Developing a Gaucher Disease Pharmacological Model of the Blood-Brain Barrier

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Introduction

- Gaucher disease (GD), a lysosomal storage disease, is a genetic disorder that causes glucocerebrosidase (GCase) to be unable to function correctly.
- GCase breaks down glucocerebroside (GluCer) and in GD, a buildup of GluCer, often leads to neuropathic conditions
- Common treatment is enzyme replacement therapy, the intravenous delivery of recombinant enzymes[5]
- Enzyme replacement therapy has an inability to treat the central nervous system because of its inability to cross the blood-brain barrier (BBB)
- In vitro studies with Gaucher afflicted BBB cellular systems are needed to test delivery of novel recombinant enzymes
- Severely limited by scarcity and expense of Gaucher endothelial cells, astrocytes, and neurons, which compose the BBB.
- Can potentially use inhibitors to lower GCase activity levels in healthy cells[1,2,3]
- Inhibitors should not affect cell health and should sustain lowered activity level[4,6]

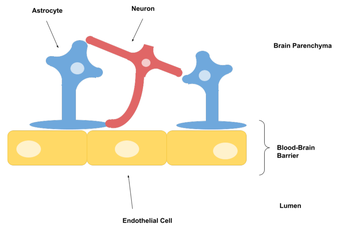

Figure 1: Diagram of the Blood-Brain Barrier

Goal

- Develop a BBB pharmacological model of Gaucher disease involving treating healthy endothelial cells, astrocytes, to exhibit Gaucher phenotype, and iPS derived Gaucher neurons and test modified GCase (modified to transcytose BBB) on the model for delivery

Methodology

- Cell culture techniques were used to grow healthy human brain macrovascular endothelial cells (HBMECs) and astrocytes and both lines were treated with conduritol beta-epoxide (CBE), an inhibitor of GCase
- Verification of Gaucher phenotype was achieved through immunofluorescence analysis of fluorescent GluCer treatment of cells after CBE treatment
- Gaucher phenotype also verified through enzymatic activity assays of cell lysates
- BBB model was built through a trans well system with CBE treated HBMEC on apical side, astrocytes on basal side of filter, and iPS derived Gaucher neurons in the basal well
- Immunofluorescence studies was used to analyze transcytosis of modified GCase in trans well and iPS derived Gaucher neurons
- Cells often treated with TNF-α, an inflammatory cytokine, to mimic inflammatory response state. Inflammation plays a key role in pathogenesis of Gaucher disease.
- Enzymes were radiolabeled with iodine-125, applied to the apical side of the trans well system for 1h, 3h, 5h or 24h following which the cells on the filter and the neurons were collected and radioactive cpms were determined.

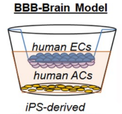

Figure 2: BBB Trans well model

Results

I. HBMECs and Astrocytes treated with CBE showed significantly decreased GCase Activity

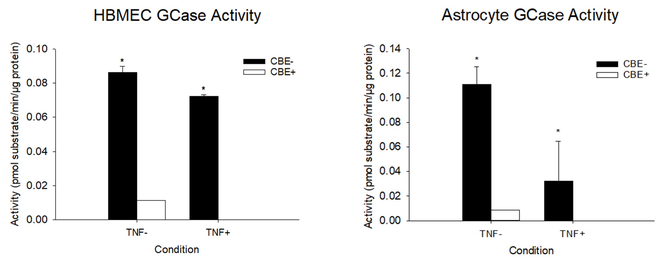

Figure 2: Enzyme activity assays on pharmacological models of HBMECs and astrocytes. HBMEC and astrocytes were incubated overnight with TNF-α at 10 ng/mL and CBE at 200 µM. Cells were then lysed for protein extraction. Amount of protein in lysates was quantified by BCA. Cell lysates were incubated for 30 min at 37 C with fluorescent 4-Methylumbelliferyl-β-D-glycopyranoside, which is a substrate for GCase. Amount of substrate cleaved was quantified by measuring fluorescence of samples with microplate spectrophotometer. Cells not treated with either TNF-α or CBE were controls. (Left) Enzymatic activity in pmol substrate cleaved per minute per µg of protein in HBMEC lysates. (Right) Enzymatic activity in pmol substrate cleaved per minute per µg of protein in astrocyte lysates. Data are mean±SEM (N=2). Significance level of p=0.05.

