

Altered Nurr1 protein expression in the hippocampal CA1 region following transient global cerebral ischemia

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Abstract. Nuclear receptor related 1 protein (Nurr1), a member of the nuclear receptor 4 family of orphan nuclear receptors, has been reported to display anti-inflammatory properties. The present study investigated the alteration of Nurr1 immunoreactivity in the gerbil hippocampus proper following 5 min of transient global cerebral ischemia. In sham operated gerbils, Nurr1 immunoreactivity was observed in pyramidal neurons in all cornu ammonis 1-3 (CA1-3) subfields of the hippocampus proper. In ischemia-operated gerbils, Nurr1 immunoreactivity was altered in the CA1 subfield. Nurr1 immunoreactivity in CA1 pyramidal neurons gradually decreased until 2 days post-ischemia, and, at 4 days post-ischemia, Nurr1 immunoreactivity was concentrated in CA1 pyramidal neurons. Additionally, Nurr1 immunoreactivity was newly expressed in microglia in the CA1 subfield at 4 days post-ischemia. Conversely, in the CA2/3 subfield, time-dependent alteration of Nurr1 immunoreactivity was not identified at any time following ischemia. These results indicated that the alteration

of Nurr1 expression in the CA1 subfield in the hippocampus may be associated with the death of CA1 pyramidal neurons.

Introduction

The hippocampus, a part of the limbic system, has been known to be one of the most vulnerable regions in the brain following transient global cerebral ischemia (tgCI) (1). Especially, tgCI leads to selective neuronal death at a few days, which is called 'delayed neuronal death, after a brief tgCI in the CA1 subfield among the four subfields (CA1-CA4) of the hippocampus proper, while the CA3 subfield is known as the most tolerant hippocampal subregion against tgCI (2,3). Until now, many studies have made an effort to explain the pathophysiology of tgCI-induced neuronal death/damage in the CA1 subfield (4-6); however, the precise underlying mechanisms of tgCI-induced selective and delayed neuronal death in the CA1 subfield have not been fully understood.

Nuclear receptor related 1 protein (Nurr1), also known as NR4A2, is a member of nuclear receptor 4 family of orphan nuclear receptors, and Nurr1 has been studied to be expressed in various types of neurons and glia in the central nervous system (7-11). The Nurr1 function has been known as a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells and participates in the development and maintenance of dopaminergic neurons in the mesencephalon (12-15). In the hippocampus, Nurr1 is expressed in pyramidal neurons in the stratum pyramidale of the hippocampus proper (CA1-CA3 subfields) (16).

Recent studies have suggested that Nurr1 in the hippocampus proper plays a major role in cognitive function and stress (10,17,18). Additionally, it has been demonstrated that Nurr1 can display neuroprotective and anti-inflammatory properties (9,19-22). Also, some studies have reported that Nurr1 expression is altered in rodent brains following cerebral ischemia (23-27). However, the expressional changes of

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Nurr1 in the hippocampus proper after tgCI have not been fully elucidated. In the present study, thus, we investigated the time-dependent changes of Nurr1 expression in the hippocampus proper after 5 min of tgCI in gerbils, which are an excellent animal model of tgCI (5,28).

Materials and methods

Experimental animals. Six-month old male Mongolian gerbils (body weight, 70–80 g) were obtained from the Experimental Animal Center, Kangwon National University, Chuncheon, Republic of Korea. Animal care and all experimental procedures were approved by Institutional Animal Care and Use Committee at Kangwon National University (approval no. KW-180124-1). We minimized numbers of the gerbils used in this study and the suffering caused by the procedures used in this experiment.

Induction of tgCI. As described in our published papers (29–32), the surgical procedure for tgCI was as follows. Briefly, gerbils (total n=103; 63 gerbils for histological analysis and 40 gerbils for western blot analysis) were anesthetized with a mixture of 2.5% isoflurane in 34% oxygen (O₂) and 66% nitrous oxide (N₂O). Blood flow to the brain was completely interrupted by bilateral common carotid artery occlusion (BCCAO) for 5 min by using aneurysm clips in order to confirm stop of blood flow in the central artery in retinae under an ophthalmoscope. Body temperature was controlled under normothermic (37±0.5°C) condition with a rectal temperature probe: Normothermia was controlled with a thermometric blanket before and during the surgery. Sham operated gerbils were subjected to the same procedure without BCCAO.

Preparation of histological sections. Brain sections containing the hippocampus were prepared from sham and ischemia operated gerbils (n=7 at each point in time) at designated times (6, 12 h, 1, 2, 3, 4, 7 and 10 days after tgCI). Brain sections from the sham operated gerbils (n=7) were prepared at 4 days after tgCI. As described in our published papers (29–32), the gerbils were intraperitoneally anaesthetized with sodium pentobarbital (60 mg/kg) (JW Pharm. Co., Ltd.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The brains were removed, postfixed with the same fixative for 8 h and cryoprotected by soaking in a 30% sucrose solution for 12 h. The brain tissues were serially sectioned into 30- μ m thickness of coronal sections in a cryostat (Leica).

