Biochemical and Biophysical Research Communications 406 (2011) 107–111

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Fluctuations in nuclear envelope's potential mediate synchronization of early neural activity

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ARTICLE INFO

Article history:
Received 30 January 2011
Available online 4 February 2011

Keywords:
Ca^{2+} release
Fluorescence voltage measurement
Neural development
Burst spike discharge
Synchronization

ABSTRACT

Neural progenitor cells and developing neurons show periodic, synchronous Ca^{2+} rises even before synapse formation, and the origin of the synchronous activity remains unknown. Here, fluorescence measurement revealed that the membrane potential of the nuclear envelope, which forms an intracellular Ca^{2+} store, changed with a release of Ca^{2+} and generated spontaneous, periodic bursts of fluctuations in potential. Furthermore, changes in the nuclear envelope's potential underlay spike burst generations. These results support the model that voltage fluctuations of the nuclear envelope synchronize Ca^{2+} release between cells and also function as a current noise generator to cause synchronous burst discharges.

1. Introduction

Neural progenitor cells in the neuroepithelium and ventricular zone show periodic, synchronous increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [1–4]. Developing neurons also show periodic rises in $[Ca^{2+}]_i$ caused by synchronous burst spike discharges [5–7]. These activities play crucial roles in the early development of the central nervous system (CNS), including proliferation, migration, neurite extension, and formation of synaptic circuits [1]. The Ca^{2+} rise may be caused by releases of Ca^{2+} from intracellular Ca^{2+} stores or depolarization-induced Ca^{2+} influx. However, the correlated Ca^{2+} rise occurs even before synapse formation and the origin of the periodic, synchronous activity remains unknown. The nuclear envelope is composed of the outer and inner membranes, and the space between them functions as a Ca^{2+} store [8–10]. Recently, it has been proposed that fluctuations in the membrane potential of the nuclear envelope mediate synchronization of Ca^{2+} release between cells by capacitative (AC) electrical coupling in the neuroepithelium [11]. The present study was undertaken to characterize the membrane potential of the nuclear envelope by measuring two kinds of fluorescence simultaneously, using an organelle-specific voltage-sensitive dye [$DiOC_5(3)$] and Ca^{2+}-sensitive probes (Fura-2 or Fura Red) in embryonic retina. Furthermore, the role of the nuclear envelope's potential in spike burst generation was investigated in developing retinal ganglion cells.

2. Materials and methods

2.1. Preparation of retina

The neural retina was isolated from a chick embryo incubated for three (E3) to 21 (P0) days at 38 °C. The optic cup was dissected out of an E3 chick embryo and the neural retina (the inner wall of the optic cup) was isolated. The diameter of the neural retina was about 400 μm. At E4-P0, the central part of the neural retina was trimmed so that the size of the retinal piece was 400 × 400 μm. The retina was positioned on the bottom of a recording chamber (volume, 0.2 ml) with the inner side up. The recording chamber was mounted on the fixed stage of an upright microscope (BX51WI, Olympus, Tokyo, Japan). The recording chamber was perfused with a normal bath solution (NBS) containing (mM): NaCl 137; KCl 5; $CaCl_2$ 2.5; $MgCl_2$ 1; HEPES 10; glucose 22; buffered to pH 7.3 by adding NaOH, at 2 ml/min. Bath solutions were changed from NBS to a test solution with solenoid valves (General Valve Corp., Fairfield, NJ). Recordings were made at 25–27 °C or 36–38 °C for oscillations.

2.2. Fluorescence microscopy

A fluorescence measurement system (OSP-3, Olympus) was used for simultaneous fluorescence recordings of Fura-2 and $DiOC_5(3)$. Fluorescence intensity was measured from a region 40 μm in diameter with a photomultiplier (H5784, Hamamatsu Photonics). Two dichroic mirrors (A10034–03, A10034–04,

Abbreviations: AC, alternating current; BK channel, big or maxi-K channel; CCh, carbamylcholine; CNS, central nervous system; $DiOC_5(3)$, 3,3'-dipentyloxacarbocyanine iodide; E3, embryonic day 3; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; $InsP_3$, inositol 1,4,5-trisphosphate; P0, postnatal day 0; Tg, thapsigargin; TTX, tetrodotoxin.

