TRPC3 mediates hyperexcitability and epileptiform activity in immature cortex and experimental cortical dysplasia

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Zhou FW, Roper SN. TRPC3 mediates hyperexcitability and epileptiform activity in immature cortex and experimental cortical dysplasia. J Neurophysiol 111: 1227–1237, 2014. First published December 18, 2013; doi:10.1152/jn.00607.2013.—Neuronal hyperexcitability plays an important role in epileptogenesis. Conditions of low extracellular calcium (Ca) or magnesium (Mg) can induce hyperexcitability and epileptiform activity with unclear mechanisms. Transient receptor potential canonical type 3 (TRPC3) channels play a pivotal role in neuronal excitability and are activated in low-Ca and/or low-Mg conditions to depolarize neurons. TRPC3 staining was highly enriched in immature, but very weak in mature, control cortex, whereas it was strong in dysplastic cortex at all ages. Depolarization and susceptibility to epileptiform activity increased with decreasing Ca and Mg. Combinations of low Ca and low Mg induced larger depolarization in pyramidal neurons and greater susceptibility to epileptiform activity in immature and dysplastic cortex than in mature and control cortex, respectively. Intracellular application of anti-TRPC3 antibody to block TRPC3 channels and bath application of the selective TRPC3 inhibitor Pyr3 greatly diminished depolarization in immature control and both immature and mature dysplastic cortex with strong TRPC3 expression. Epileptiform activity was initiated in low Ca and low Mg when synaptic activity was blocked, and Pyr3 completely suppressed this activity. In conclusion, TRPC3 primarily mediates low Ca- and low Mg-induced depolarization and epileptiform activity, and the enhanced expression of TRPC3 could make dysplastic and immature cortex more hyperexcitable and more susceptible to epileptiform activity.

low calcium; low magnesium; nonsynaptic; epilepsy; epileptogenesis

EXTRACELLULAR CALCIUM AND MAGNESIUM levels are important regulators of neuronal excitability with lower concentrations of both cations increasing excitability. Conditions with extremely low extracellular calcium (Ca; <0.2 mM) or magnesium concentration (Mg; 0 mM) have been used to study seizures and epilepsy in mature and immature animals (Albus et al. 2008; Heinemann et al. 1985, 2006; Yaari et al. 1983), with immature animals being more susceptible to seizures (Heinemann et al. 2006). Seizure activity in the low-Mg model of epilepsy is highly dependent on Ca, and vice versa (Isaev et al. 2012). Low Mg (or Ca)-induced epileptiform activity is accompanied by a decrease of Ca (or Mg) (Avoli et al. 1987; Heinemann et al. 1977; Konnerth et al. 1984; Pumain et al. 1983; Somjen 2002). Although experimental seizures cause a reduction of both extracellular Ca (Heinemann and Louvel 1983; Pumain et al. 1985) and Mg concentrations (Heinemann et al. 1977; Konnerth et al. 1984; Köhr and Heinemann 1988; Pumain et al. 1983; Somjen 2002), they do not reach the extremely low levels seen in the traditional low-Ca (<0.2 mM) or low-Mg (0 mM) models. A more physiologically relevant model involves moderate reductions in both Ca and Mg. In addition, low-Ca and low-Mg models are commonly used to induce epileptiform activity in control tissues, but the responses to low Ca and/or low Mg may be very different in pathological tissue because of altered synaptic activity and intrinsic properties of the neurons. Therefore, the first aim of the present study was to examine the effect of different combinations of Ca (0–2.0 mM) and Mg (0–1.0 mM) on membrane potential (for depolarization) and field potential (for epileptiform activity) in immature and mature control and dysplastic cortex (induced by in utero irradiation), to determine if dysplastic and immature cortex are more susceptible to epileptiform activity than control and mature cortex, respectively, in low-Ca/low-Mg conditions.

The nonselective cation channels, including transient receptor potential canonical type 3 (TRPC3), are very sensitive to divalent cations (including Ca and Mg), and decreasing concentrations of divalent cations activate whereas increasing concentrations inhibit those channels (Xiong et al. 2001; Zhou et al. 2008). TRPC3 channels are the most abundant TRPC channels in brain (Dietrich et al. 2005), and they are predominantly expressed in the developing animal and human brain (Li et al. 1999). TRPC3 channels play a pivotal role in the maintenance of cell excitability and induction of membrane depolarization, especially in developing brain (Birnbaumer 2009; Zhou et al. 2008, 2009b; Zhou 2010). Given that immature cortex has higher TRPC3 expression and is hyperexcitable and more susceptible to seizures in some (but not all, e.g., the pilocarpine-induced rat model of temporal lobe epilepsy) animal models of epilepsy (Cavalheiro et al. 1987; Curia et al. 2008; Heinemann et al. 2006), we hypothesized that higher TRPC3 expression would produce enhanced epileptiform activity in low-Ca/low-Mg conditions in immature brain. In addition, neurons in cortical dysplasia (CD) often demonstrate phenotypes that suggest persistent and abnormal expression of immature features (Cepeda et al. 2006). Therefore, we sought to determine if TRPC3 expression was enhanced in CD and if this would result in hyperexcitability and increased epileptiform activity in low-Ca/low-Mg conditions. The second aim of the present study was to examine the expression of TRPC3 channels and their roles in low Ca/low Mg-induced depolarization and epileptiform activity in immature and mature control and dysplastic cortex, to determine if alterations in TRPC3 expression make fundamental contributions to increased excitability and susceptibility to epileptiform activity in immature and dysplastic cortex.
MATERIALS AND METHODS

Animals and Irradiation

We have studied the rat model of radiation-induced CD to better understand alterations in cortex and hippocampus that may contribute to epileptogenesis (e.g., Roper et al. 1995, 1997) and memory deficits (Zhou et al. 2011; Zhou and Roper 2012). Timed-pregnant Sprague-Dawley rats were either sham-exposed (control) or exposed to 225 cGy of external irradiation on embryonic day 17 (E17). Male offspring of different ages from control and irradiated mothers were subjected to experiments. Rats were maintained on 12:12-h light-dark cycles and were given ad libitum access to food and water. All procedures followed guidelines approved by the Institutional Animal Care and Use Committee at the University of Florida.

