Research Article

Necroptosis Resumes Apoptosis in Hippocampus but Not in Frontal Cortex¹

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Abstract

Cell death subsequent to concurrent with neuroinflammation results in some damages like neuron loss and spatial memory impairment. In this study we demonstrate the temporal pattern of neuroinflammation, necroptotic and apoptotic cell deaths in hippocampus and frontal cortex following intracerebroventricular administration of lipopolysaccharide (LPS). We evaluated receptor interacting protein kinase 1 (RIP1), RIP3 and two related metabolic enzymes including glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase (GLUD) as necroptosis factors. Apoptosis pathway, antioxidant status and inflammatory cytokines were also assessed. Based on the probable role of these brain regions in working memory performance, spontaneous alternation was evaluated through the Y-maze apparatus. RIP1, RIP3, and then GLUL and GLUD, as well as apoptosis markers, inflammatory regulators and antioxidant defense demonstrated different time-dependent patterns in hippocampus and frontal cortex. Interestingly, in hippocampus but not in frontal cortex, necroptosis resumed apoptosis. Our results in behavioral section revealed that neuroinflammation along with apoptosis and necroptosis pathways could lead to reversible short-term memory impairment after LPS injection. In conclusion, it can be suggested that there is a region-specific response of cell deaths regulators activation in hippocampus and frontal cortex. In addition, elucidating the time profile of events in response to neuroinflammation would be of great help in mechanistic studies and understanding of pathways interaction.

Keywords: Necroptosis, Apoptosis, Neuroinflammation, Metabolism, Memory

Introduction

Inflammation as a host defense and tissue repair process can cause tissue damage in excessive and prolonged condition. This phenomenon in central nervous system (CNS) has been related to the onset of many neurodegenerative diseases. Many receptors that are involved in inflammation have the potential for induction of several types of programmed cell death. Tumor necrosis factor- α (TNF- α), a pleiotropic inflammatory cytokine, provokes apoptosis and necroptosis through TNF receptor 1 (TNFR1) [Fuster-Matanzo et al., 2013; Pasparakis and

Vandenabeele, 2015]. Among Toll-like receptors (TLRs), TLR4 is the most essential receptor to sense pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS). This receptor is also a vital sensor for endogenous damage-associated molecular patterns (DAMPs) released from damaged or dying cells. Recent studies show that LPS causes cell death as an agonist for TLR4 and through the amplified TLR4 mRNA level as well [Bi et al., 2014; Festoff, 2014; Panneerselvam and Ding, 2015]. Among programmed cell deaths, activated TLR pathway stimulates apoptosis and necroptosis [Gunther et al., 2015]. Necroptosis is a programmed form of necrosis. Necroptosis and apoptosis execute cell death in discrete pathways, necroptosis by necrosome formation and apoptosis by protease activation [Vasilikos et al., 2017]. The initiation of necroptosis requires receptor interacting protein kinase 1 (RIP1) and RIP3. RIP1 autophosphorylation on serine 166 can induce RIP3 autophosphorylation on serine 277. Communication of RIP1 and RIP3 is considered as a hallmark of necroptosis which results in plasma membrane rupture and cell death [Sun et al., 2017].

Inflammation, apoptosis and necrosis seem to be three sides of cell death triangle; nevertheless they interact uniquely in different conditions. While apoptosis might be initiated by proinflammatory cytokines, apoptotic cells, depending on the cell environment, can induce some anti-inflammatory mediators. Furthermore, some apoptosis inhibitors sustain inflammation pathways. In addition, apoptotic cells that cannot be cleared by phagocytes may undergo secondary necrosis and release proinflammatory mediators [Kuhn et al., 2014; Pedersen et al., 2014; Rock and Kono, 2008; Sun et al., 2015]. Necroptosis also begins and governs inflammatory signaling [Cai et al., 2016; Silke et al., 2015]. While apoptosis and necroptosis have two different signaling pathways, several studies have shown that they might mutually function as backup mechanism of cell death, as blocked apoptosis pathway can be conducted to necroptosis, or inversely, inhibition of programmed necrosis relocates necroptosis to apoptosis [Degterev et al., 2005; Gong et al., 2014; Han et al., 2009]. However, our previous study has shown that RIP1 inhibition relieved neuroinflammation, and also reduced apoptosis [Nikseresht et al., 2015].

