Hippocampal slices have long been used to investigate properties of synaptic transmission and plasticity. Here, for the first time, synapses in slices have been compared quantitatively with synapses occurring in perfusion-fixed hippocampus, which is presumed to represent the natural in vivo state. Relative to perfusion-fixed hippocampus, a remarkable 40–50% increase in spine number occurs in adult hippocampal slices, and a 90% increase occurs in slices from postnatal day 21 rats. Serial EM shows that all of the dendritic spines have normal synapses with presynaptic and postsynaptic elements; however, not all spine types are affected uniformly. Stubby and mushroom spines increase in the adult slices, and thin, mushroom- and branched spines increase in the immature slices. More axonal boutons with multiple synapses occur in the slices, suggesting that the new synapses form on preexisting axonal boutons. The increase in spine and synapse number is evident within a couple of hours after preparing the slices. Once the initial spine induction has occurred, no further change occurs for up to 13 hr in vitro, the longest time investigated. Thus, the spine increase is occurring during a period when there is little or no synaptic activity during the first hour, and the subsequent stabilization in spine synapse numbers is occurring after synaptic activity returns in the slice. These findings suggest that spines form in response to the loss of synaptic activity when slices are removed from the rest of the brain and during the subsequent 1 hr recovery period.

Key words: plasticity; dendritic spines; CA1 pyramidal cell; multiple-synapse boutons; serial electron microscopy

Hippocampal slices are widely used to study cellular mechanisms of synaptic transmission and plasticity (Bliss and Collingridge, 1993; Bindokas et al., 1998; Diamond et al., 1998; Fisahn et al., 1998; Luscher et al., 1998; Nayak et al., 1998). Many approaches have been used to optimize slice health during experimental investigations. Variables that affect slice health include the following: (1) the age of the animal; (2) the specific composition of the life-supporting media; (3) whether the slices are maintained at the interface of media and oxygen or submerged; and (4) the methods of anesthesia, killing, and cutting the slices (for review, see Garthwaite et al., 1980; Reid et al., 1988; Hajos, 1989; Aitken et al., 1995; Lipton et al., 1995). Although much is known about how different physiological or pharmacological parameters affect slices, relatively little is known about whether synapses are altered in slices.

Because of the slow diffusion of glutaraldehyde (~400 μm/hr), the middle of a brain slice remains hypoxic for at least 30 min during fixation by immersion in mixed aldehydes (Hopwood, 1967). To overcome this limitation, microwave-enhanced fixation was used to speed greatly the diffusion of glutaraldehyde to the center of the tissue (Login and Dvorak, 1985, 1994). This procedure reveals hippocampal slices that are optimally preserved throughout their depth within seconds after removal from the life-support chamber (Jensen and Harris, 1989). Using this method, synapses have been quantified in well preserved hippocampal slices after undergoing different experimental treatments, such as long-term potentiation (Shepherd and Harris, 1998; Sorra and Harris, 1998).

These earlier studies compared synapses among different hippocampal slices, all of which experienced the same conditions of preparation and maintenance in vitro. What has not been addressed quantitatively is whether synapses in slices are altered relative to their more native state in vivo. For example, during slice preparation, glial, axonal, and dendritic processes are cut. The cut processes release substances (neurotransmitters, growth factors, potassium, etc.) that can be inhibitory, stimulatory, or even toxic to the intact neurons remaining in the slice. Furthermore, all spontaneous background activity is lost when the hippocampus is removed from the rest of the brain.

The purpose of the experiments reported here was to determine whether slices have an altered complement of synapses relative to perfusion-fixed hippocampus. Several parameters were tested. Slices from both young and mature animals were tested. Slices were maintained for varying times in vitro. The brains were perfused in situ with either fixative containing 2.5% glutaraldehyde, which is standard, or 6% glutaraldehyde, which matches the concentration used to obtain rapid tissue preservation in the slices.

Serial electron microscopy was used to perform two types of analyses. One analysis used unbiased sampling to compare synapse densities in volumes of hippocampal CA1 neuropil. The other analysis used three-dimensional reconstruction of dendrites.
to compare synapse number per unit length of spiny dendrite. These complementary approaches show that neurons in hippocampal slices have more synapses than in hippocampus fixed by intravascular perfusion at both ages. These findings do not occur from an artifactual decrease in slice volume, because the changes are limited to specific subtypes of synapses.

