AMIGO is an auxiliary subunit of the Kv2.1 potassium channel

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Kv2.1 is a potassium channel α-subunit abundantly expressed throughout the brain. It is a main component of delayed rectifier current (I_K) in several neuronal types and a regulator of excitability during high-frequency firing. Here we identify AMIGO (amphoterin-induced gene and ORF), a neuronal adhesion protein with leucine-rich repeat and immunoglobin domains, as an integral part of the Kv2.1 channel complex. AMIGO shows extensive spatial and temporal colocalization and association with Kv2.1 in the mouse brain. The colocalization of AMIGO and Kv2.1 is retained even during stimulus-induced changes in Kv2.1 localization. AMIGO increases Kv2.1 conductance in a voltage-dependent manner in HEK cells. Accordingly, inhibition of endogenous AMIGO suppresses neuronal I_K at negative membrane voltages. In conclusion, our data indicate AMIGO as a function-modulating auxiliary subunit for Kv2.1 and thus provide new insights into regulation of neuronal excitability.

INTRODUCTION

Potassium channels are key determinants of neuronal excitability. Kv2.1 is an abundant voltage-dependent potassium (Kv) channel expressed in most central nervous system neurons (Hwang *et al*, 1993; Maletic-Savatic *et al*, 1995; Du *et al*, 1998). Kv2.1 channels constitute an essential component of the delayed rectifier current (I_K) and regulate excitability in several neuronal types (Murakoshi & Trimmer, 1999; Du *et al*, 2000; Malin & Nerbonne, 2002; Guan *et al*, 2007). In particular, Kv2.1 has been shown to regulate excitability during periods of high-frequency firing (Du *et al*, 2000).

The phosphorylation localization and activity of Kv2.1 are known to be strongly regulated. Under normal conditions, Kv2.1 is heavily phosphorylated and its localization is clustered (Trimmer, 1991; Misonou *et al*, 2004, 2006). Increased neuronal activity, induced by kainate seizures *in vivo* or glutamate stimulation *in vitro*, leads to dephosphorylation of Kv2.1 and dispersion of clustered Kv2.1 to diffuse somatodendritic localization (Misonou *et al*, 2004, 2006). These modifications are associated with a large hyperpolarizing shift in voltage-dependent activation of neuronal I_K, which is able to suppress neuronal activity. Interestingly, it has been proposed that Kv2.1 functions as an adjustable resistor in neuronal soma, providing a mechanism of homeostatic plasticity (Surmeier & Foehring, 2004). In addition, hypoxia/ischaemia produced by CO_2 treatment *in vivo* or chemically induced experimental ischaemia *in vitro* elicits a similar dephosphorylation and translocation of Kv2.1 (Misonou *et al*, 2005). Consequently, Kv2.1 is suggested to function as a mechanism to suppress pathological hyperexcitability of central neurons during ischaemic conditions.

Voltage-dependent potassium channels function as macromolecular complexes able to integrate a variety of signals regulating the channel activity. The ion-conducting pore of the channels is composed of four main subunits (α-subunits). The channel complex frequently contains auxiliary subunits that are diverse in structure and function. The significance of auxiliary subunits is shown in humans and experimental animals by several associated diseases, such as arrhythmogenesis, periodic paralysis, sensorineural deafness and epilepsy (Schulze-Bahr *et al*, 1997; Splawski *et al*, 1997; Abbott *et al*, 2001; Brenner *et al*, 2005). So far, auxiliary subunits have been identified only for a portion of the large group of Kv channel α-subunits. Until now, colocalization of an auxiliary subunit with Kv2.1 has not been demonstrated in brain.

AMIGO (amphoterin-induced gene and ORF) is a transmembrane protein originally identified as a gene in cultured hippocampal neurons induced by the neurite outgrowth-promoting protein HMGB1 (amphoterin). The domain structure of AMIGO comprises a small cytoplasmic region, a single transmembrane domain and an extracellular part containing both immunoglobin (Ig) and leucine-rich repeat (LRR) domains and five potential *N*-glycosylation sites. Together with its homologous proteins, AMIGO2 and AMIGO3, AMIGO defines a new family of

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cell adhesion molecules. Of these three molecules, AMIGO is predominantly expressed in the nervous system, whereas the other two members of the family are expressed more broadly (Kuja-Panula *et al*, 2003).

In this paper, we demonstrate the association and extensive colocalization of AMIGO and Kv2.1 in mouse brain and in cultured cells. The association and colocalization are preserved even during marked stimulus-induced changes in Kv2.1 channel phosphorylation and localization. Our results clearly indicate AMIGO as an integral part of the Kv2.1 channel complex that is able to affect the voltage-dependent activation of Kv2.1 and neuronal I_K. The identification of AMIGO as a Kv2.1 subunit opens up new possibilities for understanding the function and intriguing properties of Kv2.1.

