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Intermittent enzyme replacement therapy with recombinant human β-galactosidase prevents neuraminidase 1 deficiency

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Mutations in the *galactosidase β 1* (*GLB1*) gene cause lysosomal β-galactosidase (β-Gal) deficiency and clinical onset of the neurodegenerative lysosomal storage disease, GM1 gangliosidosis. β-Gal and neuraminidase 1 (NEU1) form a multienzyme complex in lysosomes along with the molecular chaperone, protective protein cathepsin A (PPCA). NEU1 is deficient in the neurodegenerative lysosomal storage disease sialidosis, and its targeting to and stability in lysosomes strictly depend on PPCA. In contrast, β-Gal only partially depends on PPCA, prompting us to investigate the role that β-Gal plays in the multienzyme complex. Here, we demonstrate that β-Gal negatively regulates NEU1 levels in lysosomes by competitively displacing this labile sialidase from PPCA. Chronic cellular uptake of purified recombinant human β-Gal (rhβ-Gal) or chronic lentiviral-mediated GLB1 overexpression in GM1 gangliosidosis patient fibroblasts coincides with profound secondary NEU1 deficiency. A regimen of intermittent enzyme replacement therapy dosing with rhβ-Gal, followed by enzyme withdrawal, is sufficient to augment β-Gal activity levels in GM1 gangliosidosis patient fibroblasts without promoting NEU1 deficiency. In the absence of β-Gal, NEU1 levels are elevated in the GM1 gangliosidosis mouse brain, which are restored to normal levels following weekly intracerebroventricular dosing with rhβ-Gal. Collectively, our results highlight the need to carefully titrate the dose and dosing frequency of β-Gal augmentation therapy for GM1 gangliosidosis. They further suggest that intermittent intracerebroventricular enzyme replacement therapy dosing with rhβ-Gal is a tunable approach that can safely augment β-Gal levels while maintaining NEU1 at physiological levels in the GM1 gangliosidosis brain.

In humans autosomal recessive mutations in the GLB1 gene give rise to a deficiency of lysosomal β-galactosidase (β-Gal), a glycosidase that participates in the degradation of multiple galactose-containing substrates (1). β-Gal deficiency gives rise to the accumulation of three biochemically distinct classes of substrates in lysosomes: GM1 and GA1 gangliosides, the glycosaminoglycan, keratan sulfate, and oligosaccharides derived from glycoprotein metabolism (2). Absent or very low levels of residual β-Gal activity result in the lysosomal accumulation of these galactose-containing substrates throughout the brain, giving rise to the progressive and fatal neurodegenerative lysosomal storage disease, GM1 gangliosidosis (3, 4). Substrate accumulation in several systemic tissues gives rise to additional chronic, debilitating symptoms (5).

Currently, no safe and effective treatment has yet been developed for GM1 gangliosidosis. A challenge for developing an effective enzyme replacement therapy (ERT) or gene therapy approach for GM1 gangliosidosis is that β-Gal has been reported to exist as a multienzyme complex in lysosomes with the chaperone protein, protective protein cathepsin A (PPCA), and neuraminidase 1 (NEU1) (1). Although NEU1 is strictly dependent upon PPCA for trafficking from the Golgi to late endosomes and for catalytic activation in lysosomes, β-Gal is only partially dependent on PPCA (1, 6, 7). Bonten *et al.* (6) suggest that recombinant human NEU1 produced in insect cells self-associates and is rapidly degraded, whereas association of NEU1 with PPCA results in the formation of stable heterodimers that preserve the sialidase activity of NEU1 (6). We have recently demonstrated that recombinant human β-Gal (rhβ-Gal) produced in Chinese hamster ovary (CHO) cells exhibits pH-dependent dynamic self-association, with the enzyme more likely being a homogenous dimer under acidic conditions, which helps to increase its thermostability (8). Our study also suggests that prolonged lentiviral-mediated GLB1 overexpression leads to accumulation of β-Gal in GM1 gangliosidosis patient fibroblasts in a prelysosomal compartment, presumably at neutral pH, which coincides with activation of an unfolded protein response and endoplasmic reticulum (ER) stress (8). This gene therapy approach to augment β-Gal levels in GM1 gangliosidosis patient fibroblasts also results in overnormalization of the mature form of the enzyme in lysosomes (8). However, it remains unknown whether chronically overnormalizing β-Gal levels in GM1 gangliosidosis patient lysosomes impacts directly on the PPCA–NEU1–β-Gal multienzyme complex and the activities of each enzyme.

Here, we hypothesized that overnormalizing β-Gal levels in GM1 gangliosidosis patient fibroblasts may have the potential to displace NEU1 from the PPCA-containing multienzyme complex and promote secondary NEU1 deficiency. First, we tested an ERT approach, in which we monitored cell surface receptor–mediated endocytosis of purified rhβ-Gal and its delivery to lysosomes in primary cultures of skin fibroblasts. We compared this ERT approach with a lentiviral-mediated

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gene therapy approach to constitutively overexpress human GLB1 cDNA, with transcription driven from the cytomegalovirus (CMV) promoter (LV–CMV–GLB1). We found that continuous ERT with rhβ-Gal or sustained GLB1 overexpression both have the potential to promote a sustained secondary NEU1 deficiency in GM1 gangliosidosis patient fibroblasts. Given that NEU1 deficiency is associated with the related degenerative lysosomal storage disease sialidosis, we developed an intermittent ERT dosing regimen in GM1 gangliosidosis patient cells and in a mouse model of the disease to safely augment β-Gal levels without promoting secondary NEU1 deficiency. Collectively, our results suggest that β-Gal acts as negative regulator of NEU1 activity by displacing the labile sialidase from the PPCA chaperone in lysosomes.

Results

Cellular uptake of purified rhβ-Gal–His_6 and rhβ-gal cannot be overnormalized in GM1 gangliosidosis patient cells

We have previously demonstrated that research-grade rhβ-Gal produced in CHO cells and purified in our laboratory exhibits CI-MPR–dependent cellular uptake in GM1 gangliosidosis patient fibroblasts (8). In this study we also characterized the glycan profile of a commercial source of rhβ-Gal–His_6 (R&D Systems) and compared this with our research-grade rhβ-Gal produced and purified in-house (8). *N*-Linked oligosaccharide profiling of peptide *N*-glycosidase F–digested glycans by capillary zone electrophoresis demonstrate that commercial rhβ-Gal–His_6 produced in CHO cells or rhβ-Gal produced and purified in our laboratory contains multiple glycan moieties (Fig. 1, *A* and *B*, *black traces*). Some of the glycans present on both enzymes can be digested to nonphosphorylated glycans after treatment with alkaline phosphatase (Fig. 1, *A* and *B*, *blue traces*). Both enzyme preparations contain similar amounts of bis-phosphorylated oligomannose (BPM7) and mono-phosphorylated oligomannose (Fig. 1, *A* and *B*). The overall amount of phosphorylated oligomannose present on commercial rhβ-Gal–His_6 and in-house purified rhβ-Gal corresponds to 2.1 and 1.5 mol/mol of enzyme, respectively (Fig. 1*C*). These results suggest that both enzyme preparations contain similar amounts of phosphorylated oligomannose, the preferred glycan species for cation-independent mannose-6-phosphate receptor (CI-MPR)–dependent cellular uptake and lysosomal targeting.

Newly synthesized β-Gal has been reported to undergo a C-terminal proteolytic maturation step in lysosomes, releasing a ~20-kDa proteolytic fragment, a process that can be monitored by Western blotting and used as an indicator of enzyme delivery to lysosomes (8). No mutant β-Gal protein can be detected in primary skin fibroblasts established from an infantile-onset GM1 gangliosidosis patient by Western blotting (GM05653; Fig. 1*D*). Following cellular uptake for 24 h in normal and GM1 gangliosidosis patient fibroblasts, the majority of internalized commercial rhβ-Gal–His_6 is detected by Western blotting as a cleaved molecule of similar size to the endogenous mature lysosomal form of β-Gal detected in untreated normal fibroblasts (GM08399; Fig. 1*D*), with only a small amount of the precursor enzyme being detected (Fig. 1*D*). This would suggest that the majority of internalized commercial rhβ-Gal–His_6 is being rapidly delivered to lysosomes and converted to the mature lysosomal species following cellular uptake in both cell lines. Appearance of the mature lysosomal rhβ-Gal–His_6 species can be completely abolished in the presence of mannose-6-phosphate (Man6P; Fig. 1*D*), a known inhibitor of CI-MPR–dependent cellular uptake (Fig. 1*D*).

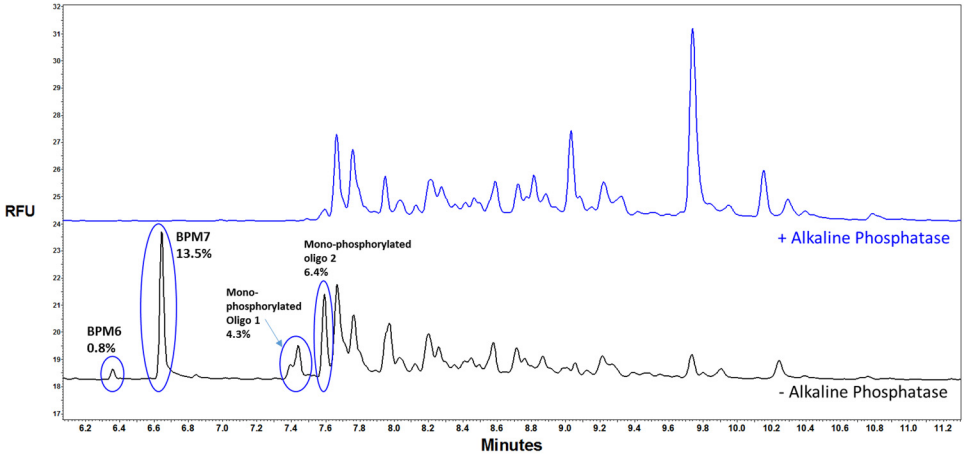
CI-MPR–mediated cellular uptake of commercial rhβ-Gal–His_6 in GM1 gangliosidosis patient fibroblasts is sufficient to increase β-Gal protein levels to levels that are comparable with endogenous β-Gal protein levels present in control fibroblasts from a normal individual, as determined by Western blotting (Fig. 1*D*). Interestingly, cellular uptake of commercial rhβ-Gal–His_6 or rhβ-Gal in normal fibroblasts results in β-Gal augmentation by only 1.56- and 2.67-fold above normal endogenous β-Gal activity levels, respectively (Fig. 1*E*). Collectively, these results suggest that our in-house purified rhβ-Gal (8) and a commercial source of rhβ-Gal–His_6 both exhibit CI-MPR–dependent cellular uptake in fibroblasts. Furthermore, the limited cellular uptake capacity for rhβ-Gal–His_6 and rhβ-Gal in normal fibroblasts differs from other purified lysosomal enzymes utilizing the CI-MPR pathway, where overnormalization of lysosomal enzyme activity by severalfold above normal can be achieved (9).

