Galectin-3 expression in hippocampal CA2 following transient forebrain ischemia and its inhibition by hypothermia or antiapoptotic agents

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Recent evidence has suggested that the hippocampal CA2 region plays an important role in the recognition process. We have reported that ischemic damage in the hippocampal CA2 region following transient ischemia is caused by apoptosis, but the underlying mechanisms are still not clear. Galectin-3 is a \(\beta\)-galactosidase-binding lectin that is important in cell proliferation and apoptotic regulation. We have also reported that galectin-3 was expressed in activated microglia in the CA1 region 96 h after transient ischemia. The aim of this study is to determine the localization and time course of galectin-3 expression in the CA2 region following transient forebrain ischemia. Galectin-3 immunostaining was observed in both interior side of CA1 region and CA2 region in hippocampus 60 h after ischemic insult. At 66 h, galectin-3 was observed in the whole CA1 region adjacent to the CA2 region in the hippocampus. Both galectin-3 expression and neuronal cell death in the CA2 region were significantly inhibited by hypothermia and by apoptosis-inhibiting reagents. These results suggest that galectin-3 in the CA2 region is expressed independent of that in the CA1 region. Protection of the expression of galectin-3 in the CA2 region might contribute toward the survival of CA2 pyramidal neurons. NeuroReport 27:311–317 Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

The hippocampal CA2 region is classically defined as the area located between the CA3 and the CA1 regions with large pyramidal cells. The hippocampus plays a key role in the acquisition and recall of episodic memory [1], and the trisynaptic circuit, entorhinal cortex layer II – dentate gyrus – CA3–CA1, provides the anatomical substrate for memory circuit functions [2,3]. The CA2 region is recognized simply as a boundary area adjacent to the CA1 and CA3 regions, but its function is not clear. However, recent evidence strongly suggests that the CA2 region plays an important role in recognizing conflict between stored and current experience, and also plays a role in sociocognitive memory processing [4,5]. These findings raise the possibility that CA2 dysfunction may affect these forms of memory processes. In fact, there is a decrease in the number of nonpyramidal neurons in the CA2 region in schizophrenia and bipolar disorder [6]. Interestingly, we and other investigators have reported that CA2 neurons are specifically damaged following transient forebrain ischemia in an animal model [7–9], although the precise underlying mechanism of CA2 neuronal death is still not clear. Clarification of the mechanism of the CA2 neuronal death might provide important information that could lead to novel therapeutic approaches in the treatment of a variety of neurological disorders.

Galectin-3 is a 30 kD \(\beta\)-galactosidase-binding lectin that binds immunoglobulin E and glycoconjugates on mammalian cell surfaces. Galectin-3 has been shown to play a pivotal role in diverse physiological functions, such as cell growth, apoptosis, and mRNA splicing, as well as in pathological processes, as an inflammatory mediator [10]. Although galectin-3 has also been associated with microglial activation in mouse brain in an experimental autoimmune disease model [11], its role in the central nervous system under pathological conditions is poorly understood. Recently, we reported that galectin-3 is upregulated only in activated microglial cells located within the hippocampal CA1 region following transient forebrain ischemia in gerbils [12]. We have also reported galectin-3 upregulation in microglial cells in the cerebellum after encephalomyocarditis virus inoculation [13].
Thus, there are strong associations of the expression and localization of galectin-3 in various central nervous diseases, such as ischemic brain injury, virus-induced encephalitis, and myelin degenerative disorders [14]. However, there are no reports investigating galectin-3 expression in the CA2 region.

The aim of this study was to investigate the underlying mechanism of CA2 degeneration. In the present study, we show that galectin-3 is transiently upregulated in the CA2 region following transient forebrain ischemia and that this upregulation is inhibited by hypothermia and neuro-protective agents.

