Cellular/Molecular

Regulation of Metabotropic Glutamate Receptor Internalization and Synaptic AMPA Receptor Endocytosis by the Postsynaptic Protein Norbin

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Group I mGluRs have diverse functions in some fundamental neuronal processes, including modulation of synaptic plasticity; and dysregulation of these receptors could lead to various neuropsychiatric disorders. Trafficking of Group I mGluRs plays critical roles in controlling the precise spatiotemporal localization and activity of these receptors, both of which contribute to proper downstream signaling. Using "molecular replacement" approach in hippocampal neurons derived from mice of both sexes, we demonstrate a critical role for the postsynaptic density protein Norbin in regulating the ligand-induced internalization of Group I mGluRs. We show that Norbin associates with protein kinase A (PKA) through its N-terminus and anchors mGluR5 through its C-terminus, both of which are necessary for the ligand-mediated endocytosis of mGluR5, a member of the Group I mGluR family. A point mutation (A687G) at the C-terminus of Norbin inhibits the binding of Norbin to mGluR5 and blocks mGluR5 endocytosis. Finally, we demonstrate an important mechanism by which Norbin regulates mGluR-mediated AMPAR endocytosis in hippocampal neurons, a cellular correlate for mGluR-dependent synaptic plasticity. Norbin, through its PKA-binding regions, recruits PKA to AMPARs on activation of mGluRs; and deletion of the PKA-binding regions of Norbin inhibits mGluR-triggered AMPAR endocytosis. We further report that Norbin is important specifically for the mGluR-mediated AMPAR endocytosis, but not for NMDAR-dependent AMPAR endocytosis. Thus, this study unravels a novel role for Norbin in the internalization of mGluRs and mGluR-mediated AMPAR endocytosis that can have clinical relevance to the function of Group I mGluRs in pathologic processes.

Key words: **endocytosis; GPCR; metabotropic glutamate receptors; Norbin; synaptic plasticity; trafficking**

Significance Statement

The postsynaptic protein Norbin interacts with mGluR5, and both of them have been implicated in disorders, such as schizophrenia. However, the mechanistic basis underlying the regulation of mGluRs by Norbin remains elusive. We have identified Norbin as an essential mediator of ligand-mediated endocytosis of Group I mGluRs. Mechanistically, Norbin N-terminus associates with protein kinase-A (PKA) and C-terminus binds to mGluR5 to coordinate receptor internalization. A point mutation NorA687G inhibits endocytosis by disrupting this interaction. Additionally, Norbin is critical for the recruitment of PKA to AMPARs on activation of Group I mGluRs that assists in mGluR-mediated AMPAR endocytosis. Thus, Norbin has a dual function in the hippocampus: regulation of mGluR internalization and PKA-dependent modulation of mGluR-mediated AMPAR endocytosis, a prerequisite for mGluR-mediated synaptic plasticity.

Introduction

Glutamate, the major excitatory neurotransmitter in the CNS, acts via two types of receptors: ionotropic and mGluRs (Pin and Duvoisin, 1995; Dhami and Ferguson, 2006). The two members of the Group I mGluR family (mGluR1 and mGluR5) are expressed at the perisynaptic region of the postsynaptic neuron and have been implicated in various forms of experience-dependent synaptic plasticity and in several neuropsychiatric disorders, such as autism, Fragile X syndrome, and others (Huber et al., 2002; Bear et al., 2004; Dolen et al., 2007; Citri and Malenka, 2008). Trafficking plays a crucial role in controlling the spatiotemporal localization and the activity of these receptors (Sallese

Received May 18, 2021; revised Nov. 15, 2021; accepted Nov. 19, 2021.

Author contributions: P.O., S.P., and S.B. designed research; P.O. and S.P. performed research; P.O. contributed unpublished reagents/analytic tools; P.O., S.P., and S.B. analyzed data; P.O. and S.B. wrote the first draft of the paper; P.O., S.P., and S.B. edited the paper; P.O. and S.B. wrote the paper.

This work was supported by Indian Institute of Science Education and Research Mohali. We thank Dr. Kathrine Roche (National Institutes of Health) and Dr. Johanna Montgomery (University of Auckland) for the generous gifts of myc-mGluR5, and myc-mGluR1 and FLAG-mGluR5 constructs, respectively; Dr. Heidi Welch (Babraham Institute) for the myc-Neurochondrin construct; and members of our laboratory for valuable input.

The authors declare no competing financial interests.

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https://doi.org/10.1523/JNEUROSCI.1037-21.2021

et al., 2000; Dale et al., 2002; Dhami and Ferguson, 2006; Bhattacharyya, 2016). Upon activation by the ligand, these receptors undergo "desensitization," a negative feedback mechanism that protects the receptor from chronic overstimulation. Subsequently, the receptor internalizes and recycles back to the cell surface, which is the mechanism for the "resensitization" of these receptors (Pandey et al., 2014; Mahato et al., 2015; Gulia et al., 2017; Sharma et al., 2018). Activation of mGluRs leads to the internalization of AMPARs, which is believed to be the cellular correlate for mGluR-dependent synaptic plasticity (Citri and Malenka, 2008; Bhattacharyya, 2016). Despite this obvious significance of trafficking of mGluRs, the molecular mechanisms governing Group I mGluR internalization and its effect in AMPAR endocytosis are not well understood.

Studies in the past have established a key role for several postsynaptic density proteins in acting as a scaffold for Group I mGluRs that link these receptors to the intracellular signaling cascades or help in sorting these receptors to appropriate subsynaptic or synaptic locations (Brakeman et al., 1997; Roche et al., 1999; Tu et al., 1999; Kitano et al., 2003; Sharma et al., 2018). One such protein that was identified in the hippocampus is Norbin, also known as Neurochondrin. Norbin is a 75 kDa protein that interacts with Group I mGluRs and positively regulates mGluR5 signaling (Wang et al., 2009). Norbin has also been implicated in synaptic plasticity and several neurodegenerative disorders, such as schizophrenia, depression, etc. (Wang et al., 2009; Matosin et al., 2015). In view of these important roles of Norbin in the CNS, we hypothesized that Norbin might play an important role in Group I mGluR internalization and in the mGluR-mediated AMPAR endocytosis.

In this study, we have used a molecular replacement strategy that allowed shRNA-mediated acute knockdown of endogenous Norbin and expression of various forms of recombinant Norbin in primary hippocampal neurons. We show here that Norbin is an important regulator of Group I mGluR endocytosis. Acute knockdown of endogenous Norbin decreased the surface expression and also blocked the agonist-induced endocytosis of Group I mGluRs. Importantly, protein kinase-A (PKA) binding to Norbin is necessary to induce the agonist-mediated internalization of mGluR5. We also found that a single residue mutation from alanine to glycine at the C-terminus of Norbin was sufficient to impair the interaction of Norbin with mGluR5 and thereby hamper the mGluR5 internalization. Finally, we showed that knockdown of Norbin blocked the mGluR-mediated AMPAR endocytosis, but it did not affect the NMDAR-triggered AMPAR endocytosis. Although knockdown of Norbin did not have any effect on the MAP-kinase signaling by Group I mGluRs or Group I mGluR-mediated Arc protein synthesis, it inhibited the increased interaction of PKA with AMPARs on activation of mGluRs. Disruption of PKA binding to Norbin inhibited the mGluR-dependent AMPAR endocytosis. Thus, our results provide compelling evidence for a dual role of Norbin: (1) as an adaptor for Group I mGluR endocytosis and (2) as an A-kinase anchoring protein that recruits PKA to AMPAR on mGluR activation and subsequently contributes to mGluR-mediated AMPAR endocytosis. Our study unravels a previously uncharacterized role for Norbin in the internalization of mGluRs and mGluR-mediated AMPAR endocytosis that might have clinical relevance to the function of Group I mGluRs in neuropsychiatric disorders.

Materials and Methods

Materials

The myc-mGluR1 and myc-mGluR5 constructs were generously gifted by Kathrine Roche (National Institute of Health). In these constructs, the myc epitope was tagged at the N-terminus of full-length mGluR1 and mGluR5. The FLAG-mGluR5 construct was obtained from Johanna Montgomery's laboratory (University of Auckland). pCMV3 myc-Neurochondrin construct was gifted by Heidi Welch (Babraham Institute). HEK293T cells were purchased from NCCS Pune. Cell culture reagents, such as media, antibiotic-antimycotic mix, and other supplements, were purchased from Invitrogen. All salts and fine chemicals, such as poly-D-lysine, cycloheximide, 5-fluoro-2′-deoxyuridine (FUDR), paraformaldehyde, and Fluoromount aqueous mounting medium, were purchased from Sigma. Protein A/G PLUS agarose beads were purchased from Santa Cruz Biotechnology, and femtoLUCENT plus-HRP kit was purchased from G-Biosciences. (*RS*)-3,5-dihydroxyphenylglycine (R,S-DHPG), NMDA, DNQX, and APV were purchased from Tocris Bioscience. Tetrodotoxin citrate (TTX) was purchased from Abcam. Anti-myc mouse monoclonal and anti-Norbin mouse polyclonal antibodies were purchased from Abcam. Anti-hemagglutinin (HA) rat monoclonal antibody was purchased from Roche. Anti-GFP mouse monoclonal and anti-FLAG rabbit polyclonal antibodies were from Sigma. Anti-GFP rabbit polyclonal antibody was purchased from Invitrogen, anti-GluA1 rabbit polyclonal antibody was purchased from Millipore, and anti-Bassoon mouse monoclonal antibody was from Enzo Life Sciences. ERK1/2 mouse monoclonal and phospho-ERK1/2 rabbit monoclonal antibodies were purchased from Cell Signaling Technology. Anti-Arc rabbit polyclonal antibody was from Synaptic Systems. Anti-β actin antibody and anti-PKAα cat antibody were obtained from Santa Cruz Biotechnology. Anti-PKA RIIα antibody was purchased from R&D Systems. AlexaFluor-conjugated secondary antibodies were purchased from Invitrogen, and HRP-conjugated secondary antibodies were purchased from Sigma.

Construct preparation

The shRNAs against Norbin were individually cloned in a multipromoter vector under the H1 promoter targeting Norbin sequences. Enhanced green fluorescent protein (eGFP) expression, which was under IRES, was used to identify transfected cells. Respective shRNAs were screened for their efficiency to knockdown the endogenous Norbin in primary neurons both by Western blotting as well as by immunocytochemistry. shRNA targeting the Norbin sequence AGGCCAAGA ATGACAGCGA (shNor) was found to be most effective and was used for this study. Replacement constructs were cloned under the ubiquitin promoter of the vector containing shNor. All the replacement constructs, shNor:Nor (full-length Norbin), shNor:NorΔN (presence of amino acids 482-712 of Norbin), shNor:NorΔC (presence of amino acids 1-481 of Norbin), shNor:NorΔPEP1 (PEP1 region of Norbin, i.e., amino acids 48-67 were deleted), shNor:NorΔPEP2 (PEP2 region of Norbin i.e., amino acids 255-274 were deleted), and shNor:NorA687G (alanine at the 687th position of Norbin was mutated to glycine) were tagged with HA epitope at the N-terminus of the protein. For generation of the above replacement constructs, silent mutations were introduced in the Norbin target region of shNor, so that shNor downregulated only the endogenous Norbin and knockdown of the replacement constructs by shNor was prevented.

Dissociated primary neuron culture and transfection

Primary neuron cultures were prepared from P0 C57BL/6 mice of both sexes as described previously with minor changes (Gulia et al., 2017; Sharma et al., 2018; Pandey et al., 2020). Briefly, hippocampi or cortex were dissected out from the pups. The tissues were dissociated in the enzymatic solution containing papain for 30 min at 37°C followed by trituration to obtain single cells. Neurons were then plated on coverslips or dishes precoated with poly-D-lysine in sodium borate (50 μg/ml poly-D-lysine + 0.1 M sodium borate) at a density of ~150,000 cells per 12 mm well in a 24 well plate. Cultures were maintained in neurobasal media supplemented with B27 and 0.5 mM glutamine. Glial growth was inhibited by adding FUDR (floxuridine) on the fourth day of culture.

Primary neurons were transfected with myc-mGluR5/myc-mGluR1, shNor, and various Norbin replacement constructs at 7-8 DIV using calcium phosphate method (Sharma et al., 2018, 2019; Pandey et al., 2020).

The transfection protocol that we used gave us 60%-65% transfection efficiency. All the experiments were performed when the cells were at 12-15 DIV.

HEK293T cell culture and transfection

HEK293T cells were maintained in DMEM supplemented with 10% FBS, antibiotic-antimycotic mix at 37°C with 95% humidity and 5% CO_2. For coimmunoprecipitation experiments, cells were transfected using polyethyleneimine (PEI) on 60 mm dishes. PEI is a stable cationic polymer that condenses DNA into positively charged particles, and the DNA-PEI complex enters into the cell through endocytosis (Sonawane et al., 2003). For transfection in 60 mm dishes, 6 µg of DNA was mixed with 18 µl of PEI in 600 µl plain DMEM. Experiments were conducted 24 h after transfection.

