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Effect of Chronic Intracerebroventricluar Administration of Lipopolysaccharide on Connexin43 Protein Expression in Rat Hippocampus

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ABSTRACT

Background: Hippocampal damages, which are accompanied by inflammation, are among the main causes of epilepsy acquisition. We previously reported that chronic intracerebroventricular (i.c.v.) injection of lipopolysaccharide (LPS) modulates epileptogenesis in rats. There is a network of gap junction channels in the hippocampus that contribute to epileptogenesis. Gap junction channels are formed by oligomeric protein subunits called connexins (Cx). Astrocytic Cx43 and neuronal Cx36 are expressed in the hippocampus. In order to find out the possible role of gap junctions in seizure-modulating effect of LPS and neuroinflammation, we studied the effect of central administration of LPS on expression of Cx36 and Cx43 in rat hippocampus. **Methods:** LPS, 2.5 μg/rat/day, was injected i.c.v. to male Wistar rats for 14 days. mRNA and protein abundance of Cx36, Cx43 and IL1-β were measured in rat hippocampus by real time-PCR, Western blot and ELISA techniques, at the beginning, in the middle, and at the end of the treatment period. **Results:** IL1-β protein level was significantly increased 6 h after first injection of LPS. Cx36 and Cx43 mRNA expression did not alter during chronic administration of LPS. A selective decrease in Cx43 protein expression was observed after 7 injections of LPS. **Conclusion:** It is suggested that Cx43 containing gap junctions in the hippocampus is down-regulated in response to chronic injection of LPS. This event can inhibit propagation of toxic and noxious molecules to neighboring cells and modulate hippocampal excitability and epileptogenesis. *Iran. Biomed. J. 16 (1): 25-32, 2012*

Keywords: Connexin36 (Cx36), Connexin43 (Cx43), Interleukin-1β (IL1-β)

INTRODUCTION

Inflammation is a hallmark of various central nervous system diseases such as multiple sclerosis, Alzheimer's disease and epilepsy [1, 2]. Gap junction channels are specialized cell-cell contacts between cells, composed of aggregates of trans-membrane hemichannels, which directly connect the cytoplasm of neighboring cells, allowing intercellular movement of ions, metabolites and second messengers [1]. Each channel consists of two hemichannels termed connexons, each of which is composed of six subunit proteins called connexin (Cx). A general consequence of brain inflammation and epilepsy is reactive gliosis characterized by proliferation and hypertrophy of astrocytes and microglia. Cx43 is the most abundant Cx expressed by astrocytes [1]. The bacterial endotoxin lipopolysaccharide (LPS) is a stimulator of microglia and used extensively as a model of neuroinflammation [3]. LPS and pro-inflammatory cytokines down-regulate astrocyte gap junction communication (GJC) and Cx43 expression *in vitro* [4-6]. Furthermore, it has recently been reported that expression of astroglial Cx43 is significantly increased after brain abscess in mice [7]. According to experimental and human evidence, neuroinflammation facilitates acquisition of seizures and epilepsy [2]. We also reported that chronic intracerebroventricular (i.c.v.) injection of LPS (2.5 μg/rat/day) modulates acquisition of epilepsy in rats [8]. One of the main brain regions involved in epilepsy is hippocampus, which has particular vulnerability to damage-induced inflammation. There is strong evidence that gap junctions play a role in the fast oscillations that precede the onset of seizures

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discharges in the hippocampus [9, 10]. In CA1 subfield of the hippocampus, parvalbumin positive GABAergic interneurons form a vast dendrodendritic network, which is responsible for synchronized oscillations in hippocampus and thereby promote inhibitory transmission [11]. Morphological [11] and electro-physiological [12] evidence indicates that electrical coupling between GABAergic interneurons in this region is mediated by Cx36. In spite of extensive *in vitro* investigations, there is no report regarding the effect of LPS and neuroinflammation on Cx43 and Cx36 expression *in vivo*. Here, we treated rats with LPS by the same previously reported protocol [8] and measured hippocampal expression of Cx36 and Cx43 at transcription and translation level at the beginning, in the middle, and at the end of 14-day injection of LPS. In order to confirm the presence of neuroinflammation, the hippocampal level of the typical pro-inflammatory mediator, IL1-β, was also measured at the above-mentioned time points.

