



## Effects of spironolactone and fludrocortisone on neuronal and glial toxicity induced by N-methyl-D-Aspartate and chloroquine in cell culture

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### ABSTRACT

Spironolactone has produced beneficial effects in animal models of neurodegenerative disorders. However, the underlying mechanisms of this agent on neurons and glia are mostly unknown. Therefore, we aimed to show the effects of spironolactone and fludrocortisone, a mineralocorticosteroid receptor agonist, on neuronal and glial toxicity induced by N-methyl-D-aspartate (NMDA) activation and chloroquine, an autophagy inhibitor, in the cell culture. We exposed the SHSY5Y neuroblastoma and 1321N1 astrocytoma exposed to NMDA (25 $\mu$ M), or chloroquine (40 $\mu$ M) for 24 and 48h to induce neuronal and glial toxicity. Spironolactone (1, 10, and 20 $\mu$ M) or fludrocortisone (300nM) were also added to the cells for 24 and 48h. Cell survival was measured using the MTT assay. Neurons and astrocytes treated with NMDA and spironolactone (1, 10, and 20 $\mu$ M) for 24 and 48h had lower cell death compared with the NMDA-treated group. Moreover, cells treated with NMDA and fludrocortisone for 24 and 48h had higher viability in comparison to the NMDA-treated group. The neuronal cells treated with chloroquine and spironolactone (10 and 20 $\mu$ M) for 24h had higher cell viability compared with the chloroquine group. Chloroquine plus spironolactone (20 $\mu$ M) treatment for 24 and 48h increased cell viability of astrocytes compared with the chloroquine-treated group. Moreover, the treatment of neurons and astrocytes with chloroquine plus fludrocortisone for 24h decreased cell death. Spironolactone and fludrocortisone protected neurons and astrocytes against NMDA- and chloroquine-induced toxicity. The mechanism of neuronal and glial protective effects of spironolactone possibly related to the inhibition of the mineralocorticosteroids. However, spironolactone might affect other non-mineralocorticoid systems.

### Keywords

Astrocytes,  
Autophagy,  
Mineralocorticoid receptors,  
Neuron,  
N-methyl-D-aspartate,  
Spironolactone

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### INTRODUCTION

Spironolactone is a potassium-sparing diuretic with beneficial effects in cardiovascular disorders [1]. This agent is a non-selective aldosterone antagonist, which affects other steroid receptors such as androgen, glucocorticoid, and progesterone receptors [2]. Spironolactone has also exerted immunomodulatory effects in the peripheral tissues and the

central nervous system (CNS) [3, 4]. Moreover, spironolactone has produced a memory-enhancing activity in human subjects [5] and suppressed neuronal injury after ischemia in animal models [6]. In a stroke model, this agent reduced the infarct size by inhibiting the mineralocorticosteroids receptors (MR)s [6]. However, there are some inconsistencies

regarding the role of MRs in neuronal survival [7, 8]. Therefore, other mechanisms other than the inhibition of MRs may be involved in the central effects of spironolactone.

MRs are mainly expressed in the kidney and are the main target of aldosterone [9]. However, some evidence shows the distribution of these receptors throughout the CNS [10]. Neuronal cells contain both the MRs and glucocorticoid receptors (GRs) [11]. MRs are extensively expressed in the hippocampus [12]. These receptors are involved in cognitive-behavioral responses and cell apoptosis in limbic structures [5]. Aldosterone, at high concentrations, activates these receptors and induces apoptosis in neurons [13]. However, aldosterone protected the rat neuronal cultures against the staurosporine-induced apoptosis [13]. Furthermore, the overexpression of the MRs in the forebrain of ischemic animals attenuated neuronal loss [14]. Glial cells also express both the MRs and the GRs [15]. However, there are limited data about the functions of the MRs on glial cells.

