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PO Box 117  
221 00 Lund  
+46 46-222 00 00

# Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome *c* and caspase-3 activation in neurons exposed to transient hypoglycemia

Michel Ferrand-Drake,<sup>\*,†</sup> Changlian Zhu,<sup>‡</sup> Gunilla Gidö,<sup>\*</sup> Anker J. Hansen,<sup>§</sup> Jan-Olof Karlsson,<sup>¶</sup> Ben A. Bahr,<sup>\*\*</sup> Naoufal Zamzami,<sup>††</sup> Guido Kroemer,<sup>††</sup> Pak H. Chan,<sup>†</sup> Tadeusz Wieloch<sup>\*</sup> and Klas Blomgren<sup>‡,§,§§</sup>

<sup>\*</sup>Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University Hospital, Lund, Sweden

<sup>†</sup>Department of Neurosurgery, Department of Neurology and Neurological Sciences and Program in Neurosciences, Stanford University School of Medicine, Stanford, California, USA

<sup>‡</sup>Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China

<sup>§</sup>Department of Pharmacology, Novo Nordisk A/S Maaloev, Denmark

<sup>¶</sup>Institute of Anatomy and Cell Biology, Göteborg University, Göteborg, Sweden

<sup>\*\*</sup>Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, Connecticut, USA

<sup>††</sup>Institut Gustave Roussy, Villejuif, France

<sup>‡‡</sup>Perinatal Center, Department of Physiology, Göteborg University, Göteborg, Sweden

<sup>§§</sup>Perinatal Center, Department of Pediatrics, Göteborg University, The Queen Silvia Children's Hospital, Göteborg, Sweden

## Abstract

Blockade of mitochondrial permeability transition protects against hypoglycemic brain damage. To study the mechanisms downstream from mitochondria that may cause neuronal death, we investigated the effects of cyclosporin A on subcellular localization of apoptosis-inducing factor and cytochrome *c*, activation of the cysteine proteases calpain and caspase-3, as well as its effect on brain extracellular calcium concentrations. Redistribution of cytochrome *c* occurred at 30 min of iso-electricity, whereas translocation of apoptosis-inducing factor to nuclei occurred at 30 min of recovery following 30 min of iso-electricity. Active caspase-3 and calpain-induced fodrin breakdown products were barely detectable in the dentate gyrus and CA1 region of the hippocampus of rat brain exposed to 30 or 60 min of insulin-induced hypoglycemia. However, 30 min or 3 h after recovery of blood

glucose levels, fodrin breakdown products and active caspase-3 markedly increased, concomitant with a twofold increase in caspase-3-like enzymatic activity. When rats were treated with neuroprotective doses of cyclosporin A, but not with FK 506, the redistribution of apoptosis-inducing factor and cytochrome *c* was reduced and fodrin breakdown products and active caspase-3 immuno-reactivity was diminished whereas the extracellular calcium concentration was unaffected. We conclude that hypoglycemia leads to mitochondrial permeability transition which, upon recovery of energy metabolism, mediates the activation of caspase-3 and calpains, promoting cell death.

**Keywords:** apoptosis-inducing factor, calpain, caspase-3, hypoglycemia mitochondrial permeability transition.

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Address correspondence and reprint requests to Michel Ferrand-Drake, Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University Hospital, 221 85, Lund, Sweden. E-mail: michelf@stanford.edu

*Abbreviations used:* Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin; AIF, apoptosis-inducing factor; CsA, cyclosporin A; DG, dentate gyrus; FBDP, fodrin breakdown product; MPT, mitochondrial permeability transition; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

Mitochondria are key regulators in the process of cell death through their capacity to release a number of pro-apoptotic proteins from their intermembrane space, such as cytochrome *c* and apoptosis-inducing factor (AIF) (Liu *et al.* 1996; Susin *et al.* 1996). One mechanism of mitochondrial membrane permeabilization involves opening of the  $\text{Ca}^{2+}$ -stimulated, cyclosporin A (CsA)-inhibitable mitochondrial permeability transition (MPT) pore (Haworth and Hunter 1980; Crompton *et al.* 1988; Broekemeier *et al.* 1989; Crompton and Costi 1990; Nicolli *et al.* 1996). During MPT, mitochondria undergo large amplitude swelling, the outer membrane disrupts and proteins residing in the intermembrane space are released (Jacotot *et al.* 1999). Release of cytochrome *c* from the mitochondria to the cytosol and subsequent complex formation among cytochrome *c*, dATP, apoptosis-activating factor-1 and procaspase-9 induces autolytic cleavage of caspase-9, which activates caspase-3 (Li *et al.* 1997). It has been confirmed that AIF can mediate neuronal cell death (Braun *et al.* 2002; Cregan *et al.* 2002; Klein *et al.* 2002; Yu *et al.* 2002; Zhang *et al.* 2002). Unlike cytochrome *c*, AIF acts in a caspase-independent manner (Susin *et al.* 1999).

Caspases have been identified as executors of apoptosis (Nicholson *et al.* 1995). Caspase-3 is the major downstream effector caspase in the brain, particularly in the immature brain (Blomgren *et al.* 2001). Calpains, another family of cysteine proteases, are calcium activated and proposed to participate in intracellular signal transduction (Saido *et al.* 1994). Calpains have mainly been implicated in excitotoxic neuronal injury and necrosis (Wang 2000). Pharmacological inhibition of calpains (Lee *et al.* 1991; Rami and Kriegstein 1993; Bartus *et al.* 1994; Hong *et al.* 1994) or caspases (Hara *et al.* 1997; Cheng *et al.* 1998; Endres *et al.* 1998) provides neuroprotection following cerebral ischemia. Calpains are strictly calcium dependent but the activity is tightly regulated by cofactors, such as calpastatin and phospholipids (Huang and Wang 2001). Furthermore, interactions between calpains and caspases have been demonstrated. Calpains have been shown to promote activation of caspase-3 (Nakagawa and Yuan 2000; Blomgren *et al.* 2001) and caspase-3 can cleave the endogenous calpain inhibitor, calpastatin (Porn-Ares *et al.* 1998; Wang *et al.* 1998; Blomgren *et al.* 1999).

Insulin-induced hypoglycemia, which constitutes an ever-present threat in the management of type I diabetes, leads to an axon-sparing dendro-somatic lesion, causing selective neurodegeneration of subpopulations in the brain (Auer *et al.* 1984b). Glutamate has been implicated in this process (Wieloch 1985). During the insult, the ionic gradients can no longer be maintained, leading to influx of  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$ ,  $\text{Cl}^{-}$  and  $\text{H}_2\text{O}$  (Siesjo and Bengtsson 1989), resulting in dendritic swelling concurrent with mitochondrial swelling (Auer *et al.* 1985a). We have demonstrated that mitochondrial swelling could be prevented by CsA, providing neuroprotection

(Friberg *et al.* 1998). This suggests that mitochondria-dependent cell death processes are activated during hypoglycemia.

