Mutant IP₃ receptors attenuate store-operated Ca²⁺ entry by destabilizing STIM–Orai interactions in Drosophila neurons

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ABSTRACT

Store-operated Ca²⁺ entry (SOCE) occurs when loss of Ca²⁺ from the endoplasmic reticulum (ER) stimulates the Ca²⁺ sensor, STIM, to cluster and activate the plasma membrane Ca²⁺ channel Orai (encoded by Olf186-F in flies). Inositol 1,4,5-trisphosphate receptors (IP₃Rs, which are encoded by a single gene in flies) are assumed to regulate SOCE solely by mediating ER Ca²⁺ release. We show that in Drosophila neurons, mutant IP₃₅R attenuates SOCE evoked by depleting Ca²⁺ stores with thapsigargin. In normal neurons, store depletion caused STIM and the IP₃₅ to accumulate near the plasma membrane, association of STIM with Orai, clustering of STIM and Orai at ER–plasma-membrane junctions and activation of SOCE. These responses were attenuated in neurons with mutant IP₃₅Rs and were rescued by overexpression of STIM with Orai. We conclude that, after depletion of Ca²⁺ stores in Drosophila, translocation of the IP₃₅ to ER–plasma-membrane junctions facilitates the coupling of STIM to Orai that leads to activation of SOCE.

KEY WORDS: Ca²⁺ signalling, Drosophila, IP₃ receptor, Orai, STIM, Store-operated Ca²⁺ entry

INTRODUCTION

Receptors that stimulate phospholipase C and, hence, formation of inositol 1,4,5-trisphosphate (IP₃) typically evoke both release of Ca²⁺ from the endoplasmic reticulum (ER) through IP₃ receptors (IP₃Rs) and Ca²⁺ entry across the plasma membrane. The latter is usually mediated by store-operated Ca²⁺ entry (SOCE), an almost ubiquitously present pathway through which empty Ca²⁺ stores stimulate Ca²⁺ entry across the plasma membrane (Putney and Tomita, 2012). The core molecular components of SOCE arestromal interaction molecule (STIM) and Orai (the gene for which is also known as Olf186-F in flies) (Hogan, 2015; Lewis, 2012). Orai forms a hexameric Ca²⁺-selective ion channel in the plasma membrane (Hou et al., 2012) and STIM is the Ca ²⁺ sensor anchored in ER membranes (Carrasco and Meyer, 2011). Ca²⁺ dissociates from the luminal EF-hand of STIM when Ca²⁺ is lost from the ER. This causes STIM to oligomerize, unmasking residues that interact with Orai, and allowing STIM to accumulate at ER–plasma-membrane junctions, where the gap between membranes is narrow enough to allow the cytosolic CAD region of STIM to bind directly to Orai (Hogan, 2015). That interaction traps STIM and Orai clusters within ER–plasma-membrane junctions and it stimulates opening of the Orai channel (Wu et al., 2014). Additional proteins also regulate SOCE, often by modulating interactions between STIM and Orai (Srikanth and Tomita, 2012) or by facilitating their interactions by stabilizing ER–plasma-membrane junctions (Cao et al., 2015) or the organization of phosphatidylinositol 4,5-bisphosphate (PIP₂)-enriched membrane domains (Sharma et al., 2013).

SOCE can be activated by thapsigargin, which depletes Ca²⁺ stores by inhibiting the ER Ca²⁺ pump, but for SOCE evoked by physiological stimuli, the Ca²⁺ stores are depleted by activation of IP₃Rs. In the present study, we use genetic manipulations in Drosophila neurons to ask whether IP₃Rs regulate SOCE solely by mediating Ca²⁺ release from the ER or whether they can also play additional roles downstream of store depletion. Drosophila is well suited to this analysis because single genes encode IP₃ R, STIM and Orai, whereas vertebrates have several genes for each of these proteins. Our results demonstrate that in Drosophila, its IP₃R contributes to assembly of the STIM–Orai complex. Comparison of results from Drosophila and vertebrates suggests that the STIM–Orai complex might assemble in plasmamembrane–ER regions equipped to allow local depletion of Ca²⁺ stores.

