Available online at www.sciencedirect.com
SCIENCE DIRECT
Brain Research 1037 (2005) 59–69
BRAIN RESEARCH
www.elsevier.com/locate/brainres

Research report

Calpain inhibitor MDL 28170 protects hypoxic–ischemic brain injury in neonatal rats by inhibition of both apoptosis and necrosis

Masanari Kawamura, Wako Nakajima, Akira Ishida*, Akiko Ohmura, Shinobu Miura, Goro Takada

Department of Pediatrics, Akita University School of Medicine, 1-1-1 Hondo, Akita, 010-8543, Japan

Accepted 17 December 2004

Abstract

MDL 28170 is a CNS-penetrating calpain inhibitor, and we examined the effects of MDL 28170 on hypoxic–ischemic brain injury in immature brain using the Rice-Vannucci model. Immediately after hypoxic exposure, 24 mg/kg of MDL 28170 was injected intraperitoneally as an initial dose, followed by 12 mg/kg every 4 h for a total dose of 60 mg/kg over 12 h post-HI. A vehicle control group received peanut oil injection instead. Macroscopic evaluation of brain injury revealed the neuroprotective effect of MDL 28170 after 12 h post-HI. Neuropathological quantitative analysis of cell death showed that MDL 28170 significantly decreased the number of necrotic cells in all the examined regions except for cingular cortex, and the number of apoptotic cells in caudate putamen, parietal cortex, hippocampus CA1, and laterodorsal thalamus. Western blots showed that MDL 28170 suppressed 145/150 kDa subunits of α-spectrin breakdown products (SBDP) in cortex, hippocampus, thalamus, and striatum, and also 120-kDa subunit of SBDP in all regions except for striatum. This suggests that MDL 28170 inhibited activation of calpain and caspase-3, respectively.

Our results indicate that post-hypoxic MDL 28170 injection is neuroprotective in HI newborn rat brain by decreasing both necrosis and apoptosis. SBDP expression also suggests that MDL 28170 injection inhibits both calpain and caspase-3 activation after HI insult.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: Immature brain; Excitotoxicity; Calpain; Caspase-3; Neuronal cell death

1. Introduction

Calpain (EC 3.4.22.17) is a calcium-activated, neutral, cytosolic cysteine protease [32] and is proposed to participate in the turnover of cytoskeletal proteins and regulation of kinases, transcription factors, and receptors [11,54]. Over the past decade, several investigators have focused on calpain-mediated proteolysis and its contribution to necrotic neuronal death in ischemic and excitotoxic neuronal injury [63]. Many studies indicate that both apoptotic and necrotic mechanisms account for neuronal death after cerebral hypoxic ischemia (HI) in different neonatal animal models [12,24,29,41,45,62], and electron microscopy also confirms that there is a morphological continuum from apoptosis to necrosis [40]. Although calpain activation is historically assumed to result in necrotic cell death [4,51,53], contribution to apoptosis has been suggested in vivo [31,39,44,55].

Calpain activation has been demonstrated in adult [17,37,47,50] and neonatal models [7] of hypoxia and ischemia. A massive release of glutamate occurring in the brain soon after ischemia causes additional cell death [48]. Elevated glutamate triggers a large influx of calcium into neurons [14], which precipitates an intracellular cascade of events including activation of calcium-dependent proteases such as calpains [42,59]. Once activated, calpains cleave

* Corresponding author. Fax: +81 18 836 2620.
E-mail address: aishida@doc.med.akita-u.ac.jp (A. Ishida).

0006-8993/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2004.12.050

structural and regulatory proteins such as αII-spectrin, βII-spectrin, and calpastatin in the cell, leading to neuronal death.

Inhibition of calpains should be effective in neonatal brain considering the high calpain content in the rapidly growing brain [6] and upregulation of calpain activity following HI [36]. Although apoptosis is more prominent as a mode of cell death after HI in neonates compared with the adult brain [5,22,41,52], necrosis predominates in almost all regions in neonatal rat model [15,56] except for cingular cortex, retrosplenial cortex, and thalamus [30,34]. Although Blomgren et al. showed that a calpain inhibitor CX295 decreased αII-spectrin degradation in vivo neonatal brain [8], the protective effect of calpain inhibitor on HI in immature brain has not vigorously studied so far. Here we used a calpain inhibitor MDL 28170 (carbobenzylzoxy-Val-Phe-H) because of its ability of penetrating the brain rapidly [26,27]. Moreover, MDL 28170 reduced infarct volume even when administered 6 h after the initiation of ischemia in the focal cerebral ischemia model in adult rats [26]. We hypothesized that MDL 28170 would protect neonatal brain after HI and inhibit both apoptosis and necrosis. We evaluated the mode of cell death using light microscopy, TUNEL staining, and electron microscopy. Different subunits of αII-spectrin break down products, i.e., 145/150 kDa and 120 kDa proteins were also detected by Western blots to measure calpain and caspase-3 activation, respectively.

2. Materials and methods

2.1. Unilateral cerebral hypoxic–ischemia model

All animals were treated in a manner that complied with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences in the Physiological Society of Japan and all experiments were performed in accordance with approved institutional animal care guidelines of Akita University School of Medicine. Wistar rats were purchased from Japan SLC (Shizuoka, Japan). Unilateral (right) cerebral hypoxic ischemia in the postnatal day (PND) 7 was based on the Rice-Vannucci model as previously reported [46,61]. In brief, PND7 rat pups were placed in a temperature-controlled incubator set to an ambient temperature 35 °C. Under anesthesia with inhaled diethyl ether, the right common carotid artery was isolated, double-ligated, and cut between the ligatures. After the surgical procedure, the animals recovered for 90 min in the temperature-controlled incubator and then were exposed to an 8% oxygen hypoxia. If an animal died before the 90 min of hypoxic period, hypoxic exposure was stopped at that time.

2.2. In vivo calpain inhibition

Preliminary experiments were done to decide the drug administration protocol. To know the toxic total amount of MDL 28170, each 80 mg/kg, 160 mg/kg, and 240 mg/kg in total was administered, referring that the optimal and lethal dose for adult rabbit when given by intravenous injection was 30 mg/kg and 60 mg/kg, respectively [33]. The total dose was divided and given intraperitoneally every 4 h until 24 h post-HI according to the half-life of MDL 28170 [26]. The pups given both 160 and 240 mg/kg MDL 28170 seized 12 h after the first injection. Next, we decided to use peanut oil as a vehicle because it was safely used for newborn rat pups [19]. Either a mixed solution of polyethylene glycol (PEG) and ethanol [33] or dimethyl sulfoxide [20] that was previously used as the vehicle prevented dams from feeding pups probably due to their specific smells. To decide the drug administration protocol, we prepared 5 groups; i.e., vehicle control group, each 60 mg/kg of MDL 28170 and 80 mg/kg up to 12 h, and each 120 mg/kg and 160 mg/kg to 24 h. The macroscopic brain injury in the right hemisphere was scored at 48 h post-HI using our rating scale (see Section 2.5) and each score was 2.8 ± 0.5, 1.4 ± 0.4, 2.3 ± 1.3, 1.9 ± 1.2, and 2.1 ± 0.6 (n = 5 in each group), therefore we adopted 60 mg/kg in total amount up to 12 h. We also confirmed that 24 mg/kg of MDL 28170 injection (the loading dose when we administered 60 mg/kg in total amount to 12 h) did not bring non-specific drug-induced hypothermia for 2 h after injection (MDL 28170 group, 36.7 ± 0.2 °C vs. vehicle control group, 36.6 ± 0.2 °C, n = 5 in each group).

