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Timing of Neuronal and Glial Ultrastructure Disruption during Brain Slice Preparation and Recovery In Vitro

JOHN C. FIALA,[1] SERGEI A. KIROV,[2] MARCIA D. FEINBERG,[1] LARA J. PETRAK,[1] PRIYA GEORGE,[1] C. ALEX GODDARD,[1] AND KRISTEN M. HARRIS[3]*
[1]Department of Biology, Boston University, Boston, Massachusetts 02215
[2]Department of Neurosurgery and Synapses and Cell Signaling Program, Medical College of Georgia, Augusta, Georgia 30912
[3]Department of Neurology and Program in Synapses and Cell Signaling, Medical College of Georgia, Augusta, Georgia 30912

ABSTRACT

Hippocampal slices often have more synapses than perfusion-fixed hippocampus, but the cause of this synaptogenesis is unclear. Ultrastructural evidence for synaptogenic triggers during slice preparation was investigated in 21-day-old rats. Slices chopped under warm or chilled conditions and fixed after 0, 5, 25, 60, or 180 minutes of incubation in an interface chamber were compared with hippocampi fixed by perfusion or by immersion of the whole hippocampus. There was no significant synaptogenesis in these slices compared with perfusion-fixed hippocampus, but there were other structural changes during slice preparation and recovery in vitro. Whole hippocampus and slices prepared under warm conditions exhibited an increase in axonal coated vesicles, suggesting widespread neurotransmitter release. Glycogen granules were depleted from astrocytes and neurons in 0-min slices, began to reappear by 1 hour, and had fully recovered by 3 hours. Dendritic microtubules were initially disassembled in slices, but reassembled into normal axial arrays after 5 minutes. Microtubules were short at 5 minutes (12.3 ± 1.1 μm) but had recovered normal lengths by 3 hours (84.6 ± 20.0 μm) compared with perfusion-fixed hippocampus (91 ± 22 μm). Microtubules appeared transiently in 15 ± 3% and 9 ± 4% of dendritic spines 5 and 25 minutes after incubation, respectively. Spine microtubules were absent from perfusion-fixed hippocampus and 3-hour slices. Ice-cold dissection and vibratomy in media that blocked activity initially produced less glycogen loss, coated vesicles, and microtubule disassembly. Submersing these slices in normal oxygenated media at 34°C led to glycogen depletion, as well as increased coated vesicles and microtubule disassembly within 1 minute. J. Comp. Neurol. 465:90–103, 2003.

Indexing terms: dendrite; dendritic spine; coated vesicles; depolymerization; excitotoxicity; glycogen; interneuron; ischemia; metabolism; microtubule; synapse; ultrastructure

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Dr. Goddard's current address: Program in Neuroscience, Harvard Medical School, Boston, MA.

*Correspondence to: Kristen M. Harris, Georgia Research Alliance Eminent Scholar in Synapses and Cell Signalling, Institute of Molecular Medicine and Genetics, 1120 15th Street, CB-2803, Augusta, GA 30912-2630. E-mail: kharris@mail.mcg.edu

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Hippocampal slices are an important tool in neuroscience research, but slices deviate in many ways from in vivo conditions. Slices develop altered levels of glutamate receptor proteins and mRNA (Taubenfeld et al., 2002) and altered expression of immediate early genes such as *c-fos* (Zhou et al., 1995; Taubenfeld et al., 2002). The intracellular concentrations and distributions of Na^+, K^+, and Ca^{2+} can be significantly different in vitro (Siklos et al., 1997). Slices can exhibit increased numbers of synapses (Wenzel et al., 1994; Kirov et al., 1999; Johnson and Ouimet, 2002) and a complete loss of dendritic microtubules (Burgoyne et al., 1982). In addition to these persistent changes, a transient drop in glycogen and ATP occurs during the initial stages of incubation (McIlwain and Tresize, 1956; Whittingham et al., 1984; Lipton, 1988; Feig and Lipton, 1990). This is accompanied by a loss of synaptic transmission that gradually recovers during the first hour in vitro (Schurr et al., 1984; Kirov et al., 1999).

Many of these changes have been attributed to the unavoidable ischemic insult of slice preparation (Newman et al., 1992; Zhou et al., 1995; Siklos et al., 1997; Taubenfeld et al., 2002). Indeed, a brief period of ischemia induces many of these same effects in vivo, including a loss of ATP and glycogen (Folbergrova et al., 1997), a loss of synaptic transmission (Xu and Pulsinelli, 1994), an influx of Ca^{2+} (Benveniste et al., 1988; Mies et al., 1993), and induction of immediate early genes (Kiessling et al., 1993). Ischemia also produces a massive synaptic release of glutamate that contributes to neuronal injury (Benviste et al., 1984,1989), but it is currently not known whether this occurs during slice preparation. Some of the effects of ischemia can be observed with electron microscopy, such as the disassembly of microtubules in CA1 pyramidal neuron dendrites (Yamamoto et al., 1986) and the long-term increase in visible glycogen granules (Koizumi, 1974).

A brief period of anoxia/hypoglycemia can trigger synaptogenesis in organotypic slice cultures (Piccini and Malinow, 2001; Jourdain et al., 2002). However, it is not known whether the synaptogenesis seen in acute slices can be attributed to the period of ischemia during slice preparation. To investigate this further we prepared for ultrastructural analysis the whole hippocampus immediately after dissection along with hippocampal slices prior to and soon after incubation. We found significant structural changes during these early stages, including disassembly of dendritic microtubules and a complete loss of visible glycogen. These ultrastructural changes recovered after 3 hours of incubation, whereas synapse number remained constant such that these slices resembled perfusion-fixed hippocampus in all aspects that were evaluated.

MATERIALS AND METHODS

Long-Evans male rats, 21 days old (P21), weighing 50–60 g were used for all experiments following National Institutes of Health guidelines for the humane care and use of laboratory animals. All protocols underwent yearly review by the Animal Care and Use Committee of Boston University. Hippocampi from three rats were used for control comparisons (Kirov et al., 1999). These animals were perfused through the heart with fixative (6% glutaraldehyde, 2% paraformaldehyde, 100 mM cacodylate buffer, 2 mM $CaCl_2$, 4 mM $MgCl_2$, at pH 7.4) under deep pentobarbital anesthesia (80 mg/kg). The same fixative was also used on dissected whole hippocampi and slices, combined with brief microwave irradiation at full power (Jensen and Harris, 1989; 700W Amana microwave oven model RS4141, Newton, IA). In the methods described below, the fixative was iced during microwave irradiation when necessary to avoid heating the tissue. The final fixative temperature was 26–32°C. Slices were prepared using three different standard protocols: chilled, warm, and ice cold with blocked synaptic transmission (Harris and Teyler, 1984; Schurr et al., 1987; Lipton et al., 1995; Kirov and Harris, 1999; Staff et al., 2000).

TABLE 1. Mean Times after Death for Completion of Each Preparation Step (± SEM in Seconds)

Preparation step	Time after death chopping (min:sec ± sec)	Time after death vibratomy (min:sec ± sec)
Brain removed	1:20 ± 04	8:28 ± 107
Right whole hippocampus fixed	2:35 ± 05	
0-min slice fixed	3:23 ± 19	17:57 ± 24
Slice incubation begun	5:43 ± 09	20:59 ± 32
1-min slice fixed		23:36 ± 31
5-min slice fixed	10:07 ± 05	26:11 ± 20
25-min slice fixed	30:10 ± 12	
30-min slice fixed		49:53 ± 17
1-h slice fixed	59:56 ± 16	
3-h slice fixed	180:05 ± 06	

[1]Means are averaged over six experiments for chopped slices, (four from chilled slice preparation and two from warm slice preparation) and over two experiments from the ice-cold condition for which a vibrating blade was used to prepare the slices.

Chilled slice preparation

Four experiments were done in which solutions and dissecting surfaces were kept on ice and the tissue chopper was kept in a refrigerator until just prior to the start of the experiment. Rats were rapidly decapitated with a guillotine and the cranium and underlying dura removed. The exposed brain was rinsed with the chilled artificial cerebrospinal fluid (ACSF; containing 117 mM NaCl, 5.3 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10 mM glucose, at pH 7.4, and bubbled with 95% O_2/5% CO_2). The brain was rapidly removed and divided along the midline. The right hippocampus was dissected out and immediately immersed in 5.5 ml of room temperature fixative in a 35-mm culture dish. This was placed on a bag of ice in the microwave oven and irradiated at full power for 60 seconds. Fixation began approximately 2 minutes 35 seconds after death (Table 1).The left hippocampus was dissected out in parallel with the right hippocampus, rinsed with chilled ACSF, and placed onto a cold Stoelting tissue chopper. Slices 400 μm thick were cut at a 70-degree angle transverse to the long axis of the middle third of the hippocampus. The first slice (0-min slice) was fixed immediately by transferring it to a 35-mm culture dish containing 5.5 ml of fixative and irradiating it for 8 seconds at full power in the microwave oven.

