Kainate receptors exhibit a highly compartmentalized distribution within the brain; however, the molecular and cellular mechanisms that coordinate their expression at neuronal sites of action are poorly characterized. Here we report that the GluK1 and GluK2 kainate receptor subunits interact with the spectrin-actin binding scaffolding protein 4.1N through a membrane-proximal domain in the C-terminal tail. We found that this interaction is important for the forward trafficking of GluK2a receptors, their distribution in the neuronal plasma membrane, and regulation of receptor endocytosis. The association between GluK2a receptors and 4.1N was regulated by both palmitoylation and protein kinase C (PKC) phosphorylation of the receptor subunit. Palmitoylation of the GluK2a subunit promoted 4.1N association, and palmitoylation-deficient receptors exhibited reduced neuronal surface expression and compromised endocytosis. Conversely, PKC activation decreased 4.1N interaction with GluK2/3-containing kainate receptors in acute brain slices, an effect that was reversed after inhibition of PKC.

Our data and previous studies therefore demonstrate that these two post-translational modifications have opposing effects on 4.1N association with GluK2 kainate and GluA1 AMPA receptors. The convergence of the signaling pathways regulating 4.1N protein association could thus result in the selective removal of AMPA receptors from the plasma membrane while simultaneously promoting the insertion and stabilization of kainate receptors, which may be important for tuning neuronal excitability and synaptic plasticity.

Kainate receptors perform roles distinct from other members of the ionotropic glutamate receptor family by regulating neural circuit activity through modulation of excitatory and inhibitory transmission and neuronal excitability (1). The cellular and molecular mechanisms regulating their diverse subcellular localization and synaptic targeting are poorly understood but are critical mediators of kainate receptor signaling in the CNS. In addition to trafficking determinants inherent to individual receptor subunits, the intracellular sorting and neuronal localization of neurotransmitter receptors are tightly regulated by association with auxiliary subunits and interacting proteins (2–5). The identification of these proteins and investigation into their role in neurotransmitter receptor trafficking has been instrumental toward understanding the cellular biology of synaptic plasticity (6). One such family of glutamate receptor interacting proteins, known as band 4.1 proteins, associate with components of the neuronal cytoskeleton and coordinate the insertion and removal of AMPA receptors (7–9), a process critical for regulating the strength of synaptic transmission (10).

The protein 4.1 family consists of four homologous proteins, termed 4.1B (brain), 4.1G (general), 4.1N (neuronal), and 4.1R (erythrocyte) (11). These proteins contain a central spectrin-actin binding domain, which allows 4.1 proteins to function as cytoskeletal adaptor proteins (12). The N- and C-terminal domains bind to the phospholipids phosphatidylinositol 4,5-biphosphate and phosphatidylserine (13, 14) in addition to a cytoskeletal adapter proteins (12). The N- and C-terminal domains bind to the phospholipids phosphatidylinositol 4,5-biphosphate and phosphatidylserine (13, 14) in addition to a
post-translational modifications. Palmitoylation of the GluK2 kainate receptor subunit promotes 4.1N association, whereas PKC phosphorylation antagonizes this interaction. These results are in contrast with previous studies on AMPA receptor association with 4.1N and suggest that common signaling pathways resulting in the covalent modification of AMPA and kainate receptors have been adapted to differentially regulate their insertion and stabilization. These two types of glutamate receptors subscribe distinct functions in the mammalian CNS, and modulation of the association with 4.1N by palmitoylation and phosphorylation could serve as one central mechanism for correlated but opposing alterations in AMPA and kainate receptor signaling.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Pharmacological Agents**—The following antibodies were used in this study: mouse anti-4.1N (611836; BD Biosciences); mouse anti-GluA1 (MAB2263; Millipore (Billerica, MA)); rabbit anti-GluK2/3 (04-921; Millipore); mouse anti-HA clone HA-7 (H6958; Sigma); mouse anti-Myc clone 9E10 (11667149001; Roche Applied Science); rabbit anti-Myc (60-549; Millipore); peroxidase-conjugated goat anti-mouse (31430; Thermo Scientific); and AF488-conjugated goat anti-rabbit, AF546-conjugated goat anti-mouse, and AF568-conjugated goat anti-rabbit (A11034, A11030, and A11036, respectively; Invitrogen). Pharmacological agents were purchased from the following: tetrodotoxin (TTX)2 (Asc-054; Ascent Scientific (Princeton, NJ)); kainate (Asc-100; Ascent Scientific); rabbit anti-Myc antibody in 10% goat serum and PBS (1:400 dilution; 350 μg/ml); rabbit anti-4.1N (611836; Millipore); kainate (Asc-100; Ascent), D-2-amino-phosphonovaleric acid and returned to the incubator for 1 h. Transfections were performed using a ratio of 1 μg of cDNA to 2 μl of reagent, and neurons were incubated for 4 h before being returned to their original wells. COS-7 and HEK293-T/17 cells were transfected using Mirus Bio Trans-IT reagent (Mirus Bio Corp., Madison, WI) at a ratio of 1 μg of cDNA to 3 μl of reagent.

**Cell ELISA**—Enzyme-linked immunosorbent assays were performed as described previously (22). COS-7 cells were plated in 12-well plates and transfected in triplicate. 48 h after transfection, cells were rinsed in PBS and fixed for 15 min in 4% paraformaldehyde in PBS followed by three washes in PBS. To label surface receptors, unpermeabilized cells were incubated with mouse anti-Myc antibody in 10% goat serum and PBS (1:400 dilution; 350 μl/well) for 1 h at room temperature. The total receptor population was labeled in parallel wells following permeabilization for 15 min in PBS containing 0.3% Triton X-100. Cells were washed three times and labeled with goat anti-mouse HRP-conjugated secondary antibody in 10% goat serum, PBS (1:1000 dilution; 350 μl/well). Following three more washes with PBS, labeled receptor protein was detected using the chromogenic HRP substrate o-phenylenediamine dihydrochloride (P9187; Sigma). After a 1-h incubation at room temperature, solution absorbance was read at 492 nm in 96-well plates using a Tecan Safire2 plate reader (Tecan Systems Inc., San Jose, CA). Solution absorbance from untransfected wells processed in parallel was subtracted from transfected wells, and the percentage of plasma membrane expression was determined by dividing the mean surface absorbance by the mean total absorbance. All wells were processed in triplicate.

