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

ORIGINAL PAPER

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

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

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

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

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

Abbreviations

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

Introduction

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

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

✉ Angela T. S. Wyse
wyse@ufrgs.br

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Animals

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

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

Experimental Protocol

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

OVX Procedure

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

Vitamin D Supplementation

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

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

Behavioral Testing

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

Open Field

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

Novel Object Recognition

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

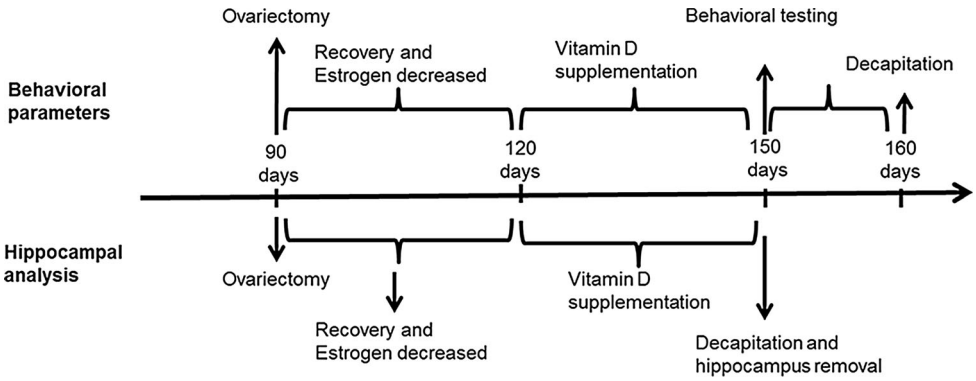


Fig. 1 Timeline of experiments. Evaluation of the effects of OVX and vitamin D supplementation on memory, phosphorylation profile of intermediate filaments and immunocontent of these proteins and others

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

Inhibitory Avoidance

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

Cytoskeleton Protein Phosphorylation

Preparation of Hippocampus Slices

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

Preincubation

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

In Vitro ^{32}P Incorporation Experiments

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

High Salt–Triton-Insoluble Cytoskeletal Fraction Preparation

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

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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

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

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

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

Protein Determination

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

Statistical Analysis

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

Results

Behavioral Effect of OVX and Vitamin D Supplementation

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

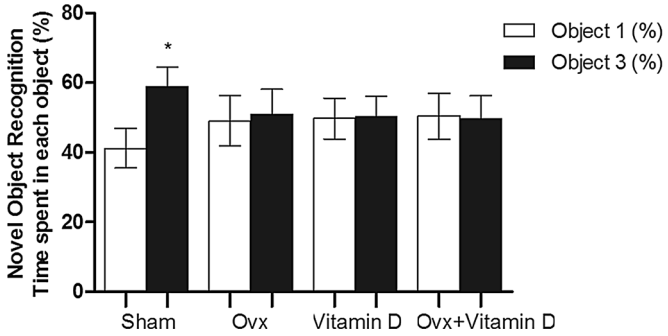
The evaluation of recognition memory was performed through the novel object recognition task (Fig. 2) of short term (tested 1 h after training) and long term (tested 7 days after training). In the training session, no difference was observed between groups on percentage of exploration time of each object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

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

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

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

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



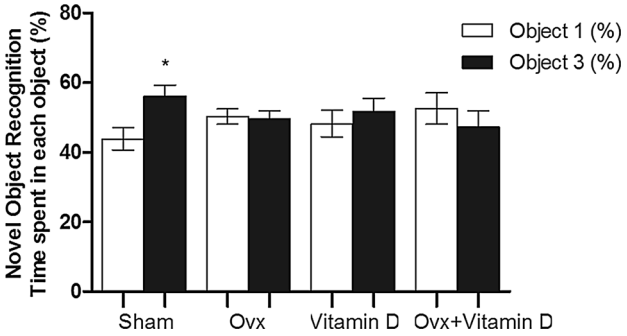


Fig. 2 Effect of OVX and vitamin D supplementation on novel object recognition performance. *Bars* represent the mean ± SEM of percentage of exploration of each object in the short-term [(1 h) **a**] and long-term [(7 days) **b**] testing session. Note 1 is the old object and 3 is the novel object. Data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