Materials and methods
Animal and in-vivo experimental procedures
Male Mongolian gerbils, weighing 65–75 g, were subjected to severe forebrain ischemia as described previously [15]. Briefly, the bilateral common carotid arteries were isolated through an anterior midcervical incision and occluded with microclips. After 5 min of forebrain ischemia, the clips were removed. Rectal temperature was maintained at 37 ± 0.3°C using a heating pad from the induction of anesthesia until 3 h following ischemia. Sham-operated animals were subjected to the same surgical manipulation, but without occlusion of bilateral common carotid arteries. After ischemic insult, animals were anesthetized with pentobarbital and perfused transcardially with saline and then with phosphate-buffered 10% formalin. Brains were removed and processed for paraffin embedding. Three micrometers coronal sections were cut at the level of the dorsal hippocampus and then used for immunohistochemical staining. The animals were housed under 12 h light/dark cycles at 22°C and were allowed free access to food and water. We fully complied with the ‘Guidelines Concerning Experimental Animals’ issued by the Japanese Association for Laboratory Animal Science.

Regulation of body temperature
Regulation of body temperature was performed as described previously [15]. The rectal temperature of gerbils was monitored. Hypothermia (31°C) was induced during ischemia by placing isoflurane-anesthetized gerbils adjacent to crushed ice in a container and a cooling fan. After 5 min of ischemia, gerbils were placed on a 40°C plate and rewarmed with a heating fan. When the rectal temperature reached 37°C, gerbils were placed on a heating pad at 37°C. After 20 min of reperfusion, anesthesia was discontinued. In the ischemic control group, the animals were placed on the heating pad and their heads were in direct contact with the pad. Rectal temperature was maintained at 36–37°C during the operation, ischemia, and 20 min following the reperfusion period, after which anesthesia was discontinued. Ninety-six hours after ischemic insult, the brains were subjected to immunostaining.

Drug administration
To evaluate the effects of apoptosis-inhibiting agents on the expression of galectin-3 in CA2 region, an inhibitor of proteolytic process of apoptosis, N-tosyl-l-phenylalanyl chloromethyl ketone (TPCK 100 mg/kg; Sigma-Aldrich, St Louis, Missouri, USA) was administered intraperitoneally 1 h before ischemic insult. Glycolysis inhibitor 2-deoxy-D-glucose (2DG; Sigma-Aldrich) was injected intraperitoneally 5 min after (4 g/kg) and 4 h after (2 g/kg) ischemic insult. Five animals per group were used for each experiment.

Immunohistochemistry
Anti-mouse galectin-3/Mac2 (Rat IgG, 14–5301) was purchased from Bay Bioscience Co. Ltd (Hyogo, Japan). The deparaffinized sections were blocked for endogenous peroxidase activity by incubation in distilled water containing 3% hydrogen peroxide for 5 min. Antigen retrieval was performed using a 0.01 M citrate buffer (pH 6.0) for antigalectin-3 antibody by the Pascal heat-induced target retrieval system (Dako, Carpinteria, California, USA). Nonspecific binding sites were blocked in 0.01 M PBS, pH 7.4, containing 2% BSA (Wako Pure Chemical, Osaka, Japan) for 60 min. Antigaglecin-3 antibodies used at a dilution of 1:100 in 2% BSA/PBS were added on the slides and incubated overnight at 4°C. Galectin-3 was detected using a biotinylated anti-rat IgG (1:200, E0468; Dako) for 30 min, followed by incubation.
H&E staining, immunohistochemical staining for galectin-3, Iba-1 and GFAP, and apoptotic DNA fragmentation detected by TUNEL staining in a low-power field and a high-power field of the hippocampus at 96 h after ischemic insult. Many damaged CA2 neurons had lost their nuclear affinity for hematoxylin and appeared as an eosinophilic ‘ghost neuron’ in H&E staining. The localization of galectin-3-positive cells coincided with that of Iba-1-positive cells. There were no GFAP-positive cells in the CA2 damaged area. Some of the TUNEL-positive CA2 neurons were distinguishable among the CA1 neurons by the shape of positive stained nuclei. Scale bars in LPF and HPF of H&E staining are 100 and 50 μm, respectively. G3, galectin-3; GF, GFAP; HE, H&E staining; HPF, high-power field; Iba, Iba-1; LPF, low-power field; TU, TUNEL staining.
Table 1 Time course of immunoreactive galectin-3 staining in the hippocampal CA2 area of gerbil brain following 5 min of transient forebrain ischemia

