# Changes in the expression of the vitamin D receptor and LVSCC-A1C in the rat hippocampus submitted to single prolonged stress

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**Abstract.** Vitamin D signaling not only controls calcium ($Ca^{2+}$) and phosphorus uptake and transport, but also correlates with neurocognitive decline and neurodegenerative diseases. Almost all actions of Vitamin D are mediated by the transcription factor, vitamin D receptor (VDR), which has been widely identified in the central nervous system. Although previous studies have substantially advanced the understanding of the action of VDR in the brain, much remains unknown concerning how VDR relates to stress. Multiple lines of evidence indicate that the downregulation of L-type voltage-sensitive $Ca^{2+}$-channels α-1C (LVSCC-A1C) by vitamin D in hippocampal neurons is able to reduce the influx and excitotoxic effects of $Ca^{2+}$ to neurons. Along these lines, the purpose of the present study was to analyze the relative expression of VDR in the hippocampus of rats exposed to single prolonged stress (SPS) as a putative animal model for human post-traumatic stress disorder (PTSD). Furthermore, changes in the levels of expression of LVSCC-A1C and $Ca^{2+}$ (neurotransmitter content) were examined during the onset periods of PTSD. The results revealed an increase in the expression of VDR at 1, 3 and 7 days post-stress compared with the control group. The intracellular free $Ca^{2+}$ levels in the hippocampus increased 1 day after SPS exposure, and then decreased gradually to the normal level at 14 days, consistent with the expression pattern of LVSCC-A1C. These results indicated that VDR may be involved in the pathogenesis of SPS rats, thereby providing an alternative preparation to search for optimal therapeutic strategies for PTSD.

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## Introduction

Post-traumatic stress disorder (PTSD) is a severe anxiety disorder that may develop following exposure to any threat or injury that results in psychological trauma. Diagnostic symptoms for PTSD include re-experiencing the original trauma through flashbacks or nightmares, avoidance of stimuli associated with the trauma and increased arousal. Single-prolonged stress (SPS), an animal model of PTSD, has been extensively developed and employed in the investigation of PTSD (1-3). The three areas of the brain whose function may be altered in PTSD have been identified as the prefrontal cortex, the amygdala and the hippocampus, among which, the hippocampus is a key organ of the limbic system involved in learning and memory, as well as being a regulatory center for the stress response (4).

Alterations in brain neurochemistry have been linked with neuropsychiatric disorders, including schizophrenia, Alzheimer's disease, depression and cognitive decline. Previous studies have identified a positive association between vitamin D signaling and cognitive function (5). Vitamin D may regulate neurotransmission, neuroprotection and neuroimmunomodulation as a neurosteroid hormone (5,6), in addition to its critical role in calcium ($Ca^{2+}$) and phosphorous regulation and skeletal mineralization (7). Hypovitaminosis D is associated with several neuropsychiatric disorders, including dementia, Parkinson's disease, multiple sclerosis, epilepsy and schizophrenia. The action of vitamin D is mediated by the vitamin D receptor (VDR), a ligand-activated transcription factor (8). VDR is considered to be a nuclear receptor that is ubiquitously expressed in a wide variety of organs or tissues, including in the muscle, adipose tissue, bone (9), cerebral cortex and hippocampus (6,10). Genetic variance in the VDR gene affects the susceptibility to age-related changes in cognitive functioning and depressive symptoms (11). The absence of VDR has been associated with neurodegenerative dementia, and VDR-knockout mouse models have revealed that genetic ablation may cause anxiety and motor disorders (12,13), indicating the essential role of VDR in the brain (14).

Several lines of evidence indicate that an alteration in neuronal $Ca^{2+}$ homeostasis may be involved in the neuroprotective actions of VDR. The appropriate $Ca^{2+}$ concentration is required for neuronal excitability. Treatment with vitamin D

decreases the density of L-type voltage-sensitive $Ca^{2+}$-channels (LVSCCs) and protects against the excitotoxicity of the rat hippocampal cells (15). Moreover, an increased function of neuronal LVSCCs is strongly linked to impaired memory and altered hippocampal synaptic plasticity in older rats (16), indicating that LVSCCs may also contribute to the pathological memory changes during the development of PTSD.

