Modification of Postsynaptic Densities after Transient Cerebral Ischemia: A Quantitative and Three-Dimensional Ultrastructural Study

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Abnormal synaptic transmission has been hypothesized to be a cause of neuronal death resulting from transient ischemia, although the mechanisms are not fully understood. Here, we present evidence that synapses are markedly modified in the hippocampus after transient cerebral ischemia. Using both conventional and high-voltage electron microscopy, we performed two- and three-dimensional analyses of synapses selectively stained with ethanolic phosphotungstic acid in the hippocampus of rats subjected to 15 min of ischemia followed by various periods of reperfusion. Postsynaptic densities (PSDs) from both area CA1 and the dentate gyrus were thicker and fluffier in postischemic hippocampus than in controls. Three-dimensional reconstructions of selectively stained PSDs created using electron tomography indicated that postsynaptic densities became more irregular and loosely configured in post-ischemic brains compared with those in controls. A quantitative

study based on thin sections of the time course of PSD modification indicated that the increase in thickness was both greater and more long-lived in area CA1 than in dentate gyrus. Whereas the magnitude of morphological change in dentate gyrus peaked at 4 hr of reperfusion (140% of control values) and declined thereafter, changes in area CA1 persisted and increased at 24 hr of reperfusion (191% of control values). We hypothesize that the degenerative ultrastructural alteration of PSDs may produce a toxic signal such as a greater calcium influx, which is integrated from the thousands of excitatory synapses onto dendrites, and is propagated to the neuronal somata where it causes or contributes to neuronal damage during the postischemic phase.

Key words: postsynaptic density; hippocampus; cerebral ischemia; electron microscope; tomography; three-dimensional reconstruction; neuronal death

Transient cerebral ischemia selectively damages CA1 pyramidal neurons ~3 d after ischemia but leaves CA3 and dentate gyrus neurons largely intact. During the 72 hr preceding cell death, the neurons destined to die have a relatively normal appearance when examined by light microscopy. However, some ultrastructural abnormalities have been reported in postischemic neurons, including disaggregation of polyribosomes and deposition of dark substances (Kirino, 1982; Pulsinelli et al., 1982; Kirino et al., 1984; Petito and Pulsinelli, 1984; Smith et al., 1984). Alterations of synaptic transmission have also been reported during the postischemic phase. A mild ischemic episode potentiates synaptic transmission (Andiné et al., 1992; Miyazaki et al., 1993, 1994; Hammond et al., 1994), whereas a more severe ischemia episode suppresses synaptic neurotransmission and results in neurobehavioral deficits during the postischemic phase (Volpe et al., 1984; Auer et al., 1989; Furukawa et al., 1990; Xu, 1995; Dalkara et al., 1996).

Several lines of evidence suggest that excitotoxicity through overactivation of glutamate receptors on synapses may be involved in ischemic neuronal death. First, neurons can be killed by exposure to glutamate (Choi, 1988). Second, extracellular glutamate

is significantly increased after depolarization during ischemia (Benveniste et al., 1984). Third, glutamate receptor blockade has been shown to be neuroprotective (for review, see Lipton, 1994). However, the increase in extracellular glutamate present at the onset of ischemia rapidly returns to control values, whereas neuronal damage occurs at ~72 hr after an ischemic episode (Kirino, 1982; Pulsinelli et al., 1982). This paradox suggests the possibility that signals triggered during ischemia may propagate progressively from synapses to the cell body to instigate delayed neuronal death during the postischemic phase.

In biochemical analyses of postsynaptic densities (PSDs), we recently reported a dramatic accumulation of *N*-ethylmaleimide-sensitive fusion protein, heat shock cognate protein 70, calcium/calmodulin-dependent protein kinase II, and protein kinase C in PSDs from the cerebral cortex of rats subjected to 15 min of transient ischemia followed by 4 hr of reperfusion (Hu et al., 1995, 1998). These biochemical changes were accompanied by robust morphological alterations in PSD structure. In this study, we conducted a detailed examination of synaptic structure in the postischemic hippocampus using quantitative and three-dimensional reconstruction methods. Based on our earlier study in cortex, we wished to determine (1) whether similar morphological alterations were observed in hippocampal synapses after ischemia; (2) how PSD modification developed over time; and (3) whether any differences existed in PSD alterations between ischemic vulnerable neurons in area CA1 compared with the relatively more resistant neurons in dentate gyrus. We demonstrate that PSD ultrastructure in both two dimensional (2D) images and

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three dimensional (3D) reconstructions is highly modified shortly after a brief ischemic episode. The modification is severe and long-lasting in hippocampal CA1 neurons but transient and less marked in dentate gyrus granule cells. These long-term synaptic ultrastructural alterations may be involved in ischemia-induced alterations in synaptic transmission and may also be early events in the initiation of neuronal damage during the postischemic phase.

