Figures and figure supplements

Nedd4 E3 ligase and beta-arrestins regulate ubiquitination, trafficking, and stability of the mGlu7 receptor

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Figure 1. mGlu7 is ubiquitinated by agonist treatment or Nedd4 expression in heterologous cells and neurons. (A) mGlu7 is ubiquitinated in HEK 293 T cells. N-terminal c-myc epitope-tagged mGlu7a (myc-mGlu7) was co-transfected with HA epitope-tagged ubiquitin (HA-Ub) in HEK 293 T cells. Cell lysates were immunoprecipitated with anti-myc antibody (9E10) and immunoblotted with anti-HA antibody. Diffuse bands of high molecular weight larger than 200 kDa representing ubiquitinated mGlu7 were detected in the lane that the co-expressed mGlu7 and Ub were loaded in. (B) HA-Ub and myc-mGlu7 were co-transfected in HEK 293 T cells. Agonist L-Glutamate (L-Glu, 1 mM) was added for 10 to 60 min before cell lysis. Bar graph below represents L-Glu-induced ubiquitination levels normalized to the 0 min timepoint sample (means ± SEM; 10 min, 1.77 ± 0.15; 30 min, 1.55 ± 0.25; 60 min, 2.27 ± 0.33; n = 5, *p<0.05, **p<0.01 versus 0 min, one-way ANOVA). (C) Cultured cortical neurons at 14 days in vitro (DIV) were incubated in ACSF (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM D-glucose, 2 mM CaCl2, 2 mM MgCl2 plus 10 μM MG132 and 52.5 μM leupeptin) for 10 min and then 400 μM L-AP4 was added for 5 min at 37°C. Endogenous mGlu7 ubiquitination in neurons was detected by immunoprecipitation using anti-ubiquitin antibody (FK2) and western blotting using anti-ubiquitin (P4D1) or anti-mGlu7 antibody. Bar graph below represents mean ± SEM (L-AP4, 2.02 ± 0.23; n = 9, **p<0.01, Student's t-test). (D) After cell surface biotinylation with membrane impermeable Sulfo-NHS-SS-Biotin, cortical neurons were treated with L-AP4 for 5 min. The surface proteins were isolated by Streptavidin-agarose beads overnight at 4°C and eluted by incubating the beads with 50 mM DTT for 30 min at 50°C. The eluted proteins (surface) and unbound lysates (internal) were further immunoprecipitated by anti-ubiquitin antibody (FK2), and western blotted with the indicated antibodies. Bar graph below represents means ± SEM of relative mGlu7 ubiquitination levels normalized to total mGlu7 and are shown as a ratio to the non-stimulated control of the surface fraction (surface, L-AP4, 1.75 ± 0.28; internal, ACSF, 0.25 ± 0.09; internal, L-AP4, 0.28 ± 0.13; n = 6, **p<0.01, one-way ANOVA). (E) Nedd4 C867S mutant was co-expressed with Ub and myc-mGlu7 in HEK 293 T cells. Ubiquitination of mGlu7 was analyzed as above. Bar graph below represents mean ± SEM of Nedd4-induced mGlu7 ubiquitination levels normalized to the vector control (Nedd4 WT, 4.51 ± 0.52; Nedd4 C867S, 1.44 ± 0.27; n = 3, **p<0.01, one-way ANOVA). (F) Cultured cortical neurons were infected with lentiviruses harboring Nedd4 shRNA (Nedd4 KD) or Figure 1 continued on next page.
non-related control shRNA (Ctl KD) for 7 days. At DIV 14, L-AP4-induced endogenous mGlu7 ubiquitination was evaluated as in Figure 1C. Bar graph represents means ± SEM (Ctl KD + L-AP4, 2.03 ± 0.41; Nedd4 KD + ACSF, 1.33 ± 0.19; Nedd4 KD + L-AP4, 1.28 ± 0.14; n = 7, **p<0.01, n.s. indicates p>0.05, one-way ANOVA). (G) Analysis of endogenous interaction between Nedd4 E3 ligase and mGlu7. Cortical neurons at DIV 14 were treated with L-AP4 for 5 min, and cell lysates were immunoprecipitated with anti-mGlu7 antibody and western blotting was carried out with the indicated antibodies. Bar graph represents mean ± SEM (L-AP4, 2.36 ± 0.36; n = 3, *p<0.05, Student’s t-test). (H) HA-tagged K48 or K63 Ub was co-transfected with mGlu7 in HEK 293 T cells. The cells were treated with MG132 (10 μM) and leupeptin (52.5 μM) for 5 hr to inhibit the proteasomal and lysosomal degradation of the receptor. The cells were stimulated with L-Glu for 5 min before harvesting. K48- or K63-mediated ubiquitination levels were quantified after normalization to total ubiquitination levels. Bar graph below represents mGlu7 band intensities normalized to Ub-WT (means ± SEM; Ub-K48, 1.01 ± 0.19; Ub-K63, 1.02 ± 0.15; n = 6, n.s. indicates p>0.05, one-way ANOVA).

