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ORIGINAL ARTICLE

Native STIM2 and ORAI1 proteins form a calcium-sensitive and thapsigargin-insensitive complex in cortical neurons

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Abstract

In non-excitatory cells, stromal interaction molecule 1 (STIM1) and STIM2 mediate store-operated calcium entry *via* an interaction with ORAI1 calcium channels. However, in neurons, STIM2 over-expression appears to play a role in calcium homeostasis that is different from STIM1 over-expression. The aim of this study was to establish the role and localization of native STIM2 in the neuronal cell. Co-immunoprecipitation experiments revealed that the interaction between endogenous STIM2 and ORAI1 was greater in a low-calcium medium than in a high-calcium medium. Using a Proximity Ligation Assay (PLA), the number of apparent complexes of endogenous STIM2 with ORAI1 was quantified. No change in the number of PLA signals was observed in the presence of thapsigargin, which depletes calcium from the endoplasmic reticulum (ER). However, the number of apparent STIM2-ORAI1 complexes increased when intracellular and subsequently ER calcium concentrations were decreased by BAPTA-AM or a low-calcium medium. Both Fura-2 acetoxymethyl ester calcium imaging and PLA in the same neuronal cell indicated that the calcium responses correlated strongly with the number of endogenous STIM2-ORAI1 complexes. The small drop in calcium levels in the ER caused by decreased intracellular calcium levels appeared to initiate the calcium-sensitive and thapsigargin-insensitive interaction between STIM2 and ORAI1.

Keywords: calcium signaling, neurons, ORAI1, proximity ligation assay, STIM2, store-operated calcium entry.

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Stromal interaction molecule 1 (STIM1), STIM2, and the ion channel protein ORAI1 are the components of store-operated calcium entry (SOCE) in non-excitable cells, a process required to refill the endoplasmic reticulum (ER) with calcium ions (Blaustein and Golovina 2001; Putney 2003). Emptying the ER of calcium causes STIM protein oligomerization and the movement of the ER membrane with these oligomers toward the plasma membrane (Liou *et al.* 2005; Zhang *et al.* 2005). At the plasma membrane, STIMs, together with ORAI1, form hetero-complexes called 'puncta' to allow calcium ions to enter the cytoplasm (Luik *et al.* 2006; Xu *et al.* 2006). Calcium levels in the ER are restored by the activity of calcium adenosine triphosphatase (Ca-ATPase) of the sarco/endoplasmic reticulum pump. This process can be inhibited by thapsigargin. However, few studies have elucidated the role of STIM proteins in neuronal calcium homeostasis (Berna-Erro *et al.* 2009; Klejman *et al.* 2009; Venkiteswaran and Hasan 2009; Gemes *et al.* 2011; Gruszczynska-Biegala *et al.* 2011; Steinbeck *et al.* 2011). Also what remains unclear is the extent to which over-expressed proteins mimic the function of endogenous proteins (Berna-Erro *et al.* 2009; Klejman *et al.* 2009; Gruszczynska-Biegala *et al.* 2011).

We previously described the complex formation of yellow fluorescent protein (YFP)-STIM2 and YFP-STIM1 with ORAI1 in primary cultures of transfected neurons (Klejman

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Abbreviations used: BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester; ER, endoplasmic reticulum; Fura-2 AM, Fura-2 acetoxymethyl ester; HBSS, HEPES buffered salt solution; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PLA, proximity ligation assay; RCP, rolling-circle product; SOCE, store-operated calcium entry; STIM, stromal interaction molecule.

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et al. 2009; Gruszczynska-Biegala *et al.* 2011). The depletion of calcium from the ER in the presence of thapsigargin increased the number of YFP-STIM1 and ORAI1 puncta, but not YFP-STIM2 and ORAI1 puncta. In contrast, YFP-STIM2-ORAI1 complexes were observed when the level of calcium ions in the extracellular medium was decreased by their chelator, ethylene glycol tetraacetic acid (EGTA). These data and single-neuron calcium imaging in transfected neurons suggested that STIMs play differential roles in calcium homeostasis in neurons (Gruszczynska-Biegala *et al.* 2011). However, these conclusions were based on tagged proteins that were over-expressed in neurons, and the localization and dynamics of the interaction between endogenous STIMs and endogenous ORAI1 proteins are not known.

As STIM2 is the dominant isoform in the brain and cortical neurons (Berna-Erro *et al.* 2009; Skibinska-Kijek *et al.* 2009; Gruszczynska-Biegala *et al.* 2011; Steinbeck *et al.* 2011) the aim of this study was to determine the role of STIM2 and whether and where the interaction between endogenous STIM2 and ORAI1 occurs in neurons. We also sought to determine how this interaction depends on cytoplasmic and ER calcium levels that are changed by thapsigargin, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), and EGTA. We first explored the association between endogenous STIM2 and ORAI1 proteins based on their co-immunoprecipitation from neuronal lysates. We then used the Proximity Ligation Assay (PLA) technique to visualize the complexes of endogenous STIM2 and ORAI1 proteins in neurons (Soderberg *et al.* 2006; reviewed in Weibrecht *et al.* 2010). Through the use of PLA, we demonstrated that the interaction between endogenous STIM2 and ORAI1 proteins occurred in neurons and was sensitive to changes in intracellular calcium levels induced by EGTA and BAPTA-AM. The data suggest that STIM2 senses and reacts to small decreases in calcium levels in the ER caused by decreased intracellular calcium levels.

Materials and methods

Primary neuron cultures

Cortical neuron cultures were prepared from embryonic day 19 Wistar rat brains. Pregnant female Wistar rats were provided by the Animal House of the Mossakowski Medical Research Centre, Polish Academy of Sciences (Warsaw, Poland). Animal care was in accordance with the European Communities Council Directive (86/609/EEC). The experimental procedures were approved by the Local Commission for the Ethics of Animal Experimentation no. 1 in Warsaw. Brains were removed from rat embryos and collected in cold Hanks solution supplemented with 15 mM HEPES buffer and penicillin/streptomycin. The cortices were isolated, rinsed three times in cold Hanks solution, and treated with trypsin for 20 min. The tissue was then rinsed in 37°C Hanks solution and dissociated by pipetting. Primary cortical neurons were plated at a density of 3×10^4 per glass well in 16-well chamber slides (Lab-Tek, Nunc, Rochester, NY, USA) coated with laminin (2 μg/mL; Roche, Mannheim, Germany) and poly-D-lysine (38 μg/mL; Sigma-Aldrich, St. Louis, MO, USA). For immunoblotting and co-immunoprecipitation, neurons were seeded on poly-D-lysine-coated Petri dishes at 8×10^6 cells/plate. Neurons were grown in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) supplemented with B27 (Invitrogen), 0.5 mM glutamine (Sigma, Poznan, Poland), 12.5 μM glutamate (Sigma), and a penicillin (100 U/mL)/streptomycin (100 μg/mL) mixture (Invitrogen). The cultures were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere.