MATERIALS AND METHODS

Ischemia model. All experimental procedures were approved by the Subcommittee on Animal Studies of the Veterans Affairs Medical Center (San Diego, CA). Male Wistar rats (250–300 gm) were fasted overnight. Anesthesia was induced with 3% halothane followed by maintenance with 1–2% halothane in an oxygen/nitrous oxide (30/70%) gas mixture. Catheters were inserted into the external jugular vein, tail artery, and tail vein to allow blood sampling, arterial blood pressure recording, and drug infusion. Both common carotid arteries were exposed and encircled by loose ligatures. Fifteen minutes before ischemia induction and 15 min after ischemia, blood gases were measured and adjusted to $\text{PaO}_2 > 90$ mmHg and PaCO_2 35–45 mmHg, pH 7.35–7.45, by adjusting tidal volume of the respirator. Bipolar EEG was recorded every 5–10 min before ischemia, continuously during the ischemic insult, and every 5 min after ischemia until the rat recovered from the anesthesia. At the beginning of a 30 min steady-state period before induction of ischemia, the inspired halothane concentration was decreased to 0.5%, and 150 IU/kg heparin was administered intravenously. Blood was withdrawn via the jugular catheter to produce a mean arterial blood pressure of 50 mmHg, and ischemia was induced by clamping both carotid arteries. Blood pressure was maintained at 50 mmHg during the ischemic period by withdrawing or infusing blood through the jugular catheter. At the end of the ischemic period, the clamps were removed, and the blood was reinfused through the jugular catheter, followed by 0.5 ml of 0.6 M sodium bicarbonate. In all experiments, temperature was maintained at 37°C before, during, and after ischemia (15 min of reperfusion). Halothane was discontinued at the end of ischemia, and all wounds were sutured. At 4 or 24 hr, 3 d, or 1 week after the ischemic episode, the animals were reanesthetized, tracheotomized, and artificially ventilated. For electron microscopic studies, the brains were perfused with ice-cold 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Sham-operated control rats were subjected to the same surgical procedures but without induction of ischemia.

Electron microscopic studies. Tissue sections from experimental and control animals were stained either by 1% ethanolic phosphotungstic acid (E-PTA) (Bloom and Aghajanian, 1966, 1968) or by the conventional osmium–uranium–lead method. Briefly, coronal brain sections were cut at a thickness of 200 μm with a Vibratome through the level of the dorsal hippocampus and post-fixed for 1 hr with 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. For conventional osmium–uranium–lead staining, sections were post-fixed for 2 hr in 1% osmium tetroxide in 0.1 M cacodylate buffer, rinsed in distilled water, and stained with 1% aqueous uranyl acetate overnight. The tissue sections were then dehydrated in an ascending series of ethanol to 100%, followed by dry acetone, and embedded in Durcupan ACM. Thin sections were counterstained with lead citrate before examination in the electron microscope. For E-PTA staining, sections were dehydrated in an ascending series of ethanol to 100% and stained for 1 hr with 1% PTA prepared by dissolving 0.1 gm of PTA in 10 ml of 100% ethanol and adding four drops of 95% ethanol. Sections were then embedded in Durcupan ACM.

EM tomography. E-PTA-stained thick sections (1 μm) were examined by a JEOL 4000EX intermediate high-voltage electron microscope at an accelerating voltage of 400 keV. Before examination in the electron microscope, 10 nm colloidal gold particles were applied to the section surface to serve as fiducial cues for subsequent alignment of images. Data for tomographic reconstruction was acquired using the single-axis tilt method. A series of images was obtained as the specimen was tilted in equal angular increments, about an axis perpendicular to the optical axis of the microscope. Each of these images represented a projection of densities in the specimen from a particular angle of view, and the volume distribution was solved using the R-weighted back-projection method. Images were typically obtained over a range of $\pm 60^\circ$ at 2° increments using film. The micrographs were digitized using a high-resolution 1000 $\times$ 1000 dpi CCD camera (Photometrics Inc.). The digitized images were

flat-fielded to correct for any unevenness in the illumination, transformed appropriately to achieve a measure of the projected density, and normalized. The tilt axis was determined, and the images were rotationally and translationally aligned using fiducial alignment and correlational techniques implemented in the SUPRIM image-processing library (Schroeter and Bretaudeau, 1996). The volume was reconstructed from the tilt series using R-weighted back-projection implemented in SUPRIM. Resulting volumes were viewed and manipulated using the program ANALYZE (Biomedical Imaging Resource, Mayo Foundation, Rochester, MN). Additional details of the tomographic method used can be found in articles by Soto et al. (1994) and Perkins et al. (1997).

Collection of data. For both 2D and 3D analyses, observations were obtained from a strip of tissue crossing the apical dendritic region of CA1 and both limbs of the dentate gyrus. PSDs were sampled from the stratum radiatum of area CA1, at a distance of ~ 200 μm above the pyramidal cell layer and from the upper limb of the dentate gyrus, within 70 μm of the granule cell layer. Distances were measured by counting grid squares in 300 mesh grids (~ 73 $\mu\text{m}/\text{grid square}$).

Quantitative analyses of thin sections. Specimens were selected for quantitative analysis based on the quality of E-PTA staining and the degree of ultrastructural preservation, as determined from conventionally stained material from the same animals. Samples were analyzed from controls ($n = 3$) and from postischemic brains at 4 hr of reperfusion ($n = 4$) and 24 hr of reperfusion ($n = 3$). Additional micrographs were obtained from the dentate gyrus from postischemic brains after 1 week of reperfusion ($n = 2$). Tissue sections were cut at a thickness of 100 nm and examined and photographed at 80 keV at a magnification of $8300\times$ with a JEOL 100CX electron microscope. For each animal, five micrographs were obtained from both CA1 and the dentate gyrus as described above. Each negative was digitized into a Macintosh computer. Using NIH Image 1.6, PSDs were first manually outlined, and then the maximal thickness, minimum thickness, length, and total area of each PSD were determined, as illustrated in Figure 3. All PSDs in which the postsynaptic density, intracleft line, and presynaptic grid (see Fig. 3) were clearly visible were chosen for analysis. PSDs were analyzed for both dentate gyrus and CA1 in control and 4 and 24 hr reperfusion conditions but only in dentate gyrus for 1 week of reperfusion. CA1 was not analyzed at this time point, because most CA1 neurons had degenerated by 1 week after ischemia. No attempt was made to perform the analysis blindly, because the morphological changes were so dramatic that the postischemic tissue was readily distinguishable from the control. The selection criterion resulted in analysis of between 45 and 70 PSDs per animal for each region. The data for the sham and 4 and 24 hr of reperfusion were analyzed using a 3 (control and 4 and 24 hr) $\times$ 2 (CA1 vs dentate gyrus) ANOVA. *Post hoc* analyses were conducted using Dunnett's test for comparison of the reperfusion conditions with the control condition. A separate ANOVA was conducted comparing the four conditions (sham, 4 and 24 hr, 3 d, and 1 week of reperfusion) analyzed for the dentate gyrus. The incidence of perforated PSDs was also determined from digitized images. A PSD was classified as perforated if it contained a discontinuity (see Fig. 7, 2D slices).

