

## Full-length article

## Cysteinyl leukotriene receptors CysLT<sub>1</sub> and CysLT<sub>2</sub> are upregulated in acute neuronal injury after focal cerebral ischemia in mice<sup>1</sup>

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### Key words

cysteinyl leukotriene receptor; cerebral ischemia; neuronal injury; mRNA expression

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### Abstract

**Aim:** To determine whether cysteinyl leukotriene receptors (CysLT<sub>1</sub> and CysLT<sub>2</sub>) are upregulated in acute neuronal injury after focal cerebral ischemia in mice, and to confirm CysLT<sub>1</sub> receptor localization. **Methods:** After permanent focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO), neurological deficits and neuron loss were determined at various time points within 48 h. The mRNA expressions of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors in the brain were analyzed by RT-PCR. CysLT<sub>1</sub> receptor localization was detected by double immunofluorescence. **Results:** Neurological deficits and neuron loss were found 6, 24 and 48 h after MCAO. The mRNA expressions of both CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors were upregulated in the ischemic hemisphere 1, 24, and 48 h after MCAO with peaks at 24 h. The CysLT<sub>1</sub> receptor was selectively localized in neurons 24 h after MCAO. **Conclusion:** CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors are upregulated in acute neuronal injury after focal cerebral ischemia, and the CysLT<sub>1</sub> receptor is localized in neurons after ischemia.

### Introduction

Cysteinyl leukotrienes (CysLT; including LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>), 5-lipoxygenase (5-LOX) metabolites of arachidonic acid, are the potent inflammatory mediators involved in various central nervous diseases, such as cerebral ischemia<sup>[1–3]</sup>, brain trauma<sup>[4]</sup>, brain tumors<sup>[5]</sup> and epilepsy<sup>[6]</sup>. After cerebral ischemia or ischemia-reperfusion, the production of CysLT in the ischemic brain is increased<sup>[1–3]</sup>. In addition, 5-LOX inhibitors ameliorate cerebral ischemic injuries via reducing the production of CysLT<sup>[7,8]</sup>. Since the actions of CysLT are mediated by activating their receptors (CysLT receptors), it is important to investigate the changes in CysLT receptors after cerebral ischemia.

Two types of CysLT receptors, CysLT<sub>1</sub> and CysLT<sub>2</sub>, have been cloned and identified as G protein-coupled receptors<sup>[9–14]</sup>. The CysLT<sub>1</sub> receptor is highly expressed in the human spleen and peripheral blood leukocytes, and weakly in the brain, lung, colon, heart, pancreas, prostate, skeletal muscle, kidney, liver, placenta and small intestine as determined by Northern blotting<sup>[9,10]</sup>. The mouse CysLT<sub>1</sub> receptor has an expression pattern distinct from that of humans; it is highly expressed in

the lung and skin, and weakly in other tissues including the brain<sup>[15]</sup>. Little is known about the expression and localization of the CysLT<sub>1</sub> receptor in the brain. Recently, we reported that the CysLT<sub>1</sub> receptor was primarily expressed in the microvascular endothelia in the human brain by immunohistochemical analyses, and its expression was increased in the neuron- and glial-appearing cells after traumatic injury<sup>[16]</sup>. Moreover, we have found that the CysLT<sub>1</sub> receptor antagonists (pranlukast and montelukast) protect against cerebral ischemic injury in rats and mice, pharmacologically suggesting that the CysLT<sub>1</sub> receptor may play a role in cerebral ischemia<sup>[17–22]</sup>.

On the other hand, the CysLT<sub>2</sub> receptor is highly expressed in the spleen, heart, adrenal glands and thymus, and weakly in the kidney, brain and peripheral blood leukocytes<sup>[23]</sup>. In the brain, the CysLT<sub>2</sub> receptor expression is found in most regions and in the spinal cord<sup>[12,13]</sup>. The expression patterns of the CysLT<sub>2</sub> receptor are similar in mouse and human tissues. We found that the CysLT<sub>2</sub> receptor was primarily expressed in the smooth muscle cells of cerebral vessels in the human brain by immunohistochemical

analyses, and its expression was also increased in the neuron- and glial-appearing cells after traumatic injury<sup>[24]</sup>. Because no selective antagonist is available for the CysLT<sub>2</sub> receptor, its role in the brain is unknown.

