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Stimulation of mGluR1/5 Improves Defective Internalization of AMPA Receptors in NPC1 Mutant Mouse

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Abstract

Niemann–Pick type C1 (NPC1) disease is characterized by neurodegeneration caused by cholesterol accumulation in the late endosome/lysosome. In this study, a defective basal and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-stimulated internalization of GluR2-containing AMPA receptors in NPC1\textsuperscript{−/−} cortical neurons was detected. Our results show that the amount of cholesterol and group I metabotropic glutamate receptors (mGluR1/5) in lipid rafts of NPC1\textsuperscript{−/−} cortical tissue and neurons are decreased and their downstream signals of p-ERK are defective, which are restored by a rebalance of cholesterol homeostasis through β-cyclodextrin (β-CD) treatment. Application of 3,5-dihydroxyphenylglycine (DHPG)—a mGluR1/5 agonist—and β-CD markedly increases the internalization of AMPA receptors and decreases over-influx of calcium in NPC1\textsuperscript{−/−} neurons, respectively. Furthermore, the defective phosphorylated GluR2 and protein kinase C signals are ameliorated by the treatment with DHPG and β-CD, respectively, suggesting an involvement of them in internalization dysfunction. Taken together, our data imply that abnormal internalization of AMPA receptors is a critical mechanism for neuronal dysfunction and the correction of dysfunctional mGluR1/5 is a potential therapeutic strategy for NPC1 disease.

Key words: β-CD, calcium imaging, GluR2, lipid raft, Niemann–Pick type C1 disease
Introduction

Niemann–Pick type C (NPC) disease is a rare neurodegenerative lysosomal storage disorder caused by mutations in NPC1 (95% of patients) or NPC2 gene (5%), leading to intracellular accumulation of unesterified cholesterol in the late endosome/lysosome (LE/LY) (Carstea et al. 1997; Xie et al. 1999; Naureckiene et al. 2000). The clinical manifestations of NPC are varied in the central nervous system (CNS) with typically neurological or psychiatric symptoms, including ataxia, tremor, dystonia, motor control problems, and seizures (Sevin et al. 2007). Although NPC1 disease has been studied for decades, the detail mechanism of the neurological disorder is not fully understood.

Previous studies confirmed an increased excitability in the hippocampus and cortex in NPC1 mutant mice (D’Arcangelo et al. 2011; Avchalumov et al. 2012). Upon a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) stimulation, NPC1 mutant (NPC1−/−) neurons have a higher and more persistent spike amplitude accompanied by an up-regulated calcium influx (D’Arcangelo et al. 2011), suggesting that postsynaptic receptors, especially AMPA receptors, are involved in abnormal neurological manifestations in NPC1 disease. AMPA receptors mediate the majority of fast excitatory transmission, and their trafficking plays a critical role in synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) (Malinow and Malenka 2002). Therefore, it is interesting to study whether the dynamics of AMPA receptors are defective in NPC1−/− neurons.

Unesterified cholesterol is highly accumulated in the LE/LY of NPC1−/− neurons, but the whole cholesterol content in neurons is unaltered compared with wild type (WT) (Karten et al. 2002; Wojtanik and Liscum 2003; Hawes et al. 2010). The cholesterol distribution on the neuronal membrane, especially in distal axons and dendrites, is dramatically reduced in NPC1−/− neurons (Karten et al. 2002; Hawes et al. 2010). As a principle element in lipid rafts, cholesterol is highly related to the function of lipid rafts (Silvius 2003). Interestingly, the dynamics of AMPA receptors located in the postsynaptic membrane are regulated by a balance of cholesterol in lipid rafts. Alteration of the cholesterol content in lipid rafts affects the stability and internalization of AMPA receptors (Hering et al. 2003; Allen et al. 2007; Martin et al. 2014). However, the relationship between the cholesterol balance and AMPA receptor trafficking in lipid rafts is not clearly elucidated yet.

Recent evidence demonstrates that the dynamics of AMPA receptors can be regulated by group I metabotropic glutamate receptors (mGluR1/5) (Snyder et al. 2001; Xiao et al. 2001). As a type of G-protein-coupled receptors, mGluR1/5 express broadly in the CNS and are critical for activity-dependent synaptic plasticity, for example, LTP and LTD induction (Conn and Pin 1997; Xiao et al. 2001; Ireland and Abraham 2002). Activation of mGluR1/5 by their specific agonists 3,5-dihydroxyphenylglycine (DHPG) increases internalization of AMPA receptors, but application of their antagonists blocks their dynamics (Nakamoto et al. 2007). Furthermore, some researches have proven that the cholesterol content in lipid rafts modulates its association with mGluR1/5 relying on the cholesterol-binding domain and affects their activities, for example, the affinity of mGluR4 with glutamate in Drosophila (Eroglu et al. 2003) and their downstream extracellular signal regulated kinase/mitogen activated protein kinase (ERK-MAPK) signaling (Kumari et al. 2013).

In this study, we investigated whether the decrease of cholesterol in lipid rafts affects the function of mGluR1/5, resulting in defective internalization of AMPA receptors in NPC1 disease by using NPC1−/− mice as well as in vitro primary neuron culture. Our data show that the defective internalization of GluR2-containing AMPA receptors is related to the dysfunction of mGluR1/5 induced by a decrease of cholesterol level in lipid rafts. Applications of DHPG and β-cyclodextrin (β-CD) restore the internalization of AMPA receptors and over-influx of calcium in NPC1−/− neurons, respectively, in which the effect of β-CD is blocked by mGluR1/5 antagonists. Taken together, our results reveal a relationship between the internalization of AMPA receptors and functions of mGluR1/5, which is regulated by the cholesterol level in lipid rafts, and provide a potential therapeutic target for disease treatment in NPC1 patients.