Slice Preparation

Coronal brain slices (400 μm) were prepared in an ice-cold cutting solution using a Vibratome. Sections from rats were collected from the rostrocaudal level of the anterior commissure to the level of the hippocampus (Paxinos and Watson 1986), which included somatosensory cortex (Lehoila et al. 2001). Slices were stored in a chamber for at least 1 h at room temperature (~23°C) before being transferred to a recording chamber.

Slice Electrophysiology

Individual slices were perfused with extracellular solution at a constant rate of 3 ml/min. Recordings were conducted using infrared-differential interference contrast video microscopy with a ×40 objective (Eclipse E600-FN; Nikon, NY) at 30°C. Signals were recorded with a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Amplitude control and data acquisition were performed using Clampex 10.1 software via Digidata 1320A (Molecular Devices). Signals were digitized at 10 kHz and analyzed offline. For whole cell recordings, pipettes filled with intracellular solution (see below) had resistances of 4–6 MΩ in the bath. Whole cell recorded neurons were only included in the analysis if the access resistance changed <15% during the experiment. Pipettes for field potential recording were filled with 2 M NaCl and had resistances of 3–4 MΩ.

We made whole cell recordings from pyramidal neurons in layer V of somatosensory cortex from control rats and in the middle region of somatosensory cortex from irradiated rats because of dyslamination. After stable baseline recordings were obtained for 10 min when extracellular Ca concentration was 2 mM and extracellular Mg concentration was 1 mM (2 Ca-1 Mg), different combinations of low Ca and low Mg concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 mM) were bath-applied for 4 min to examine the change of membrane potential. To remove the influence of synaptic activity, 50 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 1 μM picrotoxin were added to the perfusing solution. Because of difficulties in determining precisely the change of membrane potential when neurons spiked, 1 μM tetrodotoxin (TTX) was added to block action potentials.

To detect epileptiform activity, extracellular recordings of field potentials were performed. After stable baseline recordings were obtained, low concentrations of Ca and Mg were bath-applied to induce epileptiform activity. We tested combinations (0, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, and 2 mM) from low to high and found the combinations with relatively higher Ca and Mg concentrations that could induce epileptiform activity; some combinations were not tested because they would not cause epileptiform activity according to the results from tested combinations.

Solutions for Electrophysiology

Cutting solution contained (in mM) 220 sucrose, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2 CaCl2, 1 MgCl2, and 10 d-glucose. Extracellular solution contained (in mM) 124 NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2 CaCl2, 1 MgCl2, and 10 d-glucose. Low-Ca/low-Mg extracellular solution contained (in mM) A NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, B CaCl2, C MgCl2, and 10 d-glucose. The concentration of NaCl (A) was changed accordingly when the concentrations of CaCl2 (B) and MgCl2 (C) were reduced, based on the equation: A = 124 + (3[2 − B] + 3[1 − C])/2 (e.g., the concentrations of NaCl/CaCl2/MgCl2 were 127.3/0.4/0.4 mM). Note that the change of extracellular NaCl concentration from the range of 124.0–128.5 mM did not significantly change the membrane potential. Intracellular solution contained (in mM), for whole cell recordings, 125 K-gluconate, 0.1 CaCl2, 2 MgCl2, 10 HEPES, 2 MgATP, 0.3 Na, 1 GTP, 10 EGTA, and 4 Na3-phosphocreatine. The pH value was adjusted with KOH for intracellular solution for whole cell recording (pH = 7.25) and with NaOH for extracellular solutions (pH = 7.4 under the condition of bubbling with 95% O2-5% CO2). The osmolality was maintained at 280–290 mosM for intracellular solutions for whole cell recording, 300–305 mosM for extracellular solutions, and 350–360 mosM for cutting solutions.

Blockade TRPC3 Channels

Intracellular application of anti-TRPC3 antibody, an antibody against the intracellular carboxyl-terminal domain of TRPC3 channels, has been demonstrated to completely inhibit TRPC3 channels (Albert et al. 2006; Amaral and Pozzo-Miller 2007; Zhou et al. 2008, 2009b). When it is intracellularly applied, the anti-TRPC3 antibody gradually diffuses into the recorded cells to block TRPC3 channels (Albert et al. 2006). To block TRPC3 channels of whole cell recorded pyramidal neurons, intracellular solution contained 8 μg/ml anti-TRPC3 antibody. To further test antibody specificity, intracellular solution contained a mixture of 8 μg/ml anti-TRPC3 antibody and 8 μg/ml control peptide antigen; the latter was used to neutralize the antibody.

We determined whether Pyr3, a newly synthesized selective TRPC3 inhibitor (Kiyonaka et al. 2009; Shirakawa et al. 2010), could abolish low Ca/low Mg-induced depolarization and epileptiform activity. Pyr3 was applied 5 min before or during perfusion of low-Ca and low-Mg solutions.

