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# Regulation of AMPA receptor localization in lipid rafts

**Qingming Hou**[a], **Yunfei Huang**[b], **Stephen Amato**[a], **Solomon H. Snyder**[b], **Richard L. Huganir**[b], and **Heng-Ye Man**[a,*]

[a]Department of Biology, Boston University, Boston, MA 02215, USA

[b]The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

## Abstract

Lipid rafts are special microdomains enriched in cholesterol, sphingolipids and certain proteins, and play important roles in a variety of cellular functions including signal transduction and protein trafficking. We report that in cultured cortical and hippocampal neurons the distribution of lipid rafts is development-dependent. Lipid rafts in mature neurons exist on the entire cell-surface and display a high degree of mobility. AMPA receptors co-localize and associate with lipid rafts in the plasma membrane. The association of AMPARs with rafts is under regulation; through the NOS–NO pathway, NMDA receptor activity increases AMPAR localization in rafts. During membrane targeting, AMPARs insert into or at close proximity of the surface raft domains. Perturbation of lipid rafts dramatically suppresses AMPA receptor exocytosis, resulting in significant reduction in AMPAR cell-surface expression.

## Introduction

Lipid rafts are membrane microdomains enriched with glycospingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins (Allen et al., 2007; Edidin, 2003; Simons and Ehehalt, 2002). Lipid rafts are involved in multiple cellular functions including signaling (Simons and Toomre, 2000), cell polarity (Manes et al., 1999), chemotaxis (Gomez-Mouton et al., 2004), protein sorting and trafficking, including exocytosis (Gagescu et al., 2000) and endocytosis (Kasahara and Sanai, 1999). Although lipid rafts have been extensively studied in epithelial and immune cells, their roles in the neuronal system are still poorly understood. The low-density, detergent-resistant lipid microdomains can be purified by sucrose gradient centrifugation from brain and synaptosomes (Eckert et al., 2003; Suzuki et al., 2001), and were found to be present in different types of neurons, including cortical (Ma et al., 2003) and hippocampal neurons (Hering et al., 2003; Shogomori and Futerman, 2001a,b). Lipid rafts seem to be involved in multiple neuronal functions, such as dendritic–axonal protein sorting (El-Husseini Ael et al., 2001), growth cone guidance (Guirland et al., 2004), dendrite outgrowth and axonal branching (Fan et al., 2002), synapse formation (Hering et al., 2003), synaptic vesicle formation (Thiele et al., 2000), receptor clustering and synaptic plasticity (Ma et al., 2003; Zhu et al., 2006).

AMPA receptors (AMPARs), a subtype of glutamate receptors, mediate most of the excitatory synaptic transmission in the central nervous system. Studies have shown that AMPARs are undergoing constant trafficking between the plasma membrane and the intracellular



*Corresponding author. Department of Biology, Boston University, 5 Cummington St., Boston, MA 02215, USA. Fax: +1 617 353 8484.
*E-mail address:* hman@bu.edu (H.-Y. Man).

compartments (Bredt and Nicoll, 2003; Song and Huganir, 2002). Regulation of these trafficking processes will change AMPAR abundance in synapses and thus alter the strength of synaptic activity, which is believed to be the underlying mechanism of some long-term synaptic plasticity (Bredt and Nicoll, 2003; Malinow, 2003; Man et al., 2000a; Song and Huganir, 2002). Like other neurotransmitter receptors (Bruses et al., 2001) and membrane channel proteins such as potassium channels (Wong and Schlichter, 2004), AMPARs were also found in lipid raft preparations (Hering et al., 2003; Suzuki, 2002; Suzuki et al., 2001). However, the role of rafts in AMPAR localization and trafficking has not been well understood, and whether the raft residency of AMPAR is dynamically regulated remains unknown.

Here we report that in cultured hippocampal and cortical neurons, lipid rafts are dynamic microdomains that are constituted in a development-dependent manner. AMPARs associate with lipid rafts on the plasma membrane, and their residency in rafts is regulated by NMDA receptor (NMDAR) activities and NO-mediated signaling pathway. Perturbation of lipid rafts dramatically suppresses AMPAR exocytosis and reduces AMPAR cell-surface expression, indicating an important role of raft domains in AMPAR membrane insertion.

## Results

### Distribution of lipid rafts in cultured hippocampal neurons

Since cholera toxin B subunits (CTX) specifically bind to the lipid raft component GM1 (Schon and Freire, 1989), CTX is commonly used as a marker for lipid rafts. To examine the subcellular distribution of rafts in neurons, 2-wk-old cultured hippocampal neurons were incubated with FITC-conjugated CTX (CTX-FITC, 3 μg/ml) in ACSF for 10–15 min, then washed and fixed with 4% paraformaldehyde. CTX fluorescence signals were observed throughout the cell-surface, including the dendrites and the soma, in the form of small bright patches with weak labeling in between (Fig. 1A, left). The same distribution of lipid raft signals were observed when cells were incubated with CTX-FITC after fixation under non-permeant conditions. However, the pattern of raft distribution appeared drastically different when labeled under permeabilized conditions by 10 min incubation with 0.3% Triton X-100. CTX-FITC labeling on permeabilized neurons showed small, sharp, densely distributed clusters. We found that the dendritic spines were often labeled with particularly high intensity (Fig. 1A, right), suggesting important roles of rafts in spine or synapse morphology and function. The different patterns of CTX labeling following triton treatment are not likely caused by an exposure of new intracellular raft structures, since a similar pattern could be observed when cells were labeled first and then permeabilized by Triton X-100 (data not shown). Rather, it is probably due to a removal of non-raft GM1, thereby increasing the contrast between raft domains and the surrounding area (Schwarz and Futerman, 1997). We then examined raft formation and distribution during neuron development. At day 5 after plating, cultured hippocampal neurons demonstrated that CTX-FITC signals were only limited to the soma and proximal dendrites, with almost no clusters under permeant conditions. At day 10, the entire cell was labeled, but the clusters under permeant conditions were limited, localized only in the soma and basal dendrites. After 2 weeks, the clustered lipid rafts under permeant conditions occupied the whole neuron and enriched at spines (Fig. 1B).

### Lipid rafts are mobile in cultured hippocampal neurons

It has been shown that lipid rafts are highly dynamic structures (Kenworthy et al., 2004; Nichols et al., 2001), however, the dynamics of cell-surface rafts in neurons has not yet been investigated. For this purpose cultured hippocampal neurons were transfected with GFP tagged glycosyl phosphatidyl inositol (GPI)-anchored protein (GPI-GFP), a widely used lipid raft marker (Kenworthy et al., 2004; Polishchuk et al., 2004). Neurons were incubated with an anti-GFP antibody and the surface GPI-GFP was visualized by secondary antibody-conjugated

quantum dots (Qdots) (Fig. 2A). Because of its non-bleaching property, quantum dots are an ideal material for long-term continuous imaging, which has been applied successfully in the study of AMPAR and glycine receptor trafficking (Dahan et al., 2003; Ehlers et al., 2007; Tardin et al., 2003). The binding specificity of Qdots was confirmed by the lack of labeling in non-transfected cells (not shown). Under the fluorescence microscope, most of the GPI-GFP-bound dots moved constantly in either direction along the dendrites (Fig. 2B). Typically the dots traveled back and forth within a short distance, with frequent fusion and fission events occurring between dots (Fig. 2B). In addition, we found no apparent change in GPI-GFP dynamics following NMDA treatment (data not shown).