Experimental design and statistical analyses

Endocytosis assay. The myc-mGluR5/myc-mGluR1 was cotransfected with either shNor or various Norbin replacement constructs as described above, in primary hippocampal neurons. Experiments were performed 5-8 d after transfection. Live cells were incubated with anti-myc mouse monoclonal primary antibody (1:200) for 20 min at 37°C. Cells were then treated with 100 μM R,S-DHPG, a Group I mGluR specific agonist, for 5 min at 37°C. The agonist was then washed out, and cells were chased for different time periods at 37°C in plain neurobasal media in the absence of the ligand. Subsequently, cells were fixed without permeabilization using ice-cold 4% PFA for 15 min on ice. Surface receptors were labeled with a saturating concentration of goat anti-mouse Alexa-568-conjugated secondary antibody (1:100) for 1 h at 37°C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. The endocytosed receptors were then labeled by the application of goat anti-mouse Alexa-647-conjugated secondary antibody (1:750) for 1 h at 37°C. The cotransfected constructs containing GFP and HA tags were stained with the anti-GFP or anti-HA antibodies, respectively, overnight at 4°C. Subsequently, they were stained with their respective secondary antibodies. The coverslips were then mounted on glass slides and imaged under the confocal microscope. To ensure that the Alexa-647-conjugated secondary antibody did not label any detectable surface receptors in our assays, we performed control experiments to determine the saturating concentration of the first secondary antibody similar to what we have described in our earlier studies (Trivedi and Bhattacharyya, 2012; Pandey et al., 2014; Mahato et al., 2015; Gulia et al., 2017; Sharma et al., 2018, 2019; Pandey et al., 2020). The control experiments suggested that, in our assays, Alexa-647-conjugated secondary antibody did not label any detectable amount of surface receptors; thus, it stained the internalized receptors only (data not shown).

To study the role of Norbin in the mGluR-mediated AMPAR endocytosis and NMDAR-mediated AMPAR endocytosis, primary hippocampal neurons were transfected with shNor or Norbin replacement constructs as described before. Subsequently, the mGluR-mediated and NMDAR-mediated AMPAR endocytosis assays were performed using the protocol used previously (Bhattacharyya et al., 2009; Citri et al., 2009; Gulia et al., 2017; Sharma et al., 2018; Pandey et al., 2020). Briefly, cells were first preincubated in 1 μM TTX (presynaptic release blocker), 20 μM DNQX (AMPAR antagonist), and 50 μM APV (NMDAR antagonist) to study the mGluR-mediated AMPAR endocytosis or in 1 μM TTX and 20 μM DNQX to study the NMDAR-mediated AMPAR endocytosis for 30 min at 37°C. Subsequently, GluA1-containing receptors were labeled in live neurons by 15 min incubation at 37°C with a rabbit polyclonal antibody directed against the N-terminus of the GluA1 subunit (1:150). Cells were then washed with plain media, and 100 μM R,S-DHPG (for mGluR-mediated AMPAR endocytosis) or 100 μM NMDA (for NMDAR-mediated AMPAR endocytosis) was applied for 5 min. The agonist was then washed out, and cells were further incubated in the presence of appropriate antagonists for a total of 15 min at 37°C. After completion of the incubation period, cells were fixed without permeabilization using ice-cold 4% PFA for 15 min on ice. Subsequently, surface receptors were labeled by application of the saturating amount of goat anti-rabbit Alexa-568-conjugated secondary antibody (1:100) for 1 h at 37°C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min at room temperature, and the endocytosed receptors were labeled by the application of goat anti-rabbit Alexa-647-conjugated secondary antibody (1:750) for 1 h at 37°C. Staining of GFP was done by incubation with the mouse anti-GFP primary antibody (1:500) overnight at 4°C followed by application of the goat anti-mouse Alexa-488-conjugated secondary antibody (1:750) for 1 h at 37°C. Coverslips were mounted on glass slides and scanned under the confocal microscope. The saturating concentration of the Alexa-568-labeled secondary antibody that was used to label the surface GluA1-containing receptors was determined by performing control experiments similar to that we have described in our earlier studies (Gulia et al., 2017; Sharma et al., 2018; Pandey et al., 2020). These experiments also suggested that the Alexa-647-conjugated secondary antibody that was used to label the internalized receptors did not label any detectable surface receptors (data not shown).

Colocalization assay. To check whether different mutants of Norbin were targeted and localized properly at the synapse, the extent of colocalization of these mutants with the presynaptic protein Bassoon was measured. Bassoon is a core component of the active zone which acts as a marker to identify presynaptic terminals (tom Dieck et al., 1998). Briefly, different HA-tagged Norbin mutants were transfected in primary hippocampal neurons, and colocalization assays were performed on 12-15 DIV. Cells were fixed with ice-cold 4% PFA on ice for 15 min. After that, cells were permeabilized using 0.1% Triton X-100 for 30 min at room temperature. Cells were then stained with the rat anti-HA primary antibody (1:500) and mouse anti-Bassoon primary antibody (1:500) overnight at 4°C. Following primary antibody staining, cells were stained with goat anti-rat Alexa-568 (1:800)- and goat anti-mouse Alexa-647 (1:800)-conjugated secondary antibodies for 1 h at 37°C to visualize the HA-Norbin constructs and Bassoon, respectively. Finally, coverslips were mounted on glass slides and scanned under the confocal microscope.

Western blot analysis and coimmunoprecipitation assay. To check the knockdown efficiency of endogenous Norbin by shNor as well as expression of various replacement constructs of Norbin, Western blot assays were performed. For these assays, primary neurons were transfected with the appropriate constructs. At 72 h after transfection, cells were washed with ice-cold 1× PBS and lysed in RIPA lysis buffer having protease inhibitor cocktail. Samples were subsequently boiled in 5× Laemmli sample buffer at 99°C for 10 min and run on SDS-PAGE by loading equal amount of protein in each lane. They were then transferred onto a PVDF membrane and blocked with 5% skimmed milk in 0.05% PBST for 1 h at room temperature. The membrane was then incubated with either anti-Norbin mouse polyclonal antibody (1:500) or anti-HA rat monoclonal antibody (1:1000) and anti-β-actin antibody (1:1000) at 4°C overnight. β-actin was used as the loading control. After washing, membranes were incubated with the HRP-conjugated secondary antibodies (1:5000) for 45 min at room temperature. Blots were developed using femtoLUCENT plus-HRP kit, and images were acquired in ImageQuant LAS 4000.

The ability of mGluRs to upregulate the phosphorylation of ERK1/2 was investigated by transfecting the primary neurons with empty vector or shNor. Both control and shNor-transfected cells were preincubated with 100 µg/ml cycloheximide for 5 h to inhibit the synthesis of new receptors. Subsequently, 100 μM R,S-DHPG was applied for 5 min in both control cells and shNor-transfected cells to initiate the endocytosis of mGluRs. In both the conditions, one set of cells were fixed after 5 min of application of R,S-DHPG to measure the extent of upregulation of ERK1/2 phosphorylation by the mGluRs, when they were initially present at the cell surface in both control cells and shNor-transfected cells. The other set of cells were chased for 2.5 h, in absence of the ligand. Subsequently, 100 μM R,S-DHPG was applied again for 5 min, followed by fixation of cells. Both sets of cells were then lysed, and samples were run on SDS-PAGE followed by Western blotting using the same protocol as described above. The phospho-ERK1/2 and total ERK1/2 immunoblotting was performed using anti-phospho-p44/42 MAPK (ERK1/2) antibody (1:1000) and anti-p44/42 MAPK (ERK1/2) antibody (1:1000), respectively.

Coimmunoprecipitation experiments were done to check the interaction of various constructs of Norbin with mGluR5 and PKA and the

interaction of AMPA with PKA. FLAG-mGluR5 and constructs of Norbin were transfected in HEK293T cells as well as in primary hippocampal neurons, and immunoprecipitation was performed following standard procedures. Briefly, 48-72 h after transfection, cells were washed with ice-cold PBS and lysed using TAP lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1× protease inhibitor cocktail). The lysate was centrifuged at 15,000 rpm for 30 min. Then 50 μl of supernatant was collected as input. Immunoprecipitation was performed by incubating the remaining supernatant with protein A/G beads that were prepared by overnight incubation with anti-HA or anti-FLAG primary antibodies to check for the interaction of Norbin with PKA and mGluR5, respectively, or using GluA1 antibody-bound protein A/G beads to study the effect of Norbin knockdown on the interaction of AMPA with PKA. After 6-8 h, beads were washed and samples were boiled in 2× Laemmli buffer after elution. The samples were run on SDS-PAGE by loading ~50 µg of protein in each lane followed by Western blotting using the method as described above. For immunoblotting, antibodies against FLAG (1:1000), HA (1:1000), GluA1 (1:1000), Norbin (1:500), PKAα cat (1:100), and PKA RII α (1:100) were used.

Arc immunostaining assay. To check whether Norbin plays any role in the Group I mGluR-mediated *de novo* translation of Arc protein, primary hippocampal neurons were transfected with either empty vector or shNor or shNor:Nor constructs, and experiments were performed on DIV 14. Cells were preincubated in either plain media or in plain media with 100 µg/ml cycloheximide for 1 h at 37°C. Subsequently, 100 μM R, S-DHPG was applied for 5 min followed by a chase of 10 min in the absence of the ligand. Cells were fixed with 4% PFA on ice for 15 min, followed by permeabilization of the cells using 0.1% Triton X-100 for 30 min at room temperature. Cells were then incubated in anti-Arc rabbit polyclonal antibody (1:500) and anti-GFP mouse monoclonal antibody (1:500) overnight at 4°C. Following primary antibody staining, cells were stained with goat anti-rabbit Alexa-568 (1:700)- and goat anti-mouse Alexa-488 (1:700)-conjugated secondary antibodies for 1 h at 37°C to visualize Arc protein and GFP, respectively. Coverslips were then mounted on glass slides and observed under the confocal microscope.

Image acquisition and analysis

Imaging was done in a Carl Zeiss LSM 780 confocal laser scanning microscope using a 63× oil immersion objective (NA = 1.4). All experiments were repeated at least 3 times independently, and a total of 30-40 primary hippocampal neurons were imaged in each experimental group. Images from all the conditions in a particular experiment were obtained using identical parameters. All analyses were done blind using raw images, and quantitation was done using ImageJ software (National Institutes of Health) (Schneider et al., 2012). All the analysis procedures have been described in our previous study (Sharma et al., 2018). Briefly, raw images from each experiment were maximally projected and thresholded using identical values for different experimental conditions. The thresholded areas of fluorescently labeled surface and internalized receptors were subsequently measured. Internalization index for each cell was then calculated by dividing the value contributed by the internal fluorescence with the value contributed by the total fluorescence (surface + internal). These values were then normalized with respect to their controls. To measure the surface receptors in all the assays or for the measurement of the Arc fluorescence, raw images from individual experiment were maximally projected and thresholded for surface fluorescence measurement or Arc fluorescence using identical values for a particular experimental condition. The thresholded areas of fluorescently labeled surface receptors or Arc protein were divided by the cell area, which was determined by measuring the background fluorescence using a low threshold level. These values were then normalized to the average fluorescence of control cells. All the results represent dendritic values of primary hippocampal neurons, and the dendritic values were defined by the area that was 10 µm away from the soma. The extent of synaptic localization of various Norbin constructs was studied by colocalization with presynaptic Bassoon puncta along 50 µm portions of dendrites. The colocalization was quantified at a particular *Z* section of the image after thresholding the cells using identical values. To obtain the representative images, raw images were processed using Adobe Photoshop software (Adobe Systems) by using identical values of brightness and contrast. All the Western blots and immunoprecipitation experiments were also quantified using ImageJ software. Briefly, densitometric analysis was performed by selecting each band, followed by signal intensity quantitation. Data were acquired as percentage area values, and individual band intensities were normalized to β-actin in case of Western blots or to the pulled down protein in case of coimmunoprecipitation.

Statistical analysis

Each experiment was repeated 3 times, and a total of 30-40 primary hippocampal neurons were imaged in each experimental group. The quantitation of an experiment has been represented as a combined result for all the repeats of that particular experiment. Data are presented as mean ± SEM. Experimental group results were compared with each other using Student's *t* test or one-way ANOVA followed by Tukey's post-test. $p > 0.05$ was considered as nonsignificant.

Results

Norbin plays a critical role in the ligand-mediated endocytosis of Group I mGluRs

It has been reported that Norbin acts as an accessory protein to mGluR5 and it binds to the C-terminal membrane proximal region of mGluR5 (Wang et al., 2009). In order to investigate the role of Norbin in the ligand-dependent internalization of Group I mGluRs, we undertook the "molecular replacement" approach. This strategy allowed simultaneous shRNA-mediated acute knockdown of the endogenous Norbin and expression of mutant forms of recombinant Norbin in primary hippocampal neurons. There are two important advantages to this approach. First, the possibility of compensatory adaptations during synaptogenesis and synapse maturation because of the absence of the protein of interest is minimal. Second, the function of the protein of interest can be studied without having a dominant effect as required by a standard overexpression approach. We initially screened for an shRNA that could effectively knockdown the endogenous Norbin in primary neurons. After the screen, we identified an shRNA (shNor), which was highly efficient in knocking down the endogenous Norbin in primary neurons as measured through Western blot, whereas WT Norbin replacement construct (shNor:Nor; full-length Norbin) showed normal expression levels (control: 1 ± 0.07; shNor: 0.39 ± 0.09; shNor:Nor: 1.31 ± 0.01; $p = 0.031$ between control and shNor; $p = 0.047$ between control and shNor:Nor; one-way ANOVA followed by Tukey's post-test) (Fig. 1*A*,*B*). Consistent with an earlier report, shRNA-mediated knockdown of Norbin reduced the surface expression of myc-mGluR5, whereas simultaneously expressing both shNor and WT Norbin (shNor:Nor) rescued the surface expression of the receptor (control: 1.0 ± 0.03; shNor: 0.79 ± 0.03; shNor:Nor: 1.1 ± 0.06; $p = 2.380 \times 10^{-5}$ between control and shNor; $p = 0.117$ between control and shNor:Nor; one-way ANOVA followed by Tukey's post-test) (Fig. 1*C*,*D*). These results confirmed the efficacy of the shNor construct and the molecular replacement strategy in primary hippocampal neurons. However, since Norbin knockdown reduced the surface expression of myc-mGluR5, it was important for us to use an assay that allowed measurement of the proportion of surface receptors that were internalized following ligand application to study the role of Norbin in the ligand-mediated endocytosis of Group I mGluRs. For our endocytosis assay, we used a procedure that allowed staining of both ligand-mediated internalized receptors and remaining surface myc-mGluR5 that did not internalize on ligand application with different secondary antibodies.

