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**Remodeling neuronal ER-PM junctions is a conserved nonconducting function of Kv2
plasma membrane ion channels**

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Abbreviated title: Kv2 ion channel-based ER-PM junctions

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Abstract

Endoplasmic reticulum (ER) and plasma membrane (PM) form junctions crucial to ion and lipid signaling and homeostasis. The Kv2.1 ion channel is localized at ER-PM junctions in brain neurons and is unique among PM proteins in its ability to remodel these specialized membrane contact sites. Here, we show that this function is conserved between Kv2.1 and Kv2.2, which differ in their biophysical properties, modulation and cellular expression. Kv2.2 ER-PM junctions are present at sites deficient in the actin cytoskeleton, and disruption of actin cytoskeleton affects their spatial organization. Kv2.2-containing ER-PM junctions overlap with those formed by canonical ER-PM tethers. The ability of Kv2 channels to remodel ER-PM junctions is unchanged by point mutations that eliminate their ion conduction but eliminated by point mutations within the Kv2-specific PRC domain that do not impact their ion channel function. The highly conserved PRC domain is sufficient to transfer the ER-PM junction remodeling function to another PM protein. Lastly, brain neurons in Kv2 double knockout mice have altered ER-PM junctions. Together, these findings demonstrate a conserved *in vivo* function for Kv2 family members in remodeling neuronal ER-PM junctions that is distinct from their canonical role as ion-conducting channels shaping neuronal excitability.

INTRODUCTION

Specialized membrane contact sites between the endoplasmic reticulum (ER) and plasma membrane (PM), or ER-PM junctions, are a ubiquitous feature of eukaryotic cells (Henne *et al.*, 2015; Gallo *et al.*, 2016; Chang *et al.*, 2017; Saheki and De Camilli, 2017a). These specialized sites at which ER is held in close apposition (10-30 nm) to the PM represent critical platforms for mediating Ca^{2+} homeostasis and signaling events and as ER and PM lipid metabolism and transport hubs (Dickson, 2017; Balla, 2018). ER-PM junctions are typically classified based on the resident ER protein serving as the PM tether and that are members of the Extended Synaptotagmin or E-Syt (Saheki and De Camilli, 2017b), Junctophilin or JP (Takeshima *et al.*, 2015), or the Stromal Interacting Molecule or STIM (Prakriya and Lewis, 2015) families. These otherwise unrelated ER membrane proteins have a common membrane topology with a large cytoplasmic domain that mediates binding to specific classes of phospholipids in the inner leaflet of the PM (Carrasco and Meyer, 2011; Henne *et al.*, 2015). The STIM proteins can also reversibly bind to PM Orai proteins in a process triggered by ER Ca^{2+} depletion (Prakriya and Lewis, 2015). While mRNA measurements have shown that many of these ER-localized tethering proteins have high levels of expression in brain [e.g., (Nishi *et al.*, 2003; Min *et al.*, 2007; Moccia *et al.*, 2015; Takeshima *et al.*, 2015)], little is known of the cellular or subcellular localization of the corresponding proteins relative to the different classes of abundant ER-PM junctions observed in ultrastructural studies of brain neurons (Rosenbluth, 1962; Henkart *et al.*, 1976; Wu *et al.*, 2017).

Plasma membrane voltage-gated K^+ or Kv channels play crucial and diverse roles in shaping neuronal function (Trimmer, 2015). Among these, the Kv2 family contains two members: Kv2.1 and Kv2.2. Like other Kv channels, Kv2.1 and Kv2.2 are key determinants of action potential characteristics and intrinsic electrical excitability in many types of mammalian brain neurons (Du *et al.*, 2000; Malin and Nerbonne, 2002; Guan *et al.*, 2007; Johnston *et al.*, 2008; Liu and Bean, 2014; Pathak *et al.*, 2016; Honigspurger *et al.*, 2017; Palacio *et al.*, 2017),

and *de novo* mutations in Kv2.1 are associated with devastating neonatal encephalopathic epilepsies and neurodevelopmental delays (Torkamani *et al.*, 2014; Saitsu *et al.*, 2015; Thiffault *et al.*, 2015; de Kovel *et al.*, 2016). Kv2 channels are also prominently expressed in pancreatic islets (Jacobson *et al.*, 2007; Li *et al.*, 2013), smooth muscle cells (Patel *et al.*, 1997; Schmalz *et al.*, 1998), and other excitable and non-excitable cell types. In brain neurons, Kv2 channels are distinct from other Kv channels (Trimmer, 2015) in being specifically localized to high-density micron sized clusters prominent on the soma, proximal dendrites, and axon initial segment (Trimmer, 1991; Scannevin *et al.*, 1996; Du *et al.*, 1998; Murakoshi and Trimmer, 1999; Lim *et al.*, 2000; Kihira *et al.*, 2010; Mandikian *et al.*, 2014; Bishop *et al.*, 2015; Bishop *et al.*, 2018). Kv2 channels also form such clusters when exogenously expressed in cultured neurons and in heterologous cells (Scannevin *et al.*, 1996; Lim *et al.*, 2000; O'Connell and Tamkun, 2005; Mohapatra and Trimmer, 2006; O'Connell *et al.*, 2006; Tamkun *et al.*, 2007; Kihira *et al.*, 2010; Bishop *et al.*, 2015; Cobb *et al.*, 2015; Bishop *et al.*, 2018). A short Proximal Restriction and Clustering (PRC) domain within the relatively large cytoplasmic C-terminus of Kv2.1 is necessary for its clustered localization in neurons and heterologous cells (Scannevin *et al.*, 1996; Lim *et al.*, 2000), and is sufficient to transfer Kv2.1-like clustering to other Kv channels (Lim *et al.*, 2000; Mohapatra and Trimmer, 2006). A point mutation within the highly conserved PRC motif also results in loss of Kv2.2 clustering (Bishop *et al.*, 2015; Bishop *et al.*, 2018).

Immunoelectron microscopy-based studies have shown that immunoreactivity for PM Kv2.1 (Du *et al.*, 1998; Mandikian *et al.*, 2014; Bishop *et al.*, 2018) and Kv2.2 (Bishop *et al.*, 2015) is associated with subsurface cisternae, a class of ER-PM junctions that are prominent in somata of brain neurons (Rosenbluth, 1962; Henkart *et al.*, 1976; Wu *et al.*, 2017). In certain brain neurons, clusters of PM Kv2.1 channels overlay clusters of ER-localized ryanodine receptor (RyR) Ca²⁺ release channels (Antonucci *et al.*, 2001; Misonou *et al.*, 2005b; King *et al.*, 2014; Mandikian *et al.*, 2014) which are concentrated at ER-PM junctions to mediate local Ca²⁺ signaling events in diverse cell types (Franzini-Armstrong and Jorgensen, 1994; Sun *et al.*,

1995). Recent studies revealed that in addition to being localized to ER-PM junctions, exogenous expression of Kv2.1 leads to recruitment and/or stabilization of ER-PM junctions in heterologous cells and cultured hippocampal neurons or CHNs (Fox *et al.*, 2015). The ability of Kv2.1 to remodel ER-PM junctions exhibits the same phosphorylation-dependent regulation as Kv2.1 clustering (Cobb *et al.*, 2015), which itself is regulated by numerous stimuli that impact Kv2.1 phosphorylation state (Misonou *et al.*, 2004; Misonou *et al.*, 2005a; Cerda and Trimmer, 2011; Bishop *et al.*, 2015; Bishop *et al.*, 2018). It is not currently known whether the remodeling of ER-PM junctions seen upon heterologous expression of Kv2.1 is a result of the impact of the expressed channel's K⁺ conductance on the membrane potential and/or cellular ion homeostasis in the expressing cell [*e.g.*, (Felipe *et al.*, 1993; Hegle *et al.*, 2006; Jimenez-Perez *et al.*, 2016)]; established mechanisms for remodeling ER-PM junctions (Carrasco and Meyer, 2011; Henne *et al.*, 2015; Saheki and De Camilli, 2017b; Balla, 2018). Alternatively, Kv2.1 could remodel ER-PM junctions through a more direct structural role. Despite having a conserved clustered localization and a highly conserved PRC domain, Kv2.1 and Kv2.2 share only 61% overall amino acid identity, which drops to only 39% in their respective cytoplasmic C-termini that comprises about half of their primary structure. Kv2.1 and Kv2.2 have distinct biophysical properties [*e.g.*, (Dong *et al.*, 2013; Baver *et al.*, 2014)] and expression patterns [*e.g.*, (Hwang *et al.*, 1992; Hwang *et al.*, 1993a; Hwang *et al.*, 1993b; Johnston *et al.*, 2008; Li *et al.*, 2013; Bishop *et al.*, 2015; Bishop *et al.*, 2018)]. Moreover, stimuli that trigger reversible modulation of voltage activation [*e.g.*, (Baver *et al.*, 2014; Mandikian *et al.*, 2014; Bishop *et al.*, 2015)] and dispersal of clustering (Bishop *et al.*, 2015) of Kv2.1 do not have a detectable impact on Kv2.2, leading to questions as to whether Kv2.2 is also distinct from Kv2.1 in its ability to remodel ER-PM junctions. Lastly, it is not known how ablating expression of endogenous Kv2 channels impacts ER-PM junctions in brain neurons *in situ*.

Here, we define the localization of Kv2.2 relative to ER-PM junctions in brain neurons *in situ* and in culture and determine whether like Kv2.1 it also functions to remodel ER-PM

junctions. We define the relationship of Kv2-containing ER-PM junctions to the actin cytoskeleton and to other classes of molecular-defined ER-PM junctions. We employ a strategic set of point mutations in Kv2.2 and Kv2.1 to dissect the respective contributions of K⁺ conduction and clustering to the Kv2-mediated remodeling of ER-PM junctions, and also determine the domain necessary and sufficient for this function that among PM proteins is unique to Kv2.1. Finally, we use recently generated double knockout mice lacking expression of both mammalian Kv2 channel family members to determine their *in vivo* role in regulating ER-PM junctions in brain neurons *in situ*. Our results provide compelling evidence for a conserved and non-canonical role for nonconducting Kv2 channels in impacting ER-PM junctions in brain neurons and other cell types in which these ion channels are abundantly expressed.

RESULTS

Plasma membrane clusters of Kv2.2 associate with ER-PM junctions in mammalian brain neurons *in situ* and in culture, and in heterologous HEK293T cells

Kv2.2 is present in clusters on the soma, proximal dendrites and axon initial segments of mammalian brain neurons (Johnston *et al.*, 2008; Kihira *et al.*, 2010; Bishop *et al.*, 2015; Bishop *et al.*, 2018). To investigate the subcellular localization of these Kv2.2 clusters relative to native ER-PM junctions in brain neurons, we performed multiplex immunofluorescence labeling for PM Kv2.2 and ER-localized RyR Ca²⁺ release channels, which are concentrated at ER-PM junctions in many cell types including certain types of brain neurons. In mouse brain sections, somatic Kv2.2 clusters were found at/near RyR clusters in specific neuron types, including hippocampal CA1 pyramidal neurons and layer 6 neocortical neurons (Figure 1A and B). A similar juxtaposition of Kv2.2 and RyR clusters was seen in cultured hippocampal neurons or CHNs (Figure 1C). In these neurons, Kv2.2 was often found coclustered with Kv2.1 at ER-PM junctions (Figure 1C). Neurons in each preparation also contained RyR clusters that did not appear to colocalize with Kv2.2 or Kv2.1 suggesting the presence of other classes of ER-PM

junctions in these cells (Figure 1A-C). These findings demonstrate that Kv2.2 clusters localize to RyR-containing ER-PM junctions in intact mammalian brain neurons *in situ* and in culture.

We next determined whether heterologously expressed and clustered Kv2.2 localizes to ER-PM junctions. In HEK293T cells coexpressing GFP-tagged Kv2.2 and BFP-tagged SEC61 β [a general ER marker; (Zurek *et al.*, 2011)], optical sections taken through the center of cells show fingerlike projections of SEC61 β -positive ER, a subset of which were associated with PM Kv2.2 clusters that appear as discrete PM segments (Figure 1D). Three-dimensional reconstructions show that the ER projections terminating at Kv2.2-associated PM clusters were contiguous with bulk ER (Figure 1D, Supplemental Movie 1). Together these results suggest that Kv2.2 localizes to ER-PM junctions in mammalian brain neurons and when heterologously expressed in HEK293T cells.

Exogenous Kv2.2 expression remodels ER-PM junctions in cultured rat hippocampal neurons and heterologous cells

We next determined the impact of exogenous expression of recombinant Kv2.2 on ER-PM junctions in mammalian neurons and heterologous cells. We used Total Internal Reflection Fluorescence (TIRF) microscopy of living cells to selectively visualize fluorescence signals within \approx 100 nm of the coverslip, including near-PM ER (*i.e.*, ER at ER-PM junctions). In HEK293T cells expressing the fluorescent luminal ER marker DsRed2-ER5 [a general ER marker; (Day and Davidson, 2009)], the near-PM ER appeared as a highly ramified system of small reticular tubules and puncta (Figure 2A), the latter representing focal structures of cortical ER coincident with the PM or ER-PM junctions (Fox *et al.*, 2015; Chang *et al.*, 2017; Besprozvannaya *et al.*, 2018). Exogenous expression of GFP-Kv2.2 led to a remodeling of the DsRed2-ER5-positive cortical ER to form larger foci that colocalized with the PM clusters of Kv2.2 (Figure 2A and B). Cells co-expressing GFP-Kv2.2 exhibited a significant increase in both the size of ER-PM junctions (Figure 2C) and the percentage of basal cell surface area with

associated cortical ER (Figure 2D). No such changes were seen in cells expressing a related but distinct Kv channel, Kv1.4 (Figure 2A-D), which is not localized to ER-PM junctions in neurons (Trimmer, 2015). Analysis of colocalization using Pearson's Correlation Coefficient (PCC) measurements revealed that DsRed2-ER5 was significantly more colocalized with Kv2.2 than it was with Kv1.4 (Figure 2E). We also found a nearly linear relationship between the sizes of Kv2.2 clusters and ER-PM junctions (Figure 2H, red points). As previously reported (Fox *et al.*, 2015), significant increases in ER-PM junction size and ER-associated PM surface area were also observed in cells expressing Kv2.1 (Figure 2A-E). Taken together, these data demonstrate that Kv2.2 can remodel ER-PM junctions, and that this is a conserved function of Kv2 channels not shared with Kv1.4.

We next expressed DsRed2-ER5 alone or coexpressed DsRed2-ER5 with GFP-Kv2.2 in CHNs. TIRF imaging experiments revealed that GFP-Kv2.2 expression also remodeled neuronal ER-PM junctions (Figure 2F-G). Similar to HEK293T cells, we found a nearly linear relationship between the sizes of GFP-Kv2.2 clusters and ER-PM junctions in CHNs (Figure 2H, black points). These results demonstrate that exogenous expression of Kv2.2 in both HEK293T cells and CHNs is sufficient to remodel ER-PM junctions.

Kv2.2 channels associated with ER-PM junctions are on the cell surface

Given the extensive colocalization of Kv2.2 and these ER markers at ER-PM junctions, we next verified that the Kv2.2 present at these sites was in the PM by performing live cell labeling with the membrane-impermeant and Kv2-specific tarantula toxin Guangxitoxin-1E or GxTX (Herrington *et al.*, 2006). We used fluorescent GxTX conjugated to DyLight633 or GxTX-633 (Tilley *et al.*, 2014) to label cell surface Kv2.2. We first validated this approach by coexpressing BFP-SEC61 β with SEP-Kv2.1, a construct of Kv2.1 tagged with cytoplasmic mCherry and an extracellular pHluorin as a reporter of cell surface Kv2.1 (Jensen *et al.*, 2017). We observed extensive colocalization of GxTX-633 and pHluorin signals (Supplemental Figure 1), showing

that GxTX-633 is a reliable reporter for cell surface Kv2 channels. No detectable GxTX-633 labeling was observed in control HEK293T cells, or those expressing DsRed2-ER5 alone (data not shown). GxTX-633 labeling of cells coexpressing GFP-Kv2.2 and DsRed2-ER5 showed a high degree of colocalization of all three signals (Figure 3A and B). As expected, PCC measurements were slightly but significantly higher for the signals for GxTX-633 directly bound to GFP-Kv2.2 than for the GxTX-633 labeling indirectly associated with the DsRed2-ER5 present at Kv2.2-associated ER-PM junctions (Figure 3C). Taken together, these data demonstrate that the clusters of Kv2.2 associated with ER-PM junctions are on the cell surface.

Kv2.2-containing ER-PM junctions are present at sites depleted in components of the cortical actin cytoskeleton

We next determined the relationship of Kv2.2-containing ER-PM junctions to the actin cytoskeleton. In addition to its prominent clustering on the soma and proximal dendrites as shown in Figure 1, Kv2.2 is also localized to the axon initial segment or AIS (Johnston *et al.*, 2008; Sanchez-Ponce *et al.*, 2012), a subcellular compartment highly enriched for components of the actin cortical cytoskeleton including a specialized complex of spectrins and ankyrins (Leterrier, 2016). We immunolabeled brain sections for Kv2.2 and ankyrinG (ankG), which is highly expressed at the AIS. We found that in neocortical layer 5 pyramidal neurons, Kv2.2 was present in robust clusters located on the AIS, as marked by ankG (Figure 4A). However, within the AIS, Kv2.2 clusters were present at sites deficient in ankG (Figure 4A). These ankG-deficient sites or “holes” represent locations at which the ER present in the AIS, termed the cisternal organelle, comes into close apposition to the PM (Sanchez-Ponce *et al.*, 2011; King *et al.*, 2014; Schluter *et al.*, 2017).

We next immunolabeled for endogenous Kv2.2 and ankG in CHNs and found a similar relationship between the sites of Kv2.2 clustering on the AIS and regions deficient in both ankG and filamentous actin, the latter labeled with fluorescent phalloidin (Figure 4B and C). This is

apparent in line scan analyses, which revealed that the intensity profiles of the Kv2 immunolabeling and actin labeling were often negatively correlated (Figure 4). To determine whether this spatial relationship is also present in non-neuronal cells, we performed TIRF imaging on live HEK293T cells coexpressing GFP-Kv2.2, BFP-SEC61 β and mCherry-tagged actin. We found that GFP-Kv2.2 clusters and associated ER-PM junctions displayed a negatively correlated distribution with respect to cortical mCherry-actin (Figure 4D). We next coexpressed ankG-mCherry with BFP-SEC61 β and Kv2.2 and found, similar to as observed on the AIS, a negatively correlated distribution of Kv2.2 clusters and their associated ER-PM junctions with ankG (Figure 4D), showing that the distinct localization of the endogenous proteins seen on the AIS of brain neurons could be recapitulated upon exogenous expression of these proteins in heterologous cells.

The actin cytoskeleton regulates the spatial organization of Kv2.2 clusters and associated ER-PM junctions

Given the distinct spatial relationship between Kv2.2-associated ER-PM junctions and the cortical actin cytoskeleton, we next determined the impact of disrupting the organization of the actin cytoskeleton on Kv2.2-mediated ER-PM junctions. We treated cells expressing Kv2.2 with Latrunculin A (LatA) which disrupts the organization of filamentous actin (Spector *et al.*, 1983). We found LatA treatment led to a parallel reorganization of both Kv2.2 clusters and their associated ER-PM junctions (Figure 5A and B, Supplemental Movie 2). This resulted in parallel significant increases in the sizes of both Kv2.2 clusters and ER-PM junctions (Figure 5C and D), the latter reported by the DsRed2-ER5 signal coincident with the PM. The total number of ER-PM junctions in Kv2.2-expressing cells was also significantly reduced in response to LatA treatment (Figure 5E). Similar results were obtained upon LatA treatment of cells coexpressing GFP-Kv2.1 and DsRed2-ER5 (Figure 5C-E), as suggested by a previous study (Fox *et al.*, 2015). These changes were not observed in untreated cells over the course of 15 minutes (data

not shown). While LatA treatment significantly altered the spatial characteristics of Kv2.2 clusters and their associated ER-PM junctions, the extent of colocalization between GFP-Kv2.2 and DsRed2-ER5 was not significantly altered upon LatA treatment (Figure 5F). Similar results were obtained for Kv2.1 (Figure 5F). These results show that while LatA induced an apparent fusion of Kv2 clusters and associated ER-PM junctions resulting in fewer, larger structures, these changes occurred in parallel and did not affect their association *per se*. These results also suggest that the distinct and mutually exclusive localization of Kv2.2 clusters and components of the cortical actin cytoskeleton seen in brain neurons likely contributes in the organization and maintenance of these clusters and their associated ER-PM junctions.

Kv2.2-containing ER-PM junctions associate with ER-PM junctions formed by the known classes of ER-PM tethers

We next determined the relationship of Kv2.2 clusters and associated ER-PM junctions with those formed upon exogenous expression of the three other families of mammalian ER-localized ER-PM tethers. We coexpressed FP-tagged Kv2.2 and individual members of the E-Syt, JP and STIM families in HEK293T cells. In cells coexpressing the STIMs, we also induced Ca²⁺ store depletion *via* treatment with 2 μ M thapsigargin treatment for five minutes. In all cases, we observed a high degree of colocalization between clusters of Kv2.2 and these ER-PM junction tethers (Figure 6A), as demonstrated by high PCC and Mander's overlap coefficient (MOC) values (Figure 6B and C). In cells coexpressing STIM1, Kv2.2 and Orai1, store depletion resulted in not only a significant increase in colocalization of STIM1 and Orai1, but also of Orai1 and Kv2.2 (Figure 6D and F). The store depletion-induced increase in colocalization of Kv2.2 and Orai1 also occurred in the absence of STIM1 coexpression (Figure 6E and G), presumably due to endogenous STIM expression in HEK293T cells (Williams *et al.*, 2001; Soboloff *et al.*, 2006; Brandman *et al.*, 2007; Shalygin *et al.*, 2015). Together, these results show that Kv2.2 clusters can associate with ER-PM junctions formed by the three established families of ER-PM

junction tethers. Interestingly, the PCC values between Kv2.2 and these ER tethers were significantly lower than the corresponding MOC values obtained from the same cells (Figure 6B and C), suggesting that despite the extensive overlap in signal between Kv2.2 clusters and these established classes of ER-PM junctions, there are distinctions in their fine spatial organization relative to one another.

We next examined the relationship of Kv2-mediated ER-PM junctions to those formed acutely by a rapamycin-inducible system (Inoue *et al.*, 2005; Dickson *et al.*, 2016) employing ER-localized CB5-FKBP-CFP and PM-localized Lyn11-FRB (CB5/Lyn11). TIRF imaging reveals that acute treatment of HEK293T cells coexpressing CB5/Lyn11 with 5 μ M rapamycin yields robust recruitment of ER to the cell cortex (Figure 7A). HEK293T cells coexpressing Kv2.2 and CB5/Lyn11 prior to rapamycin addition exhibited CB5-FKBP-CFP fluorescence similar to other ER reporters (*e.g.*, BFP-SEC61 β , DsRed2-ER5) in being throughout the ER, and also colocalized with clustered Kv2.2 at ER-PM junctions, the latter yielding a high degree of colocalization in TIRF imaging (Figure 7B). Surprisingly, unlike the other known classes of ER-PM junctions, the rapamycin-induced CB5/Lyn11 ER-PM junctions were largely distinct and nonoverlapping from those associated with the Kv2.2 clusters (Figure 7B), as shown by the significant decrease in PCC values upon rapamycin treatment (Figure 7C). Subsequent LatA treatment impacted the spatial organization of both the Kv2.2- and CB5/Lyn11-mediated ER-PM junctions (Figure 7B). However, they remained spatially segregated such that there were no significant LatA-induced changes in PCC values between Kv2.2- and CB5 (Figure 7C). These results taken together demonstrate that in heterologous cells Kv2-associated ER-PM junctions broadly overlap with those formed by the known classes of native ER-PM junction tethers, the exception being the unique artificial ER-PM junctions formed by the rapamycin-induced association of CB5 and Lyn1. Moreover, while the actin cytoskeleton plays a role in defining the spatial boundaries of both Kv2.2- and CB5/Lyn11-mediated ER-PM junctions, disrupting the actin cytoskeleton is not sufficient to homogenize these distinct membrane contact sites.

