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Experimental Neurology 197 (2006) 495 – 504
Experimental Neurology
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Regular Article

NMDA receptor-mediated epileptiform persistent activity requires calcium release from intracellular stores in prefrontal neurons

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Received 17 March 2005; revised 18 May 2005; accepted 21 May 2005
Available online 10 November 2005

Abstract

Various normal and pathological forms of synchronized population activity are generated by recurrent excitation among pyramidal neurons in the neocortex. However, the intracellular signaling mechanisms underlying this activity remain poorly understood. In this study, we have examined the cellular properties of synchronized epileptiform activity in the prefrontal cortex with particular emphasis on a potential role of intracellular calcium stores. We find that the zero-magnesium-induced synchronized activity is blocked by inhibition of sarco-endoplasmic reticulum Ca^{2+}-ATPases, phospholipase C (PLC), the inositol 1,4,5-trisphosphate (IP3) receptor, and the ryanodine receptor. This same activity is, however, not affected by application of metabotropic glutamatergic receptor (mGluR) agonists, nor by introduction of an mGluR antagonist. These results suggest that persistent synchronized activity in vitro is dependent upon calcium release from internal calcium stores through the activation of PLC-IP3 receptor pathway. Our findings also raise the possibility that intracellular calcium release may be involved in the generation of pathologic synchronized activity in epilepsy in vivo and in physiological forms of synchronized cortical activity.

Keywords: Cortical neuron; Persistent bursting; Calcium signaling; Synaptic plasticity; Synchrony; Epilepsy

Introduction

Neural assemblies in a variety of brain areas display synchronized oscillations in response to sensory stimuli, and this synchronous activity has been suggested to underlie cognitive functions (Singer, 1993; Salinas and Sejnowski, 2001; Varela et al., 2001). An essential basis of such oscillation in the neocortex is thought to be action potential-dependent excitation among interconnected pyramidal neurons participating in local microcircuits (Steriade et al., 1993; Gray and McCormick, 1996; Sanchez-Vives and McCormick, 2000; Salinas and Sejnowski, 2001). In the prefrontal cortex (PFC) in particular, recurrent excitatory interactions are thought to account for synchronized persistent activity underlying the neural process of working memory (Goldman-Rakic, 1995).

Recurrent excitation in cortical networks is mediated by glutamatergic excitation and balanced by gamma aminobutyric acid (GABA)-ergic inhibition. Disinhibition of in vitro cortical slice preparations by blockade of $GABA_A$ receptors or enhancement of *N*-methyl-D-aspartic acid (NMDA)-mediated recurrent excitation with the removal of magnesium (Mg^{2+}) from the extracellular medium causes spontaneous synchronous burst firing which has been extensively used as models of epileptic activity (Connors, 1984; Chagnac-Amitai and Connors, 1989; Avoli et al., 1991; Traub et al., 1994; Telfeian and Connors, 1999; McCormick and Contreras, 2001; Avoli et al., 2002; Castro-Alamancos and Rigas, 2002; Cohen et al., 2002; Stoop et al., 2003).

Several lines of evidence indicate that release of intracellular Ca^{2+} stores is related to synchronous activity in cortical pyramidal neurons. For example, stimulation of the NMDA receptor to generate epileptiform activity has been shown to mobilize internal Ca^{2+} stores (Yoshimura et al., 2001). Moreover, activation of group I mGluRs through high frequency stimulation of excitatory afferents elicits propagating Ca^{2+} waves that are dependent on IP_3Rs (Nakamura et al.,

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doi:10.1016/j.expneurol.2005.05.018

2002). Although the physiological effects of the Ca^{2+} waves on the excitability of cortical neurons is not clear, it is likely that the high concentration of Ca^{2+} ions associated with these waves regulates the activity of numerous Ca^{2+}-dependent ion channels underlying inward and/or outward currents. Conversely, the population activity of pyramidal neurons during epileptiform bursting is very similar to that previously determined to be sufficient to elicit regenerative intracellular Ca^{2+} release (Nakamura et al., 2000; Larkum et al., 2003). Thus, population activity may also contribute to intracellular Ca^{2+} release and, as a result, perpetuate burst firing. How does intracellular Ca^{2+} release regulate synchronized population activity? We hypothesize that a large amount of Ca^{2+} could enter the cell through the activated NMDARs in the neuronal network, which in turn contribute to the production of IP3 by activating Ca^{2+}-sensitive PLC isoforms, resulting in activation and opening of IP3Rs and/or RyRs in the ER to release sequestered Ca^{2+} into the cytosol (Berridge et al., 2000). Here, we tested this hypothesis and examined the effects of pharmacologic agents that inhibit release of intracellular Ca^{2+} stores on the physiological properties of synchronized activity as it strengthens and evolves into persistent epileptiform activity in nominally Mg^{2+}-free solution in slice preparation of the PFC. We found that Ca^{2+} release from internal stores was essential for generation of persistent epileptiform activity in layer V pyramidal cells of the PFC.

Materials and methods

Slices preparation and physiological recording

Experiments were performed on layer V pyramidal neurons from medial prefrontal cortical slices prepared from 21 ferrets at ages of 7–14 weeks. Animals were treated according to guidelines of National Institutes of Health and in accordance with Yale's Institutional Animal Care and Use Committee regulations. The animals were deeply anesthetized with sodium pentobarbital (100 mg/kg), and the brains were removed and immediately placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 1, $NaHCO_3$ 26, and dextrose 10; pH 7.4. The tissue was trimmed, and horizontal 300 μm sections were cut on a Vibratome (Vibratome, St. Louis, MI). The slices were initially incubated in ACSF for 1 h at 35°C and then kept at room temperature. Recordings were made from slices submerged in a chamber perfused with warmed, oxygenated ACSF at a flow rate of 1.5–2 ml/min. Recordings were conducted at ~36°C.

Neurons were visualized under infrared differential interference contrast (IR-DIC) video microscopy as previously described (Gao and Goldman-Rakic, 2003). Whole-cell patch clamp recordings in current clamp mode were employed. Patch pipettes with a resistance of 5–7 MΩ were filled with intracellular solution containing (in mM): K-gluconate 120, KCl 20, EGTA 0.2, ATP-Mg 4, and HEPES 10; pH 7.3. The recordings were performed using MultiClamp 700 A amplifiers (Axon Instrument, Foster, CA). The data were filtered at 2 kHz in bridge-balance mode, and were acquired at sampling intervals of 50–100 μs with DigiData 1320 interface and pCLAMP 8.2 (Axon Instruments). Neurons were patched at the soma with a tight seal, typically at >2 GΩ seal resistance, and series resistance (10–30 MΩ) was monitored online.

