

Research Article



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Effect of vitamin D and omega-3 on the expression of endoplasmic reticulum-associated protein degradation and autophagic proteins in rat brain

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Abstract

Objectives: Valosin-containing protein (p97/VCP) and its cofactor, small p97/VCP-interacting protein (SVIP), are involved in the endoplasmic reticulum-associated degradation pathway (ERAD). We investigated the cellular localization of vitamin D receptor (VDR), ERAD, and autophagic proteins (LC3B and p62) in rat brain tissue.

Methods: There were four groups consisting of 24 Wistar albino rats: control and treatment groups for vitamin D, omega-3, and both vitamin D and omega-3. Brain tissues were stained with hematoxylin-eosin, azan trichrome, and toluidine blue for histopathological evaluation. The immunohistochemistry assay was performed for VDR, p97/VCP, SVIP, LC3B, and p62 in rat brain sections.

Results: The immunoexpression of VDR and p97/VCP was significantly increased in hippocampus and cortex of brain tissue from the vitamin D-supplemented group. Furthermore, the protein expression level of SVIP reached the highest level in vitamin D-treated group. LC3B and p62 revealed reduced expressions in vitamin D-treated group in rat brain and hippocampus, in contrast to p97/VCP, SVIP, and VDR.

Conclusions: Vitamin D and omega-3 supplementations had no negative effects at a cellular level on hippocampus and cortex of the brain tissue. Vitamin D increased the expression of the proteins that are related to the ERAD pathway, whereas it reduced the expression of the proteins in the autophagy pathway. Also, in this study, SVIP expressions were shown in rat hippocampus and cortex of the brain tissue.

Keywords: autophagy; brain; omega-3; small p97/VCP-interacting protein (SVIP); valosin-containing protein (p97/VCP); vitamin D

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Introduction

The rat hippocampus is a C-shaped structure that is settled in the caudal part of the brain. Three different subregions can be noticeable: the dentate gyrus (DG), the hippocampus proper (composed of cornu ammonis CA3, CA2, and CA1), and the subiculum [1].

Vitamin D is special because it can be produced in the skin by exposure to sunlight [2]. It has been determined that this vitamin serves many purposes than its well-known functions in bone metabolism, calcium absorption, and bone integrity [3]. Little focus has been placed on the effects of vitamin D on the growth and operation of the nervous system, although numerous papers have described the cytoprotective impacts of vitamin D and its function in the development of diabetes, cardiovascular disease, and other malignancies [4]. Vitamin D has steroid-like actions and

carries a cholesterol backbone. This fat-soluble hormone is responsible for playing a crucial part in maintaining brain health [5]. In recent years, vitamin D has been understood as an essential neuro-steroid with a variety of functions in the brain [6]. Regarding proper brain growth and function, adequate vitamin D levels may be highly significant [7, 8]. Because it may lower the incidence of central nervous system illnesses, an adequate supply of vitamin D at various life stages, especially the prenatal period, seems to be especially significant [9]. According to a study by Di Somma et al. [10], the adult brain has to have adequate levels of vitamin D in the bloodstream to maintain neurological growth. Vitamin D is essential for brain growth in fetal life. According to the study by Yates et al. [11], vitamin D insufficiency in mothers and offspring leads to developmental abnormalities, such as memory issues and learning, as well as grooming behaviors.

The omega-6 and omega-3 fatty acids are essential fatty acids that are important nutrients found in the diet [12, 13]. These critical fatty acids are concentrated at high levels in the brain, where they affect cognitive, social, and linguistic capabilities, as well as fundamental growth and development processes. A lack of alpha-linolenic acid and its omega-3 derivatives has been linked to behavioral changes, eyesight impairment, tingling in the limbs and extremities, loss of motor coordination, and growth retardation, according to previous research [14–17].

Despite the imperfection of the folding process, it is estimated that about 30 % of freshly produced proteins are misfolded [18]. Misfolded proteins are subsequently kept in the endoplasmic reticulum, where they are destroyed via the endoplasmic reticulum-associated degradation pathway (ERAD). Retrotranslocation, proteasomal degradation, and ubiquitination are some of the procedures used in ERAD to choose the target protein [19]. Misfolded proteins are targeted for proteasomal breakdown via ERAD. Misfolded substrates are removed from the ER membrane by the valosin-containing protein (p97/VCP) complex and transported to the 26S proteasome for destruction [20]. VCP, also known as p97, performs a variety of tasks by joining with more than 40 distinct adaptor proteins. Both p97/VCP and cofactors often rely on particular protein domains for these interactions. Small p97/VCP-interacting protein (SVIP) and ubiquitin ligase (gp78) are two cofactors that contain the VCP-interacting motif, which is one such domain [21, 22]. Recent research has shown that SVIP is primarily localized to the central and peripheral nervous systems [23]. p97/VCP has been related to a variety of physiological processes, including cell cycle control, Golgi biogenesis, the formation of nuclear membranes, the ubiquitin-proteasome system (UPS), apoptosis, and autophagosome maturation [24, 25].

It has been reported that groups treated with vitamin D3 in human sperm had higher levels of Hsp70 protein than control groups [26]. Given that the Hsp70 chaperone is also one of the ERAD proteins for this reason, we investigated the relationship of SVIP and p97/VCP, two ERAD proteins with vitamin D supplementation in the brain.

Cargo molecules are captured by autophagy and transported to lysosomes in the form of the double-membrane vesicles known as autophagosomes. When an autophagosome is finished, it fuses with a lysosome to generate an autolysosome. P62 and LC3B have received the most attention among autophagy markers [27–29].