RESULTS

AMIGO colocalizes with Kv2.1

AMIGO is primarily expressed in the nervous system (Kuja-Panula *et al*, 2003). To further study the localization of AMIGO in brain, we produced an affinity-purified chicken AMIGO antibody. The specificity of the AMIGO antibody was determined by several experiments (supplementary Fig S1 online).

Immunohistochemistry and western blotting with AMIGO antibody showed that spatial and temporal expression of AMIGO in mouse brain strongly resembles the expression of Kv2.1 potassium channel (supplementary Figs S2 and S3 online). We therefore examined the localization of AMIGO and Kv2.1 with double-immunohistochemical stainings. Confocal microscopy revealed a striking colocalization of AMIGO and Kv2.1 in mouse brain sections (Fig 1A,B). Both proteins were widely detected in mouse cerebral neurons. Staining was restricted to the soma and to the proximal part of neurites and was clearly clustered in nature, as previously demonstrated for Kv2.1 (Trimmer, 1991; Hwang *et al*, 1993; Du *et al*, 1998). Localization of AMIGO in mouse cerebrum was overlapping with the localization of Kv2.1 in all brain areas studied.

AMIGO and Kv2.1 are both expressed endogenously by cultured primary neurons (Maletic-Savatic *et al*, 1995; Kuja-Panula *et al*, 2003). We next studied the colocalization of AMIGO and Kv2.1 in neuronal cultures (Fig 1C). The distinctive subcellular distribution of AMIGO was preserved in cultured hippocampal neurons, as reported previously for Kv2.1 (Murakoshi & Trimmer, 1999). Staining of AMIGO and Kv2.1 showed extensive colocalization in cultured neurons as well.

Additionally, when expressed together in HEK293 cells, AMIGO and Kv2.1 colocalized extensively (Fig 1D). When transfected separately, AMIGO antibody did not recognize Kv2.1, and vice versa (supplementary Fig S1 online).

Several controls were done to ensure that the impressive colocalization of Kv2.1 and AMIGO in double immulolabellings was not due to crossreactivity of secondary antibodies or leakage between channels in confocal microscopy (supplementary Fig S1 online).

AMIGO associates with Kv2.1

We next asked whether AMIGO associates with Kv2.1. We investigated the possible interaction with immunoprecipitation of mouse brain proteins with AMIGO and Kv2.1 antibodies. As shown in Fig 2A,B, Kv2.1 was detected from AMIGO-immunoprecitated mouse brain lysate and vice versa (the first lane, indicated as non-treated). The interaction was prominent in DSP-crosslinked tissue material (Fig 2), but it could be detected even without crosslinking (supplementary Fig S4 online).

As a control, we detected the immunoprecipitated samples with Kv1.2 antibody (Fig 2C).

Stimulus-induced relocalization of AMIGO and Kv2.1

Several stimuli are able to induce marked dephosphorylation and dispersion of Kv2.1 from clusters (Misonou *et al*, 2004, 2005). To study whether the interaction of AMIGO and Kv2.1 is retained after stimulus-induced changes, we immunoprecipitated AMIGO and Kv2.1 also from brain lysates of CO_2-treated mice. CO_2 treatment has been shown to induce a change in the molecular mass of Kv2.1, owing to the dephosphorylation of the channel protein (Misonou *et al*, 2005). With western blotting, we confirmed the decreased size (from 125 to 100 kDa) of Kv2.1 after CO_2 treatment (Fig 2A, total lysate). The AMIGO antibody was also able to coimmunoprecipitate the dephosphorylated, 100 kDa form of Kv2.1 (Fig 2A, lanes indicated with CO_2). Correspondingly, the Kv2.1 antibody was able to coimmunoprecipitate AMIGO from the brain lysate of CO_2-treated mice (Fig 2B).

We also studied the distribution of AMIGO in mouse brain in response to CO_2 treatment. Distribution of AMIGO and Kv2.1 was diffuse in cortical neurons of CO_2-treated mouse (Fig 2D). However, the staining of both proteins was still restricted to the soma and the proximal part of neurites. We concluded that localization of AMIGO showed a similar, marked change in response to CO_2 treatment, as was previously reported for Kv2.1. Noticeably, AMIGO and Kv2.1 colocalized in brains from CO_2-treated animals and in brains obtained without hypoxia.

The colocalization of AMIGO and Kv2.1 was also retained in cultured cortical neurons after glutamate treatment (supplementary Fig S5 online). These experiments suggest that AMIGO and Kv2.1 are associated even when Kv2.1 is dephosphorylated, and both proteins are in their diffuse localization in the neuronal membrane.

