

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Modelo murino da mucopolissacaridose tipo I (MPS I): desenvolvimento de
vetores virais e estudo de parâmetros fisiopatológicos

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Lista de abreviaturas

AAV - vírus adeno-associado

FI - Filamento intermediário

FIV - *Feline immunodeficiency virus* (Vírus da imunodeficiência felina)

GAG - Glicosaminoglicano

GFAP - Proteína glial fibrilar ácida

GD1a - Gangliosídeo GD1a

GD1b - Gangliosídeo GD1b

GFP - *Green fluorescent protein* (Proteína verde fluorescente)

GM1 – Gangliosídeo GM1

GSL – Glicoesfigolipídeo

GT1b - Gangliosídeo GT1b

HIV - *Human immunodeficiency virus* (Vírus da imunodeficiência humana)

IDUA - α -L-iduronidase

MF - Microfilamento

MPS - Mucopolissacaridose

MoMLV - *Moloney murine leukemia virus* (Vírus da leucemia murina de Moloney)

MT - Microtúbulo

NF - Neurofilamento

Resumo

A mucopolissacaridose tipo I (MPS I) é uma doença lisossomal. A enzima envolvida com essa doença é a α -L-iduronidase (IDUA), e suas alterações levam a depósitos dos glicosaminoglicanos heparan e dermatan sulfato. As características fenotípicas da doença compreendem entre outras características malformações ósseas, hepatoesplenomegalia, problemas cardíacos, opacidade da córnea e retardo mental.

O tratamento de MPS I atualmente se baseia em reposição enzimática e transplante de medula óssea. Levando em conta que esses tratamentos ainda não levam a uma correção dos danos ao sistema nervoso central (SNC), há necessidade do desenvolvimento de novos tratamentos para MPS I. Ainda outro problema da MPS I é a falta de estudos sobre os danos fisiológicos resultantes, principalmente no SNC. Visando contribuir com estes pontos, nosso trabalho teve como objetivo (a) o desenvolvimento de três vetores virais para posteriores ensaios pré-clínicos de terapia gênica e (b) o detalhamento da caracterização fisiopatológica de camundongos representando um modelo para MPS I, enfocando alguns aspectos do SNC.

Foram produzidos três vetores virais com o cDNA da IDUA humana. O vetor baseado no vírus da imunodeficiência humana (HIV), apesar de baixos títulos virais, foi capaz de aumentar em até 60 vezes a atividade da IDUA nas células transduzidas. Um segundo vetor, baseado no vírus da leucemia murina de Moloney (MoMLV) foi capaz de transduzir células-tronco mesenquimais derivadas de medula óssea do modelo murino de MPS I, sendo assim ótima ferramenta para terapia gênica *ex vivo*. O terceiro vetor, baseado no vírus da imunodeficiência

felina (FIV) mostrou funcionalidade inferior quando comparado aos outros sistemas virais, e deve ser aperfeiçoado para utilização terapêutica.

A caracterização do modelo foi feita através do estudo da fosforilação de proteínas de neurofilamentos em diferentes estruturas do cérebro de camundongos MPS I. Os resultados mostraram, além de alterações na fosforilação direta das proteínas analisadas, uma hiperfosforilação na ERK1/2 de córtex e hipocampo. Depósitos de gangliosídeos foram investigados de forma individual no cerebelo, córtex, hipocampo e hipotálamo, sendo detectadas diferenças específicas para cada estrutura. Ainda foram realizados estudos da neurotransmissão glutamatérgica e com isso foi identificada uma diminuição na captação de glutamato em hipocampo e córtex nos camundongos MPS I, além de uma diminuição no *binding* para glutamato no hipocampo.

Como conclusão desses estudos, foram desenvolvidos vetores eficientes para transferência do gene terapêutico e, adicionalmente, os danos fisiológicos causados pela doença no SNC foram melhor elucidados no modelo murino de MPS I. As informações obtidas aqui serão de grande importância principalmente quando associadas a ensaios de terapia gênica, pois as características encontradas no SNC podem ser usadas para acompanhamentos de ensaios pré-clínicos usando os vetores desenvolvidos no trabalho.

Abstract

Mucopolysaccharidosis type I (MPS I) is a monogenic disease resulting from a defect in the gene that encodes the lysosomal hydrolase α -L-iduronidase (IDUA) and characterized by accumulation of glycosaminoglycans (GAGs) heparan and dermatan sulfate. Phenotypic characteristics involve bone malformations, hepatosplenomegaly, heart problems, corneal opacity and mental retardation.

Treatment of MPS I is currently based on enzymatic replacement and bone marrow transplantation. Since these treatments are not capable of correcting central nervous system (CNS) effects of the disease, new therapeutic approaches are needed. One of the problems in the investigation of MPS I is the paucity of studies about resulting physiological consequences, particularly those related to the CNS. Aiming to contribute to these aspects of MPS I, this study proposes (a) the development of three viral vectors to be used in gene therapy preclinical studies, and (b) the further investigation of physiopathological alterations in the CNS of a murine model of MPS I (MPS I mice).

Three viral vectors, carrying the human IDUA gene, were produced. The vector based on the human immunodeficiency virus (HIV) was produced in low titers, but induced a 60-fold increase in the baseline activity of IDUA in transduced cells. A second vector, based on the Moloney murine leukemia virus (MoMLV), was capable to transduce mesenchymal stem cells isolated from the bone marrow of MPS I mice, representing an interesting tools for *ex vivo* gene therapy protocols. The third vector, was based on the feline immunodeficiency virus (FIV), was

functionally inferior to the other two systems and needs optimization to be therapeutically useful.

The model was characterized by the investigation of phosphorylation patterns of neurofilament proteins in different regions of the brain of MPS I mice. The results showed that, besides alterations in the direct phosphorylation profile of the proteins analyzed, ERK1/2 was hyperphosphorylated in the cortex and hippocampus. Ganglioside storage was individually investigated in the cortex, cerebellum, hippocampus and hypothalamus, and specific differences were observed for each structure. An analysis of glutamatergic neurotransmission was also performed. MPS I animals showed a decrease in the glutamate uptake in the cortex and hippocampus. In addition, glutamate binding was decreased in the hippocampus.

In conclusion, the present work resulted in the development of efficient viral vectors for the transfer of the therapeutic gene, and CNS physiological damages due to the disease were characterized in the murine model of MPS I. These results will be of great importance particularly when associated to gene therapy trials, since the CNS characteristics described in this work may be used for the follow-up of preclinical assays with the viral vectors developed.

Capítulo 1

Introdução

1 Introdução

1.1 As mucopolissacaridoses

As doenças de depósito lisossomal são doenças genéticas resultantes de mutações em genes envolvidos em rotas metabólicas os quais são codificadores de hidrolases ácidas. A perda ou redução da atividade enzimática leva à disfunção celular devido ao acúmulo de substratos não degradados nos lisossomos. A maioria dessas doenças acarreta lesões de depósito global no sistema nervoso central (SNC), resultando em neurodegeneração progressiva e retardo mental (Passini *et al.*, 2003; Fuller *et al.*, 2004).

As mucopolissacaridoses (MPS) fazem parte das doenças lisossomais que constituem um grupo heterogêneo de doenças monogênicas de depósito lisossomal caracterizadas pela deficiência total ou parcial de uma enzima envolvida na rota de degradação dos glicosaminoglicanos (GAGs) (Fuller *et al.*, 2004). Na Figura 1 pode-se identificar as enzimas responsáveis por cada passo desta rota de degradação. Várias formas clínicas de MPS já foram identificadas e descritas, sendo classificadas numericamente de MPS I a MPS IX. Cada uma destas formas é resultado de mutações em genes codificantes de enzimas específicas na rota (Tabela 1).

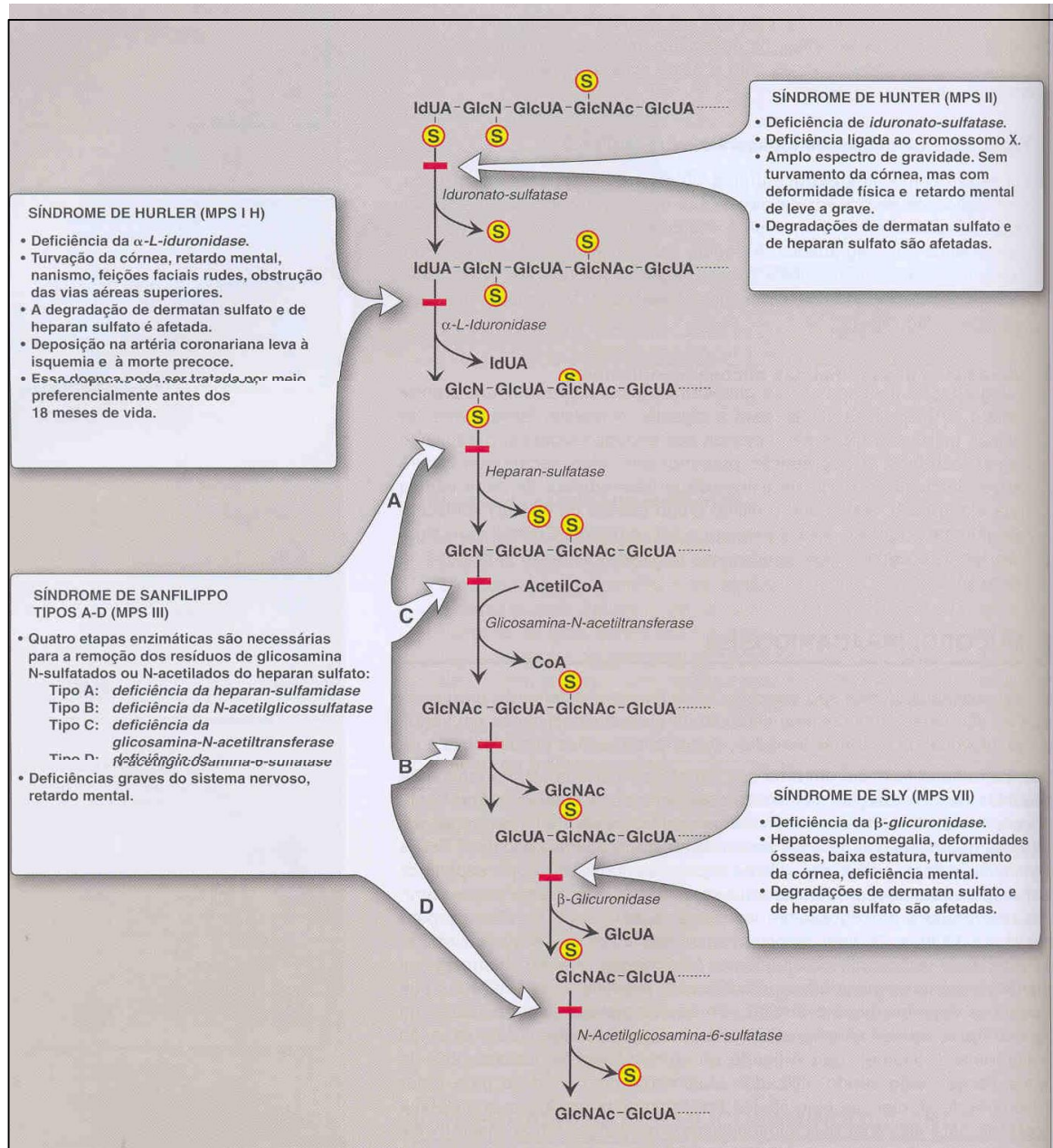


Figura 1. Rota de degradação de GAGs. Modificado de Champe *et al.* (2005).

Tabela 1. Classificação das mucopolissacaridoses. Modificado a partir de Clarke (2008).

Mucopolissacaridose	Sigla	Enzima afetada
Hurler		
Hurler-Scheie	MPS I	α -L-iduronidase
Scheie		
Hunter	MPS II	Iduronato sulfatase
Sanfilippo A	MPS III A	Heparan sulfato N-sulfatase (sulfamidase)
Sanfilippo B	MPS III B	α -N-Acetilglicosaminidase
Sanfilippo C	MPS III C	Acetiltransferase
Morquio A	MPS IV A	Galactosamina- 6-sulfatase
Morquio B	MPS IV B	α -Galactosidase
Maroteaux-Lamy	MPS VI	arilsulfatase B
Sly	MPS VII	α -Glicuronidase
MPS IX	MPS IX	Hialuronidase

A classificação e o quadro clínico das formas de MPS são determinados diretamente pelo tipo de enzima deficiente e pelo acúmulo de GAGs específicos nos lisossomos. Apesar de existirem diferentes fenótipos clínicos para o mesmo defeito enzimático, também são encontrados fenótipos semelhantes para deficiências enzimáticas diferentes (Fuller *et al.*, 2004).

As MPS são doenças raras, mas em conjunto representam uma proporção significativa das doenças metabólicas hereditárias severas, com uma incidência mundial estimada de 1:10.000 a 1:25.000 recém-nascidos vivos. Todas as MPS são doenças autossômicas recessivas, com exceção da MPS II que é ligada ao cromossomo X (Liu *et al.*, 2005).

1.2 A mucopolissacaridose tipo I

A mucopolissacaridose tipo I (MPS I) é uma doença autossômica recessiva, sendo resultante de um defeito no gene que codifica uma hidrolase lisossomal: α -L-iduronidase (IDUA). Esta enzima tem a função de degradar dois GAGs específicos, heparan e dermatan sulfato. A ausência ou a quantidade ativa diminuída desta enzima acarreta o acúmulo desses GAGs nas células (Neufeld e Muenzer, 1995). A MPS I é um dos tipos de MPS mais comuns e melhor caracterizados no Brasil, com uma incidência de 1:46.000 a 1:120.000 nascidos vivos (<http://www.mpsbrasil.org.br>).

O fenótipo da doença pode ser muito variável, desde um quadro mais severo (síndrome de Hurler), passando por um nível intermediário (Hurler-Sheie) ou mais brando (síndrome de Sheie) da patologia. Indivíduos com baixos níveis ou ausência de IDUA apresentam efeitos deletérios na infância e pré-

adolescência devido ao acúmulo dos GAGs em diferentes órgãos, incluindo o SNC, sistema reticuloendotelial e esqueleto. Outras características importantes desses pacientes são a excreção aumentada de GAGs na urina, e na maioria dos casos os pacientes apresentam disfunção neurológica progressiva. Os pacientes com MPS I apresentam sinais anormais em exames neurológicos, como nas imagens de ressonância magnética. Estas análises também são importantes para melhor compreensão dos efeitos da doença, caracterizando quais as regiões cerebrais que são mais afetadas (Matheus *et al.*, 2004). Esses pacientes são severamente afetados ainda na primeira década de vida, na maioria das vezes chegando a óbito (Zheng *et al.*, 2003).

A MPS I, assim como as outras formas, é uma doença muito complexa e caracteriza-se, principalmente, pelo depósito do heparan e dermatan sulfato, como citado anteriormente (Zheng *et al.*, 2003; Liu *et al.*, 2005). Entretanto, nessa doença sabe-se muito pouco sobre a alteração na dinâmica celular causada por esse acúmulo. Os danos neurológicos existentes nos pacientes com MPS ainda não foram totalmente elucidados. Atualmente, existe uma grande carência de biomarcadores, sejam eles bioquímicos ou moleculares, que possam nos embasar nos estudos de parâmetros fisiopatológicos da doença. A descrição desses fatores nos possibilitaria uma investigação mais aprofundada de tratamentos, além de servirem como ferramentas adicionais para o acompanhamento de estudos e aplicações pós-tratamento. Diante dessa necessidade, alguns fatores já muito estudados podem ser candidatos para um estudo mais aprofundado, como caracterização e quantificação dos depósitos de gangliosídeos e colesterol em diferentes regiões do cérebro, alterações na

fosforilação das proteínas que compõem os neurofilamentos do citoesqueleto e neurotransmissão glutamatérgica.

O fato dessas doenças lisossomais serem raras e amplamente heterogêneas quanto ao *background* genético dificulta estudos controlados em seres humanos. Para isso, dispomos de modelos animais com defeitos genéticos e que representam o fenótipo encontrado em humanos (Suzuki e Mansson, 1998). Existem modelos de doenças lisossomais induzidas que são importantes ferramentas para estudos terapêuticos e estudos básicos sobre a doença representada.

Os modelos murinos de MPS I descritos por Russel *et al.* (1998) e Zheng *et al.* (2003), e ainda um modelo imunodeficiente por Garcia-Rivera *et al.* (2007), demonstram características também presentes nos pacientes com a doença. Os animais têm deformidades ósseas, acúmulo de GAGs nos órgãos, excreção de GAGs aumentada na urina e acúmulo de gangliosídeos no cérebro.

Nosso grupo de pesquisa mantém uma colônia de camundongos de um modelo de MPS I estabelecida a partir de animais fornecidos pela Dra. Elizabeth Neufeld (UCLA, CA, EUA), conforme descrito por Zheng *et al.* (2003). As características fenotípicas desses animais, bem como nos pacientes de MPS I, são progressivas. Na colônia de MPS I observam-se, além das características descritas acima, diminuição no tempo de vida e um agravamento nos sintomas a partir dos cinco meses. Reolon *et al.* (2006) avaliaram o comportamento desses animais através de testes como exploração de campo aberto, memória no reconhecimento de objetos e esQUIVA inibitória. Os animais do modelo de MPS I avaliados nestes testes comportamentais demonstraram um comportamento

normal quanto à locomoção e ansiedade, apresentando apenas uma redução na exploração vertical no teste do campo aberto, e uma alteração na memória de longa duração quando avaliados no teste de esquiva inibitória. Os animais do modelo de Garcia-Rivera *et al.* (2007) foram avaliados quanto aos mesmos parâmetros comportamentais confirmando as alterações no comportamento anteriormente descritas. Jordan *et al.* (2005) detalharam ainda o perfil cardíaco desses animais, observando que anomalias funcionais do coração representam uma das características principais desse modelo. Este mau funcionamento se agrava entre seis e dez meses de vida. O coração apresenta-se maior e com defeitos nas válvulas e paredes cardíacas, quando comparado com o de animais normais; essas características assemelham-se às de pacientes portadores da síndrome de Hurler (MPS I-H), que desenvolvem cardiomiopatia e patologias coronárias (Hirth *et al.*, 2007).

Como já descrito nesses modelos, as células desses animais não apresentam atividade enzimática e mostram depósitos de GAGs nas células de fígado, baço, cérebro, rim, coração, entre outros (Russel *et al.*, 1998; Zheng *et al.*, 2003; Camassola *et al.*, 2005; Garcia-Rivera *et al.*, 2007). Além destas características moleculares, Reolon *et al.* (dados não publicados) descreveram um desbalanço oxidativo apresentando alterações em superóxido dismutase, catalase, glutathione peroxidase em tecidos retirados de cerebelo, pulmão, diafragma, baço, fígado e rim.