Examination of delayed neuronal death. To examine tgCI-induced delayed neuronal death in the hippocampus proper, CV staining and Fluoro-Jade B (FJB) histofluorescence staining were performed by methods as we described previously (29,30). In brief, the sections were stained with solution of CV acetate (Sigma), which was dissolved at 1.0% (w/v) and contained 0.28% glacial acetic acid, for 2 min. And the stained sections were washed and dehydrated in ethanol series. For FJB histofluorescence, the sections were serially stained with a 1% sodium hydroxide solution, a 0.06% potassium permanganate solution and a 0.0004% FJB (Histochem) solution. The reacted sections were examined using an epifluorescent

microscope (Carl Zeiss) equipped with a blue excitation light (450–490 nm) and a barrier filter.

Immunohistochemistry. As we described in our published papers (29–32), immunohistochemical staining for Nurr1 was performed as follows. In brief, the brain sections were incubated with mouse anti-Nurr1 (1:100, Thermo Fisher Scientific, Inc.) as primary antibody and exposed to biotinylated goat anti-mouse IgG and streptavidin peroxidase complex (Vector) as secondary antibody. Finally, the reacted sections were visualized with 3,3'-diaminobenzidine (in 0.1 M Tris-HCl buffer, pH 7.4).

In order to establish the specificity of Nurr1 immunoreaction, negative control test was done by using pre-immune serum instead of the primary antibody. The negative control showed no immunoreactivity in the immunostained tissues.

For the quantitative analysis of Nurr1 immunoreactivity, 6 sections were selected with 120- μ m interval per animal, and digital images of Nurr1 immunoreactivity were captured with Axio Imager 2 microscope (Carl Zeiss) equipped with a digital camera (Axiocam; Carl Zeiss). According to our published method (31), the images were calibrated into an array of 512x512 pixels corresponding to a tissue area of 140x140 μ m including the stratum pyramidale of the sham and ischemia operated gerbils. The density of all Nurr1-immunoreactive structures was evaluated as relative optical density (ROD) as follows. Optical density (RO) of all the Nurr1-immunoreactive structures were obtained after the transformation of the mean gray level using the formula: OD=log (256/mean gray level), and the OD of background was taken from the areas adjacent to the measured area. Finally, a ratio of the OD was calibrated as % (ROD) by using NIH Image 1.59 software.

Double immunofluorescence staining. To examine the cell type containing Nurr1 immunoreactivity, double immunofluorescence staining was performed after tgCI according to our published protocol (30,31). In brief, we used mouse anti-Nurr1 (1:40, Thermo Fisher Scientific, Inc.)/rabbit anti-NeuN (a marker for neurons; 1:800, Millipore), rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1, a marker for microglia; 1:100, Wako) or rabbit anti-glial fibrillary acidic protein (GFAP, a marker for astrocytes; 1:200, Millipore). The sections were incubated in the mixture of antisera and reacted in a mixture of Cy3-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA, USA) and FITC-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch). The immunoreactions were observed under a confocal MS (LSM510 META NLO, Carl Zeiss).

Western blot analysis. Nurr1 protein level in the CA1 subfield were analyzed at designated times (6, 12 h, 1, 2, 4, 7 and 10 days after tgCI) using western blot method according to our published procedure (29,30). In brief, 5 animals at each point in time were intraperitoneally anaesthetized with sodium pentobarbital (60 mg/kg), and their brains were removed. The brains were serially and transversely cut into 400- μ m thickness using a vibratome (Leica). Hippocampal CA1 fields were cut by using a surgical blade and homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl

