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TREINAMENTO EM ESTEIRA EM RATOS DIABÉTICOS: EFEITOS SOBRE O COMPORTAMENTO MOTOR E SENSORIAL, IMUNOMARCAÇÃO DE TIROSINA HIDROXILASE NA SUBSTÂNCIA NIGRA, PEPTÍDEO RELACIONADO AO GENE DA CALCITONINA NO CORNO DORSAL DA MEDULA ESPINAL E MORFOLOGIA DO NERVO SURAL

TESE DE DOUTORADO

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Treinamento em esteira em ratos diabéticos: efeitos sobre o comportamento motor e sensorial, imunomarcação de tirosina hidroxilase na substância nigra, peptídeo relacionado ao gene da calcitonina no corno dorsal da medula espinal e morfologia do nervo sural

por

Patrícia Severo do Nascimento

Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Neurociências, da Universidade Federal do Rio Grande do Sul (UFRGS), como requisito parcial para obtenção do grau de **Doutor em Neurociências.**

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LISTA DE ABREVIATURAS

5HT: 5-hidroxitriptamina, serotonina

5HT1A: receptor da serotonina tipo 1A

5HT2: receptor da serotonina tipo 2

5HT2A: receptor da serotonina tipo 2A

5HTT: transportador da serotonina

6-OHDA: 6-hidroxidopamina

ADN: ácido desoxirribonucleico

AGE's: do inglês *Advanced glycation end-products*; produtos finais da glicação

AVE's: acidentes vasculares encefálicos

CDME: corno dorsal da medula espinal

CGRP: do inglês *Calcitonin Gene-Related Peptide*; Peptídeo Relacionado ao Gene da Calcitonina

COMT: catecol O-metil-transferase

DAT: do inglês dopamine active transporter; transportador de dopamina sódio-dependente

DMT1: Diabetes Mellitus do Tipo 1

DOPA: diidroxifenilalanina

GDP: Guanosina difosfato

GFAP: do inglês Glial Fibrilar Acid Protein; proteína glial ácida fibrilar

GLUT4: do inglês Glucose Transporter type 4; transportador de glicose do tipo 4

GRD: gânglio da raiz dorsal

GS: Glicogênio Sintase

GSK3: Glicogênio Sintase Cinase 3

GTP: Guanosina trifosfato

IFN- γ: Interferon-γ

IL-1: Interleucina-1

IRS-1: do inglês insulin receptor substrate-1; substrato 1 do receptor da insulina

MAO: monoamina oxidase

mg/dL: miligramas por decilitro

MHC: do inglês Major Histocompatibility Complex; complexo principal de histocompatibilidade

MPP+: iodeto de 1-metil-4-fenilpiridínio

MPTP: 1-metil-4-fenil-1,2,3,6-tetrahidropiridina

NGF: do inglês Nerve Growth Factor; fator de crescimento neural

NT3: do inglês neurotrophin-3; Neurotrofina 3

OMS: Organização Mundial da Saúde

ONU: Organização das Nações Unidas

PI-3K: Fosfoinositídeo 3-cinase

PKB: Proteína Cinase B

PIP2: Fosfatidilinositol 4,5-bifosfato

PIP3: Fosfatidilinositol 3,4,5-trifosfato

S100B: proteína ligante de cálcio B

SNC: Sistema Nervoso Central

STZ: do inglês streptozotocina; estreptozotocina

SUS: Sistema Único de Saúde

TH: tirosina hidroxilase

TNF: do inglês Tumoral Necrosis Factor; fator de necrose tumoral

VEGF: do inglês vascular endothelial growth factor; fator de crescimento vascular endotelial

VMAT: do inglês vesicular monoamine transporter; transportador vesicular de monoaminas

VTA: do inglês ventral tegmental área; área tegmental ventral

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RESUMO

Tese de doutorado
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TREINAMENTO EM ESTEIRA EM RATOS DIABÉTICOS: EFEITOS SOBRE O COMPORTAMENTO MOTOR E SENSORIAL, IMUNOMARCAÇÃO DE TIROSINA HIDROXILASE NA SUBSTÂNCIA NIGRA, PEPTÍDEO RELACIONADO AO GENE DA CALCITONINA NO CORNO DORSAL DA MEDULA ESPINAL E MORFOLOGIA DO NERVO SURAL

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Porto Alegre, Abril de 2012.

O objetivo desta tese foi analisar os efeitos do exercício físico sobre o comportamento motor, a expressão de tirosina hidroxilase na subtsância nigra, a sensibilidade mecânica e nociceptiva, a morfologia do nervo sural e a expressão de peptídeo relacionado ao gene da calcitonina no corno dorsal da medula espinal de ratos adultos machos e diabéticos induzidos por estreptozotocina.

Para isso foram realizados três experimentos. No primeiro, foi realizado protocolo de oito semanas de treinamento em esteira em animais não diabéticos e diabéticos. Foi analisado o comportamento motor, pelo uso do teste do rotarod e campo aberto e a expressão de TH na substância nigra e área tegmental ventral desses animais. Os animais diabéticos sedentários apresentaram déficits motores e diminuição na reatividade à tirosina hidroxilase (TH) na substância nigra e área tegmental ventral. As alterações motoras e reatividade à TH na substância nigra foram prevenidas nos animais diabéticos treinados.

No segundo experimento os animais diabéticos sedentários apresentaram diminuição no limiar sensorial mecânico, avaliado pelos filamentos de von Frey, bem como diminuição da área e diâmetro das fibras mielinizadas, aumento da densidade de fibras e área ocupada pelo tecido conjuntivo, diminuição da espessura da bainha de mielina e aumento da relação g no nervo sural. Essas alterações não foram vistas nos animais diabéticos treinados. Ainda, o nervo sural dos animais diabéticos apresentou maior porcentagem de fibras mielinizadas pequenas e menor porcentagem de fibras mielinizadas de pequeno diâmetro quando comparado aos animais controles e diabéticos treinados.

No terceiro experimento demonstramos que os animais diabéticos induzidos por estreptozotocina apresentaram diminuição da sensibilidade nociceptiva, avaliada pelo teste do tail flick, assim como diminuição da expressão do peptídeo relacionado ao gene da calcitonina no corno dorsal da medula espinal. Por outro lado, os animais diabéticos treinados não mostraram alterações na sensibilidade mecânica e na expressão do mesmo peptídeo.

Estes dados demonstram que o exercício físico em esteira em ratos diabéticos é capaz de prevenir alterações no comportamento motor e sensorial, assim como as alterações morfológicas no nervo sural, na expressão de tirosina hidroxilase na substância nigra e do peptídeo relacionado ao gene da calcitonina no corno dorsal da medula espinal.

ABSTRACT

Doctoral Thesis
Programa de Pós-Graduação em Neurociências
Universidade Federal do Rio Grande do Sul

TREADMILL TRAINING IN DIABETIC RATS: EFFECTS ON MOTOR AND SENSITIVE BEHAVIOR, TYROSINE HYDROXYLASE IMMUNOREACTIVITY IN THE SUBSTANTIA NIGRA, CALCITONIN GENE-RELATED PEPTIDE IN THE DORSAL HORN OF THE SPINAL CORD AND SURAL NERVE MORPHOLOGY

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Porto Alegre, April 2012.

The aim of this thesis was to study the effects of treadmill training on the motor and sensitive behavior, the tyrosine hydroxylase immunoreactivity in the substantia nigra, the mechanical and nociceptive sensitivity, the sural nerve morphology and the calcitonin gene-related peptide immunoreactivity in the dorsal horn of the spinal cord from diabetic rats induced by streptozotocin.

For this, we made three experiments. In the first, an eight weeks treadmill training protocol was made in diabetic and non diabetic animals. Was analyzed the motor behavior using rotarod and open field tests and the immunoreactivity to tyrosine hydroxylase (TH) in the substantia nigra and ventral tegmental area of these animals. Sedentary diabetic animals showed motor deficits and a decrease in the TH immunoreactivity in the substantia nigra and in the ventral tegmental area. Treadmill training in diabetic animals prevented the motor deficits and the decrease in the tyrosine hydroxylase reactivity.

In the second experiment, sedentary diabetic animals showed a reduction in the mechanical sensitivity threshold, analyzed using von Frey filaments. Also, diabetic animals showed a decreased in the area and in diameter of the myelinated fibers, an increased in the fibers density and in the area occupied by connective tissue, a decreased myelin sheat thickness and an increased g ratio in the sural nerve. These alterations were not seen in trained diabetic animals. In addition, the sural nerve of the sedentary diabetic animals had a higher percentage of small myelinated fibers and a lower percentage of large diameter myelinated fibres than the control and trained diabetic animals.

Moreover, in the third experiment we showed that the diabetic animals induced by streptozotocin had a decrease in the nociceptive sensitivity, analyzed using the tail flick test. These animals showed a decrease in the calcitonin gene-related peptide immunoreactivity in the dorsal horn of the spinal cord. Furthermore, these decreased in the nociceptive sensitivity and in the peptide immunoreactivity were not seen in the trained diabetic rats

These data showed that treadmill training in diabetic rats is able to prevent alterations in the motor and sensitive behavior. Also, exercise is able to prevent the morphological alterations in the sural nerve, the decrease in the TH immunoreactivity in the substantia nigra and the decrease in the calcitonin gene-related peptide immunoreactivity in the dorsal horn of the spinal cord.

1 Introdução

1.1 Diabetes Mellitus do tipo 1

Segundo a Organização Mundial da Saúde (OMS, 2011), atualmente, 346 milhões de pessoas tem diabetes mellitus, e se estima que no ano de 2004 cerca de 3,4 milhões de pessoas tenham morrido devido às consequências desta patologia. Ainda, a OMS estima que o número de mortes seja duplicado entre os anos de 2005 e 2030.

O diabetes mellitus do tipo 1 (DMT1), descrito em 1922 por Frederick Banting e Charles Best, que ganharam o prêmio Nobel em Fisiologia e Medicina no ano de 1923 pela descoberta, é uma síndrome metabólica caracterizada pelo estado hiperglicêmico decorrente da deficiência absoluta do hormônio insulina por supressão das células β das ilhotas de Langerhans do pâncreas. Inicia-se antes dos 20 anos, surge em geral na infância e se torna mais grave na puberdade, correspondendo a 10% dos diabéticos. É sempre sintomático, manifestado por poliúria, polidipsia, emagrecimento, polifagia e cetoacidose (GODOY, 2006).

Três mecanismos participam da patogênese do DMT1: a predisposição genética, fatores ambientais e a autoimunidade. Os fatores genéticos são indicados pelo caráter familial e a frequência elevada da doença em determinadas regiões (nordeste europeu), sendo mais rara em negros e asiáticos (GODOY, 2006). Ainda, o DMT1 apresenta padrão complexo de associações genéticas, e os prováveis genes de suscetibilidade à doença foram mapeados em pelo menos 20 loci. Muitas dessas associações ocorrem com regiões cromossômicas, e os genes envolvidos ainda não são conhecidos. Dos diversos loci associados à doença, o mais importante é o locus da classe II do MHC (complexo principal de histocompatibilidade; HLA); de acordo com algumas estimativas, o MHC contribui para cerca de metade da suscetibilidade genética, e todos os outros genes combinados são responsáveis pela outra metade (MAITRA e ABBAS, 2005).

Existem evidências de que os fatores ambientais, especialmente as infecções, estejam envolvidos no desencadeamento da autoimunidade no DMT1. Estudos epidemiológicos sugerem a participação de alguns vírus. Dentre as infecções associadas ao DM se pode citar a caxumba, rubéola (RAMONDETTI et al., 2011) e infecção por enterovírus (COPPIETERS et al., 2012). Duas hipóteses tentam explicar como esses vírus causam lesões nas células β as levando à morte. A primeira é a de que as infecções induzem dano tecidual e inflamação, causando a liberação de antígenos das células β e o recrutamento e ativação de linfócitos e outros leucócitos inflamatórios para o tecido. A outra possibilidade que os vírus produzem proteínas que imitam auto-antígenos e que a resposta imunológica a essas proteínas apresenta reação cruzada com o tecido do hospedeiro. Entretanto, ainda não foi estabelecido se uma dessas hipóteses está realmente envolvida na patogênese do DMT1 (MAITRA e ABBAS, 2005).

O DMT1 é uma doença auto-imune na qual a destruição das ilhotas de Langerhans é causada principalmente pelos linfócitos T reagindo contra antígenos na célula β, os quais ainda não foram devidamente identificados. Apesar do início da doença ocorrer de forma abrupta, ele resulta de um ataque auto-imune crônico às células β que geralmente tem início vários anos antes da doença se tornar evidente. Quando as manifestações clássicas da doença aparecem (hiperglicemia e cetoacidose), em fase tardia, mais de 90% das células β foram destruídas (MAITRA e ABBAS, 2005).

Alguns mecanismos atuam em conjunto para a destruição das células β. Os linfócitos T reagem contra antígenos das células β: as células TCD4+ do subtipo TH1 causam dano através da ativação de macrófagos, e linfócitos T citotóxicos CD8+, matam as células β diretamente e secretando citocinas que ativam macrófagos. Dentre as citocinas envolvidas estão o interferon-γ (IFN- γ), produzidos pelas células T, e o fator de necrose tumoral (TNF) e a interleucina-1 (IL-1), produzidos pelos macrófagos ativados durante a reação imunológica.

Essas citocinas são capazes de ativar a apoptose nas células pancreáticas (MAITRA e ABBAS, 2005; MALLONE et al., 2011). Além disso, o DMT1 pode ocorrer pela presença de autoanticorpos contra as células das ilhotas de Langerhans (MANAN et al., 2010; Brezar et al., 2011).

Os critérios para o diagnóstico do DMT1 incluem: sintomas clássicos (polidipsia, poliúria, e perda de peso inexplicável) e glicose plasmática casual maior ou igual a 200 mg/dL (é considerado casual qualquer hora do dia sem considerar o tempo desde a última refeição); ou glicose plasmática em jejum maior ou igual a 126 mg/dL (é considerado jejum como ausência de ingesta calórica por pelo menos 8 horas); ou durante o teste de tolerância a glicose, os níveis de glicose plasmática devem estar maior ou igual a 200 mg/dL após 2 horas da ingesta de 75 gramas de glicose dissolvida em água (American Diabetes Assocation, 2007).

A sinalização da insulina nas células tem como objetivos a expressão gênica e o transporte de glicose para o interior das células dos tecidos muscular e adiposo. Para que isso ocorra, duas moléculas de insulina devem se ligar ao receptor tirosina-cinase, o qual possui um domínio ligando-dependente na superfície extracelular da membrana plasmática e uma enzima ativa no lado citosólico, com os dois domínios conectados por um simples segmento transmembrana. O domínio extracelular do receptor possui duas subunidades α que são os dois sítios de ligação para a insulina. Entretanto, seu domínio citosólico apresenta duas subunidades β que contém atividade tirosina-cinase, as quais, quando duas moléculas de insulina se ligam às subunidades α, tem a capacidade de se autofosforilarem. Essa autofosforilação torna a enzima capaz de fosforilar resíduos tirosina de outras proteínas-alvo. Uma dessas proteínas-alvo é o substrato 1 do receptor da insulina (IRS-1; insulin receptor substrate-1), o qual, uma vez fosforilado se torna ponto de nucleação para um complexo de proteínas que carregam a informação do receptor da insulina até os alvos-finais no citoplasma

e no núcleo, através de uma série de proteínas intermediárias. O resíduo P-Tyr do IRS-1 é ligado pelo domínio SH2 da proteína Grb2. Por sua vez, Grb2 se liga a Sos, a qual quando é ligada pela Grb2 catalisa a substituição da ligação de um GDP por GTP na proteína Ras, uma proteína da família das proteínas-G. Quando o GTP é ligado a Ras, essa pode ativar uma proteína cinase, Raf-1, a primeira de três proteínas cinases – Raf1, MEK e ERK, as quais formam uma cascata na qual cada proteína cinase ativa a próxima por fosforilação. Quando a proteína cinase ERK é ativa pela fosforilação ela medeia alguns dos efeitos biológicos da insulina, devido ao fato desta atingir o núcleo celular fosforilando proteínas tais como a Elk1, a qual modula a transcrição de cerca de 100 genes regulados pela insulina (Figura 1; NELSON et al., 2005).

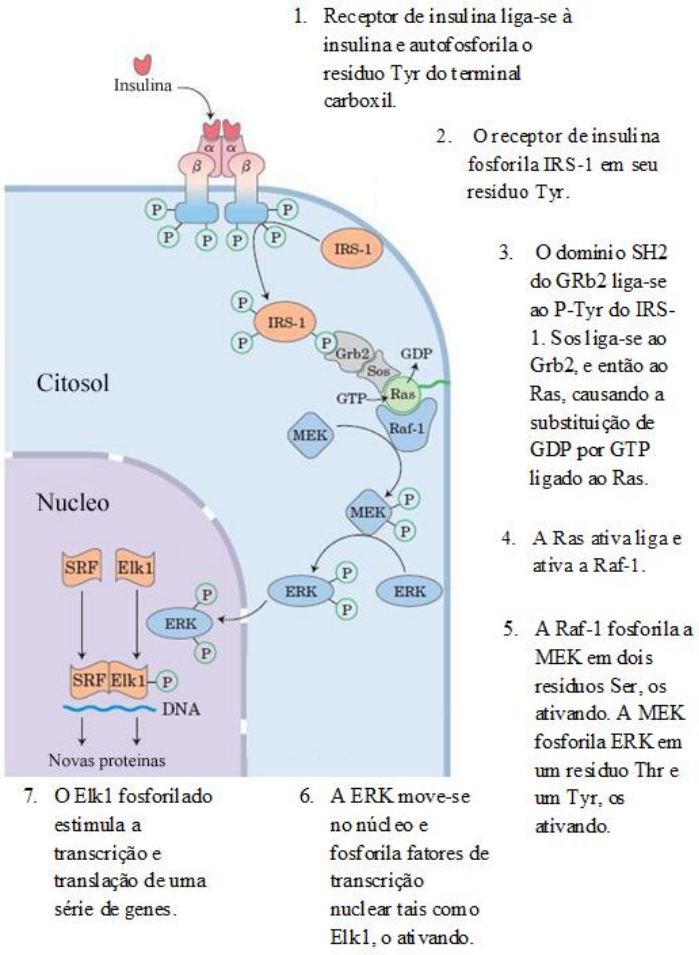

Figura 1: Desenho esquemático, representando os mecanismos de ação da insulina na expressão gênica (modificado de NELSON et al., 2005).