Os GAGs estão envolvidos em muitos processos fisiológicos e a alteração nas suas estruturas moleculares e quantidades pode comprometer a fisiologia das células (Figura 2).

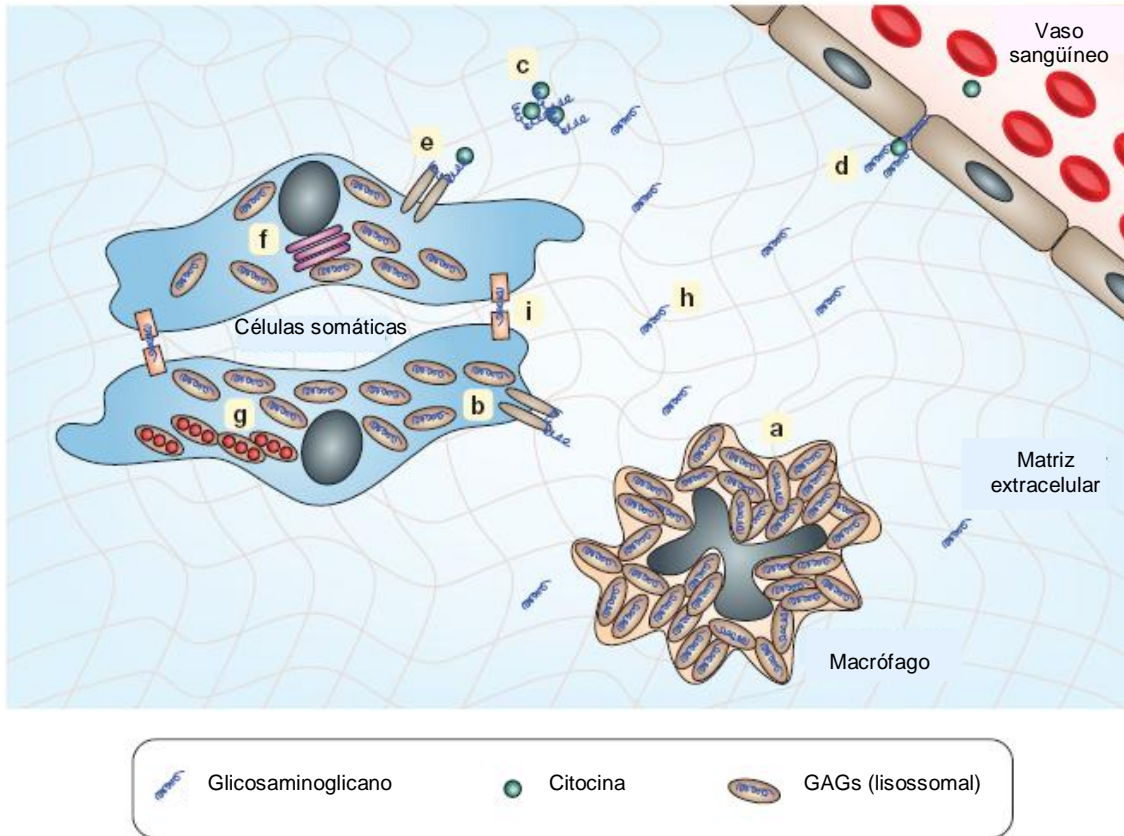


Figura 2. Representação de processos fisiológicos que podem ser alterados devido ao acúmulo de GAGs. Modificado de Clarke (2008).

1.3 Candidatos a fatores bioquímicos envolvidos com a MPS I

Apesar de vários estudos para a descrição das mucopolissacaridoses, há uma carência de parâmetros bioquímicos e moleculares que possam elucidar o mecanismo de alteração funcional neuronal, tanto dos portadores de MPS I como dos modelos animais descritos acima. São descritos a seguir possíveis candidatos, relacionados a processos fisiológicos amplamente descritos na literatura.

Gangliosídeos e colesterol

Dentro do conjunto de glicolipídeos descritos, os glicoesfilipídeos (GSLs) são os mais complexos. Eles contêm oligossacarídeos com um ou mais resíduos de ácido siálico, o que lhes confere carga total negativa. Os gangliosídeos fazem parte principalmente das membranas plasmáticas das células; entre os vários tipos existentes, os mais expressos no sistema nervoso central são os gangliosídeos GM1, GD1a, GD1b e GT1b (Zeller e Marchase, 1992). A rota metabólica de síntese ocorre, principalmente, no complexo de Golgi com a adição seqüencial de oses, a partir de nucleotídeos açúcares. A via de degradação dos GSLs envolve enzimas hidrolíticas encontradas nos lisossomos. A regulação destas rotas depende de vários fatores, incluindo o nível de expressão e das atividades das enzimas envolvidas, a necessidade de reposição na membrana plasmática e a ocorrência de estímulos externos (Maccioni *et al.*, 1999; Tettamanti, 2004).

Os gangliosídios GM1 e GM3 são GSLs que se apresentam depositados em algumas doenças lisossomais de depósito, entre elas a MPS I. Diversos estudos evidenciam o acúmulo destes GSLs em extratos de cérebro total no modelo murino de MPS I (Russel *et al.*, 1998; McGlynn *et al.*, 2004). McGlynn *et al.* (2004) demonstraram o acúmulo co-localizado de colesterol e gangliosídeos em neurônios, em um modelo murino de MPS I, através de técnicas histológicas.

As funções dos gangliosídeos ainda não estão totalmente caracterizadas e descritas. Sabe-se que, em células epidérmicas, estão envolvidos na proteção da membrana plasmática contra situações desfavoráveis, como baixo pH e enzimas de degradação. Além disso, tanto os GSLs como o colesterol estão envolvidos

nos efeitos elétricos e na concentração de íons como Ca^{+2} bem como na formação dos *rafts* de membrana, característica importante para um bom funcionamento do SNC, porque participam diretamente em rotas de sinalização (Harder e Simons, 1999). Uma possível alteração no perfil dos gangliosídeos em diferentes regiões do cérebro pode explicar defeitos principalmente na passagem de moléculas. Isso torna esses compostos importantes candidatos em estudos visando à elucidação de novos mecanismos da disfunção neuronal em indivíduos portadores de MPS I.

Componentes do citoesqueleto

O citoesqueleto das células eucarióticas compreende uma rede formada principalmente por três elementos primários: microfilamentos de actina, microtúbulos e filamentos intermediários (IF) (Dudek e Garcia, 2001).

Entre os filamentos intermediários, os neurofilamentos (NFs) são componentes importantes do citoesqueleto neuronal. Em sistemas neuronais de mamíferos, os NFs são compostos por três proteínas principais (proteínas de neurofilamentos – NFP) de massa molecular de 68, 145 e 200 kDa e são referidas como subunidades de neurofilamentos de baixo, médio e alto (*light, medium e high*) peso molecular, ou NFL, NFM e NFH, respectivamente. Essas três proteínas podem ser fosforiladas tanto *in vitro* quanto *in vivo*. O NFL é pouco fosforilado, enquanto NFM e NFH são mais fosforilados, pois possuem regiões C-terminais contendo múltiplos sítios de fosforilação. A fosforilação dessas proteínas pode regular o volume ocupado pelo NF, porque no caso das NFM e NFH existem regiões importantes que, quando fosforiladas, podem definir a projeção do

tamanho NF (Black e Lee, 1988). Ainda, os NFs participam de interações, dependentes de fosforilação, com outras estruturas do citoesqueleto. Os NFs têm sido considerados os principais determinantes do calibre de axônios, e esse calibre é que controla a condução e a velocidade da transmissão de moléculas (Tu *et al.*, 1995). Estudos na literatura sugerem o envolvimento de alterações no citoesqueleto de células neuronais em doenças neurodegenerativas, que fazem com que os neurônios percam a conectividade sináptica e a capacidade de transmitir informações (Lariviere e Julien, 2004).

Em vista da ausência na literatura de estudos sobre o envolvimento das alterações nos neurofilamentos com a doença MPS I, é de grande relevância a investigação desta relação devido à importância dos NFs para o ótimo funcionamento do SNC.

Glutamato

O aminoácido L-glutamato é o principal neurotransmissor excitatório envolvido na plasticidade sináptica do SNC de mamíferos, estando envolvido em diversas funções cerebrais tais como cognição, aprendizado, memória e formação de redes neurais durante o desenvolvimento (Izquierdo e Medina, 1997; Ozawa *et al.*, 1998; Castellano *et al.*, 2001).

Os receptores glutamatérgicos são agrupados em duas classes distintas: receptores ionotrópicos e metabotrópicos (Ozawa *et al.*, 1998). Os receptores ionotrópicos são assim denominados por permitirem a passagem de um cátion específico quando ativados por um agonista (Ozawa *et al.*, 1998), tendo sido subdivididos em N-metil-D-aspartato (NMDA), α -amino-3-hidróxi-5-metil-4-

isoxazol-ácido propiônico (AMPA) e ácido caínico (KA), de acordo com a sensibilidade a estes agonistas. Os receptores metabotrópicos (mGluR) pertencem a uma família de receptores que estão acoplados às proteínas ligantes de nucleotídeos da guanina (proteínas G), promovendo então a modulação de efetores intracelulares que por sua vez ativam e/ou inibem diversos eventos de transdução de sinal (Conn e Pin, 1997; Ozawa *et al.*, 1998).

O glutamato, após ser liberado para o espaço extracelular e exercer sua ação via seus receptores, é removido da fenda sináptica principalmente por sistemas de transporte que são dependentes de sódio, localizados nos neurônios e principalmente nas células gliais (Anderson e Swanson, 2000; Danbolt, 2001; Amara e Fontana, 2002).

Demonstramos anteriormente que os animais do modelo murino para MPS I, em testes comportamentais que avaliam a aprendizagem e memória, apresentam um prejuízo cognitivo na avaliação da memória de longa duração (Reolon *et al.*, 2006). Tendo em consideração a importância do glutamato para a formação da memória e plasticidade sináptica, a avaliação de parâmetros da sinalização glutamatérgica como a *binding* (ensaio de ligação do neurotransmissor glutamato aos seus receptores) e captação de glutamato neste modelo animal de MPS I pode ser utilizada como boa ferramenta na tentativa de explicar as alterações cognitivas encontradas nestes animais. Não há evidências desta relação na literatura.

1.4 A terapia gênica como alternativa terapêutica

A principal terapia para MPS I é o transplante de medula óssea de um

indivíduo normal para um afetado e, neste caso, a principal desvantagem é a dificuldade de encontrar doador com HLA compatível (Vellodi *et al.*, 1997; Lücke *et al.*, 2007).

A enzima recombinante humana α -L-iduronidase (Aldurazyme®, laronidase) é um medicamento em fase clínica de tratamento de reposição em pacientes com MPS I. Os pacientes tratados até o momento apresentaram melhora em geral na disposição física, na qualidade do sono e redução da hepatoesplenomegalia. Observaram ainda nos pacientes tratados uma melhora na mobilidade articular, função cardíaca, acuidade visual, opacidade da córnea e diminuição da quantidade de GAGs excretada na urina. Este tratamento, entretanto, não foi capaz de reverter os casos de comprometimento neurológico, porque a enzima só atravessa a barreira hematoencefálica quando encontrada em altas concentrações séricas, induzindo forte resposta imunológica no paciente e acarretando gastos ainda maiores (Kakkis *et al.*, 2001; Vogler *et al.*, 2005; Sifuentes *et al.*, 2007; Tokic *et al.*, 2007).

A aplicação de terapia gênica para MPS I apresenta-se como uma alternativa promissora. A terapia gênica surgiu como uma promessa para revolucionar a medicina, sendo aplicada a doenças cuja prevenção e tratamento não são plenamente obtidos por métodos tradicionais. Inicialmente este tipo de terapia foi proposta para corrigir defeitos genéticos; atualmente, estudos mostram aplicações da terapia gênica em inúmeras patologias, tais como câncer, doenças infecciosas, doenças neurodegenerativas e patologias cardíacas.

A terapia gênica permite a adição do material genético terapêutico a células somáticas humanas, suprimindo deficiências ou inibindo a expressão de certos

genes. O gene de interesse, também chamado de transgene, encontra-se em uma molécula de DNA ou RNA junto com outros elementos genéticos, como sistemas de expressão e de manutenção, que garantem sua funcionalidade.

Vetores

Os vetores podem ser usados para transferência gênica em cultura de células (*in vitro*), diretamente no indivíduo (*in vivo*) ou em células retiradas do paciente, modificadas em cultura e reintroduzidas no mesmo (*ex vivo*). Para alguns tipos de tratamentos pode ser usada ainda a transferência do vetor diretamente no local de interesse (*in situ*). Um importante passo para a terapia gênica é o desenvolvimento de vetores eficientes para a transferência gênica. O vetor ideal deve ter algumas características importantes: manter a expressão do transgene durante o tempo necessário para a patologia em tratamento; não promover reações imunológicas contra os produtos de expressão; não causar toxicidade para o paciente; manter a segurança do ambiente; ter baixo custo; não ter replicação autônoma; e ter especificidade para a célula-alvo a ser tratada (Nardi *et al.*, 2002).

Muitos laboratórios estão avaliando diferentes tipos de vetores virais e não virais para melhorar a eficiência e a estabilidade da transferência gênica. Vantagens e desvantagens destes sistemas estão resumidas na Tabela 2.

Tabela 2. Vantagens e desvantagens de transferência gênica por vetores virais e não virais (Kim *et al.*, 2001).

Sistema de transferência	Exemplos de vetores	Vantagens	Desvantagens
Viral	Adenovirus, Adeno-associados, Retrovirus, Herpes simples, etc.	Alta taxa de eficiência de transdução, expressão prolongada do transgene e infecção em variados tipos celulares.	Restrição no tamanho do transgene a ser inserido, podem estimular resposta imune, problemas na segurança, toxicidade e na sua produção em grande escala.
Não viral	Lipídio catiônico, Lipossomo, Polímero, Peptídios, etc.	Fácil manipulação, fácil produção em grande escala, flexibilidade na inserção do transgene, quanto ao tamanho, seguro, não-imunogênico e não patogênico.	Expressão por pouco tempo e instabilidade causada devido a componentes do soro quando administrados <i>in vivo</i> .

Sistemas virais

Os sistemas virais geralmente possuem maior eficiência de transfecção quando comparados com sistemas não-virais. Os vírus vêm se mostrando altamente eficientes para a transferência de ácidos nucléicos para tipos específicos de células. Diferentes tipos de vírus estão sendo adaptados como vetores, mas os estudos estão mais avançados com os retrovírus (incluindo os lentivírus), adenovírus (Ad) e vírus adeno-associados (AAV). O primeiro passo para o desenvolvimento de um vetor viral é a identificação das seqüências virais que são necessárias para a montagem das partículas virais, para o empacotamento do genoma viral dentro dessas partículas e para a transferência às células-alvo. Como próximo passo, os genes não essenciais são deletados do genoma viral para reduzir a patogenicidade e resposta imune. O genoma viral restante e o transgene serão os componentes do vetor a ser construído.

Vetores virais podem ser divididos em duas classes gerais: (a) vetores com capacidade de integração no genoma da célula hospedeira, promovendo uma expressão a longo prazo do transgene, e (b) vetores que não se integram. Exemplos de vetores que se integram são os retrovírus e adeno-associados. Atualmente, o principal vírus aplicado à terapia gênica sem a capacidade de integração é o adenovírus, o qual tem seu genoma viral mantido em forma episomal no interior da célula infectada. São descritas a seguir as principais características de cada um destes tipos de vírus.

(1) Adenovírus (Ad): são capazes de carregar transgenes de tamanho grande (maiores que 8 kb) sem afetar sua infectividade. Os Ad possuem baixa especificidade à célula hospedeira, caracterizando-se por infectar um grande

espectro de tecidos. Infectam tanto células que estão se dividindo quanto células em estado de quiescência. Foi mostrado que, apesar deles não serem vírus integrativos, a expressão do transgene transportado pode permanecer por até um ano (Smith, 2003). Entre os obstáculos à aplicação do Ad, *in vivo*, estão: a resposta imune celular contra as células transduzidas, que expressam baixos níveis de genes de origem viral, resultando na eliminação dessas células; a imunidade humoral contra o capsídeo viral, que limita aplicações repetidas do vírus; e o potencial de contaminação com adenovírus capazes de replicação (Connelly, 1999).

(2) Vírus adeno-associado (AAV): os AAV são parvovírus humanos, não patogênicos, que naturalmente requerem um *helper* vírus, o qual contém todos os genes necessários para mediar sua replicação. Seu genoma é composto de simples fita de DNA e não está envelopado. Este tipo de vetor tem um suporte de adição do transgene de aproximadamente 5,0 kb, que lhe confere uma importante limitação de uso. O vírus selvagem tem a capacidade de se integrar em uma região específica do cromossomo 19 humano. A produção de AAV em grande escala é um processo complexo e trabalhoso e sua infecção é um processo complicado (Snyder e Flotte, 2002). O uso desses vetores ainda requer muitos estudos e, esses, estarão direcionados ao desenvolvimento de vetores cada vez mais específicos em relação à região de integração (Smith, 2003; Coura e Nardi, 2007).

(3) Retrovírus: a família dos retrovírus é chamada de Retroviridae e dentro dela existem três subfamílias: oncovirinae, spumavirinae e lentivirinae. Os retrovírus são pequenos vírus de RNA que em certo momento de sua replicação encontram-

se em forma de dupla fita. Eles são encontrados em muitas espécies, incluindo humanos. Certas características dos retrovírus os fazem boas opções como vetores para terapia gênica: a expressão estável e contínua do transgene devido à integração do genoma viral no cromossomo da célula hospedeira; baixa imunogenicidade da partícula viral; grande capacidade de inserção gênica (Romano *et al.*, 2000); transmissão estável do provírus para a progênie celular (Lois *et al.*, 2002). Os retrovírus infectam as células-alvo diretamente pela interação entre a proteína do envelope viral e um “receptor” de superfície celular na célula. A seguir, o vírus é internalizado, passa pela transcrição reversa produzindo a dupla fita de DNA, que será chamada de provírus quando ocorrer a integração. Essa dupla fita é transportada para o núcleo, onde se integra de forma estável no genoma do hospedeiro (Robbins e Chivizzani, 1998).

