

Suppression of synaptic plasticity by fullereneol in rat hippocampus in vitro

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Abstract: Fullereneol, a water-soluble fullerene derivative, has attracted much attention due to its bioactive properties, including the antioxidative properties and free radical scavenging ability. Due to its superior nature, fullereneol represents a promising diagnostic, therapeutic, and protective agent. Therefore, elucidation of the possible side effects of fullereneol is important in determining its potential role. In the present study, we investigated the acute effects of 5 μ M fullereneol on synaptic plasticity in hippocampal brain slices of rats. Incubation with fullereneol for 20 minutes significantly decreased the peak of paired-pulse facilitation and long-term potentiation, indicating that fullereneol suppresses the short- and long-term synaptic plasticity of region I of hippocampus. We found that fullereneol depressed the activity and the expression of nitric oxide (NO) synthase in hippocampus. In view of the important role of NO in synaptic plasticity, the inhibition of fullereneol on NO synthase may contribute to the suppression of synaptic plasticity. These findings may facilitate the evaluation of the side effects of fullereneol.

Keywords: fullereneol, hippocampal slice, nitric oxide synthase, synaptic plasticity, oxidative stress

Introduction

Several water-soluble fullerene derivatives have been synthesized by attaching polar functional groups (eg, $-\text{OH}$, $-\text{COOH}$, $-\text{NH}_2$, and others) to the fullerene core for potential biomedical applications.¹⁻⁴ Polyhydroxylated fullerene, known as fullereneol ($\text{C}_{60}(\text{OH})_x$), has attracted considerable attention due to its antioxidative⁵⁻⁸ and antiproliferative activity.^{9,10} Hence, several studies reported that fullereneol and other fullerene derivatives may be used as drug carriers,^{11,12} neuroprotective agents,^{13,14} bioimaging contrast agents,¹⁵⁻¹⁹ and anticancer drugs.²⁰⁻²² Despite convincing evidence of the potential value of fullereneol in biomedicine,²³⁻²⁸ the data pertaining to its side effects are inadequate.

Hippocampal synapse represents/is a model system to study the mechanisms of learning and memory in the brain.²⁹ One of the classical hypotheses is that long-term potentiation (LTP), an activity-dependent strengthening of synapses, serves as a synaptic basis of learning and memory. $\text{Ca}^{2+}/\text{CaMKII}$ is a serine/threonine kinase with persistent enzymatic activity inducing LTP³⁰ and is therefore considered as a molecular switch. Evidence suggests that NO mediates hippocampal synaptic plasticity, including LTP and long-term depression and, consequently, learning and memory.³¹⁻³⁶ NO is synthesized from L-arginine by NOS in cells as a response to increased intracellular Ca^{2+} and is released upon synthesis. Our previous study has demonstrated that 5 μ M fullereneol could significantly protect hippocampal neurons against lead-induced neurotoxicity.³⁷ In the present study, we investigated the acute effects of 5 μ M fullereneol on the synaptic plasticity of CA1 area in hippocampal slices and its underlying mechanisms.

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Materials and methods

Fullerenol preparation and characterization

Fullerenol, $[C_{60}(OH)_x(ONa)_y, y \approx 6-8, x+y \approx 24]$, purchased from the Materials and Electrochemical Research Corporation (Tucson, AZ, USA), was dissolved in double-distilled water at a concentration of 5 mM (1,000 times the final concentration) and stored at 4°C. Fullerenol was ultrasonically treated for 30 minutes and dissolved in artificial cerebrospinal fluid (ACSF) (5 μ M) just before experiments. The mean diameter and zeta potential of fullerenol in water were monitored using a Zetasizer Nano ZS90 (Malvern Instruments Ltd, Malvern, Worcestershire, UK), and the morphology of fullerenol was characterized by transmission electron microscopy using JEOL-2010 TEM (Japan Electron Optics Laboratory Co. Ltd., Tokyo, Japan) at an accelerating voltage of 200 kV.

Experimental animals and treatment

Wistar rats were purchased from SLAC Laboratory Animal Limited Liability Company (Shanghai, People's Republic of China). All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No 80-23, revised in 1996) after approval from the Institutional Animal Care and Use Committee of University of Science and Technology of China. Animals were raised under controlled environmental conditions of an automatically controlled 12 hours light/dark cycles with lights on at 7.30 am, temperature of 21°C \pm 2°C, and humidity of 50% \pm 5%. Distilled water and sterilized food were available ad libitum. Females were separated after pregnancy. The pups of both sexes were used for experiments at the age of 14–21 days. All efforts were made to minimize the number of animals used and their suffering.

