PORCN Negatively Regulates AMPAR Function Independently of Subunit Composition and the Amino-Terminal and Carboxy-Terminal Domains of AMPARs

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Most fast excitatory synaptic transmissions in the mammalian brain are mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs), which are ligand-gated cation channels. Dynamic changes in AMPAR properties serve as a major mechanism governing many forms of synaptic plasticity, including homeostatic scaling and long-term depression and

INTRODUCTION

Most fast excitatory synaptic transmissions in the mammalian brain are mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs), which are ligand-gated cation channels. Dynamic changes in AMPAR properties serve as a major mechanism governing many forms of synaptic plasticity, including homeostatic scaling and long-term depression and
Finally, in HEK293T cells that lacked expression of AMPARs, expression levels in the postsynaptic density (PSD) fraction was a significant reduction in GluA2/3 but not GluA1. In this study, the inactivation of PORCN in hippocampal neurons reduced the amplitude but accelerated the decay kinetics of AMPAR-mediated synaptic currents. PORCN overexpression decreased glutamate-induced currents when exogenously expressing GluA1 homologous AMPARs (Erlenhardt et al., 2016). Whether the inhibitory effects of PORCN on AMPAR function involve interactions with a specific AMPAR subunit or particular regions of an AMPAR subunit has not been determined.

In the present study, we examined the AMPAR subunit requirement for PORCN-mediated inhibition of AMPAR function in transfected heterologous cells. We showed that PORCN inhibits glutamate-induced currents and AMPAR surface expression in an AMPAR subunit-independent manner in heterologous cells. Furthermore, the ATD and CTD of AMPARs were not required to mediate the inhibitory effect of PORCN. We used immunoprecipitation assays to show that PORCN associated with all AMPAR subunits independently of the ATD and CTD. This was consistent with the functional data. Thus, our observations strongly supported the hypothesis that PORCN regulates AMPAR trafficking to the plasma membrane through protein-protein interactions.

MATERIALS AND METHODS

HEK293T Cell Culture and Transfection

In this study, stargazin, PORCN, full-length GluA subunits, and GluA deletion constructs were expressed in HEK293T cells (CRL-11268, ATCC). First, cells were cultured in a 37°C incubator supplied with 5% CO₂. Then, cells were dissociated with 0.05% trypsin and plated on dishes at a density of 800,000 cells per 35 mm dish (counted with a μScope CellCounter Basic; C.E.T.) 24 h before transfection. A 2:3 ratio of GluA:stargazin cDNA and a 3:1 ratio of GluA-stargazin:PORCN cDNA was used as previously reported (Shi et al., 2009; Wei et al., 2017). A cDNA ratio of 3:2 was used for the coexpression of GluA1 and GluA2 as well as GluA2 and GluA3 as previously reported (Shi et al., 2009; Wei et al., 2017). In control groups, the same amount of empty vector was used instead of PORCN cDNA. Transfection was performed using polyethyleneimine (Polysciences, United States) reagents. Transfected HEK293T cells were dissociated with 0.05% trypsin and plated on pretreated coverslips that were 8 mm diameter and coated with poly-D-lysine. Electrophysiological recording or immunostaining analyses were performed on cells transfected with 4 μg of total cDNA per 35 mm dish 24–36 h after transfection. For Western blotting, cells were transfected with 6.75 μg of full-length GluA subunit or GluA deletion plasmids together with 2.25 μg of myc-PORCN or control plasmids in 60 mm dishes and harvested 48 h after transfection.

Electrophysiological Recording

Electrophysiological recording was conducted on coverslips seeded with transfected HEK293T cells maintained in an external solution of 144 mM NaCl, 10 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM d-glucose (pH 7.3–7.4, Osm 315 mOsm/kg). For whole-cell patch, microelectrodes (3–5 MΩ, World Precision Instruments) were filled with an internal solution of 145 mM KCl, 5 mM NaCl, 5 mM EGTA, 4 mM MgATP, 0.3 mM Na₂GTP, and 10 mM HEPES (pH 7.2,
Osm 305 mOsm/kg). Series resistance was compensated to 60–70%, and recordings with series resistance values greater than 20 MΩ were rejected. Glutamate-induced currents were elicited through the local administration of external solution containing 10 mM L-glutamate acid (Sigma, G8415) for 2 s using an MPS-2 perfusion instrument [Inbio Life Science Instrument Co., Ltd.; (Wu et al., 2005)]. Whole-cell voltage clamp recordings were taken with an EPC10 patch clamp amplifier (HEKA, Lambrecht, Germany). Data were analyzed using the following software packages: Clampfit 10.0 (pClamp), Prism 5 (GraphPad Prism), and Igor 6.02 (WaveMetrics).