O FIV (Vírus da Imunodeficiência Felina) e o HIV (Vírus da Imunodeficiência Humana) são retrovírus linfotróficos pertencentes à família lentivirinae. Seus genomas estão organizados como os demais retrovírus, com os genes *env*, *gag* e *pol* e apresentando ORFs (*open reading frames*) adicionais que codificam proteínas regulatórias/acessórias (Miyazawa *et al.*, 1994; Rubinson *et al.*, 2007). A utilização do FIV e do HIV como vetores de transferência gênica é considerada um avanço na tecnologia de produção de vetores (Chang *et al.*, 1999; Romano *et al.*, 2000). Poeschla *et al.* (1998) relataram que o único impedimento para infecção produtiva em células humanas pelo FIV era a baixa atividade transcricional do LTR (*Long Terminal Repeat*) em células humanas. Esse problema foi facilmente superado pela substituição da região U3 da LTR 5' (onde se encontram a maior parte dos promotores do vírus) por um promotor

constitutivo eucarioto com atividade transcricional forte.

Além disso, a substituição do envelope viral do FIV e do HIV pelo envelope do Vírus da Estomatite Vesicular (VSV-G) permitiu que o espectro de infecção desses vírus em células humanas fosse ampliado. Esse processo foi descrito inicialmente por Burns *et al.* (1993) e é conhecido como pseudotipagem, sendo amplamente utilizado na produção de vetores virais. Além da alteração no tropismo, esse procedimento garante também um aumento na estabilidade da partícula viral, permitindo melhores condições na manipulação.

Ainda não foram descritas linhagens que expressam constitutivamente as proteínas necessárias para o empacotamento de vetores baseados em FIV e HIV. Por isso, a produção de tais vetores é feita pelo método da co-transfecção de três plasmídeos descrito por Soneoka *et al.* (1995). Nesse sistema, três plasmídeos distintos são transfectados simultaneamente em células HEK 293 para a produção de vetores virais. Um dos plasmídeos (de empacotamento) codifica as proteínas necessárias para a síntese das partículas virais (genes estruturais *gag* e *pol*), sendo responsável pela produção, nas células empacotadoras, da partícula do vírus. Um outro plasmídeo (envelope) carrega a informação para a glicoproteína de envelope escolhida. O último plasmídeo (vetor de transferência) contém, além do transgene a ser transferido para a célula-alvo, as seqüências mínimas necessárias para integração do vírus e expressão desse transgene. Essa construção contém o fator de encapsidação Ψ , para que o RNA transcrito a partir desse plasmídeo seja eficientemente incorporado na partícula viral. Tal sistema garante que os genes necessários para a produção de novas partículas virais (genes estruturais existentes no primeiro plasmídeo) não sejam

encapsidados e incorporados nas células-alvo, impossibilitando a ocorrência de outro ciclo de replicação viral e com isso fazendo a sua utilização segura.

O emprego do FIV e do HIV como vetores para a terapia gênica é bastante recente, mas sua crescente importância vem sendo responsável por um aumento no número de trabalhos que o utilizam. Inúmeros estudos pré-clínicos mostraram a eficiência de vetores baseados nestes vírus nos mais diversos tipos de células, como por exemplo, a segurança e os efeitos do HIV na transdução de linfócitos humanos (Wong-Staal *et al.*, 1998; Nienhuis, 2005; Zhang *et al.*, 2008). Eles vêm sendo aplicados em doenças como diabetes, fibrose cística, desordens metabólicas e erros inatos, doenças do sistema nervoso e hematopoético, alterações na córnea e ouvido interno e câncer, entre outras (Johnston e Power, 1999; Curran *et al.*, 2000; Loewen *et al.*, 2001; Brooks *et al.*, 2002; Djalilian *et al.*, 2002; Di Natale *et al.*, 2002; Sinnayah *et al.*, 2002).

O Vírus da Leucemia Murina de Moloney (*Moloney Murine Leukemia Virus – MoMLV*) também é um retrovírus da subfamília oncovirinae, apesar de possuir as características gerais dos retrovírus. Esse vírus, ao contrário do HIV e do FIV, não se integra no genoma de células quiescentes (Silva *et al.*, 2006). A produção do MoMLV é feita somente através da transfecção do plasmídeo transferência. Ela é feita em linhagens empacotadoras já estabelecidas, que possuem todos os fatores genéticos necessários para a produção da partícula viral (Miller e Chen, 1996).

1.5 Terapia gênica para MPS I

Diversos experimentos publicados tentam estabelecer protocolos para a

terapia gênica da MPS I. Zheng *et al.* (2003) desenvolveram um protocolo pré-clínico para um modelo murino de MPS I, usando um vetor retroviral, para transdução de células de medula óssea e mostrando que tais células migram até o cérebro. Em humanos, estudos iniciais mostraram a possibilidade de transfectar fibroblastos com o gene da IDUA (Anson *et al.*, 1992), e estudos posteriores *in vitro* mostraram também a possibilidade de transfectar células CD34⁺ de medula óssea normal ou de pacientes com MPS I (Huang *et al.*, 1997). Hartung *et al.* (2004) obtiveram altos níveis de atividade de IDUA nos órgãos e plasma de animais do modelo murino de MPS I usando tratamentos com vetores adeno-associados carregando o cDNA codificante para a enzima IDUA, que se mantiveram durante cinco meses pós tratamento. Neste estudo foi observada melhora em alguns parâmetros clínicos como diminuição nos estoques de GAGs nos órgãos avaliados e excretados na urina, normalização do peso do animal e a melhora das características craniofaciais quando comparados com os animais sem tratamento. No mesmo modelo murino, Desmaris *et al.* (2004) relataram atividade da IDUA no cérebro quando os animais foram injetados com outras construções de vetores adeno-associados como o sorotipo 2 (AAV2) e 5 (AAV5) contendo o cDNA IDUA. Recentemente, o gene IDUA canino foi transferido com emprego de um retrovírus derivado de Moloney para camundongos neonatos do modelo murino de MPS I (Liu *et al.*, 2005). Os animais submetidos ao tratamento apresentaram níveis enzimáticos de IDUA capazes de prevenir os efeitos da doença.

Além dos estudos com vetores virais *in vivo*, nosso grupo de pesquisa testou outro método de transferência gênica para o modelo murino de MPS I, com

emprego de um vetor não viral carregando o cDNA IDUA. Nos animais tratados foram observados baixos níveis de atividade enzimática nos órgãos analisados; apesar dos baixos níveis, foi possível observar uma diminuição na quantidade de GAGs acumulados principalmente no baço e no fígado (Camassola *et al.*, 2005).

1.6 Células-tronco mesenquimais (MSCs) como alvo da terapia gênica

Um dos principais alvos para terapia gênica têm sido as células-tronco. Essas células são capazes de auto-renovação ilimitada ou prolongada e também podem originar outros tipos celulares num estágio de diferenciação mais avançado (Pittenger *et al.*, 1999). A célula-tronco embrionária é pluripotente e dá origem a todas as células do organismo. Em organismos adultos as células-tronco encontram-se em vários órgãos, sendo consideradas multipotentes. Na medula óssea, além das células-tronco hematopoéticas, pode ser encontrada uma outra população de células-tronco. Denominadas células-tronco mesenquimais (MSCs), elas podem diferenciar-se em múltiplas linhagens que incluem condrócitos, adipócitos, osteoblastos, miócitos e fibroblastos (Pereira *et al.*, 1995; Pittenger *et al.*, 1999). As características das MSCs e ainda as evidências mostrando serem elas capazes de migrar até o cérebro fazem com que sejam bons alvos para terapia gênica de diferentes doenças, entre elas as mucopolissacaridoses.

Em nosso grupo de pesquisa foi descrita a obtenção de cultivos celulares com características de células-tronco mesenquimais, tanto a partir de medula óssea de camundongos C57Bl/6 normais quanto de animais do modelo MPS I (Meirelles e Nardi, 2003). Estas células tiveram sua plasticidade e suas condições de expansão estabelecidas *in vitro*. Os dados revelaram que não existem

diferenças perceptíveis nas células dos dois modelos de camundongos.

Em seu conjunto, esses dados salientam a relevância de estudos visando à correção da deficiência de α -L-iduronidase neste modelo de MPS I, aplicando-se vetores virais diretamente nos animais (*in vivo*), bem como a aplicação das células-tronco mesenquimais transduzidas com tais vetores (*ex vivo*).

Observando o quadro atual da MPS I, tanto relativo ao fenótipo da doença quanto aos tratamentos existentes, torna-se clara a necessidade do desenvolvimento de vetores mais eficientes e seguros, bem como a busca por parâmetros bioquímicos que poderão esclarecer o quadro clínico da doença e proporcionar parâmetros de acompanhamento do sucesso nas terapias experimentais.

Capítulo 2

Objetivos

2. Objetivos

Um progresso significativo tem sido alcançado durante os últimos anos no planejamento da terapia gênica em termos de eficiência de transferência, direcionamento às células-alvo e segurança. Este projeto visa colaborar com a busca de informações sobre formas de transferência gênica, com o desenvolvimento de vetores para posteriores estudos pré-clínicos de terapia gênica/celular adequados para mucopolissacaridose do tipo I e, além disso, com a investigação da fisiopatologia dos camundongos do modelo de MPS I, que podem constituir importante ferramenta para a determinação do sucesso destes tratamentos.

Para isso foram propostos os seguintes objetivos específicos:

1. Construção, avaliação e comparação de três tipos de vetores retrovirais (FIV, HIV e MoMLV) de transferência gênica codificante para a enzima IDUA, a partir de métodos e elementos necessários para a expressão do transgene acima citado em diferentes linhagens celulares.
2. Avaliação dos resultados referentes à transferência gênica analisando a funcionalidade do sistema em tipos celulares diferentes, através (a) da detecção da atividade da enzima α -L-iduronidase, (b) de testes moleculares usando RT-PCR relativo quantitativo, (c) citometria de fluxo para avaliar células GFP positivas e (d) microscopia de fluorescência.
3. Caracterização qualitativa e quantitativa dos depósitos de colesterol e gangliosídeos em diferentes regiões do cérebro e tentando assim, esclarecer algum mecanismo da disfunção neuronal.
4. Análise da fosforilação de proteínas de neurofilamentos como NF-L, NFL-

M, NF-H, vimentina e GFAP, em diferentes regiões do cérebro.

5. Avaliação do sistema de captação e receptores de glutamato no córtex e hipocampo dos camundongos com MPS I.

Capítulo 3

Development of viral vectors for gene therapy of mucopolysaccharidosis
type I

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Development of viral vectors for gene therapy of mucopolysaccharidosis type I

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Abstract

Mucopolysaccharidosis type I (MPS I) is a lysosomal disorder which results from a defect in the gene that encodes the hydrolase α -L-iduronidase (IDUA). Patients with low or absent IDUA activity present harmful effects due to the accumulation of glycosaminoglycans (GAGs) in different organs, including the central nervous system. Gene therapy for MPS I is a promising alternative, and different protocols are under investigation. Although a number of viral vectors have been developed for the treatment of MPS I, there is still a need for more efficient vectors. This work aimed to develop viral vectors based on the human immunodeficiency virus (HIV) and Moloney murine leukemia virus (Mo-MLV), carrying the coding sequences for human IDUA and for the GFP (green fluorescent protein) reporter protein. Although low titers of the vectors were produced and of a low transduction efficiency, both genes were transferred into HEK 293T and mesenchymal stem cells of MPS I mice. Since even low levels of enzymatic activity in target cells representing metabolic diseases are clinically relevant, the systems represent good candidates for pre-clinical studies on gene therapy for MPS I.

Keywords: Viral vectors; Mucopolysaccharidosis I; Murine model; Mesenchymal stem cells, IDUA.

1. Introduction

Mucopolysaccharidosis type I (MPS I) is a recessive monogenic disorder which results from a defect in the gene that encodes the lysosomal hydrolase α -L-iduronidase (IDUA). The enzyme degrades the glycosaminoglycans (GAGs) heparan and dermatan sulfate (Neufeld and Muenzer, 1995). The disease may result in different characteristics, depending on the residual level of IDUA activity. Patients with low or absent IDUA enzymatic activity present harmful effects in childhood and pre-adolescence due to the accumulation of GAGs in different organs, including the central nervous system, reticuloendotelial system and skeleton. In most cases, the patients present progressive neurological dysfunction and are severely affected still in the first decade of life (Watson et al., 2006; Zheng et al., 2003;). The therapy of choice for MPS I is transplantation of bone marrow from normal individuals, but the difficulty to find donors with compatible HLA represents a major disadvantage of this approach (Lange et al., 2006; Vellodi et al., 1997). Enzymatic replacement therapy (ERT) is the currently available therapeutic alternative, still in the final phase of clinical studies. The correction of neurological problems, however, requires high concentrations of the enzyme. Furthermore, ERT is a high-cost treatment (Tokic et al., 2007; Sifuentes et al., 2007; Vogler et al., 2005; Kakkis et al., 2001). The application of gene therapy for MPS I is a promising alternative, and different protocols are under investigation (Aronovich et al., 2007; Ma et al., 2007; Di Domenico et al., 2006).

Viral systems for gene transfer usually exhibit higher efficiency as compared to nonviral ones. In a previous study, we treated MPS I mice by

intraperitoneal and intravenous injection of plasmid containing human IDUA cDNA (Camassola et al., 2005). These mice presented null enzymatic activity before treatment and low levels of the enzyme were detected in several organs analyzed after gene therapy. Although low, this enzymatic activity was able to reduce the levels of GAGs accumulated in some organs such as spleen and liver. This method, however, does not result in long-term gene transfer, which would be interesting for monogenic diseases such as MPS I.

Aiming to achieve long-term correction of target cells, different types of vectors are under research (Ma et al., 2007; Chung et al., 2007). Among the several types of viruses being adapted for gene therapy, the retrovirus family, including lentivirus, presents many advantages (Lois et al., 2002; Robbins et al., 1998). The utilization of the human immunodeficiency virus (HIV) and Moloney murine leukemia virus (MoMLV) as vectors for gene transfer is still recent, but is growing in importance (Yamamoto and Tsunetsugu, 2008; Sumimoto and Kawakami, 2007; Soneoka et al., 1995). Several pre-clinical studies have shown the efficacy of vectors based on these viruses for many types of target cells (Schambach et al., 2006; Manilla et al., 2005; Hartung et al. 2004; Zheng et al., 2003; Wong-Staal et al., 1998).

Stem cells are long-lived cells capable of self-renewal and of differentiating into mature cell types (Pittenger et al., 1999), and are currently one of the main targets of gene therapy. Mesenchymal stem cell (MSC) are particularly important in medical research, due to their extensive proliferative potential and ability to differentiate into various cell types, including chondrocytes, adipocytes, osteoblasts, myocytes and fibroblasts (Da Silva Meirelles et al., 2006; Islam et al.,

2006; Pittenger et al., 1999). We have previously described the isolation of a cell lineage with mesenchymal stem cell characteristics obtained from mice of the MPS I model, which was termed MSC-KO (Meirelles and Nardi, 2003). These cells may represent an interesting target for MPS I gene therapy.

Although a number of viral vectors have been developed for the treatment of MPS I (Camassola et al., 2005; Hartung et al., 2004; Zheng et al., 2003), there is still a need for more efficient vectors, particularly regarding the target cells. In this work, we show the development of two viral vectors carrying the coding sequences for human IDUA and for the GFP (green fluorescent protein) reporter protein. The vectors, based on the human immunodeficiency virus (HIV) and Moloney murine leukemia virus (Mo-MLV), were successfully used to transduce cells of the HEK 293T line and MSC-KO cells.

2. Material and Methods

2.1 Cell culture

HEK 293T, PT67 and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St Louis, MO) containing 10% (v/v) fetal bovine serum (FBS, Cultilab, Sao Paulo, Brazil), streptomycin (10 µg/ml) and penicillin (10 U/ml) (GIBCO, Carlsbad, CA). LTC-3 mesenchymal cells (MSC-KO cells) (Meirelles and Nardi, 2003) were grown in DMEM with 10% FBS and 2.5–3.7 g/l acid-free HEPES (Sigma Chemical Co).

2.2 Construction of transfer plasmids

The lentiviral system based on HIV was described by Rubinson et al., 2007, and for this system the human cDNA IDUA, internal ribosome entry site (IRES) and GFP genes were constructed as follows: a *Xba*I/*Bam*HI fragment containing a RSV promoter and the human IDUA cDNA pRIDUA plasmid (Camassola et al., 2005), was subcloned in the transfer plasmid pLL3.7 (Rubinson et al., 2007), between the same restriction enzyme sites. The resulting transfer plasmid was named pLLIDUA, and the system was named HIV-GFP-IDUA. The cassette IRES-GFP was also cloned into the pLR2 plasmid and the system was named HIV-GFP.

The RetroXTM System (Clontech) was used for production of the retroviral system based in MoMLV. To construct the transfer plasmid on the retroviral system, human IDUA and GFP cDNAs, previously cloned into the pTIGER backbone (www.addgene.org), were excised with *Eco*RI e purified to ligate into the transfer plasmid pLXSN cleaved by the same enzyme. The resulting plasmid was

named pLXSN-IDUA, and the systems were named MoMLV-IDUA and MoMLV-GFP.

2.3 Viral production

The HIV-based vectors were produced on HEK 293T packaging cells by co-precipitation with calcium phosphate, as described by Zufferey et al. (1997), using 4 plasmids: the transfer plasmid, pMDLg/p.RRE (packaging plasmid), pRSV.rev (HIV-1 Rev encoding plasmid) e pVSV-G (VSV-G envelope). The medium was replaced 16 h after transfection. The supernatant was harvested 24, 48 and 72 h after transient transfection, filtered through 0.45- μ m pore size filters (Millipore, Carrigtwohill, Co, Ireland) and frozen at -80°C. To determine the viral titer of the HIV-based vectors, serial dilutions of vector preparations were added to 1×10^5 target cells in the presence of 8 μ g polybrene/ml (Sigma) during 12 h at 37 °C (Salmon and Trono, 2007).

Viral MoMLV-derived particles were produced by transfection of PT67 packaging cells (Miller and Chen, 1996) with the transfer plasmid. Viral MoMLV-derived supernatants from transfectants were used to transduce PT67 cells at 3 ml/ 5×10^5 cells, followed by subculturing cells at low density to obtain clones. Individual packaging clones were collected after more than 30 days of either selection (with 0.4 mg/ml G418) or expansions, and screened for viral titer. Selectable vector was titrated in NIH3T3 cells using suitable dilutions of viral aliquots in the presence of 8 μ g/ml polybrene for 24 h, subculturing 1:10 into selective medium containing 0.4 mg/ml G418

2.4 Transduction by HIV and Moloney with IDUA and GFP cDNA

The gene transfer capacity of the vectors was tested *in vitro* by infecting MSC-KO cells and the HEK 293T cell line. A total of 1×10^5 HEK 293T cells or 5×10^4 MSC-KO cells were used in each experiment. For transduction, the culture medium was replaced with 1 ml of viral stocks for approximately 16 h in the presence of 8 $\mu\text{g}/\mu\text{l}$ polybrene. After incubation, the medium was replaced with fresh normal culture medium. Cells were analyzed by RT-PCR, fluorescence microscopy and flow cytometry, and for enzyme activity. Experiments were always performed at least in triplicate.

