Vulnerability of Mossy Fiber Targets in the Rat Hippocampus to Forebrain Ischemia

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Much of the work on forebrain ischemia in the hippocampus has focused on the phenomenon of delayed neuronal death in CA1. It is established that dentate granule cells and CA3 pyramidal cells are resistant to ischemia. However, much less is known about interneuronal involvement in CA3 or ischemic injury in the dentate hilus other than the fact that somatostatin neurons in the latter lose their immunoreactivity. We combined two sensitive methods—heat-shock protein (HSP72) immunocytochemistry and a newly developed Gallyas silver stain for demonstrating impaired cytoskeletal elements—to investigate the extent of ischemic damage to CA3 and the dentate hilus using the four-vessel-occlusion model for inducing forebrain ischemia. HSP72-like immunoreactivity was induced in neuronal populations previously shown to be vulnerable to ischemia. In addition, a distinct subset of interneurons in CA3 was also extremely sensitive to ischemia, even more so than the CA1 pyramidal cells. These neurons are located in the stratum lucidum of CA3 and possess a very high density of dendritic spines. In silver preparations, they were among the first to be impregnated as “dark” neurons, before CA1 pyramidal cells; microglial reaction was also initiated first in the stratum lucidum of CA3. Whereas CA1 damage was most prominent in the septal half of the hippocampus, hilar and CA3 interneuronal damage had a more extensive dorsoventral distribution. Our results also show a far greater extent of damage in hilar neurons than previously reported. At least four hilar cell types were consistently compromised: mossy cells, spiny fusiform cells, sparsely spiny fusiform cells, and long-spined multipolar cells. A common denominator of the injured neurons in CA3 and the hilus was the presence of spines on their dendrites, which in large part accounted for the far greater number of mossy fiber terminals they receive than their non-spiny neighbors. We suggest that the differential vulnerability of neuronal subtypes in these two regions may be attributed to their extremely dense innervation by the mossy fibers and/or the presence of non-NMDA receptor subtypes that are highly permeable to calcium. In addition, early impairment of these spiny CA3 cells and hilar neurons after ischemia may be causal to delayed neuronal death in the CA1 pyramidal cells.

[Key words: ischemia, hippocampus, dentate hilus, CA3, spiny interneurons, silver stain, heat-shock protein, microglia, kainate, mechanisms of cell death]

The pattern of neuronal death in the hippocampus following cerebral ischemia has been well described in the rat (Pulsinelli et al., 1982; Kirino et al., 1985; also see Schmidt-Kastner and Freund, 1991, for a review), gerbil (Kirino, 1982; Crain et al., 1988), primate (Zola-Morgan et al., 1992), and human (Zola-Morgan et al., 1986). Of the principal neurons, CA1 pyramidal cells are the most sensitive while CA3 pyramidal cells and dentate granule cells are resistant. It has also been shown that a subpopulation of hilar interneurons that demonstrate somatostatin immunoreactivity is vulnerable to ischemia (Johansen et al., 1987, 1992). The somatostatin neurons degenerate within 24 hr while CA1 pyramidal cells take several days to die, the mechanism of this “delayed neuronal death” (Kirino, 1982) yet to be determined.

At present, one of the most prevalent hypotheses proposed to explain the selective vulnerability of hippocampal neurons is the “excitotoxic hypothesis” of Olney (1978), which postulates that an increase in the release of excitatory amino acids leads to neuronal death by allowing a lethal entry of calcium into the postsynaptic cell via NMDA-gated ion channels (MacDermott et al., 1986; Siesjö and Bengtsson, 1989). This hypothesis is supported by the findings that CA1 has the highest density of NMDA receptors in the hippocampus (Monaghan et al., 1983; Cotman et al., 1987), and by the observations of a transient increase in glutamate release following ischemia (Benveniste et al., 1984) and that neuronal damage is attenuated by lesioning the major excitatory inputs to the hippocampus (Johansen et al., 1986; Kaplan et al., 1989). On the other hand, recent electrophysiological studies have consistently failed to demonstrate any hyperexcitability in the hippocampus prior to CA1 cell loss (Buzsáki et al., 1989; Imon et al., 1991; Jensen et al., 1991). Furthermore, many of the neuroprotective effects of NMDA antagonists in severe global ischemia (Simon et al., 1984; Gill et al., 1987; Swan et al., 1988; Ikonomidou et al., 1989; Olney et al., 1989) have now been attributed to hypothermia (Corbett et al., 1990; Buchan et al., 1991a,b; Buchan, 1992). A second hypothesis of ischemic cell vulnerability focuses on the chemical content of the affected neurons. Because an accumulation of calcium is implicated in excitotoxic cell death (Greenamyre, 1986; Choi, 1988), many studies have attempted to correlate the vulnerability of neurons with the presence or absence of the calcium-binding proteins parvalbumin and calbindin because of their calcium-buffering abilities (Nitsch et al., 1989b; Sloviter, 1989; Johansen et al., 1990). Indeed, intracel-

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lular injection of the calcium chelator BAPTA effectively protected neurons from stimulation-induced cell death *in vitro* (Scharfman and Schwartzkroin, 1989). However, studies that systematically examined the relationship between the distribution of calcium-binding proteins and neuronal vulnerability have failed to demonstrate such a relationship in either forebrain ischemia (Freund et al., 1990; Johansen et al., 1990) or epilepsy (Freund et al., 1991). Furthermore, neuronal neuropeptide content (somatostatin, neuropeptide Y, cholecystokinin, or vasoactive intestinal peptide) has also failed to predict susceptibility to an ischemic insult (Yanagihara et al., 1985; Grimaldi et al., 1990). A third hypothesis that has so far received little attention is the location of susceptible neurons within the hippocampal network being the primary determinant of ischemic vulnerability.

