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β-Glucosidase 2 (GBA2) Activity and Imino Sugar Pharmacology*

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Background: GBA2 and GBA are both β-glucosidases that degrade glucosylceramide.
Results: Conduritol B epoxide inactivates both GBA and GBA2, whereas the imino sugar *N*B-DGJ selectively inhibits GBA2.
Conclusion: *N*B-DGJ is a suitable reagent to distinguish GBA2 from GBA.
Significance: This study redefines GBA2 activity, which is relevant for clinical GBA2 measurements and imino sugar pharmacology.

β-Glucosidase 2 (GBA2) is an enzyme that cleaves the membrane lipid glucosylceramide into glucose and ceramide. The *GBA2* gene is mutated in genetic neurological diseases (hereditary spastic paraplegia and cerebellar ataxia). Pharmacologically, GBA2 is reversibly inhibited by alkylated imino sugars that are in clinical use or are being developed for this purpose. We have addressed the ambiguity surrounding one of the defining characteristics of GBA2, which is its sensitivity to inhibition by conduritol B epoxide (CBE). We found that CBE inhibited GBA2, *in vitro* and in live cells, in a time-dependent fashion, which is typical for mechanism-based enzyme inactivators. Compared with the well characterized impact of CBE on the lysosomal glucosylceramide-degrading enzyme (glucocerebrosidase, GBA), CBE inactivated GBA2 less efficiently, due to a lower affinity for this enzyme (higher K_I) and a lower rate of enzyme inactivation (k_{inact}). In contrast to CBE, *N*-butyldeoxygalactonojirimycin exclusively inhibited GBA2. Accordingly, we propose to redefine GBA2 activity as the β-glucosidase that is sensitive to inhibition by *N*-butyldeoxygalactonojirimycin. Revised as such, GBA2 activity 1) was optimal at pH 5.5–6.0; 2) accounted for a much higher proportion of detergent-independent membrane-associated β-glucosidase activity; 3) was more variable among mouse tissues and neuroblastoma and monocyte cell lines; and 4) was more sensitive to inhibition by *N*-butyldeoxynojirimycin (miglustat, Zavesca®), in comparison with earlier studies. Our evaluation of GBA2 makes it possible to assess its activity more accurately, which will be helpful in analyzing its physiological roles and involvement in disease and in the pharmacological profiling of monosaccharide mimetics.

Glucosylceramide (GlcCer)[3] is a ubiquitous eukaryotic glycosphingolipid (GSL) that is present on the cytoplasmic face of cellular membranes and on the cell surface (1–4). GlcCer is synthesized by the ceramide-specific glucosyltransferase (UGCG) (5, 6) and is degraded by glucocerebrosidase (GBA) (7) as well as by β-glucosidase 2 (GBA2), which is also known as bile acid β-glucosidase, and the nonlysosomal glucosylceramidase(Fig. 1*A*, Table 1) (8–10). GBA is a lysosomal enzyme, whereas GBA2 is present at the plasma membrane and/or the endoplasmic reticulum (8, 10, 11). These two β-glucosidases have distinct amino acid sequences and belong to different glycoside hydrolase families (Table 1). Deficiencies in GBA and GBA2 result in the accumulation of glucosylceramide, which, in the case of GBA, leads to Gaucher disease, a genetic disorder affecting the reticuloendothelial system and, in severe cases, the central nervous system (12).

GBA2-deficient mice are viable and do not exhibit overt pathology, except for male infertility (10). Nevertheless, GBA2 has recently been implicated in a number of diseases. Mutations in the *GBA2* gene have been found in patients with hereditary spastic paraplegia (13) and autosomal-recessive cerebellar ataxia (14). In zebrafish, GBA2 knockdown impaired motor behavior and axonal outgrowth of motor neurons (13). GBA2 thus appears to be essential for neuronal development. Con-

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[3] The abbreviations used are: GlcCer, glucosylceramide; AMP-DNJ, *N*-(5′-adamantane-1′-yl-methoxy)-pentyl-1-deoxynojirimycin; C12-NBD-Cer, *N*-[12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-D-erythro-sphingosine; C12-NBD-GlcCer, N-[12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)-amino]dodecanoyl]-D-glucosyl-β1–1′-sphingosine; CBE, conduritol B epoxide; GBA, glucocerebrosidase; GBA2, β-glucosidase 2, nonlysosomal glucosylceramidase, bile acid β-glucosidase; GSL, glycosphingolipid; *N*B-DNJ, *N*-butyldeoxynojirimycin, *N*B-DGJ, *N*-butyldeoxygalactonojirimycin; UCGC, glucosylceramide synthase/UDP-glucose:*N*-acylsphingosine glucosyltransferase.

