Original Article

Lipopolysaccharide causes persistent cognitive dysfunction in mice with activation of HMGB-1 and inhibition of autophagy

Han Huang[1*], Qing Zhu[1*], Cheng Zhou[2]

[1]Department of Anesthesiology, West China Second Hospital, Sichuan University, China; [2]Laboratory of Anesthesia and Critical Care Medicine, Translational Neuroscience Center, West China Hospital, Sichuan University, China. *Equal contributors.

Abstract: Background: Long-term cognitive dysfunction is a common long-term sequel in septic survivors. However, the underlying mechanism is poorly understood. In this study, we tested the long-term change in cognitive dysfunction in a mice model of Lipopolysaccharide (LPS)-induced sepsis, as well as the change in expression of High Mobility Group Box-1 (HMGB-1) and autophagy. Method: Sepsis was induced by intraperitoneal injection of LPS at the dose of 0.5 mg/kg while the naïve control animals received normal saline only. Fear-potentiated startle and water maze was test in day 9, 11 and 15 after LPS injection, to evaluate the cognitive function. Meanwhile, expression of HMGB-1 and the marker of autophagy (Light Chain 3 LC3-I and LC3-II) were evaluated by immunofluorescence staining and western blotting, respectively. Results: Compared with naïve controls, sepsis-survivor animals developed a significant cognitive dysfunction, as evidenced by decrease in testing startle response and latency in water maze test, which last for at least for 15 days after LPS injection. Meanwhile, increased expression of HMGB-1 and inhibition of autophagy were found in the hippocampus of survived mice. Conclusions: Sepsis causes long-term dysfunction in mice, which is very likely associated with persist activation HMGB-1 and inhibition of autophagy.

Keywords: Sepsis, high mobility group box-1, autophagy, cognitive dysfunction

Introduction

Sepsis patients could develop delirium [1] or other brain dysfunction [2], although there is no evidence indicating CNS infection in most of these patients. Some researchers named it as septic encephalopathy, which has been identified as an independent risk factor for increased mortality [3]. Recent studies further demonstrated that the brain dysfunction may persist, and more than one third of sepsis survivors would have mild to moderate cognitive dysfunction even 12 months after sepsis [4]. Considering the huge number of sepsis patients worldwide [5], the mechanisms underlying brain dysfunction, especially the pathogenesis of long-last cognitive dysfunction, requires intensive investigation.

Sepsis is characterised by uncontrolled systematic inflammation, which is also regarded as the trigger for sepsis-related brain dysfunction [3]. However, as indicated above, the brain dysfunction last much longer than the systematic inflammation and conventional acute inflammation markers, such as TNF-α, may not reflect its progression appropriately [6]. Recently, it has been showed that High Mobility Group Box-1 (HMGB-1), as a late inflammation modulator [7], is involved in sepsis-induced brain dysfunction [8]. However, the connection between activated HMGB-1 and impaired neuron function remains obscure. Autophagy, as an endogenous protective mechanism, has been well-explored neurological diseases, such as Alzheimer's disease [9], which has quite similar clinical manifestations to the long-term cognitive dysfunction observed in sepsis survivors. On the other hand, impaired autophagy has been related with the liver [10], kidney [11] and lung [12] injuries in sepsis patients. It is therefore rational for us to expect that the autophagy could be involved in the development of sepsis-induced brain dysfunction.

In this study, we examined the long-term change in cognitive function in mice survived sepsis and observed the change of HMGB-1 and autophagy markers in hippocampus.

Materials and methods

Animals and treatment

This study was reviewed and approved by the committee of animal welfare of Sichuan University (Chengdu, China). The C57BL/6 (weighing 18-25 g) mice were housed in standard cages in a twelve-hrs light/dark cycle, with free access to water and standard rodent chow.

Sepsis was induced by intraperitoneal injection of LPS (Sigma, USA) at 0.5 mg/kg in 100 µL diluted with normal saline. Controlled animals received the same volume of normal saline. After injection, the animals were retuned to cages for recovery. A probing cohort animals were tested first (n=10 per group), to estimate the survival rate of animals following our protocol, which was used to determine the sample sizes for further tests.

Cognitive tests

Cognitive function was tested by Fear-potentiated startle (FPS) and water maze tests. Separate cohorts of animals were used for each of the two tests, which suggest that each animal was assigned to only one of the cognitive tests only. For each test, minimal number of survived animals was 6 at each time point.