Remodeling ER-PM junctions is a nonconducting function of Kv2 channels

We next addressed whether the Kv2.2-mediated remodeling of ER-PM junctions is dependent on K^+ flux through the channels. We generated a point mutation (P412W) in the S6 transmembrane helix of Kv2.2, a position analogous to a point mutation (P404W) previously shown to eliminate conductance through Kv2.1 channels heterologously expressed in *Xenopus* oocytes (Lee *et al.*, 2003). We first expressed GFP-Kv2.2 P412W in HEK293T cells and evaluated conductance relative to wild-type GFP-Kv2.2 using voltage-clamp electrophysiology. HEK293T cells expressing GFP-Kv2 channels or GFP alone as a control were whole-cell patch clamped and held at a resting membrane potential of -80 mV. In response to positive voltage steps, delayed rectifier outward currents emerged from cells expressing GFP-Kv2.2, but not from cells expressing either GFP-Kv2.2 P412W or GFP (Figure 8A and B). As expected from previous analyses in oocytes, GFP-Kv2.1 P404W was also nonconducting when expressed in HEK293T cells (Figure 8A and C).

We next expressed GFP-Kv2.2 P412W in CHNs and found that it was localized in clusters indistinguishable from GFP-Kv2.2 (Figure 8D). The size of GFP-Kv2.2 P412W clusters was not significantly different than those of GFP-Kv2.2 (Figure 8F). We also found a lack of any significant differences in cluster size of wild-type GFP-Kv2.1 and nonconducting GFP-Kv2.1 P404W (Figure 8D and G). We next surface labeled live HEK293T cells with GxTX-633 and found no significant differences in colocalization between GxTX-633 and GFP-Kv2.2 *versus* GFP-Kv2.2 P412W (Figure 8E and H). A similar lack of significant differences was seen for GxTX labeling of GFP-Kv2.1 *versus* nonconducting GFP-Kv2.1 P404W (8E and I). These data taken together demonstrate that these Kv2 mutants lack ionic conductance but exhibit cell surface expression and clustering indistinguishable from their wild-type counterparts.

We next addressed whether the clustered but nonconducting GFP-Kv2.2 P412W mutant retained its ability to recruit/stabilize cortical ER at ER-PM junctions. Live cell TIRF imaging

showed that GFP-Kv2.2 P412W remodeled the DsRed2-ER5-labeled cortical ER into ER-PM junctions (Figure 9A). We found no significant difference between cells expressing GFP-Kv2.2 P412W versus GFP-Kv2.2 in either the size of ER-PM junctions (Figure 9C), or the surface area of the PM occupied by the cortical ER (Figure 9D). The extent of colocalization of DsRed2-ER5 with GFP-Kv2.2 P412W was also not significantly different than for GFP-Kv2.2 (Figure 9E). We next evaluated the lateral mobility of DsRed2-ER5-labeled cortical ER as an additional measure of its recruitment into ER-PM junctions (Wu *et al.*, 2014; Fox *et al.*, 2015). The mobility of PM-associated ER was significantly reduced in Kv2.2-expressing cells compared to control cells expressing DsRed2-ER5 alone (Supplemental Figure 2A-C). Cortical ER mobility was not significantly different in cells expressing the nonconducting Kv2.2 P412W mutant versus those expressing WT Kv2.2 (Supplemental Figure 2A-C). These parameters of cortical ER recruitment/stabilization were also not significantly different between WT Kv2.1 and the nonconducting Kv2.1 P404W mutant (Figure 9B-E, Supplemental Figure 2A-C). These data taken together demonstrate that the conserved function of Kv2 channels to localize to and remodel ER-PM junctions is independent of their canonical ion conducting function and is instead a distinct nonconducting function.

We next determined whether Kv2.2 clustering is necessary for remodeling of ER-PM junctions. We used a point mutant (S605A) in the Kv2.2 PRC domain that abolishes its clustering (Bishop *et al.*, 2015). Based on previous analyses of large C-terminal truncation mutants in Kv2.1 that eliminate the entire PRC domain [e.g., (VanDongen *et al.*, 1990; Scannevin *et al.*, 1996)], we expected that this point mutant would not impact the ability of Kv2.2 to function as a conducting Kv channel. To verify this, we used whole cell patch clamp recordings to compare currents from wild-type and nonclustered Kv2.2 channels in voltage clamped cells. We found that expression of GFP-Kv2.2 S605A in HEK293T cells resulted in expression of voltage-activated outward currents (Figure 9F). Neither the overall K⁺ current density nor the conductance-voltage relationships of cells expressing GFP-Kv2.2 versus GFP-

Kv2.2 S605A (Figure 9F; Table 1), or GFP-Kv2.1 versus GFP-Kv2.1 S586A (Figure 9G; Table 1) exhibited significant differences. Thus, these measurements of current density and the conductance-voltage relationship supports that Kv2 channels with these cytoplasmic point mutations that disrupt clustering do not affect the density of conducting channels on the cell surface or their gating.

We next determined the function of the nonclustering but conducting Kv2.2 S605A point mutant in remodeling ER-PM junctions. TIRF imaging revealed a diffuse localization of GFP-Kv2.2 S605A (Figure 9A). The ER-PM junction size (Figure 9C) and percentage of PM surface area occupied by cortical ER (Figure 9D) were not significantly different between cells coexpressing GFP-Kv2.2 S605A and cells expressing DsRed2-ER5 alone. This nonclustered GFP-Kv2.2 S605A mutant also had a significantly reduced colocalization with coexpressed DsRed2-ER5 relative to GFP-Kv2.2 (Figure 9E). We obtained similar results for Kv2.1 in that the ability to remodel ER-PM junctions was significantly reduced in the nonclustering but conducting GFP-Kv2.1 S586A point mutant (Figure 9B-E). Taken together, these results using this set of separation-of-function point mutants demonstrate that Kv2 channel clustering, but not conduction, is necessary for the unique ability of PM Kv2 channels to localize to and remodel ER-PM junctions, and that this conserved function of Kv2 channels is distinct and separable from its role in conducting ions.

Finally, we determined whether the highly conserved PRC domain that is remodel ER-PM junctions to another protein that lacks this function. We used a set of chimeras with C-terminal fragments of Kv2.1 containing the PRC domain appended to the C-terminus of the Kv1.5 channel (Lim *et al.*, 2000), which unlike Kv2 channels does not form PM clusters or remodel ER-PM junctions [Figure 10C, also see (Lim *et al.*, 2000; Mohapatra and Trimmer, 2006)]. We found that transfer of the entire Kv2.1 C-terminus (a.a. 411-853) or a small fragment (a.a. 536-600) containing primarily the PRC domain to Kv1.5 was sufficient for the resultant Kv1.5-Kv2.1 chimeras to now remodel ER-PM junctions (Figure 10D and E). Taken together

these results show that the highly conserved PRC domain is both necessary and sufficient for the Kv2-mediated remodeling of ER-PM junctions.

Eliminating Kv2 channel expression *in vivo* impacts RyR-containing ER-PM junctions in brain neurons

As detailed above, endogenously expressed Kv2.2 and Kv2.1 in brain neurons *in situ* and in culture colocalize with RyR-containing ER-PM junctions, and exogenously expressing either Kv2 channel remodels ER-PM junctions in CHNs and heterologous cells. We next tested whether eliminating Kv2 channel expression in knockout mice would impact the spatial organization of RyR-containing ER-PM junctions in brain neurons, taking advantage of the availability of Kv2.1 (Jacobson *et al.*, 2007; Speca *et al.*, 2014) and Kv2.2 (Hermansteyne *et al.*, 2013) knockout mice, and Kv2 double knockout mice (Bishop *et al.*, 2018). We immunolabeled Kv2.2, Kv2.1 and RyR in brain sections from these mice and from wild-type controls and analyzed RyR clusters in hippocampal CA1 pyramidal neurons, which express both Kv2.2 and Kv2.1 (Speca *et al.*, 2014; Bishop *et al.*, 2015; Palacio *et al.*, 2017; Bishop *et al.*, 2018). As shown in Figure 11, while there were no significant changes in the spatial characteristics of RyR clusters in the samples from the single Kv2.1 or Kv2.2 knockout mice when compared to those from wild-type mice, the size of RyR clusters in CA1 pyramidal neurons was significantly reduced in the samples from the double Kv2 knockout mice (Figure 11A-F). This supports an *in vivo* role for Kv2 channels in contributing to the spatial characteristics of RyR-containing ER-PM junctions in brain neurons.

DISCUSSION

Our results presented here demonstrate that both members of the Kv2 channel family have a conserved ability to remodel ER-PM junctions, which is unique among all PM proteins studied to date. We show that endogenous Kv2.2 ion channels localize to ER-PM junctions on somata,

proximal dendrites and the AIS in brain neurons. Experiments in CHNs, and in heterologous HEK293T show that Kv2.2 channels can function to remodel ER-PM junctions, and that this is a conserved and nonconducting function of mammalian Kv2 ion channels that requires an intact PRC domain. We also show that when transferred to another protein, the PRC domain can act autonomously to remodel ER-PM junctions. Moreover, elimination of Kv2 expression in knockout mice leads to altered ER-PM junctions in brain neurons. The conserved function of Kv2.2 and Kv2.1 to remodel ER-PM junctions makes the Kv2 channels the first family of PM proteins whose expression is sufficient to perform this function. Kv2-containing ER-PM junctions are found at sites deficient in components of the cortical actin cytoskeleton, which contributes to but is not the sole determinant of the overall spatial organization of Kv2 channel-containing ER-PM junctions. Kv2.2-containing ER-PM junctions are found associated with junctions containing diverse ER tethers that mediate ER to PM contacts, suggesting that ER-PM junctions formed by Kv2 channels and these ER tethers may structurally and functionally overlap in cells in which they are coexpressed. Separation-of-function mutants in Kv2.2 and Kv2.1 reveal that their conserved function in remodeling ER-PM junctions is independent of their well-established canonical function as ion conducting channels regulating electrical signaling in neurons and non-neuronal cells, but entirely dependent on an intact PRC domain and their clustering in the PM. That Kv2.2 and Kv2.1 have distinct patterns of cellular expression suggests that the highly similar yet distinct functions of these mammalian Kv2 channel paralogs, including dynamic phosphorylation-dependent regulation of their clustering (Bishop *et al.*, 2015) might distinctly impact the structure, function and regulation of ER-PM junctions in the classes of neurons and non-neuronal cells in which they are differentially expressed.

In certain brain neurons *in situ* and in hippocampal neurons in culture we found clusters of Kv2.2 at sites containing high densities of associated ER-localized RyRs, supporting that these clusters represent native Kv2.2-containing ER-PM junctions, and that these sites are associated with distinct neuronal Ca²⁺ signaling domains. Moreover, that elimination of

expression of both Kv2 channels leads to changes in the spatial organization of RyR-containing ER-PM junctions in brain neurons suggests that Kv2 channels play a role in the structural organization of these Ca²⁺ signaling microdomains. Although both Kv2.2 and Kv2.1 are unique among mammalian PM proteins in being capable of remodeling ER-PM junctions, their distinct cellular expression patterns in brain and in other mammalian tissues, together with their distinct phospho-dependent regulation, may contribute to the unique phenotypes seen in mice upon knockout of either Kv2.2 [altered sleep wake cycles (Hermansteyne *et al.*, 2010)] or Kv2.1 [neuronal and behavioral hyperexcitability (Specca *et al.*, 2014)]. The relative contribution of the separate functions of Kv2 channels, as ion conducting channels shaping membrane excitability and in impacting the structure of ER-PM junctions, to the behavioral phenotypes of these mice is as of yet unknown.

Our data using a strategically selected set of separation-of-function point mutants support that recruitment/stabilization of ER-PM junctions is a nonconducting and physical function of Kv2 channels that relies on their clustering. Both Kv2.2 and Kv2.1 are *bona fide* PM voltage-gated K⁺ channels whose ion conducting function underlies the bulk of the delayed rectifier K⁺ current in various classes of neurons (Murakoshi and Trimmer, 1999; Du *et al.*, 2000; Malin and Nerbonne, 2002; Liu and Bean, 2014). Moreover, acute pharmacological inhibition of Kv2 channels impacts neuronal excitability and shapes the characteristics of action potentials (Guan *et al.*, 2013; Liu and Bean, 2014; Kimm *et al.*, 2015; Pathak *et al.*, 2016; Honigsperger *et al.*, 2017; Palacio *et al.*, 2017). Our findings that the ability to remodel ER-PM junctions is a nonconducting function of Kv2 channels is intriguing given previous findings that the bulk of exogenous Kv2.1 expressed in either heterologous cells or neurons may be present in a nonconducting state (Benndorf *et al.*, 1994; O'Connell *et al.*, 2010; Fox *et al.*, 2013). That ion channels can have diverse nonconducting functions distinct from their canonical ion conducting roles is an emerging theme in biology, with nonconducting roles as cell adhesion molecules, as enzymes or as scaffolds for enzymes, as voltage sensors for intracellular events through

conformational coupling, etc. [reviewed in (Kaczmarek, 2006)]. Studies in pancreatic beta cells support a nonconducting function for Kv2.1 in regulating insulin secretion (Dai *et al.*, 2012), which is dependent on Kv2.1 clustering (Fu *et al.*, 2017). It is intriguing to speculate that the nonconducting role for Kv2.1 in beta cells is to impact the structure of ER-PM junctions, which have been proposed to play an important role in glucose-stimulated insulin secretion (Li *et al.*, 2016; Lees *et al.*, 2017).

The extent and nature of the physiological contribution of Kv2-mediated regulation of ER-PM junction organization in brain neurons is not known. However, recent studies employing whole exome sequencing have led to identification of encephalopathic epilepsy patients with *de novo* mutations in the KCNB1 gene that encodes Kv2.1. While the bulk of these disease-associated mutations are in the voltage-sensing and pore domains that are crucial to the canonical function of Kv2.1 as a *bona fide* Kv channel [e.g., (Torkamani *et al.*, 2014; Saitsu *et al.*, 2015; Thiffault *et al.*, 2015)], a subset are nonsense mutations that result in a truncated cytoplasmic C-terminus (de Kovel *et al.*, 2016; Marini *et al.*, 2017). While the cytoplasmic C-terminus plays a modulatory role in regulating activation gating of Kv2.1 channels (Murakoshi *et al.*, 1997; Park *et al.*, 2006; Ikematsu *et al.*, 2011), the most obvious effect of these nonsense mutations that eliminate the PRC domain is to disrupt the clustering of Kv2.1 (Scannevin *et al.*, 1996; Lim *et al.*, 2000; Bishop *et al.*, 2015; Fox *et al.*, 2015; Jensen *et al.*, 2017; Bishop *et al.*, 2018) and presumably the organization of Kv2-containing ER-PM junctions. Generating mouse models that express the separation-of-function mutations used here to selectively disrupt Kv2.1 conduction and clustering may lead to insights into the relative contributions of the separable electrical and structural roles of Kv2 channels in normal physiology, and how these distinct classes of disease-associated mutations that should impact one or the other function contribute to pathophysiology.

Our results show that both members of the Kv2 family of ion channels can in themselves remodel ER-PM junctions. As this is the first family of mammalian PM proteins found to have

this function suggests Kv2 channels use a molecular mechanism distinct from all other known classes of endogenous ER-PM junction components (*i.e.*, members of the E-Syt, JP and STIM families), which are ER tethers that bind specific lipids present in the inner leaflet of the PM, although STIM family members also exhibit conditional interaction with PM Orai proteins (Carrasco and Meyer, 2011; Henne *et al.*, 2015). That expression of either Kv2.2 and Kv2.1 is sufficient to remodel ER-PM junctions in the absence of their ion conducting functions, and *via* a mechanism that requires an intact PRC domain, suggests that both Kv2 family members act through the same mechanism. We showed here that the PRC domain is sufficient to transfer the ability to remodel ER-PM junctions, which among PM proteins is unique to Kv2 channels, to another PM protein. This suggests a model whereby the PRC domain interacts directly with an ER-localized protein or lipid binding partner to tether the PM to the ER. That we have shown here that nonconducting Kv2 channels retain their ability to remodel ER-PM junctions is consistent with a mechanism involving direct protein-protein interactions, as opposed to less direct effects of Kv channel function on cell signaling pathways, for example by influencing Ca²⁺ signaling events that lead to recruitment of ER-PM junctions, as occurs for junctions containing certain STIM and E-Syt family members (Carrasco and Meyer, 2011; Henne *et al.*, 2015; Saheki and De Camilli, 2017b; Balla, 2018). In the case of Kv2 channels, these protein-protein interactions are mediated by the PRC domain that is both necessary and sufficient for Kv2-mediated remodeling of ER-PM junctions. The abundant ER proteins VAPA and VAPB have been recently found by the Tamkun laboratory (Johnson *et al.*, 2018) and ourselves (Kirmiz *et al.*, 2018) to interact with Kv2.1 and Kv2.2. Our results herein are consistent with a model where a direct interaction between these ER VAP proteins and PM Kv2 channels mediates this class of ER-PM junctions. That Kv2 channels form clusters at ER-PM junctions in diverse cell types including brain neurons of diverse mammalian species *in situ* and in culture [*e.g.*, (Trimmer, 1991; Scannevin *et al.*, 1996; Murakoshi and Trimmer, 1999; Antonucci *et al.*, 2001; Misonou *et al.*, 2004; Misonou *et al.*, 2006; Misonou *et al.*, 2008; Kihira *et al.*, 2010; Fox *et al.*, 2013; Bishop

et al., 2015; Frazzini *et al.*, 2016; Bishop *et al.*, 2018), etc.], in spinal motor neurons (Muennich and Fyffe, 2004) and in non-neuronal heterologous cells such as human HEK293 (Bishop *et al.*, 2015; Bishop *et al.*, 2018), monkey COS-1 (Cobb *et al.*, 2015) and canine MDCK (Scannevin *et al.*, 1996) kidney cells, rat PC12 pheochromocytoma cells (Sharma *et al.*, 1993), and hamster CHO ovary cells (Cobb *et al.*, 2015) is consistent with such an underlying mechanism, as VAP proteins are highly conserved across diverse mammalian species and widely expressed in numerous cell types (Murphy and Levine, 2016). That VAPs are abundant ER proteins may be consistent with the observation that the formation of Kv2 clusters and recruitment of ER-PM junctions is not obviously saturable, such that the higher the level of Kv2.2 or Kv2.1 expression, the larger the clusters and associated ER-PM junctions (Antonucci *et al.*, 2001; Cobb *et al.*, 2015). However, it remains that other ER-localized proteins may also interact with PM Kv2 channels to mediate ER-PM junction formation in a cell-type specific manner. The conditional clustering of Kv2.1 is phosphorylation-dependent, supporting a model whereby the induction of ER-PM junction formation can be dynamically regulated, and requires sufficient Kv2.1 phosphorylation, including in critical serine residues within the PRC domain itself (Lim *et al.*, 2000; Cobb *et al.*, 2015). That the PRC domain of Kv2.2, which does not exhibit the phosphorylation-dependent clustering as does Kv2.1 (Bishop *et al.*, 2015), contains these same serine residues suggests that should phosphorylation at these sites be required for Kv2.2 clustering and ER-PM junction remodeling, then in Kv2.2, this phosphorylation is more constitutive than the dynamically-regulated phosphorylation of Kv2.1. It is intriguing that both STIM:Orai and Kv2-based ER-PM junctions are sensitive to Ca^{2+} signaling, but at a simplistic level in opposite directions and with distinct mechanisms, with the former regulated by Ca^{2+} dependent conformational changes in STIM proteins upon ER Ca^{2+} depletion leading to association with PM Orai proteins, and the latter by Ca^{2+} -calcineurin-dependent changes in Kv2.1 phosphorylation state in response to elevated $[Ca^{2+}]_i$ leading to dissociation of Kv2.1 but not Kv2.2 (Bishop *et al.*, 2015) from ER-PM junctions.

That Kv2.2-containing ER-PM junctions can colocalize with members of the E-Syt, STIM, and JP families suggests potential overlap with these distinct classes of ER-PM junctions in coexpressing mammalian cells. One explanation of these findings is that these ER-localized PM tethers, by virtue of their ER localization, are passively recruited along with other ER proteins such as Sec61 β to Kv2.2-containing ER-PM junctions. However, the lack of association of Kv2.2-containing ER-PM junctions and those generated *via* the rapamycin-triggered coupling of CB5-FKBP and Lyn11-FRB would argue against a promiscuous presence of Kv2.2 channels at any ER-PM junction, although these rapamycin-induced artificial junctions have certain characteristics, including having a narrower gap between ER and PM (Varnai *et al.*, 2007; Dickson *et al.*, 2016), than those formed by endogenous ER-PM junction components [e.g., (Wu *et al.*, 2017)]. As the tethering of E-Syts, JPs and STIMs to the PM occurs at least in part on their binding to lipids on the PM inner leaflet (Henne *et al.*, 2015), another possible explanation for the robust colocalization between Kv2.2-containing ER-PM junctions and these ER tethers is that Kv2.2 clustering results in a distinct lipid microenvironment in the PM inner leaflet at or near these clusters. Changes in the local lipid environment at/near Kv2.2 clusters could also underlie generation of ER-PM junctions at these sites, *via* recruitment of one or more lipid-binding ER-PM tethers. As noted above, these tethers in aggregate would need to have sufficiently robust expression across the numerous species and cell types in which endogenous and exogenous Kv2 channels are clustered. We note that our quantitative analyses of colocalization between Kv2.2-containing ER-PM junctions and these ER tethers suggests that despite the extensive overlap, as reported by high (≈ 1.0) MOC values, the intensity profiles of these proteins do not uniformly coincide, as reported by significantly lower paired PCC measurements (Dunn *et al.*, 2011). That there is heterogeneity in ER-PM junctions within the same cell is consistent with the variable co-occurrence of Kv2.2 and Kv2.1 clusters with RyR clusters between and within different classes of mammalian brain neurons (Antonucci *et al.*, 2001; Misonou *et al.*, 2005b; Mandikian *et al.*, 2014). This concept is further supported by the lack of colocalization between

Kv2.2-containing ER-PM junctions and those formed *via* triggered coupling of CB5/Lyn11. Recent findings (Johnson *et al.*, 2018; Kirmiz *et al.*, 2018) suggest that formation of Kv2 channel-containing ER-PM junctions involves the direct interaction of PM Kv2 channels with ER VAP proteins. Future studies defining the subcellular localization of the different members of the E-Syt, JP and STIM families endogenously expressed in mammalian brain neurons relative to those containing Kv2 channels and VAPs will lead to an increased understanding of the relationship between the native ER-PM junctions formed by these ER tethers and those containing Kv2 channels.

