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Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites

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In cells, molecular motors operate in polarized sorting of molecules, although the steering mechanisms of motors remain elusive¹. In neurons, the kinesin motor² conducts vesicular transport such as the transport of synaptic vesicle components to axons³ and of neurotransmitter receptors to dendrites⁴, indicating that vesicles may have to drive the motor for the direction to be correct. Here we show that an AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionate) receptor subunit—GluR2-interacting protein (GRIP1)—can directly interact and steer kinesin heavy chains to dendrites as a motor for AMPA receptors. As would be expected if this complex is functional, both gene targeting and dominant negative experiments of heavy chains of mouse kinesin showed abnormal localization of GRIP1. Moreover, expression of the kinesin-binding domain of GRIP1 resulted in accumulation of the endogenous kinesin predominantly in the somatodendritic area. This pattern was different from that generated by the overexpression of the kinesin-binding scaffold protein JSAP1 (JNK/SAPK-associated protein-1, also known as Mapk8ip3), which occurred predominantly in the somatoaxon area. These results indicate that directly binding proteins can determine the traffic direction of a motor protein.

The neuron, a good model of a polarized cell, is divided into two molecularly and functionally distinct domains: axons and dendrites. The precise targeting and localization of proteins within these domains are critical to every aspect of neuronal function^{1,5}. In neurons, conventional kinesin, which consists of two heavy chains (KHC: KIF5) and two light chains (KLC)^{1,2}, is a multifunctional transporter of both axonal cargo such as synapsin and GAP43 (ref. 3) and dendritic cargo such as messenger RNA⁶ and the AMPA receptor⁷ (Fig. 1a). To elucidate the mechanisms possibly regulating how kinesin moves toward axons and/or dendrites, we screened the binding regulators of kinesin in a yeast two-hybrid system⁷. By this screening, we identified the glutamate-receptor-interacting protein GRIP1 (refs 8, 9) as the strongest partner of a cargo-binding domain^{10,11} of kinesin heavy chain, and we further showed that binding proteins could regulate the polarity of kinesin traffic direction.

All KIF5 isoforms (KIF5A, KIF5B and KIF5C) contained the

minimal GRIP1-binding site (Fig. 1b). The binding site overlapped with the cargo-binding domain of the fungus kinesin, which lacks the light chains¹¹. The tails of other major neuronal KIFs, such as KIF1A, KIF1B β and KIF17 (which bound to the mLin10 PDZ domain in this assay⁷), did not bind to GRIP1. The affinity of direct KIF5–GRIP1 interaction *in vitro* by surface plasmon resonance analysis⁷ ($K_d = 1.9 \times 10^{-8}$ M) was in good agreement with that previously reported for kinesin tail binding to brain microsomes *in vitro*¹⁰. Therefore, we suggest that heavy chains of kinesin directly bind to GRIP1.

We next investigated whether kinesin transports GRIP1. We used 'kinesin-null' primary cultured extra-embryonic cells from KIF5b-