To date, the majority of studies have concentrated on the phenomenon of delayed neuronal death in the CA1 pyramidal cells. The CA3 region has been regarded to be resistant to ischemic cell damage partly due to the fact that excitatory synaptic transmission is achieved via kainate/ α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) rather than NMDA receptors (Cotman et al., 1987). And while CA1 interneurons have been shown to survive an ischemic insult (Johansen et al., 1983; Nitsch et al., 1989a), there has been no study of the fate of CA3 interneurons or dentate hilar neurons. Furthermore, the mechanism of ischemic cell damage in the hilus is still unknown. Although it is well accepted that somatostatin neurons lose their immunoreactivity, the extent of ischemic damage in other hilar cells, of which there are over 20 morphological types (Amaral, 1978), has not been investigated.

Recent studies on the role of the nonconstitutive 72 kDa heat-shock protein (HSP72) following experimental stroke have focused on its induction in the principal cells of the rat hippocampus, that is, CA1 and CA3 pyramidal cells, granule cells, and to some extent the polymorphic cells of the hilus (Nowak, 1985; Dienel et al., 1986; Vass et al., 1988; Chopp et al., 1991; Gonzalez et al., 1991; Simon et al., 1991). Although the exact role of HSP72 is still unclear (Pelham, 1986), its expression in the principal cells of the hippocampus correlates with the latter's vulnerability to cerebral ischemia (Nowak, 1985; Vass et al., 1988; Gonzalez et al., 1991; Simon et al., 1991) as well as other disease states, for example, status epilepticus (Vass et al., 1989; Sloviter and Lowenstein, 1992) and neonatal hypoxia (Dwyer et al., 1989). In the present study, we combined a recently developed silver stain for visualizing traumatized neurons (Gallyas et al., 1990; Van den Pol and Gallyas, 1990) and immunocytochemistry for HSP72 to examine the extent of damage in the dentate hilus and in CA3. Both methods allow direct visualization of compromised neurons in a Golgi-like manner. Our findings suggest that neuronal vulnerability in CA3 spiny interneurons and hilar spiny cells is a function of the density of mossy fiber innervation they receive. Ischemic cell damage in this instance is hence non-NMDA-related and not correlated with the presence or absence of somatostatin and/or calcium-binding proteins.

Materials and Methods

Induction of transient forebrain ischemia. Female Wistar rats (200–275 gm) were used in the experiments. Transient forebrain ischemia was induced using a modification (Pulsinelli and Buchan, 1988) of the four-vessel-occlusion (4VO) method of Pulsinelli and Brierley (1979). The animals were anesthetized with a mixture of ketamine (25 mg/ml),

Rompun (1.3 mg/ml), and acepromazine (0.24 mg/ml) at a surgical dose of 4 ml/kg.

The common carotids were first isolated through a ventral midline neck incision by placing atraumatic Silastic tubes around both arteries. A silk ligature was then passed anterior to the cervical and paravertebral muscles but posterior to the trachea, esophagus, external jugular veins, and common carotids. The incision was then closed and the ends of the ligature secured to the nape of the neck by tape. A second incision was made behind the occipital bone and the paraspinal muscles separated to expose the alar foramina. The vertebral arteries running in the foramina transversaria were then electrocauterized under direct visual control. Animals were allowed to recover overnight, during which food was withheld but free access to water allowed. For occlusion of the carotids, the ventral neck wound was injected with 1% lidocaine and reopened. The common arteries were then occluded by microaneurysm clips. Only animals that showed a complete loss of righting reflex throughout the duration of the carotid occlusion were included in the study. The ligature around the neck was tightened for those animals that met criterion to restrict collateral blood flow further. The microaneurysm clips were removed after either 8 min ($n = 16$) or 15 min ($n = 20$) of occlusion. Rats were monitored throughout the ischemic period and ischemia was discontinued in those animals with respiratory difficulties. At the end of the ischemic period, the ligature was cut, the wound closed, and the rats closely observed until they walked spontaneously.

Perfusion-fixation. Animals were perfused after either 12 hr [$n = 3$ (8 min), 5 (15 min)], 24 hr ($n = 4, 8$), 48 hr ($n = 6, 3$), or 96 hr ($n = 5, 5$) of reperfusion. Three different perfusion-fixation procedures were used. For demonstration of HSP72 immunocytochemistry, animals were deeply anesthetized and perfused through the aorta with 0.9% saline followed by 4% paraformaldehyde (pH 7.2). Brains were removed, postfixed for 0–4 hr, and cryoprotected in a solution of 30% sucrose in phosphate buffer (PB) overnight. Fifty micrometer sections were then cut on a freezing microtome and a 1-in-4 series collected in PB for immunocytochemistry.

For demonstration of both silver-impregnated ("dark") neurons and HSP72 immunocytochemistry, animals were perfused through the aorta first with cacodylate-buffered saline (pH 7.5) followed by a cacodylate-buffered fixative containing 4% paraformaldehyde and 5.9% calcium chloride (pH 7.5). Brains were left *in situ* for 4 hr, removed, and then postfixed in the same solution overnight. Following overnight cryoprotection in 30% sucrose, a 1-in-3 series was cut on a freezing microtome: the first section was cut at 50 μ m and collected in PB for HSP72 immunocytochemistry; the second section was cut at 75 μ m and collected in the cacodylate fixative for silver staining; and the third section was cut at 50 μ m and stored in cryoprotectant for later use.