Immunoblotting and co-immunoprecipitation

Immunoblotting was performed as previously described (Gruszczynska-Biegala *et al.* 2011) using rabbit anti-STIM2 (1 : 1000; ProSci Inc., Poway, CA, USA), mouse anti-ORAI1 (1 : 200; Sigma), rabbit anti-ORAI1 (1 : 300; Alomone Labs, Jerusalem, Israel), and rabbit anti-ORAI1 (1 : 400; Cell Signaling Technology, Danvers, MA, USA). For the co-immunoprecipitation of endogenous STIM2 and ORAI1 proteins, 8-day-old primary cortical neurons grown on Petri dishes were treated for 10 min with 2 mM $CaCl_2$ or 2 mM EGTA in HEPES Buffered Salt Solution (HBSS) and then lysed in 1 mL lysate buffer, pH 7.5, that contained 50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride supplemented with complete EDTA-free protease inhibitor cocktail (Roche). After incubation for 30 min on ice and centrifugation at 14 000 *g* for 10 min at 4°C, cleared lysates were pre-incubated with 30 μL of washed protein A-Sepharose (Roche) for 3 h at 4°C. The Sepharose and bound components were recovered by centrifugation at 14 000 *g* for 10 min at 4°C. Pre-cleared lysates were subsequently incubated overnight at 4°C on a rocking platform with 30 μL of A-Sepharose pre-incubated earlier for 3 h with 3 μg of rabbit antibody (anti-STIM2, ProSci Inc., anti-ORAI1, Alomone Labs; anti-Flag, Sigma). Precipitated samples were then washed twice with repeated centrifugation, eluted in 50 μl of 2× Laemmli Buffer, and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis with the indicated primary antibodies and then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) or horseradish peroxidase-conjugated anti-mouse IgG (Sigma). The immunoreactive bands were developed using a chemiluminescence detection kit (Pierce, Rockford, IL, USA). The optical density of the bands was estimated using a GS-800 Calibrated Densitometer and Quantity One software (Bio-Rad, Hercules, CA, USA).

Proximity ligation assay in neurons

For the analysis of cultured neurons, the cells were grown on 16-well chamber slides for 8 days or 21 days as indicated and incubated for 10 min with 2 mM $CaCl_2$, 2 mM EGTA (when required, followed by incubation with 2 mM $CaCl_2$ for 10, 25, or 40 min), or 2 μM thapsigargin (Sigma) in 0.5 mM EGTA (when required, followed by 5 min incubation with 100 μM ML-9) in HBSS. In the BAPTA-AM assays, the cells were loaded with 5 μM of the solution for 10 min at 24°C in HBSS. The PLA was performed using the Duolink II kit (Olink Bioscience, Uppsala, Sweden) and performed as described previously (Thymiakou and Episkopou 2011). After stimulation, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at 24°C, and the chambers were removed from the slides. The cells were then washed three times with PBS with agitation

Table 1 Primary rabbit, goat, and mouse antibodies used in the experiments. Secondary antibodies, called PLA probes with attached oligonucleotides, were from rabbit, goat, and mouse, respectively, and are labeled MINUS or PLUS to form RCP pairs

	ORAI1 rabbit (Cell Signaling Technology) 1 : 50	ORAI1 rabbit (Alomone Labs) 1 : 100	ORAI1 goat (Santa Cruz Biotechnology) 1 : 100	ORAI1 mouse (Sigma) 1 : 500
	Rabbit PLA probe PLUS		Goat PLA probe MINUS	Mouse PLA probe MINUS
STIM2 goat (Santa Cruz Biotechnology) 1:100 Goat PLA probe MINUS	Fig. 2, 3a	Figs 3b, 4, 5, Figure S1	–	–
STIM2 rabbit (ProSci) 1 : 500 Rabbit PLA probe PLUS	–	–	Fig. 3c	Fig. 3d

and permeabilized in PBS that contained 0.5% Triton X-100 for 10 min, followed by washing the cells twice with 0.05% Tween 20 in Tris-buffered saline buffer (TBS; 50 mM Tris-HCl [pH 7.5] and 150 mM NaCl). The cells were then blocked for 1 h with one drop of Duolink II Blocking solution in a humidified chamber at 37°C and incubated overnight at 4°C with appropriate combinations of antibodies in Duolink II Antibody Diluent solution (40 μL).

The antibodies used for the PLA were goat anti-STIM2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) combined with rabbit anti-ORAI1 (Cell Signaling Technology) or rabbit anti-ORAI1 (Alomone Labs) and rabbit anti-STIM2 (ProSci Inc.) combined with goat anti-ORAI1 (Santa Cruz Biotechnology) or mouse anti-ORAI1 (S, Sigma). See Table 1 for the antibody concentrations. After washing with wash buffer A, the cells were incubated for 1 h at 37°C with PLA probes, which are secondary antibodies (Duolink II anti-Mouse-MINUS and Duolink II anti-Rabbit-PLUS or Duolink II anti-Goat-MINUS and Duolink II anti-Rabbit-PLUS) conjugated to unique oligonucleotides. The cultures were further subjected to *in situ* PLA using a Duolink II Detection kit according to the manufacturer's instructions. Briefly, the slides were incubated with Ligation-Ligase solution in a pre-heated humidified chamber for 30 min at 37°C, followed by incubation with amplification-polymerase solution for an additional 100 min at 37°C. Finally, the cells were washed in Wash Buffer B, and the slides were mounted using Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and evaluated using an Eclipse 80i fluorescent microscope with a 40× objective (Nikon Corporation, Tokyo, Japan). Representative results are shown from experiments repeated twice. Mouse anti-STIM1 (BD Transduction Laboratories, Franklin Lakes, NJ, USA) combined with rabbit anti-ORAI1 (Alomone Labs) or rabbit anti-ORAI1 (Cell Signaling Technology) was used as a positive biological control. The incubation of L1 cell adhesion molecule antibody (Santa Cruz Biotechnology) with STIM2 antibodies was performed as a negative biological control. As a negative technical control, the primary antibodies anti-STIM2 and anti-ORAI1 were used alone, or both primary antibodies were omitted. These negative controls did not yield any significant PLA signals in either treatment condition.

Image analysis

Fluorescence microscopy signals were determined using Eclipse 80i and NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). The processed images were thresholded, and the number of *in situ* PLA signals per cell that corresponded to integrated STIM2 and ORAI1 puncta was quantified using the Particle Analysis function. The settings were kept constant for all of the images throughout the experiments. Two experiments were performed (i.e., two preparations of primary neuronal cultures). Quantifications were performed from 11 to 17 images (n) from a minimum of two slides for each culture preparation for every calcium treatment (2 mM $CaCl_2$, 5 μM BAPTA-AM, 2 μM thapsigargin with 0.5 mM EGTA, or 2 mM EGTA alone), corresponding to 100–200 cells. The number of analyzed images (n) in each experimental condition is indicated in the figure legends.

