



Co-treatment of vitamin D supplementation with enriched environment improves synaptic plasticity and spatial learning and memory in aged rats

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Abstract

Rationale and objective Environmental enrichment (EE) has been shown in old rats to improve learning and memory. Vitamin D (VitD) has also been shown to modulate age-related, cognitive dysfunction. As both EE and VitD could work to improve cognition via enhancement of neurotrophic factors, their effects might occlude one another. Therefore, a clinically relevant question is whether noted cognition-promoting effects of EE and VitD can co-occur.

Methods Aged rats were housed for 6 weeks in one of three housing conditions: environmentally enriched (EE), socially enriched (SE), or standard condition (SC). Further, a 4th group was co-treated with VitD supplementation (400 IU kg⁻¹ daily, 6 weeks) under EE conditions (EE + VitD).

Results Treatment with VitD and EE housing were associated with higher score on measures of learning and memory and exhibited lower anxiety scores compared to EE alone, SE or SC as assayed in the elevated plus maze, Morris water maze, passive avoidance, and open field tasks. Additionally, in the EE + VitD group, mRNA expression levels of NGF, TrkA, BDNF, Nrf2, and IGF-1 were significantly higher compared to expression seen in the EE group. Furthermore, field potential recordings showed that EE + VitD resulted in a greater enhancement of hippocampal LTP and neuronal excitability when compared to EE alone.

Conclusions These findings demonstrate that in aged rats exposure to EE and VitD results in effects on hippocampal cognitive dysfunction and molecular mechanisms which are greater than effects of EE alone, suggesting potential for synergistic therapeutic effects for management of age-related cognitive decline.

Keywords Age-related cognitive decline · Vitamin D · Learning and memory · Enriched environment · Neuronal excitability

Introduction

The elderly population is growing worldwide (617 million people aged ≥ 65 years). Therefore, the importance of healthy aging has become a public health precedence

for the World Health Organization (Global strategy and action plan on aging and health World Health Organization 2017). Results from studies of humans and rodent models indicate that normal aging is accompanied by decrements in perceptual abilities and a moderate degree of cognitive

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dysfunction (Gros and Wang 2018; Hertzog et al. 2008; La Spina et al. 2019; Romo-Araiza et al. 2018). While some decline of cognitive ability across a lifetime is considered normal, understanding factors which could ameliorate this decline would contribute to the improvement of aging. Human and rodent studies have noted shrinkage of the hippocampus, frontal cortex, and entorhinal cortex associated with aging which could contribute to cognitive dysfunction (Driscoll et al. 2009, 2003; Raz et al. 2005). In addition to volume reduction, age-dependent cognitive decline may be due to neuronal deficits in plasticity due to reduction in the number of synapses, alterations in the physiological processes underlying synaptic plasticity, hindered neurogenesis (Dennis et al. 2016; Mathews et al. 2017; Takei 2019), and modifications in the expression of neuroplasticity-related proteins which are changes associated with aging (Barnes 1994; Burke and Barnes 2006; Erickson and Barnes 2003; Smith et al. 2000).

Environmental enrichment (EE) has been shown to be one of the most influential external factors in inducing hippocampal plasticity and in improving learning and memory skills in experimental studies (Garthe et al. 2016; Clemenson et al. 2017; Hilal et al. 2017; Kempermann et al. 2002). The mechanisms by which EE improves learning and memory are likely multi-factorial but, at the molecular level, seem to involve neurotrophic factors which have been shown to play a significant role in promoting neuronal survival and in mechanisms underlying synaptic plasticity (Clemenson et al. 2017; Huang and Reichardt 2001). Specifically, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and insulin-like growth factor-1 (IGF-I) have been implicated in cognitive benefits of EE (Conner et al. 2009; Frielingsdorf et al. 2007; Shohayeb et al. 2018; Triviño-Paredes et al. 2016; Zigova et al. 1998).

Deletion of the murine BDNF receptor (TrkB) leads to development of impairment of long-term potentiation (LTP) and reduced neuronal survival which was associated with development of anxiety-like behavior (Bergami et al. 2008). BDNF also constitutively stimulates an antioxidant defense process by mediating the expression of a master transcription regulator known as Nrf2 which plays a role in redox homeostasis status. Low levels of BDNF inhibit Nrf2 activation which consequently leads to exposure to stress-oxidative damage (Bouvier et al. 2017). In behavioral examinations, BDNF has been shown to be important for the enhancement of learning and memory following EE (Bekinschtein et al. 2011; Jain et al. 2013; Jha et al. 2016; Kuzumaki et al. 2011; Rossi et al. 2006; Tipyasang et al. 2014).

NGF is another trophic factor shown to be important in neuronal growth, differentiation, and survival (Conner et al. 2009; Frielingsdorf et al. 2007), which has been linked to

mechanisms underlying EE-associated improvements in cognition. Housing of rats for 6 weeks in an enriched environment was associated with an increased expression of NGF and improvement of spatial and working memory (Birch et al. 2013). Blocking of endogenous NGF using specific antibodies has been shown to lead to a considerable reduction in hippocampal LTP and impaired spatial memory performance (Conner et al. 2009). There is some evidence to indicate that NGF also mediates the expression of other trophic factors such as IGF which has a crucial role in brain development and plasticity (Dyer et al. 2016). In addition to effects on cognition, EE has been shown to play a role in behaviors which influence cognition, including anxiety. Further, EE has been shown to relieve symptoms of anxiety, which was an effect associated with increases in hippocampal expression of the trophic cytokines BDNF, NGF, and IGF-1 (Zhang et al. 2018).

Vitamin D (VitD), which can stimulate trophic factors, has also been shown to play a role in cognition. VitD deficiency, especially in the elderly population, is associated with impaired learning, memory, and cognitive processes (Al-Amin et al. 2019; Llewellyn et al. 2010). Supplementation with high dose of VitD markedly improved the performance of visual (nonverbal) memory in those with primary insufficient VitD level (Pettersen 2017). Further, several studies have reported an association between low serum levels of VitD and the risk of anxiety symptoms (Han et al. 2018; Huang et al. 2014; Kim et al. 2020; Wu et al. 2016). A role for VitD in cognition and cognitive-related processes and behaviors is not unexpected as VitD acts as a potent antioxidant agent which protects cells from oxidative, stress-induced damage (Sepidarkish et al. 2019), as well as regulates the expression of proteins and neurotrophic factors involved in synaptic plasticity and physiological neuronal health (Eyles et al. 2007). Further, VitD is involved in cell differentiation (Carlberg 2016; Gil et al. 2018), neurotransmitter biosynthesis (Gezen-Ak et al. 2014b; Máčová et al. 2017), and intracellular calcium regulation (Fleet 2017).

While VitD and EE supplementation have individually been shown to enhance learning and memory and cognitive-based behaviors, as well as to stimulate processes and heighten proteins implicated in learning and memory and neurogenesis, the present study was designed to investigate for the first time the behavioral, synaptic, and molecular outcome of combining VitD (vitamin D3; 400 IU kg⁻¹ daily) and EE on cognitive dysfunction in aged rats. Cognitive performance was evaluated by a battery of behavioral tests. Synaptic plasticity and cellular excitability were examined by electrophysiology conducted in the CA1 region of hippocampal brain slices. Finally, gene expression of NGF, TrkA, BDNF, TrkB, Nrf2, and IGF-1 was evaluated by using RT-qPCR. Excitingly, our results raise the possibility that combining EE with VitD could result in heightening

the enhancement in age-related cognitive decline elicited by EE alone.

Experimental procedures

Animal subjects

In this study, we used 22-month-old Sprague–Dawley male rats. All standard conditions such as room temperature (23 ± 1 °C), humidity ($50 \pm 10\%$), 12-h dark and light cycles, and free access to water and food were maintained. Behavioral tests were performed during the dark cycle. All procedures in this work were approved by, and adhered to the ethical guidelines of the Institutional Ethics Committee (IEC) of the Kerman University of Medical Science. During the course of the experimentation, animals were monitored three times a week, and animals showing any signs of illness were excluded from the study. Animal weight and food intake were measured twice weekly.

