Dissociated Retinal Neurons Form Periodically Active Synaptic Circuits

RICHARD E. HARRIS, MARGARET G. COULOMBE, AND MARLA B. FELLER

Synapse Formation and Function Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Received 28 August 2001; accepted in final form 1 March 2002.

Harris, Richard E., Margaret G. Coulombe, and Marla B. Feller. Dissociated retinal neurons form periodically active synaptic circuits. J Neurophysiol 88: 188–195, 2002; 10.1152/jn.00722.2001. Throughout the developing nervous system, immature circuits generate rhythmic activity patterns that influence the formation of adult networks. The cellular mechanisms underlying this spontaneous activity can be studied in dissociated neuronal cultures. Using calcium imaging and whole cell recording, we showed that cultured dissociated mammalian retinal neurons form networks that produce spontaneous, correlated, highly periodic activity. As the culture matures, the spatial correlations of the periodic calcium transients evolve from being highly synchronized across neighboring cells to propagating across the culture in a wavelike manner reminiscent of retinal waves recorded in vivo. Spontaneous calcium transients and synaptic currents were blocked either by cadmium, tetrodotoxin, or the glutamate receptor antagonist 6,7-dinitroquinoxaline, indicating that the periodic activity was driven primarily by synaptic transmission between retinal ganglion cells. Evoked responses between pairs of ganglion cells exhibited paired-pulse synaptic depression, and the time constant of recovery from this depression was similar to the interval between periodic events. These results suggest that synaptic depression may regulate the frequency of network activity. Together, these findings provide insight into how networks containing primarily excitatory connections generate highly correlated activity.

INTRODUCTION

Throughout the developing nervous system, immature neural circuits spontaneously generate periodic activity patterns (Yuste 1997). There is growing evidence that this spontaneous correlated activity can play a critical role in the formation of adult networks by influencing differentiation (Berridge 1998; Spitzer et al. 2000), motility (Gomez and Spitzer 1999; Lautermilch and Spitzer 2000), and connectivity (Ben-Ari et al. 1997; Katz and Shatz 1996; Wong 1993). In the developing retina, periodic bursts of action potentials propagate across the ganglion cell layer (Meister et al. 1991), influencing the development of both circuits within the retina (Bansal et al. 2000; Sernagor and Grzywacz 1996; Sernagor et al. 2001) and retinal projections to central targets (Wong 1999).

The mechanisms underlying the spontaneous generation of periodic activity have been studied in great detail in the developing hippocampus, spinal cord, and retina. Although the specific architecture of these three circuits differs significantly, qualitatively the activity across these regions is highly similar (Feller 1999; O’Donovan 1999). Namely, spontaneous bursts of action potentials are correlated across a network of highly connected neurons causing periodic increases in intracellular calcium concentration ([Ca$^{2+}$]). These patterns are generated solely via excitatory neurotransmission. This stands in sharp contrast to “classic” periodic networks whose rhythmicity is created by reciprocal excitatory and inhibitory connections that function as a pacemaker unit (reviewed in Harris-Warrick et al. 1992) or by time variant conductances, such as hyperpolarization-induced current ($I_h$), that are involved in membrane oscillations (Angstadt and Calabrese 1989; Luthi and McCormick 1998; Pape 1996; Thoby-Brisson et al. 2000). How does a network of neurons maintain periodic activity without the aid of pacemaker circuits or conductances?

Here, we show that immature mammalian retinal neurons in culture form synthetically coupled networks that undergo periodic activity. This activity, measured as periodic compound post synaptic currents (PSCs) and [Ca$^{2+}$] transients, is correlated over large distances and is driven primarily by glutamatergic synaptic transmission between retinal ganglion cells (RGCs). We also find that evoked responses at ganglion cell-ganglion cell synapses undergo synaptic depression, and the kinetics of this recovery from synaptic depression are similar to the interval between spontaneous events. Our results provide insight into strategies used by excitatory networks to generate correlated periodic activity.