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Figure 1—figure supplement 1. Endogenous mGlu7 is ubiquitinated in cortical neurons. After cortical neurons were treated with 800 μM L-AP4 for 5 min, the lysates were immunoprecipitated with rabbit anti-mGlu7 antibody. Rabbit anti-GFP antibody was used as a negative control. Immunoprecipitates were separated by SDS-PAGE and analyzed by western blotting using the indicated antibodies.

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**Figure 2.** mGlu7 is ubiquitinated at lysine residues in both CT and iL2 domains. (A) A schematic diagram showing amino acid sequences of mGlu7 intracellular loop regions (iL1-3) and the cytoplasmic C-terminus tail (CT) of rat mGlu7a. The twelve lysine (K) residues in the CT and iL domains are displayed in red. (B) Myc-mGlu7 WT or sequential deletion mutants at the designated position were co-transfected with HA-Ub in HEK 293 T cells. Cell lysates were immunoprecipitated with anti-myc antibody and immunoblotted with anti-HA antibody. Diffuse bands of high molecular weight larger than 200 kDa represent ubiquitinated mGlu7. (C) Ubiquitination of mGlu7 WT or mutants in which the lysine (K) residues have been substituted to arginine (R) residues. Ubiquitination levels were analyzed as in Figure 1A after 5 min treatment of 1 mM L-Glu. CT 8K8R had all eight lysine residues in the mGlu7 CT mutated to arginines; IL 4K4R, all four lysine residues in the mGlu7 iL domains to arginines; 12K12R, all twelve lysine residues in the mGlu7 CT and iL domains to arginines. CT, C-terminal tail; iL, intracellular loop. Bar graph below represents mean ± SEM showing quantification of ubiquitination levels in the mutants normalized to WT (CT 8K8R, 1.09 ± 0.08; iL 4K4R, 0.97 ± 0.34; 12K12R, 0.11 ± 0.04; n = 4, **p<0.01, n.s. indicates p>0.05, one-way ANOVA). (D) Ubiquitination of mGlu7 in the intracellular loops in HEK 293 T cells. mGlu7 Δ857 does not harbor any lysine residues in the CT due to the stop codon at amino acid position 857. mGlu7 Δ857 IL 6K688/689R represents two lysine residues in the iL2 are mutated to arginines. mGlu7 Δ857/IL 4K4R or mGlu7 Δ857/IL K688/689R are mutants that combine Δ857 and IL 4K4R or IL K688/689R mutations, respectively. Bar graph below represents mean ± SEM of band intensities normalized to WT (Δ857, 0.97 ± 0.29; IL 4K4R, 0.93 ± 0.17; Δ857/IL 4K4R, 0.8 ± 0.02; Δ857/IL K688/689R, 0.17 ± 0.06; n = 4, **p<0.01, n.s. indicates p>0.05, one-way ANOVA).