Single-cell Ca^{2+} measurements

Single-cell Ca^{2+} levels in cortical neurons were recorded using the ratiometric Ca^{2+} indicator dye Fura-2 acetoxymethyl ester (Fura-2 AM). Cells grown on 16-well chamber slides were loaded with 2 μM Fura-2 AM for 30 min at 37°C in a solution that contained 145 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 10 mM glucose, 10 mM HEPES (pH 7.4), and 1 mM $MgCl_2$ (HBSS) supplemented with 2 mM $CaCl_2$ at 37°C (high-calcium medium) and then rinsed and left undisturbed for 30 min at 24°C to allow for de-esterification. Measurements of intracellular calcium levels were performed every 1 s at 37°C using an Olympus Scan^R & Cell^R imaging system that consisted of an IX81 microscope (Olympus Corporation, Tokyo, Japan), 10 × 0.40 NA UPlanSApo objective (Olympus Corporation, Tokyo, Japan), and Hamamatsu EM-CCD C9100-02 camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Intracellular Ca^{2+} levels in individual neuronal cell bodies are expressed as the F_{340}/F_{380} ratio after subtracting background fluorescence. This ratio represents the emission intensities at 510 nm obtained after excitation at 340 and 380 nm. The low-calcium medium (Ca^{2+}-free solution) contained 2 mM EGTA in the standard buffer. Data processing was performed using Olympus Cell^R software.

Statistical analysis

All of the experiments were repeated at least twice. The statistical analysis was performed using Prism version 5.02 software (GraphPad, San Diego, CA, USA). The data are expressed as the mean ± SD from n images in two separate neuronal cultures. One-way ANOVA was used to analyze sets of PLA data. Tukey's

post hoc test was used to determine statistically significant differences among groups. The statistical analyses of the co-immunoprecipitation experiments were performed using Student's *t*-test. The level of significance compared to that of controls is indicated by asterisks: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (*ns*, not significant, $p > 0.05$).

Results

Detection of STIM2–ORAI1 interactions using a co-immunoprecipitation assay

To determine whether native STIM2 is associated with native ORAI1 in neurons, co-immunoprecipitation experiments with cortical neuron lysates and anti-STIM2 or anti-ORAI1 antibody were performed. To detect possible non-specific immunoprecipitation, anti-Flag antibodies were used. Experiments were conducted using neurons treated with a medium that contained either 2 mM $CaCl_2$ or 2 mM EGTA, and the inputs and immunoprecipitates were analyzed by western blot. After immunoprecipitation with anti-STIM2 antibody, the presence of ORAI1 was detected by western blot in samples from neurons incubated under both conditions (Fig. 1a, Ca and EGTA immunoprecipitates at WB : ORAI1 blot). This indicated that a molecular complex was formed between STIM2 protein and ORAI1 in rat cortical neurons. The amount of ORAI1 that co-immunoprecipitated with STIM2 was approximately 50% higher from neurons in a low-calcium medium than in a high-calcium medium (Fig. 1a, WB : ORAI1 blot; Fig. 1b, red column, $p < 0.05$; $n = 4$). A similar protein content in both lanes was confirmed by western blotting the same membranes treated with the STIM2 antibody (Fig. 1a, immunoprecipitates at WB : STIM2). Western blots of input samples showed identical amounts of proteins in the samples (Fig. 1a, two lower blots).

In reverse experiments, in which neuronal extracts were immunoprecipitated with anti-ORAI1 antibody, STIM2 was detected in samples from both types of conditions (Fig. 1a, Ca and EGTA immunoprecipitates at WB : STIM2), confirming that a molecular complex was formed between ORAI1 and STIM2 protein. Interestingly, ORAI1 appeared to co-immunoprecipitate mainly a form of STIM2 with an apparent molecular mass of ~ 105 kDa. In ORAI1 antibody immunoprecipitates obtained from EGTA-incubated neurons, approximately 200% more STIM2 was observed compared with immunoprecipitates from neurons incubated in a high-calcium medium (Fig. 1a, immunoprecipitates at WB : STIM2; Fig. 1b, blue bar, $p < 0.05$; $n = 5$). The amount of endogenous ORAI1, which was precipitated by its antibody, was slightly higher in immunoprecipitates incubated in a low-calcium medium (Fig. 1a, Ca and EGTA immunoprecipitates at WB : ORAI1). The difference

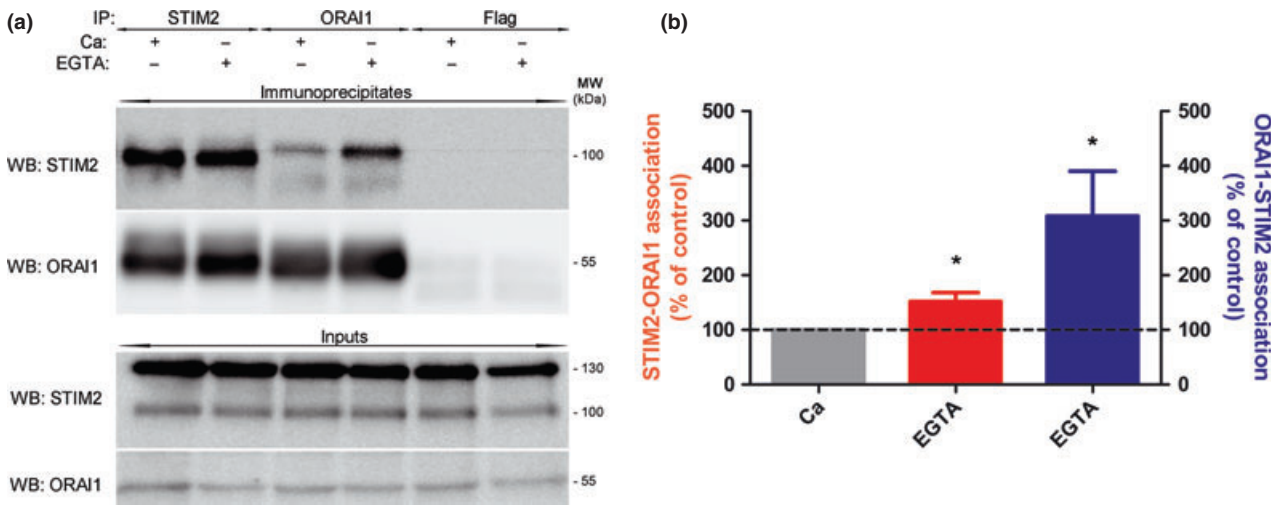

Fig. 1 Co-immunoprecipitation of endogenous stromal interaction molecule 2 (STIM2) and ORAI1 from neuronal lysates. (a) Cortical neurons were treated for 10 min with 2 mM $CaCl_2$ or 2 mM EGTA. Whole-cell lysates were immunoprecipitated (IP) with rabbit anti-STIM2 antibody or rabbit anti-ORAI1 antibody. Rabbit anti-Flag antibody was used as a negative control. The cell lysates (Inputs) and eluted fractions (Immunoprecipitates) were separated on 10% sodium dodecyl sulfate gels, blotted [western blot (WB)], and probed with anti-STIM2 and anti-ORAI1 antibodies. Notice that the cell treatment with a low-calcium medium (in the presence of 2 mM EGTA) enhanced the association between STIM2 with ORAI1. Input lanes contain 5% of the extract used for immunoprecipitation. The molecular masses of the markers run on the same gel are shown on the right. The results are representative of four (IP with STIM2 antibody) and five (IP with ORAI1 antibody) independent experiments. (b) The histogram represents the quantification of the STIM2-ORAI1 (red column) or ORAI1-STIM2 (blue column) association in neurons incubated in high-calcium medium (Ca-control) and low-calcium medium (EGTA). STIM2 or ORAI1 bands in the co-immunoprecipitates were analyzed densitometrically and normalized to the level of the loading control (i.e., bands obtained after WB with the antibody used for immunoprecipitation). The results are expressed as a percentage of control (i.e., protein association in high-calcium medium). $*p < 0.05$, compared with control.

between the amount of protein complex co-immunoprecipitated with primary anti-STIM2 and primary anti-ORAI1 antibodies is likely to reflect their different affinity and specificity for the antigens. The anti-Flag antibody that was incubated with neuronal lysates did not co-precipitate STIM2. However, trace amounts of ORAI1 were co-precipitated by anti-Flag, but this was negligible compared with the amount precipitated by a specific ORAI1 antibody. Both types of co-immunoprecipitation experiments suggested that the interaction between endogenous STIM2 and ORAI1 in primary cortical neurons depended on the levels of calcium in the medium, levels that determine intracellular calcium levels and subsequently ER calcium levels.

