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Localization of glutamate receptors to distal dendrites depends on subunit composition and the kinesin motor protein KIF17

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Abstract

Correct glutamate receptor localization in neurons is crucial for neurotransmission in the brain. Here we investigated the mechanisms underlying localization of kainate GluR5 receptors to dendrites in cultured hippocampal neurons. We find that the GluR5 distribution depends on association with GluR6 and KA2 subunits. The GluR5 subunit was expressed in distal dendrites only when GluR6 and KA2 subunits were present, whereas it was restricted to proximal dendrites in the absence of these subunits. The overlap between GluR5 distribution and the organization of microtubules in dendrites led us to examine whether KIF17, a microtubule motor protein expressed in distal dendrites, is involved in GluR5 localization to distal dendrites. We show here, for the first time that the microtubule motor protein KIF17 interacts with GluR6 and KA2 subunits and is required for GluR5 localization to distal dendrites, defining a novel mechanism that controls receptor localization in neurons.

Keywords

GluR5; GluR6 and KA2; kainate receptors; proximal and distal dendrites; Microtubule motor protein; KIF17 motor proteins; cultured hippocampal neurons; kainate receptor knock-out mice; Receptor trafficking; subunit composition

Introduction

Among the ionotropic glutamate receptor family, the function of kainate receptors is the least well understood despite their wide distribution in the brain and their critical role in synaptic transmission (Huettner, 2003) and in brain disorders such as schizophrenia (Bah et al., 2004) and autism (Jamain et al., 2002).

Kainate receptor function in neurotransmission depends upon their subunit composition and neuronal localization. Kainate receptors are composed of a specific subset of subunits termed GluR5-7 and KA1-2 (Hollmann and Heinemann, 1994). Although there is clear evidence for their distribution in the somato-dendritic compartment, their presence in the axonal compartment is controversial (Frerking and Nicoll, 2000), particularly for the GluR5 subunits because of the lack of pharmacological agents and specific antibodies (Clarke et al., 1997;

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Cossart et al., 1998; Contractor et al., 2000; Mulle et al., 2000). Overlapping expression patterns between the kainate receptor subunits indicate that the subunit composition of the native kainate receptors is diverse. It is not known, however, how subunit composition relates to subcellular localization of kainate receptors in neurons.

Current concepts about the molecular mechanisms underlying targeting of receptors has been largely based on studies of polarized epithelial cells. Until recently, most efforts have focused on identifying protein targeting sequences that direct a given receptor to axon or dendrites (Stowell and Craig, 1999; Ruberti and Dotti, 2000; Francesconi and Duvoisin, 2002, Deng et al., 2005). This view has been recently challenged with the finding that some synaptic proteins do not require a targeting sequence, but instead use their association with other proteins that possess a sorting sequence (Ahmari et al., 2000). In line with this idea, targeting of ionotropic receptors, could be determined by one subunit as has been shown for $GABA_A$ receptors in epithelial cells (Perez-Velazquez and Angelides, 1993). Although undescribed, receptor subunit composition could be an important determinant for localization of glutamate ionotropic receptors in neurons.

Although there is some evidence for a microtubule-based intracellular transport of AMPA and NMDA receptors in neurons (Setou et al., 2000; Kim and Lisman, 2001), there is no data for kainate receptors. The polarity of microtubules influences the direction of intracellular transport. In dendrites, the organization of microtubules is complex. In the proximal dendrites, defined as the half-way point between the soma and tip of dendrites, the microtubules are non-uniformly oriented, with a mixed plus- and minus-end population. In contrast, in distal dendrites, microtubules are uniformly oriented with their plus-end towards the tip of dendrites (Baas et al., 1988). Transport of receptors is accomplished by motor proteins; kinesins and dyneins (Hirokawa, 1998), which move along microtubules in a polarized-fashion. In general, dyneins move towards the minus-end of microtubules, whereas kinesins move towards the plus- or minus-end of microtubules. Based on microtubule polarity, transport in proximal dendrites can be achieved by both plus- and minus-end directed kinesins and dyneins. In contrast, transport to the distal dendrites can only be achieved by a plus-end directed kinesin. Among the kinesins that have been characterized, KIF17, a plus-end directed homodimeric protein, is a good candidate for transport of glutamate receptors to dendrites as it is expressed exclusively in the somato-dendritic compartment in both proximal and distal dendrites and has been shown to transport NMDAR2B receptors in the distal dendrites of hippocampal neurons (Guillaud et al., 2003).

Here, we investigated the intracellular distribution of GluR5 in dendrites by expressing a green fluorescent protein tagged-GluR5 (GFP-R5) in cultured hippocampal neurons. We show that GluR5 receptors are mainly localized in the somato-dendritic compartment. Since GluR5 subunits can assemble with GluR6-7 and KA1-2 subunits to form functional receptors (Herb et al., 1992; Cui and Mayer, 1999), we examined whether the subunit composition of GluR5-containing receptors could influence its subcellular localization. We compared the distribution of GluR5 receptors in neurons taken from control mice and knock-out mice for the most ubiquitous kainate receptors in the hippocampus (i.e., GluR5, GluR6 and KA2 receptors). We show that GFP-R5 distribution to distal dendrites depends on association with endogenous GluR6 and KA2 subunits. Our study also reveals that the localization of GluR5 in distal dendrites requires the motor protein KIF17, likely via the formation of a complex of GluR5/GluR6/KA2 and KIF17.

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Results

GFP is a reliable marker of the subcellular distribution of the GluR5 subunit

The lack of specific antibodies for GluR5 subunits led us to construct a GFP-tagged GluR5 in order to visualize its cellular distribution (GFP-R5, Fig. 1A). We next verified that the GFP tag did not disrupt the functional properties of GluR5 receptors in cultured hippocampal neurons. We used calcium phosphate transfection (Xia et al., 1996) to introduce cDNAs encoding GFP-R5 into hippocampal neurons. The relatively low efficiency of transfection using this method makes it possible to study single transfected neurons against a background of non-expressing neurons. We found that the distributions of GFP-R5 fusion protein and GFP alone in neurons were very distinctive. Neurons expressing GFP-R5 exhibit punctae in the soma and neurites, but GFP-R5 was never expressed within the nucleus (Fig. 1B, left panel). In contrast, neurons expressing GFP exhibit fluorescence in all neuronal compartments, including axons, dendrites, the soma and the nucleus (Fig. 1B, right panel). To show that GFP-R5 fluorescence corresponded to functional GluR5-containing receptors in neurons, we performed electrophysiological recordings from live neurons derived from knock-out mice lacking endogenous GluR5, GluR6 and KA2 receptor genes (R5-R6-KA2$^{-/-}$), thus eliminating the participation of endogenous GluR5, GluR6 and KA2 subtypes in the currents induced by the kainate receptor agonist ATPA (Paternain et al., 2000). The application of ATPA (30 μM) in the presence of an AMPA receptor antagonist, SYM 2206 (100μM), to neurons produced inward currents in GFP-R5 transfected neurons (300 ± 62.8 pA, n=12), whereas no current was detected in adjacent non-transfected neurons derived from R5-R6-KA2$^{-/-}$ knock-out mice (Fig. 1C). Similar results were obtained at DIV 7 and DIV 15, suggesting that neurons were still expressing functional GFP-R5 receptors at least 12 days after transfection. These results show that GFP-R5 form functional receptors in neurons.

In a second set of experiments, we showed that GFP-R5 proteins retain their ability to associate with GluR6 and KA2 subunits, using coimmunoprecipitation assays in HEK cells (Fig. 2A). We also determined that the GFP tag does not interfere with GluR5 cell surface expression by quantification of the cell surface expression of GFP-R5 in HEK cells using flow cytometry analysis in intact (i.e., non-permeabilized) cells (Fig. 2B). We took advantage of the GFP-tag located at the extracellular N-terminus of GluR5 to distinguish between cell surface and internal receptors and calculated a ratio between the number of surface-expressed receptors and the total pool of receptors. Two unrelated GFP-fusion proteins were used as negative controls in this assay. First, we used the GFP-NR3A NMDA receptor subunit (GFP-NR3), which is not targeted to the cell surface unless it coassembles with the NMDA NR1 subunit (Perez-Otano et al., 2001b). Second, we used a non-functional GFP-GluR2 AMPA receptor subunit (GFP-R2NF) in which the GFP tag insertion within the N-terminus disrupts the functional expression of GluR2 (see Methods). These controls were also used to show that the cell surface expression was not correlated to expression levels. As shown in Figure 2B, GFP-NR3 and GFP-R2NF are both absent from the cell surface. This result is independent on their expression levels, which is high for GFP-NR3 and low for GFP-NR2F (data not shown). The cell surface expression of GFP-R5 was ten times higher than the background surface expression of GFP-NR3 and GFP-R2NF subunits ($p < 0.01$), demonstrating that GFP-R5 is correctly located at the cell surface.