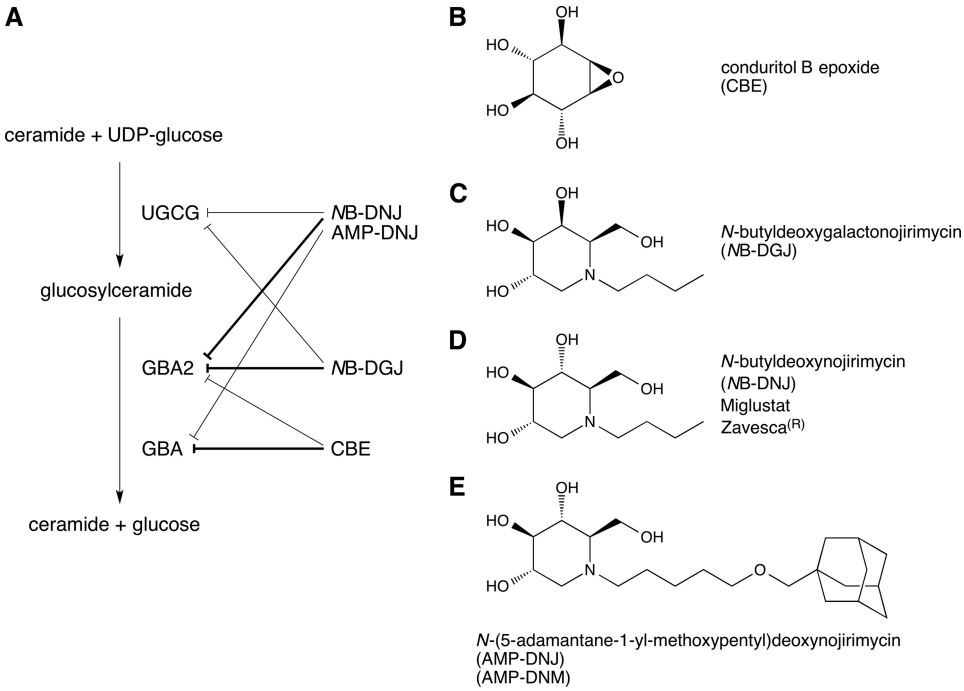

FIGURE 1. **β-Glucosidase inhibitors affecting GlcCer metabolism.** *A,* schematic representation of the biosynthesis and degradation of GlcCer, and the enzymes catalyzing these reactions, UGCG, GBA2, and GBA. Indicated is which of these enzymes are affected by the β-glucosidase inhibitors used in this study (CBE, *N*B-DGJ, *N*B-DNJ, and AMP-DNJ). Each of these compounds has the potential to hinder at least two enzymes involved in glucosylceramide biosynthesis and/or degradation. The *bold line* identifies the enzyme that is most sensitive to inhibition by a particular inhibitor. *B–E,* structural formulas of CBE, *N*B-DGJ, *N*B-DNJ, and AMP-DNJ. This study provides evidence that, in contrast to *N*B-DNJ and AMP-DNJ, *N*B-DGJ does not inhibit GBA and that CBE, in addition to blocking GBA, also can inactivate GBA2. Note that the effects of *N*B-DNJ and AMP-DNJ on GBA are primarily seen in *in vitro* assays, not in cultured cells or *in vivo*.

TABLE 1

GlcCer-metabolizing enzymes and their sensitivities to inhibition by alkylated imino sugars and CBE

IC_{50}, K_I, and k_{inact} values relate to *in vitro* enzyme activity assays. CAZy is the Carbohydrate-Active EnZymes database (94). *NA,* not applicable.

	GlcCer synthase	Glucocerebrosidase	β-Glucosidase 2
Alternative names	UDP-glucose: *N*-acylsphingosine D-glucosyltransferase; ceramide glucosyltransferase	Acid β-glucosidase	Nonlysosomal glucosylceramidase; bile acid β-glucosidase
Symbol	UGCG	GBA	GBA2
EC number	EC 2.4.1.80	EC 3.2.1.45	EC 3.2.1.45
CAZy family	GT21	GH30	GH116
Subcellular localization	Golgi	endo/lysosome	ER/plasma membrane
Inhibitor			
*N*B-DNJ (IC_{50})	20.4 μM (36) 50.0 μM (84)	520 μM (36) 424 μM (34) 400 μM (84)	310 nM (34) 141 nM (9) 230 nM (84) 12–20 nM (11) 5–7 nM (this paper)
*N*B-DGJ (IC_{50})	10.0 μM (84) 41.4 μM (36)	320 μM (84) None detected (36) None detected (this paper)	300 nM (84) 5.3 μM (9) 1–6 μM (this paper)
AMP-DNJ (IC_{50})	150 nM (84)	160–200 nM (34, 84) 48 nM (34)	1–2 nM (34, 84) 0.7 nM (this paper)
CBE	NA	+++++ (63–67) K_I = 0.14 ± 0.04 mM (this paper) k_{inact} = 0.59 ± 0.14 min^{-1} (this paper)	No inhibition (8, 34, 62) ++++/++ (35) K_I = 8.1 ± 2.8 mM (this paper) k_{inact} = 0.11 ± 0.03 min^{-1} (this paper)

versely, inducible overexpression of GBA2, resulting in lower GlcCer and higher ceramide levels, diminished the proliferation of malignant melanoma cells *in vitro*, abolished their anchorage-independent growth, and reduced tumor growth *in vivo* (15). GBA2 activity was also reported to be reduced in melanoma cells lines (15). These recent results raise the possibility that the level of GBA2 activity is one of the factors determining ceramide levels, suggesting that GBA2 may be relevant for apoptotic signaling in cancer cells. This is in accord with earlier studies on the involvement of ceramide and/or glucosylceramide in apoptosis (16–18), autophagy (19, 20), and multidrug resistance (21, 22).

The enzymes catalyzing the biosynthesis and lysosomal hydrolysis of GlcCer, UGCG, and GBA, respectively, are the subjects of multiple medicinal chemistry studies employing alkylated derivatives of the imino sugar deoxynojirimycin, deoxygalactonojirimycin (23–26), and related compounds (27–29). Inhibition of UGCG using alkylated imino sugars is the

pharmacological basis for substrate reduction therapy in type 1 Gaucher disease (30), whereas at sub-inhibitory concentrations these compounds can act as chemical chaperones for mutant forms of GBA found in Gaucher disease, facilitating protein folding (31–33). Many of the imino sugars employed in substrate reduction and chemical chaperone therapies also inhibit GBA2 (9, 34, 35). UGCG, GBA, and GBA2 differ in their sensitivities to inhibition by alkylated imino sugars (Fig. 1*A*; Table 1) so that, *in vivo*, the impact of these compounds on GlcCer and GSL levels depends greatly on their dosage. Lower doses of alkylated imino sugars primarily affect GBA2, although higher doses inhibit both GBA2 and UGCG (9, 34, 36). Accordingly, low drug doses raise GlcCer levels in peripheral tissues (9), and higher doses have this effect also in the central nervous system (9, 37, 38) but reduce GSL levels in peripheral tissues (36, 39–44). Only a very high imino sugar dose escalation reduces GSL levels in the brain (43, 45).