**MATERIALS AND METHODS**

Three studies were performed, as summarized in Table 1. All rats were of the Long-Evans strain, and all of our procedures follow National Institutes of Health guidelines and undergo yearly review by the Animal Care and Use Committee at Children’s Hospital. In total, 20 rats were used for these studies.

**Perfusion-fixed hippocampus.** All perfusion-fixed hippocampus was obtained from animals that were under deep pentobarbital anesthesia (80 mg/kg). Two different preparations of mixed aldehyde fixatives were used. For study 1, the brains from two male rats had been fixed in situ via intravascular perfusion with mixed aldehydes containing 2.5% glutaraldehyde, 2% paraformaldehyde, 1 mM CaCl₂, and 2 mM MgCl₂, pH 7.4, at 40–45°C and 4 psi pressure under deep pentobarbital anesthesia (Harris and Stevens, 1989; Harris et al., 1992). For studies 2 and 3, the fixed tissue was stored overnight in the fixative at 4°C and then microwaved for 2.5 min at 37°C using the microwave oven, which resulted in a measured final temperature that was always <5°C and usually <3°C to prevent microtubule destruction that occurs above 60°C (Jensen and Harris, 1989).

**Physiological recordings.** Physiological recordings were done to ensure slice viability (Harris and Teyler, 1984; Jackson et al., 1993; Sorra and Harris, 1998). Two concentric bipolar stimulating electrodes were positioned 600–800 μm apart in the middle of stratum radiatum on either side of a single extracellular recording electrode. Slices were judged healthy if the stimulus–response curves were sigmoidal and the half-maximal responses remained stable for at least 1 hr before fixation. At the end of each experiment, the slices were fixed in mixed aldehydes containing 2% paraformaldehyde, 6% glutaraldehyde, 1 mM CaCl₂, and 2 mM MgCl₂, pH 7.4, for 8 sec in the microwave oven, which resulted in a measured final temperature that was always <5°C and usually <3°C to prevent microtubule destruction that occurs above 60°C (Jensen and Harris, 1989).

**Light and electron microscopy.** For study 1, perfusion-fixed tissue and slices were hand processed by routine procedures as described previously (Harris and Stevens, 1989; Harris et al., 1992; Sorra and Harris, 1998). For studies 2 and 3, the fixed tissue was stored overnight in the fixative at room temperature, and then 400 μm slices were cut from the perfusion-fixed hippocampus embedded in agar and processed with the slices that had been maintained in vitro. All tissue slices were rinsed five times in buffer with repeated agitation. Each slice was manually trimmed under a dissecting microscope to a region containing only area CA1. Slices were soaked briefly in 1% osmium and 1.5% potassium ferrocyanide in 100 mM cacodylate buffer at 4°C, and then transferred to slices from each age group for in vitro perfusion experiments using a tissue chopper (Stoelting Co., Wood Dale, IL) and placed into ice-cold physiological saline containing 117 mM NaCl, 5.3 mM KCl, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 2.5 mM CaCl₂, 1.3 mM MgSO₄, and 10 mM glucose, equilibrated with 95% O₂–5% CO₂, pH 7.4. Slices were transferred in this saline via the blunt end of a glass pipette directly onto nets over wells with physiological saline, at the interface of humidified 95% O₂–5% CO₂ at 32°C in a recording chamber (Stoelting Co.), and maintained for varying times in vitro.