That LatA treatment impacted the characteristics of both Kv2- and CB5/Lyn11-containing ER-PM junctions but did not lead to their fusion suggests that the actin cytoskeleton is not the only determinant of their distinct spatial organization. The effects of actin disruption on Kv2-containing ER-PM junctions, and that they are localized to zones at the cell cortex depleted in actin and actin-interacting proteins, suggests a role for the actin cytoskeleton in shaping their spatial characteristics. This is consistent with previous studies demonstrating that Kv2.1 clusters on the axon initial segment of brain neurons are specifically localized to ankG-deficient “holes” (King *et al.*, 2014), and that disruption of the actin cytoskeleton impacts clustering of Kv2.1 (O'Connell *et al.*, 2006; Tamkun *et al.*, 2007). Recent studies reveal that the STIM1:Orai1 complex at the immune synapse (Hartzell *et al.*, 2016) and HeLa cell ER-PM junctions labeled with the reporter MAPPER (Hsieh *et al.*, 2017) are also present in actin-poor zones, and that disruption of the actin cytoskeleton altered the distribution and dynamics of these HeLa cell ER-PM junctions (Hsieh *et al.*, 2017). Depletion of ER Ca²⁺ stores can also impact ER-PM junctions *via* the conditional association of STIM1 and Orai1, which can then associate with those formed by Kv2.1 (Fox *et al.*, 2015) or, as shown here, Kv2.2. That both ER (RyR) and PM (Orai1) Ca²⁺ channels colocalize with Kv2-containing ER-PM junctions suggests a potential structural role for Kv2 channels in regulating neuronal Ca²⁺ signaling and homeostasis above and beyond their established role in impacting intracellular Ca²⁺ through their ion conducting effects on

membrane excitability. Future studies will define the respective contributions of the separate yet highly conserved conducting and nonconducting roles of Kv2 channels in impacting cellular physiology, and how this is disrupted in pathological conditions that may exert their effects through distinct impacts on these broadly and highly expressed ion channels.

Materials and methods

Preparation of mouse brain sections for immunohistochemistry

All procedures involving mice were approved by the University of California Davis Institutional Animal Care and Use Committee and were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the NIH. All mice were maintained under standard light-dark cycles and allowed to feed and drink *ad libitum*. Kv2.1-KO mice (RRID:IMSR_MGI:3806050) have been described previously (Jacobson *et al.*, 2007; Speca *et al.*, 2014), and were generated from breeding of Kv2.1^{+/-} mice that had been backcrossed on the C57/BL6J background (RRID:IMSR_JAX:000664). Kv2.2-KO mice (Hermansteyne *et al.*, 2010; Hermansteyne *et al.*, 2013) were obtained from Drs. Tracey Hermansteyne and Jeanne Nerbonne. All Kv2.2-KO mice used here were obtained from heterozygotic crosses in the C57/BL6J background (RRID:IMSR_JAX:000664). Double knockout mice for Kv2.1/Kv2.2 (Kv2 dKO) were generated by crossing Kv2.1^{+/-} and Kv2.2^{+/-} mice. Both male and female mice were used, and all were over 12 weeks old. Littermates were used when available. Mice were deeply anesthetized with 90 mg/kg Na-pentobarbital salt (Sigma Cat# P3761) in 0.9% NaCl solution through intraperitoneal injections, followed by boosts as needed. Once mice were completely anesthetized, they were transcardially perfused with 4.5 ml of ice cold PBS [150 mM NaCl, 10 mM sodium phosphate buffer (PB), pH 7.4] containing 10 U/ml heparin, followed by an ice-cold fixative solution of 4% formaldehyde (freshly prepared from paraformaldehyde, Sigma Cat# 158127) in 0.1 M sodium PB, pH 7.4 (0.1M PB), using a volume of 1 ml fixative solution per

gram of mouse weight. Following perfusions, brains were removed from the skull and cryoprotected in 10% sucrose, 0.1 M PB overnight at 4°C, then transferred to a solution of 30% sucrose, 0.1 M PB until they sank to the bottom of the tube (24–48 h). Following cryoprotection, all brains were frozen, and cut on a freezing stage sliding microtome (Richard Allen Scientific) to obtain 30- μ m-thick sagittal sections. Sections were collected in 0.1 M PB and processed for immunohistochemistry (IHC) as free-floating sections.

Multiplexed fluorescence immunohistochemistry

Multiplex immunofluorescence labeling of mouse brain sections was performed essentially as previously described (Manning *et al.*, 2012). Briefly, free-floating sections were washed 3x in 0.1 M PB plus 10 mM sodium azide at room temperature with slow agitation. All subsequent incubations and washes were at room temperature with slow agitation on an orbital platform shaker, unless stated otherwise. Sections were incubated in blocking buffer (10% goat serum in 0.1 M PB, 0.3% Triton X-100, and 10 mM sodium azide) for 1 h. Immediately after blocking, sections were incubated with primary antibody combinations (diluted in blocking buffer) overnight at 4°C. All primary antibodies used in this study have been previously described (see Table 2 for a description of primary antibodies). Following incubation, sections were washed 3 x 10 min each in 0.1 M PB and incubated for 1 h with affinity-purified goat anti-rabbit and/or goat anti-mouse IgG-subclass-specific Alexa Fluor-conjugated secondary antibodies diluted in blocking buffer, using a 1:1500 dilution for Alexa Fluor 647 conjugates, and 1:2000 dilution for Alexa Fluor 488 and 555 conjugates, all secondary antibodies from ThermoFisher. Sections were also labeled with the DNA-specific dye Hoechst 33258 (200 ng/mL, ThermoFisher Cat# H1399) during the secondary antibody step. After 3 x 10 min washes in 0.1 M PB, sections were mounted and dried onto gelatin-coated slides, treated with 0.05% Sudan Black Sudan Black (EM Sciences Cat# 21610) in 70% ethanol for 1.5 min, extensively washed in water, and mounted with Prolong Gold (ThermoFisher Cat# P36930). All immunolabeling reported for

quantification purposes are representative of three animals (biological replicates) per genotype, except for Kv2.2 KO that included brain sections from two animals. Brain sections from all biological replicates within each experiment were labeled, treated, and mounted in parallel.

All images were acquired on a Zeiss AxioObserver Z1 microscope with an X-Cite 120 lamp as the fluorescent light source and equipped with an AxioCam MRm digital camera. High-magnification optical sections were acquired using an ApoTome structured illumination system (Carl Zeiss MicroImaging) with a 63X/1.40 NA plan-Apochromat oil immersion objective. ApoTome z-stacks were acquired and processed with Axiovision 4.8.2 acquisition software (Carl Zeiss MicroImaging, RRID: SciRes_000111). All brain sections within a given experiment and immunolabeled with the same antibody cocktail were imaged under the same conditions (objective, exposure time, lamp settings, etc.). Image processing was performed in Axiovision (Carl Zeiss MicroImaging) and Fiji v2.0.0-rc-43/1.51 (NIH). All panels in a given figure were imaged and processed identically, unless otherwise noted. High-magnification ApoTome z-stacks were opened for analysis as raw image files in Fiji (NIH) using the Bio-Formats library importing plugin (Linkert et al., 2010). Quantification was performed using single optical z-sections. All statistical analyses of immunolabeling were performed in Prism (GraphPad).

Quantification of RyR immunolabeling was performed in FIJI. Images were first background subtracted; background levels were determined from “no primary antibody” immunolabeling controls for each animal, and mathematically subtracted from paired images of RyR labeling, and images were converted to 8-bit. An ROI selection was made to include cell bodies of neurons in the pyramidal cell layer of hippocampal CA1, and the image was automatically converted into a binary mask using auto local thresholding (Bernsen, 1986). RyR cluster size was quantified automatically using the “analyze particles” function in FIJI. Particles smaller than 0.06 μm^2 were excluded from this analysis.

Culture and transfection of rat hippocampal neurons

All procedures involving rats were approved by the University of California Davis Institutional Animal Care and Use Committee and were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the NIH. All rats were maintained under standard light-dark cycles and allowed to feed and drink *ad libitum*. Hippocampi were dissected from embryonic day 18 rat embryos and dissociated enzymatically for 20 min at 37 °C in 0.25% (w/v) trypsin (ThermoFisher Cat# 15050065) in HBSS and dissociated mechanically by triturating with glass polished Pasteur pipettes. Dissociated cells were suspended in Neurobasal (Invitrogen Cat# 21103-049) supplemented with 10% FBS (Invitrogen Cat# 16140071), 2% B27 (Invitrogen Cat# 17504044), 1% GlutaMAX (Invitrogen Cat# 35050061), and 0.001% gentamycin (Gibco Cat #1570-064) and plated at 60,000 cells per dish in glass bottom dishes (MatTek Cat# P35G-1.5-14-C), or number 1.5 glass coverslips, coated with poly-L-lysine (Sigma Cat# P2636). At 4-7 days *in vitro* (DIV), cytosine-D-arabinofuranoside (Millipore Cat# 251010) was added to inhibit non-neuronal cell growth. Immunofluorescence labeling was performed on CHNs at 14-18 DIV. For transfection experiments, CHNs were transiently transfected at DIV 5-10 using Lipofectamine 2000 (Lipofectamine, Invitrogen Cat# 11668019) for 1.5 hours as previously described (Lim *et al.*, 2000). Transfected CHNs were imaged 40-48 hours post transfection.

Heterologous cell culture, reagents, and transfection

HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Clone III (HyClone Cat# SH30109.03), 1% penicillin/streptomycin, and 1X GlutaMAX (ThermoFisher Cat# 35050061) in a humidified incubator at 37 °C and 5% CO₂. HEK293T cells were transfected with Lipofectamine as previously described (Bishop *et al.*, 2015). Briefly, to 6 x 10⁴ HEK293T cells were plated onto 35 mm glass bottom dishes (MatTek Cat# P35G-1.5-14-C) coated with poly-L-lysine and incubated for 20-24 hours. Cells were then transiently transfected using Lipofectamine following the manufacturer's protocol in DMEM without supplements, then

returned to regular growth media 4 hours after transfection. HEK293T cells were imaged live or subjected to fixation 40-48 hours post-transfection.

Cell fixation, immunolabeling, and fixed-cell imaging

For experiments involving imaging of fixed and immunolabeled HEK293T cells, fixation was performed as previously described (Dickson *et al.*, 2016). Briefly, cells were fixed in 3.2% formaldehyde (freshly prepared from paraformaldehyde, Sigma Cat# 158127) and 0.1% glutaraldehyde (Ted Pella, Inc., Cat # 18426) for 30 minutes at room temperature, washed 3 x 5 minutes in PBS and quenched with 1% sodium borohydride in PBS for 15 minutes at room temperature. Cells were blocked and permeabilized in 4% non-fat milk powder in PBS containing 0.5 % Triton-X 100. Neurons (CHNs) were fixed in ice-cold 4% formaldehyde, 4% sucrose in PBS for 15 minutes at 4°C, washed 3 x 5 minutes in PBS and blocked and permeabilized in 4% non-fat milk powder in PBS containing 0.1 % Triton-X 100. Primary antibody incubations were performed in blocking solution for 1 hour at room temperature. All antibodies used in this study have been previously described (see Table 2 for a description of primary antibodies). Following primary antibody incubation, and 3 x 5 minute washes in blocking solution at room temperature, coverslips were immunolabeled with species- and or mouse IgG subclass-specific Alexa Fluor-conjugated goat anti-mouse IgG subclass-specific (Manning *et al.*, 2012) or goat anti-rabbit IgG secondary antibodies (all secondary antibodies from ThermoFisher) at 1:1500 and Hoechst 33258 (200 ng/mL; ThermoFisher Cat# H1399) for one hour in blocking solution, washed 3 x 5 min in PBS, and mounted onto microscope slides using Fluoromount G (Southern Biotech Cat# 0100-01), or for samples prepared for TIRF microscopy, imaged in PBS containing ascorbate.

For conventional fluorescence imaging (used in Figures 1A-C, 4A-C, 11) images were acquired with an AxioCam MRm digital camera installed on a Zeiss AxioImager M2 microscope or with an AxioCam HRm digital camera installed on a Zeiss AxioObserver Z1 microscope with

a 63X/1.40 NA plan-Apochromat oil immersion objective or a 20X/0.8 NA plan-Apochromat objective and an ApoTome coupled to Axiovision software (Zeiss, Oberkochen, Germany). For TIRF imaging of fixed cells, imaging was identical to that used in live-cell TIRF experiments but in the absence of a heated stage/objective heater. Images were obtained with an Andor iXon EMCCD camera installed on a TIRF/widefield equipped Nikon Eclipse Ti microscope using a Nikon LUA4 laser launch with 405, 488, 561, and 647 nm lasers and a 100X PlanApo TIRF/1.49 NA objective run with NIS Elements software (Nikon). Images were collected within NIS Elements as ND2 images. For N-SIM imaging of fixed cells, images were acquired using a Hamamatsu ORCA-ER CCD camera installed on a SIM/widefield equipped Nikon Eclipse Ti microscope using an EXFO X-Cite metal halide light source and a 100X PlanApo TIRF/1.49 objective, run with NIS Elements software (Nikon). Images were collected within NIS Elements as ND2 images. SIM analysis was performed in NIS Elements. Airyscan imaging was performed with a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss), equipped with an Airyscan detection unit, with a Plan-Apochromat 63X/1.40 Oil DIC M27 objective.

Plasmid constructs

All novel constructs used in this study (DsRed-Kv2.2, GFP-Kv2.2, GFP-Kv2.2 P412W, GFP-Kv2.2 S605A, GFP-Kv2.1 P404W, GFP-Kv2.1 S586A) were generated using standard molecular biology approaches and confirmed by sequencing. DsRed-Kv2.2 and GFP-Kv2.2 were generated using Gibson assembly to insert full-length rat Kv2.2, also termed Kv2.2_{long} (Kihira *et al.*, 2010) into the GFP-C1 or DsRed-C1 vector (ClonTech) resulting in fusion of DsRed or GFP to the N-terminus of full-length rat Kv2.2. GFP-Kv2.2 S605A, GFP-Kv2.1 P404W and GFP-Kv2.1 S586A were generated *via* site directed point mutagenesis utilizing a QuikChange PCR reaction of GFP-Kv2.2 or GFP-Kv2.1 (Antonucci *et al.*, 2001), respectively, or *via* Gibson assembly. GFP-Kv2.2 P412W was generated at Mutagenex. The plasmids encoding Kv2.1 in the RBG4 vector (Shi *et al.*, 1994), Kv2.2 in the RBG4 vector (Bishop *et al.*, 2015),

Kv1.5N-Kv2.1C (Mohapatra and Trimmer, 2006), and Kv1.5N-Kv2.1PRC (Lim *et al.*, 2000) have been previously described. Plasmids encoding DsRed2-ER5 and mCherry-actin were a generous gift from Dr. Michael Davidson (Addgene plasmids # 55836 and 54965). The plasmid encoding ankG-mCherry was a generous gift from Dr. Benedicte Dargent (Addgene plasmid #42566). The plasmids encoding BFP-SEC61 β and BFP-STIM1 were a generous gift from Dr. Jodi Nunnari (University of California, Davis). The plasmid encoding GFP-JP2 was a generous gift from Dr. Fernando Santana (University of California, Davis). The plasmid encoding mCherry-E-Syt1-3 was a generous gift from Dr. Pietro De Camilli (Yale University School of Medicine). The plasmid encoding mCherry-JP4 was a generous gift from Dr. Yousang Gwack (University of California, Los Angeles). The plasmids encoding mCherry-STIM1, - STIM2 α , and - STIM2 β , and GFP-Orai1 were a generous gift from Dr. Richard Lewis (Stanford University). The plasmids encoding CFP-CB5-FKBP and Lynn11-FRB (Inoue *et al.*, 2005) were a generous gift from Dr. Eamonn Dickson.

Live cell Guangxitoxin labeling

The GxTX peptide used in surface labeling was synthesized at the Molecular Foundry of the Lawrence Berkeley National Laboratory under US Department of Energy contract no. DE-AC02-05CH11231. HEK293T cells were surface labeled with 1 μ M GxTX as previously described (Tilley *et al.*, 2014) and imaged in TIRF as described below but in physiological saline solution (4.7 mM KCl, 146 mM NaCl, 2.5 mM CaCl₂, 0.6 mM MgSO₄, 1.6 mM NaHCO₃, 0.15 mM NaH₂PO₄, 20 mM HEPES, pH 7.4) containing 8 mM glucose and 0.1 mM ascorbic acid) containing 0.1% BSA.

TIRF imaging

Total internal reflection fluorescence (TIRF) imaging was performed at the UC Davis MCB Imaging Facility. Live transfected HEK293T cells cultured on glass bottom dishes were imaged in a physiological saline solution (4.7 mM KCl, 146 mM NaCl, 2.5 mM CaCl₂, 0.6 mM MgSO₄, 1.6 mM NaHCO₃, 0.15 mM NaH₂PO₄, 20 mM HEPES, pH 7.4) containing 8 mM glucose and 0.1 mM ascorbic acid). Cells were maintained at 37°C during the course of imaging with a heated stage and objective heater. Fixed cell TIRF imaging was performed identically but in the absence of a stage and objective heaters. For experiments involving Latrunculin A (ThermoFisher Scientific, Cat# 428021100UG) treatment, Latrunculin A was diluted to 20 µM in imaging saline and added by pipette, to glass bottom dishes already containing imaging saline, to a final concentration of 10 µM. For experiments involving thapsigargin (Millipore, Cat# 586005-1MG) treatment, thapsigargin was diluted to 4 µM in imaging saline and added by pipette, to GLASS BOTTOM dishes already containing imaging saline, to a final concentration of 2 µM. For experiments involving rapamycin (Sigma, Cat# R8781-200UL) treatment, rapamycin was diluted to 10 µM in imaging saline and added by pipette to glass bottom dishes already containing imaging saline, to a final concentration of 5 µM. Images were obtained with an Andor iXon EMCCD camera installed on a TIRF/widefield equipped Nikon Eclipse Ti microscope using a Nikon LUA4 laser launch with 405, 488, 561, and 647 nm lasers and a 100X PlanApo TIRF, 1.49 NA objective run with NIS Elements software (Nikon). Images were collected within NIS Elements as ND2 images.

Cell culture and transfection for electrophysiology

All cell lines were grown in a humidified incubator at 37°C and 5% CO₂. HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone Cat # SH30109.02) and 1% penicillin/streptomycin. Transfections were performed with Lipofectamine. Cells were plated overnight prior to transfection and allowed to grow to ≈40% confluency. Lipofectamine was diluted, mixed, and incubated in Opti-MEM (Gibco Cat

#31965-062) in a 1:100 ratio for 5 minutes. Concurrently, 1 μg of plasmid DNA and Opti-MEM were mixed in the same fashion. After incubation, the DNA and Lipofectamine mixtures were combined, triturated, and allowed to incubate for 20 minutes. The transfection cocktail was added to cells for 5 hours before the media was replaced. For experiments in Figure 8, 1 μg of GFP-Kv2 or a peGFP-C1 plasmid were used. For experiments in Figure 9, 0.2 μg of GFP-Kv2 plasmids were diluted with 0.8 μg pcDNA3 plasmid.

Electrophysiology

Whole cell voltage clamp was used to measure currents from HEK293T cells expressing GFP-Kv2.2, GFP-Kv2.2 P412W, GFP-Kv2.1, GFP-Kv2.1 P404W, or GFP as a control. On the day of the experiment (two days after transfection), transiently transfected cells were detached with trypsin and plated onto cell culture-treated polystyrene dishes for electrophysiological measurements. The external (bath) solution contained (in mM): 3.5 KCl, 155 NaCl, 10 HEPES, 1.5 CaCl_2 , 1 MgCl_2 , adjusted to pH 7.41 with NaOH. The internal (pipet) solution contained (in mM): 35 KOH, 70 KCl, 50 KF, 50 HEPES, 5 EGTA adjusted to pH 7.2 with KOH. Liquid junction potential (calculated to be 7.8 mV) was not corrected for. Borosilicate glass pipettes (Sutter Instruments, Cat #BF150-110-10HP) with resistance less than 3 $\text{M}\Omega$ were used to patch the cells. Recordings were at room temperature (22–24 $^{\circ}\text{C}$). Voltage clamp was achieved with an Axon Axopatch 200B amplifier (MDS Analytical Technologies, Sunnyvale, CA) run by PATCHMASTER software, v2x90.2 (HEKA, Bellmore, NY). Holding potential was -80 mV. Capacitance and Ohmic leak were subtracted using a P/5 protocol. Recordings were low pass filtered at 10 kHz and digitized at 100 kHz. Voltage clamp data were analyzed and plotted with IgorPro software, version 7 (Wavemetrics, Lake Oswego, OR). Current amplitudes at each voltage were the average from 0.19-0.20 s after voltage step. In the experiments plotted in Figure 8, series resistance compensation was not used. The estimated series resistance in these experiments ranged from 3-8 $\text{M}\Omega$, which is predicted to result in substantial cell voltage

errors for conducting channels. For quantitative comparison of current levels and voltage activation (Figure 9), we improved control of intracellular voltage by reducing the amount of DNA transfected (described above), partially blocking the K^+ currents with tetraethylammonium (TEA) and using series resistance compensation. For experiments shown in Figure 9 on HEK293T cells expressing GFP-Kv2.2, GFP-Kv2.2 S605A, GFP-Kv2.1, or GFP-Kv2.1 S586A, the following modifications were made. The internal (pipet) solution contained (in mM): 140 KCl, 13.5 NaCl, 1.8 $MgCl_2$, 0.09 EGTA, 4 Na-ATP, 0.3 Na-GTP, and 9 HEPES, adjusted to pH 7.2 with KOH. The external (bath) solution contained (mM): 3.5 KCl, 155 TEA-Cl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose adjusted to pH 7.42 with NaOH. 155 mM extracellular TEA is predicted to inhibit at least 97% of Kv2.1 current at 0 mV [see (Ikeda and Korn, 1995; Immke *et al.*, 1999; Immke and Korn, 2000)]. A calculated liquid junction potential of 7.6 mV was corrected. Pipette tips were coated with Sylgard 184 (Dow Corning Cat #2010518, Midland, MI) and fire polished. Series resistance compensation with lag set to 10 μs was used to constrain calculated voltage error to ≤ 10 mV. Conductance was measured from the amplitude of outward tail currents averaged from the end of any capacitance transient until 2 ms after stepping to 0 mV from the indicated voltage. Fits with the fourth power of a Boltzmann distribution are described previously, where V_{mid} is the voltage where the function reaches half maximal conductance, and z is valence in units of elementary charge (e^+) of each of the four independent voltage sensors (Sack *et al.*, 2004). Conductance data shown are normalized to the maximal conductance of the Boltzmann fit.

Image analysis and statistics

All colocalization analyses were performed within Nikon NIS Elements using ND2 files. An ROI was drawn within a cell of interest and PCC and MOC values were collected. Measurements of structure sizes were quantified automatically within FIJI essentially as previously described (Dickson *et al.*, 2016). ND2 files of DsRed2-ER5 or BFP-SEC61 β collected in TIRF were

imported directly into FIJI, background subtracted, converted into an 8-bit image, and automatically converted into a binary mask using auto local thresholding (Bernsen, 1986). An ROI with identical dimensions and containing an area of $60.6 \mu\text{m}^2$ was drawn within each cell analyzed. The number of individual ER-PM junctions, average ER-PM junction size, and percent PM occupancy were quantified automatically using the “analyze particles” function in FIJI. Signals smaller than $0.04 \mu\text{m}^2$ were excluded from this analysis. An identical approach was taken in whole cell analysis.

Quantification of Kv2 cluster sizes was performed similarly. ND2 files of GFP-Kv2.2, GFP-Kv2.2 P412W, GFP-Kv2.1 or GFP-Kv2.1 P404W were collected in widefield and deconvolved in NIS elements were imported directly into FIJI, converted into an 8-bit image, and automatically converted into a binary mask using auto local thresholding (Bernsen, 1986). Kv2 cluster size was quantified automatically using the “analyze particles” function in FIJI. For scatterplot generation of ER-PM junction and Kv2 cluster sizes (Figure 3J), ND2 files were imported directly into FIJI, background subtracted using a rolling ball radius of 10 pixels and converted into an 8-bit image. Images were converted into binary masks and manually subjected to erosion operations designed to separate objects as previously described (Dickson *et al.*, 2016). Care was taken to ensure that the resulting binary image was comparable to the original image. The areas of these structures were quantified automatically using the “analyze particles” function in FIJI. Areas from 10-20 overlapping structures from each cell were paired as coordinates. In cases where more than one structure overlapped, the areas of the overlapping structures were summed as a single coordinate.