Wild-type mice treated with alkylated imino sugars do not show obvious abnormalities except impaired post-meiotic spermatogenesis, similar to GBA2-deficient mice (9, 46, 47); this pharmacological effect on male germ cell development is, however, not universal in mice and is strictly limited to a small number of inbred mouse strains (48), and it is not seen in rabbits (48) nor in man (49). In murine and feline models of Sandhoff and Niemann-Pick type C1 disease (progressive neurodegenerative conditions caused by dysfunction of the endosomal-autophagic-lysosomal system), administration of alkylated imino sugars delays disease progression, resulting in a significantly extended life span (37, 38, 43, 44, 50, 51). Clinically, *N*-butyldeoxynojirimycin (*N*B-DNJ, Zavesca®, Actelion Pharmaceuticals Ltd., Allschwil, Switzerland; Fig. 1*D*) ameliorates the pathological manifestations in type 1 Gaucher patients (52, 53) and restricts disease advancement in patients with Niemann-Pick type C1 disease (54, 55). Alkylated imino sugars thus can modulate the levels of glucosylceramide and complex GSLs, are currently in clinical use, and may be developed for additional indications (17, 56–61). Considering the biochemical activity of GBA2, its involvement in various diseases, and the clinical application and development of pharmacological agents that have overlapping effects on UGCG, GBA, and GBA2 (Fig. 1; Table 1), the characterization of the β-glucosidase activity of GBA2 is of fundamental importance.

Both GBA and GBA2 contribute to the total level of β-glucosidase activity that can be measured in tissue/cell homogenates and membrane preparations using the artificial substrate 4-methylumbelliferyl-β-D-glucoside and the fluorescent GlcCer analog C12-NBD-GlcCer. Even though GBA requires detergents to be maximally active *in vitro*, it is active under the same conditions as used for measuring GBA2 activity, *i.e.* in detergent-free membrane preparations (34, 62). Conduritol B epoxide (Fig. 1*B, CBE*) has been employed to discriminate between GBA and GBA2, because this compound is an irreversible inhibitor of GBA (63–67). GBA2 has thus been described as the membrane-associated detergent-independent β-glucosidase activity that is resistant to 1.0–2.5 mM CBE (8, 34). This pharmacological characterization of membrane-associated GBA2 is at odds with that of Matern *et al.* (35), who found that the human liver bile acid β-glucosidase activity generated by GBA2 is sensitive to inhibition by CBE, particularly in the membrane-associated state. These observations raise the possibility that the GBA2 β-glucosidase activity toward GlcCer and 4-methylumbelliferyl-β-D-glucoside is also sensitive to CBE.

To resolve the ambiguity concerning the CBE sensitivity of GBA2, we have studied GBA and GBA2 separately using biochemical and genetic strategies, and we assessed their responses to CBE and *N*-butyldeoxygalactonojirimycin (Fig. 1*C, NB-DGJ*). We found that CBE did not exclusively inhibit GBA but also reduced the membrane-associated GBA2 activity *in vitro* and in live cells. Instead, the activities of GBA and GBA2 could be very well distinguished using *N*B-DGJ. Using the latter approach, we have established that the GBA2 β-glucosidase activity in mouse tissues and cultured cells is considerably higher than the CBE-resistant β-glucosidase activity, and the sensitivity of GBA2 to inhibition by *N*B-DNJ and *N*B-DGJ differs from previous estimates.

EXPERIMENTAL PROCEDURES

Animal Tissues—Normal mouse tissues were obtained from adult C57BL/6 and CD1 mice, bred at the Carleton Animal Care Facility at Dalhousie University. Testes from adult GBA2-deficient mice (10) were provided by Yildiz Yildiz (University Clinic of Bonn, Bonn, Germany). Mice carrying a loxP-neo-loxP (lnl) cassette within intron 8 in the *Gba* gene in all tissues except the skin (68) were bred according to the guidelines of the United Kingdom Animals (Scientific Procedures) Act. Homozygous lnl/lnl mice (referred to as GBA-deficient or GBA(−) hereafter) were used at 12 days of age, because they do not survive for more than 2 weeks (68).

Cell Culture and Transfection—IMR-32 and SH-SY5Y (human neuroblastomas), RAW264.7 (murine monocyte/macrophage), THP-1 (human monocyte), and COS-7 cells (African green monkey kidney) were obtained from the American Type Culture Collection (ATCC), and cultured in DMEM with 10% fetal bovine serum (plus nonessential amino acids for the IMR-32 cells). To induce neuronal differentiation, IMR-32 and SH-SY5Y cells were cultured for 7 days in 2 mM butyric acid and 10 μM all-*trans* retinoic acid, respectively. CBE was added to the culture medium at 50 and 500 μM. For harvesting, cells were washed twice with PBS, scraped in PBS, washed once more, and stored at −80 °C until use. SH-SY5Y cells were transfected with the cDNA-encoding human GBA2 (C-terminally DYK- and Myc-tagged) in the pCMV6-Neo^R mammalian expression vector (Origene) using *Trans*IT-2020 transfection reagent (Mirus), according to manufacturer's instructions. SH-SH5Y cells stably transfected with the pCMV6-Neo^R-GBA2 construct were selected by subculturing the cells at a 1:10 ratio 24 h post-transfection in 300 μg/ml G418 sulfate (Geneticin, Invitrogen) for 2–3 weeks. Surviving colonies were picked by trypsinization and expanded in the presence of G418.