METHODS

Preparation of retinal cultures

Cultures containing neurons and glia were prepared from postnatal day 2 ferret tissue with a method that promotes the survival of RGCs (Meyer-Franke et al. 1995; Pfrieger and Barres 1997). Briefly, retinas were isolated in PBS containing gentamicin (Gibco) and were placed in Earles basic salt solution containing 15 U/ml of papain (PAP2; Worthington) and 0.04% DNase (Boehringer Mannheim). After tissue was rocked for 30 min, the supernatant was removed and replaced with an inactivating solution of trypsin inhibitor (1 mg/ml), 0.04% DNase, and ovomucoid (1 mg/ml) in neurobasal medium (Gibco). The cells were then dissociated by gentle trituration through a 1-ml pipette. The cell suspension was spun in a centrifuge (Beckman) at 800 rpm for 7 min, and the pellet was resuspended in a serum-free medium.
containing neurobasal medium, B27 additives (Gibco) glutamine, forskolin (50 μM), insulin, brain-derived neurotrophic factor (2 ng/ml), ciliary neurotrophic factor (CNTF) (200 pg/ml), fibroblast-derived growth factor (Peprotech), and gentamicin. Cells were plated on glass substrates treated with 10 μg/ml poly-d-lysine (70 kd; Sigma), and merosin or laminin (2 μg/ml) and were maintained in a humidified CO₂- O₂ incubator at 36.5 °C. For electrophysiology measurements, cells were plated at a high density of 1.0–2.0 × 10⁶ cells/cm². For routine immunohistochemistry, cells were plated at one-fifth this density.

**Immunohistochemistry**

**FIXED-CELL STAIN.** Cultures were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and were rinsed in PBS. Cells were maintained at room temperature while they were permeabilized in 0.4% Triton X for 30 min, rinsed three times in PBS, blocked in PBS containing 5% sucrose and 20% donkey serum for 1 h, and rinsed. They were incubated with primary antibodies (see below) overnight at 4 °C. They were then rinsed, blocked again as above, and incubated in secondary antibodies (donkey anti-rabbit, anti-goat or, anti-mouse conjugated to either Rhodamine Red X or Cy3 (1:100; Jackson) for 60 min at 4 °C. The primary antibodies used were polyclonal antibody markers for rabbit γ-aminobutyric acid (GABA; 1:2,000), rabbit glutamate decarboxylase (GAD-67; 1:2,000; Chemicon) and monoclonal markers for rat and mouse Thy1.1 (1:250; Chemicon) and monoclonal markers for rat and mouse Thy1.1, surface marker, the Triton permeabilizing step was omitted.

**LIVE CELL STAIN.** Live cell staining was performed with a Thy1.1 antibody to identify RGCs (Taschenberger and Granit 1995). The primary antibody was added directly to the culture medium (1:30), and antibody to identify RGCs (Taschenberger and Grantyn 1995). The cells were maintained at room temperature while they were permeabilized in 0.4% Triton X for 30 min at room temperature and were rinsed in PBS. Cells were maintained at room temperature while they were permeabilized in 0.4% Triton X for 30 min, rinsed three times in PBS, blocked in PBS containing 5% sucrose and 20% donkey serum for 1 h, and rinsed. They were incubated with primary antibodies (see below) overnight at 4 °C. They were then rinsed, blocked again as above, and incubated in secondary antibodies (donkey anti-rabbit, anti-goat or, anti-mouse conjugated to either Rhodamine Red X or Cy3 (1:100; Jackson) for 60 min at 4 °C. The primary antibodies used were polyclonal antibody markers for rabbit γ-aminobutyric acid (GABA; 1:2,000), rabbit glutamate decarboxylase (GAD-67; 1:2,000; Chemicon) and monoclonal markers for rat and mouse Thy1.1 (1:250; Chemicon) and monoclonal markers for rat and mouse Thy1.1, surface marker, the Triton permeabilizing step was omitted.

**Electrophysiological recordings**

Whole cell patch-clamp recordings were made, either in voltage-clamp or current-clamp mode, with an Axopatch 200B patch-clamp amplifier (Axon Instruments). Voltage-clamp experiments were conducted at holding voltages of −60 mV. The intracellular solution for the current-clamp experiments contained the following (in mM): 98.3 K-glucuronate, 1.7 KCl, 0.6 EGTA, 5 MgCl₂, 40.0 HEPES, 2 ATP-Na, and 0.3 GTP-Na, adjusted to pH 7.25 with KOH. The intracellular solution used for the voltage-clamp experiments contained the following (in mM): 100 Cs-glucuronate, 1.7 CsCl, 10 EGTA, 5 MgCl₂, 40 HEPES, 1 QX-314, 2 ATP-Na, and 0.3 GTP-Na, adjusted to pH 7.25 with CsOH. The external solution contained the following (in mM): 123 NaCl, 5 KCl, 3 CaCl₂, 2 MgCl₂, 10 d-glucose, and 10 HEPES, adjusted to pH 7.3 with NaOH. E_Ca was −60 mV for the above solutions. Electrode resistances varied from 2 to 5 MΩ. All recordings were performed at room temperature with perfusion rates near 1 ml/min.

