Supplemental Materials

Molecular Biology of the Cell

Fox et al.
Supplementary Figure S1. TIRF based detection of Kv2.1-ER colocalization in cultured hippocampal neurons. Cells were transfected with GFP-Kv2.1 and DsRed2-ER. The yellow represents pixels containing both the green and red fluorescence.
Supplementary Figure S2. TfR exocytosis and GFP-CLC in hippocampal neurons. Rat hippocampal neuron cultures were transfected on DIV 4-5 and imaged via TIRF on DIV 6-8 with either DsRed2-ER and TfR-SEP (A) or DsRed2-ER and GFP-CLC (B). (A) Summary figure of TfR-SEP exocytic events. Yellow dots mark events occurring within 0.3μm of the cER perimeter and blue dots mark events occurring outside of the cER perimeter. Red indicates DsRed2-ER fluorescence from a single frame. In this cell, 41 out of 51 events occurred within 0.3μm of the cER perimeter, which accounted for 25% of the footprint of the cell. Overall, 70±11% of events (n=386, from 8 cells) occurred within 0.3μm of the cER perimeter, which accounted for an average of 34±15% of the cell footprint. (B) Representative image of the association between GFP-CLC puncta and the cortical ER. Overall, 83±4% of CLC puncta (n=515, in 7 cells) were located within 0.3μm of the cER perimeter which accounted for 32±8% of the TIRF footprint.
Supplementary Figure S3

Supplementary Figure S3. Distribution of individual Qdot-labeled Kv1.3 channels relative to cER perimeter labeled with DsRed2-ER. (A) TIRF image of Qdot labeled Kv1.3 channels (blue) and DsRed2-ER fluorescence. (B and C) Individual cER and Kv1.3 fluorescent patterns, respectively. The cell perimeter is outlined. In this cell 31% of the Qdot labeled Kv1.3 were at the cER perimeter which accounted for 29.8% of the cell footprint. Overall, the area of a 0.3 μm cER perimeter occupied 40±5% of the cell footprint, while 42±14% Kv1.3 Qdots were found at this region (n=779, in 6 cells).
Supplementary Figure S4. Syntaxin4 clusters favor the cortical ER. HEK cells were transfected with Syntaxin4-HA and DsRed-ER and 12-18 hrs later labeled with AlexaFluor488-conjugated anti-HA monoclonal antibody. (A) A single TIRF image shows green puncta representing concentrations of syntaxin4 molecules that are present both near the cortical ER and in places where cER is absent. These puncta were observed with and without prior fixation indicating they do not represent antibody-induced clustering. Time-lapse imaging showed that there were two populations of syntaxin4 puncta, one highly mobile and the other relatively static. (B) Time compression indicates stable syntaxin4 localization favors the cortical ER. A 200 frame time series from the cell in (A) was compressed into a single image plane (average intensity). The highest syntaxin4 intensity is now associated with locations where there is the least movement as mobile syntaxin4 does not remain in place long enough to appear in the thresholded compression. In 13 cells co-expressing syntaxin4-HA and DsRed2-ER 68±11 % of the stable syntaxin4 clusters were within 0.3 μm of the ER while the ER perimeter represented 35±8 % of the cell footprint.
Supplementary Figure S5