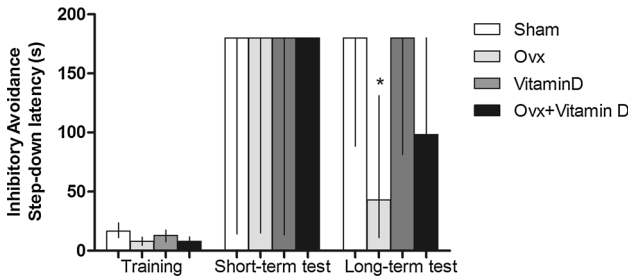


Fig. 3 Effect of OVX and vitamin D supplementation on step-down inhibitory avoidance performance. *Bars* represent the median and interquartile intervals (25–75% percentiles) of latency to step down the platform (s) in the training, short-term (1 h) and long-term (7 days) test sessions. Data were analyzed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

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

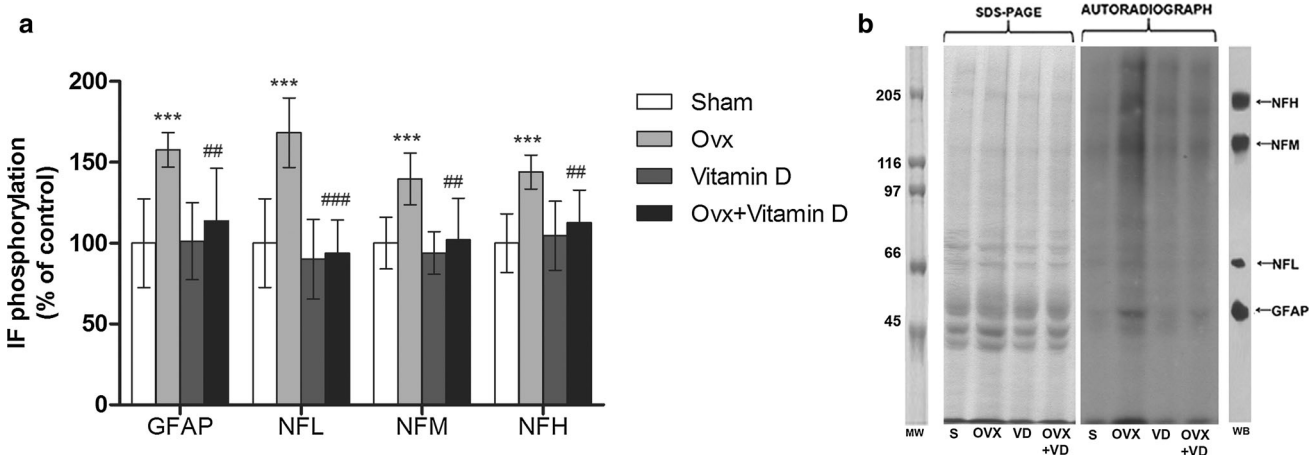


Fig. 4 Effect of OVX and vitamin D supplementation on in vitro phosphorylation of GFAP and NF subunits present in the IF-enriched cytoskeletal fraction of astrocytes and neurons, respectively, in hippocampus slices of female adult rats. *Bars* represent the mean ± SD of percentage of controls. Data were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