Taken together, these results demonstrate that GFP-R5 subunits form functional, surface expressed receptors which are able to assemble with GluR6 and KA2 subunits.

GFP-R5 distribution is polarized to the somato-dendritic compartment

We next quantified the subcellular distribution of GFP-R5 in axons and dendrites using immunocytochemistry coupled with confocal analyses in hippocampal cultures. We found that 80% of GFP-R5 transfected neurons expressed GFP-R5 exclusively in the somato-dendritic

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compartment (Figs. 3A and 3C). The remaining 20 % of neurons also expressed GFP-R5 in their axons, but only in the first 100 μm from the soma (111 ± 18.6 μm, n = 15 from 9 experiments).

To investigate whether the coassembly of GluR5 with other kainate receptor subunits influenced its polarized distribution to the somato-dendritic compartment, we compared the subcellular distribution of GFP-R5 in primary hippocampal neurons derived from control and R5-R6-KA2$^{-/-}$ mice. We first checked by western blot that R5-R6-KA2$^{-/-}$ neurons were devoid of GluR6 and KA2 protein expression (Fig. 3B, lower panel). We showed that in R5-R6-KA2$^{-/-}$ neurons, 89% of transfected neurons expressed GFP-R5 proteins exclusively in the somato-dendritic compartment (Figs. 3B and 3C). This percentage is similar to that found in control neurons (i.e., neurons co-expressing GluR6 and KA2 subunits) transfected with GFP-R5 ($p > 0.05$). Thus, GFP-R5 subunits are targeted primarily to the somato-dendritic compartment, independent of the presence of endogenous GluR5, GluR6 and KA2 subunits.

GFP-R5 distribution along the dendrites depends on GluR6 and KA2 subunits

Although the overall axon/dendrite polarized distribution of GFP-R5 does not depend on GluR6 and KA2 subunits, we show that the distribution of GFP-R5 within the dendritic compartment does depend on GluR6 and KA2 subunits. To do so, we compared GFP-R5 subunit distribution along the dendrites of control and R5-R6-KA2$^{-/-}$ cultured neurons.

We first confirmed that dendrites originating from R5-R6-KA2$^{-/-}$ and control neurons were of similar length by measuring the entire length of dendrites originating from neurons transfected with GFP (Fig. 4A). We preferred this method over the direct measure of dendrites using MAP2 antibody for two reasons. First, MAP2 labeling does not label the entire length of dendrites. Second, because of the low-efficiency of the calcium-phosphate transfection method, dendrites originating from GFP-transfected neurons can be distinguished without ambiguity from those belonging to neighboring non-transfected neurons. The results showed that the dendritic length was similar in control and R5-R6-KA2$^{-/-}$ cultures (Fig. 4A). These findings enable us to quantify the dendritic distribution of GFP-R5 as a percentage of the mean of the entire dendritic length.

In control hippocampal neurons, the distribution of GFP-R5 proteins along the dendrites extended from the soma up to 100% of the entire dendritic length (Fig. 4B). Surprisingly however, in R5-R6-KA2$^{-/-}$ neurons, the distribution of GFP-R5 receptors was significantly altered ($p < 0.001$) and extended from the soma up to only 60% of the entire dendritic length (Fig. 4B). Based on these results, we defined two dendritic compartments: the “proximal” and “distal” dendrites, corresponding to the dendritic distribution of GFP-R5 in the absence or presence of GluR5, GluR6, KA2 subunits, respectively. Hence, proximal dendrites are defined as the region from the soma extending approximately halfway down the dendrite length, while distal dendrites extended from that midpoint to the terminal end of the dendrites (Fig. 4B). Thus, the presence of endogenous GluR6 and KA2 subunits is required for GFP-R5 localization to the distal dendrites.

To further determine whether the presence of either or both GluR6 or KA2 subunits is necessary to target GFP-R5 to the distal dendrites, we compared the dendritic distribution of GFP-R5 in neurons originating from single knock-out mice for KA2 subunits (KA2$^{-/-}$), double knock-out mice for GluR5 and GluR6 subunits (R5-R6$^{-/-}$) and triple knock-out mice (R5-R6-KA2$^{-/-}$). Again, the total dendritic length was not significantly different among neurons derived from the four genotypes examined (data not shown). Furthermore, the distribution of GFP-R5 subunits in GluR5$^{-/-}$ cultures was not different from control neurons ($p > 0.05$), suggesting that endogenous GluR5 subunits do not influence GFP-R5 dendritic localization. We found that the somato-dendritic distances of GFP-R5 measured in hippocampal neurons

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originating from KA2$^{-/-}$, R5-R6$^{-/-}$ and R5-R6-KA2$^{-/-}$ mice were similar, reaching 50–55% of the entire somato-dendritic length ($p > 0.05$, Fig. 4C). These data suggest that both GluR6 and KA2 subunits are necessary for localization of GFP-R5 to the distal dendrites.

To discard the possibility that the GFP-R5 receptors that we detected by immunofluorescence in the proximal dendrites of R5-R6-KA2$^{-/-}$ neurons correspond to an internal pool of non-functional receptors, we recorded electrophysiological responses from R5-R6-KA2$^{-/-}$ neurons expressing GFP-R5 in the proximal dendrites. Following local application on dendrites of the kainate receptor agonist ATPA (30 μM), in the presence of the AMPA receptor antagonist SYM 2206 (100μM), we found that GFP-R5 receptors expressed in the dendrites elicited a response (152.2 ± 36.8 pA, n=6) after application of ATPA (Fig. 4D). This effect was due to the activation of GFP-R5 receptors since the endogenous GluR5, GluR6 and KA2 subunits were absent in neurons taken from the knock-out mice. No current was detected when ATPA was applied more distally on dendrites where no fluorescence was detected or when applied onto adjacent non-transfected neurons (data not shown). These results show that the fluorescence emitted by GFP-R5 proteins is correlated with the presence of functional channels and demonstrate that GFP-R5 proteins expressed in the dendrites are functional.

GFP-R5 localization in distal dendrites depends on the motor protein KIF17

Our results suggest that GFP-R5 localization to distal dendrites requires association with endogenous GluR6 and KA2 subunits. We next wanted to identify the molecular mechanisms underlying localization of GFP-R5/GluR6/KA2 receptor complex in distal dendrites.

Interestingly, the proximal and distal dendrites as defined by the distribution of GFP-R5 proteins, mirrors the microtubule organization in dendrites (Baas et al., 1988). Due to the uniform population of microtubules in distal dendrites which have a plus-end orientation, proteins may be transported in distal dendrites only by association with plus-end microtubule-associated motors. KIF17 is a good candidate for localization of GFP-R5 in distal dendrites because it is a plus-end microtubule-associated kinesin (Setou et al., 2000) and it is expressed in both proximal and distal dendrites (Guillaud et al., 2003) This led us to determine whether there was an interaction between KIF17 and kainate receptors.

We first compared the distribution of GFP-R5 relative to endogenous KIF17 in cultured hippocampal neurons using immunocytochemistry. We found that GFP-R5 subunits colocalized with KIF17 in the soma and dendrites of hippocampal neurons (Fig. 5).

We next determined biochemical interactions between kainate receptors and KIF17 in hippocampus, by performing coimmunoprecipitation experiments using P2 membrane preparations from mice. Using GluR6/7 and KA2 antibodies, we showed an interaction between KIF17, GluR6 and KA2 subunits. In wild-type mice, anti-KIF17 antibody coimmunoprecipitated GluR6/7 and KA2 proteins (Fig. 6A). Inversely, anti-KA2 antibodies coimmunoprecipitated KIF17 proteins (Fig. 6B, upper lane) and anti- GluR6/7 antibodies pulled-down both KIF17 and KA2 proteins (Fig. 6B, upper and lower lanes). These results demonstrate that KIF17 binds to GluR6/7 and KA2 subunits, suggesting the existence of a GluR6/7/KIF17/KA2 protein complex in hippocampus. Endogenous GluR5 was not required to form the complex since experiments performed with GluR5$^{-/-}$ mice were identical to those performed in wild-type mice (data not shown). We next checked the specificity of KIF17 interaction with GluR6 and KA2 subunits using knock-out mice (Fig. 6C). As expected, in hippocampal preparations from KA2$^{-/-}$ mice, anti-KIF17 antibodies coimmunoprecipitated GluR6/7 proteins but not KA2 proteins (Fig. 6C). Similarly, immunoprecipitation with anti-KIF17 antibody from GluR6/7$^{-/-}$ hippocampal preparations yielded KA2 immunoreactivity but no GluR6/7 immunoreactivity. To further ensure the specificity of KIF17 binding to GluR6 subunits, we also performed experiments in GluR7$^{-/-}$ mice. In absence of GluR7 subunit,

KIF17 coimmunoprecipitated KA2 and GluR6 subunits. In agreement with a previous study (Setou et al., 2002), we could not detect binding between KIF17 and the AMPA GluR2 receptors (Fig. 6C). These results suggest the existence of a specific GluR6/KA2/KIF17 protein complex in the hippocampus *in vivo*.