RESULTS

Mutant IP₃₅Rs attenuate SOCE in Drosophila

SOCE evoked by depleting ER Ca²⁺ stores with thapsigargin in Drosophila neurons was abolished by RNA interference (RNAi) treatment for STIM or Orai (see Fig. 1D; Venkiteswaran and Hasan, 2009). This is consistent with evidence that STIM and Orai are core components of SOCE. Subsequent experiments examine the role of the IP₃₅, which is encoded by a single gene (iptr) in Drosophila, in regulating SOCE. To characterize SOCE in Drosophila neurons with mutant iptr, we examined five hetero-allelic combinations of a 15-residue C-terminal deletion and three point mutations located in different parts of the IP₃₅ (Banerjee et al., 2004; Joshi et al., 2004) (Fig. 1A). We used these combinations because the adults with these mutations are viable with distinct flight phenotypes, whereas homozygotes and other hetero-allelic combinations are lethal (Joshi et al., 2004). We also used neurons heterozygous for each individual mutation. The peak Ca²⁺ signals evoked by addition of thapsigargin in Ca²⁺-free medium and the response to restoration of extracellular Ca²⁺ (SOCE) were measured in primary neuronal cultures for each genotype (Fig. 1B–D). Our use of fluo 4 fluorescence changes (AF/F₀, see Materials and Methods) to report cytosolic free Ca²⁺ concentration ([Ca²⁺]c) is vindicated by evidence that [Ca²⁺]c in unstimulated cells was unaffected by mutant IP₃₅R (Fig. S1A) and the peak fluorescence changes evoked by SOCE in wild-type neurons were only 32±14% (mean±s.d., n=9) of those evoked by saturating the indicator with Ca²⁺.

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SOCE was significantly reduced in all five itpr mutant combinations (Fig. 1D; Table S1). However, the resting $[\text{Ca}^{2+}]_c$ and thapsigargin-evoked release of Ca$^{2+}$ from intracellular stores were unaffected, confirming that mutant IP$_3$Rs selectively perturbed SOCE (Fig. 1C; Fig. S1A). The reduced SOCE in itpr mutant neurons was not, therefore, restricted to a single combination of mutant alleles. Mutant combinations in the ligand-binding domain (S224F), modulatory domain (G1891S and G2117E) and C-terminus ($\Delta$2814–2828) of the IP$_3$R all inhibited SOCE. SOCE was not significantly affected in neurons heterozygous for the individual mutants (Fig. 1D; Table S1). In adult flies, these heterozygous itpr mutants also have no significant effect on viability or flight phenotype (Banerjee et al., 2004; Joshi et al., 2004). The results establish that attenuated SOCE in Drosophila neurons is due to a perturbation of IP$_3$R function in the recessive heteroallelic mutant combinations. This conclusion is supported by evidence that RNAi-mediated knockdown of IP$_3$R also inhibited thapsigargin-evoked SOCE (Fig. 1D). It might seem surprising that so many combinations of four different itpr mutants should inhibit SOCE. However, selection of the original mutant combinations was based on flight phenotypes (Banerjee et al., 2004), and restoring SOCE can rescue these flight defects (Agrawal et al., 2010). The results so far establish that loss of IP$_3$R or mutations within IP$_3$R attenuate SOCE without affecting the Ca$^{2+}$ content of the intracellular stores. The effects are not due to loss of STIM or Orai.

**Over-expressed STIM and Orai restores SOCE in neurons with mutant IP$_3$Rs**

We used the mutant itpr combination itpr$^{-}$S224F/G1891S to examine the effects of overexpressing STIM and Orai on SOCE in cultured neurons. We chose this combination because it has been the most extensively studied of the heteroallelic mutant itpr combinations (Agrawal et al., 2010; Venkiteswaran and Hasan, 2009). The response to thapsigargin in Ca$^{2+}$-free HBM, and SOCE evoked by subsequent addition of extracellular Ca$^{2+}$ (2 mM). Rates of Ca$^{2+}$ leak and recovery from the thapsigargin-evoked Ca$^{2+}$ release, calculated from E. The colour key applies to panels E and F. STIM+, overexpression of STIM; Orai+, overexpression of Orai.
with Orai (Fig. 1D,E). This is consistent with behavioural analyses (Fig. S3A,B) (Agrawal et al., 2010). A similar restoration of SOCE upon overexpression of STIM has been reported in neurons in which IP3R expression was reduced by small interfering RNA (siRNA) (Deb et al., 2016). Hence, even though mutant IP3Rs do not affect expression of STIM or Orai, the attenuated SOCE can be compensated for by overexpressing STIM and Orai (Fig. 1D). The results so far suggest that the IP3R regulates SOCE downstream of ER Ca\(^{2+}\) release, perhaps by influencing interactions between STIM and Orai.