Housing conditions

In this study, animals were housed under one of four housing conditions: environmental enrichment (EE), social enrichment (SE), standard conditions (SC), and EE with supplementation with VitD (EE + VitD). EE cages were 50×50 cm plastic cages equipped with different colored objects that included plastic tubes, PVC pipes, and plastic huts. The objects within EE cages were changed weekly to maintain novelty. Forty old male rats (22 months) were divided randomly into 4 groups: SC, SE, EE, and EE + VitD. In the SC group, 2 old rats were housed in each standard cage (20×35 cm), whereas, in SE housing conditions, five old rats were housed with normal bedding and without any objects in 50×50 cm plastic cages. Similar to SE conditions, for EE, and EE + VitD groups, 5 rats were housed in each cage. Vitamin D supplementation (400 IU/kg/day) was given orally for 6 weeks in the EE + VitD group. After 6 weeks of SC, SE, EE, or EE + VitD animal housing, the four groups were subjected to behavioral and electrophysiological experiments, and thereafter, tissue was collected for molecular and physiological studies. During the 6 weeks of selective housing and prior to testing, we excluded 20% of animals ($n = 8$) due to age-related diseases. Accordingly, when behavioral testing was scheduled to begin, the study consisted of 32 old rats distributed within the 4 groups (SC $n = 6$, SE $n = 8$, EE $n = 9$, EE + VitD $n = 9$). Two separate personnel managed the housing conditions and behavioral assessment, ensuring that the person who completed the behavioral testing was blind to the differential housing conditions.

Sources of Vitamin D

Animals in SC, SE, and EE groups received normal diet during the 6 weeks which contained 1000 IU VitD (cholecalciferol) in each kilogram of chow. In addition to dietary VitD intake, aged rats in EE + VitD group received oral supplemental drops of VitD (400 IU kg^{-1} daily dosage) for 6 weeks (Vidrop, Medical Union Pharmaceuticals, Abu-Sultan, Ismailia, Egypt). This dose of VitD was suggested based on previous studies showing induction of neuroprotective effect of this dose in experimental animals (Hajiluian et al. 2017; Khairy and Attia 2019; Latimer et al. 2014a).

One milliliter (28 drops) of oral solution contains 2800 IU vitamin D3 (each drop contains 100 IU of vitamin D3). After calculating the dose based on animal's weight, an adjustable micropipette was used for drug delivery, the tip of the pipette was inserted at the corner of the rat's mouth, and the solutions were delivered into the mouth. Following confirmation that the animal had swallowed the solution, the remaining was emptied into the animal's mouth.

Behavioral testing

After the end of the 6 weeks of housing, rats were tested in the following behavioral tasks. An experimenter blindly tested the animals, and data were collected by a video image motion analyzer (Ethovision, Noldus Information Technology; the Netherlands).

Elevated plus maze test (EPM) and the open field test (OFT)

Expression of anxiety is believed to involve hippocampal decline and loss of cognitive functioning. Testing of effects on anxiety-like behavior was conducted using two behavioral assays, the EPM and the OFT with 6-h interval on the first day of the 7th week.

The EPM apparatus consisted of two open and two closed arms. The arms were each 50 cm long and 10 cm wide, elevated 50 cm off the ground, and the closed arms had 40 cm high walls. Each rat was placed at the junction of the 4 arms. During 5 min, the time spent in the open arms and the number of entries into each of the four arms were recorded. When all paws of the rat entered the open or closed arms, this was scored as a valid entry (Tanioka et al. 2020).

The OFT apparatus which was $90 \times 90 \times 45$ cm consisted of a 16 square grid floor with central (45×45) and peripheral regions placed in a brightly lit room (an overhead light illuminated the central squares; 60 lx in its center). Rats were individually placed at the center of the open field. During a 5-min period, the time spent within the center zone (seconds) was measured. The apparatus was thoroughly cleaned with 70% ethanol after each OFT trial (Shabani et al. 2019).

Morris water maze test (MWM)

The animals were subjected to MWM for evaluation of spatial learning and memory on days 2 and 3 of the 7th week (Fig. S1). Data were collected by a video image motion analyzer (Ethovision, Noldus Information Technology; the Netherlands). The MWM apparatus was made of a circular water pool (140 cm wide and 45 cm high). At the first session, the platform was placed 1.5 cm above the surface water and a trial was performed with visible platform. The training sessions were performed with submerged platform (1.5 cm under the surface water), and each rat was trained in 3 blocks separated by a 30-min resting period, while each block consisted of 4 trials with a 30-s interval between two trials. The location of the platform remained constant for training. Rats were allowed to swim from one of the four quadrants for 60 s while facing against the maze wall. The latency to escape and traveled distance in the three blocks (B1, B2, and B3) were recorded as indexes for evaluation of learning. After 24 h, the platform was removed, and in order to evaluate memory retention, the distance traveled in the target quadrant was recorded (Shabani et al. 2019).

Radial arm maze test (RAM)

This test was performed as has been published previously with only minor modifications (Karimi et al. 2018). Working memory was studied in an eight-arm radial maze (50 cm long and 10 cm wide). A food plate was fixed at the distal end of each arm, and a reinforcement pellet was placed on this plate. Motivation to search for food was increased by overnight fasting before the day of testing. The rats were removed from the maze after eating all four pellets. The RAM test was conducted over 3 days (days 3, 4, and 5 of the 7th week) with 3 trials in each day (Fig. S1). On day 3, 1 h after the MWM test, 3 trials of RAM were performed without overnight fasting for habituation. On days 4 and 5 of the 7th week, working memory was evaluated with overnight fasting. All spatial cues were in the same location as in trial sessions. Re-entry in an arm that was previously baited was defined as working memory error (WME). Scoring of WMEs was conducted by an investigator blinded to treatment group.

Passive avoidance test (PAT)

This test was conducted on days 5 and 6 of the 7th week (Fig. S1) as previously published (Karimi et al. 2018). One hour after the last trial of RAM, while having free access to water and food, the learning trial of the PAT was conducted. Briefly, the PAT was performed in a shuttle box

apparatus with two light and dark chambers and a guillotine door between the rooms. Step through latency (STL) was measured which is the time each rat spent in the light room before entering the dark room. PAT has three stages: adaptation, learning, and retention. In the learning trial, the animal was punished with an electrical shock (0.5 mA, 50 Hz, 2 s once) upon entrance to the dark room. The learning stage was repeated every 5 min until rats learned to avoid entering the dark room. Evaluation of retention of learning was performed 24 h after the learning stage. In adaptation and retention stages, STL time was recorded without the inclusion of the electrical shock. The maximum cutoff time for the STL was 300 s without foot shock.

Field potential recording

After the end of behavioral testing course, field potential (FP) recordings (Electromodule-R12, Science Beam, and Tehran, Iran) were performed on day 6 of the 7th week with our previously published protocol (Shabani et al. 2017).

The rats were anesthetized with urethane (1.5 g kg⁻¹), and head restraint was induced by placing them within a stereotaxic apparatus. Recordings of field potentials (fEPSPs) were conducted by insertion of bipolar stainless-steel electrodes (0.2 mm diameter, Advent, UK) in the hippocampal CA1 region (-3AP and 2L as recording site) and in the Shaffer collateral pathway (-4AP and 3L as stimulation site). Monophasic square waves were used for stimulation, and the electrical signals were amplified 1000-fold, digitized at 10 kHz, and filtered at 1–3 kHz (Esmaeili Tazangi et al. 2015; Haghani et al. 2016). Electrode placement was optimized to obtain the best possible hippocampal response. After 30 min of stability, fEPSP amplitude was recorded by progressive stimulation impulses from 25 to 1200 μ A, and input/output curves were generated. After 20 min of baseline recording, high-frequency stimulation (HFS) was delivered with 3 \times 10 trains of 20 pulses at 200 Hz in order to induce LTP. The fEPSP recording was continued for 60 min after HFS.

Tissue isolation and blood analysis

After fEPSP recordings, rats were deeply anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg), and blood samples were collected for measurement of 25 hydroxyl vitamin D3 (25OHD), calcium, and phosphorus levels in the serum (Stavenuiter et al. 2015). Following decapitation, brains were removed, the right hippocampus was isolated and immediately stored at -80 °C until processing in the real-time qPCR study.

Real-time qPCR

For determination of the mRNA expression of NGF (nerve growth factor), TrkA (tropomyosin receptor kinase A), BDNF (brain-derived neurotrophic factor), TrkB (tropomyosin receptor kinase B), Nrf2 (nuclear factor erythroid 2–related factor 2), and IGF-1 (insulin-like growth factor-1), quantitative real-time PCR was used, with amplification of GAPDH mRNA conducted as an internal control. The primer sequences used for real-time RT-PCR are shown in Table S1, and basic steps were followed which were (1) hippocampal RNA extraction using Triazole reagent (Gibco, Invitrogen Corp); (2) RNA concentration measurement using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA); (3) cDNA synthesis using a cDNA Synthesis Kit (Gibco, Invitrogen Corp); (4) quantitative real-time PCR using a Light-Cycler PCR system (Roche Diagnostics Ltd); (5) total volume of 20 μ l, including 2 μ l cDNA at a 1:10 dilution, 0.5 μ M primers, and SYBR Green I mix; (6) the amplification protocol: one incubation at 95 °C for 10 min followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; (7) melting curve analysis and subsequent sequence analysis were performed for the PCR products from each primer pair; (8) the PCR products produced both with and without RT with NGF, TrkA, BDNF, TrkB, Nrf2, IGF-1, and GAPDH primers were electrophoresed on 1.5% agarose gels and stained with ethidium bromide 9. The amounts of NGF, TrkA, BDNF, TrkB, Nrf2, and IGF-1 mRNA were normalized against mRNA levels of GAPDH in the corresponding samples (Wang et al. 2014).

