UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:BIOQUÍMICA

AVALIAÇÃO DA ATIVIDADE NEUROPROTETORA DO GANGLIOSÍDIO GM1 EM MODELO *IN VIVO* E *IN VITRO* DE TOXICIDADE DO PEPTÍDEO β-AMILOIDE.

Fernando Kreutz

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SUMÁRIO

APRESENTAÇÃO

A presente tese está organizada em seções dispostas da seguinte maneira: *Introdução, Objetivos, Resultados (Artigos I, II e III), Discussão, Conclusões, Perspectivas,* e *Referências Bibliográficas.*

Na *Introdução* são apresentados os embasamentos teóricos que conduziram a elaboração da proposta da tese e das hipóteses a serem analisadas na mesma, conforme exposto na seção *Objetivos.*

A seção *Resultados* está organizada em três Artigos, entre os quais, o primeiro está publicado, o segundo está em análise e o terceiro será submetido à publicação em revistas científicas da área. Cada um deles está organizado de acordo com os objetivos específicos propostos para a tese. Nesta seção estão detalhados os materiais e métodos utilizados para cada um dos trabalhos científicos que compõem a tese. Os três artigos que compõem esta tese foram desenvolvidos no Laboratório de Bioquímica e Biologia Celular de Lipídios e no Laboratório de Neuroproteção e Sinalização Celular, do Departamento de Bioquímica, da Universidade Federal do Rio Grande do Sul, sob orientação da Profa. Dra. Vera Maria Treis Trindade e co-orientação da Profa. Dra. Chistianne Gazzana Salbego.

Na seção *Discussão* é apresentada a interpretação geral dos resultados da tese, conforme cada um dos artigos que compõem a mesma. Na seção *Conclusões* é apresentada a conclusão final do estudo, de acordo com os objetivos iniciais traçados e os resultados obtidos. A seção *Perspectivas* apresenta os potenciais projetos a serem desenvolvidos a partir da conclusão do presente trabalho, de forma a dar continuidade à linha de pesquisa aqui desenvolvida.

Na seção *Referências Bibliográficas* é apresentada a bibliografia completa das citações científicas que compõem as seções *Introdução* e *Discussão* da presente tese.

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LISTA DE ABREVIATURAS

ADI (*Alzheimer Disease International*)

AD (Alzheimer's disease=doença de Alzheimer)

AICD (amyloid intracellular domain)

APP (amyloid precursor protein)

Aβ (beta-amiloide)

DA (Doença de Alzheimer)

GSL = Glicoesfingolipídio

ICV/icv= intracerebroventricular

βMCD= β-metil-ciclodextrina

AChE= Acetilcolinesterase

RESUMO

A doença de Alzheimer (DA) é uma desordem neurodegenerativa caracterizada por perda da memória e das demais funções cognitivas. Sua patogenia envolve a produção do peptídeo β-amiloide (Aβ), através da clivagem da proteína precursora amiloide (APP) por β- e γ-secretases (amiloidogênese), e a posterior agregação do Aβ em oligômeros e/ou fibrilas. A toxicidade do Aβ tem sido associada a diversos mecanismos, a incluir desde seu efeito oxidante e inflamatório, até sua propriedade de induzir necrose por ruptura das membranas neurais, ou apoptose via ativação de cascatas sinalizatórias. Como etapa comum a estes mecanismos de toxicidade, a interação do peptídeo com membranas neurais, em particular com o gangliosídio GM1 (constituinte dos *rafts*), parece ser indispensável ao dano neural associado ao peptídeo, bem como à ativação da cascata amiloide que caracteriza o ciclo patológico Aβ-amiloidogênese. O GM1 é um gangliosídio de membrana que, quando administrado exogenamente, apresenta propriedades neurotróficas e neuroprotetoras, inclusive em modelos de DA. Os mecanismos envolvidos nesta neuroproteção, no entanto, ainda precisam ser melhor elucidados. Com isto, o objetivo da presente tese foi avaliar o potencial efeito neuroprotetor do GM1 em modelo *in vivo* e *in vitro* de toxicidade do $A\beta_{1-42}$ fibrilado, e propor mecanismos para esta neuroproteção. Inicialmente, demonstramos que o GM1 (0,30 mg/kg), quando co-administrado (icv) com Aβ (2 nmol) previne o déficit cognitivo induzido pelo peptídeo (teste de reconhecimento de objetos), bem como a redução na atividade da Na^+,K^+-ATPase (enzima envolvida na regulação da transmissão sináptica) no hipocampo de ratos Wistar machos. Demonstrou-se, além disso, que este efeito neuroprotetor do GM1 é acompanhado de aumento nas defesas antioxidantes, em córtex e hipocampo (TRAP). Como a toxicidade do Aβ e a modulação da amiloidogênese parecem ser dependentes de alterações na estrutura dos microdomínios de membrana (*rafts*), investigamos o efeito da injeção (icv) de Aβ e do tratamento (icv) com GM1 sobre a integridade dos *rafts* e sobre a distribuição das proteínas APP e BACE1 (β-secretase) nestes microdomínios. Observamos que o Aβ promoveu um desmonte parcial nos *rafts*, acompanhado de um aumento na distribuição de APP e BACE1 nestes microdomínios, efeitos estes que foram prevenidos pelo tratamento com GM1. Em virtude de os *rafts* modularem diversas funções neurais, e de a co-localização de APP e BACE1 nos *rafts* ser um evento necessário a amiloidogênese, os resultados aqui obtidos, reforçam a ideia de que o GM1 exerça sua função neuroprotetora ao preservar a arquitetura das membranas e que o mesmo poderia frear a ativação da amiloidogênese induzida pelo Aβ, ao prevenir a redistribuição das proteínas APP e BACE1 aos *rafts* lipídicos. A fim de avaliar a atividade neuroprotetora do GM1 em modelo *in vitro* da DA, e de investigar a sua propriedade de interagir com o Aβ impedindo a interação deste às membranas neurais, avaliamos o efeito da incubação de GM1 (10, 20 e 30μM) frente à toxicidade do Aβ (0,5μM) em células de neuroblastoma humano SH-SY5Y, bem como, verificamos o efeito de uma prévia incubação Aβ-GM1 (*in vitro*) sobre a toxicidade induzida pelo peptídeo. Como resultado, observamos que o GM1 promoveu neuroproteção nas três concentrações testadas e que esta neuroproteção foi, pelo menos parcialmente, mediada por sua capacidade de interagir *in vitro* com o Aβ. Nossos dados, em conjunto, reforçam as evidências que apontam o GM1 como uma potencial droga neuroprotetora em modelos de DA.

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a loss of memory and impairment of other cognitive functions. Its pathogenesis involves the production of β-amyloid peptide (Aβ) by cleavage of the amyloid precursor protein (APP) by β- and γ-secretases (amyloidogenesis) and the subsequent aggregation of Aβ into oligomers and/or fibrils. Aβ toxicity has been associated with several mechanisms, ranging from its oxidant and inflammatory effects until their property to induce necrosis by neural membrane rupture, or apoptosis via activation of signaling cascades. As a common step to these toxicity mechanisms, the peptide interaction with neural membranes, particularly with GM1 ganglioside (component of membrane rafts), seems to be pivotal to the peptide induced neural damage, as well as the amyloid cascade activation that characterizes the pathological vicious cycle Aβ- amyloidogenesis. GM1 is a membrane ganglioside provided of neurotrophic and neuroprotective properties in AD models, when exogenously administered. The mechanisms of neuroprotection, however, need to be further elucidated. By this way, the aim of this PhD thesis was to evaluate the potential neuroprotective effect of GM1 in *in vivo* and *in vitro* models of fibrilar $A\beta_{1-42}$ toxicity, and to propose mechanisms for this neuroprotection. Initially, we demonstrated that GM1 (0.30 mg/kg.), when co-administered (icv) with Aβ (2nmol), prevents the peptide induced cognitive deficit (object recognition task), as well as Aβ induced reduction in Na^+,K^+-ATPase activity (a enzyme involved in synaptic transmission regulation) in the hippocampus from male Wistar rats. We have shown, furthermore, that this neuroprotective effect of GM1 is accompanied by an increase in antioxidant defenses in the cortex and hippocampus (TRAP). As Aβ toxicity, as well as the modulation of amyloidogenesis, appear to be dependent on changes in the structure of membrane microdomains (rafts), we have investigated the effect of Aβ icv injection and GM1icv treatment on raft integrity and on the distribution of APP and BACE1 (β-secretase) proteins in these microdomains. As result, Aβ promoted a partial raft disassemble, accompanied by an increase in the distribution of APP and BACE1 to these microdomains, effects that were prevented by GM1 treatment. Considering that rafts modulate several neural functions, and that APP and BACE1 co-localization into lipid rafts is pivotal to amyloidogenesis, our results reinforce the idea that GM1 neuroprotection is mediated by membrane architecture preservation, and that GM1 treatment could slow down the Aβ induced activation of amyloidogenesis, by prevention of APP and BACE1 redistribution into lipid rafts. In order to evaluate the GM1 neuroprotective activity in an *in vitro* AD model, and to investigate GM1 property of interacting with Aβ and preventing its interaction with neuronal membranes, we evaluated GM1 (10, 20 and 30μM) effect against Aβ (0,5 μM)-induced toxicity in SH-SY5Y human neuroblastoma cells, as well as, we verified the effect of a Aβ-GM1 *in vitro* preincubation on the peptide-induced toxicity. As our results indicate, the three tested GM1 concentrations promoted neuroprotection and this effect was, at least partially, mediated by its ability to *in vitro* interact with Aβ peptide. Our data, when taken together, reinforce the evidence suggesting GM1 as a potential neuroprotective drug in AD models.

1. INTRODUÇÃO

1.1 A Doença de Alzheimer

A doença de Alzheimer (DA), descrita inicialmente em 1906 pelo médico alemão Alois Alzheimer (Alzheimer, 1911) é uma doença neurodegenerativa caracterizada por perda progressiva, e até o momento irreversível, da memória e das demais funções cognitivas. Corresponde, atualmente, à principal forma de demência entre indivíduos com idade acima de 60 anos, atingindo atualmente em torno de 35 milhões de pacientes no mundo (Forman et al., 2004; Querfurt and LaFerla, 2010), número este que chegará a 135,5 milhões até 2050, segundo estimativa da *Alzheimer's Disease International* (ADI, 2014). No Brasil, a Associação Brasileira de Alzheimer aponta a existência de aproximadamente um milhão de pacientes diagnosticados com esta forma de demência (ABRAz, 2014).

A DA é uma doença multifatorial, visto que seu desenvolvimento e evolução envolvem tanto fatores genéticos quanto ambientais. Os principais fatores de risco associados à patologia são a idade avançada, o sexo feminino, a presença de injúria cerebral prévia, e a expressão do alelo ε4 da apolipoproteína E (Suh and Checler, 2002). Basicamente duas formas de DA são descritas: a DA do tipo familiar (5% dos casos), em que mutações no gene da Proteína Precursora Amilóide (APP) ou das enzimas secretásicas (como a presenilina) acarretam no desenvolvimento precoce do quadro demencial (pacientes com idade inferior a 50 anos já apresentando sintomas); e a DA do tipo esporádico (95% dos casos), em que a evolução é mais insidiosa, e no qual o quadro demencial manifesta-se de forma mais tardia (Cotran et al., 1994; Suh and Checler, 2002).

A evolução da DA é lenta e progressiva. O primeiro estágio clínico é caracterizado por um comprometimento leve das funções cognitivas, em que o paciente apresenta déficits sutis e intermitentes na memória de curto prazo, principalmente relacionada a pequenos eventos do cotidiano. À medida que a doença progride, as funções cognitivas e comportamentais vão sendo progressivamente afetadas, o indivíduo passa a perder a capacidade de recordar de alguns eventos distantes do passado, bem como de realizar as mais simples tarefas do dia-a-dia. Já em seu estágio terminal, a estabelecer-se em média 8 a 10 anos após seu diagnóstico clínico, o paciente atinge um estado semivegetativo, sendo incapaz de reconhecer sua identidade, de

aprender ou interagir com o meio, de comunicar-se ou de realizar qualquer espécie de tarefa (Suh and Checler, 2002; Alzheimer's Association, 2011).

O diagnóstico da DA é clínico e por exclusão, baseado principalmente na identificação e avaliação do déficit cognitivo. A confirmação do diagnóstico, no entanto, dá-se unicamente através da análise *pos-mortem* do cérebro do paciente, por meio da identificação dos principais achados morfológicos e patognomônicos para a doença de Alzheimer, a saber: a presença de placas senis extracelulares – compostas por agregados do peptídeo β-Amilóide (Aβ) –, e de emaranhados neurofibrilares – constituídos de depósito intraneural da proteína tau hiperfosforilada (Suh and Checler, 2002; Ballard et al., 2011). Alguns estudos recentes, usando imagens de MicroPET obtidos de estruturas cerebrais de ratos transgênicos indicam uma potencial confirmação de diagnóstico da DA num futuro próximo (Zimmer et al., 2014-a; 2014-b).

Embora seja descrita há mais de um século, os mecanismos envolvidos no desenvolvimento e na progressão da DA ainda não estão completamente estabelecidos. Acredita-se, no entanto, que as alterações neuroquímicas que levariam ao desenvolvimento do quadro demencial iniciem de forma precoce, pelo menos uma década antes de os sintomas cognitivos serem detectáveis (Beason-Held et al., 2013). Dentre as hipóteses de patogenia para a DA, a que tem assumindo maior importância é a da cascata amiloide e da consequente produção e depósito do peptídeo Aβ (Bloom, 2014).

1.1.1 Peptídeo β-Amiloide

O Aβ é um peptídeo constituído de 36 a 43 aminoácidos, formado pelo processamento proteolítico anormal (amiloidogênese) de uma glicoproteína de membrana chamada proteína precursora amiloide (APP). O peptídeo formado, em especial o constituído por 42 aminoácidos (Aβ1-42), apresenta, quando no meio extracelular, a propriedade de polimerizar-se ou agregar-se na forma de oligômeros e/ou fibrilas (fibrilogênese), formas nas quais passa a apresentar propriedades neurotóxicas (Haass and Selkoe, 2007; Stine et al., 2003; Suh and Checler, 2002; DaSilva et al., 2010; Querfurth and LaFerla, 2010; Selkoe, 2011). Embora o peptídeo oligomérico seja considerado de maior toxicidade, o Aβ fibrilado, por ser insolúvel, representa a principal forma de depósito do peptídeo no parênquima cerebral, formando as chamadas

placas senis, e funciona como um reservatório de liberação sustentada do peptídeo oligomérico (Figura 1) (Shankar et al., 2008; Selkoe, 2011).

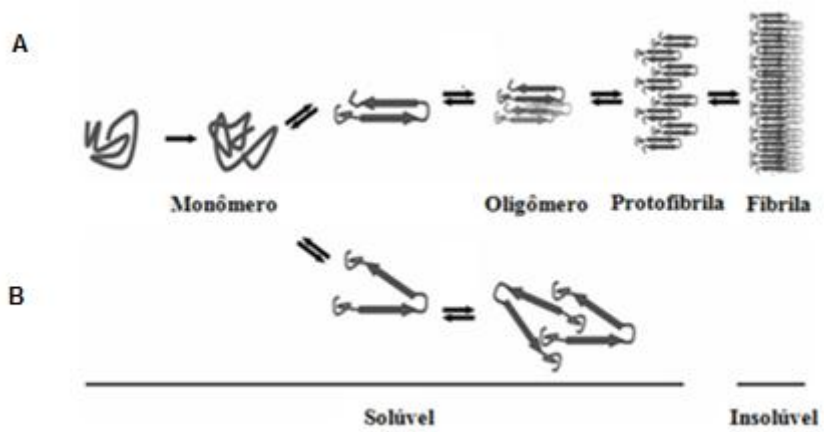

Figura 1. Processo de oligomerização e fibrilação do peptídeo β-amiloide: (A) O peptídeo β-amiloide pode sofrer um processo sequencial de agregação, formando oligômeros, protofibrilas e fibrilas insolúveis. (B) Alternativamente, o Aβ pode sofrer um processo de oligomerização indepentente, formando oligômeros que, embora não evoluam para formação de fibrilas, apresentariam considerável efeito neurotóxico (Adaptado de DaSilva et al., 2010).

Diversos mecanismos de toxicidade têm sido propostos ao Aβ, tanto em sua forma oligomérica quanto fibrilada: indução de estresse oxidativo (Butterfield DA, 2002; Giraldo et al., 2014); neuroinflamação (Hoppe et al., 2010; Bernardi et al., 2012; Frozza et al., 2013; McLarnon, 2014); dano à integridade ou alterações na estrutura/organização das membranas neurais (Sciacca et al., 2012); ativação de vias de morte celular (GSK3β) (Nassif et al., 2007; Hoppe et al., 2010; Kreutz et al., 2011; Hoppe et al.2013-a; 2013-b) e da fosforilação da proteína tau (envolvida na formação dos emaranhados neurofibrilares) (Hoppe et al., 2010; Giraldo et al., 2014); perda de funções sinápticas por alteração de proteínas sinápticas (Hoppe et al., 2013-c) e da atividade de enzimas como acetilcolinesterase (AChE) (Melo et al., 2003) e Na^+,K^+-ATPase (Bores et al., 1998; Xiao et al., 2002; Gu et al., 2004). Estes efeitos, associados a diversos outros mecanismos, culminam com o desencadear de perda de função neural e apoptose ou necrose das células nervosas (Lee et al., 2014; Liang et al., 2014).

1.1.2 Cascata amiloide e processamento da APP

A cascata amiloide representa a via de processamento da proteína precursora amiloide (APP) que culmina, em última análise, na liberação, dentre outros produtos, do peptídeo β-amiloide (Querfurth and LaFerla, 2010).

A APP, expressa de forma constitutiva nas membranas neurais, pode seguir duas vias de processamento: o processamento amiloidogênico, cujo principal produto seria o peptídeo β-amiloide; e o processamento não-amiloidogênico, via proteolítica pela qual o peptídeo não seria formado (Zhang et al., 2011-a).

O processamento amiloidogênico inicia-se por ação proteolítica de uma β-secretase, cuja principal representante é a BACE1, uma aspartil-protease de membrana que cliva a APP na extremidade N-terminal do domínio β-amiloide. Como consequência da ação da BACE1, é liberado ao meio extracelular o peptídeo sAPPβ. O fragmento da APP que permanece inserido na membrana é então alvo da ação do complexo γ-secretase (cujo centro catalítico é representado pela enzima presenilina), sendo clivado na extremidade C-terminal do domínio β-amiloide, liberando ao meio extracelular o peptídeo Aβ e, ao citosol, o domínio intracelular da APP (AICD), também provido de atividades neurotóxicas (Figura 2) (Suh and Checler, 2002; Zhang et al., 2011-a).

Já o processamento dito não-amiloidogênico tem início por uma clivagem da APP, não por β-secretase, mas sim por enzima com atividade α-secretásica, cuja principal representante é a enzima ADAM10 (Suh and Checler, 2002). O processamento por α-secretase cliva a APP dentro do domínio β-amiloide, prevenindo, desta forma, a posterior produção do Aβ. Os principais produtos da via não amiloidogênica são o fragmento solúvel da APP (sAPPα), liberado ao meio extracelular e dotado de ação neurotrófica (Figura 2) (Roch et al., 1994; Ring et al., 2007; Claasen et al., 2009; Zhang et al., 2011-a), e o peptídeo P3, que ao contrário do Aβ, é desprovido de atividades neurotóxicas (Suh and Checler, 2002; Zhang et al., 2011-a).

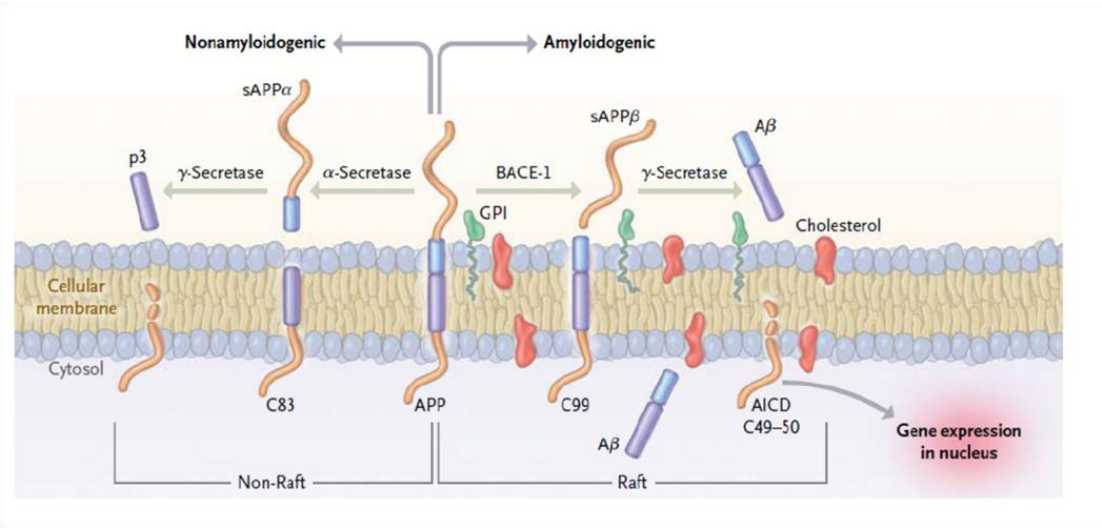

Figura 2. **Via amiloidogênica e não amiloidogênica do processamento da APP.** O processamento amiloidogênico é determinado pela clivagem sequencial da APP por β-secretase (BACE1) e γ-secretase, gerando como principal produto neurotóxico o peptídeo Aβ. A via não amiloidogênica, por outro lado, é desencadeada pela clivagem da APP por α-secretase (e sequencialmente γ-secretase), o que impede a formação do peptídeo Aβ e promove a liberação do peptídeo sAPPα, dotado de atividade neurotrófica.

Evidências sugerem que, em condições normais, apenas 10% do *pool* de APP seja dirigido à via amiloidogênica, e o restante seja processado pela via não amiloidogênica (Ehehalt et al., 2003). No entanto, fatores diversos, a incluir a idade, poderiam causar um desequilíbrio na regulação da cascata, favorecendo a via amiloidogênica em detrimento da não-amiloidogência, e desta forma, contribuir para o desenvolvimento da doença de Alzheimer ou para a progressão do estado demencial já em andamento (Guglielmotto et al., 2009; Placanica et al., 2009; Zhang et al., 2011-a; Sun et al., 2012; Saido, 2013).

1.1.3 A cascata amiloide e o peptídeo Aβ como alvos terapêuticos no tratamento da DA

Embora se considere que o β-amiloide não seja o único responsável pelas alterações neuroquímicas que desencadeiam a DA, as evidências sugerem um papel

central deste peptídeo tanto no desenvolvimento quanto na progressão desta forma de demência. Segundo Crews e Masliah (2010), o Aβ representaria a principal causa de disfunção sináptica, de morte neuronal e de declínio cognitivo em pacientes com DA.

De forma não menos importante, o peptídeo tem sido associado à progressão da doença de Alzheimer, visto ser capaz de induzir a hiperfosforilação da proteína tau (Hoppe et al., 2010; Giraldo et al., 2014) e o consequente desenvolvimento dos emaranhados neurofibrilares, assim como estimular a própria cascata amiloide, aumentando ainda mais a formação e a deposição do peptídeo no sistema nervoso central (ciclo patológico Aβ-amiloidogênese) (Peters et al., 2009; Yan and Vassar, 2014).

Desta maneira, drogas capazes de modular a cascata amiloide (reduzindo a formação do Aβ), ou de prevenir/bloquear suas propriedades neurotóxicas e sua capacidade de ativar a amiloidogênese ou os demais mecanismos envolvidos na progressão da DA, teriam um impacto expressivo na terapêutica da doença de Alzheimer (Racchi and Govoni, 2003; Bernardi et al., 2012; Frozza et al., 2013; Hoppe et al., 2013-a; 2013-b; 2013-c; Wang et al., 2013).

Neste sentido, diversos inibidores das enzimas secretásicas (BACE1 e presenilina) têm sido produzidos e testados quanto à sua capacidade de reduzir ou bloquear a produção do peptídeo Aβ. Embora mostrem-se efetivos na redução dos níveis de Aβ, tais drogas apresentam uma série de efeitos colaterais, possivelmente decorrentes da inibição da ação destas secretases sobre outros substratos fisiológicos seus. Além disso, como o diagnóstico da DA se dá quando o paciente já apresenta certo déficit cognitivo e a evolução da patologia já se apresenta em curso, a eficácia clínica destes inibidores de secretases é questionável, visto que não apresentariam efeito sobre as placas senis já formadas e depositadas (Yan and Vassar, 2014).

O melhor entendimento dos mecanismos envolvidos na regulação da cascata amiloide, no desencadear dos efeitos tóxicos do Aβ e do ciclo patológico Aβ-amiloidogênese poderiam propiciar o desenvolvimento de drogas que aliassem a capacidade de bloquear ou prevenir a toxicidade do peptídeo já depositado e de, ao mesmo tempo, frear a ativação em cadeia da cascata amiloide. Embora uma droga com tais propriedades ainda não tenha sido desenvolvida, estudos sugerem que a interação do peptídeo com as membranas neurais, particularmente com os *rafts* de membrana, e a

consequente alteração na arquitetura dos mesmos, seria um importante alvo terapêutico a ser explorado neste sentido (Lai and McLaurin, 2010).

1.2 Microdomínios de membrana *Rafts*

O conceito de organização e estrutura de membranas biológicas evoluiu de um modelo que a considerava homogênea em termos de composição lipídica e proteica, para um novo modelo que propõe sua heterogeneidade lateral, reflexo da existência de diferentes microdomínios ao longo das membranas celulares. Neste contexto, *rafts* são microdomínios de membrana enriquecidos em colesterol e esfingolipídios, formados pela interação preferencial entre o núcleo esteroidal do colesterol e o núcleo esfingoide dos diferentes esfingolipídios (Figura 3) (Simons and Ehehalt, 2002; Chauhan, 2003; Reid et al., 2007).

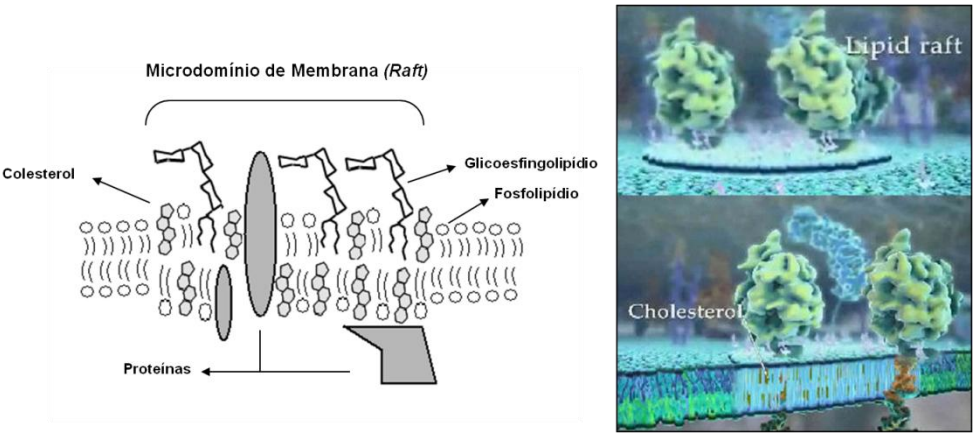

Figura 3. Estrutura e composição dos *rafts* lipídicos. *Rafts* são microdomínios de membrana enriquecidos em colesterol e esfingolipídios. Representam plataformas lipídicas com mobilidade e fluidez diferenciais, formadas e estabilizadas pela interação colesterol-esfingolipídio. São capazes de abarcar, temporária ou indefinidamente, uma série de proteínas que tenham afinidade por estes domínios de membrana, afetando sua conformação espacial (atividade) e promovendo sua compartimentalização ao longo dos diferentes domínios que compõem a membrana plasmática. (Imagem da direita capturada de: http://multimedia.mcb.harvard.edu/anim_innerlife.html)

Capazes de abarcar proteínas específicas, de forma temporária ou permanente/constitutiva, os *rafts* proporcionam um meio de compartimentalizar eventos celulares em nível de membrana, facilitando ou dificultando a interação entre proteínas diferentes, conforme a resposta celular aos mais diversos estímulos do ambiente. A localização de uma determinada proteína num microdomínio de membrana específico pode, além do efeito de compartimentalização, influenciar diretamente a atividade proteica, uma vez que diferentes ambientes lipídicos podem repercutir em alterações na estrutura tridimensional da proteína e, por conseguinte, modular sua atividade. Desta forma, são descritas enzimas cuja atividade é dependente ou pelo menos modulada segundo sua localização nos *rafts* lipídicos (Simons and Ehehalt, 2002; Chauhan, 2003; Reid et al., 2007).