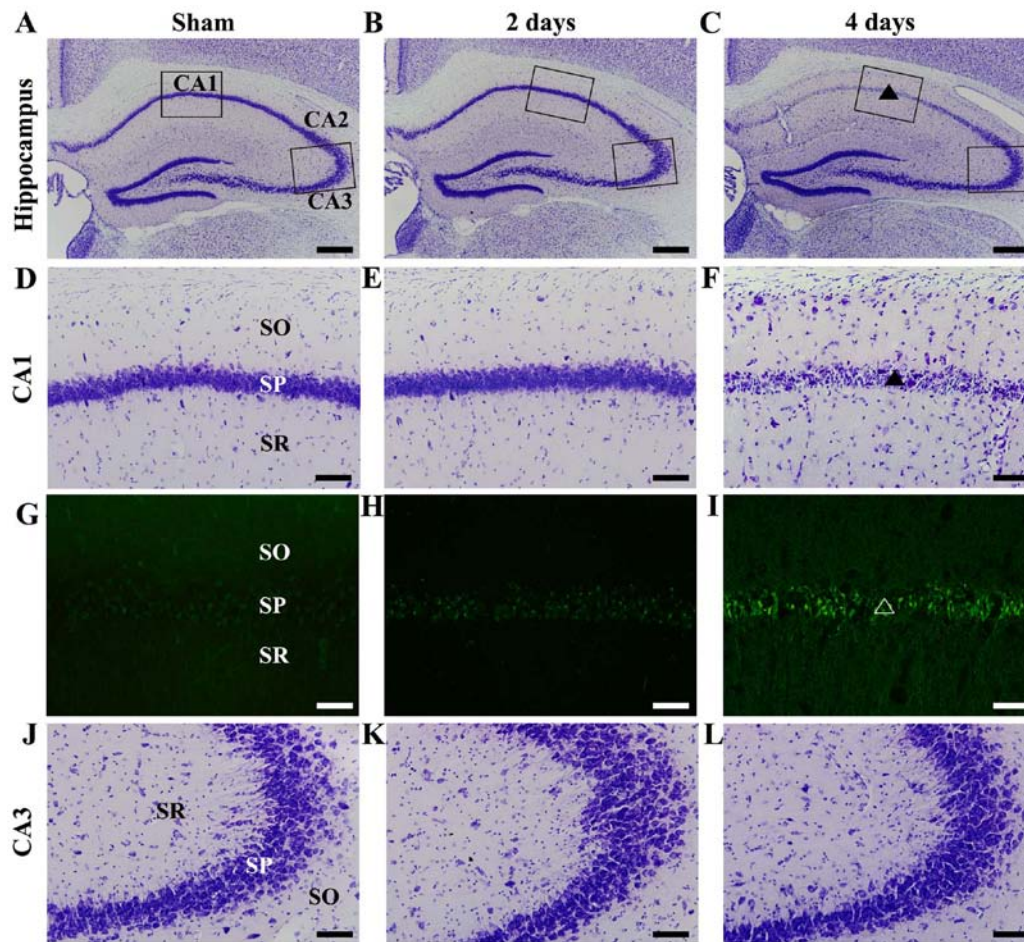


Figure 1. CV and FJB histofluorescence staining in the gerbil hippocampus. (A-C) Low magnification image of CV staining in the gerbil hippocampus of the (A) sham and (B and C) ischemia groups. The squares in the CA1 and CA3 areas indicate the positions of the high magnification images in (D-F and J-L). (D-F) High magnification images of CV staining in the CA1 subfield of the (D) sham and (E and F) ischemia groups. CV-positive cells disappear (triangle) in the SP of the CA1 subfield at 4 days after tgCI. (G-I) High magnification images of FJB histofluorescence staining in the CA1 subfield of the (G) sham and (H and I) ischemia groups. Numerous FJB-positive cells were detected (triangle) in the SP of the CA1 subfield at 4 days after tgCI. (J-L) High magnification images of CV staining in the CA3 subfield of the (J) sham and (K and L) ischemia groups. CV-positive cells were intact in the CA3 subfield until 4 days after tgCI. SO, stratum oriens; SR, stratum radiatum. Scale bar, 400 μ m (A-C) or 50 μ m (D-L). CV, Cresyle violet; FJB, Fluoro-Jade B; tgCI, transient global cerebral ischemia; SO, stratum oriens; SR, stratum radiatum; SP, stratum pyramidale; CA, cornu ammonis.

ether)-N,N,N0,N0 tetraacetic acid (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylenediamine tetraacetic acid (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol (DTT). The homogenized tissues were centrifugated at 16,000 x g for 25 min, and protein levels were determined using a Micro BCA protein assay kit (Pierce Chemical). Aliquot containing total protein (20 mg) was boiled in loading buffer containing 150 mM Tris-HCl (pH 6.8), 6% SDS, 3 mM DTT, 0.3% bromophenol blue and 30% glycerol and loaded onto 10% polyacrylamide gel. The gel was transferred to nitrocellulose transfer membranes (Pall Crop.) after electrophoresis. The background of the membrane was reduced with 5% nonfat dry milk in PBS containing 0.1% Tween 20 for 40 min, and the membrane was reacted with mouse anti-Nurr1 (1:200; Thermo Fisher Scientific, Inc.), peroxidase-conjugated goat anti-mouse IgG and ECL kit. The loading control was carried out using mouse anti- β -actin (1:5,000) antibody. Western blot analysis using homogenates at all experimental time-points was performed simultaneously. Results of the western blotting

were scanned, and the quantification of the bands was densitometrically analyzed using NIH Image 1.59 software. The quantification was represented by relative optical density (ROD). A ratio of the ROD was calibrated as %: The sham operated group was designated as 100%.