From these results, we decided the protocol as follows: immediately after the end of hypoxic exposure, the first dose, 24 mg/kg of MDL 28170, was injected intraperitoneally. Pups subsequently received 12 mg/kg of MDL 28170 every 4 h up to 12 h post-HI. MDL 28170 was diluted with peanut oil, so the injection volume was 0.05 ml per animal. Equal volume of peanut oil was injected in vehicle control group.

2.3. Tissue preparation

Pups were sacrificed at 0, 6, 12, 24, and 48 h after hypoxic exposure for Western blotting (n = 6 per time point) and histological analysis (n = 6 per time point). For Western blotting sample, we separated a hemisphere to cortex, striatum, thalamus, and hippocampus. The tissue samples were homogenized in lysis buffer and centrifuged at 23,500 × g for 60 min at 4 °C. The supernatant was decanted and stored at −70 °C until analysis. For histological study, pups were exsanguinated with PBS, then perfused and fixed with paraformaldehyde. Brains were embedded in paraffin, sectioned at a thickness of 2.5 μm, and processed for H and E, Nissl stain, and TUNEL as described previously [61]. To confirm the finding of light microscopy, additional 4 brains were processed for electron microscopy. Twenty-four and 48 h after HI, pups (n = 2 at each time point) in the vehicle control group were anesthetized with ether and perfused with 70 ml of PBS followed by 150 ml of 1% paraformaldehyde and 1.25% glutaraldehyde (GA) in 0.1 M PBS (pH 7.4). After perfusion, brains were removed and

postfixed overnight in 3% GA-PBS. Samples of cortex, hippocampus, and thalamus from right hemispheres were microdissected from each rat, postfixed for 2 h in 1% osmium tetroxide, washed with 0.2 M sodium cacodylate, dehydrated in ethanol, and embedded in epoxy resin.

2.4. In situ labeling of 3′-OH DNA ends (ApopTag)

3′-OH ends of DNA fragments were detected using the ApopTag peroxydase kit from Intergren (Norcross, GA) [30], and for antigen retrieval, we adopted pretreatment methods [21,61]. In brief, sections were deparaffinized and dehydrated in descending alcohol series, immersed in sodium citrate solution for 30 min and maintained at 80 °C in a water-bath, and incubated in a proteinase K solution for 15 min at room temperature. Then, sections were blotted and incubated with mixture of TdT enzyme and digoxigenin-tagged dUTP. The reaction products were visualized by diaminobenzidine and the sections were counterstained with methyl green.

2.5. Macroscopic evaluation of neuroprotective effect of MDL 28170

We evaluated brain injury macroscopically in all pups except for 4 brains in vehicle control group for EM before starting brain sampling (n = 12 per time point). We used the rating scale for injury as previously reported [61]. The rating scale was decided using the appearance of both hemispheres as follows: 0, no difference between the right hemisphere (ipsilateral to ligation) and left one; 1, because of edema, the right hemisphere is larger than the left one, without infarction; 2, infarction in the right hemisphere does not exceed the middle vertical line in the same hemisphere; 3, infarction in the right hemisphere exceeds the middle vertical line in the same hemisphere; 4, the whole right hemisphere is melted. To compare brain injury in the MDL 28170 injection group with the vehicle control group, analysis of variance (ANOVA) was used following a post hoc test. Data are expressed as mean ± SEM.

2.6. Quantitative neuropathological analysis of cell death

Quantitative neuropathological analysis of degenerating cells was performed using a modified method as previously reported [61]. In brief, two investigators blinded to the experimental protocol counted apoptotic and necrotic cells in caudate putamen, globus pallidus, cerebral cortex (frontal, cingulated, parietal, and retrosplenial areas), hippocampus (CA1, CA3), dentate gyrus, laterodorsal thalamus, and subiculum. The sections were selected from three levels: the anterior striatum, and the middle and posterior dorsal level of hippocampus. Each three levels was carefully matched between MDL 28170 injection group and vehicle control group. The criteria for apoptotic and necrotic cells [25,60] were adjusted between two raters in advance. Apoptotic cells were identified using the following morphological criteria; (a) intense, uniform nuclear basophilia and (b) fragmentation of the nucleus into several round and uniformly dense basophilic masses. Necrotic cells were identified by intense cytoplasmic eosinophilia accompanied by nuclear chromatin dispersion with loss of nuclear membrane integrity. Both pyknotic cells and hybrid cells that show large irregular chromatin clumps without cell membrane integrity were excluded from this analysis. To compare cell count that shows neurodegeneration in MDL 28170 injection group with vehicle control group, analysis of variance (ANOVA) was used following a post hoc test. Data are expressed as mean ± SEM.

2.7. Electron microscopy

Semithin (1 μm) sections were stained with toluidine blue and screened. An area of interest was selected and ultra thin sections were cut and placed on single-hole grids. After staining with uranyl acetate and lead citrate, the sections were examined using a JEM-1200 EX transmission electron microscope (JEOL, Japan).

2.8. Western blotting of antibody to α-spectrin (α-fodrin)

Protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin as standard, and then equal amounts of total protein (30 μg) were loaded in each lane. The Ponceau stain was used to confirm the equal amount of protein loaded. After SDS–PAGE using 7.5% Ready-gel J (Nippon BioRad Lab, Tokyo, Japan), proteins were transferred to a nitrocellulose membrane by use of Towbin transfer buffer. Blots were blocked with 5% non-fat milk with 0.1% Tween 20 in 50 mM Tris-buffered saline and probed a primary antibody for α-spectrin (FG 6090; Affiniti Research Products, UK) diluted 1:500. Immunoblots were processed with horseradish peroxidase-conjugated anti-mouse IgG; bound antibodies were detected using a chemiluminescence reagent kit (NEL 103; NEN Life Science products, Boston, MA). Proteins from human EB virus infected lymphatic corpuscles damaged by overnight starvation as positive controls of apoptotic proteins.

3. Results

3.1. Temporal pattern of macroscopic brain injury

We used 6 pups at each time point for histological analysis (group 1) and Western blotting (group 2), respectively. We evaluated brain injury macroscopically in both groups before starting brain sampling (n = 12 per time point). The scores showing brain injury in the MDL 28170 group were significantly lower from 12 to 48 h post-HI compared with vehicle controls (12 h post-HI, 0.17 ± 0.071 vs. 0.86 ± 0.20 points, $P < 0.01$; 24 h post-HI, 0.21 ±

0.096 vs. 1.7 ± 0.29 points; 48 h post-HI, 0.63 ± 0.21 vs. 2.6 ± 0.26 points, $P < 0.005$) (Fig. 1). Mortality rate of pups that underwent HI was up to 11% [group 1, 7 of 67 (10.4%); group 2, 8 of 76 (10.5%)].

3.2. Temporal and regional patterns of degenerating cells

TUNEL-stained sections show neuronal degeneration including not only apoptosis but also necrosis [15], therefore we used TUNEL-stained sections at low magnification to evaluate the distribution of degenerating cells. Before 24 h post-HI, we could hardly detect TUNEL-positive cells in cortex, hippocampus, thalamus, and in striatum in both vehicle control group and MDL 28170 groups. After 24 h post-HI, the number of TUNEL-positive cells seemed to increase in all the 4 regions in vehicle controls (Figs. 2A, B, E, and F), whereas almost no TUNEL-positive cells in MDL 28170 group (Figs. 2C, D, G, and H).