Sample Preparation for β-Glucosidase Assay—To prepare mouse tissue membranes, tissues were mechanically homogenized (Tissue-tearor; BioSpec Products, Bartlesville, OK) in 3 volumes of deionized water, centrifuged at 500 × *g* to remove tissue debris and nuclei, and centrifuged at 20,000 × *g* for 20 min at 4 °C. The pellet was washed three times in 50 mM potassium phosphate buffer, pH 5.8 (34). Membranes were resus-

pended in 3 volumes (relative to the original tissue wet weight) of the potassium phosphate buffer, frozen in liquid nitrogen, and stored at −80 °C until use. To assess β-glucosidase activities at various pH values, aliquots of brain homogenates were processed as described above but washed throughout in distilled water and resuspended in 100 mM citric acid, 200 mM disodium hydrogen phosphate buffers of pH 3–8, with increments of 0.5 pH unit.

To prepare pH 4.2 detergent extracts, mouse tissues were homogenized in citrate/phosphate buffer, pH 4.2, 1.25 mM EDTA, 0.5% Triton X-100, 0.5% sodium taurocholate (Ultrol grade, Calbiochem), and protease inhibitors (Set III, Calbiochem, 1:1,000) using a hand-held glass-Teflon homogenizer. Homogenates were incubated with gentle agitation for 30 min at 4 °C and centrifuged at 21,000 × *g* for 10 min. Supernatants were used for enzyme assays. Alternatively, tissues were homogenized in citrate/phosphate buffer, pH 4.2, devoid of detergents, and used as such or supplemented with 6 μM human saposin C (provided by Jennifer Lee and Thai Leong Yap, NHLBI, Bethesda).

Frozen cell pellets were thawed, resuspended in deionized water, incubated for 30 min at 4 °C, and homogenized by passaging 10 times through a 23-gauge needle fitted on a 1-ml syringe. Protein concentrations were determined using the BCA assay (Thermo Fisher) with BSA as standard.

β-Glucosidase Inhibitors—For inhibition studies with CBE (Toronto Research Chemicals), *N*B-DNJ, *N*B-DGJ (Toronto Research Chemicals or Actelion Pharmaceuticals Ltd., Allschwil, Switzerland), and AMP-DNJ (Cayman Chemical; Fig. 1*E*), 9 volumes of sample were mixed with either 1 volume of inhibitor stock solution prepared in 50 mM potassium phosphate buffer, pH 5.8, or citrate/phosphate buffer of desired pH. CBE-containing samples, together with control samples, were preincubated at room temperature for 30 min unless indicated otherwise. When using CBE in combination with an alkylated imino sugar, samples were first preincubated with CBE, then aliquoted, and combined with different stock solutions of *N*B-DNJ, *N*B-DGJ, or AMP-DNJ. Unless indicated otherwise, final concentrations of CBE and *N*B-DGJ were 2.5 and 0.3 mM, respectively.

β-Glucosidase Assay Using Artificial Substrate—One volume of sample was combined with 2 volumes of 4.5 mM 4-methylumbelliferyl-β-D-glucoside in 100 mM citric acid, 200 mM disodium hydrogen phosphate buffer, pH 5.8, and incubated at 37 °C for 30 or 60 min. Samples prepared in buffers of different pH values were incubated with the substrate dissolved in citrate/phosphate buffers of pH corresponding to that of the sample. Unconjugated 4-methylumbelliferone (free acid) was used as quantitative standard. Reactions were terminated by adding 20 volumes of 0.5 M sodium carbonate buffer, pH 10.7, and fluorescence was measured using a Fluoroskan Ascent FL (Thermo) or an Infinite M200 PRO (Tecan) plate reader (excitation 355 nm and emission 460 nm).

Recombinant Glucocerebrosidase Assay—Recombinant human GBA (Ceredase; Genzyme, Oxford, UK) was diluted 1:300 in citrate/phosphate buffer, pH 5.5, containing 4 mM β-mercaptoethanol and 5 mg/ml BSA. One volume of diluted Ceredase was mixed with 2 volumes of 5 mM 4-methylumbelliferyl-β-D-glucoside in citrate/phosphate buffer, pH 5.5, containing 4 mM β-mercaptoethanol, 1.25 mM EDTA, 0.5% Triton X-100, and 0.5% sodium taurocholate and incubated at 37 °C for 60 min. Reactions were terminated, and fluorescence was measured as described for β-glucosidase assays above.

β-Glucosidase Assays Using Lipid Substrate—Samples were supplemented with *N*-[12-[(7-nitro-2–1,3-benzoxadiazol-4-yl) amino]dodecanoyl]-D-glucosyl-β-1′-sphingosine (C12-NBD-GlcCer, Avanti Polar Lipids; final concentration 16 μM, from a 50× stock solution in 50% ethanol) and incubated at 37 °C for 30 min (GBA2) or 1 h (glucocerebrosidase). Reactions were terminated by adding 30 volumes of chloroform/methanol (1:2 v/v) and extracted with 20 volumes of chloroform and 20 volumes of distilled water, resulting in a phase split. The lower phase was dried under nitrogen gas, dissolved in chloroform, and spotted on HPTLC plates (Silica gel 60, EMD Chemicals/Merck), which were developed in chloroform, methanol, 20% (w/v) ammonium hydroxide (70:30:5 v/v/v) (69). *N*-[12-[(7-Nitro-2–1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-D-erythro-sphingosine (C12-NBD-Cer, Avanti Polar Lipids) was used as authentic standard. HPTLC plates were scanned with a Typhoon Variable Mode Imager (GE Healthcare) with excitation at 457 nm and emission at 526 nm. C12-NBD-GlcCer and C12-NBD-Cer bands were quantitated using ImageQuant 5.2 software (GE Healthcare).

