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

ALTERAÇÕES BIOQUÍMICAS E COMPORTAMENTAIS CAUSADAS PELA OVARIECTOMIA EM RATAS ADULTAS. EFEITO DA SUPLEMENTAÇÃO COM ANTIOXIDANTES E SOJA

Siomara da Cruz Monteiro

Orientadora: Profa. Dra. Angela Terezinha de Souza Wyse

Porto Alegre,2007

O conhecimento sem transformação
não é sabedoria.

Paulo Coelho

Á minha mãe,

por seu amor incondicional, pelo apoio e incentivo para que eu estudasse cada vez mais e por ter dedicado sua vida exclusivamente aos filhos.

AGRADECIMENTOS

A minha orientadora, Angela, pela amizade, pelos ensinamentos, pelo exemplo profissional e por ter confiado no meu trabalho.

Aos amigos do laboratório: Cristiane Matté, Cristiane Mattos, Emilene e Arthur, Francieli, Alexandra e Emílio, Daniela, Débora, Caren, Fábria, Renata, Bárbara, Vanize, Janaina, Thiago, Carolina, Andréa, Juliana, Luciene, Jaqueline Barp, Virginia, Daniel e Renan. Todos vocês, de alguma maneira, ajudaram na realização deste trabalho. Obrigada pelo apoio, pelo carinho e por terem vindo morar no meu coração para sempre.

Aos professores do grupo dos Erros Inatos do Metabolismo: Clóvis, Moacir e Dutra, pela convivência e pelos ensinamentos.

Aos amigos do Centro de Memória da PUC: Prof. Izquierdo, Lia, Martin, Janine, Juliana, Weber, Aninha, Fernando e Carol. Obrigada pela amizade, pelo ótimo convívio e pela colaboração.

Aos meus filhos, Leonardo e Maria Eduarda, pelo tempo que cederam para que eu pudesse realizar o doutorado. Vocês serão sempre a grande inspiração da minha vida.

Ao meu companheiro de vida, Carlinhos. O teu amor é fundamental para a realização dos meus projetos. Obrigada por estar sempre ao meu lado.

Aos meus avós, que aos 90 anos são o maior exemplo de garra e coragem.

Aos meus pais e a Tatinha por tudo que me ensinaram e serem ainda o meu porto seguro.

Ao Departamento de Bioquímica.

Ao CNPq, CAPES, FAPERGS, PROPESQ e UFRGS.

Resumo

Os estrógenos exercem diversas ações não reprodutivas em vários órgãos, incluindo o cérebro. Tem sido demonstrado que a privação estrogênica está implicada na patogênese de algumas doenças neurodegenerativas e na isquemia cerebral. Relatos da literatura sugerem que as mulheres menopáusicas são mais suscetíveis a esses distúrbios e ao déficit cognitivo do que as mulheres jovens. Entretanto, devido aos possíveis efeitos colaterais da terapia de reposição hormonal, tais como o câncer de mama e o aumento do risco a acidentes cerebrovasculares, cresce o número de terapias alternativas para tratar os sintomas associados à menopausa. No presente trabalho, nós investigamos o efeito da ovariectomia sobre alterações bioquímicas (Na^+,K^+-ATPase, colinesterases, gangliosídios e alguns parâmetros de estresse oxidativo) e comportamentais em ratas adultas. Também determinamos a ação do tratamento com as vitaminas E e C e da dieta de soja rica em isoflavonas sobre as alterações provocadas pela ovariectomia nos parâmetros estudados. Nossos resultados mostraram que a ovariectomia aumentou, significativamente, as atividades da acetilcolinesterase, Na^+,K^+-ATPase e catalase, e não alterou o conteúdo e o perfil dos gangliosídios, em cérebro de ratas adultas. A atividade da butirilcolinesterase sérica foi inibida pela ovariectomia. O aumento da atividade da acetilcolinesterase poderia diminuir os níveis de acetilcolina, levando à redução da transmissão colinérgica. Acreditamos que o aumento da atividade da Na^+,K^+-ATPase poderia provocar uma hiperpolarização da membrana sináptica. O aumento da atividade da catalase seria uma conseqüência do estresse oxidativo induzido pela ovariectomia. Além disso, estudos comportamentais mostraram que a ovariectomia causou um déficit na memória espacial em ratas adultas. Posteriormente, avaliamos o efeito do tratamento com as vitaminas E e C e da dieta de soja rica em isoflavonas sobre os parâmetros alterados pela ovariectomia. O tratamento crônico com as vitaminas E e C reverteu a ação da ovariectomia sobre as atividades da Na^+,K^+-ATPase e da acetilcolinesterase e sobre o déficit de memória espacial. As isoflavonas da soja reverteram a ativação da atividade da acetilcolinesterase causada pela ovariectomia e não alteraram a atividade da Na^+,K^+-ATPase. Ambos os tratamentos utilizados não modificaram a atividade da butirilcolinesterase sérica. Além disso, suplementação com isoflavonas da soja, a longo (60 dias) e a médio (30 dias) prazo, protegeram contra o déficit de memória espacial causado pela ovariectomia. Considerando que a modulação do receptor AMPA tem sido descrita como uma etapa necessária para a ativação de cascatas celulares durante o aprendizado e a formação da memória, nós também investigamos o efeito da ovariectomia e da administração das vitaminas E e C sobre a fosforilação de diferentes subunidades do receptor AMPA e a possível modulação da via ERK1/2-CREB no hipocampo. Nossos resultados mostraram que a ovariectomia aumentou os níveis de pGLU 2/3 Ser 880/891 e que o tratamento com os antioxidantes reverteu esse efeito. Não observamos modificação nos níveis de outras subunidades fosforiladas do receptor AMPA, nem na via de sinalização ERK1/2 – CREB. Esses resultados em conjunto,

mostram alguns efeitos da depleção hormonal ovariana sobre alguns parâmetros bioquímicos e comportamentais e colaboram para o entendimento dos sintomas e distúrbios neurológicos observados em algumas mulheres menopáusicas. Além disso, se confirmado em humanos, nossos dados relacionados a suplementação com as vitaminas E e C e as isoflavonas da soja podem ser uma estratégia para tratar alguns sintomas associados à menopausa.

Abstract

Estrogen also exerts diverse non-reproductive actions on multiple organs, including the brain, and it has been shown that estrogenic deprivation is implicated in the pathogenesis of neurodegenerative conditions and cerebral ischemia. There is a large body of literature to suggest that postmenopausal women are more vulnerable than younger women to such diseases and to cognitive deficits. However, due to the possible side effects of hormonal replacement therapy, such as breast cancer and increased risk of brain damages, there is a growing demand for alternative treatments of pathological processes and symptoms associated with menopause. In the present work, we investigated the effect of ovariectomy on biochemical parameters (Na^+,K^+- ATPase, cholinesterases and gangliosides), as well as on some parameters of oxidative stress and on spatial memory tasks. We also determined the actions of vitamins E and C or soy isoflavones on parameters altered by ovariectomy. Our results showed that ovariectomy increased significantly the activities of acetylcholinesterase, Na^+, K^+-ATPase and catalase, and did not alter the gangliosides content and profile, in brain of female adult rats. The activity of butyrylcholinesterase was inhibited by ovariectomy in serum. This effect on acetylcholinesterase activity could decrease acetylcholine levels, leading to reduction of cholinergic neurotransmission. The activation of Na^+,K^+-ATPase activity could cause hyperpolarization of synaptic membrane. Increased catalase activity could be a consequence of oxidative stress induced by ovariectomy. Besides, the present study reported an impairment of spatial navigation caused by ovariectomy in adult rats. Afterwards, we decided to evaluate the influence of vitamins E and C and soy isoflavone diet on parameters altered by ovariectomy. The treatment with vitamins E and C reversed the ovariectomy action on Na^+,K^+-ATPase and acetylcholinesterase activities and on spatial memory. The supplementation with soy isoflavones reversed the activation of acetylcholinestersase caused by ovariectomy and did not alter the incresead in Na^+,K^+-ATPase activity. Both treatments used in this study were unable to alter the inhibition of butyrylcholinesterase caused by ovariectomy. Besides, soy isoflavones diet, in the long term (60 days) and in the short term (30 days), protected against memory spatial deficit caused by ovariectomy. Considering that AMPA receptor modulation has been described as a necessary step to activation of cellular cascades during learning and memory formation, we also decided to investigate the effect of ovariectomy and the administration of vitamins E and C on the phosphorilation of different AMPAr subunits and on the possible modulation of the ERK1/2 – CREB signaling pathway in the hippocampus. Our results show that ovariectomy significantly increases the phosphorilation of AMPAr subunit pGLU 2/3 Ser 880/891 and the treatment with vitamins E plus C reverts this activation. We did not find any modification in the levels of other phosphorilated subunits of AMPAr and no changes were found in the levels of pERK1/2 and pCREB between groups showing that ovariectomy and the treatment with these antioxidants were unable to alter the ERK1/2 – CREB signaling pathway in the hippocampus. Taken together, our results show the effects of hormonal depletion on some biochemical

and behavioral parameters and contribute to understand the symptoms and neurological dysfunction found in some menopausal women. Assuming the possibility that these phenomena may occur in humans, these dada are very encouraging, since vitamins E plus C and soy isoflavones may constitute a good alternative to a novel therapeutic strategy to block injurious effects associated to menopause.

LISTA DE ABREVIATURAS

AChE acetilcolinesterase
AMPA receptor 4 ácido isoxazolepropionico α-amino-3-hidroxi-5-metil
BuChE butirilcolinesterase
CAT catalase
E2 17 β-estradiol
ELISA enzima imunoensaio
ERα receptor estrogênico alfa
ERβ receptor estrogênico beta
ERX receptor estrogênico X
FSH do inglês, *follicle-stimulating hormone* (hormônio folículo estimulante)
GRd glutationa redutase
GnRH do inglês, *gonadotropin-realising hormone* (hormônio liberador de gonadotrofinas)
GSH-Px glutationa peroxidase
LH do inglês, *luteinizing hormone* (hormônio luteinizante)
LPO lipoperoxidação
OVX ovariectomia
SOD superóxido dismutase
SNC sistema nervoso central
TBA-RS substâncias reativas ao ácido tiobarbitúrico
TRAP capacidade antioxidante total
TER terapia de reposição estrogênica
TRH terapia de reposição hormonal

LISTA DE FIGURAS

SUMÁRIO

I. INTRODUÇÃO

1. A Menopausa e o Efeito Neuroprotetor do Estrógeno

Com o aumento da expectativa de vida durante o último século, as mulheres passaram a viver um terço de suas vidas na menopausa. Este período caracteriza-se pelo cessar do ciclo menstrual resultando na perda da atividade folicular ovariana (*World Health Organization*, 1996).

Em uma mulher adulta com ciclo reprodutivo normal, os ovários secretam compostos esteroidais, como os estrógenos e as progesteronas, e substâncias não esteroidas, como inibinas, relaxinas, ativinas e folistatinas. A função ovariana é regulada pelo eixo hipotálamo-hipofisário-ovariano, a partir da secreção das gonadotrofinas hipofisárias, o hormônio luteinizante (LH) e o hormônio folículo estimulante (FSH), que estão sob controle da secreção pulsátil de um potente neuro-hormônio elaborado no hipotálamo, o hormônio liberador de gonadotrofinas (GnRH). Uma série de fatores hormonais e neuro-endócrinos modulam positiva ou negativamente a secreção pulsátil do GnRH, entre os quais os mecanismos de retrocontrole positivo e negativo exercidos pelas substâncias ovarianas e pelas gonadotrofinas presentes na corrente sangüinea que variam durante o ciclo menstrual (Figura 1). Após a menopausa, ambos os mecanismos são abolidos principalmente devido às baixas concentrações de estrógeno e também de progesterona e de substâncias ovarianas não esteroidais (Messinis, 2006).

Os compostos estrogênicos são secretados em grande quantidade pelos ovários e em menor proporção pelos córtices adrenais (Vermeulen, 1976). Apenas três estrogênios estão presentes no plasma feminino humano: estradiol, estrona e estriol, sendo o 17 β-estradiol o mais importante (Rodrigues et al., 1999).

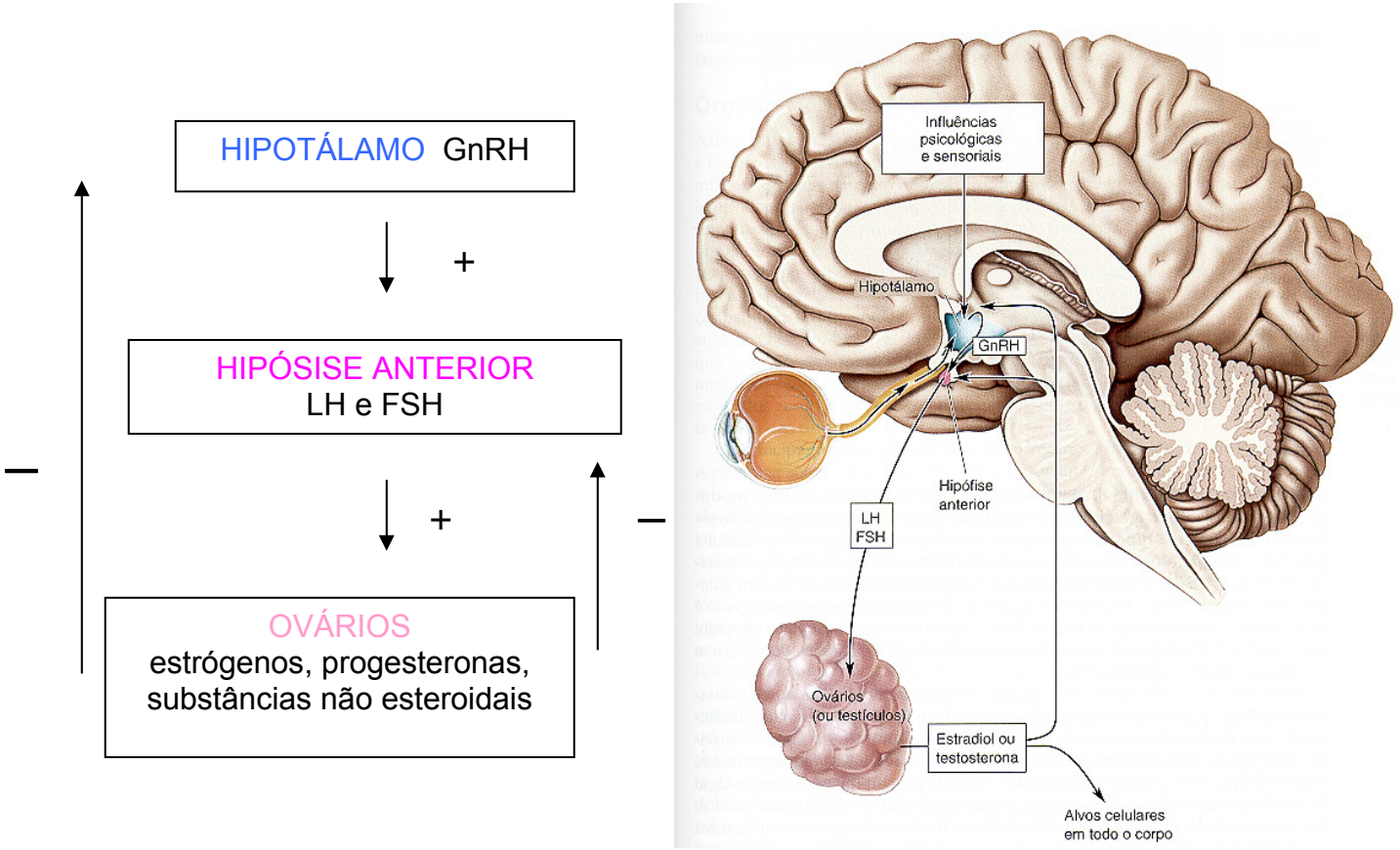


Figura 1. Eixo Hipotálamo-Hipofisiário-Ovariano e seus mecanismos de retrocontrole positivo (+) e negativo (–) (Adaptado de Bear et al., 2002).

Tem sido amplamente demonstrado que os estrógenos exercem diversas ações não reprodutivas em múltiplos sistemas fisiológicos, incluindo o ósseo, o cardiovascular, o imunitário e o sistema nervoso central (Wise, 2001). A ação estrogênica no cérebro influencia vários processos anatômicos e neuroquímicos que vão além do seu papel tradicional (Thakur & Sharma, 2006). Sua privação está implicada na patogenia de alguns distúrbios do SNC (sistema nervoso central), tais como a doença de Alzheimer e a isquemia cerebral (Tang et al., 1996; Zhang et al., 1998; Van Duijn, 1999; Waring et al., 1999; Wise, 2002; Henderson, 2006). Existem relatos na literatura sugerindo que mulheres pós-menopáusicas são mais vulneráveis do que mulheres jovens a esses distúrbios e ao déficit cognitivo (Green & Simpkins, 2000; Wise, 2003).

A descoberta dos receptores estrogênicos (ERα, ERβ, ERX e ER de membrana) demonstra que os estrógenos possuem vários alvos e diferentes mecanismos de ações (Kuiper et al., 1996; Razandi et al., 1999; Toran-Allerand et al., 2002). Os ERα estão abundantemente expressos em regiões cerebrais que controlam a reprodução, como o hipotálamo, e o ERβ no hipocampo, córtex cerebral, cerebelo, lócus ceruleus, entre outras (Mitra et al., 2003). Os efeitos neuroprotetores são específicos e doses dependentes. Níveis farmacológicos de estrógenos protegem o cérebro por mecanismos que não requerem a participação dos receptores, por meio de ações rápidas que parecem não envolver transcrição de novos genes. Entretanto, níveis fisiológicos de estradiol dependem da participação dos receptores para efetivar suas ações (Wise, 2002) e podem, entre outros efeitos, aumentar a plasticidade sináptica (McEwen et al., 1999) e elevar a

expressão de fatores de sobrevivência celular (Pike, 1999). Entretanto, a expressão dos receptores estrogênicos no cérebro muda com a idade, independente do nível de hormônio circulante.

Ratas ovariectomizadas são geralmente utilizadas como modelo animal de menopausa. A responsividade para depleção estrogênica é mais acentuada em ratas jovens com ciclos estrais regulares. Uma semana após a ovariectomia, os níveis de hormônios ovarianos já são indetectáveis no sangue (Chakraborty & Gore, 2004).

2. O Estresse Oxidativo

A geração de radicais livres é uma conseqüência natural da vida num ambiente oxidante que ocorre continuamente nas células como subprodutos do metabolismo ou durante alguns processos fisiológicos. São espécies altamente reativas que possuem um elétron desemparelhado no seu orbital mais externo. São radicais livres denominados espécies reativas de oxigênio, o radical superóxido ($O_2^{\bullet -}$), o radical hidroxila ($OH^{\bullet}$), o radical peroxila ($RO_2^{\bullet}$), o radical alcoxila ($RO^{\bullet}$). Existem também outras espécies reativas de oxigênio como o peróxido de hidrogênio (H_2O_2), o ozônio (O_3), o ácido hipocloroso (HOCl) e os singletos de oxigênio, as espécies reativas de nitrogênio como o radical óxido nítrico ($NO^{\bullet}$) e o peróxinítrico ($ONOO^-$), e as espécies reativas de enxofre ($RS^{\bullet}$) e radicais lipídicos ($ROO^{\bullet}$). Depois de formados, esses compostos produzem uma reação de oxidação em cadeia resultando na destruição, modificação ou inativação de inúmeras moléculas

(Beckman & Koppenol, 1996; Halliwell & Gutteridge, 2000). Atacam os lipídios de membrana num processo contínuo denominado lipoperoxidação (LPO) onde um radical livre reage com um composto não radical, formando outro radical livre e assim sucessivamente. Por serem formadas em grande parte por lipídios insaturados e proteínas, as membranas são particularmente vulneráveis ao ataque oxidativo. Os efeitos da LPO podem ser observados pela mudança do ambiente lipídico (modificação na fluidez e na seletividade) e também por alterações de suas proteínas (receptores e canais iônicos) (Yu, 1994).

O estresse oxidativo, a consequência do desequilíbrio entre a formação e a remoção de radicais livres (Halliwell & Whiteman, 2004), é um importante evento que está relacionado com a idade (Harman, 1992; Siqueira et al., 2005) e com a fisiopatologia de algumas condições que afetam o SNC, tais como a isquemia cerebral e algumas doenças neurodegenerativas (Halliwell & Gutteridge, 1985; Reznick & Packer, 1993). A proteção contra o dano oxidativo é providenciada pelas defesas antioxidantes, incluindo enzimas como a superóxido dismutase (SOD) que converte o $O_2^{\bullet-}$ em H_2O_2, a catalase (CAT) que é responsável pela detoxificação do H_2O_2 e a glutationa peroxidase (GSH-Px) que quebra os peróxidos derivados da oxidação de fosfolipídios de membranas, e por defesas não enzimáticas como as vitaminas A, C, E, riboflavina e tiamina, as isoflavonas, os polifenóis e alguns compostos de baixo peso molecular, que incluem a bilirrubina, α-cetoácidos, melatonina, urato, glutationa, ácido lipóico e estrógenos (Salvador & Henriques, 2004). As enzimas antioxidantes são consideradas as

primeiras defesas para proteção das macromoléculas biológicas contra o dano oxidativo (Benzi & Moretti, 1995).

O cérebro é potencialmente sensível ao estresse oxidativo devido ao grande consumo de oxigênio por unidade de massa de tecido, ao alto conteúdo lipídico das membranas neuronais (ácidos graxos de cadeia lateral poliinsaturada) que sofrem peroxidação por radicais livres, a áreas com altas concentrações de ferro que estimulam a reação de Fenton com produção de radical hidroxila, presença de aminoácidos excitatórios como o glutamato que ao desencadear mecanismos intracelulares geram espécies reativas de oxigênio e aos baixos níveis de defesas antioxidantes (Halliwell, 1996).

Inúmeros estudos evidenciam que os compostos ovarianos protegem contra o dano oxidativo. Tem sido sugerido que a perda estrogênica relacionada com a idade resulta num déficit da proteção antioxidante (Arteaga et al., 1998). Os hormônios esteróides, especialmente o estriol e o estradiol, são considerados antioxidantes naturais (Mooradian, 1993). Os estrógenos podem agir como potente antioxidantes e inibir a peroxidação lipídica (Culmsee et al., 1999). A propriedade antioxidante tem sido relacionada à presença do grupo hidrolixa no C3 localizado no anel aromático fenólico da molécula estrogênica (Behl & Lezoualc'h, 1998). Sua ação varredora de radiacais alcoxil ($RO_2^{\bullet}$) ocorre devido a doação de um hidrogênio do grupo fenólico (Figura 2) (Salvador & Henriques, 2004). Wise e colaboradores (2005) relataram que os estrógenos são capazes de reverter o efeito do estresse oxidativo em culturas de células neuronais por aumentar a concentração de glutationa, um varredor natural de radicais livres, e

que modulam a atividade de enzimas antioxidantes tais como a SOD, a CAT e a GSH-Px.

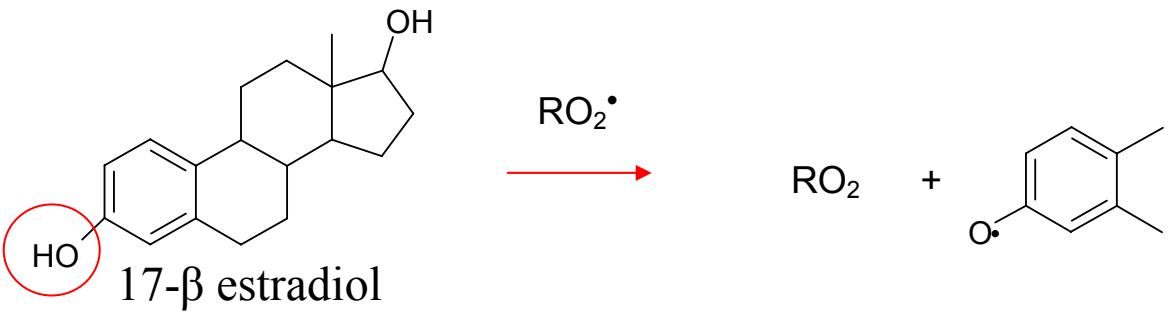


Figura 2. Estrutura química do 17β-estradiol, destacando a presença do grupamento hidroxila no anel fenólico que confere o papel antioxidante, e a formação do radical fenoxil, após a doação de hidrogênio ao radical peroxíla ($RO_2^{\bullet}$) (Adaptado de Halliwell & Gutteridge, 2000).

3. As Vitaminas E e C e as Isoflavonas de Soja

A terapia de reposição hormonal (TRH), estrógeno e progesterona associados, ou a terapia de reposição estrogênica (TRE) têm sido utilizadas para tratar os sintomas e outras condições decorrentes da menopausa. Entretanto, *The Women's Health Initiative* (Rossouw et al., 2002), alerta quanto ao uso da terapia hormonal a longo prazo em mulheres pós-menopáusicas devido os possíveis efeitos colaterais associados como o câncer de mama e o aumento ao risco de

acidentes trombo embólicos. Recentemente, Craig e colaboradores (2005) sugeriram que a combinação de estrógeno e progesterona sintéticos aumenta o risco de demência em mulheres acima de 65 anos. Além disso, Marder e Sano (2000) demonstraram que a TRH não é efetiva quando iniciada após o dano neurológico instalado. Com o objetivo de evitar estes efeitos colaterais e tendo em vista que ocorre um decréscimo no balanço antioxidante/pro-oxidante em mulheres menopáusicas quando comparadas com homens da mesma idade, suplementos nutricionais têm sido estudados para substituir ou complementar a TRH ou TRE. Existem evidências demonstrando que antioxidantes, como as vitaminas E e C, e fitoestrógenos, como as isoflavonas da soja, poderíam ser uma boa alternativa para o tratamento dos processos patológicos e sintomas associados com a menopausa (Miquel et al., 2006).

3.1. As Vitaminas E e C

A vitamina E (α-tocoferol) é uma molécula lipossolúvel concentrada no interior das membranas que tem sido considerada um antioxidante importante para o funcionamento cerebral em humanos (Vatassery, 1998). Suas propriedades antioxidantes podem prevenir doenças associadas ao estresse oxidativo como câncer e desordens neurológicas (Brigelius-Flohe et al., 2002). A vitamina E é um varredor de radical peroxil ($ROO^{\bullet}$), que são, provavelmente, os inibidores mais importantes na reação em cadeia de peroxidação lipídica em animais (Halliwell & Gutteridge, 2000). Além das propriedades antioxidantes (McCay, 1985; Carr & Frei, 1999), a vitamina E tem propriedades não antioxidantes como ações anti-

inflamatórias e antiproliferativas (Upritchard et al., 2000; Singh et al., 2005). Foi demonstrado que a suplementação de vitamina E aumenta os níveis de glutationa (GSH) e diminui a concentração de lipídios peroxidados em eritrócitos de humanos (Jain et al., 2000).

A vitamina C (ácido L-ascórbico) é encontrada em vegetais, frutas, fígado e cérebro de alguns anfíbios (du Toit et al., 2001). Os humanos não são capazes de sintetizar vitamina C, obtendo-a através da dieta. Estudos demonstraram que o ácido ascórbico é um antioxidante ativo contra radicais livres (Halliwell & Gutteridge, 2000) sendo um doador de elétrons, um agente redutor, prevenindo a oxidação de outros compostos. Essa vitamina é hidrossolúvel e possue um papel importante na regeneração da vitamina E à sua forma reduzida doando elétrons ao radical α-tocoferil prolongando seu efeito antioxidante (Figura 3).

A combinação de vitamina E e C é indicada visto que quando administrada somente a vitamina E seu efeito pode tornar-se pró-oxidante ou perder a eficácia (Yusuf et al., 2000). Evidências mostram que a vitamina E, combinada ou não com a vitamina C, pode atenuar o processo de apoptose (Qin et al., 2006), a expressão gênica e a sinalização celular (Zingg & Azzi, 2004), além de inibir a atividade da proteína quinase C (Gimeno et al., 2004) e reduzir a degeneração de células hipocampais após a isquemia cerebral (Hara et al., 1990). Estudos em ratos idosos mostram que o déficit no aprendizado motor pode ser melhorado com dietas ricas em antioxidantes (β-caroteno, vitaminas E e C) (Bickford et al., 2000). Estudos do nosso grupo de pesquisa mostraram que o tratamento com vitaminas E e C previne o déficit de aprendizado/memória causado pela homocisteína (Reis

et al., 2002) e tem efeito protetor contra o dano oxidativo cerebral causado pela administração de prolina (Delwing et al., 2005).

Há dados na literatura mostrando que os estrógenos, além de apresentarem propriedades antioxidantes, têm sido descritos por regenerar ou manter os níveis de antioxidantes endógenos como a vitamina E (Mukai et al., 1990; Ayres et al., 1996). Ratas fêmeas castradas apresentam um decréscimo nas concentrações de vitamina E nas glândulas adrenais e no fígado (Feingold et al., 1993). Por outro lado, a reposição estrogênica aumenta os níveis hepáticos e a secreção biliar de α-tocopherol em ratas ovariectomizadas (Noh et al., 1999).

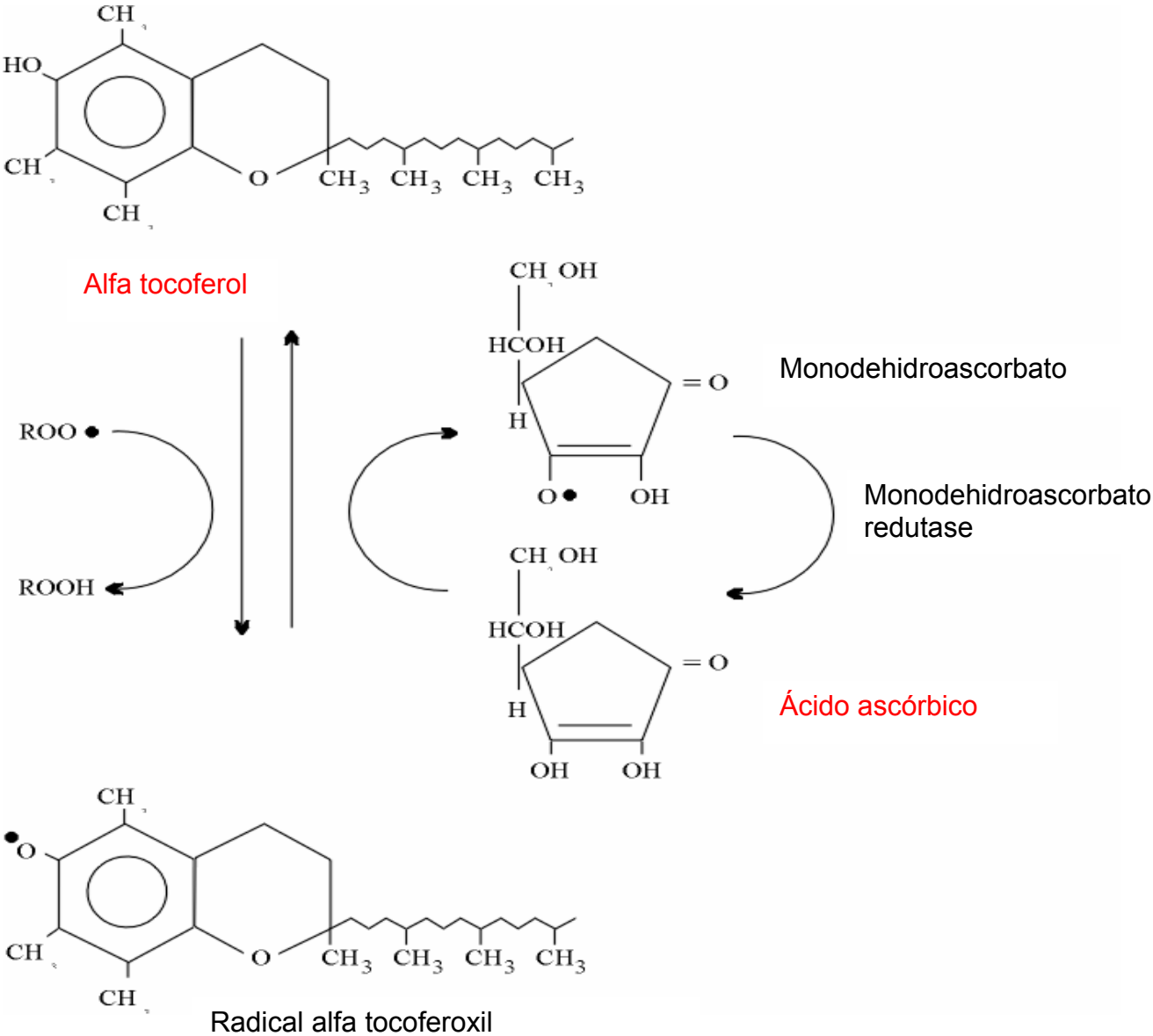


Figura 3. Reação de oxidação do α-tocoferol à radical tocoferoxil e a regeneração deste por ação do ácido ascórbico (Adaptado de Rodríguez, 1997).

3.2. As Isoflavonas da Soja

Os fitoestrógenos são compostos difenólicos, não esteroidais, derivados de plantas e com atividade estrogênica. Uma das maiores classes são as isoflavonas encontrados em grandes concentrações na soja. As principais isoflavonas estudadas são a genisteína, daidzeína e gliciteína (Sirtori et al., 2005). Os fitoestrógenos, assim como os estrógenos endógenos, entram no ambiente lipofílico do cérebro e concentram-se em regiões com abundância de receptores estrogênicos (Gamache & Acworth, 1998; Setchell, 1998; Lephart et al., 2002). São assim denominados porque reagem com os receptores ERα e, preferencialmente, com ERβ devido sua similaridade estrutural com o 17-β estradiol (Figura 4) (Setchell & Adlercreutz, 1988; Adlercreutz, 1998; Kuiper et al., 1998; Glazier & Bowman, 2001). As isoflavonas podem agir como agonistas ou antagonistas estrogênicos dependendo da dose utilizada e do tecido alvo (Miksicek, 1995). Tem sido indicado o uso de concentrações moderadas de isoflavonas, já que em altas doses elas podem aumentar o processo de apoptose e a degeneração celular em modelos animais (Cooke, 2006).

As isoflavonas podem agir como antioxidantes, direta ou indiretamente, aumentando a atividade de enzimas antioxidantes tais como CAT, SOD, GSH-Px e glutationa redutase (GSH-Rd) (Kurzer and Xu, 1997; Soulsby et al., 2004; Yousef et al., 2004; Geller & Studee, 2006). O consumo de proteína de soja quando comparada com a caseína leva a um decréscimo da peroxidação lipídica arterial (Sirtori et al., 2005) e o pré-tratamento com daidzeína mostrou-se eficaz

em proteger o dano lipídico provocado pelo etanol em jejuno de ratos (Nakagawa et al., 2006).

Embora pouco se saiba sobre o efeito potencial desses compostos sobre o aprendizado e a memória, estudos pré-clínicos e clínicos sugerem que as isoflavonas podem melhorar a função cognitiva em humanos e em ratos (Pan et al., 2000; File et al., 2001). Lee e colaboradores (2004) demonstraram que as isoflavonas da soja podem influenciar o sistema colinérgico e reduzir a perda neuronal relacionada com a idade e o declínio cognitivo em ratos. Evidenciando seu efeito neuroprotetor, tem sido descrito também que as isoflavonas utilizadas na dieta foram capazes de atenuar a fosforilação da proteína *tau* associada com a doença de Alzheimer (Kim et al., 2000). Essas substâncias estão sendo consideradas uma boa alternativa para tratar sintomas e distúrbios relacionados com a menopausa e o envelhecimento (Nakamura et al., 2000; Miquel et al., 2006).

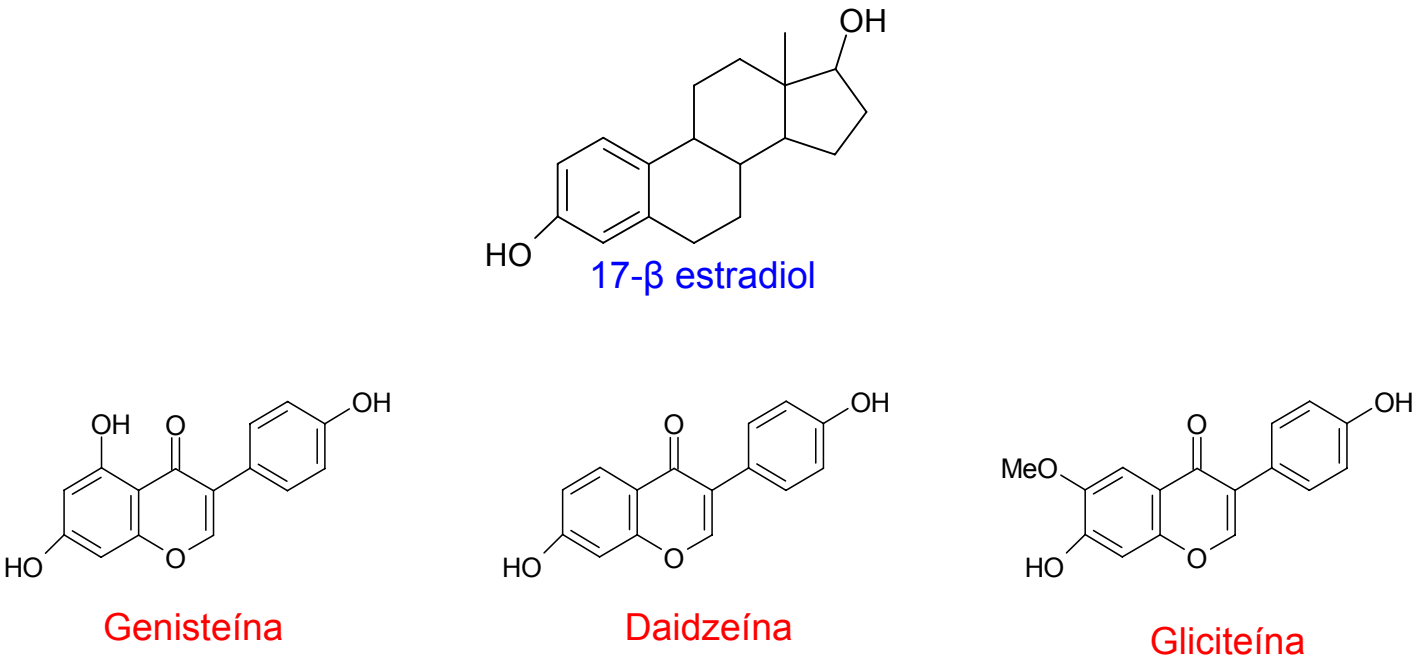


Figura 4. Estrutura química das principais isoflavonas encontradas na soja evidenciando suas similaridades estruturais com o 17 β-estradiol.

4. O Aprendizado e a Memória

Como foi mencionado, as ações dos hormônios esteróides ovarianos, como o estrógeno e a progesterona, não estão somente limitadas a neuroendocrinologia reprodutiva. A flutuação hormonal durante o ciclo reprodutivo também influencia regiões cerebrais intimamente relacionadas com o aprendizado e a memória como o córtex e o hipocampo (Walf et al., 2006). Além disso, o declínio nos níveis de estrógeno circulante durante a menopausa pode exacerbar os efeitos da idade

deixando o cérebro mais vulnerável ao desenvolvimento de distúrbios neurodegenerativos (Mhyre & Dorsa, 2006). A suplementação e reposição estrogênica pode ser cognitivamente benéfica para mulheres menopáusicas (Sherwin, 2006). Interessantemente, relatos na literatura demonstraram que as fêmeas parecem ser mais eficientes que os machos nas tarefas de reconhecimento de objetos (Eals & Silverman, 1997; James, 1997; Sutcliffe et al., 2007).

Tanto o aprendizado quanto a memória são funções básicas do SNC. O aprendizado é a aquisição de uma informação através da experiência. Já o conceito de memória inclue a aquisição, a formação, a conservação e a evocação de informações. A tarefa do labirinto aquático de Morris é adequada para avaliar a cognição em ratos, pois possuem boa capacidade de localização espacial requerida na tarefa. A memória de trabalho é a de curta duração. Mantém por pouco tempo a informação que está sendo processada no momento. Seu breve e fugaz processamento parece depender fundamentalmente dos neurônios do córtex pré-frontal (Izquierdo, 2002). O hipocampo é responsável pelo processamento das informações do tipo espacial, contextual e de trabalho (Jarrard, 1993), contendo receptores do tipo ERα e ERβ (Birzniece et al., 2006). A densidade das espinhas dendríticas hipocampais apresenta-se alterada durante o ciclo estral em ratos em resposta as mudanças cíclicas hormonais (Woolley & McEwen, 1993).

A menopausa tem sido associada com o declínio da memória e com o aparecimento de distúrbios cognitivos (Halbreich et al., 1995; Henderson, 2006).

Os efeitos da ovariectomia sobre a cognição têm sido estudados na tentativa de reproduzir os efeitos da perda hormonal ovariana na cognição humana. Recentemente Xu e Zhang (2006) mostraram que a administração de estradiol por um longo período melhora o aprendizado espacial em ratas ovarietomizadas. Estudos clínicos indicam que a TRH retarda o declínio cognitivo em mulheres pós-menopáusicas (Sherwin, 2003).

Muitos estudos têm se direcionado ao potencial dos esteróides ovarianos em facilitar o estoque de novas memórias e a proteção dos circuitos neuronais durante alguns estados debilitantes. Existem evidências de que o estrógeno está envolvido na plasticidade hipocampal e modulação da neurotransmissão (Woolley & McEwen, 1993; Baum, 2005; Bora et al., 2005; Pinkerton & Henderson, 2005), no aumento da potenciação de longa duração (Warren et al., 1995; Foy et al., 1999), na melhora na retenção da memória (Fader et al., 1999; Sandstrom & Williams, 2004), e na neurogênese (Tanapat et al., 1999). A inibição do estresse oxidativo e concomitante diminuição das espécies reativas de oxigênio têm efeitos benéficos no aprendizado e na memória. Recentemente, Quick e colaboradores (*in press*) observaram uma melhora da cognição em ratos administrando SOD sintética. Sabe-se que a ovariectomia prejudica a plasticidade sináptica hipocampal (Day & Good, 2005) e que o déficit de memória (Reis et al., 2002) e o prejuízo no metabolismo energético (Delwing et al., 2006) podem ser prevenidos pela administração de vitaminas E e C. Entretanto pouco se sabe sobre os mecanismos moleculares envolvidos nas ações benéficas do estrógenos sobre a memória e o aprendizado.

5. Os Gangliosídios

Os gangliosídios são glicoesfingolipídios, com um ácido siálico na molécula, presentes em grandes concentrações nas membranas celulares neuronais (Gottfries et al., 1996). As funções fisiológicas dos gangliosídios incluem crescimento e diferenciação celular, adesão celular e transdução de sinal (Allende & Prioa, 2002). Qualquer variação no conteúdo e na composição dos gangliosídios pode acarretar mudanças nas propriedades físicas das membranas e subseqüente disfunção neuronal (Tettamanti & Riboni, 1994). Tais variações têm sido observadas em danos cerebrais como a hipóxia (Yin et al., 2006), a isquemia (Kwak et al., 2005) e desordens neurodegenerativas (Yamamoto et al., 2006; Barrier et al., *in press*). Além disso, She e colaboradores (2005) demonstraram que os gangliosídios afetam a plasticidade sináptica em hipocampo e podem ser efetivos para atenuar déficits cognitivos em ratos. Foi demonstrado que a administração de etinilestradiol induz distintas respostas nas concentrações de gangliosídios, aumentando ou diminuindo em algumas regiões do cérebro de coelhos adultos (Islam et al., 1986). Entretanto, poucos estudos têm se direcionado ao estudo da influência da perda hormonal ovariana sobre o conteúdo e o perfil desses lipídios de membrana.

6. As Colinesterases

O sistema colinérgico possue um papel crucial na função cognitiva e tem a acetilcolina (ACh) como neurotransmissor clássico. O cérebro dos mamíferos possui dois tipos de colinesterases: a acetilcolinesterase (AChE), que hidrolisa preferencialmente a acetilcolina, e a butirilcolinesterase (BuChE), que catalisa a hidrólise da acetilcolina e de outros ésteres de colina. A AChE possui uma grande atividade catalítica com baixas concentrações de ACh enquanto que a BuChE é mais eficiente com altas concentrações de substrato (Lane et al., 2005). Dados recentes indicam que as colinesterases também estão envolvidas na modulação da glia, no fluxo sangüíneo cerebral e na fosforilação da proteína *tau* (Ballard et al., 2005). A AChE contribui para a integridade e permeabilidade da membrana sináptica durante a neurotransmissão e a condução (Grafius et al., 1971). A atividade dessa enzima, implicada em ações colinérgicas e não colinérgicas, está relacionada a distúrbios neurodegenerativos (Henderson et al., 1996). Tanto a AChE quanto a BuChE estão associadas às placas senis amilóides características da doença de Alzheimer (Mesulam & Geula, 1994; Giacobini, 2003).

A BuChE serve como um co-regulador da transmissão colinérgica (Geula & Darvesh, 2004) e é considerada um marcador periférico da AChE (Fossi et al., 1992). Têm sido descritas associações da BuChE sérica com alterações no metabolismo de lipídios (Magarian & Dietz, 1987) e com doenças cardiovasculares (Alcantara et al., 2002).

Tanto a depleção quanto a reposição estrogênica afetam o sistema colinérgico em várias regiões cerebrais (Simpkins et al., 1997; Gibbs & Aggarwal, 1998).

Recente estudo, pioneiro em mostrar a interação do estrógeno com o sistema colinérgico e seu efeito na capacidade cognitiva em humanos, demonstrou que o pré-tratamento com 17-β estradiol foi capaz de atenuar o déficit cognitivo causado por drogas anticolinérgicas em mulheres pós-menopáusicas (Dumas et al., 2006). Trabalhos do nosso grupo de estudo demonstraram que o tratamento com as vitaminas E e C preveniu o efeito inibitório da prolina sobre a atividade da AChE (Delwing et al., 2005) e a redução das atividades da AChE e BuChE pela arginina (Wyse et al., 2004).

7. A Na^+, K^+ -ATPase

Os hormônios esteróides sexuais podem gerar rápidos efeitos no cérebro modificando a estrutura e a função da membrana ou interagindo diretamente com específicas proteínas celulares de superfície (Dicko et al., 1999). Para estudos de interações neuroendócrinas a atividade enzimática da Na^+,K^+-ATPase tem sido considerada um índice adequado de atividade neural (Del Castillo et al., 1987) e, além disso, essa enzima pode ser um alvo importante para o 17 β-estradiol e moléculas com estruturas químicas semelhantes (Chen et al., 2006).

A Na^+,K^+-ATPase é uma enzima pertencente à família das P-ATPases, essencial para o funcionamento cerebral. Mantém o gradiente iônico durante a excitabilidade neuronal, hidrolizando cerca de 50% do ATP gerado no cérebro (Erecinska & Silver, 1994). A Na^+,K^+-ATPase tem sido relacionada com o

metabolismo energético (Mata et al., 1980) e a liberação de neurotransmissores (Brosemer, 1985).

A Na^+,K^+-ATPase é uma proteína heterométrica composta por duas subunidades α transmembranas, que contém os sítios de ligação para Na^+, K^+, ATP e glicosídios cardíacos, duas subunidades β regulatórias, na forma de glicoproteínas, e uma subunidade δ (Kaplan, 2002; Devlin, 2003; Erecinska et al., 2004). É responsável pela geração do potencial de membrana através do co-transporte ativo de três íons Na^+ para o meio extracelular e de dois íons K^+ para o meio intracelular (Erecinska et al., 2004) (Figura 5). A manutenção do equilíbrio eletrolítico intra e extracelular garante ao neurônio a geração do potencial de membrana a fim de manter a excitabilidade e o volume neuronal (Kaplan, 2002; Devlin, 2003).

A atividade da Na^+,K^+-ATPase é inibida por radicais livres e está reduzida na isquemia cerebral (Wyse et al., 2000) e em processos neurodegenerativos como a doença de Alzheimer (Hattori et al., 1998). Estudos realizados em nosso grupo de pesquisa mostraram que as vitaminas E e C previnem a inibição da Na^+,K^+-ATPase em hipocampo de ratos submetidos à administração aguda de homocisteína , indicando a participação do estresse oxidativo (Wyse et al., 2002). Carageorgiou e cols. (2003) observaram que a Selegilina, droga com atividades neuroprotetoras e antioxidantes, utilizada na doença de Parkinson e na depressão, diminuiu a produção de radicais livres, o que resultou na estimulação da atividade da AChE e da Na^+,K^+-ATPase. Por outro lado, a estimulação da atividade da

Na^+,K^+-ATPase está associada com um decréscimo na fluidez da membrana (Levin t al., 1990) .

