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Research Article

Induction of Exocytosis Rescues Lysosomal GM2 Accumulation in Tay-Sachs Disease

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Introduction. The Tay-Sachs disease is a progressive neurodegenerative disorder that is caused by a genetic mutation in the HEXA gene coding the lysosomal α-subunit of β-hexosaminidase A. Currently, there is no effective treatment for Tay-Sachs. Induction of exocytosis as a potential treatment approach is suggested to restore lysosomal enlargement in several lysosomal storage diseases. Here, we aimed to test the therapeutic potential of two small molecules, δ-tocopherol and hydroxypropyl-β-cyclodextrin, in fibroblast and neuroglia cells derived from *Hexa*-/-*Neu3*-/- mice and Tay-Sachs patients. *Method*. The effect of two small molecules on lysosomal enlargement and GM2 accumulation in lysosomes was examined by LysoTracker staining and immunocytochemical colocalization analysis for GM2 and LAMP1. qRT-PCR and fluorometric enzyme assay were also used to investigate the effect of combined treatment on the level of neuraminidase 1, a negative regulator of exocytosis. *Results*. Single treatment with δ-tocopherol (5-40 μM) and hydroxypropyl-β-cyclodextrin (10-50 μM) for 48 hours led to significant induction of lysosomal exocytosis. We demonstrated that the combined treatment with δ-tocopherol (10 μM) and hydroxypropyl-β-cyclodextrin (25 μM) resulted in a significant reduction of lysosomal GM2 and downregulation of lysosomal Neu1 expression. *Conclusion*. In this study, we demonstrated that inducing exocytosis by δ-tocopherol and hydroxypropyl-β-cyclodextrin might have therapeutic potential to reduce GM2 storage and pathology in Tay-Sachs cells.

1. Introduction

The Tay-Says disease (TSD) which is known as GM2-gangliosidosis, type I is caused by mutations in the HexA gene coding the α subunit of lysosomal β-hexosaminidase A (HEXA). HEXA catalyzes GM2 to GM3 conversion in the ganglioside degradation pathway, and its deficiency causes GM2 accumulation, particularly in CNS. Accumulated GM2 in neurons leads to progressive neurodegeneration in affected children. The symptoms include developmental delay, muscle weakness, vision and hearing impairment, seizures, and feeding difficulties with eventual death at the age of four [1]. Although several approaches, like enzyme replacement therapy [2], substrate deprivation therapy [3–5], and gene therapy [6–8], are carried out *in vitro* and *in vivo* experimental setups, so far there is still no effective cure for TSD patients. Hexa deficient TSD mouse model (*Hexa*-/-) did not exhibit neurological symptoms that are observed in patients. In contrast to humans, a bypass pathway for GM2 degradation mediated by neuraminidase was suggested [9, 10]. The involvement of plasma membrane-associated neuraminidase Neu3 in the bypass pathway was demonstrated by generating a *Hexa*-/-*Neu3*-/- mouse model which displays abnormal GM2 ganglioside accumulation in the CNS. The *Hexa*-/-*Neu3*-/- mice were healthy at birth but died at nearly 5 months of age due to progressive neurodegeneration with neuronal loss, Purkinje cell depletion, and neuroinflammation. Therefore, the *Hexa*-/-*Neu3*-/- mice have been introduced as a novel murine model of TSD as it displays severe neuropathology similar to TSD patients [11].

Exocytosis is a cell transport mechanism that enables neurotransmitter, cytokine, and hormone secretion out of secretory cells to the extracellular environment [12]. In nonsecretory cells, exocytosis contributes to plasma membrane repair [13], bone resorption [14], cycling/recycling proteins to the plasma membrane [15], neurite outgrowth [16], and cellular clearance [17].

Exocytosis involves the fusion of secretory vesicles or lysosomes with the plasma membrane allowing expulsion of the contents during transient cytosolic Ca^{2+} increase. The induction of exocytosis might be used as a therapeutic approach to relieve lysosomal accumulation as lysosomal contents have been found in extracellular fluids, urine, and blood of lysosomal storage disease (LSD) patients [18].

Tocopherol and cyclodextrin have been reported to be used as therapeutic small drugs to alleviate lipid accumulation and recuperate enlarged lysosomes in several LSDs as they enhance lysosomal exocytosis through increment of cytosolic and lysosomal Ca^{2+} level [19]. The exact mechanism of action is unclear, but it was reported that tocopherol isomers evoked fast Ca^{2+} responses with an increase in cytosolic Ca^{2+} [20]. It was also shown that tocopherol caused exocytosis of related vesicles, such as endocytic ones [21]. Cyclodextrins, on the other hand, have been shown to create "holes" in membrane structures by extracting cholesterol from membranes, and these disruptions in the plasma membrane are normally repaired in cells by lysosomal exocytosis [22]. It was previously shown that the repair mechanism is calcium-dependent resulting in the extrusion of lysosomal contents [22] and also cholesterol exit from cells via exosomes [23]. Thus, similar to tocopherol, cyclodextrin also induces lysosomal exocytosis in Ca^{2+}-enhanced manner, but the exact mechanism remains unknown.

A bunch of studies have demonstrated that tocopherol and cyclodextrin displayed therapeutic effects like reduction in lysosome size, lysosome distribution closer to the plasma membrane, enhanced lysosomal enzyme activity in the extracellular medium on the induced pluripotent stem cell- (iPSC-) derived neural stem cells (NSCs) from NPA, NPC1, Wolman, Batten (CLN1 and CLN2), and TSD patients [19, 24–28]. Usage of tocopherol and cyclodextrin in combination displayed synergistic effect on lysosomal exocytosis in lysosomal storage disorders. Cyclodextrin alone faces challenges due to the high concentrations required, limited pharmacokinetics, and potential toxicity. However, the unique mechanisms of action of both compounds, when combined, showed a synergistic effect in reducing cholesterol accumulation in NPC1 neural stem cells [24]. This combination demonstrated comparable efficacy to a higher concentration of cyclodextrin alone, indicating a potential therapeutic strategy for the treatment of LSDs [29].

Here, we explored the potential of combined drug treatment to induce exocytosis mechanism in fibroblast and neuroglia cells derived from *Hexa-/-Neu3-/-* mice and TSD patients. We examined whether tocopherol and cyclodextrin have a potential effect on rescuing enlarged lysosomes and GM2 ganglioside accumulation observed in TSD cells. Our study suggested that induction of exocytosis enabled remission of lysosomal enlargement and reduced GM2 accumulation in lysosomes.

2. Materials and Methods

2.1. Materials

(i) δ-tocopherol (δ-T): Sigma, CAS Number: 119-13-1

(ii) 2-Hydroxypropyl-β-cyclodextrin (CD): Sigma, CAS Number:128446-35-5

2.2. Cell Culture and Treatments

2.2.1. Fibroblast Cell Lines. Mouse fibroblasts were obtained from a skin biopsy of *Hexa-/-* and *Hexa-/-Neu3-/-* mice at the age of 5 months. Fibroblasts were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% (vol/vol) penicillin/streptomycin (Gibco) in a humidified incubator with 5% CO_2 at 37°C. Human control and Tay-Sachs fibroblasts were kindly provided by Dr. Nur Arslan, Child Neurology Department, Dokuz Eylül University.

2.2.2. Neuroglia Cell (NG) Lines. Hexa-/- NG and *Hexa-/-Neu3-/-* NG cell lines were generated from 100 mg brain tissue of 5-month-old *Hexa-/-* and *Hexa-/-Neu3-/-* mice by using adult brain dissociation kit according to manufacturer's instructions (Miltenyi Biotec, 130-107-677). After cell debris and red blood cell removal were performed, neurons were isolated by a neuron isolation kit according to the manufacturer's instructions (Miltenyi Biotec, 130-115-390). Generated cell lines were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% (vol/vol) penicillin/streptomycin (Gibco) in a humidified incubator with 5% CO_2 at 37°C.