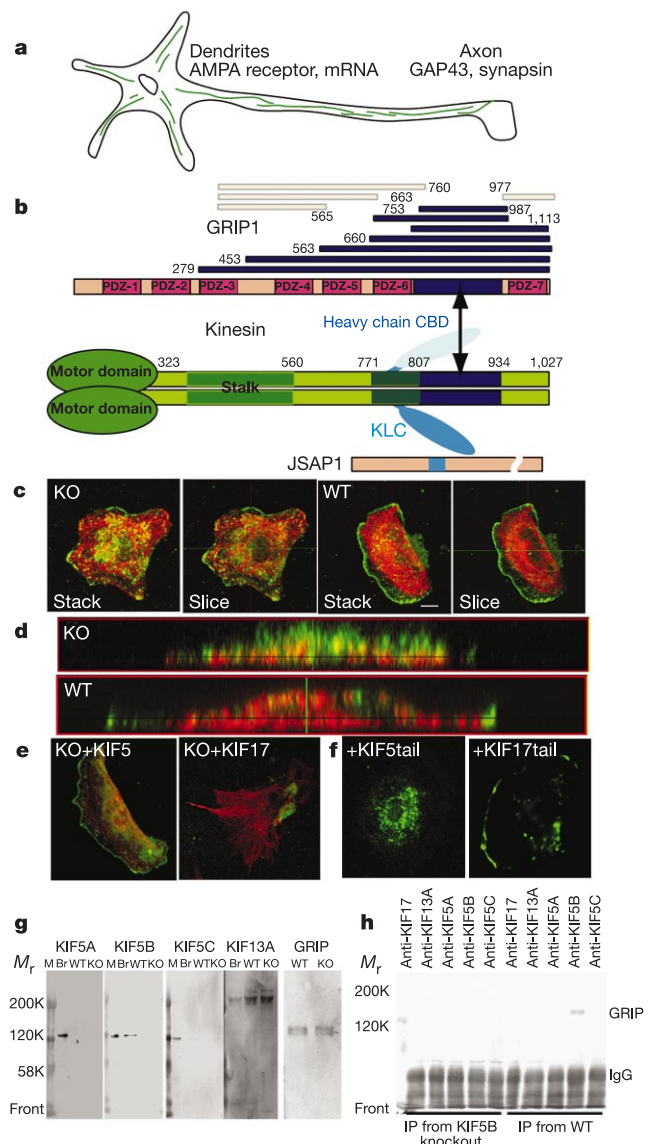


Figure 1 Kinesin binds to GRIP1, and is necessary for normal localization of GRIP1. **a**, Schematic diagram of a neuron and its reported kinesin cargo. **b**, Arrangement of GRIP1 and the kinesin binding domain. Blue boxes are the binding areas, with corresponding amino-acid positions indicated. Pale yellow boxes are areas of no binding. **c**, Aberrant localization of GRIP1 in cells lacking KIF5 (knockout cells, KO). WT, wild type. GRIP1 is green; microtubules are red. Slices are confocal plane images at one-third of cell height. Scale bar, 20 μ m. **d**, The x-z sections of the cells in **c**. **e**, KIF5, but not KIF17, rescued KIF5-knockout cells. **f**, KIF5 tail, but not KIF17 tail, disturbed the localization of GRIP1. **g**, KIFs and GRIP1 expression level in knockout and wild-type cells. **h**, GRIP1 binds KIF5B in these cells. IP, immunoprecipitate. M, molecular mass marker; Br, brain.