All the recordings included in the analysis came from RGCs. Neurons that had large somas, significant Na⁺ current, and that fired repeated action potentials in response to current steps were presumed to be RGCs (Guenther et al. 1994). Neurons that had medium to large somas, small or no Na⁺ current, and fired a single action potential or no action potential in response to step depolarization were presumed to be amacrine cells (Bieda and Copenhagen 1999; Zhou and Fain 1996).

Synaptic currents were analyzed with pClamp6 (Axon Instruments) and MiniAnalysis 4.3.2 (Synaptosoft) software. To determine the frequency of the periodic synaptic currents, we constructed power spectrum densities, typically using 100 s of data (sample frequency 2.5 kHz). To allow easier computation without altering the overall structure of the data, we first interpolated data files to 2.0 × 10⁵ points.

For the synaptic depression experiments, a standard recording electrode was filled with external solution and was used for presynaptic stimulation. Stimuli (0.5–5 mA) were delivered for 50 μs at varying intervals (Iso-flex and Master 8, AMPL). Recovery from paired-pulse depression was measured by monitoring the amplitude of evoked synaptic responses as a function of the interval between the stimulating pulses. Responses from different cell pairs were recorded at eight time intervals between 50 and 5000 ms; these were averaged, and the averages were fit to a single exponential. The error associated with the fit was estimated with an algorithm in IGORPro (WaveMetrics) that computes the SD of the fit parameters from the residuals and is based on the assumption that the residuals follow a normal distribution.

**Optical recording**

Cultures ranging from 15 to 70 days old were loaded with either fluo-3 AM or fluo-4 AM (Molecular Probes). Fifteen microliters of a 1 μg/μl solution of fluo-3 AM or -4 AM in DMSO containing 2% pluronic acid were added to 3 ml of culture media for 15 min at room temperature. After loading, the cultures were washed and perfused with external solution. Intracellular fluorophores were excited at 480 nm and imaged with either a 10× (Zeiss CP-Achromat) or 40× (Zeiss Fluar) objective. Images were captured with a CCD camera (RTD-CCD-1300; Princeton Instruments) and were typically obtained with 100-ms exposures at 1 Hz.

Fluorescence images were obtained and analyzed with MetaMorph (Universal Imaging) and IGORPro. The fractional change in fluorescence (ΔF/ΔF₀) of the transients was calculated with the equation ΔF/ΔF₀ = (F₁ − F_base)/F_base, where F₁ and F_base are the raw and baseline fluorescence values, respectively, obtained by averaging the fluorescence over the specified region of interest (ROI). For high-magnification imaging (40× objective), ROIs were defined for the soma of individual neurons; for low-magnification experiments (10× objective), ROIs were defined as 60 × 60-μm squares that encompassed roughly four neurons. No corrections were made for background fluorescence or bleaching because these factors were negligible. Typical ΔF/ΔF₀ measurements ranged from 10 to 100%.

Plots of ΔF/ΔF₀ obtained from the low-magnification images were used to construct raster plots (see Fig. 2D). Measurements of ΔF/ΔF₀ versus time were computed for each ROI (averaged over 20 × 20 μm containing ~4 cells), and the second derivative was calculated. If the second derivative was above a threshold determined by eye (typically 2.5 to 5), a vertical line was drawn in the raster plot indicating a fluorescence transient. ROIs that did not contain transients were not included in the raster plot.

To determine the frequency of the [Ca²⁺]ᵢ transients, we constructed power spectrum densities, typically using 100 s of data (sample frequency 1 Hz). See Electrophysiological recording for the procedure.