RESULTS

As has been reported previously for this ischemia model, 15 min of transient cerebral ischemia followed by up to 24 hr of reperfusion did not cause obvious abnormal morphology in hippocampal sections relative to sham-operated controls, as determined by light microscopic analysis of cresyl violet-stained sections (Fig. 1, *Sham*, 24h). However, a frank neuronal degeneration occurred selectively in CA1 pyramidal neurons ~ 3 d after the ischemic episode (Fig. 1, 3d), whereas neurons in CA3 and the dentate gyrus remained largely intact after the ischemic insult (Smith et al., 1984).

At the electron microscopic level, we found most neurons in the conventionally stained material (osmium–uranium–lead) to possess relatively normal cell membranes and normal nuclei at 4 and 24 hr of reperfusion, consistent with previous ultrastructural studies (Kirino et al., 1984; Petito and Pulsinelli, 1984; Deshpande et al., 1992; Rafols et al., 1995). No obvious breaks in the membranes of dendritic shafts or spines were seen, and presynaptic terminals, presynaptic vesicles, and PSDs were intact in the

Figure 1. Low-power light micrographs of dorsal hippocampus of control rats (*Sham*) and rats subjected to 15 min of ischemia followed by 24 hr (*24h*) and 3 d (*3d*) of reperfusion. Vibratome sections (50 μ m) were stained with cresyl fast violet. The majority of CA1 neurons at 24 hr of reperfusion look normal. A frank neuronal death was apparent after 3 d of reperfusion (*3d*). *so*, Stratum oriens; *pcl*, pyramidal cell layer; *sr*, stratum radiatum.

dendritic region of hippocampal CA1 (Fig. 2, *Sham*, *4h*, *24h*) as well as dentate gyrus (data not shown). Some PSDs from the postischemic brain appeared slightly irregular in profile compared with those in controls.

E-PTA resulted in selective staining of the postsynaptic density, the presynaptic grid, and some material in the synaptic cleft. With the exception of nuclear chromatin, other structures were not stained (Bloom and Aghajanian, 1966, 1968). In contrast to the conventionally stained material, robust alterations were apparent in postischemic tissue stained with E-PTA (Fig. 3). After

Figure 2. Electron micrographs of osmium–uranium–lead-stained synapses in the dorsal hippocampal CA1 areas from sham-operated rats (*Sham*) and rats subjected to 15 min of ischemia followed by 4 hr (*4h*) and 24 hr (*24h*) of reperfusion. The synapses (arrows) were intact, and no obvious structural alterations were seen in these osmium–uranium–lead-stained synapses after ischemia. Scale bar, 0.5 μ m.

transient ischemia, postischemic PSDs were clearly thicker and fluffier than those in controls (Fig. 3). Most control PSDs were more compact, as evidenced by their generally greater electron density relative to postischemic PSDs (Fig. 3). These changes were also present in the dentate gyrus (data not shown). Both simple (*top row*) and perforated (*bottom row*) PSDs were clearly more fluffy and curved compared with control PSDs (Fig. 3). There was also a general increase in the amount of E-PTA-stained material in the postischemic brains compared with controls. This increase did not appear to be attributable to an increase in the number of synapses in the postischemic brain (see below) but appeared to represent flocculent material that was loosely associated with or near to PSDs.

To investigate these interesting alterations further, we conducted a series of quantitative analyses of PSDs at different time points of reperfusion. Because the most obvious change in PSD

Figure 3. High-magnification electron micrographs of E-PTA-stained synapses in hippocampal CA1 regions from sham-operated rats (*Sham*) and rats subjected to 15 min of ischemia followed by 24 hr (24h) of reperfusion. Examples of simple PSDs are shown in the *top row*, and examples of perforated PSDs are shown in the *bottom row*. In comparison with the control synapses (*Sham*), postischemic PSDs were generally curved and surrounded by diffuse E-PTA-stained materials both in the single PSDs and perforated PSDs. The *inset* in the *top right panel* (*large arrow*) illustrates how PSD parameters were measured (Table 1). The area of the PSD was calculated by manually outlining the PSD (*solid white line*), whereas the *straight dotted lines* indicate the minimum (*small arrow*) and maximum thickness. Scale bar, 0.1 μ m. *Pre-*, Presynaptic specialization; *Cleft*, synaptic cleft.

Table 1. Quantification of PSDs in CA1 and DG after cerebral ischemia

Groups	Area $\times 10^3$ (nm ²)	Length (nm)	Minimum thickness (nm)	Maximum thickness (nm)
Sham				
CA1	4.03 $\pm$ 0.37	115.71 $\pm$ 4.47	21.62 $\pm$ 0.65	49.82 $\pm$ 1.89
DG	4.46 $\pm$ 0.68	112.14 $\pm$ 9.44	24.60 $\pm$ 1.71	57.80 $\pm$ 3.04
4 hr				
CA1	7.51 $\pm$ 1.23*	125.26 $\pm$ 6.66	36.59 $\pm$ 5.05*	88.17 $\pm$ 12.05*
DG	7.52 $\pm$ 2.02**	132.28 $\pm$ 22.21	34.28 $\pm$ 4.55*	80.67 $\pm$ 12.44*
24 hr				
CA1	8.26 $\pm$ 1.20*	130.27 $\pm$ 14.06	39.00 $\pm$ 0.99*	95.16 $\pm$ 1.04*
DG	6.76 $\pm$ 0.42	130.15 $\pm$ 5.02	31.51 $\pm$ 0.94**	75.71 $\pm$ 2.33**
1 week				
CA1				
DG	4.87 $\pm$ 1.34	111.94 $\pm$ 13.52	26.68 $\pm$ 3.95	68.17 $\pm$ 12.08

The samples are from sham-operated rats (sham) and rats subjected to 15 min of ischemia followed by 4 and 24 hr and 1 week of reperfusion. The data are expressed as mean $\pm$ SD from three rats in sham and 24 h groups, four rats in 4 hr group, and two rats in 1 week group. *p* values (Dunnett's test) denote significant difference between sham-operated control group and the postischemia groups.