Pharmacological compounds

The following drugs, all purchased from Sigma-RBI (St. Louis, MO), were applied to the bath in the indicated concentrations. NMDA receptor antagonist D-(−)-2-amino-5-phosphonopentanoic acid (APV), 50 μM; AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,4-dione (CNQX), 10–20 μM; sodium channel blocker tetrodotoxin (TTX), 1 μM; sarco-endoplasmic reticulum Ca^{2+} (SERCA) pump inhibitor Cyclopiazonic acid (CPA), 5–10 μM; internal Ca^{2+} store blocker Ryanodine, 5–10 μM; selective and membrane permeable IP3R inhibitor Xestospongin C (XeC), 1–2 μM; potent, membrane permeable, and IP3-independent sarco-endoplasmic reticulum Ca^{2+}-ATPases inhibitor thapsigargin, 2–4 μM; competitive metabotropic glutamate receptors (mGluR) antagonist (±)-a-Methyl-(4-carboxyphenyl) glycine ((±)-MCPG, 100 μM) and agonists L-(+)-2-Amino-4-phosphonobutyric acid (L-AP-4, 100 μM) and *trans*-(1*S*,3*R*)-1-Amino-1,3-cyclopentanedicarboxylic acid (t-ACPD, 20 μM).

For some experiments, low molecular weight heparin (1 or 2 mg/ml), a blocker of IP3R-induced-Ca^{2+} release stores (Ghosh et al., 1988; Larkum et al., 2003), or Ruthenium Red (400 μM), a blocker of ryanodine receptor (RyR) and Ca^{2+}-induced-Ca^{2+} release stores (CICR) (Caillard et al., 2000; Larkum et al., 2003), were added to the intracellular solution. Both phospholipase C (PLC) activator m-3M3FBS (25 μM) and inhibitor U 73122 (2–5 μM) were obtained from Tocris (Tocris Cookson Inc., Ellisville, MO).

Data analysis

In the zero-Mg^{2+} condition, neurons in the whole slice exhibited synchronized bursting activity. The pattern of synchronized firing could be segregated into a rhythmically alternating depolarized bursting state and hyperpolarized state. We defined the hyperpolarized state as the period during which the activity level stayed below the lower limit of depolarized state. Since in the zero-Mg^{2+} condition the firing behavior of bursting activity changed very slowly (within 35–45 min), although continuously, plus most of the drug effects were very dramatic and depressing, the effects of drug-addition could easily be distinguished within several minutes without question. One to three cells were recorded from each slice, and the depolarized bursts were manually counted before, during, and after drug application to evaluate the consistency of drug effects across tested cells. Spontaneous excitatory postsynaptic potentials (sEPSPs) events were detected and analyzed prior to (control) and after drug application, with the assistance of the software program Clampfit 9.0 (Axon Instrument, Foster, CA). For sEPSP frequencies, all events detected were used to test for statistical significance, while for sEPSP amplitudes, 20 mini-

events from each cell in control and drug application conditions, respectively, were used to avoid statistical bias. Finally, all data are presented as mean ± standard error.

Results

Multiple whole-cell recordings were performed in a total of 92 layer V pyramidal neurons in the medial PFC. Pyramidal cells were visualized and identified with the assistance of IR-DIC and on the basis of regular or intrinsic bursting firing patterns. The morphologies of recorded neurons were confirmed by biocytin labeling (data not shown). Zero-Mg^{2+} extracellular solution was applied to relieve the blockade of NMDA receptor channels and to induce synchronized bursting that transformed into persistent epileptiform activity.

Transient removal of extracellular Mg^{2+} elicits synchronized bursting and persistent epileptiform activity in the PFC in vitro

Neurons in acute brain slices were usually quiescent and stable for several hours without significant changes in their intrinsic membrane properties in regular ACSF (Fig. 1A). Under these conditions, only asynchronous spontaneous excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) were observed (Fig. 1A, inset). In our preparation, when brain slices were perfused with zero-Mg^{2+} solution, initially synchronized barrages of synaptic activity were observed, and this initial activity gradually transformed into a pattern of bursting activity. The transitory activity that was observed shortly (5–10 min) after the application of the zero-Mg^{2+} solution was comprised of large PSP discharges (Fig. 1B, inset). During continuous perfusion of zero-Mg^{2+} solution, these complex synaptic potentials gradually increased in amplitude and duration until typical seizure activity appeared after approximately 35–45 min (Fig. 1D). Spontaneous bursting or firing was not observed in control conditions in any of the pyramidal neurons recorded. The resting membrane potentials (RMP) of the recorded cells were on average −63.9 ± 0.97 mV (ranging from −58.6 to −82.4 mV, n = 42) (Table 1). Although somewhat variable from cell to cell, spontaneous bursting events, i.e., rhythmic oscillation of depolarized- and hyperpolarized-state membrane potentials, occurred at a frequency of 24.0 ± 1.46/min (ranging from 7–37/min), i.e., at periodicity of 2.5 s (0.4 Hz), when measured 20–25 min after zero-Mg^{2+} perfusion. The depolarized bursting activity lasted on average 241.7 ± 12.81 ms (range 81–462.7 ms), with an average of 1.64 ± 0.28 spikes (range 0–9) and of 11.4 ± 0.51 mV membrane potential difference (range 6–18.5 mV) relative to the hyperpolarized state, disregarding the action potentials. The frequency of synchronized bursting activity was time-dependent (Figs. 1B, C), increasing gradually until it was completely replaced by typical epileptic events (Fig. 1D). The amplitude and duration of these events, as well as the number of action potentials (APs) fired during each event, also increased in parallel to the escalation in their frequency during perfusion of the zero-Mg^{2+} solution. Burst firing was robust and synchronized for