However, no direct evidence shows the role of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors in the brain after cerebral ischemia. To clarify their role in acute ischemic neuronal injury, we investigated the temporal expressions of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor mRNA in the mouse brain from 1 h to 48 h (acute phase) after focal cerebral ischemia. We wanted to show the relationship between the temporal expressions and the development of ischemic neuronal injury. In addition, we observed the localization of the CysLT<sub>1</sub> receptor 24 h after ischemia.

## Materials and methods

**Physiological parameter monitoring** Male ICR (Institute of Cancer Research) mice weighing 25–30 g (Shanghai Experimental Animal Center, China, Shanghai, China; Certificate No. 22-001004) were used. The mice were housed under a controlled temperature, 12 h light/12 h dark cycle and allowed access to food and water *ad libitum*. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

The mice were anaesthetized with chloral hydrate (400 mg/kg, ip). A polyethylene tube was inserted into the right femoral artery for continuous monitoring of blood pressure using a computer-assisted system (PC-Lab, Kelong Inc, Nanjing, China), and for measuring arterial blood  $P_{aO_2}$ ,  $P_{aCO_2}$  and pH (Blood Gas Analyzer ABL330, Leidu Inc, Copenhagen, Kobenhavn, Denmark). Blood glucose was monitored by a one touch basic blood glucose monitoring system (Lifescan Inc, New Brunswick, New Jersey, USA). Rectal (core) temperature was measured and maintained at  $37.0 \pm 0.5$  °C with a heating pad and a heating lamp during the surgery.

**Permanent focal cerebral ischemia** After anesthesia, permanent focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) as described previously<sup>[17]</sup>. Briefly, a 6–0 nylon monofilament suture, blunted at the tip and coated with 1% poly-*L*-lysine, was inserted into the right internal carotid artery, and advanced approximately 10 mm distal to the carotid bifurcation to occlude the origin of the middle cerebral artery. In the sham-operated mice, the same procedure was done, except for the insertion of the intraluminal filament. After surgery, the mice were kept for about 2 h in a warm box heated by lamps to maintain the body temperature.

**Behavioral assessments** Neurological deficit scores were

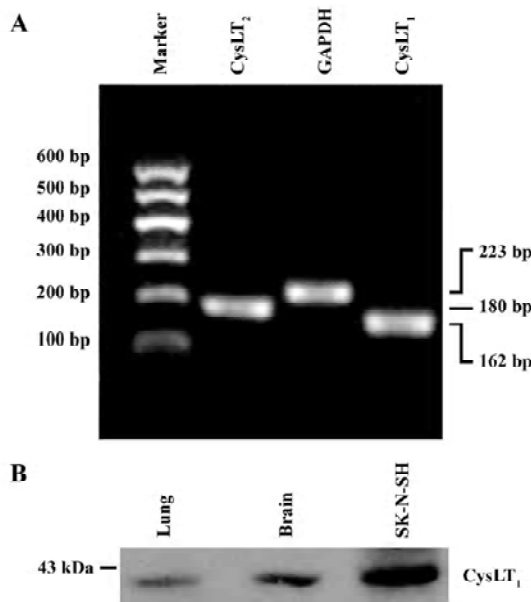
evaluated 6, 24 and 48 h after MCAO<sup>[25]</sup>: 0, no deficit; 1, flexion of contralateral forelimb upon lifting of the whole animal by the tail; 2, circling to the contralateral side; 3, falling to contralateral side; and 4, no spontaneous motor activity. An inclined board test was performed to assess balance and coordination<sup>[20]</sup> based on modification of a reported method<sup>[26]</sup>. The mice were placed on a board (50×30 cm); once they were stable, the board was inclined from horizontal to vertical. The degree at which the animal fell from the board (holding angle) was recorded. The test was repeated 3 times and the average degree was used. Behavioral assessments were not observed 1 and 3 h after ischemia because the mice did not recover completely from the anesthesia.

**Histopathology analyses** The brains were removed at 1, 3, 6, 24, and 48 h after MCAO, fixed in 4% paraformaldehyde overnight, and cut by cryostat into serial 8 μm-thick coronal sections, then stained with 1% toluidine blue. The apparently surviving neuron-appearing cells in the temporoparietal cortex III, IV layers, the hippocampal CA1 region and the striatum (1.8–2.0 mm caudal from the bregma) were counted using the ImageTool 2.0 analysis program (University of Texas Health Science Center in San Antonio, San Antonio, Texas, USA).