Immunohistochemistry

Immunohistochemistry is often used to detect and localize a specific antigen to specific cell types and to compare the protein expression levels (Klein et al. 2001; Matos et al. 2006). Immunofluorescence staining was performed as previously described (Zhou et al. 2009a, 2009b). Coronal cortical sections (45 μm) were incubated with rabbit anti-TRPC3 polyclonal antibody (2 μg/ml; Alomone Labs, Jerusalem, Israel) at 4°C for 72 h and with Alexa Fluor 488 goat anti-rabbit IgG (1:500; Molecular Probes, Invitrogen) for 2.5 h at ~23°C. For double staining of TRPC3 and biocytin in slices with biocytin-injected neurons, slices were incubated with anti-TRPC3 antibody 4°C for 72 h and then incubated with secondary antibodies for TRPC3 and biocytin (Alexa Fluor 594-conjugated streptavidin, 1:500; Molecular Probes, Invitrogen) for 2.5 h. After staining, slices were mounted on glass slides and coverslipped with fluoromount aqueous mounting medium (Sigma), and the edges were sealed with nail polish. Sections were examined with an Olympus IX81-DSU Spinning Disk Confocal Microscope (Olympus America, Melville, NY). The same staining procedures and image processing and analysis were used for sections from control and dysplastic cortex.

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Depolarization was induced by different combinations of low Ca and low Mg in a reversible fashion in pyramidal neurons from mature (postnatal day 29–31, P29–31) control and irradiated rats. In both control and irradiated rats, the depolarization increased with decreasing Ca and Mg (Fig. 1). Larger decreases of depolarization were observed in irradiated rats with increasing Ca and Mg; e.g., there was ~20-mV decrease in irradiated rats and only ~10-mV decrease in the control when conditions changed from 0 Ca-0 Mg (30.58 ± 2.64 mV in CD and 11.58 ± 1.18 mV in control) to 0 Ca-1.0 Mg (10.31 ± 1.14 mV in CD and 3.24 ± 0.42 mV in control; Fig. 1C). The magnitude of depolarization was significantly larger in irradiated rats than in the controls for all tested combinations of Ca and Mg except 2.0 Ca-0.8 Mg and 0.8 Ca-1.0 Mg (ANOVA, P < 0.01). The magnitude of depolarization was significantly larger in irradiated rats than in the controls when both Ca and Mg were 2.0 and 1.0 mM, respectively. (Fig. 1A). The magnitude of depolarization was significantly larger in irradiated rats than in the controls at 0.4 Ca-0.4 Mg (the condition that was used to induce epileptiform activity in irradiated rats in later experiments).

Low Ca/Low Mg Induces Larger Depolarization in Younger and Irradiated Rats

We observed the depolarization induced by 0.4 Ca-0.4 Mg at four ages: P8–10, P15–17, P22–24, and P29–31. In control rats, the depolarization decreased with increasing age; e.g., it was significantly smaller in the mature (P29–31, n = 7) than in the immature rats (P8–10, n = 8, P < 0.01). In irradiated rats, in contrast, the largest depolarization was observed in P15–17 rats; after P8–10, the depolarization tended to decrease with increasing ages; however, there was no significant difference among the four ages (Table 1). We compared the depolarization between the two groups of different ages. Depolarization was larger in the irradiated rats than in controls at P15–17, P22–24, and P29–31 (P < 0.05 or 0.01), respectively; however, it was not different between the two groups at P8–10 (P > 0.05). In immature (P8–10) rats, the depolarization was significantly larger in irradiated rats than in the controls when both Ca and Mg were 0 mM (control: 31.15 ± 1.94 mV, n = 7; CD: 38.49 ± 2.38 mV, n = 7, P < 0.05), whereas it was not different at other combinations. These results showed that membrane potential is very sensitive to the concentration of extracellular Ca and Mg, and responds differentially between control and −67.4 ± 1.1 mV (n = 37) in the dysplastic cortex, and they were not significantly different (P > 0.05).

### Table 1. Depolarization in cells at different ages

<table>
<thead>
<tr>
<th>Age, days</th>
<th>Control</th>
<th>CD</th>
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<tr>
<td>8–10</td>
<td>9.71 ± 0.82</td>
<td>10.65 ± 0.91</td>
</tr>
<tr>
<td>15–17</td>
<td>8.62 ± 0.85</td>
<td>11.83 ± 0.96</td>
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<tr>
<td>22–24</td>
<td>6.59 ± 0.72</td>
<td>10.41 ± 0.93</td>
</tr>
<tr>
<td>29–31</td>
<td>2.79 ± 0.31</td>
<td>9.57 ± 1.01</td>
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Values are means ± SE; n = 5–9 cells for each age. Depolarization (mV) was induced by the combination of 0.4 mM Ca and 0.4 mM Mg in pyramidal cells at different ages (days). CD, cortical dysplasia. *P < 0.05 and **P < 0.01 vs. control. °P < 0.05 and *P < 0.01 vs. control (8–10 days). †P < 0.01 vs. control (15–17 days). ‡P < 0.01 vs. control (22–24 days).
The depolarization induced by 0.4 Ca-0.4 Mg was 9.57 ± 1.01 mV (n = 7) in pyramidal neurons in mature (P29–31) rat dysplastic cortex (Fig. 3, A and E). Intracellular application of anti-TRPC3 antibody to block TRPC3 channels greatly diminished 0.4 Ca-0.4 Mg-induced depolarization (1.75 ± 0.18 mV after application of anti-TRPC3 antibody, n = 6, Fig. 3, B and E); in contrast, the depolarization was not influenced when the intracellular solution contained both anti-TRPC3 antibody and peptide antigen (n = 4, Fig. 3, C and E), suggesting that the effect of anti-TRPC3 antibody was specific. Bath application of 10 μM Pyr3, a newly synthesized selective TRPC3 inhibitor (Kiyonaka et al. 2009; Shirakawa et al. 2010), also greatly diminished the depolarization (1.83 ± 0.21 mV after Pyr3, n = 6, Fig. 3, D and E); its blocking effect was similar to that of intracellular application of anti-TRPC3 antibody. Four other concentrations (1, 3, 5, and 20 μM) of Pyr3 were tested, and we found that the TRPC3 blocking effect was dose dependent (1–10 μM) and that its effects at 10 and 20 μM were similar and maximal (n = 3–6; Fig. 4). These results suggest that anti-TRPC3 antibody and Pyr3 completely block TRPC3 channels and that low Ca/low Mg-induced depolarization in dysplastic cortex is primarily mediated by TRPC3 channels. The small, residual depolarization after TRPC3 channels are blocked may be mediated by other channels such as other nonselective cation ion channels or L-type Ca2+ channels (Pohl et al. 1992; Somjen et al. 2009).