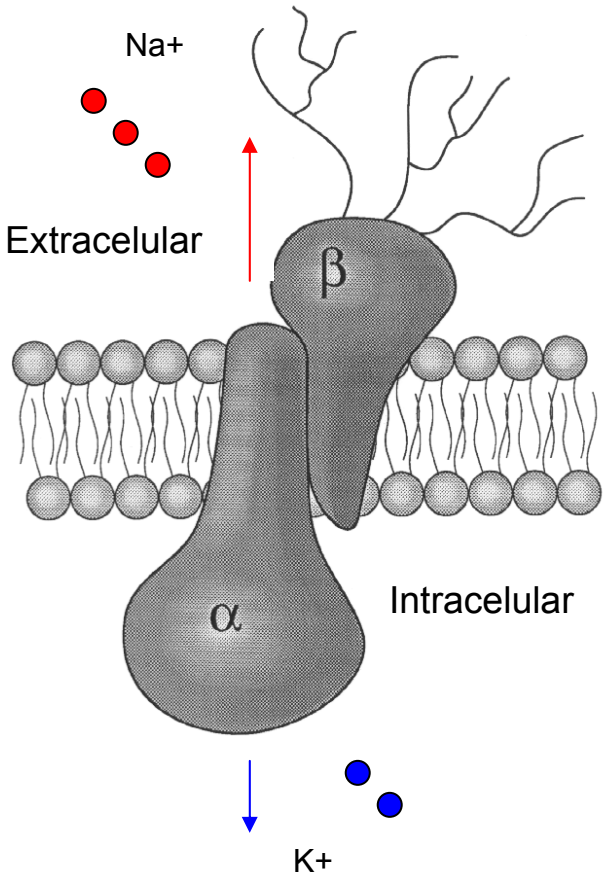


Figura 5. Representação do co-transporte ativo de três íons Na^+ para o meio extracelular e de dois íons K^+ para o meio intracelular pela Na^+,K^+-ATPase.

II. OBJETIVOS

Objetivo Geral

Considerando que (1) mulheres na menopausa são mais suscetíveis a doenças neurodegenerativas, à isquemia cerebral e ao déficit cognitivo; (2) que alterações nas funções colinérgicas e na homeostasia iônica, e o estresse oxidativo são eventos importantes associados a essas condições; (3) que as vitaminas E e C e as isoflavonas da soja têm sido descritas como alternativas a TRH; o objetivo geral do nosso estudo foi investigar alguns parâmetros bioquímicos (Na^+,K^+-ATPase, colinesterases, gangliosídios e alguns parâmetros de estresse oxidativo) e comportamentais em ratas adultas ovariectomizadas, bem como o efeito da suplementação com as vitaminas E e C e isoflavonas da soja sobre tais alterações.

Esse trabalho será dividido em seis capítulos como segue:

Capítulo I

Objetivos específicos

1. Investigar o efeito da ovariectomia sobre a atividade da AChE em homogeneizados de córtex cerebral de ratas adultas.

2. Investigar o efeito da ovariectomia sobre a atividade da BuChE, como marcador periférico da AChE, em soro de ratas adultas.

3. Avaliar o efeito da ovariectomia sobre o conteúdo de gangliosídios em córtex cerebral de ratas adultas.

Capítulo II

Objetivos específicos

1. Investigar o efeito da ovariectomia sobre a atividade da AChE em homogeneizado de hipocampo de ratas adultas.
2. Investigar o efeito da ovariectomia sobre a atividade da Na^+,K^+-ATPase em membrana plasmática sináptica de hipocampo de ratas adultas.
3. Avaliar o efeito da ovariectomia sobre alguns parâmetros de estresse oxidativo denominados TRAP (capacidade antioxidante total), TBA-RS (substâncias reativas ao ácido tiobarbitúrico), bem como a atividade das enzimas antioxidantes CAT (catalase), SOD (superóxido dismutase) e GSH-Px (glutationa peroxidase).

Capítulo III

Objetivos específicos

1. Investigar a influência do tratamento crônico com as vitaminas E e C ou com a dieta de soja rica em isoflavonas sobre a estimulação da atividade da

Na^+,K^+-ATPase em membrana plasmática sináptica de hipocampo de ratas adultas.

2. Avaliar a influência do tratamento crônico com as vitaminas E e C ou com a dieta de soja rica em isoflavonas sobre a estimulação da atividade da AChE em hipocampo de ratas adultas.
3. Determinar as ações das vitaminas e das isoflavonas da soja sobre a redução da atividade da BuChE em soro de ratas ovariectomizadas.

Capítulo IV

Objetivos específicos

1. Investigar o efeito da ovariectomia sobre a memória espacial no labirinto aquático de Morris em ratas adultas.
2. Avaliar se o tratamento crônico com as vitamina E e C altera o prejuízo na memória espacial nas ratas ovariectomizadas.

Capítulo V

Objetivos específicos

1. Investigar se o pré ou pós-tratamento com a dieta de soja rica em isoflavonas previne o prejuízo na memória espacial causado pela ovariectomia em ratas adultas.

Capítulo VI

Objetivos específicos

1. Investigar o efeito da ovariectomia e da administração das vitaminas E e C sobre a fosforilação de diferentes subunidades do receptor AMPA em homogeneizado de hipocampo de ratas adultas.
2. Avaliar o efeito da ovariectomia e posterior tratamento com as vitaminas E e C sobre a possível modulação da via de sinalização ERK1/2 – CREB em hipocampo de ratas adultas.

OBS: todos os capítulos serão apresentados na forma de artigos científicos

III. METODOLOGIA E RESULTADOS

III.1 Artigo 1

Ovariectomy enhances acetylcholinesterase activity but does not alter ganglioside content in cerebral córtex of female adult rats

Monteiro, SC, Stefanello, FM, Vianna, LP, Matté, C, Barp, J, Belló-Klein, A, Trindade, VMT, and Wyse, ATS.

Metabolic Brain Disease 20(1): 35-44, 2005.

Objetivos

1. Investigar o efeito da ovariectomia sobre a atividade da AChE em homogeneizados de córtex cerebral de ratas adultas.
2. Investigar o efeito da ovariectomia sobre a atividade da BuChE, como marcador periférico da AChE, em soro de ratas adultas.
3. Avaliar o efeito da ovariectomia sobre o conteúdo de gangliosídios em córtex cerebral de ratas adultas.

Metabolic Brain Disease, Vol. 20, No. 1, March 2005 (© 2005)
DOI: 10.1007/s11011-005-2474-1

Ovariectomy Enhances Acetylcholinesterase Activity But Does Not Alter Ganglioside Content in Cerebral Cortex of Female Adult Rats

Siomara C. Monteiro,[1] Francieli M. Stefanello,[1] Luciene P. Vianna,[1] Cristiane Matté,[1] Jaqueline Barp,[1] Adriane Belló-Klein,[1] Vera M. T. Trindade,[1] and Angela T. S. Wyse[1,2]

Received December 5, 2003; accepted May 4, 2004

In the present work we investigated the effect of ovariectomy on acetylcholinesterase (AChE) activity and ganglioside content in cerebral cortex of female rats. We also studied the activity of butyrylcholinesterase (BuChE) in serum of these animals. Adult Wistar rats were divided into three groups: (1) naive females (control), (2) sham-operated females and (3) castrated females (ovariectomy). Thirty days after ovariectomy, rats were sacrificed by decapitation without anaesthesia. Blood was collected and the serum used for BuChE determination. Cerebral cortex was homogenized to determine AChE activity and extracted with chlorophorm:methanol for ganglioside evaluation. Results showed that rats subjected to ovariectomy presented a significant increase of AChE activity, but did not change the content and the profile of gangliosides in cerebral cortex when compared to sham or naive rats. BuChE activity was decreased in serum of rats ovariectomized. Our findings suggest that the alteration in the activity of brain AChE, as well as serum BuChE activity caused by ovariectomy may contribute to the impaired cognition and/or other neurological dysfunction found in post-menopausal women.

Key words: Acetylcholinesterase; butyrylcholinesterase; gangliosides; cerebral cortex; ovariectomy; female rats.

INTRODUCTION

In adult woman with a normal reproductive cycle the estrogenic compounds are secreted in great quantity mainly by ovaries, being the 17β estradiol considered the major estrogen (Rodrigues *et al*., 1999). Estrogen exerts also diverse nonreproductive actions on multiple organs, including the brain (Wise, 2002). It has been shown that estrogen deprivation is implicated in the pathogenesis of some neurodegenerative conditions, such as Alzheimer's disease and cerebral ischemia (Tang *et al*., 1996; Van Duijn, 1999; Zhang *et al*., 1998). In this context, there is a large body of literature suggesting that post-menopausal women are more vulnerable than young women to these diseases and cognitive deficit (Green and Simpkins, 2000; Wise *et al*., 2001a,b).

[1]Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.
[2]To whom correspondence should be addressed at Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre RS Brazil. E-mail: wyse@ufrgs.br

Acetylcholinesterase (AChE) (E.C. 3.1.1.7), the enzyme involved in the hydrolysis of the neurotransmitter acetylcholine, contributes to the integrity and permeability of the synaptic membrane that occurs during neurotransmission and conduction (Grafius *et al*., 1971). This enzyme has been implicated in cholinergic and noncholinergic actions which may play a role in neurodegenerative diseases (Cummings, 2000; Henderson *et al*., 1996; Law *et al*., 2001). It has been also shown that AChE *per se* activates neuronal cell death (Calderón *et al*., 1998). On the other hand, it is known that estrogen withdrawal and replacement affect the cholinergic system in a variety of brain regions (Gibbs and Aggarwal, 1998; Simpkins *et al*., 1997).

Gangliosides are a family of sialic acid-containing glycosphingolipid present in high concentration in neural membranes. They play important roles in cell-cell interaction, cellular growth and differentiation, signal transduction, adaptation of plasma membrane to environmental variations and may be involved in neuronal development (Ando, 1983; Maccioni *et al*., 1984; Sanhoff and Van Echten, 1994). It has been proposed that gangliosides may play significant roles in memory and behavior (Rahmann, 1995). In addition, alterations in the content and composition of gangliosides have been reported in brain injuries such as hypoxia (Ramirez *et al*., 2003; Trindade *et al*., 2001, 2002), ischemia (Inokuchi *et al*., 1998), Alzheimer's disease and in other neurodegenarative disorders (Farooqui *et al*., 1988; Ohtani *et al*., 1996; Schneider *et al*., 1998; Yu and Ledeen, 1974). It was been shown that ethinylestradiol administration induces distinct responses on ganglioside concentrations, increasing or diminishing it in some regions of the forebrain of female adult rabbits, or not affecting it in others (Islam *et al*., 1986). On the other hand, there is a lack of studies analyzing the influence of female hormones reduction on neural connections which can be reflected by ganglioside content (DeKosky and Bass, 1982; Zeller and Marchase, 1992).

Considering that hormonal deprivation in post-menopausal women is implicated in the pathogenesis of cerebrovascular and Alzheimerś disease and that cholinesterases are altered in these conditions, in the present study we investigated the effect of ovariectomy on AChE activity and gangliosides content in cerebral cortex of female adult rats. We also determined BuChE activity in serum, a blood AChE marker.

MATERIALS AND METHODS

Subjects and Reagents

Female adult Wistar rats (3 months, 180–210 g BW) were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal Rio Grande do Sul, Brazil. The chemicals were purchased from Sigma, St. Louis, MO.

Experimental Treatment

Animals were randomly assigned to one of the following groups ($n = 4$): naive (control), sham (only submitted to surgery without removing of ovaries) and ovariectomized. Rats were ovariectomized by surgery removing both the ovaries under anesthesia induced by i.p. injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) to eliminate endogeneous ovarian steroids (Waynforth and Flecknell, 1992). One month after ovariectomy, rats were sacrificed by decapitation without anesthesia and the brain was immediately isolated, washed with saline solution and the cerebral cortex was dissected. Estradiol levels were evaluated in plasma after surgery by radioimmunoassay using a Biomedical kit (Biomedicals Technologies, Inc., Stoughton, MA). Estrogen levels in the ovariectomized rats were undetectable (data not shown) confirming the efficacy of the surgical procedures of ovariectomy.

Tissue Preparation

Rats were killed by decapitation without anaesthesia, the blood was rapidly collected, centrifuged at 3000 rpm for 10 min and the serum was separated and used for the BuChE assays. The brain was quickly removed and the cerebral cortex was dissected. For determination of AChE activity, cerebral cortex was homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5. For ganglioside extraction the cerebral cortex was weighed and extracted first with a 2:1 mixture of chloroform:methanol (C/M, 2:1, v/v) to a 20-fold dilution of tissue mass and centrifuged at 800 *g* for 10 min. The pellet was extracted with C/M 1:2 to a 10-fold dilution of original sample mass. The C/M extracts were combined and this pool was used for ganglioside evaluation (Roukema and Heijlman, 1970).

Acetylcholinesterase (AChE) Activity Assay

Acetylcholinesterase activity was determined according to Ellman *et al*. (1961), with some modifications (Villescas *et al*., 1981). Hydrolysis rates *v* were measured at acetylthiocholine (S) concentrations of 0.8 mM in 1 mL assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25°C. Fifty microliters of rat cerebral cortex supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30s). All samples were run in duplicate.

Butyrylcholinesterase (BuChE) Assay

BuChE activity was determined by the method of Ellman *et al*. (1961) with some modifications. Hydrolysis rate v was measured at acetylthiocholine (S) concentration of 0.8 mM in 1 mL assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Fifty microliters of rat serum was added to the reaction mixture and preincubated

for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30s) at 25°C. All samples were run in duplicate.

Ganglioside Evaluation

Aliquots from the total lipid extracts were used for ganglioside determination by the N-acetyl-neuramic acid (NeuAc) quantification with the resorcinol method described by Svennerholm (1957) and modified by Miettinen and Takki-Luukkainen (1959). Ganglioside species were analyzed by thin layer chromatography (TLC) and this technique was performed on 10 × 10 cm Merck plates of silica gel 60 using a developing tank described by Nores *et al*. (1994). Aliquots of the total lipid extracts containing 6 nmol of NeuAc suspended in C:M (1:1) were spotted on 8-mm lanes. TLC was developed, sequentially, with two mixtures of solvents, firstly C:M (4:1, v/v) and secondly C:M: 0.25% $CaCl_2$ (60:36:8, v/v). Ganglioside profile was visualized with resorcinol reagent (Lake and Goodwin, 1976; Svennerholm, 1957). The chromatographic bands were quantified by scanning densitometry at 580 nm with a CS 9301 PC SHIMADZU densitometer. Individual ganglioside values expressed as nmol ganglioside-NeuAc/mg tissue, were calculated by relating their respective percentage to the absolute total quantity of ganglioside-NeuAc. The terminology used herein for gangliosides is that recommended by Svennerholm (1963).

Protein Determination

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical Analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one way ANOVA followed by the Duncan multiple test when F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

RESULTS

Figure 1 shows the effect of ovariectomy on AChE activity from cerebral cortex of rats. Ovariectomized rats present an increase (113%) of AChE activity when compared to control (naive) or rats submitted to surgery sham [$F(2, 9) = 20.72$; $p < 0.01$].

Since in our study the ovariectomized rats present an increase of brain AChE activity and that a recent report from the literature suggested that BuChE activity could hydrolyze acetylcholine glial (Mesulam *et al*., 2002) and that this enzyme could be used as a peripheral marker of brain AChE, in the present study we also verified the activity of BuChe in serum of female adult ovariectomized rats (Fig. 2). Results showed that rats submitted to ovariectomy presented an inhibition (45%) of BuChE activity when compared to naive and sham rats [$F(2, 9) = 11.43$; $p < 0.05$].

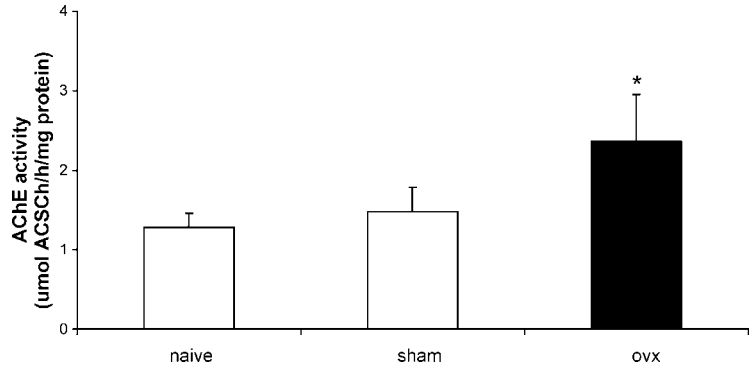


Figure 1. Effect of ovariectomy on acetylcholinesterase activity in cerebral cortex of rats. Data are expressed as mean ± S.D. for four independent experiments performed in duplicate. $^{*}p < 0.01$ compared to naive and sham groups (Duncan's multiple range test). AChE—acetylcholinesterase; ovx—ovariectomized.

Table 1 shows that ovariectomy did not cause changes in cerebral cortex weight [$F(2, 9) = 0.44$; $p > 0.05$] and total ganglioside content [$F(2, 9) = 0.54$; $p > 0.05$] in this brain structure of naive, sham and ovariectomized rats.

Thin layer chromatography (Fig. 3) shows the presence of four main cerebral gangliosides: GM1, GD1a, GD1b, and GT1b. The chromatogram reveals no difference on the ganglioside profiles between the studied groups.

DISCUSSION

Estrogen has been described to play an important role in cognitive functions and neuroprotection (Brinton, 2001; Gandy, 2003; Kampen and Sherwin, 1994). In this context, it has been shown that estrogen deprivation is implicated in the pathogenesis of neurodegenerative disorders, including stroke (Liao *et al.*, 2001) and Alzheimer's disease (Fillit,

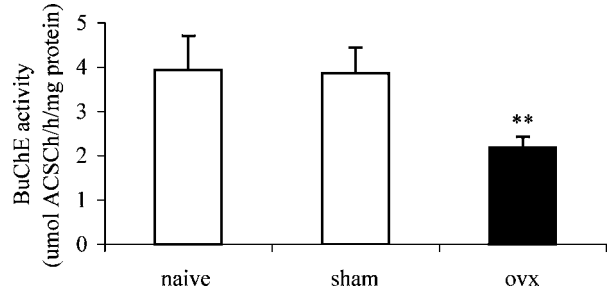


Figure 2. Effect of ovariectomy on butyrylcholinesterase activity in serum of rats. Data are mean ± S.D. for four independent experiments performed in duplicate. $^{**}p < 0.05$ compared to control (Duncan multiple range test). BuChE—butyrylcholinesterase; ovx—ovariectomized.

Table 1. Cerebral Cortex Weight and Ganglioside-NeuAc Content of Female Adult Wistar Rats

	Groups		
	Naive	Sham	Ovx
Cerebral cortex weight (mg)	668.5 ± 7.4	689.7 ± 30.9	661.0 ± 22.6
Ganglioside content (nmol NeuAc/mg tissue)	1.61 ± 0.05	1.61 ± 0.07	1.71 ± 0.10

Note. Control (naive), submitted to surgery (sham) and ovariectomized (ovx). Values are expressed as mean ± standard error; $n = 4$.

1994; Van Duijn, 1999). Evidences also show that post-menopausal estrogen replacement therapy reduces the risk and delay in the onset of these diseases (Tang *et al*., 1996; Van Duijn, 1999; Yaffe *et al*., 1998). In contrast, recent data from the literature showed that estrogen plus progestin therapy to post-menopausal women increased the risk for dementia in women aged 65 years or older and did not improve cognitive impairment in these women (Shumaker *et al*., 2003).

Reduction in cholinergic function and alteration in the content and composition of gangliosides have been reported as one of the causes of Alzheimer's disease and stroke (Bonnefont *et al*., 1998; Farooqui *et al*., 1988; Fredman, 1998; Inokuchi *et al*., 1998; Mesulam *et al*., 2002; Schneider, 1994). In addition, the interaction among estrogens, cholinergic system and especially ganglioside content has not studied.

In the present study, we investigated the effect of ovariectomy on AChE activity and on ganglioside content and profile in cerebral cortex of female adult rats. We used this animal model of steroid hormone deprivation because the ovariectomy is considered the most common animal model of post-menopausal changes in adult female rats (Savonenko and Markowska, 2003). We used cerebral cortex because the discovery of estrogen receptor, namely ER-β in this structure has provided novel sites for estrogen action in cerebral cortex (Shughrue and Merchenthaler, 2000). In addition, estrogen also appears to play a fundamental role in cortical neuroprotection, since estrogen treatment significantly reduces

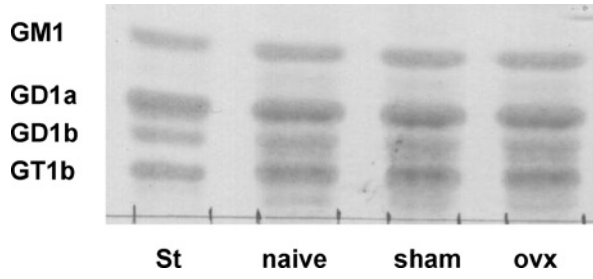


Figure 3. Thin-layer chromatography of ganglioside profile in cerebral cortex of female adult Wistar rats control (naive), submitted to surgery (sham) and ovariectomized (ovx). Ganglioside-NANA was estimated by the resorcinol-HCl reagent of Svennerholm (1957) as modified by Miettinen and Takki-Luukkainen (1959). A small volume of concentrated ganglioside extract containing 6 nmol was spotted for separation of the ganglioside fractions. The position of chromatographed ganglioside standards are indicated.

infarct size after ischemia in ovariectomized rats (Dubal *et al*., 1999). Our results showed that rats subject to ovariectomy presented a significant increase in AChE activity. However, we cannot establish at the present whether the increase of this enzyme activity following by ovariectomy would be a result of estrogen deprivation alone, since ovaries produce other substance such as progestin and inibin.

Changes in AChE activity in Alzheimer's disease patients have been previously reported (Arendt *et al*., 1992; Fishman *et al*., 1986; Gómez-Ramos and Morán, 1997). In this context, a reduction of this enzyme activity was demonstrated in cerebral cortex and hippocampus of patients affected by Alzheimer's (Fishman *et al*., 1986) and studies also show that alterations of AChE activity are associated with the cognitive alterations characteristic of these patients (Cummings, 2000; Law *et al*., 2001). On the other hand, degeneration of cholinergic nerve endings in specific regions of brain results not only in reduction of the tetrametric globular form (G4) of AChE, but also in a concomitant increase (300- to 400-fold) in the collagen-tailed form of this enzyme (Younkin *et al*., 1986). In this context, it was found that AChE (G1 globular form) is co-localized with senile plaques in the central nervous system (CNS), suggesting that this enzyme plays a role in the progressive β-amyloid aggregation and in senile plaque maturation characteristic of Alzheimer's disease (Arendt *et al*., 1992; Gómez-Ramos and Morán, 1997). In addition, recent studies suggest that amyloid-AChE complexes are formed when AChE accelarates the assembly of Aβ peptides into fibrils by interacting with the growing amyloid fibrils (Alvarez *et al*., 1997). Based on these findings, reversible inhibitors of cholinesterases have been used as cognitive stimulators in the treatment of Alzheimer's disease (Enz *et al*., 1993; Greig *et al*., 2001). Some studies also showed that ischemia transiently increases AChE activity in organotypic rat hippocampal slice cultures (Saez-Valero *et al*., 2003).

Considering that there is evidence showing that BuChe activity, which is considered a peripheral marker of neuronal AChE (Fossi *et al*., 1992), may have a role in the aggregation of AB that occurs in the early stages of senile plaque formation in Alzheimer's disease (Guillozet *et al*., 1997; Mesulam and Geula, 1994), we also examined the effect of ovariectomy on BuChE activity in serum of rats. Results showed that this enzyme activity was decreased (46%) in ovariectomized rats. The unexpected decrease of this BuChE activity in serum of ovariectomized rats may be possibly interpreted as a compensatory mechanism to decrease acetylcholine hydrolysis, since AChE activity is increased in brain. In fact, a similar pattern of these enzymes activity has been described in another study (Giacobini, 1997). In addition, other studies have reported that AChE activity is decreased in brain of patients with Alzheimeŕs disease, whereas BuChe activity is unchanged or increased (Davies and Maloney, 1976; Giacobini *et al*., 1989). So far we do not know the exact underlying mechanisms through which BuChE activity is decreased in our study.

We also showed in the present study that the content and profile of gangliosides in cerebral cortex was not changed in female rats ovariectomized. However, we cannot at this time, affirm whether the results here observed in cerebral cortex occur in other cerebral structures, because reports from literature show that estrogen administration decreases the content of total lipids in hypothalamus and increases the concentrations of gangliosides in hyppocampus, amygdaloid nucleus and olfactory bulbs, suggesting that the lipid contents and plasticity are affected differentially in the various areas of the brain by estrogen or phytoestrogen (Islam *et al*., 1986; Lephart *et al*., 2003).

Finally, it has been suggested that estrogen deprivation is likely to initiate or enhance neurodegenerative changes and to reduce the brain ability to maintain synaptic connectivity and cholinergic integrity, leading to the cognitive decline seen in post-menopausal individuals (Gandy, 2003). In this context, it has been shown that depletion of estrogen causes accumulation of Aβ peptide in the CNS of transgenic mice, which can be reversed by estradiol treatment (Zheng *et al*., 2002).

Summarizing, the present study demonstrates that female adults ovariectomized significantly increases AChE activity in cerebral cortex. This effect could decrease acetylcholine levels, leading to reduction of cholinergic neurotransmission. Assuming the possibility that these phenomena may occur in humans, our findings might be relevant to explain, at least in part, the cognitive impairment and the higher risk of neurodegenerative disease observed in post-menopausal woman.

ACKNOWLEDGMENTS

We thank Tânia Regina Gattelli Fernandes for her technical assistance. This work was supported in part by grants from CNPq—Brazil, FAPERGS, RS-Brazil, and Programa de Núcleos de Excelência-Financiadora de Estudos e Projetos (PRONEX II – FINEP-Brazil).

REFERENCES

Alvarez, A., Opazo, C., Alarcon, R., Garrido, J., and Inestrosa, N.C. (1997). Acetylcholinesterase promotes the aggregation of amyloid-beta-peptide fragments by forming a complex with the growing fibrils. *J. Mol. Biol.* **272:**348–361.

Ando, S. (1983). Gangliosides in the nervous system. *Neurochem. Int.* **5:**507–537.

Arendt, T., Bruckner, M.K., Lange, M., and Bigl, V. (1992). Changes in acetylcholinesterase and butyrylcholinesterase in Alzeimer's disease resemble embryonic development —A study of molecular forms. *Neurochem. Int.* **21:**381–396.

Bonnefont, A.B., Munoz, F.J., and Inestrosa, N.C. (1998). Estrogen protects neuronal cells from the cytotoxicity induced by acetylcholinesterase-amyloid complexes. *FEBS Lett.* **441:**220–224.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of micrograms quantities of protein utilizing the principle of protein-die-binding. *Anal. Biochem.* **72:**248–254.

Brinton, R.D. (2001). Cellular and molecular mechanisms of estrogen regulation of memory function and neuroprotection against Alzheimer's disease: Recent insights and remaining challenges. *Learn. Mem.* **8:**121–133.

Calderón, F.H., Von Bernhardi, R., De Ferrari, G., Luza, S., Aldunate, R., and Inestrosa, N.C. (1998). Toxic effects of acetylcholinesterase on neuronal and glial-like cells in vitro. *Mol. Psychiatry* **3:**247–255.

Cummings, J.L. (2000). The role of cholinergic agents in the management of behavioral disturbances in Alzheimer's disease. *Int. J. Neuropsychopharmacol.* **3:**21–29.

Davies, P., and Maloney, A.J. (1976). Selective loss of central cholinergic neurons in Alzeimer's disease. *Lancet* **2:**1403.

DeKosky, S.T., and Bass, N.H. (1982). Aging, senile dementia, and the intralaminar microchemistry of cerebral cortex. *Neurology* **32:**1227–1233.

Dubal, D.B., Shughrue, P.J., Wilson, M.E., Merchenthaler, I., and Wise, P.M. (1999). Estradiol modulates Bcl-2 in cerebral ischemia: A potencial role for estrogen receptors. *J. Neurosci.* **19:**6385–6393.

Ellman, G.L., Courtney, K.D., Andres, V., and Featherstone, R.M. (1961). A new and rapid determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7:**21–29.

Enz, A., Amstutz, R., Boddeke, H., Gmelin, G., and Malanowski, J. (1993). Brain selective inhibition of acetylcholinesterase: A novel approach to therapy for Alzeimer's Disease. *Prog. Brain Res.* **98:**431–438.

Farooqui, A.A., Liss, L., and Horrocks, L.A. (1988). Stimulation of lipolytic enzymes in Alzeimer's disease. *Ann. Neurol.* **23:**306–308.

Fillit, H. (1994). Estrogens in pathogenesis and treatment of Alzeimer's disease in postmenopausal women. *Ann. N. Y. Acad. Sci.* **743:**233–239.

Fishman, E.B., Siek, G.C., MacCallum, R.D., Bird, E.D., Volicer, L., and Marqui, J.K. (1986). Distribution of the molecular forms of acetylcholinesterase in human brain: Alterations in dementia of the Alzheimer type. *Ann. Neurol.* **19:**246–252.

Fossi, M.C., Leonzio, C., Massi, A., Lari, L., and Casini, S. (1992). Serum esterase inhibition in birds: A nondestructive biomarker to assess organophosphorus and carbamate contamination. *Arch. Environ. Contam. Toxicol.* **23:**99–104.

Fredman, P. (1998). Sphingolipids and cell signalling. *J. Inherit. Metab. Dis.* **21:**472–480.

Gandy, S. (2003). Estrogen and Neurodegeneration. *Neurochem. Res.* **28:**1003–1008.

Giacobini, E., DeSarno, P., Clark, B., and McIlbany, M. (1989). The cholinergic receptor system of the human brain: Neurochemical and pharmacological aspects in aging and Alzeimer. *Prog. Brain Res.* **79:**335–343.

Giacobini, E. (1997). From molecular structure to Alzheimer therapy. *Jpn. J. Pharmacol.* **74:**225–241.

Gibbs, R.B., and Aggarwal, P. (1998). Estrogen and basal forebrain cholinergic neurons: Implications for brain aging and Alzheimer's disease-related cognitive decline. *Horm. Behav.* **34:**98–111.

Gómez-Ramos, P., and Morán, M.A. (1997). Ultra structural localization of butyrylcholinesterase in senile plaques in the brains of aged and Alzheimer's disease patients. *Mol. Chem. Neuropathol.* **30:**161–173.

Grafius, M.A., Bond, H.E., and Millar, D.B. (1971). Acetylcholinesterase interaction with a lipoprotein matrix. *Eur. J. Biochem.* **22:**382–390.

Green, P.S., and Simpkins, J.W. (2000). Neuroprotective effects of estrogens: Potential mechanisms of action. *Int. J. Devl. Neuroscience.* **18:**347–358.

Greig, N.H., Utsuki, T., Yu, Q., Zhu, X., Holloway, H.W., Perry, T., Lee, B., Ingram, D.K., and Lahiri, D.K. (2001). A new therapeutic target in Alzheimer's disease treatment: Attention to butyrylcholinesterase. *Curr. Med. Res. Opin.* **17:**159–165.

Guillozet, A., Smiley, J.F., Mash, D.C., and Mesulam, M.M. (1997). Butyrylcholinesterase in the life cycle of amyloid plaques. *Ann. Neurol.* **42:**909–918.

Henderson, V.W., Watt, L., and Buckwalter, J.G. (1996). Cognitive skills associated with estrogen replacement in women with Alzeimer's disease. *Psychoneuroendocrinology* **21:**421–430.

Inokuchi, J., Kuroda, Y., Kosaka, S., and Fujiwara, M. (1998). L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol stimulates ganglioside biosynthesis, neurite outgrowth and synapse formation in cultured cortical neurons, and ameliorates memory deficits in ischemic rats. *Acta Biochim. Pol.* **45:**479–492.

Islam, F., Hasan, M., and Saxena, K. (1986). Isolation and estimation of gangliosides in discrete regions of the forebrain: Effects of estrogen on regional lipid profiles. *Exp. Pathol.* **29:**159–164.

Kampen, D.L., and Sherwin, B.B. (1994). Estrogen use and verbal memory in healthy postmenopausal. *Obstet. Gynecol.* **83:**979–983.

Lake, B.D., and Goodwin, H.J. (1976). Lipids. In (I. Smith, and J.W.T. Seakins, eds.), *Chromatographic and Eletrophoretic Techniques*, Vol. 1. Paper and Thin Layer Chromatography William Heinemann Medical Books Ltd., London, UK, pp. 345–366.

Law, A., Gauthier, S., and Quirion, R. (2001). Say NO Alzheimer's disease: The putative links between nitric oxide and dementia of the Alzheimer's type. *Brain Res. Rev.* **35:**73–96.

Lephart, E.D., Rhees, R.W., Setchell, K.D.R., Bu, L.H., and Lund, T.D. (2003). Estrogens and phytoestrogens: Brain plasticity of sexually dimorphic brain volumes. *J. Steroid Biochem. Mol. Biol.* **85:**299–309.

Liao, S., Chen, W., Kuo, J., and Chen, C. (2001). Association of serum estrogen level and ischemic neuroprotection in female rats. *Neurosc. Lett.* **297:**159–162.

Maccioni, H.J.F., Panzetta, P., Arrieta, D., and Caputto, R. (1984). Ganglioside glycosil transferase activities in the cerebral hemispheres from developing rat embryos. *Int. J. Develop. Neurosc.* **2:**13–19.

Mesulam, M., and Geula, C. (1994). Butyrylcholinesterase reactivity differentiates the amyloid plaques of aging from those of dementia. *Ann. Neurol.* **36:**722–727.

Mesulam, M.-M., Guillozet, A., Shaw, P., Levey, A., Duysen, E.G., and Lockridge, O. (2002). Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze. acetylcholine. *Neuroscience* **110:**627–639.

Miettinen, T., and Takki-Luukkainen, I.T. (1959). Use of butyl acetate in determination of sialic acid. *Acta Chem. Scand.* **13:**856–858.

Nores, G.A., Mitzumari, R.K., and Kremer, D.M. (1994). Chromatographic tank designed to obtain highly reproducible high-performance thin-layer chromatograms of gangliosides and neutral glycosphingolipids. *J. Chromatogr.* **686:**155–157.

Ohtani, Y., Tamai, Y., Ohnuki, Y., and Miura, S. (1996). Ganglioside alterations in the central and peripheral nervous systems of patients with Creutzfeldt-Jakob disease. *Neurodegeneration* **5:**331–338.

Rahmann, H. (1995). Brain gangliosides and memory formation. *Behav. Brain Res.* **66:**105–116.

Ramirez, M.R., Muraro, F., Zylbersztejn, D.S., Abel, C.R., Arteni, N.S., Lavinsky, D., Netto, C.A., and Trindade, V.M.T. (2003). Neonatal hypoxia-ischemia reduces ganglioside, phospholipid and cholesterol contents in the rat hippocampus. *Neurosc. Res.* **46:**339–347.

Rodrigues, H.D., Kinder, J.E., and Fitzpatrick, L.A. (1999). Treatment with 17 beta-estradiol does not influence age and weight at puberty in Bos indicus heifers. *Anim. Reprod. Sci.* **56:**1–10.

Roukema, P.A., and Heijlman, J. (1970). The regional distribution of sialoglycoproteins, gangliosides and sialidase in bovine brain. *J. Neurochem.* **17:**773–780.

Saez-Valero, J., Gonzalez-Garcia, C., and Cena, V. (2003). Acetylcholinesterase activation in organotypic rat hippocampal slice cultures deprived of oxygen and glucose. *Neurosci. Lett.* **48:**123–125.

Sanhoff, K., and Van Echten, G. (1994). Metabolism of Gangliosides: Topology, Pathobiochemistry, and Sphingolipid Activator Proteins. *Curr. Top. Membr.* **40:**75–91.

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

Schneider, J.S. (1994). The therapeutic role of gangliosides in neurological disorders. *CNS DRUGS* **1:**213–222.

Schneider, J.S., Roeltgen, D.P., Mancall, E.L., Chapas-Crilly, J., Rothblat, D.S., and Tatarian, G.T. (1998). Parkinson's disease: Improved function with GM1 ganglioside treatment in a randomized placebo-controlled study. *Neurology* **50:**1630–1636.

Shughrue, P.J., and Merchenthaler, I. (2000). Estrogen is more than a "sex hormone": Novel sites for estrogen action in the hippocampus and cerebral cortex. *Front. Neuroendocrinol.* **21:**95–101.

Simpkins, J.W., Green, P.S., Gridley, K.E., Singh, M., De Fiebre, N.C., and Rajakumar, G. (1997). Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease. *Am. J. Med.* **103:**19S–25S.

Shumaker, S.A., Legault, C., Raap, S.R., Thal, L., Wallace, R.B., Ockene, J.K., Hendrix, S.L., Jones, B.N. 3rd, Assaf, A.R., Jackson, R.D., Kotchen, J.M., Wassertheil-Smoller, S., Wactawski-Wende, J.; WHIMS Investigators. (2003). Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: A randomized controlled trial. *JAMA* **289:**2651–2662.

Svennerholm, L. (1957). Quantitative estimation of sialic acids a colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta.* **24:**604–611.

Svennerholm, L. (1963). Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10:**613–623.

Tang, M.X., Jacobs, D., Stern, Y., Marder, K., Schofield, P., Gurland, B., Andrews, H., and Mayeux, R. (1996). Effect of oestrogen during menopause on risk and age at onset of Alzeimer's disease. *Lancet* **348:**429–432.

Trindade, V.M.T., Daniotti, J.L., Raimondi, L., Chazan, R., Netto, C.A., and Maccioni, H.J.F. (2001). Effects of neonatal hypoxia/ischemia on ganglioside expression in the rat hippocampus. *Neurochem. Res.* **26:**591–597.

Trindade, V.M.T., Brusque, A.M., Raasch, J.R., Pettenuzzo, L.F., Rocha, H.P., Wannmacher, C.M.D., and Wajner, M. (2002). Ganglioside alterations in the central nervous system of rats chronically injected with methylmalonic and propionic acids. *Metab. Brain Dis.* **17:**93–102.

Van Duijn, C.M. (1999). Hormone replacement therapy and Alzeimer's disease. *Maturitas* **31**(3):201–205.

Villescas, R., Ostwald, R., Morimoto, H.D., and Bennett, E.L. (1981). Effects of neonatal undernutrition and cold stress on behavior and biochemical brain parameters in rats. *J. Nutr.* **111:**1103–1110.

Waynforth, H.B., and Flecknell, P.A. (1992). *Experimental and Surgical Technique in the Rat*, Academic Press, London, 2nd ed, pp. 276–278.

Wise, M.P., Dubal, D.B., Wilson, M.E., Rau, S.W., Böttner, M., and Rosewell, K.L. (2001a). Estradiol is a protective factor in the adult and aging brain: Understanding of mechanism derived from in vivo and in vitro studies. *Brain Res. Rev.* **37:**313–319.

Wise, P.M., Dubal, D.B., Wilson, M.E., Rau, S.W., and Liu, Y. (2001b). Estrogens: Trophic and protective factors in the adult brain. *Front. Neuroendocrinol* **22:**33–66.

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

Yaffe, K., Sawaya, G., Lieberburg, I., and Grady, D. (1998). Estrogen therapy in postmenopausal women: Effects on cognitive function and dementia. *JAMA* **279:**688–695.

Younkin, S.G., Goodridge, B., Katz, J., Lockett, G., Nafziger, D., Usiak, M.F., and Younkin, L.H. (1986). Molecular forms of acetylcholinesterases in Alzheimer's disease. *Fed. Proc.* **45:**2982–2988.

Yu, R.K., and Ledeen, R.W. (1974). Ganglioside abnormalities in multiple sclerosis. *J. Neurochem.* **23:**169–174.

Zeller, C.B., and Marchase, R.B. (1992). Gangliosides as modulators of cell function. *Am. J. Physiol.* **262:**C1341–C1355.

Zhang, Y.Q., Shi, J., Rajakumar, G., Day, A.L., and Simpkins, J.W. (1998). Effects of gender and estradiol treatment on focal brain ischemia. *Brain Res.* **784:**321–324.

Zheng, H., Xu, H., Uljon, S.N., Gross, R., Hardy, K., Gaynor, J., Lafrançois, J., Simpkins, J., Refolo, L.M., Petanceska, S., Wang, R., and Duff, K. (2002). Modulation of A (beta) peptides by estrogen in mouse models. *J. Neurochem.* **80:**191–196.

III.2 Artigo 2

Ovariectomy increases Na^+, K^+ -ATPase, acetylcholinesterase and catalase in rat hippocampus

Monteiro, SC, Matté, C, Delwing, D, and Wyse, ATS.

Molecular and Cellular Endocrinology 236: 9-16. 2005

Objetivos

1. Investigar o efeito da ovariectomia sobre a atividade da AChE em homogeneizado de hipocampo de ratas adultas.
2. Investigar o efeito da ovariectomia sobre a atividade da Na^+,K^+-ATPase em membrana plasmática sináptica de hipocampo de ratas adultas.
3. Avaliar o efeito da ovariectomia sobre alguns parâmetros de estresse oxidativo denominados TRAP (capacidade antioxidante total), TBA-RS (substâncias reativas ao ácido tiobarbitúrico), bem como a atividade das enzimas antioxidantes CAT (catalase), SOD (superóxido dismutase) e GSH-Px (glutationa peroxidase).

Molecular and Cellular Endocrinology 236 (2005) 9–16

MCE
Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Ovariectomy increases Na^+, K^+-ATPase, acetylcholinesterase and catalase in rat hippocampus

Siomara C. Monteiro, Cristiane Matté, Daniela Delwing, Angela T.S. Wyse *

Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 Anexo, CEP 90035-003 Porto Alegre, RS, Brazil

Received 4 November 2004; received in revised form 11 March 2005; accepted 23 March 2005

Abstract

In the present work we investigated the effect of ovariectomy on Na^+, K^+-ATPase and acetylcholinesterase (AChE) activities in rat hippocampus. We also studied some parameters of oxidative stress, namely total radical-trapping antioxidant potential (TRAP), thiobarbituric acid-reactive substances (TBA-RS), as well as the antioxidant enzyme activities superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities. Our hypothesis is that ovariectomy might cause alterations in essential enzyme activities necessary to brain normal functioning and that these chances could be caused by oxidative stress. Female adult Wistar rats were divided into three groups: (1) naïve (control); (2) sham-operated; and (3) ovariectomized. Thirty days after ovariectomy rats were sacrificed. Results showed that rats subjected to ovariectomy presented a significant increase in Na^+, K^+-ATPase, AChE and CAT activities, but did not change the oxidative stress parameters studied when compared to sham or naïve rats. Since ovariectomy mimics postmenopausal changes, our findings showing alteration in the activities of brain Na^+, K^+-ATPase, AChE and CAT may be related to problems in postmenopausal women.

Keywords: Na^+, K^+-ATPase; Acetylcholinesterase; Catalase; Hippocampus; Ovariectomy; Female rats

1. Introduction

In adult woman with normal reproductive cycles the estrogenic compounds are secreted in great quantity mainly by ovaries, being the 17β estradiol being considered the major estrogen (Rodrigues et al., 1999). Estrogen also exerts diverse non-reproductive actions on multiple organs, including the brain (Wise, 2002). It has been shown that estrogen deprivation is implicated in the pathogenesis of some neurodegenerative conditions, such as Alzheimer's disease and cerebral ischemia (Tang et al., 1996; Van Duijn, 1999; Zhang et al., 1998). In this context, there is a large body of literature suggesting that postmenopausal women are more vulnerable than young women to these diseases and cognitive deficit (Wise et al., 2001a, 2001b). On the other hand, recent results of The Women's Health Initiative Study show that hormonal replacement does not improve and may actually impair cognitive function in postmenopausal women (Shumaker et al., 2003).

Na^+, K^+-ATPase (E.C. 3.6.1.37) is a crucial enzyme responsible for the generation of membrane potential through the active transport of sodium and potassium ions. It is necessary to maintain the ionic gradient for neuronal excitability, consuming about 40–50% of the ATP generated in brain cells (Erecinska and Silver, 1994). Na^+, K^+-ATPase has been related to various aspects of neural function, such as innervation density (Swann et al., 1982), activity-dependent energy metabolism (Mata et al., 1980) and neurotransmitter release (Brosemer, 1985). Studies show that Na^+, K^+-ATPase activity is altered in cerebral ischemia (MacMillan, 1982; Wyse et al., 2000), in epilepsy (Grisar, 1984) and in Alzheimer's disease (Hattori et al., 1998). On the other hand, it has proposed that Na^+, K^+-ATPase activity could be a suitable index of neural activity for the study of neuroendocrine interactions (Del Castillo et al., 1987).

Acetylcholinesterase (AChE) (E.C. 3.1.1.7), the enzyme involved in the hydrolysis of the neurotransmitter

* Corresponding author. Tel.: +55 51 3316 5573; fax: +55 51 3316 5535.
E-mail address: wyse@ufrgs.br (A.T.S. Wyse).

0303-7207/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2005.03.006

acetylcholine, contributes to the integrity and permeability of the synaptic membrane that occurs during neurotransmission and conduction (Grafius et al., 1971). This enzyme has been implicated in cholinergic and non-cholinergic actions which may play a role in neurodegenerative diseases (Henderson et al., 1996; Cummings, 2000; Law et al., 2001). It has been also shown that AChE per se activates neuronal cell death (Calderón et al., 1998). On the other hand, it is known that estrogen withdrawal and replacement affect the cholinergic system in a variety of brain regions (Simpkins et al., 1997; Gibbs and Aggarwal, 1998). In addition, AChE is transiently increased in organotypic rat hippocampal ischemia in slice cultures (Saez-Valero et al., 2003) and we have recently demonstrated that ovariectomy increased this enzyme activity in cerebral cortex of female adult rats (Monteiro et al., in press).

Oxidative stress, a consequence of an imbalance between the formation and the removal of free radicals, is an important event that has been related to aging (Harman, 1992) and some neurodegenerative disorders, including epileptic seizures, multiple sclerosis and Alzheimer's disease (Halliwell and Gutteridge, 1985; Reznick and Packer, 1993). Protection against oxidative injury is provided by enzymatic and non-enzymatic antioxidant defenses. The brain is potentially sensitive to oxidative stress due to its great oxygen consumption, high-lipid content and poor activity of antioxidant defenses (Halliwell, 1996).

Considering that alterations in cholinergic functions, ion homeostasis and the imbalance between the formation and the removal of free radicals are important events that seem to be associated with common neurodegenerative disorders (Farooqui et al., 1988; Schneider, 1994; Bonnefont et al., 1998; Fredman, 1998; Inokuchi et al., 1998; Mesulam et al., 2002) and that postmenopausal women are vulnerable to neurological disorders. In the present study, we investigated the effect of ovariectomy on Na^+, K^+-ATPase and, AChE activities and on some parameters of oxidative stress, namely total radical-trapping antioxidant potential (TRAP), thiobarbituric acid-reactive substances (TBA-RS), as well as the antioxidant enzyme activities superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities in rat hippocampus. Our hypothesis is that ovariectomy might cause alterations in essential enzyme activities necessary to brain normal functioning and that these chances could be provoked by oxidative stress.

2. Materials and methods

2.1. Chemicals and animals

Female adult Wistar rats (3 months, 180–210 g BW) were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature ($22 \pm 1\,^{\circ}C$) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal Rio Grande do Sul, Brazil. The chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Experimental treatment

Animals were randomly assigned to one of the following groups: naïve (control), sham (only submitted to surgery without removing of ovaries) and ovariectomized. Rats were ovariectomized by surgery removing both the ovaries under anaesthesia induced by i.p. injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) to eliminate endogeneous ovarian steroids (Waynforth and Flecknell, 1992).

2.3. Tissue preparation

One month after ovariectomy, rats were killed by decapitation without anaesthesia.

The blood was rapidly collected, centrifuged at $1000 \times g$ for 10 min and the serum was separated and used for evaluating estrogen levels. The brain was quickly removed and the hippocampus was dissected.

For preparation of synaptic plasma membrane and determination of Na^+, K^+-ATPase activity, the hippocampus was homogenized in 10 volumes 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. For AChE assay the same structure was homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5 and centrifuged at 10 min at $1000 \times g$. The supernatant was used for the enzymatic AChE analyses. For TRAP assay the hippocampus was homogenized (1:10, w/v) in 0.1 M glycine buffer, pH 8.6, centrifuged at $750 \times g$ for 10 min and the supernatant was used to analyze. For TBA-RS assay, hipocampus was homogenized in (1:10, w/v) in 1.15% KCl. For CAT and GSH-Px assays the hippocampus was homogenized (1:10, w/v) in 10 mM potassium phosphate buffer, pH 7.6 and for SOD activity the same structure was homogenized in (1:10, w/v) 50 mM Tris–HCl containing 1 mM EDTA, pH 8.2.

2.4. Estradiol measurement and control of estrous cycle

The stage of the estrous cycle was determined by vaginal swab in naïve and sham females. The observed phases were: diestrus, when mucus, leukocytes and some nucleated cells were present (2–3 days on average); proestrus, when only nucleated cells were present (12 h); estrus, when only cornified cells were observed (24 h, the rut phase), and metaestrus, when leukocytes, cornified cells and some nucleated cells were present (Baker et al., 1979). All naïve and sham females were in the proestrus phase at the time of decapitation.

Table 1
Serum 17β-estradiol levels in different groups of female adult Wistar rats evaluated 30 days after surgery by radioimmunoassay (Adaltis Estradiol MAIA Kit)

Groups	17β-Estradiol (pg/ml)
Naïve	10.11 ± 1.42
Sham	11.67 ± 3.27
Ovariectomized	ND***

Data are reported as mean (S.E.M. for eight animals in each group.
*** $p < 0.001$ compared to naïve and sham groups (Duncan's multiple range test). ND: not detected.

Estradiol levels were evaluated in serum by radioimmunoassay using a Biomedical kit (Adaltis Estradiol Maia Kit, Italy). Estrogen levels in the ovariectomized female group were undetectable, confirming the efficacy of the surgical procedures of ovariectomy (Table 1).

2.5. Preparation of synaptic plasma membrane from hippocampus

Synaptic plasma membranes from hippocampus were prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al., 1995). The homogenate was centrifuged at 1000 × *g* for 20 min and the supernatant removed and centrifuged at 12,000 × *g* for further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1) at 0 °C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 × *g* for 2 h, the fraction between 0.8 and 1.0 mM sucrose interface was taken as the membrane enzyme preparation.

