Clustering and Activity Tuning of Kv1 Channels in Myelinated Hippocampal Axons

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Precise localization of axonal ion channels is crucial for proper electrical and chemical functions of axons. In myelinated axons, Kv1 (Shaker) voltage-gated potassium (Kv) channels are clustered in the juxtaparanodal regions flanking the node of Ranvier. The clustering can be disrupted by deletion of various proteins in mice, including contactin-associated protein-like 2 (Caspr2) and transient axonal glycoprotein-1 (TAG-1), a glycosylphosphatidylinositol-anchored cell adhesion molecule. However, the mechanism and function of Kv1 juxtaparanodal clustering remain unclear. Here, using a new myelin coculture of hippocampal neurons and oligodendrocytes, we report that tyrosine phosphorylation plays a critical role in TAG-1-mediated clustering of axonal Kv1.2 channels. In the coculture, myelin specifically ensheathed axons but not dendrites of hippocampal neurons and clustered endogenous axonal Kv1.2 into internodes. The trans-homophilic interaction of TAG-1 was sufficient to position Kv1.2 clusters on axonal membranes in a neuron/HEK293 coculture. Mutating a tyrosine residue (Tyr458) in the Kv1.2 C terminus or blocking tyrosine phosphorylation disrupted myelin- and TAG-1-mediated clustering of axonal Kv1.2. Furthermore, Kv1.2 voltage dependence and activation threshold were reduced by TAG-1 coexpression. This effect was eliminated by the Tyr458 mutation or by cholesterol depletion. Taken together, our studies suggest that myelin regulates both trafficking and activity of Kv1 channels along hippocampal axons through TAG-1.

Proper subcellular targeting of various Kv channels is critical for neuronal excitability and synaptic transmission. Molecular mechanisms underlying polarized axon-dendrite targeting of Kv channels have been extensively studied (1–10). In contrast, channel targeting in distinct membrane domains within axons is much less understood. Most axons in mammals are wrapped by layers of compact lipid membranes from myelinating glial cells, oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system. Myelinated axons form distinct membrane domains, such as nodes of Ranvier, paranodes, and juxtaparanodal (JXP), and internodal regions (11–14). In myelinated axons of both CNS and peripheral nervous system, Kv1 channels are clustered into JXP regions, controlling the fidelity of action potential conduction (11, 15–17).

Based on the findings that the JXP targeting of Kv1 channels was largely eliminated in both Caspr2 (contactin-associated protein-like 2) and TAG-1 (transient axonal glycoprotein-1) knock-out mice, it was hypothesized that Caspr2 interacts with TAG-1 and clusters Kv1 channels via a PDZ domain-containing protein (18, 19). Whereas Caspr2 is expressed only in neurons, TAG-1 is expressed in both neurons and myelinating glial cells (19–21). Caspr2 has a PDZ domain ligand at its C terminus, but deleting PDZ domain-containing proteins, such as PSD-95 (postsynaptic density-95) and/or PSD-93 (postsynaptic density-93), in mice had no effect on the Kv1 JXP targeting (22, 23). A recent study suggests that the expression of TAG-1 in myelin cells is sufficient to rescue the JXP complex and phenotypes of TAG-1 knock-out mice (24), questioning the function of axonal TAG-1. Furthermore, deleting other proteins with different functions in mice also disrupted precise Kv1 JXP clustering (25–30), suggesting that proper functions of many proteins are required. Due to the lack of a system suitable for molecular studies, the mechanism that is both necessary and sufficient to cluster Kv1 channels in JXP regions remains unclear.

Myelination of axons takes place not only in white matter but also in gray matter. Despite being less understood, gray matter myelination has significant physiological functions. Myelination of hippocampal axons may play a critical role in learning and memory. Cortical gray matter demyelination is prominent in multiple sclerosis (MS), an inflammatory demyelinating disease (31–33) that can induce and be aggravated by ion channel dysfunction (34, 35). In approximately 50% of MS patients, extensive demyelination in the hippocampus causes cognitive impairment (36, 37), most likely by altering neuronal output signals conveyed along axons. Although hippocampal neuron culture is a popular model for cell biological studies of neurons (38–40), the myelin coculture of hippocampal neurons was not yet available.

In this study, we have established a new myelin coculture of hippocampal neurons and oligodendrocytes. Myelin specifically ensheaths axons but not dendrites and segregates Kv1.2 channels and voltage-gated sodium (Nav) channels into JXP regions and nodes of Ranvier, respectively. Our study leads to a novel finding that TAG-1 is not only sufficient to initiate the clustering of Kv1.2 on axonal membranes but also regulates
Kv1.2 channel activity, in which tyrosine phosphorylation plays a critical role.

