Arch Histol Cytol, 77: 13-23 (2017)

Induction of Autophagy in the Hippocampus after Hypoxic-Ischemic Injury to Neonatal Rats

Masato Koike[1], Ai Kawahara[2], Masahiro Shibata[3], and Yasuo Uchiyama[2]

Department of Cell Biology and Neuroscience[1] and Cellular and Molecular Neuropathology[2], Juntendo University Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan; and [3]Department of Morphological Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

Summary. Neonatal hypoxic/ischemic (H/I) brain injury causes neurological impairment, including cognitive and motor dysfunction as well as seizures. Patterns of H/I injury-induced neuron death using rodent models are considered to be similar to the cases in human H/I encephalopathy. The participation of autophagy in neuron death has been a common feature in neonatal rodent models of H/I brain injury and human H/I encephalopathy when examined by immunochemical approaches for MAP1-LC3. This tendency has also been confirmed in neuronal tissue-specific Atg7 conditional knockout mice. However, while the current rat H/I model that is used for analyzing autophagy results in global damage to the ipsilateral hemisphere, it does not entirely reflect the neuropathological changes that appear in the neonatal mouse H/I model, in which the hippocampus is selectively damaged. The present study established a neonatal rat model of H/I injury with a milder ischemic insult, in which autophagy was involved in the hippocampal CA1 region after H/I injury when examined by electron microscopy, and by immunohistochemical and biochemical analyses of LC3.

Received December 1, 2016

Address for correspondence: Yasuo Uchiyama, M.D., Ph.D.
Department of Cellular and Molecular Neuropathology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
Tel: +81-3-5802-1059, Fax: +81-3-5800-0245
E-mail: y-uchi@juntendo.ac.jp

Introduction

Autophagy is a highly regulated process involving the bulk degradation of cytoplasmic macromolecules and organelles in mammalian cells via the lysosomal system (Uchiyama *et al.*, 2008b). Basal autophagy is important for the maintenance of cellular metabolic turnover and homeostasis (Kuma *et al.*, 2004; Komatsu *et al.*, 2005; Mizushima, 2007). Autophagy is also essential for maintaining neuronal homeostasis under physiological conditions, and the impairment of autophagy due to mutated autophagy-related genes (Atgs), which includes their complete loss, is associated with severe neurodegeneration in mice as well as in humans (Hara *et al.*, 2006; Komatsu *et al.*, 2006; Saitsu *et al.*, 2013; Kim *et al.*, 2016).

However, inappropriate or excessive induction of autophagy has been documented under various neurodegenerative disorders (Liu *et al.*, 2004; Koike *et al.*, 2005; Zhu *et al.*, 2007; Nixon, 2013), indicating that both impairment and excessive autophagy is associated with neurodegeneration. Autophagy is also induced by a variety of acute brain injuries (Nitatori *et al.*, 1995; Zhu *et al.*, 2005; Shacka *et al.*, 2007; Clark *et al.*, 2008; Rami *et al.*, 2008; Ginet *et al.*, 2009; Galluzzi *et al.*, 2016). The exact roles of autophagy have remained unclear in these disease situations. However,

one point of clarification for autophagy has been the neuroprotective effect in certain forms of acute brain injury, such as cocaine intoxication and hemorrhage, while autophagy triggers neuronal cell death in neonatal hypoxic-ischemic (H/I) brain injury (Galluzzi *et al.*, 2016).

Neonatal H/I brain injury causes neurological impairment, and includes cognitive and motor dysfunction as well as seizures in humans (Ferriero, 2004; Mwaniki *et al.*, 2012). The Rice-Vannucci model has been extensively used as the most clinically relevant rodent model for human hypoxic-ischemic encephalopathy (HIE) (Rice *et al.*, 1981). Morphological, biochemical, and genetic approaches to rodent H/I models have revealed that the patterns of neuron death consist of apoptosis, necrosis, and apoptosis-necrosis continuum (Northington *et al.*, 2011). Furthermore, the participation of autophagy in neuron death is a common feature in neonatal rodent models of H/I brain injury and human HIE when examined by immunochemical approaches for MAP1-LC3 (Zhu *et al.*, 2005; Koike *et al.*, 2008; Ginet *et al.*, 2014; Xie *et al.*, 2016). Importantly, such H/I neuronal death is largely prevented by neuronal tissue-specific deletion of the Atg7 gene, which is essential for autophagosome formation (Koike *et al.*, 2008; Uchiyama *et al.*, 2008a; Xie *et al.*, 2016).

Although the involvement of autophagy in neuron death after neonatal rat H/I injury has also been shown (Ginet *et al.*, 2009; Liu *et al.*, 2013; Weis *et al.*, 2014; Xu *et al.*, 2016), the current rat H/I model extensively used results of global damage in the ipsilateral hemisphere with rather apoptotic features of hippocampal CA1 pyramidal neurons. Moreover, this model does not entirely reflect the neuropathological changes demonstrated in the neonatal mouse H/I model with autophagic neuron death in the hippocampus (Koike *et al.*, 2008). The present study used a neonatal rat H/I injury with a milder ischemic insult (Uchiyama *et al.*, 2008a) that employed morphological as well as biochemical approaches to explore the involvement of autophagy in pyramidal neuron death in the hippocampal CA1 region after H/I injury.

Material and Methods

Animals

The experiments described below were performed in compliance with the regulations of Osaka University Graduate School of Medicine's Guidelines for the Care and Use of Laboratory Animals. Neonatal Wistar rats at postnatal day 3 (P3) were obtained from CLEA Japan (Tokyo, Japan) or Charles River Laboratories Japan (Yokohama, Japan) in a pathogen-free facility until the pups became P7.