The effects of various pharmacological agents on the amplitude and frequency of [Ca²⁺]ᵢ transients were measured across 3–10 cells/culture, with the results normalized to the control values for that culture. These normalized results were then averaged across cultures, and, unless otherwise noted, the significance of the pharmacological effects was assessed by a two-sided t-test.

J Neurophysiol • VOL 88 • JULY 2002 • www.jn.org
RESULTS

Retinal cultures contain predominantly ganglion and amacrine cells

Dissociated retinal cells isolated from postnatal day 2 ferrets were cultured for 15–70 days at high density. In the first few days after plating, cells extended processes that contacted neighboring cells. As the cultures matured, clusters of cells formed with a large concentration of glia (Fig. 1). Glial cells were retained in the cultures because they promote synapse formation (Meyer-Franke et al. 1995; Pfrieger and Barres 1997) and may support periodic circuit behavior (Verderio et al. 1999).

Immunohistochemical staining identified the cell types present in the cultures. Labeling with antibodies against the neuron-specific marker TeTx showed that a small percentage of cells in the culture were neurons (10.6 ± 2.91%; n = 7 culture preparations; 14–97 days in culture). The RGC-specific marker Thy1.1 identified the RGCs with staining in their somas and throughout their processes (Taschenberger and Grantyn 1995; Fig. 1A, left). Morphologically, the RGCs displayed larger soma sizes than did the other cell types, thus allowing their identification in the electrophysiology experiments. Antibodies against ChAT and the GABA-synthesizing enzyme GAD-67 identified cholinergic and GABAergic cells, respectively (Fig. 1A, middle and right). The cholinergic cells were, presumably, amacrine cells because a subset of this class provides the sole source of ACh in the retina (Famiglietti 1991; Kolb 1997; Tauchi and Masland 1984). The two main classes of GABAergic cells are amacrine and horizontal cells (Freed 1992; Massey and Redburn 1987). A horizontal-cell specific marker was not used to distinguish between these two cell types; only a few horizontal cells were seen, as identified by their characteristic morphology (Akagawa and Barnstable 1986). Accurately quantifying the percentage of neurons labeled for Thy1, ChAT, and GAD-67 was difficult because the density of neuronal cells present in the cultures was low. However, the predominant neuronal types in our cultures were amacrine cells and RGCs. All recordings described in the following experiments come from RGCs (see METHODS).

Cultured retinal neurons undergo periodic changes in \([Ca^{2+}]\)

We loaded our cultures with the calcium indicators fluo-3 AM or fluo-4 AM and monitored the spontaneous activity patterns by imaging changes in \([Ca^{2+}]\). Using short loading times, we found the contribution from glial cells was negligible. Images were captured with either a high-power (40X; Fig. 2A, top) or low-power (10X; Fig. 2A, bottom) objective and were typically obtained with 100-ms exposures at 1 Hz. The fractional change in fluorescence (ΔF/F) was plotted against time to show rhythmic fluorescence transients as seen in Fig. 2B (see METHODS). Plots of ΔF/F obtained from low-magnification images were used to construct raster plots, in which each line signified a \([Ca^{2+}]\), transient for a local region of interest (Fig. 2D; see METHODS).

Based on the spatiotemporal properties of the measured spontaneous \([Ca^{2+}]\) transients, we categorized the cultures into two groups: those that were 25–45 days in culture (DIC) and those that were 49–70 DIC. Cultures between 25 and 45 DIC displayed transient increases in fluo-3 fluorescence resulting from increases in \([Ca^{2+}]\) (16.2 ± 12.8% of the labeled cells; n = 50 cultures; Fig. 2, A and B). These \([Ca^{2+}]\) transients were highly regular, and a power spectrum analysis revealed a rhythm frequency that was uniform within a culture and that ranged from 0.10 to 0.60 Hz across cultures (corresponding roughly to interevent intervals between 2 and 10 s). In these cultures, \([Ca^{2+}]\) transients were synchronized across cells that were separated by over 1 mm (Fig. 2, C and D; for Quicktime movie, see www.biology.ucsd.edu/labs/feller/JNeurophys.html, clip1). These transients may have resulted from activity propagating >1 mm/s (Maeda et al. 1995) but appeared synchronous in our recordings because of our limited time resolution.