We hypothesized that influx of calcium and release of apoptogenic proteins through MPT would lead to activation of calpains and caspase-3 during and following hypoglycemia. Our aim was to characterize the protease activation and evaluate the effects of MPT inhibition by CsA.

## Materials and methods

### Surgical procedures

The hypoglycemia model described by Auer *et al.* (1984a) was used. For a detailed description of the surgical procedures, see Friberg *et al.* (1998). The ethical committee at the University of Lund approved all the animal experiments. Adult male Wistar rats from Møllegaard avlslaboratorium (Copenhagen, Denmark), weighing 300–340 g, were used. The animals were fasted overnight with access to water. Concomitant with the start of surgery, the rats received an i.p. injection of insulin (2 IU/kg; Actrapid; Novo A/S, Copenhagen, Denmark). Two s.c. biparietal needle electrodes were used to record a bipolar electro-encephalogram, which was monitored continuously. Isoelectric was defined as the time point when the electro-encephalogram turned isoelectric. At the onset of cerebral isoelectricity the blood pressure increased, which was compensated for by exsanguination, keeping the blood pressure between 140 and 160 mmHg. A bolus injection of 0.2 mL of 10% glucose solution, followed by infusion at a rate of 1.5 mL/h, terminated the period of isoelectricity. When the animals recovered spontaneous respiration, they were extubated and transferred to a cage with access to food and water.

### Extracellular $\text{Ca}^{2+}$ measurement

Double-barreled glass microelectrodes with a tip diameter of 5–7  $\mu\text{m}$  were used for recording extracellular  $\text{Ca}^{2+}$  concentration and DC potential shift. One barrel was filled with calcium ionophore I (Cocktail A 21048; Fluka AG, Buchs, Switzerland) and 100 mM  $\text{CaCl}_2$  and the other with 150 mM NaCl. Each barrel was connected with Ag-AgCl wires to a high input resistance amplifier. An Ag-AgCl wire was inserted s.c. into the neck for connection of the animal to the ground. The calcium electrode was calibrated in solutions containing 0.1, 0.5, 1.0 and 5.0 mM  $\text{CaCl}_2$  before and after the experiment (Hansen and Zeuthen 1981; Gido *et al.* 1994). The animal was placed in a stereotaxic instrument and a burr hole was made in the parietal cortex at the following coordinates: bregma  $-3.6$ , lateral 0.9 mm for electrode placement into the dentate gyrus (DG). The microelectrode was placed at the brain surface and lowered 3.6 mm to reach the tip of the DG. Extracellular  $\text{Ca}^{2+}$  concentrations and DC potential shifts were recorded during 30 min of isoelectricity, followed by 15 min of recovery in the animals treated with CsA ( $n = 6$ ) or vehicle ( $n = 4$ ).

### Immunohistochemistry

Animals were deeply anesthetized and perfusion fixed, following 30 or 60 min of isoelectricity or following 30 min of isoelectricity plus 30 min or 3 h of recovery, with 4% paraformaldehyde in 0.1 M

phosphate-buffered saline (PBS). The brains were rapidly removed and immersion fixed at 4°C overnight. After dehydration with graded ethanol and xylene, the brains were paraffin embedded and cut into 5-µm sections which were deparaffinized in xylene and rehydrated in graded ethanol before staining. All secondary antibodies were from Vector Laboratories (Burlingame, CA, USA). Each experimental group consisted of at least three animals.

### Active caspase-3

Sections were pre-treated with proteinase K (Roche Diagnostics, Indianapolis, IN, USA), 10 µg/mL in PBS, for 10 min at ambient temperature. Antigen recovery was performed by boiling the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min and subsequent cooling for 30 min. Non-specific binding was blocked for 30 min with 4% goat serum in PBS. Anti-active caspase-3 (no. 280, against the p17 fragment, residues 176–277; a kind gift from Dr Donald W. Nicholson, Merck Frosst Center for Therapeutic Research, Quebec, Canada) was applied diluted 1 : 500 in PBS and incubated for 60 min at room temperature, followed by biotinylated goat anti-rabbit IgG (6 µg/mL in PBS) for 60 min. Endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> in PBS for 5 min. Visualization was performed using the VECTASTAIN ABC Elite kit (Vector Laboratories) with 0.5 mg/mL 3,3'-diaminobenzidine enhanced with 15 mg/mL ammonium nickel sulfate, 2 mg/mL beta-D glucose, 0.4 mg/mL ammonium chloride and 0.01 mg/mL beta-glucose oxidase (Sigma, St Louis, MO, USA). Negative controls, where the primary antibody was omitted, were completely blank. Pre-absorption of the primary antibody with an excess recombinant active caspase-3 (MBL, Nagoya, Japan) also yielded blank stainings. The active caspase-3 used for pre-absorption was not a pure preparation, but a mixture of proteins, and the molar concentration of caspase-3 was unknown. However, the total protein content was known and, calculating as if all the protein was actually active caspase-3, we used a molar ratio of 200 : 1 for pre-absorption. Hence, the surplus of caspase-3 to antibody was less than 200-fold but the precise ratio is unknown.

### Apoptosis-inducing-factor

Antigen recovery and blocking were performed as above. Anti-AIF (Susin *et al.* 1999) was applied diluted 1 : 150 in Tris-buffered saline (TBS) containing 1% bovine serum albumin and 0.1% Triton X-100 and incubated for 60 min at room temperature, followed by a biotinylated goat anti-rabbit antibody (6 µg/mL in PBS) for 60 min. Peroxidase blocking and visualization were performed as above. Negative controls, where the primary antibody was omitted, were completely blank. The staining obtained with this AIF antibody and the D-20 antibody (2 µg/mL, sc-9416; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was also virtually indistinguishable and pre-absorption with the AIF peptide abolished staining.

### Calpain-specific fodrin breakdown product

Antigen recovery and blocking were performed as above. The anti-fodrin breakdown product (FBDP) (Bahr *et al.* 1995) was applied diluted 1 : 50 in PBS containing 0.2% Triton X-100 and incubated for 60 min at room temperature, followed by biotinylated goat anti-rabbit IgG (11 µg/mL in PBS) for 60 min. Peroxidase blocking and visualization were the same as above. Negative controls, where the primary antibody was omitted or where the primary antibody was

incubated for 1 h at room temperature with a 20-fold excess of the peptide used to raise the antibody, were completely blank.

### Cytochrome c

Antigen recovery was performed as above. Non-specific binding was blocked with 4% horse serum in PBS for 30 min. Anti-cytochrome *c* (clone 7H8.2C12; BD Pharmingen, San Diego, CA, USA), diluted 1 : 500 (2 µg/mL) in PBS, was incubated for 60 min at room temperature, followed by 60 min with a biotinylated horse anti-mouse antibody (2 µg/mL) diluted with PBS. Peroxidase blocking and visualization were the same as above. When the primary antibody was incubated with a 20-fold molar excess of cytochrome *c* from bovine heart (Sigma), the staining was abolished.