The above data suggest that GluR5 localization in distal dendrites depends on association with the GluR6/KA2/KIF17 complex. To confirm KIF17 role in GFP-R5 localization in distal dendrites, we blocked KIF17 activity by overexpression of a dominant-negative form of KIF17, KIF17 $^{\Delta 306}$, that lacks its motor domain (Guillaud et al., 2003). To ensure that each transfected neuron coexpressed both proteins, either KIF17 or KIF17 $^{\Delta 306}$ cDNA was subcloned into the GFP-R5 expression vector (see Methods). While GFP-R5 subunits were distributed both in the proximal and distal dendrites in neurons overexpressing KIF17, they were restricted to the proximal dendrites when coexpressed with KIF17 $^{\Delta 306}$ ($p < 0.001$) (Fig. 7A & B). Taken together, these data show that GFP-R5 localization in distal dendrites is KIF17-dependent and likely occurs through interaction with GluR6 and KA2 subunits.

Discussion

Using a combination of techniques including electrophysiology and immunocytochemistry combined with confocal analyses of GFP-tagged GluR5 subunits, we have shown that GluR5 receptors are mainly localized in the somato-dendritic compartment of hippocampal neurons where they form functional, surface-expressed channels. Since GluR5 subunits can form heteromers with GluR6 and KA2 subunits, we analyzed whether GluR6 and KA2 proteins played a role in GluR5 subcellular localization, by measuring GluR5 distribution in neurons originating from control and R5-R6-KA2$^{-/-}$ mice. To do so, we quantified not only the presence/absence of the GluR5 protein in dendrites but also GluR5 distribution along the somato-dendritic compartment. We showed that GluR5 distribution in dendrites is compartmentalized and its distribution to distal dendrites requires coexpression of endogenous GluR6 and KA2 subunits. In contrast, when GluR6 and KA2 subunits are knocked-out, GluR5 receptors are expressed in the proximal dendrites where they form functional channels. Our study also shows that the localization of GFP-R5 in distal dendrites requires the microtubule motor protein KIF17, likely via the formation of a complex of GluR5/GluR6/KA2 and KIF17. To our knowledge, this is the first study to show that subunit composition can influence localization of ionotropic receptors in neurons via interaction with the microtubule-motor protein KIF17, demonstrating the existence of a novel mechanism by which receptors are localized in neurons.

Dendritic Compartmentalization of GluR5 in Hippocampal Neurons

One of the most striking and surprising findings of this study is the fact that the distribution of GFP-R5 subunits in distal dendrites depends on coexpression of endogenous GluR6 and KA2 subunits, suggesting the coassembly of GluR5 with GluR6 and KA2 subunits in distal dendrites. The involvement of GluR6 subunits is not surprising considering that 90% of hippocampal neurons in culture express GluR6 mRNA (Craig et al., 1993). KA2 subunits cannot form functional channels unless they coassemble with one of the GluR5-GluR7 subunits (Herb et al., 1992; Lomeli et al., 1992; Schiffer et al., 1997). Anatomical and biochemical studies strongly suggest that KA2 coassembles with GluR6 subunits. The distribution of KA2 and GluR6/7 subunits overlaps in hippocampus *in vivo* (Gallyas et al., 2003) and *in vitro* (Roche and Huganir, 1995; Janssens and Lesage, 2001). KA2 subunits coimmunoprecipitated GluR6 but not GluR7 in hippocampus *in vivo* (Darstein et al., 2003), indicating that the two subunits assemble into the same complex. Evidence from electrophysiology support GluR5 and GluR6 coassembly into functional heteromeric channels (Cui and Mayer, 1999; Paternain et al., 2000). In the present study, we show that GFP-R5 coimmunoprecipitates with GluR6

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and KA2 in HEK 293T cells (Fig. 2A). Altogether, these results suggest the coassembly of GluR5, GluR6 and KA2 subunits into heteromeric channels.

The compartmentalization of dendrites into proximal and distal domains based on GluR5 distribution adds another degree of complexity to the classical distinction between axon and dendrites. The non-uniformity of the dendritic compartment is an emerging concept from studies of voltage-gated ion channels, including potassium (Hoffman et al., 1997), calcium (Delmas et al., 2000; Williams and Stuart, 2000) and the hyperpolarization-activated channel I_h (Magee, 1998), and also from studies of ionotropic receptors (Dodt et al., 1998; Pettit and Augustine, 2000; Andrasfalvy and Magee, 2001; Frick et al., 2001). Interestingly, a recent study showed that in the absence of endogenous $GABA_{B(2)}$ subunits, there is a redistribution of $GABA_{B(1)}$ subunits from the distal to proximal dendrites in the hippocampus (Gassmann et al., 2004), supporting the concept of a functional subdivision of dendrites into proximal and distal compartments.

What is the functional significance of proximal and distal dendrites? The proximal and distal dendrites could reflect the topographic organization of neuronal inputs, as has been documented in many areas in the brain (Smith and Bolam, 1990; Sherman and Guillery, 1996). For example, proximal and distal dendrites of dentate granule cells and CA3 hippocampal pyramidal cells are connected to the medial and lateral entorhinal area, respectively, and are associated with different functional properties (Min et al., 1998). Our results are the first to document heterogeneity of kainate receptor distribution along hippocampal dendrites that depends upon receptor subunit composition. It is well known that subunit composition determines the functional properties of ionotropic receptors (Dingledine et al., 1999). It is likely that the differential localization of GluR5 in dendrites is associated with different kainate receptors with distinct functional properties.

GluR5 localization in proximal dendrites

Our data show that GluR5 localization in the proximal dendrites does not depend on the presence of GluR6 and KA2 subunits or KIF17 motor function. Among the possible explanations, GluR5 subunits could be transported from the soma to the proximal dendrites in a kinesin-independent fashion by simple diffusion or via other motor proteins such as myosin (Bridgman, 2004). Alternatively, the composition of GluR5-containing receptors could be different in proximal dendrites and result in the association with other kinesin motor proteins. For example, GluR5 subunits could form homomeric channels or coassemble with GluR7 (Cui and Mayer, 1999) and/or KA1 subunits to form heteromeric channels. Other kinesins such as KIF21A and KIF21B (Marszalek et al., 1999), KIFC2 (Saito et al., 1997) and KIF5 (Setou et al., 2002) which are expressed in dendrites in hippocampus could be involved in the localization of GluR5 subunits in proximal dendrites, but their exact location in proximal and distal dendrites needs to be determined.

Kainate receptor interaction with KIF17 as a molecular mechanism for GluR5 localization to distal dendrites

It is interesting to find that the existence of proximal and distal compartments defined by the GluR5 distribution, mirrors the organization of microtubules described in dendrites in cultured hippocampal neurons (Baas et al., 1988). This is suggestive of a role for microtubules in GluR5 localization to distal dendrites. Three pieces of evidence suggest that GluR5 localization to distal dendrites is accomplished through the formation of a GluR5/GluR6/KA2/KIF17 interacting complex. First, GluR5 requires the coexpression of GluR6 and KA2 subunits to be localized to distal dendrites. Second, GluR6 and KA2 subunits interact with KIF17 proteins *in vivo*. Third, GluR5 localization to distal dendrites depends on the integrity of KIF17 function

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since the blockade of its activity by overexpression of a dominant-form of KIF17 lacking its motor domain prevents GluR5 from being localized in distal dendrites.