Hypoxic-Ischemic Injury

Neonatal H/I brain injury was induced in neonatal rats on P7, essentially according to the Rice-Vannucci model (Rice *et al.*, 1981), with minor modifications (Koike *et al.*, 2008; Uchiyama *et al.*, 2009; Koike *et al.*, 2013). After the rats were deeply anesthetized with isoflurane (2%), the left common carotid artery was dissected and ligated with silk sutures (6/0) at two portions with a cut between them. Following the surgical procedure, the pups were allowed to recover for 1 hour. They were then placed in chambers maintained at 37˚C through which 8% humidified oxygen (balance, nitrogen) was flowed for 30, 60, 90, or 120 min. Because H/I injury resulting from 60 min of hypoxia was optimal to produce damage mainly in the hippocampal regions (Uchiyama *et al.*, 2009), we fixed the duration of hypoxia for 60 min for all the following morphological and biochemical analyses. After hypoxic exposure for 60 min, the pups were returned to their dams and allowed to recover for 3, 8, 24, or 72 hours. At each stage, the brains were processed for biochemical and morphological analyses. Control littermates were neither operated on nor subjected to hypoxia.

Antisera

The preparation of the rabbit antibody against rat LC3 was described previously (Koike *et al.*, 2005; Lu *et al.*, 2005). Rabbit polyclonal antibodies against caspase-3 (Cell Signaling, Danvers, MA), cleaved caspase-3 (Asp175) (Cell Signaling), and cleaved caspase-7 (Asp198) (Cell Signaling), and mouse monoclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone: 6C5) (Ambion, Austin, TX), and caspase-7 (clone: 10-1-62) (BD Biosciences, San Jose, CA) were obtained commercially.

Sample preparation for histochemical and morphological analyses

Rats after H/I injury (n = 3–5 for each procedure and each stage) were deeply anesthetized with pentobarbital (25 mg/kg i.p.) and fixed by cardiac perfusion with 4% paraformaldehyde (PA) buffered with 0.1 M phosphate buffer (pH 7.2) (PB) containing 4% sucrose for light microscopy and with 2% PA-2% glutaraldehyde buffered with 0.1 M PB for ordinary electron microscopy (Koike *et al.*, 2005; Koike *et al.*, 2008). For light microscopy, brain tissues were quickly removed from the rats and further immersed in the same fixative for 2 hours at 4˚C. Samples for cryosections were embedded in OCT compound (Miles, Elkhart, IN) after cryoprotection with 15 and 30% sucrose solutions and cut into 10-µm sections with a cryostat (CM3050; Leica, Nussioch, Germany). The sections were placed on silane-coated glass slides and stored at −80˚C until used. Samples for electron microscopy were postfixed with 2% OsO_4 with 0.1 M PB, block-stained in 1% uranyl acetate, dehydrated with a graded series of alcohol, and embedded in Epon 812 (TAAB, Reading, England). For light microscopic observations, semi-thin sections were cut at 1 µm with an ultramicrotome (Ultracut N; Reichert-Nissei, Tokyo, Japan) and stained with toluidine blue. For electron microscopy, silver sections were cut with an ultramicrotome, stained with uranyl acetate and lead citrate, and observed with an electron microscope (H-7100; Hitachi, Tokyo, Japan).

Immunohistochemical analyses

For immunofluorescence, cryosections were first incubated with anti-LC3 (1:100) or anti-cleaved caspase-3 or -7 (1:100) at 4˚C overnight, followed by goat anti-rabbit IgG coupled with Alexa Fluor 488 (Invitrogen, Grand Island, NY) for 1 hour at room temperature (RT). For LC3 detection, further incubations were performed with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), and finally, with streptavidin coupled with Alexa Fluor 488 for 1 hour at RT. The sections were mounted in VECTASHIELD Mounting Medium with DAPI (Vector) and observed under a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan). For control experiments, cryosections were incubated with the non-immunized rabbit serum diluted 1:1,000, followed by respective second antibodies. Some sections were directly incubated with the second antibodies without pretreatment with the first antibodies.

Sample preparations for biochemical analyses

Anesthetized rats were euthanized by decapitation at 3, 8, 24, or 72 hours after H/I injury (n = 7 per group), and untreated control animals were euthanized on P7 (n = 6). The brains were rapidly dissected on a bed of ice. The left and right hippocampi were separately excised from the rats, frozen in liquid nitrogen, and stored at −80˚C until used.

Measurement of caspases-3 and -7 activities

For the measurement of caspases-3 and 7 activities, an Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) cleavage assay in hippocampal tissue was performed as described elsewhere (Koike *et al.*, 2003; Koike *et al.*, 2008). Briefly, each tissue was independently homogenized in 100 µl of lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 42 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5% 3-[(3-choramidopropyl)dimethylamino]propane-1-sulfonate (CHAPS), 1 mM phenylmethylsulfonylfluoride (PMSF), and protease inhibitor cocktail (Nakalai, Kyoto, Japan), pH 7.5, and spun at 10,500 g for 15 min at 4˚C. Tissue lysates (10 µl each) were incubated in an opaque 96-well plate with 190 µl of assay buffer (25 mM HEPES, 1 mM ethylenediaminetetraacetate (EDTA), 3 mM DTT, 0.1% CHAPS, 10% sucrose, and a protease inhibitor cocktail) containing 30 µM of Ac-DEVD-AMC (Peptide Institute, Inc., Minoo, Japan). The fluorescence intensity (excitation, 365 nm; emission, 465 nm) was monitored using a Spectramax microtiter plate reader (Molecular Devices, Sunnyvale, CA). The accumulation of fluorescence was linear for at least 45 min. Protein concentrations in each lysate were determined using a bicinchoninic acid assay (BCA) protein assay system (Pierce, Rockford, IL). The experiments were repeated three times with samples from each hippocampus, and the data from eight animals in each group were averaged.