MATERIALS AND METHODS

Animals. Male Wistar rats (280-320 g, the Pasteur Institute of Iran) were used throughout this study. The animals were housed in standard plexiglas cages with free access to food (standard laboratory rodent's chow) and water. The animal room temperature was maintained at 23 ± 1.0°C with a 12-h light/dark cycle (light on from 6.00 a.m.). All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) in such a way to minimize the number of animals used and their suffering. Each animal was tested once.

Materials. Ketamine (Rotex Medica, Germany), Xylazine (Chanelle, Ireland), LPS (*Escherichia coli* serotype 026:B6, Sigma, UK), Rat IL1-β ELISA kit (Koma Biotech Inc., South Korea), protease inhibitor cocktail (Roche, Germany), RNase free DNase I (Roche, Germany), first strand cDNA synthesis kit (Roche, Germany), SYBR Green I Master mix (Applied Biosystems, Warrington, UK), PVDF membrane (Roche, Germany), ECL Advance Blocking Agent (Pharmacia Amersham, UK), ECL Advance Western-Blotting detection reagents (Pharmacia Amersham, UK), mouse monoclonal anti-Cx36 (Zymed, USA), mouse monoclonal anti-Cx43 (Upstate, USA), mouse monoclonal anti-α-tubulin and peroxidase conjugated goat anti-mouse IgG (Sigma-Aldrich, Germany), X-ray film (Retina, USA), RNX-PLUS reagent (Fermentas, Ukraine) and diethyl ether (BDH Chemicals Ltd., UK), were used in this study. Other chemicals were from Applichem (Germany) and Sigma-Aldrich (USA). LPS was dissolved in PBS and prepared freshly on the day of use.

Stereotaxic surgery and LPS injection. The rats were anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). An injection guide-cannula (23 gauge) was implanted in the left lateral ventricle (coordinates: A, -0.9; L, -1.5 from bregma and V, 3.5) [8]. The cannula was fixed to the skull with dental acrylic. The animals were given 7 days for recovery after surgery, before the injection protocol was started. LPS at the dose of 2.5µg/rat was infused once daily for 14 days into the left cerebral ventricle (i.c.v., 1 µl in 3 min) via a 27-gauge cannula, which was extended 1 mm below the tip of the guide cannula.

Tissue preparation. Inflammatory mediators often reach the maximum level during 3-6 h after LPS injection [3]. Therefore, for assessment of IL1-β, the hippocampi were dissected 6 h after 1^{st}, 7^{th} and 14^{th} injection of LPS. However, to measure changes in Cx mRNA expression, the time point of 24 h after LPS injection was selected and the hippocampi were dissected 24 h after 1^{st}, 7^{th} and 14^{th} injections of LPS. All the animals and their corresponding controls were decapitated under deep ether anesthesia and their brain were removed immediately. The brains were incubated in chilled artificial cerebrospinal fluid (pH 7.3) consisted of the following composition (mM): 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 10 glucose. The hippocampi of the brains were removed and frozen immediately in liquid nitrogen and stored at -80°C. 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 analyzed for electrode position using a stereoscopic microscope (Olympus, Japan). The data of the animals in which the cannula was in the false location was not included in the results.

Hippocampal IL1-β assay. Each sample of hippocampus was homogenized with a homogenizer (Pellet Pestle, Kontes, UK) in a 1 ml ice-cold PBS (pH 7.2) containing 4% protease inhibitor cocktail, and centrifuged (Microfuge^{TM} 11, Beckman, USA) at 14000 ×g at 4 ºC for 10 min. Then, the supernatant was collected. The total protein concentration was determined by Bradford's method [13]. IL1-β level was measured by ELISA kits according to manufacturer's instructions. The concentration of the cytokine was quantified as picogram of antigen per 100 µg of total protein.

Table 1. Primers used for real-time PCR.