FPS test

The trace-fear conditioning was used to evaluate the hippocampus-dependent learning/memory. In brief, the animals were allowed to move freely in the system (Med Associates, Inc., VT, USA) for 5 min to acclimate to the testing environment with a background white noise of 65 dB. Then a loud startling stimulus noise of 105 dB was given for 40 mSec and the animal's startle response is recorded as the baseline.

After determining the baseline startle response, another 75 dB noise was given for 60 mSec followed by a foot electrical shock at 0.4 mA for 2 sec. The interval between the 75 dB noise and electrical shock was randomized between 15 to 30 sec, so called the trace fear conditioning. And this 75 dB noise-electrical shock coupling was repeated for 10 times, as the acquisition of fear conditioning. 24 hrs after the acquisition, the expression of fear conditioning was tested. The animals were placed in the same testing system and the 105 dB startling noise was given again. The startle response was recorded as testing startle response. The ratio of testing to baseline response was named as fear-conditioning potentials. The acquisition was performed on 8, 10 and 14 days after LPS injection; therefore the expression was tested on 9, 11 and 15 days after LPS injection. Animals would be sacrificed for further analysis (described below) after each test; Therefore repeated learning should not be a concern.

Water maze test

Another set of animals were used for water maze test (Pb Panlab, Barcelona, Spain). Mice were trained for hidden-platform test for 7 trials on 8, 10 and 14 days after LPS injection, respectively. And on 9, 11 and 15 days after LPS injection, probe trials were conducted with cut-off time set at 75 sec. Again, animals would be sacrificed for further analysis (described below) after each test.

Immunofluorescence staining