Trabalhos recentes indicam que diversas proteínas sinalizatórias, receptores de neurotransmissores e neurotrofinas, ou proteínas envolvidas em mecanismos patológicos tem sua função, atividade e/ou processamento fortemente influenciados por seu perfil de distribuição entre os diferentes domínios de membrana. O conhecimento dos fatores que modulam sua localização nos *rafts* pode ser explorado como maneira de modular sua atividade *in situ* (Allen et al., 2007).

Portanto, *rafts* são considerados plataformas lipídicas indispensáveis à modulação e à organização dos mais diversos processos celulares, de forma que condições que determinem uma desestruturação destes microdomínios, ou que afetem a dinâmica destas plataformas lipídicas, podem repercutir em efeitos sobre a viabilidade celular, e ter participação no desenvolvimento de diversas doenças neurodegenerativas, como é o caso da doença de Alzheimer (Michel and Bakovic, 2007).

1.2.1 Rafts e DA

Vários trabalhos têm sugerido que a DA seria acompanhada por alterações na composição lipídica das membranas neurais, o que poderia afetar a formação e a dinâmica dos *rafts* (Barrier et al., 2007). Trabalho realizado por nosso grupo demonstrou um aumento na expressão de determinados gangliosídios em modelo agudo de exposição ao peptídeo Aβ (Kreutz et al. 2011). Em contrapartida, avaliação lipídica *pos-mortem* realizada em cérebro de pacientes com DA demonstrou uma redução global no conteúdo de esfingolipídios (Svennerholm and Gottfries, 1994). Esta aparente

discrepância pode refletir diferentes estágios da patologia, onde, em um primeiro momento, a alteração no perfil lipídico das membranas poderia repercutir em alteração na composição e organização dos *raft*s (Peters et al., 2009); e em um estágio terminal, a redução global no conteúdo de esfingolipídios poderia representar um dano à estrutura das membranas neurais, repercutindo em desestruturação dos *rafts* lipídicos, e o comprometimento das vias sinalizatórias dependentes destes microdomínios (Ledesma et al., 2003; Calamai and Pavone, 2013; Hirai et al., 2013; Marin et al., 2013; Sasahara et al., 2013).

Dentre as diferentes proteínas que exercem um papel importante no desenvolvimento ou na evolução da DA, e que poderiam ser afetadas pela composição e organização lipídica das membranas, podem ser citadas: a Proteína Precursora Amilóide (APP), as enzimas β-secretase (BACE1), acetilcolinesterase (AChE) e Na^+K^+ATPase (Bores et al., 1998; ; Xiao et al., 2002; Cordy et al., 2003; Gu et al., 2004).

1.2.1.1 Rafts e a modulação do processamento da APP por β-secretase (BACE1)

Como previamente discutido, APP e BACE1 são proteínas envolvidas na cascata amiloide e responsáveis pela produção do peptídeo Aβ, elemento chave no desenvolvimento da patologia (Suh and Checler, 2002). A BACE1 tem sua atividade modulada pelo ambiente lipídico no qual está inserida, sendo sensível aos níveis de colesterol, de modo que apresenta maior atividade nos microdomínios *rafts* de membrana (Figura 4) (Cordy et al., 2003; Kalvodova et al., 2005). Desta forma, a clivagem de APP por esta enzima, e consequentemente a produção do Aβ e progressão da patologia, parece ser dependente de uma co-localização das referidas proteínas nos *rafts* de membrana, de sorte que eventos ou drogas capazes de alterar a distribuição das mesmas nos diferentes domínios de membrana poderiam ter impacto na modulação da cascata amiloide (Cordy et al., 2003; Schmechel et al., 2004; Hattori et al., 2006; Yoon et al, 2007; von Arnim et al., 2008; Won et al., 2008; Harris et al., 2009; Kojro et al., 2010; Vetrivel and Thinakaran, 2010; Marquer et al., 2011; Williamson and Sutherland, 2011).

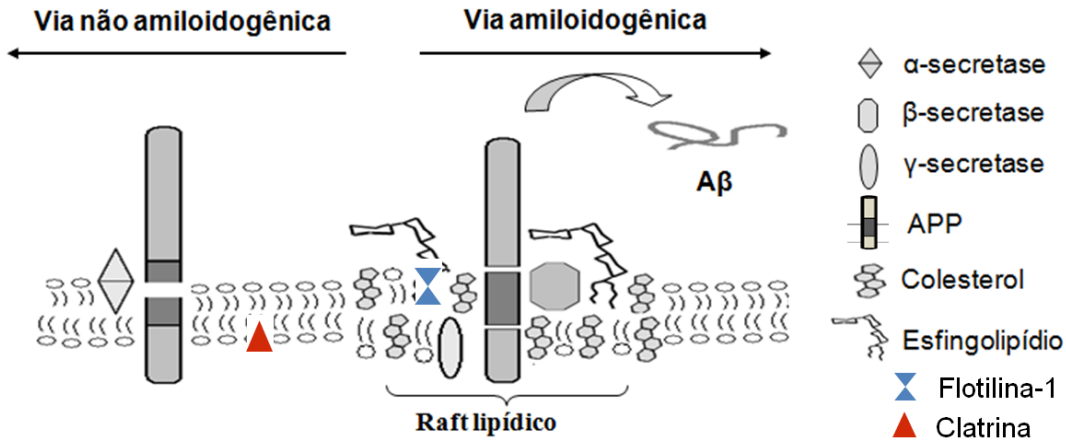

Figura 4. *Rafts* e a regulação da cascata amiloide. A cascata amiloide é regulada pela distribuição e compartimentalização das enzimas secretásicas e da proteína precursora amiloide (APP) nos diferentes domínios de membrana. Considerando que a BACE1 apresenta distribuição nos *rafts* e maior atividade quando inserida no ambiente lipídico destes microdomínios, o *pool* de APP presente nos *rafts* será alvo de β-secretase e processado pela via amiloidogênica (gerando o peptídeo Aβ), ao passo que o pool de APP presente no domínio fosfolipídico será alvo de α-secretase, sendo, portanto, processado pela via não-amiloidogênica.

1.2.1.2 Organização lipídica das membranas e as enzimas acetilcolinesterase (AChE) e Na^+K^+ATPase

A AChE é uma enzima chave na regulação da transmissão colinérgica, visto que catalisa a hidrólise de acetilcolina na fenda sináptica. Uma vez que a DA cursa com déficit colinérgico, medidas terapêuticas que visem aumentar o conteúdo de acetilcolina nas fendas sinápticas, por inibição da AChE, são muito exploradas na terapêutica do Alzheimer, embora tenha escasso efeito sobre a progressão da doença (Suh and Checler, 2002). Estudos indicam que o peptídeo β-amiloide seria capaz de aumentar a atividade acetilcolinesterásica e, desta forma, contribuir diretamente com o déficit colinérgico. Embora a AChE seja uma enzima de membrana, com uma parcela de seu pool associada

aos *rafts*, pouco é conhecido sobre o papel dos microdomínios na regulação desta enzima, e sobre o efeito do peptídeo Aβ sobre a distribuição da AChE nos diferentes microdomínios lipídicos (Melo et al., 2003; Xie et al., 2010; Hicks et al., 2011).

A Na^+,K^+-ATPase, por sua vez, é uma enzima fundamental ao equilíbrio iônico das células neurais, tendo ação direta sobre a manutenção do potencial de membrana e da transmissão sináptica (Xie and Askari, 2002; Lang, 2007). A redução da atividade desta enzima pode acarretar em acúmulo intracelular de cálcio, alterações na transmissão sináptica, e ativação de vias de morte celular (Yu, 2003). Os dados sobre a distribuição da Na^+,K^+-ATPase nos *rafts* de membrana, bem como a influência deste microambiente lipídico em sua atividade, ainda são escassos (Dalskov et al., 2005). Desta forma, embora a literatura sugira que a exposição ao peptídeo Aβ possa promover redução na atividade desta enzima (Bores et al., 1998; Xiao et al., 2002; Gu et al., 2004), não é conhecido se este efeito é mediado por alteração na estruturação dos *rafts* ou por alteração na distribuição da Na^+,K^+-ATPase nestes microdomínios de membrana.

1.2.1.3 Organização lipídica das membranas e outros processos biológicos associados à DA

Os mecanismos que regulam a dinâmica dos *rafts* e sua composição lipídica e proteica, ainda não estão completamente elucidados. Estresse oxidativo, alterações na dinâmica do citoesqueleto, processo inflamatório, alteração no processamento pós-traducional de proteínas, e outros eventos bioquímicos podem influenciar diretamente a migração de uma proteína aos *rafts* ou eventualmente comprometer a formação ou estruturação destas plataformas lipídicas. Desta forma, drogas capazes de interferir nestes eventos poderiam ter um efeito sobre a formação e estruturação dos *rafts* ou diretamente alterar a distribuição de proteínas entre os diferentes domínios de membrana, e assim exercer um papel neuroprotetor em modelos de Alzheimer, interferindo no próprio desenvolvimento e progressão da patologia (Cheng et al., 2007; Li and Gulbins, 2007; Reid et al., 2007).

1.2.2 Gangliosídios e interação do Aβ com as membranas neurais

Gangliosídios são glicoesfingolipídios (GSL) constituídos de um núcleo esfingoide (ceramida) e de uma cadeia oligossacarídica que contém, normalmente, pelo

menos um resíduo de ácido siálico (ácido N-acetil-neuramínico ou ácido N-glicolil-neuramínico). A extensão da cadeia oligossacarídica, bem como o número e a disposição dos resíduos de ácido siálico determinam a heterogeneidade nesta família de glicoesfingolipídios (Yu and Saito, 1992).

A nomenclatura dos gangliosídios, proposta por Svennerholm (1963), leva em conta o número e a disposição dos resíduos de ácido siálico, bem como a extensão da cadeia oligossacarídica. Desta forma, são denominados pela letra G (gangliosídio); seguida de letra maiúscula (M, D, T, Q, P, H ou S), que designa o número de moléculas de ácido siálico presentes na estrutura do gangliosídio (monossialo-, dissialo-, trissialo-, tetrassialo-, pentassialo-, hexassialo- ou heptassialo-, respectivamente); seguido de um algarismo que faz menção à extensão da cadeia oligossacarídica (1, 2, 3,...); e, finalmente, de uma letra minúscula (a, b ou c) que distingue estes lipídios conforme a série a que pertencem na rota de biossíntese dos gangliosídios (Svennerholm, 1963; Maccioni et al., 1999).

Desta forma, o GM1 (GM1a), protótipo da família gânglio dos GSL, corresponde ao monossialotetra-hexosilgangliosídio pertencente a série "a" da rota de síntese dos gangliosídios, sendo constituído por um resíduo de ácido siálico (unido à galactose interna por ligação glicosídica α2-3) e quatro resíduos de oses neutras unidas entre si através de ligações glicosídicas β (Figura 5) (Mocchetti, 2005).

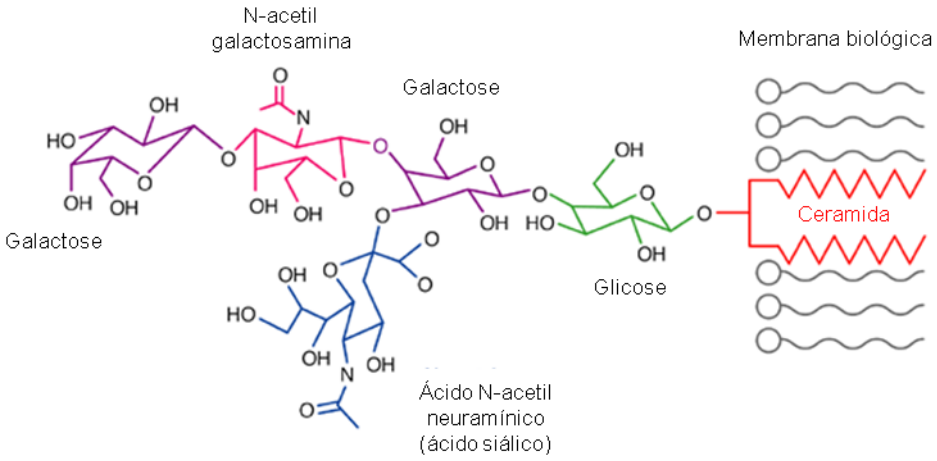

Figura 5. Estrutura química do GM1 e sua inserção na membrana biológica. O GM1 é um gangliosídio constituído de um núcleo esfingóide (ceramida), unido a uma cadeia polissacarídica que apresenta, em sua primeira galactose, uma molécula de ácido siálico (ácido N-acetil-neuramínico). Está predominantemente presente na fatia exofacial das membranas neurais, inserido na mesma através de sua porção ceramida (apolar). A cadeia polissacarídica fica, desta forma, exposta ao meio extracelular, compondo o glicocálice. fonte: http://journals.cambridge.org/fulltext_content/ERM/ERM4_21/S1462399402005057sup004.htm (15/09/2014).

Embora se distribua tanto nos domínios *raft* e quanto não-*raft* (domínio fosfolipídico), o gangliosídio GM1 apresenta maior afinidade pelos *rafts* lipídicos devido às interações entre seu núcleo esfingoide e a cadeia esteroidal do colesterol presente nestes microdomínios. Esta interação permite a concentração de diversas moléculas de GM1 em um mesmo microdomínio de membrana (formação de *clusters* de GM1). A aproximação e interação das moléculas de GM1 e de colesterol propicia a formação de pontes de hidrogênio entre a hidroxila presente na molécula de colesterol e molécula de oxigênio que liga a cadeia oligossacarídica do GM1 a seu núcleo esfingoide. Esta interação, além de fortalecer a associação GM1-colesterol, propicia uma mudança na conformação da cadeia oligossacarídica do gangliosídio, que passa a assumir uma conformação "*shovel-like*", expondo os resíduos de ácido siálico à superfície da membrana (Figura 6) (Fantini et al., 2013).

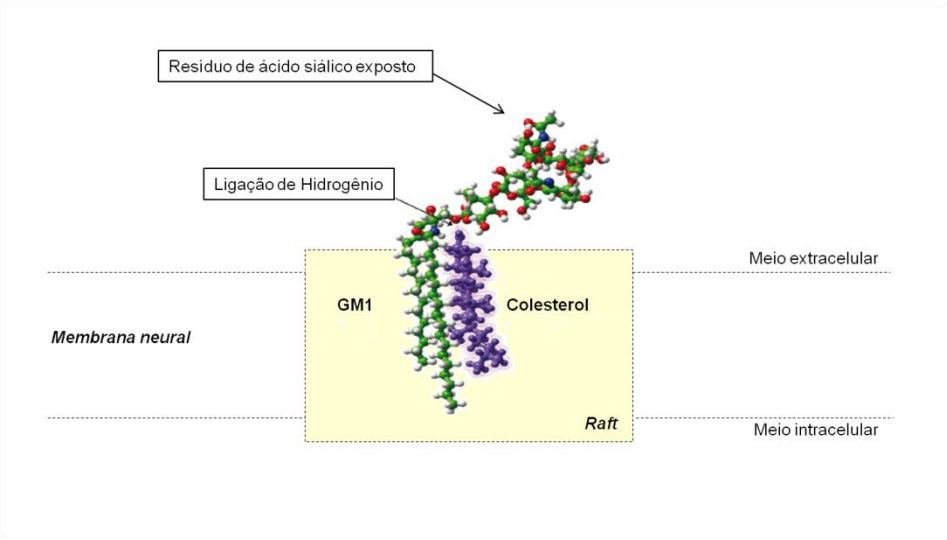

Figura 6. Modelo de interação GM1-colesterol e efeito sobre a conformação da cadeia oligossacarídica do GM1: A interação GM1-colesterol, que ocorre nos microdomínios *rafts* de membrana, é mediada por interação entre os núcleos esfingoide e esteroidal do GM1 e do colesterol, respectivamente, e pela formação de ligação de hidrogênio entre a hidroxila do colesterol e oxigênio que une a cadeia oligossacarídica do GM1 a seu núcleo esfingoide. Como resultado desta interação, ocorre alteração na conformação espacial da cadeia oligossacarídica do GM1 e maior exposição de seu resíduo de ácido siálico ao meio extracelular (Fantini et al., 2013).

O resíduo de ácido siálico, exposto quando da interação GM1-colesterol, contribui, através de sua carga negativa, para as interações eletrostáticas que direcionam os neurotransmissores à fenda pós-sináptica (Svennerholm, 1980; Fantini and Barrantes, 2009). Este mesmo resíduo de ácido siálico é responsável por mediar a interação do peptídeo Aβ com o GM1 da membrana (Matsuzaki, 2007; 2014; Matsuzaki et al. 2010).

Desta forma, os *rafts* lipídicos servem como alvo para a interação do peptídeo β-amiloide com as membranas neurais, através de sua ligação com as moléculas de GM1 presentes nestes microdomínios. A interação do peptídeo com as membranas parece mediar o processo de fibrilogênese, os danos neurais causados pelo peptídeo, e a própria evolução da DA (Matsuzaki, 2007; 2014; Matsuzaki et al. 2010). Existem evidências na literatura que associam regulação da Proteína Precursor Amilóide (APP) com a homeostasia e o metabolismo dos gangliosidíos (Grimm et al.. 2012).

A interação Aβ-GM1 é considerada um passo importante ao processo fibrilogênese, visto que o peptídeo, ainda em sua forma monomérica, tem seu processo de polimerização em oligômeros ou fibrilas acelerado pela interação com o GM1 presente nos *rafts*. Ao interagir com os *rafts*, ocorreria uma alteração na conformação estrutural do peptídeo, favorecendo a forma beta-pregueada e, em consequência, o processo de polimerização é acelerado (Figura 7) (Matsuzaki, 2007; 2014; Matsuzaki et al. 2010; Yanagisawa, 2007).

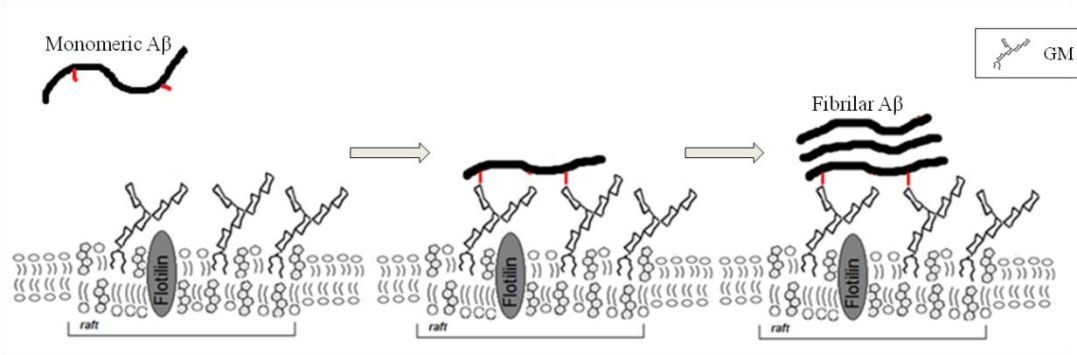

Figura 7. Interação do Aβ com as membranas e fibrilogênese. O GM1 presente nos *rafts* na forma de cluster, e com seus resíduos de ácido siálico expostos, serve como alvo para interação de monômeros do peptídeo β-amiloide. Ao interagir com o GM1, os monômeros de Aβ (desprovidos de toxicidade) sofrem uma alteração em sua conformação espacial que favorece e acelera o processo de polimerização, levando assim a formação de oligômeros ou fibrilas (fibrilogênese), caracteristicamente neurotóxicos.

A interação do peptídeo com os *rafts* também traz consequências sobre a funcionalidade destes microdomínios e, como resultado, sobre funções neurais dependentes destas plataformas lipídicas (Lai and McLaurin, 2010; Hirai et al., 2013; Marin et al., 2013; Sasahara et al., 2013). Ao interagir com os *rafts*, o Aβ promoveria dano oxidativo a estas estruturas lipídicas, o que poderia comprometer sua integridade e alterar a dinâmica destes microdomínios de membrana (Zampagni et al., 2010; Sciacca et al., 2012; Tan et al, 2013). Enzimas ou receptores dependentes do ambiente lipídico dos *rafts* teriam sua atividade alterada, o que comprometeria diversas funções neurais (Ledesma et al., 2003; Calamai and Pavone, 2013).

Além disso, a própria evolução da DA parece ser dependente da interação do peptídeo com as membranas. O ciclo patológico proposto à cascata amiloide, em que o peptídeo formado estimularia o próprio processo amiloidogênico, parece ser dependente da interação Aβ-membrana (Peters et al., 2009). Ademais, a hiperfosforilação da proteína tau, um outro evento neuroquímico que representa um passo importante no desenvolvimento e progressão da DA, parece ser acelerado em resposta à interação do peptídeo com os *rafts* de membrana (Schengrund et al., 2010).

Em consequência disso, a interação do peptídeo com os *rafts* lipídicos, especificamente com o GM1 presente nestes microdomínios, bem como as alterações na estrutura das membranas decorrentes desta interação, desempenhariam um papel chave na DA, de sorte que drogas capazes de bloquear a interação Aβ-membrana, quer pelo bloqueio dos sítios de ligação do peptídeo na membrana ou pelo sequestro do peptídeo circulante, apresentariam um efeito neuroprotetor importante com a possibilidade de frear a evolução da doença (Matsuoka et al., 2003).

1.3 Gangliosídio GM1 como agente neuroprotetor na DA

Entre as potenciais drogas neuroprotetoras de interesse em modelos de Alzheimer, cita-se o gangliosídio GM1. Estudo recente do nosso grupo (Kreutz et al., 2011), bem como outros dados da literatura (Svennerholm et al., 2002, Yang et al., 2013), atribuem a este lipídio atividade neuroprotetora em modelos de DA, quando administrado de forma exógena.

Os mecanismos associados a esta neuroproteção ainda são motivo de estudo. No entanto, diversos trabalhos sugerem a este gangliosídio atividades neurotrófica (Rabin et al., 2002; Lim et al., 2011), anti-inflamatória (Ariga and Yu, 1999; Ohmi et al., 2009; Ohmi et al., 2011) e antioxidante (Fighera et al., 2004; Gavella et al., 2007; Sokolova et al., 2007). Ao GM1 também é proposto atividade sinalizatória, sendo capaz de regular a atividade de receptores tirosina cinase (Mutoh et al., 1995; Bachis et al., 2002; Duchemin et al., 2002; Rabin et al., 2002; Mocchetti, 2005), e modular a fosforilação da GSK3β (Kreutz et al., 2011), enzima envolvida em cascata de morte celular que desempenha papel importante na DA. Além destes efeitos, o GM1 apresenta a propriedade de reverter parte dos efeitos tóxicos de neurotransmissores excitatórios

como glutamato (excitotoxicidade glutamatérgica) (Cunha et al., 1999; Dhanushkodi and McDonald., 2011); além de restabelecer a síntese de acetilcolina (um neurotransmissor cuja síntese é profundamente afetada ao longo do desenvolvimento da DA) (Fong et al., 1995; Hadjiconstantinou and Neff ,1998).

Embora o GM1 seja componente estrutural das membranas e dos *rafts* lipídicos, com a propriedade de estabilizar os microdomínios (Ohmi et al., 2011; 2012), e de interagir com uma série de proteínas, regulando sua distribuição nos *rafts* (como é o caso da APP) (Zhang et al., 2009), pouco é conhecido sobre o efeito da administração exógena deste gangliosídio na integridade e composição dos *rafts* em modelo de Alzheimer (Zhang et al., 2011-b) e, consequentemente, em sua capacidade de afetar cascatas ou enzimas que possam ser moduladas por estes microdomínios, como é o caso da cascata amiloide, e da atividade da AChE e $Na^{+}K^{+}$ATPase (Zakharova et al., 2007). Entretando, em modelo de hipóxia-isquemia, Zhang e colaboradores (2011-b) demonstraram a propriedade do GM1 de preservar a estrutura dos microdomínios de membrana.

Considerando, então, os diversos estudos que demonstram a interação Aβ-GM1 (Yahi and Fantini, 2014), e levando em conta ser o GM1 endógeno o alvo principal de interação do peptídeo com as membranas neurais, é plausível supor que o GM1 administrado exogenamente possa competir com o GM1 endógeno (de membrana) pela ligação com o Aβ, servindo, desta forma, como uma droga capaz de sequestrar ou neutralizar o peptídeo e impedir sua interação com as membranas neurais, promovendo por este meio, neuroproteção (Matsuoka et al., 2003; Bereczki et al 2011;Yuyama et al., 2014).

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar o potencial efeito neuroprotetor da administração exógena do gangliosídio GM1 em modelo *in vivo* e *in vitro* de toxicidade induzida pelo peptídeo β-amiloide (Aβ).

2.2 Objetivos Específicos

1. Investigar o efeito da administração icv do peptídeo Aβ (Aβ1-42) e do tratamento com GM1(icv) sobre estado cognitivo, sobre a atividade das enzimas AChE e Na^+,K^+-ATPase, bem como sobre a defesa antioxidante, em córtex cerebral e hipocampo de ratos Wistar machos adultos.

2. Investigar o efeito da administração icv do peptídeo Aβ (Aβ1-42) e do tratamento com GM1(icv) sobre a integridade dos *rafts* lipídicos e sobre a distribuição das proteínas amiloidogênicas APP e BACE1 nos microdomínios de membrana, em córtex cerebral e hipocampo de ratos Wistar machos adultos.

3. Avaliar o efeito da administração do gangliosídio GM1 sobre a morte celular (necrose e apoptose) induzida pelo peptídeo Aβ1-42 na linhagem de neuroblastoma humano SH-SY5Y, e investigar o papel da interação Aβ-$GM1_{exógeno}$ na neuroproteção observada.

4. RESULTADOS

Os resultados desta tese estão organizados na forma de três artigos: o primeiro publicado, o segundo em análise e o terceiro a ser submetido à publicação em revista da área.

ARTIGO I

Alterations on Na^+,K^+-ATPase and acetylcholinesterase activities induced by amyloid-β peptide in rat brain and GM1 ganglioside neuroprotective action. **Status:** Publicado no periódico *Neurochemical Research*.

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ORIGINAL PAPER

Alterations on Na^+,K^+-ATPase and Acetylcholinesterase Activities Induced by Amyloid-β Peptide in Rat Brain and GM1 Ganglioside Neuroprotective Action

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Abstract Alzheimer's disease (AD) is a neurodegenerative disorder whose pathogenesis involves production and aggregation of amyloid-β peptide (Aβ). Aβ-induced toxicity is believed to involve alterations on as Na^+,K^+-ATPase and acetylcholinesterase (AChE) activities, prior to neuronal death. Drugs able to prevent or to reverse these biochemical changes promote neuroprotection. GM1 is a ganglioside proposed to have neuroprotective roles in AD models, through mechanisms not yet fully understood. Therefore, this study aimed to investigate the effect of Aβ1-42 infusion and GM1 treatment on recognition memory and on Na^+,K^+-ATPase and AChE activities, as well as, on antioxidant defense in the brain cortex and the hippocampus. For these purposes, Wistar rats received i.c.v. infusion of fibrilar Aβ1-42 (2 nmol) and/or GM1 (0.30 mg/kg). Behavioral and biochemical analyses were conducted 1 month after the infusion procedures. Our results showed that GM1 treatment prevented Aβ-induced cognitive deficit, corroborating its neuroprotective function. Aβ impaired Na^+,K^+-ATPase and increase AChE activities in hippocampus and cortex, respectively. GM1, in turn, has partially prevented Aβ-induced alteration on Na^+,K^+-ATPase, though with no impact on AChE activity. Aβ caused a decrease in antioxidant defense, specifically in hippocampus, an effect that was prevented by GM1 treatment. GM1, both in cortex and hippocampus, was able to increase antioxidant scavenge capacity. Our results suggest that Aβ-triggered cognitive deficit involves region-specific alterations on Na^+,K^+-ATPase and AChE activities, and that GM1 neuroprotection involves modulation of Na^+,K^+-ATPase, maybe by its antioxidant properties. Although extrapolation from animal findings is difficult, it is conceivable that GM1 could play an important role in AD treatment.

Keywords Alzheimer's disease · Amyloid-β · GM1 · Na^+,K^+-ATPase · AChE · Neuroprotection

Introduction

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder that accounts for most dementia cases in humans. It is clinically characterized by progressive cognitive impairment, mainly affecting the patients' memory, motor skills and communication abilities [1, 2].

Although AD was first described one century ago, the pathological mechanisms involved in its development are not yet fully understood [2]. One of the theories for AD pathogenesis is the amyloid cascade, which involves the production of a small peptide, amyloid-β peptide (Aβ), through the cleavage of an endogenous membrane protein called APP (Amyloid Precursor Protein) by β- and γ-secretases [1, 2]. Aβ is 37-43 amino acids in length, and Aβ1-42 is considered to be the main amyloid-peptide form responsible for aggregation and senile plaque formation. Senile plaques, in turn, represent one of the main hallmarks of AD, and are responsible for many neuronal injuries observed in this neurodegenerative disease [1].

