Research Square

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Vitamin D Pretreatment Modulates PINK1/Parkin-Related Mitophagy and Attenuates Seizure-Associated Brain Injury in Pentylenetetrazole- and Kainic acid -Induced Acute Epilepsy Models

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Abstract

Objective

To investigate whether vitamin D (VitD) pretreatment influences seizure outcomes and PINK1/Parkin-related mitophagy signalling in brain tissue using two acute epilepsy mouse models.

Methods

Acute seizure models were established using pentylenetetrazol (PTZ) or kainic acid (KA). Animals were allocated to control, model, VitD pretreatment, valproate (VPA) positive control, and VitD combined with the mitochondrial fission inhibitor Mdivi-1 groups. Seizure frequency, latency and severity were evaluated using behavioural observation and electroencephalography (EEG). Hippocampal neuronal injury and network remodelling were assessed by HE, Nissl and Timm staining, while mitochondrial ultrastructure and mitophagosome formation were examined by transmission electron microscopy (TEM). Expression of mitophagy-related markers (PINK1, Parkin, Drp1, p62 and LC3) was measured using qRT-PCR and western blotting.

Results

Both PTZ and KA induced epileptiform EEG activity, hippocampal neuronal injury, abnormal mossy fibre sprouting (MFS) and altered expression of mitophagy-related markers, accompanied by mitochondrial structural abnormalities. VitD pretreatment was associated with reduced seizure frequency, prolonged seizure latency and attenuation of hippocampal injury in both models. Molecular analyses showed altered expression of PINK1/Parkin pathway markers and partial normalisation of mitophagy-related proteins following VitD pretreatment. Combined intervention with Mdivi-1 was associated with additional modulation of mitochondrial dynamics-related markers and further improvement of histopathological and ultrastructural outcomes.

Conclusions

VitD pretreatment was associated with neuroprotective effects in acute PTZ- and KA-induced seizure models and modulation of PINK1/Parkin-related mitophagy signalling. These findings support a potential role of mitochondrial quality-control pathways in the biological effects of VitD under acute seizure conditions, while further mechanistic validation is required.

Highlights

1. VitD pretreatment prolonged seizure latency and reduced seizure frequency in both pentylenetetrazole - and kainic acid -induced acute seizure models.

2. VitD mitigated hippocampal neuronal injury, mossy fiber sprouting, and mitophagosome formation following seizures.

3. Seizures activated PINK1/Parkin-mediated mitophagy, which was partially reversed by VitD pretreatment.

4. Co-treatment with mitochondrial fission inhibitor Mdivi-1 enhanced VitD-associated neuroprotection and further modulated mitophagy-related markers.

1. Introduction

Epilepsy is a chronic neurological disorder characterised by recurrent seizures resulting from abnormal, excessive neuronal activity in the brain. It affects approximately 70 million people worldwide and represents a significant public health burden [1, 2]. Although numerous antiseizure medications are available, approximately one-third of patients remain drug-resistant, highlighting the need for improved understanding of disease mechanisms and the development of complementary therapeutic strategies [3–6]. Increasing evidence suggests that epilepsy involves complex and interacting biological processes, including neuronal hyperexcitability, imbalance between excitatory and inhibitory neurotransmission, oxidative stress, neuroinflammation, and metabolic dysregulation [7, 8].

Mitochondria play a central role in neuronal energy metabolism, calcium homeostasis, and regulation of programmed cell death pathways. Neurons are particularly vulnerable to mitochondrial dysfunction due to their high metabolic demand and limited capacity for energy storage [9]. Accumulating studies indicate that mitochondrial structural damage and impaired mitochondrial function contribute to seizure susceptibility and neuronal injury [10, 11]. Mitochondrial quality-control processes, including mitochondrial fission, fusion, biogenesis, and mitophagy, are essential for maintaining cellular homeostasis under conditions of metabolic stress [9]. Mitophagy is a selective autophagic process responsible for the removal of damaged or dysfunctional mitochondria, thereby preserving mitochondrial integrity and cellular energy balance [12]. Among the pathways regulating mitophagy, the PINK1/Parkin signalling cascade is one of the most extensively studied mechanisms. Under conditions of mitochondrial stress, accumulation of PTEN-induced kinase 1 (PINK1) on the outer mitochondrial membrane promotes recruitment of the E3 ubiquitin ligase Parkin, leading to ubiquitination of mitochondrial proteins and subsequent autophagic degradation of damaged mitochondria [13]. Alterations in PINK1/Parkin-related signalling have been implicated in several neurological disorders, including Parkinson's disease, Alzheimer's disease, and epilepsy [12]. Experimental studies suggest that seizures may induce mitochondrial dysfunction and activation of mitophagy-related pathways; however, the extent to which altered mitophagy contributes to neuronal injury remains incompletely understood [11].

Vitamin D (VitD), traditionally recognised for its role in calcium and bone metabolism, has increasingly been studied as a neuroactive secosteroid with potential effects on brain function [14]. VitD receptors (VDR) are widely expressed in neurons and glial cells, suggesting broader biological functions in the central nervous system [15, 16]. Emerging evidence indicates that VitD may influence multiple processes relevant to neurological disorders, including calcium homeostasis, oxidative stress regulation, immune

modulation, neurotrophic signalling, and neuronal survival pathways [15]. Clinical and experimental studies have reported associations between VitD deficiency and increased seizure susceptibility, while VitD supplementation has been suggested to modulate seizure frequency in some patient populations [17, 18]. However, the molecular mechanisms underlying these observations remain to be clarified.