**Electrophysiology**—Whole-cell recordings were made from transfected HEK293-T/17 cells held at −70 mV as described previously (22). Currents were elicited by fast application of 10 mM glutamate using a piezoceramic system, where rise times (10–90%) ranged from 0.5 to 1.5 ms. τ values were calculated from single exponential fits of currents elicited by 100-ms glutamate applications using Clampfit10 (Molecular Devices).

**Coimmunoprecipitation of Native and Recombinant Proteins**—For analysis of endogenous proteins, mice were anesthetized using isoflurane, followed by decapitation. Brains were dissected in ice-cold PBS, and following dissection, tissue was homogenized in lysis buffer, which consisted of 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors (P2714; Sigma). Lysates were rotated end-over-end for 1 h at 4 °C to solubilize the proteins. Samples were then clarified by centrifugation at 20,000 × g for 25 min. Equal amounts of protein were precleared overnight with 50 μl of protein A/G bead slurry (20421; Thermo Scientific). Proteins were immunoprecipitated with 2 μg of mouse anti-GluA1, rabbit anti-GluK2/3,
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or mouse anti-4.1N antibodies and 50 μl of protein A/G bead slurry overnight. Bound proteins were eluted by heating samples in 2× Laemmli buffer containing β-mercaptoethanol for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted using mouse anti-GluA1, rabbit anti-GluK2/3, or mouse anti-4.1N. HRP-conjugated goat anti-mouse and anti-rabbit antibodies were from GE Healthcare. 10 μg of protein from the cell lysate was run in parallel to detect total protein expression.

Recombinant proteins were expressed in HEK293-T/17 or COS-7 cells for 48 h before cells were rinsed with ice-cold DPBS and lysed in lysis buffer as indicated previously. Crude cell lysates were then centrifuged at 20,000 × g for 25 min. Equal amounts of protein were then precleared with 50 μl of protein A/G beads for 1 h at 4°C. Proteins were immunoprecipitated using 2 μg of rabbit anti-Myc antibody and 50 μl of protein A/G beads. Bound proteins were eluted and separated as indicated previously. Proteins were detected using mouse anti-HA antibodies. 10 μg of protein from the cell lysate was run in parallel to verify equal expression of proteins between samples.

[^3H]Palmitate Labeling—[^3H]Palmitate (NET043025MC; PerkinElmer Life Sciences) was dried in a stream of N₂, to a final volume of ~0.5 ml. COS-7 cells expressing Myc-GluK2 receptors and palmitoyl acyltransferase enzymes were incubated in serum-free medium containing 2 mg/ml fatty acid-free BSA (A8806; Sigma) and 0.5 μCi of[^3H]palmitate for 4 h. Cells were rinsed and lysed in radioimmune precipitation assay buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, pH 7.5), and receptor subunits were immunoprecipitated as described above. Following SDS-PAGE, gels were fixed for 30 min (10% acetic acid, 25% isopropyl alcohol, 65% H₂O), treated with Amplify fluorographic reagent (NAMP100; GE Healthcare) for 30 min, dried under vacuum, and exposed to film at ~80°C for 4–6 weeks.

Immunocytochemistry—For analysis of endogenous proteins, neurons were fixed for 15 min in PBS containing 4% paraformaldehyde and 4% sucrose. Following three washes with PBS, neurons were permeabilized with PBS containing 2% goat serum and 0.2% Triton X-100 for 15 min. After washing, coverslips were incubated with rabbit anti-GluK2/3 antibodies (1 μg/ml) in 2% goat serum for 1 h. Coverslips were then washed and incubated with mouse anti-4.1N antibodies (1 μg/ml) in 2% goat serum for an additional 1 h. Neurons were then incubated with AF488-conjugated goat anti-rabbit and AF546-conjugated goat anti-mouse antibodies (1 μg/ml) for 1 h, washed, and then mounted onto glass slides using Prolong Gold antifade reagent.

Neurons expressing tagged kainate receptor subunits were fixed 2 days post-transfection. Following washing, surface-expressed receptors were labeled with mouse anti-Myc antibody (1 μg/ml) in PBS containing 2% goat serum for 1 h at room temperature. Cells were permeabilized for 15 min in PBS containing 2% goat serum and 0.2% Triton X-100. Intracellular receptors were then labeled with rabbit anti-Myc antibodies (1 μg/ml) diluted in PBS containing 2% goat serum for 1 h at room temperature. Antibody-labeled receptors were then incubated with AF488-conjugated goat anti-rabbit and AF546-conjugated goat anti-mouse antibodies (1 μg/ml) for 1 h. Coverslips were then mounted as described above.