**p* < 0.01.

***p* < 0.05.

morphology was the apparent change in thickness, we measured the maximum and minimum thickness for individual PSDs from each animal as well as area and length, as illustrated in Figure 3. Results of the analyses are summarized in Table 1. A 2×3

ANOVA indicated a significant interaction between hippocampal area [dentate gyrus (DG) vs CA1] and condition (sham vs 4 vs 24 hr) for maximum thickness ($F_{(2,14)} = 4.28$; *p* < 0.05) and minimum thickness ($F_{(2,14)} = 3.86$; *p* < 0.05). *Post hoc* comparisons

Figure 4. Maximum thickness of PSDs from control (C) and postischemic brains after 4 hr (4h), 24 hr (24h), and 1 week (1w) of reperfusion, plotted as a percentage of controls. Only dentate gyrus was sampled after 1 week of reperfusion, because both pyramidal cell layer and neuropil of the CA1 region have degenerated by this time point. The increase in thickness was greater and more persistent in CA1 compared with dentate gyrus.

using Dunnett's test indicated that both the minimum and maximum thicknesses of postischemic PSDs were greater than those of sham-operated controls in area CA1 at both 4 and 24 hr ($p < 0.01$, Dunnett's test) and in DG at 4 hr ($p < 0.05$) and 24 hr ($p < 0.05$). The ANOVA also showed a significant effect for area ($p < 0.01$), but the overall interaction was not significant. However, Dunnett's test indicated a significant increase in area relative to controls in CA1 at 4 hr ($p < 0.05$) and 24 hr ($p < 0.01$) and in the dentate gyrus at 4 hr ($p < 0.01$) but not at 24 hr ($p = 0.06$). No significant differences in length of PSDs were uncovered. No significant differences compared with controls were observed in the dentate gyrus for any measure at 1 week of reperfusion (Table 1).

The significant interaction between condition (sham vs 4 vs 24 hr) and region for measures of PSD thickness indicates that the alteration in PSD morphology was not equivalent between the dentate gyrus and area CA1 in the three conditions. The magnitude of morphological alteration in the dentate gyrus peaked at 4 hr of reperfusion but declined thereafter, whereas the changes in area CA1 persisted and slightly increased before cell death. This interaction is shown in graphic form in Figure 4 for maximum thickness. In comparison with controls, the increase in thickness in CA1 was 177% at 4 hr and 191% at 24 hr of reperfusion, whereas the increase in DG was 140% at 4 hr, 131% at 24 hr, and 118% at 7 d of reperfusion (Fig. 4, Table 1). After 3 d of reperfusion, >95% of CA1 neurons are degenerated (Fig. 1), and so no measurements were performed at 7 d of reperfusion for CA1. As can be seen in the frequency histograms of PSD thickness in area CA1 and dentate gyrus shown in Figure 5, nearly all synapses in CA1 showed increased thickness, whereas in the dentate gyrus, synapses on the whole tended to be thicker, especially at early time points, but there was considerable overlap in the populations from reperfused versus control brain.

The percentage of perforated PSDs as counted in randomly selected sections showed a significant increase compared with controls in CA1 ($p < 0.05$, Dunnett's test) and an insignificant increase in the dentate gyrus after transient cerebral ischemia (Table 2).

To characterize further the structural alterations observed in

Figure 5. Frequency histogram of PSD thickness in hippocampal CA1 (top panel) and dentate gyrus (bottom panel) in control (sham) and postischemic brains subjected to 4 hr (4hr), 24 hr (24hr), or 1 week (1wk) of reperfusion. The maximal thickness of most CA1 PSDs in controls was ~50 nm, whereas the thickness of most postischemic PSDs ranged from ~80 nm at 4 hr to 100 nm at 24 hr of reperfusion. The peak of the curves for the two postischemic conditions was shifted to the right compared with the controls (top panel). In contrast, in the dentate gyrus, considerable overlap was observed in the range of PSD thicknesses from all conditions. The peak of the curve was shifted to the right at 4 hr of reperfusion, although it occurred within the range of control PSD thicknesses.

Table 2. Number and perforation of synapses in CA1 and DG after cerebral ischemia

Groups	Synapses/100 μm^2		Perforation (%)	
	CA1	DG	CA1	DG
Sham	35.51 $\pm$ 2.99	43.79 $\pm$ 12.83	4.35 $\pm$ 0.62	3.63 $\pm$ 0.97
4 hr	32.943 $\pm$ 2.88	47.17 $\pm$ 6.58	5.82 $\pm$ 1.19	5.13 $\pm$ 1.27
24 hr	39.00 $\pm$ 3.40	36.46 $\pm$ 2.32	6.63 $\pm$ 0.97*	5.84 $\pm$ 2.08
7 d		38.18 $\pm$ 2.12		5.44 $\pm$ 1.99

The samples are from sham-operated rats (sham) and rats subjected to 15 min of ischemia followed by 4 and 24 hr and 7 d of reperfusion. The number of synapses per 100 μm^2 and the percent perforation are expressed as mean $\pm$ SD from three rats in each group.