Finally, eight animals were perfused with cacodylate fixative at 1 d to 1 month after 15–20 min of 4VO for demonstration of dark neurons, microglial reaction, and silver impregnation of degenerating terminals. In these cases, the brains were left *in situ* overnight after perfusion before removal from the skull. They were then left in the perfusion fixative for 1 week before sectioning at 80 μ m on a freezing microtome. Three unoperated control animals were also perfused for Timm histochemistry to demonstrate the mossy fiber termination zone (see below).

Heat-shock protein immunocytochemistry. Immunoprocessing was carried out according to the method of Gonzalez et al. (1991) following a modification of a protocol kindly provided to us by Dr. Frank Sharp (University of California at San Francisco). Tissue sections were extensively washed in PB. After 2 hr in a blocking solution of 10% normal horse serum (NHS), 0.3% Triton X-100 (TX100), 0.1% bovine serum albumin (BSA), sections were incubated for 36 hr at 4°C in the monoclonal antibody raised against HSP72 (Amersham Corp) at 1:4000 in 2% NHS, 0.3% TX100, 0.1% BSA. Sections were then rinsed in PB 3×10 min and reacted at room temperature using a Vectastain Kit (Vector Laboratories, Burlingame, CA).

"Dark" neurons. Demonstration of traumatized "dark neurons" was carried out using a recently developed silver impregnation method for demonstrating cytoskeletal damage (Gallyas et al., 1990). Sections were dehydrated in a graded 1-propanol series and incubated at 56°C for 16 hr in an esterifying solution consisting of 1.2% H_2SO_4 and 98% 1-propanol. After a 10 min treatment in 8% acetic acid, sections were developed in a silicotungstate physical developer. Development was terminated by washing in 1% acetic acid for 30 min. Sections were then dehydrated, mounted, and coverslipped with DPX.

Microglia staining. Adjacent sections from the material stained for

Figure 1. Time course of HSP72 expression: 12, 24, 28, and 96 hr after 4VO ischemia. HSP72 expression was minimum at 12 hr, and peaked at 24 hr in the hilus and 48 hr in stratum lucidum interneurons in CA3. HSP72 expression in CA1 was similar to that described by Simon et al. (1991).

“dark” neurons were used. Briefly, sections were rinsed 3×5 min in dH_2O and then left in a pretreating solution (100 ml dH_2O , 0.3 ml H_2SO_4 , 0.2 ml concentrated H_2O_2) for 16–24 hr at room temperature. After washing in dH_2O 3×5 min, sections were placed in a second pretreating solution (100 ml dH_2O , 1 gm potassium ferrocyanide, 0.1 ml concentrated H_2SO_4) for 10–30 min. Sections were then washed again in dH_2O , after which they were developed in the silicotungstate developer for the “dark” neurons. Development was terminated by washing in 1% acetic acid for 30 min.

Silver staining of degenerating terminals. The method of Gallyas et al. (1980) was used. Briefly, this consisted of treatment for 2×5 min in a preprocessing solution (2% NaOH, 2.5% NH_4OH), 10 min in an impregnating bath (0–0.8% NaOH, 2.5% NH_4OH , 0.5% AgNO_3), 3×5 min in an intermediate solution (0.5% Na_2CO_3 , 0.01% NH_4NO_3 in 30% ethanol), and 1 min in a developing solution (0.4–0.6% formaldehyde, 0.01% citric acid in 10% ethanol), followed by 3×10 min wash in 0.5% acetic acid. The sections were then mounted, dehydrated, and coverslipped using DPX.

Timm histochemistry. Timm staining according to Danscher (1981) as modified by Sloviter (1982) was performed on unoperated control rats to demonstrate the extent of the hippocampal mossy fiber projection. Rats were deeply anesthetized and perfused through the aorta with 0.37% sulfide solution (pH 7.2) for 5 min followed by 10% neutral buffered formalin (pH 7.2) for 15 min. Brains were then removed from the skull and placed in the fixative for 24 hr, after which they were transferred to 30% sucrose in PB for overnight cryoprotection. Forty micrometer sections were then cut on a freezing microtome, mounted on gelatin-coated slides, and air-dried overnight. The sections were developed at 26°C for 30–60 min in the dark in a freshly prepared developer made up of 240 ml of 50% gum arabic, 40 ml of citrate solution (9.4 gm of sodium citrate plus 10.2 gm of citric acid monohydrate), 120 ml of 5.7% hydroquinone solution, 2.0 ml of 17% silver

nitrate. After development, slides were rinsed in running water for 30 min, dehydrated, and coverslipped.

Characterization of immunolabeled and silver-impregnated neurons. Several HSP72-immunoreactive interneurons in the CA3 area and dentate hilus in addition to some silver-impregnated CA3 interneurons were drawn with the aid of a camera lucida using a $40\times$ or $100\times$ objective. Different cell types in the dentate hilus from the specimens stained for HSP72-like immunoreactivity and the “dark” neuron stain were identified according to Amaral (1978).