### Preparation of samples for measurement of caspase-3 activity or western blotting

Animals were decapitated and the brains (*n* = 5) were quickly removed and frozen on dry ice. The region of choice was dissected at –18°C and homogenized by sonication in 50 mM Tris-HCl (pH 7.3), 5 mM EDTA, 100 mM NaCl and stored at –80°C until further processing. The protein concentration of whole cell homogenates was determined (Karlsso *et al.* 1994). For western blotting, samples were mixed with an equal volume of 3 × Laemmli buffer and heated at 96°C for 5 min.

### Caspase-3-like activity assay

Samples of homogenate (50 µL) were mixed with 50 µL of extraction buffer, containing 50 mM Tris-HCl (pH 7.3), 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail (Sigma) and 0.2% CHAPS, on a microtiter plate (Microfluor; Dynatech, Chantilly, VA, USA). After incubation for 15 min at room temperature, 100 µL of peptide substrate, 50 µM Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC; Peptide Institute, Osaka, Japan) in extraction buffer without inhibitors or CHAPS but with 4 mM dithiothreitol, was added. Caspase-3-like activity was measured by the appearance of the fluorescent cleavage product, AMC, detected using a Spectramax Gemini fluorometer (Molecular Devices, Sunnyvale, CA, USA). The excitation and emission wavelengths were 380 and 460 nm. Data were collected during the linear phase of degradation and expressed as pmol AMC formed/µg protein/min.

### Western blotting

Samples containing 20 µg protein were separated on 8–16% Novex (Invitrogen, San Diego, CA, USA) Tris-glycine gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were washed in TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl and 0.05% Tween 20) and blocked with 5% milk in TBS-T. Subsequently, the membranes were incubated with the FBDP antibody (1 : 50) for 1 h at room temperature or overnight at 4°C. Visualization was performed using a peroxidase-conjugated anti-rabbit secondary antibody, Super Signal chemiluminescent substrates (Pierce, Rockford, IL, USA) and RX film (Fuji Photo Film Co., Tokyo, Japan).

### Cyclosporin A treatment

Animals were injected i.v. with a single dose of 50 mg/kg CsA (Sandimmun®; Sandoz, Basel, Switzerland) (*n* = 3) or vehicle

(7.5% ethanol and 0.85% Tween 80 in saline) ( $n = 3$ ) approximately 30 min before the onset of isoelectricity. Subsequently, the animals were perfusion fixed with 4% paraformaldehyde and further processed for immunohistochemistry. Animals were killed at 30 min or 2 days of recovery following 30 min of isoelectricity.

#### FK 506 (Tacrolimus) treatment

To control for the immunosuppressive effects of CsA, i.e. inhibition of calcineurin, a series of animals were treated with FK 506 ( $n = 3$ ). Animals were injected i.v. with 2 mg/kg as a single dose and killed at 2 days of recovery following 30 min of isoelectricity.

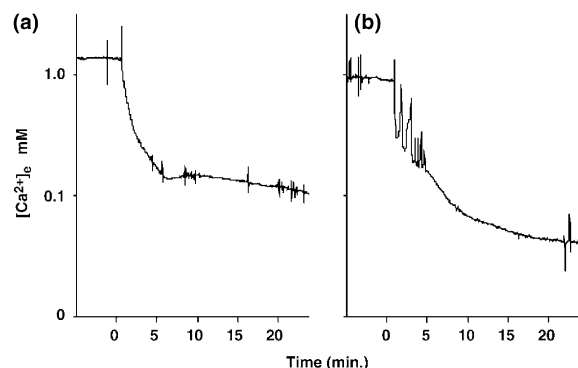
## Results

### Extracellular $\text{Ca}^{2+}$ concentration during and following hypoglycemia

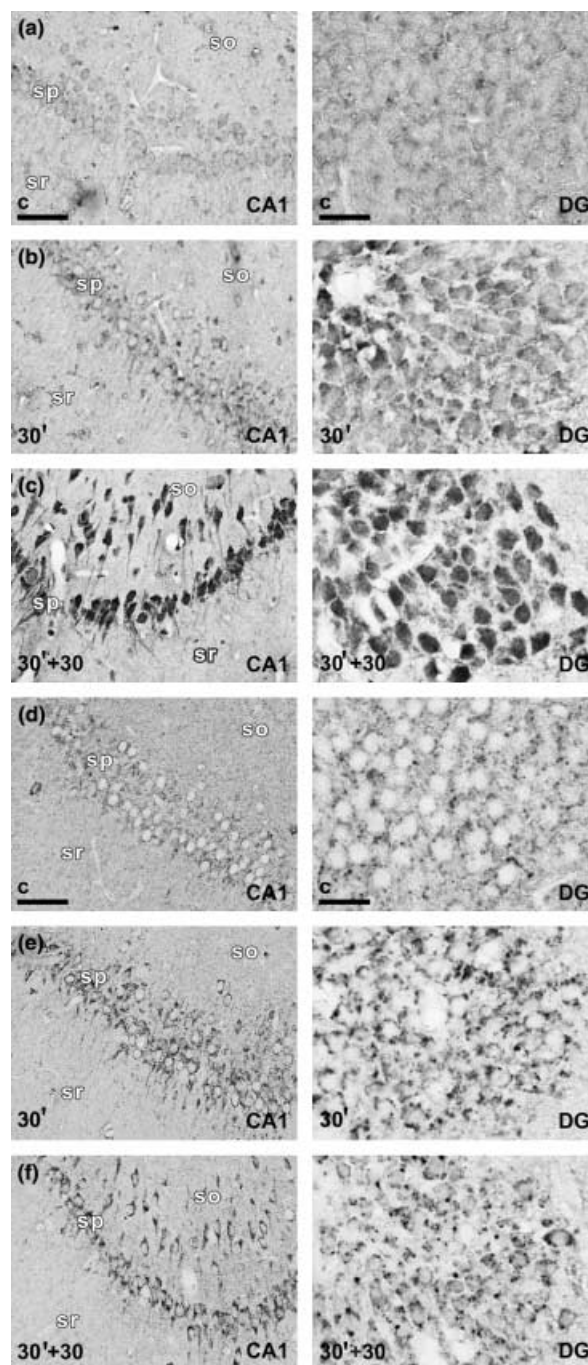
Figure 1 shows typical extracellular  $\text{Ca}^{2+}$  registrations in a vehicle-treated (Fig. 1a) and a CsA-treated (Fig. 1b) animal. In both CsA- and vehicle-treated animals the resting concentrations of extracellular  $\text{Ca}^{2+}$  were about 1 mM. At the beginning of isoelectricity and up to 10 min after, extracellular  $\text{Ca}^{2+}$  levels started to fall in both groups. Neither depolarization time nor the lowest extracellular  $\text{Ca}^{2+}$  concentration during isoelectricity differed between the groups. In animals treated with CsA or the vehicle the lowest extracellular  $\text{Ca}^{2+}$  concentration was  $0.24 \pm 0.19$  and  $0.12 \pm 0.03$  mM within a period of  $19.2 \pm 5.4$  and  $19.5 \pm 9.0$  min of depolarization, respectively. When hypoglycemia was terminated with glucose infusion, the extracellular  $\text{Ca}^{2+}$  concentration increased to about 75% of the initial concentration within 15 min (data not shown). The changes in extracellular  $\text{Ca}^{2+}$  concentrations are reflected in the change in the DC potential.