II. HBMECs and Astrocytes treated with CBE showed increased GluCer accumulation

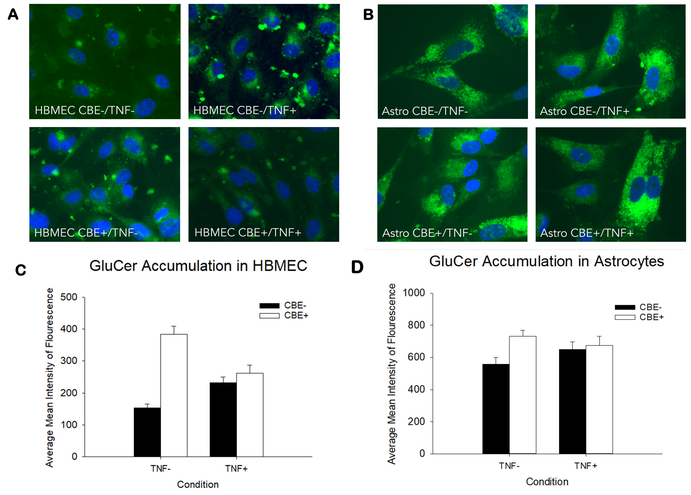

Figure 3: Lipid accumulation assay on pharmacological models of HBMECs and astrocytes. HBMEC and astrocytes were incubated overnight with TNF-α at 10 ng/mL. Cells were incubated with CBE for 72 h at 200 µM. Fluorescent lipid N-hexanoyl-NBD-Glucosylceramide, the substrate for GCase which accumulates in this disease, was added to cells at 5 µg per well for 48 hr. The level of fluorescent GluCer was visualized and quantified by microscopy and using ImagePro Analyzer. Cells without CBE and TNF treatment were controls. (A) Microscope visualization of HBMEC at 60X magnification. Green is GluCer. Blue is nuclei. (B) Microscope visualization of astrocytes at 60X magnification. Green is GluCer. Blue is nuclei. (C) Mean intensity of fluorescence in HBMEC. (D) Mean intensity of fluorescence in astrocytes. Data are mean±SEM (N=2).

III. Modified GCase transcytoses across Gaucher BBB trans well model more efficiently while GCase accumulates in model

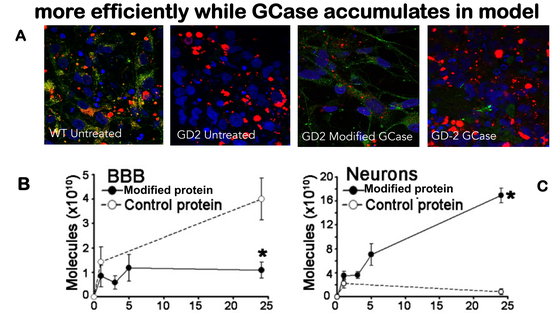

Figure 4: Transport and effect of glucocerbrosidase enzymes across the Gaucher BBB model. A BBB model of Gaucher disease was created by culturing cells on a trans well. HBMEC were grown on apical side of filter, and astrocytes on basal side. Both lines were treated with CBE at 200 µM and TNF-α at 10 ng/mL. iPS derived Gaucher neurons were grown on bottom basal well. Cells were treated with modified GCase or control Gcase radiolabeled with Iodine-125 at 3.4 µg/well. Wildtype neurons with no treatment was also included as a control. Cells were also treated with lysotracker to visualize lysosomes, where smaller lysosomes indicated a rescue of cells, and fluorescent anti-GCase antibody to visualize GCase delivery. (A) Microscope visualization of WT and iPS derived Gaucher neurons at 60X. Green is protein. Red is lysotracker (lysosomes). Blue is nuclei. (B) Molecule (protein) count determined from the radioactive cpms in BBB over time (C) Molecule (protein) count in iPS derived Gaucher neurons over time.

