An In Vitro Model of Hippocampal Sharp Waves: Regional Initiation and Intracellular Correlates

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1Toronto Western Research Institute, University Health Network, Department of Medicine, 2Division of Neurology, 3Department of Physiology, 4Institute of Biomaterials and Biomedical Engineering, and 5Epilepsy Research Program, University of Toronto, Toronto, Ontario, Canada

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Wu, Chiping, Marjan Nassiri Asl, Jesse Gillis, Frances K. Skinner, and Liang Zhang. An in vitro model of hippocampal sharp waves: regional initiation and intracellular correlates. J Neurophysiol 94: 741–753, 2005. First published March 16, 2005; doi:10.1152/jn.00086.2005. During slow wave sleep and consummatory behaviors, electroencephalographic recordings from the rodent hippocampus reveal large amplitude potentials called sharp waves. The sharp waves originate from the CA3 circuitry and their generation is correlated with coherent discharges of CA3 pyramidal neurons and dependent on activities mediated by AMPA glutamate receptors. To model sharp waves in a relatively large hippocampal circuitry in vitro, we developed thick (1 mm) mouse hippocampal slices by separating the dentate gyrus from the CA2/CA1 areas while keeping the functional dentate gyrus-CA3-CA1 connections. We found that large amplitude (0.3–3 mV) sharp wave-like field potentials occurred spontaneously in the thick slices without extra ionic or pharmacological manipulation and they resemble closely electroencephalographic sharp waves with respect to waveform, regional initiation, pharmacological manipulations, and intracellular correlates. Through measuring tissue O2,K+/H11011, and synaptic and single cell activities, we verified that the sharp wave-like potentials are not a consequence of anoxia, nonspecific elevation of extracellular K+ and dissection-related tissue damage. Our data suggest that a subtle but crucial increase in the CA3 glutamatergic activity effectively recruits a population of neurons thus responsible for the generation of the sharp wave-like spontaneous field potentials in isolated hippocampal circuitry.

INTRODUCTION

Large-amplitude EEG potentials as the result of coherent neuronal discharges occur in the hippocampus during physiological and pathophysiological conditions. For example, during slow wave sleep and consummatory behaviors, the rodent hippocampus exhibits sharp waves (SPWs) that are up to 3 mV in amplitude and often superimposed with oscillatory activity of ~200 Hz called ripples (Buzsáki 1986; Buzsáki et al. 1983, 2003; Suzuki and Smith 1987). The SPWs are thought to originate from the CA3 circuitry, and their generation is correlated with coherent discharges of CA3 neurons (Csicsvari et al. 2000; Ylinen et al. 1995) and dependent on the activity mediated by AMPA glutamate receptors (Leung and Shen 2004). The SPW-ripple complex occurs in a close temporal relation with cortical EEG spindle and delta waves, the underlying network activities are postulated to participate in memory related hippocampal synaptic plasticity and hippocampal-cortical information transfer (Siapas and Wilson 1998; Sirotta et al. 2003). In addition to physiological SPWs, interictal spikes of similar waveforms are frequently observed in the hippocampus of rodent models with chronic limbic seizures (Bragin et al. 1999, 2002a; Medvedev et al. 2000; Riban et al. 2002) and of patients with temporal lobe epilepsy (Bragin et al. 2002b; Staba et al. 2004; Wennberg et al. 2002). These interictal activities are often associated with fast ripples of 250–600 Hz (Bragin et al. 2002a; Staba et al. 2004), which are thought to be an intrinsic network property of epileptic hippocampal circuitry. The mechanisms by which the physiological SPWs and epileptic interictal activities arise from the hippocampus are not fully understood. Thus it is of great interest to model these population neuronal activities in an isolated hippocampal circuitry in vitro and to investigate the underlying cellular and neurochemical basis.

To preserve a relatively large hippocampal circuitry in vitro, we established a thick (1 mm) slice preparation from adult mice by separating the dentate gyrus from CA2/CA1 areas while keeping the functional dentate-CA3-CA1 connection (Wu et al. 2005b). While perfused in vitro, the thick slices exhibit two types of population neuronal activities without extra ionic or pharmacological manipulation, i.e., spontaneous rhythmic field potentials (SRFPs, 1–4 Hz) and large-amplitude field potentials. We have fully characterized the SRFPs, and our data suggest that they represent a CA3-driven, IPSP-based intrinsic network activity (Wu et al. 2005b). The aim of this study was to examine the large-amplitude spontaneous field potentials in detail.

We show in this study that these large-amplitude field potentials bear close resemblance to hippocampal EEG SPWs with respect to waveform, regional initiation, pharmacological manipulations, and intracellular correlates. We thus refer to these large-amplitude spontaneous field potentials as in vitro SPWs. Our data suggest that the in vitro SPWs result from the network activities of CA3 recurrent circuitry and that they may serve as a convenient model to reveal the mechanisms potentially involved in the generation of physiological SPWs and perhaps interictal epileptiform activities.

METHODS

Animals

C57Bl/6 mice at ages of 21 days to 6 mo (Charles River, Quebec, Canada) were used in these experiments. All experiments conducted

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in this study have been reviewed and approved by the animal care committee of our institution.

**Preparation of thick hippocampal slices**

The procedures to prepare thick hippocampal slices have been described in detail in our recent study (Wu et al. 2005b). Briefly, we decapitated the animal under halothane anesthesia, quickly dissected out the brain, and maintained the semi-sectioned brain in an ice-cold, oxygenated artificial cerebrospinal fluid (ACSF) for a few minutes before further dissections. We removed brain stem-thalamic tissues to expose the dorsal (dentate gyrus) side of the hippocampus and applied a fine glass probe along the hippocampal fissure to separate the dentate gyrus from the CA2/CA1 areas. By doing so, the dentate gyrus was detached from the distal portion of CA2/CA1 stratum radiatum but the dentate gyrus-CA3 tissue connection was remained. During the dissection, the brain tissue was kept in ice-cold ACSF and visualized under a dissecting scope.