*Significant difference ($p < 0.05$, Dunnett's test) between sham-operated control and the postischemia groups.

thin sections of E-PTA-stained material, we performed 3D reconstructions of E-PTA-stained PSDs contained in 1- μm -thick sections using electron tomography. The use of selective staining with E-PTA facilitates high-voltage 3D electron microscopy ex-

Figure 6. Tomographic volume reconstruction of E-PTA-stained PSDs in a 1- μm -thick section from stratum radiatum of control CA1. Only a portion of the volume is shown here. The contrast is reversed so that stained PSDs appear bright relative to unstained structures. The 3D structure of PSDs in the volume can be easily visualized. Each PSD in the volume can be viewed from any angle, as shown in the *bottom panel* for the PSD indicated by an *arrow* in the *top panel*. A 2D slice through the volume at the position of a perforation (*arrow*) is shown on the *right*, with the contrast reversed again so that the stained PSD appears *black* on *white* to facilitate comparison with thin-section images in other figures. Scale bar, 0.5 μm .

amination of synaptic densities, because it allows observation of PSD structure from any view without interference by other sub-cellular structures (Igarashi et al., 1988). We produced tomographic volume reconstructions of PSDs and presynaptic grids in thick sections from CA1 and dentate gyrus from control brains and postischemic brains at 4 and 24 hr of reperfusion. An example of a tomographic reconstruction of PSDs from control brain is shown in Figure 6. Each volume comprised $\sim 2 \mu\text{m}^3$ of tissue and contained 5–20 synapses. Approximately 30 synapses were analyzed for each condition in area CA1. The density of synapses ranged from 2–9 synapses/ μm^3 , and there was no significant difference in synaptic density between control and postischemic brains. The PSD volume was resectioned to produce 2D views similar to those in individual thin sections, to correlate 2D and 3D data from the same PSD (Fig. 6, *bottom panel*, 2D slice).

The tomographic volumes extended and supported observations made from individual thin sections (Figs. 6–8). Resectioning of tomographic volumes confirmed that the increase in PSD thickness observed in individual thin sections was not attributable

to a sectioning artifact (compare 2D slices in Figs. 6, 8) and also showed that the increase in thickness occurred in nearly every synapse visualized within the postischemic volume in area CA1, suggesting that most E-PTA-stained synapses were affected.

Three-dimensional reconstructions of PSDs revealed several additional aspects of PSD alteration in postischemic brain not apparent from the 2D images, as seen in Figure 7. Each PSD in Figure 7 was rotated to obtain an en face view of the entire PSD. Whereas both the 2D and 3D images indicated an increase in PSD thickness, the 3D reconstructions also showed that PSDs from postischemic brain were more loosely configured, irregular in shape, and fragmented than those from control brains (Fig. 7). PSDs from control sections were thin, condensed, and disk-like structures, whereas the PSDs from the postischemic hippocampus were less compact, with ragged edges. This loosening of PSD structure was most apparent in area CA1 at 24 hr of reperfusion. At 4 hr of reperfusion, PSD structure was intermediate between those of the control and 24 hr of reperfusion (Fig. 7). Three-dimensional views of PSDs also demonstrated a large variation in

Sham

4hr

24hr

Figure 7. Three-dimensional images of E-PTA-stained PSDs from tomographic volumes of control (*Sham*), 4 hr (*4hr*) and 24 hr (*24hr*) of reperfusion in CA1. Six different PSDs are shown for each condition. Each PSD was rotated individually to appear en face. The control PSDs were compact, disk-like structures with generally round edges and discrete perforations, whereas the postischemic PSDs were more loosely configured structures with irregular edges and less well defined perforations. PSDs at 4 hr of reperfusion appeared to be similar to those in control brains. The *hatching* visible in some PSDs, e.g., *top left* PSD in 24 hr, is attributable to artifacts produced in by the limited angle reconstruction. Because the specimen cannot be tilted in the electron microscope through a full 180°, resolution is degraded along the z-axis. Scale bar, 0.5 μ m.

PSD size within a given animal in both CA1 and dentate gyrus. Nearly every large synapse in both control and 24 hr reperfusion brains showed some sort of perforation; however, the perforations were more clearly defined in control brains because of their more compact structure. Perforations were not always in the center of the PSD. Small PSDs generally were not perforated or contained one small perforation but still exhibited the same loosening of PSD structure present in large synapses.

The 3D reconstructions also clarified the relationship between additional E-PTA-stained material present in the postischemic tissue. As described above, this material was present only in postischemic brains and was not always obviously connected to a PSD in individual thin sections. By resectioning the 3D reconstructions, the flocculent stained material observed near the PSD was often observed to be connected loosely to it (Fig. 8).

DISCUSSION

In this study, we demonstrated 2D and 3D ultrastructural modifications in hippocampal PSDs from CA1 and DG after a brief ischemic episode. The ultrastructural alterations of PSDs were more remarkable and persistent in CA1 than in the dentate gyrus and occurred well ahead of the degeneration observed in CA1 neurons after ischemia. Although the type of synapse served by the modified PSDs cannot be ascertained in the E-PTA material, both the 3D volume reconstructions and the frequency histograms (Fig. 5) indicated that most E-PTA-stained synapses showed an increase in thickness in area CA1. Because the majority of synapses in the molecular layer of CA1 are glutamatergic onto dendritic spines, our data suggest that at least this class of synapse is affected. We do not know whether inhibitory synapses are also affected, and this question is currently under investiga-

Figure 8. Three-dimensional volume reconstruction of an individual PSD (top left, large arrow) with associated E-PTA material loosely attached (arrowhead) from the postischemic brain after 24 hr of reperfusion. The 3D volume is shown in the left column, and 2D slices through the volume are shown in the right column. This type of configuration was not readily apparent from the 3D image alone but was easily observed after resectioning of the volume along the planes indicated. In the 2D slices, the postsynaptic density (large arrow), the synaptic cleft (small arrow), and the attached flocculent material (arrowhead) can be resolved. Similarly, the association of the flocculent material with the PSD could be missed in individual 2D sections if the plane of section were not optimal. Scale bar, 0.5 μ m.

tion. The marked ultrastructural alterations in the synapses during the postischemic phase could produce a toxic signal such as a greater calcium influx that contributes to or causes delayed neuronal death.