In contrast to these young cultures, cultures over 49 DIC displayed \([Ca^{2+}]\) transients that propagated across the cells (n = 11 cultures). This propagation is shown by a raster plot constructed from images acquired at 4 Hz (Fig. 2D, bottom). The propagating transients originated at irregular time intervals and multiple locations, suggesting that there were no “pace-maker” clusters of cells that were responsible for wave initiation (for Quicktime movie, see www.biology.ucsd.edu/labs/feller/JNeurophys.html, clip2). Moreover, all regions examined (n = 9 fields of view in each culture) contained cells that were capable of supporting propagating activity. Waves propagated at a speed of 200–500 μm/s, although analysis of the propagation speed was difficult because wavefronts were not clearly defined (Feller et al. 1997).

Periodic activity is driven primarily by glutamatergic synaptic transmission between RGCs

To determine if \([Ca^{2+}]\) transients in retinal cultures were driven by spontaneous synaptic activity, we performed whole cell recordings of RGCs. In cultures over 21 DIC, RGCs revealed robust synaptic activity. A subset of RGCs (37/137 cells, 11 cultures) displayed periodic compound PSCs manifested as a sustained (0.5–1 s) compound burst of inward currents terminated by a period of sparse activity (Fig. 3A). Power spectrum analysis identified a single prominent frequency component for each culture (Fig. 3B). Across cultures, the prominent frequency component ranged from 0.10 to 0.60 Hz.
Hz, comparable with the frequency measured for spontaneous 
Ca\(^{2+}\) transients. The range in peak frequency did not correlate 
with the age of the culture. The remaining RGCs (100/137) 
were classified as nonperiodic cells because they displayed no 
peak in the power spectrum. However a subset (27/100) of 
these nonperiodic cells did display compound PSCs.

In many preparations, the periodicity of neural activity has 
been shown to involve a time-variant intrinsic cellular conduc-
tance, such as the hyperpolarizing activated current termed \(I_h\) 
(Angstadt and Calabrese 1989; Luthi and McCormick 1998; 
Pape 1996; Thoby-Brisson et al. 2000). To determine if \(I_h\) or 
any other intrinsic conductance was involved in our regular 
activity, we performed whole cell current-clamp recordings 
(Fig. 3C). We found that RGCs displayed no changes in 
baseline membrane voltage between depolarizations (\(n = 15\) 
cells), indicating that no time-varying intrinsic conductance 
was activated. In addition, bath application of artificial cere-
brospinal fluid (ACSF) containing 2 mM Cs\(^+\), a potent antag-
onist of \(I_h\), did not prevent periodic depolarizations but did 
reduce the frequency of compound PSCs (frequency\(_{control} = 0.46 \pm 0.06; \ n = 5\) ). Given the effects of Cs\(^+\) on 
other conductances, it is difficult to interpret the change in 
frequency. However, these results indicate that \(I_h\) is not required 
for the generation of periodic activity.

We also investigated the source of the compound PSCs 
measured in RGCs. Periodic events >30 pA, which constituted 
all of the periodic compound PSCs, were blocked by Cd\(^{2+}\) 
(\(n = 6\) ), whereas spontaneous miniature events <10 pA that 
were nonperiodic, were unaltered. 6,7-Dinitro-quinoxaline 
(DNQX) abolished all synchronous periodic Ca\(^{2+}\) transients 
(\(n = 8\) cultures; Fig. 4A) and periodic compound PSCs (\(n = 6\) 
cells; Fig. 4B), indicating that the periodic events were driven 
by glutamatergic transmission. TTX, a blocker of voltage-
gated Na\(^+\) channels, also abolished these periodic Ca\(^{2+}\) tran-
sients (\(n = 5\) cultures) and compound PSCs (\(n = 7\) cells),

indicating that transmission comes from other RGCs, because 
they are the primary retinal cell type with Na\(^+\) action potential-

FIG. 2. Cultured retinal neurons display correlated 
rhythmic changes in \([Ca^{2+}]_i\). A: fluorescence (\(F\)) image of 
cultured retinal neurons loaded with fluo-3 AM visual-
ized with 40× objective. Scale bar, 10 μm. B: example of 
\(ΔF/ΔF\) trace averaged over a single soma at 26 and 49 
days in culture (DIC), recorded with 100-ms exposures at 
1-s intervals. *, \(ΔF/ΔF\) transients caused by propagating 
waves. C: fluorescence image (10× objective) of cul-
tured retinal neurons loaded with fluo-3 AM. Scale bar, 
100 μm. D: raster plots mark \([Ca^{2+}]_i\) transients averaged 
over neighboring 60 × 60-μm regions of the culture. Images 
were acquired with 100-ms exposures at 1-s (top) or 250-ms (bottom) intervals.