Derivation of K_I and k_{inact} of CBE—Brain membranes, pH 5.8, were combined in a microtiter plate with a mixture of 6,8-difluoro-4-methylumbelliferyl-β-D-glucoside (Carbosynth, Compton, Berks, UK; final concentration 300 μM, pH 5.8) and CBE (variable concentration) in a final volume of 100 μl and incubated at 37 °C in an Infinite M200 PRO instrument (Tecan). Levels of 6,8-difluoro-4-methylumbelliferone generated by β-glucosidase activity were measured every 5 min for 2 h (70), using 6,8-difluoro-4-methylumbelliferone (Invitrogen) as quantitative standard. The K_m value of the pH 5.8 β-glucosidase activity toward 6,8-difluoro-4-methylumbelliferyl-β-D-glucoside was 280 μM. Brain detergent extracts, pH 4.2, were combined with a mixture of 4-methylumbelliferyl-β-D-glucoside (final concentration 3 mM, pH 4.2) and CBE (variable concentration), incubated at 37 °C for 5, 10, 20, 40, or 80 min, and terminated as described above. The K_m value of the pH 4.2 β-glucosidase activity (GBA) toward the latter substrate was 1.7 mM, similar to earlier studies (67).

For each time point, the apparent IC_{50} values of CBE were derived via nonlinear regression to a sigmoidal dose-response (variable slope) model. In turn, the time-dependent IC_{50} data of CBE were fitted to the model of Krippendorff *et al.* (71) to derive K_I and k_{inact} values of CBE, using XLFit 5 software (IDBS, Guildford, Surrey, UK).

Western Blotting—Frozen cell pellets were thawed, resuspended, and extracted by incubating in 20 mM Tris-HCl, pH 7.9, 300 mM KCl, 10% glycerol, 0.25% Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, and protease inhibitors (Set III, Calbiochem) on ice for 15 min, and passaged 10 times through a 23-gauge needle. Insolubles were removed by centrifuging at 21,000 × *g* for 15 min at 4 °C. Samples of equal total protein content were separated by SDS-PAGE and transferred to PVDF membrane by semi-dry Western blotting in 50 mM Tris-base, 40 mM glycine, 0.0375% SDS, and 20% methanol. Blots were blocked, incubated with antibodies, and washed in 5% nonfat

milk powder in Tris-buffered saline containing 0.05% Tween 20. Primary antibodies were mouse anti-c-Myc mAb (1:1,000; THE™ cMyc Tag Antibody, Genscript) and rabbit anti-GAPDH mAb (1:1,000; clone 14C10, Cell Signaling). HRP-conjugated secondary antibodies were from Jackson ImmunoResearch. Blots were developed with SuperSignal West Pico chemiluminescent substrate (Thermo/Pierce).

Statistical Analysis and Curve Fitting—Quantitative data are presented herein as means ± S.D. and means + S.D. in line graphs and columns graphs, respectively. Quantitative data were analyzed by one-way analysis of variance and Tukey's post hoc test for multiple comparisons. IC_{50} values were calculated by fitting the enzyme activity data to a sigmoidal dose-response (variable slope) function, a two-sites model, or a biphasic bell-shaped curve using GraphPad Prism 5.0 software. To determine whether IC_{50} values calculated by nonlinear curve fitting were statistically distinct, and to compare different curve fitting models (sigmoidal dose-response/two-sites/bell-shaped), the Extra Sum-of-Squares *F*-test was applied. All statistical tests were performed with Prism 5.0 software (Graphpad); $p < 0.05$ was considered statistically significant.

RESULTS

GBA Is Not Sensitive to Inhibition by NB-DGJ—To compare and contrast GBA and GBA2, we first assessed the sensitivity of GBA to inhibition by *N*B-DGJ. Platt *et al.* (36) showed that purified human placental GBA was not affected by *N*B-DGJ. We found that clinical grade purified recombinant human GBA (Ceredase) was similarly resistant to *N*B-DGJ (Fig. 2*A*). To evaluate GBA in a more native context, we used membranes prepared from GBA2-KO mouse testes, which we assume to contain only one β-glucosidase, GBA. When assayed under conventional conditions for glucocerebrosidase (pH 5.5, detergents) (72–76), *N*B-DGJ reduced the glucocerebrosidase activity in wild-type membranes by 41% but did not have a significant effect in GBA2-KO membranes (Fig. 2*B*). We further measured β-glucosidase activity in brain membranes from wild-type mice over a wide pH range (3.0–8.0). The activity displayed a plateau at pH 3.5–4.5, increased sharply to peak at pH 5.5–6.0, and decreased to pH 8.0 (Fig. 2*C*). When assayed in the presence of *N*B-DGJ, ~26% of the total β-glucosidase activity remained, exhibiting a flat profile from pH 3.5 to 5.5 (Fig. 2*C*). In membranes prepared from GBA-deficient mouse brain, the β-glucosidase activity sharply climbed from pH 4.5 and was highest at pH 5.5–6.0, similar to the main peak of β-glucosidase activity seen in wild-type membranes. However, the GBA-deficient membranes displayed very minor enzyme activity at pH 3.5–4.5, if any (Fig. 2*D*). Moreover, in the GBA-deficient membranes, almost all β-glucosidase activity was inhibited by *N*B-DGJ (Fig. 2*D*). The GBA-deficient membranes were thus devoid of the *N*B-DGJ-resistant acidic β-glucosidase activity that was detected in wild-type membranes. These data indicate that, similar to purified GBA, membrane-associated GBA, with a pH optimum of 3.5–5.5, is not sensitive to inhibition by *N*B-DGJ.