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The mechanisms underlying Aβ-triggered neuronal toxicity and their involvement in AD cognitive decline are still uncertain. Extensive evidence, however, suggests that Aβ-induced alterations on enzymes responsible for synaptic activities and neuronal plasticity, e.g. Na^+,K^+-ATPase and acetylcolinesterase (AChE), can play an important role in the neuronal injury and cognitive decline observed in Alzheimer's disease (reviewed by Zhang et al. [3]). Therefore, it is proposed that a decrease in Na^+,K^+-ATPase (EC 3.6.1.37) activity and increase in AChE (EC 3.6.1.37) activity can have a deep impact on neuronal functionality, causing impairment to synaptic responses prior to neuronal loss [3–5]. Some reports have proposed a relationship between Na^+,K^+-ATPase activity and cholinergic function because the use of AChE inhibitors or cholinomimetic drugs seem to increase Na^+,K^+-ATPase activity [3]. Despite the uncertainty and controversy of a real association or cause-effect relationship between reduced cholinergic function and reduced Na^+,K^+-ATPase activity, these enzymes can be considered candidate targets in neuroprotection studies on AD. AChE is already used as a therapeutic target in clinical pharmacotherapy of Alzheimer, though unfortunately little success has been obtained in slowing down dementia progression [6]. Na^+,K^+-ATPase, in turn, remains little explored in clinical practice as a target for neuroprotective drugs. Its role as a target or biochemical marker, however, has already been proposed for models of AD [3] and models of other neurological diseases [7–10]. For this reason, more studies are needed to identify drugs that modulate these enzymes, preventing or reversing the damage triggered by Aβ peptide, and thus with a potential neuroprotective role in AD.

Among the several candidate drugs studied for its anti-amyloid-induced toxicity, GM1 ganglioside is getting more and more attention. GM1 is an endogenous ganglioside in neural membranes. It was firstly associated with an acceleration or increase in amyloid aggregation process [11], however, when exogenously added to culture medium of tissues exposed to amyloid peptide [12], or administered to animals injected with Aβ [13], this lipid has demonstrated neuroprotective and neurotrophic properties [14–18]. In a clinical trial with demented patients, Svennerholm et al. [19] demonstrated the effect of GM1 on the improvement of the cognitive performance, proposing then an important role of GM1 in Alzheimer's disease treatment. Although GM1 neuroprotective mechanisms are not fully elucidated, some reports suggest an antioxidant effect of this ganglioside in some animal [13] and culture models [18], and then, it is conceivable that the oxidative induced damage, triggered by amyloid peptide, could be rescued by GM1 treatment. Although the activity of Na^+,K^+-ATPase and AChE could be affected by oxidative stress [10, 20–22], the action of GM1 on Aβ-induced alterations of this enzymes remains unknown.

In view of the above, the present study is aimed to evaluate GM1 neuroprotective activity on impairment of learning ability and memory in an in vivo model of amyloid-β toxicity, and to investigate the effect of treatment with this ganglioside on Aβ-induced alterations in Na^+,K^+-ATPase and AChE activities, as well as its antioxidant properties in this model.

Materials and Methods

Amyloid Peptide and GM1 Solution Preparation

Aβ1-42 peptide or inactive control Aβ42-1 peptide (both obtained from American Peptide Co., Sunnyvale, CA, USA) were dissolved in TRIS 0.05 M (pH 7.5) prepared with sterilized bi-distilled water at a concentration of 1 mg/mL and stored at −20 °C. Aliquots of Aβ peptides were allowed to aggregate by incubation at 37 °C for 96 h before in vivo infusion (Adapted from Frozza et al. [23]).

GM1 ganglioside (Sigma, St. Louis, MO, USA) stock solution was prepared in chloroform: methanol (1:1) at 6.75 mg/mL concentration. Aliquots of this stock solution were dried in liquid nitrogen, and resuspended in TRIS 0.05 M (pH 7.5) to a final GM1 concentration of 13.5 mg/ml.

Animals

Male Wistar rats (adult, 5 months old, weighing 350–500 g) were obtained from in-house breeding colonies at the Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul (UFRGS, Porto Alegre, Brazil). Animals were housed in cages under optimum light conditions (12:12 h light–dark cycle), temperature (22 ± 1 °C), and humidity (50–60 %), with food and water provided ad libitum. All procedures used in the present study followed the "Principles of Laboratory Animal Care" from NIH publication N°85–23 and were approved by the local Animal Ethics Advisory Committee (CEUA-UFRGS, approval letter N°19055). All efforts were made to reduce the number of animals necessary and their suffering, and all animal procedures were supervised by a veterinarian.

Surgical Procedure and Drug Administration

Animals were anesthetized by a mixture of xylazine and ketamine (i.p., 10 and 90 mg/kg, respectively) and placed into a stereotaxic frame. After sterilization using standard procedures, a middle sagittal incision was made in the scalp. Bilateral holes were drilled in the skull using a dental drill over the lateral ventricles.

Injection coordinates were derived from the atlas of Paxinos and Watson [24]: 0.8 mm posterior to bregma,

1.5 mm lateral to the sagittal suture, and 3.5 mm beneath the surface of brain. The rats received a single infusion of 5 μL into each lateral ventricle of Aβ1-42 (total of 2 nmol in 10 μL). Control animals received bilateral intracerebroventricular (i.c.v.) infusions of equal volume of TRIS 0.05 M (pH 7.5) prepared with sterilized bi-distilled water (SHAM group), or GM1 water solution (GM1 control group).

Microinjections were performed using a 10-μL Hamilton syringe fitted with a 26-gauge needle. All infusions were made at a rate of 1 μL/min over a period of 5 min. At the end of the infusion, the needle was left in place for an additional 5 min before being slowly withdrawn to allow diffusion from the tip and prevent reflux of the solution [23]).

For Aβ +GM1 animal group, Aβ1-42 (total of 2 nmol in 10 μL: 5 μL in each lateral ventricle) was first injected, and 5 min later the syringe was slowly withdrawn, washed out, and then GM1 solution was injected again in the same ventricle in a dose of 0.30 mg/kg of body weight. The average volume of GM1 injection was around 3.9–5.7 μL, depending on animals' weight [25].

After the surgical procedure, the scalp was sutured and the animals were allowed to recover from the anesthesia on a heating pad to maintain body temperature at 37.5 ± 0.5 °C.

The animals were assessed on the behavioral task 1 month after surgical procedures, and were divided into five groups: (1) Sham group, which received TRIS 0.05 M i.c.v injection; (2) GM1 0.30 mg/kg group, which received just GM1 i.c.v injection; (3) Aβ group, which were injected i.c.v with 2 nmol of fibrilar Aβ1-42; (4) Aβ + GM1 0,30 mg/kg group, which were firstly injected with fibrilar Aβ1-42 followed by GM1 i.c.v administration; and (5) Aβ 42-1, which were infused with reversed Aβ peptide (Aβ 42-1).

Behavioral Analysis

This analysis was performed using the Novel Object Recognition Task (NOR) as described by Frozza et al. [23] following recently reviewed guidelines [26, 27]. This task is based on the spontaneous tendency of rodents to explore novel objects [28]. The task was performed in an apparatus made of wood covered with impermeable Formica (dimensions, 40 × 50 × 50 cm), with black floor and walls. The apparatus was used in a testing room under constant illumination. All the objects used in this experiment had similar textures, colors, and sizes, though different shapes. The objects were placed near the two corners at either end of one side of the apparatus and consisted of two cuboid glass blocks, a cylindrical bottle filled with water, and a dodecahedron-shaped block. These objects were heavy enough to prevent the rats from moving them.

One day before the tests, the rats were habituated by allowing them to explore the apparatus for 5 min without objects. On the following day, rats were acclimated in the testing room during 1 h before treatment. First, the rats completed a training session that consisted of leaving the animals in the apparatus containing two identical objects (A and A1). After training, the rats were placed in their home cages for 2 h.

The testing session to evaluate short-term recognition memory was then followed. The rats were once again allowed to explore the apparatus, but during this session the apparatus contained two dissimilar objects: the familiar object from the training session and a novel one (A and B, respectively). Long-term recognition memory was evaluated 24 h after the training session and a different pair of dissimilar objects (a familiar and a novel one, A and C, respectively) were presented. In all sessions, each rat was always placed in the apparatus facing the wall, allowed to explore the objects for 5 min and then placed again in its cage. The behavior was recorded by a video camera mounted vertically above the test arena and analyzed using appropriate video-tracking software (ANYmaze®). Each animal underwent three trials, including the training and two test sessions. The animals started to explore the objects 1 min after being placed in the apparatus.

Object recognition memory was defined as the ratio of time spent exploring the novel object (Tn) to the total amount of time spent exploring both objects (novel + familiar objects, Tn + Tf, respectively). This recognition index (RI), was thus RI = Tn/(Tn + Tf). A RI > 0.5 indicates a preferential exploration of a novel object by the animal, and this higher ratio of time spent exploring the novel object was considered to be an index of enhanced cognitive performance (memory retention) [27].

Between trials, the objects were cleaned with 10 % ethanol solution. Active exploration was defined by directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose or forepaws. Sitting on the object was not considered exploratory behavior [27].

Tissue Isolation and Process

Animals were killed by decapitation without anesthesia, 24 h after completing the behavioral tasks, the brain was removed and cerebral structures—cerebral cortex and hippocampus—were dissected.

Sample Preparation for Enzymatic Measures

For the determination of Na^+,K^+-ATPase activity, both structures were homogenized in 10 volumes (w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES (pH 7.45) and 1.0 mM EDTA, pH 7.4. Aliquots of these homogenates were used as enzyme sources [7]. For AChE activity assay, both structures were homogenized in 10 volumes (w/v) of 0.1 mM of potassium phosphate buffer,

pH 7.5 and centrifuged for 10 min at 1,000×*g*. The supernatant was used for enzymatic AChE analysis [29].

Sample Preparation for Total Radical-Trapping Antioxidant Potential Assay (TRAP)

For TRAP assay, cortex and hippocampus were homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 1,000×*g* for 10 min at 4° C, to discard nuclei and cell debris. The pellet was discarded and the supernatant was taken for biochemical assays [30].

Determination of Na^+,K^+-ATPase Activity

Na^+,K^+-ATPase activity was determined as described by Wyse et al. [7]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [31]. Enzyme specific activity was expressed as nmol Pi released per min per mg of protein.

AChE Activity Assay

Acetylcholinesterase activity was determined according to Ellman et al. [29], as described by Scherer et al. [32]. Enzyme specific activity was expressed as mmol ASCh per h per mg of protein.

Total Radical-Trapping Antioxidant Potential Assay

Trapping antioxidant potential assay, representing the total non-enzymatic antioxidant capacity of the tissue, was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis (2-amidinopropane; ABAP) at room temperature [33]. Two hundred and forty microliters of 10 mM ABAP, dissolved in 50 mM sodium phosphate buffer pH 8.6 plus 5.6 mM luminol, were added to the microplate and the background chemiluminescence was measured. Ten microliters of 300 μM trolox or supernatant were added and chemiluminescence was measured until it reached the initial levels. The addition of trolox or sample to the incubation medium reduced the chemiluminescence. The time necessary to return to the levels presented before the addition was considered to be the induction time, which is directly proportional to the antioxidant capacity of the tissue and was compared to the induction time of trolox. The results are reported as nmol of trolox per mg of protein [30].

Protein Determination

Protein was measured by the Coomassie Blue method according to Bradford [34] using bovine serum albumin as standard.

Statistical Analysis

Data were analyzed by one- or two-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparison when the *F* value was significant. All analyzes were performed using the statistical package for the social science (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

Results

Effect of GM1 on Aβ1-42 Induced Memory Impairments

As shown in Fig. 1, i.c.v infusion of fibrilar Aβ1-42 peptide caused a significant decrease in object recognition memory thirty days after the surgical procedure (mean reduction of 41 and 38.4 % in recognition index, for short-term and long-term memory trials, respectively); and the subsequently injection of a single 0.30 mg/kg dose of GM1 successfully prevented or reversed peptide-induced memory impairment observed in both short and long-term recognition memory trials. In GM1 control groups, however, no alterations were detected in the recognition index.

As an additional control for this model, we have evaluated reverse amyloid-peptide (Aβ 42-1) effect on cognition, and no alteration was found in the recognition index (Fig. 1). In addition, we assessed the effect of a smaller GM1 dose (0.15 mg/kg) on Aβ1-42-induced cognitive impairment, and no effect was observed (data not shown).

Effects of Aβ Administration and GM1 Treatment on Brain Na^+,K^+-ATPase Activity

As it can be observed in Fig. 2, i.c.v infusion of fibrilar Aβ1-42 peptide caused a 26.8 % decrease in Na^+,K^+-ATPase activity in hippocampus, though with no effects on brain cortex. GM1 treatment, in turn, partially prevented the Aβ-induced decrease in hippocampal Na^+,K^+-ATPase activity (12.7 % decrease compared to SHAM group).

Effects of Aβ Administration on Brain AChE Activity

As shown in Fig. 3, i.c.v. infusion of fibrilar Aβ1-42 peptide caused a significant increase (80.4 %) in AChE activity in brain cortex, but had no effect on hippocampus. In addition, GM1 treatment did not prevent Aβ-induced alterations on AChE and had no effect on the activity of this enzyme, neither in cortex nor in hippocampus.

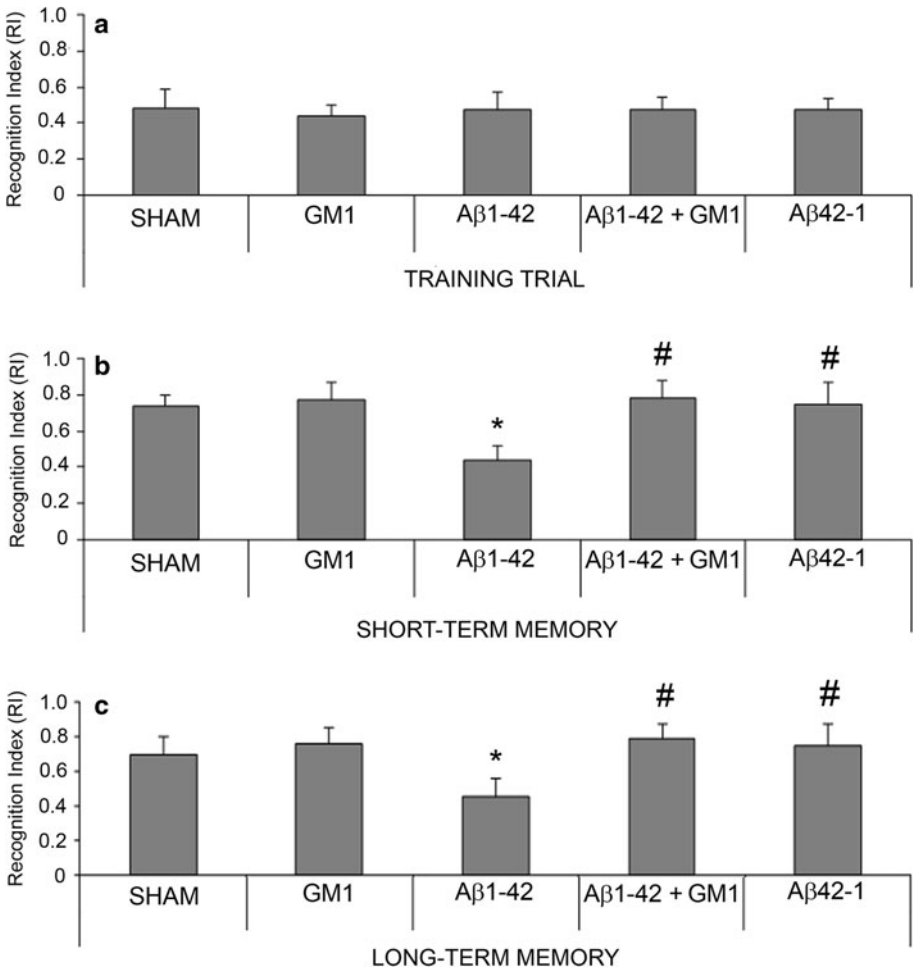

Fig. 1 Effect of Aβ1-42 infusion and GM1 acute treatment on object recognition memory. Rats were injected (2 nmol, i.c.v) with Aβ1-42 (or Aβ 42-1) and subsequently treated with one acute dose GM1 (0.30 mg/kg, i.c.v). After thirty days of surgical procedure the animals performed a novel object recognition task. Graphics represent object recognition index (RI) during 5 min in the training session (**a**), short-term memory test session performed 2 h after training (**b**), and long-term memory test session performed 24 h after training session (**c**). *Columns* indicate mean ± SD. Sample size was the following: SHAM (n = 12), GM1 control (n = 8), Aβ (n = 17), Aβ + GM1 (n = 14). *Significantly different from the SHAM group ($p < 0.05$); #Significantly different from the Aβ group ($p < 0.05$). (Two-way ANOVA, followed by Tukey test)

Effects of Aβ Administration and GM1 Treatment on Total Radical-Trapping Antioxidant Potential in Cortex and Hippocampus

As shown in Fig. 4, i.c.v infusion of fibrilar Aβ1-42 caused no alteration in this parameter in cortex, whereas in hippocampus it was observed a 25 % reduction of antioxidant defense. GM1 treatment, in turn, caused an increase in non-enzymatic radical scavenging capacity both in cortex (25 %) and in hippocampus (36 %), being able to rescue or prevent Aβ-induced decrease of this oxidative parameter.

Discussion

In the present study we evaluated the effect of fibrilar Aβ1-42 (i.c.v.) injection on recognition memory and on neurochemical parameters, such as Na^+,K^+-ATPase and AChE activities in hippocampus and brain cortex of adult male Wistar rats. We have also investigated a possible neuroprotective action of a single (i.c.v.) injection of GM1 ganglioside on cognitive deficit and enzyme activity alterations induced by amyloid peptide, as well as its antioxidant properties in this model.

Our findings showed that fibrilar Aβ1-42 (i.c.v.) injection was able to trigger a consistent recognition memory deficit in the animals, even 1 month after the surgical injection procedure, and that the administration of a single dose of GM1 significantly prevented Aβ-induced memory loss.

Our results demonstrated a neuroprotective effect of a single GM1 administration on cognitive impairment induced by the main amyloid peptide involved in neurotoxicity (Aβ1-42), 1 month after the injection and treatment procedures. Besides, since both amyloid and drug infusions were i.c.v, our model allowed us to investigate neurochemical alterations in the main brain structures associated with memory processes and affected in AD: cerebral cortex and hippocampus.

The first neurochemical parameter evaluated in this study was the effect of Aβ1-42 and/or GM1 treatment on Na^+,K^+-ATPase in cortex and hippocampus. Our findings showed that fibrilar Aβ1-42 i.c.v infusion can significantly reduce Na^+,K^+-ATPase activity in hippocampus when

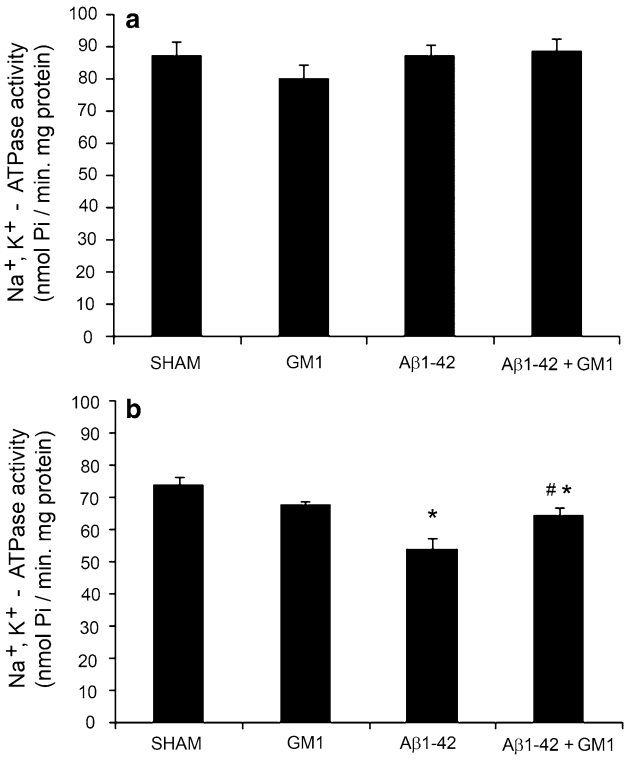

Fig. 2 Effect of Aβ1-42 and/or acute GM1 (0.30 mg/kg) i.c.v infusions on Na^+,K^+-ATPase activity in cerebral cortex (**a**) and hippocampus (**b**) of 5 month-age male Wistar rats. Data are mean ± SD for seven independent experiments (animals) performed in duplicate. $^*p < 0.01$ compared to sham group (One way ANOVA). $^{\#}p < 0.05$ compared to Aβ (Aβ1-42) group (One way ANOVA)

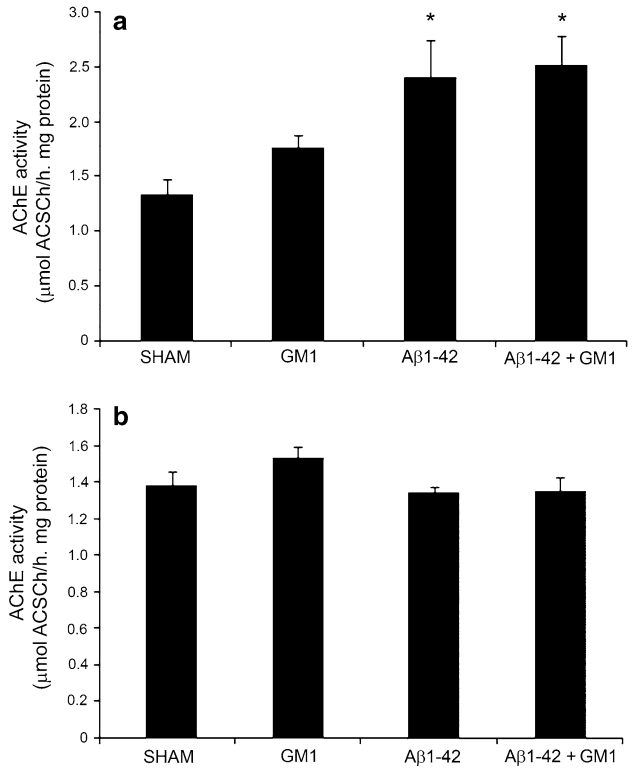

Fig. 3 Effect of Aβ1-42 and/or GM1 (0.30 mg/kg) i.c.v infusions on acetylcholinesterase activity in cerebral cortex (**a**) and hippocampus (**b**) of 5 month-age male Wistar rats. Data are mean ± SD for seven independent experiments (animals) performed in duplicate. $^*p < 0.05$ compared to sham group (One way ANOVA)

measured 1 month after the injection procedure. In the cerebral cortex, however, no alteration was observed on this enzyme. As for GM1 treatment, it was found to prevent hippocampal enzyme activity impairment.

As previously stated, Na^+,K^+-ATPase is a membrane enzyme that plays a pivotal role on the maintenance of neuronal ion balance, resting membrane potential, and thus, regulates neuronal excitability and synaptic transmission [3, 35]. It apparently plays an important role in memory, probably because of its participation in mechanisms of synaptic neuroplasticity, such as long-term potentiation [3]. In addition, Na^+,K^+-ATPase has been recently studied as a possible signaling transducer, playing other roles besides its role as a ion pump [36].

Alterations in Na^+,K^+-ATPase seem to be a common event in several neurological disorders [7–10], including Alzheimer's dementia (as reviewed by Zhang et al. [3]). Expression of this enzyme was reported to be decreased in the brain of AD patients, as revealed by post-mortem examination, and several researches have proposed that Aβ-induced neuronal damage can, at least partially, be due to alterations in Na^+,K^+-ATPase activity [3, 37, 38]. Consistent with the referred findings, a decrease in the activity of this enzyme has been reported to impair learning and memory abilities in animal models [3, 39], induce glutamate excitotoxicity [40], and trigger apoptosis in cell cultures [41].

Therefore, our findings corroborate a possible role of Aβ1-42 induced toxicity on impairing Na^+,K^+-ATPase activity, and suggest this effect can be region-dependent. GM1 neuroprotection, already reported by other studies, is here observed and proposed to be mediated, at least partially, by preventing Aβ-induced alterations on Na^+,K^+-ATPase in hippocampus. Since some reports propose that the impairment of this enzyme might act as an early event in AD [42] and have a role in the clinical course of dementia, our findings suggest that GM1 can be a disease-modifier drug [3].

Since some studies suggest a relationship between Na^+,K^+-ATPase and cholinergic function, and also that AD courses with a cholinergic deficit that plays an important role in cognitive decline [3], we investigated the effect of Aβ1-42 and/or GM1 infusions on AChE activity. This enzyme plays a pivotal role in the regulation of cholinergic neurotransmission, being responsible for the break down of acetylcholine in synaptic cleft. Consequently, and in an attempt to increase cholinergic function, AChE inhibitors represent today the first choice class of drugs for

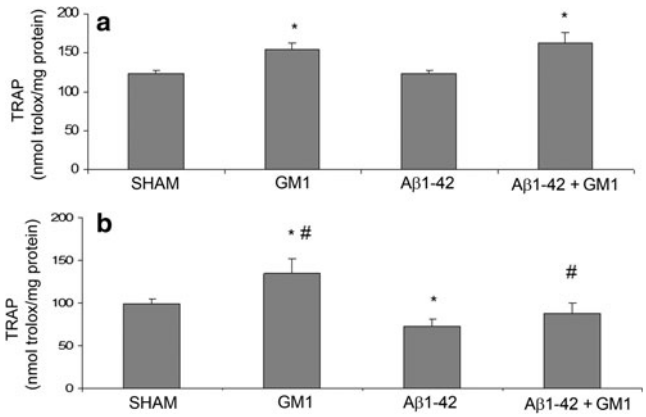

Fig. 4 Effect of Aβ1-42 and/or GM1 (0.30 mg/kg) i.c.v infusions on on total radical-trapping antioxidant potential in cerebral cortex (**a**) and hippocampus (**b**) of 5 month-age male Wistar rats. Results are expressed as mean ± SD for four independent experiments (animals). $^{*}p < 0.05$ compared to sham group (One way ANOVA, followed by Tukey test). $^{\#}p < 0.05$ compared to Abeta (Aβ1-42) group (One way ANOVA, followed by Tukey test)

AD treatment. However, these drugs have only a weak effect on cognitive function improvement, and are not effective in delaying the clinical course of dementia [6]. Anyway, some studies report that Aβ can increase AChE activity, promoting cholinergic dysfunctions that precede neuronal death and cognitive decline [5, 43].

Our findings show that fibrilar Aβ1-42 infusion increases AChE activity on cortex but has no effect on the hippocampus. GM1 treatment, however, did not prevent this alteration. Nevertheless, we can not rule out an effect of GM1 on cholinergic function [14], since its regulation involves not only AChE, but also acetylcholine synthesis enzymes, such as choline acetyltransferase, which also seems to be impaired in AD [5], and was not investigated here. Moreover, a deficit in cholinergic function can also be triggered by alterations in transport and release of neurotransmitters, or even be a consequence of death of cholinergic neurons [44].

In order to investigate a possible role of oxidative stress [13, 45] in the neuronal damage here observed, and to evaluate the potential antioxidant effect of GM1 in this model, we performed TRAP assay in cortex and hippocampus. Our results showed that i.c.v infusion of fibrilar Aβ1-42 was able to decrease radical scavenging capacity, or total nonenzymatic antioxidant defense, in hippocampus but not in cortex. GM1 administration, in turn, increased radical scavenging capacity, in both brain tissues, and prevented Aβ-induced decrease of this oxidative parameter in hippocampus. Considering that Na^+,K^+-ATPase, specially the isoforms expressed in hippocampus, is sensitive to oxidative stress[46], these results could partially explain the selective decrease of Na^+,K^+-ATPase activity in this brain region, as well as the protective effect of GM1 on this enzymatic parameter. The region-specific alteration of AChE activity induced by amyloid peptide, however, remains to be further clarified [21].

Our results provide new and important insight on the neuroprotective action of GM1, as well as on the role of Na^+,K^+-ATPase and AChE activities in Aβ induced toxicity, because it shows, for the first time, a region-specific effect of fibrilar Aβ1-42 injection and GM1 treatment on Na^+,K^+-ATPase and AChE activities, as well as in the antioxidant response potential. Although it is difficult to extrapolate animal experiment findings to human, and clinical use of GM1 is mainly made hard because of its low bioavailability [47, 48] and its suspected immunogenic potential for inducing Guillain-Barré Syndrome (not proven) [49, 50], further analyses of the neuroprotective properties of GM1 could support future studies aimed to find out ways to increase the endogenous expression of GM1 [51], develop new delivery technology to enhance brain bioavailability of this ganglioside [52, 53], or even use GM1 as a prototype for new drug design [54, 55].