The immortalized human neuroglia cells (Ng-125) obtained from the Tay-Sachs patient [30] and control human neuroglia cells (Ng-124) including cDNA clone encoding the α subunit of Hex A (pCMV-Hex α) and producing active HexA enzyme [31] were obtained and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with (10% *v*/*v*) FBS and 1% (vol/vol) penicillin/streptomycin.

To determine the minimum efficient dosage, the cells were treated with tocopherol and cyclodextrin at different dosages (tocopherol: 5 μM, 10 μM, 20 μM, and 40 μM; cyclodextrin: 10 μM, 25 μM, 50 μM, and 167 μM) [19, 28] in serum-free media at 37°C for 24 h. On day 2, the medium was replaced with DMEM supplemented with 10% FBS and cyclodextrin or tocopherol followed by incubation at 37°C for another 24 h. LysoTracker staining enables visualization of enlarged lysosomes in cells. On the day of the experiment, cells were treated with 50 nM LysoTracker Red DND-99 dye (Invitrogen, L-7528) in empty DMEM containing 1% pen/strep and incubated at 37°C for 1 hour followed by removal of medium and washing with PBS. The cells were fixed with 4% paraformaldehyde for 30 min, and after washing with PBS, the cells were mounted on slides with Fluoroshield mounting medium DAPI (Abcam). Images were obtained by using a fluorescent microscope (Olympus BX53). Intensity analysis of red fluorescence was applied by using NIH ImageJ.

2.3. Immunocytochemical Analysis. The cells were grown on microslides on a 24-well plate for 24 hours and treated with tocopherol and cyclodextrin as described above. The cells were fixed with 4% paraformaldehyde for 30 min and incubated in blocking solution (10% goat serum, 4% BSA 0.3 M glycine, and 0.3% Triton X in 1× PBS) for 1 hour. The cells were stained with the primary antibodies (anti-GM2: 1 : 250, anti-LAMP1: 1 : 250 "ab24170") and secondary antibodies

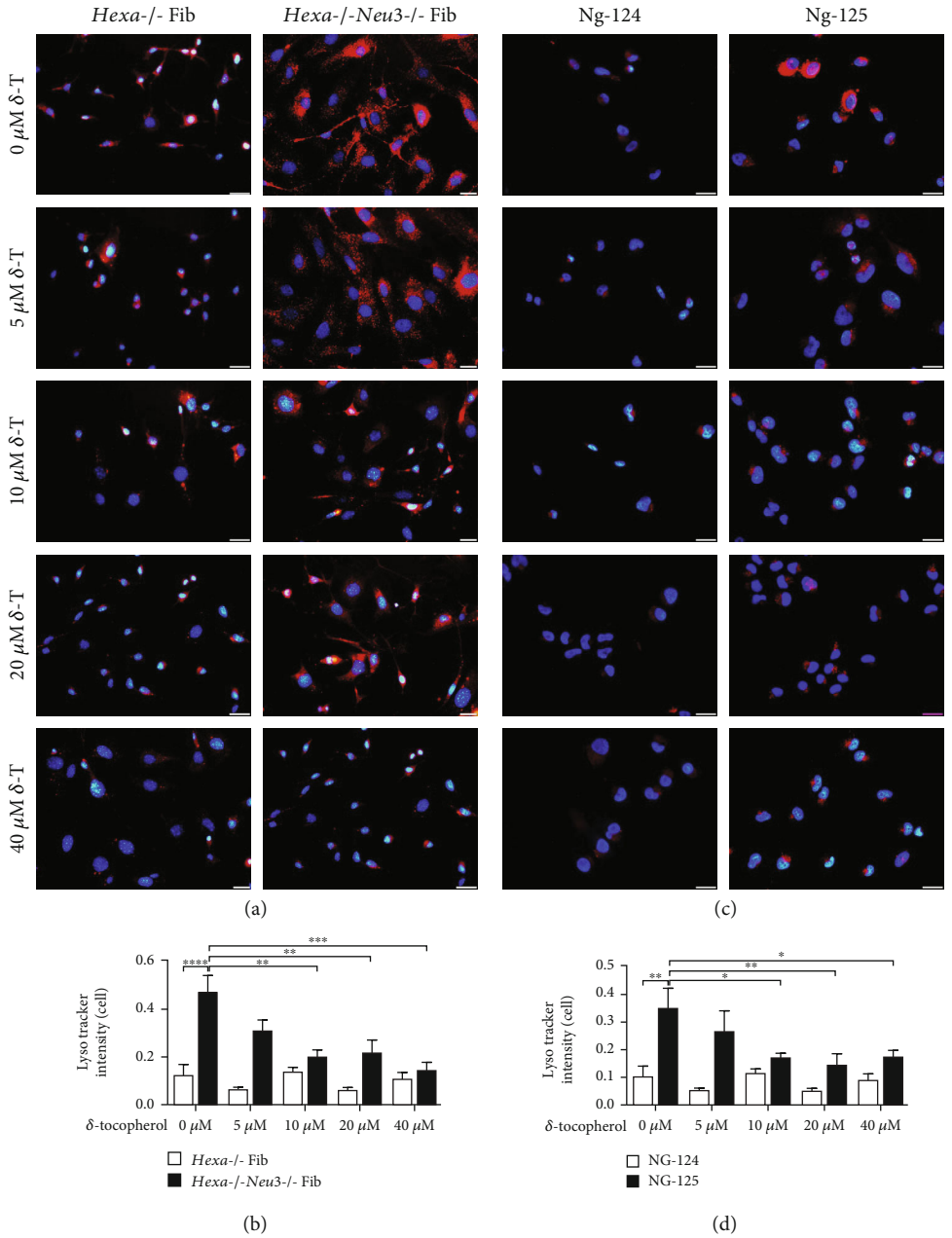

Figure 1: LysoTracker staining of (a) tocopherol- (δ-T-) treated fibroblast derived from 5-month-old *Hexa-/-* and *Hexa-/-Neu3-/-* and (c) neuroglia derived from human TSD patient. Total LysoTracker signal was measured by ImageJ, and graphics for LysoTracker intensity per cell were represented for (b) mouse fibroblast and (d) human neuroglia. Images were taken under same light intensity differing only for filter type at 100x magnification, and the data are represented as the mean ± S.E.M. Two-way ANOVA analysis was used to determine p values via GraphPad ($^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

(1 : 250, SA5-10118 Alexa Fluor®-488 and 1 : 500 ab175476, Alexa Fluor®-568), and they were mounted on slides with Fluoroshield mounting medium DAPI (Abcam). Images were obtained by using a fluorescent microscope (Olympus BX53). Colocalization analysis of red and green fluorescence was applied by using NIH ImageJ.

