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Warmer preparation of hippocampal slices prevents synapse proliferation that might obscure LTP-related structural plasticity

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Abstract

The hippocampal slice is a popular model system in which to study the cellular properties of long-term potentiation (LTP). Synaptogenesis induced by exposure to ice-cold artificial cerebrospinal fluid (ACSF), however, raises the concern that morphological correlates of LTP might be obscured, especially in mature slices. Here we demonstrate that preparation of mature hippocampal slices at room temperature (~25 °C) maintains excellent ultrastructure and a synapse density comparable to perfusion-fixed hippocampus. These results suggest that slices prepared at room temperature might provide a better basis from which to detect LTP-related changes in synapse number and morphology.
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1. Introduction

The preparation of hippocampal slices results in synaptogenesis compared to brains that are perfusion fixed *in situ* (Wenzel et al., 1994; Kirov et al., 1999). Many factors affect the amount of synaptogenesis observed in hippocampal slices including: age of the animal (Kirov et al., 1999, 2004a), time *in vitro* (Fiala et al., 2003), protein synthesis (Johnson and Ouimet, 2004), and temperature (Roelandse and Matus, 2004; Kirov et al., 2004b). In mature hippocampal slices, dendritic spines disappear immediately after preparation in ice-cold artificial cerebrospinal fluid (ACSF) and then rapidly reappear and proliferate within a few minutes of re-warming (Kirov et al., 2004b). If sucrose is added to substitute for NaCl in the ACSF, then exposure to cold temperatures does not result in total spine loss, but excess synaptogenesis still occurs when the slices are re-warmed to 32 °C in normal ACSF (Kirov et al., 2004b). Slices are prepared at cold temperatures to slow cellular metabolism and minimize neuronal disruption (Newman et al., 1992) which accompanies ischemic insult during brain dissection. Previous serial EM studies revealed more spines and synapses in slices than in perfusion-fixed hippocampus when slices were prepared at ice-cold temperatures (Kirov et al., 1999). This synaptogenic effect was seen whether the hippocampal slices were prepared with or without anesthesia, and the increase in spine and synapse number was present by 2 h and lasted for at least 13 h *in vitro*.

Slices are one of the most convenient preparations in which to investigate molecular, physiological and morphological changes associated with LTP. Changes in spine and synapse morphology are thought to underlie the enduring increase in synaptic efficacy that accompanies LTP (Fifkova and Van Harreveld, 1977; Lee et al., 1980; Chang and Greenough, 1984; Desmond and Levy, 1990; Bliss and Collingridge, 1993; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Geinisman, 2000; Popov et al., 2004; Matsuzaki et al.,

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2004). One possibility is that LTP enlarges existing synapses. Another possibility is that LTP induces new synapses. Alternatively, LTP might recruit or activate silent synapses, which would not necessarily alter synapse number or structure (Malenka and Nicoll, 1997; Malinow et al., 2000). We were concerned that slicing-induced synaptogenesis might obscure morphological changes associated with LTP (Sorra and Harris, 1998) and have tried several approaches to eliminate this potential artifact (Kirov and Harris, 1999; Fiala et al., 2003; Kirov et al., 2004a; Petrak et al., 2005). If synaptic transmission was explicitly blocked during the preparation and subsequent incubation of slices, even more synapses occurred in mature slices, although this effect was not seen prior to postnatal day 20 (Kirov and Harris, 1999; Kirov et al., 2004a). Mature hippocampal neurons are resilient and show a complete physiological recovery when they are dissected under warmer conditions (Harris and Teyler, 1984); hence, we decided to test whether a warmer dissection might ameliorate the slicing-induced synaptogenesis.

2. Materials and methods

All rats were males of the Long-Evans strain and all procedures followed National Institutes of Health guidelines and approved animal care protocols.

2.1. Perfusion-fixed hippocampus

Perfusion-fixed hippocampus was obtained from two adult rats aged 68 days (weighing 337 and 411 gm) *in situ* under pentobarbital anesthesia (80 mg/kg) via intravascular perfusion with mixed aldehydes containing 6% glutaraldehyde, 2% paraformaldehyde, 1 mM $CaCl_2$, and 2 mM $MgCl_2$, pH 7.4, at 40–45 °C and 4 psi pressure (Kirov et al., 1999).