Regarding to what explained above, overlap of neuroinflammation, apoptosis and necroptosis pathways remains a contentious area. This raises the question whether apoptosis and

necroptosis precede each other in an inflammatory context, and their fluctuations follow the same pattern. Since various destructive factors could result in inflammation and cell deaths in regions of the brain and the sensitivity of regions of the brain to detrimental elements is not clear in comparison to each other, the aim of present study was to investigate the temporal pattern of neuroinflammation, apoptosis and necroptosis in two regions of the brain: hippocampus and frontal cortex. These regions of the brain were chosen regarding their roles as central hub in spatial working memory formation [Sigurdsson and Duvarci, 2015]. For this purpose, we used LPS to induce neuroinflammation, apoptosis and necroptosis in rat brains. Based on the potential role of these pathways on working memory performance in hippocampus and frontal cortex, we also examined the changes of spontaneous alternation in LPS-injected rats.

Materials and Methods

Animals:

All experimental procedure was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences in accordance with international guidelines that were confirmed by the National Institutes of Health Publication (No. 80-23, revised 1996). The study received written approval from the neuroscience Research Center Ethics Board (code IR.SBMU.PHNS.REC.1394.60). Male Wister rats, weighting 250–280 g, were housed in standard cages under controlled temperature, humidity, and a 12-h light/dark cycle, with free access to food and water. All efforts were made to reduce animal suffering and to minimize the number of animals used.

Experimental design:

To evaluate the pattern of necroptosis, apoptosis and neuroinflammation adult male rats were assigned to eight groups of eight rats each. They were sacrificed immediately after saline microinjection (0 h) and 4, 8, 12, 24, 48, 72 and 96 h after intracerebroventricular injections of

15 µg LPS (*E. Coli* 055:B55, Sigma, USA) dissolved in 3 µl sterile saline [Iwai et al., 2008; Omidbakhsh et al., 2014]. It is worth mentioning that we had assumed sham groups at each time point with saline microinjection, but preliminary analyses determined that there were no significant differences among sham groups at each time point and 0 h. To assess the impact of LPS on spatial short-term memory, the experimental animals underwent Y-maze testing on the 2nd, 3rd and 4th days after LPS injection (n=8 per group).

Stereotaxic surgery and microinjection:

Animals were anesthetized with intraperitoneal injection of 0.2 ml mixture of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). They were then inserted with guide cannula just above the right cerebral ventricle with stereotaxic coordinates of AP -0.5 mm, L -1.5 mm, and DV 4 mm (from the skull surface) according to the rat brain atlas of Paxinos and Watson [Paxinos and Watson, 2007]. After 8 days recovery, all microinjections were performed slowly over a period of 60 s using a Hamilton syringe connected to the injection needle.

Western blot analysis:

Animals were decapitated following CO₂ asphyxiation (n=4). Hippocampi and frontal cortices were instantly removed and washed in ice-cold phosphate buffered saline (PBS). The brain tissues were isolated on ice, snap frozen in liquid nitrogen, and then stored at -80 °C. Hippocampi and frontal cortices were homogenized in a lysis buffer containing protease inhibitor. Protein concentrations of samples were determined using colorimetric method of Bradford [Bradford, 1976]. Next, proteins were separated using a SDS-PAGE gel (12%, polyacrylamide) and transferred to a Polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were probed with the following primary antibodies: TNF-α, RIP1, caspase-8, Bax, Bcl2, caspase-3, β-actin (Cell Signaling Technology, Beverly, MA, USA), cyclooxygenase-2 (COX-2) (ABR—Affinity, BioReagents, Pierce Biotechnology, Rockford, IL), Interleukin-1β (IL-1β) and RIP3 antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). HRP-linked antibody was used as a secondary antibody (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive polypeptides were detected by chemiluminescence using Electrochemiluminescence (ECL) kit (Amersham, Bioscience, Piscataway, USA). Finally, results were quantified by scan of X-ray films and analysis by ImageJ software.

Enzymes activity measurement:

All materials and reagents used in all assays were purchased from Sigma–Aldrich (St. Louis, MO, USA).