Live Labeling Internalization Assay—2 days post-transfection, coverslips were live labeled with 10 μg/ml rabbit anti-Myc antibodies prepared in 200 μl of the DMEM in which the neurons were cultured, referred to as conditioned medium, for 15 min at 37°C. Coverslips were then rinsed and returned to their original wells at 37°C. For TTX-treated neurons, coverslips were incubated in conditioned medium containing 2 μM TTX for 1 h prior to labeling with antibodies. TTX was present in all subsequent steps. For kainate stimulation, coverslips were processed in parallel to those treated with TTX alone. After antibody labeling, cells were stimulated with 10 μM kainate in conditioned medium containing 2 μM TTX for 3 min. Coverslips were then transferred into wells containing 2 μM TTX in conditioned medium for an additional 12 min. After allowing receptors to internalize for 15 min, coverslips were then fixed in cold PBS containing 4% sucrose and 4% paraformaldehyde for 15 min. Following washing, antibody-bound receptors on the neuronal surface were labeled with AF568-conjugated goat anti-rabbit antibodies (2 μg/ml) for 1.5 h at room temperature. Cells were then permeabilized with PBS containing 2% goat serum and 0.2% Triton X-100 for 15 min. Antibody-bound receptors that had internalized were then labeled with AF488-conjugated goat anti-rabbit antibodies (2 μg/ml) for 1.5 h at room temperature. After washing, coverslips were mounted as described above.

Confocal Microscopy—All images were collected on a Zeiss LSM510 META laser-scanning confocal microscope at the Northwestern University Cell Imaging Facility using a Plan-Apochromat ×63 oil immersion objective lens (numerical aperture 1.4). AF488 and was excited using the 488-nm line from an Argon laser. AF546 and AF568 were excited using the 543-nm line from a helium-neon laser. Images in each channel were acquired sequentially, and emitted photons were collected using separate photomultiplier tubes. A 505–530-nm band pass filter was used for AF488 fluorescence. A 560–590-nm band pass filter was used for AF488 fluorescence. A 560–590-nm band pass filter was used for AF488 fluorescence. A 560–590-nm band pass filter was used for AF488 fluorescence. A 560–590-nm band pass filter was used for AF488 fluorescence. A 560–590-nm band pass filter was used for AF488 fluorescence. A 560–590-nm band pass filter was used for AF488 fluorescence.

All images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Quantification of plasma membrane (PM) expression and receptor endocytosis was performed on background-subtracted images reconstructed into a stack of summed pixel intensities. Outlines were manually drawn around the cell, and integrated pixel intensities were calculated for each channel. Percentage of PM expression was calculated by dividing the surface fluorescence intensity by the summed fluorescence intensity of both the surface and intracellular channels. Endocytosis was quantified by dividing the fluorescence intensity from the internalized channel by the summed intensities of both the extracellular and internalized fluorescence to control for differences in steady-state surface expression between receptors.
RESULTS

Neuronal Kainate Receptors Interact with 4.1N—The 4.1 proteins regulate the trafficking and plasma membrane delivery of GluA1- and GluA4-containing AMPA receptors (7–9) through interactions with a cytoplasmic membrane-proximal domain (MPD) that has a modest degree of primary sequence similarity to that found in kainate receptor subunits; consequently, we postulated that kainate receptors could also associate with proteins in the 4.1 family. To test this hypothesis, we performed immunoprecipitation experiments from mouse cortex and cerebellum and hippocampus. Pull-down of GluK2/3-containing kainate receptor subunits resulted in coimmunoprecipitation of the neuron-expressed member of this family, 4.1N (Fig. 1  \(\text{A} \)). We performed parallel experiments from GluK2/3 mice and observed a strongly reduced but still detectable level of 4.1N protein, suggesting that 4.1N also interacts with GluK3-containing kainate receptors in the brain. In reciprocal immunoprecipitations, GluK2/3-containing kainate receptors were pulled down with anti-4.1N antibody (not shown). As a positive control, 4.1N also coimmunoprecipitated with GluA1-containing AMPA receptor subunits from mouse cortex, cerebellum, and hippocampus, as was described previously (Fig. 1A) (7, 9). Thus, 4.1N associates with neuronal kainate and AMPA receptors, in agreement with results from proteomic screens conducted recently (23).

We next explored the neuronal distribution of GluK2/3 kainate receptors and 4.1N proteins to determine if they reside in similar subcellular compartments. Confocal micrograph images of cultured rat hippocampal neurons stained with antibodies to GluK2/3 subunits (green) and protein 4.1N (red), demonstrating that both proteins localize to similar subcellular compartments. The boxed region is expanded in the bottom panels to illustrate the overlapping distribution of both proteins along the dendritic shaft and within spines. Scale bars, 50 and 10 \(\mu\)m. IP, immunoprecipitation.

**FIGURE 1.** Neuronal kainate receptors interact with the cytoplasmic scaffolding protein 4.1N. A, representative Western blot showing coimmunoprecipitation of 4.1N with GluK2/3-containing kainate receptors from mouse cortex (Cx) and cerebellum (Cb) and hippocampus (Hp). As a positive control, we also detected 4.1N coimmunoprecipitation with GluA1-containing AMPA receptors. B–D, representative confocal micrographs of cultured rat hippocampal neurons immunostained for endogenous GluK2/3 subunits (green) and protein 4.1N (red), demonstrating that both proteins localize to similar subcellular compartments. The boxed region is expanded in the bottom panels to illustrate the overlapping distribution of both proteins along the dendritic shaft and within spines. Scale bars, 50 and 10 \(\mu\)m. IP, immunoprecipitation.

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FIGURE 2. **Residues within the kainate receptor MPD are important determinants for plasma membrane localization.** A, schematic of an individual kainate receptor subunit illustrating the extracellular N-terminal domain (NTD), ligand binding domain (LBD), the membrane spanning segments (M1–M4), and the cytoplasmic MPD immediately following the fourth transmembrane domain. B, sequence alignment of the MPDs of the five kainate receptor subunits in addition to the GluA1 AMPA receptor subunit, demonstrating the highly conserved nature of the residues in this domain. The arrow depicts the site where splicing of kainate receptor mRNA generates alternative C-terminal tails. C, graph of the plasma membrane localization of mutated GluK2a receptors containing the depicted alanine substitutions to residues within the MPD assessed by whole-cell ELISAs in COS-7 cells. D, graph of the plasma membrane localization of GluK1 receptors with mutations in a previously identified endoplasmic reticulum retention motif (GluK1(GGAA)), demonstrating that residues within this MPD are also critical for GluK1 localization to the plasma membrane. Values shown are mean ± S.E. (error bars), and statistical significance is denoted as follows: *, p < 0.05; ***, p < 0.001.