### Association of AMPARs with lipid rafts

To examine the synaptic distribution of lipid rafts, we immunostained a synaptic marker protein PSD-95 following labeling of surface rafts with CTX-FITC. A large amount of PSD-95 puncta was found co-localized with CTX-labeled clusters in dendrites (Fig. 3A), consistent with the previous biochemical demonstration of lipid rafts from synaptosome (Suzuki, 2002). To investigate the relationship of AMPARs with lipid rafts, we sequentially incubated neurons with antibodies against GluR1 extracelluar N-terminus (anti-GluR1Nt) and CTX-FITC to double label surface AMPARs and rafts. A large portion of AMPAR puncta co-localized with or at close proximity to lipid rafts (Fig. 3B). Image analysis showed 55.6%±2.7% of AMPAR signals co-localizing with CTX fluorescence, whereas 49.3%±3.3% of CTX signals overlapping AMPAR signals (Fig. 3C). To further confirm the relationship of AMPARs with rafts, GPI-GFP, a second lipid raft marker was used. Transfection of GPI-GFP was controlled to have low expression and weak GFP signal, and surface GFP staining was performed to highlight its plasma membrane localization. Immunolabeling showed surface GPI-GFP in sharp clusters along the dendrites, which co-localize with CTX-FITC labeled puncta (Fig. 3D). In supporting the raft localization of GPI-GFP, incubation with methyl cyclodextran (MCD, 10 mM), which disrupts the integrity of lipid rafts by cholesterol extraction, dramatically reduced surface GPI-GFP clusters (Fig. 3E). When surface AMPARs and surface GPI-GFP were double labeled, co-distribution of the two immunosignals was also observed (Fig. 3F), indicating the localization of certain AMPARs in raft domains at basal conditions.

Lipid rafts are commonly purified by sucrose gradient centrifugation (Allen et al., 2007; Suzuki et al., 2001). The product of the gradient centrifugation is a mixture of rafts from plasma membrane and from intracellular organelles, and could be contaminated with other non-raft components that have similar gradient properties. Isolation of the plasma membrane rafts is therefore important in examining the raft residency of surface AMPAR as well as its dynamics. To this end, we incubated live cortical neurons with biotin-conjugated CTX B subunit (CTX-biotin) that binds to the raft lipid GM1, allowing surface rafts to be isolated with neutravidin beads following cell lysis with 1% Triton X-100 on ice. We found that the CTX-biotin precipitates were enriched in raft-residing protein Thy-1, but did not contain the non-raft protein transferrin receptors (Fig. 4A), confirming the specificity of our raft purification protocol. Importantly, we found that rafts isolated by CTX pulldown assays contained AMPARs (Fig. 4B), strongly indicating the localization of AMPARs in lipid rafts on the cell-surface, which is consistent with previous reports using the sucrose gradient method (Hering et al., 2003; Suzuki et al., 2001). AMPARs were also detected positive in CTX-biotin precipitations from synaptosome (Fig. 4B), suggesting the raft association of synaptic AMPARs. As controls, co-precipitation of AMPARs with rafts was markedly reduced by disrupting rafts with MCD treatment, or by lysing cells with buffers containing 1% sodium dodecyl sulfate (SDS) (Fig. 4B). In addition to AMPARs, some AMPAR-interacting proteins including PICK1 and NSF were also found in the CTX precipitations (Fig. 4C). However, NMDAR subunit NR2B was observed at low levels in surface rafts (Fig. 4C), suggesting a differential raft residency among subtypes of glutamate receptors.

### NMDAR activity recruits AMPARs to surface lipid rafts

The association of many proteins with rafts is dynamic and subject to regulation (Ma et al., 2003; Tansey et al., 2000). A number of previous studies have shown that NMDARs play critical roles in AMPAR trafficking and distribution. NMDA treatment, which activates the total pool of surface NMDARs, promotes AMPAR internalization (Ehlers, 2000; Lee et al., 2004) and reduces the abundance of surface AMPARs, whereas selective activation of synaptic NMDARs promotes AMPAR insertion and increases AMPAR surface expression (Lu et al., 2001). We therefore investigated whether NMDAR activation regulates AMPAR localization in rafts in the plasma membrane. Cultured cortical neurons were treated with 30 μM NMDA, then washed and incubated with CTX-biotin at 4 °C to isolate the surface rafts. 2 min and 10 min NMDA treatment increased the raft-associated AMPARs to 180.0%±5.0% ($n$ = 3) and 154.9%±19.0% ($n$ = 3) of control, respectively (Fig. 5A). This redistribution was rapid and could be observed with just 1 min NMDA treatment (data not shown). A complete abolishment of this effect by NMDAR antagonist APV (50 μM) (Fig. 5A, right) confirmed the dependence on NMDAR activity. We then examined the role of a major signaling component of NMDAR activity, the calcium/calmodulin-dependent protein kinase II (CaMKII). Application of KN-62 (10 μM) 10 min prior to and during 10 min NMDA treatment did not affect AMPAR recruitment to surface rafts compared to NMDA alone (162.6%±16.3% in NMDA; 148.6% ±20.3% in NMDA+KN-62, $n$ = 3) (Fig. 5C), indicating the involvement of other cascades. Because nitric oxide synthase (NOS) is also a major substrate for NMDAR signaling (Bredt and Snyder, 1989) and is localized in lipid rafts (Sowa et al., 2001; Yang and Rizzo, 2007), we examined the involvement of the NOS/NO cascade in AMPAR recruitment to the raft. We found that the NMDA effect was completely blocked by the NOS inhibitor L-NAME (200 μM) (184.9%±31.6% in NMDA; 103.6%±28.3% in NMDA+NAME, $n$ = 3) (Fig. 5D). Furthermore, treatment of neurons with an NO donor SNP (500 μM) for 10 min mimicked the NMDA effect in recruiting AMPARs to surface rafts (132.6%±8.8% of control, $n$ = 3), whereas L-NAME caused a modest but significant decrease in raft-residing AMPARs (86.2%±3.8% of control, $n$ = 3) (Fig. 5E). The small decrease caused by L-NAME may reflect a low level of NOS activity at basal conditions. Together, these results strongly indicate that the raft association of AMPAR is regulated by NMDAR-NOS/NO signaling pathway.

### Disruption of lipid rafts reduces AMPAR surface expression

The abundance of cell-surface AMPARs is relatively constant under steady state, but is regulated during the expression of synaptic plasticity (Collingridge et al., 2004; Malinow and Malenka, 2002). If a proportion of surface AMPARs are localized in rafts, the integrity of the lipid microdomains may be important for AMPAR surface localization. To investigate this, we incubated cultured cortical neurons with 10 mM MCD to disrupt surface lipid rafts, and the AMPARs were isolated by surface biotinylation assays. By 15 min of MCD incubation, surface AMPARs were reduced to ~60% of control, and 30 min of MCD treatment reduced the surface AMPARs to ~34% of control (Fig. 6A and B). MCD treatment did not change the total protein level of AMPARs, consistent with a previous report showing no change in total protein amounts by MCD in BHK cells (Keller and Simons, 1998). To further confirm MCD effect, we performed colorimetric assays using anti-GluR1Nt antibodies to measure AMPARs at the plasma membrane under non-permeant conditions and the total receptor abundance under permeant conditions, respectively (Man et al., 2000b). Consistently, we found a 60% reduction in surface GluR1 following 30 min MCD treatment (Fig. 6C). Because lipid rafts are connected to the submembrane cytoskeleton network, and interference of the cytoskeletal structures have been shown to affect receptor trafficking and reduce AMPAR surface expression (Zhou et al., 2001), it is therefore possible that the MCD-caused decrease of surface-localized AMPARs is only a nonspecific side effect resulting from the disruption of raft–cytoskeleton complexes. To examine the effect of MCD on cytoskeletal elements, we immunostained actin and tubulin in hippocampal neurons. We found that the morphology of actin neurofilaments and tubulin

microtubule had no obvious changes by up to 15 min MCD treatment, but was affected after 30 min MCD incubation (Fig. 6D), suggesting that the reduction in AMPAR surface expression was caused by disruption of lipid rafts at the early stage, while cytoskeleton disruption might contribute to the longer effect. We therefore limited MCD treatment to 15 min in other experiments.