**EXPERIMENTAL PROCEDURES**

**Myelin Coculture of Hippocampal Neurons and Oligodendrocytes**—Hippocampal neuron cultures were prepared as described (1). In brief, 2 days after neuron plating, 1 mM cytosine arabinose (Ara-C, Sigma) was added to the neuron medium (Neurobasal medium supplemented with B27, 0.5 mM l-glutamine, and penicillin/streptomycin (Invitrogen) to inhibit glial growth and was replaced 2 days later with fresh neuron medium. The culture medium was replenished twice a week by replacing half its volume. These neurons were usually transfected at 5–7 days in vitro (DIV) and assayed several days later or cocultured with oligodendrocytes at 14 DIV. Oligodendrocytes and precursor cells (other cell types were also present) were dissociated from the cerebellum and brain stem of day 18 rat embryos and seeded onto the hippocampal neuron culture at 14 DIV. Half of the culture medium was replaced with myelin medium, which was modified from established myelin cocultures of sensory neurons (41, 42) (50% Neurobasal medium, 50% high glucose DMEM, 0.5 mM l-glutamine, penicillin/streptomycin, 1 mM sodium pyruvate, 5 µg/ml insulin (Sigma), 2X B27 supplement, 100 µg/ml transferrin (Sigma), 100 µg/ml bovine serum albumin (Sigma), 0.2 µM progesterone (Sigma), 16 µg/ml putrescine (Sigma), 40 ng/ml sodium selenite (Sigma), 40 ng/ml triiodothyronine (Sigma), 5 µg/ml N-acetylcysteine (Sigma), 10 ng/ml biotin (Sigma), and Cellgro Trace Elements (Mediatech, Manassas, VA)). The culture medium was replenished twice a week by replacing half the volume with fresh myelin medium. The procedure for myelin coculture is illustrated in Fig. 1A. The detailed procedure is available upon request.

**Cultures of Cell Lines and Coculture with Neurons**—HEK293 cells were maintained in cell line medium (minimum essential medium with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.5 mM l-glutamine, and penicillin/streptomycin). For neuron/HEK293 cell coculture, neurons were transfected with cDNA plasmid(s) at 5 DIV and cocultured with transfected HEK293 cells for 1–2 days in neuron medium. Then the coculture was fixed and stained.

**cDNA Constructs and Transfection**—The constructs Kv1.2HA, Kv1.2HAY458A, Kv1.2HAV499A, Kvβ2, YFP-Kvβ2, YFP-Kv1.2HA, and Kv3.1bHA were described previously (3, 4). YFP-Kv1.2HAY458A was generated with a QuikChange strategy. GFP-TAG-1 and mCh-TAG-1 were constructed by inserting GFP and mCherry (a kind gift from Dr. R. Tsien), respectively, into the XhoI site engineered in the N-terminal region (between Thr36 and Phe37, right after the signal peptide) of TAG-1 (Open Biosystems, Huntsville, AL). Caspr2-mCh was made by fusing mCherry to the C terminus of Caspr2 (Open Biosystems). Contactin cDNA was a kind gift from Dr. L. Isom. Kv1.2HAFCY (43) was a kind gift from Dr. D. Ma. For transient transfection, neurons in culture at 5–7 DIV or cell lines were incubated in Opti-MEM containing 0.8 µg of cDNA plasmid and 1.5 µl of Lipofectamine 2000 (Invitrogen) for 30 min at 37 °C.

**Antibodies and Immunostaining**—Antibodies used include mouse monoclonal anti-Kv1.2, anti-Kvβ2, anti-Caspr2, anti-contactin, and anti-GFP antibodies (Antibodies Inc., Davis, CA); mouse monoclonal anti-TAG-1 4D7 (Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-HA (Covance, Emeryville, CA) antibodies; rat monoclonal anti-HA 3F10 (Roche Applied Science) and anti-myelin basic protein (MBP) (Chemicon, Temecula, CA) antibodies; mouse anti-neurofilament H nonphosphorylated (SMI-32) antibody (Covance); mouse monoclonal anti-phosphotyrosine (4G10) antibody (Millipore, Billerica, MA); mouse monoclonal anti-pan-Nav channel antibody (Sigma); rabbit polyclonal anti-MBP, anti-microtubule associated protein 2 (MA); and anti-Tau1 antibodies (Chemicon); rabbit anti-Kv1.2 and anti-Caspr2 antibodies (Alomone Laboratories, Jerusalem, Israel); rabbit anti-neurofilament 200 antibody (Sigma) and anti-GFP antibody (Invitrogen); and Cy2-, Cy3-, and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Ganglioside GM1 was labeled with cholera toxin-FITC (Sigma), and F-actin was labeled with phalloidin Alexa 546 (Invitrogen). The immunocytochemical procedures have been described (1). Briefly, neurons were stained under permeabilized conditions (with 0.2% Triton) to label total proteins and under non-permeabilized conditions (without Triton) to label the surface pool.