2.6. Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+, K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in final volume of 200 μl. The reaction was initiated by addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assay, according to the method of Tsakiris and Deliconstantinos (1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released/min/mg of protein.

2.7. AChE activity assay

Acetylcholinesterase activity was determined according to Ellman et al. (1961), with some modifications (Villescas et al., 1981). Hydrolysis rates v were measured at acetylthiocholine (S) concentrations of 0.8 mM in 1 ml of assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25 °C. Fifty microlitres of rat hippocampus supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s).

2.8. TRAP assay

TRAP, representing the total non-enzymatic antioxidant capacity of the tissue, was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azobis (2-amidinopropane) (ABAP) (Evelson et al., 2001) at room temperature. The 0.1 M glycine buffer, pH 8.6 was also used to prepare the other solutions. Four millilitres of 10 mM ABAP was added into a glass scintillation vial and the background chemiluminescence was measured. Ten microlitres of 4 mM luminol was then added and the chemiluminescence was measured. This was considered to be the initial value. Ten microlitres of 80 μM trolox (water-soluble α-tocopherol) or tissue supernatant was added and chemiluminescence was measured until it reached the initial levels. The addition of trolox or tissue supernatant to the incubation medium reduces the chemiluminescence. The time necessary for the chemiluminescence intensity to return to the initial value is considered to be the induction time. The induction time is directly proportional to the antioxidant capacity of the tissue and was compared to the induction time of trolox. The results are reported as nmol of trolox per mg protein.

2.9. TBA-RS assay

TBA-RS was determined according to the method described by Ohkawa et al. (1979). Briefly, 50 μl of 8.1% SDS (sodium dodecyl sulfate), 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 and 1.5 ml of 0.8% aqueous solution of TBA were added to 250 μl of tissue homogenate in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifugated at 1000 × *g* for 10 min. The organic layer was taken and the resulting pink stained TBA-RS was determined in a spectrophotometer at 535 nm. The acid did not produce color when tested without the addition of the supernatant, demonstrating the absence of a direct reaction to thiobarbituric acid. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve points were subjected to the same treatment as that of the supernatants. The results are reported as nmol of TBA-RS per mg protein.

2.10. CAT activity assay

CAT activity was assayed by the method of Aebi (1984), which is based on the disappearance of H_2O_2 at 240 nm. One unit of the enzyme is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity is reported as units per mg protein.

2.11. GSH-Px assay

GSH-Px activity was measured by the method of Wendel (1981), except for the concentration of NADPH, which was adjusted to 0.1 mM after previous tests performed in our laboratory. *tert*-Butyl-hydroperoxide was used as substrate. NADPH disappearance was monitored with a spectrophotometer at 340 nm. One GSH-Px unit is defined as 1 μmol of NADPH consumed per minute and specific activity is represented as units per mg protein.

2.12. SOD assay

SOD activity was measured by the method of Marklund (1985). This method is based on the autoxidation of pyrogallol, which is highly dependent on $O_2^{\bullet -}$. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation and the specific activity is reported as units per mg protein.

2.13. Protein determination

Protein was measured by the method of Lowry et al. (1951) or Bradford (1976) using bovine serum albumin as standard.

2.14. Statistical analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one-way ANOVA followed by the Duncan multiple test when *F*-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Values of $p < 0.05$ were considered to be significant.

3. Results

3.1. Effect of ovariectomy on Na^+, K^+-ATPase and AChE activities from hippocampus of female Wistar rats

Fig. 1A and B shows that the ovariectomy significantly increases by approximately 36% the activities of Na^+, K^+-ATPase (A) [$F(2, 12) = 4.657$; $^*p < 0.05$] and AChE (B) [$F(2, 12) = 4.974$; $^*p < 0.05$] in hippocampus of female adult Wistar rats, when compared to control (naïve) and submitted to surgery (sham).

3.2. Effect of ovariectomy on TRAP and TBA-RS from hippocampus of female Wistar rats

Next, we investigated the effect of ovariectomy on some parameters of oxidative stress, namely TRAP (an index of total antioxidant capacity of the tissue) and TBA-RS (an index of lipid peroxidation) in hippocampus of female adult Wistar rats. As can be observed in Fig. 2, the ovariectomy did not alter significantly TRAP (A) [$F(2, 9) = 0.049$; $p > 0.05$] and TBA-RS (B) [$F(2, 12) = 0.067$; $p > 0.05$], which suggests that ovariectomy did not induce alterations in non-enzymatic antioxidant capacity and in lipid peroxidation in this cerebral structure.

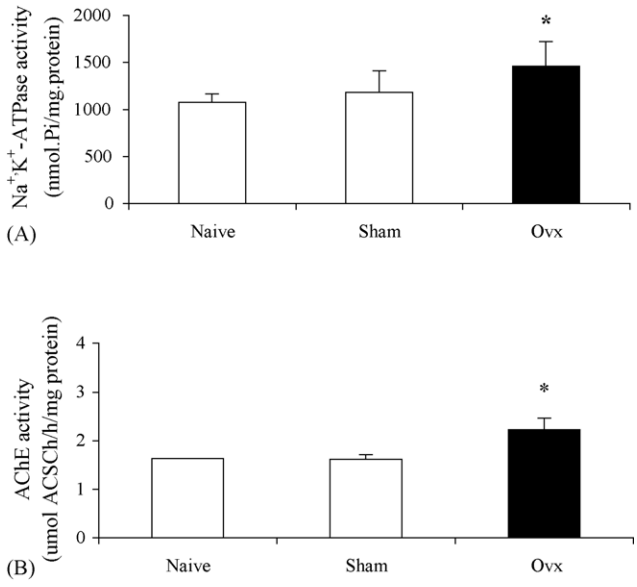


Fig. 1. Effect of ovariectomy on the activities of Na^+, K^+-ATPase (A) and acetylcholinesterase (B) from hippocampus of female adult rats. Data are expressed as mean ± S.E.M. for five independent experiments (animals) performed in duplicate. $^*p < 0.05$ compared to naïve and sham groups (Duncan's multiple range test). AChE: acetylcholinesterase; Ovx: ovariectomized.

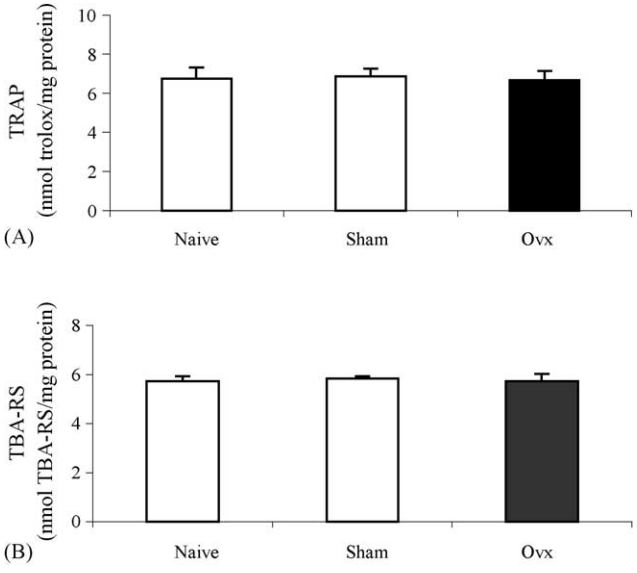


Fig. 2. Effect of ovariectomy on TRAP (A) and TBA-RS (B) from hippocampus of female adult rats. Data are expressed ±S.E.M. for four to five independent experiments (animals) performed in duplicate. $p > 0.05$ (Duncan's multiple range test). Ovx: ovariectomized.

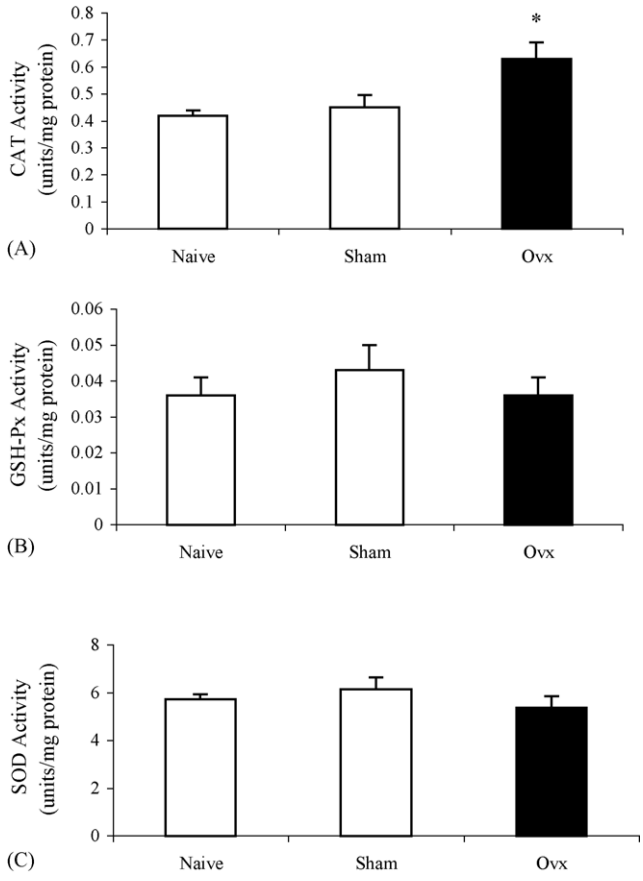


Fig. 3. Effect of ovariectomy on catalase (A), glutathione peroxidase (B) and superoxide dismutase (C) activities in hippocampus of female adult rats. Data are expressed ±S.E.M. for five independent experiments (animals) performed in duplicate. Different from naïve and sham groups $^{*}p < 0.05$ (Duncan's multiple range test). One catalase unit is defined as 1 μmol of H_2O_2 consumed per minute. One glutathione peroxidase unit is defined as 1 μmol of NADPH consumed per minute. One superoxide dismutase unit is defined as 50% inhibition of pyrogallol autoxidation. Ovx: ovariectomized; CAT: catalase; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase.

3.3. Effect of ovariectomy on CAT, GSH-Px and SOD in hippocampus of female Wistar rats

We also verified the effect of ovariectomy on hippocampal enzymatic antioxidant defenses. Post hoc analysis showed that female adult rats subjected to ovariectomy presented a significant increase (47%) in CAT activity (A) [$F(2, 12) = 5.50$; $p < 0.05$] and did not alter the activities of GSH-Px (B) [$F(2, 12) = 0.40$; $p > 0.05$] and SOD (C) $F(2, 12) = 0.83$; $p > 0.05$], when compared to the naïve and sham groups (Fig. 3).

4. Discussion

Estrogen has been described to play an important role in cognitive functions and neuroprotection (Kampen and Sherwin, 1994; Brinton, 2001; Gandy, 2003). It has been shown that estrogen deprivation is implicated in the pathogenesis of neurodegenerative disorders, including stroke (Liao et al., 2001) and Alzheimer's disease (Fillit, 1994). A growing number of studies indicate the brain as one of the body organs that suffers from the loss of estrogen in menopause and that damage from stroke and neurodegeneration in dementia may be retarded by estrogenic actions (McEwen, 2002). In this context, evidence also shows that postmenopausal estrogen replacement therapy reduces the risk and delays the onset of these diseases (Tang et al., 1996; Yaffe et al., 1998; Van Duijn, 1999). In contrast, recent data from the literature showed that estrogen plus progestin therapy to postmenopausal women increased the risk for dementia in women aged 65 years or older and did not improve cognitive impairment in these women (Shumaker et al., 2003).

In the present study, we investigated the effect of ovariectomy on activities of Na^+, K^+-ATPase, AChE and on some parameters of oxidative stress (TRAP, TBA-RS and antioxidant enzymes) in hippocampus of female adult rats. We used this animal model of steroid hormone deprivation because ovariectomy is considered the most common animal model of postmenopausal changes in adult female rats (Savonenko and Markowska, 2003). The hippocampus was used because this cerebral structure is vulnerable to brain damage, is related to memory/learning mechanisms and it was been previously shown that ovariectomy provokes memory impairment (Daniel and Dohanich, 2001; Tanabe et al., 2004; Feng et al., 2004). On the other hand, it has been shown that there are nuclear estrogen receptors present in inhibitory hippocampal interneurons (Weiland et al., 1997) and that physiological levels of estradiol promote formation of functional dendritic spines and stimulates synaptogenesis in hippocampal regions (Woolley et al., 1997).

Results showed that rats subjected to ovariectomy presented a significant increase (36%) in Na^+, K^+-ATPase activity from synaptic plasma membranes of rat hippocampus. The mechanisms for stimulation of Na^+, K^+-ATPase are not known. However, the activity of Na^+, K^+-ATPase can be modulated by several mechanisms. Diverse signal transduction pathways, leading to the formation of different mediators and the activation of a variety of kinases, regulate Na^+, K^+-ATPase activity (Bertorello and Katz, 1995). In this context, it has been shown that protein kinase C increases this enzyme activity (Xie and Cai, 2003). In addition, Na^+, K^+-ATPase activity can also be modulated by changes in the intracellular sodium concentration (Inoue and Matsui, 1991). Another possibility could be the increases in synthesis or decreases in degradation of Na^+, K^+-ATPase.

Our results also showed that rats subjected to ovariectomy presented a significant increase (35%) in AChE activity. Increased AChE activity by decreasing ACh concentrations reduces cholinergic activity in the central nervous system. Considering that hippocampus is a cholinergic area, our findings showing that AChE activity is increased in this cerebral structure of ovariectomized rats is of interest, since the stimulation of this enzyme could cause a decrease in

ACh in synaptic cleft and consequently decrease cholinergic activity. These data are in agreement with our recent study showing that AChE is increased in cerebral cortex of ovariectomized rats (Monteiro et al., in press). So far we do not know the exact underlying mechanism through which AChE activity is increased in our study.

Steroids hormones, especially estriol and estradiol, are natural antioxidants (Mooradian, 1993). There is a study providing evidence that all female brain areas increased ascorbate loss after gonadectomy, indicating enhanced oxidative stress (Kume-kick et al., 1996). Incubation of primary neuronal cultures with 17β-estradiol showed an increased survival of cells reducing lipid peroxidation (Vedder et al., 1999). These affirmations provide evidence for the hypothesis that protection against oxidative damage is afforded by ovarian sex hormones. Based on these findings and considering that oxidative stress is an important event that has been related to the pathogenesis of some conditions affecting the central nervous system, such as ischemia and neurodegenerative disorders (Reznick and Packer, 1993; Halliwell and Gutteridge, 1985), we also examined the effect of ovariectomy on TRAP and TBA-RS in hippocampus. Results showed that ovariectomy did not alter these parameters.

In order to evaluate the effect of ovariectomy on the antioxidant enzymatic defenses, we tested the effect of ovariectomy on CAT, GSH-Px and SOD activities, which are considered to be the main enzymatic antioxidant defenses in the brain against free radical production. Our results showed that ovariectomy did not alter GSH-Px and SOD activities, but significantly increased CAT activity. Considering that the antioxidant enzymes can respond to sustained oxidative stress by a compensatory increase in their activities (Travacio and Llesuy, 1996), our result of increased (47%) CAT activity due to ovariectomy could be a consequent of an enzymatic adaptation to enhanced free radical formation. Our results are in accordance with Gomez-Zubeldia et al. (2001) that observed no variations in malondialdehyde levels with a slight increase in CAT activity in erythrocytes of ovariectomized rats. These results could be explained why ovariectomy did not alter TBA-RS, an index of lipid peroxidation.

In summary, in the present study we demonstrate that ovariectomy increases significantly the activities of Na^+, K^+-ATPase, AChE and CAT in hippocampus of adult rats. Therefore, we presume that increased Na^+, K^+-ATPase and AChE activities could cause hyperpolarization of synaptic membrane and decrease in acetylcholine, decreasing cholinergic activity. Increased CAT could be a consequence of oxidative stress induced by ovariectomy. Since ovariectomy mimics postmenopausal changes, our findings showing alteration in the activities of brain Na^+, K^+-ATPase, AChE and CAT may be related to problems in postmenopausal women.

At this point, we cannot establish if the increase in Na^+, K^+-ATPase, AChE and CAT activities following ovariectomy would be a consequence of estrogen deprivation alone, since ovaries produce other substances such as progestin and inibin. Therefore, further studies will be necessary to evaluate the mechanism of these alterations.

Acknowledgements

This work was supported in part by grants from CNPq, Brazil and Programa de Núcleos de Excelência-Financiadora de Estudos e Projetos (PRONEX II, FINEP-Brazil).

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

Baker, H.J., Lindsey, J.R., Weisbroth, S.H., 1979. The laboratory rat. Biology and Diseases, vol. 1. Academic Press, New York, NY, USA.

Bertorello, A.M., Katz, A.I., 1995. Regulation of Na^+–K^+ pump activity: pathways between receptors and effectors. News Physiol. Sci. 10, 253–259.

Bonnefont, A.B., Munoz, F.J., Inestrosa, N.C., 1998. Estrogen protects neuronal cells from the cytotoxicity induced by acetylcholinesterase–amyloid complexes. FEBS Lett. 441 (2), 220–224.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of micrograms quantities of protein utilizing the principle of protein-die-binding. Anal. Biochem. 72, 248–254.

Brinton, R.D., 2001. Cellular and molecular mechanisms of estrogen regulation of memory function and neuroprotection against Alzheimer's disease: recent insights and remaining challenges. Learn. Mem. 8, 121–133.

Brosemer, R.W., 1985. Effects of inhibitors of Na^+, K^+-ATPase on the membrane potentials and neurotransmitter efflux in rat brain slices. Brain Res. 334, 125–137.

Calderón, F.H., Von Bernhardi, R., De Ferrari, G., Luza, S., Aldunate, R., Inestrosa, N.C., 1998. Toxic effects of acetylcholinesterase on neuronal and glial-like cells in vitro. Mol. Psychiatry 3, 247–255.

Chan, K.M., Delfer, D., Junger, K.D., 1986. A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal. Biochem. 157, 375–380.

Cummings, J.L., 2000. The role of cholinergic agents in the management of behavioral disturbances in Alzheimer's disease. Int. J. Neuropsychopharmacol. 3, 21–29.

Daniel, J.M., Dohanich, G.P., 2001. Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. J. Neurosci. 21, 6949–6956.

Del Castillo, A.R., Battaner, E., Guerra, M., Alonso, T., Mas, M., 1987. Regional changes of brain Na^+, K^+-transporting adenosine triphosphatase related to ovarian function. Brain Res. 416, 113–118.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 21–29.

Erecinska, M., Silver, I.A., 1994. Ions and energy in mammalian brain. Neurobiology 43, 37–71.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E.A., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem. Biophys. 388, 261–266.

Farooqui, A.A., Liss, L., Horrocks, L.A., 1988. Neurochemical aspects of Alzheimer's disease: involvement of membrane phospholipids. Metab. Brain Dis. 3 (1), 19–35.

Feng, Z., Cheng, Y., Zhang, J.T., 2004. Long-term effects of melatonin or 17 beta-estradiol on improving spatial memory performance in cognitively impaired, ovariectomized adult rats. J. Pineal. Res. 37 (3), 198–206.

Fillit, H., 1994. Estrogens in pathogenesis and treatment of Alzeimer's disease in postmenopausal women. Ann. N. Y. Acad. Sci. 743, 233–239.

Fredman, P., 1998. Sphingolipids and cell signalling. J. Inherit. Metab. Dis. 21, 472–480.

Gandy, S., 2003. Estrogen and neurodegeneration. Neurochem. Res. 28, 1003–1008.

Gibbs, R.B., Aggarwal, P., 1998. Estrogen and basal forebrain cholinergic neurons: implications for brain aging and Alzheimer's disease-related cognitive decline. Horm. Behav. 34, 98–111.

Gomez-Zubeldia, M.A., Arbues, J.J., Hinchado, G., Nogales, A.G., Millan, J.C., 2001. Influence of estrogen replacement therapy on plasma lipid peroxidation. Menopause 8, 274–280.

Grafius, M.A., Bond, H.E., Millar, D.B., 1971. Acetylcholinesterase interaction with a lipoprotein matrix. Eur. J. Biochem. 22, 382–390.

Grisar, T., 1984. Glial and neuronal Na^+–K^+ pump in epilepsy. Ann. Neurol. 16, 128–134.

Halliwell, B., Gutteridge, J.M.C., 1985. Oxygen radicals and nervous system. Trends Neurosci. 8, 22–26.

Halliwell, B., 1996. Free radicals, protein and DNA: oxidative damage versus redox regulation. Biochem. Soc. Trans. 24, 1023–1027.

Harman, D., 1992. Free radical theory of aging. Mutat. Res. 275, 257–266.

Hattori, N., Kitagawa, K., Higashida, T., Yagyu, K., Shimohama, S., Wataya, T., Perry, G., Smith, M.A., Inagaki, C., 1998. Cl^--ATPase and Na^+/K^+-ATPase activities in Alzheimer's disease brains. Neurosci. Lett. 254, 141–144.

Henderson, V.W., Watt, L., Buckwalter, J.G., 1996. Cognitive skills associated with estrogen replacement in women with Alzeimer's disease. Psychoneuroendocrinology 21, 421–430.

Inokuchi, J., Kuroda, Y., Kosaka, S., Fujiwara, M., 1998. L-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol stimulates ganglioside biosynthesis, neurite outgrowth and synapse formation in cultured cortical neurons, and ameliorates memory deficits in ischemic rats. Acta Biochim. Pol. 45, 479–492.

Inoue, N., Matsui, H., 1991. Changes in responses of Na pump isoforms to glutamate excitation of cerebral neurons during maturation in culture. Soc. Gen. Physiol. Ser. 46, 597–600.

Jones, D.H., Matus, A.I., 1974. Isolation of plasma synaptic membrane from brain by combination flotation-sedimentation density gradient centrifugation. Biochim. Biophys. Acta. 356, 276–287.

Kampen, D.L., Sherwin, B.B., 1994. Estrogen use and verbal memory in healthy postmenopausal. Obstet. Gynecol. 83, 979–983.

Kume-kick, J., Ferris, D.C., Russo-Menna, I., Rice, M.E., 1996. Enhanced oxidative stress in female rat brain after gonadectomy. Brain Res. 738, 8–14.

Law, A., Gauthier, S., Quirion, R., 2001. Say NO Alzheimer's disease: the putative links between nitric oxide and dementia of the Alzheimer's type. Brain Res. Rev. 35, 73–96.

Liao, S.L., Chen, W.Y., Kuo, J.S., Chen, C.J., 2001. Association of serum estrogen level and isquemic neuroprotection in female rats. Neurosci. Lett. 297, 159–162.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–267.

MacMillan, V., 1982. Cerebral Na^+/K^+-ATPase activity during exposure to and recovery from acute ischemia. J. Cereb. Blood Flow Metab. 2, 457–465.

Marklund, S., 1985. Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, pp. 243–247.

Mata, M., Fink, D.J., Gainer, H., Smith, C.B., Davidsen, L., Savakis, H., Schwartz, W., Sokoloff, L., 1980. Activity dependent energy metabolism rat posterior pituitary primarily reflects sodium pump activity. J. Neurochem. 34, 213–215.

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

Mesulam, M.M., Guillozet, A., Shaw, P., Levey, A., Duysen, E.G., Lockridge, O., 2002. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience 110, 627–639.

Monteiro, S.C., Stefanello, F.M., Vianna, L.P., Matté, C., Barp, J., Bellló-Klein, A., Trindade, V.M.T., Wyse, A.T.S., in press. Ovariectomy enhances acetylcholinesterase activity but does not alter ganglioside content in cerebral cortex of female adult rats. Metabolic Brain Disease.

Mooradian, A.D., 1993. Antioxidant properties of steroids. J. Steroid Biochem. Mol. Biol. 45, 509–511.

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

Reznick, A.Z., Packer, L., 1993. Free radicals and antioxidants in muscular neurological diseases and disorders. In: Poli, G., Albano, E., Dianzani, M.U. (Eds.), Free Radicals: From Basic Science to Medicine. Birkhäuser Verlag, Basel, pp. 425–437.

Rodrigues, H.D., Kinder, J.E., Fitzpatrick, L.A., 1999. Treatment with 17 beta-estradiol does not influence age and weight at puberty in Bos indicus heifers. Anim. Reprod. Sci. 56, 1–10.

Saez-Valero, J., Gonzales-Garcia, C., Cena, V., 2003. Acetylcholinesterase activation in organotypic rat hippocampal slice cultures deprived of oxygen and glucose. Neurosci. Lett. 348 (2), 123–125.

Savonenko, A.V., Markowska, A.L., 2003. The cognitive effects of ovariectomy and estrogen replacement are modulated by aging. Neurosci. 119, 821–830.

Schneider, J.S., 1994. The therapeutic role of gangliosides in neurological disorders. CNS DRUGS 1, 213–222.

Shumaker, S.A., Legault, C., Raap, S.R., Thal, L., Wallace, R.B., Ockene, J.K., Hendrix, S.L., Jones III, B.N., Assaf, A.R., Jackson, R.D., Kotchen, J.M., Wassertheil-Smoller, S., Wactawski-Wende, J., WHIMS Investigators, 2003. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the women's health initiative memory study: a randomized controlled trial. JAMA 289, 2651–2662.

Simpkins, J.W., Rajakumar, G., Zhang, Y.Q., Simpkins, C.E., Greenwald, D., Yu, C.J., Bodor, N., Day, A.L., 1997. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rats. J. Neurosurg. 87, 724–730.

Swann, A.C., Grant, S.J., Maas, J.W., 1982. Brain (Na^+, K^+)-ATPase and noradrenergic activity: effects of hyperinnervation and denervation on high-affinity ouabain binding. J. Neurochem. 38 (3), 836–839.

Tanabe, F., Miyasaka, N., Kubota, T., Aso, T., 2004. Estrogen and progesterone improve scopolamine-induced impairment of spatial memory. J. Med. Dent. Sci. 51 (1), 89–98.

Tang, M.X., Jacobs, D., Stern, Y., Marder, K., Schofield, P., Gurland, B., Andrews, H., Mayeux, R., 1996. Effect of oestrogen during menopause on risk and age at onset of Alzeimer's disease. Lancet 348, 429–432.

Travacio, M., Llesuy, S., 1996. Antioxidant enzymes and their modification under oxidative stress conditions. Free Radic. Res. Latin Am. 48, 9–13.

Tsakiris, S., Deliconstantinos, G., 1984. Influence of phosphatidilserine on ($Na^+ + K^+$)-stimulated ATPase and acetylcholinesterase activities of dog brain synaptosomal plasma membranes. Biochem. J. 22, 301–307.

Van Duijn, C.M., 1999. Hormone replacement therapy and Alzeimer's disease. Maturitas 31 (3), 201–205.

Vedder, H., Anthes, N., Stumm, G., Würz, C., Behl, C., Krieg, J.C., 1999. Estrogen hormones reduce lipid peroxidation in cells and tissue of the central nervous system. J. Neurochem. 72, 2531–2538.

Villescas, R., Ostwald, R., Morimoto, H.D., Bennett, E.L., 1981. Effects of neonatal undernutrition and cold stress on behavior and biochemical brain parameters in rats. J. Nutr. 111, 1103–1110.

Waynforth, H.B., Flecknell, P.A., 1992. Experimental and Surgical Technique in the Rat, second ed. Academic Press, London, pp. 276–278.

Weiland, N.G., Orikasa, C.M., Hayashi, S., McEwen, B.S., 1997. Distribution and hormone regulation of estrogen receptor immunoreactive

cells in the hippocampus of male and female rats. J. Comp. Neurol. 388, 603–612.

Wendel, A., 1981. Glutathione peroxidase. Methods Enzymol. 77, 325–332.

Wise, P.M., Dubal, D.B., Wilson, M.E., Rau, S.W., Liu, Y., 2001a. Estrogens: trophic and protective factors in the adult brain. Front. Neuroendocrinol. 22, 33–66.

Wise, M.P., Dubal, D.B., Wilson, M.E., Rau, S.W., Böttner, M., Rosewell, K.L., 2001b. Estradiol is a protective factor in the adult and aging brain: understanding of mechanism derived from in vivo and in vitro studies. Brain Res. Rev. 37, 313–319.

Wise, P.M., 2002. Estrogens and neuroprotection. Trends Endocrinol. Metab. 13, 229–230.

Woolley, C.S., Weiland, N.G., McEwen, B.S., Schwartzkroin, P.A., 1997. Estradiol increases the sensivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input Correlation with dendritic spine density. J. Neurosci. 17, 1848–1859.

Wyse, A.T.S., Bolognesi, G., Brusque, A.M., Wajner, M., Wannmaker, C.M.D., 1995. Na^+, K^+-ATPase activity in the synaptic plasma membrane from the central cerebral cortex of rats subjected to chemically induced phenylketonuria. Med. Sci. Res. 23, 261–262.

Wyse, A.T.S., Streck, E.L., Worm, P., Wajner, A., Ritter, F., Netto, C.A., 2000. Preconditioning prevents the inhibition of Na^+, K^+-ATPase activity after brain ischemia. Neurochem. Res. 25, 969–973.

Xie, Z., Cai, T., 2003. Na^+, K^+-ATPase-mediated signal transduction: from protein interaction to cellular function. Mol. Interv. 3 (3), 157–168.

Yaffe, K., Sawaya, G., Lieberburg, I., Grady, D., 1998. Estrogen therapy in postmenopausal women: effects on cognitive function and dementia. JAMA 279, 688–695.

Zhang, Y.Q., Shi, J., Rajakumar, G., Day, A.L., Simpkins, J.W., 1998. Effects of gender and estradiol treatment on focal brain ischemia. Brain Res. 784, 321–324.

III.3 Artigo 3

Supplementation with vitamins E plus C or soy isoflavones in ovariectomized rats: effect on the activities of Na+,K+-ATPase and cholinesterases

Monteiro, SC, Mattos, CB, Scherer, E.B.S., and Wyse, ATS.

Periódico: Metabolic Brain Disease.

Status: Aceito para publicação.

Objetivos

1. Investigar a influência do tratamento crônico com as vitaminas E e C ou com a dieta de soja rica em isoflavonas sobre a estimulação da atividade da Na^+,K^+-ATPase em membrana plasmática sináptica de hipocampo de ratas adultas.
2. Avaliar a influência do tratamento crônico com as vitaminas E e C ou com a dieta de soja rica em isoflavonas sobre a estimulação da atividade da AChE em hipocampo de ratas adultas.
3. Determinar as ações das vitaminas e das isoflavonas da soja sobre a redução da atividade da BuChE em soro de ratas ovariectomizadas.

Dear Dr. Wyse,

Thank you for submitting the revised version of manuscripts 02-093 and 02-095. Please be informed that both manuscripts have been accepted and they will be published in due course.

Happy new year to you as well!

Vilmary

From: "Angela Wyse" <00099024@ufrgs.br>
Reply-To: "Angela Wyse" <wyse@ufrgs.br>
Date: Mon, 8 Jan 2007 14:02:56 -0200
To: "Vilmary Friederichs" <vilmary.friederichs@rosalindfranklin.edu>
Subject: Re: Metabolic Brain Disease MS#02-093 and MS#02-095

Dear Dr. Vilmary,

Dear Friedrichs,

I would you appreciate very much to receive information about our manuscripts ((**MBD MS#02-093 and MBD 02-095),** which were modified according to referees sugestions.

Happy New Year for you!
Sincerely,
Dr. Angela Wyse

----- Original Message -----
From: Vilmary Friederichs
<mailto:vilmary.friederichs@rosalindfranklin.edu>

To: Angela Wyse <mailto:wyse@ufrgs.br>
Sent: Monday, December 11, 2006 4:02 PM
Subject: Metabolic Brain Disease MS#02-093 and MS#02-095
December 11, 2006
Angela T.S. Wyse
Departamento de Bioquímica
ICBS, Universidade Federal do Rio Grande do Sul
Rua Ramiro Barcelos, 2600 Anexo
CEP 90035-003
Porto Alegre, RS, Brasil

FAX # 55 51 333165535

RE: Manuscripts #02-093 and #02-095

Dear Dr. Wyse:

The manuscripts entitled, **"Supplementation with vitamins E plus C or soy isoflavones in ovariectomyzed rats: Effect on the activities of Na+, K+ - ATPase and cholinesterase"** and *"***Effect of hypermethioninemia on some parameters of oxidative stress and on Na+,K+-ATPase activity in hippocampus of rats"**, have been reviewed.

Therefore, the manuscripts are attached for your review.

Please forward the review manuscripts via email along with the copyright transfer forms.

Thank you for sending this interesting paper to our journal.

Sincerely,
David W. McCandless, Ph.D.
Editor-in-Chief
Metabolic Brain Disease

PHONE: (847) 578-3210
FAX: (847) 578-3253

Email: david.mccandless@rosalindfranklin.edu

Vilmary Friederichs
Administrative Assistant
Department of Cell Biology and Anatomy
Rosalind Franklin University of Medicine and Science
☎ (v) 847-578-3211
☎ (f) 847-578-3253
💻 vilmary.friederichs@rosalindfranklin.edu

SUPPLEMENTATION WITH VITAMINS E PLUS C OR SOY ISOFLAVONES IN OVARIECTOMIZED RATS: EFFECT ON THE ACTIVITIES OF Na^+,K^+-ATPase AND CHOLINESTERASES

Siomara C. Monteiro, Cristiane B. Mattos, Emilene B. S. Scherer and Angela T. S. Wyse.

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

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

Abstract

Since a previous study demonstrated that ovariectomized rats present an activation of Na^+,K^+-ATPase and acetylcholinesterase (AChE) activities, in the present study we investigated the influence of vitamins E plus C or soy isoflavones on the effects elicited by ovariectomy on the activities of these enzyme in hippocampus of ovariectomized rats. We also determined the effect of the same compounds on the reduction of serum butyrylcholinesterase (BuChE) activity caused by ovariectomy. Female adult Wistar rats were assigned to one of the following groups: sham (submitted to surgery without removal of the ovaries) and ovariectomized. Seven days after surgery, animals were treated for 30 days with a single daily intraperitoneous injection of vitamins E (40 mg/Kg) plus C (100 mg/Kg) or saline (control). In another set of experiments, the rats were fed for 30 days on a special diet with soy protein or a standard diet with casein (control). Rats were sacrificed after treatments and the hippocampus was dissected and serum was separated. Data demonstrate that vitamins E plus C reversed the activation of Na^+,K^+-ATPase and AChE in hippocampus of ovariectomized rats. Conversely, soy protein supplementation reversed the increase of AChE activity, but not of Na^+,K^+-ATPase activity, caused by ovariectomized group. Neither treatment was able to reverse the reduction of serum BuChE activity. Furthermore, treatments with vitamins E plus C or soy were unable to reverse the decrease in estradiol levels caused by ovariectomy. Our findings show that the treatment with vitamins E plus C significantly reversed the effect of ovariectomy on hippocampal Na^+,K^+-ATPase

and AChE activities. However, a soy diet that was rich in isoflavones was able to reverse just the increase of AChE. Neither treatment altered the reduction in serum BuChE activity. Taken together, these vitamins and soy may have a protective role against possible brain dysfunction observed in some menopause women. Vitamins E plus C and soy isoflavones may be a good alternative as a novel therapeutic strategy.

Key words Ovariectomy – Vitamins E plus C – Soy Isoflavones – Na^+,K^+-ATPase – Acetylcholinesterase – Butyrylcholinesterase.

Introduction

Adult women with normal reproductive cycles secrete a great quantity of estrogenic compounds, mainly from the ovaries, with 17β estradiol being the dominant form of estrogen in the body (Rodrigues *et al*., 1999). Estrogen also exerts diverse non-reproductive actions on multiple organs, including the brain (Wise, 2002), and it has been shown that estrogen deprivation is implicated in the pathogenesis of neurodegenerative conditions, such as Alzheimer´s disease and cerebral ischemia (Tang *et al*., 1996, Van Duijn, 1999, Zhang *et al*., 1998). Consistently, there is a large body of literature to suggest that post-menopausal women are more vulnerable than younger women to such diseases and to cognitive deficits (Green and Simpkins, 2000, Wise *et al*., 2001a, Wise *et al*., 2001b).

Hormone replacement therapy (HRT), in the form of estrogen and progesterone or estrogen alone, has been used to treat menopause symptoms and other similar conditions. However, due to the possible side effects of HRT, such as breast cancer and increased risk of thromboembolic accidents, there is a growing demand for alternatives for the treatment of pathological processes and symptoms associated with menopause (Miquel *et al*., 2006). The *Women's Health Initiative Study* (WHI, 2002) showed an increase in cardiovascular disease and breast cancer in women treated with equine estrogens and medroxyprogesterone acetate. In order to protect against injurious effects, nutritional supplements have been studied to substitute HRT. In this context, there is evidence showing that antioxidants, such as vitamins E plus C, and

phytoestrogens like isoflavones, could be a good alternative to substitute the synthetic estrogens (Miquel *et al*., 2006).

Vitamin E (α-tocopherol) has been considered a lipophilic antioxidant in humans and it is important for a normal brain function (Vatassery, 1998). Vitamin C (ascorbate), soluble in the aqueous phase, plays an important role for regenerating the vitamin E back to the reduced tocopherol. Evidences shows that α-tocopherol have properties antioxidant (McCay, 1985, Carr and Frei, 1999) and non-antioxidant such as anti-inflammatory actions (Upritchard *et al*., 2000). It has been suggested that age-related estrogen loss results in the deficit of the antioxidant protection (Arteaga *et al*., 1998). In this context, recent results from our group have shown that the impairment of spatial memory, caused by ovariectomy, in female adult rats was prevented by treatment with vitamins E plus C (Monteiro *et al*., 2005a).

Isoflavones are compounds with estrogenic activity (phytoestrogens) found almost exclusively in soybeans and in a few other legumes. The principal isoflavones are genistein, daidzein and glycitein (Sirtori *et al*., 2005). Studies show that the phytoestrogens interact with the estrogen receptors ERα and ERβ due to their structural similarity to β estradiol (Fig. 1). These substances act as antioxidants, directly or indirectly, by enhancing the enzyme activities of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase (Kurzer and Xu, 1997). Some studies have shown that soy isoflavones can improve cognitive function in humans and rats (File *et al*., 2001, Pan *et al*., 2000). Moreover, these substances are considered to be a good alternative to hormone dependent diseases and those related to aging (Nakamura *et al*., 2000).

Na^+,K^+-ATPase (E.C 3.6.1.37) is a crucial enzyme, responsible for the generation of membrane potential through the active transport of sodium and potassium ions. This enzyme is necessary to maintain the ionic gradient for neuronal excitability, consuming about 40-50% of the ATP generated in brain cells (Erecinska and Silver, 1994), and has been related to neural functions such as innervation density (Swann *et al*., 1982), activity-dependent energy metabolism (Mata *et al*., 1980) and neurotransmitter release (Brosemer, 1985). Na^+,K^+-ATPase activity is inhibited by free radicals and it is reduced in cerebral ischemia (Wyse *et al*., 2000) and in neurodegenerative diseases as Alzheimer's disease (Lees, 1993). It has also been proposed that Na^+,K^+-ATPase activity could be a suitable index of neural activity for the study of neuroendocrine interactions (Del Castillo *et al*., 1987). In addition, we have recently demonstrated that ovariectomy increases Na^+,K^+-ATPase activity in synaptic plasma membranes in rat hippocampus (Monteiro *et al*., 2005b).

The cholinergic system plays a crucial role in cognitive function. Two cholinesterases are ubiquitous constituents: acetylcholinesterase (AChE) (E.C 3.1.1.7) and butyrylcholinesterase (BuChE) (E.C 3.1.1.8). AChE has been described to contribute to the integrity and permeability of the synaptic membrane during neurotransmission and conduction (Grafius *et al*., 1971). This enzyme has been implicated in cholinergic and non-cholinergic actions, which may play a role in neurodegenerative diseases (Henderson *et al*., 1996, Cummings, 2000, Arendt *et al*., 1992). Recent evidence suggests that, in addition to AChE, BuChE catalyses the hydrolysis of the neurotransmitter, acetylcholine, and serves as a co-regulator of cholinergic transmission (Geula and Darvesh, 2004). In addition, associations of serum BuChE with alterations

in lipid metabolism (Magarian and Dietz, 1987) and coronary artery disease have been suggested (Alcantara *et al*., 2002). We have shown that ovariectomized rats present an increase of hippocampal AChE activity (Monteiro *et al*., 2005b) and a reduction of serum BuChE (Monteiro *et al*., 2005c) activity in female adult rats.

Considering that: (a) previous studies show that ovariectomy increases Na^+,K^+-ATPase and AChE activities in hippocampus of female adult rats (Monteiro *et al*., 2005b), (b) the administration of vitamins E plus C prevents the memory deficit caused by ovariectomy (Monteiro *et al*., 2005a), (c) soy isoflavones have been proposed as a good alternative to substitute the HRT (Miquel *et al*., 2006); we decided to investigate the influence of vitamins E plus C or soy isoflavones on the effects elicited by ovariectomy on Na^+,K^+-ATPase and AChE activities in hippocampus of ovariectomized rats. We also determined the actions of vitamins or soy isoflavones on serum BuChE activity of ovariectomized rats. The working hypothesis is that vitamins E plus C and isoflavones could reverse the alteration of these enzymes caused by ovariectomy.

Materials and methods

Animals and Reagents

Female adult Wistar rats (3 months, 180-210 g BW) were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto

Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room, with free access to water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal Rio Grande do Sul, Brazil.

Casein, 87% purity, was from Farmaquímica, Porto Alegre, Brazil, supplemented with 0.15% L-methionine (from Merk, Rio de Janeiro, Brazil), a mixture of minerals and vitamins (from Roche, São Paulo, Brazil) and Samprosoy 90 LH (generously supplied by EMBRAPA, Brazil). All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Experimental treatment

Eighty-day-old female rats were randomly assigned to one of the following groups: sham (only submitted to surgery, without removal of the ovaries) and ovariectomized. The stage of the estrous cycle was determined by vaginal swabs for 10 days prior to ovariectomy, to ensure that animals were cycling normally (Baker *et al*., 1979). Animals were ovariectomized by the surgical removal of both ovaries under ketamine anesthesia (90 mg/kg) and xylazine (10 mg/kg) intraperitoneous (i.p.) to eliminate endogenous ovarian steroids (Waynforth and Flecknell, 1992).

In the first set of experiments, seven days after surgery, animals were treated for 30 days with a single daily ip injection of saline (control) or vitamins E (40 mg/Kg) plus C (100 mg/Kg) (Wyse *et al*., 2002). These dosing regimes have proved effective for preventing biochemical and behavioral effects in

experimental models of metabolic diseases and ovariectomized rats (Reis *et al*., 2002, Wyse *et al*., 2002, Delwing *et al*., 2005, Monteiro *et al*., 2005a).

In other set of experiments, seven days after surgery, animals were fed for 30 days on a standard diet with casein (control) or a special diet with soy isolated protein – Samprosoy 90 LH (1.89 mg isoflavones/g soy protein). The diet contained an isoflavone mixture that included Daidzein (0.25 mg/g soy protein) and Genistein (0.23 mg/g soy protein). The soy protein dose was chosen according to a protocol establish by Reeves and colleagues (1993). Food intake and body weight were examined weekly. Both diets were isocaloric.

Tissue preparation

On the 31st day of treatment, rats were killed by decapitation without anesthesia. Brains were quickly removed and the hippocampus dissected. All sham rats were killed on the afternoon of proestrus.

For preparation of synaptic plasma membrane and determination of Na^+,K^+-ATPase activity, the hippocampus was homogenized in 10 vol. 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4.

For the AChE assay, the same structure was homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1000 X *g.* The supernatant was used for the enzymatic AChE analyses.

For the BuChE assay and the evaluation of estrogen levels, the blood was rapidly collected, centrifuged at 1000 X *g* for 10 min and the serum was separated.

Preparation of synaptic plasma membrane from hippocampus

Synaptic plasma membranes from hippocampus were prepared according to the method of Jones and Matus (1974) with some modifications (Wyse *et al*., 1995). The homogenate was centrifuged at 1000 X *g* for 20 min and the supernatant removed and centrifuged at 12,000 X *g* for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris-HCl buffer, pH 8.1), at 0°C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 x *g* for 2h, the fraction between 0.8 and 1.0 mM sucrose interface was taken as the membrane enzyme preparation.

Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris-HCl, pH 7.4, in a final volume of 200 μL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse and colleagues (2000). Released inorganic phosphate (Pi) was measured by the method of Chan and colleagues (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicate.

AChE activity assay

Acetylcholinesterase activity was determined according to Ellman and colleagues (1961), with some modifications (Villescas *et al*., 1981).

Hydrolysis rates *v* were measured at acetylthiocholine (S) concentrations of 0.8 mM in 1mL assay solutions 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 rat hippocampus supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30s). Specific enzyme activity was expressed as μmol ASCh per hour per mg of protein. All samples were run in duplicate.

BuChE activity assay

Butyrylcholinesterase activity was determined by the method of Ellman and colleagues (1961) with some modifications. Hydrolysis rate v was measured at acetylthiocholine (S) concentrations of 0.8 mM in 1mL assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Fifty microliters of rat serum was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30s) at 25°C. Specific enzyme activity was expressed as μmol ASCh per hour per mg of protein. All samples were run in duplicate.

Estradiol measurement

Serum concentration of estradiol was carried out in microplates by enzymeimmunoassay (EIA) using a Biomedical kit (BioCheck, Inc., USA). This assay uses the quantitative sandwich enzyme immunoassay technique that involves the simultaneous reaction of the measured molecules to two

monoclonal antibodies. The assay sensitivity is 1 pg/mL and, to eliminate interassay variation, all samples were assayed in a single run.

Protein determination

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one way ANOVA followed by the Duncan multiple test when F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software using a PC-compatible computer. Values of $p<0.05$ were considered to be significant.

Results

We first investigated the effect of treatment with vitamins E plus C on Na^+,K^+-ATPase and cholinesterases activities in female adult Wistar rats (Fig. 2). Figure 2A shows that animals subjected to ovariectomy presented a significant increase (54%) of hippocampal Na^+,K^+-ATPase activity and the treatment with vitamins E plus C reversed the stimulation caused by ovariectomy $F(3,13=8.26;^{**}p<0.01]$. Figure 2B shows that AChE activity was significantly increased (53%) in hippocampus of rats subjected to ovariectomy, which was reversed by vitamins E and C administration $[F(3,16)=43.44;^{***}p<0.001]$. As can be observed in figure 2C ovariectomized rats presented a reduction (33%) in serum BuChE activity and vitamins E plus C

treatment did not reverse such effect [F(3,16)=14.31; ***p<0.001]. Vitamins E plus C administration *per se* did not alter Na^+,K^+-ATPase and cholinesterases activities.

Next, we investigated the effect of the soy diet, rich in isoflavones on the activities of Na^+,K^+-ATPase and cholinesterases in hippocampus and BuChe in serum of ovariectomized female adult Wistar rats (Fig. 3). As can be observed in figure 3A, Na^+,K^+-ATPase activity was significantly increased (48%) in hippocampus of ovariectomized rats and soy treatment did not reverse such effect [F(3,12=5.04; *p<0.05]. Figure 3B shows that ovariectomy significantly increased (38%) AChE activity and soy treatment reversed this effect [F(3,16=9.30; **p<0.01]. BuChE activity (Fig. 3C) was decreased (45%) in serum of ovariectomized rats [F(3,16)=17.86; ***p<0.001] and soy diet did not reverse the effect of ovariectomy on reduction of this enzyme activity. Soy diet *per se* did not alter Na^+,K^+-ATPase and cholinesterases activities.

We observed that the animal weight gain was increased by ovariectomy in the first set of experiments, with the vitamins E plus C treatments, [F(3,16)=6.26; **p<0.01] (Table 1) and in the second set of experiments, with the diet rich in isoflavones, [F(3,16=21.77; ***p<0.001] (Table 2). As can be observed in these tables the treatment with vitamins or soy isoflavones *per se* did not alter the effect of ovariectomy on weight body of rat, when compared to controls groups.

Finally, in order to verify whether vitamins E plus C or the soy diet rich in isoflavones could alter the estradiol levels, we also measured the serum of all groups by EIA. The ovariectomy significantly decreases (98%) the estradiol levels in all ovariectomized groups, confirming the efficacy of the surgical

procedure of ovariectomy [F(7,32)=148.74; *** p<0.001]. Vitamins or soy supplementation did not revert the decrease in estradiol levels caused by ovariectomy (Fig. 4).

Discussion

The increase in female life expectancy has meant that women now live a great part of their lives beyond the cessation of their ovarian function. 17β estradiol is considered to be the major ovarian hormone and its deprivation has been implicated in some neurodegenerative conditions (Wise, 2002). A previous report demonstrated that all female brain areas have an increased ascorbate loss after gonadectomy, indicating enhanced oxidative stress (Kume-Kick *et al*., 1996). It has been suggested that steroids hormones, especially estriol and estradiol, are natural antioxidants (Mooradian, 1993). A greater decrease in the antioxidant/prooxidant balance is found in menopausal women, when compared to men of the same age, and the supplementation with antioxidants may help to protect against the antioxidant decline derived from estrogen loss (Miquel *et al*., 2006). Furthermore, the intake of antioxidant compounds could be a complement to the conventional treatments prescribed to these women.

Na^+,K^+-ATPase and AChE are essential to normal brain function and its activities are altered in cerebral ischemia (MacMillan,1982, Wyse *et al*., 2000, Saez-Valero *et al*., 2003) and in Alzheimer's disease (Hattori *et al*., 1998, Arendt *et al*., 1992, Goméz-Ramos and Morán, 1997). It has proposed that Na^+,K^+-ATPase activity could be a suitable index of neural activity for the study of neuroendocrine interactions (Del Castillo *et al*., 1987). On the other hand, it is

known that estrogen withdrawal and replacement affect the cholinergic system in a variety of brain regions (Simpkins *et al*., 1997, Gibbs and Aggarwal, 1998). BuChE may represent a complementary functional pool to AChE which may act as an enzymatic mechanism to regulate AChE levels in cholinergic brain synapses under particular conditions as Alzheimer's disease (Giacobini, 2003).