In each experiment four more slices were made during the next minute and placed immediately into chilled ACSF. These slices were then transferred to an interface recording chamber and placed onto nets over wells containing ACSF at 32 °C surrounded by humidified 95% O_2/5% CO_2. The entire process was completed as quickly as possible and the time between death and incubation of the slices averaged 5 minutes 43 seconds (Table 1). Slices were removed from the interface chamber and fixed after

5 minutes, 25 minutes, 1 hour, or 3 hours (Table 1). Field excitatory postsynaptic potentials were recorded to assess slice responsiveness for slices maintained for more than 1 hour. A concentric bipolar stimulating electrode was positioned about 400 μm from an extracellular recording electrode in the middle of the stratum radiatum of hippocampal area CA1. The stimulus-response curve was sigmoidal, and half-maximal responses were stable for an hour prior to microwave-enhanced fixation at 3 hours after death.

Warm slice preparation

Two experiments were conducted under warm dissection conditions. All surfaces and ACSF were kept warm at approximately 33°C during dissection. The tissue chopper was kept in a 37°C incubator until just prior to slicing and then removed to room temperature where the slices were prepared and transferred in warm ACSF to the standard interface incubation chamber and maintained at 32°C for the same times discussed above and indicated in Table 1.

Ice-cold slice preparation with blocked synaptic activity

Two additional experiments were done under ice-cold conditions with blocked synaptic transmission. Slices were prepared according to Staff et al. (2000) using a modified ACSF with sucrose, 0 mM Ca, and 8 mM Mg to prevent synaptic transmission (Kirov and Harris, 1999). Modified ACSF (210 mM sucrose, 2.5 mM KCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 8 mM $MgSO_4$, 10 mM glucose, pH 7.4) bubbled with 95% O_2/5% CO_2 was partially frozen prior to the start of experiment to create ice slush. The rat was anesthetized with halothane and perfused through the heart with ice-cold modified ACSF and the head was removed and immediately submerged in the modified ACSF ice slush. The brain was removed into the slush and then mounted on the ice-cold cutting platform of a vibrating-blade microtome (VT100 S, Leica Instruments, Nussloch, Germany). The brain was oriented to obtain four transverse hippocampal slices (400 μm thick) in situ. During vibratoming the entire brain was submerged in modified ACSF ice slush bubbled continuously with 95% O_2/5% CO_2. The first slice was transferred into fixative and microwaved for 8 seconds. The remaining slices were submerged in normal ACSF maintained at 34°C and bubbled with 95% O_2/5% CO_2. These slices were fixed after 1, 5, or 30 minutes of incubation in the submersion chamber (Table 1).

Electron microscopy

After fixation, the tissue from each experiment was stored overnight at room temperature in the fixative solution. The next day the tissue samples were processed for electron microscopy using standard procedures (Kirov et al., 1999). Briefly, each sample was manually trimmed to a region containing only area CA1 and processed in potassium ferrocyanide-reduced osmium, osmium, and aqueous uranyl acetate, dehydrated in a sequence of increasing acetone concentrations using microwave-enhanced processing and embedded in resins for 2 days at 60 °C (Feinberg et al., 2001).

Ultrathin (50-nm) sections were cut from the middle of the slice approximately 120–150 μm from the air surface in stratum radiatum perpendicular to the apical dendritic field, at a distance of 200 μm from the CA1 cell body layer. Sections were mounted on Pioloform-coated Synaptek slot grids (Ted Pella, Redding, CA) and stained with saturated ethanolic uranyl acetate and Reynold's lead citrate for 5 minutes each. Sections were photographed on the JEOL 1200EX or 2010 electron microscope (JEOL USA, Peabody, MA). Single sections were photographed at 6,000× magnification. Serial sections were photographed at 10,000× magnification. All negatives were digitized at 1,000 dpi on a Sprintscan 45 large-format film scanner (Polaroid, Cambridge, MA). The serial section images were aligned using *sEM Align* software (http://synapses.mcg.edu/tools/). Section dimensions were calibrated using a diffraction grating replica (Ernest Fullam, Latham, NY) photographed, and scanned with the series (Fiala and Harris, 2001b). Section thickness was calibrated by dividing the diameters of longitudinally sectioned mitochondria by the number of sections they spanned (Fiala and Harris, 2001a).

Analyses

Both single section and serial section analyses were performed using *IGL Trace* software (http://synapses.mcg.edu/tools/). Single-section images were coded prior to analysis such that the investigator was not aware of the time point being analyzed. Means and standard errors of the mean are presented for all measurements. Statistica (Statsoft, Tulsa, OK) was used to test differences among means by analysis of variance (ANOVA). Tukey's honest significance differences (HSD) test was used to compare individual time points. Three-dimensional surface reconstructions produced by *IGL Trace* were exported to *3D Studio Max* (Discreet Logic, Montreal, Canada) for rendering of final figures.

Single-section analyses were used to assess glycogen granule and coated vesicle frequency. Counting on single sections was deemed sufficient to compare relative frequencies across conditions because the objects being counted were smaller than the section thickness. Large, darkly stained granules located in the cytoplasm having the size (~20–40 nm) and appearance (Koizumi, 1974; Peters et al., 1991) of glycogen granules were counted (Fig. 1). A small or faint granule was not counted as it may have represented a ribosome or partially sectioned granule. Coated vesicles and coated pits in axonal profiles were identified by obvious clathrin-like coats containing several spokes. Coated vesicles or pits in dendritic profiles were not counted.

Microtubule analyses and reconstructions were done on series of 80–120 sections. Microtubules were identified in cross-sectioned dendrites as small circles approximately 18 nm in diameter (Fig. 1). Adjacent serial sections were used to distinguish microtubules from small cisternae of endoplasmic reticulum and other organelles. The density of microtubules was assessed in dendrites by tracing the profile of each cross-sectioned dendrite and measuring its area exclusive of any spines. The mean number of microtubules per cross-sectional area for each dendrite was calculated from 5 equally spaced measurements along a 2–4-μm segment of the dendrite. Microtubules were completely reconstructed in some dendrites by tracing each microtubule profile from section to section throughout the series. The length of microtubules was estimated using the method of Hardham and Gunning (1978) as employed by Hillman (1988), in which the number of microtubule terminations within a dendritic segment are used to esti-

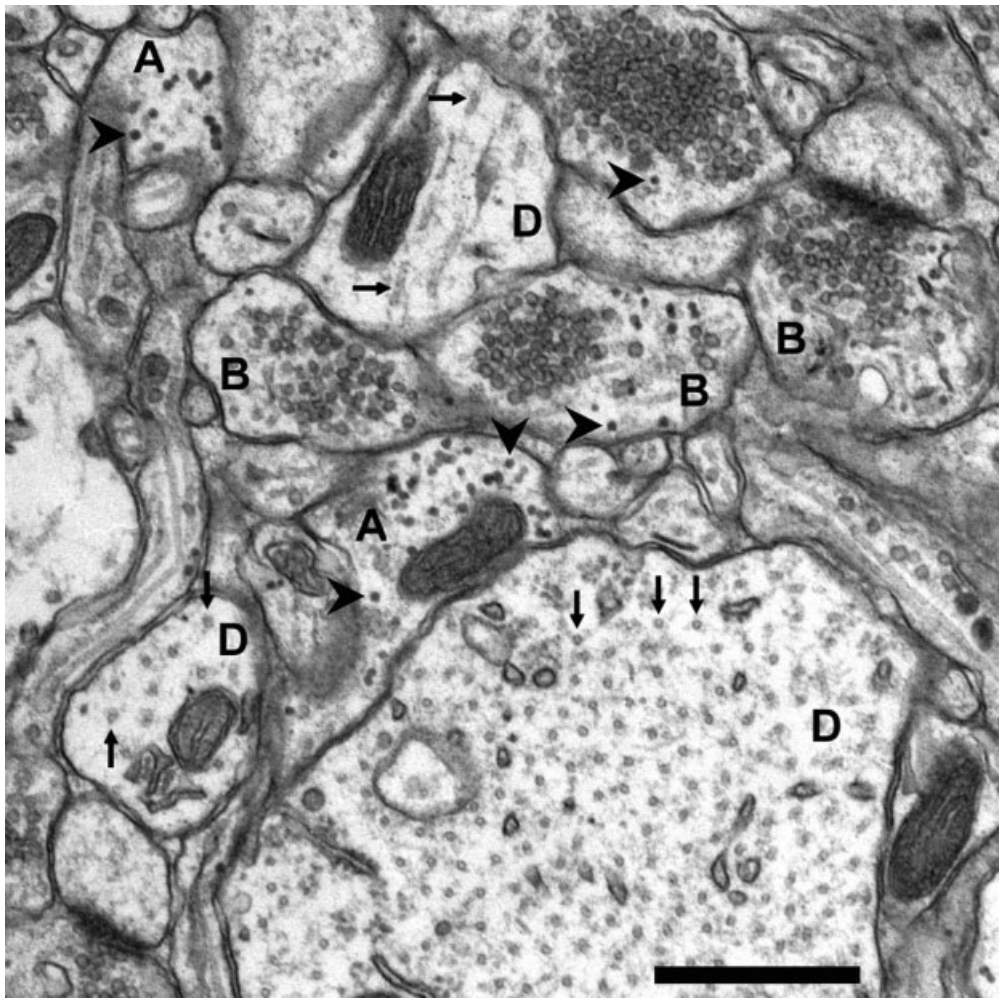

Fig. 1. Neuropil of area CA1 in perfusion-fixed hippocampus. Dendrites (D) contain axial arrays of microtubules (arrows). Glycogen granules (chevrons) are found in large numbers in astrocytes (A) and less frequently in axonal boutons (B). Scale bar = 0.5 μm.

mate the length of microtubules passing through that segment. Given the total number (N) of microtubules within the segment, the number (T) of terminations of microtubules within the segment, and the length (a) of the segment, the average length (L) of the microtubules was computed as $L = 2 \times a \times N \div T$. All the microtubules (236) in 20 spiny dendritic segments were reconstructed, as were all of the microtubules (77) in 2 aspiny segments. Unbiased stereological methods were used for analyses of synaptic densities (Fiala and Harris, 2001b). Volumes of serial sections were analyzed with the unbiased brick method to assess volume density of synapses. In perfused animals synapses were counted in 424 μm^3 from three series, and a comparable 438 μm^3 from three series were analyzed from slices prepared chilled and recovered for 3 hours. For spine density analyses, dendritic segments were reconstructed from serial sections at all time points. Along each reconstructed segment, the number of spine origins was counted exclusive of any origins that intersected the first section.