**Image Capture and Quantification**—Fluorescence images were captured with a Spot CCD camera RT slider (Diagnostic Instruments, Sterling Heights, MI) in a Zeiss upright microscope, Axioshot, using Plan Apo objectives 20×/0.75 and 100×/1.4 oil, saved as 16-bit TIFF files, and analyzed with NIH Image J and SigmaPlot 10.0 (Systat Software, Inc., Chicago, IL) for fluorescence intensity quantification. Exposure times were controlled so that the pixel intensities in dendrites and axons were below saturation, and the same exposure time was used within each group of an experiment. Only transfected neurons that had clearly separated dendrites and axons and were isolated from other transfected cells were chosen for analysis. MAP2 staining and morphology of these neurons distinguishes dendrites from axons. By laying lines along the major processes to acquire their fluorescence intensity profiles (in arbitrary units), we determined and compared channel protein levels.

**Drug Treatment**—Drugs used include methyl-β-cyclodextrin (MβCD), cytochalasin B (Sigma), and genistein (Calbiochem). In immunostaining experiments, neurons cotransfected with Kv1.2, Kvβ2, and mCh-TAG-1 were treated with the drugs for 2 h or 2–4 days. The neurons were fixed and stained under non-permeabilized conditions 6 days after the cotransfection. In whole-cell recording, HEK293 cells transfected for 2 days were treated with MβCD for 2 h before recording.

**Whole-cell Patch Clamp Recording**—The experimental procedure of whole-cell recording of HEK293 cells expressing Kv channel constructs was described previously (5). Transfected HEK293 cells were identified by fluorescence from cotransfected YFP or mCherry inserted into the N-terminal region of TAG-1 and recorded in Hanks' buffer (150 mM NaCl, 4 mM KCl, 1.2 mM MgCl2, 10 mg/ml glucose, 1 mM CaCl2, 20 mM HEPES, pH 7.4). The internal solution for electrical pipettes contained
122 mM KMeSO₄, 20 mM NaCl, 5 mM Mg-ATP, 0.3 mM GTP, and 10 mM HEPES (pH 7.2). Electrode resistance was 2–5 megaohms. Isolated cells were voltage-clamped in whole-cell mode with an Axonpatch 200B amplifier (Molecular Devices,Downingtown, PA), held at −80 mV, and given 250-ms voltage episodes from −60 to 60 mV in 10-mV increments. Voltage commands were made from pCLAMP10 software through Digidata 1440A (Molecular Devices), and currents were recorded at 5 kHz. Conductance-voltage relationships (G-V curves) for Kv1.2 channel constructs were $G = I(V_m - V_{rev})$, $V_{rev} = -95$ mV, normalized to the maximal conductance. Curves were fitted with the Boltzmann function, $G/G_{max} = 1/(1 + \exp(−(V - V_{50})/k))$, where $G_{max}$ is the maximal conductance, $V_{50}$ is the potential at which the value of the normalized conductance is 0.5, and $k$ is the slope factor. SigmaPlot10 was used for fitting. To obtain $\tau_{on}$, activation curves were fitted with a single exponential function raised to a power of 4, $I(t) = A(1 - \exp(-t/\tau_{on}))^4$. Clampfit10.0 was used for fitting.

RESULTS

A New Myelinated Coculture of Hippocampal Neurons and Oligodendrocytes—To carry out molecular studies for understanding of how myelin clusters Kv1 channels along axons, we established a new myelinated coculture system consisting of hippocampal neurons and oligodendrocytes (Fig. 1A). Hippocampal neurons were first dissociated, plated, and maintained as the regular neuron culture for 14 days. Oligodendrocytes and precursor cells (together with other cells) dissected from the brain stem and cerebellum of rat embryos were then added (Fig. 1A). After seeding into hippocampal culture at 14 DIV, some immature oligodendrocytes developed into mature myelinating cells, shown by staining for the mature myelin marker, MBP. After 3 weeks of coculture, about one-third of MBP-positive cells had multiple myelin segments, and one-third had one or two segments (data not shown).

Mature hippocampal neurons in culture develop lengthy dendrites, which are mingled with axons. To determine whether myelin specifically ensheaths the axons, we stained the coculture for MBP and an endogenous dendritic marker, MAP2. MAP2-positive myelin segments did not colocalize with MAP2-positive dendrites (Fig. 1, B and E). In contrast, most neurites ensheathed by myelin segments were neurofilament (NF)-positive axons, more than half of which showed a reduction of non-phospho-NF staining within myelin segments, as shown by the SMI-32 antibody against non-phospho-NF (Fig. 1, C–E). Moreover, myelination tended to occur at proximal but not distal axons. The commingling of axons, dendrites, and neuronal soma suggests that this coculture system mimics myelination/remyelination processes in cortical gray matter in vivo. The lack of aggregation and low density of hippocampal neurons in the coculture are ideal for studying ion channel trafficking.

