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Neuronal injury and expression of 72-kDa heat-shock protein after forebrain ischemia in the rat*

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Summary. We evaluated the relationship between the induction of the 72-kDa heat-shock protein (hsp 72) and the presence of necrotic neurons in the rat hippocampus, 48 h after an 8-min episode of forebrain ischemia in eight rates. Hsp 72 was detected using the monoclonal antibody C92 on vibratome brain tissue sections. Hematoxylin and eosin (H&E) staining on adjacent paraffin-embedded sections was used to determine histopathological features. All morphologically intact CA1/2 neurons, 70 % of which are destined to become necrotic 7 days after ischemia, exhibited intense hsp 72 staining, while necrotic or damaged neurons were devoid or low in hsp 72. Hsp 72 was also detected in CA3 neurons destined to survive 7 days after ischemia. Blood vessels positive for hsp 72 were detected in focal brain regions, in which severely damaged neurons were either devoid or low in hsp 72 staining. Occasional glial cells expressed hsp 72 in both normal and damaged brain regions. Hsp 72 response to a transient forebrain ischemia seemingly reflects differences in the selective ischemic vulnerability of CA1/2 and CA3 neurons. Further, the presence of hsp 72 within a neuron is likely only a marker of stress and is not necessarily indicative of eventual neuronal survival.

Key words: 72-kDa heat-shock protein – Neuronal necrosis – Forebrain ischemia – Immunohistochemistry – Rat

The induction of non-constitutive heat-shock proteins (hsp) has been thought to confer partial protection against anoxia and a variety of other noxious insults to cells maintained in vitro [3, 7, 8, 12–15, 17, 27]. As a result, there has been considerable interest in eliciting hsp in brain in vivo following transient ischemia [2, 4, 5, 9, 10, 22, 32], although the function of the induced hsp remains obscure. The induction of the 72-kDa hsp (hsp 72) in intact rodent brain has recently been reported [6, 18, 19, 21, 23, 24, 29]. In gerbils, subjected to brief forebrain ischemia/reperfusion, hsp 72 is induced only in neurons and its activity is minimal in hippocampal CA1 neurons destined to die; hsp 72 induction is most pronounced in dentate granule cells and CA3 neurons which are destined to survive [29]. However, recent studies in rats demonstrate a selective temporal accumulation of hsp 72 in vulnerable neurons of the dentate hilus and CA1 following global ischemia/reperfusion [24]. Moreover, hsp 72 has been observed in neurons, glia, and capillary endothelial cells in either infarcted or ischemic areas of the cerebral cortex and caudate-putamen following permanent middle cerebral artery (MCA) occlusion in rats [6]. The distribution and biological significance of hsp 72 induction, and its ability to provide a protective shield for neurons or merely being a convenient marker for early ischemic cell damage, remain to be clarified. We, therefore, investigated the relationship between hsp 72 induction and histologically evaluated cell damage in rat hippocampus following transient forebrain ischemia.

Materials and methods

Animal models

Male Wistar rates (n = 8, 220–300 g) were fasted overnight preceding surgery, but allowed free access to water. The transient forebrain ischemia model used in the present study has been described in detail by Smith et al. [25, 26]. Cerebral blood flow in forebrain structures during ischemia is nearly zero in this model [24]. Following induction of anesthesia with halothane and N_2O:O_2 (70 %:30 %), the animals were intubated and mechanically ventilated to maintain blood gases within physiological range. The core temperature of the animal was maintained at 37 °C by means of a recirculating pad. Forebrain ischemia was induced and maintained for 8 min by a combination of bilateral carotid artery clamping and a reduction of mean arterial blood pressure (n = 6). EEG and ECG were recorded during the 8 min of ischemia and the

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subsequent recovery period. Additional rats were sham-operated without the induction of ischemia ($n = 2$).

