

# Cannabidiol Post-Treatment Alleviates Rat Epileptic-Related Behaviors and Activates Hippocampal Cell Autophagy Pathway Along with Antioxidant Defense in Chronic Phase of Pilocarpine-Induced Seizure

Mahshid Hosseinzadeh<sup>1</sup> · Sara Nikseresht<sup>1</sup> · Fariba Khodaghali<sup>1,2</sup> · Nima Naderi<sup>1,3</sup> · Nader Maghsoudi<sup>1,2</sup>

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**Abstract** Abnormal and sometimes severe behavioral and molecular symptoms are usually observed in epileptic humans and animals. To address this issue, we examined the behavioral and molecular aspects of seizure evoked by pilocarpine. Autophagy can promote both cell survival and death, but there are controversial reports about the neuroprotective or neurodegenerative effects of autophagy in seizure. Cannabidiol has anticonvulsant properties in some animal models when used as a pretreatment. In this study, we investigated alteration of seizure scores, autophagy pathway proteins, and antioxidant status in hippocampal cells during the chronic phase of pilocarpine-induced epilepsy after treatment with cannabidiol. Cannabidiol (100 ng, intracerebroventricular injection) delayed the chronic phase of epilepsy. Single administration of cannabidiol during the chronic phase of seizure significantly diminished seizure scores such as mouth clonus, head nodding, monolateral and bilateral forelimb clonus and increased the activity of catalase enzyme and reduced glutathione content. Such a protective effect in the behavioral scores of epileptic rats was also observed after repeated administrations of cannabidiol at the onset of the silent phase. Moreover, the amount of Atg7, conjugation of Atg5/12, Atg12, and LC3II/LC3I ratio increased significantly in epileptic rats treated with

repeated injections of cannabidiol. In short, our results suggest that post-treatment of Cannabidiol could enhance the induction of autophagy pathway and antioxidant defense in the chronic phase of epilepsy, which could be considered as the protective mechanisms of cannabidiol in a temporal lobe epilepsy model.

**Keywords** Pilocarpine-induced seizure · Cannabidiol · Autophagy · Antioxidant status

## Introduction

One of the most common forms of partial epilepsy in humans is temporal lobe epilepsy (TLE) (Engel, 2001). Characterization of this model shows three phases: (a) a period of 24 h is known as the acute phase that extended to limbic area and causes status epilepticus (SE), (b) the second phase is a silent period where electroencephalogram and behavior are both normal and vary from 4 to 44 days, and (c) a third period known as chronic phase is characterized by spontaneous recurrent seizures (SRSs) (Cavalheiro, 1995, Arida et al. 1999). Localization of seizure foci in the limbic system, particularly in the hippocampus, is the main feature of TLE (Bartolomei et al. 2005).

Autophagy, one of the most important pathways that maintain cell homeostasis, can regulate important biological functions such as cell survival, cell death, cell metabolism, development, neuroprotection, and sometimes neurodegeneration (Hochfeld et al. 2013). In this process, cytoplasmic components are delivered to lysosomes to form autophagosomes. In this process, a group of autophagy-related proteins (Atgs) have vital roles (Levine and Klionsky, 2004, Meijer, 2003). Among these proteins, Atg5 initiates the process (Maiuri et al. 2007). The participation of two ubiquitin-like conjugation

✉ Nader Maghsoudi  
nmaghsoudi@sbmu.ac.ir

<sup>1</sup> Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>2</sup> NeuroBiology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>3</sup> Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

systems is necessary for formation of the autophagosomes. One of them is conjugation of Atg12 to Atg5 and another is binding of the phosphatidylethanolamine to LC3/Atg8 (Rubinsztein et al. 2007). The amount of LC3II (LC3-phosphatidylethanolamine conjugate) is correlated with the number of autophagosomes (Kabeya et al. 2000). In many neurodegenerative diseases like Parkinson's, Huntington's, and Alzheimer's disease (Hara et al. 2006, Komatsu et al. 2006) autophagy is a necessary route to remove abnormal proteins; autophagy is considered as a target pathway that can relieve signs of illness or prevent diseases (Berger et al. 2006, Momeni et al. 2006); so induction of autophagy may protect against a range of neurodegenerative diseases (Winslow and Rubinsztein, 2008, Zare et al. 2015).