2.2. Cold hippocampal slices

Slices were prepared from two adult rats aged 65 and 66 days old (weighing 334 and 355 gm). Animals were anesthetized with pentobarbital (80 mg/kg) and then decapitated with a guillotine. After decapitation, the left hippocampus was removed, and four to six slices (400 μm each) were cut using a tissue chopper (Stoelting Co., Wood Dale, IL) from the middle third of the hippocampus into ice-cold media containing (in mM) 116.4 NaCl, 5.4 KCl, 3.2 $CaCl_2$, 1.6 $MgSO_4$, 26.2 $NaHCO_3$, 1.0 NaH_2PO_4, and 10 D-glucose. Slices were transferred to nets positioned over wells containing media at the interface of humidified O_2 (95%) and CO_2 (5%) and maintained at 32 °C for 9–10 h *in vitro*.

2.3. Room temperature hippocampal slices

Slices were prepared from four adult rats aged 56–65 days old (weighing 263–365 gm). Animals were anesthetized with halothane and decapitated with a guillotine. After decapitation, slices were prepared identically to the cold hippocampal slices except that the ACSF was at room temperature (~25 °C). Slices were transferred to nets positioned over wells containing media at the interface of humidified O_2 (95%) and CO_2 (5%) maintained at 32 °C for 4.5 to 5.5 h *in vitro*. The entire preparation was completed within 5 min of death to minimize ischemic damage.

Physiological recordings were obtained from all slices to ensure slice viability (Harris and Teyler, 1984; Jackson et al., 1991; Sorra and Harris, 1998). Concentric bipolar stimulating electrodes were positioned in the middle of stratum radiatum within 400 microns of an extracellular recording electrode. Slices were judged healthy if the stimulus-response curves were sigmoidal and the half-maximal field potentials and the fiber volley amplitudes remained stable for at least 1 h before fixation. At the end of each experiment, the slices were fixed in mixed aldehydes containing 2% paraformaldehyde, 6% glutaraldehyde, 1 mM $CaCl_2$, and 2 mM $MgCl_2$, pH 7.4 for 8–20 s in a microwave oven, such that the final temperature did not exceed 37 °C (Jensen and Harris, 1989).

2.4. Electron microscopy and analysis

Perfusion-fixed and sliced hippocampus were either hand processed or microwave processed by routine procedures as described previously, infiltrated with Epoxy resins and then the blocks were hardened for 2 days at 60 °C (Harris and Stevens, 1989; Harris et al., 1992; Sorra and Harris, 1998; Kirov et al., 1999). The blocks were trimmed to a trapezoid in the middle of stratum radiatum of area CA1 and serial sections were cut and mounted on Pioloform-coated slot grids (Synaptek; Ted Pella Inc.). Sections were counterstained with saturated ethanolic uranyl acetate, followed by Reynolds lead citrate for 5 min each. Sections were imaged, blind to condition, on a JEOL 1230 electron microscope with a Gatan digital camera at 5000× magnification or on a JEOL 1200EX electron microscope at 4000–10,000× magnification. In total, 55 dendrites were analyzed from 8 EM series. The serial sections were aligned and dendrites were traced using the Reconstruct software (available at http://synapses.bu.edu (Fiala, 2005)). Section dimensions were calibrated using a diffraction grating replica (Ernest Fullam, Inc., Latham, NY) imaged and scanned with the series. Section thickness ranged from 45–65 nm and was calibrated using the cylindrical diameters method by dividing the diameters of longitudinally sectioned mitochondria by the number of sections they spanned (Fiala and Harris, 2001).