Target	Forward primer 5'→3'	Reverse primer 5'→3'	Amplicon (bp)
Cx36	ACTATGATTGGGAGGATCCTGTTG	CACACAAACATGGTCTGCTCATC	107
Cx43	GAAAGAGAGGTGCCCAGACATG	AGCACTGACAGCCACACCTTC	105
α–tubulin	CTGGAACCCACAGTTATTGATGAAG	CATACTCAGCACCAGCATCACC	105

Gene expression assay. The frozen hippocampi were removed from -80°C and pulverized completely. About 200 µl of chilled PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4) was added to the pulverized tissues, vortex mixed for 30 s, centrifuged and the supernatant divided in aliquot parts. One of these prepared samples was used for gene expression study and the second part for immunoblotting. According to the manufacturer′s proposal, an appropriate volume of a protease inhibitor cocktail was added to the samples, which were allocated for immunoblotting. Total cellular RNA was isolated from the hippocampus by a modification of the guanidine isothiocyanate phenol-chloroform method [14] using RNX-PLUS reagent, and then treated with 10 U RNase free DNase I. The integrity of RNA samples were determined using denaturing agarose gel electrophoresis. The concentrations of the RNAs were determined spectrophotometrically (NanoDrop, USA). The mean 260/280 ratios were 1.94 ± 0.0, while those of 260/230 were 1.98 ± 0.1. The reverse transcription reaction was performed with first strand cDNA synthesis kit using oligo-dT primer, AMV reverse transcriptase and 1µg total RNA as template, according to the manufacturer′s instructions. The concentration of synthetic cDNA was measured using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 and 280 nm. DNA samples with the A260 ⁄ A280 ratios of more than 1.5 were selected for quantitative analysis. Cx36 and Cx43 were chosen as target genes and α-tubulin was used as internal reference gene. All primers (Table 1) were designed using primer express software v.3.0 (Applied Biosystems, Foster City, CA, USA). SYBR Green I real-time PCR assay was carried out in final reaction volumes of 25 µL with 12.5 µL of SYBR Green I Master mix, 100nM of forward and reverse primers and 300ng of cDNA. Thermal cycling was performed on the ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: 10 min at 95ºC as first denaturation step followed by 40 cycles at 95ºC for 15 s and 60ºC for 1 min. Each complete amplification stage was followed by a dissociation stage at 95ºC for 15 s, 60ºC for 30 s and 5ºC for 15 s. 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 were subtracted from mCT value of the target genes to obtain ΔCT. ΔΔCT values of each sample was calculated from corresponding CT values where ΔΔCT = [mCT target (control sample) - mCT reference (control sample)] - [mCT target (test sample) - mCT reference (test sample)]. The calculated ΔΔCT was converted to ratio using the ratio formula (Ratio = $2^{-\Delta\Delta CT}$) [15]. 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. The corresponding real-time PCR efficiencies were calculated according to the slope of the standard curve and the following equation: Efficiency = $[10^{(-1/\text{Slope})}] - 1$ [16]. Data evaluation was carried out using the ABI Prism 7300 Sequence Detection System and the SDS software v.1.2.3 (Applied Biosystems, UK).

Immunoblotting. The second part of the homogenized hippocampi tissues were centrifuged at 12,000 ×g at 4°C for 10 min. The supernatant was collected and total protein concentration was determined using Bradford's method [13]. Samples were dissolved in Protein Loading Buffer and denatured at 95°C for 5 min prior to loading. Equal amounts of protein from each animal (5 µg per lane for α-tubulin, 10 µg per lane for Cx36 and 25 µg per lane for Cx43) were resolved by denaturing SDS-PAGE, 12% acrylamide and transferred to a PVDF membrane (Roche, Germany) by electroblotting (Mini Trans-Blot Electrophoretic transfer cell, Bio-Rad, USA). The membrane was blocked in Tris-buffered saline Tween-20 (TBST) buffer (100 mM Tris base, 150 mM NaCl, and 0.2% Tween 20) containing 2% ECL Advance Blocking Agent at room temperature for 60 min, rinsed briefly with TBST buffer and then incubated for 60 min with the following primary antibodies: mouse monoclonal anti-Cx36 (1:2,000), mouse monoclonal anti-Cx43 (1:10,0000) and mouse monoclonal anti-α-tubulin (1:200,000). The antibodies were diluted in blocking buffer. After washing in TBST buffer 4 times (1× for 15 min and 3× for 5 min), the membrane was incubated with peroxidase conjugated goat anti-mouse IgG (1:50,000, 1:400,000 and 1:2,000,000 for Cx36, Cx43 and α-tubulin, respectively) for 1 h, then washed with TBST buffer 4 times (1× for 15 min and 3× for 5 min) and reacted

