fiber cross-sectional area and on myofibrillar protein content of affected soleus muscle of paretic side when compared to control animals, but no difference were evidenced between paretic and non-paretic side of ischemic animals. Our protocol had been designed to allow for later observations of differences between the two sides. Although we have not demonstrated muscle atrophy on affected side our results showed a muscle hypertrophy on unaffected side which could be evidence of a possible overuse of this limb. It is well known that muscle overuse, especially in sedentary animals, can induce muscle fiber injury (28). Some classical signs of the lesion/regeneration cycle are the presence of split fibers and centralized nuclei. Previous

studies have shown that split fibers are provoked in response to stress in hypertrophied fibers (11). Thus, the presence of regenerated fibers identified in the contralateral soleus muscle is probably associated with the injury caused by its overuse.

Stroke is an important cause of long-term disability in humans and the major impairment causing disability in chronic stroke patients is motor weakness, which is due several factors including muscular atrophy of type I and II fibers, specially the last [8, 17, 23, 25]. Most chronic stroke survivors have type I fibers augmented and percentage of type II fibers reduced. Both can exhibit atrophy factor, both with atrophy factor. Such changes may be related to inactivity or transsynaptic degeneration of alpha-motoneurones as a consequence of the interruption of the corticospinal tract, collateral reinnervation process or a motor unit type transformation [13, 26]. In our study muscle modifications were slight, maybe because we used young rats. Stroke patients are generally middle-aged or old and neuromuscular plasticity is inversely correlated to age.

A limitation of our study is that we used a technique that just allows visualizing total atrophy and not allows distinguishing type I and II fibers. This was a reason because we decided to select the soleus muscle for this analysis. This muscle is extensively used for histological studies, has a greater distribution of type I fibers, is a postural muscle that displays marked changes after a period of disuse and is widely recruited during functional tasks, as the walk [20].

In our study muscle atrophy of affected limb of ischemic animals could not be displayed maybe because type II muscle fibers are predominantly atrophic after stroke and soleus muscle has mainly type I. One the other hand, affected limb disuse of ischemic animals could not be sufficient to induce the reduction of soleus area although overuse has been seeing in unaffected limb.

Weakness accompanying stroke appears to be generalized and causes impairments on the ipsilateral side as well as on the contralateral side to the stroke [5, 17]. Historically, strengthening has been excluded from neurorehabilitation programs because of the concern that high-exertion activity would increase spasticity. However, progressive resistance exercise has been used successfully to restore function either in upper and lower extremity or paretic and nonparetic limbs in long-term stroke survivors [1, 4, 7, 10, 21, 22].

To produce the best outcome after clinical stroke, rehabilitation strategy requires careful goal setting and skillful combination of different treatments. Experimental information about stroke and skeletal muscle can help to select rehabilitation strategies, particularly for chronic stroke survivors. We conclude that focal ischemia induced by ET-1 does not have long term effects on body or muscle weight and induces overuse of non-paretic limb as evidenced by hypertrophy of soleus muscle contralateral to injured hemisphere. Additional studies are necessary to determine if this model is useful to investigate the effects of strength training and functional recovery in rodents.

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Figures and Legends

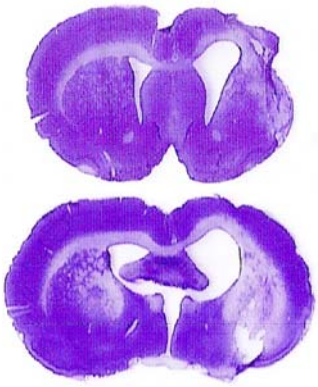

Figure 1. Representative photomicrograph of Nissl stained sections showing a typical lesion at 6 weeks post stroke. Note the tissue shrinkage tissue and enlarged ventricles.

