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Autophagy-mediated stress response in motor neurons after hypothermic spinal cord ischemia in rabbits

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Abstract

Objective: The development of spinal cord injury is believed to be related to the vulnerability of spinal motor neurons to ischemia. However, the mechanisms underlying this vulnerability are not fully investigated. Previously, we reported that spinal motor neurons are lost likely due to autophagy and that local hypothermia prevents such a spinal motor neuron death. Therefore, we investigated the role of autophagy in the normothermic and hypothermic spinal cord ischemia using an immunohistochemical analysis of Beclin 1 (BCLN1, Bcl-2 interacting protein), B cell leukemia 2 protein (Bcl-2) and γ-aminobutyric-acid type-A-receptor-associated protein (GABARAP) which are considered as autophagy related protein.

Methods: We used rabbit normothermic and hypothermic transient spinal cord ischemia models employing a balloon catheter. Neurologic function was assessed by the Johnson score, and the spinal cord was removed at eight hours and one, two and seven days after reperfusion, and morphologic changes were examined with hematoxylin-eosin staining. A Western blot analysis and histochemical study of BCLN1, Bcl-2 and GABARAP and double-label fluorescent immunocytochemical studies were performed.

Result: There were significant differences in the physiologic function between normothermic model and hypothermic model after the procedure ($P < .05$). In the normothermic model, the majority of motor neurons were selectively lost at seven days of reperfusion ($P < .001$ compared with sham group), whereas they were preserved in the hypothermic model ($P = .574$ compared with sham group). The Western blot analysis revealed that the sustained expression of autophagy markers BCLN1 and GABARAP was observed ($P < .001$ compared with sham group) and was associated with neuronal cell death under normothermic-ischemic conditions. Under hypothermic-ischemic conditions, the autophagy inhibitory protein Bcl-2 was powerfully induced

(*P* < .001 compared with sham group) and was associated with blunted expression of BCLN1 and GABARAP and neuronal cell survival. The double-label fluorescent immunocytochemical study revealed that immunoreactivitiy for BCLN1, Bcl-2 and GABARAP was induced in the same motor neurons.

Conclusion: These data suggest the prolonged induction of autophagy may be a potential factor responsible for delayed motor neuron death, while the induction of the autophagy inhibitory protein Bcl-2 by hypothermia may limit autophagy and protect against delayed motor neuron death.

Spinal cord injury following successful surgery of the thoracic aorta is a disastrous complication. The reported incidence of paraplegia in such case ranges from 2.9% to 23% [1] among patients undergoing surgery of the thoracic aorta. The reasons of spinal cord dysfunction are thought to be the induction of ischemic damage during cross-clamping. However, patients undergoing thoracic aortic aneurysm repair who awake with no neurologic deficits immediately after surgery may sometimes eventually develop delayed onset paraplegia.[2] The exact mechanism underlying this delayed vulnerability is not fully understood.

A recent study showed that neuronal survival is affected by the disturbance of the ubiquitin-proteasome pathway or the autophagy-lysosome pathway under conditions of nonlethal stress.[3] We reported that delayed and selective motor neuron death is greatly associated with the activation of autophagic signals.[4, 5, 6] In addition, we previously reported the establishment of a topical cooling model of spinal cord ischemia that inhibits delayed and selective motor neuron death.[7] As a common feature of humans and rabbits, the motor neurons are vulnerable to ischemia. The anatomical feature of rabbits, the blood flow for the spinal cord is supplied from the lumber artery. Therefore, production of the spinal cord ischemia model is possible by a simple technique without causing other organ injury.

Autophagy is an intracellular bulk degeneration process whereby cytosolic, long-lived proteins and organelles are degenerated and recycled.[8] In autophagy, cytoplasmic materials and dysfunctional organelles are sequestered by autophagosomes, and subsequently delivered to the lysosome where they are degraded by lysosomal proteases. Autophagy occurs at the basal levels, although it can be further induced by stressors, including nutrient depletion, ischemia and reperfusion.[9, 10] Autophagy plays a dual role in cell survival, promoting survival by generating free amino acids and fatty acids that can be reused to maintain mitochondrial adenosine

triphosphate production and protein synthesis, and inducing cell death under some circumstances.[11, 12, 13]