We first conducted an alanine scan of the GluK2 MPD, with relative plasma membrane localization in heterologous cells as a functional readout to identify candidate residues comprising a putative 4.1 protein association domain.

Whole-cell ELISAs performed on COS-7 cells transfected with the indicated Myc-tagged GluK2 subunits showed that substitution of Glu-841, Phe-842, Tyr-844, and Lys-848 resulted in significantly decreased surface expression (82 ± 3% for GluK2a, 12 ± 7% for E841A, 58 ± 0% for F842A, 26 ± 7% for Y844A, and 64 ± 2% for K848A; n = 3–5; *, p < 0.05; ***, p < 0.001; Fig. 2C). Unexpectedly, a double mutant containing both Y844A and K848A substitutions resulted in a similar level of PM expression as the individual K848A mutant (61 ± 6% for Y844A,K848A; n = 4; p < 0.05). The triple mutant comprised of F842A, Y844A, and K848A was almost completely absent from the surface of COS-7 cells (3 ± 1%; n = 3; p < 0.001). The analogous Y844A substitution in the GluK1 subunit also significantly reduced PM localization (6 ± 2% for GluK1 compared with 2 ± 2% for Y844A; n = 4; p < 0.001; Fig. 2D), demonstrating that these highly conserved residues are important for the surface expression of multiple kainate receptor subunits.

Kainate receptor association with auxiliary subunits and interacting proteins has been shown to modulate receptor functional properties and influence receptor localization (5). To test whether kainate receptor association with 4.1N alters receptor function, whole-cell voltage clamp recordings from transfected HEK293-T/17 cells were performed. Cells were voltage-clamped at −70 mV, and currents were elicited by fast application of 10 mM glutamate. We found that peak current amplitudes were significantly reduced for several of the trafficking mutants identified in COS-7 cell ELISAs. GluK2a receptors had a mean peak current amplitude of 5.8 ± 0.5 nA (n = 17), whereas the F842A (1.6 ± 0.4 nA; n = 10; p < 0.01), Y844A (3.4 ± 0.9 nA; n = 12; p < 0.05), Y844A,K848A (2.8 ± 0.6; n = 11; p < 0.01), and F842,Y844A,K848A mutants (0.6 ± 0.3 nA; n = 11; p < 0.01) were all significantly reduced. We were unable to eliciting currents from receptors containing the E841A substitution despite a low level of receptor surface expression in the cell ELISA experiments, suggesting that this charged juxtamembrane residue may be important for channel gating.

Because glutamate receptor kinetics can also be altered by associated proteins (4, 5), we measured desensitization rates for these receptors. Wild-type GluK2a receptors desensitized with a τ of 4.1 ± 0.1 ms (n = 17). Desensitization rates were not significantly different from GluK2 receptors for several of the trafficking mutants that exhibited significant reductions in surface localization (τ values: Y844A, 4.8 ± 0.5 ms; Y844A,K848A, 5.1 ± 0.6 ms; F842A,Y844A,K848A, 5.4 ± 0.5 ms; n = 10–12; p > 0.05; data not shown). We also tested whether overexpression of 4.1N might modulate channel properties; however, we found no evidence for any alterations in desensitization kinetics (τ value 4.1 ± 0.1 ms; n = 11; data not shown), arguing against...
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FIGURE 3. Kainate receptors associate with 4.1N through residues within the MPD. A, representative Western blots showing coimmunoprecipitation of HA-4.1N with Myc-GluK2a subunits containing alanine substitutions within the MPD from transfected heterologous cells. The bottom panel depicts the expression of the indicated receptors from the cell lysate. B, graph of the normalized immunoprecipitate/lysate density relative to wild-type GluK2a receptors. C, Western blot image demonstrating coimmunoprecipitation of HA-4.1N with Myc-GluK1-2a from transfected heterologous cells. The bottom panel depicts the amount of immunoprecipitated GluK1 protein. Values shown are mean ± S.E. (error bars); n.s., not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001. IP, immunoprecipitation; IB, immunoblot.

a role for this interaction in modulating the functional properties of kainate receptors.

To determine if reductions in membrane localization correlated with 4.1N association, we performed coimmunoprecipitation experiments from heterologous cells expressing Myc-tagged GluK2a subunits and HA-tagged 4.1N. Pull-down of GluK2a with anti-Myc antibodies resulted in robust coimmunoprecipitation of 4.1N as assayed in Western blots with anti-HA antibody (Fig. 3A). The previously identified trafficking mutants were found to have significant reductions in 4.1N coimmunoprecipitation (58 ± 9% for F842A, 46 ± 10% for Y844A, 55 ± 11% for K848A, 47 ± 10% for Y844A,K848A, and 50 ± 10% for F842A,Y844A,K848A; n = 6–10; *, p < 0.05; **, p < 0.01; ***, p < 0.001; Fig. 3A and B). Importantly, the K847A substitution neither reduced PM localization of the receptor nor altered 4.1N association relative to wild-type receptor (104 ± 14%; n = 4), arguing against a nonspecific disruption of the membrane-proximal domain structure and consequent alterations in forward trafficking. Conversely, the E841A mutation resulted in a non-significant reduction in 4.1N association (80 ± 12%; n = 6) despite a drastic reduction in PM localization of these receptors. This charged residue, which immediately follows the final transmembrane domain, might function to stabilize the intramembrane helix, as has been described previously (24). In support of this, we were unable to elicit currents from these receptors. In summary, there is general correlation between 4.1N association and plasma membrane expression of GluK2a wild-type and mutant receptors, although exceptions like E841A and the Y844A,K848A double mutant suggest that additional factors, perhaps including alterations in secondary structure or differences in affinity for other 4.1 isoforms, also play a role in the trafficking of receptors in these in vitro experiments. We note that our coimmunoprecipitation experiments do not conclusively differentiate between direct or indirect interactions between 4.1N and kainate receptor MPDs. However, our working hypothesis is that a direct interaction occurs in light of the close parallels with AMPA receptor interactions with 4.1N, which have been shown to occur within the GluA1 subunit using a yeast two-hybrid approach (7).