In the epileptiform activity of the hippocampus, the endocannabinoid system has a key role (Wallace et al. 2002, Monory et al. 2006, Ludanyi et al. 2008). Cannabidiol (CBD) as a non-psychoactive phytocannabinoid has been used for protection against epilepsy, anxiety, and psychosis in rodent models (Iuvone et al. 2009, Scuderi et al. 2009). This component is involved in many pathways such as inhibition of NF- $\kappa$ B signaling (Kozela et al. 2010) and decrease of cytokine production including tumor necrosis factor- $\alpha$  and interleukins (Kozela et al. 2010, Puffenbarger et al. 2000). On the other hand, CBD is a potent antioxidant (Fernandez-Ruiz et al. 2013). CBD also has anti-convulsive effects in some epileptic animal models like the maximal electroshock (MES), a model of partial seizure with secondary generalization, and audiogenic seizure models (Consroe and Wolkin, 1977). All these papers have studied the effects of CBD before seizure induction as pretreatment and its effect remain untested in other animal seizure models (Gordon and Devinsky, 2001). Furthermore, there is no report regarding the effect of CBD after induction of seizure as a post-treatment in behavioral scores and molecular pathways of epilepsy. Since induction of damage and abnormal sprouting in silent phase causes epileptic activities in chronic phase, improvement of behaviors in chronic phase following treatment in silent phase is critical for alleviation of epileptic-related behaviors. In this study, we investigated the therapeutic effect of CBD on the induction of seizure-related behavior, autophagy-related proteins, and antioxidant status in the chronic phase of pilocarpine-induced seizure.

## Material and Methods

### Animals

Male and female Wistar rats weighting 200–250 g were used in this study. Animals were housed at a temperature of 25  $\pm$  2 °C on a 12:12-h light/dark cycle (lights on at 08:00 a.m.) and given ad libitum access to food and water. All procedures

were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved in accordance with ethical guidelines for animal subject care and use at Neuroscience Research Center, Shahid Beheshti University of Medical Sciences.

### Surgical Procedures

Under anesthesia by ketamine (87 mg/kg intraperitoneal (i.p.), Alfasan, Woerden, Holland) and xylazine (13 mg/kg i.p., Alfasan, Woerden, Holland), (Eftekhari et al. 2014) a guide cannulae was implanted in the right ventricle of rats using stereotaxic surgery (1.6 mm lateral and 0.5 mm posterior to bregma, 4.2 mm deep from dura); (Paxinos and Watson, 2007). Seven days later, seizure was induced by i.p. injection of pilocarpine.

### Pilocarpine-Induced Seizure

Based on the method described by Pestana et al., (Pestana et al. 2010), rats were treated with pilocarpine (360 mg/kg, i.p.; Sigma-Aldrich Co. St Louis, USA) 20 min after methyl scopolamine (5 mg/kg, subcutaneously; Sigma-Aldrich Co., St. Louis, USA) administration in order to reduce the peripheral effects of pilocarpine. Seizures were allowed to last for 60 min and then were terminated by the administration of diazepam (10 mg/kg, i.p.) to reduce rate of animal death, but about 10 % of animals died because of seizure and the remaining rats all showed SE. Only rats that displayed SE (stages 3–5) were selected (Racine, 1972).

### Study Design

#### *Behavioral Studies*

Twenty-four rats were randomly selected in three distinct groups (eight rats in each group). In the first group, seizure was induced by pilocarpine injection. In the second group, 24 h after pilocarpine-induced seizure, repeated intracerebroventricular (i.c.v.) administration of CBD (100 ng (Shirazi-zand et al. 2013); Sigma-Aldrich Co., St. Louis, USA) was performed for five consecutive days. In the third group, animals received a single injection of CBD (100 ng) when the chronic phase of seizure was initiated. The onset of chronic phase was approximately 4 weeks after seizure induction by pilocarpine and was determined by daily visual observation (Table 1).

### Seizure-Related Behaviors

In order to detect the first occurrence of SRS in the chronic phase of epilepsy, rats were monitored after about 14 days post-status epilepticus induced by pilocarpine injection until

**Table 1** Behavioral study design

Groups	Surgery	Recovery (7 days)	Pilocarpine injection (360 mg/kg)	Acute phase of epilepsy (24 h)	Silent phase of epilepsy (3–4 weeks)	Onset of chronic phase of epilepsy
Pilocarpine (PLC)			+	Without any manipulation	Without any manipulation	Behavioral test
Repeated CBD injection	+	+	+	Without any manipulation	100 ng CBD (i.c.v.) in successive 5 days	Behavioral test
Single CBD injection	+	+	+	Without any manipulation	Without any manipulation	100 ng CBD (i.c.v.) 24 h later: behavioral test

occurrence of SRS (Eftekhari et al. 2014). We observed animals from the third week after pilocarpine injection to detect initiation of chronic phase. Following chronic phase onset, seizure-related behaviors were recorded for 6 consecutive days from 9:00 a.m. to 4:00 p.m. based on the method described by Racine (Racine, 1972). These behaviors were divided into four stages: stage 1, staring and mouth clonus; stage 2, automatism behavior; stage 3, monolateral forelimb clonus; and stage 4, bilateral forelimb clonus and rearing. The seizure-related behaviors were recorded during chronic phase of epilepsy. Moreover, the day onset of chronic phase of epilepsy was recorded (Table 1).

