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Research Article

Resveratrol Prevents Lipopolysaccharide-Induced Cognitive Impairment in Rats through Regulation of Hippocampal Glua1-Containing AMPA Receptors

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Abstract

Background: Evidence suggests that dysregulation in AMPA-type glutamate receptors (AMPA-Rs) has been associated with the pathogenesis of Alzheimer's disease (AD), especially during its early phase. Hence, the present study was performed to elucidate the impact of resveratrol (RV) on hippocampal expression of AMPA-Rs in a rat model of AD.

Methods: A rat model of cognitive deficits was developed by a stereotactic intracerebroventricular infusion of lipopolysaccharide (LPS) in male Wistar rats (n=24). The LPS+RV30 group (n=12) received intraperitoneal injections of RV (30 mg/kg) at 30 min, 12 h, and 24 h before LPS injection. Meanwhile, the model (LPS) and sham (SO) groups only were treated with the vehicle solution (normal saline containing 1% ethanol). One day after the LPS infusion, the mRNA expressions of AMPA-Rs subunits (*Gria1-4*) were evaluated by RT–PCR. In addition, hippocampal levels of lipid peroxidation, superoxide dismutase, and nitric oxide were assessed. Seven days after the LPS challenge, the remaining animals (n=6, each group) were subjected to the Y-maze task, and the expression and localization of GluA1-containing AMPA-Rs in their hippocampi were investigated immunohistochemically.

Results: Pretreatment with RV prevented LPS-induced cognitive dysfunction in rats and enhanced their working memory performance. Moreover, RV could moderately prevent oxidant-antioxidant imbalance in rats' hippocampi. RT-PCR results revealed that the hippocampal mRNA expression of the *Gria1* was significantly reduced, while the expressions of *Gria2* and *Gria3* were increased in LPS-challenged rats. RV significantly modulated the alteration in the *Gria1* mRNA expression; however, it could not influence the *Gria2* and *Gria3* mRNA expressions. The immunohistochemical assessment showed a significantly reduced immunoreactivity for GluA1-containing AMPA-Rs in all hippocampal subfields of the LPS group, and RV could effectively ameliorate the alteration.

Conclusion: This study is the first to report that RV could modulate GluA1-containing AMPA-Rs dysregulation in a rat AD model.

Introduction

Alzheimer's disease (AD) is a deteriorating neurologic disorder characterized by memory loss, cognitive decline, and devastating neurodegeneration. AD pathogenesis has been attributed to the presence of extracellular aggregation of beta-amyloid plaques (Aβ) and intracellular accumulation of hyperphosphorylated tau proteins. Nevertheless, synaptic alterations appear to be better associated with cognitive impairment and memory loss than histopathological markers.[1] It has been demonstrated that synaptic dysfunction can happen early in the preliminary phases of AD and may present prior to the expression of apparent atrophy.[2,3]

Glutamate (the anion of glutamic acid) is an excitatory neurotransmitter in the central nervous system mediating several physiological functions such as neural transmission, neuronal plasticity, learning, and memory process.[4] Due to the high density of glutamatergic neurons in the AD-affected brain's regions like the hippocampus, alterations in glutamate signaling play critical roles in the pathogenesis of this disorder.[5] There are two major types of synaptic receptors: G-protein-coupled (metabotropic) receptors and ligand-gated ion channels (ionotropic) that both of which are activated by glutamate.[6] The inotropic glutamate

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receptors encompass three subfamilies: N-methyl-D-aspartate (NMDA-Rs), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA-Rs), and kainite. Currently, the mechanisms of action of the available drugs for AD management are inhibition of acetylcholinesterase enzyme (galantamine, rivastigmine, donepezil) and the blockade of NMDA-Rs (memantine and ifenprodil), which highlight the importance of glutamate receptors signaling in AD management.[7,8] Unlike NMDA-Rs, the role of AMPA-Rs in the pathogenesis and management of AD has been less studied. AMPA-Rs consist of four subunits, GluA1-4 encoded by distinct genes (*Gria*1-4).[9] Evidence suggests AMPA-Rs dysregulation has been involved in the pathological features of AD, especially during its early phase.[8,10]

Nevertheless, oxidative/nitrosative stress (OS/NS) plays a prominent role in the initiation and propagation of several neurodegenerative diseases.[11] During the early and late phases of AD physiopathology, producing reactive oxygen species and nitrogen intermediates such as nitric oxides (NO) and peroxynitrite can cause neuronal damages.[12] Therefore, treatments with antioxidants such as vitamin E, minocycline, and resveratrol (RV) could protect neurons from OS/NS damage and prolong the initial step of AD.[13]

RV is a well-known biologically active compound that belongs to the polyphenols' stilbenoids group. It has gained substantial attention for several biological activities, including anti-neurodegenerative, antioxidative, anti-inflammatory, anticancer, and antimicrobial.[14] Numerous experimental studies have shown that RV may enhance the clearance of Aβ through up-regulation of the ubiquitin-proteasome system and synaptic plasticity.[15,16] In addition, it has been previously reported that RV can mediate brain functions through the facilitation of AMPA-Rs biogenesis in healthy brains.[17] However, whether RV has an impact on AMPA-Rs expression in the AD condition remains unknown.

