# Endocytosis and recycling of AMPA receptors lacking GluR2/3

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**Excitatory synapses in the mammalian brain contain two types of ligand-gated ion channels: AMPA receptors (AMPARs) and NMDA receptors (NMDARs). AMPARs are responsible for generating excitatory synaptic responses, whereas NMDAR activation triggers long-lasting changes in these responses by modulating the trafficking of AMPARs toward and away from synapses. AMPARs are tetramers composed of four subunits (GluR1–GluR4), which current models suggest govern distinct AMPAR trafficking behavior during synaptic plasticity. Here, we address the roles of GluR2 and GluR3 in controlling the recycling- and activity-dependent endocytosis of AMPARs by using cultured hippocampal neurons prepared from knockout (KO) mice lacking these subunits. We find that synapses and dendritic spines form normally in cells lacking GluR2/3 and that upon NMDAR activation, GluR2/3-lacking AMPARs are endocytosed in a manner indistinguishable from GluR2-containing AMPARs in wild-type (WT) neurons. AMPARs lacking GluR2/3 also recycle to the plasma membrane identically to WT AMPARs. However, because of their permeability to calcium, GluR2-lacking but not WT AMPARs exhibited robust internalization throughout the dendritic tree in response to AMPA application. Dendritic endocytosis of AMPARs also was observed in GABAergic neurons, which express a high proportion of GluR2-lacking AMPARs. These results demonstrate that GluR2 and GluR3 are not required for activity-dependent endocytosis of AMPARs and suggest that the most important property of GluR2 in the context of AMPAR trafficking may be its influence on calcium permeability.**

glutamate | hippocampus | plasticity | synapse | trafficking

Activity-dependent modulation of the intracellular trafficking of AMPA receptors (AMPARs) is a key mechanism underlying the changes in synaptic strength observed during several forms of long-term potentiation (LTP) and long-term depression (LTD) (1–6). Because of the critical role of AMPAR trafficking in synaptic as well as experience-dependent plasticity, over the last decade, there has been great interest in elucidating the detailed molecular mechanisms governing the directed insertion of AMPARs at synapses and their removal by endocytosis.

AMPARs are heterotetrameric complexes composed of combinations of four subunits termed GluR1–GluR4 (also known as GluRA–GluRD) (7, 8). Subunits have similar extracellular N-terminal and transmembrane domains, but differ significantly in their C-terminal cytoplasmic tail regions, resulting in binding to distinct sets of proteins (reviewed in refs. 1–8). GluR1 and GluR4 have long cytoplasmic tails, and GluR1 specifically interacts with protein 4.1N and the PDZ domain-containing protein SAP97 (1–8). In contrast, GluR2 and GluR3 have shorter tails that interact with a different subset of PDZ proteins termed GRIP/ABP and PICK1 (1–8). In addition, GluR2 interacts with *N*-ethylmaleimide-sensitive fusion (NSF) protein (1–8), which is an ATPase involved in membrane-fusion events (9).

In mature hippocampal pyramidal cells, AMPARs occur predominantly as complexes containing GluR1/2 or GluR2/3 (10). The current model of AMPAR trafficking suggests that AMPARs containing GluR1 are inserted into synapses in an activity-dependent manner, whereas GluR2/3 heteromers cycle constitutively into and out of synapses (1–6). GluR2 has specifically been proposed to play a critical role in the activity-dependent endocytosis of AMPARs and therefore various forms of LTD in the hippocampus (11–14) and the cerebellum (15–17). In addition, GluR2 may have a role in dendritic spine formation and growth due to extracellular N-terminal domain interactions (18, 19). It is therefore surprising that KO mice lacking GluR2 (GluR2KO) or both GluR2 and GluR3 (GluR2/3KO) express robust LTD in the hippocampus (20) and apparently no significant deficits in spine or synapse formation (20–22), although cerebellar LTD is absent in cells lacking GluR2 (15).

GluR2 also is a particularly critical AMPAR subunit, because it undergoes RNA editing resulting in a switch of a glutamine residue (Q607) in the pore-lining region of the subunit to an arginine (R607). This switch dramatically alters the biophysical properties of GluR2-containing AMPARs, making them impermeable to $Ca^{2+}$ and resistant to blockade by intracellular polyamines (23, 24). The RNA editing of GluR2 also is important for controlling the assembly of GluR2-containing AMPARs and their transit through the secretory pathway (25, 26). In contrast, AMPARs lacking GluR2 are highly $Ca^{2+}$-permeable and are robustly expressed in GABAergic interneurons as opposed to pyramidal neurons (23, 24).

Despite the great effort that has been exerted to examine how specific AMPAR subunits influence the trafficking of these receptors (see refs. 1–8, 22, 23), relatively few experiments have taken advantage of the available knockout (KO) mice lacking these subunits. Here, we examine the recycling- and activity-dependent endocytosis of endogenous AMPARs genetically lacking GluR2 or GluR2/3. We present evidence that the most important property of GluR2/3-lacking AMPARs for influencing their endocytosis is not their lack of interactions with GluR2/3 binding partners but rather their $Ca^{2+}$ permeability, which strongly influences the subcellular sites at which activity-dependent endocytosis occurs.

