Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós Graduação de Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Ovariectomia altera parâmetros comportamentais e neuroquímicos em hipocampo de ratas Wistar adultas: papel da suplementação com vitamina D

Cassiana Siebert

Orientadora: Profª Drª Angela Terezinha de Souza Wyse

Porto Alegre

2018

Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós Graduação de Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Ovariectomia altera parâmetros comportamentais e neuroquímicos em hipocampo de ratas Wistar adultas: papel da suplementação com vitamina D

Cassiana Siebert

Orientadora: Profª Drª Angela Terezinha de Souza Wyse

Tese apresentada ao Programa de Pós Graduação em Ciências Biológicas: Bioquímica, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas: Bioquímica

Porto Alegre, 2018.

CIP - Catalogação na Publicação

Siebert, Cassiana
Ovariectomia altera parâmetros comportamentais e neuroquímicos em hipocampo de ratas Wistar adultas: papel da suplementação com vitamina D / Cassiana Siebert. -- 2018.
136 f.
Orientadora: Angela T.S. Wyse.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2018.

1. Bioquímica. 2. Ovariectomia. 3. Vitamina D. 4. Menopausa experimental. 5. Neuroquímica. I. Wyse, Angela T.S., orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Que no fundo é simples ser feliz
Difícil é ser tão simples
(Simplicidade - O Teatro Mágico)

Dedico este trabalho às pessoas mais importantes da minha vida:
minha família.
Aos meus pais, Dianez e Celso,
Aos meus irmãos, Rochele e Tiago.

AGRADECIMENTOS

Agradeço à minha orientadora, Prof[a] Dr[a] Angela Wyse, pela oportunidade de fazer parte de seu maravilhoso grupo, pela orientação, pelos ensinamentos, pela confiança, paciência e alegria do convívio diário.

Aos colegas e amigos do laboratório 36, aos que fizeram parte e aos que fazem atualmente o laboratório um ambiente agradável e o convívio alegre. Obrigada a todos pelo auxílio, pela amizade e conhecimento compartilhado.

Agradeço em especial ao Tiago Marcon, amigo/irmão que a pós-graduação me deu. A Jana, pelos ensinamentos, parceria diária e auxílio na realização desse trabalho.

À minha família pela paciência, pelo amor, pelo apoio e incentivo em todos os momentos. Agradeço em especial, meus pais Dianez e Celso, e aos meus irmãos Rochele e Tiago, obrigada pelo amor incondicional.

À Re pelo companheirismo, cumplicidade e paciência.

À Mari Parisi pela amizade, conselhos, conversas e cumplicidade diária que tornaram tudo mais leve e feliz.

Aos amigos que Porto Alegre me deu.

Aos professores e alunos colaboradores que auxiliaram em experimentos que fazem parte dessa tese.

À Universidade Federal do Rio Grande do Sul, ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, aos funcionários da secretaria do nosso PPG e a todos os professores que contribuíram para a minha formação.

À CAPES e ao CNPq, pelo apoio financeiro.

Àqueles que de alguma forma ou de outra estiveram ao meu lado durante a realização desse trabalho.

À TODOS, muito obrigada.

SUMÁRIO

PARTE I

RESUMO

A privação estrogênica característica da menopausa pode estar relacionada com efeitos negativos para a saúde da mulher, bem como ao aumento do risco de desenvolvimento de doenças neurodegenerativas. A terapia normalmente utilizada para a menopausa é a reposição hormonal, porém efeitos adversos desta terapia são relatados. Portanto, há uma crescente busca por terapias complementares com a finalidade de minimizar os efeitos da menopausa. Neste sentido a vitamina D vem sendo proposta como possível protetora. Alterações comportamentais e bioquímicas (metabolismo energético e em importantes enzimas como acetilcolinesterase e Na^+,K^+-ATPase) já foram encontradas em estruturas cerebrais de ratas adultas submetidas à ovariectomia, um modelo de menopausa cirúrgica amplamente utilizado para mimetizar as alterações pós-menopausa. Baseado nisso, o objetivo do presente estudo foi investigar parâmetros comportamentais e bioquímicos (sistema fosforilante citoesqueletal, parâmetros inflamatórios, sistema colinérgico, de estresse oxidativo, dano a biomoléculas e comprimento de telômeros) em hipocampo de ratas adultas submetidas à ovariectomia. Níveis séricos de estradiol e calcidiol foram dosados. O papel da suplementação com vitamina D também foi investigado. A ovariectomia foi realizada em ratas Wistar adultas com 90 ou 180 dias. Trinta dias após o procedimento cirúrgico, suplementação com vitamina D na dose de 500 UI/Kg/dia por um período de 30 dias foi realizada. Resultados mostraram que a ovariectomia em ratas adultas com 90 dias provocou prejuízo na memória de reconhecimento de curto (teste 1 h após o treino) e longo prazo (teste 7 dias após o treino) na tarefa de reconhecimento de objetos, bem como na memória aversiva de longo prazo (teste 7 dias após o treino) na tarefa de esquiva inibitória. Além disso, a ovariectomia provocou um desequilíbrio no sistema fosforilante do citoesqueleto em hipocampo, evidenciado pela aumento na fosforilação da proteína glial fibrilar ácida e dos neurofilamentos de baixo peso molecular, médio peso molecular e alto peso molecular, sem alterar seus imunoconteúdos. A ovariectomia também causou aumento no imunoconteúdo da proteína cinase c-Jun-amino-terminal, da proteína cinase dependente de cálcio/calmodulina II e dos sítios repetições de aminoácidos lisina-serina-prolina, Ser55 e Ser57 fosforilados. A vitamina D reverteu os efeitos causados pela ovariectomia sobre o citoesqueleto, mas não reverteu os efeitos sobre a memória. Com relação aos parâmetros inflamatórios, a ovariectomia em ratas adultas com 90 dias causou aumento no imunoconteúdo de fosfo-NF-κb nuclear, bem como elevação dos níveis de TNF-α e interleucina-6, sem alterar interleucina 1-β. O sistema colinérgico também mostrou-se alterado através da ativação da enzima acetilcolinesterase, porém seu imunoconteúdo, e o imunoconteúdo da enzima colina acetiltransferase não apresentaram alterações. A suplementação com

vitamina D foi capaz de reverter parcialmente o aumento no fosfo-NF-κb e na interleucina-6. Os níveis de estradiol em soro das ratas submetidas à ovariectomia apresentaram-se diminuídos em relação aos controles e os níveis de calcidiol das ratas submetidas à suplementação com vitamina D apresentaram-se elevados em relação aos controles. Por fim, realizamos a ovariectomia em ratas adultas com 90 dias ou 180 dias e posterior suplementação com vitamina D. Resultados mostraram que a ovariectomia alterou a atividade de enzimas antioxidantes em ambas as idades, representada por aumento na atividade da catalase em ratas submetidas à ovariectomia com 90 dias e aumento na atividade da superóxido dismutase em ratas submetidas à ovariectomia com 180 dias. Não foram observados danos a lipídios de membrana e proteínas, porém foi observado dano ao DNA em ambas às idades. Os telômeros de ratas submetidas à ovariectomia com 180 dias apresentaram redução significativa. A suplementação com vitamina D foi capaz de reverter as alterações sobre o dano ao DNA e também no comprimento de telômeros. Juntos, nossos achados mostram alterações que podem estar presentes em mulheres na pós-menopausa e alguns efeitos benéficos da suplementação com vitamina D sobre o cérebro. Esperamos com nossos resultados auxiliar, pelo menos em parte, na compreensão da neurobiologia deste importante período na vida das mulheres, bem como abrir perspectivas para novos estudos envolvendo terapias auxiliares.

Palavras-chave: colecalciferol, hipocampo, modelo experimental de menopausa, ovariectomia, vitamina D.

ABSTRACT

The estrogen deprivation characteristic of menopause may be related to negative effects on women's health, as well as to the increased risk of developing neurodegenerative diseases. The therapy normally used for menopause is hormone replacement, however adverse effects of this therapy are reported. Therefore, there is a growing search for complementary therapies to minimize the effects of menopause. In this sense vitamin D has been proposed as a possible protective. Behavioral and biochemical changes (energy metabolism and important enzymes such as acetylcholinesterase and Na^+, K^+-ATPase) have already been found in brain structures of adult rats subjected to ovariectomy, a model of surgical menopause widely used to mimic postmenopausal changes. Based on this, the objective of the present study was to investigate behavioral and biochemical parameters (cytoskeletal phosphorylating system, inflammatory parameters, cholinergic system, oxidative stress, biomolecule damage and telomer length) in hippocampus of adult rats submitted to ovariectomy. Serum levels of estradiol and calcidiol were measured. The role of vitamin D supplementation has also been investigated. Ovariectomy was performed in adult Wistar rats at 90 or 180 days. Thirty days after the surgical procedure, vitamin D supplementation at a dose of 500 IU/kg/ day for a period of 30 days was performed. Results showed that ovariectomy in 90 day-old adult rats resulted in short-term (1 h post-training) and long-term (7-day post-training) memory impairment in the object recognition task, as well as in long-term aversive memory (7 days post-training) in the task of inhibitory avoidance. In addition, ovariectomy caused an imbalance in the phosphorylating system of the cytoskeleton in the hippocampus, evidenced by the increase in the phosphorylation of the glial fibrillary acidic protein and of the neurofilaments of low molecular weight, medium molecular weight and high molecular weight, without altering their immunocontents. Ovariectomy also caused an increase in the immunocontent of the c-Jun-amino-terminal protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II, and the lysine-serine-proline repeat, Ser55 and Ser57 phosphorylated amino acid sites. Vitamin D reversed the effects of ovariectomy on the cytoskeleton, but did not reverse the effects on memory. With regard to inflammatory parameters, ovariectomy in 90-day-old adult rats caused an increase in the immunocontent of nuclear phospho-NF-κb, as well as elevated levels of TNF-α and interleukin-6, without altering interleukin 1-β. The cholinergic system was also altered by the activation of the enzyme acetylcholinesterase, but its immunocontent and the immunocontent of the enzyme choline acetyltransferase did not present alterations. Vitamin D supplementation was able to partially reverse the increase in phospho-NF-κb and interleukin-6. Serum estradiol levels in

ovariectomized rats were decreased compared to controls and calcidiol levels of rats submitted to vitamin D supplementation were higher than controls. Finally, we performed ovariectomy in adult rats at 90 or 180 days old and subsequent vitamin D supplementation. Our results showed that ovariectomy altered the activity of antioxidant enzymes at both ages, represented by increased catalase activity in rats submitted to ovariectomy at 90 days and increase in superoxide dismutase activity in rats submitted to ovariectomy at 180 days. No damage to membrane lipids and proteins was observed, but DNA damage was observed in both ages. The telomeres of rats submitted to ovariectomy at 180 days old presented a significant reduction. Vitamin D supplementation was able to reverse changes on DNA damage and also on telomere length. Together, our findings show changes that may be present in postmenopausal women and some beneficial effects of vitamin D supplementation on the brain. We hope, with our findings, to assist in the understanding and knowledge of the neurobiology of this important period of women's lives, as well as open up perspectives for future research involving adjunct therapies.

Keywords: cholecalciferol, experimental model of menopause, hippocampus, ovariectomy, vitamin D.

LISTA DE ABREVIATURAS

ACh	acetilcolina
AChE	acetilcolinesterase
ANOVA	análise de variância
CAT	catalase
DCFH	2',7'- diclorofluoresceína
DNA	ácido desoxirribonucléico
ERN	espécies reativas de nitrogênio
ERO	espécies reativas de oxigênio
FI	filamentos intermediários
FSH	hormônio folículo-estimulante
GFAP	proteína glial fibrilar ácida
GnRH	hormônio liberador de gonadotrofinas
GPX	glutationa peroxidase
GSH	glutationa reduzida
H_2O_2	peróxido de hidrogênio
IL-1β	interleucina 1-β
IL-6	interleucina 6
cJNK	preoteína cinase-Jun amino-terminal
KSP repeats	repetições Lisina-Serina-Prolina
LH	hormônio luteinizante
LPS	lipopolissacarídeo
MAPK	proteína cinase ativada por mitógeno
MF	microfilamentos
MP	microtúbulos
NF	neurofilamentos
NF-H	neurofilamentos de alto peso molecular
NF-L	neurofilamentos de baixo peso molecular
NF-M	neurofilamentos de médio peso molecular
$^1O_2$	oxigênio singlet
$O_2^{\bullet -}$	ânion superóxido

OCl^-	ânion hipoclorito
OH•	radical hidroxila
OVX	ovariectomia
PKA	proteína cinase A
PKC	proteína cinase C
PKCaMII	proteína cinase dependente de Ca^{2+}/calmodulina
SNC	sistema nervoso central
SOD	superóxido dismutase
TBARS	substâncias reativas ao ácido tiobarbitúrico
TNF-α	fator de necrose tumoral alfa
VDR	receptor de vitamina D
VIT D	vitamina D
VIT D+OVX	vitamina D + ovariectomia

LISTA DE FIGURAS

1.INTRODUÇÃO

1.1 Menopausa e estrógenos

A menopausa é uma importante fase na senescência reprodutiva feminina. Menopausa natural é definida como a interrupção permanente da ovulação e da menstruação durante um período de 12 meses (Kato et al., 1998; Soules et al., 2001; Takahashi e Johnson, 2015). Apesar de a menopausa ser um evento fisiológico na vida da mulher, muitas experienciam esta fase precocemente (entre os 40 e 45 anos) ou prematuramente (antes dos 40 anos), de forma espontânea ou induzida por intervenções médicas como a quimioterapia, a exposição à radiação ou após ovariectomia (OVX) bilateral (Grant et al., 2015; Rocca et al., 2010; Shuster et al., 2010).

Em mulheres com o ciclo reprodutivo normal, os ovários secretam compostos esteroides, como estrógenos e progesteronas, e compostos não esteroides como inibinas, relaxinas, ativinas e folistatinas. Essa liberação é regulada pelo eixo hipotálamo-hipófise-gonadal, através da secreção das gonadotrofinas hipofisárias: o hormônio luteinizante (LH) e o hormônio folículo-estimulante (FSH), os quais estão sobre o controle da secreção do hormônio liberador de gonadotrofinas (GnRH) que é produzido e liberado pelo hipotálamo (Messinis, 2006). Diversos fatores hormonais e neuro-endócrinos modulam positivamente e negativamente a secreção do GnRH, dentre estes, os mecanismos de retrocontrole positivo e negativo exercidos pelos compostos ovarianos e pelas gonadotrofinas presentes na circulação e que variam conforme o ciclo menstrual. O período de transição para a menopausa engloba

mudanças como a elevação do FSH, e nos ovários ocorre o esgotamento dos folículos ovarianos, consequentemente diminuindo os níveis de estrógenos, progesterona e de substâncias não esteroidais na circulação, abolindo este mecanismo de retrocontrole (Honour, 2017; Messinis, 2006).

Apesar de acontecerem alterações no padrão de secreção de diversos hormônios após a menopausa, a depleção de estrógenos é a mais amplamente investigada, devido às importantes funções exercidas por este hormônio. O organismo feminino secreta três tipos principais de estrógenos: estrona, estriol e estradiol (17β-estradiol, E2 ou estrógeno), sendo este último, o principal estrógeno circulante durante a idade reprodutiva da mulher (Rettberg et al., 2014). O estriol e a estrona são encontrados em baixos níveis e possuem uma menor atividade sobre os receptores de estrogênio (RE) (Gruber et al., 2002; Morissette et al., 2008). Os estrógenos atuam principalmente como hormônios sexuais, mas também circulam por todo o organismo exercendo efeitos em vários outros órgãos e sistemas, tais como o sistema nervoso central (SNC), cardiovascular, imune e ósseo (Arnal et al., 2010; Fiocchetti et al., 2012; Wise, 2002). As ações dos estrógenos são mediadas por seus REs que possuem duas classe gerais: os REs nucleares e REs associados à membrana, ambos encontrados no cérebro (Brinton, 2009; Mitra et al., 2003). No cérebro os estrógenos são conhecidos por exercer papel neuroprotetor (Fiocchetti et al., 2012; Petrovska et al., 2012; Wise, 2002), pois são capazes de promover plasticidade sináptica e excitabilidade neural (Brinton, 2009; McEwen, 2002), induzir sobrevivência neuronal, modular marcadores sinápticos associados à cognição (Gibbs e Gabor, 2003), além de possuirem propriedades

antioxidantes (Bellanti et al., 2013) e ainti-inflamatórias (Vegeto et al., 2008). Neste sentido, dados na literatura mostram que a privação estrogênica característica da menopausa pode estar relacionada com o aumento do risco de desenvolvimento de doenças neurodegenerativas em mulheres (Henderson, 2014; Mott e Pak, 2013).

A fim de investigar os mecanismos relacionados às mudanças observadas após a menopausa em mulheres, alguns modelos animais podem ser empregados. Dentre estes modelos destaca-se a OVX, um modelo cirúrgico de depleção hormonal ovariana que consiste na remoção bilateral dos ovários, causando uma redução drástica na secreção hormonal (Conn, 2006; Diaz Brinton, 2012). A OVX é um modelo bem consolidado e amplamente utilizado para mimetizar as alterações observadas no período pós-menopausa. Estudos anteriores do nosso grupo de pesquisa já demonstraram que a OVX causa importantes alterações bioquímicas e comportamentais (memória aversiva e espacial) em ratas Wistar adultas (Ben et al., 2009a; Mackedanz et al., 2011; Monteiro et al., 2008; Monteiro et al., 2005b; Siebert et al., 2014).

O tratamento normalmente utilizado por mulheres para amenizar os sinais e sintomas da menopausa é a terapia de reposição hormonal (TRH). Os benefícios da TRH sobre a memória e funções cognitivas têm sido relatados (Sherwin, 2003; Shoupe, 2011). Por outro lado, há indícios de que esta terapia também pode causar efeitos adversos como o aumento do risco de desenvolvimento de tumores e doenças cardiovasculares (Lobo, 2007; Miquel et al., 2006). Dessa forma, a procura por alternativas de tratamento para substituir ou complementar a TRH tem crescido nos últimos anos (Ben et al., 2010; Monteiro et al., 2005a; Siebert et al., 2014).

1.2 Vitamina D

Dentre as terapias utilizadas para combater as mudanças no estado redox e funções cognitivas relacionadas à idade, por exemplo, destacam-se os compostos dietéticos e/ou farmacológicos como antioxidantes e alguns hormônios. Neste sentido, a vitamina D (VIT D), considerada um hormônio esteróide, que tem como principal função regular a homeostase do cálcio, influenciando na formação e reabsorção óssea, vem ganhando espaço como potencial agente terapêutico (Briones e Darwish, 2012; Mpandzou et al., 2016).

A VIT D é encontrada em duas formas básicas: a VIT D_2 ou ergocalciferol, proveniente de alimentos de origem vegetal, e a VIT D_3 ou colecalciferol, que pode ser proveniente da síntese cutânea endógena após exposição à radiação ultravioleta B ou através de alimentos de origem animal. A VIT D_3 endógena é sintetizada na pele por ação da radiação ultravioleta B sobre o precursor 7-deidrocolesterol (pró-vitamina D_3), convertendo este em pré-vitamina D_3, uma substância termoinstável que sofre isomerização térmica, adquirindo uma configuração mais estável na forma de VIT D_3 (colecalciferol). Na circulação sanguínea, tanto a VIT D_3 endógena quanto a proveniente da dieta se liga a uma α_1-globulina chamada proteína ligante da vitamina D (DBP) e é então transportada para o fígado. No fígado, o colecalciferol sofre hidroxilação no carbono 25 através da ação da 25-hidroxilase (CYP2R1), produzindo a 25-hidroxivitamina D_3, também chamada de calcidiol, 25-hidroxicolecalciferol ou 25(OH)D_3. Após, o calcidiol ligado a DBP retorna a corrente sanguínea para ser transportado até o rim, onde é novamente hidroxilado em seu carbono 1 pela enzima 1α-hidroxilase (CYP27B1) gerando o

calcitriol (1α,25-dihidroxivitamina D_3 ou 1α,25$(OH)_2D_3$), a forma ativa da VIT D (Figura 1)(Ferrari et al., 2017; Mostafa e Hegazy, 2015; Mpandzou et al., 2016; Pike et al., 2009).

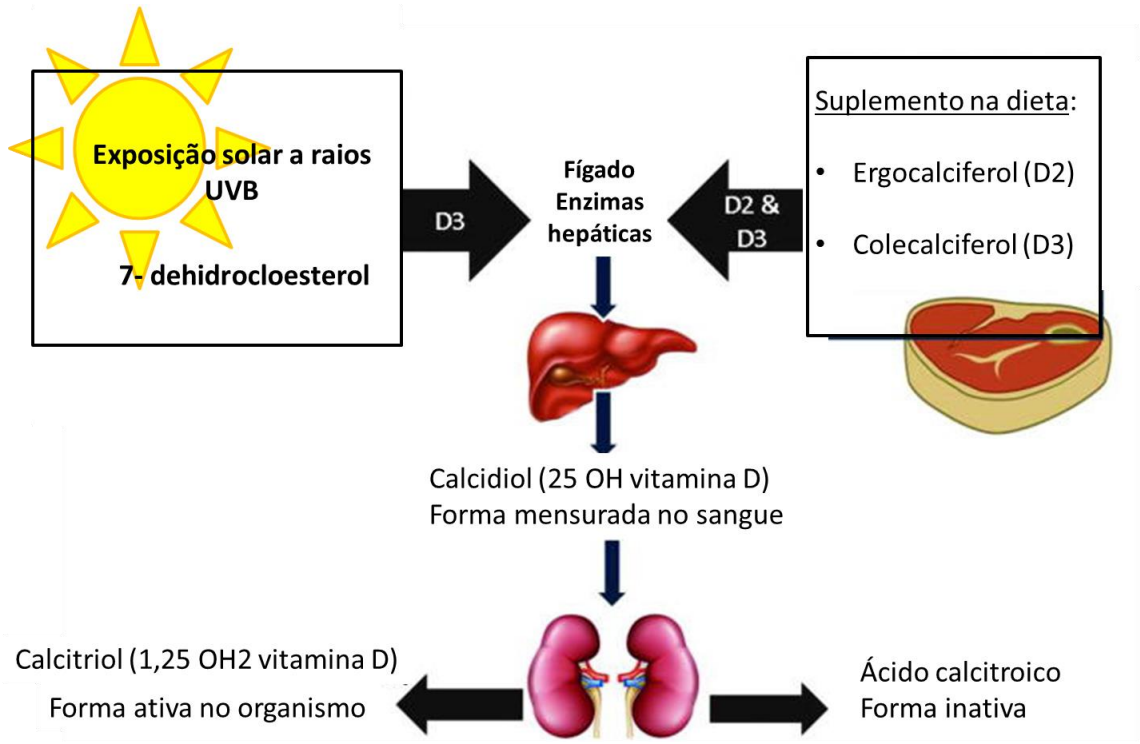


Figura 1. Síntese e metabolismo da vitamina D. Fonte: Adaptado de Mostafa e Hegazy (2015)

A VIT D possui inúmeros alvos biológicos e sua ação ocorre através de seus receptores (VDR), encontrados na maioria das células do organismo, incluindo os neurônios e células gliais (Eyles et al., 2007). Tanto em ratos quanto em humanos, o VDR parece estar localizado em regiões como amígdala, córtex e hipocampo, que são áreas cerebrais envolvidas no funcionamento cognitivo (Eyles et al., 2005; Harms et al., 2011). No SNC, a VIT D parece estar envolvida na regulação da neurotransmissão, neuroproteção, imunomodulação (Dursun et al., 2011; Spach e Hayes, 2005), bem como na

regulação da excitotoxicidade neuronal mediada pelo cálcio e redução do estresse oxidativo (Mpandzou et al., 2016).

1.3 Aprendizado e memória

Sabe-se que a flutuação hormonal que ocorre durante o ciclo reprodutivo influencia regiões cerebrais relacionadas com o aprendizado e a memória (Boulware et al., 2012; Pompili et al., 2012; Walf et al., 2006). Evidências indicam que o estradiol modula funções cognitivas em animais e humanos. Neste sentido, trabalhos mostram que a menopausa pode causar um declínio da memória e o aparecimento de distúrbios cognitivos (Halbreich et al., 1995; Henderson, 2006; Luine, 2014). O aprendizado e a memória são funções básicas do SNC. Denomina-se memória, a capacidade de adquirir, armazenar e evocar informações que posteriormente possam ser utilizadas (Izquierdo e Medina, 1997). As memórias podem ser chamadas de diferentes formas de acordo com o seu tempo de duração: quando perduram por poucos minutos ou horas, são denominadas de memórias de curta duração, e quando perduram por muitas horas, dias ou meses, são denominadas de memórias de longa duração (Alberini et al., 2006; Izquierdo et al., 1998).

O hipocampo é uma estrutura cerebral que possui importante papel nos processos relacionados à memória e aprendizagem (Ahmed et al., 2013), apresenta REs (Birzniece et al., 2006) e é altamente sensível aos efeitos da OVX (Leffa et al., 2014; Monteiro et al., 2005b; Siebert et al., 2014). Além disso, estudos anteriores já demonstraram prejuízos nas memórias espacial e

aversiva em ratas adultas ovariectomizadas (Ben et al., 2010; Monteiro et al., 2008; Monteiro et al., 2005a).

1.4 Citoesqueleto

O citoesqueleto é uma complexa rede de proteínas que determina a forma celular, fundamental para manter a homeostase celular do SNC. Seus componentes principais são os filamentos de actina ou microfilamentos (MF), os filamentos intermediários (FI) e os microtúbulos (MT) (Alberts et al., 2008). Esses filamentos são conectados entre si e suas funções coordenadas, o que permite a participação destes em diversas atividades celulares, juntamente com proteínas acessórias (Alberts et al., 2008; Bear et al., 2002).

Os neurofilamentos (NFs) são importantes FIs na fisiologia neuronal. Formados pela polimerização de três subunidades denominadas NFs de baixo, médio e alto peso molecular (NF-L, NF-M e NF-H, respectivamente), os NFs são os principais componentes do citoesqueleto de neurônios maduros (Alberts et al., 2008). A principal função do NFs consiste na manutenção do calibre axonal e dessa forma, o aumento da velocidade de condução do impulso elétrico, um processo regulado por fosforilação (Jung et al., 2000). A proteína glial fibrilar ácida (GFAP) é outro importante FI, sintetizada nos astrócitos e células de Schwann (Guo-Ross et al., 1999). A GFAP possui importante papel na modulação da motilidade e forma celular, já que fornece estabilidade estrutural aos astrócitos (Eddleston e Mucke, 1993).

A função destas proteínas em resposta a estímulos extracelulares é regulada por fosforilação, que consiste em uma modificação pós-traducional importante para as vias de transdução de sinais (Rojas et al., 2013). A fosforilação altera a função de uma proteína de forma rápida e reversível, sendo o grau de fosforilação de uma proteína alvo reflexo do balanço entre as ações contrárias de proteínas cinases e fosfatases, o que integra um conjunto de rotas de sinalização celular (Okuda et al., 2004). A fosforilação das subunidades de FIs acontece em seus domínios amino e carboxi-terminal. Proteínas cinases dependentes de segundo mensageiros, como a proteína cinase A (PKA), a proteína cinase C (PKC) e a proteína cinase dependente de cálcio e calmodulina (PKCaMII), realizam principalmente a fosforilação do domínio amino-terminal, enquanto proteínas cinases independentes de segundos mensageiros, como as proteínas cinases ativadas por mitógenos (MAPKs) e cinase dependente de ciclina 5 (Cdk5) fosforilam o domínio carboxi-terminal (Chang e Goldman, 2004; Sihag et al., 2007; Zamoner et al., 2008).

Trabalhos na literatura demonstram que proteínas do citoesqueleto estão correlacionadas com a promoção e manutenção do estresse oxidativo, bem como modulação de processos mitocondriais (de Lima Pelaez et al., 2007). Além disso, já foi demonstrado que esteróides sexuais (estrógenos e progesterona) induzem alterações no conteúdo e atividade de proteínas relacionadas ao citoesqueleto como MAP2, TAU e GFAP em células cerebrais (Hansberg-Pastor et al., 2015).

1.5 Inflamação

A inflamação é uma resposta do organismo que atua como mecanismo de defesa frente a um estímulo nocivo. Apesar de protetora no controle de infecções e promoção do reparo tecidual, a inflamação também pode ser danosa para os tecidos (Abbas e Bhawan, 2010). A resposta inflamatória envolve células e fatores solúveis e é caraterizada pela liberação de mediadores inflamatórios e recrutamento de leucócitos a partir da circulação. Tais leucócitos tornam-se então ativados no local da inflamação e liberam mais mediadores inflamatórios (Colton, 2009; Zhang, 2008). A presença de inflamação, particularmente no SNC, é chamada de neuroinflamação e é caracterizada pela ativação de micróglia e astrócitos. A neuroinflamação pode ser desencadeada por uma série de fatores, incluindo infecções, lesão cerebral traumática, presença de metabólitos tóxicos ou autoimunidade, e sua presença vêm sendo relatada em processos neurodegenerativos como doença de Parkinson e Alzheimer (Xanthos e Sandkuhler, 2014).

O fator de transcrição nucler kappa B (NF-κB) é um importante fator de transcrição relacionado à resposta celular frente a estímulos (citocinas inflamatórias, radicais livres, estresse, entre outros) e responsável pela regulação da expressão de inúmeros genes. O NF-κB é expresso no SNC e e sistema nervoso periférico, tanto por neurônio quanto por células da glia. Diversos dímeros já foram descritos no SNC, porém os mais comuns são p50-p65 e p50-p50 (O'Neill e Kaltschmidt, 1997; Shih et al., 2015). Em condições não patológicas a ativação do NF-κB pode estar envolvida na plasticidade, desenvolvimento neural e na atividade sináptica. O NF-κB é normalmente

encontrado no citoplasma ligado à proteína inibitória IkB, em um complexo inativo. Em resposta a um estímulo que ative a via do NF-kB, o IkB é fosforilado pelo complexo IKK, que o marca para degradação no proteossoma. O NF-kB é assim liberado e pode ser translocado para o núcleo da célula, onde ativa a expressão de genes específicos (Baldwin, 2001). O NF-κB é um dos reguladores mais importantes da expressão genica pro-inflamatória. A síntese de citocinas, tais como o fator de necrose tumoral alfa (TNF-α), intereucina-1-beta (IL-1β), interleucina-6 (IL-6) e interleucina-8 (IL-8), é mediada por NF-κB (Shih et al., 2015; Tak e Firestein, 2001).

As citocinas são mediadores inflamatórios secretados frente a um estímulo e desempenham seu papel na fase inicial e crônica da resposta imune e inflamatória por compartilhar sinais intracelulares com receptores, distribuirem informações sobre o tipo de infecção e recrutar células efetivas para realização da defesa do hospedeiro (Curfs et al., 1997; Turner et al., 2014). Elas são produzidas e secretadas principalmente por monócitos, macrófagos, células endoteliais, linfócitos e fibroblastos e no SNC por microglia e astrócitos ativados (Hirano e Kishimoto, 1992; Jones e Thomsen, 2013). Tais citocinas medeiam sinalização célula-célula e se ligam a receptores de superfície de alta afinidade, podendo exercer ação local ou sistêmica e influenciar a síntese de outras citocinas (Bruunsgaard, 2005).

O TNF-α, a IL-1β e a IL-6 são importantes citocinas pró-inflamatórias, liberadas frente a estímulos inflamatórios. O TNF-α é secretado principalmente por macrófagos ativados, mas também pode ser produzido por outros tipos celulares como monócitos, células T, mastócitos, células NK, fibroblastos e neurônios. O TNF-α consiste em um potente ativador do sistema imunológico e

pode induzir a ativação adicional de células ou astrócitos em repouso, bem como do NF-κB, além de ser é uma potente citocina pró-inflamatória relacionada a diversas doenças inflamatórias (Frankola et al., 2011; Olmos e Llado, 2014; Turner et al., 2014). A IL-1β é produzida por células como monócitos, macrófagos e neutrófilos em resposta a produtos bacterianos como o lipopolissacarídeo (LPS) ou outras citocinas, como o TNF-α. Aumento de seus níveis pode levar a ativação de MAPKs e do fator de transcrição NF-κB, bem como é amplamente relatado em condições neurodegenerativas (Dansokho e Heneka, 2017; Kondera-Anasz et al., 2005; Turner et al., 2014). A IL-6 é expressa por mononucleares, fagócitos, células T, células B, fibroblastos, células endoteliais, queratinócitos e células da medua óssea. Aumento de seus níveis estimula a síntese de proteínas de fase aguda, promove proliferação de linfócitos B e secreção de anticorpos (Hirota et al., 2005; Turner et al., 2014).

Estudos na literatura demonstram que a menopausa está associada a um estado inflamatório sistêmico representado pelo aumento dos níveis séricos de citocinas pró-inflamatórias como IL-1, IL-6 ou TNF-α (Cioffi et al., 2002; Yasui et al., 2006). A relação entre as diminuições hormonais associadas à menopausa e o aumento dos níveis séricos de citocinas pró-inflamatórias ainda não é totalmente compreendida (Malutan et al., 2014; Su e Freeman, 2009).

1.6 Sistema colinérgico

O sistema colinérgico é amplamente conhecido por modular funções como aprendizado e memória (Maurer e Williams, 2017; Sarter e Bruno, 1997). A acetilcolina (ACh), seus receptores e o aparato enzimático responsável por

sua síntese e degradação constituem o sistema de neurotransmissão colinérgica. A colina acetiltransferase (ChAT) é a enzima responsável pela síntese de ACh a partir de colina e acetil-CoA, posteriromente a ACh é liberada na fenda sináptica onde pode se ligar a receptores muscarínicos ou nicotínicos. Na sinapse a ACh é clivada em colina e ácido acético pela enzima acetilcolinestersae (AChE), sendo assim, ambas as enzimas são consideradas marcadores importantes dessa via, por controlar a ação da ACh (Maurer e Williams, 2017; Zimmerman e Soreq, 2006). Recentemente, estudos têm demonstrado o papel da ACh na interação do SNC com o sistema imune inato para controlar a resposta inflamatória, ressaltando seu envolvimento através da "via colinérgica anti-inflamatória" composta pelo nervo vago, pela ACh e pela subunidade α7 do receptor nicotínico de ACh (Maurer e Williams, 2017; Pavlov e Tracey, 2005; Rosas-Ballina e Tracey, 2009; Scherer et al., 2014). Essa via representa um mecanismo de resposta do SNC à presença de estímulos inflamatórios na circulação, sendo mediada pela ação do nervo vago no sentido de inibir a liberação de citocinas pró-inflamatórias e proteger contra a inflamação periférica (Maurer e Williams, 2017; Pavlov e Tracey, 2005; Tracey, 2007). Portanto, a sinalização da ACh além de essencial para o funcionamento cognitivo, bloqueia a inflamação.

O mecanismo responsável pela ação anti-inflamatória da ACh baseia-se na ativação das fibras aferentes do nervo vago por citocinas inflamatórias, as quais agem como sensor para a inflamação (Pavlov e Tracey, 2005; Zila et al., 2017). Logo, tal informação é transmitida ao SNC, que estimula o nervo vago eferente a produzir ACh, induzindo a diminuição da síntese e liberação de importantes citocinas pró-inflamatórias como o TNF-α, IL-1β e IL-6 por

macrófagos (Gwilt et al., 2007; Maurer e Williams, 2017; Parrish et al., 2008). Neste sentido, a via colinérgica anti-inflamatória representa um mecanismo fisiológico pelo qual o SNC atua com o sistema imune inato controlando a resposta inflamatória (Gallowitsch-Puerta e Pavlov, 2007).

Dados da literatura demonstram alterações de função colinérgica relacionada à OVX (Henderson, 2008). Estudos anteriores do nosso grupo de pesquisa demonstraram aumento da atividade da AChE em hipocampo de ratas adultas submetidas à OVX (Ben et al., 2009b; Monteiro et al., 2005b).

1.7 Estresse oxidativo

Espécies reativas, também chamadas de radicais livres, são definidas como qualquer espécie química que contém um ou mais elétrons desemparelhados em seu orbital mais externo, o que confere uma elevada reatividade a molécula (Halliwell e Gutteridge, 2007b). Espécies reativas podem exercer funções benéficas e/ou deletérias para sistemas vivos. Quando em baixas concentrações atuam na defesa contra agentes infecciosos e nos processos de sinalização intracelular. No entanto, em elevadas concentrações, as espécies reativas podem causar danos a biomoléculas como lipídios, proteínas e DNA, podendo resultar em perda de função celular (Halliwell, 2012; Valko et al., 2007).

As espécies reativas podem ser derivadas do oxigênio (EROs) e também do nitrogênio (ERN). As principais EROs são o ânion superóxido ($O_2^{-\bullet}$), peróxido de hidrogênio (H_2O_2), radical hidroxila ($OH^{\bullet}$), ânion hipoclorito (OCl^-

) e o oxigênio “singlet” ($^1O_2$), e dentre as ERN incluem-se óxido nítrico ($NO^{\bullet}$), óxido nitroso (N_2O) e peroxinitrito ($ONOO^-$)(Halliwell e Whiteman, 2004).

A exposição a radicais livres, a partir de uma variedade de fontes, incluindo o próprio metabolismo energético celular, levou o organismo a desenvolver uma série de mecanismos de defesa, como os antioxidantes enzimáticos e não enzimáticos. Um antioxidante é definido como uma substância que, em baixa concentração em relação a um determinado substrato, é capaz de retardar ou prevenir a oxidação do substrato oxidável (Halliwell, 1995). São exemplos de defesas antioxidantes enzimáticas a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), e de defesas antioxidantes não-enzimáticas, o ácido ascórbico (vitamina C), α-tocoferol (vitamina E), carotenóides, flavonóides entre outras (Halliwell, 2011; Halliwell e Gutteridge, 2007b). A SOD é uma metaloenzima, responsável por catalisar a reação de dismutação do ânion $O_2^{-\bullet}$, formando H_2O_2 e O_2 (Yu, 1994). O H_2O_2 resultante pode ser decomposto pelas enzimas CAT e GPx. A CAT atua na decomposição do H_2O_2 a H_2O e O_2 (Halliwell e Gutteridge, 2007b; Yu, 1994).