### Redistribution of apoptosis-inducing factor and cytochrome *c* during and following hypoglycemia

Apoptosis-inducing factor staining was weak and diffuse in the medial CA1 region of the hippocampus and in the crest of the DG on sections from the control animals (Fig. 2a). After

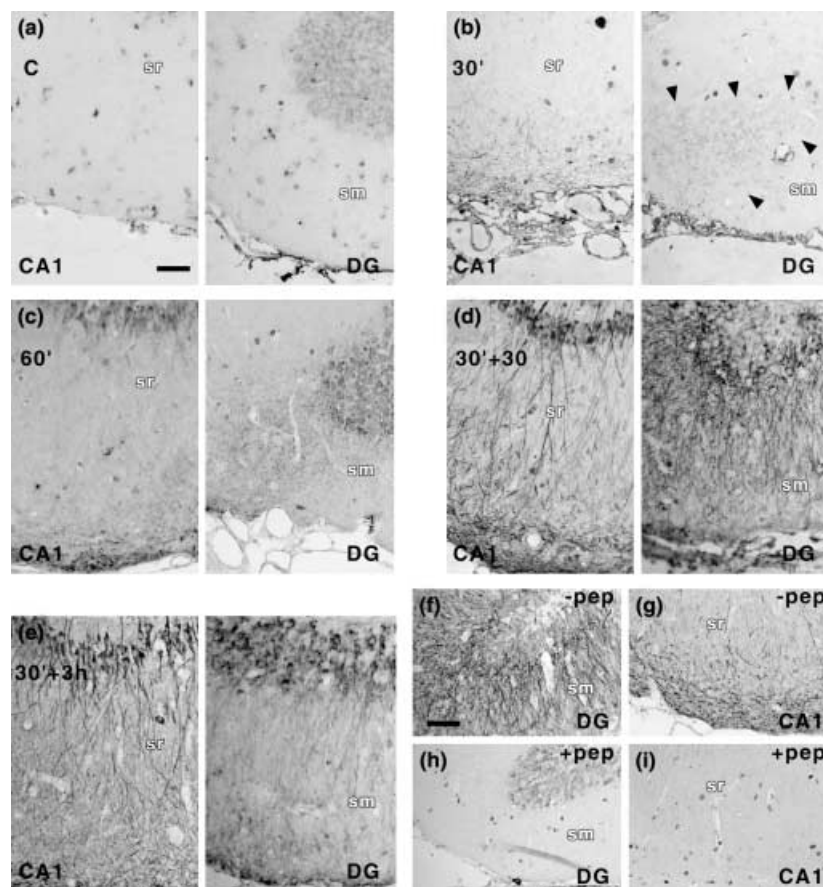


**Fig. 1** Typical recordings of the extracellular calcium concentration in the dentate gyrus in hypoglycemic rats treated with (a) vehicle or (b) cyclosporin A. Time zero indicates the onset of isoelectricity.



**Fig. 2** Photomicrographs showing (a–c) apoptosis-inducing factor or (d–f) cytochrome *c* staining in the hippocampal CA1 region and in the crest of the dentate gyrus (DG). Sections from the control (a and d), 30 min of isoelectricity (b and e) and 30 min of isoelectricity plus 30 min of recovery (c and f). c, Control; 30', 30 min of isoelectricity; 30' + 30, 30 min of isoelectricity + 30 min of recovery; sp, stratum pyramidale; so, stratum oriens; sr, stratum radiatum. Scale bars, CA1, 80  $\mu\text{m}$ ; DG, 20  $\mu\text{m}$ .

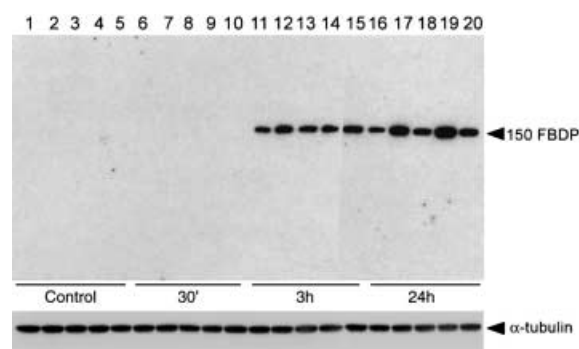
**Fig. 3** Photomicrographs from stratum moleculare (sm) of the dentate gyrus (DG) and stratum radiatum (sr) of the hippocampal CA1 region showing immunoreactivity against fodrin breakdown product (FBDP). Sections from (a) control, (b) 30 min of isoelectricity, (c) 60 min of isoelectricity, (d) 30 min of recovery following 30 min of isoelectricity and (e) 3 h of recovery following 30 min of isoelectricity. Sections from animals subjected to 30 min of isoelectricity and 30 min of recovery treated with antibody against FBDP, without antibody pre-absorption (f and g) and parallel sections treated with pre-absorbed antibody against FBDP (h and i). c, Control; 30', 30 min of isoelectricity; 30' + 30, 30 min of isoelectricity + 30 min of recovery; 60', 60 min of isoelectricity; 30' + 3 h, 30 min of isoelectricity + 3 h of recovery; - pep/+pep, without and with pre-absorption, respectively. Arrow heads demarcate weak FBDP immunoreactivity. Scale bars, (a–e), 35  $\mu$ m; (f–i), 50  $\mu$ m.



30 min of isoelectricity a slight increase in staining could be discerned in cells in both the CA1 region and in the DG, frequently localized to one pole of the nucleus (Fig. 2b). At 30 min of recovery following 30 min of isoelectricity a marked increase in staining could be seen, localized mainly to the nuclei but also to dendrites and perinuclear cytoplasm (Fig. 2c). After 60 min of isoelectricity the staining was similar to that seen after 30 min of isoelectricity (data not shown). Relatively weak, punctate staining of cytochrome *c* was seen in the medial CA1 region and in the DG in control sections (Fig. 2d). At the end of 30 min of isoelectricity a distinct staining in cells in the CA1 region and in the crest of the DG was seen, appearing in clusters around the nuclei (Fig. 2e). A similar staining pattern was detected at 30 min of recovery following 30 min of isoelectricity in both regions (Fig. 2f). Likewise, after 60 min of isoelectricity the staining looked like that after 30 min of isoelectricity (data not shown).