Recent studies have proposed that VitD may influence mitochondrial function and cellular stress responses. Experimental evidence suggests that VitD may regulate mitochondrial membrane potential, reactive oxygen species production, and cellular energy metabolism [15]. In addition, VitD has been reported to interact with intracellular signalling pathways involved in autophagy and apoptosis [14, 18]. These findings raise the possibility that VitD may influence mitochondrial quality-control processes, including mitophagy, under pathological conditions characterised by metabolic stress.

Despite growing interest in VitD as a neuroactive compound and in mitophagy as a mitochondrial quality-control mechanism in epilepsy, it remains unclear whether VitD influences seizure-associated mitophagy in vivo and whether PINK1/Parkin-related signalling contributes to this interaction. Seizure activity can induce mitochondrial stress and activate autophagy-related signalling pathways, yet the role of altered mitophagy responses across different acute seizure paradigms has not been systematically investigated. Animal models remain the most common approach for preclinical testing of candidate antiseizure therapies [19]. Importantly, pentylenetetrazol (PTZ) and kainic acid (KA) models represent distinct seizure phenotypes and neuropathological characteristics, including generalised convulsive activity and limbic system vulnerability, respectively [20, 21]. The use of both models provides a broader framework for evaluating the robustness and biological relevance of potential VitD–mitochondrial interactions .

In the present study, acute seizure mouse models were established using intraperitoneal administration (i.p.) of PTZ or KA to investigate whether VitD pretreatment influences seizure outcomes and PINK1/Parkin-related mitophagy signalling in brain tissue. We further explored the potential involvement of mitochondrial dynamics through combined intervention with the mitochondrial fission modulator Mdivi-1. The study aimed to provide mechanistic insight into the relationship between VitD and mitochondrial quality-control pathways in the context of acute seizure-induced neuronal injury, and to evaluate whether modulation of mitophagy-related signalling may contribute to the biological effects of VitD under conditions of seizure-associated metabolic stress.

2. Methods

2.1. Experimental reagents

PTZ (purity ≥ 98%, cat. no. P103065) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). $VitD_3$ (purity ≥ 98%, cat. no. V8070) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). KA (purity > 98%, cat. no. HY-N2309), valproate (VPA) (purity = 99.19%, cat. no. HY-10585), and Mdivi-1 (purity 99.96%, cat. no. HY-15886) were supplied by

MedChemExpress LLC (Monmouth Junction, NJ, USA). PINK1 polyclonal antibody (cat. no. WL04963, dilution ratio: 1:1000), Parkin polyclonal antibody (cat. no. WL02512, dilution ratio: 1:1000), and DRP1 polyclonal antibody (cat. no. WL03028, dilution ratio: 1: 2000) were acquired from Wanleibio Co., Ltd. (Shenyang, China). p62 polyclonal antibody (cat. no. 31403-1-AP, dilution ratio:1:2000), LC3 polyclonal antibody (cat. no. 14600-1-AP, dilution ratio: 1:2000), and GAPDH monoclonal antibody (cat. no. 60004-1-Ig, dilution ratio: 1:50000) were purchased from Proteintech Group, Inc. (Wuhan Sanying Biotechnology, Wuhan, China). AffiniPure goat anti-rabbit IgG (H + L) (cat. no. BA1039, dilution ratio: 1:10000) and AffiniPure goat anti-mouse IgG (H + L) (cat. no. BA1038, dilution ratio: 1:5000) were obtained from Boster Biological Technology Co., Ltd. (Wuhan, China). Total RNA extraction kit (cat. no. B518651), reverse-transcription kit (cat. no. B639252), and amplification kit (cat. no. B532955) were procured from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China).

2.2. Mice husbandry

SPF male C57BL/6J mice aged 6–8 weeks were supplied by Changchun Yisi Laboratory Animal Technology Co., Ltd (Changchun, China), license No. SCXK (Ji) 2023-0002, and housed in the Laboratory Animal Center of Jiamusi University. Environmental conditions were maintained at 20 ± 2°C and 55% ± 5% relative humidity, with a 12 h light/12 h dark cycle. Mice were group-housed (five per cage) and provided ad libitum access to standard chow and water. After a 7-day acclimation period, mice weighing 18–22 g and showing good general health were selected for experiments. The experimental protocol was approved by the Medical Ethics Committee of the Third Affiliated Hospital of Jiamusi University (Approval No. jmsukf-2023018) and was conducted in accordance with the ARRIVE guidelines.