β-Gal cellular uptake and stability in lysosomes is partially dependent upon PPCA and cannot be overnormalized

β-Gal has been proposed to associate with PPCA and NEU1 in lysosomes to form a multienzyme complex, with each enzyme deficiency being associated with a specific neurodegenerative lysosomal storage disease (summarized in Fig. 2*A*) (1). In PPCA-deficient galactosialidosis patient cells, NEU1 is strictly dependent upon PPCA for delivery to lysosomes and activation in lysosomes, thus giving rise to secondary NEU1 deficiency in the absence of PPCA (Fig. 2*A*) (1, 6, 7). In contrast, β-Gal is partially dependent upon PPCA for stability in lysosomes, giving rise to only partial β-Gal deficiency in the absence of PPCA in galactosialidosis patient cells (Fig. 2*A*) (10).

To determine whether the limited cellular uptake potential of rhβ-Gal–His_6 in fibroblasts (Fig. 1*E*) is related to its dependence upon the multienzyme complex in lysosomes, we measured the cellular uptake and decay of lysosomal-delivered rhβ-Gal–His_6 in GM1 gangliosidosis fibroblasts, normal fibroblasts, and PPCA-deficient galactosialidosis patient fibroblasts. The cellular uptake capacity of rhβ-Gal–His_6 over 24 h is partially reduced in PPCA-deficient galactosialidosis fibroblasts (GM21262; V_{max} = 2647 nmol/h/mg; Fig. 2*B*), when compared with GM1 gangliosidosis patient fibroblasts (GM05653; V_{max} = 5484 nmol/h/mg; Fig. 2*B*) and WT fibroblasts (GM08399; V_{max} = 4550 nmol/h/mg; Fig. 2*B*). The partial cellular uptake capacity of rhβ-Gal–His_6 in galactosialidosis cells can be increased to normal levels, when galactosialidosis fibroblasts are preloaded with rhPPCA–His_6 for 24 h prior to enzyme uptake (V_{max} = 5265 nmol/h/mg; Fig. 2*B*).

A



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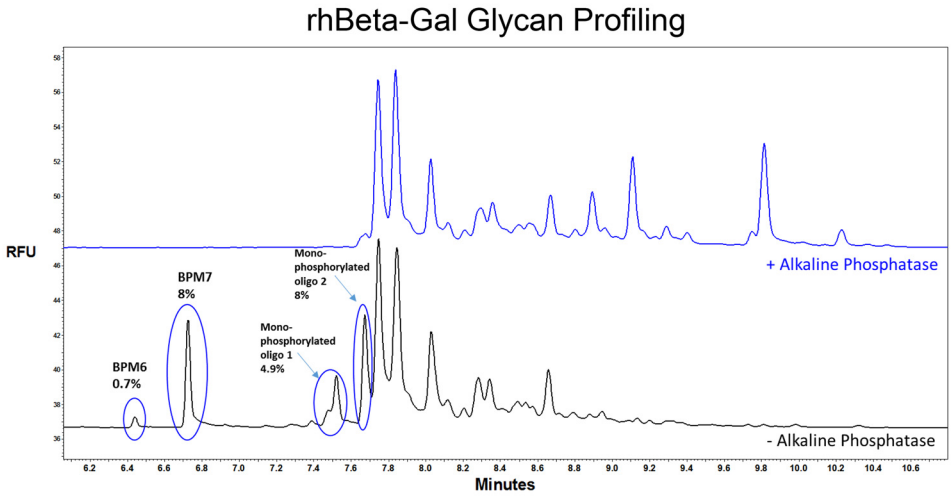
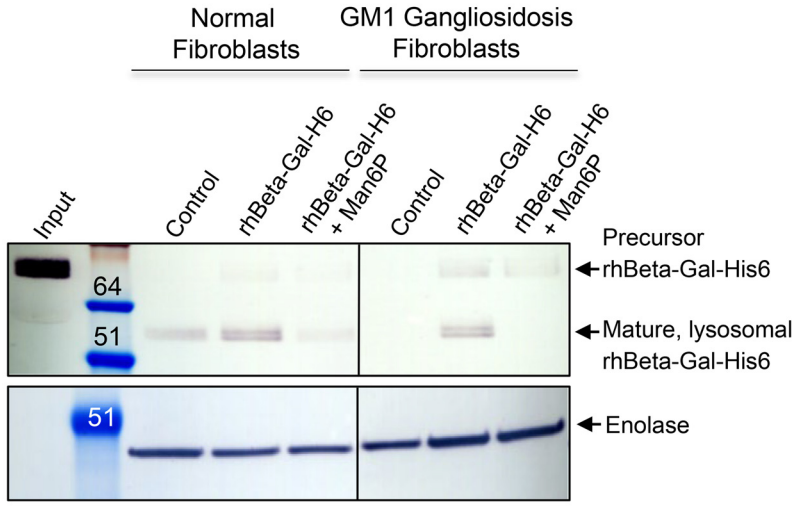


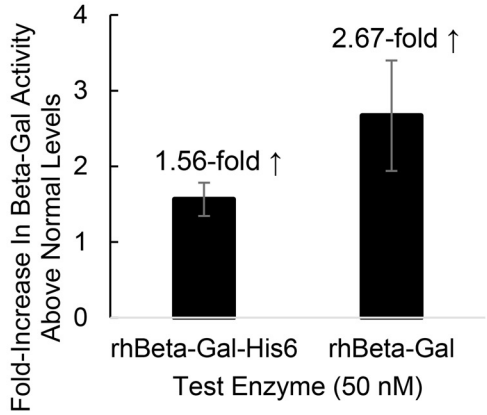
C

Product	Phosphorylated Glycan mol/mol
rhBeta-Gal	1.5
rhBeta-Gal-His6	2.0

D



E



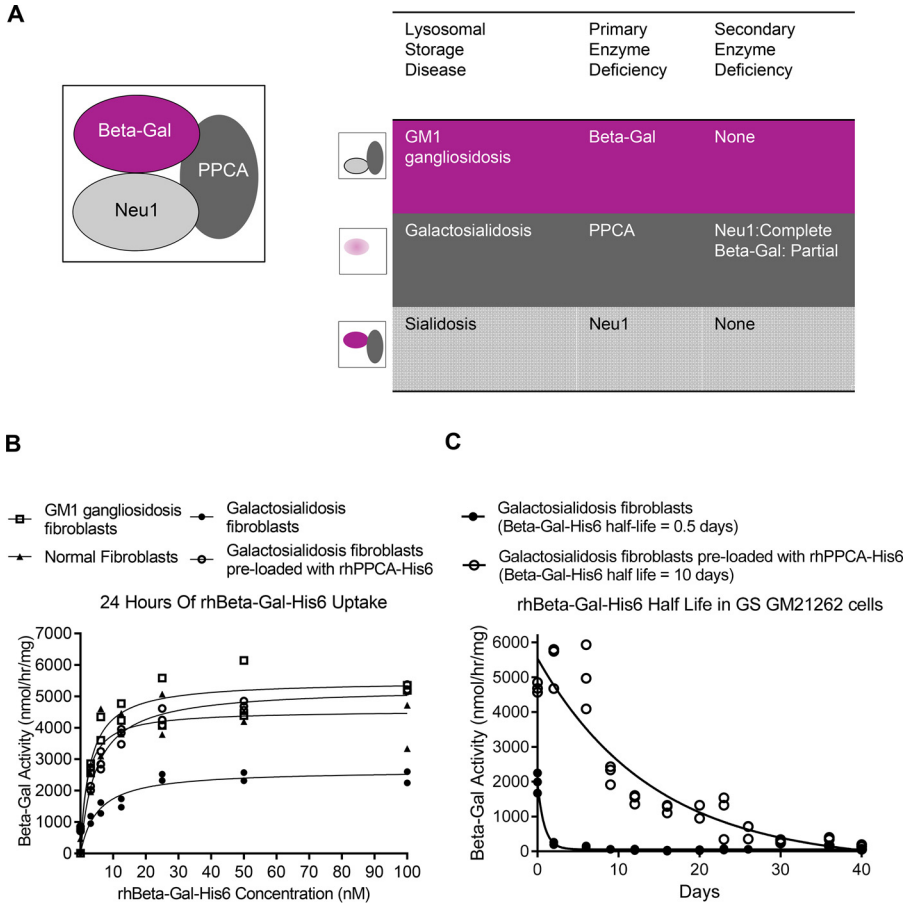


Figure 2. rhβ-Gal–His_6 cellular uptake and stability in lysosomes is partially dependent upon PPCA. *A*, schematic representation of the lysosomal PPCA–NEU1–β-Gal multienzyme complex. Deficiency of each enzyme gives rise to a specific lysosomal storage disease (1). Deficiency of PPCA chaperone in galactosialidosis patients leads to secondary NEU1 deficiency, because of its strict dependence upon PPCA for stability and only partial β-Gal deficiency. *B*, rhβ-Gal cellular uptake (24 h) in GM1 gangliosidosis fibroblasts, normal fibroblasts, and galactosialidosis fibroblasts. In some instances GS cells were also preloaded with rhPPCA–His_6 prior to uptake with rhβ-Gal, as described under "Experimental procedures." *C*, $t_{1/2}$ determination of rhβ-Gal–His_6 in galactosialidosis (*GS*) patient fibroblasts (*closed circles*) or GS fibroblasts preloaded for 16 h with 20 nM rhPPCA–His_6 (*open circles*), as described under "Experimental procedures."