Although TUNEL identified DNA fragmentation in both apoptotic and necrotic cells, the morphology differed between both types of dying cells. Therefore, we could tell whether the TUNEL-positive cells were apoptotic or necrotic at high magnification [15]. In the vehicle control group, there was a tendency that necrotic cells were dominant over apoptotic cells in cortex and striatum. In contrast, apoptotic cells were easily found in the hippocampus in vehicle controls. Although necrotic cells that showed diffusely stained chromatin (Figs. 3A, D, G, and J) were predominant, apoptotic cells with round, darkly stained chromatin of nuclei (Figs. 3B, E, H, and K) were also observed in cortex and hippocampus at 24 and 48 h post-HI. Of note, pyknotic cells, which were excluded from cell counting, were remarkable in CA1 and dentate gyrus. In the MDL 28170 group, we hardly detected degenerating cells at high magnification (Figs. 3C, F, I, and L).

3.3. Ultrastructure of degenerating neurons

In the cortex at 48 h post-HI, the necrotic cells were predominantly distributed contrasting the small number of apoptotic cells (Fig. 4A). The light microscopic features of apoptosis including regularly shaped, large round chromatin clumps, condensation of cytoplasm, and intact nuclear and cytoplasmic membrane were confirmed in all 3 regions by electron microscopy (Figs. 4C and D). Necrotic cells showing small and irregularly shaped chromatin clumps were broadly observed in the cortex and hippocampus at 48 h post-HI (Fig. 4E).

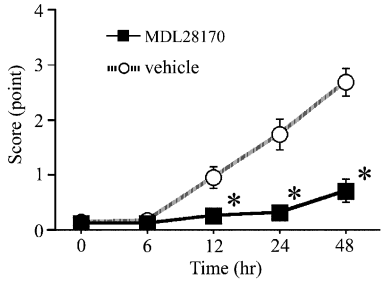


Fig. 1. Temporal pattern of macroscopic brain injury. The rating scale for injury from zero to four was decided by macroscopic observation of the appearance of the right hemisphere compared with the left one. The brain injury in MDL28170 injection group scored significantly lower than in vehicle control group from 12 h to 48 h post-HI. Difference of $P < 0.05$ (asterisk) was considered significant. Data are expressed as mean ± SEM.

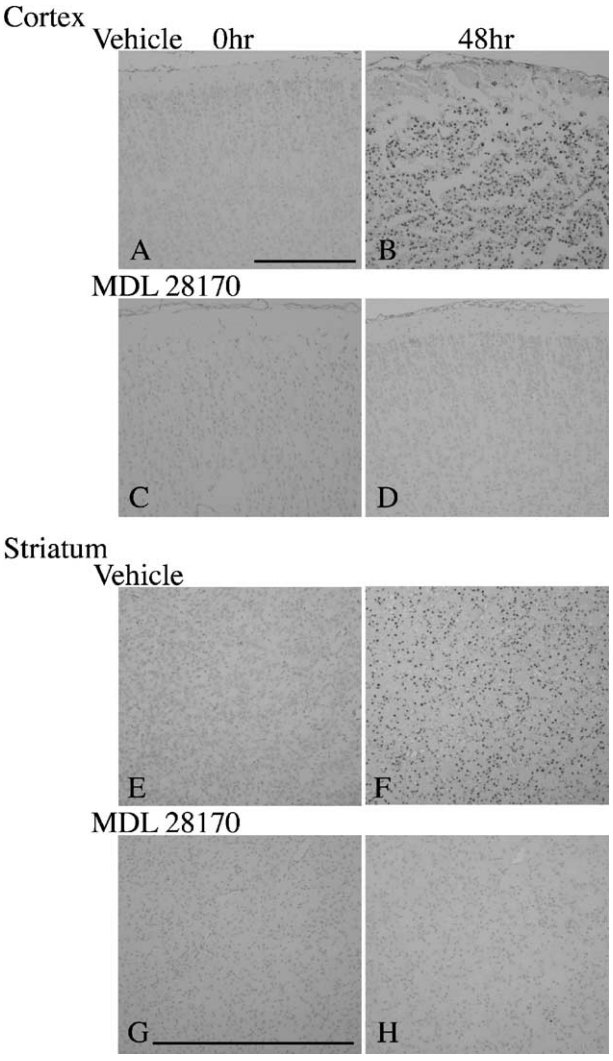


Fig. 2. Temporal pattern of brain damage after hypoxic ischemia in neonatal rats. TUNEL-stained sections at low magnification at 0 and 48 h post-HI in vehicle control group (A, B, E, and F) and in MDL 28170 injection group (C, D, G, and H). TUNEL-positive cells were not detected at 0 h post-HI in any groups in either cortex (A and C) or striatum (E and G). Although the MDL 28170 injection group showed few TUNEL-positive cells even at 48 h post-HI, a large number of TUNEL-positive cells were observed in the vehicle control group in both cortex and striatum (B and F). Scale bar: 50 μm.

3.4. Quantitative neuropathological analysis of degenerating cells

Two raters blinded to the experimental protocol counted apoptotic and necrotic cells using H and E-stained sections.