Hippocampal Culture and Calcium-Phosphate Transfection

Hippocampi were dissected from P0 pups and digested with 0.25% trypsin (Gibco, 25200072) at 37°C. Hippocampi were dissected from P0 pups and digested with 0.25% trypsin (Gibco, 25200072) at 37°C. Neurons were plated on poly-D-lysine-coated glass coverslips and maintained at 37°C in 5% CO₂ for 14 days before the experiment. The calcium-phosphate transfection method was used for the transfection of cultured neurons at 10 days in vitro. The DNA (0.5 µg per well in a 48 well plate) and Ca²⁺ were mixed and added to HBS drop by drop with a gentle vortex. After keeping the DNA/Ca²⁺/HBS mixture at room temperature for 30 min, it was added to the culture and incubated for 40 min at 37°C. Then, the culture was washed with culture medium two to three times and kept in the incubator.

Immunostaining analyses were performed as previously described (Jiang et al., 2017). In brief, transfected HEK293T cells were washed once with phosphate-buffered saline (PBS; Thermo Scientific), fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and washed three times with PBS. Then, cells were permeabilized with 0.2% Triton X-100 for 5 min at room temperature to maintain total protein analysis or were left unpermeabilized for surface protein analysis. After blocking with PBS containing 5% milk and 3% goat serum for 30 min at room temperature, cells were incubated with a primary antibody (anti-HA, 1:1000, Abmart; anti-Flag, 1:1000, Abmart) for 2 h at room temperature, washed three times with PBS, and incubated with the secondary antibody (donkey anti-mouse Alexa Fluor 546-conjugated secondary antibody, Life Technologies) for 30 min at room temperature. Fluoromount-G (Southern Biotech) was used to mount the cells on microscope slides. Images were acquired with a laser scanning confocal microscope (Olympus, FV3000) using a 60× objective lens (Olympus) and were further analyzed using the National Institutes of Health ImageJ program and Prism 5 software (GraphPad Prism).

RESULTS

PORCN Suppressed Glutamate-Induced Currents in HEK293T Cells Expressing GluA1, A2, or A3 With or Without Stargazin

To investigate whether the functional inhibition of AMPARs by PORCN depended on AMPAR subunit composition, we measured glutamate-induced currents via the whole-cell patch clamping of HEK293T cells transfected with various AMPAR subunits alone or in combination with stargazin and/or PORCN. Stargazin was used to promote AMPAR cell surface localization (Chen et al., 2000). Glutamate-induced currents were undetectable in HEK293T cells without transfection, because such cells do not normally express AMPAR subunits (Wei et al., 2016). In this study, PORCN expression significantly suppressed glutamate-induced currents mediated by GluA1 (Figure 1A), GluA2 (Figure 1B), and GluA3 (Figure 1C). The coexpression of stargazin with GluA2 or GluA3 significantly increased glutamate-induced currents compared with GluA2 or GluA3 expression alone but did not abolish the PORCN-mediated inhibition of AMPAR-mediated currents (Figures 1D–F). PORCN expression inhibited the peak amplitude of glutamate-induced currents in GluA1, GluA2, and GluA3 overexpressing cells with stargazin coexpression by 74.14 ± 14.79%, 95.39 ± 18.88%, and 79.99 ± 13.34%, respectively, and without stargazin coexpression by 60.45 ± 15.65%, 53.85 ± 20.45%, and 58.55 ± 19.44%, respectively. PORCN had similar effects on the plateau amplitude of glutamate-induced currents. In this case, percentage inhibition with stargazin coexpression was 75.39 ± 18.88%, 96.13 ± 18.72%, and 63.90 ± 30.36%, and without stargazin coexpression, it was 56.00 ± 15.30%, 68.14 ± 25.46%, and 76.37 ± 25.70%.