Identification of complexes formed by endogenous STIM2 and ORAI1 using the PLA

To confirm the interaction between endogenous STIM2 and ORAI1 proteins, we adapted the PLA to detect the complexes between endogenous proteins in neurons *in situ*. Cortical non-transfected neurons were cultured under different calcium concentrations, fixed with paraformaldehyde, blocked, and incubated with the primary antibodies anti-STIM2 and anti-ORAI1. The pair of appropriate secondary antibodies with covalently conjugated oligonucleotides, called proximity probes, was then added, the signal for which is generated only when there is tight proximity between both PLA probes. These probes were hybridized with two additional connector oligonucleotides that were added to the ligation solution that contained DNA Ligase, which allows them to ligate into a closed circle. This newly created circular DNA molecule then serves as a template for rolling-circle amplification. The amplification was primed from one of the oligonucleotides attached to the proximity probes. Rolling-circle amplification with the use of a polymerase resulted in the production of a single-strand rolling-circle product (RCP) molecule that contained concatemeric (repeated sequence) copies of the original DNA circle formed by ligation, which remained covalently attached to the proximity probe. The RCP was then detected by hybridization of complementary oligonucleotides labeled with a green fluorophore. This visualized the STIM2-ORAI1 interaction as PLA green spots. Rolling-circle products were clearly visible over the background because every RCP is capable of binding hundreds of fluorescently labeled oligonucleotides.

The incubation of non-transfected cortical neurons [8 days *in vitro* (DIV)] in a medium that contained 2 mM $CaCl_2$ led to few detectable complexes between endogenous STIM2 and ORAI1, indicated by green dots in Fig. 2a. However, with the presence of EGTA in the medium, which is known to decrease intracellular calcium levels (Tsien 1980), additional green fluorescent dots appeared, identifying newly formed STIM2-ORAI1 hetero-complexes (Fig. 2c). No significant increase in the number of PLA signals was observed after treatment with thapsigargin, which led to a decrease in calcium levels in the ER by inhibiting the ER calcium-ATPase of the sarco/endoplasmic reticulum pump (Fig. 2b).

To ensure that the PLA signals were specifically located in neuronal cells, PLA images and phase-contrast pictures of the neurons were merged. The somata and neuronal processes were simultaneously visible with PLA green dots. As shown in Fig. 2d–f, the PLA signals were mostly seen where cell bodies were present. Occasionally, single dots were detected

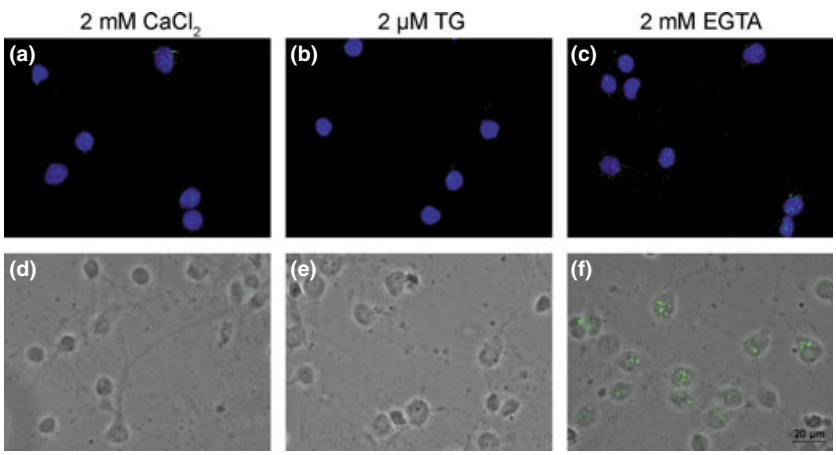

Fig. 2 Detection of interaction between stromal interaction molecule 2 (STIM2) and ORAI1 using Proximity Ligation Assay (PLA). Cortical neurons (8 DIV) were incubated with 2 mM $CaCl_2$ (a, d), 2 μM thapsigargin (TG) (b, e), or 2 mM EGTA (c, f). The cells were then fixed by the addition of paraformaldehyde, blocked for 30 min at 37°C, and incubated with the following primary antibodies: goat anti-STIM2 and rabbit anti-ORAI1. A Duolink II assay was subsequently performed according to the manufacturer's instructions. (a–c) Neurons were additionally counter-stained with the nuclear marker Hoechst dye (blue). The PLA signal, recognized as a fluorescent green dot, shows the close proximity of STIM2 and ORAI1 antigens. (d–f) The pictures show PLA signals merged with phase-contrast images from the same position. This allows the localization of the endogenous STIM2-ORAI1 complexes within neuronal cells. Notice the greater number of green dots under EGTA conditions (f) and their presence mainly in the somata. Scale bar = 20 μm for each panel.