Slice preparation and recordings

Hippocampal slices were prepared as described previously.³⁸ Briefly, after the rats were decapitated, whole brains were removed immediately and submerged in oxygenated ACSF (0°C–4°C), containing (in mM) NaCl, 124; KCl, 5; NaH₂PO₄, 1.25; NaHCO₃, 26; MgCl₂, 1.25; CaCl₂, 2.5; dextrose (pH, 7.25–7.30; osmolarity, 295–310 mOsm/kg). Coronal sections measuring 300–400 μ m in size, including sections of the hippocampus, were obtained continuously using a vibration microtome (VT1200; Leica, Wetzlar, Germany) and each slice was separated into two parts, including the right and left hippocampi. Slices were stored in ACSF at room temperature for at least 1 hour and then transferred to a recording chamber (BSC-HT; Medical Systems, Greenvale,

NY, USA), which was circulated through the chamber with ACSF (35°C) at a rate of 2 mL/min. One part was used for incubation of fullerenol (20 minutes), and the other part was incubated with normal ACSF.

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum radiatum of the CA1 area with a glass micropipette (1–3 M Ω) filled with 2 M NaCl, in response to electrical stimulation of the Schaffer collateral (SC) fibers using a bipolar stimulating electrode. Input/output curves were generated by systematic variation of the stimulus current (0.1–1.0 mA) to evaluate synaptic potency. Stimulus pulses were delivered at 0.05 Hz, and three responses were averaged at each magnitude. Paired pulse facilitation (PPF) was evaluated by delivering pairs of identical stimuli with interpulse intervals (IPI) ranging from 10 to 500 ms. The intensity of stimuli was adjusted to evoke about 40% of the maximum response of the population spike amplitude. Stimulus pairs were delivered at 0.05 Hz, and three responses were averaged at each IPI. A stable recording of LTP was obtained for 40 minutes at 0.05 Hz, followed by application of high-frequency stimulation (100 Hz; duration, 1 second). In the experiment using acute fullerenol treatment, baseline recording was obtained for 20 minutes, and then the slices were exposed to 5 μ M fullerenol solution for 20 minutes. Post-tetanic recordings were performed for 1 hour with single pulses at 0.05 Hz. The responses were normalized to baseline values.

Determination of the level of NOS and oxidative stress

After incubation with normal ACSF or fullerenol, the hippocampal tissues were removed from slices and gently homogenized (10% weight/volume) in ice-cold saline. The homogenate was centrifuged at 4,000 rpm for 15 minutes at 4°C and the supernatant collected for use. The protein concentrations were determined using a BCA protein assay kit (Beyotime, Shanghai, People's Republic of China). The activity of NOS, SOD, and the level of reduced glutathione hormone (GSH) and malondialdehyde (MDA) were measured using detection kits (Jiancheng Corp., Nanjing, People's Republic of China). All procedures were carried out according to the manufacturer's instructions. The absorbance at 530 nm (for NOS), 550 nm (for SOD), 420 nm (for GSH), and 532 nm (for MDA) was measured using an automated microplate spectrophotometer (Elx800; BioTek, Winooski, VT, USA).

Western blot

After administration of fullerenol or normal ACSF for 20 minutes, the hippocampal tissues were removed from the slices and crushed with a pestle in an ice-cold lysis buffer

(Beyotime) with protein inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) for 45 minutes. The homogenates were centrifuged at 12,000 rpm for 25 minutes at 4°C. The protein concentrations were determined using a BCA protein assay kit (Beyotime). The protein was mixed with sodium dodecyl sulfate sample buffer, heated to 100°C for 15 minutes, separated under reducing conditions on an 8% sodium dodecyl sulfate–polyacrylamide gel, and then transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membrane was incubated with antibodies against NOS (Boster, Wuhan, People's Republic of China), CaMKII (Santa Cruz Biotechnology Inc., Dallas, TX, USA), p-CaMKII (Promega, Madison, WI, USA), GAPDH (EMD Millipore), and tubulin (Beyotime) overnight at 4°C with horseradish peroxidase-linked secondary antibody (Promega) for 1 hour at room temperature. The bands were detected using chemiluminescence (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The optical density of each band was determined using ImageJ software and normalized to that of GAPDH or tubulin.