Supplementary Figure S5. Translocation of STIM1 to pre-existing cER domains following ER Ca$^{2+}$ depletion. (A-C) TIRF images of a HEK cell expressing DsRed2-ER and YFP-STIM1 at the time of cyclopiazonic acid (CPA, 10µM) addition (A), 240 seconds after CPA addition (B) and 300 seconds after CPA addition (C). White arrows point to cER domains which remain stable throughout the experiment and accumulate YFP-STIM1 following CPA addition. (D) Graph of DsRed2-ER and YFP-STIM fluorescence from the cER domain within the white circular ROI during the first 360 seconds following CPA addition. Note the dramatic increase in YFP-STIM1 fluorescence compared to the relatively stable DsRed2-ER fluorescence.
Supplementary Figure S6. TfR-SEP exocytosis in relation to cER containing STIM1 or STIM1 and ORAI1. (A) HEK cells expressing CFP-STIM1 (pseudo-colored red) and TfR-SEP (green) imaged via TIRF microscopy. In the absence of ER Ca\(^{2+}\) depletion, STIM1 imaged in TIRF serves as a cER marker. (B) HEK cells expressing STIM1 (not shown), CFP-ORAI1 (red) and TfR-SEP (green) following ER Ca\(^{2+}\) depletion with 10µM CPA. ORAI1 expression is apparent from the diffuse membrane localized fluorescence (yellow arrow) and STIM1 activation is apparent from the fluorescent ORAI1 puncta (white arrows). (C) Bar graph illustrating the percentage of TfR-SEP exocytosis events which occur within 2 pixels of the cER (black bar) and the percentage of the cell footprint that is occupied by the cER perimeter (grey bar) based on STIM1 or STIM1-ORAI1 fluorescence.
Supplementary Figure S7. Actin depolymerization does not enhance cortical ER. HEK cells expressing GFP-CB5 to mark the ER (pseudo-colored red) and RubyRed-Lifeact to bind f-actin (pseudo-colored green) were imaged at 1 Hz in TIRF following the addition of 100 nM latrunculin A. By 25 min the cortical ER present as defined by the DsRed2-ER fluorescence in the TIR-field decrease to 77±12% (mean± sd) of control (n=12). Similar results were obtained with 200 nM Swinhohide A.
Supplementary Figure S8. Relationship between actin and ER at the cell surface. HEK cells expressing DsRed2-ER and photoactivatable GFP-actin (PA-GFP-actin) were imaged in TIRF. (A and B) DsRed2-ER and GFP fluorescence 22 sec post photo-activation via 405 nm illumination in TIRF. At this time PA-GFP-actin monomers have diffused out of the TIR-field leaving behind only f-actin. (C) Image overlay. Note that the f-actin bundles and ER occupy distinct areas. (D) Profile intensity of the line shown in C. A one μm line width was used. Here the ER distribution is unrelated to the fine cortical actin distribution (top dashed line) while the ER appears to avoid areas containing actin bundles. The background GFP fluorescence is indicated by the lower dashed line.
Supplementary Figure S9. Tubulin depolymerization does not alter the cortical ER. HEK cells expressing GFP-CB5 to mark the ER (pseudo-colored red) and mCherry-tubulin (pseudo-colored green) were imaged in TIRF before and 20 min after the addition of 2.5µM colchicine. After colchicine addition the cER area visible in the TIR-illumination field was 100.5±18.7% (mean±sd) of that prior to treatment in the 5 cells examined. The standard deviation reflects the ER dynamics seen under control conditions.
Supplementary Results

TfR exocytosis also occurs at cER enriched PM domains in hippocampal neurons. We previously demonstrated that Kv2.1 clusters form trafficking hubs for Kv channels in cultured hippocampal neurons where these clusters are expressed endogenously. However, the expression of endogenous Kv2.1 is dependent on the maturation of the neurons in culture and typically does not form clusters until 9 days in vitro (DIV). In order to determine the location of TfR exocytosis in relation to cER enriched domains in the absence of Kv2.1, we transfected DIV 4-5 rat hippocampal neurons (rHN) with TfR-SEP and DsRed2-ER and imaged them on DIV 6-8. Figure S2A is a summary figure of a rHN expressing TfR-SEP and DsRed2-ER with exocytic events occurring within 0.3µm of the cER perimeter marked with yellow dots and events occurring >0.3µm from the cER perimeter marked with blue dots. In this cell, 41 out of 51 events occurred within 0.3µm of the cER perimeter, which accounted for 25% of the footprint of the cell. Overall, 70±11% of events (n=386, from 8 cells) occurred within 0.3µm of the cER perimeter, which accounted for an average of 34±15% of the cell footprint. GFP-CLC puncta similar to the ones we observed in HEK cells were present in neurons (Supplementary Figure 2B) and 83±4% (n=515, in 7 cells) were located within 0.3µm of the cER perimeter. In these cells the area within 0.3µm of the cER perimeter accounted for 32±8%. Thus cER enriched PM domains are the preferred sites of TfR exocytosis and CCP formation in cultured hippocampal neurons.