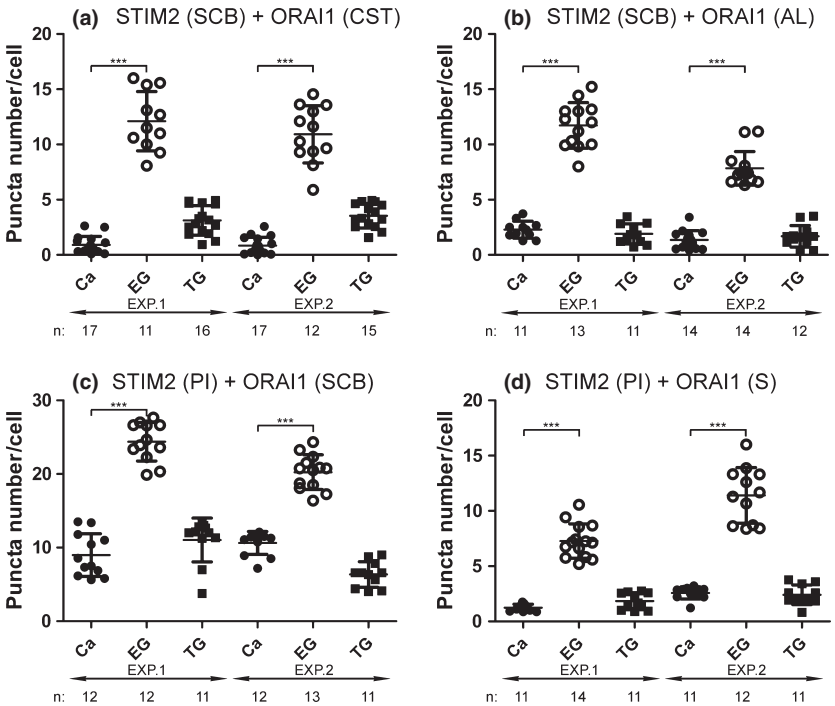

Fig. 3 Quantification of the complexes formed from endogenous stromal interaction molecule 2 (STIM2) and ORAI1 detected by Proximity Ligation Assay (PLA). Experiments were performed as described in Fig. 2. Cortical neurons (8 DIV) were incubated with 2 mM $CaCl_2$ (Ca), 2 μM thapsigargin (TG) or 2 mM EGTA (EG). Four different pairs of antibodies [goat anti-STIM2 (Santa Cruz Biotechnology), rabbit anti-ORAI1 (Cell Signaling Technology), rabbit anti-ORAI1 (Alomone Labs), rabbit anti-STIM2 (ProSci Inc.), goat anti-ORAI1 (Santa Cruz Biotechnology), mouse anti-ORAI1 (Sigma)] were used in two separate neuronal cultures/experiments. Notice that the number of endogenous STIM2-ORAI1 complexes in each case is higher in the presence of EGTA (EG) than in the presence of calcium (Ca). Eleven to 17 images (*n*) per experiment were taken for each pair of antibodies in every calcium condition (Ca, EG, and thapsigargin [TG], representing 100–200 cells). The bars indicate the mean ± SD of *n* images. The quantification of PLA signals was performed using ImageJ software to analyze neurons. ***$p < 0.001$.

in neuronal processes (data not shown). In older neurons (21 DIV), the majority of PLA signals were again observed on cell bodies. However, PLA signals were also visible in a few dendrites. The location of such dots appeared to overlap with other neuronal processes, but the phase-contrast resolution did not allow for the determination of whether it correlated with the localization of synapses (Figure S1). The quantification of the number of STIM2-ORAI1 complexes in old neuronal cultures (21 DIV) revealed an increase in the number of STIM2-ORAI1 hetero-complexes after incubation in a low-calcium medium (Figure S1b), but the increase was smaller than in 8-day-old neurons.

Comparison of PLA signals created by different pairs of anti-STIM2 and anti-ORAI1 antibodies

To confirm that the observed PLA dots represented authentic STIM2-ORAI1 complexes, one additional anti-STIM2 and three additional anti-ORAI1 antibodies were used (Table 1). This allowed us to verify the PLA signals in neurons treated with three additional different pairs of primary antibodies. All four combinations yielded identical locations of PLA signals and pattern changes between the high- and low-calcium mediums (Fig. 3). Neurons incubated in the medium with 2 mM $CaCl_2$ showed few complexes per cell. The depletion of calcium from the ER by the addition of thapsigargin to the medium did not significantly increase the number of STIM2-ORAI1 complexes. In contrast, low extracellular calcium levels triggered the formation of a large number of hetero-complexes for every pair of antibodies used (Fig. 3). Under these conditions, the PLA signal was observed in all neurons, demonstrating the general effect of a low-calcium medium on STIM2-ORAI1 complexes (Fig. 2c and f, Fig. 3; see also Fig. 5). The quantification of the number of STIM2-ORAI1 complexes revealed a two- to 13-fold increase in the low-calcium medium compared with the high-calcium medium (Fig. 3). The fold increase depended on the pair of antibodies used in the PLA experiments. Thus, the formation of STIM2-ORAI1 hetero-complexes identified by the first pair of antibodies (Fig. 2c and f) was also detected by three other pairs of antibodies, suggesting that the observed dots represented real complexes that depended on the calcium concentration.

To verify that the primary antibodies from different vendors correctly identified STIM2 and ORAI1 proteins, we used

western blot to analyze lysates of cortical neurons (Figure S2). Immunoblotting with Sigma and Alomone Labs anti-ORAI1 antibodies revealed one single ~ 50 kDa band, corresponding to the glycosylated form of the monomeric ORAI1 protein (Gwack *et al.* 2007). The ORAI1 antibody from Cell Signaling identified a ~ 35 kDa band, corresponding to the non-glycosylated form of monomeric ORAI1. STIM2 identified by the ProSci Inc. antibody migrated as a few bands, one with an apparent molecular mass of ~ 105 kDa and a more prominent ~ 120 kDa band. The latter likely represents a highly phosphorylated form of STIM2 (Williams *et al.* 2001; Smyth *et al.* 2009). The nature of the 30–35 kDa band is not known, but the specificity of this STIM2 (ProSci Inc.) antibody analyzed by western blot was previously verified (Brandman *et al.* 2007; Darbellay *et al.* 2010; El Boustany *et al.* 2010). Other antibodies could not be tested by western blot because of a heavy background. The anti-ORAI1 antibody from Santa Cruz Biotechnology has been successfully used in previous immunofluorescence experiments (Eylenstein *et al.* 2011; Gomez-Fernandez *et al.* 2012).

Calcium-dependent formation of STIM2-ORAI1 complexes in neurons

To further study the influence of intracellular [Ca^{2+}] on the STIM2-ORAI1 interaction, PLA experiments were performed in neurons treated with BAPTA-AM, a calcium chelator that penetrates live cells and decreases intracellular calcium concentrations (Fig. 4). A significant increase in the number of complexes in BAPTA-AM-treated neurons was found compared with cells in the high-calcium medium. The number of these complexes was slightly higher than in cells incubated in a low-calcium medium. These observations indicate that both treatments that affected intracellular calcium levels generated a similar effect (i.e., an increased interaction between STIM2 and ORAI1).

The calcium-dependent interaction between endogenous STIM2 and ORAI1 was further confirmed by the ability of the high-calcium medium to reverse the effect of EGTA (low-calcium medium). Neurons were first incubated in EGTA-containing medium and then incubated in a high-calcium medium for various periods of time. The PLA was then performed. The number of STIM2-ORAI1 complexes was significantly reduced after 10 min incubation in the high-calcium medium compared with incubation in EGTA-containing medium (Fig. 4). These results indicate that STIM2-ORAI1 complexes that are formed at low intracellular calcium levels dissociate when these levels increase.