## Results

**Normal Synapse and Spine Formation in the Absence of GluR2/3.** To examine the trafficking of native AMPARs lacking subunits with short cytoplasmic tails, we prepared hippocampal neuron cultures from KO mice lacking either GluR2 or GluR2 and GluR3 (20, 21). Because acute knockdown of GluR2 by using siRNA in cultured neurons has been reported to inhibit spine formation and growth (18, 19), we first examined synapse and spine density in wild-type (WT), GluR2KO, and GluR2/3KO cultures at an age [21–28 days *in vitro* (DIV)] when mature spines have formed. Synapses were identified by colocalization of puncta that contained both the presynaptic protein synapsin and the postsynaptic protein PSD-95 (Fig. 1*A1*). Hippocampal neurons derived from GluR2KO and GluR2/3KO mice expressed the same density of synapses as WT cultures (WT, 39.6 ± 5.2 per 50 $\mu$m

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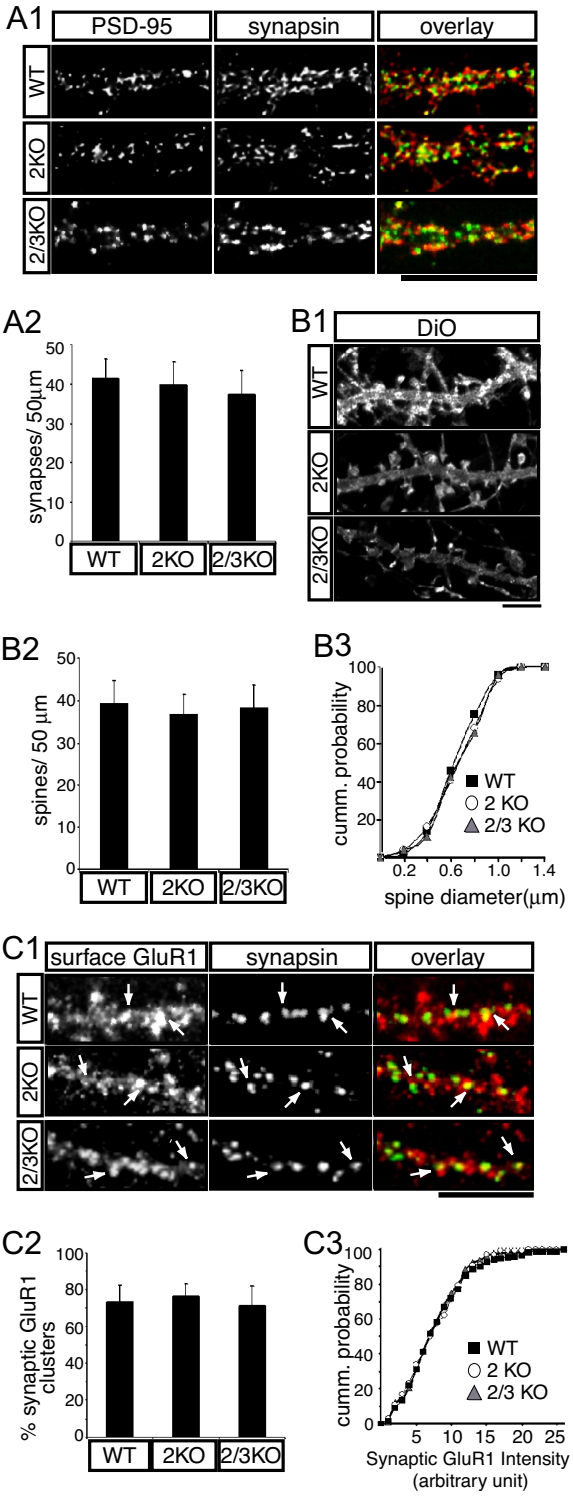


**Fig. 1.** Normal synapse and spine formation in cultured hippocampal neurons from GluR2 KO and GluR2/3 KO mice. (*A*) (*1*) Examples of dendritic staining for the postsynaptic protein PSD-95 (green) and presynaptic protein synapsin (red) in WT, GluR2KO, and GluR2/3KO neurons. (Scale bar: 10 $\mu$m.) (*2*) Quantification of synapses per 50-$\mu$m dendritic length. (*B*) (*1*) Representative dendritic segments stained with DiO to visualize spines. (Scale bar: 10 $\mu$m.) (*2*) Quantification of number of spines per 50 $\mu$m. (*3*) Cumulative frequency plot of spine head diameter. (*C*) (*1*) Examples of dendritic staining for surface GluR1 (red) and synapsin (green) (arrows, examples of synaptic AMPAR puncta). (Scale bar: 20 $\mu$m.) (*2*) Quantification of percentage of surface GluR1 puncta that are synaptic. (*3*) Quantification of surface GluR1 puncta intensity at synapses.

dendrite; GluR2KO, 36.8 ± 4.8; GluR2/3KO, 38.5 ± 5.2; $n$ = 19–22) (Fig. 1*A2*). To examine dendritic spine density and size, neuronal membranes were stained with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO), which revealed spines protruding from dendritic shafts at the same density in WT, GluR2KO, and GluR2/3KO cultures (WT, 39.6 ± 5.2; GluR2KO, 36.8 ± 4.8; GluR2/3KO, 38.5 ± 5.2; $n$ = 17–24) (Fig. 1 *B1* and *B2*). Spine diameter also was not significantly different between the KO and WT cultures (number of spines per genotype = 472–564) (Fig. 1*B3*). These results indicate that normal synapse/spine formation and growth are not dependent on GluR2 and GluR3.