Table 2. Hippocampal level of IL1- β after acute and chronic intracerebroventricular injection of LPS to rats.

Time after injection of LPS (2.5 µg/rat)	IL1-β (Pg/100 µg protein of hippocampus)		
	Sham	PBS	LPS
1st injection	18.8 ± 1.9	30.4 ± 5.0	339.0 ± 18.6***
7th injection	18.5 ± 0.8	26.8 ± 6.3	53.5 ± 12.0
14th injection	18.6 ± 1.2	33.4 ± 3.5	44.8 ± 5.6

****P*<0.001 compared to PBS and sham groups.

with ECL Advance Western-Blotting detection reagents for 4 min. An X-ray film (Retina, USA) was used for 30 s to 10 min and then developed to visualize the antibody binding. Bands were quantified by densitometry using Labworks analyzing software (Ultra Violet Products, U.K). The relative levels of Cx36 and Cx43 proteins were expressed as ratios (Cx36/α-tubulin × 100, Cx43/α-tubulin × 100).

Statistical analysis. Data are presented as mean ± S.E.M. The data were analyzed by ANOVA with Tukey post hoc test. In all experiments, $P<0.05$ was considered statistically significant.

RESULTS

Hippocampal level of IL1-β in LPS-treated rats. Hippocampal level of IL1-β significantly raised 6 h after first injection of LPS (Table 2). Chronic 7 and 14 injections of LPS did not increase IL1-β level in the hippocampus (Table 2). An increase of about 1°C in rats' body temperature was observed by first injection of LPS. Furthermore, LPS-induced mild behavioral changes such as reduced exploratory activity namely sickness behavior as reported by the other researchers [17]. However, it was not enough to affect the motor function of the animals.

Cx36 and Cx43 mRNA levels in the hippocampus of LPS-treated rats. Melting curve analysis for Cx36, Cx43 and α-tubulin gene fragments revealed unique PCR product in each reaction (Fig. 1). LPS did not change Cx36 and Cx43 mRNA levels in the hippocampus during whole period of injections (Fig. 2).

Cx36 and Cx43 protein level in the hippocampus of LPS-treated rats. Cx43 protein abundance was significantly decreased from 97.2 ± 15.6 in PBS-treated rats (control group) to 32.7 ± 18.0% in the rats treated by LPS for 7 days. However, Cx36 expression was unchanged in hippocampus during LPS injections (Fig. 3).

DISCUSSION

The present study shows that LPS increases the hippocampal level of IL-β to a 10-fold level and induces neuroinflammation 6 h after central administration. However, we did not find any raise in IL-β hippocampal level following 7 and 14 repeated injections of LPS. It has been well-documented that following LPS administration, a state of unresponsiveness to a subsequent dose of LPS develops that has been called endotoxin tolerance [18].