Endogenous Kv1.2 Channels Clustered by Myelin along Hippocampal Axons—To determine how myelin regulates endogenous Kv1 targeting, we stained cocultures for Kv1.2 and MBP. Axonal Kv1.2 channels were clustered under myelin segments of myelinating oligodendrocytes (Fig. 2, A and D) but were distributed smoothly in unmyelinated axons (Fig. 2, B and D); Nav
channels were concentrated at the axon initial segment and formed punctate clusters along the rest of the axon (Fig. 2, C and E). In mature myelin internodes, Kv1.2 clustered in putative JXP regions, often on both sides of heminodes (Fig. 2F). Whereas Kv1.2 channels localized in JXP regions, Nav channels localized in heminodes and nodes of myelinated axons (Fig. 2, F–H). Therefore, an intact node of Ranvier is not required for clustering of Kv1 channels under the myelin sheath, suggesting involvement of an intra-axonal mechanism.

Kv1.2 channels were concentrated in putative JXP regions (near the two ends) of long, thick myelin internodes but were clustered uniformly within short internodes (supplemental Fig. S1, A–C). Internode length varied (10–250 μm), probably reflecting unsynchronized differentiation of oligodendrocytes. Over time, the average length of internodes increased (44.0 ± 4.9 μm at 21 DIV, 62.0 ± 4.7 μm at 28 DIV, 106.0 ± 10.3 μm at 35 DIV), as did the percentage of internodes with Kv1.2 concentrated in JXP regions (3.8 ± 1.5% at FIGURE 2. Myelin clusters endogenous Kv1.2 channels along axons of hippocampal neurons. A, myelin segments from myelinating oligodendrocytes clustered endogenous Kv1.2 channels along the axon of a mature hippocampal neuron in culture. Coculture of hippocampal neurons and oligodendrocytes was costained for endogenous Kv1.2 (green) and MBP (red) at 28 DIV. B, endogenous Kv1.2 was distributed smoothly along unmyelinated axons of a hippocampal neuron at 28 DIV. C, distribution patterns of Kv1.2 (green) and Nav (red) channels along unmyelinated axons of a hippocampal neuron at 28 DIV. In A–C, blue arrows, axons; blue arrowheads, dendrites; black arrows, myelin segments; black arrowhead, a myelin segment of an axon that did not express Kv1.2. D, immunofluorescence intensity profiles of Kv1.2 channels along the myelinated axon in A (red) and the unmyelinated axon in B (blue). E, immunofluorescence intensity profiles of Kv1.2 (blue) and Nav (red) channels along the unmyelinated axon in C. F, axonal Kv1.2 channels (green) were clustered in putative JXP regions in an MBP-positive myelin internode (red). White arrowheads, Kv1.2 clusters in putative JXP regions. G, Nav channels (green) localized in a heminode, indicated by a white arrowhead. H, segregation of Kv1 and Nav channels into the putative JXP and nodal regions, respectively. White arrowheads, putative nodes of Ranvier. I, summary of myelin internode length and percentage of the putative Kv1.2 JXP targeting at three developmental stages (n = 4 cocultures). Scale bars, 100 μm in A–C and 20 μm in F–H. **, p < 0.001 (t test). Error bars, S.E.
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Thus, these results suggest that Kv1 channels might be first recruited into a myelin internode and then concentrated in JXP regions.

Next, we investigated whether other JXP proteins colocalized with Kv1.2 along myelinated hippocampal axons. Kvβ2, a cytosolic auxiliary subunit promoting Kv1 forward trafficking and axonal targeting (1, 44), colocalized with endogenous Kv1.2 clustering at putative JXP regions (supplemental Fig. S1E). Two JXP cell adhesion molecules, TAG-1 and Caspr2, also colocalized with Kv1.2 clusters (supplemental Fig. S1, D and F).