Quantification of CA3 and hilar damage. HSP72-immunopositive cell bodies were counted using a $10\times$ objective under light microscopy with the aid of an image analysis system (Macintosh, IMAGE software) from coronal sections obtained at four different levels according to Paxinos and Watson (1986; AP = -2.8 , -3.8 , -4.8 – -6.04 mm from bregma) along the rostral–caudal extent of the hippocampus. In area CA3, spiny interneurons in the stratum lucidum were counted and reported as mean cell number per section. In the dentate gyrus, the hilus proper (zone 4 of Amaral, 1978) was outlined and its area computed using the image analysis system; the number of labeled cells was counted in the dorsal hippocampus, and the extent of hilar involvement in each section was then expressed as the number of immunopositive cell bodies per mm^2 . In all cases, each hemisphere was treated separately in the final analysis.

Results

In sections from both unoperated and sham-operated controls neural elements did not demonstrate any HSP72 immunoreactivity. As previously described, some ependymal cells lining the ventricles (Vass et al., 1988) and capillary endothelial cells were lightly immunopositive (Gonzalez et al., 1991). Control sections incubated without the primary antibody showed no immuno-

Figure 2. Spatial distribution of ischemic damage. *A*, HSP72 immunoreactivity (15 min of ischemia, 1 d survival). Section along the longitudinal axis of the hippocampus showing dorsal-ventral gradient of ischemic cell damage in CA1. Arrowheads indicate periodicity of neuronal damage at 500–800 μ m intervals. Note uniform damage in the dentate hilus. *B*, “Dark” neuron method (15 min ischemia, 2 d survival). Coronal section at the posterior curvature. Note dorsal-ventral gradient in CA1 and the more extensive damage of hilar neurons in the ventral half of the hippocampus (large arrowhead in *A*) as revealed by both methods. *h*, hilus. Scale bar, 1 mm.

reactivity. The distribution and time course of induction of Golgi-like HSP72-immunostained neurons were similar to that observed in previous studies using 4VO (Fig. 1; Simon et al., 1991) and two-vessel occlusion (Chopp et al., 1991; Gonzalez et al., 1991) models of ischemia. In general, there was a good correspondence between the induction of HSP72 immunoreactivity and silver impregnation; that is, HSP72 immunoreactivity was only seen in those neuronal populations that were also silver stained in separate or in the same preparations (Fig. 2). In both cases, the dorsal half of CA1 was mainly affected while hilar damage extended more ventrally. Furthermore, in the section processed for HSP72 immunocytochemistry, a periodicity of staining was seen at 500–800 μ m intervals (Fig. 2*A*).

Animals subjected to 15 min of ischemia typically demonstrated hilar cell damage within 24 hr (Fig. 3*A*) and CA1 cell damage after 3–4 d (Fig. 3*B*). Damaged neurons were silver-stained in a Golgi-like manner. The general pattern of ischemic cell damage using our silver impregnation technique (M. Hsu, F. Gallyas, and G. Buzsáki, unpublished observations) is consistent with previous results (Pulsinelli et al., 1982; Schmidt-Kastner and Freund, 1991).

Impairment of CA3 interneurons after ischemia

At 1 and 2 d postischemia, a distinct population of interneurons located in the stratum lucidum of CA3 was strongly immunoreactive for HSP72 (Figs. 4*A,D*, 5). These cells could also be seen in silver-stained sections (Fig. 6). They were present throughout the septotemporal extent of the hippocampus, but the optimal area to visualize them was in the posterior hippocampus where the stratum lucidum divided the dorsal and ventral CA3 (Fig. 4*B*) and also at the occipital bend (Fig. 4*C*). In the rostral CA3 their dendrites followed the curvature of the hippocampus. Timm staining confirmed that these neurons were restricted to the termination zone of the mossy fibers (Fig. 4*E–G*). Their dendrites were oriented parallel to the pyramidal layer and spanned several hundred micrometers both in the septotemporal and transverse directions. The dendrites of these cells were covered with numerous spines (Figs. 4*D*, 5) with long necks and small spine heads. On some of the cells (Fig. 5*B*), spines could be seen even on the somata. Silver-impregnated interneurons in CA3 were very similar to those demonstrated using HSP72 immunocytochemistry. Although spines could not be

Figure 3. Ischemic cell damage in the dorsal hippocampus demonstrated by the dark neuron method. *A*, One day postischemia. Only neurons in the dentate hilus are silver-impregnated. *B*, Four days postischemia. Note Golgi-like impregnation of degenerating CA1 pyramidal cells; hilar neurons are no longer visible. Duration of ischemia, 15 min in *A* and *B*. Scale bar, 500 μ m.

clearly distinguished, it was evident that both somata and the long dendrites of these cells were restricted to the stratum lucidum. In all appearances, they resembled the calretinin-immunoreactive spiny cells described by Gulyás et al. (1992).

A three-way ANOVA was performed on HSP72-immunoreactive CA3 spiny interneurons and hilar cells (see below) with duration of carotid occlusion, survival time, and the four rostrocaudal hippocampal levels as dependent variables. Overall, there were significantly more labeled cells in the 15 min group relative to the group with the shorter occlusion time [$F(1,240) = 9.693$, $p < 0.002$]. Although the number of labeled cells peaked at 24 hr for the 8 min group, the greatest number of

cells—a twofold increase over that in the former—was observed at 48 hr postischemia in the 15 min group [$F(3,240) = 21.932$, $p < 0.0001$; Fig. 7*A*]. The number of immunoreactive cells increased with the area of stratum lucidum present at each level and thus was greatest at the two caudal levels [$F(3,240) = 11.704$, $p < 0.0001$; Fig. 7*B*]. At 96 hr postischemia, the number of labeled cells substantially decreased in both groups.