Most endogenous AMPARs in the brain exist in complexes comprising GluA1/A2 or GluA2/A3 (Wenthold et al., 1996; Lu et al., 2009). To account for this, we transfected HEK293T cells...
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FIGURE 1 | The overexpression of PORCN suppressed glutamate-induced currents mediated by GluA2 and GluA3 in HEK293T cells with or without stargazin coexpression. (A) Representative traces (left) and summary graphs (right) of the peak amplitudes and plateaus of 10 mM glutamate-induced currents in HEK293T cells transfected with GluA1 and either PORCN or a control plasmid. (B) Representative traces (left) and summary graphs (right) of the peak amplitudes and plateaus of 10 mM glutamate-induced currents in HEK293T cells transfected with GluA2 and either PORCN or a control plasmid. (C) Representative traces (left) and summary graphs (right) of the peak amplitudes and plateaus of 10 mM glutamate-induced currents in HEK293T cells transfected with GluA3 and either PORCN or a control plasmid. (D) Representative traces (left) and summary graphs (right) of the peak amplitudes and plateaus of 10 mM glutamate-induced currents in HEK293T cells transfected with GluA1 and stargazin and either PORCN or a control plasmid. (E) Representative traces (left) and summary graphs (right) of the peak amplitudes and plateaus of 10 mM glutamate-induced currents in HEK293T cells transfected with GluA2, stargazin, and either PORCN or a control plasmid. (F) Representative traces (left) and summary graphs (right) of the peak amplitudes and plateaus of 10 mM glutamate-induced currents in HEK293T cells transfected with GluA3, stargazin, and either PORCN or a control plasmid. (G) Representative traces (left) and summary graphs (right) of the peak amplitudes and plateaus of 10 mM glutamate-induced currents in HEK293T cells transfected with GluA1, GluA2, and either PORCN or a control plasmid. (H) Representative traces (left) and summary graphs (right) of the peak amplitudes and plateaus of 10 mM glutamate-induced currents in HEK293T cells transfected with GluA2, GluA3, and either PORCN or a control plasmid. In all panels, the black traces and bars represent the control condition (no PORCN expression), while the red traces and bars represent PORCN overexpression. All summary graphs show means ± SEMs; statistical comparisons were performed with a student’s t-test (*p < 0.05; **p < 0.01; ***p < 0.001).

PORCN Suppressed the Surface Delivery of GluA1, GluA2, and GluA3 in Transfected HEK293T Cells and Cultured Hippocampal Neurons

To investigate whether the PORCN-mediated inhibition of AMPAR-mediated currents was due to a reduction in surface AMPAR levels, we used quantitative immunocytochemistry to measure total and cell surface GluA protein levels in permeabilized and non-permeabilized transfected HEK293T cells, respectively. PORCN overexpression significantly reduced surface expression levels of GluA1, GluA2, and GluA3 in cells cotransfected with stargazin as indicated by decreased signals for the antibody against an extracellular epitope in non-permeabilized HEK293T cells. Signals decreased by 42.75 ± 11.09%, 59.30 ± 11.05%, and 82.11 ± 20.47%, respectively (Figures 2A1–A3). In contrast, immunostaining signals in permeabilized HEK293T cells for total GluA1, GluA2, and GluA3 levels were significantly higher in PORCN-transfected cells than in corresponding control cells (Figures 2B1–B3). This finding ruled out the possibility that the PORCN-mediated inhibition of surface GluA expression was due to a reduction in the expression of total AMPAR proteins. Thus, PORCN appeared to inhibit the plasma membrane delivery of AMPAR subunits (GluA1, GluA2, and GluA3) while increasing the total expression of these subunits in transfected HEK293T cells.
To further determine whether PORCN suppressed the surface expression of GluA subunits of AMPARs in cultured neurons, we transfected hippocampal neurons with plasmid encoding PORCN together with plasmid encoding GFP plasmids. Then, we labeled surface GluA1 and GluA2 subunits using the antibody against extracellular epitopes in transfected neurons and measured surface GluA1 and GluA2 levels using quantitative immunocytochemistry. The intensity of surface GluA1 and GluA2 puncta in non-permeabilized neurons decreased by 67.32 ± 15.92% and 51.12 ± 10.15%, respectively, while their density decreased by 33.94 ± 8.224% and 34.26 ± 7.720%, respectively (Figures 3A,B). Thus, the overexpression of PORCN significantly reduced surface GluA1 and GluA2 levels. As there were no antibodies suitable for labeling surface GluA3 levels in neurons, so we transfected the hippocampus neurons with GluA3 with a flag tag in the N terminal and labeled the surface signal using the antibody against the flag tag. The intensity of GluA3-transfected hippocampus neurons decreased by 39.44 ± 19.70%, while their density decreased by 66.95 ± 18.33% (Figure 3C). Thus, as was the case with GluA1 and GluA2, the overexpression of PORCN significantly decreased surface GluA3 levels. Overall, our data suggested that PORCN suppressed the surface expression of GluA1, GluA2, and GluA3 subunits of AMPARs in cultured neurons.
The ATD and CTD of AMPAR Subunits Are Not Required for the PORCN-Mediated Inhibition of AMPAR Delivery to the Plasma Membrane