Because GBA is often assayed in Triton X-100/taurocholate extracts from cells and tissues (72–76), we also examined the β-glucosidase activity in total homogenates from wild-type brain prepared at pH 4.2 without detergents and in pH 4.2 detergent extracts. These preparations can be assumed to contain all co-factors required for GBA activity, including saposin C. Considering the pH activity profile of GBA2 (see below), these enzyme assays were done at pH 4.2 to exclude GBA2 from contributing to the results, as recommended previously (77). *N*B-DGJ did not inhibit the β-glucosidase activity in any of the pH 4.2 preparations (Fig. 2*E*). Addition of exogenous human saposin C increased the β-glucosidase activity of crude pH 4.2 brain homogenates (without detergents) by ~50% (Fig. 2*E*). The saposin C-enhanced β-glucosidase activity was not reduced by *N*B-DGJ (Fig. 2*E*). These data show that neither GBA in its native environment nor in detergent extract was sensitive to inhibition by *N*B-DGJ; this also applied to membrane-bound GBA boosted by exogenous saposin C.

In the assays described above, β-glucosidase activities were determined using the artificial substrate 4-methylumbelliferyl-β-D-glucoside. This substrate has been used in clinical assays of GBA activity as part of the diagnosis of Gaucher disease for decades (78) and is considered to provide a highly reliable measurement of GBA activity. Nevertheless, a fluorescent GlcCer analog has been found to be more sensitive in discriminating between different mutant forms of GBA (79). Therefore, to complement the experiments performed with the artificial substrate, we assessed the β-glucosidase activity of pH 4.2 detergent extracts from wild-type brain toward a fluorescent GlcCer analog, C12-NBD-GlcCer. This sphingolipid analog was readily hydrolyzed by the pH 4.2 detergent extract (Fig. 3*A*). *N*B-DGJ did not reduce the detergent-solubilized β-glucosidase activity toward C12-NBD-GlcCer (Fig. 3*A*). Clearly, GBA activity toward the sphingolipid substrate was not affected by *N*B-DGJ. The results discussed above consistently demonstrated that GBA is not sensitive to inhibition by *N*B-DGJ.

GBA2 Has a Mildly Acidic pH Optimum—The β-glucosidase in GBA-deficient membranes was most active at pH 5.5–6.0 (Fig. 2*D*). Presuming that GBA2 is the only β-glucosidase associated with GBA-deficient membranes, this pH titration showed that GBA2 was optimally active at pH 5.5–6.0.

GBA2 Is Inhibited by NB-DGJ—*N*B-DGJ inhibited essentially all β-glucosidase associated with GBA-deficient membranes, irrespective of pH (Fig. 2*D*), demonstrating that GBA2 was efficiently inhibited by *N*B-DGJ. In wild-type membranes, the majority of the β-glucosidase activity was sensitive to inhibition by *N*B-DGJ (84% at pH 5.5 and 91% at pH 6.0) (Fig. 2*C*). Similarly, in brain homogenates and in GBA-deficient brain membranes, *N*B-DGJ inhibited the conversion of C12-NBD-GlcCer to C12-NBD-Cer at pH 5.8 by 93 and 99%, respectively (Fig. 3, *A* and *B*). Clearly, GBA2 was the predominant β-glucosidase in wild-type brain samples assayed in the absence of detergents.

GBA2 Is Sensitive to Inhibition by CBE in Vitro—Previously, CBE has been utilized with the objective to distinguish GBA and GBA2, on the basis of the hypothesis that the effects of CBE on GBA and GBA2 are mutually exclusive (8, 34, 62). Whereas GBA is sensitive to inhibition by CBE (63–67), GBA2 was considered to be resistant to 1.0–2.5 mM CBE (8, 34, 62). Accordingly, following transient transfection of COS cells with the GBA2 cDNA, the CBE-resistant β-glucosidase activity in cell lysates was increased (Fig. 4*A*), in agreement with earlier find-