Materials and Methods

Chemicals

The chemicals of (RS)-AMPA, DHPG, 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP), and (S)-(−)-a-amino-4-carboxy-2-methylbenzenecarboxylic acid (LY 367385) were purchased from Tocris Bioscience. Fura-2-AM (F1221), Neurobasal-A Medium, B-27 Supplement, Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), 1-glutamine, and penicillin–streptomycin were obtained from Thermo Fisher. Hibernate-A medium was purchased from BrainBits. OptiPrep Density Gradient Medium, DNase I, poly-γ-lysine hydrobromide (PDL), and β-CD (H107), were purchased from Sigma. Papain suspension was obtained from Worthington.

Animals

Heterozygous mice (BALB/cNctr-Npc1min/+), obtained from the Jackson Laboratory, were used to generate NPC1 homozygous (NPC1−/−) and WT (NPC1+/+) mice for experiments. Mice were kept under the controlled and standard conditions (free access to food and water at 22 °C, 12:12 h day:light cycle). This study was carried out in accordance with the recommendations of the German legislation on protection of animals and the Committee on the Ethics of Animal Experiments at the University of Rostock. The protocol was approved by the Landesamt für Landwirtschaft, Lebensmittelsicherung und Fischerei Mecklenburg-Vorpommern (LALLF M-V/7ST/7221.3-1.1-011/16).

Genotyping

For mouse genotyping, the 2-mm tails of mice were cut at postnatal day (P) 1 for the neuron culture or at P6 for lipid raft isolation and Western blots and placed into PCR tubes, then 75 μl lysis buffer (containing 25 mM NaOH and 0.2 mM ethylenediaminetetraacetic acid [EDTA], pH 12) was added for incubation at 95 °C for 30 min. After cooled to room temperature, the homogenates were neutralized by the neutralizing buffer (containing 40 mM Tris-HCl, pH 5.0). The mixture of 2 μl as template was taken together with two pairs of primers for PCR performance (Truett et al. 2000). The PCR primers for NPC1−/− mice (5′-GTCTCTGGACAGCCAACTGA-3′ and 5′-TGACCACCAGCATACTCG-3′) with a 475 bp product and for NPC1+/+ mice (5′-TCTCACAGCCACACAGTCCTC-3′ and 5′-CTCTAGGCTCATCCTGCACTG-3′) with a 173 bp were used.

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Pharmacological Treatment
NPC1+/+ and NPC1−/− mice were injected weekly with β-CD (4000 mg/kg in Ringer’s solution) by intraperitoneal injection from P7 onward as previously described (Meyer et al. 2017). The NPC1+/+ and NPC1−/− mice treated with Ringer’s solution at the same condition were used as controls (Ctrl).

Production of Astrocyte-Conditioned Medium
Astrocyte-conditioned medium was prepared as the protocol described previously (Yu et al. 2012; Feng et al. 2018). Briefly, cortices of P5 brains were separated and digested with papain (0.3% in DMEM at 37 °C for 20 min). After DMEM and DNase I were added, the cortices were triturated and centrifuged at 300 × g for 5 min. The pellets were resuspended with DMEM (containing 20% FBS) and cultured under 8% CO2 at 37 °C for 16 h. After centrifugation at 300 × g for 5 min, the supernatant was collected as the conditioned medium.

Primary Neuron Culture
Primary neuron culture was performed according to the previously described protocol (Brewer and Torricelli 2007). In brief, cortices from P1 mice were rapidly dissected from brains and digested with papain (0.2% in Hibernate-A medium for 30 min). After being digested, the suspension was added on the top of a 30% Optiprep layer in a 50% Optiprep cushion (Sigma, St. Louis, MO) for 20 min. After centrifugation, the supernatant was collected after the 30% Optiprep layer. The neurons were collected from the fractions in the gradient layers by washing with Hibernate-A medium (containing 1% B27). The neurons were plated on PDL-coated coverslips with a density of 300–400 cells/mm² for immunocytochemistry and filipin staining or 800 cells/mm² for Western blot analysis in astrocyte-conditioned medium with 8% CO2 at 37 °C. Half of the medium was changed twice per week during the culture.

AMPA Receptor Internalization Assay
The internalization assay of AMPA receptors was performed as previously described protocol (Carrodus et al. 2014). Briefly, living cortical neurons at DIV13 were incubated with anti-GluR2 antibody (10 μg/mL; MAB397, Millipore) at 37 °C for 20 min to label the N-terminal extracellular domain of GluR2. After Triton X-100 was added into the suspension, the suspension was added on the top of a 30% Optiprep layer in a 50% Optiprep cushion (Sigma, St. Louis, MO) for 20 min. After centrifugation, the supernatant was collected after the 30% Optiprep layer. The neurons were collected from the fractions in the gradient layers by washing with Hibernate-A medium (containing 1% B27). The neurons were plated on PDL-coated coverslips with a density of 300–400 cells/mm² for immunocytochemistry and filipin staining or 800 cells/mm² for Western blot analysis in astrocyte-conditioned medium with 8% CO2 at 37 °C. Half of the medium was changed twice per week during the culture.

AMPAR Receptor Internalization Assay
Images of all coverslips were obtained by the LSM780 confocal microscope with an Alexa 488: excitation wave/emission wave—488/522, Cy3: excitation wave/emission wave—561/626. For quantification, the middle images generated by z-series from at least 10 neurons of each coverslip were randomly chosen for analysis. The average fluorescence intensity of membranous and cytosolic GluR2 was measured in the same region of interest through Image J software (NIH). The internalization was evaluated by the ratio between the green and red fluorescence intensity, and the total fluorescence intensity was indicated by the sum of the green and red fluorescence intensity after normalized to control.