The Trans-homophilic Interaction of TAG-1 in Positioning Kv1.2 Clusters along Axons—To dissect how TAG-1 and Caspr2 might cluster axonal Kv1.2 channels in trans, we established a neuron/HEK293 cell coculture (Fig. 3A). Hippocampal neurons transfected with combinations of YFP-Kv1.2, Kvβ2, TAG-1, and Caspr2 were cocultured for 1–2 days with HEK293 cells expressing TAG-1 and mCherry (mCh) (Fig. 3B). HEK293 cells expressing TAG-1 clustered YFP-Kv1.2 along contacting axons from the neurons cotransfected with YFP-Kv1.2, Kvβ2, and TAG-1 (Fig. 3C). Kv1.2 was distributed smoothly along axons when expressed alone. TAG-1 in HEK293 cells became concentrated along the contacting axons (Fig. 3, C and D). In sharp contrast, TAG-1 in HEK293 cells failed to cluster YFP-Kv1.2 on contacting axons from neurons cotransfected with YFP-Kv1.2, Kvβ2, and Caspr2 (Fig. 3E). TAG-1 localization in HEK293 cells was not affected by the contacting axon (Fig. 3E). The endogenous levels of TAG-1 and Caspr2 were low in young hippocampal neurons (45), and mature neurons did not coculture well with HEK293 cells. Caspr2-mCh in HEK293 cells failed to cluster YFP-Kv1.2 along axons even in the presence of TAG-1 (supplemental Table SI). It is important to note that TAG-1 and YFP-KV1.2 colocalized even in cis along axons (Fig. 3C). Therefore, the TAG-1 expressed in HEK293 cells further directs Kv1.2 channel clusters along the contacting axons via the trans-homophilic interaction with axonal TAG-1, even in the absence of axonal Caspr2 (supplemental Table SI).

The Tyrosine Residue Tyr458 in Clustering Kv1.2 and TAG-1 along Axons—To determine how TAG-1 specifically clusters Kv1.2 channels in cis, we first examined the distribution patterns of TAG-1 and contactin. Both are GPI-anchored membrane proteins, containing Ig domains and fibronectin repeats, and belong to the same family of adhesion molecules. Contactin plays a critical role in paranode formation (11, 12, 46). When expressed in hippocampal neurons, TAG-1 was present in both
dendrites and axons and formed clusters along axons (Fig. 4, A, B, and E). In contrast, contactin distributed rather smoothly in both dendrites and axons (Fig. 4, C–E).

Next, we co-expressed Kv1.2 and TAG-1 constructs in neurons to identify the motif of channel clustering (Fig. 5A). Indeed, Kv1.2HA coclustered with GFP-TAG-1 in cis on axonal membranes (Fig. 5, B and C). Fusing GFP or mCherry to the N terminus of TAG-1 did not significantly alter its distribution pattern. We further examined whether GFP-TAG-1 could cluster Kv1.2 mutants on axonal membranes. This assay requires that Kv1.2 mutants can be targeted into axons. Our previous studies showed that disrupting the N-terminal T1 domain and truncating the C terminus eliminated axonal targeting of Kv1.2 (1). Therefore, we focused on two putative motifs in the Kv1.2 C terminus, a tyrosine-based motif (Y^458XX^902, in which X is any residue, and ^ is a bulky hydrophobic residue) and a PDZ domain-binding ligand at the extreme C terminus. When expressed in neurons, the level of Kv1.2HAY458A on axonal membranes was higher than the wild type Kv1.2HA, consistent with our previous study (1). Interestingly, Kv1.2HAY458A on the axonal surface did not cocluster well with GFP-TAG-1 (Fig. 5D). Disrupting the PDZ domain-binding motif by mutating Val^499 to Ala largely eliminated membrane targeting and axonal targeting of Kv1.2 (1). To circumvent this problem, we used Kv1.2HA fused with the forward trafficking motif from Kir2.1 at the C terminus, Kv1.2HAFCY (43). Despite the disrupted PDZ ligand at the C terminus, Kv1.2HAFCY was targeted on axonal membranes and clustered with GFP-TAG-1 (Fig. 5E). Therefore, the C-terminal tyrosine-based motif, not the PDZ domain-binding ligand, is important in TAG-1-induced clustering on the axonal surface. As a control, Kv3.1bHA was distributed smoothly along axonal membranes in the presence of GFP-TAG-1-positive clusters (Fig. 5F).

Moreover, coexpression of Caspr2-mCh surprisingly eliminated large GFP-TAG-1 clusters (Fig. 5G). Coexpression of contactin failed to cluster YFP-Kv1.2 along axons (Fig. 5H). TAG-1 clustering axonal Kv1.2 channels in cis is

**FIGURE 4.** Distinct targeting patterns of expressed TAG-1 and contactin in hippocampal neurons. TAG-1 (A and B) and contactin (C and D) were transfected into hippocampal neurons at 5 DIV. The neurons were costained with the dendritic marker MAP2 (A and C) or the axonal marker Tau1 (B and D) at 8 DIV. There was no clear signal for endogenous TAG-1 or contactin from untransfected neurons at this stage. E, fluorescence profiles of TAG-1 (A) and contactin (C) along axons (arrows). Scale bars, 50 μm.
probably a critical step in the TAG-1-mediated signaling pathway and may underlie Kv1.2 clustering on the unmyelinated side of a heminode (Fig. 2F).