**Table 1. Summary of experimental conditions**

<table>
<thead>
<tr>
<th>Study</th>
<th>Perfused</th>
<th>Early slices</th>
<th>Late slices (set 1)</th>
<th>Late slices (set 2)</th>
<th>Perfused</th>
<th>Late slices</th>
<th>Perfused</th>
<th>Late slices</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals (n)</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Age (d)</td>
<td>39, 77</td>
<td>60–65</td>
<td>55–63</td>
<td>53, 60</td>
<td>68, 68</td>
<td>65, 66</td>
<td>21, 22</td>
<td>21, 22</td>
<td>—</td>
</tr>
<tr>
<td>Weight (gm)</td>
<td>137, 310</td>
<td>279–310</td>
<td>246–310</td>
<td>236, 279</td>
<td>337, 411</td>
<td>334, 355</td>
<td>41, 55</td>
<td>52, 61</td>
<td>—</td>
</tr>
<tr>
<td>Fixative (% glutaraldehyde)</td>
<td>2.5</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>—</td>
</tr>
<tr>
<td>Time in vitro (hr)</td>
<td>—</td>
<td>1.75–2.4</td>
<td>6–13</td>
<td>4.5–5.75</td>
<td>—</td>
<td>9–10</td>
<td>—</td>
<td>9–10</td>
<td>—</td>
</tr>
<tr>
<td>Number of series</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>ASD analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA (μm²)</td>
<td>1044</td>
<td>469</td>
<td>715</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2228</td>
</tr>
<tr>
<td>HNA (μm²)</td>
<td>836</td>
<td>301</td>
<td>595</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1732</td>
</tr>
<tr>
<td>Total synapses (n)</td>
<td>465</td>
<td>256</td>
<td>395</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1116</td>
</tr>
<tr>
<td>Dendrite analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dendrites (n)</td>
<td>52</td>
<td>—</td>
<td>—</td>
<td>129</td>
<td>16</td>
<td>24</td>
<td>21</td>
<td>12</td>
<td>254</td>
</tr>
<tr>
<td>Dendrite Diameter (μm)</td>
<td>0.62 ± 0.016</td>
<td>—</td>
<td>—</td>
<td>0.62 ± 0.015</td>
<td>0.53 ± 0.02</td>
<td>0.59 ± 0.012</td>
<td>0.55 ± 0.02</td>
<td>0.52 ± 0.05</td>
<td>—</td>
</tr>
<tr>
<td>Total Length (μm)</td>
<td>155</td>
<td>—</td>
<td>—</td>
<td>254</td>
<td>107</td>
<td>125</td>
<td>155</td>
<td>68</td>
<td>864</td>
</tr>
<tr>
<td>Total Spine Synapses (n)</td>
<td>543</td>
<td>—</td>
<td>—</td>
<td>1914</td>
<td>376</td>
<td>548</td>
<td>332</td>
<td>270</td>
<td>3983</td>
</tr>
<tr>
<td>Total Shaft Synapses (n)</td>
<td>20</td>
<td>—</td>
<td>—</td>
<td>63</td>
<td>57</td>
<td>31</td>
<td>44</td>
<td>14</td>
<td>229</td>
</tr>
<tr>
<td>Total Synapses (n)</td>
<td>563</td>
<td>—</td>
<td>—</td>
<td>1977</td>
<td>433</td>
<td>579</td>
<td>376</td>
<td>284</td>
<td>4212</td>
</tr>
</tbody>
</table>

Number of series, The number of photographic series that were examined in each condition; Serial sections per series, the range in the number of sections that were in a particular series. For example, in Study 1, Late Slices (set 2), there ranged from 33 to 38 serial sections in each of the 12 series.
ment with fresh 100% resin for several hours, samples were embedded in
collin molds with the dendrites orthogonal to the cutting plane. Samples
were cured for 48 hr at 60°C.

