

Upregulation of Connexins 30 and 32 Gap Junctions in Rat Hippocampus at Transcription Level by Chronic Central Injection of Lipopolysaccharide

Mohammad Abbasian^{1,2}, Mohammad Sayyah^{*2}, Vahab Babapour³, Reza Mahdian⁴,
Samira Choopani² and Bahar Kaviani²

¹Dept. of Physiology, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran;

²Dept. of Physiology and Pharmacology, Pasteur Institute of Iran, Tehran; ³Dept. of Physiology, Faculty of Veterinary Medicine, Tehran University, Tehran; ⁴Biotechnology Research Center, Dept. of Molecular Medicine, Pasteur Institute of Iran, Tehran, Iran

Received 26 May 2012; revised 23 June 2012; accepted 26 June 2012

ABSTRACT

Background: Gap junctions composed of connexins (Cx) are functional in cell defense by propagation of toxic/death molecules to neighboring cells. Hippocampus, one of the brain regions with particular vulnerability to damage, has a wide network of gap junctions. Functional response of astrocytic Cx30 and neuronal Cx32 to hippocampal damage is unknown. **Methods:** We infused lipopolysaccharide (LPS) intracerebroventricularly (2.5 µg/rat) once daily for two weeks to create neuroinflammation. The mRNA and protein levels of the Cx were measured in the hippocampus after 1st, 7th and 14th injection by real-time PCR and Western-blot techniques. **Results:** A significant increase in Cx32 and Cx30 gene expression was observed after 7th and 14th injection of LPS with no significant change in their protein abundance. **Conclusion:** Transcriptional overexpression of hippocampal Cx30 and Cx32 could be an adaptive response to production of intracellular toxic molecules but it is not accompanied with post-transcriptional overexpression and might have no functional impact. *Iran. Biomed. J.* 16 (3): 127-132, 2012

Keywords: Connexin 30, Connexin 32, Hippocampus

INTRODUCTION

Inflammation is a hallmark of various central nervous system (CNS) diseases such as multiple sclerosis and Alzheimer's disease. Exploring the molecular mechanisms involved in cell defence during the period of neuroinflammation would help in designing new strategies to prevent inflammation-related CNS diseases. Gap junctions are specialized cell-cell contacts between eukaryotic cells, composed of aggregates of transmembrane channels. Each channel consists of two hemichannels (termed connexon), each of which is composed of six subunit proteins called connexin (Cx). Gap junctions directly connect the cytoplasm of adjacent cells and allow intercellular movement of small molecules including ions, nutrients, metabolites, second messengers and death signals [1, 2]. Gap junctions are believed to play important role in toxic effects of chemicals because

they permit the direct transfer of damage or death signals between adjacent cells. Alteration in expression and function of Cx in several brain pathologies and neurodegenerative diseases suggests that they contribute to brain damages [3]. Some studies have provided evidence that gap junction communication is associated with the spread of cell death signals, while others have equally demonstrated protective effects [3-6]. One of the main brain regions with a particular vulnerability to damage due to toxins, ischemia/hypoxia, trauma and inflammation is hippocampus. There is a wide network of gap junctions between different cell types in hippocampus [3, 7]. Hippocampal astrocytes express Cx43 and Cx30 gap junction channels and form extensive intercellular networks. *In vivo* Cx30 expression accounts for approximately 50% of astrocytic coupling in the hippocampus. While previous studies reported a decrease in expression of hippocampal Cx43 during

*Corresponding author; Tel/Fax: (+98-21) 6696 8854; E-mail: sayyahm2@pasteur.ac.ir

lipopolysaccharide (LPS) injection [4], studies on the other main astrocytic Cx, Cx30, are limited. Cx30 is widely expressed in astrocytes as a component of astrocyte/astrocyte as well as astrocyte/oligodendrocyte gap junctions or in perivascular regions localized to astrocytic endfeet [2]. In all areas of hippocampus, gap junction plaques composed of Cx30 were found at blood vessels [8]. Another important Cx hemichannel in the hippocampus is Cx32, which is predominantly expressed in oligodendrocytes (oligodendrocyte/oligodendrocyte or oligodendrocyte/astrocyte gap junctions) and parvalbumin-positive inhibitory interneurons of CA1 subfield [3, 7-9]. There is no report regarding changes of this Cx during neuroinflammation.