In the present study, we investigated the influence of vitamins E plus C or soy isoflavones on the activation of hippocampal Na^+,K^+-ATPase and AChE activities caused by ovariectomy. We used this animal model of steroid hormone deprivation because ovariectomy is considered to be the most common animal model of postmenopausal changes in adult female rats (Savonenko and Markowska, 2003). The hippocampus was used because this cerebral structure is associated with memory mechanism (Daniel and Dohanich, 2001) and ovariectomized rats present memory impairment (Monteiro *et al*., 2005a; Singh et al., 1994). Our results showed that ovariectomy significantly increased Na^+,K^+- ATPase and AChE activities (Fig. 2 and 3) in hippocampus of female rats submitted to ovariectomy. These results are in agreement with our previous studies showing that hippocampal Na^+,K^+-ATPase and AChE activities are increased in ovariectomized rats (Monteiro et al., 2005b). We also observed that vitamins E plus C were unable *per se* to affect the enzyme activities, although they markedly reversed the action of ovariectomy on Na^+,K^+-ATPase and AChE activities in hippocampus of ovariectomized rats (Fig. 2). The exact mechanism of reversal of Na^+,K^+-ATPase activities by vitamin E is unknown, however this vitamin may be important for membrane stabilization (Ekiel *et al*. 1998, Gomez-Fernandez *et al*., 1989), since Na^+,K^+-ATPase is embedded in membranes. The effect of oxidative stress should not be discarded, since it has

been shown that selegiline, an irreversible monoaminoxidase-B inhibitor used in Parkinson's disease and in depression, decreases free radical production resulting in the stimulation of brain AChE and Na^+,K^+-ATPase activities (Carageorgiou *et al*., 2003), suggesting that oxidative stress is involved in these alterations. In this context, it has been demonstrated that the administration of α-tocopherol reverses the increase of Na^+,K^+-ATPase activity provoked by chronic ethanol consumption in fish oil fed rats (Nanji and Sadrzadeh, 1994). It has also been shown that the stimulation of Na^+,K^+-ATPase activity is associated with a decrease in membrane fluidity (Levin et al., 1990) and lipid peroxidation (Nanji *et al*., 1994).

In agreement, Melo and colleagues (2003) showed that the enhancement of AChE activity induced by amyloid beta peptide is mediated by oxidative stress and that vitamin E prevents such effects. Interestingly, vitamin E plus C administration, in the same concentration used in this study, prevented the alterations caused by proline in some parameters of oxidative stress (Delwing *et al*., 2005). Since ovariectomy enhances Na^+,K^+-ATPase and AChE activities and vitamins E plus C protects against these changes, our results provide evidence for a possible role of free radicals in this phenomenon. The treatment with vitamins when ovarian hormones are depleted may be an alternative to substitute estrogen antioxidant activity.

In our study, soy protein supplementation was able to reverse the increase in AChE activity. In contrast, these substances did not reverse the activation of Na^+,K^+-ATPase activity, caused by ovariectomy (Fig. 3). The reversal of AChE activity probably indicates that isoflavones can affect brain cholinergic mechanisms, since stimulation of AChE activity provoked an

enhanced acetylcholine (ACh) hydrolysis and choline reuptake (Okuda *et al*., 2000). In agreement with our studies, soy isoflavones have been described to affect the synthesis of acetylcholine. In this context, Pan and colleagues (1999) reported that soy phytoestrogens may function as estrogen agonists in regulating choline acetyltransferase and nerve growth factor in brain of female rats.

We also determined the effect of treatment with vitamins E plus C or diet with soy rich in isoflavones on the reduction of BuChE actvity in serum of ovariectomized rats. Results showed that vitamins E plus C or the soy diet rich in isoflavones were unable to reverse the reduction of this enzyme activity, showing that the inhibitory effect of ovariectomy on this peripheral enzyme is not sensitive to the treatments used in our study.

Alpha-tocopherol is a lipid soluble vitamin that interacts with cells membranes, traps free radicals and interrupts the oxidative chain that damage cells (Ames *et al*., 1993). The resultant tocopheroxyl radical requires ascorbate (vitamin C) for its regeneration back to reduced tocopherol (Carr and Frei, 1999). The joint administration of vitamins E plus C must increase the protective action against reactive oxygen species both in the aqueous phase of the organism and in the lipid phase of the mitochondrial membranes, which are rich in polyunsaturated fatty acids quite vulnerable to oxidation (Jialal *et al*., 2001, Offerman and Medford, 1994). In addition, it has been demonstrated that castrated female rats present a decreased vitamin E concentration in serum and liver (Feingold *et al*., 1993). The antioxidant activity of estradiol is attributed to its phenolic hydroxyl (-OH) group (Fig. 1), which is capable of reducing peroxyl radicals. The regeneration of tocopherols from tocopheroxyl radicals by

estradiol has been observed under *in vitro* conditions (Mukai *et al*., 1990). It has been demonstrated that ovariectomy decreases, and estradiol replacement elevates, the tissue concentration of α-tocopherol in ovariectomized rats (Feingold *et al*., 1993). We have recently reported that an impairment of spatial navigation, caused by ovariectomy, was prevented by vitamins E plus C administration (Monteiro *et al*., 2005a). In agreement with these data, Socci and colleagues (1995) showed that chronic antioxidant treatment enhances cognitive performance of aged rats in the same behavior task.

Soy isoflavones are referred to as phytoestrogens because they bind to the estrogen receptor and can exert both agonistic and antagonistic estrogenic effects (Setchell, 2001). The phytoestrogens interact with the estrogen receptors ERα and ERβ, due to their diphenolic structural similarity to 17β-estradiol (Fig. 1) (Miksicek, 1995). These substances may improve cognitive functions by mimicking estrogen effects in the brain. Phytoestrogens have gained recognition as protective agents against diseases related to age and hormone dependent cancers (Cornwell *et al*., 2004, Aldlercreutz, 1998). It has been observed that a prolonged treatment with estradiol reduced the frequency of spontaneous oscillations and the expression/activity of Na^+,K^+-ATPase in rat uteri, indicating that this enzyme could be an important target for estrogens and estrogen-like molecules (Tsai *et al*., 2000; 2003). However, the effect of these compounds on brain function remains to be elucidated. Lephart and colleagues (2003) showed that the consumption of dietary phytoestrogen can alter hormone-sensitive hypothalamic brain volumes in rodents during adulthood. In addition, ovariectomized rats that consumed diets with soy isoflavones

demonstrated a dose-dependent improvement in their performance in radial arm maze tests (Pan *et al*., 2000).

Since reports show that estrogens may be regenerated by endogenous antioxidants (Gridley *et al*., 1997), in the present study we also verified whether vitamins E plus C or the soy diet rich in isoflavones could alter estradiol levels. We measured the serum estradiol in all groups by EIA and the results showed that ovariectomy significantly decreases (98%) the estradiol levels (Fig. 4) in all ovariectomized groups, confirming the efficacy of the surgical procedure of ovariectomy. No difference in estradiol levels was observed in the groups treated with vitamins or submitted to the soy diet rich in isoflavones.

In summary, in the present study we demonstrate that the treatment with vitamins E plus C significantly reverses the action of ovariectomy on Na^+,K^+ - ATPase and AChE activities in hippocampus of female adult rats. Conversely, a diet of soy rich in isoflavones was able to reverse only the increased AChE activity. Taken together, these compounds may have a protective role against the damage brain caused by the loss of estrogen during menopause. These data are very encouraging, since vitamins E plus C and soy isoflavones may constitute a good alternative to a novel therapeutic strategy. However, further studies are required to elucidate the exact mechanism of their action.

Acknowledgements

This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil).

References

Alcantara, V.M., Chautard-Freire-Maia, E.A., Scartezini, M., Cerci, M.S., Braun-Prado, K., and Picheth, G. (2002). Butyrylcholinesterase activity and risk factors for coronary artery disease. *Scand. J. Clin. Lab. Invest.* **62**:399-404.

Aldlercreutz, H. (1998). Epidemiology of phytoestrogens. *Bailliéres Clin. Endocrinol. Metab.* **12**:605-623.

Ames, B.N., Shigenara, M.K., and Hagen, T.M. (1993). Oxidants, antioxidants and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA.* **90**:7915-7922.

Arendt, T., Bruckner, M.K., Lange, M. and Bigl, V. (1992). Changes in acetylcholinesterase and butyrylcholinesterase in Alzeimer's disease resemble embryonic development – a study of molecular forms. *Neurochem. Int.* **21**:381-396.

Arteaga, E., Rojas, A., Villaseca, P., Bianchi, M., Arteaga, A., and Durán, D. (1998). In vitro effect of oestradiol, progesterone, testosterone, and of combined estradiol/progestins on low density lipoprotein (LDL) oxidation in postmenopausal women. *Menopause.* **6**:16-23.

Baker, H.J., Lindsey, J.R., and Weisbroth, S.H. (1979). The Laboratory Rat. In *Biology and Diseases,* (vol 1), New York, NY, USA, Academic Press.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of micrograms quantities of protein utilizing the principle of protein-die-binding. *Anal. Biochem.* **72**:248-254.

Brosemer, R.W. (1985). Effects of inhibitors of Na^+,K^+-ATPase on the membrane potentials and neurotransmitter efflux in rat brain slices. *Brain Res.* **334**:125-137.

Carageorgiou, H., Zarros, A., and Tsakiris, S. (2003). Selegiline long-term effects on brain acetylcholinesterase, (Na^+, K^+)-ATPase activities, antioxidant status and learning performance of aged rats. *Pharmacol. Res.* **48**:245-251.

Carr, A., and Frei, B. (1999). Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB J.* **13**:1007-1024.

Chan, K.M., Delfer, D., and Junger, K.D. (1986). A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. *Anal. Biochem.* **157**:375-380.

Cornwell, T., Cohick, W., and Raskin, I. (2004). Dietary phytoestrogens and health. *Phytochemistry.* **65**:995-1016.

Cummings, J.L. (2000). The role of cholinergic agents in the management of behavioral disturbances in Alzheimer's disease. *Int. J. Neuropsychopharmacol.* **3**:21-29.

Daniel, J.M., and Dohanich, G.P. (2001). Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. *J. Neurosci.* **21**:6949-6956.

Del Castillo, A.R., Battaner, E., Guerra, M., Alonso, T., and Mas, M. (1987). Regional changes of brain Na^+, K^+ - transporting adenosine triphosphatase related to ovarian function. *Brain Res.* **416**:113-118.

Delwing, D., Chiarani, F., Bavaresco, C.S., Wannmacher, C.M., Wajner, M., Dutra-Filho, C.S., and Wyse, A.T.S. (2005). Protective effect of antioxidants on brain oxidative damage caused by proline administration. *Neurosci. Res.* **52**:69-74.

Ekiel, I.H., Hughes, L., Burton, G.W., Jovall, P.A., Ingold, K.U., and Smith, I.C. (1988). Structure and dynamics of alpha-tocopherol in model membranes and in solution: a broad-line and high-resolution NMR study. *Biochemistry.* **27**:1432-1440.

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

Erecinska, M., and Silver, I.A. (1994). Ions and energy in mammalian brain. *Prog. Neurobiol.* **43**:37-71.

Feingold, I.B., Longhurst, P.A., and Colby, H.D. (1993). Regulation of adrenal and hepatic α-tocopherol content by androgens and estrogens. *Biochim. Biophys. Acta.* **1176**:192-196.

File, S.E., Jarrett, N., Fluck, E., Duffy, R., Casey, K., and Wiseman, H. (2001). Soy phytoestrogens improves human memory. *Psychopharmacology.* **157**:430-436.

Geula, C., and Darvesh, S. (2004). Butyrylcholinesterase, cholinergic neurotransmission and the pathology of Alzheimer's disease. *Drugs Today.* **40**:711-721.

Giacobini, E. (2003). Cholinesterases: New Roles in Brain Function and in Alzheimer's Disease. *Neurochem. Res*. **28**:515-522.

Gibbs, R.B., and Aggarwal, P. (1998). Estrogen and basal forebrain cholinergic neurons: implications for brain aging and Alzheimer's disease-related cognitive decline. *Horm. Behav*. **34**:98-111.

Gomez-Fernandez, J.C., Villalain, J., Aranda, F.J., Ortiz, A., Micol, V., Coutinho, A., Berberan-Santos, M.N., and Prieto, M.J. (1989). Localization of alpha-tocopherol in membranes. *Ann. N. Y. Acad. Sci.* **570**:109-120.

Gómez-Ramos, P., and Morán, M.A. (1997). Ultra structural localization of butyrylcholinesterase in senile plaques in the brains of aged and Alzheimer's disease patients. *Mol. Chem. Neuropathol*. **30**:161-173.

Grafius, M.A., Bond, H.E., and Millar, D.B. (1971). Acetylcholinesterase interaction with a lipoprotein matrix. *Eur. J. Biochem.* **22**:382-390.

Green, P.S., and Simpkins, J.W. (2000). Neuroprotective effects of estrogens: potential mechanisms of action. *Int. J. Devl. Neurosci.* **18**:347-358.

Gridley, K.E., Green, P.S., and Simpkins, J.W. (1997). Low concentrations of estradiol reduce beta-amyloid (25-35) - induced toxicity, lipid peroxidation and glucose utilization in human SK-N-SH neuroblastoma cells. *Brain Res.* **778**:158-165.

Hattori, N., Kitagawa, K., Higashida, T., Yagyu, K., Shimohama, S., Wataya, T., Perry, G., Smith, M.A., and Inagaki, C. (1998). Cl^- - ATPase and Na^+ / K^+ - ATPase activities in Alzheimer's disease brains. *Neurosci. Lett*. **254**:141-144.

Henderson, V.W., Watt, L., and Buckwalter, J.G. (1996). Cognitive skills associated with estrogen replacement in women with Alzeimer's disease. *Psychoneuroendocrinology.* **21**:421-430.

Jialal, L., Devaraj, S., and Kaul, N. (2001). The effect of alpha-tocopherol on monocyte proatherogenic activity. *Am. J. Clin. Nutr.* **131**:389S-394S.

Jones, D.H., and Matus, A.I. (1974). Isolation of plasma synaptic membrane from brain by combination flotation-sedimentation density gradient centrifugation. *Biochim. Biophys. Acta.* **356**:276-287.

Kume-kick, J., Ferris, D.C., Russo-Menna, I., and Rice, M.E. (1996). Enhanced oxidative stress in female rat brain after gonadectomy. *Brain Res.* **738**:8-14.

Kurzer, M.S.,and Xu, X. (1997). Dietary phytoestrogens. *Annu. Rev. Nutr.* **17**:353-381.

Law, A., Gauthier, S., and Quirion, R. (2001). Say NO Alzheimer's disease: The putative links between nitric oxide and dementia of the Alzheimer's type. *Brain Res. Rev.* **35**:73-96.

Lephart, E.D., Rhees, R.W., Setchell, K.D., Bu, L.H., and Lund, T.D. (2003). Estrogens and phytoestrogens: brain plasticity of sexually dimorphic brain volumes. *J. Steroid Biochem. Mol. Biol.* **85**:299-309.

Levin, G., Cogan, U., Levy, Y., and Mokady, S. (1990). Riboflavin deficiency and the function and fluidity of rat erythrocyte membranes. *J. Nutr.* **120**:857-861.

MacMillan, V. (1982). Cerebral Na^+ / K^+ - ATPase activity during exposure to and recovery from acute ischemia. *J. Cereb. Blood Flow Metab*. **2**:457-465.

Magarian, E.O., and Dietz, A.J. (1987). Correlation of cholinesterase with serum lipids and lipoproteins. *J. Clin. Pharmacol.* **27**:819-820.

Mata, M., Fink, D.J., Gainer, H., Smith, C.B., Davidsen, L., Savaki, H., Schwartz, W.J., and Sokoloff, L.. (1980). Activity-dependent energy metabolism rat posterior pituitary primarily reflects sodium pump activity. *J. Neurochem.* **34**:213-215.

McCay, P.B. (1985). Vitamin E: interactions with free radical and ascorbate. *Annu. Ver. Nutr*. **5**:323-340.

Melo, J.B., Agostinho, P., and Oliveira, C.R. (2003). Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. *Neurosci. Res.* **45**:117-127.

Miksicek, R.J. (1995). Estrogenic flavonoids: structural requirements for biological activity. *Proc. Soc. Exp. Biol. Med.* **208**:44-50.

Miquel, J., Ramírez-Boscá, A., Ramírez-Boscá, J.V., and Alperi, J.D. (2006). Menopause: a review on the role of oxygen stress and favorable effects of dietary antioxidants. *Arch. Gerontol. Geriatr*. **42**:289-306.

Monteiro, S.C., Matté, C., Bavaresco, C.S., Netto, C.A., and Wyse, A.T.S. (2005). Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. *Neurobiol. Learn. Mem.* **84**:192-199. (a)

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

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

Mooradian, A.D. (1993). Antioxidant properties of steroids. *J. Steroid Biochem. Mol. Biol.* **45**:509-511.

Mukai, K., Daifuku, K., Yokoyama, S., and Nakano, M. (1990). Stopped-flow investigation of antioxidant activity of estrogens in solution. *Biochim. Biophys. Acta.* **1035**:348-352.

Nakamura, Y., Tsuji, S., and Tonogai, Y. (2000). Determination of the levels of isoflavonoids in soybeans and soy-derived foods and estimation of isoflavonoids in the Japanese daily intake. *J. AOAC Int.* **83**:635-650.

Nanji, A.A., and Sadrzadeh, S.M. (1994). Effect of fish oil and vitamin E on ethanol-induced changes in membrane ATPases. *Life Sci.* **55**:245-249.

Nanji, A.A., Sadrzadeh, S.M., and Dannenberg, A.J. (1994). Liver microsomal fatty acid composition in ethanol-fed rats: effect of different dietary fats and relationship to liver injury. *Alcohol Clin. Exp. Res.* **18**:1024-1028.

Offerman, M.K., and Medford, R.M. (1994). Antioxidants and atherosclerosis, a molecular perspective. *Heart Dis. Stroke.* **3:**52-57.

Okuda, T., Haga, T., Kanai, Y., Endou, H., Ishihara, T., and Katsura, I. (2000). Identification and characterization of the high-affinity choline transporter. *Nat. Neurosci.* **3**:120-125.

Pan, Y., Anthony, M., and Clarkson, T.B. (1999). Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. *Pro. Soc. Exp. Biol. Med*. **221**:118-125.

Pan, Y., Anthony, M., Watson, S., and Clarkson, T.B. (2000). Soy phytoestrogens improve radial arm maze performance in ovariectomized retired breeder rats and do not attenuate benefits of 17β-estradiol treatment. *Menopause.* **7**:230-235.

Reeves, P.G., Nielsen, F.H., and Fabey, Jr. (1993). GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123**:1939-1951.

Reis, E.A, Zugno, A.I, Franzon, R., Tagliari, B., Matté, C., Lammers, M.L., Netto, C.A., and Wyse, A.T.S. (2002). Pretreatment with vitamins E and C prevent the impairment of memory caused by homocysteine administration in rats. Metab. Brain Dis. **17(3)**:211-217.

Rodrigues, H.D., Kinder, J.E., and Fitzpatrick, L.A. (1999). Treatment with 17 beta-estradiol does not influence age and weight at puberty in Bos indicus heifers*. Anim. Reprod. Sci.* **56**:1-10.

Saez-Valero, J., Gonzales-Garcia, C., and Cena, V. (2003). Acetylcholinesterase activation in organotypic rat hippocampal slice cultures deprived of oxygen and glucose. *Neurosci. Lett*. **348(2)**:123-125.

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

Setchell, K.D. (2001). Soy isoflavones – benefits and risks from nature's selective estrogen receptor modulators (SERMs*). J. Am. Coll. Nutr.* **20**:354-362.

Simpkins, J.W., Rajakumar, G., Zhang, Y.Q., Simpkins, C.E., Greenwald, D., Yu, C.J., Bodor, N., and Day, A.L., (1997). Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rats. *J. Neurosurg*. **87**:724-730.

Singh, M., Meyer, E.M., Millard, W.J., and Simpkins, J.W. (1994). Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. *Brain Res.* **644**:305-312.

Sirtori, C.R., Arnoldi, A., and Johnson, S.K. (2005). Phytoestrogens: end of a tale? *Ann. Med.* **37**:423-438.

Socci, D.J., Crandall, B.M., and Arendash, G.W. (1995). Chronic antioxidants treatment improves the cognitive performance of aged rats. *Brain Res.* **693**:88-94.

Swann, A.C., Grant, S.J., and Maas, J.W. (1982). Brain (Na^+, K^+)-ATPase and noradrenergic activity: effects of hyperinnervation and denervation on high-affinity ouabain binding. *J. Neurochem.* **38**:836-839.

Tang, M.X., Jacobs, D., Stern, Y., Marder, K., Schofield, P., Gurland, B., Andrews, H., and Mayeux, R. (1996). Effect of oestrogen during menopause on risk and age at onset of Alzeimer's disease. *Lancet.* **348**:429-432.

Tsai, M.L., Lee, C.L., Tang, M.J., and Liu, M.Y. (2000). Preferential reduction of Na^+/K^+ ATPase alpha 3 by 17 beta-estradiol influences contraction frequency in rat uteri. *Chin. J. Physiol.* **43**:1-8.

Tsai, M.L., Chang, C.C., Lee, C.L., and Huang, B.Y. (2003). The differential effects of tamoxifen and ICI 182, 780 on the reduction of Na^+/K^+ ATPase and spontaneous oscillations by 17 beta-estradiol. *Chin. J. Physiol.* **46**:55-62.

Upritchard, J.E., Sutherland, W.H.E., and Mann, J.I. (2000). Effect of supplementation with tomato juice, vitamin E, and vitamin C on LDL oxidation and products of inflammation activity in type 2 diabetes. *Diabetes Care*. **23**:733-738.

Van Duijn, C.M. (1999). Hormone replacement therapy and Alzeimer's disease. *Maturitas.* **31**:201-205.

Vatassery, G.T. (1998). Vitamin E and other endogeneous antioxidants in the central nervous system. *Geriatrics.* **53**:25-27.

Villescas, R., Ostwald, R., Morimoto, H.D., and Bennett, E.L. (1981). Effects of neonatal undernutrition and cold stress on behavior and biochemical brain parameters in rats. *J. Nutr.* **111**:1103-1110.

Waynforth, H.B., and Flecknell, P.A. (1992). Experimental and Surgical Technique. In *The rat,* (2^{nd} ed.), London, Academic Press Limited, pp. 276-278.

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

WHI. (2002). Risk and benefits of estrogen plus progestin in healthy postmenopausal women. *J. Am. Med. Assoc.* **288**:321-333.

Wise, P.M., Dubal, D.B., Wilson, M.E., Rau, S.W., and Liu, Y. (2001). Estrogens: trophic and protective factors in the adult brain. *Front. Neuroendocrinol.* **22**:33-66. (a)

Wise, M.P., Dubal, D.B., Wilson, M.E., Rau, S.W., Böttner, M., and Rosewell, K.L. (2001). Estradiol is a protective factor in the adult and aging brain: understanding of mechanism derived from in vivo and in vitro studies. *Brain Res. Ver.* **37**:313-319. (b)

Wyse, A.T.S., Bolognesi, G., Brusque, A.M., Wajner, M., and Wannmaker, C.M.D. (1995). Na^+, K^+ - ATPase activity in the synaptic plasma membrane from the central cerebral cortex of rats subjected to chemically induced phenylketonuria. *Med. Sci. Res.* **23**:261-262.

Wyse, A.T.S., Streck, E.L., Worm, P., Wajner, A., Ritter, F., and Netto, C.A. (2000). Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. *Neurochem. Res.* **25**:969-973.

Wyse, A.T.S., Zugno, A.I., Streck, E.L., Matte, C., Calcagnotto, T, Wannmacher, C.M., and Wajner, M. (2002). Inhibition of Na^+,K^+-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. *Neurochem. Res.* **27**:1685-1689.

Zhang, Y.Q., Shi, J., Rajakumar, G., Day, A.L., and Simpkins, J.W. (1998). Effects of gender and estradiol treatment on focal brain ischemia. *Brain Res.* **784**:321-324.

Figure 1 – Chemical structure of compounds considered in this article: 17β estradiol, α-tocopherol, ascorbic acid, daidzein and genistein.

Figure 2 – Effect of ovariectomy and treatment with vitamins E plus C on the activities of Na^+,K^+-ATPase (A), acetylcholinesterase (B) and butyrylcholinesterase (C) from hippocampus of female adult rats. Data are expressed as mean ± S.E.M for 4-5 independent animals performed in duplicate. ** $p<0.01$ and *** $p<0.001$ compared to sham group (Duncan's multiple range test). AChE – acetylcholinesterase; BuChE – butyrylcholinesterase; sal – saline; vit – vitamins E plus C; ovx – ovariectomized.

Figure 3 – Effect of ovariectomy and the soy diet rich in isoflavones on the activities of Na^+,K^+-ATPase (A), acetylcholinesterase (B) and butyrylcholinesterase (C) from hippocampus of female adult rats. Data are expressed as mean ± S.E.M. for 4-5 independent animals performed in duplicate. *$p< 0.05$, ** $p<0.01$ and *** $p<0.001$ and compared to sham group (Duncan's multiple range test). AChE – acetylcholinesterase; BuChE – butyrylcholinesterase; cas – casein; isofl – soy rich on isoflavones; ovx – ovariectomized.

Figure 4 – Serum estradiol levels of female adult rats evaluated by enzymeimunnoassay (BioCheck, Inc., USA). Data are expressed as mean ± S.E.M. for 5 independent animals. *** $p<0.001$ compared to sham groups

(Duncan's multiple range test). sal – saline; vit – vitamins E plus C; cas – casein; isofl – soy rich on isoflavones; ovx – ovariectomized.

Table 1 - Effect of ovariectomy and vitamins E plus C treatment on body weight of female adult rats.

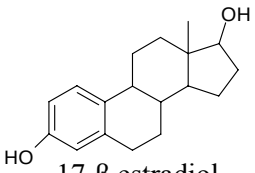
Groups	Body weigth(g) 1st day of treatment	Body weight (g) after 30 days of treatment
Sham saline	155.00±5.87	197.20±3.54
Sham vitamins	154.60±4.61	197.20±7.80
Ovx saline	156.20±7.91	231.20±8.90**
Ovx vitamins	155.40±4.03	222.40±6.47**

Data are presented as mean ± S.E.M. for 5 rats in each group. Ovariectomized rats (Ovx) were significantly different from sham groups after 30 days of treatment, **p<0.01 (ANOVA).

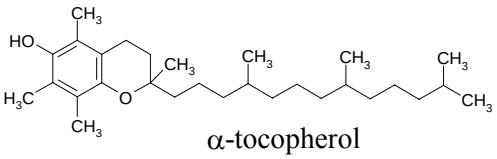
Table 2 - Effect of ovariectomy and the diet rich in isoflavones on body weight of female adult rats after 30 days of diet.

Groups	Body weight (g) 1st day of diet	Body weight (g) after 30 days of diet
Sham casein	161.20±7.85	200.80±9.11
Sham isoflavone	168.00±8.51	197.60±6.79
Ovx casein	164.00±9.02	268.20±6.49***
Ovx isoflavone	166.20±9.36	259.60±9.35***

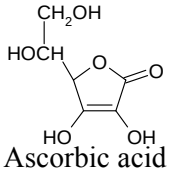
Data are presented as mean ± S.E.M. for 5 rats in each group. Ovariectomized rats (Ovx) were significantly different from sham groups after 30 days of diet, ***p<0.001 (ANOVA).



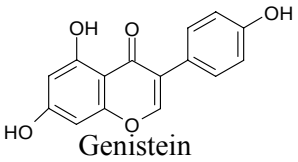
17-β estradiol



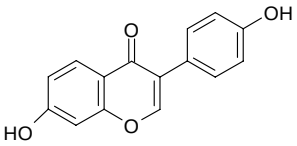
α-tocopherol



Ascorbic acid



Genistein



Daidzein

Figure 1

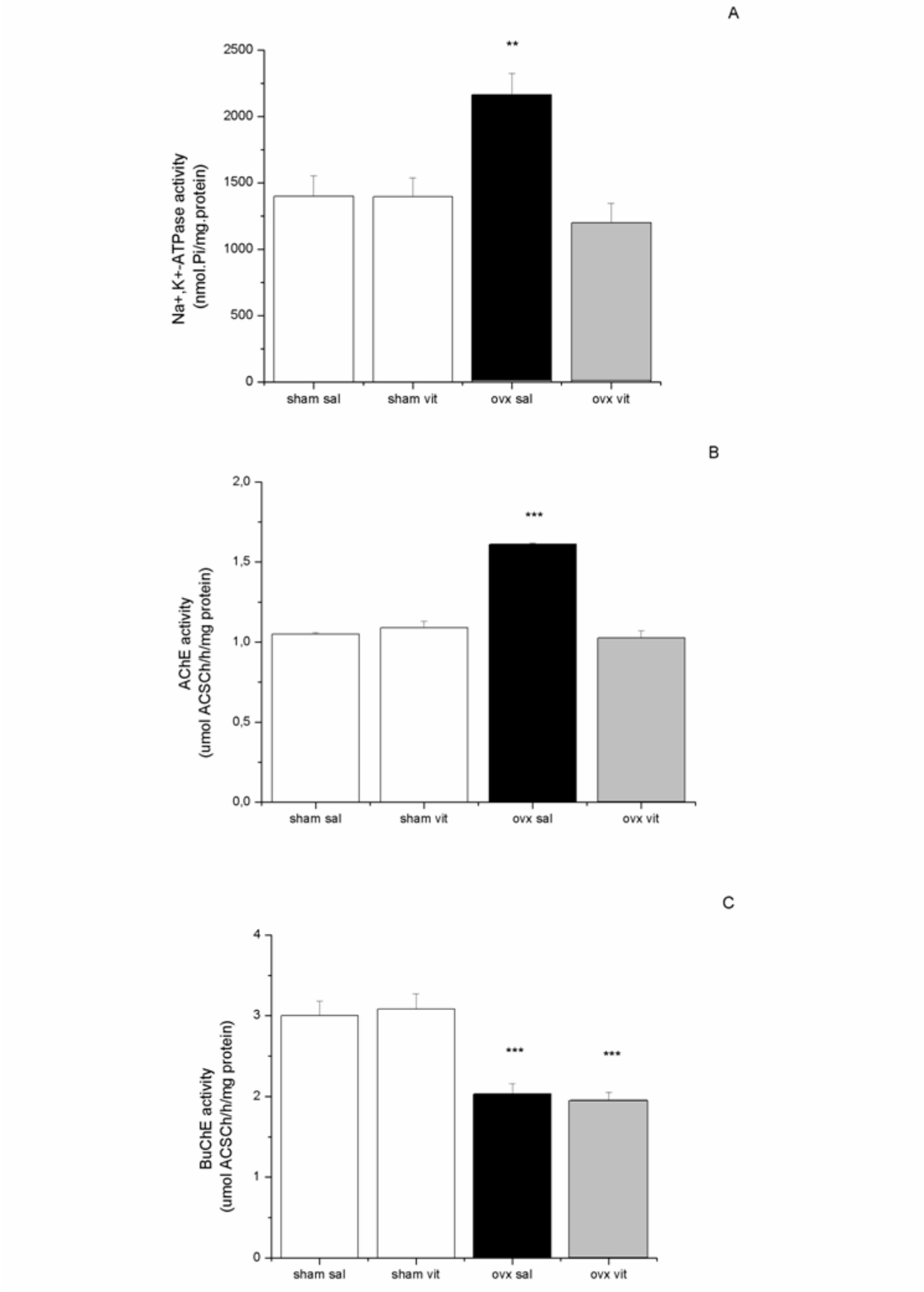

A
Na+,K+-ATPase activity
(nmol.Pi/mg.protein)
2500
2000
1500
1000
500
0
**
sham sal
sham vit
ovx sal
ovx vit
B
AChE activity
(umol ACSCh/h/mg protein)
2,0
1,5
1,0
0,5
0,0
***
sham sal
sham vit
ovx sal
ovx vit
C
BuChE activity
(umol ACSCh/h/mg protein)
4
3
2
1
0
***
***
sham sal
sham vit
ovx sal
ovx vit

Figure 2

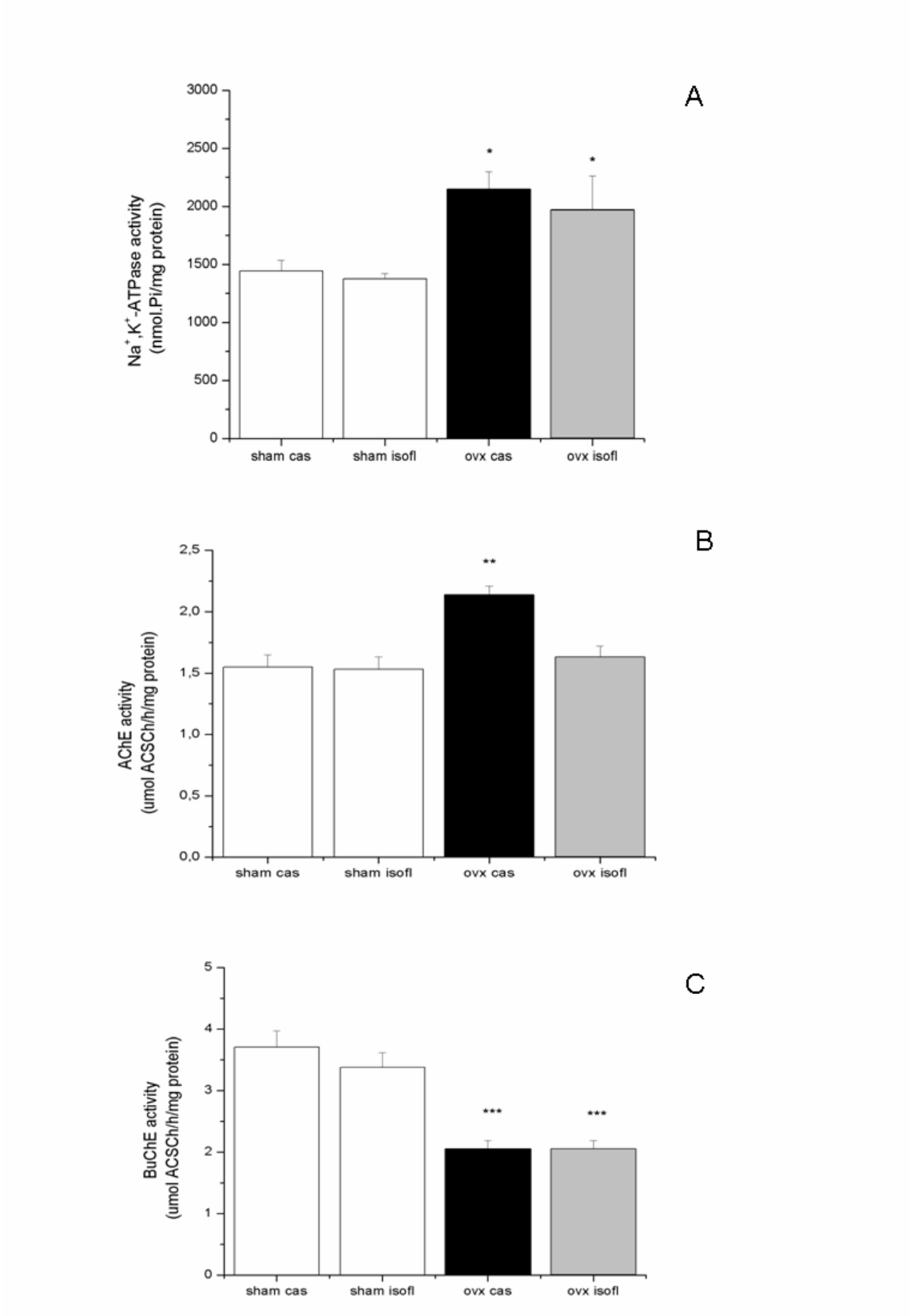

A
3000
2500
2000
1500
1000
500
0
Na+,K+-ATPase activity
(nmol.Pi/mg protein)
*
*
sham cas
sham isofl
ovx cas
ovx isofl
B
2,5
2,0
1,5
1,0
0,5
0,0
AChE activity
(umol ACSCh/h/mg protein)
**
sham cas
sham isofl
ovx cas
ovx isofl
C
5
4
3
2
1
0
BuChE activity
(umol ACSCh/h/mg protein)
***
***
sham cas
sham isofl
ovx cas
ovx isofl

Figure 3

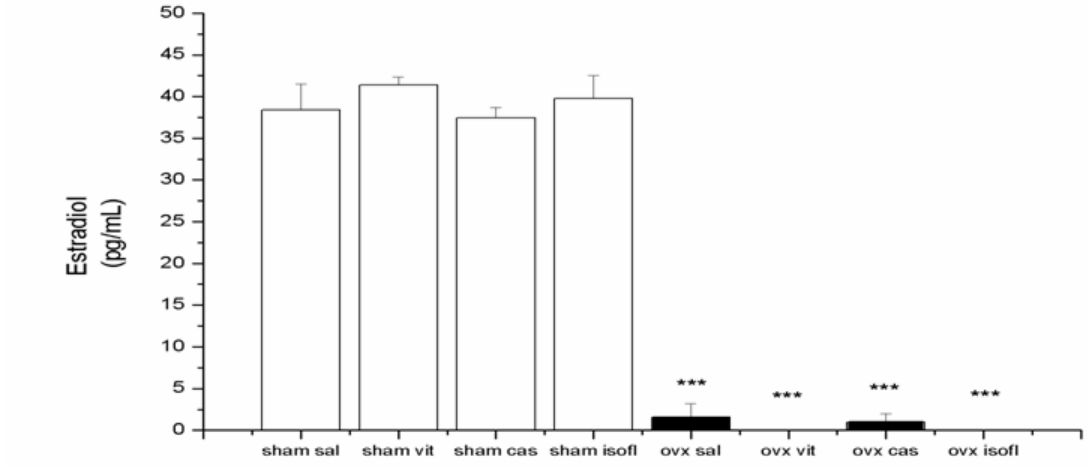

Estradiol
(pg/mL)
50
45
40
35
30
25
20
15
10
5
0
***
***
***
***
sham sal
sham vit
sham cas
sham isofl
ovx sal
ovx vit
ovx cas
ovx isofl

Figure 4

III.4 Artigo 4

Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze

Monteiro, SC, Matté, C, Bavaresco, CS, Netto, CA, and Wyse, ATS.

Neurobiology of Learning and Memory 84: 192-199. 2005

Objetivos

1. Investigar o efeito da ovariectomia sobre a memória espacial no labirinto aquático de Morris em ratas adultas.
2. Avaliar se o tratamento crônico com as vitamina E e C altera o prejuízo na memória espacial nas ratas ovariectomizadas.

.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neurobiology of Learning and Memory 84 (2005) 192–199
Neurobiology of Learning and Memory
www.elsevier.com/locate/ynlme

Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze

Siomara C. Monteiro, Cristiane Matté, Caren S. Bavaresco, Carlos Alexandre Netto, Angela T.S. Wyse *

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

Received 19 April 2005; revised 14 July 2005; accepted 2 August 2005
Available online 5 October 2005

Abstract

We investigated whether the pretreatment with vitamins E (α-tocopherol) and C (ascorbic acid) would act on ovariectomy-induced memory deficits in Morris water maze tasks. Adult female Wistar rats were divided into three groups: (1) naive (control), (2) sham (submitted to surgery without removal of ovaries) and (3) ovariectomized. Thirty days after surgery, they were trained in the Morris water maze in order to verify ovariectomy effects both on reference and working memory tasks. Results show that ovariectomized rats presented impairment in spatial navigation in the acquisition phase, as well as in the time spent in target quadrant and in the latency to cross over the location of the platform in test session, when compared to naive and sham groups (controls), in the reference memory task. Ovariectomy did not affect performance in the working memory task. Confirming our hypothesis, ovariectomized rats pretreated for 30 days with vitamins E and C had those impairments prevented. We conclude that ovariectomy significantly impairs spatial reference learning/memory and that pretreatment with vitamins E and C prevents such effect. Assuming this experimental memory impairment might mimic, at least in part, the cognitive deficit sometimes present in the human condition of lack of reproductive hormones, our findings lend support to a novel therapeutic strategy, based on vitamins E and C, to cognitive impairments in post-menopausal women.

Keywords: Ovariectomy; Memory; Morris water maze; Vitamin E and vitamin C

1. Introduction

Adult woman with normal reproductive cycles secrete a great quantity of estrogenic compounds mainly by the ovaries; 17β estradiol is the most abundant estrogen (Rodrigues, Kinder, & Fitzpatrick, 1999). Estrogen also exerts diverse non-reproductive actions on multiple organs, including the brain (Wise, 2002), and it has been shown that estrogen deprivation is implicated in the pathogenesis of neurodegenerative conditions, such as Alzheimer's disease and cerebral ischemia (Tang et al., 1996; Van Duijn, 1999; Zhang, Shi, Rajakumar, Day, & Simpkins, 1998). Consistently, there is a large body of literature suggesting that post-menopausal woman are more vulnerable than young ones to such diseases and to cognitive deficits (Green & Simpkins, 2000; Wise, Dubal, Wilson, Rau, & Liu, 2001a; Wise et al., 2001b).

The effects of ovariectomy on cognition in experimental animals have been studied in an attempt to model the human condition. Although some reports show that ovariectomy might not influence memory in rats (Iwasaki et al., 2004), others show it impairs spatial memory (Singh, Meyer, Millard, & Simpkins, 1994). However, it has also been shown that long-term (1.5–6 months)

* Corresponding author. Fax: +55 51 3316 5535.
E-mail address: wyse@ufrgs.br (A.T.S. Wyse).

1074-7427/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.nlm.2005.08.002

ovariectomy improves spatial memory in aged rats (Bimonte-Nelson et al., 2003). Despite the mechanisms involved in estrogen-induced impairments are not clearly established, there are some potential candidates. For instance, estrogens have been shown to improve memory retention (Fader, Johnson, & Dohanich, 1999; Sandstrom & Williams, 2004), as well as to promote synaptic plasticity and modulate neurotransmission (Baum, 2005; Bora, Liu, Kecojevic, Merchenthaler, & Koliatsos, in press; Pinkerton & Henderson, 2005).

There is good evidence that oxidative stress and reactive oxygen species participate in the modulation of learning/memory in many species. Studies report that stress-induced lipid peroxidation affect learning and memory performances in the rat (Abidin et al., 2004) and that a mutant of *C. elegans* with increased oxidative stress shows impairment of learning-associative behavior (Murakami & Murakami, 2005). Conversely, antioxidants have proved to prevent memory impairments in various experimental conditions, supporting a role for oxidative stress in memory modulation (Baydas, Ozer, Yasar, Tuzcu, & Koz, 2005; Bickford et al., 2000; Silva et al., 2004).

Interestingly, antioxidant actions of estrogens have long been recognized in a variety of in vitro and in vivo models (Behl, Widmann, Trapp, & Hosboer, 1995; Behl et al., 1997; Gridley, Green, & Simpkins, 1998; Behl & Moosmann, 2002). More specifically, studies show that low concentrations of estrogen can reduce brain lipid peroxidation (Gridley, Green, & Simpkins, 1997) and it was found that estrogens having an OH group at the aromatic ring have the ability to regenerate vitamin E radical back to vitamin E (Mukai, Daifuku, Yokoyama, & Nakano, 1990).

Another line of evidence supporting the role of oxidative stress on cognition emerges from studies with vitamins. Vitamin E, a peroxyl radical trapping agent, improves cognitive function of patients with temporal lobe radionecrosis (Chan, Cheung, Law, & Chan, 2004; Mecocci, Mariani, Cornacchiola, & Polidori, 2004) and may be beneficial in lowering the incidence of atherosclerotic cardiovascular diseases in patients with high risk for oxidative stress (Halliwell, 2000). As from experimental pre-clinical studies in the rat, it has been shown that age-related motor learning and memory deficits can be reversed with antioxidant-rich (β-caroteno, vitamins E and C) diets (Bickford et al., 2000) and that vitamins E and C treatment prevented deficits of learning/memory caused by homocysteine (Reis et al., 2002b).

Considering that: (a) ovariectomy might impair memory function, (b) estrogen has antioxidant actions and so affects oxidative stress, a possible modulator of memory mechanisms and (c) vitamins E and C are antioxidants that prevented memory impairment in other experimental models; we decided to evaluate the effect of ovariectomy on spatial memory tasks in rats under the influence of vitamins E and C. The working hypothesis is that ovariectomy will cause impairments in spatial tasks and that vitamins E and C would prevent such effects.

2. Materials and methods

2.1. Animals and reagents

Female adult Wistar rats obtained from the Central Animal House of the Biochemistry Department, Institute of Basic Health Sciences, at the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, were housed in groups of eight with their mothers on the day of birth. Half of them were assigned to the experimental condition and the other half served as controls; animals were weaned at 21 days after birth. Rats were maintained on a 12:12 h light/dark cycle in an air-conditioned constant-temperature (22 °C) colony room, with free access to 20% (w/w) protein commercial chow and water. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethical Committee of Universidade Federal do Rio Grande do Sul.

2.2. Control of estrous cycle

The stage of the estrous cycle was determined by vaginal swabs for 10 days prior to ovariectomy, to ensure that animals were normally cycling. The observed phases were: diestrus, when mucus, leukocytes and some nucleated cells were present (2–3 days on average); proestrus, when only nucleated cells were present (12 h); estrus, when only cornified cells were observed (24 h the rut phase), and metaestrus, when leukocytes, cornified cells and some nucleated cells were present (Baker, Lindsey, & Weisbroth, 1979).

2.3. Experimental treatment

In the first set of experiments, 80-day-old rats were randomly assigned to one of the following groups (10 animals in each group): naive (control), sham (only submitted to surgery without removal of the ovaries) and ovariectomized. Animals were ovariectomized by the surgical removal of both ovaries under ketamine anesthesia (90 mg/kg) and xylazine (10 mg/kg) i.p. to eliminate endogenous ovarian steroids (Waynforth & Flecknell, 1992). One month after ovariectomy, rats were submitted to behavioral testing.

For the second set of experiments, 80-day-old rats were divided into three groups (7–11 animals in each group): naive, sham and ovariectomized, Animals were treated for 30 days, beginning seven days after surgery,

with a single daily i.p. injection of saline (control) or vitamins E (40 mg/kg) and C (100 mg/kg) (Wyse et al., 2003). These dosing regimes have proved effective for preventing biochemical and behavioral effects in other experimental models of metabolic diseases (Delwing et al., 2003; Reis, Oliveira, Lammers, Netto, & Wyse, 2002a; Wyse et al., 2003). Twelve hours after the last injection rats were submitted to water maze testing.

2.4. Estradiol measurement

One week after surgery five animals from each group (naive, sham and ovariectomized) were killed by decapitation without anaesthesia and trunk blood was collected in plastic tubes. Serum samples were obtained by centrifugation at 3000*g* for 10 min and stored in aliquots at −20 °C until assayed. Serum estradiol levels were quantitatively measured using a commercial radioimmunoassay kit (Biomedical kit/Adaltis Estradiol Maia Kit, Italy). Quantification of estradiol is achieved by the use of iodinated radioligant (estradiol I^{125}) in conjunction with a highly specific antiserum. The range of assay detectable was 15–5000 pg/ml; assay sensitivity is 5 pg/ml and all samples were run in duplicate. Estrogen levels, in pg/ml, were: naive – 10.11 ± 1.42; sham – 11.67 ± 3.27; ovariectomized – not detected. That confirmed the efficacy of surgical procedure.

2.5. Behavioral procedures

On the 110th day of life, animals were subjected to behavioral testing. We used the Morris water maze, an apparatus widely employed for the study of spatial learning and memory tasks that depend on hippocampal function (D'Hooge & De Deyn, 2001; Morris, Garrud, Rawlins, & O'Keefe, 1982; Netto et al., 1993).

The water maze consisted of a black round tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm with water, maintained at constant temperature of 23 °C. The tank was theoretically divided into four equal quadrants for the purpose of analysis. Several distal visual cues were placed on the walls of the room. Trials were recorded by a video camera mounted above the center of the tank.

2.5.1. Reference memory task

The task consisted of six training and one test session. In the acquisition phase, rats had daily sessions of four trials per day for 6 days to find the platform, submerged 2 cm under the water surface, placed on the center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in water facing tank wall, in one of the four starting locations (N, S, W and E). The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failing to find it, they were gently guided to it; all animals were permitted to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was 15–20 min (Netto et al., 1993). One day after the last training trial, each rat was subjected to a probe trial in which the platform was removed. We measured four parameters, namely latency to cross on the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in the training sessions) and opposite quadrants. These parameters were taken as a measure for spatial memory (Netto et al., 1993).

In order to detect motor impairments that could affect performance in experimental groups, the swimming speed was calculated by taking the distance traveled in the first 15 s of the probe trial.

2.5.2. Working memory task

After 1 week, the working memory version of Morris water maze was performed. The task consisted of four consecutive trials per day, with a 30-s inter-trial interval, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every subsequent day during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance (Netto et al., 1993).

2.5.3. Open field task

The task was run in a wooden box measuring $60 \times 40 \times 50$ cm with a frontal glass wall, whose floor was divided by white lines into 12 equal squares. Animals were placed facing the rear left corner of the arena and observed for 2 min. The number of squares crossed with the four paws from one square to another was indicative of motor activity (Netto, Dias, & Izquierdo, 1986).