In control mature cortex, the depolarization induced by 0.4 Ca-0.4 Mg was smaller that in dysplastic cortex (2.79 ± 0.31 mV in control, n = 7). After intracellular application of anti-TRPC3 antibody and bath application of 10 μM Pyr3, 0.4 Ca-0.4 Mg induced depolarization of 1.86 ± 0.20 mV (n = 5) and 1.89 ± 0.23 mV (n = 5), respectively. In the immature control and dysplastic cortex, both TRPC3 antibody and Pyr3 greatly blocked 0.4 Ca-0.4 Mg-induced depolarization (not
shown). Those results indicated that residual depolarization in control was not different from the dysplastic cortex and that TRPC3-mediated depolarization is very small (1 mV) in mature control cortex.

TRPC3 Channels are Expressed in Pyramidal Neurons

TRPC3 staining was observed in all examined biocytin-injected pyramidal neurons (n = 7, Fig. 5A), but not in interneurons (n = 4, Fig. 5B), in irradiated rats at P29–31. It was weak in all examined biocytin-injected pyramidal neurons (n = 5) and was not observed in interneurons (n = 3) in control rats at P29–31. This selective expression in excitatory pyramidal neurons would have major implications for the effects of TRPC3 on the balance of excitation and inhibition in low-Ca/low-Mg conditions.

Combinations of Low Ca and Low Mg Induce Epileptiform Activity

Hyperexcitability plays a central role in the generation of epileptiform activity (Bikson et al. 1999; Hablitz 2004), and an aggregate of hyperexcitable neurons could induce epileptiform activity (Ahmed and Spencer 2004). As described above, increased depolarization was observed when Ca and Mg concentrations were decreasing, and larger depolarization was observed in irradiated rats than in controls (Fig. 1). This would result in an increased susceptibility for epileptiform activity in immature and dysplastic cortex. We determined whether different combinations of low Ca and low Mg could induce epileptiform activity when synaptic activity was blocked, unless stated otherwise.

Epileptiform activity began to appear within 5–12 min and was stable within 10–20 min after perfusion of low Ca and low Mg. The onset of epileptiform activity was variable because of different treatments (sham-irradiation or irradiation), ages, and concentrations of Ca and Mg. Usually onset took a shorter time in immature and dysplastic cortex and at lower concentrations of Ca and Mg. In cases in which epileptiform activity did not occur, the duration of perfusion was limited to 30 min. In mature (P29–31) rats, epileptiform activity was induced in dysplastic cortex when both Ca and Mg concentrations were lower than 0.5 mM, e.g., 0.4 Ca and 0.4 Mg, and at other combinations when one was higher and the other lower than 0.5 mM, e.g., 0.2 Ca and 0.8 Mg (Fig. 6B and 7). In contrast, it was induced in mature control cortex when both Ca and Mg concentrations were much lower than those used in dysplastic cortex, e.g., 0.2 Ca and 0.2 Mg.

Fig. 4. Effect of Pyr3 blockade of TRPC3 on 0.4 Ca-0.4 Mg-induced depolarization in mature dysplastic cortex. A: representative traces of different concentrations of Pyr3-induced TRPC3 blocking effect. B: pooled data show the Pyr3-induced blocking effect in a dose-dependent manner (1–10 μM), but the effects at 10 and 20 μM were similar. Note that Pyr3 at high concentrations (e.g., 10 or 20 μM) did not completely block 0.4 Ca-0.4 Mg-induced depolarization, suggesting that other channels could be involved. Pyr3 at lower concentrations (e.g., 1 μM) could not block all TRPC3. Values are means ± SE; n = 3 for 20 μM and 5–6 for others.

Fig. 5. TRPC3 staining is present in a representative recorded pyramidal neuron but not in an interneuron. Photomicrographs show that an electrophysiologically identified regular spiking pyramidal neuron (biocytin = red) from mature dysplastic cortex was stained with anti-TRPC3 (green, A), but the fast-spiking interneuron was not stained with anti-TRPC3 (B).

Fig. 6. Epileptiform activity in low-Ca/low-Mg conditions. Epileptiform activity was observed in dysplastic cortex (B) but not control cortex (A) when both Ca and Mg were 0.4 mM. The epileptiform activity was blocked by bath application of 10 μM Pyr3 5 min before 0.4 Ca-0.4 Mg perfusion (C).
0.2 Mg (Fig. 7). The combination 0.4 Ca-0.4 Mg did not induce any epileptiform activity in mature control cortex (Fig. 6A; P < 0.01, 2-tailed χ² test for comparison of control and dysplastic cortex). The amplitude (Fig. 7A) and frequency (Fig. 7B) of epileptiform activity increased with decreased Ca and Mg in the two groups. They were larger in dysplastic cortex than in the controls (all P < 0.01).

In immature rats (P8–10), some combinations, e.g., 0.5 Ca-0.5 Mg in control (n = 6) and 0.8 Ca-0.5 Mg in dysplastic cortex (n = 8), induced epileptiform activity (not shown), but those combinations did not induce any epileptiform activity in mature (P29–31) control (0.5 Ca-0.5 Mg) and dysplastic cortex (0.8 Ca-0.5 Mg) (Fig. 7). Epileptiform activity was not induced in control cortex from mature (Fig. 7) and immature rats (P8–10) in 0.2 Ca-1 Mg and 2 Ca-0 Mg conditions that are commonly used as models of seizures and epilepsy. These results are consistent with previous studies in normal rats (Heinemann et al. 2006).