Following 24 h of cellular uptake, lysosome-delivered rhβ-Gal–His_6 enzyme activity decays rapidly in PPCA-deficient galactosialidosis patient fibroblasts over a period of 40 days, with a $t_{1/2}$ corresponding to 0.5 days (Fig. 2*C*). In contrast, rhβ-Gal–His_6 stability is increased to 10 days in galactosialidosis patient fibroblasts preloaded with rhPPCA–His_6 for 24 h, prior to performing the $t_{1/2}$ experiment (Fig. 2*C*). Collectively, these experiments with PPCA-deficient galactosialidosis patient skin fibroblasts suggest that rhβ-Gal–His_6 cellular uptake and stability in lysosomes is partially dependent upon PPCA and cannot be overnormalized. These results are in agreement with those of Zhou *et al.* (10), which indicate that β-Gal activity is only partially reduced in various tissues of PPCA KO mice.

NEU1 activity in lysosomes is positively regulated by PPCA and negatively regulated by β-gal

Previous studies have demonstrated that NEU1 is strictly dependent upon its association with chaperone PPCA protein for its trafficking to lysosomes and stability (1, 6, 7). In contrast, β-Gal, which also complexes with PPCA, is only partially dependent on PPCA (1, 10). These observations led us to speculate that β-Gal mediates additional, novel roles in regulation of the multienzyme complex. A series of add-back experiments in PPCA-deficient galactosialidosis patient fibroblasts were designed to evaluate the role of β-Gal in the multienzyme complex. As expected, in the absence of PPCA, untreated galactosialidosis cells completely lack NEU1 activity (Fig. 3*B*). Cellular uptake of rhβ-Gal–His_6 alone in galactosialidosis fibroblasts

Figure 1. Cellular uptake of purified rhβ-Gal–His_6 and rhβ-Gal cannot be overnormalized in GM1 gangliosidosis patient cells. *A–C*, oligosaccharide analysis of rhβ-Gal–His_6 (*A*) or rhβ-Gal (*B*) treated without (*black trace*) or with (*blue trace*) alkaline phosphatase by capillary zone electrophoresis. The indicated tentative peak IDs assigned from co-migration with a known reference lysosomal enzyme standard were included in the run. *BPM6* or *BPM7*, bisphosphorylated oligomannose 6 or 7. *C*, calculated molar ratio of total phosphorylated glycan to protein. *D*, representative Western blotting of primary skin fibroblasts from a normal individual (WT) or an infantile-onset GM1 gangliosiodosis patient. Fibroblasts were incubated with growth medium (control) or 50 nM enzyme for 24 h in the absence in the presence of Man6P, as indicated. Precursor, nonlysosomal β-Gal, and mature lysosomal β-Gal bands are indicated. For comparison 4 ng of purified rhβ-Gal–His_6 was included on the gel as an indicator of precursor, nonlysosomal enzyme. Enolase was used as a loading control. *E*, cellular enzyme uptake capacity for rhβ-Gal–His_6 and rhβ-Gal in normal fibroblasts. Enzymes were incubated at a dose of 50 nM for 24 h, at which time cells were assayed for β-Gal activity, with activity expressed as the fold increase above endogenous levels of β-Gal activity detected in untreated normal fibroblasts.

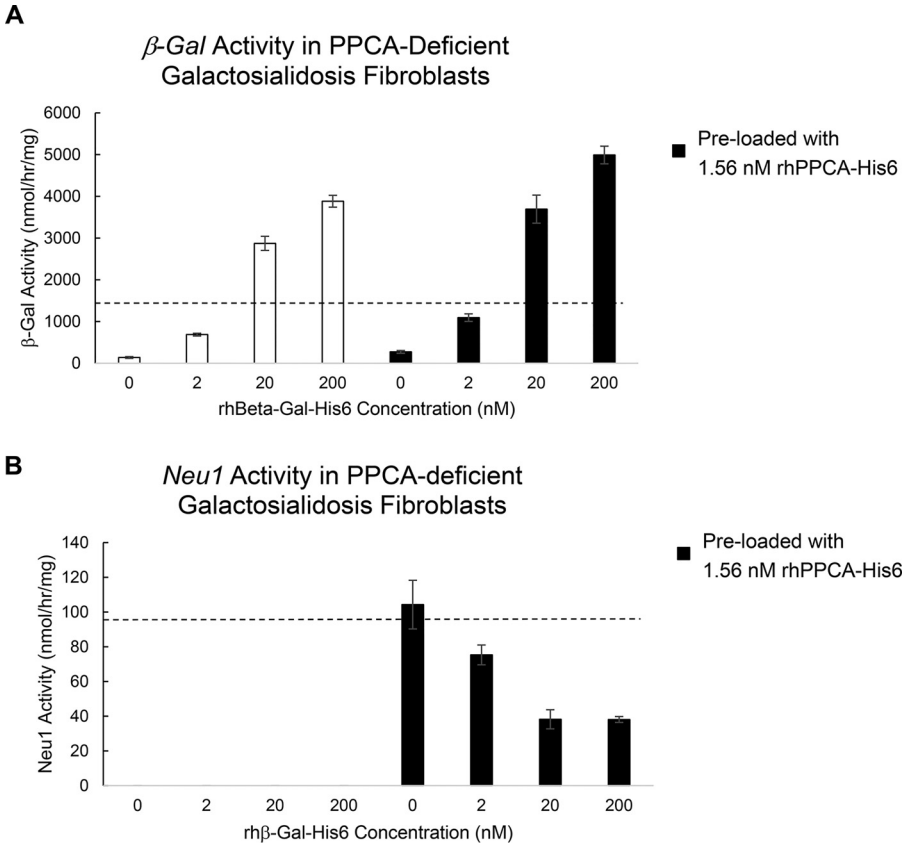


Figure 3. NEU1 activation in lysosomes is strictly dependent upon PPCA and negatively regulated by β-Gal. β-Gal activity (*A*) and NEU1 activity (*B*) in PPCA-deficient galactosialidosis patient fibroblasts following cellular uptake (24 h) with rhβ-Gal–His_6 only (*white bars*). In some instances, the cells were preloaded with 1.56 nM rhPPCA–His_6 for 16 h prior to being incubated with rhβ-Gal (*black bars*). The *dashed lines* represent the level of β-Gal activity (*A*) or NEU1 activity (*B*) detected in fibroblasts from a normal individual cultured for the same period of time.

results in a dose-dependent increase in β-Gal activity, which is increased further in cells preloaded with rhPPCA–His_6 (Fig. 3*A*). This result is not surprising in light of the partial dependence of β-Gal on PPCA for its stability in lysosomes (Fig. 2*C*) (1, 10). Cellular uptake of rhβ-Gal–His_6 in galactosialidosis patient fibroblasts does not augment NEU1 activity levels (Fig. 3*B*), suggesting that β-Gal does not positively regulate NEU1.

In galactosialidosis patient fibroblasts preloaded with rhPPCA–His_6, NEU1 activity levels are rescued close to NEU1 levels detected in fibroblasts from a normal individual (Fig. 3*B*). Interestingly, we show that this rescued NEU1 activity can be competitively reduced following endocytosis with increasing concentrations of rhβ-Gal–His_6 (Fig. 3*B*). Given that NEU1 is strictly dependent upon its association with PPCA for its activation, one can presume that increasing concentrations of lysosome-delivered rhβ-Gal–His_6 in galactosialidosis patient fibroblasts are directly or indirectly competing with and displacing the rescued NEU1 from PPCA. There are several lines of evidence to support a dynamic state, rather than a static state for the multienzyme complex. Bonten *et al.* (6) have reported that NEU1 exhibits weak self-association at acidic pH, which leads to its instability and rapid degradation, whereas association with PPCA leads to stable, long-lived PPCA-NEU1 heterodimers (6). In contrast to NEU1, our biophysical studies with rhβ-Gal suggest that β-Gal exhibits dynamic self-association and instability at neutral pH, with the enzyme being more stable as a dimer at acidic pH (8). Collectively, these results suggest that β-Gal plays a previously undescribed role in the PPCA–NEU1–β-Gal multienzyme complex, where it competes with NEU1 in a concentration-dependent manner for association with PPCA and thereby has the potential to negatively regulate sialidase levels (Fig. 3, *A* and *B*; see also Fig. 8 for a summary).

Continuous cellular uptake of rhβ-Gal–His_6 in GM1 gangliosidosis patient fibroblasts promotes a sustained secondary reduction in NEU1 activity, whereas intermittent ERT dosing does not

Our add-back studies in PPCA-deficient galactosialidosis fibroblasts (Fig. 3) suggest that β-Gal augmentation therapy has the potential to displace NEU1 from PPCA and promote secondary NEU1 deficiency. Because NEU1 deficiency is associated with the neurodegenerative lysosomal storage disease, sialidosis, we evaluated the potential for β-Gal augmentation therapy to promote secondary NEU1 deficiency in GM1 gangliosidosis patient fibroblasts. Chronic cellular uptake of rhβ-Gal–His_6 in GM1 gangliosidosis patient fibroblasts over 1 week restores β-Gal activity (Fig. 4*A*), which coincides with a dose-dependent reduction in NEU1 activity levels (Fig. 4*B*). The highest concentration of rhβ-Gal–His_6 cellular uptake tested (200 nM) coincides with almost complete loss of NEU1 activity (Fig. 4*B*), with the levels of NEU1 activity corresponding to 3%