Since little is currently known about the neurocognitive effects of VDR, it is of note to assay the expression of this receptor in the hippocampus subjected to SPS, where alterations are likely to occur during the development of PTSD. Thus, in the present study, a rat model of PTSD was built following the previously established SPS protocol (17,18), and immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and western blotting analysis approaches were used to identify the expression of VDR in the rat hippocampal cells. In addition, the expression of LVSCC-A1C protein and mRNA was examined, as well as the $Ca^{2+}$ levels. To the best of our knowledge, the present study is the first to directly assess neuronal VDR and LVSCC-A1C activities in a rat model of PTSD.

## Materials and methods

*Animals*. Young Sprague-Dawley male rats (6-7 weeks old) weighing ~200 g were obtained from the Experimental Animal Center of China Medical University (Shenyang, China). Animals were housed singly under a 12-h light/dark cycle, with food and water freely available. Following an adaptation period of 5-6 days, the experimental procedures were undertaken. All procedures were approved by the Institutional Animal Care and Use Committee (China Medical University) and were in accordance with the National Institutes of Health Guide for the care and use of laboratory animals.

*Experimental groups and the SPS model*. In total, 50 rats were randomly divided into five groups; the control group and the SPS groups of 1, 3, 7 and 14 days, with 10 rats per group. The SPS model was created as described previously, with slight modifications (17,18). Briefly, rats were restrained for 2 h inside a disposable restraint holder that was 58 mm in diameter and 150 mm in length. Next, they were individually placed in a clear acrylic container of dimensions 600 mm x 400 mm x 500 mm, which was filled two-thirds with water at 24˚C, and forced to swim for 20 min. Following a 15-min recuperation, the animals were exposed to diethyl ether until loss of consciousness and left undisturbed in their cages. The animals were then randomly assigned to one of the four SPS groups.

*Fixation and section preparation of the hippocampus*. Five rats from each group were anesthetized with pentobarbital sodium (30 mg/kg intraperitoneally; China National Medicines Corporation, Ltd., Shanghai, China) and perfused with 200 ml cold saline through the left ventricle, followed by perfusion with 300 ml of 4% cold paraformaldehyde in phosphate buffer. The whole brain was removed rapidly, dissected on ice and then fixed in the same fixative solution for 10 h at 4˚C. Following immersion in a 20% sucrose solution for 24 h, the brain was sliced into 7-$\mu$m coronal sections and stored at -70˚C.

*Immunohistochemical analysis of VDR*. The sections were treated with 5% bovine serum albumin and 0.3% Triton X-100 (Beyotime, Haimen, China) in phosphate-buffered saline (PBS) for 30 min at room temperature for blocking of non-specific staining, followed by incubation with rabbit polyclonal antibody against VDR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:200) overnight at 4˚C. Following washing with PBS, the sections were incubated with anti-rabbit immunoglobulin G horseradish peroxidase (HRP) antibody (ZSBio, Beijing, China) for 0.5 h at 37˚C. Finally, 3,3'-diaminobenzidine was used as chromogen for 10 min until the brown coloring appeared. Slices were then dehydrated and mounted with neutral gum. To assess non-specific staining, a few sections in every experiment were incubated in PBS without primary antibody.

Five slides were randomly selected from each group, and on each slide, five visual fields were randomly selected (magnification, x200). The optical density (OD) of the positive cells in each field was recorded to evaluate the average value. The OD of the VDR-immunopositive cells was analyzed using a MetaMorph/DPIO/BX41 morphology image analysis system (Olympus, Tokyo, Japan).

*Western blot analysis*. The rats of each group were decapitated rapidly and the hippocampi were dissected on ice. The samples were homogenized with loading buffer containing 200 mM Tris-buffered saline, 4% sodium dodecyl sulfate, 20% glycerol and 10% 2-mercaptoethanol, and were denatured by boiling for 3 min. The protein fraction (30 $\mu$g/lane) extracted from each sample was separated by 12% (w/v) gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a 0.45-$\mu$m polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Following blocking with 5% (w/v) skimmed milk in 0.05% TBS with Tween-20 (TBST) at room temperature for 2 h and incubation with a rabbit polyclonal antibody against VDR (Santa Cruz; 1:200) or LVSCC-A1C rabbit polyclonal antibody (Abcam, Cambridge, MA, USA; 1:200) overnight at 4˚C, the membrane was incubated with anti-mouse IgG-HRP (Santa Cruz; 1:5,000) secondary antibodies for another 2 h at room temperature. Finally, the PVDF membrane was washed three times with TBST prior to visualization using enhanced chemiluminescence (Uscnlife, Wuhan, China). For each study, a representative immunoblot from at least three independent experiments is presented.