#### Calpain activation during and following hypoglycemia

Fodrin breakdown product immunoreactivity was not seen in the stratum moleculare of the DG or stratum radiatum or the stratum moleculare of the hippocampal CA1 region in the control sections (Fig. 3a). Following 30 or 60 min of isoelectricity, weak immunoreactivity could be discerned in



**Fig. 4** An immunoblot of homogenates from the vulnerable part of the hippocampus stained with the antibody against fodrin breakdown product (FBDP). Five samples from each control and time point are shown. Lanes: 1–5, controls; 6–10, 30 min of isoelectricity; 11–15, 3 h or 16–20, 24 h of recovery following 30 min of isoelectricity. The apparent molecular mass of the FBDP is indicated (150 kDa). The membranes were stripped and probed with a  $\alpha$ -tubulin antibody as a loading control.

all these regions (Figs 3b and c, respectively). At 30 min of recovery following 30 min of isoelectricity, intense FBDP staining was seen in the DG and in the CA1 region (Fig. 3d). Conspicuous dendritic staining was still seen in the CA1

region and the DG at 3 h of recovery following 30 min of isoelectricity (Fig. 3e). The immunoreactivity seen at 30 min of isoelectricity followed by 30 min of recovery in the DG and in the CA1 region (Figs 3f and g, respectively) was completely abolished by antibody pre-absorption (Figs 3h and i). The immunohistochemical staining was confirmed on western blots stained with the FBDP antibody (Fig. 4). No bands were detected in controls or animals subjected to only 30 min of isoelectricity, whereas a prominent, single band with an apparent molecular weight of 150 kDa was detected in all samples from animals subjected to 30 min of isoelectricity followed by 3 or 24 h of recovery.

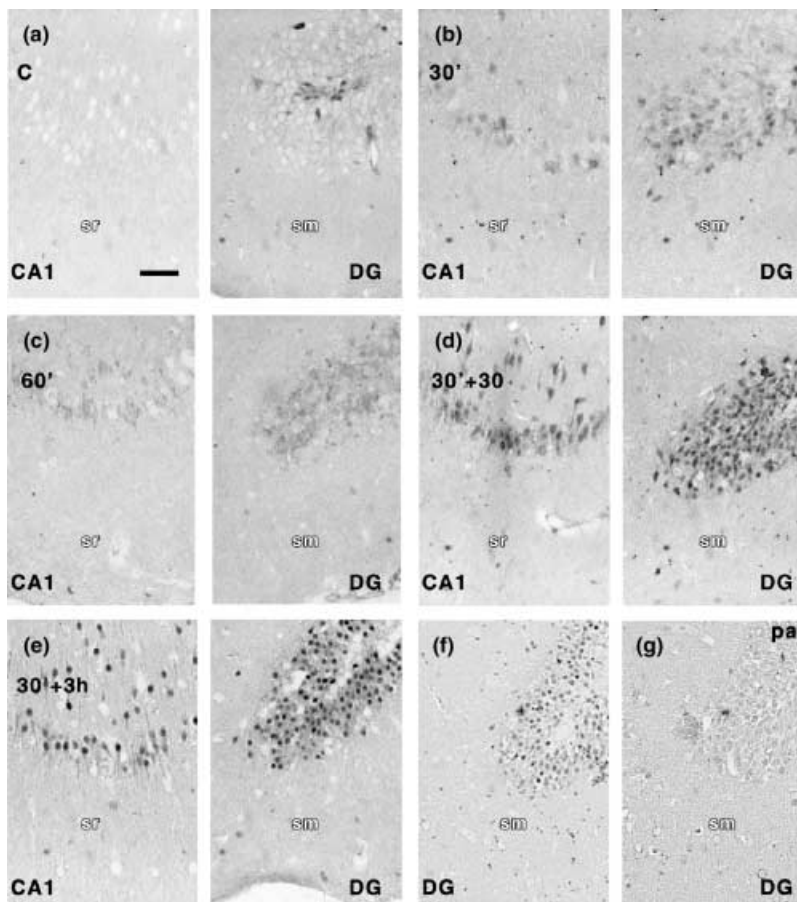
#### Caspase-3 activation during and following hypoglycemia

Using an antibody specific for activated caspase-3, we found no immunoreactivity in the hippocampal CA1 region or the crest of the DG in control sections, except for some scattered cells in the hilar region of the DG (Fig. 5a). Following 30 or 60 min of isoelectricity, caspase-3 staining was discernible but modest in the CA1 region and in the crest of the DG (Figs 5b and c, respectively). After 30 min of isoelectricity and 30 min of recovery widespread caspase-3 staining was seen in the pyramidal cell layer of the CA1

region and in the crest of the DG (Fig. 5d). The staining was even stronger after 3 h of recovery (Fig. 5e). The staining was abolished by antibody pre-absorption with active caspase-3 (Figs 5f and g). Caspase-3-like activity (DEVD-AMC cleavage) was examined in the hippocampus (Fig. 6). In the control group the mean activity was  $2.2 \pm 0.8$  pmol/ $\mu$ g protein/min. At 3 h of recovery there was a 2.3-fold, significant ( $p < 0.05$ ) increase in activity compared with the controls. By 24 h of recovery the activity had returned to control levels.

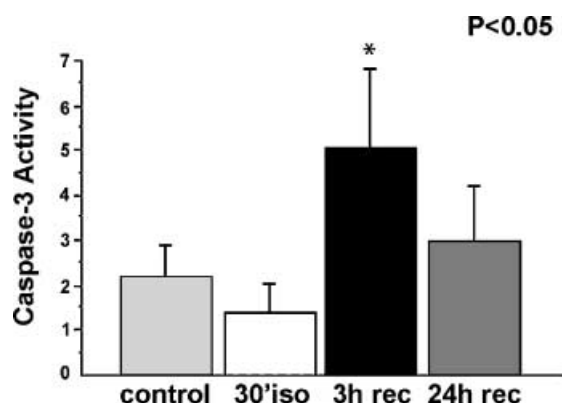
#### Cyclosporin A treatment

After 30 min of isoelectricity and 30 min of recovery, the AIF staining of vehicle-treated animals (Fig. 7a and b) was the same as in untreated animals (see Fig. 2c). Animals treated with CsA displayed substantially reduced AIF staining in the crest of the DG (Fig. 7c), whereas staining in the CA1 region was less affected (Fig. 7d). Similarly, cytochrome *c* staining was virtually identical in untreated (see Fig. 2f) and vehicle-treated animals (Figs 7e and f), typically appearing in clusters surrounding the nuclei or polarized to one side of the nuclei (Figs 7e and f). However, in animals treated with CsA, the staining was less intense and



**Fig. 5** Photomicrographs from sections of the hippocampal CA1 region and the dentate gyrus (DG) of the hippocampal formation which have been stained for caspase-3. Sections from (a) control, (b) 30 min of isoelectricity, (c) 60 min of isoelectricity, (d) 30 min of recovery following 30 min of isoelectricity and (e) 3 h of recovery following 30 min of isoelectricity. Sections from the DG following 3 h of recovery and 30 min of isoelectricity which have been stained for (f) caspase-3 antibody or (g) caspase-3 antibody pre-absorbed with active caspase-3. c, Control; 30', 30 min of isoelectricity; 30' + 30, 30 min of isoelectricity + 30 min of recovery; 60', 60 min of isoelectricity; 30' + 3 h, 30 min of isoelectricity + 3 h of recovery; sm, stratum moleculare; sr, stratum radiatum; pa, pre-absorption. Scale bar, (a–g), 45  $\mu$ m.