Immunoblot analysis

Among all samples from individual animals, pairs of the

samples with DEVD activity that was higher in the ipsilateral hippocampus than that in the contralateral (n = 5 or 6) were selected and further used for immunoblotting. The samples (15 μg for the detection of LC3 or GAPDH, 40 μg for caspases-3 and -7) were analyzed by 12.5% SDS-PAGE. The electrophoretic transfer of proteins from polyacrylamide gels to a polyvinylidenefluoride (PVDF) membrane (Immobilon-P; Millipore Co., Bedford, MA) was performed, as previously described (Koike *et al.*, 2005; Koike *et al.*, 2008). The sheets were soaked in PBS containing 0.1% Tween 20 and 5% bovine serum albumin (Sigma, St. Louis, MO) to block non-specific binding, and were then incubated overnight with anti-LC3 (1:1,000), anti-GAPDH (10 ng/ml), anti-caspase-3 (1:1,000), or anti-caspase-7 (1:1,000). The membranes were washed 3 times for 10 min in PBS containing 0.1% Tween 20 and then further incubated for 1 hour at RT with horseradish peroxidase (HRP)-labeled secondary antibody (pig anti-rabbit or goat anti-mouse IgG (DAKO, Glostrup, Denmark) diluted 1:1,000. After three 10-min washes in PBS with 0.1% Tween 20, the membranes were treated with Immobilon Western Chemiluminescent HRP Substrate (Millipore Co.) for 2 min and then observed using an LAS-3000 mini system (Fuji Photo Film, Tokyo, Japan). Immunoreactive bands were quantified using Image Gauge software (Fuji Photo Film), as described elsewhere (Zhu *et al.*, 2005). Briefly, the relative increase in LC3-II was calculated based on the optical density (OD) of the different bands in individual animals according to the following formula: OD of the ipsilateral/OD of the contralateral.

Statistical analysis

Using Kaleidagraph software (version 4.0 Mac) (Albeck Software, Reading, PA), statistical significance was analyzed via Student's *t*-test for Supplemental Figure 1P and by one-way ANOVA, as shown in Figure 4B, where all pairwise multiple comparison procedures were performed via Tukey's *post hoc* test. Data were expressed as the mean ± standard deviation (SD). We assumed statistically significant differences at $P < 0.05$.

Results

Presence of caspase-dependent and -independent neuron death in the ipsilateral hippocampus after H/I Injury

Because the hippocampus in neonatal mice is known to be vulnerable to H/I insult (Sheldon *et al.*, 2001; West *et al.*, 2006; Koike *et al.*, 2008), we sought to determine the minimal duration of hypoxia that would produce robust brain damage restricted to the hippocampus of neonatal rat brains after H/I injury. In our experience, H/I injury with a hypoxic period of 120 or 150 min, which has been defined by other studies (Ginet *et al.*, 2009; Weis *et al.*, 2014; Xu *et al.*, 2016), induced damage in the entirety of the ipsilateral hemispheres with a high mortality rate. By adjusting the duration of hypoxia, 60 min was established as the appropriate hypoxic period that would produce substantial damage mainly in the hippocampus (Uchiyama *et al.*, 2009). Therefore, the duration of H/I was performed for 60 min, and this amount of time included all the following analyses used in the present study.

The morphological and immunohistochemical changes in H/I injury-induced pyramidal neuron death were evaluated in the CA1 region of the neonatal rat hippocampus. In control neonatal rats at P7, few pyramidal neurons with pyknotic nuclei were detected in the hippocampal CA1 region (Figure 1A). As in the H/I injury model of neonatal mice (Koike *et al.*, 2008), neurodegeneration after H/I injury occurred only in the ipsilateral hippocampus, while neurons in the contralateral hippocampus appeared histologically intact and could not be distinguished from those in the untreated control brains (data not shown). As shown in toluidine blue-stained semi-thin sections, densely stained pyramidal neurons with shrunken cytoplasm were detected in the CA1 region of the ipsilateral hippocampus 3 hours after H/I injury (Figure 1B). Massive numbers of pyramidal neurons with pyknotic nuclei were detected at 8 and 24 hours after H/I injury (Figure 1C–E). At these stages, residual neurons other than neurons with pyknotic nuclei exhibited a wide variety of degenerative forms that ranged from intact to severely damaged appearances such as vacuolization (Figure 1B, C). At 72 hour after H/I injury, most pyramidal neurons possessed shrunken and fragmented nuclei, which also appeared in DAPI staining (Figure 1E, J, O). Corresponding to the morphological changes in neurons, cleaved caspase-3-positive