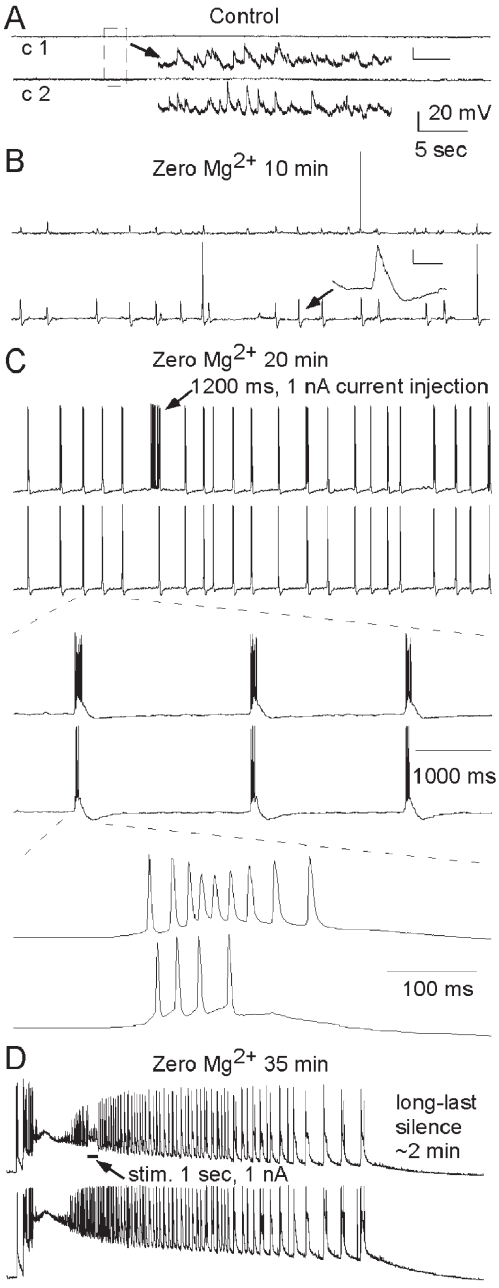

Fig. 1. Process of zero-Mg^{2+}-induced synchronized epileptiform activity in layer V pyramidal neurons of ferret prefrontal slices. (A) Examples of dual whole-cell recording from two layer V pyramidal neurons in control (normal ACSF) condition. The neurons are usually quiet and stable, with only small spontaneous EPSPs and IPSPs (inset scale bar = 0.5 mV, 500 ms). Scale bar (20 mV, 5 s) in panel B applies to panels A–D. (B and C) Perfusion of zero-Mg^{2+} solution-induced well-synchronized bursting activities. These activities were time-dependent, appeared ~5 min after perfusion of zero-Mg^{2+} solution (B) and became stronger, with periodic cycles characterized by a depolarized state accompanied by occasional burst firings and followed by a hyperpolarized state (C). The inset in panel B exhibited a large PSP (scale = 5 mV, 200 ms). The synchronized activity could not be perturbed by current injection into the soma (C). (D) Typical epileptic seizure activity appeared after 35–45 min perfusion of zero-Mg^{2+} solution. In our recording conditions, the seizure discharge lasted ~30–50 s followed by long-lasting periods of silence (~2 min).

Table 1
Drug effects on the neuronal resting membrane potentials (RMP)

Chemicals	Cell #	Action	Concentration (μM)	RMP in zero-Mg^{2+} (mV)	RMP in drugs (mV)	Difference (mV)	*P* value
Thapsigargin	12	SERCA inhibitor	2–4	−61.2 ± 2.38	−61.9 ± 2.35	−0.70 ± 0.28	0.106
CPA	11	SERCA inhibitor	5–10	−64.5 ± 2.51	−66.1 ± 2.88	−1.61 ± 0.87	0.115
Xestospongin	12	Membrane permeable IP3R inhibitor	1–2	−63.0 ± 1.86	−60.9 ± 1.60	2.11 ± 0.64	0.013 *
Ryanodine	6	Membrane permeable RyRs antagonist	5–10	−66.8 ± 1.73	−66.3 ± 1.98	0.57 ± 0.50	0.296
U 73122	7	PLC inhibitor	2–5	−67.1 ± 2.70	−66.0 ± 2.60	−0.95 ± 0.43	0.072
m-3M3FBS	5	PLC activator	25	−68.8 ± 3.35	−68.9 ± 3.34	−0.11 ± 0.65	0.873
MCPG	6	mGluR I and II antagonist	100	−64.4 ± 1.44	−63.9 ± 1.85	0.50 ± 0.79	0.593
t-ACPD	7	mGluR I and II agonist	20	−65.9 ± 1.85	−64.7 ± 2.06	1.17 ± 0.34	0.042 *
L-AP-4	5	mGluR III agonist	100	−63.0 ± 1.82	−64.6 ± 2.70	−1.57 ± 1.04	0.271
Heparin	8	Membrane impermeable IP3R antagonist	1–2 mg/ml in pipette	−65.6 ± 2.30	−65.5 ± 2.57	0.04 ± 0.45	0.935
Ruthenium red	4	Membrane impermeable RyR antagonist	400 μM in pipette	−64.6 ± 1.90	−66.8 ± 1.74	−2.15 ± 0.68	0.051

* $P < 0.05$.

about 35–45 min until it was replaced by typical epileptic ictal discharges lasting about 30–50 s (Fig. 1D). Usually, each ictal event was followed by a 2–4 min period of silence. Typical epileptic activity began abruptly with a robust long-last bursting (~2 s), followed by a long plateau potential without firing (~5 s), then a recovery period marked by a train of bursting, and finally a long-lasting period of silence (~2 min) (Fig. 1). Application of the NMDAR antagonist APV, or AMPAR antagonist DNQX, or sodium channel blocker TTX in the bath completely abolished the synchronous population activities (data not shown).

Persistent epileptiform activity is dependent upon Ca^{2+} release from internal stores

The zero-Mg^{2+}-induced synchronized bursting activity was largely and reversibly blocked by bath application of thapsigargin (2–4 μM), a potent and cell-permeable IP3-independent SERCA inhibitor. Thapsigargin had clear inhibitory effects on the synchronized activities in all neurons examined ($n = 12$) (Fig. 2A). Because thapsigargin could also affect L-type Ca^{2+} (Nelson et al., 1994) and potassium conductance (Thomas et al., 1999), we also used CPA, a specific inhibitor of SERCA that does not affect Ca^{2+} channels (Seidler et al., 1989; Nelson et al., 1994). CPA was very effective in blocking the zero-Mg^{2+}-induced activity in all of the 11 neurons tested (Fig. 2B). Although the effects of CPA on zero-Mg^{2+}-induced synchronized activity were irreversible, it affected neither the synaptic transmission nor neuronal excitability (Fig. 2B). Both thapsigargin and CPA slightly hyperpolarized the resting membrane potentials though the effects were not statistically significant perhaps due to small samples (Table 1).