Labeling of CA3 pyramidal cells was only seen in some animals with severe ischemia. In cases with shorter occlusion times, labeling was indistinguishable from the weak background labeling and contrasted sharply with that of the strongly labeled interneurons. Within the CA3 region, there was a differential vulnerability: the CA3c pyramids were more sensitive than those in CA3b or CA3a (Fig. 8*a–c*). Of the CA3c pyramidal cells, stellate-like pyramidal neurons and unaligned pyramidal cells in zone 3 of Amaral (1978) were the most sensitive to ischemia.

Impairment of hilar neurons after ischemia

In the dentate hilus, a heterogeneous population of neurons was immunoreactive for HSP72 (Fig. 9). The most common cell types labeled were the mossy cells and spiny fusiform cells (Fig. 9*C–F*; Amaral, 1978; Frotscher et al., 1991). In addition, many other cells of different morphologies were also immunoreactive. We identified six different cell types in zone 4 of Amaral (1978), all of which possessed spines on their dendrites and some on their somata. In addition to mossy cells and spiny fusiform cells, these included, in order of frequency of occurrence, sparsely spiny fusiform cells, long-spined multipolar cells, oviform cells, and spiny spheroid cells. The last of these resembled the aspiny spheroid cell at the dentate granule cell layer/hilus border (Amaral, 1978) with the exception that both soma and dendrites were covered with spines in our specimen (Fig. 9*I–K*). Granule cells were never stained.

A three-way ANOVA revealed no difference in the number of immunoreactive cells/mm² between the 8 min and 15 min groups, suggesting that the number of affected cells had already reached a maximum following 8 min of occlusion ($p = 0.3$). The number of labeled cells changed with survival time, increasing from a minimum at 12 hr postischemia, reaching a peak at 24 hr, and then tapering off thereafter [$F(3,240) = 32.182$, $p < 0.0001$; Fig. 7*A*]. This trend was true for both occlusion groups, consistent with the idea that hilar cell involvement had already reached an asymptote with the shorter occlusion time. As in CA3, the greatest number of hilar cells was seen at the more caudal levels (AP = -4.8 and -6.04 mm, respectively) with fewer cells seen at the two rostral levels [$F(3,240) = 7.744$, $p < 0.0001$; Fig. 7*B*].

HSP72 immunoreactivity was also evident in a fiber plexus in the inner molecular layer of the dentate gyrus (Fig. 8*c,d*), the terminal zone of mossy cells and other hilar cells with association projections (Han et al., 1993). No labeling was evident in

Figure 4. HSP72 immunoreactivity in CA3 stratum lucidum interneurons. *A*, Section through rostral hippocampus showing immunoreactive CA1 and CA2 (delineated by arrowheads) pyramidal cells, faint staining of CA3 pyramidal cells, and strong staining of interneurons located in the stratum lucidum. Double arrowhead, CA1–CA2 border; large arrowhead, CA2–CA3 border. *B* and *C*, Coronal sections through the posterior hippocampus where dorsal and ventral CA3 are divided by stratum lucidum (*B*) and near the occipital bend (*C*). *lu*, stratum lucidum; *r*, stratum radiatum. *D*, Stratum lucidum interneurons from another animal at higher magnification. In this section, there is very little background staining and only the interneuronal somata and their spiny dendrites are stained. *p*, pyramidal layer. Inset shows dendritic spines from one interneuron at higher magnification. Arrowheads point to spine heads. *E–G*, Timm staining of corresponding sections illustrated in *A–D* showing termination zone of the mossy fiber (*mf*) system in the stratum lucidum. *E*, Rostral section. *F* and *G*, Posterior CA3 where stratum lucidum divides dorsal from ventral CA3 (*F*) and near the occipital bend (*G*). Arrowheads, CA2–CA3 border. Scale bar: 200 μ m for *A–C*; 50 μ m for *D*; 400 μ m for *E–G*.

Figure 5. Characteristic spiny dendrites of the stratum lucidum interneurons immunostained for HSP72. *A*, Tangle of dendritic spines showing spines with long spine necks and small spine heads. *B*, Higher magnification of one such cell showing spines on the soma and proximal dendrites. *Inset* shows a closeup of a spiny distal dendrite. *C*, Camera lucida drawing of a population of such interneurons and their location in the stratum lucidum of CA3 (*lu*). *Arrowheads*, grape-like arrangement of CA3. Scale bars: *A*, 25 μ m; *B*, 100 μ m; *C*, 50 μ m.

Figure 6. Selective impairment of CA3 stratum lucidum interneurons. *A*, "Dark" neuron stain. *B*, Camera lucida drawing showing details of the cells numbered from 1 to 8 in *A*. Neurons 2, 4, and 7 are not visible in the plane of focus in *A*. Note horizontal dendrites ramifying within the stratum lucidum. *gc*, granule cell layer; *h*, hilus; *lu*, stratum lucidum; *CA3p*, CA3 pyramidal layer.

either the middle or outer thirds of the molecular layer. This HSP72-immunopositive band corresponded to the band of argyrophilia in the inner molecular layer of the dentate gyrus seen 1 week after ischemia using the Gallyas silver stain for degenerating terminals (Fig. 8*a*).