**Mutant IP3Rs attenuate the association of STIM with Orai after store depletion**

We tested whether IP3R mutations affect interactions between STIM and Orai using an ectopically expressed oOrai-CFP transgene with a pan-neuronal driver (Elav\(^{G115}\)). This allowed immunoprecipitation of Orai with an anti-GFP antibody. Expression of Orai–CFP did not restore SOCE in itpr mutant neurons (Fig. S3C,D). Treatment with thapsigargin enhanced the pulldown of STIM with anti-GFP antibody from lysates of wild-type brain, consistent with enhanced interaction between STIM and Orai after store depletion. However, the pulldown of STIM from thapsigargin-treated brains with mutant IP3R was much reduced (Fig. 2A,B). In the reciprocal immunoprecipitation using wild-type brain expressing STIM–CFP, thapsigargin increased the pulldown of Orai with the anti-GFP antibody (Fig. 2C,D). There was no detectable IP3R in this immunoprecipitate (data not shown), suggesting that any interaction between IP3R and STIM or Orai, whether direct or through other proteins, might be too weak to survive immunoprecipitation. It was impracticable to assess the effects of mutant IP3R in these immunoprecipitation experiments because STIM–YFP rescued the mutant IP3R phenotypes (Fig. 1D) (Agrawal et al., 2010). These results suggest that wild-type IP3R stabilizes interactions between STIM and Orai after depletion of Ca\(^{2+}\) stores.

To avoid reversal of attenuated SOCE in neurons with mutant IP3Rs after overexpression of STIM (Fig. 1D), we used immunostaining of fixed neurons to examine the effects of store depletion on the distribution of endogenous STIM, Orai and IP3R. We quantified the near-plasma-membrane distribution of STIM and IP3R by measuring either peripheral fluorescence in confocal sections across a mid-plane of the cell (Fig. 3A,B; Movies 1–4) or total fluorescence within a plane that included mostly plasma membrane (Fig. 3C,D) (see Materials and Methods).

In wild-type neurons, thapsigargin significantly increased the amount of STIM detected near the plasma membrane. This redistribution of STIM was attenuated in neurons with mutant IP3R (itpr\(^{S224F/G1891S}\)) (Fig. 3A,D). Store depletion also increased the intensity of IP3R immunostaining near the plasma membrane (Fig. 3A–D). There was no significant redistribution of IP3R in thapsigargin-treated neurons expressing mutant IP3R (Fig. 3A,B). Thapsigargin stimulated formation of STIM puncta in neurons expressing STIM–YFP (Fig. 3E), although the puncta were not detected with endogenous STIM. This is consistent with the effects of store depletion in mammalian cells, where STIM puncta are typically observed after overexpression of tagged STIM. The formation of STIM–YFP puncta after store depletion was significantly attenuated in Drosophila neurons with mutant IP3Rs (itpr\(^{S224F/G1891S}\)); and siRNA for the IP3R appeared to have a similar effect (Fig. 3E,F). The translocation of STIM and wild-type IP3R towards the plasma membrane after store depletion was not due to a general reorganization of the ER because co-staining of neurons for STIM and another ER protein (GFP-tagged protein disulphide isomerase, PDI–GFP) revealed that only STIM

![Fig. 2. Mutant IP3Rs attenuate association of STIM and Orai after store depletion.](image-url)
These results demonstrate that IP3R and STIM accumulate in peripheral ER near the plasma membrane after store depletion, and loss of IP3R mutations within it inhibits the translocation of STIM.