2.5 Fluorescence analyses

Transduced cells were analyzed by fluorescence microscopy and flow cytometry, five days after transduction. The pattern of GFP expression was initially evaluated by fluorescence microscopy, with an AxioVert MRC microscope (Carl Zeiss, Oberkochen, Germany) equipped with standard FITC filters (FT 510; BP 450/90; LP 520). Microphotographs were recorded using an AxioCam MRC camera (Carl Zeiss).

Fluorescence intensity and percentage of transduced cells were analyzed with a FACSCalibur (Becton Dickinson, BD, New Jersey, USA), using FL-1 channel for GFP detection. At least 10,000 events were collected, and data were analyzed using the CellQuest software (Becton Dickinson).

2.6 Analysis of enzyme activity

Around 10^5 cells were homogenized in phosphate-buffered saline with 0.02% Triton, and protein concentration was measured by the method of Peterson

(1977) using bovine serum albumin as standard. The enzyme microassay was performed using 10 μ l of the sample, 4 μ l of 2.85 nmol/l fluorogenic substrate 4-methylumbelliferyl- α -L-iduronide (Glycosynth, Cheshire, England) and 25 μ l of the 0.2 mol/l formate buffer pH 2.8. The reaction was terminated with addition of 175 μ l 0.5 mol/l glycine-NaOH buffer pH 10.3. Fluorescence was measured with 370 nm (excitation) and 460 nm (emission) on the fluorescence SpectraMax Plus384 (Molecular Devices, Toronto, Canada). Results of IDUA activity are expressed as nmoles/h/mg protein.

2.7 Relative mRNA quantification

Total RNA was harvested from cells with Trizol reagent (Invitrogen, Sao Paulo, Brazil) according to the manufacturer's instruction. The RNA was finally eluted in RNase-free water. Reverse transcription (RT) was performed using MMLV reverse transcriptase (Invitrogen) in appropriate conditions. In all reactions, cDNA was synthesized in 20 μ l from 10 μ l of total RNA from approximate 5×10^5 cells. For each reaction, 1 μ l oligo (dT)¹⁸ (500 μ M/ml), and 1 μ l dNTP (10 mM) were used. Reactions were incubated at 65°C for 10 min, then 4 μ l RT buffer (10X), 2 μ l DTT (0.1 M) and 200 U RT enzyme were added and the reaction was incubated at 42°C for 90 min.

The antisense IDUA primer was 5'CGCGGTGGGGTGTGGAAGGAGT3' and the sense primer was 5'GTAGGTACGGACTGGCGCATG3'. The antisense GFP primer was 5'CTTCAAGATCCGCCACAACAT3' and the sense primer was 5'TTACTTGACAGCTCGTCCATGC3'. The antisense GAPDH primer was 5'TCCACCACCCTGTTGCTGTA3' and the sense primer was

5'ACCACAGTCCATGCCATCAC3' (Hobson et al., 2004). PCR was carried out in a 25- μ l reaction mixture containing 100 ng cDNA quantified by Nanodrop ND-1000 (Delaware, USA), 0.4 pmol of each primer, dNTP 0.2 mM, 1 x Taq DNA polymerase buffer, and 1 U Taq DNA polymerase (Invitrogen). PCR was performed under the following conditions: initial denaturation step at 94°C for 5 min followed by 40 cycles of desnaturation at 94°C for 35 sec, annealing at 58°C for 45 sec, and extension at 72°C for 1 min. A 7 min final extension step war performed at 72°C. The PCR products were analyzed on 1.5 % agarose gel. Samples were stained with GelRed (Biotium, Hayward, CA). Agarose gels were photographed with a EC3™ 410 Imaging System (UVP, Upland, CA). The quantitative ratio between IDUA or GFP and GAPDH expression was calculated using the VisionWorksLS Software (UVP).

2.8 Statistical analysis

The results are expressed as mean \pm standard error. Comparison between groups was carried out by the Student's t test, with $p < 0,01$.

3. Results

3.1 Titration of viral vectors

The system developed in this work resulted in clones producing viral titers of 4×10^5 CFU/ml for MoMLV-IDUA and MoMLV-GFP vectors. For HIV-based vectors, lower titers were obtained, around 1×10^3 TU/ml for HIV-GFP-IDUA and 1×10^4 TU/ml for HIV-GFP.

3.2 Analysis GFP reporter gene

Transduced cells were analyzed by optical and fluorescence microscopy. Transduction of MSC-KO cells with MoMLV-GFP resulted in a low frequency of weakly fluorescent cells (Fig. 1A). For HEK 293T, however, this vector induced a high frequency of fluorescent, GFP-positive cells (Fig. 1B). No fluorescent MSC-KO cells were observed after transduction with the HIV-based vectors (not shown). The two lentiviral systems were able to transfer the GFP gene into HEK 293T cells, but with higher fluorescence level with HIV-GFP (Fig. 1C) than with HIV-GFP-IDUA (Fig. 1D).

The frequency of GFP-positive cells, as well as mean fluorescence intensity, was determined by flow cytometry of transduced cells. As shown in Table 1, whereas a higher frequency of GFP-positive cells was induced by the MoMLV-based vector, fluorescence intensity was higher in cells transduced with HIV-based vectors.

3.3 IDUA enzymatic activity

As presented in Table 2, transduction with the MoMLV-based vector resulted in a two-fold higher IDUA activity in HEK 293T cells when compared to baseline levels ($p < 0.01$). In MSC-KO cells, a modest but consistent level of enzyme activity was observed after transduction with MoMLV-IDUA.

In HEK 293T cells treated with the HIV-GFP-IDUA vector, a nearly 60-fold increase in IDUA activity was observed. This vector however had no effect on MSC-KO cells.

3.4 Expression of the IDUA and GFP gene

The expression of IDUA and GFP genes was evaluated by RT-PCR, and the results were normalized with the expression of the GAPDH constitutive gene (Table 3). HEK 293T cells have a baseline level of expression of α -L-iduronidase, which showed a two-fold increase after transduction with HIV-GFP-IDUA and a 0.5-fold increase following transduction with MoMLV-IDUA. In MSC-KO cells, which completely lack IDUA expression, transduction with MoMLV-IDUA resulted in expression of the gene. GFP expression was observed in all transduced cells.

4. Discussion

The present study introduces two different viral systems, one based on MoMLV and the other on HIV, which are able to express active amounts of IDUA.

The system used to produce HIV lentiviruses able to express IDUA cDNA and the GFP gene was developed based on a description by Rubinson et al. (2007) (modified by Vargas et al., personal communication). This viral system is characterized by high stability and capacity to transduce different cell types. The low titers observed with this lentiviral system is possibly be due to the fact that the transfer plasmid pLLIDUA was 10.3-kb long, a size considered large, which may hamper the production of viral particles (Yacoub et al., 2007).

The results showed that, in spite of low vector titers, a high IDUA activity was seen in HEK 293T transduced cells, which was nearly 60-fold higher than the baseline activity generally present in this cell type. Analyses by flow cytometry and RT-PCR also showed a high efficiency of this vector, which is probably due to the high expression potential of the RSV (Rous Sarcoma Virus) promoter used in this vector. Other studies using the RSV promoter have also shown high expression rates of the transferred gene (Chang et al., 1999; Mukhtar et al., 1996).

Although the lentiviruses HIV-GFP-IDUA and HIV-GFP showed the capacity to transduce HEK 293T cells, even at low titers, their performance was more limited in other cell types. In MSC-KO cells, which currently represent one of the main targets of cell therapy, the vectors did not induce detectable levels of any of the two proteins after transduction. These results may be explained by vector size, low titer, or a combination of both factors. Other groups, however, have also

reported a difficulty in transducing MSCs, and several studies aim at developing more efficient methods to transduce MSCs. Different modifications of the basic protocol have been attempted, such as the use of high concentrations of viral supernatants for transduction or cell centrifugation procedures (together with the supernatant, in a viral method called spin infection), among others (Jiang et al., 2008; Zhang et al., 2008).

HEK 293T cells that were treated with the virus containing MoMLV-IDUA presented around twice the baseline IDUA activity. Transduction of MSC-KO cells with MoMLV-IDUA have also resulted in low enzymatic activity, which nevertheless may be considered clinically important, particularly in gene therapy of lysosomal diseases. Other studies have shown that around 1% of the normal enzymatic level results in therapeutic benefits for metabolic diseases (Chang et al., 1999), and in the MPS I model, Di Domenico et al. (2006) reported a significant reduction of GAG storage in the organs of treated animals, although treatment with lentiviral vectors induce only 1% of the normal IDUA activity. This shows that even if the cells transduced present low IDUA activity, the system represents a good candidate for pre-clinical studies on gene therapy for MPS I.

Our results showed that, although low titers of the vectors were produced and the transduction efficiency was also low, both genes were transferred into the cells tested. The fact that even low titers of viral stocks induce enzymatic activity in target cells representing metabolic diseases is significant for the design of pre-clinical assays, increasing the safety of gene therapy.

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Table 1

Results of flow cytometry analysis of HEK 293T and MSC-KO cells transduced with MoMLV-GFP, HIV-GFP and HIV-GFP-IDUA. The results are presented as the frequency of GFP-positive cells (GFP+) and mean fluorescence intensity (MFI). Results represent mean \pm standard deviation of 3 separate analyses.

Vector	HEK 293T		MSC-KO	
	GFP+ (%)	MFI	GFP+ (%)	MFI
-	0.0	-	0.0	-
MoMLV-GFP	10,9 \pm 0,98	125.5 \pm 5.1	0.7 \pm 0.06	205.8 \pm 7.6
MoMLV-IDUA	0.0	-	0.0	-
HIV-GFP	8.67 \pm 0,46	1,324.2 \pm 6.0	0.0	-
HIV-GFP-IDUA	2.39 \pm 0.68	1,360.8 \pm 86.7	0.0	-

Table 2

IDUA enzymatic activity (nmoles/h/mg protein) in HEK 293T and MSC-KO cells transduced with MoMLV- and HIV-based vectors. Results represent mean \pm standard deviation of 4 experiments.

Vector	HEK 293T	MSC-KO
-	27.5 \pm 5.0	0
MoMLV-GFP	26.5 \pm 2.7	0
MoMLV-IDUA	46.7 \pm 6.0*	0.4 \pm 0.1
HIV-GFP	27.5 \pm 5.0	0
HIV-GFP-IDUA	1,867.0 \pm 75.0*	0

* Significantly different from non-transduced HEK 293T cells ($p < 0.01$).

Table 3

RT-PCR analysis of the expression of IDUA and GFP genes, normalized with GAPDH, in HEK 293T and MSC-KO cells transduced with MoMLV- and HIV-based vectors.

Vector	GFP/GAPDH		IDUA/GAPDH	
	HEK 293T	MSC-KO	HEK 293T	MSC-KO
None	-	-	1.14 ± 0.3	0
MoMLV-GFP	0.93 ± 0.07	1.38 ± 0.70	NT	NT
HIV-GFP	2.06 ± 0.37	NT	NT	NT
MoMLV-IDUA	NT	NT	1.66 ± 0.2	0.25 ± 0.07
HIV-GFP-IDUA	1.1 ± 0.46	NT	2.21 ± 0.5	NT

NT, not tested.

Figure captions

Fig. 1. Transduction using the retroviral and lentiviral systems. Optical and fluorescence microscopy analyses of the cells transduced with Moloney-GFP, HIV-GFP and HIV-GFP-IDUA. A and B: MSC-KO transduced with Moloney-GFP (400X magnification). C and D: HEK 293T transduced with Moloney-GFP. E and F: HEK 293T transduced with HIV-GFP. G and H: HEK 293T transduced with HIV-GFP-IDUA. (C to H: 200X magnification).

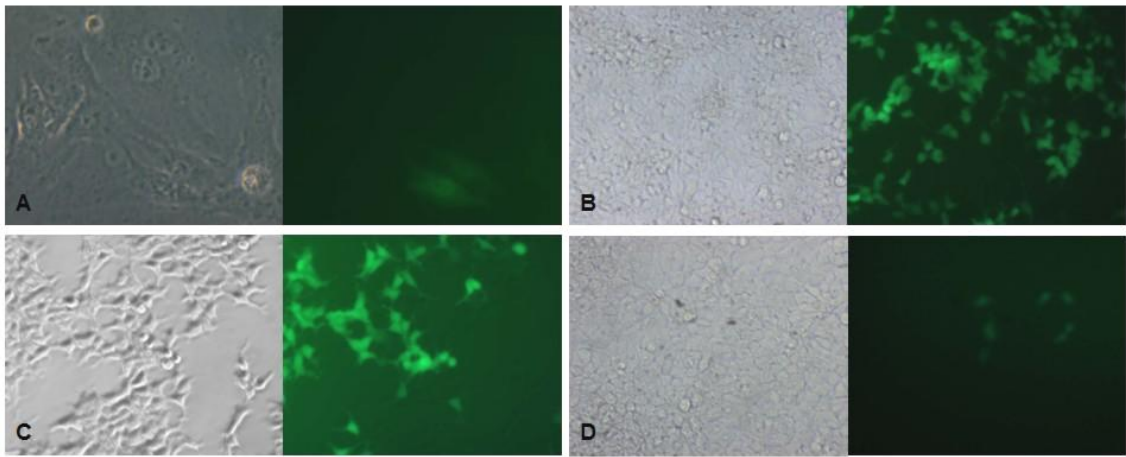


Fig. 1

Capítulo 4

Murine model of mucopolysaccharidosis type I activates ERK1/2 and affects the phosphorylation level of intermediate filaments in neural cells

Artigo submetido ao periódico *Journal of the Neurological Sciences*

MURINE MODEL OF MUCOPOLYSACCHARIDOSIS TYPE I ACTIVATES ERK
1/2 AND AFFECTS THE PHOSPHORYLATION LEVEL OF INTERMEDIATE
FILAMENTS IN NEURAL CELLS

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Running title: Mucopolysaccharidosis type I and cytoskeleton

Abstract

Mucopolysaccharidosis I (MPS I) is a lysosomal disorder characterized by a deficiency of the enzyme α -L-iduronidase (IDUA), which is responsible for the degradation of glycosaminoglycans (GAGs) leading to the accumulation of dermatan and heparan sulphate in lysosomes. In this work we investigated the *in vitro* phosphorylation of the neurofilament subunits of low and medium molecular weight (NF-L and NF-M) and glial fibrillary acidic protein (GFAP) in cerebral cortex, hippocampus and striatum of heterozygous and knockout (KO) MPS I mice. These proteins are specific intermediate filament (IF) constituents of neuronal and glial cells respectively. We also investigated the activation of the extracellular-regulated kinase (ERK), an important signal transducer in the central nervous system. Results showed hyperphosphorylation of NF-L, NF-M and GFAP in cerebral cortex, hypophosphorylation in hippocampus, while no alteration was detected in striatum of KO mice. Moreover, ERK1/2 activation was markedly increased in cerebral cortex and hippocampus of both heterozygous and KO mice. Considering that IF networks can be regulated by phosphorylation of polypeptide subunits leading to reorganization of the IF structures and that ERK plays critical roles in in the CNS, we could suppose that the altered IF phosphorylation and ERK activation could be involved in the brain damage characteristic of MPS I patients.

Keywords: MPS I; intermediate filament; brain; MAPK.

1. Introduction

The mucopolysaccharidosis (MPS) are a group of genetic diseases characterized by an excessive intralysosomal accumulation of partially degraded glycosaminoglycans (GAGs). Deficiency in the activity or inactivity of one of eleven enzymes required for GAGs degradation leads to accumulation of GAGs in lysosomes of virtually every cell of the body as well as outside of cells. Mucopolysaccharidosis type I (MPS I) is a recessive monogenic disorder resultant of a defect in the gene that encodes a lysosomal hidrolase (IDUA) [1] acting on the GAGs heparan and dermatan sulfate. MPS I is the more common and better characterized MPS type, leading to alterations in different organs, including the central nervous system, reticuloendotelial system and skeleton. In the majority of the cases the patients present progressive neurological dysfunction [2,3].

Although many efforts have been directed to develop an enzyme replacement therapy (ERT), based on intravenous injection of an active form of recombinant enzyme whose deficiency causes the disease, the biochemical basis of brain damage in MPS I is at present largely unknown [4]. In this context, Collins et al (1990) have described optic nerve atrophy and swelling in patients with all types of MPS [5]. Moreover, Walkley et al (1991) have identified the formation of focal granular enlargements containing glutamic acid decarboxilase (GAD), the enzyme responsible for synthesis of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), in various CNS regions in feline models of MPS [6].

The neuronal cytoskeleton comprises a protein network formed mainly by microtubules (MT) and neurofilaments (NF), the IFs of neurons. Neurofilaments

are composed of three different polypeptides whose approximate molecular masses are 200, 160, and 68 kDa, and are commonly referred to as heavy (NF-H), medium (NF-M) and light (NF-L) neurofilament subunits [7]. The assembly of the three NF subunits forms a typical neurofilament, in which NF-L is known to polymerize on its own, whilst NF-M and NF-H form lateral side-arms [8]. Glial fibrillary acidic protein (GFAP) is the IF of mature astrocytes and vimentin (Vim) is the IF of cells of mesenchymal origin [9, 10].

Phosphorylation and dephosphorylation of the NF proteins are complex processes orchestrated by a range of enzymes [11] which are predominantly regulated within the axonal compartment and targeted on different phosphate acceptor sites in the amino- and carboxy-terminal domains of NF subunits [12-15]. The mitogen-activated protein kinase (MAPK) signaling cascade is also implicated in the phosphorylation of KSP sites in the neurofilaments (NF) that is related with neurite outgrowth and may provide stability by rendering NFs resistant to proteolytic digestion which may occur at axonal terminals [16-18].

