SUPPLEMENTAL MATERIALS AND METHODS

Primary cultures of rat hippocampal neurons

For primary cultures of rat hippocampal neurons, hippocampi were extracted from rat embryos at E18 and deposited in trypsinization solution (0.05% trypsin, 40 µg/mL DNase I in HBSS). The tissue was incubated for 15 minutes at 37°C. Subsequently, the trypsin was inactivated with an adhesion medium (DMEM, 10% horse serum and 100 U/mL penicillin/streptomycin). The tissue was gently dissociated with Pasteur pipettes, and the obtained cells were counted in a Neubauer chamber. 350,000 cells were seeded onto a 60mm plate with 10 covers coated with poly-lysine. After 2 hours, the medium was changed to Neurobasal medium supplemented with Glutamax, B27, and antibiotics.

Biotinylation

Cell surface biotinylation was performed in wild-type and *OCRL* KO human i3 neurons differentiated for 21 days. Cells were washed with ice-cold PBS and then biotinylated using a solution of 0.2 mg/mL biotin (Thermo Fisher, #21335) at 4°C for 15 min. Subsequently, biotin was quenched by incubating the cells twice with 50 mM Tris pH 7.5 and 100 mM NaCl for 10 min on ice. The cells were then lysed with lysis buffer containing PBS, 2% Triton-X100, and protease inhibitors. After centrifugation, the lysates were incubated in rotation at 4°C for 2 h with streptavidin beads (Pierce, #20349) washed with lysis buffer. The beads were washed three times, and any remaining wash buffer was removed before resuspending the beads in the loading buffer. The biotinylated proteins were separated using SDS-PAGE followed by a western blot.

qPCR

RNA extraction and qPCR assay were performed as described (Caracci et al., 2024). Human i3 neurons were differentiated for 21 days on poly-L-ornithine-coated 6-well plates (4x105 cells/well). Total RNA was extracted in RNAse-free conditions using TRIzol reagent (Invitrogen) and 1 µg of RNA was reverse transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific #K1622). Real-time PCR was performed in a QuantStudio 3 Real-Time-PCR-System (Applied Biosystems, Thermo Fisher Scientific) using Hot FirePol Evagreen qPCR Mix, ROX (Solis Biodyne, #08-24-00001) with 200nM of ApoER2 primers. The qPCR conditions were denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The expression levels of ApoER2 were normalized to GAPDH expression using the delta–delta Cq method ($2^{-\Delta\Delta Cq}$).

Supplemental Table S1: Western blot Antibodies

Antibody	Host	Dilution	Catalog	Source
OCRL-1	Rabbit	1:1500	-	Polyclonal antibody against human OCRL was described before (Vicinanza et al., 2011)[21]
α-Tubulin	Rat	1:20000	ab6160	Abcam
β-actin-HRP	Mouse	1:20000	ab49900	Abcam
ApoER2	Rabbit	1:5000	A3481	Sigma-Aldrich
Phospho-Akt (Ser473)	Rabbit	1:1000	OMA103061	Thermo Fisher
Akt (pan)	Mouse	1:1000	2920S	Cell Signalling
Phospho-p44/42 MAPK	Rabbit	1:2000	9101S	Cell Signalling
ERK (pan)	Mouse	1:1000	610124	BD Biosciences

Supplemental Table S2: Immunofluorescence Antibodies

Antibody	Host	Dilution	Catalog	Source
EEA1	Mouse	1:500	610456	BD Biosciences
Lamp1	Mouse	1:500	H4A3	DSHB
HA	Chicken	1:500	AB3254	Millipore
HA	Rabbit	1:1000	C29F4	Cell Signalling
ApoER2	Rabbit	1:3000	A3481	Sigma-Aldrich
MAP2	Chicken	1:5000	ab5392	Abcam
Ankyrin-G	Mouse	1:100	sc12719	Santa Cruz
TfR	Mouse	1:500	G1/221/12	DSHB
SNX17	Rabbit	1:100	HPA043867	Protein Atlas
VPS26	Rabbit	1:1000	1280-1-AP	Proteintech
GM130	Mouse	1:500	610822	BD Biosciences
Phalloidin-Alexa Fluor 647	-	1:1000	A22287	Thermo Fisher
HA-Alexa Fluor 488	Goat	1:100	A21287	Thermo Fisher
Anti-mouse-IgG-Alexa Fluor 647	Goat	1:1000	A21235	Thermo Fisher
Anti-mouse-IgG-Alexa Fluor 555	Donkey	1:1000	A31570	Thermo Fisher
Anti-chicken-IgG-Alexa Fluor 647	Goat	1:1000	A21436	Thermo Fisher
Anti-chicken-IgG-Alexa Fluor 488	Goat	1:1000	A11039	Thermo Fisher
Anti-rabbit-IgG-Alexa Fluor 555	Donkey	1:1000	A31572	Thermo Fisher