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0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.02.004

Hamamatsu Photonics), a long-pass filter (A10033–63, Hamamatsu Photonics) and another photomultiplier (H5784–20, Hamamatsu Photonics) were used for simultaneous fluorescence recordings of Fura Red and $DiOC_5(3)$. LED (470 nm, 5 mW) was used as an excitation light source when spontaneous oscillations of $DiOC_5(3)$ fluorescence were recorded. For confocal fluorescence microscopy, a Nipkow-type confocal scanner (CSU10, Yokogawa, Kanazawa, Japan) and a cooled ICCD video camera were used. $DiOC_5(3)$ fluorescence intensity was measured with a photomultiplier from the eyepiece of the confocal scanner. Details were described in Ref. 19.

2.3. Electrical recording

Spike discharges and surface potentials were recorded from the inner surface of the retina with a coated silver wire electrode (ϕ 0.2 mm) and a low noise differential amplifier (CA-451F4, NF Corporation, Yokohama, Japan).

3. Results

3.1. $DiOC_5(3)$ reflects nuclear envelope's potential

In neuroepithelial cells and newborn neurons, the nuclear envelope is closely apposed to the plasma membrane [12,13] (Fig. 1A). To label the nuclear envelope, a fluorescent probe, $DiOC_5(3)$, was introduced [13]. When the neuroepithelium of an embryonic day 4 (E4) chick retina is stained with the dye, circular structures are labeled in the horizontal plane (Fig. 1B). The $DiOC_5(3)$-labeled circular structure has been identified as the nuclear envelope by enlarging the cell with a hypotonic solution [13]. In another cell type (chick red blood cell), the nuclear envelope was clearly labeled with $DiOC_5(3)$ (Supplementary Fig. S1).

$DiOC_5(3)$ fluorescence is sensitive to changes in membrane potential [13]. Since a release of Ca^{2+} from a Ca^{2+} store should cause a negative shift in the luminal potential [14], the $DiOC_5(3)$ fluorescence may be altered by a Ca^{2+} release. In the embryonic retina, stimulation of muscarinic acetylcholine receptors causes a robust release of Ca^{2+} from intracellular Ca^{2+} stores [15]. The application of a muscarinic agonist (carbamylcholine, CCh, 100 μM) to an E4 retina caused an increase in $[Ca^{2+}]_i$, as indicated by a decrease in the Fura-2 fluorescence that was excited by a wavelength of 380 nm [Fig. 1C, Fura-2 (380)]. Simultaneous measurement of $DiOC_5(3)$ fluorescence showed an increase in the $DiOC_5(3)$ fluorescence [Fig. 1C, $DiOC_5(3)$]. Decreased levels of another Ca^{2+}-sensitive fluorescent probe (Fura Red) also indicated the CCh-induced increase in $[Ca^{2+}]_i$ (Fig. 1D, Fura Red) while simultaneously recorded $DiOC_5(3)$ fluorescence showed a mirror image-like increase [Fig. 1D, $DiOC_5(3)$]. These recordings suggested that the negative voltage change caused by an efflux of Ca^{2+} was indicated by an increase in $DiOC_5(3)$ fluorescence. In the above recordings, it appeared that the increases in $DiOC_5(3)$ fluorescence preceded the decreases in Fura-2 and Fura Red fluorescence.

The CCh-induced increase in $DiOC_5(3)$ fluorescence was followed by a fall, and the Ca^{2+}-sensitive fluorescence tended to return to baseline levels (Fig. 1C and D). It is plausible that the fall in $DiOC_5(3)$ fluorescence indicates a positive shift in the luminal potential because the Ca^{2+} pump in the store membrane is electrogenic [16]; the pumping activity shifts the luminal potential in the positive direction during Ca^{2+} uptake. To test this idea, thapsigargin (Tg), an inhibitor of store Ca^{2+} pumps, was applied. The application of Tg (1 μM in a Ca^{2+} -free solution) caused an increase in $DiOC_5(3)$ fluorescence (Fig. 1E), which could be due to a basal Ca^{2+} efflux through leak channels. The increase in $DiOC_5(3)$ fluorescence was followed by a fall after the application of Tg (Fig. 1E). Since the washout of Tg and addition of external Ca^{2+} cause Ca^{2+} release-activated Ca^{2+} entry to replenish Ca^{2+} stores [17], the fall in $DiOC_5(3)$ fluorescence may reflect a positive shift in the luminal potential due to the replenishment of Ca^{2+} stores.