RESULTS

Perfusion fixation of the hippocampus in situ was used to assess ultrastructure prior to dissection (Fig. 1). Dendrites and axons in perfused hippocampus contained numerous microtubules oriented parallel to their long axes. In cross-sectioned dendrites the microtubules were arranged in an evenly spaced array occupying the central axis of the dendrite. Microtubules were always restricted to the dendritic shaft and did not enter into dendritic spines. Glycogen granules were concentrated in astrocytic processes that interdigitated between axons and dendrites. Glycogen granules were also found sparsely scattered in synaptic boutons and other parts of axons. Spiny dendrites of CA1 pyramidal neurons were devoid of glycogen granules in perfused hippocampus.

Ultrastructural changes during dissection of the hippocampus

To assess the effect of dissection, both left and right hippocampi were rapidly removed, and the right hip-

pocampus was fixed immediately by immersion with microwave irradiation. In this whole hippocampus fixed approximately 2.5 minutes after death (Table 1), dendritic microtubules were depolymerized (Fig. 2a). Although some microtubules were visible in large (3–6-μm diameter) apical dendrites, smaller diameter dendrites were completely devoid of microtubules. Microtubules in axons appeared to be unaffected. Some dendrites appeared swollen, and enlarged mitochondria occurred in both dendrites and astrocytes. No glycogen granules were observed in astrocytes, axons, or dendrites. Axons in whole-fixed hippocampus exhibited more coated vesicles and pits than perfusion-fixed hippocampus. Axonal coated vesicles represent the clathrin-mediated endocytosis of synaptic vesicle membrane following synaptic release (Heuser, 1989; Cremona and De Camilli, 1997). Serial section electron micrographs from the whole-fixed hippocampus can be compared with perfused hippocampus in supplementary material (http://synapses.mcg.edu/anatomy/bve/explorer.stm).

Immediately after the left hippocampus was dissected out, it was placed on a tissue chopper, and slices were made from the middle third. The first slice was fixed immediately without incubation (0 minutes). The ultrastructure of these first slices was similar in appearance to that of the whole-fixed hippocampus. Few microtubules were visible in small dendrites, and glycogen granules were depleted from astrocytes and synaptic boutons (Fig. 2b). Unlike the whole hippocampus, however, coated vesicles were not abundant in the 0-minute slice following chilled slice preparation (Fig. 2b).

Recovery of ultrastructure in vitro

Additional slices were incubated in an interface chamber in oxygenated ACSF at 32°C for various lengths of time prior to fixation. Slices fixed after 4–5 minutes of incubation (Table 1) showed substantial recovery of microtubules but no recovery of glycogen granules. Microtubules reappeared in an array pattern in cross-sectioned dendrites (Fig. 2c). Cross-sectioned microtubules looked normal in slices incubated for 25 minutes, but glycogen stores remained depleted (Fig. 2d). After approximately 1 hour of incubation (Fig. 2e), glycogen granules reappeared in astrocytic processes but fewer were visible than in perfusion-fixed tissue (Fig. 1).

After 3 hours, slice ultrastructure resembled that of perfusion-fixed hippocampus (Fig. 2f). Glycogen granules were concentrated in astrocytes but also occurred in axons. Unlike perfusion-fixed hippocampus, however, a few isolated granules occurred in dendritic spines and more rarely in the shafts of spiny dendrites. Serial sections of recovered slices can be compared with perfusion-fixed hippocampus in supplementary material available elsewhere (http://synapses.mcg.edu/anatomy/bve/explorer.stm).

The disruption and recovery of glycogen granules appeared to follow a similar time course whether the tissue was warm or chilled during dissection (Fig. 3a,b). Chilling the slices prior to incubation did not prevent the loss of glycogen. Glycogen stores required more than an hour of incubation to recover fully. The whole-fixed hippocampus showed a large increase in axonal coated vesicles relative to perfusion-fixed hippocampus (Fig. 3c,d; Tukey's HSD $P < 0.001$), which was similar in both warm and chilled dissections (Tukey's HSD, $P = 0.23$). With chilling, the first slice had fewer axonal coated vesicles than in warm dissection conditions (Tukey's HSD, $P = 0.018$).

Loss and recovery of the dendritic microtubules

Cross-sectioned dendrites were analyzed through serial sections to assess the degree of microtubule disruption and recovery in vitro following chilled slice preparation. In perfused hippocampus, there was a linear relationship between dendrite cross-sectional area and number of microtubules (Fig. 4). Dendrites from whole-fixed hippocampus and slices fixed prior to incubation had many fewer microtubules (Fig. 4a). After 5 minutes in vitro, dendritic microtubules reappeared in an array pattern, with almost the same linear relationship relative to dendrite size measured in perfused hippocampus. Microtubule densities were fully recovered after 3 hours (Fig. 4b).

Microtubules were reconstructed through serial sections from one chilled experiment to assess the degree of repolymerization (Fig. 5). Dendritic microtubules in a perfused hippocampus and in a slice recovered for 3 hours terminated infrequently. When present, these terminations ended in the middle of the cytoplasm rather than on mitochondria, endoplasmic reticulum, or the cell membrane. Microtubules terminated more frequently in the 5- and 25-minute slices, indicating that these microtubules were composed of shorter segments (Fig. 6). Microtubule length was estimated from the number of terminations per micron of length (see Materials and Methods). Dendritic microtubules were shorter in the slice recovered for 5 minutes than in perfused hippocampus (12.3 ± 1.1 μm versus 91.3 ± 19.6 μm; Tukey's HSD, $P = 0.04$). Microtubule lengths were not significantly different from perfused after 25 minutes (44.9 ± 25.3 μm; Tukey's HSD, $P = 0.34$) or 3 hours (84.6 ± 20.0 μm; Tukey HSD's, $P = 0.99$).

Surprisingly, microtubules were found in dendritic spines in the 5- and 25-minute slices (Figs. 5b, 7a,b). Spine microtubules often appeared to be attached to the plasmalemma of the spine head. Some were restricted to the spine head, whereas others passed through the spine neck and traversed the dendrite cylinder perpendicular to the

Fig. 2. Disruption of ultrastructure in chilled slice preparations. **a:** Whole-fixed hippocampus. Glycogen granules are absent from astrocytes (A) and other profiles. Cross-sectioned dendrites (D) contain no microtubules, but microtubules (arrow) can still be seen in axons (Ax). Some mitochondria (M) are swollen, and boutons contain many coated vesicles (bent arrows). The inset shows a coated vesicle from an adjacent section at approximately 2.4 times the magnification. **b:** 0-minute slice. In a slice fixed immediately, astrocytes (A) are essentially devoid of glycogen, although an occasional small granule (chevron) can be seen. Very few microtubules occur in dendrites (D), but microtubules are apparent in axons (Ax). **c:** 5-minute slice. Dendrites (D) in a slice incubated for 4–5 minutes exhibit axial arrays of microtubules (arrows). Glycogen granules are absent from astrocytic (A) and other profiles. **d:** 25-minute slice. Incubation for 25 minutes leads to further recovery of dendritic microtubules (arrows), but glycogen remains absent from astrocytes (A). A dendrite of an interneuron (ID) also exhibits a recovered microtubule array. **e:** 1-hour slice. One hour of incubation leads to reformation of glycogen granules (chevrons) in astrocytes (A) and axons (Ax). Cross-sectioned dendrites (D) have a normal complement of microtubules (arrows). **f:** 3-hour slice. After 3 hours in vitro the neuropil resembles that of the perfused hippocampus (Fig. 1). Glycogen granules (chevrons) are concentrated in astrocytes (A) and scattered in boutons (B). Glycogen granules occasionally occur in dendritic spines (S) as well. Dendrites (D) have microtubule arrays (arrows) indistinguishable from those in perfused hippocampus. Scale bar = 0.5 μm.