Many studies conducted have shown the relationship between ERAD and autophagic proteins. The specific mechanisms of omega-3 and vitamin D's effect on the brain are not yet elucidated. Given the significance of p97/VCP, SVIP, and LC3B, p62, the current study aimed to examine the effects of omega-3 and vitamin D on the expression of ERAD and autophagic proteins in the rat brain. We hypothesize that ERAD markers (p97/VCP and SVIP) and autophagy markers (LC3B and p62) may have a relationship when given omega-3 and vitamin D supplements.

Materials and methods

Ethical approval

The study's experimental and surgical methods were implemented in the experimental research center of Afyon Kocatepe University, Türkiye. Before the experimental applications were carried out, ethical approval was provided by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (approval no: 49533702/113).

Animal and study design

The Experimental Animals Research and Application Unit (University of Afyon Kocatepe) provided 24 Wistar albino rats for our investigation. Female Wistar albino rats (21 days old) were housed in cages under conditions of temperature (21–25 °C) and 12 h light cycle/12 h dark cycle.

Four groups of six rats each were created. Group I: The rats were used as a control group. Group II: The rats were subcutaneously injected with 120 ng/100 g/week 1,25(OH)₂D₃ (Calcijex ampule, Abbott, USA) weekly [30]. Group III: During 28 days, 1 mL/kg Omegaven (Fresenius Kabi, Austria) (100 mL of Omegaven contains 1.25–2.82 g of eicosapentaenoic acid (EPA) and 1.44–3.09 g of docosahexaenoic acid (DHA)) was applied intraperitoneally into the rats [31]. Group IV: During

28 days, the rats received daily injections of 1 mL/kg Ome-gaven and 1,25(OH)₂D₃ at a dose of 120 ng/100 g/week. At the end of the 28th day, brain tissues were obtained for histo-pathological and immunohistochemical examinations.

Animal sacrifice

After the experimental period, the Wistar albino rats were thereafter euthanized by ketamine (100 mg/kg) i.p. followed by cervical dislocation. Each rat was decapitated at the cervico-medullary junction for uniformity. The skulls were opened, and the brains were rapidly taken out. After, brain tissue samples were fixed in 10 % formalin for 48 h.

Paraffin embedding

To prepare the harvested tissue for examination by light microscopy, brain tissues were fixed in 10 % formalin at 4 °C for 24 h after the samples were washed under running tap water for 2 h. Samples were dehydrated in increasing concentrations of ethanol (Sigma Aldrich, Darmstadt, Germany) series and cleared in xylene, infiltrated with paraffin (Sigma Aldrich, Darmstadt, Germany) at 56 °C, and embedded in paraffin. The sections were cut serially in 4–5 µm thickness using a micro-tome (Leica Biosystems RM2245, Deer Park, USA).

Histological analysis

Hematoxylin-eosin (H&E), azan trichrome, and toluidine blue staining of the sections were carried out not only to evaluate the histological structure of the brain but also to investigate the hippocampus. First, we incubated sections in an oven at 56 °C for 2 h for H&E, azan trichrome, and toluidine blue staining. After that, sections were treated with xylene for 30 min and then rehydrated in the decreasing ethanol concentrations for 3 min each time. After washing sections under tap water, we stained them with Gill's hematoxylin (Merck, Darmstadt, Germany), followed by alcoholic eosin (Bio-Optica, Milano, Italy). Slides were dehydrated in rising alcohol concentrations and cleared in xylene. Then, the slides were mounted with entellan (107960, Sigma Aldrich, Darmstadt, Germany). Azan trichrome (04–011802, Bio-Optica, Milano, Italy) and toluidine blue (Atom scientific, Manchester, UK) staining were applied as described by the manufacturer. Slides were examined histologically under a light microscope (Olympus BX43, Japan) to analyze morphological changes.

Immunohistochemical examinations

The immunoexpression of the vitamin D receptor (VDR), SVIP, and p97/VCP proteins in the rat brain and hippocampus was evaluated by using immunohistochemistry. Serial sections, 4–5 µm thick, were placed on poly-L-lysine-coated slides (Marienfeld, Lauda-Königshofen, Germany) and incubated overnight at 56 °C. Tissue sections were deparaffinized in xylene before they were rehydrated in ethanol series. Antigen retrieval was applied using a microwave oven (LG MS2042D, China) in 10 mM sodium citrate buffer and left to cool for 15 min. The endogenous peroxidase activity was blocked by 3 % hydrogen peroxide in phosphate-buffered saline (PBS, BioShop, Canada) for 15 min. After washing with 1×PBS, the slides were treated with a blocking serum (Ultra V Block, Thermo Scientific™ TP-125-HL, Fremont, CA, USA) for 15 min. After that, the primer antibodies VDR (sc-124548; 1/400 dilution, Santa Cruz Biotechnology, USA), p97/VCP (ab11433; 1/700 dilution, Abcam, UK), SVIP (1/50, HPA039807, Sigma Aldrich, Germany), LC3B (NB-100–2220, 1/200 dilution, Novus Biologicals, USA) and p62 (ab91526, 1/300 dilution, Abcam, UK) were applied for an overnight period at 4 °C. The tissues were rinsed for 15 min in PBS and applied biotinylated goat anti-rabbit antibody (Thermo Scientific™ TP-125-HL, Fremont, CA, USA), and were incubated in sections for 20 min. Streptavidin peroxidase (Thermo Scientific™ TP-125-HL, Fremont, CA, USA) was applied for 20 min at room temperature. The sections were developed with 3,3'-diaminobenzidine (DAB) chromogen (ab64238, Abcam, UK). The slides were counterstained with Mayer's hematoxylin (Bio-Optica, Milan, Italy) and covered with entellan. The slides were examined and photographed under a light microscope (Olympus DP21, Japan) and a digital imaging system (Olympus DP26, Japan) using 10× and 40× objectives. Positive expressions of VDR, p97/VCP, SVIP, LC3B, and p62 were semi-quantitatively evaluated, and H-score analyses were used for the immunohistochemistry evaluation as previously described [29]. Based on the H-score analysis, the number and the intensity of the immunopositive cells in the section (five randomly selected areas) were evaluated by three observers.