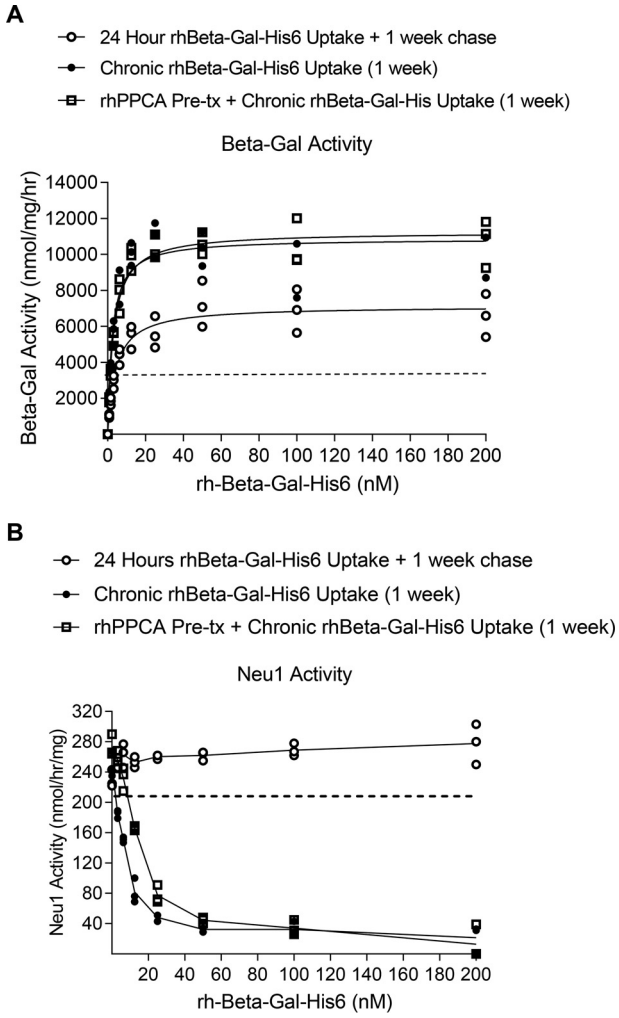


Figure 4. Chronic cellular uptake of rhβ-Gal–His_6 in GM1 gangliosidosis patient fibroblasts coincides with secondary NEU1 deficiency, which cannot be rescued with exogenous rhPPCA. β-Gal activity (*A*) and NEU1 activity (*B*) in GM1 gangliosidosis patient fibroblasts following continuous (chronic) cellular uptake of rhβ-Gal–His_6 for 1 week (*filled circles*). Alternatively, GM1 gangliosidosis patient fibroblasts were preloaded with 20 nM rhPPCA–His_6 for 16 h prior to chronic rhβ-Gal–His uptake for 1 week (*squares*). In a third group, GM1 gangliosidosis patient fibroblasts were incubated for 24 h with rhβ-Gal–His_6 and then chased in growth medium without enzyme for a further 1 week (*open circles*). The *dashed lines* represent the level of β-Gal activity (*A*) or NEU1 activity (*B*) detected in fibroblasts from a normal individual cultured for the same period of time.

of normal NEU1 activity detected in fibroblasts from a normal individual (Fig. 4*B*). Interestingly, preloading GM1 gangliosidosis patient fibroblasts with rhPPCA–His_6 does not further increase the uptake capacity of rhβ-Gal–His_6 (Fig. 4*A*) or prevent secondary NEU1 reduction (Fig. 4*B*), suggesting that factors other than PPCA are required to augment β-Gal levels in GM1 gangliosidosis patient lysosomes (Fig. 4*A*) and prevent secondary NEU1 deficiency (Fig. 4*B*).

Previous studies have suggested that the recycling time for the CI-MPR back to the cell surface for subsequent rounds of lysosomal enzyme targeting to be ~15 min (11), whereas we have previously shown that the rate of lysosomal-delivered β-Gal decay is much slower, with a $t_{1/2}$ of ~9 days (8). Therefore, with continuous exposure of GM1 gangliosidosis patient fibroblasts to rhβ-Gal over prolonged periods of time, the rate of β-Gal cellular uptake and delivery to lysosomes is likely to exceed the rate of β-Gal degradation, resulting in a net overall accumulation of β-Gal in lysosomes (Fig. 4*A*). To avoid inducing secondary NEU1 deficiency, we therefore developed an intermittent ERT dosing regimen to deliver a single pulsatile dose of rhβ-Gal–His_6 to lysosomes of GM1 gangliosidosis patient fibroblasts, followed by a 1-week chase in the absence of enzyme. Cellular uptake with rhβ-Gal–His_6 for 24 h, followed by enzyme withdrawal and a 1-week chase, is sufficient to augment β-Gal activity in GM1 gangliosidosis patient fibroblasts (Fig. 4*A*) without promoting secondary NEU1 deficiency (Fig. 4*B*).

Collectively, our results suggest that chronic cellular uptake and delivery of β-Gal to lysosomes coincides with secondary NEU1 deficiency, presumably because of NEU1 being displaced from PPCA in lysosomes by continuously delivered β-Gal (Fig. 4*B*). In contrast, intermittent dosing with rhβ-Gal–His_6 for 24 h, followed by withdrawal of the enzyme from the uptake medium and a 1-week chase, is sufficient to normalize lysosomal β-Gal levels without promoting secondary NEU1 deficiency (Fig. 4*B*). Finally, simply increasing PPCA levels in conjunction with β-Gal augmentation therapy in GM1 gangliosidosis patient fibroblasts is not sufficient to further augment β-Gal levels (Fig. 4*A*) or prevent NEU1 deficiency in GM1 gangliosidosis patient lysosomes (Fig. 4*B*), suggesting that factors in addition to PPCA are required to stabilize NEU1 levels.

Constitutive LV-mediated CMV promoter-driven GLB1 overexpression promotes a dose-dependent NEU1 deficiency

Although several studies have described GLB1 gene therapy approaches to augment *de novo* synthesis of rhβ-Gal in animal models of GM1 gangliosidosis (12–14), none of these approaches have thus far monitored the levels of other enzymes in the multienzyme complex. Chronic lentiviral-mediated, CMV promoter–driven GLB1 overexpression over a period of 8 days coincides with a dose-dependent increase in precursor and mature β-Gal protein being detected in three infantile-onset GM1 gangliosidosis patient fibroblast lines, suggestive of both prelysosomal and lysosomal accumulation of the overexpressed enzyme (Fig. 5, *A–C*, for quantification). We have shown that time-dependent accumulation of precursor, nonlysosomal rhβ-Gal coincides with up-regulation of an up-regulated unfolded protein response, as well as ER stress (8). Here we show that chronic lentiviral-mediated GLB1 overexpression and accumulation of precursor and mature rhβ-Gal over a period of 8 days also coincides with dose-dependent reduced levels of PPCA protein (Fig. 5, *A* and *D*, for quantification) and Neu1 protein by Western blotting (Fig. 5, *A* and *E*, for quantification). Lentiviral-mediated, CMV promoter-driven GLB1 gene therapy also promotes a time-dependent reduction in NEU1 protein levels, as detected by Western blotting (Fig. 6*E*).

We also monitored NEU1 activity levels in GM1 gangliosidosis patient cells following transduction with LV–CMV–GLB1. Chronic GLB1 overexpression in GM1 gangliosidosis patient fibroblasts results in dose-dependent and time-dependent increases in β-Gal activity levels being detected, with β-Gal activity corresponding to ~448% of normal β-Gal activity levels 21

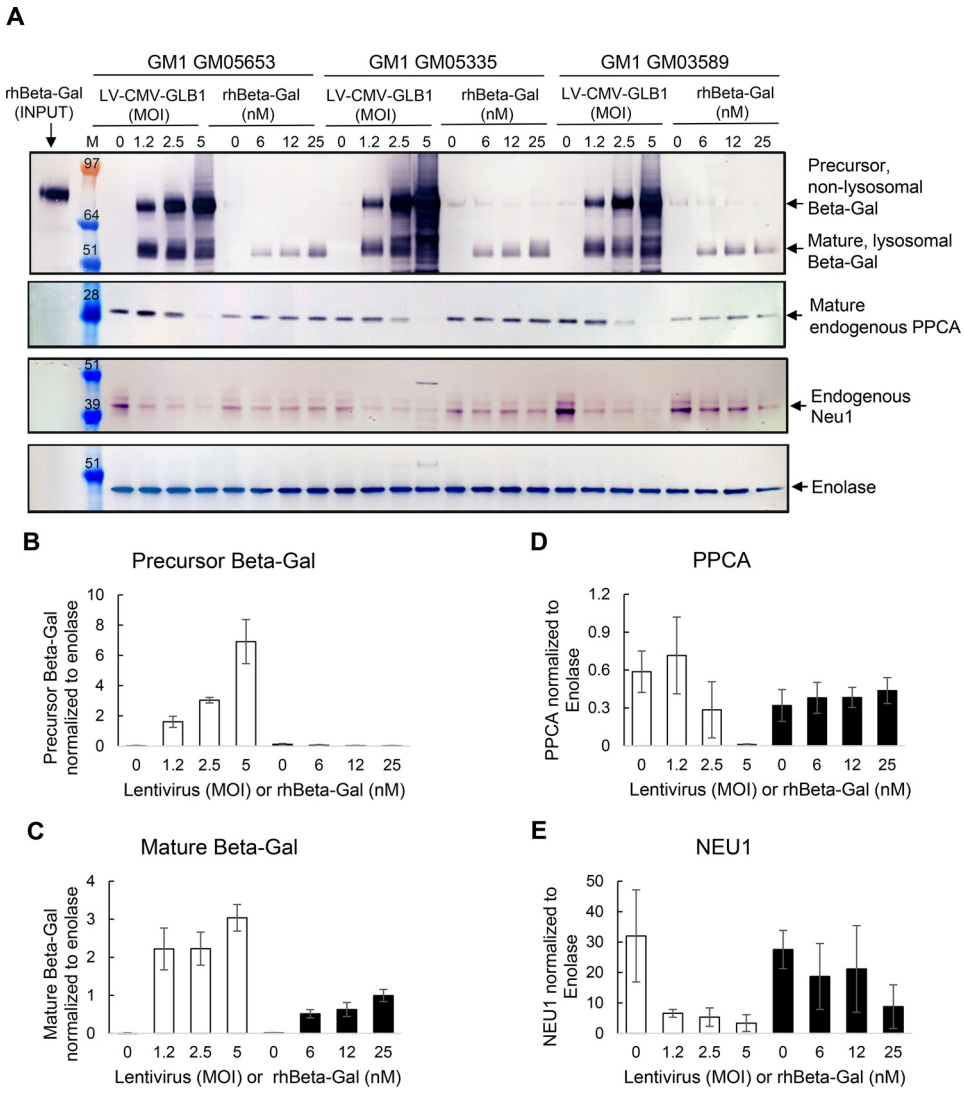


Figure 5. Constitutive lentiviral-mediated GLB1 overexpression negatively regulates PPCA and NEU1 protein levels in a dose-dependent manner, whereas intermittent ERT with rhβ-Gal does not. *A*, Western blots of cell lysates prepared from three infantile-onset GM1 gangliosidosis patient fibroblast lines 8 days after being transduced with LV–CMV–GLB1 (24 h transduction followed by 8-day chase; multiplicity of infection (MOI) = 1.25, 2.5, of 5) or 8 days after being incubated with purified rhβ-Gal (24 h of enzyme uptake followed by 8 day chase; 6.25, 12.5, or 25 nM). Note that the β-Gal panel and enolase panel shown here were also included in an earlier companion article (7), and have now been integrated with the new PPCA and Neu1 immunoblot panels. *B–E*, quantification of precursor β-Gal (*B*), mature β-Gal (*C*), PPCA (*D*), and NEU1 (*E*) protein levels detected in the three patient lines, standardized to the enolase loading control and expressed as the means ± S.D.