Em condições fisiológicas, existe um equilíbrio entre a produção de espécies reativas e as defesas antioxidantes. Entretanto, quando esse equilíbrio é rompido ocorre o chamado estresse oxidativo (Finkel e Holbrook, 2000; Gutteridge e Halliwell, 2000). O estresse oxidativo pode causar efeitos prejudiciais, tais como peroxidação de lipídios de membrana, agressão às proteínas e danos ao DNA (Silva e Coutinho, 2010; Valko et al., 2016). Qualquer célula aeróbia pode sofrer os efeitos decorrentes do estresse

oxidativo, porém, particularmente o cérebro de mamíferos possui elevada sensibilidade ao dano oxidativo (Halliwell, 2006, 2012). Dados da literatura relatam a presença de estresse oxidativo relacionada à depleção hormonal ovariana decorrente da menopausa (Doshi e Agarwal, 2013). Estudo anterior do nosso grupo de pesquisa demonstrou haver aumento de atividade antioxidante, mais especificamente da CAT frente à OVX em hipocampo de ratas adultas (Monteiro et al., 2005b), sugerindo um mecanismo compensatório frente à presença de espécies reativas.

O estresse oxidativo também parece estar relacionado à senescência celular prematura (Chen et al., 2007a; Pole et al., 2016). A análise do comprimento de telômeros como marcador de senescência celular vem sendo extensamente proposta (Butt et al., 2010; von Zglinicki e Martin-Ruiz, 2005). Os telômeros são estruturas constituídas de uma sequência repetida de DNA, que ficam localizados nas extremidades dos cromossomos e possuem como função proteger a integridade dos genomas e impedir a fusão com outros cromossomos. Com o passar da idade os telômeros das células teciduais diminuem e a quantidade de telomerase, enzima responsável por manter o comprimento de telômeros, é insuficiente para manter o comprimento dos mesmos durante as repetidas divisões celulares (Blackburn, 2000; de Jongh et al., 2017). O encurtamento de telômeros pode ser acelerado pela presença de estresse oxidativo, inflamação e proliferação celular (Pusceddu et al., 2015), o que pode aumentar a probabilidade de desenvolvimento de senescência celular e apoptose. Neste sentido, estudos clínicos e pré-clínicos já demonstraram evidências sobre a diminuição de telômeros associada à menopausa e o papel

benéfico dos estrógenos sobre a atividade da telomerase, principalmente em leucócitos (Cen et al., 2015; Gray et al., 2014; Pines, 2013; Shenassa e Rossen, 2015).

2.JUSTIFICATIVA E HIPÓTESE

Partindo do princípio de que a depleção hormonal ovariana causa importantes alterações no organismo feminino, incluindo no SNC, aumentando a suscetibilidade de desenvolvimento de déficit cognitivo, distúrbios emocionais e até uma maior predisposição ao desenvolvimento de doenças neurodegenerativas, nossa hipótese é que a suplementação com VIT D atua de forma benéfica na reversão dos efeitos comportamentais e neuroquímicos causados pela OVX.

3. OBJETIVOS

3.1 Objetivo Geral

O presente estudo teve como objetivo geral investigar a influência da OVX, um modelo animal amplamente utilizado para mimetizar alterações pós-menopausa, sobre parâmetros comportamentais (memória) e neuroquímicos (sistema fosforilante do citoesqueleto, parâmetros inflamatórios, sistema colinérgico, estresse oxidativo, dano a biomoléculas e comprimento de telômeros) em hipocampo de ratas Wistar adultas. O papel neuroprotetor da VIT D também foi investigado.

3.2. Objetivos específicos

Os objetivos específicos estão subdivididos em três capítulos, que serão apresentados na forma de artigos científicos, como segue:

Capítulo I

- Avaliar os efeitos da OVX em ratas Wistar adultas de 90 dias sobre o desempenho dos animais nas seguintes tarefas: campo aberto, reconhecimento de objetos e esquiva inibitória de curta (teste 1 hora após o treino) e longa duração (teste 7 dias após o treino);
- Verificar o perfil de fosforilação dos seguintes filamentos intermediários de astrócitos e neurônios: proteína glial fibrilar ácida (GFAP), neurofilamentos de baixo, médio e alto peso molecular (NF-L, NF-M e NF-H respectivamente) em hipocampo de ratas Wistar adultas de 90 dias submetidas à OVX;
- Avaliar o efeito da OVX em ratas Wistar adultas de 90 dias sobre o imunoconteúdo dos filamentos intermediários: GFAP, NF-L, NF-M e NF-H, bem como dos sítios de fosforilação (pSer57, pSer55 e pKSPrepeats) e de cinases envolvidas na fosforilação (p-p38, pJNK1/2, pERK1/2, PKAαsub e PKCaMIIc-sub) em hipocampo;
- Investigar o papel neuroprotetor da VIT D sobre as alterações identificadas, bem como o imunoconteúdo de seu receptor (VDR) em hipocampo de ratas Wistar adultas de 90 dias submetidas à OVX.

Capítulo II

- Avaliar o imunoconteúdo de NF-κB/p65 e fosfo-NF-κB/p65 nas frações citosólica e nuclear em hipocampo de ratas Wistar adultas submetidas a OVX com 90 dias;
- Analisar os níveis de TNF-α, IL-1β e IL-6 em hipocampo de ratas Wistar adultas submetidas à OVX com 90 dias;
- Verificar o efeito da OVX sobre parâmetros do sistema colinérgico como atividade da AChE e imunoconteúdo de AChE e ChAT em hipocampo de ratas Wistar adultas submetidas a OVX com 90 dias;
- Avaliar os níveis de estradiol e calcidiol (VIT D) em soro, bem como medir o peso corporal ao final do experimento e a ingesta alimentar de ratas Wistar adultas submetidas à OVX com 90 dias de vida;
- Investigar o possível papel protetor da suplementação com VIT D sobre os parâmetros analisados.

Capítulo III

- Avaliar parâmetros relacionados a estresse oxidativo como atividade das enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT), e oxidação do DCFH em hipocampo de ratas Wistar submetidas à OVX em duas diferentes idades: 90 dias de vida ou 180 dias de vida;
- Avaliar o conteúdo de sulfidrilas, níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) e dano ao DNA em hipocampo de ratas Wistar

submetidas à OVX em duas diferentes idades: 90 dias de vida ou 180 dias de vida;

- Analisar o comprimento de telômeros em hipocampo de ratas Wistar submetidas à OVX em duas diferentes idades: 90 dias de vida ou 180 dias de vida;
- Investigar o papel da suplementação com VIT D sobre os parâmetros acima listados.

PARTE II

1. METODOLOGIA E RESULTADOS

Os capítulos I, II, III serão apresentados na forma de artigos científicos, os quais apresentam o desenho experimental como segue:

1.1 Modelo Experimental

1.1.1 Ovariectomia

Ratas Wistar adultas (90 ou 180 dias de vida) provenientes do Biotério do Departamento de Bioquímica foram aleatoriamente divididas em quatro grupos: (1) SHAM, (2) OVX, (3) VIT D e (4) OVX+ VIT D.

A OVX consiste na retirada cirúrgica bilateral dos ovários a fim de causar a depleção dos hormônios ovarianos, mimetizando alterações observadas no período pós-menopausa. O procedimento cirúrgico foi realizado através de incisões translombares, mediante anestesia com uma mistura de cetamina e xilasina (90/20mg/Kg) e de acordo com Monteiro et al. (2005b) e Ben et al. (2009b). Os grupos SHAM e VIT D foram submetidos apenas a incisões cutâneas seguidas de sutura.

1.1.2 Suplementação com Vitamina D

Trinta dias após a OVX, as ratas foram submetidas à suplementação diária de VIT D, realizada por gavagem, por um período de 30 dias. As ratas controles receberam igual volume do veículo (propilenoglicol) utilizado para

dissolver a VIT D. Aproximadamente doze horas após a última suplementação as ratas foram submetidas aos testes comportamentais e/ou decapitadas sem anestesia para posteriores análises.

A forma suplementada de VIT D foi o colecalciferol (vitamina D_3), para que possa ser metabolizado até sua forma ativa: calcitriol. Utilizamos para este estudo a dose de 500 UI/Kg/dia de colecalciferol, que consiste em uma dose utilizada na literatura para estudos animais de diferentes modelos e em diferentes formas de administração (Chabas et al., 2013; de Souza Santos e Vianna, 2005; Féron et al., 2014; Gueye et al., 2015; Salum et al., 2013; Salum et al., 2012).

Capítulo I

Neuromol Med (2017) 19:345–356
DOI 10.1007/s12017-017-8449-7

ORIGINAL PAPER

Vitamin D_3 Reverses the Hippocampal Cytoskeleton Imbalance But Not Memory Deficits Caused by Ovariectomy in Adult Wistar Rats

Cassiana Siebert[1,4] · Paula Pierozan[1,3,4] · Janaina Kolling[1,4] · Tiago Marcon dos Santos[1,4] · Matheus Coimbra Sebotaio[1,4] · Eduardo Peil Marques[1,4] · Helena Biasibetti[1,4] · Aline Longoni[1,4] · Fernanda Ferreira[3,4] · Regina Pessoa-Pureur[3,4] · Carlos Alexandre Netto[2,4] · Angela T. S. Wyse[1,4]

Received: 19 October 2016 / Accepted: 1 July 2017 / Published online: 8 July 2017

Periódico: Neuromolecular Medicine

Status: Publicado

Neuromol Med (2017) 19:345–356
DOI 10.1007/s12017-017-8449-7

ORIGINAL PAPER

Vitamin D_3 Reverses the Hippocampal Cytoskeleton Imbalance But Not Memory Deficits Caused by Ovariectomy in Adult Wistar Rats

Cassiana Siebert[1,4] · Paula Pierozan[1,3,4] · Janaina Kolling[1,4] · Tiago Marcon dos Santos[1,4] · Matheus Coimbra Sebotaio[1,4] · Eduardo Peil Marques[1,4] · Helena Biasibetti[1,4] · Aline Longoni[1,4] · Fernanda Ferreira[3,4] · Regina Pessoa-Pureur[3,4] · Carlos Alexandre Netto[2,4] · Angela T. S. Wyse[1,4]

Received: 19 October 2016 / Accepted: 1 July 2017 / Published online: 8 July 2017

Abstract The objective of study was to investigate changes caused by ovariectomy (OVX) on aversive and non-aversive memories, as well as on cytoskeleton phosphorylating system and on vitamin D receptor (VDR) immunocontent in hippocampus. The neuroprotective role of vitamin D was also investigated. Ninety-day-old female Wistar rats were divided into four groups: SHAM, OVX, VITAMIN D and OVX + VITAMIN D; 30 days after the OVX, vitamin D supplementation (500 IU/kg), by gavage, for 30 days was started. Results showed that OVX impaired short-term and long-term recognition, and long-term aversive memories. OVX altered hippocampal cytoskeleton phosphorylating system, evidenced by the hyperphosphorylation of glial fibrillary acidic protein (GFAP), low molecular weight neurofilament subunit (NFL), medium molecular weight neurofilament subunit (NFM) and high molecular weight neurofilament subunit (NFH), and increased the immunocontent of c-Jun N-terminal protein kinases (JNK), Ca^{2+}/calmodulin-dependent protein kinase II (PKCaMII) and of the sites phosphorylated lysine–serine–proline (KSP) repeats, Ser55 and Ser57. Vitamin D reversed the effects caused by OVX on cytoskeleton in hippocampus, but it was not able to reverse the effects on memory.

Keywords Experimental menopause · Ovariectomy · Vitamin D · Short-term memory · Long-term memory · Cytoskeleton

Abbreviations

ANOVA	Analysis of variance
Ca^{2+}	Cálcio
GFAP	Glial fibrillary acidic protein
JNK	c-Jun N-terminal protein kinases
KSP	Lysine–Serine–Proline
MAPK	Mitogen-activated protein kinase
NFH	High molecular weight neurofilament subunit
NFL	Low molecular weight neurofilament subunit
NFM	Medium molecular weight neurofilament subunit
OVX	Ovariectomy
PKA	cAMP-dependent protein kinase A
PKCaMII	Ca^{2+}/calmodulin-dependent protein kinase II
VDR	Vitamin D receptor

Introduction

Natural menopause is defined as the permanent interruption of ovulation and menstruation for a period of 12 months (Soules et al. 2001; Takahashi and Johnson 2015). Although menopause is a physiological event in the woman's life, many experience it at an early phase (between ages 40 and 45) or prematurely (before age 40),

Electronic supplementary material The online version of this article (doi:10.1007/s12017-017-8449-7) contains supplementary material, which is available to authorized users.

✉ Angela T. S. Wyse
wyse@ufrgs.br

[1] Laboratório de Neuroproteção e Doenças Neurometabólicas, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2] Laboratório de Isquemia Cerebral e Neuroproteção, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[3] Laboratório de Citoesqueleto, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[4] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600, Anexo, Porto Alegre, RS CEP 90035-003, Brazil

spontaneously or induced by medical interventions as chemotherapy, radiation exposure or bilateral ovariectomy (OVX) (Grant et al. 2015; Rocca et al. 2010; Shuster et al. 2010; Rocca et al. 2012). Studies have associate the premature or early menopause with increased risk for developing of neurological diseases (Zhang et al. 2013; Scott et al. 2014) and cognitive impairment (Rocca et al. 2010), through mechanisms not fully understood.

Estrogens regulate and coordinate various cell functions, organs and genes, acting through receptors and signaling pathway, which can activate and regulate genomic and molecular responses necessary for cell survival (Rettberg et al. 2013). Experimental evidence shows that estradiol modulates cognitive function in both animals and humans (Luine 2014). For instance, it has been reported that the decrease in estrogen levels may be related to learning and memory impairments and that estrogens appear to exert neurotropic effects on brain tissue, acting indirectly to maintain cognitive functions (Luine 2014). Moreover, studies show that OVX, an animal model widely used to investigate biochemical and behavioral changes caused by estrogen deficiency (Ben et al. 2009b; Mackedanz et al. 2011; Siebert et al. 2014; Waynforth and Flecknell 1992), impairs spatial and aversive memories (Ben et al. 2010; Monteiro et al. 2008, 2005a).

The cytoskeleton consists of microtubules, intermediate filaments (IFs) and actin filaments, and it is fundamental for many complex functions performed in the cells. The IF proteins promote structural and mechanical support for the cell and are involved in numerous cellular functions, such as transport, migration, signaling and apoptosis (Pessoa-Pureur et al. 2014; Snider and Omary 2014). The neuron-specific IF, namely neurofilaments (NFs), plays a crucial role in maintaining structure and function of neurons, control of axonal caliber and consequently neural activity (Hoffman et al. 1987). NFs are divided into type IV light, medium and heavy molecular mass NF proteins (NFL, NFM and NFH, respectively). In mature astrocytes, the main IF found is the glial fibrillary acidic protein (GFAP), with functions related to the maintenance of cell shape, mechanical strength and astrocyte functions like migration/cell motility, cellular proliferation, glutamate homeostasis, neurite outgrowth and injury/protection (Middeldorp and Hol 2011; Pessoa-Pureur et al. 2014).

IF proteins are phosphorylated on their head and tail domains, and phosphorylation/dephosphorylation plays a major role in regulating the organization and function of IFs (Sihag et al. 2007; Omary et al. 2006; Grant and Pant 2000). In this context, changes in the phosphorylation profile have been associated with aggregation of aberrant IFs and seem to be associated with pathological features of several neurodegenerative diseases (Pessoa-Pureur et al. 2014; Sihag et al. 2007). Several studies have demonstrated that changes in the dynamics of cytoskeletal proteins are also involved with learning and memory process (Lamprecht 2016; Amram et al. 2016; Liebert et al. 2016). Hormones with systemic signaling activities, such as estrogen and progesterone, can regulate the activity and expression of some cytoskeletal proteins and influence several brain functions, including learning and memory (Hansberg-Pastor et al. 2015). However, to our understanding there not are data in the literature relating to estrogen deficiency, memory and cytoskeleton in the hippocampus.

Women frequently use hormone replacement therapy (HRT) to treat menopause symptoms. Despite memory and cognitive benefits have been reported after HRT (Sherwin 2003; Shoupe 2011), there is evidence that it is also able to predispose to tumor development and to increase the risk of cardiovascular disease (Miquel et al. 2006; Lobo et al. 2014). Therefore, studies that aim to find alternative therapies to replace HRT have grown in the last years (Franco et al. 2016; Al-Rahbi et al. 2014; Ben et al. 2009a; Siebert et al. 2014). In this context, the vitamin D that has numerous biological targets and acts through its receptor (VDR) found in most cells, including neurons and glia (Eyles et al. 2007; Holick 2007; Nissou et al. 2013), has been described as a potential therapeutic neuroprotective agent in several conditions (Deluca et al. 2013; Briones and Darwish 2012).

Considering that the menopause triggers a series of changes in the organism and that these alterations may be associated with memory deficits and brain alterations, we decided to investigate the effects of OVX in adult female Wistar rats on: (a) locomotor and exploratory activities, (b) aversive and recognition (short- and long-term) memories, (c) the phosphorylation profile of intermediate filaments of astrocytes and neurons in the hippocampus and (d) the possible neuroprotective action of vitamin D supplementation over these variables. The working hypothesis is that vitamin D can decrease brain changes caused by menopause.

Materials and Methods

Animals

Adult female Wistar rats were obtained from the Central Animal House of the Department of Biochemistry at the Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul (UFRGS) in Brazil. The animals were maintained in a room with light/dark cycle 12:12 h, at a constant temperature (22 °C), and had free access to water and specific diet [20% (w/w) protein commercial chow]. The care with animals followed the official governmental

guidelines issued by the Brazilian Federation of Societies for Experimental Biology, following the Guide for the Care and Use of Laboratory Animals and Arouca Law (Law n° 11.794/2008). This study was previously approved by the University Ethics Committee for the Use of Animals (CEUA) under the project (#28033).

Experimental Protocol

The experimental protocol consisted of two phases: firstly, memory function in adult female rats submitted to OVX and subsequently supplemented with vitamin D was investigated. Secondly, the phosphorylation profile and immunocontent of IF and from other proteins were studied (Fig. 1).

OVX Procedure

Ninety-day-old female Wistar rats (100 animals) were randomly divided into four groups: (1) SHAM (control: surgery without ovaries removal); (2) OVX (surgical removal of both ovaries); (3) VITAMIN D and (4) OVX + VITAMIN D. Briefly, the rats were subjected to a surgical procedure for removing both ovaries. Animals were anesthetized by intraperitoneal administration (i.p.) of ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture. The experimental model of OVX was performed as previously described (Ben et al. 2009a; Mackedanz et al. 2011). Animals submitted to this model present a significant decrease in estradiol circulating levels (Waynforth and Flecknell 1992; Monteiro et al. 2005b).

Vitamin D Supplementation

The daily supplementation of vitamin D (cholecalciferol—vitamin D_3—C9756, Sigma-Aldrich) was started 30 days after OVX through gavage, 200 µL once per day, for a period of 30 days. The control groups (SHAM and OVX) received equal volume of vehicle (propylene glycol) used to dissolve vitamin D. The dose of cholecalciferol used was 500 IU/kg/day, which was chosen based on data from literature (Gueye et al. 2015; Chabas et al. 2013; de Souza Santos and Vianna 2005; Féron et al. 2014; Salum et al. 2012, 2013). The weight of the animals was controlled weekly.

Approximately 12 h after the last administration, the rats were subjected to behavioral tests and/or decapitated without anesthesia for further tissue analysis. The estrous cycle was monitored before decapitation. SHAM rats (without ovaries removal) were decapitated in the diestrus phase, in which low plasma concentrations of estrogen are present.

Behavioral Testing

The tests were performed in different days, with an interval of 24 h, in a quiet room, with low illumination, in the order appearing below.

Open Field

The open-field test was conducted for evaluation of locomotor and exploratory activities in a black wooden box measuring 50 cm × 50 cm × 50 cm. The animals were placed facing the lower left corner of the box and observed for 5 min (Rojas et al. 2013). Behavioral parameters were recorded and subsequently elaborated with an automated activity-monitoring system (Any-maze; Stoelting, Wood Dale, IL, USA).

Novel Object Recognition

Recognition memory (short-term and long-term) was assessed by the novel object recognition task. This test is divided into two phases: In the first phase (training session), the animal was placed in the open-field box and confronted with two identical objects (object 1 and 2) by 5 min (time of object exploration was registered); in the second phase (test session), 1 h or 7 days after the training, the animal was once again placed in the box; however, one

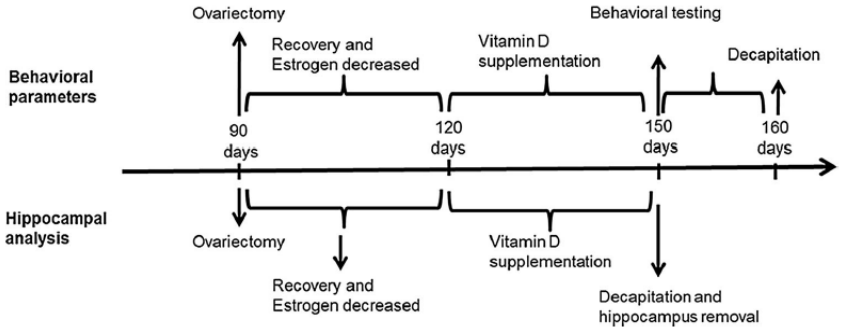


Fig. 1 Timeline of experiments. Evaluation of the effects of OVX and vitamin D supplementation on memory, phosphorylation profile of intermediate filaments and immunocontent of these proteins and others

object was substituted for a third different object (object 3) and the time spent exploring the novel object (object 3) and the familiar object (object 1) was measured for 5 min. In the test session (second phase), the discrimination index was calculated each time by the difference in the exploration time divided by the total time spent exploring the objects through the formula: Object 1-Object 3/Object 3 + Object1 (Rojas et al. 2013).

Inhibitory Avoidance

The evaluation of aversive memory was performed by the inhibitory avoidance task and run in an acrylic box (50 × 25 × 25 cm), with a platform on the left side of the box (3 cm high and 7 cm wide). The box floor was composed of a grid of parallel stainless steel bars (measuring 1.5 mm diameter) spaced 1 cm apart. In the training phase, each animal was gently placed in the platform; the latency to step down placing their four paws on the grid was measured. In the moment in which the rat stepped down from the platform, touching its paws on the grid, they received a 0.5-mA, 60-Hz foot shock for 2 s. Animals were tested for retention 1 h and 7 days after the training phase. In the test session, the rats were placed in the platform, but the foot shock was omitted; step-down latency was used as an index of retention (Arteni et al. 2003; Sanches et al. 2013).

Cytoskeleton Protein Phosphorylation

Preparation of Hippocampus Slices

Rats were euthanized by decapitation without anesthesia approximately 12 h after the last vitamin D administration, and the hippocampus was dissected under refrigeration and cut into 400-mm-thick slices with a McIlwain chopper (Pierozan et al. 2010).

Preincubation

The slices were preincubated at 30 °C for 20 min in Krebs–Hepes medium (124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na–HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$) and posteriorly at the followings protease inhibitors: 1 mM benzamidine, 0.1 mM leupeptin, 0.7 mM antipain, 0.7 mM pepstatin and 0.7 mM chymostatin (Pierozan et al. 2010).

In Vitro ^{32}P Incorporation Experiments

After the preincubation, a new incubation was carried out at 30 °C with 100 ml of the basic medium containing 100 µCi $^{[32]}$ Na_2PO_4, as previously described by (Funchal et al. 2003). The labeling reaction occurred for 30 min at 30 °C and was stopped with 1 ml of cold stop buffer containing 150 mM NaF, 5 mM, EDTA, 5 mM EGTA, Tris–HCl 50 mM, (pH 6.5) and protease inhibitors (described above). Posteriorly, slices were washed twice with stop buffer to remove excess radioactivity (Pierozan et al. 2010).

High Salt–Triton-Insoluble Cytoskeletal Fraction Preparation

Briefly, the slices, after the labeling reaction, were homogenized in 400 µL of ice-cold high salt buffer containing 5 mM KH_2PO_4 (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100 and the protease inhibitors. Posteriorly, the homogenate was centrifuged at 15.800 × g at 4 °C for 10 min. The supernatant was discarded, and the pellet was again homogenized with the same volume of the high-salt medium. The suspended pellet was centrifuged as described above and the supernatant discarded (Pierozan et al. 2010). Lastly, the final Triton-insoluble IF-enriched pellet, containing the IFs, was dissolved in 1% SDS and protein concentration was determined (Lowry et al. 1951).

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples were dissolved in 25% (v/v) of solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations of the cytoskeletal fraction were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of (Laemmli 1970). The gels were exposed to X-ray films (Kodak T-Mat) at −70 °C with intensifying screens, and thus, the autoradiography was obtained. Cytoskeletal proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

Western Blot Assays for GFAP, NFH, NFM, NFL, pERK, pJNK, p38MAPK, pPKCam, pKSP Repeats, pSer55, pSer57 and VDR Immunocontent

The homogenization of tissue slices was realized in 100 µl of a lysis solution (2 mM EDTA, 50 mM Tris–HCl, pH 6.8, 4% (w/v) SDS). After, samples were dissolved in 25% (v/v) of solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min. The proteins of homogenate (30 µg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad) for 1 h at

15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). Then, nitrocellulose membranes were washed in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5) for 10 min, followed incubation in blocking solution (TBS plus 5% defatted dried milk) by 2 h. After, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS) and incubated overnight at 4 °C in blocking solution containing some of the following monoclonal antibodies: anti-NFH (clone N52), anti-NF-150 (clone NN-18), anti-NF68 (clone NR-4), anti-GFAP (clone G-A-5), anti-pNFLSer55, anti-pNFLSer57, anti-KSPrepeats, anti-phosphoERK1/2, anti-phosphop38, anti-phosphoJNK1/2, anti-PKAαsub, anti-PKCaMIIcsub or anti-VDR. After the incubation, the blot was washed twice for 5 min with T-TBS and a new incubation in blocking solution containing peroxidase-conjugated anti-rabbit IgG or peroxidase-conjugated anti-mouse IgG for 2 h was realized. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using a chemiluminescence ECL kit (Pierozan et al. 2010; Biasibetti et al. 2016). Immunoblots were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company).

Protein Determination

The determination of total protein was performed by colorimetric method (Lowry et al. 1951) using serum bovine albumin as standard.

Statistical Analysis

The parametric data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey test, or by the Student's t test. Nonparametric data were analyzed by Kruskal–Wallis test followed by post hoc Dunn's test. Values of $p < 0.05$ were considered statistically significant. All analyzes and graphics were performed using GraphPad Prism 5.1 software program in a compatible computer.

Results

Behavioral Effect of OVX and Vitamin D Supplementation

Initially, we evaluated the effect of OVX and vitamin D supplementation in adult female Wistar rats on exploratory and locomotor activities of animals through the open-field task. We observed that the OVX and vitamin D supplementation does not alter these parameters ($p > 0.05$).

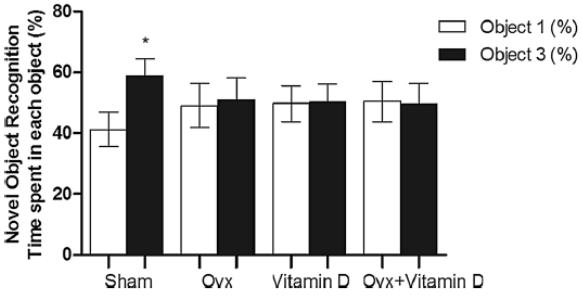
The evaluation of recognition memory was performed through the novel object recognition task (Fig. 2) of short term (tested 1 h after training) and long term (tested 7 days after training). In the training session, no difference was observed between groups on percentage of exploration time of each object and in the recognition index ($p > 0.05$). In the short-term test session, animals subjected to OVX and/or vitamin D supplementation presented an impairment in recognition memory, as evidenced by percentage of exploration time of each object (Fig. 2a). It was observed that animals subjected to OVX, vitamin D and/or OVX + vitamin D explored equally the objects [(1 and 3) $p > 0.05$], while SHAM group explore more the novelty [(3); $p < 0.05$]. The recognition index did not present significant difference ($p > 0.05$). The same pattern of alteration was observed in the long-term test. Animals subjected to OVX and/or vitamin D supplementation also explored equally the familiar object and the novel [(1 and 3) $p > 0.05$], while animals SHAM explore more the novelty [(3) Fig. 2b; $p < 0.05$]. The recognition index did not present significant difference between groups ($p > 0.05$).

The inhibitory avoidance test was performed in order to analyze the effect of OVX and/or vitamin D supplementation on aversive memory. The test was performed 1 h after the training session for evaluating the short-term memory and 7 days after training to evaluate the long-term memory. In the test session 1 h after training (recall), there was no significant difference in latency to step down from the platform between the groups ($p > 0.05$), but in the test session 7 days after training the OVX group showed an impairment in the task (Fig. 3; $p < 0.05$).

Effect of OVX and Vitamin D Supplementation on Cytoskeleton and VDR Immunocontent in Hippocampus

Regarding cytoskeleton, the effect of OVX and/or vitamin D supplementation on in vitro phosphorylation of GFAP and NF subunits present in the IF-enriched cytoskeletal fraction of astrocytes and neurons, respectively, in hippocampus of female adult rats was evaluated (Fig. 4). Results showed that OVX causes hyperphosphorylation of GFAP, NFL, NFM and NFH ($p < 0.001$). Vitamin D supplementation in OVX group reversed the increase in vitro ^{32}P incorporation in all parameters ($p < 0.01$). Western blot analysis of IFs subunits showed that neither OVX nor vitamin D supplementation changes the levels of these proteins (Supplemental Fig. 1; $p > 0.05$).

The participation of the second messenger-independent protein kinases (phosphorylate sites in the carboxyl-terminal tail domain) and second messenger-dependent



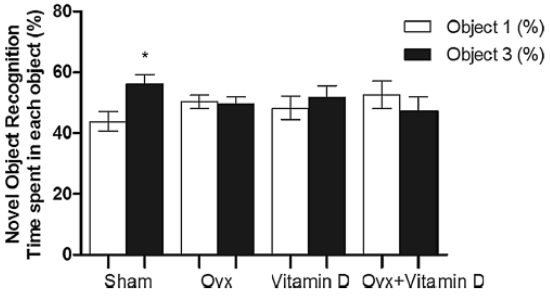


Fig. 2 Effect of OVX and vitamin D supplementation on novel object recognition performance. *Bars* represent the mean ± SEM of percentage of exploration of each object in the short-term [(1 h) **a**] and long-term [(7 days) **b**] testing session. Note 1 is the old object and 3 is the novel object. Data were analyzed between groups by one-way ANOVA ($p > 0.05$) and within the groups (objects 1 and 3) by Student's *t* test—*Different from object 1 within the respective group ($p < 0.05$); $n = 12$–13 animals per group. *OVX* ovariectomy

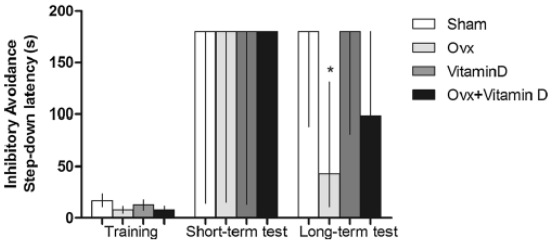
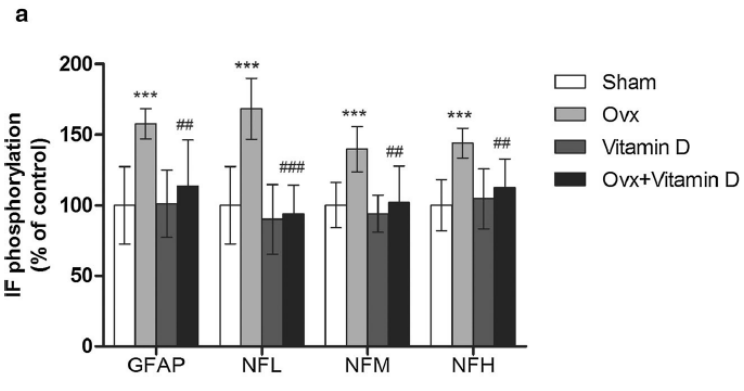


Fig. 3 Effect of OVX and vitamin D supplementation on step-down inhibitory avoidance performance. *Bars* represent the median and interquartile intervals (25–75% percentiles) of latency to step down the platform (s) in the training, short-term (1 h) and long-term (7 days) test sessions. Data were analyzed by Kruskal–Wallis test followed by Dunn's test ($n = 12$–13 animals per group) and considered significant as $p < 0.05$. *Different from SHAM group ($p < 0.05$). *OVX* ovariectomy

protein kinases (phosphorylate residues in the amino-terminal head domains) of the IF subunits also was analyzed. Western blot analysis of mitogen-activated protein kinase pathways (MAPKs) showed no difference in the immunocontents of the phosphorylated forms of extracellular signal-regulated kinases 1/2 (phosphoERK 1/2) (Fig. 5a; $p > 0.05$) and phospho-p38 (Fig. 5c; $p > 0.05$) between groups, but, c-Jun N-terminal protein kinases 1/2 (phosphoJNK 1/2) immunocontent increased in OVX group ($p < 0.001$) and vitamin D supplementation was able to reverse such effect ($p < 0.001$) at SHAM level (Fig. 5b). Regarding second messenger-dependent protein kinases, immunocontents of cAMP-dependent protein kinase A (PKA) (Fig. 6a) and Ca^{2+}/calmodulin-dependent protein kinase II (PKCaMII) (Fig. 6b) showed that the expression of PKA remains unchanged ($p > 0.05$), while the



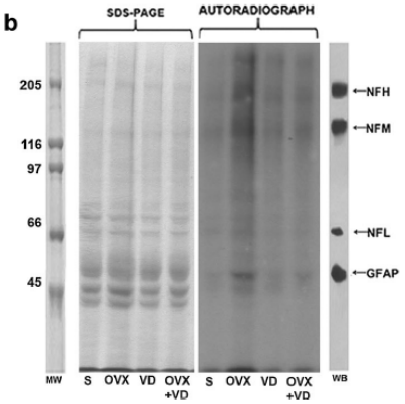


Fig. 4 Effect of OVX and vitamin D supplementation on in vitro phosphorylation of GFAP and NF subunits present in the IF-enriched cytoskeletal fraction of astrocytes and neurons, respectively, in hippocampus slices of female adult rats. *Bars* represent the mean ± SD of percentage of controls. Data were analyzed by one-way ANOVA ($n = 6$ animals per group) and was considered significant as $p < 0.05$. ***Different from SHAM group ($p < 0.001$); ##Different from OVX group ($p < 0.01$). *OVX* ovariectomy; *IF* intermediate filaments; *GFAP* glial fibrillary acidic protein; *NFL* low molecular weight neurofilament subunit; *NFM* middle molecular weight neurofilament subunit and *NFH* high molecular weight neurofilament subunit

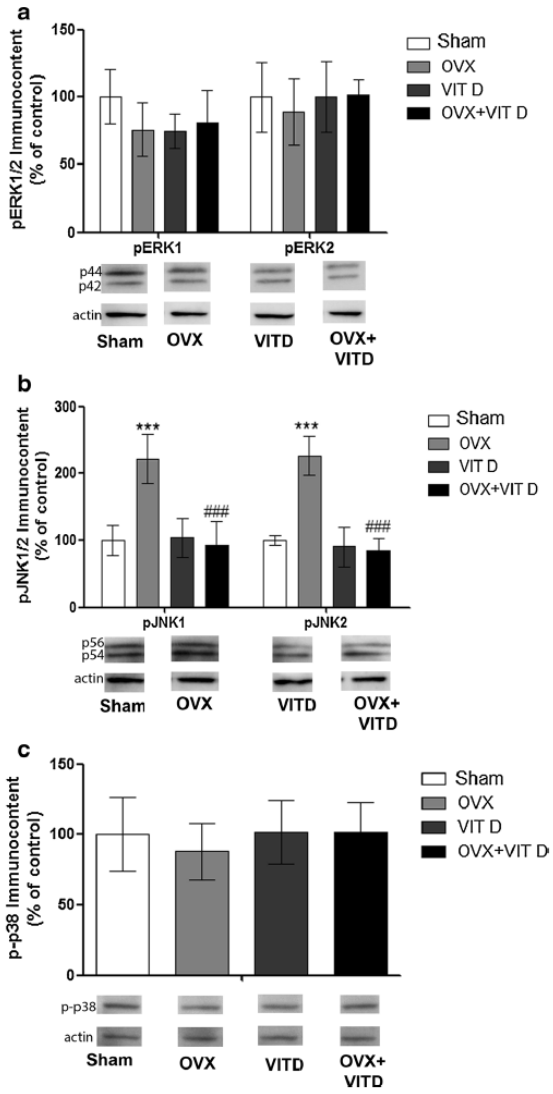


Fig. 5 Effect of OVX and vitamin D supplementation on the following second messenger-independent protein kinases immunocontent: pERK1/2 (**a**), pJNK1/2 (**b**) and p-p38 (**c**), in the cytoskeletal fraction from hippocampus slices of female adult rats. Representative Western blots of proteins studied are shown. β-Actin was used as loading control. *Bars* represent the mean ± SD of percentage of controls. Data were analyzed by one-way ANOVA ($n = 6$ animals per group) and was considered significant as $p < 0.05$. ***Different from SHAM group ($p < 0.001$);$^{###}$Different from OVX group ($p < 0.001$). *OVX* ovariectomy

expression protein of PKCaMII increased in OVX group ($p < 0.01$). Vitamin D supplementation was also able to revert this parameter at SHAM group level ($p < 0.01$).