Some combinations of low Ca and low Mg were tested when synaptic activity was not blocked. Under these conditions, we found that relatively higher concentrations of Ca and Mg could induce epileptiform activity, e.g., 0.4 Ca-0.4 Mg in mature control cortex and 0.6 Ca-0.6 Mg in mature dysplastic cortex.

These results suggest that dysplastic and immature cortex is more hyperexcitable and more susceptible to epileptiform activity than the controls and the mature specimens, respectively, in low-Ca/low-Mg conditions.

**Blocking TRPC3 Suppresses Low Ca/Low Mg-Induced Epileptiform Activity**

Field recordings showed that 0.4 Ca-0.4 Mg induced epileptiform activity in mature (P29–31) dysplastic cortex when synaptic activity was blocked (Fig. 6B), and it was completely suppressed 10–15 min after the addition of 10 μM Pyr3 (Fig. 6C). We did not observe any epileptiform activity when 10 μM Pyr3 was applied 5 min before 0.4 Ca-0.4 Mg perfusion in mature dysplastic cortex. Application of 10 μM Pyr3 also blocked 0.2 Ca-0.2 Mg-induced epileptiform activity in mature control without synaptic inputs.

Interestingly, when synaptic activity was intact, 10 μM Pyr3 abolished epileptiform activity in mature control and dysplastic cortex (all n = 3) in all low-Ca/low-Mg conditions (except 2 Ca-0 Mg in dysplastic cortex; Fig. 7) that were shown to induce epileptiform activity with intact TRPC3 channels. Pyr3 (10 μM) also abolished epileptiform activity in the 0.4 Ca-0.4 Mg condition in immature control and dysplastic cortex. Note that 10 μM Pyr3 did not show any effects on spontaneous inhibitory (sIPSCs) and excitatory postsynaptic currents (sEPSCs) in pyramidal cells from dysplastic cortex of P29–31 rats in the 0.4 Ca-0.4 Mg condition (baseline amplitude and frequency of sIPSCs: 18.9 ± 2.1 pA and 3.71 ± 0.42 Hz, n = 5; Pyr3: 19.4 ± 2.5 pA and 3.64 ± 0.51 Hz, n = 5; baseline sEPSCs: 35.6 ± 3.8 pA and 6.23 ± 0.57 Hz, n = 6; Pyr3: 37.2 ± 3.9 pA and 5.95 ± 0.52 Hz, n = 6). This suggests that TRPC3-mediated depolarization (a nonsynaptic mechanism) plays a major role in excitability in all conditions of low Ca/low Mg except 2 Ca-0 Mg, where synaptic activity is maximally enhanced by reducing the Mg²⁺ block of NMDA channels and synaptic mechanisms presumably play a major role. These data suggest that TRPC3-mediated depolarization would play a major role, but synaptic activity would play a smaller role, in the generation of epileptiform activity in the low-Ca/low-Mg conditions that are commonly observed in epilepsy patients and animal models. It may be important note that although the TRPC3 expression level is much lower in mature control than in mature dysplastic cortex, TRPC3-mediated depolarization in mature control cortex may play a role in epileptiform activity when Ca and Mg concentrations are very low (e.g., 0.2 Ca-0.2 Mg; Fig. 7), as evidenced by Pyr3-blocked epileptiform activity. However, we cannot completely exclude the role of Pyr3 on other channels activated by low Ca/low Mg. For instance, the TRPC3 blocking effect on epileptiform activity could be achieved, in part, by a potent reduction of NMDA receptor-mediated excitation when n-AP5 is not used.

Fig. 7. Intensity of epileptiform activity is increased in dysplastic cortex. A: amplitude of epileptiform activity. B: frequency of epileptiform activity. Epileptiform activity was induced by different combinations of low Ca and low Mg in cortex from mature (P29–31) rats. Both amplitude and frequency of epileptiform activity in CD were larger than in controls (all P < 0.01). Twelve of 49 combinations of low Ca and low Mg in controls and 26 of 49 combinations in CD induced epileptiform activity (n = 5–7 for each combination); the ratios were significantly different (P < 0.01, 2-tailed χ² test).
DISCUSSION

Dysplastic and Immature Cortex Are More Hyperexcitable and More Susceptible to Epileptiform Activity in Low-Ca/Low-Mg Conditions

Ca and Mg are very important for neuronal excitability, and the precise adjustment of the levels is crucial for the execution of neuronal functions. Low Ca and low Mg can induce seizures. Low Ca and low Mg are concomitantly observed in experimental models of epilepsy and in patients during epileptic seizures. Experimentally induced seizures can reduce Ca concentration to as low as 0.6 mM in pentetrazol-treated cats (Heinemann and Louvèl 1983) and to a maximal observed value of 0.1 mM in allylglycine-treated photosensitive baboon (Pumain et al. 1985). The decreased Ca concentration is accompanied by a decreased Mg concentration (Avoli et al. 1987; Heinemann et al. 1977; Konnerth et al. 1984; Pumain et al. 1983; Somjen 2002), and the Mg concentration can be as low as 0.5 mM (Köhr and Heinemann 1988). Ca and/or Mg deficits increase seizure susceptibility to proconvulsant stimuli in rats (Greenberg and Tufts 1934) and cause seizures in humans (Nuytten et al. 1991; Thiel 2006). Low Ca/low Mg concentrations ranging from 0.1 to 0.5 mM can be observed in other physiological and pathological conditions such as activation of both NMDA subtype and voltage-dependent Ca$^{2+}$ channels (Heinemann et al. 1977, 1990), iontophoretic applications of excitatory amino acids (Heinemann and Pumain 1980; Heinemann et al. 1985), repetitive electrical stimulation (Heinemann and Louvèl 1983), and spreading depression and ischemia (Ekholt et al. 1995; Hablitz and Heinemann 1994; Hansen and Zeuthen 1981).