Recent evidence has led to the suggestion that specific motor proteins could be associated with specific receptors. For example, the AMPA GluR2 receptor and the NMDA NR2B receptors are associated with KIF5 (Setou et al., 2002) and KIF17 (Setou et al., 2000), respectively. In this study we demonstrate that KIF17 is also involved in the localization of kainate receptors. In cultured hippocampal neurons, Guillaud et al. (2003) found that only 45 % of KIF17-containing vesicles are associated with NR2B subunits. It is likely that at least a proportion of the remaining KIF17-vesicles is associated with kainate receptors. This indicates that KIF17 mediates the transport of both NMDA and kainate receptors, demonstrating that KIF17 function is not restricted to a single receptor subtype. It is of interest that both NMDA and kainate receptors have been shown to mediate synaptic plasticity (Bliss and Collingridge, 1993; Bortolotto et al., 1999; Contractor et al., 2001; Schmitz et al., 2001; Vissel et al., 2001).

Experimental methods

Neuronal cultures

Primary hippocampal cultures were prepared from P0-P1 rats (Long-Evans) or mice (2–3 animals per experiment) as previously described (Boyer et al., 1998). Since there was no difference between rat and mouse cultures for dendritic length, kainate receptor expression or distribution of GFP-R5, data were pooled and referred to as control. Cells were plated at a density of 35–50 × 10^3 cells/cm^2 onto a feeder layer of hippocampal astrocytes (from the same species as the neurons) that had been laid down 3–7 days earlier and maintained in an incubator at 37°C in the presence of 5% CO_2. Cytosine-β-D-arabinoside (2.5 μM) was added after 7 days to inhibit proliferation of non-neuronal cells.

Calcium Phosphate transfection of neurons

Primary neurons growing in 24-well plates were transfected at DIV 3 using calcium phosphate precipitation (Xia et al., 1996). 1 μg of endotoxin-free DNA was used for each well.

Immunocytochemistry

Neurons were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 20 min, permeabilized in methanol for 10 min at −20°C, blocked in 10% bovine serum albumin (BSA) in PBS for 1 hr and incubated overnight at 4°C with mouse anti-MAP2 antibody (Sigma; 1:500) or rabbit anti-KIF17 antibody (Sigma; 1:200) and rabbit anti-GFP antibody (Clontech; 1:1000) or chicken anti-GFP antibody (Chemicon; 1:800) diluted in 1% BSA-PBS. The cells were then washed, incubated with biotin anti-rabbit IgG (Vector; 5 μg/ml) or biotin anti-chicken IgG (Vector; 1:500) and Cy™ 3 anti-mouse or anti-rabbit IgG (Amersham; 1μg/ml) for 1–2 h, and finally fluorescein avidin D (Vector Laboratories; 7 μg/ml) in 1% BSA-PBS for 1–2 h. The coverslips were mounted with antifading medium.

Microscopy and quantitation

Transfected neurons were analyzed at both DIV 7 and DIV 15. The choice of two time-point in culture was motivated by previous work showing a redistribution of receptors during development of hippocampal neurons in culture (Craig et al., 1993). At DIV 7, neurons are polarized with distinctive dendrites and axons (Dotti et al., 1988). At DIV 15, neurons are fully matured with a high density of dendritic spines (Boyer et al., 1998). However, we found no statistical differences for the localization of GFP-R5 between DIV 7 and 15, and data were pooled. For electrophysiological experiments, live transfected neurons were identified by the

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fluorescence emitted by GFP in the soma and dendrites. For precise measurements of dendritic and axonal lengths, we used an anti-GFP antibody to enhance the signal emitted by GFP and an anti-MAP2 antibody to distinguish between dendrites (MAP2-positive processes) and axons (MAP2-negative processes). Confocal image analysis was conducted on z-compressed image stacks of the entire depth of the transfected neuron, allowing careful examination of all GFP-labeled process. Images were captured on a Zeiss laser-scanning confocal microscope (LSM 510) using a 25 x oil immersion lens. GFP/fluorescein fluorescence was excited using a 488 nm argon/krypton laser and emission was detected with a 505–550 nm bandpass filter. Cy™3 fluorescence was excited using a 568 nm argon/krypton laser and detected with a 585 nm bandpass filter. Under these conditions, no bleed-through of fluorescence between the two channels was observed. For each set of experiments (i.e. neuronal cultures prepared, transfected and processed for immunocytochemistry at the same time) identical acquisition parameters and settings were used. For each transfected neuron, two measures were made. 1) the presence of labeled axon and/or dendrites were scored. 2) the maximal somato-dendritic (or somato-axonal) distances of GFP fluorescence were measured in the longest dendrite using the Zeiss LSM software program. This value was then expressed as a percent of the mean dendritic length calculated from GFP-transfected neurons. All observations were made blind. Statistical analyses were performed using SPSS.

Electrophysiology

Whole-cell membrane currents from hippocampal neurons were recorded at room temperature using a patch-clamp amplifier (Axopatch 200A). Patch pipettes were pulled from borosilicate glass capillaries (Corning 7740, Warner Instrument Corp, Hamden, CT) and had open tip resistances of 5–7 MΩ after being fired polished (MF-83, Narishige, Japan) and filled with a pipette solution composed of (mM): 140 CsCl, 4 NaCl, 0.5 CaCl2, 5 EGTA, 10 HEPES, 0.5 NaGTP and 2 Mg-ATP, 315 mOsm, pH 7.33 (adjusted with CsOH). Cells were constantly perfused with a HEPES-buffered external solution composed of (mM): 146 NaCl; 2.5 KCl, 1.2 $CaCl_2$, 20 mM sucrose, 20 mM glucose, 10 mM Hepes, pH 7.4 (adjusted with NaOH), 310 mOsm. In addition, the external solution contained 100 μM SYM 2206, 100 μM APV, 10 μM Bicuculline, 1 μM Strychnine and 1μM TTX. Brief pulses (100–300 ms) of the agonist solution (ATPA, 30 μM) were applied onto the neuron (either the soma or the dendrites) by pressure microejection (25 psi) using pipettes identical to the recording pipettes filled with agonist and connected to a Picospritzer II (General Valve Corporation, Fairfield, NJ). Signals were filtered at 2 kHz, digitized at 20 kHz, and stored on a computer using PClamp 8 software (Axon Instruments).

Cell surface expression

Human 293T cells (American Type Culture Collection) were transiently transfected with various cDNAs by calcium phosphate precipitation and subjected to flow cytometry two days later (Tsao and von Zastrow, 2000).

Since the GFP tag is extracellular, surface receptors were measured by indirect immunofluorescence labeling of intact cells with GFP antibody using phycoerythrein (PE) fluorophore (PE-streptavidin, Molecular Probes; 1 μg/ml). The total level of expression of GFP-tagged receptors was determined by the direct fluorescence of GFP. To eliminate dead cells, cells were stained with DAPI (5 μg/ml). Surface receptor fluorescence intensity was quantitated using a LSR 3-laser analytical flow cytometer (Becton Dickinson, Palo Alto, CA) equipped for detection of GFP, PE and DAPI. For each sample 30,000 cells were collected. Cellquest software (Becton Dickinson) was used to calculate the mean fluorescence intensity of single cells. Cells expressing GluR5 (without GFP tag) labeled with PE were used as negative controls to establish the background values of PE and GFP emission. The relative percent of the receptor on the cell surface was estimated as the ratio of PE fluorescence intensity and total

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fluorescence intensity (PE + GFP). Each ratio was then normalized to the ratio value measured for the non-functional (NF) version of GFP-R2. GFP-R2NF was constructed by inserting GFP tag in the N-terminus of GluR2. GFP-R2NF was expressed but failed to form functional receptors as determined by the lack of outward rectification measured by patch clamp in cells cotransfected with GluR4.

In vitro immunoprecipitation and immunoblotting

Glutamate receptors from transiently transfected HEK293T cells or primary cultures were immunoprecipitated, subjected to SDS-PAGE and western blot analysis (Perez-Otano et al., 2001a). The primary antibodies were all made in rabbit: anti-GFP, 1:2000, anti-calreticulin, 1:2000 (ABR), anti-GluR6/7, 1:300 and anti-KA2, 1:800 (Upstate biotechnology), anti-AMPA (anti-GluR1, 1:100, anti-GluR2/3, 1:100, Chemicon). Immunoblots were incubated with horseradish peroxide-conjugated goat antirabbit antibodies (1:5000, Biorad) for 1 hr at room temperature and developed by chemiluminescence (Amersham, Arlington Heights, IL).