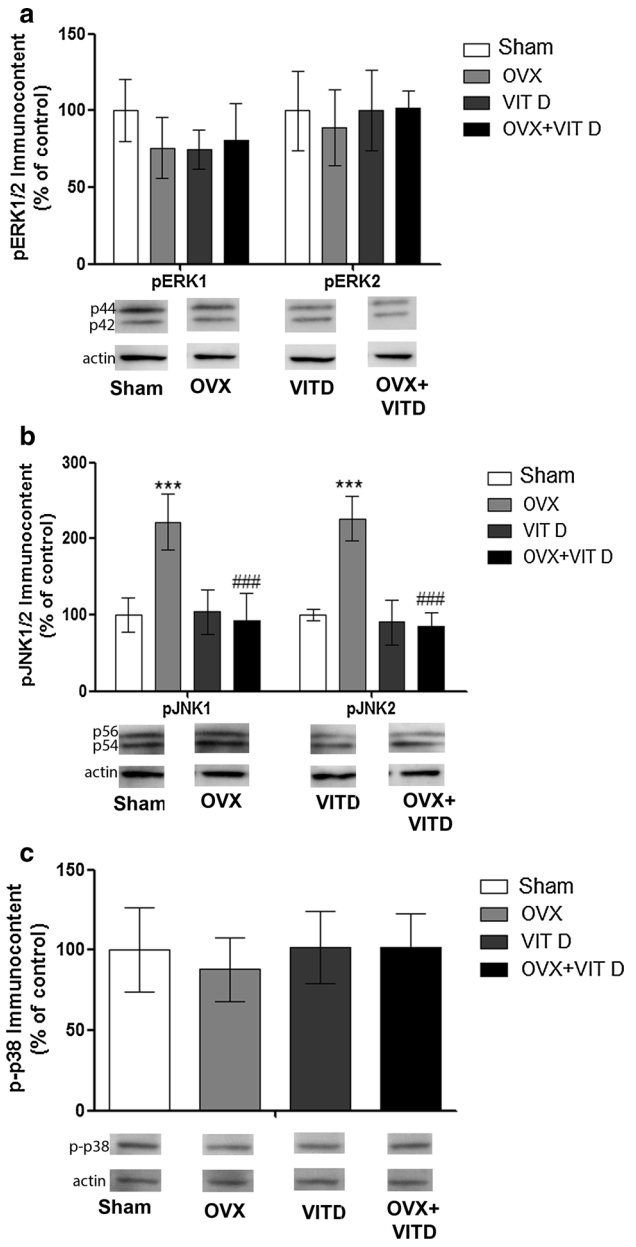


Fig. 5 Effect of OVX and vitamin D supplementation on the following second messenger-independent protein kinases immunocontent: pERK1/2 (**a**), pJNK1/2 (**b**) and p-p38 (**c**), in the cytoskeletal fraction from hippocampus slices of female adult rats. Representative Western blots of proteins studied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

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

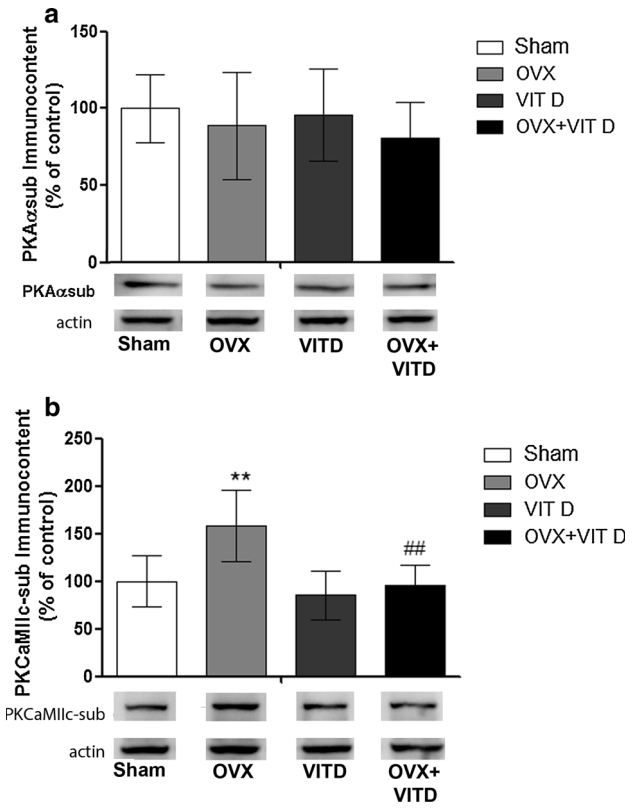


Fig. 6 Effect of OVX and vitamin D supplementation on the following second messenger-dependent protein kinases immunocontent: PKAαsub (**a**), PKCaMIIcsub (**b**), in the cytoskeletal fraction from hippocampus slices of female adult rats. Representative Western blots of proteins studied are shown. β-Actin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