2.6. Statistical analysis

Reference memory training and working memory data were analyzed by repeated measure analysis of variance (ANOVA) and data from the probe trial parameters and the open field test were analyzed by one-way ANOVA; post hoc Duncan multiple range test was run when indicated. Descriptive statistics data were expressed as mean ± SEM. Type error rate was set at 0.05 for determining statistical significance, where multiple range test was used for post hoc testing. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer.

3. Results

3.1. Experiment 1: Effect of ovariectomy on reference and working memory tasks in the Morris Water maze

Ovariectomized animals showed a lower ability to find the platform and learn its location in the 4th and 5th days of training. Two-way ANOVA (days versus groups) revealed a major days effect for both groups [$F(2,28) = 3.23$; $p < .05$] and [$F(2,28) = 8.32$; $p < .001$], with no interaction between days and groups (Fig. 1). Four parameters were evaluated in the test session, namely the latency to cross and the number of crossings on the location of the platform and the time spent in target and opposite quadrants. It is shown that ovariectomy did not affect the number of crossings on the former platform location [$F(2,28) = 0.29$; $p > .05$]. However, ovariectomized rats present a decrease on the time spent in target quadrant [$F(2,28) = 3.41$; $p < .05$] (Fig. 2A), an increase on the time spent in opposite quadrant [$F(2,28) = 4.10$; $p < .05$] (Fig. 2B) and in the latency to cross over the location of the platform [$F(2.28) = 6.90$; $p < .01$], when compared to naive and sham groups (Fig. 2C).

Ovariectomy did not affect rat performance in the working memory version of Morris water maze since the two-way ANOVA revealed no major effect [$F(2,28) = 1.09$; $p > .05$] (Table 1).

No motor deficits were found in rats performing both water maze tasks, as assessed by swim speed; the general mean, considering all experimental groups, was 28.4 cm/s, with $p > .05$.

3.2. Experiment 2: Effect of vitamins E and C on memory impairments caused by ovariectomy

Results show that vitamins E and C per se did not alter any behavioral parameter studied in control animals, but

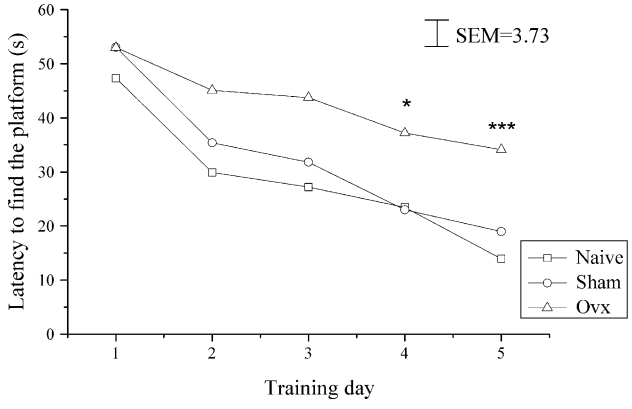


Fig. 1. Effect of ovariectomy on performance of spatial memory acquisition phase. Data are expressed as mean ± S.E.M. for 10 animals in each group. * $p < .05$ and *** $p < .001$ different from both control groups (naive and sham) on the 4th and 5th days of training sessions, respectively (ANOVA). Ovx, ovariectomized.

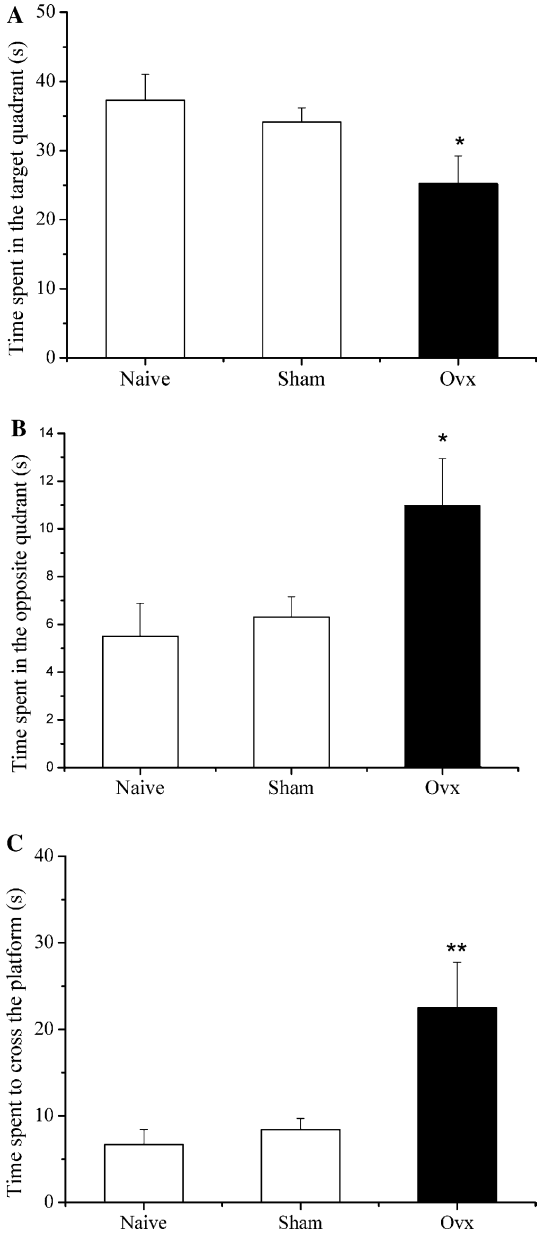


Fig. 2. Effect of ovariectomy on performance of spatial memory test session parameters, namely time spent in the target quadrant (A), time spent in opposite quadrant (B) and the latency to cross over the platform location (C). Data are expressed as mean ± S.E.M. for 10 animals in each group. * $p < .05$, different from both control groups to time spent in target and opposite quadrant and ** $p < .01$ t to the latency to cross over the location of the platform (ANOVA). Ovx, ovariectomized.

prevented the increase in latency to find the platform in the 4th and 5th days of training ([$F(5,49) = 6.054$, $p < .01$] and [$F(5,49) = 7.29$, $p < .01$] respectively, see Fig. 3) in ovariectomized rats. In addition, vitamins used also prevented the decrease in the time spent in target quadrant [$F(5,49) = 2.495$; $p < .05$] (Fig. 4A), the time spent on the opposite quadrant [$F(5,49) = 2.573$; $p < .05$] (Fig. 4B) and the latency to cross on the platform location [$F(5,49) = 4.838$; $p < .01$] (Fig. 4C).

Table 1
Effect of ovariectomy on performance in the working memory version of Morris water maze spatial task

Group	Latency to find the platform (s)			
	Trial 1	Trial 2	Trial 3	Trial 4
Naive	48.76 ± 3.09	38.46 ± 4.22	30.96 ± 5.59	30.95 ± 5.15
Sham	45.42 ± 3.55	33.00 ± 3.50	32.09 ± 2.68	21.15 ± 4.03
Ovx	50.33 ± 2.81	42.96 ± 3.35	38.05 ± 3.31	36.43 ± 5.16

Data are latencies to find the platform on each trial during the four testing days and are expressed as mean ± S.E.M for 10 animals in each group. There was no significant difference between groups, $p > .05$ (ANOVA). Ovx, ovariectomized.

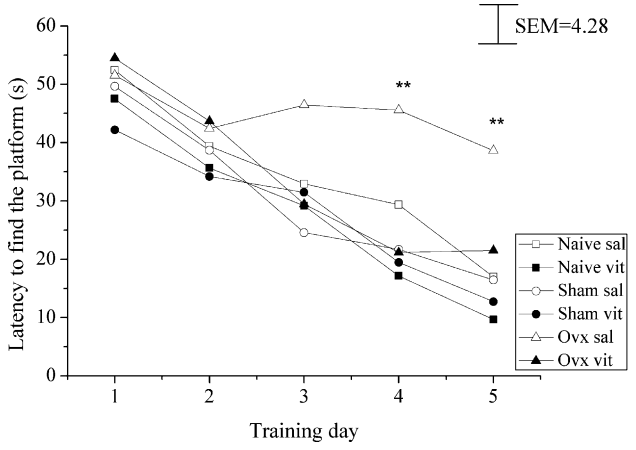


Fig. 3. Effects of ovariectomy and pretreatments with vitamins E and C on performance of spatial memory acquisition phase. Data are expressed as mean ± S.E.M. for 7–11 animals in each group. $^{**}p < .01$ different from control groups (naive and sham, either saline or vitamins) (ANOVA). Ovx, ovariectomized; sal, saline; vit, vitamins E plus C.

In order to verify whether vitamins E and C would affect motor activity, we submitted all groups (with and without vitamins treatment) to the open field task. Ovariectomy did not alter the number of crossings [$F(5,49) = 1.84$; $p > .05$] nor of rearings [$F(5,49) = 1.19$; $p > .05$] (Table 2).

Additionally, we observed that animal weight gain was affected by ovariectomy [$F(5,49] = 6.34$; $p < .01$)]. *Post hoc* analysis showed that vitamins E and C administration did not prevent the weight gain increase in ovariectomized rats (Table 3), as compared to those not receiving vitamins treatment.

4. Discussion

In the present study, we investigated the effect of ovariectomy on spatial navigation tasks in the Morris water maze, as well as the influence of vitamins E and C on such effects. This experimental condition of hormone deprivation was used because ovariectomy is the most common animal model of post-menopausal changes in adult female rats (Savonenko & Markowska, 2003). Results show that ovariectomized rats presented performance impairment in the acquisition phase (Fig. 1) and on the time spent in target quadrant and in platform location, as well as in the latency to cross over the platform location in session (Fig. 2) of reference memory task. However, no effect was found in working memory performance (Table 1). Pretreatment with vitamins E and C did not alter memory when compared to controls

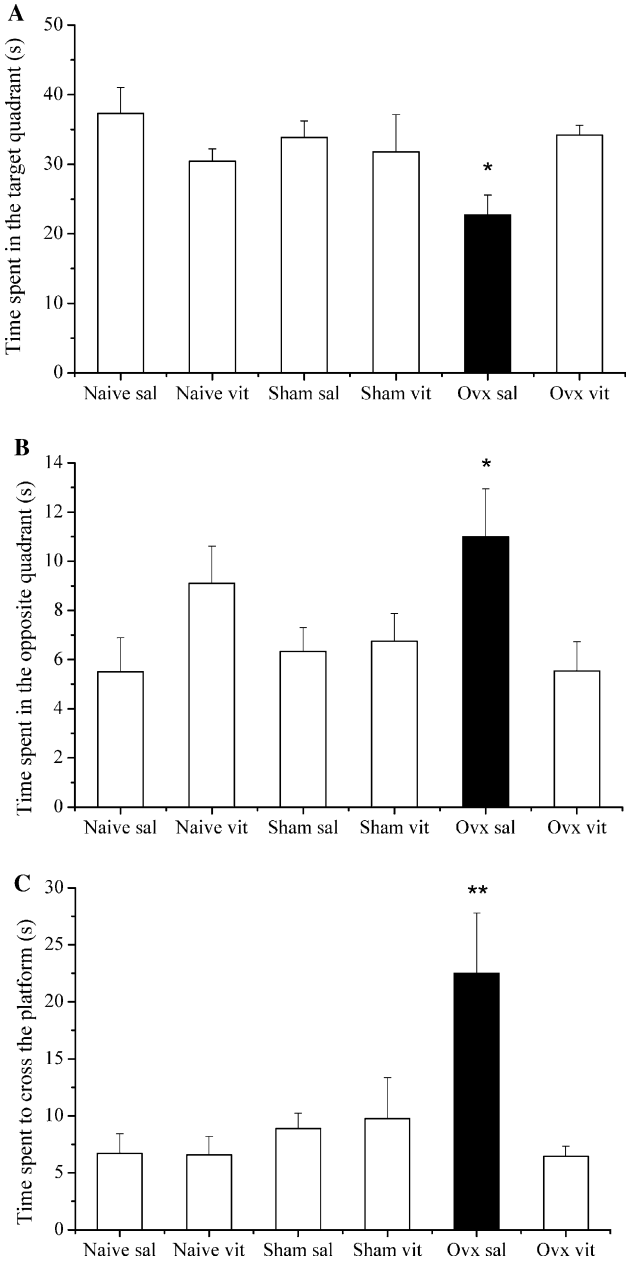


Fig. 4. Effects of ovariectomy and pretreatments with vitamins E and C on performance performance of spatial memory test session parameters, namely time spent in the target quadrant (A), time spent in the opposite quadrant (B) and the latency to cross on the location of the platform (C). Data are expressed as mean ± S.E.M. for 7–11 animals in each group. $^{*}p < .05$ different from control to time spent in target and opposite quadrant and $^{**}p < .01$ to the latency to cross over the location of the platform (ANOVA). Ovx, ovariectomized; sal, saline; vit, vitamins E plus C.

Table 2
Effect of ovariectomy and vitamins E plus C treatment on performance (number of crossings and rearings) in the open field task

Group	Number of crossings	Number of rearings
Naive sal	25.30 ± 1.95	11.10 ± 0.57
Naive vit	21.86 ± 0.74	10.14 ± 0.46
Sham sal	23.11 ± 0.99	10.11 ± 0.42
Sham vit	21.50 ± 0.82	10.38 ± 0.56
Ovx sal	21.30 ± 0.99	11.10 ± 0.43
Ovx vit	21.64 ± 0.68	10.36 ± 0.36

Data are presented as mean ± S.E.M for 7–11 rats in each group. There was no significant difference between groups, $p > .05$ (ANOVA). Ovx, ovariectomized; sal, saline; vit, vitamins E + C.

Table 3
Effect of ovariectomy and vitamins E and C treatment on body weight of 110 days age rats

Groups	Body weight (g)
Naive saline	216.20 ± 6.71
Naive vitamins	210.71 ± 7.58
Sham saline	213.11 ± 4.64
Sham vitamins	210.25 ± 4.07
Ovx saline	250.10 ± 9.72**
Ovx vitamins	245.18 ± 8.09**

Data are presented as mean ± S.E.M. for 7–11 rats in each group. Ovariectomized rats (with or without vitamins) were significantly different from naive and ham groups (controls).
** $p < .01$ (ANOVA).

(naive and sham), but prevented the memory impairment caused by ovariectomy (Fig. 3). Interestingly, vitamins treatment did not prevent the weight gain in ovariectomized rats (Table 3).

Our results are in agreement with previous studies showing that ovariectomized rats present memory/learning impairments (Singh et al., 1994). The preferred interpretation to our finding, i.e., that hormone deprivation in ovariectomized rats causes spatial memory deficits, must be tempered by the fact that estrous cycle may influence water maze behavior (Healy, Braham, & Braithwaite, 1999; Warren & Juraska, 1997; Warren & Juraska, 2000). Although we did not preclude the possibility that gonodal steroids or others substances produced by ovaries may have some influence on our results, the lack of differences between gonadally intact (naive and sham) and ovariectomized groups during the first days of training, or in the open field task, suggests that gonodal estrogens are not biasing our results. Additionally, the probe trial, in which ovariectomized rats performed poorly than controls, provided a measure of reference memory that was independent of motor performance, since swim speed did not vary between groups.

Confirming the working hypothesis, results in Fig. 3 show that vitamins E and C administration prevented spatial navigation deficits caused by ovariectomy. We are not allowed to suggest that this action of vitamins E and C was only due the its ability to scavenge free radical and/or lipid peroxidation (Ames, Shigenaga, & Hagen, 1993; Brigelius-Flohe & Taber, 1999; Burton, Wronska, Stone, Foster, & Ingold, 1990; Carr & Frei, 1999; McCay, 1985; Frei, Stocker, England, & Ames, 1990), since there are reports pointing that estrogens are regenerated by endogenous antioxidants (Gridley et al., 1997) and that antiproliferative and neuroprotective effects of vitamins E and C, independent on their antioxidant activity, have been described. In this context, vitamin E, alone or combined with vitamin C, can modulate apoptosis, increasing Bcl-2 (Barroso et al., 1997; Marsh, Laursen, Pat, Gobe, & Coombes, 2005), gene expression and cellular signaling (Zingg & Azzi, 2004). Moreover, it has been shown that vitamin E inhibits protein kinase C activity (Tasinato, Boscoboinik, Bartoli, Maroni, & Azzi, 1995; Gimeno, Zaragozá, Vina, & Miralles, 2004).

It has been demonstrated that castrated female rats (Feingold, Longhurst, & Colby, 1993) present decreased vitamin E concentrations in serum and liver, respectively; conversely, evidence also shows that estradiol replacement in ovariectomized rats increased vitamin levels (Noh, Koo, & Jeon, 1999). Other reported effects relate to vitamin E ability to reduce degeneration of hippocampal cells after cerebral ischemia (Hara, Kato, & Kogure, 1990) and to enhance the recovery of motor function after spinal cord injury (Anderson, Waters, & Means, 1988). Additionally, it has been shown that it prevents the hippocampal oxidative stress and passive avoidance memory deficits caused by sleep deprivation in mice (Silva et al., 2004).

Clinical studies suggest that vitamin E has a potential to be a complementary intervention for patients with cognitive dysfunction (Chan et al., 2004; Mecocci et al., 2004). Although it has been previously shown that vitamin E can slow progression of Alzheimer's disease (Sano et al., 1997), a recent study shows that this vitamin has no benefit in patients with mild cognitive impairment/early Alzheimer's disease (Petersen et al., 2005). Interestingly, post-menopausal women present decreased vitamin E concentrations (White et al., 2001) and study suggest that hormone replacement plus vitamin E therapy may effective in preventing atherosclerosis in post-menopausal women (Inal, Sunal, Kanbak, & Zeytinoglu, 1997).

As regards to vitamin C, it is shown that it not only recycles the vitamin E radical back to vitamin E, prolonging its antioxidant effect (Carr & Frei, 1999; Frei, Stocker, England, & Ames, 1990), but also improves endothelial function and large elastic artery compliance in estrogen-deficient post-menopausal (McSorley, Young, Bell, Fee, & McCance, 2003; Moreau, Gavin, Plum, & Seals, 2005). Thus, a combination of vitamin E and C might be used in these patients, since data show that when vitamin E is used alone, it can became prooxidant or at least lose its efficacy (Yusuf, Dagenais, Pogue, Bosch, & Sleight, 2000) what might explain the lack of its protective effects against the cognitive impair-

ment in patients with early Alzheimer's disease (Petersen et al., 2005).

In conclusion, the present study reports an impairment of spatial navigation caused by ovariectomy and that this effect was prevented by pretreatment with vitamins E and C. Assuming that hormone deprivation might also impair cognition in human beings, our results lend support to a novel therapeutic strategy, based on vitamins E and C, to cognitive deficits found in postmenopausal women.

Acknowledgment

This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nlm.2005.08.002.

References

Abidin, I., Yargicoglu, P., Agar, A., Gumuslu, S., Aydin, S., Ozturk, O., et al. (2004). The effect of chronic restraint stress on spatial learning and memory: Relation to oxidant stress. *International Journal of Neuroscience, 114*, 683–699.

Ames, B. N., Shigenaga, M. K., & Hagen, T. M. (1993). Oxidants, antioxidants and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America, 90*, 7915–7922.

Anderson, D. K., Waters, T. R., & Means, E. D. (1988). Pretreatment with alpha tocopherol enhances neurologic recovery after experimental spinal cord compression injury. *Journal of Neurotrauma, 5*, 61–67.

Baker, H. J., Lindsey, J. R., & Weisbroth, S. H. (1979). The Laboratory Rat. In *Biology and Diseases* (Vol. 1). New York NY, USA: Academic Press.

Barroso, M. P., Diaz, C. G., Lluch, G. L., Malagon, M. M., Crane, F. L., & Navas, P. (1997). Ascorbate and α-tocopherol prevent apoptosis induced by serum removal independent of Bcl-2. *Archives of Biochemistry and Biophysics, 343*, 243–248.

Baum, L. W. (2005). Sex, hormones, and Alzheimer's disease. *The Journal of Gerontology Biological Sciences and Medical Sciences, 60*, 736–743.

Baydas, G., Ozer, M., Yasar, A., Tuzcu, M., & Koz, S. T. (2005). Melatonin improves learning and memory performances impaired by hyperhomocysteinemia in rats. *Brain Research, 1046*, 187–194.

Behl, C., Widmann, M., Trapp, T., & Hosboer, F. (1995). 17-beta estradiol protects neurons from oxidative stress-induced cell death in vitro. *Biochemical and Biophysical Research Communications, 216*, 473–482.

Behl, C., Skutella, T., Lezoualc'h, F., Post, A., Widmann, M., Newton, C. J., et al. (1997). Neuroprotection against oxidative stress by estrogens: structure–activity relationship. *Molecular Pharmacology, 51*, 535–541.

Behl, C., & Moosmann, B. (2002). Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. *Free Radical Biology and Medicine, 33*, 182–191.

Bickford, P. C., Gould, T., Briederick, L., Chadman, K., Pollock, A., Young, D., Shukitt-Hale, B., & Joseph, J. (2000). Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. *Brain Research, 866*, 211–217.

Bimonte-Nelson, H. A., Singleton, R. S., Hunter, C. L., Price, K. L., Moore, A. B., & Granholm, A. C. (2003). Ovarian hormones and cognition in the aged female rat: I. Long-term, but not short-term, ovariectomy enhances spatial performance. *Behavioral Neuroscience, 117*, 1395–1406.

Bora, S. H., Liu, Z., Kecojevic, A., Merchenthaler, I., & Koliatsos, V. E. (in press). Direct, complex effects of estrogens on basal forebrain cholinergic neurons. *Experimental Neurology*.

Brigelius-Flohe, R., & Taber, M. G. (1999). Vitamin E: function and metabolism. *FASEB Journal, 13*, 1145–1155.

Burton, G. W., Wronska, U., Stone, L., Foster, D. O., & Ingold, K. U. (1990). Biokinetics of dietary RRR-α-tocopherol in the male guinea-pig at three dietary levels of vitamin C and two levels of vitamin E. *Lipids, 25*, 199–210.

Carr, A., & Frei, B. (1999). Does vitamin C act as a pro-oxidant under physiological conditions? *The FASEB Journal, 13*, 1007–1024.

Chan, A. S., Cheung, M. C., Law, S. C., & Chan, J. H. (2004). Phase II study of alpha-tocopherol in improving the cognitive function of patients with temporal lobe radionecrosis. *Cancer, 100*, 398–404.

Delwing, D., Chiarani, F., Delwing, D., Bavaresco, C. S., Wannmacher, C. M. D., Wajner, M., et al. (2003). Proline reduces acetylcholinesterase activity in cerebral cortex of rats. *Metabolic Brain Disease, 18*, 79–86.

D'Hooge, R., & De Deyn, P. P. (2001). Applications of the Morris water maze in the study of learning and memory. *Brain Research. Brain Research Reviews, 36*, 60–90.

Fader, A. J., Johnson, P. E., & Dohanich, G. P. (1999). Estrogen improves working but not reference memory and prevents amnestic effects of scopolamine of a radial-arm maze. *Pharmacology, Biochemistry and Behavior, 62*, 711–717.

Feingold, I. B., Longhurst, P. A., & Colby, H. D. (1993). Regulation of adrenal and hepatic alpha-tocopherol content by androgens and estrogens. *Biochimica et Biophysica Acta, 1176*, 192–196.

Frei, B., Stocker, R., England, L., & Ames, B. N. (1990). Ascorbate: the most effective antioxidant in the blood plasma. *Advances in Experimental Medicine and Biology, 264*, 155–163.

Gimeno, A., Zaragozá, R., Vina, J. R., & Miralles, V. J. (2004). Vitamin E activates CRABP-II gene expression in cultured human fibroblasts, role of protein kinase C. *FEBS Letters, 569*, 240–244.

Green, P. S., & Simpkins, J. W. (2000). Estrogens and estrogen-like non-feminizing compounds. Their role in the prevention and treatment of Alzheimer's disease. *Annals of the New York Academy of Sciences, 924*, 93–98.

Gridley, K. E., Green, P. S., & Simpkins, J. W. (1997). Low concentrations of estradiol reduce beta-amyloid (25–35) – induced toxicity, lipid peroxidation and glucose utilization in human SK–N–SH neuroblastoma cells. *Brain Research, 778*, 158–165.

Gridley, K. E., Green, P. S., & Simpkins, J. W. (1998). Nuclear estrogen receptor-independent neuroprotection by estratrienes: a novel interaction with glutathione. *Neuroscience, 84*, 7–10.

Halliwell, B. (2000). Why and how should we measure oxidative DNA damage in nutritional studies. How far have we come? *The American Journal of Clinical Nutrition, 72*, 1082–1087.

Hara, H., Kato, H., & Kogure, K. (1990). Protective effect of alpha-tocopherol on ischemic neuronal damage in the gerbil hippocampus. *Brain Research, 510*, 335–338.

Healy, S. D., Braham, S. R., & Braithwaite, V. A. (1999). Spatial working memory in rats: no differences between the sexes. *Proceedings, Biological Sciences/The Royal Society, 266*, 2303–2308.

Inal, M., Sunal, E., Kanbak, G., & Zeytinoglu, S. (1997). Effect of postmenopausal hormone replacement and alpha-tocopherol on the lipid profiles and antioxidant satatus. *Clinica Chimica Acta, 268*, 21–29.

Iwasaki, K., Hatip-Al-Khatib, I., Egashira, N., Akiyoshi, Y., Arai, T., Mishima, K., et al. (2004). Ovariectomy combined with amyloid beta (1–42) impairs memory by decreasing acetylcholine release and Alpha7nAChR expression without induction of apoptosis in the hippocampus CA1 neurons of rats. *Neurotoxicity Research, 6*, 299–309.

Marsh, S. A., Laursen, P. B., Pat, B. K., Gobe, G. C., & Coombes, J. S. (2005). Bcl-2 in endothelial cells is increased by vitamin E and α-lipoic acid supplementation but not exercise training. *Journal of Molecular and Cellular Cardiology, 38*, 445–451.

McCay, P. B. (1985). Vitamin E: interactions with free radical and ascorbate. *Annual Review of Nutrition, 5*, 323–340.

McSorley, P. T., Young, I. S., Bell, P. M., Fee, J. P., & McCance, D. R. (2003). Vitamin C improves endothelial function in healthy estrogen-deficient postmenopausal women. *Climacteric: The Journal of the International Menopause Society, 6*, 238–247.

Mecocci, P., Mariani, E., Cornacchiola, V., & Polidori, M. C. (2004). Antioxidants for the treatment of mild cognitive impairment. *Neurological Research, 26*, 598–602.

Moreau, K. L., Gavin, K. M., Plum, A. E., & Seals, D. R. (2005). Ascorbic acid selectively improves large elastic artery compliance in postmenopausal women. *Hypertension, 45*, 1107–1112.

Morris, R. G., Garrud, P., Rawlins, J. N., & O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature, 297*, 681–683.

Mukai, K., Daifuku, K., Yokoyama, S., & Nakano, M. (1990). Stopped-flow investigation of antioxidant activity of estrogens in solution. *Biochimica Biophysica Acta, 1035*, 348–352.

Murakami, S., & Murakami, H. (2005). The effects of aging and oxidative stress on learning behavior in *C. elegans*. *Neurobiology of Aging, 26*, 899–905.

Netto, C. A., Dias, R. D., & Izquierdo, I. (1986). Differential effect of posttraining naloxone, beta-endorphin, leu-enkephalin and electroconvulsive shock administration upon memory of an open-field habituation and of a water-finding task. *Psychoneuroendocrinology, 11*, 437–446.

Netto, C. A., Hodges, H., Sinden, J. D., Le Peillet, E., Kershaw, T., Sowinski, P., et al. (1993). Effects of fetal hippocampal field grafts on ischaemic-induced deficits in spatial navigation in the water maze. *Neuroscience, 54*, 69–92.

Noh, S. K., Koo, S. I., & Jeon, I. J. (1999). Estradiol replacement in ovariectomized rats increases the hepatic concentration and biliary secretion of alpha-tocopherol and polyunsaturated fatty acids. *The Journal of Nutritional Biochemistry, 10*, 110–117.

Petersen, R. C., Thomas, R. G., Grundman, M., Bennet, D., Doody, R., Ferrs, S., et al. (2005). Alzheimer's Disease Cooperative Study Group. Vitamin E and donepezil for the treatment of mild cognitive impairment. *The New England Journal of Medicine, 352*, 2379–2388.

Pinkerton, J. V., & Henderson, V. W. (2005). Estrogen and cognition, with a focus on Alzheimer's disease. *Seminars in reproductive medicine, 23*, 172–179.

Reis, E. A., Oliveira, L. S., Lammers, M. L., Netto, C. A., & Wyse, A. T. S. (2002a). Arginine administration inhibits hippocampal Na^+, K^+-ATPase activity and impairs retention of an inhibitory avoidance task in rats. *Brain Research, 951*, 151–157.

Reis, E. A., Zugno, A. I., Franzon, R., Tagliari, B., Matté, C., Lammers, M. L., et al. (2002b). Pretreatment with vitamins E and C prevent the impairment of memory caused by homocysteine administration in rats. *Metabolic Brain Disease, 17*, 211–217.

Rodrigues, H. D., Kinder, J. E., & Fitzpatrick, L. A. (1999). Treatment with 17 beta-estradiol does not influence age and weight at puberty in Bos indicus heifers. *Animal Reproduction Science, 56*, 1–10.

Sandstrom, N. J., & Williams, C. L. (2004). Spatial memory retention is enhanced by acute and continuous estradiol replacement. *Hormones and Behavior, 45*, 128–135.

Sano, M., Ernesto, C., Thomas, R. G., Klauber, M. R., Schafer, K., Grundman, M., et al. (1997). A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. *The Alzheimer's Disease Cooperative Study. New England Journal of Medicine, 336*, 1216–1222.

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

Singh, M., Meyer, E. M., Millard, W. J., & Simpkins, J. W. (1994). Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague–Dawley rats. *Brain Research, 644*, 305–312.

Silva, R. H., Abilio, V. C., Takatsu, A. L., Kameda, S. R., Grassl, C., Chehin, A. B., et al. (2004). Role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice. *Neuropharmacology, 46*, 895–903.

Tang, M. X., Jacobs, D., Stern, Y., Marder, K., Schofield, P., Gurland, B., et al. (1996). Effect of oestrogen during menopause on risk and age at onset of Alzeimer's disease. *Lancet, 348*, 429–432.

Tasinato, A., Boscoboinik, D., Bartoli, G. M., Maroni, P., & Azzi, A. (1995). d-α-Tocopherol inhibition of vascular smooth muscle cell proliferation occurs at physiological concentrations, correlates with protein kinase C inhibition, and is independent of its antioxidant properties. *Proceedings of the National Academy of Sciences of the USA, 92*, 12190–12194.

Van Duijn, C. M. (1999). Hormone replacement therapy and Alzeimer's disease. *Maturitas, 31*, 201–205.

Warren, S. G., & Juraska, J. M. (1997). Spatial and nonspatial learning across the rat estrous cycle. *Behavioral Neuroscience, 111*, 259–266.

Warren, S. G., & Juraska, J. M. (2000). Sex differences and estropausal phase effects on water maze performance in aged rats. *Neurobiology of Learning and Memory, 74*, 229–240.

Waynforth, H. B., & Flecknell, P. A. (1992). Experimental and Surgical Technique. In *The rat*second ed., pp. 276–278). London: Academic Press Limited.

White, E., Kristal, A. R., Shikani, J. M., Wilson, A. C., Chen, C., Mares-Perlman, J. A., et al. (2001). Correlates of serum alpha- and gamma-tocopherol in the Women's Health Initiative. *Annals of Epidemiology, 11*, 136–144.

Wise, P. M., Dubal, D. B., Wilson, M. E., Rau, S. W., & Liu, Y. (2001a). Estrogens: trophic and protective factors in the adult brain. *Neuroendocrinology, 22*, 33–66.

Wise, M. P., Dubal, D. B., Wilson, M. E., Rau, S. W., Böttner, M., & Rosewell, K. L. (2001b). Estradiol is a protective factor in the adult and aging brain: understanding of mechanism derived from in vivo and in vitro studies. *Brain Research Reviews, 37*, 313–319.

Wise, P. M. (2002). Estrogens and neuroprotection. *Trends in Endocrinology and Metabolism, 13*, 229–230.

Wyse, A. T. S., Zugno, A. I., Streck, E. L., Matté, C., Calcagnotto, T., Wannmacher, C. M. D., et al. (2003). Inhibition of Na^+, K^+-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. *Neurochemical Research, 27*, 1685–1689.

Yusuf, S., Dagenais, G., Pogue, J., Bosch, J., & Sleight, P. (2000). Vitamin E supplementation and cardiovascular events in high-risk patients: the Heart Outcomes Prevention Evaluation Study Investigators. *The New England Journal of Medicine, 342*, 154–160.

Zhang, Y. Q., Shi, J., Rajakumar, G., Day, A. L., & Simpkins, J. W. (1998). Effects of gender and estradiol treatment on focal brain ischemia. *Brain Research, 784*, 321–324.

Zingg, J. M., & Azzi, A. (2004). Non- antioxidant activities of vitamin E. *Current Medicinal Chemistry, 11*, 1113–1133.

III.5 Artigo 5

Ovariectomy impairs spatial memory: prevention and reversal by a soy isoflavone diet

Monteiro, SC, Mattos, CB, Ben, J, Netto, CA, and Wyse, ATS.

Periódico: Journal of Nutritional Biochemistry

Status: Submetido (o guia para autores do periódico Journal of Nutritional Biochemistry encontra-se no anexo 02)

OVARIECTOMY IMPAIRS SPATIAL MEMORY: PREVENTION AND REVERSAL BY A SOY ISOFLAVONE DIET

Siomara C. Monteiro, Cristiane B. de Mattos, Juliana Ben,

Carlos A. Netto and Angela T. S. Wyse.

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

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

Abstract

Since a previous study has shown that ovariectomy impairs spatial memory, we, herein, investigate the influence of pre- and post-treatment with a soy diet rich in isoflavones on the effects elicited by ovariectomy on spatial memory. Twenty-day-old female adult Wistar rats were first fed for 60 days on a standard diet with casein (control) or a soy diet rich in isoflavones. At eighty days of age, the animals were assigned to one of the following groups: sham (submitted to surgery without removal of ovaries) and ovariectomized. Seven days after surgery, the rats were submitted to behavioral testing. In another set of experiments, eighty-day-old female rats were assigned to one of the following groups: sham and ovariectomized. Seven days after surgery, animals were fed for 30 days with the same diet described above. Twelve hours after the last day of the diet, rats were submitted to water maze testing. Data demonstrate that the soy isoflavone-rich diet prevented and reversed the memory impairment caused by ovariectomy in female rats. Pre-treatment for two months before ovariectomy with the soy diet effectively prevented the increase in latency in finding the platform on the 5^{th} day of training in the ovariectomized group. Additionally, treatment with the soy isoflavone-rich diet for thirty days after ovariectomy reversed the increase in latency to find the platform in the ovariectomized group on the 5^{th} day of training, the decrease in the time spent in target quadrant, the increase in the time spent in opposite quadrant and the latency to cross the platform location. Results show that both pre- and post-treatment protected against the impairment of memory, caused by ovariectomy,

and that the dose of isoflavones used in our study was effective both in the long term (60 days) and in the short term (30 days). Based on these findings, we suggest that soy isoflavones may represent a novel therapeutic strategy to prevent or to treat cognitive symptoms found in some menopausal women.

Key words: Ovariectomy - Soy Isoflavones – Spatial Memory.

1. Introduction

Adult women with normal reproductive cycles secrete a great quantity of estrogenic compounds, mainly from the ovaries, with 17β-estradiol being the dominant form of estrogen in the body [1]. Estrogen also exerts diverse non-reproductive actions on multiple organs, such as the brain [2], and it has been shown that estrogen deprivation is implicated in the pathogenesis of neurodegenerative conditions, such as Alzheimer´s disease and cerebral ischemia [3-5]. Evidences consistently suggest that postmenopausal women are more likely than man or younger women to present cognitive deficits and neurodegenerative diseases [6-8].

Hormone replacement therapy (HRT), in the form of estrogen and progesterone or estrogen alone, has been used to treat menopause symptoms and other similar conditions. However, due to the possible side effects of HRT, such as breast cancer and the increased risk of thromboembolic accidents [9], there is a growing demand for alternatives for the treatment of pathological processes and symptoms associated with menopause. In order to protect against injurious effects, nutritional supplements have been studied to substitute HRT. In this context, there is evidence to demonstrate that phytoestrogens, such as isoflavones, could be a good alternative to substitute the synthetic estrogens [10].

Isoflavones are compounds with estrogenic activity (phytoestrogens) found almost exclusively in soybeans and in a few other legumes. The principal isoflavones are genistein, daidzein and glycitein [11]. These molecules/compounds are structurally and functionally similar to estradiol and,

thus, have many of the physicochemical and physiological properties of the estrogens [12-15], including the ability to selectively bind estrogen receptors, particularly the estrogen receptor β (ERβ) [16]. Reports from the literature indicate that soy isoflavones can have agonist or antagonist estrogenic actions, depending on the dose and the tissue specific targets [17]. Conversely, it has also shown that soy isoflavones have antioxidant activities [18-20]. Although very little is known about the potential effect of these compounds on learning/memory, clinical and preclinical studies suggest that soy isoflavones can improve cognitive function in humans and rats [21,22].

Since we recently demonstrated that ovariectomized rats present an impairment of spatial navigation memory [23], we decided to investigate the influence of pre- and post-treatment with soy isoflavones on the spatial memory alterations elicited by ovariectomy. Our hypothesis is that soy isoflavones may prevent and reverse the memory deficit caused by ovariectomy.

2. Materials and methods

2.1. Animals and Reagents

Female adult Wistar rats, obtained from the Central Animal House of the Biochemistry Department, Institute of Basic Health Sciences, at the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, were housed in groups of eight with their mothers on the day of birth. Half of them were assigned to the experimental condition and the other half served as controls; animals were weaned at 21 days after birth. Rats were maintained on a 12:12 h

light/dark cycle in an air-conditioned constant-temperature (22^{o}C) colony room, with free access to water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and the study was approved by the Ethical Committee of Universidade Federal do Rio Grande do Sul.

Casein (87% pure) was from Farmaquímica, Porto Alegre, Brazil, supplemented with 0.15% L-methionine (from Merck, Rio de Janeiro, Brazil), a mixture of minerals and vitamins (from Roche, São Paulo, Brazil) and Samprosoy 90 LH (generously supplied by EMBRAPA, Brazil). All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Experimental treatment

2.2.1. Soy isoflavone diet

The diet contained an isoflavone mixture that included Daidzein (0.25 mg/g soy protein) and Genistein (0.23 mg/g soy protein). The soy protein dose was chosen according to a protocol established by Reeves and colleagues [24]. Food intake and body weight were examined weekly. Both diets were isocaloric (Table 1).

2.2.2. Pre-treatment with soy isoflavones

Twenty-day-old female rats (10-14 animals in each group) were fed for 60 days on a standard diet with casein (control group) or a soy diet rich in isoflavones (soy-treated group). At eighty days of age the animals were randomly assigned to one of the following groups: sham (only submitted to

surgery, without removal of the ovaries) and ovariectomized. One week after ovariectomy, rats were submitted to behavioral testing.

2.2.3. Post-treatment with soy isoflavones

Eighty-day-old female rats (11-14 animals in each group) were randomly assigned to one of the following groups: sham (only submitted to surgery, without removal of the ovaries) and ovariectomized. Seven days after surgery, animals were fed for 30 days on a standard diet with casein (control group) or a soy diet rich in isoflavones (soy-treated group). Then, the rats were submitted to water maze testing.

2.3. Surgical procedures

Animals were ovariectomized by the surgical removal of both ovaries under intraperitoneous (i.p.) ketamine anesthesia (90 mg/kg) and xylazine (10 mg/kg) to eliminate endogenous ovarian steroids [25]. The stage of the estrous cycle was determined by vaginal swabs for 10 days prior to ovariectomy, to ensure that animals were cycling normally [26].

2.4. Behavioral procedures

We used the Morris water maze, an apparatus widely employed for the study of spatial learning and memory tasks that depend on hippocampal function [27-29].

The water maze consisted of a black round tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm with water and maintained at a constant temperature of 23^{o}C. The tank was theoretically divided into four equal

quadrants for the purpose of analysis. Several distal visual cues were placed on the walls of the room. Trials were recorded by a video camera mounted above the center of the tank.

Reference memory task. The task consisted of 6 training sessions and one test session. In the acquisition phase, rats had daily sessions of 4 trials per day for 5 days to find the platform, submerged 2 cm under the water surface, placed on the center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in the water facing the tank wall, in one of the 4 starting locations (N, S, W and E). The order of the starting position varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failing to find it, they were gently guided to it; all animals were permitted to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was 15-20 min [29]. One day after the last training trial, each rat was subjected to a probe trial in which the platform was removed. We measured four parameters, namely latency to cross on the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in the training sessions) and opposite quadrants. These parameters were taken as a measurement of spatial memory [29].

In order to detect motor impairments that could affect performance in experimental groups, the swimming speed was calculated by taking the distance traveled in the first 15 s of the probe trial. This result is in agreement with our previous study [23].

2.5. Statistical analysis

Reference memory training data was analyzed by repeated measures analysis of variance (ANOVA) and data from the probe trial parameters were analyzed by one-way ANOVA; a post hoc Duncan multiple range test was run when indicated. Body weight was analyzed by Student's *t* test or one-way ANOVA. Descriptive statistics data were expressed as mean $\pm$ SEM. Type error rate was set at 0.05 for determining statistical significance, where the multiple range test was used for post hoc testing. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, using a PC-compatible computer.

3. Results

We, first, investigated the effect of pre-treatment with the soy diet rich in isoflavones on the spatial memory acquisition phase of the Morris Water maze. Figure 1 shows that ovariectomized animals have a limited ability to find the platform and that the soy isoflavones diet *per se* did not alter the performance of rats, but prevented the increase in latency to find the platform in the ovariectomized group on the 5^{th} day of training [$F(3,40=6.97; p<0.001$]. Four parameters were evaluated in the test session, namely the latency to cross and the number of crossings on the location of the platform and the time spent in the target and opposite quadrants. The pre-treatment wasn't effective to prevent the impairment caused by ovariectomy in test session paramenters (data not shown).

Next, we investigated the effect of post-treatment with the soy diet rich in isoflavones on the same behavioral spatial tasks. Figure 2 shows that soy supplementation for 30 days after ovariectomy, *per se,* did not alter the performance of rats, but reversed the increase in latency to find the platform in the ovariectomized group on the 5th day of training [F(3,45=4.26; p<0.01]. In the test session, post-treatment with the soy diet effectively reversed the decrease in the time spent in the target quadrant [F(3,45)=16.54; p<0.001] (Figure 3A), the increase in the time spent in the opposite quadrant [F(3,45)=20.03; p<0.001] (Figure 3B) and the latency to cross the platform location [F(3,45)=4.03; p<0.05] (Figure 3C).

Table 2 shows that the body weight of rats fed for 60 days on the soy isoflavone diet did not differ significantly from the casein diet group, both on the first day of the diet [*t*(45)=0.541; p>0.05] and after 60 days of the diet [*t(*45)=0.519; p>0.05], suggesting that the soy isoflavone diet did not cause malnutrition in the animals. Additionally, at thirty days after ovariectomy, the rats presented a weight gain when compared to the sham group [F(3,42]=49.69; p<0.001)]. *Post hoc* analysis showed that the soy isoflavone diet did not reverse the weight gain observed in ovariectomized rats, as compared to those that received the casein diet (Table 3).

4. Discussion

Menopause has been associated with memory loss and with the development of cognitive dysfunction [30,31]. There is increasing evidence to suggest that estrogen is involved in neuronal plasticity, including increased hippocampus CA1 dendritic spine density [32], enhanced long-term potentiation [33,34], increased neurogenesis [35]. More recently, Xu and Zhang [36] have shown that long-term estradiol administration improves spatial memory in ovariectomized mice. In agreement with these data, clinical studies also indicate that HRT could delay the age-related cognitive decline in post-menopausal women [37]. Conversely, since estrogen replacement increases the risk of some cancers, HRT is not completely safe or effective as previously thought [38], the interest in therapies that retain the beneficial effects of estrogens without their adverse effects has been increased.

Isoflavones are phytoestrogens of particular interest since, like endogeneous estrogens, they enter in various brain regions abundant in ERβ [39,40], which are associated with neuroprotection [41]. It has been shown that phytoestrogens can be used as dietary supplements by peri and post-menopausal women as an alternative to HRT [10,42]; however, whether alternative soy isoflavones are beneficial to memory functioning and when soy diet supplementation should be initiated are questions that remain to be answered.

Since previous studies show that ovariectomized rats present an impairment of spatial memory [23,43,44], in the present study we investigated the influence of the soy isoflavone-rich diet on the spatial memory deficit caused by ovariectomy, using two different time protocols. This experimental condition of hormone deprivation was used because ovariectomy is the most common

animal model of postmenopausal changes in adult female rats [45]. We explored the different treatments to provide evidence to justify the use of soy isoflavones in the diet of women to protect or to ameliorate menopausal symptoms. Results show that a diet with soy, rich in isoflavones, effectively prevented and reversed the performance impairment caused by ovariectomy in the acquisition phase (Figure 1 and 2). Interestingly, treatment following ovariectomy with this diet more effective to reverse the decrease in the time spent in the target quadrant, as well as the increase in the time spent in the opposite quadrant to the platform and the latency in the time spent to cross the location of the platform, observed in the ovariectomized group (Figure 3).

Some reports have examined the influence of isoflavones on brain [22,46,47]; our results are in agreement with previous studies that show that soy isoflavones can influence learning and memory tasks. In this context, studies show that soy has a positive influence on cognitive ability in elderly male rats [48], visual spatial memory in female rats [39], as well as on cognitive deficits observed in some post-menopausal women [49].

We observed, in the present study, that ovariectomized rats presented an increase in body weight, as compared with the sham group. This result is agreement with previous studies showing that ovariectomy increases body weight [23,50,51]. No statistically significant differences were observed in food intake and body weight gain between the casein group (control) and the group treated with soy isoflavones, indicating that the soy isoflavone diet did not reverse the weight gain increase in ovariectomized rats, as compared to rats that received the casein diet. These findings agree with other studies, which

show that isoflavones did not alter the body weight gain provoked by ovariectomy [48,52]

In conclusion, our results show that the impairment in spatial navigation, caused by ovariectomy, was prevented and reversed by a soy isoflavone-rich diet. Both protocols efficiently protected against memory deficits. The preferred interpretation to our findings is that the dose of isoflavones used in this study is effective during both long-term and short-term supplementation. Assuming that hormone deprivation might also impair cognition in human beings, our results lend support to a novel therapeutics strategy, based on soy diet supplementation, to prevent or to reverse the cognitive deficits observed in post-menopausal women.

Acknowledgements

This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil).

References

[1] Rodrigues HD, Kinder JE, Fitzpatrick LA. Treatment with 17 beta-estradiol does not influence age and weight at puberty in Bos indicus heifers. Anim Reprod Sci. 1999;56:1-10.

[2] Wise PM. Estrogens and neuroprotection. Trends Endocrinol Metab. 2002;13: 229-230.

[3] Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H, Mayeux R. Effect of oestrogen during menopause on risk and age at onset of Alzeimer's disease. Lancet. 1996;348:429-432.

[4] Van Duijn CM. Hormone replacement therapy and Alzeimer's disease. Maturitas. 1999;31:201-205.

[5] Zhang YQ, Shi J, Rajakumar G, Day AL, Simpkins JW. Effects of gender and estradiol treatment on focal brain ischemia. Brain Res. 1998;784:321-324.

[6] Green PS, Simpkins JW. Neuroprotective effects of estrogens: potential mechanisms of action. Int J Devl Neurosci. 2000;18:347-358.

[7] Wise PM, Dubal DB, Wilson ME, Rau SW, Liu Y. Estrogens: trophic and protective factors in the adult brain. Front Neuroendocrinol. 2001;22:33-66. (a)

[8] Wise MP, Dubal DB, Wilson ME, Rau SW, Böttner M, Rosewell KL. Estradiol is a protective factor in the adult and aging brain: understanding of mechanism derived from in vivo and in vitro studies. Brain Res Ver. 2001;37:313-319. (b)

[09] Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J, Writing Group for the Women's Health Initiative Investigators. Risk and benefits of estrogen plus progestin in healthy postmenopausal women. J Am Med Assoc. 2002;288:321-333.

[10] Miquel J, Ramírez-Boscá A, Ramírez-Boscá JV, Alperi JD. Menopause: a review on the role of oxygen stress and favorable effects of dietary antioxidants. Arch Gerontol Geriatr. 2006;42:289-306.

[11] Sirtori CR, Arnoldi A, Johnson SK. Phytoestrogens: End of a tale? Ann Med. 2005;37:423-438.

[12] Adlercreutz H. Epidemiology of phytoestrogens. Baillieres Clin Endocrinol Metab. 1998;12;605-623.

[13] Glazier MG, Bowman MA. A review of the evidence for the use of phytoestrogens as a replacement for traditional estrogen replacement therapy. Arch Intern Med. 2001;161(9):1161-1172.

[14] Setchell KDR, Adlercreutz H. Mammalian ligants and phyto-oestrogens recent studies on their formation, metabolism and biological role in health and disease. In: Rowland IR, editor. Role of the Gut Flora in Toxicity and Cancer. New York: Academic Press; 1988. pp. 315-345.

[15] Setchell KDR. Phytoestrogens: The biochemistry, physiology and implications for human health of soy isoflavones. Am j Clin Nutr. 1998;68(6):1333S-1346S.

[16] Kuiper GG, Lemmen, JG, Carlsson B, Corton JC, Safe SH, Van der Saag PT, Van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 1998;139(10):4252-4263.

[17] Miksicek RJ. Estrogenic flavonoids: structural requirements for biological activity. Proc Soc Exp Biol Med. 1995;208:44-50.