Since no change was observed in the total AMPAR amount during raft disruption, the reduction in surface AMPARs is likely due to receptor translocation. AMPARs traffic continuously to and away from the plasma membrane via vesicle-mediated membrane exocytosis (Passafaro et al., 2001) and clathrin-dependent internalization (Man et al., 2000b). Alteration in either or both trafficking processes will change the abundance of AMPARs on the cell-surface. To investigate whether the MCD effect is due to AMPAR internalization, surface AMPARs were labeled with anti-GluR1Nt antibodies for 5 min at room temperature, and receptor endocytosis was then induced at 37 °C, with or without MCD for 15 min. The internalized receptors were visualized following a removal of the remaining surface antibodies by acid stripping (Man et al., 2007). We found that when rafts were disrupted by MCD, AMPARs were internalized to a level comparable to that of control (Fig. 7A and B), indicating that lipid rafts may not play an important role for constitutive AMPAR endocytosis. NMDAR activation has been shown to stimulate AMPAR internalization and thus reducing AMPAR surface localization (Beattie et al., 2000; Ehlers, 2000). To further examine the involvement of rafts in regulated AMPAR internalization, we first incubated neurons with MCD for 15 min to disrupt lipid rafts, followed by NMDA treatment (30 μM, 5–20 min) to induce receptor internalization. Surface biotinylation assays showed minimal effect of MCD on the rate of NMDA-induced AMPAR endocytosis (Fig. 7C). These data indicate that the machinery and dynamics of AMPAR internalization are largely intact following raft perturbation.

### Lipid rafts are important for AMPAR membrane insertion

Lipid rafts are involved in the exocytic process and play an important role in site-specific membrane targeting (Helms and Zurzolo, 2004; Salaun et al., 2004). Since our results showed no major effects of lipid rafts on AMPAR endocytosis, we reasoned that the reduction in surface AMPARs following raft disruption maybe due to a dysfunction in receptor insertion. To explore this possibility, hippocampal neurons were transfected with GPI-GFP and GluR2-HA. Surface GluR2-HAwas first blocked with anti-HA primary and non-conjugated secondary antibodies. After incubation of the cells at 37 °C to permit receptor insertion, the newly inserted receptors were detected by a second round of staining (Fig. 8A). The cell-surface localization of GPI-GFP, which displayed weak signal by controlled low expression, was highlighted by immunolabeling the surface GFP following fixation. As shown in Fig. 8B, the newly inserted GluR2-HA co-localized with surface GPI-GFP, suggesting a role of rafts in AMPAR exocytosis. To directly examine the effect of raft disruption on AMPAR insertion, some transfected neurons were incubated with antibodies on ice to block surface GluR2-HA, then transferred to 37 °C in the presence or absence of MCD to permit receptor exocytosis. Following 15 min incubation, insertion assays revealed a dramatic reduction in GluR2-HA membrane targeting in MCD-treated neurons compared to the control (Fig. 8C), strongly indicating a requirement of the integrity of surface lipid rafts in AMPAR membrane insertion.

## Discussion

In the present study, we investigated the role of lipid rafts in AMPAR distribution and trafficking. When fluorescence-conjugated CTX was used to label lipid rafts, puncta of fluorescence were used to indicate raft distribution. Image resolution prevents further elucidation on the nature of the CTX-labeled structures, but the large-sized puncta might represent clusters of individual raft domains rather than single giant rafts. In cultured neurons

lipid rafts are distributed throughout the cell-surface including the soma and dendrites. Live imaging demonstrated high mobility of the lipid microdomains. AMPARs co-localize and associate with surface lipid rafts, and their raft localization is regulated by NMDAR activity and the NOS–NO pathway. Furthermore, we found that disruption of rafts markedly reduces the abundance of surface AMPARs, which is likely caused by suppression of AMPAR insertion. We noted that, contrarily to our finding, results from a previous study indicate a role of rafts in AMPAR internalization (Hering et al., 2003). This discrepancy is likely caused by differences in the method of raft disruption. In the other study, the integrity of rafts was reduced by inhibiting cholersterol/sphingolipid synthesis by chronic drug treatment, whereas we utilized MCD for transient extraction of membrane cholesterol.

Protein localization in lipid rafts is not static. It was reported that the association of Thy-1 with rafts is transient and its residence time in rafts is regulated by raft lipid composition (Sheets et al., 1997). The raft association of the chemokine receptor CCR5 is markedly increased by IGF-I stimulation (Manes et al., 1999). In many cases, the localization of proteins in rafts is regulated either via protein–protein interactions or by protein modulation such as acylation, particularly myristoylation and palmitoylation. The AMPAR-interacting protein GRIP is recruited to lipid rafts through its association with raft-residing EphrinB (Bruckner et al., 1999). PSD-95, which is palmitoylated (El-Husseini et al., 2002) and associated with lipid rafts, recruits a potassium channel Kv1.4 to rafts via its interaction with Kv1.4 (Wong and Schlichter, 2004). The raft localization of ErbB4 upon neuregulin treatment is also mediated via its interaction with PSD-95 (Ma et al., 2003). The regulated raft localization, such as in GDNF signaling (Tansey et al., 2000), is important for receptor functioning. The GDNF receptor is a complex consisting of two components, the GPI-anchored GFRα, and RET, which is non-raft residing at basal conditions. Upon GDNF stimulation, RET translocates into raft domains where it forms a receptor complex with GFRα. We demonstrate that the localization of AMPARs in lipid rafts is also under regulation. Activation of NMDARs increases AMPAR association with rafts. The underlying mechanisms for this recruitment are unclear, but acylation of the receptor subunits (Hayashi et al., 2005; Wang et al., 2005) or palmitoylation of PSD-95 may be involved (deSouza and Ziff, 2002). Where are the newly recruited AMPARs coming from? AMPARs are not only capable of trafficking between the plasma membrane and intracellular compartments via receptor endocytosis and insertion, but can also move laterally along the cell-surface, both constitutively and in an activity-dependent manner (Tardin et al., 2003). Therefore, it is conceivable that upon NMDA stimulation, AMPARs might translocate from non-raft to raft domains via lateral movement along the plasma membrane. Secondly, because NMDAR activation promotes AMPAR membrane insertion (Lu et al., 2001; Park et al., 2004; Passafaro et al., 2001), it is possible that the newly recruited AMPARs in rafts is a result of receptor exocytosis. It is interesting to postulate that during NMDA application, the two opposite events of AMPAR internalization and insertion are activated simultaneously. The commonly observed reduction in total surface AMPAR is the net effect of a predominant extent in receptor internalization over insertion.

Studies have shown that raft and non-raft segregation is a way to regulate protein function or determine protein destination. For example, the raft-localized neuronal Src is more catalytically active than when in the soluble fraction (Mukherjee et al., 2003). In addition, amyloid precursor protein (APP) is processed differently depending on its plasma membrane location. The raft-associated APP is cleaved by beta-secretase, while the non-raft localized APP is cleaved by alpha-secretase (Ehehalt et al., 2003). It has also been reported that the transforming growth factor beta (TGF-beta) receptors follow two distinct internalization routes. While non-raft localized TGF-beta receptors undergo clathrin-dependent endocytosis that is important for intracellular signaling, the raft-associated receptors internalize via a clathrin-independent pathway, which is required for rapid receptor turnover (Di Guglielmo et al., 2003). It is therefore reasonable to postulate that the AMPARs partitioned in lipid rafts may be subject to

protein modification such as tyrosine phosphorylation (Ahmadian et al., 2004; Hayashi and Huganir, 2004), given that tyrosine kinases such as Src family kinases and insulin receptors are associated with raft domains (Kasahara et al., 2002; Vainio et al., 2002). If this is the case, then the raft-residing AMPARs may possess distinct channel properties and activity levels, and might thus serve as a cellular mechanism in the expression of synaptic plasticity.