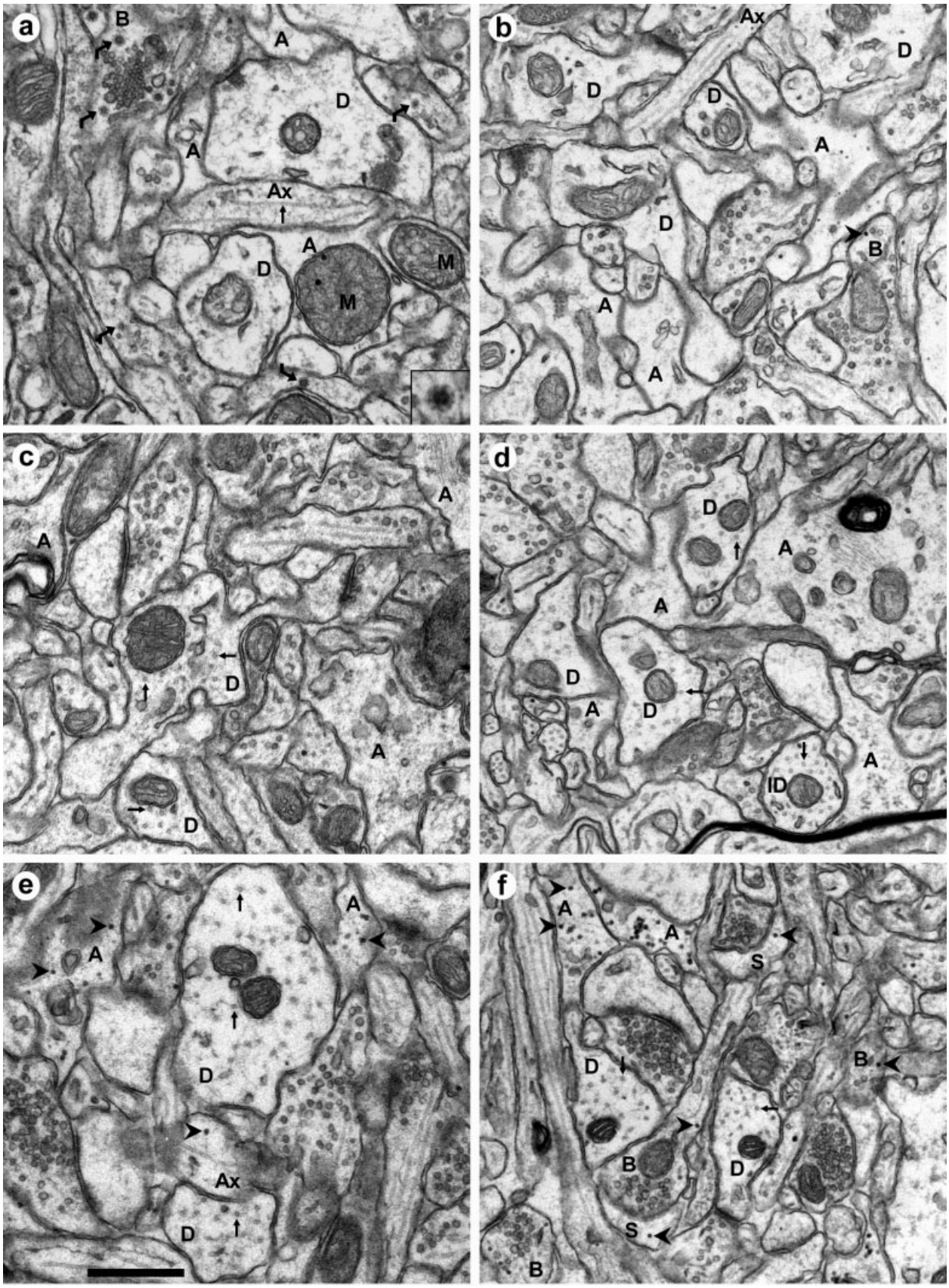
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Figure 2

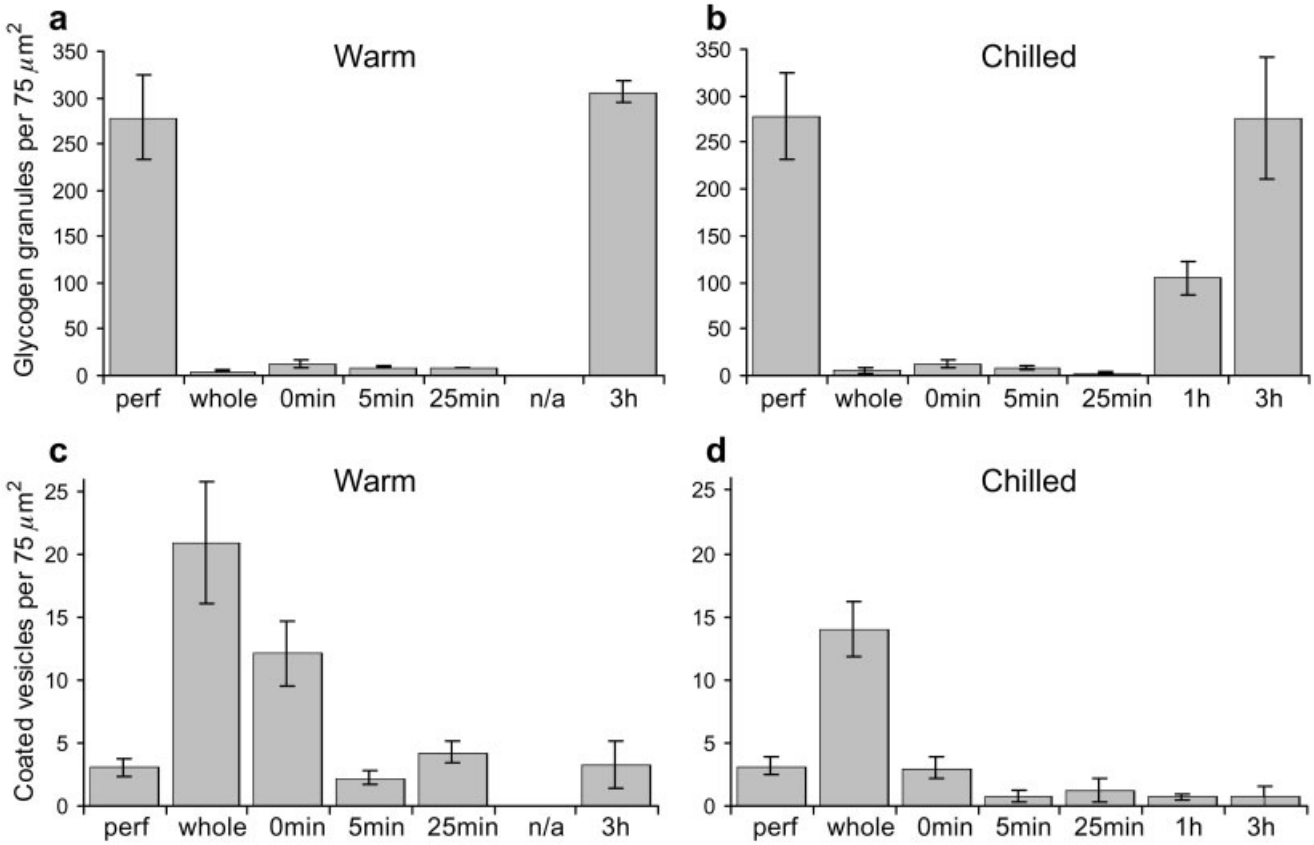

Fig. 3. Changes in glycogen granules and axonal coated vesicles during slice preparation and recovery. **a,b:** Glycogen granules were depleted relative to perfused (perf) for at least 25 minutes in both the warm and chilled dissection conditions and fully recovered after 3 hours of incubation. (The 1-hour time point was not available in the warm experiments.) **c:** Coated vesicles and pits were increased in the whole-fixed hippocampus (whole) and in the slice fixed immediately (0 minutes) in the warm dissection condition. **d:** Chilled conditions also produced more coated vesicles and pits in the whole-fixed hippocampus; however, the number of coated vesicles and pits in the first slice (0 minutes) was comparable to perfused. Three to eight 75-μm^2 sections taken from two animals were analyzed for each time point in each condition.

axial microtubule array. Occasionally, a spine microtubule entered the cylinder and continued parallel to the axial microtubules. After 5 and 25 minutes of incubation, microtubules were found in 15 ± 3% and 9 ± 4% of spines, respectively (Fig. 7c). No microtubules were observed in dendritic spines in perfusion fixed hippocampus or in slices incubated for 3 hours.

Effect of ice-cold perfusion and blocked synaptic activity

An attempt was made to prevent the structural changes by chilling the brain prior to dissection and dissecting in ice-cold medium that would block synaptic activity (Aitken et al., 1995; Lipton et al., 1995). Trauma was minimized by vibratoming slices in the ice-cold medium with surrounding brain tissue rather than chopping them from an isolated hippocampus. Vibratomed slices were submerged in oxygenated ACSF at 34°C rather than incubated in the interface chamber in order to capture the earliest events during recovery.

Glycogen granules were present in these slices when they were fixed prior to incubation (0 minutes, Fig. 8a), but as soon as slices were transferred to normal ACSF (1 minute), glycogen stores were lost and were only partially recovered after 30 minutes in vitro. Comparison of 0-minute slices with perfused hippocampus reveals that some glycogen may have been lost during the 18 minutes of dissection and vibratomy (Table 1). However, glycogen may also have recovered more quickly because the counts at 30 minutes in the submerged slices are comparable to counts at 1 hour in interface slices (Fig. 3b). Qualitatively, this recovery appeared to favor axonal glycogen rather than astrocytic glycogen as seen in interface slices (Fig. 2e,f).