After separating the dentate gyrus, we glued the brain tissue onto an agar block and obtained vibrotome sections of 1 mm thickness along the transverse plane of the hippocampus. Because the CA2-CA1 s. radiatum regions of the ventral hippocampus are wider than that in the middle-dorsal hippocampus, brain slices of 0.8 mm thickness that contained the ventral hippocampal tissues were used in some experiments. After slicing, cortical tissue was surgically removed. The removal of cortical tissue together with the dentate gyrus separation produced a “C” shaped hippocampal tissue strip, which allowed a direct exposure of its deeper layers to the oxygenated ACSF. The thick slices were maintained in warm ACSF (32–33°C) for 1–6 h before recordings. The ACSF contained (in mM) 3.5 KCl, 1.25 NaH2PO4, 125 NaCl, 25 NaHCO3, 2 CaCl2, 1.3 MgSO4, and 10 glucose. The pH of the ACSF was ~7.4 when aerated with 95% O2-5% CO2.

To prepare conventional ventral hippocampal slices of 0.5 mm thickness, we made horizontal vibrotome cuts from the basal brain (2 slices per half brain). These slices were similarly maintained in vitro as described above.

**Perfusion apparatus**

We used a submerged perfusion chamber with inner dimensions of 3.5 × 5 × 20 mm (depth × width × length; Wu et al. 2002). The slice was held on a stainless steel fine mesh via six to eight mosquito pins or a frame made of fine stainless steel wires. The mesh was set ~1.5 mm above the bottom of the chamber to effectively perfuse the bottom side of the slice. The ACSF was warmed and perfused to the slice at 32–33°C. A water bath underneath the recording chamber was set at 32°C via an automatic temperature control unit. This setting allowed warm and humidified 95% O2-5% CO2 pass over the perfusate to increase local oxygen tension in the recording chamber. The slice was perfused at a rate of ~15 ml/min, but at a minimal submergence level, to achieve effective exchanges between the oxygenated ACSF and the perfused thick slice. By placing a fine temperature probe in the ACSF near the perfused slice, we verified that the perfusate temperature was at ~32°C.

To conduct brief hypoxia, we perfused the slice with an ACSF that was aerated with 95% N2-5% CO2 rather than 95% O2-5% CO2 for 5–6 min. During the hypoxic episode, the humidified 95% O2-5% CO2 that passed over the perfusate was replaced with 95% N2-5% CO2 (Chung et al. 1998; Perez-Velazques and Zhang 1994).

**Extracellular recording and afferent stimulation**

Extracellular electrodes were pulled from thin wall glass tubes (1.5 mm OD; World Precision Instruments, Sarasota, FL). The resistance of these electrodes was ~2 MΩ after being filled with a solution that contained 150 mM NaCl and 2 mM HEPES (pH 7.4). Extracellular signals were recorded via an Axoclamp-2B (Axon Instruments, Union City, CA) and/or an extracellular amplifier (Model 3000, A-M Systems, Carlsborg, WA). The frequency range of input signals of these amplifiers was 0–3 kHz, and output signals were amplified for a total gain of 2000–5000 times before digitization (Digidata 1200 or 1300A, Axon Instruments). Data acquisition and storage were done using PCLAMP software (version 8, Axon Instruments).

For afferent stimulation, we placed a bipolar tungsten wire (50 μm diam) electrode in the middle molecular layer of the dentate gyrus or s. radiatum of the CA3 or CA1 area. Constant current pulses of 0.1 μA and 20–150 μA were generated by a Grass stimulator (Model S88) and delivered through an isolation unit every 30 s. We routinely evoked synaptic field potentials from the CA3 and/or CA1 areas to assess the general functionality of thick slices. Data were collected for further analyses if the evoked synaptic field potentials were stable and their amplitudes were >0.5 mV after the afferent stimulation at near maximal intensities (±100 μA).

**Whole cell patch recordings**

A “blind” method (Zhang et al. 1991, 1994) was used to approach individual cell in thick slices (Wu et al. 2002, 2005b). Patch pipettes were pulled from the thin wall glass tubes. The resistance of these pipettes was 4–5 MΩ after being filled with a solution that contained 120 mM potassium gluconate, 2 mM HEPES, 0.1 mM EGTA, and 0.5% neurobiotin (pH 7.25 and 280–290 mOsm). Single cell activities were recorded using Axopatch-200B or dual amplifier 700A (Axon Instruments).

To monitor slow Ca2+-dependent K+ currents, we used a patch pipette solution containing 150 mM potassium methysulfate, 2 mM HEPES, and 0.1 mM EGTA (Zhang et al. 1994). To effectively buffer intracellular calcium, we used a patch pipette solution that contained 130 mM potassium methylsulfate, 10 mM K-EGTA, 1 mM CaCl2, and 10 mM HEPES (Zhang et al. 1995).

In some experiments, we monitored neuronal discharges via cell-attached, voltage-clamp recordings, which cannot provide a full voltage control of the recorded neuron but allow noninvasive measurements of the timing of spike currents (Frickier et al. 1999; Verheugen et al. 1999; Wu et al. 2002, 2005b).

**Oxygen measurements**

The preparation of O2-sensitive carbon fiber electrodes has been described in detail in our recent study (Wu et al. 2005b). Briefly, a carbon fiber (~10 μm diam; Goodfellow) was sealed into a glass electrode with epoxy, and the carbon fiber was set to protrude the glass electrode tip by ~20 μm. The other end of carbon fiber was adhered (Silver Print, GC Electronics, Rockford, IL) to a copper wire, which was connected to a polarographic amplifier (Chemical Microsensor 1201, Diamond General, Ann Arbor, MI). During oxygen measurements, a voltage of –0.85 V was applied between the carbon fiber electrode and an Ag/AgCl reference electrode. Carbon fiber electrodes were calibrated in warmed ACSFs (~32°C) that were aerated with 95% N2-5% CO2, 25% O2-5% CO2-70% N2, 50% O2-5% CO2-45% N2, or 95% O2-5% CO2, respectively. The corresponding O2 levels were 32.0 ± 3.2, 126.5 ± 4.7, 242.4 ± 24.3, and 360.9 ± 18.4 mmHg (n = 8 electrodes). These values could be fitted via a linear regression line with r = 0.992 and slope = 3.5.

