Kv2 channel–AMIGO β-subunit assembly modulates both channel function and cell adhesion molecule surface trafficking

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ABSTRACT

The Kv2 channels encode delayed rectifier currents that regulate membrane potential in many tissues. They also have a non-conducting function to form stable junctions between the endoplasmic reticulum and plasma membranes, creating membrane contact sites that mediate functions distinct from membraneexcitability. Therefore, proteins that interact with Kv2.1 and Kv2.2 channels can alter conducting and/or non-conducting channel properties. One member of the AMIGO family of proteins is an auxiliary β-subunit for Kv2 channels and modulates Kv2.1 electrical activity. However, the AMIGO family has two additional members of ~50% similarity that have not yet been characterized as Kv2 β-subunits. In this work, we show that the surface trafficking and localization of all three AMIGOs are controlled by their assembly with both Kv2 channels. Additionally, assembly of each AMIGO with either Kv2.1 or Kv2.2 hyperpolarizes the channel activation midpoint by ~10 mV. However, only AMIGO2 significantly slows inactivation and deactivation, leading to a prolonged open state of Kv2 channels. The co-regulatory effects of Kv2s and AMIGOs likely fine-tune both the electrical and non-electrical properties of the cells in which they are expressed.

KEY WORDS: Kv2, AMIGO, Trafficking, Electrophysiology, ER–PM junction, Membrane localization

INTRODUCTION

β-subunits of the voltage-gated potassium channel (Kv) superfamily are important regulators of neuronal system function. Indeed, this diverse class of proteins can modulate almost every aspect of Kv channel physiology, including subunit assembly, trafficking, protein stability, conduction, localization and pharmacology (Li et al., 2006; Pongs and Schwarz, 2010; Sun et al., 2012). For example, the classical Kv auxiliary subunits, Kvβ1.1–1.3 and Kvβ2.1 are soluble proteins that interact with the cytoplasmic domains of Kv1 channels (Heinemann et al., 1996; Rhodes et al., 1997). They confer dramatic effects on channel gating, in some cases inducing fast inactivation in otherwise non-inactivating channels (Englund et al., 1995; Leicher et al., 1996). Recently, amphotericin-induced gene and ORF (AMIGO)1 was identified as a β-subunit of Kv2.1 and Kv2.2 channels (Peltola et al., 2011; Bishop et al., 2018). This work showed that AMIGO1 assembles with both Kv2 isoforms in neurons and transfected human embryonic kidney (HEK)293 cells and induces a hyperpolarizing shift in the voltage dependence of Kv2.1. Furthermore, trafficking and localization of Kv2.1 and Kv2.2 were unchanged by assembly with AMIGO1, but both Kv2s increased the surface expression of AMIGO1 and redistributed it to large clusters, which are characteristic of Kv2 channel surface localization.

The clustered pattern of Kv2s is due to a phosphorylation-regulated interaction with an endoplasmic reticulum (ER) protein, VAPA/B (Johnson et al., 2018; Kirmiz et al., 2018). Interaction between cell surface Kv2s and VAPA/B brings the ER into close apposition with the plasma membrane (PM), forming an increasingly appreciated organelle, called the ER–PM junction (Fox et al., 2015; Johnson et al., 2018; 2019). In an electron microscopy (EM) study of rat brains, 90% of subsurface cisterns of ER membrane were positive for Kv2.1 channels (Du et al., 1998). Another EM study that heterologously expressed Kv2.1 in HEK293 cells found that these Kv2.1 clusters were localized to the PM surface (Fox et al., 2015), which has also been observed via fluorescent labeling of surface channels in live cells (O’Connell and Tamkun, 2005; O’Connell et al., 2006; Fox et al., 2013; Johnson et al., 2018). ER–PM junctions represent ~12% of the total neuronal surface in vivo (Wu et al., 2017) and regulate many cellular processes, including endo- and exocytosis (Deutsch et al., 2012; Feinshreiber et al., 2009; 2010; Fox et al., 2015; Fu et al., 2017a,b), Ca2+ signaling and store refilling (Fox et al., 2015; O’Dwyer et al., 2020; Vierra et al., 2019), and neuron–glia and neuron–neuron interactions (Du et al., 1998; Wu et al., 2017; Cserép et al., 2019). The consequences of AMIGO localization to Kv2-induced ER–PM junctions for each of these functions are not well understood.

In addition to regulating cell function via ER–PM junction formation, Kv2 channels are important regulators of neuronal excitability. They underlie most of the delayed rectifier current in central neurons (Murakoshi and Trimmer, 1999) and control neuronal excitability under conditions of high-frequency firing (Du et al., 2000; Misonou et al., 2005; Park et al., 2006). In the mouse hippocampus, Kv2 channel expression is higher in CA1 than in CA2 and confers differences in the firing patterns of pyramidal neurons between these two areas (Palacio et al., 2017). Indeed, the entire complement of ion channel and auxiliary subunit expression in an individual neuron will determine its unique firing patterns.

Although the assembly of Kv2 channels and AMIGO1 is well documented (Peltola et al., 2011; Bishop et al., 2018), nothing is known about the assembly of Kv2s with other members of the AMIGO family. The AMIGO family consists of three members (AMIGO1, AMIGO2 and AMIGO3), all of which are type-I single-pass transmembrane proteins (Kuja-Panula et al., 2003). All three AMIGOs have an extracellular domain, which contains six leucine-rich repeats and an immunoglobulin-like domain, that allows them to act as homophilic and heterophilic cell adhesion molecules.
(Kajander et al., 2011; Kuja-Panula et al., 2003). AMIGO1 is almost exclusively expressed in the brain, while AMIGO2 and AMIGO3 are more widespread, but also enriched in the brain (Kuja-Panula et al., 2003). Likewise, AMIGO2 and AMIGO3 show widespread expression in tissues in which AMIGO1 is low or absent (Kuja-Panula et al., 2003). Interestingly, Kv2 channels have perhaps the widest tissue distribution of any Kv channel, being expressed in cells as diverse as cortical, hippocampal and spinal motor neurons (Bishop et al., 2018; Du et al., 1998; Hwang et al., 1993; Muennich and Fyffe, 2004; Trimmer, 1991), vascular smooth muscle cells (Amberg and Santana, 2006; O’Dwyer et al., 2020), retinal ganglion cells (Yazulla and Studholme, 1998) and pancreatic beta cells (Fu et al., 2017a;b; Greitzer-Antes et al., 2018; MacDonald, 2011; Yoshida et al., 2010). Therefore, the consequences of AMIGO interactions with Kv2 channels have implications for cell function far beyond the nervous system.

Functionally, AMIGOs have been implicated in early axon guidance, growth, survival and adult axon growth inhibition (Chen et al., 2012; Kuja-Panula et al., 2003; Ahmed et al., 2013; Soto et al., 2019). Interestingly, AMIGO2 has also been implicated in enhanced growth and survival in a variety of cells (Ono et al., 2003; Hossain et al., 2011; Rabenau et al., 2004; Fontanals-Cirera et al., 2017). This increase in cell viability may be due to an interaction between AMIGO2 and PDK1 (also known as PDPK1), a kinase that activates Akt in cell survival pathways (Park et al., 2015). Although the residues involved in AMIGO1–Kv2.1 interaction are unknown, electrophysiology studies with chimeras between AMIGO1 and a neuronal cell adhesion molecule (NCAM) suggest that the AMIGO1 transmembrane domain is required for interaction with Kv2.1 (Peltola et al., 2011). The transmembrane domain of the AMIGO family is ~48% conserved (see Fig. S3), supporting the possibility that the other AMIGO family members could also act as β-subunits for the Kv2 family.

Given the lack of detailed information regarding the structural basis of the Kv2–AMIGO1 interaction, it is difficult to predict whether AMIGO2 and AMIGO3 will assemble with and exert similar functional effects on the Kv2 channels. The aim of the present study was to investigate whether AMIGO2 and AMIGO3 also act as auxiliary β-subunits for Kv2.1 and Kv2.2 and to further characterize the effects of interaction on AMIGO localization and Kv2 electrical activity. The results of this work reveal that all three AMIGOs assemble with both Kv2 channels. The interaction of Kv2 channels with the AMIGOs appears to have isoform-specific effects on AMIGO trafficking, but minimal effects on Kv2 trafficking and localization. Furthermore, while all three AMIGOs modulate Kv2 channel activation voltage sensitivity and kinetics, the association of AMIGO2 also specifically slows channel inactivation and deactivation. These results indicate that the three AMIGOs assemble with both Kv2.1 and Kv2.2 to alter both channel-conducting functions and the composition of Kv2 surface clusters.

