



Neuroprotective effects of lovastatin in the pilocarpine rat model of epilepsy according to the expression of neurotrophic factors

Pooyan Moradi¹ · Mahin Ganjkhani¹ · Iraj Jafari Anarkooli² · Alireza Abdanipour²

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Abstract

Studies have suggested that neurotrophic factors (NTFs) are involved in the status epilepticus development. This indicates their essential role in mediating acquired epileptic conditions. Therefore, modulating the expression of NTFs may inhibit seizure-induced cell loss in the epileptic lesions. In this study, we examined the anti-apoptotic, anti-necrotic and regulatory effects of lovastatin on the expression of NTFs in the pilocarpine rat model of temporal lobe epilepsy (TLE). A total of 32 male Wistar rats were divided into 4 groups ($n = 8$ per group): i) normal; ii) non-treated epileptic group [intraperitoneal (i.p.) administration of 350–400 mg/kg pilocarpine]; iii) treatment group (pilocarpine-treated rats treated followed by 5 mg/kg lovastatin); and iv) vehicle epileptic rats treated with Carboxymethyl cellulose (CMC). Animals that had a behavioral score of 4–5 according to the Racine scale were selected for study participation. Three days after the first seizure, pilocarpine-treated rats received i.p. injections of lovastatin for 14 days. The rats were killed and prepared for histopathologic analysis as well as real-time RT-PCR 17 days after the first seizure. The results of this study showed increased mRNA expression of glial cell line-derived neurotrophic factor (GDNF) and Ciliary neurotrophic factor (CNTF) and decreased expressions of Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4) mRNA in the epileptic rats treated with lovastatin. Histological analysis of neurodegeneration in the brain sections showed that the number of hippocampal apoptotic and necrotic cells significantly decreased in the treatment groups. Furthermore, numerical density of neurons per area was significantly higher in the treated groups compared with the untreated groups. Collectively, the results of this study have shown that lovastatin could attenuate seizure-induced expression of neurotrophic factors and consequently reduce hippocampal cell death in the pilocarpine rat model of TLE.

Keywords Lovastatin · Neurotrophic factors · Temporal lobe epilepsy · Pilocarpine

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- ✉ Mahin Ganjkhani
ghanjkhani@zums.ac.ir
- ✉ Alireza Abdanipour
abdani.anatomy@yahoo.com;
abdani.anatomy@zums.ac.ir

¹ Department of Physiology and Pharmacology, School of Medicine, Zanzan University of Medical Sciences (ZUMS), P.O. Box 45139-56184, Zanzan, Iran

² Department of Anatomy, School of Medicine, Zanzan University of Medical Sciences (ZUMS), P.O. Box 45139-56184, Zanzan, Iran

Introduction

Epilepsy is a chronic neurological disorder effecting millions of people around the world. There are different classifications and subtypes described for Epilepsy but temporal lobe epilepsy (TLE) is known to be most common and severe form of epilepsy accounting for almost 30% present of patients diagnosed with epilepsy (Télliez-Zenteno and Hernández-Ronquillo 2012). Hippocampal sclerosis, neurodegeneration, and a substantial reorganization of hippocampal circuits are among the neuropathological changes observed in hippocampal tissues obtained from patients with TLE (Buckmaster 2004; Strine et al. 2005). Although there has been a great improvement in treating various types of epileptic disorders, individuals suffering from TLE are often refractory to the antiepileptic drugs (AEDs), raising a need for alternative

approaches to treat and prevent this neurological disorder (Kalilani et al. 2018).

Neurotrophic factors (NTFs) are a group of growth factors promoting differentiation and survival in many different neuronal populations. They are also involved in regulating neurogenesis, synaptic plasticity and maintaining the activity of nervous system (Ivanisevic and Saragovi 2013; Maness et al. 1994). Neurotrophic factors are generally divided in three main families: (1) neurotrophins, (2) glial cell-line derived neurotrophic factor family ligands (GFLs) and (3) neuropoietic cytokines. Neurotrophin family of neurotrophic factors includes Brain-derived neurotrophic factor (BDNF), Nerve growth factor (NGF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4). Glial cell line-derived neurotrophic factor (GDNF) and Ciliary neurotrophic factor (CNTF) are the key members of GFLs and neuropoietic cytokines, respectively (Deister and Schmidt 2006; Henderson 1996). It has been widely reported that neurotrophic factors are one of the key mediators in epileptogenesis, inducing their effects via various signaling pathways and biomolecules (Simonato et al. 2006; Simonato and Zucchini 2010). The expression of the neurotrophic factors changes in different types of epileptic disorders, particularly in temporal lobe epilepsy. Different animal models of epilepsy show that each neurotrophic factor can exert anti-epileptic or pro-epileptic features. Interestingly a specific neurotrophic factor can exhibit both anti-epileptic and pro-epileptic effects in different time points of epilepsy progression. However, this endogenous protective responses are not sufficient to repair seizure-induced damage or to bring back normal activity to injured hippocampal regions (Iughetti et al. 2018; Kandratavicius et al. 2013; Simonato et al. 2006). To tackle this problem, numerous studies have been implemented to inhibit epileptic disorders by direct infusion of favorable neurotrophic factors to chronically injured hippocampus in order to repair damaged tissues and inhibit epileptogenesis (Falcicchia et al. 2018; Paolone et al. 2019). However, direct infusion of drugs has its own complications both in animal and clinical studies. Hence, treating epilepsy and associated neural damages by indirect therapies could be better option for treating epilepsy and associated neuropathological changes.