We postulated that 4.1N might also associate with additional kainate receptor subunits based on the high degree of sequence homology between their MPDs (Fig. 2B). We chose to examine the GluK1-2a subunit, which only contains two amino acids following the MPD, resulting in a short 16-amino acid C-terminal tail (Fig. 2B) to determine if the MPD is sufficient to mediate interaction with 4.1N. If more distal sites within the GluK2a subunit were involved in 4.1N, then we probably would not detect association with GluK1-2a. Pull-down of Myc-tagged GluK1-2a resulted in coimmunoprecipitation of HA-4.1N (Fig. 3C), demonstrating that this short initial segment of the C-terminal tail is sufficient for 4.1N association. These findings indicate that 4.1N associates with multiple kainate receptor subunits dependent upon discrete residues within the MPD and that a largely overlapping set of residues are important for the surface localization of these receptors.

Receptors with Decreased 4.1N Association Exhibit Reduced Expression on the Neuronal Plasma Membrane—We next tested if the MPD mutations that reduced 4.1N association and impacted PM localization in heterologous cells had analogous effects on the neuronal localization of these receptors. Cultured hippocampal neurons expressing Myc-tagged wild-type and mutant GluK2a receptors were stained for visualization of the surface (red) and intracellular receptor pools (green) with confocal microscopy. Wild-type receptors exhibited a very high level of PM localization in neurons (60 ± 2%; n = 21; Fig. 4, A and B), consistent with earlier studies (25, 26). Similar to the results obtained in COS-7 cells, many of the 4.1N binding mutants exhibited significant reductions in PM localization (percentage of plasma membrane expression, 32 ± 4% for F842A; 20 ± 4% for Y844A; 28 ± 3% for Y844A,K848A and...
17 ± 3% for F842A,Y844A,K848A; n = 13–20; *** p < 0.001; Fig. 4, A and B). The K848A mutant, which exhibited modest reductions in surface expression in surface expression in heterologous cells, exhibited a non-significant reduction in neuronal membrane localization (48 ± 3; n = 14). We also tested the GluK2a(K847A) mutant, which exhibited normal levels of 4.1N association and surface localization in COS-7 cells, and found that this receptor was expressed at wild-type levels on the neuronal surface (55 ± 3%; n = 10).

Neuronal Distribution of GluK2a Is Altered When 4.1N Binding Is Reduced—During our analysis of receptor surface localization, we observed that two mutants, Y844A and Y844A,K848A, exhibited a dramatic and readily apparent redistribution of surface-expressed receptors (Fig. 4C). Despite an almost complete loss of surface-expressed receptors from dendritic shafts, a subset of spines contained bright surface puncta. To quantitate this effect, we calculated a spine/shaft ratio of peak fluorescence intensity for both channels, corresponding to the PM and intracellular populations of receptors (Fig. 4, D and E). Both populations of wild-type GluK2a receptors exhibited similar spine/shaft fluorescence ratios (Fig. 4E; 1.0 ± 0.1 for the intracellular channel compared with 1.1 ± 0.1 for the surface channel; n = 76), indicating that the wild-type receptor was not preferentially localized to either of the two domains. The F842A mutation, which shows mild reductions in 4.1N association, had a slight, but not significant increase in surface spine fluorescence (2.4 ± 0.5) with minimal effects on intracellular distribution (0.8 ± 0.1; n = 46). Consistent with our qualitative assessment, the Y844A and Y844A,K848A mutants exhibited marked increases in surface spine/shaft ratios on PM (Y844A, 19.9 ± 8.2; n = 21; Y844A,K848A, 20.7 ± 6.0; n = 25); thus, essentially all receptor proteins on the PM were localized to spines rather than dendritic shafts. This disequilibrium was not present for intracellular receptors because the Y844A mutant had a similar level of intracellular spine/shaft fluorescence to GluK2a (1.3 ± 0.2; n = 21), and the Y844A,K848A mutations resulted in a modest but not significant 3-fold enhancement in intracellular spine/shaft expression (3.1 ± 0.5; n = 25). These experiments demonstrate multiple roles for 4.1 proteins in promoting both the forward trafficking of receptors to the neuronal PM and the maintenance of an extrasynaptic pool of surface-expressed receptors on the dendritic shaft.

Knockdown of 4.1N Decreases Plasma Membrane Localization—Multiple 4.1 isoforms are expressed in the brain (27, 28), and it is therefore possible that mutation of residues within the MPD altered kainate receptor association with other neu-
4.1N Association Is Important for Regulated Endocytosis of Kainate Receptors—The decreased PM localization of these receptors could be caused by disrupted binding to the spectrin and actin cytoskeleton, which is important for regulating receptor endocytosis (29). This is the case for the GluK2b kainate receptor splice variant, where plasma membrane stability and receptor exocytosis are regulated by association with the actin-binding protein profilin IIa (30). To test whether 4.1N association regulates kainate receptor endocytosis, we compared internalization of Myc-GluK2a receptors and a mutant receptor with reduced 4.1N association (Y844A,K848A) and a 4.1N construct resistant to shRNA-mediated degradation (4.1N rescue), or both. The merged images show both the surface-expressed receptors (red/orange) and intracellular populations (green). Scale bar, 50 μm. C, quantification of receptor surface expression for the conditions illustrated in B. Values shown are mean ± S.E. (error bars). *** p < 0.001.