Statistical analyses

The data are presented as the mean \pm SEM, and all calculations were made using GraphPad Prism (version 6.07). The Student's unpaired *t*-test was used for comparison of body weight, food intake, serum calcium, and phosphorus between rats with normal VitD and rats with supplementary VitD. We used a one-way ANOVA to evaluate EPM, OFT,

PA, swimming speed, swimming in the correct quadrant, and qRT-PCR data. For comparison of traveled distance and time in 3 blocks of the MWM test, WMEs in the RAM task, and to evaluate differences in fEPSP slope in I/O curves, we used a two-way ANOVA. For comparison of the time and interaction (time and group) effects on fEPSP changes after delivery of HFS, a two-way repeated-measure ANOVA was used. Tukey's correction post-hoc test was used for confirmation of differences occurring between groups. For all data, differences were considered significant at the level of $p < 0.05$.

Results

A timeline of behavioral experiments across days and time/rest between different tests as well as the time of electrophysiology and tissue isolation in EE + VitD group has been shown in Fig S1.

Physiological factors and blood analysis

For evaluation of possible effects of vitamin D supplementation on animal physiological factors (Fig. 1), we measured daily food intake and body weight and for comparisons, divided the animals into two groups: 3 non-vitamin D groups ($n = 23$) and EE + VitD ($n = 9$). Our results showed that in spite of a tenfold increase in daily intake of VitD in the supplemented group, there were no significant differences in food intake [$F(3, 28) = 0.02, p = 0.9$] or body weight between the groups [$F(3, 168) = 1.060, p = 0.3678$]. At the beginning of the 6 weeks of housing, old male SD rats (22 months) weighed ~ 500 g, and by the end of the 6 weeks of housing and before behavioral testing, animals in all groups had gained ~ 30 – 60 g, and there were no significant changes in daily food intake and body weight throughout the experiment between the groups (Fig. 1a–c).

To verify that the provision of a tenfold increase in daily vitamin D resulted in heightening VitD status, blood levels of 25OHD as well as calcium and phosphorus were

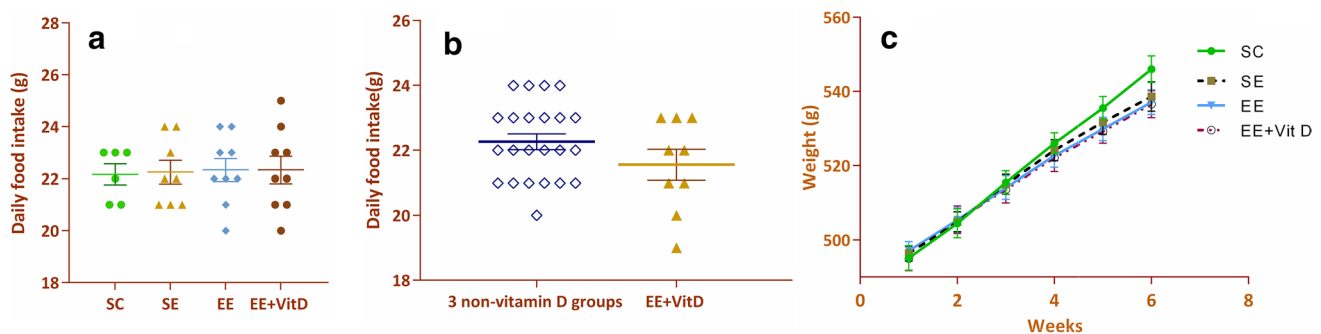


Fig. 1 Effects of SE, EE, and EE + VitD on food intake and body weight in aged rats (a–c). The values are shown as mean \pm SEM (SC $n = 6$, SE $n = 8$, EE $n = 9$, EE + VitD $n = 9$). One-way ANOVA with Tukey's correction post-hoc test

measured in all animals (Fig. 2). We found that the serum levels of calcium (10.6 ± 0.12 vs 11.12 ± 0.37 mg/dL) and phosphorus (5.9 ± 0.19 vs 5.7 ± 0.25 mg/dL) were unchanged with the VitD supplementation (Fig. 2a, b). However, we found an approximately twofold increase in 25OHD levels (31.45 ± 1.67 vs 16.02 ± 0.37 ng/mL) in the group receiving VitD supplementation when compared to levels seen in the normal VitD group (Fig. 2c).

In addition, the one-way ANOVA analysis showed that there were no significant differences in the serum levels of calcium and phosphorus between the four groups [$F(3, 28) = 0.76, p = 0.52$] and [$F(3, 28) = 0.46, p = 0.70$] respectively (Fig. 2d, e), and we found a significantly higher level of 25OHD in the EE + VitD group relative to SC, SE, and EE groups [Fig. 2f; $F(3, 28) = 55.55, p < 0.0001$].

Behavioral tests

Aged rats exposed to 6 weeks of EE with VitD supplementation displayed improved learning and memory as well as reductions in anxiety-like behavior when compared to performance seen in the EE group when behavior was evaluated in the MWM, PAT, EPM, and OFT tasks.

Aged rats housed under SE conditions spent a greater amount of time in the open arms in the EPM when compared to the time spent by the SC group (SE: 94.6 ± 4.7 s

or $31.5 \pm 1.5\%$; SC: 52.3 ± 10.1 s or $17.4 \pm 3.3\%$; $p < 0.05$; Fig. 3a, b). Aged rats housed under EE conditions also spent a significantly higher amount of time in the open arms when compared to the SC group (EE: 88.6 ± 9.2 s or $29.5 \pm 3\%$; $p < 0.05$). The EE group receiving VitD supplementation exhibited a significantly higher time spent in the open arms (130.6 ± 9.9 s or $43.5 \pm 3.3\%$) when compared to that seen in the EE group (88.6 ± 9.2 s; $p < 0.01$) and the SE group [94.6 ± 4.7 s; $p < 0.05, F(3, 28) = 11.97, p < 0.0001$]. Our results suggest that SE and EE housing do decrease anxiety in old animals, and co-treatment with VitD supplementation can enhance the reduction of anxiety-like behavior. There were no significant differences between the groups in the number of entries into the open and close arms [$F(3, 28) = 0.02, p = 0.99$] and [$F(3, 28) = 0.45, p = 0.71$] respectively (Fig. 3c, d).

Anxiety-like behavior was also monitored with the OFT. As shown in Fig. 4a, b, the SE (30.8 ± 2.4 s or $10.2 \pm 0.8\%$) and EE (34.1 ± 2.2 s or $11.3 \pm 0.7\%$) groups exhibited a significantly ($p < 0.05, p < 0.01$) higher amount of time in the center when compared to that shown by SC aged rats (17.3 ± 3.2 s or $5.7 \pm 1\%$). The group receiving co-treatment of VitD and EE displayed a significantly greater amount of time exploring the center of the maze (45.3 ± 3.8 s or $15.1 \pm 1.2\%$) when compared to that shown by the EE ($p < 0.05$) and SE (30.8 ± 2.4 s; $p < 0.01$) groups [$F(3, 28) = 12.90, p < 0.0001$].