The O2 measurements were made from the CA3 or CA1 proximal s. radiatum area of perfused thick slices. For assessing O2-depth profile, the carbon electrode was advanced into the slice at an angle of ~75° vertical to the horizontal plane and in a stepwise manner via using a micromanipulator. The exact distance of the carbon electrode tip traveled in the slice could not be determined because of tissue dimpling associated with electrode advancement. The estimated travel distance of the carbon electrode tip was ~100 μm per step.
Measurements of extracellular $K^+$

$K^+$-selective electrodes were prepared as previously described (Wu et al. 2002). Briefly, these electrodes were pulled from the thin wall glass tubes with a tip diameter of $\sim$20 $\mu$m. One barrel of the two-barrel electrode was filled at the tip with potassium ionophore cocktail A (Fluka, Buchs, Switzerland) and back-filled with 200 mM KCl. The reference barrel was filled with 200 mM NaCl. Signals were recorded using a differential amplifier designed for ion-selective electrodes (input impedance of $>10^{14}$ $\Omega$; A-M Systems). The sensitivity of the $K^+$-electrodes was 66.2 $\pm$ 2.8 mV for a 10-fold change in KCl concentrations (from 3.5 to 35 mM). Calibration solutions were similar to the ACSF with KCl substituted for corresponding moles of NaCl.

Data analyses

Evoked synaptic field potentials and basic intracellular parameters were measured as previously described (Wu et al. 2002, 2005b; Zhang et al. 1994, 1995, 1998b). Spectral analyses were conducted using Micro-Origin (version 6, Origin Lab Corp., Northampton, MA) or PCLAMP software (version 9, Axon Instruments). If needed, original data were treated with a band-pass filter (Bessel, 50–1,000 Hz) to reveal the rhythmic signals that were associated with the ripple-like oscillations. Event detection function (threshold search method, PCLAMP software, version 9) was used to measure the amplitudes, durations, interevent intervals of spontaneous field potentials, and the correlated cellular activities. If necessary, the original data were treated with a band-pass filter (0.2–1,000 Hz) to decrease the DC shift and high-frequency noise. Time-frequency analyses (Akay 1997) of spontaneous field potentials were made from 3-min extracellular data segments, and the time resolution of these analyses was 0.5 s (Wu et al. 2005b). No digital filtering was applied to the original data before these analyses. Statistical significance was determined using Student t-test or one-way ANOVA (SigmaStat or Origin). Mean and SE were calculated and presented throughout the text except where indicated.

RESULTS

Thick hippocampal slices were prepared from C57Bl/6 mice at ages of 21 days to 6 mo. A majority of extracellular and single cell recordings were conducted in the CA3 area except where indicated. All recordings were made at the perfusate temperature of $\sim$32°C.

General features of in vitro SPWs

In nearly every thick slice ($n > 500$) successfully prepared, extracellular recordings in the CA3 or CA1 area revealed SRFPs with amplitudes of 30–300 $\mu$V and occurrence frequencies of 1–4 Hz. In $\sim$40% of the thick slices examined, the SRFPs were intermingled with another type of field potentials. The latter were larger (amplitudes of 0.3–3 mV) and less frequent (occurrence frequencies of 0.02–0.2 Hz) than the SRFPs (Fig. 1A). We refer to these large-amplitude spontaneous field potentials as in vitro SPWs because they resemble closely the major features of EEG SPWs observed from intact animals. The in vitro SPWs occurred without extra ionic or pharmacological manipulation and they could be continuously monitored for up to 2 h with a relatively constant rate and waveform in individual thick slice (Fig. 1B).

The in vitro SPWs recorded from the CA3 and CA1 areas share a similar waveform polarity with the evoked synaptic field potentials—they exhibited positive (upward) or negative (downward) waveforms as recorded from the somatic (stratum pyramidale) or apical dendritic (s. radiatum) layer, respectively. Peri-somatic SPWs were often superimposed with oscillatory activities (Fig. 1A), referred to as ripple-like oscillations. Time-frequency analyses (Akay 1997; Wu et al. 2005b) revealed a stable frequency distribution of the ripple-like oscillations over consecutively recorded in vitro SPWs. Each SPW event was associated with high-frequency signals of 100–300 Hz, which were surrounded by strong rhythmic signals of $\leq$80 Hz (Fig. 1C). Because the extracellular recordings of ripple-like oscillations were dependent on the electrode position and a clear temporal relation between the ripple-like oscillations and associated single cell activities was often difficult to reveal without substantially filtering of the original data (band-pass filter of 50–250 Hz), we did not focus on these oscillatory activities in these experiments.

In the thick slices exhibiting baseline SPWs, single afferent stimulation could induce SPW-like field responses. The evoked responses appeared in the decay phase of primarily evoked synaptic field potentials, and their waveforms were similar to spontaneous SPWs. The threshold to evoke these SPW-like responses in the CA3 area were 45–55 $\mu$A (constant current pulses of 0.1 ms, $n = 6$ slices), which were higher than that needed to evoke primary synaptic potentials in the same slices (20–30 $\mu$A). These SPW-like responses behaved in an all-or-none like fashion, i.e., their waveforms and amplitudes were relatively constant when evoked by the threshold or super-threshold stimulus (Fig. 1D). These observations suggest that the generation of in vitro SPWs may result from a subtle but critical increase in glutamatergic drive (see DISCUSSION).

We noticed a close temporal relation between the in vitro SPWs and SRFPs. The onset of individual SPW was often preceded by a SRFP event, and the SRFPs occurring after individual SPW were increased in amplitude and decreased in frequency (Fig. 1A). These observations suggest that generation of these two distinct population activities may share a common local network (see DISCUSSION).

In vitro SPWs are intrinsic to isolated hippocampal circuitry

To control the possible hypoxic influence on the generation of in vitro SPWs, we measured tissue $O_2$ from SPW-exhibiting thick slices via using $O_2$-sensitive carbon fiber microelectrodes (Jiang et al. 1991; Mulkey et al. 2001; Wu et al. 2005b). One example of such measurements is shown in Fig. 2A, where a carbon electrode was placed in the CA2/CA1 area to record tissue $O_2$ and a NaCl-filled glass electrode was positioned in the CA3 area to monitor in vitro SPWs. The carbon electrode was advanced in a stepwise manner from the top surface toward the deeper layers of the slice and at an angle of $\sim$75° relative to horizontal plane. The $O_2$ measurements collected at different slice depths showed an asymmetrical U-shaped profile—higher $O_2$ levels at the top and bottom layers and the lowest but stable $O_2$ level at the middle layers of the slice. We observed the U-shaped $O_2$-depth profile in all SPW-exhibiting thick slices examined ($n = 11$). The $O_2$ levels measured from top and middle layers of these slices were 289.3 $\pm$ 32.8 and 35.6 $\pm$ 15.8 mmHg, respectively (Fig. 2B).