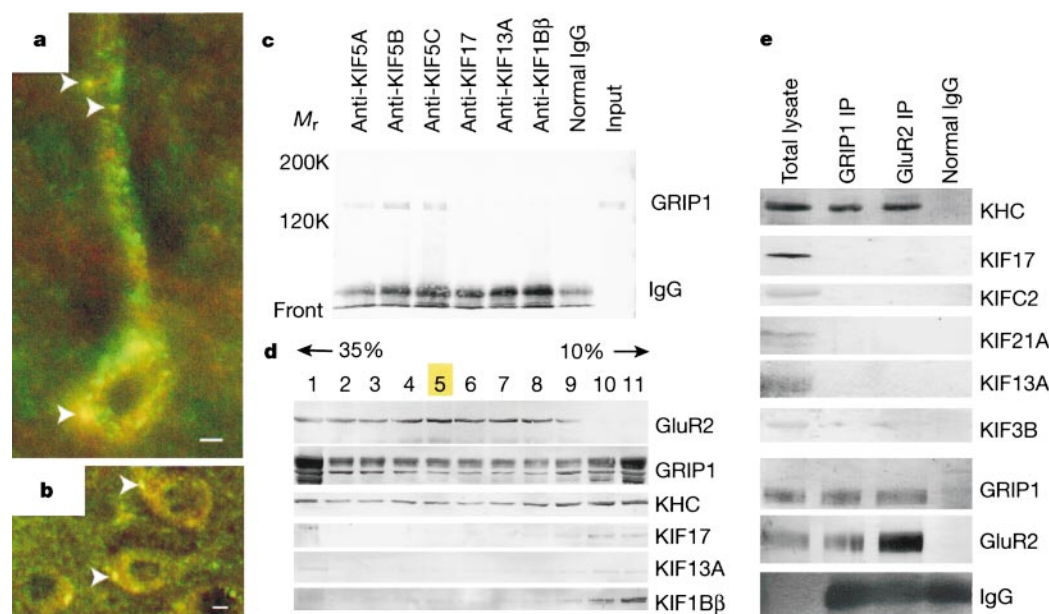


Figure 2 Kinesin-GRIP1-GluR2 complex *in vivo*. **a**, Kinesin (KIF5A, red) and GRIP1 (green). They co-localized in the soma and dendritic shafts (indicated with arrowheads), but not on the small scattered green dots (perhaps synaptic) or the red fibre that crosses (perhaps axonal). Scale bar, 2 μ m. **b**, The signal of KIF5A (in red) in the soma is related to Golgi (arrowheads), with β -COP (green). Scale bar, 2 μ m. **c**, Immunoprecipitation of

GRIP1 with KIFs. **d**, Kinesin-GRIP1-GluR2 transport vesicles. The middle fraction (5) was previously characterized as GRIP-GluR2-positive transporting vesicles¹⁸. KHC shows a wide, overlapping distribution. **e**, Immunoprecipitation of the kinesin-GRIP1-GluR2 complex.

knockout mice and control wild-type littermates¹². Both knockout and wild-type cells expressed GRIP1 equally (Fig. 1g), consistent with the previous description of embryonic GRIP expression¹³. If kinesin indeed transports GRIP1, then a lack of kinesin might result in a mislocalization of GRIP1 in these cells. Native GRIP1 clustered abnormally at the perinuclear region in knockout cells, which was different from the case in wild-type cells (Fig. 1c). In KIF5b-

knockout cells, most GRIP1 disappeared from the periphery, and appeared as relatively abundant intracellular clusters in the centre of a cell. In *x-z* cell sections, we could see a larger amount of intracellular stacked GRIP1 at the centre of knockout cells compared with wild-type (Fig. 1d) cells. This effect is specific because a lack of kinesin does not affect the bulk delivery systems of the endoplasmic reticulum, the Golgi, the *trans*-Golgi network (TGN)

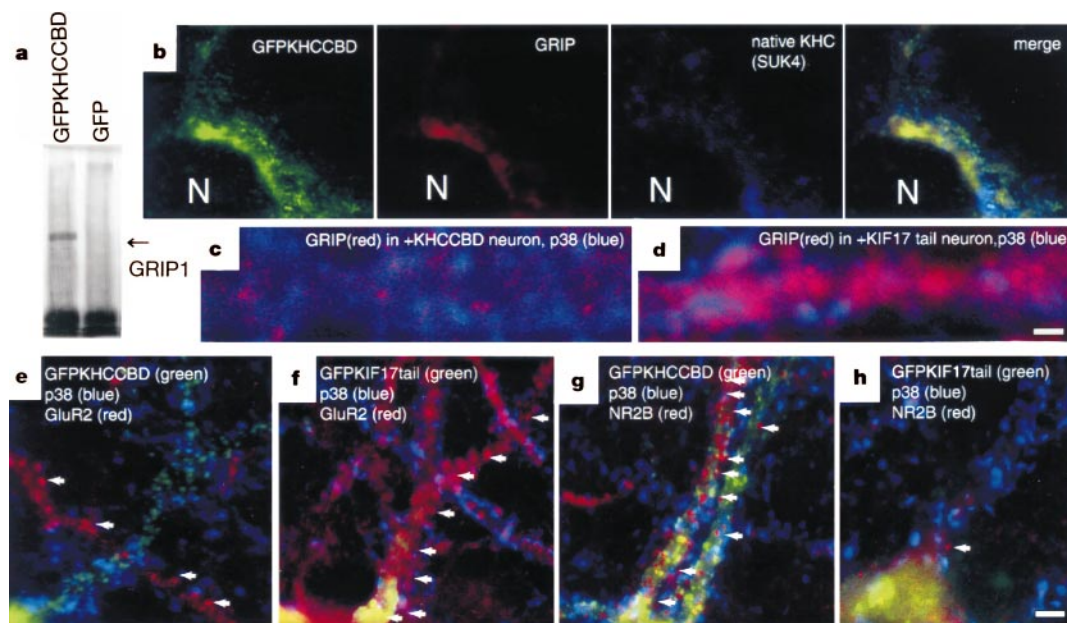


Figure 3 KHCCBD relocates GRIP1 and GluR2. **a**, GFP-KHCCBD binds native GRIP1 in neurons. **b**, GFP-KHCCBD and endogenous GRIP1 in the soma. The fourth panel is the merged image of the other three. Co-localization is observed around the nucleus (N). **c**, GRIP1 in distal dendrites. Synaptophysin (p38) labels presynapses. **d**, Control. Scale bar, 1 μ m. **e**, Dominant negative effects of GFP-KHCCBD. p38 is also slightly affected,