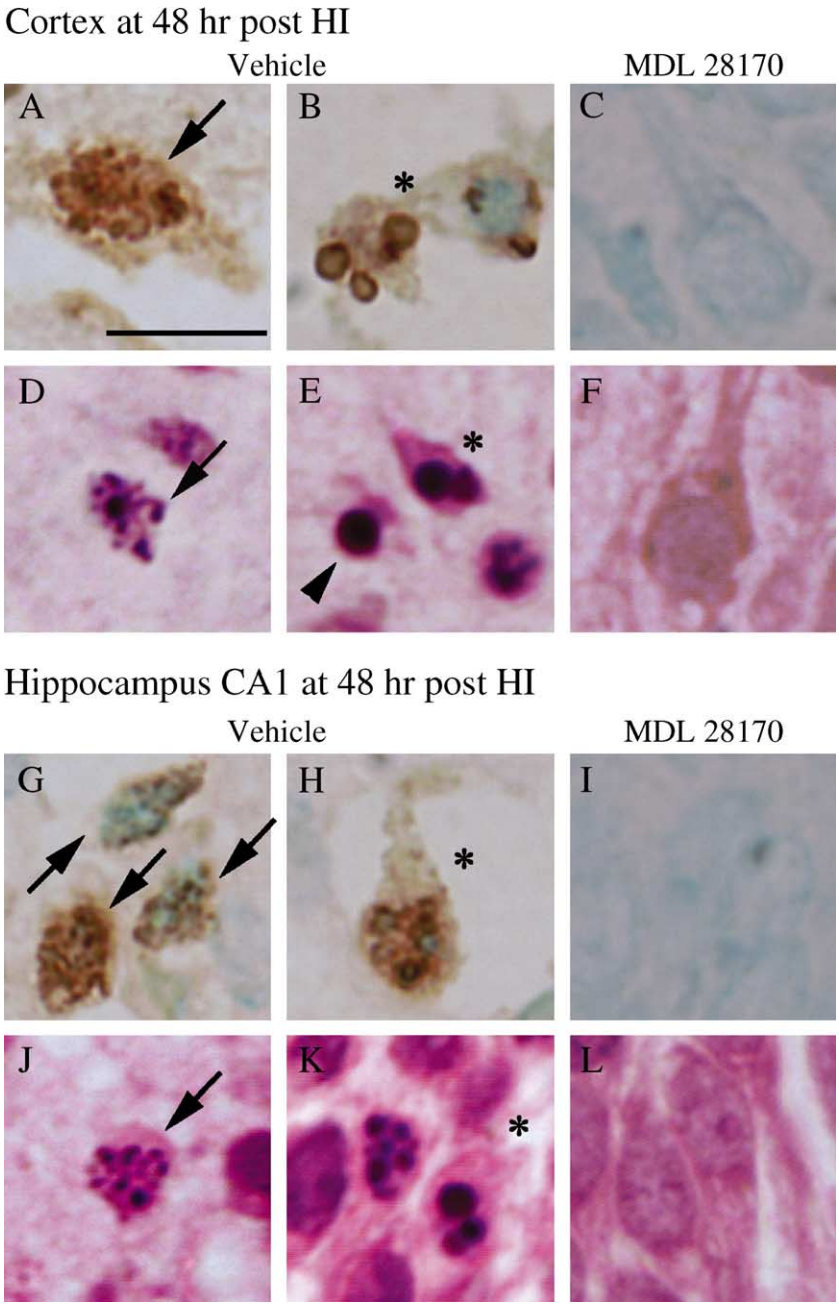


Fig. 3. High magnification images of degenerating neurons at 48 h post-HI in the right hemispheres in the cortex (A–F) and hippocampus (G–L). TUNEL-stained sections (A–C and G–I) and H and E-stained sections (D–F and J–L) are shown. In the vehicle control group, TUNEL-positive cells which represent a feature of necrotic cell (arrow) in cortex (A) and hippocampus (G) show irregularly, diffusely stained small chromatin clumps, contrasting with apoptotic morphology (asterisk) that shows large, round, darkly stained chromatin of nuclei (B and H). Necrotic cells (D and J) and apoptotic cells (E and K) are also shown in H and E-stained sections. MDL 28170 injection inhibited both necrosis and apoptosis. Normal cells are shown as TUNEL-negative cells (C and I) and normal morphology in H and E-stained sections (F and L). Scale bar: 10 μm.

Fig. 5 shows the averaged number of the two raters in the 11 brain regions examined at 0–48 h post-HI. In vehicle controls, the number of necrotic cells increased dramatically after 24 h post-HI. The absolute number of necrotic cells in frontal cortex, parietal cortex, and caudate putamen after 24 h post-HI was also larger than that in other regions. In the cingular cortex, retrosplenial cortex, CA1 in hippocampus, laterodorsal thalamus, and in subiculum, the number of apoptotic cells was relatively larger compared with that of necrotic cells in vehicle controls. This finding agreed with previous reports [30,46,56,61]. MDL 28170 injection significantly decreased the number of necrotic cells in all the examined regions except for cingular cortex. In addition, the number of apoptotic cells in the MDL 28170 group was significantly smaller compared with the vehicle controls in the following regions; parietal cortex at

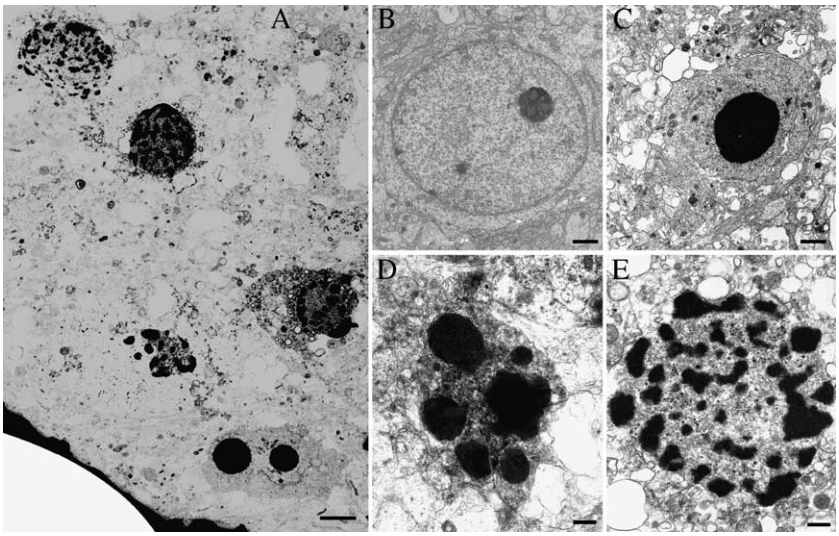
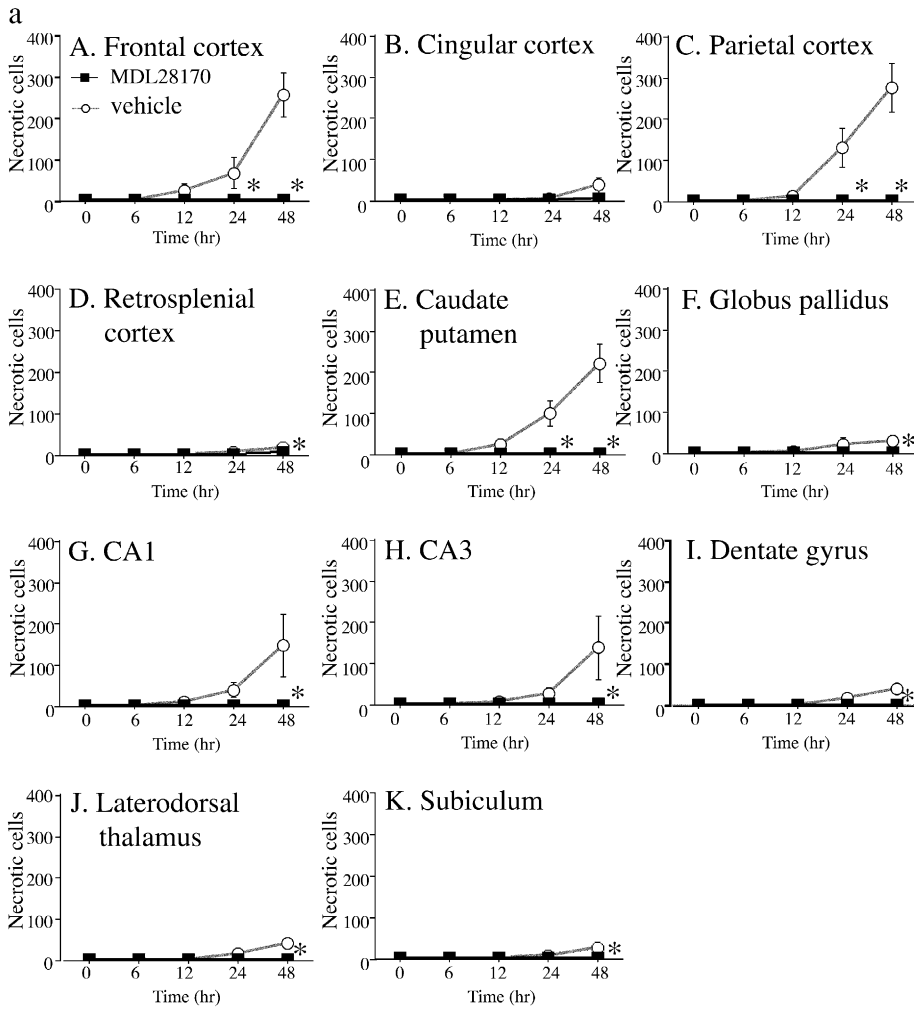


Fig. 4. Ultrastructure of degenerating neurons in cortex at 24 h post-HI (B and C) and 48 h post-HI (A, D, and E) in the vehicle control group. Necrosis was the predominant form of neurodegeneration at 48 h post-HI (A). Apoptotic cells were infrequently observed in contrast with the large number of necrotic cells (A). Normal neuron is shown in B for reference. The nucleus of neuron at a middle stage of apoptosis (C) shows round chromatin clump surrounded by an intact nuclear envelope. Plasma membrane integrity was preserved. The condensed, shrunken cell at more advanced stage of apoptosis loses plasma membrane integrity (D). Necrotic neuron shows condensation of chromatin into numerous, irregular small clumps (E). Scale bars: A, 2 μm; D and E, 500 nm; B and C, 1 μm.