Kainate Receptors

Association regulation of kainate receptor endocytosis, we compared internalization of Myc-GluK2a receptors and a mutant receptor with reduced 4.1N association (Y844A,K848A) and a 4.1N construct resistant to shRNA-mediated degradation (4.1N rescue), or both. The merged images show both the surface-expressed receptors (red/orange) and intracellular populations (green). Scale bar, 50 μm. C, quantification of receptor surface expression for the conditions illustrated in B. Values shown are mean ± S.E. (error bars). *** p < 0.001.

Coexpression of the 4.1N shRNA with Myc-GluK2 greatly reduced 4.1N expression in transfected HEK293 cells, and a 4.1N rescue construct containing silent point mutations rendering it resistant to shRNA-mediated degradation (9). As was previously reported, this 4.1N shRNA construct significantly reduced 4.1N expression and surface receptors were differentially labeled with fluoro-rors. The decreased PM localization of these receptors could be caused by disrupted binding to the spectrin and actin cytoskeleton, which is important for regulating receptor endocytosis (29). This is the case for the GluK2b kainate receptor splice variant, where plasma membrane stability and receptor exocytosis are regulated by association with the actin-binding protein profilin IIa (30). To test whether 4.1N association regulates kainate receptor endocytosis, we compared internalization of Myc-GluK2a receptors and a mutant receptor with reduced 4.1N association (Y844A,K848A) and a 4.1N construct resistant to shRNA-mediated degradation (4.1N rescue), or both. The merged images show both the surface-expressed receptors (red/orange) and intracellular populations (green). Scale bar, 50 μm. C, quantification of receptor surface expression for the conditions illustrated in B. Values shown are mean ± S.E. (error bars). *** p < 0.001.

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FIGURE 5. Knockdown of 4.1N reduces GluK2 expression on the neuronal plasma membrane. A, representative Western blots demonstrating that 4.1N protein is reduced by 4.1N shRNA 11 and rescued by a siRNA-resistant construct in HEK293-T/17 cells. Tubulin immunoreactivity was used as a loading control in these experiments. B, cultured hippocampal neurons expressing wild-type GluK2a receptors and 4.1N shRNA, a 4.1N construct resistant to shRNA-mediated degradation (4.1N rescue), or both. The merged images show both the surface-expressed receptors (red/orange) and intracellular populations (green). Scale bar, 50 μm. C, quantification of receptor surface expression for the conditions illustrated in B. Values shown are mean ± S.E. (error bars). *** p < 0.001.

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Kainate Receptors

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The distal cysteine residues at positions 858 and 871 were confirmed to be the major sites of palmitoylation because alanine substitutions prevented [3H]palmitate incorporation even when neuronal palmitoyl acyltransferase enzymes were overexpressed (Fig. 7A).

Proximal palmitoylation of the GluA1 MPD antagonizes 4.1N association, but no analogous cysteine exists in GluK2a subunits, raising the possibility that distal palmitoylation may play an alternative role in 4.1N association. Pull-down of Myc-tagged GluK2a(C858A,C871A) resulted in significantly less coimmunoprecipitation of HA-4.1N compared with wild-type subunits (18% vs. 4% control; p < 0.001; Fig. 7, B and C). This result demonstrates that, unlike proximal palmitoylation within the MPD of the GluA1 receptor subunit, distal palmitoylation of GluK2a promotes association with 4.1N. These results prompted us to examine the plasma membrane localization of these palmitoylation-incompetent receptors in hippocampal neurons. Similar to mutations within the MPD, there was a significant reduction in surface expression of GluK2a(C858A,C871A) (39% vs. 14% control; p < 0.01; Fig. 7, D and E), possibly due to alterations in receptor endocytosis. Similar to the effect of mutations within the MPD, non-palmitoylated receptors exhibited normal levels of basal internalization (27% vs. 20% control; n = 20) and a high degree of constitutive endocytosis when action potentials were blocked with TTX (25% vs. 20% control; Fig. 7, F and G). Exogenous application of kainate during action potential blockade did not increase receptor internalization, similar to what was seen with MPD mutations (31% vs. 20% control; Fig. 7D). Thus, whereas proximal palmitoylation of AMPA receptors antagonizes 4.1N binding, this modification is instead permissive for GluK2 MPD association with 4.1N and consequent stabilization on the plasma membrane.

PKC Phosphorylation Promotes Dissociation of 4.1N—One functional consequence of GluK2a palmitoylation is that non-palmitoylated receptors were found to be better substrates for PKC phosphorylation (18), thus raising the possibility that 4.1N association is also regulated by phosphorylation. To determine whether neuronal kainate receptor interaction with 4.1N is regulated in a phosphorylation-dependent manner, we performed coimmunoprecipitation experiments from acute brain slices, in which endogenous proteins are more likely to be localized to synaptic locations found in the intact brain. Acute slices were perfused with ACSF in a heated chamber containing vehicle, the PKC activator phorbol 12-myristate 13-acetate (PMA), or
the PKC inhibitor GF109203X. After 15 min, GluK2/3 subunits were immunoprecipitated from the homogenized tissue, and 4.1N association was determined by Western blot. Activation of PKC with 200 nM PMA resulted in a 52% reduction in 4.1N that was coimmunoprecipitated with GluK2/3-containing kainate receptors compared with vehicle-treated controls (\(n=3\); Fig. 8, A and B). Conversely, inhibition of PKC with 500 nM GF109203X increased the amount of 4.1N pulled down with GluK2/3 by 55% (\(n=3\); PMA versus GF109203X; \(p<0.05\); Fig. 8, A and B). These results demonstrate that PKC phosphorylation negatively regulates neuronal kainate receptor interaction with 4.1N.