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

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ARTIGO II

Ganglioside GM1 administration prevents Aβ-induced alterations on lipid raft integrity and on the distribution of amyloidogenic proteins along membrane microdomains in rat cortex and hippocampus. **Status:** Submetido ao periódico *Molecular Neurobiology*.

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Dear Dr Trindade,

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Ganglioside GM1 administration prevents Aβ-induced alterations on lipid raft integrity and on the distribution of amyloidogenic proteins (APP and BACE1) along membrane microdomains in rat cortex and hippocampus.

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by cerebral deposition of amyloid-beta (Aβ) peptides. The generation of (Aβ) (amyloidogenesis) begins with cleavage of amyloid precursor protein (APP) by β-secretase (BACE1) enzyme. Alterations in membrane architecture are proposed to mediate Aβ-induced toxicity and AD development, since lipid rafts are the main target for Aβ induced membrane peroxidation, and modulate amyloidogenesis through regulation of BACE1 and APP interactions. In the present study, the effect of fibrilar Aβ1-42 ICV injection on lipid raft integrity and on amyloidogenic protein distribution along membrane microdomains in cortex and hippocampus of Wistar rats was assessed, as well as the effect of GM1, a ganglioside with neuroprotective action on these parameters. As a result, Aβ induced partial disruption of lipid rafts from cerebral cortex and hippocampus, as well as an enhancement of amyloidogenic protein distribution to lipid rafts from cortex. GM1 treatment, in turns, prevented these biochemical alterations, preserving lipid raft structure and avoiding enhanced raft association of BACE1 and APP. Given the important role of lipid rafts on signaling and neuronal functions, and that amyloidogenesis seems to be dependent of APP and BACE1 co-localization into these membrane microdomains, our results reinforce the idea that membrane architecture alterations caused by fibrilar Aβ, could promote neuronal function loss and mediate the pathological cycling events involved in AD progression. Since GM1 treatment prevented raft disruption and alteration in amyloidogenic protein distribution, we suggest that the neuroprotective action of this ganglioside could also have an effect on modulation of amyloidogenesis and disease progression.

Key words: Amyloid-peptide (Aβ), GM1, neuroprotection, lipid rafts, APP, BACE1

Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia affecting millions of patients worldwide. It is clinically characterized by a progressive and still irreversible decline of memory and other cognitive functions. Its pathogenesis involves the production of amyloid peptide (Aβ) through the cleavage of Amyloid Precursor Protein (APP) by β-secretase (BACE1) and γ-secretase enzymes, the subsequent aggregation of this peptide into oligomers or fibrils, and deposition of insoluble fibrils in senile plaques. The whole process, named amyloid cascade, is believed to be involved not only with AD development, but also with its progression [1, 2].

Among the drugs proposed to promote neuroprotection in AD's models, GM1 – a ganglioside that composes neural membranes - has been extensively investigated [3-5]**.** In a previous study, we demonstrated that the ICV administration of GM1 prevented the cognitive deficit induced by fibrilar Aβ ICV injection in male Wistar rats [6]. However, further elucidations of GM1 neuroprotection and its effect on amyloid cascade, are necessary.

The mechanisms involving Aβ induced toxicity and the regulation of the amyloidogenic process are still not fully understood. Evidence suggests that neuronal membrane architecture, especially lipid raft dynamics and organization, plays a major role in the pathogenesis of AD [7-10].

Lipid rafts are defined as membrane microdomains enriched in cholesterol and sphingolipids (such as gangliosides), that play a pivotal role in the orchestration of multiple cellular functions. They act as specialized lipid platforms that modulate membrane protein activities via promotion of differential protein segregation along cell membranes and on the regulation of protein-lipid interactions. Thus, the shift of a protein in or out of lipid rafts can dramatically change its functionality, by restricting its interaction with partner-proteins, co-factors or substrates and by direct modulation of its activity by provision of new membrane lipid environment [11-15].

Raft analysis is traditionally based on extractive methods that use non-ionic detergent (Triton X-100) solubilization of non-raft membrane domains and the subsequent isolation and purification of rafts by density gradient centrifugation. Alternatively, free-detergent methods were developed in an attempt to avoid the theoretical detergent-induced false association of proteins to lipid raft. Control of the process and identification of the fractions corresponding to lipid rafts are based on the

main biochemical characteristics of these membrane microdomains: high cholesterol concentration, low protein content, high concentration of sphingolipids (mainly GM1), presence of flotillin1 (a marker protein for lipid rafts) and absence or lower level of clathrin (a protein mainly associated to non-raft domains) [13, 16, 17].

The role played by lipid rafts in AD is still a matter of study. Considering that they are pivotal for several cellular functions, including regulation of neurotransmitter receptor activity and neuroplasticity process, the integrity of these specialized lipid platforms is crucial for a proper cognitive function [14, 18]. Rafts, especially those enriched in GM1, represent a target for Aβ interaction, and as a result, they are the main site to the oxidative damage induced by the peptide [19].

Besides, rafts could be a site of amyloidogenesis, regulating secretase activity and, consequently, APP cleavage into amyloid peptide [7, 20]. Thereby, the distribution of amyloidogenic proteins along the different membrane microdomains seems to influence the pathogenesis of AD [21]. In addition, rafts are proposed to mediate amyloid peptide polymerization, serving as a seed for oligomer and fibril formation [22-24]. In short, alterations in membrane architecture could have a deep impact on the onset and progression of AD [8, 25].

In view of the above, and considering data showing that amyloid peptide could affect membrane lipid composition and fluidity [4, 26, 27], the aims of this study were: i) to investigate the effect of Aβ and/or GM1 ICV injections on neural membrane raft integrity and ii) to evaluate the effect of these procedures on amyloidogenic proteins (APP and BACE1) distribution in membrane microdomains.

Materials and methods

Amyloid Peptide and GM1 solution Preparation

Aβ1-42 peptide (obtained from American Peptide Co., Sunnyvale, CA, USA) was dissolved in TRIS 0.05M (pH 7.5) prepared with sterilized bi-distilled water at a concentration of 1mg/mL and stored at −20°C. Aliquots of Aβ peptides were allowed to aggregate by incubation at 37°C for 96 h before in vivo infusion adapted from Frozza et al. [28].

GM1 ganglioside (Sigma, St. Louis, MO, USA) stock solution was prepared in chloroform:methanol (1:1) at 6.75mg/mL concentration. Aliquots of this stock solution

were dried in liquid nitrogen, and resuspended in TRIS 0.05M (pH 7.5) to a final GM1 concentration of 13.5mg/ml.

Animals

Male Wistar rats (adult, 5 months old, weighing 350–500g) were obtained from in-house breeding colonies at the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. The animals were housed in cages under optimum light conditions (12:12 h light–dark cycle), temperature (22 ± 1°C), and humidity (50 to 60%), with food and water provided *ad libitum*. All procedures used in the present study observed the "Principles of Laboratory Animal Care" from NIH publication No 85–23 and were approved by the local Animal Ethics Advisory Committee (CEUA-UFRGS, approval letter N°19055). All efforts were made to reduce the number of animals necessary and their suffering, and all animal procedures were supervised by a veterinarian.

Surgical Procedure and Drug Administration

Surgical procedures and drug administration were performed according to Kreutz et al. [6]. Briefly, the animals were anesthetized with xylasine and ketamine (10mg/kg and 90mg/kg, respectively) and placed in a stereotaxic frame. Stereotaxic surgery was performed, using the following injection coordinates: 0.8 mm posterior to bregma, 1.5 mm lateral to the sagittal suture, and 3.5 mm beneath the surface of brain.

Rats received a single 5μL Aβ1-42 infusion into each lateral ventricle (total of 2nmol in 10μL).Control animals received bilateral intracerebroventricular (ICV) infusions of equal volume of TRIS 0.05M (pH 7.5) prepared with sterilized bi-distilled water (SHAM group), or GM1 water solution (GM1 control group).

Microinjections were performed using a 10-μL Hamilton syringe fitted with a 26-gauge needle. All infusions were made at a rate of 1μL/min over a period of 5min. At the end of the infusion, the needle was left in place for an additional 5min before being slowly withdrawn to allow diffusion from the tip and prevent reflux of the solution [28].

For Abeta+GM1 animal group, Aβ1-42 (total of 2nmol in 10μL: 5μL in each lateral ventricle) was first injected, and 5 minutes later the syringe was slowly withdrawn, washed out, and then GM1 solution was injected into the same ventricle at a

concentration of 0.30mg/kg of body weight. The average volume of GM1 injection was around 3.9 – 5.7μL, depending on the animals' weight [6].

After the surgical procedure, the scalp was sutured and the animals were allowed to recover from the anesthesia on a heating pad to maintain body temperature at 37.5±0.5°C.

Animals were then divided into 4 groups: (1) Sham group, which received TRIS 0.05M ICV injection; (2) GM1 0.30mg/kg group, which received just GM1 ICV injection; (3) Aβ group, which were injected ICV with 2nmol of fibrilar Aβ1-42; and (4) Aβ+ GM1 0.30mg/kg group, which were firstly injected with fibrilar Aβ1-42 followed by GM1 ICV administration. One month after the surgical procedures, Aβ-induced cognitive deficit and GM1 neuroprotection were confirmed as previously demonstrated [6].

Tissue Isolation

The animals were killed by decapitation without anesthesia one month after the surgical procedures. The brain was removed, and the cerebral structures – cerebral cortex and hippocampus – were dissected.

Raft Extraction and Isolation

For raft extraction, two methods were employed: Triton X-100 and detergent-free methods.

Triton X-100 method for raft extraction

One hundred milligram of tissue (cortex or hippocampus) was homogenized in 500 μl of detergent lysis buffer [1X PBS (pH 8.0), 1% Triton X-100, 1% protease inhibitor cocktail, 1mM PMSF, 5mM NaF, 1mM sodium orthovanadate]. The homogenate was kept on ice for 30 min, and then centrifuged at 1,000xg for 10 minutes at 4 °C. The supernatant, free of nuclear fraction and cellular debris, was separated, and the extraction procedure was repeated with the pellet. The pool of supernatants was then immediately subjected to discontinuous sucrose gradient [29].

Detergent Free Method for Raft Extraction

One hundred milligram of tissue (cortex) was homogenized in 500 μl of detergent free lysis buffer [1X PBS (pH 8.0), 1% protease inhibitor cocktail [1mM

PMSF, 5mM NaF, 1mM sodium orthovanadate, 1mM $CaCl_2$, 1mM $MgCl_2$]. The homogenate was then centrifuged at 1,000xg for 10 minutes at 4°C. The supernatant was removed and the pellet was subjected again to extraction process. The pooled supernatants were then subjected to discontinuous sucrose gradient [29].

Discontinuous Sucrose Gradient

Two hundred twenty-five microliters of the extracts were placed on the bottom of centrifuge tubes (55KRPM) and gently mixed with an equal volume of an 85% (w/v) sucrose-TBS solution. Upon this mixture 3 mL of 35% (w/v) sucrose-TBS solution and 675 µL of 5% (w/v) sucrose-TBS solution were sequentially overlaid. The gradient was subjected to ultracentrifugation at 200,000xg for 18h at 4°C. After centrifugation, 14 aliquots (fractions) of 260 µL were collected from the top to the bottom of the centrifuge tube (14^{th} and 15^{th} aliquots/fractions were collected together). Each fraction was then subjected to biochemical evaluation for identification and characterization of raft and non-raft fractions [29].

Biochemical Evaluation of Sucrose Gradient Fractions

Cholesterol Evaluation

Cholesterol content distribution through the different sucrose fractions were determined by fluorimetric method, using an Amplex Red Cholesterol Assay kit (#10007640 Cayman Chemicals) and 50µL of each fraction.

Protein Measurement

Protein content from each fraction was measured using modified Lowry method. Samples (15µL) were previously mixed with SDS (2% final concentration) to avoid Lowry reactant precipitation induced by Triton X-100 [30].

GM1 dot blot analysis

For GM1 content determination by dot blot, a 2 µL aliquot from each sucrose fraction was carefully loaded on a nitrocellulose membrane. After overnight drying, the membranes were incubated (1h) with blocking solution containing bovine albumin (5%) and 0.1% Tween-20 in Tris–buffered saline (TBS; 50 mM Tris–HCl, 1.5% NaCl, pH 7.4). The membranes were incubated overnight with choleric toxin, and subsequently submitted to overnight incubation with anti-choleric toxin antibody (1:1000). The

membranes were finally incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000), and chemioluminescence (ECL) was detected using X-ray films (Kodak X-Omat) [31].

Western blot detection of flotillin, clathrin, APP and BACE1:

Equal volumes of each sucrose fraction were homogenized with a 5X LAEMLI buffer and β-mercaptoethanol, and then resolved by 8% SDS–PAGE. Protein was transferred onto nitrocellulose membranes (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) at 50mV for 14 h. After 1 hour incubation at 4^{o}C in blocking solution containing 5% non-fat milk and 0.1% Tween-20 in Tris–buffered saline (TBS; 50 mM Tris–HCl, 1.5% NaCl, pH 7.4), membranes were incubated overnight with the appropriate primary antibody diluted in the same blocking solution. Primary antibodies against the following proteins were used: anti-flotillin-1 (1:1000) (cat #3244 Cell Signaling Technology, Danvers, MA, USA), anti-clathrin (1:1000) (cat #2410 Cell Signaling Technology, Danvers, MA, USA), anti-APP (1:1000) (cat 51-2700, Invitrogen Corporation, Camarillo, CA, USA), and anti-BACE1 (1:1000) (cat MAB5308 Merck Millipore, Darmstadt, Germany). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit (1:1000) (cat NA934V GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) or anti-mouse antibody (1:5000) (cat #AP12P, Merck Millipore, Darmstadt, Germany). Chemioluminescence (ECL) was detected using X-ray films (Kodak X-Omat).

Cyclodextrin-induced dismount of lipid rafts

One hundred milligram of cerebral cortex was sliced (100nm) in both directions using a chopper. Tissue was incubated with a β-Methyl-cyclodextrin (βMCD) (C4555, Sigma Aldrich, St Louis, MO, USA) solution (20 or 50mM, prepared in TRIS 0.05M, adapted from Ilangumaran and Hoessli [32] for 1h at 37°C and under constant agitation. After the incubation period, the sample was centrifuged at 10,000xg for 15min. The pellet was suspended in 0.05M TRIS buffer and centrifuged again. The final pellet was then suspended in lysis buffer containing Triton X-100 and finally subjected to the raft extraction technique described above. The extract obtained was first subjected to protein measurement [30] and cholesterol content quantification (Amplex Red Cholesterol Assay kit). Then, an aliquot was subjected to discontinuous sucrose gradient for

separation and purification of raft fractions (according to the corresponding item above). From the obtained fractions, cholesterol and protein contents were measured and western blot was performed for analysis of flotillin and clathrin distribution (as described above).

Results

Standardization and Comparison of two Techniques for Raft Extraction

Standardization of raft extraction was performed comparing two different techniques: Triton X-100 and detergent-free extractive methods. Cerebral cortex was used for these procedures.

After density gradient centrifugation, the efficiency of raft extraction and purification processes could be compared by visualization of a sharper floating opaque band – corresponding to raft fraction – in the interface between the 5% and the 35% sucrose gradient phases obtained by Triton X-100 extractive method (Fig. 1).

From the top of the centrifuge tube, 14 fractions were collected and assessed according to the parameters for raft fraction characterization or identification: Cholesterol level, Flotillin and Clathrin immunocontent, and GM1 content.

As shown in Fig. 2, Triton X-100 method promoted a better raft extraction procedure, as evidenced by the higher concentration of cholesterol (A) in the upper gradient fractions, as well as greater flotilin distribution (raft marker) in the first low density gradient fractions and clathrin (non-raft marker) in the high density fractions (B). GM1, however, was similarly distributed along gradient in both evaluated methods (C).

These results allowed us to choose Triton X-100 method as the most suitable for rafts extraction in our model, as well as enabled the identification of the first gradient fractions as corresponding to raft ones.

Evaluation of Aβ and GM1 effect on raft integrity

Once determined the best method for raft extraction, we assessed the effect of fibrilar Aβ1-42 ICV injection and GM1 treatment (ICV) on the integrity of lipid rafts obtained from cerebral cortex and hippocampus of male Wistar rats, using cholesterol and flotilin/clathrin distribution as biochemical parameters.

As shown in Fig. 3, Aβ caused a partial disassembly/disruption of lipid rafts, as evidenced by the triggered dispersion of cholesterol (A) and flotilin/clathrin (B) along sucrose gradient fractions from cerebral cortex. GM1 treatment, in turn, promoted repair or preservation of raft structure, avoiding Aβ induced alterations in membrane architecture. Similar results were observed in hippocampus (Fig. 4).

Raft disassembling/disruption induced by β-Methyl-Cyclodextrin

β-Methyl-Cyclodextrin (βMCD) in vitro treatment was employed to promote raft dismount, and to serve as a control of microdomain disassembling process. Because of its bigger tissue mass, in this experiment just cerebral cortex was employed.

The same analytical parameters were used to assess raft integrity: cholesterol and protein distribution along gradient fractions.

As shown in Fig. 5, 20mM βMCD caused a slight change in cholesterol and flotillin/clathrin distribution, as evidenced by a dispersion of these biochemical markers along gradient fractions. Similarly to the previously observed Aβ-induced effect, 20mM βMCD did not cause a complete excision of cholesterol (A) or flotillin (B) from the upper gradient fractions, indicating that lipid raft dismounting was just partial. In contrast to these observations, 50mM βMCD caused a shift of cholesterol and flotillin out from the upper fractions, indicating a complete disruption of raft microdomains.

Considering that βMCD-triggered raft disruption is mediated by membrane cholesterol sequestering/excision effect, we measured membrane cholesterol content after 20mM and 50mM βMCD treatments. As shown in Fig. 5C, the lowest βMCD concentration caused no significant reduction in total membrane cholesterol, whereas 50mM βMCD brought about a 31,5 % reduction in this biochemical parameter.

Effect of Aβ peptide and GM1 ganglioside treatment on APP and BACE1 association with lipid rafts

Once demonstrated the association of APP and BACE1 with lipid rafts (Supplemental data, Fig. S1 and S2), we investigated the effect of Aβ ICV injection and GM1 treatment on the distribution of these amyloidogenic proteins through the different gradient fractions, in cerebral cortex and hippocampus.

As shown in Fig 6, Aβ caused alterations in APP and BACE1 distribution along sucrose gradient fractions, which were most evident in the cerebral cortex. GM1

treatment reduced the association of APP with lipid raft and practically excluded BACE1 from the raft fractions, preventing the increase in BACE1 association with the upper fractions triggered by amyloid peptide.

4. Discussion

Since the first report of Aβ interaction with GM1 containing rafts, and its proposed effect in toxic amyloid fibril and oligomers formation, the role of lipid rafts in AD pathogenesis has been extensively explored, although with no definite conclusions [8, 9].

On the one hand, some studies suggest that the interaction of peptide with rafts is crucial for the triggering of its toxic effects, such as kinase activation, the consequent hyperphosphorylation of tau protein and neuronal death [33]. Consistent with these results, blocking of membrane GM1 negative charges or depletion in ganglioside and/or cholesterol membrane content reduced peptide-induced toxicity [34]. These data suggest, at first sight, that lipid raft disruption could play a protective role by eliminating the major site for Aβ interaction with neural membranes, including slowing the conversion of Aβ from the monomeric form (non-toxic) to oligomeric or fibrillated ones (higher toxicity) [23].

On the other hand, and in contrast to previous reported evidences, other studies propose that lipid raft disruption could mediate the cognitive impairments of AD [27, 35, 36], since these lipid platforms play an essential role in the regulation of cellular functions. Thus, the preservation of lipid raft structure could mediate neuroprotection [37, 38].

The main inconsistency in the interpretation of the role of lipid rafts in AD concerns the difficulty to compare or correlate the results from different models or studies due to differences related to the used methodologies, such as the limitations of these methods and the different biochemical parameters used to assess lipid raft fractions. For this reason, in the beginning of our study we compared the effectiveness of two methods for lipid raft extraction (Triton X-100 and detergent-free method) in our experimental model, using a set of parameters that accurately determined the lipid raft fractions. The Triton X-100 method was found to be more efficient in lipid raft extraction, since it enabled better cholesterol and flotillin/clathrin distribution profiles

along sucrose gradient. The superiority of Triton X-100 methodology was also visible: the characteristic opaque floating band between 5% and 30% sucrose phases. GM1 distribution along gradient fractions was similar for the two tested methods, suggesting that this parameter may not be the most appropriate and effective for identification and evaluation of lipid raft fractions. At first sight, these data seem to be discrepant with findings from a previously published study where the detergent-free method was found more appropriate [29]. However, we should take into consideration that differences in animal age could cause alterations in lipid membrane composition or organization that could impact the effectiveness of extraction methods in our experimental model.

Using the Triton X-100 method, we evaluated the effect of fibrilar Aβ1-42 and GM1 treatment on membrane microdomain integrity. As shown by our results, Aβ ICV injection caused a partial disruption of membrane microdomains, as evidenced by the dispersion of cholesterol and flotillin along gradient. Treatment with GM1 was able to prevent or revert this effect. These alterations were similarly observed both in cerebral cortex and hippocampus, the main brain structures affected by Alzheimer´s disease. In order to confirm whether this dispersion in cholesterol and flotillin distribution could really indicate partial disruption of the lipid raft, we compared this data with raft disruption caused by different doses of βMCD. As demonstrated, 20mM βMCD caused dispersion in cholesterol and flotillin distribution along sucrose gradient, while 50mM βMCD promoted the complete shift of raft markers to the denser fractions. These results indicate that the first βMCD concentration promotes a partial disassembly of lipid rafts (since it was still possible to observe flotillin in upper gradient fractions) while the last one caused complete raft disruption. Aβ effect on raft architecture was similar to the one promoted by 20mM βMCD.

These findings are consistent with studies that suggest the Aβ effect of reducing neuronal membrane fluidity [27] or lipid raft dynamics [39, 40], and also with a study that demonstrates lipid raft disruption in the cortex of human patients with Alzheimer's disease [35]. The present results, however, has firstly demonstrated a fibrilar Aβ induced disassembly of lipid raft, and a GM1 protective or restoring effect, in the main brain structures associated with memory functions.GM1 effect of preventing Aβ-induced lipid raft disruption is consistent with previous data showing that this ganglioside may repair raft structure after hypoxia-ischemia event [41].

The mechanisms involved in Aβ and GM1 effect on lipid raft integrity remain to be elucidated. Since previous studies suggested that Aβ could affect lipid metabolism or

cholesterol and ganglioside contents [4, 42, 43, 44], and considering the role of these lipids in maintaining raft structure [45-49], we investigated Aβ and GM1 treatment effects on total cholesterol and GM1 membrane content (Supplemental data, Fig. S3). Surprisingly, no change in these parameters was observed, suggesting that changes on raft structures triggered by Aβ, as well as GM1-promoted conservation of microdomain architecture, could not involve significant change in membrane lipid levels (consistent with results of raft disassembling promoted by 20mM MβCD), but rather alterations in lipid distributions along membrane.

Additionally, Zampagni and colleges [19] showed that interaction of amyloid peptide with rafts triggers microdomain-target oxidative damage, what suggests that reactive oxygen species generation could be involved in membrane architecture changes [50]. Consistent with this hypothesis, we previously demonstrated that GM1 neuroprotection observed in this model is associated with reversal of Aβ-induced decrease in antioxidant defenses [6].

Another proposed role for rafts in Alzheimer's disease is amyloid cascade regulation by segregation of amyloidogenic enzymes and modulation of BACE1 activity. As suggested by studies, APP amyloidogenic cleavage by BACE1 is dependent on a raft co-localization of these proteins, since lipid raft environment favors secretase activity [51, 52] and the proper interaction between BACE1 and APP [7, 53]. It is suggested that in normal conditions even 10% of APP is concentrated into rafts [54] and, similarly, a pool of BACE1 is also associated with these microdomains [55]. An increase in distribution of these proteins to lipid rafts could promote amyloidogenesis [51, 56], and by this way, drugs able to prevent raft association of APP and BACE1 could slow down amyloid production and Alzheimer's disease progression [21, 57].

In order to assess the association of APP and BACE1 with rafts, in our model, we compared the detergent and detergent-free methods to rule out the hypothesis that the localization of these proteins in microdomains could be a detergent artifact. As a result, both methodologies enabled the identification of APP and BACE1 pool in lipid raft fractions (Supplemental data, Fig. S1). To confirm this result, we investigated the effect of partial or total raft disruption induced by MβCD and found that this cholesterol sequestering drug was able to cause the shift of these proteins out of raft fractions, confirming the hypothesis that, under normal conditions, they are partially microdomain associated (Supplemental data, Fig. S2).

We demonstrated here that fibrilar Aβ injection changed APP and BACE1 distribution along membrane domains, increasing their association with lipid rafts, specifically in cerebral cortex. Consistent with our findings, Peters and colleges [44], showed that Aβ interaction with membranes increases amyloid deposition by stimulation of amyloidogenesis, corroborating the idea that neuronal membranes could be involved not only in amyloid toxicity and neuronal death, but also in the pathological vicious cycle triggered by amyloid peptide and consequent AD progression [25].

The finding of an Aβ induced partial disruption of lipid microdomains could, at first sight, suggest a reduction in protein raft association. We hypothesize, however, that the partial disruption of raft structures could promote redistribution of amyloidogenic proteins in the remaining intact lipid rafts by lateral diffusion or even by membrane raft endocytic recycling process, which would explain our findings. Consistent with this idea, Kahether and Hass [58] have demonstrated that partial disruption of lipid rafts, by mild reduction of cholesterol content, enhances APP cleavage by BACE1, whereas total disruption of membrane microdomains by higher cholesterol reduction impairs amyloidogenic processing.

Interestingly, the Aβ effect on amyloidogenic protein distribution was prevented by GM1 treatment. Although the consequence of this effect on BACE1 activity was not evaluated here, we can hypothesize that the effect of GM1 in preventing Aβ-induced APP/BACE1 shift into lipid rafts could reduce amyloidogenesis. Dissociation of BACE1 from lipid rafts could mildly reduce its activity and, most importantly, change the substrate targets for this enzyme, preventing its action on APP (raft dependent event) and allowing its interaction with other substrates that eventually do not depend on lipid raft environment. This effect, if confirmed, would represent an enormous advance for therapeutic modulation of BACE1, since a selective reduction in its tendency for APP cleavage could theoretically avoid many adverse effects inherent to BACE1 inhibition [59]. Considering that GM1 also possesses neurotrophic and neuroprotective effects [60, 61], our findings suggest that GM1 could represent an important disease modifying drug in Alzheimer's disease.

The mechanisms involved in GM1 effect of preventing the enhancement of APP/BACE1 shift into raft were not evaluated. Tan and colleagues [62] have shown, however, that oxidative stress increases BACE1 raft association. The antioxidant properties of GM1[6, 63] could be involved in the effects observed here.

Considering the importance of rafts to neural functions such as neuroplasticity, synaptic transmission, and the regulation of protein receptors and enzyme activity [12, 14, 49], we suggest that cognitive deficit induced by fibrilar Aβ and GM1 neuroprotection [6] could be mediated by the alterations in raft architecture demonstrated here.

In conclusion, our results reinforce the importance of membrane architecture alterations in the toxicity triggered by amyloid peptide, and propose that GM1 neuroprotection could be partially mediated by the preservation of raft integrity. Since GM1 also prevented Aβ-induced redistribution of amyloidogenic proteins into lipid rafts, we may suggest that this ganglioside could also break down the pathological vicious cycle involving membrane-Aβ interaction and amyloidogenic cascade activation (Fig.7).

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Conflict of interest: The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1 Sucrose density gradient and fraction collection from Triton X-100 and detergent-free extracts. Arrow indicates the raft fraction.

Fig. 2 Standardization and comparison of Triton X-100 and detergent-free methods for lipid raft extraction: A) Cholesterol concentration in the different sucrose gradient fractions, B) Flotilin and Clathrin distribution along sucrose gradient fraction and C) GM1 immunocontent along gradient fractions.

Fig. 3 Aβ and GM1 Effects on Raft Integrity from Cortex Neural Membranes. A) Cholesterol content distribution along sucrose gradient fractions and B) Flotilin and Clathrin distribution.

Fig. 4 Aβ and GM1 Effects on Lipid Raft Integrity from Hippocampus Membranes. A) Cholesterol content distribution along sucrose gradient fractions and B) Flotillin and Clathrin distribution.