The bacterial endotoxin LPS is a stimulator of microglia and is used extensively as a model of neuroinflammation [10, 11]. Neuroinflammation is associated with alterations in Cx expression [12-15]. LPS and proinflammatory cytokines down-regulate astrocyte gap junction communication and Cx43 expression [5, 16-18]. In spite of extensive investigations on Cx43 hemichannel communication during inflammatory states, Cx30 and Cx32 hemichannels have received less attention. It has been reported that global ischemia induces a selective increase in Cx32 protein abundance in hippocampal CA1 before onset of neuronal death [19]. Acute hypoxia causes an increase in Cx30 protein and mRNA levels in the developing rat brain as well [20]. However, there is no *in vivo* study indicating the behavior of Cx30 and Cx32 gap junctions in response to brain insult and inflammation.

In the present study, we examined the changes of Cx30 and Cx32 expression in rat hippocampus at transcription and translation level consequent to acute and chronic intracerebroventricular (i.c.v.) injection of LPS.

MATERIALS AND METHODS

Animals. Male Wistar rats (250-300 g, Pasteur Institute of Iran) were used. The animal experiment protocol was approved by the Review Board and Ethics Committee of Pasteur Institute of Iran (Tehran) and conformed to the European Communities Council

Directive of November 1986 (86/609/EEC) in such a way to minimize the number of animals used and their suffering.

Stereotaxic surgery and LPS injection. The rats were stereotaxically implanted with a cannula in the left lateral ventricle according to the previously described method [21]. The animals were given 7 days of recovery after surgery, before the injection protocol was started. LPS at the dose of 2.5 µg/rat was infused once daily i.c.v. for 14 days. For each experimental group, a sham (cannula-implanted non-injected) and a control (cannula-implanted PBS-injected) groups were considered.

Tissue collection. The hippocampi were dissected 24 h after 1st, 7th and 14th injection of LPS as previously described [21]. The rest of the brains were placed in 10% formalin for at least 3 days at room temperature, cut into 10-µm thick slices and qualitatively examined for cannula position using a stereoscopic microscope (Olympus, Japan). The data of the animals, in which the cannula was in the false place, were not included in the results.

Gene expression assay. Tissue preparation, RNA extraction and cDNA synthesis were performed according to the previously described method [21]. Expression of Cx30 and Cx32 in the hippocampus was measured by real-time PCR technique. The procedure has been mentioned in detail previously [22]. Briefly, α -tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal reference genes. All primers (Table 1) were designed using primer express software (v.3.0, Applied Biosystems, Foster City, CA, USA). The specificity of the primers for their target sequences was checked on NCBI website (www.ncbi.nlm.nih.gov/blast). SYBR Green I real-time PCR assay was also carried out. The extent of gene expression was calculated using comparative threshold cycle. The mean threshold cycle (mCT) was obtained from duplicate amplifications during the exponential phase of amplification. Then, mCT of reference genes was subtracted from mCT value of the target genes to obtain Δ CT. $\Delta\Delta$ CT values of each sample was calculated from corresponding CT values, where $\Delta\Delta$ CT = [mCT target (control sample) - mCT

Table 1. Primers used for real-time PCR.

| Target | Forward primer 5'→3' | Reverse primer 5'→3' | Amplicon (bp) |
|-------------------|---------------------------|---------------------------|---------------|
| Cx30 | AATGTGGCCGAGTTGTGTTACC | AAGCTGGTGATGGCATTCTGAC | 161 |
| Cx32 | CGGCATCTGCATTATCCTCAAC | CAGCAGCTTGTTGATCTCATTCTG | 163 |
| α -tubulin | CTGGAACCCACAGTTATTGATGAAG | GGCATAGTTATTGGCAGCATCCTTC | 159 |
| GAPDH | AGTCAAGGCTGAGAATGGGAAG | CATACTCAGCACCAGCATCACC | 160 |