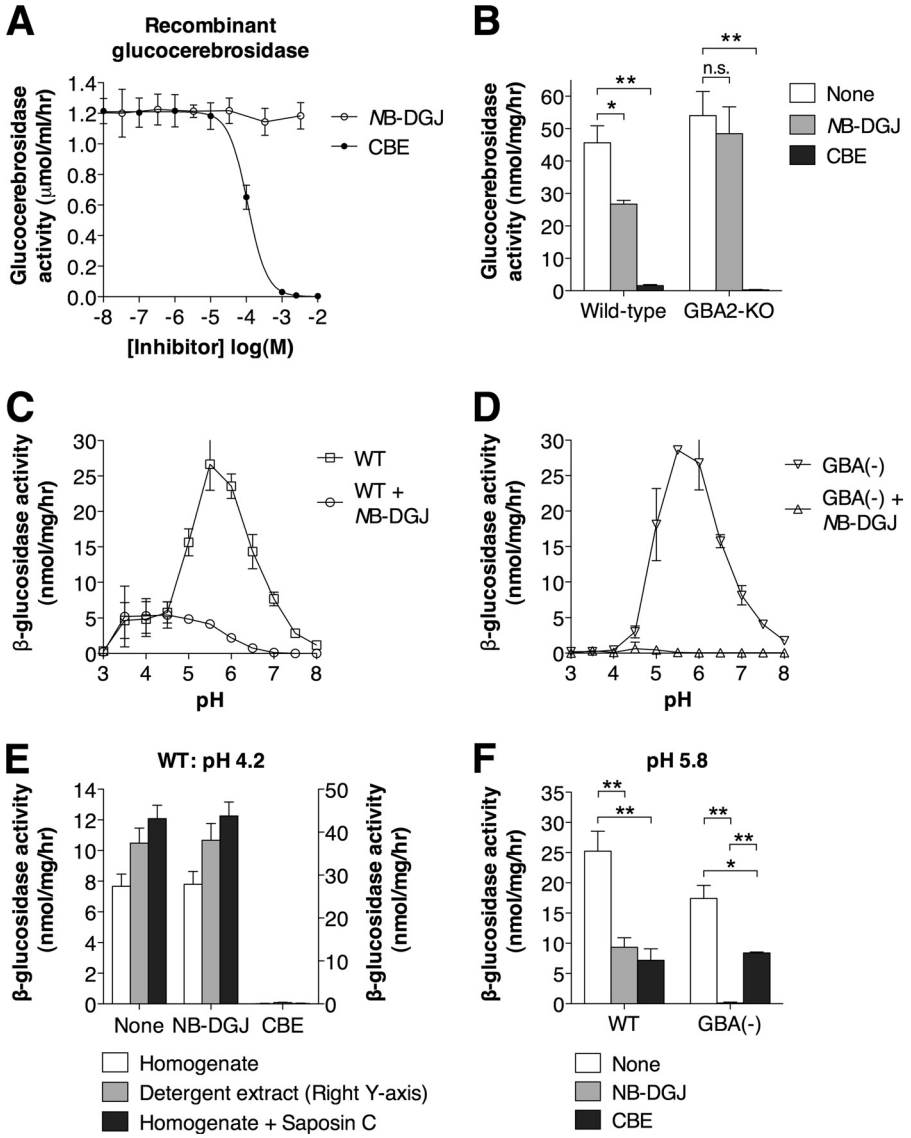

FIGURE 2. **Comparison of the effects of *N*B-DGJ and CBE on the β-glucosidase activities of GBA and GBA2.** *A,* titration of highly purified clinical grade GBA (Ceredase) with *N*B-DGJ and CBE. *N*B-DGJ (up to 3.3 mM) did not affect the enzyme, whereas CBE abolished its activity with an apparent IC_{50} of 110 μM. *B,* testis membranes from wild-type and GBA2-KO mice were assayed for glucocerebrosidase activity as described for Ceredase, either in the presence of 0.3 mM *N*B-DGJ or after preincubation in 2.5 mM CBE. In wild-type membranes, *N*B-DGJ and CBE reduced the glucocerebrosidase activity to 59 and 3% of the values obtained for membranes incubated without any inhibitor, respectively. In GBA2-deficient membranes (containing only GBA), *N*B-DGJ had no significant impact on the enzyme activity, although CBE completely suppressed the β-glucosidase activity. *C* and *D,* membranes from adult wild-type (*C*) and GBA-deficient (*D*) mice were assayed for β-glucosidase activity at different pH values, in the absence or presence of *N*B-DGJ. Whereas wild-type membranes contained an *N*B-DGJ-resistant β-glucosidase activity, *N*B-DGJ inhibited essentially all β-glucosidase activity in GBA-deficient membranes. *E,* different pH 4.2 preparations from wild-type mouse brains were assayed for β-glucosidase activity either in the presence of *N*B-DGJ or after preincubation with CBE. *N*B-DGJ did not affect the enzyme activity, irrespective of the addition of exogenous saposin C. CBE fully inhibited the enzyme activity in all preparations. *F,* comparison of the impact of *N*B-DGJ and CBE on the β-glucosidase activity in brain membranes prepared from GBA(−) mice and age-matched wild-type mice ($n = 3$ or 4). Despite the absence of GBA from the GBA(−) membranes, CBE reduced the β-glucosidase activity in these membranes by 52%. *, $p < 0.01$; **, $p < 0.001$; *n.s.*, not significant.

ings (8). We made similar observations in stably transfected SH-SY5Y cell lines that overexpress GBA2 (Fig. 4*B*), supporting the notion that GBA2 activity is resistant to CBE. We therefore expected that the β-glucosidase activity of GBA-deficient membranes, *i.e.* GBA2, would not be affected by CBE. This was, however, not what we found. CBE reduced the β-glucosidase activity of GBA-deficient membranes by 52% (Fig. 2*F*). Similarly, in brain homogenates and in GBA-deficient brain membranes, CBE inhibited the conversion of C12-NBD-GlcCer to C12-NBD-Cer at pH 5.8 by 79 and 61%, respectively (Fig. 3, *A* and *B*). These results showed that GBA2 was sensitive to inhibition by CBE *in vitro*.

We next assayed the β-glucosidase activity in membranes prepared from a number of tissues from wild-type mice for sensitivity to inhibition by either *N*B-DGJ or CBE. The β-glucosidase activity in testis, lung, brain, and liver was reduced by 69–93% using CBE and by 50–84% using *N*B-DGJ (Fig. 5, *left panel*). The effects of these two inhibitors were not mutually exclusive but had a significant overlap, with 43–66% of the membrane-associated β-glucosidase activity being sensitive to inhibition by both CBE and *N*B-DGJ (Table 2). We obtained similar results for monocyte/macrophage (RAW and THP-1) and neuroblastoma cell lines (IMR-32 and SH-SY5Y) (Fig. 5, *right panel*). In homogenates of these cells, 6–36% of the β-glu-