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Figure 3. β-arrestin 1 (β-ar1), β-arrestin 2 (β-ar2), and Nedd4 are recruited to mGlu7 by agonist stimulation within 5 min. (A–F) Time-course interaction among mGlu7, Nedd4, and β-arrestins. HEK 293 T cells were co-transfected with FLAG-mGlu7, HA-Nedd4, and β-arrestin 1-GFP (A) or β-arrestin 2-GFP (B). In panel E, myc-mGlu7, HA-Nedd4, and β-arrestin 1-FLAG were utilized. L-Glu (1 mM) was administered for the indicated time before cell lysis. Cell lysates were immunoprecipitated with anti-FLAG antibody and western blotting was carried out with the indicated antibodies. Time-course binding of β-arrestins to mGlu7 (C), Nedd4 to mGlu7 (D), β-arrestin 1 to Nedd4 (F) were quantified and presented as mean ± SEM (β-ar1, 5 min, 1.79 ± 0.31; 15 min, 1.11 ± 0.23; 30 min, 0.73 ± 0.12; 60 min, 0.52 ± 0.17; β-ar2, 5 min, 1.36 ± 0.10; 15 min, 1.36 ± 0.15; 30 min, 1.01 ± 0.18; 60 min, 0.96 ± 0.38; D, β-ar1, 5 min, 2.36 ± 0.57; 15 min, 1.16 ± 0.31; 30 min, 0.61 ± 0.14; 60 min, 0.72 ± 0.49; β-ar2, 5 min, 2.20 ± 0.36; 15 min, 1.97 ± 0.68; 30 min, 1.96 ± 0.62; 60 min, 1.83 ± 0.49; F, β-ar1, 1 min, 1.67 ± 0.12; 10 min, 1.72 ± 0.19; 30 min, 1.11 ± 0.17; 120 min, 0.71 ± 0.13; n = 3–4, *p<0.05 versus 0 min, Student’s t-test). (G) Endogenous β-arrestin 1, Nedd4, and mGlu7 are present in the same complex. After cortical neurons were treated with 400 μM L-AP4 for 5 min, the lysates were immunoprecipitated with anti-β-arrestin 1 antibody or anti-GFP antibody as a control. Arrow and arrowhead indicate β-arrestin 1 and immunoglobulin heavy chain (IgH), respectively. (H) Bar graph represents mean ± SEM showing quantification of mGlu7 or Nedd4 binding levels to β-arrestin 1 (mGlu7 L-AP4, 1.56 ± 0.19; Nedd4 L-AP4, 1.46 ± 0.13; n = 5, *p<0.05 versus vehicle, Student’s t-test). (I) myc-mGlu7 and HA-Nedd4 were co-transfected either with pSuper β-arrestin 1, 2 shRNA or pSuper control shRNA in HEK 293 T cells. Three days after transfection, 1 mM L-Glu was treated for 5 min and cell lysates were immunoprecipitated using anti-myc antibody. Immunoprecipitates were resolved by SDS-PAGE and analyzed by western blotting using the indicated antibodies. (J) Bar graph represents mean ± SEM showing quantification of Nedd4 binding to mGlu7 levels normalized to untreated Ctrl KD (mGlu7 + L-AP4, 1.56 ± 0.19; Nedd4 L-AP4, 1.46 ± 0.13; n = 5, *p<0.05 versus vehicle, Student’s t-test). (K) β-arrestins are involved in agonist-induced Nedd4 recruitment to mGlu7 in neurons. Cultured cortical neurons were infected with lentiviruses harboring β-arrestin 1 or 2 shRNA (β-ar1 or 2 KD), or non-related target shRNA (Ctrl KD) for 7 days. Both β-arrestin 1 and 2 shRNA were co-infected for β-ar1/2 KD lane. After treatment with 400 μM L-AP4 for 5 min, the lysates were immunoprecipitated with anti-mGlu7 antibody and endogenous Nedd4 binding was analyzed by western blotting. (L) Bar graph represents mean ± SEM showing quantification Figure 3 continued on next page
of Nedd4 binding levels to mGlu7 (Ctl KD + L-AP4, 1.54 ± 0.11; β-arr1 KD + L-AP4, 1.99 ± 0.29; β-arr2 KD + L-AP4, 1.73 ± 0.16; β-arr1/2 KD, 1.15 ± 0.07; n = 4, *p<0.05 versus vehicle in Ctl KD, Student’s t-test). (M) β-arrestins mediate agonist-induced ubiquitination of mGlu7 in neurons. Cultured cortical neurons were infected with β-arrestins shRNA lentiviruses and then incubated with L-AP4 (400 μM) as panel K. Cell lysates were immunoprecipitated with anti-ubiquitin antibody (FK2) and western blotting was performed with anti-mGlu7 antibody. (N) Bar graph represents mean ± SEM showing mGlu7 ubiquitination levels normalized to Ctl KD (β-arr1 KD, 0.77 ± 0.06; β-arr2 KD, 0.74 ± 0.08; β-arr1/2 KD, 0.50 ± 0.03; n = 4, **p<0.01, *p<0.05, Student’s t-test). DOI: https://doi.org/10.7554/eLife.44502.007
Figure 3—figure supplement 1. Binding of β-arrestins to mGlu7. GFP-tagged β-arrestin 1 or 2 was co-transfected with either FLAG-tagged mGlu7 or FLAG-tagged β2AR in HEK 293 T cells. The lysates were immunoprecipitated with anti-FLAG antibody and western blotting was carried out with the indicated antibodies.
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Figure 3—figure supplement 2. The binding affinity of Nedd4 and β-arrestin 1 to mGlu7 is not altered by ubiquitination site mutations of mGlu7. mGlu7 WT, IL 4K4R, Ct 8K8R, or 12K12R was expressed on cultured cortical neurons via lentivirus-mediated transduction. After treatment with 400 μM L-AP4 for 5 min, co-immunoprecipitation assay was carried out using anti-myc antibody. Arrow and arrowhead indicate β-arrestin 1 and immunoglobulin heavy chain (IgH), respectively. Bar graph in the right panel represents mean ± SEM showing quantification of Nedd4 or β-arrestin 1 binding levels in the mutants normalized to WT (Nedd4, CT 8K8R, 1.05 ± 0.03; IL 4K4R, 1.04 ± 0.10; 12K12R, 1.05 ± 0.14; β-arrestin 1, CT 8K8R, 0.85 ± 0.09; IL 4K4R, 1.05 ± 0.18; 12K12R, 1.13 ± 0.15; n = 3, n.s. indicates p>0.05, Student’s t-test).