Immunofluorescence

After 20 minutes incubation of normal ACSF or fullerenol, hippocampus slices were fixed with 4% paraformaldehyde in phosphate-buffered saline, blocked, and permeabilized

with 5% goat serum and 0.3% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA), all in phosphate-buffered saline. Hippocampal sections (40 μm) were sliced using a microtome (CM1950; Leica). For NOS immunofluorescence, the sections were incubated with mouse anti-NOS2 antibody (Boster) overnight at 4°C and with DAPI for 10 minutes at room temperature. The binding was visualized using the Alexa Fluor-conjugated secondary antibodies (Abcam, Cambridge, MA, USA). Sections were observed by confocal microscopy (Carl Zeiss Inc., Thornwood, NY, USA).

Data analysis

The slope of fEPSP represented the maximal slope obtained from the first deflection of the potential. Results of Western blot were statistically analyzed and compared by Student's *t*-test (paired, two-tailed). Other results were statistically analyzed using analysis of variance with Tukey's test. Data are presented as mean \pm standard error of mean. $P < 0.05$ was considered statistically significant.

Results

Characterization of fullerenol

The images obtained using transmission electron microscopy revealed that fullerenol (5 μM) was easily dispersed and aggregated in water (Figure 1A). The hydrodynamic size of

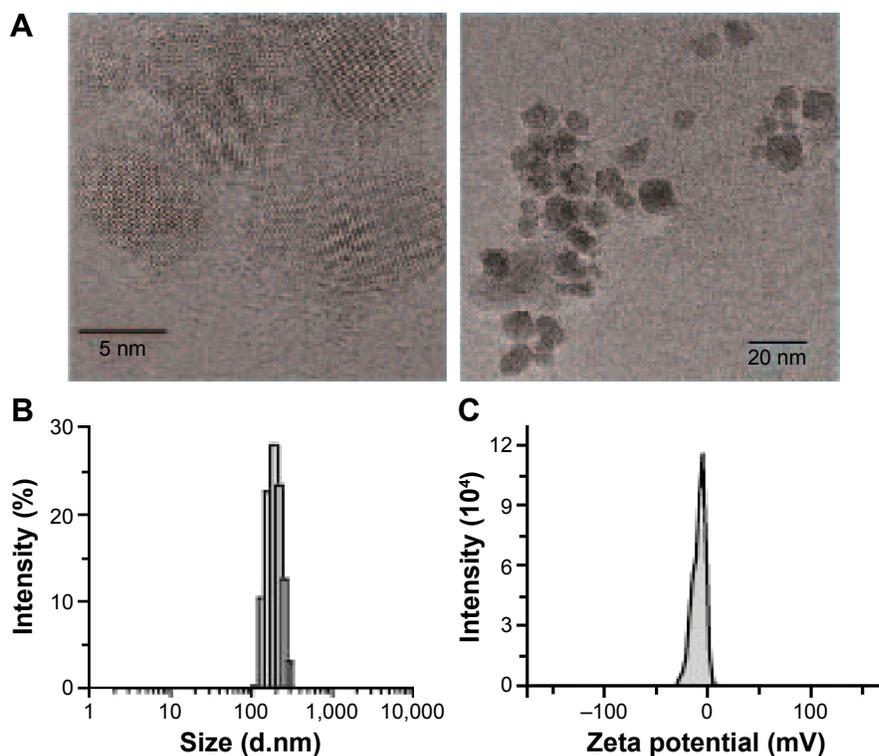


Figure 1 Characterization of fullerenol.

Notes: Transmission electron microscopic image of smaller fullerenol cores (A, left) and larger aggregates (A, right, 1 μM fullerenol), size distributions (B), and zeta potentials (C) of fullerenol in water.

fullerenol was 173.53 ± 3.29 nm (Figure 1A and B), and the surface zeta potential of fullerenol was -22.72 ± 3.87 mV (Figure 1C).