but GluR2 reduction is more significant. Arrows indicate the non-transfected dendrites, as an internal control. **f**, Arrows indicate normal GluR2 clusters in the dendrites of transfected neurons. **g**, GFP-KHCCBD does not affect NR2B. **h**, The GFP-KIF17 tail affects NR2B. The arrow indicates an NR2B cluster.

and the plasma membrane, nor does it affect localization of these molecules in the cells of the knockout mice¹². KIF5, but not KIF17, could rescue this phenotype (Fig. 1e). Exogenous KIF5 tail expression in wild-type cells also resulted in the abnormal clustering of GRIP1 (Fig. 1f). KIF5B antibody effectively immunoprecipitated GRIP1 from wild-type cells but not from knockout cells (Fig. 1h). Thus, kinesin can efficiently target GRIP1 to the cell periphery.

We then investigated the function of the kinesin–GRIP complex in neurons. Kinesin exists in both the somatodendritic area and axons¹⁴ and Golgi-related structures in the soma¹⁵ *in vivo*. GRIP1 localizes in Golgi-like structures in the soma, in vesicles in dendritic shafts, and in post-synaptic density (PSD)⁹. Kinesin and GRIP1 tend to co-localize in dendritic shafts and somatic Golgi-related structures in brain sections (Fig. 2a, b). GluR2 is known to exist in synapses and dendritic shafts¹⁶. A large population of the intracellular (including extrasynaptic) GluR2 forms a protein complex with GRIP1 (ref. 9). Moreover, scaffolding protein complexes can work as adapters for receptor traffic by motors^{7,17}. Therefore, we analysed the kinesin–GRIP–GluR2 complex *in vivo*. Antibodies against all the KIF5 isozymes immunoprecipitated GRIP from brain lysates (Fig. 2c). Kinesin exists in the subcellular fraction enriched in GluR2–GRIP-positive transporting vesicles¹⁸ (Fig. 2d). Antibodies to GRIP1 and GluR2 pulled kinesin down from this fraction (Fig. 2e). Thus, the GRIP1–GluR2 complex is one of the binding partners of kinesin in dendrites.

Having found that GRIP1 is a kinesin-binding scaffolding protein for GluR2 *in vivo*, we investigated whether the KIF5–GRIP complex transports GluR2, using a KIF5 dominant negative construct in neurons. We investigated the effect in cultured neurons of a construct (green fluorescent protein (GFP)–KHCCBD KIF5A807–1027) that contains the GRIP1-binding site, but lacks the KLC-binding site. When we immunoprecipitated the GRIP1–GFP–KHCCBD complex with an anti-GFP antibody, a significant

amount of GRIP1 was detected in the precipitate from cells expressing GFP–KHCCBD (Fig. 3a), as expected. GRIP1 and GFP–KHCCBD co-localized in the soma (Fig. 3b). The GFP–KHCCBD construct competed with the intrinsic KIF5–GRIP1 complex (Fig. 3b), and reduced the amounts of GRIP1 (Fig. 3c) and GluR2 in the synapses (Fig. 3e). Control KIF17 tail expression did not abolish GluR2 in the synapses (Fig. 3f). KIF5 dominant negative constructs did not significantly affect NMDA (N-methyl-D-aspartate) receptors in these cells (Fig. 3g), whereas KIF17 dominant negative constructs (Fig. 3h) did affect these receptors. Thus we showed that GluR2 is a passenger of the kinesin–GRIP1 transport machinery.

