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The role of Kif5B in axonal localization of Kv1 K^+ channels

Jacqueline Rivera,* Po-Ju Chu,*,† Tommy L. Lewis Jr and Don B. Arnold
University of Southern California, Department of Biological Sciences, Division of Molecular and Computational Biology, and Program in Neuroscience, 1050 Childs Way MCB 204, Los Angeles, CA 90089–2910, USA

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Abstract

Here we present evidence that the kinesin, Kif5B, is involved in the transportation and axonal targeting of Kv1 channels. We show that a dominant negative variant of Kif5B specifically blocks localization to the axon of expressed, tagged versions of Kv1.3 in cultured cortical slices. In addition, the dominant negative variant of Kif5B blocks axonal localization of endogenous Kv1.1, Kv1.2, and Kv1.4 in cortical neurons in dissociated cultures. We also found evidence that Kif5B interacts with Kv1 channels. Endogenous Kv1.2 colocalized with Kif5B in cortical neurons and coimmunoprecipitated with Kif5B from brain lysate. The T1 domain of Shaker K^+ channels has been shown to play a critical role in targeting the channel to the axon. We have three pieces of evidence to suggest that the T1 domain also mediates interaction between Kv1 channels and Kif5B: Addition of the T1 domain to a heterologous protein, TfR, is sufficient to cause the resulting fusion protein, TfRT1, to colocalize with Kif5B. Also, the T1 domain is necessary for interaction of Kv1.3 with Kif5B in a coimmunoprecipitation assay. Finally, dominant negative variants of Kif5B block axonal targeting of TfRT1, but have no effect on dendritic localization of TfR. Together these data suggest a model where Kif5B interacts with Kv1 channels either directly or indirectly via the T1 domain, causing the channels to be transported to axons.

Introduction

Voltage-gated K^+ channels of the Kv1 family are localized in axons, where they modulate action potential propagation and neurotransmitter release (Sheng *et al*., 1992; Wang *et al*., 1994; Veh *et al*., 1995; Debanne *et al*., 1997). Disruption of the function of these channels leads to increases in axonal excitability, which can cause ataxia (Browne *et al*., 1994) and seizures (Smart *et al*., 1998). Recent work has shown that targeting of Kv1 channels to the surface of the axon is dependent on signals within the T1 tetramerization domain (Gu *et al*., 2003; Rivera *et al*., 2005). The T1 domain is also capable of causing transport vesicles to traffic to the axon (Rivera *et al*., 2005), which suggests that motor proteins may play a role in targeting mediated by this domain.

Although specific motor proteins have yet to be implicated in the transport of axonal ion channels, kinesins have been linked to transport of three dendritic channels: Kif5A–C have been associated with transport of the AMPA receptor GluR2 and Kif17 has been associated with transport of both the NMDA receptor, NR2B, and the K^+ channel, Kv4.2 (Setou *et al*., 2000; Setou *et al*., 2002; Guillaud *et al*., 2003; Chu *et al*., 2006). Kif5B is also capable of transporting axonal proteins, including the nonchannel transmembrane proteins, ApoER2 and APP (Kamal *et al*., 2000; Kamal *et al*., 2001; Verhey *et al*., 2001). The decision of Kif5A–C to go to the axon or the dendrite appears to be influenced by the adaptor protein with which they interact. Association of Kif5A–C with the PDZ domain protein, GRIP, results in dendritic localization of the complex, whereas interaction of Kif5A–C with JSAP1 causes that complex to localize to axons (Setou *et al*., 2002).

In this paper we explore the role of Kif5B in transport and axonal targeting of Kv1 channels. Our results indicate that Kif5B is necessary for localization of Kv1 channels to axons in mammalian neurons. A dominant negative variant of Kif5B, but not one of Kif17, prevents axonal localization of both endogenous and expressed Shaker K^+ channels. In addition, Kv1.2 and Kif5B likely interact. They colocalize in dissociated neurons in culture and coimmunoprecipitate from brain lysate. Results from coimmunoprecipitation experiments in COS cells and colocalization experiments in cultured neurons indicate that this interaction is likely mediated by the T1 domain. Finally, dominant negative experiments indicate that Kif5B plays an essential role in axonal targeting of heterologous proteins mediated by the T1 domain. Together, these results suggest a model where the T1 domain mediates axonal targeting, at least in part, by causing the Kv1 channels to associate with Kif5B.

Materials and methods

Preparation of dissociated cell cultures

Briefly, rats pregnant with E18 embryos were asphyxiated with CO_2. Embryos were removed and their cortices were dissected in Hank's Balanced Salt Solution (Invitrogen). Cortices were dissociated by incubating in papain enzyme solution [100 mM $CaCl_2$, 50 mM EDTA, 0.1% β-mercaptoethanol (ICN), 100 U papain (Sigma) in EBSS, final pH 7.4] for 30 min. Dissociated cortical neurons were plated on polylysine-coated glass coverslips at a density of 1×10^5 neurons per well in neurobasal media (Invitrogen) supplemented with 10 mL/L glutamax (Invitrogen), 1 μg/mL gentamicin (Invitrogen), 20 mL/L B-27 supplement (Invitrogen) and 50 mL/L fetal bovine serum (Invitrogen). The media was changed after one hour to neurobasal

Correspondence: Dr Don B. Arnold, as above.
E-mail: darnold@usc.edu

*J.R. and P.-J.C. contributed equally to this work.

†*Present Address*: Department of Life Sciences, Chang Gung University, Kwei-Shan, Taoyuan, Taiwan, Republic of China.

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media without serum and every four days thereafter. Dissociated cell cultures at 14–16 DIV and COS-7 cells were transfected with DNA constructs using either the Calphos transfection system (BD biosciences) or Effectene transfection reagent (Qiagen), respectively, using procedures suggested by the respective manufacturers.

Immunocytochemisty of dissociated neuronal cultures

The cells were fixed with 4% paraformaldehyde for 5 min and washed with PBS. This was followed by a permeabilization step and a blocking step with blocking solution (1% bovine serum albumin, 5% normal goat serum, 0.1% Triton X-100 in PBS). After blocking, primary antibody was diluted in blocking solution and added for 30–120 min. Secondary antibody was diluted in blocking solution and added for 30 min in the dark. Primary antibody dilutions were as follows; rabbit anti-GFP 1 : 2000 (BD Biosciences), mouse anti-MYC 1 : 1000 (Covance), mouse anti-HA 1 : 1000 (Covance), rabbit anti-Kv1.1 1 : 2000 (Alomone Laboratories), rabbit anti-Kv1.2 (Alomone Laboratories), rabbit anti-Kv1.4 (Alomone Laboratories), mouse anti-Kif5B 1 : 200 (Sigma), mouse anti-Tau (Chemicon) and mouse anti-Synaptophysin (Sigma). Antibody labelling was then visualized by incubating cells with Alexa 488, Alexa 594, and Alexa 647-conjugated secondary antibodies (Molecular Probes).

Slice immunocytochemistry

Culturing of slices was described previously (Arnold & Clapham, 1999). Following incubation for four hours the slices were transfected using the Helios gene gun (Biorad, Richmond, California). Slices were then stained for total protein after incubation for 3 days. Slices were fixed with 2.5% paraformaldehyde and 4% sucrose for 30 min, incubated in blocking solution (2% bovine serum albumin, 10% normal goat serum, 0.25% Triton X-100 in PBS) for one hour followed by incubation with primary antibody for one hour. Primary antibodies used were as follows; rabbit anti-GFP 1 : 2000 (BD Biosciences), chicken anti-βGal 1 : 1000 (ICL), and mouse anti-HA 1 : 500 (Covance). They were then incubated in secondary antibody for one hour. Antibody labelling was visualized by Alexa 594, Alexa 488, and Alexa 647-conjugated secondary antibodies (Molecular Probes). Slices were then cleared with xylene and mounted.

Induction of expression

Constructs driven by the pIND promoter (Invitrogen) were cotransfected into neurons in cortical slices with the pVgRXR vector and incubated for 24 h. They were subsequently induced using ponasterone A (Invitrogen), which was added to a final concentration of 5 μM in the slice culture medium or the medium of the dissociated cultures. Immunocytochemisty was performed after an additional 48 h of incubation.