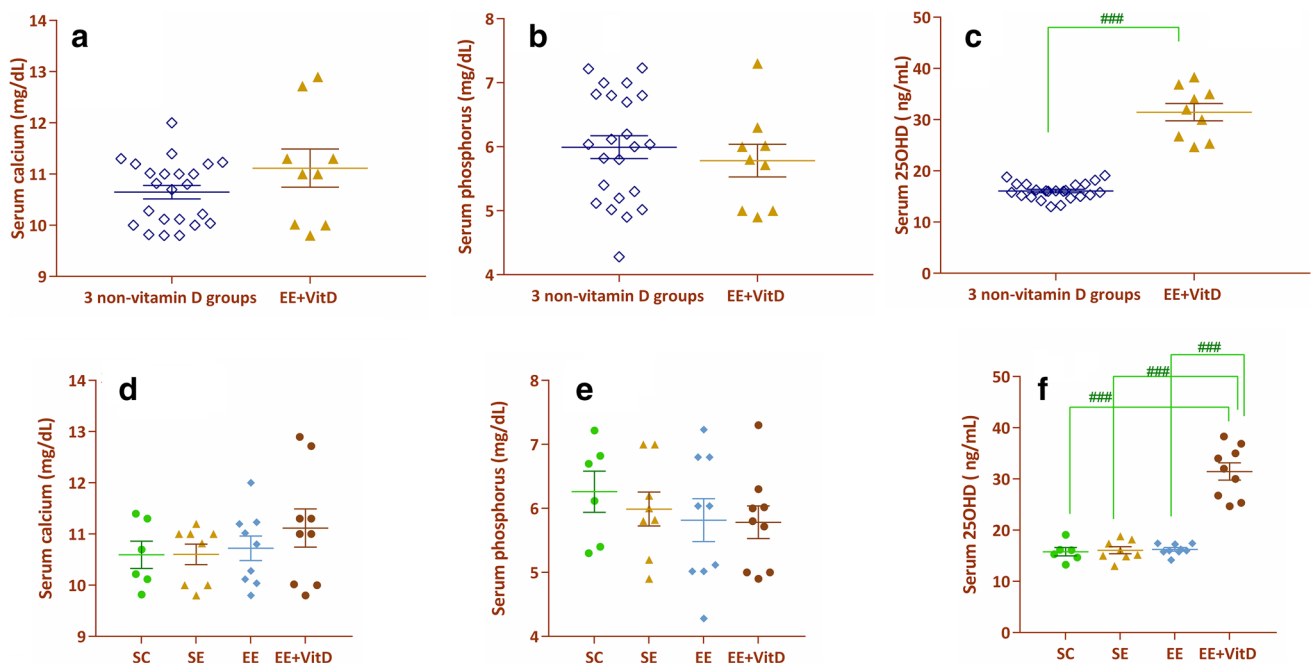
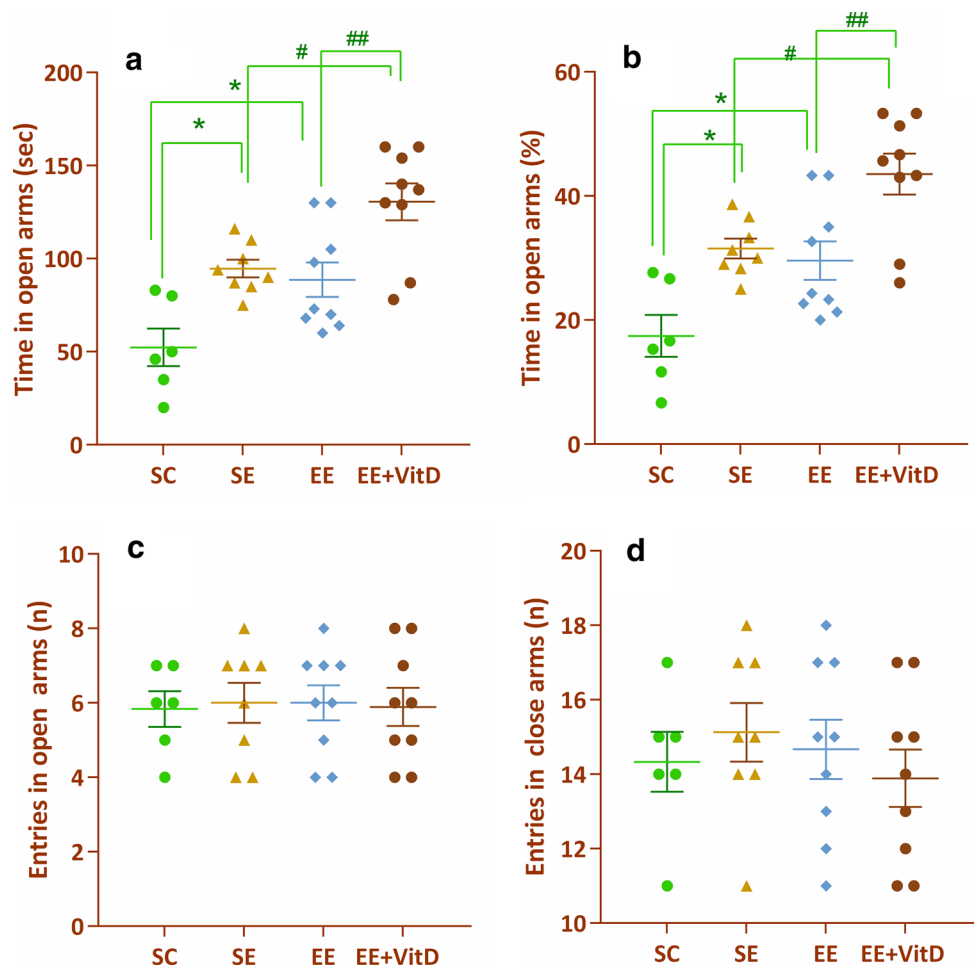


Fig. 2 Effects of vitamin D supplementation on the serum levels of calcium, phosphorus, and 25OHD in aged rats. Unpaired *t*-test between 3 non-vitamin D groups and EE + VitD (a–c). One-way ANOVA analysis with Tukey’s correction post-hoc test between SC,

SE, EE, and EE + VitD (d–f) (### $p < 0.001$ compared to EE + VitD). The values are shown as mean \pm SEM (SC $n = 6$, SE $n = 8$, EE $n = 9$, EE + VitD $n = 9$)

Fig. 3 Effects of SE, EE, and EE + VitD on anxiety-like behavior in aged rats as assayed by the elevated plus maze. The time spent in the open arms was greater in SE and EE when compared to SC (* $p < 0.05$). The time spent in the open arms was also significantly higher in the EE + VitD group when compared to EE alone (## $p < 0.01$) and SE (# $p < 0.05$) (a and b). The number of entries into the open and close arms are shown (c and d). The values are shown as mean \pm SEM (SC $n = 6$, SE $n = 8$, EE $n = 9$, EE + VitD $n = 9$). One-way ANOVA with Tukey’s correction post-hoc test were used



To assess the progression of spatial learning, traveled distance and time to find the platform in the MWM during the training phase were analyzed for each rat across 12 trials

conducted in 3 blocks. Progression in spatial learning was determined by decreases in traveled distance and latency to reach the platform. When taken together, the data indicated

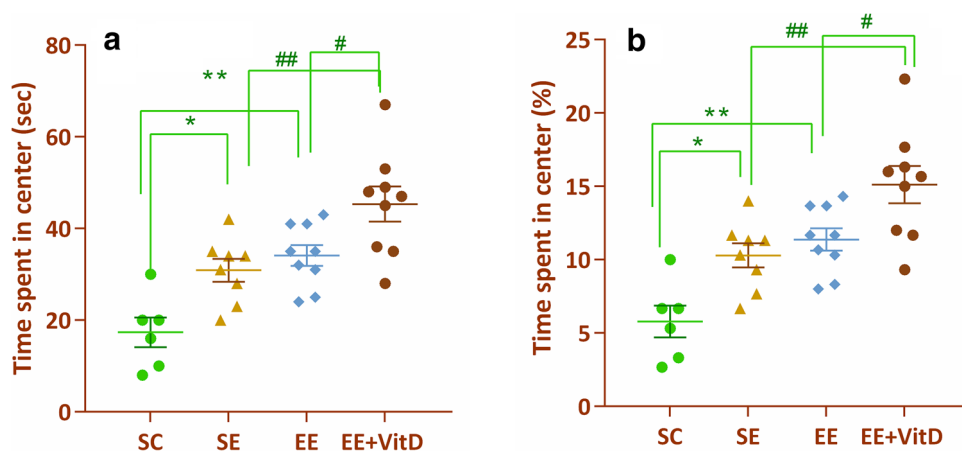


Fig. 4 Effects of SE, EE, and EE + VitD on anxiety behavior in the open field test. One-way ANOVA analysis with Tukey’s correction post-hoc test showed the time spent in the central zone was significantly greater in the SE and EE groups when compared to SC (* $p < 0.05$, ** $p < 0.01$). VitD supplementation in combination with

EE resulted in significantly higher time spent in the central zone when compared to EE and SE (# $p < 0.05$, ## $p < 0.01$) (a and b). The values are shown as mean \pm SEM (SC $n = 6$, SE $n = 8$, EE $n = 9$, EE + VitD $n = 9$)

that SE and EE were significantly different in B1 and B2 trials in aged rats when compared to similar parameters seen in the SC group, and there were no significant differences between SE and EE in these measures in any of the 3 trial blocks (Fig. 5a, b).

VitD supplementation resulted in a significantly reduced traveled distance and time to find the platform in 3 blocks when compared to similar measures in the SC group. When compared to findings in the EE group, the VitD+EE group only showed a difference in a decrease in traveled distance and latency in block 2 [Distance: 375 ± 58.9 vs 770 ± 70.3 cm; $F(3, 12) = 22.86$, $p < 0.0001$, and Latency: 24 ± 2.9 vs 35.7 ± 2.1 s; $F(3, 12) = 16.42$, $p = 0.0002$].