Além disso, IRS-1 fosforila PI-3K (fosfoinositídeo 3-cinase), o qual converte o lipídeo de membrana PIP_2 (fosfatidilinositol 4,5-bifosfato) em PIP_3 (fosfatidilinositol 3,4,5-trifosfato). Quando se liga ao PIP_3, a PKB (proteína cinase B) é fosforilada e ativa. Uma vez que PKB é ativa, ela causa a fosforilação da proteína GSK3 (glicogênio sintase cinase 3), inativando-a. A proteína GSK3 na sua forma não-fosforilada é ativa e causa a fosforilação da enzima glicogênio sintase (GS), que por sua vez, quando fosforilada é inativa. Portanto, a inativação da GSK3 pela fosforilação não pode converter a GS em sua forma inativa, permanecendo essa na forma ativa, contribuindo para a síntese de glicogênio a partir da glicose. Além disso, a PKB no músculo causa o movimento dos transportadores de glicose do tipo 4 (GLUT4; transportador de glicose do tipo 4) de estoques vesiculares internos em direção à membrana plasmática, estimulando a entrada de glicose na célula (Figura 2; NELSON et al., 2005).

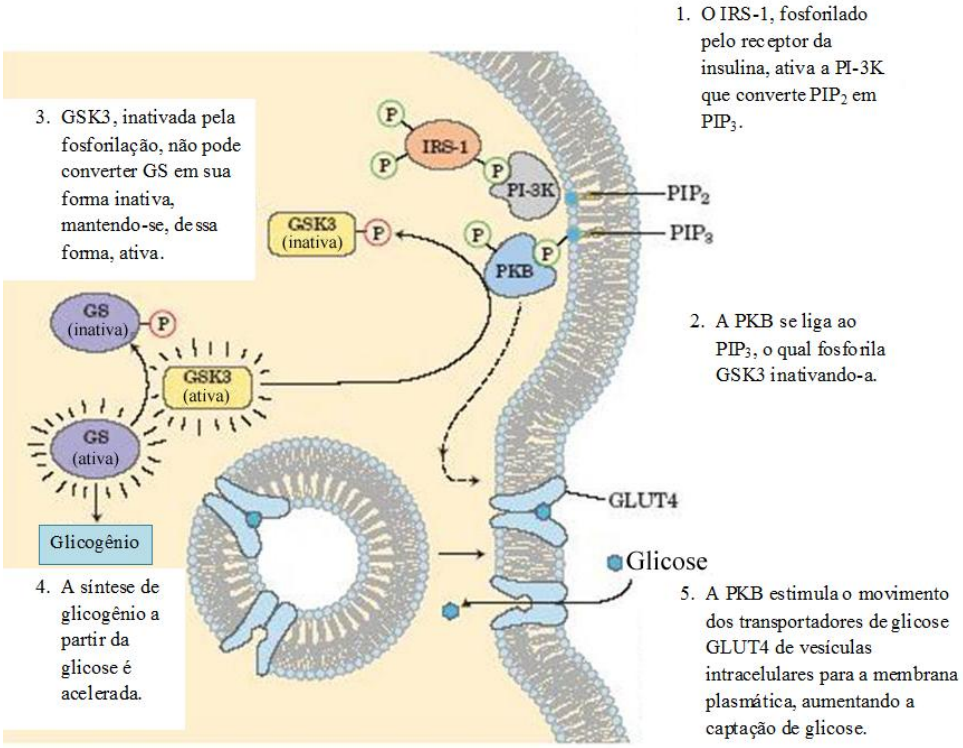

Figura 2: Desenho esquemático mostrando as ações da insulina sobre o transporte de glicose e síntese de glicogênio (modificado de Nelson et al., 2005).

Com a ausência da insulina, causada pela destruição das células β-pancreáticas, ocorre hiperglicemia crônica, sendo esta a principal causadora das complicações do diabetes, tais como retinopatia, nefropatia, neuropatia (BROWNLEE, 2001) e encefalopatia diabética (SIMA et al., 2009). Os danos celulares causados pela hiperglicemia são atribuídos a quatro principais fatores: aumento do fluxo na via do poliol, aumento da formação AGE's (produtos finais da glicação), ativação das isoformas da PKC e aumento do fluxo da via da hexosaminase (BROWNLEE, 2001).

1.2 Encefalopatia diabética

Os tipos 1 e 2 do diabetes mellitus são amplamente associados a sérias complicações secundárias, as quais afetam os rins (SNYDER e PENDERGRAPH, 2005) , a retina (REGATIERI et al., 2012), os nervos periféricos (BOULTON et al., 2004; BOULTON et al., 2005; TESFAYE et al., 2010) e a vasculatura (RUDERMAN et al., 1992; *THE DIABETES CONTROL AND COMPLICATIONS TRIAL RESEARCH GROUP*, 1993; VAS et al., 2012).

Além dessas alterações, atualmente, as complicações no sistema nervoso central (SNC) de pacientes diabéticos são reconhecidas e aceitas como disfunções relacionadas ao diabetes mellitus, e são denominadas encefalopatias diabéticas (BIESSELS et al., 2002). Essas alterações são evidentes em crianças diabéticas nas quais o DMT1 afetou a estrutura e função encefálica. Essas crianças apresentam déficits de atenção, memória, funções executivas alteradas, velocidade de processamento menor e desempenho escolar não tão satisfatório quanto ao dos seus colegas (NORTHAM et al., 2001; SCHOENLE et al., 2002).

Estudos por imageamento por ressonância magnética tem correlacionado alterações cognitivas e alterações estruturais. Estes mostram diminuições no volume de substância branca na região parahipocampal, assim como nos lobos temporais e frontais doze anos após o diagnóstico do DMT1 em pacientes jovens, e diminuição do volume da substância cinzenta do

tálamo, hipocampo e córtex insular (NORTHAM et al., 2009). Da mesma forma, foi relatada diminuição na densidade da substância cinzenta dos giros temporal, angular, médio-temporal, médio-frontal e tálamo em adultos diabéticos quando comparados aos sujeitos não-diabéticos (MUSEN et al., 2006).

Em modelos animais, outras estruturas encefálicas apresentam alterações morfológicas induzidas pelo DMT1. O córtex cerebral e o tronco encefálico apresentaram diminuições no conteúdo de serotonina (5HT) e seus receptores ($5HT_{1A}$ e $5HT_2$; SANDRINI et al., 1997); na córtex cerebral e no cerebelo houve aumento do conteúdo de proteína glial ácida fibrilar (GFAP, do inglês *Glial Fibrilar Acid Protein*) e proteína ligante de cálcio B (S100B) indicando reação astrocitária (BAYDAS et al., 2003); ocorreram diminuições na densidade de espinhos dendríticos dos neurônios piramidais do hipocampo, córtex pré-frontal e occipital (MARTÍNEZ-TELLEZ et al., 2005); ocorreu aumento da expressão gênica de acetilcolinesterase e do receptor dopaminérgico D2 assim como diminuição da expressão gênica do receptor dopaminérgico D1 no cerebelo (PEEYUSH et al., 2010).

Notavelmente, ao mesmo tempo em que o DMT1 causa alterações morfológicas, modifica também a função e o comportamento. Tem sido demonstrado em ratos diabéticos alterações na memória espacial (DE SENNA et al., 2011; PIAZZA et al., 2011), na atividade vertical e locomoção (MOREIRA et al., 2007) e na disfunção motora (ABRAHAM et al., 2010; ANU et al., 2010; PEEYUSH et al., 2010).

Interessantemente, antes mesmo do aparecimento dos sintomas, se considera o DM como um fator de risco importante para o desenvolvimento da doença de Alzheimer (DAVIGLUS et al., 2010), e se tem relacionado os sinais da doença de Parkinson com o diabetes (ARVANITAKIS et al., 2007). Tanto a doença de Alzheimer, quanto a doença de Parkinson são relacionadas com o aumento da idade (BENNETT et al., 1996; SALVATORE et al., 2009; DAVIGLUS et al., 2010), e, em ambos os casos ocorre morte neuronal e déficits

motores (WIRTHS e BAYER, 2008; JOLIVALT et al., 2010). Outra semelhança das complicações dessas patologias é o estresse oxidativo (BROWNLEE, 2001; CHAN et al., 2010; BUTTERFIELD e SULTANA, 2011), o qual contribui para a morte neuronal na córtex cerebral (MASTROCOLA et al., 2005), acelera a formação de placas senis no hipocampo (MURAKAMI et al., 2011), e causa morte dos neurônios dopaminérgicos na substância nigra *pars compacta* (CHAN et al., 2010; GARCIA-GARCIA et al., 2012).

Juntamente com as alterações morfológicas e estruturais causadas pela hiperglicemia ocorrem alterações funcionais, as quais refletem diretamente as primeiras. Em alterações hipocampais são demonstradas alterações de memória espacial, testada pela tarefa de reconhecimento do novo objeto, na qual animais induzidos por indução de estreptozotocina (STZ) apresentam preferência diminuída pelo objeto recolocado (REVSIN et al., 2009; DE SENNA et al., 2011; PIAZZA et al., 2011). No cerebelo, a expressão gênica diminuída dos genes da superóxido dismutase, da glutationa peroxidase, do receptor da serotonina tipo 2A ($5HT_{2A}$) e do transportador da serotonina (5HTT) é caracterizada por alterações motoras, nas quais os animais diabéticos induzidos por STZ apresentam redução no tempo de permanência no rotarod, indicando perda de coordenação motora (ABRAHAM et al., 2010).

Contribuindo para o aparecimento das alterações morfológicas e funcionais, ocorrem alterações vasculares decorrentes da hiperglicemia, sendo esta um determinante para as complicações microvasculares causadas pelo diabetes (STEPHENSON et al., 1994). A glicose em excesso causa danos vasculares em retinas (DURHAM & HERMAN 2011), nos glomérulos renais (FIORETTO & MAUER, 2007), nos nervos periféricos (TESFAYE et al., 2005) e também no SNC (VAN DUINKERKEN et al., 2009). A hiperglicemia é também associada à doença macrovascular aterosclerótica acelerada que afeta vasos que suprem o coração, os membros inferiores e o cérebro, e, como resultado, os pacientes com diabetes mellitus apresentam maior risco de infarto do miocárdio (BRINDISI et al., 2010), amputações

de membros inferiores (LÓPEZ-DE-ANDRÉS et al., 2011) e acidentes vasculares encefálicos (AVE's; CANTÚ-BRITO et al., 2010).

O dano mediado pela glicose em retinas, glomérulos renais, nervos assim como no SNC apresenta características patofisiológicas comuns. Em tecidos não dependentes da insulina para o transporte de glicose, a hiperglicemia causa a entrada da glicose na célula, ocorre então abnormalidades no fluxo sanguíneo e aumenta a permeabilidade vascular. Ao mesmo tempo, a diminuição da atividade de vasodilatadores, o aumento da atividade de vasoconstritores, a elaboração de fatores de permeabilidade vascular, como o fator de crescimento vascular endotelial (VEGF, do inglês *vascular endothelial growth factor*), e danos na matriz extracelular contribuem para um irreversível aumento na permeabilidade vascular. Posteriormente ocorre a perda de células da microvasculatura por necrose ou apoptose, e há progressiva oclusão capilar, devido à superprodução de matriz extracelular induzida por fatores de crescimento, como o fator de crescimento transformante-β (TGF- β; do inglês *transforming growth factor-β*). Todos esses faotores levam à formação de edema, isquemia e neovascularização induzida pela hipóxia, causando danos celulares (BROWNLEE 2001).

Dessa forma, em pessoas de idade avançada, a hiperglicemia decorrente do diabetes causa lesões endoteliais e celulares, contribuindo para o aparecimento precoce das doenças de Alzheimer e Parkinson.

1.3 Substância nigra

Uma estrutura encefálica muito importante para o controle do comportamento motor é a susbtância nigra, a qual é um complexo nuclear profundo à *crus cerebri* em cada pedúnculo cerebral do mesencéfalo. Consiste da *pars compacta, pars reticulata* e a *pars lateralis* (Figura 3). A *pars compacta* e a *pars lateralis* correspondem ao grupo de células dopaminérgicas A9,

as quais, juntamente com o núcleo retrobulbar (A8) abrangem a população neuronal dopaminérgica do mesencéfalo e são a fonte do sistema de dopamina mesoestriatal que se projetam ao estriado. A *pars compacta* de cada lado é contínua com sua contraparte contralateral através do grupo dopaminérgico tegmental ventral A10, também conhecido como núcleo paranigral ou área tegmental ventral (VTA, do inglês *ventral tegmental área;* OADES e HALLIDAY, 1987). Essa é a fonte do sistema de dopamina mesolímbico, a qual supre o estriado ventral e partes vizinhas do estriado dorsal, além dos córtices pré-frontal e cingulado anterior. Os neurônios da *pars compacta* e do núcleo paranigral (A10) também contêm colecistocinina ou somatostatina (STANDRING, 2008).

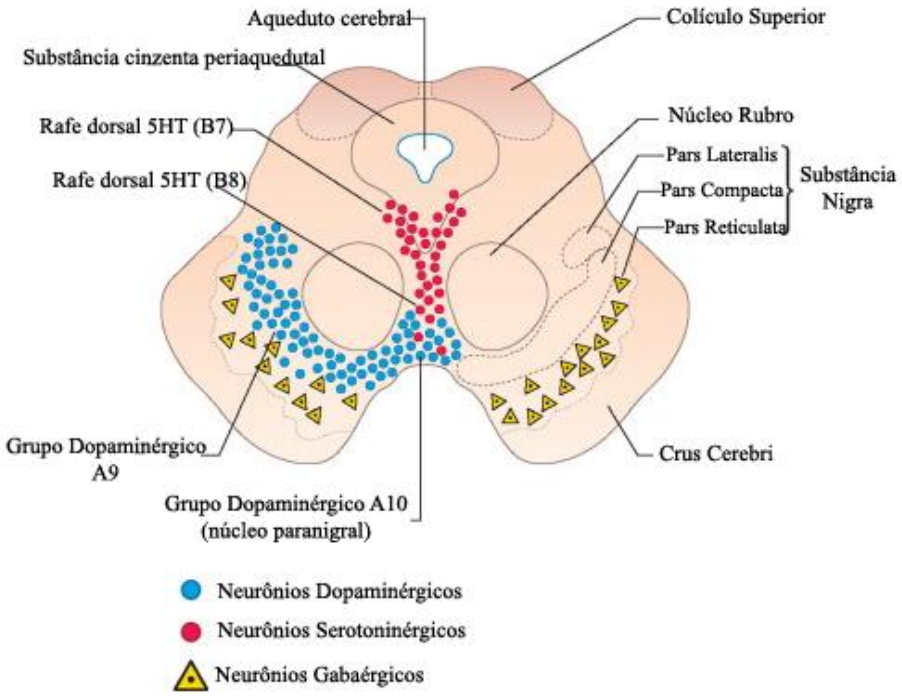

Figura 3: Desenho esquemático representando uma secção transversal no mesencéfalo para mostrar a substância nigra e suas regiões, e os grupos celulares dopaminérgicos, serotoninérgicos e gabaérgicos (modificado de Standring, 2008).

A catecolamina dopamina, neurotransmissor também classificado como "neurotransmissor de molécula-pequena", é sintetizada a partir do aminoácido tirosina. O primeiro passo da reação é catalisado pela enzima tirosina hidroxilase (TH), a qual utiliza

oxigênio como co-substrato e tetrahidrobiopterina como cofator para a síntese de diidroxifenilalanina (DOPA). Finalmente, a enzima DOPA descarboxilase remove uma molécula de dióxido de carbono, formando o neurotransmissor dopamina (Figura 4; PURVES et al., 2005).

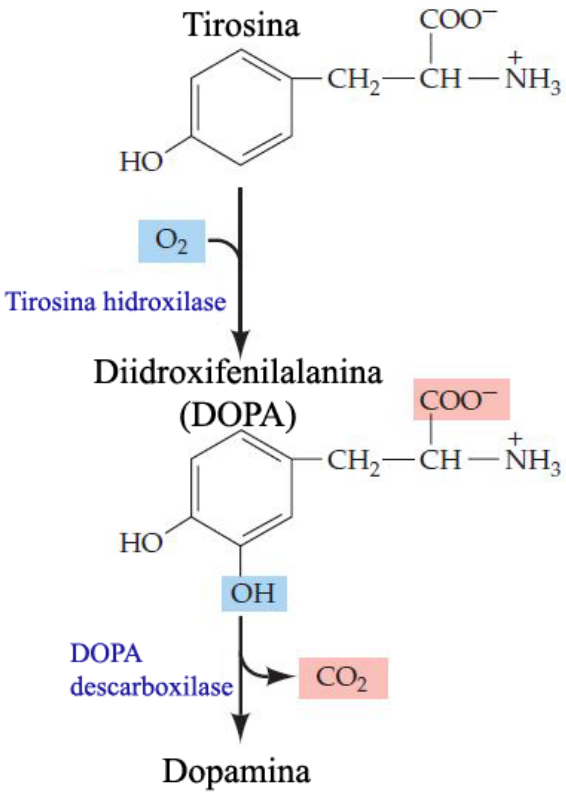

Figura 4: Desenho esquemático mostrando a síntese da dopamina a partir do aminoácido tirosina (modificado de Purves et al., 2005).

Seguida a síntese, a qual ocorreu no citoplasma do terminal sináptico, o neurotransmissor é armazenado em vesículas sinápticas pela ação de transportadores vesiculares de monoaminas (VMAT, do inglês *vesicular monoamine transporter*). A ação da dopamina na fenda sináptica é terminada pela sua recaptação, para o interior do terminal sináptico ou pelas células gliais circundantes à sinapse, por transportadores de dopamina sódio-dependentes (DAT, do inglês *dopamine active transporter*). Por outro lado, o catabolismo da dopamina ocorre pela ação das enzimas monoamina oxidase (MAO) e/ou

catecol O-metil-transferase (COMT). Assim como os neurônios, as células gliais contêm MAO mitocondrial e COMT citoplasmática (PURVES et al., 2005).

Os neurônios dopaminérgicos localizados nessa estrutura são susceptíveis aos danos causados tanto por drogas exógenas, como por exemplo, o 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP; PORRAS et al., 2012), iodeto de 1-metil-4-fenilpiridínio (MPP^+; ARVIN et al., 2000), rotenona (LAX et al., 2012), paraquat (BAGATINI et al., 2011; SRIVASTAVA et al., 2012) e a 6-hidroxidopamina (6-OHDA; DUTRA et al., 2012), como pelo avanço da idade (MCCORMACK et a., 2004).

A lesão neuronal na substância nigra, causada por qualquer desses agentes exógenos, depleta a dopamina e aparecem os quatro sinais clássicos da doença de Parkinson: bradicinesia, distúrbios da marcha, rigidez e tremores (GARCIA-RUIZ et al., 2011). Dessa forma, estudos experimentais, os quais utilizam os modelos animais da doença de Parkinson com as drogas MPTP, 6-OHDA (LALOUX et al., 2012), MPP^+ (ALCARAZ-ZUBELDIA et al., 2009), rotenona (LAX et al., 2012), paraquat (BAGATINI et al., 2011) avaliam o comportamento motor desses animais.