Fullerenol suppressed PPF and LTP in the hippocampus

Effects of fullerenol ($5 \mu\text{M}$) exposure on basic synaptic transmission and short-term potentiation in the CA1 area of hippocampus were examined. After incubation with fullerenol for 20 minutes, no significant differences were observed in input/output curves between the control and fullerenol-treated groups ($n=6$, $P>0.05$, Figure 2A and B), suggesting that fullerenol exerted no recognizable effect on the basic synaptic transmission in hippocampus. We then investigated the effect of fullerenol on short-term synaptic plasticity. PPF is the facilitation of a second response when a synapse is stimulated twice with a short interstimulus interval (20–400 ms). The ratio of amplitude of fEPSP induced by second pulse stimuli to that of first pulse stimuli was measured upon increased IPI (10–500 ms) (Figure 2C).

The average peak facilitation in the control group was $201\% \pm 18\%$ at 30 ms IPI ($n=8$) and $151\% \pm 8\%$ in fullerenol-treated group at 40 ms IPI ($n=8$). Fullerenol significantly decreased the peak of PPF ($P<0.05$), suggesting that fullerenol suppressed the short-term synaptic plasticity from SC to CA1.

LTP is a long-lasting alteration in synaptic strength and plays a crucial role in learning and memory.³⁹ We further investigated the acute effect of fullerenol on LTP in CA1 area. Fullerenol significantly suppressed the high-frequency stimulation-induced potentiation of excitatory postsynaptic potential (EPSP) in CA1 (control, $180\% \pm 12\%$; fullerenol, $117\% \pm 7\%$, $n=8$, $P<0.001$, Figure 2D), indicating that fullerenol suppressed the long-term synaptic plasticity in CA1. We also tested the effect on LTP of $1 \mu\text{M}$ fullerenol; a lower concentration. Our data showed that fullerenol had no significant effect on the fEPSP slope in CA1 (control, $160\% \pm 19\%$, $n=7$; fullerenol, $163\% \pm 17\%$, $n=9$, $P>0.05$, Figure 2E), suggesting that the effect of fullerenol on LTP is concentration dependent.

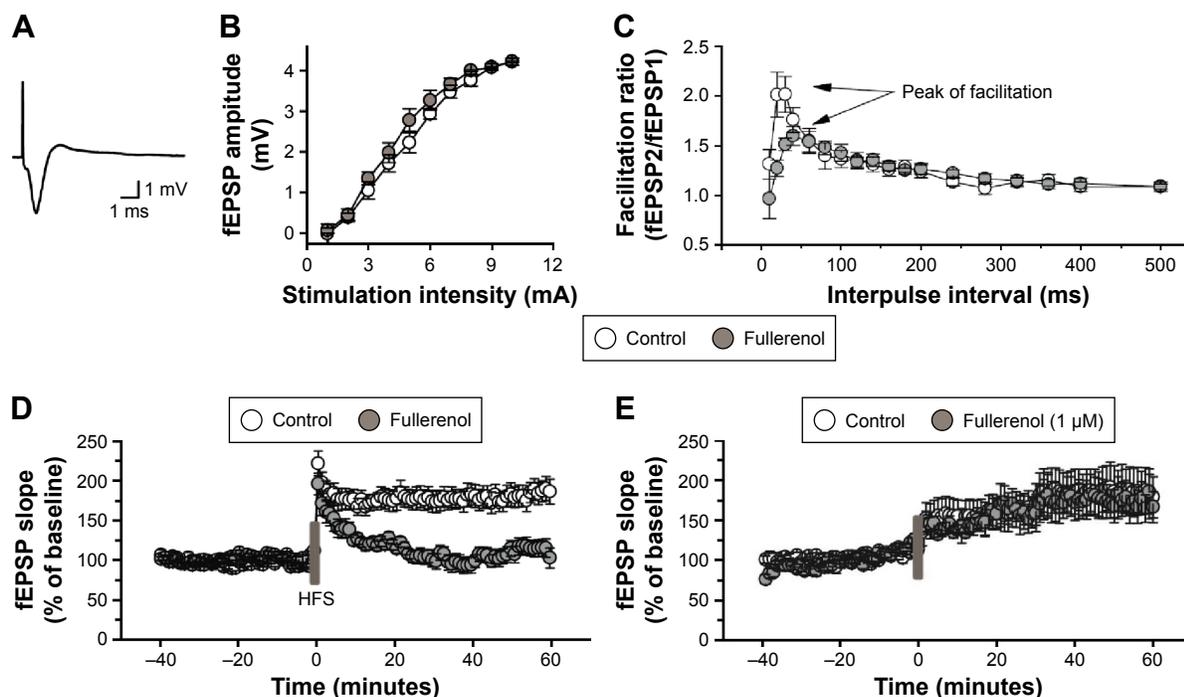


Figure 2 Effects of fullerenol on synaptic plasticity in the CA1 area.