Next, we investigated which AMPAR regions were required for the PORCN-mediated inhibition of AMPAR plasma membrane delivery. The removal of the LBD or transmembrane domain of AMPARs results in a complete loss of receptor function, so we focused the analysis on the ATD and CTD regions of AMPARs.

To this end, we constructed thirteen plasmids expressing full or deletion constructs according to our previously reported protocol for GluA1-ATD deletion constructs (Wei et al., 2016, 2017). We generated GluA2-ΔATD and GluA3-ΔATD deletion constructs using strategies similar to those adopted previously for GluA1 (Sheng et al., 2018). We fused all GluA-ΔATD constructs with a flag tag immediately downstream from the signal peptide separated by a GGQ spacer, and we fused GluA CTD deletion constructs with an HA tag at the extreme C-terminus separated by a GGQ spacer. Then, we measured the ligand-gated currents of cells expressing the various fragments of AMPARs employed in our previous function assay.

Our results demonstrated that glutamate-induced currents were significantly higher in control cells expressing stargazin and GluA1/2/3-ΔATD constructs and in control cells expressing GluA1/2/3-ΔCTD deletion constructs GluA1/2-ΔC, GluA1/2/3-Δ824, and GluA3-ΔMPR than in corresponding cells coexpressing PORCN. Percentage inhibitions for GluA1-ΔATD (69.87 ± 15.46%), GluA1-Δ824 (65.66 ± 15.35%), GluA1-ΔC (89.81 ± 19.65%), GluA2-ΔATD (79.53 ± 17.53%), GluA2-Δ824 (83.63 ± 19.53%), GluA2-ΔC (81.52 ± 31.68%), GluA3-ΔATD (82.47 ± 25.50%), GluA3-Δ824 (86.10 ± 18.90%), and GluA3-ΔMPR (67.98 ± 15.30%) have been shown in Figures 4A–C. Consistent with these results, the surface expression of AMPARs in non-permeabilized HEK293T cells transfected with GluA1/2/3-ΔATD or CTD deletion constructs was significantly suppressed by the overexpression of PORCN. Percentage inhibitions for GluA1-ΔATD (45.22 ± 8.90%), GluA1-Δ824 (50.15 ± 8.97%), GluA1-ΔC (80.55 ± 14.46%), GluA2-ΔATD (84.74 ± 10.79%), GluA2-Δ824 (85.62 ± 11.50%), GluA2-ΔC (82.23 ± 12.84%), GluA3-ΔATD (50.73 ± 10.06%), GluA3-Δ824 (52.84 ± 16.18%), and GluA3-ΔMPR (47.09 ± 13.62%) have been given in Figures 5A–C.