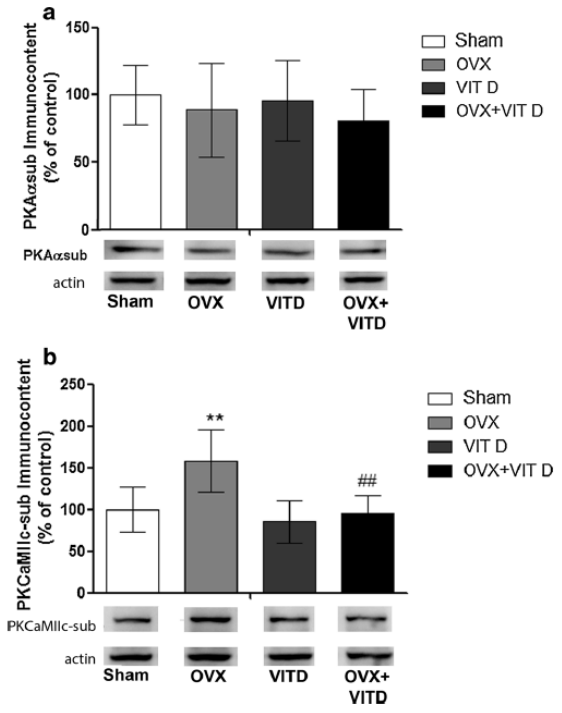


Fig. 6 Effect of OVX and vitamin D supplementation on the following second messenger-dependent protein kinases immunocontent: PKAαsub (**a**), PKCaMIIcsub (**b**), in the cytoskeletal fraction from hippocampus slices of female adult rats. Representative Western blots of proteins studied are shown. β-Actin was used as loading control. *Bars* represent the mean ± SD of percentage of controls. Data were analyzed by one-way ANOVA ($n = 6$ animals per group) and was considered significant as $p < 0.05$. **Different from SHAM group ($p < 0.01$);$^{##}$Different from OVX group ($p < 0.01$). *OVX* ovariectomy

In order to check the phosphorylating sites targeted by protein kinases, we investigated the expression protein of phosphoKSPrepeats (phosphorylates carboxyl-terminal domain of NFM and NFH), phosphoSer57 and phosphoSer55 (phosphorylates amino-terminal domain of NFL). In Fig. 7, we observed that OVX increased phosphorylation level of phosphoKSPrepeats ($p < 0.01$), phosphoSer55 ($p < 0.01$) and phosphoSer57 ($p < 0.001$). Vitamin D was able to reverse such effects, reducing this activation when compared to SHAM groups (pKSPrepeats, $p < 0.01$; pSer55 and pSer57, $p < 0.001$). All the immunoblots can be visualized in Supplemental Fig. 2.

Lastly, to investigate the action of vitamin D, we evaluated the protein expression of VDR in hippocampus of rats subjected to OVX and/or vitamin D supplementation. Western blot analysis showed that expression of the receptor does not change between the experimental groups (Supplemental Fig. 3; $p > 0.05$). Figure 8 shows a summary of the results found in this work.

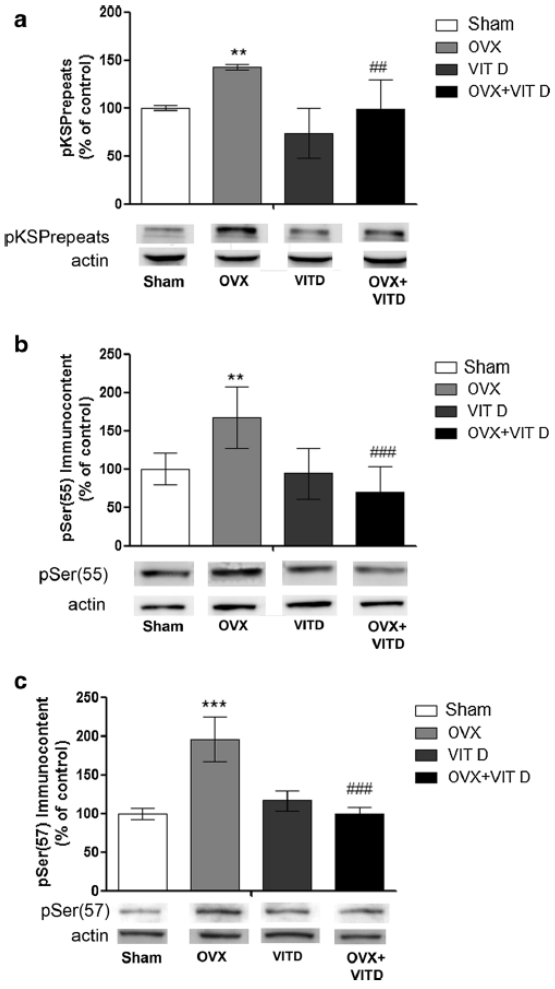


Fig. 7 Effect of OVX and vitamin D supplementation on the following phosphorylation sites immunocontent: pKSP repeats (**a**), pSer55 (**b**) and pSer57(**c**), in the cytoskeletal fraction from hippocampus slices of female adult rats. Representative Western blots of proteins studied are shown. β-Actin was used as loading control. *Bars* represent the mean ± SD of percentage of controls. Data were analyzed by one-way ANOVA ($n = 6$ animals per group) and was considered significant as $p < 0.05$. ***Different from SHAM group ($p < 0.001$); **Different from SHAM group ($p < 0.01$);$^{###}$Different from OVX group ($p < 0.001$);$^{##}$Different from OVX group ($p < 0.01$). *OVX* ovariectomy

Discussion

In this study, initially, we investigated the effect of experimental menopause on behavioral parameters. We observed no locomotor or exploratory deficits in open-field test, but evaluation of recognition memory showed an impairment in the short-term memory (tested 1 h after training) and long-term memory (tested 7 days after training) of animals subjected to experimental menopause, which was represented by similar exploration percentage of both objects (familiar and new). Based on the natural tendency of rodents to explore new objects in comparison with what is already familiar (Ennaceur and Delacour 1988), our result demonstrated an impairment in recognition memory. Furthermore, we observed the presence of negative recognition index in some groups (short- and long-term memory), showing the preference of these groups to the familiar object; however, this preference was not statistically significant.

Regarding aversive memory, assessed by the inhibitory avoidance task, we observed that ovariectomized rats present a significant impairment in long-term aversive memory (tested 7 days after training), but not in the short-term testing (tested 1 h after training). Considering that memories are divided into short-term memory, lasting minutes to hours, and long-term memory, lasting days, weeks, and even a lifetime (Bailey et al. 1996; Izquierdo and Medina 1997; Routtenberg 2008), in our study we chose a 7-day period of long-term memory because previously we showed that rats subjected to OVX had an impairment in aversive memory at 24 h after training session (Ben et al. 2010). Thus, we can suggest that the OVX causes impairment in the retention of long-term aversive memory, detected 24 h after the training and persisting for at a minimum 7 days.

Estrogen appears to have a beneficial effect on hippocampal-dependent memory (Luine 2008). Estriol, a type of estrogen, seems to be involved with differentiation and plasticity of hippocampal neurons (Audesirk et al. 2003; Solum and Handa 2002; Luine 1997), suggesting that hippocampus may be an important target of this hormone (Juraska et al. 1989; Woolley and McEwen 1992; Mukai et al. 2010; Maki 2005).

The phosphorylating system associated with cytoskeletal IF proteins in hippocampus was also evaluated. Initially, we investigated in vitro phosphorylation of NF subunits and GFAP present in the IF-enriched cytoskeletal fraction of neurons and astrocytes, respectively, in hippocampus of female adult rats; results showed that the modulation of OVX causes hyperphosphorylation of GFAP and NFs subunits without changing the IFs immunocontent. It is known that second messenger-dependent protein kinases phosphorylated specific sites located on the head domain of IF subunits (Zheng et al. 2003). Consistent with this, results showed that PKCAMII are activated by OVX, suggesting that this protein kinase is involved in GFAP and NFL phosphorylation. Moreover, NFLSer55 and NFLSer57 appeared to be specific sites targeted by OVX, and PKCaMII is the most prominent protein kinase mediating this effect. The phosphorylation of these specific sites is relevant for filament assembly (Gill et al. 1990), and

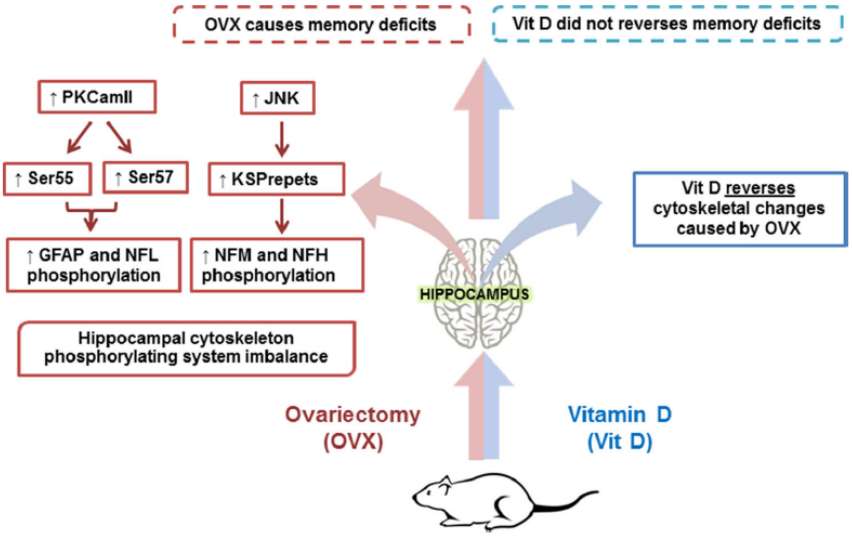


Fig. 8 Schematic figure of the main findings

dysregulation of the dynamics of phosphorylation/dephosphorylation can interfere with the functions of the neural cytoskeleton. Moreover, phosphorylation of the head domain of homopolymeric filaments like GFAP is known to play a special role in dividing cells. Therefore, abnormal phosphorylation of GFAP could lead to the disassembly of GFAP contributing to disruption of cell homeostasis (Gill et al. 1990).

KSP repeats on NFM and NFH tail domains are hyperphosphorylated in the hippocampus by OVX. Hyperphosphorylation of KSP repeats in neuronal IFs is considered an important event promoting the aggregation between NFs and causing the formation of agglomerates into the axons (Holmgren et al. 2012). These agglomerates interfere with NF axonal transport and can explain, at least in part, the behavioral deficits associated with OVX. Since these sites are phosphorylated by second messenger-independent protein kinases MAPK, we searched for the protein kinases activated by OVX and we found that JNK are phosphorylated/activated in the hippocampus. We could, therefore, propose that the tail domains of these NF subunits are phosphorylated by JNK.

Female sex hormones can modify the size, morphology and function of neural cells, and these changes are due to modification in the neuronal and glial cytoskeleton (Hansberg-Pastor et al. 2015). Several reports have demonstrated that estrogen plays an important role in regulating the cytoskeleton dynamics (Sanchez et al. 2009; Kramár et al. 2013; Giretti and Simoncini 2008). Estrogen is a key modulator of cell morphology and movement, and some of these events lead to cytoskeletal rearrangement by changes in the phosphorylation state (Hansberg-Pastor et al. 2015). Since this hormone has an important role in cytoskeleton regulation, the lack of estrogen could be deleterious for the cytoskeletal dynamics and knowledge of the mechanisms by which sex hormones OVX modulation on cytoskeletal may be important to understand the changes of learning and memory during the different stages of life.

After performing the surgical procedure (OVX) and before the assessment of behavioral and cytoskeletal tests, vitamin D supplementation was performed in the animals studied. Results showed that vitamin D was able to reverse the changes caused by OVX on cytoskeleton, but it was not able to improve the performance of animals in behavioral tasks. In addition, we observed an effect of vitamin D *per se* on recognition memory. These results are interesting to our understanding that there are not data in literature relating vitamin D supplementation, memory and ovariectomy. On the other hand, a study using adult male Wistar rats in a streptozotocin-induced diabetes model showed that cholecalciferol, at doses of 500 IU/kg/day, promoted recovery of recognition memory; however, in this study the treatment time was 10 weeks and only males were used, which did not suffer hormonal fluctuations as in females (Alrefaie and Alhayani 2015).

In order to verify whether the neuroprotection of vitamin D on cytoskeleton is due to changes in protein expression of its receptor, we analyzed the VDR immunocontent in hippocampus. The results showed that the VDR did not change in the experimental groups studied. VDR is widely distributed in the brain, particularly in hippocampus (Langub et al. 2001; Lardner 2015), a region involved in learning and memory. But in our study, results suggest that protective actions of vitamin D are not through changes in protein expression of receptors.

It has been reported that vitamin D acts through VDR modulating a complex signaling system involving rapid formation of second messengers, activation of protein kinases and the opening of Ca^{2+} channels. Considering the relevance of Ca^{2+} and second messengers on the modulation of the phosphorylating system associated with the cytoskeleton, it is likely that vitamin D is upstream of complex membrane initiated signaling pathways leading to prevention of effects caused by OVX. Consistent with this, it has been shown that the vitamin D has modulatory effects on cytoskeleton, acting through Ca^{2+} overload and adenylyl cyclase (Zanatta et al. 2011; Zamoner et al. 2008).

In conclusion, the present study showed that OVX causes an impairment in recognition and aversive memories, besides an imbalance in the hippocampal cytoskeleton phosphorylating system, causing hyperphosphorylation of IFs and changes in proteins related to this system. Vitamin D prevented cytoskeleton changes but not memory deficits (Fig. 8). Therefore, more studies are necessary to understand the neuroprotective effects of this vitamin on behavior which remain controversial. Taken together, our findings show changes that may be present in postmenopausal women and we hope to help, at least in part, in understanding the neurobiology of this important period in women's lives.

Acknowledgements This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—Brazil).

Compliance with Ethical Standards

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

Ethical Approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Human and Animal Rights All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

References

Al-Rahbi, B., Zakaria, R., Othman, Z., Hassan, A., Mohd Ismail, Z. I., & Muthuraju, S. (2014). Tualang honey supplement improves memory performance and hippocampal morphology in stressed ovariectomized rats. *Acta Histochemica, 116*(1), 79–88. doi:10.1016/j.acthis.2013.05.004.

Alrefaie, Z., & Alhayani, A. (2015). Vitamin D_3 improves decline in cognitive function and cholinergic transmission in prefrontal cortex of streptozotocin-induced diabetic rats. *Behavioural Brain Research, 287*(1), 156–162.

Amram, N., Hacohen-Kleiman, G., Sragovich, S., Malishkevich, A., Katz, J., Touloumi, O., et al. (2016). Sexual divergence in microtubule function: The novel intranasal microtubule targeting SKIP normalizes axonal transport and enhances memory. *Molecular Psychiatry*. doi:10.1038/mp.2015.208.

Arteni, N. S., Salgueiro, J., Torres, I., Achaval, M., & Netto, C. A. (2003). Neonatal cerebral hypoxia-ischemia causes lateralized memory impairments in the adult rat. *Brain Research, 973*(2), 171–178.

Audesirk, T., Cabell, L., Kern, M., & Audesirk, G. (2003). β-estradiol influences differentiation of hippocampal neurons in vitro through an estrogen receptor-mediated process. *Neuroscience, 121*(4), 927–934.

Bailey, C. H., Bartsch, D., & Kandel, E. R. (1996). Toward a molecular definition of long-term memory storage. *Proceedings of National Academy of Sciences U S A, 93*(24), 13445–13452.

Ben, J., Soares, F. M., Cechetti, F., Vuaden, F. C., Bonan, C. D., Netto, C. A., et al. (2009). Exercise effects on activities of Na(+), K(+)-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats. *Brain Research, 1302,* 248–255. doi:10.1016/j.brainres.2009.09.013.

Ben, J., Soares, F. M., Scherer, E. B., Cechetti, F., Netto, C. A., & Wyse, A. T. (2010). Running exercise effects on spatial and avoidance tasks in ovariectomized rats. *Neurobiology of Learning and Memory, 94*(3), 312–317. doi:10.1016/j.nlm.2010.07.003.

Biasibetti, H., Pierozan, P., Rodrigues, A. F., Manfredini, V., & Wyse, A. T. (2016). Hypoxanthine intrastriatal administration alters neuroinflammatory profile and redox status in striatum of infant and young adult rats. *Molecular Neurobiology*. doi:10.1007/s12035-016-9866-6.

Briones, T. L., & Darwish, H. (2012). Vitamin D mitigates age-related cognitive decline through the modulation of pro-inflammatory state and decrease in amyloid burden. *Journal of Neuroinflammation, 9,* 244. doi:10.1186/1742-2094-9-244.

Chabas, J.-F., Stephan, D., Marqueste, T., Garcia, S., Lavaut, M.-N., Nguyen, C., et al. (2013). Cholecalciferol (vitamin D_3) improves myelination and recovery after nerve injury. *PLoS ONE, 8*(5), e65034. doi:10.1371/journal.pone.0065034.

de Souza Santos, R., & Vianna, L. M. (2005). Effect of cholecalciferol supplementation on blood glucose in an experimental model of type 2 diabetes mellitus in spontaneously hypertensive rats and Wistar rats. *Clinica Chimica Acta, 358*(1), 146–150. doi:10.1016/j.cccn.2005.02.020.

Deluca, G. C., Kimball, S. M., Kolasinski, J., Ramagopalan, S. V., & Ebers, G. C. (2013). Review: The role of vitamin D in nervous system health and disease. *Neuropathology and Applied Neurobiology, 39*(5), 458–484. doi:10.1111/nan.12020.

Ennaceur, A., & Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research, 31*(1), 47–59.

Eyles, D., Almeras, L., Benech, P., Patatian, A., Mackay-Sim, A., McGrath, J., et al. (2007). Developmental vitamin D deficiency alters the expression of genes encoding mitochondrial, cytoskeletal and synaptic proteins in the adult rat brain. *Journal of Steroid Biochemistry and Molecular Biology, 103*(3–5), 538–545. doi:10.1016/j.jsbmb.2006.12.096.

Féron, F., Marqueste, T., Bianco, J., Gueye, Y., Chabas, J.-F., & Decherchi, P. (2014). Repairing the spinal cord with vitamin D: A promising strategy. *Biologie aujourd'hui, 208*(1), 69–75. doi:10.1051/jbio/2014008.

Franco, O. H., Chowdhury, R., Troup, J., Voortman, T., Kunutsor, S., Kavousi, M., et al. (2016). Use of plant-based therapies and menopausal Symptoms: A systematic review and meta-analysis. *JAMA, 315*(23), 2554–2563. doi:10.1001/jama.2016.8012.

Funchal, C., de Almeida, L. M., Oliveira Loureiro, S., Vivian, L., de Lima Pelaez, P., Pessutto, F. D. B., et al. (2003). In vitro phosphorylation of cytoskeletal proteins from cerebral cortex of rats. *Brain Research. Brain Research Protocols,11*(2), 111–118.

Gill, S. R., Wong, P. C., Monteiro, M. J., & Cleveland, D. W. (1990). Assembly properties of dominant and recessive mutations in the

small mouse neurofilament (NF-L) subunit. *Journal of Cell Biology, 111*(5 Pt 1), 2005–2019.

Giretti, M. S., & Simoncini, T. (2008). Rapid regulatory actions of sex steroids on cell movement through the actin cytoskeleton. *Steroids, 73*(9–10), 895–900. doi:10.1016/j.steroids.2008.01.011.

Grant, M. D., Marbella, A., Wang, A. T., Pines, E., Hoag, J., Bonnell, C., et al. (2015). *Menopausal symptoms: Comparative effectiveness of therapies* (AHRQ Comparative Effectiveness Reviews). Rockville (MD): Agency for Healthcare Research and Quality (US). Report No.: 15-EHC005-EF.

Grant, P., & Pant, H. C. (2000). Neurofilament protein synthesis and phosphorylation. *Journal of Neurocytology, 29*(11–12), 843–872.

Gueye, Y., Marqueste, T., Maurel, F., Khrestchatisky, M., Decherchi, P., & Feron, F. (2015). Cholecalciferol (vitamin D(3)) improves functional recovery when delivered during the acute phase after a spinal cord trauma. *Journal of Steroid Biochemistry and Molecular Biology, 154,* 23–31. doi:10.1016/j.jsbmb.2015.06.007.

Hansberg-Pastor, V., Gonzalez-Arenas, A., Pina-Medina, A. G., & Camacho-Arroyo, I. (2015). Sex hormones regulate cytoskeletal proteins involved in brain plasticity. *Frontiers in Psychiatry, 6,* 165. doi:10.3389/fpsyt.2015.00165.

Hoffman, P. N., Cleveland, D. W., Griffin, J. W., Landes, P. W., Cowan, N. J., & Price, D. L. (1987). Neurofilament gene expression: a major determinant of axonal caliber. *Proceedings of National Academy of Sciences U S A, 84*(10), 3472–3476.

Holick, M. F. (2007). Vitamin D deficiency. *New England Journal of Medicine, 357*(3), 266–281. doi:10.1056/NEJMra070553.

Holmgren, A., Bouhy, D., & Timmerman, V. (2012). Neurofilament phosphorylation and their proline-directed kinases in health and disease. *Journal of Peripheral Nervous System, 17*(4), 365–376. doi:10.1111/j.1529-8027.2012.00434.x.

Izquierdo, I., & Medina, J. H. (1997). Memory formation: The sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiology of Learning and Memory, 68*(3), 285–316. doi:10.1006/nlme.1997.3799.

Juraska, J. M., Fitch, J. M., & Washburne, D. L. (1989). The dendritic morphology of pyramidal neurons in the rat hippocampal CA3 area. II. Effects of gender and the environment. *Brain Research, 479*(1), 115–119.

Kramár, E. A., Babayan, A. H., Gall, C. M., & Lynch, G. (2013). Estrogen promotes learning-related plasticity by modifying the synaptic cytoskeleton. *Neuroscience, 239,* 3–16. doi:10.1016/j.neuroscience.2012.10.038.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.

Lamprecht, R. (2016). The role of actin cytoskeleton in memory formation in amygdala. *Frontiers in Molecular Neuroscience, 9,* 23. doi:10.3389/fnmol.2016.00023.

Langub, M. C., Herman, J. P., Malluche, H. H., & Koszewski, N. J. (2001). Evidence of functional vitamin D receptors in rat hippocampus. *Neuroscience, 104*(1), 49–56.

Lardner, A. L. (2015). Vitamin D and hippocampal development-the story so far. *Frontiers in Molecular Neuroscience, 8,* 58. doi:10.3389/fnmol.2015.00058.

Liebert, A. D., Chow, R. T., Bicknell, B. T., & Varigos, E. (2016). Neuroprotective effects against POCD by photobiomodulation: Evidence from assembly/disassembly of the cytoskeleton. *Journal of Experimental Neuroscience, 10,* 1–19. doi:10.4137/JEN.S33444.

Lobo, R. A., Davis, S. R., De Villiers, T. J., Gompel, A., Henderson, V. W., Hodis, H. N., et al. (2014). Prevention of diseases after menopause. *Climacteric: The journal of the International Menopause Society, 17*(5), 540–556. doi:10.3109/13697137.2014.933411.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.

Luine, V. N. (1997). Steroid Hormone modulation of hippocampal dependent spatial memory. *Stress, 2*(1), 21–36.

Luine, V. N. (2008). Sex steroids and cognitive function. *Journal of Neuroendocrinology, 20*(6), 866–872. doi:10.1111/j.1365-2826.2008.01710.x.

Luine, V. N. (2014). Estradiol and cognitive function: Past, present and future. *Hormones and Behavior, 66*(4), 602–618. doi:10.1016/j.yhbeh.2014.08.011.

Mackedanz, V., Mattos, C. B., Feksa, L. R., Wannmacher, C. M., & Wyse, A. T. (2011). Ovariectomy alters energy metabolism in rat striatum: Effect of supplementation with soy diet rich in isoflavones. *Metabolic Brain Disease, 26*(2), 97–105. doi:10.1007/s11011-010-9216-8.

Maki, P. M. (2005). Estrogen effects on the hippocampus and frontal lobes. *International Journal of Fertility Women's Medicine, 50*(2), 67–71.

Middeldorp, J., & Hol, E. M. (2011). GFAP in health and disease. *Progress in Neurobiology, 93*(3), 421–443. doi:10.1016/j.pneurobio.2011.01.005.

Miquel, J., Ramírez-Boscá, A., Ramírez-Bosca, J. V., & Alperi, J. D. (2006). Menopause: A review on the role of oxygen stress and favorable effects of dietary antioxidants. *Archives of Gerontology and Geriatrics, 42*(3), 289–306. doi:10.1016/j.archger.2005.08.005.

Monteiro, S. C., de Mattos, C. B., Ben, J., Netto, C. A., & Wyse, A. T. S. (2008). Ovariectomy impairs spatial memory: prevention and reversal by a soy isoflavone diet. *Metabolic Brain Disease, 23*(3), 243–253. doi:10.1007/s11011-008-9093-6.

Monteiro, S. C., Matté, C., Bavaresco, C. S., Netto, C. A., & Wyse, A. T. S. (2005a). Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. *Neurobiology of Learning and Memory, 84*(3), 192–199. doi:10.1016/j.nlm.2005.08.002.

Monteiro, S. C., Matte, C., Delwing, D., & Wyse, A. T. (2005b). Ovariectomy increases Na + , K + -ATPase, acetylcholinesterase and catalase in rat hippocampus. *Molecular and Cellular Endocrinology, 236*(1–2), 9–16. doi:10.1016/j.mce.2005.03.006.

Mukai, H., Kimoto, T., Hojo, Y., Kawato, S., Murakami, G., Higo, S., et al. (2010). Modulation of synaptic plasticity by brain estrogen in the hippocampus. *Biochimica et Biophysica Acta, 1800*(10), 1030–1044. doi:10.1016/j.bbagen.2009.11.002.

Nissou, M. F., Brocard, J., El Atifi, M., Guttin, A., Andrieux, A., Berger, F., et al. (2013). The transcriptomic response of mixed neuron-glial cell cultures to 1,25-dihydroxyvitamin d3 includes genes limiting the progression of neurodegenerative diseases. *Journal of Alzheimer's Disease, 35*(3), 553–564. doi:10.3233/JAD-122005.

Omary, M. B., Ku, N. O., Tao, G. Z., Toivola, D. M., & Liao, J. (2006). "Heads and tails" of intermediate filament phosphorylation: multiple sites and functional insights. *Trends in Biochemical Sciences, 31*(7), 383–394. doi:10.1016/j.tibs.2006.05.008.

Pessoa-Pureur, R., Heimfarth, L., & Rocha, J. B. (2014). Signaling mechanisms and disrupted cytoskeleton in the diphenyl ditelluride neurotoxicity. *Oxidative Medicine and Cellular Longevity, 2014,* 458601. doi:10.1155/2014/458601.

Pierozan, P., Zamoner, A., Soska, A. K., Silvestrin, R. B., Loureiro, S. O., Heimfarth, L., et al. (2010). Acute intrastriatal administration of quinolinic acid provokes hyperphosphorylation of cytoskeletal

intermediate filament proteins in astrocytes and neurons of rats. *Experimental Neurology, 224*(1), 188–196. doi:10.1016/j.expneurol.2010.03.009.

Rettberg, J. R., Yao, J., & Brinton, R. D. (2013). Estrogen: A master regulator of bioenergetic systems in the brain and body. *Frontiers in Neuroendocrinology*. doi:10.1016/j.yfrne.2013.08.001.

Rocca, W. A., Grossardt, B. R., Miller, V. M., Shuster, L. T., & Brown, R. D., Jr. (2012). Premature menopause or early menopause and risk of ischemic stroke. *Menopause, 19*(3), 272–277. doi:10.1097/gme.0b013e31822a9937.

Rocca, W. A., Grossardt, B. R., & Shuster, L. T. (2010). Oophorectomy, menopause, estrogen, and cognitive aging: the timing hypothesis. *Neurodegenerative Diseases, 7*(1–3), 163–166. doi:10.1159/000289229.

Rojas, J. J., Deniz, B. F., Miguel, P. M., Diaz, R., Hermel, E. E., Achaval, M., et al. (2013). Effects of daily environmental enrichment on behavior and dendritic spine density in hippocampus following neonatal hypoxia-ischemia in the rat. *Experimental Neurology, 241,* 25–33. doi:10.1016/j.expneurol.2012.11.026.

Routtenberg, A. (2008). The substrate for long-lasting memory: If not protein synthesis, then what? *Neurobiology of Learning and Memory, 89*(3), 225–233. doi:10.1016/j.nlm.2007.10.012.

Salum, E., Kals, J., Kampus, P., Salum, T., Zilmer, K., Aunapuu, M., et al. (2013). Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats. *Diabetes Research and Clinical Practice, 100*(2), 243–249. doi:10.1016/j.diabres.2013.03.008.

Salum, E., Kampus, P., Zilmer, M., Eha, J., Butlin, M., Avolio, A. P., et al. (2012). Effect of vitamin D on aortic remodeling in streptozotocin-induced diabetes. *Cardiovascular Diabetology, 11,* 58. doi:10.1186/1475-2840-11-58.

Sanches, E. F., Arteni, N. S., Scherer, E. B., Kolling, J., Nicola, F., Willborn, S., et al. (2013). Are the consequences of neonatal hypoxia-ischemia dependent on animals' sex and brain lateralization? *Brain Research, 1507,* 105–114. doi:10.1016/j.brainres.2013.02.040.

Sanchez, A. M., Flamini, M. I., Fu, X. D., Mannella, P., Giretti, M. S., Goglia, L., et al. (2009). Rapid signaling of estrogen to WAVE1 and moesin controls neuronal spine formation via the actin cytoskeleton. *Molecular Endocrinology, 23*(8), 1193–1202. doi:10.1210/me.2008-0408.

Scott, E. L., Zhang, Q. G., Vadlamudi, R. K., & Brann, D. W. (2014). Premature menopause and risk of neurological disease: basic mechanisms and clinical implications. *Molecular and Cellular Endocrinology, 389*(1–2), 2–6. doi:10.1016/j.mce.2014.01.013.

Sherwin, B. B. (2003). Estrogen and cognitive functioning in women. *Endocrine Reviews, 24*(2), 133–151.

Shoupe, D. (2011). Individualizing hormone therapy to minimize risk: Accurate assessment of risks and benefits. *Womens Health (Lond), 7*(4), 475–485. doi:10.2217/whe.11.42.

Shuster, L. T., Rhodes, D. J., Gostout, B. S., Grossardt, B. R., & Rocca, W. A. (2010). Premature menopause or early menopause: Long-term health consequences. *Maturitas, 65*(2), 161–166. doi:10.1016/j.maturitas.2009.08.003.

Siebert, C., Kolling, J., Scherer, E. B., Schmitz, F., da Cunha, M. J., Mackedanz, V., et al. (2014). Effect of physical exercise on changes in activities of creatine kinase, cytochrome c oxidase and ATP levels caused by ovariectomy. *Metabolic Brain Disease, 29*(3), 825–835. doi:10.1007/s11011-014-9564-x.

Sihag, R. K., Inagaki, M., Yamaguchi, T., Shea, T. B., & Pant, H. C. (2007). Role of phosphorylation on the structural dynamics and function of types III and IV intermediate filaments. *Experimental Cell Research, 313*(10), 2098–2109. doi:10.1016/j.yexcr.2007.04.010.

Snider, N. T., & Omary, M. B. (2014). Post-translational modifications of intermediate filament proteins: Mechanisms and functions. *Nature Reviews Molecular Cell Biology, 15*(3), 163–177. doi:10.1038/nrm3753.

Solum, D. T., & Handa, R. J. (2002). Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus. *Journal of Neuroscience, 22*(7), 2650–2659.

Soules, M. R., Sherman, S., Parrott, E., Rebar, R., Santoro, N., Utian, W., et al. (2001). Executive summary: Stages of reproductive aging workshop (STRAW) Park City, Utah, July, 2001. *Menopause, 8*(1072-3714 (Print)), 402–407.

Takahashi, T. A., & Johnson, K. M. (2015). Menopause. *Medical clinics of North America, 99*(3), 521–534. doi:10.1016/j.mcna.2015.01.006.

Waynforth, H. B., & Flecknell, P. A. (1992). *Experimental and surgical technique in the rat* (2nd ed.). New York: Academic press.

Woolley, C. S., & McEwen, B. S. (1992). Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *Journal of Neuroscience, 12*(7), 2549–2554.

Zamoner, A., Pierozan, P., Vidal, L. F., Lacerda, B. A., Dos Santos, N. G., Vanzin, C. S., et al. (2008). Vimentin phosphorylation as a target of cell signaling mechanisms induced by 1α,25-dihydroxyvitamin D_3 in immature rat testes. *Steroids, 73*(14), 1400–1408. doi:10.1016/j.steroids.2008.07.002.

Zanatta, L., Zamoner, A., Zanatta, A. P., Bouraima-Lelong, H., Delalande, C., Bois, C., et al. (2011). Nongenomic and genomic effects of 1α,25(OH)2 vitamin D3 in rat testis. *Life Sciences, 89*(15–16), 515–523. doi:10.1016/j.lfs.2011.04.008.

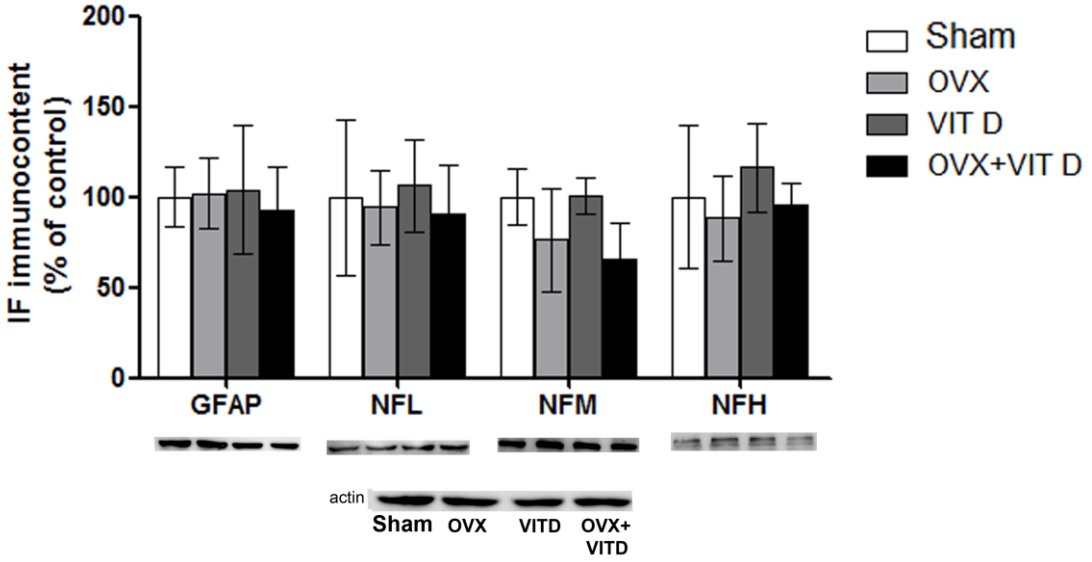
Zhang, Q. G., Wang, R. M., Scott, E., Han, D., Dong, Y., Tu, J. Y., et al. (2013). Hypersensitivity of the hippocampal CA3 region to stress-induced neurodegeneration and amyloidogenesis in a rat model of surgical menopause. *Brain, 136*(Pt 5), 1432–1445. doi:10.1093/brain/awt046.

Zheng, Y. L., Li, B. S., Veeranna, & Pant, H. C. (2003). Phosphorylation of the head domain of neurofilament protein (NF-M): a factor regulating topographic phosphorylation of NF-M tail domain KSP sites in neurons. *Journal of Biological Chemistry, 278*(26), 24026–24032. doi:10.1074/jbc.M303079200.

Vitamin D_3 reverses the hippocampal cytoskeleton imbalance but not memory deficits caused by ovariectomy in adult Wistar rats

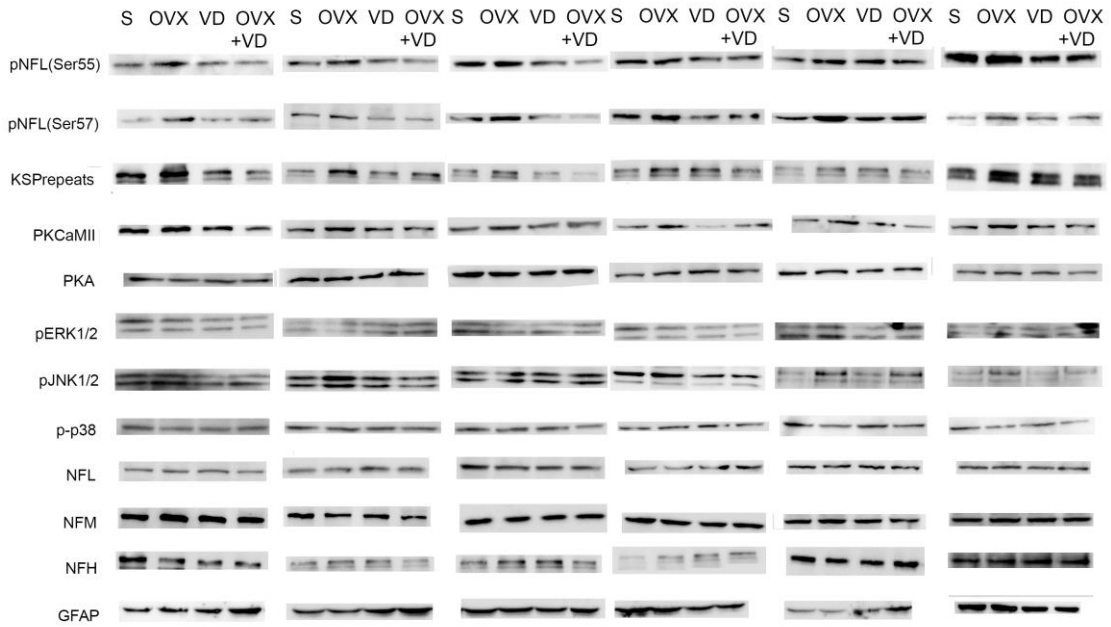
Cassiana Siebert[a], Paula Pierozan[a,c], Janaina Kolling[a], Tiago Marcon dos Santos[a], Matheus Coimbra Sebotaio[a], Eduardo Peil Marques[a], Helena Biasibetti[a], Aline Longoni[a], Fernanda Ferreira[c], Regina Pessoa-Pureur[c], Carlos Alexandre Netto[b], Angela T.S. Wyse[a*]

Eletronical Supplementary Material

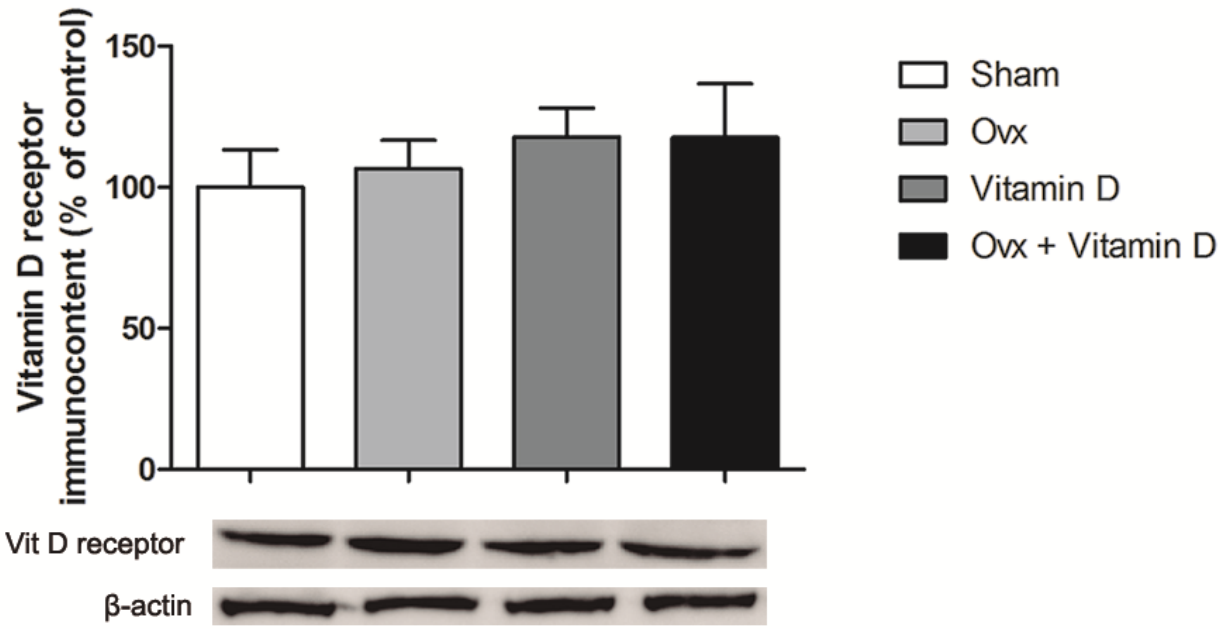


Supplemental Fig 1 Effect of OVX and vitamin D supplementation on immunocontent of intermediate filament in the cytoskeletal fraction (a) from hippocampus slices of female adult rats Representative Western blots of proteins studied are shown (b). β-actin was used as loading control. Bars represent the means±SD of percentage of controls. Data were analyzed by one-way ANOVA (n= 6 animals per group) and was considered significant as $p<0.05$. OVX – ovariectomy; IF – intermediate filaments; GFAP – glial fibrilar

acid protein; NFL – low molecular weight neurofilament subunit; NFM - middle molecular weight neurofilament subunit and NFH - high molecular weight neurofilament subunit



Supplemental Fig 2 Immunoblots of all proteins investigated in hippocampus of adult ovariectomized rats and vitamin D supplemented. β-actin was used as loading control. OVX – ovariectomy; VD- vitamin D.