### Molecular Studies

In a set of experiments, the animals were divided into six groups as follows: (1) Sham group: animals received pilocarpine vehicle, saline (i.p.), and 1  $\mu$ l CBD vehicles dimethylsulfoxide 10 % and phosphate buffer solution 90 % (i.c.v.); (2) Pilocarpine group: i.p. injection of pilocarpine with no further treatment; (3) Single injection of CBD group: animals received single injection of CBD (100 ng, 1  $\mu$ l, i.c.v.); (4) Repeated injections of CBD group: animals received repeated injections of CBD for 5 consecutive days (100 ng, 1  $\mu$ l, i.c.v.); (5) Pilocarpine and single injection of CBD group: animals received single injection of CBD (as described earlier) in the onset seizure chronic phase; and (6) Pilocarpine and repeated injections of CBD group: animals received repeated injections of CBD 24 h following pilocarpine injection (as described earlier).

### Western Blot Analysis for Quantification of Atg7, Atg5-12, Atg12, and LC3-II in Rat Hippocampus

Hippocampal tissues were collected in the onset of the chronic phase of epilepsy. Animals were sacrificed by decapitation and hippocampi immediately transferred to liquid nitrogen, frozen, and then stored in  $-80$  °C until the day of assessment. Tissues were homogenized with lysis buffer containing protease inhibitor cocktail. After

centrifuging, the supernatants were transferred to the new tubes. Protein concentration was determined by the Bradford protein assay method (Bradford, 1976). Equal amounts of protein samples and pre-stained protein ladder were electrophoretically separated on SDS polyacrylamide gels, and then transferred to a polyvinylidene fluoride (PVDF) membrane. The blots were incubated in skimmed milk in Tris-buffer saline Tween 20 (TBST) for 75 min at room temperature on a shaker, and then incubated overnight with primary antibody against Atg12 (1:1000; Cell Signaling, USA); Atg7 (1:1000; Cell Signaling, USA); and LC3-II (1:1000; Cell Signaling, USA) at 4 °C. The blots were rinsed 10 min with TBST three times and incubated with secondary antibody for 90 min. After washing with TBST, protein bands were visualized using the enhanced chemiluminescence assay (ECL, Amersham), then the images were analyzed by an image analyzer, ImageJ software, to subtract background and to perform densitometry. All western blots were replicated three times. Beta-actin was used as internal control.

### Measurement of Catalase Activity and Reduced Glutathione Content

According to Aebi method (Aebi, 1984),  $H_2O_2$  could be decayed by catalase activity. To determine catalase activity spectrophotometrically, 60  $\mu$ g of total protein of each sample was added to  $H_2O_2$  and then its decomposition was monitored at 240 nm. And in order to measure reduced glutathione content, the method of Ellman (Ellman, 1959) was applied.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.01. Independent sample *t* test and one-way ANOVA followed by Tukey post-test were used for comparison between two and three or more groups, respectively. A *p* value less than 0.05 was considered statistically significant. Data were shown as mean  $\pm$  S.E.M.