Notes: (A) Representative trace of fEPSP recorded from SC–CA1 synapses. (B) Input/output curves after incubation of normal ACSF (six slices from six rats) and $5 \mu\text{M}$ fullerenol (six slices from six rats). Data were analyzed using a two-way repeated measures ANOVA, $P=0.081$. (C) Mean PPF of fEPSP slope after incubation with normal ACSF (eight slices from eight rats) and $5 \mu\text{M}$ fullerenol (eight slices from eight rats). Data were analyzed using a one-way repeated measures ANOVA followed by Tukey's post hoc test of the significance of PPF peaks, $P=0.021$. (D) LTP was induced by HFS (5 trains of 20 pulses at 200 Hz separated by 1 second, repeated six times at intervals of 1 minute) after treatment with normal ACSF (eight slices from eight rats) or fullerenol ($5 \mu\text{M}$, eight slices from eight rats). A two-way repeated measures ANOVA followed by Tukey's post hoc test was used ($P=0.0004$). (E) LTP induced after treatment with normal ACSF (seven slices from seven rats) or $1 \mu\text{M}$ fullerenol (nine slices from nine rats). A two-way measures ANOVA followed by Tukey's post hoc test was used ($P=0.9133$). Fullerenol dissolved in ACSF was perfused 20 minutes prior to LTP induction. Data are presented as mean \pm SEM.

Abbreviations: ACSF, artificial cerebrospinal fluid; LTP, long-term potentiation; HFS, high-frequency stimulation; fEPSP, field excitatory postsynaptic potential; PPF, paired pulse facilitation; ANOVA, analysis of variance; SC, schaffer collateral; SEM, standard error of mean.

Suppression of synaptic plasticity independent of p-CaMKII

During synaptic potentiation, phosphorylation of CaMKII is essential for LTP induction.^{30,40} To investigate the mechanism of LTP suppression by fullereneol, we tested the level of p-CaMKII protein in hippocampus using Western blot. After 20 minutes of treatment with fullereneol (5 μ M), the level of p-CaMKII/total CaMKII of fullereneol group was $161.11\% \pm 57.22\%$ (control set as 1, $P > 0.05$) (Figure 3). Although p-CaMKII showed little increase in fullereneol-treated group, no statistically significant differences were observed (Figure 3). These results suggested that fullereneol suppressed LTP independent of p-CaMKII level.

Fullereneol downregulated NOS activation and expression

NO plays a critical role in the LTP of SC-CA1 pathway of hippocampus. Therefore, we tested the changes in hippocampal NOS after fullereneol treatment. Incubation with fullereneol for 20 minutes decreased the NOS in the hippocampus (Figure 4A) and reduced the total NOS to about 73% of the control level ($n=5$, Figure 4B). We further investigated