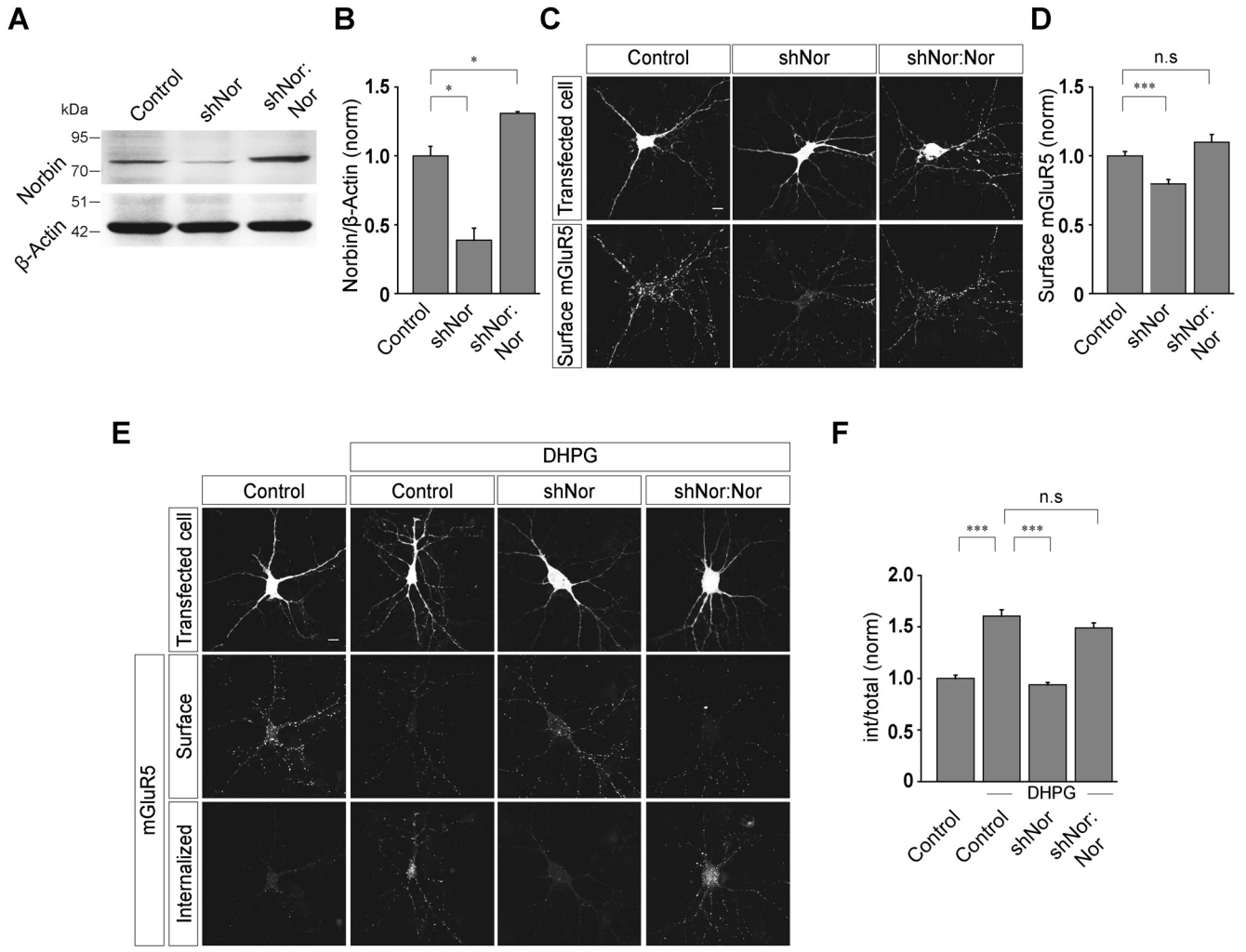

Figure 1. Knockdown of endogenous Norbin decreases the surface expression and inhibits the ligand-mediated internalization of mGluR5. ***A***, ***B***, Western blot (***A***) and quantitation of Western blots (***B***) showing efficient knockdown of the endogenous Norbin and replacement of the endogenous Norbin with full-length Norbin. ***C***, ***D***, Representative images (***C***) and quantitation of surface myc-mGluR5 (***D***) showing that surface myc-mGluR5 expression was reduced because of the knockdown of the endogenous Norbin by shNor compared with control cells, and this reduction was rescued by expression of WT Norbin. ***E***, Representative examples of surface and internalized myc-mGluR5, 30 min after application of 100 μM R,S-DHPG in control cells, shNor-expressing cells, and shNor and WT Norbin-expressing cells. ***F***, Quantitation of the endocytosis index suggested that knockdown of endogenous Norbin inhibited the R,S-DHPG-mediated internalization of myc-mGluR5 and expression of WT Norbin rescued the normal endocytosis of the receptor. Data are mean ± SEM collected from three independent experiments. Scale bar, 10 μm. ***$p < 0.001$; *$p < 0.05$; n.s., $p > 0.05$.

Subsequently, we measured the proportion of surface receptors that endocytosed following ligand application (see Materials and Methods). Primary hippocampal neurons were cotransfected with myc-mGluR5 and shNor or shNor:Nor constructs. Live neurons expressing myc-mGluR5 were labeled with anti-myc primary antibody. Subsequently, application of 100 μM R,S-DHPG, an agonist of Group I mGluRs, resulted in the internalization of myc-mGluR5 in 30 min in control cells. We chose 30 min as the time point because our earlier studies suggested that R,S-DHPG-mediated internalization of myc-mGluR5 reaches maximum level at 30 min after ligand application (Mahato et al., 2015). On the other hand, acute knockdown of the endogenous Norbin resulted in the inhibition of the R,S-DHPG-mediated internalization of myc-mGluR5, and most of the receptors were observed to be localized at the cell surface in cells expressing shNor (control: 1 ± 0.03; control + DHPG: 1.6 ± 0.06; shNor + DHPG: 0.94 ± 0.02, $p = 3.609 \times 10^{-13}$ between control and control + DHPG; $p = 9.992 \times 10^{-16}$ between control + DHPG and shNor + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 1*E*,*F*). This inhibition in the endocytosis of myc-mGluR5 because of the knockdown of the endogenous Norbin was rescued by the expression of recombinant WT Norbin (shNor-resistant), indicating that the observed effect was not a result of some nonspecific effect of shNor (shNor:Nor + DHPG: 1.49 ± 0.05; $p = 0.163$ between control + DHPG and shNor:Nor + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 1*E*,*F*). We subsequently investigated the role of Norbin, if any, in the ligand-mediated endocytosis of the other member of the Group I mGluR family, mGluR1. Knockdown of endogenous Norbin reduced the surface expression of myc-mGluR1 that was rescued by expression of the WT Norbin. On the other hand, 100 μM R,S-DHPG-mediated internalization of myc-mGluR1 was inhibited on knockdown of the endogenous Norbin, and replacement of the endogenous Norbin with WT Norbin rescued the normal internalization of the receptor (data not shown). Thus, these results suggest that Norbin plays a critical role in the surface stabilization as well as in the ligand-mediated internalization of both members of the Group I mGluR family (i.e., mGluR5 and mGluR1). Since mGluR5

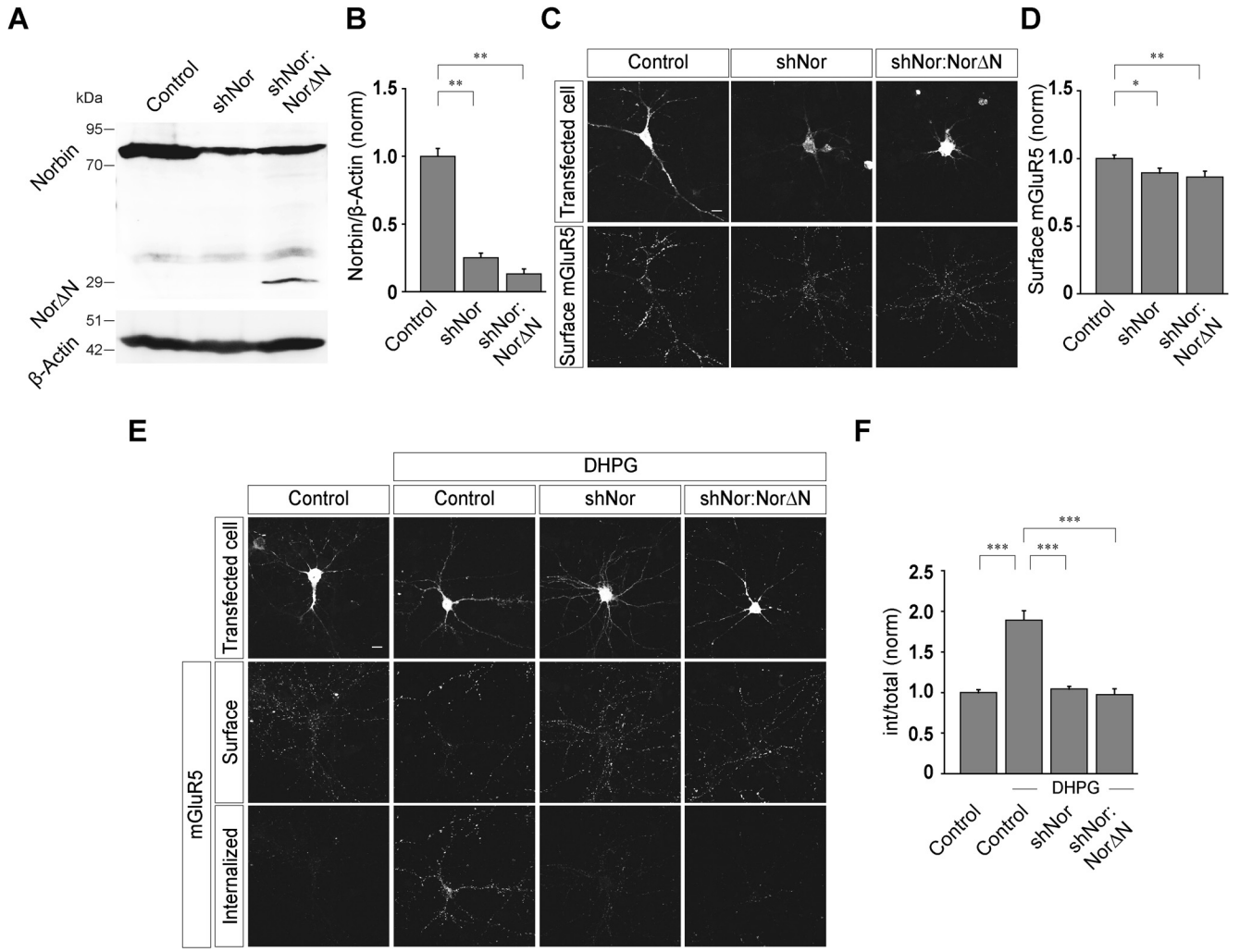

Figure 2. Role of N-terminal region of Norbin in surface stabilization as well as in ligand-mediated endocytosis of mGluR5. ***A***, ***B***, Western blot (***A***) and quantitation of Western blots (***B***) showing effective knockdown of endogenous Norbin by shNor and expression of the NorΔN replacement construct. ***C***, ***D***, Representative images (***C***) and quantitation (***D***) of surface myc-mGluR5 suggested that the surface localization of myc-mGluR5 decreased in shNor-expressing cells and expression of NorΔN replacement construct could not rescue the surface expression of myc-mGluR5. ***E***, ***F***, Representative cells (***E***) and quantitation (***F***) of 100 μM R,S-DHPG-induced internalization of myc-mGluR5, suggesting that knockdown of endogenous Norbin led to the inhibition of ligand-mediated endocytosis of myc-mGluR5, and expression of the NorΔN replacement construct could not rescue the normal endocytosis of the receptor. Data are mean ± SEM collected from three independent experiments. Scale bar, 10 μm. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

together with Norbin has been highlighted in various neuropsychiatric disorders, we concentrated on mGluR5 for the rest of the study.

PKA binding at the N-terminal region of Norbin is required for the ligand-mediated endocytosis of mGluR5

Norbin is a neuronal cytoplasmic protein with no obvious domain structures. However, it has been reported that Norbin acts as a high-affinity PKA RIIα subunit-specific A-kinase anchoring protein through its N-terminal region (Hermann et al., 2015). Furthermore, PKA modulates the mGluR5 function by directly phosphorylating the receptor (Uematsu et al., 2015). In order to investigate whether binding of PKA with Norbin is important for the ligand-mediated endocytosis of mGluR5, we first deleted the N-terminal region of Norbin and replaced the endogenous Norbin with a form of Norbin lacking the N-terminal region (NorΔN; presence of amino acids 482-712 of Norbin). NorΔN showed expression in primary neurons, but its amount of expression was lower than the endogenous Norbin (control: 1 ± 0.06; shNor: 0.25 ± 0.03; shNor:NorΔN: 0.13 ± 0.04; $p = 0.008$ between control and shNor; $p = 0.006$ between control and shNor:NorΔN; one-way ANOVA followed by Tukey's post-test) (Fig. 2*A*,*B*). Knockdown of the endogenous Norbin decreased the surface expression of myc-mGluR5. However, unlike WT Norbin, NorΔN did not rescue the surface expression of myc-mGluR5 (control: 1.0 ± 0.03; shNor: 0.89 ± 0.04; shNor:NorΔN: 0.86 ± 0.04; $p = 0.021$ between control and shNor; $p = 0.006$ between control and shNor:NorΔN; one-way ANOVA followed by Tukey's post-test) (Fig. 2*C*,*D*). Subsequently, we investigated the effect of the N-terminal region deletion of Norbin on the ligand-mediated endocytosis of mGluR5. Unlike full-length Norbin, NorΔN replacement construct did not rescue the inhibition in the R,S-DHPG-mediated endocytosis of myc-mGluR5 caused by the knockdown of endogenous Norbin (control: 1 ± 0.04; control + DHPG: 1.89 ± 0.11; shNor + DHPG: 1.04 ± 0.03; shNor:NorΔN + DHPG: 0.97 ± 0.07; $p = 1.942 \times 10^{-10}$ between control and control + DHPG; $p = 2.771 \times 10^{-10}$ between control + DHPG and shNor + DHPG; $p = 1.11 \times 10^{-8}$ between control + DHPG and shNor:NorΔN + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 2*E*,*F*). Since the NorΔN construct did not express properly in

neurons, the inability of this construct to rescue the surface expression and ligand-mediated endocytosis of myc-mGluR5 was probably because of its insufficient expression in neurons.