Modification of PSDs

The finding of a robust ultrastructural modification of postischemic PSDs stained with E-PTA was unexpected because several previous ultrastructural studies had not reported or found such noticeable changes in PSDs stained with osmium-heavy metal in tissue from several transient ischemic models, including the model used in the present study (von Lubitz and Diemer, 1983; Kirino et al., 1984; Petito and Pulsinelli, 1984; Deshpande et al., 1992). These inconsistencies may be attributable to the fact that general heavy metal staining obscures the synaptic modifications occurring in postischemic tissue. Alternatively, it is possible that

E-PTA stains different components in the PSD than osmium-uranium-lead methods. It has been known for a long time that PSDs stained with E-PTA are shorter and probably wider than those stained with the osmium-heavy metal method, although E-PTA does not stain the plasma membranes adjacent to the PSDs (Jones et al., 1974; Burry and Lasher, 1978). PTA, a heteropolyacid, preferentially reacts with protein(s) rich in basic amino acid residues, including lysine, arginine, and histidine, such as collagen or histones (Bloom and Aghajanian, 1968), whereas conventional heavy metal staining preferentially stains a wide array of lipids and cytoskeletal and cytoplasmic structures (Burry and Lasher, 1978). The staining of different components by these two methods may explain why such a robust ultrastructural alteration in postischemic PSDs stained with E-PTA has not been previously noted in the studies using heavy metal-stained sections

in postischemic brain tissues (von Lubitz and Diemer, 1983; Kirino et al., 1984; Petito and Pulsinelli, 1984; Deshpande et al., 1992; Tomimoto and Yanagihara, 1992; Rafols et al., 1995).

The use of electron tomography combined with EPTA staining proved to be a useful method for observing the 3D morphology of individual PSDs and presynaptic grids without having to resort to serial sectioning techniques. Large numbers of synapses can be reconstructed concurrently and because the entire PSD is contained within a single section, there are minimal sectioning and alignment distortions. The combination of selective staining and tomographic reconstruction produced gray scale volumes that required no additional segmentation. Thus, the fine structure of individual PSDs was clearly visible, and the uncondensed, flocculent structure of PSDs was readily apparent in the postischemic brain. This type of fragmented configuration was not as apparent in the 2D images of PSDs from thin sections. The resulting volume reconstructions could also be sectioned in any arbitrary plane, facilitating correlation of the 2D and 3D images.

PSDs are composed of a cytoskeletal frame, in which proteins, including neurotransmitter receptors, ion channels, and adaptor proteins, are anchored and targeted (Harris et al., 1992; Harris and Kater, 1994; Kennedy, 1994; Suzuki, 1994; Klauck and Scott, 1995). The ischemia-induced increases in intracellular calcium and production of free radicals activate proteases and result in partial degradation of certain cross-linker proteins in PSDs such as spectrin and microtubule-associated protein 2 (Yoshimi et al., 1991; Robert-Lewis et al., 1994). In addition, unfolding proteins induced by ischemia may aggregate and change the construction of PSDs. These may result in the loosening of the PSD frame structure, rendering them more diffuse and more irregular.

Transient cerebral ischemia induced a significant increase in perforated synapses from 4.35 to 6.63% in the CA1 but not in dentate gyrus at 24 hr of reperfusion. This finding suggests that the neurons react to ischemia more severely in CA1 than in the dentate gyrus, consistent with analysis of other PSD parameters in this study. It has been suggested that perforated synapses are more efficient in synaptic transmission than simple synapses (for review, see Edwards, 1995). However, it is unclear that an increase of only ~2% of perforated synapses without a change in total synapse number would contribute to the neuronal vulnerability in CA1 neurons. It is possible that the increase in synaptic perforation of synapse is a reactive process to neuronal depolarization or the calcium influx induced by ischemia (Siesjö, 1988). Alternatively, as observed in the 3D reconstructions, PSDs from postischemic CA1 were more irregular in profile and less condensed compared with their control counterparts. The impression garnered from the 3D reconstructions was not that there was an increase in discrete perforations in PSDs from postischemic tissue, but that there was a general loosening of E-PTA-stained material, leading to discontinuities in PSD structure. Thus this fragmentation could lead to an apparent, rather than a real, increase in the number of perforated synapses in a 2D analysis.

Significance of the synaptic changes

In our previous paper, we found that several protein kinases and protein ATPases were translocated to the synapses in cortical tissue after ischemia (Hu et al., 1998). Because only 5–20% of neocortical neurons die after ischemia, we suggested that the changes in the synapses might be attributable to a plastic synaptic response, i.e., kinase translocation to the PSDs to modulate receptor function. One objective of the present investigation was to examine the possible association between these synaptic

changes and neuronal death after transient cerebral ischemia. In the ischemic model used in this study, CA1 neurons die after 2 d of reperfusion, whereas dentate granule cells survive. Two questions are raised by our findings. First, do the observed synaptic changes represent a pathological or physiological phenomenon? Second, do the observed synaptic changes contribute to neuronal cell death?