Because some neurons in the cultures were GABAergic

FIG. 3. Spontaneous periodic compound postsynaptic currents (PSCs) and 
postsynaptic potentials (PSPs) measured in cultured neurons. A: continuous 
whole cell voltage-clamp recordings of a retinal ganglion cell (RGC). Cell was 
held at −60 mV. Top: expansion of a single compound PSC. B: power 
spectrum density computed from a whole cell voltage-clamp trace. The spec-
tral peak for the cell was 0.195 Hz. C: continuous whole cell current-clamp 
recording from a RGC resting at −50 mV. The action potential peaks are 
truncated and shown at top.
the remaining six cultures, curare did not reduce the amplitude or frequency significantly [$\Delta F/F_{\text{curare}}/\Delta F/F_{\text{control}} = 0.80 \pm 0.33; P > 0.2; n = 6$ and frequency$_{\text{curare}}$/frequency$_{\text{control}} = 0.87 \pm 0.13; P > 0.05; n = 5$]. Consistent with this finding, the bath application of cholinergic antagonists did not alter the amplitude of synaptic currents (PSC$_{\text{DBE}}$/PSC$_{\text{control}} = 0.99 \pm 0.24; P > 0.5; n = 6$ cells). These results indicate that cholinergic amacrine cells had a variable contribution to the spontaneous activity but, in most cultures studied, were not required for the generation of periodic activity.

In summary, our results indicate that periodic activity is mediated primarily by a glutamatergic, synaptically coupled RGC network, although, in some cultures, cholinergic amacrine cells can contribute substantially. Although GABAergic neurotransmission correlates with glutamatergic activity, GABAergic transmission does not influence the temporal pattern of glutamate release or the size of the $[Ca^{2+}]_i$ increases in retinal neurons.

**Recovery from synaptic depression is similar to the periodicity of spontaneous activity**

In the hippocampus (Staley et al. 1998) and spinal cord (Fedirchuk et al. 1999; Tabak et al. 2000), synaptic depression plays a critical role in generating rhythmic activity in vivo. To test if this mechanism might be involved in generating periodic activity in our retinal cultures, we examined paired-pulse synaptic depression. An extracellular pipette was used to stimulate a single visualized RGC to release glutamate to a nearby postsynaptic cell that was in whole cell configuration. This elicited inward PSCs (Fig. 5A) that were blocked by TTX (100 nM; $n = 5$ cells) or by DNQX (25 μM; $n = 5$ cells; data not presented). The time course of recovery from synaptic depression correlates with the frequency of rhythmic events. A: time course of recovery from synaptic depression is measured as the ratio of the amplitudes of the second PSC to the first (%PPD = $1 - B/A$) averaged over all experiments. Solid line, single exponential fit; error bars, SD. Inset: whole cell voltage-clamp recording of pairs of PSCs evoked by extracellular stimulation of a neighboring RGC. Cells were held at $-50 \text{ mV}$. B: continuous whole cell voltage-clamp recording from a RGC. Inset: power spectrum density for the cell.

**FIG. 5.** Time course of recovery from synaptic depression correlates with the frequency of rhythmic events. A: time course of recovery from synaptic depression is measured as the ratio of the amplitudes of the second PSC to the first (%PPD = $1 - B/A$) averaged over all experiments. Solid line, single exponential fit; error bars, SD. Inset: whole cell voltage-clamp recording of pairs of PSCs evoked by extracellular stimulation of a neighboring RGC. Cells were held at $-50 \text{ mV}$. B: continuous whole cell voltage-clamp recording from a RGC. Inset: power spectrum density for the cell.
shown). When two stimuli were delivered at intervals of <5 s, the second response showed depression (Fig. 5A, inset). Using pairs of stimuli with increasing interstimulus intervals, we found the time course of recovery from depression, as determined by a single exponential fit, to have a τ of 2.0 ± 0.3 s (Fig. 5A), similar to that seen in several other synapses (for recent examples, see Dittman and Regehr 1998; Parker 2000; Staley et al. 1998; Tsodyks and Markram 1997). This recovery time is similar to the intervals between periodic compound PSCs recorded in these same cultures (2.0 ± 0.3 s; n = 6 cells; Fig. 5B). This result is consistent with the hypothesis that the kinetics of recovery from synaptic depression set the frequency of the periodic cells. However, to establish a causal relationship, additional experiments that test whether altering the recovery from synaptic depression affects the periodicity of the synaptic activity of the entire network are required.