| Time course (h) | Galectin-3 |
|----------------|-----------|
| Sham-operation | 0         |
| 24             | 0         |
| 36             | 0         |
| 48             | 0         |
| 54             | 3.3±4.7   |
| 60             | 5.0±4.1   |
| 66             | 23.8±5.9  |
| 72             | 22.5±0.7  |
| 96             | 25.6±5.6  |
| 2 weeks        | 3.8±4.3   |

Cell numbers of immunoreactive galectin-3 in the hippocampal CA2 area at each time point were counted. Results were expressed as mean±SD in 3–10 animals.

Data were collected from 3–13 animals. Data are expressed as the mean±SD. Statistical analysis was carried out using analysis of variance with multiple-comparison correction using the Tukey test. A value of P value less than 0.05 was considered significant.

Results
Galectin-3 in gerbil CA1 and CA2 hippocampus following transient ischemia is easily recognized in immunohistochemical staining for galectin-3 because galectin-3 is not expressed in other brain tissues, except ependymal cells lining the lateral ventricles (Fig. 1). The ependymal cells are strongly positive for galectin-3 in sham-operated animals and thus used as an internal positive control.

Many damaged CA2 neurons had lost their nuclear affinity for hematoxylin and appeared as an eosinophilic ‘ghost neuron’ in HE staining. The localization of galectin-3-positive cells coincided with that of Iba-1-positive cells. There were no GFAP-positive cells in the CA2 damaged area. Some of the TUNEL-positive CA2 neurons were distinguishable among the CA1 neurons by the shape of positive stained nuclei (Fig. 2).

The time course of immunostaining for galectin-3 in gerbil hippocampal CA2 sector is summarized in Table 1 and the representative photographs of galectin-3 immunostaining are shown in Fig. 3. Immunostaining of galectin-3 was not observed at 24, 36, and 48 h after ischemic insult, or in the sham-operated group, but was faintly apparent at 54 and 60 h, and strongly apparent at 66, 72, and 96 h after ischemia. Then, the staining was decreased at 2 weeks (Table 1). Galectin-3 immunostaining was observed only in the interior sidecell of the CA1 region and the CA2 sector in the hippocampus at 60 h after ischemic insult (Fig. 1). At 66 h, galectin-3 was observed in the entire CA1 region adjacent to the CA2 sector in the hippocampus (Fig. 1). This indicates that galectin-3 immunostaining in CA1 and CA2 occurs independently. This is followed by a staining area developing throughout the CA1 and CA2 regions. These results suggest that the galectin-3 activating pathway in the CA2 region is regulated independent of the CA1 region.

The expression of galectin-3 in the CA2 region after transient ischemia was almost completely prevented by intra-ischemic hypothermia (Fig. 4). Intraperitoneal administration of TPCK (100 mg/kg) 1 h before ischemic insult and intraperitoneal administration of 2DG, administered 5 min (4 g/kg) and 4 h (2 g/kg) after ischemic insult, strongly inhibited the galectin-3 expression in CA2. These interventions also suppressed galectin-3 expression in CA1 in the same manner (data not shown).

Discussion
Although there is an extensive literature describing the importance of the CA1 region in memory function, very little is known about the role of the CA2 region in hippocampal function [16,17]. Recently, DeVito and colleagues reported that CA2 may be involved in social recognition memory and memory for temporal order [18]. Recent evidence strongly suggested that the CA2 region plays a key role in resolve conflict between stored and current experience, as well as being a critical hub for socio-cognitive memory processing [4,5]. Furthermore, dysfunction of the CA2 region may contribute toward the significant deficits in both social behavior and episodic memory of schizophrenic and bipolar disorder patients [6]. Therefore, understanding the mechanisms of CA2 dysfunction might provide key insights into understanding memory disorders and may lead to novel treatments for schizophrenic or bipolar patients.