In the intracellular Ca^{2+} signaling pathway, SERCA pumps control the refilling process of depleted Ca^{2+} stores following release through IP3Rs or RyRs (Berridge, 1998; Fill and Copello, 2002). We therefore examined the effects of inhibiting Ca^{2+} release. Bath application of the potent and selective membrane permeable IP3Rs blocker Xestospongin C (Gafni et al., 1997) completely blocked the synchronized activity in all cases tested ($n = 12$; Fig. 3) although resting membrane potentials were significantly depolarized on average by 2.11 ± 0.64 mV (Table 1, $P < 0.05$). It should be noted that Xestospongin C, like the SERCA inhibitors, effectively blocked the synchronized activity induced by zero-Mg^{2+}, while leaving the NMDAR- and AMPAR-mediated spontaneous EPSPs (sEPSPs) unperturbed (Figs. 2A, B and 3B insets). The amplitudes and frequencies of the sEPSPs were not significantly different during control and drug application periods ($n = 7$, $P = 0.558$ for frequency and $P = 0.989$ for amplitude, respectively) (Fig. 3B). Spike numbers during the spike train induced by current injection (+2 nA, 2 s) were also similar with 58 in the control condition and 57 following Xestospongin C application, respectively.

Ryanodine (5–10 μM), an agent that acts on RyRs on the endoplasmic reticulum (ER) to block CICRs (Rousseau et al., 1987; Larkum et al., 2003), completely and reversibly prevented the activities in 4 of 6 cells tested (Fig. 4). The remaining 2 cells only exhibited very weak inhibition. Unlike Xestospongin C, ryanodine not only significantly depressed the sEPSP amplitudes ($P < 0.001$) but also reduced the frequencies of spontaneous activities ($P < 0.01$), suggesting both pre- and postsynaptic actions.

In contrast to bath application, intracellular loading of the membrane impermeable antagonists of IP3Rs (heparin, $n = 4$) or RyRs (ruthenium red, $n = 4$) in individual neurons failed to eliminate the synchronized activity. Interestingly, heparin in all 4 cases at 2 mg/ml (but not at 1 mg/ml, $n = 4$) and ruthenium red in 3 of the 4 cells tested blocked the generation of APs by the barrage of PSPs associated with the epileptiform activity (Fig. 5). Action potential generation in response to intracellular current injection and other intrinsic properties of these neurons remained normal (Larkum et al., 2003), indicating the applied agents were not compromising the physiological state of the neurons, or changing the voltage threshold of the action potentials. These data suggest that the synchronized activity is

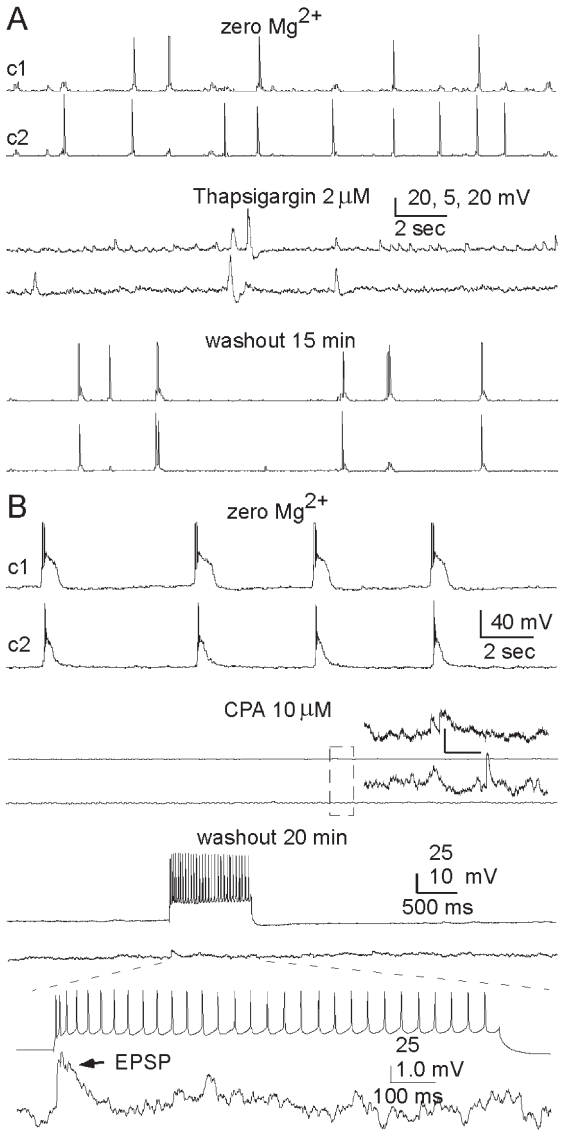

Fig. 2. SERCA-ATPase inhibitors are very effective in blocking the zero-Mg^{2+}-induced synchronized activity. (A) Bath application of thapsigargin (2 μM) largely blocked the synchronized activity in a paired recording, with very good recovery after a 10–15-min washout period. (B) The potent SERCA-ATPase inhibitor CPA (10 μM) completely and irreversibly blocked the synchronized activity, without affecting synaptic transmission. The inset shows the spontaneous NMDAR- and AMPAR-mediated EPSPs (inset, scale = 1 mV, 200 ms). Although the synchronized activity could not be recovered after CPA application (~20 min), neuronal excitability and synaptic transmission seem to be unaffected as shown in the monosynaptic connected pair (bottom panel).

network-dependent such that interference with Ca^{2+} release in a single cell had no noticeable effect on the activity.

To assess the variability of drug action in individual neurons, we plotted the rate of synchronized events versus time to evaluate how fast and consistently the drugs blocked the events across cells (Fig. 6). All four drugs were very effective in blocking synchronized burst activity. CPA exhibited the most rapid and consistent effects although these were irreversible in most of the cells examined, even after a 20-min wash (not shown). In contrast, thapsigargin's effects were more variable, slower, and only partially recovered after 10 min wash-in of zero-Mg^{2+}. Xestospongin effectively blocked the burst activity, and recovery was complete and rapid with little variability among tested cells. Ryanodine, as stated above, was very effective in majority of the cells (4/6) and bursting activity recovered completely in the wash-out period.