Neuropathological signs of cell damage

As early as 1 d after ischemia, the dendrites of some immunolabeled neurons in the hilus and CA3 appeared pathological. Many of the spiny dendrites contained uneven beads or large vacuoles with little or no cytoplasmic bridges (Fig. 10*A,B*). In silver-impregnated material, these beaded dendrites could also be seen (Fig. 10*C*), again with incomplete or complete interruption of the cytoplasm. As described above, visualization of dendrites of spiny neurons in CA3 was more difficult after longer durations of ischemia using HSP72 immunocytochemistry. Similarly, silver-impregnated neurons were rarely seen with the "dark" neuron method after 15 min of ischemia. In this context, it is important to emphasize that following more severe ischemia (>20 min) both HSP72 immunocytochemistry and the "dark" neuron method failed to stain CA1 neurons, even though these cells are known to degenerate (Gonzalez et al., 1991; M. Hsu

and G. Buzsáki, unpublished observations). Support for the permanent damage/loss of spiny CA3 cells comes from silver staining of reactive microglia. At 1 month postischemia, reactive microglia were present both in the cell layer and neuropil of CA1 in animals that showed bilateral cell loss but not in sham-operated controls (not shown). In addition, there was intense microglia staining in the stratum lucidum of CA3 as well as in the hilus (Fig. 8*e,f*). These microglia were bigger in size and darker, and had more processes than other microglia seen occasionally in other regions, indicating that they were activated microglia.

Discussion

The present results show that ischemic damage in the hilus is more extensive and involves many more cell types than previously shown. Even more important, they demonstrate ischemic vulnerability in area CA3 that has never been described before; that is, a subpopulation of interneurons restricted to the stratum lucidum is selectively damaged in the absence of any other CA3 involvement. Because both the hilar cells and CA3 interneurons possess spines on their dendrites and are thus densely innervated by the mossy fibers, we suggest that the

Figure 7. IISP72-immunopositive cell counts in the dentate hilus and CA3. Hilus cell counts are expressed as number of cells/mm²; CA3 cell counts are absolute numbers. *A*, Change in number of labeled hilar spiny cells (*left*) and CA3 spiny interneurons at different survival times. *B*, Change in hilar (*left column*) and CA3 (*right column*) cell counts in four sections (*graph legend*) along the rostral-caudal extent of the hippocampus (AP = -2.8, -3.8, -4.8, and -6.04 mm).

Figure 8. Neuronal degeneration in ischemia. *a*, Gallyas stain for degenerating terminals from an animal 7 d after 20 min of 4VO ischemia. Note silver deposits in the inner molecular layer of the dentate gyrus (arrowheads) and involvement of CA3c pyramidal cells (white arrow). *b–d*, HSP72 immunoreactivity. *B*, CA3c pyramidal cell. *C*, Unaligned stellate-like CA3c pyramidal cell (arrow). Arrowheads show dense staining of fibers in the inner molecular layer corresponding to silver deposits in *a*. *g*, granule cell layer. *d*, Osmium-intensified section at higher magnification showing immunolabeled fibers in the inner molecular layer (white arrowheads). *h*, hilus. *e* and *f*, Microglia staining showing microglia (arrows) only in the stratum lucidum (*lu*) in CA3 at low (*e*) and high (*f*) magnification. *r*, stratum radiatum; *p*, pyramidal layer. Scale bars: *a–d*, 100 μ m; *e*, 40 μ m; *f*, 20 μ m. Scale bar in *b* applies to *b–f*.

Figure 9. Hilar cells showing HSP72 immunoreactivity. *A*, Low-magnification photomicrograph showing extensive involvement of many cell types in the hilus. *B*, Oviiform cell (arrow); unaligned pyramidal cell (arrowhead). *C*, Mossy cells (arrows). *D*, Higher magnification of large mossy cell on the left in *C* showing characteristic “moss” on proximal dendrites (arrowheads). *E*, Spiny fusiform cell located below the granule cell layer. *F*, Spiny dendrites from the spiny fusiform cell in *E*. *G*, Sparsely spiny fusiform cell. *H*, Long-spined multipolar cell. *I*, Spheroid cell at the granule cell/hilus border (arrow) and pyramidal cells (white arrow and white double arrowhead). *J* and *K*, Spines on the soma (*J*) and dendrites (*K*) of cell in *I* (arrowheads). Scale bar: 100 μ m for *A* and *I*; 50 μ m for *B*, *C*, *E*, and *H*; 10 μ m for *D*, *F*, *J*, and *K*; 25 μ m for *G*.

degree of mossy fiber innervation predicts ischemic vulnerability in these two regions.

Ischemic damage in CA3 interneurons and hilar neurons

Previous studies have demonstrated a loss of somatostatin immunoreactivity in the dentate hilus after ischemia (Johansen et al., 1987, 1992; Grimaldi et al., 1990) and epilepsy (Sloviter, 1983; Sloviter and Nilaver, 1987). In addition, vulnerability of hilar mossy cells has also been implicated. To date, degenerating terminals located in the inner molecular layer have been the only evidence for the degeneration of mossy cells in the ischemic or epileptic hippocampus (Sloviter, 1987; Crain et al., 1988). However, apart from mossy cells, several other hilar cell types and modified pyramidal cells also project to the inner molecular layer of the dentate gyrus of the rat (Li et al., 1992; Han et al., 1993). Our results using three different techniques provide positive evidence that mossy cells are indeed compromised: degenerating terminals were found in the inner molecular layer of the dentate gyrus, many "dark" neurons in the hilus had the morphology of mossy cells, and the characteristic thorny excrescences revealed by HSP72 immunocytochemistry positively identified these cells as mossy cells. In fact, mossy cells were the most frequently immunolabeled cells.