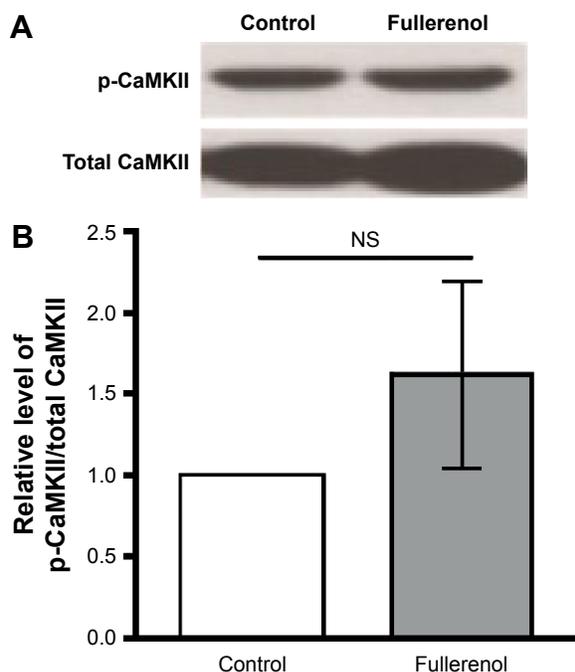


Figure 3 Fullereneol exhibited limited effect on p-CaMKII protein in the hippocampus. **Notes:** (A) Representative Western blots of p-CaMKII and total CaMKII of hippocampal tissues harvested from control samples and samples incubated with fullereneol for 20 minutes. (B) Summarized Western blot results of p-CaMKII in slices normalized to total CaMKII in the hippocampal tissues harvested from control samples (four rats) and samples incubated with fullereneol for 20 minutes (four rats). A two-tailed Student's *t*-test was used ($P=0.934$). GAPDH served as a loading control. Data are presented as mean \pm SEM. NS denotes $P > 0.05$. **Abbreviations:** NS, nonsignificant; SEM, standard error of mean.

the activity of NOS following fullereneol treatment of hippocampus. The NOS activity in the fullereneol-treated group was significantly lower than in the control (control, 1.21 ± 0.09 U/mg protein; fullereneol, 1.64 ± 0.06 U/mg protein, $n=5$, $P < 0.01$, Figure 4C). These results suggest that fullereneol downregulated NOS, which may contribute to fullereneol-induced suppression of synaptic plasticity.

Effects of fullereneol on oxidative stress

In the present study, we tested various parameters related to oxidative stress, including the level of MDA and GSH and the activity of SOD. MDA is one of the major products of lipid peroxidation, and, therefore, indicates the rate and intensity of lipid peroxidation in the hippocampus. Our results show that the MDA concentration was significantly lower in the fullereneol-treated group (control, 6.35 ± 0.11 nmol/mg protein;¹ fullereneol, 3.80 ± 0.41 nmol/mg protein, $n=5$, $P < 0.01$, Figure 5A). The SOD concentration and GSH activity directly reflect tissue antioxidant levels. Our results showed no significant differences in the activity of SOD between the fullereneol-treated and control groups (control, 39.80 ± 0.64 U/mg protein; fullereneol, 39.78 ± 0.81 U/mg protein, $n=5$, $P > 0.05$, Figure 5B). The GSH levels of the two groups showed a similar trend (control, 39.78 ± 0.81 mg/g protein; fullereneol, 39.80 ± 0.64 U/mg protein, $n=5$, $P > 0.05$, Figure 5C).

Discussion

Our study provides direct evidence suggesting suppression of short- and long-term synaptic plasticity by acute fullereneol. First, it is widely recognized that PPF is a form of short-term, activity-dependent synaptic plasticity.⁴¹ It is attributed to residual intraterminal free Ca^{2+} derived from the first action potential facilitating release of neurotransmitters by the second stimulus.⁴² Our data showed that incubation with 5 μ M fullereneol for 20 minutes significantly decreased the PPF, which suggested that fullereneol treatment decreased the presynaptic neurotransmitter release.⁴³ Additional experiments are needed to elucidate the mechanism of inhibition of the PPF. Second, LTP represents an important form of long-term synaptic plasticity.⁴⁴ Our study showed that 5 μ M fullereneol inhibits LTP in hippocampus. However, the inhibition of LTP by fullereneol is concentration-dependent, because 1 μ M fullereneol has no significant effect on the LTP. To the best of our knowledge, this is the first evidence of suppression of synaptic plasticity by fullereneol.

It is noteworthy that p-CaMKII plays a vital role in LTP induction. Although fullereneol suppressed LTP, no significant changes in p-CaMKII/CaMKII were observed in the