The blocks were trimmed to contain a region spanning the width of
the slices and in the middle of stratum radiatum midway between area CA3
and the subiculum. Then, several 1-µm-thick and 60-nm-thin test sections
were taken spanning the full width of the slices. Thick sections were
stained with 1% toluidine blue to guide subsequent trimming. Thin
sections were mounted on Ploioform-coated (SPI Supplies, West Ches-
ter, PA) slot grids (Synaptek; Ted Pella Inc.) and counter stained with
saturated ethanolic uranyl acetate, followed by Reynolds lead citrate,
each for 5 min. Sections were examined with a JEOL (Peabody, MA)
1200EX electron microscope to choose an area midway between the air
and net surfaces of the hippocampal slice for subsequent serial thin
sectioning. At an optimal depth between 100 and 200 µm from the cut
surfaces, excellent tissue preservation was found, as evidenced by well
preserved dendrites, with intact mitochondria, microtubules, and synap-
as, and the relative absence of dark or swollen neuronal processes (see
Figs. 2–5). A square diamond trimming tool (Electron Microscopy Sci-
ences, Fort Washington, PA) was used to prepare a small trapezoidal
area < 100 µm on a side for study 1 and < 50 µm on a side for studies 2
and 3. Serial thin sections were cut on the Leica (Mahrern, PA) Ultraci-
microtome, mounted, and counter stained as above for the test
thins. Individual grids were placed in grid cassettes (Advance Microscopy
Techniques, Danvers, MA), stored in numbered gelatin capsules (Elec-
tron Microscopy Sciences), and mounted in a rotating stage to obtain
uniform orientation of the sections on adjacent grids. All studies were
photographed at 4000 – 6000 magnification for each series in viewing
S ultramicrotome, mounted, and counter stained as above for the test
thins.

The series of sections were photographed at 4000 – 6000 × magnifica-
tion for study 1 perfused, early, and set 1 of the late slices, and at 10,000 ×
magnification for the second set of late slices in study 1 and all conditions
in studies 2 and 3. Calibration grids (Ernest Fullam Inc., Latham, NY)
were photographed with each series. In total, 35 EM series were ana-
lyzed, ranging from 25 to 134 serial sections (Table 1).

Adjustable synapse density analysis. The adjusted synapse density (ASD)
was computed for the conditions of study 1, as outlined in Table 1, using
the following equation (for review, see Harris, 1994; Sorra and Harris,
1998):

\[
ASD(\#\text{synapses/100} \mu\text{m}^2) = \frac{(n_{\text{syn}}/\text{HNA}) \times \text{mean}(1/n_{\text{syn}})}{(1/st)}
\]

Synapse number (n_{syn}) was computed by counting the number of
postsynaptic densities (PSDs) occurring within a sample area (SA) or on
two of the four lines defining the sample area. Synapses were counted if
the PSD was evident on the sample section or on the adjacent section for obliquely
cut synapses. Because synapse density is markedly influenced by ele-
ments occurring nonuniformly in the SA (i.e., myelinated axons, cell
bodies, and large dendrites with section profiles > 0.94 µm²), the areas of
these elements were measured and subtracted from the sample areas to
obtain the homogeneous neuropil area (HNA). Approximately half of
each sample field was analyzed for synapses by two investigators.

PSDs have different shapes and sizes, and the probability of capturing
them on a single sample section differs in proportion to the number of
sections they occupy. Thus, the number of sections each synapse occup-
ced was counted, and the average number of sections (n_{sec}) was com-
puted for each synapse type described in Results. The mean inverse of
n_{sec} was used to adjust for any differences in viewing probability, thereby removing potential size, shape, or orientation biases.

Sampling is also affected differentially by section thickness. Every
effort was made to obtain uniform section thickness at the time of cutting
(platinum-colored sections in the diamond boat); however, the same
section colors are not necessarily the same section thickness (Peachey,
1958). A better estimate of section thickness was obtained for each series
by measuring the diameters (d) of longitudinally sectioned mitochondria
or dendrites, counting the number of serial sections they occupied (n),
and computing section thickness (st) as: st = d/n.

Dendritic analysis. Spine number per unit length of dendrite was com-
puted for lateral apical dendritic segments only (having a diameter of < 1
µm), because the primary apical dendrites were too large and infrequent


to obtain useful statistics (Table 1). Furthermore, the primary apical
dendrites were far more spiny, and thus could not be grouped with the
lateral dendrites (data not shown). Dendritic segment lengths equaled
the number of sections they spanned multiplied by section thickness for
cross-sectioned dendrites. Segment lengths were computed with the

RESULTS

Physiological analysis

The physiological responses were monitored for a couple of hours
in slices at both ages, beginning 15–20 min after they were
transferred to the recording chamber (Fig. 1). No physiological
responses were elicited in slices during the first 10–15 min,
regardless of the stimulus intensity delivered and regardless of
whether there was anesthesia before guillotining. During the next
30 min, there was a dramatic recovery of the field EPSP (IEPS),
which was stabilized by ~35 min in adult and by ~1 hr in young
hippocampal slices. This recovery phase is typical of all slice
experiments, and the data are presented only to emphasize the
dramatic physiological events taking place after slicing.