Statistical analysis

Data analysis was done using GraphPad Prism 8.4.2 software (San Diego, CA, USA). The multiple comparison tests were done after a two-way ANOVA to determine the significance of the data between groups. The statistical significance level was accepted as $p < 0.05$. All data were represented as mean ± standard error.

Results

Histopathological results of rat brains in experimental groups

Hematoxylin-eosin (Supplementary Material, Figure S1), azan trichrome (Supplementary Material, Figure S2), and toluidine blue-stained (Supplementary Material, Figure S3) sections of the hippocampus and cortex of the brain are arranged normally in the control, vitamin D, omega-3, and vitamin D+omega-3 groups. The sample is made up of the cornu ammonis CA1, CA2, CA3, and DG areas (Supplementary Material, Figures S1–S3). None of the rats used in the study exhibited gliosis, atrophy, or neuronal cell death.

Cellular expressions of vitamin D receptor

We first conducted a thorough analysis of VDR localization. Although the immunostaining of VDR was seen in all groups, the staining intensity was increased in the neuronal cells of the hippocampus and cortex of brain sections in the vitamin D treated group compared to the

control group. Additionally, moderate immunoexpression of VDR was detected in vitamin D+omega-3, and this expression was decreased in the hippocampus and cortex of the brain in the control and omega-3 groups (Figure 1). The H-score analysis revealed that the immunoexpression level of VDR was significantly increased in the rat hippocampus and the cortex of the brain from the vitamin D-treated group compared to the control ($p < 0.05$). There was no significant difference considering the expression of VDR in omega-3 and vitamin D+omega-3 groups compared to the control group ($p > 0.05$) (Figure 2).

Altered expression of ERAD (p97/VCP and SVIP) markers in brain tissue

In addition to VDR, we have also investigated the expression of ERAD markers in brain tissues. The immunolocalization of p97/VCP (Figure 3) and SVIP (Figure 4) was detected in the neuronal cells in the hippocampus and the cortex of the rat brain in all groups. The H-score analysis revealed that the immunoexpression level of p97/VCP was significantly boosted in the rat hippocampus and cortex of the brain from the

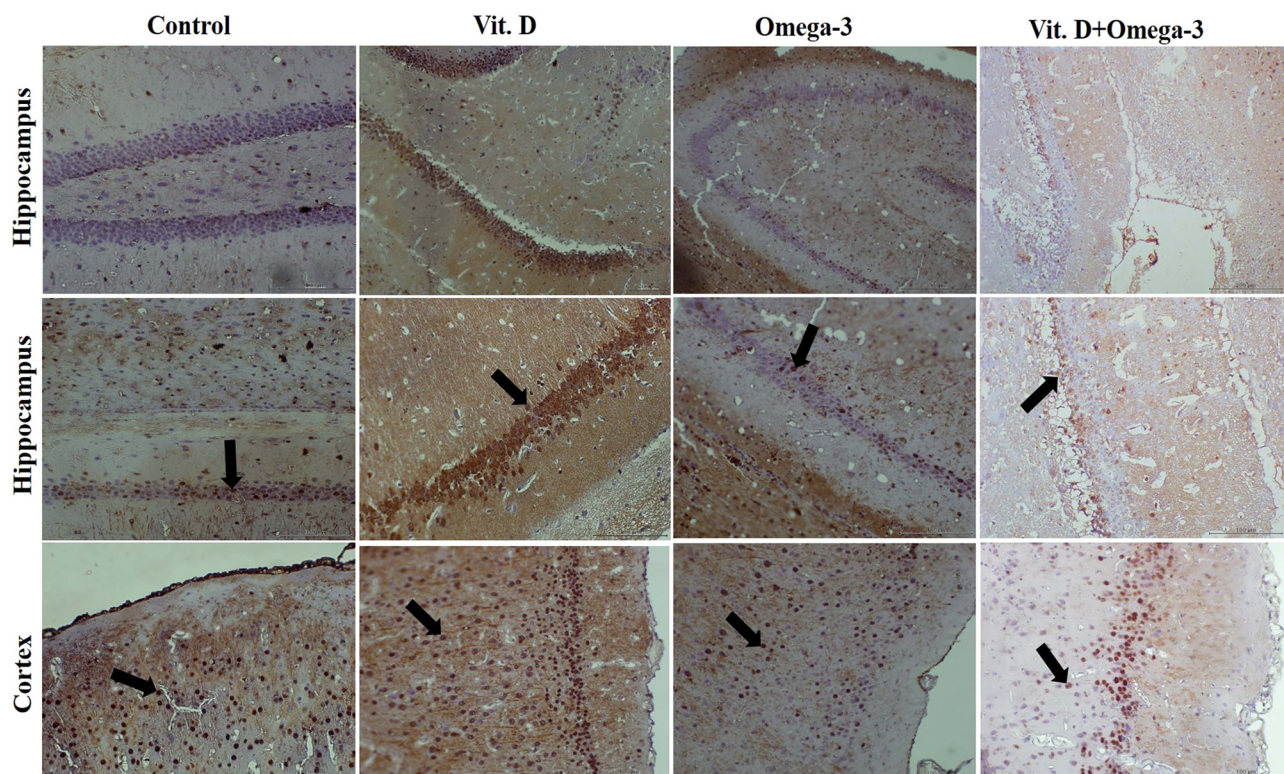


Figure 1: Immunolocalization of VDR protein in the hippocampus and cortex of rat brain in control and vitamin D, omega-3, and vitamin D+omega-3 treatment groups. Black arrows indicate the VDR-positive cells. Three independent experiments were performed. The micrographs were taken with 10× and 40× objectives. Scale bars: 100 μm, 200 μm. VDR, vitamin D receptor.