In addition to mossy cells, many other different cell types were also immunoreactive for HSP72, including, in order of frequency of occurrence, spiny and sparsely spiny fusiform cells, long-spined multipolar cells, oviform cells, and spiny spheroid cells (Amaral, 1978; Ribak and Seress, 1988).

The mechanism of ischemic cell damage in the hilus is not well understood. In our study, a common feature of the hilar neurons immunoreactive for HSP72 was the presence of spines on their dendrites and/or cell bodies. It has recently been suggested that the somatostatin neurons correspond to the sparsely spiny fusiform cells (Han et al., 1993) originally described by Amaral (1978). However, the sparsely spiny fusiform cells comprised only a small portion of HSP72-immunoreactive neurons; the majority of the immunolabeled cells included several other types of spiny neurons. In contrast, none of the previously identified aspiny cells in the hilus (e.g., dentate basket cell, small aspiny multipolar cell, aspiny stellate cell) were immunoreactive for HSP72 or impregnated with silver. Many of the immunolabeled dendrites, especially at later survival periods (2 and 4 d), were swollen and vacuolated, reflecting overt damage of the cell membrane. With longer postischemic times, the spines lost their structural integrity and were no longer visible. Unevenly beaded and irregularly varicose dendrites were also apparent in our silver-stained material, providing further evidence that these cells were in the process of disintegration. These degenerative changes were similar in the spiny interneurons of the CA3 stratum lucidum and hilar neurons. Nevertheless, we should add that none of the described morphological alterations are unambiguous indicators of irreversible cell damage. Many neurons may recover despite serious injury to the dendritic spines and shafts. We can only conclude by inference that HSP72 expression and/or silver impregnation of neurons are predictors of eventual cell loss.

Several groups have proposed that neurons containing various calcium-binding proteins that buffer the cells against calcium increases are resistant to ischemic insults or to epilepsy (Scharfman and Schwartzkroin, 1989; Sloviter, 1989; Léránth and Ribak, 1991). However, neither the resistant CA3 pyramidal cells nor the vulnerable hilar somatostatin and mossy cells contain

Figure 10. Pathological dendrites from degenerating hilar neurons. *A* and *B*, HSP72 immunoreactivity. *A*, Vacuolated dendrites (arrowheads). *B*, Beaded dendrites (arrowheads). Double arrowhead points to a swollen spinehead. *C*, "Dark" neuron stain for traumatized neurons showing beaded dendrites (arrowheads). Note complete interruption of cytoplasm between the heads. Scale bar: 10 μ m for *A* and *B*; 25 μ m for *C*.

any known calcium-binding proteins. Both the resistant granule cells and the sensitive CA1 pyramidal cells, on the other hand, contain calbindin. Hippocampal neurons containing a second calcium-binding protein, parvalbumin, are resistant to ischemia. Parvalbumin-immunoreactive neurons in the striatum and reticular nucleus of the thalamus, however, are especially vulnerable (Freund et al., 1990). In our study, the spiny cells in the stratum lucidum and the long-spined multipolar cells in the hilus most likely correspond to the calretinin-immunoreactive

cells of Gulyás et al. (1992). These cells are vulnerable to ischemia (T. F. Freund, unpublished observations) and have not been shown to be colocalized with somatostatin. Furthermore, hilar mossy cells as well as the other cells we described using HSP72 immunocytochemistry do not contain calretinin. Even among calretinin-containing neurons, vulnerability to ischemia was not equal: aspiny calretinin-positive neurons found outside the stratum lucidum or the hilus were much less affected by ischemia (Freund, unpublished observations). Finally, somatostatin cells located outside the hilus are resistant. Thus, the heterogeneity of ischemia-sensitive neurons provides further proof that neither calcium-binding protein nor neuropeptide content reliably predicts ischemic vulnerability. We suggest that the common denominator shared by the ischemia-susceptible neurons in the hilus and CA3 region is the dense mossy fiber innervation they receive.

Possible explanations for differential sensitivity to mossy fiber-mediated ischemic injury

The mossy fiber system provides the major excitatory input to neurons in the hilus, pyramidal cells in CA3, and the spiny interneurons in the stratum lucidum of CA3 (Lorente de Nó, 1934; Gulyás et al., 1992). Excitatory synaptic transmission in this pathway is mediated primarily through kainate or AMPA receptors (Monaghan et al., 1983; Cotman et al., 1987). The mossy fiber termination zone in the stratum lucidum has very high levels of kainate receptors but low levels of NMDA sites (Cotman et al., 1987). This pathway has so far received little attention in investigations into the pathological mechanisms of ischemic cell damage perhaps because of the role accorded to NMDA receptors in mediation of ischemic cell death. However, even in area CA1, the exclusive involvement of NMDA receptors in mediating ischemic cell damage has been challenged recently. Much of the neuroprotective effects of NMDA antagonists in severe global ischemia (Simon et al., 1984; Gill et al., 1987; Swan et al., 1988; Ikonomidou et al., 1989; Olney et al., 1989) have now been attributed to hypothermia (Freund et al., 1989; Buchan and Pulsinelli, 1990; Corbett et al., 1990; Buchan et al., 1991a; Buchan, 1992). The emerging role of AMPA receptors in mediating ischemic cell death is supported by recent findings that administration of the AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*) quinoxaline offers neuroprotection (Buchan et al., 1991b; Buchan, 1992; Diemer et al., 1992; Nellgard and Wieloch, 1992).