AMIGO alters voltage-dependent activation of Kv2.1

To examine the effects of AMIGO on Kv2.1 channel current, we expressed Kv2.1 either alone or in combination with AMIGO in HEK293 cells and recorded ionic currents in the whole-cell voltage-clamp configuration. As a control, we expressed Kv2.1 with the non-related membrane protein NCAM.

Empty-vector-transfected HEK293 cells did not express detectable levels of Kv2.1 (supplementary Fig S1 online) and showed no significant outward currents (Fig 3A, left panel). Transfection of cells with Kv2.1 alone or Kv2.1 with AMIGO resulted in sustained outward currents in response to depolarizing voltage steps. Current amplitudes at maximal membrane depolarization (+100 mV) were not significantly different. However, coexpression of AMIGO significantly increased the current at membrane potentials from −40 mV to +20 mV. The ratio of average currents ($I_{AMIGO+Kv2.1}$ to $I_{Kv2.1}$) at different membrane potentials is presented in Fig 3B and shows that the effect of AMIGO on Kv2.1 currents was highest at the threshold of activation. Without AMIGO, the Kv2.1 current was activated at around −20 mV ($I_{−20 mV} = 258 \pm 35$ pA, $n = 16$). When AMIGO was present, the current at −20 mV was already three times higher ($I_{−20 mV} = 796 \pm 61$ pA, $n = 14$). Transfection of NCAM with Kv2.1 did not affect the currents recorded.
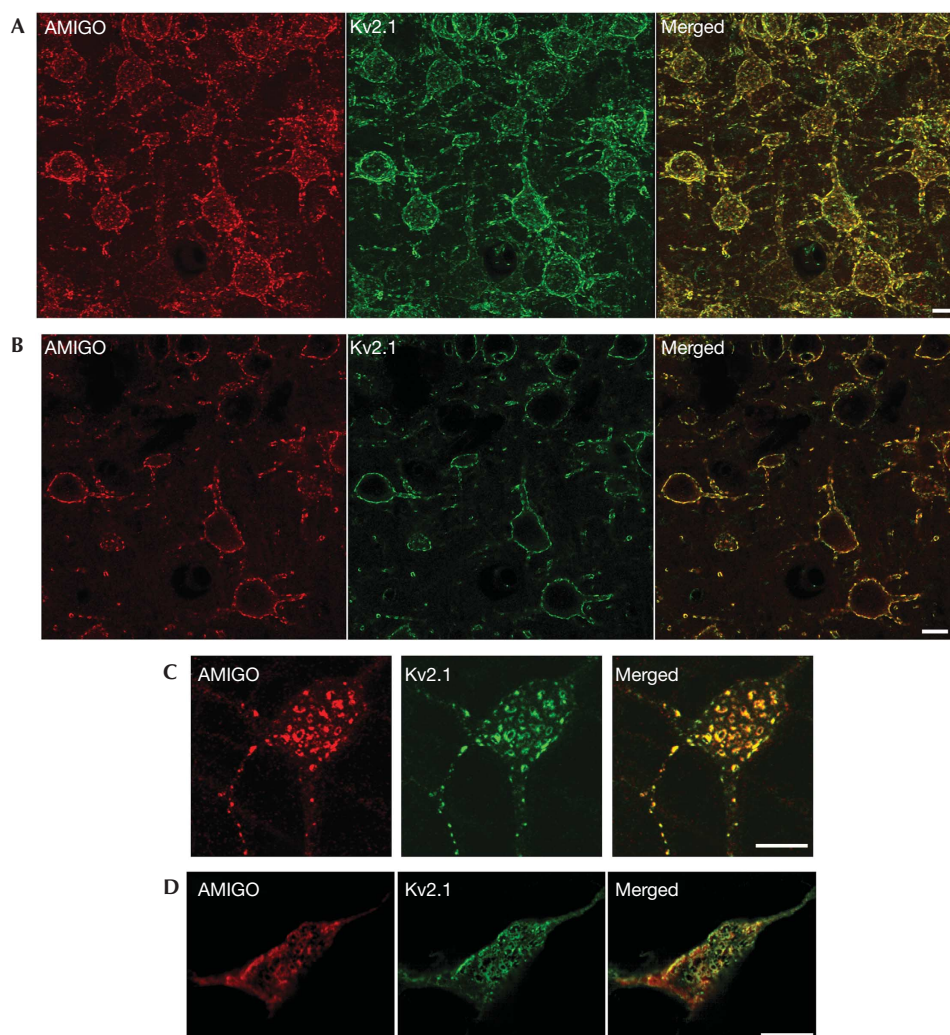
Fig 1 | Colocalization of AMIGO and Kv2.1. Samples were double labelled with AMIGO (red, left panels) and Kv2.1 K89/34 (green, middle panels) antibodies. The overlay of images is presented in the right panels (merged). (**A, B**) Colocalization of AMIGO and Kv2.1 in mouse brain. Projection of confocal stack (**A**) and a single confocal plane (**B**) from the cerebral cortex. (**C**) Colocalization of AMIGO and Kv2.1 in 24 days *in vitro* cultured hippocampal neurons. Single confocal plane from the basal surface of the cell. The projection of the confocal stack is presented in supplementary Movie S1 online. (**D**) Colocalization of AMIGO and Kv2.1 in transfected HEK293 cells. Single confocal plane from the basal surface of the cell. Scale bar, 10 μm. AMIGO, amphoterin-induced gene and ORF; Kv, voltage-dependent potassium channel.