**RT-PCR** The ischemic and the contralateral non-ischemic hemispheres were dissected on ice 1, 3, 6, 24 and 48 h after MCAO, and stored at -80 °C for RNA extraction. Total RNA was extracted using Trizol reagents (Invitrogen, Carlsbad, California, USA) according to the manufacture's protocol. For cDNA synthesis, aliquots of total RNA (1 μg) were mixed with 1 mmol/L dNTP, 0.2 μg random primer, 20 U RNase, 200 U M-MuLV reverse transcriptase in 20 μL of the reverse reaction buffer. The mixture was incubated at 42 °C for 60 min, and then 70 °C for 10 min to inactivate the reverse transcriptase. As a negative control, the reaction was performed at the absence of reverse transcriptase.

PCR was performed in a 40 μL reaction mixture containing PCR buffer, 4 μL cDNA, 200 μmol/L dNTP, 1.5 mmol/L MgCl<sub>2</sub>, 10 pmol of each primer and 1 U of Taq DNA polymerase (Takara, Kyoto, Japan). PCR reactions were performed as follows: 94 °C for 2 min followed by 35 cycles of 93 °C for 10 s, 66 °C for 30 s and 72 °C for 30 s, and finally extended at 72 °C for 10 min. The primer sequences for mouse CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors<sup>[13]</sup> were synthesized by Sangon Biotech Co (Shanghai, China) as follows: mCysLT<sub>1</sub>(+): 5'-CAA CGA ACT ATC CAC CTT CAC C-3', mCysLT<sub>1</sub>(-): 5'-AGC CTT CTC CTA AAG TTT CCA C-3'; mCysLT<sub>2</sub>(+): 5'-GTC CAC GTG CTG CTC ATA GG-3', mCysLT<sub>2</sub>(-): 5'-ATT GGC TGC AGC CAT GGT C-3'. GAPDH was used as the

control; its primer sequences were GAPDH (+): 5'-GTC GGT GTGAACGGATTTGG-3' and GAPDH (-): 5'-GCTCCTGGAAGA TGG TGA TGG-3'. PCR products in 10  $\mu$ L were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and photographed. The optical density of the bands was determined by an image analysis system (Bio-Rad, Hercules, California, USA). The PCR product sizes of CysLT<sub>1</sub>, CysLT<sub>2</sub> receptors and GAPDH were 162 bp, 180 bp and 223 bp, respectively (Figure 1A). CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor mRNA levels were normalized to the level of GAPDH mRNA.



**Figure 1.** RT-PCR products (A) and Western blotting results using the antibody against CysLT<sub>1</sub> receptor protein (B). The sizes of RT-PCR products of GAPDH, CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor mRNA from the mouse brain are 223 bp, 162 bp and 180 bp, respectively (A). Western blotting analysis using a polyclonal rabbit anti-human CysLT<sub>1</sub> antibody reveals a band that is closed to 43 kDa in the samples from the mouse lung, mouse brain and human neuroblastoma cell line SK-N-SH cells (B).

**Double immunofluorescence** To visualize the localization of the CysLT<sub>1</sub> receptor, double immunofluorescence was employed. Briefly, after blocking non-specific binding of IgG with 5% normal goat serum for 2 h at room temperature, the brain sections were incubated overnight at 4 °C with a mixture of rabbit polyclonal anti-human CysLT<sub>1</sub> receptor antibody (1:150, Cayman Chemicals, Ann Arbor, MI, USA) and mouse monoclonal antibody against neuronal nuclei (NeuN; 1:200; Chemicon, Temecula, California, USA), a specific marker of neurons, or against a glial fibrillary acidic protein (GFAP; 1:800; Chemicon, USA), a specific marker of

astrocytes. Then, the sections were incubated with the mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG (1:100; Chemicon, USA).

To confirm the specificity of the polyclonal rabbit anti-human CysLT<sub>1</sub> receptor antibody used in the double immunofluorescence of the mouse brain tissue, we collected protein samples of cultured human neuroblastoma cell line SK-N-SH cells (purchased from the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) as well as a normal mouse lung and cerebral cortex. Protein samples (50  $\mu$ g) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes then reacted with the rabbit polyclonal anti-human CysLT<sub>1</sub> receptor antibody (1:2000), and with the peroxidase-conjugated goat anti-rabbit IgG (1:2000) after repeated washing. Finally the protein bands were visualized by enhanced chemiluminescence. The result showed that a band was close to 43 kDa in the mouse cerebral cortex, the same as that in the SK-N-SH cells (Figure 1B). This was consistent with previously published results using the same polyclonal antibody<sup>[27,28]</sup>. Thus, the antibody was usable for immunohistochemistry of mouse brain tissue.