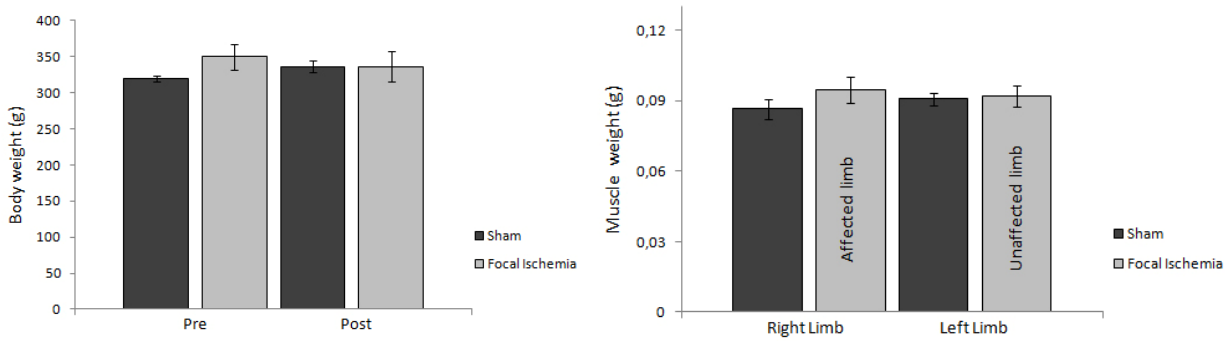

Figure 2: A: Body weight (g); B: Muscle weight (g). Data are reported as means ± SEM.

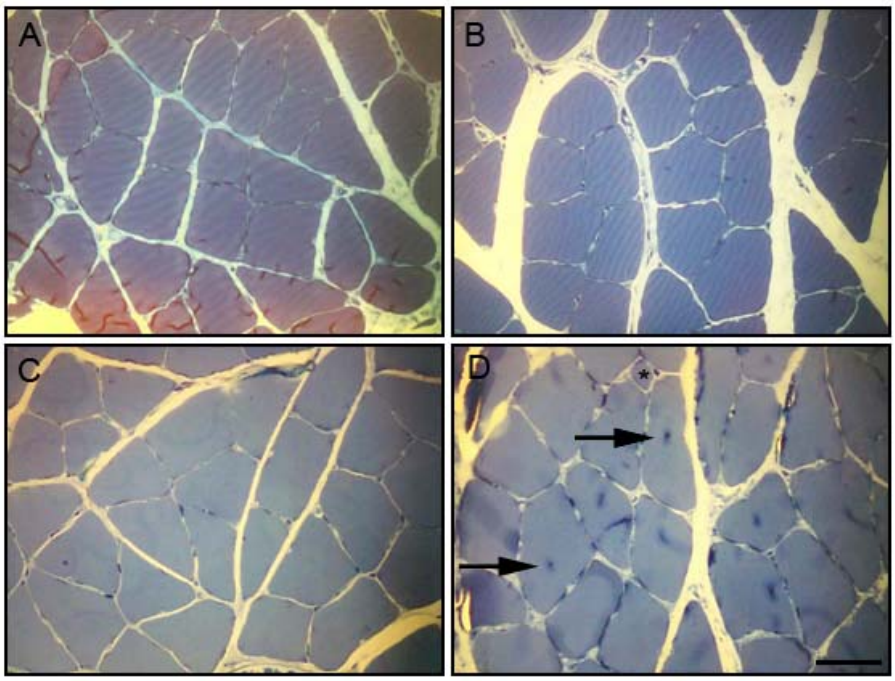

Figure 3: Digitized images of transverse-semithin sections (1 μm) obtained from soleus muscles. A and B: right and left soleus muscle of sham group showing a normal aspect. C: affected limb of focal ischemia group presenting normal aspect and D: unaffected limb of focal ischemia group showing normal fibers variable in size, splits (asterisk) and fibers with centralized nuclei (arrows). Scale bar corresponds to 50 μm.

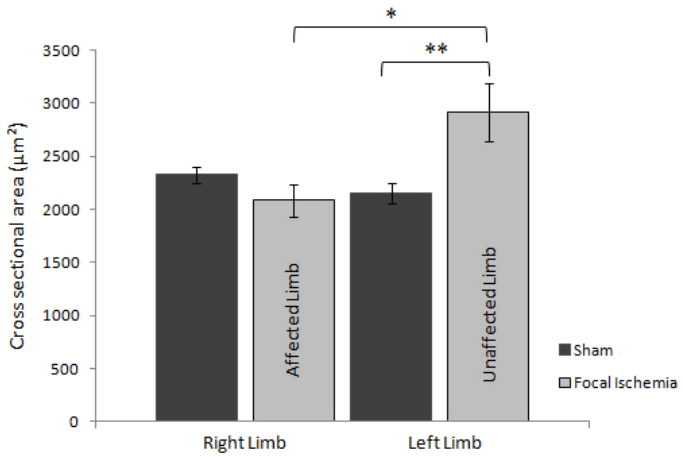

Figure. 4: Cross sectional area of soleus mean fiber area of right and left hind limbs of sham (n=5) and focal ischemia (n=5) groups. Data are expressed as means ± SEM. (*p=0.03, ** p=0.01).