The Tyrosine Residue Tyr458 in Kv1.2 Clustering along Myelinated Hippocampal Axons—To determine whether the Tyr458-based motif is crucial for myelin-regulated Kv1 targeting, we transfected hippocampal neurons with YFP-tagged channel constructs and examined channel localization after the axons were myelinated. In controls, soluble YFP was transfected into cultured hippocampal neurons at 7 DIV, which were then cocultured with oligodendrocytes at 14 DIV for 2 weeks. Many YFP-expressing axons were myelinated, but YFP localization was not affected by myelin (Fig. 6A). In neurons expressing YFP-Kv1.2, myelin segments formed either near the axon initial segment (Fig. 6B, top) or along the middle of the axon (Fig. 6B, bottom) concentrated axonal YFP-Kv1.2 into putative inter-
In some myelinated axons, YFP-Kv1.2 was further clustered to the two ends of myelin segments, the putative JXP regions (Fig. 6B, bottom). In contrast, localization of YFP-Kv1.2Y458A was much less affected by myelin (Fig. 6C). Therefore, in vitro myelination regulates axonal localization of overexpressed Kv1.2 channels, which requires the tyrosine residue Tyr458, consistent with its role in TAG-1-induced clustering.

Potential TAG-1-initiated Signaling in Clustering Axonal Kv1 Channels—How does TAG-1 cluster Kv1 channels on axonal membranes? Because TAG-1 does not have a cytoplasmic domain and no previous study shows that its Ig domains or fibronectin repeats can bind to the extracellular portion of Kv1.2 channels, it is unlikely that TAG-1 directly binds to the channel. Instead, TAG-1 is a GPI-anchored cell adhesion molecule (47, 48) associated with lipid rafts containing sphingolipid and cholesterol. GPI-anchored cell adhesion molecules may cluster lipid rafts and hence recruit signaling molecules (49). Therefore, we wondered whether TAG-1 could cluster with lipid rafts. When expressed in hippocampal neurons, TAG-1 indeed highly colocalized with ganglioside GM1, a major component of lipid rafts, revealed by cholera toxin-FITC (Fig. 7A). The staining was performed under non-permeabilized conditions. Importantly, TAG-1 expression significantly increased
the levels of ganglioside GM1 on both dendritic and axonal membranes (Fig. 7).

Antibody-mediated cross-linking of TAG-1 activates Src family tyrosine kinases (50). To assess whether tyrosine phosphorylation is involved in coclustering of TAG-1 and Kv1.2, we cotransfected neurons with Kv1.2HA and mCh-TAG-1 and stained for phosphotyrosine using an anti-phosphotyrosine antibody, 4G10. Remarkably, the coclusters highly colocalized with phosphotyrosine (Fig. 8, A and B). This result suggests that tyrosine phosphorylation plays an important role in the TAG-1-induced clustering of Kv1.2. Using cholera toxin-FITC to label lipid rafts and phalloidin Alexa 546 to label F-actin, we found that they partially colocalized with Kv1.2/TAG-1 clusters (Fig. 8, B–D).

Next, we assessed the effects of disrupting lipid rafts (by MβCD), F-actin (by cytochalasin B), or tyrosine phosphorylation (by genistein). Long term (4 days) but not short term (2 h) MβCD treatment significantly decreased Kv1.2HA clustering on neuronal membranes (Fig. 8E), suggesting that lipid rafts initiate but do not maintain Kv1.2HA clusters. Cytochalasin B (2 h) largely eliminated Kv1.2HA clusters on the axonal surface (Fig. 8E), suggesting the actin cytoskeleton is critical for maintaining Kv1.2HA clusters. Genistein (2 days) decreased the coclustering of TAG-1 and Kv1.2HA and increased the level of Kv1.2HA on axonal membranes (Fig. 8, E and F). Thus, tyrosine phosphorylation is required for Kv1 clustering, consistent with the critical role of the tyrosine-based motif (Tyr458) at the Kv1.2 C terminus. It is important to note that in the present study, despite the long incubation time, the drugs were used at low concentrations. Because protein trafficking takes time, it is appropriate to use the long term treatment in our study, which is actually consistent with many previous studies successfully using long term drug treatment to alter actin cytoskeleton, lipid rafts, and tyrosine phosphorylation (51–55).

TAG-1 Coexpression and Kv1.2 Channel Activity—To determine whether TAG-1 coexpression regulates channel activity, we performed whole-cell voltage clamp recording on HEK293 cells transfected with Kv1.2 channel constructs. Under our experimental conditions, the outward currents (carried by endogenous Kv channels) of untransfected HEK293 cells were...
less than 0.1 nA (for the voltage command of +60 mV), which was \(-5\%\) of overexpressed Kv1.2 constructs and thus ignored. Coexpression of mCh-TAG-1 altered the G-V curve by increasing the slope factor \(k\), rendering the channel less voltage-dependent and lowering the activation threshold (Fig. 9, A–C, and supplemental Table SII). Thus, the channels are more

![Diagram](image-url)