**RESULTS**

**The three AMIGOs colocalize with Kv2 channels in surface clusters**

Previous work demonstrated that AMIGO1 is a β-subunit for Kv2s (Bishop et al., 2018; Peltola et al., 2011); however, it is unknown whether other members of the AMIGO family can act as β-subunits. To determine whether AMIGO2 and AMIGO3 might also interact with Kv2s, we co-expressed GFP–Kv2.1 or GFP–Kv2.2 and one of the three Ruby2-tagged AMIGOs in HEK293 cells to assess colocalization between the proteins. When AMIGOs were expressed alone, surface localization was uniform across the PM surface, as shown by the images and line scans of the basal cell surface in Fig. 1A. However, when AMIGOs were co-expressed with Kv2.1, surface localization of all three AMIGOs redistributed to micron-sized clusters formed by Kv2.1 (Fig. 1B). Line scans below the images in Fig. 1B show nearly perfect overlap of fluorescence peaks between each AMIGO (magenta) and Kv2.1 (green), which is vastly different from the uniform distribution seen in Fig. 1A. Likewise, when AMIGOs were co-expressed with Kv2.2, all three AMIGOs redistributed to micron-sized clusters on the surface (Fig. 1C). When line scans across clusters were plotted, peaks of AMIGO and Kv2.2 fluorescence also overlapped. When Kv2.1 or Kv2.2 were expressed alone (Fig. 1B,C, bottom right), the surface localization was not qualitatively different from that when co-expressed with AMIGOs. To determine whether AMIGOs were actually colocalized in surface clusters, we measured the Spearman’s rank correlation coefficient for each cell. This coefficient scales from −1 to 1, which indicate negative correlation and positive correlation, respectively. Indeed, as suggested by the images, all three AMIGOs were significantly positively correlated with both Kv2.1 and Kv2.2 (P<0.0001; Fig. 1D). We observed similar colocalization in clusters on the PM of rat hippocampal neurons (Fig. S1).

Altogether, these data indicate that the surface localization of all three AMIGOs was altered by the presence of both Kv2s in both HEK cells and rat hippocampal neurons. Co-expression with the Kv2s caused the three AMIGOs to redistribute to clusters that overlapped with Kv2 clusters, suggesting that an interaction with Kv2 channels causes the redistribution. Previous studies showed that Kv2 clusters are always associated with the ER (Fox et al., 2015; Du et al., 1998; Johnson et al., 2018; Kirmiz et al., 2018; Mandikian et al., 2014), which is retained even with co-expression of AMIGO1 (Bishop et al., 2018). Therefore, we believe that each of the clusters presented in Fig. 1 and Fig. S1 likely represents an ER–PM junction.

**Kv2s increase the surface expression of AMIGO isoforms**

While Kv2s altered the surface localization of AMIGOs, we also wanted to address the influence of co-expression on surface trafficking of each AMIGO. When AMIGO1 and AMIGO2 were expressed alone, some surface localization was observed as seen in the optical slices in Fig. 2AB (left images). However, internal vesicles containing each AMIGO were prominent, as indicated by the punctate fluorescence in the middle of these cells and the large peaks in the corresponding line scans. In contrast, when expressed alone, very little AMIGO3 was expressed on the cell surface. As seen in the left image of Fig. 2C and its corresponding line scan, the majority of AMIGO3 fluorescence was internal and little fluorescence overlapped with the membrane marker PH–GFP. Intracellular AMIGO3 was widespread, with very few bright puncta, indicating that the majority of intracellular AMIGO3 was likely to be localized within the ER membrane (Fig. 2C, left image).

Co-expression with Kv2.1 increased the surface trafficking of all three AMIGOs (Fig. 2A–C, middle), as indicated by overlapping peaks in fluorescence at the edge of the line scans. Whereas the effect on surface trafficking of AMIGO1 and AMIGO2 was robust, much of AMIGO3 was still retained within the cell, suggesting that the affinity of Kv2.1 for AMIGO3 may be lower. Likewise, co-expression with Kv2.2 appeared to increase the surface trafficking of all three AMIGOs (Fig. 2A–C, right), although to perhaps a lesser degree than Kv2.1 in the case of AMIGO1 and AMIGO2. Kv2.2 on its own does not traffic as efficiently to the cell surface (Fig. 2F), suggesting that the affinity of Kv2.2 for AMIGO3 may be lower.
which might explain this diminished effect on AMIGO surface expression.

By taking a ratio of surface density over total density for each AMIGO, we quantified the observed changes in AMIGO localization while controlling for differences in protein expression between cells. In this measurement, ratios greater than one indicate preferential surface expression and ratios less than one indicate preferential internal expression (Fig. 2D). Using Student’s t-tests against a test mean of one, we determined the preference of each AMIGO for surface or internal localization. When expressed alone, AMIGO1 showed a significant preference for surface localization (Fig. 2D, blue box, \( P < 0.0005 \)). This preference was maintained when AMIGO1 was co-expressed with either Kv2.1 (\( P < 0.0005 \)) or Kv2.2 (\( P < 0.0005 \)), and represented a significant increase in surface trafficking from AMIGO1 expression alone (Kv2.1 \( P < 0.0005 \), Kv2.2 \( P < 0.05 \)). Expressed alone, AMIGO2 showed a slight, but not significant, preference for surface localization (Fig. 2D, green box, \( P = 0.096 \)). However, AMIGO2 showed a significant preference for surface localization when co-expressed with either Kv2.1 (\( P < 0.0005 \)) or Kv2.2 (\( P < 0.0005 \)), which were significant improvements from expression alone (\( P < 0.0005 \)).

In contrast, AMIGO3 alone showed a significant preference for internal localization (Fig. 2D, orange box, \( P < 0.0005 \)). With Kv2 co-expression, AMIGO3 surface localization increased with Kv2.1 (\( P < 0.0005 \)) and Kv2.2 (\( P < 0.005 \)), which was significantly different from AMIGO3 alone (\( P < 0.0005 \)). These data further suggest that all three AMIGOs are capable of interaction with Kv2s, because the surface trafficking of each AMIGO was increased by co-expression with a Kv2. Although Kv2 co-expression significantly increased the surface trafficking of AMIGOs, the AMIGO family did not appear to alter the surface trafficking of Kv2s (Fig. 2E,F).

All three AMIGOs assemble with both Kv2.1 and Kv2.2
The colocalization and trafficking effects we observed above are consistent with the hypothesis that all three AMIGO isoforms act as auxiliary subunits for the Kv2 channels, at least within the Kv2 channel clusters. To further support the idea that the AMIGOs and Kv2s co-assemble, we performed co-immunopurification (Co-IP) experiments on HEK293 cells transfected with Kv2 \( \alpha \)-subunits alone or in the presence of AMIGO1–GFP, AMIGO2–GFP or AMIGO3–GFP. The literature indicates that much of the Kv2.1
channel is resistant to solubilization with non-ionic detergents used for immunopurification (IP) (Scannevin et al., 1996; Chung and Li., 2005), and Movie 1 shows that the clustered Kv2.1 channels representing ER–PM junctions are resistant to detergent solubilization. This video shows that, after the addition of 1% TX-100/EDTA solubilization buffer to live HEK293 cells expressing GFP–Kv2.1, the GFP–Kv2.1 clusters remain intact but assume a globular morphology as the surrounding membrane is solubilized. Thus, any immunopurified Kv2.1 protein likely represents non-clustered Kv2.1, which can comprise up to half of the cell surface channel. Owing to their lower membrane density, these non-clustered channels are not apparent in Figs 1 and 2.
In each of the co-expression conditions, an antibody against the Kv2 α-subunit co-immunopurified an ∼80 kDa protein that was detected by a GFP antibody via western blotting (Fig. 3A,B, asterisks). Importantly, no bands were detected by the same GFP antibody when Kv2.1 or Kv2.2 was expressed alone (Fig. 3C,D). The AMIGO1–GFP bands detected by the GFP antibody were also detected by a monoclonal antibody against AMIGO1 itself, supporting the idea that the GFP antibody successfully detected our transfected AMIGO constructs (Fig. S2A,B). The bands detected by the GFP antibody were of reasonable sizes for all three AMIGOs fused to a single GFP protein. It should be noted that we did detect endogenous AMIGO1 protein in our HEK293 cell lysates, albeit at very low levels. Indeed, endogenous AMIGO1 was only detectable in our Co-IP starting material when we loaded 10× the amount of sample, and it was not detectable in the material purified by either the anti-Kv2.1 or anti-Kv2.2 antibody (Fig. S2C,D). Therefore, we believe that, when transfected alone, the majority of Kv2.1 and Kv2.2 channels are not assembled with endogenous AMIGO1 and that, when co-transfected, the tagged AMIGOs outcompete any endogenous AMIGOs for assembly with the channels. Whether this result is simply explained by the low abundance of endogenous AMIGOs in HEK293 cells or due to more complex regulation of Kv2–AMIGO assembly would be interesting to investigate in the future. It should also be noted that although the results of these Co-IP experiments show proof of principle for the co-assembly of AMIGOs and Kv2 channels in the same complex, they are not quantitative, and therefore determination of the relative abundance of co-assembled AMIGO/Kv2 awaits further investigation.