2.3. Preparation of Major Reagents and Model Establishment

Ten milliliters of sterile saline were mixed with 100 mg of PTZ powder to obtain a PTZ solution at 10 mg/mL. Five milliliters of sterile saline were mixed with 10 mg of KA powder to obtain a KA solution at 2 mg/mL. Twenty milligrams of VitD powder were dissolved in 4 mL of DMSO to prepare a 5 mg/mL stock solution; one part of this stock was combined with nineteen parts of corn oil to yield a final VitD working solution of 0.25 mg/mL. Ten milliliters of sterile saline were mixed with 300 mg of VPA powder to obtain a VPA solution at 30 mg/mL. Ten milligrams of Mdivi-1 powder were dissolved in 0.25 mL of DMSO to prepare a 40 mg/mL stock solution; one part of this stock was combined with nineteen parts of corn oil to yield a final Mdivi-1 working solution of 2 mg/mL. Throughout the procedure, all solutions were protected from light and stored wrapped in aluminum foil. In the present study, VitD and Mdivi-1 were initially dissolved in a minimal volume of DMSO and then diluted to their final concentrations with physiologically inert corn oil containing 5% (v/v) DMSO; this dose has been repeatedly shown to exert no influence on neurological behavior, histology, or biochemical indices in mice [22]. The corn oil used has also been verified in multiple epilepsy studies to have no effect on neuronal excitability. All reagents were freshly prepared immediately before use [23].

A single i.p. injection of PTZ at 60 mg/kg [24] or KA at 20 mg/kg [25] was used to establish an acute generalized tonic–clonic seizure model or an acute TLE model, respectively. Mice that failed to develop seizures or died during model construction were excluded from the analysis.

2.4 Experimental Groups

A total of 176 mice were selected; 14 died during model induction and treatment, leaving 162 animals available for statistical analysis. All mice were allocated to nine groups (n = 18 each) by random-number tables, ensuring no systematic differences in baseline body weight or locomotor activity and minimizing selection bias. Behavioral testing and all subsequent tissue-based assays were conducted with the investigators blinded to group assignment until data collection and analysis were complete. In the VitD pre-treatment groups, VitD (1 mg/kg) was administered subcutaneously 40 min before PTZ or KA injection. In the positive-control VPA groups, VPA (300 mg/kg) was given intraperitoneally 2 min after PTZ or KA [26]. For the combined-intervention group, Mdivi-1 (20 mg/kg) [27] was injected intraperitoneally at 24 h and again at 30 min before PTZ or KA; VitD (1 mg/kg) was then administered subcutaneously 40 min before PTZ or KA; finally, PTZ or KA was injected intraperitoneally 30 min after the last Mdivi-1 dose [27]. A flowchart of the experimental groups and treatment timeline is illustrated in Fig. 1.

2.5 Behavioral Observations

PTZ model: A single i.p. injection of PTZ (60 mg/kg) [24] was given, followed by continuous observation for 60 min to record seizure frequency and latency. Seizures graded > stage III on the Racine scale were considered successful model establishment [28]. KA model: A single i.p. injection of KA (20 mg/kg) [25] was given, and animals were monitored for 60 min to record seizure frequency and latency. Seizures graded > stage III indicated successful modeling. After modeling, seizure severity in each group was evaluated with the Racine scale: 0 = no convulsion; I = facial twitching; II = nodding or tail jerking; III = forelimb clonus; IV = bilateral forelimb extension with rearing; V = generalized tonic–clonic fall [28]. Seizure frequency: total number of seizures recorded during the 60-min period. Seizure latency: time interval from PTZ or KA injection to the first observable seizure. Behavioral assessments were performed twice. During model induction and intervention, the first author, Jiahao Liu, conducted the initial Racine-scale evaluation. The corresponding author, Professor Jin Guo, subsequently scored seizure behaviors from recorded videos. To maximize objectivity and minimize bias, Professor Jin Guo remained unaware of group assignments. The independent scores obtained by both investigators were identical.

2.6 Electroencephalogram (EEG) recordings to document seizure severity

Twenty-four hours after modeling and intervention, three mice from each group were randomly selected and anesthetized with intraperitoneal tribromoethanol. After induction of anesthesia, the animals were secured in a stereotaxic frame, the scalp was shaved, and the skin was incised to expose and disinfect

the skull. Using Bregma as the reference point, recording electrodes were implanted and fixed at the following coordinates: Hippocampus: Anterior–Posterior − 3.0 mm, Medial–Lateral ± 3.0 mm, Dorsal–Ventral − 3.0 mm [29]. This site targets the vicinity of the dentate gyrus, a focus of high-frequency epileptiform discharges, facilitating the detection of typical epileptic activity such as spike waves [29]. Prefrontal motor cortex: Anterior–Posterior + 3.0 mm, Medial–Lateral − 2.0 mm, Dorsal–Ventral − 1.5 mm [16]. This location lies in the posterior part of the frontal lobe adjacent to the primary motor cortex and effectively monitors the propagation of seizure activity from limbic structures, including the hippocampus, to the neocortex; the recorded signals are tightly synchronized with behavioral motor manifestations. The mice were then placed in a recording chamber and allowed to recover from anesthesia. A recovery period of at least 4 h was provided, and complete recovery was defined as the resumption of spontaneous grooming and free exploration [16]. This ensured that subsequent EEG recordings reflected the true physiological state rather than residual anesthetic effects. EEGs were acquired for 1 h using a Z2N-F-20-C amplifier (Shanghai Nuocheng Electric Co., Ltd.) and NCERP software, with the following settings: paper speed 6 cm/s, sensitivity 50 μV/cm, and a 50 Hz notch filter enabled.