Statistical analysis. The data shown here represent the means \pm SEM. Differences of the means among the groups were statistically analyzed by analysis of variance (ANOVA) with Duncan's post hoc test in order to elucidate ischemia-related differences among experimental groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

tgCI-induced delayed neuronal death. tgCI-induced delayed neuronal death in the hippocampus proper (CA1-3 subfields) was examined at 2 days and 4 days after tgCI using CV staining and FJB histofluorescence staining. In the sham group, cells in the CA1-3 subfields were easily stained with CV. In particular, pyramidal neurons of the stratum pyramidale of the CA1-3

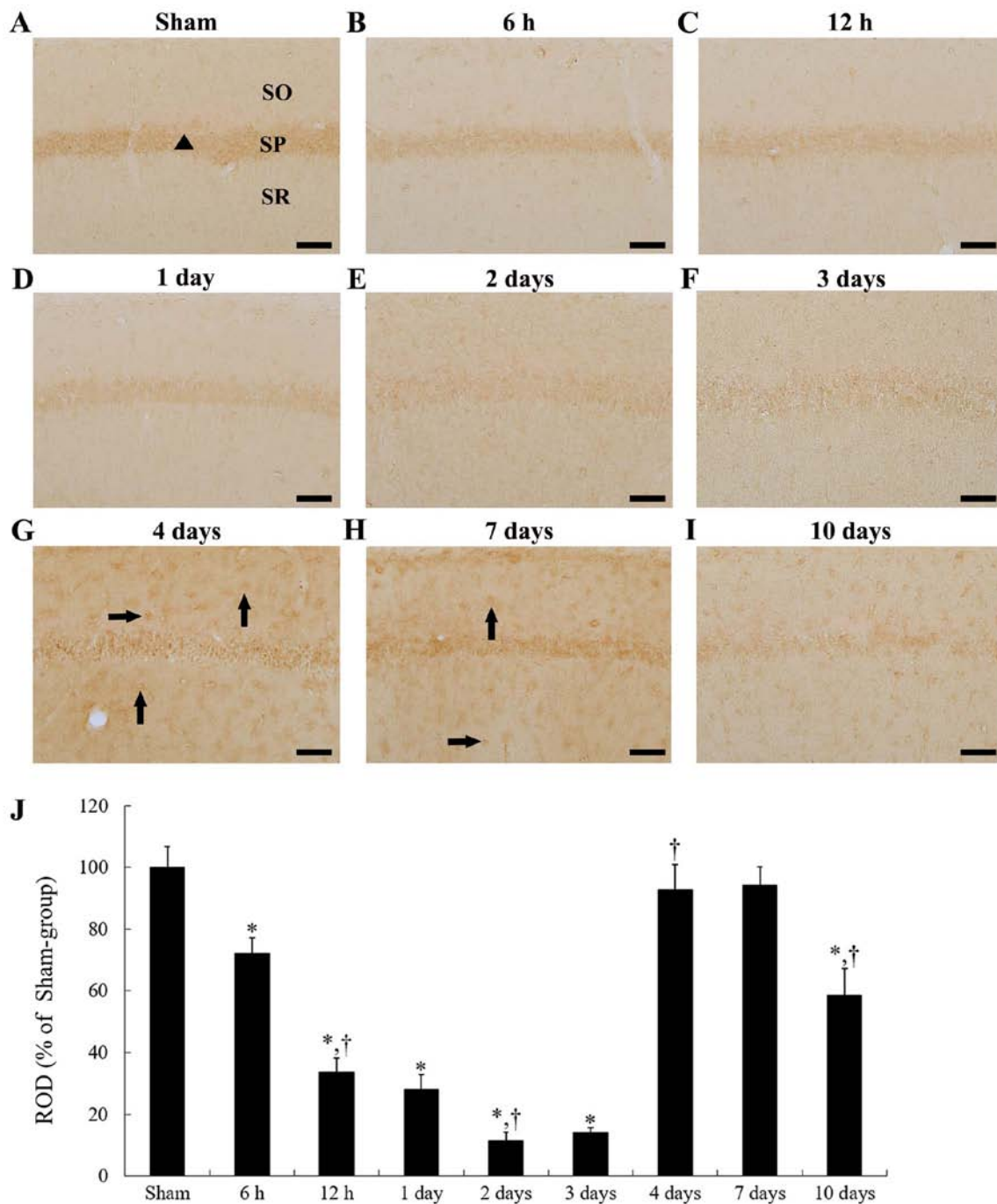


Figure 2. Immunoreactivity of Nurr1 in the CA1 subfield of the (A) sham and (B-I) ischemia groups. Nurr1 immunoreactivity in the sham group was observed in CA1 pyramidal neurons (triangle). In the ischemia group, Nurr1 immunoreactivity in CA1 pyramidal neurons gradually decreased until 2 days and 3 days post-ischemia. At 4 days and 7 days post-ischemia, Nurr1 immunoreactivity was increased in non-pyramidal cells (arrows) of the CA1 subfield. Subsequently, Nurr1 immunoreactivity in the CA1 subfield was decreased again at 10 days after tgCI. Scale bar, 50 μ m. (J) ROD as % of Nurr1-immunoreactive