Coimmunoprecipitation and Western blotting

COS cells were cultured in 60-mm dishes, grown to approximately 80% confluent and transfected with Effectene transfection reagent (Qiagen) using 0.5 μg of each expression plasmid according to the manufacturer's protocol. Cells were then washed twice with PBS 48 h after transfection and lysed in ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% (v/v) NP-40) with protease inhibitors (0.12 mg/mL PMSF, 2 μg/mL leupeptin, 1 μg/mL aprotinin and 1 μg/mL pepstatin A). The following steps were carried out at 4 °C unless otherwise noted. After cell lysates were incubated for 1 h, insoluble material was removed by centrifugation at 16 000 × *g* for 15 min, and the supernatants normalized for protein content were precleared with protein A-agarose (ImmunoPure immobilized protein A, Pierce) for 1 h. Rabbit anti-GFP (BD Biosciences) or normal rabbit IgG (BD Biosciences) were added to precleared supernatants and incubated with inversion overnight. Protein A-agarose was added to the immune complexes and the mixtures were further incubated with inversion for 3 h followed by centrifugation at 9000 × *g* for 5 min. The precipitates were washed three times with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.1% (v/v) NP-40 and eluted in SDS sample buffer [125 mM Tris-HCl, pH 6.8, 80 mM EDTA, 4% SDS, 30% (v/v) glycerol, and 0.1 mg/mL bromophenol blue]. For immunoprecipitation of endogenous Kv1.2 from brain lysate, the preparation of postnuclear supernatant (PNS) and immunoprecipitation reaction were performed as described previously (Cavalli *et al*., 2005) with modification. Briefly, brains from postnatal 14-day-old rats were homogenized in ice-cold homogenization buffer (8% sucrose, 20 mM beta-glycerophosphate, 3 mM imidazole, pH 7.4) with protease inhibitors. The brain PNS was collected by removing pellets under centrifugation of 1000 × *g* for 10 min. The PNS was further diluted in lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 20 mM Tris-HCl, pH 7.5, with protease inhibitors) and incubated at 4 °C for 1 h. Extracts were then clarified twice by centrifugation at 15 000 × *g* for 5 min to remove insoluble material. After preclearance with protein A-agarose for 1 h, the resulting brain lysate was incubated with rabbit Kv1.2 polyclonal antibody (Alomone Laboratories), anti-GFP (BD Biosciences) or normal rabbit IgG with inversion overnight. Protein A-agarose was added to the immunocomplexes and the mixtures were further incubated with inversion for 3 h followed by centrifugation at 9000 × *g* for 5 min. The precipitates were washed five times with PBS and eluted in SDS sample buffer.

For Western blotting, protein samples were resolved by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad). The resulting blots were blocked for 1 h in Tris-buffered saline with Tween-20 [TBST; 500 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween-20] containing 5% nonfat dry milk. The blots were incubated with specific primary antibodies; rabbit anti-Kif5B (1 : 5000, gift from R. Vale), or mouse anti-HA (1 : 1000, Covance) at 4 °C for overnight, washed three times in TBST, and followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies; goat anti-mouse (1 : 10 000, Jackson ImmunoResearch) or donkey anti-rabbit (1 : 5000, Amersham Biosciences) at room temperature. After washing three times in TBST, the blots were visualized using Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences) followed by exposure to BioMax Light films (Kodak).

Colocalization of proteins

Colocalization was indicated with the help of a colocalization plugin for ImageJ. This program considered a pixel to be colocalized if the ratio of the less intense signal was > 50% of the stronger signal and both signals were over a threshold of 50. Colocalized pixels were marked in white.

DNA constructions

All constructs used were in the mammalian expression vector, GW, a CMV promoter-based mammalian expression vector. MYC-Kv1.3 was

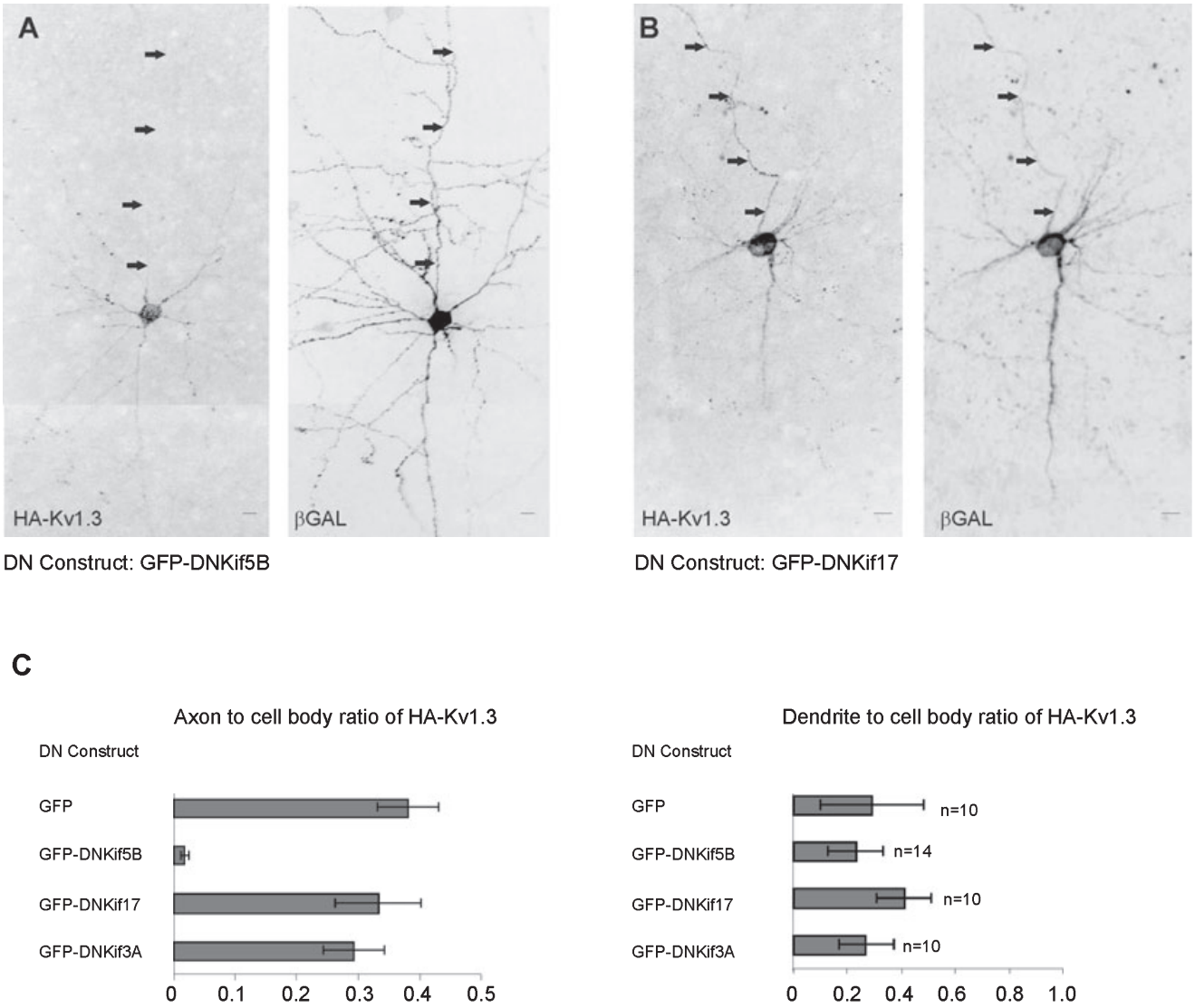
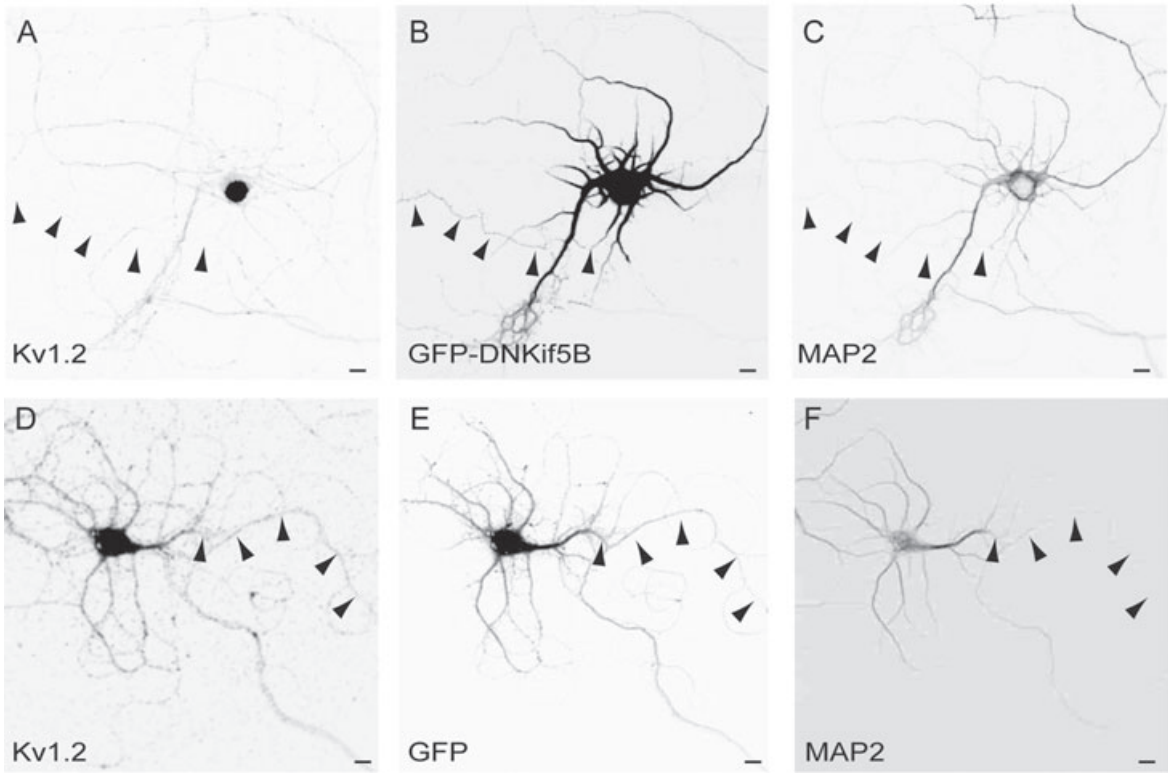