## Results

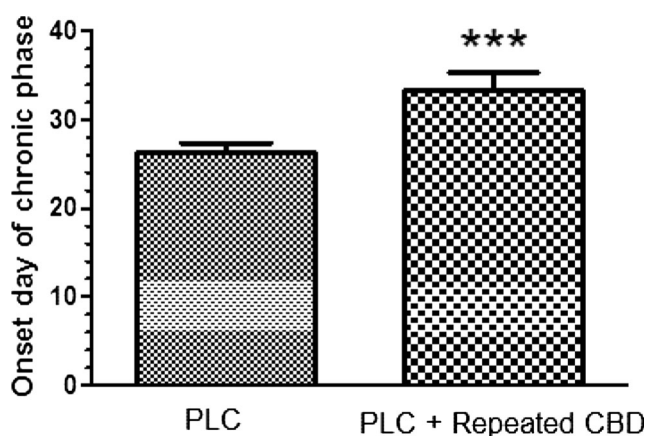
### Behavioral Results

#### *CBD Delayed the Day Onset of Chronic Phase of Epilepsy*

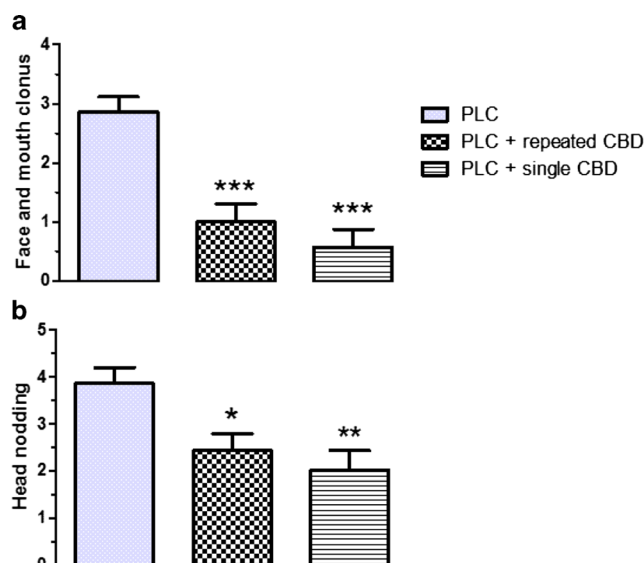
CBD was administered 24 h after pilocarpine-induced SE and repeated for 5 successive days and significantly postponed the day of initiation of chronic phase of seizure ( $p < 0.001$ ). The SRS onset was occurred between the 30th and 37th day after repeated injections of CBD in pilocarpine-induced seizure rats compared to the 25th and 27th day in epileptic rats without CBD treatment ( $26.29 \pm 0.42$ ; Fig. 1). In other words, 24 h after pilocarpine injection, the silent phase of seizure was initiated in animals and administration of CBD following initiation of silent phase of seizure for 5 consecutive days, postponed the initiation of chronic phase of seizure.

#### Attenuation of Behavioral Scores by Post-Treatment of CBD

All the rats in the pilocarpine group exhibited facial and mouth clonus but the frequency of this behavior was significantly different among groups [ $F(2,23) = 17.59$ ,  $p < 0.0001$ ; Fig. 2a]. Further analysis by Tukey's post test revealed a significant decrease both in rats that received repeated i.c.v. injections of CBD ( $p < 0.001$ ) and in rats that received single dose of CBD ( $p < 0.001$ ), suggesting CBD could attenuate the severity of this seizure behavior. Furthermore, there was a significant difference in the frequency of head nodding among groups receiving either



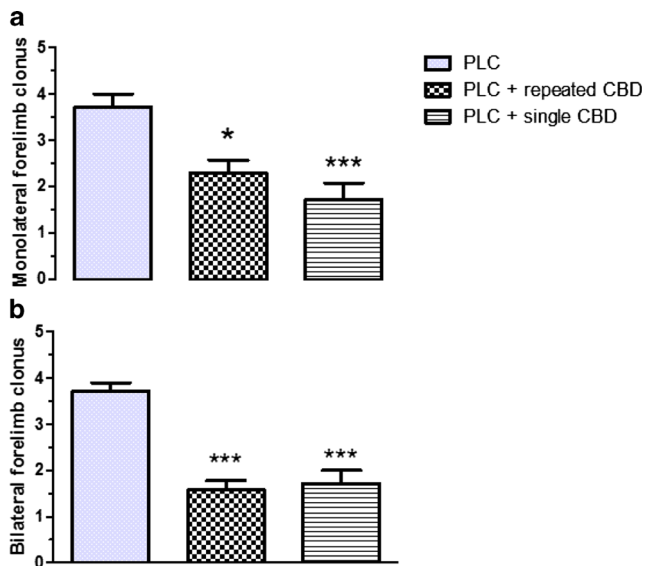
**Fig. 1** Repeated injection of cannabidiol (CBD; 100 ng/rat, i.c.v. injection) delayed the initiation of chronic phase of seizure. The onset day of the chronic phase of epilepsy and occurrence of spontaneous recurrent seizure was significantly delayed compared to epileptic rats with no CBD treatment, pilocarpine (PLC). \*\*\* $p < 0.001$  significant difference compared with the PLC group



**Fig. 2** **a** Effect of cannabidiol (CBD; 100 ng/rat, i.c.v. injection) treatment on the number of face and mouth clonus in chronic phase of pilocarpine (PLC)-induced seizure. There is a significant reduction in the number of face and mouth clonus in both groups that were treated with cannabidiol compared with the pilocarpine group. **b** Moreover, CBD both in single and in repeated administration significantly reduced number of head nodding compared with PLC group. Each bar represents mean  $\pm$  SEM of data from eight rats. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  significant difference compared with the PLC group

repeated or single injection of CBD and rats receiving no further treatment after pilocarpine-induced SE [ $F(2, 23) = 6.41$ ,  $p < 0.01$ ; Fig. 2b]. Post hoc analysis showed that CBD injection caused a decrease in the number of head nodding both in single ( $p < 0.01$ ) and in repeated ( $p < 0.05$ ) administration of CBD compared to the epileptic rats with no treatment (Fig. 2b). CBD could also significantly change the occurrence of monolateral forelimb clonus [ $F(2,23) = 10.88$ ,  $p < 0.0001$ ; Fig. 3a]. Post hoc analysis revealed that both single-dose injection of CBD in the chronic phase of seizure ( $p < 0.001$ ) and repeated injections of CBD after pilocarpine-induced SE ( $p < 0.05$ ) could significantly diminish the number of monolateral forelimb clonus compared to the group receiving no CBD treatment (Fig. 3a).