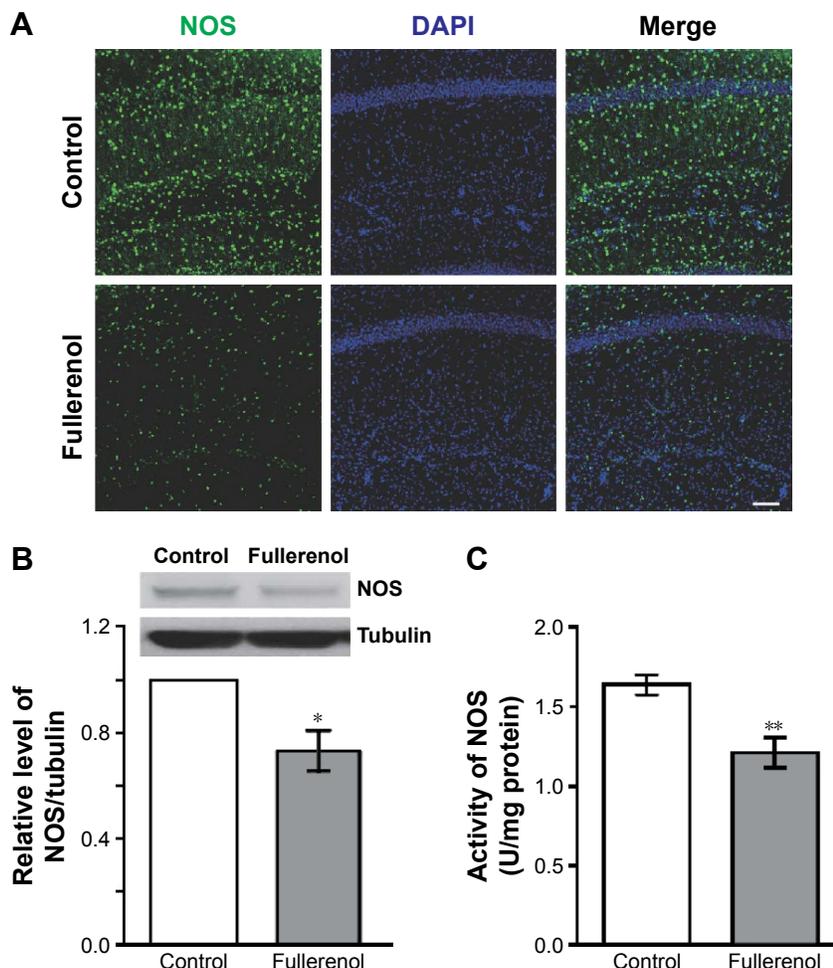


Figure 4 Fullerenol downregulated NOS in hippocampus. **Notes:** (A) NOS (green) in the hippocampal neurons treated with normal ACSF (control) or fullerenol was analyzed by immunofluorescence using anti-NOS antibody. DAPI (blue) was used to label cell nuclei. The fluorescence ratio of NOS/DAPI was 61.11% in the control and 17.89% in the fullerenol-treated group. Scale bar =100 μ m. (B) NOS level in the hippocampus after incubation with ACSF (five rats) or fullerenol (five rats) was determined by Western blot. The bottom panel shows the level of NOS/tubulin compared with the average value of control (ACSF) set at 1. Data were derived from five different samples and analyzed using two-tailed Student's *t*-test, $P=0.026$. (C) NOS activity of hippocampus after incubation of normal ACSF (five rats) or fullerenol (five rats) was detected using a protein assay kit. Data were analyzed using a one-way repeated measures ANOVA followed by Tukey's post hoc test, $P=0.005$. *Denotes $P<0.05$; **denotes $P<0.01$. Data are presented as mean \pm SEM. **Abbreviations:** ANOVA, analysis of variance; ACSF, artificial cerebrospinal fluid; DAPI, 4',6-diamidino-2-phenylindole; SEM, standard error of mean.