- **Glutamate-ammonia ligase (GLUL)** activity in purpose brain tissues was measured based on Kingdon et al. method, in which the ADP production is associated with the synthesis of glutamine and linked to the oxidation of β -Nicotinamide adenine dinucleotide solution (β -NADH) in the presence of phosphoenolpyruvate, pyruvate kinase, and lactic dehydrogenase [Kingdon et al., 1968]. Absorbance of samples was recorded at 340 nm in presence of substrate including imidazole HCl buffer, sodium glutamate solution, adenosine 5'-triphosphate solution (ATP), $MgCl_2$, KCl, NH_4Cl , phospho(enol)pyrovate solution, β -NADH, and pyruvate kinase/lactic dehydrogenase enzyme solution. Condition of the reaction mixture was constant ($T = 37^\circ C$ and $pH 7.1$).
- **Glutamate dehydrogenase (GLUD)** activity was spectrophotometrically determined based on oxidation of the reduced co-enzyme NADH [Doherty, 1970]. In this assay, reaction mixture contained potassium phosphate buffer $pH 7$, NADH, NH_4Cl , 2-Oxo-glutarate. Finally, the absorbance of samples was recorded at 340 nm.

Antioxidant status evaluation:

- **Lipid peroxidation** concentration was determined based on double heating method [Draper and Hadley, 1990], reaction between thiobarbituric acid (TBA) and Malondialdehyde (MDA) produces purple color which is measured spectrophotometrically. For this purpose, Hippocampus or frontal cortex homogenate equivalent to 200 μg of total protein was mixed with 2.5 ml of trichloroacetic acid (TCA, 10 % w/v) solution and then was boiled to $100^\circ C$ for 15 min. After cooling, the mixture was centrifuged at 3000 rpm for 10 min. Next, 2 ml of each sample transferred to a test tube containing 1 ml of TBA solution (0.67 % w/v). Then, each tube was placed in hot plate for 15 min. After cooling to room temprature, the absorbance was measured at 532 nm.
- **Superoxide dismutase (SOD)** activity was measured based on the method described by Kakkar et al. [Kakkar et al., 1984]. First, we prepared assay mixture containing 0.052 M

phosphate buffer pH 8.3, 186 μ M phenazinemethosulphate, and 300 μ M nitrobluetetrazolium. After adding the homogenized tissues to the assay mixture, reaction was started by adding 780 μ M NADH. Strength of color was measured at 560 nm.

- **Catalase** activity was measured by adding of H₂O₂ (0.01 M) to 60 μ g of total protein of each homogenized tissues samples according to the method of Aebi [Aebi, 1984]. Disintegration of H₂O₂ by the activity of catalase was monitored at 240 nm spectrophotometrically.
- **Reduced Glutathione** level was determined based on Ellman's method [Ellman, 1959] and the absorbance was determined at 412 nm.

Immunofluorescence:

It has become evident that RIP3 expression is a specific marker of non-apoptotic cell death [Roychowdhury et al., 2012], collected data by Western blot analysis were confirmed using Immunofluorescence technique. Animals were perfused transcardially with PBS (pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia (n=4). The whole brains were then removed and post fixed in 4% paraformaldehyde for 24 h and subsequently fixed in paraffin for histopathological studies and sectioned at a thickness of 5 μ m. Coronal sections of hippocampi were rehydrated and rinsed in running tap water. After antigen retrieval, the brain sections were premeabilized then blocked with goat serum and incubated with RIP3-specific antibody (1:250 dilution) overnight at 4 °C followed by fluorescent secondary antibody. Data analysis was conducted using a fluorescence microscope (Olympus IX71, Japan).

Y-Maze spontaneous alternation test:

Spontaneous alternation evaluates working memory performance through in a symmetrical Y-maze apparatus which does not require any reward or punishment [Kimura et al., 2010; Ohno et al., 2004]. Experimental animals were allowed to explore through the maze during 8 minutes. Two parameters were recorded visually, total number of arms entered and the sequence of entries. In this protocol, alternation is representation of all three different arms in a triple set.

Percentage of alternation is the number of triads containing entries into all three arms divided by the maximum possible alternations (the total number of arms entered minus 2) X 100.

Data analysis:

All data are represented as the mean $\pm$ SEM. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test to investigate the difference among groups. The statistical significances were achieved when $p < 0.05$.