In Fig 3D, voltage-dependent activation of Kv2.1 was studied with a tail current protocol. Normalized conductance–voltage relationships clearly demonstrated the effect of AMIGO on Kv2.1 activation. The membrane potential of half-maximal activation ($V_{1/2}$) was $+21.5 \pm 1.0$ mV ($n = 8$) for Kv2.1 only, and $+6.2 \pm 1.7$ mV ($n = 8$) for Kv2.1 with AMIGO. AMIGO thus shifted the voltage-dependent activation of Kv2.1 to more hyperpolarized potentials ($\Delta V_{1/2} = 15.3$ mV, $P = 0.00001$). Transfection of the control protein NCAM with Kv2.1 did not affect the tail currents. Essentially, the same effect of AMIGO on the Kv2.1 activation was found when the normalized conductance–voltage relationship was constructed from the sustained currents evoked by depolarization steps (Fig 3C).

Our electrophysiological recordings with AMIGO and NCAM fusion protein constructs revealed that the AMIGO transmembrane domain was critical for the modulation of Kv2.1 gating, whereas the extracellular and intracellular domains were required for the full functional effect (supplementary Fig S6 online).

AMIGO affects voltage-dependent activation of neuronal I_K

Kv2.1 is an essential component of delayed rectifier currents (I_K) in hippocampal neurons (Murakoshi & Trimmer, 1999; Du *et al*, 2000). Accordingly, guangxitoxin, an inhibitor of Kv2.1/Kv2.2 channels, clearly decreased the delayed rectifier current density in cultured hippocampal neurons (supplementary Fig S7 online). At membrane potentials ranging from -20 mV to $+60$ mV, the current density was decreased 24–37% from control. At -20 mV, the average current density was 8.93 ± 0.93 pA/pF in control cells ($n = 8$) and 5.93 ± 0.72 pA/pF in guangxitoxin-treated cells ($n = 8$, $P = 0.023$).