The bilateral forelimb clonus was also significantly changed among groups [ $F(2,23) = 27.52$ ,  $p < 0.001$ ; Fig. 3b]. Further analysis revealed that both single administration of CBD during chronic phase ( $p < 0.001$ ) and repeated administration of CBD after pilocarpine-induced SE ( $p < 0.001$ ) could significantly reduce the number of bilateral forelimb clonus compared with the group that received no CBD treatment (Fig. 3b). Thus, repeated and single i.c.v. injection of CBD caused a decrease in the frequency of epileptic behaviors in the chronic phase of epilepsy.



**Fig. 3** Cannabidiol (CBD; 100 ng/rat, i.c.v. injection) caused a decrease in the occurrence of both monolateral (a) and bilateral (b) forelimb clonus compared to the pilocarpine (PLC) group during chronic phase of PLC-induced seizure. Each bar represents mean  $\pm$  SEM of data from eight rats. \* $p < 0.05$  and \*\*\* $p < 0.001$  significant difference compared with the PLC group

## Molecular Results

### Repeated Administration of CBD Induced the Level of Autophagy-Related Proteins

The autophagy-related markers were evaluated in order to see whether the protective effect of CBD treatment is through altering the autophagy-related markers. The Western blot technique was used to measure levels of autophagy-related proteins in rat hippocampus. No significant changes were observed in the level of autophagy-related cytoplasmic proteins between sham- and pilocarpine-injected groups suggesting that pilocarpine-induced seizure could not affect this pathway or its effect was reduced in the chronic phase of epilepsy on the level of autophagy-related proteins. In addition, injection of CBD alone could not change the rate of any of the measured cytoplasmic proteins compared to either the sham group or seizure group. However, an increased level of autophagy-related proteins was observed in epileptic rats treated with repeated injections of CBD compared to both sham and seizure (no CBD treatment) groups (Fig. 4a). A significant increase of Atg7 in the hippocampal tissue of epileptic rats treated with repeated administration of CBD was observed compared to sham ( $p < 0.001$ ) and pilocarpine alone ( $p < 0.01$ ) groups [ $F(5,17) = 16.48$ ,  $p < 0.001$ ; Fig. 4b]. The rate of Atg5–Atg12 conjugate was also significantly different among groups [ $F(5,17) = 4.36$ ,  $p < 0.01$ ; Fig. 4c]. The pilocarpine-induced SE group that was treated with repeated injections of CBD showed a significant increase in the level of Atg5–Atg12 conjugation compared to the sham ( $p < 0.05$ ) and