Results

Influence of LPS on necroptosis markers during 4 days

Hippocampus: As shown in Fig. 1a-c, RIP1 and RIP3 augmentation started at 8 h ($p < 0.001$). Necroptosis markers reached the highest level 24 h after LPS injection ($p < 0.001$). Although the temporal patterns of RIP1 and RIP3 show a significant increase at 8, 12, and 24 h ($p < 0.001$), there was a significant difference in the amount of each protein between the time point of 8 and 24 h ($p < 0.001$), confirmed an augmented rate of necroptosis cell death in hippocampus during 16 hours. Following the peak, decrease of RIP1 and RIP3 returned to their basal levels at 96. Consistent with Western blot findings, our data from immunofluorescence method also revealed the highest level of RIP3 24 h after LPS injection (Fig. 1d).

Frontal cortex: Different temporal patterns of necroptosis markers were depicted in frontal cortex region (Fig. 1e-g). The increase of RIP1 and RIP3 started at 8 h ($p < 0.001$) and lasted until 24 h subsequent to LPS injection ($p < 0.001$). Necroptosis regulatory factors returned to their basal level 48 h after LPS injection. Non-significant difference of the RIP1 and RIP3 protein levels among the time points of 8, 12, and 24 h revealed a wide peak of these protein levels during 16 hours.

Effect of LPS injection on GLUL and GLUD1 enzyme activity

Hippocampus: GLUL enzyme activity increased at 8 h compared to zero time point significantly ($p < 0.05$). Augmentation of the GLUL activity continued getting the highest level at 48 h ($p < 0.01$)

and then declined to its basal level 96 h after LPS injection (Fig. 2a). GLUD followed the same pattern and reached its peak at 48 h ($p<0.001$) (Fig. 2b).

Frontal cortex: A different time-dependent pattern was observed in frontal cortex. GLUL activity augmentation started at 8 h ($p<0.05$) and continued to 12 and 24 h after LPS injection ($p<0.001$), then dropped from 48 until 96 h following LPS injection (Fig. 2c). GLUD followed the same pattern to reach the highest level and decline to its basal level (Fig. 2d).

Impact of LPS on apoptosis regulatory factors during 4 days following LPS injection

Hippocampus: As shown in Fig. 3a-d, apoptosis regulatory factors have been induced 8 h following LPS microinjection. Bax/Bcl2 ratio touched the highest level at 8 h ($p<0.001$) and then the decrease of this ratio was detected at 12 ($p<0.01$) and 24 h. Despite reduction of Bax/Bcl2 ratio level, second augmentation was observed at 48 h ($p<0.001$). Following the second induction of Bax/Bcl2 ratio, proteins moved to their basal levels (Fig. 3b). Cleaved caspase-3 had also a number of changes during 4 days following LPS injection, two peaks, first at 8 h ($p<0.001$) and second at 48 h ($p<0.001$). Subsequent to the highest level of cleaved caspase-3, two reductions were found, first at 12 ($p<0.01$) and 24 h and second at 72 ($p<0.5$) and 96 h (Fig. 3c). Cleaved caspase-8 pursued the same pattern similar to cleaved caspase-3, the highest protein level at 8 ($p<0.001$) and 48 h ($p<0.001$) after LPS injection then shrinking to basal level (Fig. 3d). Frontal cortex: This region had a different time-dependent manner during 4 days following LPS injection. All apoptosis markers including Bax/Bcl2 ratio, cleaved caspase-3, and cleaved caspase-8 increased significantly at 4 h compared to zero time point ($p<0.001$, $p<0.05$, and $p<0.001$ respectively) then all of them returned to basal level at 24 h (Fig. 3e-h).

Influence of LPS on inflammatory mediators during 4 days

Hippocampus: TNF- α , as a necroptosis and apoptosis initiator, elevated at 4 h ($p<0.05$) and stayed in high levels from 8 to 72 h following LPS injection ($p<0.001$). Other inflammatory mediators, IL-1 β and COX-2, rose following TNF- α increase (Fig. 4a-d).

Frontal cortex: TNF- α enhanced at 4 h ($p<0.01$) and continued an increased trend at 8 and 12 h ($p<0.001$), finally TNF- α decreased to its basal level 48 h after LPS injection. IL-1 β and COX-2 followed parallel patterns subsequent to the TNF- α alteration (Fig. 4e-h).

Impact of LPS on oxidative stress relative enzymes activity, GSH Content, and MDA concentration

As was supposed, LPS injection provoked an oxidative stress manifestation by changing activity of oxidative stress-related enzymes SOD and catalase, concentration of GSH, and levels of MDA in the hippocampus and frontal cortex of rats in a time-dependent manner.