In the context of AD pathogenesis, neuroinflammation can commence even prior to the emergence of Aβ plaques processing and contribute to evoke the subsequent OS/NS, synaptic dysfunction, and cognitive impairment.[18] Accordingly, animal models of neuroinflammation induced by lipopolysaccharide (LPS) are broadly used to investigate the disease process and therapeutic agents.[19] Hence, the present study aimed to elucidate the effects of RV pretreatment on hippocampal expression and activation of AMPA-Rs in LPS-induced cognitive impairment in rats.

Methods

Chemical and reagents

RV (R5010; CAS No: 501-36-0; purity≥ 99%), LPS (L3129; Escherichia coli; O127: B8), phosphate-buffered saline (PBS) tablets (P4417), goat serum (G6767), 3,3′-Diaminobenzidine (D8001), sodium pentobarbital (P3761; CAS No: 57-33-0), 2-Thiobarbituric acid (T5500; CAS No: 504176; purity≥ 98%), and bovine serum albumin (A9418; CAS NO: 9048-46-8) were purchased from Sigma Aldrich Company (St Louis, MO, USA). Vanadium III chloride (1.12393.0025; CAS No: 7718-98-1; purity = 97 %), ortho-Phosphoric acid (1.00573, CAS No: 7664-38-2; purity= 85%) and 1-Butanol (1.00988; CAS No: 71-36-3) were acquired from Merck Company (Darmstadt, Germany). Zinc sulfate (326190500; CAS No: 7446-20-0) was obtained from BIOCHEM Chemopharma (Cosne-Coura-sur-Loire, France).

Triton® X-100 (TR04441000) was obtained from Scharlau Company (Barcelona, Spain). Tris Base (Hydroxymethyl aminomethane; B111102), total RNA extraction kit (A101231; LOT No: 1951), and cDNA synthesis kit (A101161; LOT No: 1636) were purchased from Parstous Company (Mashhad, Iran) and SYBR Premix Ex Taq (Tli RNaseH Plus) Master Mix (RR820L; LOT No: AK9601) was procured from Takara Bio Company (TaKaRa, Dalian, China). Diethylpyrocarbonate (DEPC) treated water (CAS No: CH8143) and DNAse I (DNase1, RNase-free; CAT No: MO5401) were procured from SinaClon Knowledge Base Company (Tehran, Iran). Primary rabbit polyclonal antibody to glutamate receptor 1(GluA1) (ab31232) and secondary goat Anti-Rabbit IgG H&L (HRP) (ab7090) were purchased from ABCAM Company (California, USA). Protein (NS-15073) and superoxide dismutase (SOD) (NadoxTM, NS-15032) assay kits were bought from Navand Salamat Company (Urmia, Iran).

Animals

The animal investigations were reviewed and approved by the Institutional Ethics Committee of Birjand University of Medical Sciences (Ir.bums.REC.1398.119). Male Wistar rats (10-week old) were obtained from the Research Centre of Experimental Medicine of Birjand University of Medical Sciences, Iran. Animals were kept in a temperature (24 ± 2 ºC) and humidity (30- 35%) -controlled environment with a 12-h light/dark cycle (6 a.m. to 6 p.m.). During the study period, all rats had *ad-libitum* access to food (Behparvar Co, Iran) and water.

Study design and LPS administration

This experiment consisted of three groups of male Wistar rats (n=12 each): sham-operated (SO), LPS-induced AD model (LPS), and LPS-injected rats intraperitoneally (i.p.) pretreated with RV at the dose of 30 mg/kg (LPS+RV30). The sterile and freshly prepared artificial cerebrospinal fluid (aCSF, pH= 7.4) containing 140 mM NaCl, 25 mM $CaCl_2$, 3 mM KCl, 1.2 mM Na2Hpo4 and 1mM $MgCl_2$ was used to dissolve the LPS.[20]

A rat model of LPS-induced cognitive impairment was developed by performing stereotactic surgery under sodium pentobarbital anesthesia (45 mg/kg, i.p.).[21] Briefly, after anesthesia, a single dose of Bupivacaine: Lidocaine mixture (1:1, 0.2 mL) was also injected subcutaneously to induce analgesia in rats. Then, each rat was placed over a heating pad (709-0250; Narco BioSystems, Austin, USA), and its skull was fixed on a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The scalp was shaved, disinfected,

and then a midline incision was made from the line of the eyes to the neck's base. The injection sites (0.8 mm posterior to the bregma; ±1.8 mm lateral to the midline; and 3.8 mm ventral to the skull surface)[22] were labeled with ink and drilled (0.5 mm diameter) cautiously up to the level of dura mater. Subsequently, 10 µL of LPS (dissolved in the aCSF (50 µg/20µL)) was bilaterally injected into the lateral ventricle at a rate of 1µL /min using a 26G cannula needle and a syringe pump (EN-300, New Era Pump Systems, Farmingdale, NY, USA).[23] After each ICV infusion, the cannula was left for 5 min to prevent backflow. The SO rats were given an infusion of the aCSF (10 µL /ventricle) instead of the LPS solution.