Beclin 1 (BCLN1) is part of the Class Ⅲ PI3K complex that participates in autophagosome formation, mediating the localization of other autophagy proteins to the preautophagosomal membrane.[14, 15] B cell leukemia 2 protein (Bcl-2) is key regulator of apoptosis and autophagy. Anti-apoptotic Bcl-2 family proteins can bind the autophagy essential protein BCLN1 and inhibit BCLN1-dependent autophagy.[16, 17, 18] However, the function of BCLN1 and Bcl-2 in spinal cord ischemia-induced neurodegeneration is not completely understood. γ-aminobutyric-acid type-A-receptor-associated protein (GABARAP) is a mammalian Atg8-related protein that localizes to autophagosomal membranes following post-translational modifications and has been shown to be an autophagosomal marker in mammals.[19] Using the rabbit spinal cord ischemia model, we previously reported the overexpression of GABARAP following transient ischemia.[6] Therefore, in the present study, we hypothesized that motor neuron cells, which eventually die of acute spinal cord ischemia in this model demonstrate the prior induction of BCLN1, Bcl-2 and GABARAP. In addition, differences in their expression level under moderate hypothermia were assessed.

Materials and methods

Animal models

The animals were treated in accordance with the declaration of Helsinki and the guiding principles for the care and use of animals during the experiment. The animal care committee of the Kyushu University School of Medicine approved the experimental and animal care protocols.

Forty-five Japanese domesticated white rabbits weighing 2-3 kg were used in this study and divided into three groups: a normothermic ischemia group (group N), a hypothermic ischemia group (group H) and a sham control group. Anesthesia was induced via the intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane inhalation with 100% oxygen. A 4-Fr. pediatric catheter (CI-300, Harmac Medical Products, Inc., USA) was inserted through the femoral artery and advanced 15 cm forward into the abdominal aorta. Then, a balloon was inflated and 15 minutes transient ischemia was performed. The balloon was deflated after 15 minutes ischemia and the catheter was immediately removed. Preliminary investigations conducted via laparotomy confirmed that the distal end of the balloon of the catheter was positioned 0.5 ~ 1.5 cm immediately distal to the left renal artery.[4-7] The aortic pressures was continuously monitored both at the proximal and distal positions of the balloon during the experiment. When the balloon of the catheter was inflated in the abdominal aorta, the systemic blood pressure of the rabbits did not change. In addition, the arterial pressure of distal end of the catheter fell to near zero, and no pulsation was recorded. The arterial blood pressure of this portion decreased for 15 minutes and returned to the normal level after deflation of the balloon. The body temperature was maintained at 37℃ with a heating pad and monitored using a rectal thermistor during procedure and subsequent ischemia. Group H was operated in the same method with the cooling pad.[7] The cooling pad was attached to the lumber region (L1-5) of the naked skin. We confirmed the localized cooling effect on the temperature of the rectum (34.85 ± 0.21 versus 31.35 ± 0.30, $P < .001$). The animals were sacrificed with deep anesthesia with sodium pentobarbital (100 mg/kg administered intravenously) at eight hours and one, two and seven days after reperfusion (n = 5 each group at each time point). In the sham control group, the animals were sacrificed after seven days (n = 5) after reperfusion

following the insertion of the catheter without inflating the balloon. With using the plunger of a 1-ml syringe, the spinal cords were quickly removed immediately after sacrifice. The tissue samples used for the Western blot analysis and immunohistochemical studies were frozen and stored at -80℃. The samples used for histology were fixed via immersion in 4% paraformaldehyde in 0.1 M phosphate buffer then stored at 4℃ for one week; they were then cut transversely at approximately the L2 or L3 level and embedded in paraffin.

Neurological assessment

The neurologic function was evaluated before the rabbits were sacrificed at seven days after reperfusion. The rabbits were classified according to a five-point scale devised by Johnson et al.[20] as follows: 0: Hind-limb paralysis; 1: Severe paralysis; 2: Functional movement, no hop; 3: Ataxia, disconjugate hop; 4: Minimal ataxia; and 5: A normal function. Two individuals without knowledge of the treatment graded the neurologic function independently.

Histological study

In order to determine the pathological changes in the spinal cord following ischemia, we performed hematoxylin-eosin staining of a set of sections examined using light microscopy and counted the number of intact large motor neuron cells in the ventral gray matter region in five sections per animal. An observer who was unaware of the animal groups and neurological outcomes examined each slide (×100). The neurons were considered "dead" if the cytoplasm was diffusely eosinophilic and "viable" if the cells demonstrated basophilic stippling (that is, contained Nissl substance), on hematoxylin-eosin staining.