The $O_2$ levels measured from the perfused slices might be overestimated or underestimated due to potential drifts in background signals of the carbon fiber electrodes. To control this, we exposed the SPW-exhibiting thick slices to brief ($\sim$5
min) hypoxia (Chung et al. 1998; Perez-Velazques and Zhang 1994) and measured the resulting change in tissue O₂ from the middle layer of thick slices. The hypoxic episode caused a significant drop in baseline tissue O₂ (by 31.1 ± 9.3 mmHg, n = 5 slices, P = 0.002, paired t-test) and a reversible suppression of in vitro SPWs (Fig. 2C). Collectively, these data indicate that there was no anoxic zone in the SPW-exhibiting thick slices and the SPWs observed from thick slices are not a consequence of hypoxia.

We noticed that the in vitro SPWs were followed by small and reversible decreases in tissue O₂ (Fig. 2C). As measured from 26 SPW events (n = 5 slices) that occurred in relative isolation without overlaps in the associated O₂ signals, the mean decrease in tissue O₂ was 10.5 ± 3.0 mmHg per SPW event (P = 0.0016, paired t-test). We observed a greater decrease in tissue O₂ (by 22.4 ± 2.9 mmHg, P = 0.013, independent t-test) in the same slices after repetitive afferent stimulation (20 Hz for 200 ms). The latter observations are in keeping with a previous study in olfactory cortical slices (Fujii et al. 1982). Because the in vitro SPWs were correlated with coherent neuronal discharges and dependent on the AMPA glutamatergic activity (see Figs. 5 and 6), the SPW-associated decrease in tissue O₂ may largely reflect an activity-dependent increase in tissue O₂ consumption (Fujii et al. 1982).

Do the in vitro SPWs result from nonspecific ionic alterations particularly a rise of extracellular K⁺ and subsequent neuronal depolarization? To address this possibility, we measured extracellular K⁺ from SPW-exhibiting slices using ion-selective electrodes (Wu et al. 2002, 2005b). As measured from the CA3 proximal s. radiatum at the middle layer of thick slices, the basal extracellular K⁺ was 3.92 ± 0.15 mM (n = 3 slices), close to the KCl concentration of the perfusate (3.5 mM). There was no detectable rise of extracellular K⁺ that preceded the in vitro SPWs. However, an elevation of extracellular K⁺ by 0.5–1.2 mM was readily noticeable after individual SPWs and the elevated K⁺ signals gradually returned to
the baseline level in 10–20 s (Fig. 2E). While the mechanisms underlying SPW-associated K\(^+\) signals remain to be examined, the activity-dependent increases in extracellular K\(^+\) have been observed from conventional hippocampal slices or cultured hippocampal slices, and both glial and neuronal ionic buffering systems are thought to play important roles in determining the amplitude and recovery rate of extracellular K\(^+\) (Kann et al. 2003; Walz and Wuttke 1999). It is conceivable that similar mechanisms may account for the SPW-associated changes in extracellular K\(^+\).

CA3 and CA1 neurons in the SPW-exhibiting thick slices exhibited stable intracellular activities when monitored via the whole cell recordings. Basic intracellular parameters for CA3 pyramidal neurons were: resting membrane potentials of \(-66.8 \pm 1.1\) mV, input resistance of \(89.3 \pm 5.3\) M\(\Omega\), action potential amplitude of \(104.5 \pm 2.4\) mV, and action potential half-width of \(1.21 \pm 0.05\) ms \((n = 35\) neurons). The intracellular parameters for putative CA3 interneurons were: resting membrane potentials of \(-53.6 \pm 2.0\) mV, input resistance of \(165.0 \pm 23.3\) M\(\Omega\), action potential amplitude of \(77.4 \pm 5.6\) mV, and action potential half-width of \(0.80 \pm 0.15\) ms \((n = 7\) neurons). These measurements are in keeping with a previous study in conventional mouse hippocampal slices (Shuttleworth and Connor 2001). The recorded CA3 pyramidal neurons and inhibitory interneurons did not display evident depolarizing shifts that occurred before the appearance of local SPWs. Collectively, these observations suggest that the initiation of in vitro SPWs is unlikely caused by a substantial rise in extracellular K\(^+\).

We produced thick slices after separating the dentate gyrus from the CA2/CA1 areas to directly perfuse the deep layers of the thick slices with oxygenated ACSF. We have shown previously that such separation is essential to provide adequate oxygenation of the entire thick slice (Wu et al. 2005b). Although the dentate gyrus-CA3–CA1 connections remain functional in the thick slices (Wu et al. 2005b), the procedure of
dentate gyrus separation may disturb the hilar-CA3 circuitry and thus contribute to the generation of in vitro SPWs. To address this possibility, we prepared conventional ventral hippocampal slices (0.5 mm thickness, without dentate gyrus separation) from 3-mo-old mice and monitored spontaneous extracellular potentials from the CA3 and CA1 areas. These slices showed no baseline SPW when perfused with standard ACSF (see Methods). However, self-sustained, large-amplitude (0.5–2 mV) field potentials were observed in 5 of 38 slices after high-frequency afferent stimulation (80 Hz, 1 s; Fig. 3A). These induced field potentials were similar in waveform to the spontaneous in vitro SPWs observed from thick slices. Yee et al. (2003) have reported that similar high-frequency afferent stimulation induces recurrent field bursts in conventional rat hippocampal slices. We have shown recently that SPW-like field potentials occur spontaneously in conventional ventral hippocampal slices prepared from 8- to 10-mo-old rats (Wu et al. 2005a). Taken together, it appears that SPW-like field potentials can occur in conventional hippocampal slices, although their incidence rates were very low in our experiments. Based on these observations, we argue that the disturbance associated with dentate gyrus separation is unlikely to be responsible for the generation of in vitro SPWs.