After completing cognitive test at each time point, half of the tested mice were scarified with overdose pentobarbital injection and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M PBS at pH 7.4. Then brains were harvested and fixed in 4% PFA at 4°C. The 25-µm coronal cryostat brain sections were collected and incubated with antibody for HMGB-1 (1:200, Abcam, Cambridge, MA, USA) overnight at 4°C. After washing with PBS with Triton, the slides were incubated with fluorochrome-conjugated secondary antibodies (Millipore, United Kingdom). Finally, the slides were stained and mounted with VECTASHIELD Mounting Medium (Vector Lab, USA) to visualize the nuclei. The immunofluorescence images were taken using an Olympus microscope at the same exposure time and were quantified using ImageJ (NIH, USA). Data of fluorescence intensity are normalized against basal value of naive control and are expressed as a percentage of that in naive control. The numbers of total cells with positive staining for HMGB-1 and cells with only cytoplasm staining of HMGB-1 were counted in 10 randomly selected vision field for each slides, respectively. The ratio of cells with positive cytoplasm staining was calculated and analysed.
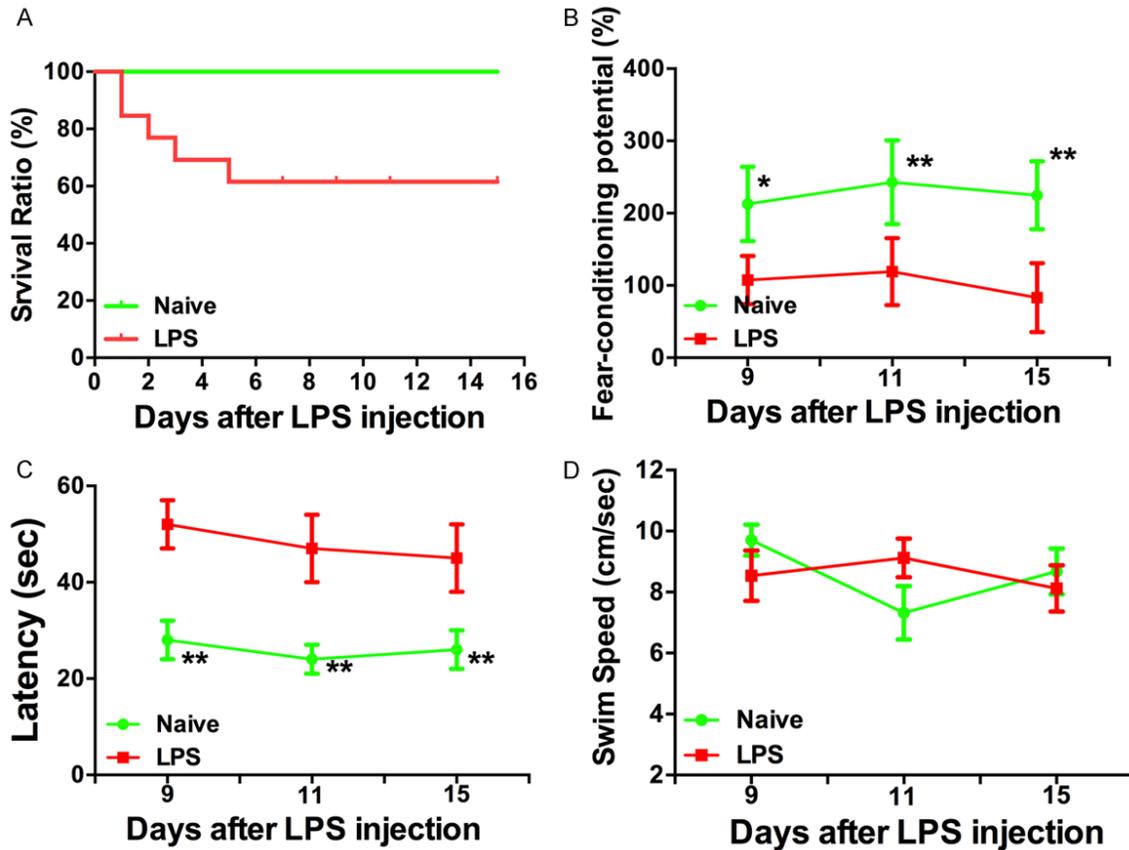
Figure 1. Cognitive change in survived mice. A. LPS injection caused a 40% mortality (n=10, P<0.01); B. Fear-conditioning potential was significantly decreased in survived mice, as compared with naïve controls at all the tested time points (n=10 in naïve; n=7 in group LPS at 11 days after LPS injection while n=6 in group LPS at 9 and 15 days after LPS injection); C. The latency for finding the platform in the water maze was significantly delayed in animals receiving LPS injection, as compared with naïve controls (n=10 in naïve; n=6 in group LPS at all the tested time points); D. There was no difference in swimming speed between group LPS and naïve control (n=10 in naïve; n=6 in group LPS at all the tested time points) (P<0.05 vs. LPS; **P<0.001 vs. LPS).