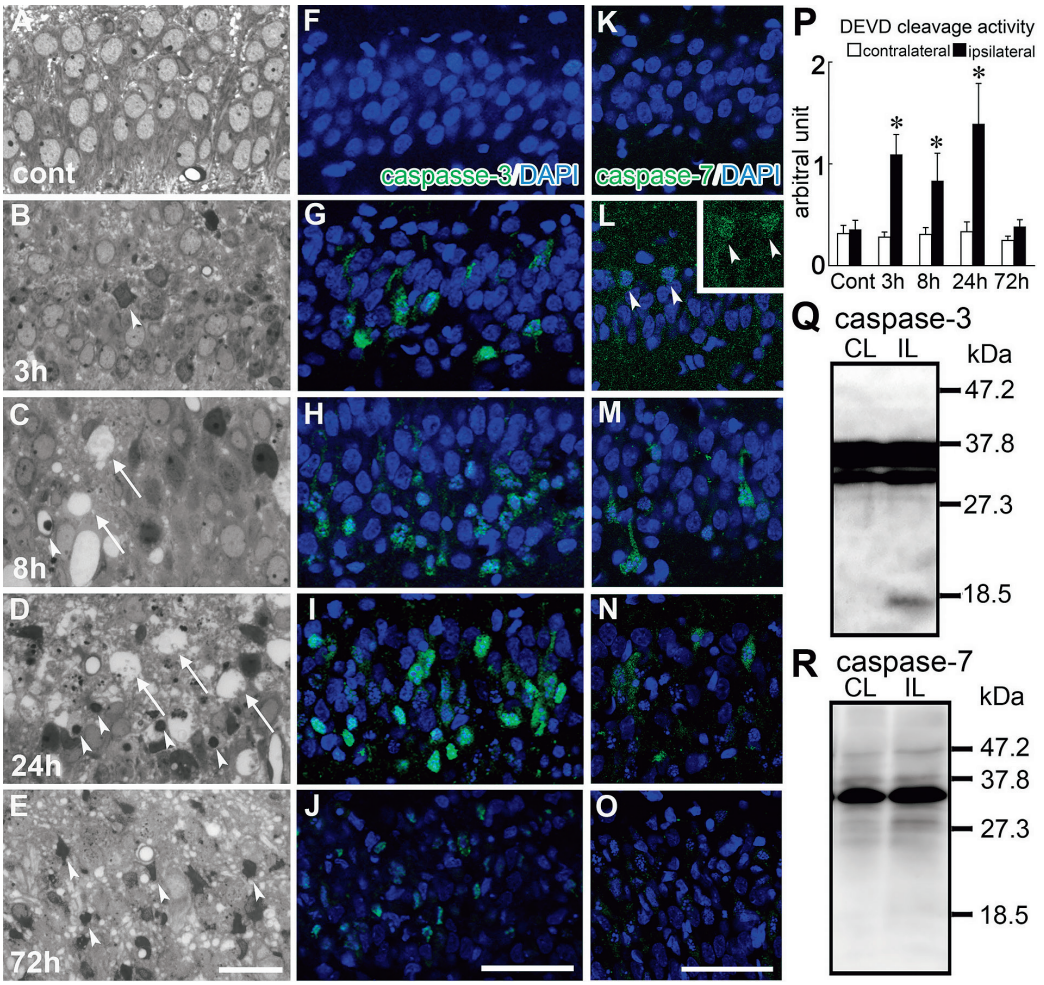

Fig. 1. Caspase-dependent and -independent pyramidal neuron death in the CA1 region of the ipsilateral hippocampus of a neonatal rat after H/I injury. (A–O) Histological sections of the CA1 pyramidal layer of the left hippocampus of an untreated control rat at P7 (A, F, K) and an ipsilateral hippocampus obtained at 3 (B, G, L), 8 (C, H, M), 24 (D, I, N) and 72 hours (h) (E, J, O) after H/I injury. (A–E) Semi-thin sections stained with toluidine blue. Arrowheads in B–E show shrunken cells or cells with pyknotic nuclei. Arrows in C and D show vacuolization due to a loss of the cytoplasm and organelles. (F–O) Immunohistochemistry for cleaved caspases-3 (F–J) and -7 (K–O) (green). Sections were counterstained with DAPI (blue). Inset in L shows neurons weakly immunopositive for cleaved caspase-7 (arrowheads). Scale bars: 50 μm. (P) Proteolytic activity of DEVDase for caspases-3 and -7 in the ipsilateral and contralateral hippocampi 3, 8, 24, and 72 after H/I injury. The activity in a non-treated hippocampus was used as a control (cont). The final value is presented as the mean ± SD for three animals. *$P < 0.001$ (Student's *t*-test) for a comparison of the values between the ipsilateral and contralateral sides. (Q, R) Western blotting of caspases-3 and -7 in the contralateral (CL) and ipsilateral (IL) hippocampi of rat brains at 24 hours following H/I injury.

pyramidal neurons in the CA1 region of the ipsilateral hippocampus first appeared in 3 hours and peaked 24 hours after H/I injury (Figure 1F–J). Cleaved caspase-7-positive CA1 pyramidal neurons were also detected in the ipsilateral hippocampus 3 to 24 hours after H/I injury, but these were much fewer in number (Figure 1K–O). Similar to the previous study on mouse neonatal H/I injury, neurons positive for cleaved caspase-3 or -7 did not always coincide with those having pyknotic nuclei in the ipsilateral CA1 pyramidal layer, indicating the presence of caspase-dependent and -independent

neuron death in this area after ischemic insult. Corresponding to the immunohistochemistry for cleaved caspase-3 or -7, DEVDase activity was increased in the ipsilateral hippocampus from 3 to 24 hours after H/I injury (Figure 1P). In Western blotting, the cleaved form of caspase-3, but not caspase-7, was clearly detected in the ipsilateral hippocampus 24 hours after H/I Injury (Figure 1Q).

Ultrastructure of dying neurons in the ipsilateral hippocampus after H/I Injury

Electron microscopic observations showed that CA1 pyramidal neurons from the control neonatal rats exhibited normal morphology among various perikaryal organelles and nuclei (Figure 2A). As in the H/I injury model of neonatal mice (Koike *et al.*, 2008), dying pyramidal neurons with typical apoptotic features were not observed in the CA1 region of the ipsilateral hippocampus after H/I injury. In the early stages after H/I insult, degenerating neurons demonstrated nuclei with small patches of clumped chromatin that often became electron dense and profiles of the neuronal cell bodies appeared shrunken (Figure 2B–D). These dying neurons were occupied by various types of vacuoles and vesicles, and some possessed double membranes such as autophagosomes (Figure 2B–E). In later stages, the dying neurons often possessed nuclei with envelopes that were broken and contained numerous autophagosomes and autolysosomes (Figure 2F). These results suggest that autophagy is induced in neuron death in the neonatal rat CA1 pyramidal layer after H/I injury.

Immunohistochemistry and Western blotting for LC3 in the ipsilateral hippocampus after H/I Injury

To further understand the involvement of autophagy in the hippocampus after H/I injury, we performed immunohistochemical and biochemical examinations of LC3, which is required for autophagy (Kabeya *et al.*, 2000). It is well known that LC3 has two forms: cytosolic (LC3-I) and membrane-bound (LC3-II). The cytosolic form is converted into the latter when autophagy is induced (Kabeya *et al.*, 2000). Double immunostaining for LC3 and DAPI revealed that ipsilateral CA1 pyramidal neurons became intensely immunopositive for LC3 in the perikarya at 3, 8 and 24 hours