[18] Soulsby ME, Phillips B, Chowdhury P. Brief communication: Effects of soy-protein diet on elevated brain lipid peroxide levels induced by simulated weightlessness. Ann Clin Lab Sci. 2004;34(1):103-106.

[19] Yousef MI, Kamel KI, Esmail AM, Baghdadi HH. Antioxidant activities and lipid lowering effects of isoflavone in male rabbits. Food Chem Toxicol. 2004;42(9):1497-1503.

[20] Geller SE, Studee L. Soy and red clover for mid-life and aging. Climateric. 2006;9(4):245-63.

[21] File SE, Jarrett N, Fluck E, Duffy R, Casey K, Wiseman H. Eating soya phytoestrogens improves human memory. Psychopharmacology. 2001;157:430-436.

[22] Pan Y, Anthony M, Clarkson TB. Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. Pro Soc Exp Biol Med. 1999;221:118-125.

[23] Monteiro SC, Matté C, Bavaresco CS, Netto CA, Wyse ATS. Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. Neurobiol Learn Mem. 2005;84:192-199.

[24] Reeves PG, Nielsen FH, Fabey Jr, GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 1993;123:1939-1951.

[25] Waynforth HB, Flecknell PA. Experimental and Surgical Technique. In: The rat. 2nd ed. London: Academic Press Limited; 1992. pp. 276-278.

[26] Baker HJ, Lindsey JR, Weisbroth SH. The Laboratory Rat. In: Biology and Diseases. vol 1. New York, NY, USA: Academic Press; 1979.

[27] D'Hooge R, De Deyn PP. Applications of the Morris water maze in the study of learning and memory. Brain Res Brain Res Rev. 2001;36:60-90.

[28] Morris RG, Garrud P, Rawlins JN, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. Nature. 1982; 297:681-683.

[29] Netto CA, Hodges H, Sinden JD, Le Peillet E, Kershaw T, Sowinski P, Meldrum BS, Gray, JA. Effects of fetal hippocampal field grafts on ischaemic-induced deficits in spatial navigation in the water maze. Neuroscience. 1993;54:69-92.

[30] Henderson VW. Estrogen, cognition and woman's risk of Alzheimer's disease. The Am J Med. 1997;103:11-18.

[31] Halbreich U, Lumley LA, Palter S, Manning C, Gengo F, Joe SH. Possible acceleration of age effects on cognition following menopause. J Psychiatr Res. 1995;29:153-163.

[32] Woolley CS, McEwen BS. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J. Comp Neurol.1993;336:293-306.

[33] Foy MR, Xu J, Xie X, Brinton RD, Thompson RF, Berger TW. 17β-Estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J Neurophysiol. 1999;81:925-929.

[34] Warren SG, Humphreys AG, Juraska JM, Greenough WT. LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. Brain Res. 1995;703:26-30.

[35] Tanapat P, Hastings NB, Reeves AJ, Gould E. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci. 1999;19:5792-5801.

[36] Xu X, Zhang Z. Effects of estradiol benzoato on learning-memory behavior and synaptic structure in ovariectomized mice. Life Sci. 2006;79:1553-1560.

[37] Sherwin BB. Estrogen and cognitive functioning in women. Endocr Ver. 2003;24:133-151.

[38] Hays J, Ockene JK, Brunner RL , Kotchen JM, Manson JE, Patterson RE, Aragaki AK, Shumaker SA, Brzyski RG, LaCroix AZ, Granek IA, Valanis BG. Women's Health Initiative Investigators. Effects of estrogen plus progestin on health-related quality of life. N Engl J Med. 2003;348:1839-1854.

[39] Lund TD, West TW, Tian Y, Bu LH, Simmons DL, Setchell KDR, Adlercreutz H, Lephart ED. Visual spatial memory is enhanced in female rats (but inhibited in males) by dietary soy phytoestrogens. BMC Neurosci. 2001;2:20.

[40] Patisaul HB, Melby M, Whitten PL, Young LJ. Genistein affects ERβ- but not ERα-dependent gene expression in the hypothalamus. Endocrinology. 2002;143:2189-2197.

[41] Sawada H, Ibi M, Kihara T, Urushitani M, Akaike A, Shimohama S. Estradiol protects mesencephalic dopaminergic neurons from oxidative stress-induced neuronal death. J Neurosci Res. 1998;54:707-719.

[42] Lephart ED, West TW, Weber KS, Rhees RW, Setchell KDR, Adlercreutz H, Lund TD. Neurobehavioral effects of dietary soy phytoestrogens. Neurotoxicol Teratol. 2002;24:5-16.

[43] Feng Z, Cheng Y, Zhang J. Long-term effects of melatonin or 17β-estradiol on improving spatial memory performance in cognitively impaired, ovariectomized adult rats. J Pineal Res. 2004;37:198-206.

[44] Singh M, Meyer EM, Millard WJ, Simpkins JW. Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. Brain Res. 1994;644:305-312.

[45] Savonenko AV, Markowska AL. The cognitive effects of ovariectomy and estrogen replacement are modulated by aging. Neuroscience. 2003;119:821-830.

[46] Lephart ED, Rhees RW, Setchell KDR, Bu LH, Lund TD. Estrogens and phytoestrogens: brain plasticity of sexually dimorphic brain volumes. J Steroid Biochem Mol Biol. 2003;85:299-309.

[47] Fader AJ, Johnson PE, Dohanich, GP. Estrogen improves working but not reference memory and prevents amnestic effects of scopolamine of a radial-arm maze. Pharmacol Biochem Behav. 1999;62:711-717.

[48] Lee YB, Lee HJ, Won MH, Hwang IK, Kang TC, Lee JY, Nam SY, Kim SY, Kim KS, Sohn HS. Soy isoflavones improve spatial delayed matching-to-place performance and reduce cholinergic neuron loss in elderly male rats. J Nutr. 2004;134:1827-1831.

[49] Kritz-Silverstein D, Von Muhlen D, Barrett-Connor E, Bressel MA. Isoflavones and cognitive function in older women: the soy and postmenopausal health in aging (SOPHIA) study. Menopause. 2003;10:196-202.

[50] Torto R, Boghossian S, Dube MG, Kalra PS, Kalra SP. Central leptin gene therapy blocks ovariectomy-induced adiposity. Obesity (Silver Spring). 2006; 14(8):1312-1319.

[51] Iwamoto J, Takeda T, Sato Y, Shen CL, Yeh JK. Effect of pre- and post-surgery treatment with risedronate on trabecular bone loss in ovariectomized rats. Exp Anim. 2006;55(5):457-466.

[52] Choi EJ, Lee BH, Lee k, Chee KM. Long-term combined administration of quercetin and daidzein inhibits quercetin-induced suppression of glutathione antioxidant defenses. Food Chem Toxicol. 2005;43:793-798.

Figure 1 – Effect of ovariectomy and pre-treatment with a soy diet rich in isoflavones on performance of spatial memory acquisition phase. Data are expressed as means ± S.E.M for 10-14 independent animals in each group. *** $p<0.001$ compared to other groups (sham casein, sham isoflavone and ovx isoflavone) (ANOVA). Ovx, ovariectomized; cas, casein; isofl, isoflavones.

Figure 2 – Effect of ovariectomy and post-treatment with a soy diet rich in isoflavones on performance of spatial memory acquisition phase. Data are expressed as means ± S.E.M for 11-14 independent animals in each group. ** $p<0.01$ compared to other groups (sham casein, sham isoflavone and ovx isoflavone) (ANOVA). Ovx, ovariectomized; cas, casein; isofl, isoflavones.

Figure 3 - Effect of ovariectomy and post-treatment with a soy diet rich in isoflavones on performance of spatial memory test session parameters, namely time spent in the target quadrant (A), time spent in the opposite quadrant (B) and the latency to cross on the location of the platform (C). Data are expressed as means ± S.E.M for 11-14 independent animals in each group. *** $p<0.01$ and * $p<0.05$ compared to other groups (sham casein, sham isoflavone and ovx isoflavone) (ANOVA). Ovx, ovariectomized; cas, casein; isofl, isoflavones.

Table 1 – Nutritional composition of the diets (g/kg).

	Casein group	Isoflavone group
Casein (87% protein) $^{\phi}$	211	-
Soy rich in isoflavones	-	206
Sucrose	100	100
Fat (corn oil)	70	70
Salt mix *	35	35
Vitamin mix $^{\#}$	12.5	12.5
Non-nutritive fiber	50	50
Metionine	1.5	1.5
BHT	0.014	0.014
Carbohydrate (corn starch)	520	525

$^{\phi}$ Casein, pure 87% (from Farmaquímica, Porto Alegre, Brazil).

* Salt and $^{\#}$ vitamin composition are according to Horwitz (Horwitz W. Official methods of analysis of the Association of Official Analytical Chemists. Association of Official Analytical Chemists, Washington, D.C., 1980.

* Mineral mixture (from Roche, São Paulo, Brazil; mg/100g of ration): 557 NaCl; 3.2 KCl; 1556 KH_2PO_4; 229 $MgSO_4$; 1536 $CaCO_3$; 108 $FeSO_4.7H_2O$; 16 $MnSO_4.H_2O$; 2.2 $ZnSO_4.7H_2O$; 1.9 $CuSO_4.5H_2O$; 0.09 $CaCl_2.6H_2O$.

Vitamin mixture (from Roche, São Paulo, Brazil; mg/100g of ration): 4 vitamin A; 0.5 vitamin D; 10 vitamin E; 0.5 menadione; 200 choline; 10 *p*-aminobenzoic acid (PABA); 10 inositol; 4 niacin; 4 pantothenic acid; 0.8 riboflavin; 0.5 thiamin; 0.5 pyridoxine; 0.2 folic acid; 0.04 biotin; 0.003 vitamin B12.

Energy for both diets was 4.3 kcal/g of diet.

Table 2 - Effect of the pre-treatment with the isocaloric diets on body weight (g) and food consumption (g/day) of female rats.

	body weight		food consumption
Groups	1st day of diet 20-day-old female rats	after 60 days of diet 80-day-old female rats	
Casein	106.43±1.74	189.59±1.74	17.50±0.31
Isoflavone	105.25±1.35	191.21±1.77	17.22±0.37

Data are presented as mean ± S.E.M. for 10-14 rats in each group. There was no significant difference between groups (Student's *t* test).

Table 3 - Effect of ovariectomy and the post-treatment with soy isoflavones on body weight (g) and food consumption (g/day) of female adult rats after 30 days of diet.

	body weight		food consumption
Groups	1st day of diet	after 30 days of diet	
Sham casein	186.45±2.69	206.45±1.58	24.60±0.87
Sham isoflavone	189.75±2.11	209.50±1.94	23.91±0.63
Ovx casein	192.73±1.88	236.00±3.33***	24.25±0.57
Ovx isoflavone	192.67±2.87	240.67±2.82***	23.76±0.59

Data are presented as mean ± S.E.M. for 11-14 rats in each group. Ovariectomized rats (Ovx) were significantly different from sham groups after 30 days of diet, ***p<0.001 (ANOVA).

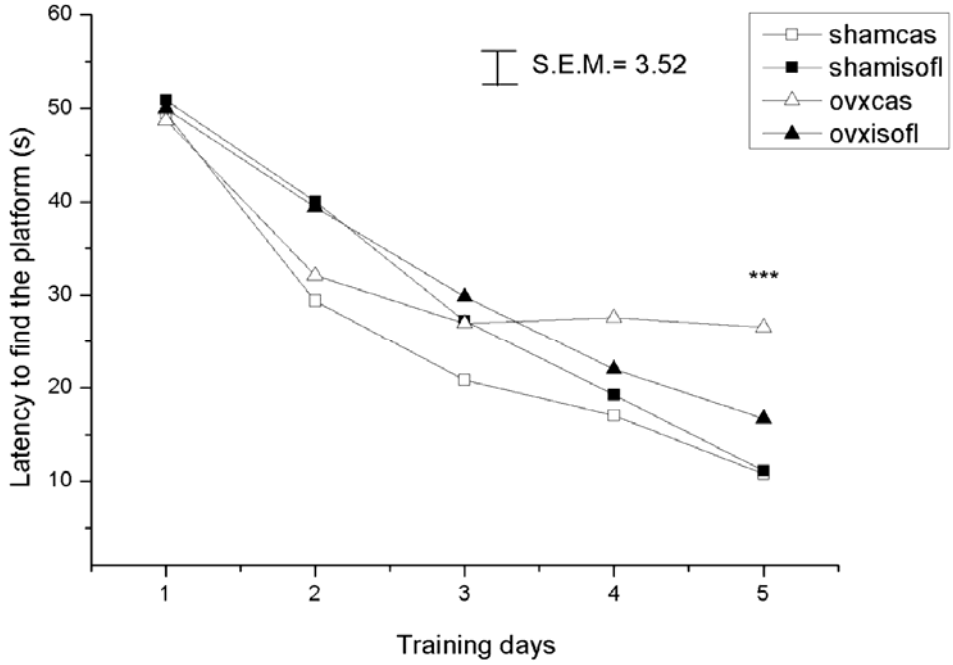

60
50
40
30
20
10
Latency to find the platform (s)
S.E.M.= 3.52
shamcas
shamisofl
ovxcas
ovxisofl
***
1
2
3
4
5
Training days

Figure 1

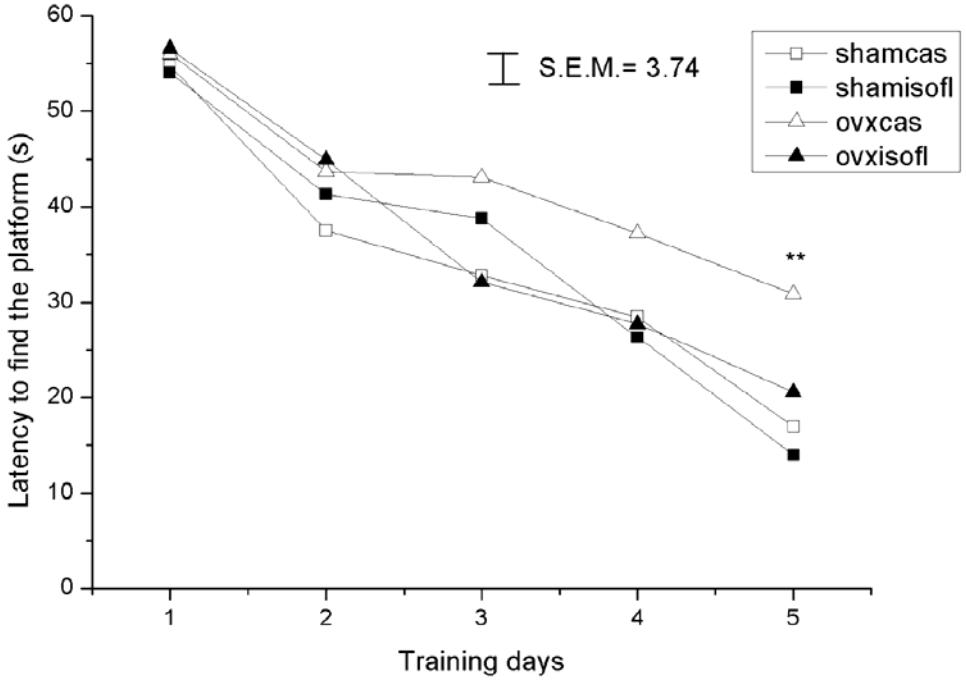

Latency to find the platform (s)
Training days
S.E.M.= 3.74
shamcas
shamisofl
ovxcas
ovxisofl
**
0
10
20
30
40
50
60
1
2
3
4
5

Figure 2

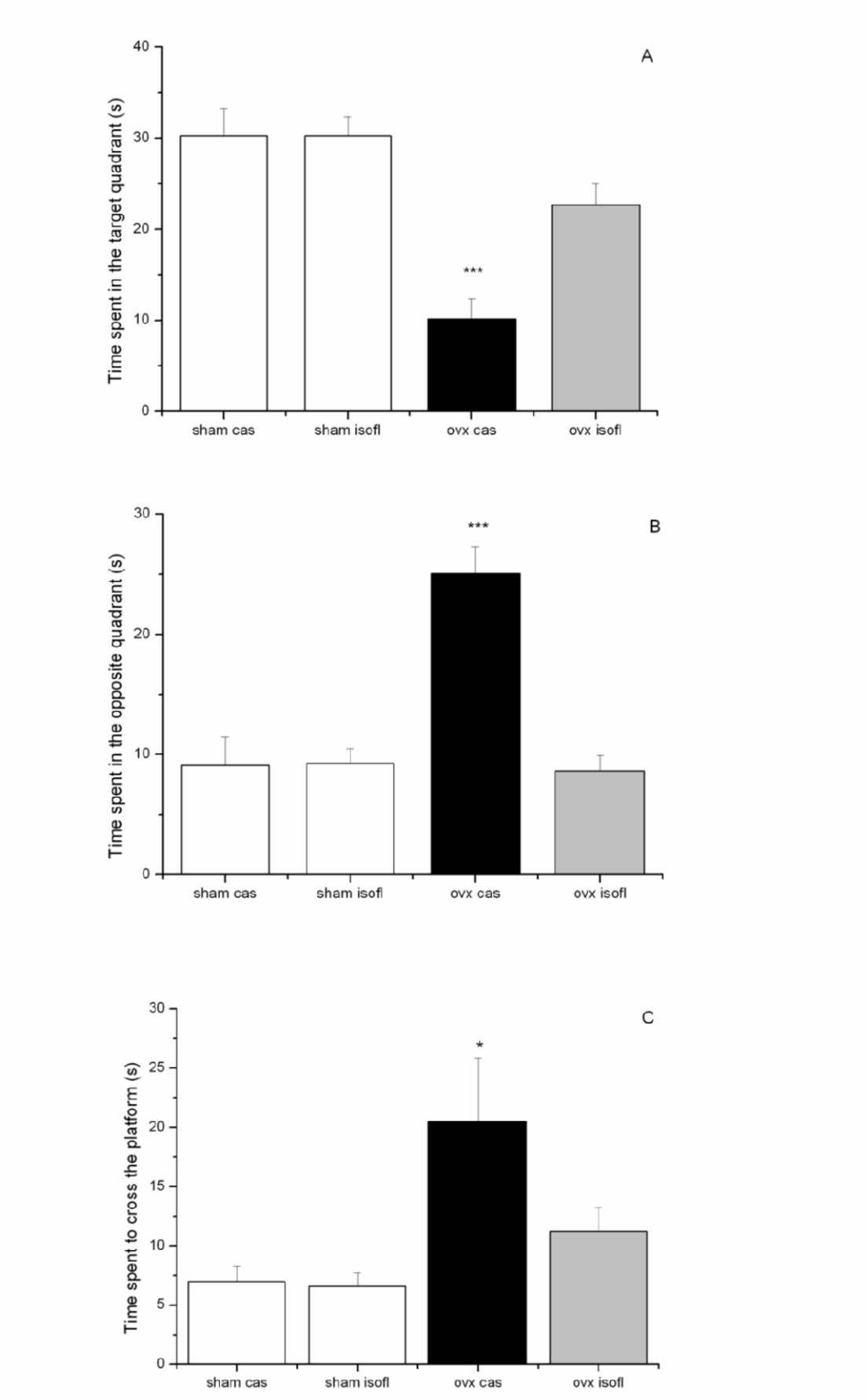

A
Time spent in the target quadrant (s)
40
30
20
10
0
***
sham cas
sham isofl
ovx cas
ovx isofl
B
Time spent in the opposite quadrant (s)
30
20
10
0
***
sham cas
sham isofl
ovx cas
ovx isofl
C
Time spent to cross the platform (s)
30
25
20
15
10
5
0
*
sham cas
sham isofl
ovx cas
ovx isofl

Figure 3

III.6 Artigo 6

Change in hippocampal pGluR-2/3 AMPA subunit following ovariectomy is reversed by vitamin E plus C

Monteiro, SC, Cammarota, M, Izquierdo, I, Silva, AFS, Bevilaqua, LMR and Wyse, ATS.

Periódico: Neuroscience.

Status: Submetido (o guia para autores do periódico Neuroscience Biochemistry encontra-se no anexo 02).

Objetivos

1. Investigar o efeito da ovariectomia e da administração das vitaminas E e C sobre a fosforilação de diferentes subunidades do receptor AMPA em homogeneizado de hipocampo de ratas adultas.
2. Avaliar o efeito da ovariectomia e posterior tratamento com as vitaminas E e C sobre a possível modulação da via de sinalização ERK1/2 – CREB em hipocampo de ratas adultas.

CHANGE IN HIPPOCAMPAL pGLUR-2/3 AMPA SUBUNIT FOLLOWING OVARIECTOMY IS REVERSED BY VITAMIN E PLUS C

Siomara C. Monteiro [1], Martín Cammarota[2], Iván Izquierdo[2], Ana Flávia Shürmann da Silva [2], Lia R. M. Bevilaqua [2] and Angela T. S. Wyse [1].

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

[2] Centro de Memória, Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil.

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

Abstract

Estrogen deprivation has been implicated in the pathogenesis of different neurodegenerative conditions, such as Alzheimer´s disease and cerebral ischemia. It is known that estrogenic steroids modify the biochemical and morphological properties of neuronal cells in different areas of the brain. Hippocampal functionality is modulated by estrogens and it is know that these hormones have profound impact in plasticity, learning and memory, maybe controlling state of signiling pathways that can be altered by the deleterious effects of oxidative stress. Recently, we demonstrated that vitamins E plus C reversed the memory impairment caused by ovariectomy in female adult rats. In this study, we investigate the effect of ovariectomy and the administration of vitamins E plus C on the phosphorilation of different AMPAr subunits and on the possible modulation of the ERK1/2 – CREB signaling pathway in the hippocampus. Our results show that ovariectomy significantly increases the phosphorilation of AMPAr subunit pGLU 2/3 Ser 880/891 and the treatment with vitamins E plus C reverts such effect. Do not find any modification in the levels of other phosphorilated subunits of AMPAr as pGLUR1 Ser 845, pGLUR1 Ser 831 and pGLUR1 Ser 836. Besides, no changes were found in the levels of pERK1/2 and pCREB between groups showing that ovariectomy and the treatment with these antioxidants were unable to alter the ERK1/2 – CREB signaling pathway in the hippocampus. The modulation of NMDA receptor is a necessary upstream step for the activation of cellular cascades during learning. Assuming the possibility that these phenomena may occur in humans, our

findings may lead to the development of a novel therapeutic strategy, based on antioxidants, to reverse cognitive deficits found in postmenopausal women.

Key words: ovariectomy; Vitamins E plus C; AMPA

Introduction

Adult women with normal reproductive cycles secrete a great quantity of estrogenic compounds, mainly from the ovaries (Rodrigues et al., 1999). In addition to its role in reproduction, estrogen also exerts diverse non-reproductive actions on multiple organs, including the brain (Wise, 2002). Actually, estrogen deprivation has been implicated in the pathogenesis of different neurodegenerative conditions, such as Alzheimer´s disease and cerebral ischemia (Tang et al., 1996; Zhang et al., 1998). It is known that estrogenic steroids modify the biochemical and morphological properties of neuronal cells in different areas of the brain (Wong et al., 1996; Brinton, 2001; Murphy and Andrews, 2000). For example, hippocampal functionality is modulated by estrogens in adult female rats and it is known that these hormones have profound impact in plasticity and learning and memory (Wise et al., 2001; Daniel and Dohanich, 2001), maybe controlling the activation state of signaling pathways that can be altered by the deleterious effects of oxidative stress.

Inhibition of cellular oxidative stress and the concomitant diminution in reactive oxygen species (ROS) levels have beneficial effects in learning and memory in different animal species. In fact, it has been known for long that the learning impairment associated with normal aging as well as with different environmental pollutants involves increased generation of ROS (Manikandan et al., 2006; Cui et al., 2006) and it has been suggested that a diet rich in nutritional sources of antioxidants reverses the age-associated decline in cognitive function (Bickford et al., 2000). Recently it was reported that

administration of a superoxide dismutase mimetic improved cognition in mice (Quick et al., *in press*).

Estrogens have beneficial effects on oxidative stress, synaptic plasticity and learning and memory processes (Cordoba et al., 1997; Gupta et al., 2001; Dykens et al., 2005; Barron et al., 2006; Daniel, 2006; Miquel et al., 2006; Zurkovsky et al., 2007) and because of that some have speculated about a possible causal relationship among these phenomena (Lopez-Jaramillo and Teran, 1999; Toran-Allerand, 2005). In this respect, it is known that ovariectomy hampers hippocampal synaptic plasticity (Day and Good, 2005) and results from our group and other labs indicate that the ovariectomy-induced impairment in memory retention can be blocked by the antioxidant vitamin E and C (McCay, 1985; Carr and Frei, 1999; Reis et al., 2002; Monteiro et al., 2005). However, little is known about the molecular mechanisms involved in the promnesic actions of estrogens.

Since it has been suggested that many of the central events induced by estrogen, including its effect of long-term potentiation and depression, involve activation of the NMDA and AMPA subtypes of glutamate receptors and the ERK1/2 pathway (Kim et al., 2002; Gureviciene et al., 2003; Zamani et al., 2004; Smith and McMahon, 2005), we decided to investigate the effect of ovariectomy and of the administration of vitamins E and C on the phosphorylation of different AMPAr subunits and on the possible modulation of the ERK1/2 – CREB signaling pathway in the hippocampus.

Experimental Procedures

Animals and Reagents

Female adult Wistar rats obtained from the Central Animal House of the Biochemistry Department, Institute of Basic Health Sciences, at the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, were housed in groups of eight with their mothers on the day of birth. Half of them were assigned to the experimental condition and the other half served as controls; animals were weaned at 21 days after birth. Rats were maintained on a 12:12 h light/dark cycle in an air-conditioned constant-temperature (22^{o}C) colony room, with free access to 20% (w/w) protein commercial chow and water. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethical Committee of Universidade Federal do Rio Grande do Sul.

Anti-ERK and anti-pERK 1/2 (Thr202/Tyr204) were obtained from Cell Signaling Technology (Beverley, MA, USA); anti-CREB, anti-pCREB (Ser133) were from New England Biolabs (USA). Anti–pGluR-1 (Ser831), anti-pGluR-1 (Ser845), anti-pGluR-1 (Ser863) and anti-pGluR-2/3 (Ser880/Ser891) were obtained from Santa Cruz Biotechnology (USA). Horseradish peroxidase-coupled anti-IgG antibody was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The West Pico chemiluminescent kit was obtained from Pierce (Rockford, IL, USA). Other drugs, kits and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

Experimental treatment

Eighty-day-old rats were randomly assigned to one of the following groups: sham (only submitted to surgery without removal of the ovaries) and ovariectomized. Animals were ovariectomized by the surgical removal of both ovaries under ketamine anesthesia (90 mg/kg) and xylazine (10 mg/kg) *i.p.* to eliminate endogenous ovarian steroids (Waynforth and Flecknell, 1992). The stage of the estrous cycle was determined by vaginal swabs for 10 days prior to ovariectomy, to ensure that animals were cycling normally (Baker et al., 1979).

Seven days after surgery, sham and ovx animals (n=5) were treated for 30 days, with a single daily i.p. injection of saline (control) or vitamins E (40 mg/Kg) and C (100 mg/Kg) (Wyse et al., 2002). These dosing regimes have proved effective for preventing biochemical and behavioral effects in experimental models (Reis et al., 2002; Delwing et al., 2005; Monteiro et al., 2005). Twelve hours after the last injection rats were killed in the proestrous and the hippocampus was dissected.

Immunoblot experiments

To perform immunoblot experiments, the hippocampus was homogenized in 10 volumes 0.1mM potassium phosphate buffer, pH7.5. Equal amounts of cell proteins (20μg/lane) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinyledilene difluoride (PVDF) membranes. Protein loading and electrobloting efficiency were verified by Ponceau S staining, and the membrane was then blocked in Tween-Tris buffered saline (TTBS; 100mM Tris-HCl, pH7.5, containing 0.9%NaCl and 0.1%Tween-20) containing 5%albumin and incubated overnight with the primary antibody to be tested. The membrane was washed in TTBS and incubated with

horseradish peroxidase-coupled anti-IgG antibody, washed again and the immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis of the films was performed with the Opti-Quant® software. Blots were developed to be linear in the range used densitometry (Cammarota et al., 1997; 1998).

Protein determination

Protein contents of each sample were measured as described by Bradford et al. (1976), using bovine serum albumin as standard for data normalization.

Statistical analysis

Data were analyzed by one way ANOVA followed by the Duncan multiple test when F-test was significant and are expressed as means ± S.E.M. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Values of $p<0.05$ were considered to be significant.

Results

In order to investigate if ovarian hormone depletion is associated with changes in the phosphorilation state of different AMPA receptor, we first investigated the effect of ovariectomy on the phosphorylation of different AMPAr subunits in hippocampus of female adult rats. Using immunoblot experiments with specific antibody, as can be seen in Fig. 1, the levels of pGluR-2/3 (Ser880/Ser891) are significantly increased (85%) in ovariectomized group.

Moreover, when the treatment with vitamins E plus C reverted this increased of pGluR-2/3 (Ser880/Ser891) $F(3,12=14.29;p<0.001]$. No differences in pGluR-1 (Ser831), pGluR-1 (Ser845), pGluR-1 (Ser863) were observed among the groups (data not shown).

Next, we investigated on the possible modulation of the ERK1/2 – CREB signaling pathway in the hippocampus of female ovariectomized rats. Fig. 2 shows that the ovariectomy and the treatment with vitamins E plus C are unable to alter the pERK1/2 (A), $F(3,16=2.08;p>0.05]$, neither pCREB (B), $F(3,16=0.33;p>0.05]$, in the hippocampus.

Discussion

Gonadal steroid hormones influence central nervous system functioning through a variety of different mechanisms. Clinical evidence indicates that estrogenic steroids can improve cognitive functions in postmenopausal women (Henderson, 1997) and induce changes in neuronal excitability across nongenomic mechanisms involving a direct interaction with sites of plasma membranes to regulate ion channels and neurotransmitter transporters (Wong et al., 1996). Besides, estrogen affects the microstructure of different brain regions (Wang et al., 2002) and can bind to estrogen receptors - ERs (ERα and ERβ) to initiate rapid signaling events at or near the plasma membrane (Mhyre and Dorsa, 2006). The estrogenic depletion by ovariectomy results in loss of spines from some hippocampal cells in adult rats and the exogeneous estrogen can restore this effect (Wooley and McEwen, 1993). Zurkovsky and colleagues (2007) showed that estrogen can modulate learning in female rats by acting

directly at distinct memory systems. The neuroprotection of estrogen has been accepted but the mechanism remains unclear.

It has been suggested that steroids hormones, especially estriol and estradiol, are natural antioxidants (Mooradian, 2003). The antioxidant activity of estradiol is attributed to its phenolic hydroxyl (-OH) group, which is capable of reducing peroxyl radicals (Behl and Lezoualc'h, 1988). A greater decrease in the antioxidant/prooxidant balance is found in menopausal women, when compared to men of the same age, and the supplementation with antioxidants may help to protect against the antioxidant decline derived from estrogen loss (Miquel et al.,2006). Furthermore, the intake of antioxidant compounds could be a complement to the conventional treatments prescribed to these women. The Women's Health Initiative Memory Study (WHIMS) alerts against the use of hormone therapy in long-term treatment for postmenopausal women. Taken together, estrogen and progestin, could increase the risk of stroke, cardiovascular disease and breast cancer (Shumaker et al., 2003).

Alpha-tocopherol is a lipid soluble vitamin that interacts with cells membranes, traps free radicals and interrupts the oxidative chain that damage cells (Ames et al., 1993). The resultant tocopheroxyl radical requires ascorbate (vitamin C) for its regeneration back to reduced tocopherol (Carr and Frei, 1999). The joint administration of vitamins E plus C must increase the protective action against ROS both in the aqueous phase of the organism and in the lipid phase of the mitochondrial membranes, which are rich in polyunsaturated fatty acids quite vulnerable to oxidation (Jialal et al. 2001; Kim et al., 2002). In addition, it has been demonstrated that castrated female rats present a decreased vitamin E concentration in serum and liver (Feingold et al. 1993). We

reported that an impairment of spatial navigation, caused by ovariectomy, was prevented by vitamins E plus C administration (Monteiro et al., 2005). In agreement with these data, Socci and colleagues (1995) showed that chronic antioxidant treatment enhances cognitive performance of aged rats in the same behavior task.

In the present study we investigated the effect of ovariectomy and the administration of vitamins E plus C on the phosphorylation of different AMPAr subunits and on the possible modulation of the ERK1/2 – CREB signaling pathway in the hippocampus. We used this animal model of steroid hormone deprivation because ovariectomy is considered to be the most common animal model of postmenopausal changes in adult female rats (Savonenko and Markowska, 2003). The hippocampus was used because this cerebral structure is associated with memory mechanism (Daniel and Dohanich, 2001). Our results show that ovariectomy significantly increases the phosphorilation of AMPAr subunit pGLU 2/3 Ser 880/891 (Fig.1). Do not find any modification in the levels of other phosphorilated subunits of AMPAr as pGLUR1 Ser 845, pGLUR1 Ser 831 and pGLUR1 Ser 836 (data not shown). We also found that the treatment with vitamins E plus C reverts the activation of AMPAr subunit pGLU 2/3 Ser 880/891 (Fig.1) Evidences has been showed that a increase of [(3)H] AMPA-specific binding in prefrontal and cingulated cortices, and the nucleus accubens of ovariectomized rats when compared with intact controls, which was corrected by estradiol treatment (Le Saux et al., 2006). The exact reversal mechanism of the activation of AMPAr subunit pGLU 2/3 Ser 880/891 by vitamin E is unknown, however it is known that this antioxidant may be important for membrane stabilization (Singh et al., 1994; Gomez-Fernandez et

al., 1989). Besides, no changes were found in the levels of pERK1/2 and pCREB between groups showing that ovariectomy and the treatment with these antioxidants were unable to alter the ERK1/2 – CREB signaling pathway in the hippocampus (Fig.2).

In summary, our results showed that ovariectomy significantly increases the phosphorilation of AMPAr subunit pGLU 2/3 Ser 880/891 and the treatment with vitamins E plus C reverts such effect. No changes were found in the levels of pERK1/2 and pCREB between groups showing that ovariectomy and the treatment with these antioxidants were unable to alter the ERK1/2 – CREB signaling pathway in the hippocampus. Understanding the molecular mechanism that vitamins E plus C reverse memory deficit and signaling pathways may lead to the development of alternative treatments for postmenopausal women. Assuming that hormone deprivation might also impair cognition in human beings, our results lend support to a novel therapeutic strategy, based on vitamins E and C, to cognitive deficits found in postmenopausal women.

Acknowledgements

This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil), FAPERGS, RS-Brazil, and Programa de Núcleos de Excelência-Financiadora de Estudos e Projetos (PRONEX II – FINEP-Brazil).

References

Ames BN, Shigenara MK, Hagen TM (1993) Oxidants, antioxidants and the degenerative diseases of aging. Proc Natl Acad Sci USA 90:7915-7922.

Baker HJ, Lindsey JR, Weisbroth SH (1979) The Laboratory Rat. In: Biology and Diseases, vol 1. New York, NY, USA: Academic Press.

Barron AM, Fuller SJ, Verdile G, Martins RN (2006) Reproductive hormones modulate oxidative stress in Alzheimer's disease. Antioxid Redox Signal 8(11-12):2047-2059.

Behl C, Lezoualc'h F (1998) Estrogens with an intact phenolic group prevent death of neuronal cells following glutathione depletion. Restor Neurol Neurosci 12(2-3):127-134.

Bickford PC, Gould T, Briederick L, Chadman K, Pollock A, Young D, Shukitt-Hale B, Joseph J (2000) Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. Brain Res 866(1-2):211-217.

Bradford MM (1976) A rapid and sensitive method for the quantification of micrograms quantities of protein utilizing the principle of protein-die-binding. Anal Biochem 72:248-254.

Brinton, RD (2001) Cellular and molecular mechanisms of estrogen regulation of memory function and neuroprotection against Alzheimer's disease: recent insights and remaining challenges. Learn Mem 8(3):121-133.

Carr A, Frei B (1999) Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J 13:1007-1024.

Cammarota M, Paratcha G, Levi de Stein M, Bernabeu R, Izquierdo I, Medina JH (1997) B-50/GAP-43 phosphorylation and PKC activity are increased in

rat hippocampal synaptosomal membranes after an inhibitory avoidance training. Neurochem Res 22:499-505.

Cammarota M , Bernabeu R, Levi De Stein M, Izquierdo I, Medina JH (1998) Learning-specific, time-dependent increases in hippocampal Ca2+/calmodulin-dependent protein kinase II activity and AMPA GluR1 subunit immunoreactivity. Eur J Neurosci 10:2669-2676.

Cordoba Montoya DA, Carrer HF (1997) Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats. Brain Res 778(2):430-438.

Cui M, Yang Y, Yang J, Zhang J, Han H, Ma W, Li H, Mao R, Xu L, Hao W, Cao J (2006) Enriched environment experience overcomes the memory deficits and depressive-like behavior induced by early life stress. Neurosci Lett 404(1-2):208-212.

Daniel JM, Dohanich GP (2001) Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. J Neurosci 21(17):6949-6956.

Daniel JM (2006) Effects of oestrogen on cognition: what have we learned from basic research? J Neuroendocrinol 18(10):787-795.

Day M, Good M (2005) Ovariectomy-induced disruption of long-term synaptic depression in the hippocampal CA1 region in vivo is attenuated with chronic estrogen replacement. Neurobiol Learn Mem 83:13-21.

Delwing D, Chiarani F, Bavaresco CS, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse ATS (2005) Protective effect of antioxidants on brain oxidative damage caused by proline administration. Neurosci Res 52:69-74.

Dykens JA, Moos WH, Howell N (2005) Development of 17alpha-estradiol as a neuroprotective therapeutic agent: rationale and results from a phase I clinical study. Ann N Y Acad Sci 1052:116-135.

Feingold IB, Longhurst PA, Colby HD (1993) Regulation of adrenal and hepatic α-tocopherol content by androgens and estrogens. Biochim Biophys Acta 1176: 192-196.

Gomez-Fernandez JC, Villalain J, Aranda FJ, Ortiz A, Micol V, Coutinho A, Berberan-Santos MN, Prieto MJ (1989) Localization of alpha-tocopherol in membranes. Ann N Y Acad Sci 570:109-120.

Gupta RR, Sen S, Diepenhorst LL, Ruddick CN, Maren S (2001) Estrogen modulates sexually dimorphic contextual fear conditioning and hippocampal long-term potentiation (LTP) in rats(1). 888(2):356-365.

Gureviciene I, Puolivali J, Pussinen R, Wang J, Tanila H, Ylinen A (2003) Estrogen treatment alleviates NMDA-antagonist induced hippocampal LTP blockade and cognitive deficits in ovariectomized mice. Neurobiol Learn Mem 79:72-80.

Henderson VW (1997) Estrogen, cognition and woman's risk of Alzheimer's disease. The Am J Med 103:11-18.

Jialal L, Devaraj S, Kaul N (2001) The effect of alpha-tocopherol on monocyte proatherogenic activity. Am J Clin Nutr 131:389S-394S.

Kim JS, Kim HY, Kim SH, Shin HK, Lee SH, Lee YS, Son H (2002) Enhancement of rat hippocampal long-term potentiation by 17 beta-estradiol involves mitogen-activated protein kinase-dependent and independent components. Neurosci Lett 332:65-69.

Le Saux M, Estrada-Camarena E, Di Paolo T (2006) Selective estrogen receptor-alpha but not-beta agonist treatment modulates brain alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. J Neurosci Res 84(5):1076-1084.

Lopez-Jaramillo P, Teran E (1999) Improvement in functions of the central nervous system by estrogen replacement therapy might be related with an increased nitric oxide production. Endothelium 6(4):263-266.

Manikandan S, Padma MK, Srikumar R, Jeya Parthasarathy N, Muthuvel A, Sheela Devi R (2006) Effects of chronic noise stress on spatial memory of rats in relation to neuronal dendritic alteration and free radical-imbalance in hippocampus and medial prefrontal cortex. Neurosci Lett 399(1-2):17-22.

McCay PB (1985) Vitamin E: interactions with free radicals and ascorbate. Annu Rev Nutr 5:323-340.

Mhyre AJ, Dorsa DM (2006) Estrogen activates rapid signaling in the brain: role of estrogen receptor alpha and estrogen receptor beta in neurons and glia. Neuroscience 138(3):851-858.

Miquel J, Ramírez-Boscá A, Ramírez-Boscá JV, Alperi JD (2006) Menopause: a review on the role of oxygen stress and favorable effects of dietary antioxidants. Arch Gerontol Geriatr 42(3):289-306.

Monteiro SC, Matté C, Bavaresco CS, Netto CA, Wyse ATS (2005) Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. Neurobiol Learn Mem 84:192-199.

Mooradian AD (1993) Antioxidant properties of steroids. J. Steroid Biochem. Mol Biol 45:509-511.

Murphy DD, Andrews SB (2000) Culture models for the study of estradiol-induced synaptic plasticity. J Neurocytol 29(5-6):411-417.

Offerman MK, Medford RM (1994) Antioxidants and atherosclerosis, a molecular perspective. Heart Dis Stroke 3:52-57.

Quick KL, Ali SS, Arch R, Xiong C, Wozniak D, Dugan LL (*in press*) A carboxyfullerene SOD mimetic improves cognition and extends the lifespan of mice. Neurobiol Aging.

Reis EA, Zugno AI, Franzon R, Tagliari B, Matté C, Lammers ML, Netto CA, Wyse ATS (2002) Pretreatment with vitamins E and C prevent the impairment of memory caused by homocysteine administration in rats. Metab Brain Dis 17(3):211-217.

Rodrigues HD, Kinder JE, Fitzpatrick LA (1999) Treatment with 17 beta-estradiol does not influence age and weight at puberty in Bos indicus heifers. Anim Reprod Sci 56:1-10.

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

Shumaker SA, Legault C, Raap SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, 3rd Jones BN, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende S: WHIMS Investigators (2003) Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. JAMA 289:2651-2662.

Singh M, Meyer EM, Millard WJ, Simpkins JW (1994) Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. Brain Res 644:305-312.

Smith CC, McMahon LL (2005) Estrogen-induced increase in the magnitude of long-term potentiation occurs only when the ratio of NMDA transmission to AMPA transmission is increased. J Neurosci 25(34):7780-7791.

Socci DJ, Crandall BM, Arendash GW (1995) Chronic antioxidant treatment improves the cognitive performance of aged rats. Brain Res 693(1-2):88-94.

Wang H, Isaksson E, Von Schoultz B, Cline JM, Sahlin L (2002) The effect of long-term treatment with steroid hormones or tamoxifen on oestrogen receptors (alpha and beta) in the endometrium of ovariectomized cynomolgus macaques. J Endocrinol 175(3):673-681.

Waynforth HB, Flecknell PA (1992) Experimental and Surgical Technique. In: The rat. 2nd ed. London: Academic Press Limited. pp 276-278.

Wise PM, Dubal DB, Wilson ME, Rau SW, Böttner M, Rosewell KL (2001) Estradiol is a protective factor in the adult and aging brain: understanding of mechanism derived from in vivo and in vitro studies. Brain Res Ver 37:313-319.

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

Wong M, Thompson TL, Moss RL (1996) Nongenomic actions of estrogen in the brain: physiological significance and cellular mechanisms. Crit Rev Neurobiol 10(2):189-203.

Woolley CS, McEwen BS (1993) Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J Comp Neurol 336:293-306.

Wyse ATS, Zugno AI, Streck EL, Matte C, Calcagnotto T, Wannmacher CM, Wajner M (2002) Inhibition of Na^+,K^+-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. Neurochem Res 27:1685-1689.

Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H, Mayeux R (1996) Effect of oestrogen during menopause on risk and age at onset of Alzeimer's disease. Lancet 348:429-432.

Toran-Allerand CD (2005) Estrogen and the brain: beyond ER-alpha, ER-beta, and 17 beta-estradiol. Ann N Y Acad Sci 1052:136-144.

Zamani MR, Levy WB, Desmond NL (2004) Estradiol increases delayed, *n*-methyl-D-aspartate receptor-mediated excitation in the hippocampal CA1 region. Neuroscience 129:243-254.

Zhang YQ, Shi J, Rajakumar G, Day AL, Simpkins JW (1998) Effects of gender and estradiol treatment on focal brain ischemia. Brain Res 784:321-324.

Zurkovsky L, Brown SL, Boyd SE, Fell JA, Korol DL (2007) Estrogen modulates learning in female rats by acting directly at distinct memory systems. Neuroscience 144:26-37.

Figure 1 – Effect of ovariectomy and treatment with vitamins E plus C on the levels of pGluR-2/3 (Ser880/Ser891) in hippocampus of female adult rats. (A) Representative immunoblot showing an increase of pGluR-2/3 (Ser880/Ser891). (B) Densitometric analyses. Data are expressed as mean ± S.E.M for 5 independent animals performed in duplicate. *** $p<0.001$ compared to sham group (Duncan's multiple range test). sal – saline; vit – vitamins E plus C; ovx – ovariectomized.

Figure 2 - Effect of ovariectomy and treatment with vitamins E plus C on the levels of pERK1/2 – pCREB signaling pathway in hippocampus of female adult rats. (A) Representative immunoblot and densitometric analyses shows no changes in pERK1/2. (B) Representative immunoblot and densitometric analyses shows no changes in pCREB. Data are expressed as mean ± S.E.M for 5 independent animals performed in duplicate. $p>0.05$ compared to sham group (Duncan's multiple range test). sal – saline; vit – vitamins E plus C; ovx – ovariectomized.