Western blotting

For the rest half of animals, fresh hippocampus tissues were collected and were frozen in liquid nitrogen until analysis. In brief, hippocampus was mechanically homogenized in lysis buffer and the protein concentration of its supernatant was determined by the BCA method (Pierce, Rockford, IL, USA). The supernatant containing 40 μg of protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Primary antibodies included LC3 (Cell signalling technology, USA), and β-actin (Abcam, Cambridge, MA, USA). The membrane was then incubated with an HRP-conjugated anti-rabbit secondary antibody (1:20,000; Cell signalling technology, USA) for 1 h and the blot was developed with a Supersignal chemiluminescence detection kit (Pierce, Illinois, USA). The immune blotting was visualized with a Kodak X-ray Processor 102 (Eastman Kodak, Rochester, NY, USA) and analyzed with ImageJ (NIH, USA).

Statistical analysis

All data were expressed as mean ± SD unless otherwise specified. Difference in survival rate was analysed with Kaplan survival analysis. Mann-Whitney T test was used for cognitive test. Immunofluorescence and band intensity from western blotting was analysed with ANOVA analysis followed by post hoc analysis when indicated. The ratio of cells with positive cytoplasm staining for HMGB-1 was analysed by chi-square test. All the statistical analysis was performed with GraphPad Prism Software (Ver 6.0, USA). A *p* value of less than 0.05 was considered to be statistically significant.
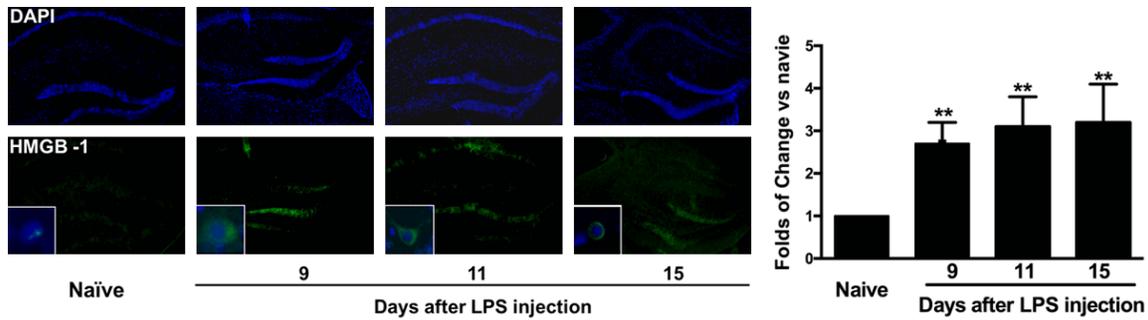
Figure 2. Sepsis induced a persistent activation of HMGB-1 in the hippocampus of survived mice. The expression of HMGB-1 was significantly increased in the hippocampus of survived mice (magnification ×40). It is further found that HMGB-1 was translocated into nuclei after LPS insult as showed in the merged imaging on the lower left corner (magnification ×400) (N=10 in naïve and n=6 in group LPS at each test time point; $P<0.05$ vs. LPS; **$P<0.001$ vs. LPS).
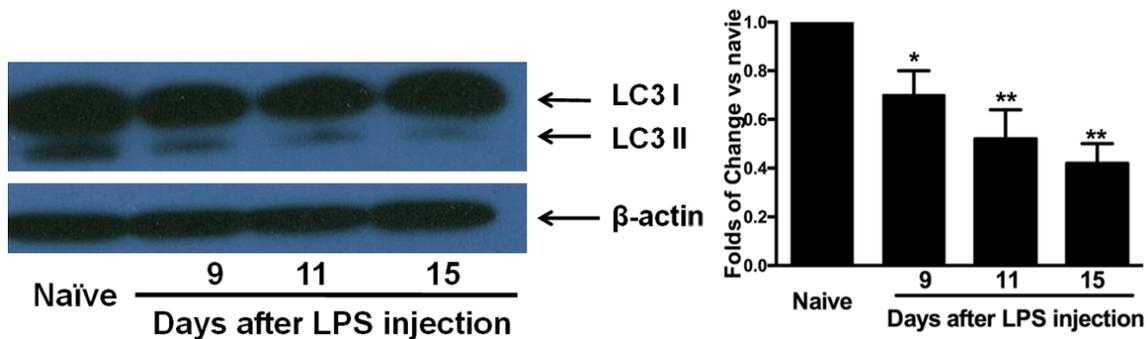

Figure 3. Sepsis caused a persist inhibition of autophagy in the hippocampus of survived mice. The ratio of Light Chain II (LC II) to Light Chain I (LC I) was significantly decreased in mice receiving LPS insult, for at least 15 days (N=10 in naïve and n=6 in group LPS at each test time point; $P<0.05$ vs. LPS; **$P<0.001$ vs. LPS).

Results

LPS injection caused a 40% mortality in mice (**Figure 1A**) and all the death occurred within 5 days after LPS insult. At 7th day after LPS injection, all the survived animals regained normal motor function, with no evidence of poor grooming, huddling, or abnormal activity.

Compared with the naïve animals, the sepsis survivors showed a more-than-50% decrease in fear-conditioning potentials in expression of trace-fear conditioning (**Figure 1B**), which suggest a defect in hippocampus-dependent learning/memory process. Similar defect was also observed in water maze test, evidenced as a more-than-2-fold increase in the latency for finding the platform (**Figure 1C**). However, the swimming speeds, automatically measured by the software, were similar between the two groups (**Figure 1D**), which excluded the possi-

bility that the difference in cognitive tests resulted from the impaired locomotors function.

At each test time point, the expression of HMGB-1 in hippocampus was observed with immunofluorescence staining. Concomitant with the development of cognitive dysfunction, the expression of HMGB-1 increased more than two folds compared with naïve control (**Figure 2**). It was further notified that HMGB-1 was actively translocated from cytoplasm into nuclei (as indicated by the inserts in **Figure 2**, where it would work as a transcription factor to initiate downstream cellular signalling. The percentage of cells with cytoplasm staining for HMGB-1 was significantly higher in animal received LPS injection (37.8%, 32.6% and 29.7% for 9,11 and 15 days after LPS injection), compared with naïve animals (7.68%, $P<0.01$).