Lipid Raft Isolation and Cholesterol Detection
Lipid rafts were isolated from cortices of mice at different postnatal days or neurons according to the protocol described previously (Lemaire-Vieille et al. 2013). Briefly, after homogenized with glass tissue grinders, the cortices or neurons were extracted in ice-cold lysis buffer (150 mM NaCl, 25 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% Triton X-100) for 2 h. After detection of the concentration of homogenates, Triton X-100 was added into the homogenate with a proper ratio of 5:1 (5 μL Triton X-100 with 1× protein in weight) for maximum solubilization (Ostermeyer et al. 1999). Then, 1 mL extraction was mixed with 2 mL 60% OptiPrep and loaded on the bottom of transparent tubes (#344500; Beckman Coulter). Furthermore, 60% OptiPrep was diluted by gradient buffer (150 mM NaCl, 25 mM Tris-HCl, and 5 mM EDTA) to 30% and 5%. Next step, 6 mL 30% and 2.5 mL 5% OptiPrep were loaded on the top of the mixture, followed by a centrifugation (100,000 × g; Rotor: SW40Ti; ultracentrifuge: Optima L80) at 4 °C for 24 h. The total 11 fractions (each 1 mL) were collected gently from the top to bottom. Same volume of each fraction was analyzed by Western blots.

To quantify the concentration of cholesterol from each fraction in lipid rafts or non-raft fractions, Amplex Red Cholesterol Assay kit (Thermo Fisher) was utilized according to the instructions of the manufacturer. Protein concentrations of fractions were determined by BCA Protein Assay Kit (Pierce).

Western Blot Analysis
The tissue and neurons were homogenized in radioimmunoprecipitation assay buffer (20 mM Tris, 137 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% natriumdeoxycholate, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM ethyleneglycol-bis(2-aminoethylether)-N,N,N’,N’-tetra acetic acid, 1 mM Na2O, 25 mM natriumpyrophosphate, protease, and phosphatase inhibitors) and then separated by 4–15% SDS gel (Criterion TGX Precast Protein Gels; Bio-Rad), followed by transferred to nitrocellulose membranes. After blocked with 3% bovine serum albumin (BSA) in 0.1% tris-buffered saline with Tween 20 (TBST), the membranes were incubated overnight at 4 °C with primary mouse anti-GluR2 (MAB397; 1:1000, Abcam), anti-ERK (#4696; 1:1000, Cell Signaling Technology), anti-mGluR1 (#610964; 1:1000), anti-β-TrCP1 (21C9C5; 1:1000, Cell Signaling Technology), anti-p-ERK (#9101s; 1:1000, Cell Signaling Technology), anti-p-GluR2 (ab52180; 1:1000, Abcam), and anti-phospho-(Ser) protein kinase C (PKC) substrate (#2261,
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D'Arcangelo et al. 2011). In brief, neurons (DIV13) were washed three times with warm HEPES buffer (incubated at 37°C; 151 mM NaCl, 1.25 mM NaH2PO4, 10 mM HEPES, 2.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, pH 7.4 with NaOH). Then, the cells were loaded with 5 μM Fura-2-AM in HEPES buffer (made from a 5 mM Fura-2-AM stock solution in DMSO) at 37°C for 30 min in the dark, followed by three times washing and 40-min incubation with HEPES buffer in the dark for de-esterification of the Fura-2-AM. The coverslips were then placed in an Axioskop2-Fsmot microscope (Carl Zeiss AG) and superfused with HEPES buffer at room temperature. Fluorescent recordings were performed at a frequency of 1 Hz using the software TillVision (v4.5.56; TILL Photonics GmbH) in combination with a Polychrome V (TILL Photonics GmbH) and a SensiCam camera (PCO AG). Excitation wavelength at 340 nm for calcium-bound Fura-2 and at 380 nm for calcium-free Fura-2 was used, and emission was measured at a wavelength of 510 nm. During the recordings, combinations of the following substances were applied via the HEPES buffer superfusing the cells, respectively: 1 μM thapsigargin, 100 μM AMPA, 100 μM DHPG, 10 μM MPEP, and 100 μM LY367385. The ratio of the fluorescence intensities at 340 and 380 nm (after background correction) was calculated. Mean fluorescent ratios over time were calculated from single recordings under the same conditions and visualized as an overlay using GraphPad Prism 7 (GraphPad Software Inc.) for comparison. Area under the curve (AUC) was calculated for the comparison of the calcium concentration with Excel (Microsoft).

Statistical Analysis
Data were analyzed by GraphPad 7 (GraphPad Software Inc.) and represented as mean ± SEM. Data were analyzed with two-tailed unpaired Student’s t-test between two groups and with two-way ANOVA among three or more groups. Post hoc statistics were carried on using Tukey’s or Bonferroni’s multiple comparison tests. The detail information of statistical analyses was listed in Supplementary Table 1. P value less than 0.05 was considered as statistical significant.