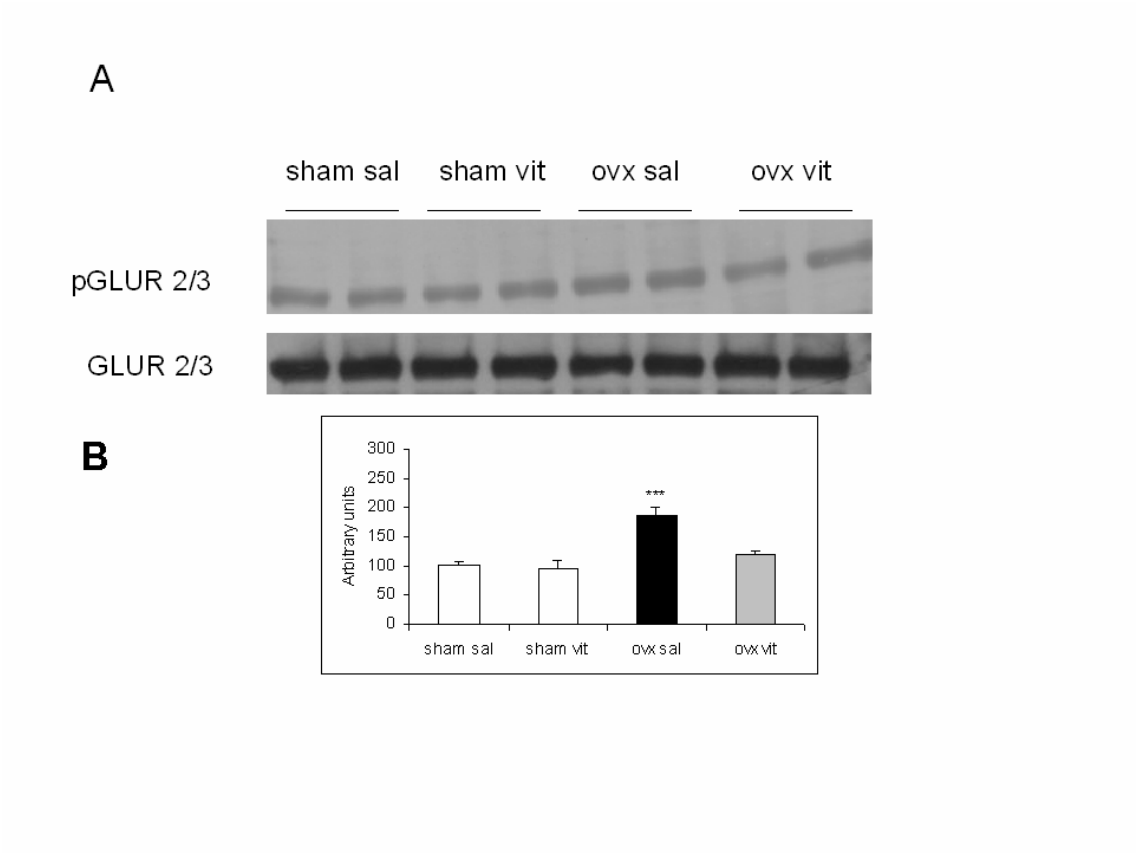
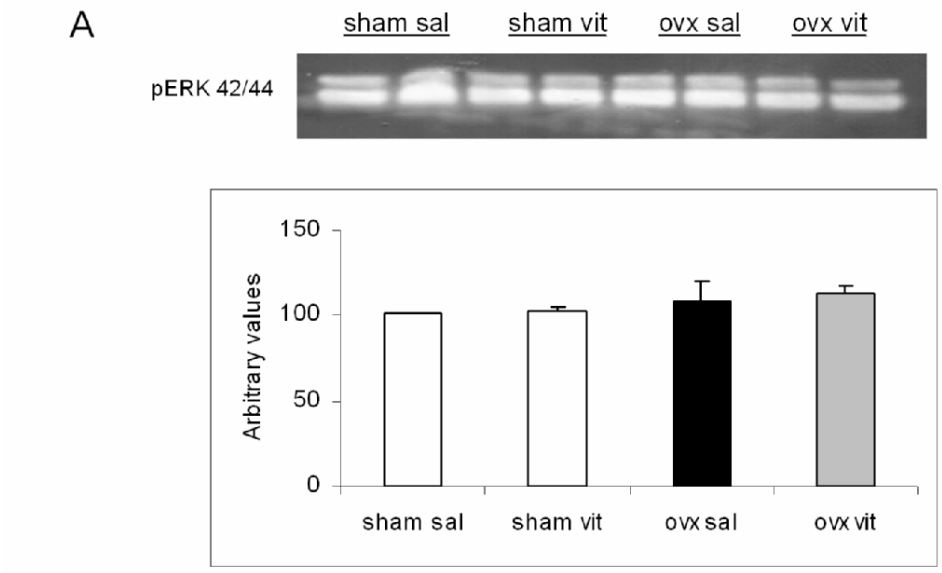

A
sham sal
sham vit
ovx sal
ovx vit
pGLUR 2/3
GLUR 2/3
B
Arbitrary units
300
250
200
150
100
50
0
***
sham sal
sham vit
ovx sal
ovx vit

Figure 1


A
sham sal
sham vit
ovx sal
ovx vit
pERK 42/44
Arbitrary values
150
100
50
0
sham sal
sham vit
ovx sal
ovx vit

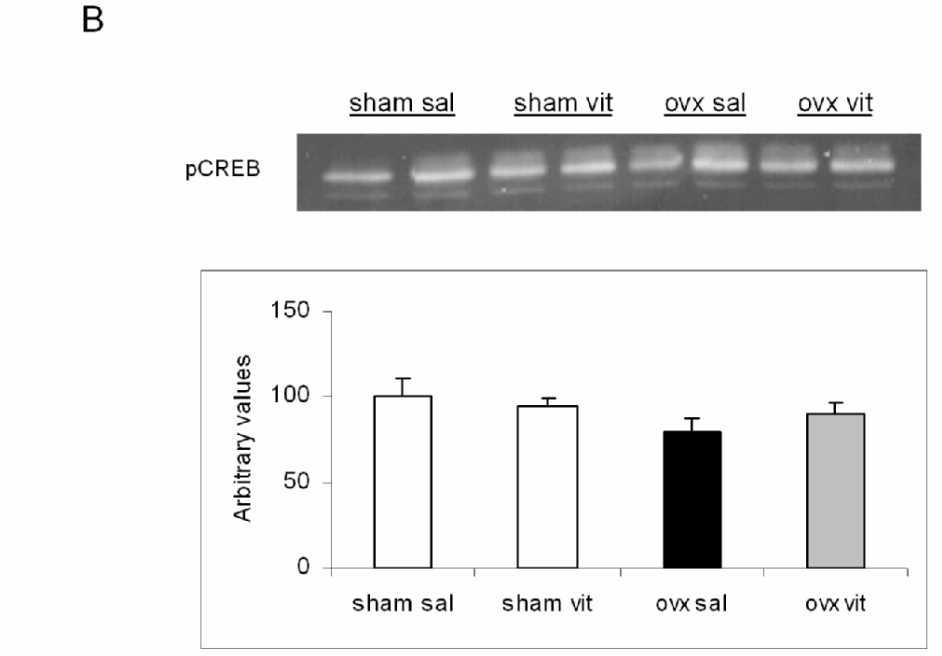

B
sham sal
sham vit
ovx sal
ovx vit
pCREB
Arbitrary values
150
100
50
0
sham sal
sham vit
ovx sal
ovx vit

Figure 2

IV. DISCUSSÃO

Iniciamos nossos estudos investigando o efeito da ovariectomia sobre a atividade da AChE e o conteúdo e o perfil de gangliosídios em córtex cerebral de ratas adultas. Utilizamos a ovariectomia para deprivação hormonal ovariana por ser considerado o modelo animal que melhor reproduz as mudanças pós-menopáusicas em ratas fêmeas adultas (Savonenko & Markowska, 2003). O córtex cerebral foi utilizado nestes experimentos por apresentar ERβ (Mitra et al., 2003) e ser uma estrutura importante nos mecanismos de memória. Sabe-se que regiões cerebrais que expressam altas concentrações de AChE parecem ser as primeiras estruturas que sofrem dano em doenças neurodegenerativas e um aumento na atividade dessa enzima promove um conseqüente aumento na hidrólise e recaptação da ACh (Gómez-Ramos & Morán, 1997; Okuda et al., 2000). Nossos resultados mostraram que as ratas submetidas à ovariectomia apresentaram um aumento na atividade da AChE.

A hipótese colinérgica para demência sugere que esta, com déficits na memória, no aprendizado e mudanças no comportamento, seja causada, pelo menos em parte, por um decréscimo nos níveis de ACh no cérebro (Ballard et al., 2005). Uma redução na atividade dessa enzima foi demonstrada em córtex cerebral e hipocampo de pacientes afetados por doenças neurodegenerativas (Fishman et al., 1986), sugerindo que alterações na atividade da AChE pode estar associada com as alterações cognitivas características dessas doenças (Cummings, 2000; Law et al., 2001). Por outro lado, a degeneração dos terminais nervosos colinérgicos em regiões cerebrais específicas resulta numa redução da forma globular tetramétrica (G4) associada à membrana da AChE e

também, num concomitante aumento nas formas A12 e A8 dessa enzima (Younkin et al., 1986). Neste contexto, a forma globular G1 está co-localizada nas placas senis no SNC, sugerindo que essa enzima possue uma função na agregação progressiva β-amilóide e na maturação das placas senis características da doença de Alzheimer (Arendt et al., 1992; Gómez-Ramos & Móran, 1997). Baseado nesses relatos, inibidores reversíveis das colinesterases têm sido usados como estimuladores cognitivos no tratamento dessa doença (Greig et al., 2001).

Tendo em vista que existem evidências demonstrando que a BuChE é considerada um marcador periférico da AChE, neste trabalho também examinamos o efeito da ovariectomia sobre a atividade da BuChE sérica. Nossos resultados mostraram que a ovariectomia causou um decréscimo na atividade da BuChE em soro de ratas adultas. Esse resultado pode ser interpretado como um mecanismo compensatório à diminuição da hidrólise de ACh, já que a atividade da AChE está aumentada no cérebro. De fato, o mesmo padrão de atividade das colinesterases já fora descrito em outro estudo (Giacobini, 1997). Relatos da literatura demonstraram que, mesmo com a alteração da atividade da AChE em pacientes com a doença de Alzheimer, a atividade da BuChE permanecia inalterada ou aumentada (Davies & Maloney, 1976; Giacobini et al., 1989).

Demonstramos também nesse estudo, que o conteúdo e o perfil de gangliosídios não se modificam em córtex cerebral de ratas ovariectomizadas. Os gangliosídios estão presentes em altas concentrações nas membranas cerebrais e possuem uma função importante na interação célula-célula, no crescimento e diferenciação celular, na transdução de sinal e na adaptação da

membrana plasmática às variações ambientais (Maccioni et al., 1984; Sanhoff & Van Echten, 1994). Sabe-se que o estradiol mantém a integridade e a plasticidade dos neurônios agindo como um fator trófico na estrutura celular (Naftolin et al., 1996). Não podemos afirmar que o resultado encontrado nesse estudo também ocorra em outras estruturas cerebrais. Estudos mostraram que a administração de estradiol diminui o conteúdo total de lipídios no hipotálamo e aumenta as concentrações de gangliosídios no hipocampo, núcleo amigdalóide e bulbo olfatório, sugerindo que o conteúdo de lipídios e a plasticidade estão diferencialmente afetados nas várias regiões cerebrais por estrógenos e fitoestrógenos (Islam et al., 1986; Lephart et al., 2003).

Concluímos que a privação hormonal ovariana, principalmente refletindo os efeitos da falta de estrógenos, mas também das outras substâncias ovarianas, aumenta a atividade da AChE em córtex cerebral de ratas, o que pode resultar num decréscimo nos níveis de ACh levando a uma redução da neurotransmissão colinérgica. Um mecanismo compensatório pode ser observado com a redução da atividade da BuChE no soro das ratas ovariectomizadas. O conteúdo e o perfil de gangliosídios não estão alterados no córtex cerebral das ratas submetidas à ovariectomia.

Tendo em vista que além das alterações colinérgicas, a homeostasia iônica e o desequilíbrio entre a formação e remoção de radicais livres são eventos importantes associados à fisiopatologia de alguns distúrbios neurodegenerativos (Erecinska et al., 2004; Ballard et al., 2005; Siqueira et al., 2005) e que mulheres pós-menopáusicas estão mais suscetíveis a desenvolver doenças neurológicas (Wise, 2001; Miquel et al; 2006), nossos próximos objetivos foram investigar o efeito da ovariectomia sobre a atividade da Na^+,K^+-

ATPase e da AChE e sobre alguns parâmetros de estresse oxidativo denominados: TRAP (capacidade total antioxidante), TBA-RS (substâncias reativas ao ácido tiobarbitúrico), assim como, sobre a atividade das enzimas antioxidantes CAT (catalase), SOD (superóxido dismutase) e GSH-Px (glutationa peroxidase) em hipocampo de ratas adultas.

O hipocampo foi a estrutura cerebral usada, pois além de ser vulnerável ao dano cerebral, possue ERα e ERβ expressos, e está intimamente relacionado com mecanismos de memória e aprendizado que serão posteriormente estudados (Weiland et al., 1997; Daniel & Dohanich, 2001). Níveis fisiológicos de estradiol estimulam a sinaptogênese em regiões hipocampais (Wooley et al., 1997). Além disso, Saez-Valero e colaboradores (2003) mostraram que a isquemia transiente aumenta a atividade da AChE em culturas organotípicas de células do hipocampo.

Nossos resultados mostraram que a ovariectomia aumenta a atividade da Na^+,K^+-ATPase em membranas plasmáticas de hipocampo de ratas. Sabe-se que a atividade dessa enzima pode ser modulada por vários mecanismos. Algumas vias de transdução de sinal, que levam à formação de mediadores e à ativação de quinases, regulam a atividade da Na^+,K^+-ATPase (Bertorello and Katz, 1995). Nesse contexto, tem sido demonstrado que a proteína quinase C aumenta a atividade desta ATPase (Xie & Cai, 2003). Além disso, a atividade da Na^+,K^+-ATPase também pode ser modulada por mudanças na concentração intracelular de sódio (Inoue & Matsui, 1991). Outra possibilidade poderia ser um aumento na síntese e na degradação da enzima, provocados pela deficiência hormonal, já que a Na^+,K^+-ATPase é considerada um índice adequado de atividade neural para estudos de interações neuro-endócrinas (Del Castillo et

al., 1987). Também observamos um aumento na atividade da AChE no hipocampo das ratas submetidas à ovariectomia. Uma atividade aumentada da AChE promove um decréscimo nos níveis de ACh reduzindo a atividade colinérgica no SNC. Não sabemos o exato mecanismo pelo qual as atividades dessas enzimas estão aumentadas. Efeitos decorrentes do estresse oxidativo não devem ser descartados já que dados da literatura demonstram que o α-tocoferol reverteu o aumento na atividade da Na^+,K^+-ATPase provocado pelo consumo crônico de etanol em ratos (Nanji & Sadrzadeh, 1994).

Os hormônios esteróides são considerados antioxidantes naturais (Mooradian, 1993). Um estudo evidencia que todas as áreas cerebrais aumentam a perda de ascorbato após a gonadectomia (Kume-Kick et al. 1996) e outro trabalho demonstra que a incubação de culturas neuronais primárias com 17 β-estradiol aumenta a sobrevivência celular reduzindo a lipoperoxidação (Vedder et al., 1999). Tais afirmações direcionam para a hipótese de que a proteção contra o estresse oxidativo pode ser perdida com a depleção dos hormônios ovarianos, principalmente os estrógenos. Baseado nestes relatos e considerando que o estresse oxidativo é um evento importante relacionado com a patogenia de algumas doenças que afetam o SNC (Halliwell & Gutteridge, 2000), nós examinamos o efeito da ovariectomia sobre alguns parâmetros de estresse oxidativo em hipocampo de ratos.

A ovariectomia não foi capaz de alterar o TRAP, o TBA-RS e nem a atividade das enzimas SOD e GSH-Px. Somente a atividade da CAT mostrou-se alterada no hipocampo do grupo ovariectomizado. Considerando que as enzimas antioxidantes podem suportar o estresse oxidativo por um aumento compensatório de suas atividades (Travacio & Llesuy, 1996), a estimulação

observada na atividade da CAT poderia ser interpretado como conseqüência da adaptação enzimática a um possível aumento na produção de radicais livres. Nossos resultados estão em concordância com os de Gómez-Zubeldia e colaboradores (2001) que não observaram variação nos níveis de malondialdeído e um aumento na atividade da CAT em eritrócitos de ratas ovariectomizadas.

Em resumo, neste segundo grupo de estudos observamos um aumento nas atividades da Na^+,K^+-ATPase e da AChE, podendo causar uma hiperpolarização das membranas sinápticas e um decréscimo nas concentrações de ACh com subseqüente perda da atividade colinérgica. Também observamos um aumento da CAT, interpretado como uma conseqüência inicial do estresse oxidativo provocado pela perda dos hormônios ovarianos, em hipocampo de ratas adultas submetidas à ovariectomia.

Dando continuidade aos nossos estudos e considerando que a ovariectomia aumentou as atividades das enzimas Na^+,K^+-ATPase e da AChE hipocampais e diminuiu a atividade da BuChE sérica, decidimos investigar a influência do tratamento as vitaminas E e C e da dieta de soja rica em isoflavonas sobre as alterações enzimáticas observadas em hipocampo de ratas adultas ovariectomizadas.

A suplementação com esses compostos, os quais são antioxidantes, tem sido considerada um complemento aos tratamentos convencionais, prescritos para mulheres menopáusicas, com o objetivo de proteger contra o declínio antioxidante derivado da perda estrogênica (Miquel et al., 2006).

Nossos resultados mostraram que as vitaminas E e C são capazes de reverter a ativação das atividades da Na^+,K^+-ATPase e AChE em hipocampo

de ratas adultas no grupo ovariectomizado. Tsai e colaboradores (2000; 2003) observaram que o tratamento prolongado com estradiol reduz a freqüência das oscilações espontâneas e a expressão/atividade da Na^+,K^+-ATPase em útero de ratas, indicando que esta enzima pode ser um importante alvo para moléculas estrogênicas. O exato mecanismo pelo qual as vitaminas revertem o efeito da Na^+,K^+-ATPase é desconhecido. Entretanto, a vitamina E pode ser importante para a estabilização das membranas (Ekiel et al., 1988; Gómez-Fernandez et al., 1989), local onde Na^+,K^+-ATPase está inserida. Além disso, a redução na fluidez das membranas pode estar associada com uma elevação na atividade das enzimas que estão associadas às membranas (Levin et al., 1990). Como já havíamos citado, um estudo demonstrou que a administração de α-tocoferol reverteu a ativação da Na^+,K^+-ATPase provocada pelo tratamento crônico com álcool em ratos alimentados com óleo de peixe (Nanji & Sadrzadeh, 1994), mostrando a participação do estresse oxidativo. Corroborando com os nossos resultados, Melo e colaboradores (2003) mostraram que o aumento da atividade da AChE induzida pelo peptídio β-amilóide é mediado pelo estresse oxidativo, visto que foi prevenido pela administração de vitamina E. Interessantemente, a administração de vitamina E e C nas mesmas concentrações utilizadas no nosso estudo, preveniu as alterações causadas pela prolina em alguns parâmetros de estresse oxidativo (Delwing et al., 2005). Desde que a ovariectomia estimula as atividades da Na^+,K^+-ATPase e da AChE e que as vitaminas E e C protegem contra essas mudanças enzimáticas, nossos resultados geram evidências para uma possível participação dos radicais livres nesse fenômeno.

As isoflavonas da soja são fitoestrógenos de particular interesse porque, como os estrógenos endógenos, entram no cérebro e atuam em regiões com abundância de ER (Lund et al., 2001; Patisaul et al. 2002). Essas substâncias têm sido utilizadas como suplementos em dietas de mulheres pós e peri-menopáusicas (Lephart et al., 2002; Miquel et al., 2006). No nosso estudo, a dieta de soja rica em isoflavonas foi capaz de reverter somente a estimulação da AChE em hipocampo de ratas ovariectomizadas. Pan e colaboradores (1999) comunicaram que as isoflavonas da soja podem funcionar como agonistas estrogênicos regulando a enzima colina acetiltransferase em cérebro de ratas (Pan et al., 1999).

Nenhum dos tratamentos utilizados foi capaz de reverter a diminuição da atividade da BuChE em soro de ratas ovariectomizadas. O efeito inibitório da ovariectomia sobre essa enzima periférica não é sensível aos tratamentos usados nesse estudo.

Nesse trabalho também investigamos os níveis de 17 β-estradiol em ratas ovariectomizadas que receberam o tratamento com as vitaminas E e C ou com as isoflavonas da soja. Medimos os níveis de estradiol sérico em todos os grupos por ELISA. Como esperado, a ovariectomia reduziu os níveis de 17 β-estradiol. Observamos que os tratamentos com as vitaminas E e C ou com as isoflavonas da soja não foram capazes de alterar os níveis desse hormônio esteróide no soro.

Em resumo, o tratamento com as vitaminas E e C é capaz de reverter a ação da ovariectomia sobre as atividades da Na^+,K^+-ATPase e da AChE e a dieta de soja rica em isoflavonas foi eficaz em reverter somente a atividade da AChE, em hipocampo de ratas adultas.

Posteriormente utilizamos a tarefa do labirinto aquático de Morris para avaliar o efeito da ovariectomia sobre a memória espacial em ratas adultas. O efeito do tratamento crônico com as vitaminas E e C também foi estudado. O labirinto aquático é uma tarefa adequada para avaliar cognição em ratos já que os mesmos são bons nadadores, possuem aversão pela água e apresentam uma boa capacidade de localização requerida nessa tarefa. O paradigma consiste numa piscina circular preenchida com água que contém uma plataforma submersa. Na primeira fase da tarefa, durante um número adequado de dias de treinamento, os animais aprendem, guiados por pistas colocadas nas paredes da sala (mapeamento ou estratégia espacial), o local da plataforma de escape (D'Hooge & De Deyn, 2001).

Nossos primeiros resultados mostram que as ratas ovariectomizadas apresentaram um déficit na fase de aquisição da tarefa, permanecendo menos tempo no quadrante alvo onde estava localizada a plataforma de escape, demorando mais tempo para cruzar o local da plataforma e ficando mais tempo no quadrante oposto ao da plataforma. Entretando, a memória de trabalho, tarefa que avalia a memória de curta duração, não foi alterada nas ratas ovariectomizadas comparadas às ratas controles (naive e sham). Nossos resultados estão de acordo com estudos recentes que demonstram um prejuízo da memória em ratas ovariectomizadas (Singh et al., 1994; Sato et al., 2003; Heikkinen et al., 2004; Wallace et al., 2006). Acreditamos que a ovariectomia causa um déficit na memória espacial de referência, independente das habilidades motoras do animal, visto que não foram encontradas alterações no campo aberto e na velocidade do nado.

Tem sido descrito que os estrógenos melhoram a retenção da memória (Sandstrom & Williams, 2004) e promovem a plasticidade sináptica modulando a neurotransmissão (Baum, 2005). Mulheres pós-menopáusicas apresentam um déficit cognitivo (Wise, 2001) e um decréscimo nas concentrações de vitamina E (White et al., 2001). Os estrógenos estão descritos por regenerar antioxidantes endógenos (Gridley et al., 1997). Existem evidências de que o estresse oxidativo participa na modulação do aprendizado e da memória. A lipoperoxidação induzida pelo estresse afetou as performances de aprendizado/memória em ratos (Abidin et al., 2004). Dietas com antioxidantes (β-caroteno, vitaminas E e C) reverteram o déficit no aprendizado motor relacionado com a idade (Bickford et al.,2000) e o tratamento com as vitaminas E e C foi capaz de prevenir o prejuízo na memória causado pela hiper-homocisteinemia (Reis et al., 2002).

Na próxima etapa do presente trabalho avaliamos o efeito da administração de vitamina E e C na prevenção do déficit de memória observado nas ratas ovariectomizadas. Estudos clínicos demonstraram que a vitamina E pode ser utilizada com sucesso como uma intervenção complementar em pacientes com disfunção cognitiva (Chan et al., 2004; Meccoci et al., 2004) e que pode diminuir a progressão da doença de Alzheimer em pacientes afetados com grau moderado (Sano et al., 1997). A vitamina C foi administrada concomitantemente com o objetivo de prolongar o efeito antioxidante da vitamina E reciclando-a a sua forma reduzida (Carr & Frei, 1999). Petersen e colaboradores (2005) demonstraram que a vitamina E não apresentava benefícios em pacientes com a doença de Alzheimer com déficit cognitivo severo. A vitamina E quando foi administrada sozinha pode

apresentar efeitos pró-oxidantes ou perder sua eficácia (Inal et al., 1997). Além disso, relatos da literatura mostraram que ratas castradas tiveram um decréscimo nas concentrações de vitamina E (Feingold et al., 1993) no soro e no fígado e que a reposição estrogênica aumentou os níveis desse antioxidante (Noh et al., 1999).

Confirmando nossa hipótese, o tratamento com as vitamina E e C foi capaz de prevenir o efeito da ovariectomia a partir do quarto dia de treinamento na fase de aquisição do Water Maze, e nos testes denominados tempo de permanência no quadrante alvo, tempo de permanência no quadrante oposto e latência para cruzar o local da plataforma. Sabe-se que as ratas ovariectomizadas apresentam um aumento do peso corporal quando comparadas com as ratas com gônadas intactas (Torto et al. 2006; Iwamoto et al., 2006). Porém, esse tratamento não previniu o ganho de peso observado no grupo ovariectomizado.

Desde que a ovariectomia prejudicou a memória espacial e a dieta de soja rica em isoflavonas reverteu a ativação da AChE em hipocampo de ratas adultas, dando continuidade ao trabalho, nós investigamos a influência do pré e pós-tratamento com a suplementação de isoflavonas da soja sobre os efeitos da depleção hormonal ovariana na memória espacial de referência. Embora, pouco se sabe sobre o efeito desses fitoestrógenos sobre o aprendizado/memória, estudos pré-clínicos e clínicos sugerem que as isoflavonas da soja podem melhorar a função cognitiva em humanos e em ratos (File et al., 2001; Pan et al., 1999). Porém, não existe padronização de um tratamento adequado quanto a dose e tempo de administração desses compostos.

Considerando que as isoflavonas são benéficas para a memória, pergunta-se quando deveria ser começado o tratamento? Baseado nesse questionamento, nós exploramos dois tempos de tratamento com o objetivo de fornecer mais informações quanto ao uso em dietas para melhorar ou aliviar os sintomas da menopausa relacionados com o declínio da memória. O labirinto aquático de Morris foi utilizado para medida da memória espacial de referência. Primeiro, nós utilizamos o pré-tratamento durante dois meses com dieta de soja rica em isoflavonas, dos 20 até os 80 dias de idade. Observamos que ocorreu uma prevenção do prejuízo na performance no quinto dia da fase de aquisição, do decréscimo do tempo no quadrante alvo e do aumento do tempo no quadrante oposto, observado no grupo ovariectomizado. A dieta de soja rica em isoflavonas foi capaz de prevenir os efeitos da ovariectomia nos parâmetros onde as ratas ovariectomizadas apresentaram um déficit de memória espacial. Numa segunda etapa, estudamos o efeito desse tratamento pós-ovariectomia. As ratas foram ovariectomizadas aos 80 dias de idade e 7 dias após foram submetidas à dieta. Interessantemente, a dieta enriquecida com isoflavonas foi capaz de reverter o prejuízo no desempenho das ratas no quinto dia da fase de aquisição, o decréscimo do tempo no quadrante alvo, o aumento do tempo no quadrante oposto e o aumento da latência para achar o local da plataforma, observado no grupo ovariectomizado.

Nossos resultados estão de acordo com alguns estudos recentes que demonstraram a influência positiva das isoflavonas da soja sobre a capacidade cognitiva em ratos machos idosos (Lee et al., 2004), sobre a memória visual espacial em ratas fêmeas (Lund et al., 2001), bem como o déficit cognitivo observado em mulheres pós-menopáusicas (Kritz-Silverstein et al., 2003).

Nesse estudo realizamos o controle da ingestâo e do peso corporal das ratas durante os tratamentos. Não encontramos diferenças estatísticas entre os grupos quanto ao consumo de ração e o ganho de peso. Ambas as dietas são isocalóricas. A dieta de soja rica em isoflavonas não foi capaz de alterar o ganho de peso das ratas ovariectomizadas com os protocolos utilizados.

Resumidamente, esses resultados demonstram que a dieta rica em isoflavonas foi capaz de prevenir e reverter o déficit de memória espacial observado no grupo ovariectomizado. Com a dose de isoflavonas utilizada, tanto a suplementação a longo quanto a médio prazo, demonstraram ser eficientes nos parâmetros analisados.

Por fim, considerando que o receptor AMPA está envolvido nos mecanismos de formação de memória e que as vitaminas E e C foram capazes de proteger contra o déficit de memória provocado pela ovariectomia, nós avaliamos o efeito da ovariectomia e do posterior tratamento com esses antioxidantes sobre a fosforilação de diferentes subunidades do receptor AMPA. É sabido que a funcionalidade hipocampal pode ser modulada pelos estrógenos no cérebro de ratas adultas e que esses hormônios atuam na plasticidade, no aprendizado e na memória (Daniel & Dohanich, 2001; Zamani et al., 2004), possivelmente ativando vias de sinalização que podem estar alteradas pelo estresse oxidativo. A inibição do estresse oxidativo tem efeitos benéficos no aprendizado e na memória (Reis et al., 2002). Tem sido sugerido que o uso de uma dieta rica em antioxidantes reverte o declínio associado com idade (Bickford et al., 2000). Muitos dos eventos onde ocorrem a participação dos estrógenos, incluindo os efeitos na potenciação e depressão de longa duração, envolvem a ativação de diferentes subtipos dos receptores

glutamatérgicos AMPA e NMDA e a participação da via de sinalização ERK1/2 – CREB (Kim et al.,2002; Gureviciene et al., 2003).

Nossos resultados mostraram que a ovariectomia aumentou os níveis da subunidade pGLU 2/3 Ser 880/891 do receptor AMPA e que a administração das vitaminas E e C reverteu essa ativação. Não encontramos diferença nos níveis de outras subunidades do receptor AMPA como pGLUR1 Ser 845, pGLUR1 Ser 831 e pGLUR1 Ser 836, nem no pERK1/2 e pCREB, demonstrando não ser esta a via de sinalização envolvida, nos grupos estudados com ou sem o tratamento com antioxidantes.

O entendimento dos mecanismos celulares pelo quais a ovariectomia afeta as vias de sinalização hipocampais, as quais são importantes para os mecanismos de memória poderá levar ao desenvolvimento de tratamentos para melhorar a cognição em mulheres pós-menopáusicas. Le Saux e colaboradores (2006) demonstraram recentemente que o ERα está envolvido na modulação do receptor AMPA. Além disso, o uso das vitaminas E e C pode ser uma alternativa, já que reverte o aumento dos níveis fosforilados de uma subunidade específica do receptor glutamatérgico AMPA provocado pela ovariectomia.

Esses resultados em conjunto, mostram alguns efeitos da depleção hormonal ovariana sobre alguns parâmetros bioquímicos e comportamentais e colaboram para o entendimento dos sintomas e distúrbios neurológicos observados em algumas mulheres menopáusicas. Além disso, se confirmados em humanos, nossos dados relacionados com a suplementação de vitaminas E e C e isoflavonas da soja podem ser uma estratégia para tratar alguns sintomas associados à menopausa.

V. CONCLUSÕES

A ovariectomia em ratas adultas:

- Diminuiu os níveis de estradiol no soro
- Aumentou a atividade da AChE em córtex cerebral e diminuiu a atividade da BuChE sérica
- Não alterou o conteúdo e o perfil de gangliosídios em córtex cerebral
- Aumentou as atividades da Na^+,K^+-ATPase e da AChE em hipocampo
- Estimulou a atividade da CAT em hipocampo e não alterou os demais parâmetros de estresse oxidativo analisados (TRAP, TBA-RS, SOD e GSH-Px)
- Provocou um prejuízo na memória espacial no labirinto aquático de Morris e não alterou a memória de trabalho
- Aumentou os níveis da subunidade pGLU 2/3 Ser 880/891 do receptor AMPA em hipocampo. Não alterou os níveis de outras subunidades do receptor AMPA como, pGLUR1 Ser 845, pGLUR1 Ser 831 and pGLUR1 Ser 836
- Não modificou os níveis de pERK1/2 e pCREB, demonstrando não ser está a via de sinalização envolvida na ativação da subunidade pGLU 2/3 Ser 880/891 do receptor AMPA.

O tratamento com as vitamina E e C em ratas adultas:

- Reverteu o déficit de memória e o aumento das atividades da Na^+,K^+-ATPase e da AChE em hipocampo de ratas ovariectomizadas
- Reverteu o aumento nos níveis da subunidade pGLU 2/3 Ser 880/891 do receptor AMPA em hipocampo de ratas ovariectomizadas.
- Não alterou os níveis séricos da BuChE e do estradiol causados pela ovarectomia, bem como o aumento do peso corporal observado nas ratas ovariectomizadas.

A dieta de soja rica em isoflavonas em ratas adultas:

- Reverteu a estimulação da atividade da AChE, mas não alterou a atividade da Na^+,K^+-ATPase em hipocampo de ratas ovariectomizadas.
- Preveniu e reverteu o déficit de memória espacial, observada no labirinto aquático de Morris, causado pela ovariectomia em ratas.
- Não alterou os níveis séricos da BuChE e do estradiol causados pela ovarectomia, bem como o aumento do peso corporal observado nas ratas ovariectomizadas.

Esses resultados em conjunto, mostram alguns efeitos da depleção hormonal ovariana sobre alguns parâmetros bioquímicos e comportamentais e colaboram para o entendimento dos sintomas e distúrbios neurológicos observados em algumas mulheres menopáusicas. Além disso, se confirmados em humanos, nossos dados relacionados com a suplementação de vitaminas E

e C e isoflavonas da soja podem ser uma estratégia para tratar alguns sintomas associados à menopausa.

VI. PERSPECTIVAS

1. Estudar outras vias de sinalização em ratas ovariectomizadas e o efeito da suplementação com as vitaminas E e C e isoflavonas de soja sobre as possíveis alterações encontradas.
2. Verificar se as vitaminas E e C e as isoflavonas da soja previnem o déficit de memória em outras tarefas comportamentais causado pela ovariectomia.
3. Estudar detalhadamente os mecanismos de alteração da Na^+,K^+-ATPase, AChE, BuChE e CAT.

VI. REFERÊNCIAS BIBLIOGRÁFICAS

Abidin, I, Yargicoglu, P, Agar, A, Gumuslu, S, Aydin, S, Ozturk, O, and Sahin, E. The effect of chronic restraint stress on spatial learning and memory: relation to oxidant stress. Int. J. Neurosci. 114(15):683-699. 2004.

Adlercreutz, H. Epidemiology of phytoestrogens. Baillieres Clin. Endocrinol. Metab. 12(4);605-623. 1998.

Alcantara, VM, Chautard-Freire-Maia, EA, Scartezini, M, Cerci, MS, Braun-Prado, K, and Picheth, G. Butyrylcholinesterase activity and risk factors for coronary artery disease. Scand. J. Clin. Lab. Invest. 62:399-404. 2002.

Allende, ML, and Prioa, R. Lubricating cell signaling pathways with gangliosides. Curr. Opin. Struct. Biol. 12:587-582. 2002.

Arendt, T, Bruckner, MK, Lange, M, and Bigl, V. Changes in acetylcholinesterase and butyrylcholinesterase in Alzeimer's disease resemble embryonic development – a study of molecular forms. Neurochem. Int. 21:381-396. 1992.

Arteaga, E, Rojas, A, Villaseca, P, Bianchi, M, Arteaga, A, and Durán, D. In vitro effect of oestradiol, progesterone, testosterone, and of combined estradiol/progestins on low density lipoprotein (LDL) oxidation in postmenopausal women. Menopause. 6:16-23. 1998.

Ayres, S, Tang, M, and Subbiah, MT. Estradiol-17β as an antioxidant: some distinct features when compared with common fat-soluble antioxidants. J. Lab. Clin. Med. 128(4):367-375. 1996.

Ballard, CG, Greig, NH, Guillozet-Bongaarts, AL, Enz, A, and Darvesh, S. Cholinesterases: roles in the brain during health and disease. Curr. Alzheimer Res. 2(3):307-318. 2005.

Barrier, L, Ingrand, S, Damjanac, M, Bilan, AR, Hugon, J, and Page, G. Genotype-related changes of ganglioside composition in brain regions of transgenic mouse models of Alzheimer's disease. Neurobiol. Aging. *In press.*

Baum, LW. Sex, hormones, and Alzheimer's disease. J. Gerontol. A. Biol. Sci. Med. Sci. 60(6):736-743. 2005.

Bear, MF, Connors, BW, and Paradiso, MA. Sexo e Sistema Nervoso. In: Neurociências: desvendando o sistema nervoso. Estados Unidos da América: ARTMED Editora S.A. Tradução da 2ª edição americana. p.555. 2002.

Beckman, JS, and Koppenol, WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am. J. Physiol. 271:C1424-1437. 1996.

Behl, C, and Lezoualc'h, F. Estrogens with an intact phenolic group prevent death of neuronal cells following glutathione depletion. Restor. Neurol. Neurosci. 12:127-134. 1998.

Benzi, G, and Moretti, A. Age- and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and glutathione system. Free Radic. Biol. Med. 19:77-101. 1995.

Bertorello, AM, and Katz, AI. Regulation of Na^+,K^+ pump activity: pathways between receptors and effectors. News Physiol. Sci. 10:253-259. 1995.

Bickford, PC, Gould, T, Briederick, L, Chadman, K, Pollock, A, Young, D, Shukitt-Hale, B, and Joseph, J. Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. Brain Research. 866:211-217. 2000.

Birzniece, V, Backstrom, T, Johansson, IM, Lindblad, C, Lundgren, P, Lofgren, M, Olsson, T, Raqaqnin, G, Taube, M, Turkmen, S, Wahlstrom, G, Wang, MD, WihlbaCk, AC, and Zhu, D. Neuroactive steroid effects on cognitive functions with a focus on the serotonin and GABA systems. Brain Res. Brain Res. Rev. 51(2):212-239. 2006.

Bora, SH, Liu, Z, Kecojevic, A, Merchenthaler, I, and Koliavtos, VE. Direct, complex effects of estrogens on basal forebrain cholinergic neurons. Exp. Neurol. 194(2):506-522. 2005.

Brigelius-Flohe, R, Kelly, FJ, Salonen, JT, Neuzil, J, Zingg, JM, and Azzi, A. The European perspective on vitamin E: current knowledge and future research. Am. J. Clin. Nutr. 76(4):703-716. 2002.

Brosemer, RW. Effects of inhibitors of Na^+,K^+-ATPase on the membrane potentials and neurotransmitter efflux in rat brain slices. Brain Res. 334:125-137. 1985.

Carageorgiou, H, Zarros, A, and Tsakiris, S. Selegiline long-term effects on brain acetylcholinesterase, (Na+,K+)-ATPase activities, antioxidant status and learning performance of aged rats. Pharmacol. Res. 48(3):245-51. 2003.

Carr, A, and Frei, B. Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J. 13:1007-1024. 1999.

Chakraborty, TR, and Gore, AC. Aging-related changes in ovarian hormones, their receptors, and neuroendocrine function. Exp. Biol. Med. (Maywood). 229:977-987. 2004.

Chan, AS, Cheung, MC, Law, SC, and Chan, JH. Phase II study of alpha-tocopherol in improving the cognitive function of patients with temporal lobe radionecrosis. Cancer. 100:398-404. 2004.

Chen, JQ, Contreras, RG, Wang, R, Fernandez, SV, Shoshani, L, Russo, IH, Cereijido, M, and Russo, J. Sodium/potasium ATPase (Na(+),K(+)-ATPase) and ouabain/related cardiac glycosides: a new paradigm for development of anti-breast cancer drugs? Breast Cancer Res. Treat. 96:1-15. 2006.

Cooke, GM. A review of the animal models used to investigate the health benefits of soy isoflavones. J. AOAC. Int. 89(4):1215-1227. 2006.

Cornwell, T, Cohick, W, and Raskin, I. Dietary phytoestrogens and health. Phytochemistry. 65:995-1016. 2004.

Craig, MC, Maki, PM, and Murphy, DGM. The Women's Health Initiative Memory Study: findings and implications for treatment. Lancet Neurol. 4:190-194. 2005.

Culmsee, C, Vedder, H, Ravati, A, Junker, V, Otto, D, Ahlemeyer, B, Krieg, JC, and Krieglstein, J. Neuroprotection by estrogens in a mouse model of focal cerebral ischemia and in cultured neurons: evidence for a receptor-independent antioxidative mechanism. J. Cereb. Blood Flow Metab. 19:1263-1269. 1999.

Cummings, JL. The role of cholinergic agents in the management of behavioral disturbances in Alzheimer's disease. Int. J. Neuropsychopharmacol. 3:21-29. 2000.

Daniel, JM, and Dohanich, GP. Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. J. Neurosci. 21:6949-6956. 2001.

Davies, P, and Maloney, AJ. Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet. 2:1403. 1976.

Day, M, and Good, M. Ovariectomy-induced disruption of long-term synaptic depression in the hippocampal CA1 region in vivo is attenuated with chronic estrogen replacement. Neurobiol. Learn. Mem. 83:13-21. 2005.

Del Castillo, AR, Battaner, E, Guerra, M, Alonso, T, and Mas, M. Regional changes of brain Na^+, K^+ - transporting adenosine triphosphatase related to ovarian function. Brain Res. 416:113-118. 1987.

Delwing, D, Chiarani, F, Bavaresco, CS, Wannmacher, CM, Wajner, M, Dutra-Filho, CS, and Wyse, ATS. Protective effect of antioxidants on brain oxidative damage caused by proline administration. Neurosci. Res. 52:69-74. 2005.

Delwing, D, Tagliari, B, Chiarani, F, Wannmacher, CM, Wajner, M, and Wyse, ATS. Alpha-tocopherol and ascorbic acid administration prevents the impairment of brain energy metabolism of hyperarginenic rats. Cell. Mol. Neurobiol, 26(2):177-189. 2006.

Devlin, TM. Membranas biológicas: estrutura e transporte em membranas. In: Manual de bioquímica com correlações clínicas. Estados Unidos da América: editora Edgard Blücher LTDA. Tradução da 5ª edição americana. p.462-463. 2003.

D'Hooge, R, and De Deyn, PP. Applications of the Morris water maze in the study of learning and memory. Brain Res. Rev. 36:60-90. 2001.

Dicko, A, Morissette, M, Ben Ameur, S, Pézolet, M, and Di Paolo, T. Effect of estradiol and tamoxifen on brain membranes: investigation by infrared and fluorescence spectroscopy. Brain Res. Bull. 49(6):401-405. 1999.

Dumas, J, Hancur-Bucci, C, Naylor, M, Sites, C, and Newhouse, P. Estrogen treatment effects on anticholinergic-induced cognitive dysfunction in normal postmenopausal women. Neuropsychopharmacology. 31(9):2065-2078. 2006.

Du Toit, DR, Volsteedt, Y, and Apostolides, Z. Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. Toxicology. 166(1-2):63-69. 2001.

Eals, MS, and Silverman, I. The hunter-gatherer theory of spatial sex differences: proximate factors mediating the female advantage in the recall of object arrays. Ethol. Sociobiol. 15:95-105. 1997.

Ekiel, IH, Hughes, L, Burton, GW, Jovall, PA, Ingold, KU, and Smith, IC. Structure and dynamics of alpha-tocopherol in model membranes and in solution: a broad-line and high-resolution NMR study. Biochemistry. 27:1432-1440. 1988.

Erecinska, M, Cherian, S, and Silver, IA. Energy metabolism in mammalian brain during development. Prog. Neurobiol. 73:397-445. 2004.

Erecinska, M, and Silver, IA. Ions and energy in mammalian brain. Prog. Neurobiol. 43:37-71. 1994.

Fader, AJ, Johnson, PE, and Dohanich, GP. Estrogen improves working but not reference memory and prevents amnestic effects of scopolamine of a radial-arm maze. Pharmacol. Biochem. Behav. 62:711-717. 1999.

Feingold, IB, Longhurst, PA, and Colby, HD. Regulation of adrenal and hepatic alpha-tocopherol content by androgens and estrogens. Biochim. Biophys. Acta. 1176(1-2):192-196. 1993.

File, SE, Jarrett, N, Fluck, E, Duffy, R, Casey, K, and Wiseman, H. Eating soya phytoestrogens improves human memory. Psychopharmacology. 157:430-436. 2001.

Fishman, EB, Siek, GC, MacCallum, RD, Bird, ED, Volicer, L, and Marqui, JK. Distribution of the molecular forms of acetylcholinesterase in human brain: alterations in dementia of the Alzheimer type. Ann. Neurol. 19:246-252. 1986.

Fossi, MC, Leonzio, C, Massi, A, Lari, L, and Casini, S. Serum esterase inhibition in birds: a nondestructive biomarker to assess organophosphorus and carbamate contamination. Arch. Environ. Contam. Toxicol. 23:99-104. 1992.

Foy, MR, Xu, J, Xie, X, Brinton, RD, Thompson, RF, and Berger, TW. 17β-Estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J. Neurophysiol. 81:925-929. 1999.

Gamache, PH, and Acworth, IN. Analysis of phytoestrogens and polyphenols in plasma, tissue, and urine using HPLC with coulometric array detection. Proc. Soc. Exp. Biol. Med. 217(3):274-280. 1998.

Geller SE, and Studee L. Soy and red clover for mid-life and aging. Climateric. 9(4):245-263. 2006.

Geula, C, and Darvesh, S. Butyrylcholinesterase, cholinergic neurotransmission and the pathology of Alzheimer's disease. Drugs Today. 40:711-21. 2004.

Giacobini, E. From molecular structure to Alzheimer therapy. Jpn. J. Pharmacol. 74:225-241. 1997.

Giacobini, E. Cholinesterases: New Roles in Brain Function and in Alzheimer's Disease. Neurochem. Res. 28:515-522. 2003.

Giacobini, E, DeSarno, P, Clark, B, and McIlhany, M. The cholinergic receptor system of the human brain: neurochemical and pharmacological aspects in aging and Alzheimer. Prog. Brain Res. 79:335-343. 1989.

Gibbs, RB, and Aggarwal, P. Estrogen and basal forebrain cholinergic neurons: implications for brain aging and Alzheimer's disease-related cognitive decline. Horm. Behav. 34:98-111. 1998.

Gimeno, A, Zaragoza, R, Vina, JR, and Miralles, VJ. Vitamin E activates CRABP-II gene expression in cultured human fibroblasts, role of protein kinase C. FEBS Lett. 569(1-3):240-244. 2004.

Glazier MG, and Bowman MA. A review of the evidence for the use of phytoestrogens as a replacement for traditional estrogen replacement therapy. Arch. Intern. Med. 161(9):1161-1172. 2001.

Gomez-Fernandez, JC, Villalain, J, Aranda, FJ, Ortiz, A, Micol, V, Coutinho, A, Berberan-Santos, MN, and Prieto, MJ. Localization of alpha-tocopherol in membranes. Ann. N. Y. Acad. Sci. 570:109-120. 1989.

Gómez-Ramos, P, and Morán, MA. Ultra structural localization of butyrylcholinesterase in senile plaques in the brains of aged and Alzheimer's disease patients. Mol. Chem. Neuropathol. 30:161-173. 1997.

Gomez-Zubeldia, MA, Arbues, JJ, Hinchado, G, Nogales, AG, and Millan, JC. Influence of estrogen replacement therapy on plasma lipid peroxidation. Menopause. 8:274-280. 2001.

Gottfries, CG, Karlsson, I, and Svennerholm, L. Membrane components separate early-onset Alzheimer's disease from senile dementia of the Alzheimer type. Int. Psychogeriatr. 8:365-372. 1996.

Grafius, MA, Bond, HE, and Millar, DB. Acetylcholinesterase interaction with a lipoprotein matrix. Eur. J. Biochem. 22:382-90. 1971.

Green, PS, and Simpkins, JW. Neuroprotective effects of estrogens: potential mechanisms of action. Int. J. Devl. Neurosci. 18:347-358. 2000.

Greig, NH, Utsuki, T, Yu, Q, Zhu, X, Holloway, HW, Perry, T, Lee, B, Ingram, DK, and Lahiri, DK. A new therapeutic target in Alzheimer's disease treatment: attention to butyrylcholinesterase. Curr. Med. Res. Opin. 17:159-165. 2001.

Gridley, KE, Green, PS, and Simpkins, JW. Low concentrations of estradiol reduce beta-amyloid (25-35) - induced toxicity, lipid peroxidation and glucose utilization in human SK-N-SH neuroblastoma cells. Brain Res. 778:158-165. 1997.

Gureviciene, I, Puolivali, J, Pussinen, R, Wang, J, Tanila, H, and Ylinen, A. Estrogen treatment alleviates NMDA-antagonist induced hippocampal LTP blockade and cognitive deficits in ovariectomized mice, Neurobiol. Learn. Mem. 79:72-80. 2003.

Halbreich, U, Lumley, LA, Palter, S, Manning, C, Gengo, F, and Joe, SH. Possible acceleration of age effects on cognition following menopause. J. Psychiatr. Res. 29:153-163. 1995.

Halliwell, B. Free radicals, protein and DNA: oxidative damage versus redox regulation. Biochem. Soc. Trans. 24:1023-1027. 1996.

Halliwell, B, and Gutteridge, JMC. Oxygen radicals and nervous system. Trends Neurosci. 8:22-26. 1985.

Halliwell, B, and Gutteridge, JMC. Free radicals in biology and medicine. Oxford University Press Inc. New York, 3rd edition, 2000.

Halliwell, B, and Whiteman, M. 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. 2004.

Hara, H, Kato, H, and Kogure, K. Protective effect of alpha-tocopherol on ischemic neuronal damage in the gerbil hippocampus. Brain Research. 510:335-338. 1990.

Harman, D. Free radical theory of aging. Mutant Res. 275:257-266. 1992.

Hattori, N, Kitagawa, K, Higashida, T, Yagyu, K, Shimohama, S, Wataya, T, Perry, G, Smith, MA, and Inagaki, C. Cl^- - ATPase and Na^+ / K^+ - ATPase activities in Alzheimer's disease brains. Neurosci. Lett. 254:141-144. 1998.

Henderson, VW, Watt, L, and Buckwalter, JG. Cognitive skills associated with estrogen replacement in women with Alzeimer's disease. Psychoneuroendocrinology. 21:421-430. 1996.

Henderson, VW. The neurology of menopause. Neurologist. 12(3):149-59. 2006.

Heikkinen, T, Puolivali, J, and Tanila, H. Effects of long-term ovariectomy and estrogen treatment on maze learning in aged mice. Exp. Gerontol. 39(9):1277-1283. 2004.

Inal, M, Sunal, E, Kanbak, G, and Zeytinoglu, S. Effect of postmenopausal hormone replacement and alpha-tocopherol on the lipid profiles and antioxidant satatus. Clin Chim Acta. 268(1-2):21-29. 1997.

Inoue, N, and Matsui, H. Changes in responses of Na pump isoforms to glutamate excitation of cerebral neurons during maturation in culture. Soc. Gen. Physiol. Ser. 46:597-600. 1991.

Islam, F, Hasan, M, and Saxena, K. Isolation and estimation of gangliosides in discrete regions of the forebrain: effects of estrogen on regional lipid profiles. Exp. Pathol. 29(3):159-164. 1986.

Iwamoto, J, Takeda, T, Sato, Y, Shen, CL, and Yeh, JK. Effect of pre- and post-surgery treatment with risedronate on trabecular bone loss in ovariectomized rats. Exp Anim. 55(5):457-466. 2006.

Izquierdo, I. Os tipos e as formas de memória. In: Memória. ARTMED Editora S.A. Porto Alegre, RS. Brasil. p.19-33. 2002.

Jain, SK, Mc Vie, R, and Smith, T. Vitamin E supplementation restores glutathione and malondialdehyde to normal concentrations in erythrocytes of type 1 diabetic children. Diab. Care. 23:1389-1394. 2000.

James, TWK. Sex differences in remembering the location of objects in an array; location shifts versus location exchanges. Evol. Human Behav. 18:155-163. 1997.

Jarrard, LE. On the role of the hippocampus in learning and memory in the rat. Behav. Neural. Biol. 60(1):9-26. 1993.

Kaplan, JH. Biochemistry of Na^+,K^+-ATPase. Annu. Rev. Physiol. 65:817-849. 2002.

Kim, JS, Kim, HY, Kim, SH, Shin, HK, Lee, SH, Lee, YS, and Son, H. Enhancement of rat hippocampal long-term potentiation by 17 beta-estradiol involves mitogen-activated protein kinase-dependent and independent components, Neurosci. Lett. 332:65-69. 2002.

Kim, H, Xia, H, Li, L, and Gewin, J. Attenuation of neurodegeneration-relevant modifications of brain proteins by dietary soy. Biofactors. 12:243-250. 2000.

Kritz-Silverstein, D, Von Muhlen, D, Barrett-Connor, E, and Bressel, MA. Isoflavones and cognitive function in older women: the soy and postmenopausal health in aging (SOPHIA) study. Menopause. 10:196-202. 2003.

Kuiper, GG, Enmark, E, Pelto-Huikko, M, Nilsson, S, and Gustafsson, JA. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA. 93(12):5925-5930. 1996.

Kuiper, GG, Lemmen, JG, Carlsson, B, Corton, JC, Safe, SH, Van der Saag, PT, Van der Burg, B, and Gustafsson, JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 139(10):4252-4263. 1998.