The GluK2a subunit contains two serine residues, one within the MPD (Ser-846) and the other adjacent to a palmitoylated cysteine residue (Ser-868), that act as the predominant sites of PKC phosphorylation (20). To determine whether PKC phosphorylation within the MPD regulates 4.1N association, Ser-846 was substituted for either alanine to block PKC phosphorylation or aspartate to mimic the phosphorylated state. Receptors were expressed in heterologous cells, and 4.1N association was determined by coimmunoprecipitation with GluK2a-containing kainate receptors compared with vehicle-treated controls (\(n=3\); \(p<0.05\); Fig. 8, A and B). These results demonstrate that PKC phosphorylation negatively regulates neuronal kainate receptor interaction with 4.1N.

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**Figure 7. Palmitoylation is critical for GluK2-containing kainate receptor surface expression and regulated endocytosis by influencing 4.1N association.** A, autoradiographic signal from covalent attachment of \(^{3}H\)palmitate to GluK2a receptors. Wild-type and mutant receptors were coexpressed with the indicated palmitoyl acyltransferase enzymes (DHHC, −) in heterologous cells and labeled with \(^{3}H\)palmitate. B, representative Western blot showing reduced coimmunoprecipitation of HA-4.1N with the C858A,C871A mutant from heterologous cells. C, quantification of normalized coimmunoprecipitation of 4.1N with the indicated receptors. D, confocal micrographs of the merged surface (red) and intracellular (green) populations of receptors expressed in cultured hippocampal neurons. Scale bar, 50 μm. E, quantification of the surface localization of receptors from D, F, confocal micrographs of transfected hippocampal neurons. Receptors were live labeled and allowed to endocytose for 15 min in the absence or presence of 2 μM TTX and 10 μM kainate where indicated. Scale bar, 50 μm. G, quantification of the percentage of receptors that underwent endocytosis for the conditions indicated. Values shown are mean ± S.E. (error bars). **, \(p<0.01\); ***, \(p<0.001\). IP, immunoprecipitation; IB, immunoblot.
Association was determined by coimmunoprecipitation. The phosphomimic substitution S846D did not affect 4.1N binding compared with wild-type receptors (99 ± 26%; n = 5; Fig. 8, C and D), but occluding PKC phosphorylation with the S846A mutation significantly increased the amount of 4.1N association (258 ± 67%; n = 5; p < 0.05; Fig. 8, C and D).

Pharmacological activation of PKC in acute brain slices decreased the interaction between GluK2/3 and 4.1N, and thus a phosphomimetic substitution at the critical serine residues would be predicted to impair surface expression of those receptors by blocking 4.1N association. The GluK2a PKC phosphomimic mutants S846D, S868D, and S846D,S868D were expressed in cultured hippocampal neurons, and surface (red) and intracellular (green) receptor pools were labeled and imaged using confocal microscopy. Receptors containing the S846D substitution were expressed at lower levels on the PM (29 ± 3%; n = 15; p < 0.001; Fig. 8, E and F), suggesting that phosphorylation of this domain antagonized 4.1N association in neurons. The distal phosphomimetic substitution S868D had a modest effect on GluK2 surface expression (49 ± 5%; n = 10;
Mimicking PKC phosphorylation of both residues (S846D,S868D) strongly decreased receptor localization to the plasma membrane (10 ± 2%; n = 16; p < 0.001; Fig. 8, E and F), similar to previously reported results (20). We conclude that PKC phosphorylation of both proximal and distal sites synergistically controls kainate receptor surface expression by regulating association with 4.1N.

**DISCUSSION**

In this study, we show that kainate receptors associate with the neuronal scaffolding protein 4.1N and that this interaction is critical for surface expression of receptors, probably through regulation of their constitutive cycling from the plasma membrane. A domain immediately following the final transmembrane segment is key to the association with 4.1N, which is tightly controlled by receptor modification by both palmitoylation and phosphorylation.

We found that the membrane-stabilizing association with 4.1N is promoted by distal palmitoylation of GluK2-containing kainate receptors and opposed by PKC phosphorylation. Although our data do not rule out an indirect interaction with 4.1N as part of a larger macromolecular signaling complex, the analogous membrane-proximal domains in GluA1 and GluA4 AMPA receptors are known to directly interact with 4.1 proteins (7, 8). Kainate receptor endocytosis and removal from the neuronal surface during long term depression involves PKC activation (32, 34–36), whereas PKC phosphorylation of GluA1-containing AMPA receptors has an opposite effect. Proximal phosphorylation of the GluA1 C terminus promotes 4.1N association, which is antagonized by palmitoylation within this domain (9). How these modulatory systems impact synaptic signaling is less clear because gene-targeted mice lacking 4.1G and with reduced 4.1N expression exhibit no apparent deficits in NMDA receptor-dependent long term potentiation (37). AMPA and kainate receptors perform different functions in the CNS; in part, this arises from very distinct gating kinetics of postsynaptic receptors, which have important consequences on neuronal excitability, particularly during repetitive activation (38). In addition, synaptic expression is both developmentally (39) and differentially regulated by protein interactions (19, 36). This raises the intriguing possibility that the interplay between palmitoylation and phosphorylation, by altering receptor association with 4.1N, may be a critical mechanism for fine tuning synaptic transmission.