Next, we tested the hypothesis that Ca^{2+}-sensitive PLC isoforms are involved in the production of synchronized activity by applying a PLC activator and inhibitor, respectively (Fig. 7).

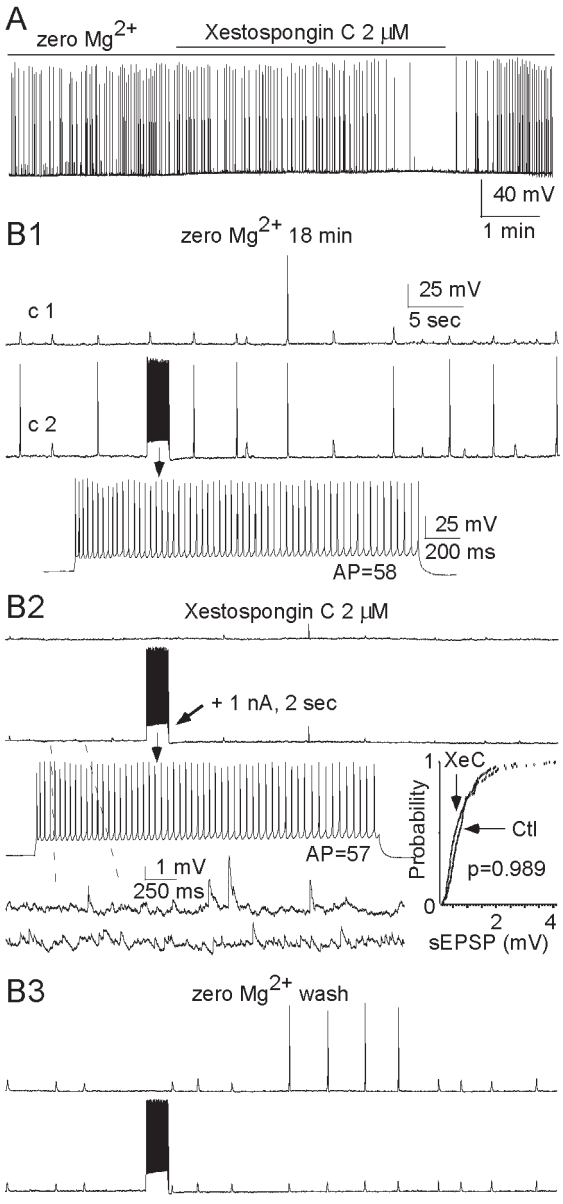

Fig. 3. The epileptiform-synchronized activity depends upon Ca^{2+} release from internal stores, possibly through activation of IP3Rs. (A) Zero-Mg^{2+}-induced synchronized activity was effectively and reversibly blocked by application of the selective and potent IP3R inhibitor Xestospongin C (2 μM). (B) A synchronized neuronal pair showing that, although Xestospongin C was effective in blocking the synchronized activity, it affected neither neuronal excitability (spike number = 58 in control condition and 57 in Xestospongin C) nor synaptic transmission. Expanded segments in panel B2 exhibited that the spontaneous NMDAR- and AMPAR-mediated EPSPs, neither the frequency nor the amplitude, were perturbed by application of Xestospongin C in the bath (n = 7, P = 0.558 for frequency and P = 0.989 for amplitude).

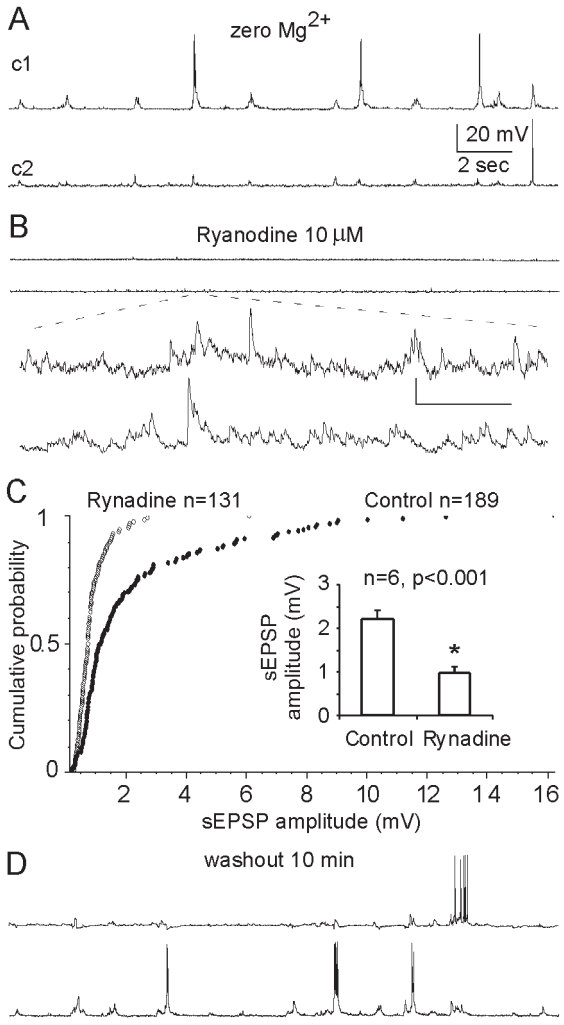

Fig. 4. Ryanodine receptors inhibited the zero-Mg^{2+}-induced synchronized activity by reducing glutamate release and responsiveness in both pre- and postsynaptic sites. (A) Perfusion of zero-Mg^{2+} solution-induced neuronal synchronization in a neuronal pair without direct synaptic connections. (B and D) Bath application of ryanodine (10 μM) successfully and reversibly blocked the synchronized activity (inset scale = 1 mV, 250 ms). (C) Ryanodine significantly reduced the amplitude (n = 6, P < 0.001) and frequency (P = 0.009) of spontaneous EPSPs.