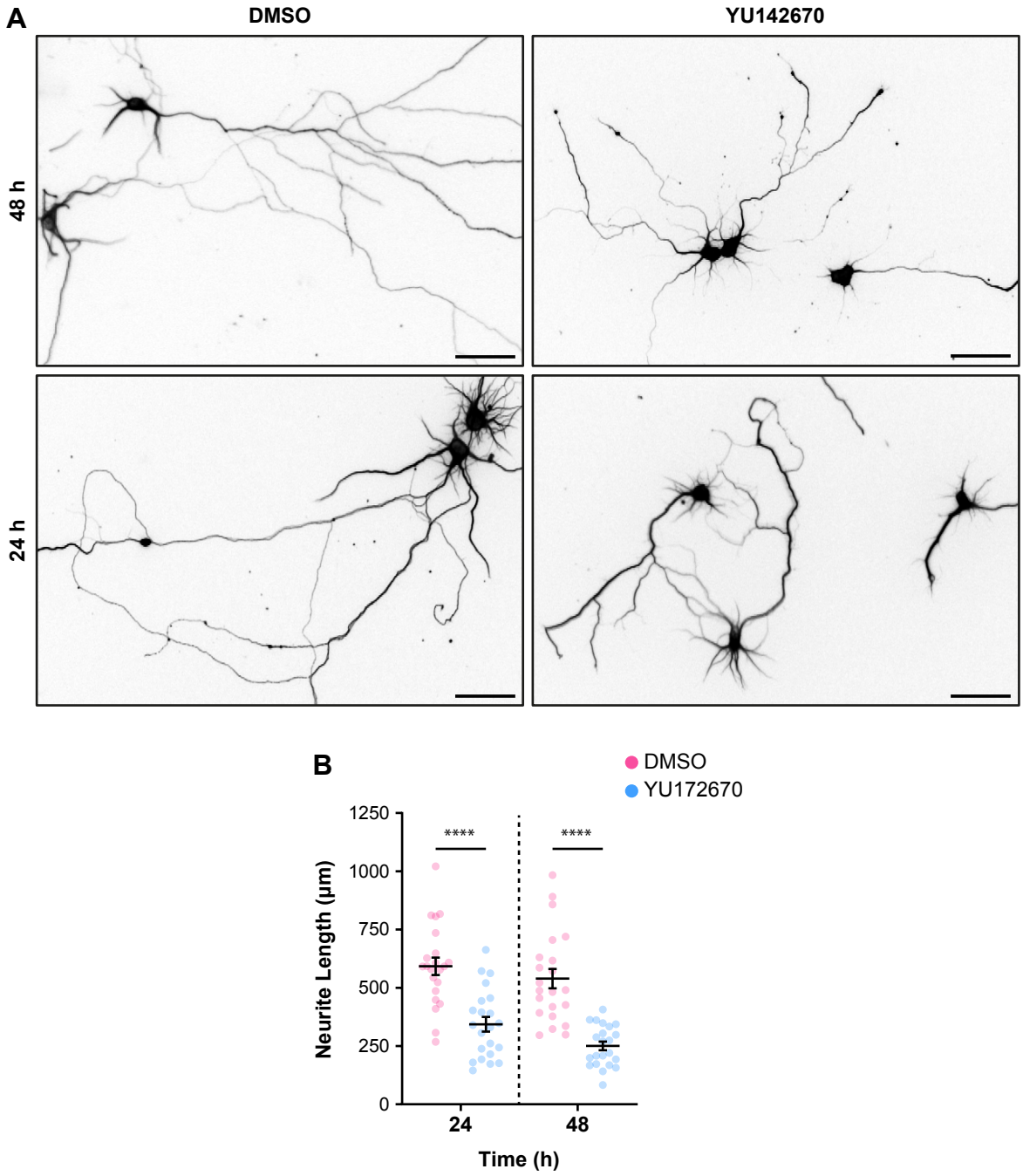

Figure S1. Pharmacological inhibition of OCRL1 in rat hippocampal neurons reduces neurite length. Primary cultures of rat embryonic (E18) hippocampal neurons were seeded on coverslips coated with poly-L-lysine. (**A**) After 3 days in vitro, neurons were treated with DMSO or 50uM YU1426701 for 24 or 48 h, fixed and stained for βIII tubulin. Images were captured using a wild-field microscope and analyzed with Fiji. Scale bar = 50 µm. (**B**) Neurite length was measured from βIII tubulin signal using Neuron J. Data from 22 neurons cells from 3 experiments were analyzed, and statistical significance was calculated using ANOVA with Sidak's multiple comparisons test. **** $p < 0.0001$.

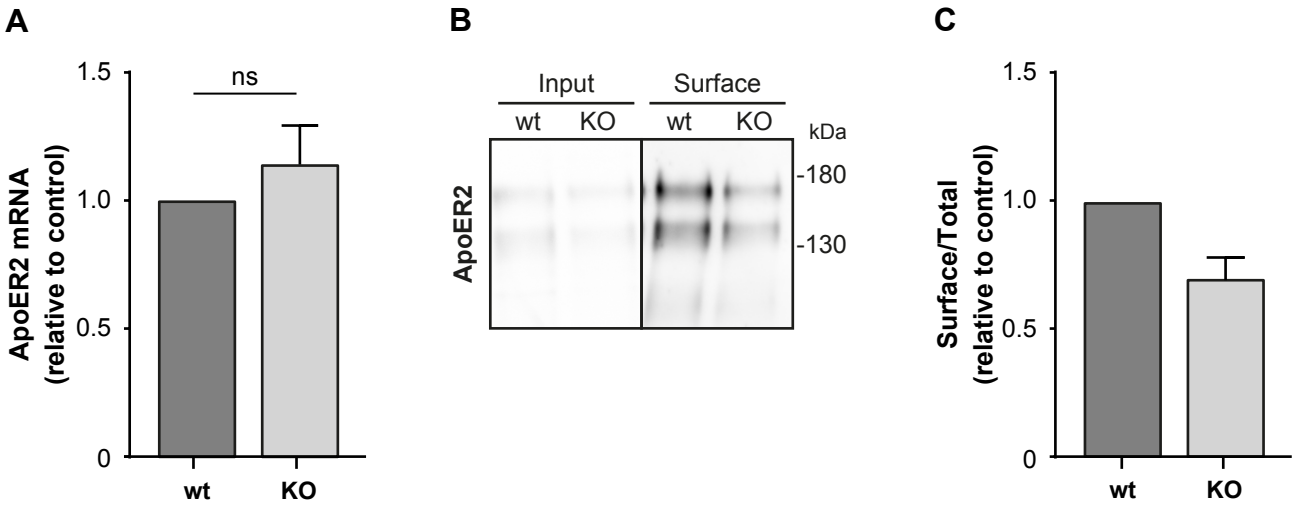

Figure S2. mRNA and surface ApoER2 levels in *OCRL* KO i3 neurons. (**A**) Relative expressions of ApoER2 mRNA from wt and *OCRL* KO i3 neurons differentiated for 21 days. Expression levels were normalized to GAPDH expression using the delta-delta Cq method (2-ΔΔCq). Data are presented as the mean±SEM, n=3, Mann-Whitney t-test.Biotinylation was performed after 21 days of differentiation. (**B)** i3 neurons (wt or *OCRL* KO) were biotinylated at 4ºC for 15 min. Proteins bound to biotin were separated with streptavidin beads. Input and Surface proteins were analyzed by western blot. (**C**) The signal from ApoER2 was measured with Fiji, and the ratio of surface vs input was determined and normalized to wt. Since only two experiments were performed, no statistic analysis was calculated.