Results
Defective Internalization of AMPA Receptors in NPC1−/− Cortical Neurons
To verify the hypothesis that abnormal cholesterol accumulation in NPC1−/− neurons induces the defective internalization of AMPA receptors, we utilized GluR2 as a marker to trace the spatiotemporal dynamics of GluR2-containing AMPA receptors due to its wide co-assembling into AMPA receptors in the cortex (Jonas and Burnashev 1995; Isaac et al. 2007). As the first step, we measured GluR2 protein in the cortex tissue at different stages and in cultivated cortical neurons of the NPC1−/− mouse. The results showed no significant difference in the amount of GluR2 protein between WT and NPC1−/− mice (Fig. 1A,B). Furthermore, to analyze internalization of GluR2-containing AMPA receptors, an internalization assay with the dual-color fluorescent immunostaining technique for labeling the same protein in living cells (Lin et al. 2000) was utilized to study the basal and AMPA-stimulated GluR2 internalization by comparing the fluorescence intensities between the membrane surface and cytosol in cortical neurons. Under the basal condition, we found increased membranous/cytosolic GluR2 ratios in NPC1−/− neuronal bodies and dendrites compared with WT Ctrl (Fig. 1C,E). Upon AMPA stimulation, which increases AMPA receptor endocytosis (Hering et al. 2003), the internalization of GluR2 in neuronal bodies and dendrites increased dramatically compared with basal conditions, still the membranous/cytosolic ratios between NPC1−/− and WT in neuronal bodies and dendrites were significantly different (Fig. 1D,E). Of note, the total fluorescence intensity of membranous and cytosolic GluR2 between NPC1−/− and WT neurons in the basal and AMPA-stimulated states showed no alteration (Fig. 1F), in consistent with the results of Western blots (Fig. 1A,B). Taken together, our data suggest that GluR2 protein level in NPC1−/− neurons is not changed when compared with WT, but its internalization is defective in both basal and AMPA-stimulated states in NPC1−/−.

Cholesterol Disturbance in Lipid Rafts Affects Distribution and Function of mGluR1/5 in NPC1−/− Mice
Next step, we further investigated the possible mechanisms involved in the defective internalization of GluR2. The dynamics of AMPA receptors are highly modulated by mGluR1/5 (Snyder et al. 2001; Xiao et al. 2001), which are located mainly in postsynaptic regions (Nusser et al. 2003). Therefore, considering the disturbed cholesterol balance in NPC1 disease, we hypothesized that mGluR1/5 functions in NPC1−/− neurons are influenced by the alteration of cholesterol content in lipid rafts, which accounts for the defective internalization of GluR2. Interestingly, the amounts of mGluR1/5 proteins in the cortex tissue and cultivated neurons were not different between NPC1−/− and WT mice (Fig. 2A–C). In line with previous reports, the amount of mGluR1 in neurons was really weak (Shigemoto et al. 1992; Romano et al. 1995; Ferraguti et al. 1998; Lopez-Bendito et al. 2002). To identify whether the association of mGluR1/5 with lipid rafts is affected, lipid rafts from the NPC1−/− cortex at different stages and cultivated neurons were isolated and fractionated. While total cholesterol remained unchanged (Fig. 2E), the ratio of cholesterol content between the lipid raft fractions (caveolin-1-positive fraction 3 and 4 in Fig. 2D) and non-raft fractions (TFR-positive fraction 8–11 in Fig. 2D) was diminished at P35 and...
Figure 1. Defective internalization of GluR2-containing AMPA receptors in NPC1<sup>−/−</sup> cortical neurons. (A, B) Western blots analyses (A) and quantification (B) of GluR2 in the cortex of WT and NPC1<sup>−/−</sup> mice at P9, P35, and P65 and of WT and NPC1<sup>−/−</sup> cortical neurons. GAPDH is used as a loading Ctrl (n = 3 mice/group). n.s., no significance (unpaired Student’s t-test). (C, D) Representative images of the basal internalization (C) and AMPA-stimulated internalization (D) of GluR2 in WT and NPC1<sup>−/−</sup> cortical neurons (DIV13). Membranous GluR2 is indicated by green, cytosolic GluR2 by red, and nuclei with DAPI in blue. Scale bar: 5 μm. (E, F) Quantification of GluR2 internalization as indicated by the ratio between membranous (green) and cytosolic (red) fluorescence intensity of GluR2 (E) and of normalized total fluorescence intensity of membranous (green) and cytosolic (red) GluR2 (F) in the basal and AMPA-stimulated internalization (100 μM AMPA at 30 min) in WT and NPC1<sup>−/−</sup> neuronal bodies (body) or dendrites (dendrite) (DIV13). n = 9 culture preparations from 9 WT/NPC1<sup>−/−</sup> mice, respectively. n.s., no significance. ***P < 0.001 (two-way ANOVA).

P65 cortical tissues and cultivated neurons compared with WT (Fig. 2F), suggesting that cholesterol accumulation in the LE/LY in NPC1<sup>−/−</sup> mice induces less amount of cholesterol in lipid rafts located in the neuronal membrane.

In order to test whether the amount of mGluR1/5 in lipid rafts is altered, we examined the protein ratios of mGluR1/5 in lipid rafts and non-lipid rafts. In cortical tissue, the ratio changed similarly to the alteration of cholesterol contents, for example, not changed at P9 but down-regulated at P35 and more severely at P65 when compared with WT (Fig. 2G,H for mGluR1; Fig. 2G,I for mGluR5), while caveolin-1 and TfR had no change (Fig. 2J,K). However, due to the abundantly expressed mGluR5 but poorly expressed mGluR1 in cortical neurons (Fig. 2A), we only observed the decreased mGluR5 in cultured neuronal lipid rafts (Fig. 2G,J) and no alteration in levels of caveolin-1 and TfR (Fig. 2J,K), which is consistent with the findings in the cortical tissue.