Coefficient of variation is defined as standard deviation of intensity divided by mean intensity as previously described (Bishop *et al.*, 2015; Jensen *et al.*, 2017). Quantification of coefficient of variation and intensity measurements were collected in FIJI. An ROI was drawn around a cell and standard deviation of intensity, and mean intensity values were collected.

For line scan analysis of fluorescence intensity, raw intensity values were collected within FIJI and normalized to the maximum value collected.

Analysis of DsRed2-ER5 velocity was performed in MATLAB (MathWorks) using the PIVlab toolkit (Thielicke and Stamhuis, 2014) as previously described (51). Briefly, successive frames (captured at 31.25 Hz) of DsRed2-ER5 expression in HEK293T cells transfected with DsRed2-ER5 alone or cotransfected with GFP-Kv2.2, GFP-Kv2.2 P412W, GFP-Kv2.1 or GFP-Kv2.1 P404W were collected in TIRF. Images were converted into BMP file format and 1 out of every 10 frames (creating a time lapse of 320 ms) were imported into PIVlab. Contrast limited adaptive histogram equalization (contrast enhancement) was engaged, and frame pairs were analyzed with 3 successive passes, utilizing interrogation areas of 64, 32, and 16 pixels. From an ROI drawn within the center of each cell analyzed, average velocity magnitude values (reported as pixels per frame) were collected.

For all analysis, values were imported into GraphPad Prism for presentation and statistical analysis as noted. For IHC experiments, we define biological replicates as individual animals. The datasets in this manuscript involving IHC contain biological replicates. For experiments performed with cells in culture, we define biological replicates as experiments performed on different days, and technical replicates as experiments performed on the same day. The datasets in this manuscript involving cells in culture contain biological and/or technical replicates.

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Table 1. Biophysical characteristics of Kv2 currents from nonclustering mutants

Sample	I_K at +50 mV (pA/pF)	V_{mid}	n (cells)
GFP-Kv2.2	70.39 ± 41.67	17.34 ± 3.08 mV	6
GFP-Kv2.2 S605A	51.11 ± 36.34^a	13.43 ± 3.10 mV ^c	5
GFP-Kv2.1	68.89 ± 17.95	-10.09 ± 2.70 mV	5
GFP-Kv2.1 S586A	80.89 ± 23.85^b	-8.76 ± 4.90 mV ^d	4

p values (two tailed, unpaired t-test)

a. 0.434 versus Kv2.2

b. 0.438 versus Kv2.1

c. 0.067 versus Kv2.2

d. 0.649 versus Kv2.1

Table 2. Antibody information

Antigen and antibody name	Immunogen	Manufacturer information	Concentration used	Figures used
Kv2.2 (N372B/60)	Fusion protein aa 717-907 of rat Kv2.2 long isoform	Mouse IgG2a mAb, NeuroMab catalog #73-360, RRID:AB_2315867	Purified, 10 µg/mL (1-cortex, 11), 20 µg/mL (1-CA1), 15 µg/mL (12)	1, 4 (CHN), 10, 11
Kv2.2 (N372C/51)	Fusion protein aa 717-907 of rat Kv2.2 long isoform	Mouse IgG1 mAb, NeuroMab catalog #75-358, RRID:AB_2315866	Purified, 10 µg/mL	4 (IHC)
Kv2.1 (K89/34)	Synthetic peptide aa 837-853 of rat Kv2.1	Mouse IgG1 mAb, NeuroMab catalog #73-014, RRID:AB_1067225	Tissue culture supernatant, 1:5	1, 4 (CHN), 10
Kv2.1 (KC)	Synthetic peptide aa 837-853 or rat Kv2.1	Rabbit pAb, In-house (Trimmer Laboratory), RRID:AB_2315767	Affinity-purified, 1:150	1, 4 (IHC), 11
Ryanodine Receptor (34C)	Partially purified chicken pectoral muscle ryanodine receptor	Mouse IgG1 mAb, Developmental Studies Hybridoma Bank, RRID:AB_528457	Concentrated tissue culture supernatant, 1 µg/mL	1 (CHN)
Kv1.5e	Synthetic peptide aa 271-284 of rat Kv1.5	Rabbit pAb, In-house (Trimmer Laboratory), RRID:AB_2722698	Affinity-purified, 1:100	10
Ryanodine Receptor (34C)	Partially purified chicken pectoral muscle ryanodine receptor	Mouse IgG1 mAb, ThermoFisher catalog #MA3-925 RRID:AB_2254138	Purified, 2.5 µg/mL (1-cortex), 1 µg/mL (1-CA1, 12)	1 (IHC), 11
AnkyrinG (N106/36)	Full-length recombinant human	Mouse IgG2b mAb, NeuroMab catalog #75-147, RRID:AB_10675130	Purified, 10 µg/mL	4 (CHN)
AnkyrinG (N106/65)	Full-length recombinant human	Mouse IgG2b mAb, NeuroMab catalog #75-147, RRID:AB_10675130	Purified, 5 µg/mL	4 (IHC)
MAP2	Purified microtubule associated protein from rat brain	Chicken pAb, EnCor catalog #CPCA-MAP2, RRID:AB_2138173	Purified IgY fraction, 1:5000	1

Figure Legends

FIGURE 1. Kv2.2 associates with ER-PM junctions in mammalian brain neurons *in situ* and in culture, and in heterologously expressing HEK293T cells. (A-C) Single z-stack images of multiplex immunofluorescence labeling of adult mouse brain sections showing neocortex (A) and hippocampal CA1 region (B) immunolabeled for Kv2.2 (green) and RyR (magenta), or cultured hippocampal neurons (C) immunolabeled for Kv2.2 (green), Kv2.1 (blue) and RyR (red), as indicated. Scale bar in Kv2.2 neocortex panel is 10 μm and holds for all brain panels. Scale bar in MAP2 CHN panel is 10 μm and holds for all CHN panels in that row. Scale bar in Kv2.2 magnified inset is 2.5 μm and holds for all panels in that row. Panels to the right of each set of images are the corresponding normalized fluorescence intensity values across the individual line scans depicted by the white line in the merged images. (D) Images of fixed HEK293T cells coexpressing GFP-Kv2.2 (green) and BFP-SEC61 β (magenta). The top row shows a single N-SIM optical section taken through the center of a cell. The scale bar is 1.25 μm . The bottom rows show a 2D projection of a 3D reconstruction (middle row), and a single orthogonal slice through the 3D reconstruction (bottom row) of a cell imaged with a Zeiss Airyscan microscope. Scale bar in the GFP-Kv2.2 panel of the 3D reconstruction is 2.5 μm and holds for all panels in bottom two rows. Panels to the right of each set of rows are the corresponding normalized fluorescence intensity values across the individual line scans depicted by the arrows (top) or white line (bottom) in the merged images.

FIGURE 2. Exogenous Kv2 expression remodels ER-PM junctions in HEK293T cells and cultured rat hippocampal neurons. (A-B) TIRF images of live HEK293T cells expressing DsRed2-ER5 (magenta) either alone (A), or coexpressed with (in green) GFP-Kv2.2, GFP-Kv2.1, or GFP-Kv1.4, as indicated (B). Scale bar in (A) is 5 μm and holds for all panels. (C) Graph of mean ER-PM junction (EPJ) size per cell measured from HEK293T cells coexpressing DsRed2-ER5 and GFP-Kv2.2, GFP-Kv2.1, or GFP-Kv1.4, or expressing DsRed2-ER5 alone

(control). (D) Graph of percent of the PM area per cell occupied by cortical ER measured from HEK293T cells coexpressing DsRed2-ER5 and GFP-Kv2.2, GFP-Kv2.1, or GFP-Kv1.4, or expressing DsRed2-ER5 alone (control). (E) Graph of Pearson's Correlation Coefficient (PCC) values between DsRed2-ER5 and GFP-Kv2.2, GFP-Kv2.1, or GFP-Kv1.4 measured from HEK293T cells coexpressing DsRed2-ER5 and GFP-Kv constructs. The dashed line denotes a PCC value of 0.5. Bars on all graphs are mean \pm SD. See Supplemental Tables 1-3 for values and statistical analyses for Figures 2C-E. (F) TIRF image of a live CHN expressing DsRed2-ER5 (magenta) alone. (G) TIRF image of a live CHN coexpressing DsRed2-ER5 (magenta) and GFP-Kv2.2 (green). Scale bar in DsRed2-ER5 panel is 10 μ m and holds for all panels in that row. Magnified images shown in bottom row. Scale bar in DsRed2-ER5 magnified inset panel is 2.5 μ m and holds for all panels in that row. (H) Scatter plot shows sizes of Kv2.2 clusters and associated ER-PM junctions (EPJs, as reported by DsRed2-ER5 in TIRF) in HEK293T cells (red points) and CHNs (black points). n = 3 cells each.

FIGURE 3. ER-PM junction-localized Kv2.2 channels are expressed on the cell surface. (A) TIRF images of a live HEK293T cell expressing GFP-Kv2.2 (green) and DsRed2-ER5 (red), and surface labeled for Kv2 channels with GxTX-633 (blue). Heat map shows overlap of GFP-Kv2.2 and GxTX-633 pixels. Scale bar is 5 μ m. (B) Fluorescence intensity values across the individual line scan depicted by the white line in the merged image. (C) Graph of PCC values between GxTX and Kv2.2 or DsRed2-ER5 measured from live HEK293T cells surface labeled with GxTX-633 and coexpressing GFP-tagged Kv2.2 and DsRed2-ER5. Bars are mean \pm SD. See Supplemental Table 5 for values and statistical analyses for Figure 3C.

FIGURE 4. Kv2-mediated ER-PM junctions are located at sites depleted in components of the cortical actin cytoskeleton. (A) Mouse brain section immunolabeled for Kv2.2 (green), Kv2.1 (red), and ankG (blue). Scale bar for large image is 20 μ m, and for Kv2.2 inset is 3 μ m and

holds for all inset panels. (B) Projected z-stack of optical sections taken from a CHN immunolabeled for Kv2.2 (green), Kv2.1 (red), and ankG (blue). Scale bar for large image is 20 μm , and for Kv2.2 inset is 3 μm and holds for all inset panels. (C) Single optical section taken from the cell body of a CHN immunolabeled for Kv2.2 (green), Kv2.1 (red), and labeled for F-actin with phalloidin (blue). Scale bar for merged panel is 10 μm holds for all panels in set. Panels below each set of images show the corresponding normalized fluorescence intensity values across the line scans indicated in the merged images in that column. (D) TIRF images of live HEK293T cells coexpressing GFP-Kv2.2 (green) and BFP-SEC61 β (blue) in conjunction with (in red) mCherry-actin (top row) or ankG-mCherry (bottom row). Scale bar for GFP-Kv2.2 panel in top row is 5 μm and holds for all panels in set. Panels to the right of each row show the corresponding normalized fluorescence intensity values across the line scan depicted by the white line in the merged images.

FIGURE 5. Disrupting the actin cytoskeleton impacts spatial organization of Kv2.2-mediated ER-PM junctions. (A-B) TIRF images of a live HEK293T cell coexpressing GFP-Kv2.2 (green) and DsRed2-ER5 (magenta), prior to (Rest) and 15 min after Latrunculin A (After LatA) treatment. Scale bar in GFP-Kv2.2 Rest panel is 5 μm and holds for all panels. Graphs below show values measured from cells before (Rest) and after a 15-minute treatment (After LatA) with 10 μM LatA. (C) Mean Kv2.2 cluster size per cell. (D) Mean ER-PM junction (EPJ) size per cell. (E) Number of ER-PM junctions per cell. (F) PCC values between Kv2.2 and DsRed2-ER5. See Supplemental Tables 6-9 for values and statistical analyses for Figures 5C-F.

FIGURE 6. Kv2.2-containing ER-PM junctions colocalize with multiple components of mammalian ER-PM junctions. (A) TIRF images of live HEK293T cells coexpressing DsRed- or GFP-tagged Kv2.2 and various members of the E-Syt, JP and STIM families of ER-localized PM tethers. Scale bar in top left GFP-Kv2.2 panel is 10 μm and holds for all panels. Heat maps

show pixel overlap of Kv2.2 and ER-PM tether signals. STIM-containing cells were treated with 2 μ M thapsigargin for 5 minutes prior to imaging. (B) Graph of PCC values between Kv2.2 and ER-PM tethers. Bars are mean \pm SD. (C) Graph of MOC values between Kv2.2 and ER-PM tether signals. Bars are mean \pm SD. See Supplemental Table 10 for values and statistical analyses for Figure 6B and C. (D) TIRF images of a live HEK293T cell coexpressing BFP-STIM1 (blue), DsRed-Kv2.2 (green), and GFP-Orai1 (red) prior to (Rest) and immediately after 5 min of treatment with 2 μ M Thapsigargin (+Thap). Scale bar in top left DsRed-Kv2.2 panel is 5 μ m and holds for panels in D, E. (E) TIRF images of a live HEK293T cell coexpressing DsRed-Kv2.2 (green) and GFP-Orai1 (magenta) prior to (Rest) and immediately after 5 min of treatment with 2 μ M Thapsigargin (+Thap). (F) Graph of PCC values between Orai1 and Kv2.2 (black) or STIM1 (red) measured from cells with BFP-STIM1 coexpression before (Rest) and after (+Thap) Thapsigargin treatment. (G) Graph of PCC values between Orai1 and Kv2.2 measured from cells without BFP-STIM1 coexpression before (Rest) and after (+Thap) Thapsigargin treatment. Bars on all graphs are mean \pm SD. See Supplemental Table 11 for values and statistical analyses for Figure 6F and G.

FIGURE 7. Acutely formed ER-PM junctions are distinct from Kv2.2-containing ER-PM junctions. (A) TIRF images of CFP fluorescence in a live HEK293T cell coexpressing CFP-CB5-FKBP and Lyn11-FRB before (Rest) and immediately after treatment with 5 μ M rapamycin (+Rap). Scale bar is 5 μ m and holds for both panels. Graph to right of panels shows fluorescence intensity of CFP-CB5-FKBP across the individual line scan depicted by the white lines before (Rest) and immediately following treatment with 5 μ M rapamycin (+Rap). (B) TIRF images of a live HEK293T cell coexpressing DsRed-Kv2.2 (green), CFP-CB5-FKBP (magenta), and Lyn11-FRB. Top row. Prior to rapamycin treatment (Rest). Middle row. Same cell immediately following 5 μ M rapamycin treatment (+Rap). Bottom row. Same cell after subsequent 15-minute treatment with 10 μ M LatA (+LatA). Panels to the right of each row are

the corresponding normalized fluorescence intensity values across the individual line scans depicted by the white line in the merged images. Scale bar is 10 μm and holds for all panels. (C) Graph of PCC values between DsRed-Kv2.2 and CFP-CB5-FKBP. Bars are mean \pm SD. See Supplemental Table 12 for values and statistical analysis.

FIGURE 8. Mutations that eliminate K^+ conductance do not impact Kv2.2 channel clustering. (A) Exemplar whole-cell voltage clamp recordings (left) and corresponding graphs of current levels versus command voltage (right) of HEK293T cells expressing GFP (control), GFP-Kv2.2, GFP-Kv2.2 P412W, GFP-Kv2.1, or GFP-Kv2.1 P404W. Recordings shown are representative responses to 100 ms steps from -100 mV to -40, 0 and +40 mV. Note the lack of outward currents in control GFP-Kv2.2 P412W, and GFP-Kv2.1 P404W recordings. (B-C) Summary graphs showing whole cell current at +40 mV for cells expressing Kv2.2 (B) or Kv2.1 (C) isoforms. See Supplemental Table 13 for values and statistical analyses for Figure 8B-C. (D) Deconvolved widefield image of a live CHN expressing GFP-Kv2.2 P412W or GFP-Kv2.1 P404W. Scale bar is 5 μm and holds for both panels. (E) TIRF images of live HEK293T cells expressing GFP-Kv2.2 P412W or GFP-Kv2.1 P404W and surface labeled with GxTX-633. Scale bar in the Kv2.2 P412W panel is 5 μm and hold for all panels. (F-G) Graphs of mean cluster size per cell measured from CHNs expressing GFP-Kv2.2 or GFP-Kv2.2 P412W (F), or GFP-Kv2.1 or GFP-Kv2.1 P404W (G). Bars are mean \pm SD. See Supplemental Table 14 for values and statistical analyses for Figure 8F-G. (H-I) Graph of PCC values between Kv2 and GxTX from HEK293T cells expressing GFP-Kv2.2 or GFP-Kv2.2 P412W (H), or GFP-Kv2.1 or GFP-Kv2.1 P404W (I). Bars are mean \pm SD. See Supplemental Table 15 for values and statistical analyses for Figure 8H-I.

FIGURE 9. Separation of function point mutations show that clustering, but not conduction, is necessary for Kv2-mediated remodeling of ER-PM junctions. (A) TIRF images of live HEK293T

cells expressing GFP-tagged Kv2.2 mutants (nonconducting P412W and nonclustering S605A in green) and DsRed2-ER5 (magenta). Scale bar is 5 μ m and holds for all panels. (B) TIRF images of live HEK293T cells expressing GFP-tagged Kv2.1 mutants (nonconducting P404W and nonclustering S586A in green) and DsRed2-ER5 (magenta). (C-E) Comparisons of cells expressing wild-type and mutant Kv2 isoforms (Kv2.2 P412W, Kv2.2 S605A, Kv2.1 P404W, or Kv2.1 S586A); control refers to cells expressing DsRed2-ER5 alone. (C) Mean ER-PM junction (EPJ) size per cell. (D) Percent PM per cell occupied by cortical ER. (E) PCC values between DsRed2-ER5 and wild-type and mutant Kv2 isoforms. Bars on all graphs are mean \pm SD. See Supplemental Table 16-18 for values and statistical analyses. (F, G) Exemplar whole-cell voltage clamp recordings (left) and graphs of the corresponding normalized conductance-voltage relationship from HEK293T cells expressing GFP-Kv2.2 or GFP-Kv2.2 S605A (F), and GFP-Kv2.1 or GFP-Kv2.1 S586A (G). Different colors represent data from distinct cells. Recordings shown are representative responses to 200 ms steps from -100 mV to -40, 0 and +40 mV. Note the lack of a significant impact of the declustering point mutation on the properties of the whole cell currents. See Table 1 for values and statistical analyses for whole cell current density and midpoint of voltage activation, respectively.

FIGURE 10. The PRC domain can act autonomously to transfer the ER-PM junction remodeling function of Kv2 channels to another PM protein. TIRF images of fixed and immunolabeled HEK293T cells coexpressing BFP-SEC61 β (magenta) and in green either Kv2.2 (A), Kv2.1 (B), Kv1.5 (C), Kv1.5N-Kv2.1C (D), or Kv1.5N-Kv2.1PRC (E). Scale bar in (A) is 10 μ m and holds for all panels.

FIGURE 11. Genetic ablation of Kv2.2 and Kv2.1 alters RyR localization in mouse brain neurons. (A-D) Single optical sections of CA1 hippocampus from mouse brain sections from wild-type (A, WT), Kv2.1 knockout (B, Kv2.1KO), Kv2.2 knockout (C, Kv2.2KO), or Kv2.1 and

Kv2.2 double knockout (D, Kv2 dKO) mice immunolabeled for RyR (red), Kv2.2 (green), and Kv2.1 (blue). (A) RyR, Kv2.2, and Kv2.1 immunolabeling from WT mouse. (B) RyR and Kv2.2 immunolabeling from Kv2.1 KO mouse. (C) RyR and Kv2.1 immunolabeling from Kv2.2 KO mouse. (D) RyR immunolabeling from Kv2.1/Kv2.2 dKO mouse. Scale bar in (A) is 10 μm and holds for all panels in set. Panels to the right of each row are the corresponding normalized fluorescence intensity values across the individual line scans depicted by the white line in the merged images. (E) Enlarged selections of RyR-labeling of WT and Kv2.1/Kv2.2 dKO images as indicated by boxes in panels (A) and (D), respectively. Scale bar in WT RyR inset panel is 1.25 μm and holds for both panels. (F) Graph of mean RyR cluster size measured from each genotype. Bars are mean \pm SD. See Supplemental Table 20 for values and statistical analyses.

Supplemental Movie 1. Rotating 3D reconstruction of a fixed HEK293T cell expressing GFP-Kv2.2 (left panel, green) and BFP-SEC61 β (middle panel, magenta). Merged image is shown in right panel.

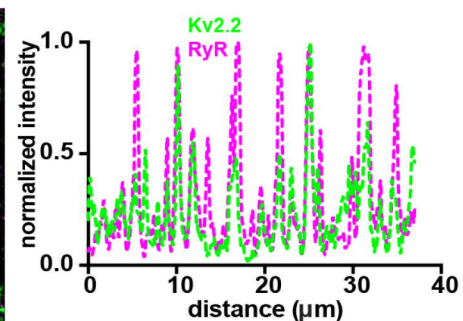
Supplemental Movie 2. Time lapse movie (15 minutes compressed into 10 seconds) of TIRF imaging of the effects of 10 μM LatA treatment of a live HEK293T cell expressing GFP-Kv2.2 (left panel, green) and DsRed2-ER5 (middle panel, red). Merged image is shown in the right panel.

Supplemental Figure Legends.

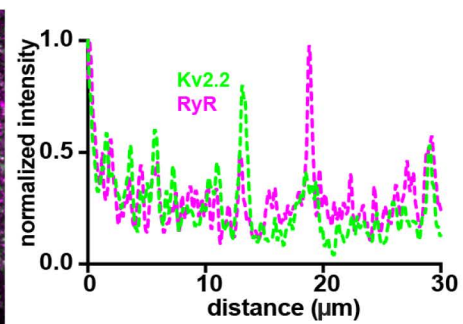
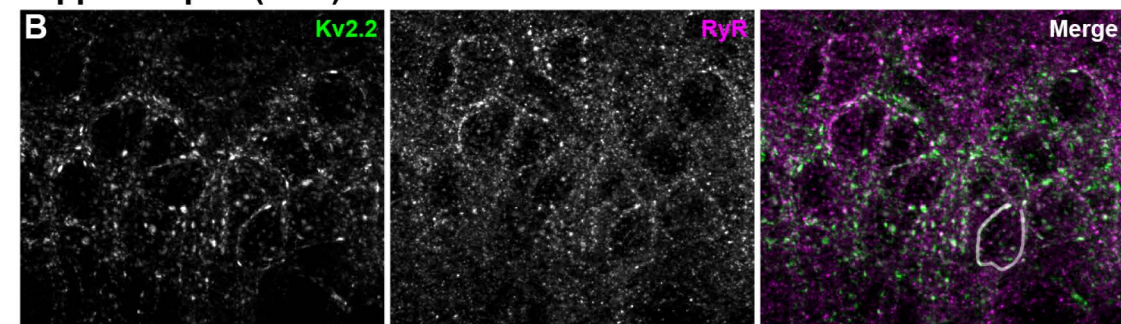
Supplemental Figure 1. Cell surface SEP-Kv2.1 at ER-PM junctions can be efficiently labeled with GxTX-633. Top panels. TIRF images of live HEK293T cells coexpressing SEP-Kv2.1 and BFP-SEC61 β and surface labeled with GxTX-633. The merged image shows SEP-Kv2.1 (pHluorin), BFP-SEC61 β , and GxTX-633. Scale bar is 5 μ m and holds for all panels. Heat map shows overlap of SEP-Kv2.1 (pHluorin) and GxTX-633 pixels. Bottom left panel shows the normalized fluorescence intensity values across the line scan depicted by the white line in the merged image. Graph on bottom right shows PCC values between GxTX and SEP-Kv2.1 or BFP-SEC61 β . Bars are mean \pm SD. See Supplemental Table 4 for values and statistical analyses.