To determine whether PORCN suppressed the surface expression of the various fragments of AMPARs employed in cultured neurons, we transfected neurons with plasmid encoding PORCN, together with GluA1-ΔATD, GluA1-ΔC, GluA2-ΔATD, GluA2-ΔC, GluA3-ΔATD, GluA3-Δ824, or GluA3-ΔMPR and labeled the surface signal using the antibody against the flag tag in the N terminal of deletion constructs. Quantitative immunocytochemistry showed that the overexpression of PORCN suppressed the surface expression of all deletion constructs. Decreases in the intensity of

Supplementary Figure S1A
abolish the interaction with N4.1 in GluA1 (Lin et al., 2009; deleted, as this is known to disrupt the palmitoylation site and domain (Granger et al., 2013). In addition, we constructed removed two serine phosphorylation sites and the PDZ-binding CTD deleted. To delete the amino acid 824 from the CTD, we had the ATD and various CTDs or the amino acid 824 from the mutated versions of GluA1, GluA2, or GluA3. These plasmids results show means ± SEMs; statistical comparisons were performed with a student’s t-test (**p < 0.01; ***p < 0.001).
FIGURE 4 | The ATD and CTD of GluAs are not required for the inhibitory effect of PORCN on glutamate-induced currents. (A) Representative traces (A1) and summary graphs (A2) of the normalized peak amplitudes of 10 mM glutamate-induced currents in HEK293T cells transfected with full-length GluA1 or GluA1 deletion constructs (GluA1-ΔATD, GluA1-Δ824, and GluA1-ΔC), stargazin, and either PORCN or a control plasmid. (B) Representative traces (B1) and summary graphs (B2) of the normalized peak amplitudes of 10 mM glutamate-induced currents in HEK293T cells transfected with full-length GluA2 or GluA2 deletion constructs (GluA2-ΔATD, GluA2-Δ824, or GluA2-ΔC), stargazin, and either PORCN or a control plasmid. (C) Representative traces (C1) and summary graphs (C2) of the normalized peak amplitudes of 10 mM glutamate-induced currents in HEK293T cells transfected with full-length GluA3 or GluA3 deletion constructs (GluA3-ΔATD, GluA3-Δ824, and GluA3-ΔMPR), stargazin, and either PORCN or a control plasmid. All summary graphs show means ± SEMs; statistical comparisons were performed with a student’s t-test (*p < 0.05; **p < 0.01; ***p < 0.001).

GluA1-ΔATD (66.47 ± 13.30%), GluA1-ΔC (83.47 ± 17.66%), GluA2-ΔATD (79.84 ± 13.89%), GluA2-ΔC (74.20 ± 11.99%), GluA3-ΔATD (75.61 ± 19.31%), GluA3-Δ824 (69.23 ± 14.05%), GluA3-ΔMPR (75.72 ± 13.44%) varied. Decreases in the density of GluA1-ΔATD (25.37 ± 11.17%), GluA1-ΔC (51.10 ± 11.19%), GluA2-ΔATD (40.54 ± 10.29%), GluA2-ΔC (26.60 ± 9.540%), GluA3-ΔATD (53.14 ± 10.26%), GluA3-Δ824 (50.29 ± 11.66%), GluA3-ΔMPR (62.02 ± 8.041%) also varied (Figures 6A1, A2, B1, B2, C1–C3). Taken together, our results demonstrated that neither the ATD nor the CTD of AMPAR subunits GluA1-3 were required for the PORCN-mediated functional inhibition of AMPARs.

The Interaction of PORCN With AMPARs Was Independent of Their ATD or CTD

The PORCN-mediated functional inhibition of AMPARs is thought to be caused by an interaction of PORCN with these receptors (Schwenk et al., 2012; Erlenhardt et al., 2016). We used immunoprecipitation assays to search for regions in AMPAR subunits that interacted with PORCN. We used an anti-myc antibody to immunoprecipitate myc-PORCN in HEK293T cells transfected with full-length GluA2 or GluA2 deletion constructs (GluA2-ΔATD, GluA2-Δ824, or GluA2-ΔC), stargazin, and either PORCN or a control plasmid. (C) Representative traces (C1) and summary graphs (C2) of the normalized peak amplitudes of 10 mM glutamate-induced currents in HEK293T cells transfected with full-length GluA3 or GluA3 deletion constructs (GluA3-ΔATD, GluA3-Δ824, and GluA3-ΔMPR), stargazin, and either PORCN or a control plasmid. All summary graphs show means ± SEMs; statistical comparisons were performed with a student’s t-test (*p < 0.05; **p < 0.01; ***p < 0.001).