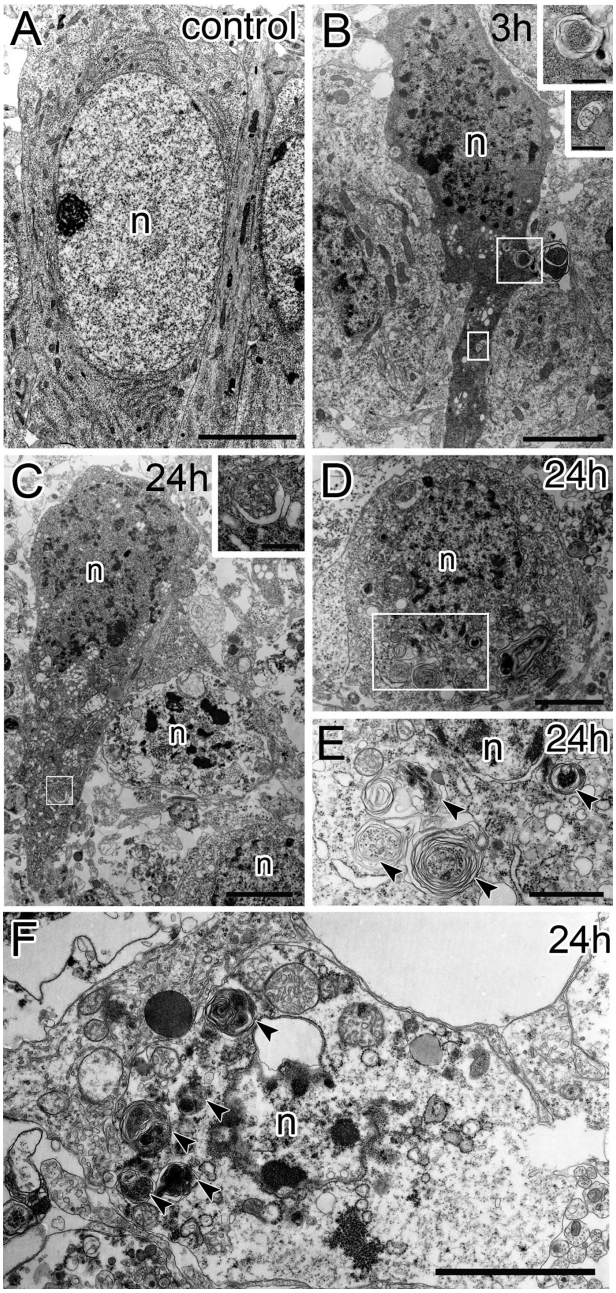

Fig. 2. Electron micrographs of CA1 pyramidal neurons in the hippocampus of an untreated control rat at P7 and in the ipsilateral hippocampus of a neonatal rat 3, 8 and 24 hours after H/I injury. Squared areas in B, C and D are enlarged and shown in the insets in B, C and E, respectively. Arrowheads indicate autophagosomes in the perikarion of the ipsilateral dying neurons showing nuclei with small patches of chromatin clumping (n). Scale bars: 5 μm (A); 3 μm (B–D); 1 μm (E, F); 0.5 μm (insets in B and C).

after H/I injury (Figure 3 A, C, E). Most neurons that showed intense and/or dot-like immunoreactivity for LC3 possessed shrunken or irregularly shaped nuclei. The CA1 pyramidal neurons in the contralateral hippocampus at the corresponding time points showed only weak and diffuse staining for LC3 in the perikarya (Figure 3 B, D, F). Western blotting showed that the protein amounts of membrane-bound LC3-II had increased in the ipsilateral neonatal rat hippocampi relative to those of the contralateral ones at 3, 8 and 24 hours after H/I injury, respectively (Figure 4A, B). These results further confirmed the induction of autophagy in the dying pyramidal neurons of the neonatal rat hippocampus after H/I injury.

Discussion

The present study used histochemical, ultrastructural and biochemical analyses to demonstrate the involvement of autophagy in the hippocampal pyramidal neuron death of neonatal rats after H/I injury. Recent studies on H/I brain injury using neonatal rats have focused on producing damage that was limited to the ipsilateral hemisphere via the use of a hypoxic period of 120 min or more (Ginet *et al.*, 2009; Liu *et al.*, 2013; Carloni *et al.*, 2014; Weis *et al.*, 2014; Demarest *et al.*, 2016; Xie *et al.*, 2016). The present study was an attempt to produce damage restricted to the ipsilateral hippocampus with a shorter hypoxic period: H/I injury produced by 60 min of hypoxia (Uchiyama *et al.*, 2009).

Neuron death in the pyramidal layer of neonatal mouse brains after H/I injury is known to occur by both caspase-dependent and -independent pathways (Zhu *et al.*, 2005; West *et al.*, 2006; Koike *et al.*, 2008). Another study, however, has shown that pyramidal neurons throughout the ipsilateral hippocampus of neonatal rat brains were immunopositive for active caspase-3 48 hours after H/I insult (Liu *et al.*, 2004). By contrast, in our neonatal rat model of H/I injury, only some of the dying neurons were positive for active caspase-3 in the CA1 pyramidal layers of the ipsilateral hippocampus at all time periods after H/I insult. Caspase-7, which is structurally and functionally similar to caspase-3 (Lakhani *et al.*, 2006), is known to compensate for the lack of caspase-3 in mouse brains (Houde *et al.*, 2004). As far as could be ascertained, only one study has shown activated caspase-7 in rat traumatic brain injury *in vivo* (Larner *et al.*, 2005). The present study is the first to demonstrate the presence of dying neurons positive for active caspase-7 following neonatal rat H/I injury, although the number of these neurons was much diminished compared with the number of active caspase-3-positive neurons. The limited population of active caspase-3- or -7-positive CA1 pyramidal neurons in the ipsilateral hippocampus following neonatal rat H/I injury reflected the presence of caspase-dependent and -independent neuron death. Based on these results, it seems likely that our neonatal rat model of H/I injury corresponds well to the neonatal mouse model used in our previous study (Koike *et al.*, 2008).