The activity of autophagy was also tested. As showed in **Figure 3**, the ratio of LC3 II-to-LC3 I, the marker of autophagy level, was increased more than 30% compared with naïve control, at each time point.

Discussion

In this study, we successfully demonstrated that 1. Sepsis causes long-term activation of HMGB-1 within hippocampus which was synchronised with the development of cognitive dysfunction in sepsis-survivor animals; 2. Accompanying the cognitive dysfunction, decreased autophagy level was also observed in hippocampus.

Previous study has showed that blood HMGB-1 concentration peaked much later than commonly-used inflammatory markers, such as TNF-α and IL-1β, which therefore was identified as a modulator for delayed or late inflammation [13]. Our results showed that activation of HMGB-1 in CNS last even longer than previously reported serum HMGB-1 level [6]. And translocation of HMGB-1 into nuclei was also observed in our study. Taking together, our results showed that HMGB-1 was persistently activated in hippocampus after sepsis. At the same time, cognitive dysfunction developed although the acute inflammation had resolved and the animals regained full recovery physically and no impairment in motor skills was observed, which was very similar to the long-term cognitive deficient observed in otherwise normal human sepsis survivors. Previous study had indicated that HMGB-1 played an important role in sepsis-related brain dysfunction. By utilizing neutralizing antibody again HMGB-1, sepsis-induced brain dysfunction could be prevented [14]. But clinical use of HMGB-1 neutralizing antibody may not be feasible in a short future. Therefore, it is important for us to find out how did the activated HMGB-1 lead to brain dysfunction before clinically-applicable intervention could be identified.

Toll-Like Receptor 4 (TLR-4) [15] and Receptor for Advanced Glycan Endproduct (RAGE) [16] have been identified as two major receptors for HMGB-1, and the activation of both TLR-4 and RAGE has been linked to modulation of autophagy. Autophagy, from the *Latin* of "eating itself", plays a vital role for survival in life-threatening challenges, such as stress, trauma or infection, by preserving energy substrate for vital biological activities [17]. As mentioned before, the decreased level of autophagy has been found in septic lungs, liver and kidneys, which was associated with the injured pulmonary, hepatic and renal functions, respectively [10-12]. On the other hand, decreased autophagy has also been found in Alzheimer's disease [18], which is characterized by cognitive dysfunction, similar to the observed brain dysfunction in septic survivors. Therefore, it is rational for us to expect that autophagy could contribute to the sepsis-induced brain dysfunction. In fact, our data clearly demonstrated that there is a sustained inhibition of autophagy in septic brain, which is accompanied by the cognitive dysfunction. Therefore, the activated HMGB-1 could bind to TLR-4 and/or RAGE to induce long-term inhibition of autophagy, which in turn may account for the impaired brain function in sepsis survivors. Therefore, modulation of autophagy could be a potential therapeutic target for sepsis induced brain dysfunction.

The limitation of this study is, to more accurately evaluate the autophagy, "autophagic flux" should be assessed, which would answer whether the decrease in autophagy markers (as the case in our study) was due to decreased formation of autophagosomes or, contrarily, an accelerated autophagic flux downstream of the "autophagosome" stage (leading to increased clearance autophagosomes. To validate this, blockers of the autophagic flux (such as bafilomycin) were often used in *in vitro* study [19], which was seldom given in *in vivo* model (the animal model we used in our study) because it is very difficult to maintain a stable drug concentration with single or repeated i.p injection. Of note, using markers (such as LC3) for autophagy assessment is general acceptable [10-12]. At the same time, future studies should focus on the testing the effect of autophagy enhancer on reserving or preventing sepsis-induced long-term brain dysfunction.

In conclusion, we demonstrated that long-term cognitive dysfunction might develop in a mice model of LPS-induced sepsis, as observed in human septic survivors. And inhibition of autophagy by persistent activation of HMGB-1 may play a major role in this sepsis-induced brain dysfunction.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Cheng Zhou, Laboratory of Anesthesia and Critical Care Medicine, Translational Neuroscience Center, West China Hospital, Sichuan University, No. 1, Ke Yuan Si Lu, Gao Xin District, Chengdu 610000, Sichuan, China. Tel: 86-28-8516-4040; Fax: 86-28-8516-4040; E-mail: zhouc@163.com

References

[1] Shehabi Y, Riker RR, Bokesch PM, Wisemandle W, Shintani A, Ely EW; SEDCOM (Safety and Efficacy of Dexmedetomidine Compared With Midazolam) Study Group. Delirium duration and mortality in lightly sedated, mechanically ventilated intensive care patients. Crit Care Med 2010; 38: 2311-8.