structures in the CA1 subfield after tgCI (n=7 at each time point). Bars indicate the means \pm SEM. *P<0.05 vs. sham group; †P<0.05 vs. previous time point. tgCI, transient global cerebral ischemia; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; CA, cornu ammonis; Nurr1, nuclear receptor related 1 protein.

subfields were large and pyramidal in shape (Fig. 1A, D and J). In the ischemia group, no difference in CV staining in the hippocampus proper was found at 2 days after tgCI, compared to the sham group (Fig. 1B, E and K). On the other hand, most of CV-positive pyramidal neurons in the stratum pyramidale of the CA1 subfield were dead at 4 days after tgCI (Fig. 1C and F). At this point of time, many FJB positive cells were observed in the stratum pyramidale of the CA1 subfield (Fig. 1I). However, CV-positive pyramidal neurons in the CA2/3 subfield were not

damaged at 4 days after tgCI (Fig. 1C and L). In addition, any FJB positive cells were not found in the CA2/3 subfield (data not shown). This result is consistent with the results of our previous studies (29-31).

tgCI-induced change of Nurr1 immunoreactivity in CA1 subfield. In the sham group, Nurr1 immunoreactivity was primarily shown in pyramidal neurons of the stratum pyramidale of the hippocampus proper (CA1-3 subfields) (Fig. 2A).