Fig. 5 βMCD effect on raft integrity and membrane lipid composition. A) Cholesterol content distribution along sucrose gradient fractions, B) Flotilin and Clathrin distribution along gradient fractions, and C) Total membrane cholesterol content. Data (n=4) were analyzed by one-way ANOVA, followed by Tukey's test, using GraphPad Prism 6.0 software. Data were considered statistically significant when $p < 0.05$.

Fig. 6 Effect of Aβ and GM1 treatment on APP and BACE1 association to lipid raft in A) Cerebral cortex and B) Hippocampus.

Fig. 7: Proposed mechanism for amyloid induced membrane damage/alterations and GM1 neuroprotective action. Fibrilar Aβ causes partial disruption/disassembling of membrane rafts and redistribution of APP and BACE1 into the remaining intact membrane microdomains. The co-localization of APP and BACE1 into rafts favors their interaction and amyloidogenesis, what is believed to enhance Aβ deposition. The pathological vicious cycle triggers an increased damage of membrane architecture, with lipid raft disruption, the consequent loss of microdomain signaling regulatory roles, and cognitive impairment. GM1 treatment prevents Aβ effects on neural membranes, preserving lipid raft integrity and avoiding the shift of amyloidogenic proteins into microdomains. Exogenously administered GM1 is proposed to break amyloid vicious cycle.

Figure 1

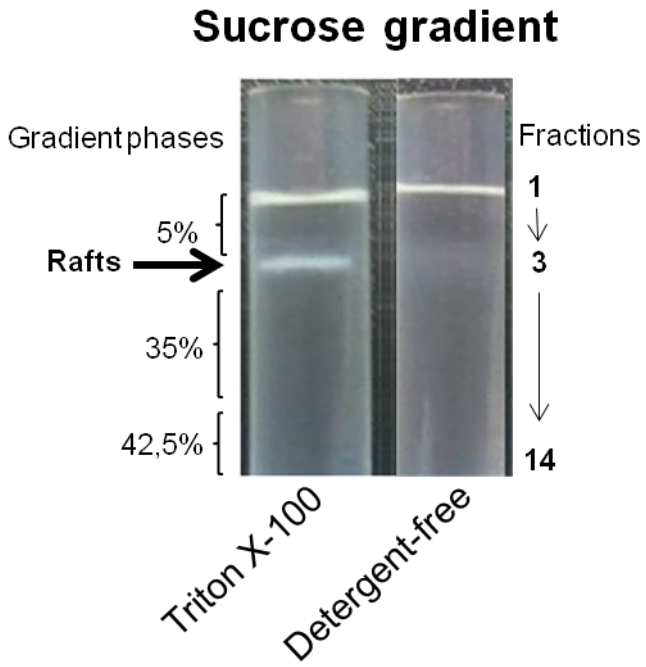

Sucrose gradient
Gradient phases
Fractions
1
5%
Rafts
3
35%
42,5%
14
Triton X-100
Detergent-free

Figure 2

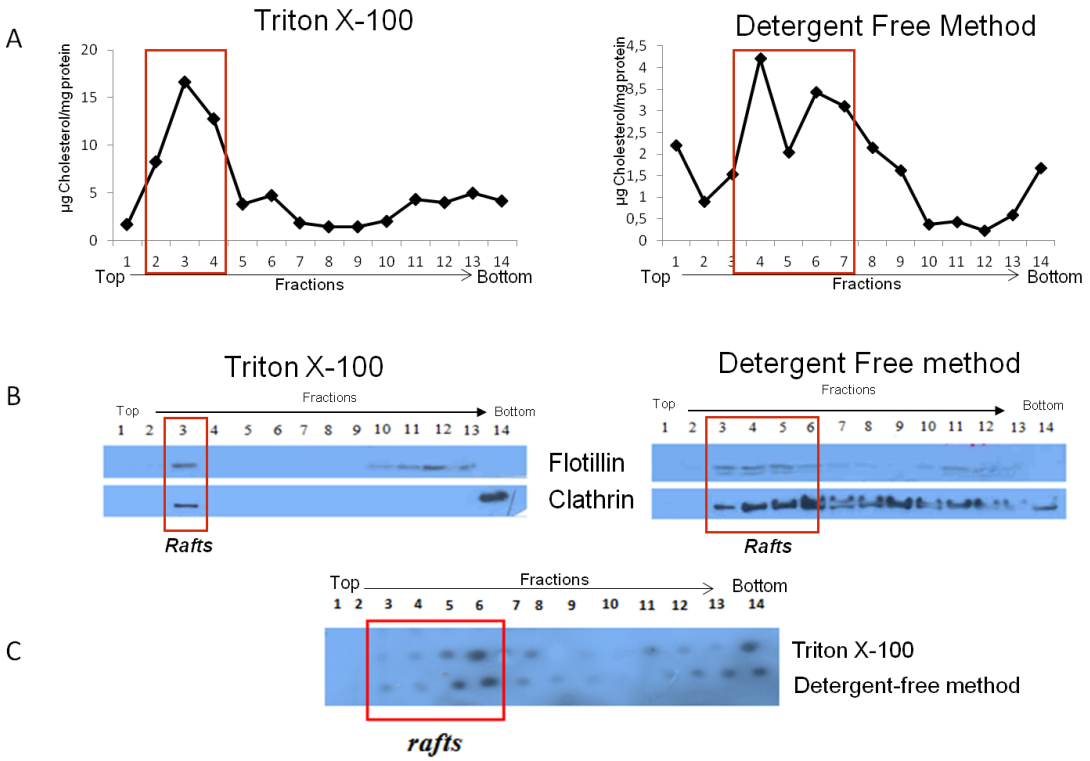

A
Triton X-100
µg Cholesterol/mg protein
Top
Bottom
Fractions
Detergent Free Method
B
Triton X-100
Detergent Free method
Flotillin
Clathrin
Rafts
C
rafts
Triton X-100
Detergent-free method

Figure 3

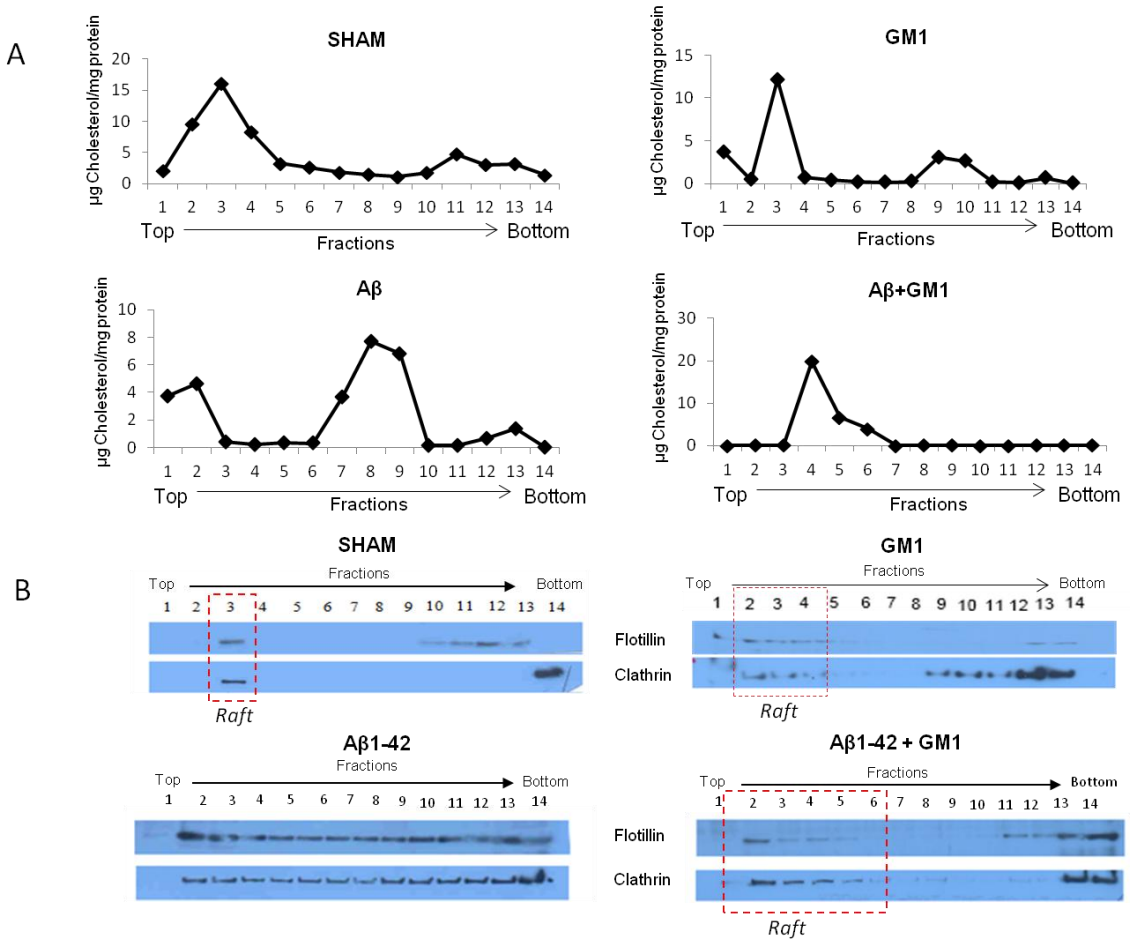

A
SHAM
GM1
Aβ
Aβ+GM1
µg Cholesterol/mg protein
Top
Bottom
Fractions
B
SHAM
GM1
Aβ1-42
Aβ1-42 + GM1
Flotillin
Clathrin
Raft

Figure 4

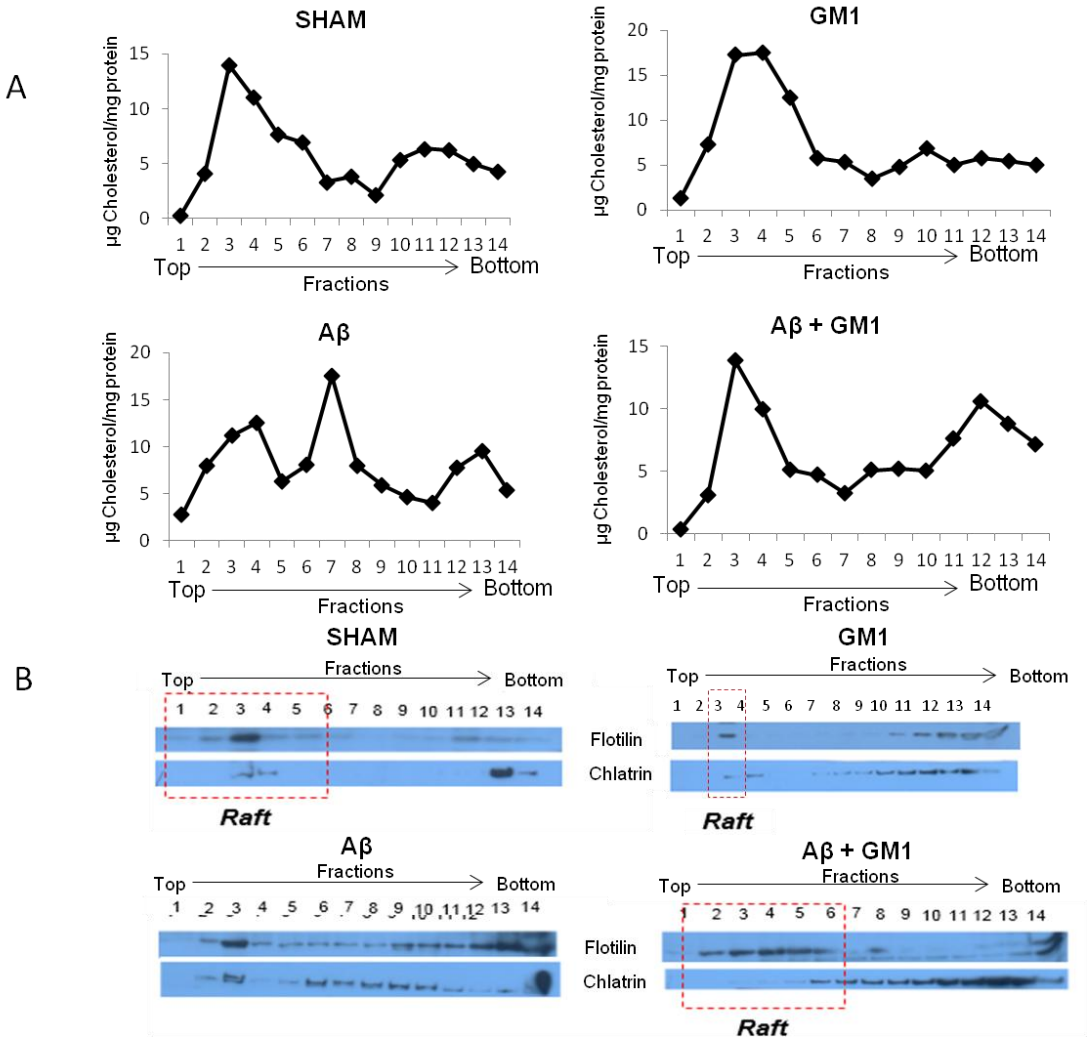

A
SHAM
GM1
Aβ
Aβ + GM1
µg Cholesterol/mg protein
Top
Bottom
Fractions
B
SHAM
GM1
Aβ
Aβ + GM1
Top
Bottom
Fractions
Flotilin
Chlatrin
Raft

Figure 5

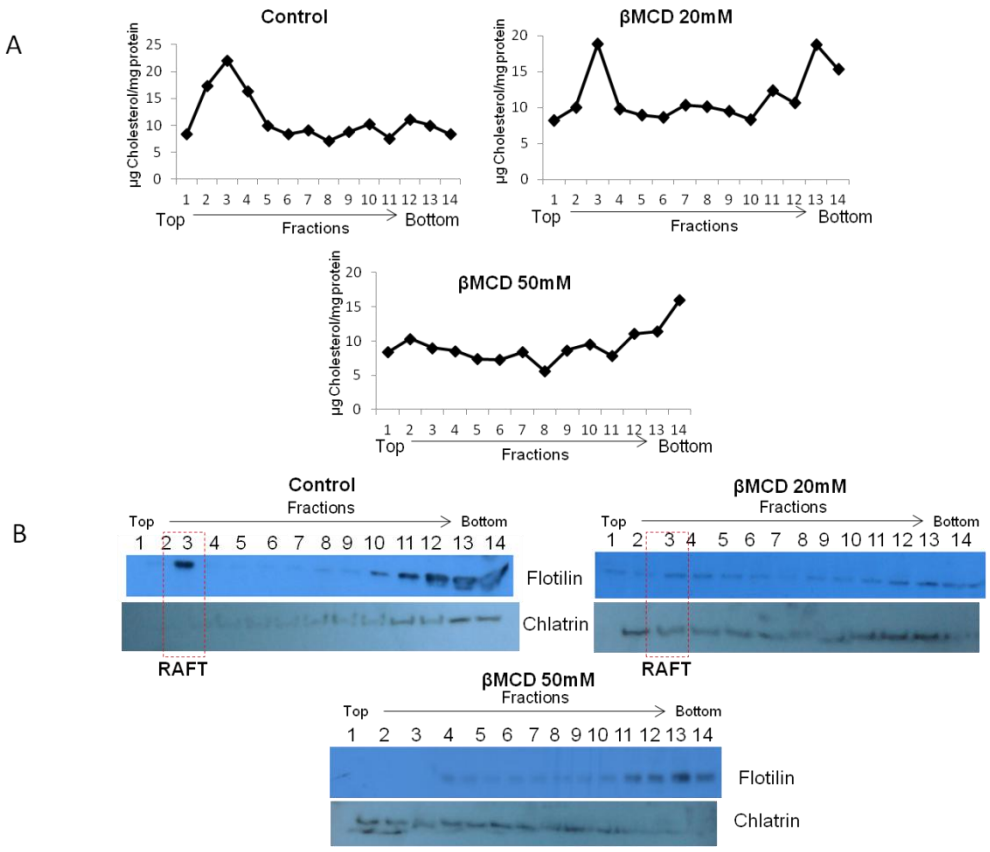

A
Control
βMCD 20mM
βMCD 50mM
µg Cholesterol/mg protein
Top
Fractions
Bottom
B
Control
βMCD 20mM
βMCD 50mM
Fractions
Top
Bottom
Flotilin
Chlatrin
RAFT
C
Cholesterol content
(Percentage of the control)
Control
βMCD 20mM
βMCD 50mM
*

Figure 6

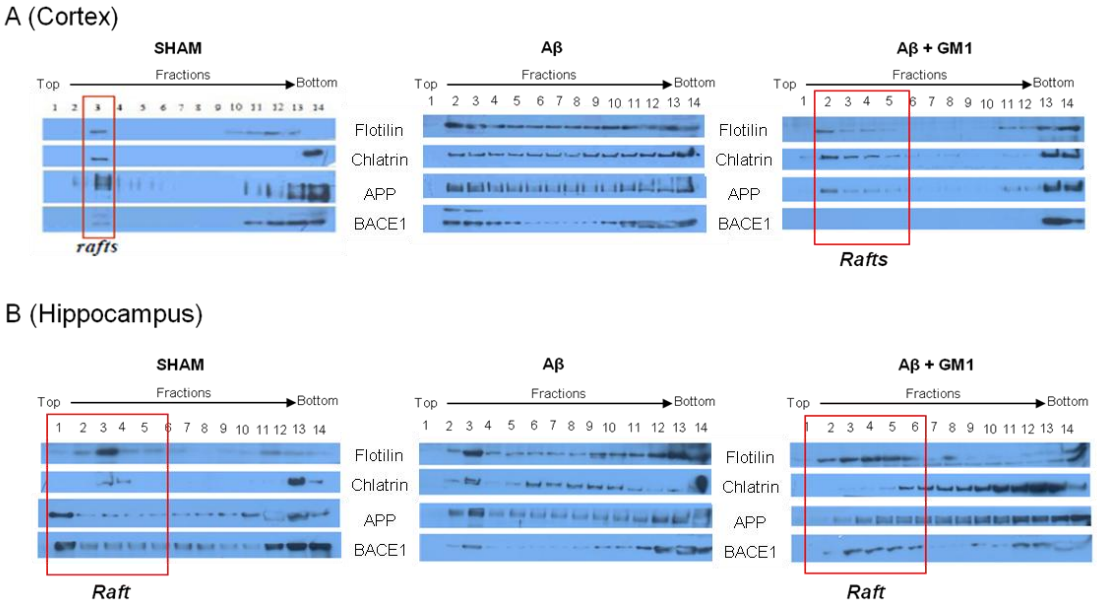
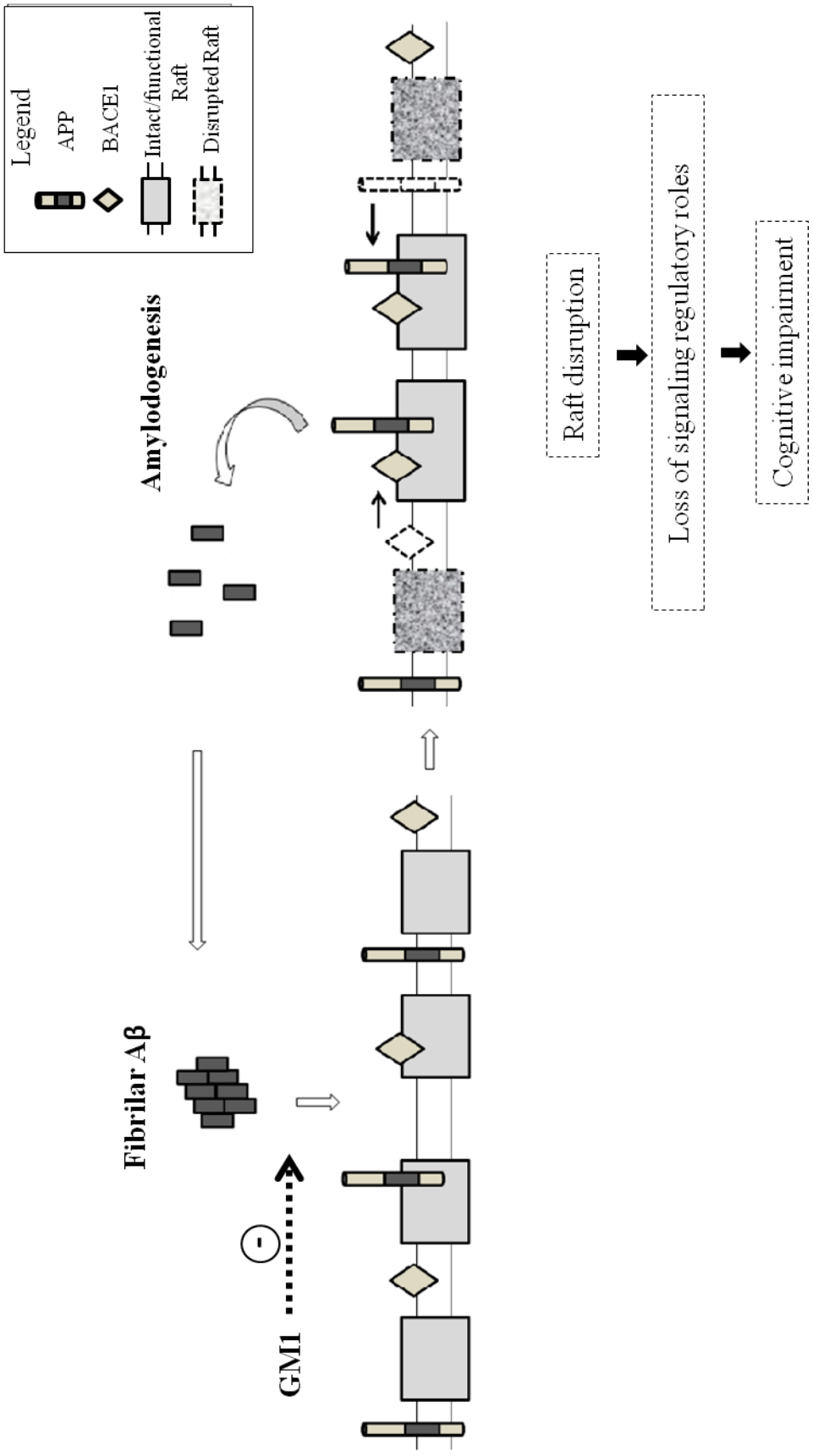

A (Cortex)
SHAM
Aβ
Aβ + GM1
Top
Fractions
Bottom
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Flotilin
Chlatrin
APP
BACE1
rafts
Rafts
B (Hippocampus)
SHAM
Aβ
Aβ + GM1
Raft
Raft

Figure 7


Legend
APP
BACE1
Intact/functional Raft
Disrupted Raft
Amylodogenesis
Fibrilar Aβ
GM1
-
Raft disruption
Loss of signaling regulatory roles
Cognitive impairment

Supplemental data

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Ganglioside GM1 administration prevents Aβ-induced alterations on lipid raft integrity and on the distribution of amyloidogenic proteins (APP and BACE1) along membrane microdomains in rat cortex and hippocampus.

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Supplemental data

Materials and methods

Evaluation of APP and BACE1 distribution to lipid rafts

The presence of APP and BACE1 in lipid rafts was assessed in cerebral cortex using Triton X-100 and detergent-free methods. As shown in Fig. S2, both extraction methods enabled the identification of APP and BACE1 in the raft fractions.

To further investigate the raft association of APP and BACE1, we investigated the effect of partial disassembly and total disruption of lipid raft, induced by in vitro incubation of cerebral cortex slices with βMCD (20mM and 50mM, respectively), in the distribution of these proteins along gradient. For this purpose, we used the detergent method, due to its better extraction efficiency. As seen in Figure S3, 20mM βMCD caused a partial dissociation of APP and BACE1 from lipid rafts, whereas 50mM βMCD completely eliminated these proteins from the upper gradient fractions.

Lipid extraction

The cerebral cortex and hippocampus were 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 xg for 10 minutes. 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.

Total gangliosides and cholesterol determinations

Aliquots from the total lipid extracts were used for ganglioside determination by N-acetyl-neuraminic acid (NeuAc) quantification using the thiobarbituric acid assay described by Skoza and Mohos (1976). Cholesterol was quantified in aliquots from total lipid extracts according to the Trinder-enzymatic technique (Bergmeyer, 1974), respectively.

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Figure Legends

Fig. S1 Evaluation of APP and BACE1 raft association by Triton X-100 and Detergent Free Methods.

Fig. S2 βMCD effect on APP and BACE1 association to lipid raft.

Fig. S3 Neural membrane cholesterol (A) and ganlioside (B) content from cerebral cortex and hippocampus. Cholesterol content is expressed as μg cholesterol/mg protein, and ganglioside as nmol NeuAc/mg protein. Data are presented as mean S.D. for 4 animals in each group, and analyzed by one-way ANOVA, followed by Tukey's test, using GraphPad Prism 6.0 software. Data were considered statistically significant when $p < 0.05$.