Western blot analysis

In order to investigate changes in the BCLN1, Bcl-2 and GABARAP expressions, we examined a Western blot analysis. The tissue samples were homogenized in a lysis buffer (1 μg/mL of aprotinin, 0.01 mol/L of Tris-HCl, pH 7.5, 0.1 mol/L of NaCl and 1 mmol/L of EDTA), and the homogenates were centrifuged at 7,000 g for 15 minutes at 4℃. Assays to determine the protein concentrations of the samples were subsequently performed by comparing the results to a known concentration of bovine serum albumin using a kit (BCA protein assay reagent kit #23225, Pierce, IL, USA). We performed sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a 15% polyacrylamide gel under nonreducing conditions. Then, we boiled the protein samples at 100℃ in 5% β-mercaptoethanol 2.5% SDS and 2.5% SDS, and lysates equivalent to 20 μg of protein obtained from each sample were run on the gel for 90 minutes at 20 mA, together with a size marker (dual colored protein, Biorad). The electrophoresis running buffer contained 0.1% SDS, 250 mmol/L of glycine and 25 mmol/L of Trisbase. The proteins on the gel were then transferred to a polyvinylidene fluoride membrane (Invitrogen, Carlsbad, CA, USA) using a transfer buffer consisting of 20% methanol, 0.4% SDS, 39 mmol/L of glycine and 48 mmol/L of Trisbase.

The membranes were placed in 4% powdered milk in phosphate-buffered saline (PBS) for blocking nonspecific binding after the transfer. After that, the membranes were incubated with primary antibodies at 1:1000 dilution for 20 hours at 4℃. The primary antibodies used were as follows: mouse monoclonal anti-BCLN1 antibody (TA502527; OriGene Technologies, Inc., Rockville, MD, USA), goat polyclonal anti-Bcl-2 antibody (TA302955; OriGene Technologies, Inc., Rockville, MD, USA) and goat polyclonal anti-GABARAP antibody (SC-9190; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Then, the membranes were incubated with

horseradish peroxidase-conjugate anti-mouse IgG (#7076; Cell Signaling Technology, Danvers, MA, USA) and horseradish peroxidase-conjugate anti-goat IgG (PI-9500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:5000 dilution in PBS for 90 minutes after washing PBS, respectively. The blots were developed using the ECL Plus detection method (RPN2132; Amersham Bioscience, Buckinghamshire, England). Another membrane was stained in a similar way without the primary antibodies to ascertain the specific binding of the antibodies for the proteins. The images of the Western blot study were quantified by plotting a two-dimensional densitogram using the image analysis program Image J, version 1.63 (Research Services Branch, NIMH, National Institutes of Health).

BCLN1, Bcl-2 and GABARAP immunocytochemistry

To investigate changes in the expressions of BCLN1, Bcl-2 and GABARAP, we also performed an immunohistochemical study in five sections per animals (×200). With use of 0.1 M phosphate-buffered saline (PBS), spinal cord sections were rinsed for 20 minutes. The sections were blocked in 2% normal horse serum for two hours at room temperature and incubated with primary antibodies in 0.3% Triton-X 100 and 10% normal horse serum or 10% normal rabbit serum and for 20 hours at 4℃, respectively. The primary antibodies were the same as those used for the Western blot analysis noted above, and each dilution was as follows: antibody against BCLN1 at 1:200, antibody against Bcl-2 at 1:100 and antibody against GABARAP at 1:200.

The sections were exposed to 10% methanol and 0.3% H_2O_2 for 20 minutes for quenching the endogenous peroxidase activity, and washed in PBS. After that, the sections incubated for three

hours with biotinylated anti-goat IgG (PK-6105; Vector Laboratories, Burlingame, CA, USA) and biotinylated anti-mouse IgG (PK-6102; Vector Laboratories, Burlingame, CA, USA) at 1:200 dilution in PBS containing 0.018% normal horse and rabbit serum, respectively. The samples were then subsequently incubated with avidin-biotin-horseradish peroxidase complex (PK-6102; Vector Laboratories, Burlingame, CA, USA). The samples were colorized with DAB/H2O2 solution, and the cytoplasm was counterstained with hematoxylin. A set of sections were stained in a similar way without the primary antibodies in order to ascertain the specific binding of the antibodies for the proteins.