Both low-Ca (<0.2 mM) and low-Mg (0 mM) models of seizures can mimic the extremes reached during experimentally induced seizures in vitro and in vivo (Albus et al. 2008; Heinemann et al. 1985; Yaari et al. 1983). Low Ca can induce epileptiform activity in normal cortical (Heinemann et al. 1977, 2006) and hippocampal slices (Heinemann et al. 2006; Konnerth et al. 1986; Jefferys and Haas 1982; Yaari et al. 1983) and in vivo after treatment with Ca$^{2+}$ chelating agents (Feng and Durand 2003). Similar epileptiform activity induced by omitting Mg has been reported in normal cortical and hippocampal slices (Dreier and Heinemann 1991; Stanton et al. 1987), in slices of human epileptogenic neocortex (Avoli et al. 1987, 1991), and in experimental CD in the perinatal freeze lesion model (Redecker et al. 2005). Those models have commonly used low Ca or low Mg and normal concentrations of other ions, but not the combinations of low Ca and low Mg.

The success of low Ca (or Mg) models of epilepsy is highly dependent on Mg or Ca (Iisaev et al. 2012). First, Ca and Mg act antagonistically at all chemically mediated synapses (Somjen 2002). Synaptic activity is enhanced when Ca is raised and is depressed when Ca is reduced (Balestrino et al. 1986); activity is depressed when Mg is raised and is enhanced when Mg is lowered (Rausche et al. 1990). In the traditional low-Ca model, a nonsynaptic epilepsy model, synaptic activity is completely blocked at a Ca concentration of <0.3 mM when Mg is within normal ranges (Jones and Heinemann 1987) or higher (Jefferys and Haas 1982). In the low-Mg model, synaptic activity is greatly enhanced at 0 mM Mg when Ca is within normal ranges. The net effect of combinations of low Ca and low Mg on synaptic activity would depend on the interplay between low Ca-induced inhibition and low Mg-induced facilitation on synaptic activity.

CD is a major cause of intractable epilepsy in humans. In utero irradiated rats are used as a model of CD (Roper 1998) and most closely resemble type IB CD in humans (Blümcke et al. 2011). Our previous histological studies have demonstrated the loss of both interneurons (Deukmedjian et al. 2004; Roper et al. 1999; Zhou and Roper 2010, 2011) and GABAergic presynaptic terminals (Zhou and Roper 2010) and an increase of the density of glutamatergic terminals in dysplastic cortex (Zhou and Roper 2010). Electrophysiological studies have further confirmed an imbalance between excitatory and inhibitory synaptic inputs to pyramidal neurons (Chen and Roper 2003; Zhu and Roper 2000) and to GABAergic interneurons in irradiated rats with CD (Xiang et al. 2006; Zhou et al. 2009a; Zhou and Roper 2011). The imbalance favors inhibition to result in a decreased excitability in interneurons (Zhou and Roper 2011); in contrast, it favors excitation to result in an increased excitability in pyramidal neurons (Zhu and Roper 2000). We have performed these electrophysiological studies under conditions where Ca concentration is 2 mM and Mg concentration is 1 mM; the present study showed that spontaneous epileptiform activity was not observed in this condition.

In the present study, synaptic activity was pharmacologically blocked in most experiments when we examined the effect of low Ca/low Mg on depolarization and epileptiform activity to investigate nonsynaptic mechanisms. In these conditions, depolarization of pyramidal cells and susceptibility to epileptiform activity increased with decreasing Ca and Mg, whereas they decreased with increasing Ca and Mg.

Based on the above findings, moderate reductions in Ca and Mg can be seen acutely during seizures and epileptiform activity and chronically in some types of epileptogenic lesions. Therefore, a more physiologically relevant model involves moderate reductions in both Ca and Mg in both control and pathological tissues. We examined the effect of different combinations of Ca (0–2.0 mM) and Mg (0–1 mM) on membrane depolarization and epileptiform activity in the absence of synaptic activity. In mature control rat cortex, we tested low Ca (<0.2 mM) or low Mg (0 mM), widely used as in vitro epilepsy models, and we did not observe any epileptiform activity. For the low-Mg condition, the blockade of synaptic activity probably explains the failure of initiation of epileptiform activity in our study. For the low-Ca condition, the depolarization might be too small to initiate epileptiform activity (~3 mV when Ca was 0 mM and Mg was 1 mM; see Fig. 1C). Other explanations are that the cortex has lower cell packing density than hippocampus, where those models work very well, and that the additional conditions used in previous studies (Heinemann et al. 1977, 2006), such as hyposmolar solutions that reduce extracellular space and high concentrations of potassium that increase cell excitability, might be required to initiate epileptiform activity. In mature control cortex, some combinations, such as 0.2 Ca-0.4 Mg and 0.4 Ca-0.2 Mg, could initiate epileptiform activity when synaptic activity was blocked, and higher concentrations (e.g., 0.4 Ca-0.4 Mg) initiated epileptiform activity when synaptic activity was intact. Those combinations were within or close to physiologically relevant ranges of Ca and Mg concentration in experimental models of epilepsy and in patients.
Mechanisms of Hyperexcitability in Low-Ca and Low-Mg Conditions

In the immature control cortex, larger depolarization and greater susceptibility to epileptiform activity were observed compared with mature control cortex. In mature irradiated rats, we demonstrated that the dysplastic cortex was more hyperexcitable and more susceptible to epileptiform activity than mature control cortex in low-Ca/low-Mg conditions. For instance, in dysplastic cortex, higher Ca and Mg concentrations (e.g., 0.4 Ca-0.6 Mg) induced epileptiform activity with a blockade of synaptic activity, and even higher concentrations (0.6 Ca and 0.6 Mg) initiated epileptiform activity in dysplastic cortex when synaptic activity was not blocked. Those combinations tested in dysplastic cortex fell within the range of Ca and Mg levels that have been demonstrated in experimental models of epilepsy and in patients. Previous studies have shown no difference in neuronal density in irradiated rat (Deukmedjian et al. 2004; Roper et al. 1999); therefore, alterations of intrinsic membrane properties would most likely contribute to the differences between the two groups when synaptic activity is blocked, as discussed below.