Hippocampus: SOD and catalase had two augmentations at 4 and 24 h ($p<0.05$) and a transient drop at 8 h ($p<0.05$) while GSH decreased at 8 and 48 h ($p<0.05$). MDA levels showed two amplifications at 8 and 48 h ($p<0.05$) (Fig. 5a-d).

Frontal cortex: The results showed reduction of SOD and catalase activity, decline of GSH concentration and augmentation of MDA levels (Fig. 5e-h).

Spontaneous alternation changes during 4 days following LPS injection

As shown in Fig. 6, our data in Y-maze test showed that LPS significantly impaired spontaneous alternation on the 3rd day ($p<0.01$) of testing, while it had no significant effects on the 2nd and 4th days. LPS had no effect on the total number of arm entries.

Discussion

In the present study, necroptosis regulatory markers including RIP1 and RIP3, their downstream signaling elements, GLUL and GLUD activity, along with apoptosis and antioxidant defense markers, and also spatial short-term memory performance were evaluated in an inflammatory context time-dependently. This study focused on two regions of the brain, hippocampus and frontal cortex, respecting their roles as central hub in spatial working memory formation. Based on our results, hippocampus and frontal cortex showed two different temporal patterns in cell

deaths, neuroinflammation and antioxidant defense after LPS microinjection. Moreover, hippocampus demonstrated the second apoptosis markers amplification after necroptosis.

Necrosis is commonly regarded as an accidental unregulated form of cell death whereas necroptosis cell death is a form of programmed necrosis controlled by RIP1 and RIP3 kinases [Vandenabeele et al., 2010]. Although it has been shown that various destructive factors such as inflammation, toxic elements, trauma, and ischemia could result in necroptosis cell death in hippocampal and cerebral neurons [Liu et al., 2014; Qinli et al., 2013; You et al., 2008], the sensitivity of these two regions of the brain to detrimental elements is not clear in comparison to each other. Based on our results, despite different distances of hippocampus and frontal cortex from lateral brain ventricle, initiation of necroptosis in these two regions was simultaneous. Programmed necrosis was terminated in two different time points in these two areas. Necroptosis markers remained active in a wide range of time in hippocampus while cortical region experienced less contact with necroptosis regulatory factors. Since prolonged exposure to cell death-associated molecules could be destructive, it might be speculated that hippocampus is on the verge of more destruction compared to the frontal cortex.

Programmed necrosis is considered to be an energy dependent process [Yin et al., 2015]. Communication between RIP3, as a main necroptosis regulator, and GLUL and GLUD, as metabolic enzymes was shown in 2009 [Zhang et al., 2009]. This was also confirmed in necroptosis cell death in our previous study [Nikseresht et al., 2015]. GLUL, a cytosolic enzyme, catalyzes glutamate (Glu) to form glutamine (Gln) and Gln is transferred into the mitochondria and turns into Glu. GLUD1, a mitochondrial matrix enzyme, converts Glu to α -ketoglutarate. GLUL and GLUD1 are essential for the use of amino acid Glu or Gln as substrates for ATP production by means of oxidative phosphorylation [Zhang et al., 2009]. Since RIP3 directly interacts with GLUL and GLUD1, RIP3 may govern the cell's metabolic state. Moreover, knockdown of GLUL or GLUD1 partially inhibits TNF-induced necroptosis [Declercq et al., 2009]. Our results demonstrated that GLUL and GLUD1 were activated following augmented RIP3 protein level in hippocampal and cortical tissues. Similar to RIP3 activation in an extended time in hippocampus in comparison with frontal cortex, time frame of hippocampi's metabolic enzymes activity elevation were longer than frontal cortices'.