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Figure 3—figure supplement 3. Co-immunoprecipitation assay to supplement Figures 3K and L. Bar graph represents mean ± SEM showing quantification of binding affinity between Nedd4 and mGlu7 normalized to Ctl KD (β-arr1/2 KD, 0.84 ± 0.23; Ctl KD + L-AP4, 1.73 ± 0.14; β-arr1/2 KD + L-AP4, 0.74 ± 0.06; n = 3, *p<0.05 versus vehicle in Ctl KD, n.s. indicates p>0.05, Student’s t-test).

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Figure 3—figure supplement 4. Ubiquitination assay to supplement Figures 3M and N. Bar graph represents mean ± SEM showing mGlu7 ubiquitination levels normalized to Ctl KD (β-arr1/2 KD, 1.15 ± 0.09; Ctl KD + L-AP4, 1.42 ± 0.07; β-arr1/2 KD + L-AP4, 1.06 ± 0.09; n = 3, *p<0.05 versus vehicle in Ctl KD, n.s. indicates p>0.05, Student’s t-test).
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Figure 4. Mapping of binding domains between mGlu7 and Nedd4 or β-arrestins. (A) GST pull-down experiments to identify the interaction domains of mGlu7 with β-arrestin 1 or Nedd4. Total rat brain extract was subjected to a pull-down assay using immobilized GST fusion proteins containing the mGlu7 IL 1–3 domains, mGlu7a CT, or mGlu7b CT. After washing, bound proteins were analyzed by SDS-PAGE and western blotting using the indicated antibodies. The amount of GST fusion protein as bait was verified by Coomassie Brilliant Blue (CBB) staining. (B) Direct binding assay between mGlu7 and Nedd4 or β-arrestins. Recombinant His-tagged Nedd4, β-arrestin 1, or β-arrestin 2 proteins were purified using Ni-NTA resin. The purified proteins were incubated at 4°C for 2 hr with immobilized GST-mGlu7 IL or CT domains. After washing, bound proteins were analyzed by SDS-PAGE and western blotting using the indicated antibodies. (C) Mapping of binding domain of Nedd4 to mGlu7. Schematic diagrams of Nedd4 where each domain has been deleted are shown. ΔC2, amino acids (aa) 1–151; ΔC2-WW1/2, aa 1–380; ΔWW3, aa 381–454; ΔWW4, aa 455–506; ΔHECT, aa 507–900; Δ denotes deletion. (D) mGlu7 binds to the HECT domain of Nedd4. myc-mGlu7 was co-transfected with the indicated FLAG-Nedd4 domain deletion constructs in HEK 293 T cells. Immunoprecipitation was carried out with anti-myc antibody, and bound proteins were detected with anti-FLAG antibody.