Glial cells have vital roles in the physiological function of the CNS. Therefore, glial dysfunction contributes to numerous neurological disorders [16]. Astrocytes are glial cells with crucial importance in several brain functions, including the regulation of neurotransmitters such as glutamate [16]. Glutamate has a unique role in neuronal function and neurotoxicity [16]. The high concentrations of glutamate in the extracellular space and overactivation of N-methyl-D-aspartate (NMDA) receptors play a leading role in neurodegenerative disorders [17]. There are some conflicting results regarding the interaction of NMDA receptors and MRs. It has been shown that the interaction of NMDA receptors and MRs affected the function of the hippocampus [18]. A study showed that MRs are essential for the survival of hippocampal neurons [19]. Moreover, MR activation decreased the overall activity of NMDA receptors [20]. On the other hand, Xiao et al. showed that NMDA-induced neurotoxicity is independent of MRs [21]. Glutamate receptors and MRs have a substantial role in the pathogenesis of the neurodegenerative disorder. Therefore, we proposed that MR modulators may affect NMDA-induced neuronal and glial loss.

According to new evidence, autophagy is another system that plays an important role in neuronal survival and neurodegeneration [22]. Autophagy-lysosome is a de novo system that is responsible for the degradation and clearance of the misfolded proteins and damaged organelles [23]. Autophagy inhibitors, such as chloroquine, induce cell apoptosis [24, 25]. Some evidence has shown that the MRs modulators affected autophagy. Aldosterone and spironolactone stimulate autophagy flux [26, 27]. Moreover, aldosterone activates oxidative stress and autophagy in renal podocytes [28]. In contrast, the inhibition of autophagy increased the toxicity of aldosterone on these peripheral cells [28]. It has been proposed that autophagy may suppress MR toxic effects in the peripheral tissues [28]. However, spironolactone and MRs agonists' effects on neuronal and glial autophagy are mostly unidentified. Therefore, this study aimed to show the neuronal and glial protective effects of spironolactone and

fludrocortisone, an MR agonist, against NMDA activation and autophagy inhibition in cell culture.

## MATERIALS AND METHODS

### Materials

The human SH-SY5Y neuroblastoma and 1321N1 astrocytoma cell lines were purchased from the Pasteur Institute (Tehran, Iran). Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12), fetal bovine serum (FBS), and Penicillin-Streptomycin were obtained from Gibco® life technologies™ (USA). NMDA, spironolactone, fludrocortisone, chloroquine, and Dimethyl sulfoxide (DMSO) were procured from the Sigma-Aldrich (USA). Spironolactone and fludrocortisone were dissolved in cell culture medium (FBS 1%) plus DMSO (1%). We considered the cell culture medium (FBS 1%) plus DMSO (1%) as the control group.

### Neuronal and astrocyte cell Cultures

The SH-SY5Y neuronal cells and 1321N1 astrocyte cells were seeded on a plate until they become confluent. Then the cells at the density of  $1 \times 10^5$  cells/well were transferred to the 96-well plates containing DMEM/F12 (1:1), fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were maintained in a humidified atmosphere of 95% air /5% CO<sub>2</sub> and at 37°C during the experiment.

### Study design and treatments

The optimum concentration of each agent was defined according to the concentrations used in a pilot study and previous investigations [29]. In two separate sets of experiments, neuronal and astrocyte, cells were exposed to the NMDA (25µM) or chloroquine (40µM) for 24 and 48h. The treatment groups for the NMDA set of experiment were as follow: 1) control, 2) NMDA (25µM) plus DMSO (1%), 3) NMDA plus spironolactone (1 µM), 4) NMDA plus spironolactone (10 µM), 5) NMDA plus spironolactone (20 µM), 6) NMDA plus fludrocortisone (300 nM), 7) NMDA plus spironolactone (10µM) plus fludrocortisone (300nM). The treatment groups for the chloroquine set of experiment were as follow: 1) control, 2) Chloroquine (40µM) plus DMSO (1%), 3) chloroquine (40µM) plus spironolactone (1 µM), 4) chloroquine (40µM) plus spironolactone (10 µM), 5) chloroquine (40µM) plus spironolactone (20 µM), 6) chloroquine (40µM) plus fludrocortisone (300nM), and 7) chloroquine (40µM) plus spironolactone (10 µM) plus fludrocortisone (300nM).

### Cell Viability Assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure the cell viability of neurons and astrocytes. Briefly, the MTT reagent (5 mg/ml) was added to the wells contained the cells and treatments and incubated for 4h. After dissolving the precipitate in DMSO (100µl), the absorbance of each well was measured at 570 nm by a microplate reader (Synergy HT, Bio-

tek®). Higher absorbance showed a higher level of living cells.