In vivo immunoprecipitation

P2 membrane proteins were prepared from P0-P1 mice hippocampi as follows. Using a Polytron, hippocampi were homogenized twice in 5 ml of ice-cold 10 mM Tris.HCl/0.1 mM EDTA, pH 8.0, containing 320 mM sucrose with a 20-sec interval between burst. The tissue homogenate was centrifuged at 700 x *g* for 10 min at 4°C. The pellet was rehomogenized and spun again. The supernatants were combined and centrifuged at 37,000 *g* at 4°C for 40 min. The pellet was resuspended in 20 mM Tris.HCl/4 mM EDTA, pH 8.0. The P2 membrane preparation was solubilized using non-denaturing conditions as described (Luo et al., 1997). The solubilized membrane preparations were preincubated with proteinA/G sepharose during 5 hours at 4°C to preclear the preparation, then incubated with antibody precoupled to proteinA/G sepharose overnight at 4°C. The immunopellet was washed 5 times with 1 ml of binding buffer. The proteins were solubilized in 2 x sample-loading buffer, heated at 70°C for 10 min and separated on SDS-polyacrylamide gels.

Generation of mutant expression plasmids

We used the unedited GluR5-2a isoform cDNA generously provided by P. Seeburg (Max Planck Institute, Heidelberg, Germany). The GFP-R5 construct was made by inserting EGFP (Clontech, Palo Alto, CA) in- frame at the extracellular N-terminus between the fifth and sixth amino acids after the predicted signal peptide cleavage site. KIF17 cDNA (Setou et al., 2000) was isolated from RT-PCR of brain RNA. The dominant-negative form of KIF17 cDNA (KIF17 $^{\Delta 306}$) which lacks the microtubule binding domain (a.a. 1–306) (Guillaud et al., 2003) was generated by PCR using modified primer set and sequenced. Both KIF17 and KIF17 $^{\Delta 306}$ were modified to contain the Kozak sequence at the translation start sites (Kozak, 1999) and separately subcloned into pcDNA 3.1 vector (Invitrogen, Carlsbad, CA). Whole expression cassettes, including CMV promoter, cDNA, and BGH polyadenylation sequence from pcDNA 3.1 vector, were then subcloned into the GFP-R5 expression vector away from cloning site to express both GFP-R5 and KIF17 or GFP-R5 and KIF17 $^{\Delta 306}$ from the same vector.

Generation of kainate receptor-deficient mice

R5-R6-KA2$^{-/-}$ mutant mice were generated by crossing mice deficient for GluR5 (Mulle et al., 2000), GluR6 (Mulle et al., 1998) and KA2 (Contractor et al., 2003). The absence of GluR5 (Mulle et al., 2000), GluR6 (Mulle et al., 1998) and KA2 subunits (Contractor et al., 2003 and see Fig. 3B) has been verified in the corresponding knock-out mice. GluR6/7$^{-/-}$ mice were generated by crossing mice deficient for GluR6 and GluR7 (unpublished). The absence of GluR6 and GluR7 proteins was verified by the absence of GluR6/7 immunoreactive band

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detected on Western blots from GluR6/7$^{-/-}$ hippocampi (data not shown). The genetic background of the control mice was isogenic 129SvEv. Each knock-out mouse used in the study was genotyped by Southern blot analysis of tail DNA.

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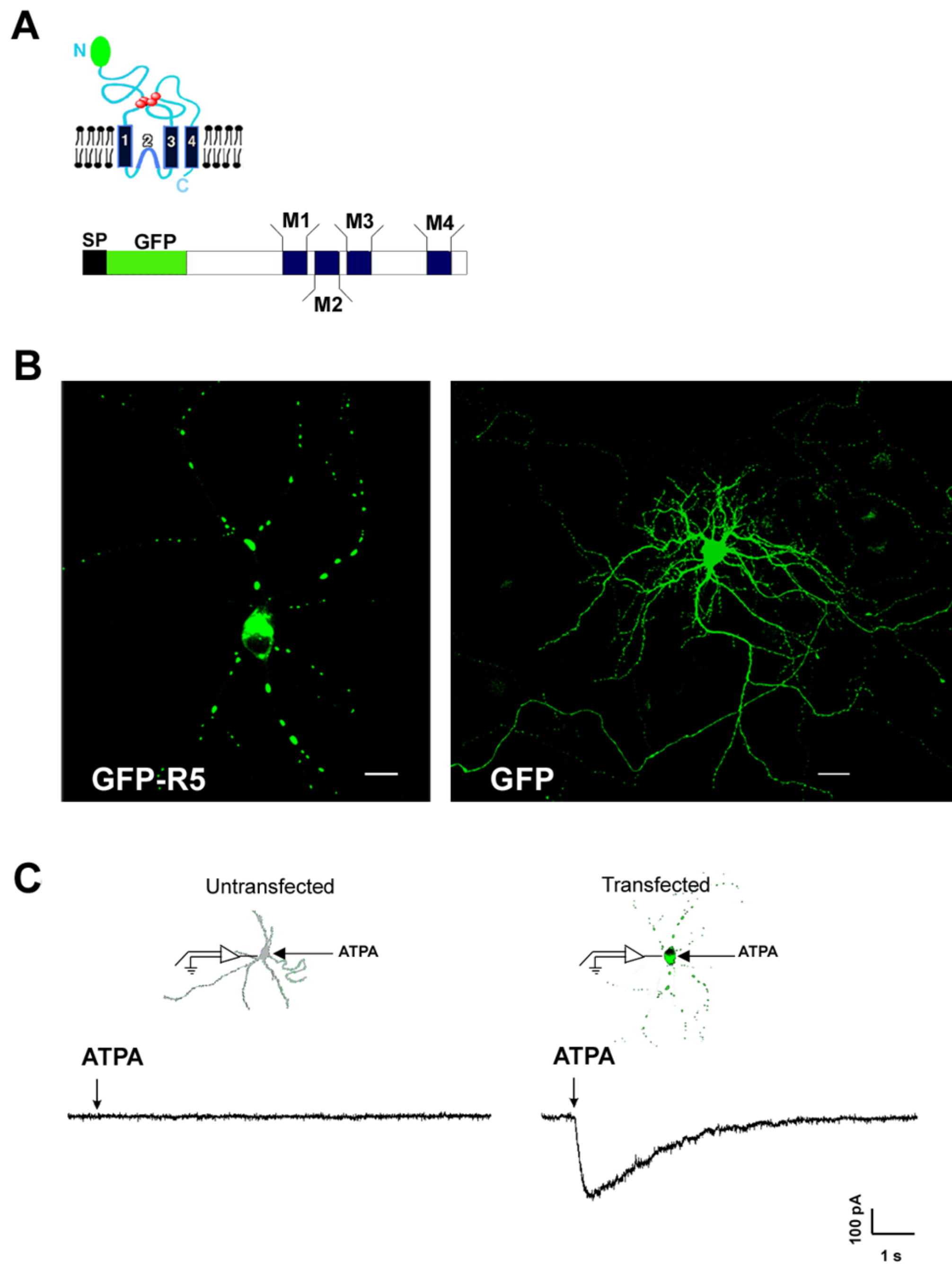

Fig. 1.

GFP-R5 forms functional receptors in hippocampal neurons. (A) Schematic diagram of GFP-R5 structure. The GFP tag (green bar) was inserted in the N-terminal extracellular domain between the fifth and sixth amino acids after the predicted signal peptide (SP). (B) The punctate distribution of GFP-R5 subunits (left panel) in neurons contrasts with the diffuse distribution of GFP in all the neuronal compartments (right panel). These two confocal images represent a single focal plane taken at the level of the nucleus. Note the absence of GFP-R5 expression in the nucleus when compared to GFP-expressing neurons. Scale bar, 20 μm. (C) GFP-R5 subunits form functional channels in cultured primary hippocampal neurons. At DIV7, application of ATPA (30 μM), in the continuous presence of SYM2206 (100 μM) onto neurons

originating from R5-R6-KA2$^{-/-}$ deficient mice, elicited no current in untransfected neurons (n=12) whereas an inward current was observed in the soma of a GFP-R5 transfected neuron (n=14). Similar results were obtained at DIV15 in untransfected (n= 2) and transfected cells (n= 4).