All three AMIGOs enhance voltage-dependent parameters of Kv2 activation

After showing that each AMIGO isoform can assemble with each Kv2 α-subunit, we investigated the effects of each AMIGO on Kv2.1 and Kv2.2 channel electrical function. Although the majority of Kv2 channels expressed in heterologous cells are non-conducting and do not contribute to whole-cell ionic currents, there is a large population of conducting channels that reside mostly outside of clusters (Fox et al., 2013). Therefore, the electrophysiological results herein describe the effect of the AMIGOs on conducting channels that mostly reside outside of clusters. First, we assayed voltage-dependent activation, a classical measure of ion channel function that helps predict how channels will respond to dynamic membrane potentials seen in vivo. Currents carried by Kv2 subunits alone or Kv2 subunits plus each AMIGO were qualitatively similar and resembled classic delayed rectifier currents (Fig. 4A,B). Differences in current magnitude in Fig. 4, and throughout, are due to variable expression of the Kv channels in individual cells as opposed to population-level differences between conditions. Our voltage-clamp protocol to assay channel activation included a tail current step to −40 mV, which was used to measure the extent of channel activation in the preceding voltage step (Fig. 4C,D, insets). The Kv2.1 and Kv2.2 current magnitudes at the beginning of the tail currents were normalized to their maximum values and plotted against voltage as shown in Fig. 4C,D, respectively. We fit each data set with a single Boltzmann function (Fig. 4C,D, solid line curves) to estimate two voltage-dependent parameters of channel gating: activation midpoint ($V_{1/2}$) and slope factor ($k$). $V_{1/2}$ contains
important information regarding the free energy of the closed and open states of the channel, and shifts in \( V_{1/2} \) can underlie dramatic changes in neuronal firing properties. Indeed, the midpoint of Kv2.1 activation was shifted to more hyperpolarized potentials by AMIGO1, AMIGO2 and AMIGO3 (\( P<0.0005 \)), consistent with the reported effect of AMIGO1 on Kv2.1 activation midpoint (Peltola et al., 2011). We also analyzed the effect of each AMIGO on the \( V_{1/2} \) of Kv2.2, which has not yet been characterized in the literature. The midpoint of Kv2.2 activation was also shifted to more hyperpolarized potentials by all three AMIGO isoforms (\( P<0.0005 \)).

In addition to activation midpoint, we also compared the slope factors of the Boltzmann fits shown in Fig. 4C,D. The slope factor is a measure of the voltage dependence of channel opening, with steeper curves having smaller slope factors and greater voltage dependence. Each of the AMIGOs tended to steepen the Kv2.1 and Kv2.2 activation curve, and, indeed, the slope factors under these conditions also decreased (Fig. 4C,D and Table 1). Interestingly, the change in slope factor conferred by AMIGO1 was not significant for either Kv2 channel, whereas the changes conferred by AMIGO2 and AMIGO3 were (\( P<0.05 \)). These data, along with the observed changes in \( V_{1/2} \), reveal that assembly with each of the three AMIGOs modulates Kv2.1 and Kv2.2 activation gating, either by lowering the energy of activation, increasing the voltage dependence of opening, or both.

**The AMIGOs alter Kv2 activation kinetics**

In addition to channels’ voltage dependence, the kinetics of opening and closing also govern the behavior of channels in physiological systems. An analysis of the effects of AMIGOs on Kv2 channel kinetics has not been undertaken, and, therefore, we analyzed the time dependence of the current traces from Fig. 4. A comparison of normalized current traces during a step to +20 mV are shown in Fig. 5A and reveal that each of the three AMIGOs enhanced activation kinetics of Kv2.1 such that maximum current was reached earlier. We fit Kv2.1-activating currents with a single exponential function as illustrated in Fig. S4 and plotted the resulting time constants as a function of voltage (Fig. 5B). The time constants of Kv2.1 activation were faster when co-expressed with each of the AMIGOs at some voltages. For example, at +20 mV, the time constant of Kv2.1 activation was 13.9±0.9 ms, and co-expression with each of the three AMIGOs significantly decreased this time.
constant ($P<0.005$). Results from the same analysis with Kv2.2 are shown in Fig. 5C,D. The speeding of Kv2.2 kinetics was more dramatic for AMIGO2 than for the other two isoforms. Indeed, Kv2.2 activation at +20 mV proceeded with a time constant of 14.1±1.9 ms, and this rate was sped up significantly by AMIGO2 ($P<0.05$), but not AMIGO1 ($P=0.2$) or AMIGO3 ($P=0.06$).

Increased activation kinetics were only observed at voltages at which voltage-dependent gating is near maximal, and thus this observation cannot explain the changes in voltage-dependent activation observed in Fig. 4. The shape of the plots in Fig. 5B,D suggested that AMIGOs may alter the voltage dependence of Kv2 activation kinetics. Therefore, we used the method of Scholle et al., 2004 to fit the plots in Fig. 5B,D with an exponential function (Eqn 1) and compared the equivalent charge ($z$) between conditions. The equivalent charge measured in this way is a somewhat arbitrary descriptor of the voltage dependence of the process. However, a change in $z$ can indicate an increase or decrease in voltage dependence of channel behavior, which may hint at a real physical mechanism. In general, steeper curves will have higher $z$ values. The fits in Fig. 5B,D appeared steeper when Kv2.1 or Kv2.2 was co-expressed with each AMIGO. This was especially evident when either Kv2 was expressed with AMIGO2, which slowed activation kinetics below 0 mV but sped activation kinetics above 0 mV, causing the fits to intersect (Fig. 5B,D, green curves versus black curves). As summarized in Table 1, the equivalent charge of Kv2.1 and Kv2.2 activation kinetics tended to increase in the presence of each AMIGO isoform; however, the comparisons did not reach the level of statistical significance. Together, these results show that although AMIGOs do speed Kv2 activation kinetics at extreme voltages, a change in the voltage dependence of Kv2 kinetics cannot alone explain the shifts in activation midpoints seen in Fig. 4 and in previous work (Peltola et al., 2011).

**AMIGO2 specifically reduces Kv2 inactivation**

Most voltage-gated ion channels undergo both voltage- and time-dependent inactivation after opening. Kv2 channels are well known for their slow inactivation, which helps maintain action potential (AP) firing fidelity at high frequencies (Du et al., 2000). Therefore, changes in the voltage or time dependence of Kv2 inactivation may perturb neuron firing *in vivo*. Previous studies on AMIGO1 and Kv2.1 did not address whether AMIGO1 modulated channel inactivation (Peltola et al., 2011). We assayed voltage-dependent inactivation (Peltola et al., 2011). We assayed voltage-dependent inactivation in HEK293 cells with and without AMIGO isoforms.