7. DISCUSSÃO

Esta tese teve como objetivo principal estudar os efeitos do tratamento por meio de tarefas de habilidade e de repetição sobre parâmetros comportamentais (aspectos motores e funcionais) e aspectos teciduais de ratos *Wistar* submetidos à lesão do SNP e SNC. O enfoque comportamental é voltado para a habilidade de alcance e preensão do membro anterior. Sabe-se que a ativação neuronal e a atividade muscular coordenada são fundamentais para a funcionalidade da extremidade superior, e que esta se encontra prejudicada mediante lesões centrais e periféricas (FARR & WHISHAW, 2002; DUFF, 2005).

Uma série de testes comportamentais já foi proposta a fim de avaliar a condição sensório-motora em animais (veja SCHALLERT, 2006 para revisão). O teste do cilindro é vastamente utilizado para mensurar o uso assimétrico do membro anterior. Para tal, o animal é posicionado dentro de um cilindro de acrílico transparente apoiado sobre uma mesa de vidro. A filmagem dos movimentos exploratórios do animal é feita por uma vista inferior obtida por meio de um espelho posicionado sob a mesa. A análise das imagens em câmera lenta permite ao examinador quantificar o uso de cada segmento durante os movimentos de elevação corporal e apoio dos membros anteriores (SCHALLERT et al., 2000).

Enquanto o cilindro permite avaliação da motricidade ampla, o teste do *Staircase* foi desenvolvido para avaliar a motricidade fina dos membros anteriores. Este teste permite a avaliação precisa da habilidade motora durante os movimentos de alcance e preensão unilateral (MONTOYA et al., 1991). Existe uma grande variabilidade acerca do desempenho de diferentes linhagens de roedores no teste do *Staircase* (NIKKHAH et al., 1998). Dessa forma, o objetivo 1 desta tese foi caracterizar a fase de aquisição desta tarefa, estabelecendo o tempo e critérios ideais de treinamento.

Conforme descrito no capítulo 1, semelhante a outras linhagens, o critério de apanhar e consumir no mínimo 12 das 21 esferas fornecidas pode ser adotado para ratos *Wistar* (WHISHAW et al., 1997; BIERNASKIE et al., 2004; GALTREY & FAWCETT, 2007). Embora a execução ideal possa ser obtida com ambos os membros anteriores, é aconselhável a escolha do membro dominante para a quantificação do desempenho motor. Estender por mais de duas semanas o tempo de treinamento nesta tarefa pode ser útil para garantir a estabilidade de desempenho, bem como, para maximizar o aproveitamento de animais, uma vez que o tempo para aquisição da tarefa pode ser variável.

O objetivo primordial do teste do *Staircase* é a quantificação das esferas comestíveis apanhadas e consumidas. Todavia, por meio de adaptações na execução do teste, pode-se ter melhor avaliação da qualidade do movimento. Isso pode ser conseguido por meio do uso de esferas de diferentes cores a cada degrau, bem como por meio da análise de imagem durante a realização do teste (KLOTH et al., 2006; CLARKE et al., 2007). Os dados obtidos por meio do estudo referido no capítulo 1 foram de grande valia para a execução dos estudos seguintes.

Conforme descrito nos capítulos 2 e 3 desta tese, o segundo e o terceiro trabalhos buscaram verificar os efeitos do tratamento por meio de tarefas de habilidade e de repetição na recuperação motora/funcional, além de investigar as adaptações teciduais relacionadas à reabilitação.

Os resultados do capítulo 2 mostram que, após lesão do SNP, a atividade referida como “de repetição” resultou em aceleração da regeneração, conforme evidenciado pelo aumento do diâmetro axonal e espessura da bainha de mielina. Ambas as tarefas (de habilidade e de repetição) ocasionaram aceleração da recuperação motora,

avaliada por meio do teste do *Staircase* e do teste do cilindro. A reinervação e recuperação da função eram esperadas, tendo em vista que o modelo utilizado induz ruptura axonal, mas com preservação do tecido conjuntivo (axonotmese) (SEDDON, 1943; BURNETT & ZAGER, 2004; RODRIGUEZ et al., 2004).

Sabe-se que o processo de regeneração em nervos periféricos é relacionado à experiência sensório-motora pós-lesão (DUFF, 2005), e que atividades motoras com menor sobrecarga muscular induzem melhor recuperação quando comparadas ao treinamento com carga excessiva (GUTMANN & JAKOUBEK, 1963; VAN MEETEREN et al., 1998; VAN DER KOOI et al., 2005; ILHA et al., 2008). Embora a sobrecarga muscular deva ser evitada, há necessidade de um grau mínimo de despolarização e contração muscular a fim de favorecer o processo de regeneração (CUP et al., 2007). Talvez este limite mínimo tenha sido atingido por meio da terapia da tarefa de repetição, ao contrário do que ocorreu com tarefa de habilidade.