Consistent with the results of our previous study, there was a marked increase in E-PTA-stained material in the PSDs from postischemic brain, particularly in area CA1. However, from the 3D reconstructions of synapses, we found that PSDs are not only increased in thickness, as was seen in 2D views, but appear to be disintegrating in area CA1. This type of alteration is much less noticeable and more transient in the dentate gyrus as well as in the neocortex (data not shown). The degenerative changes observed in CA1 synapses in this study suggest to us that the translocation of protein ATPase and protein kinases reported in our previous paper may also be related to degenerative changes (Hu et al., 1998). Both heat-shock cognate protein 70 and *N*-ethylmaleimide-sensitive fusion protein function as protein chaperones to prevent protein misfolding during their assembly. In addition, both protein kinase C and calcium/calmodulin-dependent protein kinase II are inactive after translocation to the PSDs after ischemia (Hu et al., 1995). These results suggest that accumulation of proteins in the PSDs may be attributable to protein unfolding or denaturing to expose their hydrophobic segments during ischemia, thereby causing interprotein aggregation in the PSDs after ischemia. The increased amount of material associated with PSDs in the postischemic brain could thus represent denatured and aggregated proteins. Consistent with this hypothesis, we recently found that PSD proteins from the CA1 region are dramatically and persistently ubiquitinated, indicating that the proteins in the PSDs are going to degrade (our unpublished data). Thus, transient ischemia may result in degradation of synaptic proteins by ubiquitin-dependent proteinases, leading to dysfunctional synaptic transmission at the altered synapse. Physiological studies suggest that synaptic transmission is depressed during the postischemic phase after 15 min of global ischemia (Volpe et al., 1984; Auer et al., 1989; Furukawa et al., 1990; Xu, 1995; Dalkara et al., 1996), further suggesting that the synaptic changes observed in the present study are a pathological phenomenon.

The observations in the present study that the degenerative changes in morphology are more robust and long-lived in vulnerable CA1 neurons than in resistant dentate gyrus neurons and occur well before CA1 neurons degenerate raise the question of whether this synaptic alteration may contribute to cell death. The morphological analyses conducted in the present study indicate that the majority of synapses in area CA1 are affected. There are ~10,000 dendritic spines per CA1 neuron, and >90% of excitatory synapses occur on dendritic spines (Andersen et al., 1990; Harris and Kater, 1994). Thus, if alterations in synaptic structure and biochemistry result in a toxic signal, e.g., greater calcium influx, from these changed synapses either because of nonspecific leakage or altered glutamatergic transmission, the damage signal could be integrated from several thousands of synapses and propagate to the dendritic trunk and, eventually, the cell body to cause cell death. This would be consistent with the observations by many investigators that a damage signal propagates from the dendrites to the somata of vulnerable neurons (Kirino et al., 1984; Petito and Pulsinelli, 1984; Tomimoto and Yanagihara, 1992). This is also consistent with the theory of an overload in

intracellular calcium (Choi, 1988; Siesjö, 1988). Because the changes in PSD structure were observed to be greater and more prolonged in CA1 neurons compared with dentate gyrus neurons, the cumulative effects of PSD damage would be expected to be greater in CA1 than in dentate gyrus, thereby contributing to their selective vulnerability.