To elucidate the role of endogenous AMIGO in I_K, we inhibited the expression of AMIGO with inhibitory RNA (iRNA) in cultured
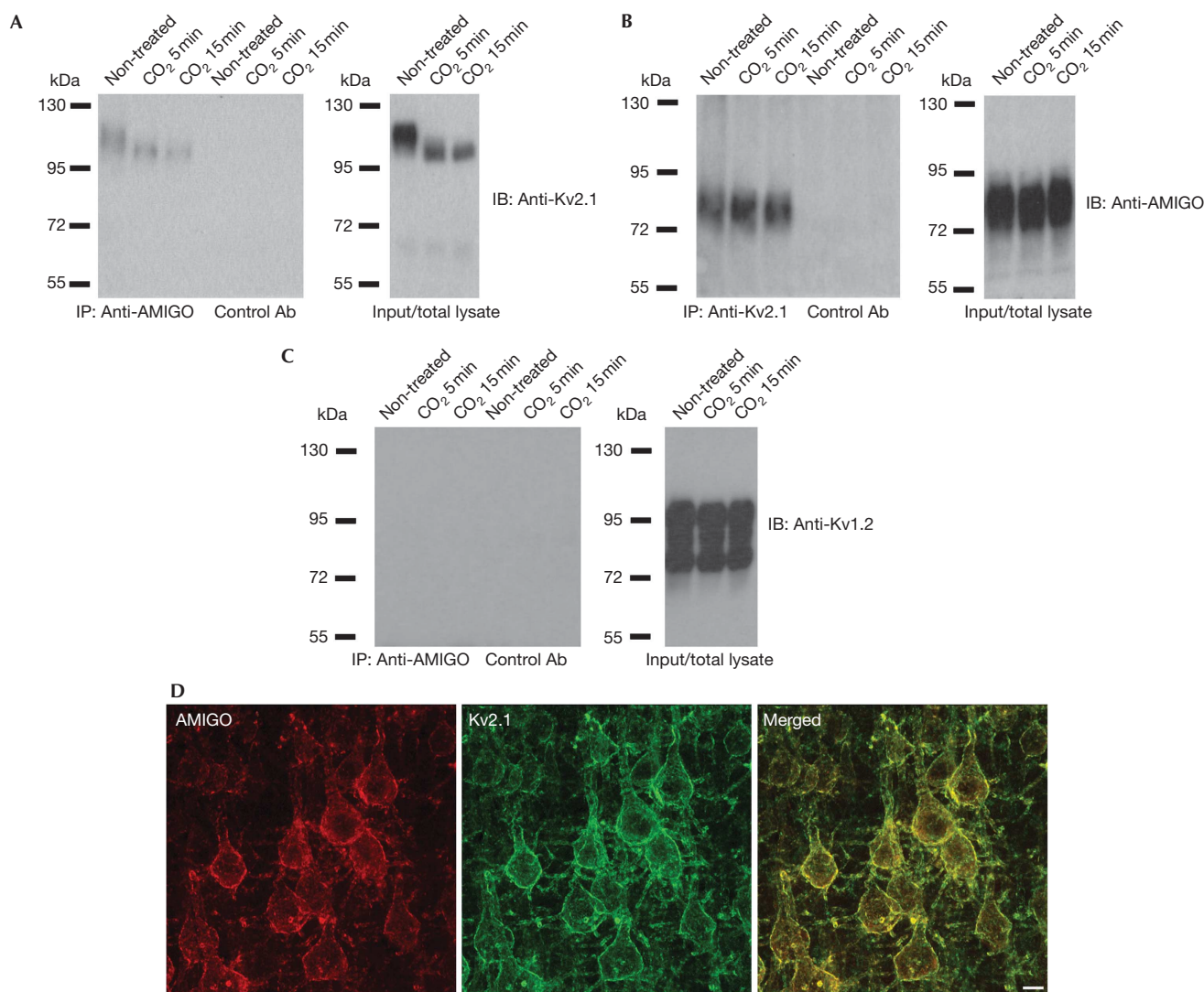
Fig 2 | Colocalization and association of AMIGO and Kv2.1 is retained during stimulus-induced dephosphorylation and dispersion of Kv2.1 in brain. Brain samples from non-treated and CO_2-treated (5 min or 15 min) mice were immunoprecipitated with anti-Kv2.1 (K39/25), anti-AMIGO or control antibodies from the same species. (**A**) AMIGO antibody coimmunoprecipitates Kv2.1 from brain samples of non-treated and CO_2-treated mice. Notice the reduced size of Kv2.1 after CO_2 treatment due to dephosphorylation. (**B**) Kv2.1 antibody coimmunoprecipitates AMIGO from brain samples of non-treated and CO_2-treated mice. (**C**) Control blot detected with Kv1.2 antibody. (**D**) Double staining with AMIGO (red) and Kv2.1 K89/34 (green) antibodies in cerebral cortex of CO_2-treated (2 min) mouse. The overlay of images is presented in the right panel. Scale bar, 10 μm. Ab, antibody; AMIGO, amphoterin-induced gene and ORF; IB, immunoblotting; IP, immunoprecipitation; Kv, voltage-dependent potassium channel.

hippocampal neurons and measured the sustained outward currents in response to depolarizing voltage steps. After iRNA treatment, a clearly reduced amount of AMIGO protein in cell lysates was confirmed with western blotting (supplementary Fig S1 online). Consistent with our data from heterologous expression system in HEK293 cells, the currents were similar in response to strong membrane depolarization, but inhibition of AMIGO significantly decreased the current at the threshold of activation. At membrane potentials ranging from -40 mV to -20 mV, the current density was decreased 39–47% from control, whereas at membrane potentials over -10 mV the current was close to control values (Fig 4A,B). At -20 mV, the average current density was 8.38 ± 1.25 pA/pF in control cells ($n = 16$) and 5.13 ± 0.67 pA/

pF in AMIGO-inhibited cells ($n = 16$, $P = 0.029$). The normalized conductance–voltage relationships (Fig 4C) clearly demonstrated that voltage-dependent activation of neuronal I_K was altered when AMIGO was inhibited.