AMPAR-mediated synaptic responses and synaptic GluR1 content assessed by immunogold staining are reduced in CA1 pyramidal cells from GluR2KO mice (21, 22). We therefore examined the amount of GluR1 at synapses in GluR2KO and GluR2/3KO cultures by staining surface GluR1 clusters and counterstaining for synapsin. GluR1 surface staining was punctate (Fig. 1*C1*), and the percentage of GluR1 puncta that were synaptic did not differ among WT, GluR2KO, and GluR2/3KO cultures (WT, 73.1 ± 9.1%; GluR2KO, 76.3 ± 7.2%; GluR2/3KO, 71.5 ± 10.3%; $n$ = 12–16) (Fig. 1*C2*). Quantification of the amount of surface GluR1 at individual synapses also revealed no difference between the three cell types (number of puncta per genotype = 572–626) (Fig. 1*C3*). These results indicate that AMPARs lacking GluR2 and GluR3, which presumably are GluR1 homomers because GluR4 is present at very low levels in mature cultured neurons (27), can be inserted into synapses as efficiently as AMPARs containing GluR2 and/or GluR3.

**Normal NMDA Receptor (NMDAR)-Induced AMPAR Endocytosis in the Absence of GluR2/3.** To examine the hypothesis that GluR2 and GluR3 play specific requisite roles in mediating AMPAR endocytosis, we stained surface GluR1 in live neurons and then applied NMDA to trigger endocytosis. After 15 min, remaining surface and internalized AMPARs were labeled (see *Materials and Methods*). This procedure permits calculation of the proportion of surface AMPARs that were internalized and triggers the endocytosis of dendritic and presumably synaptic AMPARs in a manner that mimics NMDAR-dependent LTD (28). Consistent with previous studies (12, 14, 28, 29), brief NMDA application elicited robust internalization of AMPARs in WT neurons (internalization index: 2.7 ± 0.5, $n$ = 48) (Fig. 2 *A* and *C*). Surprisingly, the same amount of AMPAR internalization was triggered by NMDA in cultures prepared from the GluR2KO and GluR2/3KO mice (2.9 ± 0.4, $n$ = 52; 2.6 ± 0.4, $n$ = 49, respectively) (Fig. 2 *B* and *C*). These results demonstrate that neither GluR2 nor GluR3 is required for NMDAR-driven endocytosis of AMPARs and that NMDAR activation drives endocytosis of homomeric GluR1 AMPARs in a manner comparable to AMPARs containing GluR2/3.

A previous study using siRNA to knock down GluR2 in cultured neurons reported a large increase in constitutive endocytosis of GluR1-containing AMPARs to a degree that occluded further NMDA-triggered endocytosis (14). We found that basal endocytosis of AMPARs in the GluR2KO and GluR2/3KO cultures was only slightly enhanced (internalization index: GluR2KO, 1.3 ± 0.1, $n$ = 62; GluR2/3KO, 1.3 ± 0.1, $n$ = 59; $P < 0.01$) (data not shown) compared with WT neurons (WT, 1.0 ± 0.1, $n$ = 53) (data not shown), an increase that was not sufficient to affect the AMPAR endocytosis triggered by NMDA application in the cells lacking GluR2/3.

Internalization of AMPARs in cultured neurons as well as during LTD occurs through dynamin-dependent, clathrin-mediated endocytosis (1–6). A key step in this process is the binding of the clathrin adaptor protein complex AP2 to AMPARs. All AMPAR subunits contain putative AP2-binding sites in their intracellular tails, but it is unclear whether GluR1 binds to AP2 as efficiently as GluR2 and GluR3 (12, 13). It also has been suggested that the LTD that is generated in GluR2/

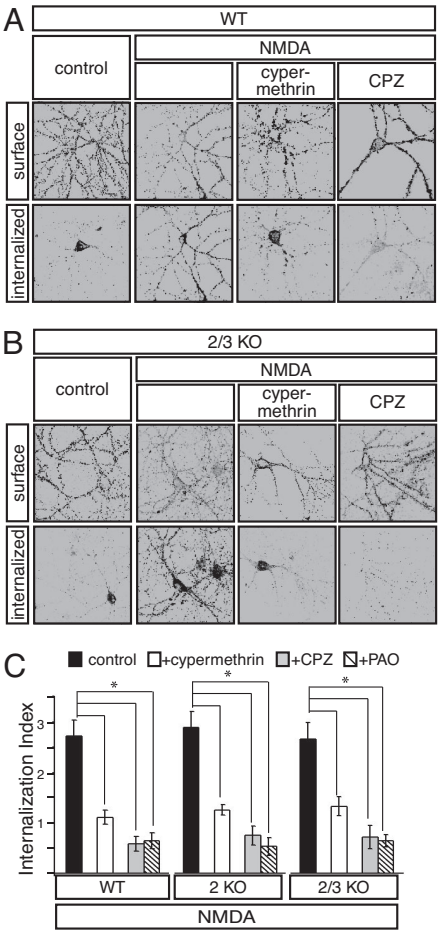


**Fig. 2.** NMDA-induced endocytosis of AMPARs lacking GluR2 or GluR2/3 is blocked by inhibitors of calcineurin or clathrin. (*A* and *B*) Examples of surface and internalized GluR1 immunoreactivity at steady state (control), 15 min after NMDA treatment alone, or 15 min after NMDA treatment after preincubation with cypermethrin or CPZ. (*C*) Quantification of magnitude of AMPAR internalization. *, $P < 0.01$.

