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Increased cyclin D1 in vulnerable neurons in the hippocampus after ischaemia and epilepsy: a modulator of *in vivo* programmed cell death?

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Abstract

Several observations suggest that delayed neuronal death in ischaemia, epilepsy and other brain disorders includes an apoptotic component, involving programmed cell death (PCD). PCD is hypothesized to result, in part, from aberrant control of the cell cycle. Because they are instrumental in mitosis, cyclins D are key markers to evaluate whether neurons indeed progress into the cell cycle in situations of pathology. Therefore, we investigated in rat brains, the expression of cyclins D in the delayed neuronal death that occurs following transient global ischaemia and kainate-induced seizures. Following a four-vessel occlusion insult, quantitative *in situ* hybridization revealed a highly significant and persistent 100% increase of cyclin D1 mRNA in the vulnerable pyramidal neurons of the CA1 hippocampal region. Ischaemia also induced a smaller and transient cyclin D1 mRNA increase in the resistant CA3 area and dentate gyrus. In contrast, the cyclin D2 and D3 mRNAs, expressed constitutively in the adult rat hippocampus, were not upregulated. Following kainate-induced seizures, cyclin D1 mRNA was induced in the vulnerable CA3 region, and to a lesser extent, in non-vulnerable regions. Cyclin D1 immunohistochemistry revealed increased protein levels in the cytoplasm and nucleus of neurons commited to die after ischaemia. Double labelling experiments indicate that cyclin D1 is also expressed in reactive astrocytes but not in microglial cells. Finally, we report that in neurons, cyclin D1 expression peaks before nuclear condensation and the appearance of DNA fragmentation. We propose that cyclin D1, when expressed at high levels in lesioned neurons, may act as a modulator of apoptosis.

Introduction

Following transient forebrain ischaemia, delayed neuronal degeneration occurs in the striatum, thalamus, cortex and CA1 area of the hippocampus (Kirino, 1982; Pulsinelli, 1985). It is generally accepted that ischaemic cell death results, at least in part, from glutamate excitotoxicity (Choi, 1988; Coyle & Puttfarcken, 1993). Cell death in the nervous system can occur through necrosis or apoptosis, two morphologically distinct cell deaths that involve either passive or active cellular events, respectively. Recent works based on morphological and biochemical criteria suggest that the process of delayed neuronal death in ischaemia has features of apoptosis (for review see Charriaut-Marlangue *et al*., 1996) and programmed cell death (PCD) [for review see MacManus & Linnick, 1997].

Little is known about the molecular mechanisms involved in PCD, but the process is hypothesized to result, in part, from aberrant control of the cell cycle (Colombel *et al*., 1992; Lee *et al*., 1993; Ucker, 1991). Although direct evidence for this hypothesis is still sparse, a number of results converge to reinforce it: commitment to apoptosis often takes place during the G1 phase of the cell cycle (Ucker, 1991); ultrastructural aspects of apoptotic and dividing cells share common morphological features (Ucker, 1991; Wyllie, 1980); non-proliferating cells, e.g. differentiated prostate cells, can synthesize DNA after stimulation to undergo cell death (Colombel *et al*. 1992); the c-myc protooncogene and the antitumour p53 protein (Evan *et al*., 1992; Ryan *et al*., 1993) are involved in PCD, suggesting that PCD is associated with abnormal or conflicting signals for cell cycle activation and cell cycle arrest. Examples of a potential link between cell cycle and cell death in differentiated neurons are further provided by the expression of the SV40 large tumour antigen (SV 40 T antigen) in transgenic mice. Expression of the T antigen often leads to the formation of tumours in a variety of tissues, but when directed to horizontal neurons of the retina (Hammang *et al*., 1993), Purkinje cells of the cerebellum (Feddersen *et al*., 1992) or rod receptors (Al-Ubaidi *et al*., 1992), the differentiated neurons die.

Brain ischaemia is followed by an induction of transcription factors (for review see Khrestchatisky *et al*., 1996) and growth factors (Lindvall *et al*., 1992; Finklestein *et al*., 1990; Kiyota *et al*., 1991; Kumon *et al*., 1993; Shozuhara *et al*., 1992; Wiessner *et al*., 1993). The ectopic and atypical expression of the latter, some of which are mitogenic, may promote entry and progression of neurons into the cell cycle (Khrestchatisky *et al*., 1996). This may be antagonized by the effects of the tumour suppressor gene p53 whose expression is upregulated in vulnerable areas after ischaemia (Li *et al*., 1995; Manev *et al*., 1994).

Cyclins D1, D2 and D3 play a key role in the G1 phase progression and act primarily as growth factor sensors (for review see Sherr, 1993). Cyclins are believed to associate primarily with cyclin-

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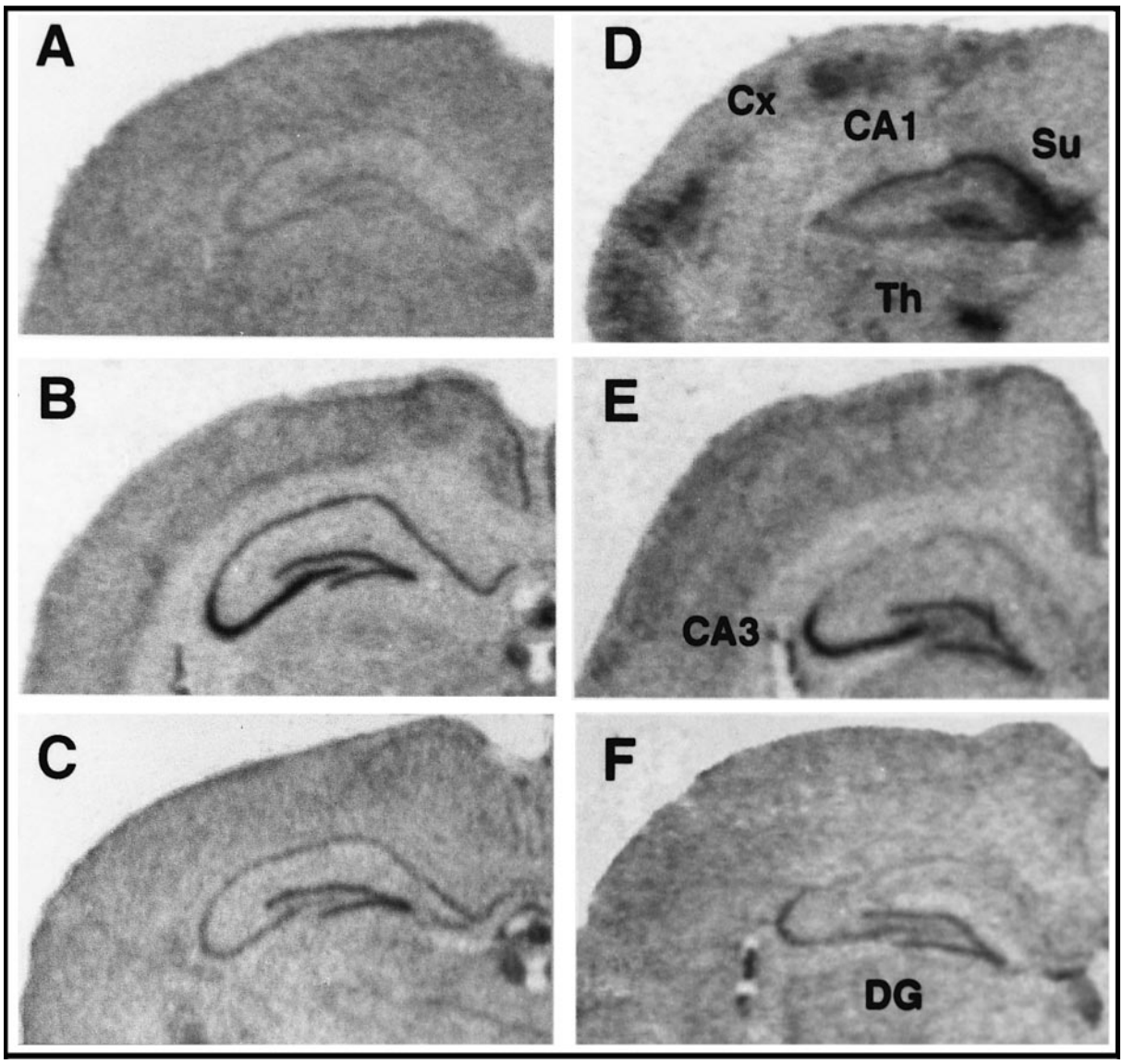


FIG. 1. Increased cyclin D1 mRNA expression, in regions of neuronal death, following transient global cerebral ischaemia. Autoradiograms illustrating the expression of cyclin D1 (A, D), D2 (B, E) and D3 (C, F) following *in situ* hybridization on coronal brain sections at the hippocampal level of control rats (A, B, C) or 96 h following transient ischaemia (D, E, F). Note the dramatic increase of cyclin D1 mRNA in regions of neuronal death after ischaemia, e.g. the CA1 region, cortex (Cx) and thalamus (Th) [D]. No increase in cyclin D2 and D3 mRNAs is detected in the vulnerable areas. Abbreviations: Su, subiculum of the hippocampus, CA3 region of the hippocampus; DG, dentate gyrus of the hippocampus.

dependent kinases (cdks) to phosphorylate Rb, although cyclin D1 may also act independently from cdks (Zwijsen *et al*., 1997). Phosphorylated Rb releases E2F and allows progression from the G1 to the S phase of the cell cycle. Cyclins D are therefore key markers to evaluate, in situations of pathology, whether terminally differentiated neurons indeed progress into the cell cycle. Evidence obtained *in vitro* suggests that it is indeed the case. In sympathetic neurons deprived of NGF, PCD is associated with cyclin D1 upregulation (Freeman *et al*., 1994). Furthermore, cyclin D1 transfection into neuroblastoma cells induces cell death with all the characteristics of PCD (Kranenburg *et al*., 1996).

To investigate the potential role of cyclins D in selective and delayed neuronal death, we studied the expression of cyclins D1, D2 and D3 following transient global ischaemia and kainate-induced seizures, two models of *in vivo* excitotoxic neuronal death. We show an upregulation of cyclin D1 in vulnerable neurons after ischaemia and seizures.

Materials and methods

Animal models

Experiments involving animals were approved by the French ethical science committee (Statement no. 04223). Rats had access to food and water *ad libitum* and were housed under a 12 h light–dark cycle at 22–24 °C.