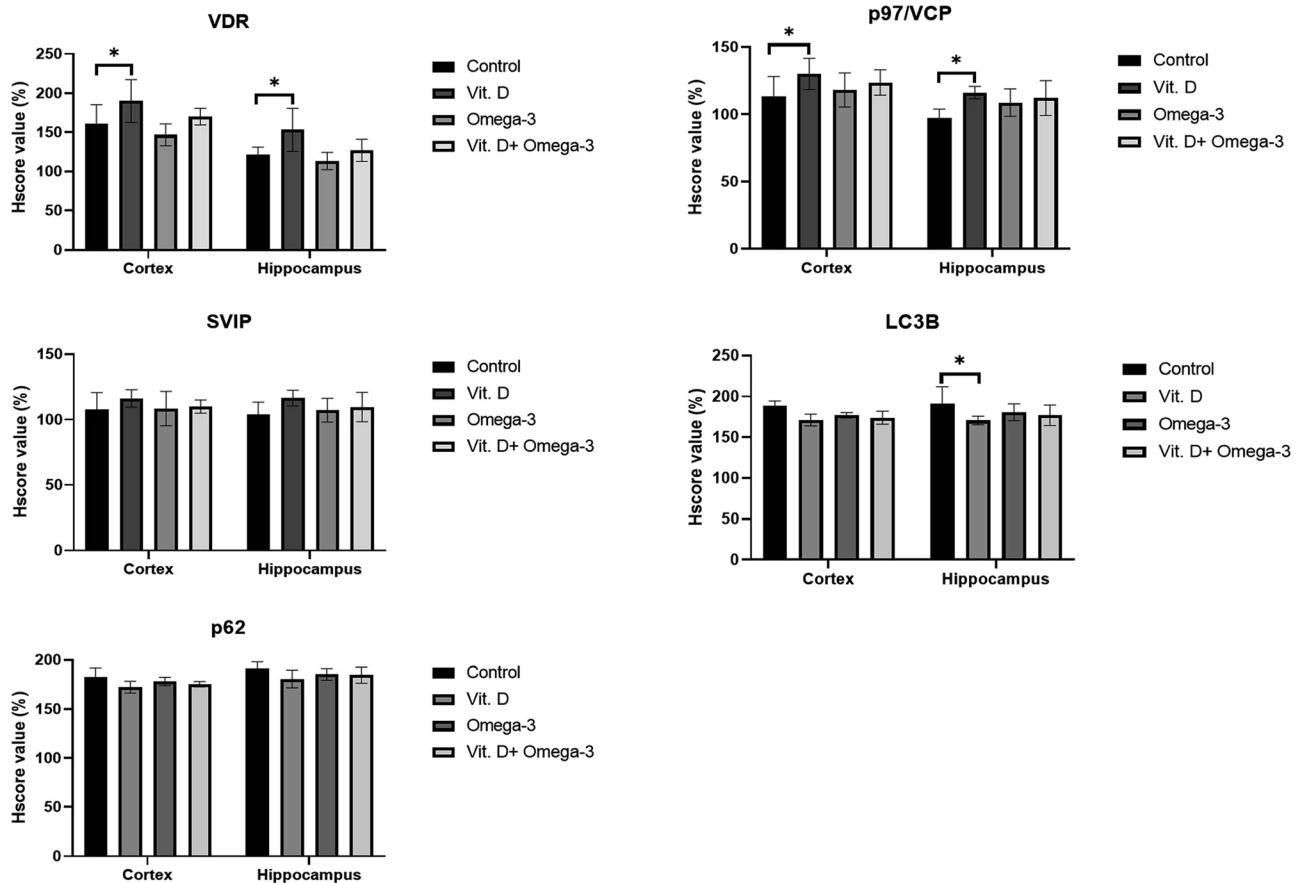


Figure 2: The H score analysis of the immunoexpression levels of VDR, p97/VCP, SVIP, LC3B and p62 in the hippocampus and cortex of rat brain in control and vitamin D, omega-3, and vitamin D+omega-3 treatment groups. The immunoexpression levels of VDR and p97/VCP were considerably raised in the rat hippocampus and cortex of the brain from the vitamin D-treated group compared to the control. The immunoexpression level of LC3B was significantly decreased in the rat hippocampus of the brain from the vitamin D-treated group compared to the control group. Three independent experiments were performed. The data are demonstrated as mean $\pm$ SEM. * $p < 0.05$. VDR, vitamin D receptor.

vitamin D-treated group compared to the control group ($p < 0.05$). The immunoexpression levels of p97/VCP did not show any significant differences in omega-3 and vitamin D+omega-3 groups compared to the control group ($p > 0.05$). Furthermore, the SVIP immunoexpression was higher in the vitamin D group compared to others. However, there was no significant difference considering the expression of SVIP in vitamin D, omega-3, and vitamin D+omega-3 groups compared to the control group ($p > 0.05$) (Figure 2).