Immunohistochemical technique and histopathological evaluation

The immunohistochemical technique used to detect hsp 72 was essentially as described in detail by Vass et al. [29]. Forty-eight hours following recirculation all rats were given an overdose of pentobarbital and the brains were fixed by transcardiac perfusion with 100 mM sodium phosphate buffer (pH 7.4) followed by 4% paraformaldehyde in buffer. Brains were removed and placed in the same fixative overnight at 4 °C. The brains were then transferred to 10 mM phosphate-buffered saline (PBS). Coronal slices (3 mm) of the cerebral hemispheres were made using a rodent brain matrix. Coronal sections (50 μm) were cut on a vibratome and reacted immunohistochemically using a mouse monoclonal antibody C92 (Amersham, RPN 1197, Cleveland, OH) to hsp 72. Biotinylated sheep anti-mouse IgG was used as the second antibody. Sections were incubated with streptavidin "bridge" 1:200 in PBS 1 h, followed by biotinylated horseradish peroxidase 1:400 in PBS 1 h. Peroxidase was detected with diaminobenzidine. Sections were

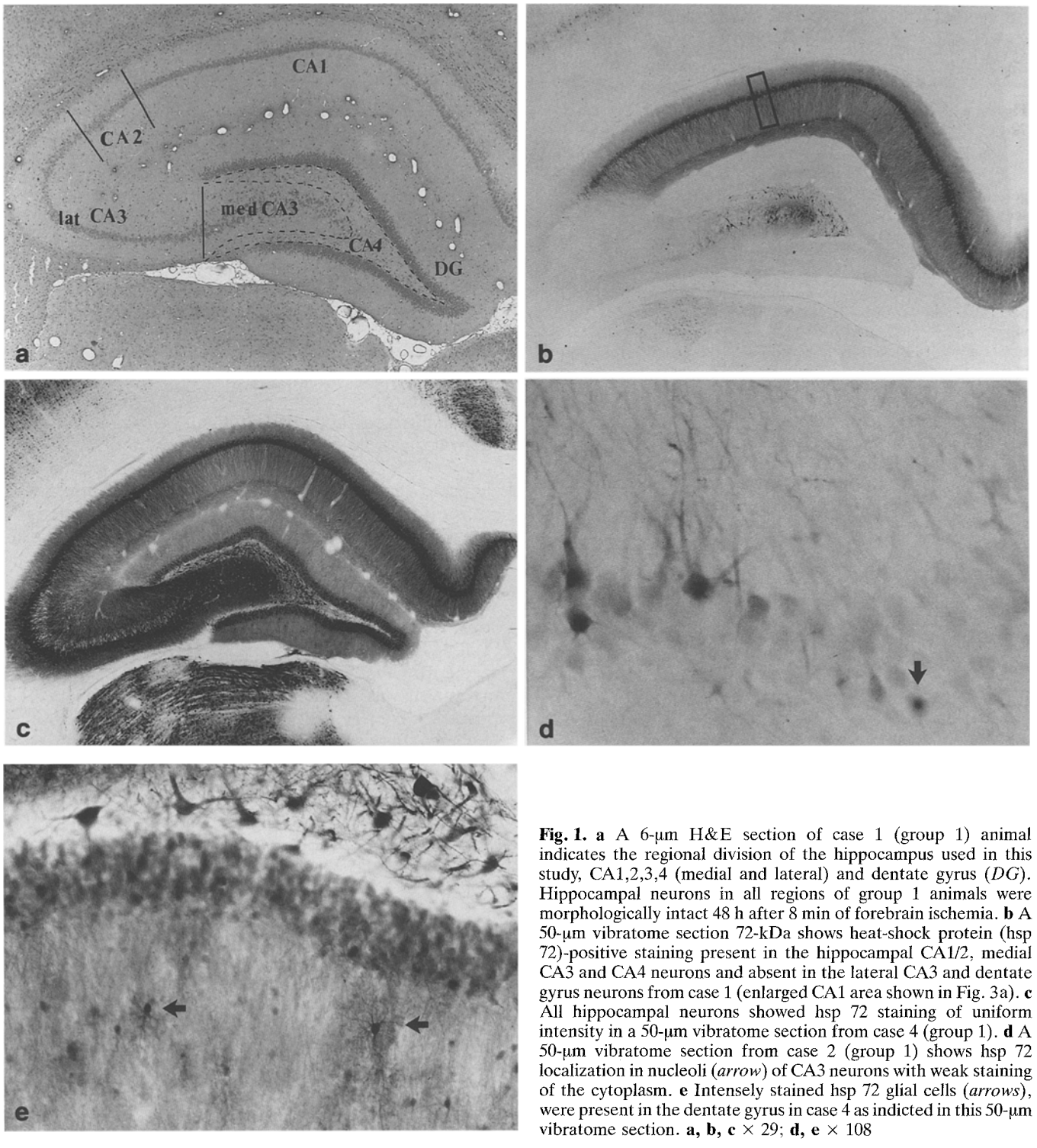

Fig. 1. a A 6-μm H&E section of case 1 (group 1) animal indicates the regional division of the hippocampus used in this study, CA1,2,3,4 (medial and lateral) and dentate gyrus (*DG*). Hippocampal neurons in all regions of group 1 animals