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

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

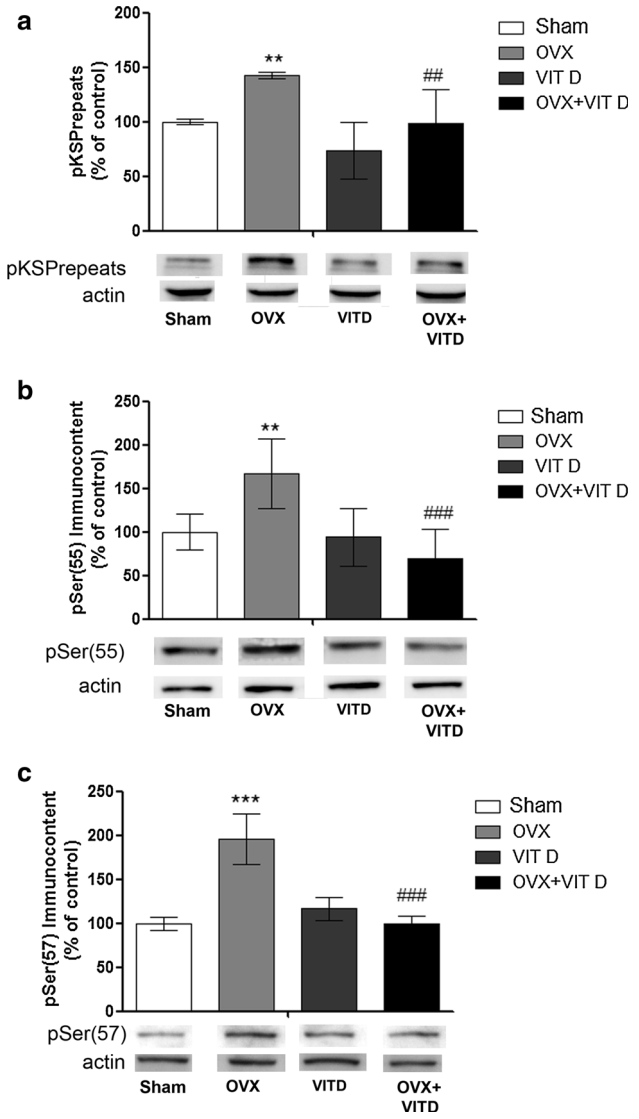


Fig. 7 Effect of OVX and vitamin D supplementation on the following phosphorylation sites immunocontent: pKSP repeats (**a**), pSer55 (**b**) and pSer57(**c**), in the cytoskeletal fraction from hippocampus slices of female adult rats. Representative Western blots of proteins studied are shown. β-Actin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

Discussion

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

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

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

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

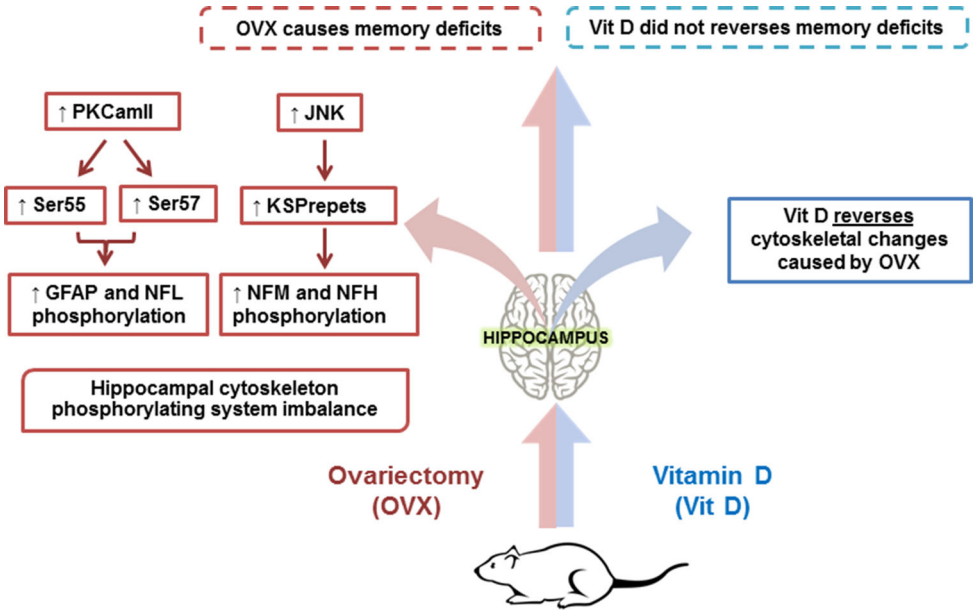


Fig. 8 Schematic figure of the main findings

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

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

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

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

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

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

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

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

Compliance with Ethical Standards

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

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

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

References

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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