Mutant IP3Rs attenuate formation of Orai puncta at the plasma membrane

Using an antibody to endogenous Orai, we observed that depletion of Ca²⁺ stores with thapsigargin stimulated formation of Orai puncta in neurons expressing wild-type IP3R, but not in neurons expressing mutant IP3Rs (itpr<sup>S224F/G1891S</sup>) (Fig. 4A). Furthermore, the sparse Orai puncta that did form in neurons with mutant IP3Rs were both smaller and less intensely stained than in neurons with wild-type IP3Rs (Fig. 4B–D). Overexpression of STIM had no effect on the formation of Orai puncta in neurons with either genotype, although it partially restored SOCE (Fig. 1D). However, after overexpression of both STIM and Orai, store depletion stimulated formation of Orai puncta that were similar in neurons with wild-type or mutant IP3Rs.
The ability of overexpressed STIM and Orai to rescue formation of Orai puncta in neurons with mutant IP₃Rs coincides with a similar rescue of SOCE in mutant neurons (Fig. 1D) and of flight in flies with mutant IP₃R (Fig. S3A,B). These results suggest that overexpression of STIM with Orai can override the requirement of IP₃R for formation of Orai puncta or SOCE after store depletion. However, when STIM and Orai are expressed at native levels in Drosophila, the interaction between them, the formation of Orai puncta and the activation of SOCE are enhanced by IP₃R.

DISCUSSION

Before STIM was identified as the Ca²⁺ sensor that regulates SOCE, IP₃Rs were speculated to adopt this role (Irvine, 1990). However, thapsigargin evokes SOCE in avian DT40 cells lacking IP₃Rs (Sugawara et al., 1997) (Fig. 5A) and SOCE can be functionally reconstituted with Orai and IP₃R for formation of Orai puncta or SOCE after store depletion. However, when STIM and Orai are expressed at native levels in Drosophila, the interaction between them, the formation of Orai puncta and the activation of SOCE are enhanced by IP₃R.

In some mammalian cells, IP₃Rs have been shown to colocalize with Orai1 (Lur et al., 2011) and to interact with STIM1, Orai1 and transient receptor potential canonical channels (TRPCs) (Hong et al., 2011), but there is no functional evidence that IP₃Rs directly contribute to SOCE mediated by Orai. Block of SOCE by an antagonist of IP₃Rs, 2-aminoethoxydiphenyl borate (2-APB), was originally suggested to reflect IP₃R coupling to a SOCE channel (Ma et al., 2000), but it is now attributed to direct inhibition of STIM and Orai by 2-APB. However, most analyses of SOCE use thapsigargin to completely deplete intracellular Ca²⁺ stores, and overexpressed proteins to track movements of Orai and STIM. These exaggerated conditions successfully identify key features of

![Fig. 4. Mutant IP₃Rs attenuate clustering of Orai after store depletion.](image-url)

(A) Typical confocal images from neurons with or without thapsigargin (TG) treatment and immunostained for endogenous Orai. The effects of the mutant IP₃R combination (S224F/G1891S, red boxes), and overexpression of STIM alone (STIM⁺) or with Orai (STIM⁺/Orai⁺) are shown. Scale bar: 5 µm. The representative confocal images show z-stacks of the deconvoluted sections. Each section was analysed individually for the summary analyses. (B–D) Summary data for the number of puncta/cell (B), intensity of fluorescence within puncta (C) and size of puncta (D). The colour key applies to all three panels. In B–D, the box represents the 25–75th percentiles, and the median is indicated. The whiskers show the 10–90th percentiles. Outliers are represented by dots. **P<0.01, Kruskal–Wallis test, followed by Wilcoxon signed-rank post-hoc test (>150 cells from at least three independent experiments).
SOCE, but they might override more subtle modulatory influences, including, for example, the contribution of IP$_3R$ to recruitment of STIM1 to ER–plasma-membrane junctions (Hogan, 2015; Park et al., 2009). We used DT40 cells with and without IP$_3R$1 (encoded by itpr1) and examined SOCE after graded depletion of intracellular Ca$^{2+}$ stores to assess whether partially depleted stores might more effectively activate SOCE in the presence of IP$_3R$. However, the relationship between store depletion and SOCE was unaffected by expression of IP$_3R$1 (Fig. 5A). Hence, there is no compelling evidence to suggest that the contribution of IP$_3R$1 to SOCE in Drosophila is a feature shared with vertebrates.