In polarized cells such as epithelial cells and neurons, lipid rafts are believed to be part of the carrier that delivers membrane proteins to specific sites (Helms and Zurzolo, 2004; Simons and Ikonen, 1997). Rafts may also participate directly in exocytosis (Salaun et al., 2004). Indeed, it was reported that the exocytotic components such as syntaxin, SNAP-25 and VAMP2 are associated with lipid rafts (Chamberlain et al., 2001). Furthermore, lipid rafts associate with both tubulin and actin (Gomez-Mouton et al., 2001; Palestini et al., 2000), components of two major cytoskeletal structures by which cargo vesicles move toward and fuse with the plasma membrane. For neurotransmitter receptors, nicotinic receptors were reportedly exocytosed via raft-dependent transportation (Marchand et al., 2002). Our receptor insertion results demonstrated that the newly inserted AMPARs co-localize with surface rafts, suggesting that either the surface rafts act as docking sites for AMPAR exocytosis, or the vesicles for AMPAR delivery contain raft domains. Furthermore, both NSF, which is involved in AMPAR membrane targeting (Noel et al., 1999), and GRIP1, which has been implicated as a KIF5 motor driver protein transporting AMPARs along the microtubule track to the somadendritic domain (Setou et al., 2002), are associated with lipid rafts shown by the present work and results from others (Suzuki et al., 2001). Also, we found that the NMDA-caused AMPAR enrichment in surface rafts is NOS dependent, which can be mimicked by an NO donor. This is consistent with our previous work showing that *S*-nitrosylation of NSF via NMDAR–calcium–NOS pathway is involved in AMPAR insertion (Huang et al., 2005). All these observations support the importance of raft–AMPAR association in the sorting and targeting of AMPARs to the somadendritic membrane in neurons.

## Experimental methods

### Neuronal cultures and transfection

Primary cortical and hippocampal neurons were prepared from E18 rat embryos. Cortical neurons were plated on 60 mm dishes ($4–6 \times 10^6$) and fed twice a week with glia-conditioned medium for 2–3 weeks. Low-density ($0.3 \times 10^6$ per 60 mm dish) hippocampal neurons were grown on coverslips in Banker style. Transfections were performed on 12–14-day-old hippocampal neurons using Lipofectamine 2000.

### Immunocytochemistry and cholera toxin (CTX) cell-surface labeling

2-week-old low-density hippocampal neurons on coverslips were fixed in 4% paraformaldehyde in PBS for 10 min. Cell-surface GPI-GFP, GluR2-HA or endogenous AMPARs were labeled with anti-GFP, anti-HA or anti-GluR1Nt antibodies, respectively, under non-permeant conditions.

FITC-conjugated cholera toxin B subunit (CTX-FITC) which binds specifically to GM1 was used as a marker of surface lipid rafts. Following a rinse inACSF (150 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4), live or fixed neurons were incubated with CTX-FITC (3 μg/ml) in ACSF at room temperature for 10–15 min. Cells were then washed 3 times with ACSF and fixed with 4% paraformaldehyde for 10 min. For double labeling of surface rafts and AMPARs, cells were fixed and incubated sequentially with CTX-FITC (or anti-GFP antibodies in GPI-GFP transfected neurons) and anti-GluR1N antibodies.

Images for AMPAR co-localization with lipid raft markers including CTX and GPI-GFP were collected by confocal microscopy, and analyzed with Image-J software. Other immunostainings were examined using a fluorescence microscope with 63× objective.

### Qdots live imaging of surface GPI-GFP

Hippocampal neurons expressing GPI-GFP were incubated with anti-GFP antibodies (1:500) for 2–5 min at room temperature. After washing, cells were incubated with quantum dots (Qdots) conjugated with secondary antibody (1:5000) for 1–2 min at room temperature. Cells were washed again and transferred to an imaging chamber in ACSF with temperature controlled at 35 °C on a Zeiss fluorescence microscope.

### Lipid raft preparation by CTX-biotin pull-down assays

2–3 week-old primary cortical neurons were incubated with 10 mM MCD for 15 min, or treated with 30 μM NMDA for 2–10 min at 37 °C, and were then incubated with CTX-biotin (3–5 μg/ml) in ACSF for 15 min at 10 °C. After washing twice with ACSF, cells were lysed on ice in PBS containing 1% Triton X-100. The lysates were rotated at 4 °C for 1 h and then centrifuged at 10,000 ×*g* for 10 min at 4 °C. The CTX-biotin-associated surface rafts in the supernatants were precipitated with 40 μl of neutravidin beads. The proteins in rafts were eluted with 2× sample buffer, and visualized by western blotting. In some experiments, the cells were lysed first and then incubated with CTX-biotin to pull-down all rafts.

### AMPAR Internalization assays

Hippocampal neurons were incubated with anti-GluR1Nt antibodies (1:100) for 5 min at room temperature. After washing, cells were transferred to 37 °C for 15 min, with or without 10 mM MCD, to induce receptor internalization. Following a 5min ×2 acid strip (0.5 M NaCl, 0.2 M acetic acid) on ice to remove surface labeling, neurons were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and incubated with a fluorescence-conjugated secondary antibody for visualization. For the biochemical experiments, cortical neurons were treated with 10 mM MCD for 15 min, washed, and treated with 30 μM NMDA for different periods of time. Cells were then subjected to surface biotinylation assays as described previously (Man et al., 2007). Briefly, cells were incubated at 10 °C with 1 mg/ml sulfo-NHS-SS-biotin in ACSF for 30 min, and lysed in RIPA buffer (PBS containing 1% Triton X-100, 0.5% DOC and 0.1% SDS) following 3 washes. Biotinylated surface proteins were precipitated with immobilized neutravidin beads and AMPARs were probed with anti-GluR1 C-terminal antibodies.

For colorimetric assays, cortical neurons were cultured in 12-well plates. After incubation with 10 mM MCD for 30 min, cells were fixed and either the total or the cell-surface AMPARs were labeled with anti-GluR1 N-terminal antibodies under permeant and non-permeant conditions, respectively. Following incubation with Horseradish peroxidase (HRP)-conjugated secondary antibodies, HRP substrate *O*-phenylenediamine dihydrochloride (OPD) was added (1 ml/well) for 2–5 min and the reaction was stopped with 0.2 ml 3N HCl. The color density of the supernatant was measured by a spectrophotometer at 492 nm (Man et al., 2000b).

### AMPAR insertion assays

Cultured hippocampal neurons were transfected with GluR2-HA and GPI-GFP. The GPI-GFP construct was scaled down during transfection to limit its expression level. 2 days after transfection, neurons were incubated with anti-HA antibodies (1:200) for 40 min at 10 °C, washed, and incubated with non-conjugated anti-mouse IgG (1:100) at 10 °C for 45 min. Following 3 washes, cells were then transferred to 37 °C to permit receptor exocytosis. After 10 min fixation, cells were stained again with anti-HA (1:400) and Cy3-conjugated anti-mouse

(1:700) antibodies. In some experiments surface GPI-GFP was also stained following fixation with an FITC-conjugated anti-GFP antibody under non-permeant conditions.

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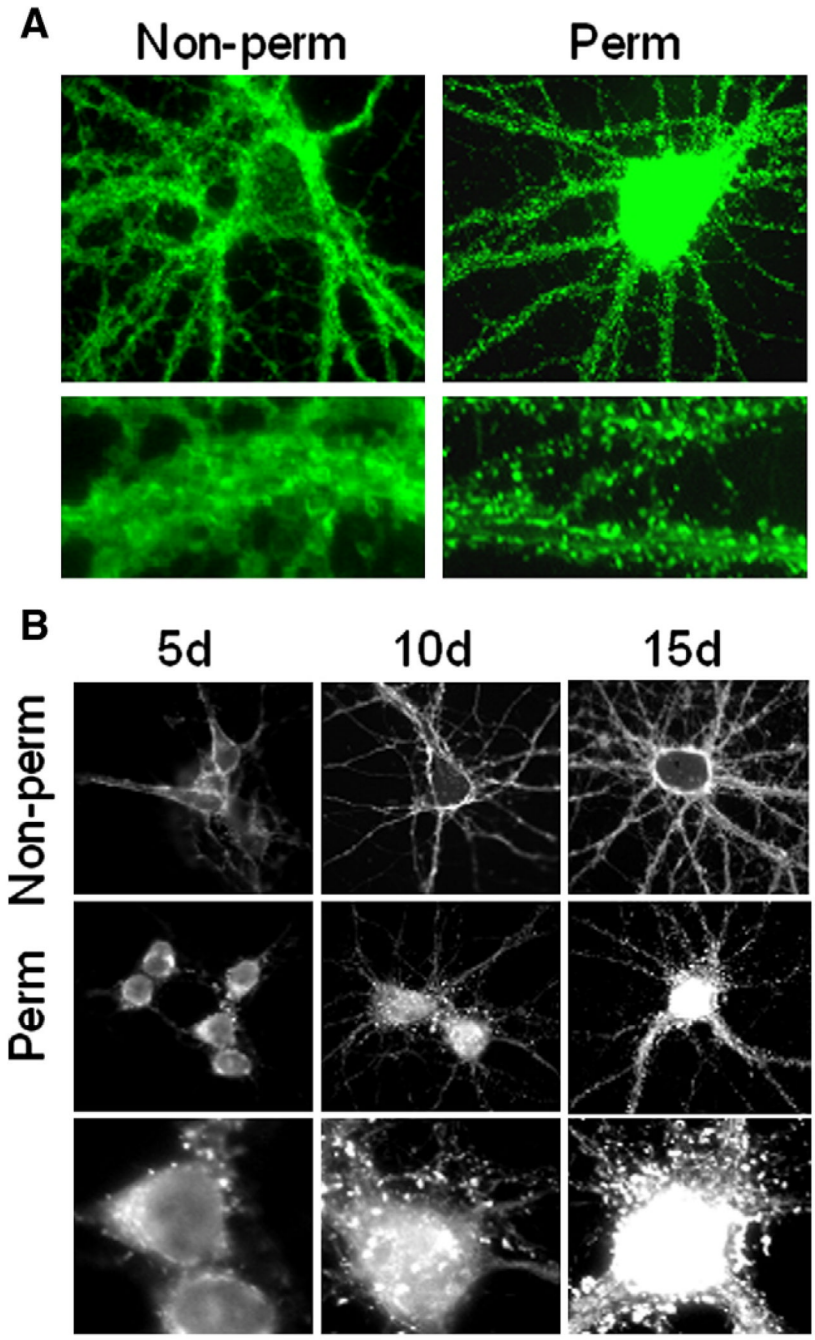