**FIGURE 8.** Tyrosine phosphorylation and related signaling events in TAG-1-mediated clustering of Kv1.2 along axons. Hippocampal neurons were cotransfected with Kv1.2HA, Kvβ2, and mCh-TAG-1 (red) at 5 DIV, fixed, and stained 3–5 days later. A, phosphotyrosine (phospho-Y) was highly enriched in the clusters containing both Kv1.2 and TAG-1. Transfected hippocampal neurons were stained with rabbit anti-Kv1.2 (green) and mouse anti-phosphotyrosine, 4G10 (blue), antibodies under permeabilized conditions. White arrows, clusters containing Kv1.2HA, mCh-TAG-1, and phosphotyrosine. The fluorescence intensity plots were given under high magnification images of three axonal segments. B, percentage of colocalization of phosphotyrosine (revealed by the 4G10 antibody), surface GM1 (revealed by the cholera toxin-FITC (CT-FITC) under non-permeabilized conditions), and F-actin (revealed by phalloidin Alexa 546), with clusters containing both Kv1.2 and TAG-1. Values are mean ± S.E. (error bars). **, \(p < 0.01\) (t test). C, colocalization of surface GM1 with TAG-1/Kv1.2 clusters on axonal membranes. Transfected hippocampal neurons were stained with an anti-HA antibody (green) and cholera toxin-FITC (blue) under non-permeabilized conditions. White arrows, clusters containing Kv1.2HA, mCh-TAG-1, and CT-FITC. D, fluorescence profiles along the axon in C, E, differential effects of depleting cholesterol, depolymerizing F-actin, and inhibiting tyrosine kinases on TAG-1-induced clustering of Kv1.2HA on axonal membranes. The neurons were treated with 5 mM MβCD for 2 h or 4 days, 10 \(\mu\)M cytochalasin B for 2 h, or 20 \(\mu\)M genistein for 2 days or left untreated. F, anti-HA fluorescence intensities revealing the surface levels of Kv1.2HA on axonal membranes. *, \(p < 0.05\); **, \(p < 0.01\). One-way analysis of variance was used, followed by Dunnett’s test for \(p\) values.
likely to regulate the resting membrane potential and to prevent spontaneous depolarization events. In contrast, MβCD decreased slope factor $k$ and activation constant $\tau_{on}$ and increased the activation threshold in the presence of Kvβ2 (Fig. 9, A–C, and supplemental Table SII). Kv1.2HAY458A channel kinetics were much less affected by MβCD (Fig. 9, B and C). As expected, current amplitudes differed under these conditions, reflecting the regulation of channel expression and trafficking (Fig. 9C). Importantly, TAG-1 also regulates Kv1 channel kinetics, most likely by changing the lipid composition of the microenvironment.

**DISCUSSION**

In this study, using different culture methods, including a new myelin coculture of hippocampal neurons and oligodendrocytes, we examined the underlying mechanism and potential physiological consequence of Kv1 channel clustering in myelinated axons. Based on the results, we propose a new model in which the TAG-1 aggregation induced by trans-homophilic interaction clusters lipid rafts and recruits Kv1 channels that are phosphorylated at Tyr$^{458}$ and anchored to the actin cytoskeleton (Fig. 9, D and E). Due to lipid composition in the clusters, Kv1 channels are probably fine tuned to play an important role in maintaining the local resting membrane potential and constraining aberrant action potential firing resulting from small spontaneous depolarization events.

Different from highly organized myelin segments in white matter in vivo, the length of myelin segments varied in our coculture. The discrepancy may result from the following possibilities. First, in this coculture, axons, dendrites, and soma are...
Kv1.2 Clustering in Myelinated Hippocampal Axons

commingled. Thus, it may mimic the myelination and/or remyelination process in cortical gray matter, different from white matter myelination. Second, the development of myelinating oligodendrocytes may be less synchronized in vitro. Third, like other myelin cocultures, neurons were dissociated, losing the anatomical structure of the neural network in vivo. These may also contribute to the difference between the precise JXP clustering of Kv1.2 in white matter axons in vivo and the clustering patterns of Kv1.2 along myelin segments in our coculture (Fig. 2I). Nonetheless, in the coculture, Kv1 and Nav channels are clearly segregated in the putative JXP regions and nodes of Ranvier, respectively (Fig. 2, F and H). The discrepancy between in vivo and in vitro targeting of Kv1 channels may actually provide novel mechanistic insights into myelin-regulated channel targeting.