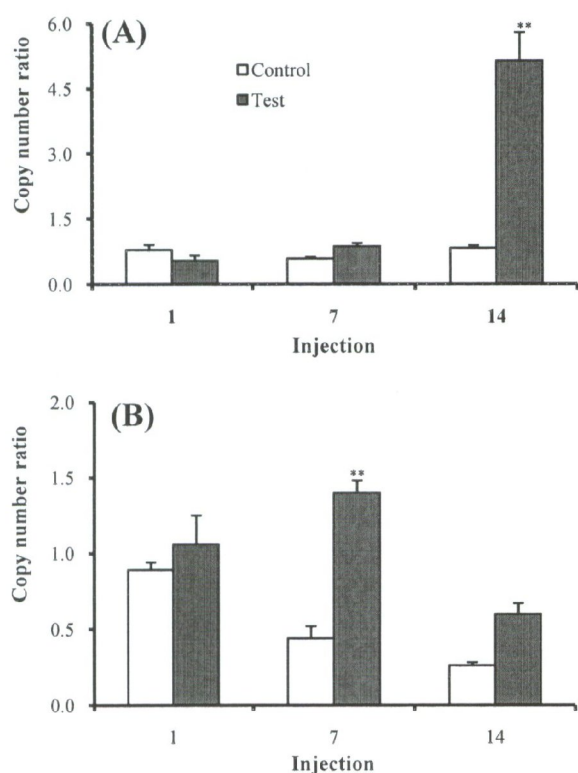


Fig. 1. Cx30 (A) and Cx32 (B) mRNA level in the hippocampus of the rats after daily i.c.v. injection of LPS. Connexin mRNA levels were normalized to α -tubulin and GAPDH mRNA level. Data are expressed as means \pm S.E.M. (n = 5). ** $P < 0.001$ compared to respective control group.

reference (control sample)] - [mCT target (test sample) - mCT reference (test sample)]. The calculated $\Delta\Delta CT$ was converted to ratio using the ratio formula (Ratio = $2^{-\Delta\Delta CT}$) [22]. Dissociation curve analysis was performed for each amplification reaction to detect any possible primer dimers or non-specific PCR product. Before using comparative threshold cycle method, amplification efficiency of each gene was determined from the standard curve drawn by plotting the logarithmic input amount of template DNA versus the corresponding CT values.

Immunoblotting. Protein expression of Cx30 and Cx32 in the hippocampus was determined by Western-blot technique, which is previously explained in detail [22]. In brief, total protein concentration in homogenized hippocampi was determined. Equal amounts of protein from each animal (5 μ g per lane for α -tubulin, 10 μ g per lane for Cx30 and Cx32) were transferred to a PVDF membrane (Roche, Germany) by electroblotting. The membrane was blocked in TBST buffer (100 mM Tris base, 150 mM NaCl, and 0.2%

w/v Tween 20) and then incubated with the following primary antibodies: mouse monoclonal anti-Cx30 and anti-Cx32 (diluted 1:200,000, Invitrogen, USA) and mouse monoclonal anti- α -tubulin (diluted 1:200,000, Invitrogen, USA). After washing, the membrane was incubated with peroxidase conjugated goat anti-mouse IgG (diluted 1:100,000, 1:100,000, and 1:2,000,000 for Cx30, Cx32 and α -tubulin, respectively; Sigma-Aldrich, Germany), then washed with TBST buffer and reacted with enhanced chemiluminescence Advance Western-blotting detection reagents (Pharmacia Amersham, UK). Bands were visualized on X-ray film and quantified by densitometry. The relative levels of Cx30 and Cx32 proteins were expressed as ratios (Cx30/ α -tubulin $\times 100$ and Cx32/ α -tubulin $\times 100$).

Statistical analysis. The data were analyzed by ANOVA with Tukey's post hoc test and presented as mean \pm S.E.M. (n = 5). In all experiments, $P < 0.05$ was considered statistically significant.