**Fig. 1.**

Distribution of lipid rafts in cultured hippocampal neurons. Lipid rafts were labeled with FITC-conjugated CTX B subunits (CTX-FITC). (A) Rafts show a sharp clustered pattern under permeabilized condition (Perm) compared to the non-permeant labeling (Non-perm). (B) The formation and distribution of lipid rafts changed during neuronal development. Rafts were labeled with CTX-FITC in neurons at day 5, 10 and 15 *in vitro*. Brighter fluorescence signals were observed in more mature neurons. Under permeabilized conditions (Perm), raft clusters localized only around the soma at day 5, but covered the whole cell at day 15. The soma was shown in higher magnification for clarity (bottom panel).

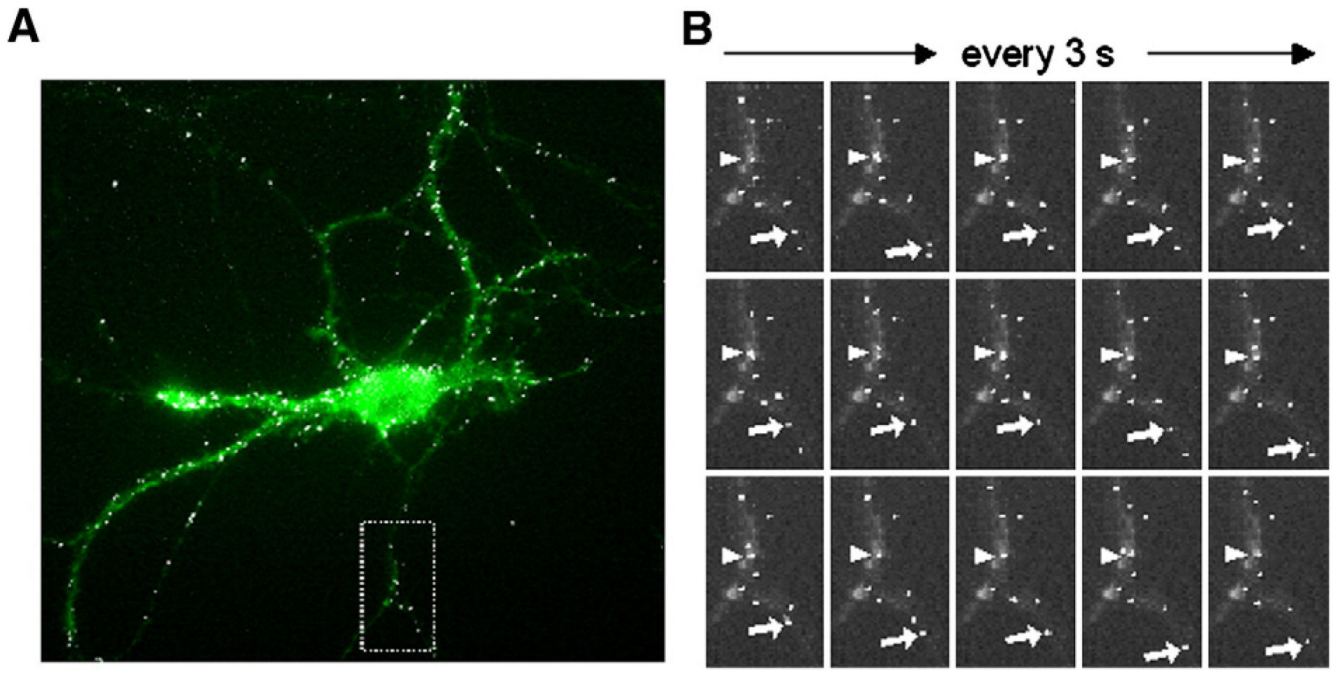


**Fig. 2.**

Live imaging the mobility of a raft marker protein GPI-GFP. (A) Hippocampal neurons were transfected with the raft-associated protein GPI-GFP. Cells were incubated with anti-GFP antibodies (1:500), washed and then incubated with a secondary antibody conjugated with fluorescent Qdots. Green fluorescence indicates GPI-GFP and surface Qdots are shown in white. (B) A small boxed region of the neuron which was imaged every 3 s is shown in high magnification. Most dots moved constantly along the dendrites within short distances (arrow). Fusion and separation of Qdots, as indicated by the arrowhead, were also frequently observed.