### Statistical analysis

The results were analyzed using the one-way analysis of variance (ANOVA) followed by the LSD test. The  $P < 0.05$  was considered statistically significant. The SPSS software (version 23) was used to analyze the data.

## RESULTS

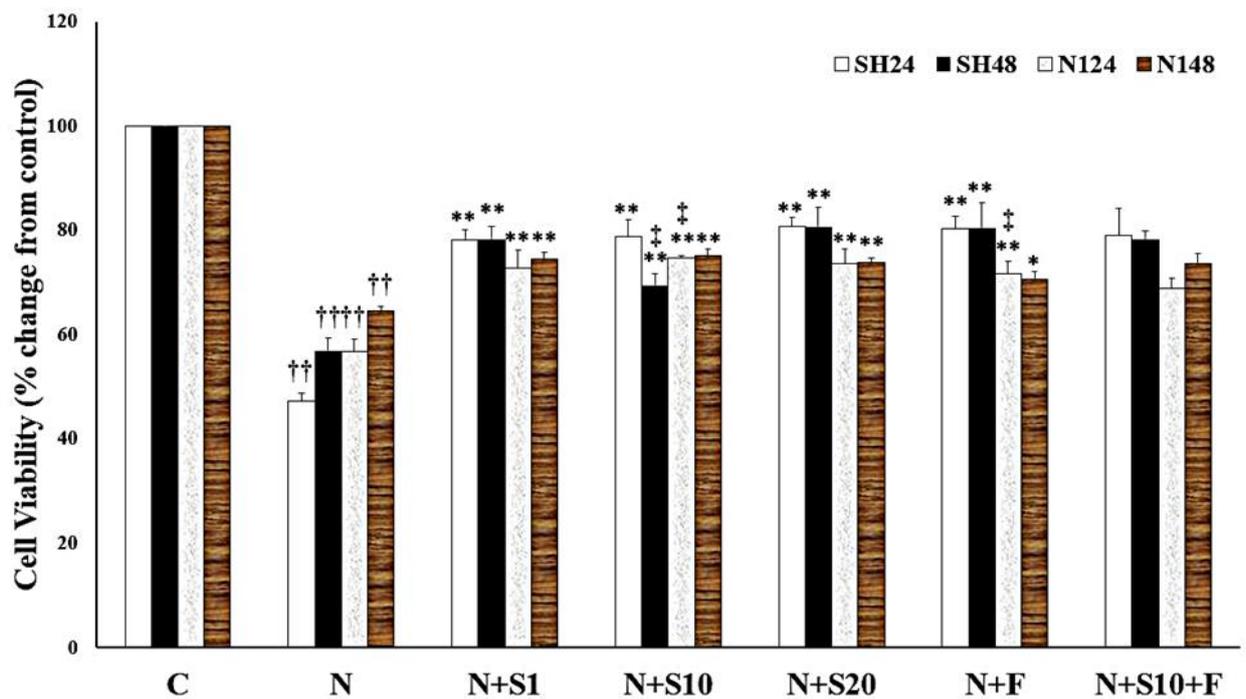
Neuroprotective and glioprotective effects of spironolactone and fludrocortisone against NMDA

Treatment of SHSY5Y neuronal and 1321N1 astrocyte cultures with NMDA ( $25\mu\text{M}$ ) plus DMSO (1%) for 24 and 48h significantly decreased the cell viability in comparison to the control groups ( $P < 0.001$ ) (Fig. 1). Neuronal and astrocyte cells treated with NMDA plus spironolactone (1, 10, and  $20\mu\text{M}$ ) for 24 and 48h had lower cell death compared with the NMDA-treated group ( $P < 0.001$ ) (Fig. 1). Moreover, cells treated with NMDA plus fludrocortisone for 24 and 48h had higher viability in comparison to the NMDA-treated group ( $P < 0.05$ ) (Fig. 1). The treatment of neuronal cells with NMDA plus spironolactone ( $10\mu\text{M}$ ) plus fludrocortisone for 48h reduced cell toxicity in comparison to the NMDA plus spironolactone ( $10\mu\text{M}$ ) group. The treatment of astrocytes with NMDA plus spironolactone ( $10\mu\text{M}$ ) plus fludrocorti-