Altered expression of autophagy (LC3B and p62) markers in brain tissue

We examined the expression of autophagic markers in rat brain cortex and hippocampus in addition to ERAD markers. Autophagic protein expressions LC3B (Figure 5) and p62 (Figure 6) tended to decrease in vitamin D and omega-3 treated groups compared to control. The H-score analysis

revealed that the immunoexpression level of LC3B was significantly decreased in the hippocampus of the brain from the vitamin D-treated group compared to the control group ($p < 0.05$). There was no significant difference considering the expression of LC3B in omega-3 and vitamin D+omega-3 groups compared to the control group ($p > 0.05$). Furthermore, p62 was decreased in the hippocampus and the cortex of the rat brain from the vitamin D-treated group compared to the control group. However, the immunoexpression levels of p62 showed no significant differences in vitamin D, omega-3, and vitamin D+omega-3 groups compared to the control group ($p > 0.05$) (Figure 2).

Discussion

In the present study, we examined the effects of vitamin D and omega-3 supplements on the ERAD and autophagic protein expressions in the hippocampus and cortex of the

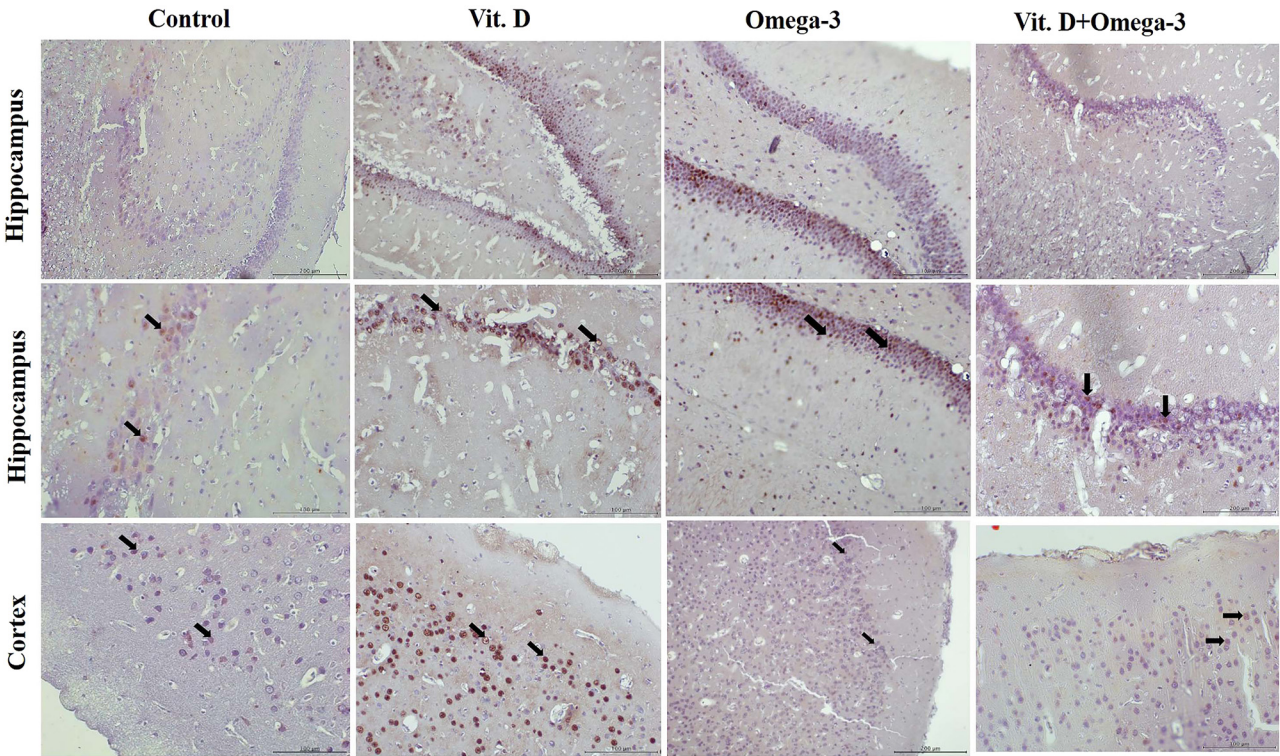


Figure 3: Immunolocalization of p97/VCP protein in the hippocampus and cortex of rat brain in control and vitamin D, omega-3, and vitamin D+omega-3 treatment groups. Black arrows indicate the p97/VCP-positive cells. Three independent experiments were performed. The micrographs were taken with 10× and 40× objectives. Scale bars: 100 µm, 200 µm.