Four-vessel occlusion forebrain ischaemia

Transient forebrain ischaemia was induced by a four-vessel occlusion as described previously by Pulsinelli & Brierley (1979). On the first day, male Wistar rats (300–320 g, Charles River, France) were anaesthetized with intraperitoneal 3% chloral hydrate (300 mg/kg). Both vertebral arteries were coagulated and clamps set on both carotid arteries. The next day, the carotid arteries were clamped for 20 min. Only rats with unresponsiveness and loss of the righting reflex during carotid clamping were included in the study. Rats with only

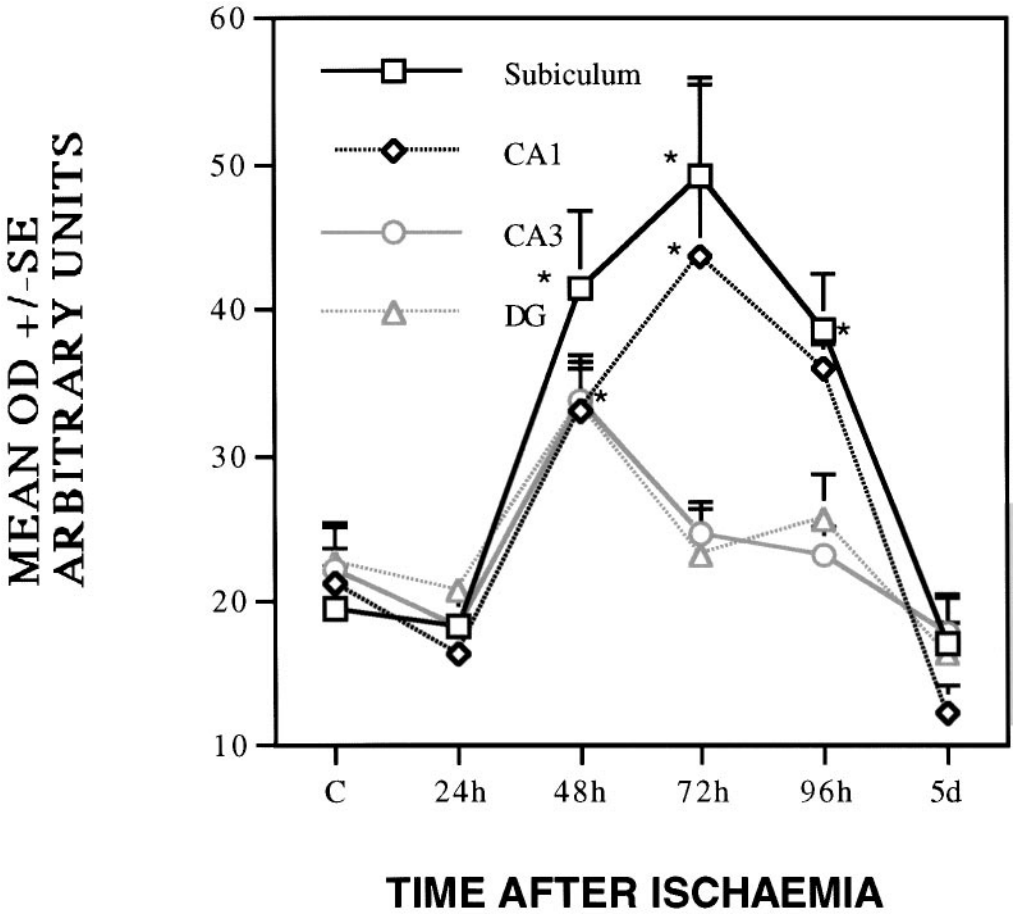


FIG. 2. Cyclin D1 mRNA levels following transient global cerebral ischaemia in the hippocampus. Horizontal axis: time after ischaemia; vertical axis: mean optical density (OD) ± standard error (SE). C: sham-operated rats; h, hours and d, days after ischaemia. Stars indicate a significant difference ($P < 0.05$, Fischer test) when compared to control values. Note the dramatic increase of the cyclin D1 mRNA level in the CA1/subiculum vulnerable region.

electrocoagulated vertebral arteries were used as control (sham operated rats). A total of 67 rats was used in this study. For the *in situ* hybridization experiments, at least three rats per time point were analysed 24, 48, 72 and 96 h post-ischaemia. Rats at 1 h ($n = 2$), 4 ($n = 2$), 8 ($n = 2$), 16 ($n = 1$) and 5 days ($n = 1$) after recirculation were also analysed. Rats were anaesthetized, intracardially perfused with 400 mL of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS); brains were removed rapidly, postfixed for 24 h in 4% PFA and cryoprotected for 24 h in 20% sucrose and then frozen in isopentane at –70 °C. Serial cryostat coronal sections (15 μm) were collected on gelatin chrom-alum-coated slides and stored at –70 °C.

Intra-amygdaloid injections of kainate

For seizure-induced selective lesions of the hippocampal CA3 area, adult male Wistar rats (180–200 g) were anaesthetized with chloral hydrate, and kainate (Sigma, St. Louis, Missouri, USA; 1.2 μg in 0.3 μL of phosphate buffer, pH = 7.4) was unilaterally injected in the amygdala as previously described (Ben-Ari *et al*., 1980). Animals showing typical kainate-induced limbic motor seizures for at least 2 h upon recovery from anaesthesia were kept for the experiments. They were killed 8, 16, 24 h, 3 and 14 days after the onset of seizures. Control animals were similarly injected with 0.3 μL phosphate buffer. Rats were killed, and their brains removed and immediately frozen and processed as described above.

Radiolabelled RNA probes for cyclin D1, D2 and D3

^{35}S-UTP-labelled (1000 Ci/mmol; Amersham, UK) sense and antisense cRNA probes were transcribed *in vitro* from linearized pBluescript plasmids containing rat cyclin D1 (209 bp), D2 (158 bp) and D3 (515 bp) inserts with T7 or T3 RNA polymerases (Pharmacia LKB Biotech., Uppsala, Sweden), using a riboprobe kit (Promega Corp., Madison, Wisconsin, USA). Specific activities of sense and antisense probes were in the range of $1–1.5 \times 10^7$ cpm/μg of RNA. Before hybridization, the larger cyclin D3 cRNAs were hydrolysed for 30 min in 0.4 M Na_2Co_3 to generate fragments of about 150 bases.

In situ *hybridization*

Brain sections were processed at different times (1, 4, 8, 16, 24, 48, 72, 96 h, and 5 days) after recirculation as previously described (Fontaine & Changeux, 1989). Briefly, frozen sections were brought to room temperature (RT), fixed in cold 4% PFA, and then rinsed in glycine phosphate buffer, acetylated, dehydrated, delipidated and incubated with 5×10^4 cpm/μL antisense or sense riboprobes overnight at 55 °C. Slides were then rinsed in 4 × SSC, treated with RNAse A for 30 min and washed with increasingly stringent conditions up to 0.1 × SSC at 60 °C for 30 min. Sections were apposed to Biomax (Kodak, Rochester, New York, USA) film and then processed for emulsion autoradiography (NTB2, Kodak) with respective exposure times of 3 days and 2–3 weeks. Two types of control experiments were performed: sense probes were used on ischaemic rat tissues at

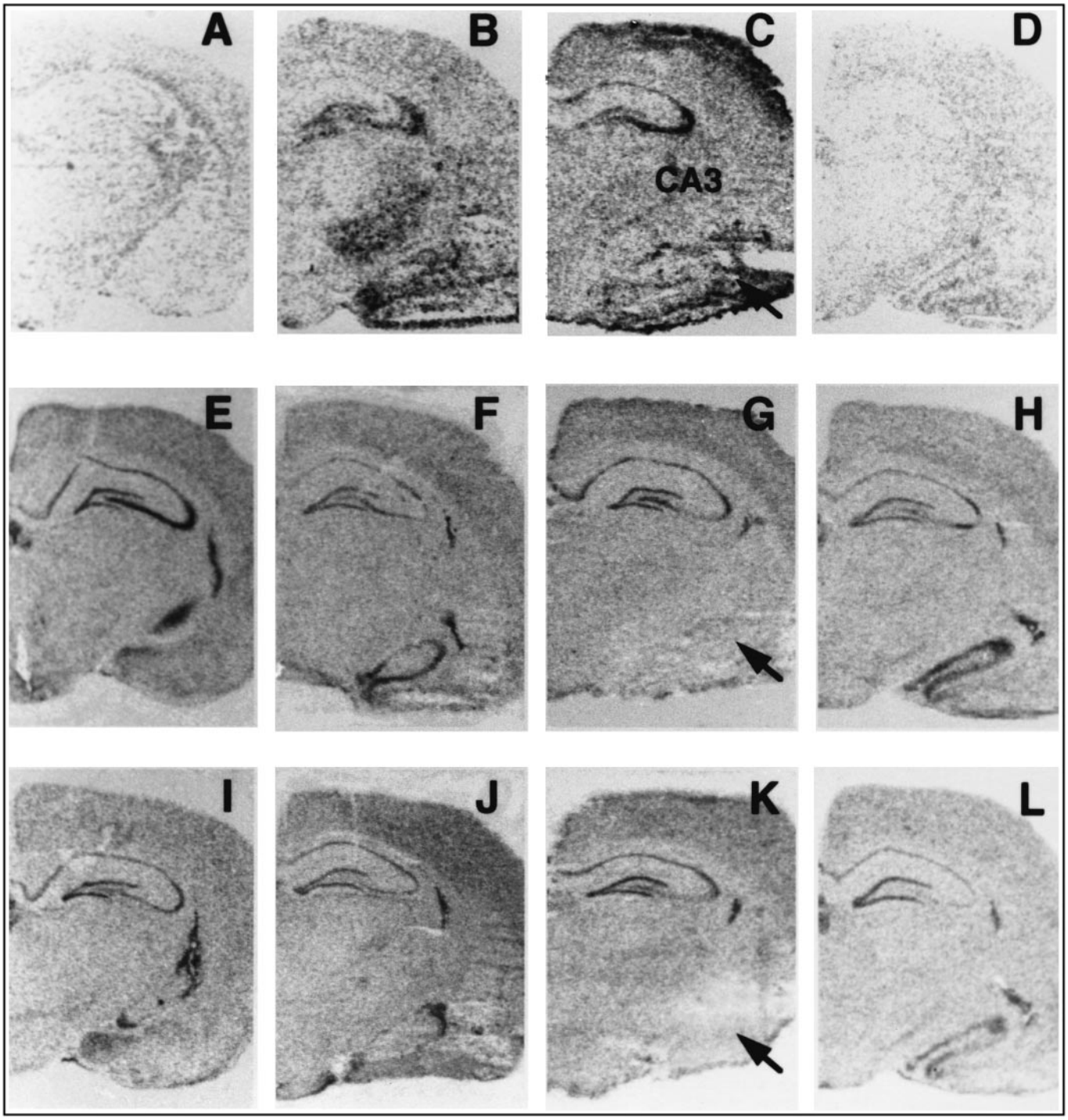


FIG. 3. Increased cyclin D1 mRNA expression but not D2 or D3 after kainate-induced seizures. Autoradiograms illustrating the expression of cyclin D1 (A–D), D2 (E–H) and D3 (I–L) mRNAs following *in situ* hybridization on coronal brain sections at the hippocampal level of control rats (A, E, I) or 24 h (B, F, J), 3 days (C, G, K) and 14 days (D, H, L) following kainate. Arrows point to the site of injection. Note in (B) and (C) the increased expression of cyclin D1 mRNA in the vulnerable CA3 region of the hippocampus. No increase of cyclin D2 and D3 mRNAs is detected in this region.

different times after recirculation (24, 48, 72, 96 h), sense and antisense probes were used on brain sections from sham-operated animals at different times (1, 4, 8, 16, 24, 48, 72, 96 h and 7 days).