Bath application of PLC activator, m-3M3FBS (n = 5), significantly enhanced the synchronized activity by increasing the frequencies, but not durations, of depolarized states. The enhancing effects could be completely abolished either by co-application of Xestospongin C or by introducing the PLC inhibitor U 73122 immediately following application of m-3M3FBS (Fig. 7). Drugs that were effective in regulating of PLC- or IP3-mediated Ca^{2+} release did not exhibit significant effects on neuronal excitabilities: M-3M3FBS and Xestospongin C did not affect spike number, amplitude, or half-width of action potentials (see expanded segments in Figs. 7B, C). Moreover, the PLC inhibitor U 73122 (2–5 μM, n = 7) was very effective in blocking zero-Mg^{2+}-induced synchronized activities (Figs. 7 and 8), without having a significant effect on resting membrane potential (Table 1), synaptic transmission (Fig. 8B, inset), or neuronal excitability (Figs. 8A–C).

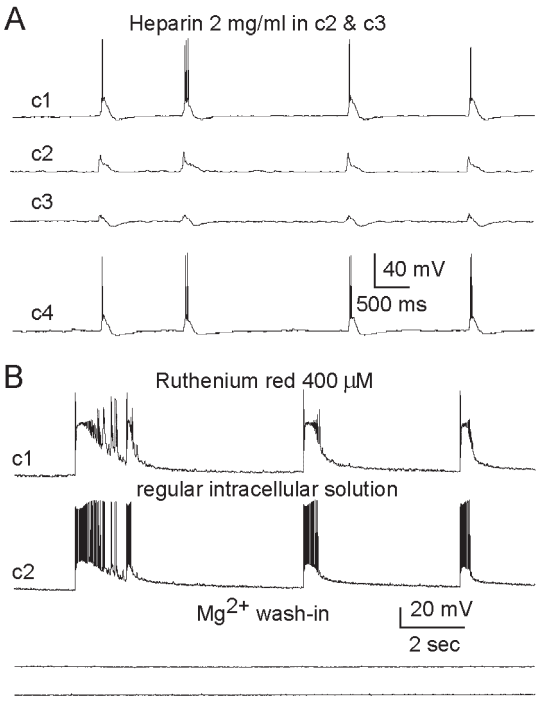

Fig. 5. Intracellular application of the membrane impermeable antagonists of IP3Rs or RyRs failed to eliminate the epileptiform activity but blocked the generation of APs. (A) IP3R blocker heparin (2 mg/ml, n = 4) had no effect on the synchronized activity but blocked the spike-generation when loaded into the cells with recording pipettes (c2 and c3). (B) Similarly, the RyR blocker ruthenium red (400 μM in recording pipette) had also no clear effects on synchronized activity other than blocking spikes.

To explore whether IP3 production may be supplemented by activation of mGluRs, either the mGluR group I and II agonist t-ACPD (20 μM, n = 7), the mGluR group III agonist L-AP-4 (100 μM, n = 5), or the mGluR group I and II antagonist (±)-MCPG (100 μM, n = 6) were applied to the bath (Table 1). None of these pharmacologic applications produced significant effects

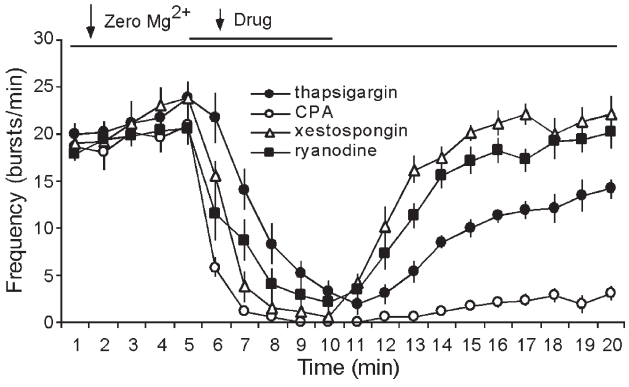

Fig. 6. Summary of drug effects on synchronized burst activity. The average burst frequency before, during, and after drug applications across cells from different animals is shown. All four drugs were effective in blocking the bursting events. Thapsigargin's effects were variable, slow, and only partially recovered. CPA produced fast, consistent blockade of bursting activity that was irreversible. In contrast, Xestospongin effectively blocked the burst activity, but activity recovered quickly and consistently across tested cells. Ryanodine was effective in a majority of cells tested (4/6) and was reversible.