Statins, or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are medications used to treat dyslipidemia and reduce the risk of atherosclerosis. Over time, an extensive literature has been developed on neuroprotective effects of statins, unrelated to their cholesterol-reducing properties. Previous studies have shown statins can reduce vascular inflammatory response, stimulate angiogenesis, mediate cytokine production and decrease oxidative stress (McFarland et al. 2014; Wood et al. 2014). Evidence from recent research suggests that statins could induce neuroprotection in different animal models of epilepsy. It has been shown that statins induce neurogenesis, inhibits mossy fiber

sprouting and reduce apoptosis in the hippocampal formation (Lee et al. 2008; Lu et al. 2007; Rangel et al. 2005). Interestingly, preliminary data have shown that statins are able to modify the expression of neurotrophic factors in different animal models of neurological disorders (Roy et al. 2015; Yang et al. 2012). To date, the particular mediators of neuroprotective effects of statins on epilepsy are still unknown. Here, we tested anti-apoptotic and anti-necrotic features of lovastatin in animal model of temporal lobe epilepsy (TLE) with the consideration of neurotrophic factors expression.

Materials and methods

Animal study design

The environment, housing, freedom of movement, food, water and care provided for each animal in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines (Hollands 1986). We divided the 32 rats into 4 groups ($n = 8$ animals per group) as follows: i) normal rats (without any treatment); ii) non-treated epileptic rats (received 350–400 mg/kg pilocarpine); iii) epileptic rats treated with 1% carboxymethyl cellulose (CMC) as the vehicle; and iv) epileptic rats treated with once daily lovastatin (5 mg/kg) administered 3 days after the first seizure up to 14 days (treatment group).

Pilocarpine model of temporal lobe epilepsy (TLE)

The pilocarpine rat model of TLE is among the most well recognized models for human epilepsy. Rats injected with pilocarpine, develop cholinergic effects and recurrent seizures, followed by the appearance of chronic epilepsy (Leroy et al. 2003). Furthermore, animals that receive systemic administration of pilocarpine tend to show behavioral alterations and partial seizures that culminate in limbic SE (Cavalheiro et al. 1991). A total of 32 adult male Wistar rats that weighed 250–300 g were housed under controlled standard conditions that consisted of a standard 12-h light/dark cycle, room temperature (RT) of 21–22 °C, and 45%–55% humidity. Rats were given ad libitum access to food and water. The rat epilepsy model was performed by an intracellular injection of pilocarpine as described previously (Abdanipour et al. 2011). Briefly, 30 min before the pilocarpine injection, these rats received methylscopolamine bromide (1 mg/kg s.c.) to reduce the side effects of the intraperitoneal (i.p.) injection of pilocarpine hydrochloride (350–400 mg/kg). One hour after the first epileptic incidence, each affected rat received an injection of 2.5 mg/kg diazepam. We administered the Racine behavioral test to evaluate the epileptic rats. Animals that scored 4,5 were selected for the study (Racine 1972). After 17 days, the

animals were killed for tissue studies and gene expression analysis. Researchers have stated that structural changes in the hippocampus and changes in the expression of genes occur during the second and third weeks after the onset of epileptic seizures (Hansen et al. 2014; Jongbloets et al. 2015).