Figure S1

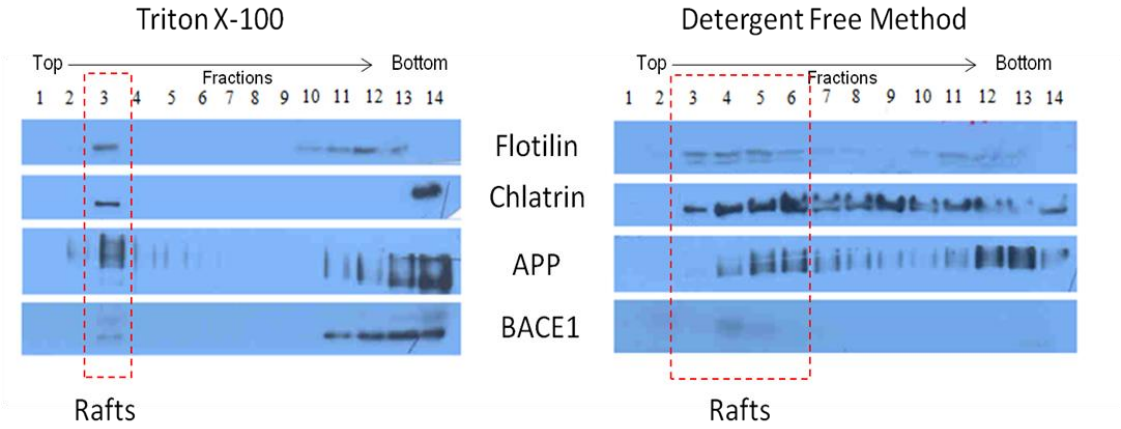
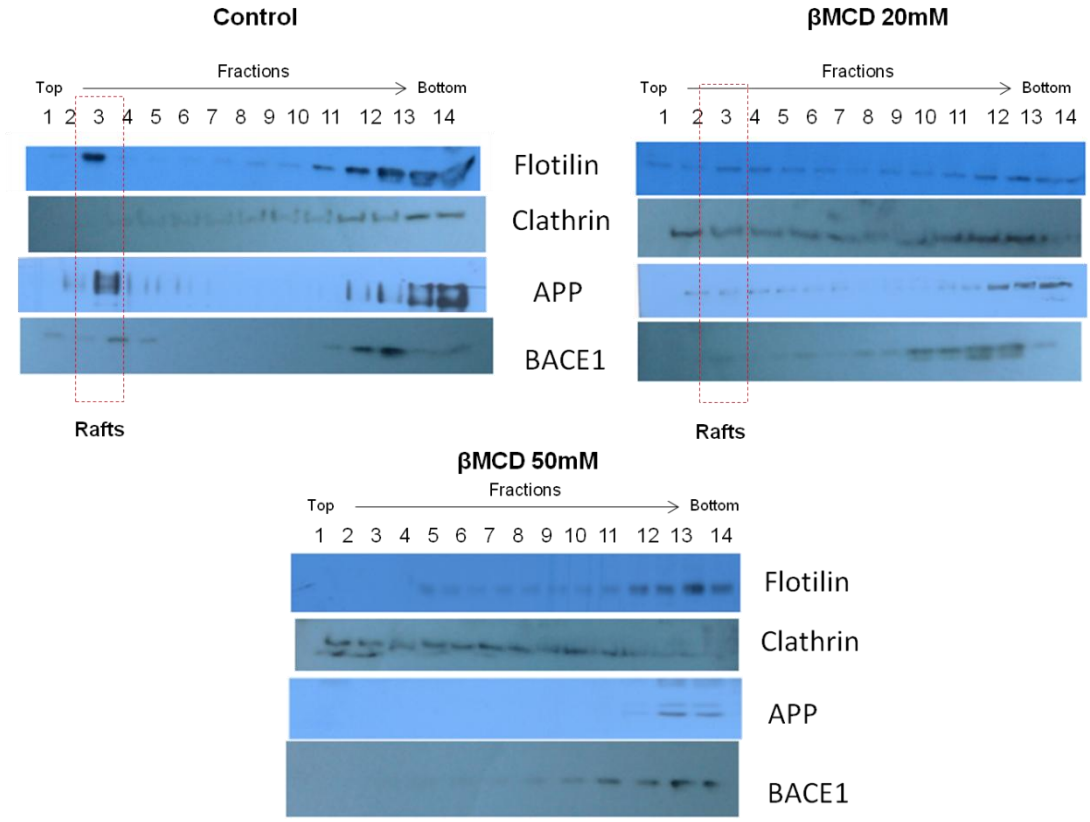

Triton X-100
Top
Fractions
Bottom
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Rafts
Flotilin
Chlatrin
APP
BACE1
Detergent Free Method
Top
Fractions
Bottom
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Rafts

Figure S2


Control
Fractions
Top
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1 2 3 4 5 6 7 8 9 10 11 12 13 14
Flotilin
Clathrin
APP
BACE1
Rafts
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Bottom
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Rafts
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1 2 3 4 5 6 7 8 9 10 11 12 13 14
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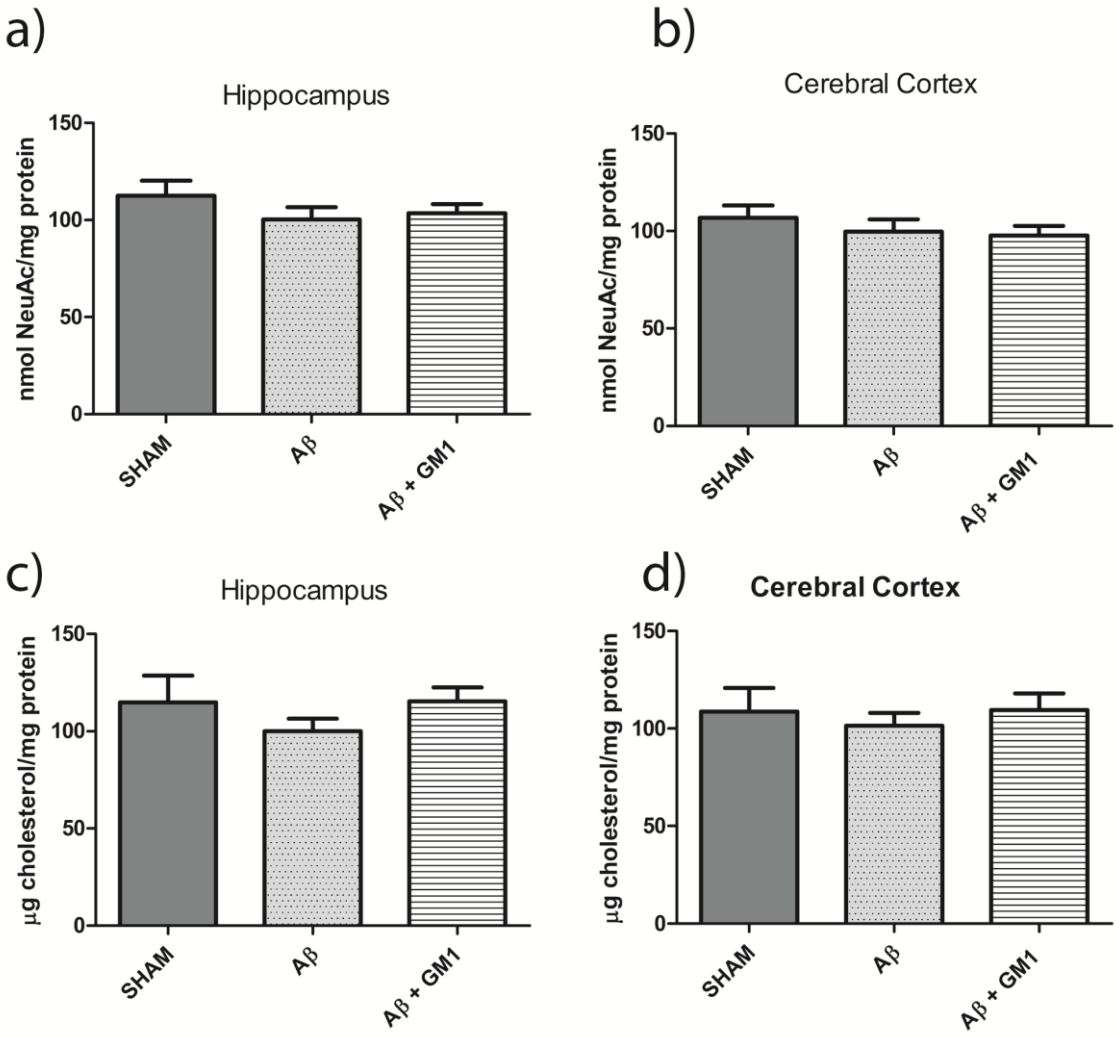
Clathrin
APP
BACE1

Figure S3


a)
Hippocampus
nmol NeuAc/mg protein
150
100
50
0
SHAM
Aβ
Aβ + GM1
b)
Cerebral Cortex
nmol NeuAc/mg protein
150
100
50
0
SHAM
Aβ
Aβ + GM1
c)
Hippocampus
µg cholesterol/mg protein
150
100
50
0
SHAM
Aβ
Aβ + GM1
d)
Cerebral Cortex
µg cholesterol/mg protein
150
100
50
0
SHAM
Aβ
Aβ + GM1

ARTIGO III

GM1 neuroprotection against Aβ-peptide toxicity involves exogenous Aβ–GM1 interaction and prevention of peptide binding to neuronal membranes. **Status:** A ser submetido ao periódico *Neurobiology of Disease*

GM1 neuroprotection against Aβ-peptide toxicity involves exogenous Aβ–GM1 interaction and prevention of peptide binding to neuronal membranes

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Abstract

Amyloid peptide (Aβ), produced by cleavage of neuronal membrane protein APP (Amyloid Precursor Protein), is involved in Alzheimer's disease (AD) pathogenesis. The interaction of Aβ with neuronal membranes, specifically with membrane microdomain (raft) components, seems to be crucial for amyloid polymerization into oligomers or fibrils, Aβ triggering of cell death and the pathological vicious cycle between Aβ deposition and enhancement of amyloid peptide production. Thus, drugs able to prevent peptide interaction with membrane rafts could play a neuroprotective role and have disease modifying properties. Since previous studies have identified GM1 ganglioside as the raft component responsible for Aβ interaction, and evidences suggest a neuroprotective role for exogenously administered GM1, the present study aimed to evaluate exogenous GM1 neuroprotective property against fibrilar Aβ toxicity in human neuroblastoma cells. As a result, GM1 was able to prevent peptide-induced cell death (necrosis and apoptosis), when co-administered with the peptide. In order to investigate whether this neuroprotective action of GM1 could be mediated by sequestering of fibrilar Aβ and avoidance of its interaction with neuronal membranes, the effect of previous *in vitro* incubation of fibrilar Aβ and GM1 on peptide toxicity was assessed, and *in vitro* Aβ-GM1 incubation was found to prevent peptide-induced cell death. The interaction between Aβ and GM1 was confirmed by co-sedimentation and dot blot analysis. In conclusion, the present results reinforce the idea of GM1 neuroprotective roles in AD models, when administered as an exogenous drug, and propose a mechanism for this neuroprotection, proposing GM1 as a promising disease-modifying drug for AD.

Key Words: Alzheimer's disease, Amyloid peptide (Aβ), GM1 ganglioside, neuroprotection

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive loss of memory and impairment of other cognitive functions. The main histological and biochemical hallmark of this dementia (Mattson, 2004) disease are senile plaques, mainly composed of Aβ. This peptide, formed by the amyloidogenic processing of membrane amyloid precursor protein (APP) by secretase enzymes (Querfurth and LaFerla, 2010), is promptly polymerized into oligomers or fibrils with neurotoxic properties, playing a key role in AD development and progression (Suh and Checler, 2002; Haas and Selkoe, 2007; DaSilva et al., 2010; Bloom, 2014).

Therapeutics for AD are still limited, being restricted to palliative drugs that act on cognitive functions and have little or no effect on disease progression. For this reason, there is a growing interest in the identification of drugs with disease modifying properties, such as reduction of Aβ production, enhancement of its clearance, or blockage of peptide-induced neurotoxicity (Matsuoka et al., 2003; Racchi and Govoni, 2003; Jackson, 2014; Schneider et al., 2014).

Several different mechanisms are proposed for Aβ toxicity, and there is no consensus on which would be the most important one (Suh and Checler, 2002; Kayed and Lasagna-Reeves, 2013). Evidence suggests, however, that the toxicity and the pathological vicious cycle triggered by the peptide are dependent on Aβ interaction with neuronal membranes, more precisely with GM1 ganglioside molecules clustered into membrane lipid microdomains (rafts) (Peters et al., 2009; Lai and McLaurin, 2010; Zampagni et al., 2010). Therefore, drugs that can directly interact with Aβ and block peptide interaction sites with membranes could avoid amyloid induced toxicity by means of a sequestration or neutralization mechanism, thus representing a promising alternative therapy for AD (Matsuoka et al., 2003; Evangelisti et al., 2013).

Several studies have proposed neuroprotective properties to exogenously administered GM1, suggesting that this ganglioside could reduce Aβ toxicity. The mechanisms involved in this neuroprotection, however, remain a matter of investigation (Sokolova et al., 2007; Kreutz et al., 2011; Yang et al., 2013).

Among the different experimental models to study AD and neuroprotective drugs, the exposure of neuronal cell cultures to Aβ peptide represents a valuable tool. SH-SY5Y is a human neuroblastoma cell line largely used for this purpose (Zampagni et al., 2010; Kouyoumdijan et al., 2013; Matsuzaki 2014; Stockburger et al., 2014; Yahi and Fantini, 2014) including the study of APP processing regulation and its link to ganglioside metabolism (Grimm et al., 2012).

In view of the above mentioned, the aim of this study was to evaluate GM1 neuroprotective effect on Aβ induced toxicity in SH-SY5Y cell cultures. Since both *in vivo* and *in vitro* Aβ-GM1 interaction are widely proposed (Fantini et al., 2013; Yahi and Fantini, 2014), this study also attempted to investigate whether interaction between Aβ and exogenously administered GM1 could mediate ganglioside neuroprotection, by a neutralization or sequestration effect of GM1 on amyloid peptide.

2. Materials and Methods

2.1 Amyloid Peptide and GM1 Solution Preparation

Aβ1-42 peptide (obtained from American Peptide Co., Sunnyvale, CA, USA) was dissolved in TRIS 0.05 M (pH 7.5) prepared with sterilized bi-distilled water at a concentration of 1 mg/mL and stored at −20°C. Aliquots of Aβ peptides were allowed to aggregate by incubation at 37°C for 96 h before its use in the experiments (Kreutz et al., 2013).

GM1 ganglioside (Sigma, St. Louis, MO, USA) stock solution was prepared in chloroform:methanol (1:1) at 6.75 mg/mL concentration. Aliquots of this stock solution were dried in liquid nitrogen, and solubilized in TRIS 0.05 M (pH 7.5) to a final GM1 concentration of 13.5 mg/mL.

2.2 In vitro Co-incubation of Aβ and GM1

Fibrilar Aβ (0.5 μM) and GM1 (10 μM) were incubated together (*in vitro*), in TRIS 0.05 M at 37^{o}C under constant agitation. After 1h incubation, the sample was centrifuged (10,000xg, for 20 min) to promote fibrilar Aβ sedimentation. The supernatant (GM1 solution) was discarded and the pellet was washed twice in TRIS

0.05 M with sequential centrifugation. The final pellet, composed of fibrilar Aβ, probably interacted with GM1 molecules (Aβ-GM1^{i}), was reserved for subsequent toxicity evaluation in culture cells and for investigation of GM1 immunoreactivity by dot blot analysis.

As a control experiment, fibrilar Aβ solution (0.5 μM, in TRIS 0.05M) was incubated in the absence of GM1, for 1h at 37^{o}C. The solution was subjected to the same procedures of centrifugation and washing with 0.05M TRIS buffer. The final pellet (Aβ^{i}) was reserved for control experiments in the subsequent culture experiments.

2.2.1 Dot Blot from Aβ-GM1 pellet

The final pellet obtained by in vitro incubation of Aβ and GM1 was suspended in 5 μL of a buffer composed of 4% sodium dodecyl sulfate, 2 mM EDTA, 50 mM Tris, and then carefully loaded on a nitrocellulose membrane, for dot blot analysis of GM1. After overnight drying, the membrane was incubated (1h) with blocking solution containing bovine albumin (5%) and 0.1% Tween-20 in Tris–buffered saline (TBS; 50 mM Tris–HCl, 1.5% NaCl, pH 7.4). Membrane was then incubated overnight with choleric toxin (Sigma, St. Louis, MO, USA), and subsequently submitted to overnight incubation with anti-choleric toxin antibody (1:1000) (kindly provided by H.J. Maccioni, Universidad Nacional de Córdoba, Argentine). The membranes were finally incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000) (Sigma, St. Louis, MO, USA), and chemioluminescence (ECL) was detected using X-ray films (Kodak X-Omat) (Ilangumaran et al., 1997).

2.3 Cell Culture

Human neuroblastoma cells, SH-SY5Y (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) were maintained in a 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®/Invitrogen, São Paulo, SP, Brazil) supplemented with 10% fetal bovine serum (FBS) (Cripion Biotecnologia Ltda., Andradina, SP, Brazil), 26 μM of sodium bicarbonate (Merck, Darmstadt, Germany), 0.28 μg/μL of gentamicin and 1 μL/mL of amphotericin B, at 37 °C under a 5% CO2 humidified atmosphere. The culture medium was replaced every 3 days and cells were subcultured when they reached 90% of confluence (Lopes et al., 2010).

2.3.1 Cell Culture Treatments and Cell Viability Analysis

Cells were seeded in 96 well plates at a density of $5x10^4$ cells per well, and 24 h after cell plating, the medium was changed to 1:1 DMEM:F12 mixture supplemented with 1% fetal bovine serum. The cultures were then incubated with fibrilar Aβ peptide (0.25 μM, 0.5 μM, 1 μM, and 2 μM) and/or GM1 (10 μM, 20 μM, and 30 μM) for 48 h. In order to measure Aβ induced toxicity and GM1 neuroprotection, the cells were incubated with propidium iodide (PI) (5 μM) (Sigma, St. Louis, MO, USA) and Hoechst 33342 (HO) (5 μg/ml) (Invitrogen, São Paulo, SP, Brazil) for 30 min (Macklis and Madison, 1990). The cells were then observed under an inverted microscope (Nikon Eclipse TE 300) and two different images (PI and HO stained cells) were captured and analyzed (qualitatively and quantitatively) with software Image J. After overlay of the images, the cells were classified as follows: live (cells with round and regular nuclei stained only by HO), apoptotic (cells with condensed and/or fragmented nuclei stained by HO) and necrotic (cells with red spherical or vesicle nuclei stained by PI and with red cytoplasm). For apoptosis computing, cells in initial apoptosis (bright blue nuclei) and cells in late apoptosis (bright blue and red nuclei – purple in the merged images) were included. The percentage of living and death cells (necrotic plus apoptotic ones) was established and cell death was differentiated into necrotic cells and apoptotic ones (initial plus late apoptosis) taking into account the morphology and staining of the cells. For cell death measurement, three independent experiments were performed, each one composed of 3 replicates (3 culture wells) per experimental group. From each culture well, four microscopic fields were photographed, and the mean percentage of live/dead (apoptotic/necrotic) cells were considered for statistical analysis.

2.3.2 Toxicity Assay of the Aβ Previously Incubated with GM1

In order to evaluate the effect of previous *in vitro* Aβ-GM1 interaction on peptide toxicity, the pellet obtained after Aβ-GM1 incubation (Aβ-$GM1^i$), as well as the pellet obtained by Aβ incubation in absence of GM1 ($A\beta^i$) – the control of incubation procedures-, were suspended in DMEM:F12/SFB (1:1/1%) and used to replace culture medium.

For cell viability analysis, SH-SY5Y cells were seeded in 96 well plates, as previously described. After 24h of plating, the experiment was divided into 4 groups:

a. Control: Cells have their medium changed for DMEM:F12/SFB (1:1/1%);

b. Aβ 0.5 μM: Cells have their medium changed for DMEM:F12/SFB (1:1/1%) and were incubated with the Aβ not submitted to incubation procedures, at final concentration of 0.5 μM, as previously described;

c. $A\beta^{i}$: Cells have their medium discarded and replaced by suspension of $A\beta^{i}$ pellet with DMEM:F12/SFB (1:1/1%);

d. Aβ-$GM1^{i}$: Cells have their medium discarded and replaced by suspension of Aβ-$GM1^{i}$ pellet with DMEM:F12/SFB (1:1/1%)

Cellular viability was assessed after 48h by PI uptake and Hoescht staining, as previously described.

2.4 Statistical Analysis

Data are expressed as mean ± S.D. and analyzed by one-way or two-way analysis of variance (ANOVA) followed by Tukey's test, using GraphPad Prism 6.0 software. Differences between mean values were considered significant when $p < 0.05$.

3. Results

3.1 Toxicity of Aβ peptide and GM1 neuroprotection in SH-SY5Y cultures

In order to reproduce Aβ toxicity in SH-SY5Y cell cultures and choose the most appropriate Aβ concentration, a death-concentration curve study was performed. As shown in Fig.1, Aβ triggered cell death in a concentration-related way (r^2=0.9367, P value<0.0001, F=281.4). Differential analysis of cell death revealed that Aβ triggered both necrosis and apoptosis (necrosis: r^2=0.8225, P value<0.0001, F=88.02; apoptosis: r^2=0.8944, P value<0.0001, F=161.0), with apoptosis being the most relevant event.

Once defined the death-concentration curve, 0.5μM Aβ was elected for the subsequent experiments of GM1 neuroprotection.

3.2 Evaluation of GM1 neuroprotective effect on Aβ-induced toxicity

As shown in Fig. 2, the three tested GM1 concentrations (10 µM, 20 µM, and 30 µM) promoted equivalent neuroprotective effects, preventing Aβ-induced cell death (either total cell death, necrosis or apoptosis).

3.3 Evaluation of previous in vitro Aβ-GM1 co-incubation on peptide induced toxicity

In order to propose a mechanism for GM1 neuroprotection, the effect of previous Aβ+GM1 co-incubation on peptide-induced toxicity was evaluated. As shown in Fig.3, previous in vitro co-incubation of Aβ (0.5µM) and GM1 (10µM) ($A\beta GM1^{i}$) prevented the peptide property of inducing cell death in this model (either for total cell death, necrosis or apoptosis).

3.4 Determination of in vitro Aβ-GM1 interaction

Aβ-GM1 interaction after in vitro co-incubation was evaluated by dot blot analysis. The pellet obtained after centrifugation of Aβ + GM1 mixture was suspended, as previously described, and submitted to dot blot immunodetection of GM1. As shown in Fig. 4, the obtained pellet presented immunoreactivity to choleric toxin, indicating co-sedimentation of Aβ and GM1 and supporting the idea of peptide-ganglioside interaction.

4. Discussion

AD neurodegeneration involves progressive loss of neural functions and the consequent neuronal death. Although several pathogenic hypotheses have been proposed and diverse elements or factors have been attributed to AD development, amyloid cascade and its main product, amyloid peptide, are still believed to play a central role in AD pathogenesis (Querfurth and LaFerla, 2010; Bloom, 2014).

Although the oligomeric form of the peptide has been proposed to be more toxic than fibrils, fibrilar Aβ is the preferential form of amyloid deposition into cerebral

parenchyma. In addition, fibrilar Aβ has been suggested to function as a source for sustained release of oligomeric peptides, and has also proinflammatory, oxidant and membrane structure perturbing effects (Suh and Checler, 2002; Haas e Selkoe, 2007; DaSilva et al., 2010; Sciacca et al., 2012). For these reasons, the fibrilar form of Aβ1-42 was selected in the present study.

As observed in the first results (Fig.1), a linear correlation for cell death (total cell death, necrosis or apoptosis) was found for the tested fibrilar Aβ concentrations.

GM1 ganglioside is a component of neural membranes and is an important component of lipid rafts (Ohmi et al., 2011; 2012). It performs several cellular functions and has demonstrated neuroprotective effects in models of AD or other forms of neurodegeneration, when exogenously administered (Mocchetti, 2005; Zhang et al., 2009; Zhang et al., 2011; Calamai and Pavone, 2013). This neuroprotective effect of GM1 had already been observed in animal models of Aβ toxicity (Kreutz et al., 2013; Yang et al., 2013) or had been demonstrated in rat organotypic hippocampal-slice cultures (Kreutz et al., 2011). In this study, the referred effect was tested in a culture model of human neuroblastoma cells and the results were interesting and consistent compared to the study that used PC12 cells (Sokolova et al., 2007). The incubation of cell cultures with GM1 prevented Aβ induced cell death when GM1 and the peptide were co-administered (Fig. 2). This effect was similarly observed with the three tested GM1 concentrations, what might indicate a saturating effect of the neuroprotective mechanism at concentrations above 10 μM. GM1 neuroprotection involved both prevention of necrosis and apoptosis, resulting in a deep reduction of Aβ-induced total cell death, in such a way that a prevention or blockage of peptide toxicity could be suggested.

The mechanisms involved in GM1 neuroprotection against Aβ-induced toxicity are still speculative. Previous studies demonstrated neurotrophic roles for GM1 ganglioside, proposing GM1 modulation in the release and biological response of neurotrophins (Duchemin et al., 2002; Rabin et al., 2002; Mocchetti, 2005; Lim et al., 2011). In addition, previous studies conducted by our research group proposed the role of GM1 signaling properties, as well as GM1 antioxidant effect, on the triggered neuroprotection in models of Aβ-induced toxicity (Kreutz et al., 2011; 2013).

However, the idea of a GM1-induced neuroprotection in AD models may seem a little contradictory, since numerous studies suggest an important role of GM1 in the modulation of Aβ polymerization process (Matsuzaki, 2007; 2014; Matsuzak et al., 2010; Yanagisawa, 2007), and evidences point neural membrane GM1 as the main binding site for Aβ peptide (Evangelisti et al., 2013).

The interaction of Aβ with neuronal membrane GM1 is considered a key event in the neurodegenerative process (Evangelisti et al., 2013). Peptide binding seems to be specifically addressed to raft associated GM1 gangliosides, since the cholesterol enriched-environment of these microdomains favors Aβ interaction through modulation of GM1 oligosaccharide chain conformation and through the clustering of several GM1 molecules into one membrane domain (Fantini et al., 2013).

Therefore, the binding of Aβ peptide with GM1 containing lipid rafts is believed to elicit local perturbation of membrane architecture/structure (Sciacca et al., 2012; Evangelisti et al., 2014), changes in diffusion properties of raft components (Calamai and Pavone, 2013; Sasahara et al., 2013), and damage of the structure and functions of these membrane microdomains (Ledesma et al., 2003; Hirai et al., 2013). Aβ-induced alterations in rafts – triggered by GM1 interaction – could mediate the property of this peptide to activate some cell death signaling pathways such as those of GSK3β (Sui et al., 2006; Kreutz et al., 2011, Frozza et al.,2013;) and tau proteins (Schengrund, 2010; Hoppe et al. 2010).

In addition, it is proposed that Aβ interaction with membranes is involved in the activation of the amyloidogenic processing, in the enhancement of amyloid deposition, and in the pathological vicious cycle that mediates the progression of AD neurodegeneration (Peters et al., 2009).

In view of the above mentioned, some researchers have proposed the use of exogenous Aβ binding molecules as a tool to avoid peptide interaction with membranes, and the consequent triggering of neuronal damage (Matsuoka et al., 2003; Bereczki et al 2011; Kouyoumdjian et al., 2013; Yuyama et al., 2014) The present study investigated the Aβ sequestration/neutralization role of exogenous GM1 ganglioside in the observed neuroprotection.

For this purpose, Aβ peptide was co-incubated *in vitro* with GM1 and then pelleted by centrifugation, prior to its administration to cell cultures. As shown in the results, the previous co-incubation procedure changed Aβ capacity of inducing cell death, preventing peptide-induced toxicity. To rule out loss of peptide content due to centrifugation steps as an explanation for reduction in toxicity response, the experiments involved an additional control group, corresponding to Aβ peptide subjected to the same centrifugation procedures but with no incubation with GM1 ($A\beta^{i}$). As shown by the results, there was apparently a loss in Aβ content during the centrifugation processes. However, it was not responsible for the marked decrease in neurotoxicity observed after Aβ-GM1 incubation ($A\beta GM1^{i}$).

In order to assess the interaction between Aβ peptide and GM1, the present study investigated their co-sedimentation after centrifugation step. As shown in the dot blot experiment, GM1 co-sediments with fibrilar amyloid peptide, suggesting that direct interaction or complexation between them under these experimental conditions might play a role in neuroprotection.

Our findings are consistent with previous studies that proposed the ability of exogenous GM1 to act as an Aβ-sequestering molecule (Matsuoka et al., 2003). Our study, however, first demonstrated this effect with the already fibrillated amyloid peptide. In addition, considering that Aβ-GM1 interaction was promoted *in vitro* and before peptide incubation with the cultures, our results enabled us to better evaluate the role of peptide-sequestering effect of GM1 in the observed neuroprotection, since it ruled out the contribution of neurotrophic effect of direct GM1 administration to neuronal cultures.

In conclusion, our results suggest that GM1 neuroprotective effect against fibrilar Aβ1-42 toxicity involves sequestration or neutralization of amyloid peptide, the consequent blockage of its interaction with neuronal membranes and the avoidance of all downstream mechanisms that link membrane-Aβ interaction with neuronal damage, cell death and disease progression (Fig. 5).

Based on these findings, as well as on data proposing neurotrophic, signaling and cognitive modulating effect of GM1 (Fong et al., 1997; Hadjiconstantinou and Neff, 1998; Mocchetti, 2005), we propose this ganglioside – or some of its chemical

analogues (Wu et al., 2005; Biraboneye et al., 2009) – as a promising disease modifying drug for Alzheimer's disease.

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Conflict of interest: The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1: Aβ cell death concentration curve. A) Representative photomicrographs of cells exposed to different Aβ concentrations or control conditions and marked with propidium iodide (PI), Hoescht stain, and merged image; B) Quantification of total cell death percentage, defined as the percentage of cells stained with one or both cell death markers; C) Quantification of cell necrosis (%), defined as the percentage of cells stained by PI; D) Quantification of cell apoptosis (%), defined as the percentage of cells with condensed and/or fragmented nuclei stained by HO. Significantly different from control cultures (one-way ANOVA followed by Tukey test, $p < 0.05$).

Figure 2: GM1 neuroprotective effect against Aβ-induced cell death. A) Representative photomicrographs of cells exposed to control conditions, Aβ (0.5μM) and/or GM1 (10, 20 or 30μM), and marked with propidium iodide (PI), Hoescht stain, and merged image; B) Quantification of total cell death percentage, defined as the percentage of cells stained with one or both cell death markers; C) Quantification of cell necrosis (%), defined as the percentage of cells stained by PI; D) Quantification of cell apoptosis (%), defined as the percentage of cells with condensed and/or fragmented nuclei stained by HO. Bars represent the mean + S.D. *Significantly different from control cultures (two-way ANOVA followed by Tukey test, $p < 0.05$), # significantly different from Aβ group (two-way ANOVA followed by Tukey test, $p < 0.05$).