The $DiOC_5(3)$ fluorescence might reflect mitochondrial membrane potentials. To examine this possibility, a specific mitochondrial fluorescent probe (rhodamine 123) was introduced to label granular structures (Supplementary Fig. S2). The application of FCCP (10 μM), a protonophore and uncoupler of oxidative phosphorylation in mitochondria, caused a continuous increase in rhodamine 123 fluorescence (Fig. 1F). This increase was likely due to mitochondrial depolarization, which causes dequenching and release of the dye from mitochondria [18]. $DiOC_5(3)$ fluorescence, however, showed only a transient change during FCCP application (Fig. 1F). This suggested that $DiOC_5(3)$ fluorescence does not reflect the mitochondrial membrane potential. As another possibility, $DiOC_5(3)$ fluorescence might reflect plasma membrane potentials. To test this possibility, a high-K^+ solution (50 mM) was applied to depolarize the plasma membrane. $DiOC_5(3)$ fluorescence, however, only transiently decreased during continuous depolarization of the plasma membrane (Supplementary Fig. S3). Thus, $DiOC_5(3)$ fluorescence does not directly indicate the plasma membrane potential. These lines of evidence suggest that $DiOC_5(3)$ fluorescence reflects the membrane potential of the Ca^{2+} store.

3.2. Spontaneous oscillations of $DiOC_5(3)$ fluorescence

The recordings in Fig. 1 were performed at 25–27 °C. When the recording temperature was raised to 36–38 °C, $DiOC_5(3)$ fluorescence showed spontaneous, periodic increases in an E4 retina (Fig. 2A). The spontaneous oscillations of $DiOC_5(3)$ fluorescence, however, might have been due to factors other than ionic movements, such as intrinsic properties of the dye or instability of the optical recording systems. To examine this possibility, electrical activities were recorded from the inner surface of the retina, simultaneous with $DiOC_5(3)$ fluorescence. The electrical recording showed that the surface potential oscillated synchronously with the $DiOC_5(3)$ fluorescence oscillations (Supplementary Fig. S4). This strongly supports the idea that the spontaneous oscillations of $DiOC_5(3)$ fluorescence result from ionic movements, although the mechanism for the generation of surface potential was not studied in detail in the present study.

The spontaneous, periodic increases in $DiOC_5(3)$ fluorescence were also recorded from the ganglion cell layer at E13–16 (Fig. 2B). At E11 and afterward, the ganglion cells generate a tetrodotoxin (TTX)-sensitive action potential (Supplementary Fig. S2 in Ref. 19), and they show spontaneous, periodic bursts of multiple spikes at 36–38 °C (Fig. 2C). To find the temporal relationship between the burst spike discharges and the $DiOC_5(3)$ fluorescence increase, spike discharges were simultaneously recorded with $DiOC_5(3)$ fluorescence in four retinas. In these experiments, intensity of the excitation light was minimized because the irradiation was harmful to the spiking activity. Fig. 2D shows a raw recording of spike discharges (top trace), the rectified waveform (middle trace), and $DiOC_5(3)$ fluorescence (bottom trace). These recordings clearly show that the onset of burst spike discharges corresponded with the onset of $DiOC_5(3)$ fluorescence increases. The recordings from the other three retinas also show that the burst discharges coincided with the rises in $DiOC_5(3)$ fluorescence (Supplementary Fig. S5). This temporal relationship suggests that the $DiOC_5(3)$ fluorescence increase is involved in the generation of spike bursts. Conversely, it might also be supposed that the $DiOC_5(3)$ fluorescence increase was caused by action potentials or synaptic potentials. However, the spontaneous, periodic increases in $DiOC_5(3)$ fluorescence were recorded even in the presence of TTX (1 μM) and antagonists of fast excitatory transmitter receptors (5 μM MK-801 for NMDA receptors, 100 μM CNQX for non-NMDA receptors, and