Apoptosis detection by TUNEL assay and acridine orange (AO) staining

We used Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay to detect seizure-induced apoptotic cells in CA1–4 areas of hippocampal formation. Acridine orange (AO) staining were also performed in order to detect necrosis in similar epileptic regions of hippocampus. At 17 days after the first SE (after the last injection of lovastatin), the animals were anesthetized with ketamine (44 mg/kg) and xylazine (13 mg/kg), transcardially perfused with 20 ml heparinized saline, and then perfused with 4% paraformaldehyde in PBS (pH 7.4). The brain tissues were immersed in the same fixative for the next 24 h (Cavazos et al. 1994). For each animal, we selected one hemisphere of 10 different sections, 10 μm thickness, located 2800–3800 mm distance from the bregma for histological evaluation by acridine orange (AO) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining. Cell morphological changes in the necrosis were assessed by AO staining. The paraffin sections were dewaxed and dehydrated in a graded ethanol series (50%, 70%, 85%, 95%, 100%). Then, the sections were permeabilized by incubation in PBS with 0.1% Triton X-100 and 0.1% sodium citrate for 15 min, and in 20 $\mu\text{g}/\text{ml}$ proteinase K for 15 min (Yeh et al. 2008). For AO staining, the sections were incubated in 6 $\mu\text{g}/\text{ml}$ AO (Sigma) in 0.1 M citric acid and 0.2 M Na_2HPO_4 (pH 2.6) for 30 min at RT. The stained sections were observed under a fluorescent microscope. We counted total cells and the number of necrotic cells with orange/yellow stained cytoplasm were assessed. The necrotic cells had a nuclear morphology which resembled the viable cells without any condensed chromatin and fragmented nuclei. For TUNEL staining, the sections were assayed with an In Situ Cell Death Detection Kit (Roche, Germany) in accordance with the manufacturer's instructions. TUNEL-positive cells were exposed to 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Germany) chromogen and counterstained with hematoxylin. Quantification of TUNEL-positive cells was measured in all hippocampus regions (CA1, CA2, CA3, and CA4). Neuronal apoptosis was determined by the TUNEL assay.

Real-time RT-PCR

Real-time RT-PCR was carried out with prepared cDNA from all of the experimental groups. Total RNA from hippocampal

tissues were isolated by TRIzol® (Invitrogen/Life Technologies). We used 1000 ng of purified RNA to synthesize 20 μl of cDNA according to a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany) in accordance with the manufacturer's instructions. The cDNA was used to quantify mRNA levels of the neurotrophins *BDNF*, *GDNF*, *CNTF*, *NGF*, *NT-3*, and *NT-4*. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for normalization. Table 1 lists the primer sequences. We performed the PCR reaction in a 12.5 μl final reaction volume that contained forward and reverse primers (200 nM each), cDNA (0.5 μl ; 25 ng of RNA samples), SYBR® Green I (6.5 μl ; Fermentas; Thermo Fisher Scientific, Inc.) and nuclease-free water up to final volume for 40 cycles at 95 °C for 15 s followed by 60 °C for 1 min. Relative changes in target mRNA levels were determined using the $\Delta\Delta\text{C}_q$ method (Xu et al. 2017).

Statistics

Statistical analyses were performed with SPSS version 15. All data are presented as mean \pm standard error of mean (SEM) from independent experiments that were repeated 5 times. One-way ANOVA followed by Tukey's post hoc were used for data comparison between the groups. The level of significance was set at $P \leq 0.05$.

Results

Pilocarpine-treated rats

We found that within 5 min after the pilocarpine injection, the rats developed peripheral side effects of cholinergic signs that included diarrhea, salivation, and lacrimal gland activation. During the following 15–20 min, the rats exhibited signs of head nodding, scratching, masticatory jaw movements, chewing, and exploratory behavior. Recurrent seizures began approximately 49.37 ± 2.99 min after pilocarpine administration. We observed that approximately 60% of the animals exhibited seizures after the pilocarpine injection. The initial acute insult was followed by a seizure-free phase (latent) and finally a chronic period.

Detection of apoptotic cells (TUNEL assay)

Figures 1a and 2 show the results. The percentage of apoptotic cells were calculated in the CA1, CA2, CA3, and CA4 regions of the hippocampus. Figure 2a shows the mean percentage of apoptotic cells in the treated CA1 (44.62 ± 3.7), CA2 (33.08 ± 3.08), CA3 (43.13 ± 3.17), and CA4 (40.16 ± 4.76) groups as well as for the non-treated CA1 (62.42 ± 3.66), CA2 (67.11 ± 3.36), CA3 (64.51 ± 2.76), and CA4

Table 1 Primer sequences and PCR parameters. Primers for amplification of target sequences, their Gen Bank accession number and size of the fragment amplified are presented