Activated YFP-STIM1 accumulates at discrete pre-existing cER domains. Our ultrastructural study of cortical ER in HEK cells suggested that some of the cER we observe in TIRF actually forms close physical contact with the plasma membrane. To study these apparent connections in greater detail with TIRF microscopy we expressed YFP-STIM1, which is a well characterized marker for ER-PM contacts, along with DsRed2-ER. YFP-STIM1 is distributed throughout the ER under normal resting conditions, but displays a remarkable translocation to sites of contact between the ER and PM under conditions of ER Ca\(^{2+}\) depletion. Supplementary Figure 4A illustrates both DsRed2-ER and YFP-STIM1 fluorescence under normal conditions. The YFP signal was weak compared to the DsRed2-ER signal and the contrast has been adjusted so that very little overlap is apparent. Cyclopiazonic Acid (CPA, 10µM) was added at 0 seconds to deplete ER Ca\(^{2+}\) by inhibiting the sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA). In Supplementary Figure 4B-C, YFP-STIM accumulated at discrete cER domains after 240 and 300 seconds of CPA treatment, respectively. Importantly, the intensity of DsRed2-ER in these regions did not increase appreciably as indicated in Supplementary Figure 4D. Since STIM1 translocation to areas of ER-PM contact is thought to be mediated in part by direct interaction with the inner leaflet of the PM (Carrasco and Meyer, 2011), this suggests that these domains are close enough for STIM1 to contact the membrane without greatly changing the position of the ER in the TIR evanescent excitation field which decays exponentially as the distance from the coverslip increases.
**TfR exocytosis occurs frequently at STIM1-ORAI1, ER-PM contacts.** To determine if ER-PM contacts are involved in the localization of trafficking events near the cER perimeter, we repeated our TfR exocytosis study using STIM1 as an ER marker and ORAI1 as a marker for ER-PM contacts. ORAI1 is a PM Ca\(^{2+}\) channel which is localized to ER-PM contacts by interactions with activated STIM1. Supplementary Figure 5A illustrates the diffuse ER localization of CFP-STIM1 (red) prior to activation by ER Ca\(^{2+}\) depletion. Using inactive STIM1 as a marker for the cER we observed 74 ± 5% (n=4, 179 events) of TfR-SEP exocytosis events occurred within 0.3 µm (2 pixels) of the cER perimeter while the cER perimeter only accounted for 20±4% of the cell footprint. Supplementary Figure 5B illustrates the punctuate localization of CFP-ORAI1 after activation of STIM1 (not shown) by CPA. ORAI1 typically has a diffuse PM localization and only localizes into puncta following physical interaction with STIM1, so that the ORAI puncta in Supplementary Figure 5B are definitively sites of ER-PM contact. When we used ORAI1 puncta as a marker of ER-PM contacts we observed 67 ± 5% (n=5, 150 events) of TfR exocytic events occurred within 0.3 µm (2 pixels) of the ORAI1 puncta while the puncta only accounted for only 15 ± 3% of the cell footprint. In this case, it is likely that not all of the cortical ER is marked by ORAI1 clusters, perhaps accounting for the lower percentage of TfR exocytic event occurring there.
Supplementary Methods

Neuronal culture

Neurons from cryo-preserved E18 rat hippocampal dissociations were plated at a density of ~15,000 - 30,000 cells/cm² on poly-D-lysine coated glass-bottom dishes (Matek) and cultured in glial-cell conditioned neurobasal medium containing B27 supplement (Invitrogen) as previously described (O’Connell et al., 2006). Animals were deeply anesthetized with isoflurane and euthanized by decapitation according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Colorado State University. Transfections were performed after 4-8 days in culture with 2.0 μl of Lipofectamine 2000 (Invitrogen) using 0.75 μg of Kv channel-expressing plasmid DNA, 25ng of GFP-CLC, 100ng of DsRed2-ER or 500ng of TfR-SEP in 100 μl OptiMem (Invitrogen) according to the manufacturer's directions. When necessary, 0.5 μg of pSec Bir A expressing DNA was co-transfected. Two-hours after transfection, the culture medium was replaced with fresh Neurobasal/B27 media. Neurons were imaged 24-48 hours post-transfection in neuronal imaging saline consisting of (in mM) 126 NaCl, 4.7 KCl, 2.5 CaCl₂, 0.6 MgSO₄, 0.15 NaH₂PO₄, 0.1 ascorbic acid, 8 glucose, and 20 HEPES, pH7.4.