Por outro lado, os resultados referentes ao capítulo 3 demonstram que atividades que envolvem habilidade motora induzem maior plasticidade sináptica no córtex sensório-motor e hipocampo ipsilaterais, conforme evidenciado pela maior expressão de sinapsina e PSD-95 nessas estruturas. Essas proteínas foram escolhidas a fim de avaliar os componentes pré e pós sináptico, respectivamente. A Sinapsina-I é uma proteína envolvida na plasticidade sináptica, específica de neurônios pré-sinápticos (FERREIRA & RAPOPORT, 2002). Sua expressão pode ser induzida pela atividade motora em condições de normalidade e após lesão encefálica (GÓMEZ-PINILLA et al., 2002; VAYNMAN et al., 2004; KLINTSOVA et al., 2004; PLOUGHMAN et al., 2009), possivelmente por meio da ação do BDNF (JOVANOVIC et al., 2000). Por outro lado, a PSD-95 pode ser utilizada como um marcador de neurônios pós-sinápticos, uma

vez que ancora receptores excitatórios na membrana neuronal (KORNAU et al., 1995; KEITH & HUSSEINI, 2008). A densidade pós-sináptica também é uma estrutura passível de adaptações por meio da atividade. Estudos anteriores relatam que a densidade de PSD-95 pode estar diminuída após lesão hipóxico-isquêmica (HU et al., 1998) e aumentada mediante atividade motora (DIETRICH et al., 2005; XU & RUAN, 2009).

Além dos componentes pré e pós-sináptico, os quais são elementos evidentes do complexo, tem-se dado atenção especial ao papel de um terceiro componente sináptico, a astroglia circundante, que atuaria regulando o microambiente extracelular (FELLIN et al., 2006). A GFAP é o marcador mais amplamente utilizado para identificar a reatividade astrocitária. Se, por um lado sua expressão normalmente está aumentada após lesão do SNC (RIDET et al., 1997), a reatividade astroglial também tem sido reportada como participante da plasticidade neuronal em animais saudáveis (SIREVAAG et al., 1991) e após lesão (BRIONES et al., 2006). Em nossos resultados observamos que os níveis de GFAP encontravam-se aumentados no córtex sensório-motor após a isquemia, algo que, de acordo com a literatura, era esperado. Todavia, a intensificação da expressão de GFAP ocorreu após o treinamento de habilidade somente no hipocampo, o que se justifica, tendo em vista o aspecto cognitivo associado a este tipo de tarefa motora.

Embora a expressão de diferentes proteínas "sinápticas" tenha apontado para o favorecimento da plasticidade neuronal, seja por sinaptogênese ou por aumento da eficácia sináptica, nenhum grau de recuperação motora foi observado nos animais tratados. Isso pode ter ocorrido, em parte, devido à adoção de compensações de movimento durante a tarefa de habilidade. Após o AVE, humanos e roedores adotam

posturas compensatórias no movimento de alcance, o que pode ocasionar limitação na recuperação funcional (MICHAELSEN et al., 2001; FARR & WHISHAW, 2002; LEVIN et al., 2009; METZ et al., 2005).

Outra possibilidade, que justificaria a ausência de recuperação motora, poderia ser o dano excessivo produzido pelo modelo utilizado em nosso estudo. A associação de duas injeções corticais a uma injeção estriatal de 800 pmol de ET-1 induz prejuízo motor em longo prazo e possui grande semelhança ao AVE que ocorre em humanos, mas induz grandes volumes de infarto encefálico (WINDLE et al., 2006).

No capítulo 3, avaliamos os efeitos, em longo prazo, da isquemia encefálica sobre aspectos morfológicos do músculo sóleo ipsilateral e contralateral ao hemisfério lesado. O objetivo dessa avaliação surgiu pelo fato da fraqueza e atrofia musculares serem fatores limitantes da recuperação de pacientes após o AVE (BOHANNON, 1995; BOHANNON et al., 1995). Dessa forma, um modelo de induzisse prejuízo motor em longo prazo, associado a modificações na estrutura muscular poderia ser útil para o estudo do fortalecimento como forma de reabilitação após a isquemia.