**Statistical analysis** Data are presented as mean  $\pm$  SD. Differences between groups were analyzed by one-way ANOVA (Student-Newman-Keuls) using the SPSS software package (Version 10.0 for Windows; SPSS, Chicago, Illinois, USA). *P* < 0.05 was considered statistically significant.

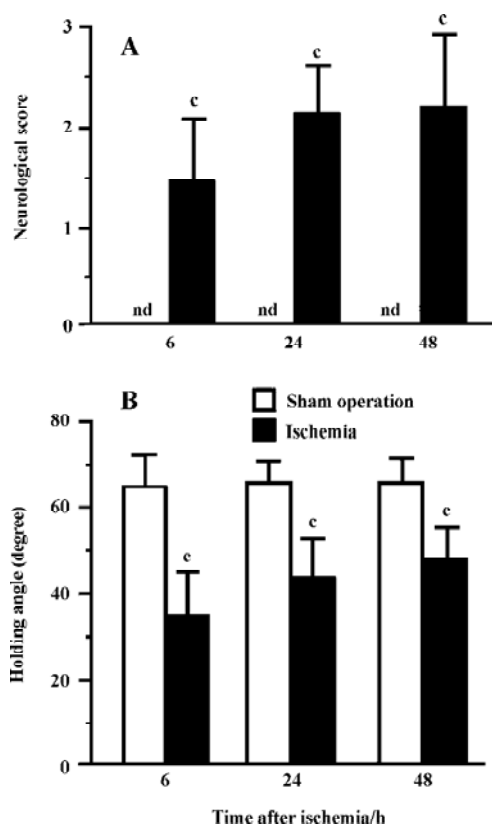
**Results**

**Physiological variables** There was no significant difference in mean arterial blood pressure, *P*<sub>aO<sub>2</sub></sub>, *P*<sub>aCO<sub>2</sub></sub>, arterial blood pH, and blood glucose between 30 min before and 30 min after ischemia (Table 1).

**Ischemic injury** The neurological deficit score was increased (Figure 2A) and the holding angle in the inclined board test was reduced (Figure 2B) 6, 24, and 48 h after MCAO. In the cortex, the hippocampal CA1 region and the striatum of the ischemic hemisphere, no apparent change was observed, and the number of neuron-appearing cells was unchanged at 1 and 3 h after MCAO. The Nissl bodies in the neurons disappeared and the neurons shrunk at 6 h after MCAO; most of neurons were destroyed and left vacuoles at 24 and 48 h after MCAO (Figure 3A). The number of neuron-appearing cells in these regions was significantly reduced at 6, 24, and 48 h after MCAO (Figure 3B). As the control, the cell number in the contralateral non-ischemic hemispheres had no significant change within 48 h after MCAO.

**Table 1.** Physiological variables before and after cerebral ischemia in mice. Data are mean±SD. *n*=7 mice in each group. MAP: mean arterial pressure.

Variable		Sham operation	Ischemia
MAP (mmHg)	before ischemia	86.7±6.8	87.4±5.7
	30 min after ischemia	83.8±6.3	90.0±6.2
<i>P</i> <sub>aO<sub>2</sub></sub> (mmHg)	before ischemia	122±6	123±4
	30 min after ischemia	120±4	121±3
<i>P</i> <sub>aCO<sub>2</sub></sub> (mmHg)	before ischemia	36.1±1.1	35.7±1.3
	30 min after ischemia	37.3±2.1	36.1±1.6
Glucose (mmol/L)	before ischemia	6.27±1.40	5.59±0.69
	30 min after ischemia	5.89±0.88	5.93±0.83
pH	before ischemia	7.37±0.01	7.36±0.04
	30 min after ischemia	7.35±0.02	7.35±0.04



**Figure 2.** Neurological deficits after focal cerebral ischemia in mice. Permanent focal cerebral ischemia was induced by MCAO. Neurological score (A) and holding angle in an inclined board test (B) were determined starting 6 h after MCAO when the mice recovered from anesthesia. Data are reported as mean±SD. *n*=12 mice in each group.  $^{\circ}P<0.01$  vs the sham-operated group, one-way ANOVA. nd, not detectable.