Gene	Gene Accession no.	Sense 5 → 3	Anti-sense 5 → 3
<i>NT-3</i>	NM_031073	GAGGGACCATTCGAGGTGAC	CCAGGGACGTCGACATGAAG
<i>NT-4</i>	NM_013184	ATCAGAGGACCCCTGACTTAC	GATACGGTGCTCAGGATAGA
<i>BDNF</i>	NM_001270636.1	TTGAGCACGTGATCGAAGAG	CTCCAGCAGAAAGAGCAGAG
<i>GDNF</i>	NM_019139.1	CGGACGGGACTCTAAGATGA	ATATTGGAGTCACTGGTCAGC
<i>NGF</i>	NM_001277055.1	TCATCCACCCACCCAGTCTTCC	TAATGTTACCTCGCCCAGCAC
<i>CNTF</i>	NM_013166.1	GAGCAAACACCTCTGACCCT	CAACGATCAGTGCTTGCCAC
<i>GAPDH</i>	NM_017008.4	TTGTCAGCAATGCATCCTGCAC	GTCTGGGATGGAATTGTGAG

Primers were designed by Gene Runner 3.05 software (Product by: info@genfanavaran.com). *NT-3*, Neurotrophin 3; *NT-4*, Neurotrophin 4; *BDNF*, Brain-derived neurotrophic factor; *GDNF*, Glial cell line-derived neurotrophic factor; *NGF*, Nerve growth factor; *CNTF*, Ciliary neurotrophic factor; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase

(60.7 ± 2.75) groups. The number of apoptotic cells in the CA1, CA2, and CA3 regions significantly decreased in the lovastatin treated group compared with the non-treatment group ($P \leq 0.05$). Figure 2 shows TUNEL staining of the cells and hippocampal tissues.

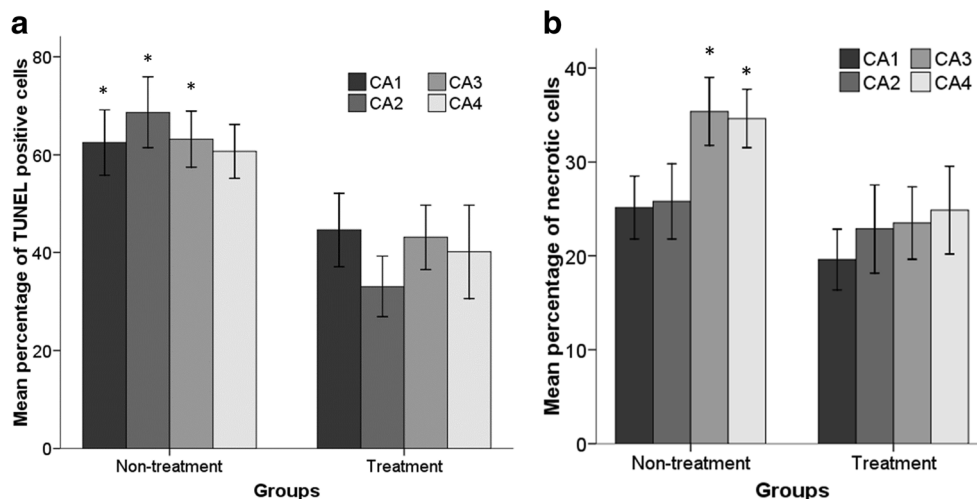
Detection of necrotic cells (acridine orange staining)

Figures 1b and 3 show the results. The percentage of necrotic cells were calculated in the CA1, CA2, CA3, and CA4 regions of the hippocampus. Figure 2b shows the mean percentage of necrotic cells in the treated CA1 (19.6 ± 1.61), CA2 (22.87 ± 2.34), CA3 (23.5 ± 1.93), and CA4 (24.86 ± 2.34) groups and non-treatment (CA1 (25.14 ± 1.68), CA2 (25.8 ± 2.00), CA3 (35.39 ± 1.8), and CA4 (34.63 ± 1.64) groups. The numbers of necrotic cells in the CA3 and CA4 regions significantly decreased in the lovastatin treated group compared with the non-treatment group ($P \leq 0.05$). Figure 3 shows the acridine positive cells.

Gene expression

The changes in expressions of *BDNF*, *GDNF*, *CNTF*, *NGF*, *NT-3*, and *NT-4* mRNA in the experimental groups were examined by using quantitative real-time RT-PCR. The results were separately presented relative to the normal (Fig. 4) and non-treatment (Fig. 5) groups. The lovastatin treated group (relative to the normal group) had significantly increased expression of *GDNF* (3.51 ± 0.27) and *CNTF* (2.00 ± 0.2) compared with the non-treatment group. The non-treatment group (relative to the normal group) had significantly increased expressions of *BDNF* (3.2 ± 0.33), *NGF* (0.22 ± 0.01), *NT-3* (5.5 ± 0.32), and *NT-4* (0.75 ± 0.04) compared with the lovastatin treated group (Fig. 4). In the group treated with lovastatin (relative to the non-treatment group), we observed significantly increased expressions of *BDNF* (0.38 ± 0.03), *GDNF* (3.5 ± 0.27), and *CNTF* (1.39 ± 0.07) compared with the normal group ($P \leq 0.05$; Fig. 5). There was no significant change in *NGF* mRNA expression relative to both the non-treatment and normal groups.