Inhibition of Orai clustering in Drosophila neurons with mutant IP$_3R$s is reminiscent of the effects of septin depletion in mammalian cells. Septin 4 concentrates PIP$_2$ around Orai1 and facilitates recruitment of STIM1 through its polybasic cytoplasmic tail (Sharma et al., 2013). Assembly of STIM–Orai complexes at PIP$_2$-enriched plasma membrane domains concentrates the complexes at regions best equipped to sustain production of the IP$_3$ that evokes Ca$^{2+}$ release from stores. Such colocalization of Ca$^{2+}$ release and SOCE might be important because activation of SOCE by physiological stimuli probably requires substantial local depletion of intracellular Ca$^{2+}$ stores (Bird et al., 2009; Luik et al., 2008). However, Drosophila STIM lacks the polybasic tail through which mammalian STIM1 binds to PIP$_2$ (Huang et al., 2006). Association of Drosophila STIM with IP$_3R$, which might itself bind to PIP$_2$ (Lupu et al., 1998), could serve a function analogous to PIP$_2$-mediated targeting of STIM1 in mammals. Recent evidence demonstrating a link between Septin 7, IP$_3R$ and SOCE (Deb et al., 2016) suggests that IP$_3R$ might influence STIM–Orai interactions within a larger macromolecular complex. We speculate that interaction of mammalian STIM1 with PIP$_2$ might ensure that intracellular stores locally depleted of Ca$^{2+}$ by IP$_3R$ are effectively localized to STIM–Orai complexes (Fig. 5B). Translocation of both IP$_3R$ and STIM to ER–plasma-membrane junctions after store depletion, where IP$_3R$ facilitates the interaction of STIM with Orai, might fulfill a similar role in Drosophila (Fig. 5B).

**MATERIALS AND METHODS**

**Drosophila strains**

Single-point mutants of the itpr gene were characterized as described previously (Joshi et al., 2004; Srikanth et al., 2004). UAS transgenic strains were generated by injecting Drosophila embryos with a pUAST construct. All fly strains, including Elav$^{GAL4}$ (pan neuronal, Bloomington Stock Center, Indiana University, Bloomington, IN), and RNAi lines for itpr (no. 1063, National Institute of Genetics, Japan), STIM (no. 47073) and Orai (no. 12221) were procured from the Vienna Drosophila Resource Centre, Austria. The Canton-S strain was used as the wild-type control.

**Measurements of [Ca$^{2+}$]$_c$ in primary cultures of Drosophila neurons**

Materials, unless stated otherwise, were from ThermoFisher Scientific (Waltham, MA). Methods for primary cultures were adapted from Wu et al. (1983). The brain and ventral ganglia from Drosophila third-instar larvae were dissociated by incubation for 20 min at 25°C in Schneider’s medium containing collagenase (0.75 µg/µl) and dispase (0.4 µg/µl, Roche, Burgess Hill, UK). After centrifugation (600 g for 5 min), cells were plated onto poly-l-lysine-coated coverslips in HEPES-buffered medium (HB1, in mM: HEPES (30), NaCl (150), KCl (5), MgCl$_2$ (1), CaCl$_2$ (1), sucrose (35), pH 7.2) or (for most experiments) Dulbecco’s modified Eagle’s medium (DMEM) with F12 and Glutamax-I, NaHCO$_3$ and sodium pyruvate, and supplemented with 20 mM HEPES (pH 7.2) and 10% fetal bovine serum. This enriched medium substantially reduced the heterogeneity of the Ca$^{2+}$ signals between neurons. All culture media contained 50 units/ml penicillin, 50 µg/ml streptomycin and 10 µg/ml amphotericin B. Cells were incubated at 25°C in humidified air with 5% CO$_2$. After 14–16 h, cells were loaded with fluo 4 by incubation at 25°C for 30 min with fluo 4-AM (2.5 µM) and Pluronic F-127 (0.02%) in HB1. Medium was then replaced with HB1, and after a further 10–30 min, with Ca$^{2+}$-free HB1 containing 0.5 mM EGTA. Cells were immediately imaged at 15-s intervals with excitation at 488 nm and emission at 520 nm using a Nikon TE2000 microscope with a 60×1.4 NA objective, Evolution QEi CCD camera and QED imaging software (Media Cybernetics, Rockville, MD). Background fluorescence (measured from an area without cells) was subtracted from all measurements before calculation of $\Delta F/F_0$. 