days after infection with the highest dose of lentivirus tested (Multiplicity of Infection = 1.25; Fig. 6*A*). The increased β-Gal activity detected in the chronic GLB1 gene therapy-treated cells (Fig. 6*A*) coincides with a dose-dependent and time-dependent reduction in NEU1 activity, down to pathological levels that are associated with sialidosis (Fig. 6*C*). After 21 days of GLB1 overexpression, the level of NEU1 activity is somewhat increased at the lower doses of virus, when compared with the 7-day time point (Fig. 6*C*). This could potentially be due to nontransduced cells outgrowing the culture, which was not evaluated further here. To control for potential lentivirus-mediated toxicity, we also assessed NEU1 protein levels in GM1 gangliosidosis patient fibroblasts transduced with lentivirus expressing the GFP reporter gene, with its expression being driven by the CMV promoter. Transduction of GM1 gangliosidosis patient fibroblasts with LV–CMV–GFP coincides with a time-dependent increase in GFP protein levels by Western blotting (Fig. 6*E*), without any noticeable impact on NEU1 protein levels (Fig. 6*E*). Collectively, these results (Figs. 5 and 6) suggest that similar to chronic cellular uptake of rhβ-Gal (Fig. 4), chronic viral-mediated GLB1 overexpression results in continuous delivery of β-Gal to lysosomes, which coincides with reduction in NEU1 activity and NEU1 protein levels down to pathological levels associated with sialidosis (1).

In contrast to the gene therapy approach, a pulsatile ERT approach with rhβ-Gal is sufficient to augment β-Gal activity in GM1 gangliosidosis patient fibroblasts (Fig. 6*B*) and restore NEU1 activity and protein levels over time (Fig. 6, *D* and *E*). Cellular uptake of rhβ-Gal for 24 h followed by an 8-day chase results in detection of only the mature from of rhβ-Gal by Western blotting (Fig. 5, *A* and *C*, for quantification). Cellular uptake of rhβ-Gal for 24 h results in β-Gal activity being

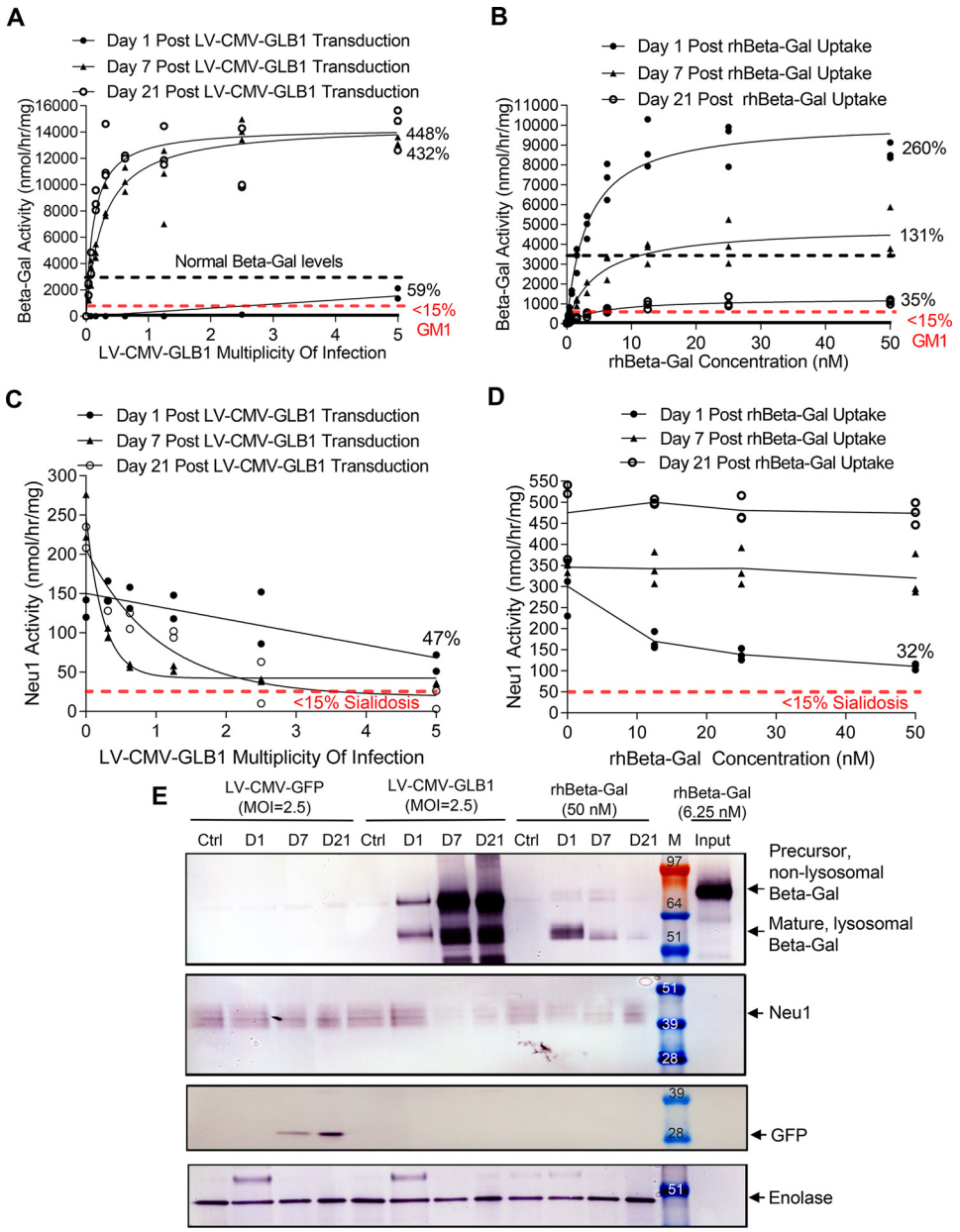


Figure 6. Constitutive lentiviral-mediated GLB1 overexpression promotes secondary NEU1 deficiency, whereas an intermittent ERT dosing regimen does not. *A–D*, GM1 gangliosidosis patient fibroblasts were transduced with LV–CMV–GLB1 for 24 h (*A* and *C*) or incubated with rhβ-Gal for 24 h (*B* and *D*). After 24 h, the cells were washed; chased for 1, 7, or 21 days; and then assayed for β-Gal activity (*A* and *B*) or NEU1 activity (*C* and *D*). A *red dashed line* on each graph represents the theoretical threshold, where the majority of lysosomal enzyme deficiencies are associated with onset of lysosomal storage disease. *E*, GM1 gangliosidosis patient fibroblasts (GM05653) were transduced for 24 h with LV–CMV–GFP or LV–CMV–GLB1 (multiplicity of infection (MOI) = 2.5) or incubated with rhβ-Gal (50 nM) for 24 h. The cells were then washed; chased in growth medium for 1, 7, or 21 days; and then analyzed by Western blotting for β-Gal and NEU1 protein levels, along with the GFP reporter and enolase loading control. rhβ-Gal (6.25 nM) was also included on the gel as an indicator of the precursor nonlysosomal form of β-Gal (*arrow*). The mature lysosomal β-Gal band is also indicated with an *arrow*. Note that the β-Gal, GFP, and enolase panels shown here were also included in an earlier companion manuscript (7) and have now been integrated with the new Neu1 immunoblot panel.

augmented to ~260% of β-Gal activity levels detected in normal fibroblasts at the highest dose of enzyme tested (50 nM; Fig. 6*B*). Following 24 h of cellular uptake with rhβ-Gal, washout of the enzyme, and a chase for 7 days, β-Gal activity can still be readily detected in GM1 gangliosidosis patient fibroblasts, with enzyme levels remaining normalized at the highest dose of enzyme tested (50 nM; 131% of β-Gal activity detected in normal fibroblasts; Fig. 6*B*). After 21 days, the level of β-Gal activity has decayed further, with the level of β-Gal activity corresponding to 35% of β-Gal activity detected in normal fibroblasts (Fig. 6*B*). The slow decay of lysosomal-delivered rhβ-Gal can also be observed by Western blotting, with the mature form of β-Gal being detected in GM1 gangliosidosis patient cells 21 days following cellular uptake with rhβ-Gal (Fig. 6*E*). These results are in agreement with our previous findings, which suggest that purified rhβ-Gal delivered to lysosomes by CI-MPR–mediated cellular uptake exhibits a $t_{1/2}$ of ~9 days, which coincides with slow decay of enzyme activity over several weeks (8). Furthermore, we have previously shown that the long $t_{1/2}$ of lysosomal-delivered rhβ-Gal is sufficient to

maintain substrate clearance in GM1 gangliosidosis patient cells for several weeks and that as little as 14% of normal β-Gal levels are sufficient to prevent substrate clearance (8).