*RT-PCR*. Total mRNA was extracted from the hippocampus using the TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers were designed by Shenggong Biotech Co., (Shanghai, China) according to the serial number from Genbank, and are shown in Table I. GAPDH mRNA used as an internal control was co-amplified with VDR or LVSCC-A1C mRNA. The products were observed following electrophoresis on a 1.2% agarose gel, and the density of each band was analyzed with the Gel Image Analysis system (Tanon 2500R; Tanon Science & Technology Co., Ltd., Shanghai, China). The levels of VDR and LVSCC-A1C mRNA were determined by calculating the density ratio of VDR or LVSCC-A1C mRNA to GAPDH mRNA.

Table I. Oligonucleotide sequences and product sizes of the primers.

| Gene | Primer | Sequence | Product size, bp |
|---|---|---|---|
| VDR | Forward | GTCTGCAGCGTGTTGGATAG | 157 |
| | Reverse | ATGACTCTACCCACGGCAAG | |
| LVSCC-A1C | Forward | AATCTGACGGGAAAAAAGATGAA | 447 |
| | Reverse | TCCTGTCGACTCCTTAGTTAATCCT | |
| GAPDH | Forward | ACGCCAGTAGACTCCACGAC | 178 |
| | Reverse | ATGACTCTACCCACGGCAAG | |

VDR, Vitamin D receptor; LVSCC-A1C, L-type voltage-sensitive $Ca^{2+}$-channels α-1C.

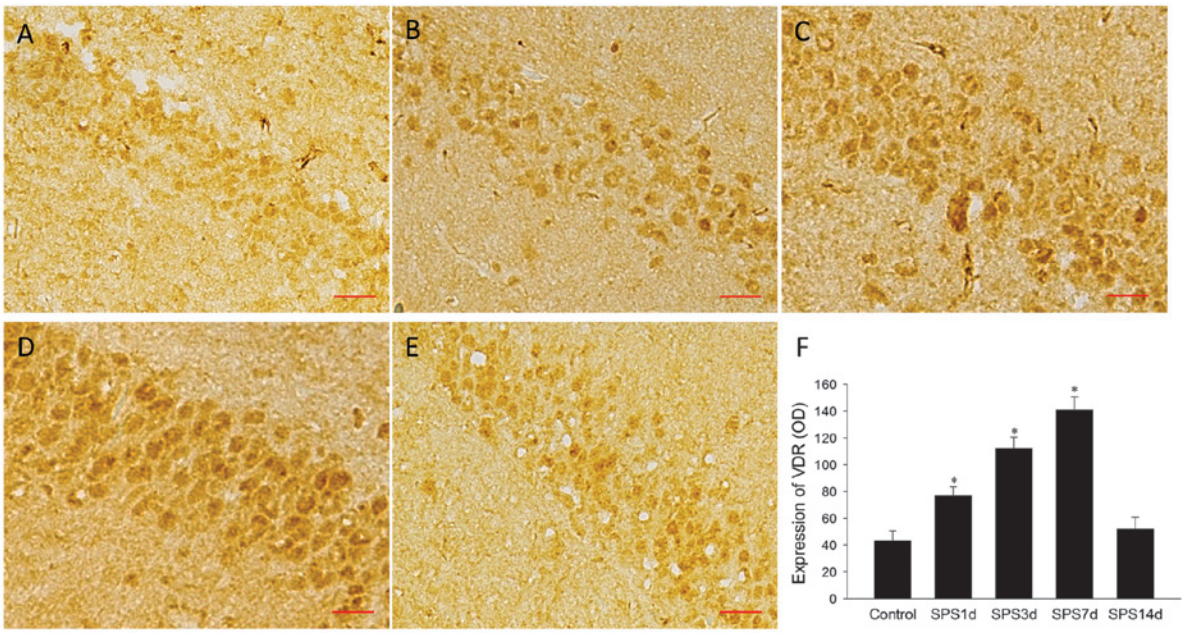


Figure 1. Immunohistochemistry of VDR expression in the CA1 of the hippocampus. (A) Control group; (B) SPS 1 day group; (C) SPS 3 day group; (D) SPS 7 day group; and (E) SPS 14 day group. Scale bar, 20 $\mu$m (magnification, x200). (F) Quantitative analysis of the mean ODs. *P<0.05 vs. the control group. The data represent the mean ± standard deviation (n=5 per group). VDR, vitamin D receptor; SPS, single prolonged stress; OD, optical density.