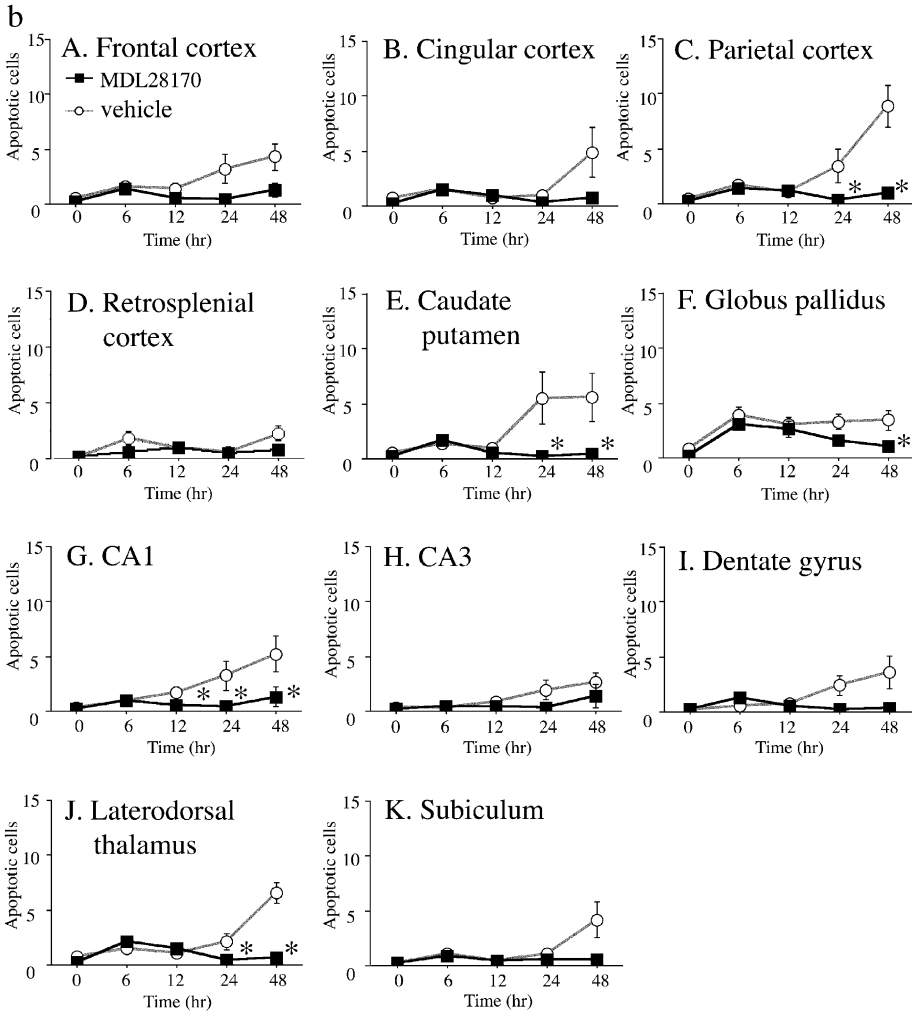


Fig. 5. Quantitative analysis of regional and temporal patterns of necrotic (a) and apoptotic cells (b). Closed square shows the MDL28170 group and open circle denotes the vehicle control group. Necrotic cells (a) and apoptotic cells (b) were counted in each brain region from 0 h to 48 h post-HI. Ten fields in each region were evaluated and 500 nuclei were counted in H and E-stained sections. The number of necrotic cells was almost null at 0 h and 6 h, after 24 h those increased over 100 in frontal cortex, parietal cortex, and caudate putamen. At 48 h post-HI, the number of necrotic cells was significantly smaller in the MDL28170 injection group than in the vehicle control group in all regions except for cingular cortex (a). MDL28170 significantly inhibited the number of apoptotic cells in the parietal cortex at 24 h and 48 h post-HI (b. C), in caudate putamen at 24 h and 48 h (b. E), in hippocampus CA1 (b. G) from 12 h to 48 h post HI, and laterodorsal thalamus (b. J) at 24 h and 48 h post-HI. Difference of $P < 0.05$ (asterisk) was considered significant. Data are expressed as mean ± SEM.

24 h and 48 h post HI, caudate putamen at 24 h and 48 h, hippocampus CA1 from 12 h to 48 h, and laterodorsal thalamus at 24 h and 48 h. It was notable that there was a tendency that the number of both necrotic and apoptotic cells was smaller in the MDL 28170 group even at 48 h post-HI in all regions.

3.5. Western blot analysis of αII-spectrin breakdown products

Nonerythroid αII-spectrin is a cytoskeletal protein that is broken down by caspases and calpains during cell death [59]; cleavage by either of these enzymes yields different-sized products. Full-length αII-spectrin migrates as a 240-kDa peptide on Western blots. When it is cleaved by caspase-3, the products are 150- and 120-kDa peptides, but when cleaved by calpain, the products are 150 and 145 kDa [32,38,59]. This αII-spectrin breakdown pattern is regarded as a marker for both caspase and calpain activation in neuronal death.

Fig. 6 demonstrates temporal changes of cleavage of αII-spectrin. In vehicle controls, both SBDP145/150 kDa doublet and SBDP120 kDa appeared after 6 h post-HI in cortex, hippocampus, and thalamus (Figs. 6A, B, and D). MDL 28170 injection seemed to suppress 145/150 kDa subunits after 6 h post-HI till 48 h in cortex (Fig. 6A), whereas MDL 28170 seemed to suppress 145/150 kDa subunit at 48 h post-HI in the other 3 regions. In contrast, in striatum in vehicle controls, 145/150 kDa subunits were observed at 6 and 48 h post-HI but the 120-kDa subunit was not detected throughout the experiment (Fig. 6C), which was different SBDP expression from other 3 regions (Figs. 6A, B, and D). It was remarkable that MDL 28170 seemed to inhibit the 120-kDa subunit even