Following the removal of the platform, spatial memory can be evaluated as the percentage of distance and time spent in the correct quadrant. The greater the percentage of time spent and the path length indicates memory enhancement. The percentage of distance ($43.6 \pm 2.4\%$ vs $30.1 \pm 1.8\%$) and the time spent in the correct quadrant ($31.3 \pm 1.7\%$ vs $20.5 \pm 2.9\%$; $p < 0.05$) were significantly greater in the aged rat under EE housing conditions when compared to findings in aged rats under SC housing (Fig. 5c, d). Rats treated with VitD supplementation exhibited a significantly greater percentage of traveled distance ($58.7 \pm 3.1\%$ vs $43.6 \pm 2.4\%$; $p < 0.01$) and time in the target quadrant when compared to similar parameters in the EE group (42.5 ± 2.6 vs $31.3 \pm 1.7\%$; $p < 0.05$) (Fig. 5c, d). The speed

of swimming was also monitored as speed of swimming would be expected to decline with age as has been reported in other studies (Briones and Darwish 2012). However, there were no differences seen when this parameter was compared between SC, SE, and EE groups. Interestingly, VitD supplementation was found to be associated with a significant increase in the speed of swimming (30.3 ± 2.3 cm/s) relative to rats in EE (20.6 ± 2.2 cm/s), SE ($p < 0.05$), and SC groups ($p < 0.01$; Fig. 5e). Because the time spent to find the platform is affected by the speed of swimming, our findings of traveled distance in the correct quadrant, and total distance traveled to reach the visible platform in the 3 acquisition trials, suggest that these two measures might be superior in detecting a difference in spatial learning and memory improvement than the time.

The RAM test was used across 3 consecutive days to assess spatial working memory. Two-way ANOVA analysis showed that SE housing did not result in significant differences in working memory in aged animals when compared to SC conditions (Fig. 6). Additionally, EE housing and EE+VitD treatment did not result in significant changes in WMEs on the first day of assessment. However, in the 2nd day of testing, we found a significant decrease in WMEs in EE (3.9 ± 0.7 ; $p < 0.01$) and EE+VitD (3.3 ± 0.8) groups compared to SC (7.2 ± 0.8 ; $p < 0.001$). On the 3rd testing day, WMEs were also significantly lower in EE ($p < 0.05$) and EE+VitD ($p < 0.01$) groups when compared to errors

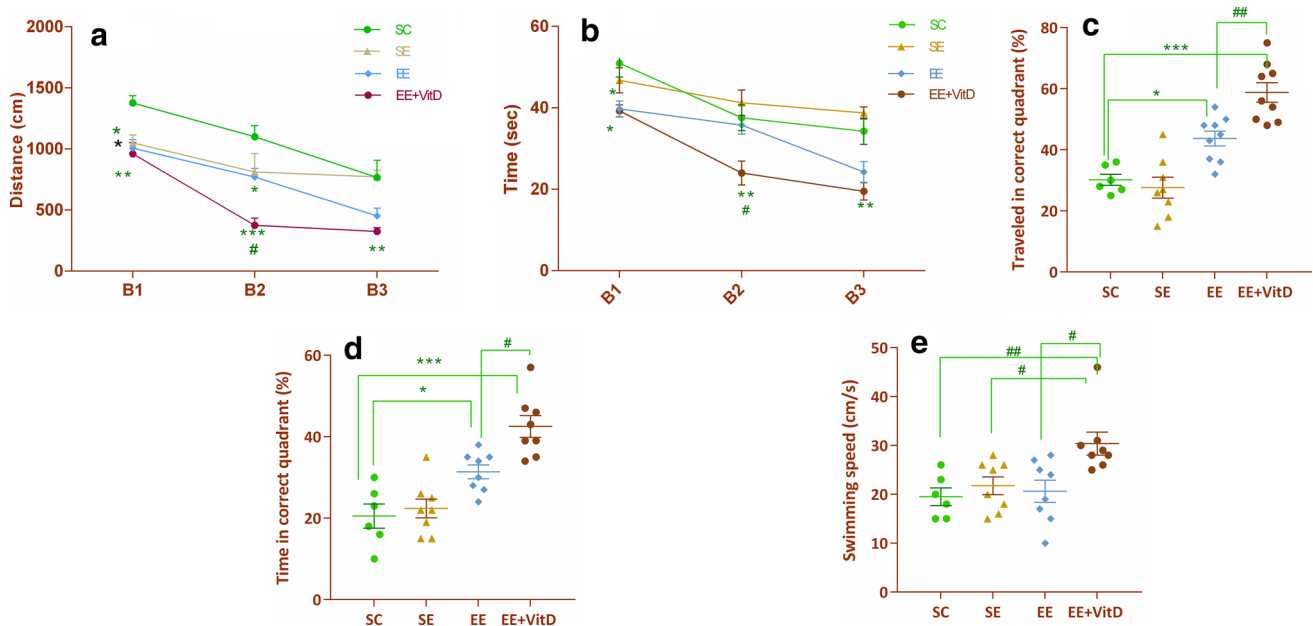


Fig. 5 Effects of SE, EE, and EE+VitD on spatial learning and memory in the Morris water maze. After VitD supplementation, traveled distance and latency time significantly decreased in block 2 when compared to EE alone (two-way ANOVA) (a and b). VitD supplementation was associated with an increase in the percentage of distance and

latency time in the correct quadrant (c and d) (one-way ANOVA with Tukey's correction post-hoc test). VitD supplementation increased swimming speed in aged rats (e) ($*p < 0.05$, $***p < 0.001$ compared to SC; $\#p < 0.05$, $##p < 0.01$ compared to EE+VitD). The values are shown as mean \pm SEM (SC $n = 6$, SE $n = 8$, EE $n = 9$, EE+VitD $n = 9$)

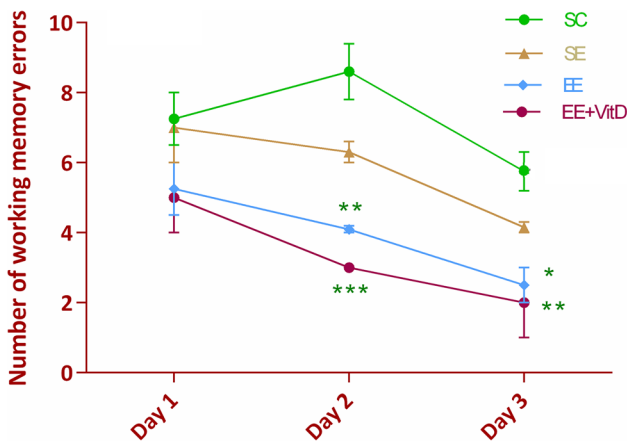
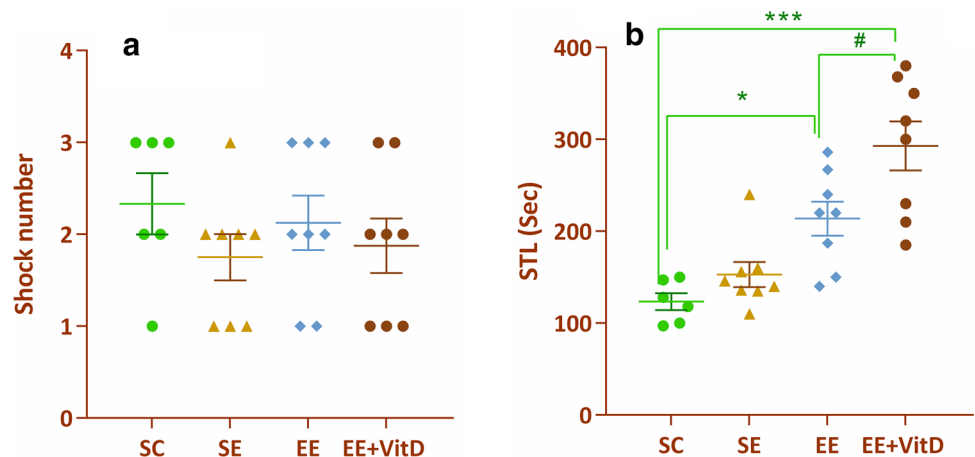


Fig. 6 Effects of SE, EE, and EE+VitD on spatial working memory in the radial arm maze compared with SC. The values are shown as mean±SEM. The working memory errors (WMEs) over 3 consecutive days are shown. There was a significantly lower amount of WMEs in EE and EE+VitD on days 2 and 3, when compared to WMEs made in the SC group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Two-way RM ANOVA (SC $n = 6$, SE $n = 8$, EE $n = 9$, EE+VitD $n = 9$) were used

seen in SC [$F(3, 4) = 16.94, p = 0.0097$]. Learning of the PAT appeared to be similar in all groups, and there were no significant differences between the groups in the shock number applied (1–3 foot shocks) (Fig. 7a).