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Figure 5. β-arrestins and Nedd4 regulate endocytosis of mGlu7 in neurons. (A) The endocytosis of mGlu7 was analyzed by an antibody uptake internalization assay. myc-mGlu7 was co-transfected with Nedd4 WT, C867S mutant, or vector control (Vec) in cultured hippocampal neurons. Two days after transfection, neurons were labeled with anti-myc antibody for 10 min, and returned to conditioned media for 15 min at 37°C. Neurons were fixed and incubated with Alexa Fluor 568-conjugated secondary antibody (red) to label surface-expressed receptors before permeabilization. After permeabilization with 0.25% Trion X-100 for 5 min, neurons were then incubated with Alexa Fluor 488-conjugated secondary antibody (green) to label the internalized receptors. Merged images are presented in which the red signal represents the surface mGlu7 and the green signal represents the internalized mGlu7. Scale bar, 20 μm. (B) Summary histograms quantifying the internalized mGlu7 from panel A are present as the ratio of the internalized population compared with total (surface + internalized) population measured using Metamorph software. Scatter plots show mean ± SEM (Vec, 1.00 ± 0.04; Nedd4 WT, 1.23 ± 0.04; Nedd4 C867S, 1.02 ± 0.04; n > 35, **p<0.01, one-way ANOVA). (C) myc-mGlu7 was co-transfected with pSuper-Ctl shRNA (Ctl KD) or pSuper-Nedd4 shRNA (Nedd4 KD) in cultured hippocampal neurons. Internalization of mGlu7 was analyzed in the absence or presence of 400 μM L-AP4 for 15 min at 37°C. (D) Summary histograms quantifying the internalized mGlu7 from panel C. Scatter plots show mean ± SEM (Vec, 1.00 ± 0.06; Vec + L-AP4, 1.39 ± 0.14; Nedd4 KD, 0.86 ± 0.06; Nedd4 KD + L-AP4, 0.94 ± 0.09; n > 10, *p<0.05, n.s. indicates p>0.05, one-way ANOVA). (E) myc-mGlu7 and β-arrestin 1 or 2 were co-expressed and the internalized mGlu7 was analyzed as above. (F) Summary histograms quantifying the internalized mGlu7 from panel E. Scatter plots show mean ± SEM (Vec, 1.00 ± 0.05; β-arrestin 1, 1.26 ± 0.07; β-arrestin 2, 1.30 ± 0.07; n > 30, **p<0.01, one-way ANOVA).

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Ubiquitination is required for endocytosis of endogenous mGlu7. Cortical neurons were treated with 0.1 μM MLN7243 for 18 hr to inhibit ubiquitination. After treatment with 800 μM L-AP4 for 15 min, surface biotinylation assay was performed. Treatment of MLN7243 in cortical neurons resulted in increased surface expression and decreased ubiquitination of endogenous mGlu7.

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Figure 5—figure supplement 1. Ubiquitination is required for endocytosis of endogenous mGlu7. Cortical neurons were treated with 0.1 μM MLN7243 for 18 hr to inhibit ubiquitination. After treatment with 800 μM L-AP4 for 15 min, surface biotinylation assay was performed. Treatment of MLN7243 in cortical neurons resulted in increased surface expression and decreased ubiquitination of endogenous mGlu7.

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Figure 5—figure supplement 2. Separated images that supplement the merged images shown in Figure 5. Surface-expressed and internalized mGlu7 signals in Figures 5A, C and E were separated, and individual images were presented in A, B, and C, respectively.