[2] Iwashyna TJ, Ely EW, Smith DM, Langa KM. Long-term cognitive impairment and functional disability among survivors of severe sepsis. JAMA 2010; 304: 1787-94.

[3] Russell JA, Walley KR. Update in sepsis 2012. Am J Respir Crit Care Med 2013; 187: 1303-7.

[4] Pandharipande PP, Girard TD, Jackson JC, Morandi A, Thompson JL, Pun BT, Brummel NE, Hughes CG, Vasilevskis EE, Shintani AK, Moons KG, Geevarghese SK, Canonico A, Hopkins RO, Bernard GR, Dittus RS, Ely EW; BRAIN-ICU Study Investigators. Long-term cognitive impairment after critical illness. N Engl J Med 2013; 369: 1306-16.

[5] Adhikari NK, Fowler RA, Bhagwanjee S, Rubenfeld GD. Critical care and the global burden of critical illness in adults. Lancet 2010; 376: 1339-46.

[6] Terrando N, Rei Fidalgo A, Vizcaychipi M, Cibelli M, Ma D, Monaco C, Feldmann M, Maze M. The impact of IL-1 modulation on the development of lipopolysaccharide-induced cognitive dysfunction. Crit Care 2010; 14: R88.

[7] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999; 285: 248-51.

[8] Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, Susarla SM, Ulloa L, Wang H, DiRaimo R, Czura CJ, Roth J, Warren HS, Fink MP, Fenton MJ, Andersson U, Tracey KJ. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. Proc Natl Acad Sci U S A 2004; 101: 296-301.

[9] Shin JY, Park HJ, Kim HN, Oh SH, Bae JS, Ha HJ, Lee PH. Mesenchymal stem cells enhance autophagy and increase beta-amyloid clearance in Alzheimer disease models. Autophagy 2014; 10: 32-44.

[10] Carchman EH, Rao J, Loughran PA, Rosengart MR, Zuckerbraun BS. Heme oxygenase-1-mediated autophagy protects against hepatocyte cell death and hepatic injury from infection/sepsis in mice. Hepatology 2011; 53: 2053-62.

[11] Hsiao HW, Tsai KL, Wang LF, Chen YH, Chiang PC, Chuang SM, Hsu C. The decline of autophagy contributes to proximal tubular dysfunction during sepsis. Shock 2012; 37: 289-96.

[12] Lo S, Yuan SS, Hsu C, Cheng YJ, Chang YF, Hsueh HW, Lee PH, Hsieh YC. Lc3 overexpression improves survival and attenuates lung injury through increasing autophagosomal clearance in septic mice. Ann Surg 2013; 257: 352-63.

[13] Ulloa L, Messmer D. High-mobility group box 1 (HMGB1) protein: friend and foe. Cytokine Growth Factor Rev 2006; 17: 189-201.

[14] Chavan SS, Huerta PT, Robbiati S, Valdes-Ferrer SI, Ochani M, Dancho M, Frankfurt M, Volpe BT, Tracey KJ, Diamond B. HMGB1 mediates cognitive impairment in sepsis survivors. Mol Med 2012; 18: 930-7.

[15] Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 2007; 27: 135-44.

[16] Kang R, Tang D, Lotze MT, Zeh HJ 3rd. RAGE regulates autophagy and apoptosis following oxidative injury. Autophagy 2011; 7: 442-4.

[17] Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol 2010; 221: 3-12.

[18] Wong M. Mammalian target of rapamycin (mTOR) pathways in neurological diseases. Biomed J 2013; 36: 40-50.

[19] Roux C, Aligny C, Lesueur C, Girault V, Brunel V, Ramdani Y, Genty D, Driouich A, Laquerrière A, Marret S, Brasse-Lagnel C, Gonzalez BJ, Bekri S. NMDA receptor blockade in the developing cortex induces autophagy-mediated death of immature cortical GABAergic interneurons: An ex vivo and in vivo study in Gad67-GFP mice. Exp Neurol 2015; 267: 177-93.