Apesar do DMT1 causar alterações encefálicas em regiões responsáveis pelo controle motor (KONO e TAKADA, 1994; BAYDAS et al., 2003; PEEYUSH et al., 2010) e essas alterações motoras terem sido demonstradas em humanos (CAVANAGH et al., 1992; VAN DEURSEN e SIMONEAU, 1999) e em animais (ABRAHAM et al., 2010; PEEYUSH et al., 2010; DE SENNA et al., 2011) e apesar de a substância nigra ser vulnerável a insultos exógenos e metabólicos, assim como os causados pelo aumento da idade (EMBORG et al., 1998; MCCORMACK et al., 2004), não foram encontrados na literatura estudos sobre os efeitos do DMT1 sobre a substância nigra, comportamento motor e os efeitos do exercício físico sobre essa estrutura, sendo esse o objeto de estudo do primeiro artigo dessa tese.

1.4 Sistema somatossensorial

Os sistemas sensoriais são encarregados de recolher informações tanto do meio que nos rodeia quanto do interior do organismo e de transmiti-las ao SNC para seu processamento e análise. A informação do meio externo é utilizada para a regulação da função dos órgãos internos; manutenção da vigília e para o controle dos movimentos. Por sua vez, a informação que provém do interior do organismo (vísceras, vasos sanguíneos, músculos e articulações) é utilizada para a regulação da temperatura corporal, pressão arterial, frequência cardíaca, respiração, produção de movimentos reflexos, isto é, está fundamentalmente relacionada com a manutenção de parâmetros fisiológicos do organismo, a homeostase (PAZO, 2004).

O processamento sensorial de estímulos externos inicia-se pela ativação de uma população diversificada de receptores cutâneos e subcutâneos. Esses são classificados como mecanoceptores, nociceptores e termoceptores. Quatro tipos de receptores encapsulados transmitem ao SNC informações relacionadas ao tato, pressão e vibração: os corpúsculos de Meissner, os corpúsculos de Paccini, os discos de Merkel e os corpúsculos de Ruffini, que são inervados por axônios mielinizados, relativamente largos, classificados como fibras Aβ, os quais possuem seus grandes corpos celulares localizados no gânglio da raiz dorsal (GRD). Essas fibras apresentam uma velocidade de condução entre 30 e 70 m/s. Além disso, os fusos musculares e órgãos tendinosos de Golgi, os proprioceptores, enviam informações acerca de forças mecânicas originadas no próprio corpo, como a posição dos membros no espaço. Esses receptores são inervados por fibras do tipo Ia e II, cuja velocidade de condução é de cerca de 70 e 120 m/s (PURVES et al., 2005; PARENT, 1996).

Especializações como nociceptores e termoceptores são denominadas terminações nervosas livres, pois a região receptora é o próprio terminal axonal que se ramifica na derme e na epiderme (PURVES et al., 2005). Essas fibras são classificadas como fibras Aδ, pouco

mielinizadas, e C, não-mielinizadas, as quais apresentam uma velocidade de condução de 20 e 2 m/s respectivamente, estão distribuídas por toda a pele e transduzem informações sobre dor, temperatura e tato grosseiro. Essas fibras apresentam seus corpos celulares pequenos, localizados no gânglio da raiz dorsal (PARENT, 1996).

Uma vez que um estímulo mecânico tenha causado um potencial de ação em um receptor, a fibra nervosa do neurônio de primeira ordem, que traz a informação dos mecanoceptores, ao entrar no corno dorsal da medula espinal, bifurca-se em ramos ascendentes e descendentes, os quais enviam ramos colaterais para um ou dois segmentos espinais. Alguns desses ramos penetram no corno dorsal da medula espinal (CDME) e estabelecem sinapse com neurônios localizados principalmente nas lâminas III e IV de Rexed (Figura 5). Estas sinapses medeiam, principalmente, reflexos segmentares, tal como o reflexo miotático. No entanto, o principal ramo dos axônios que chegam à medula espinal ascende ipsilateralmente ao longo das colunas dorsais da medula espinal até atingir o bulbo, onde estabelece contato com neurônios de segunda ordem dos núcleos grácil e cuneiforme. Os axônios dos núcleos da coluna dorsal projetam-se na porção dorsal de cada lado da parte inferior do tronco encefálico, onde formam o trato interno arqueado, que cruza a linha média, formando o lemnisco medial. Os axônios do lemnisco medial alcançam o núcleo ventral posterior lateral (NVPL) do tálamo, cujas células são os neurônios de terceira ordem do sistema coluna dorsal – lemnisco medial, os quais enviam as informações ao córtex sensorial, que por sua vez, integrará as informações (PURVES et al., 2005; KANDEL et al., 2003).

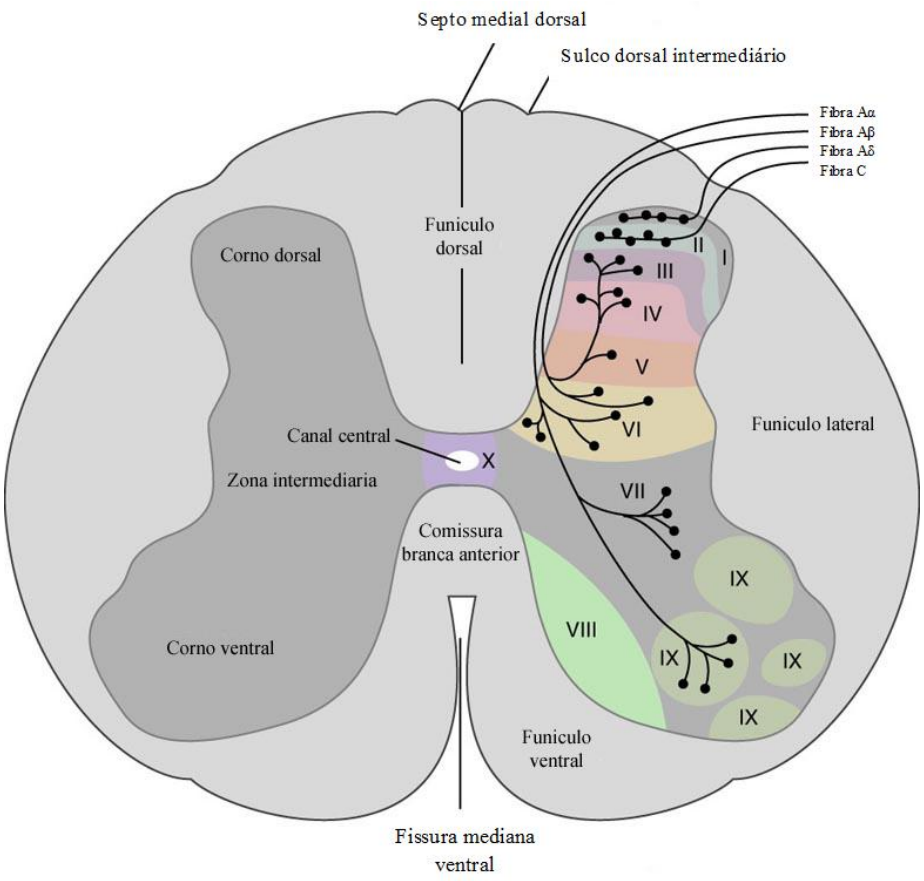

Figura 5: Desenho esquemático da secção transversal da medula espinal, mostrando as lâminas de Rexed nas quais os neurônios de primeira ordem fazem sinapse (adaptado de Squire et al., 2002).

Assim como os outros neurônios sensoriais nos GRD's, os axônios das células nervosas nociceptivas entram na medula espinal através das raízes dorsais. Ao atingirem o corno dorsal da medula espinal, os axônios se ramificam em colaterais ascendente e descendente, constituindo o trato de Lissauer, que interconecta diferentes segmentos medulares. Ao penetrarem na substância cinzenta as fibras Aδ e C fazem sinapse com neurônios na lâmina I de Rexed (zona marginal) e lâmina II (substância gelatinosa). A informação da lâmina II de Rexed é transmitida a neurônios de projeção de segunda ordem nas lâminas IV, V e VI (PURVES et al., 2005; KANDEL et al., 2003).

Esses neurônios sensoriais de primeira ordem fazem sinapse no corno dorsal da medula espinal (CDME) nas lâminas I, II e V, utilizam a substância P, a somatostatina, a

colecistocinina e o peptídeo relacionado ao gene da calcitonina (CGRP: do inglês *Calcitonin Gene-Related Peptide*) como neuromoduladores (LEVINE et al., 1993).

O CGRP é um neuropeptídeo formado por 37 aminoácidos e é envolvido na modulação dos estímulos nociceptivos. Os terminais nervosos que contém CGRP são em sua grande maioria os não-mielinizados e as fibras mielinizadas de pequeno diâmetro, isto é, as fibras do tipo C e as Aδ (MCNEILL et al., 1988), e são concentrados nas lâminas I e II e na região reticulada da lâmina V do CDME (CARLTON et al, 1988). Dessa forma, quando o estímulo nociceptivo despolariza o nociceptor, ocorre a liberação de CGRP nas lâminas I e II do CDME, que se conectam com os neurônios de segunda ordem nas lâminas IV – VI, os quais cruzam a linha média e ascendem até o tronco encefálico e o tálamo. Essas fibras, juntamente com os axônios dos neurônios de segunda ordem da lâmina I, formam o trato espinotalâmico, a principal via ascendente para informação sobre dor e temperatura. No tálamo, essas fibras fazem sinapse com neurônios do núcleo ventral posterior medial (NVPM) e NVPL. Neurônios no NVPM recebem informação nociceptiva da face, enquanto que os neurônios do NVPL recebem informações nociceptivas do resto do corpo. Então, a informação é transmitida ao córtex somatossensorial primário, o qual realizará o processamento da informação nociceptiva (Figura 6; PURVES et al., 2005; KANDEL et al., 2003).

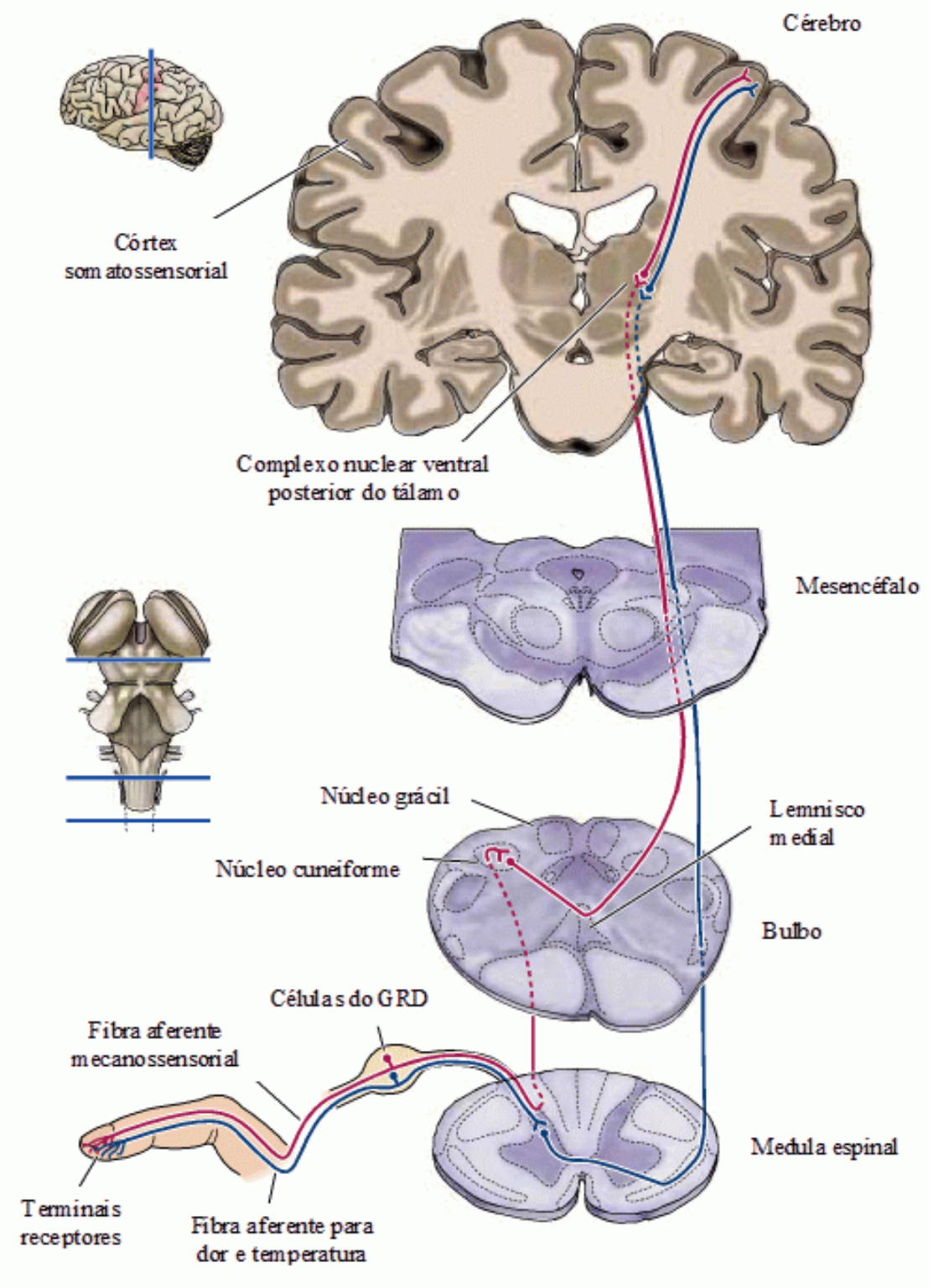

Figura 6: Desenho esquemático das vias somatossensoriais. Em azul via espinotalâmica, em vermelho, a via da coluna dorsal, lemnisco medial (modificado de Purves et al., 2005).

1.5 Neuropatia diabética

As neuropatias que se desenvolvem em consequência do diabetes são conhecidas por serem heterogêneas devido aos seus sintomas, padrões de envolvimento neurológico, risco e alterações patológicas (TESFAYE et al., 2010). Após várias tentativas de classificação, como por exemplo, neuropatia generalizada ou focal/multifocal (mononeuropatia múltipla, lombossacral, torácica, cervical), a classificação em dois subgrupos se tornou convincente (THOMAS 1997; BOULTON et al., 2005).

As polineuropatias diabéticas atípicas são diferentes das típicas em vários pontos, como na patogênese da doença, suas manifestações, associações e mecanismos. Podem se desenvolver em qualquer momento no curso do diabetes, o aparecimento dos sintomas pode ser agudo, subagudo ou crônico, sendo monofásico ou flutuante ao longo do tempo. Devido à inconstância das características das polineuropatias atípicas, estas são menos estudadas e caracterizadas (TESFAYE et al., 2010).

Por outro lado, a polineuropatia diabética (PND) típica é crônica, simétrica, sensorial-motora e dependente do comprimento do nervo e é a variedade mais comum (DYCK et al., 1993). Essa, por sua vez, se desenvolve em ambiente com hiperglicemia de longa data, sendo assim, associada com desarranjos metabólicos (aumento do fluxo na via do poliol, acumulação de produtos finais da glicação, estresse oxidativo e alterações lipídicas), com retino e nefropatia, e é considerada como um fator de risco para doenças cardiovasculares (DYCK et al., 1999; TESFAYE et al., 2005). Alterações funcionais como as alterações de sensibilidade térmica, mecânica, nociceptiva (DREL et al., 2007) e vibratória (MASER et al., 1989; JENSEN et al., 1991; BALDUCCI et al., 2006), e no comportamento motor tais como diminuição na locomoção (MOREIRA et al., 2007;) e incoordenação motora (ABRAHAM et al., 2010) são características da PND.

As alterações em nervos periféricos são causadas pelos mesmos efeitos já citados da hiperglicemia, a qual produz estresse oxidativo, ativa a via do poliol, causa a glicação não-enzimática de proteínas da matriz extracelular, causando danos em células endoteliais, que, por sua vez, diminui o fluxo sanguíneo para a vasa nervorum, causando um estado de hipóxia-isquemia no nervo, que culmina com alterações morfológicas e funcionais importantes (SIMA e SUGIMOTO, 1999).

Morfologicamente, a neuropatia diabética ocorre em nervos periféricos, como o nervo ciático (SELAGZI et al., 2008) e nervo sural (MALIK et al., 2001) e é caracterizada por ocorrer degeneração e regeneração tanto de fibras mielinizadas, como de não-mielinizadas (BRITLAND et al., 1990), atrofia axonal, com redução do tamanho da fibra (SIMA e SUGIMOTO, 1999), desmielinização (DYCK e GIANNINI, 1996), edema nodal (YAGIHASHI et al., 1990). Ainda, se notam alterações em proteínas do citoesqueleto, como diminuição da quantidade de neurofilamentos axonais (YAGIHASHI et al., 1990) e densidade de neurofilamentos em fibras mielínicas e amielínicas (SCOTT et al.,1999), fosforilação aberrante de neurofilamentos (FERNYHOUGH et al., 1999), diminuição da quantidade de microtúbulos em fibras mielínicas grandes e pequenas, denotando a diminuição do tamanho da fibra nervosa, bem como diminuição da velocidade de condução, assim como transporte axonal anormal (SCOTT et al., 1999).

Essas fibras nervosas que foram afetadas pelo estado hiperglicêmico e que trazem a informação sensorial das terminações nervosas livres ou de receptores sensoriais localizados na pele possuem seus corpos neuronais localizados no gânglio da raiz dorsal (GRD). Uma característica importante da estrutura do GRD é a presença de capilares fenestrados, diferentemente do SNC o qual apresenta capilares contínuos, uma das características da barreira hemato-encefálica, a qual forma uma barreira para a difusão de moléculas grandes

dos vasos para o espaço extracelular, tornando os neurônios localizados nesta região mais susceptíveis às variações de glicose plasmática (ALLEN e KIERNAN, 1994).

Notavelmente, foram demonstradas alterações fisiopatológicas nos neurônios localizados no GRD, causadas pelo aumento da concentração sanguínea de glicose, tais como degeneração do aparelho de Golgi (KAMIYA et al., 2006), diminuição do volume do soma (SIDENIUS e JAKOBSEN, 1980; DO NASCIMENTO et al., 2010), indução de apoptose nessas células (SCHMEICHEL et al., 2003), presença de lipofuscina, um pigmento formado a partir da peroxidação de lipídeos e associado ao envelhecimento celular precoce, em lisossomos secundários (SASAKI et al., 1997) e déficits de fatores neurotróficos, o que contribui para a indução da apoptose (ISHII, 1995; FERNYHOUGH et al., 1998; KAMIYA et al., 2006).

Em situações de lesões dolorosas, ou dor neuropática, como, por exemplo, quando há lesão do nervo ciático, ocorre um aumento do conteúdo dos neuromoduladores liberados pelas fibras Aδ e C dos neurônios sensoriais no CDME, isto é, ocorre aumento da liberação de CGRP, caracterizando o estado de hiperalgesia (ZHENG et al., 2008). Em contrapartida, situações de hipoalgesia, na qual o indivíduo não responde à estimulação nociceptiva, são associadas com a depleção de CGRP dos terminais nervosos, diminuindo sua liberação no CDME, como ocorre na neuropatia causada pelo diabetes (DIEMEL et al., 1992; DIEMEL et al., 1994).