Supplemental Fig 3 Effect of OVX and vitamin D supplementation on VDR immunocontent in hippocampus of female adult rats. Results are expressed as mean ± SD. Data were analyzed by one-way ANOVA (n = 6 animals per group) and was considered significant as $p<0.05$. VDR- vitamin D receptor; OVX- ovariectomy

Capítulo II

Vitamin D partially reverses the increase in p-NF-κB/p65 immunocontent and interleukin-6 levels, but not in acetylcholinesterase activity in hippocampus of adult female rats ovariectomized

Cassiana Siebert[1], Carolina Gessinger Bertó[1], Fernanda Silva Ferreira[1], Daniella de Souza Moreira[1], Tiago Marcon dos Santos[1], Angela T.S. Wyse[1*]

Periódico: International Journal of Developmetal Neuroscience

Status: Submetido

Vitamin D partially reverses the increase in p-NF-κB/p65 immunocontent and interleukin-6 levels, but not in acetylcholinesterase activity in hippocampus of adult female ovariectomized rats

Cassiana Siebert[1], Carolina Gessinger Bertó[1], Fernanda Silva Ferreira[1], Daniella de Souza Moreira[1], Tiago Marcon dos Santos[1], Angela Terezinha de Souza Wyse[1*]

[1]Laboratório de Neuroproteção e Doenças Neurometabólicas, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.

*Address reprint request to: Dr. Angela T. S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Phone 55 51 3316 5573, Fax 55 51 3316 5535, E-mail: wyse@ufrgs.br

ABBREVIATIONS LIST

ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
ChAT	Choline acetyltransferase
CNS	Central nervous system
HRT	Hormone replacement therapy
IL-1β	Interleukin-1β
IL-6	Interleukin 6
NF-kB	Nuclear factor-kappa B
OVX	Ovariectomy
OVX+VIT D	Ovariectomy + Vitamin D supplementation
p-NF-kB	phosphorylated Nuclear factor-kappa B
TNF-α	Tumor necrosis factor-α
VDR	Vitamin D receptor
VIT D	Vitamin D

ABSTRACT

The aim of this study was to verify the effects of ovariectomy (OVX) and/or vitamin D supplementation (VIT D) on inflammatory and cholinergic parameters in hippocampus, as well as on serum estradiol and VIT D levels of rats. Ninety-day-old female Wistar rats were randomly divided into four groups: SHAM, OVX, VIT D or OVX+VIT D. Thirty days after OVX, VIT D (500 IU/kg/day) was supplemented by gavage, for 30 days. Approximately 12 hours after the last VIT D administration, rats were decapitated without anesthesia; the hippocampus and serum were obtained for further analysis. Results showed that OVX rats presented a decrease in estradiol levels when compared to control (SHAM). There was an increase in VIT D levels in the groups submitted to VIT D supplementation. OVX increased the immunocontent of nuclear p-NF-κB/p65, TNF-α and IL-6 levels. VIT D partially reversed the increase in p-NF-κB/p65 immunocontent and IL-6 levels. Regarding cholinergic system, OVX caused an increase in acetylcholinesterase activity without changing acetylcholinesterase and choline acetyltransferase immunocontents. VIT D did not reverse the increase in acetylcholinesterase activity caused by OVX. These results demonstrate that OVX alters inflammatory and cholinergic systems parameters and that VIT D supplementation at the dose used, partially reversed the increase in immunocontents of p-NF-Kb/p65 and IL-6 levels, but it was not able to reverse the other parameters studied. We hope to assist in changes understanding that occurs after menopause and open new perspectives for research involving VIT D therapies.

Keywords: Vitamin D; inflammation; acetylcholinesterase; cholinergic system; choline acetyltransferase; ovariectomy.

1. Introduction

17β-Estradiol (estrogen, estradiol) is a steroid hormone secreted mainly by ovaries and known to exert anti-inflammatory and neuroprotective functions in several organs and systems, including central nervous system (SNC) (Pozzi et al., 2006; Pratap et al., 2016; Villa et al., 2016). The estrogenic deprivation that occurs in menopause may be associated with cognitive impairment and increased risk for neurodegenerative diseases development (Georgakis et al., 2016; Henderson, 2014). In addition, the abrupt reduction of circulating estrogen caused by surgical menopause has been related to increasing in body fat, drop in basal metabolism and also increase of inflammatory mediators (Ben-Shmuel et al., 2015; Pacifici et al., 1989; Van Pelt et al., 2015; Vegeto et al., 2006; Vieira-Potter et al., 2015).

Nuclear factor-kappa B (NF-κB) is an important mediator of the responses triggered by inflammation and oxidative stress (Lawrence, 2009; Sies et al., 2017; Sunday et al., 2007). It is a nuclear transcription factor and its activation is related to transcription regulation of a large number of genes, including proinflammatory cytokines, chemokines, stress-response proteins and anti-apoptotic proteins (Lawrence, 2009; Li and Verma, 2002). Proinflammatory cytokines may be secreted by activated microglia in response to several inflammatory/oxidative stimulus and increased levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), Interleukin-1β (IL-1β) and Interleukin 6 (IL-6) have been related to neurodegenerative conditions (Colonna and Butovsky, 2017; Kempuraj et al., 2017). In this context, literature studies have associated the ovariectomy (OVX), a surgical model of menopause, with the presence of an inflammatory phenotype (Au et al., 2016).

Acetylcholine (ACh) is an important neurotransmitter in the central and peripheral nervous system. ACh together with the enzyme acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) constitute of brain cholinergic system, known to modulate several important functions, such as learning and memory (Perry et al., 1999; Peter et al., 2016; Sarter and Bruno, 1997). In this way, recently we have shown that OVX impairs memory and increases AChE activity in hippocampus of adult rats (Monteiro et al., 2008; Monteiro et al., 2005; Siebert et al., 2017). It has been shown that the cholinergic system is associated with inflammation mechanisms through the "cholinergic anti-inflammatory pathway", suggesting that ACh would act through the transmission of neural signals via the vagus nerve to inhibiting the release of peripheral cytokines (Han et al., 2017; Pavlov and Tracey, 2005; Rosas-Ballina and Tracey, 2009).

Hormone replacement therapy (HRT) is widely prescribed to postmenopausal women for treatment and relief of signs and symptoms. Although effective in some cases, adverse effects of this therapy are widely reported (Sohrabji and Bake, 2006), therefore, it is very important the search for alternative and/or complementary therapies in order to obtain better results for the general health of women in the postmenopausal period. In this context, vitamin D (VIT D) is considered a steroid hormone beneficial for calcium homeostasis with antioxidant and anti-inflammatory activities (Di Somma et al., 2017; Hajiluian et al., 2017; Longoni et al., 2017). In humans, the main sources of VIT D are the endogenous formation as result of ultraviolet B radiation exposure (Barberger-Gateau, 2014; Di Somma et al., 2017; Mpandzou et al., 2016; Stroud et al., 2008) and the diet, which has become an important

alternative source of this vitamin. Studies described in the literature have already shown some benefits of VIT D supplementation on changes associated with OVX (Babaei et al., 2017; Li et al., 2016; Siebert et al., 2017).

In this work we investigate the effects of OVX on inflammatory and cholinergic system in hippocampus of adult female Wistar rats. Serum levels of estradiol and VIT D, as well as body weight were evaluated. The possible neuroprotective role of VIT D supplementation on changes caused by OVX was also investigated. Our hypothesis is that OVX causes changes related to hippocampal inflammatory and cholinergic systems, and that VIT D supplementation acts in a beneficial way in reversing such changes.

2. Material and Methods

2.1. Animals

Ninety-day-old female Wistar rats were obtained from the Central Animal House of the Department of Biochemistry at the Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. Animals were housed in plastic cages and maintained at a constant temperature (22 °C) in a light/dark cycle 12:12 h with free access to water and receiving specific commercial chow (pelleted food containing in its composition 2.000 IU/kg of VIT D3). The ethical standards followed the official governmental guidelines issued by the Brazilian Federation of Societies for Experimental Biology, following the Guide for Care and Use of Laboratory Animals and Arouca Law (Law n° 11.794/2008). Animal experimentation protocols had been approved by the University Ethics Committee for the Use of Animals (CEUA) under the project #28033.

2.2. Experimental groups

Female Wistar rats (90-day-old) were randomly divided into four groups: (1) SHAM (submitted to surgery without ovaries removal); (2) OVX (submitted to surgical removal of both ovaries); (3) VIT D or (4) OVX+VIT D. The summary of the experimental design can be seen in Fig 1.

2.3. Ovariectomy procedure

Ninety-day-old female Wistar rats were anesthetized by intraperitoneal (i.p.) administration of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) and subsequently submitted to a surgical procedure for removing both ovaries (OVX) according to Mackedanz et al. (2011) in order to eliminate endogenous ovarian steroids and mimics the effects observed after menopause.

2.4. Vitamin D supplementation

Thirty days after the OVX, the animals received the daily supplement with VIT D (200 µL) for 30 days by gavage. Control groups (SHAM and OVX) received an equal volume of vehicle (propylene glycol). Based on previous work the dose of VIT D (cholecalciferol – vitamin D3; Sigma-Aldrich®) used was 500 IU/kg/day (Chabas et al., 2013; de Souza Santos and Vianna, 2005; Gueye et al., 2015; Salum et al., 2013; Siebert et al., 2017). Approximately 12 hours after the last administration, rats were decapitated without anesthesia and the blood and hippocampus were removed for further analysis.

2.5. Evaluation of daily food consumption and body weight

The daily food consumption was evaluated through the control of the weight of food (g) ingested daily per rat (15 days of evaluation). For this, the amount of food consumed per box (g) was divided by the number of animals per box, generating an estimate of daily food consumption (g). We also performed the control of the body weight (g) of animals before OVX, during treatment with VIT D (weekly, for VIT D dose adjustment) and at the end of experiment.

2.6. Serum estradiol and calcidiol levels measurements

The hormonal determinations were performed in serum. Blood was collected and centrifuged at 1000xg for 10 min at 4°C, and serum was removed by suction. Estradiol levels were detected by radioimmunoassay method and calcidiol (25(OH)D) levels were detected by the chemiluminescent method. The results were calculated as picogram per ml (pg/ml) for estradiol levels and nanogram/ml (ng/ml) for calcidiol levels.

2.7. Cytokine levels measurements

The hippocampus was homogenized in 1:5 (w/v) saline solution (0.9% NaCl). The homogenate was centrifuged at 800×g for 10 min at 4 °C, and the supernatant was used for analysis. TNF-α, IL-1β and IL-6 levels in the hippocampus were measured by kit rat high-sensitivity enzyme-linked immunoabsorbent assays (ELISA) with commercially available kits (Sigma-Aldrich®).

2.8. Acetylcholinesterase activity measurement

The hippocampus was homogenized in 10 volumes (1:10, w/v) of 0.1 mM potassium phosphate buffer (pH 7.5) and centrifuged at 1000xg for 10 min at 4 °C. AChE activity was determined in the supernatant according to the method of Ellman et al. (1961), with modifications (Scherer et al., 2010). Hydrolysis of rates were measured at acetylthiocholine concentration of 0.8 mM in 300 µL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic acid - DTNB) at 25 °C. Fifty microliters of hippocampus supernatant was added to reaction mixture and incubated for 3 min. The hydrolysis was monitored by the formation of the thiolate di-anion of DTNB at 412 nm for 2–3 min, measured at intervals of 30 s.

2.9. Cellular preparation for cytosolic and nuclear detection of NF-κB/p65 immunocontent

Cellular preparation for detection of cytosolic and nuclear fractions of NF-κB/p65 and NF-κB/p65 phosphorylated subunit (p-NF-κB/p65) was realized as described by da Cunha et al. (2012). Hippocampus was homogenized in hypotonic lysis buffer (300 µL) containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 5 mM NaF, 1 mM sodium orthovanadate plus protease inhibitor cocktail. Subsequently, the samples homogenate were lysed with 18 µL 10% IGEPAL. The homogenate was then centrifuged (14.000×g, 30s, 4 °C), the supernatants containing the cytosolic fraction were stored at −80 °C and the nuclear pellet was resuspended in 200 µL ice-cold hypertonic extraction buffer containing 10 mM HEPES (pH 7.9), 0.40 M NaCl, 1.5 mM MgCl2, 10 mM KCl,

0.5 mM PMSF, 1 mM DTT, 5 mM NaF, 1 mM sodium orthovanadate, 0.25 mM EDTA, 25% glycerol plus protease inhibitor cocktail. The extract resulting was mixed for 40 min and centrifuged at 14.000×g, 10 min, 4 °C. The supernatants containing nuclear protein were stored at −80 °C. Lastly, aliquot of samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, and 50 mM Tris-HCl, pH 6.8, for electrophoresis analysis.

2.10. Tissue preparation for detection of acetylcholinesterase and choline acetyltransferase immunocontents

The hippocampus was homogenized in 200 uL of a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4% sodium dodecyl sulfate (SDS). The samples were dissolved 1:1 in Laemmli buffer 2x containing 40% glycerol, 5% 2-mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and 10 % SDS, and boiled for 5 min. The samples were stored at −80 °C (Biasibetti-Brendler et al., 2017).

2.11. Western blotting analysis of NF-κB/p65, acetylcholinesterase and choline acetyltransferase

Total protein homogenate were separated by 10 % SDS–PAGE (20 μg/lane of total protein and 10 μg/lane of total protein for nuclear fraction of NF-κB/p65) and transferred (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). Blot was then incubated overnight at 4°C in a blocking solution containing 5% bovine serum albumin (BSA) and the following diluted antibodies: mouse anti-β-actin (1:1000, Sigma-

Aldrich), rabbit anti-AChE (1:1000, Santa Cruz Biotechnology), goat anti-ChAT (1:1000, Sigma-Aldrich), rabbit anti-NF-κB/p65 (1:1000; Santa Cruz Biotechnology) or rabbit anti-p-NF-κB/p65 (1:1000; Santa Cruz Biotechnology). For detection of AChE immunocontent, the blot was washed twice for 5 min with 0.05% Tween-20 Tris-buffered saline (T-TBS) and twice for 5 min with Tris-buffered saline (TBS), followed by incubation for 2 hours in antibody solution containing non-conjugated anti-rabbit IgG diluted 1:1000. Later, the blot was washed as above described and then incubated for 1h in a solution containing streptavidin-HRP (Cell Signaling Technology) diluted 1:3000. For the other antibodies, the blot was washed twice for 5 min with T-TBS and twice for 5 min with TBS, followed by incubation for 2 hours with peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology) diluted 1:1000 for β-actin, anti-rabbit IgG (Cell Signaling Technology) diluted 1:2000 for NF-κB/p65 and anti-goat IgG diluted 1:3000 for ChAT. The blot was developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected by ImageQuant LAS 4000, GE Healthcare Life Sciences (Biasibetti-Brendler et al., 2017).

2.12. Protein determination

The determination of total protein was performed by a colorimetric method (Lowry et al., 1951), using serum bovine albumin as standard.

2.13. Statistical analysis

The data were analyzed by two-way analysis of variance (ANOVA) followed by post hoc Tukey´s test. Values of $p<0.05$ were considered

statistically significant. All analyses and plots were performed using GraphPad Prism 5.1 software program in a compatible computer.

3. Results

3.1. Serum levels of estradiol and calcidiol, body weight and daily food intake of Wistar rats submitted to OVX and/or VIT D supplementation

Serum estradiol levels were measured in order to confirm the efficacy of OVX. As can be observed in Table 1, estradiol levels were significantly altered by OVX [$F(1,19)=11.82$, $p=0.0028$] and there was no effect of VIT D supplementation [$F(1,19)=0,6919$, $p=0.4158$] nor interaction [$F(1,19)=1.493$, $p=0.2366$]. Post hoc Tukey´s test analysis revealed that OVX and OVX+VIT D groups present a decrease in estradiol levels compared to non-ovariectomized groups SHAM and VIT D ($p<0.05$). The measure of serum calcidiol levels was performed in order to confirm the efficacy of VIT D supplementation in increasing their circulating levels (Table 1). Serum calcidiol levels was altered by VIT D supplementation [$F(1,18)=20.89$, $p=0.0002$]. There was no effect of OVX [$F(1,18)=1.138$, $p=0.3001$], and no interactions [$F(1,18)=0.09612$, $p=0.7601$]. Post hoc Tukey´s test analysis revealed that there was an increase in calcidiol levels in VIT D and VIT D+OVX groups when compared to groups SHAM and OVX that received vehicle ($p<0.05$).

We also analyzed the body weight and food intake of rats. Table 1 showed that the body weight of the rats at the end of experiment was altered by OVX [$F(1,8)=31.15$, $p=0.0005$]. There was no effect of VIT D supplementation [$F(1,8)=0.035$, $p=0.8554$] and no interactions [$F(1,8)=0.854$, $p=0.3743$]. Post

hoc Tukey´s test analysis revealed that OVX and OVX+VIT D groups present an increase in body weight compared to controls groups SHAM and VIT D ($p<0.05$). Daily food intake was not altered by OVX [$F(1,20)=0.8781$, $p=0.3599$], nor by VIT D supplementation [$F(1,20)=0.6652$, $p=0.4243$], and no interactions were seen [$F(1,20)=0.1593$, $p=0.6940$].

3.2. Effect of OVX and/or VIT D supplementation on parameters related to hippocampal inflammation in adult Wistar rats

Fig 2 shows the effect of OVX and/or VIT D supplementation on immunocontent of cytosolic and nuclear fraction of NF-κB/p65 and p-NF-κB/p65. Two-way ANOVA revealed a significant interaction between OVX and vehicle administration on nuclear fraction of p-NF-κB/p65 immunocontent [$F(1,19)=10.19$, $p=0.0037$; Fig 2d]. There was no effect of OVX, neither of VIT D supplementation on this parameter [$F(1,19)=2.957$, $p=0.1018$; $F(1,19)=01045$, $p=0.9196$, respectively; Fig 2d]. Analyzing the interaction, post hoc Tukey´s test analysis revealed that the immunocontent of nuclear fraction of p-NF-κB/p65 was significantly increased by OVX in the groups that received vehicle administration (SHAM and OVX; $p<0.01$; Fig 2d). VIT D supplementation partially reversed the increase in nuclear fraction of p-NF-κB/p65 caused by OVX. No alterations caused by OVX were observed on immunocontent of cytosolic fraction of NF-κB/p65 and p-NF-κB/p65, as well as in the immunocontent of nuclear fraction NF-κB/p65 [$F(1,20)=0.4756$, $p=0.4984$; $F(1,20)=0.01656$; $p=0.8989$; $F(1,20)=0.5836$; $p=0.4543$, respectively]. There was no effect of VIT D on these parameters [cytosolic fraction of NF-κB/p65: $F(1,20)=0.3884$, $p=0.5402$; cytosolic fraction of p-NF-κB/p65: $F(1,20)=0.01660$,

p=0.8988; nuclear fraction of NF-κB/p65: F(1,20)=0.02862, p=0.8674] and no interactions were observed [cytosolic fraction of NF-κB/p65: F(1,19)=0.01182, p=0.9145; cytosolic fraction of p-NF-κB/p65: F(1,19)=0.3543, p=0.5584; nuclear fraction of NF-κB/p65: F(1,19)=0.5134, p=0.4824] (Fig 2a, Fig 2b and Fig 2c).

Since active form NF-κB promotes gene expression of proinflammatory molecules, we evaluated the effect of OVX and/or VIT D supplementation on TNF-α, IL-1β and IL-6 in hippocampus of adult rats. Fig 3 shows that OVX induced an increase in TNF-α (Fig 3a) and IL-6 (Fig 3c) levels in rat hippocampus [F(1,16)=30.97, p<0.0001 and F(1,16)=5.25, p=0.0358, respectively], but not in IL-1β levels (Fig 3b) [F(1,16)=1.444, p=0.2470]. There was no effect of VIT D supplementation on these parameters [TNF-α:F(1,16)=0.1020, p=0.7536; IL-1β: F(1,16)=2.424, p=0.1390; IL-6: F(1,16)=0.01644, p=0.8990] and interaction was observed only in IL-6 [F(1,16)=8.820, p=0.0090]. Post hoc Tukey's test analysis revealed that the OVX and OVX+VIT D groups present an increase of TNF-α levels compared to SHAM (p<0.01; Fig 3a). Regarding IL-6 levels (Fig 3c), OVX group presents an increase in this parameter compared to SHAM (p<0.01) and VIT D supplementation seems to partially reverse this effect (equal to SHAM and OVX).

3.3. Effect of OVX and/or VIT D supplementation on parameters related to the cholinergic system in hippocampus of adult Wistar rats

In order to investigate whether OVX and/or VIT D supplementation would affect the cholinergic system, we evaluated some parameters related to this system in hippocampus of adult rats. Fig 4a shows a significant effect by OVX

on AChE activity [F(1,20)=22.35, p<0.0001]. There was no effect of the VIT D [F(1,20)=2.556, p=0.1255] and no interaction [F(1,20)=3.015, p=0.0979]. Post hoc Tukey´s test demonstrated an increase in AChE activity in OVX and OVX+VIT D groups (p<0.01). We also analyzed the immunocontent of AChE and ChAT in hippocampus of adult rats submitted to OVX and/or VIT D supplementation. OVX did not exert effects on immunocontent of AChE (Fig 4b) and ChAT (Fig 4c) [F(1,16)=0.6787, p=0.4221 and F(1,12)=0.09496, p=0.7633, respectively]. VIT D supplementation also did not affect this immunocontent [AChE: F(1,16)=0.1485, p=0.7050; ChAT: F(1,12)=0.3154, p=0.5847] and there was no interaction [AChE: F=(1,16)=0.9222, p=0.3512; ChAT: F(1,12)=1.215, p=0.2919].

4. Discussion

Changes in estrogens levels seem to affect several brain processes, including cognition (Au et al., 2016; Heberden, 2017; McEwen et al., 2012). Previous studies from our research group and others have already shown that OVX is associated with alterations in energy metabolism and cytoskeleton in hippocampus, as well as with learning and memory impairment in adult rats (Cui et al., 2017; Siebert et al., 2014; Siebert et al., 2017). In the present study, we investigated some biochemical parameters related to inflammation and cholinergic system in hippocampus of adult female Wistar rats submitted bilateral ovarian withdrawal (OVX) followed by VIT D supplementation. The body weight, food intake and serum estradiol and calcidiol levels were also evaluated.

In this study OVX significantly increased body weight, which corroborates with the literature data (Ben et al., 2010; Fang et al., 2015; Iwasa et al., 2011; Mackedanz et al., 2011; Monteiro et al., 2008; Russell et al., 2017). VIT D supplementation, at the dose used in our study, was not able to act on body weight gain since the animals of the OVX + VIT D group also presented na increase in body weight when compared to the SHAM group. Excessive body weight gain related to estrogen deprivation may predispose to the development of obesity, insulin resistance, cardiovascular and chronic diseases (Ferrara et al., 2002; Rogers et al., 2009; Tchernof and Despres, 2000), which are associated with a decline in quality of a woman's life (Lobo et al., 2014). In order to verify that the body weight gain of the OVX animals in this study is related to the increase in dietary intake, we evaluated the daily food intake per animal (g). Although the literature suggests that increase in body weight caused by OVX would be due to hyperphagia (Iwasa et al., 2011; Jiang et al., 2008), our data did not show changes in daily food intake among groups. It is necessary to emphasize that the measure of food consumption in this study was controlled during a short period (about two weeks), concomitant with vehicle or VIT D administration, and consists of a mean estimate of the food consumption per box.

Surgical removal of ovaries causes an abrupt decrease in serum estradiol levels. In agreement with previous reports (Iwasa et al., 2011; Monteiro et al., 2007; Waynforth and Flecknell, 1992), the present study revealed that rats submitted to OVX present a significant decrease in serum estradiol, confirming the efficacy of the surgical procedure. In addition, in order to verify whether the dose of VIT D supplemented was effective in increasing

serum VIT D levels, we performed the calcidiol measurement in serum of animal. Results showed that 500 IU/Kg/day VIT D supplementation increased serum calcidiol levels in the supplemented groups. The calcidiol is the most abundant form of VIT D and the best indicator for assessing VIT D status (Maeda et al., 2014).

Although the association between menopause and an increased risk of neurological disorders is widely described and investigated, the mechanisms involved are still not fully understood. Considering that neuroinflammation is described as a common feature of various diseases affecting the brain (Simen et al., 2011) and postmenopausal decrease in estrogens levels may be related to alterations in inflammatory status (Au et al., 2016), we investigated some inflammatory parameters in hippocampus of adults rats submitted to OVX and/or VIT D supplementation. The evaluation of cytoplasmatic and nuclear NF-κB/p65 was performed by analyzing the immunocontents of NF-κB/p65 and p-NF-κB/p65. OVX increased nuclear p-NF-κB/p65 immunocontent. VIT D *per se* had no effect on NF-κB/p65 immunocontent, but partially reversed this parameter. NF-κB is an important transcription factor activated in inflammatory situations. It is a heterodimer situated in the cytoplasm and bound to inhibitory proteins (IκB) inactivated. Phosphorylation process induces activation and translocation of NF-κB to the nucleus and thus transcriptional activation of specific target genes, such as inflammatory mediators, including cytokines, chemokines and cell adhesion molecules occurs (McKay and Cidlowski, 1999; Shih et al., 2015; Sunday et al., 2007; Tak and Firestein, 2001). This translocation can be a mechanism to cellular responses to oxidants or inflammatory and immune stimulus (Barnes, 1997; Morgan and Liu, 2011).

The NF-KB activation can stimulate the secretion of inflammatory cytokines, which are molecules involved in signaling during the immune response (Lawrence, 2009; Tak and Firestein, 2001). Therefore, we analyzed the levels of the proinflammatory cytokines TNF-α, IL-1β and IL-6 in hippocampus. Results showed that OVX provoked an increase in proinflammatory cytokines TNF-α and IL-6. VIT D *per se* had no effect on these parameters and partially reversed the increase in IL-6 levels. Proinflammatory cytokines TNF-α, IL-1 and IL-6 produced by microglia are considered classic mediators of inflammation (Wang et al., 2015). Estrogens exert anti-inflammatory actions in several systems, therefore our results agree with other studies in the literature that report the presence of inflammatory changes in brain of animals submitted to OVX (Benedusi et al., 2012; Kireev et al., 2014; Wang et al., 2016; Xu et al., 2015) and although the mechanisms involved in the activation of the immune system in adult Wistar rats submitted to OVX need to be better studied, our results suggest that the increase in cytokines levels observed could be closely related to NF-κB activation.

Acetylcholine (ACh) is an important cerebral neurotransmitter of the nervous system and is involved in learning and memory processes (Maurer and Williams, 2017; Mohapel et al., 2005). ChAT is the enzyme responsible for the synthesis of ACh from acetyl-coenzyme A and choline. AChE is the enzyme responsible for the hydrolysis of ACh in acetate and choline. ACh, together with its receptors and the enzymes AChE and ChAT constitute the cholinergic neurotransmission system, which has recently received great attention in relation to its role in anti-inflammatory pathways (Han et al., 2017; Maurer and Williams, 2017). In this context, we investigated the activity and immunocontent

of AChE, as well as ChAT immunocontent in hippocampus of adult rats submitted to OVX and/or VIT D supplementation. OVX increases AChE activity without altering the AChE and ChAT immunocontents. VIT D *per se* had no effect on these parameters and was also not able to reverse the activation of AChE activity. Our results allow us to suggest that the increase in AChE activity without changing ChAT immunocontent may be cause a reduction of cholinergic neurotransmission due to a decrease in the levels of available ACh in the synaptic cleft, corroborating with memory deficits already described in this model (Siebert et al., 2017) and may be related to the inflammatory changes observed in this study.

Although beneficial effects of VIT D supplementation have already been described (Barberger-Gateau, 2014; Feron et al., 2014; Gregoriou et al., 2017), few studies specifically address the effects of VIT D supplementation in the form of cholecalciferol in hippocampus, especially using a OVX model. In this study, VIT D supplementation partially reversed the increase in p-NF-KB/p65 immunocontent and IL-6 levels in hippocampus of adult female Wistar rats submitted to OVX. Therefore, we may suggest that the VIT D dose used could help improve inflammatory parameters. Regarding the cholinergic system, we did not observe a reversion in AChE activity by VIT D, therefore the dose used was not effective in improving this parameter. Other beneficial actions of VIT D in this dose and in this model have already been reported in previous studies (Siebert et al., 2017).

In summary, our results revealed that OVX causes an imbalance in the inflammatory and cholinergic hippocampal systems of adult Wistar rats, but the mechanism by which such changes occur is still not completely clear. Although

effective in increasing serum calcidiol levels, VIT D supplementation was not fully effective in reversing the inflammatory and cholinergic changes in the hippocampus. We hope, with our findings, to assist in the understanding and knowledge of brain alterations that may be present in postmenopausal women. They may open perspectives for future research in order to better understand the cerebral changes observed in the postmenopausal period. A summary of the results of this work may be found in Fig 7.

Acknowledgements

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) and INCT (EN 465671/2014-4)/CNPq-Brazil.

Compliance with Ethical Standards

Animal experimentation protocols had been approved by the University Ethics Committee for the Use of Animals (CEUA) under the project (#28033). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest

All authors declare no conflict of interest.

References

Au, A., Feher, A., McPhee, L., Jessa, A., Oh, S., Einstein, G. (2016) Estrogens, inflammation and cognition. Front Neuroendocrinol 40, 87-100.

Babaei, P., Shirkouhi, S.G., Hosseini, R., Soltani Tehrani, B. (2017) Vitamin D is associated with metabotropic but not neurotrophic effects of exercise in ovariectomized rats. Diabetol Metab Syndr 9, 91.

Barberger-Gateau, P. (2014) Nutrition and brain aging: how can we move ahead? Eur J Clin Nutr 68, 1245-1249.

Barnes, P.J. (1997) Nuclear factor-kappa B. Int J Biochem Cell Biol 29, 867-870.

Ben-Shmuel, S., Scheinman, E.J., Rashed, R., Orr, Z.S., Gallagher, E.J., LeRoith, D., Rostoker, R. (2015) Ovariectomy is associated with metabolic impairments and enhanced mammary tumor growth in MKR mice. J Endocrinol 227, 143-151.

Ben, J., Soares, F.M., Scherer, E.B., Cechetti, F., Netto, C.A., Wyse, A.T. (2010) Running exercise effects on spatial and avoidance tasks in ovariectomized rats. Neurobiol Learn Mem 94, 312-317.

Benedusi, V., Meda, C., Della Torre, S., Monteleone, G., Vegeto, E., Maggi, A. (2012) A lack of ovarian function increases neuroinflammation in aged mice. Endocrinology 153, 2777-2788.

Biasibetti-Brendler, H., Schmitz, F., Pierozan, P., Zanotto, B.S., Prezzi, C.A., de Andrade, R.B., Wannmacher, C.M.D., Wyse, A.T.S. (2017) Hypoxanthine Induces Neuroenergetic Impairment and Cell Death in Striatum of Young Adult Wistar Rats. Mol Neurobiol.

Chabas, J.-F., Stephan, D., Marqueste, T., Garcia, S., Lavaut, M.-N., Nguyen, C., Legre, R., Khrestchatisky, M., Decherchi, P., Feron, F. (2013) Cholecalciferol (vitamin D_3) improves myelination and recovery after nerve injury. PLoS One 8, e65034-e65034.

Colonna, M., Butovsky, O. (2017) Microglia Function in the Central Nervous System During Health and Neurodegeneration. Annu Rev Immunol 35, 441-468.

Cui, J., Guo, Y., Yang, W., Zhao, X., Yu, F., Tang, W., Pang, B., Su, X. (2017) Effects of exercise on learning and memory, oxidative stress and nNOS expression in marginal division of striatum of the ovariectomized rats. J Sports Med Phys Fitness.

da Cunha, A.A., Ferreira, A.G., Loureiro, S.O., da Cunha, M.J., Schmitz, F., Netto, C.A., Wyse, A.T. (2012) Chronic hyperhomocysteinemia increases inflammatory markers in hippocampus and serum of rats. Neurochem Res 37, 1660-1669.

de Souza Santos, R., Vianna, L.M. (2005) Effect of cholecalciferol supplementation on blood glucose in an experimental model of type 2 diabetes mellitus in spontaneously hypertensive rats and Wistar rats. Clinica Chimica Acta 358, 146-150.

Di Somma, C., Scarano, E., Barrea, L., Zhukouskaya, V.V., Savastano, S., Mele, C., Scacchi, M., Aimaretti, G., Colao, A., Marzullo, P. (2017) Vitamin D and Neurological Diseases: An Endocrine View. Int J Mol Sci 18.

Ellman, G.L., Courtney, K.D., Andres, V., Jr., Feather-Stone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7, 88-95.

Fang, J., Yang, L., Zhang, R., Zhu, X., Wang, P. (2015) Are there differences between Sprague-Dawley and Wistar rats in long-term effects of ovariectomy as a model for postmenopausal osteoporosis? Int J Clin Exp Pathol 8, 1491-1502.

Feron, F., Marqueste, T., Bianco, J., Gueye, Y., Chabas, J.F., Decherchi, P. (2014) [Repairing the spinal cord with vitamin D: a promising strategy]. Biol Aujourdhui 208, 69-75.

Ferrara, C.M., Lynch, N.A., Nicklas, B.J., Ryan, A.S., Berman, D.M. (2002) Differences in adipose tissue metabolism between postmenopausal and perimenopausal women. J Clin Endocrinol Metab 87, 4166-4170.

Georgakis, M.K., Thomopoulos, T.P., Diamantaras, A.A., Kalogirou, E.I., Skalkidou, A., Daskalopoulou, S.S., Petridou, E.T. (2016) Association of Age at Menopause and Duration of Reproductive Period With Depression After Menopause: A Systematic Review and Meta-analysis. JAMA Psychiatry 73, 139-149.

Gregoriou, E., Mamais, I., Tzanetakou, I., Lavranos, G., Chrysostomou, S. (2017) The Effects of Vitamin D Supplementation in Newly Diagnosed Type 1 Diabetes Patients: Systematic Review of Randomized Controlled Trials. Rev Diabet Stud 14, 260-268.

Gueye, Y., Marqueste, T., Maurel, F., Khrestchatisky, M., Decherchi, P., Feron, F. (2015) Cholecalciferol (vitamin D(3)) improves functional recovery when delivered during the acute phase after a spinal cord trauma. J Steroid Biochem Mol Biol 154, 23-31.

Hajiluian, G., Abbasalizad Farhangi, M., Nameni, G., Shahabi, P., Megari-Abbasi, M. (2017) Oxidative stress-induced cognitive impairment in obesity can be reversed by vitamin D administration in rats. Nutr Neurosci, 1-9.

Han, B., Li, X., Hao, J. (2017) The cholinergic anti-inflammatory pathway: An innovative treatment strategy for neurological diseases. Neurosci Biobehav Rev 77, 358-368.

Heberden, C. (2017) Sex steroids and neurogenesis. Biochem Pharmacol 141, 56-62.

Henderson, V.W. (2014) Alzheimer's disease: review of hormone therapy trials and implications for treatment and prevention after menopause. J Steroid Biochem Mol Biol 142, 99-106.

Iwasa, T., Matsuzaki, T., Kinouchi, R., Gereltsetseg, G., Murakami, M., Nakazawa, H., Fujisawa, S., Yamamoto, S., Kuwahara, A., Yasui, T., Irahara, M. (2011) Effect of immune stress on body weight regulation is altered by ovariectomy in female rats. J Reprod Immunol 91, 41-47.