2.7 Hematoxylin and eosin (HE) staining for hippocampal morphology

Twenty-four hours after modeling and intervention, three mice from each group were randomly selected, anesthetized, and subjected to thoracotomy. A needle was inserted into the left ventricle, and the right atrial appendage was incised for exsanguination. The animals were first perfused with physiological saline until the effluent was clear, followed by 4% paraformaldehyde until limb rigidity was observed. The brains were rapidly removed, rinsed in saline, and fixed in 4% paraformaldehyde. After fixation, the tissue was embedded in paraffin. Serial coronal sections (3 non-consecutive slices, 120 μm apart) were obtained from − 1.8 mm to − 2.4 mm posterior to bregma. The sections were deparaffinized, rehydrated, and stained with HE. After differentiation, bluing, dehydration, and clearing, the slices were mounted with neutral balsam. Hippocampal neuronal morphology was examined under an inverted microscope.

2.8 Nissl staining to evaluate neuronal survival/injury

Twenty-four hours after modeling and intervention, three mice from each group were randomly selected and anesthetized. Following cardiac perfusion with physiological saline and then paraformaldehyde, the brains were removed and fixed. The tissue was routinely embedded in paraffin, and continuous coronal sections 5 μm thick were prepared. After deparaffinization and rehydration, the sections were stained with 0.5% toluidine blue, differentiated in ethanol, dehydrated through graded alcohols, cleared in xylene, and mounted with neutral balsam. The distribution and morphology of Nissl bodies in hippocampal neurons were examined under a light microscope to evaluate neuronal survival and injury.

2.9 Timm staining was used to assess mossy fiber sprouting (MFS) in the dentate gyrus

Twenty-four hours after modeling and intervention, three mice from each group were randomly selected, anesthetized, and transcardially perfused with physiological saline followed by 1% sodium sulfide and then 4% paraformaldehyde. The brains were removed, post-fixed in 4% paraformaldehyde overnight, and transferred to 30% sucrose until they sank. Horizontal sections (30 μm) containing the hippocampus were cut on a cryostat. The sections were processed for Timm staining by incubating in the dark at 40°C for 40 min in the silver-developing solution, then rinsed, dehydrated, cleared, and mounted. MFS in the dentate gyrus was examined and evaluated under a microscope.

2.10 Transmission electron microscopy (TEM) for mitochondrial ultrastructure and mitophagosomes

Twenty-four hours after modeling and intervention, three mice from each group were randomly selected, anesthetized, and their brains were removed to obtain 1 mm^3 hippocampal tissue blocks. The samples were doubly fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated in a graded ethanol series, infiltrated and embedded in epoxy resin, and then cut into ultrathin sections. After double staining with uranyl acetate and lead citrate, the sections were examined and imaged under a TEM to observe the ultrastructure of mitochondria and autophagosomes in neurons. Autophagosomes containing mitochondria were identified as degenerating mitochondria enclosed by double membranes, or as autolysosomes formed by fusion with lysosomes in which mitochondrial contents were degraded [30].

2.11 Quantitative real-time PCR (qRT-PCR) for mitophagy-related mRNA expression

Twenty-four hours after modeling and intervention, three mice from each group were randomly selected, anesthetized, and their brains were removed to isolate the bilateral hippocampal tissue. Total RNA was extracted using a commercial RNA isolation kit, and the concentration and purity of each sample were determined with a NanoDrop UltraC FL micro-spectrophotometer. All samples exhibited A260/A280 ratios between 1.9 and 2.1, indicating high RNA purity. RNA integrity was further verified by 1.5% agarose gel electrophoresis, which revealed sharp 28S and 18S ribosomal RNA bands without degradation, confirming intact RNA. Subsequently, 1 μg of total RNA was reverse-transcribed to synthesize first-strand cDNA. Quantitative real-time PCR was performed on an SLAN-96S real-time PCR system using the primers listed in Table 1 to quantify the mRNA levels of PINK1, Parkin, Drp1, p62, and LC3, with β-actin serving as the endogenous reference for normalization. Relative gene expression was calculated using the 2^−ΔΔCt method [31]. All primers were designed and synthesized based on the corresponding CDS sequences obtained from the NCBI database. The primer sequences are listed in Table 1.

Table 1
Primer sequences for qRT-PCR

Target Gene	Primer Sequence
PINK1-F	TATCTCGGCAGGTTCCTCCA
PINK1-R	AAGCTGCTTGGGACCATCTC
Parkin-F	CCTGCAAACAAGCAACCCTC
Parkin-R	TCACCACTCATCCGGTTTGG
Drp1-F	GGCAACTGGAGAGGAATGCT
Drp1-R	CTTGCAACTGGAACTGGCAC
p62-F	GCACAGGCACAGAAGACAAGAG
p62-R	CCCACCGACTCCAAGGCTATC
LC3-F	GCCTTCTTCCTGCTGGTCAAC
LC3-R	TCCGTCTTCATCCTTCTCCTGTTC

2.12 Western blot (WB) analysis for mitophagy-related proteins

Twenty-four hours after modeling and intervention, three mice were randomly selected from each group, anesthetized, and their brains were harvested to dissect the bilateral hippocampi and partial cerebral cortex. The tissue samples were lysed and centrifuged; the supernatants were collected for protein concentration determination using the BCA assay, and subsequently stored at -80°C after denaturation. Protein separation was then performed using SDS-PAGE, followed by transfer to PVDF membranes. After blocking, the membranes were sequentially incubated with primary antibodies (overnight at 4°C) and secondary antibodies (at room temperature for 1 hour). Finally, the proteins were visualized by chemiluminescence, and band intensities were analyzed using ImageJ software to calculate the relative expression levels of target proteins. To determine an appropriate loading control, we evaluated the expression stability of GAPDH in brain tissues from the normal control, epilepsy model, and intervention groups through preliminary experiments. No significant fluctuations in GAPDH expression were observed among the three groups; therefore, it was selected as the internal reference for protein quantification in this study. All target protein signals were normalized to GAPDH to correct for variations in total protein loading.