No tratamento do paciente com DMT1 é incluída a realização de exercício físico (THE DIABETES CONTROL AND COMPLICATIONS TRIAL RESEARCH GROUP, 1993). No entanto, pouco se sabe sobre seus efeitos sobre as sensibilidade mecânica e nociceptiva, ou sobre a morfologia do nervo sural e sobre a imunorreatividade ao CGRP no CDME, sendo esses, alvo de estudo apresentados nos artigos 2 e 3 dessa tese.

1.6 Exercício físico e diabetes

Em humanos, além do controle dos níveis glicêmicos, a auto-administração de insulina, e a educação do paciente sobre a dieta, o exercício físico tem sido preconizado como parte da abordagem terapêutica convencional do diabetes do tipo 1 (The Diabetes Control and Complications Trial Research Group, 1993). Este deve ser incentivado em indivíduos com DMT1 pelas mesmas razões que deve ser encorajado em indivíduos não diabéticos: diminuição da velocidade de progressão e dos fatores de risco para doença cardiovascular, como hiperlipidemia, coagulação sanguínea anormal, hipertensão, intolerância à glicose e obesidade (PAFFENBARGER, 1980; SCHNEIDER et al., 1986; WASSERMAN e ZINMAN, 1994).

A atividade física é definida como um movimento corporal produzido pela contração de músculos esqueléticos, gastando mais energia do que no estado de repouso. Exercício físico é caracterizado como uma modalidade da atividade física, sendo este, planejado, estruturado e realizado de forma repetitiva com a finalidade da melhora de um ou mais componentes da aptidão física. Tem sido utilizado com a finalidade de controlar a glicemia, auxiliar no controle do peso corporal e reduzir os riscos de doenças cardiovasculares (CENTERS FOR DISEASE CONTROL AND PREVENTION, 1996). Para isso o exercício físico deve ser realizado por pelo menos 150 min/semana, com atividade aeróbica de intensidade moderada (50-70% da freqüência cardíaca máxima) e/ou 90 min/semana de exercício aeróbico vigoroso (70% ou mais da frequência cardíaca máxima), devendo ser realizado 3 dias/semana, com intervalos de no máximo 2 dias sem exercício físico (American Diabetes Association, 2007).

Em ratos diabéticos, os efeitos do exercício físico sobre a frequência cardíaca (HOWARTH et al, 2005), disfunção autonômica e do miocárdio (DE ANGELIS et al., 2000; TAKEDA et al., 1988), transportadores musculares de glicose (RODNICK et al., 1992;

SLENTZ et al., 1992), síntese de ácidos graxos livres (FIEBIG et al., 2001) e metabolismo do glicogênio (TAN et al., 1984) têm sido amplamente estudados. Também se tem demonstrado efeitos sobre a modulação do estresse oxidativo no córtex cerebral (LAPPALAINEN et al., 2008), sobre a melhora da memória espacial, as quais são relacionadas com alterações morfológicas hipocampais (DE SENNA et al., 2011) e sobre melhora da função cognitiva, relacionadas com a neurogênese hipocampal e aumento da expressão do fator de crescimento neural (NGF; do inglês nerve growth factor; CHAE et al., 2009) em ratos diabéticos. Uma vez que as habilidades motoras são influenciadas e controladas pela substância nigra, e como não foram encontrados trabalhos na literatura que mostrassem os efeitos do exercício físico sobre a função motora, bem como a expressão de TH na substância de ratos diabéticos, este é foi objetivo do 1° artigo apresentado nessa tese.

Além disso, um estudo com humanos demonstrou que a realização de exercício físico é capaz de diminuir a incidência de neuropatia diabética e aumentar a velocidade de condução do nervo fibular, assim como a sensibilidade vibratória (BALDUCCI et al., 2006). Em ratos, se tem demonstrado que o exercício físico melhora a função do membro inferior e os parâmetros morfométricos do nervo ciático lesionado por esmagamento (MALYSZ et al., 2010) e do músculo sóleo (MALYSZ et al., 2011), aumenta o volume dos neurônios do tipo A do GRD de ratos diabéticos (DO NASCIMENTO et al., 2010), atenua a hiperalgesia térmica (ROSSI et al., 2011), protege contra deficits na velocidade de condução nervosa e retarda o aparecimento de hipersensibilidade tátil (SHANKARAPPA et al., 2011), no entanto, não foram encontrados estudos na literatura que mostrassem os efeitos do exercício físico sobre a morfologia do nervo sural de ratos diabéticos, assim como os efeitos do exercício sobre a sensibilidade nociceptiva e expressão de CGRP no CDME de ratos diabéticos, sendo esses os objetivos do 2° e 3° artigos, respectivamente, que compõem essa tese.

1.7 Modelos animais para o estudo do diabetes

Para o estudo do DMT1 em animais, a STZ tem sido usada amplamente. Esta é um antibiótico derivado do *Streptomyces acromogenes* cujo mecanismo de destruição das células β-pancreáticas acarreta no impedimento da produção da insulina. Possuindo afinidade pelos transportadores de glicose do tipo 2 (GLUT2), a STZ é transportada pela membrana plasmática das células β-pancreáticas, age toxicamente sobre elas causando a metilação do ADN e a formação de espécies reativas de oxigênio, culminando na ativação da morte celular programada dessas células (MURATA et al., 1999; LENZEN, 2008; Figura 7).

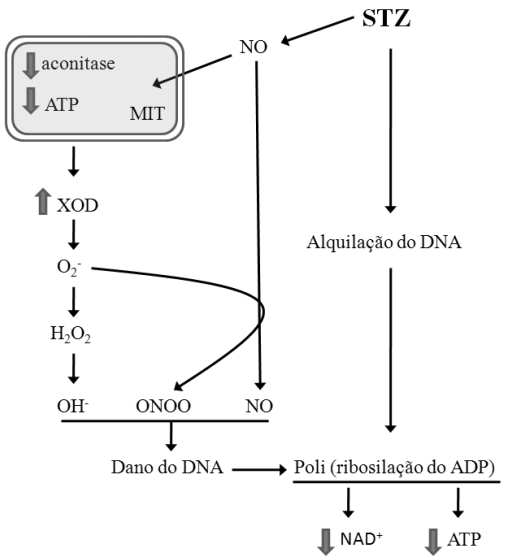

Figura 7: Desenho esquemático das ações da STZ nas células β-pancreáticas, a qual causa estresse oxidativo e dano no DNA. MIT: mitocôndria; XOD: xantina oxidase; O_2^-: superóxido; H_2O_2 peróxido de hidrogênio; OH^-: íon hidroxila; ONOO: peroxinitrito; NO: óxido nítrico (modificado de SZKUDELSKI, 2001).

A administração de uma única dose de STZ em ratos, resulta na produção de diabetes do tipo 1, com marcante hiperglicemia, semelhante ao diabetes do tipo 1 em humanos. Doses únicas de STZ, entre 40 e 60 mg/kg da massa corporal são suficientes para induzir o DMT1 (SZKUDELSKI, 2001). A administração intravenosa pela veia caudal é a forma mais utilizada devido ao menor risco de mortalidade dos animais e maior confiabilidade do seu

efeito diabetogênico, embora a mesma severidade do diabetes possa ser atingida veia sublingual ou injeção intraperitoneal de STZ (DELFINO et al., 2002).

Nos modelos experimentais a STZ causa hiperglicemia, hiperosmolaridade plasmática, hipoinsulinemia, hiperfagia, polidipsia e perda da massa corporal (SERINO et al., 1998), características do estado diabético. A simplicidade desse modelo o tornou um modelo animal muito comum para o estudo do diabetes e suas complicações, inclusive o estudo das encefalopatias (PIAZZA et al., 2011) e da neuropatia periférica (KISHI et al., 2002).

2 Justificativa

O diabetes aumenta o índice de mortalidade, além de aumentar o custo financeiro para a sociedade e os sistemas de saúde. Esse conhecimento vem determinando a necessidade dos serviços públicos de saúde a estruturação adequada e criativamente para conseguir enfrentar o problema com eficácia e eficiência (PORTAL DO SUS). Ainda no ano de 2007 a Assembleia Geral da Organização das Nações Unidas (ONU) aprovou a Resolução nº 61/225, considerando o diabetes um problema de saúde pública e conclamou aos países e governos a definirem políticas e suporte adequados para os portadores da doença.

Considerando essa patologia como a nova epidemia mundial, sua incidência aumentada a cada ano e o custo, devido ao tratamento direto da doença assim como o de suas comorbidades, que traz ao sistema único de saúde, se preconiza a prevenção de suas comorbidades, uma vez que o aparecimento dessas é indiretamente proporcional à qualidade de vida do indivíduo (BROWN et al., 2000).

Assim, exercício aeróbico tem sido empregado e estimulado como agente preventivo e terapêutico no diabetes mellitus e outras patologias. No entanto, enquanto o estudo sobre os efeitos do exercício físico no diabetes mellitus do tipo 2 é amplamente estudado, pouco se tem explorado os efeitos do treinamento no diabetes mellitus do tipo 1. Dessa forma, essa tese justifica-se por buscar maior compreensão dos mecanismos neurobiológicos envolvidos nos efeitos do exercício sobre o comportamento motor e na neuropatia sensorial, morfologia do nervo sural e expressão de CGRP no CDME de ratos diabéticos. A hipótese de que o exercício produz efeitos sobre a expressão de tirosina hidroxilase na substância nigra, na morfologia do nervo sural e na expressão do peptídeo relacionado ao gene da calcitonina no corno dorsal da medula espinal foi testada.

3 Objetivos

3.1 Objetivo geral

Este trabalho teve como objetivo geral analisar os efeitos do exercício físico em esteira sobre o comportamento motor e imunorreatividade à tirosina hidroxilase na substância nigra e área tegmental ventral, bem como sobre a sensibilidade mecânica e nociceptiva, morfometria do nervo sural e imunorreatividade ao peptídeo relacionado ao gene da calcitonina no corno dorsal da medula espinal de ratos machos adultos diabéticos induzidos por estreptozotocina.

3.2 Objetivos específicos

i. Avaliar o comportamento motor, pela análise em campo aberto e rotarod, de ratos machos adultos diabéticos induzidos por estreptozotocina, submetidos a 8 semanas de treinamento em esteira (Artigo 1);

ii. Avaliar o conteúdo de tirosina hidroxilase imunoreativa na substância nigra e área tegmental ventral de ratos machos adultos diabéticos induzidos por estreptozotocina, submetidos a 8 semanas de treinamento em esteira (Artigo 1);

iii. Avaliar os efeitos de 8 semanas de treinamento em esteira sobre a sensibilidade mecânica, pelo uso dos filamentos de von Frey, de ratos machos adultos diabéticos induzidos por estreptozotocina (Artigo 2);

iv. Avaliar os efeitos de 8 semanas de treinamento em esteira sobre a morfometria do nervo sural incluindo área e diâmetro da fibra mielinizada, densidade de fibras mielinizadas, área ocupada por tecido conjuntivo, espessura da bainha de mielina e índice de mielinização em ratos machos adultos diabéticos induzidos por estreptozotocina (Artigo 2);

v. Avaliar os efeitos de 8 semanas de treinamento em esteira sobre a sensibilidade nociceptiva, usando o teste do tail-fick, em ratos machos adultos diabéticos induzidos por estreptozotocina (Artigo 3);

vi. Avaliar os efeitos de 8 semanas de treinamento em esteira sobre o conteúdo do peptídeo relacionado ao gene da calcitonina imunoreativo no corno dorsal da medula espinal de ratos adultos diabéticos induzidos por estreptozotocina (Artigo 3).

4 Métodos e Resultados

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BRAIN RESEARCH

Research Report

Treadmill training improves motor skills and increases tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta in diabetic rats

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ABSTRACT

The aim of this study was to evaluate the effects of treadmill training on motor skills and immunoreactivity to tyrosine hydroxylase in the substantia nigra pars compacta and ventral tegmental area from diabetic rats induced by streptozotocin. Male Wistar rats were divided into three groups: control, diabetic and trained diabetic. Treadmill training was performed for 8 weeks. Blood glucose concentrations and body weight were evaluated 48 h after diabetes induction and every 30 days thereafter. Motor skills were evaluated on the rotarod and open field tests. Then, animals were transcardially perfused and the brains were post-fixed, cryoprotected and sectioned in a cryostat. Immunohistochemistry for tyrosine hydroxylase analyses was done in the ventral tegmental area and in the substantia nigra. Motor skills showed that diabetic animals had a decrease in the latency to fall and enhanced number of falls in the rotarod test compared to control and trained diabetic animals. In the open field, diabetic animals had a decrease in the number of crossed squares, rearings and spent a less time moving compared to control and trained diabetic animals. In diabetic animals, optical densitometry of immunohistochemistry showed that tyrosine hydroxylase reaction decreased in the ventral tegmental area and in the neurons and process in the substantia nigra. In the later region, that decrease was reversed by treadmill training. In conclusion, we demonstrated that treadmill training can reverse the loss of the motor skills, which was correlated to tyrosine hydroxylase immunoreactivity in the substantia nigra of diabetic animals without pharmacological treatment.

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1. Introduction

Diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, heart, blood vessels and nerve fibers (American Diabetes Association, 2010a). Diabetic neuropathy is highly prevalent and causes particularly significant morbidity to affected patients (Tesfaye et al., 2010). Moreover, streptozotocin (STZ)-induced diabetes in rats causes degenerative changes in the autonomic nervous system (Schaan et al., 2004), sensory neurons (Sidenius and Jakobsen, 1980; Fernyhough et al., 1999; Zherebitskaya et al., 2009; do Nascimento et al., 2010), and brain structures, such as the cerebellum (Anu et al., 2010) and the substantia nigra pars compacta (SN_{pc}; Figlewicz et al., 1996), causing deficits in the autonomic, sensory and motor systems.

The SN_{pc} and the ventral tegmental area (VTA) are motor structures that provide largely dopaminergic inputs to the cortex, striatum and to a lesser extent, pallidum (Paxinos, 1995). These structures are vulnerable to damage caused by exogenous toxins (McCormack et al., 2004), by aging, causing motor impairment (Emborg et al., 1998; Stark and Pakkenberg, 2004), and also by hyperglycemia of diabetes in rats (Figlewicz et al., 1996). Moreover, tyrosine hydroxylase (TH), which catalyzes the conversion of L-tyrosine to L-dopa and is the initial and rate-limiting step in the biosynthesis of catecholamines, has been used for the study of dopaminergic neurons (Nakashima et al., 2009).

Although the beneficial effects of regular physical exercise are well-known and used as part of the treatment of diabetic patients (American Diabetes Association, 2010b), few data on its efficacy in human diabetic neuropathy have been reported (Balducci et al., 2006). In addition, some studies in rats have shown the benefits of treadmill training in diabetes-induced cardiovascular and autonomic dysfunction (De Angelis et al., 2000; Harthmann et al., 2007), as well as in sensory neuropathy (do Nascimento et al., 2010). However, there are no data available on the effectiveness of treadmill training on motor deficits caused by diabetes in animals. Thus, the aim of this study was to evaluate the effects of a treadmill training protocol on motor skills and immunoreactivity to tyrosine hydroxylase (TH-ir) in the SN_{pc} and ventral tegmental area (VTA) of rats with STZ-induced diabetes.

2. Results

2.1. Body weight and blood glucose concentrations

There were no differences in the body weight between the C (298±5.1), D (295±4.6) and TD (305.8±6.5) groups 48 h before diabetes induction ($P>0.05$). Moreover, 30, 60 and 90 days after diabetes induction, rats from the D (253.3±16.7; 238±16; 237.7±15.7 respectively) and TD groups (281.3±5.6; 269.7±9; 277.7±11 respectively) showed lower body weight than the C group (351.3±3.9; 383.7±3.2; 406±2.9 respectively; $P<0.001$; Table 1).

As expected, 48 h after diabetes induction, blood glucose was higher in the diabetic groups (D and TD; 380.2±22.1 and 365.2±17.1 respectively) vs. the C group (86.3±4.6; $P<0.001$). In addition, the D and TD groups showed higher blood glucose than C group 30 (526±23.5; 485.1±37.3; 89.8±2.5 respectively; $P<0.001$), 60 (521.5±11.5; 512±17.6; 88.8±2.2 respectively; $P<0.001$) and 90 (514.7±18.7; 500.7±22.4; 94±2.7 respectively; $P<0.001$) days later. However, there were no differences between the D and TD groups in any of these variables ($P>0.05$; Table 1).

2.2. Motor skills

2.2.1. Rotarod test

Animals from group D presented a lower latency to fall (37.5±3.2) as compared to those in the C (56.6±1.7; $P<0.001$) and TD groups (53.4±2.3; $P<0.001$). There were no differences between the C and TD groups ($P>0.05$; Fig. 1a). In addition, the D group (4.2±0.3) was seen to fall more frequently than the C (0.8±0.3; $P<0.001$) and TD (1.7±0.5; $P<0.001$) groups. However, there were no differences between the C and TD groups ($P>0.05$; Fig. 1b).

2.2.2. Open field test

The number of squares crossed by animals from the D group (10.1±1.4) was lower than in the C (22.1±3.5; $P<0.05$) and TD groups (29.4±3.9; $P<0.001$). There were no differences between the C and TD groups ($P>0.05$; Fig. 2a). Furthermore, in the open field, the D group spent less time (15.3±2.4) moving than the C (33.7±3.1; $P<0.05$) and TD groups (34.2±4.8; $P<0.001$). There were no differences between the C and TD groups ($P>0.05$;

Table 1 – Time course changes in body weight and blood glucose in the studied rats.

Body weight and blood glucose in the studied groups								
Group	48 h		30 days		60 days		90 days	
	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)
C	298.0±5.1	86.3±4.6	351.3±3.9	89.8±2.5	383.7±3.2	88.8±2.2	406±2.9	94±2.7
D	295.0±4.6	380.2±22.1*	253.3±16.7*	526.0±23.5*	238.0±16*	521.5±11.5*	237.7±15.7*	514.7±18.7*
TD	305.8±6.5	365.2±17.1*	281.3±5.6*	485.1±37.3*	269.7±9*	512.0±17.6*	277.7±11*	500.7±22.4*

C: control group; D: diabetic group; TD: trained diabetic group. Body weight: repeated measures ANOVA group effect [$F_{(2,15)}=48.208$; $P<0.001$], time effect [$F_{(3,45)}=42.702$; $P<0.001$], time vs. group interaction [$F_{(6,45)}=42.702$; $P<0.001$]. Glycemia: repeated measures ANOVA group effect [$F_{(2,15)}=405.504$; $P<0.001$], time effect [$F_{(3,45)}=22.611$; $P<0.001$], time vs. group interaction [$F_{(6,45)}=5.313$; $P<0.001$].
* corresponds to $P<0.001$ compared to the C group.