*Intracellular free $Ca^{2+}$ assay*. The rats of each group were decapitated rapidly and the hippocampi were dissected on ice. A cell suspension of $10^6$-$10^7$ cells/ml was achieved with a routine method and loaded with 1 mmol/l fura-2-acetoxymethyl ester (Beyotime) for 35 min, and then analyzed with a spectrofluorometer (F-4500FL Fluorescence Spectrophotometer; Hitachi, Tokyo, Japan), following the manufacturer's instructions.

*Statistical analysis*. Data are presented as the mean ± standard deviation and were analyzed with SPSS software (version 20.0; IBM, Armonk, NY, USA). A one-way analysis of variance (ANOVA) with post hoc Tukey's test was used to determine statistical significance. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Immunohistochemical analysis of VDR expression*. Following SPS stimulation, the hippocampi from the treated and non-treated rats were analyzed with immunohistochemical staining, as shown in Fig. 1A-E. Positive immunohistochemical cells stained with the antibody against VDR were brown, the majority revealing strong nuclear and cytoplasmic staining; however, certain cells had extremely light staining, indicating variations in the levels of VDR expression among neurons. The evaluation of VDR expression by the mean ODs indicated a significant change in the SPS 1, 3 and 7 day groups compared with the control group (P<0.05; Fig. 1F). The peak of the increase was at SPS 7 days. The immunoreactivity then decreased significantly to its normal level at SPS 14 days. (P>0.05 vs. control).

*Western blotting analysis of VDR and LVSCC-A1C proteins*. The VDR, LVSCC-A1C and β-actin proteins were detected at 51, 249 and 36 kDa, respectively (Fig. 2A), and the mean values of the band densities of the control group were set as 100%. The data were expressed as normalized ODs. The OD value of the VDR bands had a significant increase at 1, 3 and 7 days in the SPS groups compared with the control group (P<0.05; Fig. 2B), while at 14 days it returned to its normal value compared with

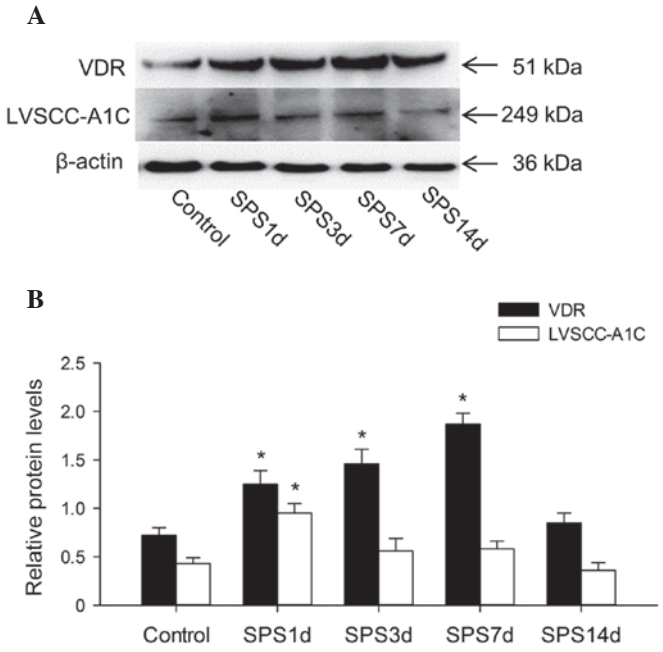


Figure 2. (A) Western blot analysis demonstrating the expression of VDR and LVSCC-A1C proteins in the hippocampus of the control and SPS 1, 3, 7 and 14 day groups. β-actin was used as a loading control. (B) The relative levels are presented as the mean ± standard deviation, n=5 per group. VDR, vitamin D receptor; LVSCC-A1C, L-type voltage-sensitive calcium-channels α-1C; SPS, single prolonged stress.