Immunohistochemical and biochemical approaches for LC3 have shown that autophagy is involved in neuron death following H/I brain injury to neonatal rats when a hypoxic period is loaded for 120 min or more (Ginet *et al.*, 2009; Liu *et al.*, 2013; Carloni *et al.*, 2014; Weis *et al.*, 2014; Demarest *et al.*, 2016; Xie *et al.*, 2016). Moreover, the induction of autophagy is known to differ according to brain region (Weis *et al.*, 2014) and dying pyramidal neurons in the hippocampal CA1 region exhibit strong apoptotic characteristics as opposed to autophagic neuron death (Ginet *et al.*, 2009). In contrast, our neonatal rat model of H/I injury used a milder hypoxic insult to induce autophagy in the dying pyramidal neurons of the ipsilateral hippocampal CA1 region, which is consistent with our previous study using neonatal mice for H/I injury (Koike *et al.*, 2008). In the present ultrastructural study, however, the early stages of dying CA1 pyramidal neurons were characterized by striking features of autophagic cell death (Clarke, 1990) such as massive vacuolization that included autophagosomes and nuclei with small patches of clumped chromatin. The morphological features of the apoptosis-necrosis continuum of dying CA1 neurons include broken nuclear membranes, nuclei with coarsely clumped chromatin, and electron-lucent cytoplasm with swollen mitochondria in the later stages (Sheldon *et al.*, 2001). The present study showed that these neurons also contain numerous autolysosomes. These results indicate that the progression of pyramidal neuron death is aligned with the maturation of the autophagic process. Considering that CA1 pyramidal neurons in the ipsilateral hippocampus become significantly positive for active caspase-3 following H/I injury to neonatal rats with prolonged hypoxia (Liu *et al.*,

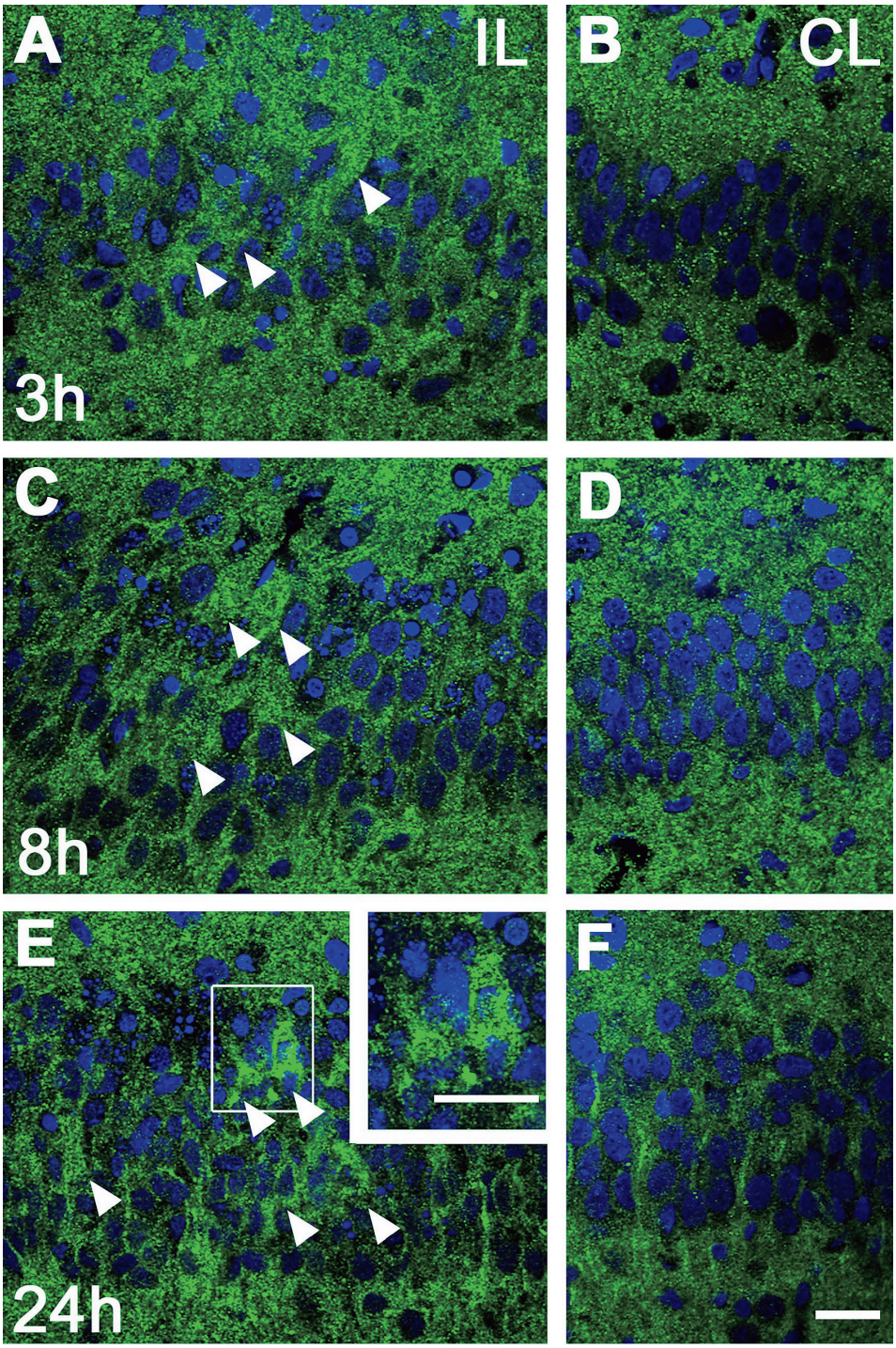

Fig. 3. Double staining of LC3 (green) and DAPI (blue) in the CA1 pyramidal layer of the ipsilateral (IL) (A, C, E) and contralateral (CL) (B, D, F) hippocampi of neonatal rat brains 3 (A, B), 8 (C, D), and 24 (E, F) hours following H/I injury. Neurons with intense granular staining for LC3 are indicated by arrowheads, and those in the boxed areas of E are enlarged and shown in the inset. Scale bars: 20 μm.