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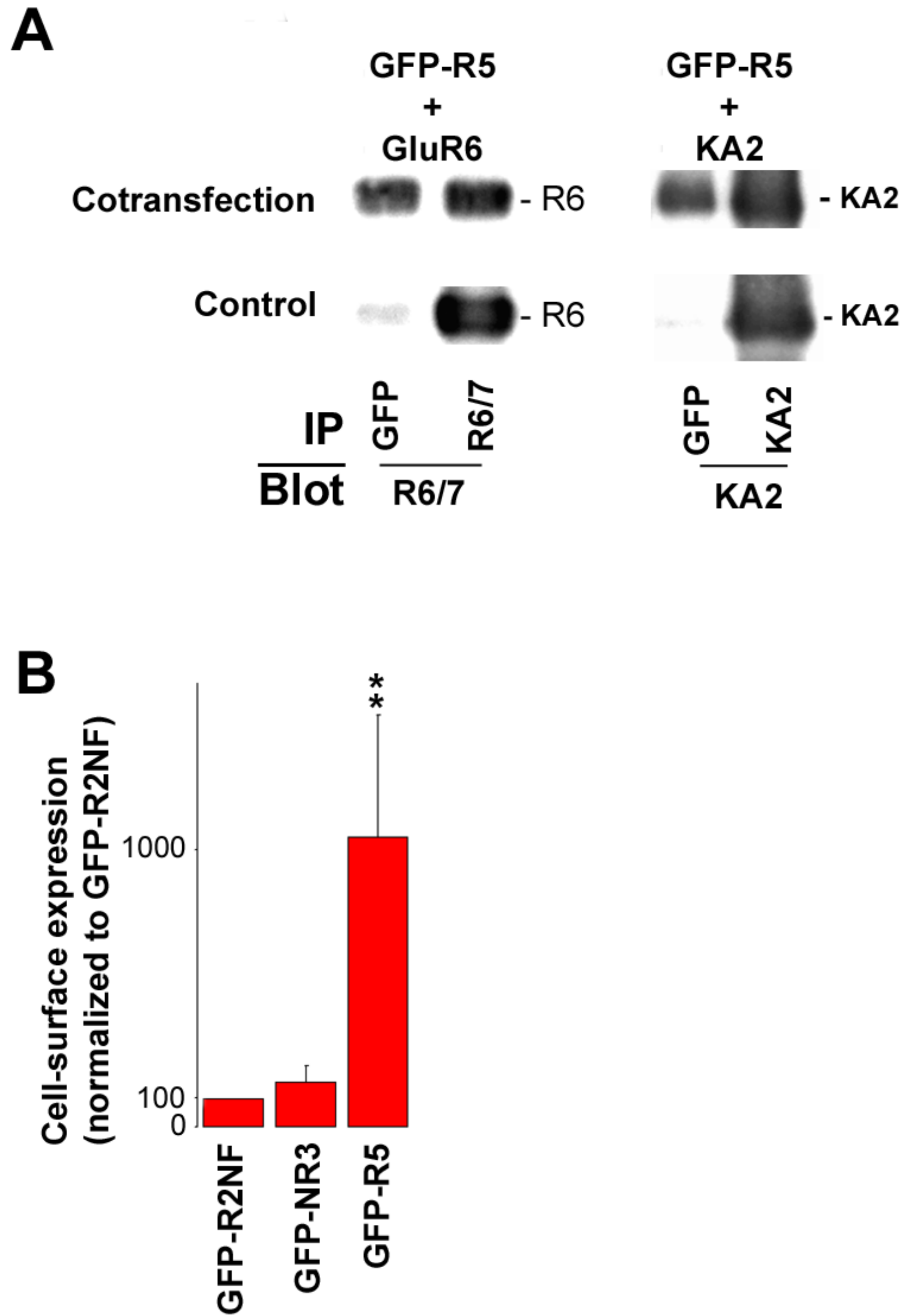

Fig. 2.
GFP –R5 shows normal assembly properties and surface expression. (A) GFP-R5 subunits co-assemble with GluR6 (left panel) and KA2 subunits (right panel). HEK 293T cells were cotransfected with the combinations of kainate receptor subunits indicated above each lane. Lysates were immunoprecipitated (IP) and immunoblotted with antibodies as indicated. Lower panel shows the corresponding control, where we checked that mixing lysates from cells expressing individual subunits (as indicated above) only resulted in background coimmunoprecipitation (n=5 independent experiments). (B) GFP-R5 subunits are expressed at the cell surface as measured by flow-cytometry (see Methods). Values represent the mean

(± S.E.M) of 4 independent experiments. ** $p < 0.01$ when compared to GFP-R2NF (Mann-Whitney post-hoc test).

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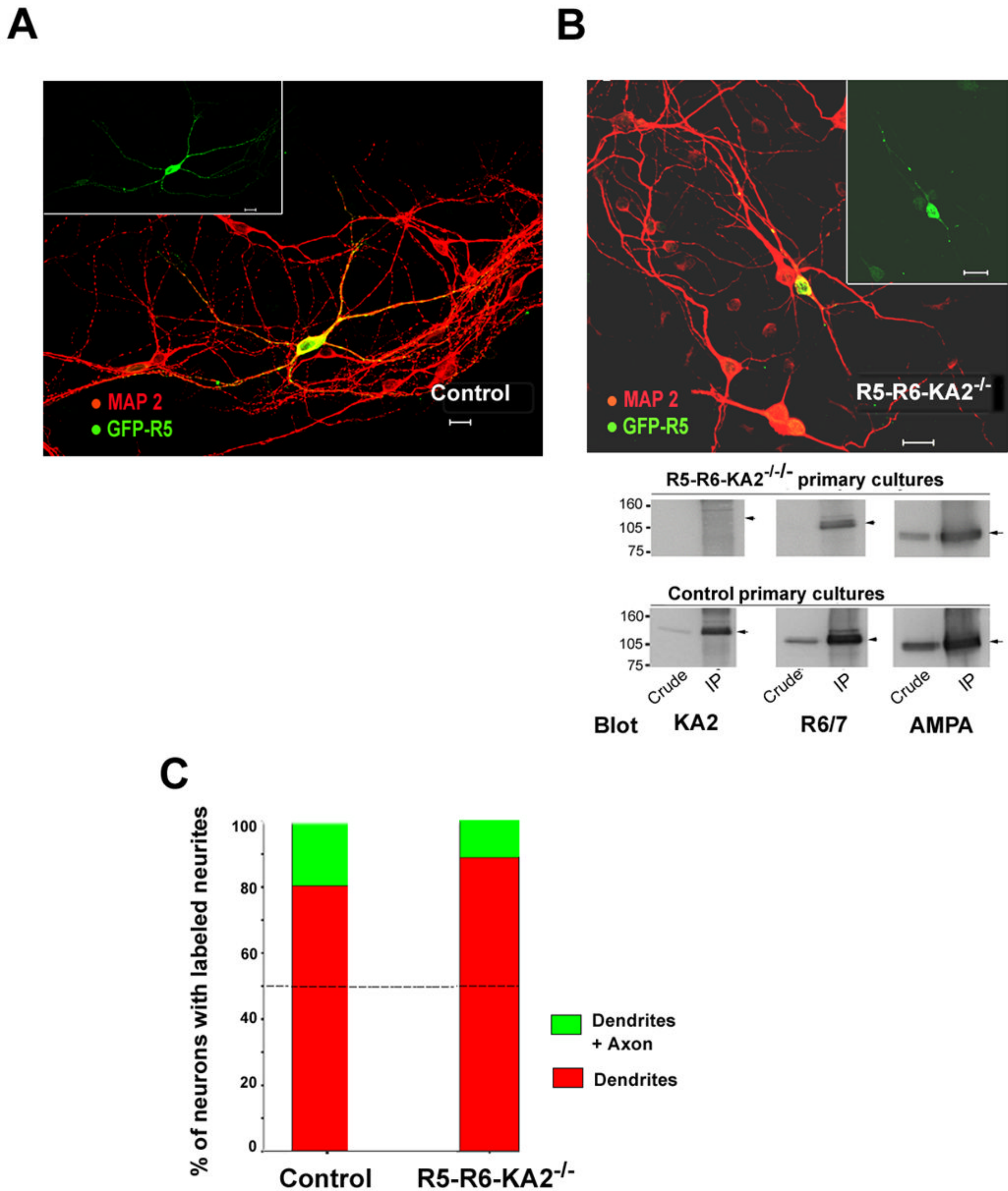

Fig. 3.