DISCUSSION

We have identified AMIGO as an integral component of the Kv2.1 channel complex in mouse brain. Our observation that Kv2.1 is in complex with AMIGO indicates that the full understanding of the properties and function of the Kv2.1 channel requires experimental systems in which AMIGO is present. The wide presence of AMIGO and Kv2.1 in cerebral neurons indicates that the channel complex contributes to the fundamental properties of a neuron.
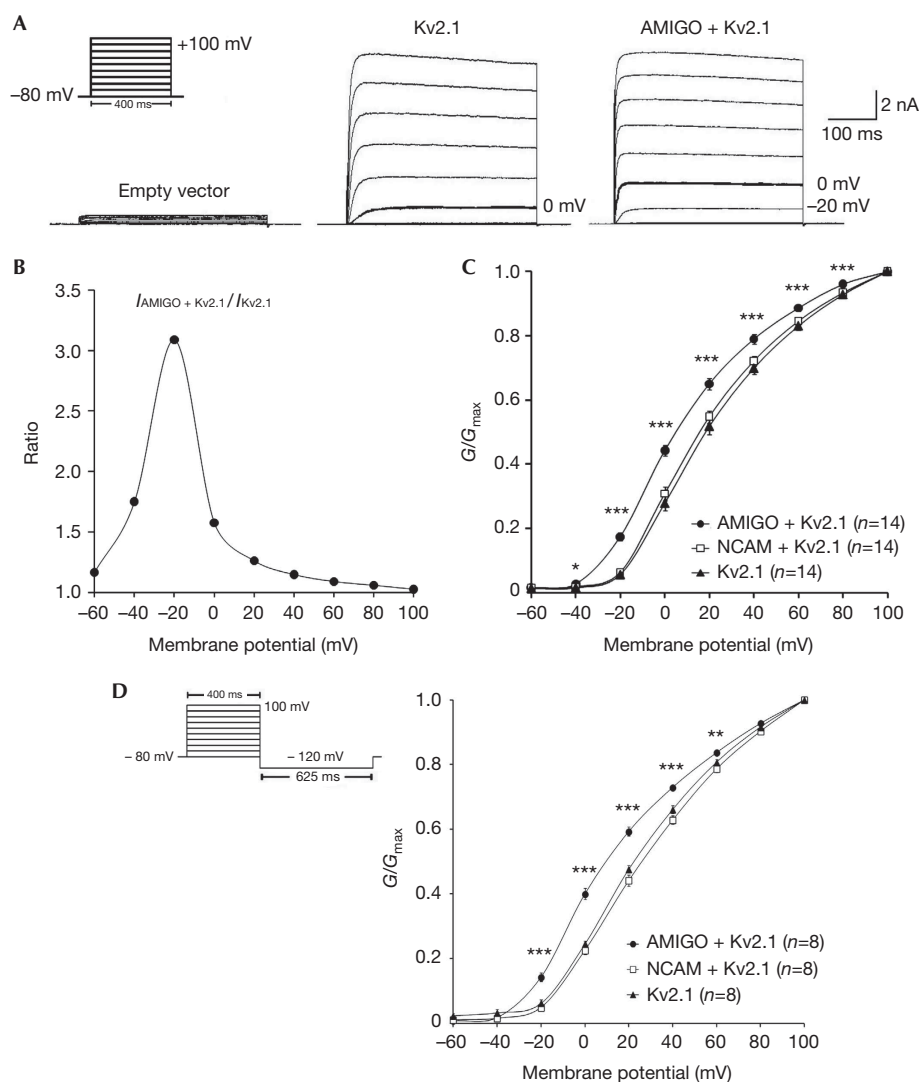
Fig 3 | AMIGO alters voltage-dependent activation of Kv2.1 in HEK cells. Currents were measured from transfected HEK293 cells. (**A**) Representative whole-cell current traces from HEK293 cells transfected with control vector, Kv2.1 or AMIGO + Kv2.1. Pulse protocol is indicated as an inset. (**B**) Ratio of sustained outward currents at different membrane potentials from cells expressing AMIGO + Kv2.1 to that from cells expressing Kv2.1 only. (**C**) Normalized conductance (G)–voltage relationship of sustained outward currents for Kv2.1 (filled triangles), AMIGO + Kv2.1 (filled circles) or NCAM + Kv2.1 (open squares). (**D**) Normalized conductance–voltage relationship of tail currents for Kv2.1, AMIGO + Kv2.1 or NCAM + Kv2.1 (symbols as for part C). Pulse protocol is indicated as an inset ($***P < 0.005$, $**P < 0.02$, $*P < 0.05$). Error bars represent s.e.m. AMIGO, amphoterin-induced gene and ORF; Kv, voltage-dependent potassium channel; NCAM, neural cell adhesion molecule.

The presence of AMIGO in Kv2.1 channel complex was demonstrated with coimmunoprecipitation and colocalization. With these experiments, we cannot exclude the possibility that the interaction between AMIGO and Kv2.1 is indirect. However, the interaction is permanent in nature as it cannot be disrupted by stimulus-induced dispersal of Kv2.1 at the cell membrane. Furthermore, AMIGO is able to modify the channel function.