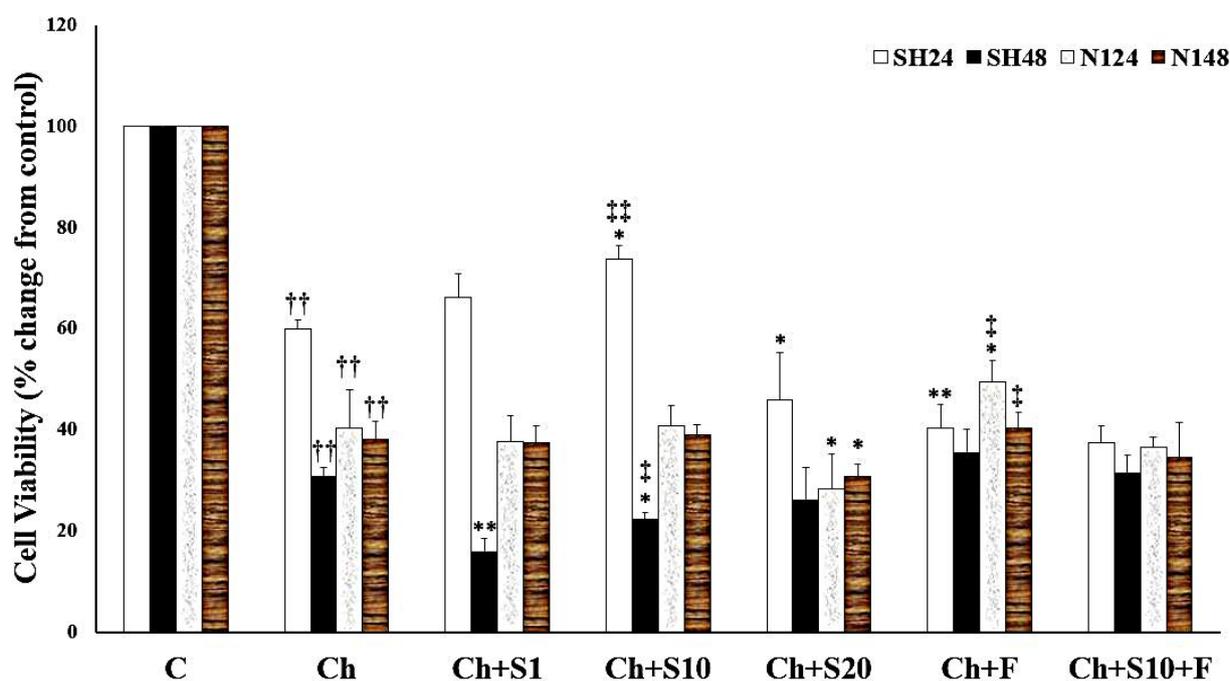
sone for 24h showed a decreased cell viability compared with both NMDA plus spironolactone ( $10\mu\text{M}$ ) ( $P = 0.004$ ) and NMDA plus fludrocortisone ( $P = 0.017$ ) groups.

### Neuroprotective and glioprotective effects of spironolactone and fludrocortisone against chloroquine

SHSY5Y neuronal and 1321N1 astrocyte cells treated with chloroquine ( $40\mu\text{M}$ ) plus DMSO (1%) for 24 and 48h had lower cell viability compared with the control group ( $P < 0.001$ ) (Fig. 2). The neuronal cells treated with chloroquine plus spironolactone (10 and  $20\mu\text{M}$ ) for 24h had higher cell viability in comparison to the chloroquine plus DMSO (1%) group ( $P = 0.001$ ) (Fig. 2). The neuronal cells treated with chloroquine plus spironolactone (1 and  $10\mu\text{M}$ ) for 48h had higher cell viability in comparison to the chloroquine plus DMSO (1%) group ( $P < 0.001$ , and  $p = 0.007$ , respectively) (Figure 2). The addition of spironolactone (1 and  $10\mu\text{M}$ ) to chloroquine for 24 and 48h did not affect astrocytes viability in comparison to the chloroquine plus DMSO group ( $P > 0.05$ ) (Fig. 2). In contrast, chloroquine plus spironolactone ( $20\mu\text{M}$ ) treatment for 24 and 48h increased cell viability of astrocytes compared with chloroquine plus DMSO (1%) ( $P = 0.003$ , and  $P = 0.012$ , respectively). Moreover, treatment of neurons and astrocytes with chloroquine plus fludrocortisone for 24h decreased cell death compared with the chloroquine plus DMSO groups ( $P < 0.001$ , and  $P = 0.016$ ,