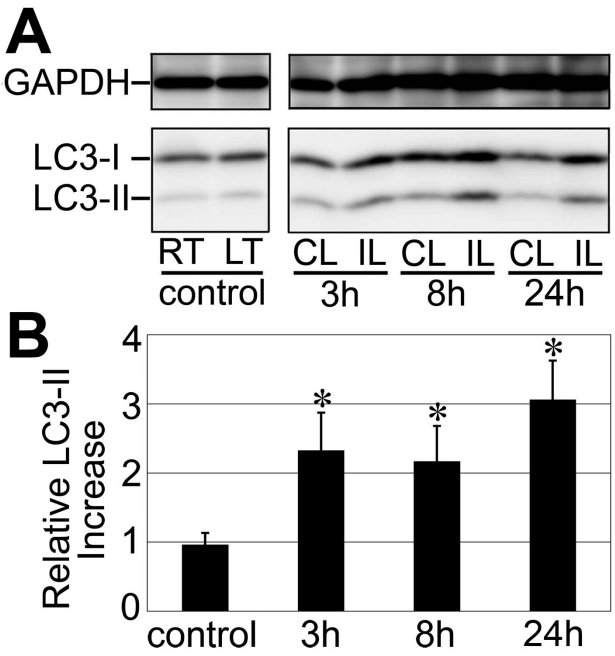

Fig. 4. (A) Western blotting of LC3 in the untreated left (LT) and right (RT) hippocampi (control) and the ipsilateral (IL) and contralateral (CL) hippocampi of neonatal rat brains at 3, 8, and 24 hours after H/I injury. Protein bands of GAPDH were used for loading controls to normalize signals from LC3. (B) Quantification of A. The ratios of the amounts of LC3-II between the ipsilateral and contralateral sides were calculated at each corresponding time. The final values represent the mean ± SD for three animals. *$P < 0.001$ (ANOVA) for a comparison of the values between the control and the experimental groups.

2004; Ginet *et al.*, 2009), it seems likely that the cell death mode after H/I injury depends on the duration of the ischemic stimuli.

Several genetic studies, including those by our group, have used brain-specific Atg7-deficient mice to provide strong evidence for the protective role that autophagy inhibition exerts in response to neonatal mouse H/I brain injury (Koike *et al.*, 2008; Uchiyama *et al.*, 2009; Xie *et al.*, 2016). Therefore, one of the important therapeutic strategies for the treatment of neonatal H/I brain injury would be pharmacological inhibition of autophagy-induced neuron death. The neonatal rat H/I brain injury model of mild ischemic insult that was used in the present study would be beneficial to any evaluation of the neuroprotective effect of chemical agents that modulate autophagy in the hippocampus.

Acknowledgements

The authors wish to thank K. Ikeue, A. Koseki and E. Oiki for their technical assistance. This work was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (16GS0315 for Y. U., M. S., and M. K.) and from the Japan Society for the Promotion of Science (JSPS), Scientific Research on Innovative Areas (23111004 for Y. U. and 15H01388 for M. K.) and the Private University Research Branding Project (M. K.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Carloni S, Albertini MC, Galluzzi L, Buonocore G, Proietti F, Balduini W: Increased autophagy reduces endoplasmic reticulum stress after neonatal hypoxia-ischemia: role of protein synthesis and autophagic pathways. *Exp Neurol* 255: 103-112 (2014).

Clark RS, Bayir H, Chu CT, Alber SM, Kochanek PM, Watkins SC: Autophagy is increased in mice after traumatic brain injury and is detectable in human brain after trauma and critical illness. *Autophagy* 4: 88-90 (2008).

Clarke PG: Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol (Berl)* 181: 195-213 (1990).

Demarest TG, Waite EL, Kristian T, Puche AC, Waddell J, McKenna MC, Fiskum G: Sex-dependent mitophagy and neuronal death following rat neonatal hypoxia-ischemia. *Neuroscience* 335: 103-113 (2016).

Ferriero DM: Neonatal brain injury. *N Engl J Med* 351: 1985-1995 (2004).

Galluzzi L, Bravo-San Pedro JM, Blomgren K, Kroemer G: Autophagy in acute brain injury. *Nat Rev Neurosci* 17: 467-484 (2016).

Ginet V, Puyal J, Clarke PG, Truttmann AC: Enhancement of autophagic flux after neonatal cerebral hypoxia-ischemia and its region-specific relationship to apoptotic mechanisms. *Am J Pathol* 175: 1962-1974 (2009).

Ginet V, Spiehlmann A, Rummel C, Rudinskiy N, Grishchuk Y, Luthi-Carter R, Clarke PG, Truttmann AC, Puyal J: Involvement of autophagy in hypoxic-excitotoxic neuronal