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FIGURE 5 | The ATD and CTD of GluAs are not required for the inhibitory effect of PORCN on the membrane expression of AMPARs in transfected HEK cells.  
(A) Representative images and quantification of the puncta intensity of the surface expression of GluA1 deletion constructs (A1: GluA1-ΔATD, A2: GluA1-Δ824, A3: GluA1-ΔC) and stargazin and transfected with either PORCN or a control plasmid. (B) Representative images and quantification of the puncta intensity of the surface expression of GluA2 deletion constructs (B1: GluA2-ΔATD, B2: GluA2-Δ824, B3: GluA2-ΔC) and stargazin and transfected with either PORCN or a control plasmid. (C) Representative images and quantification of the puncta intensity of the surface expression of GluA3 deletion constructs (C1: GluA3-ΔATD, C2: GluA3-Δ824, C3: GluA3-ΔMPR) and stargazin and transfected with either PORCN or a control plasmid. All summary graphs show means ± SEMs; statistical comparisons were performed with a student’s t-test (**p < 0.01; ***p < 0.001).

We performed similar experiments with GluA2 using an anti-GluA2 antibody that recognized the transmembrane region of GluA2 present in all GluA2 deletion constructs. GluA2-Δ824, GluA2-ΔATD, GluA2-ΔC, and the full-length GluA2 protein bound to myc-PORCN (Figure 7B). The results of similar experiments performed with GluA3 using an anti-flag antibody revealed that GluA3-Δ824, GluA3-ΔATD, and GluA3-ΔMPR as well as the full-length GluA3 protein bound to myc-PORCN (Figure 7C). Collectively, our data indicated that neither the ATD nor the CTD were involved in the interaction of PORCN with AMPARs, which suggested that other regions in AMPARs, such as the LBD and transmembrane domains, might be involved.

DISCUSSION

The trafficking of AMPARs to the plasma membrane determines the synaptic strength at excitatory synapses, and auxiliary subunits are key regulators of the intracellular and membrane delivery of AMPARs. PORCN, an auxiliary subunit of AMPARs, controls surface AMPAR levels in both transfected heterologous cells and in neurons (Schwenk et al., 2012; Erlenhardt et al., 2016). Here, we demonstrated that PORCN inhibits the ligand-gated currents and surface expression levels of GluA1, GluA2, and GluA3 in transfected HEK293T cells. This finding of subunit independence supported the previous finding that the inactivation of PORCN in hippocampal neurons reduced the total levels of GluA1, GluA2, and GluA3 (Erlenhardt et al., 2016). This inhibition required neither the ATD nor the CTD of these AMPAR subunits. Moreover, the interaction of PORCN with AMPARs was independent of the ATD and CTD of these AMPAR subunits. Thus, PORCN inhibited the function of AMPARs in a subunit-independent manner that did not involve the ATD or CTD of AMPARs.