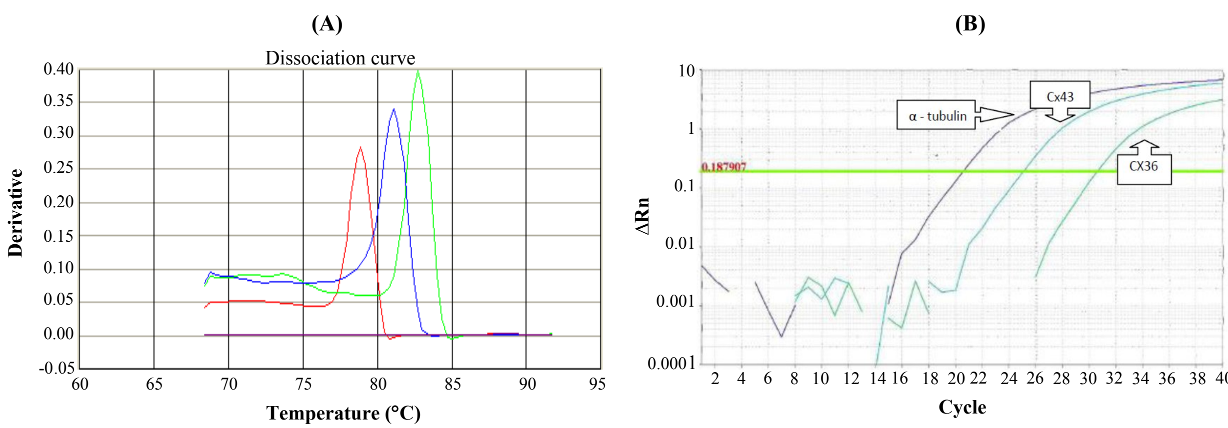

Fig. 1. (A) Melting curve analysis for Cx36, Cx43 and α-tubulin gene fragments detected by the real-time PCR assay. Each peak represents a unique PCR product in each reaction. Tm: melting temperature. (α-tubulin Tm = 81.3°C Cx36 Tm = 79.4°C Cx43 Tm = 82.6°C). **(B)** Amplification plots of the target and reference genes (Cx43, Cx36, α-tubulin) in the real-time PCR assay. mCT: mean threshold cycle. (α-tubulin mCT = 20.69, Cx36 mCT = 31.29 and Cx43 mCT = 25.22).

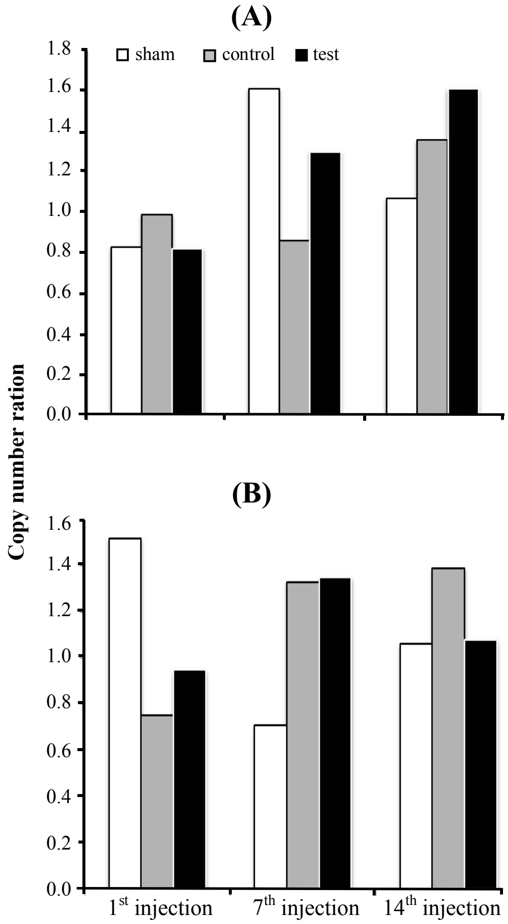

Fig. 2. Hippocampal Cx36 **(A)** and Cx43 **(B)** gene expressions in rats after acute and chronic intracerebroventricular injection of LPS. The expression was normalized to α- tubulin. There is no significant difference between groups.

In this phenomenon, the systemic levels of TNF-α, IL-β and IL-6 are diminished in endotoxin tolerant animals in response to a subsequent challenge with the same endotoxin [19]. It seems that in our study a kind of endotoxin tolerance has been occurred by repeated injection of LPS, and therefore no increase in cytokine levels is observed.