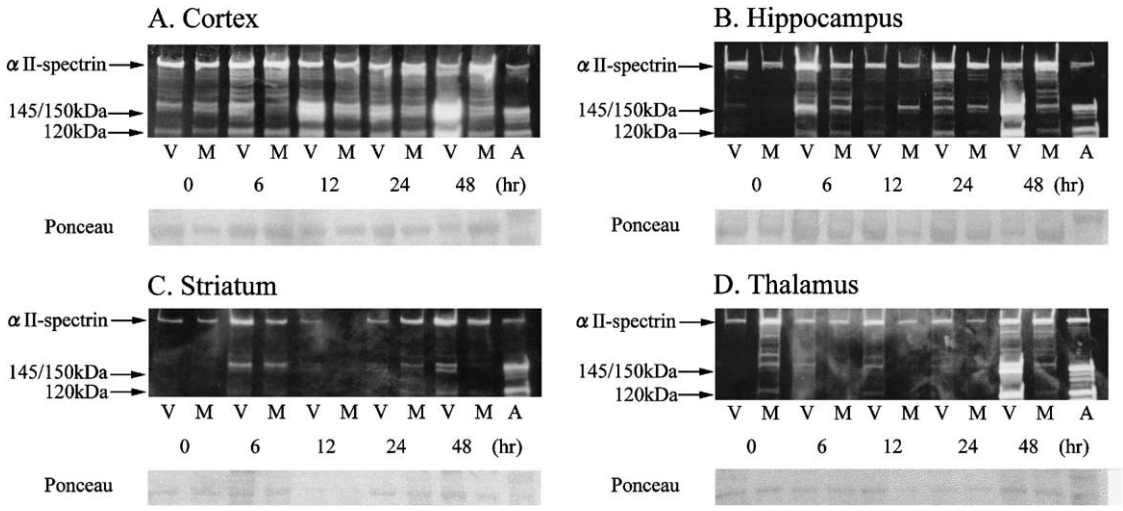


Fig. 6. Western blots of spectrin proteolysis in cortex, hippocampus, striatum, and thalamus (A–D) (Lane V, vehicle control group; Lane M, MDL 28170 injection group; Lane A, proteins from human EB-virus infected lymphatic corpuscles damaged by overnight starvation). Proteins from apoptotic lymphoblast show intense bands of 145/150 kDa doublet and 120 kDa (Lane A). In the vehicle control group, both SBDP145/150 kDa doublet and SBDP120 kDa appeared after 6 h post-HI in cortex, hippocampus, and thalamus (A, B, and D). In the striatum, SBDP145/150 kDa doublet was observed, but the 120-kDa subunit was not detected till 48 h post-HI (C). MDL 28170 injection seemed to suppress 145/150 kDa subunits after 6 h post-HI in the cortex (A). In other 3 regions (B–D), MDL 28170 seemed to suppress 145/150 kDa subunit at 48 h post-HI. MDL 28170 also seemed to inhibit the 120-kDa subunit at 48 h post-HI in all regions except for the striatum (A, B, and D). Ponceau shows that the equal amount of protein was applied to each lane.

at 48 h post-HI in all regions except for striatum (Figs. 6A, B, and D).

4. Discussion

This study is the first to our knowledge to show the neuroprotective effect of MDL 28170 in the neonatal HI rat model. MDL 28170 significantly reduced the score rating macroscopic brain injury from 12 h to 48 h post-HI compared with vehicle controls. Quantitative neuropathological analysis showed that the number of necrotic cells in all examined brain regions decreased except for cingular cortex. Suppression of SBDP145/150 kDa doublet by MDL 28170 in cortex, hippocampus, thalamus, and striatum at 48 h post-HI also suggests that MDL 28170 inhibited calpain activation causing necrotic cell death. Moreover, the number of apoptotic cells in the MDL 28170 group was significantly smaller in parietal cortex, caudate putamen, CA1 in hippocampus, laterodorsal thalamus compared with vehicle controls by neuropathological quantification. Western blots supported the finding by suppression of SBDP 120 kDa, which reflects apoptotic pathway, in cortex, hippocampus, and thalamus at 48 h post-HI. In the present study, we only used SBDP expression for detecting cell degeneration but did not quantify spectrin cleavage.

Calpain-mediated proteolysis is a feature of some adult animal models of neurodegenerative disease; i.e., a model of motor nerve terminal injury [35], focal cerebral ischemia [17], and head trauma [49]. Administration of a calpain inhibitor zVF to adult cerebral ischemia model decreased cerebral infarction volume at 72 h post-ischemia and SBDP150 kDa at both 0.5 and 1 h post-ischemia [17]. Bartus et al. also showed that a calpain inhibitor AK 295 decreased infarct volume at 21 h post-ischemia even when administered at 1.25 h in adult rat model [2]. On the other hand, Alzheimer's disease model mice treated by a calpain inhibitor E64 [3] showed improvement of synaptic and cognitive function evaluated 5 months after initiation of drug administration. In terms of calpain inhibitors, calpain inhibitor I and II, E 64 and leupeptin cannot cross cell walls or likely the blood–brain barrier [27]. Therefore, AK 295, AK 275, and CX 295 that are oxoamide inhibitor molecules were developed [2]. These calpain inhibitors possess sufficient potency, solubility, and membrane permeability, and neuroprotective effect has shown in adult cerebral ischemia models [2], trauma models [49]. MDL 28170 rapidly penetrates cells and blood–brain barrier [27] and acts as a competitive inhibitor of calpains [28]. The half-life of activity is approximately 2 h and maximal activity was obtained at 30 min after a single intravenous bolus injection when administered in adult rat [26]. It has been observed that MDL 28170 is effective in inhibiting the cell degeneration in vitro [1,9,10,16,43] as well as in vivo [26]. Due to the technical difficulty of measuring the activation state of calpains accurately in vivo, experiments designed to examine calpain activity often rely on measurements of the breakdown products of a calpain's preferred substrates, spectrin [32,54,59]. Spectrin breakdown products are the most widely used marker of calpain and caspase activity. It has previously shown that necrosis leads to calpain-mediated SBDP150/145 kDa doublet whereas apoptosis produces the calpain-mediated fragments as well as caspase-3-mediated SBDP150 kDa and SBDP120 kDa [32,59].

The pattern of brain injury after HI in newborn rats in the present study was similar to previous reports [46,56,57]. The

cortex, striatum, CA1 hippocampus, and thalamus were particularly sensitive to HI exposure. In our study and in other ischemia models, necrosis predominated in the ischemic core, whereas apoptosis occurred primarily in the penumbra [12,13,23]. Our electron microscopic finding at 24 h and 48 h post-HI confirmed that necrosis was the predominant form of cell death although apoptosis contributed to neuronal injury after HI. The quantitative neuropathological analysis of cell death showed that MDL 28170 decreased the number of necrotic cells at 48 h post-HI in all regions except for cingular cortex, which is a region that necrosis was hardly detected. Inhibition of SBDP145/150 kDa doublet at 48 h post-HI in examined 4 regions supported that MDL 28170 suppressed the necrotic pathway. On the other hand, the quantification showed that the number of apoptotic cells was significantly smaller in parietal cortex, CA1 hippocampus, caudate putamen, and laterodorsal thalamus at 48 h, and this was consistent with inhibition of SBDP 120 kDa in MDL 28170 group in cortex, hippocampus, and thalamus. Although MDL 28170 decreased the number of apoptotic cells after 24 h post-HI in striatum, inhibition of SBDP 120 kDa was not shown. Moreover, in the striatum, SBDP 120 kDa was hardly detected throughout the experiment in both groups. Because striatum is a region where necrosis is superior to apoptosis, the amount of apoptotic cell would be too small to detect apoptotic change in the striatal tissue homogenates. It would also be the case in regions that show different results between pathologic scores and Western blots.

Our study is the first report to show the expression of SBDP 120 kDa subunits after HI insult in vivo condition. The MDL 28170 is neuroprotective when administered after hypoxic exposure in newborn rats by decreasing not only necrosis but also apoptosis. The short half-life of MDL 28170 would have made it difficult to use for small animals because of technical difficulty in getting line for continuous injection; however, for human infant, this cannot be problematic. In the past decade, involvement of apoptosis after HI attracted attention, but still necrosis is the major form of cell death after HI insult in newborn brain. Although necrosis has been assumed to irreversible form of cell death [25], morphological and biochemical evidence has accumulated showing continuum of cell death [18,30,40]. In addition, the cell death mode switch from necrosis to apoptosis in brain has proposed in vitro using cultured cortical neuron [58]. Taken together, our results are important to show the possibility that we could intervene in necrosis as well as in apoptosis after HI insult.