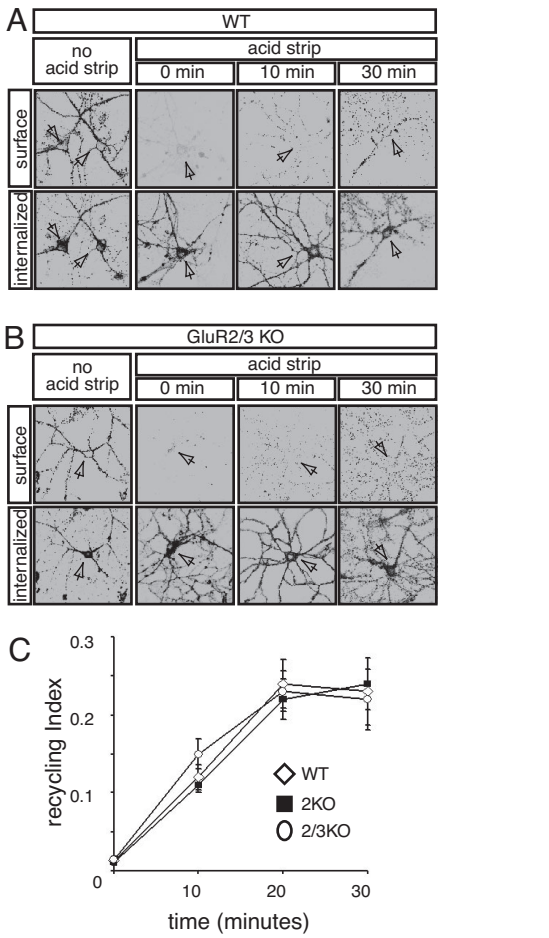


**Fig. 3.** Normal recycling of AMPARs lacking GluR2 or GluR2/3. (*A* and *B*) Examples of surface and internalized GluR1 immunoreactivity 15 min after NMDA treatment and 0, 10, or 30 min after acid wash to strip antibody from surface receptors. (*C*) Quantification of the time course of AMPAR recycling after NMDA-induced internalization.

3KO mice may, in part, be independent of AMPAR endocytosis (14, 20). Therefore, it was important to test whether the internalization of AMPARs lacking GluR2/3, presumably GluR1 homomers, is mediated by clathrin-dependent endocytosis. To address this question, we pretreated cells with 20 $\mu$M chlorpromazine (CPZ) or 10 $\mu$M phenylarsine oxide (PAO), two inhibitors of clathrin-mediated endocytosis (30–32). Both drugs reduced basal AMPAR endocytosis in WT neurons as well as GluR2KO and GluR2/3KO neurons (CPZ: WT, 0.6 ± 0.2; GluR2KO, 0.6 ± 0.2; GluR2/3KO, 0.6 ± 0.2; PAO: WT, 0.6 ± 0.2; GluR2KO, 0.5 ± 0.3; GluR2/3KO, 0.7 ± 0.2) (data not shown) and completely prevented the increase in endocytosis normally triggered by NMDAR activation (CPZ plus NMDA: WT, 0.6 ± 0.3; GluR2KO, 0.8 ± 0.2; GluR2/3KO, 0.7 ± 0.2; PAO plus NMDA: WT, 0.6 ± 0.2; GluR2KO, 0.5 ± 0.2; GluR2/3KO, 0.7 ± 0.1; $n = 18–32$) (Fig. 2). These results suggest that, similar to the endocytosis of AMPARs containing GluR2/3, the constitutive and NMDAR-trigged endocytosis of AMPARs lacking GluR2/3 is clathrin-dependent.

The activity-dependent internalization of AMPARs as well as NMDAR-dependent LTD both require activity of the calcium/calmodulin-dependent protein phosphatase calcineurin (28, 29, 33). To examine whether NMDAR-triggered internalization of AMPARs lacking GluR2/3 also requires calcineurin, we pretreated cultures with the calcineurin inhibitor cypermethrin (0.5 $\mu$M). This manipulation blocked the large increase in AMPAR endocytosis normally induced by NMDAR activation in WT as well as GluR2KO and GluR2/3KO cultures (WT, 1.1 ± 0.1; GluR2KO, 1.3 ± 0.1; GluR2/3KO, 1.3 ± 0.2; $n = 17–25$) (Fig. 2). Thus, the NMDAR-triggered endocytosis of AMPARs lacking GluR2/3, like that of GluR2-containing AMPARs, depends on calcineurin.