Jiang, J.M., Sacco, S.M., Ward, W.E. (2008) Ovariectomy-induced hyperphagia does not modulate bone mineral density or bone strength in rats. J Nutr 138, 2106-2110.

Kempuraj, D., Thangavel, R., Selvakumar, G.P., Zaheer, S., Ahmed, M.E., Raikwar, S.P., Zahoor, H., Saeed, D., Natteru, P.A., Iyer, S., Zaheer, A. (2017) Brain and Peripheral Atypical Inflammatory Mediators Potentiate Neuroinflammation and Neurodegeneration. Front Cell Neurosci 11, 216.

Kireev, R.A., Vara, E., Vina, J., Tresguerres, J.A. (2014) Melatonin and oestrogen treatments were able to improve neuroinflammation and apoptotic processes in dentate gyrus of old ovariectomized female rats. Age (Dordr) 36, 9707.

Lawrence, T. (2009) The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 1, a001651.

Li, Q., Verma, I.M. (2002) NF-kappaB regulation in the immune system. Nat Rev Immunol 2, 725-734.

Li, S., Niu, G., Wu, Y., Du, G., Huang, C., Yin, X., Liu, Z., Song, C., Leng, H. (2016) Vitamin D prevents articular cartilage erosion by regulating collagen II turnover through TGF-beta1 in ovariectomized rats. Osteoarthritis Cartilage 24, 345-353.

Lobo, R.A., Davis, S.R., De Villiers, T.J., Gompel, A., Henderson, V.W., Hodis, H.N., Lumsden, M.A., Mack, W.J., Shapiro, S., Baber, R.J. (2014) Prevention of diseases after menopause. Climacteric : the journal of the International Menopause Society 17, 540-556.

Longoni, A., Kolling, J., Siebert, C., Dos Santos, J.P., da Silva, J.S., Pettenuzzo, L.F., Meira-Martins, L.A., Goncalves, C.A., de Assis, A.M., Wyse, A.T. (2017) 1,25-Dihydroxyvitamin D3 prevents deleterious effects of homocysteine on mitochondrial function and redox status in heart slices. Nutr Res 38, 52-63.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193, 265-275.

Mackedanz, V., Mattos, C.B., Feksa, L.R., Wannmacher, C.M., Wyse, A.T. (2011) Ovariectomy alters energy metabolism in rat striatum: effect of supplementation with soy diet rich in isoflavones. Metab Brain Dis 26, 97-105.

Maeda, S.S., Borba, V.Z., Camargo, M.B., Silva, D.M., Borges, J.L., Bandeira, F., Lazaretti-Castro, M., Brazilian Society of, E., Metabology (2014) Recommendations of the Brazilian Society of Endocrinology and Metabology (SBEM) for the diagnosis and treatment of hypovitaminosis D. Arq Bras Endocrinol Metabol 58, 411-433.

Maurer, S.V., Williams, C.L. (2017) The Cholinergic System Modulates Memory and Hippocampal Plasticity via Its Interactions with Non-Neuronal Cells. Front Immunol 8, 1489.

McEwen, B.S., Akama, K.T., Spencer-Segal, J.L., Milner, T.A., Waters, E.M. (2012) Estrogen effects on the brain: actions beyond the hypothalamus via novel mechanisms. Behav Neurosci 126, 4-16.

McKay, L.I., Cidlowski, J.A. (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr Rev 20, 435-459.

Mohapel, P., Leanza, G., Kokaia, M., Lindvall, O. (2005) Forebrain acetylcholine regulates adult hippocampal neurogenesis and learning. Neurobiol Aging 26, 939-946.

Monteiro, S.C., de Mattos, C.B., Ben, J., Netto, C.A., Wyse, A.T.S. (2008) Ovariectomy impairs spatial memory: prevention and reversal by a soy isoflavone diet. Metab Brain Dis 23, 243-253.

Monteiro, S.C., Matte, C., Delwing, D., Wyse, A.T. (2005) Ovariectomy increases Na+, K+-ATPase, acetylcholinesterase and catalase in rat hippocampus. Mol Cell Endocrinol 236, 9-16.

Monteiro, S.C., Mattos, C.B., Scherer, E.B., Wyse, A.T. (2007) Supplementation with vitamins E plus C or soy isoflavones in ovariectomized rats: effect on the activities of Na(+), K (+)-ATPase and cholinesterases. Metab Brain Dis 22, 156-171.

Morgan, M.J., Liu, Z.G. (2011) Crosstalk of reactive oxygen species and NF-kappaB signaling. Cell Res 21, 103-115.

Mpandzou, G., Ait Ben Haddou, E., Regragui, W., Benomar, A., Yahyaoui, M. (2016) Vitamin D deficiency and its role in neurological conditions: A review. Rev Neurol (Paris) 172, 109-122.

Pacifici, R., Rifas, L., McCracken, R., Vered, I., McMurtry, C., Avioli, L.V., Peck, W.A. (1989) Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin 1 release. Proc Natl Acad Sci U S A 86, 2398-2402.

Pavlov, V.A., Tracey, K.J. (2005) The cholinergic anti-inflammatory pathway. Brain Behav Immun 19, 493-499.

Perry, E., Walker, M., Grace, J., Perry, R. (1999) Acetylcholine in mind: a neurotransmitter correlate of consciousness? Trends Neurosci 22, 273-280.

Peter, J., Lahr, J., Minkova, L., Lauer, E., Grothe, M.J., Teipel, S., Kostering, L., Kaller, C.P., Heimbach, B., Hull, M., Normann, C., Nissen, C., Reis, J., Kloppel, S. (2016) Contribution of the Cholinergic System to Verbal Memory Performance in Mild Cognitive Impairment. J Alzheimers Dis 53, 991-1001.

Pozzi, S., Benedusi, V., Maggi, A., Vegeto, E. (2006) Estrogen action in neuroprotection and brain inflammation. Ann N Y Acad Sci 1089, 302-323.

Pratap, U.P., Patil, A., Sharma, H.R., Hima, L., Chockalingam, R., Hariharan, M.M., Shitoot, S., Priyanka, H.P., ThyagaRajan, S. (2016) Estrogen-induced neuroprotective and anti-inflammatory effects are dependent on the brain areas of middle-aged female rats. Brain Res Bull 124, 238-253.

Rogers, N.H., Perfield, J.W., 2nd, Strissel, K.J., Obin, M.S., Greenberg, A.S. (2009) Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. Endocrinology 150, 2161-2168.

Rosas-Ballina, M., Tracey, K.J. (2009) Cholinergic control of inflammation. J Intern Med 265, 663-679.

Russell, A.L., Grimes, J.M., Cruthirds, D.F., Westerfield, J., Wooten, L., Keil, M., Weiser, M.J., Landauer, M.R., Handa, R.J., Wu, T.J., Larco, D.O. (2017) Dietary

Isoflavone-Dependent and Estradiol Replacement Effects on Body Weight in the Ovariectomized (OVX) Rat. Horm Metab Res 49, 457-465.

Salum, E., Kals, J., Kampus, P., Salum, T., Zilmer, K., Aunapuu, M., Arend, A., Eha, J., Zilmer, M. (2013) Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats. Diabetes Research and Clinical Practice 100, 243-249.

Sarter, M., Bruno, J.P. (1997) Cognitive functions of cortical acetylcholine: toward a unifying hypothesis. Brain Res Brain Res Rev 23, 28-46.

Scherer, E.B., da Cunha, M.J., Matte, C., Schmitz, F., Netto, C.A., Wyse, A.T. (2010) Methylphenidate affects memory, brain-derived neurotrophic factor immunocontent and brain acetylcholinesterase activity in the rat. Neurobiol Learn Mem 94, 247-253.

Shih, R.H., Wang, C.Y., Yang, C.M. (2015) NF-kappaB Signaling Pathways in Neurological Inflammation: A Mini Review. Front Mol Neurosci 8, 77.

Siebert, C., Kolling, J., Scherer, E.B., Schmitz, F., da Cunha, M.J., Mackedanz, V., de Andrade, R.B., Wannmacher, C.M., Wyse, A.T. (2014) Effect of physical exercise on changes in activities of creatine kinase, cytochrome c oxidase and ATP levels caused by ovariectomy. Metab Brain Dis 29, 825-835.

Siebert, C., Pierozan, P., Kolling, J., Dos Santos, T.M., Sebotaio, M.C., Marques, E.P., Biasibetti, H., Longoni, A., Ferreira, F., Pessoa-Pureur, R., Netto, C.A., Wyse, A.T.S. (2017) Vitamin D3 Reverses the Hippocampal Cytoskeleton Imbalance But Not Memory Deficits Caused by Ovariectomy in Adult Wistar Rats. Neuromolecular Med.

Sies, H., Berndt, C., Jones, D.P. (2017) Oxidative Stress. Annu Rev Biochem 86, 715-748.

Simen, A.A., Bordner, K.A., Martin, M.P., Moy, L.A., Barry, L.C. (2011) Cognitive dysfunction with aging and the role of inflammation. Ther Adv Chronic Dis 2, 175-195.

Sohrabji, F., Bake, S. (2006) Age-related changes in neuroprotection: is estrogen pro-inflammatory for the reproductive senescent brain? Endocrine 29, 191-197.

Stroud, M.L., Stilgoe, S., Stott, V.E., Alhabian, O., Salman, K. (2008) Vitamin D - a review. Aust Fam Physician 37, 1002-1005.

Sunday, L., Osuna, C., Krause, D.N., Duckles, S.P. (2007) Age alters cerebrovascular inflammation and effects of estrogen. Am J Physiol Heart Circ Physiol 292, H2333-2340.

Tak, P.P., Firestein, G.S. (2001) NF-kappaB: a key role in inflammatory diseases. J Clin Invest 107, 7-11.

Tchernof, A., Despres, J.P. (2000) Sex steroid hormones, sex hormone-binding globulin, and obesity in men and women. Horm Metab Res 32, 526-536.

Van Pelt, R.E., Gavin, K.M., Kohrt, W.M. (2015) Regulation of Body Composition and Bioenergetics by Estrogens. Endocrinol Metab Clin North Am 44, 663-676.

Vegeto, E., Belcredito, S., Ghisletti, S., Meda, C., Etteri, S., Maggi, A. (2006) The endogenous estrogen status regulates microglia reactivity in animal models of neuroinflammation. Endocrinology 147, 2263-2272.

Vieira-Potter, V.J., Padilla, J., Park, Y.M., Welly, R.J., Scroggins, R.J., Britton, S.L., Koch, L.G., Jenkins, N.T., Crissey, J.M., Zidon, T., Morris, E.M., Meers, G.M., Thyfault, J.P. (2015) Female rats selectively bred for high intrinsic aerobic fitness are protected from ovariectomy-associated metabolic dysfunction. Am J Physiol Regul Integr Comp Physiol 308, R530-542.

Villa, A., Vegeto, E., Poletti, A., Maggi, A. (2016) Estrogens, Neuroinflammation, and Neurodegeneration. Endocr Rev 37, 372-402.

Wang, W.Y., Tan, M.S., Yu, J.T., Tan, L. (2015) Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann Transl Med 3, 136.

Wang, Y., Xu, Y., Sheng, H., Ni, X., Lu, J. (2016) Exercise amelioration of depression-like behavior in OVX mice is associated with suppression of NLRP3 inflammasome activation in hippocampus. Behav Brain Res 307, 18-24.

Waynforth, H.B., Flecknell, P.A. (1992) Experimental and surgical technique in the rat 2nd ed ed.

Xu, Y., Sheng, H., Tang, Z., Lu, J., Ni, X. (2015) Inflammation and increased IDO in hippocampus contribute to depression-like behavior induced by estrogen deficiency. Behav Brain Res 288, 71-78.

Figures Legends

Figure 1. Timeline of experimental design. OVX- ovariectomy; VIT D – vitamin D

Figure 2. Effect of OVX and/or VIT D supplementation on cytosolic and nuclear fraction of NF-κB/p65 and p-NF-κB/p65 in hippocampus of adult female Wistar rats. NF-κB/p65 citosolic fraction (a), p-NF-κB/p65 citosolic fraction (b), NF-κB/p65 nuclear fraction(c), p-NF-κB/p65 nuclear fraction (d). Results are expressed as percentage of control. Uniformity of gel loading was confirmed with β-actin as standard. Data are mean ± SD for five to six animals in each group (two-way ANOVA followed by Tukey's test). **p < 0.01, different from SHAM. OVX - ovariectomy; VIT D - vitamin D; NF-κB/p65 - nuclear factor-kappa B; p-NF-κB/p65 - phosphorylated nuclear factor-kappa B

Figure 3. Effect of OVX and/or VIT D supplementation on proinflammatory cytokines levels in hippocampus of adult female Wistar rats. (a) TNF-α levels, (b) IL-1β levels and (c) IL-6 levels. Results are expressed as pg/mg protein. Data are mean ± SD for five animals in each group (two-way ANOVA followed by Tukey's test). **p < 0.01, different from SHAM. OVX - ovariectomy; VIT D - vitamin D; IL-6 - interleukin-6; TNF-α - tumor necrosis factor alpha; IL-1 β - interleukin-1 beta

Figure 4. Effect of OVX and/or VIT D supplementation on cholinergic parameters in hippocampus of adult female Wistar rats. (a) acetylcholinesterase

activity, (b) acetylcholinesterase immunocontent and (c) choline acetyltransferase immunocontent. Results are expressed as µmol AcSCh/h/mg protein (a) or percentage of control (b and c). Uniformity of gel loading was confirmed with β-actin as standard. Data are mean ± SD for four to six animals in each group (two-way ANOVA followed by Tukey's test). **p<0.01 and ***p<0.001, different from SHAM. OVX - ovariectomy; VIT D - vitamin D; AChE - acetylcholinesterase; ChAT – choline acetyltransferase; AcSCh - acetylthiocholine

Figure 5. Representative figure of the main results

Table 1. Serum levels of estradiol and calcidiol, body weight and daily food intake of Wistar rats submitted to OVX and/or VIT D supplementation

Parameters	Groups			
	SHAM	OVX	VIT D	OVX+VIT D
Serum estradiol levels (pg/ml)	30.69±11.29	13.84±5.66*	23.26±10.78	15.25±5.81*
Serum calcidiol levels (ng/ml)	49.96±4.38	64.24±31.65	100.56±20.30*	108.41±29.71**&
Final body weight (g)	250.33±1.52	296.66±22.05***#	258.33±3.05	291.33±10.21**#
Daily food intake (g)	15.93±0.95	16.70±2.07	16.63±1.25	16.94±1.08

Note: Data are expressed as mean ± SD for 5-6 animals per group (Two-Way ANOVA followed by *post hoc* Tukey´s test).

* significantly different from SHAM ($p<0.05$).

** significantly different from SHAM ($p<0.01$).

*** significantly different from SHAM ($p<0.001$).

significantly different from VIT D ($p<0.05$).

& significantly different from OVX ($p<0.05$).

Fig 1

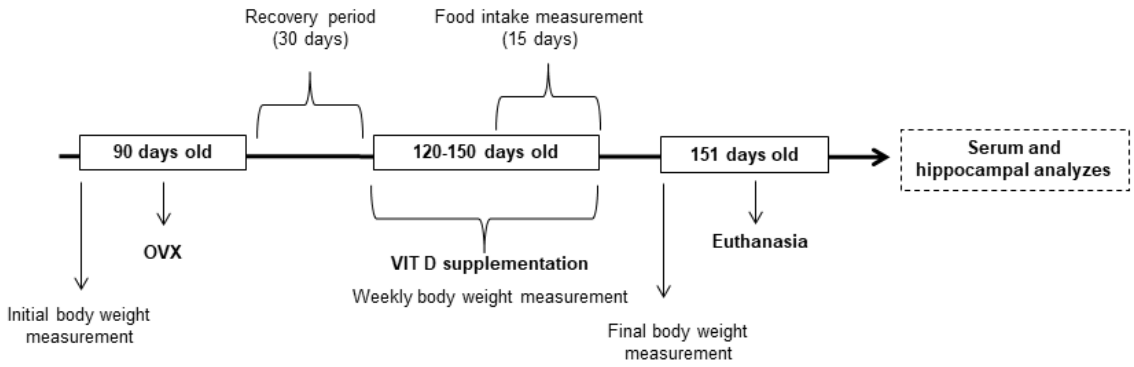

Recovery period
(30 days)
Food intake measurement
(15 days)
90 days old
120-150 days old
151 days old
Serum and
hippocampal analyzes
OVX
VIT D supplementation
Euthanasia
Weekly body weight measurement
Initial body weight
measurement
Final body weight
measurement

Fig 2

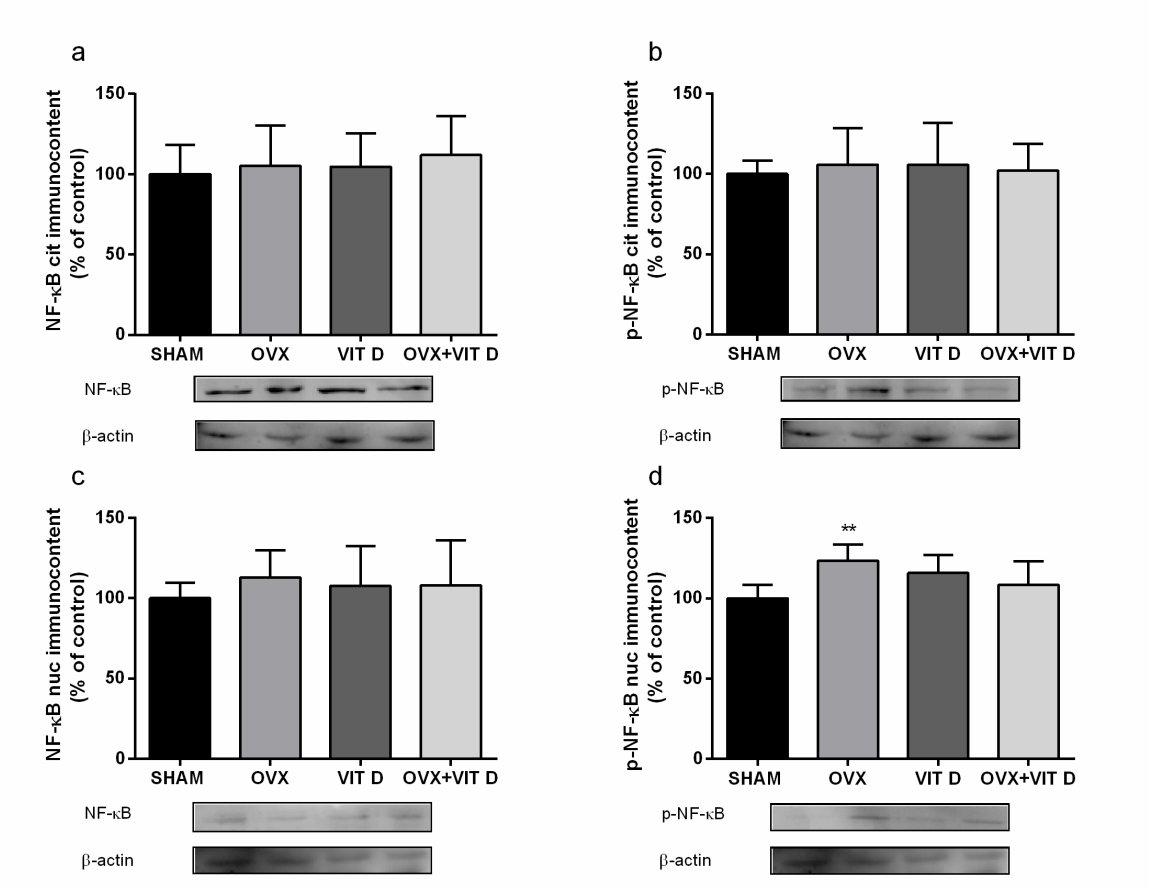

a
NF-κB cit immunocontent
(% of control)
150
100
50
0
SHAM
OVX
VIT D
OVX+VIT D
NF-κB
β-actin
b
p-NF-κB cit immunocontent
(% of control)
p-NF-κB
c
NF-κB nuc immunocontent
(% of control)
d
p-NF-κB nuc immunocontent
(% of control)
**

Fig 3

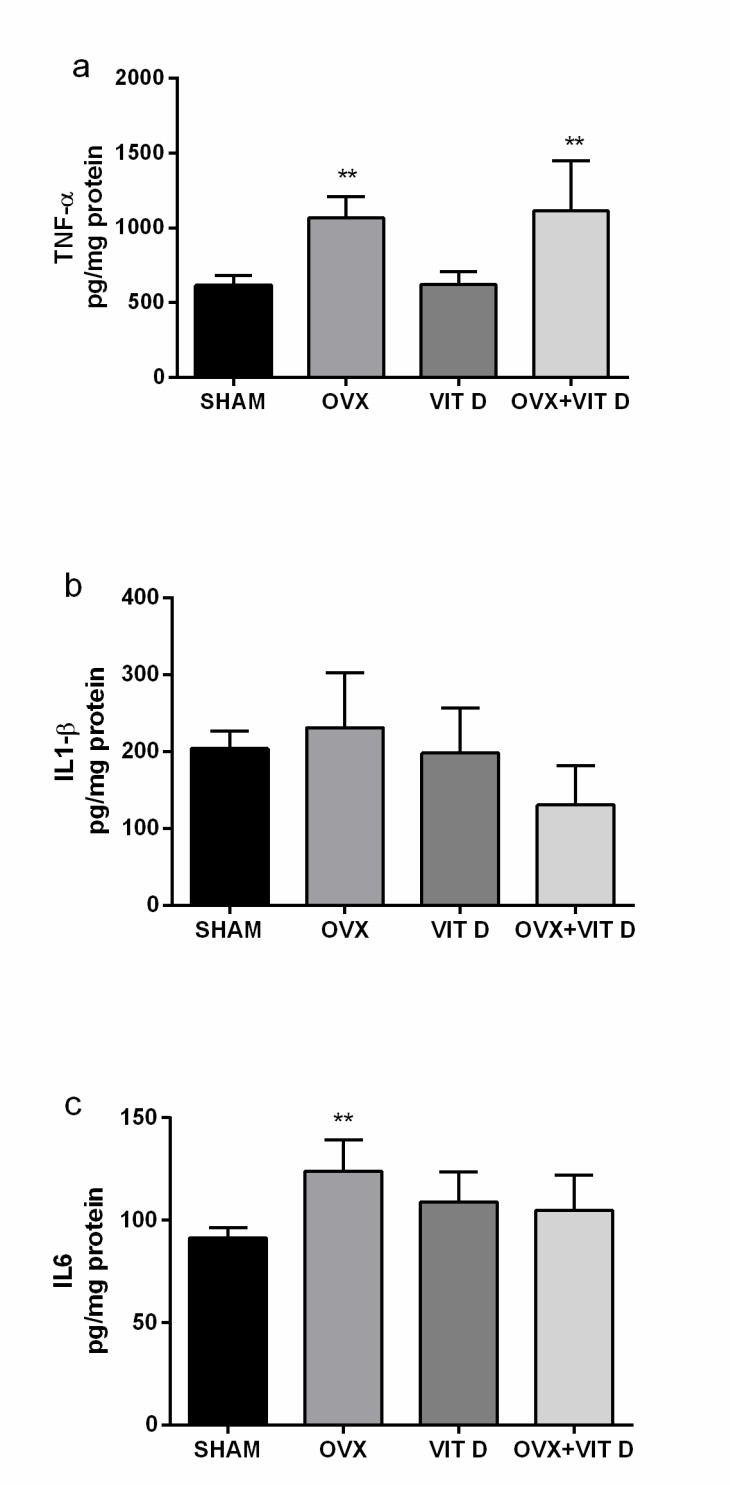

a
TNF-α
pg/mg protein
2000
1500
1000
500
0
**
**
SHAM
OVX
VIT D
OVX+VIT D
b
IL1-β
pg/mg protein
400
300
200
100
0
SHAM
OVX
VIT D
OVX+VIT D
c
IL6
pg/mg protein
150
100
50
0
**
SHAM
OVX
VIT D
OVX+VIT D

Fig 4

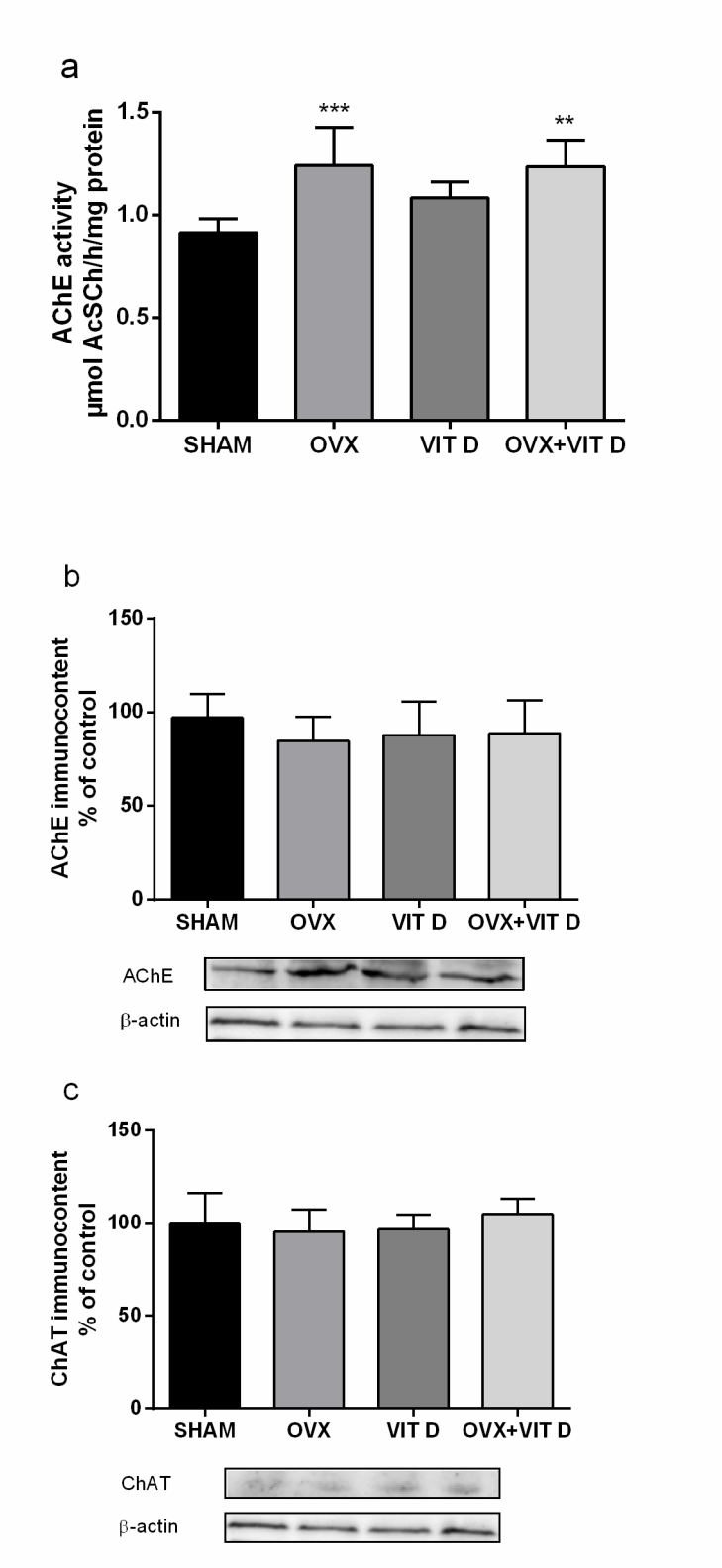
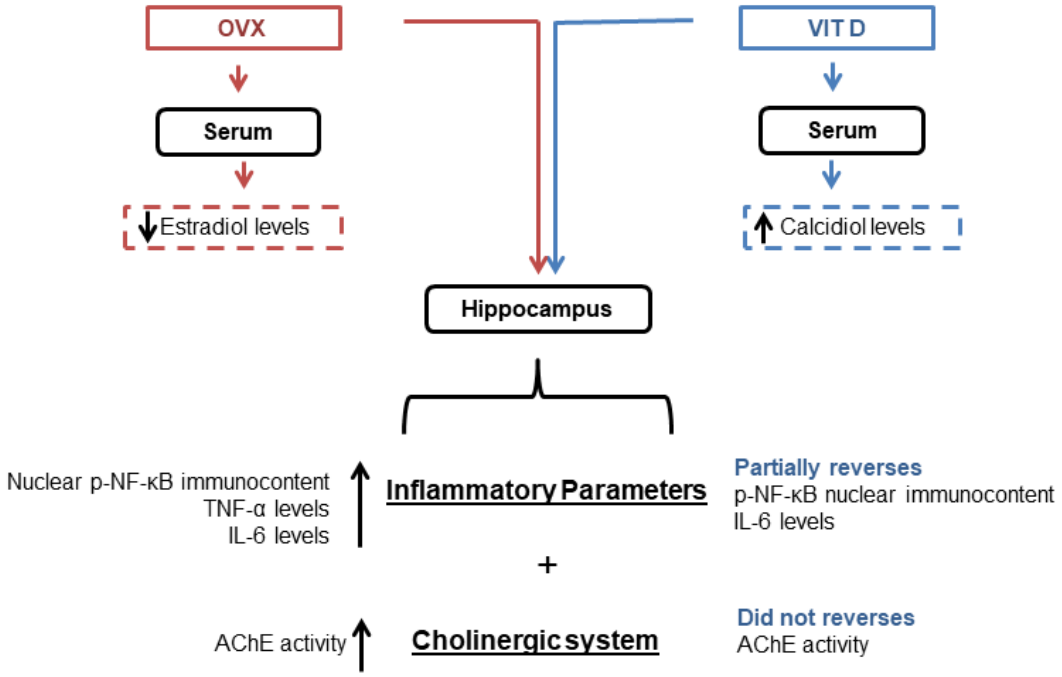

a
AChE activity
µmol AcSCh/h/mg protein
1.5
1.0
0.5
0.0
***
**
SHAM
OVX
VIT D
OVX+VIT D
b
AChE immunocontent
% of control
150
100
50
0
SHAM
OVX
VIT D
OVX+VIT D
AChE
β-actin
c
ChAT immunocontent
% of control
150
100
50
0
SHAM
OVX
VIT D
OVX+VIT D
ChAT
β-actin

Fig 5


OVX
Serum
Estradiol levels
VIT D
Serum
Calcidiol levels
Hippocampus
Nuclear p-NF-κB immunocontent
TNF-α levels
IL-6 levels
Inflammatory Parameters
Partially reverses
p-NF-κB nuclear immunocontent
IL-6 levels
+
AChE activity
Cholinergic system
Did not reverses
AChE activity

Capítulo III

Neurotoxicity Research
https://doi.org/10.1007/s12640-018-9909-z

ORIGINAL ARTICLE

Vitamin D Supplementation Reverses DNA Damage and Telomeres Shortening Caused by Ovariectomy in Hippocampus of Wistar Rats

Cassiana Siebert [1] · Tiago Marcon dos Santos [1] · Carolina Gessinger Bertó [1] · Mariana Migliorini Parisi [2] · Ritiéle Pinto Coelho [3] · Vanusa Manfredini [3] · Florencia M. Barbé-Tuana [2] · Angela T. S. Wyse [1]

Received: 13 November 2017 / Revised: 25 April 2018 / Accepted: 25 April 2018

Periódico: Neurotoxicity Research

Status: Publicado

Neurotoxicity Research
https://doi.org/10.1007/s12640-018-9909-z

ORIGINAL ARTICLE

Vitamin D Supplementation Reverses DNA Damage and Telomeres Shortening Caused by Ovariectomy in Hippocampus of Wistar Rats

Cassiana Siebert[1] · Tiago Marcon dos Santos[1] · Carolina Gessinger Bertó[1] · Mariana Migliorini Parisi[2] · Ritiéle Pinto Coelho[3] · Vanusa Manfredini[3] · Florencia M. Barbé-Tuana[2] · Angela T. S. Wyse[1]

Received: 13 November 2017 / Revised: 25 April 2018 / Accepted: 25 April 2018

Abstract

The aim of this study was to investigate the effect of ovariectomy (OVX), a surgical model of menopause, and/or vitamin D (VIT D) supplementation on oxidative status, DNA damage, and telomere length in hippocampus of rats at two ages. Ninety-day-old (adult) or 180-day-old (older) female Wistar rats were divided into four groups: SHAM, OVX, VIT D, and OVX + VIT D. Thirty days after OVX, rats were supplemented with VIT D (500 IU/kg) by gavage, for a period of 30 days. Results showed that OVX altered antioxidant enzymes, increasing the activities of catalase in adult rats and superoxide dismutase in older rats. VIT D *per se* increased the activities of catalase and superoxide dismutase in older rats, but not in adult rats. VIT D supplementation to OVX (OVX + VIT D) rats did not reverse the effect of OVX on catalase in adult rats, but it partially reversed the increase in superoxide dismutase activity in older rats. OVX increased DNA damage in hippocampus of adult and older rats. VIT D *per se* reduced DNA damage, and when associated to OVX, it partially reversed this alteration. Additionally, OVX caused a telomere shortening in older rats, and VIT D was able to reverse such effect. Taken together, these results demonstrate that surgical menopause in rats causes hippocampal biochemical changes and VIT D appears, at least in part, to act in a beneficial way.

Keywords Vitamin D · DNA damage · Telomere length · Antioxidant enzymes · Experimental menopause · Ovariectomy

Abbreviations

ANOVA	Analysis of variance
CAT	Catalase
CNS	Central nervous system
DCF	2′7′dichlorofluorescein
DCFH	2′7′dichlorofluorescin
DCFH-DA	2′7′dichlorofluorescein diacetate
DI	Damage index
DTNB	5,5′-dithio-bis (2-nitrobenzoic acid)
gDNA	Genomic DNA
GPX	Glutathione peroxidase
HRT	Hormone replacement therapy
OVX	Ovariectomy
OVX + VIT D	Ovariectomy + vitamin D supplementation
qPCR	Real-time polymerase chain
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
VDR	Vitamin D receptor
VIT D	Vitamin D
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TNB	5-thio-2-nitrobenzoic acid

✉ Angela T. S. Wyse
wyse@ufrgs.br

[1] Laboratório de Neuroproteção e Doenças Neurometabólicas, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, Porto Alegre, RS 90035-003, Brazil

[2] Laboratório de Biologia Molecular e Bioinformática, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, Porto Alegre, RS 90035-003, Brazil

[3] Grupo de Pesquisa em Estresse Oxidativo, Programa de Pós-Graduação em Bioquímica, Universidade Federal do Pampa, BR 472, Km 585, 118, Uruguaiana, RS 97500-970, Brazil

Introduction

Menopause is a physiological state in women's lives. It occurs naturally, diagnosed after 12 months of amenorrhea without pathological cause, but, it can also be induced by chemotherapy, radiation exposure, or surgery, such as ovariectomy

(Grant et al. 2015; Shuster et al. 2010). The real effects of surgical menopause are not fully understood, but it is believed to be associated with increased long-term health risk factors (Henderson and Sherwin 2007; Rocca et al. 2010; Shuster et al. 2010).

Evidence shows that estrogenic deprivation characteristic of menopause may be related to increased risk of developing neurodegenerative diseases in women, highlighting the possible neuroprotective role of estrogens (Georgakis et al. 2016; Henderson 2014). Furthermore, the involvement of estrogens in the protection against oxidative imbalance is well recognized (Borras et al. 2010; Xiao et al. 2017). Accentuated estrogen's reduction has been related to an imbalance between the production of oxidant species and antioxidant defenses, leading to oxidative stress (Doshi and Agarwal 2013). Although the low concentration of oxidative species is important in the intracellular signaling, high concentrations may cause damage to biomolecules such as lipids, proteins, and DNA, which may contribute to damage and cellular function loss (Halliwell 2012; Valko et al. 2016). Elevated levels of reactive oxygen species (ROS) induce the oxidation of guanine bases (Cadet and Wagner 2013) and single and double breaks in the DNA (Nathan and Cunningham-Bussel 2013; Tamm et al. 2008). Consequently, progressive accumulation of DNA damage is related to premature induction of senescence and the appearance of an early disease-dependent phenotype (Bhatia-Dey et al. 2016; Chen et al. 2007).

Among senescence, telomere lengths have been proposed as a biomarker of cellular aging (Sanders and Newman 2013; von Zglinicki and Martin-Ruiz 2005). Telomeres are ribonucleoprotein structures located at the end of linear eukaryotic chromosomes whose function is attributed to protection of genome integrities (Blackburn 2000; O'Sullivan and Karlseder 2010). The telomeres are composed of a tandemly repeated hexamer DNA sequence (5′-TTAGGG-3′) and naturally undergo shortening under physiological conditions (O'Callaghan and Fenech 2011). However, the premature or accelerated shortening rate has been considered a marker of cellular senescence (Bernadotte et al. 2016).

Menopausal women usually perform hormone replacement therapy (HRT) for the substitution of endogenous estrogens; however, it is known that this practice is not free of adverse effects. Therefore, the search for alternative treatments to replace or complement the HTR used by menopausal women has increased in the last years (Ben et al. 2010; Monteiro et al. 2005a; Siebert et al. 2014). Vitamin D (VIT D) is considered a steroid hormone with important function in calcium homeostasis. The main source of VIT D is its endogenous formation in cutaneous tissues as a result of exposure to ultraviolet B radiation (Mpandzou et al. 2016; Stroud et al. 2008). However, diet has become an important alternative source of VIT D. VIT D has numerous biological targets and acts through its receptor (VDR), found in most body cell (Eyles et al. 2007). In both rats and humans, VDR appears to be localized in brain key area regions, such as amygdala, cortex, and hippocampus, which are involved in cognitive functioning (Eyles et al. 2005; Harms et al. 2011; McGrath et al. 2004).

In this work, we investigate the effects of ovariectomy (OVX-surgical menopause model), performed at two different ages of female Wistar rats: 90 days old (adult) and 180 days old (older), on oxidative stress parameters, DNA damage index, and relative telomere length in hippocampus, a brain structure sensitive to effects caused by this model (Monteiro et al. 2005b; Siebert et al. 2014). The neuroprotective effect of VIT D supplementation was also evaluated. Our hypothesis is that VIT D could reverse some alteration caused by OVX.