Surface plasmon resonance experiments have identified that the N-terminal region of Norbin contains two distinct peptides PEP1 (48-67 amino acids of Norbin) and PEP2 (255-274 amino acids of Norbin), which are involved in its interaction with the RIIα subunit of PKA (Hermann et al., 2015). Since the entire N-terminal deletion construct of Norbin did not express properly in primary neurons, we made two deletion replacement constructs of Norbin to investigate whether binding of PKA with Norbin is essential for the ligand-mediated endocytosis of mGluR5. In one replacement construct, the PEP1 region was deleted (NorΔPEP1; PEP1 region of Norbin, i.e., amino acids 48-67 were deleted) and in the other PEP2 region was removed (NorΔPEP2; PEP2 region of Norbin, i.e., amino acids 255-274 were deleted). We first examined the effect of replacing the endogenous Norbin with NorΔPEP1 on the surface expression of myc-mGluR5. NorΔPEP1 did not rescue the decrease in the surface expression of myc-mGluR5 caused by the knockdown of endogenous Norbin (control: 1.0 ± 0.02; shNor: 0.82 ± 0.03; shNor:NorΔPEP1: 0.82 ± 0.04; $p = 6.292 \times 10^{-7}$ between control and shNor; $p = 5.356 \times 10^{-4}$ between control and shNor:NorΔPEP1; one-way ANOVA followed by Tukey's post-test) (Fig. 3*A*,*B*). NorΔPEP1 also did not rescue the R,S-DHPG-mediated internalization of myc-mGluR5. No significant internalization of myc-mGluR5 was observed subsequent to the application of 100 μM R,S-DHPG in shNor:NorΔPEP1-expressing cells, similar to what was observed in Norbin knockdown cells (control: 1 ± 0.03; control + DHPG: 1.64 ± 0.08; shNor + DHPG: 1.06 ± 0.04; shNor:NorΔPEP1 + DHPG: 0.99 ± 0.05; $p = 1.637 \times 10^{-11}$ between control and control + DHPG; $p = 7.85 \times 10^{-9}$ between control + DHPG and shNor + DHPG; $p = 1.022 \times 10^{-9}$ between control + DHPG and shNor:NorΔPEP1 + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 3*E*,*G*). Similar to NorΔPEP1, NorΔPEP2 also did not rescue the decrease in the myc-mGluR5 surface expression because of the knockdown of the endogenous Norbin (control: 1.0 ± 0.02; shNor: 0.87 ± 0.03; shNor:NorΔPEP2: 0.83 ± 0.04; $p = 6.208 \times 10^{-4}$ between control and shNor; $p = 1.751 \times 10^{-4}$ between control and shNor:NorΔPEP2; one-way ANOVA followed by Tukey's post-test) (Fig. 3*C*,*D*). Interestingly, unlike NorΔPEP1, NorΔPEP2 partially rescued the R,S-DHPG-mediated endocytosis of myc-mGluR5 (control: 1 ± 0.04; control + DHPG: 1.62 ± 0.09; shNor + DHPG: 0.96 ± 0.04; shNor:NorΔPEP2 + DHPG: 1.2 ± 0.07; $p = 9.289 \times 10^{-9}$ between control and control + DHPG; $p = 2.083 \times 10^{-9}$ between control + DHPG and shNor + DHPG; $p = 2.257 \times 10^{-4}$ between control + DHPG and shNor:NorΔPEP2 + DHPG; $p = 0.006$ between shNor + DHPG and shNor:NorΔPEP2 + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 3*F*,*H*). NorΔPEP1 and NorΔPEP2 replacement constructs showed expression similar to endogenous Norbin in Western blots (control: 1 ± 0.04; shNor: 0.37 ± 0.1; shNor: NorΔPEP1: 0.94 ± 0.07; shNor: NorΔPEP2: 1.19 ± 0.22; $p = 0.028$ between control and shNor; $p = 0.537$ between control and shNor:NorΔPEP1; $p = 0.492$ between control and shNor:NorΔPEP2; one-way ANOVA followed by Tukey's post-test) (Fig. 3*I*,*J*). We subsequently studied the effect of deletion of the PEP1 and PEP2 regions of Norbin individually on the Norbin and PKA interaction. Our data suggested that deletion of both PEP1 and PEP2 domains of Norbin disrupted the binding of PKA to Norbin, suggesting that these two regions of Norbin play a critical role in the interaction of PKA with Norbin (Norbin + PKA: 1 ± 0.04; NorΔPEP1 + PKA: 0.19 ± 0.05; NorΔPEP2 + PKA: 0.15 ± 0.05; $p = 0.006$ between Norbin + PKA and NorΔPEP1 + PKA; $p = 0.005$ between Norbin + PKA and NorΔPEP2 + PKA; one-way ANOVA followed by Tukey's post-test) (Fig. 3*K*,*L*). These results suggest that PKA binding at the N-terminal region of Norbin is required for the ligand-dependent internalization of mGluR5.

Interaction of mGluR5 with C-terminal region of Norbin is necessary for the ligand-mediated internalization of mGluR5

It has been reported that the C-terminal region of Norbin interacts with several transmembrane proteins, including mGluR1 and mGluR5 (Wang et al., 2009). Therefore, we subsequently investigated whether the C-terminal region of Norbin plays any role in the ligand-mediated internalization of mGluR5 by replacing the WT Norbin with a form of Norbin that lacked the C-terminal region of the protein (NorΔC; presence of amino acids 1-481 of Norbin). NorΔC appeared to express properly as observed by the Western blot (control: 1 ± 0.05; shNor: 0.43 ± 0.01; shNor:NorΔC: 1.02 ± 0.06; $p = 0.009$ between control and shNor; $p = 0.838$ between control and shNor:NorΔC; one-way ANOVA followed by Tukey's post-test) (Fig. 4*A*,*B*). Expression of this replacement construct (shNor:NorΔC) did not rescue the decrease in the surface expression of myc-mGluR5 because of the knockdown of the endogenous Norbin (control: 1.0 ± 0.03; shNor: 0.85 ± 0.04; shNor:NorΔC: 0.73 ± 0.03; $p = 0.002$ between control and shNor; $p = 1.32 \times 10^{-9}$ between control and shNor:NorΔC; one-way ANOVA followed by Tukey's post-test) (Fig. 4*C*,*D*). Importantly, NorΔC did not rescue the ligand-mediated internalization of myc-mGluR5. No significant internalization of myc-mGluR5 was observed subsequent to the application of 100 μM R,S-DHPG in 30 min in shNor:NorΔC-expressing cells, similar to what was observed in Norbin knockdown cells (control: 1 ± 0.04; control + DHPG: 1.62 ± 0.08; shNor + DHPG: 1.07 ± 0.03; shNor:NorΔC + DHPG: 1.04 ± 0.05; $p = 1.974 \times 10^{-10}$ between control and control + DHPG; $p = 6.391 \times 10^{-9}$ between control + DHPG and shNor + DHPG; $p = 5.429 \times 10^{-8}$ between control + DHPG and shNor:NorΔC + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 4*E*,*F*). These results suggest that, similar to the N-terminal region, C-terminal region of Norbin also plays a critical role in the ligand-induced internalization of mGluR5.

In order to investigate whether binding of mGluR5 with Norbin is critical for the ligand-mediated internalization of mGluR5, we made a Norbin replacement construct wherein alanine residue at the 687th position of full-length Norbin was mutated to glycine (NorA687G). WT Norbin showed prominent interaction with mGluR5, but in case of the point mutant, this interaction was hampered, suggesting that alanine 687 in Norbin plays a critical role in the interaction of Norbin with mGluR5 (mGluR5 + Norbin: 1 ± 0.08; mGluR5 + NorA687G: 0.29 ± 0.06; $p = 4.62 \times 10^{-4}$ between mGluR5 + Norbin and mGluR5 + NorA687G; unpaired *t* test) (Fig. 5*A*,*B*). This replacement construct showed expression similar to the WT Norbin in Western blot (control: 1 ± 0.07; shNor: 0.43 ± 0.04; shNor:NorA687G: 0.96 ± 0.13; $p = 0.002$ between control and shNor; $p = 0.819$ between control and shNor:NorA687G; one-way ANOVA followed by Tukey's post-test) (Fig. 5*C*,*D*). Expression of this replacement construct was unable to rescue the decrease in the surface myc-mGluR5 expression because of the knockdown of the endogenous Norbin (control: 1.0 ± 0.04; shNor: 0.85 ± 0.03; shNor:

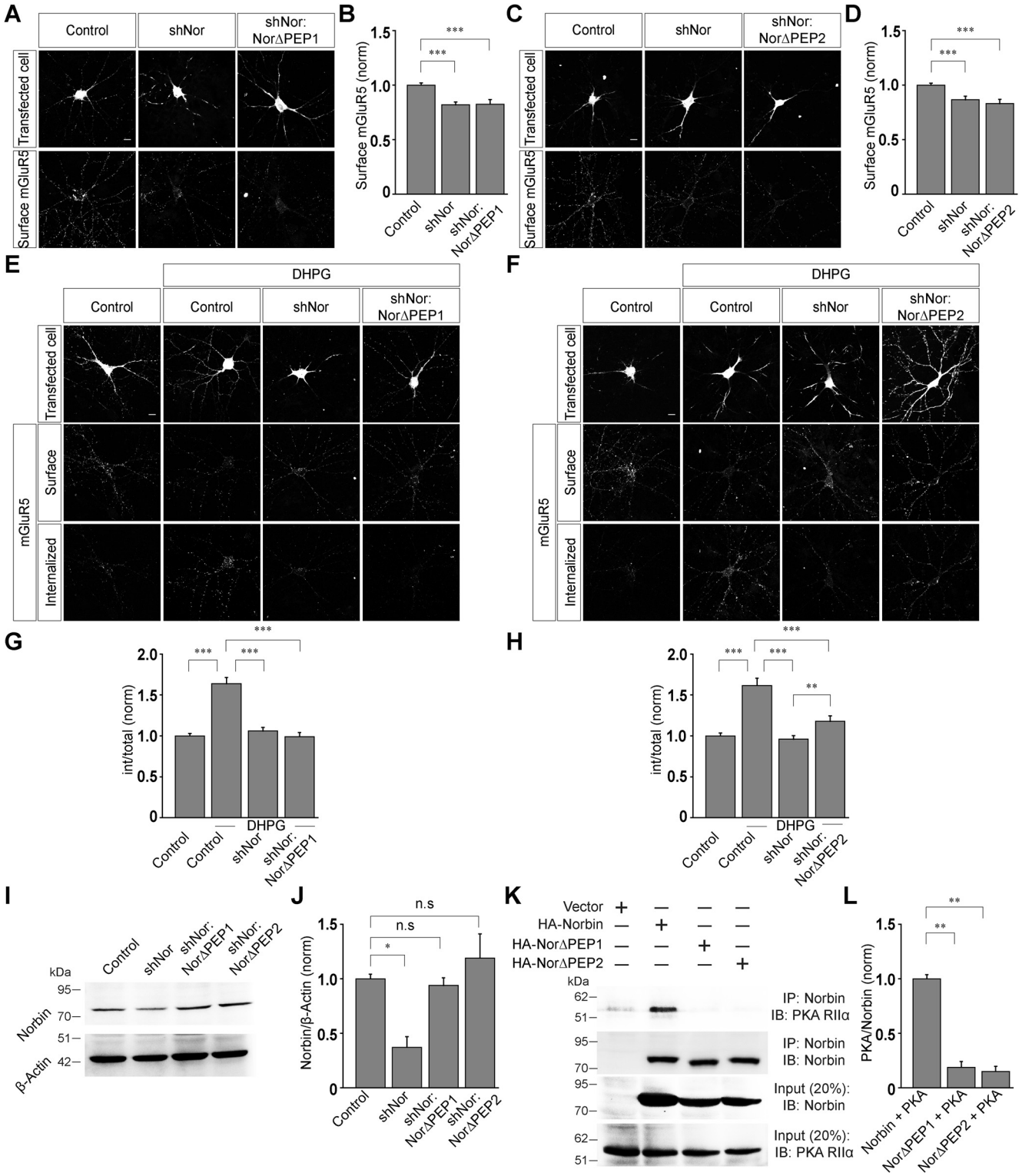

Figure 3. Binding of PKA to Norbin is necessary for the ligand-induced endocytosis of mGluR5. ***A***, ***B***, Deletion of PEP1 region of Norbin reduced the surface expression of myc-mGluR5, as shown in the representative images (***A***) and quantitation (***B***) of surface myc-mGluR5 in cells expressing shNor or shNor and NorΔPEP1. ***C***, ***D***, Deletion of the PEP2 region of Norbin also decreased the surface expression of myc-mGluR5, as shown in the representative images (***C***) and quantitation (***D***) of surface localized myc-mGluR5 in control cells, shNor-expressing cells, and shNor- and NorΔPEP2-expressing cells. ***E***, ***G***, Representative images (***E***) and quantitation (***G***) of ligand-mediated endocytosis of myc-mGluR5 in control cells, and cells expressing shNor or shNor and NorΔPEP1 suggested that NorΔPEP1 could not rescue the inhibition in the ligand-mediated internalization of myc-mGluR5 because of the knockdown of the endogenous Norbin. ***F***, ***H***, Representative images (***F***) and quantitation (***H***) of ligand-mediated myc-mGluR5 endocytosis in control cells and cells expressing shNor, or shNor and NorΔPEP2 suggested that NorΔPEP2 partially rescued the ligand-mediated endocytosis of myc-mGluR5. ***I***, ***J***, Western blot (***I***) and quantitation of Western blots (***J***) showing the knockdown of endogenous Norbin by shNor and expression of NorΔPEP1 and NorΔPEP2 replacement constructs. ***K***, ***L***, Coimmunoprecipitation assay (***K***) and quantitation of the assay (***L***) demonstrating that deletion of both PEP1 and PEP2 regions of Norbin disrupted the binding of PKA to Norbin. Data are mean ± SEM collected from three independent experiments. Scale bar, 10 μm. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n. s., $p > 0.05$. IB, Immunoblot; IP, immunoprecipitate.