All dendrites were less than 1 micron in diameter and hence lateral rather than apical dendrites. Dendritic segments that were in cross section and traversed the entire series were chosen for analysis. One cold slice had 10 dendritic segments measuring (mean ± sem) 0.62 ± 0.11 μm in diameter and 6.3 ± 1.29 μm in length and the other cold slice had 14 dendritic segments at 0.57 ± 0.03 μm in diameter and 4.39 ± 0.35 μm in length for a total summed length of 124 microns. In each of the two perfusion fixed samples, there were 8 dendritic segments for a total of 16 with mean diameters of 0.54 ± 0.04 and 0.53 ± 0.03 μm, respectively, and mean lengths of 6.99 ± 0.18 and 6.35 ± 0.63 μm for a summed length of 107 microns in perfusion fixed hippocampus. There were 3, 4, 4, and 4 for a total of 15 dendritic segments from the four room-temperature slices. These averaged 0.51 ± 0.08, 0.66 ± 0.04, 0.65 ± 0.04, and 0.59 ± 0.05 μm in diameter, and 7.96 ± 0.17, 8.97 ± 0.79, 7.83 ± 0.24, 6.81 ± 0.63 μm in length for a summed length of 118 μm in the room temperature slices. Hence the sampled dendritic segments were of comparable dimensions. Spine density was calculated for all conditions by counting the number of dendritic protrusions and dividing by the length of each dendritic segment. All of these dendrites were analyzed blind as to condition, as part of other studies.

3. Results

3.1. Tissue quality and spine types

The tissue preservation was excellent in all three conditions (Fig. 1), showing few or no signs of ischemic or slicing-related damage (i.e. dark degenerating processes, swollen mitochondria, loosely organized and excessively thickened PSDs, depletion of synaptic vesicles). Synapses and spines of all types were detected in all three conditions.

3.2. Spine density

Three-dimensional reconstructions are illustrated from dendrites having the mean spine density in cold slices, perfusion fixed hippocampus and room temperature slices (Fig. 2A). Spine densities from the two cold slices and perfusion fixed hippocampi were compared to those from the room

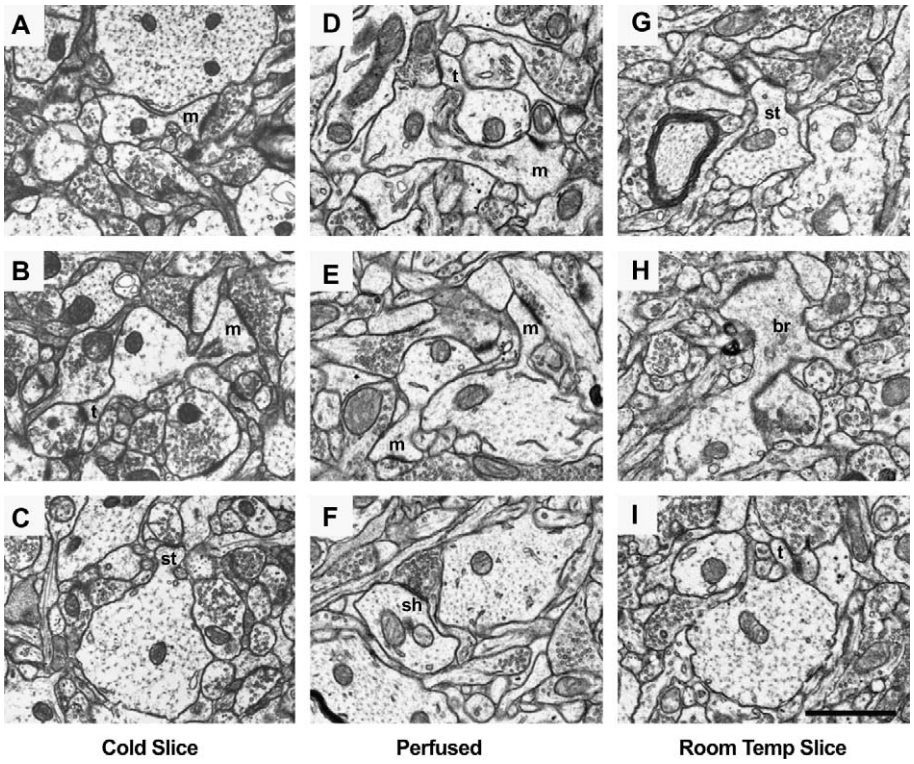

Fig. 1. Neuropil from stratum radiatum of area CA1 is comparable across the three conditions of slices prepared under: (A–C) ice-cold conditions (cold slice), (D–F) perfusion-fixed hippocampus *in situ* (perfused), and (G–I) slices prepared at room temperature (room temp slice). m = mushroom, t = thin, st = stubby, sh = shaft, br = branched. Scale bar = 0.5 μm.