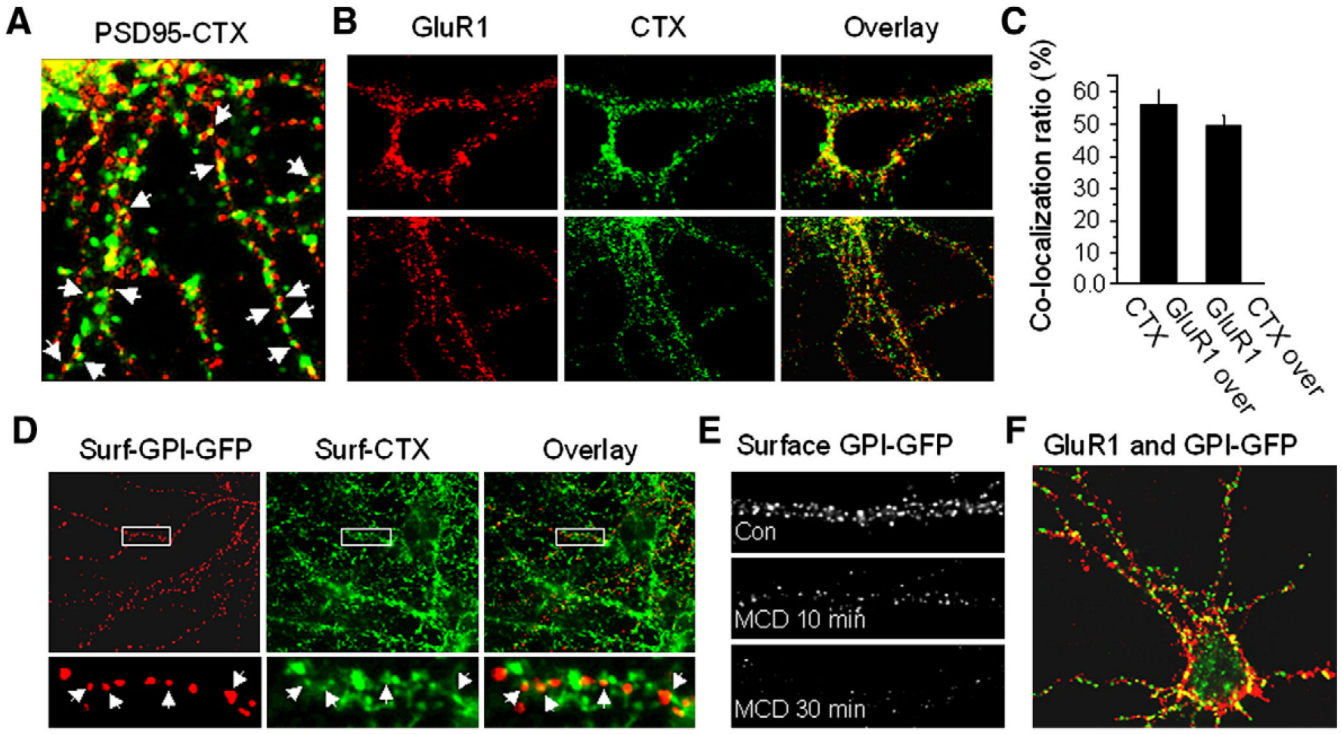


**Fig. 3.**

Co-localization of AMPARs with lipid rafts. (A) Double labeling of PSD-95 and CTX-FITC. Neurons were fixed and incubated with CTX-FITC (green) to label surface rafts. PSD-95 (red) was then immunostained following permeabilization. (B) Cell-surface AMPARs were immunolabeled with an antibody against the extracellular N-terminus of AMPAR GluR1 subunits (GluR1), while the surface rafts were labeled with CTX-FITC (CTX). AMPARs and lipid rafts show a high degree of co-localization (Overlay). (C) Quantification of GluR1-CTX co-localization. 55.6%±2.7% of GluR1 signals co-localizing with CTX fluorescence, whereas 49.3%±3.3% of CTX pixels containing GluR1 signal. (D) Hippocampal neurons were transfected with GPI-GFP as a raft marker. Because the expression of GPI-GFP was controlled at low level, the GFP signal was almost invisible with short exposure at imaging. Surface GPI-GFP was visualized after immunostaining using anti-GFP antibodies (red). A double labeling with CTX-FITC showed the clusters of surface GPI-GFP co-localizing with CTX signals, consistent with their raft localization. (E) Disruption of rafts with 10 mM MCD removed the surface localization of GPI-GFP. (F) Double staining of surface GluR1 (red) and surface GPI-GFP (green) demonstrated an overlap between AMPARs and lipid rafts.

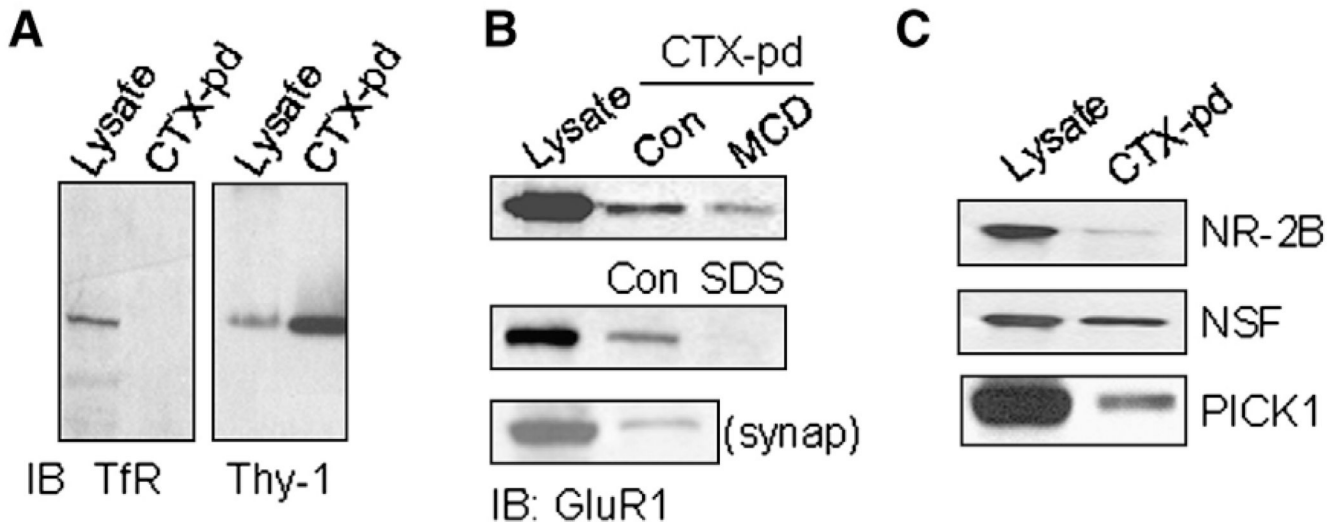


**Fig. 4.**

Isolation of surface lipid rafts in cultured cortical neurons with CTX pulldown assays. (A) Cultured cortical neurons were incubated with biotin-conjugated CTX B subunits (CTX-biotin). Following washing, cells were lysed with 1% Triton X-100 on ice, and cell-surface lipid rafts were isolated by neutravidin beads. Transferrin receptors (TfR), a typical non-raft protein, were not detected in the CTX pulldown sample (CTX-pd) (left), whereas a typical raft-associated protein Thy-1 was enriched in the pulldown complex. (B) AMPAR association with lipid rafts. GluR1 subunits were detected in the purified surface rafts (Con). The amount of raft-associated GluR1 was dramatically reduced in neurons pretreated with MCD (top panel), and was completely abolished by addition of 1% SDS in the lysis buffer (middle panel). GluR1 was also found in rafts from synaptosome preparation (synap) (bottom panel). (C) AMPAR-interacting proteins NSF and PICK1, and a minimal level of NMDAR subunit NR-2B, were found in the CTX-biotin precipitates.