We wondered why kinesin does not transport the GluR2–GRIP complex into the axons. We had regarded kinesin as a major motor for axonal transport supplying materials into axons that are more than 99% of the volume of mature mammalian neurons *in vivo*. So we further dissected the molecular details of the GluR2–GRIP1–kinesin complex. The minimal kinesin-binding domains of GRIP1 (753–987) should be free from retention or reactivation with other possible binding partners (this construct lacks the binding sites for GluR2, self-multimerization sites and other potential regulator RasGEF-binding sites¹⁹, and did not bind endogenous GRIP1; Fig. 4m). This construct of GRIP1 delocalized kinesin predominantly in the somatodendritic area, but not significantly in axons (86% of the GRIP1 fragment signal co-localized with kinesin. Some 27% of the GRIP fragment and 39% of kinesin were located in dendrites (Fig. 4a–d). In contrast, the minimal binding domain of JSAP1 (mouse Syd2, JIP3), which functions as a binding partner of kinesin via KLC^{17,20,21}, delocalized kinesin predominantly in axons and soma (Fig. 4i–l) as a good control (92% of the JSAP1 fragment signal co-localized with kinesin; 4% of JSAP1 and 4% of kinesin were located at dendrites). Microtubules have their positive ends directed to the distal end in both axons and distal dendrites, but

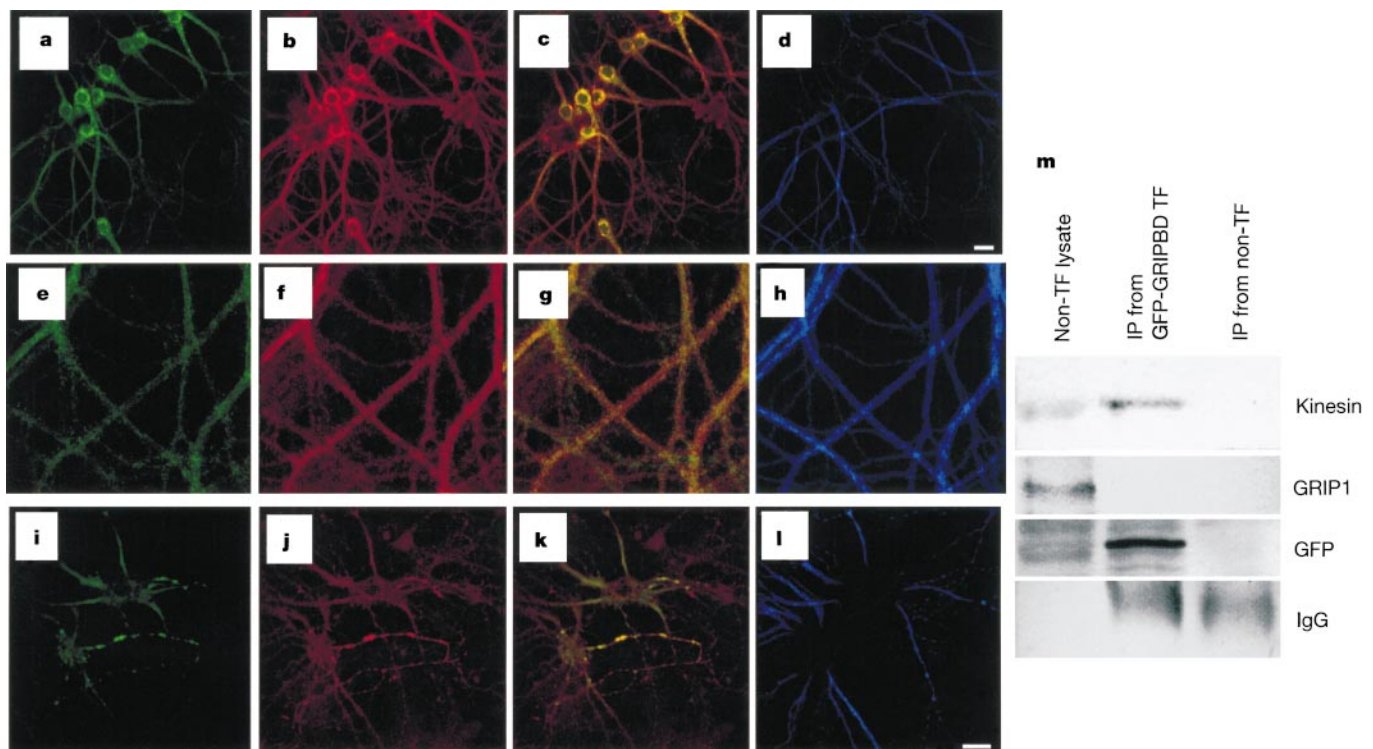


Figure 4 The GRIP1 kinesin-binding site recruits kinesin to the soma and dendrites. **a**, The Kinesin-binding domain of GRIP1–GFP (green). **b**, Intrinsic kinesin (red). **c**, GRIP1 and kinesin (merged, yellow) in the somatodendritic area. **d**, Dendrites stained by anti-MAP2 (blue). Scale bar, 10 μ m. **e–h**, Higher magnification of **a–d**, respectively. **i**, JSAP1