### Temporal expressions of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor mRNA

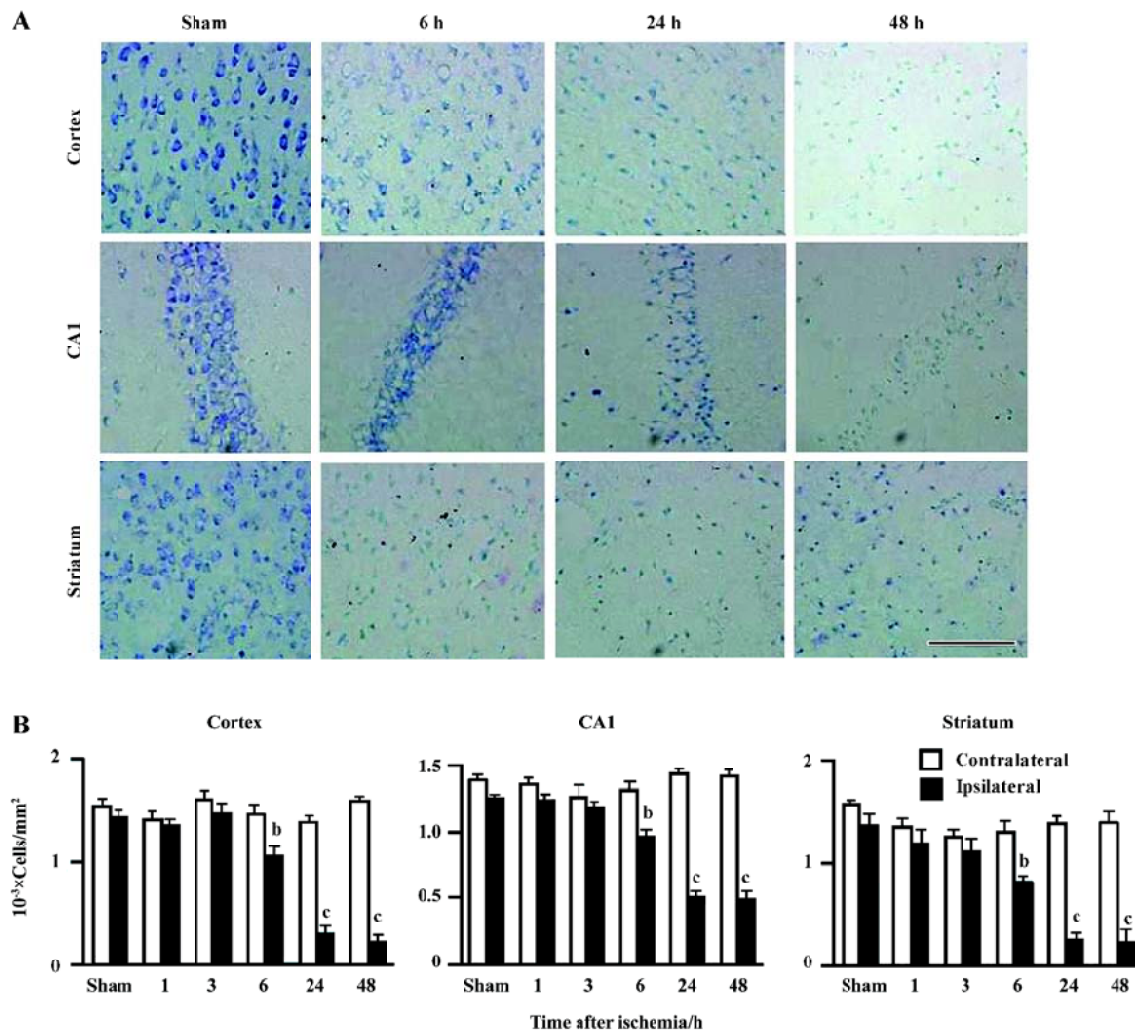
The expression of CysLT<sub>1</sub> receptor mRNA in the ischemic hemisphere initially increased at 1 (1.9-fold) and 3 h (1.6-fold), partially recovered at 6 h, and largely increased again 24 (4.5-fold) and 48 h (3.8-fold) after MCAO (Figure 4A,C). Similarly, the expression of CysLT<sub>2</sub> receptor mRNA in the ischemic hemisphere significantly increased at 1 h (2.2-fold), partially recovered at 3–6 h, then largely increased again 24 (5.3-fold) and 48 h (3-fold) after MCAO (Figure 4B, 4D).

**CysLT<sub>1</sub> receptor localization** Double immunofluorescence staining showed that the CysLT<sub>1</sub> receptor was localized in the NeuN-positive neurons in the cortex, the hippocampal CA1 region and the striatum of the ischemic hemispheres at 24 h after MCAO (right panels in Figure 5A); almost none were detected in the hemisphere from the sham-operated mice (left panels in Figure 5A). On the other hand, the CysLT<sub>1</sub> receptor was not localized in GFAP-positive astrocytes in these regions in both the sham-operated and ischemic hemispheres (Figure 5B).