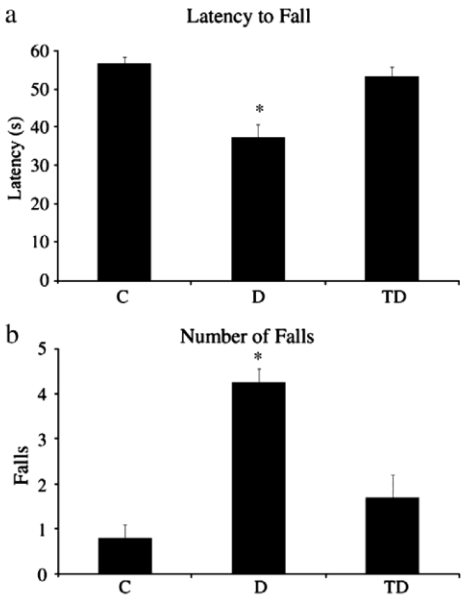

Fig. 1 – a: Latency to fall in the rotarod test. One way ANOVA [$P<0.001$; $F_{(2,25)}=16, 548$] with Bonferroni *post hoc* test. * corresponds to $P<0.001$ compared to the C and TD groups. b: Number of falls in the rotarod test. One way ANOVA [$P<0.001$; $F_{(2,25)}=19, 70,120$] with Bonferroni *post hoc* test. * corresponds to $P<0.001$ compared to the C and TD groups.

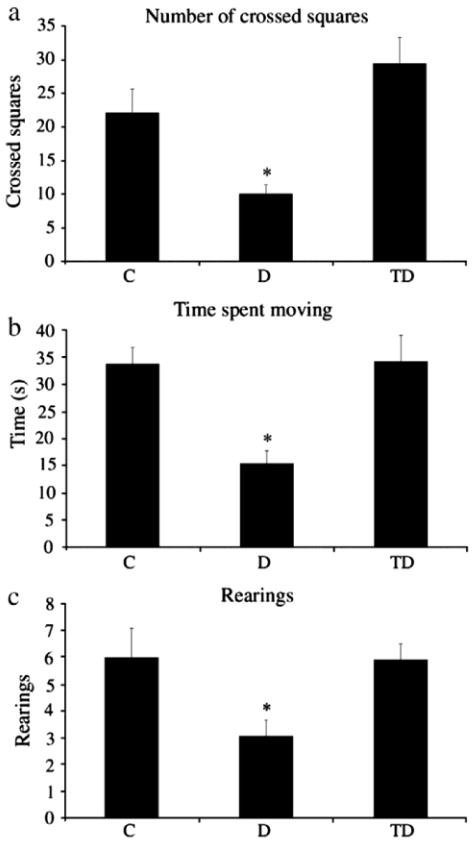

Fig. 2 – a: Number of crossed squares in the open field. One way ANOVA [$P<0.001$; $F_{(2,30)}=11, 9815$] with Bonferroni *post hoc* test. * corresponds to $P<0.05$ compared to the C and TD groups. b: Mean time spent moving in the open field. One way ANOVA [$P<0.001$; $F_{(2,30)}=9, 8645$] with Bonferroni *post hoc* test. * corresponds to $P<0.05$ compared to the C and TD groups. c: Number of rearings in the open field. One way ANOVA [$P<0.001$; $F_{(2,30)}=5, 3100$] with Bonferroni *post hoc* test. * corresponds to $P<0.05$ compared to the C and TD groups.

Fig. 2b). The D group was seen to rear (3.1±0.6) less frequently than the C (6.0±1.1; $P<0.05$) and TD (5.9±0.6; $P<0.05$) groups. There were no differences between the C and TD groups ($P>0.05$; Fig. 2c).

2.3. *Optical densitometry of TH-ir*

The OD analysis of the VTA showed that the TH-ir was lower in the neurons and processes from the D group (0.44±0.01) than in group C (0.51±0.01; $P<0.05$). However, there were no differences between the TD (0.5±0.02) and C groups ($P=1.0$), or between the TD and D groups ($P=0.08$; Fig. 3a).

Interestingly, the OD analysis of the SN_{pc} showed that the TH-ir of neurons and processes in the D group (0.35±0.01) was lower than in the C (0.42±0.01; $P<0.05$) and TD groups (0.43± 0.01; $P<0.05$). However, there were no differences between C and TD groups ($P>0.05$; Fig. 3b). Images from the groups are shown in Fig. 3c.

3. Discussion

The present study showed that treadmill training alone, with no pharmacological intervention, can reverse the loss of motor skills previously induced by STZ in rats, an improvement that was associated with tyrosine hydroxylase immunoreactivity changes in the substantia nigra and ventral tegmental area.

As expected, diabetic rats induced by STZ displayed higher blood glucose levels and lower body weights when compared to control animals. The treadmill training did not reduce blood glucose nor body weights, which is in accordance with previous results from our (do Nascimento et al., 2010) and other group (Midaoui et al., 2006), showing that physical training alone is not able to significantly improve metabolic control in these animals.

In the rotarod test, diabetic animals performed less well than the control and trained diabetic animals, showing lower latency to fall and a greater number of falls during the test. This task tests locomotion and coordination (Dunham and Miya, 1957); thus, it is evident that diabetic animals had a decrease in the motor coordination, affecting motor systems, as previously shown (Peeyush et al., 2009; Abraham et al., 2010). Interestingly, trained diabetics performed as well as

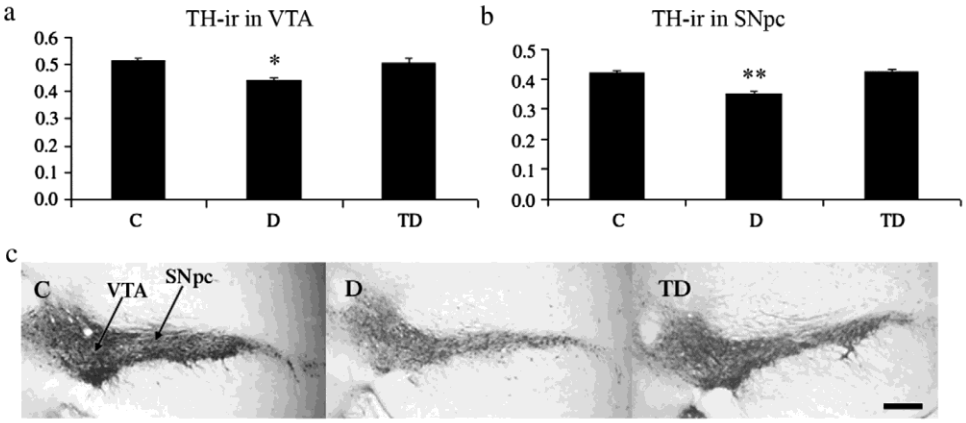

Fig. 3 – a: TH immunoreactivity (TH-ir) in the ventral tegmental area (VTA). One way ANOVA [$P<0.05$; $F_{(2,15)}=4,681$] with Bonferroni *post hoc* test. * corresponds to $P<0.05$ compared to the C group. b: TH immunoreactivity (TH-ir) in the substantia nigra pars compacta (SN_{pc}). One way ANOVA [$P<0.001$; $F_{(2,15)}=8,912$] with Bonferroni *post hoc* test. ** corresponds to $P<0.05$ compared to the C and TD groups. c: Digitalized images of coronal sections of substantia nigra pars compacta (SN_{pc}) and ventral tegmental area (VTA) in C, D and TD animals showing the TH immunoreactivity in neurons and processes. Note the decreased immunoreaction in the VTA and SN_{pc} from the D animal. In the image from the TD animal, note the lower immunoreaction in the VTA, compared to C animal, and the immunoreaction in the SN_{pc} which is similar to the C animal. Scale bar: 300 μm.

nondiabetic rats in this test, showing that exercise was able to reverse motor dysfunction and coordination deficits determined by diabetes, a finding not described before.

In the open field task, diabetic animals were seen to spend less time moving, crossed fewer squares and reared less frequently than the animals in the C and TD groups. All of these results demonstrate that diabetic animals were bradykinetics, resulting in a less exploratory behavior. Our results from both motor tasks, as well as the modification in the TH-ir from neurons and processes of SN_{pc} in STZ-diabetic rats suggest the involvement of the motor centers of the brain in the altered motor activity.

Additionally, in our study, the diabetic animals were seen to have a lower TH-ir in the VTA, probably giving rise to lower production of dopamine. However, although treadmill training improved motor skills, it was unable to reverse the decrease in TH-ir in the VTA. Moreover, the VTA plays a central role in multiple critical brain functions, including cognition, motivation, reward (Nieoullon, 2002; Wise, 2004; Fields et al., 2007) and together with the SN_{pc} influences locomotor activity (Paxinos, 1995; Schultz, 2007). However, there are differences in the morphological and electrophysiological properties of the dopaminergic neurons in these two regions, such as in the ionic channels (Neuhoff et al., 2002; Khaliq and Bean, 2010), which can cause different responses to injury and physical activity. In addition, although the treadmill training did not completely reverse the decrease in the VTA-ir, there was a strong trend toward normal values.

The SN_{pc} provides dopaminergic inputs to the cortex, striatum and pallidum, which facilitate most loops and outputs in the extrapyramidal motor system (Paxinos, 1995). However, the untrained diabetic rats had lower TH-ir in the SN_{pc}, which is in agreement with a previous study, in which diabetic animals were found to have lower TH mRNA levels in the SN_{pc}/VTA (Figlewicz et al., 1996). This decrease in TH reaction could be explained by changes in the total number of cells, in the total number of immunoreactive cells, in the immunostained area and/or by changes in intracellular immunoreactivity, as observed in an animal model of Parkinson's disease (Xavier et al., 2005). Interestingly, hyperglycemia causes oxidative stress and mitochondrial dysfunction (Mastrocola et al., 2005), leading to vascular damage and consequently hypoxia in the brain (Muresanu et al., 2010), which may contribute to neuronal death or reduced dopamine production, causing a decrease in the TH-ir in neurons and processes. Thus, in our study, STZ-diabetic rats presented motor alterations that were modified by treadmill training which recuperates TH-ir in the SN_{pc}, contributing to the maintenance of the extrapyramidal motor system of these rats.

On the other hand, brain derived neurotrophic factor (BDNF) is a neurotrophin that is enhanced by physical exercise in the hippocampus and is associated with the object recognition memory (Hopkins and Bucci, 2010) and improvement in the spatial memory (Khabour et al., 2010). Exercise alters the BDNF expression in the spinal cord of adult rats (Macias et al., 2007), in the cerebellum and motor cortex (Klintsova et al., 2004). In addition, BDNF also regulates early postnatal cell death in the SN_{pc} (Oo et al., 2009), and exercise exerts a neuroprotective effect in an animal model of Parkinson's disease (Yoon et al., 2007; Tajiri et al., 2010). Given this, we hypothesized that the improvement in the motor skills and in the TH-ir provided by the treadmill training in the STZ-diabetic rats could be caused by the BDNF downstream effects.

3.1. *Conclusions*

In summary, our results show that diabetes induced by STZ causes motor abnormalities and reduced TH-ir in the SN_{pc}.

Treadmill training promotes an increase in motor skills and behavior, which is accompanied by changes in TH-ir in the SN_{pc}, but not in the VTA.

4. Experimental procedures

4.1. Animals

Thirty three male Wistar rats (12 weeks old) from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul) were housed under standard laboratory conditions with food and water available *ad libitum* and maintained under a 12:12 light/dark cycle (lights on at 8:00 h). All efforts were made to minimize the number of animals studied and their suffering. The animals were cared for in accordance with Arouca Brazilian law (11794/2008) and the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary Surgery, University of Buenos Aires, and the International Brain Research Organization, and in compliance with the National Institute of Health's Guidelines for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985). This study was previously approved by the Ethical Committee from UFRGS under the protocol number 2008-062.

4.2. Experimental design

The rats were divided in three groups as follows: non-diabetic rats (C), diabetic rats (D) and diabetic rats submitted to treadmill training (TD). For analyses of motor skill in the rotarod, 10 animals were used in group C, 8 animals in group D and 10 animals in group TD. In the open field, 9 animals were used in group C, 13 animals in group D and 11 animals in group TD. Six animals per group were randomly selected for immunohistochemistry studies.

4.3. Diabetes induction

After an overnight fasting period (6 h), the rats received a single intravenous injection of STZ (50 mg/kg of body weight; Sigma Chemical Co., USA) diluted in 10 mM citrate buffer, pH 4.5. Non-diabetic animals received only citrate buffer (Junod et al., 1969; do Nascimento et al., 2010). Blood glucose concentrations were evaluated in blood collected from the rat-tail using test strips (Performa, Roche, Indianapolis, USA). Diabetes was defined as a fasting glucose >300 mg/dL in tail vein blood 48 h after STZ injection (Junod et al., 1969). Body weights and blood glucose concentrations were measured 48 h after the induction of diabetes and every 30 days thereafter.

4.4. Maximal exercise test

At the 4th week after diabetes induction, all animals underwent adaptation to a treadmill originally designed for human use (Runner, Brazil) and modified for use in rats during 10 minutes at 5 m/min for 4 days. On the 5th day, the rats were submitted to a maximal exercise test (MET), consisting of a graded exercise on the treadmill, with speed increments of 5 m/min every 3 minutes, starting at 5 m/min and continuing up to the maximal intensity attained by each rat, and was stopped when each animal remained more than 50% of the time without giving signs of intention to advance (Melo et al., 2003; Rodrigues et al., 2007; Ilha et al., 2008; do Nascimento et al., 2010). The values obtained in the MET were used to plan the treadmill training program, which started in the 5th week after diabetes induction. In order to correct the exercise intensity, a second MET was performed in the fifth training week.

4.5. Treadmill training

Exercise was performed on a treadmill twice a day, with an interval of 4 h between each session, 5 days per week (Tancrède et al., 1982), and the training intensity increased gradually, according to the MET results. During the first week, the running sessions lasted 10 min, and the duration of each increased each week, reaching 60 min in the 7th week, which was maintained until the 8th week. Moreover, each training session consisted of a warm-up period, a main period and a cooling-off period. During the warm up period, the rats ran 15% of the session time at 30% of the maximum velocity determined by the MET; in the main period, the rats ran 70% of the session time at 60% of the maximum velocity; and in the cooling-off period, the rats ran 15% of the session time at 30% of the maximum MET values.

4.6. Motor skills

4.6.1. Rotarod test

On the day after the last session of treadmill training, the rats were trained to remain on the rota rod apparatus (Insight, Brazil) with the speed adjusted to 12 rpm for 60 s. The following day, the selected rats were tested in the apparatus with the speed adjusted to 16 rpm for 5 sixty-second trials (modified from Linck et al., 2009). The latency to fall (data presented as the mean of the 5 trials) and the number of falls were evaluated.

4.6.2. Open field test

The rats were gently placed in the corner of a 40 cm × 50 cm × 60 cm box, in which the floor was divided into 12 squares, and then filmed with a digital camcorder (DCR-SR47, Sony, Japan) for 3 min (modified from Moreira et al., 2010). The number of crossings from one square to another, the time spent moving, and the number of rearings were counted.

4.7. Immunohistochemical procedure

One day after the analyses of the motor skills, rats were anesthetized with sodium thiopental (i.p; 50 mg/kg; Cristalia, Brazil). Heparin (1000 IU; Cristalia, Brazil) was injected into the left cardiac ventricle, then the animals were transcardially perfused through the left ventricle using a peristaltic pump (Control Company, Brazil, 20 mL/min) with 400 mL of 0.9% saline solution, followed by 400 mL of a fixative solution 4% paraformaldehyde (Synth, Brazil) in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were removed

from the skulls, post-fixed in the same solution at room temperature for 4 h and cryoprotected by immersion in a 15% and 30% sucrose (Synth, Brazil) solution in PB at 4 °C until they sank. After these procedures, the brains were quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and kept in a freezer (–70 °C) for further analyses.

Coronal sections (50 μm) from VTA and SN_{pc} were obtained from each brain using a cryostat (CM1850, Leica, Germany) at –20 °C and collected in a PB saline (PBS), pH 7.4. These areas were identified using Paxinos and Watson's Atlas (1998). The free-floating sections were pre-treated with 3% hydrogen peroxide for 30 min, carefully washed and treated with 2% bovine serum albumin (Inlab, Brazil) in PBS containing 0.4% Triton X-100 (PBS-Tx) for 30 min and incubated with monoclonal TH antibody (Sigma Chemical Co., USA) raised in mice, diluted 1:2000 in PBS-Tx for 48 h at 4 °C.

Sections were again washed in PBS-Tx and incubated in an anti-mouse antibody conjugated with peroxidase (Sigma Chemical Co., USA) diluted 1:200 in PBS-Tx for 2 h at room temperature. The reaction was revealed in a medium containing 0.06% 3,3′-diaminobenzidine (DAB, Sigma Chemical Co., USA) dissolved in PBS for 10 min and then 1 μL of 3% H_2O_2/mL was added to the DAB medium for an additional 10 min. Finally, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with Entellan (Merck, Germany) and coverslips. Control sections were prepared omitting the primary antibody by replacing it with PBS.

4.8. *Optical densitometry*

Semi-quantitative densitometric analysis was used to measure the intensity of the TH immunoreaction using a Nikon Optiphot-2 microscope (40×, Japan) coupled to a Micrometrics camera (Accu Scope, USA) and Image Pro Plus Software 6.0 (Media Cybernetics, USA). The digitized images obtained from the selected areas were converted to an 8-bit gray scale (0–255 gray levels). All lighting conditions and magnifications were held constant. Picture elements (pixels) employed to measure optical density were obtained from squares measuring 9680 μm^2 (area of interest, AOI) overlaid on the gray scale image. Both left and right sides of each brain were used. For each rat, 10 measures were taken from the VTA and 10 measures each from the medial, lateral and intermediary regions of the SN_{pc}. The results shown for the SN_{pc} were the total mean value from the three studied regions.

Background staining subtraction and correction were done in accordance with our previous published protocol (Xavier et al., 2005).

The optical density (OD) was calculated using the following formula:

$$OD(x,y) = -\log[(INT(x,y)-BL)] / (INC-BL)$$

Where "OD(x,y)" is the optical density at pixel(x,y), "INT(x,y)" or intensity is the intensity at pixel(x,y), "BL" or black is the intensity generated when no light goes through the material and "INC" is the intensity of the incidental light.

4.9. *Statistical analysis*

Blood glucose and body weight data were analyzed using repeated measures analysis of variance (ANOVA), and differences between the groups were assessed using the Bonferroni *post-hoc* test. Data obtained from motor skills tests, as well as optical densitometry of TH-ir were analyzed using one-way ANOVA and Bonferroni *post-hoc* test. Statistical significance was set at $P<0.05$. Data were run on Statistica 6.0 software package (StatSoft, Inc., USA). All data are represented by the mean ± standard error of mean (SEM).