kinesin-binding domain (green). **j**, Kinesin (red). The JSAP1 kinesin-binding domain predominantly accumulates intrinsic kinesin in axon-like processes. **k**, Merged images of **i** and **j**. **l**, Dendritic marker map2 (blue). **m**, The GFP-minimal kinesin-binding domain of GRIP did not bind to native GRIP. TF, transfected.

microtubule polarity is mixed in proximal dendrites²². Thus, the 'plus-end-directed' motor kinesin can function in both directions^{5,7}. It has been confirmed that kinesin exists in axons, soma and dendrites under normal conditions^{14,23}, so the unbalanced ratio of the dendritic driver GRIP1 and axonal driver JSAP1 may cause the unbalanced redistribution of kinesin in this assay.

Findings on unique dendritic plus-end-directed motors such as KIF21B²⁴ have suggested that a 'smart motor' may differentiate dendrite-directed microtubules from axonal microtubules⁵. In this context, our results provide the evidence that a 'smart motor complex' may recognize the difference between the dendrite-directed and axon-directed microtubules; that is, smart units can be constructed of various complexes of the motor and its drivers. We examined the default microtubule preference of the kinesin motor engine without drivers in another study (T. Nakata and N.H., manuscript in preparation). Kinesin-binding proteins may also contribute to enhancing, converting or masking the properties of molecular engines. This concept allows the various cargoes to be carried by a limited number of motors to different directions at different times. Most of the other KHC binding partners previously reported, such as kinectin²⁵ or MyoVA²⁶, are mainly dendritic. Moreover, most of the KLC partners, such as JSAP^{21,17} and amyloid precursor protein²⁷, have been considered axon directed, implying that kinesin transport can be generally categorized as the KHC pathway for somatodendritic dominance, and the KLC pathway for somatoaxonal dominance. In summary, we have provided evidence that GRIP1 enables kinesin to transport dendritic proteins such as AMPA receptor subunit GluR2. □

Methods

Molecular biology constructs

KIF5A (808–1027) was used for yeast two-hybrid screening. Tail constructs of KIF5s, such as KIF5A (808–967), KIF5A (808–934), KIF5B (808–963 end), KIF5B (808–935), KIF5C (808–957 end), and KIF5C (808–935), were also positive for GRIP (279–1,113) binding. Other KIF constructs (KIF1A (400–end), KIF1B α (830–end), KIF1B β (830–end) and KIF17 (939–end)), or binding domain separation mutants (KIF5A (808–877), KIF5A (877–1,027), KIF5A (953–1,027), and KIF5B (808–883)) were negative for GRIP (279–1,113 end) binding, as negative controls. We identified JSAP1 as the strongest binding partner for KLC. We excluded the JSAP1 data from this paper because it has been reported recently²¹. Full-length GRIP1 and JSAP1 were cloned and sequenced from the mouse brain library⁷. GFP-fusion GRIP mutants were subcloned from pB42AD clones into the pEGFP2 vector (Clontech) using *EcoRI*–*XhoI* sites. Deleted or tagged constructs of KIFs were obtained from Hirokawa laboratory stocks. The Sindbis virus was constructed following the manufacturer's instruction (Sinrep system, Invitrogen). GFP, Myc and haemagglutinin tags were attached to GRIP1 or KIF amino-terminal ends, and virus titres were normalized using their titre on BHK cells.