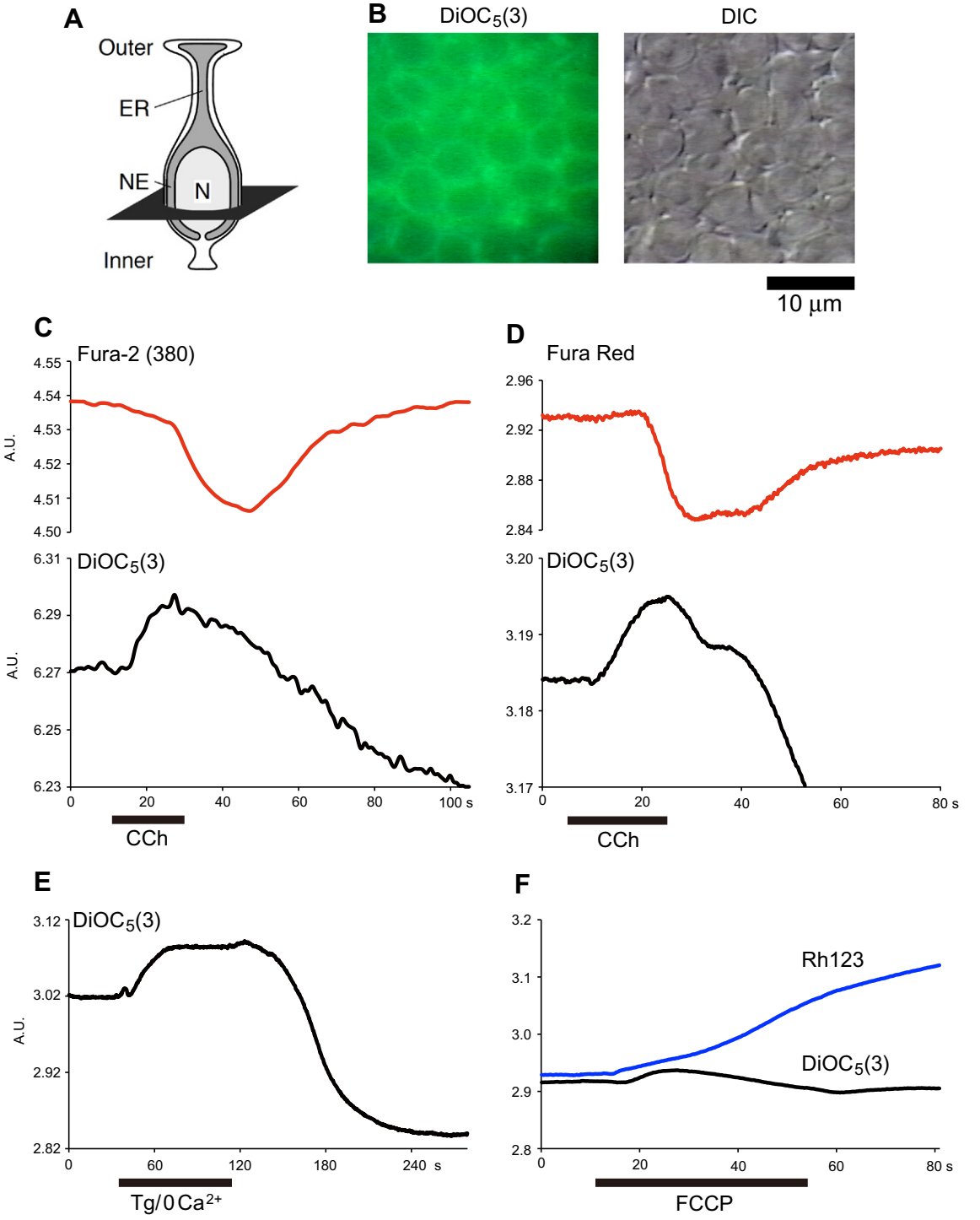

Fig. 1. Fluorescence measurements of nuclear envelope potential. (A) A schematic drawing of a neuroepithelial cell (cited from Ref.13). ER: endoplasmic reticulum, N: nucleoplasm, NE: nuclear envelope. (B) $DiOC_5(3)$ fluorescence image and Nomarski optics (DIC) view of horizontal plane of inner layer of an E4 retina (5 µm inside of the inner surface). (C) Simultaneous fluorescence recordings of Fura-2 and $DiOC_5(3)$. Fura-2 was excited by a wavelength of 380 nm. Carbamylcholine (CCh, 100 µM) was bath-applied during the bar. (D) Simultaneous fluorescence recordings of Fura Red and $DiOC_5(3)$. (E) $DiOC_5(3)$ fluorescence response to thapsigargin (Tg, 1 µM in a Ca^{2+}-free solution). (F) Rhodamine 123 and $DiOC_5(3)$ fluorescence responses to FCCP (10 µM). Recordings in C–F were taken from E4 retinas.

10 µM DHβE for nicotinic acetylcholine receptors) (Supplementary Fig. S6). Thus, it is likely that voltage changes of the nuclear envelope underlie the burst spike discharges.