β-CD is an effective drug to treat neurodegeneration and prolong life span in NPC1<sup>−/−</sup> mice (Davidson et al. 2009; Liu et al. 2009; Ramirez et al. 2010) based on replacing the functions...
Figure 2. Decreased amount of cholesterol and mGluR1/5 in lipid rafts in NPC1−/− mice. (A–C) Western blot analyses (A) and quantification of mGluR1 (B) and mGluR5 (C) in the cortex tissue of P9, P35, and P65 (n = 3 mice/group), and cortical neurons (DIV13; n = 3 culture preparations of WT/NPC1−/− mice, respectively) between WT and NPC1−/− mice. n.s., no significance (unpaired Student's t-test). (D) Representative Western blots of 11 fractions isolated from the cortex of WT mice at P9 by ultracentrifugation. Lipid raft fractions (LR, fractions 3 and 4) are indicated by lipid raft marker (caveolin-1) and non-lipid raft fractions (non-LR, fractions 8–11) by TfR. (E, F) The total cholesterol amount (E) and the amount of cholesterol in lipid raft fractions indicated by the ratio of the cholesterol between LR and non-LR (F) at P9, P35, and P65 mice (n = 3 mice/group) and cortical neurons (DIV13; n = 3 culture preparations from 3 WT/NPC1−/− mice, respectively). n.s., no significance (unpaired Student's t-test). (G–K) Western blot analyses (G) and quantification of mGluR1 (H) and mGluR5 (I) (indicated by the ratios of LR and non-LR fractions), and quantification of caveolin-1 (J) and TfR (K) (as loading Ctrl) in LR and non-LR fractions of the cortex of P9, P35, and P65 mice (n = 3 mice/group) and cortical neurons (DIV13; n = 3 culture preparations from 3 WT/NPC1−/− mice, respectively). In (G), to compare protein differences, the fractions of the same order from WT and NPC1−/− were loaded in parallel. n.s., no significance. *P < 0.05, **P < 0.01 (unpaired Student's t-test).
Interestingly, phosphorylated ERK1/2 (Thr-202/Tyr-204) were shown to be involved in the regulation of mGluR1/5 function also affects ERK–MAPK signaling.

Since the defective internalization of AMPA receptors in NPC1−/− mice was found (Fig. 3C), we further investigated whether β-CD regulates the internalization of AMPA receptors in both bodies and dendrites of NPC1−/− neurons but not in WT; however, both were blocked by the antagonists of mGluR1—LY367385 and mGluR5—MPEP, respectively (Fig. 6A,C). Underlying AMPA stimulation, the treatment of β-CD also increased internalization of AMPA receptors in bodies and dendrites of NPC1−/− neurons but still not in WT neurons, which were both partially blocked by MPEP/LY367385 (Fig. 6B,D), suggesting that β-CD can restore the defective internalization of NPC1−/− neurons.

### Stimulation of mGluR1/5 Improves Defective Internalization of AMPA Receptors

Since previous studies reported that stimulation of mGluR1/5 up-regulated the internalization of AMPA receptors in hippocampal neurons (Snyder et al. 2001; Xiao et al. 2001), we further investigated whether the reduced internalization of GluR2-containing AMPA receptors is related to mGluR1/5 in NPC1 mice. We applied the specific mGluR1/5 agonist DHPG with a concentration of 100 μM to cultivated cortical neurons for different incubation time to investigate its effect on internalization of AMPA receptors. Our results demonstrated that upon DHPG stimulation, the internalization of GluR2-containing AMPA receptors in the basolateral state of the NPC1−/− neurons is significantly improved, as indicated by a decreased ratio between the membranous and cytosolic GluR2 fluorescence intensity when compared with untreated NPC1−/− neurons (Fig. 5A,C,D). Of note, the ratios of membranous/cytosolic GluR2 fluorescence intensity were not changed (Fig. 5A,D,E).

### β-CD Improves Defective Internalization of AMPA Receptors by Partially Modulating mGluR1/5

Since β-CD can modulate the internalization of AMPA receptors in neurons (Hering et al. 2003), we further investigated whether β-CD regulates the internalization of AMPA receptors in NPC1−/− neurons via modulating mGluR1/5. In the basal state, the β-CD treatment increased internalization of AMPA receptors in both bodies and dendrites of NPC1−/− neurons but not in WT; however, both were blocked by the antagonists of mGluR1—LY367385 and mGluR5—MPEP, respectively (Fig. 6A,C). Underlying AMPA stimulation, the treatment of β-CD also increased internalization of AMPA receptors in bodies and dendrites of NPC1−/− neurons but still not in WT neurons, which were both partially blocked by MPEP/LY367385 (Fig. 6B,D), suggesting that β-CD can restore the defective internalization of NPC1−/− neurons in basal and AMPA-stimulated states, which is at least partially mediated through mGluR1/5 function.