### Table 1. Parameters from electrophysiological experiments of Kv2.1 and Kv2.2 expressed in HEK293 cells with and without AMIGO isoforms

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Parameters from activation protocol</th>
<th>Parameters from inactivation protocol</th>
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<tbody>
<tr>
<td></td>
<td>$V_{1/2 \text{ act}}$ (mV)</td>
<td>Slope factor ($k_{\text{act}}$)</td>
</tr>
<tr>
<td>Kv2.1</td>
<td>$-2.4\pm1.1$ (9)</td>
<td>9.6±0.4</td>
</tr>
<tr>
<td>+AMIGO1</td>
<td>$-8.5\pm1.2$ (7)</td>
<td>8.7±0.4</td>
</tr>
<tr>
<td>+AMIGO2</td>
<td>$-10.3\pm1.2$ (8)</td>
<td>6.5±0.6</td>
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<tr>
<td>+AMIGO3</td>
<td>$-9.1\pm1.2$ (9)</td>
<td>7.1±0.3</td>
</tr>
<tr>
<td>Kv2.2</td>
<td>$-2.2±0.9$ (12)</td>
<td>7.7±0.5</td>
</tr>
<tr>
<td>+AMIGO1</td>
<td>$-7.8±1.4$ (9)</td>
<td>7.1±0.6</td>
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<tr>
<td>+AMIGO2</td>
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<td>6.0±0.3</td>
</tr>
<tr>
<td>+AMIGO3</td>
<td>$-9.0±1.4$ (9)</td>
<td>5.3±0.4</td>
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Bold text indicates significant difference compared to Kv2.1 or Kv2.2 expressed alone. $z_{\text{act}}$ refers to the voltage dependence of activation kinetics, as described in the text.

* at +20 mV; **, at −40 mV; *, after 2.5s step to +40 for Kv2.1 or +30 mV for Kv2.2; #, at +40 mV.
inactivation using a three-pulse protocol, as shown in Fig. 6A. The currents carried by Kv2.1 and Kv2.2 showed slow inactivation over pulse 2, as expected for Kv2 channels. Representative pulse 3 currents from these experiments are shown in Fig. 6B,C and show the amount of current remaining after pulse 2 at various voltages. We plotted the fraction of current remaining against the pulse 2 voltage for Kv2.1 plus/minus each AMIGO in Fig. 6D and for Kv2.2 plus/minus each AMIGO in Fig. 6E. Both Kv2.1 and Kv2.2 alone showed U-shaped inactivation that is characteristic of Kv2.1 channels (Fig. 6D,E, black squares) (Klemic et al., 1998). To minimize our analysis of the U-shaped inactivation, we restricted Boltzmann fits of the inactivation plots to cover voltages between −80 and +10 mV (Fig. 6D,E, solid line curves). The midpoints of Kv2 channel inactivation did not change by more than 5 mV with the addition of any single AMIGO isoform. Whereas some of the small differences between inactivation midpoints reached statistical significance, others did not. Furthermore, we found that the midpoint of inactivation was sensitive to the voltage range used for the fit due to the U shape of the data. Therefore, we did not deem changes in inactivation midpoint meaningful in Fig. 6, and do not mention them further. The values from the fits are presented in Table 1.

Fig. 6. AMIGO2 specifically reduces the extent of Kv2 inactivation. (A) Three-pulse protocol and representative currents recorded to measure voltage-dependent inactivation from Kv2.1 and Kv2.2. Pulse 1 and 3 were to +40 mV. Every other sweep is shown for clarity. Dashed box indicates the region of the current traces shown in B and C. (B) Representative currents illustrating the amount of Kv2.1 current remaining in pulse 3 after inactivation during pulse 2 when expressed alone or with each AMIGO isoform. The voltages indicated refer to the pulse 2 voltage that preceded the indicated pulse 3 trace. (C) Representative currents from Kv2.2 in pulse 3 of the inactivation protocol when expressed alone or with each AMIGO isoform. (D) Voltage dependence of Kv2.1 inactivation determined from the fraction of the pulse 1 current remaining in pulse 3 after inactivation. AMIGO2 specifically reduced inactivation at voltages between −20 mV and +60 mV as indicated by the green asterisks ($P<0.05$). For example, at +40 mV: Kv2.1=0.43±0.02 ($n=10$), +AMIGO1=0.46±0.02 ($n=9$), +AMIGO2=0.62±0.01 ($n=8$), +AMIGO3=0.59±0.03 ($n=8$); one-way ANOVA, $F=20.9$, $P<0.0005$ with pairwise post Tukey tests: $P=0.7$, $P<0.0005$, $P=0.05$, respectively, for each AMIGO compared to Kv2.1 alone. (E) Voltage dependence of Kv2.2 inactivation with and without each AMIGO. AMIGO2 specifically reduced inactivation between −10 mV and +30 mV, as indicated by the green asterisks ($P<0.05$). At +30 mV, the inactivated fractions were Kv2.2=0.47±0.03 ($n=10$), +AMIGO1=0.47±0.03 ($n=8$), +AMIGO2=0.59±0.03 ($n=8$), +AMIGO3=0.49±0.04 ($n=9$); one-way ANOVA, $F=3.2$, $P<0.05$ with pairwise post Tukey tests: $P=0.99$, $P<0.05$, $P=0.97$ compared to Kv2.2 alone, respectively.
Although the shifts conferred by the AMIGOs on Kv2 inactivation midpoint were difficult to interpret, we were immediately struck by the absolute reduction in inactivation conferred by AMIGO2, as is evident in the plots in Fig. 6D,E (green squares). In order to quantify this change in inactivation extent, we compared the fraction of current remaining for each Kv2 isoform alone or with each AMIGO at individual voltages. For example, after the pulse 2 step to +40 mV, Kv2.1 had 0.43±0.02 of its maximal current remaining (Fig. 6D, black squares). Assembly with either AMIGO1 or AMIGO3 did not change the available fraction of Kv2.1 current (P=0.07 and P=0.05, respectively). However, assembly of Kv2.1 with AMIGO2 increased the available fraction to 0.62±0.01 (Fig. 6D, green squares, P<0.0005). In fact, AMIGO2 increased the available fraction of Kv2.1 current at all voltages between −20 mV and +60 mV. AMIGO2 also conferred reduced inactivation to Kv2.2 to a similar extent between −10 mV and +30 mV (Fig. 6E). The available fraction of Kv2.2 current after the +30 mV step did not change when co-expressed with AMIGO1 or AMIGO3, and so the reduction of inactivation was again specific to AMIGO2. Taken together, these data show that although none of the AMIGOs dramatically altered the voltage sensitivity of Kv2.1 or Kv2.2 inactivation, AMIGO2 specifically reduced inactivation at multiple voltages for both channels.

AMIGO2 slows inactivation and deactivation

To further explore the mechanism underlying the effect of AMIGO2 on Kv2.1 and Kv2.2 inactivation, we compared the kinetics of Kv2 channel inactivation in the presence and absence of each AMIGO isoform. Normalized traces of Kv2.1 and Kv2.2 current decay over 2.5 s at +40 mV are shown in Fig. 7A,C, respectively, and reveal that AMIGO2 slowed the decay of both Kv2 channels’ inactivation kinetics (green traces). We quantified the time course of inactivation at each voltage by fitting a single exponential function to the decay of the Kv2 current at various voltages. A summary of the time constants of inactivation for Kv2.1 and Kv2.2 is shown in Fig. 7B,D, respectively, reinforcing that AMIGO2 slowed Kv2.1 inactivation at all voltages between −10 mV and +60 mV, as indicated by the green asterisks (P<0.05).