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Artigo 2: Aceito para publicação na revista *Muscle and Nerve*

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Diabetes increases mechanical sensitivity and causes morphological abnormalities in the sural nerve which are prevented by treadmill training

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Diabetes increases mechanical sensitivity and causes morphological abnormalities in the sural nerve which are prevented by treadmill training

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Running title: Treadmill training and sural nerve of diabetic rats.

Diabetes increases mechanical sensitivity and causes morphological abnormalities in the sural nerve which are prevented by treadmill training

ABSTRACT:

Introduction: We evaluated the effects of treadmill training on mechanical sensitivity and sural nerve morphology in diabetic rats. Methods: rats were divided into three groups: control (C), diabetic (D) and trained diabetic (TD). Training was performed for 8 weeks. Mechanical sensitivity was evaluated using von Frey filaments. Sural nerves analysis included fibre area, diameter, density of myelinated fibres, area occupied by connective tissue, myelin sheath thickness and the g ratio. Results: The D animals showed a reduced mechanical sensitivity threshold. Morphometry showed that the D group had smaller myelinated fibre area, diameter, higher density of fibres and area occupied by connective tissue, smaller myelin sheath thickness and higher g ratio. The D group had a higher percentage of small myelinated fibres and a lower percentage of large diameter myelinated fibres than the C and TD groups. Conclusion: training prevents functional and morphological abnormalities in the sural nerve caused by diabetes.

List of Keywords:

Diabetes

Diabetic neuropathy

Mechanical sensitivity threshold

Treadmill training

Sural nerve morphometry

List of abbreviations:

DPN**:** distal polyneuropathy

STZ: streptozotocin

UFRGS: Universidade Federal do Rio Grande do Sul

C: Control group

D: Diabetic group

TD: trained diabetic group

MET: maximal exercise test

PB: phosphate buffer

OsO_4: Osmium tetroxide

ANOVA: analysis of variance

SEM: standard error of mean

1. Introduction

Diabetic neuropathies are the most frequently seen complications of the diabetes. Among them, the most common are chronic sensorimotor distal polyneuropathy (DPN) and the autonomic neuropathies. In clinical practice, DPN is defined as the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of the other causes. Moreover, up to 20% of patients with diabetes may experience symptoms, the most frequent being burning pain, electrical or stabbing sensations, paresthesia, hyperesthesia, deep pain and allodynia. These symptoms are experienced in the feet and lower limbs, although in some cases the hands may also be affected[1].

Morphologically, DPN is characterised by alterations in the peripheral nerve fibres, as well as degeneration and regeneration in both myelinated and unmyelinated fibres in humans[2,3]. Moreover, studies in rats have shown that diabetes causes morphological alterations, such as decreased axonal diameter in the sciatic nerve[4], decreased area and myelin sheath thickness in the sural nerve[5], alterations to the cytoskeletal components in the dorsal root ganglia and in the sural nerve[6], functional alterations such as reduced motor and sensory nerve conduction velocity[7], as well as thermal hyperalgesia and mechanical allodynia[8].

Currently, programmed physical exercise is recommended for diabetic patients in order to prevent and treat the complications of diabetes[9]. We have previously shown that physical training in diabetic rats is able to cause morphological alterations in the L5 dorsal root ganglia neurons[10], accelerate hindlimb motor function recovery in injured rats[4], improve soleus muscle morphology in nerve regeneration[11], improve motor skills and increase tyrosine hydroxylase immunoreactivity in the substantia nigra[12] and prevent the cognitive deficits and astroglial alterations induced by diabetes[13].

However, little is known about the effects of physical training on mechanical sensitivity and sural nerve morphology. Thus, the aim of this study was to evaluate the effects

of eight weeks of treadmill training on the mechanical sensitivity, evaluated using von Frey filaments, and on sural nerve morphology in rats subjected to diabetes induced by streptozotocin (STZ). Our hypothesis is that diabetes increases the mechanical sensitivity due to alterations in the number of small and large myelinated fibres in the sural nerve, and that treadmill training is able to prevent increased mechanical sensitivity, as well as the morphological alterations.

2. Methods

1.1 Animals

Thirty male Wistar rats (12 weeks old) from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul) were housed under standard laboratory conditions with food and water available *ad libitum* and maintained under a 12:12 light/dark cycle (lights on at 8:00h). All efforts were made to minimize the number of animals studied and their suffering. The animals were cared for in accordance with Arouca Brazilian law (11794/2008) and the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary Surgery, University of Buenos Aires, and the International Brain Research Organization, and are in compliance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1985). This study was previously approved by the Ethical Committee of the UFRGS under the protocol number 2008-062.

1.2 Experimental design

The rats were divided in three groups: non-diabetic rats (C), diabetic rats (D) and diabetic rats submitted to treadmill training (TD). For the analyses of mechanical sensitivity, 10 animals were used per group, and for the morphometrical studies, 5 animals were randomly selected per group.

1.3 Diabetes induction

After an overnight fasting period (6 h), rats from the D and TD groups received a single intravenous injection of STZ (50 mg/kg of body weight; Sigma Chemicals Co., USA) diluted in 10 mM citrate buffer, pH 4.5, while the C group received only citrate buffer[10,12]. Blood collected from the rat-tail was used to measure blood glucose concentrations using test strips (Performa, Roche, USA). Diabetes was defined as a fasting glucose > 300 mg/dL in tail vein blood 48h after STZ injection[14]. Body weights were measured before diabetes induction and every 30 days thereafter, and blood glucose concentrations were measured 48h after the induction of diabetes and every 30 days thereafter.

1.4 Maximal exercise test and treadmill training

At the 4th week after diabetes induction, all animals underwent adaptation to a treadmill originally designed for human use (Runner, Brazil) and modified for use by rats during 10 minutes at 5 m/min for 4 days. On the 5th day the rats were submitted to a maximal exercise test (MET), consisting of a graded exercise on the treadmill, with speed increments of 5 m/min every 3 minutes, starting at 5 m/min and continuing up to the maximal intensity attained by each rat, and was stopped when each animal remained more than 50% of the time without giving signs of intention to advance[10,12,15,16]. The values obtained in the MET were used to plan the treadmill training program, which started in the 5th week after diabetes induction. In order to correct the exercise intensity, a second MET was performed in the 5th training week.

Exercise was performed on a treadmill twice a day, with an interval of 4 h between each session, 5 days per week and the training intensity was increased gradually, according to the MET results. During the first week, the running sessions lasted 10 min, and the duration of each increased each week, reaching 60 min in the 7th week, which was maintained until the 8th week. Moreover, each training session consisted of a warm-up period, a main period and a

cooling-off period. During the warm up period, the rats ran 15% of the session time at 30% of the maximum velocity determined by the MET; in the main period the rats ran 70% of the session time at 60% of the maximum velocity; and in the cooling-off period the rats ran 15% of the session time at 30% of the maximum MET values[12].

1.5 Mechanical sensitivity analysis

To assess mechanical sensitivity using a series of von Frey filaments, the rats were acclimatized for 20 min in a box (35 X 31 X 10 cm) with a mesh floor. The filaments were applied, in a consecutive sequence (8, 10, 15, 26, 60 e 100 g), three times, to the plantar surface of the left and right hind paw pads. Vertical elevation of the paw immediately upon removal of the testing hair was considered to be a positive response. To confirm the response, following a positive response, this filament was replaced by a weaker one. In the absence of paw withdrawal, filaments of increasing weight were sequentially applied[17]. This test was done twice, before the induction of diabetes and the day after the last training session. The data shown are the mean of the responses from the left and right hind paws in grams.

1.6 Histological and morphometric analysis procedure

One day after the analysis of mechanical sensibility, the rats were anesthetized with sodium thiopental (i.p; 50 mg/kg; Cristalia, Brazil). Heparin (1000 IU; Cristalia, Brazil) was injected into the left cardiac ventricle, then the animals were transcardially perfused through the left ventricle using a peristaltic pump (Control Company, Brazil, 20 mL/min) with 400 mL of 0.9% saline solution, followed by 400 mL of a fixative solution 4% paraformaldehyde (Synth, Brazil) in 0.1 M phosphate buffer, pH 7.4 (PB). The right sural nerves were removed, post-fixed for 1 h at room temperature in 0.5% glutaraldehyde (Sigma Chemicals Co., USA) and 2% paraformaldehyde (Synth, Brazil) in a 0.1M (PB), pH 7.4, then washed in PB 0.1 M and post-fixed in 1% OsO_4 (Sigma Chemicals Co., USA) in PB for 1h at room temperature.

Sural nerves were then washed in PB 0.1M and dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, USA), embedded in resin (Durcupan, ACM-Fluka, Switzerland), kept in vacuum for 24 h and afterwards put in resin blocks and polymerised for 48h at 60°C.

For the morphometric studies, the nerves were transversally sectioned using an ultramicrotome (Leica, Austria), after which semithin sections (1 μm) were stained with toluidine blue (Merck, Germany) diluted in 1% sodium tetraborate (Ecibra, Brazil). Images of the semithin sections were captured and digitalized using a Nikon Eclipse E-600 microscope (Tokyo, Japan; initially 1000X and further amplified 200% for analysis) coupled to a Pro-Series High Performance CCD camera with Image Pro Plus Software 6.0 (Media Cybernetics, USA).

For the 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 nerve core in order to obtain a representative area per nerve segment[15]. Both large and small myelinated fibres were used in the morphometric evaluation and the items measured included the average myelinated fibre area (μm^2), the fibre diameter (μm), the density of myelinated fibres (fibres/mm^2), the percentage of area occupied by connective tissue (considering 1561 μm^2 to be 100% of the analyzed area in each studied nerve), the average myelin sheath thickness (μm) and the g ratio (the quotient axon diameter/fibre diameter, a measurement of the degree of myelination). The average myelin sheath thickness was estimated using the measurement tools found in Image Pro Plus software. The sizes of the areas were estimated using a point-counting technique[15,18] involving grids with a point density of 1 point per 1.55 μm^2 and the following equation:

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

Where Â is the area, Σp the sum of the points, and a/p the area/point value (1.55 μm^2). To facilitate comparisons with previous studies and the interpretation of results, areas were recalculated in order to correspond to the diameters of (imaginary) circles with equivalent areas. To characterize the distribution of the fibres in each group, the fibres were classified according to their diameter, ranging from 1 to 12 μm. Then, frequency histograms were drawn with the percentage of fibres per diameter[19].

1.7 Statistical analysis

Blood glucose, body weight and mechanical sensitivity data were analyzed using repeated measures analysis of variance (ANOVA), and differences between the groups were assessed using the Bonferroni *post-hoc* test. Morphometric data were analyzed using one-way ANOVA and the Bonferroni *post-hoc* test. Statistical significance was set at $p < 0.05$. Data were run on Statistica 6.0 software package (StatSoft, Inc., USA). Data are represented by the mean ± standard error of mean (SEM).

3. Results

1.8 Body weight and blood glucose concentrations

The body weight and blood glucose concentrations are shown in the Table 1. Blood glucose concentration was higher in D and TD when compared to the C group at all experimental periods, with no differences being observed between D and TD. Body weights were lower in D and TD compared to C group at all experimental periods, with no differences being observed between D and TD.

1.9 Mechanical sensitivity

Regarding mechanical sensitivity, there were no differences between the C (60 ± 0 g), D (49.8 ± 5.1 g) and TD (56.6 ± 3.4 g) groups before diabetes induction. However, 90 days after diabetes induction there was difference between the D (43 ± 5.7 g) group compared to C

(60 ± 0 g) and TD (60 ± 0 g) groups ($p < 0.001$). Moreover, there were no differences between C and TD groups 90 days after diabetes induction (Figure 1).

1.10 Morphometric analysis

One-way ANOVA showed that the myelinated fibre area was smaller in the D (26.5 ± 0.8 μm^2) group than in the C (35.2 ± 1.9 μm^2) and TD (33.9 ± 2.8 μm^2) groups ($p < 0.05$). Moreover, there were no differences between the C and TD groups (Figure 2).

Analysis showed that the fibre diameter in the D group (5.5 ± 0.7 μm) was smaller than that of the C (6.2 + 0.1 μm; $p < 0.05$) group. Moreover, there were no difference between the C and TD (6 ± 0.2 μm) groups (Figure 3).

In the analysis of the frequency histograms (Figure 4), statistics showed that the percentage of fibres with diameters of 2 μm and 6 μm was higher in the D group (2.3 ± 0.36; 20.8 ± 1.1 respectively) than in the C (0.5 ±0.2; 13.5 ± 1.34 respectively) and TD (0.44 ± 0.4; 13.12 ± 1.3 respectively) groups ($p < 0.01$), and there were no differences between the C and TD groups. In addition, the percentage of fibres with a diameter of 9 μm was lower in the D group (5.26 ± 0.82) than in the C (14.28 ± 1.37) and TD (12.06 ± 1.32) groups ($p < 0.001$). Moreover, there was no difference between the C and TD groups.

In the D (21075 ± 388) group there was an increase in the density of myelinated fibres, i.e, there were more myelinated fibres/mm^2 than in the C (17620 ± 680) group ($p < 0.01$), while there was no difference between the C and the TD (19655 ± 925) group (Figure 5). Moreover, the D (45.6 ± 0.8) group showed an increase in the area occupied by connective tissue compared to the C (40.3 ± 1.3; $p < 0.05$) and TD (39.3 ± 1.4; $p < 0.001$) groups, while there was no difference between C and TD groups (Figure 6).

In addition, the myelin sheath thickness was smaller in the D group (0.85 ± 0.03 μm) than in the C (1.14 ± 0.06 μm; $p < 0.001$) and TD (1.09 ± 0.02 μm; $p < 0.001$) groups. There were no differences between the C and TD groups (Figure 7). Furthermore, the g ratios of the

C (0.63 ± 0.01; $p < 0.05$) and TD (0.63 ± 0.01; $p < 0.05$) groups were smaller than that of the D group (0.68 ± 0.01; Figure 8).

Representative images from the C, D and TD groups are shown in Figure 9 A, B and C.

4. Discussion

The present study showed that STZ increases the mechanical sensitivity in rats and that this can be modified by eight weeks of treadmill training. Moreover, the results suggest that the reduced mechanical sensitivity threshold may be related to the morphological alterations in the sural nerve, such as myelinated fibre area, fibre diameter, density of myelinated fibres in the nerve, myelin sheath thickness and the degree of myelination, which were also prevented by the treadmill training.

As our and other groups have previously shown, rats with STZ-induced diabetes display higher blood glucose concentrations and lower body weights than control animals, confirming their catabolic state, as previously reported[20]. Moreover, treadmill training was neither able to reduce the blood glucose concentrations nor alter the body weight[10,12,21]. The long-term persistance of high blood glucose concentrations in diabetic animals is in accordance with data previously reported in rats[4, 10,11,12,22] and type 1 diabetic patients[23].

Indeed, streptozotocin induces an insulin-deficient state[14], similar to type 1 diabetes in humans, which, unlike type 2 diabetes, an insulin-resistant state[24], does not usually benefit from exercise interventions intended to control blood glucose levels[25]. Interestingly, in the present study, multiple functional and structural neural abnormalities induced by diabetes were reversed by exercise training, although there was no apparent reduction in blood glucose concentration. Given that exercise has been shown to improve insulin sensitivity and facilitate muscle metabolism[26], increase adipocyte insulin-stimulated glucose uptake[27], enhance muscle insulin receptor autophosphorylation[28] and membrane GLUT4 translocation and expression[29],

increase capillary supply and blood flow capacity to the skeletal muscle[30,31], and activate neurotrophic factors (nerve growth factor[32,33], brain derived neurotrophic factor[34,35] and neurtrophin-3[36]), we hypothesize that the improvements observed in the present study were induced by such multiple beneficial effects of exercise training despite the fact the blood glucose levels remained unaltered.

In our study, the von Frey filament test showed that the diabetic rats were more sensitive to mechanical stimulus, i.e., the diabetic rats responded to the thin filaments while control and trained diabetic rats responded to the thicker ones. Our results are in agreement with others which showed that diabetes causes sensory abnormalities, including tactile allodynia[37,39]. The reduced mechanical threshold, measured using von Frey filaments, as reported in this study, and the spontaneous discharge of Aδ and C-fibres recorded from the tibial nerve previously reported in STZ-diabetic rats are changes that may contribute towards increased mechanical sensitivity[40], and may reflect the ongoing state of various stages of afferent nerve regeneration and/or degeneration[41]. Interestingly, we have shown that treadmill training was able to prevent abnormal mechanical sensitivity, a previously unreported finding.

Furthermore, the morphometrical studies showed that the sural nerve of the diabetic animals had a smaller area and diameter of the myelinated fibres, increased density of myelinated fibres and an increased area occupied by connective tissue. Moreover, the myelin sheath was thinner in diabetics as compared to the control and trained diabetic animals, and there was a poor myelination in the diabetic animals. These data are in agreement with previous studies which showed that diabetes causes considerable damage to the peripheral nerve fibres[5,41,42] and also reinforces the beneficial effects of exercise, independently of blood glucose reduction.

Moreover, the g ratio reflects the degree of myelination, i.e., the higher g ratio, the less myelination. Values for the g ratio between 0.6 and 0.7 would provide the best and maximum

conduction velocity of a myelinated fibre[43]. Although the values found in this study were within this range, sedentary diabetic animals had a higher g ratio than the control and trained diabetic animals, showing that three months of hyperglycemia were sufficient to cause a decrease in conduction velocity of the myelinated fibre in the sural nerve, and that two months of exercise were able to prevent the altered conduction velocity. Although this effect of exercise on the degree of fibre myelination was greater in animals in which the sciatic nerve had been crushed[15], it was also shown in the present study, where the nerve damage was induced only by metabolic derangements.

Nevertheless, a decrease in the fibre area, in the fibre diameter and myelin sheath thickness, corroborated by an increase in the density of fibres and the area occupied by connective tissue in a nerve should lead to reduced sensitivity, the opposite of what occurred in our study. However, when examining the frequency histogram of the sural nerves from the sedentary diabetic rats, one can see that there was a higher percentage of smaller myelinated fibres compared to the control and trained diabetic rats. On the other hand, in the sedentary diabetic rats the percentage of the larger myelinated fibres (9 μm in diameter) was lower.

Moreover, the small nerve fibres correspond to the Aδ fibres, which carry the pain signal to the dorsal horn of the spinal cord. However, the larger calibre Aβ fibres, which carry the mechanical signal, form a synaptic contact on inhibitory interneurons, competing with the pain stimuli reaching the projection neuron[44]. In our study, the percentage of small fibres was higher in the sedentary diabetic rats, which explains the fact that these animals were more sensitive to the mechanical stimulus applied using the von Frey filaments, causing increased mechanical sensitivity. On the other hand, treadmill training prevented abnormal mechanical sensitivity and maintained the fibre distribution in the trained diabetic animals similar to that of the control animals.