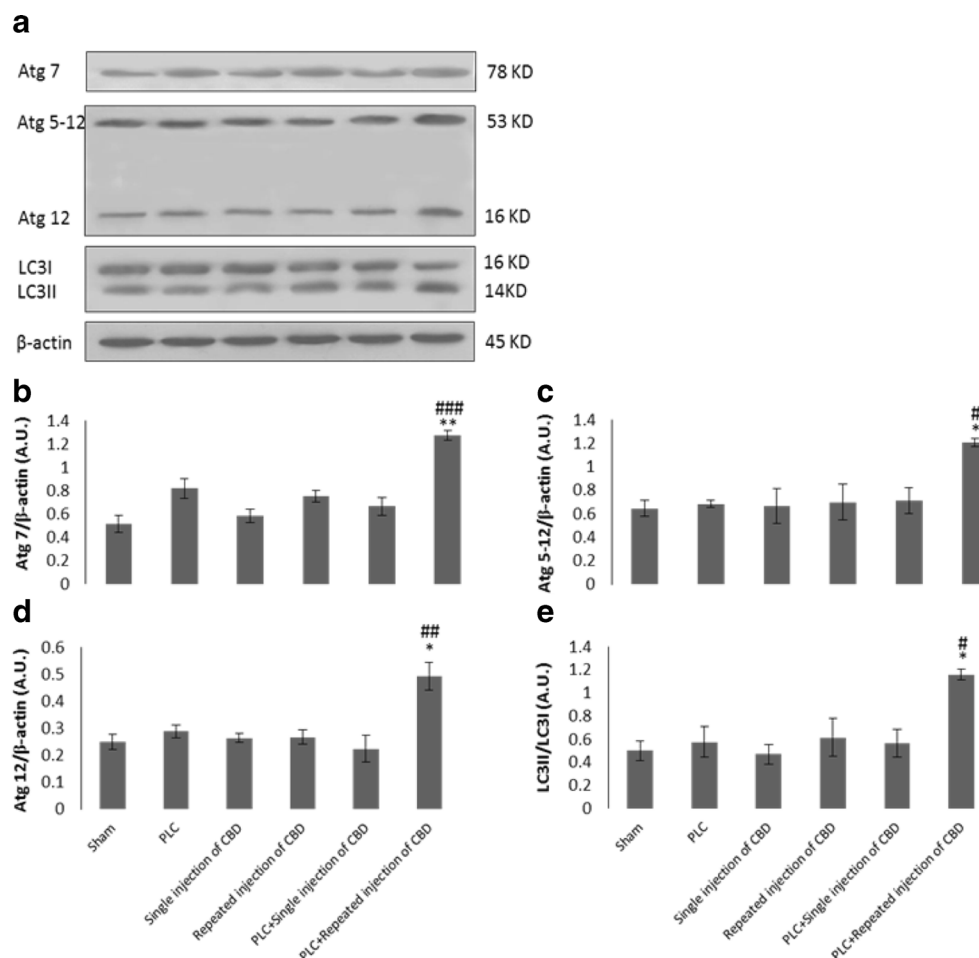
seizure group that received pilocarpine alone ( $p < 0.05$ ) (Fig. 4c). Likewise, significant changes were observed in the amount of Atg12 among designated groups [ $F(5,17) = 7.73$ ;  $p < 0.001$ ; Fig. 4d]. A significant enhancement of Atg12 was seen subsequent to repeated injections of CBD in epileptic rats compared to the sham ( $p < 0.01$ ) and seizure ( $p < 0.05$ ) groups (Fig. 4d). To evaluate the autophagic activity, the induction of LC3II was also investigated and a significant change was observed among groups [ $F(5,17) = 5.14$ ,  $p < 0.001$ ; Fig. 4e]. A remarkable upregulation of LC3II/LC3I level in epileptic rats treated with repeated injections of CBD was observed compared to both sham ( $p < 0.05$ ) and seizure ( $p < 0.05$ ) groups (Fig. 4e). In conclusion, repeated injections of CBD in the silent phase of epilepsy enhanced autophagy in the chronic phase of epilepsy while single administration of CBD at the initiation of chronic phase did not change the amount of autophagy-related proteins in the hippocampus of epileptic rats, which was obtained 24 h after CBD injection.

### CBD Induced Catalase Activity and Augmented the GSH Level

Based on our results, the activity of catalase and GSH content decreased in pilocarpine-induced epilepsy group ( $p < 0.05$  and  $p < 0.01$ , respectively) and administration of a single dose of CBD in epileptic rats at the beginning of chronic phase returns this reduction to the control level. So, these results suggest that CBD could improve epileptic-induced damages in cells by activation of antioxidant status (Table 2).

## Discussion

Repeated daily administration of CBD for 5 successive days immediately after initiation of silent phase of seizure postponed the initiation of chronic phase of seizure and significantly reduced the frequency of various epileptic behaviors described in the Racine scale (Racine, 1972). The decrease in epilepsy-related behavior scores were concurrent with an increase in the amount of autophagy-related proteins and enhancement of antioxidant defense in hippocampus of epileptic rats with repeated injections and single administration of CBD, respectively. It was shown that deficit in autophagy like knock-out of Atg5 or Atg7 causes behavioral deficits (Hara et al. 2006, Komatsu et al. 2006). Enhancement of autophagy-related proteins were related to neural plasticity (Otabe et al. 2014), and as behavior would be related to neural plasticity, so autophagy by this mechanism may affect epilepsy-related behaviors. We observed that both single and repeated injections of CBD could diminish epileptic-related behaviors, but enhancement of autophagy was observed only in epileptic rats with repeated injections of CBD; the dissimilarity might be the result of time or the number of injections. We speculate



**Fig. 4** Autophagy-related proteins level increased in epileptic rats treated with repeated injection of cannabidiol (CBD; 100 ng/rat, i.c.v. injection). **a** Representative image of rates of autophagy markers in hippocampal tissues in sham, pilocarpine (PLC), single and repeated injection of CBD, and PLC plus single and repeated injection of CBD were measured by Western blotting.  $\beta$ -actin was used as internal control. **b** Quantitative analysis of Atg7 demonstrated a significant increase in PLC + repeated injection of CBD compared with sham ( $p < 0.001$ ) and pilocarpine rats ( $p < 0.01$ ). **c** Epileptic rats treated with repeated injection of CBD showed increase level of Atg5/12 conjugation compared with sham and

pilocarpine group ( $p < 0.05$ ). **d** Quantitative analysis of western blotting showed increased induction of Atg12 in PLC + repeated injection of CBD compared with sham ( $p < 0.01$ ) and pilocarpine group ( $p < 0.05$ ). **e** Elevated rate of LC3II/LC3I in PLC + repeated injection of CBD compared with sham and pilocarpine groups ( $p < 0.05$ ). Each bar represents mean  $\pm$  SEM of data from three rats. \* $p < 0.05$ , \*\* $p < 0.01$  significant difference compared with the PLC group. # $p < 0.05$ , ## $p < 0.01$ , and ### $p < 0.001$  significant difference compared with the sham group