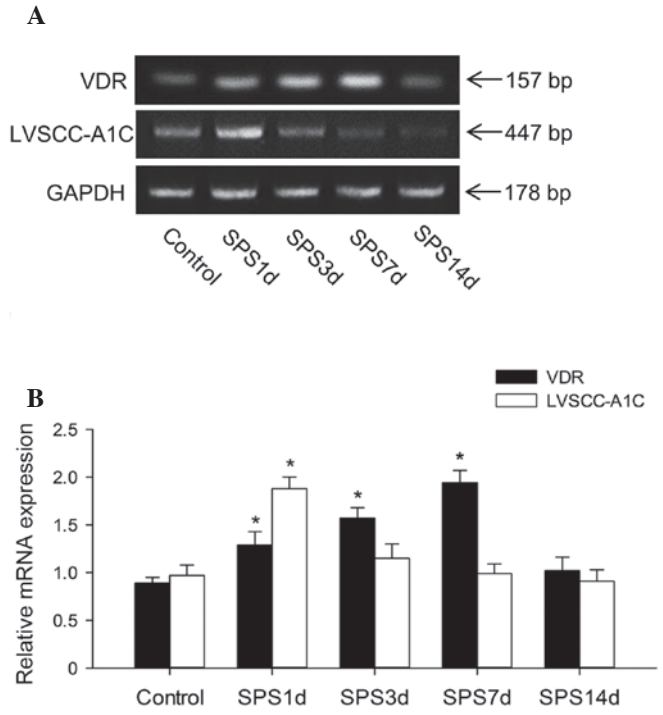


Figure 3. Reverse transcription-polymerase chain reaction demonstrating the mRNA expression of (A) VDR, LVSCC-A1C and GAPDH, and (B) their relative levels from five different experiments *P<0.05 vs. the control group. VDR, vitamin D receptor; LVSCC-A1C, L-type voltage-sensitive calcium-channels α1C; SPS, single prolonged stress.

the control group (P>0.05). The OD value of LVSCC-A1C was upregulated at day 1 (P<0.05 vs. control), and then had a significant reduction at 3, 7 and 14 days (P>0.05 vs. control; Fig. 2B).

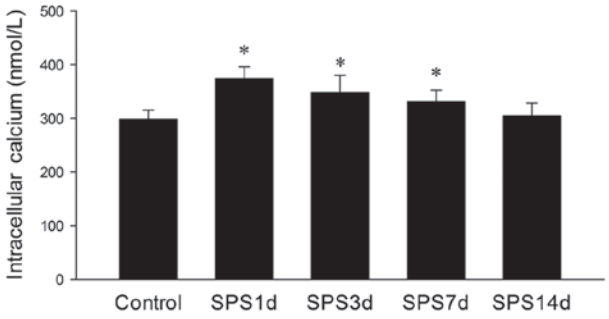


Figure 4. Changes of intracellular $Ca^{2+}$ in nmol/l in the various groups. *P<0.05 vs. the control group. SPS, single prolonged stress.

*RT-PCR results*. To further confirm the changes in VDR and LVSCC-A1C expression caused by SPS exposure, RT-PCR analysis was performed (Fig. 3A). The levels of VDR and LVSCC-A1C mRNA were normalized with GAPDH mRNA. In the analysis of VDR, the one-way ANOVA with post hoc analysis revealed that there were significant differences between the SPS 1, 3 and 7 day groups and the control group, respectively (P<0.05; Fig. 3B). Immediately following SPS, the bands of the 1 day group demonstrated a significant upregulation of the LVSCC-A1C mRNA level in the hippocampus compared with the unexposed control group (P<0.05), which was then downregulated to its normal level at 3 days (P>0.05).

*Free $Ca^{2+}$ concentration in the hippocampus* . Following SPS exposure, the rats had an increased intracellular free $Ca^{2+}$ level in the hippocampal neurons compared with the control group. The increase peaked 1 day following exposure to SPS, and then decreased to normal levels at 14 days (Fig. 4).

## Discussion

In this study, the detection of the free $Ca^{2+}$ content in the hippocampal neurons revealed $Ca^{2+}$ overload 1 day after SPS stimulation, which gradually decreased to the normal levels at 14 days. Further analysis of LVSCC-A1C, one of the main $Ca^{2+}$ regulatory proteins in the central nervous system (CNS), revealed that the expression of LVSCC-A1C in the hippocampus markedly increased 1 day after SPS stimulation, indicating that the LVSCC-A1C content changed synchronously with the change of $Ca^{2+}$ concentration. This occurred as the increased intracellular free $Ca^{2+}$ levels in the hippocampal neurons induced overexpression of the LVSCC-A1C protein after SPS exposure, and the increased LVSCC-A1C caused increased $Ca^{2+}$ influx as a positive feedback. VDR expression was also demonstrated to have increased significantly at day 1, and reached its peak 7 days after SPS stimulation, synchronously with the downregulation of intracellular $Ca^{2+}$ level 1 day after SPS. Notably, the expression of LVSCC-A1C also reached its peak at day 1 and then decreased significantly. Thus, it was postulated that the increased VDR expression in the hippocampus may interact with LVSCC-mediated $Ca^{2+}$ dysregulation during the development of PTSD. Therefore, VDR may also be important for modulating $Ca^{2+}$ homeostasis, which is well recognized to be critical in neuroprotection.