It is important to point out that a partial reduction in NEU1 activity is observed in GM1 gangliosidosis patient cells immediately following cellular uptake with rhβ-Gal at day 1, particularly at the highest concentration tested (50 nM), corresponding to 32% of NEU1 activity detected in normal fibroblasts (Fig. 6*D*). This phenomenon could potentially occur at regions in close proximity to the site of rhβ-Gal administration in clinical trials for intracerebroventricular (ICV)–ERT in GM1 gangliosidosis patients. From a safety standpoint, after removing rhβ-Gal from the uptake medium and a 7- or 21-day chase, NEU1 activity levels (Fig. 6*D*) and NEU1 protein levels (Fig. 6*E*) are restored to normal levels. Collectively, these results suggest that with an intermittent ERT dosing regimen, rhβ-Gal has the potential to promote secondary NEU1 deficiency immediately following its cellular uptake and delivery to lysosomes. The 21-day chase studies shown in Fig. 6 (*B* and *D*) suggest that regularly spaced intervals between rhβ-Gal dosing helps to restore NEU1 activity levels over time, while maintaining β-Gal at levels that are sufficient to mediate substrate turnover. This is due to the slow decay of lysosomal-delivered β-Gal (~9 days), which coincides with very slow decay of the enzyme and substrate clearance for several weeks (8).

NEU1 activity is up-regulated in GLB1 KO mice, which can be restored to normal levels with weekly ICV dosing with rhβ-gal

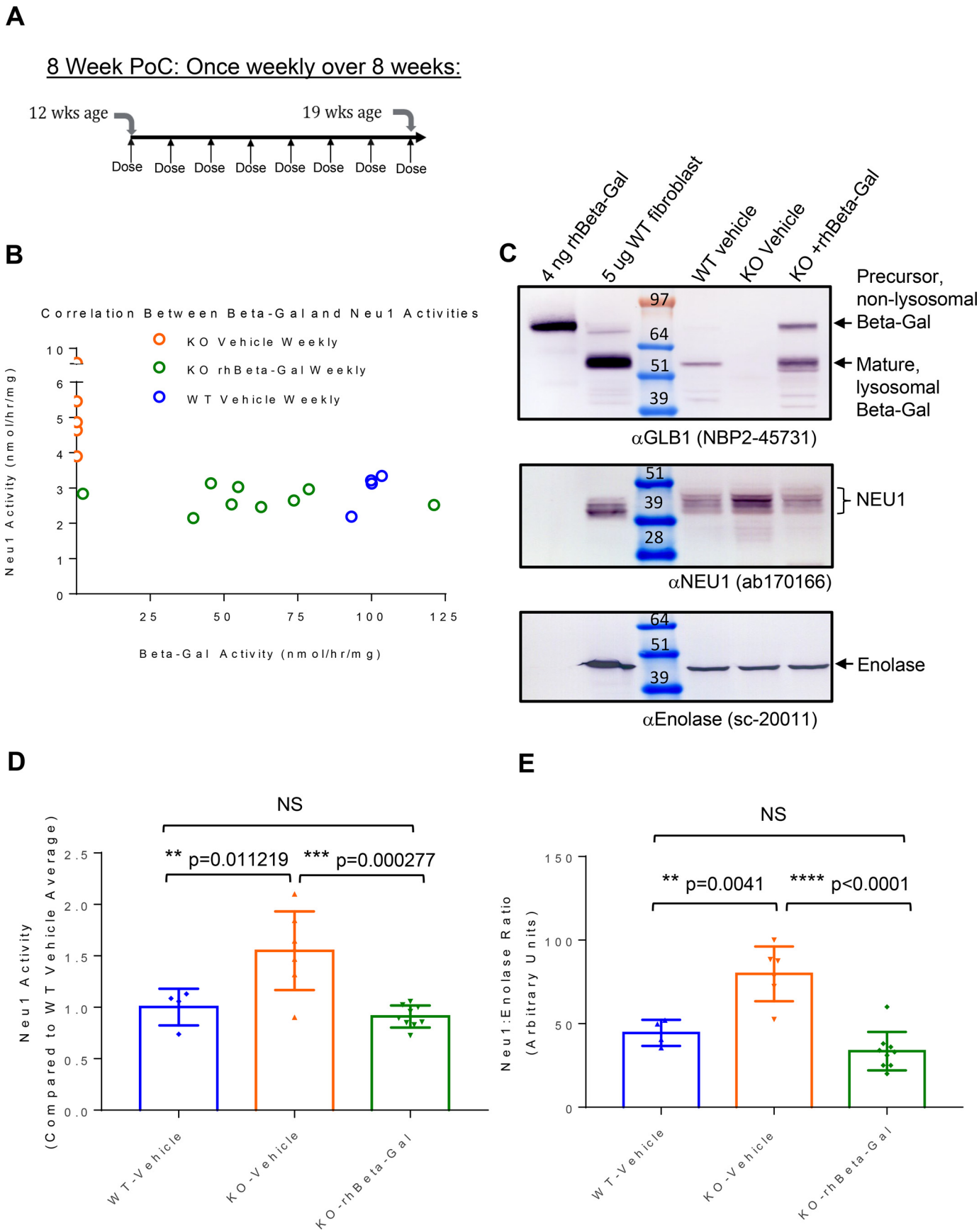
Our chronic β-Gal ERT augmentation studies in GM1 gangliosidosis patient cells (Fig. 4) and chronic gene therapy-mediated GLB1 overexpression studies in GM1 gangliosidosis patient cells (Figs. 5 and 6) suggest that continual β-Gal delivery to lysosomes has the potential to negatively regulate NEU1 activity and potentially promote a secondary NEU1 deficiency. In contrast, development of an intermittent ERT dosing regimen in GM1 gangliosidosis patient cells with a pulsatile dose of rhβ-Gal, followed by a 7-day "drug holiday" is sufficient to augment lysosomal β-Gal activity without promoting NEU1 deficiency for prolonged periods (Fig. 6, *B* and *D*). We therefore evaluated a weekly ICV dosing regimen with rhβ-Gal in a GLB1 KO mouse model of GM1 gangliosidosis (Fig. 7*A*) in an attempt to augment β-Gal levels in the brain in sufficient amounts to clear pathological substrates without promoting secondary NEU1 deficiency. Interestingly, NEU1 activity (Fig. 7, *B* and *D*, for quantification) and NEU1 protein levels (Fig. 7, *C* and *E*, for quantification) were significantly elevated in vehicle-treated GLB1 KO mouse brain tissue, when compared with vehicle-treated WT mouse brain tissue. These results are in agreement with our add-back studies in galactosialidosis patient cells (Fig. 3), further suggesting that β-Gal acts to negatively regulate NEU1 levels in the multienzyme complex, and in β-Gal-deficient GLB1 KO mice, NEU1 levels become elevated. Weekly ICV dosing with rhβ-Gal for 8 weeks commencing at 12 weeks of age (Fig. 7*A*) was sufficient to normalize β-Gal activity (Fig. 7*B*, *green circles*) and β-Gal protein levels (Fig. 7*C*) in brain tissues from rhβ-Gal–treated GLB1 KO mice (Fig. 7*B*, *green circles*) to similar β-Gal levels observed in WT-vehicle-treated mice (Fig. 7*B*, *blue circles*). Furthermore, weekly ICV dosing with rhβ-Gal is sufficient to restore NEU1 levels to normal levels, with the level of NEU1 activity (Fig. 7, *B* and *D*) and NEU1 protein (Fig. 7, *C* and *E*, for quantification) in the ERT-KO mice being lowered to similar NEU1 levels detected in the vehicle-treated WT group. Collectively, these results suggest that pulsatile ERT dosing with rhβ-Gal can safely augment β-Gal activity in patient cells without promoting secondary NEU1 deficiency for prolonged periods, an obvious safety advantage over gene therapy approaches, which have the potential to chronically disrupt the NEU1 from PPCA and promote secondary lysosomal enzyme deficiency.

Discussion

Our results suggest that although a chronic ERT dosing approach (Fig. 4), and a stable gene therapy approach (Fig. 6, *A*, *C*, and *E*) can augment β-Gal levels in GM1 gangliosidosis patient fibroblasts, both approaches also have the potential to promote secondary NEU1 deficiency, when β-Gal levels are augmented for prolonged periods. To circumvent this we developed an intermittent ERT dosing strategy to permit a pulsatile dose of lysosomal-delivered β-Gal to decay slowly over several weeks, which coincides with NEU1 activity levels being restored to normal levels over time (Figs. 4 and 6, *B* and *D*). A gene therapy approach could conceivably also be employed, where the dose and duration of β-Gal expression levels are regulated to ensure that NEU1 activity levels are not disrupted.

Add-back studies with purified rhPPCA and purified rhβ-Gal in PPCA-deficient galactosialidosis patient cells uncover novel insights into the PPCA–β-Gal–Neu1 multienzyme complex, suggesting that although NEU1 is strictly dependent upon PPCA for stability in lysosomes, β-Gal acts to negatively regulate NEU1, possibly by directly or indirectly competitively displacing this labile sialidase from PPCA (Fig. 3). Although our results in PPCA-deficient galactosialidosis patient fibroblasts suggest that β-Gal is partially dependent on PPCA for its stability in lysosomes (Figs. 2, *B* and *C*, and 3*A*), factors other than PPCA regulate this glycosidase, because cellular uptake of exogenous rhPPCA in GM1 gangliosidosis patient cells does not help to further increase rhβ-Gal cellular uptake (Fig. 4*A*).

Interestingly, we found that GLB1 KO mice exhibit significantly elevated levels of NEU1 in their brain tissue, when compared with WT control mice (Fig. 7, *B* and *C*, for quantification). These results are in line with the cell-based assays in this study, further supporting the contention that β-Gal acts as a negative regulator of NEU1 levels, presumably by displacing NEU1 from PPCA. Although it is not clear whether elevated NEU1 levels contribute to disease pathogenesis, overexpression of NEU1 has been proposed to contribute to impaired myogenesis in C2C12 cells and progression of atherosclerosis (15, 16). In contrast, a lack of NEU1 is associated with the severe neurodegenerative lysosomal storage disease, sialidosis. Substrates for NEU1 include LAMP-1 (17) and amyloid precursor protein (18), which have both been proposed to contribute to neurodegenerative disease (18). These observations warrant careful monitoring of NEU1 levels during ERT clinical trials with rhβ-


A
8 Week PoC: Once weekly over 8 weeks:
12 wks age
19 wks age
Dose Dose Dose Dose Dose Dose Dose Dose
B
Correlation Between Beta-Gal and Neu1 Activities
KO Vehicle Weekly
KO rhBeta-Gal Weekly
WT Vehicle Weekly
Neu1 Activity (nmol/hr/mg)
Beta-Gal Activity (nmol/hr/mg)
C
4 ng rhBeta-Gal
5 ug WT fibroblast
WT vehicle
KO Vehicle
KO +rhBeta-Gal
Precursor, non-lysosomal Beta-Gal
Mature, lysosomal Beta-Gal
αGLB1 (NBP2-45731)
NEU1
αNEU1 (ab170166)
Enolase
αEnolase (sc-20011)
D
NS
** p=0.011219
*** p=0.000277
Neu1 Activity (Compared to WT Vehicle Average)
WT-Vehicle
KO-Vehicle
KO-rhBeta-Gal
E
NS
** p=0.0041
**** p<0.0001
Neu1:Enolase Ratio (Arbitrary Units)
WT-Vehicle
KO-Vehicle
KO-rhBeta-Gal

Gal or with gene therapy for GM1 gangliosidosis to ensure that NEU1 levels remain at physiological levels.