Supplemental Figure 2. Both wild-type and nonconducting Kv2 channel mutants stabilize ER-PM junctions in HEK293T cells. (A) TIRF images of DsRed2-ER5 expressed in live HEK293T cells with and without coexpression of wild-type and mutant Kv2 channel isoforms as labeled. Scale bar is 2.5 μ m and holds for all panels. (B) Kymographs of DsRed2-ER5 mobility from regions indicated by the lines in the adjacent panels. (C) Graph of ER-PM junction (EPJ) velocity (as reported by DsRed2-ER5 in TIRF). Bars are mean \pm SD. See Supplemental Table 19 for values and statistical analyses.

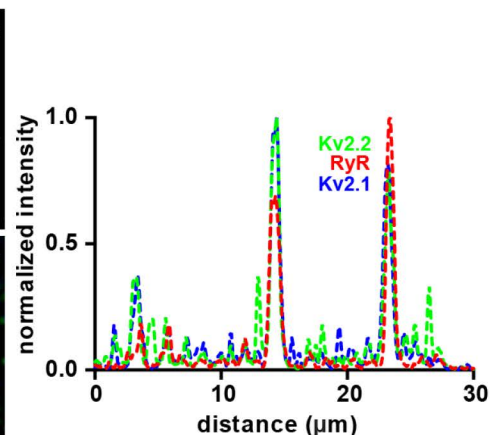
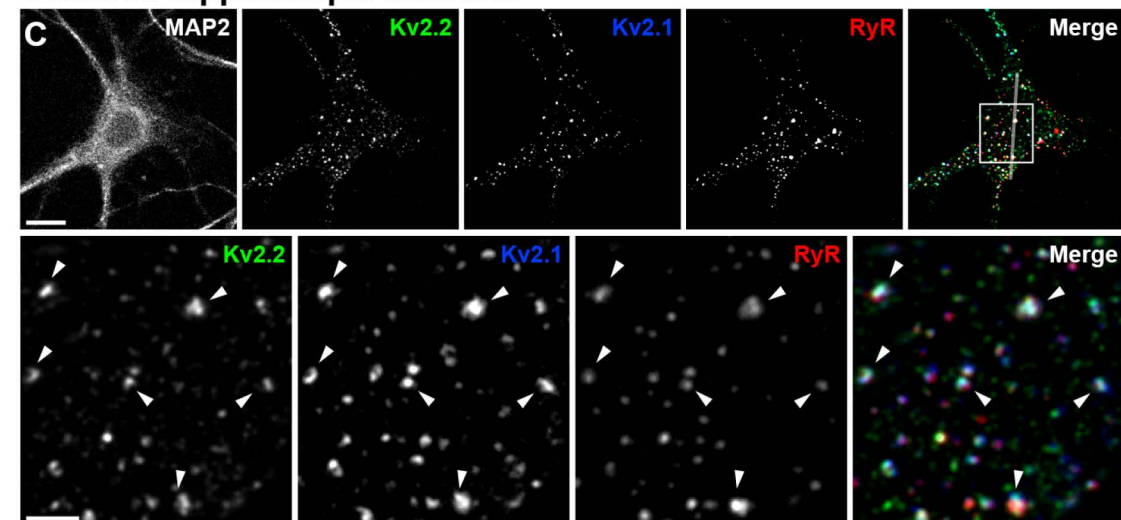
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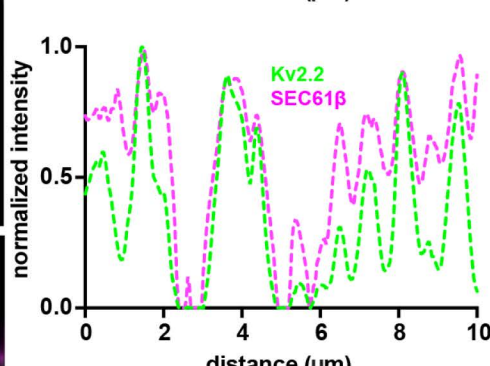
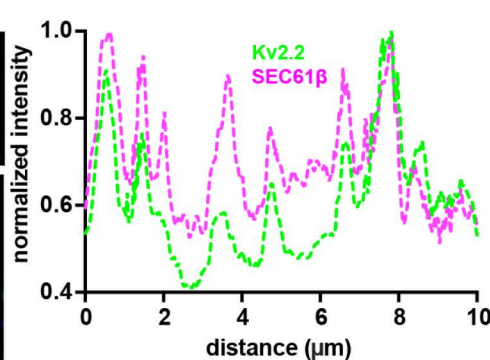
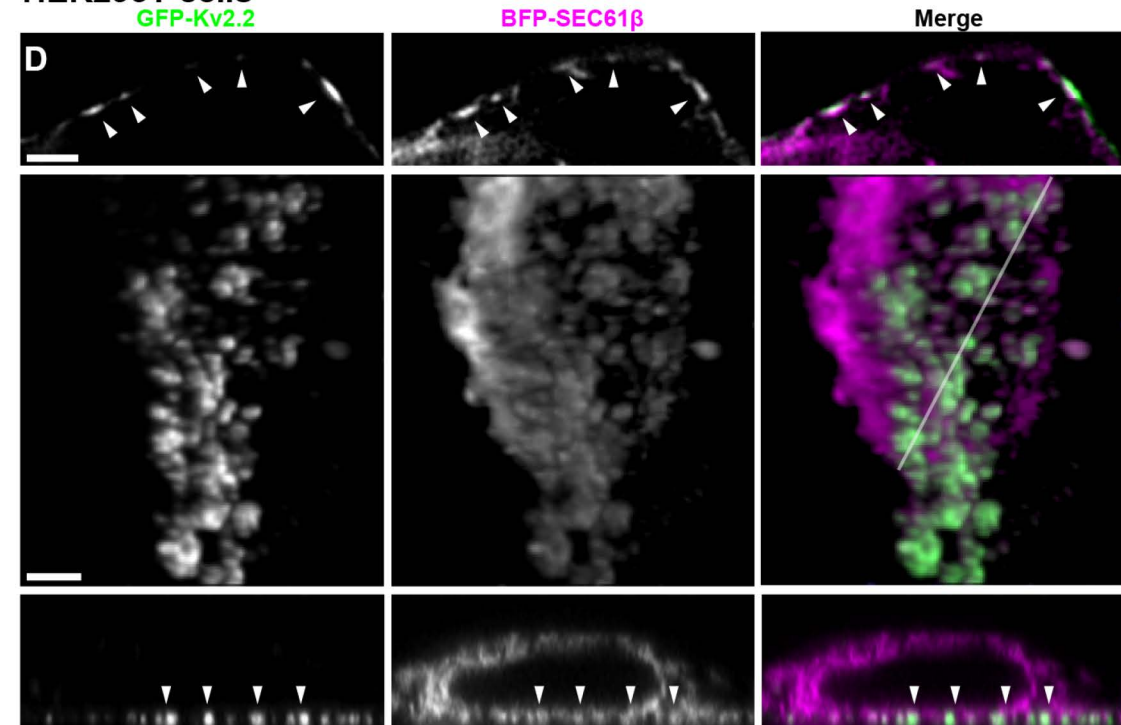
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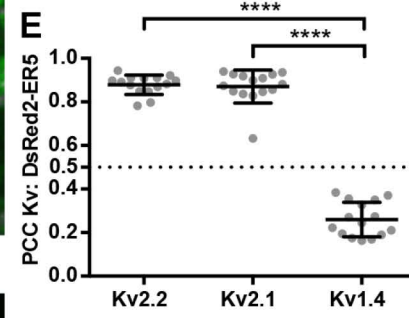
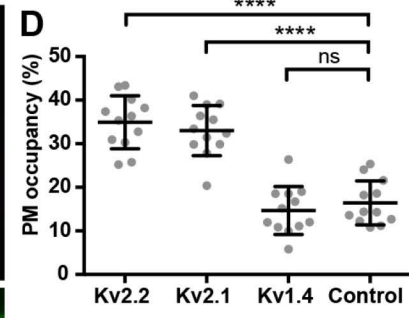
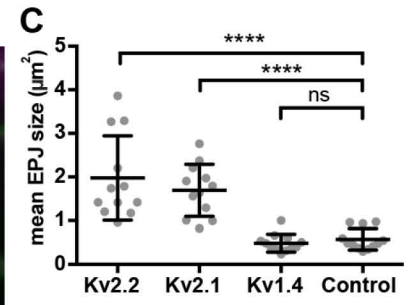
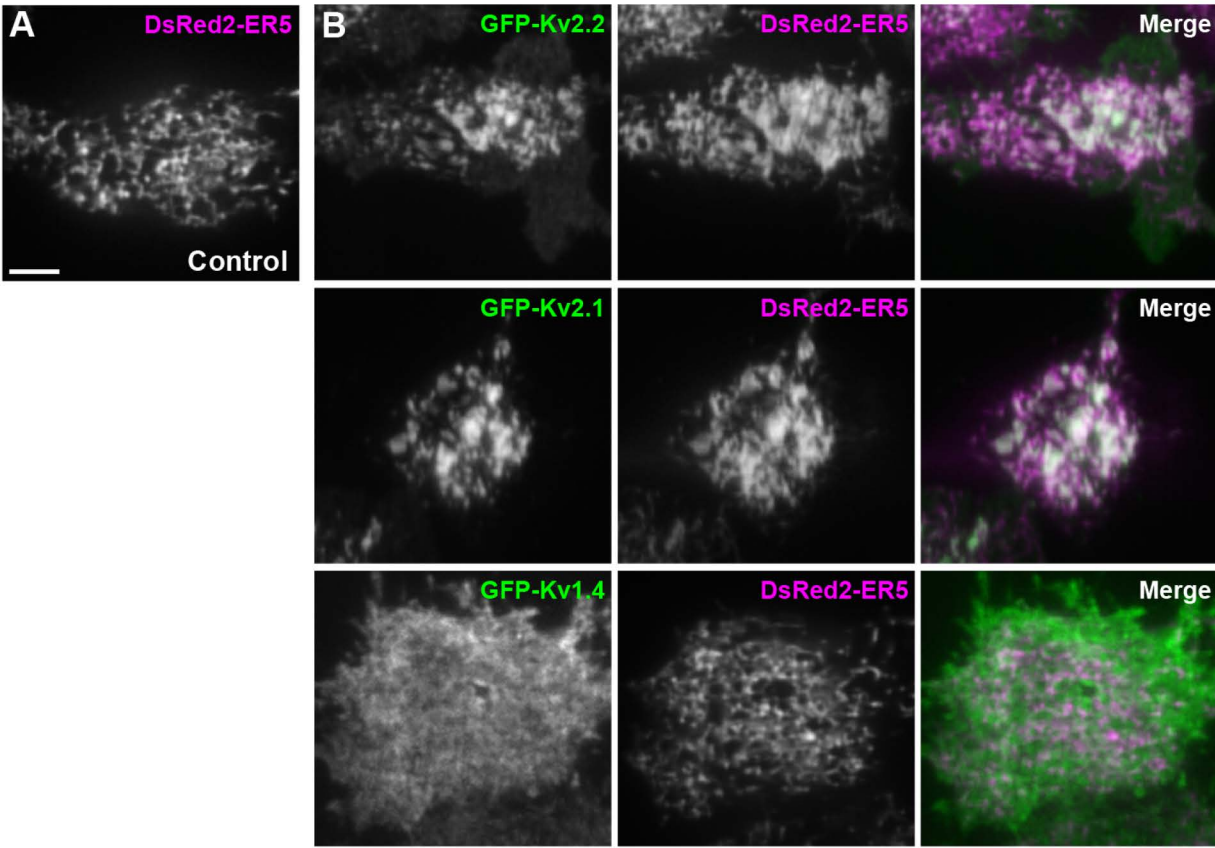
Cultured hippocampal neurons



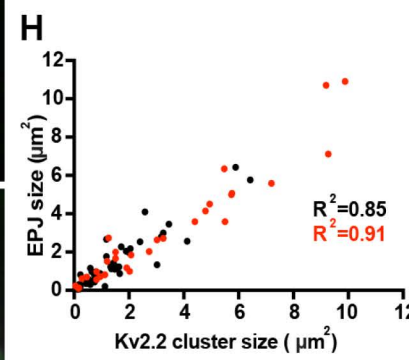
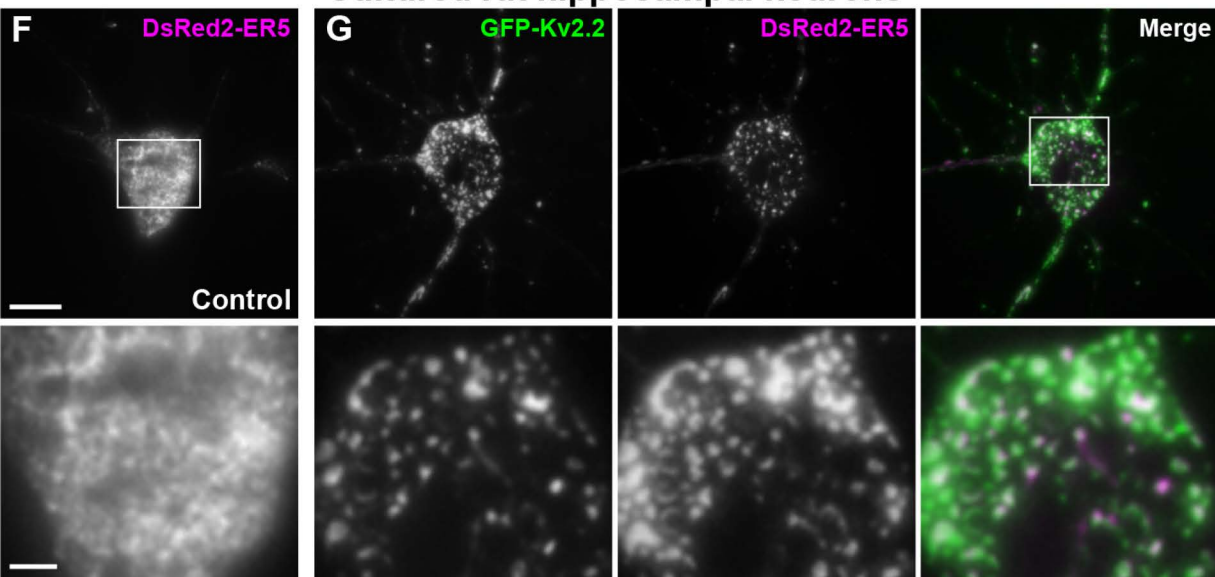
HEK293T cells

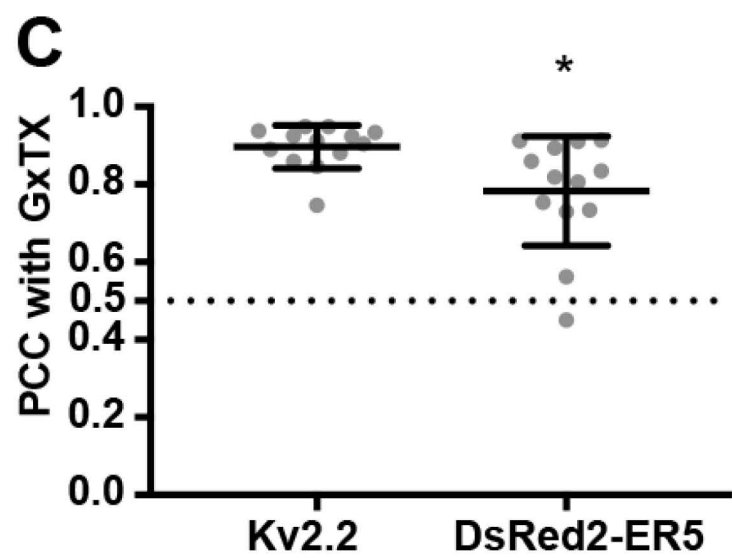
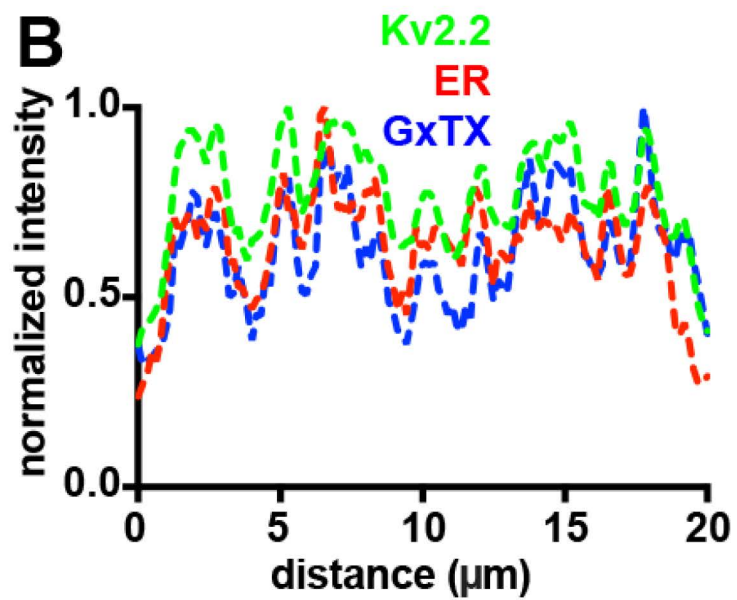
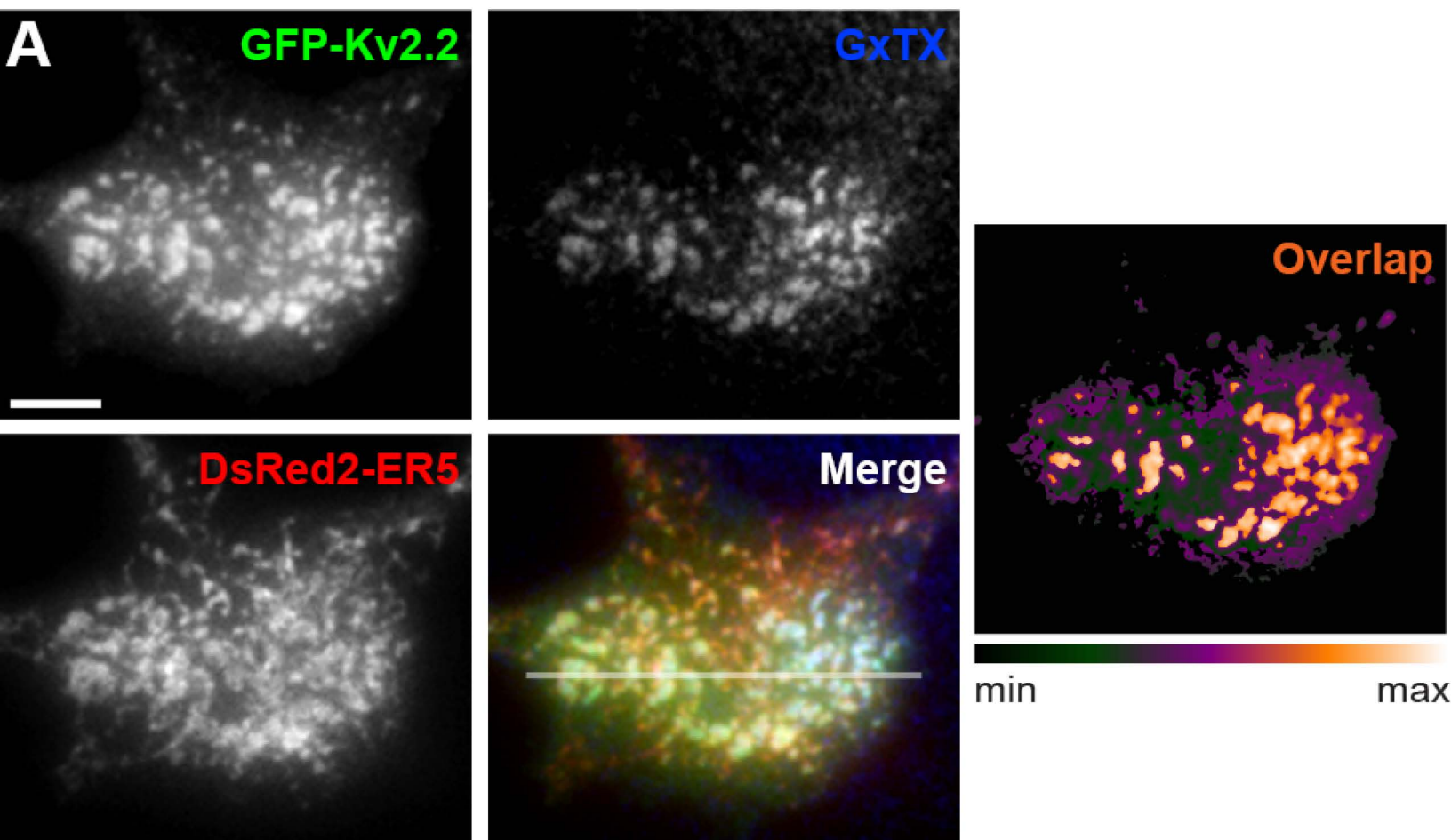


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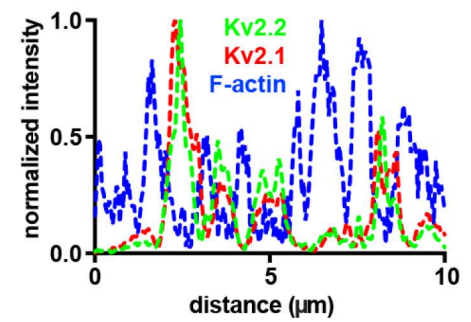
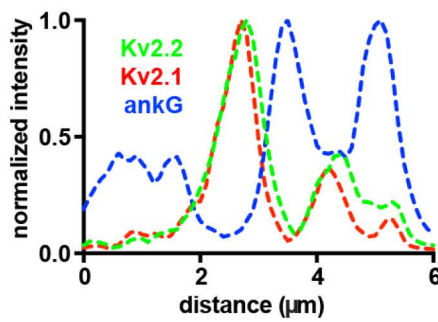
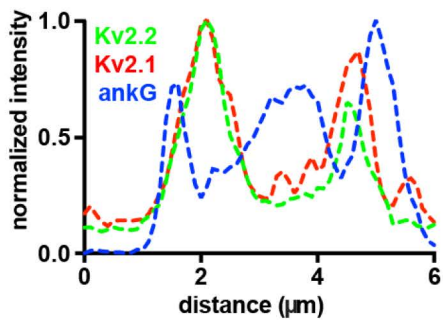
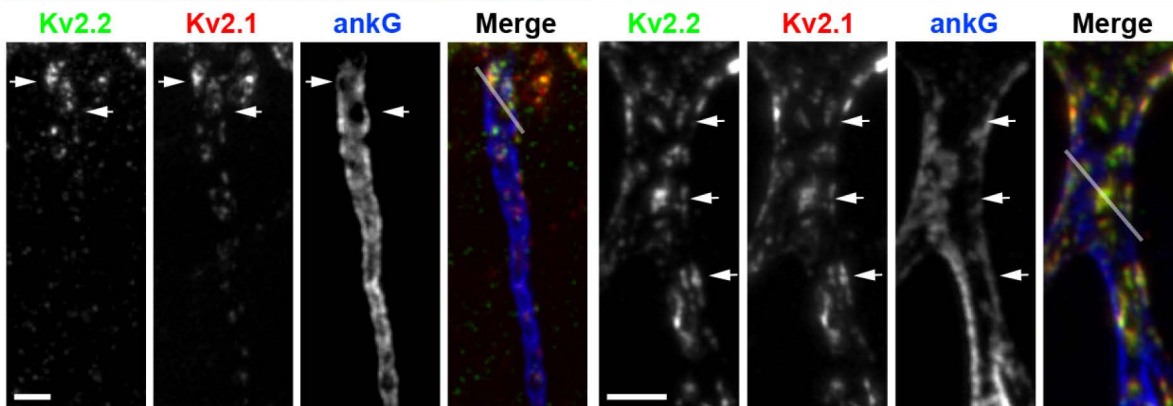
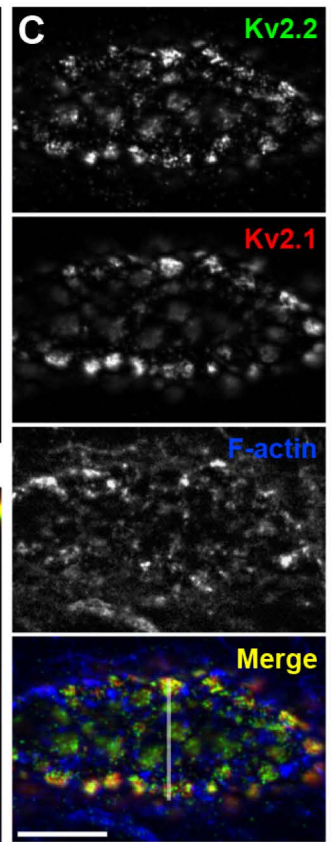
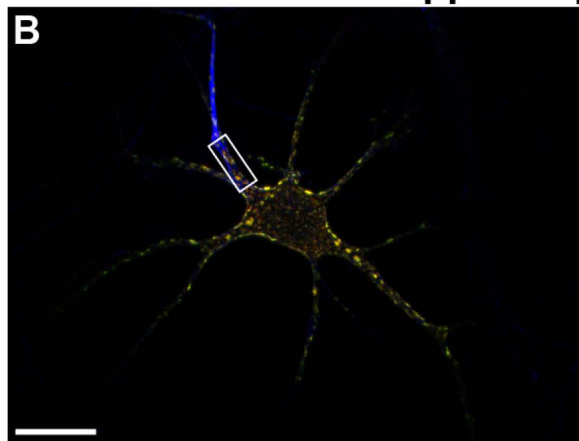
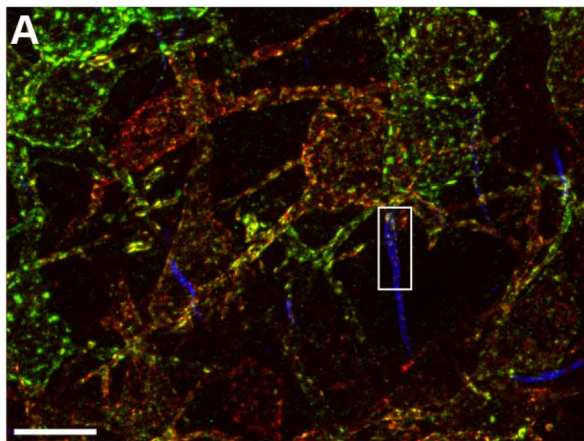
Cultured rat hippocampal neurons