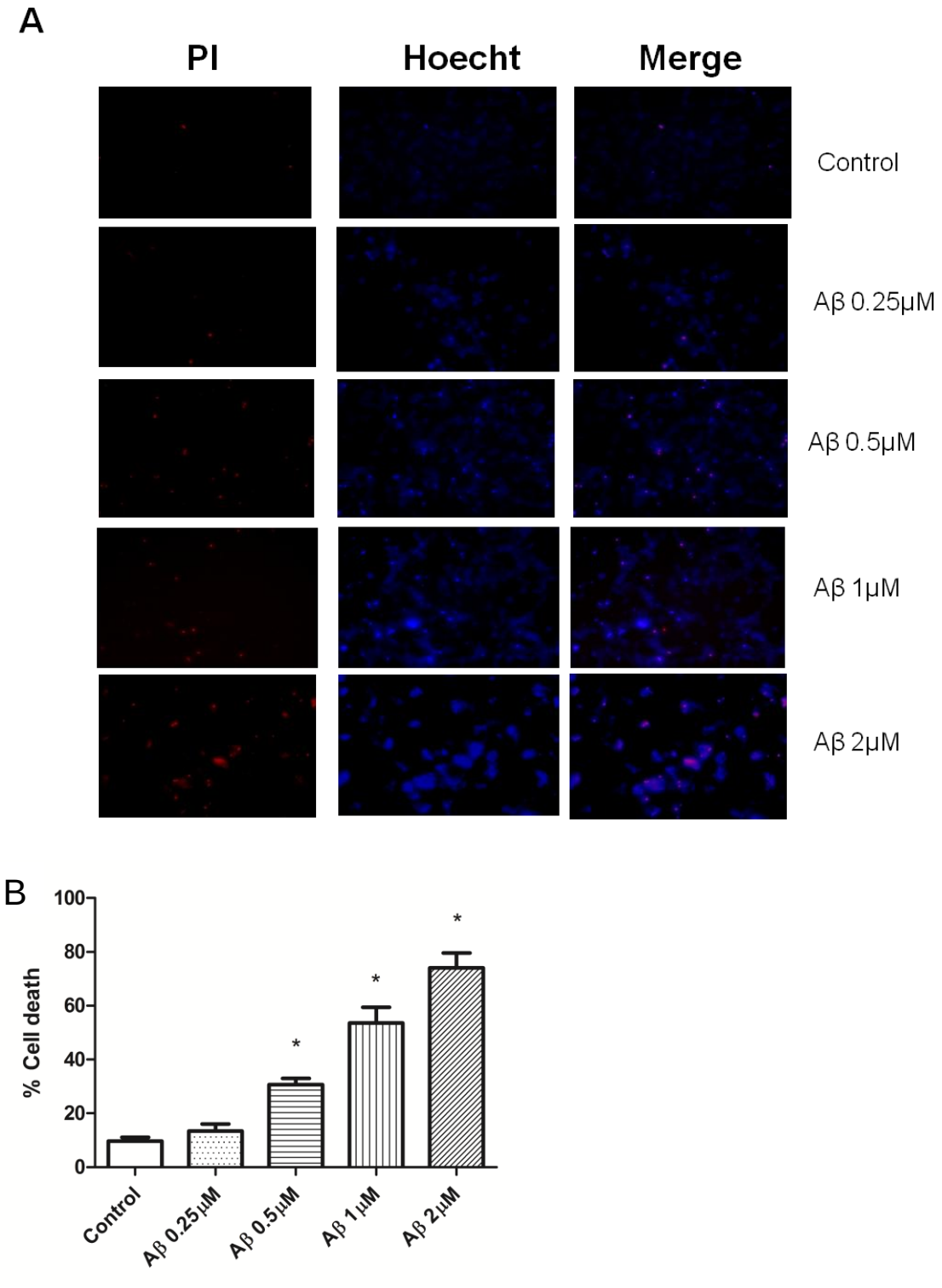
Figure 3: Effect of previous Aβ-GM1 in vitro incubation on peptide induced toxicity. A) Representative photomicrographs of cells exposed to control conditions, Aβ (0.5μM), $A\beta^{i}$ (peptide subjected to centrifugation procedures without GM1 incubation) and $A\beta GM1^{i}$ (Aβ peptide incubated with GM1 prior to exposure to cell cultures), and marked with propidium iodide (PI), Hoescht stain, and merged image; B) Quantification of total cell death percentage, defined as the percentage of cells stained with one or both cell death markers; C) Quantification of cell necrosis (%), defined as the percentage of cells stained by PI; D) Quantification of cell apoptosis (%), defined as the percentage of cells with condensed and/or fragmented nuclei stained by HO. Bars represent the mean

+ S.D. *Significantly different from control cultures (two-way ANOVA followed by Tukey test, $p < 0.05$), #significantly different from Aβ group (two-way ANOVA followed by Tukey test, $p < 0.05$), &significantly different from $A\beta^{i}$ group (two-way ANOVA followed by Tukey test, $p < 0.05$).

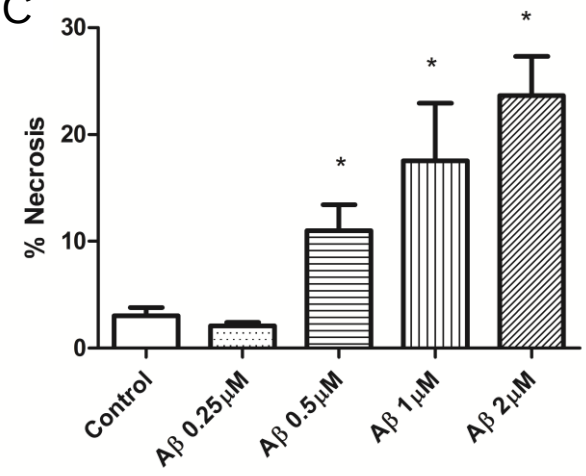
Figure 4: Co-sedimentation of Aβ and GM1 ganglioside after *in vitro* incubation. The imunocontent of GM1 was determined in fibrilar Aβ sediment by dot blot. Representative image from 3 independent experiments.

Figure 5: Proposed mechanism of GM1 neuroprotection by Aβ peptide binding and sequestration. (A) Aβ peptide interacts with neuronal membranes, specifically with raft associated GM1. This interaction perturbs membrane architecture, bringing alterations to integrity and dynamics of lipid raft, and promotes loss of cellular functions which ends in neuronal death. As a consequence of Aβ-membrane interaction and alterations in raft dynamics, the peptide induces its own production by promoting amyloidogenesis (pathological vicious cycle). (B) Exogenously administered GM1 interacts with Aβ and blocks peptide binding to membrane. Prevention of Aβ interaction with membranes preserves membrane architecture and raft structure, avoiding the triggering of cell death and activation of the pathological vicious cycle.

Figure 1


A
PI
Hoecht
Merge
Control
Aβ 0.25μM
Aβ 0.5μM
Aβ 1μM
Aβ 2μM
B
% Cell death
100
80
60
40
20
0
*
*
*
Control
Aβ 0.25μM
Aβ 0.5μM
Aβ 1μM
Aβ 2μM

C


% Necrosis
30
20
10
0
*
*
*
Control
Aβ 0.25μM
Aβ 0.5μM
Aβ 1μM
Aβ 2μM

D

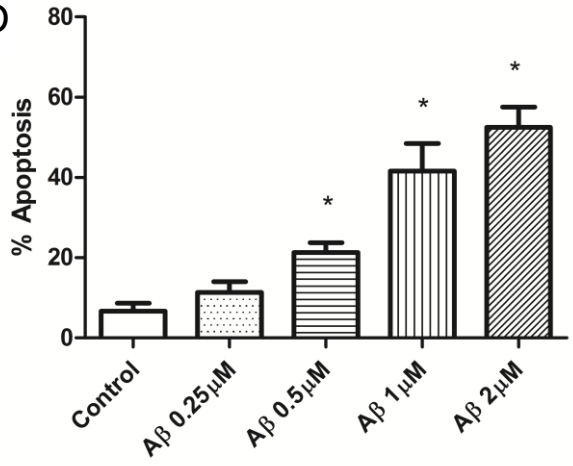
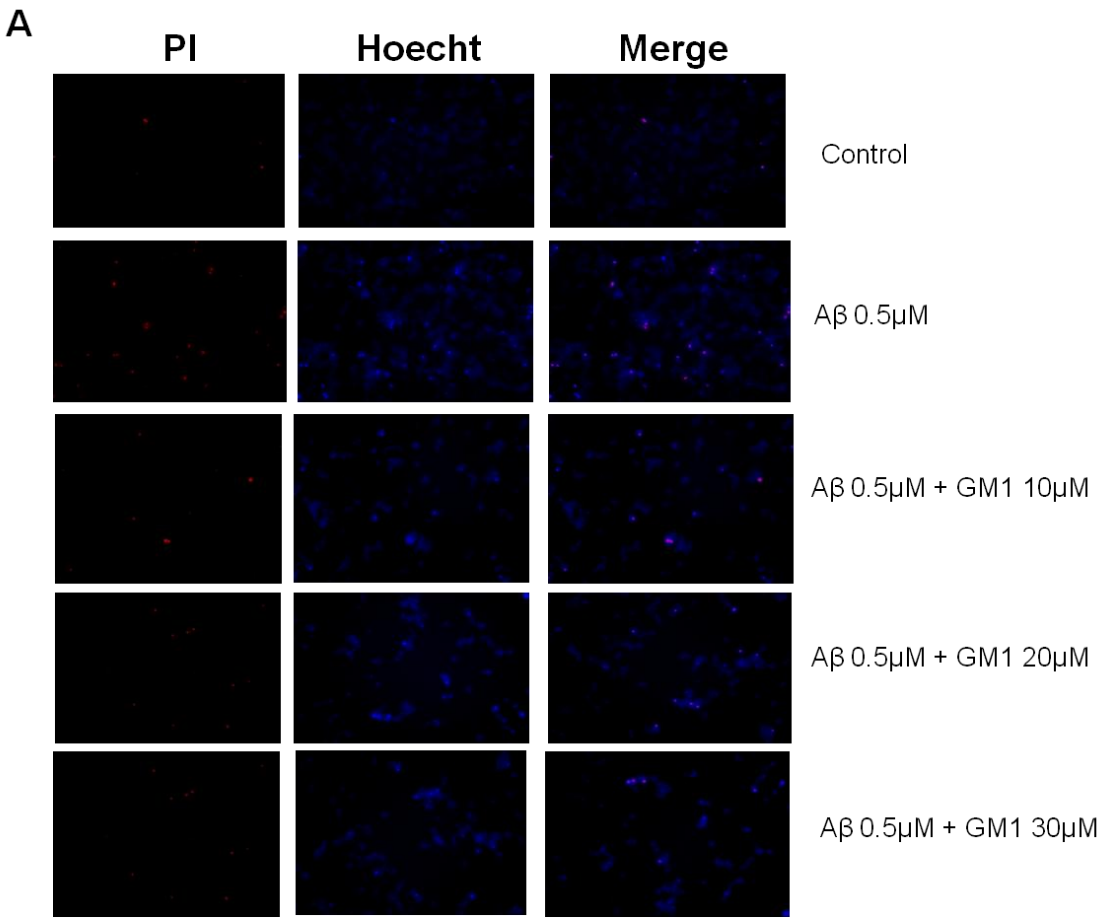
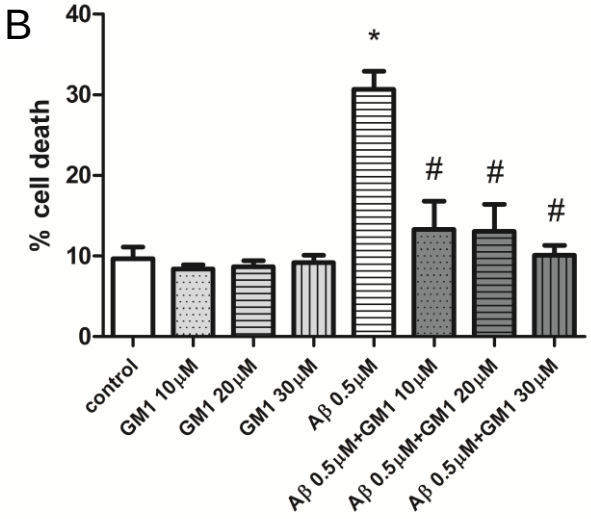
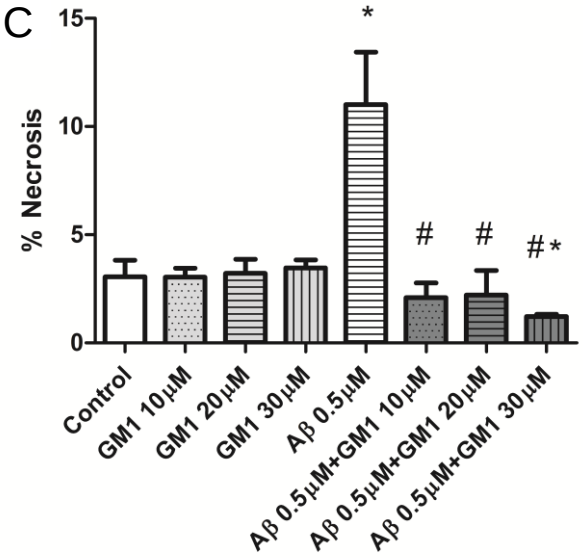

% Apoptosis
80
60
40
20
0
*
*
*
Control
Aβ 0.25μM
Aβ 0.5μM
Aβ 1μM
Aβ 2μM

Figure 2


A
PI
Hoecht
Merge
Control
Aβ 0.5μM
Aβ 0.5μM + GM1 10μM
Aβ 0.5μM + GM1 20μM
Aβ 0.5μM + GM1 30μM


B
% cell death
40
30
20
10
0
*
#
#
#
control
GM1 10μM
GM1 20μM
GM1 30μM
Aβ 0.5μM
Aβ 0.5μM+GM1 10μM
Aβ 0.5μM+GM1 20μM
Aβ 0.5μM+GM1 30μM


C
% Necrosis
15
10
5
0
*
#
#
#*
Control
GM1 10μM
GM1 20μM
GM1 30μM
Aβ 0.5μM
Aβ 0.5μM+GM1 10μM
Aβ 0.5μM+GM1 20μM
Aβ 0.5μM+GM1 30μM

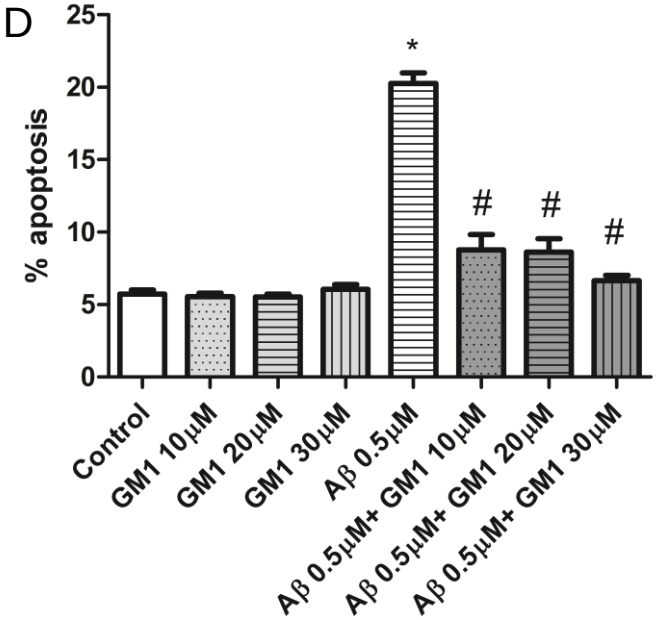
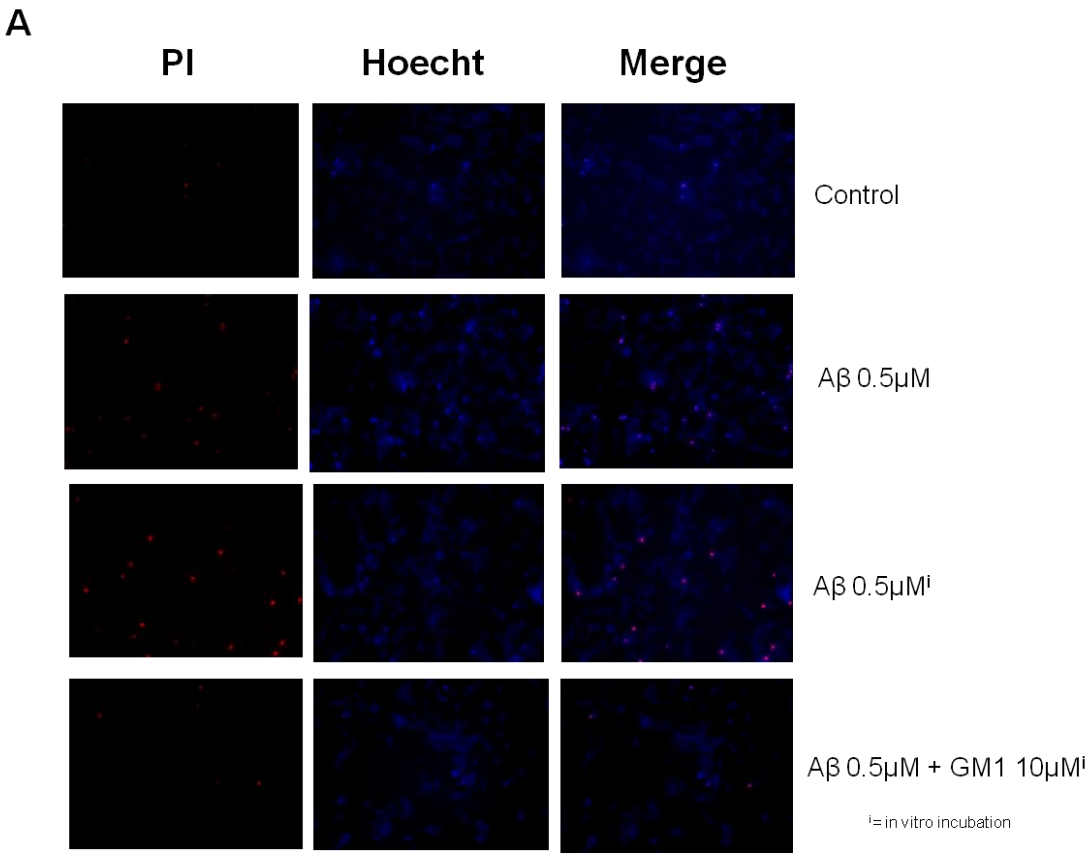
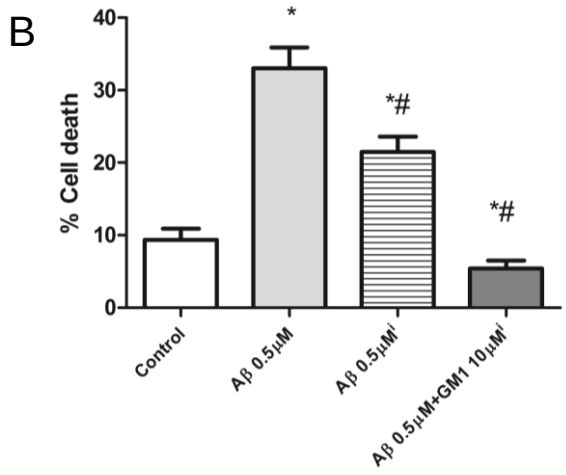
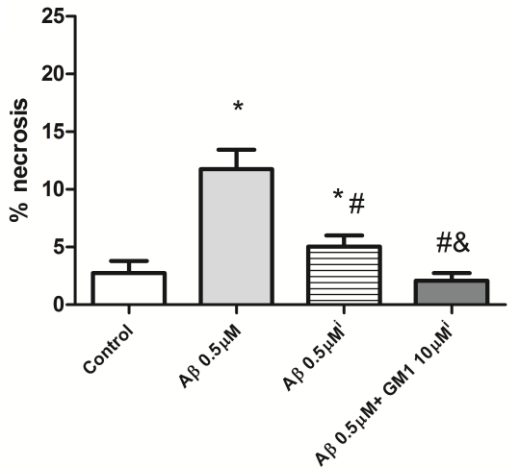

D
% apoptosis
25
20
15
10
5
0
*
#
#
#
Control
GM1 10μM
GM1 20μM
GM1 30μM
Aβ 0.5μM
Aβ 0.5μM+ GM1 10μM
Aβ 0.5μM+ GM1 20μM
Aβ 0.5μM+ GM1 30μM

Figure 3


A
PI
Hoecht
Merge
Control
Aβ 0.5μM
Aβ 0.5μM[i]
Aβ 0.5μM + GM1 10μM[i]
[i]= in vitro incubation


B
% Cell death
40
30
20
10
0
*
*#
*#
Control
Aβ 0.5μM
Aβ 0.5μM[i]
Aβ 0.5μM+GM1 10μM[i]

C


% necrosis
25
20
15
10
5
0
*
* #
#&
Control
Aβ 0.5μM
Aβ 0.5μM
Aβ 0.5μM+ GM1 10μM

D

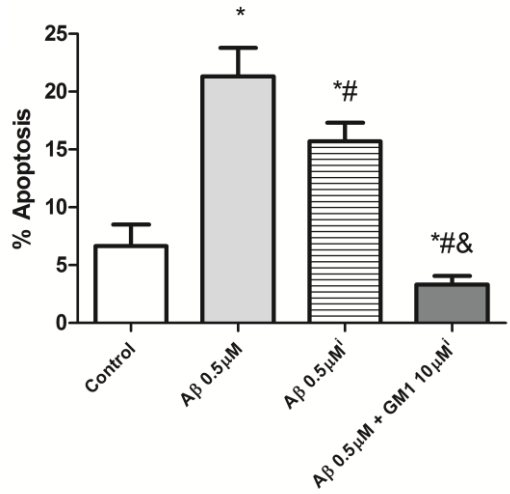

% Apoptosis
25
20
15
10
5
0
*
*#
*#&
Control
Aβ 0.5μM
Aβ 0.5μM
Aβ 0.5μM + GM1 10μM

Figure 4

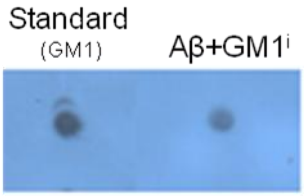
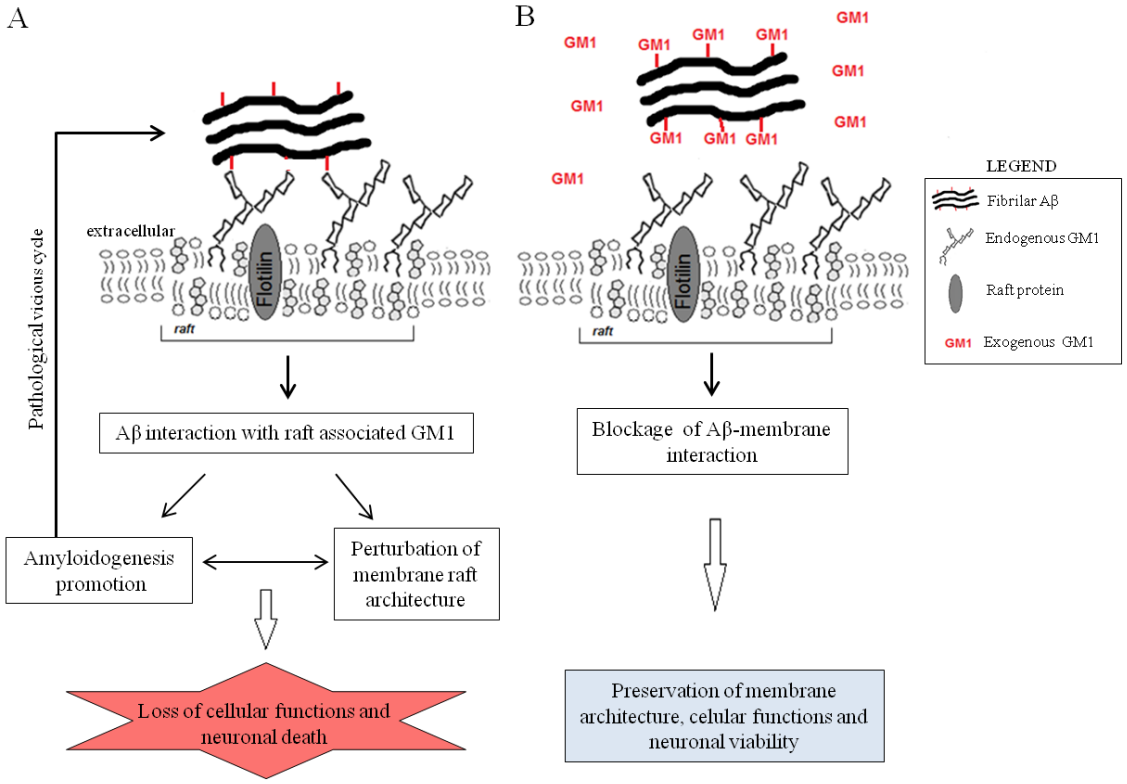


Figure 5


A
B
GM1
Pathological vicious cycle
extracellular
Flotilin
raft
Aβ interaction with raft associated GM1
Amyloidogenesis promotion
Perturbation of membrane raft architecture
Loss of cellular functions and neuronal death
Blockage of Aβ-membrane interaction
Preservation of membrane architecture, celular functions and neuronal viability
LEGEND
Fibrilar Aβ
Endogenous GM1
Raft protein
GM1 Exogenous GM1

4. DISCUSSÃO

A doença de Alzheimer, conforme discutido na introdução desta tese, é uma doença neurodegenerativa que evolui de forma progressiva e irreversível, levando o paciente a um estado demencial caracterizado por severa perda de memória e grave comprometimento das demais funções cognitivas, a incluir aprendizado, fala e comportamento (Suh and Checler, 2002; De-Paula et al., 2012).

O tratamento atual da doença de Alzheimer, preconizado pelos protocolos clínicos, é ainda muito limitado, restringindo-se a fármacos anticolinesterásicos ou inibidores dos receptores NMDA (memantina), cujo efeito sobre o estado cognitivo é praticamente paliativo (Jackson, 2014; Schneider et al., 2014). O desenvolvimento de novos fármacos, idealmente dotados da propriedade de modificar a evolução da patologia, esbarra na dificuldade de se obter um diagnóstico precoce (prévio ao estabelecimento do dano cognitivo) e, principalmente, de se definir um alvo terapêutico que seja efetivo em prevenir o desenvolvimento da doença, ou frear a evolução da patologia já em curso, restaurando o estado cognitivo do paciente ou prevenindo o avanço do processo neurodegenerativo (Citron, 2010; Blennow et al., 2014; Zimmer et al., 2014-a; 2014-b).

Desta necessidade nasce o incessante interesse em desvendar os mecanismos neuroquímicos associados ao desenvolvimento e à progressão da doença de Alzheimer. O peptídeo Aβ, considerado o principal produto neurotóxico da cascata amiloide, é amplamente explorado em modelos experimentais para o estudo da DA e, embora não seja o único responsável pelo desenvolvimento do estado demencial associado a esta patologia, apresenta propriedades e características que o destacam como um forte candidato a alvo terapêutico no desenvolvimento de novas drogas (Crews and Masliah, 2010; Lannfelt et al., 2014).

Conforme previamente discutido, diversos mecanismos de toxicidade têm sido propostos ao Aβ, e uma importante característica que o destaca no desenvolvimento da DA é a capacidade de estimular sua própria produção, através do favorecimento do processamento amiloidogênico da proteína precursora amiloide (APP). Cria-se, desta forma, um ciclo vicioso, em que o peptídeo formado, e depositado na forma de placa ou polimerizado na forma de oligômeros solúveis, estimula sua própria produção e deposição, aumentando a extensão do dano neurológico gerado (Peters et al., 2009; Yan

and Vassar, 2014). O desenvolvimento de drogas que sejam, ao mesmo tempo, efetivas em prevenir a toxicidade do peptídeo já gerado e de frear a ativação da cascata amiloide, depende da identificação de um mecanismo comum que sirva como alvo terapêutico. Evidências sugerem que a interação do peptídeo com as membranas neurais seja um evento chave tanto à toxicidade induzida pelo peptídeo quanto para o desencadear do ciclo patológico característico da doença (Peters et al., 2009; Lai and McLaurin, 2010). Desta forma, o bloqueio da interação do peptídeo com as membranas neurais, ou a prevenção das alterações na arquitetura das membranas induzida por esta interação, representa uma propriedade interessante a ser analisada ao se avaliar uma droga neuroprotetora (Matsuoka et al., 2003).

Dentre as inúmeras drogas neuroprotetoras propostas em modelos de DA, o gangliosídio GM1 vem assumindo crescente importância (Svennerholm et al., 2002; Kreutz et al., 2011; Yang et al., 2013). Como componente endógeno das membranas neurais, ou como droga exogenamente administrada, o GM1 desempenha funções neuroprotetoras e neurotróficas pelos mais variados mecanismos (Fong et al., 1995; Mutoh et al., 1995; Cunha et al., 1999; Ariga and Yu, 1999; Bachis et al., 2002; Duchemin et al., 2002; Rabin et al., 2002; Fighera et al., 2004; Mocchetti, 2005; Gavella et al., 2007; Sokolova et al., 2007; Ohmi et al., 2009; Lim et al., 2011; Ohmi et al., 2011).

A fim de investigar a ação neuroprotetora do GM1, o artigo I desta tese avaliou o efeito de sua administração icv (0,30mg/kg) sobre o dano cognitivo induzido pela injeção (icv) do peptídeo Aβ1-42 fibrilado (2nmol) em ratos Wistar machos e adultos. Trinta dias após a cirurgia estereotáxica, o estado cognitivo dos animais foi avaliado pelo teste comportamental de reconhecimento de objetos. Conforme mostraram os resultados, a administração de GM1 preveniu substancialmente o déficit cognitivo induzido pelo peptídeo, tanto no que se refere à memória de curto prazo quanto à memória de longo prazo. Estes dados estão de acordo com outros trabalhos que, em modelos *in vitro* ou *in vivo*, demonstraram efeito neuroprotetor da administração deste gangliosídio frente à toxicidade de outros fragmentos do peptídeo β-amiloide (Kreutz et al., 2011; Yang et al., 2013).