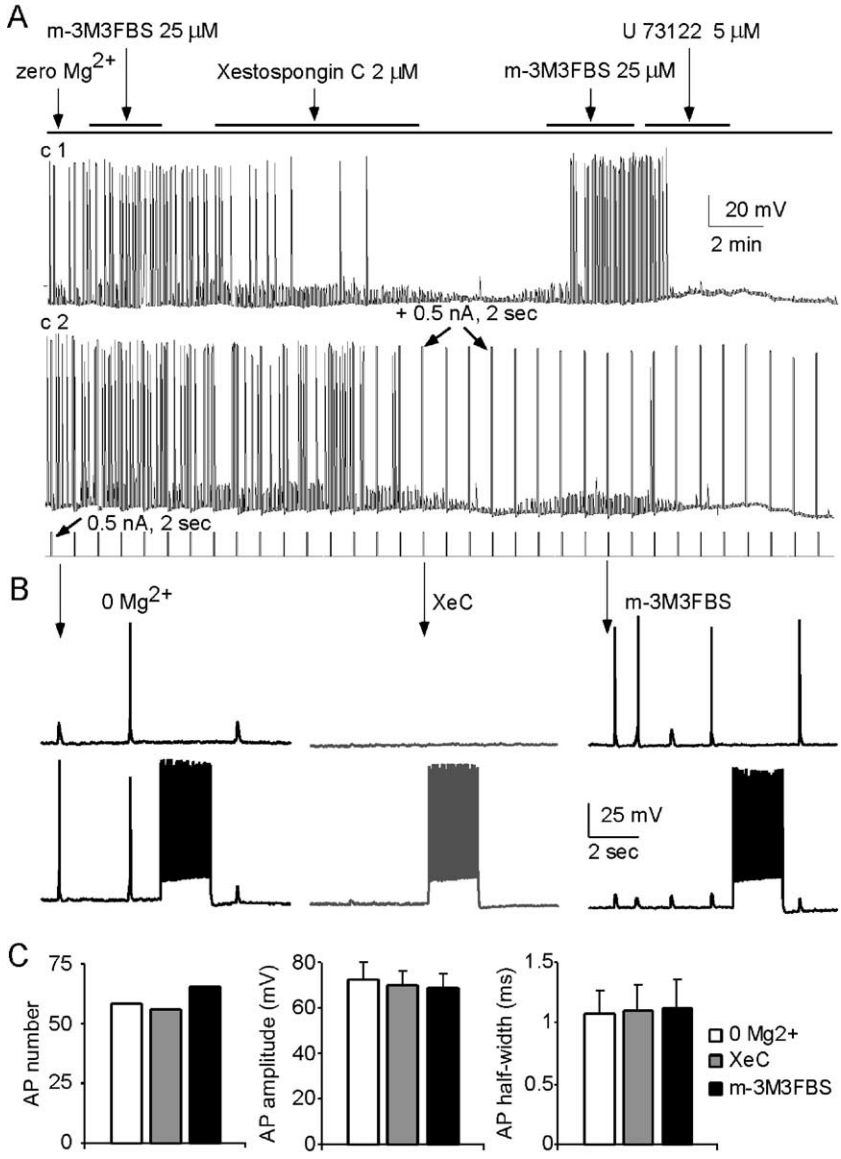

Fig. 7. Sequential drug applications showing the involvement of both PLC and IP3Rs in synchronized activity on two simultaneously recorded pyramidal neurons. (A) Perfusion of zero-Mg^{2+} solution-induced synchronized activities and these activities were significantly enhanced by bath application of the PLC activator m-3M3FBS (25 μM). The enhanced activities were in turn completely abolished either by co-application of IP3R inhibitor Xestospongin C (2 μM), or followed by application of PLC inhibitor U 73122 (5 μM). Note that intermittent positive currents (+0.5 nA, 2 s) were injected onto the second cell to monitor the excitability changes during the drug application. (B) Expanded segments showing that none of these drugs had significant effects on neuronal excitability. (C) Measurements of action potentials (AP) in the spike-train indicating the absence of any measurable effect on number, amplitude, or half-width of action potentials during the drug applications.

on the synchronized activity (data not shown), indicating that in the zero-Mg^{2+} condition Ca^{2+} influx through activated NMDAR is sufficient to induce population activity, while the supplementary role of mGluRs exhibited in other conditions is not necessary for the induction of synchronized activity.

Discussion

We have employed multiple whole-cell recording combined with pharmacological applications to address the intracellular signaling mechanisms of synchronized activity induced by transient removal of extracellular Mg^{2+} in the prefrontal cortex. Application of glutamate receptor agonists and antagonists indicated that the synchronized population activities were generated by recurrent synaptic activity mediated by NMDARs and AMPARs, and were not modulated by mGluRs. Pharmacological manipulation of intracellular Ca^{2+} signaling revealed a dependency of synchronized activity on Ca^{2+} release from internal calcium stores and provided evidence suggesting that this release was mediated through activation of the PLC-IP3R pathway.

The consistent inhibitory actions of two potent SERCA inhibitors, thapsigargin and CPA, strongly indicate that the process of Ca^{2+} release from internal stores is involved in generation or maintenance of synchronized activity as SERCA pumps replenish depleted Ca^{2+} stores following

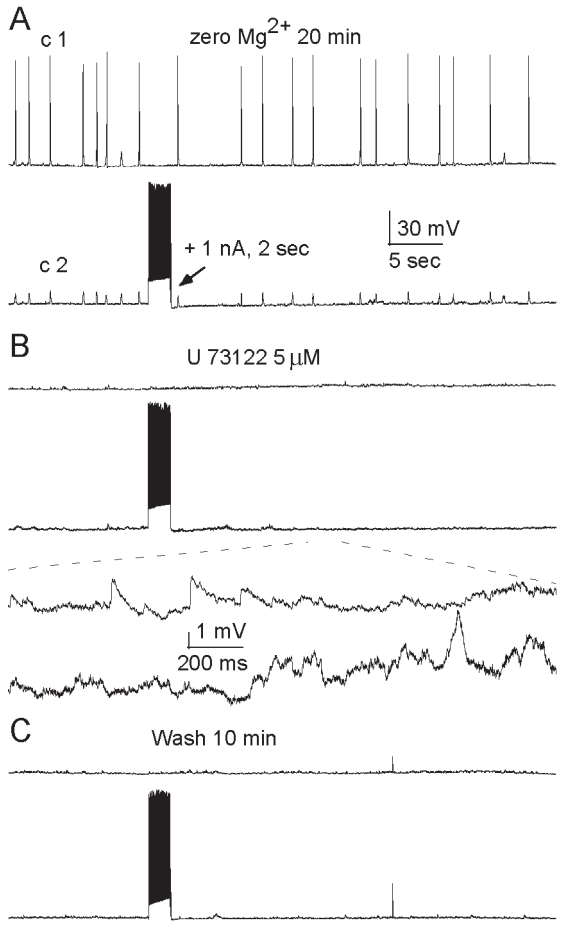

Fig. 8. The synchronized activity depends on the activation of PLC. (A) The zero-Mg^{2+}-induced synchronized activity was not affected by current injection in a single cell. (B and C) PLC inhibitor U 73122 (5 μM) effectively abolished the synchronized activity, without disturbing the synaptic transmission and cell excitability.

release. The specificity of thapsigargin towards SERCAs is well established although at low concentrations (1–4 μM) the inhibition of Ca^{2+} flow through SERCAs may not be complete (Treiman et al., 1998). However, in addition to inhibiting the store-operated Ca^{2+} entry (Mason et al., 1991; Mikoshiba and Hattori, 2000), thapsigargin has been shown to block the L-type Ca^{2+} channels in GH3 pituitary cells (Nelson et al., 1994) and to reduce potassium conductance in rat lens. It is unlikely that thapsigargin's action in slice preparation was due to an effect on Ca^{2+} channels because CPA, a SERCA inhibitor without these confounding actions (Suzuki et al., 1992; Nelson et al., 1994), also blocked synchronous activity. The inhibition of synchronized activity following CPA application probably is mediated solely by inhibition of the SERCA. The specific action of the potent IP3R inhibitor Xestospongin C lends further support for the hypothesis that internal Ca^{2+} release is necessary for the maintenance of synchronized activity, possibly through the activation of IP3Rs. It should be noted that a recent study indicated that Xestospongin C could equally inhibit both IP3Rs and ER Ca^{2+} pumps (De Smet et al., 1999), raising the possibility that Xestospongin C's inhibitory action may be mediated through blocking the ER pumps, as well as via inhibition of IP3Rs. However, the effectiveness of a PLC activator and a PLC inhibitor in induction and prevention, respectively, of the zero-Mg^{2+}-induced synchronized activity, clearly implicates the importance of IP3R pathway in stimulation of Ca^{2+} release from internal stores (Berridge, 1998). This conclusion is further supported by the fact that the drug effects were present without a significant impact on synaptic transmission and neuronal excitability.