temperature slices (Fig. 2B, One-way ANOVA $F(2,52) = 10.86$, $p < 0.001$). As previously reported, the cold slices had a higher spine and synapse density (4.50 ± 0.20 spines/μm) than the perfusion fixed hippocampus (3.49 ± 0.31 spines/μm; Post hoc Tukey's method, $p < 0.02$). In contrast, spine density along dendrites from slices prepared at room temperature (3.05 ± 0.18 spines/μm) did not differ significantly from the perfusion-fixed dendrites (3.49 ± 0.31; Post hoc Tukey's method, $p = 0.46$).

4. Discussion

The hippocampal slice is one of the most common preparations used to study the physiological and morphological properties of LTP. Our previous findings showing slicing-induced synaptogenesis was of particular concern when attempting to learn whether LTP might be accompanied by an increase in structural synaptic density (Sorra and Harris, 1998; Kirov et al., 1999). Here we explored the possibility that quickly chopping slices at a warmer temperature, under light halothane anesthesia, might avoid excessive spinogenesis. We found that dendrites, axons, and synapses were comparable to perfusion-fixed hippocampus both in tissue quality and spine density at 4–5 h *in vitro*, a time when synapse density had reached plateau in mature slices prepared under ice-cold conditions (Kirov et al., 1999). Thus, hippocampal slices prepared at warmer temperatures might provide a better basis from which to discover whether there are LTP-related effects on spine and synapse morphology in the mature hippocampus. Whether a similar approach will be useful in immature slices remains to be determined.

Multiple factors could contribute to changes in synapse density during hippocampal slice recovery. One factor might be a homeostatic synaptogenesis compensating for loss of input during a "silent" recovery period of 40 min to 1 h *in vitro* (Kirov et al., 1999). In support of this hypothesis, blocking synaptic transmission induces more spines and synapses in slices (Kirov and Harris, 1999; Kirov et al., 2004a; Petrak et al., 2005). However, this additional up-regulation in spine number was not present during postnatal days (PND) 1–15, when no or few spines populate the developing dendrites (Fiala et al., 1998; Kirov et al., 2004a). A homeostatic up-regulation seems insufficient to account for the long-lasting increase in spines in mature slices prepared under ice-cold conditions because the room-temperature slices also undergo a silent recovery period without excessive spine proliferation.

In addition to the synaptic silencing, preparation in ice-cold ACSF also induces reversible cytoskeletal destabilization, dendritic beading and spine loss. Inhibition of the Na^+-K^+-ATPase by reduced ATP, glycogen and temperature during brain slicing may be an additional factor (Fiala et al., 2003; Kirov et al., 2004b). Ionic imbalance, particularly calcium changes, during ice-cold slice preparation may also trigger the excess proliferation of spines on mature dendrites. Depending on incubation conditions, total spine number may