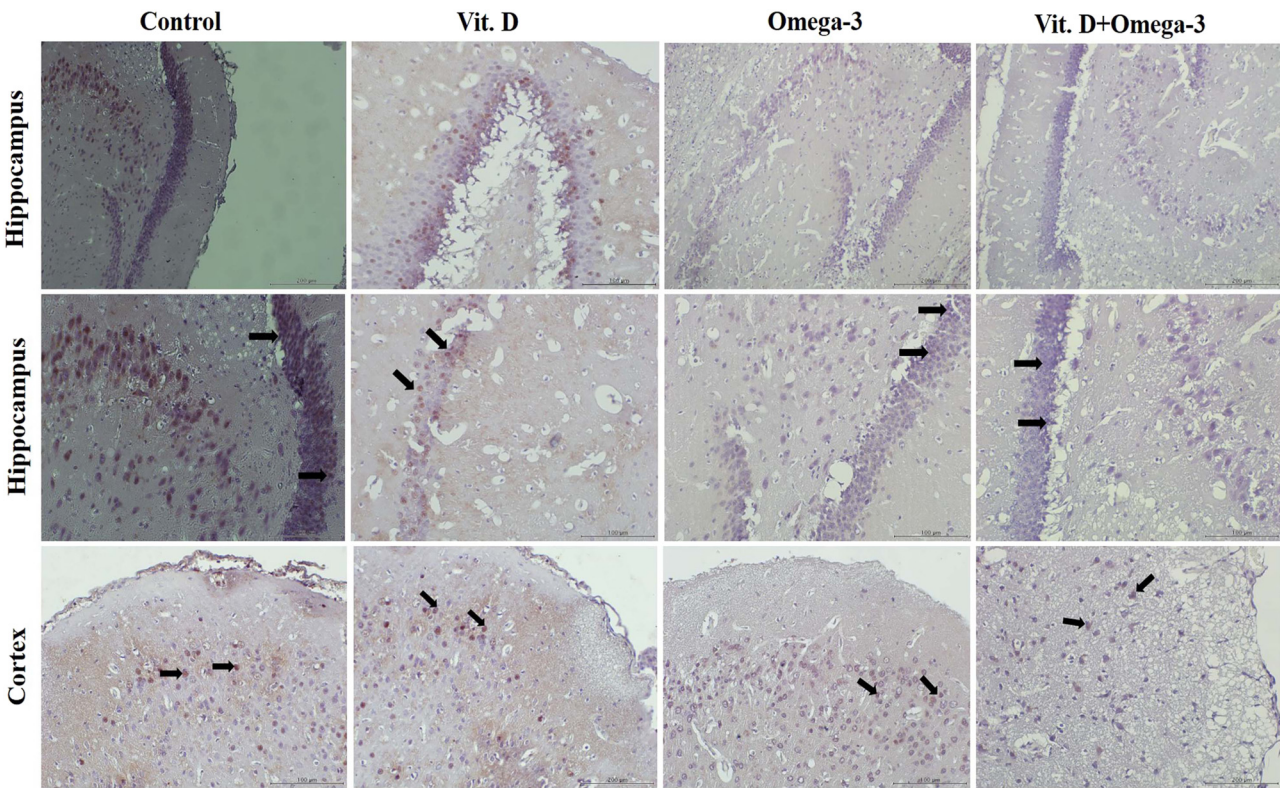


Figure 4: Immunolocalization of SVIP protein in the hippocampus and cortex of rat brain in control and vitamin D, omega-3, and vitamin D+omega-3 treatment groups. Black arrows indicate the SVIP-positive cells. Three independent experiments were performed. The micrographs were taken with 10× and 40× objectives. Scale bars: 100 µm, 200 µm.

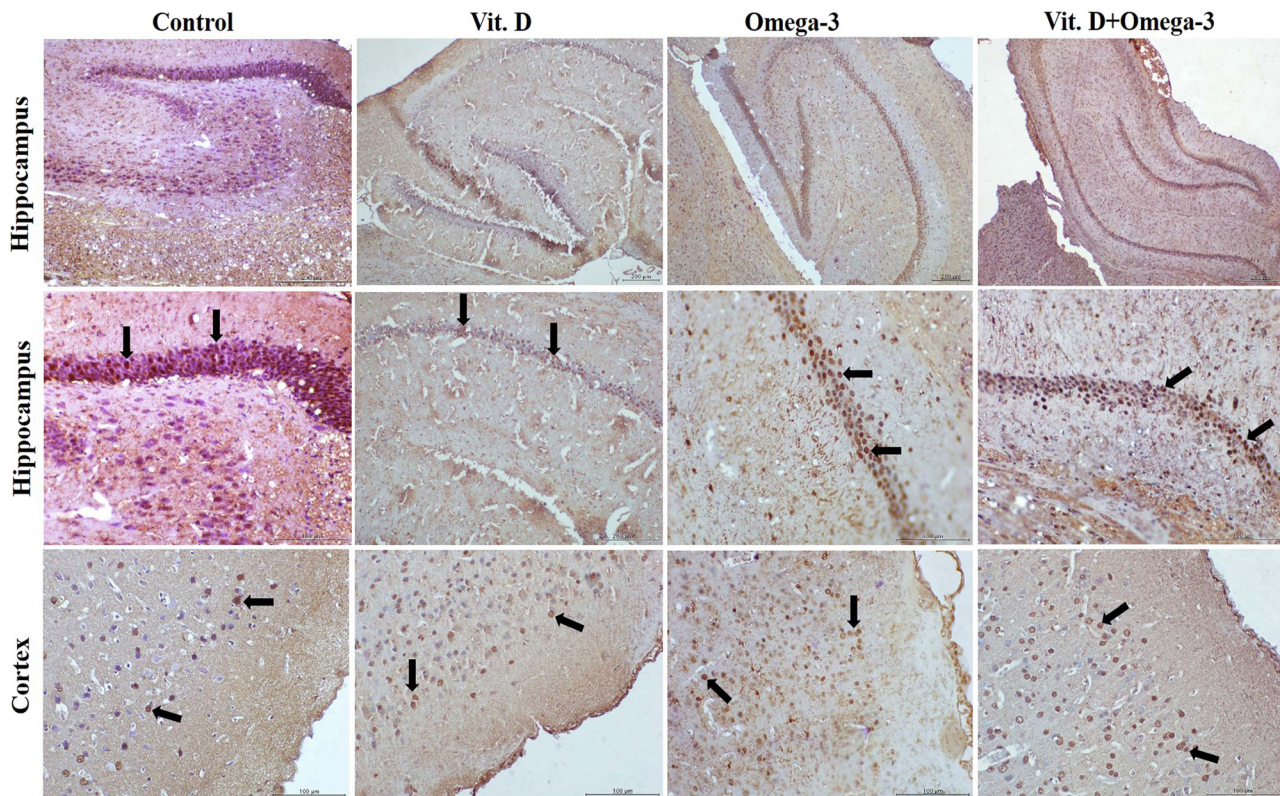


Figure 5: Immunolocalization of LC3B protein in the hippocampus and cortex of rat brain in control and vitamin D, omega-3, and vitamin D+omega-3 treatment groups. Black arrows indicate the LC3B-positive cells. Three independent experiments were performed. The micrographs were taken with 10× and 40× objectives. Scale bars: 100 μ m, 200 μ m.