**Table 2** The effect of CBD on catalase activity and GSH contents in hippocampal tissue of rats in the chronic phase of pilocarpine-induced seizure

Groups	Catalase (% of control/sham)	GSH (% of control/sham)
Sham	100 $\pm$ 6.46	100 $\pm$ 2.94
Pilocarpine	68.34 $\pm$ 4.44*	80.34 $\pm$ 2.4**
Single injection of CBD	105.78 $\pm$ 6.00###	97.92 $\pm$ 3.4###
Repeated injection of CBD	103.60 $\pm$ 5.74###	96.1 $\pm$ 2.4#
Pilocarpine + single injection of CBD	102.46 $\pm$ 3.19###	97.56 $\pm$ 2.5###
Pilocarpine + repeated injection of CBD	74.83 $\pm$ 4.65*	86.17 $\pm$ 2.2*

Each point shows the mean  $\pm$  SEM ( $n = 3$ )

PLC pilocarpine, CBD cannabidiol

\* $p < 0.05$ ; \*\* $p < 0.01$  compared to sham

# $p < 0.05$ ; ## $p < 0.01$  compared to pilocarpine rats

that the number of CBD injections and long time interval between CBD injection and behavioral tests in repeated CBD-injected animals resulted in the maintenance of enhanced rate of autophagy-related proteins; while in single injection of CBD, the single injection was not able to raise the level of autophagy-related proteins.

The antiseizure activity of CBD has been shown previously in many studies. CBD pretreated rats (100 mg/kg) show anti-convulsant effects of CBD by reduction in seizure severity, tonic-clonic seizures, and mortality in pentylenetetrazole-induced seizure (Jones et al. 2010). CBD also reduces seizure activity in MES (Consroe and Wolkin, 1977). Moreover, in vitro investigations using the  $Mg^{2+}$ -free and 4-AP models have shown that CBD could decrease epileptiform local field potential (LFP) burst amplitude and burst duration in rat hippocampal slices (Jones et al. 2010). In the penicillin model of partial seizure, pretreatment of CBD has an anticonvulsant effect and considerably reduces the rate of mortality and decreases the percentage of animals showing tonic-clonic seizures (Jones et al. 2012). The CBD-induced reduction in epileptiform activity in hippocampal region suggests that further investigation is required for evaluation of CBD effects on the other animal models related to temporal lobe epilepsy.

The reasons for choosing CBD doses were mainly based on previous studies of our lab. Shirazi-zand et al. (Shirazi-zand et al. 2013), showed that CBD at the dose of 200 ng/mouse could protect mice against both types of acute pentylenetetrazole- and maximal electroshock-induced seizure. Moreover, CBD at 100 ng/mouse was almost protective (with 90 % inhibition) against electroshock-induced seizure. In the present study, treatment of rat with CBD (200 ng/rat) caused an increase in mortality rate especially in repeated administration (data not shown). But, treatment with CBD at the dose of 100 ng/rat was both safe and effective against seizure. There are few studies that use CBD through i.c.v. administration. In a study carried out on sleep by Murillo-Rodríguez et al. (Murillo-Rodríguez et al. 2006), CBD was administered at a 100 times higher dose (10  $\mu$ g, i.c.v.) than the dose administered in our study to modulate sleep in rats. In another study by Liput et al., (Liput et al. 2013), treatment with CBD gel that resulted in CBD plasma levels of approximately 100 ng/ml was used as a target concentration for development of an optimum result against ethanol intoxication. In vitro studies on neuroprotective effects of CBD generally use various concentrations ranged from 0.1 to 10  $\mu$ M (approximately 30–300 ng/ml) to obtain optimum results (Castillo et al. 2010). And finally, microinjections of CBD (1.5, 3, and 6 nmol) directly into the ventrolateral periaqueductal gray had antinociceptive activity and caused a decrease on the firing activity of the ON cells (Maione et al. 2011). The exact mechanism of CBD still is unknown. Some of possible mechanisms of CBD include: no affinity for CB1, a possible role for 5HT (1A) and CB2 receptors, effect on TRPV1 receptor