2.4. qRT-PCR Analysis. Total RNA was extracted from cell pellets collected from human and mouse fibroblast and neuroglia cells by using TRIzol reagent (Geneaid), and cDNA synthesis was done by using a reverse transcription kit (Applied Biosystems). Expression analysis of the Neu1 sialidase was performed by using Roche LightCycler 480 SYBR Green I Master Mix according to the manufacturer's instructions. GAPDH gene expression was used as an endogenous control. The following pairs of primers were used for expression analysis: M-Neu1F: 5′-TCATCGCCATGAGGAGGTCCA-3′, M-Neu1R: 5′-AAAGGGAATGCCGCTCACTCCA-3′;

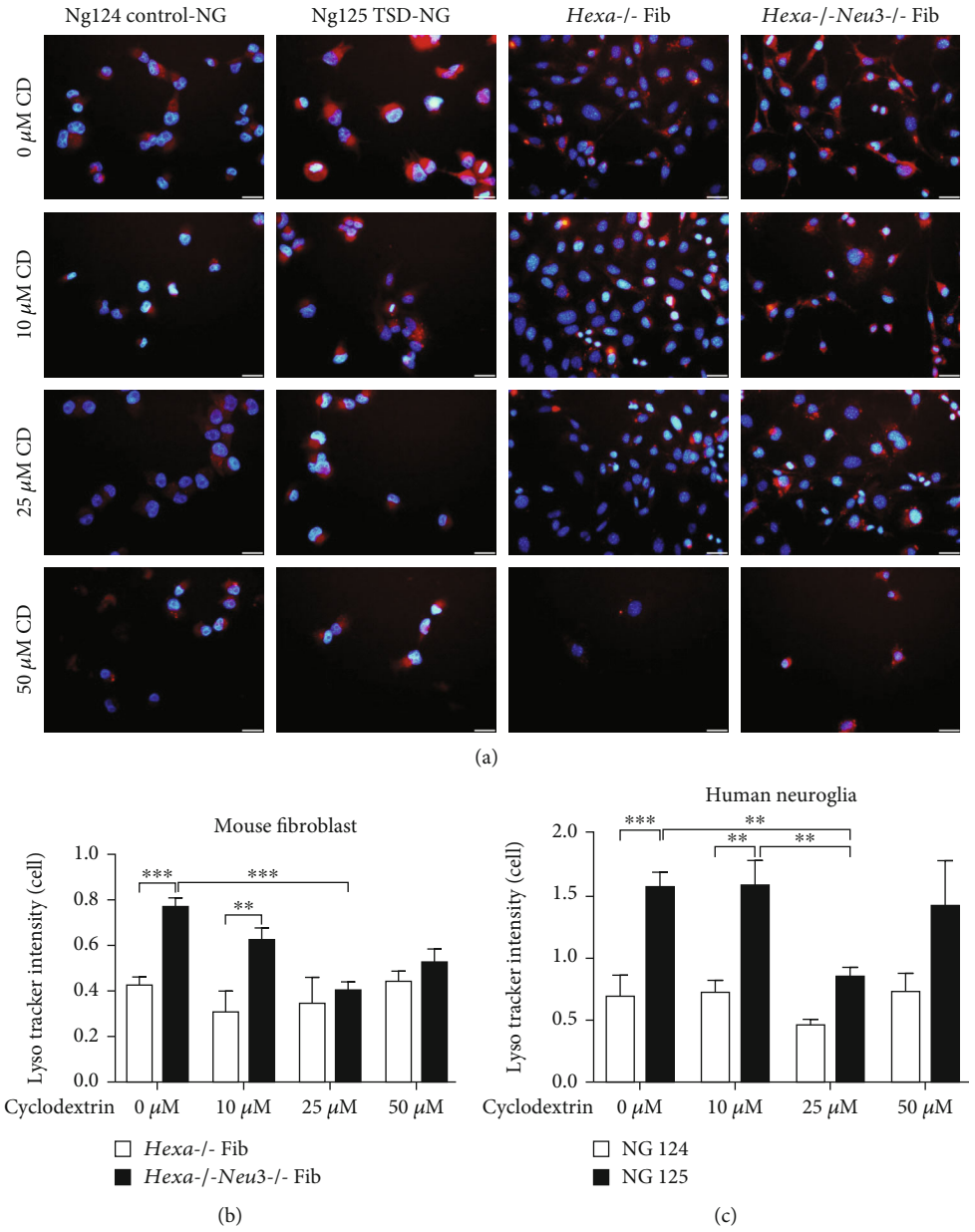

Figure 2: LysoTracker staining of cyclodextrin- (CD-) treated fibroblast derived from 5-month-old *Hexa*-/- and *Hexa*-/-*Neu3*-/- and neuroglia derived from human TSD patient (a). Total LysoTracker signal was measured by ImageJ, and graphics for LysoTracker intensity per cell were represented for (b) mouse fibroblast and (c) human neuroglia. Images were taken under same light intensity differing only for filter type at 100x magnification, and the data are represented as the mean ± S.E.M. Two-way ANOVA analysis was used to determine p values via GraphPad ($^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

M-GAPDHF: 5′-CCCCTTCATTGACCTCAACTAC-3′, M-GAPDHR: 5′-ATGCATTGC TGACAATCTTGAG-3′; H-Neu1F: 5′-GACCGGGCTCTGGTATTCAG-3′, H-Neu1 R: 5′-GGTTTCGGGCATTGATGACG-3′; and H-GAPDHF: 5′-GTCTCCTCTGACTT CAACAGCG-3′, H-GAPDHR: 5′-ACCACCCTGTTGCTG TAGCCAA-3′.

2.5. Enzyme Assay. 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MUNANA) sodium salt hydrate (Sigma, US) was used as a substrate to measure the NEU1 sialidase activity in the mouse and human fibroblast and neuroglia cells lysed and supernatant. The cells were sonicated at 60 V for 10 s. Growth medium was obtained as supernatant from 24-hour-treated cells and centrifuged at 12000 g for 10 minutes. 10 μl of cell mixture and 50 μl of media were incubated with 0.5 mM substrate in a 0.5 M sodium acetate buffer (pH 4.5) at 37°C for 1 hour. 3.9 ml 0.2 M glycine buffer pH 10.8 was added to stop the reaction. Samples were analyzed using a spectrofluorometer (Shimadzu, Japan) at an excitation wavelength of 365 nm and emission wavelength of 445 nm. Protein concentration was determined by the Bradford assay (Sigma, US), and specific enzyme activity was calculated.