rat brain. We demonstrated for the first time the expression of p97/VCP and SVIP proteins in rat brains with vitamin D and omega-3 supplements. In addition, we present evidence that vitamin D supplements importantly changed both the expression level of ERAD and autophagy proteins in rat brains. The histopathological results revealed that the cellular architectures of the hippocampus and cortex were normal throughout the groups.

Circulating 25(OH) vitamin D penetrates glial cells and neuronal cells after crossing the blood-brain barrier, where it is transformed into 1,25(OH)₂D, the active form of vitamin D [32]. The vitamin D receptor, through which vitamin D works in the brain, is a nuclear steroid receptor. It has been discovered that the brain produces and destroys vitamin D. Additionally, VDR is present in several parts of the brain and necessary for vitamin D to have its effects [33].

For the brain to develop into its full functional potential, omega-3 fatty acids are crucial. Neurodevelopmental disorders in humans have been related to low maternal n-3 polyunsaturated fatty acids (PUFA) intake [34]. Concerns about the potential negative consequences of low n-3 PUFA consumption on human child neurodevelopment [35] and the prevalence of neurodevelopmental illnesses, including Autism

spectrum disorder and schizophrenia [36], have been raised globally.

Moreover, the central nervous system's microglia, or nonneuronal cells, also contain nuclear VDRs [37]. Neuroprotection has been associated with the activation of the vitamin D pathway [38]. In the post-mortem human brain, neurons and glial cells express VDR, showing that vitamin D can be locally activated [39, 40]. The central nervous system has been shown to express the vitamin D receptor and the enzymes that bioactivate vitamin D, notably in regions where neurodegenerative diseases are prevalent, such as the hippocampus. Gezen-Ak et al. [41] have investigated the presence of 25-OH vitamin D-24-hydroxylase enzyme required for vitamin D catabolism in cortical and hippocampal neurons. They also contrast the expression of 24-hydroxylase and VDR in cortical and hippocampal neurons. They concluded that increased 24-hydroxylase and VDR gene expression may imply a greater requirement for vitamin D in the hippocampus and the possible impacts of vitamin D shortage on cognitive decline, neurodegeneration, and Alzheimer's disease. To determine the VDR expression in the brain, we performed immunohistochemistry. The cellular localization of VDR was shown in the neuronal cells of the