Fig. 1 The effects of lovastatin on inhibition of apoptosis (TUNEL assay). **a** the mean percentage of apoptotic cells in the experimental groups. **b** the mean percentage of necrotic cells (acridine positive cells). Apoptotic and necrotic cells were calculated in the 4 regions (CA1, CA2, CA3 and CA4) of hippocampus. The bars indicate the mean \pm SEM; * $P \leq 0.05$ compared to lovastatin treated group



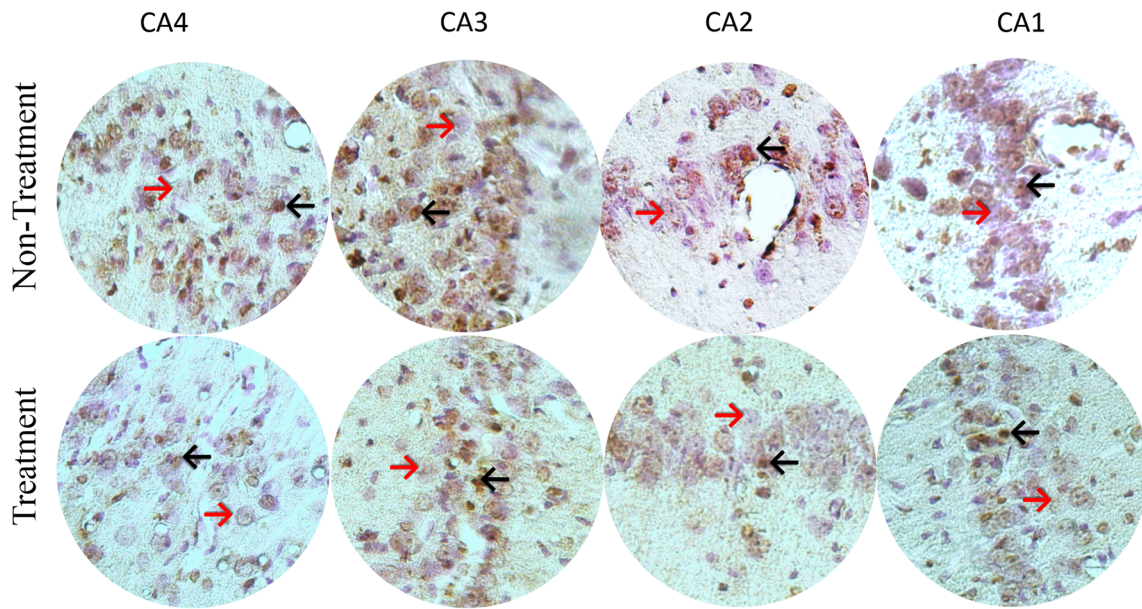


Fig. 2 Representative Images of TUNEL staining in the (CA1, CA2, CA3 and CA4) regions of hippocampus. The photomicrographs shows TUNEL positive cells in the experimental groups. Black and red arrows

shows DNA fragmentations and non-fragmented respectively. Hematoxylin was used for counterstaining. Magnification: 200 ×

Discussion

In the present study, our findings showed that mRNA levels of neurotrophic factors are modified after inducing temporal lobe epilepsy (TLE). Further, we showed that treating epileptic animals with lovastatin regulated the expression of neurotrophic factors. There was an increase in both GDNF and CNTF mRNA expressions in epileptic animals treated with lovastatin for 14 days post TLE. Also there was a significant decrease in mRNA expression of BDNF, NT-3, and NT-4 relative to the

non-treatment groups. In addition, we observed significant levels of apoptosis and necrosis in CA1–4 areas of hippocampus in animals treated with pilocarpine. In this study we showed that treating with lovastatin for 14 days after TLE, could significantly decrease apoptosis and necrosis in CA1–4 areas of hippocampus. Taken together, we suggest that lovastatin induces its neuroprotective effects by regulating the expression of neurotrophic factors in the hippocampal formation.