were morphologically intact 48 h after 8 min of forebrain ischemia. **b** A 50-μm vibratome section 72-kDa shows heat-shock protein (hsp 72)-positive staining present in the hippocampal CA1/2, medial CA3 and CA4 neurons and absent in the lateral CA3 and dentate gyrus neurons from case 1 (enlarged CA1 area shown in Fig. 3a). **c** All hippocampal neurons showed hsp 72 staining of uniform intensity in a 50-μm vibratome section from case 4 (group 1). **d** A 50-μm vibratome section from case 2 (group 1) shows hsp 72 localization in nucleoli (*arrow*) of CA3 neurons with weak staining of the cytoplasm. **e** Intensely stained hsp 72 glial cells (*arrows*), were present in the dentate gyrus in case 4 as indicted in this 50-μm vibratome section. **a, b, c** × 29; **d, e** × 108

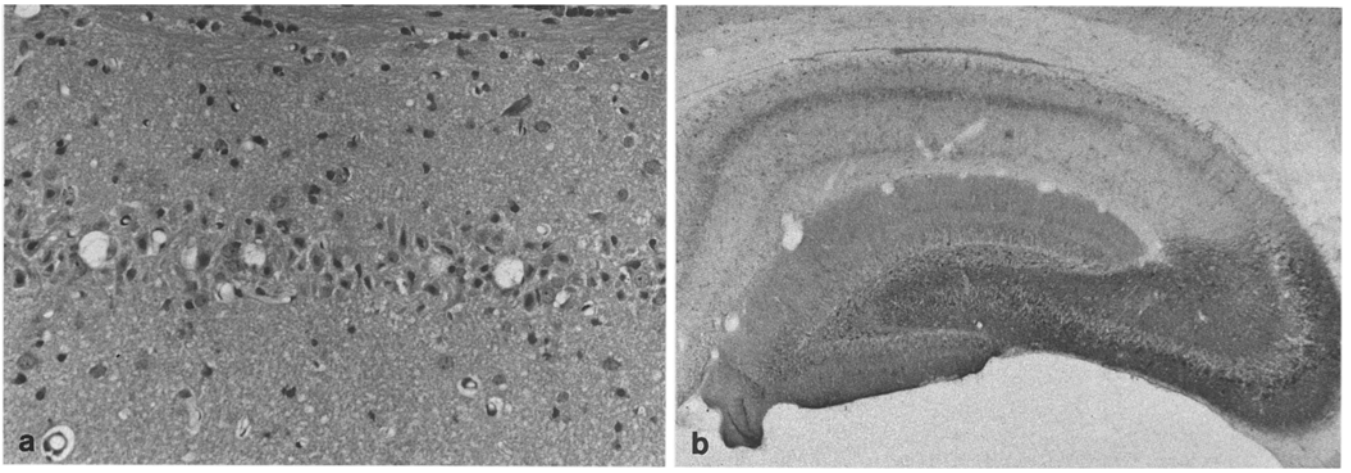

Fig. 2. **a** A 6-μm H&E section from case 5 (group 2), shows severe histological damage of CA1/2 hippocampal neurons. **b** Intense hsp 72-positive staining (50-μm vibratome section) was present in CA3, CA4 neurons and dentate gyrus from this case. **a** × 53; **b** × 31

gelatin mounted on slides for light microscopic evaluation. Control sections were run on each experiment without primary antibody to rule out cross reactivity with anti-mouse secondary antibody and endogenous rat Ig. Irrelevant primary antibody was not used.