### Discussion

In the present study, the most important finding is that both the expressions of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor mRNA in the ischemic brain are upregulated in the acute phase of focal cerebral ischemia. The increased expressions are temporally related to neuronal injury, including neurological deficits and regional neuron loss. These results suggest that both CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors may be involved in acute ischemic neuronal injury.

Because neuronal injury occurs initially in the acute phase after cerebral ischemia, in this study, we focus on the temporal changes in CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor mRNA as well as the neuronal injury within 48 h after focal cerebral ischemia. Neurological deficits and neuron loss appeared from 6 to 48 h (Figures 2, 3). Both CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor mRNA were initially increased at 1 h, and largely increased at 24 and 48 h after ischemia with peaks at 24 h (Figure 4). We can not explain why both the receptor mRNA expressions temporally increased, however, the initial increase might be induced by excitotoxicity that occurs at minute to hour after cerebral ischemia, and might mediate the initiation of delayed responses (hour to day), such as neuronal injury and inflammation<sup>[29]</sup>. As supporting evidence, we found that microinjection of *N*-methyl-*D*-aspartate (an exogenous excitatory acid) into the mouse cortex induced CysLT<sub>1</sub> receptor expression (unpublished observations). On the other hand, the delayed increase of the 2 receptor mRNA expressions (24 and 48 h after MCAO) might be induced in response to sec-

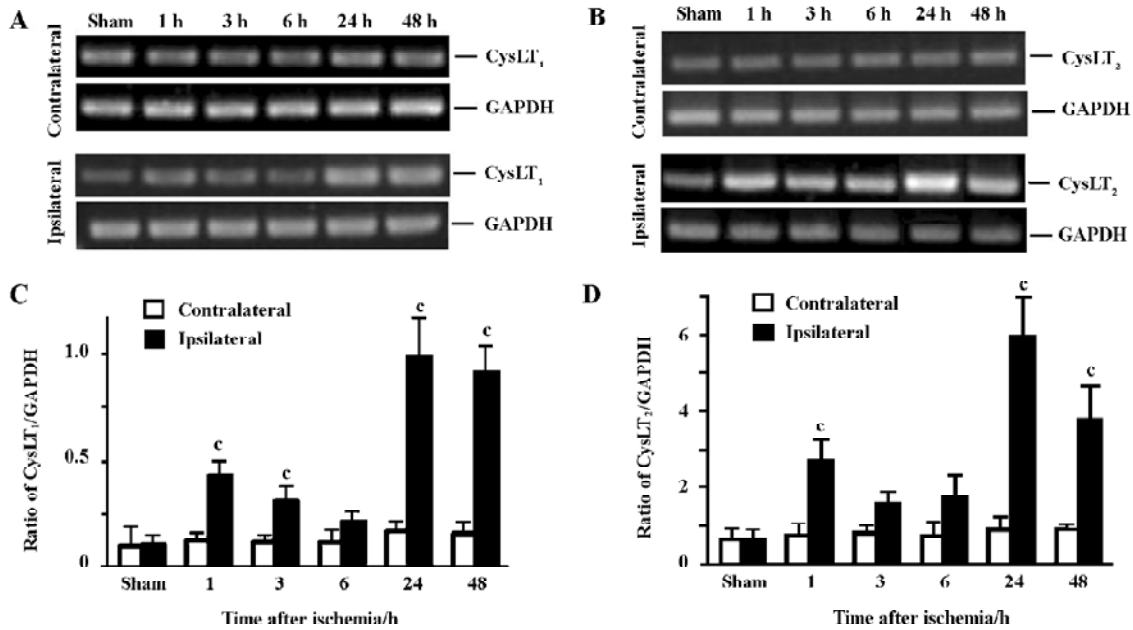


**Figure 3.** Time-course of histopathological changes after focal cerebral ischemia in mice. Microphotographs showing histopathological changes in the cortex, the hippocampal CA1 region and the striatum 6, 24, and 48 h after MCAO (A). The summarized data of the number of the apparently surviving neuron-appearing cells (B). Mean±SD. *n*=8 mice in each group. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs the sham-operated group, one-way ANOVA. Scale bars=50 μm.

ondary inflammation, and might mediate late responses like neuronal injury. The possible mediation was suggested by a close relationship between the increased expressions and the neuronal injury 24 and 48 h after MCAO in the present study. Of course, ischemic neuronal injury is caused by more complex systems, not by the CysLT<sub>1</sub> or CysLT<sub>2</sub> receptor alone.