Neurotrophic factors are essential for survival of the neuronal populations. They are involved in neural development

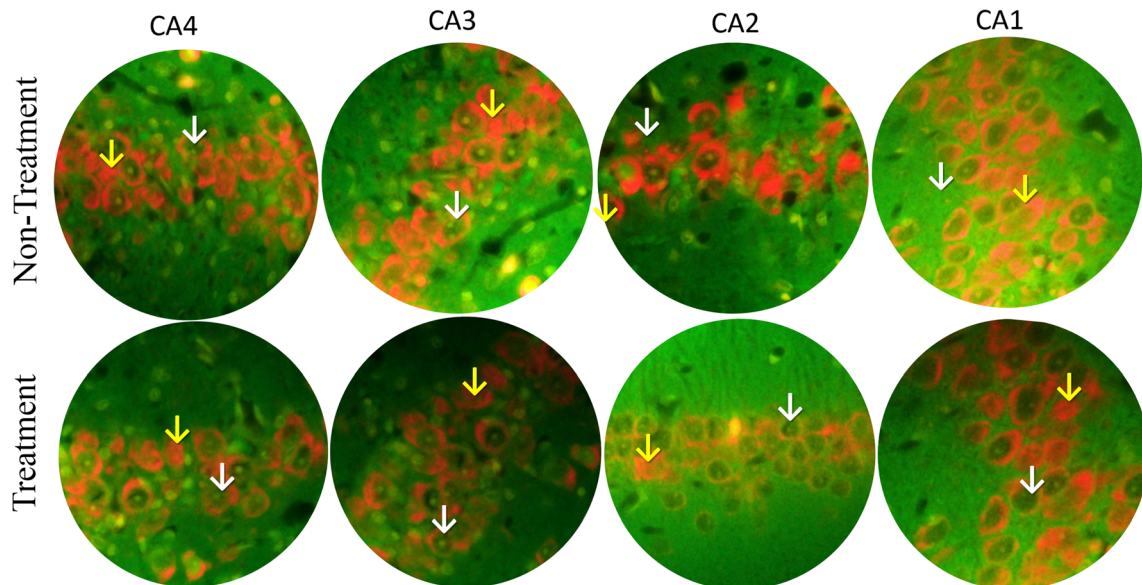


Fig. 3 Representative Images of acridine staining in the (CA1, CA2, CA3 and CA4) regions of hippocampus. The photomicrographs shows acridine positive cells in the experimental groups. Yellow and white arrows shows death and intact cells respectively. Magnification: 200 ×