Considerando os dados da literatura que indicam alteração na atividade das enzimas Na^+,K^+-ATPase e AChE como eventos a mediarem o dano cognitivo associado a DA (Pákáski and Kálmán, 2008; Zhang et al., 2013), investigamos o efeito da injeção

de Aβ e do tratamento com GM1 sobre a atividade destas enzimas. Como resultado, observamos que peptídeo β-amiloide promoveu redução na atividade da Na^+,K^+-ATPase e aumento na atividade da AChE no hipocampo e no córtex cerebral, respectivamente. O tratamento com GM1, por sua vez, foi capaz de prevenir parcialmente a redução na atividade da Na^+,K^+-ATPase, sem contudo, interferir no efeito do peptídeo sobre a atividade da AChE.

Considerando a importância da Na^+,K^+-ATPase na regulação da transmissão sináptica e nos mecanismos de neuroplasticidade e formação de memória (Hernandez, 1992; Zhang et al., 2013), bem como os indícios já relatados na literatura quanto à importância da redução na atividade desta enzima no desenvolvimento do dano cognitivo associado a DA (Mark et al., 1995; Chauhan et al., 1997; Kairane et al., 2002; Zhang et al., 2013), os resultados aqui obtidos reforçam a ideia de que o GM1 apresente ação neuroprotetora em modelos de DA, quando administrado exogenamente.

Embora a ativação da AChE, observada no córtex cerebral em resposta ao peptídeo β-amiloide, não tenha sido revertida ou prevenida pelo tratamento com GM1, não descartamos a participação deste gangliosídio na regulação da atividade colinérgica, visto que outros trabalhos sugerem o efeito deste sobre a regulação da atividade da colina-acetil transferase, enzima envolvida na síntese de acetilcolina (Hadjiconstantinou and Neff, 1998).

Como forma de propor um mecanismo para o efeito do peptídeo e do GM1 sobre a atividade da Na^+,K^+-ATPase, avaliamos a capacidade antioxidante, por meio da técnica de TRAP, em córtex e hipocampo. Como resultado, observamos que o peptídeo reduziu a capacidade antioxidante, especificamente no hipocampo, e que o GM1 promoveu aumento neste parâmetro, prevenindo ou revertendo o efeito do Aβ sobre as defesas antioxidantes. Como a literatura propõe que a Na^+,K^+-ATPase seja afetada em sua atividade pelo estresse oxidativo (Hieber et al., 1991), é possível sugerir que o efeito do GM1 sobre a atividade desta enzima se dê por modulação das respostas antioxidantes.

Como previamente discutido, grande parte dos efeitos neurotóxicos induzidos pelo peptídeo são dependentes de sua interação com as membranas neurais e talvez decorrentes de alterações na estrutura ou arquitetura das mesmas. Desta forma, o Artigo II desta tese avaliou o efeito do peptídeo β-amiloide e da administração conjunta de GM1 sobre a integridade e a composição dos *rafts* de membrana.

Como há diferentes técnicas para extração e purificação de *rafts*, envolvendo basicamente o uso ou não de detergentes não-iônicos no processo extrativo, e considerando que os diferentes métodos de extração e purificação podem apresentar diferenças importantes em termos de efetividade, a depender do modelo e das condições a serem empregados (London and Brown, 2000; Allen et al., 2007; Persaud-Sawin et al., 2009), iniciamos esta etapa do estudo comparando e avaliando a eficiência das técnicas de extração de *rafts* com ou sem o emprego do detergente Triton X-100, seguidas da separação e purificação dos domínios *rafts* por meio de um gradiente de sacarose e fracionamento em 14 frações. Para análise da eficiência extrativa, avaliou-se em cada fração obtida do gradiente, os principais parâmetros utilizados para identificação dos domínios *rafts*: alto conteúdo de colesterol e baixo conteúdo de proteínas, presença da proteína marcadora de *raft* (flotilina) e ausência ou menor conteúdo relativo da proteína de domínio não-*raft* (clatrina), bem como maior concentração do gangliosídio GM1 (London and Brown, 2000; Macdonald and Pike, 2005; Allen et al., 2007).

Observamos, como resultado, que no presente modelo, a técnica com utilização de detergente Triton X-100 foi superior àquela sem emprego do detergente na extração e purificação dos microdomínios *rafts* de membrana, como evidenciado pela obtenção frações com maior concentração de colesterol e de flotilina na porção superior do gradiente (menor densidade). A distribuição do gangliosídio GM1, no entanto, não se mostrou um bom parâmetro, visto que não permitiu identificar diferenças significativas entre as duas metodologias.

Uma vez tendo sido eleita a técnica com emprego de Triton X-100 para extração e purificação de *rafts*, partiu-se para a análise do efeito da administração icv de Aβ e do tratamento conjunto com GM1 (icv) sobre a integridade dos microdomínios de membrana obtidos a partir de córtex cerebral e hipocampo dos ratos. Para análise da integridade dos *rafts*, os mesmos parâmetros de avaliação dos microdomínios foram avaliados, com exceção da distribuição de GM1 – por não ter se mostrado um bom marcador neste modelo. Conforme mostraram os resultados, a injeção icv do peptídeo Aβ1-42 fibrilado promoveu, tanto em córtex quanto em hipocampo, um desmonte parcial dos *rafts*, evidenciado pela dispersão na distribuição de colesterol e de flotilina ao longo do gradiente. Este efeito, no entanto, foi prevenido pela administração conjunta de GM1 (0,30mg/kg).

A fim de confirmar se os achados bioquímicos aqui observados são realmente indicativos de um desmonte parcial dos microdomínios de membrana, comparamos os mesmos com o efeito obtido pela incubação de fatias de córtex cerebral com β-metil-ciclodextrina (βMCD), uma droga capaz de desestabilizar e desmontar os *rafts* lipídicos ao remover o colesterol das membranas (Riddell et al., 2001; Allen et al., 2007). Duas concentrações de βMCD foram testadas (20 mM e 50 mM): a primeira promoveu uma dispersão nos marcadores de *raft* (colesterol e flotilina) ao longo do gradiente (de forma semelhante aos resultados obtidos com o peptídeo Aβ), enquanto que a maior concentração promoveu um deslocamento completo destes marcadores das frações superiores (*rafts*) às frações inferiores do gradiente. A fim de confirmar estes resultados, realizamos a dosagem do conteúdo total de colesterol das membranas expostas à βMCD, e observamos que apenas na concentração superior desta droga obteve-se uma redução significativa (31,5%) no conteúdo de colesterol. Estes achados nos permitem concluir que apenas na concentração de 50 mM de βMCD houve um desmonte completo dos *rafts* lipídicos, e que na concentração de 20 mM desta droga o desmonte dos *rafts* (evidenciado pela dispersão dos marcadores) não foi completo, mas sim parcial, visto que nas frações superiores (correspondentes aos *rafts*) ainda foi possível observar um *pool* de colesterol e de flotilina ali concentrado.

O desmonte parcial dos *rafts* induzido pelo peptídeo β-amiloide, observado no presente modelo, é condizente com dados prévios da literatura que apontam o efeito deste peptídeo em reduzir a fluidez das membranas neurais (Sasahara et al., 2013) e afetar a dinâmica dos microdomínios de membrana (Calamai and Pavone, 2013; Hirai et al., 2013). Confirmando, ainda, nossos resultados, Ledesma e colaboradores (2003) observaram desmonte dos *rafts* lipídicos em córtex cerebral de pacientes humanos com doença de Alzheimer (análise *post mortem*).

Nossos dados, no entanto, demonstraram, pela primeira vez, o efeito da forma fibrilada do peptídeo Aβ1-42 sobre a integridade dos microdomínios e o efeito protetor do GM1 sobre este parâmetro.

Considerando as diversas funções exercidas pelos microdomínios de membranas (Tsui-Pierchala et al., 2002; Allen et al., 2007; Sebastiao et al., 2013; Head et al., 2014), as alterações na arquitetura das membranas neurais aqui observadas podem mediar o dano neural e cognitivo desencadeado pelo peptídeo β-amiloide, bem como o efeito neuroprotetor do gangliosídio GM1.

Como previamente comentado, a distribuição das proteínas APP e BACE1 nos diferentes domínios de membrana parece modular ou regular a cascata amiloide (Vetrivel and Thinakaran, 2010). A BACE1 apresenta maior atividade quando inserida nos microdomínios de membrana (Cordy et al., 2003; Kalvodova et al., 2005), e promove a clivagem da APP mais eficientemente quando co-localizada com a mesma nos *rafts* (von Arnim et al., 2008; Vetrivel and Thinakaran, 2010). Desta forma, o pool de APP presente nos *rafts* seria encaminhado ao processamento amiloidogênico – gerando o peptídeo Aβ –, ao passo que o pool presente no domínio fosfolipídico seria processado pela via não-amiloidogênica. Em condições normais, considera-se que a maior parte do pool de APP, presente nas membranas, esteja distribuído no domínio fosfolipídico, e que sua migração aos *rafts* seja um fator facilitador da amiloidogênese (Ehehalt et al., 2003; Bhattacharyya et al., 2013).

Considerando que os dados aqui apresentados indicam o efeito do Aβ em alterar a arquiteturas dos *rafts*, o próximo passo a ser analisado foi o efeito do peptídeo Aβ e tratamento conjunto com GM1 sobre a distribuição das proteínas amiloidogênicas nos diferentes domínios de membrana.

Como a utilização de detergente pode, em teoria, alterar a distribuição de determinadas proteínas nas diferentes frações de membrana, gerando falsos resultados quanto a sua distribuição nos domínios *rafts* (Munro, 2003), iniciamos esta fase do estudo comparando a distribuição de APP e BACE1 nas diferentes frações de membrana obtidas a partir das técnicas de extração de *rafts* com e sem detergente. Como evidenciaram os resultados, ambas as metodologias permitiram a obtenção de uma fração de APP e de BACE1 nos *rafts*, o que vem a demonstrar que a localização das mesmas nos microdomínios de membrana não é artefato da técnica empregada. A fim de reforçar esta constatação, avaliamos o efeito do desmonte dos *rafts*, induzido por βMCD, na distribuição das proteínas amiloidogênicas. Como evidenciaram os resultados, a extração de colesterol das membranas e o consequente desmonte dos microdomínios, promoveram a redistribuição de APP e BACE1 para as frações não *raft* do gradiente, o que corrobora com a ideia de que estas proteínas sejam, pelo menos em parte, associadas aos microdomínios de membrana.

Demonstrada a localização de APP e BACE1 nos *rafts*, investigamos o efeito do peptídeo e do tratamento com GM1 sobre a distribuição destas proteínas

amiloidogênicas nos diferentes domínios de membrana. Como demonstraram os resultados, o Aβ promoveu redistribuição de APP e BACE1 para os *rafts* que se mantiveram intactos, aumentando a associação destas proteínas nos microdomínios de membrana, especificamente em córtex cerebral.

Estes resultados sugerem que o efeito do peptídeo em favorecer o processamento amiloidogênico da APP, conforme sugerido por Peters e colaboradores (2009), seja mediado por alterações na arquitetura das membranas neurais e redistribuição das proteínas amiloidogênicas aos *rafts* lipídicos, onde encontrariam o ambiente adequado a sua interação.

O tratamento com GM1, por sua vez, preveniu a redistribuição de APP e BACE1 aos *rafts* lipídicos, sugerindo que este gangliosídio poderia, além de seu efeito neuroprotetor, frear a ativação da amiloidogênese induzida pelo peptídeo Aβ, o que, uma vez confirmado, representaria um enorme avanço na identificação de drogas capazes de alterar a evolução da DA.

Os mecanismos envolvidos no efeito do Aβ e do tratamento com GM1 sobre a arquitetura das membranas neurais não foram avaliados. A constatação de que os níveis de colesterol e de gangliosídios na membrana não foram significativamente alterados como efeito do tratamento com o Aβ descarta a possibilidade de que os efeitos aqui observados na integridade dos microdomínios sejam decorrentes de uma ruptura das membranas neurais. Por outro lado, dados sugerem que o peptídeo, ao interagir com os *rafts*, induziria dano oxidativo às membranas neurais, dirigido especificamente aos microdomínios de membrana (Zampagni et al., 2010), de modo que a propriedade do GM1 de aumentar as defesas antioxidantes – conforme demonstrado no Artigo I desta tese – poderia desempenhar um papel importante na manutenção da integridade das membranas neurais. O efeito do peptídeo em alterar a distribuição das proteínas amiloidogênicas nos diferentes domínios de membrana, bem como a propriedade do GM1 de prevenir este efeito, também podem ser mediados por alterações no estado redox, visto que, segundo Tan e colaboradores (2013), a distribuição de BACE1 nos microdomínios de membrana seria alterada em resposta a estresse oxidativo, aumentando sua localização nos *rafts* e favorecendo o processamento amiloidogênico da APP.

Os danos oxidativos e a própria ativação da amiloidogênese, induzidos pelo Aβ, parecem ser dependentes da interação deste peptídeo com componentes lipídicos

constituintes dos *rafts* (Zampagni et al., 2010; Peters et al., 2009). Considerando que o GM1 é reportado como o principal componente dos *rafts* a mediar a interação com o Aβ (Yahi and Fantini, 2014), é possível que os efeitos neuroprotetores observados nesta tese sejam decorrentes da capacidade de o GM1 (exogenamente administrado) interagir com o peptídeo, bloqueando seus sítios de ligação à membrana biológica, e prevenindo, desta forma, o desencadear do dano neurológico e das alterações de membrana aqui observados.

Desta forma, o artigo III desta tese teve como principal objetivo avaliar o efeito da interação Aβ-$GM1_{exógeno}$ sobre a toxicidade induzida pelo peptídeo, utilizando para isto, de um modelo de toxicidade do peptídeo Aβ em cultura de células de neuroblastoma humano (SH-SY5Y).

Para tanto, iniciamos esta fase do estudo avaliando a toxicidade de diferentes concentrações do peptídeo Aβ (0,25, 0,5, 1 e 2 μM) e a potencial neuroproteção induzida por distintas concentrações de GM1 (10, 20 e 30 μM), administradas exogenamente às culturas. Como demonstraram nosso resultados, o peptídeo induziu morte celular (apoptose e necrose) de forma concentração-dependente, e o GM1, quando co-administrado às culturas, promoveu neuroproteção nas três concentrações testadas, prevenindo a morte neural (apoptose e necrose) induzida pelo peptídeo.

Para avaliar se o efeito neuroprotetor do GM1, neste modelo *in vitro* de toxicidade do Aβ, seria mediado por sua capacidade de interagir com o peptídeo e bloquear sua interação com as membranas neurais, servindo como forma de sequestrar o peptídeo Aβ, avaliamos o efeito da complexação *in vitro* Aβ-GM1, confirmada pelo experimento de co-sedimentação e dot-blot, sobre a capacidade de o peptídeo induzir morte celular.

Como observado em nossos resultados, a complexação Aβ-GM1 preveniu a posterior toxicidade do peptídeo as células SH-SY5Y.

Estes resultados estão de acordo com dados prévios da literatura que demonstram a propriedade de lipossomas contendo GM1 em reduzir a toxicidade do peptídeo em cultura células (Bereczki et al 2011; Kouyoumdjian et al., 2013), bem como de reduzir os níveis de Aβ em cérebro de animais transgênicos (Yuyama et al., 2014), reforçando a ideia de que o gangliosídio GM1 poderia, enquanto droga exógena, servir como forma de quelar ou sequestrar o peptídeo β-amiloide, prevenindo os efeitos decorrentes de sua interação com as membranas neurais (Matsuoka et al., 2003).

Embora os dados aqui obtidos, em conjunto com evidências já reportadas na literatura, sugiram um importante papel neuroprotetor do gangliosídio GM1, sendo capaz de reduzir ou bloquear a toxicidade do peptídeo depositado na forma de fibrilas e de possivelmente prevenir as alterações na arquitetura de membrana que levariam ao estímulo da cascata amiloide, o emprego clínico do GM1 como alternativa terapêutica em pacientes humanos esbarra em dois pontos importantes: sua baixa biodisponibilidade central (Orlando et al., 1979; Ghidoni et al., 1989) e o suposto efeito imunogênico atribuído a este gangliosídio (Kaida et al., 2009; Schneider et al., 2013). Tais limitações, no entanto, poderiam ser contornadas pela tecnologia farmacêutica (Maysinger et al., 1989) e/ou pelo emprego de análogos sintéticos com maior biodisponibilidade e menor risco de desencadear resposta autoimune (Wells et al., 1996; Wu et al., 2005; Biraboneye et al., 2009).

5. CONCLUSÕES

Esta tese investigou e avaliou a atividade neuroprotetora do gangliosídio GM1 frente à toxicidade induzida pelo peptídeo Aβ1-42 fibrilado, utilizando-se de dois modelos experimentais: modelo *in vivo*, com utilização de ratos machos adultos (Wistar) injetados (icv) com o peptídeo e/ou com GM1; e modelo *in vitro*, com utilização de linhagem de neuroblastoma humano (SH-SY5Y) expostas ao peptídeo.

Os resultados aqui observados permitem concluir que o GM1, quando administrado exogenamente, apresenta importante atividade neuroprotetora, conforme ficou evidenciado pelos seguintes resultados:

- Prevenção ou redução do déficit cognitivo induzido pelo peptídeo Aβ1-42 fibrilado em ratos machos adultos (Wistar);
- Prevenção parcial do efeito do Aβ sobre a atividade da enzima Na^+,K^+-ATPase em hipocampo;
- Aumento das defesas antioxidantes não enzimáticas, e redução do efeito do Aβ sobre este parâmetro, conforme demonstrado pela técnica de TRAP, em córtex e hipocampo dos animais;
- Preservação da arquitetura das membranas neurais, conforme evidenciado pelo efeito do gangliosídio em prevenir o desmonte parcial dos *rafts* lipídicos induzido pelo Aβ, tanto em córtex cerebral quanto em hipocampo;
- Prevenção da morte celular (necrose e apoptose) induzida pelo peptídeo Aβ1-42 fibrilado em modelo de cultura de células SH-SY5Y.

Além disso, os resultados apresentados nesta tese sugerem que as alterações na arquitetura das membranas neurais, além de terem possível participação nos danos neurológicos associados ao peptídeo β-amiloide, estariam envolvidas no efeito do peptídeo em ativar a cascata amiloide, ao promover a redistribuição das proteínas amiloidogênicas aos microdomínios de membrana. Como a administração de GM1 preveniu o efeito do peptídeo sobre a distribuição de APP e de BACE1 nos *rafts*, é possível propor que este gangliosídio teria um importante efeito sobre o ciclo patológico Aβ-amiloidogênese, interferindo, desta forma, em mecanismos envolvidos na progressão da DA (Figura 8).

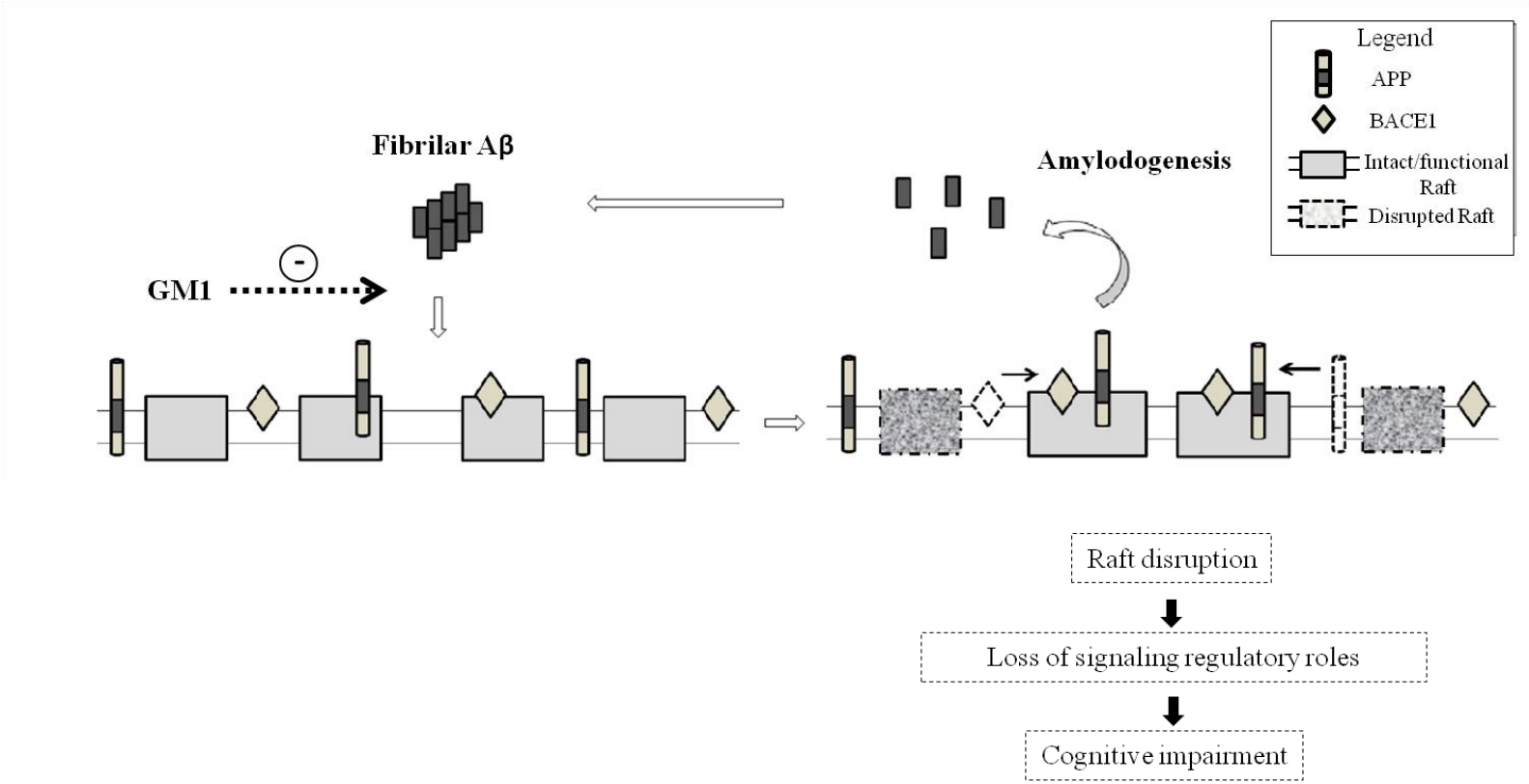


Figura 8: Mecanismo proposto para a toxicidade do peptídeo Aβ e neuroproteção com GM1, envolvendo alterações na arquitetura das membranas neurais. O peptídeo fibrilado causa desmonte parcial dos *rafts* lipídicos e a redistribuição da APP e BACE1 nos microdomínios que restam intactos. A co-localização de APP e BACE1 nos *rafts* favorece sua interação e a amiloidogênese, aumentando a deposição de Aβ. O ciclo patológico formado desencadeia aumento do dano à arquitetura das membranas neurais, com desmonte dos *rafts,* a consequente perda do papel regulatório dos mesmos sobre as mais diversas vias sinalizatórias, e, finalmente, a perda cognitiva. O tratamento com GM1 previne os efeitos do Aβ sobre as membranas neurais, preservando a integridade dos *rafts* e prevenindo o aumento na distribuição das proteínas amiloidogênicas aos microdomínios de membrana. Ao GM1 exogenamente administrado é proposto, aqui, o papel de frear o ciclo patológico Aβ-amiloidogênese.

E por fim, conforme evidenciado pelo estudo com células SH-SY5Y, a atividade neuroprotetora do GM1 parece ser mediada por sua capacidade de interagir e quelar ou sequestrar o Aβ extracelular, prevenindo, possivelmente, sua interação com as membranas biológicas (Figura 9).

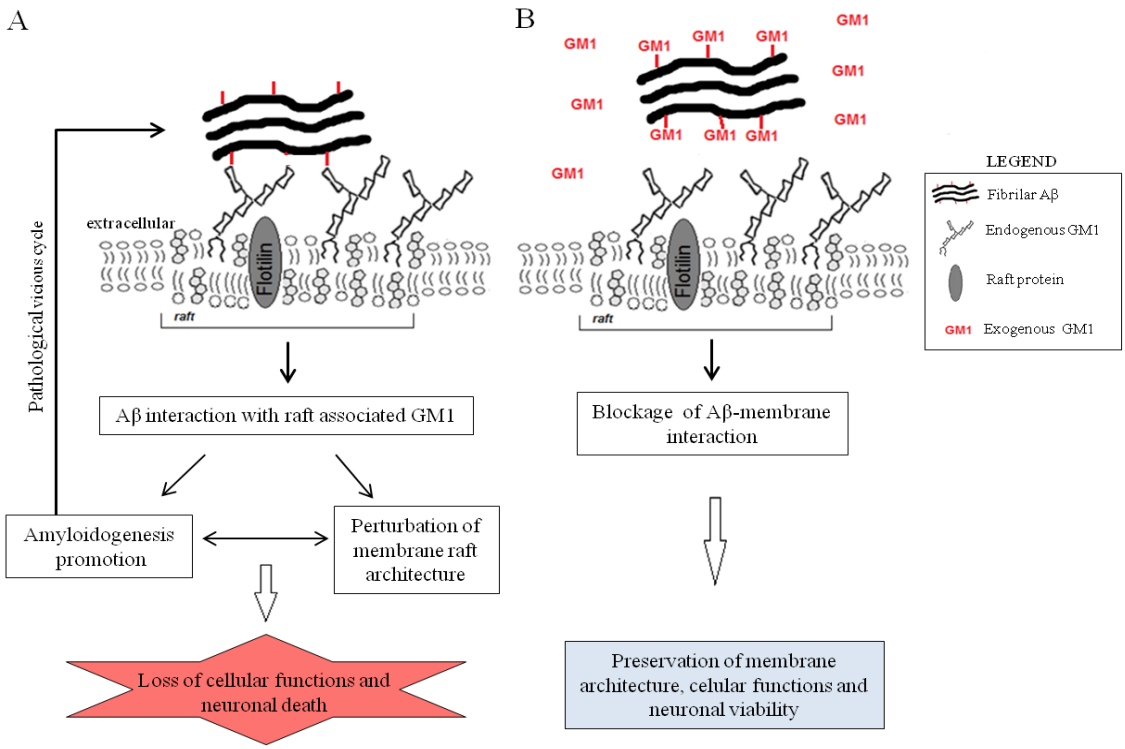


Figura 9. Mecanismo proposto de neuroproteção do GM1 através da ligação e sequestro do peptídeo Aβ. (A) O Aβ interage com as membranas neurais, especificamente com o GM1 associado aos *rafts*. Esta interação perturba a arquitetura das membranas, gerando alterações na integridade e na dinâmica dos *rafts* lipídicos, e acarretando em perda de funções celulares que levam a morte neural. Como consequência da interação Aβ-membrana e da alteração na dinâmica dos *rafts*, o peptídeo induz sua própria produção ao favorecer a amiloidogênese (ciclo vicioso). (B) O GM1, administrado exogenamente, interage com o Aβ e bloqueia a ligação do peptídeo às membranas neurais. Ao prevenir a interação do Aβ às membranas, o GM1 preserva a arquitetura das membranas e a estrutura dos *rafts*, evitando o desencadeamento de morte celular bem como a ativação do ciclo patológico Aβ-amiloidogênese.

Mais estudos são necessários, no entanto, para melhor avaliar o potencial do gangliosídio GM1 enquanto alternativa terapêutica ao tratamento da DA.

6. PERSPECTIVAS

As principais perspectivas de estudo que surgem ao término desta tese são:

- Avaliar o efeito do Aβ1-42 e do tratamento com GM1 sobre a integridade e dinâmica dos *rafts* lipídicos de células SH-SY5Y, utilizando de método de FRET com a marcação dos microdomínios através de anticorpo anti-flotilina1 e anti-GM1.

- Investigar o efeito do peptídeo e do tratamento com GM1, também em células SH-SY5Y, sobre a co-localização das proteínas APP e BACE1 com os marcadores de *rafts* (técnica de FRET).

- Investigar o efeito do peptídeo e do tratamento com GM1 sobre a atividade das enzimas BACE1 e ADAM10, em células SH-SY5Y expostas ao peptídeo Aβ1-42, como forma de investigar seu real efeito sobre a cascata amiloide.

- Avaliar o efeito do GM1 sobre o aumento na fosforilação da proteína tau induzida pelo peptídeo Aβ em células SH-SY5Y, como forma de reforçar as evidências de que o gangliosídio interferiria nos mecanismos associados à progressão da DA.

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