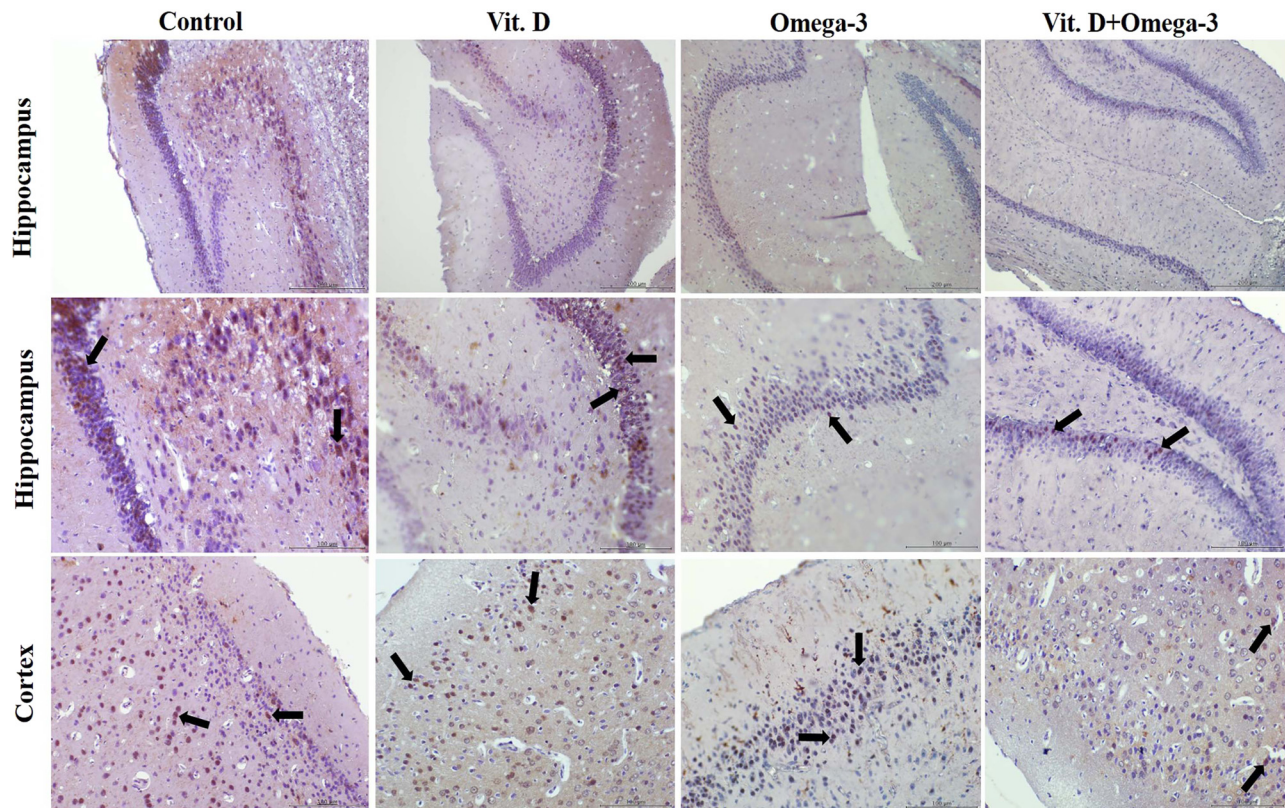


Figure 6: Immunolocalization of p62 protein in the hippocampus and cortex of rat brain in control and vitamin D, omega-3, and vitamin D+omega-3 treatment groups. Black arrows indicate the p62-positive cells. Three independent experiments were performed. The micrographs were taken with 10× and 40× objectives. Scale bars: 100 μm, 200 μm.

hippocampus and cortex of the rat brain. Additionally, the immunoexpression of VDR was significantly increased in the vitamin D-treated group.

The valosin-containing protein, which is ubiquitously expressed and supports numerous cellular processes in a cofactor-dependent manner, is encoded by the VCP gene. p97/VCP performs apoptosis, mitochondrial quality control, and protein homeostasis maintenance [24]. The ubiquitin-proteasome system and autophagy lysosomal systems work in conjunction with p97/VCP to regulate the cell cycle, produce organelles, sort vesicles, and maintain protein homeostasis, among other cellular activities [42]. Endoplasmic reticulum-associated protein production is decreased by VCP knockdown in primary cultured hippocampal neurons, which reduces the development of dendritic spines [43, 44]. The multisystem disease known as inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia, which is defined by inclusion bodies in the brain or muscle, is related to mutations in the human VCP [45, 46]. According to Hirabayashi et al. [47], p97/VCP serves as both a pathogenic effector for several neurodegenerative symptoms and a recognition factor for proteins that are folded improperly. For the

therapy of neurodegenerative diseases, p97/VCP may be the perfect molecular target.

SVIP has been associated with several physiological functions, including the regulation of autophagy, vacuole formation, and ERAD suppression. It serves as the adaptor protein for the multifunctional p97/VCP [23, 48, 49]. The study conducted by Y. Wang et al. [23] has shown that SVIP is primarily localized in the central and peripheral nervous systems. According to Jiawen Wu et al. [50], SVIP and p97/VCP were seen to co-localize in the cell bodies of neurons. It is important to note that they also discovered that in compact myelin, where p97/VCP was not present, SVIP co-localizes with myelin basic protein.

The molecular effects of 1,25(OH)₂ D₃ on the unfolded protein response (UPR), ERAD, and androgenic signaling were studied by Erzurumlu et al. [51]. In LNCaP prostate cancer cells, they discovered that 1,25(OH)₂ D₃ distinctly controlled the inositol-requiring enzyme 1 (IRE1) and protein kinase RNA-like ER kinase (PERK) branches of the UPR and adversely affected the expression level of ERAD components.

A recent study has indicated that SVIP plays a regulatory function in p97 subcellular localization and is a new regulator of autophagy [23]. Moreover, p97/VCP eases the

degradation of misfolded proteins through UPS and ERAD, and it is essential for aggresome formation and protein aggregate trafficking. It has been previously reported that p97/VCP is in the regulation of autophagy [52]. Previous studies have indicated that vitamin D3 decreases cell apoptosis, protects cell survival, and reduces disease severity by autophagy regulation. Also, the study showed that vitamin D3 treatment develops autophagy by decreasing the expression of p62, LC3-II, and Beclin-1 proteins in the myocardium of rats treated with vitamin D3 [53]. In the present study, LC3B and p62 revealed reduced expressions in vitamin D-treated group compared to control in the rat brain and hippocampus.

In conclusion, we have identified the cellular localization and immunoexpression of ERAD pathway proteins (p97/VCP and SVIP) and autophagic proteins (LC3B and p62) in the rat hippocampus and cortex of the brain tissue. The immunolocalization of p97/VCP and SVIP was detected in the neuronal cells of the hippocampus and cortex of the brain. Additionally, it was established that vitamin D increased the immunoexpression of the ERAD pathway's p97/VCP and SVIP proteins. Also, in this study, SVIP expressions were shown in the rat hippocampus and cortex of the brain tissue. Autophagic protein expressions LC3B and p62 tended to decrease in vitamin D and omega-3 groups compared to control. Accordingly, a negative correlation between ERAD markers (SVIP, p97/VCP) and autophagic markers (LC3B, p62) was observed, recommending a relationship between ERAD and autophagy.

In summary, the current study represented that vitamin D supplements activated the VDR pathway. To define autophagic flux and protein activities, cell culture studies with varied inhibitors and activators of ERAD and autophagy will absolutely explain the interrelationship between them with supplementations. To conclude, our results suggest that ERAD and autophagy protein expressions differ between the experimental groups. Therefore, we can recommend that more research is required to ascertain and determine the relationship between vitamin D and omega-3 supplementation and the pathways.

Research ethics: This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (Decision number: 49533702/113).

Informed consent: Not applicable.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Use of Large Language Models, AI and Machine Learning

Tools: None declared.

Conflict of interest: The authors state no conflict of interest.

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Data availability: The raw data can be obtained on request from the corresponding author.

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