The functional role of NF phosphorylation is to date not completely clear. However, phosphorylation of N-terminal domain of NF-L and other IF subunits has been related to their association into filamentous structures [11]. It has been demonstrated that *in vitro* phosphorylation of carboxy-terminal domains of NF-H and NF-M straightens individual neurofilaments and promotes their alignment into bundles [19], whereas *in vivo* phosphorylation of these proteins is associated with an increased interneurofilament spacing [20]. As a consequence, NF-H and NF-M carboxy-terminal side arms extend and form crossbridges among neurofilaments and other cytoskeletal elements [21]. Moreover altered protein phosphorylation

has been associated with neurodegeneration [22] and alterations in the phosphorylation level of cytoskeletal proteins is considered a critical event in CNS pathology [23]. On the other hand, misregulation of the activity of kinases and phosphatases that use IF as substrate is a common feature in neurodegeneration [8, 24]. In this context, Nayeem et al (2007) [25] have described hyperphosphorylation of the microtubule-associated protein tau and NF in cerebella of phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-deficient mice, a condition associated with neurodegeneration, and this effect has been associated with ERK activation.

In order to better understand the influence of the deficiency of IDUA, the condition causing MPS I, on the IF-associated phosphorylating level and the activation of a MAPK pathway in different cerebral structures, a MPS I knock-out (KO) mouse model was utilized. The use of transgenic models to study IF phosphorylation has been revealing, not only in functional terms but also in highlighting important differences between tissues and cell-culture systems [26]. We then evaluated the IF phosphorylating system, and ERK activation in slices of different cerebral structures, hippocampus, striatum and cortical cells of wild type (WT), heterozygous and IDUA-KO mice.

2. Methods

2.1. Animals

C57Bl/6 knockout mice deficient for α -L-iduronidase (IDUA-KO) represent a murine model for human MPS I. IDUA-KO were derived from animals kindly provided by Dr Elizabeth Neufeld (UCLA, Los Angeles, CA, USA). The mice were produced by targeted disruption of the murine IDUA gene [27]. The colony is maintained by breeding heterozygous animals, and homozygous mutants were identified at birth by polymerase chain reaction. The animals were kept in standard conditions on a 12-h light/12-h dark cycle with water and food available *ad libitum* and were used in the experiments at 5 months of age. The experimental protocol followed the “Principles of Laboratory Animal Care” (NIH publication 85-23, revised 1985) and was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul.

2.2. Intermediate filament phosphorylation

Cerebral cortex, hippocampus and striatum from mice were dissected on ice and cut into 400 μ m thick slices with a McIlwain chopper. Tissue slices were initially preincubated at 30°C for 20 min in a Krebs-Hepes medium containing 124 mM NaCl, 4mM KCl, 1.2 mM MgSO₄, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1mM CaCl₂, and the following protease inhibitors: 1 mM benzamidine, 0.1 μ M leupeptin, 0.7 μ M antipain, 0.7 μ M pepstatin and 0.7 μ M chymostatin. After preincubation, the medium was changed and incubation was carried out at 30°C with 100 μ l of the basic medium containing 80 μ Ci of [³²P] orthophosphate (CNEN,

São Paulo, Brazil). The labeling reaction was allowed to proceed for 30 min at 30 °C and stopped with 1 ml of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, 50 mM Tris-HCl, pH 6.5), and the protease inhibitors described above. After treatment, IF-enriched cytoskeletal fractions were obtained by extraction in a high salt Triton-containing solution obtained from cerebral cortex, hippocampus and striatum of WT, heterozygous or IDUA-KO mice [28]. Briefly, after the labelling reaction, slices were homogenized in 400 µl of ice-cold high salt buffer containing 5 mM KH₂PO₄, (pH 7.1), 600 mM KCl, 10 mM MgCl₂, 2 mM EGTA, 1 mM EDTA, 1 % Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 15800 x g for 10 min at 4 °C, in an Eppendorf centrifuge, the supernatant discarded and the pellet homogenized with the same volume of the high salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The high-salt Triton-insoluble IF-enriched pellet, containing NF subunits, vimentin and GFAP, was dissolved in 1% SDS.

2.3. Total tissue homogenate

In experiments designed to study ERK activation, total tissue homogenate was obtained by homogenizing tissue slices in a lysis solution containing 2 mM EDTA, 50 mM Tris-HCl, pH 6.8, 4%(w/v) SDS. Protein concentration was determined using serum bovine albumin as the standard in both cytoskeletal fraction and total tissue homogenate [29].

2.4. Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min, were analyzed by 10 % polyacrylamide gels (SDS-PAGE) according to the discontinuous system of Laemmli (1970) [30] and transferred to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). In the experiments on *in vitro* ³²P incorporation, the gels containing the IF-enriched cytoskeletal fraction were exposed to X-ray films (T-mat G/RA) at -70 °C with intensifying screens, and finally the autoradiograms were obtained and quantified as described below.

2.5. Western blot analysis

In experiments designated to study the MAPK cascade, total tissue homogenate was processed to western blot analysis using the monoclonal antibodies anti-ERK1/2 or anti-phospho ERK1/2 (Cell Signaling Technology, Boston, MA, USA). The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4°C in blocking solution containing the following monoclonal antibodies: anti-ERK1/2 or anti-phospho ERK1/2 diluted 1:2000. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in TBS containing peroxidase conjugated anti-rabbit IgG 1:2000 (Amersham, (Oakville, Ontario, Canada). The blot was then developed using a chemiluminescence ECL

kit (Amersham, (Oakville, Ontario, Canada). All other reagents were of analytical grade and purchased from Sigma Chemical Company or Amersham Biosciences. Autoradiograms and Western blots were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an OptiQuant version 02.00 software (Packard Instrument Company).

2.6. Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison tests when the F-test was significant. All analysis were performed using the GraphPAD InStat Software version 1.12a.

3. Results and Discussion

In order to verify if the phosphorylation level of the IF subunits recovered in the cytoskeletal fraction was altered in different cerebral structures, the *in vitro* incorporation of ^{32}P -orthophosphate into GFAP, vimentin, NF-L and NF-M was analyzed in slices of cerebral cortex, hippocampus and striatum of WT, heterozygous and IDUA-KO mice. We could not observe differences in the phosphorylation of the IF subunits of heterozygous as compared to WT mice in the structures studied (Figure 1 A, B and C). However, cerebral cortex of KO animals presented hyperphosphorylation of both astrocyte IF subunits, GFAP and vimentin as well as NF-L and NF-M, the IF characteristic of neurons (Figure 1A). Conversely, we evidenced hypophosphorylation of the astrocyte and neuronal IF subunits in striatum and hippocampus of these animals (Figure 1B and C).

Cytoskeletal proteins constitute an important target of phosphorylating/dephosphorylating enzymes acting in response to physiological extracellular signals as well as to drugs or endogenous metabolites accumulating in pathological conditions [11]. In this report we demonstrate that IDUA-KO mice present altered activity of the phosphorylating system which includes the endogenous kinase and phosphatase activities associated with the high-salt Triton insoluble cytoskeletal proteins in cerebral cortex, hippocampus and striatum. This high-salt cytoskeletal fraction is an important tool to assess the activity of the endogenous phosphorylating system associated with the cytoskeleton, since it is enriched in IF proteins and in IF-associated kinases and phosphatases are present in the intact cell [28].

In vivo phosphorylation of NF is slow and orchestrated by a range of enzymes. Although the physiological roles of NF phosphorylation have not yet been completely clarified, several evidences point to involvement of different protein kinases in regulating the axonal calibre, the assembly/disassembly ability, the velocity of axonal transport and susceptibility to calcium-mediated protease digestion [11, 18]. Moreover, phosphatases do play an importante role in the labile stages of axonal outgrowth and in the more stable phases of mature axon function during impulse conduction. Since native NFs are abundantly phosphorylated, phosphatases may be the primary enzymes regulating cytoskeletal protein interactions, particularly during more labile stages of axon outgrowth [11]. We propose that IF hyperphosphorylation observed in cerebral cortex as well as hypophosphorylation evidenced in hippocampus and striatum of IDUA-KO mice are potentially deleterious to neural cell function. This is supported by our previous evidences of altered kinase and phosphatase activities associated with the IF-enriched cytoskeletal fraction in neurometabolic disorders [31-33]. We also observed morphological alterations in cultured astrocytes and C6 glioma cells by interfering with GFAP and actin organization, with loss of actin stress fibres in astrocytes [34, 35].

In order to verify if the activities of MAPKs were altered we tested the ERK pathway. The immunocontent of total ERK (p42 and p44) was similar in all the structures studied from WT, heterozygous and KO mice (Figure 2A). In addition, we observed a significant increase in p42 and p44 ERK phosphorylation in cerebral cortex and hippocampus of the heterozygous and IDUA-KO as compared to WT, however no differences were observed in striatum (Figure 2B).

Interestingly, although the heterozygous animals do not present the MPS I characteristic phenotype, Erk activation showed in the cerebral cortex and hippocampus of these mice suggest that this signaling pathway could be mediating cerebral dysfunctions other than altered IF phosphorylation.

Concerning Erk activation and IF hypophosphorylation in KO hippocampal slices, we could not exclude the possibility of Erk-induced phosphatase activation, leading to IF hypophosphorylation. In this context, Wang et al. (2007) have described that JNK activation was related to overexpression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in intestinal epithelial cells [36]. In addition, Chappell et al. (2005) demonstrated ERK activation and increase in both PTEN levels and phosphorylation in 293 HEK fibroblasts [37].

Activation of phospho-ERK levels has been related to morphological differentiation and maturation of hippocampal astrocytes [38]. Activation of ERK1/2 is involved in the formation of fibrillar and toxic amyloid-beta peptides, which are considered responsible for Alzheimer disease neurodegeneration [39]. In addition, we have recently described increased IF phosphorylation mediated by phospho ERK1/2 in cerebral cortex of hypothyroid rats (unpublished results). However it is interesting to note that our present results do not show a direct relationship between IF hyperphosphorylation and ERK activation in the different structures. This is evident in striatum, where IF are hypophosphorylated while ERK pathway is not activated. Indeed, in hippocampus, IF are hypophosphorylated while ERK is activated. We are not able to address the reasons associated with these changes, but regardless the molecular mechanisms underlying such effects, it is important to consider that ERK activation may mediate important consequences.

In this context, MAPK cascade is involved in regulating *in vivo* NF tail domain phosphorylation within axons, possibly during the period of axon outgrowth [11, 40]. In addition, it is described a possible linkage between glutamate transporters and MAPK cascade, suggesting that these transporters can act as receptors transmitting extracellular glutamate signal to intracellular messengers [41]. In this context, activation of phospho-ERK levels has been related to morphological differentiation and maturation of hippocampal astrocytes [38] and increased long-term potentiation (LTP) stimulation in hippocampal slices of hypothyroid rats [42].

The human pathology caused by deficient IDUA activity is related to the accumulation of GAGs in specific organs, such as the spleen, liver, skeleton, nervous and reticuloendothelial systems. In severe cases patients have progressive neurological dysfunction and die in the first decade of life [43]. There are evidences that GAGs could be related to kinase activation and cytoskeleton phosphorylation in astrocytes. In this context, hyaluronan, one of the major GAGs in the extracellular matrix, binding to astrocytes stimulated Rac1-dependent PKN α kinase activity (Rac1-activated serine/threonine kinase) which, in turn, up-regulated the phosphorylation of the cytoskeletal protein, cortactin, and attenuated the ability of cortactin to cross-link F-actin. This event plays a pivotal role in cytoskeleton activation and astrocyte migration [44]. Moreover, Bourguignon et al. (2004) described that hyaluronan promotes phospholipase C-regulated Ca²⁺ signaling and cortactin-cytoskeleton interaction that were required for a variety of cellular functions [45]. Taking into account that in the murine model of MPS I used in this study, the KO animals presented increased GAG levels as compared to WT mice, the excess of GAGs described in brain [46] could be associated with ERK

activation and cytoskeletal alterations observed in IDUA-KO mice. However, concerning the ERK activation observed in hippocampus and cerebral cortex from heterozygous animals, the underlying link with IF phosphorylation remains to be identified. Nevertheless, we can not exclude the possibility that other mechanisms could be activated by this signaling pathway and might be somehow affecting CNS physiology despite of the absence of phenotypic alterations in these mice.

4. Conclusion

In the KO model of MPS I there is a disturb in the phosphorylation level of IF proteins that are important molecular alterations in cerebral cortex, striatum and hippocampus. Also, ERK1/2 are activated in cerebral cortex and hippocampus both in KO and in heterozygous mice. The role of these alterations on the impairment of development and cognitive functions observed in MPS I should be further evaluated.

Acknowledgements

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Legends

Figure 1 Phosphorylation of intermediate filament subunits in different cerebral structures of wild type, heterozygous and IDUA-KO mice. (A) cerebral cortex, (B) striatum and (C) hippocampus. Tissue slices were incubated in the presence of ^{32}P orthophosphate for 30 min. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into the intermediate filament subunits was measured. WT wild type; He, heterozygous; KO, IDUA-KO mice. Data are reported as means \pm S.E.M. of 6 animals and expressed as arbitrary units. Statistically significant differences from controls, as determined by one-way ANOVA followed by Tukey–Kramer multiple comparison test were indicated: * $P < 0.001$.

Figure 2. Activity of ERK 1/2 in different cerebral structures of wild type, heterozygous and IDUA-KO mice. (A) total ERK levels, (B) phosphoERK levels. The immunocontent of total- and phospho- ERK1/2, was measured in the homogenate of slices of cerebral cortex, striatum and hippocampus of wild type (WT), heterozygous (He) and IDUA-KO (KO) mice. Densitometric data of total- and phospho-ERK1/2 are shown. Representative immunoblots are presented. Scans from 6 different animals from each group were quantified. Results are expressed as mean \pm S.E.M. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. * $P < 0.001$; ** $P < 0.0001$.

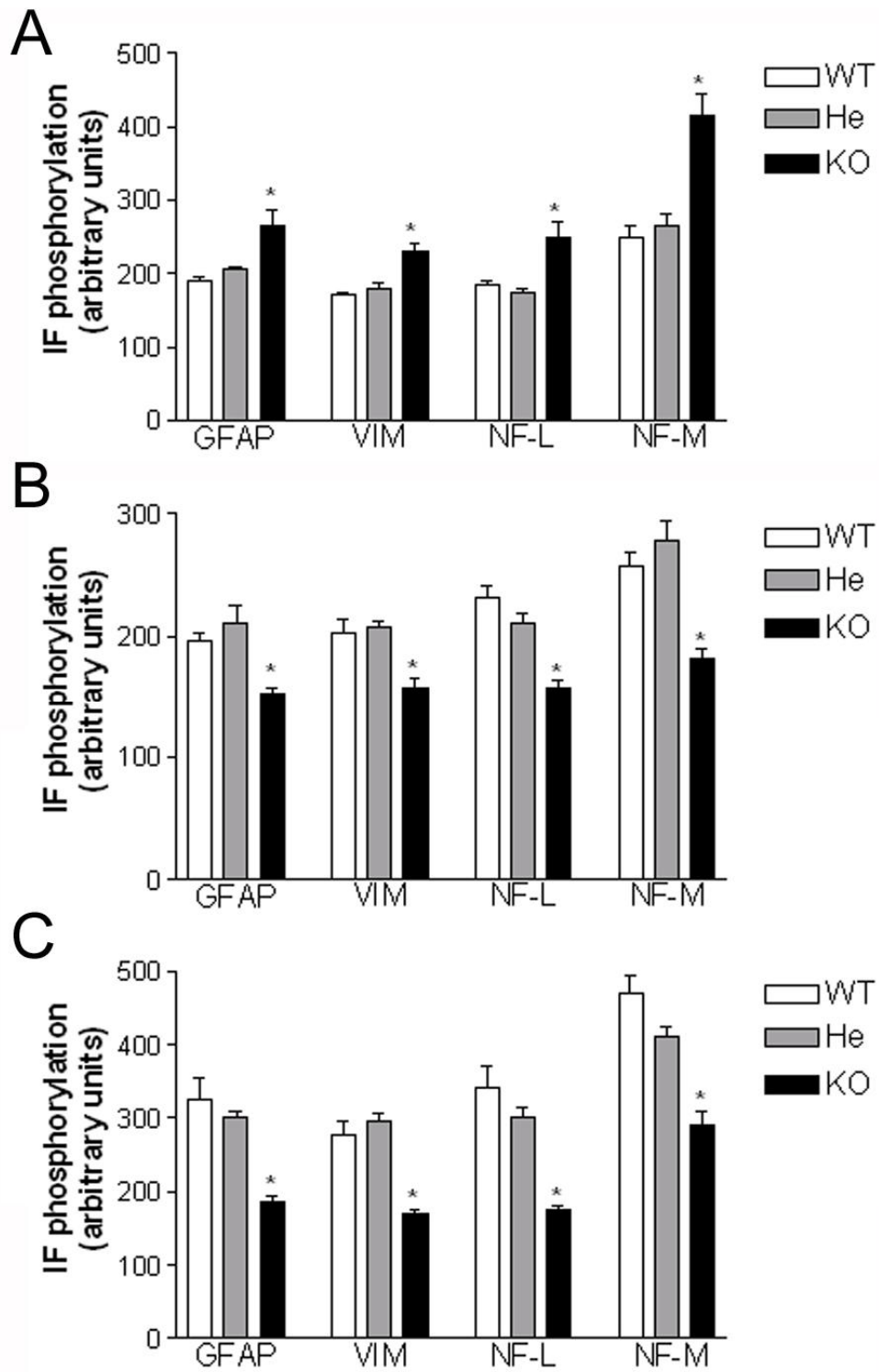


Figure 1

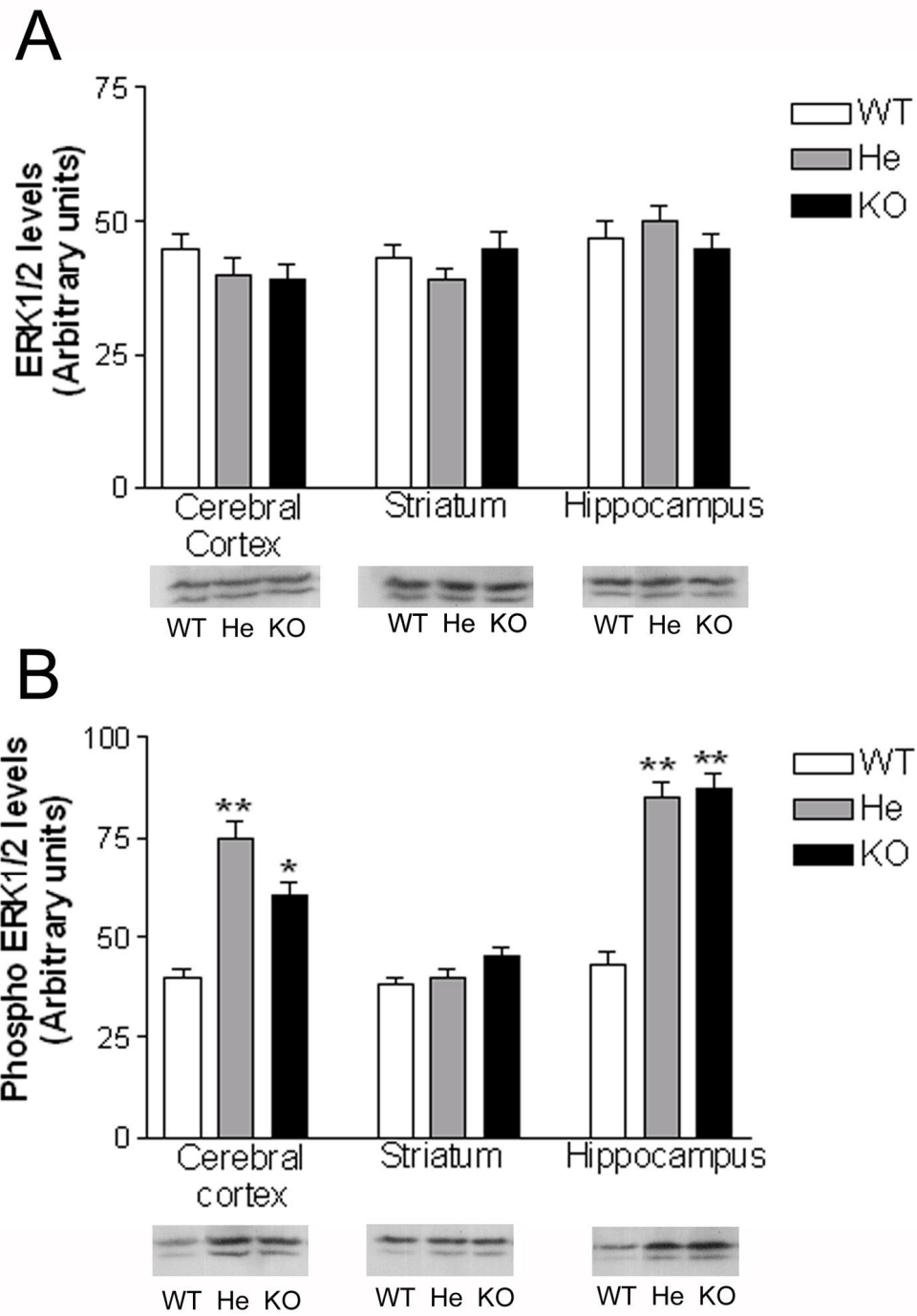


Figure 2

Capítulo 5

Evaluation of ganglioside content and profile in different brain structures of murine model of mucopolysaccharidosis type I (MPS I)

Artigo a ser submetido ao periódico *International Journal Development Neuroscience*.