**Recycling of AMPARs to the Surface in the Absence of GluR2/3.** The cytoplasmic tails of GluR2/3 bind to a set of proteins, GRIP/ABP and PICK1, that do not bind to GluR1/4 (1–8). Furthermore, the membrane-proximal region of the GluR2 tail contains a binding site for NSF (1–8), a protein extensively studied because of its role in membrane-fusion events (9). Because the NSF–GluR2 interaction may help maintain AMPARs at synapses (12, 34) and because interactions with GRIP/ABP and PICK1 may have diverse roles in stabilizing AMPARs at synapses or in intracellular compartments (1–6), we examined the cycling of AMPARs lacking GluR2/3 back to the plasma membrane after internalization. This assay was performed by "stripping" the antibody off AMPARs remaining on the surface 15 min after NMDA treatment and then waiting 0–30 min for internalized AMPARs to reappear on the surface, at which point remaining internalized and recycled AMPARs were labeled (Fig. 3). This procedure permitted a "recycling index" to be

calculated (see *Materials and Methods*) (Fig. 3*C*). In WT neurons, ≈10% of internalized AMPARs recycled to the surface at 10 min, and this recycling reached a plateau of ≈20% at 20 min (Fig. 3). The recycling of AMPARs lacking GluR2 or GluR2/3 was indistinguishable from WT AMPARs (Fig. 3*C*). Thus, the protein–protein interactions involving GluR2/3 are not required for the cycling of internalized AMPARs back to the plasma membrane.

**The Calcium Permeability of AMPARs Influences the Subcellular Localization of AMPAR Endocytosis.** Examining NMDAR-triggered endocytosis of AMPARs as well as the recycling of internalized AMPARs back to the plasma membrane revealed no differences between GluR2/3-containing and GluR2/3-lacking AMPARs. These results suggest that differences in the protein interactions of GluR2/3 versus GluR1 do not significantly influence these trafficking events. The other major difference between GluR2-containing and GluR2-lacking AMPARs is their permeability to $Ca^{2+}$. Previously, we found that AMPAR endocytosis triggered by the application of AMPA was due to activation of voltage-dependent $Ca^{2+}$ channels and was largely restricted to the soma and proximal dendrites (28). In contrast, NMDA application caused robust endocytosis throughout the dendritic tree at synapses due to $Ca^{2+}$ entry through NMDARs (28). We therefore examined whether the application of AMPA caused a different spatial pattern of AMPAR endocytosis in cells lacking GluR2 compared with WT cells. Consistent with previous results (28), a brief application of AMPA to WT neurons caused robust endocytosis of AMPARs in the soma (4.3 ± 0.4, $n = 21$) but minimal endocytosis in dendrites (1.24 ± 0.2) (Fig. 4). This AMPA-induced AMPAR endocytosis was largely blocked by nominally removing $Ca^{2+}$ from the extracellular media (soma, 1.4 ± 0.2; dendrites, 0.9 ± 0.2; $n = 22$) or application of the $Ca^{2+}$ channel-blocker cadmium (200 $\mu$M) (soma, 1.3 ± 0.3; dendrites, 0.9 ± 0.2; $n = 19$) (Fig. 4). The responses of GluR2KO and GluR2/3KO neurons were dramatically different in that AMPA application caused robust AMPAR endocytosis in both the soma (GluR2KO, 4.3 ± 0.3, $n = 22$; GluR2/3KO, 4.7 ± 0.2, $n = 23$) and the dendrites (GluR2KO, 2.9 ± 0.4; GluR2/3KO, 3.1 ± 0.3), and this pattern of endocytosis was unaffected by the application of cadmium (soma: GluR2KO, 3.9 ± 0.4, $n = 22$; GluR2/3KO, 3.9 ± 0.4, $n = 24$; dendrite: GluR2KO, 2.8 ± 0.4; GluR2/3KO, 2.9 ± 0.4) but was greatly reduced by nominal removal of extracellular $Ca^{2+}$ (soma: GluR2KO, 1.8 ± 0.3, $n = 22$; GluR2/3KO, 1.9 ± 0.3, $n = 24$; dendrite: GluR2KO, 1.6 ± 0.3; GluR2/3KO, 1.6 ± 0.2) (Fig. 4). These results strongly suggest that $Ca^{2+}$ entry through the $Ca^{2+}$-permeable GluR2-lacking AMPARs is responsible for the endocytosis of AMPARs in response to AMPA application in the GluR2 and GluR2/3KO neurons.

Hippocampal pyramidal cells primarily express heteromeric GluR1/2 and GluR2/3 AMPARs at their synapses (1–6, 9), raising the question of whether our observations using GluR2KO and GluR2/3KO neurons have physiological relevance. To address this issue, we examined AMPA-induced endoctyosis of AMPARs in GABAergic neurons, which express a high proportion of GluR2-lacking AMPARs (23, 24). GABAergic cells were readily identified by strong immunoreactivity for the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) (Fig. 5*A*, closed arrows) and were interspersed with pyramidal cells that did not express GAD (Fig. 5*A*, open arrows). Direct comparison between GAD-positive and GAD-negative cells on the same coverslips revealed that basal AMPAR endocytosis was enhanced in both the dendrites and somas of GAD-positive cells compared with GAD-negative cells (soma, 1.8 ± 0.5; dendrite, 1.9 ± 0.5; $n = 19$) (Fig. 5*B*). Application of AMPA caused further robust internalization of AMPARs in both the dendrites and somas of GAD-positive neurons (soma, 3.5 ± 0.5; dendrite, 3.2 ± 0.2; $n = 22$) but primarily in the somas of GAD-negative neurons (soma, 4.5 ± 0.5; dendrite, 1.3 ± 0.5; $n = 18$) (Fig. 5*B*). These results are extremely similar to those observed in cultures prepared from GluR2KO and GluR2/3KO mice and provide further evidence that the $Ca^{2+}$ permeability of GluR2-lacking AMPARs is a key property controlling their endocytosis.