Auxiliary subunits of ion channels might have several roles in the channel complex. They are shown to affect the subunit assembly, transporting, protein stability, conduction properties, localization and pharmacological properties of the channel (Li *et al*, 2006; Pongs & Schwarz, 2010). Here, AMIGO is shown to affect the voltage-dependent activation of Kv2.1. In HEK293

cells, the presence of AMIGO enhanced the Kv2.1 current most in response to modest depolarization. Correspondingly, in cultured hippocampal neurons, inhibition of AMIGO decreased the I_K current in response to modest depolarization at negative membrane potentials, around the action potential threshold. This might have fundamental effects on the integration of excitatory inputs during high-frequency transmission (Du *et al*, 2000). Damping of somatodendritic excitability at the negative membrane voltages might also be an important protective mechanism in response to ischaemia (Misonou *et al*, 2005), where association of AMIGO and Kv2.1 is preserved.

The transmembrane protein AMIGO brings a novel extracellular component to the Kv2.1 channel complex. The
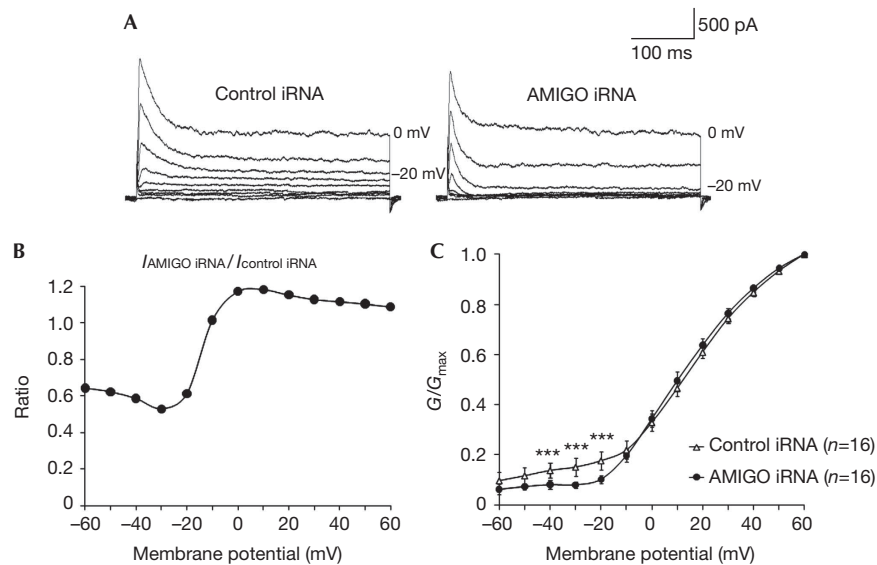
Fig 4 | Inhibition of AMIGO in cultured hippocampal neurons alters the voltage-dependent activation of delayed rectifier currents. (A) Representative whole-cell current traces from AMIGO or control iRNA inhibited neurons at depolarization steps from -70 to 0 mV. **(B)** Ratio of average currents ($I_{\text{AMIGO iRNA}}$ to $I_{\text{control iRNA}}$) at different membrane potentials. Current is expressed as a current density. **(C)** Normalized conductance (G)–voltage relationship for AMIGO-inhibited neurons (●) or control neurons (Δ) (***$P < 0.005$). Error bars represent s.e.m. Similar results were obtained in three independent experiments. AMIGO, amphoterin-induced gene and ORF.

extracellular part of AMIGO contains LRR and Ig domains and provides a cell adhesion motif (Kuja-Panula et al, 2003). Cell adhesion molecules are well known as ion channel auxiliary subunits for sodium channels. β-Subunits of sodium channels are multifunctional. They serve both as modulators of channel gating and as cell adhesion molecules, affecting cell–cell interaction and binding to the extracellular matrix and cytoskeletal proteins (Brackenbury et al, 2008). It is attractive to speculate whether AMIGO contributes to interaction of Kv2.1 with extra- or intracellular structures associated with Kv2.1 clusters.

Potassium channels are potential therapeutic targets for several diseases concerning neuronal excitability, such as epilepsy, stroke and psychiatric disorders (Wickenden, 2002). AMIGO as a new auxiliary subunit provides possibilities to modulate the Kv2.1 channel function and serves as a potential new drug target.

METHODS

Antibodies. The chicken polyclonal antibody to AMIGO was produced against the cytoplasmic tail of mouse AMIGO (residues 394–492, Agrisera, Sweden). The antibody was purified from egg yolk by ammonium sulphate precipitation and affinity purification with recombinant cytoplasmic AMIGO. The mouse monoclonal antibodies to Kv2.1 (K89/34 and K39/25), Kv1.2 (K14/16) and PSD-95 (K28/43) were obtained from the UC Davis/NIH NeuroMab Facility. The rabbit polyclonal antibody to Kv2.1 was obtained from Sigma-Aldrich (P9607).