2.13 Data Analysis

To objectively evaluate the histological results, this study employed a blinded analysis. All sections were randomly coded by an independent investigator to ensure that the evaluator remained unaware of group allocations during scoring. Unblinding and statistical analysis were conducted only after completion of all data collection. WB signals were captured using the Tanon 4200 chemiluminescence imaging system

(Tanon Science & Technology Co., Ltd., Shanghai, China). Protein band intensities were quantified using ImageJ and normalized to the internal reference protein for each sample. Seizure frequency and latency were manually recorded. All data are expressed as mean ± standard deviation (SD) and were analyzed and graphed using GraphPad Prism 10.0. Intergroup differences were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistical significance was defined as $P < 0.05$.

3. Results

1.1 Behavioral Performance

As shown in Fig. 2, compared with the normal control group, mice in the PTZ group sequentially exhibited mild restlessness, facial and forelimb focal clonic seizures following drug administration, which rapidly progressed to generalized tonic–clonic seizures, followed by a brief postictal suppression period. Mice in the KA group predominantly displayed sniffing behaviour, facial muscle twitching, and rhythmic chewing movements. Focal seizures (e.g., forelimb clonus) were generally less severe than those observed in the PTZ group, although severe cases also progressed to generalized tonic–clonic seizures and showed a longer recovery period.

Compared with the PTZ group (seizure frequency: 6.0 ± 1.6 episodes/h; seizure latency: 5.21 ± 1.76 min/h), the KA group showed a numerically higher seizure frequency (7.1 ± 1.5 episodes/h, $P = 0.2817$) and a significantly longer seizure latency (15.96 ± 2.48 min/h, $P < 0.0001$). Relative to the PTZ group, the VitD + PTZ group showed a lower seizure frequency (4.7 ± 1.2 episodes/h, $P = 0.1574$) and a significantly prolonged seizure latency (8.47 ± 1.16 min/h, $P = 0.0017$). Similarly, the VitD + KA group showed a lower seizure frequency (5.9 ± 1.1 episodes/h, $P = 0.2131$) and a slightly prolonged seizure latency (17.01 ± 1.68 min/h, $P = 0.5785$) compared with the KA group. Overall, VitD pretreatment was associated with a trend towards reduced seizure frequency and prolonged seizure latency in both models, with a comparatively larger magnitude of change observed in the PTZ model.

3.2 EEG Monitoring of Seizure Severity in Mice

EEG recordings showed that mice in the normal control group exhibited regular waveforms with low amplitudes. In contrast, both PTZ and KA groups displayed typical epileptiform discharges, characterised by high-amplitude spikes and sharp waves, increased slow-wave activity, and disorganised waveforms, confirming successful establishment of the acute seizure models. Compared with the PTZ group (epileptiform discharge frequency: 35 ± 7 events; total seizure duration: 12.14 ± 4.38 min), the KA group (epileptiform-discharge frequency: 46 ± 9 events; total seizure duration: 15.28 ± 6.30 min) showed a numerically higher frequency of epileptiform discharges and longer total seizure duration.

VitD pretreatment alone did not significantly alter epileptiform activity compared with the corresponding model groups. In contrast, the positive control treatment VPA reduced spike and sharp wave activity. The

combined VitD + Mdivi-1 group showed a lower frequency of epileptiform discharges and shorter seizure duration compared with VitD pretreatment alone (Fig. 3).

3.3 Morphological Changes in the Hippocampal CA3 Region of Mice Observed by HE Staining

Histopathological examination of the hippocampus showed that neurons in the CA3 region of the normal control group exhibited intact cellular morphology, clear structure, and regular arrangement. In contrast, both PTZ and KA groups showed disrupted neuronal organisation, reduced cell density, and increased numbers of neurons with condensed nuclei (pyknotic morphology) in the CA3 region. The KA group showed a comparatively greater degree of structural disruption than the PTZ group.

Following pharmacological intervention, the VPA group showed partial preservation of neuronal arrangement compared with the model groups. VitD pretreatment was associated with partial improvement in neuronal morphology in both seizure models, with a comparatively greater degree of preservation observed in the PTZ model. The combined VitD + Mdivi-1 group showed comparatively improved cellular organisation and fewer neurons with morphological characteristics consistent with neuronal injury compared with VitD pretreatment alone (Fig. 4).

3.4 Observation of Neuronal Morphology in the Hippocampal CA3 Region of Mice by Nissl Staining

Nissl staining showed that neurons in the hippocampal CA3 region of the normal control group contained abundant Nissl bodies, characterised by dense distribution and well-defined morphology. In contrast, both PTZ and KA groups showed reduced Nissl body density, decreased staining intensity, and a more dispersed distribution pattern within the CA3 region, suggesting neuronal structural alterations. The KA group showed a comparatively greater reduction in Nissl staining intensity than the PTZ group.