Antibodies

Anti-KIF antibodies were obtained from Hirokawa laboratory stocks^{7,14}. SUK-4 was purchased from Covance, and H2 from Chemicon. The goat anti-GluR2 antibody was purchased from Santa-Cruz²⁸. The anti-GluR2 polyclonal antibody for immunoprecipitation was from Chemicon. The anti-NR2B polyclonal antibody was a gift from M. Mishina. The anti-Pan GRIP was a gift from M. Sheng, and the anti-GRIP1 and GRIP2 antibodies were gifts from R. L. Huganir. The anti-GRIP1 monoclonal antibody was purchased from Beckton Dickinson. The anti-MAP2 antibody, the anti- β -COP antibody, and anti-synaptophysin antibody were purchased from Sigma. The anti- α -tubulin monoclonal antibody was from Serotec. For secondary antibodies, Alexa 488, 568, goat anti-rabbit or mouse immunoglobulin- γ (Molecular Probe), Alexa 568 donkey anti-goat antibody (Molecular Probe), fluorescein isothiocyanate donkey anti-mouse antibody (Jackson Laboratories), anti-mouse Cy5 antibody (Amersham Pharmacia), and ALP-goat anti-mouse, goat anti-rabbit, and donkey anti-goat (Chapel) antibodies were used.

Immunoprecipitations

For brain analysis, about 10 μ g of affinity-purified rabbit anti-KIF antibodies precoupled with protein-A beads was mixed with 400 μ g of solubilized lysates at 4°C overnight (for KIF5s, antibodies against its rod were used). The beads were centrifuged for 2 min and washed with Triton-Tris-EDTA buffer three times. GRIP2 was weakly detected in the precipitates of kinesin, but the significance of this association was not investigated further. For subcellular fractionation and immunoprecipitation of the GRIP–GluR2 transport complex *in vivo*, we followed a previously described method¹⁸ without major modifications.

Primary culture analysis

Extra-embryonic cell culture and rescue were carried out as previously described¹². Biochemical analysis of dominant negative constructs was from mid-density-cultured rat

brain neurons²⁹ in 10-cm dishes for 4 weeks. Results of morphological analysis with respect to the effects of KIF5 dominant negative constructs on GRIP1 and GluR2 in neurons^{5,7} were presented. Images were processed with NIH image for numerical analysis¹². The co-localization of the molecules analysed was determined by dividing the pixel number with both signals by that of a single signal filtered into a normalized bitmap at a median threshold. The titres of the viruses were adjusted to the level where about 70% of the cells showed GFP expression.

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Rapid regulation of steroidogenesis by mitochondrial protein import

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Most mitochondrial proteins are synthesized on cytoplasmic ribosomes and imported into mitochondria^{1–3}. The imported proteins are directed to one of four submitochondrial compartments—the outer mitochondrial membrane, the inner mitochondrial membrane, the intramembraneous space, or the matrix—where the protein then functions. Here we show that the steroidogenic acute regulatory protein (StAR), a mitochondrial protein required for stress responses, reproduction, and sexual differentiation of male fetuses^{4–7}, exerts its activity transiently at the outer mitochondrial membrane rather than at its final resting place in the matrix. We also show that its residence time at this outer membrane and its activity are regulated by its speed of mitochondrial import. This may be the first example of a

mitochondrial protein exerting its biological activity in a compartment other than that to which it is finally targeted. This system enables steroidogenic cells to initiate and terminate massive levels of steroidogenesis within a few minutes, permitting the rapid regulation of serum steroid hormone concentrations.

Steroid hormones, which are essential for life in all vertebrates, are made from cholesterol. The first and rate-limiting step in steroidogenesis is conversion of cholesterol to pregnenolone by the mitochondrial cholesterol side-chain cleavage enzyme, P450_{scc}, located on the matrix side of the inner mitochondrial membrane (IMM)⁸. Steroidogenic cells store minimal amounts of hormone; the regulation of steroid secretion and serum concentrations is primarily at the level of steroid hormone synthesis. Because of the powerful actions of steroid hormones and the toxicities resulting from their overproduction, vertebrates have evolved mechanisms for the rapid induction and termination of their synthesis. Tropic hormones stimulate transcription of genes encoding steroidogenic enzymes, providing a slow regulation that determines the identity and capacity of steroidogenic cells. However, the ability to induce steroidogenesis 10–100-fold within minutes and terminate this synthesis rapidly is regulated at the level of cholesterol flow from the outer mitochondrial membrane (OMM) to the IMM⁶. This acute response requires StAR^{4,5}, which can confer an acute steroidogenic response to non-steroidogenic cells co-transfected with the P450_{scc} system⁵. StAR mutations cause potentially lethal congenital lipoid adrenal hyperplasia, in which virtually no steroids are produced⁵. This disease develops in two stages, corresponding to the loss of the acute and then the chronic steroidogenic response to tropic stimuli⁷.