The novel finding is that the expression of CysLT<sub>2</sub> receptor mRNA is upregulated after focal cerebral ischemia. However, because neither selective antagonists nor specific antibodies (for rats and mice) are available for studying the CysLT<sub>2</sub> receptor, we can not explore more properties of this receptor in mice or rats with cerebral ischemia. In peripheral

tissues, the CysLT<sub>2</sub> receptor mediates endothelial and macrophage activation, mast cell interleukin-8 secretion, and fibrosis<sup>[30]</sup>, but it is not known how the CysLT<sub>2</sub> receptor regulates the central nervous function. Recently, we showed that the CysLT<sub>2</sub> receptor expression increased in neuron-appearing cells as detected by immunohistochemical staining in human brain specimens from patients with traumatic brain injury<sup>[24]</sup>, but the implication is still unclear. We also found that PC12 cells transfected with the CysLT<sub>2</sub> receptor were more sensitive than those transfected with the CysLT<sub>1</sub> receptor to the *in vitro* ischemic-like injury induced by oxygen-glucose deprivation<sup>[31]</sup>, suggesting that the CysLT<sub>2</sub> receptor may play a more important modulating role in ischemic



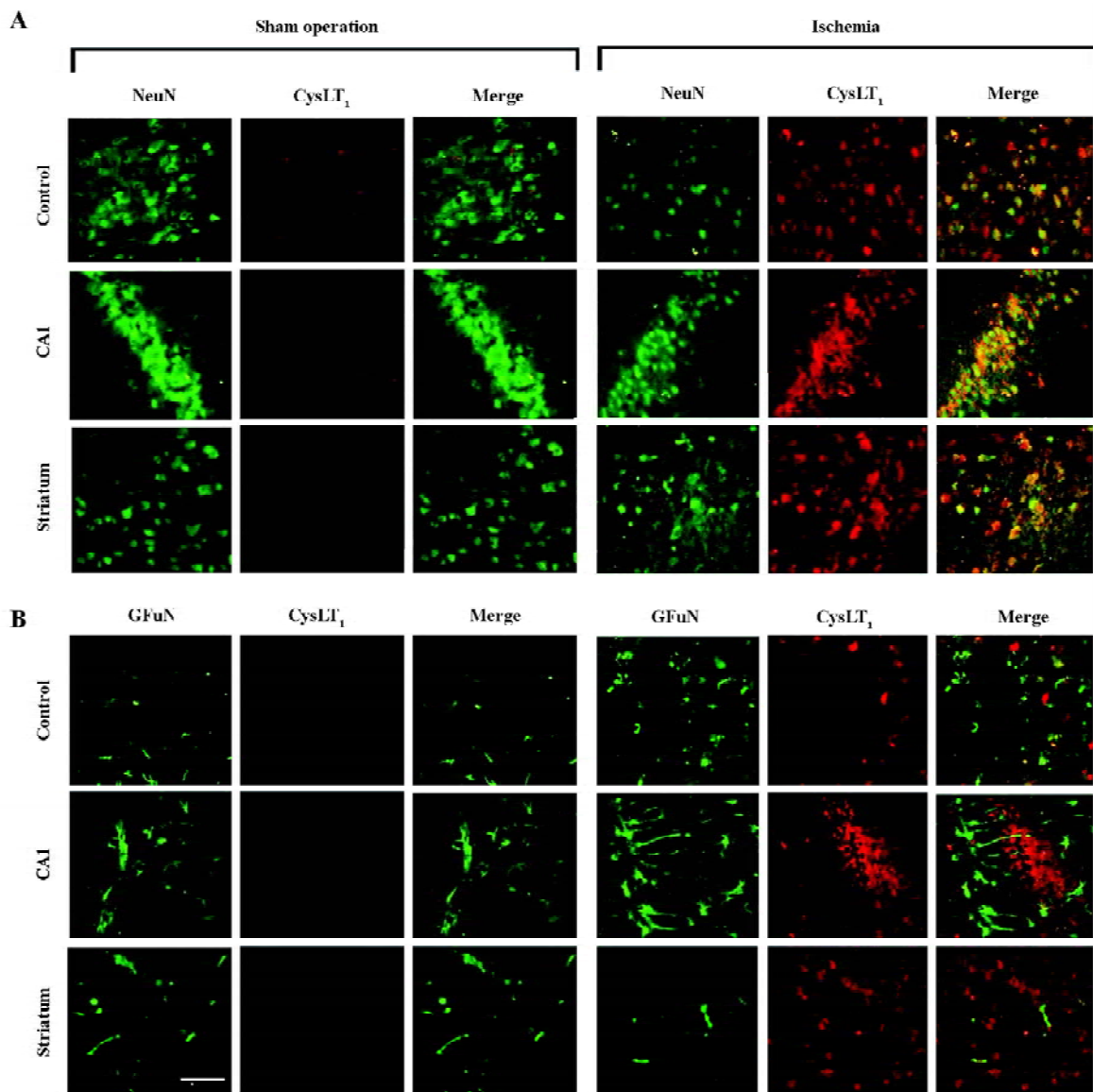
**Figure 4.** Time course of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor mRNA expressions in the ischemic and the contralateral non-ischemic hemispheres of mice. CysLT<sub>1</sub> (A) and CysLT<sub>2</sub> receptor (B) mRNA were detected by RT-PCR. The summarized data for CysLT<sub>1</sub> (C) and CysLT<sub>2</sub> receptor (D) mRNA expressions. Mean±SD. *n*=8 mice in each group. <sup>c</sup>*P*<0.01 vs the sham-operated group, one-way ANOVA.