Experimental protocols

The stock solution of RV was prepared by dissolving 50mg RV in 1mL ethanol. Then, the stock solution was diluted with 0.9% normal saline until the relative injection volume for each rat contained less than 1% ethanol. Therefore, the 0.9% normal saline solution containing 1% ethanol was used as a vehicle. The treatment was started at 30 min, 12 h, and 24 h before the ICV infusion of LPS with i.p. injections of RV (LPS+RV30) or vehicle (SO and LPS groups).[24] The RV dose (30 mg/kg) was selected based on pilot experiments as well as previous studies where the RV exhibited neuroprotective effects.[25,26] In order to avoid RV elimination due to its rapid metabolism and low bioavailability, three repeated injections of RV were performed.[27]

One day after the LPS infusion, half of the rats in each group (n=6) were randomly selected, anesthetized with sodium pentobarbital (50 mg/kg, i.p.),[23] and sacrificed for biochemical and molecular experiments.[24] The remaining animals were used for the behavioral test (Y-maze) and immunohistochemical (IHC) experiments. The animal experiments schedule is shown in Figure 1.

Y-maze test

On the 7th day after the LPS challenge, the short-term memory was tested by Y-maze,[28] which measures the hippocampus's spatial memory function. Briefly, rats were placed on the equilateral triangular center of the apparatus and left to move freely into the maze for 8 min. An accepted arm entry was considered when all four paws of the rat were within an arm. Spontaneous alternation behavior was defined as the consecutive entry sequence into all three arms. It worth be noted that to prevent intra-maze cues, the apparatus' arms were cleaned with a glass cleaner spray before the next animal was tested.

The percentage alternation (memory index or alternation score) was calculated with the following formula:
Memory index = [(number of alternations)/ (total arm entries-2) ×100].

Tissue collection and sample preparation

As mentioned above, 24 h after the ICV LPS infusion, six rats from each group were randomly selected, sacrificed and their brains were gently removed and placed on an ice-cold dissection tray pre-washed with DEPC treated water. The hippocampi were dissected out, and the right hippocampus was transferred into RNAse-free microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at −80 °C for molecular experiments. The left hippocampus was weighed and homogenized in 10 volumes of ice-cold PBS (pH 7.2-7.4) for estimating protein, lipid peroxidation, SOD activity, and nitric oxide content.

Seven days after LPS injection and following the Y-maze test, the remaining animals (n=6/group) were considered for IHC investigation. They were sacrificed under anesthesia with pentobarbital (50 mg/kg, i.p) and transcardially perfused with cold 0.9% saline (5 mL) followed by 4% paraformaldehyde fixative solution (20 mL, pH=7.4).[29] Then, their brains were carefully removed and post-fixed in 4% paraformaldehyde solution at 4 °C for 48 h.

RNA isolation and cDNA synthesis

The total RNA was extracted from the hippocampal tissues using a commercially available total RNA extraction kit (Parstus, Mashhad, Iran). The RNA quality was checked by measuring optical density (OD) in an Epoch Microplate Spectrophotometer (Biotek™, Winooski, VT, USA) at the 260/280 nm ratio. Samples with the ratio ≥2.0 were considered to be of sufficient quality for further analysis. In addition, RNA integrity was checked by agarose (1.2%) gel electrophoresis to visualize the staining intensity of the major ribosomal RNA (rRNA) bands (28S and 18S).[30] RNA samples were treated with DNAse I and reverse transcribed to the single-stranded cDNA using random hexamer and oligo $(dt)_{16}$ primers following the instructions of the Easy cDNA synthesis kit (Parstus, Iran). cDNA samples were stored at −20 °C.

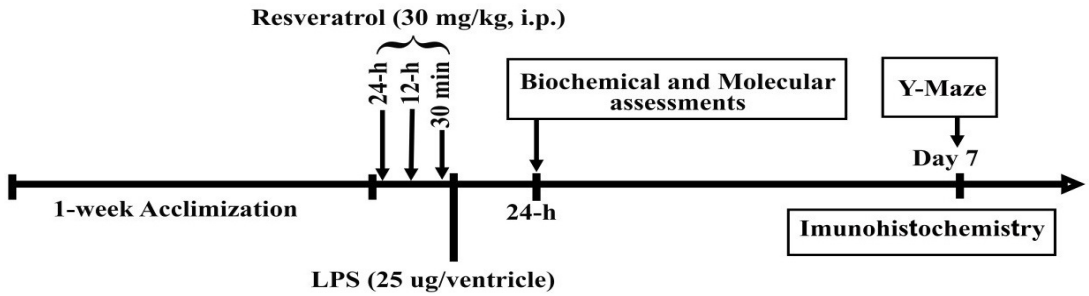

Figure 1. Schematic diagram of the experimental design

Reverse transcription-polymerase chain reaction (RT-PCR)

Real-time PCR was carried out to investigate hippocampal mRNA expression of AMPA-Rs subunits *Gria1* (GluA1), *Gria2* (GluA2), *Gria3* (GluA3), and *Gria4* (GluA4). The list of all primer sequences is shown in Table 1. RT-PCR was run in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA). The mRNA expression levels were estimated using an intercalating dye-based PCR master mix (Tli RNaseH Plus, TaKaRa, China). The RT-PCR assay was initiated with 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C, 30 sec at 60°C.

Amplification of each gene was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as a housekeeping gene. The relative difference in the target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (StepOne™ Software, V2.3, USA) and expressed as the fold change after GAPDH normalization.[30,31] All reactions were run in duplicate.

Biochemical assessments

The protein content of hippocampal tissue extracts (supernatants) was determined using a commercially available kit (Navand Salamat, Iran).