FIG. 1. A dominant negative variant of Kif5B, but not one of Kif17, blocks axonal localization of introduced, tagged Kv1.3. (A) A pyramidal cell from rat brain cortex transfected with HA-Kv1.3 driven by an inducible promoter and a dominant negative variant of Kif5B (GFP-DNKif5B) and β-galactosidase (βGAL) driven by constitutive promoters. For 24 h GFP-DNKif5B and βGAL alone were expressed, after which time expression of HA-Kv1.3 was induced. Subsequently the slice was incubated for an additional 48 h with all three proteins expressing. Comparison of HA-Kv1.3 staining with that of βGAL reveals that very little of the channel was present in the axon (indicated by arrows), although the channel was present in the soma and dendrites. Note that all immunostaining was carried out using permeabilization. (B) Cortical pyramidal cell expressing HA-Kv1.3, GFP-DNKif17, and βGAL in an experiment similar to that of A. HA-Kv1.3 staining was prominent in the axon and the soma and less prominent in the dendrites. Note that the amount of βGAL staining varied from neuron to neuron, but did not correlate with the localization of HA-Kv1.3. (C) In order to quantify the extent to which HA-Kv1.3 localized to axons in the presence of different dominant negative kinesin constructs we measured the ratio of the expression level of the channel in the axon vs. the cell body (ACR). The ACR of HA-Kv1.3 when coexpressed with GFP-DNKif5B (0.02 ± 0.007, $n = 14$) was significantly lower ($P < 0.001$) than that of the channel when coexpressed with either GFP (0.38 ± 0.05, $n = 10$), GFP-DNKif17 (0.33 ± 0.07, $n = 10$), or GFP-DNKif3A (0.29 ± 0.05, $n = 10$), indicating that GFP-DNKif5B blocked localization of the channel to the axon. In contrast, the dendrite to cell body ratio (DCR) of HA-Kv1.3 (0.2 ± 0.1) was not significantly different ($P > 0.1$) from that of the channel when expressed with either GFP (0.3 ± 0.2), GFP-DNKif3A (0.3 ± 0.1) or GFP-DNKif17 (0.4 ± 0.1). Error bars represent standard error of the mean. Scale bars, 10 μm.

generated by inserting a double MYC epitope tag (EQKLISEEDL) encoded by two double stranded oligos after amino acid 315 in Kv1.3. For HA-Kv1.3, the MYC tag was replaced with a double HA epitope tag (YPYDVPDYASL) encoded by two double-stranded oligos. Additionally, Asn237 and Asn238 were mutated to Gly and Ser, respectively, to remove glycosylation sites in order to improve antibody recognition of the HA tags. In addition to the two mutated glycosylation sites, the pore mutation W386F, which has been shown to eliminate currents in the Shaker K^+ channel (Perozo *et al.*, 1993), was introduced to HA-Kv1.3. To generate the dominant negative Kif5B construct GFP-DNKif5B, we replaced the motor domain, amino acids 2–366, with GFP. YFP-Kif5B was made by adding YFP to the N-terminus of the wild-type kinesin. For HA-Kif5B, the YFP molecule was replaced with a double HA-epitope tag. To generate GFP-Kv1.3, full-length Kv1.3 was tagged with GFP at the N-terminus. GFP-Kv1.3ΔTI was created from GFP-Kv1.3 by deleting amino acids 2–158 of Kv1.3. TfR-HA and TfRT1-HA were generated from TfR-GFP and TfRT1-GFP (8) by replacing GFP with a double HA tag.