Densitometric analysis of the hybridization signals on the autoradiograms was performed using a Samba/200S image analysis system (TITN Alcatel, France). Statistical analysis (Fischer test) was performed with data combined from three animals regardless of the degree of severity of ischaemia at each of the following time points: 24, 48, 72 and 96 h.

Immunohistochemistry of cyclin D1

Cyclin D1 immunohistochemistry was performed on 30 μm floating sections prepared from the brains of ischaemic and sham-operated rats at 24, 72 and 96 h after recirculation. Briefly, endogenous

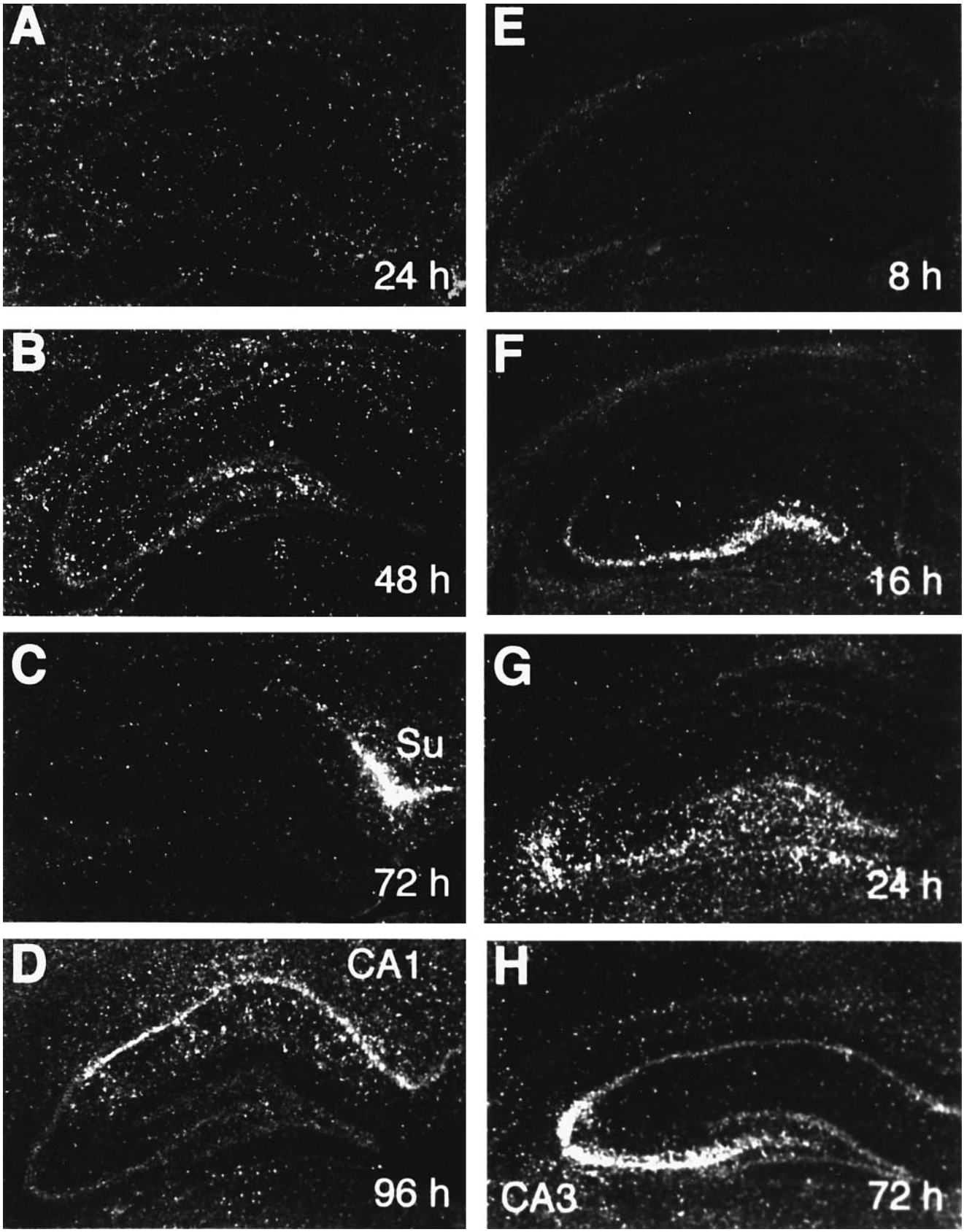
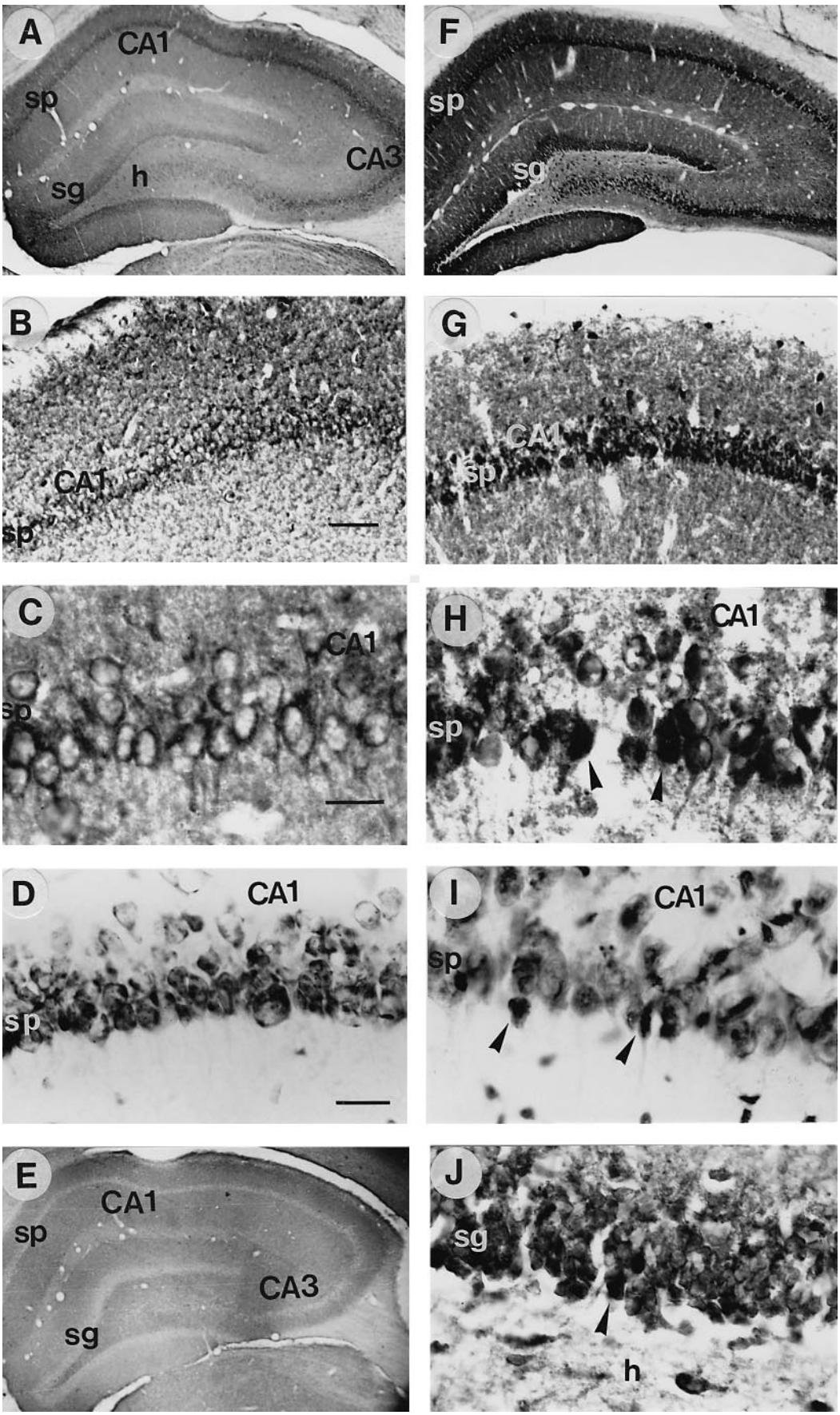


FIG. 4. Comparison of cyclin D1 mRNA expression following transient global cerebral ischaemia and kainate-induced seizure as a function of time on emulsioned-coated sections. Coronal sections through the hippocampus in tissues at different times (in h) after ischaemia (A–D) or after kainate (E–H). Following ischaemia, note in (C) and (D) the homogeneous signal for cyclin D1 mRNA in the vulnerable stratum pyramidale of CA1 and the subiculum (Su). After seizures, note (G, H) the increased expression of cyclin D1 mRNA in the vulnerable stratum pyramidale of CA3, ipsilateral to the site of kainate injection. In some cases (16 h, F) the increased cyclin D1 mRNA in the stratum pyramidale of CA3 was bilateral and the hippocampus contralateral to the site of kainate injection is shown in (F).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peroxidases were inactivated by incubating the sections for 10 min in 0.1% H_2O_2 at RT. Tissues were blocked and permeabilized for 30 min in 1.5% goat serum, 0.2% Triton X-100 in PBS. After rinsing in PBS, the sections were incubated overnight at 4 °C with an affinity-purified cyclin D1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted 1:1000 in PBS/