Tissue quality and synapse composition

Light microscopy revealed well preserved tissue in the CA1
pyramidal cell layer and in stratum radiatum (Fig. 2). Optimal

tissue quality occurred in the middle 200 µm of the slice (Fig. 3a),
which was unchanged up to 13 hr in vitro, the longest time studied
in these experiments (Figs. 4, 5). If a particular slice had many
dark, degenerating processes, shrunken or highly vacuolated
and swollen dendrites, disrupted microtubules, and distended mito-
chondria, the tissue quality was judged unsuitable for quantitative
analysis (Fig. 3b). Excellent tissue preservation was a prerequisite
for all samples in the perfusion and slice conditions of these
Quantitative analysis of synapses

The ASDs in the neuropil of perfusion-fixed hippocampus were compared with slices fixed early or late after the onset of incubation in vitro (Table 1, Study 1). The ASD of 213 ± 17 synapses/100 μm³ in the perfusion-fixed hippocampus was significantly less than the ASDs of 322 ± 22 synapses/100 μm³ in the early slices and 338 ± 32 synapses/100 μm³ in the late slices (p < 0.02) (Fig. 6). The ASDs from the early and the late slices did not differ significantly, suggesting that synapse number is maximally elevated by ~2 hr in vitro and then remains stable for the duration (up to 13 hr).

The results from the ASD analyses were confirmed in the dendrite analysis. The number of synapses per unit length of dendrite was also found to be greater in the slices than in perfusion-fixed hippocampus (Fig. 7). In adult study 1, dendritic segments in the perfused hippocampus had 3.5 ± 0.2 spines/μm, in contrast with 4.9 ± 0.2 spines/μm in the slices (p < 0.001) (Fig. 7a). In adult study 2, the dendritic segments in the perfused hippocampus had 3.5 ± 0.3 spines/μm, in contrast with 4.5 ± 0.2 spines/μm in the slices (p < 0.01) (Fig. 7b). The difference was even greater at postnatal day 21 (P21), wherein the dendrites in the perfused hippocampus had only 2.2 ± 0.2 spines/μm, in contrast with 4.2 ± 0.5 spines/μm in the slices (p < 0.001) (Fig. 7c).

Spine and synapse specificity

The increase in synapse number in slices was not uniform across different types of dendritic spines. At all ages, thin spines predominated in both the perfused and slice conditions (Fig. 8), and in the adults, there was no significant difference in thin spines between perfused and slice conditions. There were more mushroom and stubby spines (Fig. 8a,b) and fewer asymmetric shaft synapses in the adult slices (Fig. 8b). At P21, thin, mushroom, and stubby spines occurred in approximately equal proportions in the perfused brain; however, in the slices, there was a dramatic increase in both the thin and mushroom spine categories (Fig. 8c). Branched spines were rare at all ages, and there was a small significant increase in the slices at P21 only (p < 0.05) (Fig. 8c). Additionally, five filopodia were observed at P21 (data not shown).

In studies 2 and 3, synapses were characterized as having macular or perforated PSDs. In the adults, only the macular PSDs increased significantly in slices (Fig. 9a). In contrast, the P21 slices showed a large increase in macular and perforated PSDs (Fig. 9b). In study 1, the presynaptic axons associated with every synapse were followed through serial sections to identify whether there was only one PSD [single-synapse bouton (SSB)] or more than one PSD [multiple-synapse bouton (MSB)]. There were more MSBs in the slices (p < 0.03) (Fig. 10).

DISCUSSION

Hippocampal slices have 40–90% more dendritic spines, depending on age, than comparable regions of hippocampus fixed by perfusion in vivo. The relative increase in dendrite spininess was detected within 2 hr after slicing. Serial EM shows that all of the dendritic spines have normal synapses with presynaptic and postsynaptic elements; however, not all spine types are affected uniformly. Stubby and mushroom spines increase in the adult slices, and thin, mushroom, and branched spines increase in the immature slices. MSBs increase preferentially in the slices, suggesting that the new synapses formed on preexisting axonal boutons.