### Up-regulated Calcium Influx into NPC1−/− Neurons Is Restored via Modulating mGluR1/5

AMPA receptors play a critical role in modulating synaptic transmission (Collingridge et al. 1983) and cytosolic calcium balance (Liu and Zukin 2007; Joshi et al. 2011). Therefore, we examined intracellular calcium in cultivated cortical neurons (DIV13) utilizing Fura-2-AM. After equilibration, the baseline value ([Ca2+]i) of the defective internalization of AMPA receptors in NPC1−/− neurons was lower than that in WT neurons when thapsigargin was added into the bath buffer (2–12 min). Furthermore, the stimulation with AMPA (100 μM; from 12 to 32 min in Fig. 7A) induced a quick and high release of calcium lasting for a long period in both NPC1−/− and WT neurons. However, the cytosolic calcium concentration ([Ca2+]i) in NPC1−/− neurons was higher than that in WT neurons upon AMPA stimulation (Fig. 7A,C,D), in line with the data published before (D’Arcangelo et al. 2011), suggesting that more AMPA receptors were blocked in the membrane of NPC1−/− neurons under the stimulation, resulting in stronger calcium influx. Moreover, when DHPG together with AMPA was applied into the bath buffer, the [Ca2+]i in NPC1−/− was restored to the normal WT level (Fig. 7A,C,D), while there is no difference in WT neurons in the condition with or without DHPG (Fig. 7A,C,D), supporting that DHPG can restore the defective internalization of AMPA receptors, resulting in the lower calcium influx into NPC1−/− neurons.
we further investigated whether application of β-CD can also restore the increased calcium influx in NPC1−/− neurons. After treatment with β-CD and/or MPEP/LY367385, the \([\text{Ca}^{2+}]\) of cells were measured. When without AMPA stimulation (2–12 min in Fig. 7E), the cellular \([\text{Ca}^{2+}]\), between the treated and untreated Ctrl was not different (Fig. 7E, H) and also in thapsigargin-induced calcium release from the internal store (Fig. 7E, F). However, upon AMPA stimulation (12–32 min), in NPC1−/− neurons, the calcium influx in β-CD-treated cells was restored to the WT level (Fig. 7E, G, H), which was partially blocked by the incubation with MPEP/LY367385 (Fig. 7E, G, H). In WT neurons, the calcium influx was not changed, but MPEP/LY367385 treatment led to a higher calcium influx (Fig. 7E, G, H). Taken together, the results demonstrate that the defective internalization of AMPA receptors leads to an over-influx of calcium into NPC1−/− neurons upon AMPA stimulation, which can be restored by DHPG or β-CD. Of note, the recovery function of β-CD can be partially inhibited by MPEP/LY367385, suggesting that at least the effect of β-CD is partially mediated by regulation of mGluR1/5.

**The Mechanism Involved in Regulation of mGluR1/5 for Defective Internalization of AMPA Receptors**

In order to investigate the downstream signals involved in defective internalization of GluR2 in NPC1−/− neurons, we examined phosphorylation of GluR2 at Ser880 position (p-GluR2) based on
The amount of p-GluR2 was down-regulated in NPC1−/− both WT and NPC1−/− demonstrated that DHPG treatment increased p-GluR2 level in the effect of DHPG on p-GluR2 was also investigated, which et al. 2004; Park et al. 2009). Western blot analyses showed that cultivated neurons (DIV13; PKC is inhibited in NPC1 disease (Walter et al. 2009; Tamari et al. 2013; Peter et al. 2017).

In this study, the relationship and mechanism between abnormal accumulation of cholesterol and defective synaptic plasticity in NPC1 disease was investigated. Our data showed that the internalization of GluR2-containing AMPA receptors is disturbed in NPC1−/− neurons (Fig. 1), which is correlated with the downregulated distribution in lipid rafts and dysfunction of mGluR1/5 (Figs 2–4). Stimulation of mGluR1/5 dramatically increases internalization of AMPA receptors and decreases the over-influx of calcium in NPC1−/− neurons (Figs 5 and 7). Furthermore, β-CD treatment improves internalization of AMPA receptors and calcium balance via mGluR1/5 (Figs 6 and 7). Finally, PKC and p-

GluR2, as the downstream signals, participate in the defective internalization of GluR2-containing AMPA receptors (Fig. 8). Our study fulfills the missing gap between cholesterol imbalance and the aberrant postsynaptic receptor dynamics, revealing the defective internalization of postsynaptic receptors as a new insight for discovering aberrant synaptic plasticity in NPC1 disease.

AMPA receptors play an essential role for brain functions, for example, memory, learning, and cognition, and mediate the majority of fast excitatory neurotransmission in the CNS (Henley and Wilkinson 2013). Dynamic regulation of AMPA receptors at synapses induces the long-lasting changing of synaptic strength (Luscher et al. 1999), for example, LTP and LTD (Chater and Goda 2014). The defective internalization of AMPA receptors in NPC1−/− neurons may explain the sustained activation found in the hippocampus and cortex (D’Arcangelo et al. 2011; Avchalumov et al. 2012). However, a few studies reported an impaired synaptic transmission in both glutamatergic and GABAergic synapses in NPC1−/− mice (Xu et al. 2010, 2011; Feng et al. 2018), mainly induced by the defective vesicle exocytosis at the presynapse (Karten et al. 2006; Xu et al. 2010). Therefore, the aberrant synaptic plasticity in NPC1 disease is a complicated process involving the presynapse and/or postsynapse.

In this study, GluR2 was used as a target to study the dynamics of AMPA receptors based on its assembling into most AMPA receptors compared with other subunits (GluR1, GluR3, or GluR4) (Jonas and Burnashev 1995). Although GluR2-containing AMPA receptors are characterized being Ca2+ impermeable (Hollmann et al. 1991; Verdoorn et al. 1991; Burnashev et al. 1992), over-influx of calcium into NPC1−/− neurons upon AMPA stimulation suggests that the defective internalization is a general defect, in which not only GluR2-containing AMPA receptors but also other
Stimulation of mGluR1/5 by DHPG increases internalization of GluR2-containing AMPA receptors. (A, B) Representative images of neuron bodies and dendrites in the basal (A) and AMPA-stimulated (100 μM, 30 min) internalization (B) of GluR2 in WT and NPC1<sup>−/−</sup> cortical neurons with or without DHPG treatment (100 μM, 30 min). Membranous GluR2 is indicated by green, cytosolic GluR2 by red, and nuclear by DAPI as blue. Scale bar: 5 μm. (C, D) The quantification of GluR2 internalization as indicated by the ratio between surficial and cytosolic fluorescence intensity of GluR2 in the basal state of neuronal bodies (C) and dendrites (D) (DIV13) upon DHPG treatment (100 μM, at 5, 15, and 30 min). (n = 4 culture preparations from 4 WT/NPC1<sup>−/−</sup> mice, respectively). **P < 0.05, ***P < 0.001 (two-way ANOVA). (E, F) Quantification of GluR2 internalization as indicated by the ratio between surficial and cytosolic fluorescence intensity of GluR2 in neuronal bodies (E) and dendrites (F) upon AMPA (100 μM) and DHPG (100 μM) treatment for 30 min (DIV13) (n = 3 culture preparations from 3 WT/NPC1<sup>−/−</sup> mice, respectively). n.s., no significance. **P < 0.01, ***P < 0.001 (two-way ANOVA).