GluK1-, GluK2-, and GluK5-containing kainate receptors undergo PKC-mediated phosphorylation, which has divergent effects on their surface expression and synaptic localization (19, 32, 35, 36). PKC inhibition decreased the EPSC\textsubscript{KA} at mossy fiber-CA3 synapses within the hippocampus, which was attributed to phosphorylation-dependent regulation of GRIP/PICK1 association at distal sites of the GluK1 and GluK2 C-terminal tail (19). Conversely, PKC inhibition increased the EPSC\textsubscript{KA} at this same synapse by preventing interaction of GluK5 with SNAP25 (36), suggesting that receptor phosphorylation by PKC can have opposing effects on synaptic transmission and is dependent on both receptor subunit combination and phosphorylation site dynamics. Additionally, PKC activation promotes the endocytosis and removal of kainate receptors from the plasma membrane in cultured hippocampal neurons (32) and leads to long term depression of EPSC\textsubscript{KA} in LII/III of the perirhinal cortex (34). These disparate observations can be explained mechanistically by our current findings, in which proximal PKC phosphorylation antagonizes kainate receptor association with 4.1N, leading to alterations in surface localization, PM destabilization, and subsequent endocytosis, whereas phosphorylation at the extreme C terminus promotes stabilization of receptors within the synapse through interactions with synaptic scaffolding proteins. Our working model postulates that interaction with 4.1N is critical for initial delivery and stabilization of receptors that are further recruited by additional protein-protein interactions within the postsynaptic density during synaptic plasticity. Regulated endocytosis of AMPA receptors at extrasynaptic sites has been shown to be required for long term potentiation (40, 41), suggesting that 4.1N-mediated regulation of kainate receptor endocytosis might be important for the expression of kainate receptor-dependent plasticity.

Phosphorylation is a well studied mechanism regulating the dynamics of glutamate receptor trafficking, synaptic plasticity, and memory (42, 43), but much less is known regarding the regulation of the addition and removal of palmitate from proteins (44). Alterations in neuronal activity result in differential palmitoylation of a variety of neuronal proteins (45), suggesting that, like phosphorylation, palmitoylation is dynamically controlled and allows for activity-dependent regulation of both subcellular protein distribution and protein-protein interactions (46). It is unclear, however, how kainate receptor palmitoylation regulates PKC phosphorylation. The addition of palmitate to this positively charged region of GluK2a would presumably tether the C-terminal tail closely to the plasma membrane, which may prevent PKC from recognizing serine substrates. Conversely, it is possible that PKC phosphorylation, through introduction of negatively charged moieties, prevents palmitoyl acyltransferase enzymes from recognizing modifiable cysteine residues. The dynamic nature of this interplay suggests that association of AMPA and kainate receptors with 4.1N is tightly regulated to balance neuronal excitability.

Recently, GluK2-containing kainate receptor surface expression has been shown to be regulated by the post-translational addition of SUMO, a small protein that can direct modified proteins to various subcellular compartments, including endosomes (47). Agonist stimulation promotes the SUMOylation of GluK2 receptors by stimulating PKC phosphorylation of residues within the C-terminal tail and causes subsequent endocytosis of receptors (48, 49). As a result, kainate receptor SUMOylation is involved in long term depression of kainate receptor signaling. Our findings raise the intriguing possibility that the decoupling of receptors from the cytoskeleton through PKC phosphorylation and loss of 4.1N association may work in parallel to direct SUMOylated receptors into endosomal compartments. Conversely, 4.1N interaction and SUMOylation may influence separate receptor populations once distinct intracellular signaling cascades are initiated. It will be important to explore how these post-translational modifications work in concert to direct kainate receptor expression and regulate cellular excitability.
Kainate Receptor Trafficking and Cytoskeletal 4.1N

Additional 4.1 proteins, 4.1B and 4.1G, are expressed in the brain (27, 28), and it remains unclear if these 4.1 isoforms have specific functions in directing kainate receptor expression. 4.1N knockdown experiments demonstrate that kainate receptor interaction with this particular isoform is important for their dendritic localization in hippocampal neurons. The low level of GluK2 surface expression observed following 4.1N depletion could be the result of other 4.1 isoforms promoting the forward trafficking and surface stabilization of receptors.

The exceptions we noted between plasma membrane expression and 4.1N association in COS-7 cells suggests that other factors are at play in the MDP that influence GluK2 trafficking. Other members of the 4.1 protein family are obvious candidates because these proteins also associate kainate receptors in heterologous expression systems (data not shown). 4.1 protein isoforms are localized to distinct subcellular compartments, including axons (50, 51), and may play distinct roles in localizing kainate receptors to presynaptic terminals, where they function as autoreceptors to regulate neurotransmitter release (52). GluK3-containing receptors require dendritic endocytosis for their polarized expression at presynaptic terminals (53), raising the possibility that specific 4.1 isoforms are important for axonal targeting of these receptors by regulating their endocytosis.

Despite strong expression of 4.1N within dendritic spines, inhibiting kainate receptor interaction with 4.1N causes a loss in surface expression along the dendritic shaft but only at a subset of spines. The surface expression within spines may be due to kainate receptor interaction with other postsynaptic scaffolding proteins, such as GRIP or PSD-95 (19, 54). 4.1N interaction is critical for maintaining a pool of surface receptors along the dendritic shaft and extrasynaptic membrane that might then be recruited to synapses during synaptic potentiation in a manner similar to what has been reported for AMPA receptors (55, 56). Kainate receptor-mediated signaling during synaptic plasticity may therefore be regulated by association with 4.1N, which can be bidirectionally controlled by multiple post-translational modifications. This mechanism, which is regulated in an opposing manner with AMPA receptors, would allow for the simultaneous control of both receptor families to coordinate synaptic signaling kinetics.

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