Correlation between calcium response and number of endogenous STIM2-ORAI1 complexes identified by PLA in the same neuron

The above results suggest that the STIM2-ORAI1 interaction depends on intracellular calcium concentrations. To verify these observations, we sought to determine whether a

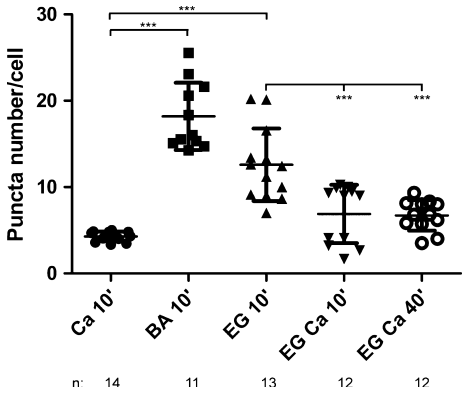

Fig. 4 Calcium-dependent formation of stromal interaction molecule 2 (STIM2)-ORAI1 complexes in neurons. Eight-day-old neurons were treated with 2 mM $CaCl_2$ (Ca), 5 μM of the calcium-chelator BAPTA-AM (BA), 2 mM EGTA (EG), or 2 mM EGTA followed by re-incubation with 2 mM $CaCl_2$ for 10 or 40 min (EG Ca). After fixation, protein interactions were visualized by the Proximity Ligation Assay (PLA) using goat anti-STIM2 (Santa Cruz Biotechnology) and rabbit anti-ORAI1 (Alomone Labs) antibodies and quantified. The number of PLA signals per cell from two independent experiments is expressed as the mean ± SD. Notice that BAPTA-AM treatment enhanced the number of STIM2-ORAI1 complexes even more than EGTA treatment. Complex formation was also reversed. ***$p < 0.001$.

correlation exists between decreased cytosolic calcium levels and an increased number of STIM2-ORAI1 complexes. Two methods were used in the same neurons: calcium imaging and a subsequent PLA. Intracellular calcium levels were first measured using a Fura-2 AM indicator in individual cells (Fig. 5a). Neurons were loaded with Fura-2 AM in a high-calcium medium, washed for 30 min, and incubated in the high-calcium medium. Their subsequent incubation in a low-calcium medium led to a decrease in intracellular free calcium. The neurons were then fixed, and PLA was performed to identify STIM2-ORAI1 complexes (Fig. 5b). Variations were observed in the number of PLA signals (Fig. 5c, red bars) and cytosolic calcium levels in response to the low-calcium medium (Fig. 5c, blue bars). However, in at least 70% of the cells analyzed, the higher number of STIM2-ORAI1 complexes that formed was associated with a higher calcium response to decreased calcium levels in the medium. The responses shown in Fig. 5c as Delta Ratio values are defined as the difference between the F_{340}/F_{380} ratio at the beginning of experiments in the high-calcium medium (Ca^{2+}) and the F_{340}/F_{380} ratio of the experiments in the low-calcium medium (EGTA). Given the linear distribution of the values, Pearson's correlation was used. In all three experiments shown in Fig. 5, we found strong correlations between the number of complexes and calcium response ($0.5 <$ Pearson's correlation coefficient $[r] < 1$). The r values for the examined neurons were the following: Experiment 1, 0.8447 (***$p = 0.0005$); Experiment 2, 0.5113 ($p = 0.1595$, ns; but when excluding neuron no. 21,

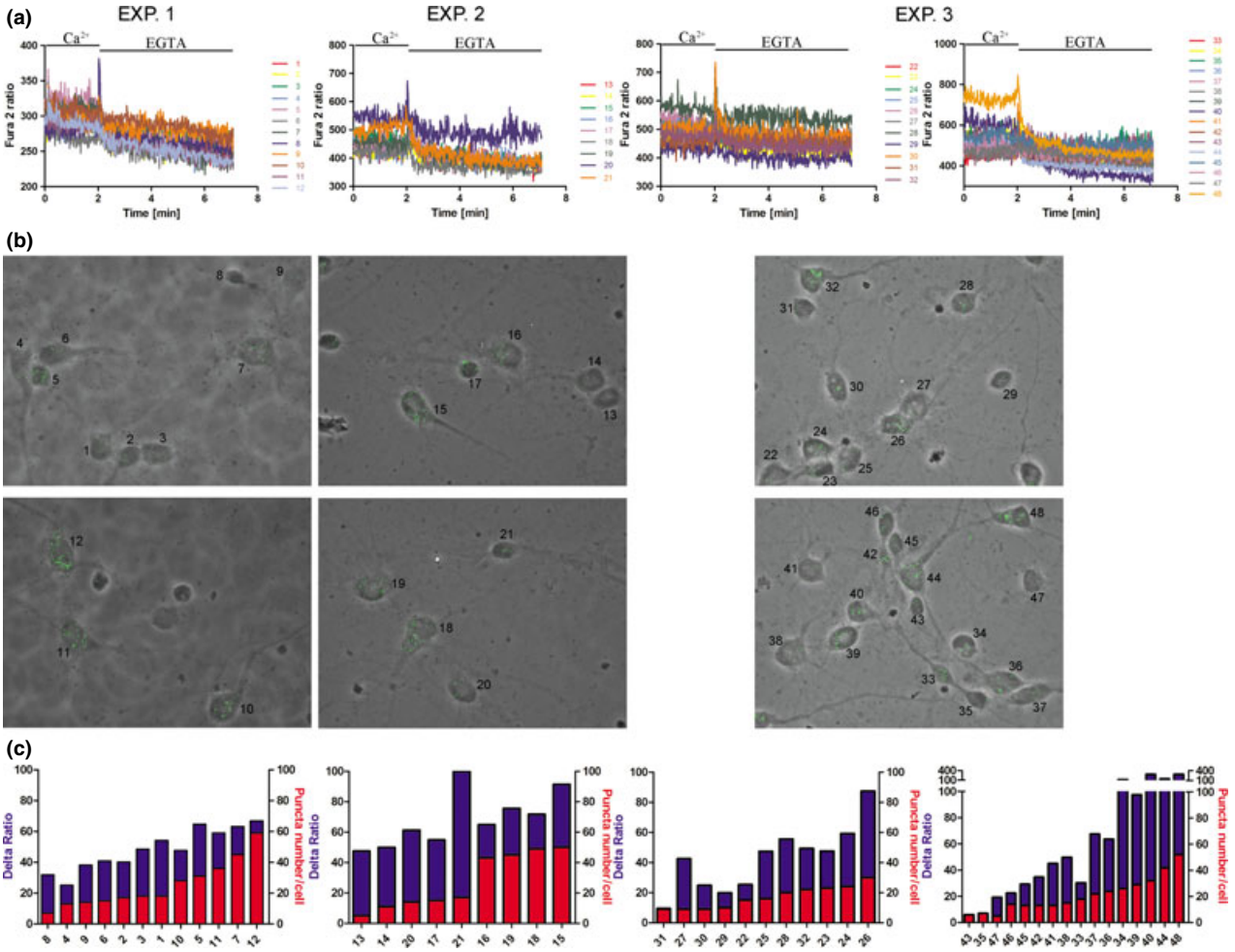

Fig. 5 Correlation between calcium response and number of endogenous stromal interaction molecule (STIM2)-ORAI1 complexes identified by the Proximity Ligation Assay (PLA) in the same neuron. (a) Raw traces of cytosolic calcium levels (ratio of F_{340}/F_{380}) in 48 individual non-transfected neurons. Each of three experiments began in the presence of 2 mM $CaCl_2$ and continued by an exchange to a medium with 2 mM EGTA. (b) After calcium measurements, neurons were fixed and probed with a pair of goat anti-STIM2 (Santa Cruz Biotechnology) and rabbit anti-ORAI1 (Alomone Labs) antibodies. The PLA was then performed as described in Fig. 2. The pictures show PLA signals merged with phase-contrast images of the cells. The number identifies the cell in which the calcium level was measured, as shown by one of the traces in (a). (c) Summary of numbered neurons, in which both calcium imaging (a) and the PLA (b) were performed. Blue columns show calcium responses to the decreased calcium level in the medium. Responses are shown as Delta Ratio values, which were calculated as the difference between the F_{340}/F_{380} ratio at the beginning of experiments (Ca^{2+}) and the F_{340}/F_{380} ratio in the low-calcium medium (EGTA). Red columns show the number of endogenous STIM2-ORAI1 complexes in the low-calcium medium in the same cell. For clarity, the red bars overlap with the blue bars. The bars are arranged in the order of increasing puncta numbers. The higher number of endogenous STIM2-ORAI1 complexes was strongly positively correlated with a larger calcium response in three independent experiments (Pearson correlation test, $0.5 < r < 1$).