If a relatively large hippocampal circuitry renders the spontaneous occurrence of in vitro SPWs, one would expect to observe SPWs from all layers of the thick slices. To address this issue, we placed one extracellular electrode in the CA3 area to monitor local SPWs as a reference and positioned another extracellular electrode in the CA2/CA1 area to record coherent SPWs at different slice depths. The second electrode was advanced from slice top layer toward deeper layers as described in the preceding text. As shown in Fig. 3B, the SPWs recorded from a constant CA3 site were minimally changed in amplitude and waveform, whereas coherent SPWs recorded from different depths of CA2/CA1 areas showed a bell-shaped amplitude distribution, i.e., larger SPWs in the middle layers and smaller SPWs in top and bottom layers of thick slices (n = 4). Thus the entire hippocampal tissue of thick slices appeared to be involved in the generation of in vitro SPWs. The inevitable tissue damage associated with the slicing procedure may explain the smaller SPWs observed from the top and bottom layers of thick slices.

Regional initiation and spread of in vitro SPWs

Previous in vivo studies indicate that the time lag between CA1 and CA3 SPWs is in accordance with the population activity mediated by the Schaffer collateral projection (Buzsáki 1986; Ylinen et al. 1995). By monitoring SPW-associated single unit discharges from the hippocampus of behaving rats, Csicsvari et al. (2000) have shown that pyramidal neurons in CA3a-b areas exhibit an early onset of discharges by 6–20 ms in comparison with discharges of CA1 pyramidal neurons.

We conducted multiple extracellular recordings together with surgical cuts to assess the regional initiation and spread of in vitro SPWs. We found that SPWs were robust in the CA3 and CA1 areas but were absent or with small amplitudes (<0.2 mV) in the dentate gyrus of the same slices (n = 5; Fig. 4A and B). Single afferent stimulation applied to the mid-molecular layer of dentate gyrus could evoke SPW-like responses in the CA3 but not in the dentate gyrus area (Fig. 4C). Moreover, coherent CA3 and CA1 SPWs remained in the thick slices in which the dentate gyrus tissue was surgically removed immediately after slicing (n = 10; Fig. 4D, left). Thus the thick slices...
Pharmacological manipulations of in vitro SPWs

Leung and Shen (2004) have shown that in intact rats intrahippocampal injections of AMPA/kainate receptor antagonists significantly attenuate hippocampal EEG irregular activities and SPWs, whereas the similar applications of the N-methyl-D-aspartate (NMDA) receptor antagonist AP5 are less effective in attenuating these EEG activities. To explore the roles of different glutamate receptors in the generation of in vitro SPWs, we perfused thick slices with the AMPA receptor antagonist CNQX, the NMDA receptor antagonist AP5 or the group I-II metabotropic receptor antagonist MCPG. During the perfusions of 0.5 μM CNQX (n = 3), the occurrence frequency, but not the amplitude, of in vitro SPWs was decreased before a complete abolishment (Fig. 5A). These observations are in keeping with the idea that the generation of SPWs in thick slices may be initiated by a subtle but crucial increase in glutamatergic drive. Perfusions of CNQX at 5–10 μM (n = 6 slices) caused a rapid and reversible blockade of the SPWs (Fig. 5B). Perfusion of the thick slices with 100 μM AP5 (n = 5 slices) or 2 mM MCPG (n = 4 slices) did not abolish CA3 SPWs but reduced their incidences or amplitudes in 2 slices (Fig. 5B). Based on these observations, we suggest that glutamatergic drive mediated by AMPA receptors is essential for the generation of SPWs in thick slices, whereas the activities mediated by NMDA and metabotropic glutamate receptors may be modulating factors.

Buzsáki (1986) has shown that systemic applications of pentobarbital or diazepam in intact rats suppress hippocampal EEG SPWs. To explore the influence of GABAergic inhibition of in vitro SPWs, we first examined effects of methohexital, a fast-acting anesthetic barbiturate that has been shown to enhance the GABA_A receptor–mediated synaptic activities in rat hippocampal slices (Zhang et al. 1998a). Perfusion of thick slices with methohexital at 1 μM (8–9 min) reduced the incidence of CA3 SPWs (from 3.7 ± 0.7 to 1.4 ± 0.1 events per minute, P = 0.009, paired t-test, n = 5 slices) but without significant changes in their waveforms (Fig. 5C). SPW amplitudes and half-widths were 0.8 ± 0.2 mV and 53.3 ± 3.5 ms in baseline controls and 0.9 ± 0.2 mV and 59.5 ± 2.7 ms after methohexital applications (P = 0.8 and 0.16, paired t-test), respectively. At 10 μM, methohexital applications reversibly abolished CA3 SPWs (n = 3 slices). Perfusions of diazepam at 1 μM (9–12 min) reduced the incidence of CA3 SPWs (3.3 ± 0.1 and 1.4 ± 0.1 events per minute before and after diazepam application, P = 0.026, n = 3 slices) and decreased their durations (half-width 59.3 ± 4.3 ms and 34.4 ± 1.9 ms before and after diazepam, P < 0.0001). In contrary, perfusion of thick slices with the GABA_A receptor antagonist bicuculline at 2 μM (6–8 min) increased the amplitude and half-width of CA3 SPWs (0.5 ± 0.1 mV and 46.2 ± 3.3 ms before, 0.7 ± 0.1 mV and 59.1 ± 2.8 ms after bicuculline, n = 5 slices, P = 0.026 and P < 0.0001). Bicuculline applications also increase the frequency of the ripple like oscillations (93–180 Hz before and 225–420 Hz after bicuculline, n = 3 slices; Fig. 5D). Applications of bicuculline at 10 μM abolished SPWs, and robust bursting field potentials (amplitudes of 2–3 mV and durations of 400–800 ms) were observed during washing (n = 3 slices). Collectively, the observations above are in keeping with previous in vivo studies (Buzsáki 1986; Leung and Shen 2004) and suggest that a stable generation of in vitro SPWs requires a balanced interaction between glutamatergic excitation and GABAergic inhibition.
Intracellular correlates of in vitro SPWs

Through simultaneous extra- and intracellular recordings in anesthetized rats, Ylinen et al. (1995) have shown that CA1 pyramidal neurons display EPSPs/discharges in correlation with local EEG SPWs and that CA1 inhibitory interneurons exhibit increased discharges during SPW-ripples. To date, little is known about the intracellular activities of hippocampal CA3 neurons that are correlated with EEG SPWs. We conducted simultaneous extracellular and single cell recordings in the CA3 area of thick slices to explore intracellular correlates of in vitro SPWs. The distance between these two recording sites was estimated to be \(0.5\) mm. Individual CA3 neurons were initially monitored via cell-attached voltage-clamp recordings (Fricker et al. 1999; Verheugen et al. 1999; Wu et al. 2002, 2005b), and their identity (pyramidal neuron versus inhibitory interneuron) was verified in subsequent whole cell recordings.