RESULTS

No significant change was observed between sham and control groups. Therefore, the results of sham group are not shown.

Elevation of Cx30 and Cx32 mRNA levels in LPS-treated rats. Melting curve analysis for Cx30, Cx32, GAPDH and α -tubulin gene fragments revealed a unique PCR product in each reaction. Each peak represented a unique PCR product in each reaction. Melting temperatures of 80.5°C for GAPDH, 81.3°C for α -tubulin, 79.8°C for Cx30, and 79.0°C for Cx32 were obtained. The amplification curves of the both reference genes (α -tubulin, GAPDH) have crossed the threshold line at the same point. mCT of 21.95 for GAPDH and α -tubulin, 25.61 for Cx32, and 27.92 for Cx30 were obtained. Cx30 mRNA expression was not changed after the 1st and 7th injection of LPS; however, it was significantly increased after the 14th injection with $P < 0.001$ (Fig. 1A). LPS was also capable to significantly elevate the expression of Cx32 mRNA after 7 injections ($P < 0.001$). It reached the control level at 14th injection of LPS (Fig. 1B).

No change in Cx30 and Cx32 protein expression by LPS. Hippocampal Cx30 and Cx32 protein abundance did not change during acute and chronic injection of LPS. Although a decrease in Cx30 and Cx32 protein expression was observed after 14 injections of LPS, it was not statistically significant (Fig. 2).