The remaining coronal slices from these rat brains were fixed in 10% buffered formalin for a minimum of 1 week, embedded in paraffin, and sections (6 μm) adjacent to those cut on the vibratome, were stained with H&E for histopathological evaluation. Figure 1a illustrates the regional division of the hippocampus used in this study.

Results

Sham-operated control animals exhibited no damage to neurons and no specific staining for hsp 72. Control sections without primary antibody incubations were totally negative. Data from the six experimentals rats subjected to 8 min of forebrain ischemia are divided into two groups: group 1, minimal neuronal necrosis in CA1/2 hippocampus (<10% necrotic neurons); Group 2, severe neuronal necrosis in CA1/2 hippocampus (>75% necrotic neurons).

Group 1 (cases 1–4)

Hippocampal neurons showed little histological damage in the group 1 animals (Fig. 1a). Pronounced hsp 72 staining of the cytoplasm and processes in CA1/2 and CA4 neurons was present in all four rats. Cell bodies of the hsp 72-positive CA1 pyramidal cells were closely packed in the stratum pyramidale. The majority had round light staining nuclei surrounded by a relatively darker cytoplasm. The numerous dendritic processes of these cells were aligned in parallel stacks (see Fig. 3a). Hsp 72 staining was absent in lateral CA3 and the dentate gyrus (DG) neurons in case 1 (Fig. 1b), but was present in the other three cases. Some CA3 neurons (cases 2, 3; Fig. 1d) revealed hsp 72-positive staining of nucleoli with weak cytoplasmic staining of the lateral CA3 area. Hsp 72 expression in all of the hippocampal neurons was remarkably strong and of uniform intensity in case 4 (Fig. 1c). No immunoreactive capillaries could be found in any of these cases. No immunoreactive glial cells could be found in cases 1–3, but intensely stained hsp 72 glial cells and their processes were occasionally present in the dentate gyrus and CA1 regions in case 4 (Fig. 1e).

Group 2 (case 5, 6)

Severe histological damage in CA1/2 (Fig. 2a) and CA4 neurons was noted, but no obvious damage in the majority of CA3 neurons and dentate gyrus, was detected in case 5. Hsp 72 detection was minimal in necrotic hippocampal CA1/2 neurons, intense in CA3 and CA4 neurons, with dentate gyrus neurons also showing hsp 72-positive staining (Fig. 2b). Both immunohistochemistry and H&E staining showed clearly demarcated zones between normal and necrotic neurons. In case 6, neuronal necrosis was present in CA1/2. A few hsp 72-positive CA1/2 neurons were detected where the membrane of the soma was unclear, the cytoplasm was shrunken and weakly stained, and the processes consistently demonstrated structural change (Fig. 3b). In this animal neuronal processes were present that were positively stained for hsp 72 in the absence of detectable somatic staining (Fig. 3c). Hsp 72-immunoreactive capillaries and isolated immunoreactive glial cells, with or without obvious processes, were also detected in the necrotic regions in these two rats.

Discussion

Our data indicate that 48 h after a global ischemic event (8 min), morphologically intact neurons in the CA1/2 hippocampus contain hsp 72. Although these CA1/2 neurons appear morphologically intact, approximately 70% are destined to progress to necrosis 7 days after 8-min ischemia [1, 25]. Thus, the presence of hsp 72 in CA1/2 does not necessarily provide or imply protection