Following pharmacological intervention, the VPA group showed partial preservation of Nissl body density and distribution compared with the model groups. VitD pretreatment was associated with partial preservation of Nissl body morphology in both seizure models, with a comparatively greater degree of preservation observed in the PTZ model. The combined VitD + Mdivi-1 group showed comparatively higher Nissl body density and more regular distribution compared with VitD pretreatment alone (Fig. 5).

3.5 Assessment of MFS in the Dentate Gyrus by Timm Staining

Timm staining showed minimal Timm-positive granules in the dentate gyrus of the normal control group, with MFS rarely observed. In contrast, both PTZ and KA model groups exhibited increased Timm-positive staining in the dentate gyrus. Dense brownish-black granule deposits formed continuous bands with higher staining intensity than the surrounding tissue background. The KA group showed a comparatively higher band density than the PTZ group.

Following pharmacological intervention, both VitD pretreatment and VPA treatment were associated with reduced Timm staining intensity compared with the model groups. Timm-positive signals in the dentate gyrus showed a more dispersed distribution pattern, with previously dense continuous bands appearing less compact and more discontinuous. The combined VitD + Mdivi-1 group showed comparatively lower Timm staining intensity and reduced band continuity compared with VitD pretreatment alone (Fig. 6).

3.6 Observation of Mitochondrial Ultrastructure and Mitophagy in Hippocampal Neurons by TEM

TEM revealed that mitochondria in hippocampal neurons of the normal control group exhibited regular morphology with intact membranes and clearly defined cristae, and no mitophagosome-like structures were observed. In contrast, both PTZ and KA model groups showed mitochondrial structural alterations, including mitochondrial swelling, disrupted or reduced cristae, and increased cytoplasmic vacuole-like structures. Double-membrane vesicles containing mitochondrial components, morphologically consistent with mitophagosome-like structures, were observed in neuronal cytoplasm (indicated by red arrows in the Fig. 7).

Compared with the PTZ group, the KA group showed a comparatively greater degree of mitochondrial structural alteration, including increased mitochondrial vacuolisation and a higher number of mitophagosome-like structures. Following pharmacological intervention, both VitD and VPA treatments were associated with partial preservation of mitochondrial ultrastructure, including reduced mitochondrial swelling and comparatively clearer cristae morphology. The VitD + Mdivi-1 group showed comparatively fewer mitochondria with structural alterations and a reduced number of mitophagosome-like structures compared with VitD pretreatment alone (Fig. 7).

3.7 qRT-PCR Analysis of Gene mRNA Expression in Mouse Brain Tissue

Compared with the normal control group, relative mRNA expression levels of PINK1, Parkin, Drp1, and LC3 were higher in both PTZ and KA groups, whereas p62 mRNA expression showed a lower level. The KA group showed comparatively larger changes in these markers than the PTZ group.

Following VitD pretreatment and VPA treatment, relative mRNA expression levels of PINK1, Parkin, Drp1, and LC3 were were lower compared with the corresponding model groups, while p62 mRNA expression showed a higher level. Compared with VitD pretreatment alone, the VitD + Mdivi-1 group showed comparatively lower mRNA expression levels of PINK1, Parkin, Drp1, and LC3 and comparatively higher p62 mRNA expression (Fig. 8).

3.8 WB Analysis of Protein Expression in Mouse Brain Tissue

Compared with the normal control group, both PTZ and KA groups showed higher protein levels of PINK1, Parkin, Drp1, and LC3-II/LC3-I ratio, together with lower p62 protein levels. The KA group showed

comparatively larger changes in these protein markers than the PTZ group.

Following VPA treatment, protein levels of Parkin and Drp1 were lower in the PTZ group, whereas the KA group showed lower expression levels of PINK1, Parkin, Drp1, and LC3-II/LC3-I ratio compared with the corresponding model group. After VitD pretreatment, protein expression levels of PINK1, Parkin, and Drp1 were lower compared with the model groups. No clear change in p62 protein expression was observed following VPA or VitD treatment. Compared with VitD pretreatment alone, the VitD + Mdivi-1 group showed comparatively lower protein expression of Parkin, Drp1, and LC3-II/LC3-I ratio, together with comparatively higher p62 protein levels (Fig. 9).

4. Disscussion

Epilepsy is a complex neurological disorder involving multiple interacting mechanisms, including neuronal hyperexcitability, imbalance between excitatory and inhibitory neurotransmission, oxidative stress, and neuroinflammation [32]. Increasing evidence indicates that mitochondrial dysfunction and dysregulated autophagy contribute to seizure-associated neuronal injury. Using two widely established acute seizure mouse models, PTZ and KA, the present study investigated whether vitamin D pretreatment influences seizure-associated mitochondrial stress and PINK1/Parkin-related mitophagy signalling in the hippocampus. The results show that VitD pretreatment was associated with attenuation of seizure-related hippocampal structural alterations and partial normalisation of mitophagy-related molecular markers, supporting a potential role for mitochondrial quality-control pathways in the neuroprotective actions of VitD.

PTZ, a non-competitive GABA receptor antagonist, induces generalised tonic–clonic seizures by reducing inhibitory neurotransmission [33], whereas KA, a glutamate receptor agonist, models key pathological features of TLE, including selective hippocampal neuronal vulnerability and MFS [34]. In the present study, both models produced characteristic behavioural seizures and epileptiform EEG discharges consistent with previous reports [35], supporting the reliability of the experimental paradigms.