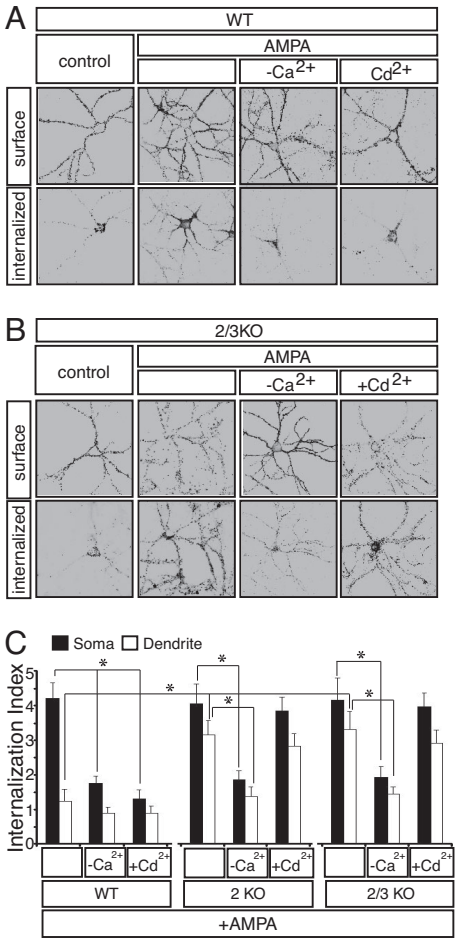


**Fig. 4.** AMPARs lacking GluR2 exhibit robust dendritic endocytosis in response to AMPA. (*A* and *B*) Examples of surface and internalized GluR1 immunoreactivity at steady state (control), 15 min after AMPA treatment alone, or 15 min after AMPA treatment in $Ca^{2+}$-free media or after preincubation with cadmium. (*C*) Quantification of magnitude of AMPA-induced AMPAR internalization in the soma (filled bars) or in distal dendrites (open bars) after various treatments. *, $P < 0.01$.

## Discussion

The molecular mechanisms underlying the activity-dependent trafficking of AMPARs toward and away from synapses have received great attention because of their importance in mediating various forms of synaptic- and experience-dependent plasticity (1–8). Based on their interactions with different sets of cytoplasmic proteins and their distinct trafficking behavior when overexpressed, specific AMPAR subunits have been proposed to play very different roles in mediating the delivery of AMPARs to the synapse during LTP and the removal of AMPARs from the synapse during LTD. Whereas GluR1 has been suggested to be critically involved in the activity-dependent delivery of AMPARs during LTP, GluR2, which shares many but not all of its properties with GluR3, has been suggested to be particularly important for mediating both the constitutive and activity-dependent endocytosis of AMPARs (1–6, 11–17). In addition, it

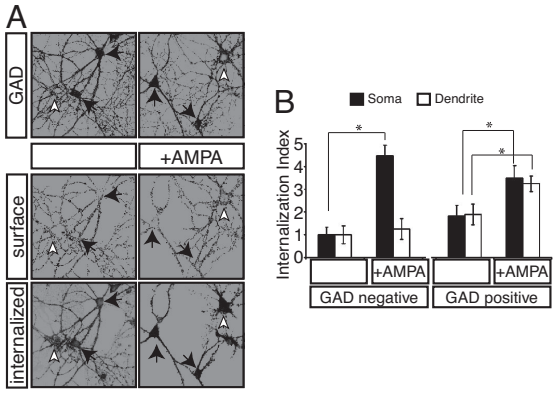


**Fig. 5.** AMPA-induced AMPAR endocytosis in the dendrites of GABAergic cells. (*A*) (*Upper*) Example of GAD staining identifying GABAergic inhibitory neurons (closed arrows) and excitatory neurons lacking GAD staining in the soma (open arrows; staining around the soma represents presynaptic terminals of GABAergic cells). (*Lower*) Examples of surface and internalized GluR1 immunoreactivity at steady state (*Left*) and 15 min after AMPA treatment (*Right*) in GAD-positive (closed arrows) and GAD-negative (open arrow) cells. (*B*) Quantification of basal and AMPA-induced AMPAR internalization at the soma (filled bars) and distal dendrites (open bars) of GAD-negative and GAD-positive cells. *, $P < 0.01$ between control and AMPA-treated cultures.

has been suggested to be important for spine formation and growth (18, 19).