**Fig. 7. AMIGO2 specifically slows Kv2 inactivation and deactivation kinetic.** (A) Representative traces of normalized Kv2.1 currents during a 2.5 s step to +40 mV with and without AMIGO co-expression. Traces are normalized to their maximum values. (B) Summary of Kv2.1 inactivation kinetics with and without three AMIGOs. Time constants are from single exponential fits to the current decay traces. At +40 mV, Kv2.1 τ=3.2±0.1 s (n=10); one-way ANOVA, F=28.8, P<0.0005 with post hoc Tukey tests: +AMIGO1 τ=3.2±0.2 s, N=9, P=0.99; +AMIGO2 τ=5.7±0.28 s, N=8, P=0.0005; +AMIGO3 τ=3.9±0.2 s, N=9, P=0.11. AMIGO2 slowed Kv2.1 inactivation at all voltages between −10 mV and +60 mV, as indicated by the green asterisks (P<0.05). (C) Representative traces of normalized Kv2.2 currents during a 2.5 s step to +40 mV with and without AMIGO co-expression. (D) Voltage dependence of Kv2.2 inactivation kinetics obtained from single exponential fits to the 2.5 s current decay in the presence and absence of each AMIGO. At +30 mV, Kv2.2 τ=3.6±0.3 s (n=10); one-way ANOVA, F=6.03, P=0.005 with post hoc Tukey tests: +AMIGO1 τ=3.6±0.3 s, n=8, P=0.99; +AMIGO2 τ=5.4±0.4 s, n=8, P=0.005; +AMIGO3 τ=3.9±0.4 s, n=9, P=0.86. AMIGO2 slowed Kv2.2 inactivation kinetics obtained from single exponential fits to the current decay traces. At +40 mV, Kv2.2 τ=3.9±0.2 s, N=9, P=0.11. **AMIGO2 slowed** Kv2.2 inactivation at all voltages between −10 mV and +30 mV, as indicated by the green asterisks (P<0.05). (E) Representative traces of Kv2.1 deactivation time course at −40 mV after a depolarizing step to +30 mV in the presence and absence of each AMIGO. Currents were normalized to the current at the beginning of the −40 mV step. (F) Summary of Kv2.1 deactivation kinetics at −40 mV under the four conditions. Kv2.1 deactivation at −40 mV (18.6±1.0 ms, n=9) was slowed by co-expression of AMIGO2 (60.7±7.0 ms, P<0.0005, n=8) but not AMIGO1 (23.2±1.3 ms, P=0.8, n=7) or AMIGO3 (29.2±1.2 ms, P=0.14, n=9) (one-way ANOVA, F=28.8, P<0.0005). **AMIGO2 slowed** Kv2.2 deactivation kinetics at −40 mV under the four conditions. Kv2.2 deactivation at −40 mV (19.5±1.5 ms, n=12) was slowed by co-expression of AMIGO2 (70.7±11.2 ms, P<0.0005, n=8) but not AMIGO1 (25.1±2.1 ms, P=0.9, n=9) or AMIGO3 (32.6±4.8 ms, P=0.3, n=9) (one-way ANOVA, F=17, P<0.0005). ***P<0.0005.
inactivation for both Kv2.1 and Kv2.2. Indeed, at +40 mV, Kv2.1 alone inactivated with a time constant of 3.2±0.1 s and AMIGO2, but not AMIGO1 or AMIGO3, significantly increased this parameter \((P<0.0005)\). The results for Kv2.2 were similar, although at extremely depolarized potentials the differences disappeared. These kinetic results match well with the AMIGO effects on the inactivated fraction (Fig. 6) and suggest that the inhibitory effects of AMIGO2 on Kv2 channel inactivation are mainly due to a slowing of the inactivation process.

As mentioned previously, channel inactivation proceeds in a time- and voltage-dependent manner after channel activation. The loss of current observed during channel inactivation is distinct from the loss of current observed during channel deactivation, which refers specifically to the process of channel closing. Therefore, an assessment of the effects of the AMIGOs on deactivation could shed light on the mechanism underlying the observed effects on channel inactivation. We analyzed the kinetics of the tail currents collected in Fig. 4, which report channel closing during deactivation. Normalized tail currents recorded at −40 mV are shown in Fig. 7E for Kv2.1 with and without each AMIGO isoform. We fit the tail currents and compared the decay time constants (Fig. 7F) and found that AMIGO2 slowed Kv2.1 deactivation significantly \((P<0.0005)\). Although AMIGO1 and AMIGO3 also tended to slow the time constant of tail current decay, they did not confer statistically different values compared to Kv2.1 alone. Comparable results were observed for Kv2.2 tail currents in the presence and absence of each AMIGO (Fig. 7G,H). The observation that AMIGO2 slows both the inactivation and deactivation of Kv2 channels suggests a general increase in the stability of the open state of the channel when AMIGO2 is bound, rather than a direct interference with the inactivation process.

**DISCUSSION**

Kv β-subunits are well known for their modulatory effects on channel function and localization. Our results show that all three AMIGOs do indeed modulate the activation of both Kv2.1 and Kv2.2 channels, and AMIGO2 specifically had an additional dramatic effect on channel inactivation and deactivation. The effects of assembly on auxiliary subunit trafficking are often overlooked. Here, we demonstrate that both Kv2s confer increased trafficking and a clustered localization to all three AMIGOs. Given the AMIGO family’s widespread physiological functions, such as neurite growth and cell survival (Kuja-Panula et al., 2003; Chen et al., 2012; Ono et al., 2003), assembly with Kv2 channels likely modulates some of these functions as well. Therefore, the interaction between Kv2 channels and each AMIGO represents a co-regulatory mechanism for both electrical and non-electrical cell properties.

The effects of the AMIGOs on voltage-dependent activation observed in this work agree with previously published data on AMIGO1 (Peltola et al., 2011). Namely, each AMIGO conferred a hyperpolarizing shift to Kv2 channel activation midpoint (Fig. 4). In addition to shifting the activation midpoint, AMIGO2 and AMIGO3 also altered the slope factor of the Kv2.1 and Kv2.2 activation curves, indicating increased voltage dependence of channel opening. This observed increase in voltage sensitivity could be due to multiple mechanisms, which were not rigorously tested in this work, and a molecular description of the effects of the AMIGOs on Kv2 channel activation will require additional experiments. For example, it is possible that the changes in channel activation are due to altered voltage dependence of the Kv2 voltage-sensing machinery. However, we think that this mechanism is unlikely. Kv2.1 channels have a characteristic separation of the Q–V and G–V curves (O’Connell et al., 2010; Tilley et al., 2019), and thus a leftward shift in G–V (as seen here) could be caused by increased coupling between voltage sensing and pore opening without a shift in the Q–V curve. A similar mechanism has been proposed to explain the effects of leucine-rich-repeat-containing protein 26 (LRRC26) on the large conductance potassium channel (BK). This AMIGO-like auxiliary subunit of BK causes a dramatic increase in the coupling between voltage sensitivity and pore opening (Yan and Aldrich, 2010). In addition to speeding activation, AMIGO2 specifically slowed inactivation and deactivation of Kv2 channels by about twofold and threefold, respectively (Fig. 7). This effect is also qualitatively similar to the effect of LRRC26 on BK (Yan and Aldrich, 2010), further supporting a similar mechanism of action for AMIGO2 on Kv2 channels. However, there is clearly a dissociation between the effects of the AMIGOs on channel opening and closing, because all three isoforms shifted the G–V relationship to similar extents, while only AMIGO2 altered inactivation and deactivation kinetics. Mapping the interaction sites of the AMIGOs with Kv2 along with additional electrophysiological studies may shed light on the differences conferred by each AMIGO isoform.

The potassium voltage-gated channel subfamily E (KCNE) proteins are another family of type-1 transmembrane proteins that act as Kv channel auxiliary subunits. Although the stoichiometry of interaction between KCNE and Kv channels is still under debate, it is well accepted that each channel can bind multiple KCNE subunits (Plant et al., 2014; Solé et al., 2020; Nakajo et al., 2010), and a single KCNE subunit is sufficient to maximally alter Kv1.3 inactivation kinetics (Solé et al., 2020). The stoichiometry of the AMIGO-Kv2 interaction has not yet been addressed to our knowledge; however, like KCNEs, each Kv2 channel may bind multiple AMIGO auxiliary subunits. The consequences of simultaneous interaction with multiple AMIGOs, which could have different functional effects (Figs 6 and 7), should be addressed in future studies. Could the AMIGO2 effects on inactivation and deactivation outcompete the effects of AMIGO1 and AMIGO3? How do the stoichiometry and functional effects change Kv2 function in cells in which AMIGOs and KCNEs are both present?

The effects of the AMIGOs on channel kinetics observed in this work are particularly interesting in terms of Kv2 function in the brain. As mentioned in the Introduction, Kv2 channels are not recruited for single AP repolarization, but are thought to be important for maintaining polarized membrane potentials during high-frequency firing. Faster Kv2 activation and slower inactivation, as seen in this work, would result in more outward K⁺ current at a given firing frequency. This hyperpolarizing current could enhance the recovery of Na⁺ channels from inactivation during the interspike interval, leading to an overall increase in firing frequency. Indeed, this hypothesis is in line with the observed requirement for Kv2 in preventing depolarization block during high-frequency firing in cervical ganglion neurons, CA1 pyramidal neurons and cortical neurons (Guan et al., 2013; Liu and Bean, 2014). Alternatively, the increased hyperpolarizing current contributed by Kv2s associated with AMIGOs could halt firing by preventing sufficient depolarization for AP initiation. The overall result of AMIGO assembly with Kv2s on neuronal firing properties will likely vary on a cell-by-cell basis and will be determined by the cell’s full complement of ion channels and the neuronal inputs it receives.