Evaluation of ganglioside content and profile in different brain structures of murine model of mucopolysaccharidosis type I (MPS I)

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Abstract

Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disease characterized by a deficiency of the enzyme α -L-iduronidase (IDUA), resulting in cerebral accumulation of glycosaminoglycans (GAGs) heparan and dermatan sulphate and a progressive neurological dysfunction. The physiopathology of the MPS I is still uncertain; however other groups have reported alterations total brain lipid extract, as consequence of the accumulation of GAGs. The objective of this work was evaluating the concentration and distribution of ganglioside and the cholesterol content in cortex, cerebellum, hippocampus and hypothalamus in the murine model of MPS I. The experiment compared a group of mice MPS I and a wild type group (WT), it observed itself an increase in the total content of ganglioside of selective form for cortex and cerebellum of mice KO, while was verified an accumulation of GM3 and GM2 exclusively in hippocampus and hypothalamus of the group MPS I. The analysis of the content of cholesterol did not reveal significant difference between the groups MPS I and WT. In view of the importance of the lipid of membrane in the cellular biology, and of the accounts that suggest a function for apoptotic to the GM3, these finds can be related with the neurological dysfunction presented by patients with mucopolysaccharidosis I.

Keywords: Mucopolysaccharidosis, MPS I, ganglioside, GM3, GM2.

1. Introduction

Mucopolysaccharidosis (MPS) belong to the class of lysosomal diseases, a varied group of monogenic lysosomal storage diseases that are typically characterized by the total or partial deficiency of an enzyme involved in the degradation pathway of glycosaminoglycans (GAGs) (Fuller et al., 2004). Several clinical manifestations of MPS have been characterized and described, and are numerically classified from MPS I to MPS IX. Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease, which results from a defect in the gene that codifies a lysosomal hydrolase, α -L-iduronidase (IDUA). This enzyme degrades two particular GAGs, heparan and dermatan sulfate. The absence or the reduced active amount of this enzyme leads to a storage of these GAGs in cells (Neufeld and Muenzer, 1995). The disease's phenotype may vary considerably, from a more severe (Hurler syndrome), to an intermediate level (Hurler-Scheie syndrome) or mild (Scheie syndrome) manifestation of the pathology. The patients have deleterious effects in childhood and preadolescence due to the underlying accumulation of GAGs in different organs, including the SNC, reticuloendothelial system and skeleton. In most cases the patients present progressive neurological dysfunction. These patients are severely affected as early as in their first 10 years of life, and death is a very common outcome (Zheng et al., 2003). This form of the disease, as well as its other manifestations characterized, is very complex. It is most typically defined by storage sites of heparan and dermatan sulfate as previously mentioned (Liu et al., 2005; Zheng et al., 2003). Nevertheless, little is known about the changes in cell dynamics caused by this accumulation.

Nowadays, a significant lack of biomarkers is observed, whether

biochemical or molecular biology levels. These biomarkers would work as a starting point for the studies on physiopathological parameters of the disease. The description of these factors would permit to conduct more thorough investigations of the possible treatment strategies, and would also provide additional tools for the follow-up stages of these studies and assist in the development of post-treatment applications. In this scenario, some factors previously studied may become the object of further research, like the characterization and quantification of membrane lipid components (ganglioside and cholesterol) in different brain structures.

In the set of glycosphingolipids described, gangliosides are the most complex ones. They contain oligosaccharides with one or more sialic acid residues, which accounts for their total negative charge. Gangliosides are mainly present in plasmatic membrane of cells and are highly expressed in vertebral central nervous system where they are involved in neural development (synaptogenesis myelinogenesis). They are also related to cell differentiation and proliferation and to signal transduction. Of the several types characterized, the most significantly gangliosides expressed in the central nervous system are GM1, GD1a, GD1b, and GT1b (Zeller and Marchase, 1992). Gangliosides GM1 and GM3 are glycosphingolipids that build up when some lysosomal storage diseases occur, among them MPS I. Some studies have demonstrated the accumulation of these gangliosides in total brain from the murine MPS I model (McGlynn et al., 2004; Russel et al., 1998).

The present study analyzes the ganglioside content and profile in the cortex, cerebellum, hippocampus and hypothalamus of MPS I rats in comparison to wild type animals. An increase in total ganglioside contents were observed in

cortex and cerebellum of MPS I rats while ganglioside profile in these regions was not modified. On other hand, hippocampal and hypothalamic ganglioside contents of MPS I rats were similar to the respective wild-type while their profiles revealed the presence of GM3 and GM2 gangliosides in these MPS I brain structures. Taken together these data, it could be suggested that the alterations detected are region specific. This study also showed that the level of cholesterol, important lipid for membrane fluidize, was not altered in the brain structures evaluated, for healthy and for MPS I animals.

2. Experimental procedures

2.1. Animal model

C57Bl/6 knockout mice deficient for α -L-iduronidase (IDUA-KO) represent a murine model for human MPS I. MPS I mice were derived from animals kindly provided by Dr Elizabeth Neufeld (UCLA, Los Angeles, CA, USA). The mice were produced by targeted disruption of the murine IDUA gene (Zheng et al., 2003). The colony is maintained by breeding heterozygous animals, and homozygous mutants are identified at birth by polymerase chain reaction. The animals were kept in standard conditions and were used in the experiments at 5 months of age.

2.2. Materials

GM3, GM2, GM1 and GD1a gangliosides and thiobarbituric acid were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Silica-gel 60 thin-layer chromatography (TLC) sheets were supplied by Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical grade.

2.3. Lipid extraction

The animals were decapitated and cortex, cerebellum, hippocampus and hypothalamus were removed, weighed and homogenized in a 2:1 mixture of chloroform:methanol (C:M, 2:1, v/v) to a 20-fold dilution of tissue mass and centrifuged at 800 x g for 10 min. The pellet was re-homogenized in C:M (1:2) to a 10-fold dilution of original sample mass (Folch et al., 1957). The C:M extracts were combined and this pool was used for the following determinations.

2.4 Ganglioside evaluation

Aliquots from the total lipid extracts were used for ganglioside determination by the N-acetyl-neuramic acid (NeuAc) quantification with the thiobarbituric acid method described by Skoza and Mohos (1976). Ganglioside species were analyzed by thin layer chromatography (TLC) performed on 10 X 10 cm Merck plates of silica gel 60 using a developing tank described by Nores et al. (1994). Aliquots of the total lipid extracts containing 16 nmol of NeuAc suspended in 10 μ L C:M (1:1) were spotted on 8 mm lanes. TLC was developed, sequentially, with two mixtures of solvents, firstly C:M (4:1, v/v) and secondly C:M: 0.25% CaCl₂ (60:36:8, v/v). Ganglioside profile was visualized with resorcinol reagent (Lake and Goodwin, 1976; Svennerholm L, 1957). The chromatographic bands were quantified by scanning densitometry at 580 nm with a CS 9301 PC SHIMADZU densitometer. The terminology used herein for gangliosides is that recommended by Svennerholm (1963).

2.5 Cholesterol quantification

Aliquots of total lipid extract were evaporated, suspended in isopropanol and quantified according to the Trinder enzymatic method (Bergmeyer, 1974).

2.6 Protein quantification

Protein sediment obtained after lipid extraction was dissolved with NaOH 1N and measured by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as standard.

2.7. Statistical analysis

The results are expressed as mean \pm standard error. Comparison between groups was carried out by the Student's t test, with $p < 0.05$.

3. Results

3.1. Ganglioside analysis

The cortex, cerebellum, hippocampus and hypothalamus of seven MPS I and five wild type animals were used in this experiment. For hippocampus and hypothalamus, two pools of specimens from seven MPS I animals and two pools of five wild type animals were analyzed.

Samples of different brain structures, both for wild type and MPS I animals were collected and prepared for the determination of the total ganglioside contents. Fig. 1 shows a significant increase in ganglioside ($p < 0.05$) for the cortex and cerebellum of MPS I animals. As for hippocampus and hypothalamus, the amount of gangliosides was similar to that observed in the same structures of wild type animals.

After evaluation of total ganglioside content, all structures underwent more specific analyses. Lipid extracts were analyzed by thin layer chromatography to identify the ganglioside species. The posterior chromatogram densitometry analyses shows the proportion of each species, comparing the structures of wild-type and of MPS I animals.

The four main gangliosides present in the CNS (GM1, GD1a, GD1b and GT1b) were detected in the cerebral structures studied. The profile of the different ganglioside species from wild-type and of MPS I animals was apparently unaffected on cortex and cerebellum (Fig. 2A, 2B and Fig. 3). Relating these data to total ganglioside contents it can be observed an increase on all gangliosides species of mice MPS I cortex and cerebellum. The gangliosides GM3 and GM2 were found exclusively on hippocampus and hypothalamus of MPS I group (Fig.

2C). In spite of this the individual ganglioside proportion on hippocampus and hypothalamus was not changed comparing wild-type and of MPS I mice (Fig. 3)

3.2. Cholesterol quantification

The level of cholesterol in brain structures was quantified as described in the Materials and Methods section. Cholesterol levels don't show significance statistical in MPS I animals than in wild type animals (Fig. 4).

4. Discussion

Due to the fact that mucopolysaccharidosis type I is a lysosomal disease characterized by neurodegeneration, it becomes an ideal candidate object for the study of neurological parameters. MPS I and other lysosomal diseases that also lead to neurodegeneration present characteristics that deserve to be studied in more detail. Considering the importance of the roles played by membrane lipids, and that studies have demonstrated the existence of GM2 and GM3 storage sites in total brain extracts of murine models with MPS I, as well as of cholesterol storage (McGlynn et al., 2004; Russel et al., 1998), detailed investigations on ganglioside compositions are seen as a requirement to elucidate issues surrounding the model.

Our data for hippocampus and hypothalamus specimens reveal a greater build-up of GM2 and of GM3 in these structures. Sohn et al. (2006) demonstrated that the increase in GM3 may be directly associated with oxidative glutamate toxicity. . In ours study, glutamate can be involved in these process, as the uptake of glutamate and the number of glutamate receptors is reduced, as described in previous experiments (unpublished data). Nevertheless, generally speaking this increase in GM3 may be caused by the inhibition of enzymes responsible for glycosphingolipids degradation, induced by GAGs stored in cells (McGlynn et al., 2004). As GM3 expression occurs predominantly during the embryo development, a GM3 profile deregulation can be involved in the slow neural development in MPS I patients may be explained in the light of GM3 accumulation (Ikeno et al., 1982).

The results obtained in the present study reveal an increase in total ganglioside content for the cortex and cerebellum of MPS I mice. Preliminary

investigations studied the activation of the extracellular signal-regulated protein kinases (ERK). These studies observed that phosphorylation of ERK1/2 was markedly higher in the cortex of MPS I mice as compared to that wild type animals (unpublished data). According to Singleton et al. (2000), GM1 modulates the phosphorylation of ERK1/2. The increased total amount of gangliosides in the cortex of MPS I animals, including that of GM1, confirms the data previously obtained for ERK1/2.

It is also known that glycosphingolipids are involved in electric effects and in the concentration of ions like Ca^{+2} , as well as in the formation of membrane rafts, a feature that has proved its importance in the proper function of CNS signaling routes. Thus, changes in glycosphingolipids structure and amount may influence these processes (Harder and Simons, 1999). The findings described in the present study for the brain structures of MPS I animals may explain behavioral data already obtained for these animals, as described by Reolon et al., (2006). These animals presented a reduction in vertical exploration in open-field tests, as well as changes in long-term memory, when evaluated using the inhibitory avoidance test.

The quantification of cholesterol levels in the different brain structures did not present any differences between healthy and MPS I animals. According to the study by McGlynn et al. (2004), cholesterol storage sites were observed side by side with GM2 and GM3 accumulation in immunohistochemical evaluation of neurons from MPS I animals, which however was not observed in our results. The animals used in the study by McGlynn et al. (2004) were between 4 and 7 months of age and considering that the characteristics of the MPS I tend to vary considerably with age in MPS I models (Zheng et al., 2003; Russel et al., 1998),

the experiments with these types of analyses have to be carried out with animals of identical or similar ages. This avoids the variations that may have influenced the analyses conducted by McGlynn et al. (2004).

Studies on gangliosides in MPS I models show the occurrence of widespread ganglioside storage sites, mentioning total brain extracts, not each structure in detail (Russel et al., 1998). As a whole, the results obtained in the present study afford to conclude that ganglioside content and profile may be efficiently employed as follow-up parameters in MPS I models, during and after preclinical tests of gene therapy, cellular therapy, and enzyme replacement therapy. Apart from this, ganglioside storage sites are also important in investigations of new mechanisms of neuronal dysfunction in MPS I individuals.

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Figure captions

Fig. 1. Total ganglioside contents in the different brain structures analyzed. KO: Knockout; WT: Wild type. $p < 0.05$.

Fig. 2. Images of the separation of different gangliosides of the brain structures using thin-layer chromatography. (A) cortex; (B) cerebellum; (C) hippocampus and hypothalamus. HT: Hypothalamus; KO: Knockout; WT: Wild type.

Fig. 3. Densitometry analysis of TLC based on the lipid extracts for the different brain structures. KO: Knockout; WT: Wild type.

Fig. 4: Cholesterol levels in the different brain structures analyzed. KO: Knockout; WT: Wild type.