This may be due to the use of the different neuronal types and with different AMPA concentration and incubation time. Moreover, β-CD was utilized to restore the cholesterol homeostasis through intraperitoneal injection in NPC1<sup>−/−</sup> mice, which increases the amounts of cholesterol and mGluR1/5 in lipid rafts and improves the defective internalization of AMPA receptors. Even though β-CD doubles life span and restores cholesterol level in nearly all tissues and organs to WT levels.
Figure 6. β-CD improves the defective internalization of AMPA receptors by modulating mGluR1/5. (A, B) Representative images of neuron bodies and dendrites in the base (A) and AMPA-stimulated (100 μM, 30 min) internalization (B) with or without β-CD and/or MPEP (MP) and LY367385 (LY36) treatment. β-CD: 0.2 mM incubated for 48 h; MPEP: 10 μM for 72 h; LY367385: 100 μM for 72 h. Membranous GluR2 is indicated by green, cytosolic GluR2 by red, and nuclear by DAPI as blue. Scale bar: 5 μm.

(C, D) The quantification of GluR2 internalization as indicated by the ratio between membranous and cytosolic fluorescence intensity of GluR2 in neuronal bodies and dendrites in the base state (C) and AMPA-stimulated (100 μM) state (D) with or without β-CD and/or MP and LY36 treatment (DIV13; n = 3 culture preparations from 3 WT/NPC1−/− mice each). β-CD: 0.2 mM, 48 h; MPEP: 10 mM, 72 h; LY367385: 100 μM, 72 h. n.s., no significance. *P < 0.05, **P < 0.01, ***P < 0.001 (two-way ANOVA).
Figure 7. Restoring the up-regulated calcium influx into NPC1−/− neurons under the AMPA stimulation via modulating mGluR1/5. (A) Quantification of calcium concentration measured by 340/380 ratio with Fura-2 in WT and NPC1−/− neurons with or without DHPG (100 μM) treatment. 0–2 min: equalization time; 2–12 min: basal recording with the application of thapsigargin (1 μM); 12–32 min: AMPA-stimulated (100 μM) recording with or without DHPG treatment. Black color, WT neurons; red color, NPC1−/− neurons; brown color, WT neurons treated with DHPG; green color, NPC1−/− neurons treated with DHPG. (B) The quantitation of the AUC of the thapsigargin-induced peak (in A) and of the AMPA-induced peak (2 in A) with or without DHPG (n = 3 culture preparations from 3 WT/NPC1−/− mice, respectively). n.s., no significance.  *P < 0.05, **P < 0.01 (two-way ANOVA). (C) Representative calcium images labeled as “a” and “b” in (A). Scale bar: 50 μm. (D) The quantification of calcium concentration measured by 340/380 ratio with Fura-2/AM in WT and NPC1−/− neurons with or without β-CD and MPEP (MP) and LY367385 (LY36) treatment. 0–2 min: equalization time; 2–12 min: basal recording with the application of thapsigargin; 12–32 min: AMPA-stimulated recording with or without β-CD and MP/LY36 treatment. AMPA: 100 μM; β-CD: 0.2 mM incubated for 48 h; MPEP: 10 μM for 72 h; LY367385: 100 μM for 72 h. Different color indicates distinct states. Black color, WT neurons; red color, NPC1−/− neurons; brown color, WT neurons treated with β-CD; green color, NPC1−/− neurons treated with β-CD; blue color, WT neurons treated with β-CD and MP/LY36; purple color, NPC1−/− neurons treated with β-CD and MP/LY36. (E-G) The quantitation of the AUC of the thapsigargin-induced peak (1 in E) and of the AMPA-induced peak (2 in E) with or without β-CD and MP/LY36 treatment (n = 3 culture preparations from 3 WT/NPC1−/− mice each). n.s., no significance.  *P < 0.05, **P < 0.01, ***P < 0.001 (two-way ANOVA). (H) Representative calcium images labeled as “a” and “b” in (E). Scale bar: 50 μm.

some studies reported that the neurodegeneration cannot be fully restored by β-CD treatment via intraperitoneal injection. This is most likely due to difficulty of β-CD crossing the blood–brain barrier (BBB) in adult mice (Liu et al. 2009, 2012). The recovery effect in our study with treatment from P7 until P65 is probably due to incomplete closure of the BBB during the early development, as it was suggested that β-CD crosses the BBB and reaches the brain in infant stage of mice (Liu 2012).