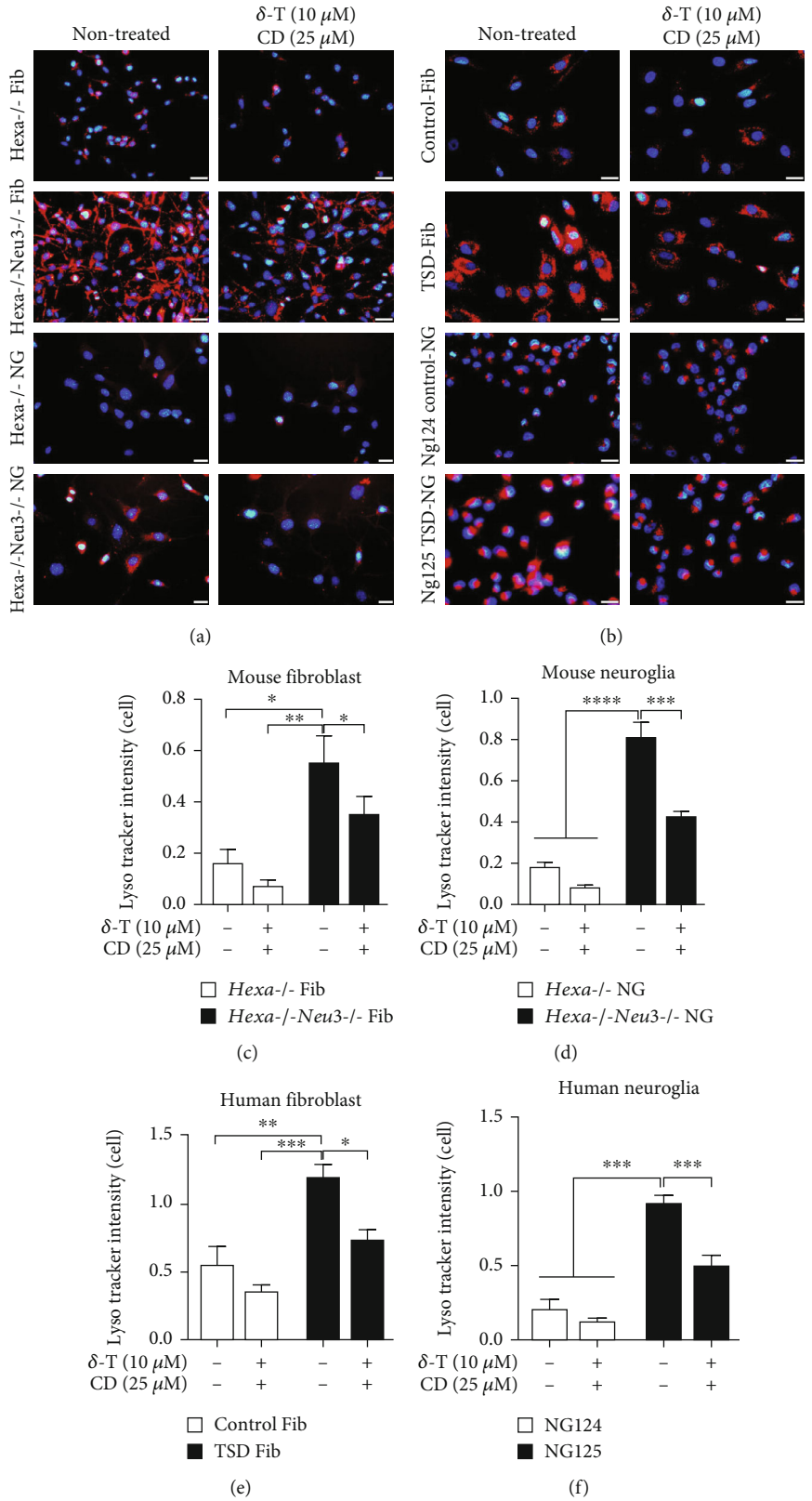

Figure 3: LysoTracker staining of tocopherol- (δ-T-) and cyclodextrin- (CD-) treated fibroblast and neuroglia derived from (a) 5-month-old *Hexa-/-* and *Hexa-/-Neu3-/-* and (b) from human TSD patient. Total LysoTracker signal was measured by ImageJ, and graphics for LysoTracker intensity per cell were represented for (c) mouse fibroblast, (d) mouse neuroglia, (e) human fibroblast, and (f) human neuroglia. Images were taken under the same light intensity differing only for filter type at 100x magnification, and the data are represented as the mean ± S.E.M. Two-way ANOVA analysis was used to determine p values via GraphPad ($^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

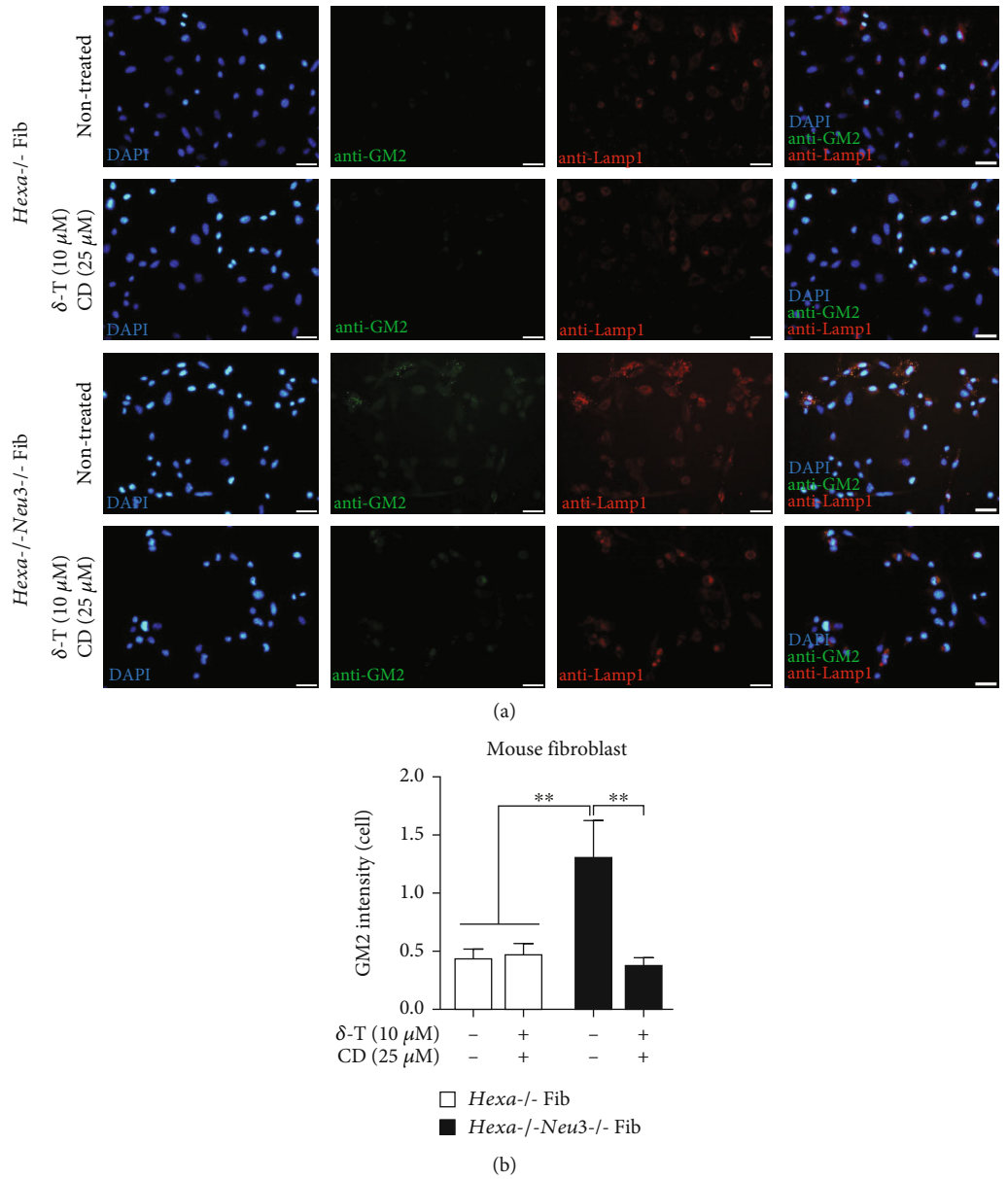

Figure 4: Immunocytochemical colocalization analysis images for tocopherol- (δ-T-) and cyclodextrin- (CD-) treated fibroblast derived from 5-month-old *Hexa-/-* and *Hexa-/-Neu3-/-* mice. The cells were stained with anti-GM2 antibody (green; GM2 ganglioside), anti-Lamp1 (red; lysosomal marker), and DAPI (blue; nucleus) (a). Total GM2 intensity was measured by ImageJ, and graphics for intensity per cell were represented (b). Images were taken under the same light intensity differing only for filter type at 100x magnification, and the data are represented as the mean ± S.E.M. Two-way ANOVA analysis was used to determine p values via GraphPad ($^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

3. Results

3.1. Dose-Dependent Tocopherol and Cyclodextrin Treatment Rescue Lysosomal Storage in Cells. To detect the effect of tocopherol and cyclodextrin on the lysosomal burden, fibroblast and neuroglia derived from *Hexa-/-*, *Hexa-/-Neu3-/-* mice, and human TSD patients were treated with tocopherol and cyclodextrin. Untreated *Hexa-/-Neu3-/-* fibroblast and Ng125 TSD-Ng cell lines showed increased levels of LysoTracker staining, indicating the enlarged lysosomal compartments (Figures 1 and 2). Treatment of the cells with 10, 20, and 40 μM tocopherol resulted in a significant reduction in the number of lysosomes in *Hexa-/-Neu3-/-* fibroblast (Figures 1(a) and 1(b)) and in Ng125 TSD-Ng (Figures 1(c) and 1(d)) compared to the untreated cells. Similarly, we determined decreased lysosome enlargement in human neuroglia and mouse fibroblasts after 48 hours of 25 μM and 50 μM cyclodextrin treatment. However, 50 μM cyclodextrin had a cytotoxic effect on these cell lines and caused increased cell death (Figure 2). Thus, the minimum efficient treatment dosage was determined as 10 μM tocopherol and 25 μM cyclodextrin for further experiments. Combined