**CA3 pyramidal neurons**

**CELL-ATTACHED RECORDINGS.** When monitored at resting potentials (0 holding voltage), CA3 pyramidal neurons barely fired during SRFPs but exhibited a cluster of spike currents in phase with local SPWs (Fig. 6A). Each spike current was composed with a downward transient (half-width \(\approx 0.4\) ms) followed by a small upward component. In a set of 13 stable recordings achieved, CA3 pyramidal neurons fired 1–3 \((n = 4)\) or 5–12 spike currents \((n = 9)\) during SPWs. The SPW-correlated spike currents showed evident adaptation, and their intervals were 3–12 ms during the early phase \((\leq 50\) ms) of SPWs and \(\geq 50\) ms during the late phase of SPWs.

**WHOLE CELL RECORDINGS.** Monitored via the whole cell recording at the resting potential, CA3 pyramidal neurons exhibited excitatory postsynaptic potentials (EPSPs) or EPSPs-discharges in correlation with local SPWs \((n = 32\) paired recordings; Fig. 6A). When monitored at depolarizing potentials \((-55\) to \(-45\) mV), all CA3 pyramidal neurons discharged repeatedly in phase with local SPWs. Dual whole cell recordings revealed that CA3 pyramidal neurons discharged coherently in phase with local SPWs \((n = 4\) pairs, Fig. 6B), but discharges induced by intracellular current injections did not produce detectable field potentials nor interrupt the on-going local SPWs. These observations suggest that the in vitro SPWs represent a network-driven phenomenon as the result of coherent discharges by a large number of CA3 pyramidal neurons.

After each SPW, CA3 pyramidal neurons exhibited a slow hyperpolarization (Fig. 6, A and B) or an outward current \((I_{SPW})\) in voltage-clamp recordings (Fig. 7A). The extrapolated reversal
potential of the $I_{SPW}$ was more negative than $-80$ mV ($n = 3$ pyramidal neurons), whereas in the same neurons the Cl$^{-}$-dominant, SRFP-correlated synaptic currents (Wu et al. 2002, 2005b) were reversed at $-65$ to $-70$ mV (Fig. 7B). These observations suggest that the $I_{SPW}$ is likely mediated by a K$^+$ current.

Repetitive discharges or depolarizing stimulation are known to elicit slow afterhyperpolarization (sAHP) in hippocampal pyramidal neurons. The sAHP is mediated by a Ca$^{2+}$-dependent K$^+$ current ($I_{sAHP}$), thought to be activated via Ca$^{2+}$ entry through voltage-gated channels and a subsequent rise in intracellular Ca$^{2+}$ (Sah 1996; Storm 1990). The SPW-correlated discharges in CA3 pyramidal neurons may similarly raise intracellular Ca$^{2+}$, hence producing $I_{sAHP}$-like currents. To test this, we examined intracellular calcium dependence of $I_{SPW}$ by whole cell dialysis of CA3 pyramidal neurons with an internal solution that contained 10 mM EGTA (see METHODS). Intracel-

![FIG. 6. SPW-correlated discharges in CA3 pyramidal neurons. Data were collected via simultaneous extracellular and single cell recordings from 3 thick slices. A: CA3 pyramidal neuron was monitored initially by cell-attached voltage-clamp recordings (0 holding voltage, top) and by whole cell current-clamp recording at resting potential (bottom). Representative events (arrow) are shown in fast sweeps at right. B: whole cell current-clamp recordings were made simultaneously from 2 CA3 pyramidal neurons together with extracellular monitoring of local SPWs. Resting potentials of the recorded neurons are indicated. SPW-associated discharges observed from a CA3 interneuron. Interneuron was initially recorded via cell-attached voltage-clamp recordings (0 holding potential, top) and recorded via whole cell current-clamp recording at resting potential (bottom).](image1)

![FIG. 7. Activation of a Ca$^{2+}$-dependent K$^+$ current ($I_{SPW}$) after in vitro SPWs. A: whole cell voltage-clamp recordings were made from a CA3 pyramidal neuron together with extracellular monitoring of local SPWs. Neuron was held at different voltages as indicated. Filled circles denote peaks of $I_{SPW}$ currents. Arrows represent representative synaptic current that correlated with SRFPs. B: peaks (mean ± SE) of $I_{SPW}$ and SRFP-correlated synaptic currents (PSCs) were plotted vs. holding voltages. Lines through data points were computed via linear regression function. C: CA3 pyramidal neuron was dialyzed with an internal solution that contained 150 mM K-methylsulfate, 2 mM HEPES, and 0.1 mM EGTA. Neuron was held at $-55$ mV throughout recording period. Correlated extra- and intracellular activities were collected shortly ($\leq 2$ min, left) and $\geq 15$ min (middle) after formation of the whole cell recording. The shown $I_{sAHP}$ (right) was collected from the same neuron after $>15$ min of whole cell dialysis. D: another CA3 pyramidal neuron was dialyzed with an internal solution that contained 150 mM K-methylsulfate, 10 mM HEPES, and 10 mM EGTA. The extra- and intracellular activities were similarly collected and shown.](image2)
Intracellular dialysis with EGTA caused a time-dependent decrease in the \( I_{\text{SPW}} \), from an initial amplitude of 112.0 ± 20.6 to 17.2 ± 4.9 pA after 15–20 min of dialyses (\( n = 8 \) neurons, \( P = 0.0016 \), paired \( t \)-test). The \( I_{\text{AHP}} \) evoked by constant depolarizing pulses (60 mV, 400 ms, Zhang et al. 1994, 1995) was similarly decreased in the same neurons, from an initial amplitude of 78.0 ± 13.5 to 15.8 ± 13.7 pA after EGTA dialyses (\( P = 0.017 \), Fig. 7D). In contrary, both the \( I_{\text{SPW}} \) and \( I_{\text{AHP}} \) remained relatively stable in the control recordings without excessive buffering of intracellular calcium (Fig. 7C). Their amplitudes, measured after 15–20 min of whole cell dialyses (\( I_{\text{SPW}} \) of 55.7 ± 6.7 pA and \( I_{\text{AHP}} \) of 110.3 ± 32.3 pA, \( n = 10 \) and 5) were significantly greater than the currents recorded from the EGTA dialyzed CA3 pyramidal neurons (\( P = 0.025 \), independent \( t \)-test). These observations suggest that the \( I_{\text{SPW}} \) is likely mediated by a \( \text{Ca}^{2+} \)-dependent \( K^+ \) current.