Of the three isoforms, AMIGO2 had the most dramatic effects on Kv2.1 and Kv2.2 channel kinetics (Fig. 7). In the hippocampus, Kv2 channels and AMIGO2 have somewhat reciprocal expression...
patterns, with Kv2.1 and Kv2.2 more highly expressed in CA1 and CA3 (Palacio et al., 2017), and AMIGO2 highly expressed in CA2 (Laememans et al., 2013; Lein et al., 2007). These observations suggest that AMIGO2 may not regulate Kv2 channel electrical function extensively in the hippocampus. An AMIGO2 knockout mouse has been characterized as having perturbed neuronal morphology in the retina and associated vision impairments (Soto et al., 2019). Whether these effects can be attributed to the regulatory effects of AMIGO2 on Kv2 currents is unknown, but Kv2.1 is also highly expressed in the retina (Gayet-Primo et al., 2018). According to the Human Protein Atlas, AMIGO2 is also found in peripheral tissues that express Kv2 channels, such as endocrine and muscle cells. Whether the dramatic effects of AMIGO2 on Kv2 electrical properties regulate the functions of Kv2-containing cells in these tissues will be interesting to investigate.

In addition to AMIGO-dependent changes in Kv2 electrical function, we also identified Kv2-dependent changes in AMIGO localization and trafficking. Expressed alone in HEK293 cells, surface AMIGO proteins were distributed evenly across the cell surface. When co-expressed with Kv2 channels, each of the three AMIGOs was trafficked more efficiently to the PM and underwent re-localization to ER–PM junctions, as was previously observed with AMIGO1 and Kv2 channels (Bishop et al., 2018). Redistribution of AMIGOs to Kv2 clusters on the cell surface is likely to be due to co-assembly of the two proteins in the same complex, as supported by the Co-IP results in this work. The Kv2.1–AMIGO1 interaction was proposed to occur directly via the transmembrane (TM) domain of AMIGO1 and an unidentified TM domain of Kv2.1 (Peltola et al., 2011). Because the TM domains of the three AMIGOs are fairly homologous (Fig. S3, yellow; 48% identical, 81% similar, UniProt), interaction between each AMIGO and Kv2 likely occurs via a similar mechanism, although the specific residues involved in interaction have yet to be determined.

In our Co-IP experiments, both the GFP-tagged and endogenous AMIGO1 appeared as doublets (Fig. 3 and Fig. S2, respectively). The lower of the two GFP–AMIGO1 bands was preferentially co-immunopurified by both anti-Kv2.1 and anti-Kv2.2 antibodies, suggesting that the channels assemble more favorably with one AMIGO form over the other. Early work with AMIGO1 suggests that the two molecular masses represent different glycosylation states of the protein at any of five predicted N-linked glycosylation sites (Kajander et al., 2011). Perhaps the lower-molecular-mass version that assembles with Kv2 channels is not fully glycosylated. Whether assembly with Kv2.1 alters AMIGO1 glycosylation or, once assembled, the complex no longer traffics through the classic Golgi pathway, as has been suggested (Jensen et al., 2017), remains an open question.

Each of the AMIGO isoforms showed variable levels of surface expression, and, of the AMIGOs, AMIGO3 showed the lowest surface expression, with much of the internal AMIGO3 appearing to reside in the ER. Although an ER-retention motif has not yet been identified in any of the AMIGOs, interestingly, AMIGO3 has an additional six amino acids in the proximal C-terminus (Fig. S3, cyan). These six amino acids contain a di-arginine motif, which is a sequence involved in general ER retention (Sharma et al., 2010). Internal AMIGO1 and AMIGO2 appeared to be localized in trafficking vesicles, which could represent a forward trafficking or recycling pool. Future studies may wish to determine in which pool these vesicles belong, and whether assembly with Kv2 channels prolongs their lifetime at the PM.

The effect of Kv2 channels on AMIGO surface expression represents a gain-of-function phenotype that likely has downstream effects due to AMIGO functions at the cell surface. For example, AMIGO2 localization to the PM may upregulate lipid-dependent phosphorylation of Akt, leading to increased growth and survivability (Park et al., 2015). Likewise, because all three AMIGOs are thought to act as cell adhesion molecules (Kuja-Panula et al., 2003), increased surface expression could increase the size, number or location of cell–cell adhesions. Indeed, EM micrographs of Kv2.1 clusters in the rat hippocampus have been identified as sites of astrocyte adhesion (Du et al., 1998), and, more recently, it was reported that microglia often contact neurons at Kv2.1 clusters (Cserép et al., 2019). Although neither of these neuron–glia adhesions have been attributed to AMIGO family interactions, it is tempting to speculate that AMIGOs residing in Kv2 clusters play a role in adhesion.

Until recently, the Kv2 literature and AMIGO literature have been largely distinct bodies of work. The finding (Peltola et al., 2011) that AMIGO1 is a Kv2.1 auxiliary subunit marks the merging of the two fields. In this work, we further intertwine the AMIGO and Kv2 fields by showing functional interactions between all members of both families. This result will inevitably lead to new and renewed interest in various aspects of each family’s physiological function. For example, could AMIGOs reduce or exacerbate effects of the known pathogenic de novo Kv2.1 mutations (Kang et al., 2019; Kovel et al., 2017; Thiffault et al., 2015)? Are Kv2 channels somehow involved in the activity dependence of AMIGO expression (Ono et al., 2003)? What are the consequences of concentrating each of the three AMIGOs at the extracellular surface of Kv2-induced ER–PM junctions? These and other questions await future investigation.

MATERIALS AND METHODS

DNA constructs

The original AMIGO1, AMIGO2 and AMIGO3 constructs were obtained from the DNASU plasmid repository (plasmid IDs HsCD0029615, HsCD00513136 and HsCD00512989, respectively). Fluorescent protein fusion constructs were generated by PCR-based addition of restriction sites to the ends of each AMIGO family insert (Table S1). Fluorescent protein vectors were cut with the same enzymes and each insert was ligated into the recipient vector, such that the fluorescent protein (GFP–N1 or Ruby2–N1) was fused to the C-terminus of AMIGO. Full-length, codon-optimized Kv2.2 (accession number NM_054000) was synthesized by GeneWiz and inserted into a pEGFP-C1 expression vector (Clontech) via EcoRI and Sall restriction sites. A non-tagged Kv2.2 plasmid was made by removing the GFP from the N-terminus of Kv2.2 in this construct. GFP–Kv2.1 and Kv2.1–loopBAD (Kv2.1–LB) have been described previously (O’Connell and Tamkun, 2005; Tamkun et al., 2007). A human baintin ligase, hBiRA, was a gift from Alice Ting (Chen et al., 2005). For additional details, see Table S1.

Cell culture and transfection

HEK293 cells (CRL-1573, American Type Culture Collection, Manassas, VA, checked for mycoplasma when received and used for only ten to 12 passages) were maintained in 10 cm dishes (#229620, CellTreat, Pepperell, MA) at 37°C under 5% CO₂ in Dulbecco’s modified Eagle medium (#10-013-CV, Corning, Corning, NY) supplemented with 10% fetal bovine serum (#S12450, R&D Systems, Minneapolis, MN). For transfections, cells were trypsinized and electroporated (GenePulse Xcell, BioRad, Berkeley, CA) with either Kv2 α-subunits (500 ng per dish), AMIGO subunits (300 ng per dish) or a combination of both. In some experiments a GFP-tagged pleckstrin-homology domain construct was used as a marker to facilitate measurements of fluorescence associated with the PM. For IP experiments, the Kv2.1–LB and untagged Kv2.2 constructs were used to allow for the detection of GFP-tagged AMIGO subunits with an anti-GFP antibody.
Hippocampal cultures of neurons were isolated from E18 Sprague Dawley rat brains of both sexes. Pregnant rats were deeply anesthetized with isoflurane, as outlined in a protocol approved by the Institutional Animal Care and Use Committee of Colorado State University (protocol ID 15-6130A). Hippocampi were dissociated and cultured as previously described for neurons (Bartlett and Banker, 1984; Brewer et al., 1993). Cultures were seeded on glass-bottom 35 mm dishes (with No. 1.5 coverglasses; MatTek, Ashland, MA), coated with poly-L-lysine (Sigma-Aldrich, St Louis, MO) in borate buffer, and in a medium composed of Neurobasal Medium (Gibco/Thermo Fisher Scientific, Waltham, MA), B27 Plus Supplement (Gibco/Thermo Fisher Scientific), penicillin/streptomycin (Cellgro/Mediatech, Manassas, VA) and GlutaMAX (Gibco/Thermo Fisher Scientific).