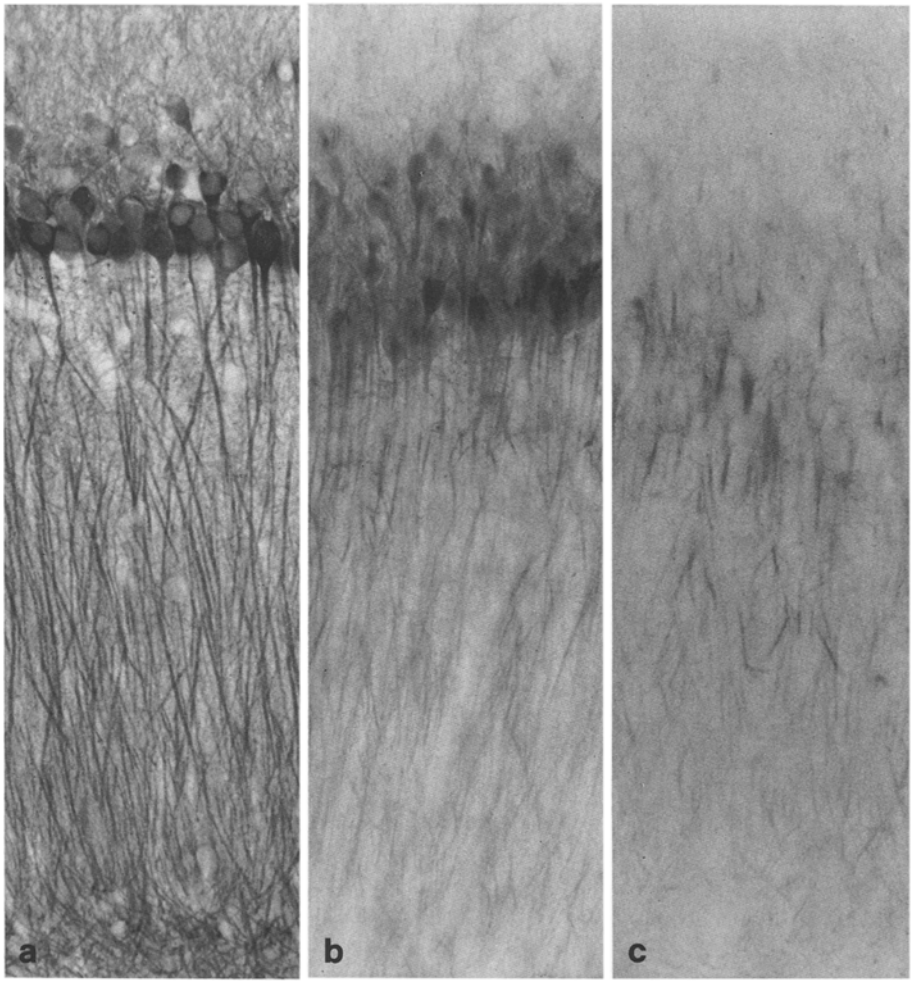

Fig. 3. **a** Intact hsp 72-positive staining CA1 neurons (enlargement of boxed area Fig. 1b) with light staining round nuclei surrounded by relatively darker staining cytoplasm from case 1 are present in this vibratome section. **b** 50 μm vibratome section indicates a few hsp 72-positive CA1/2 neurons with unclear somatic membranes and shrunken, weakly staining cytoplasm (case 6). **c** Case 6 also exhibited neuronal processes that were positively stained for hsp 72 in the absence of detectable somatic staining. **a, b, c** × 114

against delayed ischemic necrosis. Less than 10 min of forebrain ischemia infrequently produces neuronal necrosis in CA3 hippocampus 7 days after ischemia [16, 25]. In five of the six rats, we found that CA3 neurons express hsp 72. Thus, in CA3 neurons, expression of hsp 72 is not associated with neuronal necrosis. The presence of hsp 72 in CA3 neurons may be a stress response and is not a marker of lethal damage.

Minimal or no hsp 72 was detected in necrotic neurons. The lack of hsp 72 activity in necrotic neurons (cases 5, 6) 48 h after ischemia, may be due either to no production or the loss of protein from these cells. Abnormal hsp 72 transcription or translation, or both, may underlie the failure to detect hsp 72 staining [20]. Although there is evidence that the intensity of hsp expression is proportional to the inducing stress [12], this may not be the case for cells that are so severely damaged that they undergo early (≤48 h) ischemic necrosis. Cells suffering severe lethal damage early after ischemia may not express hsp 72 [7, 29]. Some necrotic neurons, however, show evidence of hsp 72 expression only in neuronal processes and the intensity of hsp 72 staining is much less than that found in morphologically intact neurons. The presence of hsp 72 in neuronal processes may be explained by the movement of hsp into the axon from the cytoplasm [28]. The observation of light hsp 72 staining in neuronal processes in *morphologically* damaged neurons is consistent with in situ hybridization mRNA results [23], which demonstrate hsp 72 induction in CA1/2 neurons after ischemia, persisting up to the death of these neurons.