The possible contribution of RyRs' should also be considered, since Xestospongin C in the micromolar range has also been shown to have a modest effect on RyRs (Gafni et al., 1997). Indeed, ryanodine blocked the synchronized activity in some neurons, suggesting a role of RyRs in this activity. This partial effect is similar to the partial and cell-type specific inhibitory effect of RyR blockade on intracellular Ca^{2+} waves (Larkum et al., 2003). However, action mechanism of RyRs might be distinct from that of IP3R. For example, activation of RyRs has been shown to affect both excitatory (Emptage et al., 2001; Simkus and Stricker, 2002) and inhibitory (Savic and Sciancalepore, 1998; Llano et al., 2000; Bardo et al., 2002; Galante and Marty, 2003) synaptic release presynaptically (Bouchard et al., 2003), although such a claim remains disputed (Carter et al., 2002). Our results, showing that ryanodine reduced both sEPSP amplitude and frequency, also strongly suggest that ryanodine affects synaptic transmission through actions on both pre- and postsynaptic sites. It is thus possible that the inhibition of synchronized activity by ryanodine is attributable to either a reduction of glutamate release through activation of presynaptic RyRs and/or a reduced responsiveness to released glutamate. Yet, several studies suggest that mobilizing ryanodine-sensitive stores could also occlude the release of Ca^{2+} by IP3Rs (Khodakhah and Armstrong, 1997; Nakamura et al., 2000; Power and Sah, 2002). Therefore, the two-receptor pathways might interact in a complex manner to regulate Ca^{2+}-signaling pathways and modulate synchronized activity (Ehrlich and Bezprozvanny, 1994). In summary, our results are in agreement with a previous study showing that two of the major mechanisms for Ca^{2+} homeostasis, i.e., thapsigargin-sensitive SERCA system and the IP3R/RyR-mediated CICR system, might be altered in epileptogenesis (Pal et al., 2001).

The involvement of intracellular Ca^{2+} release-dependent processes in the generation and perpetuation of synchronized activity is also supported by the fact that the rising phase of each Ca^{2+} spike coincides with a burst of APs (Robinson et al., 1993; Badea et al., 2001; Ikegaya et al., 2004) and is consistent with the Ca^{2+} transient detected by Ca^{2+} imaging in epileptic events (Badea et al., 2001) and in the upstate of medium striatal neurons (Kerr and Plenz, 2004). However, some studies suggest that synchronized activity is due to flow of Ca^{2+} through membrane channels and therefore independent of the CICR. For example, a study in dissociated neurons concluded that the Ca^{2+} oscillation caused by removal of Mg^{2+} was due to influx through NMDARs (Nunez et al., 1996); another in cultured cells indicated that

L-type Ca^{2+} channels were involved (Wang and Gruenstein, 1997). The discrepancies in findings may in part be due to the differential action of drugs in different preparations. In our slice preparation, the efficacy of several drugs that interfere with release of intracellular Ca^{2+} stores in inhibiting the synchronized activities without having significant effects on NMDAR-mediated spontaneous EPSPs indicates that influx of Ca^{2+} through membrane channels, if involved at all, is not necessary for blockade of synchronized activity.

Implications for physiological forms of regenerative activity in cortical networks

Highly interconnected networks of neurons in the brain can generate a wide variety of synchronized activities, including those underlying epileptic seizures (Traub and Wong, 1982; Connors, 1984; McCormick and Contreras, 2001). Indeed, throughout the neocortex, the spiking activity of populations of neurons has been found to exhibit various patterns of synchrony during both spontaneous activity and under sensory stimulation (Gray et al., 1989; Steriade and Contreras, 1998; Tsodyks et al., 2000). The mechanisms of inducing synchronous firing in populations of neurons have not been as yet directly addressed experimentally, although recent studies have provided some insights (Sanchez-Vives and McCormick, 2000; Cossart et al., 2003; Shu et al., 2003). Although loss of afferent input in slice preparation limits the evaluation of the role of cortical circuitry in generation of synchronized activity, slice preparation is still very useful for understanding the synaptic and cellular mechanisms underlying synchronized activity. In general, groups of neurons can become synchronized either via direct synaptic connections within the group or as the result of stimulation by common inputs. Since most of the synapses a cortical cell receives originate from local neurons in the same cortical area (DeFelipe and Farinas, 1992), it is reasonable to assume that much of the observed synchronization is generated locally as a consequence of population dynamics (Salinas and Sejnowski, 2001). It is widely accepted that synchronized epileptiform activity in cortical slices can be induced by low Mg^{2+} medium (Traub and Wong, 1982; Gutkin et al., 2001), as well as by barrages of PSPs, similar to those described in slow oscillation (Sanchez-Vives and McCormick, 2000; Shu et al., 2003). Although the properties of epileptiform activity are significantly different from slow oscillation in several respects, including induction conditions, time-course, and frequency of up-state, there are some important similarities. For example, both are initiated by layer V pyramidal neurons and depend on activation of NMDARs and AMPARs in the recurrent network. Indeed, slow oscillations are very easily converted into epileptic seizure (Sanchez-Vives and McCormick, 2000; McCormick and Contreras, 2001). It is therefore reasonable to assume that similar intracellular signaling pathways including those that involve release of intracellular Ca^{2+} stores may mediate the two processes. Additional experiments are needed to explore in greater depth the degree to which physiologic and pathologic forms of population activity are related at the intracellular level.

Acknowledgments

This paper is dedicated to the memory of Dr. Patricia S. Goldman-Rakic, a great neuroscientist and mentor. We are grateful to Drs. L.D. Selemon, T. Koos, C.D. Paspalas, and N. Kabbani for comments on the manuscript. This work was supported by P50 MH44866, RO1 MH38546, Essel Foundation to PGR, and NARSAD Young Investigator Award to WJG.

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