At DIV5, neurons were transfected using DNA, Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY) and OptiMEM for experiments in 48 h. Neurons were transfected with either GFP–Kv2.1 or GFP–Kv2.2 α-subunits (500 ng per dish) and one of the three Ruby2–AMIGO subunits (300 ng per dish). After 48 h, neurons were transfected to an imaging saline composed of 126 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 0.6 mM MgSO₄, 0.15 mM NaH₂PO₄, 0.1 mM ascorbic acid, 8 mM glucose and 20 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, 300 mOsm/l.

Microscopy

Spinning disk confocal microscopy was used for most imaging experiments. HEK293 cells were electroporated as described above and plated at a low density on glass-bottom dishes (#P35G-1.5-14-C, MatTek, Boston, MA) coated with Matrigel (#354230, BD Biosciences, Franklin Lakes, NJ) in a maintenance medium for 18–24 h. Cells were briefly removed from incubation for medium exchange to HEK293 imaging saline, which contains the following: 146 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 0.6 mM MgSO₄, 1.6 mM NaHCO₃, 0.15 mM NaH₂PO₄, 0.1 mM ascorbic acid, 8 mM glucose and 20 mM Hepes, pH 7.4. After medium exchange, cells were immediately placed on the heated microscope stage and allowed to equilibrate for 10 min before imaging.

Spinning disk confocal microscopy was performed on a Yokogawa (Musashino, Japan)-based CSU1X system with an Olympus (Tokyo, Japan) IX83 inverted stand, and coupled to an Andor (Abingdon, UK) laser laser launching containing 405, 488, 568 and 637 nm diode lasers, 100–150 mW each. Images were collected using an Andor iXon EMCCD camera (DU-897) and 100× Plan Apo, 1.4 NA objective. This system is equipped with the ZDC constant focus system and a Tokai Hit chamber and objective heater. Images were collected using MetaMorph software (version 7.8.13.0).

Total internal fluorescence (TIRF) microscopy was used to identify transfected cells for electrophysiological experiments. TIRF microscopy was performed on a Nikon (Tokyo, Japan) Eclipse Ti microscope equipped with 100× epi-diopter lenses at 405, 488, 561 and 640 nm. Cells were imaged using a 100× Plan Apo TIRF, 1.49 NA objective and captured by an Andor iXon EMCCD DU-897 camera using appropriate band-pass filters.

Co-IP

HEK293 cells were transfected with either Kv2.1–LB or Kv2.2 alone, or one of the Kv2 α-subunits along with either AMIGO1–GFP, AMIGO2–GFP or AMIGO3–GFP. After 16–24 h, cells were washed 1× with cold PBS then scraped in 1 ml lysis buffer (50 mM Tris base, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100, pH 7.2)+Pierce Protease Inhibitor Cocktail (A23955, Thermo Fisher Scientific). After 20 min, the lystate was centrifuged at 3000 g for 10 min to remove insoluble material, and the supernatant was pre-cleared with Protein G Sepharose Beads (#6511, BioVision, Milpitas, CA) for 1 h. Beads were removed by centrifugation and the pre-cleared lysate split into two aliquots for positive and negative IP. The positive IP samples were incubated with 1 µl IP antibody (mouse anti-Kv2.1 or mouse anti-Kv2.2, clones K89/34 and K37/89, respectively; both knockout validated; Neuromab, Davis, CA) for 16 h at 4°C before adding 50 µl Protein G Sepharose Beads. The negative IP samples received no antibody. An equivalent volume of beads was added to the negative IP samples at the same time, and all samples were incubated for 4 h at 4°C. The beads were collected by centrifugation at 1000 g for 3 min and the supernatants saved. The beads were washed 3× with 100 µl lysis buffer+Protease Inhibitor Cocktail before resuspending directly in 2× sample buffer (#1610737, BioRad, Hercules, CA)+β-mercaptoethanol and boiled for 10 min. Starting material and supernatant fractions were diluted in lysis buffer and sample buffer+β-mercaptoethanol and boiled for 10 min, after which proteins from all samples were separated on SDS-polyacrylamide gels (#5671034, BioRad). AMIGOs were much more abundant in the starting material and supernatants compared to the IP fractions. Therefore, the relative loading of the gel lanes was not equivalent. Indeed, the IP lanes were loaded with ~600 more cell equivalents than the supernatant and starting material lanes. After separation, protein was transfected to a nitrocellulose membrane. To confirm protein transfer, membranes were stained briefly with Ponceau S-Stain (P7170-1L, MilliporeSigma, Burlington, MA) before blocolding in PBS-Tween+5% w/v powdered milk, followed by incubation in primary antibodies (1:1000) for 1 h. The following primary antibodies were used for detection on nitrocellulose membranes: mouse anti-Kv2.1 (K89/34, Neuromab), mouse anti-Kv2.2 (K37/89, Neuromab), mouse anti-AMIGO1 (L86A/37, knockout validated, Neuromab) and rabbit anti-GFP (TP-401, Torrey-Pines, Secaucus, NJ). Blots were washed 3× with PBS-Tween then incubated in appropriate mouse or rabbit secondary antibodies conjugated to horseradish peroxidase (115-035-003, Jackson ImmunoResearch, West Grove, PA or GtxRb-003-JHRPX, ImmunoReagents, Raleigh, NC) at 1:10,000 in PBS-Tween+5% goat serum for 45 min. After washing, secondary antibodies were visualized with Super Signal Substrate (#34095, Thermo Fisher Scientific) for up to 5 min.

Electrophysiology

HEK293 cells were transfected with either Kv2.1–LB or GFP-Kv2.2 alone, or along with one of the three AMIGO constructs tagged with mRuby2. hBirA was co-transfected in experiments using Kv2.1–LB to biotinylate the channel, enabling its visualization as described below. Transfected cells were plated at low density on Matrigel-coated glass-bottom dishes. The next day, cells were bathed in extracellular solution containing the following: 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4, 340 mOsm/l. Cells expressing Kv2.1–LB were labeled with a streptavidin-conjugated fluorophore (#29041, Biotium, Hayward, CA) for 10 min in extracellular solution before washing and proceeding with electrophysiology. Cells expressing Kv2 α-subunits and AMIGOs were identified by fluorescence of protein tags and/or the streptavidin-fluorophore. Pipettes were pulled from thin-walled borosilicate glass and had tip resistances of 1–3 MΩ when filled with internal solution containing the following: 4 mM NaCl, 150 mM KCl, 1 mM MgCl₂, 0.5 mM EGTA, 10 mM HEPES, pH 7.4, 310 mOsm/l. Whole-cell voltage-clamp experiments were performed at room temperature with an Axopatch200B amplifier and Digidata 1550A using Clampex 10.6 software (Molecular Devices, Silicon Valley, CA). Data were acquired at 20 kHz and low-pass filtered at 5 kHz. At least 80% series resistance prediction and compensation were used to mitigate voltage error and all recordings were leak subtracted using the P/4 method. To investigate channel activation, cells were depolarized from a holding potential of −80 mV to between −80 and +70 mV for 250 ms in 10 mV increments (every other sweep is shown in figures for clarity). After each depolarizing pulse, the membrane was stepped to −40 mV for 250 ms to probe channel deactivation in tail currents before repolarizing to −80 mV. The tail currents were fit with a single exponential function and extrapolated backwards to obtain the instantaneous current at −40 mV associated with each of the variable voltage pulses. These currents were normalized (I/Imax) and fit with a single Boltzmann function to estimate channel activation parameters. The time constants of single exponential fits of tail currents were used as a measure of channel deactivation kinetics. To estimate the kinetics of channel activation, we fit the ‘late’ phase of the depolarizing step currents with single exponential functions as illustrated in Fig. S4. The time constants of these fits were used as a measure of channel activation kinetics at different voltages. To estimate the voltage dependence of activation kinetics, we fitted time constant (τ)–V plots with the exponential equation:

$$
\tau = \frac{A e^{-\frac{V - \alpha}{\beta}}}{1 + B}
$$
functions were fit to the data between compared to the pre-pulse for each variable voltage. Single Boltzmann channel inactivation was taken as the fraction of current in the test pulse inactivation, followed by a 100 ms test pulse to +40 mV. The amount of inactivation by fitting inactivating currents from 250 ms after the end of state (Klemic et al., 1998; Carrillo, et al., 2013). We also probed the kinetics of inactivation by fitting inactivating currents from 250 ms after the end of the pre-pulse to the end of the 2500 ms variable pulse.