Several lines of evidence from our study suggest that tyrosine phosphorylation may play a central role in myelin-mediated clustering of axonal Kv1 channels. A tyrosine residue, Tyr458, in the C terminus of Kv1.2 was important for TAG-1-induced clustering of Kv1.2 along axons (Fig. 5 and supplemental Table S1). This residue was also critical for myelin-mediated clustering of YFP-Kv1.2 (Fig. 6). Moreover, anti-phosphotyrosine staining showed that phosphorylated tyrosine highly colocalized with TAG-1 and Kv1.2 clusters (Fig. 8, A and B). Finally, inhibiting tyrosine kinase activity not only increased axonal levels of Kv1.2HA but also decreased the coclustering of Kv1.2HA and TAG-1 (Fig. 8, E and F). Our results are consistent with a previous study showing that Kv1.2 can be phosphorylated by tyrosine kinase at Tyr458 via activation of the acetylcholine receptor, and the phosphorylation regulates the binding to contactin, an actin-binding protein (56). It will be of interest to determine whether TAG-1- and acetylcholine receptor-induced phosphorylation of Kv1.2 share the same signaling pathway in future studies.

Our model suggests the possibility of redundancy at the level of intra-axonal signaling. Despite being highly localized, the signaling pathway may not be unique for TAG-1. It is possible that a tyrosine kinase can be activated by multiple different receptors. In fact, JXP Kv1 channels were not completely eliminated in either Caspr2 or TAG1 knock-out mice (18, 19), which is consistent with our model. Kv1 JXP targeting apparently does not require the direct physical interaction between Kv1 and TAG1 or Caspr2. Actually, both TAG-1 and Caspr2 associate with Kv1 in brain lysates but not in the lysates of transfected cell lines (18, 19), indicating that they do not directly interact with Kv1 channels. Furthermore, both PSD-93 and PSD-95 are dispensable for Kv1 JXP targeting (22), also suggesting that another mechanism is involved.

What is the role of Caspr2 in clustering Kv1 channels in myelinated axons? Previous studies showed that at the JXP, Kv1 channels associated with the complex of Caspr2 and TAG-1, which bind to TAG-1 on the myelin membrane (12, 18, 19). However, Caspr2 was not required in TAG-1-initiated clustering of Kv1.2 in vitro (Fig. 3 and supplemental Table S1). Therefore, Caspr2 may be involved in later steps of the Kv1 JXP targeting or in placing the TAG-1/Kv1 clusters into the JXP. In fact, in Caspr2 knock-out mice, Kv1 channels are distributed along the internode (18, 19). This provides another explanation for the internodal targeting of Kv1.2 in short myelin segments observed in our coculture (Fig. 2I and supplemental Fig. S1, A–C). In the coculture, the availability of essential components in the JXP complex might not be synchronized as in vitro, resulting in diverse targeting patterns of Kv1 channels along myelin segments. Furthermore, a recent study showed that selective somatodendritic endocytosis underlies the axonal targeting of Caspr2, in contrast to the direct delivery of axonal Kv1 channels (47). These studies indicate that Caspr2 is certainly important for the Kv1 JXP targeting but may be not essential in the initial clustering.

Consistent with this study, TAG-1 knock-out mice indeed had disrupted juxtaparanodes (57). These mice also had impairment of learning and memory (57), which are usually associated with the function of hippocampus. A recent study suggests that only the glial TAG-1 is required for the JXP targeting of Kv1 channels (24), whereas our studies suggest the trans-homophilic interaction of TAG-1 is critical for the placement of Kv1.2 clusters on axonal membranes (Fig. 3 and supplemental Table S1). The discrepancy may result from dynamic assembly and functional redundancy of the JXP components. Another recent study identified TAG-1 as a candidate autoantigen recognized by both autoantibodies and T helper 1/17 cells in MS patients, suggesting that a TAG-1-specific T-cell response contributes to the development of gray matter pathology in both murine experimental autoimmune encephalomyelitis and MS (58). Moreover, TAG-1 also interacts with L1-CAM, β-integrin, neural cell adhesion molecule, proteoglycans, and amyloid precursor protein (59–62). Interestingly, recent studies show that SLEEPLESS, a GPI-anchored protein in the Ly-6/neurotoxin family, is crucial for sleep in Drosophila, by regulating the expression levels, localization, and activity of Shaker (Drosophila homolog of Kv1 channels) (63, 64). Given that lipids can regulate Kv channel activity (65, 66), our data raise an intriguing possibility that the GPI-anchored proteins, TAG-1 and SLEEPLESS, may regulate Kv1.2 channel activity by altering lipid composition. However, it is currently too difficult to directly record JXP Kv1 channels under the myelin sheath. Therefore, the exact pathway through which TAG-1 regulates Kv1 channel targeting and function will remain an interesting topic for future investigation.

This study is the first report demonstrating myelin formation along hippocampal axons in a dissociated culture. The findings and techniques reported here lay a foundation for molecular studies of myelin-regulated trafficking of axonal ion channels in central neurons to better understand cortical gray matter demyelination and devise strategies to treat cognitive deficits in MS and other demyelinating diseases.