The mossy fiber system also contains a high concentration of zinc (Danscher, 1981; Gaarskjaer, 1986). There is evidence that zinc may regulate both excitatory and inhibitory synaptic transmission (Assaf and Chung, 1984; Aniksztejn et al., 1987; Westbrook and Mayer, 1987; Xie and Smart, 1991), and zinc neurotoxicity has been demonstrated in cell cultures (Choi et al., 1988) and implicated in the selective death of dentate hilar neurons after cerebral ischemia (Tonder et al., 1990).

Even though mossy fiber system involvement in non-NMDA-mediated ischemic cell death is likely, it is not clear why different subpopulations within the hilus and CA3 neurons (stratum lucidum spiny interneurons vs. pyramidal cells and other CA3 interneurons) are not equally sensitive to ischemia. We suggest that this differential vulnerability may be due to differences in mossy fiber innervation and/or different expression of glutamate receptor subtypes in the postsynaptic cells.

First, the density of mossy fiber innervation onto hilar cells and CA3 stratum lucidum interneurons is far greater than onto

the CA3 pyramidal cells. A granule cell sends at least seven collaterals off its principal axon through the hilus forming contacts with many hilar neurons before making its way to CA3 (Claiborne et al., 1986). In area CA3, Gulyás et al. (1992) calculated that up to 10,000–20,000 mossy fibers terminated onto a single stratum lucidum spiny interneuron whereas only 10–50 mossy fibers terminated onto a CA3 pyramidal cell (Claiborne et al., 1986). Our observation that CA3c pyramidal cells were also irreversibly damaged following longer durations of ischemia provides further support for the importance of the density of mossy fiber innervation because pyramidal cells in the CA3c subregion possess substantially more thorny excrescences and receive a greater number of mossy fiber terminals than their counterparts in the CA3b and CA3a subregions (Claiborne et al., 1986). Based on the currently available experimental evidence, we suggest that vulnerability of neurons in the hilus and CA3 region is a function of the density of mossy fiber innervation they receive. From this perspective, the circuitry in which the vulnerable neurons are located is the primary factor determining cell death, whereas other factors such as intracellular calcium buffering capacity probably play a secondary role.

An alternative explanation for the differential sensitivity of the mossy fiber targets may be sought in the different receptor subtypes of the vulnerable and resistant cell populations. Different cell targets of the mossy fiber system may express different subunit combinations of the same receptor complex that are either Ca^{2+} permeable or Ca^{2+} impermeable. It has recently been shown that the calcium permeability of kainate/AMPA-gated channels depends on the differential expression of the glutamate receptor subunits GluR1, GluR2, and GluR3 (Hollmann et al., 1991; Petralia and Wenthold, 1992). Coexpression of GluR2 with either GluR1 or GluR3 results in Ca^{2+} -impermeable channels, while channels expressing GluR1, GluR3, or GluR1 plus GluR3 form Ca^{2+} -permeable channels. This suggestion is supported by electrophysiological studies showing the presence of two types of kainate responses in hippocampal neurons, a Ca^{2+} -impermeable type I response that had linear current–voltage properties and resembled GluR1 plus GluR2 or GluR3 plus GluR2 responses and a Ca^{2+} -permeable type II response that was sigmoidal and resembled GluR1, GluR3, and GluR1 plus GluR3 responses (Iino et al., 1990). Spiny hilar neurons showed slow kinetics while aspiny hilar neurons showed fast kinetics in response to kainate (Livsey and Vicini, 1992). Finally, in area CA3, McBain and Dingledine (1993) were able to identify two types of interneurons based on their different kainate responses.

Combining the above information with our present findings, we suggest that the differential vulnerability seen between spiny interneurons and pyramidal cells in area CA3 and spiny and aspiny neurons in the hilus may be due to the absence of the GluR2 subunit in the vulnerable populations. Since channels lacking GluR2 subunits allow large influxes of Ca^{2+} on an order equivalent to NMDA ionophores (Hollmann et al., 1991), they may contribute to excitotoxicity. Along the same lines, Pellegrini-Giampietro et al. (1992) report a reduced GluR2 expression in postischemic CA1 pyramidal cells preceding the increase in Ca^{2+} influx in the CA1 cells. Direct confirmation of our hypothesis requires the demonstration of a lack of GluR2 subunits on the vulnerable cell populations and the selective survival of neurons expressing GluR2 subunits after ischemia or a differential calcium permeability in the vulnerable versus resistant neuronal populations that are targets of the mossy fiber system.

Implications for delayed neuronal death in CA1 pyramidal neurons

It has been suggested that delayed neuronal death in area CA1 is dependent upon a functional CA3 input (Benveniste et al., 1989). In light of the present findings, we speculate that the impairment of an important control mechanism in the CA3 region and hilus (spiny stratum lucidum and spiny hilar neurons) precedes and may be causal to delayed neuronal loss in CA1. This hypothesis may be tested by demonstrating the prevention of CA1 delayed neuronal death by pharmacological protection of the hilar and CA3 spiny cell types. In conclusion, the present findings demonstrate that substantially greater number of neuron types are involved in ischemia than previously thought. A common feature of these neurons is the dense innervation of their spiny dendrites by the mossy terminals of granule cells. Since the neuronal circuits of which they are a part (hilus → CA3) comprise the major input to CA1, examination of their causal involvement in the delayed neuronal death of CA1 pyramidal cells is warranted.

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