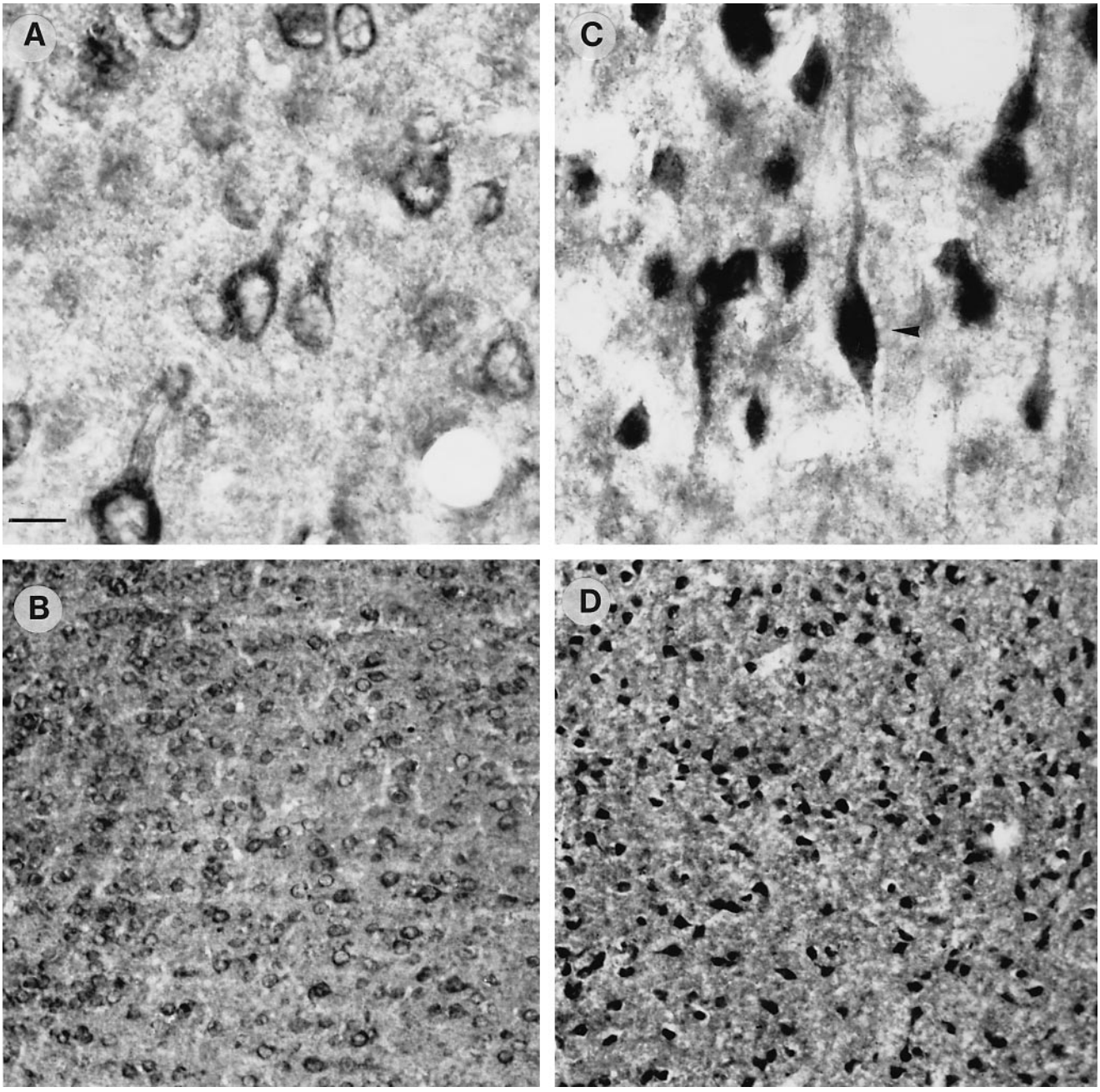
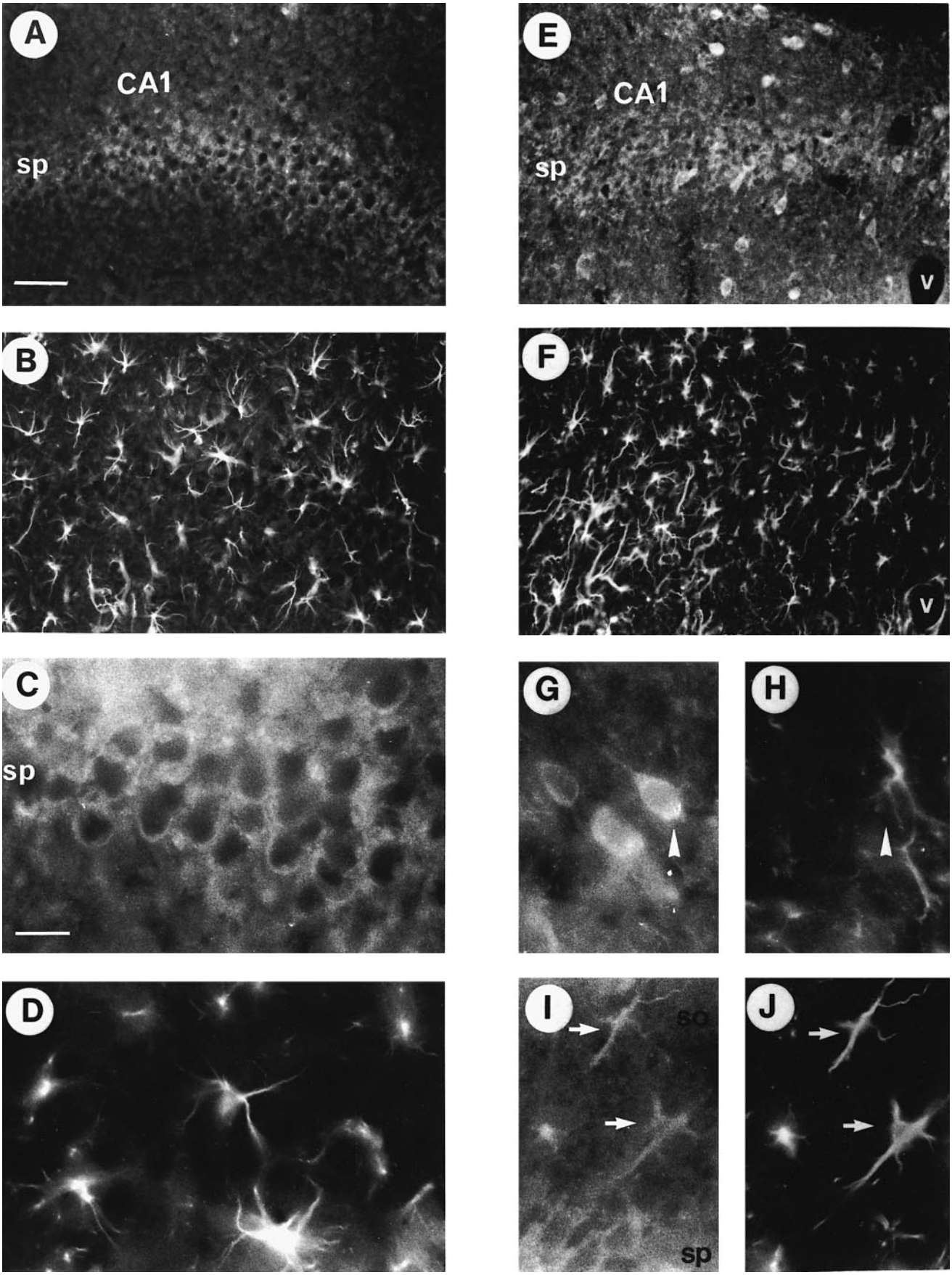


FIG. 6. Cyclin D1 protein is expressed in neurons of the cortex and thalamus, and upregulated after transient global ischaemia. Cyclin D1 immunoreactivity on coronal sections through the cortex (A, C) and the thalamus (B, D) before (A, B) and 72 h after ischaemia (C, D). (A), (B) The cyclin D1 immunoreactivity is restricted to the cytoplasm of neurons, while in (C) and (D) the immunoreactivity is increased in the cytoplasm and present in the nucleus (black arrowhead in C). Scale bars in (A), (C) = 6 μm and (B), (D) = 380 μm.

FIG. 5. Cyclin D1 protein is expressed in neurons of the hippocampus and is increased after ischaemia. Cyclin D1 immunoreactivity (A–C, E–H and J) on coronal hippocampal sections. (D), (I) Corresponding Nissl-stained sections of the CA1 region. (A)–(E) Sham-operated rats; (F)–(J) 72 h after ischaemia. (B), (C) Magnifications of (A) and (G); (H), (J) magnifications of (F). (E) The polyclonal cyclin D1 antibody was preadsorbed with the cyclin D1 peptide. (C) Note the restricted cytoplasmic D1 immunoreactivity in pyramidal neurons of the CA1 area in control animals compared to the increased immunoreactivity in pyramidal neurons after ischaemia (H). Note that cyclin D1 labelling is no more restricted to the cytoplasm but is also present in the nuclei (black arrowheads). (D) The pyramidal layer of CA1 is intact, while in (I) some neurons already exhibit nuclear condensation (black arrowheads). Abbreviations: sg, stratum granulosum; sp, stratum pyramidale; CA1 region of the hippocampus; CA3 region of the hippocampus; h, hilus of the hippocampus. Scale bars in (B), (G) = 25 μm; (C), (D), (H), (I) = 12.5 μm.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gelatine 0.5%/BSA 1%/triton 0.2%. The following day, sections were rinsed in PBS, incubated for 2 h with a biotinylated goat anti-rabbit antibody diluted 1:400 (Vector, Burlingame, CA, USA) at RT and rinsed with PBS. The sections were then incubated for 1 h at RT in Vectastain ABC peroxidase standard solution (Vector), rinsed in PBS and stained for 10 min using diaminobenzidine (DAB). Controls were performed omitting the first antibody and by neutralizing the cyclin D1 antibody with a 10-fold excess (weight/weight) of immunizing peptide for 1 h at 30 °C. Following neutralization, the protocol described above was used.