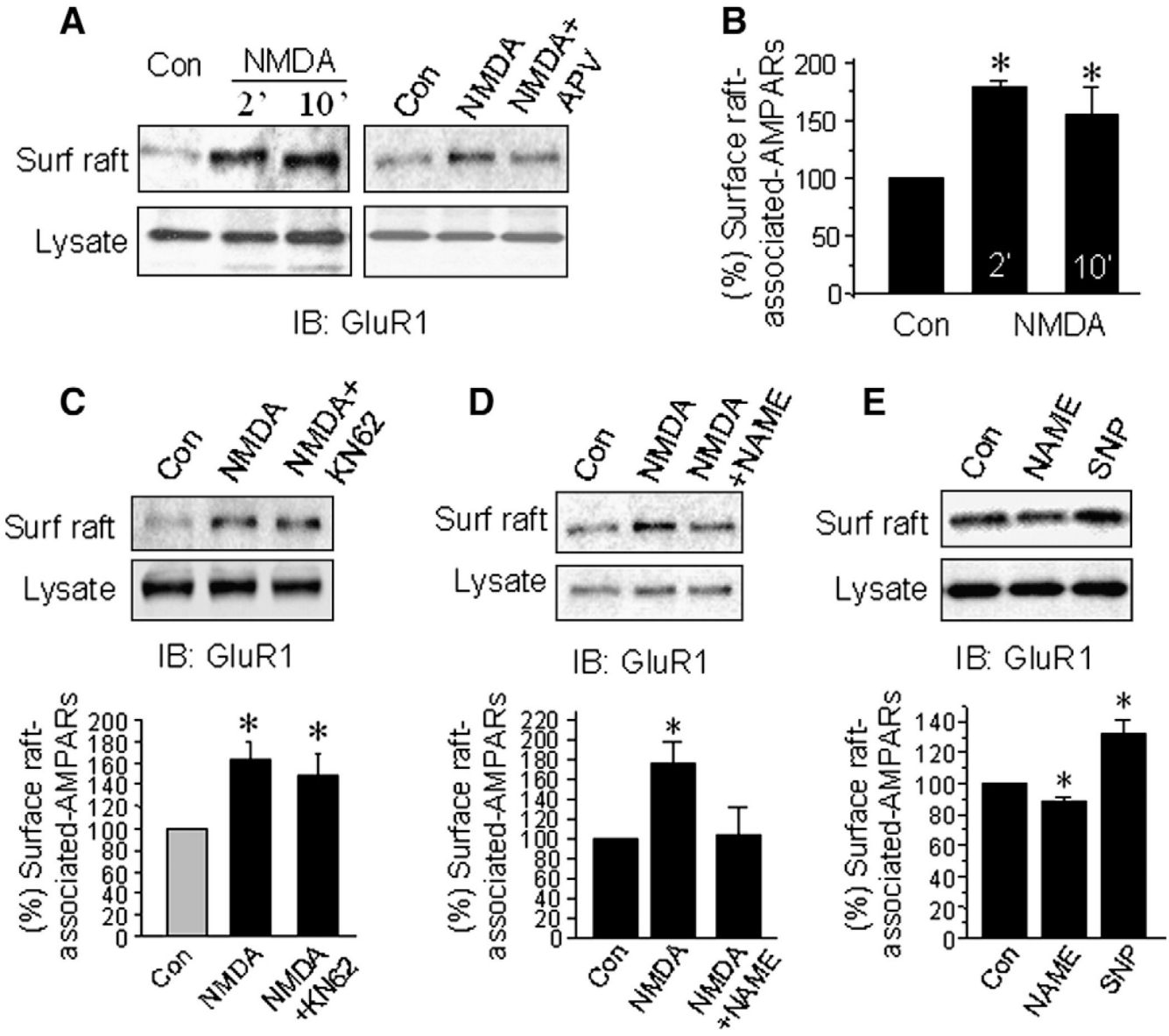


**Fig. 5.**

NMDAR activity recruits AMPARs to surface lipid rafts. Cultured cortical neurons were treated with 30 μM NMDA, and surface (Surf) rafts were isolated by CTX-biotin pulldown assays. AMPARs were recruited to raft domains by 2 or 10 min NMDA treatments (A left and B). The NMDA effect was completely blocked by NMDAR antagonist APV (A right). Whereas no effect was found when CaMKII activity was inhibited by KN-62 (10 μM) (C) ($n$ = 3, *$p$<0.05 compared to the control, $t$ test), the NMDA-induced AMPAR translocation was blocked by the NOS inhibitor L-NAME (200 μM) (D), indicating the involvement of NO pathway. Consistently, the amount of raft-associated AMPARs was decreased with 10 min L-NAME (200 μM) treatment at basal condition ($n$ = 3, *$p$<0.05, $t$ test), while a marked increase in raft-AMPARs was induced in cells treated with an NO donor SNP (500 μM, 10 min) (E) ($n$ = 3, *$p$<0.05, $t$ test).

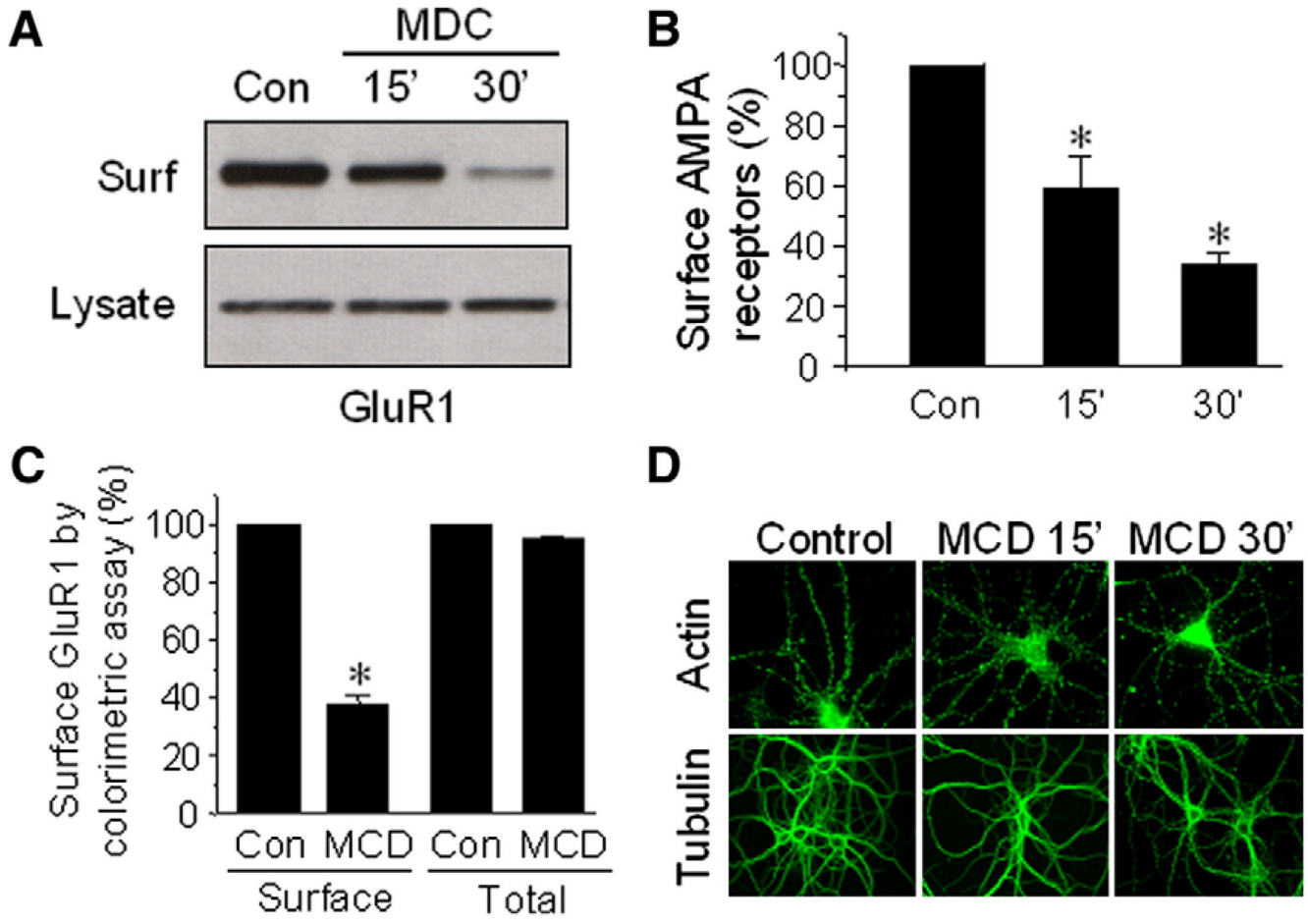


**Fig. 6.**

Disruption of lipid rafts reduces AMPAR surface expression. Cortical neurons were incubated with 10 mM MCD, and the cell-surface AMPARs were isolated by surface biotinylation assays. 15 and 30 min MCD treatment dramatically reduced the abundance of surface AMPARs ($n$ = 3, *$p$<0.05, $t$ test) (A and B). Consistently, colorimetric assays also revealed a marked reduction of surface AMPARs by 30 min MCD incubation (37.6%±3.1% of control, $n$ = 3, *$p$<0.05, $t$ test) (C). Immunostaining of actin and tubulin showed no obvious morphological alterations following 15 min MCD incubation (MCD 15'), but disorganization of the cytoskeletal elements was observed after 30 min treatment (MCD 30') (D).