**DISCUSSION**

In this study, neural network behavior was examined in dissociated cell cultures generated from mammalian retinas. These retinal cultures consisted of periodically active networks whose pattern evolved with the age of the culture. In young cultures, imaging revealed near-synchronous \([Ca^{2+}]\) transients in cells that were correlated across much of the culture. In contrast, older cultures exhibited \([Ca^{2+}]\) transients that propagated across many cells at velocities similar to the “waves” seen in the intact developing retina (Wong 1999). Our study focused on the younger cultures in an effort to determine the cellular mechanisms underlying the periodic synchronous activity. These cultures contained periodically active networks that were synaptic coupled via excitatory glutamatergic and cholinergic transmission and whose periodicity was similar to the recovery kinetics from synaptic depression.

**Comparison with in vivo circuits**

Spontaneous periodic activity correlated across neighboring cells, either through mechanisms that lead to synchronous activation or to propagation, can be found throughout the developing nervous system (for reviews, see Ben-Ari et al. 1997; Feller 1999; O’Donovan 1999; Yuste 1997). In the developing mammalian retina, rhythmic trains of action potentials propagate across the RGC layer, causing periodic increases in \([Ca^{2+}]\). Here we compare the circuit responsible for generating correlated activity in the culture dish to that of the intact developing retina.

Spontaneous activity in culture consists of periodic increases in intracellular calcium driven by barrages of excitatory synaptic input from a variety of cell types. Periodic depolarizations and compound PSCs are TTX- and DNQX-dependent mechanisms, indicating that glutamatergic transmission between RGCs is the primary source of excitatory coupling. In some cultures, cholinergic amacrine cells also provide a significant percentage of the excitatory input. In addition, GABA A receptor-mediated currents contribute to the compound PSCs recorded in culture; however, blocking GABA A receptors did not alter the periodicity of spontaneous activity. Hence, although GABAergic interneurons participate in the spontaneously active network, their input is not required for the generation of periodic activity.

In the intact developing retina, RGCs fire periodic propagating bursts of action potentials with a periodicity on the order of 1–2 min. This spontaneous activity is mediated by synaptic transmission between retinal interneurons, either cholinergic amacrine cells or bipolar cells, and RGCs. Retinal interneurons are known to release transmitter in a TTX-independent, graded-release manner. In contrast to the activity observed in culture, waves persist in the presence of TTX (Stellwagen et al. 1999) and even in the absence of RGCs (Stellwagen et al. 2000). The details of the circuit underlying the spontaneous generation of correlated activity in the intact retina changes with development. Early on, cholinergic synaptic transmission between amacrine and RGCs is required for the waves (Feller et al. 1996). Later, bipolar cell synaptic input to ganglion cells plays more of a predominant role (Bansal et al. 2000; Wong et al. 2000; Zhou and Zhao 2000). Although there is a substantial amount of GABA A receptor-mediated current recorded during waves (Feller et al. 1996), blockade of GABA A receptors does not affect the spatiotemporal properties of retinal waves (e.g., wave propagation speed, size, and periodicity) during the period of development when retinal waves are mediated by activation of nAChRs (Stellwagen et al. 1999), similar to the role of GABA A receptors in culture. However, at older ages in the intact retina, blockade of GABA A receptors will significantly alter the observed firing patterns (Wong et al. 2000).

Although the circuit produced in the culture dish differs from that in the intact mammalian retina, the observation that developing RGCs form periodically active networks allows us to use an in vitro model to study the mechanisms that may underlie the periodic propagating activity patterns seen in vivo. Our results are consistent with the hypothesis that these correlated activity patterns emerge from a network containing excitatory synaptic connections. One hypothesis we have explored in culture is whether the periodicity of the activity is determined by synaptic depression.