Extremely low Ca or low Mg can induce pronounced membrane depolarization with superimposed prolonged bursts of action potentials (Heinemann et al. 2006; Hille 2001; Shuai et al. 2003; Xiong et al. 1997; Zhou et al. 2008), and aggregates of hyperexcitable neurons can initiate epileptiform activity in mature and immature hippocampus and cortex (Feng and Durand 2003; Heinemann et al. 1977, 2006; Jefferys and Haas 1982; Konnerth et al. 1986; Yaari et al. 1983). Mechanisms underlying low Ca or low Mg-induced hyperexcitability and epileptiform activity are unclear. Previous studies have suggested that low Ca and low Mg reduce surface-charge screening, leading to a hyperpolarizing shift of half-activation of \( I_{Na} \) that results in a reduced firing threshold (~5 mV) and increases excitability (Hille 2001; Isaev et al. 2012; Mody et al. 1987). Other mechanisms may be involved, such as low Mg-induced facilitation of the activation of NMDA receptors (Avoli et al. 1991; Köhr and Heinemann 1988; Stanton et al. 1987) and voltage-dependent Ca\(^{2+}\) channels. Mg\(^{2+}\) is less effective than Ca\(^{2+}\) in surface-charge screening (Jefferys and Haas 1982) and more effective in the activation of NMDA receptors and Ca\(^{2+}\) channels (Köhr and Heinemann 1988). Those channels only contribute a small component of low Ca/low Mg-induced depolarization and inward current in cardiac myocytes (Bosteels et al. 1999; Mubagwa et al. 1997), where TRPC3 is highly expressed (Clapham 2003; Venkatachalam and Montell 2007). The roles of those channels in low Ca/low Mg-induced epileptiform activity have been studied by others; e.g., activation of L-type Ca\(^{2+}\) channels may contribute to low Mg-induced epileptiform activity in hippocampus (Polh et al. 1992), but it does not mediate low Ca-induced epileptiform activity (Bikson et al. 1999). Other studies have demonstrated that low Ca can excite hippocampal pyramidal neurons through activation of novel and unidentified nonselective cation channels that are not blocked by specific inhibitors of known voltage-gated or ligand-gated ion channels (Chu et al. 2003; Xiong et al. 1997; Xiong and MacDonald 1999). A computer modeling study showed that activation of nonselective cation channels in low Ca is sufficient to generate epileptiform activity (Somjen et al. 2009). Another study in cultured pyramidal neurons revealed that activation of those channels could induce depolarization in low Ca and low Mg, suggesting that those channels could constitute a major component of enhanced neuronal excitability during epileptic seizures (Xiong et al. 2001). In the present study, we identified TRPC3 as the nonselective cation channel that mediated low Ca/low Mg-induced depolarization and epileptiform activity in immature and mature control and dysplastic cortex.

Increased Expression of TRPC3 Channels Underlie Increased Depolarization and Epileptiform Activity in Immature and Dysplastic Cortex in Low-Ca/Low-Mg Conditions

TRPC3 channel proteins are predominantly expressed in developing brain. TRPC3 channels are the most abundant TRPC channels in brain (Dietrich et al. 2005). TRPC3 mRNA is enriched in the immature and mature human (Riccio et al. 2002; Zhu et al. 1996) and animal brain (Mori et al. 1998); however, TRPC3 channel proteins, in contrast, are low in adult human (44 year old) and adult animal brain (Li et al. 1999; Strübing et al. 2003). TRPC3 channel proteins are predominantly expressed in the developing rat and mouse cortex and other brain regions from around E15 to P20, a critical period in the development of brain function (Li et al. 1999), and are highly expressed in the 19-wk-old human fetus (Li et al. 1999).

Immature animals and humans are more susceptible to seizures compared with adults in many animal models of epilepsy (Ben-Ari and Holmes 2006; Heinemann et al. 2006). However, in the pilocarpine-induced rat model of epilepsy, the susceptibility increases with age and maturity of brain neural circuits important for generation and propagation of seizure activity (Cavalheiro et al. 1987; Curia et al. 2008). Epileptiform activity is often more prevalent and more robust in in vitro studies from immature human and animal hippocampus and neocortex compared with mature specimens (Heinemann et al. 2006; Patrylo et al. 1996; Roper et al. 1993; Tasker et al. 1992). In addition, a number of studies have demonstrated the preservation of immature properties in animal and human CD and have proposed these features as contributing to the epileptic phenotype (Cepeda et al. 2007; DeFazio and Hablitz 2000). Mechanisms underlying the increased susceptibility to seizure generation are unclear. Does the high TRPC3 expression in normal immature animals and humans contribute to the increased susceptibility observed in this study? Is TRPC3 channel expression high in dysplastic cortex as in immature cortex? We found that the answers to both questions are “yes.” Our present study showed that TRPC3 staining was highly enriched in both immature control and mature (as well as immature) dysplastic cortex but very weak in mature control cortex. Our finding that TRPC3 channels are expressed in a narrow age window in control rats is consistent with a previous study (Li et al. 1999). High TRPC3 expression in mature irradiated rats may be due to an arrest of the developmental pattern of TRPC3. Arrested development has also been used to explain the seizure phenotype (Cepeda et al. 2007; DeFazio and Hablitz 2000).