Although necroptosis and apoptosis might be triggered by the same death receptor agonists, the onset of apoptosis is notably faster than necroptosis in response to the similar stimuli [Degterev et al., 2005]. Interestingly, our data showed that apoptosis and necroptosis began simultaneously in hippocampus while apoptosis occurred before necroptosis in frontal cortex. These two regions of the brain also showed two different time-dependent patterns after apoptosis initiation. Hippocampus had the second amplification after returning Bax/Bcl2 ratio and cleaved caspase-3 to their basal levels. Perhaps intensive damage following necroptosis leads to apoptosis. Moreover, apoptosis is known to negatively regulate necroptosis via active caspase-8, which is able to cleave necroptosis regulators, including RIP1 and RIP3. In addition, inhibition of caspase-8 activity causes cells to express RIPK3 [Newton, 2015]. In the present study, hippocampal caspase-8 had two peaks. Unlike what was expected in hippocampus, when the first peak of caspase-8 was observed, necroptosis regulators started to increase while its second peak resulted in necroptosis down regulation. In frontal cortex, caspase-8 was stimulated earlier than RIP1 and RIP3. However, activated caspase-8 was not able to suppress the onset of necroptosis. These data imply that apoptosis and necroptosis may occur concurrently in hippocampal and cortical cells. In view of the fact that apoptosis regulators have two peaks in hippocampus, and hippocampal cells experience apoptosis condition for a longer time compared to cortical cells, it can be suggested that there is a region-specific response in the elevation of apoptosis regulators. In agreement with our findings, it has been reported that in lead-induced caspase-3 activation condition in rat brain, further effects in hippocampus and fewer effects in the frontal cortex were observed. Therefore, it seems that that hippocampus is more susceptible to apoptosis compared to other regions of the brain [Kiran Kumar et al., 2009].

As mentioned above, we observed elevated activity of GLUL and GLUD enzymes following LPS injection. Use of glutamate and glutamine amplifies reactive oxygen species (ROS) production that occurs as a consequence of energy metabolism [Declercq et al., 2009]. Antioxidants are defense molecules against ROS, which can lead to lipid peroxidation and necroptosis. The relative importance of this molecule depends on the type, place, and target of ROS-induced damage [Halliwell, 1995; Vandenabeele et al., 2010]. The results of present study indicated that

LPS, in hippocampus, caused transient upregulation of oxidative stress related enzymes including SOD and catalase before the onset of apoptosis both in first and second amplifications. Activity of these enzymes decreased with elevation of apoptosis regulatory markers. It seems that augmented activity of antioxidant agents might try to prevent apoptosis. Likewise, according to a recent study, enrichment of antioxidant defense activity and reduction of oxidative stress result in apoptosis prevention [Fernandez-Millan et al., 2015]. Although antioxidant have been found to be involved in necroptosis inhibition [Canli et al., 2016], in this study, SOD and catalase activity augmentation occurred concurrent with necroptosis. It might happen because of intensive damage which could not be broken by antioxidant activity. In frontal cortex, SOD and catalase activity declined along with upregulation of apoptosis regulatory factors. It is remarkable that GSH content decreased and MDA concentration increased following apoptosis markers activation both in hippocampus and frontal cortex. Interestingly, MDA was produced as a necroptosis product in these two areas. In line with these data, SOD activity decreased in the striatum while increased in the frontal cortex following toluene exposure. Different alterations in SOD activity in various parts of the brain may represent different capacities in the special brain regions [Kodavanti et al., 2015].

Derived component from the outer membrane of Gram-negative bacteria, LPS, is capable of inducing an immune response through the activation of TLR-4 that results in increasing the levels of several proinflammatory cytokines including IL-1 β and TNF- α directly within the CNS [Block et al., 2007; Erickson and Banks, 2011]. Our data revealed that TNF- α elevated following LPS injection then, IL-1 β and COX-2 protein level enhanced in both hippocampus and frontal cortex. Key inflammatory cytokines, TNF- α , IL-1 β , and COX-2 reduction to their basal levels took longer time in hippocampus than frontal cortex. Interestingly, following apoptosis markers, necroptosis and inflammatory hallmarks also returned to their basal level. It showed that hippocampal and cortical cells didn't go into a vicious cycle after LPS single dose.

Systemic injection of LPS was applied to induce acute neuroinflammation in various brain regions by Noh et al; they reported that LPS injection could bring about IL-1 β rise in hippocampus and frontal cortex while TLR-4 protein expression in frontal cortex was higher than hippocampus. In addition, their data showed that acute neuroinflammation model by

systemic LPS injection could not induce cognitive dysfunction [Noh et al., 2014]. While Noh et al. suggested non-significant changes of spatial memory following LPS injection, study of Joshi et al. showed working memory impairment by LPS administration through cholinergic dysfunction in hippocampus and prefrontal cortex [Joshi et al., 2014]. In behavioral test, our data represented spatial short-term memory impairment 3 days but not 2 days after intracerebroventricular injection of LPS; the reason might be integration of neuroinflammation, apoptosis and necroptosis pathways before 3rd day. On the other hand, the results demonstrated that there was no significant difference between saline and 4 days following LPS microinjection group. It can be speculated that a single dose of LPS induced transient memory impairment and following the termination of cell deaths and neuroinflammation, memory failure reverted. Another study has also shown that LPS-induced impairments in spatial learning and memory could be reversible depending on LPS doses [Bian et al., 2013].