Fig. 5. Coordination of store depletion and SOCE in insects and vertebrates. (A) DT40-KO or DT40-IP$_3$R1 cells were incubated with different concentrations of a reversible inhibitor of the ER Ca$^{2+}$ pump, cyclopiazonic acid (CPA, 0.1–3 µM) for 15 min in Ca$^{2+}$-free HBS. In parallel wells, the peak increase in [Ca$^{2+}$]$_c$ evoked by ionomycin (1 µM, to determine the Ca$^{2+}$ content of the intracellular stores) or restoration of extracellular Ca$^{2+}$ (1.5 mM, to determine SOCE) were measured. Results (means±s.e.m., n=3, with three replicates in each) show the relationship between store depletion and SOCE for the two cell lines. (B) Substantial loss of Ca$^{2+}$ from the ER causes STIM to cluster and assemble with Orai at ER–plasma-membrane junctions. The polybasic cytoplasmic tail of vertebrate STIM1 binds to PIP$_2$ within the plasma membrane and contributes to its targeting to junctions. Drosophila STIM lacks a PIP$_2$-binding motif, but, after store-depletion, Drosophila STIM moves to ER–plasma-membrane junctions, and so too does IP$_3R$ where it might bind to PIP$_2$. Physiological stimuli, through IP3, probably trigger the large decrease in luminal [Ca$^{2+}$] needed to activate STIM1 in only a subset of the ER. Targeting of vertebrate STIM1 to plasma membrane enriched in the PIP$_2$, from which IP$_3$ is synthesized, ensures that the machinery needed to locally deplete Ca$^{2+}$ stores remains closely associated with essential components of the SOCE pathway. We speculate that in Drosophila, association of IP$_3$R with STIM and Orai at ER–plasma-membrane junctions might fulfill a similar role.
where \( F_0 \) is the initial fluorescence and \( AF \) is the difference between basal and peak fluorescence.

**Measurements of SOCE in DT40 cells**

Avian DT40 cells in which endogenous IP₃R genes are disrupted (DT40-KO cells) (Sugawara et al., 1997) or the same cells stably expressing rat IP₃R1 (DT40-IP₃R1) were used to determine the contribution of IP₃R to SOCE in calibrated to \([\text{Ca}^{2+}]_c\) from:

\[
\text{excitation 485 nm, emission 525 nm}\]

was recorded at 1.44-s intervals at area 96-well plates. After centrifugation (300 g, 2 min), re-suspended in HBS (5×10⁶ cells/ml) and distributed (50 µl/well) into polystyrene-coated half-area 96-well plates. After centrifugation (300 g, 2 min) fluorescence (excitation 485 nm, emission 552 nm) was recorded at 1.44-s intervals at 20°C in a FlexStation 3 plate-reader. Fluorescence signals (F) were calibrated to \([\text{Ca}^{2+}]_c\)

\[
\frac{[\text{Ca}^{2+}]_c}{[\text{Ca}^{2+}]_{\text{in}}] = \frac{F_{\text{min}}}{F_{\text{max}} - F}
\]

where, \( F_{\text{min}} \) and \( F_{\text{max}} \) are the fluorescence values determined in parallel wells by addition of Triton X-100 (0.1% w/v) and either BAPTA (10 mM) for \( F_{\text{min}} \) or CaCl₂ (10 mM) for \( F_{\text{max}} \), and \( K_{\text{Ca}} = 345 \) nM.