death. *Autophagy* 10: 846-860 (2014).
Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N: Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441: 885-889 (2006).
Houde C, Banks KG, Coulombe N, Rasper D, Grimm E, Roy S, Simpson EM, Nicholson DW: Caspase-7 expanded function and intrinsic expression level underlies strain-specific brain phenotype of caspase-3-null mice. *J Neurosci* 24: 9977-9984 (2004).
Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19: 5720-5728 (2000).
Kim M, Sandford E, Gatica D, Qiu Y, Liu X, Zheng Y, Schulman BA, Xu J, Semple I, Ro SH, Kim B, Mavioglu RN, Tolun A, Jipa A, Takats S, Karpati M, Li JZ, Yapici Z, Juhasz G, Lee JH, Klionsky DJ, Burmeister M: Mutation in ATG5 reduces autophagy and leads to ataxia with developmental delay. *Elife* 5: e12245 (2016).
Koike M, Shibata M, Ezaki J, Peters C, Saftig P, Kominami E, Uchiyama Y: Differences in expression patterns of cathepsin C/dipeptidyl peptidase I in normal, pathological and aged mouse central nervous system. *Eur J Neurosci* 37: 816-830 (2013).
Koike M, Shibata M, Ohsawa Y, Nakanishi H, Koga T, Kametaka S, Waguri S, Momoi T, Kominami E, Peters C, Figura K, Saftig P, Uchiyama Y: Involvement of two different cell death pathways in retinal atrophy of cathepsin D-deficient mice. *Mol Cell Neurosci* 22: 146-161 (2003).
Koike M, Shibata M, Tadakoshi M, Gotoh K, Komatsu M, Waguri S, Kawahara N, Kuida K, Nagata S, Kominami E, Tanaka K, Uchiyama Y: Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. *Am J Pathol* 172: 454-469 (2008).
Koike M, Shibata M, Waguri S, Yoshimura K, Tanida I, Kominami E, Gotow T, Peters C, von Figura K, Mizushima N, Saftig P, Uchiyama Y: Participation of autophagy in storage of lysosomes in neurons from mouse models of neuronal ceroid-lipofuscinoses (Batten disease). *Am J Pathol* 167: 1713-1728 (2005).
Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K: Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441: 880-884 (2006).
Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T: Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* 169: 425-434 (2005).
Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N: The role of autophagy during the early neonatal starvation period. *Nature* 432: 1032-1036 (2004).
Lakhani SA, Masud A, Kuida K, Porter GA, Jr., Booth CJ, Mehal WZ, Inayat I, Flavell RA: Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* 311: 847-851 (2006).
Larner SF, McKinsey DM, Hayes RL, KK WW: Caspase 7: increased expression and activation after traumatic brain injury in rats. *J Neurochem* 94: 97-108 (2005).
Liu CL, Siesjo BK, Hu BR: Pathogenesis of hippocampal neuronal death after hypoxia-ischemia changes during brain development. *Neuroscience* 127: 113-123 (2004).
Liu Y, Shoji-Kawata S, Sumpter RM, Jr., Wei Y, Ginet V, Zhang L, Posner B, Tran KA, Green DR, Xavier RJ, Shaw SY, Clarke PG, Puyal J, Levine B: Autosis is a Na+, K+-ATPase-regulated form of cell death triggered by autophagy-inducing peptides, starvation, and hypoxia-ischemia. *Proc Natl Acad Sci U S A* 110: 20364-20371 (2013).
Lu Z, Dono K, Gotoh K, Shibata M, Koike M, Marubashi S, Miyamoto A, Takeda Y, Nagano H, Umeshita K, Uchiyama Y, Monden M: Participation of autophagy in the degeneration process of rat hepatocytes after transplantation following prolonged cold preservation. *Arch Histol Cytol* 68: 71-80 (2005).
Mizushima N: Autophagy: process and function. *Genes Dev* 21: 2861-2873 (2007).
Mwaniki MK, Atieno M, Lawn JE, Newton CR: Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review. *Lancet* 379: 445-452 (2012).
Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibanai K, Kominami E, Uchiyama Y: Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci* 15: 1001-1011 (1995).

Nixon RA: The role of autophagy in neurodegenerative disease. *Nat Med* 19: 983-997 (2013).

Northington FJ, Chavez-Valdez R, Martin LJ: Neuronal cell death in neonatal hypoxia-ischemia. *Ann Neurol* 69: 743-758 (2011).

Rami A, Langhagen A, Steiger S: Focal cerebral ischemia induces upregulation of Beclin 1 and autophagy-like cell death. *Neurobiol Dis* 29: 132-141 (2008).

Rice JE, 3rd, Vannucci RC, Brierley JB: The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol* 9: 131-141 (1981).

Saitsu H, Nishimura T, Muramatsu K, Kodera H, Kumada S, Sugai K, Kasai-Yoshida E, Sawaura N, Nishida H, Hoshino A, Ryujin F, Yoshioka S, Nishiyama K, Kondo Y, Tsurusaki Y, Nakashima M, Miyake N, Arakawa H, Kato M, Mizushima N, Matsumoto N: De novo mutations in the autophagy gene WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood. *Nat Genet* 45: 445-449 (2013).

Shacka JJ, Lu J, Xie ZL, Uchiyama Y, Roth KA, Zhang J: Kainic acid induces early and transient autophagic stress in mouse hippocampus. *Neurosci Lett* 414: 57-60 (2007).

Sheldon RA, Hall JJ, Noble LJ, Ferriero DM: Delayed cell death in neonatal mouse hippocampus from hypoxia-ischemia is neither apoptotic nor necrotic. *Neurosci Lett* 304: 165-168 (2001).

Uchiyama Y, Koike M, Shibata M: Autophagic neuron death in neonatal brain ischemia/hypoxia. *Autophagy* 4: 404-408 (2008a).

Uchiyama Y, Koike M, Shibata M, Sasaki M: Autophagic neuron death. *Methods Enzymol* 453: 33-51 (2009).

Uchiyama Y, Shibata M, Koike M, Yoshimura K, Sasaki M: Autophagy-physiology and pathophysiology. *Histochem Cell Biol* 129: 407-420 (2008b).

Weis SN, Toniazzo AP, Ander BP, Zhan X, Careaga M, Ashwood P, Wyse AT, Netto CA, Sharp FR: Autophagy in the brain of neonates following hypoxia-ischemia shows sex- and region-specific effects. *Neuroscience* 256: 201-209 (2014).

West T, Atzeva M, Holtzman DM: Caspase-3 deficiency during development increases vulnerability to hypoxic-ischemic injury through caspase-3-independent pathways. *Neurobiol Dis* 22: 523-537 (2006).

Xie C, Ginet V, Sun Y, Koike M, Zhou K, Li T, Li H, Li Q, Wang X, Uchiyama Y, Truttmann AC, Kroemer G, Puyal J, Blomgren K, Zhu C: Neuroprotection by selective neuronal deletion of Atg7 in neonatal brain injury. *Autophagy* 12: 410-423 (2016).

Xu Y, Tian Y, Tian Y, Li X, Zhao P: Autophagy activation involved in hypoxic-ischemic brain injury induces cognitive and memory impairment in neonatal rats. *J Neurochem* 287: 32861-32873 (2016).

Zhu C, Wang X, Xu F, Bahr BA, Shibata M, Uchiyama Y, Hagberg H, Blomgren K: The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. *Cell Death Differ* 12: 162-176 (2005).

Zhu JH, Horbinski C, Guo F, Watkins S, Uchiyama Y, Chu CT: Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. *Am J Pathol* 170: 75-86 (2007).