Fluorescence double-labeling study for BCLN1, Bcl-2 and GABARAP

Spinal cords sections were prepared as described above. The sections were blocked using 10% horse serum before the application of the primary antibodies. The sections were then incubated with Bcl-2 goat monoclonal antibodies at 1:100 dilution simultaneously with BCLN1 and GABARAP goat polyclonal antibodies at 1:100 dilution simultaneously with BCLN1. The primary antibodies were incubated overnight at 4℃and detected using goat anti-mouse lgG linked with green-fluorescent Alexa Flour 488 1:50 (A11001; Invitrogen, Carlsbad, CA, USA) and donkey anti-goat lgG linked with orange-fluorescent Alexa Flour 555 1:50 (A-21432; Invitrogen, Carlsbad, CA, USA). The slides were mounted in aqueous mounting media with DABCO and observed using fluorescein microscopy (×400).

Statistical analysis

The neurologic score, the cell numbers and the optical density values of the Western blots were analyzed by ANOVA for quantitative analyses. *P*-value of less than 0.05 was considered to be

statistically significant. Parametric data are presented as the mean ± standard deviation.

Results

Neurological assessment

The results of the neurological assessment are shown in Table 1. A normal neurologic function (score of 5) was observed in all sham operated rabbits (n=5). Both groups N (n=20) and H (n=20) were divided based on their reperfusion times (8 hours, 1 day, 2 days and 7 days, respectively). In group N at two days after reperfusion (n=5), all rabbits had a disconjugate hop or no hop. In group N at seven days after reperfusion (n=5), all rabbits exhibited worse scores than those of at two days. Group H rabbits had almost a normal neurologic function after reperfusion. There were significant differences in the neurological function between group N and group H seven days after reperfusion ($P<0.05$, respectively).

Histological study

The results of cell counting in the ventral gray matter region on the sections obtained in this study are shown in Figures 1. In the sham operated control group, no significant changes were observed in the motor neurons (29.2 ± 3.605 cells per slide; Fig. 1a, A and D). In the normothermic group, approximately 70% of the motor neurons were lost at seven days after reperfusion in the ventral gray matter (8.0 ± 3.16 cells per slide, $P < .001$; Fig. 1a, C), although almost all motor neurons were intact at two days after reperfusion (24.6 ± 3.43 cells per slide, $P = .0825$; Fig. 1a, B). On the other hand, in the hypothermic group, almost all motor neurons in the ventral gray matter were preserved at seven days after reperfusion (27.6 ± 3.36 cells per slide,

P = .574; Fig. 1a, F). Dorsal horn neurons were intact following transient ischemia (data not shown). Therefore, the selective loss of motor neurons and the protection of motor neurons by hypothermia were confirmed, according to our previous reports.[5-8]

Western blot analysis

The results of the Western blot analysis are shown in Figure 2. For BCLN1, a weak band of a molecular weight of 52 kDa was detectable in the sham operated group. This band was enhanced strongly at eight hours and preserved until two days after reperfusion in the N group (Fig. 2, A). In the H group, this band was enhanced at eight hours, then returned to the almost same level as that observed in the sham operated group, one day after reperfusion (Fig. 2, B). With respect to Bcl-2, a weak band of a molecular weight of 26 kDa was detectable in the sham operated group. There was no statistical significant difference. (Fig. 2, A). In the H group, this band was enhanced strongly at eight hours and preserved until two days after reperfusion (Fig. 2, B). Regarding GABARAP, a weak band of a molecular weight of 13 kDa was detectable in the sham operated group. This band was enhanced strongly at eight hours and preserved until two days after reperfusion in the N group (Fig. 2, A). In the H group, this band was enhanced at eight hours, then returned to the almost same level as that observed in the sham operated group one day after reperfusion (Fig. 2, B). The membranes without the primary antibodies exhibited no bands (data not shown). Using a ANOVA, we found that the levels of BCLN1, Bcl-2 and GABARAP were significantly increased at eight hours after reperfusion in both the N and H groups (*P < .05, **P < .001) (Fig. 3). In addition, comparing the N and H groups, each protein demonstrated significant differences in their expression level after one day (p < 0.05).

Histochemical study

Immunoreactive BCLN1, Bcl-2 and GABARAP of the spinal cords are shown in Figure 3. The spinal cords of the sham control group at seven days after reperfusion did not show BCLN1 (Fig. 3a, A), Bcl-2 (Fig. 3b, A) or GABARAP (Fig. 3c, A) immunoreactivity in any cells. The motor neurons selectively exhibited strong immunoreactivity for BCLN1 (Fig. 3a, B and F), Bcl-2 (Fig. 3b, B and F) and GABARAP (Fig. 3c, B and F) at eight hours after reperfusion in both the N and H groups. In the N group, the immunoreactivity of the motor neurons for BCLN1 and GABARAP was preserved after two days (Fig. 3a and 3c, C and D), while that for Bcl-2 was decreased at one day (Fig. 3b, C and D). In the H group, the immunoreactivity of the motor neurons for BCLN1 and GABARAP was decreased at one day (Fig. 3a and 3c, G and H), while that for Bcl-2 was preserved after two days (Fig. 3b, G and H).