Similar to the inhibitory effect of ABHD6, another auxiliary subunit of AMPARs (Schwenk et al., 2012; Wei et al., 2016, 2017), the inhibitory effect of PORCN on cell surface levels of GluA1, GluA2, and GluA3 in transfected HEK293T cells does not require the presence of either stargazin (γ-2, Figure 1C) or γ-8 (Erlenhardt et al., 2016). In contrast, the expression of CNIH-2, another auxiliary AMPAR subunit, in HEK cells slows the deactivation of AMPARs comprising GluA1, A2, or their combination; however, γ-8 expression reverses the effect of CNIH-2 on GluA2-containing AMPARs but not GluA1 homomers (Herring et al., 2013). Thus, multiple classes of auxiliary AMPAR proteins can mediate AMPAR trafficking to the plasma membrane.
FIGURE 6 | The ATD and CTD of GluAs are not required for the inhibitory effect of PORCN on the surface expression of AMPARs in cultured neurons. (A1,A2) Representative images and quantification of the intensity and density of the surface expression of GluA1 deletion constructs (A1: GluA1-ΔATD, A2: GluA1-ΔC) in cultured neurons expressing GluA1 deletion constructs. (B1,B2) Representative images and quantification of the intensity and density of the surface expression of GluA2 deletion constructs (B1: GluA2-ΔATD, B2: GluA2-ΔC) in cultured neurons expressing GluA2 deletion constructs. (C1–C3) Representative images and quantification of the intensity and density of the surface expression of GluA3 full length and deletion constructs (C1: GluA3-ΔATD, C2: GluA3-Δ824, C3: GluA3-ΔMPR) in cultured neurons expressing GluA3 deletion constructs. The white lines in the images represent scale bars (scale bars = 5 µm). All summary graphs show means ± SEMs; statistical comparisons were performed with a student’s t-test (*p < 0.05; **p < 0.01; ***p < 0.001).
FIGURE 7 | The binding of PORCN to AMPARs is independent of the AMPAR ATD or CTD. (A) Pulldown of GluA1, GluA1-ΔATD, GluA1-Δ824, and GluA1-ΔC expressed in transfected HEK293T cells together with the pulldown of myc-tagged PORCN with an anti-myc antibody. (B) Pulldown of GluA2, GluA2-ΔATD, GluA2-Δ824, and GluA2-ΔC expressed in transfected HEK293T cells together with the pulldown of myc-tagged PORCN with an anti-myc antibody. (C) Pulldown of GluA3, GluA3-ΔATD, GluA3-Δ824, and GluA3-ΔMPR expressed in transfected HEK293T cells together with the pulldown of myc-tagged PORCN with an anti-myc antibody.

Our data demonstrated that the ATD and CTD of AMPAR subunits GluA1, GluA2, and GluA3 were not required for the PORCN-mediated inhibition of AMPAR function or for the PORCN–AMPAR interaction. The ATD and CTD of AMPARs play substantial roles in the membrane trafficking of this receptor (Xia et al., 2002; Granger et al., 2013). Notably, multiple sites or regions in the CTD of AMPARs undergo protein modifications, such as nitrosylation, palmitoylation, ubiquitination, and phosphorylation/dephosphorylation [see (Diering and Huganir, 2018) for a detailed review]. A sophisticated molecular replacement strategy has been used to show that the PDZ binding motif in the AMPAR CTD is crucial for the synaptic delivery of AMPARs to the postsynaptic plasma membrane during both the basal state and long-term potentiation (Sheng et al., 2018). The interaction of other auxiliary subunits with the AMPAR CTD is essential. For example, ABHD6 reduces the surface expression levels of AMPARs in heterologous cells by binding to their cytoplasmic region (Wei et al., 2016, 2017). Another recent report indicated that the AMPARs ATD is involved in AMPAR trafficking. In the AMPAR complete knockout background, GluA1 or GluA2 expression resulted in the full or partial restoration of AMPAR-mediated synaptic transmission in Schaffer collateral pathways, while the expression of corresponding ATD deletion...
constructs did not rescue this transmission (Diaz-Alonso et al., 2017; Watson et al., 2017).

The exact molecules mediating this GluA-ATD interaction are unknown, but promising proteins associated with the GluA-ATD, including neuronal pentraxins, have been reported (O’Brien et al., 1999, 2002; Sia et al., 2007; Chang et al., 2010; Gu et al., 2013; Pelkey et al., 2015; Lee et al., 2017). In this study, AMPAR subunits, even in the absence of the ATD or CTD (for example, GluA-∆C and GluA-∆ATD), constituted functional receptors in transfected HEK293T cells as indicated by their surface expression and capability to mediate glutamate-induced currents. Interestingly, PORCN inhibited the membrane expression of AMPARs and ligand-mediated glutamate-induced currents. Interestingly, PORCN constituted functional receptors in transfected HEK293T cells 1 mutants (endogenous AMPARs with various GluA1 CTD deletion mutants) that the expression levels of these AMPAR mutants were 2 agreed with previous work, which showed that normal AMPAR-subunit functionally interact remains to be identified. This issue must be addressed through further systematic molecular and cellular biology studies.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

MWe, LY, and CZ designed the research. MWe and LY performed the research. MWe, MWa, JW, FS, YY, MS, SW, Mel, HW, MiL, WL, and YG analyzed the data. CZ and LY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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