A modified thiobarbituric acid reactive species (TBARS) test was used to evaluate hippocampal lipid peroxidation products by estimating malondialdehyde (MDA). Each sample was mixed with 0.67% thiobarbituric acid (1:2 v/v) and 1% o-Phosphoric acid (1:6 v/v) and placed in a boiling water bath for 45 min. Then, the test tubes were immediately chilled by placing them into an ice bath. After cooling, to increase the test sensitivity, 800 µL 1-butanol was added to each test tube, shaken well, and centrifuged (5000 g at 4 °C for 20 min). The resulting supernatants were collected (200µL) and transferred into a 96-well plastic plate. The plate was immediately read at 532 nm, and values were expressed as nmol/mg protein.[32]

Superoxide dismutase (SOD) activity was evaluated using a WST-1 based SOD inhibition assay kit (Navand Salamat, Iran). Briefly, 50 µL of each sample (supernatant) was added (in duplicate) to each well of a 96-well plate, mixed with 250 µL of the reaction mixture (provided in the kit), and incubated at room temperature for 5 min. Eventually, the plate was read at 405 nm, and values were expressed as U/mg protein.[33]

According to the Griess method, the hippocampal NO content was estimated by determining the accumulated NO metabolites (nitrate and nitrite). Firstly, samples were deproteinized by adding zinc sulfate (15 mg/mL, 1:8 v/v) and centrifuged at 14000 g for 20 min. Next, 100 µL of each deproteinized supernatant was added (in duplicate) to separate wells of a 96-well plate, mixed with 100 µL of vanadium III chloride (8 mg/mL) and 100 µL of Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% ortho-phosphoric acid]. Finally, the plate was incubated at 37°C for 30 min, and its absorbance was read at 530 nm. The levels of nitrite plus nitrate were determined from a $NaNO_2$ (0-150 µM) standard curve.[34]

Immunohistochemical (IHC) assessment of GluA1

After analyzing the gene expression results, to investigate whether the observed change in *Gria1* mRNA expression can also be seen in the translated protein (GluA1) and pinpoint what region of the hippocampus exhibited the change, an IHC assay was performed.

The fixed brain tissues were embedded in paraffin wax and sectioned coronally at 5-7 µm thickness using a sliding microtome (Leitz 1512, Italy). Slides were deparaffinized, rehydrated and rinsed in PBS. Antigen retrieval was performed by boiling the slides in citrate buffer (10 mM, pH 6.0) for 15-20 min. After cooling (30 min at room temperature), slides were washed with TBST (Tris-buffered saline plus 0.025% Triton X 100) and blocked with 10% goat serum in TBS for 2 h at room temperature. Thereupon, sections were incubated with the primary rabbit polyclonal anti-GluA1 primary antibody (ab31232, ABCAM, 1:100) at 4 °C for 12 h. Next, slides were rinsed two times with TBST, and immersed in freshly prepared 0.3% H_2O_2 in methanol solution for 15 min to block endogenous peroxidase activity. The slides were washed

Table 1. Primer pairs used for reverse transcription-polymerase chain reaction (RT-PCR).

Name (Target)[a]	Sequences[b] (5'->3')	Target region[c]
Gria1 *(Rattus norvegicus* glutamate ionotropic receptor AMPA type subunit 1) [GluA1]	F: ATGTGGCAGGCGTGTTCTA R: TCGACTCGCTACGGGATTTG	2598-2694 of NM_ 031608.2
Gria2 (*Rattus norvegicus* glutamate ionotropic receptor AMPA type subunit 2) [GluA2]	F: CGAGGGCTACTGTGTTGAC R: TGCAATGTCAGCTTTCCCGTA	1755-1918 of NM_001083811.1 1755-1918 of NM_017261.2
Gria3 *(Rattus norvegicus* glutamate ionotropic receptor AMPA type subunit 3) [GluA3]	F: AGTTCGGAAGTCCAAGGGAT R: CAGGCGTTCCTAATGCTGAG	2210-2370 of NM_ 032990.2 2210-2370 of NM_001112742.1
Gria4 *(Rattus norvegicus* glutamate ionotropic receptor AMPA type subunit 4) [GluA4]	F: GTTCGAGAGGAAGTCATCGC R: ACAAGACCACACTGACACCAT	1965-2137 of NM_001113184.1 1965-2137 of NM_ 017263.2
GAPDH *(Rattus norvegicus* glyceraldehyde-3-phosphate dehydrogenase)	F: GTCATCCCAGAGCTGAACGG R: ACTTGGCAGGTTTCTCCAGG	730-835 of NM_001394060.2 727-832 of NM_017008.4

[a]Name of the primer pair/target gene and full name of the target gene (protein names that differ from the gene name are written in square brackets).
[b]Nucleotide sequences of the forward (F) and reverse (R) primers.
[c]Region of the target gene amplified by the primer pair.

Table 2. Effects of pretreatment with resveratrol (RV, 30 mg/kg, i.p.) on hippocampal stress oxidative markers 24-h following intracerebroventricular infusion of lipopolysaccharide

Groups	MDA (nmol/mg protein)	SOD (U/mg protein)	NO (nmol/mg protein)
Sham-operated	0.33±0.02	64.66± 4.27	5.01± 0.89
LPS	0.64±0.04 ***	39.16 ± 3.03***	11.33±1.21***
LPS+RV (30 mg/kg)	0.39±0.04*,###	55.66± 4.32**, ###	9.33± 1.03***,#

MDA: malondialdehyde; SOD: superoxide dismutase; NO: nitric oxide. Values are expressed as mean±S.D., n=6 animals per group. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ versus sham-operated group; # $P<0.05$ and ### $P<0.001$versus LPS group in the Tukey's multiple-range test.