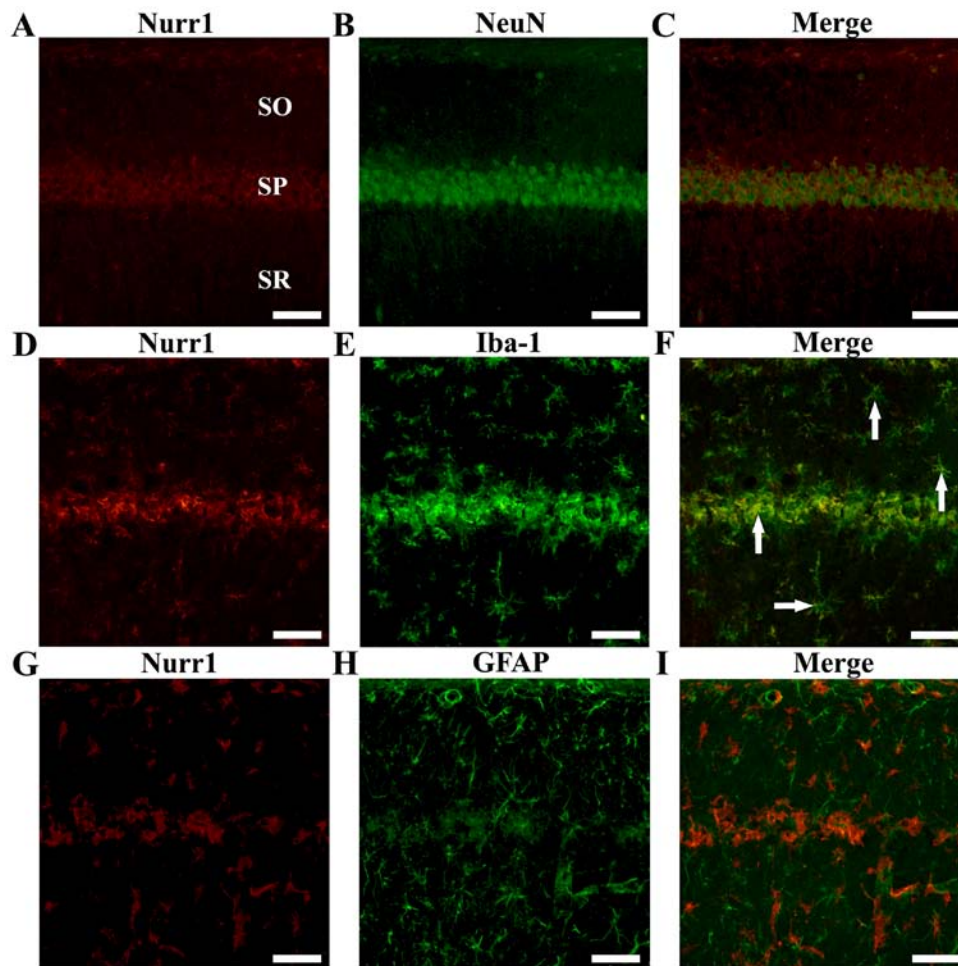


Figure 3. Double immunofluorescence staining for (A, D and G) Nurr1 (red), (B) NeuN (green) for neurons, (E) Iba-1 (green) for microglia or (H) GFAP (green) for astrocytes, and (C, F and I) merged images in the CA1 subfield. Nurr1-immunoreactive cells were colocalized with NeuN-immunoreactive neurons in the sham operated group. At 4 days after tgCI, Nurr1-immunoreactive cells expressed Iba-1 immunoreactivity (arrows) in the CA1 subfield of the ischemia operated group. Scale bar, 50 μ m. tgCI, transient global cerebral ischemia; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; CA, cornu ammonis; Nurr1, nuclear receptor related 1 protein; NeuN, neuronal nuclei; Iba-1, allograft inflammatory factor 1.

In the CA1 subfield of the ischemia group, Nurr1 immunoreactivity in the CA1 pyramidal neurons began to decrease from 6 h after tgCI, and the immunoreactivity was gradually decreased with a time-dependent manner until 3 days after tgCI (Fig. 2B-F and J). Especially, at 2 and 3 days after tgCI, Nurr1 immunoreactivity in the CA1 pyramidal neurons was hardly detected (Fig. 2E and F). However, Nurr1 immunoreactivity was increased in the CA1 subfield at 4 and 7 days after tgCI (Fig. 2G, H and J). At these points of time, Nurr1-immunoreactive cells were observed in the stratum pyramidale of the CA1 subfield, and Nurr1 immunoreactivity was detected in non-pyramidal cells of the stratum oriens and radiatum in the CA1 subfield (Fig. 2G and H). And then, Nurr1 immunoreactivity was decreased in the CA1 subfield at 10 days after tgCI (Fig. 2I and J).

Nurr1 expression in neurons or microglia. As shown in Fig. 2A, Nurr1 immunoreactivity was observed in the stratum pyramidale of the CA1 subfield of the sham operated group. To confirm whether Nurr1 is expressed in pyramidal neurons, double immunofluorescence staining was performed for Nurr1/NeuN (neurons). We found that Nurr1-immunoreactive cells were colocalized

with NeuN-immunoreactive neurons in the CA1 subfield of the sham operated group (Fig. 3A-C).

In addition, as shown in Fig. 2F and G, Nurr1 immunoreactivity was newly expressed in non-pyramidal cells in the CA1 subfield at 4 days after tgCI. To identify the cell type, double immunofluorescence staining was performed for Nurr1/Iba-1 (microglia) and Nurr1/GFAP (astrocytes). We found that Nurr1-immunoreactive cells were colocalized with Iba-1-immunoreactive microglia, not with GFAP-immunoreactive astrocytes, in the CA1 subfield at 4 days after tgCI (Fig. 3D-I).

tgCI-induced change of Nurr1 immunoreactivity in CA2/3 subfield. Unlike the tgCI-induced change of Nurr1 immunoreactivity in the CA1 subfield, Nurr1 immunoreactivity in the CA2/3 subfield was not significantly changed at any times after tgCI (Fig. 4A-D).

tgCI-induced change of Nurr1 protein level. Nurr1 protein level in the CA1 field was significantly changed with time after tgCI (Fig. 5), showing that the pattern of change of Nurr1 protein level was similar to that observed in the immunohistochemical