Immunohistochemistry. C57BL6 mouse brains were obtained by pentobarbital anaesthesia or by pentobarbital anaesthesia and CO_2 treatment for 2 min. Brains were fixed by 4% paraformaldehyde perfusion and immersion fixation overnight (4% paraformaldehyde). For immunofluorescent staining, brains were cryoprotected (30% sucrose), frozen and cut into 40-μm floating sections.

Cryosections were blocked and permeabilized with 2% BSA and 0.5% Triton X-100 in PBS. Primary antibodies were incubated simultaneously in 2% BSA and 0.1% Triton X-100 in PBS overnight at 4 °C and detected with Alexa Fluor 546/555-conjugated goat anti-chicken and Alexa Fluor 488-conjugated goat/donkey anti-mouse antibodies (2 μg ml^{-1}, Invitrogen).

Primary neurons. Primary hippocampal and cortical neurons were prepared from E17 rat embryos. Cells were cultured in neurobasal medium (Gibco) with B27 supplement (Gibco) and penicillin–streptomycin and used at 14–24 days in vitro (DIV) for treatments and/or immunocytochemistry.

Coimmunoprecipitation experiment. Mice were subjected to CO_2 inhalation for 2 min and decapitated heads were retained at room temperature for 5 or 15 min. For non-treated samples, mice were decapitated without CO_2 treatment and the brains were dissected directly. Crosslinking was carried out with 0, 1 or 3 mM DSP (Pierce) in 20 mM HEPES, pH 7.4, 0.15 M $NaCl_2$, Complete protease inhibitor cocktail (Roche) and Phosstop cocktail (Roche). Brains were homogenized with rotor–stator homogenizer and left shaking at $+4$ °C for 2 h. Excess of crosslinker was blocked with 50 mM glycine at $+4$ °C for 30 min. Homogenates were lysed by adding 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate and left shaking at $+4$ °C for 3 h. Lysates were centrifuged twice at 10,000 g for 15 min. Immunoprecipitation (0.5 ml input lysate) was performed with 6 μg chicken anti-AMIGO, using 6 μg non-related chicken IgY as a control, or with 4 μg mouse anti-Kv2.1 (K39/25), using 4 μg non-related mouse IgG as a control. IgY was pulled down with chicken IgY precipitation agarose (Millipore) and mouse IgG with protein-G agarose (Upstate). After several washes (20 mM HEPES, pH 7.4, 0.15 M $NaCl_2$, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate), immunoprecipitated proteins were eluted by boiling in Laemmli buffer and were subjected to western blotting.

Cell culture, transient transfection. HEK293 cells (American Type Culture Collection) were transiently transfected with recombinant complementary DNAs inserted into pLEN–CMV plasmids using Fugene HD transfection reagent (Roche). The cDNAs used for immunocytochemistry were wild-type non-tagged mouse Kv2.1 and wild-type non-tagged mouse AMIGO. The cDNAs used for electrophysiology were wild-type N-terminally EGFP-tagged mouse Kv2.1 and wild-type non-tagged mouse AMIGO. All cells were used within 48–72 h after transfection.

Electrophysiology. Currents were recorded in the whole-cell voltage-clamp configuration from transfected HEK293 cells and cultured hippocampal neurons. The extracellular solution for HEK293 cells contained (in mM) 160 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose and 10 HEPES, pH 7.3. In tail current recordings (Fig 3D), the extracellular solution contained (in mM) 125 NaCl, 40 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose and 10 HEPES, pH 7.3. The pipette solution contained (in mM) 160 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 EGTA, 10 glucose and 10 HEPES, pH 7.3. *In vitro* hippocampal neurons (16 DIV) were treated with lentiviruses (600,000 transforming units per 40,000 cells) expressing AMIGO short hairpin RNA or with control virus expressing scrambled short hairpin RNA (Supplementary information online). The extracellular solution for hippocampal neurons contained (in mM) 124 NaCl, 4.8 KCl, 1.9 $CaCl_2$, 1.9 $MgCl_2$, 9.6 glucose, 9.6 HEPES, pH 7.3 and 1 μM tetrodotoxin. The pipette solution contained (in mM) 123 KCl, 1.9 $MgCl_2$, 0.9 $CaCl_2$, 4.7 EGTA, 9.3 glucose and 9.3 HEPES, pH 7.3. The cells were held at −80 mV and step depolarized from −80 mV for 400 ms with depolarizing 20 mV increments for HEK293 and 10 mV increments for hippocampal neurons until +100 mV. In tail current recordings, tail voltage was −120 mV. The interpulse interval was 5 s. The sustained current (I) was determined as the mean current amplitude between 310 and 360 ms. Tail current amplitude was measured 1 ms after the depolarization step.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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