Acknowledgments

We thank Drs. K. Kawamura and S. Chida for technical assistance in electron microscopic study and Dr. M.V. Johnston for critical reading of the manuscript.

References

[1] L. Arlinghaus, S. Mehdi, K.S. Lee, Improved posthypoxic recovery with a membrane-permeable calpain inhibitor, Eur. J. Pharmacol. 209 (1991) 123–125.
[2] R.T. Bartus, N.J. Hayward, P.J. Elliott, S.D. Sawyer, K.L. Baker, R.L. Dean, A. Akiyama, J.A. Straub, S.L. Harbeson, Z. Li, et al., Calpain inhibitor AK295 protects neurons from focal brain ischemia. Effects of postocclusion intra-arterial administration, Stroke 25 (1994) 2265–2270.
[3] F. Battaglia, F. Trinchese, S. Liu, S. Walter, R.A. Nixon, O. Arancio, Calpain inhibitors, a treatment for Alzheimer's disease: position paper, J. Mol. Neurosci. 20 (2003) 357–362.
[4] E. Bednarski, P. Vanderklish, C. Gall, T.C. Saido, B.A. Bahr, G. Lynch, Translational suppression of calpain I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices, Brain Res. 694 (1995) 147–157.
[5] E.J. Beilharz, C.E. Williams, M. Dragunow, E.S. Sirimanne, P.D. Gluckman, Mechanisms of delayed cell death following hypoxic–ischemic injury in the immature rat: evidence for apoptosis during selective neuronal loss, Brain Res. Mol. Brain Res. 29 (1995) 1–14.
[6] K. Blomgren, J.O. Karlsson, Developmental changes of calpain and calpastatin in rabbit brain, Neurochem. Res. 14 (1989) 1149–1152.
[7] K. Blomgren, S. Kawashima, T.C. Saido, J.O. Karlsson, A. Elmered, H. Hagberg, Fodrin degradation and subcellular distribution of calpains after neonatal rat cerebral hypoxic–ischemia, Brain Res. 684 (1995) 143–149.
[8] K. Blomgren, C. Zhu, X. Wang, J.O. Karlsson, A.L. Leverin, B.A. Bahr, C. Mallard, H. Hagberg, Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia–ischemia: a mechanism of "pathological apoptosis?" J. Biol. Chem. 276 (2001) 10191–10198.
[9] J.R. Brorson, C.J. Marcuccilli, R.J. Miller, Delayed antagonism of calpain reduces excitotoxicity in cultured neurons, Stroke 26 (1995) 1259–1266 (discussion 1267).
[10] H. Caner, J.L. Collins, S.M. Harris, N.F. Kassell, K.S. Lee, Attenuation of AMPA-induced neurotoxicity by a calpain inhibitor, Brain Res. 607 (1993) 354–356.
[11] S.L. Chan, M.P. Mattson, Caspase and calpain substrates: roles in synaptic plasticity and cell death, J. Neurosci. Res. 58 (1999) 167–190.
[12] C. Charriaut-Marlangue, I. Margaill, A. Represa, T. Popovici, M. Plotkine, Y. Ben-Ari, Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis, J. Cereb. Blood Flow Metab. 16 (1996) 186–194.
[13] M. Chopp, Y. Li, Apoptosis in focal cerebral ischemia, Acta Neurochir., Suppl. (Wien) 66 (1996) 21–26.
[14] D.E. Croall, G.N. DeMartino, Calcium-activated neutral protease (calpain) system: structure, function, and regulation, Physiol. Rev. 71 (1991) 813–847.
[15] C. de Torres, F. Munell, I. Ferrer, J. Reventos, A. Macaya, Identification of necrotic cell death by the TUNEL assay in the hypoxic–ischemic neonatal rat brain, Neurosci. Lett. 230 (1997) 1–4.
[16] K. Hiramatsu, N.F. Kassell, K.S. Lee, Improved posthypoxic recovery of synaptic transmission in gerbil neocortical slices treated with a calpain inhibitor, Stroke 24 (1993) 1725–1728.
[17] S.C. Hong, Y. Goto, G. Lanzino, S. Soleau, N.F. Kassell, K.S. Lee, Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia, Stroke 25 (1994) 663–669.
[18] S.T. Hou, J.P. MacManus, Molecular mechanisms of cerebral ischemia-induced neuronal death, 93–148. Write to the Help Desk NCBI|NLM|NIH Department of Health and Human Services Privacy Statement|Freedom of Information Act|Disclaimer.
[19] A. Ishida, W.H. Trescher, M.S. Lange, M.V. Johnston, Prolonged suppression of brain nitric oxide synthase activity by 7-nitroindazole