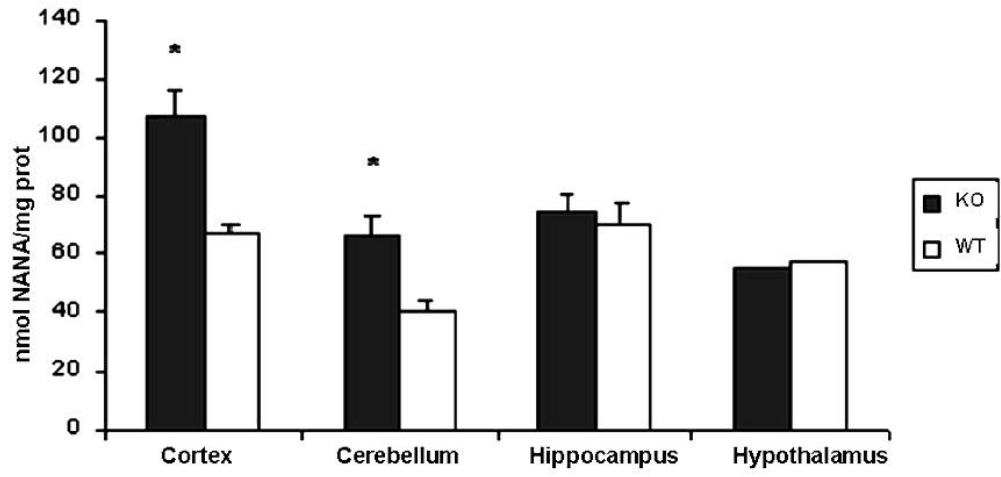


Fig. 1

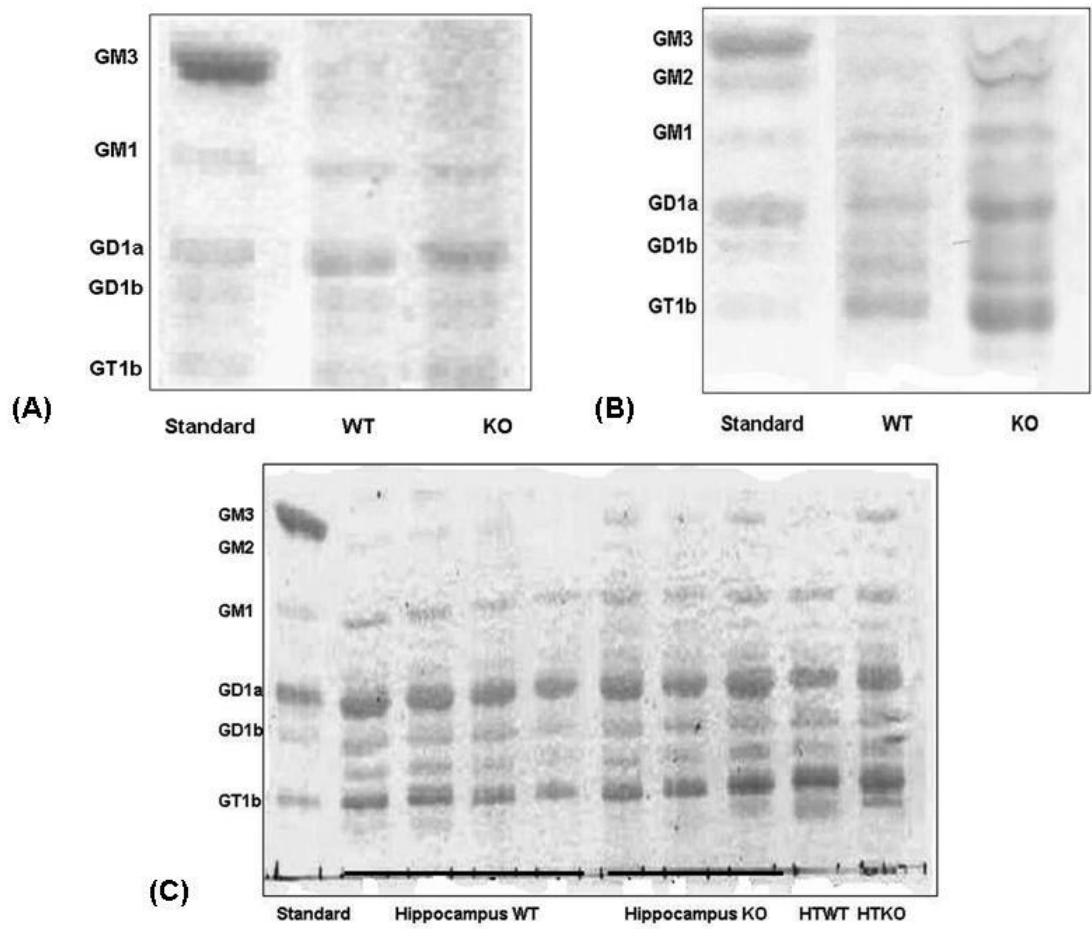


Fig. 2

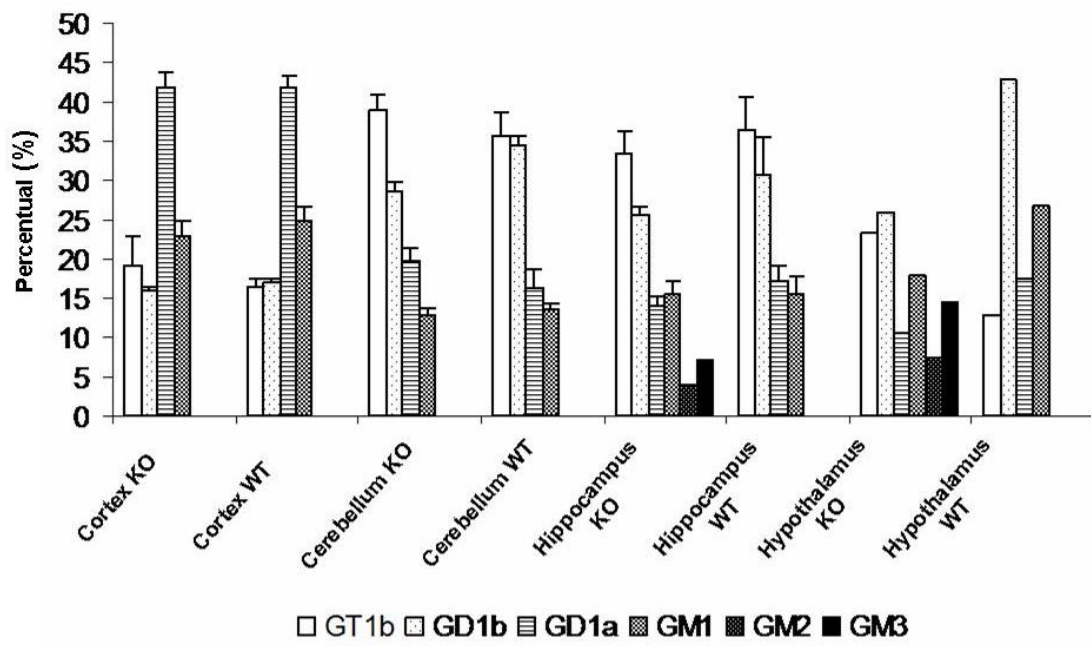


Fig. 3

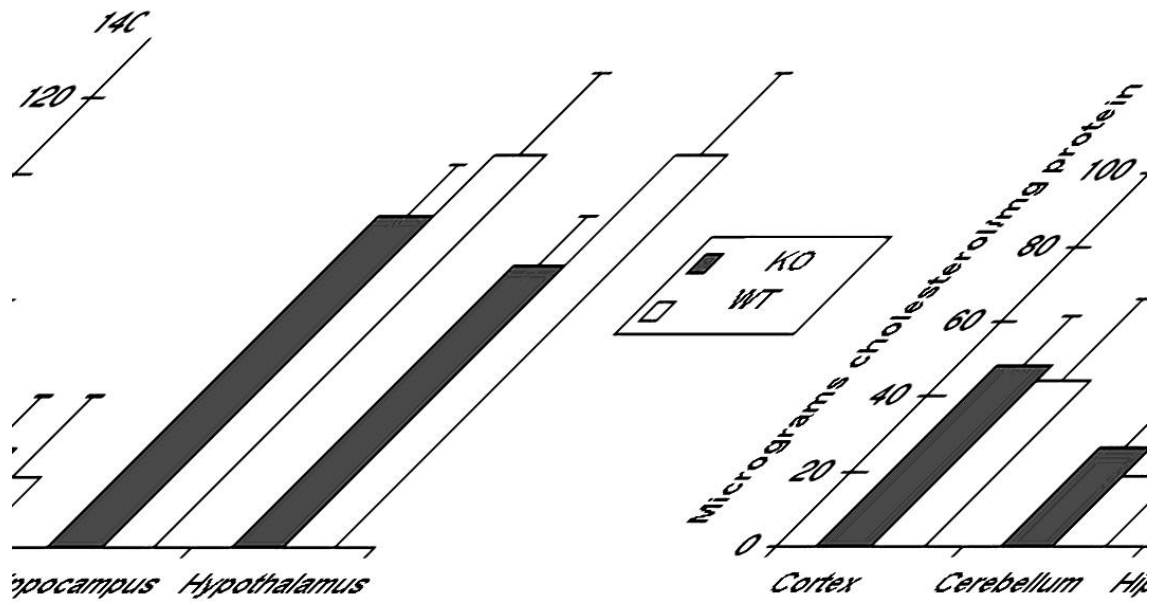


Fig. 4

Capítulo 6

Glutamatergic synapses in murine model of mucopolysaccharidosis type I
(MPS I)

Brief Communication a ser submetido ao Molecular Genetics and Metabolism.

Glutamatergic synapses in murine model of mucopolysaccharidosis type I
(MPS I)

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Abstract

Patients with mucopolysaccharidosis type I (MPS I) presents neurodegeneration of the central nervous system. In the murine model of MPS I, deficits of long term memory and learning may also be observed. In this work, we analyzed glutamate binding and uptake in the cortex and hippocampus of MPS I and wild type mice. MPS I animals showed a decrease in the glutamate uptake in the cortex and hippocampus. In addition, glutamate binding was decreased in the hippocampus but not in the cortex. These results show that glutamatergic neurotransmission is altered in MPS I and we suggest that impairment of main excitatory neurotransmitters may play a role in the behavioral alterations observed in this disorder.

Keywords:

Mucopolysaccharidosis type I, murine model, glutamate uptake, glutamate receptors, lysosomal storage diseases.

Introduction

Lysosomal storage diseases are genetic diseases that result from mutations in genes coding for acid hydrolases, involved in different metabolic pathways. Loss or reduction in enzyme activity leads to cell dysfunction, due to the storage of substrates that are not degraded in the lysosome. Most of these diseases involve abnormal substrate storage in the central nervous system (CNS), which results in progressive neurodegeneration and mental retardation [1]. Mucopolysaccharidosis type I (MPS I) is a recessive monogenic disorder due to mutations in the gene that encodes the lysosomal hydrolase α -L-iduronidase (IDUA), which degrades heparan and dermatan sulphate. In severe cases, patients have progressive neurological dysfunction and may die in the first decade of life [2].

Glutamate is the main excitatory neurotransmitter of the CNS in mammals, and this amino acid participates of the important functions such as cognition, learning and memory, as well as in the formation of neural networks during development [3 – 5]. However, impairment in the glutamatergic synapse can lead to neuronal death being involved in the pathophysiology of many acute and chronic neurodegenerative diseases [6, 7].

In a previous study, we showed that mice with MPS I presented low exploratory activity associated with impairment in the long-term memory in the inhibitory avoidance task [8]. However, the participation of neurotransmitters systems as in the behavioral alterations as in the neurodegeneration observed in MPS I are poorly investigated so far.

Considering that glutamate is essential for the maintenance of brain homeostasis and functions such as learning and memory, this study aimed to

investigate if important parameters of glutamatergic signaling could be altered in two important brain areas (hippocampus and cortex) from wild type and MPS I mice.

Materials and methods

Animals

C57Bl/6 knockout mice deficient for α -L-iduronidase (MPS I), representing a murine model for human MPS I, were used in this study. The colony was derived from animals kindly provided by Dr Elizabeth Neufeld (UCLA, Los Angeles, CA, USA) [9].

Glutamate uptake into brain slices

The animals were decapitated, their brains immediately dissected out and humidified with Hank's balanced salt solution (HBSS, Sigma Chemical Co., St Louis, MO) containing (in mM): 137 NaCl; 0.63 Na₂HPO₄; 4.17 NaHCO₃; 5.36 KCl; 0.44 KH₂PO₄; 1.26 CaCl₂; 0.41 MgSO₄; 0.49 MgCl₂ and 1.11 glucose, pH 7.2. The hippocampus and cortex were dissected out and placed onto Petri dishes with HBSS. Hippocampal and parietal cortex slices (0.4 mm) were obtained using a McIlwain tissue chopper and transferred to 24-well culture plates. Slices were maintained at 35° C in HBSS during 15 minutes in HBSS (pre-incubation period). After pre-incubation the medium was changed and glutamate uptake was started by adding 100 μ M L-[³H] glutamate to the slices. Glutamate uptake into hippocampal slices was performed in five minutes while cortical slices were incubated during seven minutes. The glutamate uptake was finished by aspiration of the medium followed by two washes with cold HBSS in the absence of sodium (replaced by N-methyl-d-glucamine). Sodium-independent uptake was carried out

4°C in HBSS without sodium at the same conditions described above. Both specific and non-specific uptake assays were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409). Specific uptake was considered to be the difference between total and nonspecific binding. Protein content was measured following the method described by Peterson [10].

Preparation of Murine Cortex and Hippocampus Membrane

All binding assays were performed using brain synaptic membranes prepared as described [11], and stored at - 70°C. On the day of the binding assay, the membranes were rapidly thawed in a water bath (37°C), homogenized with 3 volumes of 5 mM Tris-HCl, pH 7.4, and centrifuged 3 times at 27,000 x g for 15 min.

[3H]-Glutamate binding assay

Membranes were incubated in 0.5 ml reaction mixture containing 50 mM Tris/acetate, pH 7.4, and 40 nM [3H]-glutamate. Incubation was carried out at 30°C for 30 min, and the reaction was stopped by centrifugation at 27,000 X g for 10 min. The pellet was quickly and carefully washed with ice-cold Milli-Q water. SDS (0.1%) and scintillation liquid were added to the dried pellet and radioactivity incorporated was determined in a liquid scintillation counter (Wallac 1409). Binding was then immediately started by adding brain membranes. In all binding experiments, nonspecific binding was determined by adding 1000 times nonradioactive glutamate to the medium in a parallel assay. Nonspecific binding

typically amounted to 20% of total binding. Specific binding was considered to be the difference between total and nonspecific binding.

Statistical analysis

The results were analyzed using Student's t-test (unpaired). Values of $P < 0.05$ were considered statistically significant.

Results

Glutamate uptake was performed in slices from hippocampus and parietal cortex of wild type and MPS I mice at different ages. MPS I mice with 5 months-old showed a decrease (15%) in the glutamate uptake in the parietal cortex slices compared to wild type mice (Fig 1 A, white bars). Besides, a more pronounced decrease in the glutamate uptake (32%) was observed in the hippocampal slices from MPS I mice with 5 months-old (Fig. 1 A, black bars). MPS I mice with 10 months-old also presented alterations in the glutamate uptake. Slices of parietal cortex from MPS I mice presented a decrease (33.5%) in the glutamate uptake (Fig. 1B, white bars) while slices from hippocampus presented 36% of reduction in the glutamate uptake (Fig. 1B, black bars).

Glutamate binding was performed in synaptic membranes from hippocampus and parietal cortex of wild type and MPS I mice with 5-months old. Synaptic membranes prepared from the parietal cortex of MPS I mice did not shown any differences compared to wild type membranes in the glutamate binding (Fig. 1C, white bars). However, membranes prepared from the hippocampus of MPS I mice presented a decrease (37%) in the glutamate binding compared to wild type (Fig. 1C, black bars).

Discussion

MPS I mice have been shown to present cognitive deficits, changes in oxidative stress parameters, ganglioside storage, and more specifically glycoaminoglycan storage [8, 16, 17]. Patients with MPS I present alterations in brain imaging analyses show abnormalities in the cerebral activity verifying deficits of memory and learning [12, 13]. Our results agree with these findings, specially relative to the behavioral problems observed in these animals and that will be able to explain those cerebral abnormalities in the MPS I patients. Confirming our results regarding the binding, Liu et al, 2007 described a system of gene therapy for the murine model of MPS VI the system was based in virus adenoassociated, the results reverted some of the characteristics of the disease and between them the restore of the levels of glutamate receptors NMDA (NMDA, N-methyl-D-aspartate) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) described in the same work. That work confirms that in mucopolysaccharidosis have alterations in the synaptic neurotransmission [18].

The decreased uptake of glutamate may be due to several reasons, such as modifications in the distribution of receptors and transporters. In MPS I animals, the uptake was reduced in the structures analyzed, when compared to the same structures of normal mice. The binding analyses conducted to assess total glutamate receptor distribution showed that the decreased uptake may be due to the low level of receptors. This decreased uptake may also have an important role in gangliosides storage, as already described for this animal model [17]. According to Shon *et al.* (2006) [19], glutamate alters the synthesis of GM3 ganglioside, and may also lead to an up-regulation of GM3 levels.

The decrease in glutamate uptake may be due to the decreased number of receptors, which explains memory and learning dysfunctions in this murine MPS I model.

Acknowledgements

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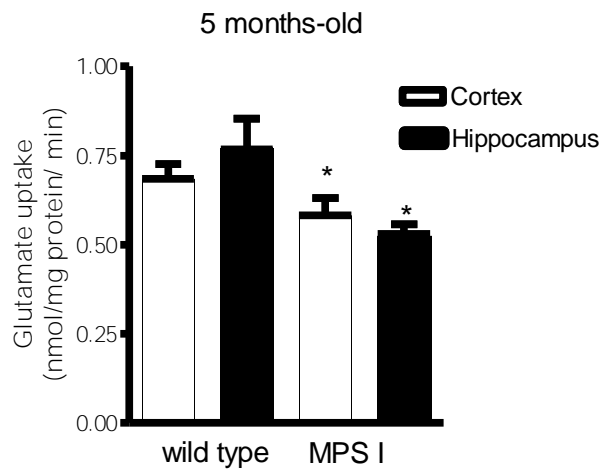
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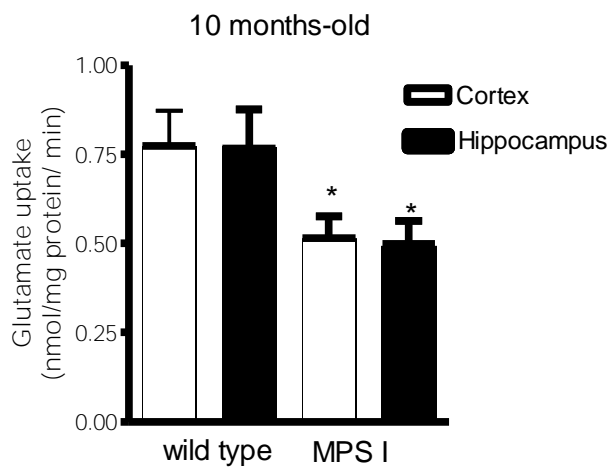
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Fig. 1. Glutamate uptake and binding in cortex and hippocampus specimens from wild type and MPS I animals. A) Glutamate uptake in the cortex and hippocampus of 5-month-old animals (n = 8 wild type; n = 7 MPS I animals) ($p < 0.05$). B) Glutamate uptake in the cortex and hippocampus of 10-month-old animals (n = 8 wild type; n = 7 MPS I animals) ($p < 0.05$). C) Glutamate binding in the cortex (n = 4 wild type; n = 3 MPS I animals) and hippocampus (n = 7 wild type; n = 7 MPS I animals) of 10-month-old animals ($p < 0.05$).

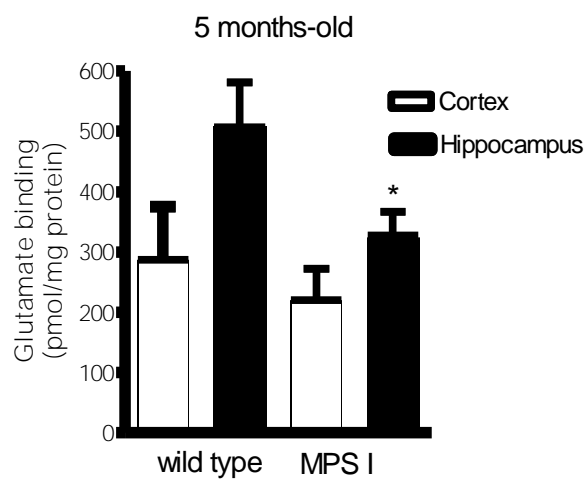
A



B



C



Capítulo 7

Informações complementares aos resultados

Desenvolvimento de um vetor lentiviral baseado no vírus da imunodeficiência felina (FIV)

O FIV (vírus da imunodeficiência felina) é um retrovírus linfotrófico pertencente à família Lentivirinae. Seu genoma está organizado como o dos demais retrovírus, contendo os genes *env*, *gag* e *pol*, e apresentando ORFs (open reading frames) adicionais que codificam proteínas regulatórias ou acessórias (Miyazawa *et al.*, 1994). A utilização do FIV como vetor de transferência gênica é considerada um avanço na tecnologia de produção de vetores (Romano *et al.*, 2000). Poeschla *et al.* (1998) relataram que o único impedimento para infecção produtiva em células humanas pelo FIV era a baixa atividade transcricional do LTR (*Long Terminal Repeat*) em células humanas. Esse problema foi facilmente superado pela substituição da região U3 da LTR 5' (onde se encontra a maior parte dos promotores do vírus) por um promotor constitutivo eucarioto com atividade transcricional forte. Além disso, a substituição do envelope viral do FIV pelo envelope do vírus da estomatite vesicular (VSV-G) permitiu que o espectro de infecção desse vírus em células humanas fosse ampliado.