Material and Methods

Animals and Reagents

Female Wistar rats (90 or 180 days old) were obtained from the Central Animal House of the Department of Biochemistry at the Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. Animals were housed in plastic cages and maintained at a constant temperature (22 °C) in a light/dark cycle 12:12 h with free access to water and protein commercial chow containing 2.000 IU/kg of vitamin D3 (cholecalciferol) in its composition. The ethical standards followed the official governmental guidelines issued by the Brazilian Federation of Societies for Experimental Biology, following the Guide for Care and Use of Laboratory Animals and Arouca Law (Law no. 11.794/2008). Animal experimentation protocols had been approved by the University Ethics Committee for the Use of Animals (CEUA) under the project (#28033). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except Platinum® Taq DNA polymerase enzyme (Invitrogen, USA).

Experimental Groups

Considering that Wistar rats reach sexual maturity from the sixth week of life (Sengupta 2013), we chose to perform OVX in adult Wistar rats at two different ages: 90 or 180 days old. Ninety- or 180-day-old female Wistar rats were randomly divided into four groups: (1) SHAM (control: surgery without ovaries removal), (2) OVX (surgical removal of both ovaries), (3) VIT D, and (4) OVX + VIT D. The timeline of the experimental protocol used can be seen in Fig. 1.

OVX Procedure

Animals were anesthetized by intraperitoneal (i.p.) administration of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) and subsequently subjected to a surgical procedure

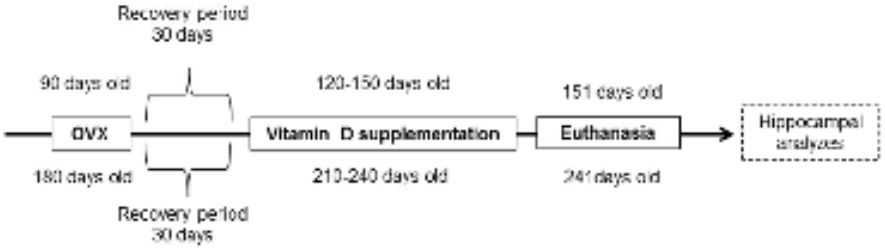


Fig. 1 Timeline of experimental design for adult (upper) or older (down) female rats

for removing both ovaries as previously described (Ben et al. 2009; Mackedanz et al. 2011). Studies from our and other groups have already shown that OVX causes a significant decrease in estradiol circulating levels (Monteiro et al. 2005b; Waynforth and Flecknell 1992), which confirms the effectiveness of the procedure for this purpose.

Vitamin D Supplementation

After full recuperation from OVX (30 days), animals received for a 30-day period, daily supplementation with VIT D (cholecalciferol–vitamin D3; 200 μL once per day) by gavage. Control groups (SHAM and OVX) received an equal volume of vehicle (propylene glycol). Based on previous work (Chabas et al. 2013; de Souza Santos and Vianna 2005; Gueye et al. 2015; Salum et al. 2013), the dose of VIT D used was 500 IU/kg/day. Animals' weight was controlled weekly.

Approximately 12 h after the last administration, rats were decapitated without anesthesia, and the hippocampus was removed for further tissue analysis. The rats of the SHAM group were decapitated at the diestrus stage of the estrous cycle, where low plasma concentrations of estrogen are present.

Tissue Preparation to Measure of Oxidative Stress

The hippocampus was homogenized in ten volumes (1:10, *w*/*v*) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl and centrifuged at 800×*g* for 10 min at 4 °C. The supernatant was immediately frozen for subsequent oxidative stress assays.

Superoxide Dismutase Assay

The assay for the measurement of the antioxidant enzyme superoxide dismutase (SOD) activity is based on the autoxidation ability of the reagent pyrogallol (1,2,3-trihydroxybenzene) in the presence of superoxide (substrate for SOD). The inhibition of this autoxidation occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm (Marklund 1985). A calibration curve was performed with purified SOD as standard. The results were reported as units/mg protein.

Catalase Assay

The assay for the measurement of the antioxidant enzyme catalase (CAT) activity is based on the consumption of H_2O_2 measured at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL (Aebi 1984). CAT activity was expressed as CAT units/mg protein. One CAT unit was defined as 1 mmol of H_2O_2 consumed per minute.

2′7′dichlorofluorescin Oxidation Assay

The production of ROS was measured based on the oxidation of 2′7′-dichlorofluorescein (LeBel et al. 1992). Hippocampus supernatant (60 μL) was incubated with 240 μL of 100 μM 2′7′dichlorofluorescein diacetate (DCFH-DA) solution for 30 min at 37 °C in the dark. DCFH-DA is cleaved by cellular esterases, and the resultant 2′7′ dichlorofluorescin (DCFH) is oxidized by ROS present in samples. This reaction produces 2′7′ dichlorofluorescein (DCF), a fluorescent compound, which was measured at 488-nm excitation and 525-nm emission. The production of reactive species was calculated as nmol DCF/mg protein.

Thiobarbituric Acid Reactive Substances Assay

The index of lipid peroxidation was determined by TBARS according to the method described by Ohkawa et al. (1979). Hippocampus supernatant was mixed with 20% trichloroacetic acid (TCA) and 0.8% thiobarbituric acid (TBA) and heated in a boiling water bath for 60 min. TBARS were determined by absorbance at 535 nm. The results are calculated as nmol TBARS/mg protein.

Sulfhydryl Content

The sulfhydryl content is inversely correlated to oxidative damage present in proteins. This assay is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid-DTNB) by thiols, generating a yellow derivate 5-thio-2-nitrobenzoic acid (TNB) whose absorption was measured spectrophotometrically at 412 nm (Aksenov and Markesbery 2001). The results are calculated as nmol TNB/mg protein.

Comet Assay

The alkaline comet assay was performed in duplicate as described by Singh et al. (1988) in accordance with general guidelines for use of the comet assay (Bajpayee et al. 2005; Hartmann et al. 2003; Tice et al. 2000). Homogenized tissues were suspended in agarose and spread onto glass microscope slides pre-coated with agarose. Agarose cell suspension was allowed to set at 4 °C for 5 min. To examine DNA damage, slides were incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0, and 1% Triton X-100 with10% DMSO) in order to remove proteins, leaving DNA as "nucleoids". Next, slides were placed in a horizontal electrophoresis chamber, covered with a fresh solution (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow DNA unwind and the expression of alkali-labile sites. Electrophoresis was performed for 20 min (25 V; 315 mA; 0.9 V/cm). Slides were then neutralized, washed in bidistilled water, and stained using a silver staining protocol. After overnight drying at room temperature, slides were analyzed using an optical microscope. A total of 100 comets (50 comets from each of the two replicate slides) were arbitrarily chosen and analyzed. Comets were visually scored from 0 (no migration) to 4 (maximal migration) according to tail length. From this, a DNA damage index (DI) was created for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration) (Tice et al. 2000). Slides were analyzed by at least two different operators blinded for the experimental identification of the groups. Scores are presented as median values.

Telomere Length Determination

The relative quantification of telomere length was performed by real-time polymerase chain (qPCR). For each sample, two consecutive reactions were performed, a telomeric (T) and single copy gene (36B4) control amplification, as previously reported (Cawthon 2002) with modifications (Barbe-Tuana et al. 2016).

Briefly, after euthanasia, a small portion of the hippocampus tissue was snap frozen in liquid N_2. Genomic DNA (gDNA) was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) (Chomczynski and Sacchi 1987), and gDNA (25 ng/reaction) was used as template for measurement of relative telomere length. We used already published oligonucleotide primers (O'Callaghan and Fenech 2011), specific for rodent single copy gene (36B4, S) or human/rodent telomeres (T) detection.

In each run, standard curves were performed for single copy constitutive gene (S) and telomeres (T). Reactions were done using the Platinum® Taq DNA polymerase enzyme (Invitrogen, USA) in a StepOnePlus™ apparatus (Applied Biosystems). We included two controls per plate, a negative control to detect any possible contamination, and a randomly chosen sample.

The results were analyzed when the efficiency of the reaction was 80–110%, and linear regression coefficient of the standard curve was $R^2 \geq 0.985$. Triplicates with difference ≥ 0.5 threshold cycles (Ct) were discarded and repeated. The T/S ratio was calculated by the delta delta Ct method (each sample relative to the control group mean) using StepOnePlus™ (Software v2.2.2, Applied Biosystems). The coefficient of variation (CoV = standard deviation/mean) was calculated to monitor the inter-plate variation. The relative measurement of telomere length was expressed as a mean value of the sample by the T/S ratio.

Protein Determination

The determination of total protein was performed by colorimetric method (Lowry et al. 1951), using serum bovine albumin as standard.

Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. Non-parametric data (telomere length) were analyzed by Kruskal-Wallis test followed by post hoc Dunn's test or Mann-Whitney *U* test. Values of $p < 0.05$ were considered statistically significant. All analyses and plots were performed using GraphPad Prism 5.1 software program in a compatible computer.

Results

Tables 1 and 2 show the effect of OVX and/or VIT D supplementation on oxidative status parameters in rats submitted to OVX at two different ages: 90 or 180 days old, respectively. Results showed that OVX at 90-day-old rats increased the activity of the antioxidant enzyme CAT ($p < 0.05$), but did not alter the activity of the antioxidant enzyme SOD ($p > 0.05$). VIT D *per se* did not alter these parameters ($p > 0.05$); however, when associated with OVX (OVX + VIT D group), maintained increase in CAT activity observed in OVX group ($p < 0.05$; Table 1). On the other hand, OVX in 180-day-old rats present a different pattern of changes on enzymatic antioxidant defenses (Table 2). At this age, OVX group showed an increase in SOD activity ($p < 0.005$) and no change in CAT activity ($p > 0.05$); VIT D *per se* increased SOD and CAT activities ($p < 0.005$; $p < 0.05$, respectively), and when the supplementation was associated with OVX (OVX + VIT D), it partially reversed the increase in the SOD activity caused by OVX. Regarding the reactive species levels, lipid damage, and protein damage (DCFH oxidation, TBARS levels, and sulfhydryl content, respectively), the results showed that both OVX and VIT D supplementation did not alter these parameters in both ages ($p > 0.05$, Tables 1 and 2).

Table 1 Parameters of oxidative stress in hippocampus of adult ovariectomized rats and/or vitamin D supplemented

Parameters	Groups			
	SHAM	OVX	VIT D	OVX + VIT D
SOD activity (units/mg protein)	12.07 ± 0.89	10.99 ± 1.02	12.21 ± 1.07	11.21 ± 0.98
CAT activity (units/mg protein)	0.57 ± 0.15	0.83 ± 0.20*	0.66 ± 0.16	0.82 ± 0.11*
DCFH oxidation (nmol/mg protein)	379.18 ± 71.22	364.96 ± 46.07	325.80 ± 34.60	342.46 ± 65.51
TBARS levels (nmol/mg protein)	1.57 ± 0.34	1.54 ± 0.22	1.34 ± 0.14	1.37 ± 0.04
Sulfhydryl content (nmol TNB/mg protein)	56.69 ± 20.90	46.98 ± 6.74	41.21 ± 7.93	47.36 ± 4.13

Data are expressed as mean ± SD for 5–8 animals per group (one-way ANOVA followed by post hoc Tukey's test)

Adult rats submitted to OVX or SHAM at 90 days old

*Significantly different SHAM ($p < 0.05$)

Subsequently, we evaluated the DNA damage index in hippocampus from Wistar rats submitted to OVX at 90 (adult rats, Fig. 2a) or 180 (old rats, Fig. 2b) days old and VIT D supplemented. The results followed the same pattern in both ages. OVX significantly increased the DNA damage index (Fig. 2a, b; $p < 0.001$) when compared to control (SHAM). VIT D *per se* decreased DNA damage index (Fig. 2a: $p < 0.05$ and 2b: $p < 0.001$), but when was associated with OVX (OVX + VIT D), it partially reversed the DNA damage caused by OVX (Fig. 2a, b, different from SHAM and OVX groups, $p < 0.001$).

Finally, we performed analysis of the telomeres length (T/S ratio) in hippocampus of rats submitted to OVX at 90 or 180 days old with or without VIT D supplementation (Fig. 3a, b, respectively). Figure 3a shows no statistical difference in telomeres length between the groups ($p > 0.05$) of rats submitted to OVX at 90 days old; however, we observed a tendency of decrease in telomeres length in OVX and VIT D groups. When this parameter was analyzed in rats submitted to OVX at 180 days old, the results showed that OVX rats have shorter telomeres ($p < 0.005$; Fig. 3b); VIT D *per se* did not alter telomere length ($p > 0.05$), and when associated with OVX (OVX + VIT D), VIT D supplementation was able to reverse the telomere shortening observed ($p < 0.005$). Considering that the telomeres length suffers influence of normal aging, we performed additional analysis of this parameter between the ages studied (Table 3). The results showed that animals submitted to OVX at 180 days old presented telomeres shorter than those submitted to OVX at 90 days old ($p < 0.05$).

Discussion

In the present study, we investigated some biochemical parameters associated with oxidative stress, DNA damage, and telomere length in hippocampus of female Wistar rats after bilateral ovarian withdrawal (OVX) at two different ages: 90 or 180 days old, followed by VIT D supplementation. The hippocampus was the brain structure used because is associated with memory mechanism, and previous studies show that OVX causes memory impairment (Monteiro et al. 2008;

Table 2 Parameters of oxidative stress in hippocampus of older ovariectomized rats and/or vitamin D supplemented

Parameters	Groups			
	SHAM	OVX	VIT D	OVX + VIT D
SOD activity (units/mg protein)	8.92 ± 0.96	12.08 ± 1.98**	12.6 ± 1.59**	10.59 ± 1.47
CAT activity (units/mg protein)	0.52 ± 0.05	0.45 ± 0.12	0.71 ± 0.12*	0.54 ± 0.10[a]
DCFH oxidation (nmol/mg protein)	222.78 ± 40.82	225.75 ± 25.81	225.86 ± 15.09	229.05 ± 38.51
TBARS levels (nmol/mg protein)	3.17 ± 0.16	3.16 ± 0.20	2.90 ± 0.38	2.93 ± 0.33
Sulfhydryl content (nmol TNB/mg protein)	44.76 ± 14.74	47.39 ± 12.78	46.18 ± 13.16	55.65 ± 11.61

Data are expressed as mean ± SD for 6–8 animals per group (one-way ANOVA followed by post hoc Tukey's test)

Older rats submitted to OVX or SHAM at 180 days old

*Significantly different from SHAM ($p < 0.05$)

**Significantly different from SHAM ($p < 0.005$)

[a]Significantly different from VIT D ($p < 0.005$)

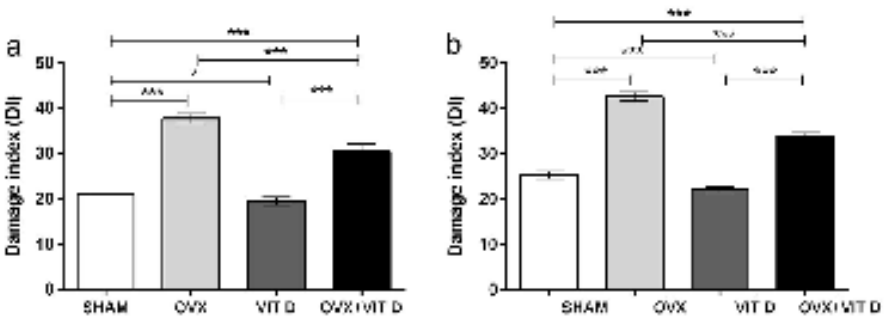


Fig. 2 Effect of ovariectomy and vitamin D supplementation on DNA damage from adult [OVX at 90 days old (a)] and older rats [OVX at 180 days old (b)]. Results are expressed as mean ± S.D. for 6–8 animals each group. $^{*}p < 0.05$; $^{***}p < 0.001$ (one-way ANOVA followed by post hoc Tukey's test). OVX ovariectomy, VIT D vitamin D, DI damage index

Siebert et al. 2017; Su et al. 2012) and alters some biochemical parameters, including energy metabolism in this brain structure of adult rats (Siebert et al. 2014).

Initially, we investigated some oxidative stress markers such as the activities of antioxidant enzymes SOD and CAT, reactive oxygen species production, and damage to lipid and protein in the hippocampus. Results showed that OVX alters enzymatic antioxidant defenses, but did not change other parameters related to oxidative stress analyzed (levels of reactive species, protein damage, and lipids) at both ages. Rats submitted to OVX at 90 days old present an increase in CAT activity, and rats submitted to OVX at 180 days old present an increase in SOD activity, suggesting that the effect of OVX on these enzymes depend on the age of rats. SOD catalyzes the reaction of dismutation of superoxide ($O_2^{\bullet -}$) in oxygen (O_2) and hydrogen peroxide (H_2O_2). CAT is one of the enzymes responsible for detoxifying H_2O_2 (Gruber et al. 2013; Halliwell and Gutteridge 2007b). CAT activation observed in rats submitted to OVX at 90 days old may be an adaptive mechanism for H_2O_2 presence that is being detoxified and therefore is not detected by oxidation of DCFH, a measure used for the detection of reactive oxygen species. On the other hand, the SOD activation observed in rats submitted to OVX at 180 days old may be resulting in an increase in H_2O_2 which was also not detected by the oxidation of DCFH and did not result in the CAT activation. This H_2O_2, produced as a result of the SOD activation, may be following the Fenton or Haber-Weiss reaction, forming the hydroxyl ($OH^{\bullet}$) radicals. The $OH^{\bullet}$ radical is highly reactive; it can react and alter cellular structures and influence enzymes, membranes, or nucleic acids (Martindale and Holbrook 2002). Evidence indicates that the continuous presence of reactive species may lead to upregulation of antioxidant enzymes as adaptive cellular strategy for oxidative stress (Finkel and Holbrook 2000; Halliwell and Gutteridge 2007a; Halliwell and Whiteman 2004; Poljsak and Milisav 2013). Therefore, we cannot rule out the presence of oxidative stress in these animals. Since the DCFH oxidation was not altered, the increase in antioxidants enzymes activities may be reflecting a compensatory mechanism for the production of reactive species present over time after OVX. We also observed that VIT D *per se* increased SOD and CAT activities in older rats, but when it was associated with OVX (OVX + VIT D), the activities of SOD and CAT decreased; therefore, when the VIT D was supplemented to rats with intact ovaries, there was an increase in activities of antioxidant defenses of CAT and SOD.

Considering the neuroprotective properties already described for estrogens and also VIT D (Annweiler et al. 2014; Arevalo et al. 2015; Green and Simpkins 2000; Kesby et al. 2011), we investigated the presence of DNA damage index in hippocampus of rats submitted to OVX at 90 or 180 days old and subsequent VIT D supplementation. Results showed that OVX caused an increase in DNA damage index at the same pattern of alteration at the two ages studied. VIT D *per se* reduced the DNA damage index and partially reversed the effect of OVX on this parameter. Since that oxidative stress may promote damage to lipids and proteins as well as DNA (Halliwell 2007; Silva and Coutinho 2010), and that in our study we observed that OVX provokes alteration in antioxidant enzymes and damage to DNA, but not to lipid and protein (measured by TBARS and sulfhydryl contents), we could not

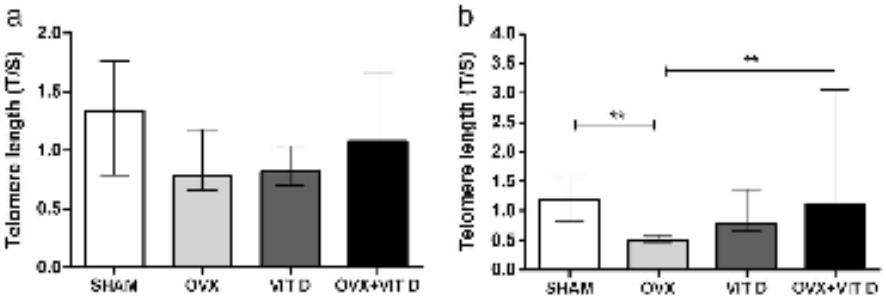


Fig. 3 Effect of ovariectomy and vitamin D supplementation on telomere length from adult [OVX at 90 days old (a)] and older rats [OVX at 180 days old (b)]. Results are expressed as median and interquartile range for 6–8 animals each group. $^{**}p < 0.005$ (Kruskal-Wallis followed by post hoc Dunn's test). OVX ovariectomy, VIT D vitamin D

Table 3 Analysis of the telomeres length between the ages

	Adult	Older
SHAM	1.33 (0.78–1.76)	1.18 (0.83–1.57)
OVX	0.78 (0.66–1.171)	0.49 (0.47–0.58)*
VIT D	0.82 (0.69–1.03)	0.79 (0.66–136)
OVX + VIT D	1.07 (0.83–1.66)	1.12 (0.94–3.06)

Data are expressed as median and interquartile range for 5–8 animals per group (Mann-Whitney *U* test)

Adult: rats submitted to OVX or SHAM at 90 days old

Older: rats submitted to OVX or SHAM at 180 days old

*$p < 0.05$

exclude other mechanisms that may be occurring due to a direct or indirect effect of oxidative stress in ovariectomized animals (suggested by adaptation of antioxidant enzymes). It is also important to remember that in our study, the surgical procedure of OVX was performed 2 months before of the decapitation of the animals; therefore, the changes observed can have been accumulated over the 60 days of OVX.

The relationship between DNA damage and aging has been widely studied (Fei Fang et al. 2016; Ribezzo et al. 2016; Schumacher et al. 2008). Both oxidative stress and DNA damage can cause shortening of telomeres and consequently accelerate senescence (Bernadotte et al. 2016; Correia-Melo et al. 2014); therefore, in this study, we also investigated the telomere length in hippocampus of Wistar rats submitted to OVX at 90 and 180 days old and subsequent VIT D supplementation. In the animals submitted to OVX at 90 days old, the OVX and VIT D *per se* groups presented a tendency of the decreased telomere lengths, but this was not significant. In 180-day-old rats submitted to OVX, we observed that OVX significantly reduced the telomere length, and the VIT D supplementation (OVX + VIT D) returns this change at SHAM-group level. In addition, our results show that normal aging of the animals of this study does not cause telomere shortening, represented by similar mean telomere length of SHAM animals at both ages. Nonetheless, OVX is a factor that stimulates telomeres attrition of rats submitted to OVX at 180 days old when compared to the rats submitted to OVX at 90 days old ($p < 0.05$). These observed telomeres shortening can be explained by the presence of DNA damage, since that the presence of oxidative DNA damage can accelerate this process (Goronzy et al. 2006; Yip et al. 2017). In addition, the OVX may be causing a reduction in the activity or in overall levels of telomerase, resulting in shortening of telomeres. The critical shortening of telomeres renders the end of the chromosome unprotected, thus, occurs subsequent DNA damage responses and activation of signaling pathways that induce replicative senescence or apoptosis (Artandi and Attardi 2005; Smogorzewska and de Lange 2002). These events may compromise homeostasis and tissue function eventually leading to organism aging, signaling senescence (Chen et al. 2007).

We also explore the effects of VIT D supplementation on hippocampus in 90 or 180 days old rats submitted to OVX. We observed some effects of VIT D *per se* and others resulting from its association with OVX, reversing OVX effects. The different actions of VIT D in the body have been studied, including regulation of neurotransmission, neuroprotection, and immunomodulation (Dursun et al. 2011; Spach and Hayes 2005), as well as in the regulation of calcium-mediated neuronal excitotoxicity and reduction of oxidative stress (Mpandzou et al. 2016; Tarbali and Khezri 2016). The beneficial effects of VIT D supplementation in ovariectomized rats observed in this study on DNA damage in both ages and telomere shortening in older rats could open perspectives for new studies to discover the mechanisms involved in these actions.

These data together suggest an imbalance in the antioxidant system that could corroborate, at least in part, with DNA damage and telomeres shortening observed in OVX group. VIT D appears to be beneficial in reversing, principally, the effects of OVX in older rats. Although hormonal changes due to OVX are well described in the literature, our results do not allow us to discuss the mechanisms involved without further studies addressing the more specific mechanisms of action. Our results constitute a preclinical study that opens perspectives for other studies involving OVX effects and auxiliary therapies. We hope, with our findings, to assist in the understanding and knowledge of brain alterations that may be present in postmenopausal women and in this way contribute to the advancement of research in this area.

Funding Information This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) and INCT (EN 465671/2014-4)/CNPq-Brazil.

Compliance with Ethical Standards

Animal experimentation protocols had been approved by the University Ethics Committee for the Use of Animals (CEUA) under the project (#28033). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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

References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Aksenov MY, Markesbery WR (2001) Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 302:141–145

Annweiler C, Karras SN, Anagnostis P, Beauchet O (2014) Vitamin D supplements: a novel therapeutic approach for Alzheimer patients. Front Pharmacol 5:6. https://doi.org/10.3389/fphar.2014.00006

Arevalo MA, Azcoitia I, Garcia-Segura LM (2015) The neuroprotective actions of oestradiol and oestrogen receptors. Nat Rev Neurosci 16:17–29. https://doi.org/10.1038/nrn3856

Artandi SE, Attardi LD (2005) Pathways connecting telomeres and p53 in senescence, apoptosis, and cancer. Biochem Biophys Res Commun 331:881–890. https://doi.org/10.1016/j.bbrc.2005.03.211

Bajpayee M, Pandey AK, Parmar D, Mathur N, Seth PK, Dhawan A (2005) Comet assay responses in human lymphocytes are not influenced by the menstrual cycle: a study in healthy Indian females. Mutat Res 565:163–172. https://doi.org/10.1016/j.mrgentox.2004.10.008

Barbe-Tuana FM et al (2016) Shortened telomere length in bipolar disorder: a comparison of the early and late stages of disease. Rev Bras Psiquiatr 38:281–286. https://doi.org/10.1590/1516-4446-2016-1910

Ben J, Soares FM, Cechetti F, Vuaden FC, Bonan CD, Netto CA, Wyse AT (2009) Exercise effects on activities of Na(+),K(+)-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats. Brain Res 1302:248–255

Ben J, Soares FM, Scherer EB, Cechetti F, Netto CA, Wyse AT (2010) Running exercise effects on spatial and avoidance tasks in ovariectomized rats. Neurobiol Learn Mem 94:312–317. https://doi.org/10.1016/j.nlm.2010.07.003

Bernadotte A, Mikhelson VM, Spivak IM (2016) Markers of cellular senescence. Telomere shortening as a marker of cellular senescence. Aging (Albany NY) 8:3–11. https://doi.org/10.18632/aging.100871

Bhatia-Dey N, Kanherkar RR, Stair SE, Makarev EO, Csoka AB (2016) Cellular senescence as the causal nexus of aging. Front Genet 7:13. https://doi.org/10.3389/fgene.2016.00013

Blackburn EH (2000) Telomeres and telomerase Keio. J Med 49:59–65

Borras C, Gambini J, Lopez-Grueso R, Pallardo FV, Vina J (2010) Direct antioxidant and protective effect of estradiol on isolated mitochondria. Biochim Biophys Acta 1802:205–211. https://doi.org/10.1016/j.bbadis.2009.09.007

Cadet J, Wagner JR (2013) DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. Cold Spring Harb Perspect Biol 5 doi:https://doi.org/10.1101/cshperspect.a012559

Cawthon RM (2002) Telomere measurement by quantitative. PCR Nucleic Acids Res 30:e47–e447

Chabas J-F, Stephan D, Marqueste T, Garcia S, Lavaut MN, Nguyen C, Legre R, Khrestchatisky M, Decherchi P, Feron F (2013) Cholecalciferol (vitamin D_3) improves myelination and recovery after nerve injury. PLoS One 8:e65034–e65034. https://doi.org/10.1371/journal.pone.0065034

Chen JH, Hales CN, Ozanne SE (2007) DNA damage, cellular senescence and organismal ageing: causal or correlative? Nucleic Acids Res 35:7417–7428. https://doi.org/10.1093/nar/gkm681

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159. https://doi.org/10.1006/abio.1987.9999

Correia-Melo C, Hewitt G, Passos JF (2014) Telomeres, oxidative stress and inflammatory factors: partners in cellular senescence? Longev Healthspan 3:1. https://doi.org/10.1186/2046-2395-3-1

Doshi SB, Agarwal A (2013) The role of oxidative stress in menopause. J Midlife Health 4:140–146. https://doi.org/10.4103/0976-7800.118990

Dursun E, Gezen-Ak D, Yilmazer S (2011) A novel perspective for Alzheimer's disease: vitamin D receptor suppression by amyloid-beta and preventing the amyloid-beta induced alterations by vitamin D in cortical neurons. J Alzheimers Dis 23:207–219. https://doi.org/10.3233/JAD-2010-101377

Eyles DW, Smith S, Kinobe R, Hewison M, McGrath JJ (2005) Distribution of the vitamin D receptor and 1 alpha-hydroxylase in human brain. J Chem Neuroanat 29:21–30. https://doi.org/10.1016/j.jchemneu.2004.08.006

Eyles D, Almeras L, Benech P, Patatian A, Mackay-Sim A, McGrath J, Feron F (2007) Developmental vitamin D deficiency alters the expression of genes encoding mitochondrial, cytoskeletal and synaptic proteins in the adult rat brain. J Steroid Biochem Mol Biol 103:538–545. https://doi.org/10.1016/j.jsbmb.2006.12.096

Fei Fang E, Scheibye-Knudsen M, Chua K, Mattson M, Croteau D (2016) Nuclear DNA damage signaling to mitochondria in aging. Nat Mol Cell Biol 17:308–321

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247. https://doi.org/10.1038/35041687

Georgakis MK, Thomopoulos TP, Diamantaras AA, Kalogirou EI, Skalkidou A, Daskalopoulou SS, Petridou ET (2016) Association of age at menopause and duration of reproductive period with depression after menopause: a systematic review and meta-analysis. JAMA Psychiatry 73:139–149. https://doi.org/10.1001/jamapsychiatry.2015.2653

Goronzy JJ, Fujii H, Weyand CM (2006) Telomeres, immune aging and autoimmunity. Exp Gerontol 41:246–251. https://doi.org/10.1016/j.exger.2005.12.002

Grant MD et al. (2015). In: Menopausal symptoms: comparative effectiveness of therapies. AHRQ comparative effectiveness reviews. Rockville (MD)

Green PS, Simpkins JW (2000) Neuroprotective effects of estrogens: potential mechanisms of action. Int J Dev Neurosci 18:347–358

Gruber J, Fong S, Chen CB, Yoong S, Pastorin G, Schaffer S, Cheah I, Halliwell B (2013) Mitochondria-targeted antioxidants and metabolic modulators as pharmacological interventions to slow ageing. Biotechnol Adv 31:563–592. https://doi.org/10.1016/j.biotechadv.2012.09.005

Gueye Y, Marqueste T, Maurel F, Khrestchatisky M, Decherchi P, Feron F (2015) Cholecalciferol (vitamin D(3)) improves functional recovery when delivered during the acute phase after a spinal cord trauma. J Steroid Biochem Mol Biol 154:23–31. https://doi.org/10.1016/j.jsbmb.2015.06.007

Halliwell B (2007) Oxidative stress and cancer: have we moved forward? Biochem J 401:1–11. https://doi.org/10.1042/BJ20061131

Halliwell B (2012) Free radicals and antioxidants: updating a personal view. Nutr Rev 70:257–265. https://doi.org/10.1111/j.1753-4887.2012.00476.x

Halliwell B, Gutteridge JM (2007a) Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. Oxford University Press, New York

Halliwell B, Gutteridge JMC (2007b) Free radicals in biology and medicine, 4th edn. Oxford University Press, Oxford

Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142:231–255. https://doi.org/10.1038/sj.bjp.0705776

Harms LR, Burne TH, Eyles DW, JJMG (2011) Vitamin D and the brain. Best Pract Res Clin Endocrinol Metab 25:657–669. https://doi.org/10.1016/j.beem.2011.05.009

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR, 4th International Comet Assay Workshop (2003) Recommendations for conducting the in vivo alkaline comet assay. 4th International Comet Assay Workshop Mutagenesis 18:45–51

Henderson VW (2014) Alzheimer's disease: review of hormone therapy trials and implications for treatment and prevention after menopause. J Steroid Biochem Mol Biol 142:99–106. https://doi.org/10.1016/j.jsbmb.2013.05.010

Henderson VW, Sherwin BB (2007) Surgical versus natural menopause: cognitive issues. Menopause 14:572–579. https://doi.org/10.1097/gme.0b013e31803df49c

Kesby JP, Eyles DW, Burne TH, McGrath JJ (2011) The effects of vitamin D on brain development and adult brain function. Mol Cell Endocrinol 347:121–127. https://doi.org/10.1016/j.mce.2011.05.014

LeBel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2', 7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5:227–231

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Mackedanz V, Mattos CB, Felksa LR, Wannmacher CM, Wyse AT (2011) Ovariectomy alters energy metabolism in rat striatum: effect of supplementation with soy diet rich in isoflavones. Metab Brain Dis 26: 97–105. https://doi.org/10.1007/s11011-010-9216-8

Marklund SL (1985) Pyrogalol autoxidation. In: Handbook for Oxygen Radical Research

Martindale JL, Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192:1–15. https://doi.org/10.1002/jcp.10119

McGrath JJ, Feron FP, Burne TH, Mackay-Sim A, Eyles DW (2004) Vitamin D3-implications for brain development. J Steroid Biochem Mol Biol 89-90:557–560. https://doi.org/10.1016/j.jsbmb.2004.03.070

Monteiro SC, Mattè C, Bavaresco CS, Netto CA, ATS W (2005a) Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. Neurobiol Learn Mem 84:192–199. https://doi.org/10.1016/j.nlm.2005.08.002

Monteiro SC, Matte C, Delwing D, Wyse AT (2005b) Ovariectomy increases Na+, K+-ATPase, acetylcholinesterase and catalase in rat hippocampus. Mol Cell Endocrinol 236:9–16

Monteiro SC, de Mattos CB, Ben J, Netto CA, ATS W (2008) Ovariectomy impairs spatial memory: prevention and reversal by a soy isoflavone diet. Metab Brain Dis 23:243–253. https://doi.org/10.1007/s11011-008-9093-6

Mpandzou G, Ait Ben Haddou E, Regragui W, Benomar A, Yahyaoui M (2016) Vitamin D deficiency and its role in neurological conditions: a review. Rev Neurol (Paris) 172:109–122. https://doi.org/10.1016/j.neurol.2015.11.005

Nathan C, Cunningham-Bussel A (2013) Beyond oxidative stress: an immunologist's guide to reactive oxygen species. Nat Rev Immunol 13:349–361. https://doi.org/10.1038/nri3423

O'Callaghan NJ, Fenech M (2011) A quantitative PCR method for measuring absolute telomere length. Biol Proced Online 13:3. https://doi.org/10.1186/1480-9222-13-3

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358

O'Sullivan RJ, Karlseder J (2010) Telomeres: protecting chromosomes against genome instability. Nat Rev Mol Cell Biol 11:171–181. https://doi.org/10.1038/nrm2848

Poljsak B, Milisav I (2013) Aging, oxidative stress and antioxidants. Oxidative stress and chronic degenerative diseases - a role for antioxidants. doi:https://doi.org/10.5772/51609

Ribezzo F, Shiloh Y, Schumacher B (2016) Systemic DNA damage responses in aging and diseases. Semin Cancer Biol 37-38:26–35. https://doi.org/10.1016/j.semcancer.2015.12.005

Rocca WA, Grossardt BR, Shuster LT (2010) Oophorectomy, menopause, estrogen, and cognitive aging: the timing hypothesis. Neurodegener Dis 7:163–166. https://doi.org/10.1159/000289229

Salum E, Kals J, Kampus P, Salum T, Zilmer K, Aunapuu M, Arend A, Eha J, Zilmer M (2013) Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats. Diabetes Res Clin Pract 100:243–249. https://doi.org/10.1016/j.diabres.2013.03.008

Sanders JL, Newman AB (2013) Telomere length in epidemiology: a biomarker of aging, age-related disease, both, or neither? Epidemiol Rev 35:112–131. https://doi.org/10.1093/epirev/mxs008

Schumacher B, Garinis GA, Hoeijmakers JH (2008) Age to survive: DNA damage and aging. Trends Genet 24:77–85. https://doi.org/10.1016/j.tig.2007.11.004

Sengupta P (2013) The laboratory rat: relating its age with human's. Int J Prev Med 4:624–630

Shuster LT, Rhodes DJ, Gostout BS, Grossardt BR, Rocca WA (2010) Premature menopause or early menopause: long-term health consequences. Maturitas 65:161–166. https://doi.org/10.1016/j.maturitas.2009.08.003

Siebert C, Kolling J, Scherer EBS, Schmitz F, da Cunha MJ, Mackedanz V, de Andrade RB, Wannmacher CMD, Wyse ATS (2014) Effect of physical exercise on changes in activities of creatine kinase, cytochrome c oxidase and ATP levels caused by ovariectomy. Metab Brain Dis 29:825–835. https://doi.org/10.1007/s11011-014-9564-x

Siebert C, Pierozan P, Kolling J, dos Santos TM, Sebotaio MC, Marques EP, Biasibetti H, Longoni A, Ferreira F, Pessoa-Pureur R, Netto CA, Wyse ATS (2017) Vitamin D3 reverses the hippocampal cytoskeleton imbalance but not memory deficits caused by ovariectomy in adult Wistar rats. NeuroMolecular Med 19:345–356. https://doi.org/10.1007/s12017-017-8449-7

Silva JP, Coutinho OP (2010) Free radicals in the regulation of damage and cell death—basic mechanisms and prevention. Drug Discov Ther 4:144–167

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Smogorzewska A, de Lange T (2002) Different telomere damage signaling pathways in human and mouse cells. EMBO J 21:4338–4348

de Souza SR, Vianna LM (2005) Effect of cholecalciferol supplementation on blood glucose in an experimental model of type 2 diabetes mellitus in spontaneously hypertensive rats and Wistar rats. Clin Chim Acta 358:146–150. https://doi.org/10.1016/j.cccn.2005.02.020

Spach KM, Hayes CE (2005) Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice. J Immunol 175: 4119–4126

Stroud ML, Stilgoe S, Stott VE, Alhabian O, Salman K (2008) Vitamin D—a review. Aust Fam Physician 37:1002–1005

Su J, Sripanidkulchai K, Hu Y, Wyss JM, Sripanidkulchai B (2012) The effect of ovariectomy on learning and memory and relationship to changes in brain volume and neuronal density. Int J Neurosci 122: 549–559. https://doi.org/10.3109/00207454.2012.690795

Tamm C, Zhivotovsky B, Ceccatelli S (2008) Caspase-2 activation in neural stem cells undergoing oxidative stress-induced apoptosis. Apoptosis 13:354–363. https://doi.org/10.1007/s10495-007-0172-7

Tarbali S, Kheiri S (2016) Vitamin D3 attenuates oxidative stress and cognitive deficits in a model of toxic demyelination Iran. J Basic Med Sci 19:80–88

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221

Valko M, Jomova K, Rhodes CJ, Kuca K, Musilek K (2016) Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. Arch Toxicol 90:1–37. https://doi.org/10.1007/s00204-015-1579-5

Waynforth HB, Flecknell PA (1992) Experimental and surgical technique in the rat. 2nd ed edn

Xiao H, Fau-Deng M, Deng M, Fau-Yang B, Yang B, Fau-Hu Z, Hu Z, Fau-Tang J, Tang J (2017) Pre-treatment of 17beta-estradiol attenuates cerebral-ischemia-induced blood-brain barrier disruption in aged rats: involvement of antioxidant signaling. Neuroendocrinology 106:20–29. https://doi.org/10.1159/000455866

Yip BW, Mok HO, Peterson DR, Wan MT, Taniguchi Y, Ge W, Au DW (2017) Sex-dependent telomere shortening, telomerase activity and oxidative damage in marine medaka Oryzias melastigma during aging. Mar Pollut Bull 124:701–709. https://doi.org/10.1016/j.marpolbul.2017.01.021

von Zglinicki T, Martin-Ruiz CM (2005) Telomeres as biomarkers for ageing and age-related diseases. Curr Mol Med 5:197–203

PARTE III

1. DISCUSSÃO

Os processos relacionados ao envelhecimento e à menopausa humana estão associados ao aumento de diversos fatores de risco para a qualidade de vida e saúde geral da mulher. Investigar a fisiopatologia de tais eventos é algo complexo, portanto, a utilização de modelos experimentais em roedores a fim de mimetizar as alterações presentes no período pós-menopausa têm sido amplamente empregados. Embora alguns mecanismos possam diferir em se tratando de uma espécie para outra, a utilização de tais modelos proporcionam oportunidades para estudarmos elementos importantes envolvidos nos processos reprodutivos e de envelhecimento, abrindo perspectivas para a melhor compreensão de tais eventos, bem como possibilitando a utilização de intervenções no sentido de prevenir e/ou reverter alguns efeitos proporcionando melhora na qualidade de vida durante esse período tão longo da vida das mulheres (Koebele e Bimonte-Nelson, 2016).