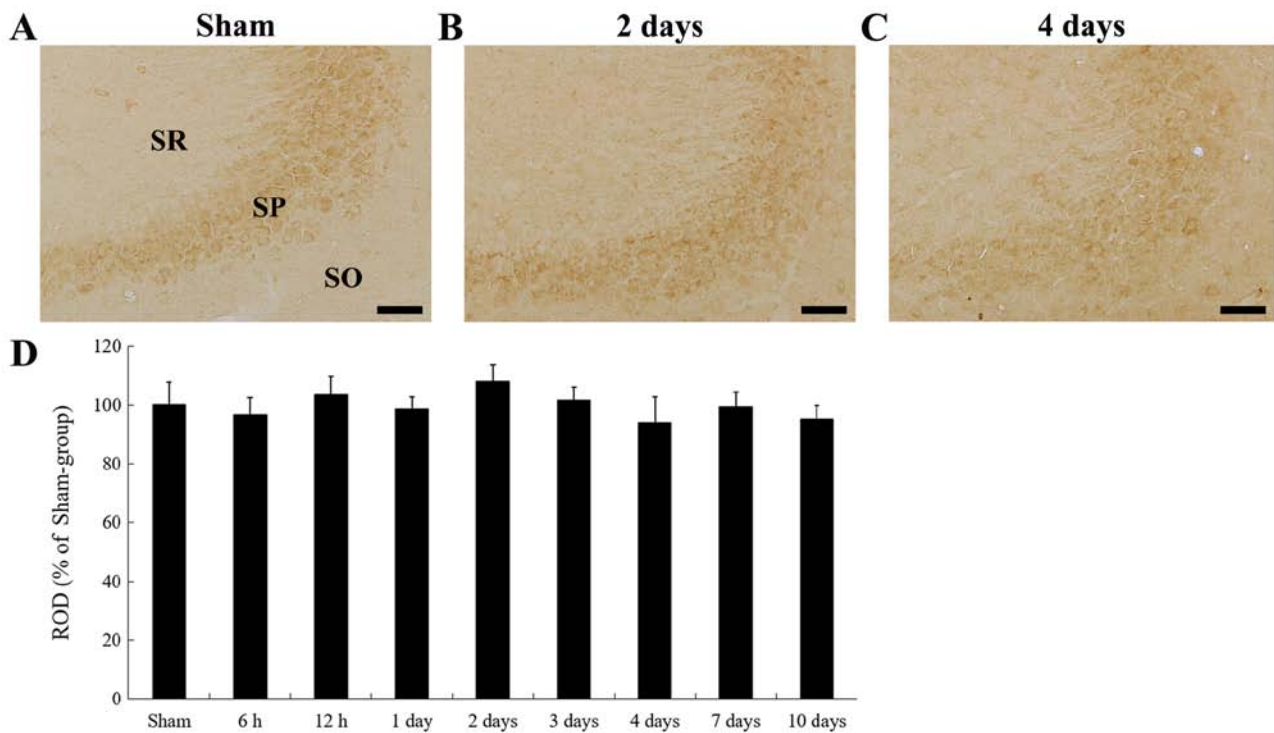


Figure 4. Immunoreactivity of Nurr1 in the CA3 subfield of the (A) sham and (B and C) ischemia groups. Nurr1 immunoreactivity in CA3 pyramidal neurons was not altered at (B) 2 and (C) 4 days following tgCI. Scale bar, 50 μ m. (D) ROD as % of Nurr1-immunoreactive structures in the CA3 subfield after tgCI (n=7 at each time point). Bars indicate the means \pm SEM. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; Nurr1, nuclear receptor related 1 protein; CA, cornu ammonis; tgCI, transient global cerebral ischemia.

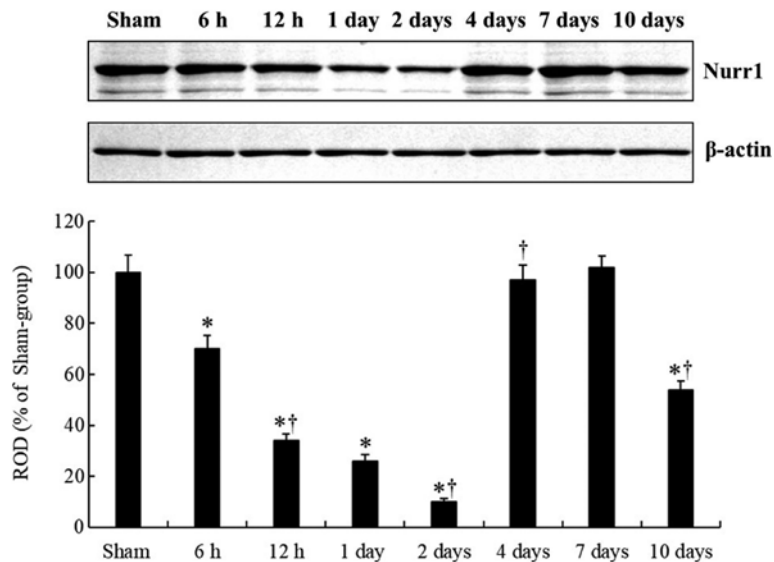


Figure 5. Western blot analysis of Nurr1 in the cornu ammonis 1 subfield of the sham-operated and ischemia-operated gerbils. ROD as % value of immunoblot band is represented (n=5 at each time point). *P<0.05 vs. sham group; †P<0.05 vs. previous time point. Bars indicate the means \pm SEM. Nurr1, nuclear receptor related 1 protein.

data. Nurr1 protein level was significantly decreased at 6 h after tgCI compared to that of the sham operated group, and it was gradually decreased until 2 days after tgCI. At 4 days after tgCI, Nurr1 protein level was significantly increased, compared to that at 2 days after tgCI. At 7 days after tgCI, Nurr1 protein level was not different from that at 4 days after tgCI. And then, Nurr1 protein level was significantly decreased at 10 days after tgCI, compared to that at 7 days after tgCI.

Discussion

In this study, we examined neuronal death in the CA1 subfield after tgCI and found that CA1 pyramidal neurons did not die until 2 days after tgCI; however, CA1 pyramidal neurons died at 4 days post-tgCI. On the other hand, pyramidal neurons in the CA3 subfield did not die at any time after tgCI. These findings are consistent with results of previous studies (30,32).