The expression of hsp 72 differs in CA3 and CA1/2 neurons. The differential CA1/2, CA3, hsp 72 response is consistent with selective vulnerability of hippocampal neurons to ischemic cell damage [11, 26]. Our data is also consistent with those of Gonzalez et al. [7], in which hsp 72 induction was shown to reflect the selective vulnerability of hippocampal cells to ischemic injury. All morphologically intact CA1/2 neurons contain hsp 72, while morphologically intact CA3 neurons contain hsp 72, while morphologically intact CA3 neurons (in one animal) lacked hsp 72. Within a range of stress less than that required to induce early (<48 h) necrosis or disrupt all protein expression, we assume that the expression of hsp 72 in intact neurons is proportional to the stress [12]. Thus, the absence of hsp 72 in intact CA3 neurons and the ubiquitous presence of hsp 72 in intact CA1/2 neurons may imply that CA1/2 neurons have a lower threhold than CA3 neurons for hsp 72 expression. Although the intensity of hsp 72 staining was not quantified, sections from animals in group 1 revealed a lower intensity of hsp 72 staining in CA3 than in CA1/2 in

three of four animals. We speculate, that, because CA1/2 neurons are more sensitive and vulnerable to damage than CA3 neurons, the former therefore express more hsp 72.

The intracellular distribution of hsp 72 is different for the CA3 and CA1/2 neurons, and may reflect the time course of hsp 72 expression after the ischemic event. In rat embryo fibroblast, rat kidney, baby hamster kidney and HeLa cells, early after synthesis, hsp 72 is found in the nucleus and concentrated in nucleoli [30, 31]. At later times, the protein accumulates in the cytoplasm, suggesting movement of hsp 72 from the nucleus to the cytoplasm. In our in vivo studies, hsp 72 staining was intense in the cytoplasm axon and dendrites and not in the nucleoli of CA1/2 pyramidal cells. In contrast, hsp 72 staining was detected in nucleoli in the lateral portion of CA3 neurons (cases 2, 3) with weaker staining in the cytoplasm. Cytoplasmic hsp 72 staining in CA3 neurons is also found in cases 3–6. If the in vitro observations of differential intracellular staining are applicable to the in vivo case, the staining of nucleoli detected in CA3 suggests, that expression of hsp 72 in CA3 may occur later than in CA1/2. Alternatively, it may mean that the damage to the cell is more mature or advanced in CA1/2 than in CA3 hippocampus. An earlier expression of hsp 72 in CA1/2 than in CA3 hippocampus, is consistent with recent data published by Simon et al. [24].

Immunoreactive hsp 72 has been reportetd in glial and endothelial cells in the region of an infarction produced by middle cerebral artery occlusion in the rat [6]. Our data demonstrate that immunoreactive hsp 72 is induced in blood vessels in specific areas where hippocampal neurons are severely damaged and are either devoid or low in production of hsp 72 (Cases 5, 6). Immunoreactive hsp 72 glial cells, with or without processes, were occasionally found in both normal and damaged neuronal areas of CA1 and dentate gyrus in three of the six cases, frequently in association with immunoreactive blood vessels in damaged neuronal areas (Case 5, 6). The reason why isolated immunoreactive glial cells exist is unclear.

In summary, hsp 72 is expressed in CA1/2 neurons destined to die after a transient forebrain ischemic event. Severely damaged or necrotic neurons are mostly either devoid or low in hsp 72. Neurons in CA3 hippocampus destined to survive may or may not express hsp 72. Therefore, CA1/2 and CA3 neurons may differ in the expression of hsp 72 in response to a transient forebrain ischemic event, and hsp 72 is, likely, only a marker of stress rather than an indicator of neuronal survival or necrosis.

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