The presence of VDR in the CNS was first identified in 1982 (19). There is now ample evidence that VDR and its cascade enzymes are distributed in different regions of the brain, and that the VDR signaling system acts within the CNS as a neurosteroid with multiple actions (20,21). However, evidence indicating a correlation between VDR and stress is limited. It is unclear whether VDR is involved in neurogenesis or $Ca^{2+}$ homeostasis in response to stress. Little is known about whether and how disturbing the function of the VDR affects the development of post-traumatic stress disorder, since the regulators of VDR expression in the CNS remains unknown (22). However *in vitro* evidence indicates that there is cross-talk between the VDR and glucocorticoid receptors in the hippocampus, and that vitamin D is involved in neuronal differentiation and/or apoptosis in this region (23). Mice lacking VDR have exhibited substantial behavioral impairment and increased anxiety (24).

It is now better recognized that vitamin D, the ligand of VDR, exhibits a role in the nervous system. In a number of studies, it has been indicated that vitamin D in the brain regulates neurotrophic factor expression, oxidative stress mechanisms and $Ca^{2+}$ homeostasis (25-31). Vitamin D may affect neuronal plasticity processes and increase neurite outgrowth when added to cultured hippocampal cells (28). It has been revealed that vitamin D upregulates the expression of microtubule-associated protein-2 and growth-associated protein-43 in cultured cortical neurons (32). Maternal hypovitaminosis D decreases the expression of proteins involved in cytoskeleton maintenance, including neurofilaments, tubulin, actin and glial fibrillary acidic protein (33,34). More recently, studies have indicated a potential beneficial role of vitamin D in cognitive function and neuroprotective effects beyond classical mineral homeostasis. Defects in the vitamin D signaling system have been associated with various neuropsychiatric disorders (35-37). Human vitamin D deficiency may result in an active mood disorder and worse cognitive functioning (38,39). In animals, it has been demonstrated that prenatal vitamin D deficiency resulted in alterations in brain morphology, learning and memory (40,41). In addition, mice lacking a functional VDR gene exhibited anxiety-like behavior, indicating that vitamin D may affect cognitive functioning and the prevalence of depressive symptoms (13).

The vitamin D signaling system is essential in overall $Ca^{2+}$ homeostasis. Acute exposure to $1,25(OH)_2D_3$ increases the mean open time and plasma membrane $Ca^{2+}$ permeability of the LVSCC in the short term, thereby easing the requirement for $Ca^{2+}$ influx (42). VSCCs mediate the influx of $Ca^{2+}$ in response to membrane depolarization, and regulate intracellular functions, including excitation-secretion, gene transcription, neurotransmitter release and cell differentiation. The neuroprotective effects of vitamin D appear to be exerted via the regulation of $Ca^{2+}$ homeostasis and the synthesis of neurotrophins, which support the survival of existing neurons and the growth and differentiation of new neurons (26,27,30,43). Previous studies have indicated that $Ca^{2+}$ dysregulation is involved in the aging brain and in Alzheimer's disease, giving rise to the '$Ca^{2+}$ hypothesis of brain aging and dementia' (44). $Ca^{2+}$ overload and the dysregulation of $Ca^{2+}$ signaling in the nerve cells in PTSD patients have been shown to increase the cytotoxicity of the brain neurons (45). The detrimental effects of excessive $Ca^{2+}$ on memory formation and cognitive functioning are widely acknowledged (46-49). Administration of vitamin D or its metabolites may decrease neuronal death in rat hippocampal cultures, elicited by $Ca^{2+}$-mediated neurotoxicity through the downregulation of LVSCC and increased VDR levels (26,32).

To date, the pathogenesis of PTSD is far from definite. PTSD may result from a series of biochemical and physiological abnormalities in the brain, which leads to dysfunction of the hippocampus. Collectively, the findings of this study provide a novel perspective on the pathogenesis of PTSD. Further evidence of the actions of VDR in the hippocampus may indicate its potential effects in a wide range of neuropsychiatric disorders.

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