**Immunoprecipitation, western blotting and immunocytochemistry**

For immunoprecipitation analyses, neuronal cultures were stimulated, washed and lysed in cold PBS (pH 7.4) containing 1% NP-40 and 5 mM EDTA, Roche protease inhibitor tablet and 10 µM MG-132. The lysate was homogenized by passage through a 26 G needle, mixed (30 min, 4°C) and after centrifugation (14,000 g, 20 min), the supernatant (0.5 µg protein/µl in PBS) was incubated with Dynabeads (2 mg) bound to anti-GFP antibody (15 µg, #A-11122). After 18–20 h at 4°C, the beads were washed and lysed according to the manufacturer’s protocol, and used for western blotting. The antibodies used were: *Drosophila* STIM (1:10; Abexome, Bangalore, India) (Agrawal et al., 2010), GFP (1:5000; #SC9996, Santa Cruz Biotechnology, Dallas, TX) and tubulin (1:5000; #E7, Developmental Studies Hybridoma Bank, University of Iowa, IA). HRP-conjugated anti-rabbit-IgG (#32260; Thermo Scientific), anti-mouse-IgG (#70767; Cell Signaling Technologies, Danvers, MA) and anti-rat-IgG (#012030003; Jackson ImmunoResearch, West Grove, PA) secondary antibodies were used and visualized with SuperSignal West Dura Extended Duration Substrate. For immunostaining, methods were adapted from Wegener et al. (2004). Cultured neurons were treated with thapsigargin and then fixed (30 min, 25°C, 3.5% paraformaldehyde and 0.5% glutaraldehyde in PBS), washed three times (PBS with 0.5% BSA, 0.05% Triton X-100 and 0.05% glycerol) and permeabilized (1 h, PBS with 5% BSA, 0.5% Triton X-100, 0.5% glycerol). Cells were incubated for 12 h with primary antibody (rabbit for *Drosophila* IP₃R (1:300) (Srikanth et al., 2004), mouse for *Drosophila* STIM (1:10) (Agrawal et al., 2010) and rat for *Drosophila* Orai (1:1000) (Pathak et al., 2015)). Cells were then washed, incubated (30 min, 4°C) with appropriate secondary antibody (1:500) conjugated to Alexa Fluor 488 (#A11088; Thermo Scientific), Alexa Fluor 594 (#20185) or Alexa Fluor 633 (#A201948) and washed. Images were acquired using an Olympus laser-scanning confocal microscope with 60×1.3 NA oil immersion objective. All images were corrected for background by subtraction of fluorescence recorded outside the cell.

**Image analysis**

Confocal images were deconvoluted using Huygens 4.5 software (SVI, The Netherlands) as described previously (Deb et al., 2016). To quantify the peripheral fluorescence of immunostained IP₃R in each neuron, most of which have a near-circular profile (Fig. 3A), an automated algorithm (Matlab) was used to identify the confocal section with the maximal perimeter. Within this section, which we describe as the ‘mid-section’, the centre of the cell was identified and an average radius calculated (r). Fluorescence intensities were then calculated for the central circular region (with r2) and for the remaining peripheral annulus. The ratio (peripheral fluorescence to total fluorescence) was then used to report the redistribution of IP₃R.

To quantify near-plasma-membrane immunostaining of STIM and IP₃R, we used Orai immunostaining to manually identify the confocal section within which most plasma membrane apposed the coverslip (Fig. 3C). The fluorescence intensity within this optical section relative to that from the entire cell was used to report the near-plasma-membrane distribution.

To quantify the distribution of STIM–YFP (Fig. 3G,H) and Orai (Fig. 4) puncta, every confocal section (~15 sections/cell) was analysed (Deb et al., 2016). Puncta were identified automatically (Matlab) as fluorescence spots that exceeded average cellular fluorescence by at least 1.9× the s.d. and occupied a square with sides of 1–12 pixels (1 pixel=103 nm×103 nm) with a circularity of 0–0.3. Analysis of sequential sections within the z-stack allowed non-redundant counting of puncta, from which the total number of puncta/cell was determined. The section in which a punctum had the brightest intensity was used for analysis, and then normalized to the mean intensity of Orai for the cell.

**Statistical analysis**

Most data were analysed using non-parametric methods (Kruskal–Wallis test for variance followed by Wilcoxon signed-rank post-hoc tests). These data are presented as box and whisker plots showing medians, 25–75th percentiles (boxes), 10–90th percentiles (whiskers) and points for values beyond the 10th and 90th percentiles. Student’s t-tests were used for statistical analysis of western blots (Fig. 2B) and Ca²⁺ signals in DT40 cells (Fig. 5A).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

S.C., B.K.D. and T.C. performed experiments with *Drosophila*. V.K. performed experiments with DT40 cells. S.C., G.H. and C.W.T. designed and interpreted experiments, analysed data and wrote the paper with input from all authors.

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