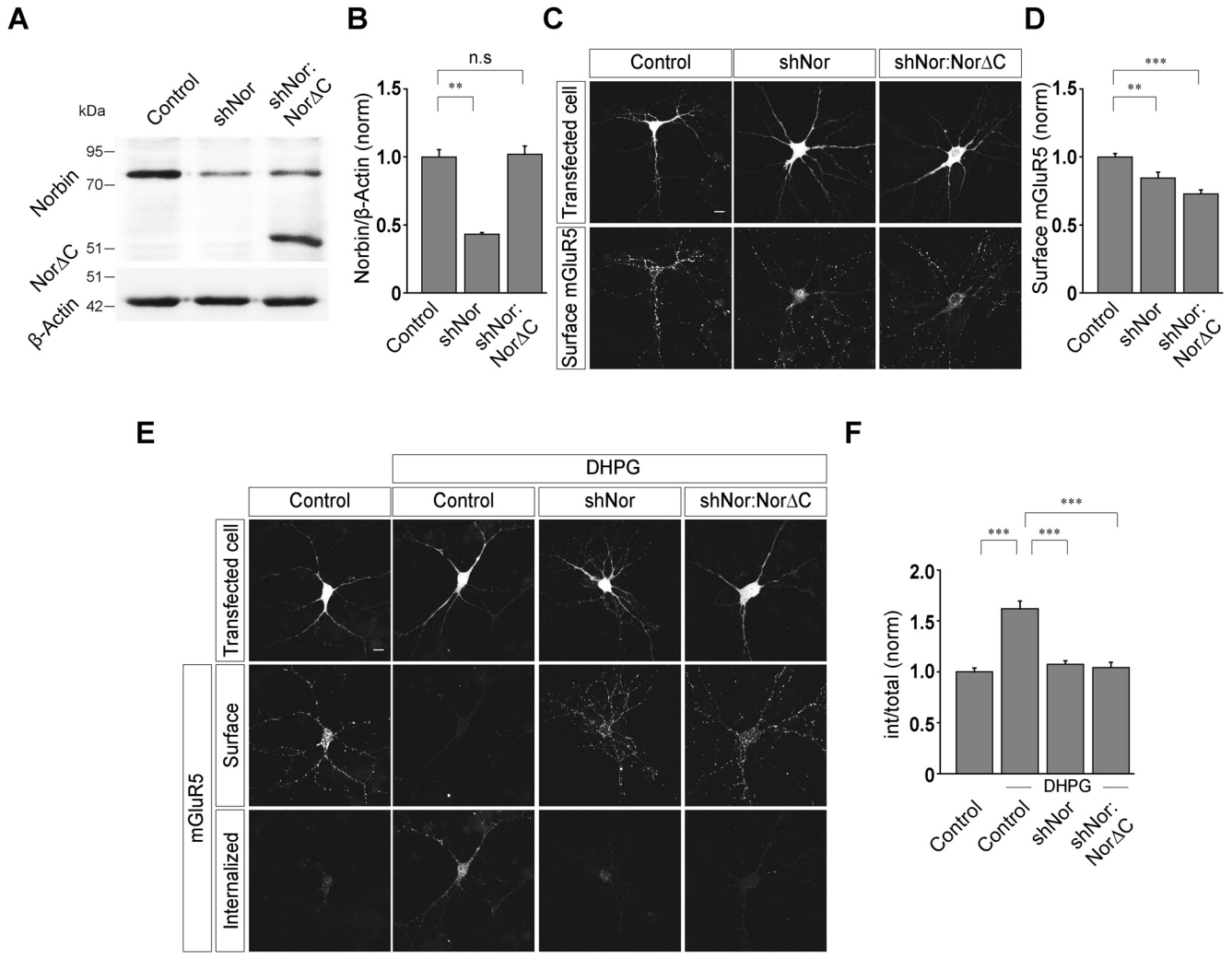

Figure 4. C-terminal region of Norbin plays a critical role in the surface expression and ligand-induced endocytosis of mGluR5. ***A***, ***B***, Acute knockdown of endogenous Norbin and replacement of endogenous Norbin with NorΔC, as shown by the Western blot (***A***) and quantitation of the Western blots (***B***). ***C***, ***D***, Representative images (***C***) and quantitation (***D***) showing a reduction in the surface expression of myc-mGluR5 in shNor and shNor:NorΔC-transfected cells. ***E***, ***F***, Representative images (***E***) and quantitation (***F***), suggesting that knockdown of endogenous Norbin resulted in the inhibition of ligand-mediated endocytosis of myc-mGluR5, and expression of the NorΔC replacement construct could not rescue the endocytosis. Data are mean ± SEM collected from three independent experiments. Scale bar, 10 μm. ***$p < 0.001$; **$p < 0.01$; n.s., $p > 0.05$.

NorA687G: 0.75 ± 0.05; $p = 0.008$ between control and shNor; $p = 3.714 \times 10^{-4}$ between control and shNor:NorA687G; one-way ANOVA followed by Tukey's post-test) (Fig. 5*E*,*F*). Interestingly, unlike WT Norbin, NorA687G could not rescue the ligand-mediated endocytosis of myc-mGluR5. In shNor:NorA687G-transfected cells, the receptors did not endocytose on application of 100 μM R,S-DHPG (control: 1 ± 0.05; control + DHPG: 2.07 ± 0.19; shNor + DHPG: 0.91 ± 0.03; shNor:NorA687G + DHPG: 1.09 ± 0.11; $p = 8.639 \times 10^{-7}$ between control and control + DHPG; $p = 1.645 \times 10^{-7}$ between control + DHPG and shNor + DHPG; $p = 3.48 \times 10^{-5}$ between control + DHPG and shNor:NorA687G + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 5*G*,*H*). Hence, binding of mGluR5 with Norbin is indeed critical for the endocytosis of the receptor. Together, our results suggest that both binding of PKA at the N-terminal region of Norbin and interaction of mGluR5 at the C-terminus of Norbin are necessary for the ligand-mediated endocytosis of mGluR5.

Expression and synaptic localization of mutants of Norbin

In order to observe whether the Norbin mutants that we have used in this study were targeted and localized at the synapse in primary hippocampal neurons, we first checked the expression profile of each one of them. These constructs contain HA tag at the N-terminus; thus, they produce recombinant proteins on expression that are fused with HA at the N-terminus of the protein (Fig. 6*A*). Our data suggested that the expression patterns of the NorΔPEP1, NorΔPEP2, NorΔC, and NorA687G proteins were very similar to the expression pattern of the WT Norbin replacement protein (Fig. 6*B*). On the other hand, the NorΔN protein was mislocalized, and it was predominantly present at the cell body of the neuron. Unlike other replacement constructs, not much expression of the NorΔN was observed in the dendritic region of the neurons (Fig. 6*B*). We subsequently investigated whether these mutants of Norbin were localized at the synapse. The proportion of synapses containing detectable amount of these mutants of Norbin were quantified by staining for HA-containing clusters and counterstaining for Bassoon, a core component of the active zone that is commonly used to identify presynaptic terminals (tom Dieck et al., 1998). Our data suggested that NorΔPEP1, NorΔPEP2, NorΔC, and NorA687G localized at the synapse similar to the WT Norbin protein. Expectedly, the NorΔN protein, which did not target properly at the dendrites and was mostly localized in the cell body region, did not show

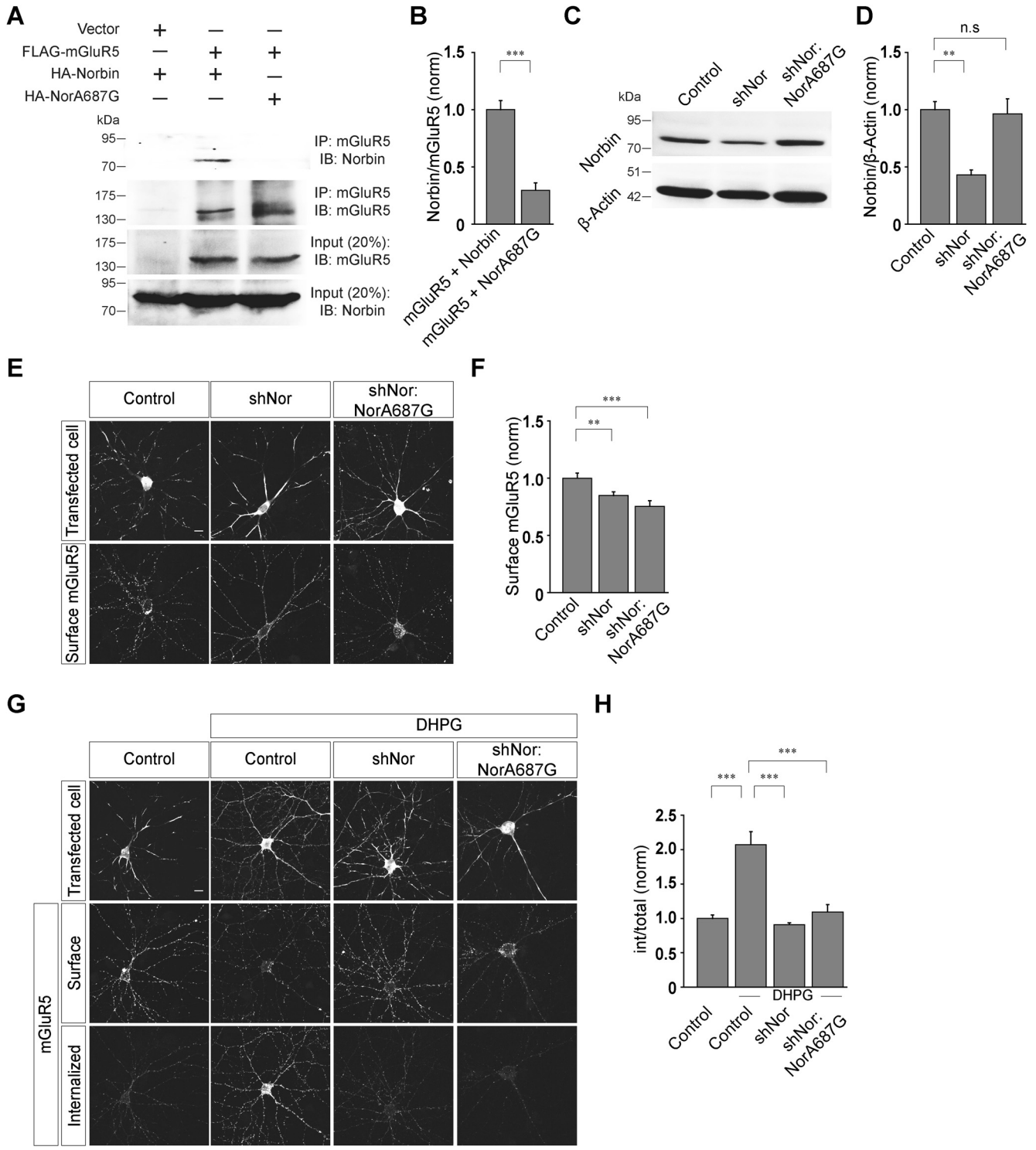

Figure 5. A687G mutation in Norbin disrupts its binding to mGluR5 and blocks ligand-mediated endocytosis of mGluR5. ***A***, Coimmunoprecipitation assay demonstrating that point mutation from alanine 687 to glycine in full-length Norbin disrupted the binding of Norbin to mGluR5. ***B***, Quantitation of the coimmunoprecipitation assays. ***C***, ***D***, Western blot (***C***) and quantitation of the Western blots (***D***) showing the knockdown of endogenous Norbin by shNor and expression of the NorA687G replacement construct. ***E***, ***F***, Representative images (***E***) and quantitation (***F***) of surface myc-mGluR5 in control cells and cells expressing shNor or shNor:NorA687G. ***G***, ***H***, Representative images (***G***) and quantitation (***H***) of ligand-mediated endocytosis of myc-mGluR5 in control cells and cells expressing shNor or shNor:NorA687G showing an inhibition in the ligand-mediated endocytosis of myc-mGluR5 in both shNor and shNor:NorA687G-expressing cells. Data are mean ± SEM collected from three independent experiments. Scale bar, 10 μm. ***$p < 0.001$; **$p < 0.01$; n.s., $p > 0.05$.

much colocalization with Bassoon (wt-Norbin: 89.72 ± 1.89; NorΔN: 16.93 ± 4.52; NorΔPEP1: 89.23 ± 2.05; NorΔPEP2: 83.49 ± 2.9; NorΔC: 84.46 ± 2.01; NorA687G: 88.83 ± 2.21; $p = 3.823 \times 10^{-14}$ between wt-Norbin and NorΔN; $p = 0.859$ between wt-Norbin and NorΔPEP1; $p = 0.067$ between wt-Norbin and NorΔPEP2; $p = 0.06$ between wt-Norbin and NorΔC; $p = 0.76$ between wt-Norbin and NorA687G) (Fig. 6*C*, *D*). These results together suggested that deletion of the N-terminal domain of Norbin mislocalized the protein. All other Norbin constructs were targeted properly at the synapse.