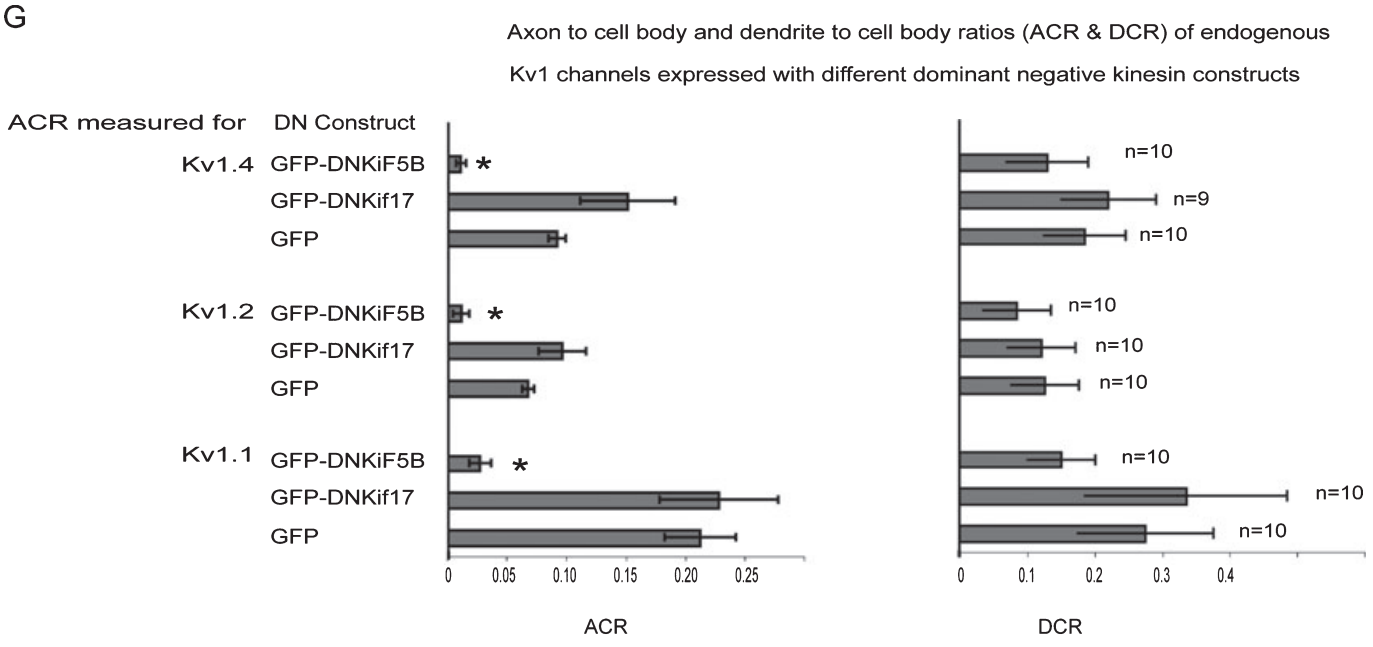


FIG. 2. A dominant negative variant of Kif5B blocks axonal localization of endogenous Kv1.1, Kv1.2 and Kv1.4. (A–C) A cortical neuron in dissociated culture transfected with GFP-DNKif5B and, after 48 h, fixed and stained with anti-Kv1.2 (A), anti-GFP (B) and anti-MAP2 (C). The axon (arrowheads) was identified as being GFP-DNKif5B-positive and MAP-2-negative. GFP-DNKif5B causes endogenous Kv1.2 to be absent from the axon (black arrowheads). Note that processes labelled in A that are not found in B or C are likely axonal projections from neurons with somata out of the field of view. (D–F) Neurons transfected with GFP expressed endogenous Kv1.2 throughout the cell, including the axon. Scale bars, 10 μm. (G) Axon to cell body ratio (ACR) measured for Kv1.1, Kv1.2, and Kv1.4 in cells expressing either GFP, GFP-DNKif5B or GFP-DNKif17. The ACR for each channel was considerably lower in cells expressing GFP-DNKif5B than in cells expressing either GFP or GFP-DNKif17. In contrast, the DCR for each channel is not significantly different in cells expressing GFP-DNKif5B than in cells expressing either GFP or GFP-DNKif17. Prior to staining, cells were permeabilized. Error bars represent standard error of the mean. Scale bars, 10 μm. *indicates that a value is significantly different ($P < 0.01$) from the adjacent value(s).

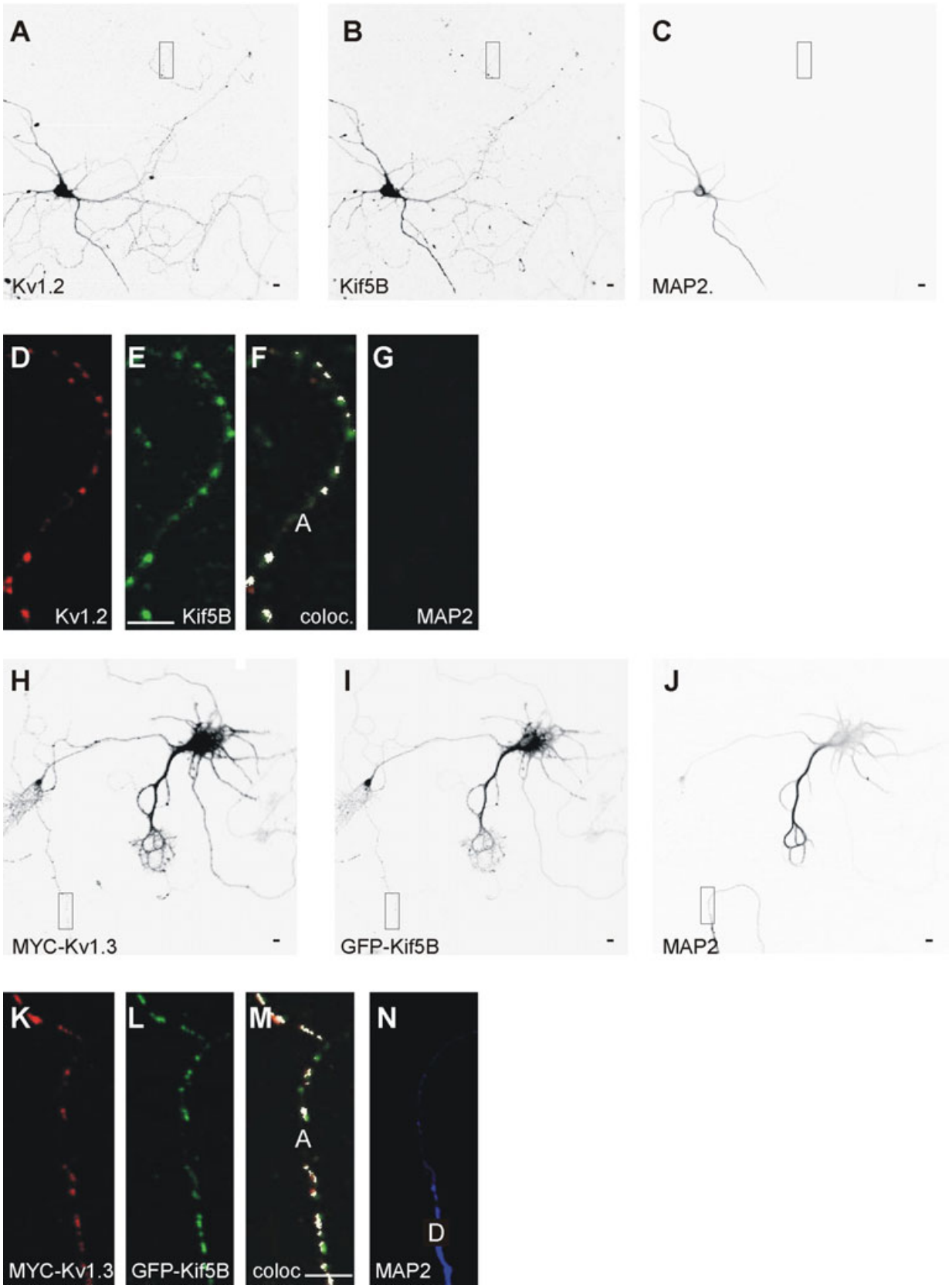


FIG. 3. Kif5B colocalizes with Kv1.2 and Kv1.3. (A–G) Cortical neuron in dissociated culture stained for endogenous Kv1.2 and endogenous Kif5B. (D–G) High power images of regions shown inside boxes in (A–C), respectively. White regions in (F) indicate areas of colocalization of Kv1.2 with Kif5B. (H–J) Introduced MYC-Kv1.3 colocalizes with introduced YFP-Kif5B as indicated by white regions in (M). (K–N) High power images of regions shown inside boxes in H–J. The axon is labelled with A and is MAP2 negative (F and M), whereas the dendrite is marked with D and is MAP2 positive (N). Scale bars, 5 μm.

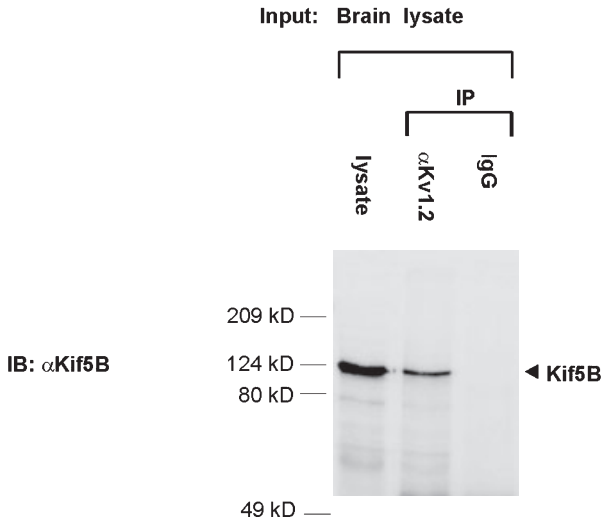


FIG. 4. Kv1 channels coimmunoprecipitate with Kif5B from brain lysate. Proteins from rat brain lysate were immunoprecipitated either with anti-Kv1.2 or with control IgG and the coimmunoprecipitated proteins were labelled on a Western blot with anti-Kif5B. The presence of a band corresponding to Kif5B in the immunoprecipitation lane, but not in the control lane indicates that Kv1.2 coimmunoprecipitates with Kif5B.