REFERENCES

- Andersen P, Raastad M, Storm JF (1990) Excitatory synaptic integration in hippocampal pyramids and dentate granule cells. *Cold Spring Harb Symp Quant Biol* 55:81–86.
- Andiné P, Jacobson I, Hagberg H (1992) Enhanced calcium uptake by CA1 pyramidal cell dendrites in the post-ischemic phase despite subnormal evoked field potentials: excitatory amino acid receptor dependency and relationship to neuronal damage. *J Cereb Blood Flow Metab* 12:773–783.
- Auer RN, Jensen ML, Whishaw IQ (1989) Neurobehavioral deficits due to ischemic brain damage limited to half of the CA1 sector of the hippocampus. *J Neurosci* 9:1641–1647.
- Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus following transient cerebral ischemia monitored by intracerebral microdialysis. *J Neurochem* 43:1369–1374.
- Bloom FE, Aghajanian GK (1966) Cytochemistry of synapses: a selective staining method for electron microscopy. *Science* 154:1575–1577.
- Bloom FE, Aghajanian GK (1968) Fine structural and cytochemical analysis of staining of synaptic junctions with phosphotungstic acid. *J Ultrastruct Res* 22:361–375.
- Burky RW, Lasher RT (1978) A quantitative electron microscopic study of synapse formation in dispersed cell cultures of the rat cerebellum stained either by Os-UL or by E-PTA. *Brain Res* 147:1–15.
- Choi DW (1988) Calcium mediated neurotoxicity and diseases of the nervous system. *Trends Neurosci* 11:465–469.
- Dalkara T, Ayaata C, Demirci M, Erdemli G, Onur R (1996) Effects of cerebral ischemia on *N*-methyl-D-aspartate and dihydropyridine sensitive calcium currents: an electrophysiological study in the rat hippocampus in situ. *Stroke* 27:127–133.
- Deshpande J, Bergstedt K, Linden T, Kalimo H, Wieloch T (1992) Ultrastructural changes in the hippocampal CA1 region following transient cerebral ischemia: evidence against programmed cell death. *Exp Brain Res* 88:91–105.
- Edwards FA (1995) Anatomy and electrophysiology of fast central synapses lead to a structural model for long term potentiation. *Physiol Rev* 75:759–787.
- Furukawa K, Yamana K, Kogure K (1990) Post-ischemic alterations of spontaneous activities in rat hippocampal CA1 neurons. *Brain Res* 530:257–260.
- Hammond C, Crépel V, Ben-Ari Y (1994) Anoxic LTP sheds light on the multiple facets of NMDA receptors. *Trends Neurosci* 17:497–503.
- Harris KM, Kater SB (1994) Dendritic spines: cellular specializations and imparting both stability and flexibility to synaptic function. *Annu Rev Neurosci* 17:341–371.
- Harris KM, Jensen FE, Tsao B (1992) Three-dimensional structure of dendritic spine and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. *J Neurosci* 12:2685–2750.
- Hu BR, Wieloch T (1995) Persistent translocation of Ca^{2+} /calmodulin-dependent protein kinases II to synaptic junctions in the vulnerable hippocampal CA1 region following transient cerebral ischemia. *J Neurochem* 64:277–284.
- Hu BR, Kamme F, Wieloch T (1995) Alterations of Ca^{2+} /calmodulin-dependent protein kinase II and its messenger RNA in the rat hippocampus following normal and hypothermic ischemia. *Neuroscience* 67:1003–1016.
- Hu BR, Park M, Martone ME, Fischer WH, Ellisman MH, Zivin JA (1998) Assembly of proteins to postsynaptic densities after transient cerebral ischemia. *J Neurosci* 18:625–633.
- Igarashi H, Nasu F, Inomata K, Arai T (1988) High-voltage electron microscopy of E-PTA-stained synaptic junctions in the rat frontal cortex. *Brain Res* 473:565–568.
- Jones DG, Dittmer MM, Reading LC (1974) Synaptogenesis in guinea-pig cerebral cortex: a glutaraldehyde-PTA study. *Brain Res* 70:245–259.
- Kennedy MB (1994) The biochemistry of synaptic regulation in the central nervous system. *Annu Rev Biochem* 63:571–600.
- Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res* 239:57–69.
- Kirino T, Tamura A, Sato K (1984) Delayed neuronal death in the rat hippocampus following transient forebrain ischemia. *Acta Neuropathol (Berl)* 64:139–147.
- Klauck TM, Scott JD (1995) The postsynaptic density: a subcellular anchor for signal transduction enzymes. *Cell Signal* 7:747–757.
- Lipton SA (1994) Prospective for clinically tolerated NMDA antagonists: open-channel blockers and alternative redox states of nitric oxide. *Trends Neurosci* 16:527–532.
- Miyazaki S, Katayama Y, Furuichi M, Kinoshita K, Kawamata T, Tsubokawa T (1993) Post-ischemic potentiation of Schaffer collateral CA1 pyramidal cell responses of the rat hippocampus in vivo. Involvement of *N*-methyl-D-aspartate receptors. *Brain Res* 611:155–159.
- Miyazaki S, Katayama Y, Furuichi M, Kano T, Yoshino A, Tsubokawa T (1994) *N*-Methyl-D-aspartate receptor-mediated prolonged afterdischarges of CA1 pyramidal cells following transient cerebral ischemia in the rat hippocampus in vivo. *Brain Res* 657:325–329.
- Perkins G, Renken C, Martone ME, Young SJ, Ellisman M, Frey T (1997) Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts. *J Struct Biol* 119:260–272.
- Petito CK, Pulsinelli WA (1984) Delayed neuronal recovery and neuronal death in rat hippocampus following severe cerebral ischemia: possible relationship to abnormalities in neuronal processes. *J Cereb Blood Flow Metab* 4:194–205.
- Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol* 11:491–499.
- Rafols JA, Daya AM, O'Neil BJ, Krause GC, Neumar RW, White BC (1995) Global brain ischemia and reperfusion: Golgi apparatus ultrastructure in neurons selectively vulnerable to death. *Acta Neuropathol (Berl)* 90:17–30.
- Robert-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Simon R (1994) Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *J Neurosci* 14:3934–3944.
- Rothman SM, Olney JW (1995) Excitotoxicity and the NMDA receptor—still lethal after eight years. *Trends Neurosci* 18:57–58.
- Schroeter JP, Bretauiere JP (1996) SUPRIM: easily modified image processing software. *J Biol Struct* 116:131–137.
- Siesjö BK (1988) Historical overview. Calcium, ischemia, and death of brain cell. *Ann NY Acad Sci* 522:638–661.
- Smith ML, Bendek G, Dahlgren N, Rosen I, Wieloch T, Siesjö BK (1984) Models for studying long-term recovery following forebrain ischemia in the rat. A 2-vessel occlusion model. *Acta Neurol Scand* 69:385–401.
- Soto GE, Young SJ, Martone ME, Deerinck TJ, Lamont S, Carragher BO, Hama K, Ellisman MH (1994) Serial section electron tomography: a method for three-dimensional reconstruction of large structures. *NeuroImage* 1:230–243.
- Suzuki T (1994) Protein kinases involved in the expression of long-term potentiation. *Int J Biochem* 26:735–744.
- Tomimoto H, Yanagihara T (1992) Electron microscopic investigation of the cerebral cortex after cerebral ischemia and reperfusion in the gerbil. *Brain Res* 598:87–97.
- Volpe BT, Pulsinelli WA, Tribuna J, Davis HP (1984) Behavioral performance of rats following transient forebrain ischemia. *Stroke* 15:558–562.
- von Lubits DKJE, Diemer NH (1983) Cerebral ischemia in the rat: ultrastructural and morphometric analysis of synapses in stratum radiatum of the hippocampal CA-1 region. *Acta Neuropathol (Berl)* 61:52–60.
- Xu ZC (1995) Neurophysiological changes of spiny neurons in the rat neostriatum after transient forebrain ischemia: an in vivo intracellular recording and staining study. *Neuroscience* 67:823–836.
- Yoshimi K, Takeda M, Nishimura T, Kudo T, Nakamura Y, Tada K, Iwata N (1991) An immunocytochemical study of MAP2 and clathrin in gerbil hippocampus after cerebral ischemia. *Brain Res* 560:149–158.