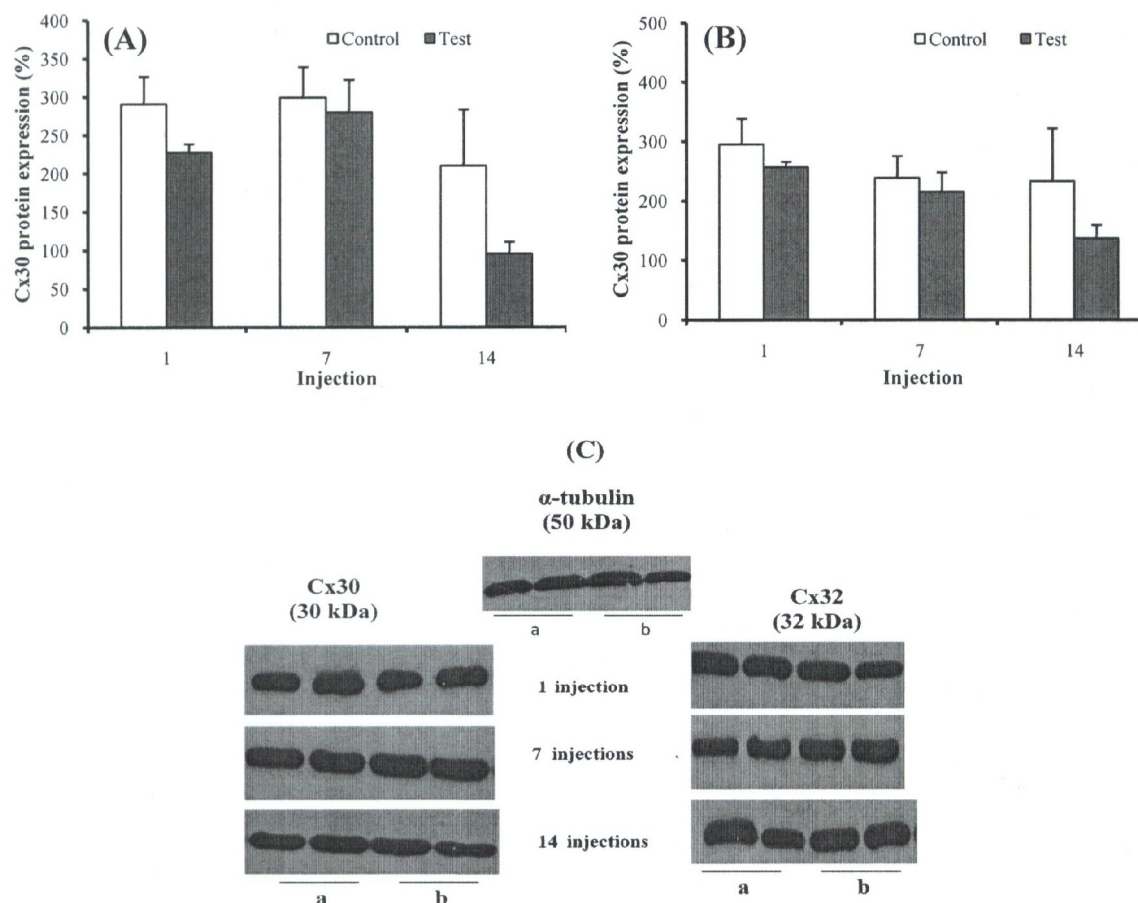


Fig. 2. Cx30 (A) and Cx32 (B) protein levels in the hippocampus of the rats after daily i.c.v. injection of lipopolysaccharide (LPS). (C) Immunoblots of Cx30, Cx32 and α -tubulin in LPS-treated samples. Each immunoblotting was performed in duplicate to increase the reliability of the measurements. (a), control; (b), test. Connexin protein levels were normalized to α -tubulin protein level. Data are expressed as means \pm S.E.M. (n = 5).

DISCUSSION

Our data indicate a significant increase in Cx30 and Cx32 gene expression, but no change in their protein abundance following acute and chronic central injection of LPS.

Numerous CNS disorders including multiple sclerosis and Alzheimer's disease are typified by neuroinflammation. Yet treatment is directed mainly at blocking the symptoms, not the underlying cause. The course of inflammation is a critical period. If the events occurring during this period are explored, it would help in designing new strategies to prevent the inflammation-related diseases. Many of CNS inflammatory diseases, including multiple sclerosis, Alzheimer's disease and cerebral ischemia are associated with changes in gap junction intercellular

communication as reflected by alterations in dye coupling and Cx expression [4]. Cx30 and Cx32 are among the main Cx expressed in astrocytes and oligodendrocytes, respectively. However, there are negligible studies regarding the role of gap junctions composed of these Cx in inflammatory state [20]. It is reported that acute hypoxia is associated with a 2-4-fold increase in Cx30 protein expression in the hippocampi of rats during first two postnatal weeks, but no change was observed in older rats [20]. Moreover, expression of Cx30 in mice is significantly enhanced till 7 days after brain abscess over long distances, extending from primary site of infection [14]. It remains to be explored whether the overexpression of Cx30 is associated with inflammation exert helpful or hurtful effects. In double-knockout mice for Cx30 and Cx43 (Cx30^{-/-}

Cx43^{-/-}), a decrease in astroglial glutamate and potassium clearance leads to increased neuronal excitability, suggesting a major role for astrocytic networks in glutamate clearance, potassium buffering and extracellular space-volume regulation during basal synaptic activity [23]. We previously demonstrated that chronic i.c.v. injection of LPS, as used in the present study, induces hippocampal inflammation [21]. In this study, we observed about 4-fold increase in expression of Cx30 mRNA consequent to 14 days i.c.v. injection of LPS. Nonetheless, no change was detected in Cx30 protein abundance during this period. Therefore, it seems that chronic central injection of LPS has no functional effect on Cx30 gap junctions.

Cx32 is abundantly expressed in mammalian brain. In hippocampus, Cx32 is expressed predominantly in oligodendrocytes [7] and parvalbumin-positive inhibitory interneurons of CA1 subfield [19]. Expression of Cx32 gap junction protein is increased selectively in CA1 GABAergic interneurons after global ischemia and transgenic Cx32-null mice exhibit enhanced vulnerability to global ischemia-induced neuronal death, indicating a protective role for Cx32 gap junctions against ischemia-induced cell death [19]. These observations suggest that Cx32 gap junctions are over expressed under brain damages as a part of adaptive processes to reduce damages and protect the hippocampal neurons. In the present study, Cx32 mRNA expression is upregulated after chronic injection of LPS. However, we could not detect any changes in Cx32 protein abundance during LPS injection period. Lack of correlation between mRNA and protein level of Cx, observed in our study, has been reported by other researchers [19]. Oguro et al. [19] found a marked reduction in Cx32 mRNA abundance and at the same time a marked increase in Cx32 protein level in mouse hippocampus after global ischemia. Our observation might be related to rapid turn-over of Cx proteins [4]. Moreover, LPS might affect the stability of Cx proteins as reported by others [24]. Furthermore, half life of Cx32, the main Cx in the liver, is reduced during liver inflammation, induced by LPS [25, 26].