Although the PPCA–NEU1–β-Gal multienzyme complex has been proposed to contain all three enzyme components (1) (summarized in Fig. 8*A*), a recent study from the d'Azzo laboratory has proposed that the multienzyme complex exists in a dynamic state, which is in constant flux and sensitive to the concentrations of the various enzyme components (6). Our results in this study point toward two distinct pools of PPCA being available in lysosomes that can bind to Neu1, as well as β-Gal. We propose that both pools of PPCA share a common binding site for β-Gal and NEU1, which can be competitively displaced by either of the two enzymes (Fig. 8). When β-Gal dimer and NEU1 monomer are present in equimolar amounts in lysosomes, PPCA is associated equally with both enzymes, resulting in normal physiological levels of both enzymes (Fig. 8*B*). In GM1 gangliosidosis cells, β-Gal is absent, which may lead to increased association of NEU1 with PPCA (Fig. 7*C*). This model could potentially explain why we observe significantly elevated levels of NEU1 activity in GLB1 KO mice, when compared with WT controls (Fig. 7, *B* and *C*, for quantification). Our results suggest that chronic delivery of β-Gal to lysosomes leads to dose-dependent displacement of NEU1 from PPCA by the excess β-Gal and onset of secondary NEU1 deficiency (Fig. 8*D*). This can be avoided with an intermittent ERT dosing regimen, which permits the slow decay of lysosomal-delivered β-Gal over several weeks, allowing newly synthesized PPCA chaperone protein to be readily available to associate with NEU1 to promote its stability (Fig. 8*E*).

An additional safety issue to consider for GM1 gangliosidosis gene therapy clinical trials is the propensity for β-Gal to mislocalize in the ER, when overexpressed. Previous studies have demonstrated that endogenous mutant missense β-Gal localizes poorly to lysosomes of human and feline GM1 gangliosidosis fibroblasts and instead localizes to the ER, which coincides with increased levels of GRP78/BiP and PDI protein levels, suggestive of an up-regulated unfolded protein response (19). The same study suggests that rhβ-Gal stably overexpressed at supranormal levels in feline GM1 gangliosidosis fibroblasts predominantly co-localizes with PDI, suggestive of ER retention (19). Our recently published results (8) are in agreement with these observations, with time-dependent increased accumulation of the precursor form of rhβ-Gal in cells stably overexpressing GLB1, coinciding with increased PDI/Grp78 protein levels and CHOP translocation to the nucleus (8). We link this to dynamic self-association of rhβ-Gal, which occurs more at neutral pH and leads to instability of the monomer, whereas under acidic conditions we observe predominantly a stable dimer (10). In this study we show that chronic delivery of β-Gal to lysosomes of GM1 gangliosidosis patient cells, either by chronic gene therapy or chronic ERT, can promote secondary NEU1 deficiency. Collectively, these observations emphasize the importance of carefully titrating the extent and duration of GLB1 gene expression, when employing gene therapy approaches to treat GM1 gangliosidosis and that a tunable ERT approach may help to circumvent the ER and directly deliver a long-lived β-Gal dimer to lysosomes (8). Furthermore, to avoid displacing NEU1 from the PPCA multienzyme complex, our results presented here suggest that a tunable, intermittent ERT dosing regimen with purified rhβ-Gal can safely augment β-Gal activity in GM1 gangliosidosis patient lysosomes without promoting secondary NEU1 deficiency for prolonged periods.

Experimental procedures

Materials

Rhβ-Gal protein was purified from supernatants of stably transfected Chinese hamster ovary (CHO) cells generated with the GS Mammalian Gene Expression System (Lonza Biologics) as previously described (8). rhβ-Gal–His_6 and rhPPCA–His_6 were purchased from R&D Systems. Antibodies for Western blotting were purchased from a variety of vendors: anti–β-Gal (Novus, NBP2-45731; and R&D Systems, MAB6464), anti-enolase (Santa Cruz Biotechnology, sc-15343), anti-PPCA (Abcam, ab111752), and anti-NEU1 (Abcam, ab170166). Fibroblast cells lines used in this paper were all obtained from the Coriell Institute for Medical Research: GM1 gangliosidosis (GM05653), galactosialidosis (GM21262), and WT (GM08399).

Glycan analysis of rhβ-gal and rhβ-Gal-His_6

Approximately 80 μg of β-Gal were digested using peptide:*N*-glycosidase F overnight. The recovered oligosaccharides were derivatized by reductive amination with the fluorescent dye 8-aminopyrene-1,3,6-trisulfonic acid. The oligosaccharides were resuspended in 3 μl of the supplied 8-aminopyrene-1,3,6-trisulfonic acid in citric acid followed by a vigorous mixing. Then 7 μl of sodium cyanoborohydride was added, followed by another vigorous mixing. The labeling reaction was incubated at 37 °C overnight. Excess dye is removed from the labeling mixture by packing a low speed Sephadex G10 gel column followed by addition of the labeling reaction amd spinning for 3 min at less than 1000 rpm. Oligosaccharide profiling experiments were done to monitor the global oligosaccharide content of β-Gal. Profiling glycans by capillary electrophoresis consists of releasing oligosaccharides enzymatically using the endoglycosidase peptide:*N*-glycosidase F, followed by a precapillary

Figure 7. NEU1 activity is up-regulated in GLB1 KO mice, which can be restored to normal levels with weekly ICV dosing with rhβ-Gal. *A*, summary of the 8-week short-term proof-of-concept (*PoC*) ICV-ERT studies evaluated in the GM1 gangliosidosis mouse model. The 8-week proof-of-concept study commenced at 12 weeks of age, with mice receiving weekly ICV dosing (100 μg/dose) with rhβ-Gal or vehicle for 8 weeks. The mice were taken down 24 h after the final ICV dose of enzyme. *B*, correlation between NEU1 activity (*y* axis) and β-Gal activity (*x* axis) in brain homogenates prepared from individual mice in the 8-week proof-of-concept study. WT vehicle, $n = 4$; KO vehicle, $n = 5$; KO-rh β-Gal, $n = 9$. *C*, Western blotting of β-Gal protein levels and NEU1 protein levels in pooled brain homogenates prepared from the left hemisphere of WT or GLB1 KO mice treated with vehicle or rhβ-Gal, as summarized for *A*. For comparison, 4 ng of purified rhβ-Gal I is included on the gel as an indicator of precursor, nonlysosomal enzyme. Also included is 5 μg of cell lysate prepared from WT human fibroblasts, as an indicator of mature β-Gal successfully delivered to lysosomes. Enolase was used as a loading control. Note that the β-Gal and enolase panels shown here was also included in an earlier companion article (7), which has now been integrated with the new Neu1 immunoblot panel. *D*, Neu1 activity expressed as fold above normal levels in the three test groups. *E*, quantification of Neu1 protein levels in brain homogenates from Western blots of individual samples, standardized to an enolase loading control. WT vehicle, $n = 4$; KO vehicle, $n = 5$; KO-rhβ-Gal, $n = 9$. ***, statistically significant with *p* values indicated. *NS*, not significant.