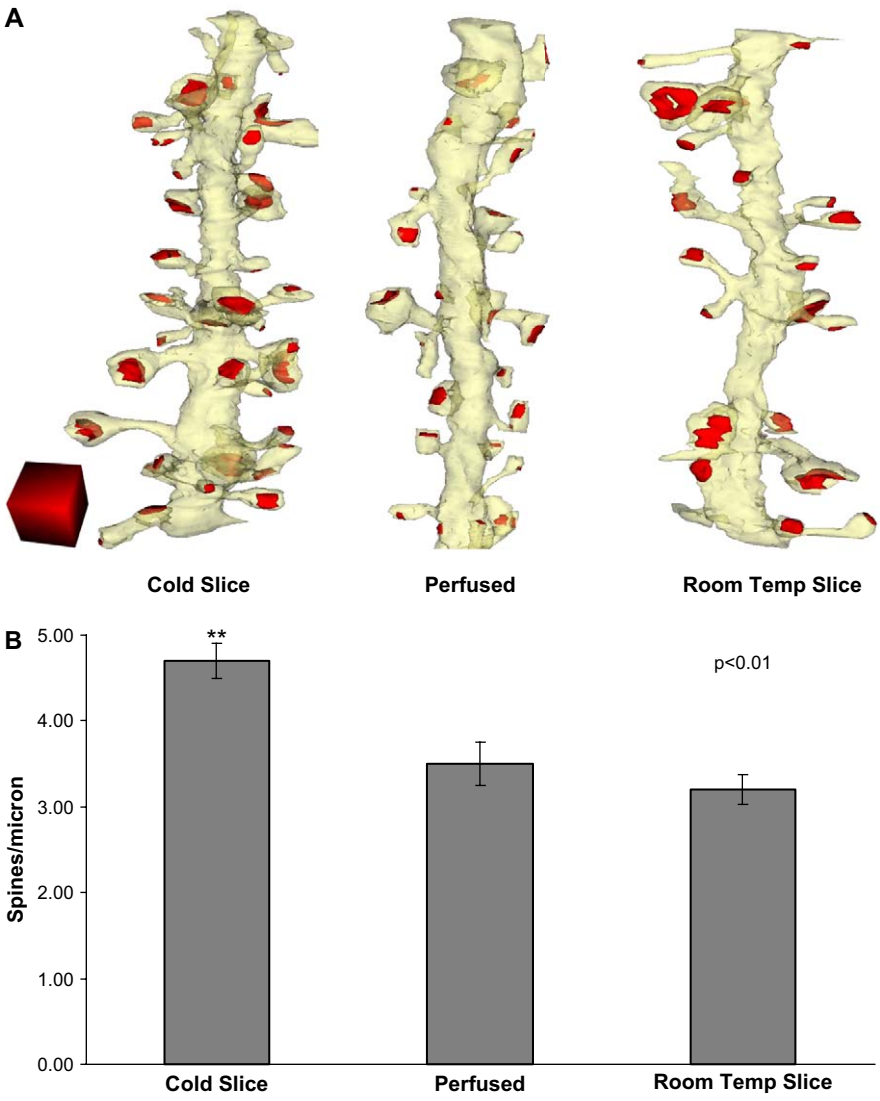

Fig. 2. Spine density in hippocampal slices prepared at room temperature is comparable to perfusion fixed hippocampus. (A) Three-dimensional reconstructions of dendrites with a mean spine density from each of the three conditions. Scale cube 1.0 μm^3. (B) Dendrites from ice cold slice preparations had a higher spine and synapse density compared to perfusion-fixed hippocampus ($p < 0.02$), while dendrites from room temperature hippocampal slices were comparable to perfusion-fixed hippocampus.

be further regulated by subsequent recovery of synaptic activity or induction of LTP. More synapses might be preserved during blockade of synaptic activity (Kirov and Harris, 1999) while excess synapses might be eliminated through competition when normal network activity returns during recovery or with LTP. Slices prepared at room temperature may undergo some loss of synaptic input and metabolic stress; however, they may avoid the excessive spine loss that occurs at colder temperatures because there is less inhibition of the Na^+-K^+-ATPase at room temperature. Although we cannot know for sure from these experiments whether there was dynamic spine loss, proliferation and elimination in the room temperature slices, the net synapse and spine density was comparable to perfusion fixed hippocampus. This could be an important advantage if one considers that dendrites might only support a limited maximum number of synapses. If cold-slicing were to induce enough new spines to reach this ceiling, then LTP might not be able to further induce synapses. Instead, LTP might either recruit or eliminate the new synapses. Under this scenario, it would be quite difficult to detect a net shift in synapse number during LTP, even though the effective synapse number had changed.

Sorra and Harris (1998) reported that there were no significant differences in spine density 2 h after tetanic stimulation compared to low-frequency stimulation (LFS) in the same slice. These slices were prepared with ice-cold ACSF. Both the LTP and LFS values for spine density were 4–5 spines/micron (Sorra and Harris, 1998), which is substantially higher than the average of 3–3.5 spines/micron found along CA1 lateral dendrites in perfusion fixed hippocampus. Thus, slicing-induced synaptogenesis may have obscured LTP-related plasticity and further studies are needed to determine the extent and timeline of morphological changes underlying LTP in mature hippocampal slices prepared at room temperature.

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