Trabalhávamos com a hipótese de que a isquemia cerebral induziria atrofia muscular no lado contralateral ao hemisfério lesado. Essa proposição não foi confirmada. Entretanto, observou-se aumento da área média transversa das fibras musculares no lado ipsilateral à lesão. Isso sugere que, embora o desuso não tenha sido suficiente para ocasionar atrofia muscular no lado contralateral, houve sobrecarga no membro posterior ipsilateral ao hemisfério lesado, como forma de compensar o déficit sensório-motor do membro parético.

A atrofia muscular pode ser observada em pacientes após longo prazo de disfunção ocasionada pelo AVE. A atrofia pode ser observada nas fibras do tipo I e II,

predominantemente no último tipo (SLAGER et al., 1985; BOHANNON et al., 1987; HACHISUKA et al., 1997; PATTEN & LEXELL, 2004). Isso pode estar relacionado à degeneração dos motoneurônios alfa secundária à lesão da via piramidal ou a modificações específicas na junção neuromuscular (DATTOLA et al., 1993; TOFFOLA et al., 2001). Uma limitação de nosso estudo é que a técnica utilizada não permite a diferenciação entre fibras do tipo I e II. Este fator poderia mascarar a atrofia ou a redução no número de um determinado tipo de fibra em detrimento a outro.

Dessa forma, observamos que as lesões utilizadas como modelos experimentais nesta tese (lesão do plexo braquial por esmagamento e a isquemia focal induzida por ET-1) causam prejuízo motor e funcional, os quais podem ser avaliados de forma fidedigna utilizando-se o teste do *Staircase* em ratos *Wistar*. Além disso, a isquemia focal por ET-1 induziu modificações musculares em longo prazo, conforme discutido no capítulo 4. Na lesão do SNP (capítulo 2) a recuperação motora foi acelerada pelo tratamento e a avaliação morfológica evidenciou efeitos positivos do tratamento por meio da tarefa de repetição. Por outro lado, na lesão do SNC (capítulo 3) não houve recuperação espontânea ou induzida pelo tratamento de reabilitação. Porém, observamos que os parâmetros teciduais avaliados evidenciaram efeitos positivos do tratamento por meio de tarefa de habilidade. Esses resultados ampliam os conhecimentos acerca da lesão no SNP e no SNC, sobre formas de avaliação comportamental (motora e funcional) e sobre efeitos das tarefas utilizadas para reabilitação.

8. CONCLUSÕES

Os dados obtidos por meio dos trabalhos desenvolvidos nesta tese permitem concluir que:

- Ratos *Wistar* apresentam bom desempenho no teste do *Staircase*, podendo esta ferramenta ser utilizada com fidedignidade para avaliação independente da habilidade de alcance e preensão, desde que observados os critérios adequados de execução;

- As atividade motoras propostas (tarefas de habilidade e de repetição) induzem efeitos distintos de regeneração no SNP e SNC, sendo que a recuperação funcional nem sempre está correlacionada às adaptações teciduais decorrentes do treinamento motor:

  - Em nervos periféricos, a regeneração é favorecida pela atividade muscular, resultante da tomada de peso no membro afetado, durante a tarefa de repetição;

  - No SNC a plasticidade encefálica é favorecida pela associação entre aspectos motores, cognitivos e perceptivos durante o treinamento motor de habilidade do membro anterior;

- O modelo de isquemia focal induzida por ET-1 utilizado neste trabalho induz déficit sensório-motor prolongado (6 semanas), o qual é evidente no membro anterior. No membro posterior, o desuso não é suficiente para induzir atrofia do músculo sóleo contralateral ao hemisfério lesado. Todavia, ocorre aumento na área total de fibras musculares no membro posterior ipsilateral ao hemisfério lesado, 6 semanas após induzida a isquemia;

9. PERSPECTIVAS

- Investigar possíveis diferenças obtidas na recuperação da função, por meio do tratamento de reabilitação em diferentes tarefas de habilidade (com e sem restrição das compensações de movimento);

- Pesquisar o papel de outras estruturas e vias relacionadas à atividade motora, como cerebelo e medula espinal, após isquemia encefálica e treinamento de reabilitação do membro anterior;

- Verificar os efeitos da lesão no SNP e das tarefas utilizadas para reabilitação em neurônios da medula espinal;

- Analisar a morfologia de músculos localizados no membro anterior relacionados ao movimento de alcance e preensão, utilizando técnicas que permitam a diferenciação entre fibras do tipo I e II (identificação do grau de atrofia, bem como, da redução no número de ambos os tipos de fibras).

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