Defective cholesterol in lipid rafts of NPC1−/− mice (Fig. 2) can seriously affect functions of lipid rafts, such as membrane trafficking, actin cytoskeleton, and signal transduction (Simons and Toomre 2000; Simons 2002; Hering et al. 2003). Lipid rafts bind various molecules to modulate the synaptic plasticity and can be served as a useful platform for discovering new candidates for the treatment of neurological diseases (Ehehalt et al. 2003; Hashimoto et al. 2003; Fortin et al. 2004; Lauren et al. 2009). Relying on their CRAC motif and cholesterol content in lipid rafts, mGluR1/5 in lipid rafts can adjust their downstream signals (Kumari et al. 2013). Therefore, mGlurR1/5 was selected as potential targets in NPC1 disease, and our data showed an important role of them on internalization of AMPA receptors in NPC1 disease. Moreover, mGlurR1/5 have been widely studied in other CNS disorders, for example, activation of mGluR1/5 protects neurons from excitotoxicity (Baskys et al. 2005); the removal of AMPA receptors from the postsynaptic membrane by mGluR activation induces LTD (Xiao et al. 2001) and eliminates spine formation (Hasegawa et al. 2015). Therefore, mGlurR1/5 possesses great potential for the treatment of NPC1 disease. However, mGlurR1 and mGlur5 have distinct functions in modulating synaptic activity (Calo et al. 2005; Zhang et al. 2008) and different expression patterns in various regions of the brain (Romano et al. 1995; Ferraguti et al. 2009).
Figure 8. Analyses of p-GluR2 and p-PKC substrates and possible mechanism of defective internalization of AMPA receptors in NPC1 disease. (A–C) Western blot analyses (A) and quantification of phosphorylated GluR2 (p-GluR2; B) and phosphorylated PKC (p-PKC) substrates (C) in WT and NPC1−/− neurons upon β-CD and DHPG treatment at DIV13 (A). The bands over 55 kDa were quantified as p-PKC substrates. Total GluR2 and GAPDH as the loading Ctrl (n = 3 culture preparations from 3 WT/NPC1−/− mice, respectively). n.s., no significance. **P < 0.01, ***P < 0.001 (two-way ANOVA). (D) Possible mechanism involved in defective internalization of AMPA receptors in NPC1 disease. The Npc1 mutation leads to aberrant accumulation of cholesterol in the LE/LY, resulting in decreasing cholesterol content in lipid rafts located in the postsynaptic neuronal membrane and triggering the disassociation of mGluR1/5 within lipid rafts, followed by deactivating the downstream PKC. The influenced PKC decreases the phosphorylation of GluR2 and affects the release of GluR2 from the complex ABP/GRIP (facilitating GluR2 stability in the neuronal membrane), resulting in defective internalization of GluR2.

1998; Lopez-Bendito et al. 2002); therefore, the precise regulation of individual mGluR1 and mGluR5 on synaptic activity in NPC1 disease should be further investigated.

In this study, we confirmed that the decreases of phosphorylated PKC substrates and p-GluR2, a downstream target of phosphorylated PKC, can be reversed through the stimulation upon mGluR1/5 by DHPG (Fig. 8A–C). Therefore, a possible mechanism involved in regulating internalization of GluR2-containing AMPA receptors in NPC1 disease is proposed (Fig. 8D). The Npc1 mutation leads to aberrant accumulation of cholesterol in the LE/LY, resulting in decreasing cholesterol content in lipid rafts located in the postsynaptic neuronal membrane and triggering the disassociation of mGluR1/5 within lipid rafts, followed by deactivating the downstream PKC (Mao et al. 2008). The deactivated PKC decreases the phosphorylation of GluR2, which affects the release of GluR2 from the complex AMPA receptor-binding protein/glutamate receptor-interacting protein (ABP/-GRIP; facilitating GluR2 stability in the neuronal membrane) (Lu and Ziff 2005), resulting in defective internalization of GluR2.

In summary, we first found the defective internalization of GluR2-containing AMPA receptors in NPC1−/− cortical neurons and confirmed mGluR1/5 as the candidates that are responsible for defective internalization. Furthermore, application of DHPG and β-CD improves the internalization and decreases the over-influx of calcium via modulating mGluR1/5. Finally, the defective phosphorylated GluR2 and PKC signals can also be improved by DHPG and β-CD, indicating an involvement of them in the internalization dysfunction. Therefore, we confirm that abnormal internalization of AMPA receptors is a critical mechanism for neuronal dysfunction and the use of drugs to target mGluR1/5 is a promising therapeutic strategy for NPC1 disease.

Supplementary Material
Supplementary Material is available at Cerebral Cortex online.

Author’s Contribution
X.F., F.Y., M.R., A.R., and J.L. designed the study; X.F. and F.Y. participated in the cortical neuron culture; X.F., M.R., and M.J.F. performed the calcium imaging experiment; X.F. performed the lipid rafts isolation; A.W., A.U.B., M.W., and A.G. performed the pharmacological treatment of NPC1 mutant mouse; X.F., Z.W.,
and M.R. perform the data acquisition and data analysis; X.F., M.R., Z.W., and J.L. drafted the manuscript; X.F., F.Y., M.R., Z.W., A.U.B., A.H., and J.L. helped editing the manuscript.

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References
Chater TE, Goda Y. 2014. The role of AMPA receptors in postsynaptic mechanisms of synaptic plasticity. Front Cell Neurosci. 8:401.

Joshi DC, Singh M, Krishnamurthy K, Joshi PG, Joshi NB. 2011. AMPA induced Ca\(^{2+}\) influx in motor neurons occurs through voltage gated Ca\(^{2+}\) channel and Ca\(^{2+}\) permeable AMPA receptor. Neurochem Int. 59:913–921.


Li Z, Ji GC, Neugebauer V. 2011. Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior. J Neurosci. 31:1114–1127.


Simons K. 2002. Lipid rafts and membrane trafficking. J Gen Physiol. 120:4A.