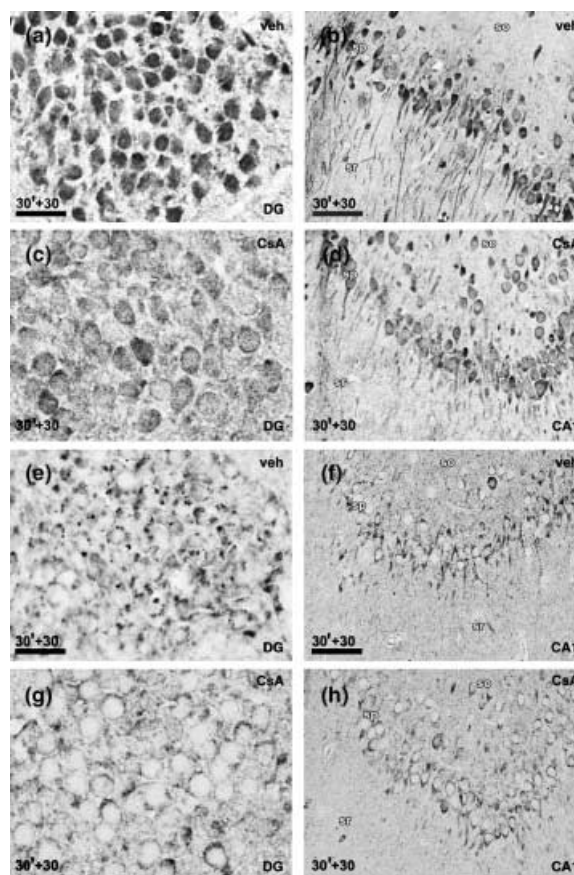
**Fig. 6** Caspase-3 activity in whole cell homogenates of the vulnerable part of the hippocampus, from control brains or brains from animals subjected to 30 min of isoelectricity followed by 0, 3 or 24 h of recovery. The data are expressed in pmol AMC formed/min/μg protein and as means  $\pm$  SD ( $n = 5$ ). \* $p < 0.05$  denotes Mann-Whitney U and Bonferroni correction. 30' iso, 30 min of isoelectricity; 3 and 24 h rec, 3 and 24 h of recovery following 30 min of isoelectricity, respectively.

considerably less clustering of cytochrome *c* staining was seen in the crest of the DG (Fig. 7g), whereas the changes in cytochrome *c* staining in the CA1 region were less pronounced (Fig. 7h).

The intense FBDP staining, indicating calpain activation, in the DG and the CA1 region at 30 min of recovery was also seen in the vehicle-treated animals (Fig. 8a and b). Interestingly, the FBDP staining was completely abolished after CsA treatment in both the DG and the CA1 region (Figs 8c and d). Similarly, the caspase-3 staining in both the DG and the CA1 region (Figs 8e and f) was abolished by CsA treatment (Figs 8g and h).

#### FK 506 treatment

After 30 min of isoelectricity and 2 days of recovery, most cells in the crest of the DG and in the medial CA1 region showed signs of neurodegeneration, similar to previous investigations (Auer *et al.* 1985b; Friberg *et al.* 1998). Vehicle-treated animals displayed scattered, more or less pyknotic, AIF- and cytochrome *c*-positive cells in both the CA1 region and the DG (Figs 9a, b, m and n, respectively). The number of cells was lower than at 30 min of recovery. Treatment with FK 506 produced a modest decrease in the number of AIF- and cytochrome *c*-positive cells (Figs 9i, j, u and v, respectively), whereas CsA treatment completely abolished them (Figs 9e, f, q and r, respectively). The number of FBDP-positive cells in the vehicle-treated animals at 2 days of recovery was also lower than at 30 min of recovery and the conspicuous dendrite staining was much reduced (Figs 9c and o). FK 506 treatment partly reduced the FBDP staining in some animals (Figs 9k and w), whereas CsA treatment completely abolished it (Figs 9g and s). The number of cells positive for active caspase-3 in the vehicle-



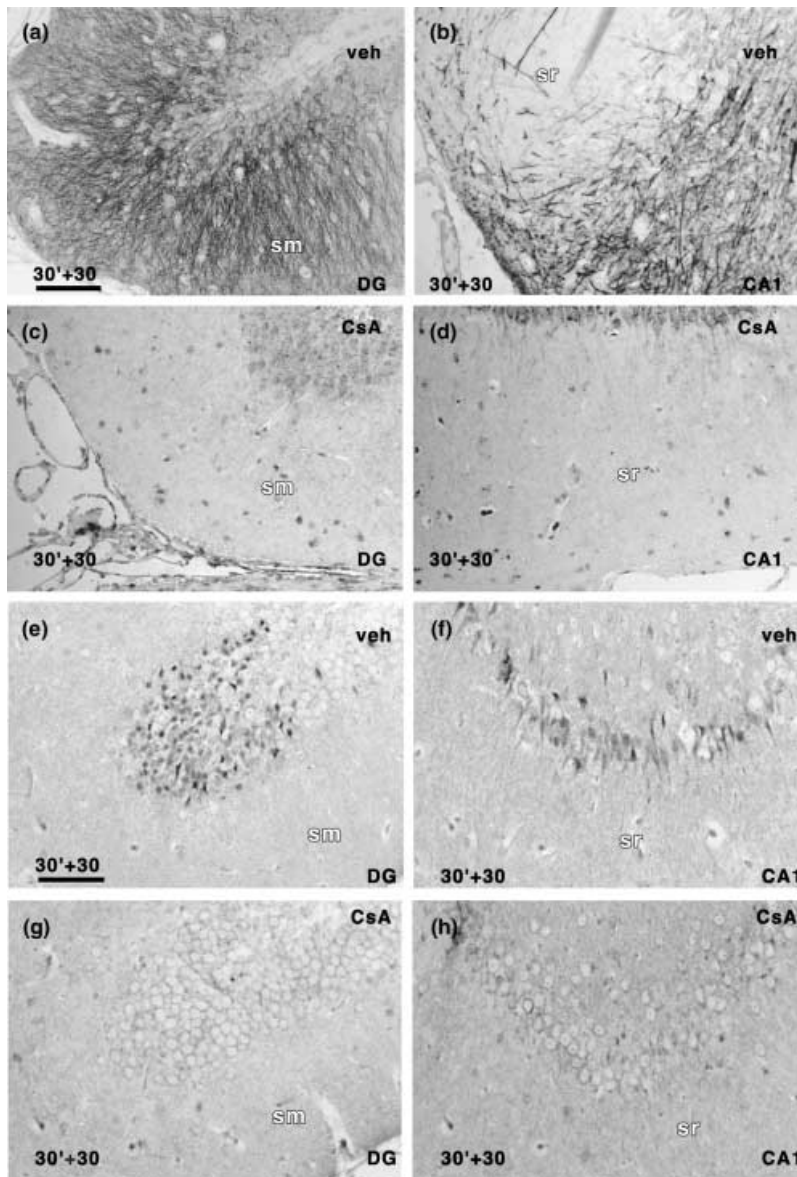
**Fig. 7** Photomicrographs of sections from the crest of the dentate gyrus (DG) and the medial hippocampal CA1 region stained with the apoptosis-inducing factor (a–d) or the cytochrome *c* (e–h) antibody. Animals were treated with vehicle solution (veh) (a, b, e and f) or cyclosporin A (CsA) (c, d, g and h), subjected to 30 min of isoelectricity and allowed 30 min of recovery. 30' + 30, 30 min of isoelectricity + 30 min of recovery; sp, stratum pyramidale; so, stratum oriens; sr, stratum radiatum. Scale bars, CA1, 20 μm; DG, 80 μm.