The mechanism and site of action of StAR are controversial. StAR is produced as a pre-protein of relative molecular mass 37,000

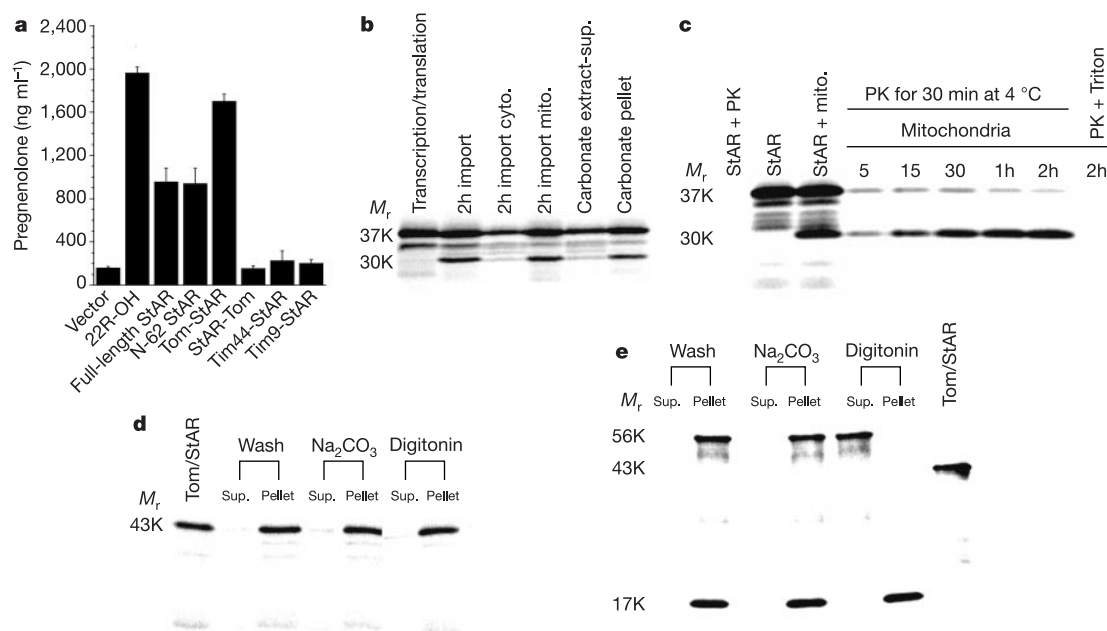


Figure 1 Affixing StAR to the outer mitochondrial membrane increases activity. **a**, Steroidogenic activity (accumulated pregnenolone secreted after 48 h), of COS-1 cells co-transfected with the F2 fusion of the cholesterol side-chain cleavage system and with vectors expressing the indicated constructions. 22R-OH, steroidogenesis from control cells incubated with 22-R-OH cholesterol; all other data show steroidogenesis from endogenous cellular cholesterol. **b**, Import of [³⁵S]StAR into MA-10 mitochondria. After 2 h some of the M_r 37K cell-free translation product is incorporated into the membrane and cleaved to 30K, which remained membrane-associated. **c**, Import of StAR into MA-10

mitochondria. Extramitochondrial 37K StAR is partially sensitive to proteinase K (PK), but intramitochondrial 30K StAR accumulates with time and remains protease-protected without Triton X-100. **d**, Import of Tom/StAR. Tom/StAR of M_r 43K associates with mitochondria but is not imported; carbonate and digitonin extractions show association with OMM (cyto., cytoplasm; mito., mitochondria; sup., supernatant). **e**, Digitonin selectively solubilizes the IMM. MA-10 mitochondria were prepared, washed and serially extracted with carbonate and digitonin; the indicated fractions were separated by gel electrophoresis and immunoblotted with a mixture of antisera to human P450_{scc} and to human Tom20.