(TBST) and incubated with the secondary antibody (goat anti-rabbit IgG-HRP, 1:200, ab7090, ABCAM) for 1-h at room temperature. The slides were washed again (TBS), and signals were visualized using 3,3'-diaminobenzidine (0.03%). Lastly, sections were counterstained with hematoxylin, dehydrated, cleared, and mounted with Entellan and coverslip. Three random slides from each rat (n=18/ group) were evaluated under a light microscope (Euromex-CMEX-10, Netherland), and images were taken using a digital camera (Image Focus v2, Netherland) connected to the microscope. The immunohistochemical quantification of images was performed and represented as OD by image analysis software (Image J, 1.48, NIH, USA) in different hippocampal subfields.[35]

Statistical analysis

Statistical analysis of data was performed with the SPSS software (version 22) using ANOVA, Tukey's, and Dunnett T3 post-hoc multi-comparison tests when appropriate. Data are presented as mean ± SD. Statistical significance was considered for $P < 0.05$ in all statistical evaluations.

Results

Effect of RV on the hippocampal stress oxidative markers

The results of hippocampal levels of oxidative parameters (MDA, SOD, and NO) are shown in Table 2. The extent of lipid peroxidation as measured by the MDA level showed a sharp increase following LPS administration ($p < 0.001$). The MDA level of the RV pretreated animals was statistically lower than SO rats ($p <0.001$). However, the MDA level in the LPS+RV30 group remained statistically higher than the SO group ($p = 0.021$).

Compared to the SO group, the LPS infusion produced a notable reduction in hippocampal SOD activity ($p < 0.001$). RV administration could effectively prevent the hippocampal SOD decrease in the LPS-treated rats ($p < 0.001$). However, its level was still significantly lower than the SO group ($p = 0.002$).

Compared to the SO group, the hippocampal NO content in the LPS group dramatically was increased ($p < 0.001$). RV administration could significantly (but slightly) prevent NO elevation ($p = 0.04$) in the LPS-challenged rats. However, the hippocampal NO content of the LPS+RV30 group was still statistically higher than the normal values ($p <0.001$).

Effect of RV on the hippocampal mRNA expression of Gria1-4

The $2^{-\Delta\Delta Ct}$ method was used to compute the relative expression of *Gria1-4* genes. LPS administration downregulated ($p < 0.001$) the relative *Gria1* gene expression, while upregulated the expressions of *Gria2* and *Gria3* ($p < 0.001$ both) in the rats' hippocampi. However, *Gria4* expression was unchanged following LPS administration (Figure 2). Pretreatment with RV only influenced the *Gria1* gene expression change in the LPS-treated animals. As illustrated in Figure 2 (A), pretreatment with RV increased hippocampal mRNA expression of *Gria1* in LPS-treated animals ($p = 0.008$). Compared to the LPS group, the mRNA expression of *Gria3* in the LPS+RV30 group tended to downregulate (Figure 2, C), but it was not statistically significant ($p = 0.07$). RV administration could not influence the elevated mRNA expression of *Gria2* in the LPS-treated rats (Figure 2, B).

Effects of RV on working memory

When one-way ANOVA analyzed the performance of rats in the Y-maze task, a significant difference ($p < 0.001$) in terms of alternation score (memory index) was found between the studied groups. Rats in the LPS group had a significantly ($p < 0.001$) lower memory index than the SO group (Figure 3).

RV pretreatment (30 mg/kg) significantly ($p = 0.03$) improved memory index in LPS-challenged rats. However, the memory index of the LPS+RV30 group was still lower than that of the SO group ($p = 0.003$).

Effects of RV on GluA1 immunohistochemistry

The expression and localization of GluA1-containing AMPA-Rs in hippocampal tissues were assessed immunohistochemically. As noticed from Figure 4, prominent expression of GluA1 was detected on different hippocampal subfields, including CA1, CA3, and dentate gyrus of the SO group. In contrast, the expression of GluA1 was significantly attenuated by LPS administration in all the studied regions. Pretreatment with RV (30 mg/kg, i.p.) caused significant increases in GluA1 expression in all the studied hippocampal subfields compared to the LPS group ($p<0.05$ all).

Discussion

The results of this study provide new evidence that RV treatment can positively modulate the altered hippocampal