treated animals was similar to or even higher than at 30 min of recovery (Figs 9d and p), supporting earlier findings that activation of caspase-3 is delayed and sustained (Blomgren *et al.* 2001). As in the case of the other markers, FK 506 treatment only slightly reduced the caspase-3 staining (Figs 9l and x), whereas little or no caspase-3 immunoreactivity was seen after CsA treatment (Figs 9h and t).

#### Discussion

We demonstrated previously that CsA, but not the immunosuppressor and calcineurin inhibitor FK 506, completely prevented cell death in the crest of the DG (Friberg *et al.* 1998). Concomitantly, mitochondrial, but not dendritic, swelling was prevented. This strongly implicates a critical role for MPT in the neuronal mitochondrial swelling seen during severe hypoglycemia and for the development of





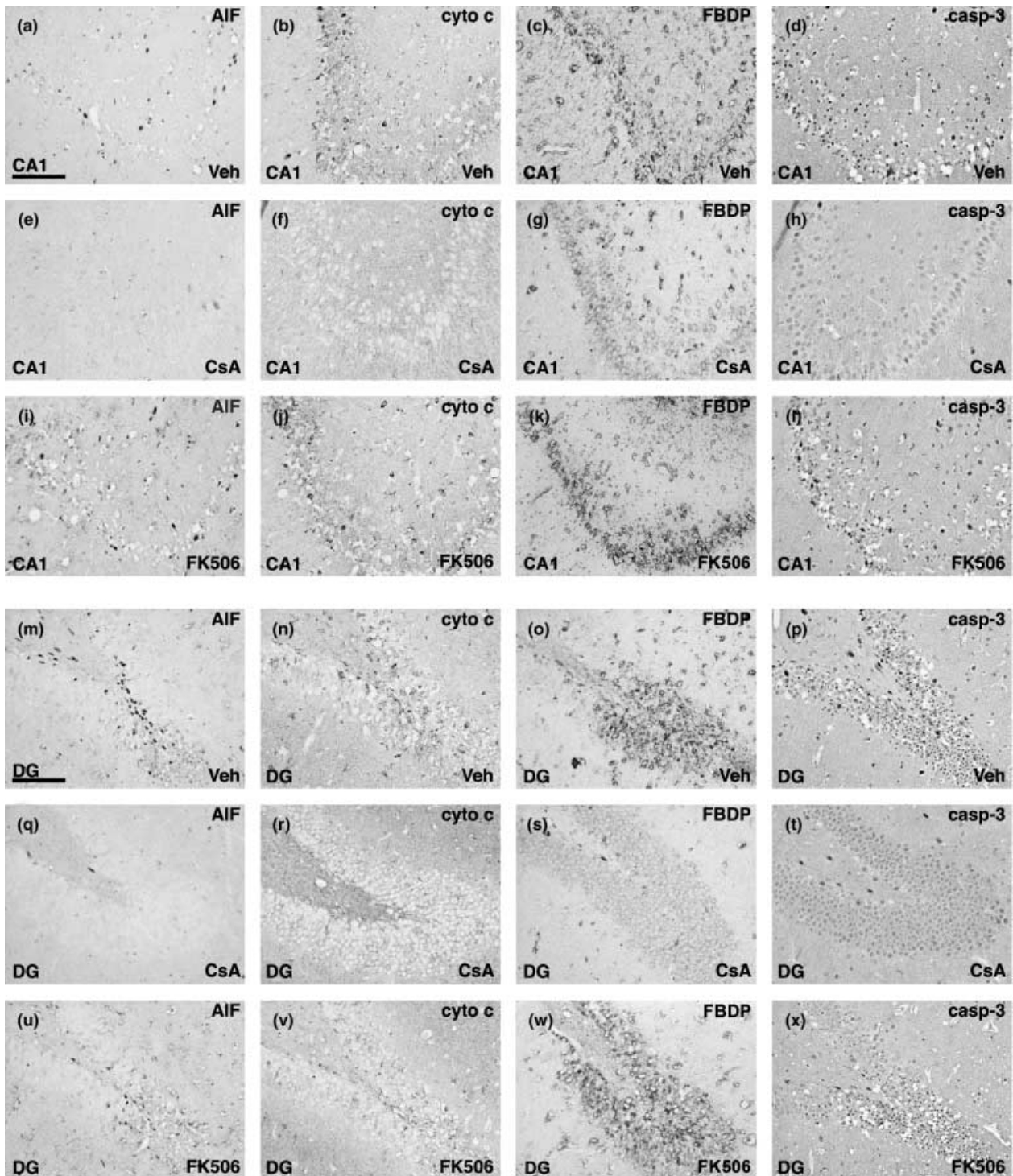
**Fig. 8** Photomicrographs of sections from stratum moleculare (sm) and the dentate granule cells of the dentate gyrus (DG) and stratum radiatum (sr) of the hippocampal CA1 region stained with (a–d) the fodrin breakdown product or (e–h) the caspase-3 antibody. Immunostaining following 30 min of isoelectricity and 30 min of recovery from animals treated with the vehicle (veh) (a, b, e and f) or cyclosporin A (CsA) (c, d, g and h). 30' + 30, 30 min of isoelectricity + 30 min of recovery. Scale bars, 60  $\mu$ m.