In vitro microglia-astrocyte co-culture studies have shown that LPS stimulates microglia to release pro-inflammatory factors and these factors (TNF-α, Nitric oxide and IL-β) significantly reduce astrocyte GJC concomitant with a decrease in Cx43 protein levels [4-6, 20, 21]. In this regard, Cx43 protein content was reduced to 54% of control level after LPS treatment, while Cx43 mRNA remained unchanged [21]. In other similar study, LPS decreased Cx43 expression at both protein and mRNA levels in rat primary astrocytes culture [6]. Despite these *in vitro* investigations, there is little information regarding the effect of neuro-inflammation induced by LPS on astrocyte gap junctions and Cx expression *in vivo*. In a recent study, expression of Cx43 in mice is significantly enhanced 3 days after experimental brain abscess over long distances extending from the primary site of infection [7]. To our knowledge, our study is the first *in vivo* study on the effect of LPS on brain Cx expression. We could not find any detectable change in mRNA level of Cx43 in the hippocampus during administration of LPS. However, LPS induced a marked reduction in Cx43 protein abundance in the hippocampus after 7 injections of LPS. This finding is consistent with the *in vitro* findings and indicates that down-regulation of Cx43 expression by LPS is regulated at the translation or post-translation level [20]. In our study, the inhibitory effect of LPS on Cx43 expression occurred after chronic (7 days) administration of LPS, when no detectable increase in hippocampal level of IL-β was observed. Therefore, down-regulation of Cx43 expression by LPS is unlikely to be related to pro-inflammatory mediators such as IL-β; and other possible mechanism(s) might be involved. Further complementary investigations are required to clarify this issue.

It is not known whether the changes in GJC and Cx expression associated with the inflammation has helpful or hurtful effects. Experimental evidence indicates that Cx43 containing gap junctions exacerbate central nervous system tissue damage by potentially propagating the spread of toxic/stress molecules to neighboring cells and amplify neuronal damage after brain insults [1, 22, 23]. For instance, pharmacological blockade of Cx43 hemichannels by mimetic peptides abolishes neuronal death induced by TNF-α and IL-1β *in vitro* [24]. On the other hand, astrocytic gap junctions may be beneficial to prevent the propagation of apoptotic and noxious stimuli to distant tissues. In this regard, Cx43+/- mice, which have significantly reduced Cx43 expression, showed a significant increase in infarct lesions and enhanced apoptosis after brain ischemia compared to wild-type (Cx43+/+) mice, suggesting that GJC plays a neuroprotective role [25, 26].

It has been suggested that blockade of glial gap junctions can result in impaired spatial buffering and accumulation of extracellular potassium and glutamate, which, in turn, increase neuronal excitability and lead to seizures [27, 28]. In line with this finding, in Cx43 and Cx30 knockout mice, threshold of epileptiform discharge is reduced [29] and hippocampal synaptic transmission and neuronal excitability are increased due to decreased astroglial glutamate and potassium clearance [30]. These evidences in conjunction with the role of neuroinflammation as a causative factor in epileptogenesis [2] suggest that decrease in Cx43 protein expression during neuroinflammation might