IV. Modified GCase shows high colocalization with lysosomes in iPS derived Gaucher neurons

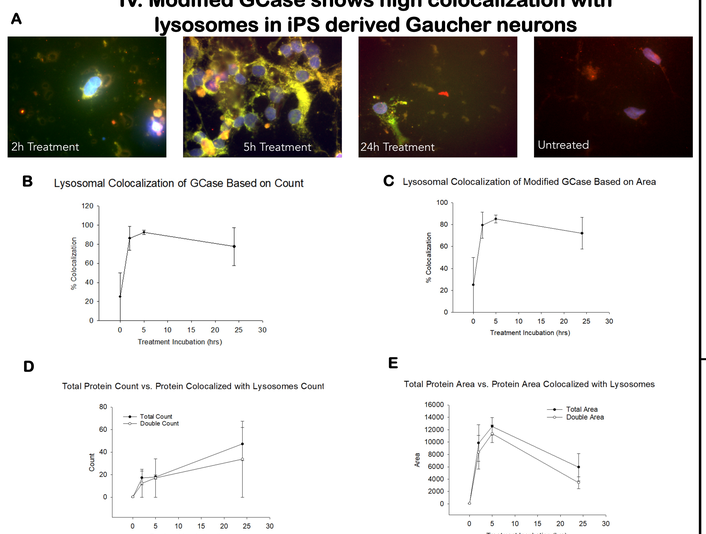

Figure 5: Lysosomal colocalization study of modified GCase in iPS derived neurons. iPS derived Gaucher neurons were treated with TNF-α at 10 ng/mL and were treated with modified GCase at 3.4 µg/well, with treatment incubations of 2h, 5h, and 24h. Cells were then treated with anti-GCase and LAMP-1 antibodies for protein and lysosomal staining respectively. Level of protein accumulation and colocalization with lysosomes was visualized and quantified using microscopy. Untreated neurons were control. (A) Microscope visualization of iPS derived Gaucher neurons at 60X. Green is protein. Red is lysosomes. Yellow is colocalized protein. Blue is nuclei. (B) Lysosomal colocalization of modified GCase based on fluorescent object count. (C) Lysosomal colocalization of modified GCase based on area of fluorescence. (D) Total protein count versus protein colocalized with lysosomes count. (E) Total protein fluorescent area versus protein colocalized with lysosomes fluorescent area. Data are mean±SEM (N=2)

V. Modified GCase shows no significant increase of cytotoxicity in iPS derived Gaucher neurons

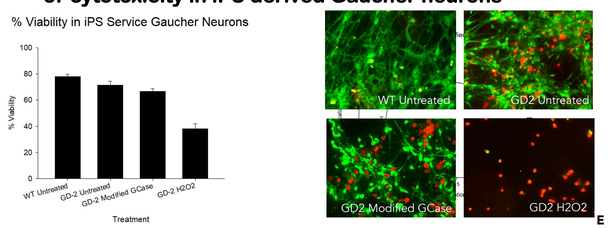

Figure 6: Toxicity study of modified GCase enzyme in iPS derived neurons. iPS derived Gaucher neurons and Wildtype neurons were untreated, treated with 10 µg of modified GCase for 48 h, or hydrogen peroxide for 48 h. After incubation with treatments or lack of treatment, calcein AM and EthD-1 were used to stain cells green if live, or red if dead respectively. Number of live cells, dead cells, and thus viability was visualized and quantified by microscopy. Wildtype untreated was the positive control while hydrogen peroxide treated GD2 was the negative control. (Left) % Viability of each condition. (Right) Microscope visualization of neurons at 20X. Green is live cell. Red is dead cell. Data are mean±SEM (N=3)

Conclusions

- Treatment with CBE lowered GCase activity and increased GluCer accumulation in both HBMEC and astrocyte cell types.
- An in vitro model of the Gaucher BBB was established using CBE treated HBMEC and astrocytes with iPS derived Gaucher neurons
- Transport of modified GCase, modified to transcytose more efficiently, was effectively tested on Gaucher BBB trans well model
- More efficient transcytosis was observed with modified GCase with a higher accumulation of control GCase in BBB and a higher accumulation of modified GCase in neurons.
- High colocalization with lysosomes, primary destination of GCase, was seen with modified GCase in iPS derived Gaucher neurons, and it was also effective at lowering lysosomal size.
- No significant effect on cell viability was exhibited with treatment of modified GCase in iPS derived Gaucher neurons.
- Overall model provides a promising step towards testing potential therapeutics for Gaucher disease.
- Future work can include testing GluCer clearance of modified GCase in neurons

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Acknowledgments

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