Image capture and analysis

All imaging was carried out on a Biorad MRC-1024 confocal microscope. Each cell was imaged as a stack of optical sections, 1 μm in depth apart. All calculations were performed on compressed images. For each cell, images of expressed constructs were taken using the 488, 568 and 647 nm laser lines at the same settings. Each construct was imaged in cells that were taken from at least three different slices.

For neurons in a slice, the axon was identified as a single process that (i) projected in a direction opposite to that of the cortical surface and the apical dendrite and (ii) was clearly longer than any dendrite (Arnold & Clapham, 1999). To quantify the degree of localization of Kv1 channels to axons, the axon to cell body ratio (ACR) was measured. The ratio of the expression level of Kv1 in the axon vs. the cell body was calculated as follows. The average magnitude/-pixel of fluorescence associated with Kv1 staining was measured in the cell body and at 25 μm from the cell body on the axon for cells in both dissociated culture and in slices. For the axonal measurement, a line approximately 5 μm long (centred at 25 μm from the cell body) was drawn parallel to and overlaying the axon and the average magnitude/pixel along that line was measured. In order to measure the dendrite to cell body ratio (DCR) we measured the average magnitude/pixel of fluorescence at 25 μm from the cell body on the apical dendrite in neurons in slices, and on the three most prominent dendrites on neurons in dissociated culture and compared the respective measurements to the magnitude/pixel of the cell body. To quantify the degree of polarization in the distribution of a particular protein we calculated the axon to dendrite ratio (ADR). ADR was defined as the ratio of the amount of protein in the axon vs. that in the dendrites normalized by the ratio of the volume of the axon vs. that of the dendrite as determined by the ratio of total anti-GFP staining in each compartment (Rivera *et al*., 2003). This normalization was used because the axonal compartments tended to be much smaller than the dendritic compartments and could vary greatly in size. The background fluorescence was subtracted from each measurement. All measurements were taken with ImageJ. Only expression levels of proteins detected by the same antibodies were compared.

Results

Dominant negative variants of Kif5B block localization of Kv1 channels

To understand better the molecular mechanisms that underlie targeting of Kv1 channels to axons, we sought to identify the kinesin isoform responsible for transporting these channels. Approximately 45 kinesin isoforms are known to be present in rats, mice and humans (Miki *et al*., 2005). Experiments performed in squid suggest that of these Kif5B might be the most likely to transport Kv1 channels. It was found that the homologue of Kif5B copurified from squid axoplasm with 50 nm vesicles containing a K^+ channel homologous to mammalian Kv1 channels (Clay & Kuzirian, 2002). Because this connection between the kinesin and the channel might have been conserved throughout evolution, we tested whether Kif5B is involved in transport of Kv1 channels in mammalian neurons.

To test whether Kif5B plays a role in transport of Kv1 channels in mammalian neurons we asked whether a GFP-tagged, dominant negative variant of the kinesin, GFP-DNKif5B, blocked localization of introduced, tagged Kv1.3 (HA-Kv1.3) in pyramidal cells in slices of rat cortex. Note that we used the slice culture preparation because it represents a closer approximation to *in vivo* than dissociated cultures. GFP-DNKif5B consists of wild-type Kif5B with the motor domain deleted and replaced with GFP. A similar mutant has been shown to block localization of the AMPA receptor GluR2 (Setou *et al*., 2002). Because this molecule blocks the function of Kif5B by forming inactive heterodimers with endogenous Kif5B molecules, it must be expressed at a level sufficient to bind to the majority of endogenous Kif5B in order for function of the kinesin to be disrupted (Chu *et al*., 2006). For this reason, we first expressed GFP-DNKif5B under the control of a constitutive promoter in neurons for 24 h, and subsequently induced expression of HA-Kv1.3 in the same cells for 48 h using an ecdysone-sensitive inducible promoter. Following expression of GFP-DNKif5B, Kv1.3 was largely absent from the axon, consistent with GFP-DNKif5B blocking transport of the channel in the axon, and in dramatic contrast to the distribution of the channel following expression of either GFP (data not shown) or GFP-DNKif17 (Fig. 1A and B). Note that in each case the axon was identified as being (i) considerably longer than dendrite and (ii) projecting in a direction directly opposite to that of the apical dendrite (Arnold & Clapham, 1999). Note also that staining was performed using a permeabilization step so that total HA-Kv1.3 is stained.

We further assessed whether transport to axons had been blocked by calculating the ratio of the expression level of the induced channel at a point 25 μm into the axon with the expression level of the same channel in the cell body, a value that we termed the axon to cell body ratio or ACR. Note that because this value is normalized by the amount of channel expressed in the cell body it is relatively independent of the expression level. We found that the ACR of HA-Kv1.3 following expression of GFP-DNKif5B was 0.02 ± 0.007 ($n = 14$; Fig. 1C). This value is significantly lower than those obtained for the same ratios following expression of either GFP-DNKif17 (0.33 ± 0.07, $n = 10$), GFP-DNKif3A (0.29 ± 0.05, $n = 10$) or GFP alone (0.38 ± 0.05, $n = 10$; Fig. 1C) indicating that significantly less Kv1.3 was present in the axon in the first case than in the latter three ($P < 0.001$ for all three comparisons). To assess the effect of GFP-DNKif5B on dendritic localization of HA-Kv1.3, we calculated a dendrite to cell body ratio (DCR), in which we measured