To confirm that the increased cyclin D1 immunoreactivity in ischaemic rat brains was specific, we also used a mouse monoclonal anti-cyclin D1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 (IgG = 0.1 μg/mL) incubated overnight at RT. Sections were revealed using $2^0/_{00}$ $NiSO_4$ DAB as chromogen. Controls were performed omitting the first antibody or by using a non-immune mouse-purified immunoglobulin IgG (Sigma) at the same working concentration as the cyclin D1 monoclonal antibody.

Double labelling combining cyclin D1 and neural as well as nuclear markers

Cyclin D1 immunofluorescence was performed on floating sections prepared from the brains of ischaemic and sham-operated rats at 72 and 96 h after recirculation. Briefly, tissues were blocked, the sections were incubated with the cyclin D1 rabbit polyclonal antibody (see above) diluted 1:500 overnight at 4 °C. The following day, sections were rinsed and incubated for 1 h with a biotinylated goat anti-rabbit antibody and rinsed with PBS. The sections were then incubated for 30 min at RT in Avidin D-FITC (Vector) diluted 1:400, or Avidine D-Rhodamine (Vector) diluted 1:400 rinsed in PBS and mounted. Different neural markers were used: an anti-GFAP mouse monoclonal, diluted 1:500 (Sigma) to label astrocytes, revealed by an anti-mouse TRITC diluted 1:400; isolectin B4 (Bandeirae Simplicifolia)-FITC labelled (Sigma) at 20 μg/mL to identify microglial cells; Hoechst 33258 (1 μg/mL) to label nuclei. For double staining of cyclin D1 and GFAP, both antibodies were co-incubated overnight followed by incubation with the biotinylated goat anti-rabbit antibody, and subsequently by Avidine D-FITC combined with anti-mouse TRITC; for cyclin D1 and lectin B double staining, the cyclin D1 immunoreaction procedure described above was revealed by Avidine D-Rhodamine and sections were incubated for 2 h at RT in PBS/triton 0.1%/lectin-FITC, rinsed in PBS and mounted; for the cyclin D1/Hoechst double staining, the cyclin D1 immunoreaction was revealed by Avidine D-FITC and sections were incubated for 2 min in Hoechst/PBS, rinsed in PBS and mounted. Controls were performed omitting the first antibody (D1 or GFAP) or both of them (D1 and GFAP).

Combined TUNEL or immunohistochemistry and in situ *hybridization for cyclin D1 mRNA*

In situ hybridization was performed as above except that the dehydration and delipidation steps were performed after the immunohistochemistry or TUNEL procedures. Following the last wash in 0.1 × SSC, hybridized sections (24, 72 h after recirculation) were incubated with antibodies against different cell type markers, or processed for *in situ* end labelling of fragmented DNA as described above.

For the TUNEL staining (72 h after recirculation), tissues were processed according to the method of Gavrieli *et al*. (1992). Briefly, sections were permeabilized overnight in 1% Triton X-100 in PBS, treated with proteinase K for 8 min at RT. Endogenous peroxidases were inactivated with 0.1% H_2O_2 for 10 min at RT. Terminal deoxynucleotidyl Transferase (TdT, Gibco BRL, Gaithersburg, MD, USA) and biotinylated dUTP were then added to the sections. These were incubated in a humid atmosphere at 37 °C for 90 min. The reaction was terminated by transferring the slides in 2 × SSC for 15 min at RT. After 10 min immersion in 2% bovine serum albumin, the sections were incubated with the streptavidin–biotine peroxidase complex (Vector) for 2 h at RT. Labelling was revealed with diaminobenzidine/$2^0/_{00}$ $NiSO_4$. Controls were performed on sham-operated rats. Controls for eventual background were assessed by omitting the TdT. After revelation, sections were processed for autoradiography. When $NiSO_4$ DAB was used, sections were covered with a thin film of 1% Pro-Celloidine (Fluka, Switzerland) diluted in 50% ethanol/50% diethyl-ether (v/v) to protect the emulsion (NTB2, Kodak) from interaction with $NiSO_4$ DAB.

Pathology

Sections adjacent to those processed for *in situ* hybridization and immunohistochemistry were stained with cresyl violet to evaluate cell pathology following ischaemia and seizures.

Results

Neuronal damage following ischaemia or kainate induced seizures

Three days after transient forebrain ischaemia, Nissl staining revealed typical morphological signs of delayed neuronal death in the hippocampal CA1 pyramidal layer, and in the subiculum (Fig. 5I compared to 5D) in agreement with data already published elsewhere (Pulsinelli *et al*., 1982). At 5 days post-ischaemia, a loss of staining was clearly visible in the pyramidal layer of the CA1 region.

We included in our study another *in vivo* model of excitotoxic delayed neuronal death wherein kainate-induced seizures were associated with selective degeneration of the CA3 pyramidal neurons (Ben-Ari *et al*., 1980). Nissl staining revealed clear morphological signs of neuronal death in the CA3 pyramidal layer 16 h after kainate, hence earlier than the ischaemia-induced lesions.

Upregulation of cyclin D1 mRNA in vulnerable regions of neuronal death following forebrain ischaemia

On autoradiograms, cyclin D1 mRNA was barely detectable in the brains of sham-operated animals (Fig. 1A). At 48 h post-ischaemia,

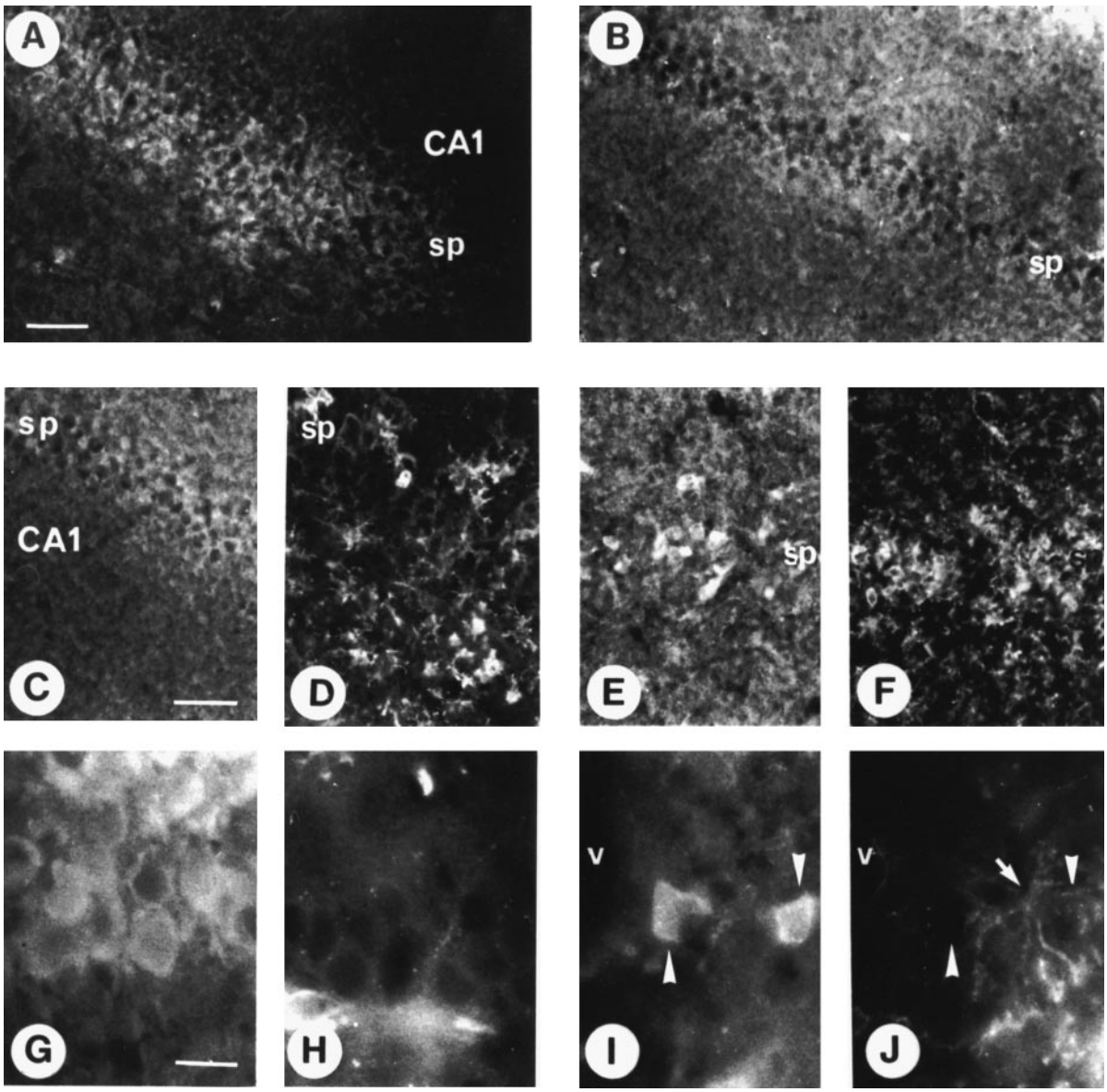
FIG. 7. Expression of cyclin D1 protein in astrocytes after transient global ischaemia. Double labelling experiments on hippocampal coronal sections. (A)–(D) Sham-operated rats; (E)–(J): 96 h after ischaemia. Double staining combining immunofluorescence of cyclin D1 (A, C, E, G, I) and GFAP (B, D, F, H, J). (C), (D) Magnifications of (A) and (B), respectively. (G), (I) Magnifications of (E). (H), (J) Magnifications of (F). In the stratum pyramidale (sp) of CA1 note the increased GFAP immunoreactivity after ischaemia (F) compared to control (B). (F) A vessel (V) is surrounded by an astrocyte. In control animals, the cyclin D1 positive cells in the pyramidal layer are labelled only in the cytoplasm with an absence of signal in the nucleus (C); these cells are not co-labelled with GFAP (D). Following ischaemia, pyramidal neurons of the stratum pyramidale (sp) of CA1 express cyclin D1 in the cytoplasm and the nucleus (G, white arrowhead), while the GFAP-labelled cells are not labeled in the nucleus (H, white arrowhead). In the stratum oriens (so), white arrows point to cyclin D1 positive cells (I) that co-express GFAP (J). Scale bars in (A), (B), (E), (F) = 21 μm and (C)–(J) = 7 μm.

cyclin D1 mRNA was induced in the hilus of the hippocampus, near the granular cell layer, a region rich in somatostatinergic interneurons whose death precedes that of the CA1 pyramidal neurons (Johansen *et al.*, 1987). At 72 h, cyclin D1 mRNA was detected in the subiculum, in the medial part of the CA1 region and in the thalamus. At 96 h (Fig. 1D), the signal extended to the lateral part of the CA1 area, and was obvious in the thalamus and the deep layers of the neocortex; the signal was also detectable, although less markedly, in other hippocampal layers (CA3 pyramidal layer and granular cell layer). At 5 days, labelling had disappeared in all hippocampal layers. The increased labelling observed in the CA1/subiculum region was confirmed by optical density quantification on the autoradiograms (Fig. 2). The cyclin D1 hybridization signal was increased in the CA1 and subiculum at 48 h (70 and 100%, respectively), and was maximal at 72 h (100 and 150%, respectively). Labelling decreased at 96 h and thereafter presumably due to the loss of the pyramidal cells. These results were statistically significant at 72 h in the CA1/subiculum area and in the subiculum at 48 and 96 h as compared to control values. At 48 h, there was a statistically significant 50% increase of cyclin D1 labelling in the granular and CA3 pyramidal layers. However, this increase was transient and returned to control values in the non-vulnerable areas.