Figure 2. Toluidine blue-stained sections from area CA1 in slices maintained in vitro. a. In study 1, the sections were cut parallel to the long axis of the apical dendritic arbors of the CA1 pyramidal cells for the perfused, the early, and the first set of late slices. b. For the 12 series photographed from the second two late slices in study 1 and for all of the slices in studies 2 and 3, the sections were cut perpendicularly to the apical dendritic arbor to obtain mostly cross-sectioned dendrites, which are optimal for computing the spine number per unit length of dendrite. The air and net surfaces of the slices are labeled, and the double arrow indicates the region of optimal tissue preservation in the middle 200 μm of the slice. The trapezoids illustrate the approximate location of subsequent serial thin sections. Scale bar: a, b, 100 μm.
Figure 3. Neuropil from within the middle of stratum radiatum of hippocampal slices. Both slices are photographed at 168–176 μm from the air surface of the slice. 

\( a \), In this adult hippocampal slice, the dendrites and axons are well preserved, with distinct microtubules (arrows), clear cytoplasm, nonswollen mitochondria (arrowheads), and a uniform distribution of vesicles in presynaptic axonal boutons (sv). Some examples of longitudinally sectioned mushroom (m) and stubby (s) spines are also evident. 

\( b \), This slice was rejected because of poor tissue quality, as evidenced by numerous shrunken and dark processes (d), swollen mitochondria (arrowheads), regions of unidentifiable whorls of membrane (large arrow), and synaptic vesicular clumping (svc) in presynaptic axonal boutons. There are some dendrites of good quality interspersed among the degenerating processes; however, it was often difficult to follow the spines past darkened processes, and many of the spines emerging from apparently healthy dendrites synapsed with dark degenerating axons. Scale bar: \( a, b \), 1 μm.
Figure 4. Representative neuropil in the middle of stratum radiatum of area CA1 from each experimental condition. Per fused conditions are on the left, and slice conditions are on the right. a, Study 1, adult hippocampus perfused with 2.5% glutaraldehyde. b, Study 1, adult early slice. c, Study 2, adult hippocampus perfused with 6% glutaraldehyde. d, Study 2, adult late slice. e, Study 3, P21 hippocampus perfused with 6% glutaraldehyde. f, Study 3, P21 late slice. Sample lateral dendrites are indicated by asterisks. In all cases, the tissue was judged suitable for quantitative analysis of synapses by the overall high quality of tissue preservation. Scale bar: a–f, 0.5 μm.
Figure 5. Synapses in area CA1 from perfusion-fixed hippocampus (left) and a hippocampal slice maintained in vitro for 13 hr before fixation (right). These particular examples were selected to illustrate longitudinally sectioned spines, dendrite origins, and presynaptic boutons. a, Perfusion-fixed thin spine (t). b, In vitro thin (t) and stubby (s) spines. c, Perfusion-fixed mushroom spine. d, In vitro mushroom spine. e, Perfusion-fixed stubby spine on an MSB. f, Another mushroom spine from an in vitro slice. Scale bar: a–f, 0.5 μm.
Several potential artifacts can be ruled out as explanations for these findings. Less than 5 min were needed to prepare the hippocampal slices, and perfusion fixation required 4–5 min of previous anesthesia and 30 sec before the fixative cleared the brain. Previous studies have shown there are no changes in synapse number with brief periods of hypoxia–ischemia such as these (Hu et al., 1998). Thus, the relative increase in spines is unlikely to result from subtle differences in anoxia during slice preparation versus perfusion fixation. In studies 2 and 3, anesthesia was introduced before slice preparation to control for this potential variable relative to perfusion-fixed brain, and still there were more spines in the slices. Thus, the relative increase in spine number in slices was not, instead, an artifactual loss of spines during anesthesia in the perfusion-fixed brains. All of the slices had a central core of well preserved neuropil (Reid et al., 1988; Jensen and Harris, 1989). This excellent tissue quality, as well as the elevated spine densities, were maintained for at least 13 hr in vitro, the longest time studied.