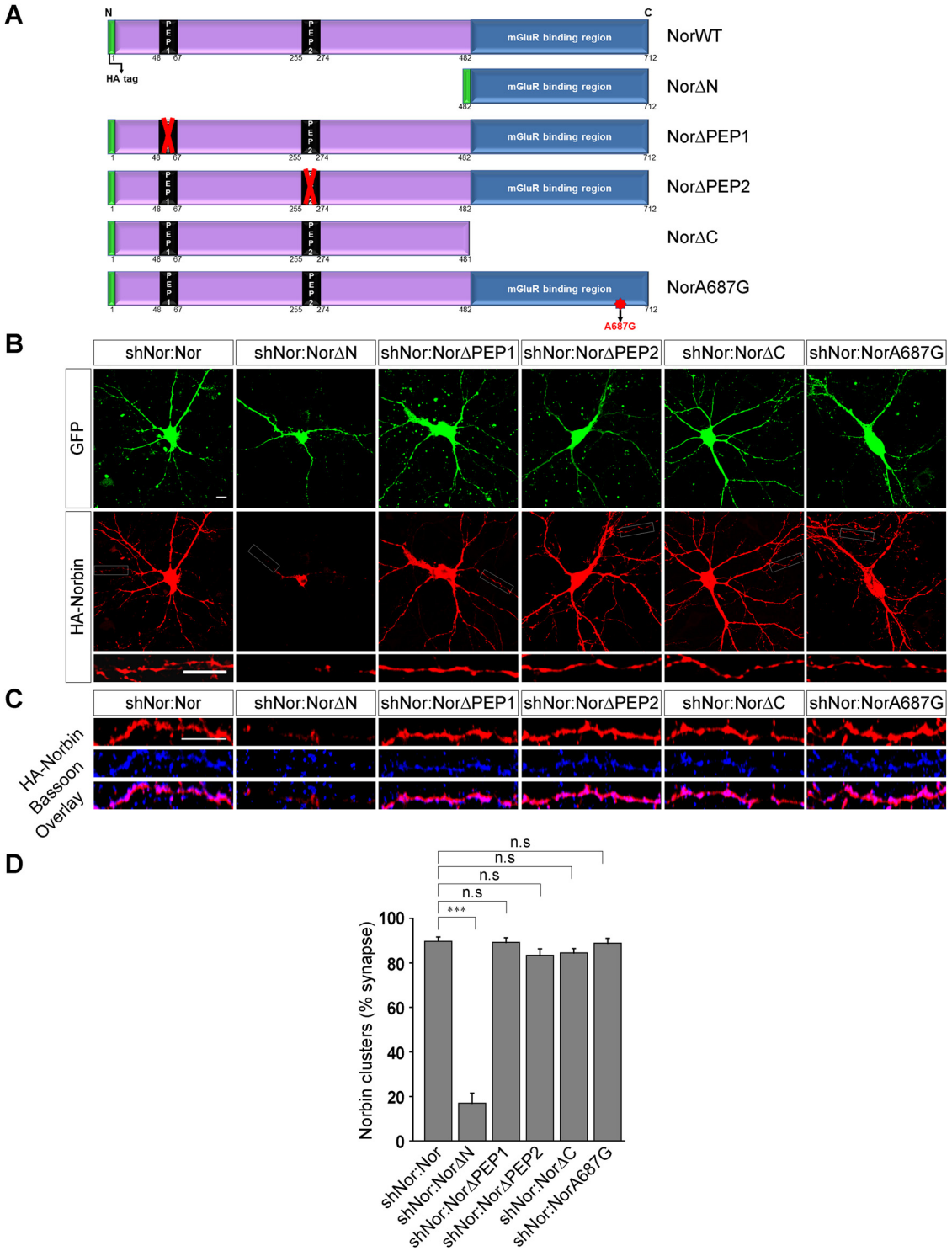

Figure 6. Targeting and synaptic localization of Norbin constructs. ***A***, Schematic of various Norbin constructs. ***B***, Representative images showing that WT Norbin was targeted to the dendrites. NorΔPEP1, NorΔPEP2, NorΔC, and NorA687G showed an expression pattern similar to that of the WT Norbin replacement protein. In contrast, NorΔN did not target properly to the dendrites of the neuron. ***C***, Representative images showing the colocalization of Bassoon with various forms of Norbin. All the constructs of Norbin except NorΔN were found to colocalize with Bassoon. NorΔN did not show much colocalization with Bassoon. ***D***, Quantitation also suggested that all the constructs of Norbin except NorΔN were localized at the synapse to the similar extent. Data are mean ± SEM. Scale bar, 10 µm. $^{***}p < 0.001$; n.s., $p > 0.05$.

Norbin specifically regulates the mGluR-mediated AMPAR endocytosis

Rapid endocytosis of surface AMPARs can be triggered in cultured hippocampal neurons by application of various glutamate receptor agonists, including glutamate itself, NMDA, AMPA, and Group I mGluR agonists (Biou et al., 2008; Bhattacharyya et al., 2009; Citri et al., 2009, 2010). mGluR-mediated AMPAR endocytosis is believed to be the cellular correlate for the mGluR-dependent synaptic plasticity (Citri and Malenka, 2008; Gladding et al., 2009; Niswender and Conn, 2010; Bhattacharyya, 2016). Because the goal of this study was to elucidate the role of Norbin in Group I mGluR internalization and in turn its effect

on the mGluR-dependent endocytosis of AMPARs, we initially studied the effect of acute knockdown of Norbin on the mGluR-mediated synaptic AMPAR endocytosis. We performed these experiments using the protocol that results in the endocytosis of synaptic AMPARs on activation of Group I mGluRs (Gulia et al., 2017; Sharma et al., 2018; Pandey et al., 2020). As opposed to its effect on mGluR surface localization, knockdown of endogenous Norbin did not have any effect on the surface expression of GluA1-containing AMPARs in primary hippocampal neurons (control: 1.0 ± 0.02; shNor: 0.97 ± 0.03; shNor:Nor: 1.0 ± 0.03; $p = 0.478$ between control and shNor; $p = 0.958$ between control and shNor:Nor; one-way ANOVA followed by Tukey's post-test) (Fig. 7*A*,*B*). On the other hand, we found that knockdown of Norbin with shNor caused an almost complete inhibition of mGluR-triggered internalization of GluA1-containing receptors (control: 1 ± 0.03; control + DHPG: 1.65 ± 0.08; shNor + DHPG: 1.05 ± 0.06, $p = 2.319 \times 10^{-10}$ between control and control + DHPG; $p = 1.427 \times 10^{-7}$ between control + DHPG and shNor + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 7*C*,*E*). This reduction in AMPAR endocytosis was rescued by expression of recombinant WT Norbin (shNor:Nor + DHPG: 1.82 ± 0.11; $p = 0.202$ between control + DHPG and shNor:Nor + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 7*C*,*E*). AMPAR endocytosis is greatly enhanced not only by activation of mGluRs, but also by activation of NMDARs in a manner that presumably mimics NMDAR-triggered LTD (Citri and Malenka, 2008). We therefore examined whether knockdown of Norbin influences the NMDAR-dependent AMPAR endocytosis as well. In contrast with its clear effects on mGluR-triggered AMPAR endocytosis, neither expression of shNor nor recombinant WT Norbin with shNor had a detectable effect on the endocytosis of AMPARs on application of 100 μM NMDA in primary hippocampal neurons (control: 1 ± 0.07; control + NMDA: 1.73 ± 0.09; shNor + NMDA: 2.03 ± 0.16; shNor:Nor + NMDA: 2.04 ± 0.19; $p = 8.341 \times 10^{-8}$ between control and control + NMDA; $p = 0.116$ between control + NMDA and shNor + NMDA; $p = 0.153$ between control + NMDA and shNor:Nor + NMDA; one-way ANOVA followed by Tukey's post-test) (Fig. 7*D*,*F*). Therefore, Norbin appears to be critical specifically for the endocytosis of synaptic AMPARs triggered by the activation of Group I mGluRs, but not for NMDAR-mediated AMPAR endocytosis.

Group I mGluR-mediated signaling and Arc protein synthesis is not dependent on Norbin

We subsequently investigated the reason for the inhibition of the mGluR-mediated AMPAR endocytosis in the absence of endogenous Norbin. Group I mGluRs undergo desensitization and rapid internalization on ligand exposure and subsequently, recycle back to the cell membrane in 2.5 h (Dhami and Ferguson, 2006; Pandey et al., 2014; Mahato et al., 2015; Sharma et al., 2018). On recycling to the plasma membrane, these receptors are known to resensitize (i.e., functionally recover) such that their responsiveness toward the agonist is restored (Sharma et al., 2018). To investigate the effects of Norbin knockdown on the signaling by Group I mGluRs, we studied the ability of the receptors to induce the second messenger responses on binding with the ligand in control cells and Norbin knockdown cells. Group I mGluRs upregulate the phosphorylation of MAPK/ERK1/2 on activation (Kim et al., 2008; Gladding et al., 2009; Sharma et al., 2018). In control cells, application of 100 μM R,S-DHPG increased the phosphorylation of ERK1/2. Expectedly, the receptors that recycled back to the cell surface in 2.5 h also upregulated the phosphorylation of ERK1/2 on application of 100 μM R,S-DHPG, suggesting that the receptors that came back to the cell surface through normal recycling route were resensitized (control: untreated: 1 ± 0.3; DHPG: 2.39 ± 0.2; 2.5 h recycling: untreated: 1.39 ± 0.06; DHPG: 3.38 ± 0.45; $p = 0.018$ between untreated and DHPG; $p = 0.012$ between 2.5 h recycling untreated and 2.5 h recycling DHPG; $p = 0.268$ between untreated and 2.5 h recycling untreated; one-way ANOVA followed by Tukey's post-test) (Fig. 8*A*,*B*). In shNor-transfected cells, initial application of 100 μM R,S-DHPG resulted in the increase in the phosphorylation of ERK1/2. Unexpectedly, the receptors that did not internalize on ligand application were also able to increase the phosphorylation of ERK1/2, on activation with 100 μM R,S-DHPG, 2.5 h after first application of the ligand (shNor: untreated: 1 ± 0.04; DHPG: 1.71 ± 0.05; 2.5 h: untreated: 1.02 ± 0.12; DHPG: 2.03 ± 0.21; $p = 3.764 \times 10^{-4}$ between untreated and DHPG; $p = 0.013$ between 2.5 h untreated and 2.5 h DHPG; $p = 0.892$ between untreated and 2.5 h untreated; one-way ANOVA followed by Tukey's post-test) (Fig. 8*C*,*D*). These results suggest that knockdown of Norbin did not have any effect on the signaling by Group I mGluRs and the receptors that did not internalize on R,S-DHPG application in shNor-transfected cells were still able to activate the second messenger response in the absence of Norbin.

It has been reported that mGluRs mediate AMPAR endocytosis through Arc protein present in the neuron and stimulate the local synthesis of Arc that results in the long-term increase in the AMPAR endocytosis rate (Waung et al., 2008; Niere et al., 2012). In order to investigate whether Norbin controls the Group I mGluR-mediated local protein synthesis, we studied the effect of endogenous Norbin knockdown on the mGluR-mediated Arc protein synthesis in primary hippocampal neurons. In control cells, application of 100 μM R,S-DHPG for 5 min led to the increase in the Arc protein expression compared with untreated cells, whereas preincubation of cells with cycloheximide inhibited the increase in the Arc protein expression on application of 100 μM R,S-DHPG (control: untreated: 1 ± 0.07; DHPG: 1.62 ± 0.12; DHPG + CHX: 0.94 ± 0.08; $p = 5.089 \times 10^{-5}$ between untreated and DHPG; $p = 0.585$ between untreated and DHPG + CHX; one-way ANOVA followed by Tukey's post-test) (Fig. 8*E*,*F*). Importantly, in shNor-transfected cells, application of 100 μM R,S-DHPG for 5 min also increased the expression of Arc protein, which was inhibited in cells preincubated with cycloheximide (shNor: untreated: 1 ± 0.06; DHPG: 1.69 ± 0.13; DHPG + CHX: 1.12 ± 0.11; $p = 1.448 \times 10^{-5}$ between untreated and DHPG; $p = 0.307$ between untreated and DHPG + CHX; one-way ANOVA followed by Tukey's post-test) (Fig. 8*E*,*G*). Furthermore, shNor:Nor-transfected cells also showed the increase in Arc protein expression on application of 100 μM R,S-DHPG for 5 min and cells preincubated with cycloheximide did not show that increase (shNor:Nor: untreated: 1 ± 0.1; DHPG: 1.52 ± 0.09; DHPG + CHX: 0.95 ± 0.1; $p = 2.093 \times 10^{-4}$ between untreated and DHPG; $p = 0.724$ between untreated and DHPG + CHX; one-way ANOVA followed by Tukey's post-test) (Fig. 8*E*,*H*). Thus, these experiments suggest that Norbin does not play any role in the mGluR-mediated Arc protein synthesis.