$r = 0.88$, $^{**}p = 0.0039$); Experiment 3, 0.8758 ($^{***}p = 0.0004$) and 0.8654 ($^{***}p < 0.0001$). These data suggest that a STIM2-ORAI1 complex formed as a result of decreased cytosolic calcium levels.

Biological and technical PLA controls

When a STIM2 antibody was replaced with a STIM1 antibody in the PLA, the experiments could be considered positive biological controls because the interaction between STIM1 and ORAI1 proteins has been shown to increase in neurons after thapsigargin treatment (Klejman *et al.* 2009; Gruszczynska-Biegala *et al.* 2011; Mitchell *et al.* 2012) and other cell types (Mercer *et al.* 2006; Peinelt *et al.* 2006; Soboloff *et al.* 2006; Zhang *et al.* 2006; Jardin *et al.* 2008). The neurons were incubated under four different conditions: in a medium that contained 2 mM $CaCl_2$, 2 mM EGTA, 2 μM thapsigargin, or 2 μM thapsigargin, followed by the addition of the SOCE inhibitor ML9 (Fig. 6). As expected,

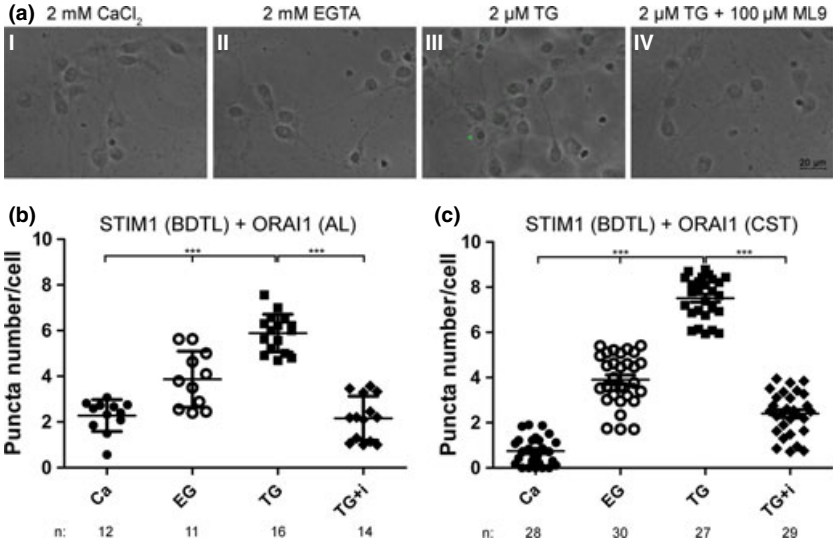

Fig. 6 Quantification of the complexes formed from endogenous stromal interaction molecule 1 (STIM1) and ORAI1 detected by Proximity Ligation Assay (PLA). Experiments were performed as described in Fig. 2, but anti-STIM1 antibody was used instead of anti-STIM2. Cortical neurons were treated with 2 mM $CaCl_2$ (Ca), 2 mM EGTA (EG), 2 μM thapsigargin (TG), or 2 μM thapsigargin, followed by 5 min incubation with 100 μM ML-9 (TG+i), and then probed with a pair of STIM1 (BD Transduction Laboratories) and (a, b) ORAI1 (Alomone Labs) or (c) ORAI1 (Cell Signaling Technology) antibodies. (a) The pictures show PLA signals merged with phase-contrast images from the same position. Notice the strongest effect of thapsigargin on the endogenous STIM1-ORAI1 hetero-complex (III) and its inhibition by ML9 (IV). Scale bar = 20 μm for each panel. (b, c) Bars represent averages from (b) 11–16 and (c) 27–30 (*n*) images taken in three independent experiments. ***$p < 0.001$.

the largest number of endogenous STIM1 and ORAI1 hetero-complexes was detected after SOCE induction by thapsigargin (Fig. 6aIII, b, c). The subsequent incubation of these cells with ML9 decreased the number of complexes nearly to the state before thapsigargin stimulation, demonstrating the reversibility of this process. The presence of EGTA in the medium increased the number of dots, but to a lesser extent than in the presence of thapsigargin (Fig. 6). This is opposite to the effects of EGTA and thapsigargin media on STIM2/ORAI1 hetero-complexes shown in Figs 2 and 3. These results demonstrate that the PLA distinguishes the biological activity of similar proteins, such as STIM1 and STIM2, and detects changes in the interaction in response to different factors (i.e., thapsigargin, EGTA, and ML9).

To confirm the specificity of the antibodies and the PLA process itself, several control experiments were conducted. In one PLA experiment, the cells were incubated in a medium that contained either 2 mM $CaCl_2$ or 2 mM EGTA, fixed, and probed with STIM2 and L1 cell adhesion molecule antibodies. The pictures show that PLA signals merged with 4′,6-diamidino-2-phenylindole (DAPI) staining to better visualize single dots (Figure S3a). As expected, these biological controls yielded no fluorescent dots in the majority of the cells. In rare cases, a few were randomly distributed within neuronal cells. The number of these complexes did not increase in the presence of a low-calcium medium (compare Figure S3aii and ai). These data also show that EGTA itself did not induce the formation of PLA signals.

Assays without primary antibodies but with both PLA probes (rabbit-PLUS and mouse-MINUS or rabbit-PLUS and goat-MINUS) were used as a technical control. No signals were detected, demonstrating the specificity of the PLA technique (Figure S3bi and ii). Additional negative controls were performed using single primary STIM2 or ORAI1 antibodies, followed by the incubation of two respective PLA probes. This resulted in no PLA signals or in a negligible number (Figure S3biii and iv), again demonstrating the specificity of this technique.

Discussion

This study provides the first demonstration of complex formation between endogenous STIM2 and ORAI1 proteins in primary cortical neurons. Co-immunoprecipitation indicated an increase in the interaction between STIM2 and ORAI1 under low-calcium conditions. Recent studies that used the co-immunoprecipitation method found that endogenous STIM2 and ORAI1 interacted with each other in the ER in HEK293 cells and upon the depletion of acidic stores in human platelets (Bandyopadhyay *et al.* 2011; Zbidi *et al.* 2011). Studies of non-excitable transfected cells showed that STIM2 can interact with ORAI1 store-dependently and -independently (Parvez *et al.* 2008; Graham *et al.* 2011). The form of STIM2 with an apparent molecular mass of ~ 105 kDa was suggested to be responsible for store-dependent calcium entry (Graham *et al.* 2011). However, our immunoprecipitation

experiments indicated that mainly an apparent 105 kDa form of STIM2 associates with ORAI1 in cortical neurons. Endogenous STIM2 can also exist in two forms: phosphorylated (with an apparent molecular mass ~ 120 kDa) and non-phosphorylated (Williams *et al.* 2001; Smyth *et al.* 2009). If so, then mainly non-phosphorylated STIM2 appears to be able to interact with ORAI1 in cortical neurons.