The STL time was significantly greater in EE (213.8 ± 18.4 s, $p < 0.05$) and in EE + VitD group (292.9 ± 26.6 s, $p < 0.001$) when compared to this value in the SC group (123.3 ± 9.2 ; Fig. 7b), and rats in the SE group did not exhibit a significantly different latency to entry into the dark room than rats under the SC housing protocol. Further, treatment with the combination of VitD + EE housing resulted in a significantly greater STL time (292.9 ± 26.6 s) than that seen in the EE group (213.8 ± 18.4 s, $p < 0.05$; Fig. 7b), suggesting an improvement in fear-memory performance beyond that seen with EE alone.

Fig. 7 Effects of SE, EE, and EE + VitD on passive avoidance memory. The difference in numbers of shocks applied is shown (a). Treatment with VitD and EE housing resulted in a significantly higher STL time when compared to STL values seen in the EE group (b). The values are shown as mean±SEM. Significant differences with respect to the SC (* $p < 0.05$, *** $p < 0.001$) and EE + VitD (# $p < 0.05$) are shown

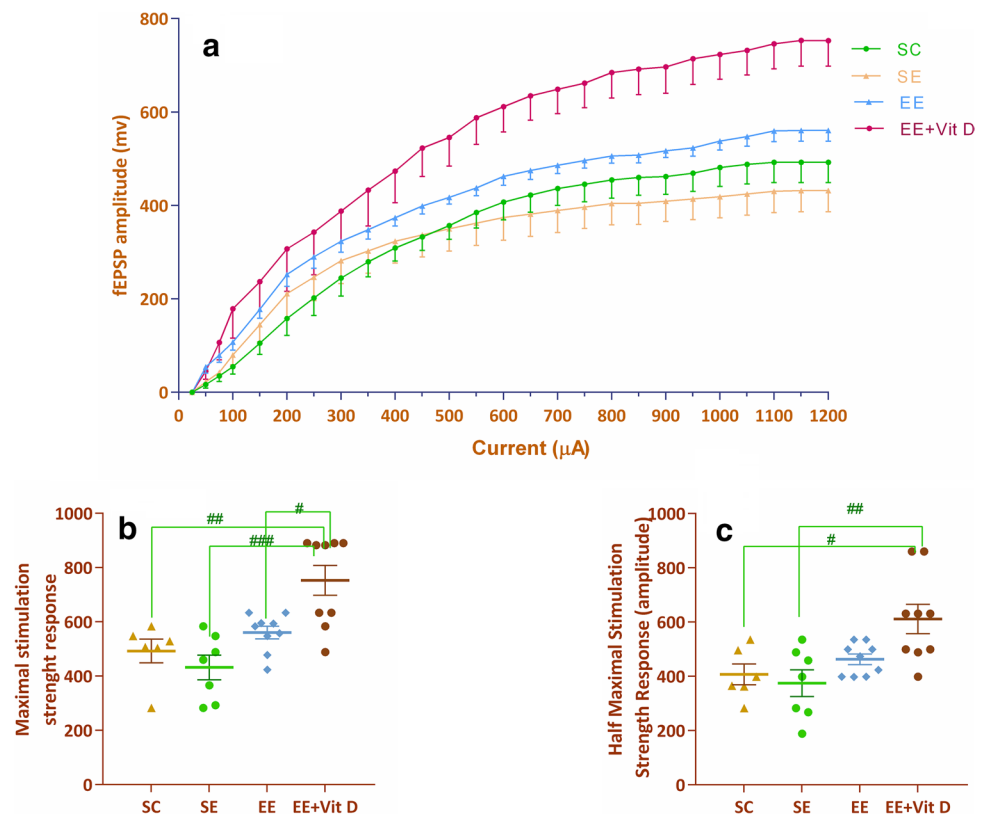


Field potential recording

For evaluation of neuronal excitability in the CA1 region of the hippocampus, an input/output (I/O) curve was generated by monitoring fEPSP responses to progressive increases in the current stimulation intensity (25–1200 μ A). The slope of the I/O curve in the EE + VitD group exhibited a significant left shift relative to that seen in the other groups (Fig. 8a). Therefore, more detailed comparisons of the maximum fEPSP slope from the I/O curve of each group were conducted which showed a significant increase in the maximum responses in the EE + VitD group (752.9 ± 54.9) when compared to findings in the SC (492 ± 43.5 ; $p < 0.01$), SE (431 ± 45.2 ; $p < 0.001$), and EE (560 ± 23.3 ; $p < 0.05$) groups [$F(3, 27) = 10.62, p < 0.0001$; Fig. 8b]. Further analysis showed that the half-maximal stimulation strength responses were significantly greater in the EE + VitD group (611.4 ± 54.1) with respect to those in the SC (407 ± 37 ; $p < 0.05$) and SE (374.4 ± 49 ; $p < 0.01$) groups [$F(3, 27) = 6.281, p = 0.0023$; Fig. 8c]. While previous studies have shown that VitD can recover excitability in aged rats (Latimer et al. 2014b), our data raise the intriguing possibility that excitability can be further heightened by adding EE to VitD treatment.

As the co-treatment of VitD and EE could improve learning and memory to a greater degree than EE alone, we focused on synaptic plasticity differences between EE and EE + VitD. Long-term potentiation in the CA1 region of the hippocampus is believed to mechanistically underlie cognitive processes, and therefore, we determined whether LTP varied across different housing conditions. The obtained results showed that aged rats under EE housing displayed increased LTP ($157.5 \pm 2.3\%$), when compared to that seen under SE ($125.1 \pm 5.6\%$; $p < 0.01$) and SC ($112.5 \pm 3\%$; $p < 0.001$) housing. Furthermore, the addition of VitD resulted in a heightened increase which was significant ($181.3 \pm 7.9\%$), relative to EE alone [$p < 0.01$; $F(3, 27) = 29.71, p < 0.0001$; Fig. 9a–c].

Fig. 8 Co-treatment of VitD and EE improved neuronal excitability of CA1 neurons in aged rats. The slope of the I/O curve in the EE + VitD group displays a significant left-ward shift relative to the other groups (two-way ANOVA) (a). One-way ANOVA showed significant differences in the maximum and half-maximal stimulation strength responses in the EE + VitD group ($\#p < 0.05$, $\#\#\#p < 0.001$) (b and c). The values are shown as mean \pm SEM (SC $n = 6$, SE $n = 7$, EE $n = 9$, EE + VitD $n = 9$)



Quantitative real-time RT-PCR

We found that different housing conditions were associated with significant differences in mRNA expression levels of NGF [$F(3, 28) = 23.82$, $p < 0.0001$], TrkA [$F(3, 28) = 27.82$; $p < 0.0001$], BDNF [$F(3, 28) = 24.28$, $p < 0.0001$], TrkB [$F(3, 28) = 16.80$, $p < 0.0001$], Nrf2 [$F(3, 28) = 16.63$, $p < 0.0001$], and IGF-1 [$F(3, 28) = 12.44$, $p < 0.0001$] in aged rats. Rats housed under the SC protocol exhibited significantly lower levels of mRNA for these 6 proteins than levels seen in either the EE group or the EE + VitD groups (Fig. 10a–f). SE housing conditions were associated with higher levels of mRNA for BDNF, Nrf2, and IGF-1 when compared to levels seen in the SC group ($p < 0.05$). Additionally, the combination of VitD and EE housing resulted in significantly enhanced expression mRNA levels of NGF, TrkA, BDNF ($p < 0.001$), Nrf2 ($p < 0.01$), and IGF-1 ($p < 0.05$) from levels seen in the EE group.

Discussion

The present study investigated whether the addition of VitD to enrichment of the environment can improve hippocampal-based cognition and CA1 synaptic plasticity in aged rats more than either EE or SE alone. We found that 6 weeks of SE significantly decreased signs of anxiety-like behavior

as assayed in the EPM and OFT tasks, whereas it did not improve learning and memory abilities monitored by the MWM and RAM. Exposure to EE was associated with a significant increase in learning and memory performance as confirmed by the MWM, RAM, and PAT behavioral tests. Our findings are in line with those from other studies which have shown a determinative role of EE in preventing several aspects of cognitive impairment and in enhancing spatial memory performance in behavior tasks including the MWM test, an object/odor recognition task, and RAM (Cortese et al. 2018; Frick et al. 2003). However, for the first time, we have shown that EE animals who received the addition of VitD supplement exhibited enhanced performance in learning and memory tasks as assayed in the MWM and PAT tests. The increased swim speed due to vitamin D supplementation suggests wider physiological changes; thus, future studies on this parameter are recommended. Further, co-treatment of VitD and EE was associated with reduced anxiety-like behaviors when compared to EE alone in aged rats based on the increase in the time spent in the central part of the OFT or open arms of the EPM which has been suggested to reflect a decrease in anxiety (Prut and Belzung 2003; Wang et al. 2019). As anxiety-related behaviors are linked to hippocampal neurogenesis (Marques et al. 2016; Revest et al. 2009), neurogenesis could play a role in our findings.