Slices fixed prior to incubation had coated vesicle frequencies comparable to that of perfused hippocampus (Fig. 8b). With submersion in warm ACSF, the frequency of coated vesicles increased by 1 minute and remained elevated for the entire 30 minutes of incubation (Tukey's HSD, $P < 0.05$). This contrasted with the interface slices in which coated vesicles remained at a relatively low level throughout the incubation period (Fig. 3c,d).

Dendritic microtubules appeared better preserved with ice-cold preparation. In the 0-minute slice, many dendrites, especially larger (>0.6-μm-diameter) ones, had normal-appearing microtubule arrays. When slices were

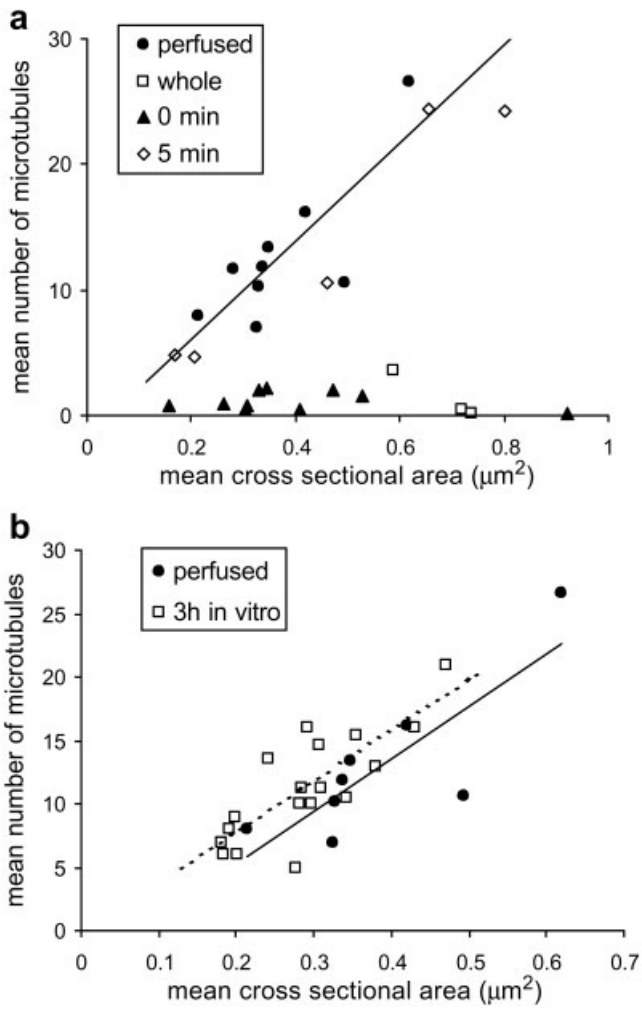

Fig. 4. **a,b:** Loss and recovery of microtubule arrays in dendrites. Each point in the graphs represents the mean number of microtubules and mean diameter from five measurements along a dendritic segment reconstructed through serial sections from one chilled dissection experiment. In perfused hippocampus there was a linear relationship of microtubules to the cross-sectional area of the pyramidal neuron dendrites (solid lines). In the whole hippocampus (whole) and the first slice fixed immediately (0 minutes), microtubules were mostly absent from cross-sectioned dendrites. The microtubule arrays recovered after 5 minutes in vitro and exhibited the same linear relationship at 3 hours (dashed line) as in perfused.

submerged in warm ACSF, dendritic microtubules disassembled further, occasionally accompanied by signs of swelling in dendritic organelles. Because serial sections were not available for these slices, a single-section analysis of the linear relationship between dendrite cross-sectional area and the number of microtubules was used to show that microtubules were disassembled in 1- and 5-minute slices, but recovered by 30 minutes in vitro (Fig. 8c).

Spine and synapse density in recovered slices

Synaptogenesis was evaluated in slices by comparing them with perfused hippocampus. No changes were evident at any stage of recovery in these slices (Fig. 9). Spiny dendrites in three slices incubated for 3 hours had 2.96 ± 0.07 spines/μm. This did not differ significantly from the 2.55 ± 0.23 spines/μm observed in three perfused hippocampi [ANOVA $F(1,4) = 2.86$, $P = 0.17$]. Likewise, the volume density of synapses was the same in both perfused hippocampus and 3-hour slices [313 ± 44 versus 321 ± 30 synapses/100 μm^3; ANOVA $F(1,4) = 0.03$, $P = 0.88$].

Interneuron dendrites

Interneuron dendrites in the hippocampus are characterized by a predominance of synapses on the shaft of the dendrite rather than spine synapses (Gulyás et al., 1999). Dendrites with a predominance of shaft synapses were occasionally encountered in the serial section electron micrographs from this study. Unlike spiny dendrites, the interneuron dendrites in perfused hippocampus contained glycogen granules scattered in the cytoplasm (Fig. 10a). During warm or chilled brain dissection, interneuron dendrites lost all their glycogen granules and microtubules (Fig. 10b). After 5 minutes of incubation, cytoplasmic glycogen granules were absent from interneuron dendrites but microtubule arrays appeared to have recovered (see the dendrite labeled "ID" in Fig. 7b). After 3 hours in vitro, large numbers of glycogen granules were again found in the cytoplasm of interneuron dendrites (Fig. 10c). Glycogen granules were also concentrated in rare protrusions from these dendrites (Fig. 10c).

There were too few interneuron dendrites in the data to perform a systematic analysis of their microtubular arrays and lengths. However, two interneurons were reconstructed, one from perfusion-fixed hippocampus and another from a chilled slice recovered for 3 hours in the interface chamber. In the 6.4-μm segment of dendrite from perfusion-fixed hippocampus only 1 microtubule of 37 terminated (Fig. 10d), suggesting that microtubules segments were very long (474 μm). In contrast, the dendrite reconstructed from the 3-hour slice had 9 of 40 microtubules terminating in 4.6 μm, for an estimated microtubule length of only 41 μm (Fig. 10e). This observation suggests that interneuron microtubules had not fully recovered after 3 hours. Other explanations are possible, however, such as different microtubule segment lengths associated with different types of interneurons.

DISCUSSION

Ultrastructural changes observed during slice preparation, including glycogen loss, microtubule dissassembly, and coated vesicle increases, are consistent with the consequences of ischemia. The whole-fixed hippocampus exhibited the most dramatic changes. Fixation of whole hippocampus was started within 2–3 minutes, but complete fixation of the center of the tissue may have taken longer. Whole hippocampus was also not chilled below room temperature prior to fixation. The duration of oxygen and glucose deprivation at a relatively warm temperature probably contributed to the severity of the ultrastructural disruptions. Slices prepared in warm conditions were initially similar to whole-fixed hippocampus. Astrocytic glycogen and dendritic microtubules subsequently recovered in vitro with the restoration of oxygen and glucose. This is consistent with chemical analyses showing that there is an initial loss of glycogen in slices that slowly recover in vitro (McIlwain and Tresize, 1956; Lipton, 1988).