CA3 inhibitory interneurons

Putative GABAergic inhibitory interneurons were recorded from the peri-somatic CA3 areas. Monitored via cell-attached voltage-clamp recordings, CA3 interneurons fired 1–3 spike currents in correlation with local SRFPs and a cluster of 8–15 spike currents in phase with local SPWs (\( n = 3 \), Fig. 6C). Monitored via whole cell recordings at resting potentials, CA3 interneurons (\( n = 7 \)) exhibit repeated discharges in correlation with local SPWs but without evident post-SPW hyperpolarization (Fig. 6C). Cross-correlation analyses did not reveal a clear temporal relation between interneuronal discharges and local ripple like oscillations when the original data were analyzed in a relatively wide frequency range (50–1,000 Hz). Because only a limited number of CA3 interneurons were recorded in the present experiments, we did not conduct further data analyses about the SPW-correlated interneuronal rhythmicity.

Discussion

In vitro SPWs represent an intrinsic network activity of isolated CA3 circuitry

Several recent studies have reported that conventional slices prepared from the ventral (but not dorsal) hippocampus of adult rats (Colgin et al. 2004; Kubota et al. 2003; Papatheodoropoulos and Kostopoulos 2002) and mice (Maier et al. 2002, 2003) can exhibit spontaneous field rhythms of 2–4 Hz. These field rhythms are similar to the SRFPs we observed from the whole mouse hippocampal isolate and thick mouse hippocampal slices, i.e., they are of CA3 origin and correlated largely with GABA\(_A\) inhibitory postsynaptic potentials (IPSPs) in pyramidal neurons. Thus the generation of low frequency (<4 Hz), and IPSP-correlated rhythmic field potentials appears to be a common feature of isolated rodent hippocampal circuitry. We show here that, besides the SRFPs, the CA3 circuitry of thick mouse hippocampal slices is capable of exhibiting SPW-like spontaneous field potentials. The in vitro SPWs and SRFPs display a close temporal relation in their appearance, but they are distinct in several aspects. The in vitro SPWs are greater in amplitude, longer in duration, and less frequent in occurrence than the SRFPs. The intracellular correlates of these two types of population neuronal activities are distinct, i.e., the in vitro SPWs are in phase with EPSP discharges in pyramidal neurons, whereas the SRFPs are correlated largely with GABA\(_A\) IPSPs in pyramidal neurons. The intracellular correlates of in vitro SPWs are in agreement with the intracellular study of EEG SPWs in intact rat hippocampus (Ylinen et al. 1995). Thus the CA3 circuitry of adult mouse hippocampus, when isolated in a relatively large form in vitro, is capable of generating both inhibition- and excitation-based spontaneous population rhythmic activities.

The in vitro SPWs are not a consequence of hypoxia because the thick slices are adequately oxygenated and brief hypoxia reversibly abolishes the in vitro SPWs. The generation of in vitro SPWs is unlikely triggered by a nonspecific elevation of extracellular K\(^+\), because the onset of in vitro SPWs is not preceded by a detectable rise of extracellular K\(^+\) signals (Fig. 2E) and depolarizing shifts in CA3 pyramidal neurons. The generation of in vitro SPWs cannot be attributed to a disturbance associated with the dentate gyrus separation because similar field potentials can occur in conventional hippocampal slices either spontaneously (Wu et al. 2005a) or after high-frequency afferent stimulation (Rafiq et al. 1993; Stasheff et al. 1985; Yee et al. 2003) (Fig. 3A). Considering that single afferent stimulation can evoke the SPW like responses in the thick slices (Fig. 1D), we suggest that the in vitro SPWs represent an intrinsic property of the CA3 circuitry.

Buzsáki et al. (1989) have postulated that in the intact brain, the hippocampus is under inhibitory control by subcortical afferent inputs and that removal of such tonic inhibition may allow a high level of synchronized network activity to occur in the deafferented hippocampus. Via intracellular recordings and labeling of CA3 pyramidal neurons from intact rats, Li et al. (1994) have shown that the areas of highest density of CA3 recurrent axon collaterals are always several hundred microns anterior or posterior to the cell body. The CA3 recurrent circuitry is known to be capable of generating synchronized population events in conventional slices (Miles and Wong 1987). It is conceivable that the thick slices encompass a relatively large of CA3 recurrent circuitry than conventional slices but without the inhibitory influences from the extrahippocampal structures, thus increasing the propensity of the CA3 recurrent circuitry to generate in vitro SPWs. However, an increase in the size of the CA3 recurrent network is not a sole explanation, because only ~40% of thick slices exhibit in vitro SPWs in our experiments, whereas nearly every thick slice exhibits the IPSP-based SRFP. Considering that the SRFPs and in vitro SPWs display a close temporal relation in their appearance (Fig. 1, A and B) and that single afferent stimulation can induce the SPW-like responses (Fig. 1D), we present the following hypothesis to account for SPW generation in thick slices.