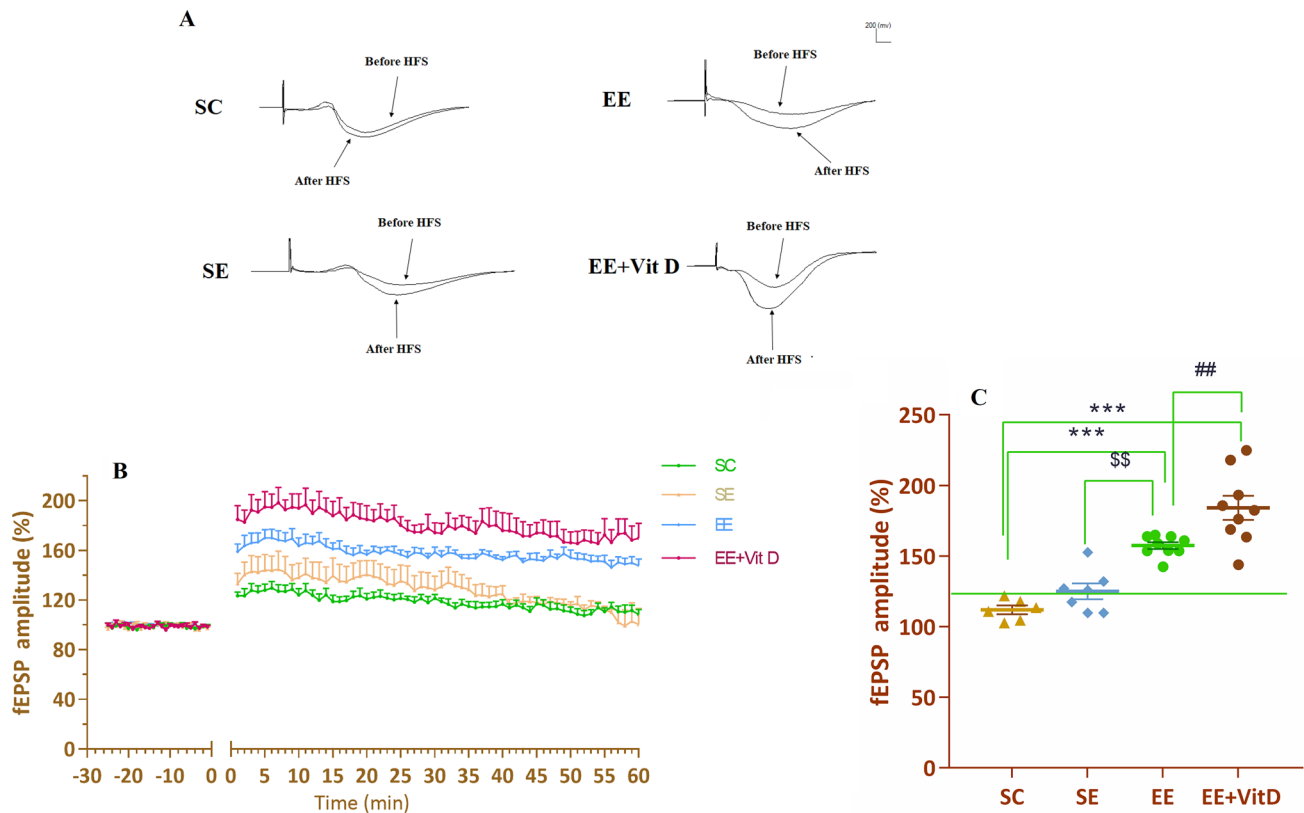


Fig. 9 EE + VitD enhanced LTP in aged rats. The sample traces are presented before and after high-frequency stimulation (HFS; 200 Hz) which occurred at time 0 (a). The normalized fEPSP amplitude to baseline before HFS, and 60 min after HFS. (b). The means of per-

cent changes of fEPSP 60-min post-HFS are presented (c). Significant differences with respect to the SC (** $p < 0.001$), EE + VitD (## $p < 0.05$), and (\$ $p < 0.01$). Data are presented as mean \pm SEM (SC $n = 6$, SE $n = 7$, EE $n = 9$, EE + VitD $n = 9$)

Neurogenesis can be affected by several extrinsic parameters including diet, drugs, physical activity, and environmental conditions (Bayat et al. 2015; Shohayeb et al. 2018; Triviño-Paredes et al. 2016). An environment enriched by physical and social interactions has been shown to enhance age-related neurogenesis and ameliorate reductions in synaptic plasticity, which likely contribute to cognitive improvements as well as diminished anxiety-like behaviors (Bayat et al. 2015; Clemenson et al. 2017; Hilal et al. 2017). We found that improvements in cognitive behavioral tests and synaptic mechanisms were elicited from EE alone, but that addition of VitD resulted in heightening this improvement, suggesting that the mechanism underlying the cognitive-based benefits of EE are distinct from those mediated by VitD or that potentiation is possible and that the effects of the two can be synergistic rather than occluding one another. The mechanisms underlying the EE enhancement in cognition and synaptic activity and those underlying VitD are unknown. However, our molecular findings suggest a potential role of BDNF, IGF-I, NGF, and their related receptors (TrkA and TrkB) as their mRNA expression was greatest when EE was supplemented with VitD.

The peptides NGF, IGF, and BDNF positively affect neurogenesis by modulating neuronal stem cell proliferation, differentiation, and survival (Acheson et al. 1995; Bekinschtein et al. 2011; Scharfman et al. 2005). NGF and BDNF bind with high affinity to two types of tropomyosin-related kinase (Trk) receptors TrkA and TrkB (Bekinschtein et al. 2011). There is a positive correlation between hippocampal NGF expression throughout life and hippocampal plasticity and hippocampal-dependent memory. In addition, promotion of cholinergic septal neurons which provide input to the hippocampus (Conner et al. 2009) has been shown to be mediated by activation of TrkA receptor (Latina et al. 2017; Sanchez-Ortiz et al. 2012). Activation of TrkB receptor induces neuronal synaptic activity through induction of the transcription factor, cAMP response element binding (CREB) which is a key player in the signaling process underlying normal learning and memory (Hu et al. 2013; Sakamoto et al. 2011). The critical role of BDNF for memory-related processes underlying LTP, synaptic plasticity, and synaptic consolidation has been well-established (Bramham and Messaoudi 2005) as well as the importance of this factor in the regulation