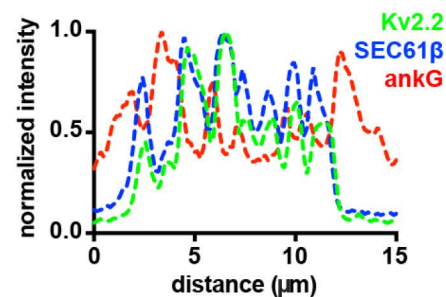
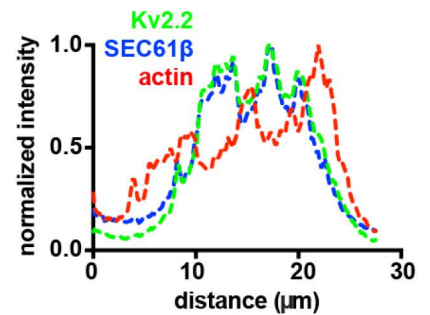
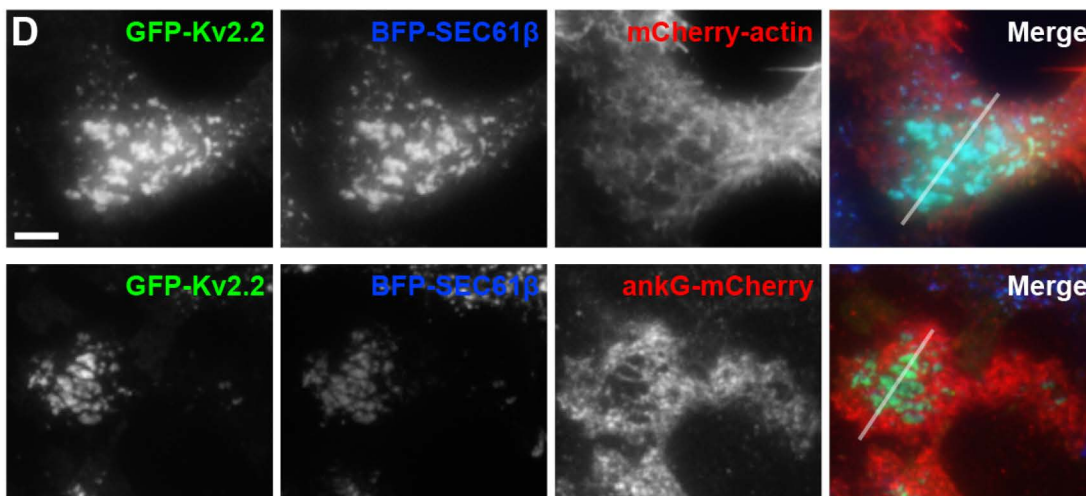


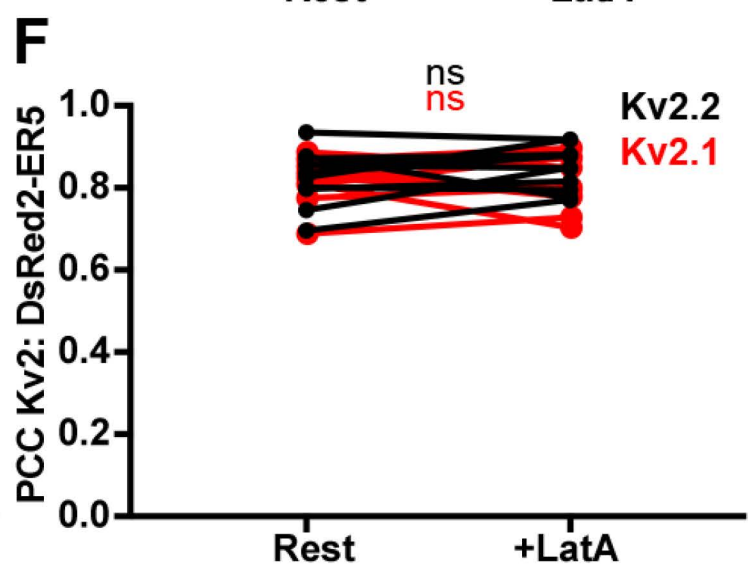
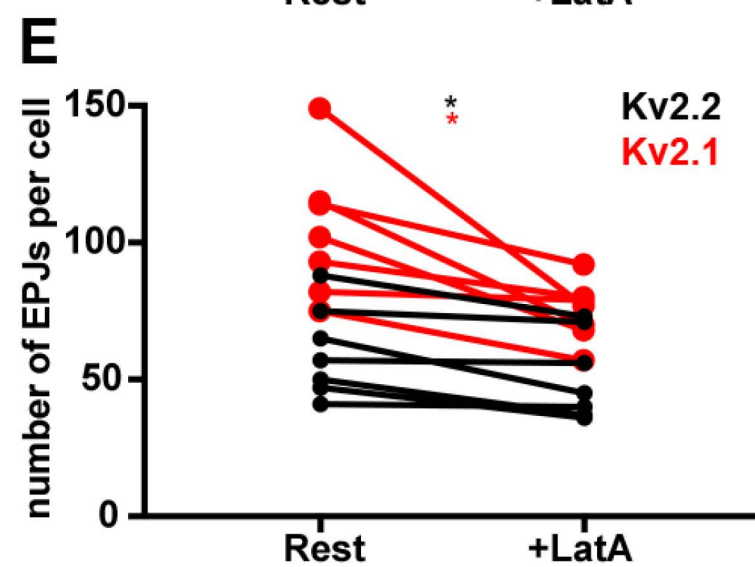
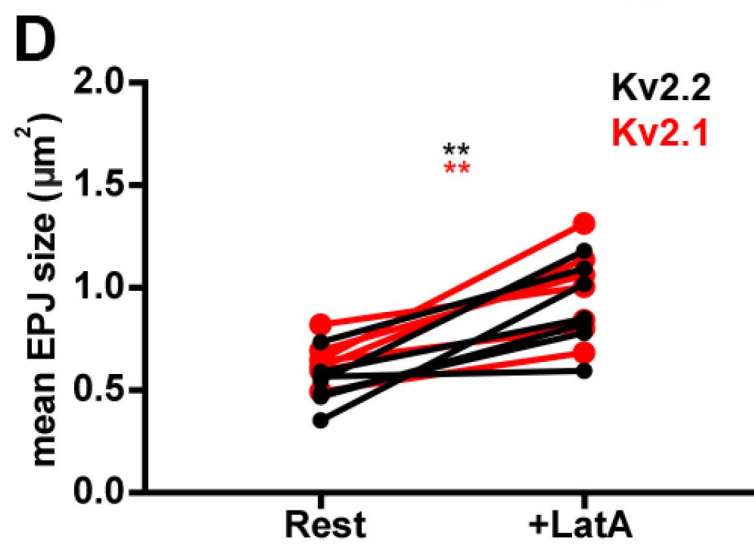
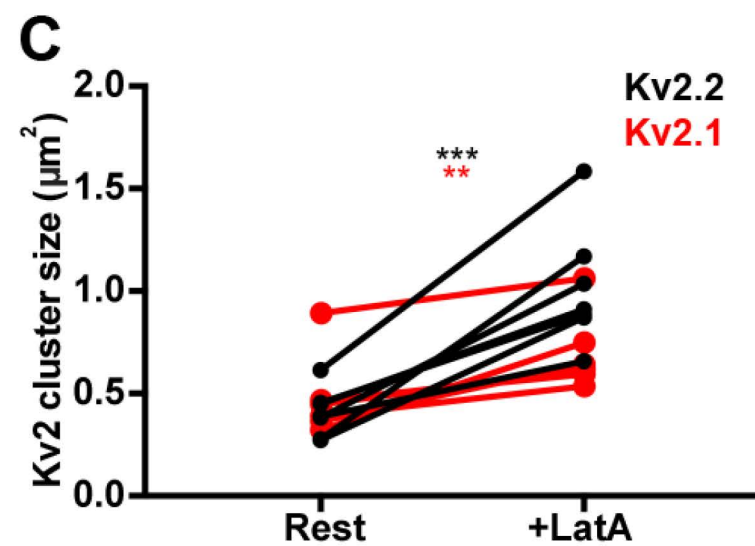
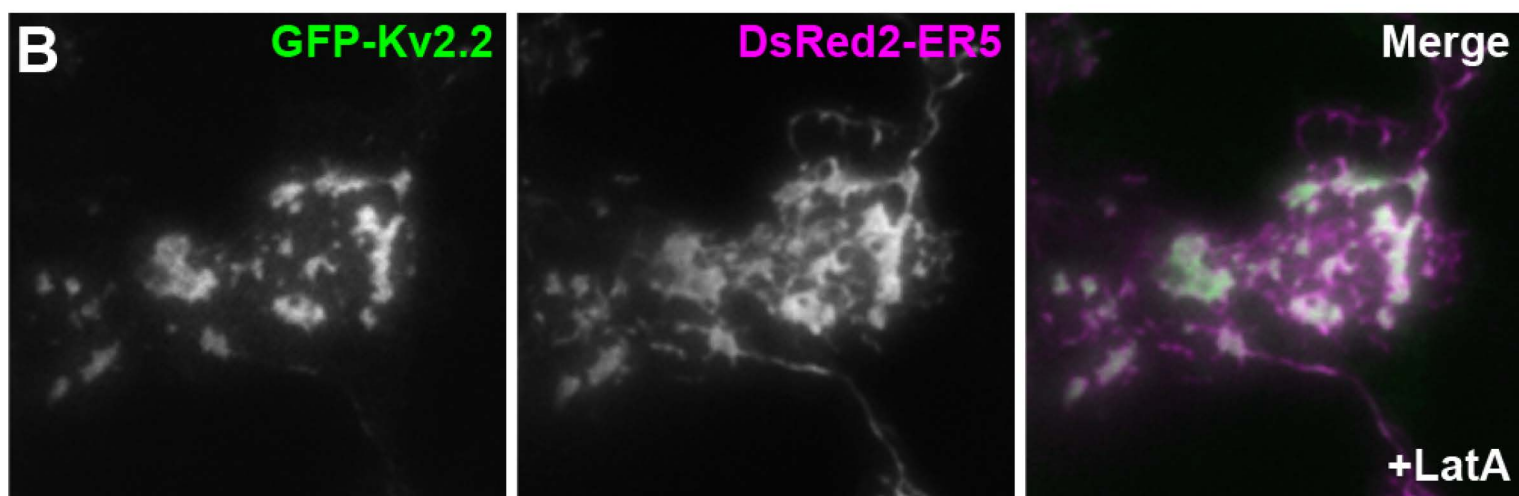
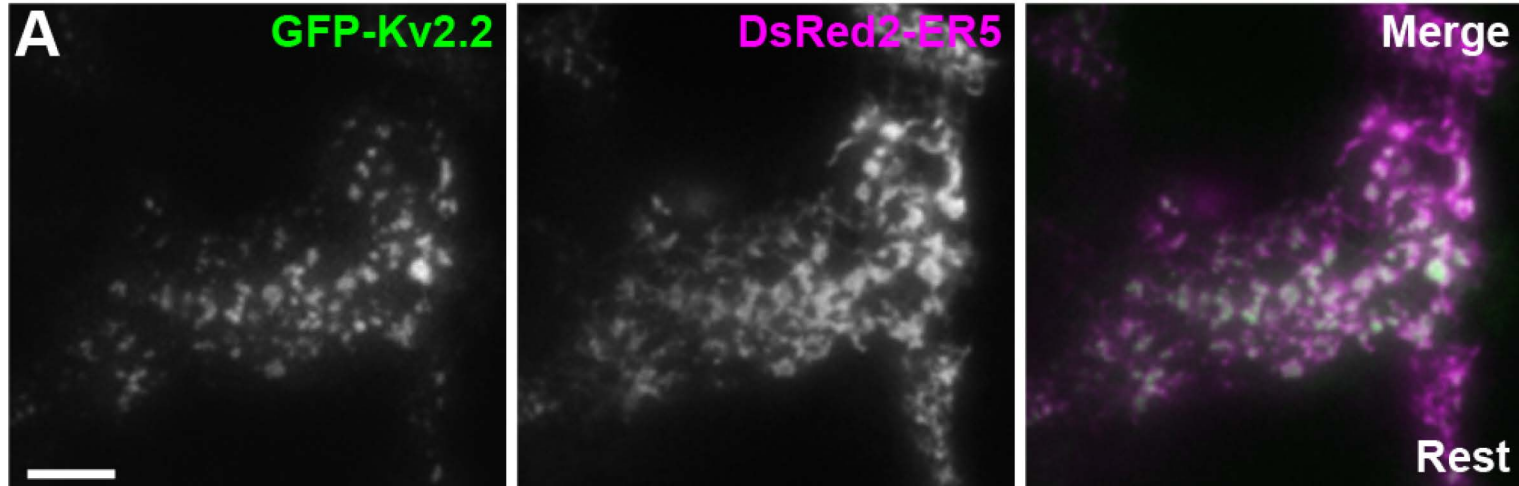
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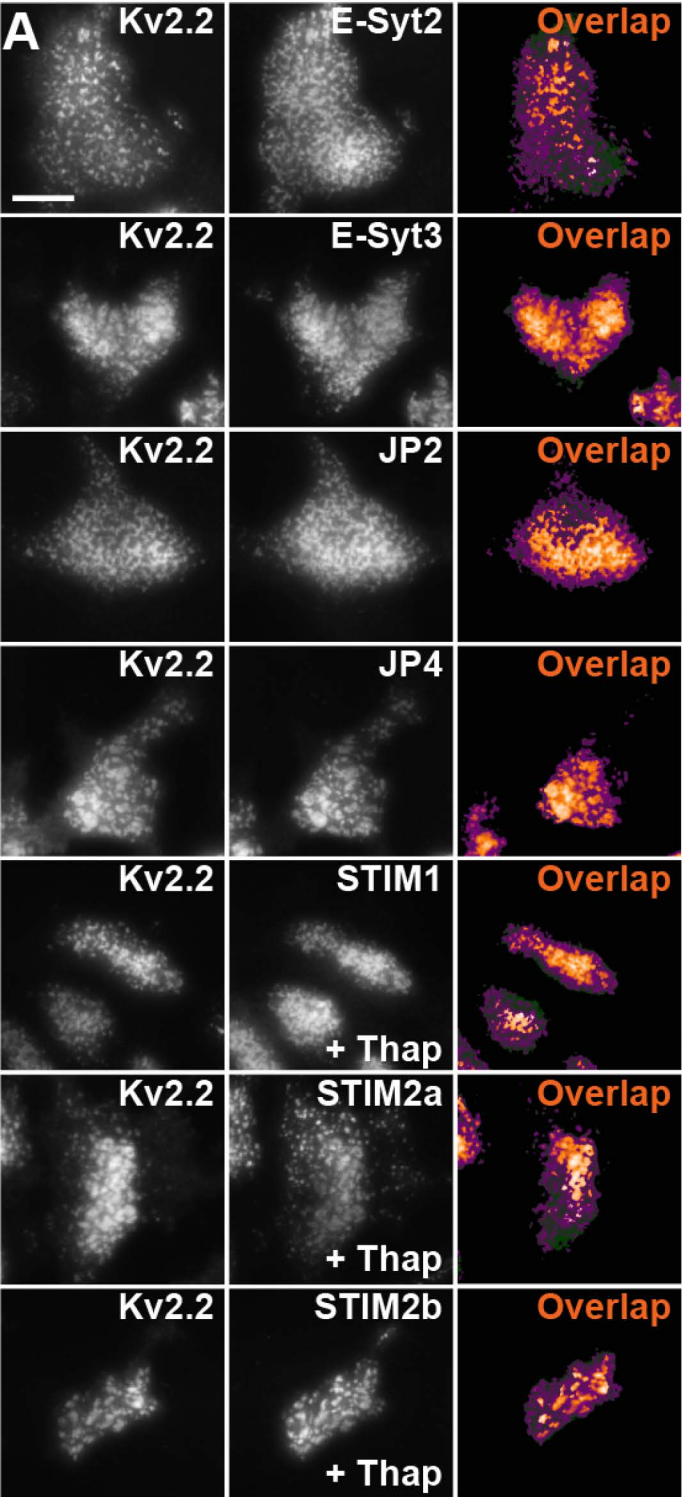
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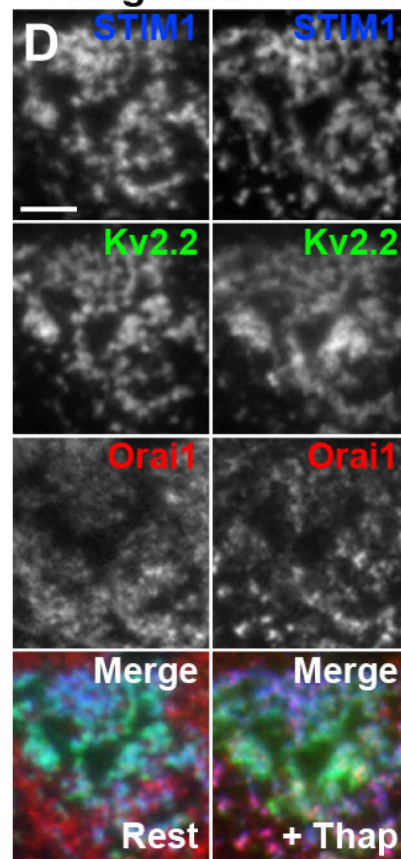
HEK293T cells



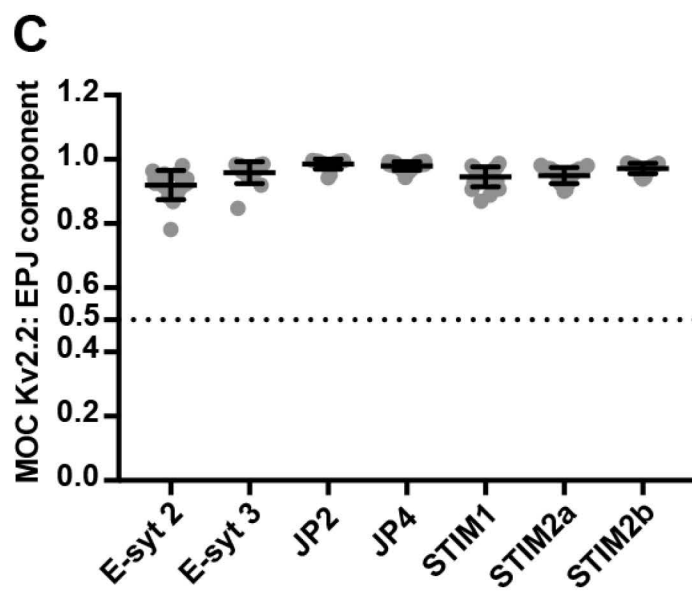
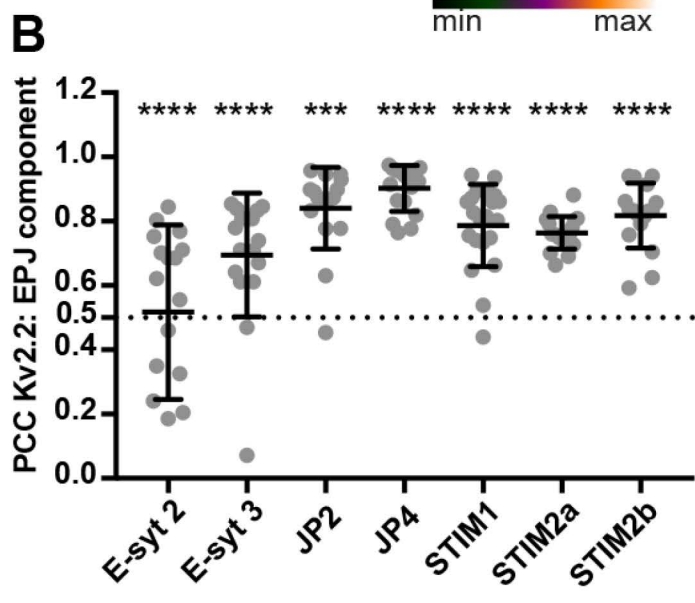
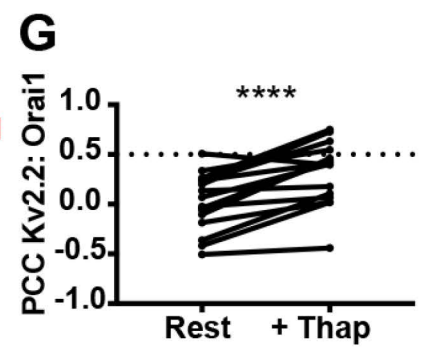
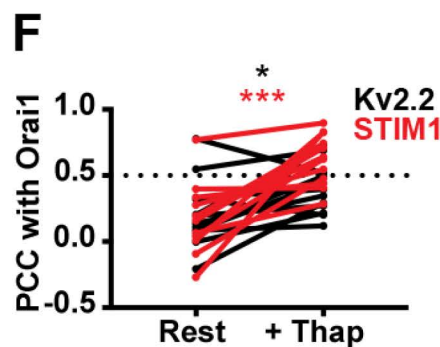
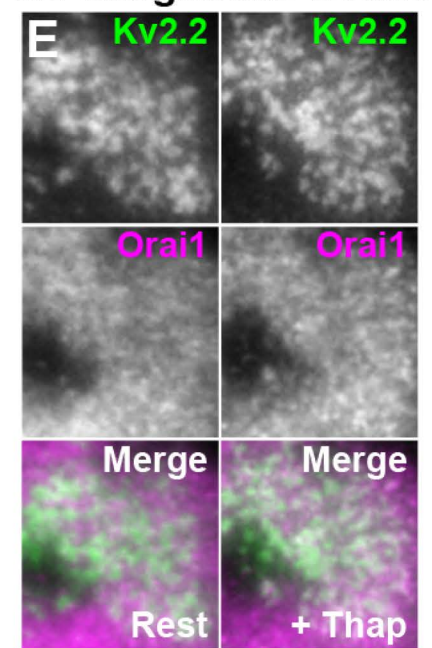