Ainda não foram descritas linhagens que expressam constitutivamente as proteínas necessárias para o empacotamento de vetores baseados em FIV. Por isso, a produção de tais vetores é feita pelo método da co-transfecção de três plasmídeos. Nesse sistema, três plasmídeos distintos são transfectados simultaneamente em células HEK 293T para a produção de vetores virais. O plasmídeo de empacotamento codifica as proteínas necessárias para a síntese das partículas virais (genes estruturais *gag* e *pol*), sendo responsável pela

produção, nas células empacotadoras, da partícula do vírus. O plasmídeo de envelope carrega a informação para a glicoproteína de envelope escolhida. O último plasmídeo (vetor) contém, além do transgene a ser transferido para a célula-alvo, as seqüências mínimas necessárias para integração do vírus e expressão do transgene. Essa construção contém o fator de encapsidação Ψ , para que o RNA transcrito a partir desse plasmídeo seja eficientemente incorporado na partícula viral. Tal sistema garante que os genes necessários para a produção de novas partículas virais (genes estruturais existentes no primeiro plasmídeo) não sejam encapsidados e incorporados nas células-alvo, impossibilitando a ocorrência de outro ciclo de replicação viral e, com isso, garantindo a segurança na utilização do vetor.

O emprego do FIV como vetor para terapia gênica é bastante recente, e sua crescente importância é refletida no aumento do número de trabalhos que o utilizam. Inúmeros estudos pré-clínicos têm mostrado a eficiência de vetores baseados neste vírus nos mais diversos tipos de (Johnston e Power, 1999; Curran *et al.*, 2000; Brooks *et al.*, 2002; Djalilian *et al.*, 2002; Di Natale *et al.*, 2002; Sinnayah *et al.*, 2002).

Nenhum trabalho até o momento descreveu o desenvolvimento de um sistema baseado em FIV para terapia gênica da MPS I. Um dos objetivos desta tese foi o desenvolvimento de um vetor baseado em FIV.

O cDNA da IDUA humana, o gene GFP e a seqüência IRES foram clonados dentro do plasmídeo de transferência pTIGER plasmídeo de transferência, ele foi adquirido da empresa ADDGENE (<http://www.addgene.org>). Os insertos contendo o IRES-GFP, derivado do plasmídeo pRL2 e o cDNA IDUA

(obtido a partir do plasmídeo pLLIDUA) foram clonados no pTIGER com as enzimas *Bam*HI e *Nhe*I. O plasmídeo de transferência resultante foi denominado de pTIGER-IDUA.

Os vetores foram produzidos por co-precipitação com fosfato de cálcio como descrito por Pear *et al.* (1993). O plasmídeo envelope, codificando a proteína VSV-G, foi usado para a produção viral e o plasmídeo pCF1-A foi usado como plasmídeo empacotador (Poeschla *et al.*, 1998). O meio foi substituído 16 h depois da transfecção, e o sobrenadante foi trocado por meio DMEM fresco. A coleta do sobrenadante foi feita 48 horas pós transfecção, filtrado com membranas de 0,45 µm e congelados no – 80°C. Para determinar os títulos de partículas infectantes do vetor baseado em FIV-GFP-IDUA, diluições seriais da preparação do vetor foram adicionadas, por 12 h a 37°C, a cultura contendo 1×10^5 de células HEK 293T na presença de 8 µg/ml de polibreno, após as 12 h o sobrenadante foi retirado e substituído por DMEM fresco .

Após a transdução das células, a avaliação das células expressando GFP foi feita com o microscópio de fluorescência AxioVert MRC (Carl Zeiss, Oberkochen, Alemanha) equipado com um conjunto de filtros FITC (FT 510; BP 450/90; LP 520). Fotomicrografias foram obtidas usando o sistema de captura de imagem AxioCam MRC (Carl Zeiss, Oberkochen, Germany).

As células também foram analisadas por citometria de fluxo no aparelho FACSCalibur (Becton Dickinson, BD, New Jersey, EUA).

Após identificação da presença de células GFP-positivas, as culturas foram avaliadas quanto à atividade da IDUA. Aproximadamente 10^5 células foram homogeneizadas em solução de lise contendo 0,02% Triton X-100 em PBS. Após,

as amostras foram submetidas a um microensaio enzimático, usando 10 μL de amostra, 4 μL substrato fluorogênico 4-methylumbelliferyl- α -L-iduronide (2.85 nmol/L) (Glycosynth, Cheshire, England) e 25 μL de tampão formato (0.2 mol/L, pH 2.8). A reação foi parada com a adição de 175 μL de tampão glicina-NaOH (0.5 mol/L, pH 10.3), a fluorescência foi medida no fluorímetro SpectraMax Plus384 (Molecular Devices, Toronto, Canadá), com 370 nm de excitação e 460 nm de emissão. A concentração de proteína total foi medida pelo método de Peterson (1977), usando albumina bovina como padrão. Os resultados da atividade da IDUA foram expressos como nmoles/h/mg proteína.

A microscopia de fluorescência e citometria de fluxo não revelaram presença de células GFP-positivas nas culturas de HEK 293T transduzidas com FIV-IDUA.

A dosagem enzimática das células que receberam o FIV está representada na figura 1. A atividade enzimática das células transduzidas com o vetor contendo IDUA mostrou-se três vezes maior que a atividade basal das células HEK 293T não transduzidas.

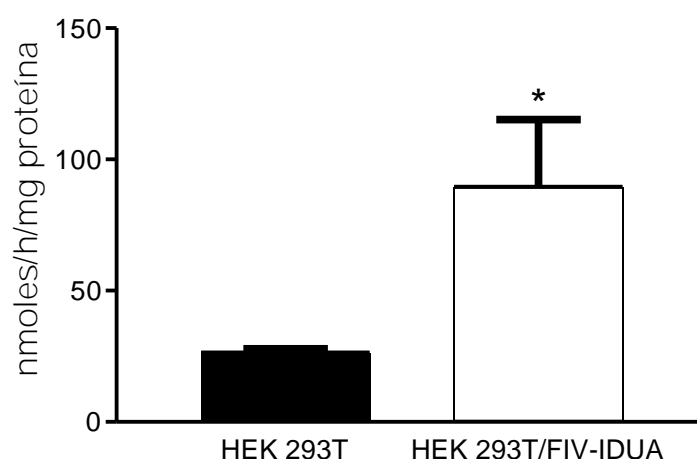


Figura 1 - Média dos valores da atividade enzimática da IDUA. HEK 293T, em experimentos realizado em triplicata. As células transduzidas com o vírus FIV-IDUA (HEK 293T/FIV-IDUA) mostraram atividade significativamente maior que células não transduzidas (HEK 293T, atividade basal). * $p < 0,0001$.

Para uma utilização ótima da terapia gênica, ainda são essenciais estudos básicos focados no desenvolvimento de vetores eficazes e ao mesmo tempo a serem utilizados em modelos animais e futuramente em humanos. Para isso, um dos objetivos desta tese foi o desenvolvimento de vetores para terapia gênica de MPS I.

Os resultados relacionados ao desenvolvimento do vetor baseado em FIV sugerem que a construção do plasmídeo de transferência pTIGER-IDUA está na forma correta, tanto no aspecto estrutural quanto funcional. O aumento na atividade enzimática da IDUA em comparação com células não transduzidas é uma evidência de que a clonagem IDUA-IRES-GFP está expressando o gene IDUA de forma correta. A ausência de células GFP-positivas possivelmente indica uma menor sensibilidade dos métodos de detecção da expressão do gene

repórter. Entretanto, este resultado, somado ao fato de que o outro lentivírus empregado neste trabalho (HIV) resultou em um aumento de 60 vezes na atividade da IDUA em células HEK 293T (capítulo 3), indicam a necessidade de aperfeiçoar este sistema para uma maior expressão dos dois genes.

Capítulo 8

Discussão

Discussão

Nosso modelo murino já foi utilizado para alguns estudos de protocolos pré-clínicos no tratamento da mucopolissacaridose tipo I. Os ensaios de terapia por reposição enzimática foram testados nesse modelo e atualmente o tratamento já se encontra em fase clínica. Já foram testados protocolos de transferência gênica não viral e viral, e mostraram-se bons resultados quanto ao desempenho dos vetores usados. Os dois tipos de tratamento, entretanto, têm sido limitados pela resposta imunológica induzida contra o transgene, mas em alguns ensaios de terapia gênica a resposta é ainda maior por ser induzida contra aos próprios vetores, fazendo com que as células transduzidas sejam eliminadas do organismo (Lowenstein *et al.*, 2007). Esta situação, portanto, resulta na necessidade de vetores mais seguros e ao mesmo tempo eficientes para o tratamento da MPS I.

Os vetores virais desenvolvidos nesse trabalho podem constituir boas ferramentas para estudos de protocolos pré-clínicos para MPS I, já que apesar de baixos títulos virais mostraram eficiência na expressão do transgene. Além disto, o vetor retroviral mostrou capacidade de transduzir células-tronco mesenquimais, importantes alvos da terapia gênica. O emprego do vetor retroviral resultou em baixa atividade enzimática nos animais transduzidos, o que entretanto não é um fator limitante para doenças metabólicas e especificamente para MPS I, já que as células possuem a capacidade de internalizar a enzima IDUA contida no meio extracelular usando os receptores manose-6-fosfato (Unger *et al.*, 1994).

O vetor baseado em FIV (sistema Felix) mostrou funcionalidade inferior quando comparado com os outros sistemas virais desenvolvidos aqui. As partículas virais desse vetor foram produzidas usando o plasmídeo empacotador

de um outro sistema FIV, pois o plasmídeo de empacotamento do sistema Felix TIGER não estava disponível quando construímos o plasmídeo de transferência (pTIGER-GFP-IDUA). Esse plasmídeo de transferência poderá ser testado com o plasmídeo de empacotamento especificamente descrito para o sistema, uma vez que o mesmo encontra-se agora comercialmente disponível através da empresa ADDGENE. Além deste fator, entretanto, o empacotamento também pode estar sendo influenciado pelo grande tamanho do inserto correspondente ao transgene. Quando comparamos os sistemas baseados em HIV e FIV podemos observar uma maior eficiência do vetor baseado em HIV quanto à atividade da IDUA; o promotor usado para o sistema FIV é mais fraco como já descrito (Chen *et al.*, 2008).

Os vetores virais com capacidade integrativa ainda estão sendo estudados quanto à sua integração no genoma das células. O objetivo de estudos com terapia gênica é não modificar o perfil de expressão gênica da célula além da ativação do transgene a ser transferido (Hackett *et al.*, 2007). Estudos para o desenvolvimento de vetores mais seguros e eficientes tornam-se cada vez mais necessários.

Apesar de todo esse esforço concentrado no desenvolvimento de vetores mais seguros e eficientes nos ensaios pré-clínicos, o acompanhamento dos mesmos é geralmente concentrado em análises da atividade enzimática, expressão do transgene, conteúdo de glicosaminoglicanos, testes comportamentais, função cardíaca e análises de esqueleto. Pelo que conhecemos hoje sobre MPS I, entretanto, principalmente dos modelos animais usados que são ferramentas importantes nesses estudos, tornam-se cada vez mais

necessários maiores estudos de pesquisa básica para esclarecermos a situação fisiopatológica das células MPS I, principalmente do sistema nervoso central. Assim, neste estudo, além do desenvolvimento dos vetores virais, também tivemos como objetivo aprofundar a caracterização do modelo murino de MPS I, e para isso analisamos alguns parâmetros bioquímicos do mesmo.

Nas análises de citoesqueleto observamos algumas alterações como descrito no capítulo 4. A ERK1/2 encontrada no hipocampo pode estar sendo fosforilada por outras quinases e com isso alterando os processos normais de aprendizado e memória, dos quais ela faz parte diretamente (Bekinschtein *et al.*, 2008). Além disso, a ERK1/2 tem papel importante no desencadeamento de novos processos relacionados com a sobrevivência celular, indicando assim uma possível explicação para esse tipo de doença neurodegenerativa (Zhuang e Schnellmann, 2006). Ainda em se tratando do hipocampo, não achamos uma associação direta da fosforilação das proteínas de neurofilamentos com a fosforilação da ERK1/2, indicando, como esperado, um sistema complexo de sinalização celular onde outras vias podem estar agindo sozinhas e/ou em sinergismo com a ERK1/2 no controle da fosforilação dessas proteínas. Para uma maior compreensão desse sistema, maiores estudos com outras vias de sinalização celular são necessários.

No mesmo estudo, descrevemos uma hiperfosforilação nas proteínas dos neurofilamentos no córtex, e hiperfosforilação da ERK1/2, podendo haver uma relação direta entre esses resultados. Para melhores esclarecimentos quanto ao córtex são necessários estudos mais detalhados, por tratar-se de uma estrutura maior e mais complexa. Ainda, os resultados encontrados para ERK1/2 poderiam

indicar uma alteração na regulação funcional da proteína, mas os tipos de análises realizadas não foram capazes de indicar especificamente quais sítios das proteínas estão sendo fosforilados.

As alterações encontradas na ERK1/2 são a nível pós-traducional, já que não há alterações quanto à quantidade da proteína. Estes resultados sugerem uma possível alteração na regulação funcional da ERK1/2.

GFAP é uma proteína específica de células gliais e sua alteração pode indicar que, além de neurônios, outros tipos celulares estão modificados neste quadro patológico. Isto pode ser explicado por alterações em nível basal, podendo assim corresponder aos próprios depósitos de GAGs. Para relacionar as alterações no hipotálamo nesse modelo murino, testes comportamentais deverão ser feitos para auxiliar na compreensão funcional dessas alterações. Estas análises devem incluir, por exemplo, testes de ansiedade, depressão e comportamento agressivo.

Com base em todas as alterações encontradas com as investigações de citoesqueleto podemos concluir que as alterações apresentam-se dependentes da estrutura analisada. Concluindo, os resultados obtidos nas análises do citoesqueleto indicam que as alterações neuronais do modelo podem estar sendo influenciadas por distúrbios em rotas de sinalização responsáveis pela sobrevivência celular e transporte axonal.

Foram identificados GM3 e GM2 em hipocampo e hipotálamo, como detalhado no capítulo 5. A presença de GM3 pode ser devida à inibição das enzimas que o degradam, o que seria causado pelo depósito principal da doença, os glicosaminoglicanos. A presença desse depósito de gangliosídeo pode ser

também devida a um aumento na sua síntese, envolvendo modificações das enzimas da rota de síntese. Como já descrito para doenças neurodegenerativas, muito comumente observa-se morte celular, e esta aparece diretamente ligada a desbalanços oxidativos, alterados possivelmente devido à presença de GM3. Em um trabalho recentemente realizado em colaboração com nosso grupo de pesquisa, já foram descritos desbalanços oxidativos para este modelo murino de MPS I. As análises foram feitas em alguns órgãos incluindo cérebro, permitindo a identificação de alterações que podem indicar morte celular (Reolon *et al.*, comunicação pessoal).

Analisamos a neurotransmissão glutamatérgica através dos experimentos de aptação de glutamato e *binding*. Com isso vimos que existe uma diminuição na captação tanto no córtex como no hipocampo nos animais MPS I, além de uma diminuição no *binding*. A diminuição da captação de glutamato tanto em córtex como hipocampo nos animais MPS I pode estar ocorrendo devido a uma alteração na quantidade ou ativação nos receptores de glutamato. A diminuição da ativação dos receptores pode também acontecer quando existem alterações na fosforilação necessária para tal. Com a baixa captação, mais glutamato estará na fenda pré-sináptica e as moléculas podem estar entrando no neurônio e não ativando seus receptores na membrana. Assim, ao invés de uma excitotoxicidade glutamatérgica que normalmente ocorre pelo glutamato, o dano pode ser mesmo pela toxicidade oxidativa que leva à morte neuronal (Sohn *et al.*, 2006).

Ainda, essa toxicidade oxidativa do glutamato associa-se diretamente com o gangliosídeo GM3. Através da associação direta com o glutamato, o gangliosídeo GM3 pode levar à morte celular e ainda, ao ser metabolizado, pode

ser precursor na liberação de ceramida e com isso induzir apoptose, já que a ceramida é um esfingolípido pró-apoptótico (Sohn *et al.*, 2006; Teichgräber *et al.*, 2008).

A neurotransmissão glutamatérgica também depende da fosforilação de seus transportadores, o que pode estar ligado aos dados que já descrevemos sobre fosforilação e principalmente a ERK1/2 (Danbolt, 2001).

Ao contrário dos artigos anteriormente descritos com esse modelo de MPS I, identificamos diferenças no perfil de gangliosídeos. Os trabalhos demonstram que os depósitos de GM3 e GM2 ocorrem em extratos de cérebro total, mas nenhum deles mostra a caracterização do perfil gangliosídico para cada estrutura.

Os proteoglicanos de membrana são moléculas importantes por estarem envolvidas em muitas funções celulares como rotas de sinalização, adesão, *rafts* de membrana, matriz extracelular e manutenção de receptores (Bernfield *et al.*, 1999). Na estrutura molecular desses proteoglicanos de membrana, um componente importante é o heparan sulfato, um dos principais GAGs acumulados nas células de MPS I. Na literatura não existem estudos de detalhamento da composição molecular das membranas neuronais derivadas de indivíduos com MPS I. Cabe salientar que, a partir dos resultados obtidos em nossos trabalhos, serão ainda mais importantes estudos desse tipo.

O presente estudo é um ótimo exemplo das necessidades atuais de associar a pesquisa aplicada com a pesquisa básica em um mesmo modelo de doença. Todos os pontos levantados nesse projeto fazem parte de um grande conjunto de aspectos que deverão ser melhor caracterizados na MPS I, assim como nos outros tipos de mucopolissacaridoses. Com os dados obtidos aqui,

grupos de pesquisa poderão fazer acompanhamentos detalhados em protocolos pré-clínicos de terapia gênica, celular e reposição enzimática, além de enriquecer as pesquisas sobre biologia básica da célula.

Capítulo 9

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