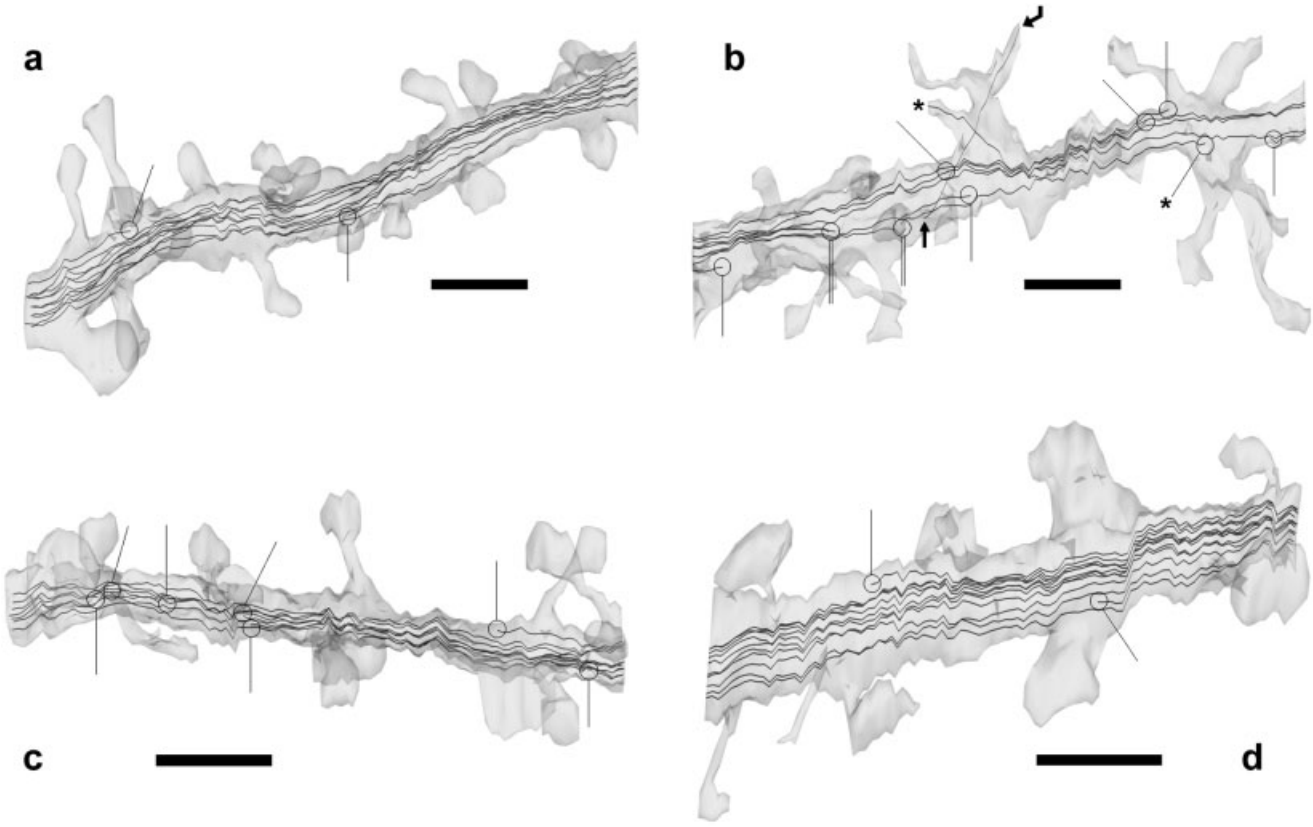

Fig. 5. Three-dimensional reconstructions of dendritic segments and their microtubules. Reconstructed microtubules appear jagged because of small misalignments between sections. Each line from a circle indicates a microtubule termination. **a:** Perfused. A very regular bundle of 12 microtubules has only two terminations within this segment. **b:** 5-minute slice. After a brief recovery in vitro, microtubules are highly disordered in a dendrite from a chilled slice preparation. This segment contains a large number of terminations and two microtubules that originate in spines. One microtubule extends from the spine tip (bent arrow) across the dendrite to the cell membrane on the opposite side (arrow). Another microtubule extends from a spine tip (asterisk) into the dendrite, becoming parallel to the axial microtubules before terminating in the cytoplasm (asterisked termination). **c:** 25-minute slice. Microtubules are more ordered after 25 minutes in vitro, but there are still a large number of terminations. **d:** 3-hour slice. After 3 hours, microtubules have recovered to the normal parallel order with few terminations. Scale bars = 1 μm.

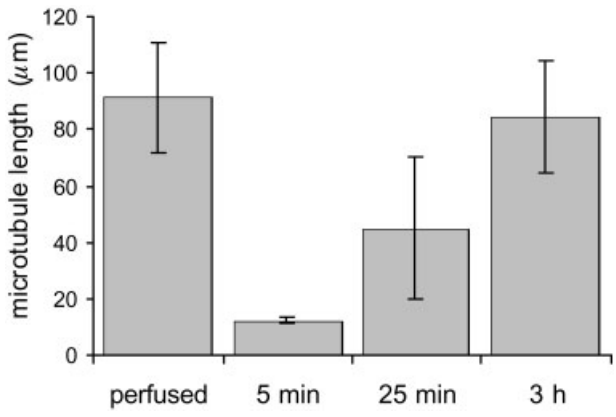

Fig. 6. Recovery of microtubule lengths in slices prepared under chilled conditions. All of the microtubules in five dendrites were reconstructed for each time point in one experiment.

Chilling the hippocampus during dissection and slicing did not prevent the loss of glycogen or the disassembly of dendritic microtubules but forestalled the increase in coated vesicles otherwise observed in the first slice. Keeping the brain ice-cold throughout dissection and slice preparation further preserved glycogen and microtubules, but submersion in warm ACSF produced immediate glycogen and microtubule loss along with increased coated vesicles. Chemical analyses also show that slices prepared under ice-cold conditions experience a dramatic drop in energy levels as soon as they are warmed to a physiological incubation temperature (Whittingham et al., 1984; Feig and Lipton, 1990). The cause of this energy drop during rewarming has not been adequately explained, and consequently it is unclear how to prevent it. The drop in slice energy does not appear to be synaptically mediated because recovery of slices in media containing 0 mM Ca^{2+} and 10 mM Mg^{2+} to block all synaptic transmission does not prevent it (Feig and Lipton, 1990).

Glycogen can be better preserved during slice preparation by the prior administration of anesthetics (McIlwain and Tresize, 1956), but anesthetics ultimately do not block

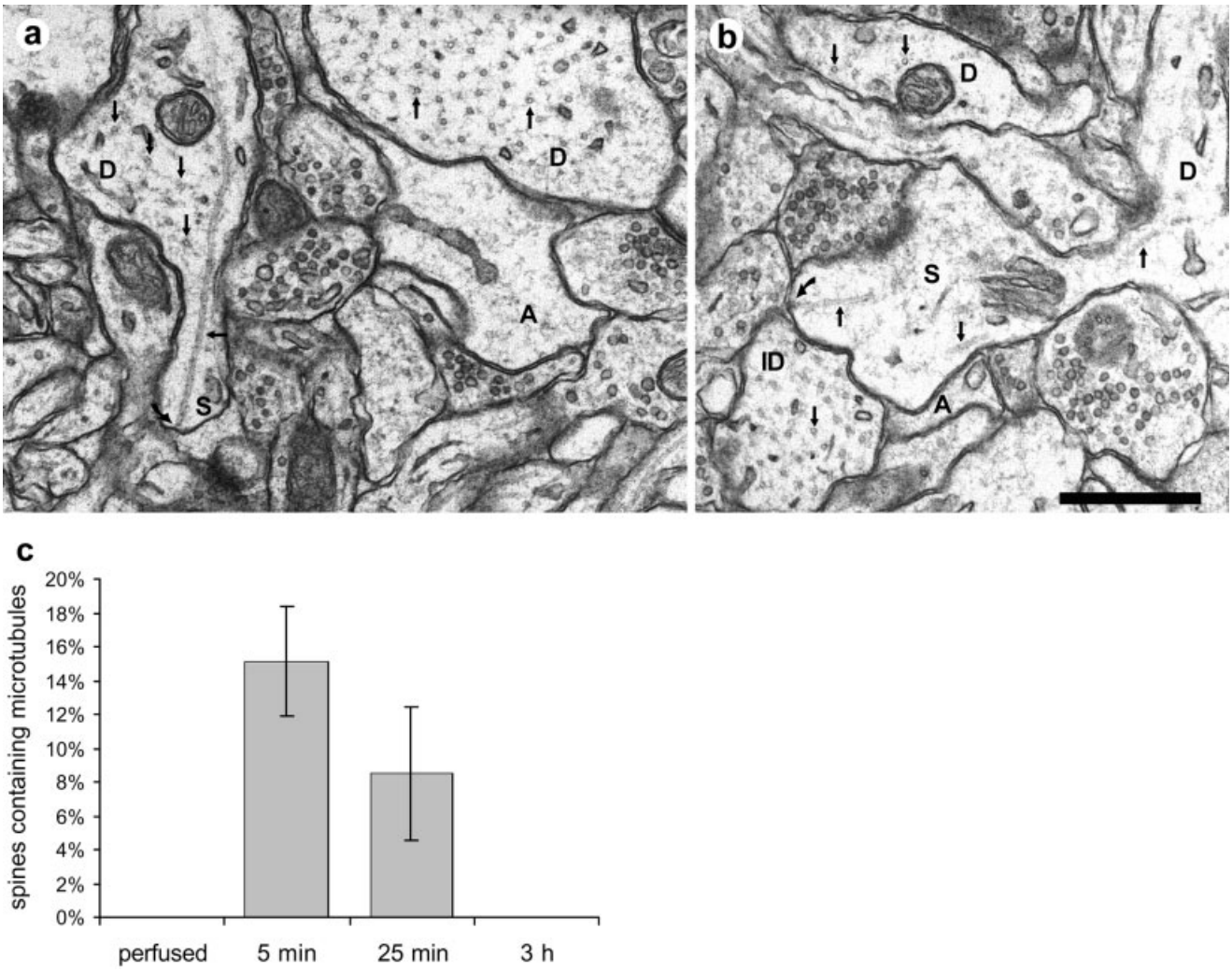

Fig. 7. Aberrant microtubules in dendritic spines during early slice recovery following chilled dissection. **a:** A slice incubated for just 5 minutes has no glycogen in astrocytes (A), but there are axial microtubules (arrows) in dendrites (D). In addition, a microtubule in a spine (S) extends from an origin (curved arrow) on the plasmalemma of the spine head into the dendrite perpendicular to the axial microtubules. **b:** Another example from this slice shows multiple microtubules (arrows) in a large spine (S), including one that attaches at the plasmalemma (curved arrow). Normally oriented microtubules are apparent in other spiny dendrites (D) and in a nonspiny interneuron dendrite (ID). **c:** In an analysis of five reconstructed dendritic segments from each condition, no microtubules were found in spines in perfused (n = 86) or in slices maintained for 3 hours in vitro (n = 70). However, spine microtubules were found in slices incubated for only 5 or 25 minutes (n = 74 and n = 56 spines, respectively). Scale bar = 0.5 μm in b (also applies to a).

the loss of energy stores, e.g., when cold slices are subsequently rewarmed (Feig and Lipton, 1990). In slices recovered in the interface chamber, glycogen loss and recovery occurred primarily in astrocytes. Astrocytic energy sources appear to be required for the recovery of synaptic function in interface slices (Schurr et al., 1997). This may explain why the time required for visible recovery of glycogen is approximately the same as the time required for full recovery of evoked synaptic transmission in interface slices (~60 minutes; Kirov et al., 1999).