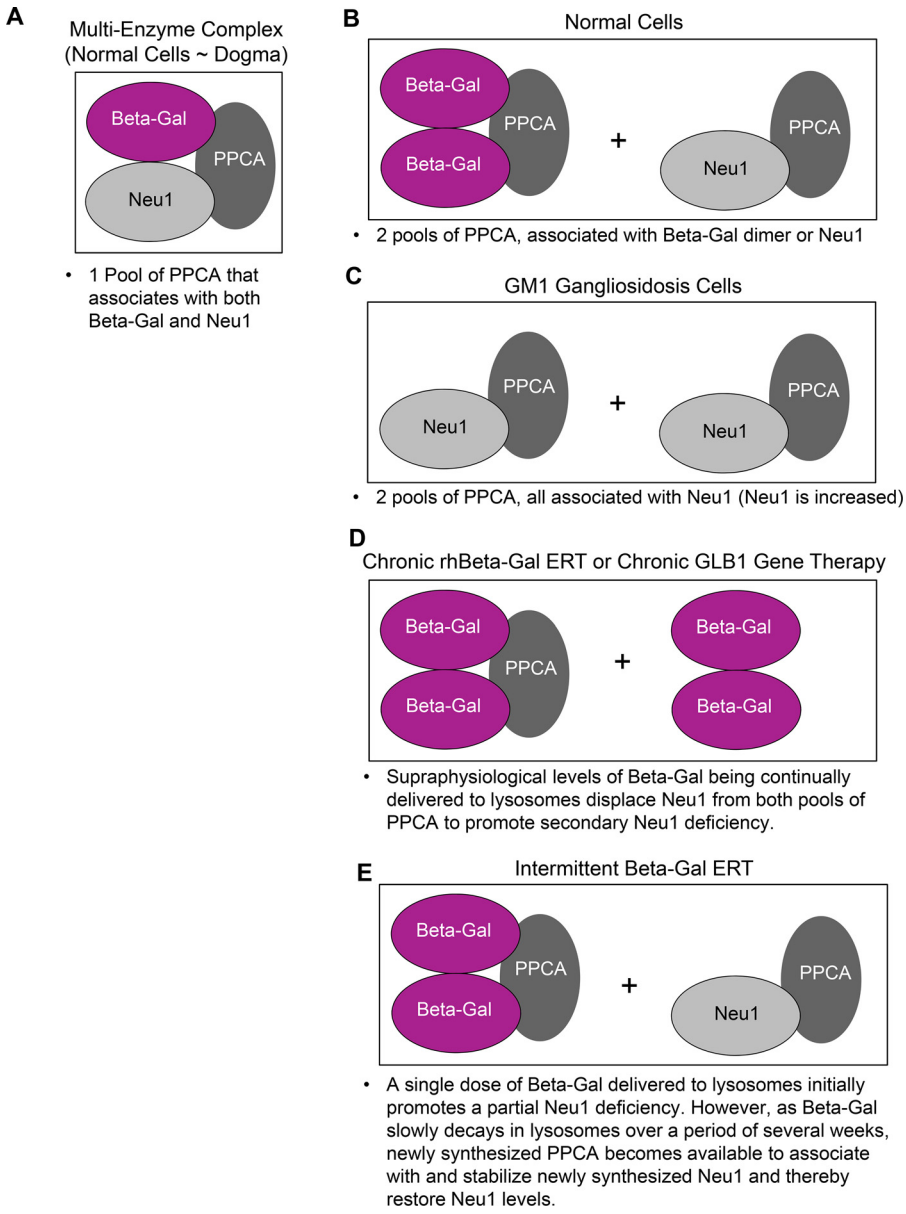


Figure 8. Summary of the findings of this study. *A*, β-Gal is reported to exist in a multienzyme complex with PPCA and NEU1 in lysosomes, with NEU1 being strictly dependent upon PPCA chaperone for its stability and activity in lysosomes and β-Gal only being partially dependent upon PPCA (1). However, we have recently demonstrated that β-Gal is most likely to be a stable dimer in the acidic environment of the lysosome (7), suggesting that the multienzyme complex may in fact be more dynamic and heterogeneous than represented here. *B–D*, our cellular uptake results suggest that NEU1 and β-Gal compete with each other for association with PPCA, resulting in two pools of PPCA, either associated with β-Gal dimer or NEU1. *B*, our results suggest that in normal cells β-Gal dimer and NEU1 are expressed at levels where both enzymes can associate with PPCA, resulting in physiological levels of β-Gal and NEU1. *C*, in the absence of β-Gal in GM1 gangliosidosis patient cells, more PPCA can associate with NEU1, resulting in the potential for NEU1 to be overnormalized. In support of this we observed a significantly increased level of NEU1 activity (Fig. 7*D*) and NEU1 protein (Fig. 7*E*) in GLB1 KO mouse brain homogenates above normal WT controls. *D*, in contrast, in GM1 gangliosidosis patient fibroblasts continually overexpressing β-Gal from the CMV promoter (chronic GLB1 gene therapy; Fig. 6*A*, 6*C*) or in cells chronically endocytosing rhβ-Gal from the cell surface (chronic rhβ-Gal ERT; Fig. 4), lysosome-delivered β-Gal has the potential to displace NEU1 from PPCA chaperone and promote secondary NEU1 deficiency. Furthermore, in our gene therapy studies in patient cells, chronic overexpression of β-Gal coincides with a reduction in PPCA levels as well as NEU1 levels (Fig. 5*A*, 5*D*), suggesting that overexpressed β-Gal has the potential to also negatively regulate PPCA chaperone, in addition to NEU1. *E*, to avoid disrupting NEU1 from PPCA, we developed an intermittent ERT dosing strategy to augment β-Gal levels in GM1 gangliosidosis patient cells without promoting secondary NEU1 deficiency. We show in GM1 gangliosidosis patient cells that cellular uptake of rhβ-Gal over 24 h can normalize β-Gal activity levels (Fig. 6*B*), which coincides with a partial reduction in NEU1 activity (Fig. 6*D*). Following withdrawal of the rh β-Gal from the uptake medium and a 3-week chase, the lysosomal-targeted rhβ-Gal slowly decays in GM1 gangliosidosis patient skin fibroblasts (Fig. 6*B*), which coincides with restoration of NEU1 activity to normal levels (Fig. 6*D*). Weekly ICV-ERT dosing with rhβ-Gal is also sufficient to normalize β-Gal levels in brain tissue of a mouse model of GM1 gangliosidosis and helps to restore NEU1 activity to normal levels (Fig. 7).

derivatization with a charged fluorophore. This labeling reaction occurs through reductive amination by reaction of the amino group of the fluorophore dye and the aldehyde of the reducing termini of the released oligosaccharides. Once labeled, the oligosaccharides are separated electrophoretically and detected by laser-induced fluorescence. An example oligosaccharide profile derived from recombinant β-Gal generates a complex electropherogram with at least 24 oligosaccharide peaks. In some instances, oligosaccharides were treated with alkaline phosphatase prior to electrophoresis.

Electrophoresis was performed on the CESI 8000 Plus (Sciex) using laser-induced fluorescence. The laser excitation wavelength was 488 nm. A 65-cm N-CAP coated capillary with a 50-μm inner diameter was used along with a Sciex kit supplied N-CAP buffer. The capillary was prerinsed with buffer at 40 p.s.i. for 1 min. A "water pillow" was injected at 0.5 p.s.i. for 20 s, followed by sample injections at 0.8 p.s.i. for 5.0 s. A second "water pillow" was injected at 0.1 p.s.i. for 10 s following the sample loading. Separation voltage was set to 21 kV for 20 min. The capillary temperature was set to 25 °C. The raw data were processed using 32 Karat software.

Analysis of β-gal uptake in patient fibroblasts

Patient fibroblast lines were obtained from the Coriell Institute for Medical Research and maintained in GM1 complete medium: Eagle's minimum essential medium (ATCC, catalog no. 30-2003) supplemented with 15% fetal bovine serum (not heat-inactivated), 1 × pen/strep (Thermo, catalog no. 15140122), and 1× GlutaMAX (Thermo, catalog no. 35050-061). Cellular uptake experiments with rhβ-Gal–His_6 or rhβ-Gal were performed for 24 h in the presence of absence of 8 mM Man6P. β-Gal activity in cell lysates was monitored by activity against 4-methylumbelliferyl-β-D-galactopyranoside fluorogenic substrate (Sigma, catalog no. M1633). The cell lysates were incubated with 4 mM 4-methylumbelliferyl-β-D-galactopyranoside fluorogenic substrate in assay buffer (100 mM sodium citrate, 250 mM NaCl, 1% Triton X-100, 0.2% BSA, pH 4.5) and then incubated for 40 min at 37 °C. The reaction was terminated with stop buffer (0.5 M glycine, 0.3 M NaOH, pH 10.3), and fluorescence was read on Spectramax i3 plate reader (Molecular Devices) with excitation emission of 355/460 nm, respectively. Serial dilutions of 4-methylumbelliferone (4MU; Sigma, catalog no. 1381) were used to establish a standard curve and calculate β-Gal activity in samples. To determine the V_{max} and K_{uptake}, enzyme activity individual β-Gal activity values were plotted using Michaelis–Menten analysis within GraphPad Prism software, with a single value assigned. For $t_{1/2}$ determination GM05653 cells were incubated with a low nanomolar dose of rhβ-Gal (6.25 nM) for 18 h and then washed several times to remove any noninternalized enzyme, and fresh growth medium was added to chase. β-Gal activity was assayed roughly every 1–2 weeks, over a period of 6 weeks. $t_{1/2}$ was determined by plotting β-Gal activity into GraphPad Prism software and using nonlinear exponential decay regression.

For preloading experiments with rhPPCA–His_6, the cells were incubated with the chaperone protein for 16 h and then rinsed several times to remove all traces of rhPPCA–His. Preloaded cells were then incubated with rhβ-Gal–His_6, as described above.

Animals and ICV cannula implantation

The GLB1 null mouse (GLB1 KO; GM1 mouse) was generated as described by Hahn *et al*. (20). Founder mice were transferred from Sandra d'Azzo's laboratory at St. Jude Children's Research Hospital (Memphis, TN, USA) to the Jackson Laboratories for rederivation prior to importation to the BioMarin vivarium at the Buck Institute for Research and Aging (Novato, CA, USA). The studies conducted herein were approved by the Institutional Animal Care and Use Committee of the Buck Institute. All details of ICV dosing with rhβ-Gal in GLB1 KO mice and β-Gal activity measurements in mouse brain tissue have been previously described in the companion article (7).

NEU1 activity determination, Western blotting

For detection of Neu1 activity, the cells were lysed in water on ice for 10 min and then immediately incubated with 4-methylumbelliferyl-α-D-*N*-actylneuraminic acid sodium salt (Sigma, catalog no. M8639) for 1 h at 37 °C. The reaction was stopped with stop buffer (0.5 M $NaHCO_3$, pH 10.7), and fluorescence was read at excitation/emission of 355/460 nm, respectively. Serial dilutions of 4MU standards were utilized to generate a standard curve to calculate Neu1 activity.

For detection of Neu1 activity in tissue samples, brain tissue was homogenized in HPLC-grade water with a Bullet Blender (Next Advance) in a cold room set to 4 °C. The homogenate was immediately assayed for Neu1 activity as described above. To prepare the 4MU-Neu1 substrate 15 mg of 4-methyumbelliferyl-α-D-*N*-acetylneuraminic acid, sodium salt was dissolved in 15 ml of cell-culture water in a 50-ml Falcon tube. 15 ml of water-saturated methyl-*tert*-butyl ether was then added, and the tube was shaken vigorously. The upper phase was then discarded. This process was repeated this with a further 15 ml of water-saturated ether two more times (a total of three extractions). The 4MU-NEU1 substrate was then place on ice, with nitrogen gas bubbling through the substrate solution for ~2 h, until the solution had lost almost all of the ether smell. The substrate was stored in aliquots (500 μl) at −80 °C. NEU1 protein was also examined by Western blotting using a NEU1 antibody from Abcam (catalog no. ab170166).

Data availability

All data are contained within the article.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ERT, enzyme replacement therapy; ICV, intracerebroventricular; CHO, Chinese hamster ovary; β-Gal, β-galactosidase; PPCA, protective protein cathepsin A; NEU1, neuraminidase 1; Man6P, mannose-6-phosphate;

CI-MPR, cation-independent mannose 6-phosphate receptor; rh, recombinant human; ER, endoplasmic reticulum; CMV, cytomegalovirus; 4MU, 4-methylumbelliferone.

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