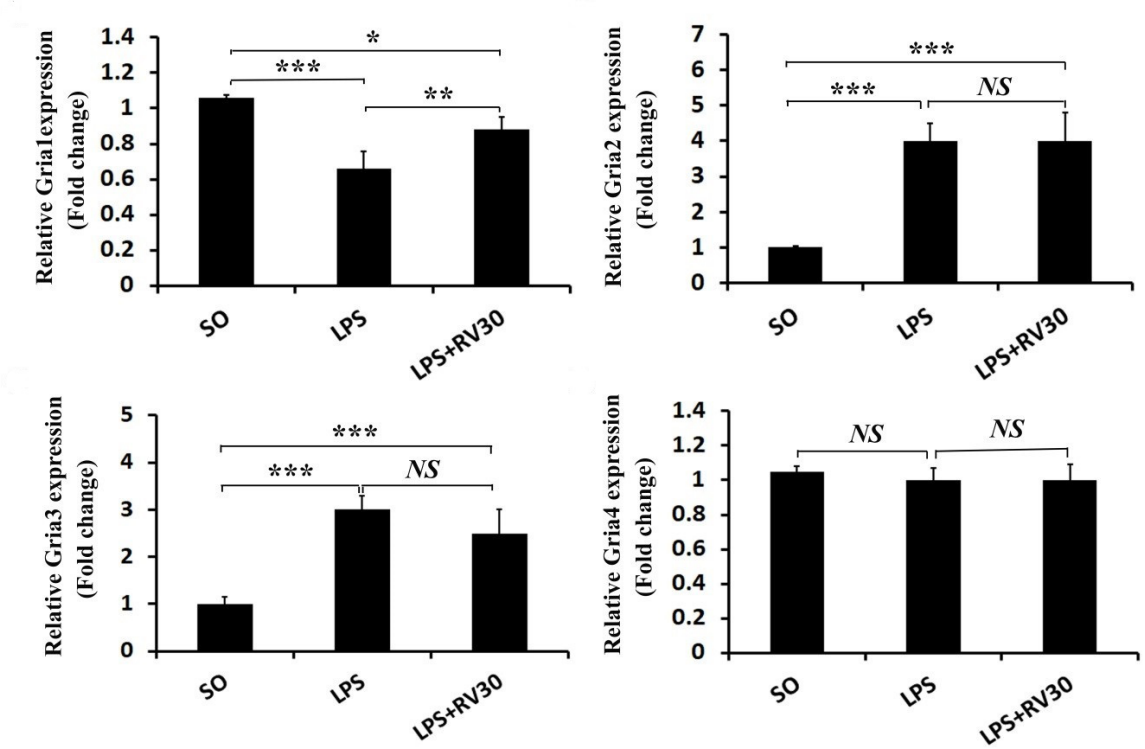

Figure 2. Effect of pretreatment with resveratrol (RV; 30 mg/kg, i.p.) on hippocampal mRNA expression of *Gria*1-4 genes (AMPA receptor subunits 1-4) against GAPDH. The results are presented as the mean ± S.D., n = 6 (in duplicate). SO: Sham-operated rats received vehicle (i.c.v); LPS: rats received LPS (50 µg/rat, i.c.v.); LPS+RV30: rats received resveratrol at the dose of 30 mg/kg (i.p.) 24 h, 12 h, and 30 min before LPS injection. NS: none significant. Differences were defined as significant as *$p < 0.5$, **$p < 0.01$, and ***$p < 0.001$ between groups in the Dunnett's T3 post hoc test.

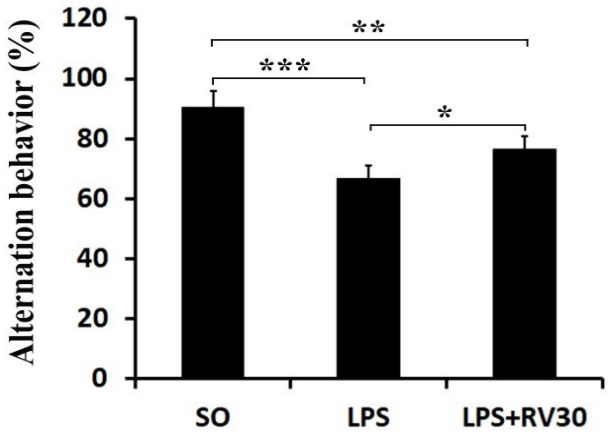

Figure 3. Protective effect of resveratrol (30 mg/kg, i.p.) on spontaneous alterations (%) in lipopolysaccharide (LPS)-challenged rats. Memory index (spontaneous alterations) was evaluated by the Y-maze test 7 days after LPS injection. SO: Sham-operated rats received vehicle (i.c.v); LPS: rats received LPS (50 µg/rat, i.c.v.); LPS+RV30: rats received resveratrol at the dose of 30 mg/kg (i.p.) 24 h, 12 h, and 30 min before LPS injection. Values are expressed as mean ± S.D., n = 6 rats per group. Differences were defined as significant as *$p < 0.5$, **$p < 0.01$, and ***$p < 0.001$ between groups in the Tukey's multiple-range test.

AMPA-Rs expression (GluA1 subunit) in a rat model of AD. Pretreatment with RV prevented LPS-induced cognitive dysfunction in rats, as evidenced by improved working memory performance in the Y-maze task. Moreover, RV could moderately prevent oxidant-antioxidant imbalance induced by LPS in rats' hippocampi.

Previous studies have demonstrated the deleterious effects of LPS on cognitive performance along with several pathological events (oxidative stress, inflammation, mitochondrial dysfunction) that are also evident in neurodegenerative diseases such as AD. Therefore, this model is widely used for studying AD pathology.[23,36-38] Several clinical and experimental studies have shown that RV could improve cognitive function and memory.[39-41] Li and coworkers reported that a single pretreatment with RV (30 mg/kg, i.p.) at 1 or 4 h before focal cerebral ischemia significantly inhibited the hippocampal CA1 neuronal death and improved spatial cognition in rats.[42] Prior mechanistic studies have shown that RV can prevent cognitive impairment and neurodegeneration by modulating several pathways, including oxidative stress,[43] Aβ clearance,[44] inflammation,[45] autophagy,[46] mitochondrial functions,[47] gut microbiota composition,[48] and neuronal cell death.[49] In the present study, we focused on the effects of RV on OS/NS and AMPA-Rs trafficking in LPS-induced cognitive impairment rats.