Another question is whether the slices experienced more shrinkage than in perfusion-fixed brain. If so, this would artificially produce a higher spine density in the slices. Several observations rule against this potential artifact. First, there is a spine and synapse specificity to the effect. If there were a generalized shrinkage in the slices, with no other change, then all types of synapses should be affected uniformly. Wenzel et al. (1994) also found a specific effect of slicing in adult hippocampus, wherein somatic spines were found on dentate granule cells from adult slices, which do not normally occur in hippocampus perfusion-fixed in vivo. Second, the dendrite diameters were uniform across conditions, showing that no generalized shrinkage of individual processes occurred (data not shown). Jensen and Harris (1989) also found a constancy in mitochondrial diameters across the slice and perfused conditions. Other studies have found that the extracellular space (ECS) should occupy ~20% of the brain volume (Nicholson and Sykova, 1998); however, some of the ECS is lost during processing of perfusion-fixed tissue, thereby accounting for a net 5–15% overall tissue shrinkage (Hillman and Deutsch, 1978; Cragg, 1980; Schuz and Palm, 1989). Similarly, EMs from the hippocampal slices show very little extracellular space. The living slices were originally cut at ~400 µm thickness, as determined by the Vernier scale on the tissue chopper, but were ~350 µm thick after processing (Fig. 2h), accounting for a 12.5% shrinkage that is comparable to perfusion-fixed tissue.

The ASD analysis was used to obtain an unbiased estimate of synapse densities in the slices and perfused hippocampus. This analysis could be influenced by other factors, such as subtle changes in the density of small glial processes or dendrite diameter, which could alter the relative density of synapses. This potential artifact was minimized by computing the HNA. In addition, the dendrite analysis of spine number per unit length is not sensitive to these potential volume artifacts. Because both approaches revealed the same results, it strengthens the findings from all of the ASD analyses.

The increase in MSBs in slices is consistent with other studies showing that when spines form quickly they make new synapses with preexisting axonal boutons (Woolley et al., 1996).
be interesting to learn whether there is a significant portion of nonsynaptic boutons in perfusion-fixed brain that could be the source of the nonsignificant trend toward an increase also in the SSBs.

The increase in stubby spines in the adult slice preparations recapitulates processes that occur during hippocampal development (Harris and Stevens, 1989; Harris et al., 1992; Fiala et al., 1998). During the first postnatal week, hippocampal synapses are recruited to dendritic shafts via filopodia. Then, shaft synapses give rise to stubby spines, which predominate for the next few weeks. As the animals mature, the shaft and stubby spine synapses are markedly reduced while thin and mushroom spines are formed, and eventually the thin spines become the dominant spine shape. Curiously, in slices from P21, the thin and mushroom spines reach numbers comparable with those found in the mature hippocampus in vivo and in vitro. These findings emphasize that spine induction is more profound in the developing slices than in mature slices, possibly accelerating an ongoing developmental process.

Together, these arguments support the conclusion that new spines are formed in hippocampal slices. One possible mechanism is that spines are induced by excessive synaptic activation when glutamate and potassium ions are released from the cut processes. This possibility seems unlikely because recent findings show that many more spines are induced when synaptic transmission is completely blocked in slices (Kirov and Harris, 1998). These findings are consistent with observations from developing cortex, which show that blocking synaptic transmission results in spinier developing dendrites (Dalva et al., 1994; Rocha and Sur, 1995). Conversely, excessive activation caused by elevated NMDA results in spine loss (Segal, 1995; Halpain et al., 1998). The spine induction observed in our experiments occurs within the first 2 hr, pointing to the loss of spontaneous synaptic activity when slices are removed from the rest of the brain and the “synaptically silent” recovery period as the candidate mechanisms for induction of new spines in slices.

There are several ways in which the new spines could form. One possibility that has been widely suggested, without convincing morphological evidence, is that new spines form from the splitting of previously existing spine synapses. This process is unlikely because branched or “splitting” spine heads never share the same presynaptic axon in hippocampal area CA1 in vivo or in slices.
REFERENCES


Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis. Nature 394:680–683.