(Burstein, 2015), enhancement of adenosine signaling through inhibition of its uptake (Carrier et al. 2006), antagonism of GPR55 receptor (Chiurchiù et al. 2014), inhibition of catabolic enzyme fatty acid hydrolase that degrades endocannabinoids and by this mechanism could promote their actions (Bisogno et al. 2001). Cannabinoid receptor-independent mechanisms might also be involved in antiseizure effects of CBD. On the other hand, the anti-oxidative action of CBD was proved against some neurodegenerative diseases like Parkinson (Garcia-Arencibia et al. 2007) and Alzheimer (Iuvone et al. 2004). The CA1 and CA3 areas of hippocampus are among the most important regions for which seizure causes cell death and neuronal loss (Freitas et al. 2005). Generation of reactive oxygen species (ROS) (Freitas et al. 2005, Tejada et al. 2007), toxicity of glutamate (Voutsinos-Porche et al. 2006), and consumption of ATP (Gupta et al. 2001) occur in animals with pilocarpine-induced SE (Freitas et al. 2005, Tejada et al. 2007), and all of these factors can induce autophagy and influence cell's antioxidant defense. There are controversial reports about the role of autophagy in epilepsy. A significant increase in LC3II level occurred about 4 h following the focal injection of kainic acid in hippocampal cells (Wallace et al. 2002). Augmentation of LC3II/LC3I ratio in striatal cells also reported (Monory et al. 2006). Activation of autophagic signaling also has been noted in pilocarpine-induced seizure (Wallace et al. 2002, Ludanyi et al. 2008). In mice with deletion of Atg7 and deficiency in autophagy activity, spontaneous seizures occur thus it could be suggested that impaired autophagy might be one of the mechanisms of epilepsy (Wallace et al. 2002), but some reports show that a decrease in autophagy could enhance neurodegeneration in some conditions (Underwood et al. 2010). In these circumstances, oxidative stress can influence autophagy process. On the other hand, an inhibitory role of ROS and reactive nitrogen species on the autophagic pathway was also shown (Dong et al. 2013) suggesting that pharmacological stimulation of autophagic machinery constitutes a hopeful clinical strategy for the treatment of neurodegenerative disorders suggesting the role of autophagy in the inhibition of epileptogenesis and that epilepsy might occur when autophagy is inactivated. Pilocarpine-induced seizure might enhance autophagy-related proteins with a peak 24 h after pilocarpine injection (Cao et al. 2009). Moreover, it was reported that up to 48 h after pilocarpine-induced seizure, increase in some autophagy-related proteins occurred (Benz et al. 2014). In our study, no change was observed in the amount of autophagy-related proteins in epileptic rats during the chronic phase of SE, which is in agreement with that study. Besides, we would propose that this controversy could be due to the fact that induction of autophagy might occur after pilocarpine-induced seizure but this augmentation is reduced in the chronic phase of epilepsy.

As mentioned earlier, oxidative stress and ROS production will occur following epilepsy and CBD through enhancement of autophagy in hippocampus and as a potent antioxidant might affect glutamate excitotoxicity by inhibiting NMDA receptors (Jones et al. 2012) and decreasing cytoplasmic  $Ca^{2+}$  concentration in hippocampal neurons (Ryan et al. 2009). Schiavon et al. indicate that repeated CBD administration at a lower dose (3 mg/kg) increases cell proliferation (Schiavon et al. 2015), so it could be suggested that one of the protective mechanisms of CBD in epilepsy in repeated—but not single—injection protocol might be due to induction of autophagy signaling in the hippocampal cells and its protective effect on epilepsy-related behaviors in single injection might be affected by other pathways besides autophagy. One of the main pathways, as mentioned earlier, might be the antioxidant property of CBD that by this mechanism could act as a neuroprotection component against pilocarpine-induced seizure. Other pathways might be through PPAR $\gamma$  (O'Sullivan et al. 2009), MAPK and JAK/STAT (Kozela et al. 2010), ERK and PI3K/Akt (Puffenbarger et al. 2000).

We can consider that a vicious cycle could occur if autophagy is not induced or impairment of this pathway happens. Thus, it might be proposed that seizures could trigger pathological alterations associated with autophagy impairment and use of components activating autophagy might be useful. Thus, the use of autophagy modulators as drugs or dietary component may become a useful tool to be used early in the course of epilepsy to prevent the onset of seizure relapse. On the other hand, CBD as an antioxidant might prevent neuronal injury and improve control of seizure. Finally, we suggested that future studies will be required to investigate the exact mechanism of CBD on seizure-related behaviors and investigating induction of autophagy in different times after CBD injection.

## Conclusion

Our results suggested an improvement in seizure-related behaviors both in single administration of CBD during the chronic phase and in repeated administration of CBD at the onset of the silent phase. Moreover, significant increase in the level of autophagy and activation of antioxidant pathway were observed following administration of CBD at the onset of chronic phase. Based on our knowledge regarding the protective role of autophagy and antioxidant pathway in other neurodegenerative diseases, we suggest that autophagy induction and activation of antioxidant defense by CBD could be involved, at least in part, in protection against pilocarpine-induced seizure.

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