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Figure 6. β-arrestins and Nedd4 regulate ERK and JNK signaling of mGlu7 in neurons. (A) Cultured hippocampal neurons were infected with control (Ctl KD), β-arrestin 1 (β-arr1 KD), β-arrestin 2 (β-arr2 KD), or Nedd4 KD shRNA lentiviruses for 7 days. At DIV 14, neurons were treated with 400 μM L-AP4 for 0, 5, 20 min, and neuronal lysates were separated by SDS-PAGE and probed using the indicated antibodies. (B) Bar graph represents mean ± SEM of pERK band intensities normalized to the control lane (Ctl KD, 5 min, 1.60 ± 0.20; 20 min, 1.34 ± 0.17; β-arr1 KD, 0 min, 1.02 ± 0.25; 5 min, 1.25 ± 0.28; 20 min, 0.92 ± 0.26; β-arr2 KD, 0 min, 0.89 ± 0.19; 5 min, 0.64 ± 0.10; 20 min, 0.53 ± 0.10; Nedd4 KD, 0 min, 0.78 ± 0.20; 5 min, 0.58 ± 0.12; 20 min, 0.56 ± 0.11; n = 8, *p<0.05, n.s. indicates p>0.05, Student’s t-test). (C) Bar graph represents mean ± SEM of pJNK band intensities normalized to the control lane (Ctl KD 5 min, 1.48 ± 0.14; 20 min, 1.36 ± 0.19; β-arr1 KD 0 min, 1.52 ± 0.21; 5 min, 1.77 ± 0.24; 20 min, 1.50 ± 0.19; β-arr2 KD 0 min, 2.15 ± 0.33; 5 min, 2.03 ± 0.36; 20 min, 2.19 ± 0.41; Nedd4 KD 0 min, 1.83 ± 0.32; 5 min, 2.07 ± 0.36; 20 min, 1.92 ± 0.33; n = 7, *p<0.05, n.s. indicates p>0.05, Student’s t-test).

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Figure 7. Nedd4-mediated or agonist-stimulated mGlu7 degradation occurs via both the proteasomal and lysosomal degradation pathways. (A) myc-mGlu7 and FLAG-Nedd4 WT or C867S were co-transfected in HEK 293 T cells. Thirty-six hours after transfection, cell lysates were analyzed by western blotting using the indicated antibodies. (B) Following transfection, cells were starved overnight in serum-free DMEM culture medium and then treated with 1 mM L-Glu for 1 hr. Cell lysates were analyzed by western blotting using the indicated antibodies. (C) HEK 293 T cells transiently expressing myc-tagged mGlu7 was incubated with 100 μM MG132 or 52.5 μM leupeptin (Leu) for 2 hr and then L-Glu was added for 1 hr. Cell lysates were analyzed by SDS-PAGE and western blotting using the indicated antibodies. (D) Summary histograms quantifying mGlu7 expression in panel C. Bar graph represents mean ± SEM of band intensities normalized to control lane (DMSO + L-Glu, 0.74 ± 0.09; MG132, 1.15 ± 0.11; MG132 + L-Glu, 1.14 ± 0.15; Leu, 1.01 ± 0.12; Leu + L-Glu, 1.23 ± 0.18; n = 5, *p<0.05, n.s. indicates p>0.05, Student’s t-test). (E) FLAG-tagged Nedd4 WT or Nedd4 C867S was expressed on cultured cortical neurons via lentiviruses. After treatment with 0.5 μg/ml cycloheximide for 21 hr, the neurons were incubated with 100 μM MG132 or 52.5 μM Leu for 4 hr and then 400 μM L-AP4 was added for 1 hr. Cell lysates were analyzed by western blotting using the indicated antibodies. (F) Summary histograms quantifying mGlu7 expression in panel E. Bar graph represents mean ± SEM of band intensities normalized to control lane (Nedd4 C867S, 0.98 ± 0.07; Nedd4 WT, 0.81 ± 0.09; Nedd4 WT + MG132, 1.06 ± 0.13; Nedd4 WT + Leu, 1.00 ± 0.13; n = 5, *p<0.05, n.s. indicates p>0.05, Student’s t-test).

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