3.3. Periodic bursts of fluctuations in $DiOC_5(3)$ fluorescence

As a link between the nuclear envelope potential and synchronous spike discharges, fluctuations in the nuclear envelope's potential were considered. Fluorescence imaging with a Nipkow-type confocal scanner and a video camera has shown that $DiOC_5(3)$ fluorescence oscillates synchronously between cells [13]. Connecting a photomultiplier to the confocal scanner revealed that the periodic increases in $DiOC_5(3)$ fluorescence are repeats of bursts of high frequency fluctuations in $DiOC_5(3)$ fluorescence [19]. The bursts of $DiOC_5(3)$ fluorescence fluctuations recorded from E3–9 retinas are shown in Supplementary Fig. S7a. The bursts contained

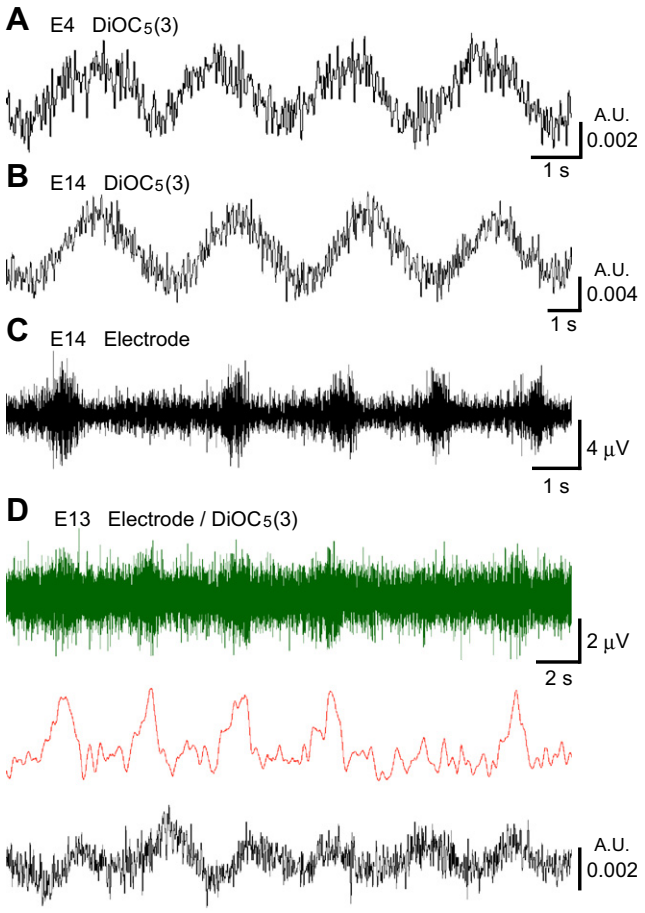

Fig. 2. Spontaneous oscillations of $DiOC_5(3)$ fluorescence and burst spike discharges. (A) Spontaneous oscillations of $DiOC_5(3)$ fluorescence recorded from an E4 retina. (B) Spontaneous oscillations of $DiOC_5(3)$ fluorescence recorded from an E14 retina. (C) Spontaneous burst spike discharges recorded from an E14 retina. (D) Simultaneous recordings of burst spike discharges and $DiOC_5(3)$ fluorescence. A raw recording of spike discharges (top), the rectified waveform (middle) and $DiOC_5(3)$ fluorescence (bottom).

fluctuations of more than 100 Hz (Supplementary Fig. S7b). In one case at E3, a single peak appeared around 300 Hz in the power spectrum (Supplementary Fig. S8). It is proposed that the fluctuation in $DiOC_5(3)$ fluorescence is due to voltage fluctuations caused by an efflux of Ca^{2+} and counter-movements of other ions such as K^+ (Supplementary Fig. S9). The bursts of $DiOC_5(3)$ fluorescence fluctuations were recorded from the inner layer about 5 μm below the inner surface, where S-phase cells in the cell cycle or newborn ganglion cells are positioned at E3–5 [20], and the ganglion cell layer is formed at E9 [21]. At stages later than E9, however, the confocal microscopy was not applicable to the ganglion cell layer because the thickness of the nerve fiber layer became >10 μm [21], and it was impossible to record fluorescence from the layers beneath the nerve fiber layer.