Nesse contexto no presente estudo utilizamos a OVX bilateral em ratas Wistar adultas como um modelo animal de depleção ovariana a fim de mimetizar alterações decorrentes da menopausa (Koebele e Bimonte-Nelson, 2016; Savonenko e Markowska, 2003). A OVX causa uma rápida diminuição na secreção dos hormônios ovarianos. Dados na literatura mostram que cerca de uma semana após o procedimento cirúrgico os níveis circulantes de estradiol já se apresentam significativamente reduzidos (Monteiro et al., 2005c; Savonenko e Markowska, 2003).

A suplementação com VIT D tem sido amplamente utilizada como terapia auxiliar em diversas condições, sobretudo na menopausa, onde a maior

parte dos estudos aborda seus efeitos sobre o metabolismo ósseo (Harvey e Cooper, 2016; Sullivan et al., 2017). A VIT D é uma molécula lipossolúvel que atravessa facilmente a barreira cérebro-sangue podendo, portanto exercer efeitos sobre o cérebro (Nair e Maseeh, 2012). Neste sentido, resolvemos investigar os efeitos da suplementação com VIT D em ratas adultas submetidas à OVX sobre o cérebro, mais especificamente sobre o hipocampo. Com base na literatura, escolhemos a dose de 500 UI/Kg/dia de colecalciferol (VIT D) para utilizarmos em nosso estudo (Chabas et al., 2013; Salum et al., 2013). Considerando que nossos animais recebem suplementação com VIT D na dieta padrão (cerca de 2000 UI/KG de ração), escolhemos tal dose, considerada relativamente baixa e que poderia se somar com a dieta minimizando os riscos de malefícios em decorrência de possível toxicidade.

A diminuição precoce e prolongada de hormônios ovarianos na circulação, particularmente de estradiol, parece estar relacionada com efeitos neurológicos negativos (Brann et al., 2007; Scott et al., 2012). A relação entre o aumento no déficit cognitivo e a menopausa é amplamente relatada, porém os mecanismos envolvidos ainda não são totalmente conhecidos. No presente estudo, iniciamos investigando os efeitos da menopausa experimental em ratas Wistar adultas sobre parâmetros comportamentais. Não foram observados déficits locomotores ou exploratórios no teste de campo aberto, mas a avaliação da memória de reconhecimento mostrou comprometimento na memória de curto prazo (teste 1 h após a sessão de treino) e de longo prazo (teste 7 dias após a sessão de treino) em animais submetidos à menopausa experimental, o que foi representado por percentual de exploração semelhante de ambos objetos (familiar e novo). Com base na tendência natural dos

roedores para explorar novos objetos em comparação com o que já é familiar (Ennaceur e Delacour, 1988), nosso resultado demonstrou haver comprometimento na memória de reconhecimento. Em relação à memória aversiva, avaliada pela tarefa de esquiva inibitória, observamos que as ratas ovariectomizadas apresentaram comprometimento significativo da memória aversiva de longo prazo (teste 7 dias após a sessão de treino), mas não no teste de curto prazo (teste 1 h após a sessão de treino). Considerando que as memórias são divididas em memória de curto prazo, com duração de minutos a horas e memória de longo prazo, com duração de dias, semanas e até uma vida inteira (Bailey et al., 1996; Izquierdo e Medina, 1997; Routtenberg, 2008), em nosso estudo escolhemos avaliar a memória de longo prazo 7 dias após a sessão de treino, pois dados anteriores do nosso grupo de pesquisa já demonstraram que ratas submetidas a OVX apresentam comprometimento na memória aversiva 24 horas após a sessão de treino (Ben et al., 2010). Assim, podemos sugerir que a OVX causa comprometimento na retenção de memória aversiva de longo prazo, detectada 24 horas após a sessão de treino e persistindo durante pelo menos 7 dias. Prejuízos na memória de reconhecimento relacionada à OVX em camundongos já foram relatadas, porém a alteração dependeu do tempo de avaliação: quando avaliados 12 semanas após a OVX os animais apresentaram déficit na memória de reconhecimento e quando avaliados anteriormente, 6 semanas após a OVX tal alteração de memória não foi observada (Bastos et al., 2015). Além disso, Diz-Chaves et al. (2012) mostraram que o tratamento com valerato de estradiol melhora a memória de ratas OVX, demonstrando ação benéfica dos estrógenos sobre a mesma.

A suplementação com VIT D não foi capaz de melhorar o desempenho dos animais nas tarefas comportamentais. Além disso, a VIT D *per se* alterou a memória de reconhecimento. Fedotova et al. (2016), em um estudo utilizando ratas Wistar ovariectomizadas, mostraram que a VIT D, na forma de colecalciferol em doses elevadas administrada de forma isolada ou em conjunto com 17β-estradiol, possui efeito antidepressivo em ratas fêmeas adultas. Além disso, Alrefaie e Alhayani (2015), em um estudo com ratos Wistar machos adultos em um modelo de diabetes induzido por estreptozotocina mostrou que o colecalciferol na dose de 500 UI/kg/dia, promoveu a recuperação da memória de reconhecimento. No entanto, neste estudo, o tempo de tratamento foi de 10 semanas e foram utilizados apenas machos que não sofrem flutuações hormonais como as fêmeas. Portanto, podemos sugerir que, talvez, a dose e o tempo de tratamento utilizados neste estudo podem não ter sido suficientes para alcançar efeitos sobre os parâmetros de memória analisados.

Estudos prévios já demonstraram alterações nas atividades de importantes enzimas cerebrais, como enzimas antioxidantes, AChE, citocromo c oxidase e Na^+,K^+-ATPase, bem como alterações comportamentais no modelo de OVX em ratas adultas. Baseado nisto, estendemos nossos estudos, focando especificamente em hipocampo, uma importante estrutura cerebral relacionada a processos de memória e altamente sensível aos efeitos dos estrógenos (Ben et al., 2009b; Monteiro et al., 2005b; Monteiro et al., 2005c; Siebert et al., 2014). Buscamos melhor compreender os mecanismos envolvidos nas alterações encontradas até o momento e outras, bem como

investigar os efeitos da suplementação com VIT D sobre os parâmetros investigados.

A partir destes resultados e outros da literatura que confirmam que a menopausa pode ocasionar distúrbios de memória e aumentar o risco de desenvolvimento de neurodegeneração, avaliamos parâmetros relacionados ao sistema fosforilante do citoesqueleto em hipocampo. O citoesqueleto é importante na organização celular e especificamente os neurofilamentos estão relacionados com a fragilidade celular, visto que são responsáveis por fornecer resistência mecânica para as células (Fuchs e Cleveland, 1998; Omary, 2009; Omary et al., 2004). Além disso, distúrbios na homeostase do citoesqueleto parecem estar associados à neurodegeneração (Afreen et al., 2017; Zetterberg, 2016). Iniciamos investigando a fosforilação in vitro de subunidades de NFs e GFAP presentes na fração citoesquelética enriquecida com FIs de neurônios e astrócitos, respectivamente. Os resultados mostraram que a OVX causa aumento da fosforilação de GFAP e NFs sem alterar o imunoconteúdo destes FIs. Sabe-se que as proteínas cinases dependentes de segundos mensageiros fosforilaram locais específicos localizados nos domínios amino-terminal de subunidades de FIs (Zheng et al., 2003). Consistente com isto, nossos resultados mostraram que a PKCaMII é ativada pela OVX, sugerindo que tal proteína cinase está envolvida na hiperfosforilação da GFAP e NF-L detectada nesses animais. Além disso, NFLSer55 e NFLSer57 parecem ser os sítios de fosforilação alvos da OVX, sendo a PKCaMII a proteína cinase responsável por mediar este efeito. A fosforilação destes locais específicos é relevante para a montagem dos filamentos, e a desregulação na dinâmica de fosforilação/desfosforilação pode interferir no desempenho das funções do

citoesqueleto neuronal. Além disso, a fosforilação anormal da GFAP pode levar à desmontagem da mesma, contribuindo para perturbações da homeostase celular (Gill et al., 1990).

Sítios KSP, localizados no domínio carboxi-terminal de NF-M e NF-H também apresentaram-se hiperfosforiladas no hipocampo em decorrência da OVX. Hiperfosforilação de sítios KSP “repeats” em FIs neuronais é considerado um evento importante por promover a agregação entre NFs, causando a formação de aglomerados em axônios (Holmgren et al., 2012). Estes aglomerados interferem no transporte axonal de NFs, e podem explicar, pelo menos em parte, os déficits comportamentais associados com a OVX. Levando em consideração que estes sítios são fosforilados por proteínas cinases independentes de segundo mensageiro MAPKs, pesquisamos por proteínas cinases ativadas pela OVX e identificamos JNK fosforilada/ativada no hipocampo. Portanto, podemos propor que os domínios carboxi-terminais de subunidades de NFs (NF-M e NF-H) estão sendo fosforilados por JNK.

Hormônios sexuais femininos podem modificar o tamanho, morfologia e função das células neurais e essas mudanças são devido a modificações no citoesqueleto neuronal e glial (Hansberg-Pastor et al., 2015). Estudos demonstram que os estrógenos desempenham importante papel na regulação da dinâmica do citoesqueleto (Giretti e Simoncini, 2008; Kramár et al., 2013; Sanchez et al., 2009). Além disso, o estrogênio é um modulador chave da morfologia celular e movimento, sendo estes eventos responsáveis pelo rearranjo do citoesqueleto em decorrência de mudanças no estado de fosforilação (Hansberg-Pastor et al., 2015). Uma vez que este hormônio tem um papel importante na regulação do citoesqueleto, a falta de estrogênio

poderia ser prejudicial para sua dinâmica. O conhecimento desses mecanismos utilizados pelos hormônios sexuais para modular o citoesqueleto e, portanto, a plasticidade sináptica pode ser importante para entender as alterações de aprendizado e memória presentes na menopausa.

A suplementação com VIT D reverteu as alterações em nível de citoesqueleto causadas pela OVX. Foi relatado que a VIT D atua modulando um sistema de sinalização complexo envolvendo rápida formação de segundos mensageiros, ativação de proteínas cinases e abertura de canais de Ca^{2+}. Considerando a relevância do Ca^{2+} e dos segundos mensageiros sobre a modulação do sistema de fosforilação associado ao citoesqueleto, é provável que a VIT D atue em vias de sinalização protegendo contra efeitos da OVX. De acordo com isso, já foi descrito que a VIT D tem efeitos moduladores sobre o citoesqueleto, agindo através de sobrecarga de Ca^{2+} e adenilil ciclase (Zanatta et al., 2011; Zamoner et al., 2008).

A fim de verificar se a dose de VIT D utilizada seria suficiente para causar aumento nos VDRs, avaliamos o imunocoteúdo de VDR hipocampal. Os resultados mostraram que o imunoconteúdo de VDR não altera entre os grupos experimentais estudados. O VDR é amplamente distribuído no cérebro, particularmente no hipocampo (Langub et al., 2001; Lardner, 2015). Porém, nossos resultados nos permitem sugerir que as ações neuroprotetoras da VIT D em nível de citoesqueleto no hipocampo não ocorrem através de alterações no imunoconteúdo de VDR.

Em conjunto nossos resultados iniciais mostram que a OVX causa comprometimento das memórias de reconhecimento e aversiva, além de um desequilíbrio no sistema de fosforilação do citoesqueleto em hipocampo. A

dose de VIT D que utilizamos não foi capaz de reverter os parâmetros relacionados à memória, porém se mostrou efetiva em reverter as alterações citoesqueletais encontradas nos animais OVX, portanto, seguimos nosso estudo utilizando esta mesma dose, a fim de investigar outros benefícios desta suplementação.

Sabe-se que os estrógenos afetam múltiplos sistemas corporais, sendo considerados então com ação pleiotrópica. Situações que causem alterações nos níveis fisiológicos circulantes dos estrógenos como menopausa natural, administração de estrógenos exógenos ou remoção cirúgica dos ovários, por exemplo, têm sido relacionada com alterações do sistema imune, bem como de processos cognitivos e neurodegeneração (Au et al., 2016). Procurando investigar outros mecanismos relacionados às alterações já observadas em decorrência deste modelo e considerando a relação entre o aumento de mediadores inflamatórios e a presença de neurodegeneração, seguimos nossa investigação analisando parâmetros inflamatórios em hipocampo. A ativação do fator de transcrição NF-κB é um importante evento na regulação da resposta inflamatória, portanto, avaliamos esta ativação através da análise do imunoconteúdo de NF-κB/p65 e fosfo-NF-κB/p65 em frações hipocampais citoplasmáticas e nucleares. Em nosso estudo, OVX causou um aumento do imunoconteúdo de fosfo-NF-κB/p65 nuclear. O NF-κB é um importante fator de transcrição ativado em situações de inflamação. O NF-κB é um heterodímetro situado no citoplasma e ligado a proteínas inibitórias (IκB) inativadas. O processo de fosforilação induz a ativação e translocação de NF-κB para o núcleo e, portanto, a ativação transcricional de genes alvos específicos, como mediadores inflamatórios, incluindo citocinas, quimiocinas e moléculas de

adesão celular (Barnes, 1997; Sunday et al., 2007; Wang et al., 2015). Essa translocação pode ser um mecanismo de resposta celular a oxidantes ou a estímulos inflamatórios e imunes (Benedusi et al., 2012; Wang et al., 2016). O NF-κB é responsável pelo início da aceleração de vários processos neurodegenerativos como nas doenças de Parkinson, doença de Alzheimer e infecções virais. Muitos estudos clínicos ou utilizando modelos experimentais descrevem um aumento de NF-κB em condições neuropatológicas (Grilli e Memo, 1999).

A ativação do NF-κB pode estimular a secreção de citocinas inflamatórias, que são moléculas envolvidas na sinalização durante a resposta imune (Lawrence, 2009; Wang et al., 2015). Portanto, analisamos os níveis das citocinas pró-inflamatórias TNF-α, IL-1β e IL-6 em hipocampo. Os resultados mostraram que a OVX causou um aumento nos níveis das citocinas pró-inflamatórias TNF-α e IL-6, sem alterar significativamente os níveis de IL-1β. As citocinas investigadas são consideradas mediadoras clássicas da inflamação (Xu et al., 2015). Nossos resultados concordam com outros estudos na literatura que relatam a presença de alterações inflamatórias em cérebro de animais submetidos à OVX (Kireev et al., 2014; Maurer e Williams, 2017; Xu et al., 2015). Neste sentido, sugerimos que o aumento nos níveis de citocinas pró-inflamatórias observado nos animais submetidos ao nosso modelo de OVX pode estar intimamente relacionado com a ativação NF-κB. A VIT D *per se* não teve efeito sobre tais parâmetros e a suplementação com VIT D em ratas OVX reverteu parcialmente o aumento nos níveis de fosfo-NF-κB e IL-6.

Benefícios da suplementação com VIT D sobre a inflamação já foram relatados (Chirumbolo et al., 2017; Hansdottir e Monick, 2011; Mousa et al., 2017). Estudos sugerem que os benefícios da VIT D sobre respostas inflamatórias e imunes é apoiado pela presença do VDR na maioria das células imunes, incluindo monócitos, macrófagos e linfócitos T e B ativados (Gysemans et al., 2005; Mousa et al., 2017). Estudos *in vitro* relatam que a forma ativa da VIT D (1,25-dihidroxivitamina D_3) inibe a expressão de citocinas pró-inflamatórias, além de estimular a produção de citocinas anti-inflamatórias, atuando como reguladora da atividade das células imunes (Guillot et al., 2010; Lemire, 1992). Além disso, um trabalho utilizando o modelo de doença de Parkinson em comundongos mostrou que a suplementação com VIT D diminuiu a expressão de citocinas pró-inflamatórias, aumentou a expressão de citocinas anti-inflamatórias, bem como atenuou a ativação de células microgliais em estriado e substância nigra (Calvello et al., 2017). Somado a isso, uma possível ação da VIT D como inibidora do fator de transcrição NF-κB tambem já foi relatada (Al-Rasheed et al., 2015; de Jongh et al., 2017). Tais evidências mostrando ações benéficas da VIT D no controle da inflamação podem explicar a reversão parcial no imunoconteúdo de NF-κB e nos níveis de IL-6.

A ACh é um neurotransmissor cerebral importante do SNC e envolvida em processos de aprendizagem e memória. A ACh, juntamente com seus receptores e as enzimas ChAT (responsável pela síntese de Ach a partir de acetil-coenzima A e colina), e a AChE (responsável pela hidrólise de ACh em acetato e colina), fazem parte do sistema de neurotransmissão colinérgica, que recentemente recebeu grande atenção em relação à sua atuação na via anti-

inflamatória (Maurer e Williams, 2017; Pavlov e Tracey, 2005). Estudos anteriores já demonstraram aumento da atividade da AChE, em hipocampo de ratas adultas submetidas a OVX (ref), portanto, estendemos tal investigação analisando além da atividade enzimática, o imunoconteúdo de tal enzima bem como o imunoconteúdo da ChAT. Nossos achados demonstraram que a ativação da AChE observada neste modelo é independente de alterações no imunoconteúdo da enzima e também do imunoconteúdo da ChAT. O aumento da atividade de AChE sem alteração no imunoconteúdo de ChAT pode causar uma redução da neurotransmissão colinérgica devido a uma diminuição nos níveis de ACh disponível na fenda sináptica, corroborando com os déficits de memória já descritos neste modelo, bem como para alterações inflamatórias observadas, já que a ACh pode atuar no sentido de atenuar a inflamação. A VIT D *per se* não teve efeito nesses parâmetros e também não foi capaz de reverter a ativação na atividade AChE, demonstrando não exercer efeitos sobre o sistema colinérgico.

Nesta parte do trabalho dosamos os níveis de estradiol em soro ao final do experimento (60 dias após a OVX) e observamos redução significativa dos níveis de estradiol nos animais submetidos à OVX, confirmando a eficácia da cirurgia para o objetivo proposto. Da mesma forma, para verificar se a dose utilizada de VIT D estaria causando alterações nos níveis de VIT D circulantes, dosamos os níveis de calcitriol em soro. Resultados mostraram aumento dos níveis de calcitriol em soro de ratas suplementadas com VIT D, mostrando que tal dose foi capaz de alterar os níveis circulantes da vitamina. O peso corporal ao final do experimento e a ingestão de alimentos (avaliação por 15 dias) também foram analisados. O aumento do peso corporal é uma característica

comum da OVX. A OVX aumentou significativamente o peso corporal final, corroborando com os dados da literatura (Ben et al., 2010; Fang et al., 2015; Mackedanz et al., 2011; Monteiro et al., 2008; Russell et al., 2017). A suplementação com VIT D na dose utilizada não foi capaz de atuar sobre o ganho de peso corporal, uma vez que os animais do grupo OVX + VIT D também apresentaram aumento no peso corporal quando comparados ao grupo SHAM. O excesso de ganho de peso corporal relacionado à privação de estrógenos pode predispor ao desenvolvimento de várias doenças crônicas como obesidade, resistência à insulina e doenças cardiovasculares (Ferrara et al., 2002; Rogers et al., 2009; Tchernof e Despres, 2000) que estão associadas a um declínio na qualidade da vida da mulher (Lobo et al., 2014). Para verificar se o ganho de peso corporal dos animais OVX estava relacionado ao aumento da ingestão alimentar, avaliamos a ingestão diária de ração por animal (gramas). Embora a literatura sugira que o aumento do peso corporal causado pela OVX seria devido à hiperfagia (Iwasa et al., 2011; Jiang et al., 2008), nossos dados não mostraram mudanças na ingestão diária de ração entre os grupos. É necessário enfatizar que a medida do consumo alimentar no presente estudo foi controlada por um curto período de tempo (cerca de duas semanas), concomitante à administração de veículo ou VIT D, e consiste em uma estimativa média do consumo de alimentos por caixa.

Seguimos nossa busca por alterações que poderiam estar relacionadas ao que já foi encontrado até o momento, e investigamos o papel da suplementação com VIT D sobre parâmetros oxidativos em hipocampo. Estudo anterior do nosso grupo de pesquisa já demonstrou aumento na atividade da CAT em decorrência da OVX em ratas adultas (90 dias de vida), sem alteração

dos demais parâmetros relacionados ao estresse oxidativo investigados, como as atividades da SOD, glutationa peroxidase (GPX) e TRAP. Neste sentido, ampliamos a investigação analisando parâmetros de estresse oxidativo em ratas adultas submetidas à OVX em duas diferentes idades: 90 dias ou 180 dias (a fim de compreender se o procedimento cirúrgico realizado mais tardiamente exerceria efeitos distintos sobre o hipocampo destes animais) e estendemos a investigação analisando danos a biomoléculas como proteínas, lipídios e DNA, bem como comprimento de telômeros.

Nossos resultados ao final do experimento mostraram (60 dias após o procedimento cirúrgico) que a OVX em ratas adultas com 90 dias causou aumento na atividade da CAT, sem alterar a atividade da SOD. Por outro lado, em ratas adultas submetidas à OVX com 180 dias houve um aumento na atividade da SOD sem alteração na atividade da CAT, o que demonstra um padrão diferente de ativação nas enzimas antioxidantes causada pela OVX em diferentes idades. Curiosamente, em ambas as idades a OVX não causou alterações detectáveis nos níveis de espécies reativas pelo ensaio de oxidação do DCFH, o que não exclui a presença de outras espécies reativas não detectáveis através deste ensaio.

A SOD é a enzima responsável por catalisar a reação de dismutação do ânion $O_2^{\bullet-}$ em oxigênio (O_2) e H_2O_2. A CAT, bem como a GPX, é responsável pela detoxificação do H_2O_2 (Gruber et al., 2013; Halliwell e Gutteridge, 2007a). Portanto, o aumento na atividade da CAT observado em ratas submetidos à OVX aos 90 dias pode representar um mecanismo adaptativo à possível presença de H_2O_2, que está sendo detoxificado e, portanto, não é detectado

pela oxidação de DCFH, uma medida usada para a detecção de espécies reativas de oxigênio. Por outro lado, a ativação da SOD observada em ratas submetidos à OVX com 180 dias de idade pode resultar em um aumento no H_2O_2 que também não foi detectado pela oxidação da DCFH e não resultou na ativação da CAT. Este H_2O_2, produzido como resultado da ativação de SOD pode estar seguindo a reação de Fenton ou Haber-Weiss, formando os radicais HO•. O radical HO• é altamente reativo; ele pode reagir e alterar estruturas celulares próximas, causando danos a proteínas, membranas ou ácidos nucleicos (Martindale e Holbrook, 2002; Silva e Coutinho, 2010). Evidências indicam que a presença contínua de espécies reativas pode levar à elevação das enzimas antioxidantes como estratégia celular adaptativa para o estresse oxidativo (Finkel e Holbrook, 2000; Halliwell e Whiteman, 2004; Poljsak e Milisav, 2013). Portanto, não podemos descartar a presença de estresse oxidativo nesses animais, uma vez que a oxidação de DCFH não foi alterada. O aumento nas atividades das enzimas antioxidantes observado em nosso modelo pode refletir um mecanismo compensatório para a produção de espécies reativas presentes ao longo do tempo após a OVX. Também observamos que a VIT D *per se* aumentou as atividades da SOD e da CAT em ratas submetidas à OVX com 180 dias, o que nos mostra que a VIT D quando suplementada em animais com ovários intactos e em idade mais avançada causa aumento nas atividades das defesas antioxidantes CAT e SOD.

Sabe-se que em condições fisiológicas existe um equilíbrio entre a produção de espécies reativas e as defesas antioxidantes. O estresse oxidativo, conhecido como o desequilíbrio entre a produção de espécies reativas e as defesas antioxidantes (Finkel e Holbrook, 2000; Gutteridge e

Halliwell, 2000), está envolvido em diversas condições e patologias (Doshi e Agarwal, 2013; Halliwell, 2006). Espécies reativas podem reagir com proteínas, lipídios e também com o DNA e o RNA levando a mutações somáticas e distúrbios de transcrição (Delanty e Dichter, 1998). Nossos resultados não demonstraram alterações no conteúdo de sulfidrilas e TBARS, medidas de dano a proteínas e lipídios, respectivamente, em ambas as idades. Porém, considerando que o DNA é uma biomolécula altamente sensível ao estresse oxidativo e também inflamação, investigamos a possível presença de dano ao DNA em hipocampo. Os resultados mostraram que a OVX causou um aumento no índice de dano ao DNA nas duas idades estudadas (OVX com 90 dias e 180 dias). A VIT D *per se* reduziu o índice de dano ao DNA e quando associada à OVX reverteu parcialmente o efeito da OVX sobre este parâmetro. Uma vez que o estresse oxidativo pode promover danos aos lipídios, proteínas e DNA e que em nosso estudo observamos que OVX provocou alterações em enzimas antioxidantes, e dano ao DNA, mas não a lipídios e proteínas, não podemos excluir outros mecanismos que possam estar ocorrendo devido a um efeito direto ou indireto do estresse oxidativo em animais ovariectomizados (sugerido pela adaptação de enzimas antioxidantes). Também é importante lembrar que em nosso estudo o procedimento cirúrgico de OVX foi realizado dois meses antes da eutanásia dos animais, portanto, as mudanças observadas podem ter sido acumuladas ao longo dos 60 dias da depleção hormonal ovariana. As principais causas de dano ao DNA incluem o acúmulo de espécies reativas e/ou deficiência no reparo do DNA. Defeitos na conformação normal do DNA são observados na presença de dano e se o dano não sofrer reparo pode ocorrer mutação ou bloqueio da replicação do DNA (Bruning et al., 2014). O

dano ao DNA não é causado somente pelo estresse oxidativo, mas é considerado um marcador biológico sensível ao estresse oxidativo.

O envelhecimento é um processo natural do organismo, porém danos oxidativos, disfunções mitocondriais, inflamação e encurtamento de telômeros podem acelerar esse processo levando a senescência celular. Além disso, a relação entre o dano ao DNA e o envelhecimento tem sido amplamente estudada (Bernadotte et al., 2016; Correia-Melo et al., 2014; Fei Fang et al., 2016; Ribezzo et al., 2016; Schumacher et al., 2008). Considerando nossos resultados até o momento, e a fim de verificar se o procedimento cirúrgico de remoção bilateral dos ovários causa alterações no fenótipo senescente, realizamos a análise do comprimento de telômeros em hipocampo dos animais submetidos à OVX com 90 ou 180 dias de idade.

Resultados mostram que nos animais submetidos à OVX com 90 dias de idade, os grupos OVX e VIT D *per se* apresentaram tendência à redução do comprimento dos telômeros, mas isso não foi significativo. Em ratos de 180 dias submetidos à OVX, observamos que a OVX reduziu significativamente o comprimento dos telômeros e a suplementação com VIT D reverteu este parâmetro. Este encurtamento de telômeros observado pode ser explicado pela presença de dano do DNA, uma vez que a presença de dano oxidativo ao DNA pode acelerar esse processo (Goronzy et al., 2006; Yip et al., 2017). O encurtamento crítico dos telômeros torna o fim do cromossomo desprotegido, assim, ocorrem respostas subsequentes ao dano do DNA e a ativação das vias de sinalização que induzem senescência replicativa ou apoptose (Artandi e Attardi, 2005; Smogorzewska e de Lange, 2002). Esses eventos podem

comprometer a homeostase e a função do tecido, levando ao envelhecimento do organismo, sinalizando a senescência (Chen et al., 2007b).

Evidências de que a VIT D pode influenciar o envelhecimento em humanos através de ação sobre os telômeros já foram reportadas (de Jongh et al., 2017). Estudos recentes em mulheres na pré e pós-menopausa mostraram associação entre altas concentrações de calcidiol e maior comprimento de telômeros em leucócitos (Liu et al., 2013; Richards et al., 2007) e tal achado não foi reproduzido em mulheres adultas-jovens (Williams et al., 2016). Os efeitos positivos da VIT D sobre o comprimento dos telômeros podem levar à integridade e estabilidade genética, o que pode resultar em melhora na longevidade (de Jongh et al., 2017). Para o nosso conhecimento, até o momento a associação entre OVX, suplementação com VIT e comprimento de telômeros em hipocampos ainda não foi investigada na literatura, portanto, nosso resultado é pioneiro em demonstrar tal relação.

Por fim, mesmo que a associação entre menopausa e um risco aumentado de transtornos neurológicos seja amplamente descrita e investigada, os mecanismos envolvidos ainda não são totalmente compreendidos e os dados na literatura ainda são conflitantes. Adicionalmente, embora efeitos benéficos da suplementação com VIT D já tenham sido descritos, poucos estudos abordam especificamente os efeitos da suplementação com VIT D na forma de colecalciferol em parâmetros hipocampais, especialmente no modelo OVX em ratas Wistar adultas. Esperamos, com nossos achados, auxiliar na melhor compreensão das alterações cerebrais que possam estar presentes em mulheres na pós-menopausa. Nossos resultados abrem perspectivas para pesquisas futuras a

fim de melhor compreender as alterações cerebrais observadas no período pós-menopausa, bem como para futuras terapias auxiliares.

2.CONCLUSÕES

Os resultados da presente tese permitem concluir que:

- *A OVX em ratas fêmeas adultas com 90 dias de vida*:

- Diminui os níveis de estradiol circulantes em soro;
- Prejudicou a memória de reconhecimento de curto e longo prazo;
- Prejudicou a memória aversiva de longo-prazo;
- Causou hiperfosforilação de FIs de neurônios e astrócitos em hipocampo;
- Alterou o imunoconteúdo de sítios de fosforilação e cinases envolvidas na fosforilação de FIs do citoesqueleto em hipocampo;
- Aumentou o imunoconteúdo de fosfo-NF-κB nuclear em hipocampo;
- Aumentou os níveis das citocinas pró-inflamatórias TNF-α e IL-6;
- Aumentou a atividade da AChE em hipocampo;
- Causou aumento de peso corporal;
- Aumentou a atividade da CAT em hipocampo;
- Causou dano ao DNA em hipocampo.

- *A OVX em ratas fêmeas adultas com 180 dias de vida*:

- Aumento a atividade da SOD em hipocampo;
- Causou dano ao DNA em hipocampo;
- Causou encurtamento de telômeros em hipocampo.

- *A suplementação com vitamina D em ratas adultas submetidas a OVX com 90 dias:*

- Exerceu efeito *per se* na memória de reconhecimento de curta e longa duração;
- Reverteu a hiperfosforilação de FIs de neurônios e astrócitos em hipocampo;
- Reverteu o aumento no imunoconteúdo de sítios de fosforilação e cinases envolvidas na fosforilação de FIs do citoesqueleto em hipocampo;
- Reverteu parcialmente o imunoconteúdo de fosfo-NF-κB e níveis de IL-6 em hipocampo;
- Reverteu o dano ao DNA em hipocampo;
- Exerceu efeito *per se* reduzindo índice de dano ao DNA;

- *A suplementação com vitamina D em ratas adultas submetidas a OVX com 180 dias:*

- Reverteu parcialmente a ativação na SOD em hipocampo;
- Reverteu parcialmente o dano ao DNA;
- Exerceu efeito *per se* reduzindo o índice de dano ao DNA.
- Reverteu a diminuição no comprimento de telômeros.

2.1 CONCLUSÃO GERAL

Em conjunto, os resultados desse trabalho mostram que a OVX causa prejuízos comportamentais, altera a homeostase do citoesqueleto, do sistema inflamatório e colinérgico, bem como causa alterações em enzimas antioxidantes, dano ao DNA e redução do comprimento de telômeros em hipocampo de ratas Wistar adultas submetidas à OVX. Nossos achados podem estar relacionados com alguns dos efeitos neurológicos negativos observados em mulheres após a menopausa. A suplementação com VIT D foi capaz de reverter total ou parcialmente algumas das alterações hipocampais encontradas nos animais submetidos à OVX. Neste sentido, a utilização da suplementação com VIT D como terapia para tratar efeitos da menopausa parece promissora, porém necessita de estudos complementares a fim de melhor determinar a dose e tempo de tratamento.

3. PERSPECTIVAS

- ✓ Avaliar parâmetros de metabolismo energéticos, função mitocondrial e morfologia do hipocampo;
- ✓ Analisar níveis de neurotrofinas em hipocampo (NGF, GDNF e NT-3);
- ✓ Investigar os efeitos do tratamento concomitante com estrogênio e VIT D;

REFERÊNCIAS BIBLIOGRÁFICAS

Abbas, O., Bhawan, J. (2010) Cutaneous perineural inflammation: a review. J Cutan Pathol 37, 1200-1211.

Afreen, S., Riherd Methner, D.N., Ferreira, A. (2017) Tau45-230 association with the cytoskeleton and membrane-bound organelles: Functional implications in neurodegeneration. Neuroscience 362, 104-117.

Ahmed, H.H., Estefan, S.F., Mohamd, E.M., Farrag Ael, R., Salah, R.S. (2013) Does melatonin ameliorate neurological changes associated with Alzheimer's disease in ovariectomized rat model? Indian J Clin Biochem 28, 381-389.

Al-Rasheed, N.M., Al-Rasheed, N.M., Bassiouni, Y.A., Hasan, I.H., Al-Amin, M.A., Al-Ajmi, H.N., Mohamad, R.A. (2015) Vitamin D attenuates pro-inflammatory TNF-alpha cytokine expression by inhibiting NF-small ka, CyrillicB/p65 signaling in hypertrophied rat hearts. J Physiol Biochem 71, 289-299.

Alberini, C.M., Milekic, M.H., Tronel, S. (2006) Mechanisms of memory stabilization and de-stabilization. Cell Mol Life Sci 63, 999-1008.

Alberts, A., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. (2008) Molecular biology of the cell. Garland Science, New York.

Alrefaie, Z., Alhayani, A. (2015) Vitamin D_3 improves decline in cognitive function and cholinergic transmission in prefrontal cortex of streptozotocin-induced diabetic rats. Behavioural Brain Research 287, 156-162.

Arnal, J.F., Fontaine, C., Billon-Gales, A., Favre, J., Laurell, H., Lenfant, F., Gourdy, P. (2010) Estrogen receptors and endothelium. Arterioscler Thromb Vasc Biol 30, 1506-1512.

Artandi, S.E., Attardi, L.D. (2005) Pathways connecting telomeres and p53 in senescence, apoptosis, and cancer. Biochem Biophys Res Commun 331, 881-890.

Au, A., Feher, A., McPhee, L., Jessa, A., Oh, S., Einstein, G. (2016) Estrogens, inflammation and cognition. Front Neuroendocrinol 40, 87-100.

Bailey, C.H., Bartsch, D., Kandel, E.R. (1996) Toward a molecular definition of long-term memory storage. Proc Natl Acad Sci U S A 93, 13445-13452.

Baldwin, A.S., Jr. (2001) Series introduction: the transcription factor NF-kappaB and human disease. J Clin Invest 107, 3-6.

Barnes, P.J. (1997) Nuclear factor-kappa B. Int J Biochem Cell Biol 29, 867-870.

Bastos, C.P., Pereira, L.M., Ferreira-Vieira, T.H., Drumond, L.E., Massensini, A.R., Moraes, M.F., Pereira, G.S. (2015) Object recognition memory deficit and depressive-like behavior caused by chronic ovariectomy can be transitorialy recovered by the acute activation of hippocampal estrogen receptors. Psychoneuroendocrinology 57, 14-25.

Bear, M.F., Connors, B.W., Paradiso, M.A. (2002) Neurociências: desvendando o sistema nervoso, 2.ed. ed, Porto Alegre.

Bellanti, F., Matteo, M., Rollo, T., De Rosario, F., Greco, P., Vendemiale, G., Serviddio, G. (2013) Sex hormones modulate circulating antioxidant enzymes: impact of estrogen therapy. Redox Biol 1, 340-346.

Ben, J., Soares, F.M., Cechetti, F., Vuaden, F.C., Bonan, C.D., Netto, C.A., Wyse, A.T. (2009a) Exercise effects on activities of Na(+),K(+)-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats. Brain Res 1302, 248-255.