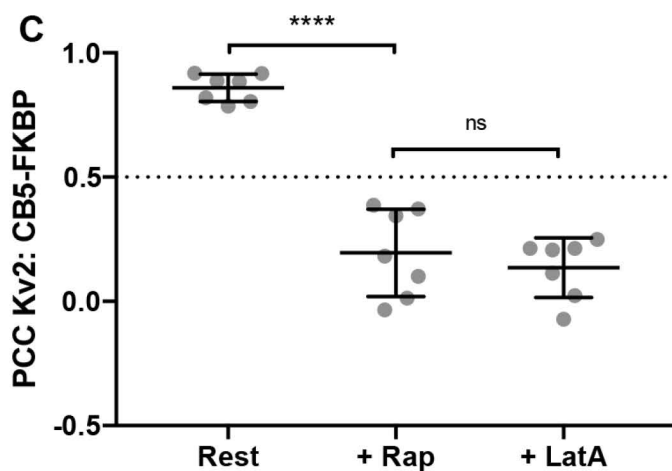
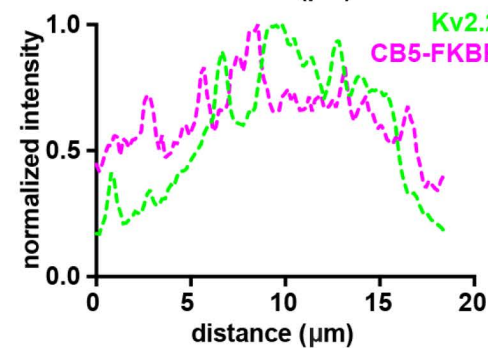
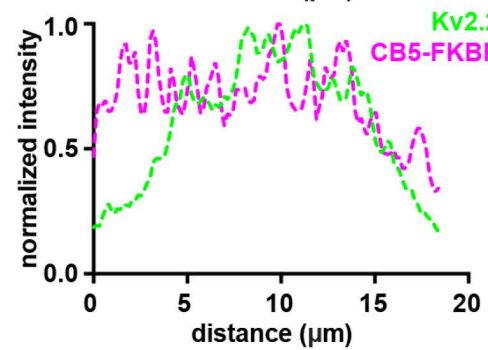
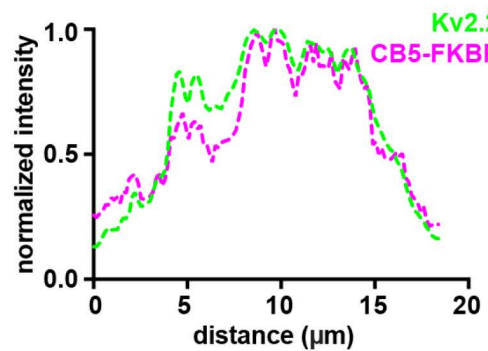
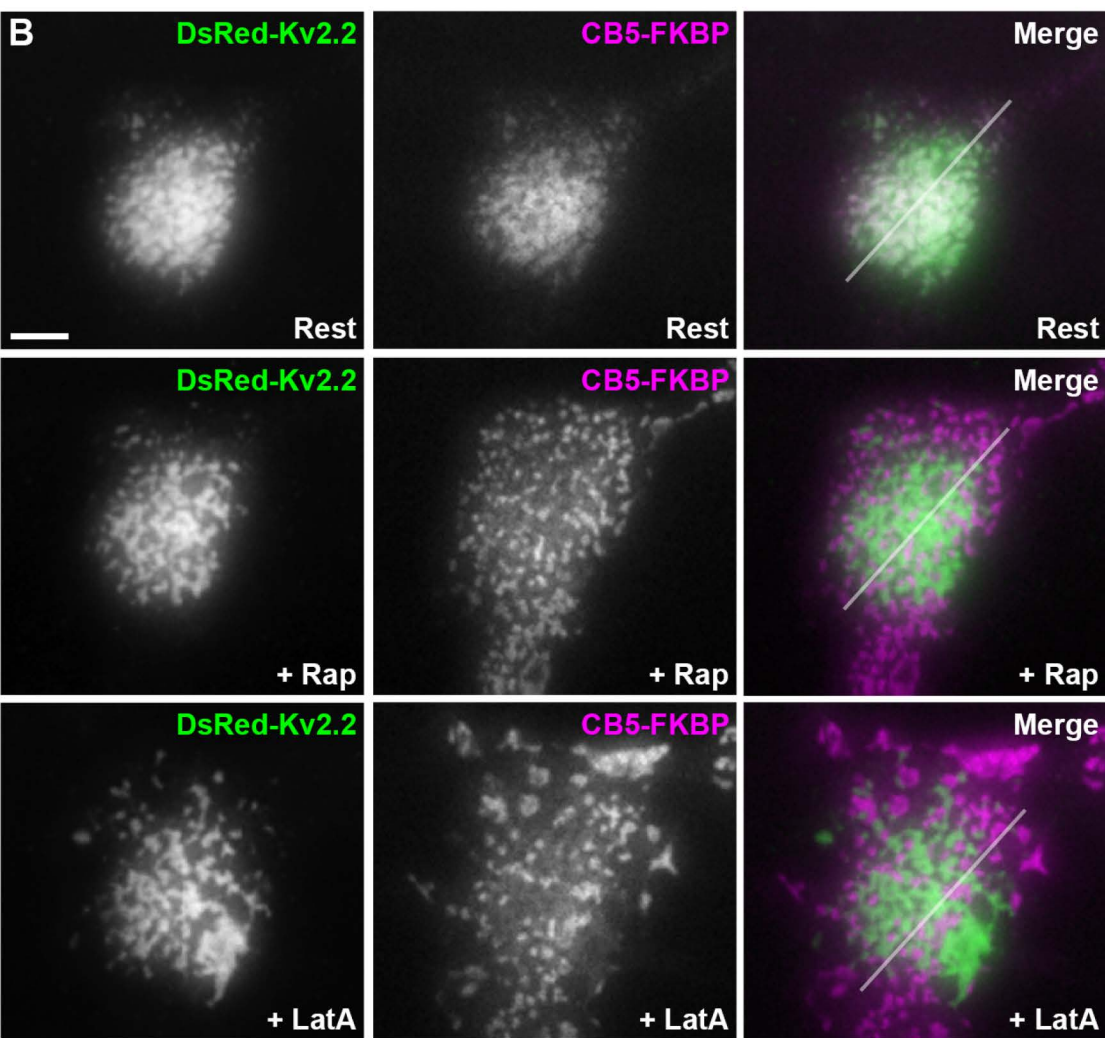
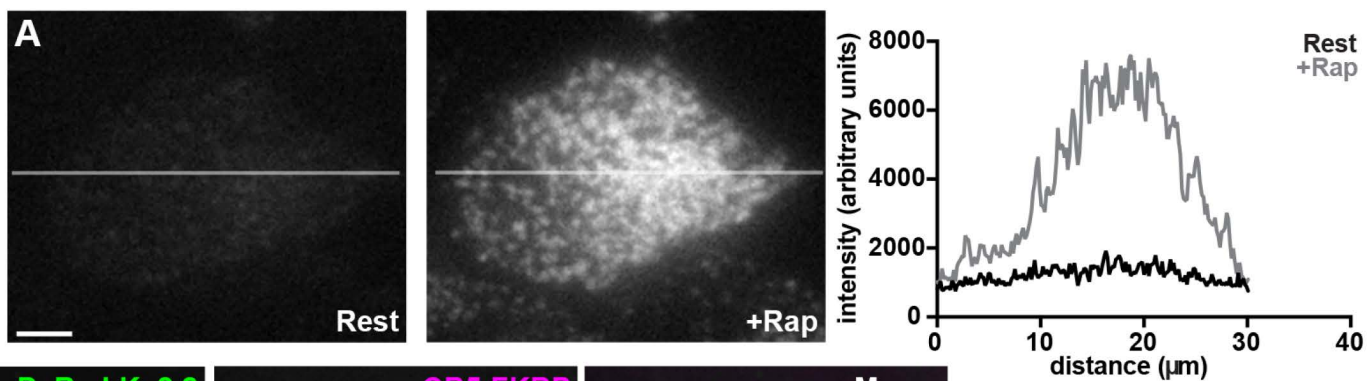
+ Exogenous STIM1



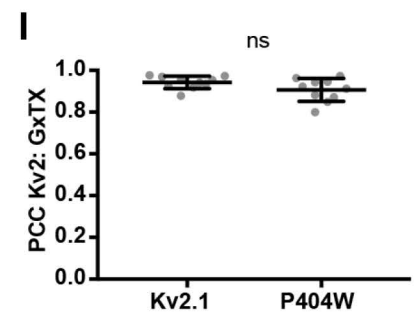
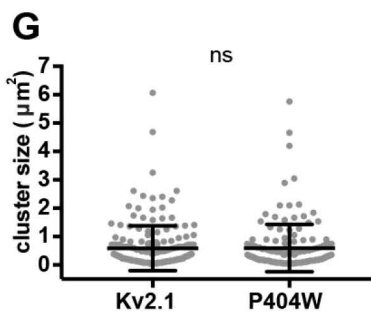
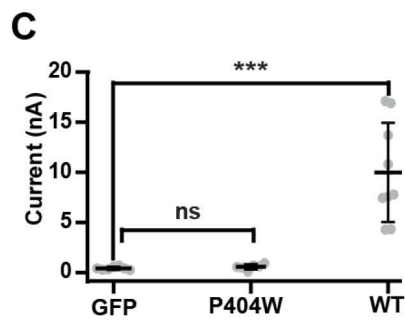
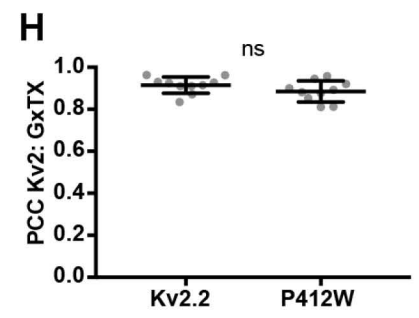
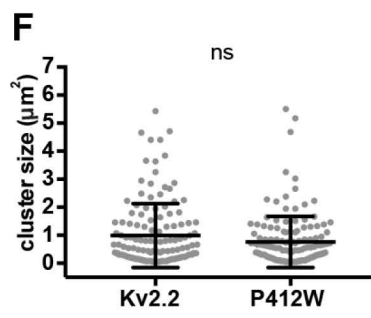
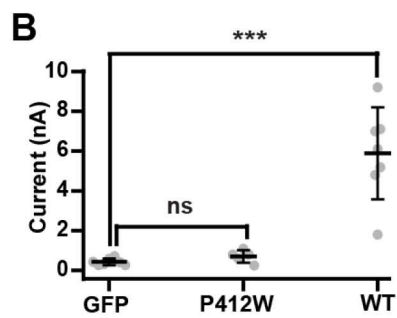
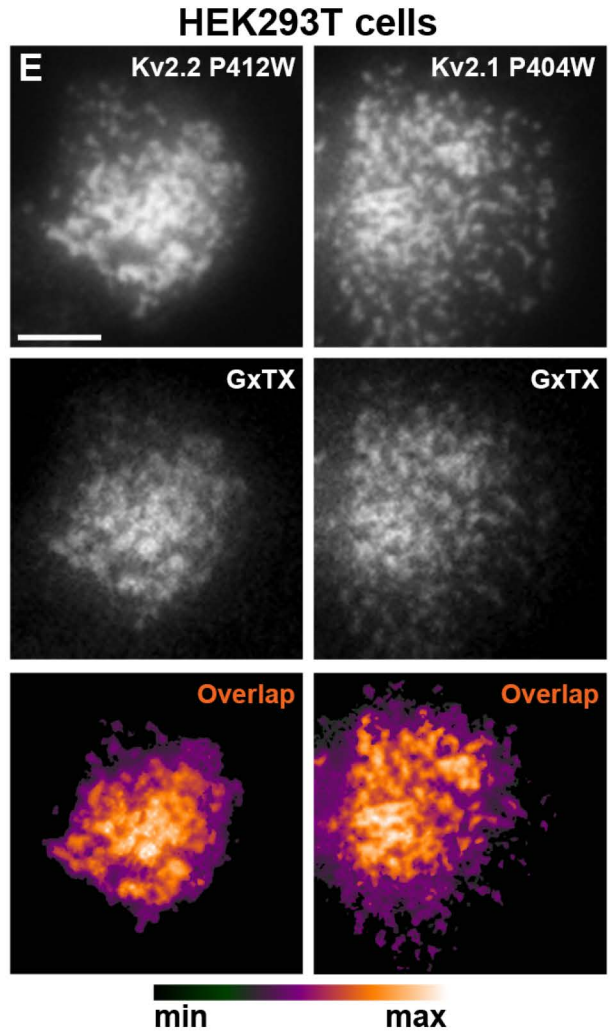
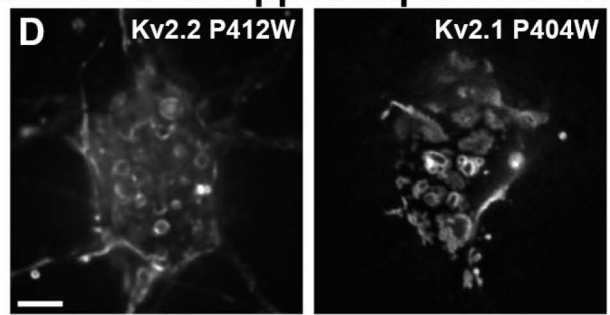
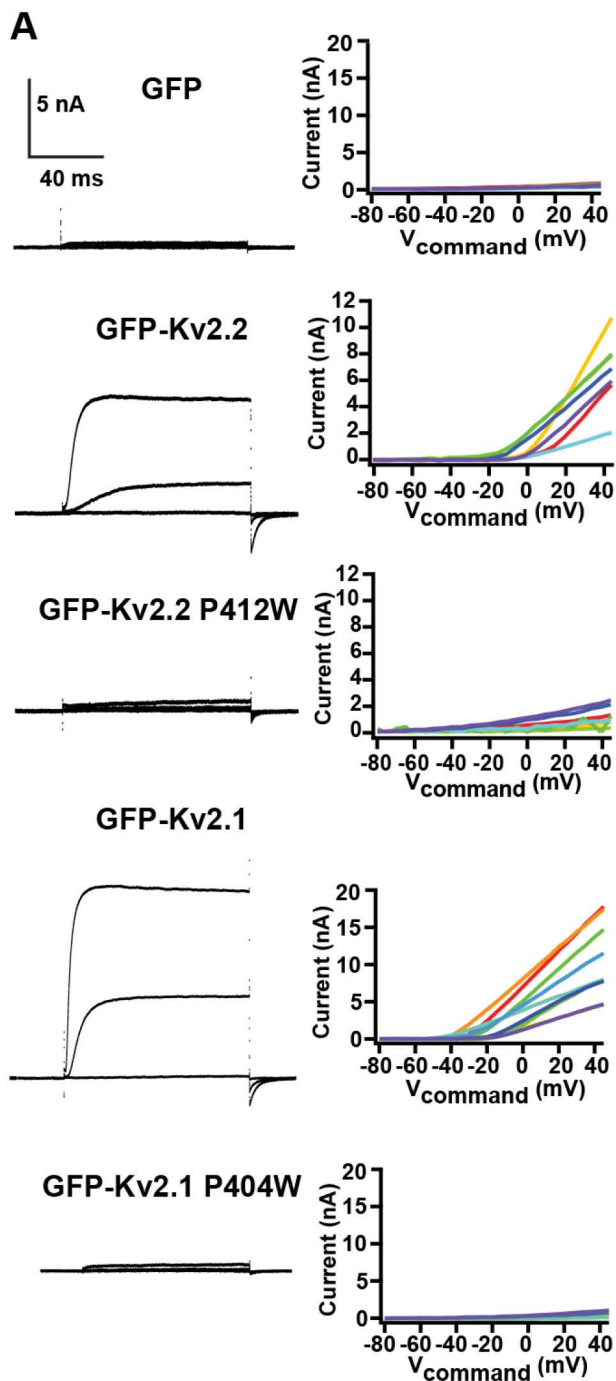
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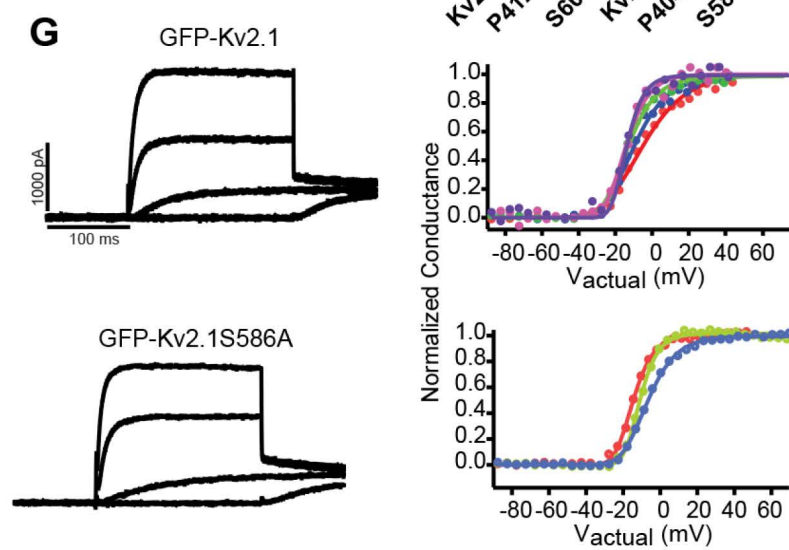
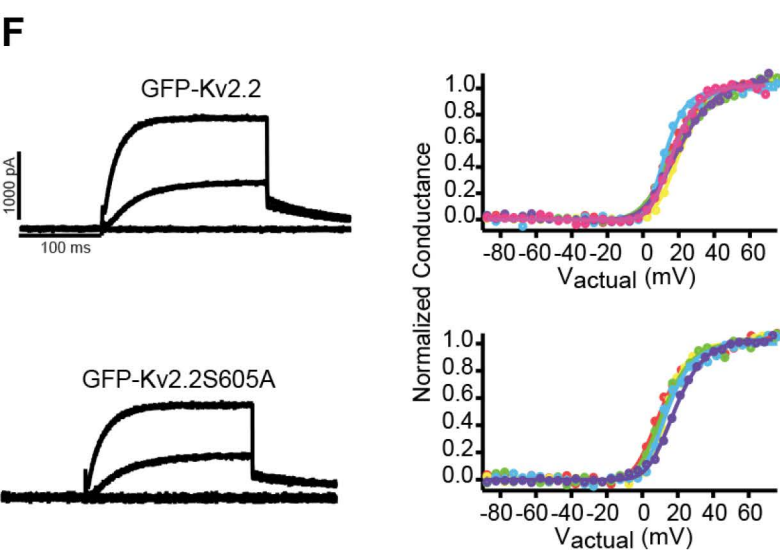
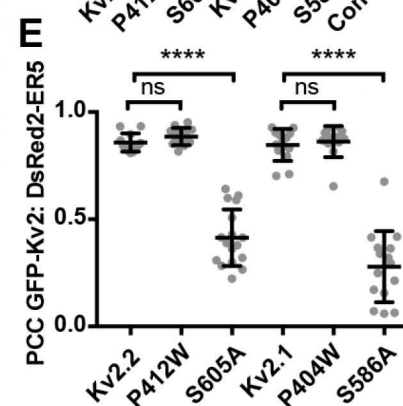
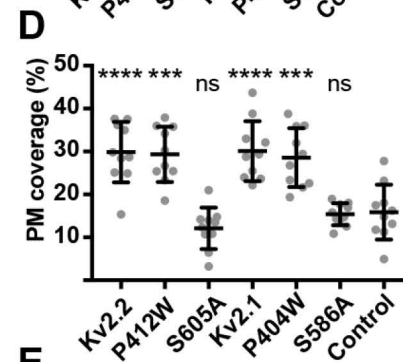
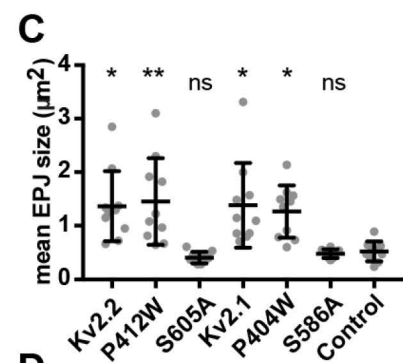
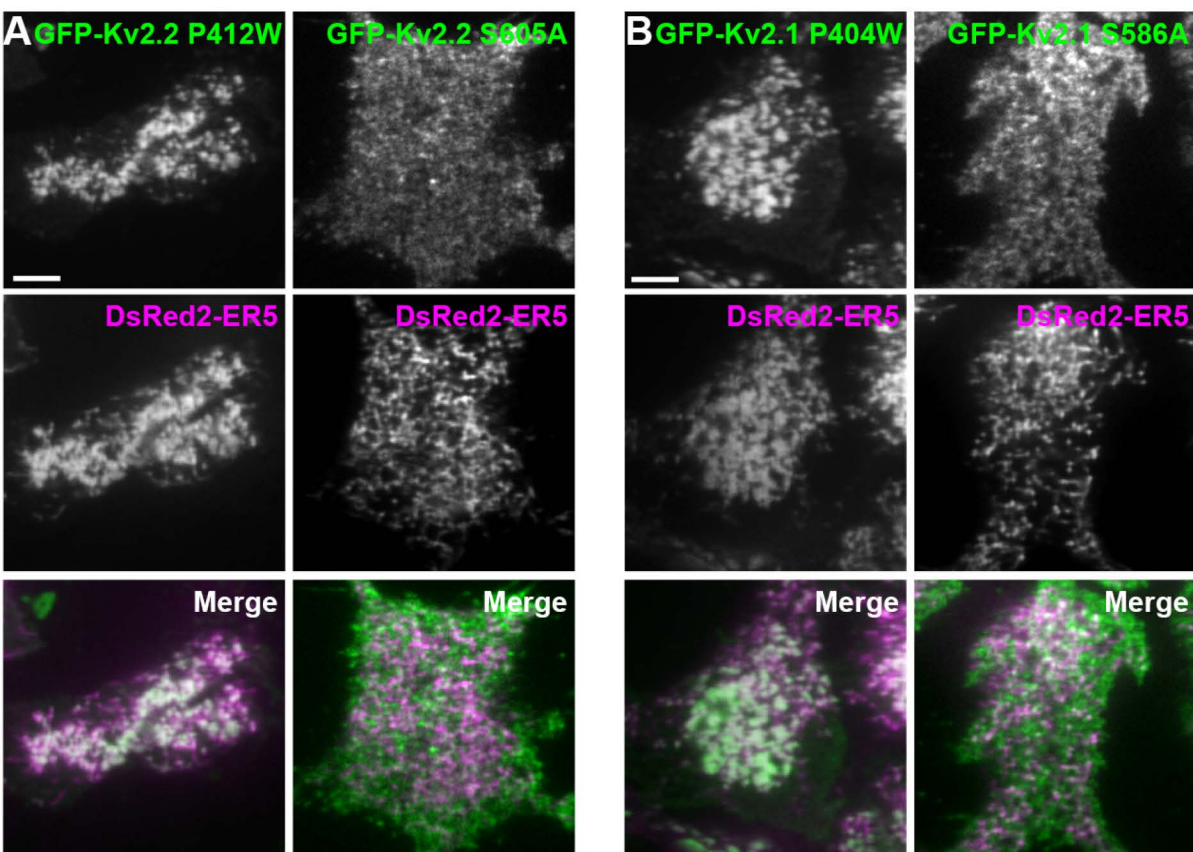