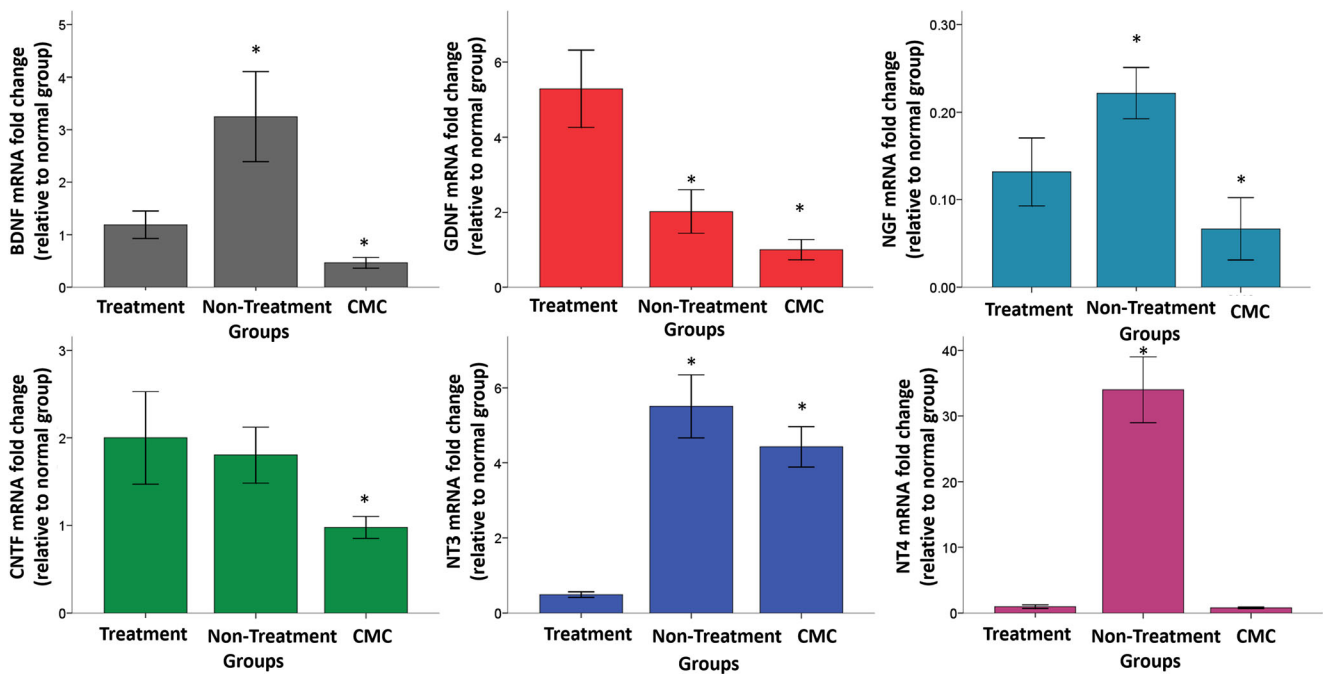


Fig. 4 Quantitative real-time RT-PCR results, relative to normal group. mRNA is presented as relative expression normalized to *GAPDH* mRNA amplification. The bars indicate the mean \pm SEM; * (compared with treatment group), $P \leq 0.05$

and plasticity in developing and mature nervous system. Proliferation, maturation and normal function of neurons are highly dependent on neurotrophic factors. All these critical roles, are indicators of essential involvement of this group of growth factors in different neuronal populations (Friedman 2012; Johnson and Tuszynski 2008). Acute or chronic

changes in the expression of neurotrophic factors may result in neurodegeneration, excitotoxicity, aberrant axonal sprouting and extensive cell loss (Iughetti et al. 2018; Sampaio et al. 2017). As stated before, expression level of different neurotrophic factors is altered in different neurological disorders including epilepsy, resulting in pro-epileptic or

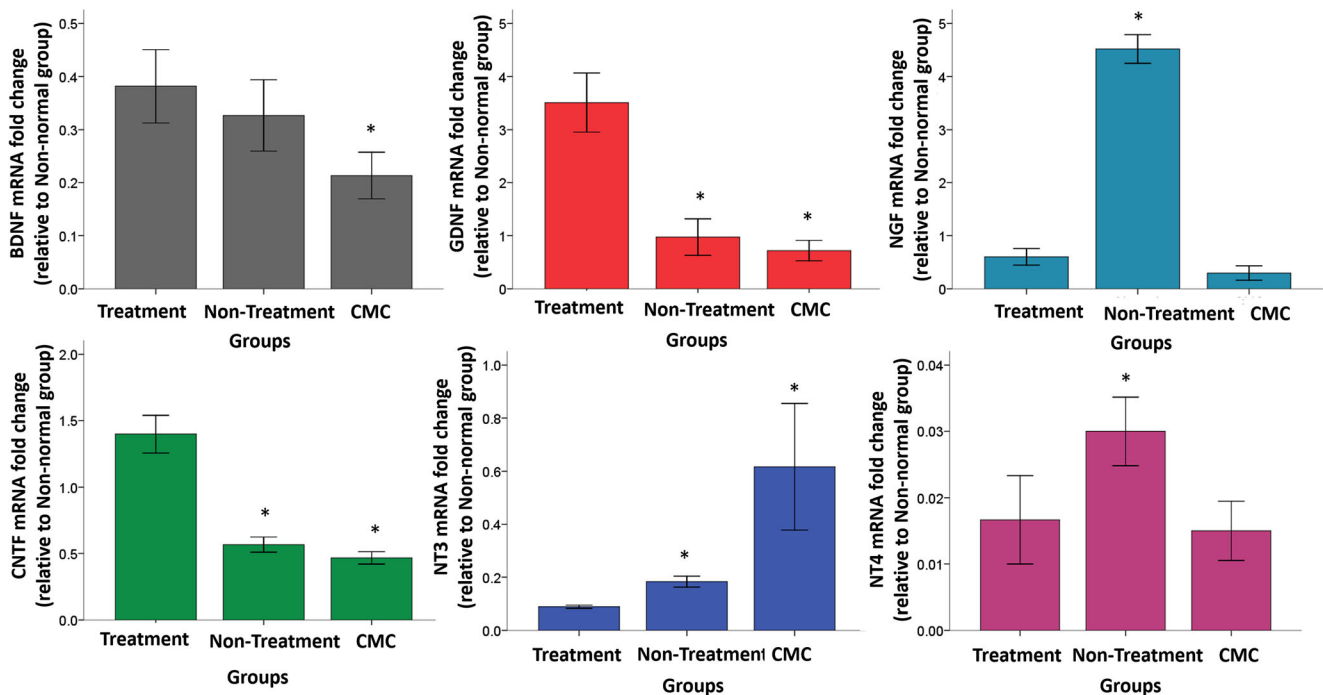


Fig. 5 Quantitative real-time RT-PCR results, relative to non-treatment group. mRNA is presented as relative expression normalized to *GAPDH* mRNA amplification. The bars indicate the mean \pm SEM; * (compared with treatment group), $P \leq 0.05$