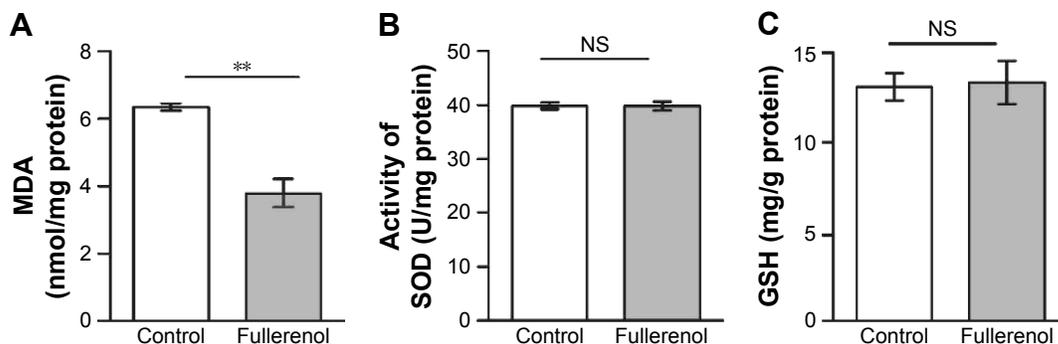


Figure 5 Effects of fullerenol on oxidative stress in hippocampus. **Notes:** Effects of fullerenol on MDA content (A, five rats, using a one-way repeated measures ANOVA followed by Tukey's post hoc test, $P=0.0003$), SOD activity (B, five rats, using a one-way repeated measures ANOVA followed by Tukey's post hoc test, $P=0.991$), and GSH level (C, six rats, using a one-way repeated measures ANOVA followed by Tukey's post hoc test, $P=0.643$). Data are presented as mean \pm SEM. **Denotes $P<0.01$; NS denotes $P>0.05$. **Abbreviations:** GSH, glutathione; MDA, malondialdehyde; ANOVA, analysis of variance; NS, nonsignificant; SEM, standard error of mean.

present study suggesting that fullerenol acted independently of p-CaMKII. Meanwhile, NO generated by NOS formed cyclic guanosine monophosphate via soluble guanylyl cyclase⁴⁵ and, in turn, activated cyclic guanosine monophosphate-dependent protein kinases. Behavioral experiments demonstrated that NO transmission was essential for short-³³ and long-term memory.⁴⁶ LTP in CA1 region of hippocampus is predominantly associated with memory formation. Several studies have shown NO affects LTP. NO released from postsynaptic neurons serves as a retrograde messenger and acts directly on the presynaptic neurons to produce LTP.^{47–50} Our results showed that fullerenol significantly inhibited the activity and expression of NOS in hippocampus. A previous study showed that fullerenol directly scavenged NO.⁷ Other studies showed that fullerene derivatives such as tris-malonyl-C₆₀ also inhibited NOS activity and reduced the maximal velocity of product formation in rat pituitary cells in vitro.⁵¹ Fullerenol-induced reduction of NO synthesis and scavenging of NO may mediate the suppression of synaptic plasticity.

Oxidative stress plays a critical role in cognitive functions,^{52,53} including hippocampal synaptic plasticity. Our previous study of quantum dots indicated that this nanoparticle impaired synaptic transmission and plasticity in the rat dentate gyrus area of hippocampus via oxidative stress.⁵⁴ Fullerenol may suppress the synaptic plasticity in hippocampus by triggering oxidative stress. However, such a mechanism was not possible in our experimental conditions since incubation with fullerenol for 20 minutes significantly reduced the concentration of MDA, suggesting that fullerenol inhibits oxidative damage in hippocampal neurons in vitro. Abundant evidence indicated that fullerenol exerted protective effects in neurons by scavenging free radicals,^{8,37,55} blocking glutamate receptors, and lowering the intracellular Ca²⁺ concentration.⁵⁶ It is noteworthy that the antioxidative effect of fullerenol in the present experimental conditions did not depend on GSH or SOD levels.

Fullerenol has been investigated for potential application in chemotherapy, radiobiology,⁵⁷ and as an agent targeting neurodegenerative diseases^{13,58} or inducing neuroprotection.^{8,37,56} Despite the convincing evidence suggesting the potential biomedical applications of fullerenol, our knowledge of the possible side effects, especially in neural tissues, is incomplete. Our previous study showed that fullerenol not only protects hippocampal neurons from lead-induced damage but also induces cell death in certain doses.³⁷ In the present study, fullerenol suppressed synaptic

plasticity and downregulated NOS, which is similar to the acute side effects of fullerenol in hippocampal tissue. Long-term studies investigating fullerenol suppression of synaptic plasticity in vivo are needed.

Conclusion

In the present study, we have shown that acute treatment with 5 μ M fullerenol suppressed short- and long-term synaptic plasticity, indicated by decreased PPF and LTP in hippocampal slices. Furthermore, the suppression by fullerenol was independent of the inhibition of p-CaMKII. Fullerenol significantly reduced the activity and quantity of NOS, which was probably responsible for the suppression of synaptic plasticity. Therefore, the study findings suggest the possible side effects of fullerenol and partly illustrate the underlying mechanisms, with potential implications for biomedical applications.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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