Proposed mechanisms for SPW generation in vitro

We hypothesize that CA3 recurrent circuitry of thick slices may have a basal activity state in which SRFPs exist alone. In this basal state, only a small number of CA3 pyramidal neurons discharge sparsely, which in turn preferentially excites local inhibitory interneurons via the CA3 recurrent circuitry (Cohen and Miles 2000; Fricker and Miles 2000) or CA1 interneurons via the Schaffer collateral pathway. The activated interneurons synchronize likely via mechanisms of mutual inhibition and electrical communication (Maier et al. 2003; Skinner et al. 1999; Zhang et al. 1998b) and impose IPSPs onto a large
number of local pyramidal neurons thus producing SRFPs. A subtle but crucial increase of overall glutamate excitation in the CA3 recurrent circuitry may allow more pyramidal neurons to fire. Under this condition, the discharges of some CA3 pyramidal neurons may be synchronized and subsequently recruit a large number of CA3 pyramidal neurons and inhibitory interneurons, thus producing SPWs. Synchronous discharges by population of CA3 pyramidal neurons have been postulated as the basis of hippocampal EEG sharp waves (Buzsáki 1986; Csicsvari et al. 2000).

Modeling studies have shown that large-amplitude rhythmic population activities can emerge from a recurrent network if some fraction of the cells discharge or burst spontaneously. Specifically, Traub et al. (1989) showed that some bursting cells may act as initiators in a model network to recruit more excitatory as well as inhibitory cells into the discharge mode. It may also be that as the glutamatergic drive increases in the recurrent network, the larger number of firing excitatory neurons could ignite a population burst via nonlinear interactions in the network circuitry (Latham et al. 2000; Tsodyks et al. 2000). Our present data provide a basis for the examination of these hypotheses in modeling studies.

We found that the SPW-associated discharges in CA3 pyramidal neurons exhibit an evident adaptation pattern (Fig. 6A), which is a necessary feature for large-amplitude population bursts to occur in recurrent model networks (Latham et al. 2000). Hippocampal pyramidal neurons are known to exhibit strong firing adaptation when activated by intracellular stimulation, and several voltage-dependent or independent K+ currents are thought to play important roles in firing adaptation (Storm 1990). We speculate that similar ionic mechanisms may participate in the firing adaptation of the CA3 pyramidal neurons observed during the in vitro SPWs, including the Ca2+-dependent $I_{\text{SPW}}$. In addition to intrinsic ionic currents, it is conceivable that the SPW-associated discharges in CA3 pyramidal neurons are also influenced by synaptic kinetics. Tsodyks et al. (2000) have shown that synaptic depression can be an important characteristic underlying population synchrony in model networks. The intrinsic ionic currents and synaptic inputs that control the SPW-associated discharge patterns in hippocampal neurons deserve a thorough investigation in further studies. Because of the complex nonlinear nature of network activities, a combined experimental and modeling approach, particularly exploring the spatial-temporal properties of the in vitro SPWs (Dzhala and Staley 2003), may help to elucidate the fundamental principles that control the generation of synchronous bursts in the CA3 recurrent circuitry.

Significance and limitation of in vitro mode of hippocampal SPWs

During slow wave sleep and consummatory behaviors, the rodent hippocampus exhibits irregular EEG activities (main frequencies of 2–3 Hz; Jarosiewicz et al. 2002; Leung et al. 1982) and SPW-ripple complex (Buzsáki 1986; Buzsáki et al. 1983, 2003; Suzuki and Smith 1987). The generation mechanisms of hippocampal EEG irregular activities are largely unknown, but accumulating evidence indicates that EEG SPWs originate from the CA3 network as cortical and subcortical inputs to the hippocampus are reduced during slow wave sleep (Buzsáki 1980; Bragin et al. 1995; Chrobak and Buzsáki 1994; Csicsvari et al. 2000). We show in this study that SPW-like field potentials arise spontaneously in thick hippocampal slices and the in vitro SPWs share some common features with the EEG SPWs with respect to regional initiation and spread, pharmacological manipulations and intracellular correlates. Moreover, the coexistence of the in vitro SPWs and SRFPs (1–4 Hz) in thick slices mimics to some extent the intermingled EEG irregular activities and SPW-ripple complex seen in intact animals. We therefore suggest that the in vitro SPWs could possibly serve as a convenient model for exploring the cellular and neurochemical basis that may be involved in the generations of EEG SPWs in intact animals.

However, several differences exist between the in vitro and in vivo SPWs. For example, the in vitro SPWs are greater in amplitude and longer in duration than the rat hippocampal EEG SPWs (Buzsáki 1986; Buzsáki et al. 1983; Suzuki and Smith 1987). SPW-associated discharges of CA3 pyramidal neurons occur with higher frequencies in thick slices (see RESULTS) than those observed from intact animals (Csicsvari et al. 2000). CA3 pyramidal neurons exhibit a profound post-SPW hyperpolarization as the result of SPW-correlated bursting discharges (Fig. 6A and B). This hyperpolarization is much greater than that observed from the rat hippocampal CA1 pyramidal neurons in vivo (Ylinen et al. 1995). Because rat CA1 pyramidal neurons do not exhibit bursting discharges during EEG SPWs (Ylinen et al. 1995), the ionic currents underlying the in vivo and in vitro post-SPW hyperpolarizations may be different. The relatively large amplitude of in vitro SPWs may be partly due to a high level of background excitability and synaptic activities of the isolated hippocampal circuitry as suggested by the previous study in deafferented rat hippocampus (Buzsáki et al. 1989). The hippocampal EEG SPW-ripple complex is noticeably greater in mice than in rats (Buzsáki et al. 2003). This difference is thought to result from the larger “packing density” of neuronal elements in the mouse brain (Insauti 1993) and may partly account for the relatively large in vitro SPWs we observed from thick mouse hippocampal slices. We also recognize that the in vitro SPWs are similar to hippocampal epileptiform interictal activities in terms of CA3 origin, dependence on the glutamatergic drive and intracellular correlation (Borck and Jefferys 1999; Motalli et al. 2002; Nagao et al. 1996; Yee et al. 2003). Further experiments by preparing thick slices from animals with chronic limbic seizures (Bragin et al. 1999; Bouilleret et al. 1999, 2000) may help to determine the possible relation of in vitro SPWs and epileptiform interictal activities.

In summary, we suggest that the CA3 circuitry, when isolated in a relatively intact form, is capable of exhibiting SPW like spontaneous field potentials. While the physiological relevance of the in vitro SPWs remains to be further verified, this in vitro model may be of great value in elucidating the cellular and neurochemical basis that underlie spontaneous population neuronal discharges in the hippocampus.

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