The distribution of GFP-R5 is polarized to the somato-dendritic compartment. Panel A and B illustrate neurons taken from control (A) and R5-R6-KA2$^{-/-}$ cultures (B), expressing GFP-R5 in the somato-dendritic compartment (yellow = colocalization of MAP2 and GFP). Scale bar, 20 μm. (Lower panel) Endogenous GluR6 and KA2 subunits are expressed in control but absent in R5-R6-KA2$^{-/-}$ hippocampal cultures. Lysates from hippocampal cultures were immunoblotted directly (crude) or IP with antibodies as indicated and then immunoblotted with the same antibody used for IP. 0.3 % of lysates from hippocampal cultures (2500 × 10^3 hippocampal cells) was loaded directly on the western blot gel (crude) while the remaining lysates were immunoprecipitated (IP) with the same antibody used for immunoblotting. Note

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in R5-R6-KA2$^{-/-}$ primary cultures the absence of KA2 subunits. Note also that the remaining immunoreactive band detected in R5-R6-KA2$^{-/-}$ cultures with the anti-GluR6/7 antibody corresponds to GluR7 subunits. No GluR6/7 immunoreactivity was detected in GluR6/7$^{-/-}$ knock-out mice (data not shown). Molecular size markers (in kilodaltons) are shown on the left. Scale bar, 20 μm. (C) The quantitative analysis of GFP-R5 dendrite/axon polarity in control and R5-R6-KA2$^{-/-}$ hippocampal cultures. The graph indicates the percent of transfected neurons exhibiting dendritic labeling only (red) or dendritic with axon labeling (green) (n = 50 neurons, 9 independent experiments).

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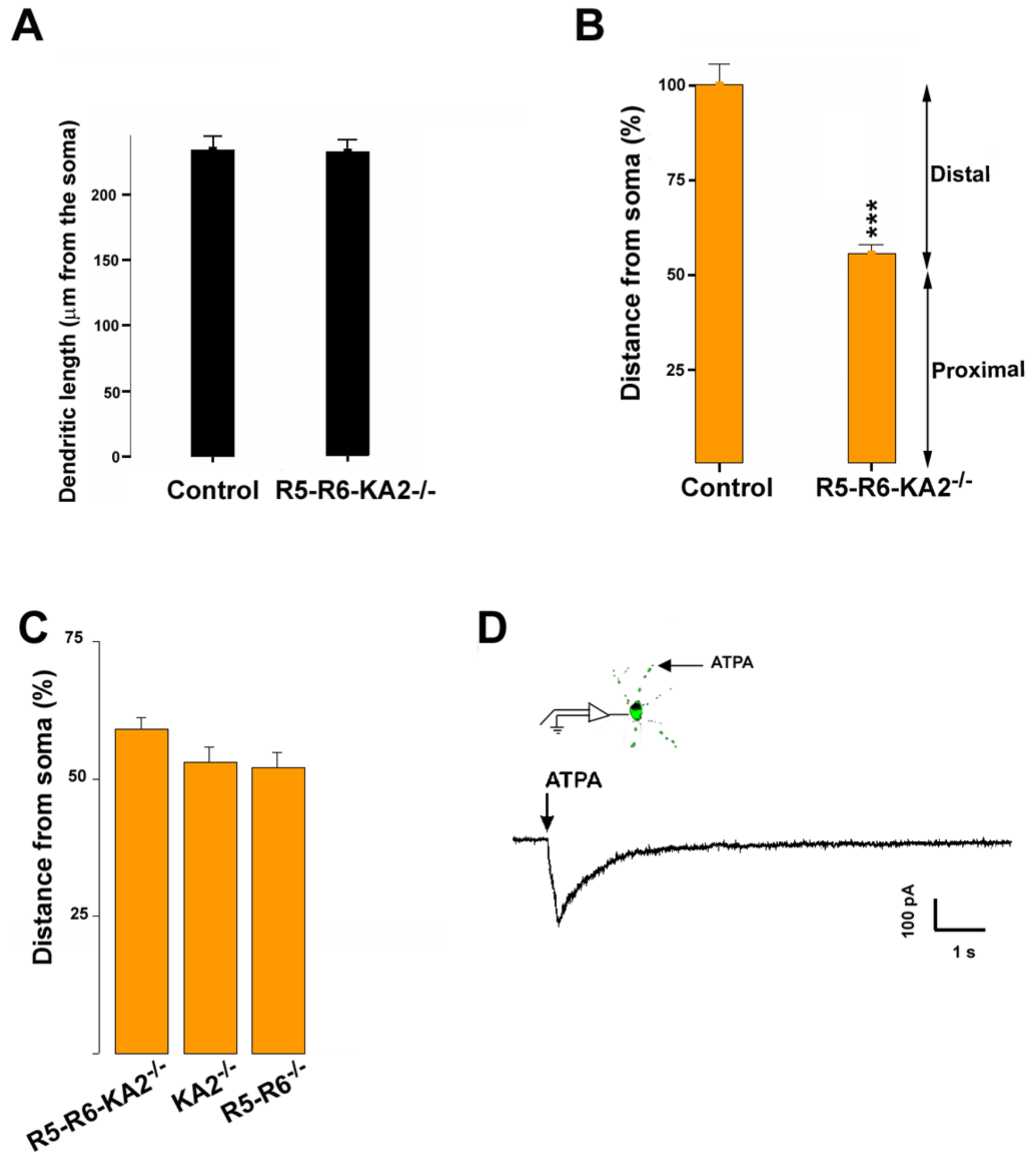

Fig. 4.

The dendritic distribution of GFP-R5 depends on GluR6 and KA2 subunits. (A) The total dendritic length, as measured by the extent of GFP-labeling in dendrites, does not differ between hippocampal neurons cultured from control and R5-R6-KA2$^{-/-}$ mice. Data are derived from the same cultures as in Figure 3C and represent the mean (± S.E.M) somato-dendritic distance measured for each neuron. (B) Quantitative analysis of the dendritic location of GFP-R5. Data are derived from the same cultures reported in panel A. The distance from soma was reported as a percentage of the mean maximal dendritic length calculated from panel A. *** $p < 0.001$ when compared to control hippocampal cultures (Dunnett post-hoc test). A representation of proximal and distal dendrites defined from GFP-R5 distribution is also

depicted on the right axis. (C) Both GluR6 and KA2 subunits are required for GFP-R5 localization to distal dendrites. The dendritic location of GFP-R5 was similar in neurons originating from R5-R6-$KA2^{-/-}$, $KA2^{-/-}$ and R5-$R6^{-/-}$ knock-out mice ($p > 0.05$), and the mean ± S.E.M dendritic location was reported for each condition as a percentage of the maximal dendritic length (n = 70–120 neurons per condition from 3 independent experiments). (D) GFP-R5 receptors expressed in the proximal dendrites of R5-R6-$KA2^{-/-}$ hippocampal neurons are functional. To ensure that only dendritic GFP-R5 receptors were activated by ATPA, the tip of the application pipette was positioned less than 5 μm away from the dendrite with the soma located upstream of the application pipette. Whole-cell currents were evoked by local application of ATPA (30 μM) in the presence of SYM2206 (100 μM), to the proximal dendrite (100 μm from the soma) of hippocampal neurons expressing GFP-R5 (n=6).

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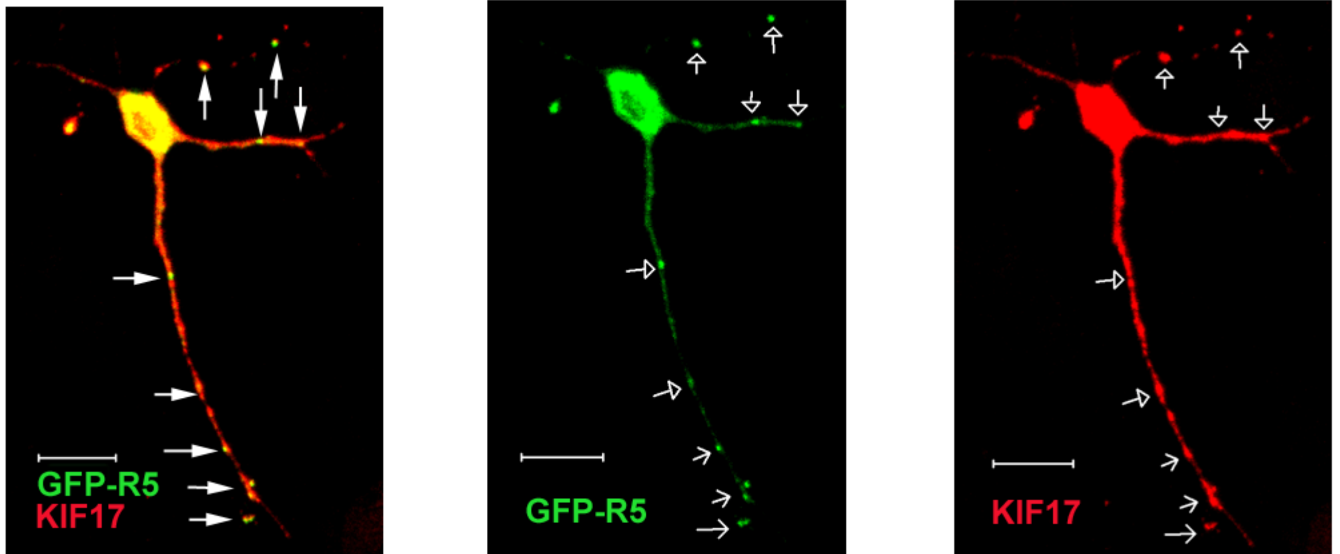

Fig. 5.
Colocalization of GFP-R5 and KIF17 in hippocampal neurons. Immunocytochemistry in control neurons shows colocalization (arrows) of GFP-R5 (green) and endogenous KIF17 (red) proteins. Scale bar, 20 μm.