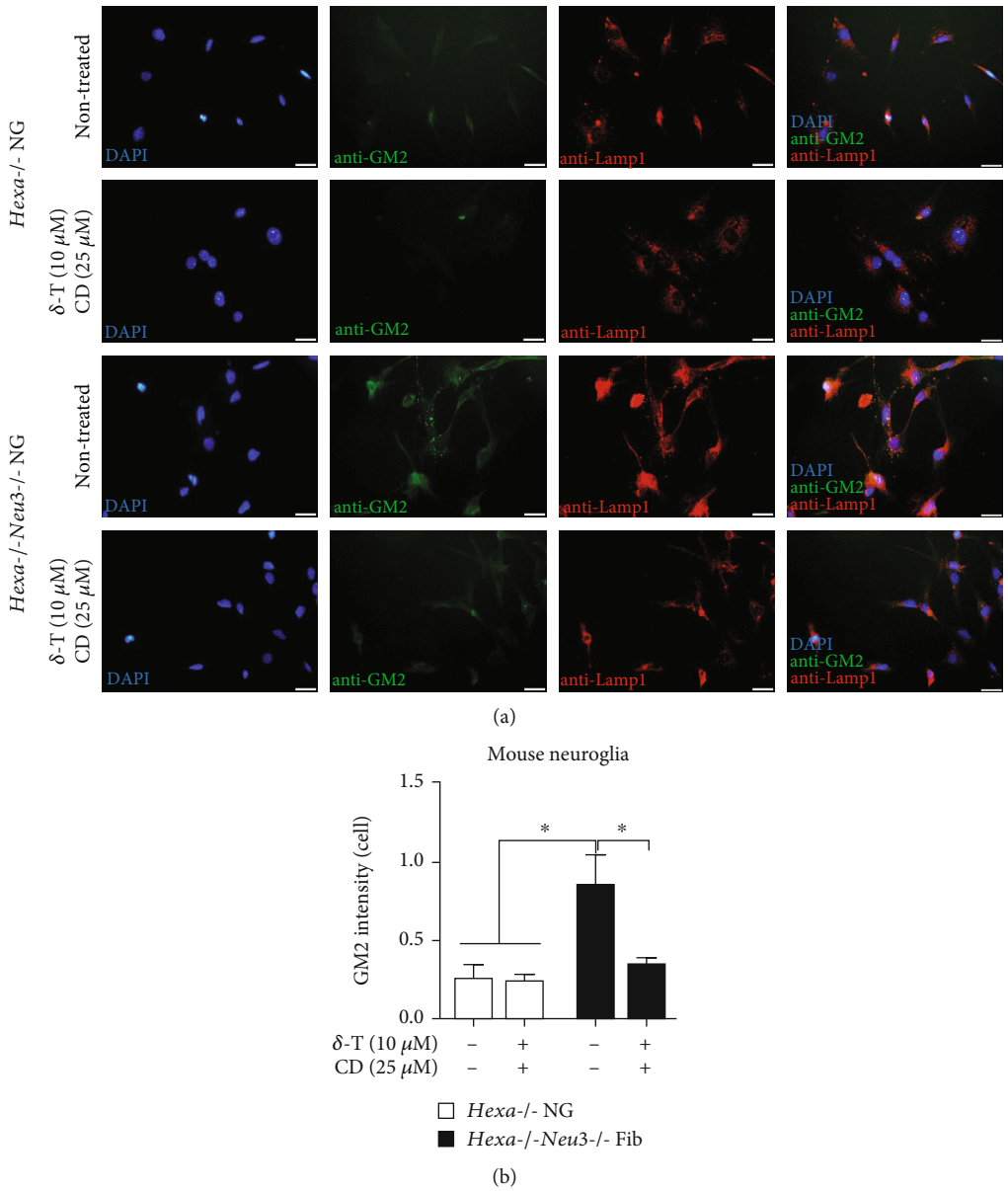

Figure 5: Immunocytochemical colocalization analysis images for tocopherol- (δ-T-) and cyclodextrin- (CD-) treated neuroglia derived from 5-month-old *Hexa*-/- and *Hexa*-/-*Neu3*-/- mice. The cells were stained with anti-GM2 antibody (green; GM2 ganglioside), anti-Lamp1 (red; lysosomal marker), and DAPI (blue; nucleus) (a). Total GM2 intensity was measured by ImageJ, and graphics for intensity per cell were represented (b). Images were taken under the same light intensity differing only for filter type at 100x magnification, and the data are represented as the mean ± S.E.M. Two-way ANOVA analysis was used to determine p values via GraphPad ($^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

treatment of tocopherol and cyclodextrin was investigated to improve efficacy and reduce the dosage required to achieve the maximal response. LysoTracker staining revealed that combined treatment significantly decreased lysosomal accumulation in the fibroblast and neuroglia derived from *Hexa*-/-*Neu3*-/- mice and Tay-Sachs patients (Figure 3). The human and mouse TSD neuroglia cell lines displayed more effective response to exocytosis induction (Figures 3(d) and 3(f)).

3.2. Combined Treatment with Tocopherol and Cyclodextrin Reduces GM2 Accumulation in Lysosomes. To assess the potential therapeutical effect of these small molecules on lysosomal GM2 accumulation, we performed immunocytochemistry for GM2 ganglioside in the tocopherol and cyclodextrin-treated fibroblast and neuroglia derived from *Hexa*-/-*Neu3*-/- mice and TSD patients. Combined treatment of 10 μM tocopherol and 25 μM cyclodextrin significantly reduced GM2 accumulation in all cell lines studied (Figures 4(b), 5(b), and 6(b)). Human neuroglia cells displayed a decrease in GM2 accumulation, but it was not statistically significant (Figure 7(b)). Parallel to LysoTracker staining, a reduction of Lamp1 signal was also observed after