PKA binding to AMPARs through Norbin is critical for the mGluR-mediated AMPAR endocytosis

Since knockdown of the endogenous Norbin did not affect the MAP-kinase signaling by Group I mGluRs or mGluR-mediated Arc protein synthesis, it was important to investigate why knockdown of Norbin inhibited the mGluR-mediated AMPAR

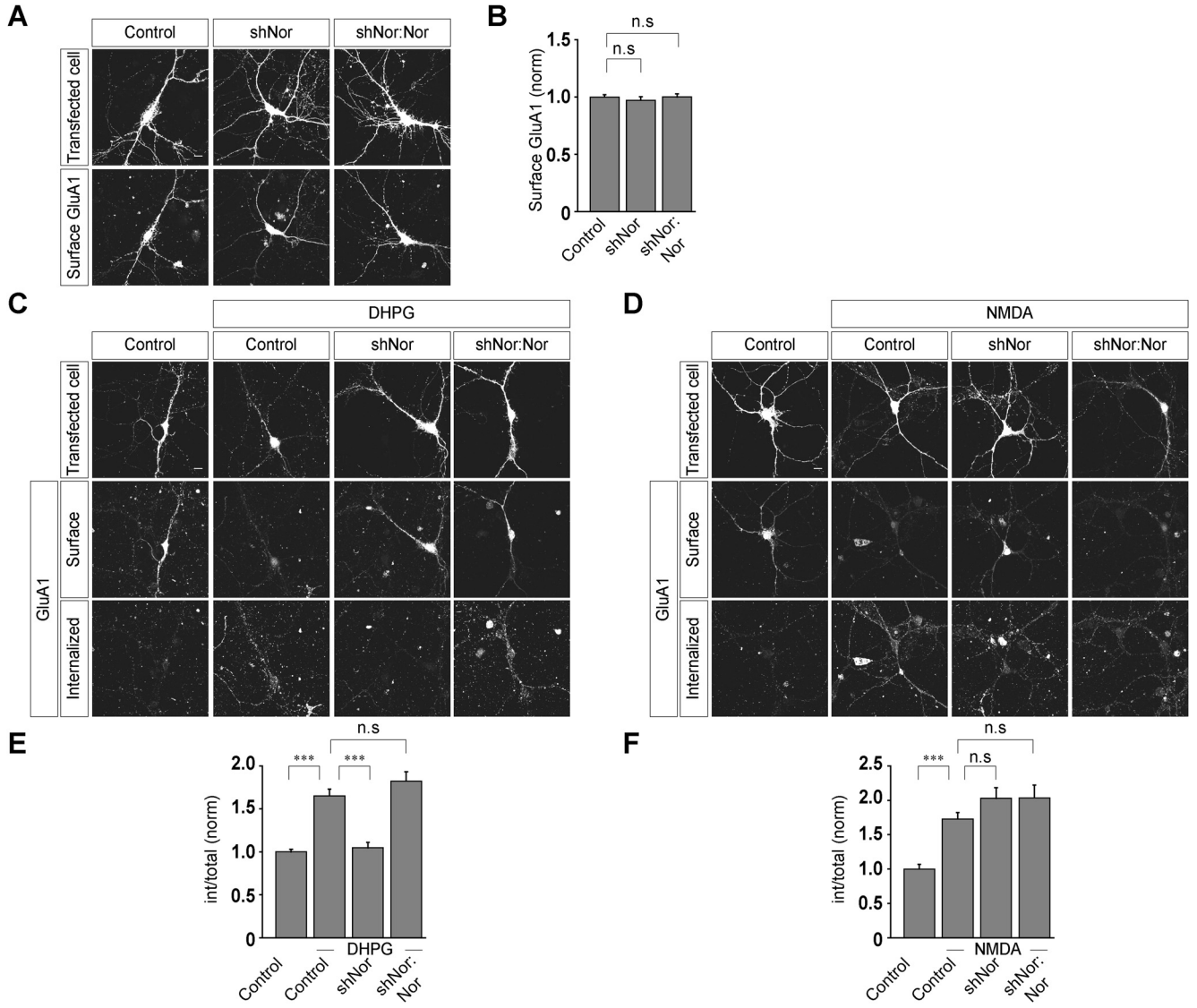

Figure 7. Norbin specifically regulates mGluR-mediated AMPAR endocytosis. ***A***, ***B***, Representative images (***A***) and quantitation of surface GluA1-containing receptors (***B***) showing that knockdown of endogenous Norbin and replacement with WT Norbin had no effect on the surface expression of GluA1-containing receptors. ***C***, ***E***, Representative cells (***C***) and quantitation of mGluR-mediated AMPAR endocytosis (***E***) suggested that, in control cells, the receptors internalized when cells were treated with 100 μM R,S-DHPG for 5 min. In contrast, endocytosis of GluA1-containing receptors was inhibited in Norbin knockdown cells. Expression of WT Norbin rescued the R,S-DHPG-mediated endocytosis of AMPARs. ***D***, ***F***, Representative images (***D***) and quantitation of NMDAR-triggered AMPAR endocytosis (***F***) suggested that application of 100 μM NMDA for 5 min triggered the internalization of GluA1-containing receptors in control cells, and this internalization was not affected in shNor-transfected cells, and cells transfected with shNor and full-length Norbin. Data are mean ± SEM collected from three independent experiments. Scale bar, 10 μm. ***$p < 0.001$; n.s., $p > 0.05$.

endocytosis. mGluR activation is known to upregulate PKA signaling in neurons, and Group I mGluRs promote AMPAR phosphorylation through the activation of PKA (Kim et al., 2008; Dell'anno et al., 2013). It has been reported that AMPAR endocytosis is dependent on the phosphorylation of AMPAR by PKA (Roche et al., 1996; Lee, 2006). Since Norbin is an A-kinase anchoring protein that binds to the RIIα regulatory subunit of PKA, we wanted to see whether Norbin plays any role in the association of PKA with AMPARs. In control cells, GluA1-containing receptors showed basal interaction with PKA, which increased significantly on application of 100 μM R,S-DHPG. Importantly, in shNor-transfected cells, application of 100 μM R,S-DHPG did not increase the interaction of PKA with GluA1-containing receptors. The increased interaction of PKA with GluA1-containing receptors subsequent to the application of 100 μM R,S-DHPG was restored on expression of the WT Norbin replacement construct. On the other hand, cells transfected with NorΔPEP1 or NorΔPEP2 replacement constructs did not show increased interaction of PKA with GluA1-containing receptors on application of 100 μM R,S-DHPG (control: 1 ± 0.11; control + DHPG: 1.64 ± 0.05; shNor: 0.89 ± 0.04; shNor + DHPG: 0.8 ± 0.06; shNor:Nor: 0.93 ± 0.05; shNor:Nor + DHPG: 1.88 ± 0.12; shNor:NorΔPEP1: 0.92 ± 0.09; shNor:NorΔPEP1 + DHPG: 1.06 ± 0.03; shNor:NorΔPEP2: 0.91 ± 0.03; shNor:NorΔPEP2 + DHPG: 0.87 ± 0.04; $p = 0.007$ between control and control + DHPG; $p = 0.271$ between shNor and shNor + DHPG; $p = 0.002$ between shNor:Nor and shNor:Nor + DHPG; $p = 0.229$ between shNor:NorΔPEP1 and shNor:NorΔPEP1 + DHPG; $p = 0.435$ between shNor:NorΔPEP2 and shNor:NorΔPEP2 + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 9*A*,*B*). These results suggest that activation of Group I mGluRs results in the increased interaction of PKA with

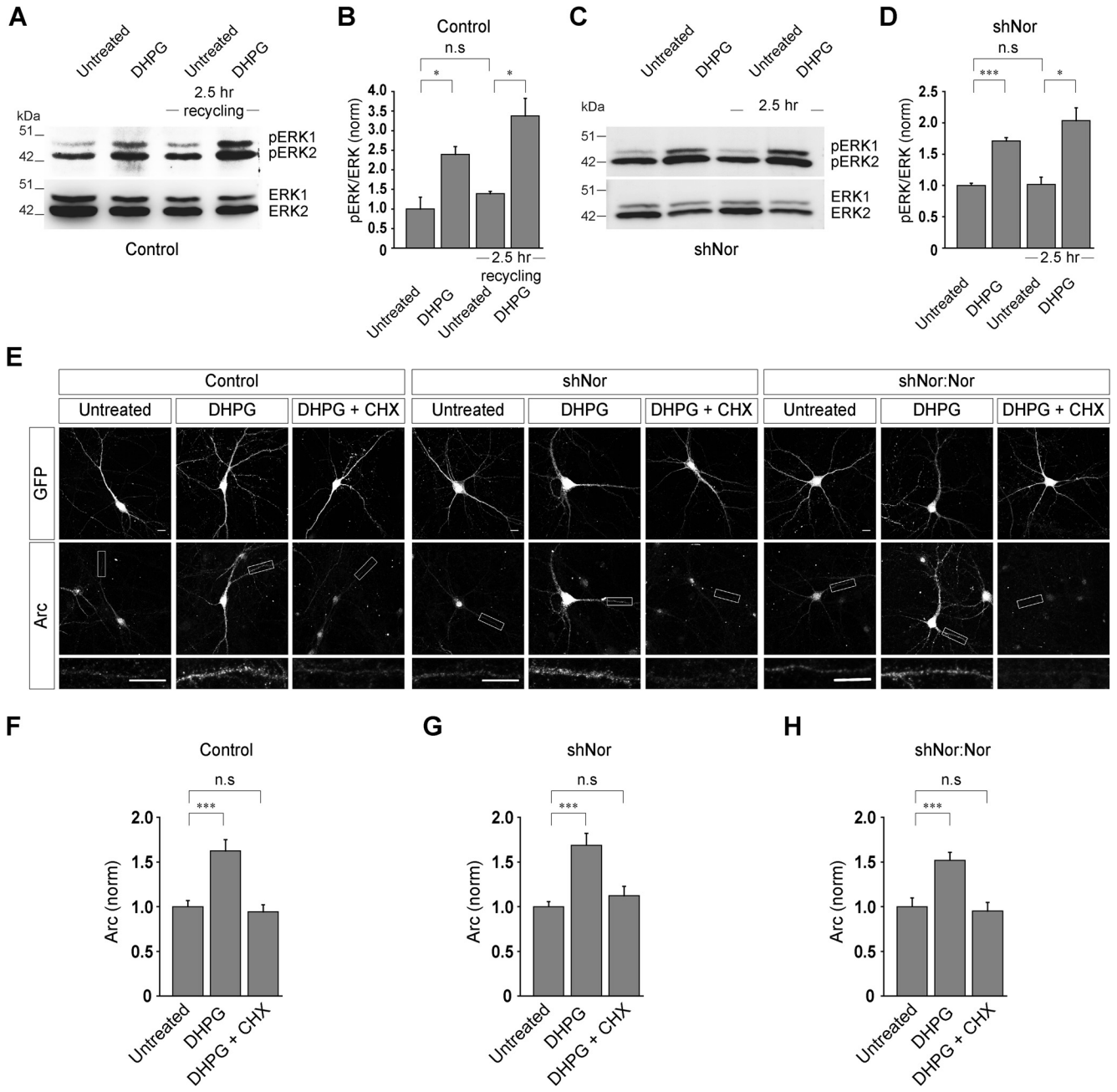

Figure 8. Norbin does not affect the signaling by Group I mGluRs and mGluR-mediated Arc protein synthesis. ***A***, ***B***, Western blot (***A***) and quantitation of the Western blots (***B***) showing that, in control cells, application of 100 μM R,S-DHPG for 5 min increased the phosphorylation of MAP kinases. The receptors that recycled to the cell surface in 2.5 h also induced phosphorylation of MAP kinases on application of the ligand. ***C***, ***D***, Western blot (***C***) and quantitation of the Western blots (***D***) suggested that, in Norbin knockdown cells, initial application of 100 μM R,S-DHPG for 5 min increased the phosphorylation of MAP kinases, and the receptors that could not internalize because of the knockdown of endogenous Norbin were still able to increase the phosphorylation of MAP kinases on application of the ligand. ***E***, Representative images showing that expression of shNor or shNor:Nor did not have any effect on the Group I mGluR-mediated Arc protein synthesis. ***F-H***, Quantitation of the Arc protein expression in control cells (***F***), shNor-expressing cells (***G***), and shNor:Nor expressing cells (***H***) suggested that, in all these cells, Arc protein synthesis increased on application of 100 μM R,S-DHPG and preincubation of these cells with cycloheximide inhibited this increase. Data are mean ± SEM collected from three independent experiments. Scale bar, 10 μm. ***$p < 0.001$; *$p < 0.05$; n.s., $p > 0.05$.

AMPARs, and Norbin acts as a critical mediator in this process.