Collectively, these data suggest that LPS could induce enhancement of RIP1 and RIP3 protein levels, RIP3 downstream events including GLUL and GLUD enzyme activity, apoptosis regulatory factors, antioxidant defense, and inflammatory cytokines in a time-dependent manner in hippocampus and frontal cortex (Fig. 7). It could also influence spontaneous alternation behavior as an index of spatial short-term memory. Different properties of hippocampus and frontal cortex suggest that the similar molecular pathways are not in fact the same in different regions of the brain. In addition, hippocampus is more vulnerable to destructive conditions. In conclusion, clarifying the trend of events and disturbing successive events in response to neuroinflammation might be an approach to control neuroinflammation-induced diseases.

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Abbreviation	Full title
COX-2	Cyclooxygenase 2
GLUD	Glutamate dehydrogenase
GLUL	Glutamate-ammonia ligase
GSH	Glutathione
IL-1 β	Interleukin 1 beta
MDA	Malondialdehyde
RIP1	Receptor interacting protein 1
RIP3	Receptor interacting protein 3
SOD	Superoxide dismutase
TNF- α	Tumor necrosis factor- α

Fig. 1 Protein quantification by the use of Western blotting (a and e) with RIP1 and RIP3-specific antibodies in hippocampus (b and c) and in frontal cortex (f and g). β -actin assumed as an internal control and all protein bands normalized with β -actin (one representative Western blot is shown) (D) Representative images of immunofluorescent stained brain sections from LPS-injected rat. The brains were fixed, sectioned, and stained with anti-RIP3 antibody and secondary antibody Alexa Fluor 488-conjugated (green) and DAPI (blue), ($n=4$ for each group, scale bar 50 μ m). Each point shows the mean $\pm$ SEM. (ns=non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ * vs. 0 h and # vs. 8 h)

Fig. 2 Analysis of the activity of GLUL and GLUD in the hippocampus (a, b) and frontal cortex (c, d). (n=4 for each group). Data are mean $\pm$ SEM. (* p <0.05, ** p <0.01, *** p <0.001 * vs. 0 h)

Fig. 3 Proteins were quantified by Western blot technique (a and e). Protein levels of Bax/Bcl2 ratio, cleaved caspase-3 and cleaved caspase-8 were measured in hippocampus (b-d) and in frontal cortex (f-h). (n=4 for each group). Each point shows the mean $\pm$ SEM. (* p <0.05, ** p <0.01, *** p <0.001 * vs. 0 h)

Hippocampus

Fig. 4 Protein measurement by Western blot technique (a and e). Protein levels of TNF- α , IL-1 β and COX-2 were quantified in hippocampus (b-d) and in frontal cortex (f-h). (n=4 for each group). Each point shows the mean $\pm$ SEM. (* p <0.05, ** p <0.01, *** p <0.001 * vs. 0 h)

Fig. 5 Analysis of the activity of SOD and catalase in the hippocampus (a, b) and frontal cortex (e, f) and the content of GSH and MDA in the hippocampus (c, d) and frontal cortex (g, h) showed alterations in LPS injected rats in a time-dependent manner. (n=4 for each group). Data are mean $\pm$ SEM. (*p<0.05, **p<0.01 * vs. 0 h)

Fig. 6 Memory impairment was induced by LPS in a time dependent manner. (a) Spatial short-term memory was studied immediately after saline injection and also 2, 3 and 4 days after LPS injection using Y-maze. (b) No significant differences in total arm entries among groups. (n=8 for each group). Data are mean $\pm$ SEM. (**p < 0.01 * vs. saline group)

Fig. 7 Different temporal profiles of neuroinflammation, necroptosis and apoptosis pathways in hippocampus and frontal cortex in various time points after LPS injection. (TNF- α , Caspase-3 and RIP3 were assumed as inflammation, apoptosis and necroptosis markers respectively.)

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