Glutamate release is minimized by chilled preparation and interface recovery

Synaptic vesicles that have fused with the presynaptic plasma membrane are thought to be retrieved through clathrin-mediated endocytosis (Heuser, 1989; Cremona and De Camilli, 1997). Thus, an increase in axonal coated vesicles was evidence of an increase in synaptic transmission. This transmission was likely glutamatergic given the nature of the majority of axons in stratum radiatum of CA1. Widespread glutamate release in the CA1 region is known to occur as a consequence of ischemia (Benveniste et al., 1984,1989). In addition, activation of glutamate receptors, especially those permeable to Ca^{2+}, has been shown to play a role in slice injury caused by preparation (Feig and Lipton, 1990).

Cold preparation initially prevented glutamate release. Axonal endocytosis in 0-minute chilled or ice-cold slices did not exceed that of the deeply anesthetized perfusion-fixed hippocampus. This is consistent with the finding

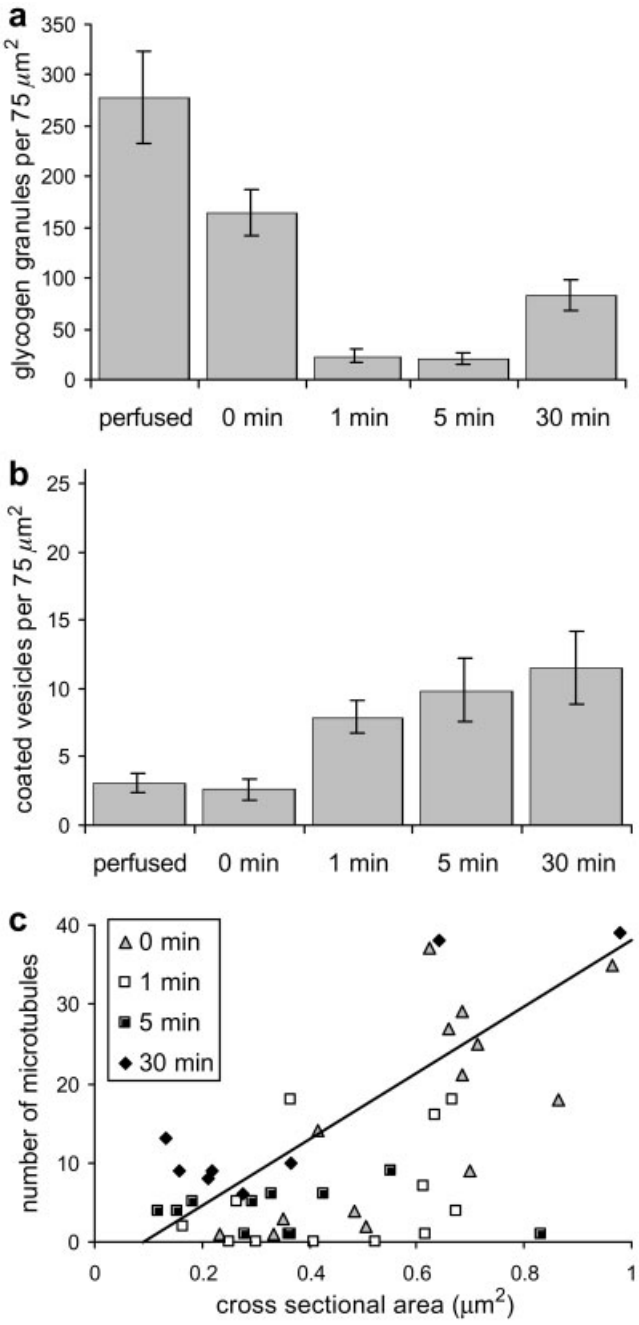

Fig. 8. Glycogen, coated vesicle, and microtubule changes with ice-cold perfusion prior to dissection. **a:** Although glycogen granules were present in the first slice (0 minutes), they quickly disappeared during the first minute of incubation and began to recover by 30 minutes, sooner than in the warm or chilled conditions. **b:** Coated vesicles were as infrequent as in perfused hippocampus prior to incubation but increased in conjunction with the loss of glycogen. **c:** Single-section measurements of the microtubule densities in cross-section dendrites. The line shows the linear relationship from perfused hippocampus (Fig. 4). Microtubules were relatively preserved in larger dendrites with ice-cold dissection, but small dendrites showed some loss of microtubules that was exacerbated on rewarming. Cross-sectional microtubule arrays recovered after 30 minutes. Six to thirteen 75-μm^2 sections taken from two animals were analyzed for each time point.

that hypothermia protects the brain from injury during ischemia (Barone et al., 1997). Either hypothermia (31°C) prior to dissection or chilling (21°C) slices immediately after chopping leads to healthier looking CA1 pyramidal neurons in vitro (Newman et al., 1992). However, preparation of slices by vibratomy in ice-cold conditions does not have this same beneficial effect on CA1 appearance after incubation in vitro (Feig and Lipton, 1990).

Axonal endocytosis increased dramatically in ice-cold slices recovered in the submerged condition but not in warm or chilled slices recovered in the interface chamber. The reason for different amounts of synaptic release in the three conditions is unclear. Ice-cold slices experienced colder temperatures and longer preparation times and were vibratomed instead of chopped. Very cold (<10°C) temperatures can lead to glutamate-mediated injury whereas milder (17°C) temperatures do not (Emery and Lucas, 1995). Ice-cold slices also had residual glycogen stores prior to incubation. If slices are dependent on glycogen for synaptic transmission, then the presence of residual glycogen stores could facilitate the onset of synaptic transmission in recovering slices.

Another reason for the difference in glutamate release may be the type of incubation chamber. Slices incubated in interface chambers are metabolically different from submerged slices (Croning and Haddad, 1998). Interface slices appear to rely more on astrocyte-supported aerobic metabolism (Schurr et al. 1997,1999), wheras submerged slices can function with only anaerobic glycolysis (Tian and Baker, 2000; Yamane et al., 2000). In addition, submerged slices have a continuously developing edema (Siklos et al., 1997). Such edema was not visible in our interface slices. The cytoplasm of dendrites, axons, and astrocytes was as dense as that of perfused hippocampus (Fig. 2f versus Fig. 1), and the density of dendritic microtubules was as great in 3-h slices as in perfused (Fig. 4b).

Dendritic microtubules can reassemble locally

The length of microtubules in neuronal dendrites has been a subject of debate. Some have suggested that individual microtubules in vivo extend from the soma to the tips of the smallest dendritic branches (Hillman, 1988). Recent studies have shown that dendritic microtubules are created in the soma and transported into dendrites as short segments (Stevens et al., 1988; Wang et al., 1996; Yu et al., 2000). Our reconstructions in perfused hippocampus and recovered slices reveal that microtubules in small-diameter spiny dendrites of pyramidal neurons are segments 85–90 μm long, much longer than the ~25 μm obtained by reconstructing a dendrite of a retinal ganglion neuron (Sasaki et al., 1983).

Microtubules are relatively unstable polymers that can be depolymerized by changes in temperature, lowered concentration of constituents GTP and tubulin, or elevated levels of divalent cations (Weisenberg and Deery, 1981; Mandelkow et al., 1991). Warm and cold dissections had similar effects on dendritic microtubule disassembly such that temperature alone was probably not a factor in depolymerization. The rapid disassembly and reassembly of microtubules was consistent with the rapid elevation and recovery of cytoplasmic Ca^{2+} concentrations observed during in vitro ischemia (Zhang and Lipton, 1999). Microtubule disassembly occurred in 0-minute slices from chilled and ice-cold preparations without indication of increased

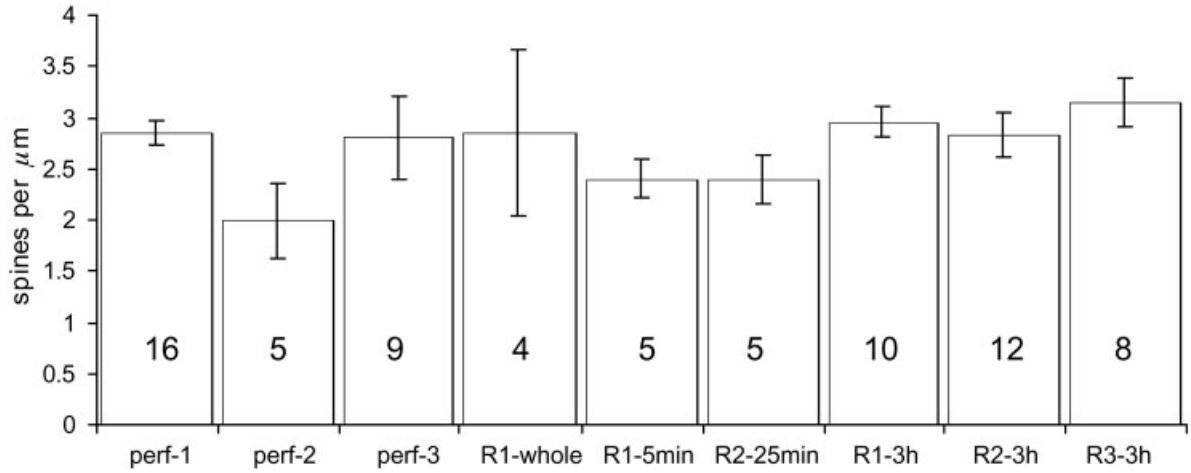

Fig. 9. Spine density on reconstructed dendrites. Dendritic segments 4–11 μm long were reconstructed through serial sections and spine origins counted along the length. Shown is the mean and standard error for each set of dendrites from each series analyzed. The number of segments reconstructed is given on each bar. The 6 animals from which each series was taken are indicated by the names on the horizontal axis (perf-1, perf-2, perf-3, and R1, R2, and R3 for slices).