4. Discussion

As an explanation for the synchronization of $DiOC_5(3)$ fluorescence oscillation, the capacitative (AC) electrical coupling model has been proposed, wherein voltage fluctuations of the nuclear envelope produce alternating currents (AC). The resulting voltage fluctuations can be synchronized between cells by capacitative electrical coupling when the nuclear envelope is closely apposed to the plasma membrane and the cells are tightly packed [11]. Since the nuclear envelope is closely apposed to the plasma membrane in neuroepithelial cells and developing ganglion cells and these cells are tightly packed (Fig. 1A and B, and Supplementary Fig. S10), it is plausible that the voltage fluctuations of the nuclear envelope are synchronized between the cells by capacitative AC electrical coupling (Supplementary Fig. S11a). If the capacitative currents (I c) cross the plasma membrane, voltage-dependent Na^+ channels could thereby be activated to produce Na^+ currents (I_{Na}), which may flow through gap junctions, leading to burst spike discharges in multiple neurons. However, this could be the case during a limited period in the course of CNS development because distances between neurons become larger after the loss of neurons due to natural cell death and successive gliosis. At that point, capacitative AC electrical coupling becomes impossible. In fact, the ganglion cells are dispersed (Supplementary Fig. S12), and the spontaneous spike discharges become asynchronous at postnatal day 0 (P0) in accordance with the sudden reduction in spontaneous synchronous activity [6] (Supplementary Fig. S13). Separation of the nuclear envelope from the plasma membrane would also render AC electrical coupling impossible (Supplementary Fig. S11b).

The ganglion cells at E13–18 show spontaneous, synchronous increases in $[Ca^{2+}]_i$ [6]. Wong et al. showed that glutamate antagonists lower the frequency of spike bursts [6]. However, the authors mentioned both that "the modulation of frequency by the application of antagonists did not affect the synchronization of activity between cells; all cells participated in correlated bursting regardless of the frequency of bursts" and that "although neurotransmission plays a major role in potentiating and modulating bursting activity, neurotransmission is unlikely to mediate the synchronization of activity between cells" [6]. The present study showed that burst spike discharges occur with increases in $DiOC_5(3)$ fluorescence, which suggests that synchronous voltage fluctuations of the nuclear envelope function as current noise generators to trigger burst discharges (Supplementary Fig. S11c). It is likely that the depolarization by glutamate may facilitate or modulate the generation of action potentials (Supplementary Fig. S11c).

The present study may provide insight into the generation of synchronous spikes in other parts of the developing CNS. In newborn mouse cerebral cortices, tightly synchronized oscillatory discharges are induced by CCh and blocked by atropine, suggesting that muscarinic receptors are required to generate this activity [22]. CCh also induced synchronous burst discharges in the retina without spontaneous activity (Supplementary Fig. S14) and accelerated $DiOC_5(3)$ fluorescence bursting [19]. These facts suggest that the activation of muscarinic receptors causes Ca^{2+} release and voltage fluctuations of the nuclear envelope, which could evoke burst discharges. The synchronous discharges in newborn mouse cortices are inhibited by quinidine [22]. Quinidine also inhibits spontaneous $DiOC_5(3)$ fluorescence increases [13]. Since quinidine is a membrane-permeant blocker of BK (big or maxi-K) channels, the BK channels in the nuclear envelope may be essential for the release of Ca^{2+} as well as the generation of voltage fluctuations [13,19].

Activation of G protein-coupled receptors including muscarinic receptors may produce inositol 1,4,5-trisphosphate ($InsP_3$). If $InsP_3$ diffuses through gap junctions, an increase in $[Ca^{2+}]_i$ might be synchronized between cells [23,24]. However, agonist-induced increases in $[Ca^{2+}]_i$ are less synchronous than spontaneous Ca^{2+} oscillations [13], suggesting that the diffusion of $InsP_3$ is not responsible for the synchronicity. Gap junctions may mediate the synchronization of spike discharges, as gap junction blockers suppress spontaneous burst activity [6,7]. However, the gap junction lowers input resistance of the cell, which leads to stabilization of the membrane potential [19]. Thus, the gap junction itself cannot be the origin of spike generation, although action currents (I_{Na}) may flow through gap junctions when a cell fires (Supplementary Fig. S11b). Pacemaker-like neurons may also provide extensive

excitatory input [5]. Alternatively, the present study suggests that voltage fluctuations of the nuclear envelope underlie the generation of synchronous burst discharges before action potentials are generated by synaptic inputs.

Acknowledgments

I thank Dr. Yoshio Maruyama for helpful discussions. This work was supported by the Japan Spina Bifida and Hydrocephalus Research Foundation (JSBHRF), NISSAN Science Foundation, Strategic Promotion System for Brain Science, Special Coordination Funds for Promoting Science and Technology (SCF), Japan Society for the Promotion of Science (JSPS) and the Ministry of Education, Culture, Sports, Science and Technology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.004.

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