**Figure 1.** The protective effects of spironolactone and fludrocortisone against N-methyl-D-aspartate (NMDA)-induced toxicity in SHSY5Y neuronal and 1321N1 astrocyte cultures. The cells exposed to the administered agents for 24 and 48h and the cell viability was measured using the MTT assay. Data were shown as mean + standard deviation of four experiment. ††: shows P-value lower than 0.001 compared with the control group, \* and \*\*:  $P < 0.05$  and  $P < 0.001$  compared with the NMDA group, and ‡:  $P < 0.001$  compared with the NMDA + spironolactone ( $10\mu\text{M}$ ) + fludrocortisone-treated group. N: NMDA ( $25\mu\text{M}$ ), S: Spironolactone (1, 10, and  $20\mu\text{M}$ ), and F: fludrocortisone ( $300\text{nM}$ ). SH 24 and SH48: SHSY5Y neuronal cells treated for 24 and 48h, N124 and N148: 1321N1 astrocyte cells treated for 24 and 48h



**Figure 2.** The protective effects of spironolactone and fludrocortisone against chloroquine-induced toxicity in SHSY5Y neuronal and 1321N1 astrocyte cultures. The cells exposed to the administered agents for 24 and 48h and the cell viability was measured using the MTT assay. Data were shown as mean + standard deviation of four experiment. ††: shows P-value lower than 0.001 compared with the control group, \* and \*\*: P<0.05 and P<0.001 compared with the chloroquine group, and ‡ and ‡‡: P<0.05 and P<0.001 compared with the chloroquine + spironolactone (10 $\mu$ M) + fludrocortisone-treated group. Ch: chloroquine (40 $\mu$ M), S: Spironolactone (1, 10, and 20 $\mu$ M), and F: fludrocortisone (300nM). SH 24 and SH48: SHSY5Y neuronal cells treated for 24 and 48h, N124 and N148: 1321N1 astrocyte cells treated for 24 and 48h.

respectively) (Fig. 2). The addition of fludrocortisone to chloroquine for 48h did not affect cell viability in comparison to the chloroquine-treated group (P>0.05) (Fig. 2). Furthermore, treatment of neurons with chloroquine plus spironolactone (10 $\mu$ M) plus fludrocortisone for 24 decreased cell viability (P<0.001) while the treatment for 48h increased cell viability (P=0.004) compared with the chloroquine plus spironolactone groups (Fig. 2). Astrocytes treated with chloroquine plus spironolactone (10 $\mu$ M) plus fludrocortisone for 24 and 48h had lower cell viability compared with the chloroquine plus fludrocortisone-treated groups (P=0.001, and P=0.042, respectively) (Fig. 2).

## DISCUSSION

The present study showed that spironolactone protected neurons and astrocytes against the NMDA activation- and autophagy inhibition-induced toxicity. Although several studies have shown the beneficial effects of spironolactone in animal models of stroke [30] and neuropathic pain[4], there are limited data regarding the neuronal and glial protective effects of this agent. A study by Sun and his colleagues showed that spironolactone reduced the neuropathic pain by inhibiting the microglia and suppressing the pro-inflammatory cytokines in mice [4]. Moreover, Frieler et al.

have shown that spironolactone exerted anti-inflammatory and microglial-suppressive activities and protected animals in a stroke model [31]. Another study has proposed that the beneficial effects of spironolactone in a stroke model are related to the suppression of epidermal growth factor in the CNS [6]. Our study showed that spironolactone protected neurons and astrocytes, and these direct neuronal and astrocytes protective effects might contribute to its beneficial changes in cerebrovascular disorders.