Gap junction communication can be regulated at several levels, including changes in Cx transcription, translation, stability, post translational processing and channel gating [4]. However, post-translational processes are thought to be major factors in regulating Cx levels and functional coupling [4]. Therefore, our results suggest no functional modulation of Cx30 and Cx32 gap junction coupling by LPS. Nevertheless, effect of LPS on Cx30 and Cx32 channel gating should be determined to further elucidate effect of LPS on gap junction communication. Therefore, further studies in other models of neuroinflammation are required to

explore whether Cx30 and Cx32 gap junctions play a role in the process of neuroinflammation.

ACKNOWLEDGEMENTS

Financial support of Pasteur Institute of Iran (grant No. 472) is acknowledged.

REFERENCES

1. Condorelli DF, Trovato-salinaro A, Mudo G, Mirone MB, Belluardo N. Cellular expression of connexins in the rat brain: neuronal localization, effects of kainate-induced seizures and expression in apoptotic neuronal cells. *Euro J Neurosci.*2003 Oct;18(7):1807-27.
2. Söhl G, Maxeiner S, Willecke K. Expression and functions of neuronal gap junctions. *Nat Rev Neurosci.*2005 Mar;6(3):191-200.
3. Rouach N, Avignone E, Meme W, Koulakoff A, Venance L, Blomstrand F, et al. Gap junctions and connexin expression in the normal and pathological central nervous system. *Biol Cell.*2002 Nov;94(7-8):457-75.
4. Kielian T. Glial connexins and gap junctions in CNS inflammation and disease. *J Neurochem.*2008 Aug;106(3):1000-16.
5. Froger N, Orellana JA, Cohen-Salmon M, Ezan P, Amigou E, Saez JC et al. Cannabinoids prevent the opposite regulation of astroglial connexin43 hemichannels and gap junction channels induced by pro-inflammatory treatments. *J Neurochem.*2009 Dec;111(6):1383-97.
6. Froger N, Orellana JA, Calvo CF, Amigou E, Kozoriz MG, Naus CC, et al. Inhibition of cytokine-induced connexin43 hemichannel activity in astrocytes is neuroprotective. *Mol Cell Neurosci.*2010 Sep;45(1):37-46.
7. Rash JE, Yasumura T, Davidson KG, Furman CS, Dudek FE, Nagy JI. Identification of cells expressing Cx43, Cx30, Cx26, Cx32, and Cx36 in gap junctions of rat brain and spinal cord. *Cell Commun Adhes.*2001;8(4-6):315-20.
8. Gosejacob D, Dublin P, Bedner P, Huttman K, Zhang J, Tress O et al. Role of astroglial connexin30 in hippocampal gap junction coupling. *Glia.* 2011 Mar;59(3):511-9.
9. Xu G, Wang W, Harold K, Zhou M. Electrical coupling of astrocytes in rat hippocampal slices under physiological and simulated ischemic conditions. *Glia.*2010 Mar;58(4):481-93.
10. Ambrosini A, Louin G, Croci N, Plotkine M, Jafarian-Tehrani M. Characterization of a rat model to study acute neuroinflammation on histopathological, biochemical and functional outcomes. *J Neurosci Methods.*2005 Jun;144(2):183-91.
11. Turrin NP, Gayle D, Ilyin SE, Flynn MC, Langhans W, Schwartz GJ et al. Pro-inflammatory and anti-inflammatory cytokine mRNA induction in the periphery and brain following intraperitoneal