Conclusions: our results showed that diabetes causes an increase in mechanical sensitivity that was accompanied by morphological abnormalities in the sural nerve. Treadmill training prevents functional abnormalities and maintains normal sural nerve morphology.

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Table 1. Time course changes in body weight and blood glucose in the studied rats.

Table 1 Body weight and blood glucose in the studied groups

Group	*48 hs*		*30 days*		*60 days*		*90 days*	
	Weight (g)	**Glycemia (mg/dL)**	**Weight (g)**	**Glycemia (mg/dL)**	**Weight (g)**	**Glycemia (mg/dL)**	**Weight (g)**	**Glycemia (mg/dL)**
C	299 ± 3	83 ± 3	347.6 ± 3	89 ± 2	374 ± 4	88 ± 1.5	395.8 ± 5	93 ± 2
D	295 ± 10	374±14***	262±9.5***	510±21***	254±12***	509±14***	256±13***	496±25***
TD	300 ± 6.7	355±12***	280±7***	450 ± 33***	271±7***	521±16***	286±12***	513±19***

C: control group; D: diabetic group; TD: trained diabetic group. ***: corresponds to $p<0.001$ compared to the C group. Body weight: Repeated measures ANOVA group effect [$F(2,27)=41.859$; $p<0.001$], time effect [$F(3,81)=6.215$; $p<0.001$), time vs group interaction [$F(6,81)=40.005$; $p<0.001$]. Glycemia: Repeated measures ANOVA group effect [$F(2,27)=472.005$; $p<0.001$], time effect [$F(3,81)=27.694$; $p<0.001$], time vs group interaction [$F(6,81)=7.308$ $p<0.001$].

Figure Legends:

Figure 1: Mechanical sensitivity before and 90 days after diabetes induction. *** corresponds to $p < 0.001$ compared to C and TD groups 90 days after diabetes induction.

Figure 2: myelinated fibre area in μm^2 from C, D and TD groups. * corresponds to $p < 0.05$ compared to C and TD groups.

Figure 3: myelinated fibre diameter in μm from C, D and TD groups. * corresponds to $p < 0.05$ compared to C group.

Figure 4: frequency histograms from control (A), diabetic (B) and trained diabetic rats (C).

Figure 5: density of myelinated fibres (fibres/mm^2) from C, D and TD goups. ** correspond to $p < 0.01$ compared to C group.

Figure 6: area occupied by connective tissue (%) from C, D and TD groups. * corresponds to $p < 0.05$ compared to C group. *** corresponds to $p < 0.001$ compared to TD group.

Figure 7: myelin sheath thickness in μm from C, D and TD groups. *** corresponds to $p<0.001$ compared to C and TD groups.

Figure 8: g ratio from C, D and TD groups. * corresponds to $p < 0.05$ compared to C and TD groups.

Figure 9: representative images from the C (A), D (B) and TD (C) groups respectively. Scale bar: 100 μm.

Figure 1

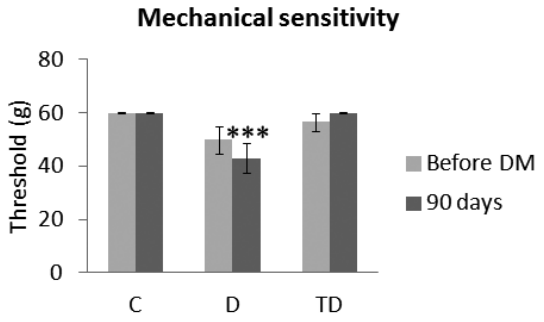


Figure 2

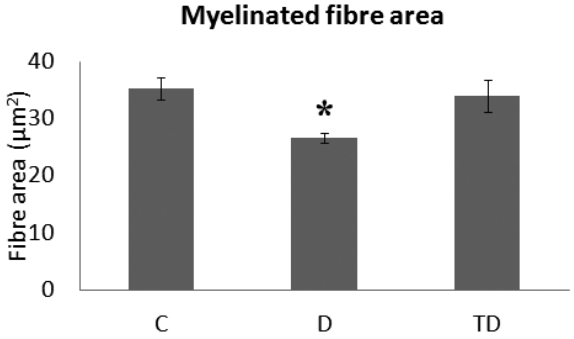


Figure 3

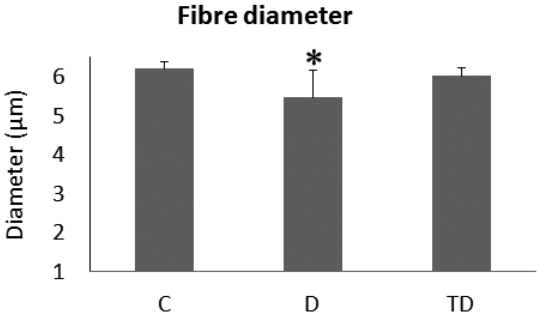


Figure 4

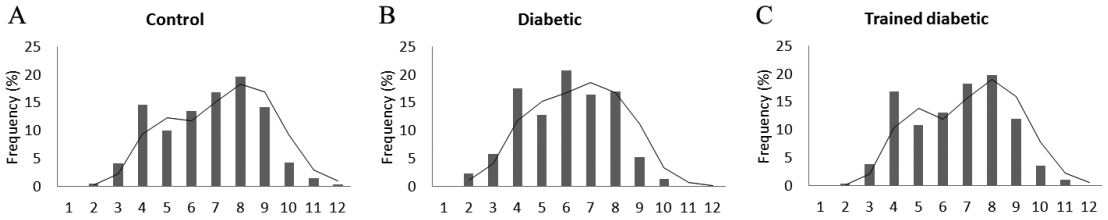


Figure 5

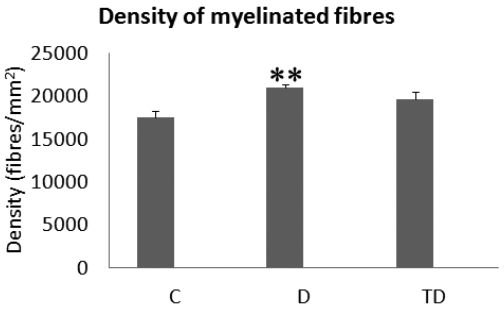


Figure 6

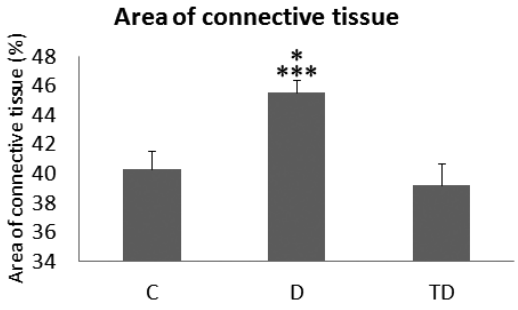


Figure 7

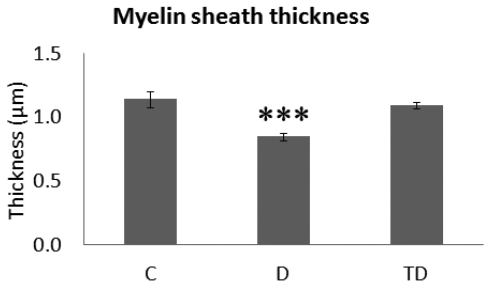


Figure 8

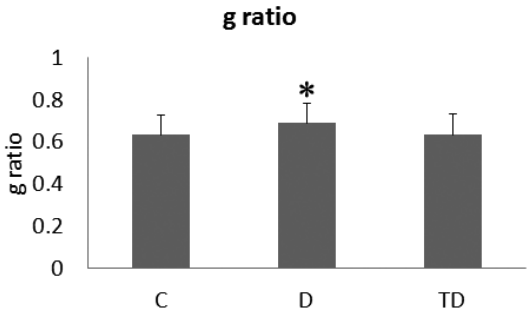
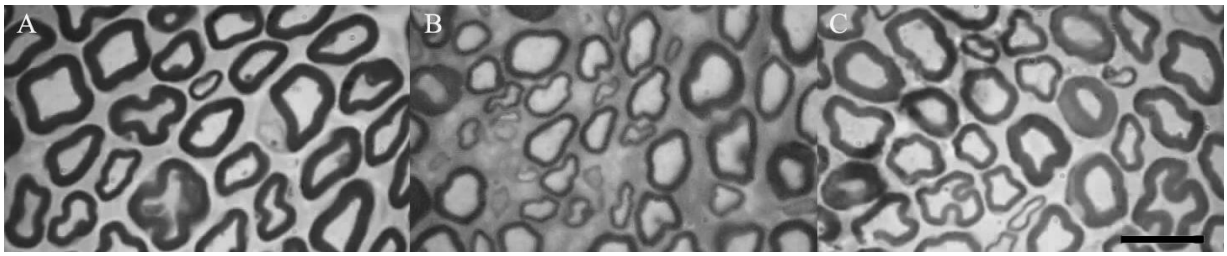


Figure 9



Artigo 3: Aceito para publicação na revista *Clinics*

Clinics

Exercise prevents hypoalgesia and increases the level of calcitonin gene-related peptide in the dorsal horn of the spinal cord of diabetic rats

--Manuscript Draft--

Manuscript Number:	
Full Title:	Exercise prevents hypoalgesia and increases the level of calcitonin gene-related peptide in the dorsal horn of the spinal cord of diabetic rats
Short Title:	Exercise and CGRP in the spinal cord
Article Type:	Original Study
Section/Category:	Basic Research
Keywords:	Hypoalgesia, Tail-flick test, CGRP content, dorsal horn of spinal cord, Diabetic neuropathy
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Exercise prevents hypoalgesia and increases the level of calcitonin gene-related peptide in the dorsal horn of the spinal cord of diabetic rats

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Running title: Exercise and CGRP in the spinal cord

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INTRODUCTION: The aim of this study was to evaluate the effects of treadmill training on nocicpetive sensitivity and immunoreactivity to caltonin gene-related peptide (CGRP-ir) in the dorsal horn of the spinal cord from diabetic rats. METHODS: Male Wistar rats were divided into three groups: control, diabetic and trained diabetic. Treadmill training was performed for 8 weeks. Blood glucose concentrations and body weight were evaluated 48 h after diabetes induction and every 30 days thereafter. Nociceptive sensitivity was evaluated using the tail-flick apparatus. Then, animals were transcardially perfused and the spinal cords were post-fixed, cryoprotected and sectioned in a cryostat. Immunohistochemistry for CGRP analysis was done in the dorsal horn of the spinal cord. RESULTS: Nociceptive sensitivity analysis showed that, compared to the control and trained diabetic animals, the latency to deflect the tail on the apparatus was longer for diabetic animals. Optical densitometry showed a decreased CGRP-ir in the dorsal horn of the spinal cord in diabetic animals which was reversed by treadmill training. CONCLUSION: We demonstrated that treadmill training can prevent the nociceptive hypoalgesia and reverse decreased CGRP-ir in the dorsal horn of the spinal cord of diabetic animals without pharmacological treatment.

Keywords: Hypoalgesia, Tail-flick test, CGRP content, dorsal horn of spinal cord, Diabetic neuropathy

1. Introduction

One of the most common complications of diabetes mellitus is chronic sensorimotor distal simetric polyneuropathy, which sequelae include painless foot ulceration and occasionally lower limb amputations (1). A mutifactorial pathogenesis have been advocated for diabetic neuropathy development, including hyperglycemic-triggered events causing formation of advanced glycation end products, activation of the polyol pathway (2) and excess of reactive oxygen species formation (3, 4), vascular factors (5), growth factors (6) and immune mechanisms (7). All these factors contribute to microvascular and neuronal damage, including sensory neurons (8, 9).

Sensory neurons, located in the dorsal root ganglia, are also affected by chronic hyperglycemia, resulting in a decrease in the production (10- 12) of the most abundant peptide involved in processing nociceptive information, the calcitonin gene-related peptide (CGRP; 13, 14), consequently there is an alteration in the sensory behavior, contributing to sensory loss and its consequences.

Recently, several studies have shown the beneficial effects of physical exercise in patients (15) and rats with diabetes (9, 16- 18). However, as physical exercise has been indicated for people with diabetes, and little is known about the effects of exercise on the processing of nociceptive information, the aim of this study is to investigate the effects of treadmill training on nocicpetive sensitivity and CGRP levels in the dorsal horn of the spinal cord of male diabetic rats.

2. Methods

2.1. Animals

Thirty male Wistar rats (12 weeks old) from a local breeding colony (ICBS, UFRGS) were housed under standard laboratory conditions with food and water available *ad libitum*

and maintained under a 12:12 h light/dark cycle. All efforts were made to minimize the number of animals studied and their suffering. The animals were cared for in accordance with Arouca Brazilian law (11794/2008) and the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary Surgery, University of Buenos Aires, and the International Brain Research Organization, and are in compliance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1985). This study was previously approved by the Ethical Committee of the UFRGS under the protocol number 2008-062.

2.2. Experimental design

The rats were divided into three groups: non-diabetic rats (C), diabetic rats (D) and diabetic rats submitted to treadmill training (TD) for 8 weeks. For the nociceptive analysis using the tail-flick test, 10 animals were used per group. For immunohistochemistry studies, 6 animals were randomly selected per group.

2.3. Diabetes induction

After an overnight fasting period (6 h), rats from the D and TD groups received a single intravenous injection of streptozotocin, STZ (50 mg/kg of body weight; Sigma Chemicals Co., USA) diluted in 10 mM citrate buffer, pH 4.5, while the C group received only citrate buffer (9, 17). Blood collected from the rat tail was used to measure blood glucose concentrations using test strips (Performa, Roche, USA). Diabetes was defined as a fasting glucose > 300 mg/dL in tail vein blood 48h after STZ injection (19). Body weights were measured before diabetes induction and every 30 days thereafter, and blood glucose concentrations were measured 48 h after the induction of diabetes and every 30 days thereafter.

2.4. Maximal exercise test and treadmill training

In the 4th week after diabetes induction, all animals underwent adaptation to a treadmill originally designed for human use (Runner, Brazil) and modified for use by rats by walking for 10 minutes at 5 m/min for 4 days. On the 5th day the rats were submitted to a maximal exercise test (MET), consisting of a graded exercise on the treadmill, with speed increments of 5 m/min every 3 minutes, starting at 5 m/min and continuing up to the maximal intensity attained by each rat, and was stopped when each animal remained more than 50% of the time without giving signs of intention to advance (20, 21). The values obtained in the MET were used to plan the treadmill training program, which started in the 5th week after diabetes induction. In order to correct the exercise intensity, a second MET was performed in the 5th training week.

Exercise was performed on a treadmill twice a day, with an interval of 4 h between each session, 5 days per week and the training intensity was increased gradually, according to the MET results. During the first week, the running sessions lasted 10 min, with increases in duration each week, reaching 60 min in the 7th week, which was maintained until the 8th week. Moreover, each training session consisted of a warm-up period, a main period and a cooling-off period. During the warm up period, the rats ran 15% of the session time at 30% of the maximum velocity determined by the MET; in the main period the rats ran 70% of the session time at 60% of the maximum velocity; and in the cooling-off period the rats ran 15% of the session time at 30% of the maximum MET values (17).

2.5. Nociceptive sensitivity analysis

Nociceptive sensitivity was assessed using the tail-flick apparatus. Rats were wrapped in a towel and placed on the apparatus. The light source positioned below the tail was focused on a point 2–3 cm rostral to the tip of the tail (22). Deflection of the tail activated a photo cell and automatically terminated the test. Moreover, every week the animals were exposed to the tail-flick apparatus to familiarize them with the procedure, since the novelty of the apparatus

can itself induce antinociception (23). The tail-flick latency represented the period of time from the beginning of the test to the tail deflection. Final data are presented as a percentage of the control group, which was assigned a value of 100%, and the values are expressed as mean ± SEM.

2.6. Immunohistochemical Procedure

One day after the nociception analysis, rats were anesthetized with sodium thiopental (i.p; 50 mg/kg; Cristalia, Brazil). Heparin (1000 IU; Cristalia, Brazil) was injected into the left cardiac ventricle, then the animals were transcardially perfused through the left ventricle using a peristaltic pump (Control Company, Brazil, 20 mL/min) with 400 mL of 0.9% saline solution, followed by 400 mL of a fixative solution 4% paraformaldehyde (Synth, Brazil) in 0.1 M phosphate buffer, pH 7.4 (PB). The lumbar spinal cords were removed, post-fixed in the same solution at room temperature for 4 h and cryoprotected by immersion in a 15 and 30% sucrose (Synth, Brazil) solution in PB at 4 °C until they sank. After these procedures, the spinal cords were quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and kept in a freezer (- 70 °C) for further analyses.

Transverse sections (35 µm) from the lumbar spinal cords were obtained using a cryostat (CM1850, Leica, Germany) at -20 °C and collected in a PB saline (PBS), pH 7.4. The free-floating sections were pre-treated with 3% hydrogen peroxide for 30 min, carefully washed and treated with 2% bovine serum albumin (Inlab, Brazil) in PBS containing 0.4% Triton X-100 (PBS-Tx; Sigma Chemical Co, USA) for 30 min and incubated 48 h with polyclonal CGRP-antibody (1:2250; courtesy of Dr. Rodrigo, Cajal Institute, Spain) with gentle stirring at 4 °C. The primary antibody was then removed and the sections washed in PBS-Tx for 30 min. Subsequently, the sections were immersed in a secondary antibody (goat anti-rabbit lgG; Sigma Chemical Co., USA) diluted 1:50 in PBS-Tx, for 2 h at room temperature with gentle stirring. After washing with PBS-Tx 3 times for 15 min, a soluble

complex of horseradish peroxidase rabbit anti-horseradish peroxidase (Sigma Chemical Co., USA) diluted 1:500 was applied for 1h and 30 min at room temperature. The reaction was revealed in a medium containing 0.06% 3,3′-diaminobenzidine (DAB, Sigma Chemical Co., USA) dissolved in PBS for 10 min and then 1 μL of 3% H_2O_2/mL was added to the DAB medium for an additional 10 min. Finally, the sections were rinsed in PBS, mounted on glass slides, dehydrated in ethanol, cleared with xylene and covered with Entellan (Merck, Germany) and coverslips. Control sections were prepared omitting the primary antibody by replacing it with PBS.

2.7. Optical Densitometry

Semi-quantitative densitometric analysis was used to measure the intensity of the CGRP immunoreaction using a Nikon Optiphot-2 microscope (100x, Japan) coupled to a Micrometrics camera (Accu Scope, USA) and Image Pro Plus Software 6.0 (Media Cybernetics, USA). The digitized images obtained from the dorsal horn of the spinal cords were converted to an 8-bit gray scale (0–255 gray levels). All lighting conditions and magnifications were held constant. Picture elements (pixels) employed to measure optical density were obtained from squares measuring 1160 μm^2 (area of interest, AOI) overlaid on the gray scale image. Both left and right sides of each spinal cord were used. For each rat, 10 measures were taken from the dorsal horn of the spinal cord. The results shown for the spinal cords were the total mean value from the three studied regions.