In our study, fludrocortisone, an aldosterone agonist with little activity on glucocorticoid receptors [32], partially inhibited the neuronal and glial protective effects of spironolactone against the NMDA-induced cell toxicity. Therefore, spironolactone interaction with other systems may have a role in its neuronal and astrocytic protective effects against NMDA-induced toxicity. Recent studies have demonstrated that spironolactone reduced free radicals in the ischemic tissues [33] and increased the expression of neuroprotective growth factors in astrocytes [34]. Moreover, spironolactone has decreased the expression of NMDA receptors and suppressed the activation of these receptors in mice [4]. A study by Sun et al. have suggested that microglial inhibition may also influence spironolactone effects on the NMDA receptors in neurons [4]. Our study showed that spironolactone

reduced the detrimental effects of NMDA receptor activation on neurons. The activation of the NMDA receptors causes the influx of cations, especially calcium into neurons and glial cells [35]. The elevated level of intracellular calcium activates the apoptotic pathways in neurons [35]. This process is called excitotoxicity and is an essential characteristic of neurodegenerative disorders [36]. The results of the present study may imply that the desirable effects of spironolactone in neurodegenerative disorders may be related to the suppression of NMDA receptors and excitotoxicity. Moreover, the protective effects of spironolactone against NMDA over-activation are not entirely dependent on MRs.

In the present study, fludrocortisone exerted neuroprotective and astrocyte protective activity against the NMDA-induced toxicity. Previous studies have shown that MR activation increased the anti-apoptotic factors such as Bcl2 and Bcl-XL and protected the rat hippocampus from the kainate-induced neurotoxicity [37, 38]. Furthermore, the MR agonists exerted an anti-apoptotic action in primary neuronal culture [39]. Lai et al. showed that MR activation is essential for the survival of hippocampal neurons [19]. Recent evidence has demonstrated that MR receptors in the brain activate anti-apoptotic signaling mechanism [40]. Aldosterone also protected spinal neurons against glutamate neurotoxicity [41]. It has been documented that MRs modulate glutamate neurotransmission in the hippocampus [42]. Moreover, these receptors tune the synaptic NMDA receptors [43]. Therefore, the activation of the MRs may be beneficial against neurotoxic and gliotoxic insults, though this system may produce detrimental effects on neuronal vasculature [6].

Recent studies have shown that autophagy is closely related to neurotoxicity and neurodegenerative disorders [44]. Correia et al. have documented that autophagy deficit preceded the formation of the neurofibrillary tangle [45]. Moreover, autophagy suppression led to neurodegeneration in animal models [46]. The impaired autophagy in astrocytes also influences neuronal survival and increases the neurological deficit in animal models [47]. The MR agonists and antagonists affect autophagy in the peripheral tissues [27, 48]. However, there is limited information regarding the MR effects on autophagy in neurons and astrocytes. Our study showed that both spironolactone and fludrocortisone protected the neuronal and glial cells against autophagy inhibition. Previous researches have shown that spironolactone restores the normal function of autophagy and protects peripheral cells from mechanical stress [27]. Aldosterone increased the autophagy markers in podocyte cell culture [26]. Moreover, autophagy inhibition enhances the toxic effects of aldosterone on podocytes [26]. However, the MR function in the CNS is different from the peripheral tissues. On the contrary, MR activation may enhance autophagy and protect neurons and astrocytes. Moreover, both spironolactone and fludrocortisone may enhance autophagy machinery and protect neurons and astrocytes against insults. In contrast, the use of spironolactone plus fludrocortisone reduced the effects of each agent. This may imply that over-activation of autophagy

by two drugs may exert a toxic effect on neurons and astrocytes. Moreover, the results of this study may demonstrate that the MRs may have a complex interaction with autophagy. Spironolactone, at lower concentrations, may only affect the autophagy-induced neurotoxicity and not the astrocyte viability. In contrast, spironolactone, at higher concentrations, had modest effects on neurons and more marked effects on astrocytes. Accordingly, spironolactone may influence different autophagy mechanisms in neurons and astrocytes. Another possibility may be different processes for the regulation of autophagy in neurons and astrocytes.

This study had some limitations. We did not measure the intracellular mechanisms involved in the NMDA activation- and autophagy inhibition-induced neuronal and glial toxicity. It is desirable in future studies to investigate the effects of spironolactone and fludrocortisone on cellular machinery responsible for these processes.

### CONCLUSION

The mechanism of neuronal and glial protective effects of spironolactone possibly related to the MRs inhibition. However, other systems rather than MRs may be involved in the spironolactone protective effects against NMDA activation- and autophagy inhibition-induced neuronal and astrocyte toxicity.

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### CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article

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