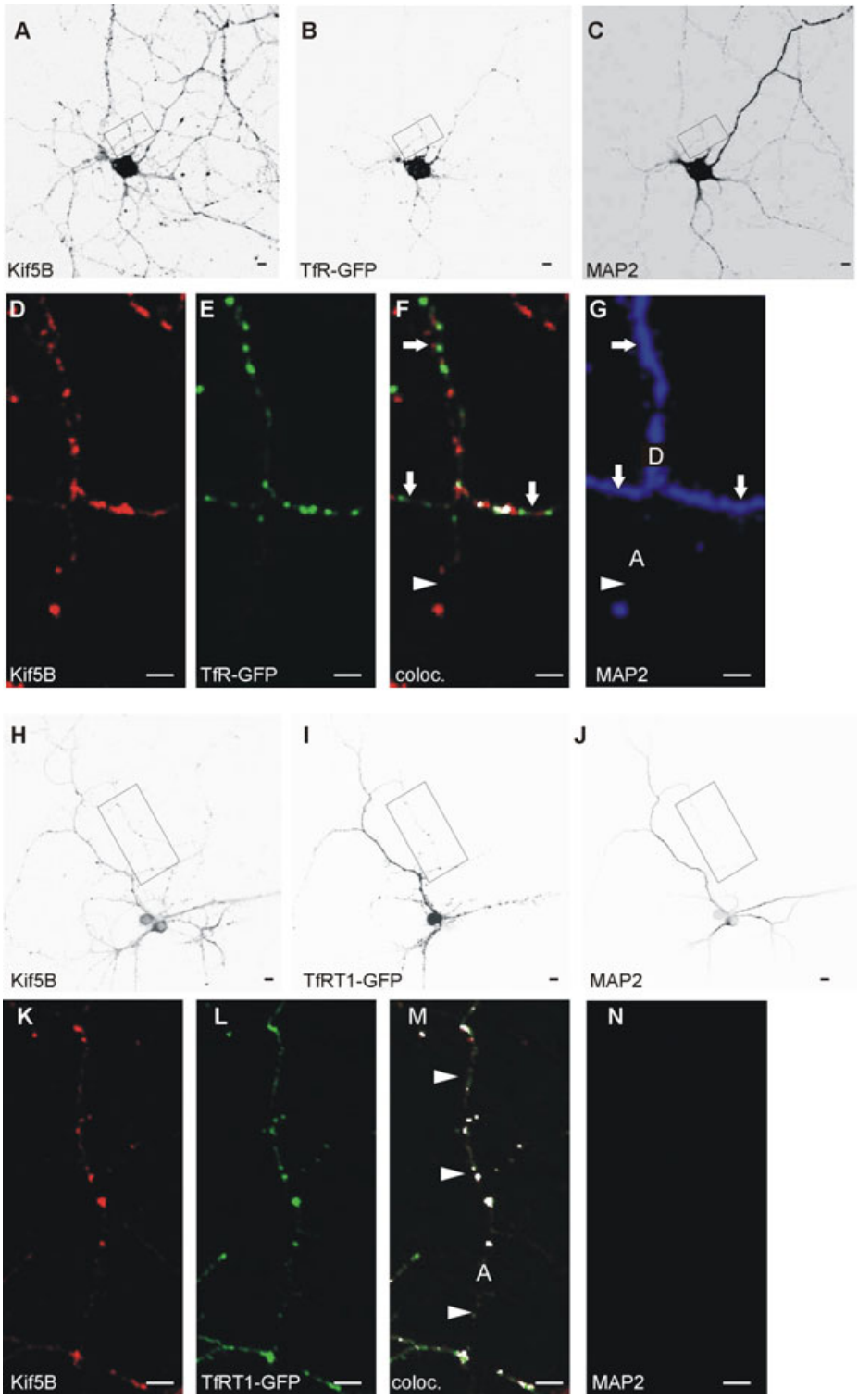


FIG. 5. TfRT1-GFP colocalizes with Kif5B but TfR-GFP does not. (A–G) Cortical neuron in dissociated culture transfected with TfR-GFP. Colabelling for TfR-GFP and for endogenous Kif5B indicates that the two proteins largely do not colocalize (white areas in F). (H–N) Introduced TfRT1-GFP colocalizes with endogenous Kif5B in a cortical neuron in dissociated culture (white areas in M). Dendrite is labelled with D and is MAP2 positive, whereas the axon is labelled with A and is MAP2 negative. (G and M) Arrows point to dendrite and arrowheads point to axon (F, G and M). Scale bars, 10 μm.

the average density of HA-Kv1.3 at 25 μm from the cell body in the apical dendrite and compared the average of those values with the average density in the cell body. We found that the DCR of HA-Kv1.3 was lower in the presence of GFP-DNKif5B (0.2 ± 0.1) vs. coexpression with GFP (0.3 ± 0.2), with GFP-DNKif17 (0.4 ± 0.1), or with GFP-DNKif3A (0.3 ± 0.1). However, in each case the difference was not statistically significant ($P > 0.1$). We conclude from these data that Kif5B is necessary for localization to the axon,

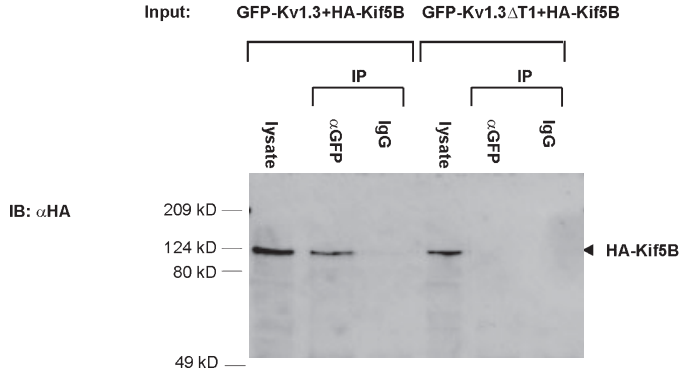


FIG. 6. The T1 domain is necessary for GFP-Kv1.3 to coimmunoprecipitate HA-Kif5B. GFP-Kv1.3 coimmunoprecipitates HA-Kif5B when both proteins are exogenously expressed in COS cells. Conversely, GFP-Kv1.3ΔT1, which lacks the T1 domain, does not coimmunoprecipitate HA-Kif5B under similar conditions, indicating that the T1 domain is necessary for Kv1.3 to coimmunoprecipitate Kif5B. GFP antibodies were used to immunoprecipitate GFP-Kv1.3 and GFP-Kv1.3ΔT1, HA antibodies were used to detect HA-Kif5B.

but has a minor role, if any, in localization of protein to dendrites. Note that previously we showed that the GFP-DNKif17 construct used in Fig. 1 disrupted dendritic localization of Kv4.2 and that GFP-DNKif5B disrupted localization of GluR2 indicating that both constructs likely block transport mediated by the corresponding kinesins (Chu *et al*., 2006). Moreover, GFP-DNKif5B did not block transport of Kv4.2, indicating that it did not block transport nonspecifically (Chu *et al*., 2006). The results shown in Fig. 1 are thus consistent with Kif5B being responsible for transport of Kv1.3 in axons.

In order to test further whether Kif5B transports Kv1 channels, we asked whether GFP-DNKif5B could block axonal localization of endogenous Kv1 channels. To do so we expressed GFP-DNKif5B in dissociated cultures of cortical neurons for 48 h. Subsequently, we stained the cells for endogenous Kv1.1, Kv1.2, or Kv1.4 and examined the resulting expression patterns for the presence of the endogenous channel in axons. As controls we examined the effects of GFP-DNKif17 and GFP alone on localization of the channels. Note that dissociated cultures were used because staining for endogenous proteins in them is easier than in cultured slices. As with introduced, tagged Kv1.3, endogenous Kv1.1, Kv1.2 and Kv1.4 expressed at very low levels in axons following expression of GFP-DNKif5B (Fig. 2A–C; data not shown). In contrast, the three channels were present in axons following expression of GFP-DNKif17 or GFP (Fig. 2D–F; data not shown). To describe these results in a quantitative manner, we calculated the ACR, as in Fig. 1, for introduced HA-Kv1.3. We found that, as with the introduced construct, the ACR for Kv1.1 was dramatically less in cells expressing GFP-DNKif5B (0.03 ± 0.01, $n = 10$) than in cells expressing either GFP alone (0.21 ± 0.03, $n = 10$) or GFP-Kif17 (0.23 ± 0.05, $n = 10$), a statistically significant difference ($P < 0.01$; Fig. 2G). Similar results were obtained for Kv1.2 and Kv1.4; GFP-DNKif5B blocked localization of the endogenous channels to the axon, but GFP and GFP-DNKif17 did not in both cases (Fig. 2G). Note that for the data shown in Fig. 2, we identified the axon by counterstaining the cell for endogenous MAP2 and identifying processes that were unstained. However, we saw similar effects of GFP-DNKif5B on endogenous Kv1.2 in cell counterstained for the axonal marker Tau (data not shown). Moreover, we found that GFP-DNKif5B did not block axonal localization of Synaptophysin indicating that it did not block localization of all axonal proteins (data not shown). To examine the effects of dominant negative kinesins on dendritic targeting we calculated the DCR of endogenous Kv1.1, Kv1.2, and Kv1.4 as in Fig. 1. We found that when expressed with GFP-DNKif5B endogenous Kv1.1 had a lower DCR (0.15 ± 0.05) than when expressed with either GFP (0.28 ± 0.1) or with GFP-DNKif17 (0.33 ± 0.15). However, these differences were not statistically significant ($P > 0.1$), indicating that Kif5B does not dramatically affect dendritic localization of Kv1 channels. Similar results were found for Kv1.2 and Kv1.4 (Fig. 2G).