hypoglycemic neuronal damage. The dendritic swelling was unaffected by CsA (Friberg *et al.* 1998), demonstrating that the collapse of ion homeostasis across the plasma membrane still occurred, which includes calcium and sodium entry into cells concomitant with water (Siesjo and Bengtsson 1989). The decreased levels of extracellular calcium, and concomitant increased levels of intracellular calcium, which occur in the presence of CsA do not lead to cell damage or degeneration. It is, therefore, reasonable to assume that events immediately downstream from the mitochondria lead to cell death, including the activation of proteases. In this study we showed that redistribution of AIF and cytochrome *c* occurred and that calpains and caspase-3 were activated following hypoglycemia in cells that subsequently will succumb. This redistribution of mitochondrial, pro-apoptotic

proteins and the activation of proteases was inhibited by CsA but not by FK 506 in the DG, a region which is completely protected by CsA treatment. In the hippocampal CA1 region, the neuroprotective effect of CsA is incomplete (Friberg *et al.* 1998), which fits with the finding that the effect of CsA on the redistribution of cytochrome *c* and AIF was less pronounced in this region. Taken together, this implies a strong coupling between MPT, protease activation and hypoglycemic cell death in the brain.

#### Calpain activation

We know from previous studies that, at the end of 30 min of isoelectricity, the mitochondria in the dentate granule cells are grossly swollen in the perikarya and along the dendrites. This swelling is presumably due to the dissipation of the



**Fig. 9** Photomicrographs of sections from (a–l) the hippocampal CA1 region and (m–x) the dentate gyrus (DG) taken from animals treated with the vehicle (veh) (a–d and m–p), cyclosporin A (CsA) (e–h and q–t) or FK 506 (i–l and u–x) and subjected to 30 min of isoelectricity

ionic gradients across the cell membrane, which leads to an influx of calcium, sodium ions and water into the cells (Harris *et al.* 1984). Glutamate receptor antagonists mitigate

and allowed 48 h of recovery. Sections were stained for apoptosis-inducing factor (AIF) (a, e, i, m, q and u), cytochrome *c* (cyto *c*) (b, f, j, n, r and v), fodrin breakdown product (FBDP) (c, g, k, o, s and w) and active caspase-3 (casp-3) (d, h, l, p, t and x). Scale bars, 200  $\mu$ m.

hypoglycemic damage in the striatum (Wieloch 1985), DG and CA1 region (Papagapiou and Auer 1990; Nellgard and Wieloch 1992). This led to the hypothesis of glutamate

toxicity in hypoglycemic damage (Wieloch 1985), which stated that calcium overload due to NMDA receptor activation contributes to cell death (Siesjo *et al.* 1995). Downstream from the increase in intracellular calcium levels, degradative processes, including proteolysis, have been implicated in neuronal cell death (Siman and Noszek 1988; Seubert *et al.* 1989). Given the fact that calpains have an absolute requirement for  $\text{Ca}^{2+}$  for activity (Huang and Wang 2001), it is somewhat surprising that only weak FBDP immunostaining was seen following 30 and 60 min of hypoglycemia, considering that the cells were flooded with  $\text{Ca}^{2+}$  (Siesjo and Bengtsson 1989). Apparently, it was not the total time spent with increased intracellular  $\text{Ca}^{2+}$  levels that determined the extent of calpain activation, because 60 min of isoelectricity produced little or no FBDP staining, whereas 30 min of isoelectricity plus 30 min of recovery produced massive FBDP staining. This strongly indicates that events other than  $\text{Ca}^{2+}$  influx during recovery are critical for calpain activation. Cysteine proteases, like calpains and caspases, require a reduced cysteine residue at their active site to maintain proteolytic activity. During hypoglycemia, the intracellular redox state is shifted towards oxidation, which is the opposite of what happens during ischemia (Siesjo 1988). The relatively low levels of FBDP during hypoglycemia, despite high levels of intracellular calcium, might be explained by oxidation of the active site cysteine, known to inhibit calpain activity (Di Cola and Sacchetta 1987; Guttman and Johnson 1998). Once normoglycemia is restored, and the cellular redox state is normalized, calpains may become activated. This activation could be further amplified by the degradation of calpastatin, the endogenous inhibitor of calpain and a substrate of both calpains and caspase-3 (Emori *et al.* 1987; Porn-Ares *et al.* 1998; Wang *et al.* 1998; Blomgren *et al.* 1999). The fact that CsA, but not FK 506, prevented calpain activation strongly suggests that MPT somehow mediates the observed calpain activation following hypoglycemia. This mechanism is still elusive. It is possible that CsA enhances the mitochondrial uptake and retention of calcium and thereby prevents calpain activation during recovery, as indicated in a recent study where CsA prevented calpain activation following microcystin-induced MPT and cell death in hepatocytes (Ding *et al.* 2002). Alternatively, other factors may be released through MPT during hypoglycemia, which mediate calpain activation during recovery.

### Caspase-3 activation

As mentioned earlier, it has been shown that caspase-3 is activated following MPT through the release of cytochrome *c* from the mitochondria and the subsequent formation of an 'apoptosome' protein complex, which transforms pro-caspase-3 to caspase-3. Furthermore, calpains may activate caspase-3, either directly or via caspase-12 (Nakagawa and Yuan 2000; Blomgren *et al.* 2001). dATP is an absolute

requirement for the formation of the apoptosome complex (Li *et al.* 1997). In our investigation, caspase-3 activity, as judged by DEVD-AMC cleavage, was unaffected (or even somewhat reduced) during hypoglycemia, despite a modest increase in immunoreactivity for active caspase-3. This absence of caspase-3 activation during hypoglycemia under conditions which evidently lead to cytochrome *c* release could, therefore, be due to the low levels of ATP preventing apoptosome formation. This is also supported by the increased DEVD-AMC cleavage seen after 3 h of recovery, when the ATP levels are partially replenished (Agardh *et al.* 1978). Mitochondrial ATP-dependent potassium channels may also be involved, since activation of these has been demonstrated to protect against ischemia-induced death by a mechanism involving suppression of Bax translocation and cytochrome *c* release (Liu *et al.* 2002). The relatively oxidative environment during hypoglycemia, as mentioned above, may also serve to inhibit cysteine proteases like caspases.

In conclusion, we propose that mitochondria undergo MPT during hypoglycemia. Due to oxidative conditions in the neurons, calpains are not activated despite increased cellular calcium levels. The low levels of ATP during hypoglycemia prevent the formation of the apoptosome and the activation of caspase-3. When normoglycemia is restored, the intracellular redox environment and ATP levels are normalized, leading to calpain activation (fodrin degradation) concomitant with activation of caspase-3. Both calpain- and caspase-3-mediated cellular proteolysis may contribute to cell death induced by hypoglycemia.

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