We reported that transient forebrain ischemia induced CA2 neuronal degeneration [9]. Furthermore, we reported that galectin-3 is expressed in microglia located in the hippocampal CA1 region following transient ischemia [12]. In the present paper, we have shown that the galectin-3 is transiently upregulated after transient forebrain ischemia in the CA2 region. It has been well recognized that galectin-3 is a microglia-selective cell surface marker [19]. Galectin-3 immunostaining was observed only in the interior side of CA1 and CA2 regions in the hippocampus 60 h after ischemic insult. Galectin-3 was observed in the whole CA1 region adjacent to the CA2 region 66 h after ischemic insult. These results suggest that galectin-3 in the CA2 region is expressed independent of that in the CA1 region.

Galectin-3 is a β-galactosidase-binding protein and growing evidence suggests that galectins may be involved in fine tuning of the inflammatory response...
Time course of representative microphotographs of immunohistochemical staining for galectin-3 in gerbil hippocampal CA2 sector at 0 (sham), 48, 60, and 96 h after ischemic insult. No Galectin-3 expression was detected in CA2 at 0 (sham) and 48 h. Microphotographs in the right column (HPF) show a high-power field of the rectangle on the left (LPF). Ependymal cells lining the lateral ventricles are strongly positive for galectin-3 as an internal positive control. Scale bars in LPF and HPF of sham are 200 and 50 μm, respectively. HPF, high-power field; LPF, low-power field.
Intraischemic hypothermia and preadministration of TPCK and 2DG strongly inhibited the ischemia-induced galectin-3-positive cells in the CA2 region 96 h after ischemic insult. Data are presented as mean±SD. Numbers in parentheses indicate animal numbers in each group. *Statistically different from the ischemic control (P<0.05), determined by ANOVA. ANOVA, analysis of variance; 2DG, 2-deoxy-D-glucose; ischemic cont., ischemic control; sham-op, sham operation; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone.

We observed a transient increase in galectin-3 expression in the CA2 region following transient ischemia, which occurred independent of that in the CA1 region. Following transient ischemia, galectin-3 expression, as well as neuronal cell death, in the CA2 region was significantly inhibited by hypothermia and by apoptosis-inhibiting reagents. Thus, protecting galectin-3 expression in the CA2 region might be correlated with protecting against neuronal death of CA2 pyramidal neurons.

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**Conflicts of interest**

There are no conflicts of interest.