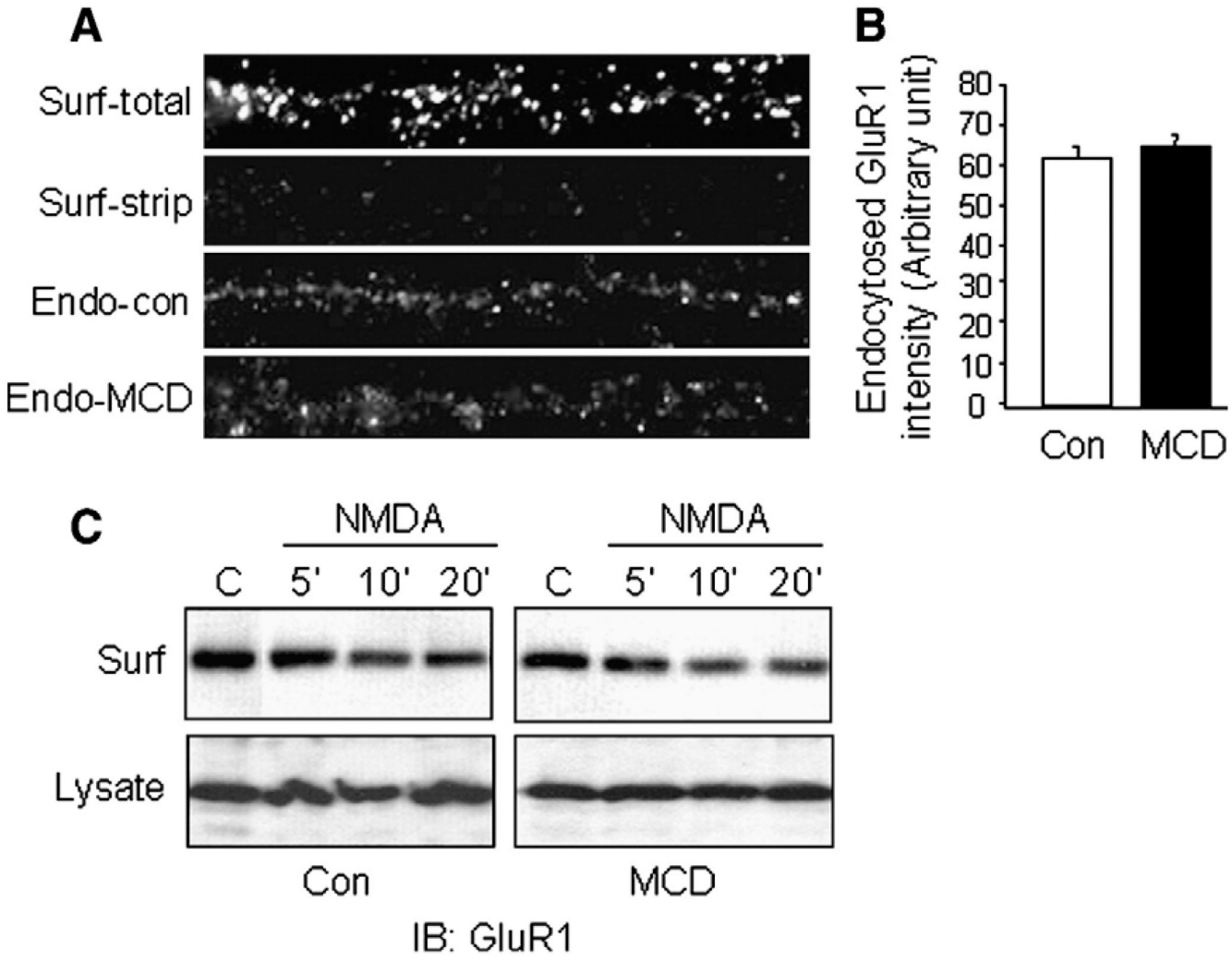


**Fig. 7.**

Effects of raft disruption on AMPAR endocytosis. (A) Surface AMPARs were labeled with anti-GluR1 N-terminal antibodies (1:100, 5 min) at room temperature. As controls, one coverslip was directly stained to show total surface GluR1 levels (Surf-total); another coverslip was incubated with acidic stripping buffer to remove the surface antibodies and then stained with a secondary antibody (Surf-strip). Other coverslips were transferred to 37 °C for 15 min, with or without 10 mM MCD, to allow receptor endocytosis. The internalized AMPARs were detected following acid stripping. GluR1 showed similar levels of internalization between the control (Endo-con) and the MCD-treated (Endo-MCD) cells. (B) Average intensities of internalized GluR1 in dendrites. MCD treatment (64.8±2.7, $n$ = 20) showed no difference compared to the control (62.0±2.6, $n$ = 20). (C) Effect of raft disruption on NMDA-induced AMPAR internalization. Cortical neurons were incubated with MCD for 15 min, then treated with 30 μM NMDA for various periods of time as indicated. Surface biotinylation assays showed a similar reduction of surface AMPARs in both control (left) and MCD-treated cells (right).

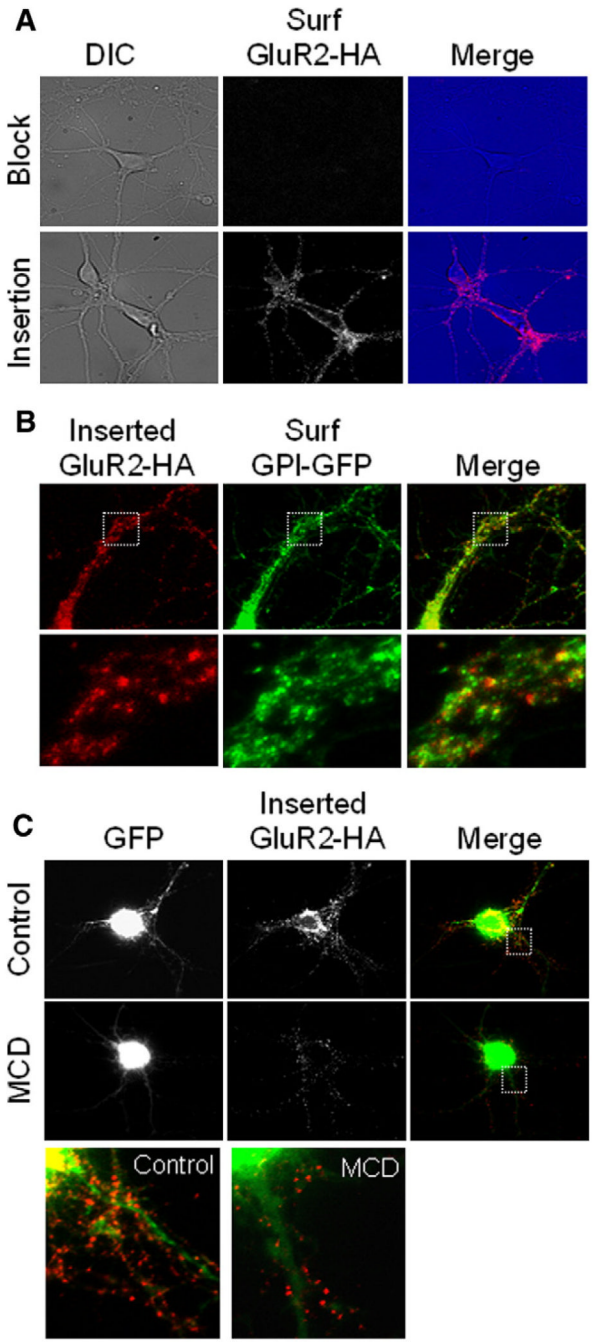


**Fig. 8.**

Disruption of lipid rafts suppresses AMPAR membrane insertion. Cultured hippocampal neurons were transfected with GluR2-HA and raft marker GPI-GFP. The existing surface GluR2-HA was blocked at 10 °C with an anti-HA primary antibody and a non-conjugated secondary antibody. Following incubation at 37 °C to allow receptor exocytosis, the newly inserted GluR2-HA was visualized with a second round of immunostaining. (A) As a control, surface GluR2-HA was completely blocked (upper panel) and the newly inserted receptors were observed following 37 °C incubation for 15 min (Lower panel). (B) In neurons transfected with GluR2-HA and GPI-GFP, the newly inserted surface GluR2-HA (red) and surface GPI-GFP (green) were labeled by immunostaining and a selected area was enlarged for clarity

(lower panel). Note that the newly inserted GluR2-HA co-localized with raft marker GPI-GFP, indicating a role for lipid rafts in AMPAR exocytosis, probably as docking sites. (C) Disruption of lipid rafts suppresses AMPAR insertion. Hippocampal neurons were transfected with GluR2-HA together with GFP. Following blocking of the existing surface GluR2-HA, cells were transferred to 37 °C, with or without 10 mM MCD, to allow receptor insertion. Note that in the MCD-treated neurons, the GluR2-HA surface insertion was dramatically reduced compared to the control. A small region was enlarged for clarity (Bottom).