By combining the PLA with phase-contrast microscopy, we showed the location of hetero-complexes formed by STIM2 and ORAI1 in single neuronal cells. The presence of these complexes mainly in the somata indicated where neuronal calcium entry *via* ORAI1 channels likely occurs in 8-day-old neurons. In 21-day-old neurons, the majority of the PLA signals were again observed on cell bodies, but a few were also observed in dendrites (Figure S1). The location of the PLA signals that overlapped other neuronal processes may suggest that STIM2-ORAI1 complexes also appear in synaptic regions. However, the issue of whether these complexes are present in synaptic regions will be verified using 21-day-old neuronal cultures co-stained with synaptic markers. The PLA procedure used in this study excludes the staining for the pre-synaptic and post-synaptic markers without major modifications.

The present assay also allowed us to quantify the apparent number of STIM2-ORAI1 hetero-complexes per cell and detect their dynamics. Our earlier observations with ectopically expressed YFP-STIM2 and ORAI1 indicated that their interaction increased when neurons were transferred to a low-calcium medium (Gruszczynska-Biegala *et al.* 2011), a condition commonly used in the study of ORAI channel activation (Brandman *et al.* 2007; Bird *et al.* 2008). In this study using the PLA, we found that this phenomenon also occurred with endogenous proteins. The data showed that STIM2 responded to changes in intracellular calcium levels by interacting with ORAI1. We detected an average 6.5-fold increase in the number of STIM2-ORAI1 hetero-complexes per cell in low-calcium media, but no increase was observed when SOCE was induced by thapsigargin treatment. Endogenous STIM1 protein, which is homologous to STIM2, behaved differently. The number of STIM1 hetero-complexes with endogenous ORAI1 increased significantly in the presence of thapsigargin, but to a much smaller extent in the low-calcium medium. This shows that the difference in the physiological activity of two very similar proteins can be distinguished by the PLA. These observations indicate that endogenous STIM1 and STIM2 have different functions in neurons. This was suggested previously in studies of over-expressed tagged proteins (Berna-Erro *et al.* 2009; Gruszczynska-Biegala *et al.* 2011; Steinbeck *et al.* 2011).

Proximity Ligation Assay data rely on the use of a pair of specific antibodies from two different species. Thus, the PLA data can be considered reliable only when the specificity of both primary antibodies is unequivocally established. To confirm the specificity of the antibodies and the PLA process itself, several control experiments were conducted (see Fig. 6 and Figure S3). The data indicated that the PLA signals were specific. However, these experiments could not exclude the possibility that one of the primary antibodies used in the initial experiment (Fig. 2) exhibited cross-reactivity with another protein. To exclude this possibility and to confirm that the PLA signals represented authentic STIM2-ORAI1 hetero-complexes, three additional pairs of primary antibodies were used (Table 1). All four combinations yielded identical PLA signal locations (i.e., mainly in the somata) and identical vectors of change that depended on the calcium level in the medium (Fig. 3). In the low-calcium medium, the number of PLA dots was greater than the number observed in a high-calcium medium or in the presence of thapsigargin.

As mentioned earlier, in non-excitatory cells, STIM2 was shown to control both store-independent, which is critical for maintaining basal intracellular calcium homeostasis, and store-dependent (SOCE) modes of ORAI1 activation (Brandman *et al.* 2007; Parvez *et al.* 2008; Graham *et al.* 2011). The former is generally defined as thapsigargin-insensitive, and the latter is generally defined as thapsigargin-sensitive calcium influx from the extracellular environment. This study provided a novel finding, in which the increased association between endogenous STIM2 and ORAI1 in cortical neurons occurred in the presence of BAPTA-AM or in a low-calcium medium, but not in the presence of thapsigargin. One interpretation for these observations is that STIM2 forms a hetero-complex with ORAI1 in neurons to allow calcium entry in reaction to subtle changes in the calcium level in the ER. Exposing cells to the extracellular calcium-chelator EGTA or intracellular calcium-chelator BAPTA-AM depletes not only cytosolic calcium but also induces, mainly by leakage, a passive depletion of intracellular calcium stores (Hoth and Penner 1992; Maloney *et al.* 1999; Bird *et al.* 2008). The calcium-dependent interaction between STIM2 and ORAI1 proteins in neurons was confirmed by dissociation of the complexes formed with low cellular [Ca^{2+}] when cytoplasmic [Ca^{2+}] increased. In addition, the number of endogenous STIM2-ORAI1 complexes correlated strongly with the calcium response to the low-calcium medium. Namely, the greater number of complexes was positively associated with the response to decreased calcium levels in the medium in the same cell. These findings indicate that decreased cytosolic calcium levels are a key factor that determines STIM2-ORAI1 complex formation.

Altogether, our findings demonstrate that endogenous STIM2 can physically interact and form hetero-complexes with endogenous ORAI1 in a calcium-dependent but thapsigargin-insensitive manner, suggesting that these proteins are involved in the regulation of basal intracellular calcium levels in rat cortical neurons. A subtle decrease in calcium in the ER, such as the one experimentally induced by BAPTA-AM or EGTA, results in the activation of STIM2 rather than STIM1. This is likely attributable to the lower [Ca^{2+}]

sensitivity of STIM2 compared with STIM1 (Brandman *et al.* 2007). Thus, the STIM2-ORAI1 interaction allows the influx of calcium to restore calcium levels in the cytoplasm and refill the calcium pool stored in the ER. When the calcium pool in the ER is significantly decreased (e.g., by thapsigargin), a STIM1-ORAI1 complex is formed. STIM1 aggregates approximately 70-fold faster than STIM2 (Stathopulos *et al.* 2009) and saturates ORAI1 binding sites. The precise mechanism of the STIM2-ORAI1 interaction still needs to be established, because the dysregulation of neuronal calcium homeostasis has been suggested to play a role in the pathogenesis of ischemia (Berna-Erro *et al.* 2009), Alzheimer's disease (Bojarski *et al.* 2009), epilepsy (Steinbeck *et al.* 2011), and Huntington's disease (Wu *et al.* 2011; Vigont *et al.* 2012). The possible contribution of STIM2 to these changes has been suggested in some of these works. The newly described crystal structure of ORAI1 will help understand the details of its interaction with STIM proteins (Hou *et al.* 2012).

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Author contributions

J. G.-B. designed and performed the experiments, analyzed the data, and wrote the manuscript. J. K. designed the experiments, analyzed the data, and wrote the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Detection of interaction between STIM2 and ORAI1 using the PLA.

Figure S2. Staining of endogenous ORAI1 and STIM2 in lysates of primary cortical neurons using antibodies from different vendors.

Figure S3. Positive and negative controls for the PLA.

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