Cyclin D2 and D3 mRNAs were detected in the hippocampi of sham-operated rats (Fig. 1B,C). The cyclin D2 signal was prominent in area CA3 (Fig. 1B), while the cyclin D3 signal was prominent in the dentate gyrus (Fig. 1C). At 96 h post-ischaemia, cyclins D2 (Fig. 1E) and D3 (Fig. 1F) were not upregulated in the CA1 region. The strong cyclin D2 and D3 labelling in the CA3 sector and granular cell layer, respectively, persisted at 48, 72, 96 h and 5 days after ischaemia. In contrast with cyclin D1, no increased signal was detected at different times post-ischaemia in the striatum, cortex or thalamus.

Upregulation of cyclin D1 mRNA in vulnerable regions of neuronal death after kainate-induced seizure

On autoradiograms, cyclin D1 mRNA was not detected in the brains of sham-injected animals (Fig. 3A). At 24 h after kainate injection, cyclin D1 mRNA was induced in area CA3 (Fig. 3B), persisted at 3 days (Fig. 3C) and had disappeared at 14 days (Fig. 3D). A less intense signal was also observed in the stratum granulosum (Fig.



3B,C) as well as in the CA1 stratum pyramidale (Fig. 3C). In general, the labelling in the hippocampus was ipsilateral to the injected amygdala, but in some cases (data not shown) it was detected bilaterally, probably due to the propagation to the contralateral side of kainate-induced epileptiform activity. Cyclin D1 labelling was also detected at 24 h and 3 days in the amygdala, around the site of kainate injection (Fig. 3C), and the thalamus at 24 h (Fig. 3B).

Cyclin D2 and D3 mRNAs were detected in the hippocampi of sham-injected rats (Fig. 3E,I). The cyclin D2 signal was prominent in area CA3 (Fig. 3E), while the cyclin D3 signal was prominent in the dentate gyrus (Fig. 3I). Twenty-four hours and 3 days after the onset of seizures, cyclin D2 labelling faded in area CA3, ipsilateral to the side of injection, due to CA3 pyramidal cell loss (Fig. 3F–H). Similarly, the cyclin D3 signal started to fade away in CA3 from 24 h onwards and had virtually disappeared at 14 days (Fig. 3J–L).

Comparison of cyclin D1 mRNA expression in vulnerable regions of neuronal death after ischaemia and kainate-induced seizure on emulsion-coated sections

Following ischaemia, no hybridization signal was detected at 24 h post-ischaemia in the hippocampus on emulsion-coated sections (Fig. 4A). A scattered spotty labelling was detected at 48 h in the stratum oriens, stratum radiatum and alveus (Fig. 4B); a more homogeneous, concentrated signal was observed in the hilus, at the inner lining of the granular cell layer (Fig. 4B). At 72 h, a homogeneous labelling was observed in the neuronal layer of the subiculum and the medial part of the CA1 stratum pyramidale (Fig. 4C). However, at this time point there was individual variability in the extent of CA1 labelling. At 96 h (Fig. 4D), the spotty labelling persisted in the entire CA1 region; the homogeneous signal faded in the neuronal layer of the subiculum while it extended toward the lateral part of the CA1 stratum pyramidale.

In the kainate-treated animals, no signal was detected 8 h after kainate injection on emulsioned sections (Fig. 4E–H). At 16, 24 and 72 h post-kainate (Fig. 4F–H) labelling was apparent in the vulnerable CA3 stratum pyramidale.

Increased cyclin D1 mRNA is associated with increased cyclin D1 protein

Cyclin D1 immunohistochemistry was performed with a polyclonal (Figs 5 and 6) and monoclonal antibody (not shown). In the hippocampus (Fig. 5A,B), the cortex (Fig. 6A) and thalamus (Fig. 6B) of sham-operated rats, a basal expression of cyclin D1 protein, as revealed by the polyclonal antibody, was identified in the cytoplasm of most neurons. At 24 h after ischaemia, in the hippocampus, no obvious increased signal was detected in the strata pyramidale and