Background staining subtraction and correction were done in accordance with our previous published protocol (24). The optical density (OD) was calculated using the following formula:

$$OD(x,y)=-\log[(INT(x,y)-BL)]/(INC-BL)$$

Where “OD(x,y)” is the optical density at pixel(x,y), “INT(x,y)” or intensity is the intensity at pixel(x,y), “BL” or black is the intensity generated when no light goes through the material and “INC” is the intensity of the incidental light.

2.8. Statistical analysis

Blood glucose and body weight data were analyzed using repeated measures analysis of variance (ANOVA), and differences between the groups were assessed using the Bonferroni *post-hoc* test. Data obtained from the nociceptive test, as well as optical densitometry of CGRP-ir were analyzed using one-way ANOVA and the Bonferroni *post-hoc* test. Statistical significance was set at $P<0.05$. Data were run on the Statistica 6.0 software package (StatSoft, Inc., USA). All data are represented by the mean ± standard error of mean (SEM).

3. Results

3.1. Body weight and blood glucose concentrations

Before diabetes induction there were no differences in the body weight between the C (298 ± 5 g), D (307 ± 16 g) and TD (297 ± 9 g) groups ($P>0.05$). Moreover, 30, 60 and 90 days after the diabetes induction, rats from the D (269 ± 10 g; 256 ± 11 g; 254 ± 11 g, respectively) and TD (281 ± 11 g; 270 ± 8 g; 290 ± 17 g, respectively) groups showed lower body weights than the C group (351 ± 4 g; 384 ± 3 g; 406 ± 3 g respectively; $P<0.001$).

Two days after diabetes induction, blood glucose concentrations were higher in the D (389 ± 21 mg/dL) and TD (352 ± 20 mg/dL) groups compared to the C (86 ± 5 mg/dL) group ($P<0.001$). The blood glucose concentrations were still higher 30, 60 and 90 days in the D (525 ± 25 mg/dL; 534 ± 7 mg/dL; 527 ± 26 mg/dL, respectively) and TD (438 ± 46 mg/dL; 525 ± 22 mg/dL; 535 ± 27 mg/dL, respectively) groups after the diabetes induction, compared

to the C group (90 ± 2 mg/dL; 89 ± 2 mg/dL; 94 ± 3 mg/dL, respectively; $P<0.001$). There were no differences between the D and TD groups in any of those times.

3.2. Nociceptive sensitivity

The latency to the tail deflection of the C group was expressed as 100 %. Group D took 154.7 % more time to deflect the tail than the C group ($P<0.001$), and 120 % more time to deflect the tail than the TD group ($P<0.001$). Moreover, the TD group took just 34.7 % more time to deflect the tail than the C group ($P>0.05$; Figure 1).

3.3. Optical densitometry of CGRP-ir

The OD analysis of the dorsal horn of the spinal cord showed that the CGRP-ir was lower in the D (0.056 ± 0.004) group than the C (0.098 ± 0.003; $P<0.001$) and TD groups (0.096 ± 0.01; $P<0.05$). Moreover, there was no difference between the C and TD groups ($P>0.05$; Figure 2). Representative images are shown in Figure 3.

4. Discussion

In the present study we have shown that only treadmill training, with no pharmacological intervention, can prevent the nociceptive loss caused by the hyperglycemic state in rats with STZ-induced diabetes. This beneficial effect was associated with changes to calcitonin gene-related peptide immunoreactivity in the dorsal horn of the spinal cord.

As expected, diabetic rats display lower body weights and higher blood glucose levels than non-diabetic rats (9, 17). Moreover, treadmill training did not interfere in the body weight or blood glucose levels in rats (9, 17, 25) and in type 1 diabetic patients (26). It is well known that STZ induces an insulin-deficient state similar to type 1 diabetes in humans, which is not usually improved by exercise interventions (27), which, unlike type 2 diabetes, an insulin-resistant state (28), does not usually benefit from exercise interventions intended to control blood glucose levels.

In the analysis of nociceptive sensitivity, measured using the tail-flick test, diabetic rats showed an increase in the latency to deflect the tail from the apparatus, which indicates an STZ-induced loss in sensitivity. This data is in accordance with previous studies which found deficits in the motor and sensory nerve conduction in animals (29, 30), as well as thermal (30, 31) and nociceptive hypoalgesia (31). Moreover, our finding is also in agreement with clinical data showing that diabetes in human s can cause nociceptive hypoalgesia (32- 34), which is an important determinant of lower limb ulcers and amputations.

However, diabetic rats submitted to the treadmill training displayed a latency to deflect the tail from the apparatus similar to that of the non-diabetic animals, showing that the training is able to prevent alterations in the sensitivity behavior of diabetic animals, data previously unreported in the literature. In addition, we examined the effects of the training on the CGRP-ir in the dorsal horn of the spinal cord, and the data show that the physical exercise can maintain the content of this peptide in the spinal cord similar to that of control animals.

It is well known that the diabetes causes a reduction in the content of CGRP in peripheral nerves (10, 11, 35), as well as in the dorsal root ganglia neurons and in the dorsal horn of the spinal cord (36). Although physical training has been considered in the treatment of diabetic patients for some time, it was not known, until now, if training was able to prevent alterations in the nociceptive signaling and CGRP content in the spinal cord. The benefits of treadmill training in these parameters could arise from the effects of the nerve growth factor (NGF) and neurotrophin-3 (NT-3). These neurotrophic factors promote neuronal survival and differentiation, by activating the phosphatidylinositol 3-kinase signaling (PI3-K). Moreover, there are various studies showing that treadmill training increases the level of NGF in the sensory neurons (37) and in the hippocampus (38, 39), of NT-3 in the spinal cord (40) and of NGF and NT-3 in soleus muscle (41, 42).

Conclusions: we conclude that treadmill training is able to prevent the nociceptive hypoalgesia caused by diabetes, and this improvement is related to the content of CGRP in the dorsal horn of the spinal cord.

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Figure Legends:

Figure 1: Nociceptive sensitivity from C, D and TD groups. One-way analysis of variance (ANOVA), followed by the Bonferroni's *post hoc* test. * corresponds to $P < 0.001$ compared to C and TD groups.

Figure 2: CGRP immunoreactivity in the dorsal horn of the spinal cord from C, D and TD animals. One-way analysis of variance (ANOVA), followed by the Bonferroni's *post hoc* test.* corresponds to $P < 0.05$ compared to C and TD.

Figure 3: Representative digitalized images of transverse sections of spinal cord in C, D and TD animals showing the CGRP immunoreactivity in the dorsal horn. Note the decreased immunoreaction in the D group. Scale bar: 100 μm.

Figure 1

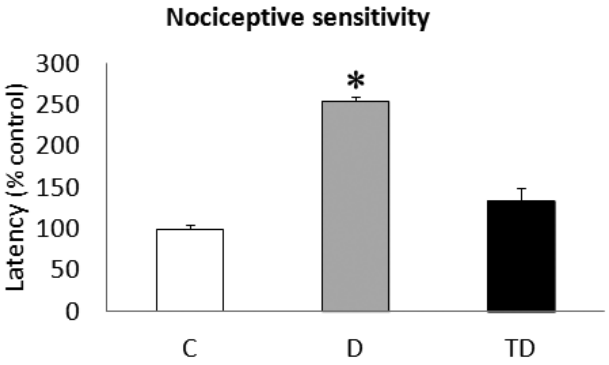


Figure 2

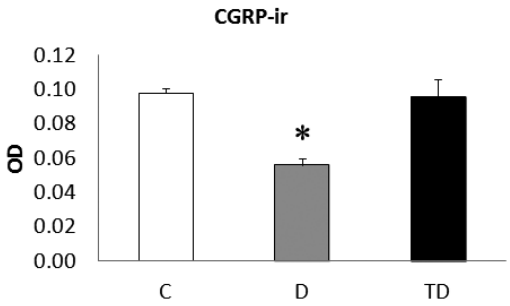
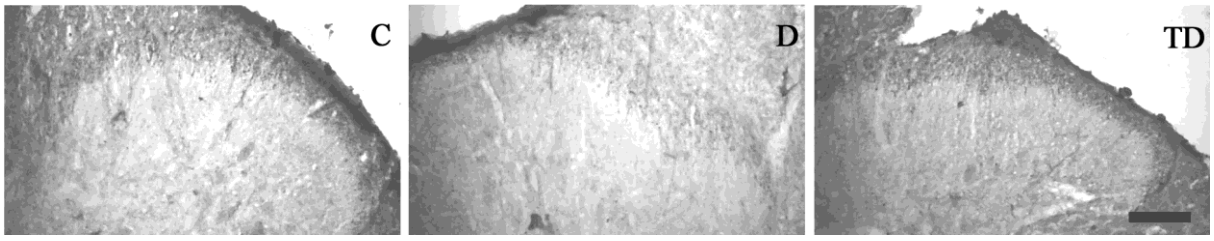


Figure 3



5 Discussão

Este trabalho teve como objetivo geral analisar os efeitos do exercício físico em esteira sobre o comportamento motor e imunorreatividade à tirosina hidroxilase na substância nigra e área tegmental ventral, bem como sobre a sensibilidade mecânica e nociceptiva, morfometria do nervo sural e imunorreatividade ao peptídeo relacionado ao gene da calcitonina no corno dorsal da medula espinal de ratos machos adultos diabéticos induzidos por estreptozotocina.

A necessidade do entendimento da forma e meios pelos quais o exercício físico pode contribuir para a prevenção ou atraso do aparecimento das complicações decorrentes do diabetes se torna cada vez mais importante, uma vez que na atualidade o exercício físico regular se encontra indissociável dos conceitos de saúde e qualidade de vida (SCLIAR, 2007).

No primeiro artigo apresentado nesta tese, publicado na revista *Brain Research* no ano de 2011, mostrou os efeitos de 8 semanas de exercício físico em esteira sobre o comportamento motor, analisados pelos testes no campo aberto e rotarod, e a imunorreatividade à TH na substância nigra e área tegmental ventral de ratos diabéticos induzidos pela STZ. Neste trabalho demonstramos que o exercício físico é capaz de prevenir o aparecimento de alterações motoras em ratos diabéticos, o que não foi demonstrado nos animais diabéticos e sedentários. As alterações demonstradas no teste do rotarod, tais como a diminuição na latência de queda e o aumento do número de quedas do aparato, demonstram óbvios déficits de coordenação e equilíbrio nos animais diabéticos, os quais não foram prevenidos pelo treinamento em esteira.

O aparelho do rotarod vem sendo amplamente utilizado em estudos que necessitam caracterizar as habilidades motoras em animais (SHIOTSUKI et al., 2010), inclusive no estudo das alterações motoras decorrentes do DMT1 em ratos (PEEYUSH et al., 2010). Já havia sido demonstrado que o diabetes causa alterações motoras importantes em pessoas e animais (BALDUCCI et al., 2006; ANU et al., 2006), no entanto, essas alterações foram relacionadas com alterações em nervos motores (BALDUCCI et al., 2006) e fraqueza

muscular (LORD et al., 1993). Embora o diabetes seja considerado como uma fator de risco para a doença de Parkinson (SUN et al., 2012), as alterações motoras decorrentes dessa doença não haviam sido, até o momento, correlacionadas com alterações na neurotransmissão na substância nigra de animais diabéticos, como demonstramos no artigo 1 desta tese. Os parâmetros analisados no campo aberto, demonstrados no mesmo artigo, como o número de quadrantes cruzados, o tempo gasto em movimento e o número de *rearings*, demonstram bradicinesia nos animais diabéticos. Estes dados reforçam a relação existente entre o diabetes e a doença de Parkinson, uma vez que a bradicinesia é uma das alterações marcantes na doença de Parkinson (BÉNÉ et al., 2009).

Ademais, o dado marcante deste trabalho foi o de que o exercício físico, mesmo na ausência de tratamento farmacológico, foi capaz de prevenir as alterações motoras funcionais e ao mesmo tempo, foi capaz de reverter a diminuição da imunorreatividade à TH na substância nigra dos ratos diabéticos, dado este ainda não mostrado na literatura. Uma vez que a TH é uma enzima fundamental na síntese de dopamina, quando mostrada a diminuição da sua reação nos ratos diabéticos sedentários, se pode inferir que houve diminuição da síntese de dopamina naquela região encefálica. Por outro lado, o treinamento em esteira normalizou a reação, ficando a marcação da imunorreatividade à TH nos ratos diabéticos treinados semelhante à dos animais controle.

A melhora das habilidades motoras e da imunorreatividade à TH mostrada nos animais exercitados pode ter sido causada pelos efeitos do fator neurotrófico derivado do encéfalo (BDNF, do inglês *brain derived neurotrophic factor*), uma neurotrofina importante para a manutenção de neurônios relacionados com o controle motor, a qual é capaz de promover a sobrevivência desses neurônios (KOLIATSOS et al., 1993). Além disso, já haviam demonstrado previamente que o exercício é capaz de alterar os níveis dessa neurotrofina no

hipocampo (CHAE et al., 2009), na medula espinal (MACIAS et al., 2007), no cerebelo e no córtex motor (KLINTSOVA et al., 2004).

Estes dados renovam a importância da prática de exercício físico na presença do diabetes com o objetivo de retardar o aparecimento e prevenção de alterações motoras, dentre elas incoordenações e bradicinesias, as quais contribuem para as quedas, frequentes entre pessoas que tem diabetes e idade avançada.

No segundo artigo desta tese mostramos alteração na sensibilidade mecânica, ocorrendo hipersensibilidade nos animais diabéticos, e alterações morfológicas no nervo sural, tais como diminuição na área e diâmetro da fibra mielinizada, aumento da densidade de fibras mielinizadas/mm^2, aumento da área ocupada por tecido conjuntivo, diminuição da espessura da bainha de mielina das fibras mielinizadas e aumento do índice de mielinização (relação g) no grupo diabético sedentário. Alterações morfológicas em nervo periférico haviam sido demonstradas em outros estudos (WATTIG et al., 1989; XU et al., 2011; KAMIYA et al., 2009). Nossos novos achados, por outro lado, mostraram que o exercício físico em esteira é capaz de impedir essas alterações no nervo sural.

Ainda, foi constatada, no nervo sural dos animais diabéticos sedentários, uma alteração na quantidade de fibras mielinizadas grandes e pequenas, isto é, os animais diabéticos apresentaram maior porcentagem de fibras de pequeno calibre e menor porcentagem de fibras mielinizadas de grande calibre.

As fibras mielinizadas de pequeno calibre representam fibras Aδ responsáveis pela transdução do sinal nociceptivo e os levam ao CDME, principalmente na lâmina I de Rexed. Por outro lado, as fibras mielinizadas de maior calibre representam as fibras Aβ que levam a informação dos mecanoceptores até, principalmente, à lâmina V de Rexed ao CDME. Ambas fazem sinapse no CDME em um neurônio de projeção que envia a informação aos centros superiores e em um interneurônio inibitório, o qual faz sinapse no mesmo neurônio de

projeção, o inibindo (PAXINOS e MAI, 2003). Dessa maneira, conforme a teoria das comportas da dor proposta por Melzack e Wall em 1965, enquanto o estímulo mecânico excita o interneurônio inibitório, impedindo a ativação do neurônio de projeção, o estímulo nocicpetivo inibe o interneurônio inibitório, permitindo que o sinal nociceptivo atinja os centros superiores pela ativação direta do neurônio de projeção (MCMAHON e KOLTZENBURG, 2005). Portanto, como os animais diabéticos mostraram maior quantidade de fibras mielinizadas de pequeno calibre, a partir disso podemos explicar o motivo pelo qual esses animais se mostraram mais sensíveis aos estímulos aplicados pelos filamentos de von Frey.

Em adição, se torna importante enfatizar o efeito do exercício físico sobre a sensibilidade mecânica, uma vez que nos animais diabéticos treinados não ocorreu a alteração na sensibilidade mecânica assim como na distribuição das fibras mielinizadas pequenas e de maior calibre.

Estes dados, apresentados no artigo 2 desta tese, se tornam importantes para o entendimento de como o exercício físico pode se tornar um aliado para o portador de diabetes, uma vez que 65% dos portadores de diabetes tipo 1 e 2 apresentarão algum sinal de neuropatia periférica (DYCK et al., 1992).

No 3° artigo apresentado nessa tese, vimos que o diabetes foi capaz de reduzir a sensibilidade nociceptiva desses animais, ou seja, os animais diabéticos demoravam mais tempo para responder à estimulação sensorial nociceptiva. Fato que comumente ocorre na neuropatia diabética periférica indolor, na qual a pessoa perde a sensibilidade dolorosa o que contribui de forma significativa para o desenvolvimento das lesões nos pés, as quais podem resultar em amputações (BRITLAND et al., 1990). Estes dados, em conjunto com os dados do artigo 2 mostram que ao mesmo tempo em que ocorre uma perda da sensibilidade nociceptiva,

há aumento da sensibilidade mecânica nos ratos diabéticos e sedentários, mostrando importante alteração sensorial no diabetes.

Além disso, foi demonstrado que em lesões de nervos periféricos, ao mesmo tempo em que há destruição das fibras do tipo C e Aδ na lâmina II do CDME, há também um brotamento central das fibras Aβ de mecanoceptores da lâmina V em direção à lâmina II do CDME, ocorrendo uma reorganização estrutural nos nervos periféricos na qual, mesmo com diminuição da quantidade de fibras C, há ocorrência de dor provocada por fibras Aβ (WOOLF et al., 1992). Nós pensamos que este mesmo mecanismo pode estar envolvido na transmissão de estímulos mecânicos e nociceptivos no diabetes.

Embora tenham ocorrido essas alterações sensoriais nos animais diabéticos, o mesmo não ocorreu nos ratos treinados em esteira, ressaltando, mais uma vez, os efeitos que o exercício físico pode trazer à pessoa diabética.

6 Conclusões e perspectivas

Os resultados apresentados nesta tese nos permitem concluir que:

- O exercício físico em esteira em ratos diabéticos mantém a coordenação motora, a mobilidade, a atividade exploratória do animal, a sensibilidade aos estímulos mecânicos e nociceptivos;
- Essas alterações comportamentais nos animais diabéticos, assim como os efeitos do exercício físico são relacionados com alterações na imunorreatividade à tirosina hidroxilase na substância nigra, na morfologia do nervo sural e na imunorreatividade ao peptídeo relacionado ao gene da calcitonina no corno dorsal da medula espinal.

Como perspectivas futuras sugerimos que sejam estudados:

- Os efeitos do exercício físico em ratos diabéticos sobre a expressão de neurotrofinas dependentes de atividade (BDNF, NGF e NT3) no SNC e periférico;
- A ativação da morte neuronal por apoptose, através da imunomarcação de caspases no SNC e periférico de ratos diabéticos submetidos ao exercício físico;
- Estudar ultraestuturalmente a substância nigra e medula espinal dos ratos diabéticos submetidos ao treinamento em esteira.

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