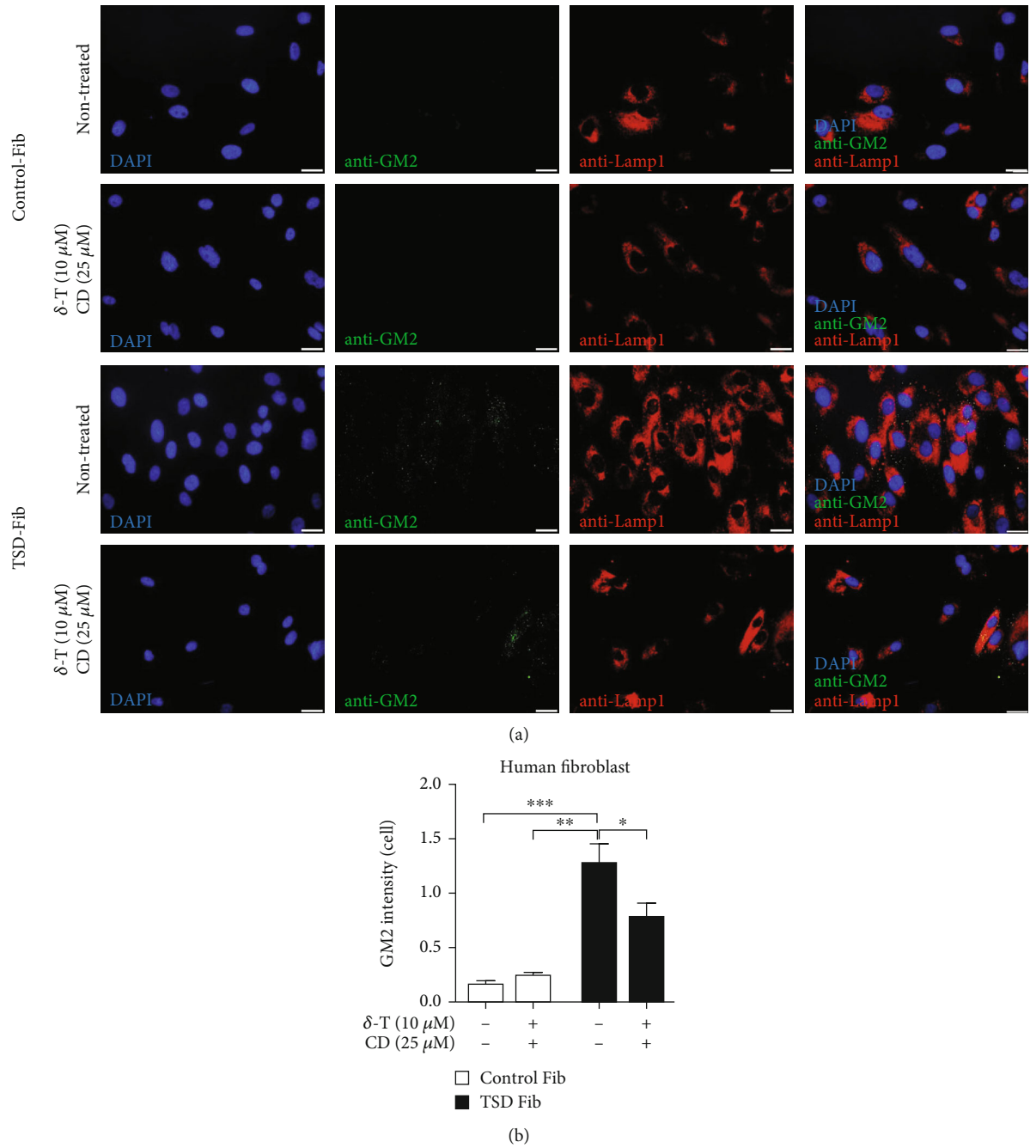

Figure 6: Immunocytochemical colocalization analysis images for tocopherol- (δ-T-) and cyclodextrin- (CD-) treated fibroblast derived from human TSD patient. The cells were stained with anti-GM2 antibody (green; GM2 ganglioside), anti-Lamp1 (red; lysosomal marker), and DAPI (blue; nucleus) (a). Total GM2 intensity was measured by ImageJ, and graphics for intensity per cell were represented (b). Images were taken under same light intensity differing only for filter type at 100x magnification, and the data are represented as the mean ± S.E.M. Two-way ANOVA analysis was used to determine p values via GraphPad ($^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

the same combined treatment. Taken together, the results demonstrate a synergistic effect of combined treatment of tocopherol and cyclodextrin on the reduction of lysosomal GM2 accumulation in TSD fibroblast and neuroglia.

3.3. Combined Treatment of Tocopherol and Cyclodextrin Inhibits Neu1 Gene Expression. It was reported that lysosomal neuraminidase 1 (Neu1), which catalyzes the cleavage of terminal sialic acid residues, deglycosylates lysosomal membrane protein (LAMP1) and impairs lysosomal exocytosis [32]. To assess whether combined treatment of tocopherol and cyclodextrin alters the Neu1 expression levels, we also analyzed the fibroblast and neuroglia *of Hexa-/-Neu3-/-* and TSD patients with qRT-PCR. As expected, we

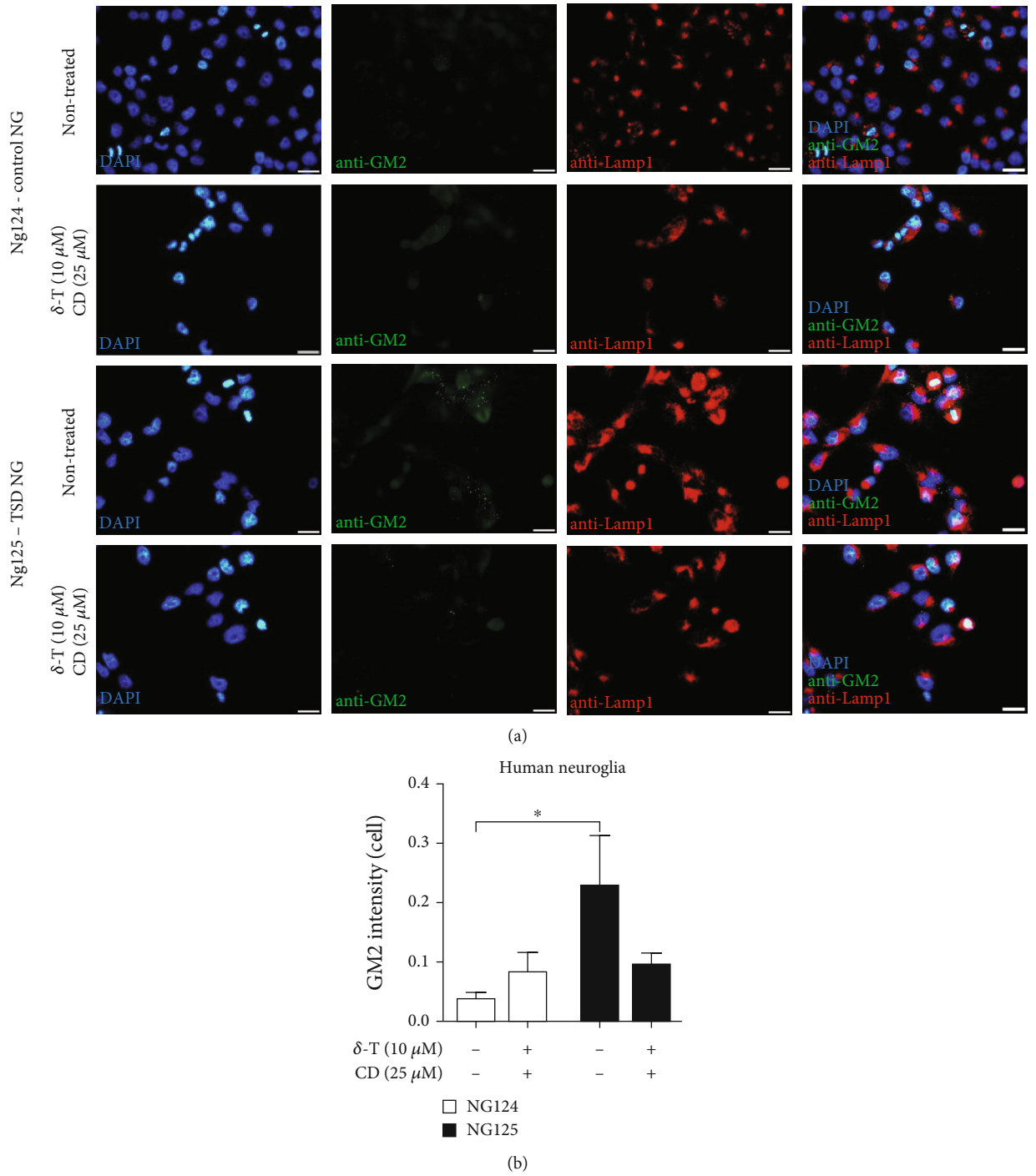

Figure 7: Immunocytochemical colocalization analysis images for tocopherol- (δ-T-) and cyclodextrin- (CD-) treated control (Ng124) and human TSD neuroglia (Ng125). The cells were stained with anti-GM2 antibody (green; GM2 ganglioside), anti-Lamp1 (red; lysosomal marker), and DAPI (blue; nucleus) (a). Total GM2 intensity was measured by ImageJ, and graphics for intensity per cell were represented (b). Images were taken under the same light intensity differing only for filter type at 100x magnification, and the data are represented as the mean ± S.E.M. Two-way ANOVA analysis was used to determine p values via GraphPad ($^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

observed an increased level of Neu1 gene expression in mouse and human TSD cells when compared to the control group. Notably, the combined treatment of tocopherol and cyclodextrin reduced the level of Neu1 expression in all cell lines studied compared to untreated ones except *Hexa*-/-*Neu3*-/- fibroblast (Figure 8).