**References**

1. Bird CM, Burgess N. The hippocampus and memory: insights from spatial processing. Nat Rev Neurosci 2008; 9:182–194.
2. Marr D. Simple memory: a theory for archicortex. Philos Trans R Soc Lond B Biol Sci 1971; 262:23–81.
3. Nakazawa K, Quirk MC, Chitwood RA, Watanabe M, Yeckel MF, Sun LD, et al. Requirement for hippocampal CA3 NMDA receptors in associative memory recall. Science 2002; 297:211–218.
4. Wintzer ME, Boehringer R, Polygalov D, McHugh TJ. The hippocampal CA2 ensemble is sensitive to contextual change. J Neurosci 2014; 34:3056–3066.
5. Hitti FL, Siegelbaum SA. The hippocampal CA2 region is essential for social memory. Nature 2014; 508:88–92.
6. Benes FM, Kwok EW, Vincent SL, Todtenkopf MS. A reduction of nonpyramidal cells in sector CA2 of schizophrenics and manic depressives. Biol Psychiatry 1998; 44:85–97.
7. Ito U, Spatz M, Walker JT Jr, Klatzo I. Experimental cerebral ischemia in mongolian gerbils. I. Light microscopic observations. Acta Neuropathol 1975; 32:209–223.
8. Brown AW, Levy DE, Kubik M, Harrow J, Plum F, Brierley JB. Selective chromatolysis of neurons in the gerbil brain: a possible consequence of "epileptic" activity produced by common carotid artery occlusion. Ann Neurol 1979; 5:127–138.
9. Iwai T, Niwa M, Hara A, Mori H, Uematsu T, Sakai N. DNA fragmentation in the CA2 sector of gerbil hippocampus following transient forebrain ischemia. Brain Res 2000; 857:275–278.
10. Sundblad V, Croci DO, Rabinovich GA. Regulated expression of galectin-3 a multifunctional glycan-binding protein in haematopoietic and non-haematopoietic tissues. Histol Histopathol 2011; 26:247–265.
11. Reichert F, Rotshenker S. Galectin-3/MAC-2 in experimental allergic encephalomyelitis. Exp Neurol 1999; 160:508–514.
12. Satoh K, Niwa M, Gods W, Brih NH, Nakashima M, Takamatsu M, Hara A. Galectin-3 expression in delayed neuronal death of hippocampal CA1 following transient forebrain ischemia, and its inhibition by hypothermia. Brain Res 2011; 1382:266–274.
13. Kobayashi K, Niwa M, Hoshi M, Saito K, Hisamatsu K, Katano Y, et al. Early microlesion of viral encephalitis confirmed by galectin-3 expression after a virus inoculation. Neurosci Lett 2015; 592:107–112.
14. Niwa M, Maruyama T, Hisamatsu K, Kobayashi K, Miyazaki T, Hirata A, et al. The role of microglial galectin-3 in central nervous system disease. In: Giffard ER, editor. Microglia: physiology, regulation and health implications. New York, USA: Nova Science Publish; 2015. pp. 205–216.
15. Hara A, Yoshimi N, Mori H, Iwai T, Sakai N, Yamada H, Niwa M. Hypothalamic prevention of nuclear DNA fragmentation in gerbil hippocampus following transient forebrain ischemia. Neurol Res 1995; 17:461–464.
16. Kartsoulis LD, Rudge P, Stevens JM. Bilateral lesions of CA1 and CA2 fields of the hippocampus are sufficient to cause a severe amnesic syndrome in humans. J Neurol Neurosurg Psychiatry 1995; 59:95–98.
Blum S, Moore AN, Adams F, Dash PK. A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *J Neurosci* 1999; 19:3535–3544.

DeVito LM, Konigsberg R, Lykken C, Sauvage M, Young WS, Eichenbaum H. Vasopressin 1b receptor knock-out impairs memory for temporal order. *J Neurosci* 2009; 29:2676–2683.

Lalancette-Hébert M, Gowing G, Simard A, Weng YC, Kriz J. Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. *J Neurosci* 2007; 27:2596–2605.

Rabinovich GA, Toscano MA. Turning ‘sweet’ on immunity: galectin-glycan interactions in immune tolerance and inflammation. *Nat Rev Immunol* 2009; 9:338–352.

Lalancette-Hébert M, Swarup V, Beaulieu JM, Bohacek I, Abdelhamid E, Weng YC, et al. Galectin-3 is required for resident microglia activation and proliferation in response to ischemic injury. *J Neurosci* 2012; 32:10393–10395.

Hara A, Niwa M, Nakashima M, Iwai T, Uematsu T, Yoshimi N, et al. Protective effect of apoptosis-inhibitory agent, N-tosyl-l-phenylalanyl chloromethyl ketone against ischemia-induced hippocampal neuronal damage. *J Cereb Blood Flow Metab* 1998; 18:819–823.

Niwa M, Hara A, Iwai T, Nakashima M, Yoshimi N, Mori H, et al. Prevention of ischemia-induced hippocampal neuronal damage by 2-deoxy-D-glucose in gerbils. *Life Sci* 1999; 64:PL193–PL198.

Yanashita S, Miyamoto O, Janjua NA, Tomizawa K, Matsu H, Nakamura T, et al. Role of the hippocampal CA2 region following postischemic hypothermia in gerbil. *Mol Brain Res* 2003; 111:8–16.

Niwa M, Hara A, Iwai T, Nakashima M, Yano H, Yoshimi N, et al. Relationship between magnitude of hypothermia during ischemia and preventive effect against post-ischemic DNA fragmentation in the gerbil hippocampus. *Brain Res* 1998; 794:338–342.