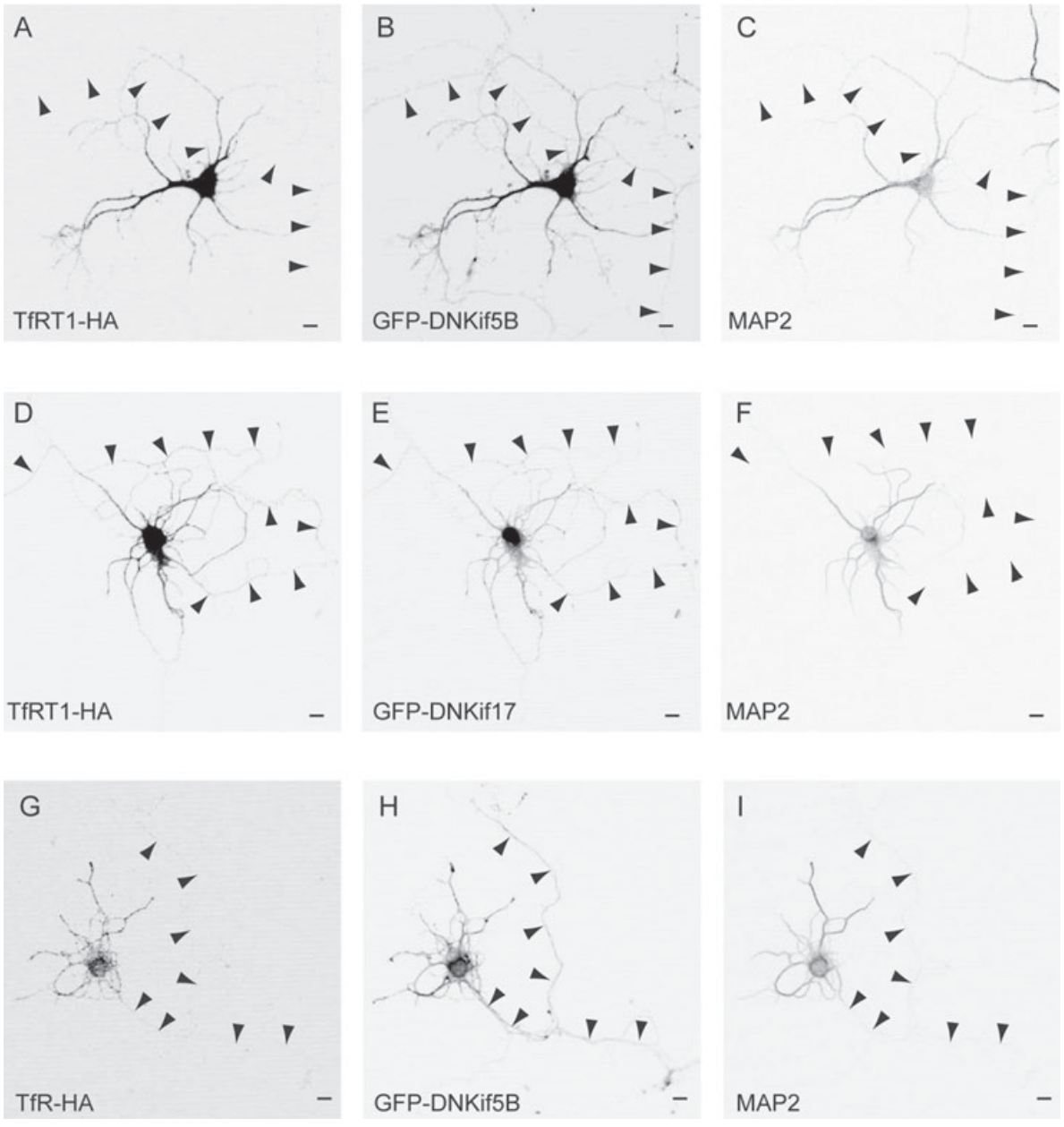
Kif5B colocalizes and coimmunoprecipitates with Kv1 channels

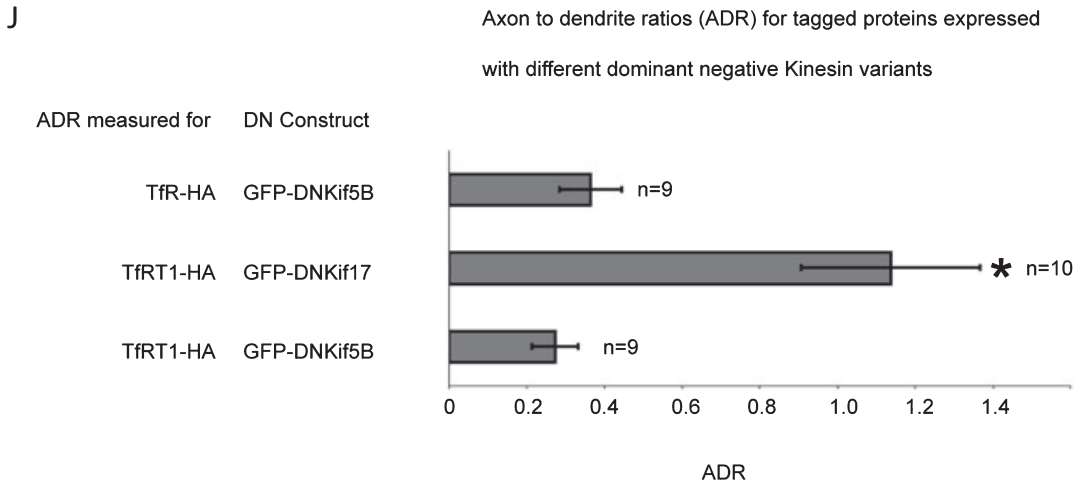
If Kif5B mediates transport of Kv1 channels, one would expect that these channels and the kinesin would colocalize during the transport process. To test for colocalization of Kv1.2 and Kif5B we stained dissociated cultures of cortical neurons using antibodies that recognize native forms of the two proteins. We found that there is considerable overlap between the expression patterns of Kv1.2 and Kif5B (Fig. 3A–G). In addition we found that introduced MYC-Kv1.3 colocalizes with introduced YFP-Kif5B (Fig. 3H–N). These results are consistent with Kv1 channels associating with Kif5B. To test for this interaction in a more direct manner, we asked whether Kv1.2 coimmunoprecipitated with Kif5B from native brain tissue from rats. We found that Kv1.2 antibodies coimmunoprecipitated Kif5B as determined by Western blot (Fig. 4), indicating that the two proteins are part of the same complex.

Kif5B plays a role in axonal targeting of heterologous proteins by the T1 domain

The previous results suggest that Kv1 channels interact either directly or indirectly with Kif5B. Because the T1 tetramerization domain mediates axonal targeting of Kv1 channels, it is logical that this region might mediate interaction with Kif5B. To explore the role of the T1 domain in mediating association with Kif5B, we asked whether attaching the T1 domain to a heterologous protein (the Transferrin receptor, TfR) could cause that protein (TfRT1) to associate with Kif5B. Previously, it has been shown that TfRT1 fusion proteins are present in axons, in contrast to TfR alone, which localizes specifically to dendrites (Gu *et al*., 2003; Rivera *et al*., 2005). We found that in dissociated cultures of cortical neurons, puncta stained for TfR-GFP show very little overlap with puncta stained for endogenous Kif5B, indicating that the kinesin is likely not responsible for transport of exogenous TfR (Fig. 5A–G). In contrast, TfRT1-GFP demonstrates striking colocalization with endogenous Kif5B (Fig. 5H–N). Similar results were obtained with YFP-Kif5B, which colocalized almost 100% with TfRT1-HA, but largely did not colocalize with TfR-HA (data not shown). These results show that addition of the T1 domain can cause a heterologous protein to colocalize with Kif5B, which is consistent with the T1 domain mediating association either directly or indirectly with the kinesin.

To test further the importance of the T1 domain for interaction of Kv1 channels with Kif5B, we asked whether the T1 domain of Kv1.3 is necessary for the channel to coimmunoprecipitate Kif5B in COS cells. Initially we showed GFP-Kv1.3 coimmunoprecipitated HA-Kif5B when both proteins were introduced into COS cells (Fig. 6). We then introduced the construct GFP-Kv1.3ΔT1, which lacks the T1 domain, into COS cells along with HA-Kif5B and tested whether the two proteins coimmunoprecipitate. We found that GFP-Kv1.3ΔT1 did not coimmunoprecipitate HA-Kif5B in dramatic contrast to the wild-type GFP-Kv1.3 (Fig. 6). As a control we showed that GFP-Kif5B coimmunoprecipitates MYC-Kv1.3 when the two





proteins are expressed in COS cells. In contrast, GFP-Kif17 does not coimmunoprecipitate MYC-Kv1.3 under similar circumstances, showing that the two proteins likely do not interact (data not shown). These experiments provide further evidence that Kv1 channels interact with Kif5B either directly or indirectly through the T1 domain.