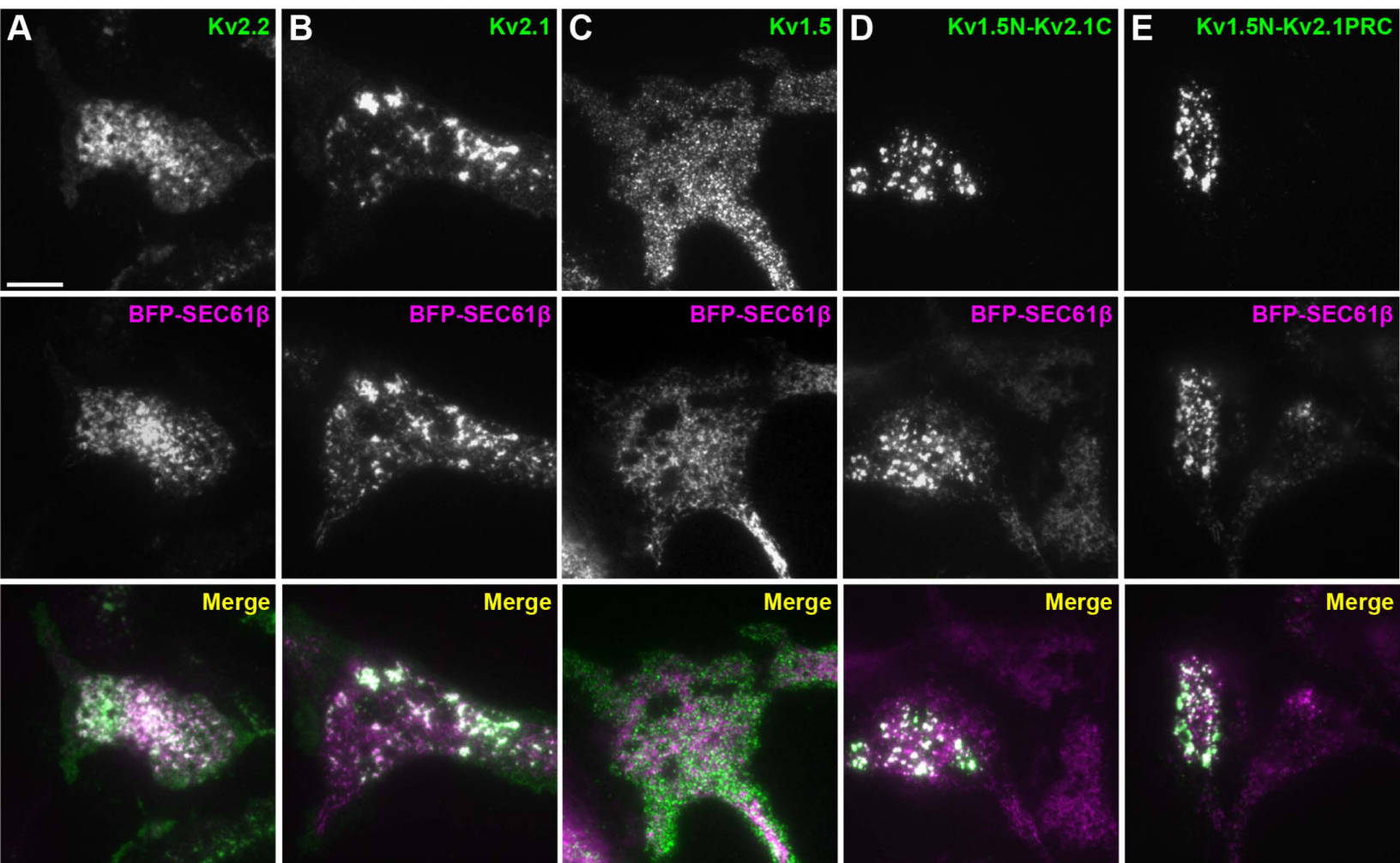
CB5-FKBP

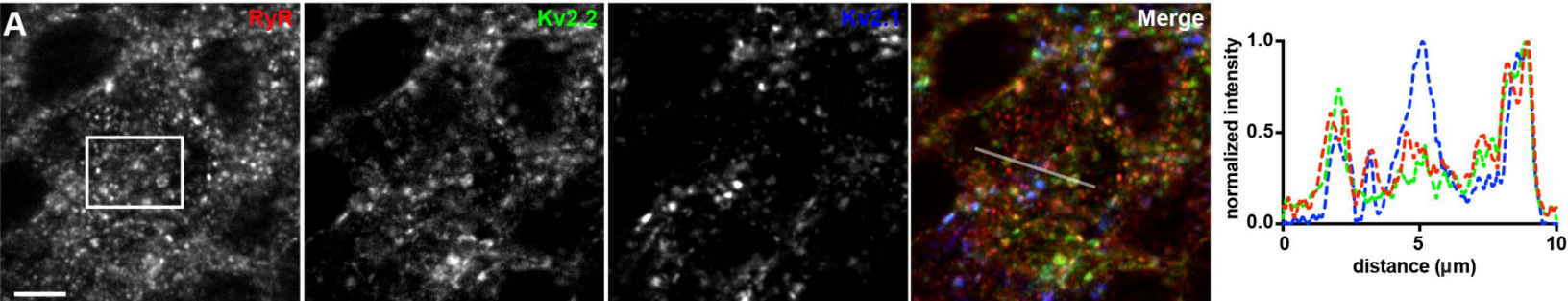
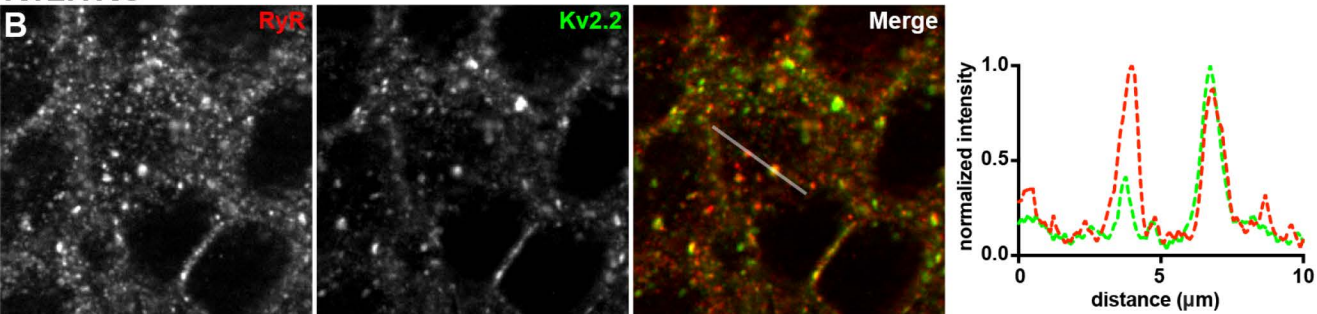
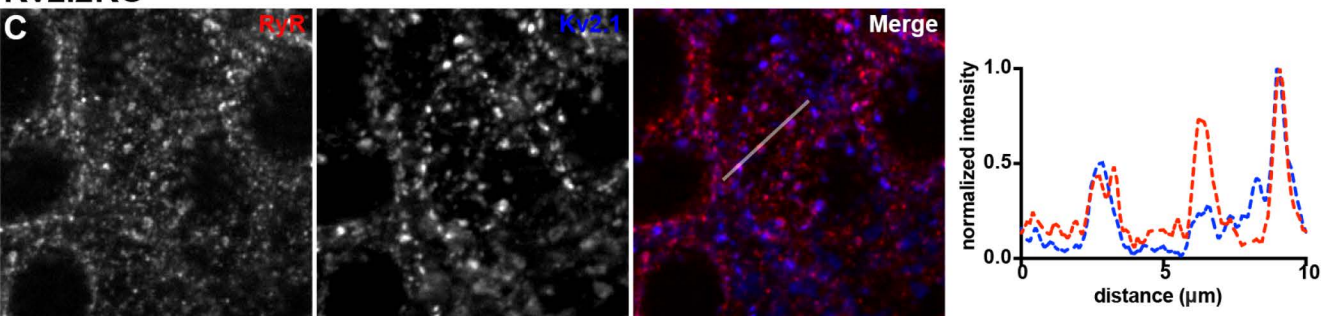
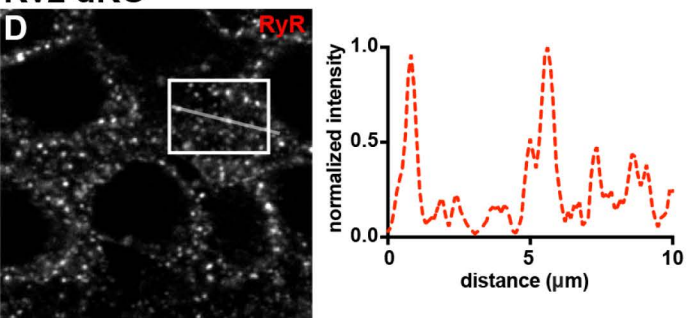
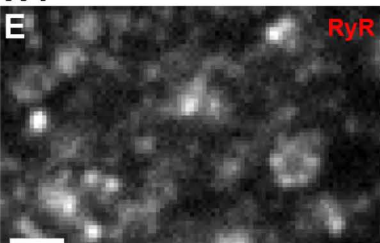
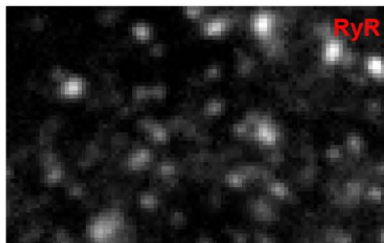
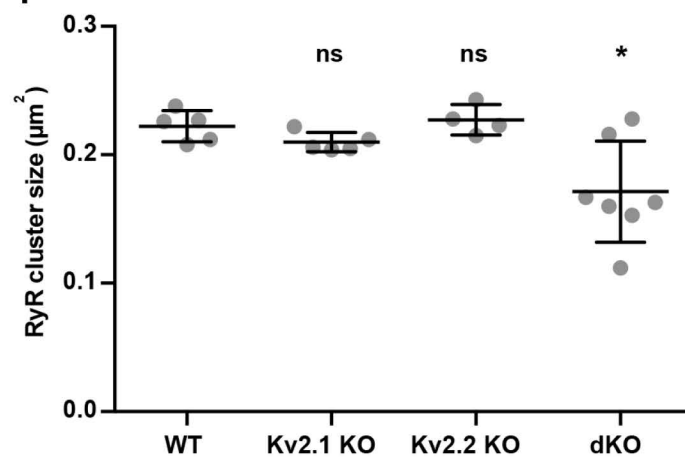


Cultured rat hippocampal neurons







WT**Kv2.1KO****Kv2.2KO****Kv2 dKO****WT****Kv2 dKO****F**

Supplemental Table 1. Figure 2C

Sample	ER-PM junction size (μm^2)	n number	p-value (two-tailed, unpaired t-test)
Kv2.2	1.98 ± 0.97	12 cells	vs. control, ****, 7.164×10^{-5}
Kv2.1	1.69 ± 0.60	12 cells	vs. control, ****, 4.512×10^{-6}
Kv1.4	0.49 ± 0.20	12 cells	vs. control, ns, 0.3602
control	0.57 ± 0.25	12 cells	N/A

Supplemental Table 2. Figure 2D

Sample	PM occupancy (%)	n number	p-value (two-tailed, unpaired t-test)
Kv2.2	34.9 ± 6.1	12 cells	vs. control, ****, 4.680×10^{-8}
Kv2.1	33.0 ± 5.8	12 cells	vs. control, ****, 1.634×10^{-7}
Kv1.4	14.7 ± 5.5	12 cells	vs. control, ns, 0.4256
control	16.4 ± 5.0	12 cells	N/A

Supplemental Table 3. Figure 2E

Sample	PCC Kv: DsRed2-ER5	n number	p-value (two-tailed, unpaired t-test)
Kv2.2	0.88 ± 0.04	15 cells	vs. Kv1.4, ****, 2.585×10^{-21}
Kv2.1	0.87 ± 0.08	15 cells	vs. Kv1.4, ****, 5.768×10^{-19}
Kv1.4	0.26 ± 0.08	15 cells	N/A

Supplemental Table 4. Supplemental Figure 1

Sample	PCC with GxTX	n number	p-value (ANOVA followed by Tukey's multiple comparisons test)
SEP-Kv2.1 (pHluorin)	0.85 ± 0.06	10 cells	vs. mCherry, ns, 0.5102; vs. SEC61 β , *, 0.0396
SEP-Kv2.1 (mCherry)	0.86 ± 0.06	10 cells	vs. SEC61 β , *, 0.0259
SEC61 β	0.78 ± 0.11	10 cells	N/A

Supplemental Table 5. Figure 3C

Sample	PCC with GxTX	n number	p-value (two-tailed, unpaired t-test)
Kv2.2	0.90 ± 0.06	13 cells	vs. DsRed2-ER5, *, 0.01211
DsRed2-ER5	0.78 ± 0.14	13 cells	N/A

Supplemental Table 6. Figure 5C

Sample	Size (µm ²)	n number	p-value (two tailed, paired t-test)
Kv2.2 clusters rest	0.41 ± 0.12	7 cells	vs. +LatA, ***, 0.0007
Kv2.2 clusters +LatA	1.01 ± 0.30	7 cells	N/A
Kv2.1 clusters rest	0.48 ± 0.19	7 cells	vs. +LatA, **, 0.0019
Kv2.1 clusters +LatA	0.69 ± 0.18	7 cells	N/A

Supplemental Table 7. Figure 5D

Sample	Size (µm ²)	n number	p-value (two tailed, paired t-test)
Kv2.2-EPJ rest	0.48 ± 0.19	7 cells	vs. +LatA, **, 0.0043
Kv2.2-EPJ +LatA	0.69 ± 0.18	7 cells	N/A
Kv2.1-EPJ rest	0.64 ± 0.10	7 cells	vs. +LatA, **, 0.0039
Kv2.1-EPJ +LatA	0.98 ± 0.22	7 cells	N/A

Supplemental Table 8. Figure 5E

Sample	Number of ER:PM junctions	n number	p-value (two tailed, paired t-test)
Kv2.2-EPJ rest	104.29 ± 24.81	7 cells	vs. +LatA, *, 0.0150
Kv2.2-EPJ +LatA	74.71 ± 11.04	7 cells	N/A
Kv2.1-EPJ rest	60.43 ± 16.69	7 cells	vs. +LatA, *, 0.0161
Kv2.1-EPJ +LatA	51.14 ± 15.74	7 cells	N/A

Supplemental Table 9. Figure 5F

Sample	PCC Kv2:DsRed2-ER5	n number	p-value (two tailed, paired t-test)
Kv2.2 rest	0.84 ± 0.052	10 cells	vs. +LatA, ns, 0.2505
Kv2.2 +LatA	0.85 ± 0.055	10 cells	N/A
Kv2.1 rest	0.82 ± 0.058	10 cells	vs. +LatA, ns, 0.4408
Kv2.1 +LatA	0.81 ± 0.064	10 cells	N/A

Supplemental Table 10. Figure 6B and C

Sample	PCC with Kv2.2	MOC with Kv2.2	n number	PCC vs. MOC, p-value (two-tailed, paired t-test)
E-Syt2	0.52 ± 0.27	0.92 ± 0.045	17 cells	****, 6.229x10 ⁻⁶
E-Syt3	0.69 ± 0.19	0.98 ± 0.034	17 cells	****, 5.690x10 ⁻⁶
JP2	0.84 ± 0.13	0.99 ± 0.015	17 cells	***, 1.057x10 ⁻⁴
JP4	0.90 ± 0.071	0.98 ± 0.013	18 cells	****, 7.894x10 ⁻⁵
STIM1	0.79 ± 0.13	0.94 ± 0.031	21 cells	****, 2.070x10 ⁻⁶
STIM2a	0.76 ± 0.051	0.95 ± 0.025	19 cells	****, 1.1x10 ⁻¹⁴
STIM2β	0.82 ± 0.10	0.97 ± 0.016	17 cells	****, 2.949x10 ⁻⁶

Supplemental Table 11. Figure 6F and G

Sample	PCC with Orai1	n number	p-value (two tailed, paired t-test)
STIM1 Rest	0.17 ± 0.26	12 cells	N/A
STIM1 +Thap	0.61 ± 0.19	12 cells	vs. Rest, ***, 0.0005092
Kv2.2 Rest + STIM1	0.21 ± 0.26	12 cells	N/A
Kv2.2 +Thap + STIM1	0.39 ± 0.19	12 cells	vs. Rest, *, 0.02501
Kv2.2 Rest no STIM1	0.018 ± 0.29	16 cells	N/A
Kv2.2 +Thap no STIM1	0.32 ± 0.31	16 cells	vs. Rest, ****, 0.00003238

Supplemental Table 12. Figure 7C

Sample	PCC Kv2: CB5-FKBP	n number	p-value (two tailed, paired t-test)
Kv2.2 Rest	0.86 ± 0.055	7 cells	N/A
Kv2.2 +Rap	0.19 ± 0.18	7 cells	vs. Rest, ****, 0.00006706
Kv2.2 +Rap +LatA	0.14 ± 0.12	7 cells	vs. +Rap, ns, 0.3099

Supplemental Table 13. Figure 8B and C

Sample	Current at +40 mV (nA)	n number	p-value (two-tailed, unpaired t-test)
GFP-Kv2.2	5.88 ± 2.31	7 cells	vs. GFP, ***, 0.000762
GFP-Kv2.2 P412W	0.70 ± 0.31	5 cells	vs. GFP, ns, 0.123
GFP-Kv2.1	9.98 ± 4.93	9 cells	vs. GFP, ***, 0.000399
GFP-Kv2.1 P404W	0.57 ± 0.27	6 cells	vs. GFP, ns, 0.287
GFP	0.42 ± 0.16	7 cells	N/A

Supplemental Table 14. Figure 8F and G

Sample	Cluster size (μm^2)	n number	p-value (two-tailed, unpaired t-test)
GFP-Kv2.2 P412W	0.76 ± 0.91	3 cells	vs. GFP-Kv2.2, ns, 0.0637
GFP-Kv2.2	0.99 ± 1.14	3 cells	N/A
GFP-Kv2.1 P404W	0.59 ± 0.84	3 cells	vs. GFP-Kv2.1, ns, 0.9441
GFP-Kv2.1	0.59 ± 0.79	3 cells	N/A

Supplemental Table 15. Figure 8H and I

Sample	PCC with GxTX	n number	p-value (two-tailed, unpaired t-test)
GFP-Kv2.2 P412W	0.88 ± 0.050	10 cells	vs. GFP-Kv2.2, ns, 0.1531
GFP-Kv2.2	0.91 ± 0.039	10 cells	N/A
GFP-Kv2.1 P404W	0.91 ± 0.055	10 cells	vs. GFP-Kv2.1, ns, 0.0863
GFP-Kv2.1	0.94 ± 0.030	10 cells	N/A

Supplemental Table 16. Figure 9C

Sample	ER-PM junction size (μm^2)	n number	p-value (ANOVA followed by Tukey's multiple comparisons test)
GFP-Kv2.2	1.36 ± 0.65	10 cells	vs. control, *, 0.01308
GFP-Kv2.2 P412W	1.45 ± 0.81	10 cells	vs. Kv2.2 ns, 0.9998; vs. control, **, 0.004206
GFP-Kv2.2A S605A	0.40 ± 0.11	10 cells	vs. control, ns, 0.9990
GFP-Kv2.1	1.38 ± 0.79	10 cells	vs. control, *, 0.01048
GFP-Kv2.1 P404W	1.26 ± 0.49	10 cells	vs. Kv2.1, ns, 0.9989; vs. control, *, 0.04155
GFP-Kv2.1 S586A	0.47 ± 0.081	10 cells	vs. control, ns, >0.9999
Control	0.52 ± 0.19	10 cells	N/A

Supplemental Table 17. Figure 9D

Sample	PM coverage (%)	n number	p-value (ANOVA followed by Tukey's multiple comparisons test)
GFP-Kv2.2	29.8 ± 7.0	10 cells	vs. control, ****, 5.507×10^{-5}
GFP-Kv2.2 P412W	29.4 ± 6.4	10 cells	vs. Kv2.2, ns, >0.9999; vs. control, ***, 0.0001027
GFP-Kv2.2 S605A	12.1 ± 4.8	10 cells	vs. control, ns, 0.8047
GFP-Kv2.1	30.1 ± 7.0	10 cells	vs. control, ****, 3.3938×10^{-5}
GFP-Kv2.1 P404W	28.6 ± 6.8	10 cells	vs. Kv2.1, ns, 0.9977; vs. control, ***, 0.0002994
GFP-Kv2.1 S586A	15.4 ± 2.6	10 cells	vs. control, ns, >0.9999
Control	15.9 ± 6.4	10 cells	N/A

Supplemental Table 18. Figure 9E

Sample	PCC with DsRed2-ER5	n number	p-value (ANOVA followed by Tukey's multiple comparisons test)
GFP-Kv2.2	0.86 ± 0.04	12 cells	N/A
GFP-Kv2.2 P412W	0.88 ± 0.04	12 cells	vs. GFP-Kv2.2, ns, 0.9943
GFP-Kv2.2 S605A	0.44 ± 0.14	12 cells	vs. GFP-Kv2.2, ****, $<1 \times 10^{-15}$ vs. GFP-Kv2.2 P412W, ****, $<1 \times 10^{-15}$
GFP-Kv2.1	0.86 ± 0.06	12 cells	N/A
GFP-Kv2.1 P404W	0.86 ± 0.07	12 cells	vs. GFP-Kv2.1, ns, >0.9999
GFP-Kv2.1 S586A	0.26 ± 0.17	12 cells	vs. GFP-Kv2.1, ****, $<1 \times 10^{-15}$ vs. GFP-Kv2.1 P404W, ****, $<1 \times 10^{-15}$

Supplemental Table 19. Supplementary Figure 2C

Sample	ER-PM junction velocity ($\mu\text{m}/\text{sec}$)	n number	p-value (ANOVA followed by Tukey's multiple comparisons test)
GFP-Kv2.2	0.069 ± 0.019	3 cells	vs. control, ****, 1.358×10^{-11}

GFP-Kv2.2 P412W	0.067 ± 0.018	3 cells	vs. Kv2.2, ns, 0.9796; vs. control, 1.038x10 ⁻¹²
GFP-Kv2.1	0.062 ± 0.015	3 cells	vs. control, ****, 7.15x10 ⁻¹³
GFP-Kv2.1 P404W	0.066 ± 0.017	3 cells	vs. Kv2.1, ns, 0.8041; vs. control, ****, 8.49x10 ⁻¹³
Control	0.093 ± 0.020	3 cells	N/A

Supplemental Table 20. Figure 11F

Sample	RyR cluster size (μm ²)	n number	p-value (two tailed, unpaired t-test)
WT	0.22 ± 0.012	3 animals	N/A
Kv2.1 KO	0.21 ± 0.0075	3 animals	vs. WT, ns, 0.0884
Kv2.2 KO	0.22 ± 0.012	2 animals	vs. WT, ns, 0.5506
Kv2 dKO	0.17 ± 0.039	3 animals	vs. WT, *, 0.0199