protects against cerebral hypoxic–ischemic injury in neonatal rat, Brain Dev. 23 (2001) 349–354.
[20] Q. Jiang, P.K. Stys, Calpain inhibitors confer biochemical, but not electrophysiological, protection against anoxia in rat optic nerves, J. Neurochem. 74 (2000) 2101–2107.
[21] Y. Jiao, Z. Sun, T. Lee, F.R. Fusco, T.D. Kimble, C.A. Meade, S. Cuthbertson, A. Reiner, A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections, J. Neurosci. Methods 93 (1999) 149–162.
[22] M.V. Johnston, Excitotoxicity in neonatal hypoxia, Ment. Retard. Dev. Disabil. Res. Rev. 7 (2001) 229–234.
[23] Y. Li, M. Chopp, C. Powers, N. Jiang, Apoptosis and protein expression after focal cerebral ischemia in rat, Brain Res. 765 (1997) 301–312.
[24] A. Macaya, Apoptosis in the nervous system, Rev. Neurol. 24 (1996) 1356–1360.
[25] G. Majno, I. Joris, Apoptosis, oncosis, and necrosis. An overview of cell death, Am. J. Pathol. 146 (1995) 3–15.
[26] C.G. Markgraf, N.L. Velayo, M.P. Johnson, D.R. McCarty, S. Medhi, J.R. Koehl, P.A. Chmielewski, M.D. Linnik, Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats, Stroke 29 (1998) 152–158.
[27] S. Mehdi, Cell-penetrating inhibitors of calpain, Trends Biochem. Sci. 16 (1991) 150–153.
[28] S. Mehdi, M.R. Angelastro, J.S. Wiseman, P. Bey, Inhibition of the proteolysis of rat erythrocyte membrane proteins by a synthetic inhibitor of calpain, Biochem. Biophys. Res. Commun. 157 (1988) 1117–1123.
[29] H. Mehmet, X. Yue, M.V. Squier, A. Lorek, E. Cady, J. Penrice, C. Sarraf, M. Wylezinska, V. Kirkbride, C. Cooper, et al., Increased apoptosis in the cingulate sulcus of newborn piglets following transient hypoxia–ischaemia is related to the degree of high energy phosphate depletion during the insult, Neurosci. Lett. 181 (1994) 121–125.
[30] W. Nakajima, A. Ishida, M.S. Lange, K.L. Gabrielson, M.A. Wilson, L.J. Martin, M.E. Blue, M.V. Johnston, Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat, J. Neurosci. 20 (2000) 7994–8004.
[31] R. Nath, K.J. Raser, K. McGinnis, R. Nadimpalli, D. Stafford, K.K. Wang, Effects of ICE-like protease and calpain inhibitors on neuronal apoptosis, NeuroReport 8 (1996) 249–255.
[32] R. Nath, K.J. Raser, D. Stafford, I. Hajimohammadreza, A. Posner, H. Allen, R.V. Talanian, P. Yuen, R.B. Gilbertsen, K.K. Wang, Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis, Biochem. J. 319 (Pt. 3) (1996) 683–690.
[33] R.W. Neumar, D.J. DeGracia, L.L. Konkoly, J.I. Khoury, B.C. White, G.S. Krause, Calpain mediates eukaryotic initiation factor 4G degradation during global brain ischemia, J. Cereb. Blood Flow Metab. 18 (1998) 876–881.
[34] F.J. Northington, D.M. Ferriero, L.J. Martin, Neurodegeneration in the thalamus following neonatal hypoxia–ischemia is programmed cell death, Dev. Neurosci. 23 (2001) 186–191.
[35] G.M. O'Hanlon, P.D. Humphreys, R.S. Goldman, S.K. Halstead, R.W. Bullens, J.J. Plomp, Y. Ushkaryov, H.J. Willison, Calpain inhibitors protect against axonal degeneration in a model of anti-ganglioside antibody-mediated motor nerve terminal injury, Brain 126 (2003) 2497–2509.
[36] K. Ostwald, H. Hagberg, P. Andine, J.O. Karlsson, Upregulation of calpain activity in neonatal rat brain after hypoxic–ischemia, Brain Res. 630 (1993) 289–294.
[37] K. Ostwald, M. Hayashi, M. Nakamura, S. Kawashima, Subcellular distribution of calpain and calpastatin immunoreactivity and fodrin proteolysis in rabbit hippocampus after hypoxia and glucocorticoid treatment, J. Neurochem. 63 (1994) 1069–1076.
[38] B.R. Pike, X. Zhao, J.K. Newcomb, R.M. Posmantur, K.K. Wang, R.L. Hayes, Regional calpain and caspase-3 proteolysis of alpha-spectrin after traumatic brain injury, NeuroReport 9 (1998) 2437–2442.
[39] B.R. Pike, X. Zhao, J.K. Newcomb, K.K. Wang, R.M. Posmantur, R.L. Hayes, Temporal relationships between de novo protein synthesis, calpain and caspase 3-like protease activation, and DNA fragmentation during apoptosis in septo-hippocampal cultures, J. Neurosci. Res. 52 (1998) 505–520.
[40] C. Portera-Cailliau, D.L. Price, L.J. Martin, Excitotoxic neuronal death in the immature brain is an apoptosis–necrosis morphological continuum, J. Comp. Neurol. 378 (1997) 70–87.
[41] M.R. Pulera, L.M. Adams, H. Liu, D.G. Santos, R.N. Nishimura, F. Yang, G.M. Cole, C.G. Wasterlain, Apoptosis in a neonatal rat model of cerebral hypoxia–ischemia, Stroke 29 (1998) 2622–2630.
[42] A. Rami, Ischemic neuronal death in the rat hippocampus: the calpain–calpastatin–caspase hypothesis, Neurobiol. Dis. 13 (2003) 75–88.
[43] A. Rami, D. Ferger, J. Krieglstein, Blockade of calpain proteolytic activity rescues neurons from glutamate excitotoxicity, Neurosci. Res. 27 (1997) 93–97.
[44] S.K. Ray, G.G. Wilford, D.C. Matzelle, E.L. Hogan, N.L. Banik, Calpeptin and methylprednisolone inhibit apoptosis in rat spinal cord injury, Ann. N. Y. Acad. Sci. 890 (1999) 261–269.
[45] S. Renolleau, D. Aggoun-Zouaoui, Y. Ben-Ari, C. Charriaut-Marlangue, A model of transient unilateral focal ischemia with reperfusion in the P7 neonatal rat: morphological changes indicative of apoptosis, Stroke 29 (1998) 1454–1460 (discussion 1461).
[46] J.E.d. Rice, R.C. Vannucci, J.B. Brierley, The influence of immaturity on hypoxic–ischemic brain damage in the rat, Ann. Neurol. 9 (1981) 131–141.
[47] J.M. Roberts-Lewis, M.J. Savage, V.R. Marcy, L.R. Pinsker, R. Siman, Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain, J. Neurosci. 14 (1994) 3934–3944.
[48] S.M. Rothman, J.W. Olney, Glutamate and the pathophysiology of hypoxic–ischemic brain damage, Ann. Neurol. 19 (1986) 105–111.
[49] K.E. Saatman, H. Murai, R.T. Bartus, D.H. Smith, N.J. Hayward, B.R. Perri, T.K. McIntosh, Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 3428–3433.
[50] T.C. Saido, M. Yokota, S. Nagao, I. Yamaura, E. Tani, T. Tsuchiya, K. Suzuki, S. Kawashima, Spatial resolution of fodrin proteolysis in postischemic brain, J. Biol. Chem. 268 (1993) 25239–25243.
[51] P. Seubert, K. Lee, G. Lynch, Ischemia triggers NMDA receptor-linked cytoskeletal proteolysis in hippocampus, Brain Res. 492 (1989) 366–370.
[52] R.S. Sidhu, U.I. Tuor, M.R. Del Bigio, Nuclear condensation and fragmentation following cerebral hypoxia–ischemia occurs more frequently in immature than older rats, Neurosci Lett. 223 (1997) 129–132.
[53] R. Siman, J.C. Noszek, Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo, Neuron 1 (1988) 279–287.
[54] H. Sorimachi, S. Ishiura, K. Suzuki, Structure and physiological function of calpains, Biochem. J. 328 (Pt. 3) (1997) 721–732.
[55] M.K. Squier, A.C. Miller, A.M. Malkinson, J.J. Cohen, Calpain activation in apoptosis, J. Cell. Physiol. 159 (1994) 229–237.
[56] J. Towfighi, N. Zec, J. Yager, C. Housman, R.C. Vannucci, Temporal evolution of neuropathologic changes in an immature rat model of cerebral hypoxia: a light microscopic study, Acta Neuropathol. (Berl.) 90 (1995) 375–386.
[57] W.H. Trescher, S. Ishiwa, M.V. Johnston, Brief post-hypoxic–ischemic hypothermia markedly delays neonatal brain injury, Brain Dev. 19 (1997) 326–338.
[58] H. Ueda, R. Fujita, Cell death mode switch from necrosis to apoptosis in brain, Biol. Pharm. Bull. 27 (2004) 950–955.
[59] K.K. Wang, Calpain and caspase: can you tell the difference? Trends Neurosci. 23 (2000) 20–26.

[60] A.H. Wyllie, J.F. Kerr, A.R. Currie, Cell death: the significance of apoptosis, Int. Rev. Cytol. 68 (1980) 251–306.
[61] N. Yasuoka, W. Nakajima, A. Ishida, G. Takada, Neuroprotection of edaravone on hypoxic–ischemic brain injury in neonatal rats, Brain Res. Dev. Brain Res. 151 (2004) 129–139.
[62] X. Yue, H. Mehmet, J. Penrice, C. Cooper, E. Cady, J.S. Wyatt, E.O. Reynolds, A.D. Edwards, M.V. Squier, Apoptosis and necrosis in the newborn piglet brain following transient cerebral hypoxia–ischaemia, Neuropathol. Appl. Neurobiol. 23 (1997) 16–25.
[63] P. Yuen, K.K. Wang, Calpain inhibitors: novel neuroprotectants and potential anticataract agents, Drugs Future 23 (1998) 741–749.