Kume-Kick, J, Ferris, DC, Russo-Menna, I, and Rice, ME. Enhanced oxidative stress in female rat brain after gonadectomy. Brain Res. 738:8-14. 1996.

Kurzer, MS, and Xu, X. Dietary phytoestrogens. Annu. Rev. Nutr. 17:353-381. 1997.

Kwak, DH, Kim, SM, Lee, DH, Kim, JS, Kim, SM, Lee, SU, Jung, KY, Seo, BB, and Choo, YK. Differential expression patterns of gangliosides in the ischemic cerebral cortex produced by middle cerebral artery occlusion. Mol. Cells. 20(3):354-360. 2005.

Lane, RM, Potkin, SG, and Enz, A. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int. J. Neuropsychopharmacol. 9(1):101-124. 2005.

Law, A, Gauthier, S, and Quirion, R. Say NO Alzheimer's disease: The putative links between nitric oxide and dementia of the Alzheimer's type. Brain Res. Brain Res. Rev. 35:73-96. 2001.

Lee, YB, Lee, HJ, Won, MH, Hwang, IK, Kang, TC, Lee, JY, Nam, SY, Kim, KS, Kim, E, Cheon, SH, and Sohn, HS. Soy isoflavones improve spatial delayed matching-to-place performance and reduce cholinergic neuron loss in elderly male rats. J. Nutr. 134:1827-1831. 2004.

Lephart, ED, Rhees, RW, Setchell, KDR, Bu, LH, and Lund, TD. Estrogens and phytoestrogens: brain plasticity of sexually dimorphic brain volumes. J. Steroid Biochem. Mol. Biol. 85: 299-309. 2003.

Lephart, ED, West, TW, Weber, KS, Rhees, RW, Setchell, KDR, Adlercreutz, H, and Lund, TD. Neurobehavioral effects of dietary soy phytoestrogens. Neurotoxicol. Teratol. 24:5-16. 2002.

Le Saux, M, Estrada-Camarena, E, and Di Paolo, T. Selective estrogen receptor-alpha but not-beta agonist treatment modulates brain alpha-amino-3-hydroxy-5-

methyl-4-isoxazolepropionic acid receptors. J. Neurosci. Res. 84(5):1076-1084. 2006.

Levin, G, Cogan, U, Levy, Y, and Mokady, S. Riboflavin deficiency and the function and fluidity of rat erythrocyte membranes. J. Nutr. 120:857-861. 1990.

Lund, TD, West, TW, Tian, Y, Bu, LH, Simmons, DL, Setchell, KDR, Adlercreutz, H, and Lephart ED. Visual spatial memory is enhanced in female rats (but inhibited in males) by dietary soy phytoestrogens. BMC Neurosci.1;2:20. 2001.

McEwen, BS, Tanapat, P, and Weiland, NG. Inhibition of dendritic spine induction on hippocampal CA1 pyramidal neurons by a nonesteroidal estrogen antagonist in female rats. Endocrinology. 140:1044-1047. 1999.

Maccioni, HJF, Panzetta, P, Arrieta, D, and Caputto, R. Ganglioside glycosil transferase activities in the cerebral hemispheres from developing rat embryos. Int. J. Develop. Neurosc. 2:13-19. 1984.

Magarian, EO, and Dietz, AJ. Correlation of cholinesterase with serum lipids and lipoproteins. J. Clin. Pharmacol. 27:819-820. 1987.

Marder, K, and Sano, M. Estrogen to treat Alzheimer's disease: too little, too late? So what's a woman to do? Neurology. 54(11):2035-2037. 2000.

Mata, M, Fink, DJ, Gainer, H, Smith, CB, Davidsen, L, Savakis, H, Schwartz, W, and Sokoloff, L. Activity dependent energy metabolism rat posterior pituitary primarily reflects sodium pump activity. J. Neurochem. 34:213-215. 1980.

McCay, PB. Vitamin E: interactions with free radical and ascorbate. Annu. Ver. Nutr. 5:323-340. 1985.

Mecocci, P, Mariani, E, Cornacchiola, V, and Polidori, MC. Antioxidants for the treatment of mild cognitive impairment. Neurol. Res. 26(5):598-602. 2004.

Melo, JB, Agostinho, P, and Oliveira, CR. Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. Neurosci. Res. 45:117-127. 2003.

Messinis, IE. Ovarian feedback, mechanism of action and possible clinical implications. Hum. Reprod. Update. 12(5):557-571. 2006.

Mesulam, M, and Geula, C. Butyrylcholinesterase reactivity differentiates the amyloid plaques of aging from those of dementia. Ann. Neurol. 36:722-727. 1994.

Mhyre, AJ, and Dorsa, DM. Estrogen activates rapid signaling in the brain: role of estrogen receptor α and estrogen receptor β in neurons and glia. Neuroscience. 138:851-858. 2006.

Miksicek, RJ. Estrogenic flavonoids: structural requirements for biological activity. Proc. Soc. Exp. Biol. Med. 208:44-50. 1995.

Miquel, J, Ramírez-Boscá, A, Ramírez-Boscá, JV, and Alperi, JD. Menopause: a review on the role of oxygen stress and favorable effects of dietary antioxidants. Arch. Gerontol. Geriatr. 42(3):289-306. 2006.

Mitra, SW, Hoskin, E, Yudkovitz, J, Pear, L, Wilkinson, HÁ, Hayashi, S, Pfaff, DW, Ogawa, S, Rohrer, SP, Schaeffer, JM, McEwen, BS, and Alves, SE. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology. 144(5):2055-2067. 2003.

Mooradian, A.D. Antioxidant properties of steroids. J. Steroid Biochem. Mol. Biol. 45:509-511. 1993.

Mukai, K, Daifuku, K, Yokoyama, S, and Nakano, M. Stopped-flow investigation of antioxidant activity of estrogens in solution. Biochim. Biophys. Acta. 1035:348-352. 1990.

Naftolin, F, Leranth, C, Horvath, TL, and Garcia-Segura, LM. Potencial neuronal mechanisms of estrogen actions in synaptogenesis and synaptic plasticity. Cell. Mol. Neurobiol. 16: 213-223. 1996.

Nakagawa, K, Adachi, J, Wong, MC, and Ueno, Y. Protective effect of daidzein against acute ethanol-induced lipid peroxidation in rat jejunum. Kobe J. Med. Sci. 52(5):141-149. 2006.

Nakamura, Y, Tsuji, S, and Tonogai, Y. Determination of the levels of isoflavonoids in soybeans and soy-derived foods and estimation of isoflavonoids in the Japanese daily intake. J. AOAC Int. 83:635-650. 2000.

Nanji, AA, and Sadrzadeh, SM. Effect of fish oil and vitamin E on ethanol-induced changes in membrane ATPases. Life Sci. 55:245-249. 1994.

Noh, SK, Koo, SI, and Jeon, IJ. Estradiol replacement in ovariectomized rats increases the hepatic concentration and biliary secretion of α-tocopherol and polyunsaturated fatty acids. J. Nutr. Biochem. 10:110-117. 1999.

Okuda, T, Haga, T, Kanai, Y, Endou, H, Ishihara, T, and Katsura, I. Identification and characterization of the high-affinity choline transporter. Nat. Neurosci. 3:120-125. 2000.

Pan, Y, Anthony, M, and Clarkson, TB. Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. Pro. Soc. Exp. Biol. Med. 221:118-125. 1999.

Pan, Y, Anthony, M, Watson, S, and Clarkson, TB. Soy phytoestrogens improve radial arm maze performance in ovariectomized retired breeder rats and do not attenuate benefits of 17 beta-estradiol. Menopause. 7(4):230-250. 2000.

Patisaul, HB, Melby, M, Whitten, PL, and Young, LJ. Genistein affects ERβ- but not ERα-dependent gene expression in the hypothalamus. Endocrinology. 143:2189-2197. 2002.

Petersen, RC, Thomas, RG, Grundman, M, Bennet, D, Doody, R, Ferrs, S, Galasko, D, Jin, S, Kaye, J, Levey, A, Pfeiffer, E, Sano, M, van Dyck, CH, and Thal, LJ. Alzheimer's Disease Cooperative Study Group. Vitamin E and donepezil for the treatment of mild cognitive impairment. N. Engl. J. Med. 352:2379-2388. 2005.

Pike, CJ. Estrogen modulates neuronal Bel-xl expression and β-amyloid-induced apoptosis:relevance to Alzheimer's disease. J. Neurochem. 72:1552-1563. 1999.

Pinkerton, JV, and Henderson, VW. Estrogen and cognition, with a focus on Alzheimer's disease. Semin. Reprod. Med. 23(2):172-179. 2005.

Qin, F, Yan, C, Patel, R, Liu, W, and Dong, E. Vitamins C and E attenuate apoptosis, beta-adrenergic receptor desensitization, and sarcoplasmic reticular

Ca2+ ATPase downregulation after myocardial infarction. Free Rad. Biol. Med. 40(10):1827-1842. 2006.

Quick, KL, Ali, SS, Arch, R, Xiong, C, Wozniak, D, and Dugan, LL. A carboxyfullerene SOD mimetic improves cognition and extends the lifespan of mice. Neurobiol. Aging. *In press*.

Razandi, M, Pedram, A, Greene, GL, and Levin, ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERα and ERβ expressed in Chinese hamster ovary cells. Mol. Endocrinol. 13:307-319. 1999.

Reis, EA, Zugno, AI, Franzon, R, Tagliari, B, Matté, C, Lammers, ML, Netto, CA, and Wyse, ATS. Pretreatment with vitamins E and C prevent the impairment of memory caused by homocysteine administration in rats. Metab. Brain Dis. 17(3):211-217. 2002.

Reznick, AZ, and Packer, L. Free radicals and antioxidants in muscular neurological diseases and disorders. In: Free Radicals: From Basic Science to Medicine. Poli, G, Albano, E, and Dianzani, M. (eds.). Birkhäuser Verlag, Basel, pp. 425-437. 1993.

Rodrigues, HD, Kinder, JE, and Fitzpatrick, LA. Treatment with 17 beta-estradiol does not influence age and weight at puberty in Bos indicus heifers. Anim. Reprod. Sci. 56:1-10. 1999.

Rodríguez, GP. Funciones de la vitamina E en la nutrición humana. Rev. Cubana Aliment. Nutr. 11(1):46-57. 1997.

Rossouw, JE, Anderson, GL, Prentice, RL, LaCroix, AZ, Kooperberg, C, Stefanick, ML, Jackson, RD, Beresford, SA, Howard, BV, Johnson, KC, Kotchen, JM, and

Ockene, J, Writing Group for the Women's Health Initiative Investigators. Risk and benefits of estrogen plus progestin in healthy postmenopausal women. J. Am. Med. Assoc. 288:321-333. 2002.

Saez-Valero, J, Gonzalez-Garcia, C, and Cena, V. Acetylcholinesterase activation in organotypic rat hippocampal slice cultures deprived of oxygen and glucose. Neurosci. Lett. 48: 123-125. 2003.

Salvador, M, and Henriques, JAP. In: Radicais livres e a resposta celular ao estresse oxidativo. 1ª edição. Canoas, RS: Editora da Ulbra. 2004.

Sandstrom, NJ and Williams, CL. Spatial memory retention is enhanced by acute and continuous estradiol replacement. Horm. Behav. 45(2):128-135. 2004.

Sanhoff, K, and Van Echten, G. Metabolism of Gangliosides: Topology, Pathobiochemistry, and Sphingolipid Activator Proteins. In: Current Topics in Membranes, vol.40, pp.75-91. 1994.

Sano, M, Ernesto, C, Thomas, RG, Klauber, MR, Schafer, K, Grundman, M, Woodbury, P, Growdon, J, Cotman, C W, Pfeiffer, E, Schneider, LS, and Thal, LJ. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer´s disease. The Alzheimer's Disease Cooperative Study. N. Engl. J. Med. 336(17):1216-1222. 1997.

Sato, T, Teramoto, T, Tanaka, K, Ohnishi, Y, Irifune, M, and Nishikawa, T. Effects of ovariectomy and calcium deficiency on learning and memory of eight-arm radial maze in middle-aged female rats. Behav. Brain Res. 142:207-216. 2003.

Savonenko, AV, and Markowska, AL. The cognitive effects of ovariectomy and estrogen replacement are modulated by aging. Neuroscience. 119(3):821-830. 2003.

Setchell, KDR. Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones. Am. J. Clin. Nutr. 68(6):1333S-1346S. 1998.

Setchell, KDR, and Adlercreutz, H. Mammalian ligants and phyto-oestrogens recent studies on their formation, metabolism and biological role in health and disease. In: Role of the Gut Flora in Toxicity and Cancer. Rowland IR (ed.). New York: Academic Press. pp. 315-345. 1988.

She, JQ, Wang, M, Zhu, DM, Sun, LG, and Ruan, DY. Effect of ganglioside on synaptic plasticity of hippocampus in lead-exposed rats in vivo. Brain Res. 1060:162-169. 2005.

Sherwin, BB. Estrogen and cognitive functioning in women. Endocr. Ver. 24:133-151. 2003.

Sherwin, BB. Estrogen and cognitive aging in women. Neuroscience. 138(3):1021-1026. 2006.

Simpkins, JW, Rajakumar, G, Zhang, YQ, Simpkins, CE, Greenwald, D, Yu, CJ, Bodor, N, and Day, AL. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rats. J. Neurosurg. 87:724-30. 1997.

Singh, U, Devaraj, S, and Jialal, I. Vitamin E, oxidative stress, and inflammation. Annu. Rev. Nutr. 25:151-174. 2005.

Singh, M, Meyer, EM, Millard, WJ, and Simpkins, JW. Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. Brain Res. 644:305-312. 1994.

Siqueira, IR, Fochesatto, C, Andrade, A, Santos, M, Hagen, M, Bello-Klein, A, and Netto, CA. Total antioxidant capacity is impaired in different structures from aged rat brain. Int. J. Devl. Neuroscience. 23:663-671. 2005.

Sirtori, CR, Arnoldi, A, and Johnson, SK. Phytoestrogens: End of a tale? Ann. Med. 37:423-438. 2005.

Soulsby, ME, Phillips, B, and Chowdhury, P. Brief communication: Effects of soy-protein diet on elevated brain lipid peroxide levels induced by simulated weightlessness. Ann. Clin. Lab. Sci. 34(1):103-106. 2004.

Sutcliffe, JS, Marshall, KM, and Neil, JC. Influence of gender on working and spatial memory in the novel object recognition task in the rat. Behav. Brain Res. 177:117-125. 2007.

Tanapat, P, Hastings, NB, Reeves, AJ, and Gould E. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J. Neurosci. 19:5792-5801. 1999.

Tang, MX, Jacobs, D, Stern, Y, Marder, K, Schofield, P, Gurland, B, Andrews, H, and Mayeux, R. Effect of oestrogen during menopause on risk and age at onset of Alzeimer's disease. Lancet. 348:429-432. 1996.

Tettamanti, G, and Riboni, L. Ganglioside turnover and neural function: a new perspective. In: Svennerholm L, Asbury AK, Reisfeld RA, Sandhoff, K, Suzuki, K, Tettamanti, G, Toffano, G (eds.). Progress in brain research, vol. 101. Amsterdam: Elsevier Science BV. p. 77-100. 1994.

Thakur, MK, and Sharma, PK. Aging of brain: role of estrogens. Neurochem. Res. 31:1389-1398. 2006.

Toran-Allerand, CD, Guan, X, MacLusky, NJ, Horvath, TL, Diano, S, Singh, M, Connolly, ES Jr, Nethrapalli, IS, and Tinnikov, AA. ER-X: a novel plasma-membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J. Neurosci. 22:8391-8401. 2002.

Torto, R, Boghossian, S, Dube, MG, Kalra, PS, and Kalra, SP. Central leptin gene therapy blocks ovariectomy-induced adiposity. Obesity (Silver Spring). 14(8):1312-1319. 2006.

Travacio, M, and Llesuy, S. Antioxidant enzymes and their modification under oxidative stress conditions. Free Radic. Res. Latin Am. 48:9-13. 1996.

Tsai, ML, Chang, CC, Lee, CL, and Huang, BY. The differential effects of tamoxifen and ICI 182, 780 on the reduction of Na^+/K^+ ATPase and spontaneous oscillations by 17 beta-estradiol. Chin. J. Physiol. 46:55-62. 2003.

Tsai, ML, Lee, CL, Tang, MJ, and Liu, MY. Preferential reduction of Na^+/K^+ ATPase alpha 3 by 17 beta-estradiol influences contraction frequency in rat uteri. Chin J Physiol. 43:1-8. 2000.

Upritchard, JE, Sutherland, WHE, and Mann, JI. Effect of supplementation with tomato juice, vitamin E, and vitamin C on LDL oxidation and products of inflammation activity in type 2 diabetes. Diabetes Care. 23:733-738. 2000.

Van Duijn, CM. Hormone replacement therapy and Alzeimer's disease. Maturitas. 31:201-205. 1999.

Vatassery, G.T. Vitamin E and other endogeneous antioxidants in the central nervous system. Geriatrics. 53:25-27. 1998.

Vedder, H, Anthes, N, Stumm, G, Wurz, C, Behl, C, and Krieg, JC. Estrogen hormones reduce lipid peroxidation in cells and tissues of the central nervous system. J. Neurochem. 72(6):2531-2538. 1999.

Vermeulen, A. The hormonal activity of the postmenopausal ovary. J. Clin. Endocrinol. Metab. 42(2):247-253. 1976.

Walf, AA, Rhodes, ME, and Erye, CA. Ovarian steroids enhance object recognition in naturally cycling and ovariectomized, hormone-primed rats. 86(1):35-46. 2006.

Wallace, M, Luine, V, Arellanos, A, and Frankfurt, M. Ovariectomized rats show decreased recognition memory and spine density in the hippocampus and prefrontal cortex. Brain Res. 1126:176-182. 2006.

Waring, SC, Rocca, WA, Petersen, RC, O'Brien, PC, Tangalos, EG, and Kokmen, E. Postmenopausal estrogen replacement therapy and risk of AD: a population-based study. Neurology. 52:965-970. 1999.

Warren, SG, Humphreys, AG, Juraska, JM, and Greenough, WT. LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. Brain Res. 703:26-30. 1995.

Weiland, NG, Orikasa, CM, Hayashi, S, and McEwen, BS. Distribution and hormone regulation of estrogen receptor immunoreactive cells in the hippocampus of male and female rats. J. Comp. Neurol. 388(4): 603-612. 1997.

White, E, Kristal, AR, Shikani, JM, Wilson, AC, Chen, C, Mares-Perlman, JA, Masaki, KH, and Caan, BJ. Correlates of serum alpha- and gamma-tocopherol in the Women's Health Initiative. Ann. Epidemiol. 11(2):136-144. 2001.

Wise, PM. The “menopause” and the aging brain: causes and repercussions of hypoestrogenicity. Biogerontology. 2(2):113-115. 2001.

Wise, PM. Estrogens and neuroprotection. Trends Endocrinol. Metab. 13:229-230. 2002.

Wise, PM. Estrogens: protective or risk factors in brain function? Prog. Neurobiol. 69(3):181-191. 2003.

Wise, PM, Dubal, DB, Rau, SW, Brown, CM, and Suzuki, S. Are estrogens protective or risk factors in brain injury and neurodegeneration? Reevaluation after The Women’s Health Initiative. Endocr. Rev. 26:308-312. 2005.

Woolley, CS, and McEwen, BS. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J. Comp. Neurol. 336:293-306. 1993.

Woolley, CS, Weiland, NG, McEwen, BS, and Schwartzkroin, PA. Estradiol increases the sensivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input. Correlation with dendritic spine density. J. Neurosci. 17: 1848-1859. 1997.

World Health Organization 1996. Research on the Menopause in the 1990s. Technical report Scr. 866, Geneva, Switzerland.

Wyse, ATS, Stefanello, FM, Chiarani, F, Delwing, D, Wannmacher, CM, and Wajner, M. Arginine administration decreases cerebral cortex acetylcholinesterase and serum butyrylcholinesterase probably by oxidative stress induction. Neurochem Res. 29(2):385-389. 2004.

Wyse, ATS, Streck, EL, Worm, P, Wajner, A, Ritter, F, and Netto, CA. Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. Neurochem. Res. 25:969-973. 2000.

Wyse, ATS, Zugno, AI, Streck, EL, Matte, C, Calcagnotto, T, Wannmacher, CM, and Wajner, M. Inhibition of Na^+,K^+-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. Neurochem. Res. 27:1685-1689. 2002.

Yamamoto, N, Van Nostrand, WE, and Yanagisawa, K. Further evidence of local ganglioside-dependent amyloid beta-protein assembly in brain. Neuroreport. 17(16):1735-1737. 2006.

Yin, J, Hashimoto, A, Izawa, M, Miyazaki, K, Chen, GY, Takematsu, H, Kozutsumi, Y, Susuki, A, Furuhata, K, Cheng, FL, Lin, CH, Sato, c, Kitajima, K, and Kannagi, R. Hypoxic culture induces expression of sialin, a sialic acid transporter, and cancer-associated gangliosides containing non-human sialic acid on human cancer cells. Cancer Res. 66(6):2937-2945. 2006.

Younkin, SG, Goodridge, B, Katz, J, Lockett, G, Nafziger, D, Usiak, MF, and Younkin, LH. Molecular forms of acetylcholinesterases in Alzheimer's disease. Fed. Proc. 45:2982-2988. 1986.

Yousef, MI, Kamel, KI, Esmail, AM, and Baghdadi, HH. Antioxidant activities and lipid lowering effects of isoflavone in male rabbits. Food Chem. Toxicol. 42(9):1497-1503. 2004.

Yu, BP. Cellular defenses against damage from reactive oxygen species. Physiol. Rev. 74(1):139-162. 1994.

Yusuf, S, Dagenais, G, Pogue, J, Bosch, J, and Sleight, P. Vitamin E supplementation and cardiovascular events in high-risk patients: the Heart Outcomes Prevention Evaluation Study Investigators. N. Engl. J. Med. 342:154-160. 2000.

Xie, Z, and Cai, T. Na^+, K^+ - ATPase-mediated signal transduction: from protein interaction to cellular function. Mol. Interv. 3(3):157-168. 2003.

Xu, X, and Zhang Z. Effects of estradiol benzoato on learning-memory behavior and synaptic structure in ovariectomized mice. Life Sci. 79:1553-1560. 2006.

Zamani, MR, Levy, WB, and Desmond, NL. Estradiol increases delayed, *n*-methyl-D-aspartate receptor-mediated excitation in the hippocampal CA1 region. Neuroscience. 129:243-254. 2004.

Zhang, YQ, Shi, J, Rajakumar, G, Day, AL, and Simpkins, JW. Effects of gender and estradiol treatment on focal brain ischemia. Brain Res. 784:321-324. 1998.

Zingg, JM., and Azzi, A. Non-antioxidant activities of vitamin E. Curr. Med.Chem. 11:1113-1133. 2004.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM CB: BIOQUÍMICA

ATA DE DEFESA DE TESE DE DOUTORADO DE
SIOMARA DA CRUZ MONTEIRO
NÚMERO 164

Aos quinze dias do mês de março do ano dois mil e sete, no Auditório do Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul (UFRGS), realizou-se a defesa de Tese de Doutorado de **SIOMARA DA CRUZ MONTEIRO**, intitulada ***"Alterações bioquímicas e comportamentais causadas pela ovariectomia em ratas adultas: efeito da suplementação com antioxidante e soja"***, orientada pela Doutora Angela Terezinha de Souza Wyse, apresentada de acordo com o artigo 31 do Regimento do Programa. Às quatorze horas (14:00 h), o Doutor Carlos Alberto Saraiva Gonçalves, Coordenador do Programa de Pós-Graduação do Programa, abriu os trabalhos. Em seguida apresentou ao público presente os membros da Banca Examinadora, passando, logo após, a palavra a SIOMARA DA CRUZ MONTEIRO, para que apresentasse seu trabalho de Tese de Doutorado. Após isso, iniciou-se a Defesa da Tese. A Doutora Angela Terezinha de Souza Wyse, Presidente da Banca Examinadora, passou a palavra ao primeiro membro da Banca, Doutora SOLANGE CRISTINA GARCIA POMBLUM (Departamento de Análises Clínicas e Toxicológicas-Centro de Ciências da Saúde/Universidade Federal de Santa Maria). Após, fez uso da palavra o segundo membro da Banca, Doutor ALDO BOLTEN LUCION (Departamento de Fisiologia-Instituto de Ciências Básicas da Saúde/Universidade Federal do Rio Grande do Sul). A seguir, fez uso da palavra o terceiro membro da Banca, Doutora CARLA DALMAZ (Programa de Pós-Graduação em Ciências Biológicas: Bioquímica-Instituto de Ciências Básicas da Saúde/Universidade Federal do Rio Grande do Sul). Os examinadores mantiveram diálogo com a candidata. A Doutora ANGELA TEREZINHA DE SOUZA WYSE, Presidente da Banca Examinadora, comunicou aos presentes que a Banca iria proceder ao ato de atribuição de conceitos, reunindo-se em sessão secreta. Para tanto, os trabalhos foram interrompidos por dez (10) minutos. Após este intervalo, a Banca emitiu os seguintes conceitos: Doutora SOLANGE CRISTINA GARCIA POMBLUM - conceito final: "A", Doutor ALDO BOLTEN LUCION - conceito final: "A" e Doutora CARLA DALMAZ - conceito final: "A". A candidata fez jus ao grau de **DOUTOR** ***em Ciências Biológicas: Bioquímica***. Finalmente o Doutor Carlos Alberto Saraiva Gonçalves encerrou os trabalhos, dos quais lavrei a presente ata, que vai assinada pelos membros examinadores e pelo Coordenador do Pós-Graduação do Programa.

SOLANGE CRISTINA GARCIA POMBLUM
DEPTO. DE ANÁLISES CLÍNICAS
E TOXICOLÓGICAS-CCS/UFSM

ALDO BOLTEN LUCION
DEPARTAMENTO DE FISIOLOGIA-
ICBS /UFRGS

CARLA DALMAZ
PPG EM C.B.: DE BIOQUÍMICA -
ICBS/UFRGS

ANGELA TEREZINHA DE SOUZA WYSE
DEPARTAMENTO DE BIOQUÍMICA-ICBS/UFRGS
PRESIDENTE DA BANCA

CARLOS ALBERTO SARAIVA GONÇALVES
COORDENADOR DO P.P.G. EM C.B.: BIOQUÍMICA-
ICBS/UFRGS

The *Journal of Nutritional Biochemistry* has been accepted for coverage in MEDLINE. Issues will be covered beginning with the January 2003 issue.

Guide for Authors

The editors of *The Journal of Nutritional Biochemistry (JNB)* welcome the submission of original manuscripts on experimental and clinical nutrition as it interfaces with biochemistry, molecular biology, physiology, pharmacology, and toxicology. The scope of the journal includes the broad area of *in vitro* and *in vivo* studies of mechanistic aspects of nutritional sciences. The criteria for acceptance of papers submitted for publication are originality, quality and clarity of the content. All contributions must be based on original, unpublished research and will be peer reviewed. All authors bear responsibility for ensuring the integrity and quality of their reported research. It is the author's responsibility to secure permission to use figures or tables that have been published elsewhere.

Contributions may be classified as original research, review, rapid communication or methodological articles. Most review articles are invited by the editor. Authors interested in submitting a review article should contact the editorial office. Rapid publication of original manuscripts is a goal of the journal. Manuscripts must be written in English. Each manuscript is considered for publication with the understanding that it has not been submitted to any other journal. Upon acceptance for publication, papers are subject to editorial review and revision.

Contact Address:
Dr. Bernhard Hennig, Editor-in-Chief
The Journal of Nutritional Biochemistry
University of Kentucky
900 Limestone Street
Rm. 599 Wethington Health Sciences Building
Lexington, KY 40536-0200
E-mail address: JNB@uky.edu

Submission Guidelines
All manuscripts must be submitted via the Elsevier Editorial System (EES) at http://ees.elsevier.com/jnb/ . Authors may send queries concerning the submission process, manuscript status or journal procedures to the Editorial Office (JNB@uky.edu). All correspondence regarding submitted manuscripts will be through e-mail. Authors who are unable to provide an electronic version or have other circumstances that prevent online submission must contact theEditorial Office prior to submission to discuss alternate options.

A manuscript submission through EES consists of a minimum of four distinct files: a cover letter; an abstract; 3-5 suggested reviewers; and the manuscript. EES accepts files from a broad range of word processing applications. All four files must be typed in 12-point type, double-spaced with one-inch margins, and all pages should be numbered consecutively. The file should follow the general instructions on style/arrangement, and, in particular, the reference style. The file should use the wrap-around end-of-line

feature, i.e., returns at the end of paragraphs only. Place two returns after every element, such as title, headings, and paragraphs.

In addition, Tables and Figures should be included as separate, individual files.

Revised manuscripts should also be accompanied by a file (separate from the cover letter) with responses to reviewers' comments. All files should be labeled with appropriate and descriptive file names (e.g., SmithText.doc, Fig1.eps, Table3.doc). The text, tables and graphics must be submitted as separate files. Complete instructions for electronic artwork submission are accessible via the JNB home page (http://journals.elsevierhealth.com/periodicals/jnb/). The web site guides authors through the creation and uploading of the various files. The preferred file format is **Microsoft Word.** Please note that PDF files are not allowed for submission. When the submission files are uploaded, the system automatically generates an electronic (PDF) proof which is then used for review.

Cover Letter
Provide a cover letter indicating the name, mailing address, telephone, fax number, and e-mail address of the corresponding author. The cover letter must state that: all authors listed have contributed to the work, all authors have agreed to submit the manuscript to JNB, no part of the work has been published before, except in abstract form, and all human and animal studies have been reviewed by the appropriate ethics committees. The editor reserves the right to reject manuscripts that do not comply with the above-mentioned requirements.

Suggested Reviewers
Provide a list of 3 to 5 suggested reviewers for your manuscript. Please be sure to give complete contact information with the e-mail address being the most important.

Manuscript Outline
The manuscript should include the text, references, and figure/ table legends. Do not include the figures or tables in this file.

Title page

Please provide the following:
â€¢ The first name, middle initial, and the last name of all authors
â€¢ The name and address of the corresponding author to who reprint requests should be sent
â€¢ Each author's institutional affiliation(s)
â€¢ A running title of up to 50 characters;
â€¢ Grants, sponsors, and funding sources
â€¢ Up to six key words

Abstract

â€¢ Provide an abstract of a single paragraph with up to 250 words summarizing the hypothesis tested, experimental design, results, and conclusions. Do not cite references and avoid abbreviations.

Text

â€¢ Start the text on a new page. Arrange the text into four parts: Introduction, Methods and Materials, Results, and Discussion.

SI Units

â€¢ All laboratory data should be presented in SI units. See Young, DS. Implementation of SI units for clinical laboratory data. J Nutr Biochem 1990; 1: 599-633.

References

â€¢ References should follow the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals". References should be numbered sequentially in the order of their citation in the text, e.g., [1, 2], and appear at the end of the main text. Style references as follows:

For journal articles:

Brown M, Evans M, McIntosh M. Linoleic acid partially restores the triglyceride content of conjugated linoleic acid-treated cultures of 3T3-L1 preadipocytes. J Nutr Biochem. 2001;12:381-7.

For article or chapter in edited book:

Hennig B, Toborek M, Boissonneault GA. Lipids inflammatory cytokines, and endothelial cell injury. In: Gershwin ME, German JB, Keen CL, editors. Nutrition and Immunology: Principles and Practice. New Jersey: Humana Press Inc.; 2000. pp. 203-20.

For books:

Abbas AK, Lichtman AH, Pober JS. Cellular and molecular immunology. 4th ed. Philadelphia: WB Saunders; 2000 [chapter 11].

Illustrations

To properly submit digital artwork, please see "Artwork Instructions" on http://ees.elsevier.com/jnb/ or http://authors.elsevier.com for details on image formats, sizing, naming conventions, preparation, and file delivery of your digital artwork. Digital artwork that does not conform to these instructions will be rejected.

Revised Manuscripts

Please provide a separate file that clearly addresses the reviewers concerns. Once a revised manuscript is accepted for publication, a proof is prepared and submitted for final review to the corresponding author. Subsequently, the corrected proof will be published in *JNB* online as an 'article-in-press' available for immediate citation. The authors are solely responsible for the accuracy of their articles. Once a manuscript is

selected for inclusion in an issue, the article will be updated with volume, issue, and page information.

Scientific Correspondence

Letters to the Editor will be considered for publication at the discretion of the editor. Submission of a letter constitutes permission for publication. Letters are subject to editing and abridgement.

Reprints

An order form will be sent to the corresponding author from the publisher at the time your manuscript begins production.

Conflict of Interest Policy

Authors are required to disclose commercial or similar relationships to products or companies mentioned in or related to the subject matter of the article being submitted. Sources of funding for the article should be acknowledged in a footnote on the title page. Affiliations of authors should include corporate appointments relating to or in connection with products or companies mentioned in the article, or otherwise bearing on the subject matter thereof. Other pertinent financial relationships, such as consultancies, stock ownership or other equity interests or patent-licensing arrangements, should be disclosed to the Editor-in-Chief in the cover letter at the time of submission. Such relationships may be disclosed in the Journal at the discretion of the Editor-in-Chief in footnotes appearing on the title page.

Copyright

All manuscripts accepted for publication become the sole property of the Publisher. Before publication authors are requested to assign copyright to Elsevier. As an author, you retain rights for large number of author uses, including use by your employing institute or company. These rights are retained and permitted without the need to obtain specific permission from Elsevier. The copyright transfer form is sent to authors with proofs.

Sponsored Articles:

The Journal of Nutritional Biochemistry offers authors or their institutions the option to sponsor non-subscriber access to their articles on Elsevier's electronic publishing platforms. For more information please click here.

Guide for Authors

I GENERAL

II. SUBMISSION OF MANUSCRIPTS **SUBMISSION OF MANUSCRIPTS**

III. PREPARATION OF MANUSCRIPTS **PREPARATION OF MANUSCRIPTS**

1. *Neuroscience* publishes original research on any aspect of the scientific study of the nervous system. **Papers most suitable for publication are those that report new observations that directly contribute to our understanding of how the nervous system works. .**

2. *Section Editors*. With the rapid growth of neuroscience into diverse areas ranging from molecular biology to cognitive science, the accurate and fair assessments of papers for publication require the Chief and Associate Editors to seek advice from Section Editors representing all major areas of research. Section Editors suggest appropriate reviewers and also recommend an editorial decision based on the reviews. Section Editors have been appointed in the areas of Behavioral Neuroscience, Cellular, Clinical, Cognitive, Developmental, Molecular, Neuroanatomy, Neuropharmacology, Pain Mechanism, Systems Neuroscience and Sensory Systems. A special section editor has also been assigned to commission Reviews and Special Issues and another Section Editor is a special statistical consultant.

3. *The Editorial Board*. The international Editorial Board, which is appointed by the Publications Committee of IBRO, consists of specialists in all areas of neuroscience. Each paper is typically evaluated by at least two Editors or ad hoc reviewers. Papers are accepted by the Chief and Associate Editors in consultation with the appropriate Section Editor. **The review and decision process is identical regardless of whether the Chief or Associate Editor oversees the review process.** All manuscripts are subject to any modifications required by the Journal Office to conform to Journal policy.

4. *Types of papers*

(a) **Research papers.** These are full-length papers describing original research. There are no specific page limits although authors are encouraged to be as concise as possible and to use as few, high quality illustrations as necessary to adequately document their findings.

(b) **Rapid Reports.** These are short communications that describe outstanding new discoveries. They will be reviewed by the appropriate Section Editor and either the Chief or Associate Editor and an initial decision will be made as to whether the paper warrants processing as a Rapid Report. The Editors will aim for a two-week time to decision once a paper is accepted as a Rapid Report. This decision will be based on whether the paper reports a particularly important series of findings that are likely to have high impact on the field. Papers of the type that are typically published in Science or Nature are sought for this category. Papers that are not deemed acceptable for review under this category will be returned to the authors. All accepted Rapid Reports will be first published on the Neuroscience website http://www.neuroscience-ibro.com/

within 22 working days of acceptance, as a fully-citable article in ScienceDirect between 24-34 working days, and in the print version between 38-48 working days.

(c) **Reviews** (previously know as Commentaries). These are short articles (3000-10,000 words in length), not exhaustive reviews, that are intended to either draw attention to developments in a specific area of research, to bring together observations that seem to point the field in a new direction, to give the author's personal views on a controversial topic, or to direct soundly based criticism at some widely held dogma or widely used technique in neuroscience. Reviews may also provide an historical perspective on an area of neuroscience research. Authors should make their Review understandable to a broad spectrum of neuroscientists. Potential authors are invited to submit a letter of interest to the Section Editor for Reviews and Special Issues or to the Chief or Associate Editors indicating the topic of a potential Review. Proposals for reviews or commentaries should also contain an outline of the contents, including an abstract (<200 words), a list of 10 relevant articles including 5 from the proposer's own research, and a brief statement on why now is a good time to review the topic in question. Reviews will not be accepted for editorial processing unless pre-approved for submission.

(d) **Letters to the Editors.** A limited amount of space will be available for comments about important scientific points that arise out of papers previously published in *Neuroscience*. Such comments should be sent to the Chief or Associate Editor and they should be no longer than 1000 words excluding references. The Chief and Associate Editor reserve the right to invite replies to such comments by the authors of the original papers.

â€¢ Submission of any form of paper to *Neuroscience* implies that it represents original research not previously published, in whole or in part (except in the form of an abstract) and that it is not being considered for publication elsewhere. Each manuscript should be accompanied by a signed cover letter in which the corresponding author states: "The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed".

â€¢ All submissions to *Neuroscience* must contain experiments that conform to the ethical standards printed below. To confirm your agreement with this, you are required to include the following statement in your cover letter indicating your agreement with these standards: "I have read and have abided by the statement of ethical standards for manuscripts submitted to *Neuroscience*."

ETHICAL STANDARDS:

â€¢ The authors declare that all experiments on human subjects were conducted in accordance with the Declaration of Helsinki http://www.wma.net/ and that all procedures were carried out with the adequate understanding and written consent of the subjects.

â€¢ The authors also certify that formal approval to conduct the experiments described has been obtained from the human subjects review board of their institution and could be provided upon request.

â€¢ If the studies deal with animal experiments, the authors certify that they were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC).

â€¢ The authors also certify that formal approval to conduct the experiments described has been obtained from the animal subjects review board of their institution and could be provided upon request.

â€¢ The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

â€¢ If the ethical standard governing the reported research is different from those guidelines indicated above, the authors must provide information in the submission cover letter about which guidelines and oversight procedures were followed.

â€¢ The Editors reserve the right to return manuscripts in which there is any question as to the appropriate and ethical use of human or animal subjects.

â€¢ All papers must be prepared according to the instructions given in Section III. All manuscripts should be submitted through our web based submission system, Editorial Manager (http://nsc.editorialmanager.com/). Authors are strongly encouraged to use this web submission system. However, for those who are unable to submit via the web, please contact the *Neuroscience* Journal Office at neuroscience@journal-office.com 525 B Street, San Diego, CA 92101. FAX: 619-699-6855.

Main Editors:

Section Editors:

(e) **Cognitive**: Dr. Jerome N. Sanes, Department of Neuroscience, Brown Medical School, Box 1953, Providence, RI 02912, USA
(f) **Developmental**: Dr. John L. R. Rubenstein, Nina Ireland Distinguished Professor in Child Psychiatry, Nina Ireland Laboratory of Developmental Neurobiology, Center for Neurobiology and Psychiatry, Genetics, Development and Behavioral Sciences Building, 1550 4th Street, 2nd Floor South, Room GD 284C, University of California at San Francisco, San Francisco, CA 94143-2611, USA
(g) **Molecular**: Dr. Werner Sieghart, Brain Research Institute, University of Vienna, Division of Biochemistry and Molecular Biology, Spitalgasse 4, A-1090 Vienna, Austria
(h) **Neuroanatomy**: Dr. Charles R. Gerfen, NIMH, Lab of Systems Neuroscience, Building 36, Room 2D-30 MSC 4075, 36 Convent Drive, Bethesda, MD 20892-4075, USA
(i) **Neuropharmacology**: Dr. Yoland Smith, Yerkes National Primate Research Center, Emory University, 954 Gatewood Road NE, Atlanta, GA 30329, USA
(j) **Systems Neuroscience**: Dr. Miles Herkenham, NIMH, Section on Functional Neuroanatomy, Bldg 36, Rm 2D15 36, Convent Dr, MSC 4070, Bethesda, MD 20892-4070, USA
(k) **Systems Neuroscience**: Dr. Asla Pitkanen, University Kuopio, Al Virtanen Institute, PO Box 1627 FIN-70211, Kuopio, Finland
(l) **Systems Neuroscience**: Dr. Minoru Kimura, Division of Neurophysiology, Graduate School, Kyoto Prefectural University of Medicine Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto, 602-8566 Japan
(m) **Pain Mechanisms**: Dr. Linda S. Sorkin, Department of Anesthesiology, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0818, USA
(n) **Sensory Systems**: Dr. Richard Weinberg, University of North Carolina, Department of Cell Biology and Anatomy, CB 7090, Chapel Hill, NC 27599, USA
(o) **Statistical Consultant**: Dr. Viktorie Vlachova, Department of Cellular Neurophysiology, Institute of Physiology AS CR, Videnska 1083, 14220 prague 4, Czech Republic

â€¢ Cover illustrations. Authors are encouraged to submit visually and scientifically interesting figure(s) representative of their data, though not necessarily as they appear in the manuscript, for potential cover illustrations. The use of cover illustrations is at the discretion of the Editors.

â€¢ Illustrations for the cover should be related to the author's submitted article, but would not necessarily be included in the paper. We invite authors to produce exciting graphical images that represent the subject matter of their accepted article. Cover art should be sent to:
Trisha Stucker
Neuroscience Journal Office
525 B Street, Suite 1900
San Diego
CA 92101
U.S.A

â€¢ When art is submitted, please insure that the manuscript reference number is included on all materials. Covers should be submitted in digital format. Cover art should be formatted for the entire 8 1/2 X 11 inch cover and should be submitted in a resolution

of at least 300 dpi. Covers can be submitted as TIFF, Photoshop, JPEG or Powerpoint formats.

1. *Organization*

(a) All manuscripts must be typewritten with **double-spacing** throughout and with margins at least 2.5 cm wide. Pages should be numbered in succession, the title page being no. 1

(b) The title page should include the title, the authors' names and affiliations, full contact details (address, telephone and fax numbers, and **e-mail address**) of the corresponding author, any necessary footnotes, and the appropriate **Section Editor** to whom the manuscript should be sent for handling.

(c) A **comprehensive list of abbreviations** used in the main text should be put on a separate page that follows the title page.

(d) Each paper must begin with a brief Abstract of no more than 300 words. The abstract should summarize the goals of the research and the results obtained. The abstract should conclude with a final statement summarizing the major conclusions in such a way that the implications of the work to the field would be clear to a general neuroscience reader. **Abbreviations must be kept to a minimum in the abstract.**

(e) Authors are asked to include a maximum of **six key words** (not used in the title) at the foot of the abstract.

(f) **Preparation of Research Papers:**
Research papers should be organized in the following four main sections:

(i) *Introduction*. This should provide the scientific rationale for the research that is reported. The heading "Introduction" should be omitted and no results should be presented.
(ii) *Experimental procedures*. Procedures used in the research should be described in sufficient detail to permit the replication of the work by others. Previously published procedures should be referenced and briefly summarized. The source of all materials, including animals and human tissue, must be provided. The location of each supplier should be detailed on first use in the text.
(iii) *Results*. This section presents findings without discussion of their significance. Subsections should be used in order to present results in an organized fashion.
(iv) *Discussion*. This section presents the authors' interpretations of their findings and an assessment of their significance in relation to previous work. Avoid repetition of material presented in the Results section.

(g) Preparation of Rapid Reports:

Rapid Reports are short reports of original research focused on an outstanding finding whose importance means that it will be of interest to scientists in all areas of neuroscience. They do not normally exceed **3 pages** of *Neuroscience*, and have **no more than 30** references. They begin with a fully referenced paragraph of not more than 180 words, aimed at a broad audience of neuroscientists. This paragraph contains a

summary of the background and rationale for the work, followed by a one-sentence statement of the main conclusions starting "We have found that ?" or equivalent phrase. The rest of the text is typically about 1,500 words long, starting with a further brief paragraph of introductory material if the author requires it, not repeating information in the summary paragraph. Any discussion at the end of the text should be as succinct as possible. Letters typically have 3 or 4 small figures. Word counts refer to the text of the paper. References, title, author list and acknowledgements are **not** included in total word counts.

(h) **Preparation of Reviews**:
Reviews should have an introductory section, followed by several information presentation sections and then end with a conclusion section. Section headings should be used to organize the presentation of information.

(i) **Other features of manuscript preparation** i. *Personal Communications* may be used only when written authorization from the communicator is submitted with the original manuscript; they may be mentioned only in the text and in the following form: (G. H Orwell, Department of Psychiatry, University of Washington, personal communication)
(ii) Unpublished or submitted experiments by one of the authors may be mentioned only in the text, not in the References. Initials, as well as surnames, must be given for authors whose unpublished experiments are quoted: (M.L. King, unpublished observations)
(iii) *Acknowledgements*. It is the corresponding author's responsibility to insure that individuals who are acknowledged for assistance or for providing comments on the manuscript are agreeable to being acknowledged in this way.
(iv) *References*. The reference list should be included at the end of the main text. A paper which has been accepted for publication but which has not appeared may be cited in the reference list with the abbreviated name of the journal followed by the words "in press".
(v) *Tables and figures* should be included on separate pages placed at the end of the manuscript. Their desired approximate locations should be indicated in the text. Each figure must be accompanied by an explanatory legend in a separate section entitled Figure Legends. In general, tables and figures should be constructed so that they, together with their captions and legends, will be intelligible with minimal reference to the main text.

2. **Style**

(a) Manuscripts should be written in English in a concise and understandable style. Technical jargon or "laboratory slang" should not be used. It is the responsibility of the corresponding author to ensure that the manuscript is written in a style that is grammatically correct and free of spelling or other typographical errors. The Editorial Office reserves the right to revise the wording of manuscripts accepted for publication in *Neuroscience*.

(b) The excessive use of abbreviations in the text is strongly discouraged. In order to aid communication between scientists of different disciplines, authors should only use abbreviations sparingly and should always define the abbreviation when first used in the text by placing it in parentheses after the full term, e.g. acetylcholinesterase (AChE).

The abbreviations should then be used consistently thereafter and appear at least twice in the text.

(c) Symbols for physical units should be restricted to the Systems Internationale (S.I.) Units.

(d) Drug names should be the official or approved names; trade names or common names may be given in brackets where the drug is first mentioned. The manufacturer's name must be given. The doses of the drugs should be given as unit weight/unit body weight, e.g. mmol/kg or mg/kg.

(e) Authors must be consistent in the style of their figures. This includes, but is not restricted to, the use of symbols and lettering size, type, style and positioning on the figures.

3. **Reference format**

(a) In the text, references should be quoted as the name of the first author and year in chronological order. Multiple authors are indicated by "et al.", except when there are only two authors, in which case both names are written. For example, The pattern of the pathology instead represents a synaptically connected network of neurons (Braak and Braak, 1991; Morris, 1997). This hypothesis was recently proposed by Nagy et al. (1997).

(b) The reference list should be on a separate page at the end of the manuscript, **in alphabetical order** and arranged as follows: authors' names and initials, year, title of the article, abbreviated title of the journal, volume, first and last page numbers. Journal titles should be abbreviated according to the rules adopted in the fourth edition of the *World List of Scientific Periodicals* (Butterworths, 1965). Note that first and last pages are given in full. For example, Nagy ZA, Esiri MM, Cato A-M, Smith AD (Cell cycle markers in the hippocampus in Alzheimer's disease. Acta Neuropath 94:6-15.1997).

(c) References to books should include the authors' names and initials, year, title of book, volume, publisher, place of publication and page numbers. Where relevant, the title of a paper within a book, and the editor's name(s) should be given. For example, Morris JH (1997) Alzheimer's disease. In: The neuropathology of dementia, vol. 2 (Esiri MM, Morris JH, eds), pp 70-121 Cambridge: Cambridge University Press.

4. **Presentation of data in tables or figures**

(a) It is the policy of *Neuroscience* to publish high-quality half-tone (black and white or color plates) and line art illustrations. Authors must submit high-quality original figures. New policies have been put into place to evaluate the quality of illustrations at several stages during the review process. If the quality of illustrations is deemed unacceptable, authors will be requested to create new illustrations prior to review or acceptance of the paper. Color plates or line drawings will be charged to the authors. The exact cost will be given on request. Typical pricing is as follows: First page is Euros 273.00 (approx. USD300) and subsequent pages are Euros 182.00 (approx. USD200)

(b) **Preparation of figures**

Please refer to the detailed guide on electronic artwork submission which is available at: http://authors.elsevier.com/artwork
Files should be submitted in the following formats: TIFF, EPS or PDF. TIFF and PDF files should be supplied at the following minimum resolutions:
Line artwork = 1000 dpi;
Halftone artwork = 300 dpi (without lettering) and 500 dpi (with lettering);
Color artwork = 300 dpi (without lettering) and 500 dpi (with lettering);
Combination artwork = (line/tone) = 500 dpi
MS Office files (Word, PowerPoint, Excel) are also accepted provided the artwork placed into MS Office applications is at the appropriate minimum resolution as above. Color images should be provided in CMYK colorspace.

Authors must clearly describe in the Experimental Methods section all manipulations made to digital data that were collected as images, and images which have been scanned and printed for publication.