Kalueff et al. demonstrated that subcutaneous administration of 1,25(OH)D antagonised PTZ-induced seizures, delayed seizure onset, reduced seizure duration, and decreased mortality, with effects reported to be independent of systemic calcium regulation [36]. Consistent with these observations, the present study showed that VitD pretreatment was associated with prolonged seizure latency and reduced seizure frequency in both PTZ- and KA-induced models, with comparatively larger behavioural changes observed in the PTZ model. The relatively smaller magnitude of change observed in the KA model may reflect the more complex excitotoxic and inflammatory processes associated with KA-induced hippocampal injury. The dose (1 mg/kg) and 40-minute pretreatment interval applied in this study were informed by the work of Kalueff et al., who proposed that rapid actions of 1,25(OH)D may involve non-genomic steroid signalling mechanisms [36]. Supporting this concept, previous studies have reported that 1,25(OH)D can activate intracellular signalling pathways within minutes via membrane-associated

receptors or interactions with membrane lipid microdomains, thereby influencing neuronal excitability [37].

As a direct electrophysiological indicator of seizure activity and neuronal network synchrony, EEG provides an objective measure of seizure severity [38]. In the present study, EEG recordings showed that mice in the normal control group exhibited stable background rhythms with low amplitude. In contrast, both PTZ and KA groups displayed characteristic epileptiform discharges, including high-amplitude spikes, sharp waves, and disrupted rhythmic activity, consistent with previously reported features of acute seizure models [39]. The KA model group showed a higher frequency of spike and sharp wave discharges together with longer cumulative seizure duration than the PTZ group. These electrophysiological features corresponded with more pronounced structural alterations observed in the KA model, including increased MFS and more extensive neuronal morphological disruption, which are consistent with electrophysiological characteristics reported in TLE [40].

Although VitD pretreatment prolonged seizure latency in behavioural observations, its effect on spike and sharp wave activity detected by surface EEG was relatively modest in this study. This partial dissociation between behavioural and electrophysiological findings may suggest that the acute effects of VitD preferentially influence seizure threshold or neuronal network excitability, thereby delaying the onset of behavioural seizures, while exerting limited immediate modulation of already-established synchronous epileptiform discharges [41]. Alternatively, VitD may influence seizure propagation rather than the initial generation of epileptiform activity, which would require higher spatial resolution electrophysiological approaches, such as depth electrode recordings, for further clarification [16]. In contrast, treatment with VPA reduced spike and sharp wave activity, consistent with its established role in stabilising neuronal excitability and suppressing epileptiform discharges [26].

Histological and ultrastructural analyses, including HE staining, Nissl staining, Timm staining, and TEM, showed that VitD pretreatment was associated with attenuation of seizure-related neuronal structural alterations in the hippocampal CA3 region, reduced loss of Nissl bodies, decreased MFS intensity, and fewer mitophagosome-like structures. These effects appeared more evident in the PTZ model than in the KA model. Furthermore, combined administration of the mitochondrial fission inhibitor Mdivi-1 with VitD was associated with additional preservation of neuronal ultrastructure and further modulation of mitophagy-related markers, supporting a potential contribution of mitochondrial quality-control pathways to the observed neuroprotective effects of VitD.

Mitophagy is an important mitochondrial quality-control process responsible for the removal of damaged mitochondria; however, dysregulated mitophagy has been associated with neuronal metabolic dysfunction and increased vulnerability to cell injury under pathological conditions [42]. In the present study, both PTZ and KA models showed higher mRNA and protein expression levels of PINK1, Parkin, Drp1, and LC3-II/LC3-I ratio, together with lower p62 expression, a molecular profile commonly associated with increased mitophagy-related signalling. These molecular changes were accompanied by increased numbers of mitophagosome-like structures observed by TEM, suggesting an association

between seizure activity and altered mitochondrial quality-control processes. Following VitD pretreatment, expression patterns of mitophagy-related markers showed partial normalisation, together with a reduction in mitophagosome-like structures, supporting a potential modulatory role of VitD in seizure-associated mitochondrial stress responses. One possible mechanism is that VitD influences transcriptional regulation of PINK1 and Parkin through vitamin D receptor (VDR)-mediated genomic signalling pathways [43]. In addition, VitD has been reported to regulate intracellular calcium homeostasis, which may influence mitochondrial membrane stability and susceptibility to mitochondrial dysfunction under excitotoxic conditions [44]. These mechanisms may collectively contribute to modulation of mitochondrial quality-control processes in the context of seizure-related neuronal stress.

To further explore the potential involvement of mitochondrial dynamics in the observed neuroprotective effects, the present study included combined intervention with Mdivi-1, a commonly used inhibitor of mitochondrial fission that targets the GTPase activity of dynamin-related protein 1 (Drp1), a key regulator of mitochondrial morphology and mitophagy-related processes [45]. Dysregulated mitochondrial fission has been implicated in neuronal injury following seizures, and modulation of Drp1 activity may influence mitochondrial quality-control pathways under conditions of metabolic stress. Co-administration of Mdivi-1 with VitD pretreatment was associated with additional improvements across several outcome measures compared with VitD alone. In particular, the combined intervention was associated with further attenuation of epileptiform EEG activity, greater preservation of hippocampal neuronal morphology (HE, Nissl, and Timm staining), and improved mitochondrial ultrastructural integrity (TEM). At the molecular level, combined treatment was associated with further modulation of mitophagy-related markers, including PINK1, Parkin, Drp1, p62, and LC3, compared with VitD pretreatment alone. Collectively, these findings are consistent with the concept that mitochondrial quality-control processes contribute to seizure-associated neuronal injury and may represent one component of the neuroprotective effects observed following VitD pretreatment.