To investigate further the connection between Kif5B and axonal targeting mediated by the T1 domain, we asked whether Kif5B is necessary for axonal targeting of TfRT1-HA. We used a paradigm similar to that illustrated in Fig. 1 where individual dominant negative kinesin variants were expressed in cortical neurons in dissociated culture for approximately 24 h and, subsequently, TfRT1-HA was expressed in the same cells for 48 h. We found that when GFP-DNKif5B was expressed prior to induction of TfRT1-HA the latter protein was almost completely absent from the axon (Fig. 7A–C). However, although TfRT1-HA was absent from the axon, it was clearly present in dendrites, indicating that protein transport in general was not blocked (Fig. 7A–C). These experiments indicate that Kif5B is necessary for axonal targeting mediated by the T1 domain. In contrast, when GFP-DNKif17 was tested in a similar paradigm, it did not block axonal localization of TfRT1-HA. In this case TfRT1-HA was clearly present in the axon as well as the dendrite indicating that the effect seen with GFP-DNKif5B could not be achieved with expression of arbitrary dominant negative kinesin variants (Fig. 7D–F). To determine whether Kif5B is necessary for transport of TfR to dendrites, we expressed TfR-HA following GFP-DNKif5B in the same manner as in the previous experiments. We found that it was expressed throughout dendrites in much the same manner as TfR has previously been found to localize (Fig. 7G–I), indicating that Kif5B is not necessary for transport of TfR to dendrites (West *et al*., 1997).

To quantify these observations, we calculated the relative amount of either TfR-HA or TfRT1-HA in the axon vs. the dendrite. This number, the axon to dendrite ratio (ADR), is a measure of the ratio of intensity of staining in the axon vs. that in the dendrite normalized by the ratio of the size of the axon to that of the dendrites. An ADR of 1 indicates that a protein is relatively evenly distributed between axons and dendrites, while ADR less than 1 indicates that a protein is localized to dendrites. The ADR for TfRT1-HA following expression of GFP-DNKif5B was 0.27 ± 0.03 ($n = 9$), which was very similar to the ADR of TfR-HA under similar circumstances (0.36 ± 0.02, $n = 9$). In contrast, the ADR of TfRT1-HA expressed after GFP-DNKif17 was 1.1 ± 0.2 ($n = 10$), which is significantly different from the ADR of TfRT1-HA expressed after GFP-DNKif5B ($P < 0.01$), and indicates nonspecific targeting (Fig. 7J). These results indicate that Kif5B is necessary for the T1 domain to mediate axonal targeting of a heterologous protein.

Discussion

In this paper we provide evidence that Kv1 channels are transported to axons by the kinesin isoform, Kif5B. We found that dominant negative variants of Kif5B block axonal targeting of both introduced and endogenous Kv1 channels. In contrast, dominant negative variants of Kif5B do not have a statistically significant effect on dendritic localization of the protein. Furthermore, Kv1.2 colocalizes with Kif5B in neurons and coimmunoprecipitates with the kinesin from brain lysate. Experiments in COS cells show that Kv1.3 channels coimmunoprecipitate with Kif5B.

We also investigated the role of Kif5B in axonal targeting mediated by the T1 domain. We found that the T1 domain was both sufficient to cause TfRT1 to colocalize with Kif5B in dissociated cortical neurons and necessary for Kv1.3 to coimmunoprecipitate with Kif5B in COS cells. In addition, dominant negative variants of Kif5B prevented the T1 domain from mediating axonal targeting of TfRT1. These results suggest a model where the T1 domain mediates interaction (either directly or indirectly) with Kif5B, which, in turn, plays a vital role in axonal targeting mediated by the domain. This model contrasts with a model for dendritic trafficking of the K^+ channel Kv4.2 suggested by our recent work (Chu *et al*., 2006). The Kv4.2/Kif17 model has Kif17 attaching at the extreme C-terminus of Kv4.2, a site distinct from the dendritic targeting motif. In addition, Kif17 does not appear to play an indispensible role in dendritic targeting as it is not necessary for dendritic targeting of heterologous proteins by the motif.

A possible explanation for how the T1 domain mediates axonal targeting becomes apparent if we propose that Kif5B acts as a 'smart motor', which is defined as a motor protein that traffics autonomously to a specific subcellular compartment (Burack *et al*., 2000). In this case, if Kif5B were to traffic specifically to axons, then merely attaching the kinesin to Kv1 would be sufficient for the T1 domain to mediate axonal targeting. Although truncated Kif5B shows a preference for axons, endogenous Kif5B is found in both axons and dendrites, so it is unlikely that it is a smart motor (Niclas *et al*., 1994; Nakata & Hirokawa, 2003). However, recent results have shown that Kif5B in combination with an adaptor protein can act as a 'smart motor complex' (Setou *et al*., 2002). That is, Kif5B in combination with the minimal binding domain of the adaptor protein GRIP traffics preferentially to dendrites, while Kif5B in combination with the minimal binding domain of JSAP1 preferentially traffics to the axonal compartment. Thus, the T1 domain of Kv1 channels might mediate axonal targeting by attaching to a smart motor complex consisting of Kif5B and one or more adaptor proteins. One group of proteins that could play a role in modulating Kif5B function are Kvbeta subunits. It has been shown that binding of Kvbeta to T1 is essential for the domain to mediate axonal targeting of CD4T1 (Gu *et al*., 2003). Our result that Kv1.3 can bind to Kif5B in COS cells would suggest that Kvbeta is not necessary for binding between the channel and the kinesin, as Kvbeta subunits are not expressed at high levels endogenously in COS cells (Bekele-Arcuri *et al*., 1996). However, this result does not suggest that Kvbeta is not part of the complex containing the channel and Kif5B. Although examining the role of Kvbeta in axonal targeting mediated by the T1 domain was beyond the scope of this study, defining its role will be essential to understanding the mechanisms underlying axonal targeting of Kv1 channels.

Ultimately, trafficking to specific compartments in neurons must depend on the ability of certain motor proteins, in combination with

FIG. 7. A dominant negative variant of Kif5B blocks axonal trafficking mediated by the T1 domain. (A–C) TfRT1-HA localizes exclusively in the soma and dendrites of a cortical neuron in dissociated culture when its expression was induced following expression of GFP-DNKif5B. (D–F) TfRT1-HA localizes in the axon as well as the soma and dendrites of a cortical neuron when induced following expression of GFP-DNKif17. (G–I) TfR-HA localizes exclusively to the soma and dendrites when induced following expression of GFP-DNKif5B. (J) When expressed following GFP-DNKif5B, TfRT1 has an ADR of 0.27 ± 0.03 ($n = 9$), indicating that it is localized to dendrites. Conversely, TfRT1 has an ADR of 1.1 ± 0.2 ($n = 10$) when expressed following expression of GFP-DNKif17, indicating that it localizes to the axon as well as to dendrites. TfR-HA has an ADR of 0.36 ± 0.02 ($n = 9$) when expressed following expression of GFP-DNKif5B indicating that it is localized to dendrites. Thus, GFP-DNKif5B prevents the T1 domain from causing TfRT1 to localize in axons. Scale bars, 10 μm. *indicates that a value is significantly different ($P < 0.01$) from the adjacent value(s).

interacting proteins, to distinguish between axonal and dendritic microtubules. Thus, two critical questions to answer in the future are; (i) what are the features that allow axonal microtubules to be distinguished from those found in dendrites and *vice versa* and (ii) how are a particular set of features recognized by motor proteins that traffic specifically to a given compartment? The results presented in this paper will enable us to begin to address these questions in the context of axonal targeting of Kv1 channels.

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Abbreviations

ACR, axon to cell body ratio; ADR, axon to dendrite ratio; DCR, dendrite to cell body ratio.

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