Some previous studies have reported that brain ischemic insults cause changes of *Nurr1* expression in various rodent models of cerebral ischemia (23,25,27). However, it is unclear if *Nurr1* has a neuroprotective or detrimental effect against cerebral ischemia injury. It has been reported that a level of *Nurr1* mRNA increased in the cerebral cortex following permanent middle cerebral artery occlusion (MCAO) in rats (25). They have also shown that *Nurr1* mRNA is induced in pyramidal neurons of the rat hippocampal CA1 and CA3 subfields at 1 h after ischemic insult induced by MCAO (25). Recently, Zhang and Yu (27) have reported that protein expression and mRNA transcription of *Nurr1* are significantly enhanced in the mouse brain following transient focal ischemia by MCAO, showing that the infarct volume is significantly reduced in *Nurr1* knockout mice, compared with wild-type mice, after transient focal ischemia. They have urged that an increase of *Nurr1* is associated with the progression of cerebral ischemia-reperfusion injury and that upregulated *Nurr1* is involved in neuronal apoptosis and mitochondrial injury (27). Conversely, other previous studies have reported that cerebral ischemic insults decrease *Nurr1* expression in brains (23,24,26). Erdo *et al* (23) have demonstrated that *Nurr1* protein expression is down-regulated in the ipsilateral basal ganglia after transient focal ischemia induced by MCAO in mice. Additionally, it has been reported that *Nurr1* mRNA expression is not changed in the pyramidal cell layer in the CA1 subfield of the gerbil hippocampus until 24 h after 5 min of tgCI, and then decreased at 72 h after tgCI (24). Recently, Xie *et al* (26) have reported that mRNA and protein levels of *Nurr1* begin to decrease immediately after ischemic damage, reaching a minimum at 12 h and increase at 48 h in the rat brain including the hippocampus following transient MCAO. They have also shown that increased expression of *Nurr1* can inhibit TNF- α expression in microglia, reduce ischemia-induced neuroinflammatory and cytotoxic response in neurons, and decrease infarct volume at an early stage after transient MCAO (26). In this study, we found that *Nurr1* immunoreactivity, which was shown in pyramidal neurons of the CA1 subfield, gradually decreased with a time-dependent manner after tgCI, and that *Nurr1* immunoreactivity in the CA1 pyramidal neurons was markedly weak at 2 and 3 days after tgCI. In addition, *Nurr1* protein expression in the CA1 subfield was gradually decreased until 2 days after tgCI. However, we found that tgCI-induced change of *Nurr1* immunoreactivity in the CA3 subfield was not shown at any time after tgCI. It has been reported that CA3 subfield is known to be a tolerant subfield following tgCI (3,29). Thus, it can be postulated that tgCI-induced reduction of *Nurr1* expression in pyramidal neurons of the CA1 subfield may be closely involved with a process of the tgCI-induced death of CA1 pyramidal neurons.

In the present study, we first found that *Nurr1* immunoreactivity was detected in non-pyramidal cells in the strata oriens and radiatum of the CA1 subfield at 4 days after tgCI. We examined the type of these non-pyramidal cells and found that they were microglia. It has been reported that *Nurr1* expression in microglia is regulated in response to inflammation (33). Additionally, it has well been known that *Nurr1* can attenuate expressions of proinflammatory and neurotoxic mediators in microglia through its ability to decrease NF κ B activation (8,9,20). Additionally, some studies have suggested that

beneficial effects of microglia, such as removal of cell debris and production of neurotrophic factors, are effectively played in the ischemic brain regions following ischemic insults (34,35). Thus, it is likely that newly expressed *Nurr1* in microglia in strata oriens and radiatum of the CA1 subfield may be related with a beneficial effect of microglia following tgCI.

In summary, *Nurr1* immunoreactivity was significantly changed in the CA1 subfield, not the CA3 subfield, following tgCI, and *Nurr1* immunoreactivity was newly expressed in microglia of ischemic CA1 subfield at 4 days after tgCI. These results indicate that tgCI-induced alteration of *Nurr1* expression may be closely related with tgCI-induced neuronal death in the hippocampal CA1 subfield.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

TKL, CWP and YEP performed the measurements. DWK, JCL, HAL and GEY analyzed and interpreted data. JHP, JHA, MHW and CHL made substantial contributions to conception and design, and were involved in drafting and revising the manuscript, and interpreting all data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of this experiment was approved by Institutional Animal Care and Use Committee (IACUC) at Kangwon National University (approval no. KW-180124-1). This protocol adhered to guidelines from the current international laws and policies in the 'Guide for the Care and Use of Laboratory Animals'.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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