Fluorescence double-labeling study

The results of Bcl-2 and BCLN1 double-staining immunohistochemistry and GABARAP and BCLN1 double-staining immunohistochemistry are shown in Figure 4. In both groups, Bcl-2 (Fig. 4a and 4b, A) and GABARAP (Fig. 4a and 4b, D) were detected using orange-fluorescent Alexa Flour 555 (red), while BCLN1 (Fig. 4a and 4b, B and E) was detected using green-fluorescent Alexa Flour 488 (green); these molecules were preferentially expressed in the cytoplasm of the motor neurons. The merged image was shown to be double-positive in Figure 4a and 4b, C and F. In the motor neurons, BCLN1, Bcl-2 and GABARAP were expressed and well colocalized in the cytoplasm in both groups.

Discussion

This is the first report to show the expression profiles of BCLN1, Bcl-2 and GABARAP following normothermic and hypothermic transient spinal cord ischemia in rabbits. Normothermic transient ischemia induced selective delayed motor neuronal death and affected the expression profiles of BCLN1, Bcl-2 and GABARAP. Although selective delayed motor neuronal death was not induced under hypothermic ischemia, the profiles of these proteins differed from those observed during normothermic transient ischemia. The prolonged expression of BCLN1 and GABARAP was observed until two days after reperfusion following normothermic ischemia, while the prolonged expression of Bcl-2 was observed until two days after reperfusion following hypothermic ischemia.

Recently, 35 autophagy-related (ATG) genes were identified whose products appear to be related to the process of autophagy. It was found that the molecular basis of autophagy may well be highly consevsed from yeast to humans.[8] BCLN1 is part of the Class Ⅲ PI3K complex that participates in autophagosome formation, mediating the localization of other autophagy proteins to the preautophagosomal membrane.[15] The upregulation of BCLN1 in the ischemic penumbra has been reported in a focal cerebral ischemia model, suggesting the role of autophagy as either a mechanism for recycling injured cells and reducing damage or a process leading to cell demise.[21] In the present study, BCLN1 was upregulated in the motor neurons following transient ischemia in the spinal cord. This upregulation was prolonged in the normothermic group and fell to the basal level in the hypothermic group. As such, the upregulation of BCLN1 observed in the present study may be considered to prolong the activity of autophagy in motor neurons following normothermic transient ischemia in the spinal cord.

B cell leukemia 2 protein (Bcl-2) is a key regulator of apoptosis and autophagy.[17] In the present study, Bcl-2 was upregulated in the motor neurons following transient ischemia, and this upregulation was prolonged in the hypothermic group. Bcl-2 family proteins can bind the autophagy essential protein BCLN1 and inhibit BCLN1-dependent autophagy in yeast and mammalian cells.[17] In addition, mild hypothermia reduced both cell death and the autophagy in cardiomyocyte.[22] Recent study have also reported that mild hypothermia reduces ischemic neuron death by increasing Bcl-2 expression in rats.[23] These results suggest that hypothermia controls autophagy in a restrained manner and prevents selective delayed motor neuronal death in the spinal cord following hypothermic transient ischemia by increasing Bcl-2 expression.

γ-aminobutyric-acid type-A-receptor-associated protein (GABARAP) is a mammalian Atg8-related protein that localizes to autophagosomal membranes following post-translational modifications and has been shown to be an autophagosomal marker in mammals.[19] In the present study, GABARAP was upregulated in the motor neurons following transient ischemia in the spinal cord. This upregulation was prolonged in the normothermic group and fell to the basal level in the hypothermic ischemia group. These results suggest that the upregulation of GABARAP reflects the activity of autophagy in the motor neurons following transient ischemia.

In the present study, BCLN1, Bcl-2 and GABARAP were upregulated in the motor neurons at an early phase following transient ischemia in the spinal cord, and a difference in the expression profile was noted between the normothermic and hypothermic groups. Therefore, one can speculate that whether autophagy plays a beneficial or detrimental role in motor neurons following transient spinal cord ischemia depends on the characteristics of the stress and the balance of the expressions of BCLN1, Bcl-2 and GABARAP.