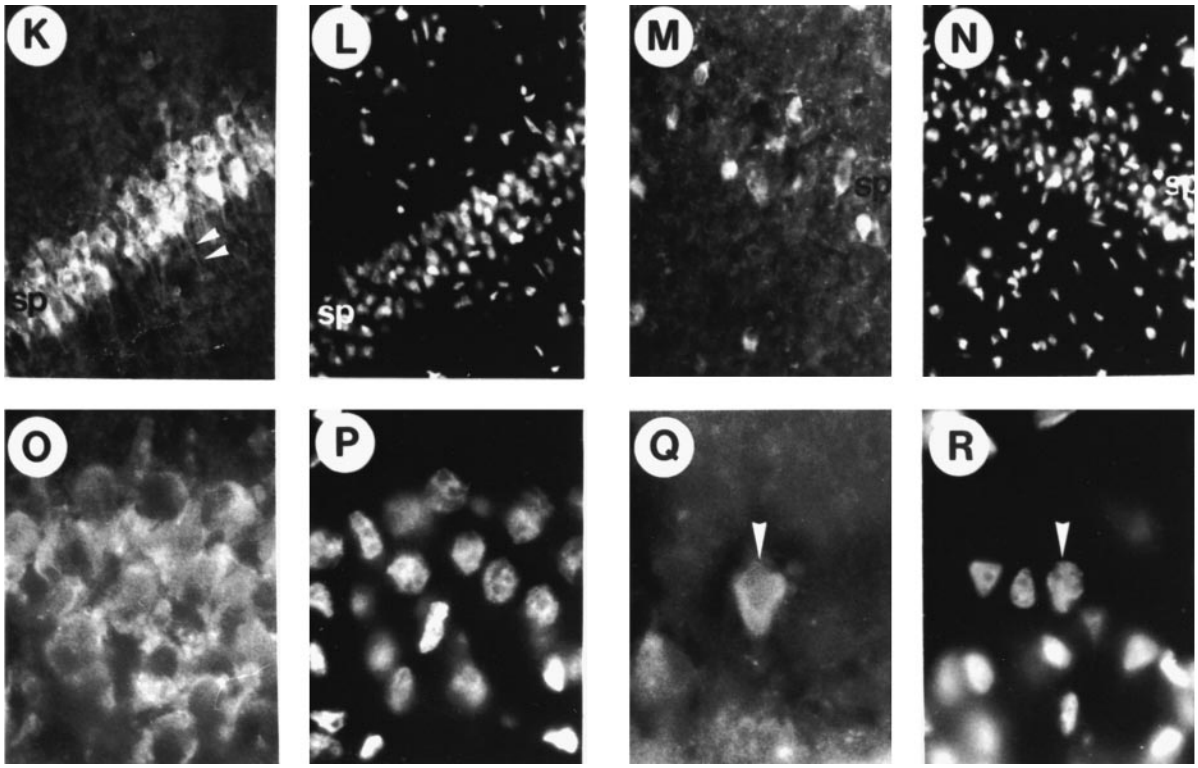


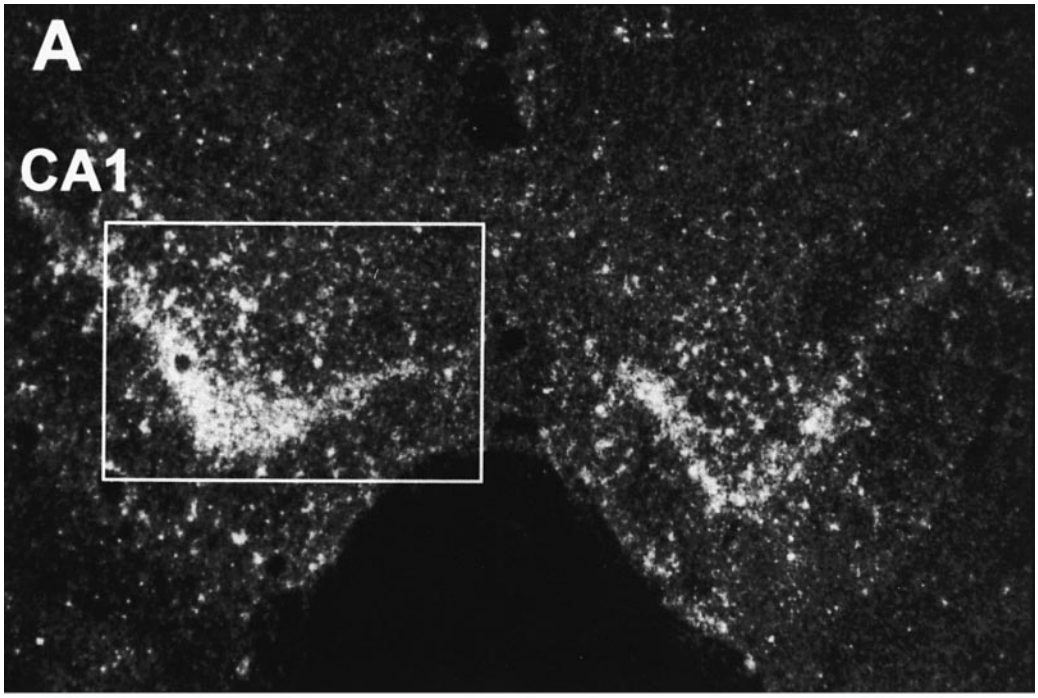
FIG. 8. After transient global ischaemia, the peak of cyclin D1 protein expression precedes complete microglial invasion of the stratum pyramidale. Double labelling experiments on hippocampal coronal sections. (A), (B) Sham-operated rats; (C)–(R) 72 h after ischaemia. (A)–(J) Double staining combining cyclin D1 immunofluorescence (A, C, E, G, I) and isolectin B4 (B, D, F, H, J), a microglial marker. (G), (H) Magnification of (C), (D) and (I), (J) magnification of (E), (F), respectively. Note the absence of microglial marker expression before ischaemia (B), while after ischaemia, the lectin B labelling is increased (D, F). Many cyclin D1-positive cells (G), but only a few microglial cells are visible in the stratum pyramidale (D, H). In the stratum pyramidale (sp) of animals with more severe hippocampal damage, only a few cells express cyclin D1 (E, I, white arrowheads). The microglial reaction (F) is higher than in (D) but still no cyclin D1/isolectin B4 co-staining is visible (I, J, white arrowheads for cyclin D1 and white arrow for microglia). Note in (J) the isolectin B4 microglial labelling in the vessel (v). (K)–(R) Double staining combining cyclin D1 immunofluorescence (K, O, M, Q) and Hoechst staining (L, N, P, R), a nuclear marker. (O), (P) Magnification of (K), (L) and (Q), (R) magnification of (M), (N), respectively. Many neurons (K, O) express cyclin D1 and the cellular density outside the stratum pyramidale is low (L). In animals with more severe hippocampal damage, few cells express cyclin D1 (M, Q) in the stratum pyramidale (sp), and the cellular density outside the stratum pyramidale is high (N). The white arrowhead points to a cyclin D1-positive cell in the stratum pyramidale, with cytoplasmic and nuclear staining (Q), but no nuclear condensation (R). Note in (K) the cyclin D1 staining of the apical dendrite of a pyramidal neuron (white arrowheads). Scale bars in (A)–(F) and (K)–(N) = 22 μm; (G)–(J) and (O)–(R) = 7 μm.

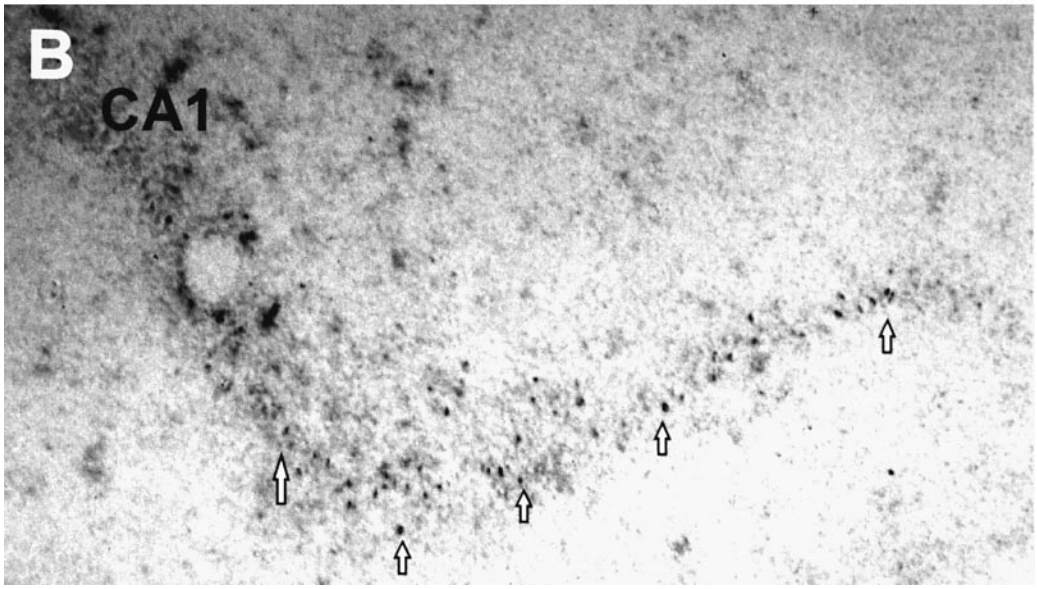
granulosum. In the hippocampus, 72 h after ischaemia, an increased cyclin D1 immunoreactivity was clearly visible in neurons of the strata pyramidale and granulosum. The apical dendrites of pyramidal neurons were also immunostained in the ischaemic brains (Figs 5G and 9K). The morphology of neurons expressing cyclin D1 was preserved, and no signs of apoptosis, e.g. cell shrinkage or nuclear condensation, were observed. After ischaemia, scattered cells were stained in the stratum oriens (Fig. 5G). Seventy-two hours after ischaemia, the cyclin D1 labelling was no longer restricted to the cytoplasm but was also detected in cell nuclei (Fig. 5H and also see Fig. 9Q). This nuclear staining was identified in the vulnerable pyramidal neurons of CA1 and also in the CA3 pyramidal and granular neurons (Fig. 5F,J). Neutralization of the cyclin D1 antibody with the corresponding control peptide (Fig. 5E) as well as omission of the primary antibody (not shown) abolished staining. The cortex and thalamus are two regions of neuronal death. In these structures, 72 h after ischaemia, an increased cyclin D1 immunoreactivity was clearly visible in neurons, both in the cytoplasm and the nucleus (Fig. 6C, black arrowhead; Fig. 6D).

Similar patterns of expression were observed when using a monoclonal cyclin D1 antibody at 72 and 96 h (data not shown).

Expression of cyclin D1 in non-neuronal cells

To assess whether astrocytes also expressed cyclin D1, double stainings for cyclin D1 and GFAP proteins (Fig. 7) were performed. In sham-operated rats, no cyclin D1/GFAP-positive cells were identified in the stratum pyramidale (Fig. 7C,D). Immunostaining for GFAP at 72 h (not shown) and 96 h (Fig. 7B) after ischaemia showed a glial reaction in the stratum oriens and radiatum as well as in the stratum pyramidale (Fig. 7B). At 96 h, in the stratum oriens, double immunostainings with the cyclin D1 and GFAP antibodies revealed astrocytes expressing cyclin D1 (Fig. 7I,J).





Seventy-two hours after ischaemia, few cells were double labelled with the cyclin D1 cRNA probe and the GFAP antibody. The bodies of the large GFAP-positive cells exhibited dense silver grains surrounded by a filamentous GFAP staining (not shown).

To assess whether microglial cells expressed cyclin D1, double stainings with a cyclin D1 antibody and lectin B were performed (Fig. 8A–J). In sham-operated rats, no microglial cells were detected in the stratum pyramidale (Fig. 8B). Seventy-two hours after ischaemia, lectin B staining revealed an intense microglial reaction around and within the stratum pyramidale of CA1 (Fig. 8D,F). While numerous cyclin D1-positive cells were detected in the stratum pyramidale (Fig. 8C,G), only a few microglial cells were visible (Fig. 8D,H). In animals with more severe hippocampal damage, where few remaining cells in the stratum pyramidale expressed cyclin D1 (Fig. 8E,I), the microglial reaction (Fig. 8F,J) was greater than in less damaged hippocampus (Fig. 8D). However, once again, no co-labelling was detected in the stratum pyramidale (Fig. 8I,J).

Glial and microglial reactions were also assessed indirectly with the Hoechst staining, a dye for the nuclei of all cell types. When after ischaemia, many neurons expressed cyclin D1 (Fig. 8K,O), the cellular density revealed by Hoechst outside the stratum pyramidale was low (Fig. 8L,P). In contrast, when few cells expressed cyclin D1 protein (Fig. 8M,Q), the cellular density outside the stratum pyramidale was high (Fig. 8N,R).

Time window of neuronal cyclin D1 expression

In order to study the time window of cyclin D1 expression in the process of neuronal demise following ischaemia, we analysed (i) cell morphology using cresyl violet staining on adjacent sections, (ii) the state of the nuclei using Hoechst staining, and (iii) assessed chromosomal DNA fragmentation using TUNEL staining. Seventy-two hours after ischaemia, cresyl violet staining and cyclin D1 immunohistochemistry on adjacent sections revealed that when neurons expressed cyclin D1, cresyl violet staining was still prominent. In contrast, in animals with more severe hippocampal damage, the loss of cresyl violet staining was associated with low levels of cyclin D1 immunoreactivity (data not shown). Combining Hoechst staining of the nuclei with cyclin D1 immunofluorescence (Fig. 8K–R) indicated that in general the cyclin D1 protein was expressed in cells whose nucleus was not yet condensed (Fig. 8Q,R). Combining *in situ* hybridization with TUNEL at 72 h post-ischaemia indicated that while numerous cells were densely labelled by the cyclin D1 probe in the subiculum and CA1 pyramidal layer (Fig. 9A), only a few cells were TUNEL positive (Fig. 9B,C).

Discussion

Our results indicate that cyclin D1 mRNA, but not D2 or D3, are upregulated in regions of neuronal death, notably the CA1 and CA3 areas after ischaemic and epileptic insults, respectively. In contrast, in resistant neurons, cyclin D1 was upregulated transiently and to a lesser extent. Furthermore, cyclin D1 immunoreactivity, normally expressed in the cytoplasm of mature neurons, was increased in the cytoplasm and the nucleus of neurons after ischaemia. Double labelling experiments indicated that although cyclin D1 protein was also induced in astrocytes, it was not present in reactive microglial cells. Finally, we showed that cyclin D1 induction in vulnerable neurons occurred mainly before nuclear condensation and the appearance of chromosomal DNA fragmentation. These observations suggest that cyclin D1 upregulation may be an important signal in the molecular cascade of events that lead to excitotoxic cell death.