In line with previous studies, our results showed that the LPS administration significantly increased MDA and NO's hippocampal levels and markedly reduced SOD activity. Nerve cell membranes have markedly higher contents of polyunsaturated fatty acids such as arachidonic and linoleic acids than other cells; in consequence, one of

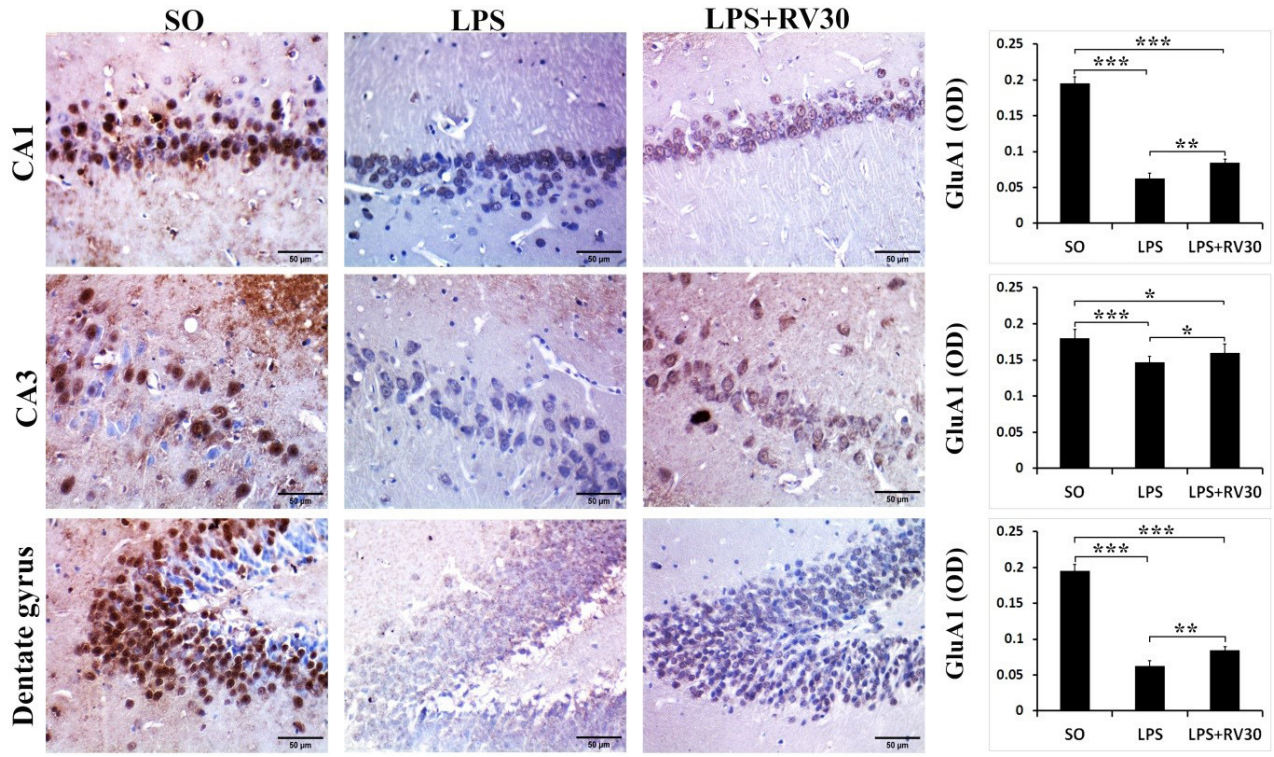

Figure 4. Immunohistochemical evaluation of GluA1-containing AMPA receptors in different hippocampal subfields (CA1, CA3, and dentate gyrus) (400X, scale-bars = 50 µm). The bar charts represent the mean optical density (OD) of positive immunoreactivity. Values are expressed as mean ± S.D., n = 18 sections per group. SO: Sham-operated rats received vehicle (i.c.v); LPS: rats received lipopolysaccharide (50 µg/rat, i.c.v.); LPS+RV30: rats received resveratrol at the dose of 30 mg/kg (i.p.) 24 h, 12 h, and 30 min before LPS injection. Differences were defined as significant as *$p < 0.5$, **$p < 0.01$, and ***$p < 0.001$ between groups in the Tukey's multiple-range test.

the most common oxidative damage in these cells is lipid peroxidation. If lipid peroxidation due to low antioxidant capacities or a damaged antioxidant defense system is not controlled, it will proliferate unabated and lead to cell death.[50] The brain is also more vulnerable to OS/NS damage because it has a weaker antioxidant system (low activities of SOD, catalase, and glutathione peroxidase) than other organs in the body.[51] The activity of a group of metal enzymes with antioxidant properties is considered as an indicator of the balance of the oxidant-antioxidant system. SOD is a group of enzymes that catalyzes the conversion of superoxide radicals (O_2^-) to O_2 and H_2O_2, contributing to maintaining the low levels of cellular free radicals. It has been shown that the SOD enzyme enhances neural survival and protection against oxidative damage as its deficiency could accelerate memory impairment, Aβ aggregation, and tau hyperphosphorylation in the AD mouse model.[52] RV's *in vivo* and *in vitro* neuroprotective and antioxidant properties have been extensively studied.[53] RV can rapidly cross the blood-brain barrier and exert its local antioxidant and anti-inflammatory effects.[54] Our findings were broadly in line with a recently published study conducted by Rao and coworkers, in which seven days of pretreatment with RV (10-20 mg/kg, i.p.) decreased MDA level and increased SOD activity in total brain homogenates of colchicine induced–AD rats.[55] Moreover, the neuroprotective activity of RV in other pathological conditions such as diabetes has been investigated by Sadi and Konat, and their results showed that RV treatment enhanced antioxidant activity and decrease lipid peroxidation in the frontal cortex of diabetic rats.[56]