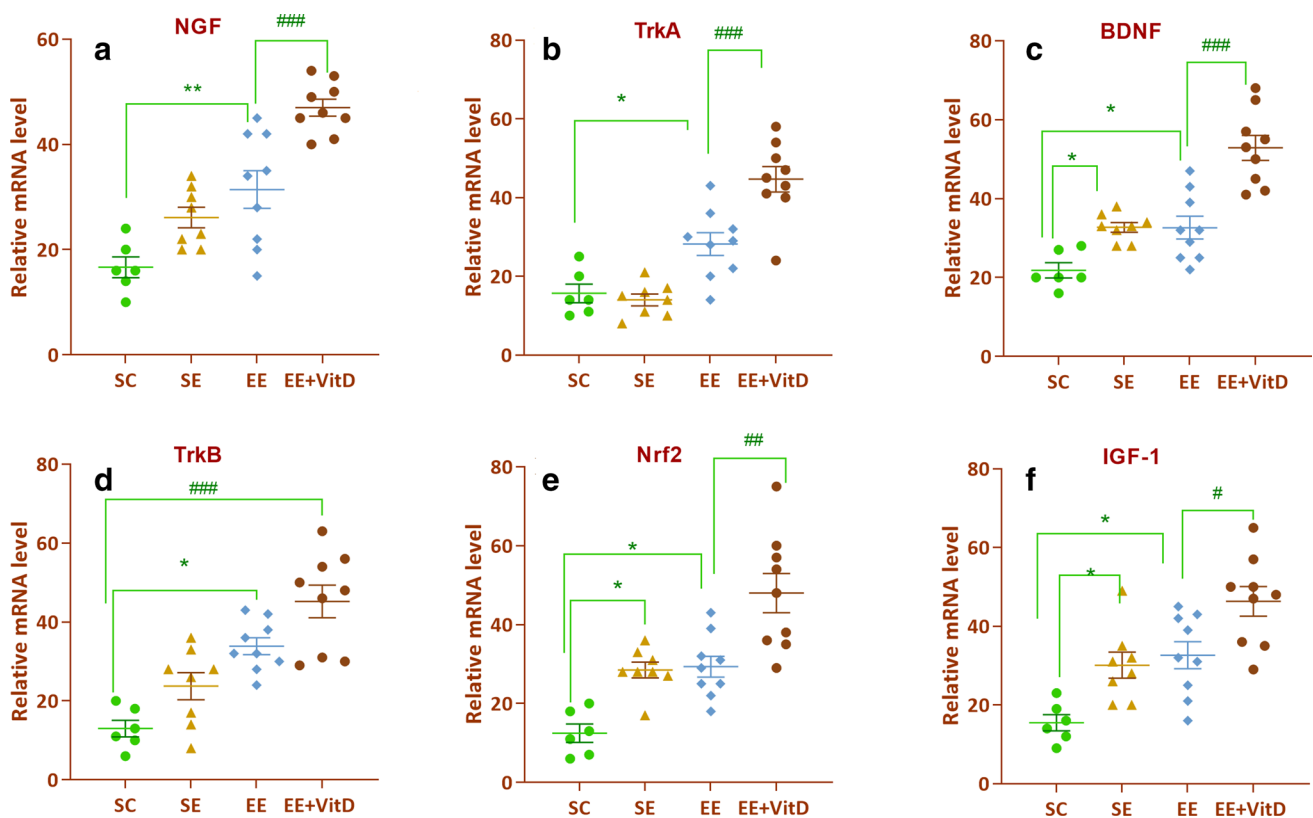


Fig. 10 mRNA expression analysis by quantitative real-time RT-PCR. mRNA expression levels of NGF, TrkA, BDNF, TrkB, Nrf2, and IGF-1 in the SC group were significantly lower than those in the EE and EE+VitD group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

compared with SC). Significant elevation in expression levels of (NGF, TrkA, BDNF (### $p < 0.001$)), Nrf2 (## $p < 0.01$), and IGF-1 (# $p < 0.05$) were noted in the EE+VitD when compared to findings seen in the EE group (a–f)

of trafficking (Caldeira et al. 2007), phosphorylation (Lin et al. 1998), and expression levels of NMDARs which play a vital role in synapse strengthening (Suen et al. 1997). BDNF can also increase the number, size, and complexity of dendritic spines, likely through upregulation of actin polymerization (Alonso et al. 2004; Horch and Katz 2002; Rex et al. 2007). BDNF is considered a key regulator of environmental-induced neurogenesis since enriched BDNF heterozygous knockout mice did not show improvement in hippocampal neurogenesis (Rossi et al. 2006). Further, EE has been shown to increase the level (Kuzumaki et al. 2011) and protein content (Lazarov et al. 2005) of hippocampal BDNF. In addition, BDNF, Nrf2, and IGF-1 expression modify anxiety-like behavior (Baldini et al. 2013; Hill and Martinowich 2016; Mitschelen et al. 2011; Zhou et al. 2019), which is supported by our findings that a significant increase in BDNF, NGF, and IGF-1, and Nrf2 was associated with a significant reduction in anxiety in SE, EE, and EE + VitD groups when compared to animals in SC. The findings of the study also revealed that compared to EE alone, administration of VitD with EE was associated with a significant increase in the expression

of all aforementioned target genes with the exception of TrkB. Relevant to this point, the beneficial effects of vitamin D supplementation on primary hippocampal and cortical neuron culture were shown to be associated with a significant increase in NGF release (Gezen-Ak et al. 2011, 2014a). Furthermore, the antiaging, anti-oxidation activity of VitD has been suggested to be mediated via upregulation of Nrf2 gene (Chen et al. 2019). Finally, there is a positive correlation between VitD supplementation and the expression of proteins and neurotrophic factors involved in synaptic plasticity and physiological neuronal health (Eyles et al. 2007).

In order to clarify the effects of SC, SE, EE, and/or EE + VitD on hippocampal functions underlying learning and memory, we also examined LTP and CA1 neuronal excitability as previous work had shown that synaptic plasticity and neuronal excitability decrease with aging (Burke and Barnes 2010). EE + VitD was associated with a greater degree of LTP than that seen with EE alone. In our work, neuronal excitability in the CA1 region was monitored by generation of input/output curves using field potential recordings. Significant differences between SC, SE, and EE

in neuronal excitability were not detected. However, steeper I/O slopes (leftward shift) in the EE + VitD group were seen which indicated an increase of neuronal excitability. Our data are supported by findings that VitD supplementation causes a significantly steeper I/O slope in hippocampal neurons in slices that was indicative of higher neuronal excitability (Latimer et al. 2014b). Although we did not examine neurogenesis, it has been established that newborn neurons exhibit high excitability which has been shown to underlie heightened hippocampal activity (Goncalves et al. 2016). Accordingly, we speculate that the increase of neuronal excitability in EE + VitD animals could involve an increase in hippocampal neurogenesis following VitD supplementation.

VitD regulates several crucial physiologic processes in the CNS including cell differentiation (Carlberg 2016; Gil et al. 2018), neurotransmitter biosynthesis, and neurotrophic release (Gezen-Ak et al. 2014b; Máčová et al. 2017). VitD supplementation decelerates age-related reductions of neuronal density (Landfield and Cadwallader–Neal 1998). Although there is very little information about the regulatory role of VitD on calcium in the CNS, in the periphery, a calcium regulatory role of VitD has been identified (Fleet 2017). As aging is associated with a significant upregulation of voltage-operated calcium channels (Landfield et al. 1992; Moyer et al. 1992), and impairment of calcium homeostasis is now considered as one of the pathological mechanisms involved in several neurodegenerative diseases (Wang and Sun 2010), since chronic treatment with VitD has been shown to downregulate hippocampal L-type voltage-sensitive calcium channels (VSCC) (Brewer et al. 2001), VitD-mediated reductions in calcium could play a role in slowing degeneration. Further, the antioxidant properties of VitD have been well-established as there are many reports which confirm its cell-protecting activity against oxidative damage (Sepidarkish et al. 2019).

When VitD was combined with EE, we saw significant improvements in several anxiety and cognitive-based tests. Cognitive performance is affected by the level of VitD as deficiency of this hormone, especially in the elderly population, is associated with impaired learning, memory, and cognitive ability (Al-Amin et al. 2019; Llewellyn et al. 2010). VitD supplementation has been shown in aged rats to prevent cognitive impairment and improve hippocampal plasticity via upregulating either the expression of some key genes involved in neuroplasticity or upregulating the major neurotransmitter receptors such as dopamine, serotonin, and glutamate (Latimer et al. 2014a). Whatever the mechanism underlying the effects of VitD, the exciting conclusion from our study is that the effects of VitD on improving memory and learning performance, as well as up-regulating the expression of neurotrophic factors and related receptors and enhancing synaptic activity, are synergistic with those

of EE. Although there are studies regarding the enhancer role of VitD or EE on neuronal plasticity and neurogenesis, to the best of our knowledge, this is the first report of the simultaneous effects of such a combination on neurogenesis and hippocampal plasticity. Our study has several limitations, including the daily oral gavage of vitamin D which can be a stress-inducing technique. In addition, the use of only male rats is a limitation, because the effect observed in male rats may not be the same in female rats, and, therefore, the possibility of a sex-based difference needs to be explicitly studied. Finally, inclusion of a vitamin D-only control group or vitamin D + SC/SE groups within this study potentially would have allowed a better understanding of the interactions of VitD supplementation with differential housing.

Conclusion

In summary, the results presented in this study demonstrate that co-treatment of VitD supplementation and enriched environment in aged rats improves cognitive dysfunction to a greater extent than social or environmental enrichment. Our finding suggests that this neurohormone exerts its beneficial function by increasing synaptic plasticity, neuronal excitability, and gene expression of NGF, TrkA, BDNF, Nrf2, and IGF-1, which likely reduce the negative effects of aging on cognition.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00213-021-05853-4>.

Author contribution MB has conceived and designed the concept and road map of the study, searched the literature collected the data, and drafted the manuscript. KAK, MH, ABH, AK, GB, and EH helped design the study, searched the literature, reviewed the manuscript, and helped with the revision of the manuscript. MS has critically reviewed the manuscript, designed the study, and helped in manuscript preparation. He is the archival author and attests to the integrity of the original data and the analysis reported in this manuscript. All authors have made substantive contribution and attest to approving the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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