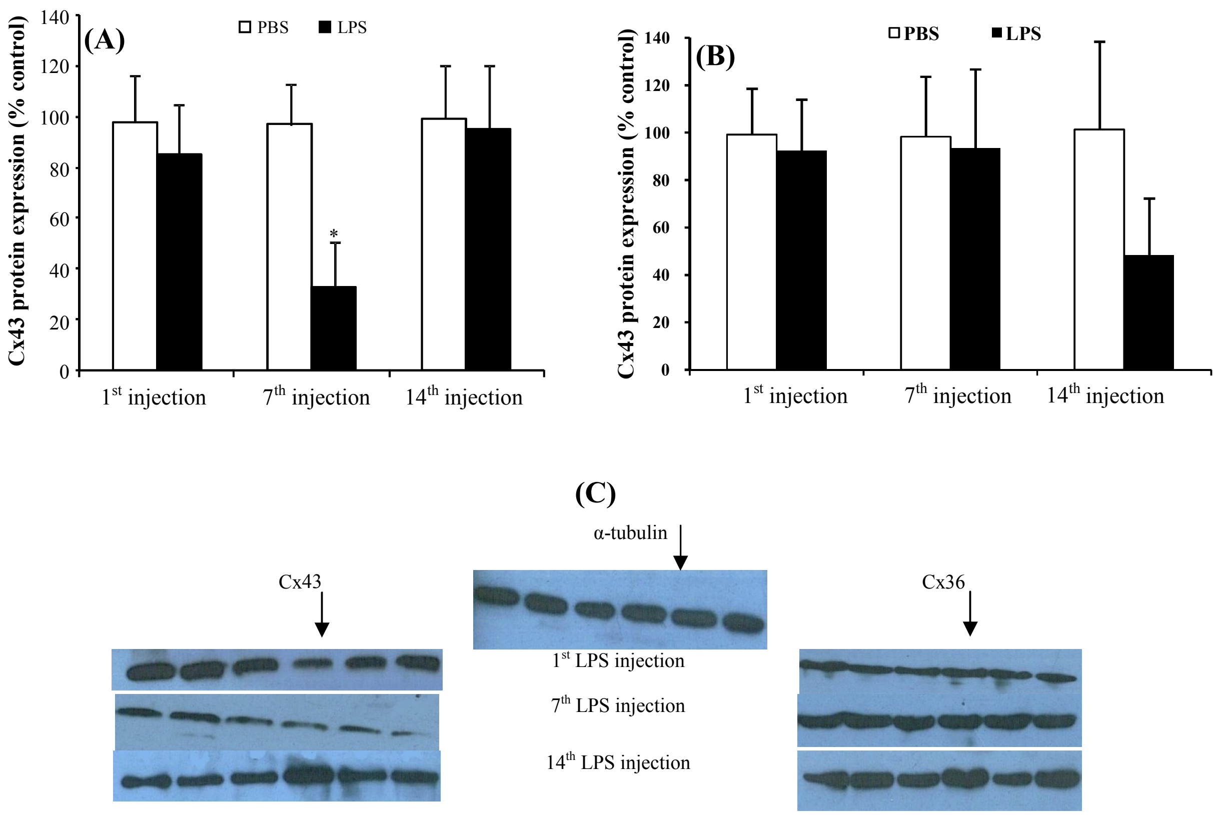

Fig. 3. Hippocampal Cx43 **(A)** and Cx36 **(B)** protein expressions in rats after acute and chronic intracerebroventricular injection of LPS. Cx36 and Cx43 protein levels were normalized to that of α-tubulin protein. Data are expressed as means ± S.E.M (n = 6). Panel **C** shows representative immunoblots of Cx43 and Cx36 in LPS-treated samples. *: *P*<0.05 compared to control PBS group.

play a role in facilitation of epilepsy. However, there are contradictory results indicating an anticonvulsant role for LPS [31-33]. Moreover, application of Cx43 mimetic peptide as a gap junction blocker has been shown to arrest spontaneous seizures [34] and seizure-induced secondary lesion spread [35]. In the present study, LPS inhibited Cx43 expression after 7 i.c.v. injections. We previously observed that 7 i.c.v. injections of LPS inhibit focal seizures development in rats [8]. Therefore, the possibility that down-regulation of Cx43 containing gap junctions, observed in the present study, may have a role in anti-epileptigenic activity of LPS can be suggested.

Cx36 is thought to be the main Cx mediating the electrical coupling between GABAergic interneurons in the CA1 area of the hippocampus. Global ischemia induces a selective upregulation of Cx36 gap junction protein in the CA1, which contributes to the survival of GABAergic interneurons [36]. Moreover, it has been shown that Cx36 hemichannels release ATP during KCl-induced depolarization in primary cortical neuron cultures, which leads to protection against ischemia [37]. However, we found no detectable alteration in mRNA and protein level of Cx36 in the hippocampus by LPS. Therefore, it is suggested that Cx36 hemichannels in the hippocampus are not affected by LPS and might not be involved in modulation of epileptogenesis by LPS.

In conclusion, consistent with *in vitro* studies, protein expression of astrocytic Cx43 hemichannels is down-regulated in the hippocampus by chronic central administration of LPS. The potential role of this change in contribution of LPS to epileptogenesis needs to be clarified by further studies.

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