It is important to note that Mdivi-1 is widely recognised as an inhibitor of mitochondrial fission rather than a highly specific inhibitor of mitophagy. Therefore, the present findings should be interpreted as supporting a potential involvement of mitochondrial dynamics and PINK1/Parkin-related signalling pathways, rather than demonstrating a definitive causal role of PINK1/Parkin-dependent mitophagy in mediating the protective effects of VitD. The present data suggest that modulation of mitochondrial stress responses may represent one mechanistic pathway through which VitD contributes to neuroprotection under acute seizure conditions.

Based on the overall findings, this study proposes a conceptual framework linking VitD pretreatment with mitochondrial quality-control processes, as illustrated in Fig. 10. VitD pretreatment was associated with reduced PINK1/Parkin-related signalling activity and partial normalisation of mitophagy-associated molecular markers following PTZ- or KA-induced seizures. These observations raise the possibility that VitD may influence early mitochondrial stress responses and thereby contribute to preservation of neuronal energy homeostasis and structural integrity during acute seizure-related injury.

Nevertheless, the precise molecular mechanisms linking VitD signalling to regulation of PINK1 expression remain to be elucidated. Potential pathways may involve genomic regulation mediated via the vitamin D receptor (VDR), as well as indirect modulation through upstream metabolic signalling pathways such as AMPK. In addition, the present study focused on acute responses assessed 24 hours after seizure induction. Whether VitD influences mitochondrial quality-control pathways during epileptogenesis, or in chronic epilepsy models characterised by recurrent spontaneous seizures, requires further investigation.

This study has several limitations. First, the present work primarily focused on the classical PINK1/Parkin signalling pathway, whereas mitophagy can also be regulated through Parkin-independent mechanisms, including pathways mediated by FUNDC1 and BNIP3 [46, 47]. Whether VitD influences these alternative mitophagy-related pathways remains to be determined. Second, although Mdivi-1 was used as a pharmacological tool to support functional interpretation, more direct genetic evidence-such as conditional knockout of PINK1 or VDR in specific neuronal populations-would provide stronger mechanistic validation of the proposed regulatory framework. Third, as the present study focused on the hippocampus, a brain region particularly vulnerable to seizure-associated injury, the potential effects of VitD on mitochondrial quality-control pathways in other key brain regions involved in epileptic networks, including the cortex and thalamus, warrant further investigation.

5. Conclusion

In summary, this study shows that VitD pretreatment is associated with neuroprotective effects in both PTZ- and KA-induced acute mouse seizure models, including prolonged seizure latency, reduced seizure frequency, attenuation of hippocampal structural alterations, and reduced formation of mitophagosome-like structures. These effects were accompanied by modulation of PINK1/Parkin-related signalling and mitophagy-associated molecular markers. Combined administration of the mitochondrial fission inhibitor Mdivi-1 was associated with additional preservation of neuronal ultrastructure and further normalisation of mitophagy-related markers, supporting a potential role for mitochondrial quality-control processes in the observed protective effects of VitD. Taken together, the present findings suggest that VitD, acting as a neuroactive secosteroid hormone, may contribute to neuronal resilience under acute seizure conditions through modulation of mitochondrial stress responses. These results provide experimental evidence supporting the potential relevance of VitD in neuroprotective nutritional strategies targeting mitochondrial function. Further studies are required to clarify the molecular mechanisms linking VitD signalling to regulation of PINK1 expression and to determine whether similar effects are observed in chronic epilepsy models or during epileptogenesis.

Abbreviations

VitD, Vitamin D; PTZ, Pentylenetetrazole; KA, Kainic acid; VPA, Valproate; ASMs, Anti-seizure medications; DRE, Drug-resistant epilepsy; TLE, Temporal lobe epilepsy; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; BBB, Blood–brain barrier; VDR, Vitamin D receptor; i.p., intraperitoneal administration; EEG,

Electroencephalogram; HE, Hematoxylin and eosin; MFS, Mossy fiber sprouting; TEM, Transmission electron microscopy; qRT-PCR, Quantitative real-time PCR; WB, Western blot; s.c., Subcutaneous administration

Declarations

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CRediT authorship contribution statement

Jiahao Liu: Writing-original draft, Formal analysis, Validation, Writing-review & editing. **Yuanyuan Liu:** Formal analysis, Data curation. **Ruting Fu:** Writing-review & editing, Conceptualization, Formal analysis. **Liya Fang:** Writing-original draft, Formal analysis, Software. **Deming Kong:** Writing-review & editing. **Luchuan Wang**: Data curation, Formal analysis. **Pei Zeng:** Formal analysis, Data curation. **Xinru Zhu:** Formal analysis. **Youdi Fu:** Formal analysis. **Jin Guo:** Conceptualization, Resources, Funding acquisition, Supervision. **Shaobo Zhou:** Supervision, Conceptualization, Writing-review & editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author, Jin Guo and Shaobo Zhou, upon reasonable request.

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