Upon stimulation with 10 μM tocopherol and 25 μM cyclodextrin, the Neu1 sialidase enzyme activity was

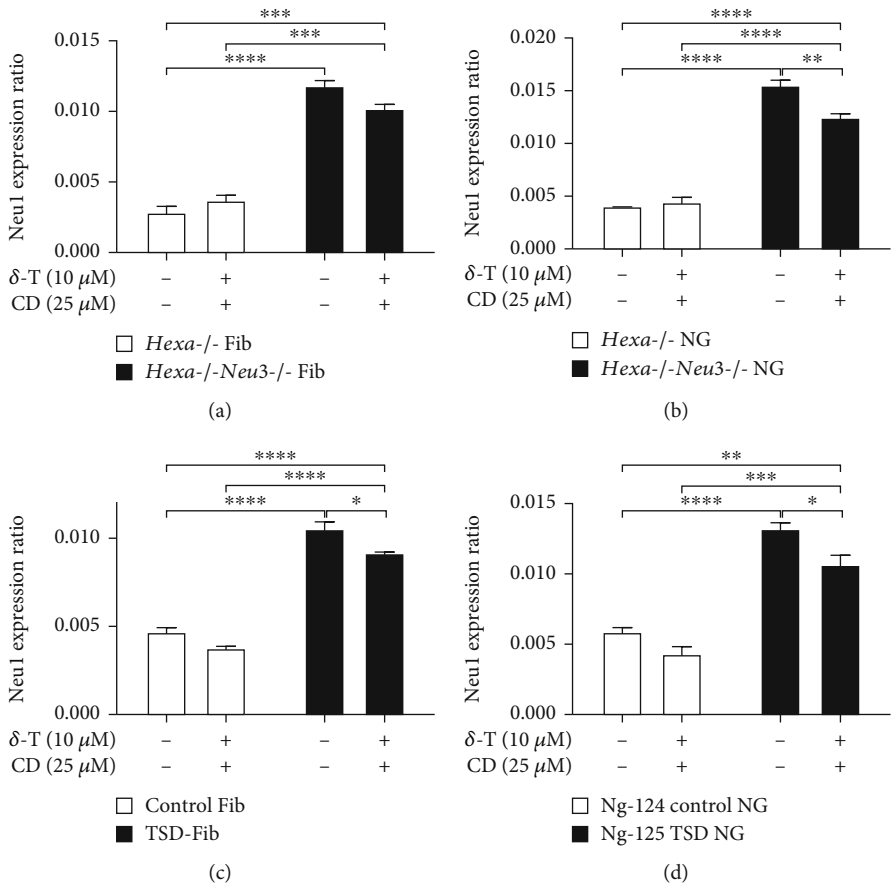

Figure 8: Neu1 gene expression in the tocopherol- (δ-T-) and cyclodextrin- (CD-) treated (a) mouse fibroblast, (b) mouse neuroglia, (c) human fibroblast, and (d) human neuroglia. Expression ratios were calculated by ΔCT method, and percent ratios were represented. One-way ANOVA analysis was used to determine p values via GraphPad. Data are reported as mean ± SEM ($n = 3$; $^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

measured in the fibroblast and neuroglia of *Hexa-/-Neu3-/-* mice and TSD patients. Sialidase activity significantly decreased in the treated cells possibly through lysosomal exocytosis (Figure 9). Consistent with our data, we also detected higher Neu1 activity in the culture media supporting the existence of exocytosis (Figure 10).

4. Discussion

GM2-gangliosidosis type I along with other LSDs displays an accumulation of endogenous substrates in lysosomes causing lysosomal dysfunction that can subsequently alter many cellular processes [33]. Previously reported studies on LSDs showed abnormalities in lysosomal pH regulation, synaptic release, endocytosis, vesicle maturation, autophagy, exocytosis, and Ca^{2+} homeostasis among other biochemical alterations [34].

Lysosomal exocytosis is a Ca^{2+}-regulated mechanism carried out for specialized physiological processes like plasma membrane repair, bone resorption by osteoclasts, antigen presentation, and hematopoietic stem cell differentiation [35, 36]. Despite the heterogeneity of the LSDs, regulation of exocytosis has been suggested as a potential therapy in patients [14, 17]. Tocopherol and cyclodextrin are very well-known modulators of lysosomal exocytosis as they increase cytosolic Ca^{2+} and reduce lysosomal accumulation and enlargement [24, 25, 37]. Although tocopherol and cyclodextrin induce exocytosis through different mechanisms of action, combined treatment of the two drugs may contribute to their therapeutic efficacy in reducing lysosomal expansion, as shown in TSD neural stem cells [28]. Previously, the synergistic effect of these two molecules on lysosomal exocytosis was demonstrated on NPA neural stem cells [24]. Accordingly, we focused on reducing lysosomal enlargement and accumulation by inducing exocytosis in GM2-gangliosidosis type I cells. Firstly, we analyzed the effect of tocopherol and cyclodextrin on fibroblast and neuroglia derived from *Hexa-/-Neu3-/-* mice and human TSD patients. We showed that tocopherol was effective in reducing lysosomal enlargement at 10 μM and higher concentrations in *Hexa-/-Neu3-/-* fibroblast and human neuroglia (Figure 1). In addition, cyclodextrin was effective at both 25 μM and 50 μM concentrations, but we observed decrease in cell number in *Hexa-/-Neu3-/-* fibroblast and human neuroglia at 50 μM cyclodextrin (Figure 2). This cytotoxic impact may be related to cyclodextrin's ability to bind and extract cholesterol from membranes [22]. Higher cyclodextrin concentrations may result in a greater number of "holes" in plasma membrane structures, making plasma membrane

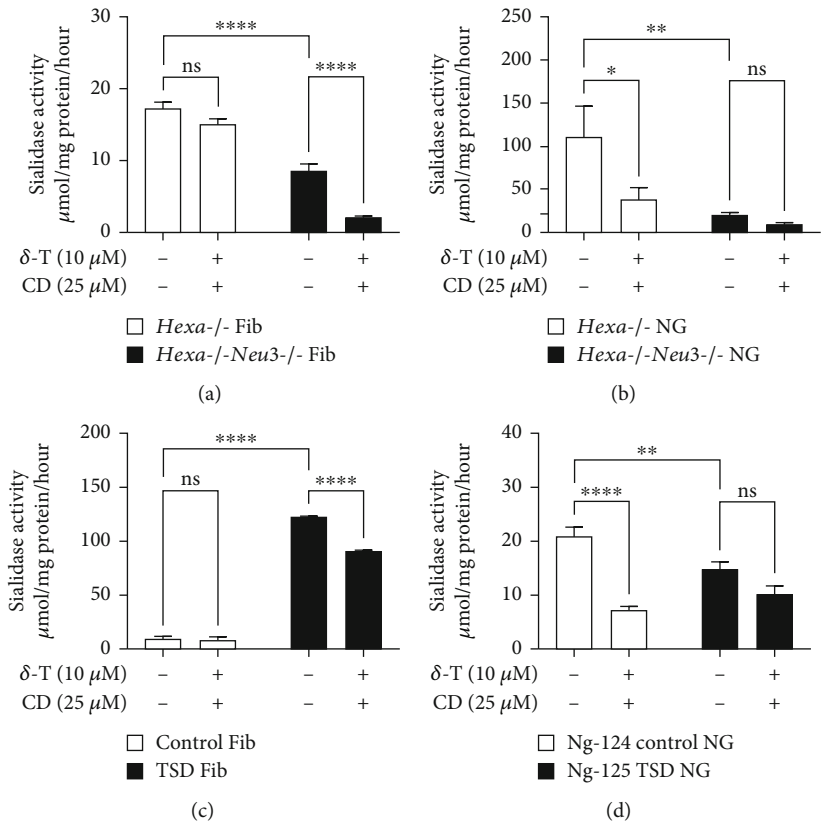

Figure 9: Sialidase activity in (a) mouse fibroblast, (b) mouse neuroglia, (c) human fibroblast, and (d) human neuroglia cells after 24-hour tocopherol (δ-T) and cyclodextrin (CD) treatment. Two-way ANOVA analysis was used to determine p values via GraphPad. Data are reported as mean ± SEM ($n = 2$; $^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