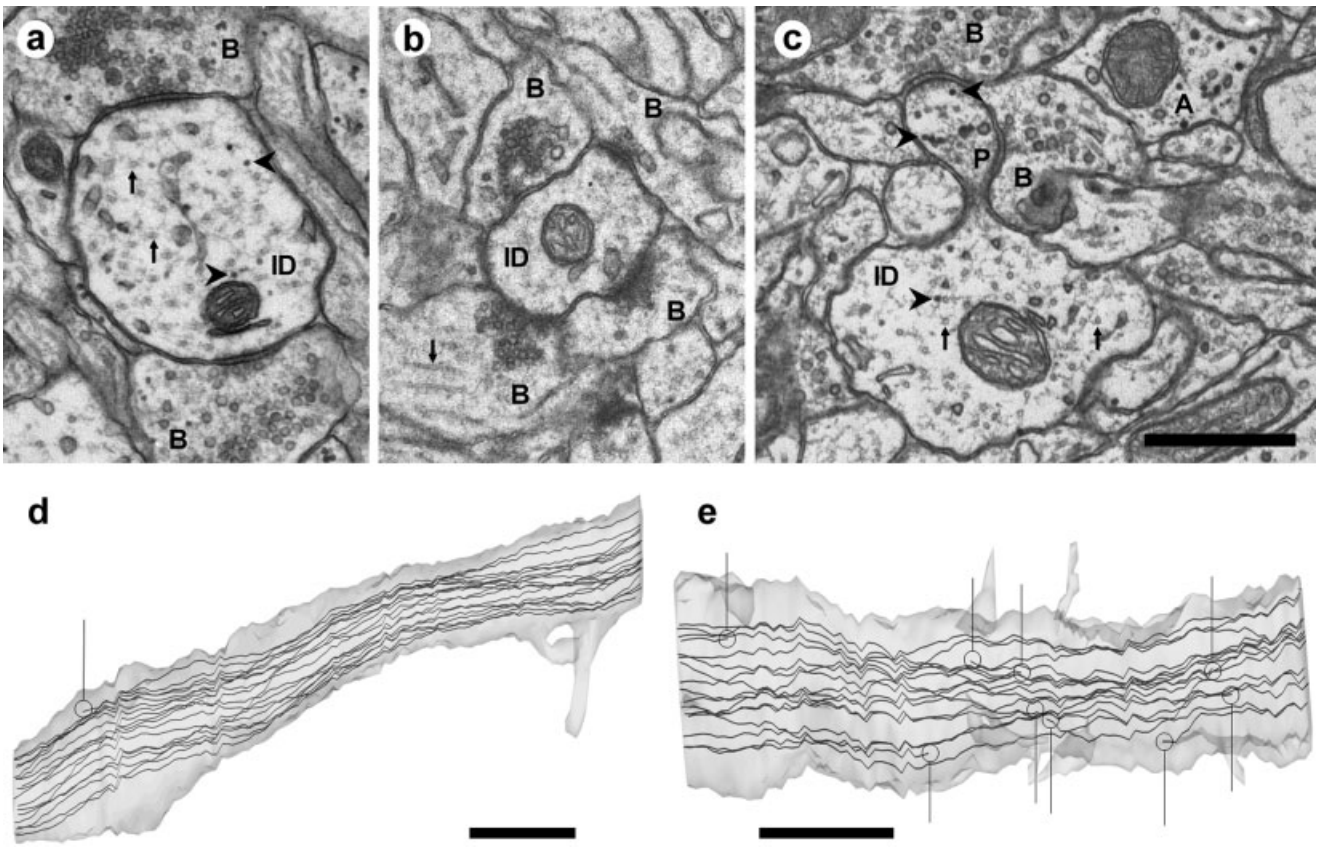

Fig. 10. Loss and recovery of microtubules in interneuron dendrites from chilled slice preparations. **a:** Perfused. An interneuron dendrite (ID) in perfusion-fixed hippocampus shows characteristic shaft synapses with axonal boutons (B). The dendrite contains an axial array of microtubules (arrows) and glycogen granules (chevrons). **b:** Whole-fixed hippocampus. An interneuron dendrite (ID) has no microtubules. Microtubules (arrow) can be seen in one of the synaptic boutons (B) that surround the dendrite. **c:** 3-hour slice. After 3 hours in vitro, interneuron dendrites have a normal axial array of microtubules (arrows) and cytoplasmic glycogen granules (chevrons). In this section, glycogen granules are concentrated in a rare protrusion (P) that extends from the dendrite and makes synaptic junctions with 2 boutons (B). **d:** Perfused. Reconstruction of the microtubules in a segment of interneuron dendrite from perfusion-fixed hippocampus reveals only one termination. **e:** 3-hour slice. Reconstruction of the microtubules in an incubated interneuron dendrite reveals many terminations. Only half of the reconstructed microtubules in each dendrite are shown in d and e so that the terminations can be clearly seen. Scale bars = 0.5 γm in c (also applies to a,b) ; 1 μm in d,e.

glutamate release, even though a major mode of Ca^{2+} entry in ischemia is glutamate receptors (Zhang and Lipton, 1999); however, the increased release may have occurred earlier during dissection.

The rapid reassembly of microtubules in these dendrites after complete disassembly was remarkable. The dendrites were at least 200 μm from the soma such that segments nucleated in the soma would need at least 20 minutes to appear, given maximum microtubule transport/growth rates of 5–10 μm/min (Schulze and Kirschner, 1988; Troutt et al., 1990). Other studies have shown that cell processes severed from the soma can locally assemble microtubules (Banks et al., 1975; McNiven and Porter, 1988). Because dendrites lack γ-tubulin for nucleating new microtubules (Baas and Joshi, 1992), such reassembly may occur on small, preserved fragments of microtubules (Baas and Heidemann, 1986). Such small fragments were not apparent in our micrographs, but they may have been too short and misoriented relative to dendrite cross sections to be readily identified.

Another surprising finding was the formation of microtubules in spines during recovery. Microtubules are not found in these types of spines in perfusion-fixed brain (Spacek, 1985; Peters et al., 1991; Fiala and Harris, 1999). Live-imaging studies similarly show that microtubules are restricted to dendritic shafts in these neurons (Kaech et al., 2001). In agreement with these data we did not observe microtubules in spines of perfusion-fixed hippocampus or in recovered slices. However, our observations suggest that microtubules occur briefly in dendritic spines during slice recovery, as has been observed previously in cortical slices (Westrum et al., 1980). Rather than having a functional purpose, as some have suggested (Van Rossum and Hanisch, 1999), spine microtubules may be artifacts of local microtubule reassembly. They are quickly eliminated as slices recover, perhaps by influxes of calcium in spines as synaptic activity resumes.

Slices can recover to a state resembling perfused hippocampus

Slices prepared from P21 rats using chilled dissections did not exhibit significant synaptogenesis after 3 hours in vitro. This contrasts with previous results from our laboratory and others that detected synaptogenesis in adult slices within 1–3 hours in vitro (Wenzel et al., 1994; Kirov et al., 1999; Johnson and Ouimet, 2002). Kirov et al. (1999) found an increase in spine density in acute P21 slices after 9 hours in vitro, but they did not examine earlier time points. The present results are consistent with those of Kirov et al. (1999) if synaptogenesis is significantly slower in P21 slices than in slices from adults. P21 slices might experience less glutamate release during preparation or during incubation in vitro. In the chilled slices of the present study, the ultrastructural evidence was that glutamate release remained at or below that seen in deeply anesthetized perfusion-fixed hippocampus in the first slice and subsequent incubation (Fig. 3d).

Whatever the reason for reduced synaptogenesis at 3 hours, it appears that under certain conditions acute slices can be recovered to an ultrastructural state that closely resembles that preserved by fixation in situ. This has important implications for both animal and human studies. Normally, human brain tissue is fixed as soon as possible after surgery (Morin et al., 1997). No matter how short the delay, this immediate fixation does not provide conditions during which the neurons can recover from ischemic injury. Recovering surgical tissue in vitro may improve the quality of ultrastructural studies of human brain.

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