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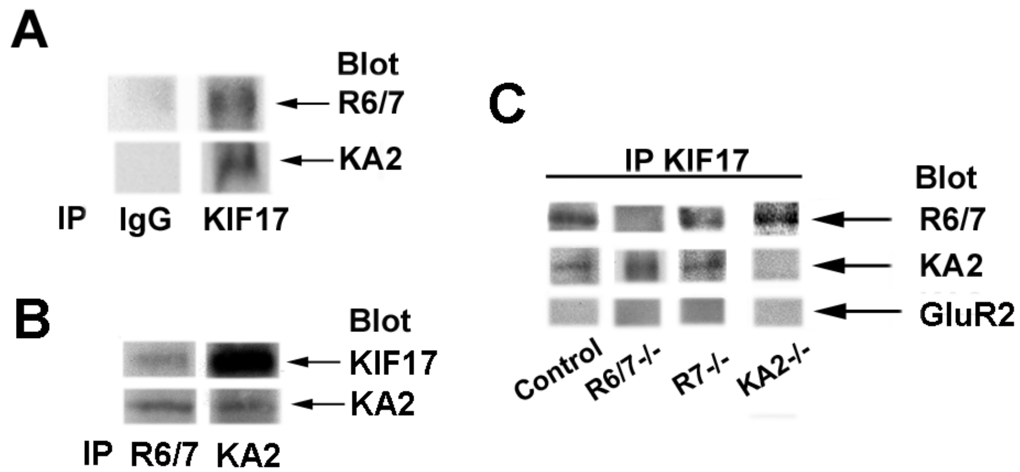

Fig. 6.

In vivo interaction of GluR6 and KA2 subunits with KIF17 in mouse hippocampi. (A) KIF17 coimmunoprecipitates GluR6 and KA2 subunits. Membrane proteins from 6 hippocampi were IP with an anti-IgG or anti-KIF17 antibody (20 µl) and immunoblotted with anti-GluR6/7 antibodies (upper panel) or anti-KA2 antibodies (lower panel). No signal was detected when membranes were IP with an anti-IgG antibody. (B) GluR6 and KA2 subunits coimmunoprecipitate KIF17 proteins. Hippocampi membrane proteins were IP with anti-R6/7 (5 µg, left panel) or anti-KA2 antibodies (10 µg, right panel) and immunoblotted with anti-KIF17 antibodies (1:1000, upper panel). The same immunoblot was then reprobed with anti-KA2 antibodies (lower panel) revealing that GluR6/7 subunits interact with both KIF17 and KA2 proteins. (C) KIF17 interaction with GluR6 and KA2 subunits is specific. Immunoprecipitation from $KA2^{-/-}$ hippocampi with anti-KIF17 antibodies yielded GluR6/R7 subunits but no band immunoreactive for KA2 was detected. In $GluR7^{-/-}$ mice, KIF17 coimmunoprecipitated KA2 subunits and GluR6 subunits. In $GluR6/7^{-/-}$ preparations, KIF17 coimmunoprecipitated KA2 subunits but no band immunoreactive for GluR6 or GluR7 was detected. We also verified that KIF17 did not bind to the AMPA GluR2 subunits by showing that no immunoreactive band for GluR2 antibodies (1:200, Chemicon) could be detected after immunoprecipitation of KIF17 from wild-type (WT) and knock-out mice.

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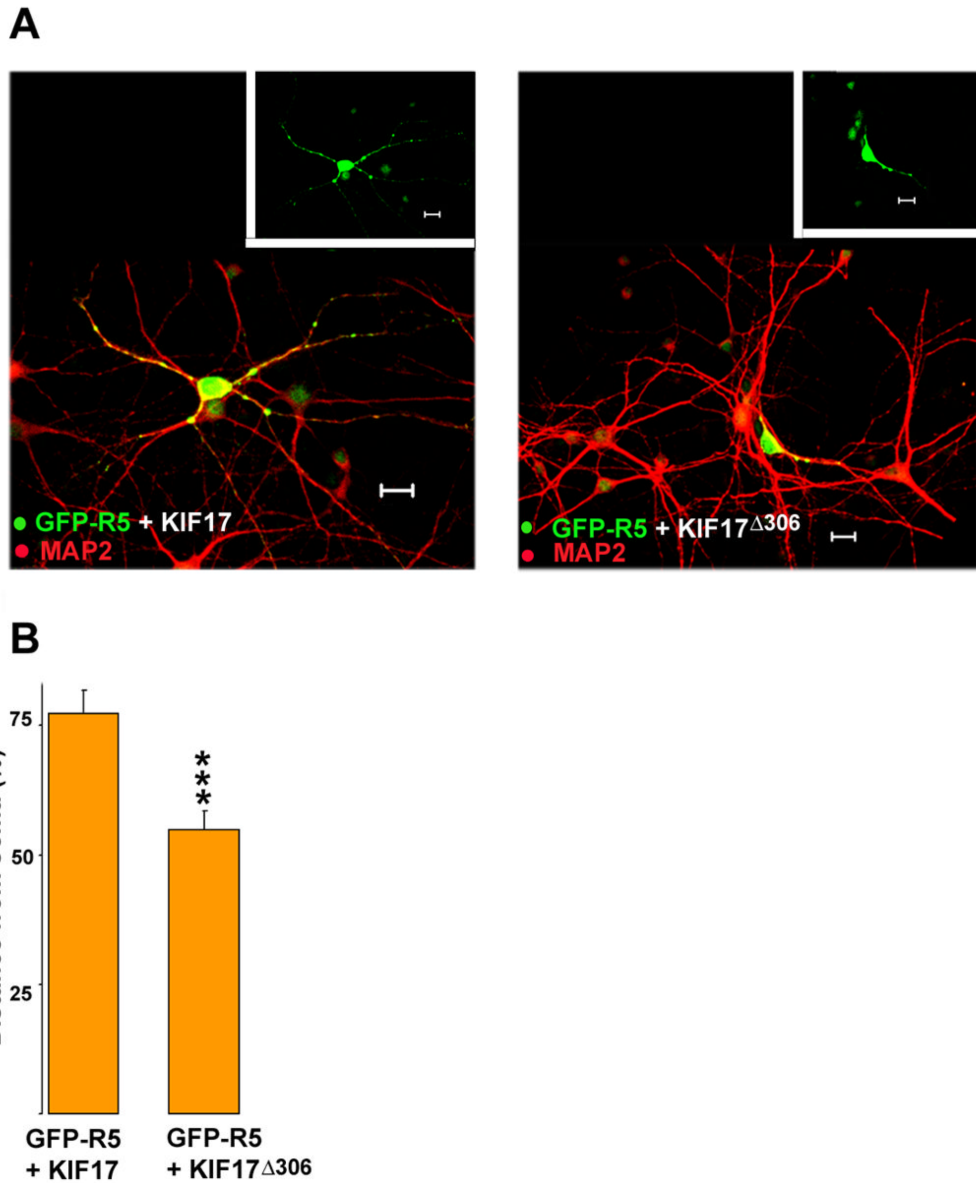

Fig. 7.

KIF17 inhibition blocks GFP-R5 localization in distal dendrites. (A) Illustration of a neuron coexpressing GFP-R5 and KIF17 (left panel) or GFP-R5 and KIF17 $^{\Delta 306}$ (right panel). GFP-R5 distribution in dendrites immunoreactive for MAP2 (red) extends in proximal and distal dendrites in neurons coexpressing KIF17, whereas it is restricted to proximal dendrites in neurons coexpressing the dominant-negative KIF17 $^{\Delta 306}$. Scale bar, 20 μm. (B) Quantitative analysis of the dendritic location of GFP-R5 when coexpressed with KIF17 or KIF17 $^{\Delta 306}$. *** $p < 0.001$ when compared to GFP-R5 coexpressed with KIF17 (n= 30–50 neurons from 2 independent experiments).