To address the importance of GluR2 and GluR3 in controlling spine formation and AMPAR trafficking, we took advantage of the availability of KO mice lacking GluR2 or GluR2/3 (20, 21). When placed in culture, an extensively used preparation for studying these phenomena (1–6), neurons from these mice developed synapses and dendritic spines indistinguishably from WT neurons. In GluR2- and GluR2/3-lacking neurons, NMDAR activation enhanced AMPAR endocytosis in a manner that also was indistinguishable from that observed in WT neurons. Importantly, in both WT and GluR2/3KO neurons, this NMDAR-triggered AMPAR internalization depended on calcineurin activity and clathrin-mediated endocytosis. Furthermore, the internalized GluR2/3-lacking AMPARs recycled to the plasma membrane identically to WT AMPARs. These results unequivocally demonstrate that GluR2 and GluR3 are not required for spine formation and growth or the activity-dependent endocytosis of AMPARs. They also are consistent with the ability to generate robust LTP and LTD in the GluR2KO and GluR2/3KO mice (20, 21) and the lack of any detectable changes in spine density and synapse size in GluR2KO mice as assessed by serial EM reconstruction of hippocampal (22) and cerebellar (35) tissue.

The major difference that was observed in GluR2-lacking versus WT AMPARs was that, in response to the application of AMPA, endocytosis of GluR2-lacking AMPARs occurred robustly throughout the dendritic tree, whereas in WT GluR2-containing AMPARs, endocytosis was largely restricted to the soma and proximal dendrites. This difference was likely due to the high $Ca^{2+}$ permeability of GluR2-lacking AMPARs, which provided a local source for $Ca^{2+}$ not available to WT AMPARs containing GluR2. A similar spatial pattern of AMPAR endocytosis was observed in GABAergic neurons, which express high levels of GluR2-lacking AMPARs (23, 24). These observations suggest that the types of stimuli that elicit endocytosis of AMPARs in GABAergic inhibitory neurons will differ from those that elicit AMPAR internalization in pyramidal neurons, a difference that may profoundly affect neural circuit function during adaptive and pathological forms of plasticity.

Methodological differences in the approaches used to study the importance of GluR2/3 in controlling AMPAR endocytosis likely influence the conclusions that have been reached. The trafficking behavior of overexpressed recombinant subunits in cultured neurons and hippocampal slice cultures has provided much of the data supporting the idea that GluR2 is critically important for endocytosis and LTD. This approach permits elegant molecular manipulations of the recombinant proteins, including epitope tagging and specific mutagenesis (e.g., see refs. 11, 14, 36). Caveats to this approach include the possibility that the overexpressed subunits saturate the endogenous trafficking machinery and thus do not behave identically to endogenous AMPARs. In addition, homomeric AMPARs, which normally compose only a small proportion of endogenous AMPARs, are often studied, and their behavior may not be identical to heteromeric AMPARs. Another important approach has been to use small peptides or expression of C-terminal tails that presumably interfere with specific subunit protein–protein interactions (e.g., see ref. 36). This permits the examination of endogenous AMPARs but has the limitation that the interpretation of any result depends on the specificity of the peptide's actions.

The approach used here, studying endogenous AMPARs genetically lacking specific subunits, allows an unequivocal answer to the question of whether GluR2 and GluR3 and their specific interacting protein partners are necessary for the endocytosis and recycling of AMPARs. The results, however, cannot address the question of whether these subunits normally do or do not play an important role in modifying AMPAR trafficking. When studying any genetically modified preparation, it is impossible to rule out that some sort of "compensation" occurred, such that the normal critical role of the missing or modified protein was masked. It seems unlikely that the core cellular mechanisms involved in AMPAR endocytosis could have been modified in the GluR2/3KO cells to such a degree that specific GluR2/3-dependent protein interactions, which normally play a critical role in this process, are no longer required. However, this possibility cannot be ruled out.

An issue that we did not address is whether the intracellular trafficking of AMPARs that did not recycle to the surface after endocytosis is affected by the presence or absence of GluR2/3. This topic is important because both the type of stimuli used to elicit AMPAR endocytosis (29) and the subunit composition of the AMPAR (14) have been suggested to influence whether internalized AMPARs recycle to the plasma membrane or are diverted to late endosomes or lysosomes. It also is important to note that the detailed trafficking behavior of AMPARs may be cell type-specific. In contrast to LTD in hippocampal CA1 pyramidal cells (20), LTD in cerebellar Purkinje cells clearly appears to be dependent on GluR2 and its interactions with PICK1 (15, 16). Thus, it may prove difficult to generate general principles about AMPAR trafficking that apply to all neuronal cell types.

## Materials and Methods

**Cell Culture.** Primary hippocampal cultures were prepared from littermate embryonic day 19 WT, GluR2KO, and GluR2/3KO mice (20, 21) kindly provided by Zhengping Jia (Hospital for Sick Children, Toronto, ON, Canada). To examine GABAergic cells (Fig. 5), cultures were prepared from postnatal day 0 or 1 mice. Cells were plated at medium density (≈50,000 cells per 12-mm well) on poly-D-lysine-coated coverslips. Cultures were maintained in minimal essential medium (MEM) supplemented with 0.5 mM glutamine and N-2 for up to 4 weeks. Glial growth was inhibited by adding 5-fluoro-2′-deoxyuridine after 1 week.