repair more difficult. In clinical studies, the safety of cyclodextrin usage as a pharmaceutical excipient is dependent on the route of administration and dosages. Orally administered cyclodextrins, owing to their bulky and hydrophilic nature, are essentially nontoxic. Intravenous administration of cyclodextrin is generally deemed safe; however, excessive dosages can be harmful. While some *in vitro* studies have indicated hemolytic effects of cyclodextrins, the toxicological significance of *in vivo* studies is considered negligible when evaluating cyclodextrin toxicity profiles [38, 39].

It has been reported that tocopherol and cyclodextrin enabled the reduction of storage material in several LSD models such as decreasing lipid accumulation and reducing lysosomal enlargement in NPC [19]. A combined administration of tocopherol and cyclodextrin was also performed to increase the efficacy. Our data showed a significant decrease in lysosomal enlargement in both mouse and human TSD cell models, but the decrease was more significant in neuroglia cell lines (Figure 3). We also observed that the treatment resulted in a significant decrease in the level of GM2 accumulation in fibroblast and neuroglia derived from *Hexa-/-Neu3-/-* mice (Figures 4 and 5) and human TSD patients (Figures 6 and 7). Thus, we demonstrated that GM2 ganglioside can be exported from cells through lysosomal exocytosis, and inducing lysosomal exocytosis reduces GM2 ganglioside accumulation in TSD cell models. For human neuroglia, the decrease was not statistically significant and we assume that this can be caused by the difference in the severity of the GM2 accumulation and variable responses of different cell types to drug treatment.

It has been reported that Neu1 sialidase negatively regulates the exocytosis mechanism by catalyzing deglycosylation of LAMP1 protein and *Neu1-/-* mice displayed enhanced lysosomal exocytosis of catalytically active hydrolases [32, 40, 41]. In addition, our group previously showed significantly increased Neu1 expression and specific activity in the brain of 5-month-old *Hexa-/-Neu3-/-* mice compared to age-matched *WT* and *Hexa-/-* mice (unpublished data) (Figure S1). Consistent with that, here we also showed significantly increased Neu1 expression in untreated mouse and human TSD cells compared to healthy controls (Figure 8) which might cause impairment in lysosomal exocytosis. When we analyzed the effect of combined drug treatment on Neu1 expression, we noticed a significant decrease in the expression level of Neu1 sialidase in mouse and human TSD cell models except for *Hexa-/-Neu3-/-* fibroblast (Figure 8). Downregulation of Neu1 gene expression in treated TSD cell models might be related to the interrelation between Neu1 sialidase and exocytosis pathway. Neu1 is predominantly located in lysosomes and also found in plasma membrane structure [42]; thus, inducing exocytosis by combined drug treatment resulted in a decrease in Neu1 gene expression. However, how Neu1 mRNA expression is affected transcriptionally is not understood mechanistically, and this

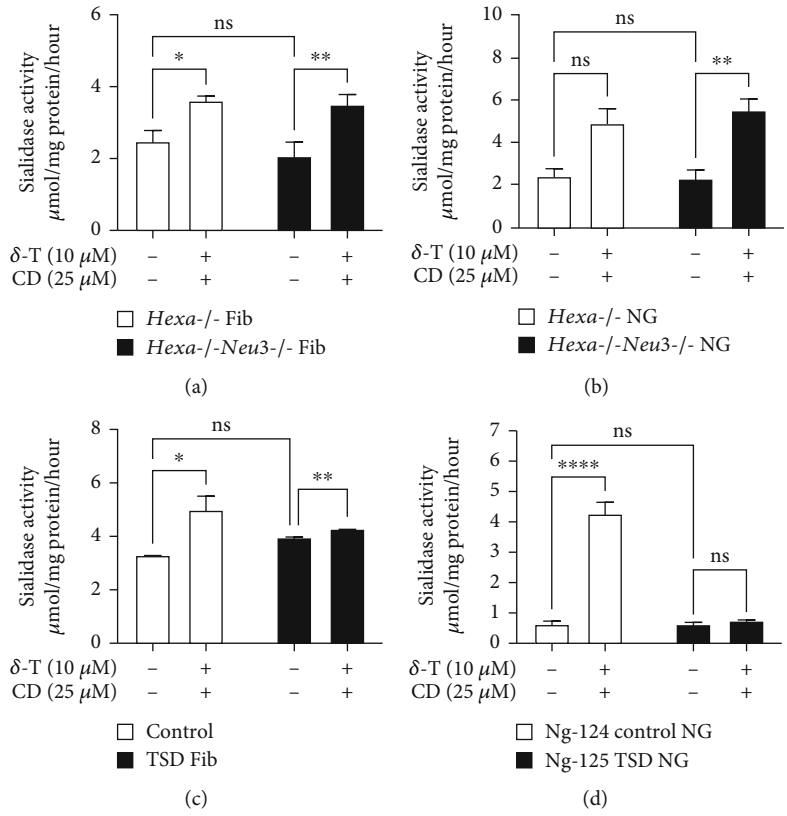

Figure 10: The activity of sialidase in culture media after 24-hour tocopherol- (δ-T-) and cyclodextrin- (CD-) treated (a) mouse fibroblast, (b) mouse neuroglia, (c) human fibroblast, and (d) human neuroglia cells. Two-way ANOVA analysis was used to determine p values via GraphPad. Data are reported as mean ± SEM ($n = 2$; $^{*}p < 0.05$, $^{**}p < 0.025$, and $^{***}p < 0.01$).

suggestion requires further investigation. When we analyzed the enzymatic activity of the NEU1 sialidase, combined drug treatment reduced NEU1 enzyme activity which is consistent with lower gene expression of Neu1. Our data supports that the inhibition of exocytosis by Neu1 sialidase was reduced by combined treatment and might result in an additive effect on the induction of lysosomal exocytosis in treated cells.

Overall, our data suggest that combined treatment of tocopherol and cyclodextrin downregulates Neu1 gene expression and enhances lysosomal exocytosis. We demonstrated that induction of lysosomal exocytosis leads to the reduction of abnormal lysosomal enlargement and GM2 accumulation in fibroblast and neuroglia derived from *Hexa-/-Neu3-/-* mice and human TSD patients. Our findings provide promising evidence for further investigation of targeting the lysosomal exocytosis pathway in the *Hexa-/-Neu3-/-* mouse model as a therapeutic approach to alleviate GM2 accumulation *in vivo*.

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1: (A, B) Neu1 gene expression and (C, D) specific Neu1 activity in brains of 2 and 5 months *WT*, *Hexa-/-*, and *Hexa-/-Neu3-/-*. Expression ratios were calculated by Δ CT method, and percent ratios were represented. One-way ANOVA analysis was used to determine p values via GraphPad. Data are reported as mean ± SEM ($n = 3$; $^{**}p < 0.025$, $^{***}p < 0.01$, and $^{****}p < 0.0001$). *(Supplementary Materials)*

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