Immunohistochemistry is often used to compare protein expression levels between experimental groups (Klein et al. 2001; Matos et al. 2006). However, it is not as sensitive to minor differences in protein expression as Western blots. In this study, the differences in TRPC3 expression were so
different between immature and mature controls and between dysplastic and control mature cortex that Western blot techniques did not seem necessary. In addition, immunohistochemistry allowed us to determine the identity of the cells that expressed TRPC3 (i.e., pyramidal neurons). However, it is possible that Western blotting could show subtle differences between age groups, etc., that were beneath our levels of detection with immunohistochemistry alone.

**Low Ca and low Mg activate TRPC3 channels.** Normal baseline Ca and Mg levels are 1.2–1.5 mM in cortex and hippocampus (Heinemann et al. 1977; Heinemann and Pumain 1980; Somjen 2002). Ca and Mg at 1–2 mM is often used in in vitro slice electrophysiological experiments. Previous studies have demonstrated that when Ca and Mg levels are within normal ranges, the activities of unidentified nonselective cation channels are greatly inhibited (Somjen et al. 2009; Xiong et al. 1997), and only a relatively small tonic current is permitted to flow through them to add to the resting conductance of the cell (Somjen et al. 2009). Those channels are activated to different extents when Ca and/or Mg are low (Albert et al. 2006; Thorneloe and Nelson 2004; Xiong et al. 1997, 2001; Zhou et al. 2008) and are maximally activated when both are maximally low, e.g., 0 mM (Xiong et al. 1997). The activation induced by reducing Ca (or Mg) alone can be counterbalanced by normal or higher Mg (or Ca) (Xiong et al. 1997), suggesting that the combination of low Ca and low Mg could be an optimal condition to induce depolarization and epileptiform activity due to the activation of TRPC3 channel proteins. When Ca and Mg are at normal levels, the contribution of TRPC3 to membrane potential and excitability should be negligible, even though it is highly expressed in immature and dysplastic cortex. This is supported by current and previous (Zhu and Roper 2000) findings that baseline membrane potentials at 2 mM Ca and 1 mM Mg were similar (~70 mV) in pyramidal neurons in both control and dysplastic cortex. In low-Ca/low-Mg conditions, TRPC3 channels are activated, and their role in low Ca and Mg-induced depolarization and susceptibility to epileptiform activity would be different between treatment groups (control and dysplastic cortex) and ages (immature and mature) because of differential expression of TRPC3 channels. We found that low Ca/low Mg induced larger depolarization and epileptiform activity in immature and dysplastic cortex because of higher TRPC3 expression.

**Possible role of TRPC3 in low Ca/low Mg-induced depolarization and epileptiform activity.** We propose that increased TRPC3 expression may have an important role in the transition from the interictal to the ictal state. The low Ca/low Mg observed in epileptic and other physiological or pathological conditions would activate TRPC3 channels. This activation would in turn cause membrane depolarization that could facilitate the activation of NMDA receptors, voltage-gated Ca^2+ channels, and persistent TTX-sensitive Na^+ channels, leading to additional decreases in Ca and Mg and greater activation of TRPC3 channels. These voltage-independent, TRPC3-mediated depolarizing currents induced by low Ca and Mg could constantly depolarize these neurons and drive these neurons toward their action potential threshold as demonstrated in substantia nigra pars reticulata GABAergic neurons (Zhou et al. 2008) when other voltage-dependent depolarizing ion channels (e.g., the persistent TTX-sensitive Na^+ channels) are not active at more hyperpolarized membrane potentials (less than ~50 mV) (Atherton and Bevan 2005). An aggregate of neurons with pronounced membrane depolarization, observed in the present study and others (Heinemann et al. 2006; Hille 2001; Shuai et al. 2003; Xiong et al. 1997; Zhou et al. 2008) could initiate epileptiform activity (Feng and Durand 2003; Heinemann et al. 1977; Jefferys and Haas 1982; Konnerth et al. 1986; Yaari et al. 1983). The enhanced depolarization is accompanied by an increased intracellular concentration of Ca^2+ and Na^+, which in turn activates Ca^2+- and Na^+- activated K^+ currents that cause hyperpolarization to counteract depolarization to terminate seizures (Timofeev et al. 2004). Our present study revealed that once the TRPC3 expression level is high enough (such as in immature and dysplastic cortex) and both Ca and Mg levels are low enough (such as 0.4 Ca-0.4 Mg in dysplastic cortex) to activate TRPC3, an aggregate of neurons will depolarize profoundly and initiate epileptiform activity. Conversely, blocking TRPC3 greatly diminished low Ca/low Mg-induced depolarization and completely abolished epileptiform activity.

In conclusion, we have demonstrated that TRPC3 primarily mediates low Ca and low Mg-induced depolarization and epileptiform activity and that enhanced expression of TRPC3 makes immature and dysplastic cortex more hyperexcitable and more susceptible to epileptiform activity. The combination of low Ca and low Mg is a physiologically relevant condition that may lead to unique insights into factors that underlie the transition to the ictal state. Findings from this study have added to our understanding of mechanisms of hyperexcitability and epileptogenesis in immature and dysplastic cortex and may provide avenues for novel new therapies in the future.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

F.-W.Z. and S.N.R. conception and design of research; F.-W.Z. performed experiments; F.-W.Z. analyzed data; F.-W.Z. and S.N.R. interpreted results of experiments; F.-W.Z. prepared figures; F.-W.Z. and S.N.R. drafted manuscript; F.-W.Z. and S.N.R. approved final version of manuscript.

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