Upregulation of cyclin D1 mRNA and protein after excitotoxic neuronal death

While the cyclin D1 protein was detected in the cytoplasm of granule and pyramidal cells, basal expression of cyclin D1 mRNA was barely detectable in the hippocampi of control animals in agreement with Wiessner *et al*. (1996) who failed to detect cyclin D1 mRNA in the hippocampus using oligonucleotide probes. In embryonic brains used as positive controls, the riboprobe we used in this study revealed a strong labelling in the ventricular zone and retinas (Timsit *et al*., 1995), in agreement with Sicinski *et al*. (1995). The widespread

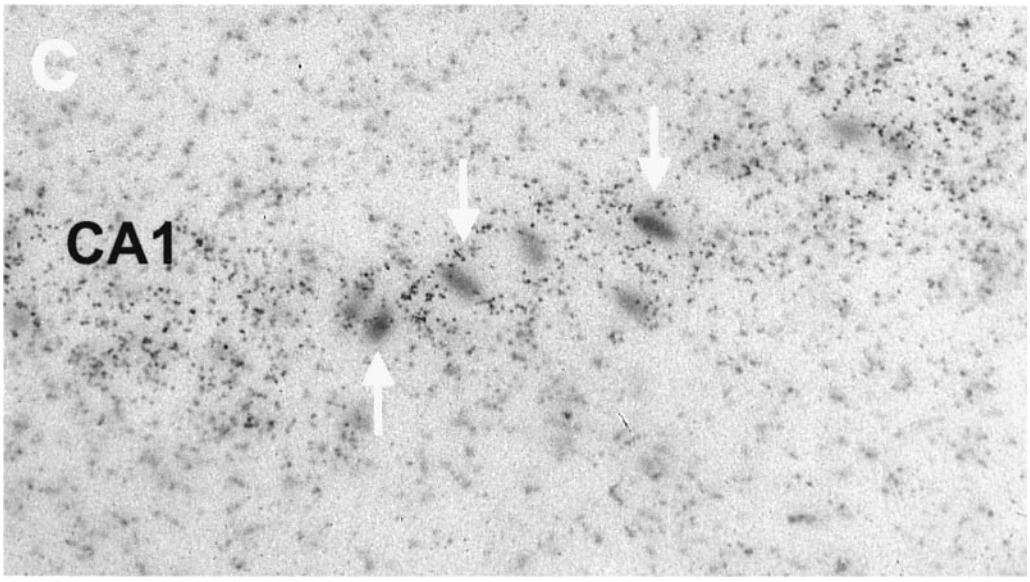


FIG. 9. Cyclin D1 mRNA is expressed mostly in TUNEL-negative cells. Double staining combining cyclin D1 *in situ* hybridization and TUNEL staining 72 h following transient global cerebral ischaemia. Coronal sections through the hippocampus. (A) Dark field photomicrograph of an emulsioned section showing the white silver grains of the cyclin D1 labelling in the subiculum/CA1 region of the hippocampus. (B) Higher magnification of the box shown in (A), bright field photomicrograph showing TUNEL-positive cells (small arrows). (C) Magnification of (A) and (B). Note that TUNEL-positive cells (white arrows) are devoid of silver grains.

distribution of cyclin D2 and D3 mRNA, and of cyclin D1 protein in granular and pyramidal cells of the hippocampus suggests a role for cyclins D in terminally differentiated neurons. Whether cyclin D2 and D3 protein are targeted to the nucleus or not remains to be determined. In agreement with our results, cyclin D2 mRNA was shown to be expressed in post-mitotic neurons during and after embryogenesis (Ross *et al*., 1996).

In ischaemic rats, we find that cyclin D1 mRNA is selectively upregulated (over 100%) in regions of neuronal death, but neither cyclin D2 or D3 mRNA were enhanced. Wiessner *et al*. (1996) also reported an increase in cyclin D1 mRNA after global ischaemia in the CA1 region. After kainate-induced seizures, an upregulation of cyclin D1 mRNA was observed in the vulnerable CA3 area, 16 h after the onset of seizures, hence earlier than in the ischaemia model. This is of interest as in the seizure model that we utilized, apoptosis is observed at the ultrastructural level from 18 h on (Pollard *et al*., 1994). Again, cyclin D1 mRNA was also upregulated, albeit at lower levels, in the resistant stratum pyramidale CA1 and in the stratum granulosum. In another model of kainic acid treatment, Liu *et al*. (1996) showed increased cyclin D1 immunoreactivity in the entire hippocampus. Our observations cannot be ascribed to astrocytic and microglial reactions, as in our model astrocytic and microglial reactions only start 3 days after kainate injection (Represa *et al*., 1995). Most likely this upregulation of cyclin D1 mRNA following seizures occurs in degenerating CA3 pyramidal neurons.

Increased cyclin D1 mRNA expression in regions of neuronal death paralleled increased cyclin D1 protein at a time when a major drop in global protein synthesis has been described (Dienel *et al*., 1980; Nowak, 1985; Thilmann *et al*., 1986). Only a few proteins are known to be synthesized during ischaemia. Some of them, e.g. bax, p53, fos, jun (for review see MacManus & Linnick, 1997) are considered to be involved in apoptosis. In this setting, the increased cyclin D1 protein in neurons scheduled to die stresses the potential role of cyclin D1 in neuronal death.

Expression of cyclin D1 in astrocytes following ischaemia

Double labelling experiments using a GFAP antibody revealed that besides neurons, astrocytes expressed cyclin D1 mRNA and protein. The increase of cyclin D1 in astrocytes could be related either to proliferation or glial reaction. In contrast, double labelling experiments with isolectin B4 did not reveal any expression of cyclin D1 in activated microglial cells. Furthermore, the peak of cyclin D1 protein expression in the stratum pyramidale preceded its complete invasion by microglial cells and occurred mainly in neurons. Our results differ from those of Wiessner *et al*. (1996) showing cyclin D1 mRNA expression in Ox42-positive cells after ischaemia. This discrepancy might be explained either by the absence of cyclin D1 mRNA translation in microglial cells or by the different approaches that were used. In agreement with our results, Li *et al*. (1997) showed that cyclin D1 protein expression was not increased in microglial cells identified with isolectin B4 following focal cerebral ischaemia. Taking into account these data, we conclude that in our experimental model of ischaemia, cyclin D1 is mainly expressed in two cell types: neurons and astrocytes.

Cyclin D1 immunoreactivity has a defined time-window of induction in neurons in the vulnerable areas following ischaemia

We attempted to define the time window during which cyclin D1 was induced in neurons scheduled to die. Cyclin D1 protein was expressed beyond 24 h after ischaemia. Seventy-two hours after ischaemia, in hippocampi with severe damage that showed poor Nissl staining, cyclin D1 immunoreactivity was low in the remaining neurons. In contrast in hippocampi with moderate damage, neurons exhibited Nissl staining and cyclin D1 immunoreactivity was high. The morphology of these cyclin D1-positive neurons was well preserved. The nucleus in these cells was not condensed and cyclin D1 mRNA accumulated essentially in TUNEL-negative cells. Similarly, Li *et al*. (1997) did not find cyclin D1 protein in TUNEL-positive cells following focal ischaemia. Hence, cyclin D1 is expressed in neurons commited to die while retaining their morphological integrity and mainly at a stage preceding DNA condensation and fragmentation. Our observations are in agreement with those of Deshmuckh *et al*. (1996) showing that cyclin D1 is increased in neurons commited to die despite the use of caspase inhibitors that prevent neuronal apoptosis. This indicates that cyclin D1 expression precedes the commitment and execution phases of cell death associated with caspase activation.

Increased cyclin D1 expression in neuronal death: cause or effect?

In vivo, direct evidences on the role of cyclin D1 during apoptosis are lacking. In mouse Lurcher and Staggerer cerebellar mutants, granular cells and inferior bulbar neurons commited to die express cyclin D and proliferating cell nuclear antigen (PCNA) and incorporate BrdU, suggestive of a successful entry into S phase prior to cell death (Herrup & Busser, 1995). Following focal cerebral ischaemia, Li *et al*. (1997) showed that cyclin D1 protein was increased in neurons. However, these were not morphologically identified as apoptotic or necrotic, leading to the conclusion that cyclin D1 might be associated with DNA repair in neurons that are not irreversibly damaged. This may indeed be the case in our ischaemia model in the CA3 pyramidal neurons and dentate gyrus where we find a transient increase in cyclin D1 expression, of lower magnitude than in the vulnerable regions. In the ischaemia model used in our study, we show that in the vulnerable areas, e.g. CA1, cyclin D1 is expressed at high levels in most neurons scheduled to die. Importantly, the increase is dramatic and sustained in their nuclei. This nuclear localization of cyclin D1 is reminiscent of that in cells that progress from the G1 to S phase (Sewing *et al*., 1993). Based on the differences we observe in the cyclin D1 increase between the vulnerable and resistant neurons, we propose that increased nuclear cyclin D1 contributes to delayed neuronal death through a gene dosage effect. As a protooncogene (Sherr, 1996), cyclin D1 fulfills the criteria defined by Heintz (1993), suggesting that in differentiated neurons, PCD is an alternative effector pathway induced by the same molecular events leading to neoplastic transformation in dividing cells. There is now a large body of evidence to suggest that cell cycle proteins are involved in neuronal cell death (reviewed in Khrestchatisky *et al*., 1996). Importantly, *in vitro* studies also suggest that increased cyclin D1 expression might play a critical role in PCD. Park *et al*. (1997) showed that cyclin-dependent kinase inhibitors as well as dominant negative cdk 4 and cdk6 promote the survival of NGF-deprived sympathetic neurons. Furthermore, Kranenburg *et al*. (1996) showed that increased expression of cyclin D1 in a neuroblastoma cell line is able to induce a Rb-dependent cell death with all the characteristics of PCD. Evidence has recently emerged indicating that Rb cleavage near the C-terminus is a crucial event in apoptosis and that the regions cleaved are binding sites for E2F-1, MDM2 and cyclins D (Jänicke *et al*., 1996). Because increased expression of cyclin D1 occurs in neurons before morphological features of apoptosis, e.g. nuclear condensation, cyclin D1 might belong to a group of apoptosis modulators. These include p53, bax, bad, bcl-2, bcl-X_L, gadd45 and PCNA, shown to be expressed before cell death commitment

(MacManus & Linnick, 1997), and with which cyclin D1 could be involved. For example, p53 modulates cyclin D1 activity, by controlling cyclin D1 expression (Spitkovsky *et al*., 1995), or by controlling p21 expression, a cyclin D1 inhibitor (for review see Sherr, 1993).

Taken together, the *in vivo* and *in vitro* observations reported above suggest that increased nuclear cyclin D1 could promote unscheduled progression of differentiated neurons into the G1 phase of the cell cycle, and could participate, in combination with other apoptosis modulators, in their commitment to cell death.

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Abbreviations

DAB, diaminobenzidene; PBS, phosphate-buffered saline; PCD, programmed cell death; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde.

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