In conclusion, this study demonstrated that immunoreactivity for BCLN1, Bcl-2 and GABARAP

was induced at an early phase in the same motor neurons and confirmed a difference in the expression profiles of these molecules between the normothermic and hypothermic groups. The prolonged induction of BCLN1 and GABARAP proteins at the early stage after reperfusion indicates a stress response and is a possible factor responsible for selective motor neuron death following transient spinal cord ischemia. Meanwhile, the prolonged induction of Bcl-2 may prevent selective motor neuron death following transient spinal cord ischemia in rabbits.

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1

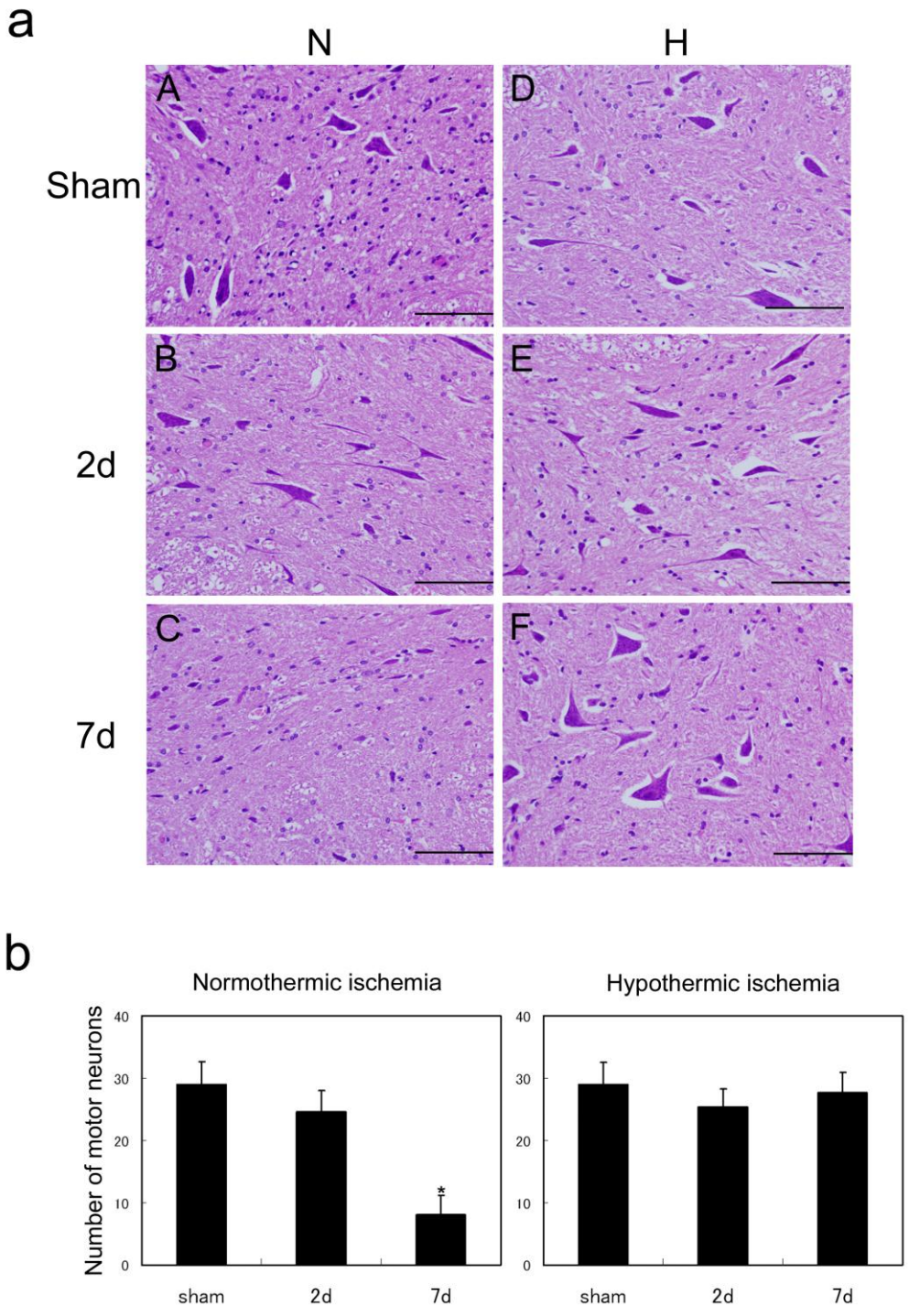
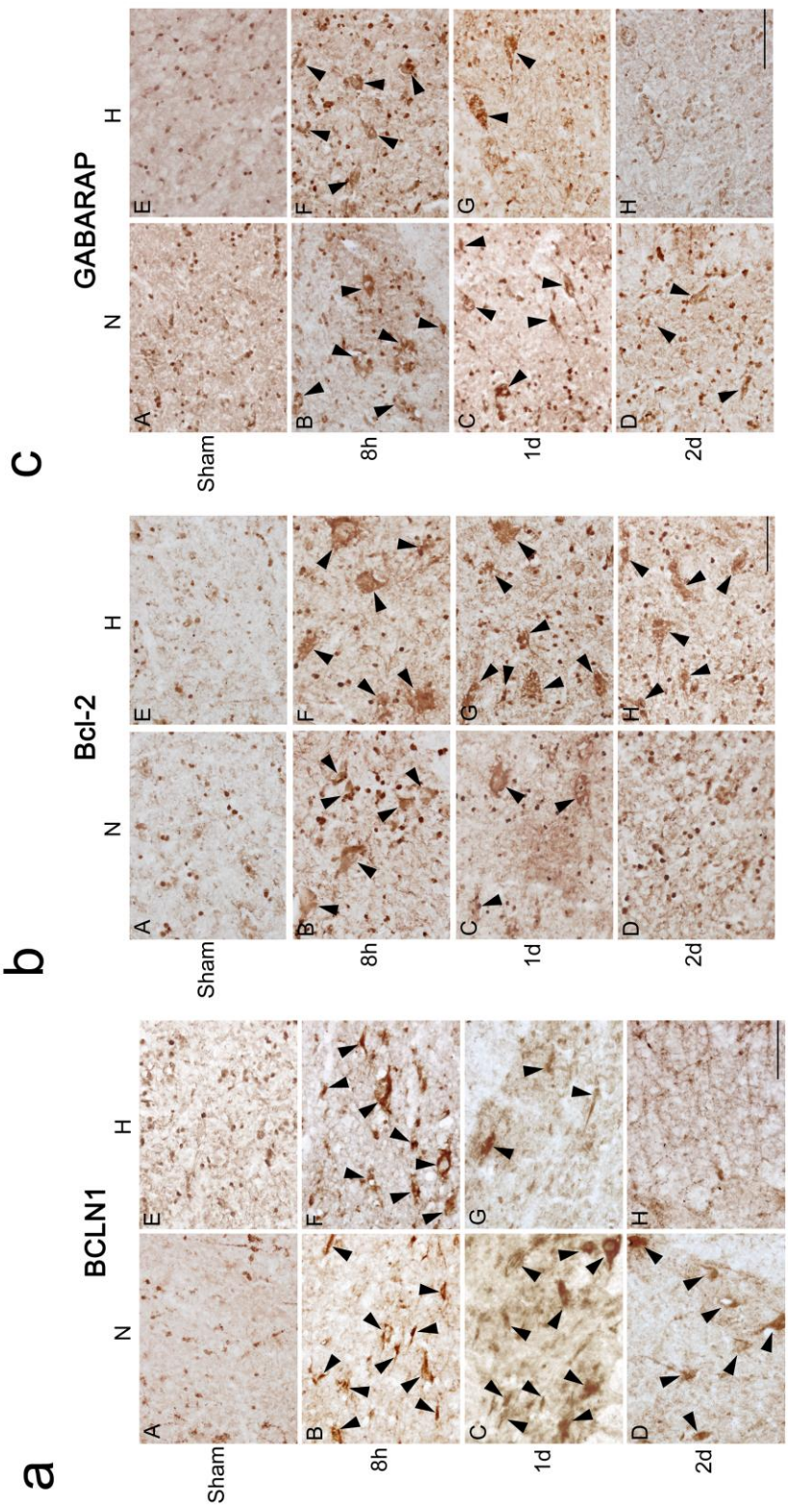

a
N
H
A
D
Sham
B
E
2d
C
F
7d
b
Normothermic ischemia
Hypothermic ischemia
Number of motor neurons
40
30
20
10
0
*
sham
2d
7d
sham
2d
7d

Figure1

A
S
8h
1d
2d
BCLN1
Bcl-2
GABARAP
Actin
Rerative optical density
sham
8h
1d
2d
B
S
8h
1d
2d
BCLN1
Bcl-2
GABARAP
Actin
Relative optical density
sham
8h
1d
2d