neuronal injury.

Another important finding is that the CysLT<sub>1</sub> receptor is localized in the neurons in the ischemic brain and may mediate neuronal injury as confirmed by CysLT<sub>1</sub> receptor antagonism. For detecting CysLT<sub>1</sub> receptor localization, we performed double immunofluorescence using a polyclonal rabbit anti-human CysLT<sub>1</sub> receptor antibody, which was also specific to mouse brain tissue (Figure 1B). Much less CysLT<sub>1</sub> receptor is expressed in the normal brain from the sham-operated mice, but it was highly expressed in the neurons in the ischemic cortex, the hippocampal CA1 region and the striatum 24 h after focal cerebral ischemia. Furthermore, the CysLT<sub>1</sub> receptor was less expressed in astrocytes. A similar pattern of CysLT<sub>1</sub> receptor localization was also found in the rat brain after transient focal cerebral ischemia in our recent study (unpublished observations). In that study, we found that the CysLT<sub>1</sub> receptor was primarily expressed in neurons, not in astrocytes, in the cortex 24 h after transient focal cerebral ischemia, while it was expressed in the astrocytes, not in neurons, in the boundary zone adjacent to the ischemic core 14 d after ischemia. The present finding further indicated the localization of the CysLT<sub>1</sub> receptor in the striatum and the hippocampal CA1 region in addition to the cortex in a mouse model of permanent focal cerebral ischemia without reperfusion. Both the results from the focal ischemic mice

and rats indicate that the CysLT<sub>1</sub> receptor may mediate neuron injury in the acute phase of ischemia. This mediation has been suggested by the neuroprotective effect of selective CysLT<sub>1</sub> receptor antagonists as we previously reported<sup>[17–20]</sup>, although the antagonists may have other non-specific effects attenuating ischemic injury. The CysLT<sub>1</sub> receptor regulates vascular leak, bronchoconstriction, dendritic cell maturation and migration, macrophage activation, hematopoietic progenitor cell recruitment, eosinophil and mast cell cytokine secretion, and smooth muscle proliferation in peripheral tissues<sup>[30]</sup>. Our finding indicates a role of the CysLT<sub>1</sub> receptor in ischemic neuronal injury.

In summary, the present study shows that CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors are upregulated in acute neuronal injury after focal cerebral ischemia; The CysLT<sub>1</sub> receptor is localized in neurons after ischemia. Since inflammation is one of the post-ischemic events in the brain<sup>[29]</sup> and pro-inflammatory CysLT are produced in the brain after cerebral ischemia<sup>[1–3]</sup>, it is necessary to clarify the modulating roles of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors in cerebral ischemia. Our findings of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor expression will contribute to understanding the mechanisms of cerebral ischemic injuries, including neuronal injury, and to developing new therapeutics for the treatment of ischemic stroke.



**Figure 5.** Localization of the CysLT<sub>1</sub> receptor in the neurons (A) and astrocytes (B) in the brain 24 h after ischemia in mice. The CysLT<sub>1</sub> receptor is weakly expressed in NeuN-positive neurons (A) and GFAP-positive astrocytes (B) in the cortex, the hippocampus CA1 region and the striatum from the sham-operated mice (left panels). It is strongly expressed in the neurons (right panels in A) but less in astrocytes (right panels in B) in these regions of the brain in the ischemic mice. The same experiments were repeated at least 3 times with similar results. Scale bars=50 μm.

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