**Synaptic depression as a rhythm-generating mechanism**

Rhythm generation is a prominent feature of the nervous system. Rhythmic motor behaviors such as walking, feeding, swimming, and respiration involve central pattern generators. In contrast, the periodic activity seen in dissociated cultures does not require the existence of inhibitory synapses or intrinsic conductances that cause membrane potentials of individual neurons to oscillate (Bacci et al. 1999; Misgeld et al. 1998; Murphy et al. 1992; Nunez et al. 1996; Robinson et al. 1993; Senn et al. 1998). In particular, we found that, although GABA A receptors are activated synchronously with glutamate receptors, bath application of the GABA A receptor antagonist picrotoxin had little effect on the periodicity of compound PSCs and \([Ca^{2+}]\) transients (Fig. 4). In addition, whole cell current-clamp recordings revealed no substantial afterhyperpolarizations following spontaneous depolarizations, indicating that the periodicity of the events was not determined by oscillations in the membrane potentials of ganglion cells (Fig. 3C). Rather, the periodic activity was prevented by blockers of glutamatergic synaptic transmission, and, in some cultures, cholinergic transmission, indicating that the spontaneous activity is mediated exclusively by excitatory synaptic transmission.

We presented preliminary evidence that synaptic depression may play a role in setting the periodicity of the spontaneous
activity in our dissociated cultures (Senn et al. 1998; Traub et al. 1989). We found that the time course of recovery from synaptic depression closely matched the mean interval we measured between compound PSCs or $[Ca^{2+}]_i$ transients (Fig. 5). To demonstrate that the frequency of events is determined by the kinetics of recovery from synaptic depression, pharmacological manipulations that alter the kinetics of recovery without changing the efficacy of synaptic transmission are required.

Different versions of this depression mechanism have been proposed to regulate the temporal structure of spontaneous correlated activity in vivo. In the developing spinal cord, the depression that limits the length of bursts of action potentials recorded from motoneurons manifests itself as a decrease in synaptic strength (Fedirchuk et al. 1999; Tabak et al. 2000). In hippocampal networks, periodic activity is thought to be modulated by synaptic depression via postsynaptic mechanisms (Traub et al. 1989) or presynaptic mechanisms, such as the depletion of glutamate pools (Bains et al. 1999; Staley et al. 1998). In the developing mammalian retina, this form of network depression has been described as a refractory period, a finite period of time after activation of a region of the retina during which that region cannot participate in subsequent waves (Butts et al. 1999; Feller et al. 1997). In the case of the intact retina, the period of network depression is on the order of 30–40 s. Hence, if synaptic depression is responsible for this refractory period, then the mechanism would be different from the fast recovery observed in culture. Recovery from depression at synapses in hippocampal neurons can last on the order of 30 s when the readily releasable pool has been fully depleted (Liu and Tsien 1995; Stevens and Wesseling 1999). In addition, the long recovery time seen in the intact retina may be a reflection of the kinetics of depression at TTX-independent graded-release synapses (von Gersdorff and Matthews 1997) that provide the synaptic input to RGCs during waves in contrast to the TTX-dependent release observed in culture. Thus it is possible that synaptic depression sets the slow periodicity of the spontaneous activity seen in vivo.

In summary, we have demonstrated that cultured retinal neurons can act as a model system for studying the cellular mechanisms underlying spontaneous correlated periodic activity. A culture that would better represent the in vivo retinal circuit would be enriched for graded-release retinal interneurons. Whether this culture will generate activity with similar patterns remains to be determined.

We thank Rukmini Mirotnik and Joshua H. Singer for critical reading of the manuscript.

This work was supported by a National Institutes of Neurological Disorders and Stroke Intramural Research Program. R. Harris was supported by a National Institute of Neurological Disorders and Stroke Intramural Research Program Research Project Postdoctoral Fellowship. Present addresses: R. E. Harris, Division of Rheumatology, Immunology, and Allergy, Georgetown University Medical Center, 3800 Reservoir Road NW—LL Gorman Bldg., Washington, DC 20007-2197; M. B. Feller, Neurobiology Section 0357, Division of Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92030-0357.

REFERENCES