**Immunocytochemistry.** For immunostaining with synaptic markers (Fig. 1), neurons were fixed at 21–28 DIV in paraformaldehyde (PFA)-sucrose (4% PFA and 4% sucrose in PBS). Cells were permeabilized in 0.25% Triton X-100 and stained with rabbit anti-PSD-95 (Invitrogen) and mouse anti-synapsin (BD

Biosciences PharMingen). To visualize synaptic AMPAR clusters, live neurons were incubated at 4°C for 30 min with saturating levels of an antibody directed against the N-terminal domain of GluR1 (Calbiochem), fixed in PFA-sucrose, and counterstained with mouse anti-synapsin. Primary antibodies were visualized with Alexa 488- or 568-conjugated secondary antibodies and mounted in Mowiol (Hoechst Pharmaceuticals). For membrane staining, 28 DIV neurons were fixed for 15 min at 37°C in PFA-sucrose and incubated overnight at 4°C with Vybrant DiO (Invitrogen) according to the manufacturer's instructions.

To measure AMPAR internalization, surface AMPARs were labeled in live neurons by 15-min incubation (in regular Tyrodes' solution: 119 mM NaCl, 5 mM KCl, 25 mM Hepes, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 2 $\mu$M glycine) with a saturating concentration of the GluR1 N-terminal antibody. After washout, cells were preincubated for 5 min in an appropriate antagonist mixture (1 $\mu$M TTX and 100 $\mu$M LY341495, as well as 20 $\mu$M DNQX to block AMPARs for NMDA application or 100 $\mu$M 2-amino-5-phosphonovaleric acid (APV) to block NMDARs for AMPA application; all from Tocris) before treatment with agonists (100 $\mu$M NMDA or AMPA for 3 min; Calbiochem and Tocris, respectively). After agonist washout, neurons were maintained at 37°C for 15 min before fixation in PFA-sucrose. Antagonists were present during the entire experiment. Surface receptors were visualized by using saturating levels of anti-rabbit Alexa 568-conjugated secondary antibodies (Invitrogen), followed by permeabilization and staining of internalized receptors with cy5-conjugated anti-rabbit antibody (Jackson ImmunoResearch). Inhibitors of clathrin-dependent endocytosis (20 $\mu$M CPZ or 10 $\mu$M PAO; Calbiochem and Sigma–Aldrich, respectively) or calcineurin (1 $\mu$M cypermethrin; Tocris) were added 15 min before NMDA application and were present during the entire experiment. For experiments involving AMPA application in nominally $Ca^{2+}$-free media, cells were bathed in Tyrodes' solution in which $CaCl_2$ was replaced by 2 mM $SrCl_2$ (Sigma–Aldrich). $Ca^{2+}$ channels were blocked by adding 200 $\mu$M $CdCl_2$ (Sigma–Aldrich) to the antagonist mixture 5 min before AMPA treatment.

To measure AMPAR recycling (Fig. 3), neurons were treated with NMDA, and surface AMPARs were allowed to internalize for 15 min before cells were placed on ice for 5 min. Surface antibodies were stripped by applying ice-cold acidic solution (0.5 M NaCl and 0.2 M acetic acid) for 2 min. Cells were washed in Tyrodes' solution and returned to 37°C for 0–30 min before fixation. Internalized and recycled receptors were visualized as described in the preceding paragraph. The GAD antibody to visualize GABAergic cells was developed by David Gottlieb (Washington University, St. Louis, MO) and was obtained from the Developmental Studies Hybridoma Bank.

**Image Acquisition and Analysis.** Coverslips were mounted in Mowiol (Hoechst Pharmaceuticals), and images were acquired by using a Zeiss LSM 510 laser scanning confocal microscope using a ×63 or ×40 oil-immersion Plan Achromat objective. Images were analyzed with Metamorph image analysis software (version 6.1, Universal Imaging) and processed by using Adobe Photoshop software for presentation (Adobe Systems). Each experimental manipulation and analysis was performed on a minimum of three different cover slips (average seven to eight coverslips) prepared from a minimum of three different animals (average of four animals). *n* refers to the number of cells examined if not stated otherwise. Data are presented as mean ± SEM. Group results were compared by using Student's *t* test.

To measure AMPAR internalization, images for all conditions in individual experiments were analyzed by using identical acquisition parameters. Images from each experiment were thresholded, and total staining intensity of surface and internalized receptors was measured by using the Integrated Morphometry Analysis protocol (Metamorph; Universal Imaging). For experiments comparing somatic versus dendritic localization of internalized AMPARs, areas were manually drawn ≈20 $\mu$m around the soma, and staining intensity inside (somatic) and outside (dendritic) the selected area were measured. Synaptic GluR1 puncta were defined by visual colocalization with presynaptic synapsin puncta, and synapses were defined by visual colocalization of PSD-95 puncta with synapsin puncta. Spine density was assessed by visually counting bulbous dendritic protrusions along 50-$\mu$m portions of dendrites. The width of spine heads was assessed by measuring the length of an axis manually laid through the widest point of randomly selected spine heads. The internalization index refers to intracellular fluorescence divided by total fluorescence normalized to untreated WT neurons. The recycling index was calculated as the ratio of surface fluorescence divided by the total fluorescence and normalized to WT non-acid-washed neurons.

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