Figure2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BCLN1
N
H
A
E
Sham
B
F
8h
C
G
1d
D
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2d
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Bcl-2
N
H
A
E
Sham
B
F
8h
C
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1d
D
H
2d
c
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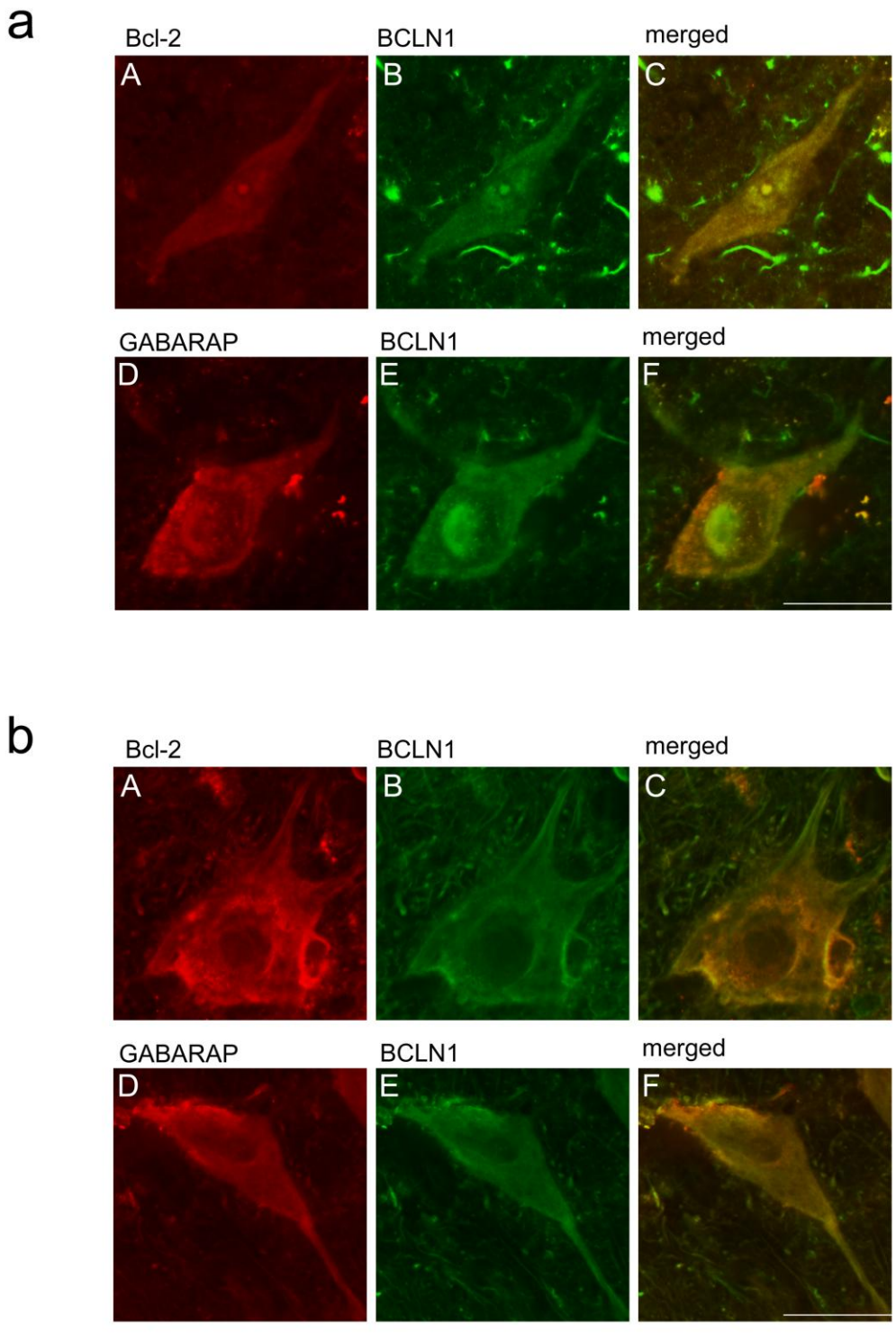
H
2d

Figure3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BCLN1
merged
A
B
C
GABARAP
BCLN1
merged
D
E
F
b
Bcl-2
BCLN1
merged
A
B
C
GABARAP
BCLN1
merged
D
E
F

Figure4