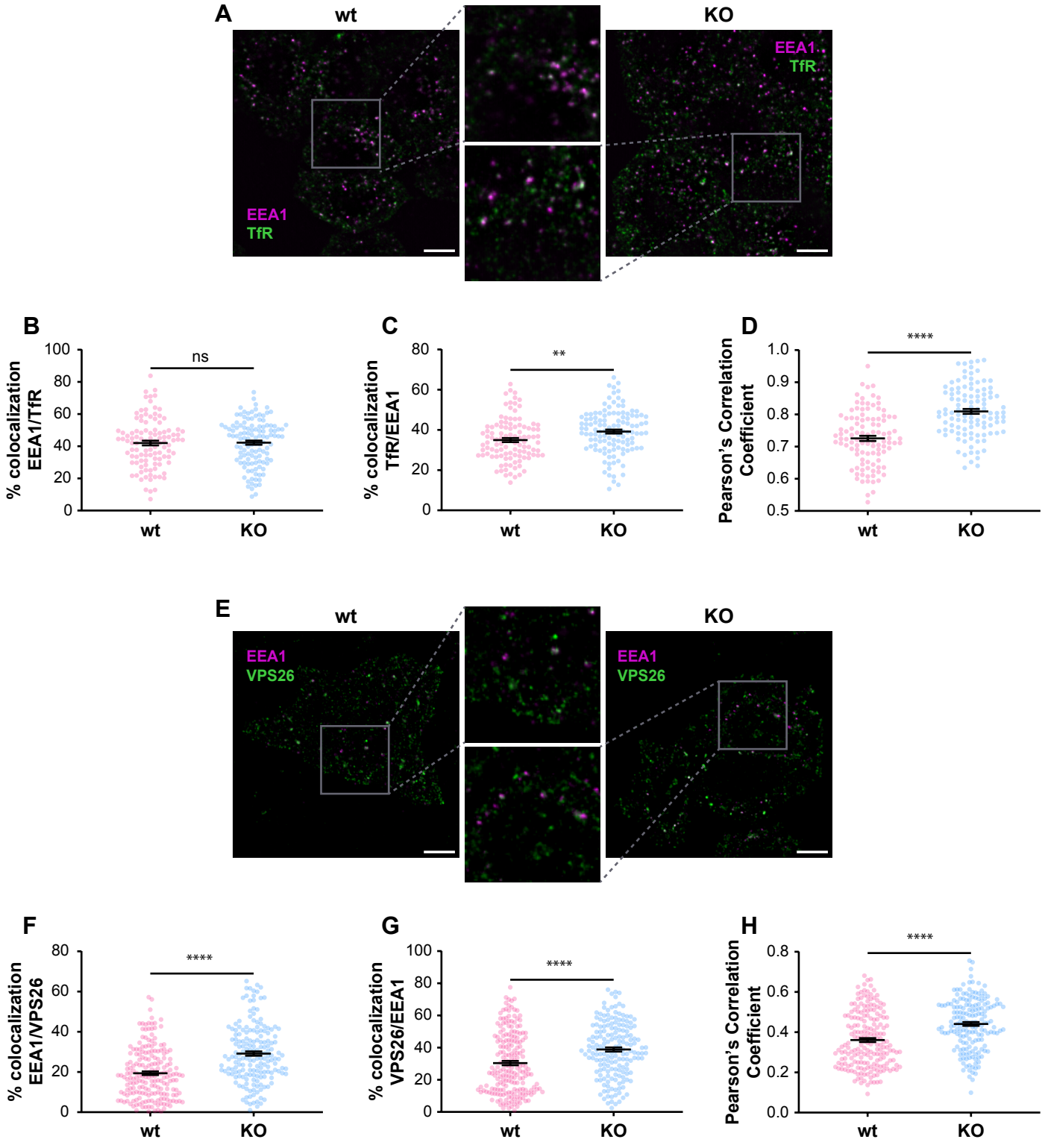

Figure S3. Loss of OCRL1 disturbs intracellular traffic. H4 cells were fixed, and stained for EEA1 (magenta) and indicated proteins (green): (**A**) Transferrin Receptor (TfR); (**E**) VPS26. Confocal images were deconvolved and analyzed with ImageJ. Scale bar indicates 10 µm. (**B, C, F, G**) Manders coefficients were analyzed for EEA1 and each protein (corresponding images above). (**D, H**) Pearson's Correlation Coefficient (PCC) were analyzed for EEA1 and each protein (corresponding images above). Error lines represent mean±SEM. t-student's test was calculated from 100 cells from 2 experiment. ns $p > 0.05$, ** $p < 0.01$, **** $p < 0.0001$.