- administration of bacterial lipopolysaccharide. *Brain Res Bull.*2001 Mar;54(4):443-53.
12. Garg S, Syed MM, Kielian T. Staphylococcus aureus-derived peptidoglycan induces Cx43 expression and functional gap junction intercellular communication in microglia. *J Neurochem.*2005 Oct;95(2):475-83.
 13. Nakase T, Söhl G, Theis M, Willecke K, Naus CC. Increased apoptosis and inflammation after focal brain ischemia in mice lacking connexin43 in astrocytes. *Am J Pathol.*2004 Jun;164(6):2067-75.
 14. Karpuk N, Burkovetskaya M, Fritz T, Angle A, Kielian T. Neuroinflammation leads to region-dependent alterations in astrocyte gap junction communication and hemichannel activity. *J Neurosci.*2011 Jan;31(2):414-25.
 15. Ohsumi A, Nawashiro H, Otani N, Ooigawa H, Toyooka T, Shima K. Temporal and spatial profile of phosphorylated connexin43 after traumatic brain injury. *J Neurotrauma.*2010 Jul;27(7):1255-63.
 16. Me'me W, Calvo C, Froger N, Ezan P, Amigou E, Koulakoff A et al. Proinflammatory cytokines released from microglia inhibit gap junctions in astrocytes: potentiation by β -amyloid. *FASEB J.*2006 Jan;20:494-6.
 17. Hinkerohe D, Smikalla D, Schoebel A, Haghikia A, Zoidl G, Haase CG et al. Dexamethasone prevents LPS-induced microglial activation and astroglial impairment in an experimental bacterial meningitis co-culture model. *Brain Res* 2010 May;1329:45-54.
 18. Liao CK, Wang SM, Chen YL, Wang HS, Wu JC. Lipopolysaccharide-induced inhibition of connexin43 gap junction communication in astrocytes is mediated by downregulation of caveolin-3. *Int J Biochem Cell Biol.*2010 May;42(5):762-70.
 19. Oguro K, Jover T, Tanaka H, Lin Y, Kojima T, Oguro N, et al. Global ischemia-induced increases in the gap junctional proteins connexin 32 (Cx32) and Cx36 in hippocampus and enhanced vulnerability of Cx32 knock-out mice. *J Neurosci.*2001 Oct;21(19):7534-42.
 20. Zeinieh MP, Talhouk RS, El-Sabban ME, Mikati MA. Differential expression of hippocampal connexins after acute hypoxia in the developing brain. *Brain Dev.*2010 Nov;32(10):810-7.
 21. Sayyah M, Kaviani B, Khoshkholgh-Sima B, Bagheri M, Olad M, Choopani S et al. Effect of chronic intracerebroventricular administration of lipopolysaccharide on connexin43 protein expression in rat hippocampus. *Iran Biomed J.*2012 Jan;16(1):25-32.
 22. Akbarpour B, Sayyah M, Babapour V, Mahdian R, Beheshti S, Kamyab AR. Expression of connexin 30 and connexin 32 in hippocampus of rat during epileptogenesis in the kindling model of epilepsy. *Neurosci Bull.* 2012;In press.
 23. Pannasch U, Vargová L, Reingrube J, Ezan P, Holcman D, Giaume C et al. Astroglial networks scale synaptic activity and plasticity. *Proc Natl Acad Sci USA.*2011 May;108(20):8467-72.
 24. Chanson M, Derouette JP, Roth I, Foglia B, Scerri I, Dudez T, Kwak BR. Gap junctional communication in tissue inflammation and repair. *Biochim Biophys Acta.*2005 Jun;1711(2):197-207.
 25. Gingalewski C, Wang K, Clemens MG, De Maio A. Posttranscriptional regulation of connexin 32 expression in liver during acute inflammation. *J Cell Physiol.*1996 Feb;166(2):461-7.
 26. De Maio A, Gingalewski C, Theodorakis NG, Clemens MG. Interruption of hepatic gap junctional communication in the rat during inflammation induced by bacterial lipopolysaccharide. *Shock.*2000 Jul;14:53-9.