We subsequently investigated whether binding of Norbin with PKA is important for the mGluR-mediated AMPAR endocytosis. Application of 100 μM R,S-DHPG led to the internalization of GluA1-containing receptors in control cells. Knockdown of the endogenous Norbin inhibited the AMPAR endocytosis, and replacement of endogenous Norbin with NorΔPEP1 could not rescue the mGluR-mediated AMPAR endocytosis (control: 1 ± 0.05; control + DHPG: 1.86 ± 0.16; shNor + DHPG: 0.89 ± 0.07; shNor:NorΔPEP1 + DHPG: 1.07 ± 0.06; $p = 2.816 \times 10^{-7}$ between control and control + DHPG; $p = 9.889 \times 10^{-7}$ between control + DHPG and shNor + DHPG; $p = 2.073 \times 10^{-5}$ between control + DHPG and shNor:NorΔPEP1 + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 9*C*,*D*). Similar to NorΔPEP1, NorΔPEP2 also did not rescue the R,S-DHPG-mediated endocytosis of GluA1-containing receptors (control: 1 ± 0.06; control + DHPG: 1.53 ± 0.13; shNor + DHPG:

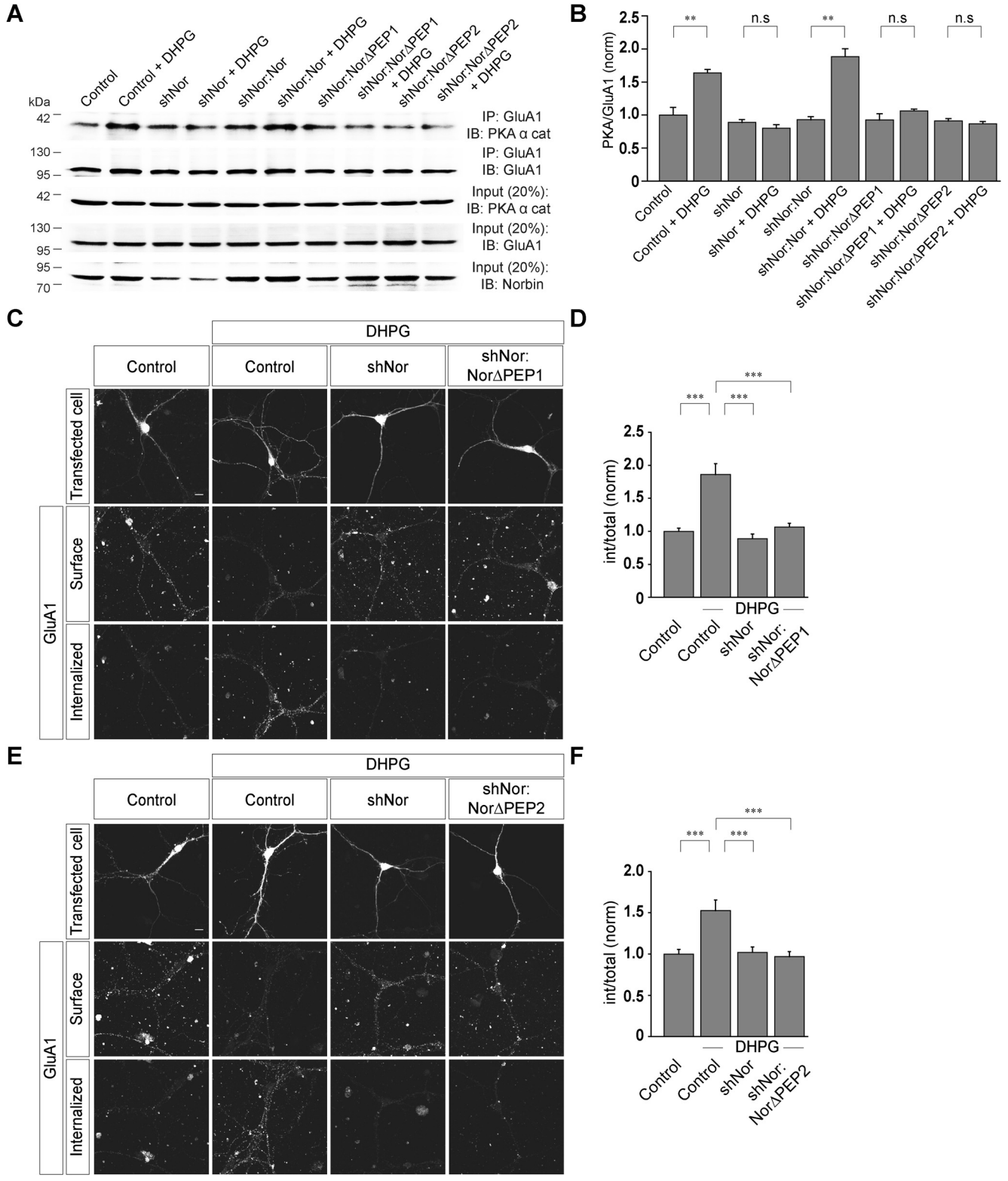

Figure 9. PKA binding to AMPARs through Norbin is essential for the mGluR-mediated AMPAR endocytosis. ***A***, ***B***, Coimmunoprecipitation assay (***A***) and quantitation of the coimmunoprecipitation assays (***B***) showing the basal interaction of PKA catalytic subunit with AMPARs in control cells, which was increased on activation of Group I mGluRs by 100 μM R,S-DHPG for 5 min. On the other hand, cells in which endogenous Norbin was knocked down, application of 100 μM R,S-DHPG was unable to recruit PKA to AMPARs. Increased interaction of PKA to AMPARs on application of 100 μM R,S-DHPG was rescued by replacing the endogenous Norbin with WT Norbin, but not with either NorΔPEP1 or NorΔPEP2. ***C***, ***D***, The inhibition of the mGluR-mediated AMPAR endocytosis because of the knockdown of the endogenous Norbin was not rescued by replacing the endogenous Norbin with NorΔPEP1, as observed from representative images (***C***) and quantitation of the endocytosis index (***D***). ***E***, ***F***, Representative images and quantitation of the endocytosis index suggested that NorΔPEP2 replacement construct also did not rescue the inhibition in the mGluR-mediated AMPAR endocytosis because of the knockdown of endogenous Norbin. Data are mean ± SEM collected from three independent experiments. Scale bar, 10 μm. ***$p < 0.001$; **$p < 0.01$; n.s., $p > 0.05$.

1.02 ± 0.07; shNor:NorΔPEP2 + DHPG: 0.97 ± 0.06; $p = 1.463 \times 10^{-4}$ between control and control + DHPG; $p = 8.593 \times 10^{-4}$ between control + DHPG and shNor + DHPG; $p = 1.098 \times 10^{-4}$ between control + DHPG and shNor:NorΔPEP2 + DHPG; one-way ANOVA followed by Tukey's post-test) (Fig. 9*E*,*F*). Together, these results suggest that activation of Group I mGluRs led to enhanced interaction of PKA with the AMPARs, which is essential for the mGluR-mediated AMPAR endocytosis; and Norbin acts as a scaffolding protein which recruits PKA to the AMPARs subsequent to the activation of Group I mGluRs.

Discussion

In the last few years, Group I mGluRs have emerged as potential therapeutic targets for the treatment of several psychiatric disorders, including Fragile X syndrome, autism, and schizophrenia (Huber et al., 2002; Bear et al., 2004; Dolen et al., 2007; Citri and Malenka, 2008). Trafficking of these receptors plays critical roles in regulating their proper spatiotemporal localization in the neuron as well as the activity of these receptors. Thus, inappropriate trafficking of the receptor could result in abnormal signaling with pathologic consequences. Because of these reasons, understanding the molecular mechanisms that govern the Group I mGluR trafficking and their effect in the brain has emerged as a major area of research. In this study, we reported a novel role for Norbin in the ligand-mediated internalization of Group I mGluRs and mGluR-dependent AMPAR endocytosis, which is the cellular correlate for the mGluR-mediated synaptic plasticity. Norbin is a 75 kDa scaffolding protein, which is known to interact with mGluR5; and it is reported that Norbin KO mice display a behavioral phenotype related to schizophrenia (Wang et al., 2009). However, it is currently unknown how Norbin binding affects the internalization and signaling of these receptors. Previous reports have shown that overexpression of Norbin increases the surface localization of mGluR5 (Wang et al., 2009). Moreover, until now, Norbin is not known to affect the agonist-mediated internalization of any GPCR. On the basis of a series of mutant Norbin constructs, primarily using a strategy in which mutant forms of Norbin replace endogenous Norbin that has been knocked down by shRNA, we have demonstrated that Norbin plays a critical role in surface stabilization as well as in the ligand-mediated internalization of Group I mGluRs. Knockdown of endogenous Norbin caused a significant reduction in the ligand-induced endocytosis of mGluR5. Norbin also affected the surface expression of mGluR5 independent of its effects on endocytosis. Prior studies from our laboratory had established a key role for two scaffolding proteins, tamalin and sorting nexin 1, in the ligand-mediated endocytosis and recycling of Group I mGluRs, respectively (Sharma et al., 2018; Pandey et al., 2020). Interestingly, none of them affected the surface expression of mGluRs in our assays, but Norbin is the first protein that intricately regulates both the surface stability and ligand-mediated internalization of Group I mGluRs.

We found that the N-terminal region of Norbin has an important role to play in targeting this protein to the dendritic regions of the neuron and for its localization at the synapse. N-terminal region of Norbin harbors two distinct peptides (PEP1 and PEP2) through which PKA interacts with Norbin (Hermann et al., 2015). In contrast to WT Norbin, replacement of endogenous Norbin with NorΔPEP1 and NorΔPEP2 could not rescue the block in mGluR5 endocytosis caused by shNor, suggesting that PKA binding to Norbin through PEP1 and PEP2 is important for ligand-mediated endocytosis of mGluR5. However, it is important to note that NorΔPEP2 could partially rescue the endocytosis of mGluR5, indicating that Norbin might have differential binding affinities for PKA through each of these peptides; and the partial rescue in case of NorΔPEP2 could be a result of stronger affinity of PKA for the PEP1 region compared with the PEP2 region of Norbin. This is something that can be evaluated in the future using more specific and robust measurements of binding affinities for these two mutant forms of Norbin for PKA. The C-terminal region of Norbin has been shown to be important for its interaction with various GPCRs, including mGluR5 (Wang et al., 2009). Consequently, deletion of the C-terminal region of Norbin could not rescue the decrease in surface expression as well as block in the ligand-mediated mGluR5 endocytosis. Importantly, we found a critical residue alanine at the 687th position in the C-terminal region of Norbin, which when mutated to glycine, led to a strong impairment of Norbin's interaction with mGluR5. However, whether Norbin interacted with mGluR5 directly or indirectly could not be concluded from these coimmunoprecipitation experiments. Interestingly, this single residue mutation was unable to rescue the decrease in surface expression of mGluR5, and it could not reverse the effect of Norbin knockdown on ligand-mediated mGluR5 endocytosis. The simplest model to explain our results is that C-terminal of Norbin positions it in the vicinity of mGluR5 and N-terminal interacts with PKA to assist in mGluR5 internalization.

We also studied the role of Norbin in mGluR-dependent AMPAR endocytosis, which is a prerequisite for mGluR-mediated synaptic plasticity. Our data suggested that, unlike its effect on mGluR surface expression, knockdown of Norbin had no effect on the surface expression of AMPARs, suggesting that its effect on the surface localization is specific for Group I mGluRs. We showed that the role of Norbin in the endocytosis of AMPARs is highly specific. Knockdown of Norbin markedly reduced the mGluR-mediated AMPAR endocytosis but did not influence NMDAR-triggered AMPAR endocytosis. This observation is not surprising since mGluR-mediated LTD involves signaling cascades that are distinct from those underlying NMDAR-triggered LTD (Citri and Malenka, 2008; Bhattacharyya et al., 2009; Citri et al., 2010; Bhattacharyya, 2016). We also found that the Group I mGluRs that could not internalize in the absence of endogenous Norbin were still able to induce the ERK signaling cascades on application of R,S-DHPG. An earlier report suggested that overexpression of Norbin resulted in the increased mGluR5 signaling in HEK293T cells, whereas we did not see any effect on mGluR signaling on knockdown of endogenous Norbin in primary neurons (Wang et al., 2009). This discrepancy in results could be because of different cellular backgrounds (i.e., HEK293T cells vs primary neurons), or it could also be because of different approaches used to manipulate Norbin (i.e., overexpression vs acute knockdown). mGluR-mediated local Arc protein synthesis has been reported to be critical for the long-term increase in the AMPAR endocytosis rate. Our data suggested that Norbin did not play any role in controlling the synthesis of Arc protein by mGluRs. Therefore, it was very interesting to find that, although mGluR-mediated signaling and

mGluR-mediated Arc protein synthesis were normal in Norbin knockdown cells, still mGluR-mediated AMPAR endocytosis was inhibited in these cells. In this study, we have proposed that Norbin acts as a central regulator of mGluR-mediated AMPAR endocytosis by recruiting PKA to AMPARs on mGluR activation. This is supported by the observation that interaction of PKA with GluA1-containing AMPARs is enhanced on activation of Group I mGluRs and the presence of Norbin, that has the PKA binding sites is essential for this process. The increased interaction of PKA with GluA1-containing receptors on activation of Group I mGluRs probably results in the phosphorylation of critical residue(s) of the receptor, which might be essential for the endocytosis of the receptor. But this hypothesis needs to be investigated in the future. In addition, our data suggest that mGluR-mediated AMPAR endocytosis is inhibited in the presence of Norbin that lacks PKA binding domains, PEP1 and PEP2. Together, these results suggest that, although mGluR-mediated Arc protein synthesis might be critical for the mGluR-mediated AMPAR endocytosis, recruitment of PKA to the GluA1-containing receptors by Norbin is also essential for this process. Thus, this study provides a novel mechanism for the regulation of mGluR-mediated AMPAR endocytosis. Further research is needed to find out the substrates of PKA and other interacting partners of Norbin. In addition, further study of the regulation of Group I mGluRs and mGluR-dependent AMPAR endocytosis by Norbin and its interacting proteins is of great importance. Our study demonstrated that a well-orchestrated relationship between Group I mGluRs, Norbin, PKA, and AMPARs is integral for the normal functioning of the brain, and it can have clinical relevance to the function of Group I mGluRs in neuropsychiatric disorders.

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