Analysis and statistics

Images were processed and analyzed using ImageJ (v1.52p). All images are single z-planes, unless otherwise noted. Graphs and statistics in Figs 1, 2 and S1 were generated using GraphPad Prism 9.0.

Images in Fig. 1 that assess subunit assembly were from the basal cell surface. Line scans were drawn such that multiple clusters would be included. Fluorescent values from the line scans were standardized to the maximum measured fluorescence in order to be comparable between image channels. To measure colocalization between Kv2s and AMIGOs, a z-plane at the basal surface of HEK cells (Fig. 1) or neurons (Fig. S1) was assessed using the Coloc 2 plugin for ImageJ. Spearman’s ranked correlation coefficients were measured for each cell and were then compiled for one-sample Student’s t-tests against a test mean of zero.

Surface trafficking (Fig. 2) was measured from single z-planes at the center of each cell. A region of interest (ROI) was hand drawn around each cell, and the total fluorescence was measured for each channel. Using cells expressing PH–GFP to mark the membrane, the average width of the membrane was measured to be seven pixels. Seven pixels were then subtracted from the perimeter of the cell ROI to measure total fluorescence which was not on the PM. By subtracting the internal fluorescence from the total fluorescence, the fluorescence on the surface was determined. In the case of AMIGO3, for which little fluorescence was on the surface, PH–GFP was co-expressed to determine the location of the PM. These measurements were performed in ImageJ.

Fluorescence density in arbitrary units (AU)/µm² was calculated by dividing the sum of fluorescence in each ROI by the area of that ROI in µm². By using a ratio of densities, we eliminate the bias of differences in protein expression between cells. A surface trafficking index was determined by dividing the surface fluorescence density by total fluorescence density. This measure indicates the preference of a protein for surface expression (if greater than one) or intracellular expression (if less than one). The surface trafficking index was compared to a test mean of one using a one-sample t-test (t) to determine whether the protein preferred to localize to the surface or intracellularly. To determine if co-expression significantly increased surface trafficking index, one-way ANOVAs with post hoc Tukey tests were used.

Western blot images were acquired on a UVP ChemStudio imaging system with VisionWorks software (Analytik-Jena, Jena, Germany). Images of blots were cropped to molecular mass ROIs and contrasted in ImageJ. In Fig. 3B, mild gamma correction was used to better show bands in the starting material and supernatant fractions.

Electrophysiological data were analyzed using MATLAB (MathWorks) and plotted using Origin2018b software. Data shown are mean±s.e.m. Data points with no error bars have standard error smaller than the symbol size. One-way ANOVA (F) was used to test for significance between multiple groups, α=0.05. Post hoc Tukey’s tests were performed to determine significance between each group, α=0.05. Sample sizes (n) are included in figure legends and represent data from at least two biological replicates.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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References


Fig. S1. AMIGOs co-localize with Kv2 channels on the surface of rat hippocampal neurons. (A) Representative images of center z-planes of DIV7 neurons expressing AMIGO1 (left, blue box), AMIGO2 (middle, green box) or AMIGO3 (right, orange box). These data show all 3 AMIGOs have large intracellular expression in the ER and in vesicles. AMIGO2 shows the most expression on the plasma membrane, indicated by the ring of fluorescence on the edge of the cell. Representative images of a center z-plane of neurons expressing Kv2.1 (B) or Kv2.2 (C) (green) and AMIGO1 (magenta) show co-localization of AMIGO1 and Kv2 clusters on the plasma membrane when co-expressed. Notably, AMIGO1 surface trafficking does not appear to improve as much as observed in HEK cells (Figure 2). Co-expression of AMIGO2 and Kv2.1 (D) or Kv2.2 (E) also shows co-localization in surface clusters. AMIGO2 surface expression seemed to improve with both Kv2s. Co-expression of AMIGO3 and Kv2.1 (F) or Kv2.2 (G) also shows co-
Localization in surface clusters. AMIGO3 surface expression seemed to improve with Kv2.1, but not Kv2.2. Overlapping fluorescence between the 2 proteins is indicated by white pixels. H) Spearman’s ranked correlation coefficient was calculated from basal surface clusters of neurons. Box plots depict median, interquartile range, and the min and max values for each dataset. All datasets were significantly higher than 0, indicating that AMIGOs are positively correlated with Kv2s (p < 0.0001). Medians are 0.7891, 0.7931, 0.7439, 0.7142, 0.8126, and 0.7221, respectively. Medians and t-tests were performed on datasets containing 20, 17, 22, 16, 8 and 8 cells respectively. Scale bars are 5 µm.
**Fig. S2. Co-Immunopurification control conditions**

**A)** Top - A monoclonal AMIGO1 antibody also detects an approximately 80 kDa band after immunopurification with the Kv2.1 IP antibody (asterisk).  

+IP Beads = material pulled down by Kv2.1 antibody; -IP Beads = material pulled down in absence of IP antibody; +IP Sup. = material remaining in supernatant after pull down with IP antibody; -IP Sup. = material remaining in supernatant after pull down in absence of IP antibody; 

Start. Mat. = material in pre-cleared lysate before IP. Bottom - Kv2.1 itself was enriched in the +IP Beads sample indicating successful pull-down. 

**B)** Top - Anti-AMIGO1 antibody detected AMIGO1-GFP pulled down by Kv2.2 antibody (asterisk). The lane labels are the same as in A but a Kv2.2-
specific antibody was used. **Bottom**- The Kv2.2 IP antibody detects Kv2.2 enriched in the pull down fraction. **C-D)** **Top** - Endogenous AMIGO1 was undetectable after Co-IP of cells transfected with either Kv2.1 (C) or Kv2.2 (D) alone. However, endogenous AMIGO1 was observed in the starting material from these experiments when loaded at 10x our standard amount (right blots). **Bottom** – Kv2 alpha subunits were successfully enriched in their respective pull down fractions in Co-IP experiments from cells transfected with only Kv2.1 (C) or Kv2.2 (D). The same bands were observed in the starting material when the blots were exposed longer (right blots, C and D, respectively). Three biological replicates were used for each condition.
Fig. S3. Alignment of protein sequences from human isoforms of AMIGO1, AMIGO2 and AMIGO3. Transmembrane domains are highlighted in yellow. The 6 amino acids which might be responsible for ER retention in AMIGO3 are highlighted in cyan. * indicates fully conserved residue, : indicates conservation in amino acids with high similarity, and . indicates conservation between groups with low similarity.
**Fig. S4. Fitting the late phase of Kv2 current activation** Example exponential fits (red) to raw data (black) used to obtain the data shown in Figure 6 of the main text. To avoid fitting artifacts induced by the sigmoidal current time course, fits were restricted to the portion of current between the inflection point and the max.
**Movie 1. Kv2.1 clusters are detergent insoluble structures.** HEK cells expressing GFP-Kv2.1 were imaged at 1.1Hz with the focal plane at the basal surface following the addition of lysis buffer (50 mM Tris Base, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100, pH 7.2) at 20°C. At 1.5 min after this detergent addition the membrane and nonclustered GFP-Kv2.1 begin to solubilize. The GFP-Kv2.1 clusters first aggregate and then assume a globular shape but do not solubilize even after 5 min. Non-solubilized GFP-Kv2.1 aggregates can be seen both diffusing through the solution and adhered to the substrate.
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