anti-epileptic effects. These neuroprotective responses are not sufficient to completely retard epileptogenesis; therefore neural damage would be still life-threatening. Hence, extrinsic treatment of damaged epileptic regions would be a more efficient approach to manage neuropathological alterations. It should be kept in mind that treating epilepsy by modifying the expression of neurotrophic factors is a challenging approach since each distant growth factor may induce “good” or “bad” effects on the epileptic regions. Results from our study provided preliminary evidence suggesting that epileptic animals treated with lovastatin showed significant neurological improvement at two weeks post TLE. Interestingly, treating with lovastatin also modified the seizure-induced expression of NTFs in the CA1–4 regions of the hippocampus. The neurological improvements recorded in this research are along with the findings from previous studies reporting significant preventative effects of statins on epileptic structural alterations (Lee et al. 2012; McFarland et al. 2014). Furthermore, results from real-time PCR experiment showed that treating with lovastatin up-regulated the expression of GDNF and CNTF mRNAs and down-regulated the expression of BDNF, NT-3 and NT-4 mRNAs in the CA1–4 regions, two weeks post TLE. Here, we suggest that reduction in apoptotic and necrotic lesions of hippocampus is probably due to the lovastatin’s capacity to regulate the expression of different neurotrophic factors. Up-regulation of GDNF and CNTF can be considered as one of the anti-epileptic and neuroprotective features of lovastatin. GDNF therapy and its anti-epileptic effects has been shown in recent studies. Continues administration of encapsulated GDNF to the targeted areas, was successful to induce neuroprotection and reduced seizures in pilocarpine model of epilepsy (Paolone et al. 2019). Downstream intracellular molecular mechanisms activated by GDNF, maintain viability and inhibit apoptosis via Ret-mediated signaling cascades in different types of neurons and glial cells. Preserving AMPA receptor levels and GluR2 subunits are the suggested molecular events accompanied with GDNF therapy (Shishkina et al. 2018). CNTF, another potential anti-epileptic agent, has been shown to inhibit epileptogenesis and reduce cell loss mainly by activation of astrocytes and stimulating them to migrate towards the neural populations. Further, pretreatment with CNTF, diminished structural alterations after Kainic Acid induced epilepsy (Bechstein et al. 2012). Hippocampal neuroprotection observed in experimental groups can be also result of down-regulation of pro-epileptic neurotrophic factors such as BDNF, in animals treated with lovastatin. Effects of BDNF induction and its coupled receptor (TrkB) on the development of epilepsy has been extensively studied in different animal models of epilepsy (Heinrich et al. 2011; Ryuta and Yuji 2005; Scharfman 2005). However, there are contradictory results in the recent studies showing that BDNF can induce anti-epileptic effects via chronic and targeted delivery to the epileptic regions. It has

been suggested that BDNF induces its anti-epileptic effects by activation of other neuropeptides such as neuropeptide Y (Falcicchia et al. 2018; Iughetti et al. 2018). Finally we observed down-regulation of NT-3 and NT-4 in epileptic rats treated with lovastatin. There are limited studies on the role of NT-3 and NT-4 in epileptogenesis, making it hard to draw a conclusion from our results. NT-3, by itself, induces axonal sprouting in the mossy fiber system. However, it decreases kindling-induced mossy fiber sprouting when combined with kindling (Xu et al. 2002).

Collectively, in this study we suggest that lovastatin-induced modification of neurotrophic factors is a probable underlying cause for neurologic improvement in the epileptic areas of hippocampus. Our data has added to a growing body of literature on distinct neuroprotective effects of statins on several neurological disorders including TLE. However, it should be kept in mind that different statins exert various types of neuroprotection and each distinct NTF can induce variable effects in different animal models for human diseases. Additional studies are necessary to validate the conclusions drawn from this study and should be devoted to investigation of the possible mediators of statin-induced expression of neurotrophic factors and also to further investigation of neuroprotective features of neurotrophic factors.

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Author contributions Pooyan Moradi: Executing the research project, edited the manuscript; Mahin Ganjkhani: supervised experiments; Iraj Jafari Anarkooli: Advisor; Alireza Abdanipour: designed the study, analyzed the data, wrote the manuscript, and supervised experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Ethics approval and consent to participate All experimental protocols were approved by the Zanjan University of Medical Sciences ethics committee.

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