Our findings also revealed that RV pretreatment significantly decreased the hippocampal NO content in LPS-challenged rats. In the CNS, NO is involved in many physiological functions such as synaptic plasticity, the sleep-wake cycle, and neurosecretion. However, it also contributes to several neurodegenerative-associated mechanisms such as neuronal death, apoptosis, necrosis, and autophagy.[11] Microglial cells play a pivotal role in the LPS-induced neuroinflammation rat model. They are activated following exposure to LPS and release proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1β, monocyte chemotactic protein 1 (MCP-1), prostaglandin E2, and NO.[57] Interestingly, it has been reported that RV could alleviate the production of NO, TNF-α, IL-6, and MCP-1 in LPS-activated microglia.[58] Therefore, we can speculate that RV decreased hippocampal MDA level by enhancing the activity of the antioxidant enzyme SOD, and reduced NO content through its anti-inflammatory properties.

Wang *et al.*[17] have found a marked increase in AMPARs expression in the hippocampus, prefrontal cortex, and cerebellum of healthy mice five hours after RV injection (30 mg/kg, i.p.).The scenario suggests that the observed beneficial effects of RV are not limited to its antioxidant properties. In addition, it has been previously reported that

RV could modulate the hippocampal NMDA receptors expression in an AD-like animal model.[59] To the best of our knowledge, no one has studied the effects of RV on AMPA-Rs trafficking in the AD hippocampus. We found that the hippocampal mRNA expression of the *Gria1* gene was significantly reduced, while the expressions of subunits *Gria2* and *Gria3* were increased in LPS-challenged rats. The decrease in *Gria1* mRNA expression was significantly inhibited by RV pretreatment. However, RV could not influence the *Gria2* and *Gria3* expressions. The inotropic glutamatergic receptors, including AMPA, Kinate, and NMDA, are essential for synaptic transmission and proper cognitive function.[60] As mentioned in the introduction, AMPA receptors consist of four subunits (GluA1–4) which can form both homotetramers and heterotetramers structures. However, GluA1 and GluA2 are mostly expressed in the hippocampus and cerebral neocortex.[61] It has been previously noted that upon activation of microglia in conditions like AD, the GluA2 expression significantly increases, while GluA1, GluA3, and GluA4 significantly decrease.[62] Previous studies have shown that GluA1 plays a distinctive role in the memory process.[63] Recently, researchers have shown that AMPA-Rs in CA1 pyramidal cells tend to shift from synapses to intracellular compartments in AD.[64] In addition, there is evidence showing that ubiquitination of the GluA1 subunit during aging may be an underlying mechanism of AD.[65] Such findings highlight the importance of the GluA1 subunit in different neurological pathologies such as AD.

It is well known that overstimulation of glutamatergic receptors causes excitotoxicity that eventually leads to neuronal impairment and cell death. In different neurodegenerative disorders, the excitotoxicity phenomenon is observed. Therefore, the use of glutamatergic receptor antagonists (such as memantine) is one of the therapeutic approaches in managing AD.[5] Karthick and colleagues, in an elegant study, investigated the effects of a single i.p. administration of RV (20 mg/kg) on hippocampal NMDA-Rs subunits (NR2A and NR2B) expression in ibotenic acid-induced excitotoxicity in rats. They found that intrahippocampal injection of ibotenic acid (an analog of glutamate) significantly down-regulated the mRNA expression of NR2A and up-regulated NR2B mRNA expression. Surprisingly, RV treatment reversed the alterations in NR2A/NR2B mRNA expression.[59] We showed that RV could also positively regulate GluA1 containing AMPA-Rs in an AD-like rat model. Hence, we can add this property to other mechanisms through which RV exerts its neuroprotective effects in AD.

Conclusion

Identifying and understanding how the synaptic function is controlled by nutrition and supplementation in the early stage of neurodegenerative diseases may give us clues to prolonging the prodromal stages of these diseases. The present study is the first to report that RV could positively modulate GluA1-containing AMPA-Rs dysregulation in a rat model of cognitive impairment. There are still several questions that must be answered to understand the potential crosstalk between mechanisms through which natural compounds such as RV could modulate synaptic function in the early stage of AD.

Ethical Issues

This study was approved by the Ethics Committee of Birjand University of Medical Sciences (permit code: Ir.bums.REC.1398.119).

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Author Contributions

MHT and MH conceptualized and designed the research, SH and MH performed the animal experiment. MM and MH performed biochemical, molecular and histopathological tests. MHT and MH analyzed the data and edited the manuscript draft. All authors read and approved the final manuscript.

Conflict of Interest

The authors report no conflicts of interest.

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