Ben, J., Soares, F.M., Scherer, E.B., Cechetti, F., Netto, C.A., Wyse, A.T. (2010) Running exercise effects on spatial and avoidance tasks in ovariectomized rats. Neurobiol Learn Mem 94, 312-317.

Ben, J., Soares, F.M.S., Cechetti, F., Vuaden, F.C., Bonan, C.D., Netto, C.A., Wyse, A.T.d.S. (2009b) Exercise effects on activities of Na+,K+-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats. Brain Res 1302, 248-255.

Benedusi, V., Meda, C., Della Torre, S., Monteleone, G., Vegeto, E., Maggi, A. (2012) A lack of ovarian function increases neuroinflammation in aged mice. Endocrinology 153, 2777-2788.

Bernadotte, A., Mikhelson, V.M., Spivak, I.M. (2016) Markers of cellular senescence. Telomere shortening as a marker of cellular senescence. Aging (Albany NY) 8, 3-11.

Birzniece, V., Backstrom, T., Johansson, I.M., Lindblad, C., Lundgren, P., Lofgren, M., Olsson, T., Ragagnin, G., Taube, M., Turkmen, S., Wahlstrom, G., Wang, M.D., Wihlback, A.C., Zhu, D. (2006) Neuroactive steroid effects on cognitive functions with a focus on the serotonin and GABA systems. Brain Res Rev 51, 212-239.

Blackburn, E.H. (2000) Telomeres and telomerase. Keio J Med 49, 59-65.

Boulware, M.I., Kent, B.A., Frick, K.M. (2012) The impact of age-related ovarian hormone loss on cognitive and neural function. Curr Top Behav Neurosci 10, 165-184.

Brann, D.W., Dhandapani, K., Wakade, C., Mahesh, V.B., Khan, M.M. (2007) Neurotrophic and neuroprotective actions of estrogen: basic mechanisms and clinical implications. Steroids 72, 381-405.

Brinton, R.D. (2009) Estrogen-induced plasticity from cells to circuits: predictions for cognitive function. Trends Pharmacol Sci 30, 212-222.

Briones, T.L., Darwish, H. (2012) Vitamin D mitigates age-related cognitive decline through the modulation of pro-inflammatory state and decrease in amyloid burden. J Neuroinflammation 9, 244.

Bruning, J.G., Howard, J.L., McGlynn, P. (2014) Accessory replicative helicases and the replication of protein-bound DNA. J Mol Biol 426, 3917-3928.

Bruunsgaard, H. (2005) Physical activity and modulation of systemic low-level inflammation. J Leukoc Biol 78, 819-835.

Butt, H.Z., Atturu, G., London, N.J., Sayers, R.D., Bown, M.J. (2010) Telomere length dynamics in vascular disease: a review. Eur J Vasc Endovasc Surg 40, 17-26.

Calvello, R., Cianciulli, A., Nicolardi, G., De Nuccio, F., Giannotti, L., Salvatore, R., Porro, C., Trotta, T., Panaro, M.A., Lofrumento, D.D. (2017) Vitamin D Treatment Attenuates Neuroinflammation and Dopaminergic Neurodegeneration in an Animal Model of Parkinson's Disease, Shifting M1 to M2 Microglia Responses. J Neuroimmune Pharmacol 12, 327-339.

Cen, J., Zhang, H., Liu, Y., Deng, M., Tang, S., Liu, W., Zhang, Z. (2015) Anti-aging effect of estrogen on telomerase activity in ovariectomised rats--animal model for menopause. Gynecol Endocrinol 31, 582-585.

Chabas, J.-F., Stephan, D., Marqueste, T., Garcia, S., Lavaut, M.-N., Nguyen, C., Legre, R., Khrestchatisky, M., Decherchi, P., Feron, F. (2013) Cholecalciferol (vitamin D_3) improves myelination and recovery after nerve injury. PLoS One 8, e65034-e65034.

Chang, L., Goldman, R.D. (2004) Intermediate filaments mediate cytoskeletal crosstalk. Nat Rev Mol Cell Biol 5, 601-613.

Chen, J.H., Hales, C.N., Ozanne, S.E. (2007a) DNA damage, cellular senescence and organismal ageing: causal or correlative? Nucleic Acids Res 35, 7417-7428.

Chen, J.H., Ozanne Se Fau - Hales, C.N., Hales, C.N. (2007b) Methods of cellular senescence induction using oxidative stress. Methods Mol Biol 371, 179-189.

Chirumbolo, S., Bjorklund, G., Sboarina, A., Vella, A. (2017) The Role of Vitamin D in the Immune System as a Pro-survival Molecule. Clin Ther 39, 894-916.

Cioffi, M., Esposito, K., Vietri, M.T., Gazzerro, P., D'Auria, A., Ardovino, I., Puca, G.A., Molinari, A.M. (2002) Cytokine pattern in postmenopause. Maturitas 41, 187-192.

Colton, C.A. (2009) Heterogeneity of microglial activation in the innate immune response in the brain. J Neuroimmune Pharmacol 4, 399-418.

Conn, P.M. (2006) Handbook of models for human aging. Elsevier Academic Press, Amsterdam ; Boston.

Correia-Melo, C., Hewitt, G., Passos, J.F. (2014) Telomeres, oxidative stress and inflammatory factors: partners in cellular senescence? Longev Healthspan 3, 1.

Curfs, J.H., Meis, J.F., Hoogkamp-Korstanje, J.A. (1997) A primer on cytokines: sources, receptors, effects, and inducers. Clin Microbiol Rev 10, 742-780.

Dansokho, C., Heneka, M.T. (2017) Neuroinflammatory responses in Alzheimer's disease. J Neural Transm (Vienna).

de Jongh, R.T., van Schoor, N.M., Lips, P. (2017) Changes in vitamin D endocrinology during aging in adults. Mol Cell Endocrinol 453, 144-150.

de Lima Pelaez, P., Funchal, C., Loureiro, S.O., Heimfarth, L., Zamoner, A., Gottfried, C., Latini, A., Wajner, M., Pessoa-Pureur, R. (2007) Branched-chain amino acids accumulating in maple syrup urine disease induce morphological alterations in C6 glioma cells probably through reactive species. Int J Dev Neurosci 25, 181-189.

de Souza Santos, R., Vianna, L.M. (2005) Effect of cholecalciferol supplementation on blood glucose in an experimental model of type 2 diabetes mellitus in spontaneously hypertensive rats and Wistar rats. Clinica Chimica Acta 358, 146-150.

Delanty, N., Dichter, M.A. (1998) Oxidative injury in the nervous system. Acta Neurol Scand 98, 145-153.

Diaz Brinton, R. (2012) Minireview: translational animal models of human menopause: challenges and emerging opportunities. Endocrinology 153, 3571-3578.

Diz-Chaves, Y., Kwiatkowska-Naqvi, A., Von Hulst, H., Pernia, O., Carrero, P., Garcia-Segura, L.M. (2012) Behavioral effects of estradiol therapy in ovariectomized rats depend on the age when the treatment is initiated. Exp Gerontol 47, 93-99.

Doshi, S.B., Agarwal, A. (2013) The role of oxidative stress in menopause. J Midlife Health 4, 140-146.

Dursun, E., Gezen-Ak, D., Yilmazer, S. (2011) A novel perspective for Alzheimer's disease: vitamin D receptor suppression by amyloid-beta and preventing the amyloid-beta induced alterations by vitamin D in cortical neurons. J Alzheimers Dis 23, 207-219.

Eddleston, M., Mucke, L. (1993) Molecular profile of reactive astrocytes--implications for their role in neurologic disease. Neuroscience 54, 15-36.

Ennaceur, A., Delacour, J. (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 31, 47-59.

Eyles, D., Almeras, L., Benech, P., Patatian, A., Mackay-Sim, A., McGrath, J., Feron, F. (2007) Developmental vitamin D deficiency alters the expression of genes encoding mitochondrial, cytoskeletal and synaptic proteins in the adult rat brain. J Steroid Biochem Mol Biol 103, 538-545.

Eyles, D.W., Smith, S., Kinobe, R., Hewison, M., McGrath, J.J. (2005) Distribution of the vitamin D receptor and 1 alpha-hydroxylase in human brain. J Chem Neuroanat 29, 21-30.

Fang, J., Yang, L., Zhang, R., Zhu, X., Wang, P. (2015) Are there differences between Sprague-Dawley and Wistar rats in long-term effects of ovariectomy as a model for postmenopausal osteoporosis? Int J Clin Exp Pathol 8, 1491-1502.

Fedotova, J., Dudnichenko, T., Kruzliak, P., Puchavskaya, Z. (2016) Different effects of vitamin D hormone treatment on depression-like behavior in the adult ovariectomized female rats. Biomed Pharmacother 84, 1865-1872.

Fei Fang, E., Scheibye-Knudsen, M., Chua, K., Mattson, M., Croteau, D. (2016) Nuclear DNA damage signaling to mitochondria in aging. Nat Mol Cell Biol 17, 308-321.

Féron, F., Marqueste, T., Bianco, J., Gueye, Y., Chabas, J.-F., Decherchi, P. (2014) [Repairing the spinal cord with vitamin D: a promising strategy]. Biologie aujourd'hui 208, 69-75.

Ferrara, C.M., Lynch, N.A., Nicklas, B.J., Ryan, A.S., Berman, D.M. (2002) Differences in adipose tissue metabolism between postmenopausal and perimenopausal women. J Clin Endocrinol Metab 87, 4166-4170.

Ferrari, D., Lombardi, G., Banfi, G. (2017) Concerning the vitamin D reference range: pre-analytical and analytical variability of vitamin D measurement. Biochem Med (Zagreb) 27, 030501.

Finkel, T., Holbrook, N.J. (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408, 239-247.

Fiocchetti, M., Ascenzi, P., Marino, M. (2012) Neuroprotective effects of 17beta-estradiol rely on estrogen receptor membrane initiated signals. Front Physiol 3, 73.

Frankola, K.A., Greig, N.H., Luo, W., Tweedie, D. (2011) Targeting TNF-alpha to elucidate and ameliorate neuroinflammation in neurodegenerative diseases. CNS Neurol Disord Drug Targets 10, 391-403.

Fuchs, E., Cleveland, D.W. (1998) A structural scaffolding of intermediate filaments in health and disease. Science 279, 514-519.

Gallowitsch-Puerta, M., Pavlov, V.A. (2007) Neuro-immune interactions via the cholinergic anti-inflammatory pathway. Life Sci 80, 2325-2329.

Gibbs, R.B., Gabor, R. (2003) Estrogen and cognition: applying preclinical findings to clinical perspectives. J Neurosci Res 74, 637-643.

Gill, S.R., Wong, P.C., Monteiro, M.J., Cleveland, D.W. (1990) Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NF-L) subunit. J Cell Biol 111, 2005-2019.

Giretti, M.S., Simoncini, T. (2008) Rapid regulatory actions of sex steroids on cell movement through the actin cytoskeleton. Steroids 73, 895-900.

Goronzy, J.J., Fujii, H., Weyand, C.M. (2006) Telomeres, immune aging and autoimmunity. Exp Gerontol 41, 246-251.

Grant, M.D., Marbella, A., Wang, A.T., Pines, E., Hoag, J., Bonnell, C., Ziegler, K.M., Aronson, N. (2015), Menopausal Symptoms: Comparative Effectiveness of Therapies, Rockville (MD).

Gray, K.E., Schiff, M.A., Fitzpatrick, A.L., Kimura, M., Aviv, A., Starr, J.R. (2014) Leukocyte telomere length and age at menopause. Epidemiology 25, 139-146.

Grilli, M., Memo, M. (1999) Nuclear factor-kappaB/Rel proteins: a point of convergence of signalling pathways relevant in neuronal function and dysfunction. Biochem Pharmacol 57, 1-7.

Gruber, C.J., Tschugguel, W., Schneeberger, C., Huber, J.C. (2002) Production and actions of estrogens. N Engl J Med 346, 340-352.

Gruber, J., Fong, S., Chen, C.B., Yoong, S., Pastorin, G., Schaffer, S., Cheah, I., Halliwell, B. (2013) Mitochondria-targeted antioxidants and metabolic modulators as pharmacological interventions to slow ageing. Biotechnol Adv 31, 563-592.

Gueye, Y., Marqueste, T., Maurel, F., Khrestchatisky, M., Decherchi, P., Feron, F. (2015) Cholecalciferol (vitamin D(3)) improves functional recovery when delivered during the acute phase after a spinal cord trauma. J Steroid Biochem Mol Biol 154, 23-31.

Guillot, X., Semerano, L., Saidenberg-Kermanac'h, N., Falgarone, G., Boissier, M.C. (2010) Vitamin D and inflammation. Joint Bone Spine 77, 552-557.

Guo-Ross, S.X., Yang, E.Y., Walsh, T.J., Bondy, S.C. (1999) Decrease of glial fibrillary acidic protein in rat frontal cortex following aluminum treatment. J Neurochem 73, 1609-1614.

Gutteridge, J.M., Halliwell, B. (2000) Free radicals and antioxidants in the year 2000. A historical look to the future. Ann N Y Acad Sci 899, 136-147.

Gwilt, C.R., Donnelly, L.E., Rogers, D.F. (2007) The non-neuronal cholinergic system in the airways: an unappreciated regulatory role in pulmonary inflammation? Pharmacol Ther 115, 208-222.

Gysemans, C.A., Cardozo, A.K., Callewaert, H., Giulietti, A., Hulshagen, L., Bouillon, R., Eizirik, D.L., Mathieu, C. (2005) 1,25-Dihydroxyvitamin D3 modulates expression of chemokines and cytokines in pancreatic islets: implications for prevention of diabetes in nonobese diabetic mice. Endocrinology 146, 1956-1964.

Halbreich, U., Lumley, L.A., Palter, S., Manning, C., Gengo, F., Joe, S.H. (1995) Possible acceleration of age effects on cognition following menopause. J Psychiatr Res 29, 153-163.

Halliwell, B. (1995) Antioxidant characterization. Methodology and mechanism. Biochem Pharmacol 49, 1341-1348.

Halliwell, B. (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97, 1634-1658.

Halliwell, B. (2011) Free radicals and antioxidants - quo vadis? Trends Pharmacol Sci 32, 125-130.

Halliwell, B. (2012) Free radicals and antioxidants: updating a personal view. Nutr Rev 70, 257-265.

Halliwell, B., Gutteridge, J.M.C. (2007a) Free radicals in biology and medicine, 4th ed. Oxford University Press, Oxford ; New York.

Halliwell, B., Gutteridge, J.M.C. (2007b) Free radicals in biology and medicine, New York.

Halliwell, B., Whiteman, M. (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142, 231-255.

Hansberg-Pastor, V., Gonzalez-Arenas, A., Pina-Medina, A.G., Camacho-Arroyo, I. (2015) Sex Hormones Regulate Cytoskeletal Proteins Involved in Brain Plasticity. Front Psychiatry 6, 165.

Hansdottir, S., Monick, M.M. (2011) Vitamin D effects on lung immunity and respiratory diseases. Vitam Horm 86, 217-237.

Harms, L.R., Burne, T.H., Eyles, D.W., McGrath, J.J. (2011) Vitamin D and the brain. Best Pract Res Clin Endocrinol Metab 25, 657-669.

Harvey, N.C., Cooper, C. (2016) High-dose vitamin D supplementation does not alter bone mass or muscle function over 1 year in postmenopausal women. Evid Based Med 21, 30.

Henderson, V.W. (2006) The neurology of menopause. Neurologist 12, 149-159.

Henderson, V.W. (2008) Cognitive changes after menopause: influence of estrogen. Clin Obstet Gynecol 51, 618-626.

Henderson, V.W. (2014) Alzheimer's disease: review of hormone therapy trials and implications for treatment and prevention after menopause. J Steroid Biochem Mol Biol 142, 99-106.

Hirano, T., Kishimoto, T. (1992) Molecular biology and immunology of interleukin-6. Res Immunol 143, 723-724.

Hirota, Y., Osuga, Y., Hirata, T., Harada, M., Morimoto, C., Yoshino, O., Koga, K., Yano, T., Tsutsumi, O., Taketani, Y. (2005) Activation of protease-activated receptor 2 stimulates proliferation and interleukin (IL)-6 and IL-8 secretion of endometriotic stromal cells. Hum Reprod 20, 3547-3553.

Holmgren, A., Bouhy, D., Timmerman, V. (2012) Neurofilament phosphorylation and their proline-directed kinases in health and disease. J Peripher Nerv Syst 17, 365-376.

Honour, J.W. (2017) Biochemistry of the menopause. Ann Clin Biochem, 4563217739930.

Iwasa, T., Matsuzaki, T., Kinouchi, R., Gereltsetseg, G., Murakami, M., Nakazawa, H., Fujisawa, S., Yamamoto, S., Kuwahara, A., Yasui, T., Irahara, M. (2011) Effect of immune stress on body weight regulation is altered by ovariectomy in female rats. J Reprod Immunol 91, 41-47.

Izquierdo, I., Barros, D.M., Mello e Souza, T., de Souza, M.M., Izquierdo, L.A., Medina, J.H. (1998) Mechanisms for memory types differ. Nature 393, 635-636.

Izquierdo, I., Medina, J.H. (1997) Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiol Learn Mem 68, 285-316.

Jiang, J.M., Sacco, S.M., Ward, W.E. (2008) Ovariectomy-induced hyperphagia does not modulate bone mineral density or bone strength in rats. J Nutr 138, 2106-2110.

Jones, K.A., Thomsen, C. (2013) The role of the innate immune system in psychiatric disorders. Mol Cell Neurosci 53, 52-62.

Jung, C., Yabe, J.T., Lee, S., Shea, T.B. (2000) Hypophosphorylated neurofilament subunits undergo axonal transport more rapidly than more

extensively phosphorylated subunits in situ. Cell Motil Cytoskeleton 47, 120-129.

Kato, I., Toniolo, P., Akhmedkhanov, A., Koenig, K.L., Shore, R., Zeleniuch-Jacquotte, A. (1998) Prospective study of factors influencing the onset of natural menopause. J Clin Epidemiol 51, 1271-1276.

Kireev, R.A., Vara, E., Vina, J., Tresguerres, J.A. (2014) Melatonin and oestrogen treatments were able to improve neuroinflammation and apoptotic processes in dentate gyrus of old ovariectomized female rats. Age (Dordr) 36, 9707.

Koebele, S.V., Bimonte-Nelson, H.A. (2016) Modeling menopause: The utility of rodents in translational behavioral endocrinology research. Maturitas 87, 5-17.

Kondera-Anasz, Z., Sikora, J., Mielczarek-Palacz, A., Jonca, M. (2005) Concentrations of interleukin (IL)-1alpha, IL-1 soluble receptor type II (IL-1 sRII) and IL-1 receptor antagonist (IL-1 Ra) in the peritoneal fluid and serum of infertile women with endometriosis. Eur J Obstet Gynecol Reprod Biol 123, 198-203.

Kramár, E.A., Babayan, A.H., Gall, C.M., Lynch, G. (2013) Estrogen promotes learning-related plasticity by modifying the synaptic cytoskeleton. Neuroscience 239, 3-16.

Langub, M.C., Herman, J.P., Malluche, H.H., Koszewski, N.J. (2001) Evidence of functional vitamin D receptors in rat hippocampus. Neuroscience 104, 49-56.

Lardner, A.L. (2015) Vitamin D and hippocampal development-the story so far. Front Mol Neurosci 8, 58.

Lawrence, T. (2009) The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 1, a001651.

Leffa, D.D., Damiani, A.P., Damazio, D.D., Guerra, N.P., Moretti, M., de Brito, G.G., Boeck, C.R., Gavioli, E.C., de Andrade, V.M. (2014) Long-term effects of ageing and ovariectomy on aversive and recognition memory and DNA damage in the hippocampus of female rats. Acta Neuropsychiatr 26, 161-169.

Lemire, J.M. (1992) Immunomodulatory role of 1,25-dihydroxyvitamin D3. J Cell Biochem 49, 26-31.

Liu, J.J., Prescott, J., Giovannucci, E., Hankinson, S.E., Rosner, B., Han, J., De Vivo, I. (2013) Plasma vitamin D biomarkers and leukocyte telomere length. Am J Epidemiol 177, 1411-1417.

Lobo, R.A. (2007) Menopause and stroke and the effects of hormonal therapy. Climacteric 10 Suppl 2, 27-31.

Lobo, R.A., Davis, S.R., De Villiers, T.J., Gompel, A., Henderson, V.W., Hodis, H.N., Lumsden, M.A., Mack, W.J., Shapiro, S., Baber, R.J. (2014) Prevention of diseases after menopause. Climacteric : the journal of the International Menopause Society 17, 540-556.

Luine, V.N. (2014) Estradiol and cognitive function: past, present and future. Horm Behav 66, 602-618.

Mackedanz, V., Mattos, C.B., Feksa, L.R., Wannmacher, C.M., Wyse, A.T. (2011) Ovariectomy alters energy metabolism in rat striatum: effect of supplementation with soy diet rich in isoflavones. Metab Brain Dis 26, 97-105.

Malutan, A.M., Dan, M., Nicolae, C., Carmen, M. (2014) Proinflammatory and anti-inflammatory cytokine changes related to menopause. Prz Menopauzalny 13, 162-168.

Martindale, J.L., Holbrook, N.J. (2002) Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192, 1-15.

Maurer, S.V., Williams, C.L. (2017) The Cholinergic System Modulates Memory and Hippocampal Plasticity via Its Interactions with Non-Neuronal Cells. Front Immunol 8, 1489.

McEwen, B. (2002) Estrogen actions throughout the brain. Recent Prog Horm Res 57, 357-384.

Messinis, I.E. (2006) Ovarian feedback, mechanism of action and possible clinical implications. Hum Reprod Update 12, 557-571.

Miquel, J., Ramírez-Boscá, A., Ramírez-Bosca, J.V., Alperi, J.D. (2006) Menopause: A review on the role of oxygen stress and favorable effects of dietary antioxidants. Archives of Gerontology and Geriatrics 42, 289-306.

Mitra, S.W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H.A., Hayashi, S., Pfaff, D.W., Ogawa, S., Rohrer, S.P., Schaeffer, J.M., McEwen, B.S., Alves, S.E. (2003) Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 144, 2055-2067.

Monteiro, S.C., de Mattos, C.B., Ben, J., Netto, C.A., Wyse, A.T.S. (2008) Ovariectomy impairs spatial memory: prevention and reversal by a soy isoflavone diet. Metab Brain Dis 23, 243-253.

Monteiro, S.C., Matté, C., Bavaresco, C.S., Netto, C.A., Wyse, A.T.S. (2005a) Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. Neurobiology of Learning and Memory 84, 192-199.

Monteiro, S.C., Matte, C., Delwing, D., Wyse, A.T. (2005b) Ovariectomy increases Na+, K+-ATPase, acetylcholinesterase and catalase in rat hippocampus. Mol Cell Endocrinol 236, 9-16.

Monteiro, S.C., Stefanello, F.M., Vianna, L.P., Matte, C., Barp, J., Bello-Klein, A., Trindade, V.M., Wyse, A.T. (2005c) Ovariectomy enhances acetylcholinesterase activity but does not alter ganglioside content in cerebral cortex of female adult rats. Metab Brain Dis 20, 35-44.

Morissette, M., Le Saux, M., D'Astous, M., Jourdain, S., Al Sweidi, S., Morin, N., Estrada-Camarena, E., Mendez, P., Garcia-Segura, L.M., Di Paolo, T. (2008) Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. J Steroid Biochem Mol Biol 108, 327-338.

Mostafa, W.Z., Hegazy, R.A. (2015) Vitamin D and the skin: Focus on a complex relationship: A review. J Adv Res 6, 793-804.

Mott, N.N., Pak, T.R. (2013) Estrogen signaling and the aging brain: context-dependent considerations for postmenopausal hormone therapy. ISRN Endocrinol 2013, 814690.

Mousa, A., Naderpoor, N., Johnson, J., Sourris, K., de Courten, M.P.J., Wilson, K., Scragg, R., Plebanski, M., de Courten, B. (2017) Effect of vitamin D supplementation on inflammation and nuclear factor kappa-B activity in overweight/obese adults: a randomized placebo-controlled trial. Sci Rep 7, 15154.

Mpandzou, G., Ait Ben Haddou, E., Regragui, W., Benomar, A., Yahyaoui, M. (2016) Vitamin D deficiency and its role in neurological conditions: A review. Rev Neurol (Paris) 172, 109-122.

Nair, R., Maseeh, A. (2012) Vitamin D: The "sunshine" vitamin. J Pharmacol Pharmacother 3, 118-126.

O'Neill, L.A., Kaltschmidt, C. (1997) NF-kappa B: a crucial transcription factor for glial and neuronal cell function. Trends Neurosci 20, 252-258.

Okuda, S., Roozendaal, B., McGaugh, J.L. (2004) Glucocorticoid effects on object recognition memory require training-associated emotional arousal. Proc Natl Acad Sci U S A 101, 853-858.

Olmos, G., Llado, J. (2014) Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. Mediators Inflamm 2014, 861231.

Omary, M.B. (2009) "IF-pathies": a broad spectrum of intermediate filament-associated diseases. J Clin Invest 119, 1756-1762.

Omary, M.B., Coulombe, P.A., McLean, W.H. (2004) Intermediate filament proteins and their associated diseases. N Engl J Med 351, 2087-2100.

Parrish, W.R., Rosas-Ballina, M., Gallowitsch-Puerta, M., Ochani, M., Ochani, K., Yang, L.H., Hudson, L., Lin, X., Patel, N., Johnson, S.M., Chavan, S., Goldstein, R.S., Czura, C.J., Miller, E.J., Al-Abed, Y., Tracey, K.J., Pavlov, V.A.

(2008) Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling. Mol Med 14, 567-574.

Pavlov, V.A., Tracey, K.J. (2005) The cholinergic anti-inflammatory pathway. Brain Behav Immun 19, 493-499.

Petrovska, S., Dejanova, B., Jurisic, V. (2012) Estrogens: mechanisms of neuroprotective effects. J Physiol Biochem 68, 455-460.

Pike, C.J., Carroll, J.C., Rosario, E.R., Barron, A.M. (2009) Protective actions of sex steroid hormones in Alzheimer's disease. Front Neuroendocrinol 30, 239-258.

Pines, A. (2013) Telomere length and telomerase activity in the context of menopause. Climacteric 16, 629-631.

Pole, A., Dimri, M., Dimri, G.P. (2016) Oxidative stress, cellular senescence and ageing. AIMS Molecular Science 3, 300.

Poljsak, B., Milisav, I. (2013) Aging, Oxidative Stress and Antioxidants.

Pompili, A., Arnone, B., Gasbarri, A. (2012) Estrogens and memory in physiological and neuropathological conditions. Psychoneuroendocrinology 37, 1379-1396.

Pusceddu, I., Farrell, C.J., Di Pierro, A.M., Jani, E., Herrmann, W., Herrmann, M. (2015) The role of telomeres and vitamin D in cellular aging and age-related diseases. Clin Chem Lab Med 53, 1661-1678.

Rettberg, J.R., Yao, J., Brinton, R.D. (2014) Estrogen: a master regulator of bioenergetic systems in the brain and body. Front Neuroendocrinol 35, 8-30.

Ribezzo, F., Shiloh, Y., Schumacher, B. (2016) Systemic DNA damage responses in aging and diseases. Semin Cancer Biol 37-38, 26-35.

Richards, J.B., Valdes, A.M., Gardner, J.P., Paximadas, D., Kimura, M., Nessa, A., Lu, X., Surdulescu, G.L., Swaminathan, R., Spector, T.D., Aviv, A. (2007) Higher serum vitamin D concentrations are associated with longer leukocyte telomere length in women. Am J Clin Nutr 86, 1420-1425.

Rocca, W.A., Grossardt, B.R., Shuster, L.T. (2010) Oophorectomy, menopause, estrogen, and cognitive aging: the timing hypothesis. Neurodegener Dis 7, 163-166.

Rogers, N.H., Perfield, J.W., 2nd, Strissel, K.J., Obin, M.S., Greenberg, A.S. (2009) Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. Endocrinology 150, 2161-2168.

Rojas, J.J., Deniz, B.F., Miguel, P.M., Diaz, R., Hermel Edo, E., Achaval, M., Netto, C.A., Pereira, L.O. (2013) Effects of daily environmental enrichment on

behavior and dendritic spine density in hippocampus following neonatal hypoxia-ischemia in the rat. Exp Neurol 241, 25-33.

Rosas-Ballina, M., Tracey, K.J. (2009) Cholinergic control of inflammation. J Intern Med 265, 663-679.

Routtenberg, A. (2008) The substrate for long-lasting memory: if not protein synthesis, then what? Neurobiol Learn Mem 89, 225-233.

Russell, A.L., Grimes, J.M., Cruthirds, D.F., Westerfield, J., Wooten, L., Keil, M., Weiser, M.J., Landauer, M.R., Handa, R.J., Wu, T.J., Larco, D.O. (2017) Dietary Isoflavone-Dependent and Estradiol Replacement Effects on Body Weight in the Ovariectomized (OVX) Rat. Horm Metab Res 49, 457-465.

Salum, E., Kals, J., Kampus, P., Salum, T., Zilmer, K., Aunapuu, M., Arend, A., Eha, J., Zilmer, M. (2013) Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats. Diabetes Research and Clinical Practice 100, 243-249.

Salum, E., Kampus, P., Zilmer, M., Eha, J., Butlin, M., Avolio, A.P., Podramagi, T., Arend, A., Aunapuu, M., Kals, J. (2012) Effect of vitamin D on aortic remodeling in streptozotocin-induced diabetes. Cardiovasc Diabetol 11, 58.

Sanchez, A.M., Flamini, M.I., Fu, X.D., Mannella, P., Giretti, M.S., Goglia, L., Genazzani, A.R., Simoncini, T. (2009) Rapid signaling of estrogen to WAVE1 and moesin controls neuronal spine formation via the actin cytoskeleton. Mol Endocrinol 23, 1193-1202.

Sarter, M., Bruno, J.P. (1997) Cognitive functions of cortical acetylcholine: toward a unifying hypothesis. Brain Res Brain Res Rev 23, 28-46.

Savonenko, A.V., Markowska, A.L. (2003) The cognitive effects of ovariectomy and estrogen replacement are modulated by aging. Neuroscience 119, 821-830.

Scherer, E.B., Loureiro, S.O., Vuaden, F.C., da Cunha, A.A., Schmitz, F., Kolling, J., Savio, L.E., Bogo, M.R., Bonan, C.D., Netto, C.A., Wyse, A.T. (2014) Mild hyperhomocysteinemia increases brain acetylcholinesterase and proinflammatory cytokine levels in different tissues. Mol Neurobiol 50, 589-596.

Schumacher, B., Garinis, G.A., Hoeijmakers, J.H. (2008) Age to survive: DNA damage and aging. Trends Genet 24, 77-85.

Scott, E., Zhang, Q.G., Wang, R., Vadlamudi, R., Brann, D. (2012) Estrogen neuroprotection and the critical period hypothesis. Front Neuroendocrinol 33, 85-104.

Shenassa, E.D., Rossen, L.M. (2015) Telomere length and age-at-menopause in the US. Maturitas 82, 215-221.

Sherwin, B.B. (2003) Estrogen and cognitive functioning in women. Endocr Rev 24, 133-151.

Shih, R.H., Wang, C.Y., Yang, C.M. (2015) NF-kappaB Signaling Pathways in Neurological Inflammation: A Mini Review. Front Mol Neurosci 8, 77.

Shoupe, D. (2011) Individualizing hormone therapy to minimize risk: accurate assessment of risks and benefits. Womens Health (Lond) 7, 475-485.

Shuster, L.T., Rhodes, D.J., Gostout, B.S., Grossardt, B.R., Rocca, W.A. (2010) Premature menopause or early menopause: long-term health consequences. Maturitas 65, 161-166.

Siebert, C., Kolling, J., Scherer, E.B., Schmitz, F., da Cunha, M.J., Mackedanz, V., de Andrade, R.B., Wannmacher, C.M., Wyse, A.T. (2014) Effect of physical exercise on changes in activities of creatine kinase, cytochrome c oxidase and ATP levels caused by ovariectomy. Metab Brain Dis 29, 825-835.

Sihag, R.K., Inagaki, M., Yamaguchi, T., Shea, T.B., Pant, H.C. (2007) Role of phosphorylation on the structural dynamics and function of types III and IV intermediate filaments. Exp Cell Res 313, 2098-2109.

Silva, J.P., Coutinho, O.P. (2010) Free radicals in the regulation of damage and cell death - basic mechanisms and prevention. Drug Discov Ther 4, 144-167.

Smogorzewska, A., de Lange, T. (2002) Different telomere damage signaling pathways in human and mouse cells. EMBO J 21, 4338-4348.

Soules, M.R., Sherman, S., Parrott, E., Rebar, R., Santoro, N., Utian, W., Woods, N. (2001) Executive summary: Stages of Reproductive Aging Workshop (STRAW). Fertil Steril 76, 874-878.

Spach, K.M., Hayes, C.E. (2005) Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice. J Immunol 175, 4119-4126.

Su, H.I., Freeman, E.W. (2009) Hormone changes associated with the menopausal transition. Minerva Ginecol 61, 483-489.

Sullivan, S.D., Lehman, A., Nathan, N.K., Thomson, C.A., Howard, B.V. (2017) Age of menopause and fracture risk in postmenopausal women randomized to calcium + vitamin D, hormone therapy, or the combination: results from the Women's Health Initiative Clinical Trials. Menopause 24, 371-378.

Sunday, L., Osuna, C., Krause, D.N., Duckles, S.P. (2007) Age alters cerebrovascular inflammation and effects of estrogen. Am J Physiol Heart Circ Physiol 292, H2333-2340.

Tak, P.P., Firestein, G.S. (2001) NF-kappaB: a key role in inflammatory diseases. J Clin Invest 107, 7-11.

Takahashi, T.A., Johnson, K.M. (2015) Menopause. Med Clin North Am 99, 521-534.

Tchernof, A., Despres, J.P. (2000) Sex steroid hormones, sex hormone-binding globulin, and obesity in men and women. Horm Metab Res 32, 526-536.

Tracey, K.J. (2007) Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest 117, 289-296.

Turner, M.D., Nedjai, B., Hurst, T., Pennington, D.J. (2014) Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta 1843, 2563-2582.

Valko, M., Jomova, K., Rhodes, C.J., Kuca, K., Musilek, K. (2016) Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. Arch Toxicol 90, 1-37.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39, 44-84.

Vegeto, E., Benedusi, V., Maggi, A. (2008) Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. Front Neuroendocrinol 29, 507-519.

von Zglinicki, T., Martin-Ruiz, C.M. (2005) Telomeres as biomarkers for ageing and age-related diseases. Curr Mol Med 5, 197-203.

Walf, A.A., Rhodes, M.E., Frye, C.A. (2006) Ovarian steroids enhance object recognition in naturally cycling and ovariectomized, hormone-primed rats. Neurobiol Learn Mem 86, 35-46.

Wang, W.Y., Tan, M.S., Yu, J.T., Tan, L. (2015) Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann Transl Med 3, 136.

Wang, Y., Xu, Y., Sheng, H., Ni, X., Lu, J. (2016) Exercise amelioration of depression-like behavior in OVX mice is associated with suppression of NLRP3 inflammasome activation in hippocampus. Behav Brain Res 307, 18-24.

Williams, D.M., Palaniswamy, S., Sebert, S., Buxton, J.L., Blakemore, A.I., Hypponen, E., Jarvelin, M.R. (2016) 25-Hydroxyvitamin D Concentration and Leukocyte Telomere Length in Young Adults: Findings From the Northern Finland Birth Cohort 1966. Am J Epidemiol 183, 191-198.

Wise, P.M. (2002) Estrogens and neuroprotection. Trends Endocrinol Metab 13, 229-230.

Xanthos, D.N., Sandkuhler, J. (2014) Neurogenic neuroinflammation: inflammatory CNS reactions in response to neuronal activity. Nat Rev Neurosci 15, 43-53.

Xu, Y., Sheng, H., Tang, Z., Lu, J., Ni, X. (2015) Inflammation and increased IDO in hippocampus contribute to depression-like behavior induced by estrogen deficiency. Behav Brain Res 288, 71-78.

Yasui, T., Uemura, H., Tomita, J., Miyatani, Y., Yamada, M., Kuwahara, A., Matsuzaki, T., Maegawa, M., Tsuchiya, N., Yuzurihara, M., Takeda, S., Irahara, M. (2006) Association of interleukin-8 with hot flashes in premenopausal, perimenopausal, and postmenopausal women and bilateral oophorectomized women. J Clin Endocrinol Metab 91, 4805-4808.

Yip, B.W., Mok, H.O., Peterson, D.R., Wan, M.T., Taniguchi, Y., Ge, W., Au, D.W. (2017) Sex-dependent telomere shortening, telomerase activity and oxidative damage in marine medaka Oryzias melastigma during aging. Mar Pollut Bull.

Yu, B.P. (1994) Cellular defenses against damage from reactive oxygen species. Physiol Rev 74, 139-162.

Zamoner, A., Pierozan, P., Vidal, L.F., Lacerda, B.A., Dos Santos, N.G., Vanzin, C.S., Pessoa-Pureur, R. (2008) Vimentin phosphorylation as a target of cell signaling mechanisms induced by 1alpha,25-dihydroxyvitamin D3 in immature rat testes. Steroids 73, 1400-1408.

Zetterberg, H. (2016) Neurofilament Light: A Dynamic Cross-Disease Fluid Biomarker for Neurodegeneration. Neuron 91, 1-3.

Zhang, C. (2008) The role of inflammatory cytokines in endothelial dysfunction. Basic Res Cardiol 103, 398-406.

Zheng, Y.L., Li, B.S., Veeranna, Pant, H.C. (2003) Phosphorylation of the head domain of neurofilament protein (NF-M): a factor regulating topographic phosphorylation of NF-M tail domain KSP sites in neurons. J Biol Chem 278, 24026-24032.

Zila, I., Mokra, D., Kopincova, J., Kolomaznik, M., Javorka, M., Calkovska, A. (2017) Vagal-immune interactions involved in cholinergic anti-inflammatory pathway. Physiol Res 66, S139-S145.

Zimmerman, G., Soreq, H. (2006) Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. Cell Tissue Res 326, 655-669.