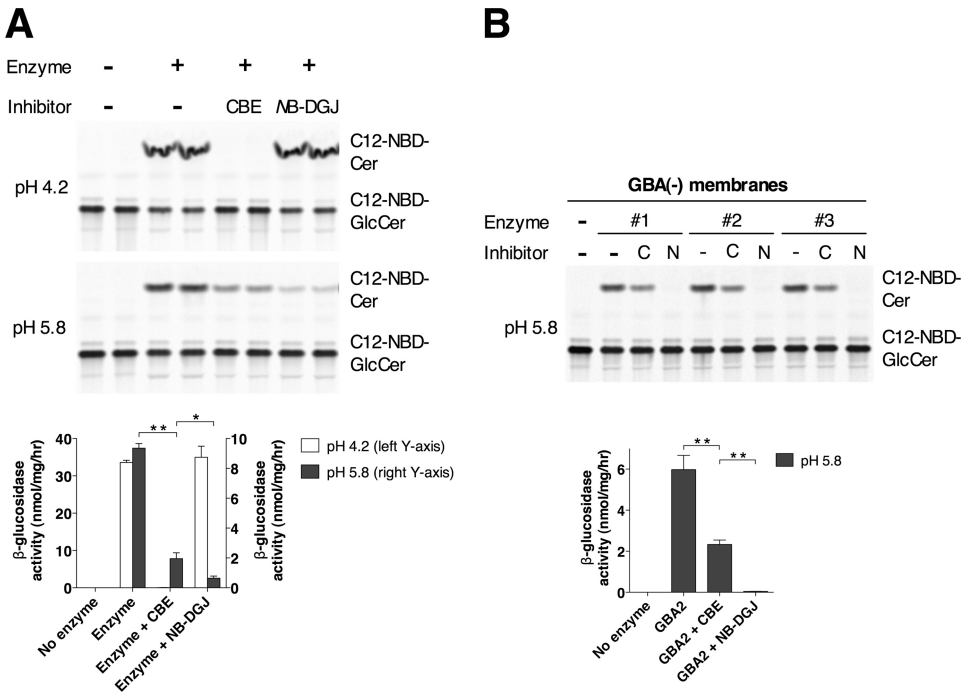

FIGURE 3. **Impact of *N*B-DGJ and CBE on β-glucosidase activities toward C12-NBD-GlcCer.** *A,* different preparations from wild-type brains were incubated with C12-NBD-GlcCer in the absence or presence of either 2.5 mM CBE or 0.3 mM *N*B-DGJ, and the reaction products were separated via HPTLC. *Upper panel,* pH 4.2 detergent extract. *Lower panel,* pH 5.8 homogenate. *Graph*, quantitation of TLC data ($n = 3$). *B,* membranes prepared from GBA-deficient brains at pH 5.8 were incubated with C12-NBD-GlcCer in the absence or presence of either CBE (*C*) or *N*B-DGJ (*N*), and the reaction products were separated via HPTLC. *Graph*, quantitation of TLC data ($n = 3$). *, $p < 0.01$; **, $p < 0.001$.

cosidase activity was sensitive to inhibition by CBE as well as *N*B-DGJ (Table 2). For mock- and GBA2-transfected COS-7 cells, the overlap between *N*B-DGJ-sensitive and CBE-sensitive β-glucosidase activities was 37 and 42%, respectively (Table 2), irrespective of the large difference in the total β-glucosidase activity in these cells (Fig. 4*A*). Neuronal differentiation of SH-SY5Y cells enhanced the total and *N*B-DGJ-sensitive β-glucosidase activities (Fig. 4*B*) and also increased the overlap between *N*B-DGJ-sensitive and CBE-sensitive β-glucosidase activities from 6 to 35% (Table 2). Thus, we consistently found an overlap between *N*B-DGJ-sensitive and CBE-sensitive β-glucosidase activities. Having established that GBA is not affected by *N*B-DGJ (see above), these results indicate that in various wild-type mouse tissues and cell lines a proportion of GBA2 activity was sensitive to inhibition by CBE.

CBE Inhibits GBA2 in an Irreversible, Time-dependent Fashion—CBE is an irreversible, mechanism-based inactivator of GBA (63–67). Inhibitors of this type engage with their target enzymes in two steps, first binding reversibly and then forming a covalent bond, which renders the enzyme persistently inactive (Fig. 6*A*) (80, 81). The formation of the covalent inhibitor-enzyme bond is not instantaneous but time-dependent. The degree of inhibition achieved by mechanism-based enzyme inactivators is therefore a function of time. For these inhibitors, IC_{50} values decrease with increasing incubation time. The reversible and covalent enzyme-inhibitor interactions can be characterized by two time-invariant parameters, the inhibition constant (K_I), which reflects the affinity of the initial reversible association of inhibitor and enzyme, and the rate of covalent bond formation, *i.e.* the rate of enzyme inactivation (k_{inact}) (80, 81).

To establish whether CBE affects GBA2 in an enduring fashion, we preincubated adult mouse brain membranes with CBE, washed out the CBE by repeated dilution and sedimentation, and determined what proportion of the remaining β-glucosidase activity was sensitive to inhibition by *N*B-DGJ. Membranes that had been preincubated with CBE, and subsequently depleted for CBE, had lost up to 95% of GBA2 activity compared with control membranes (Fig. 6*B*). Clearly, transient exposure to CBE was sufficient to reduce GBA2 activity.

We determined K_I and k_{inact} of CBE toward GBA2 and GBA, by directly measuring the effect of CBE on the reaction progress (71). Without preincubating the enzymes with CBE, they were exposed to a fixed level of substrate and a variable concentration of CBE. Levels of product formed were measured at multiple time points (71). For both enzymes, apparent IC_{50} values of CBE decreased over time (Fig. 6, *C, D,* and *F*). Time-dependent IC_{50} values toward GBA2 were higher than those toward GBA (Fig. 6, *C, D,* and *F*). The temporal decline of apparent IC_{50} values (Fig. 6*F*) was fitted to a general model that specifies the relationship between time-dependent IC_{50} values, K_m, K_I, and k_{inact} for mechanism-based inhibitors (71). The K_I value of GBA2 was considerably higher than that of GBA, whereas the k_{inact} value of GBA2 was lower compared with that of GBA (Table 1). Wild-type membranes, pH 5.8, in which most β-glucosidase activity is due to GBA2, exhibited K_I and k_{inact} values that were close to those of GBA-deficient membranes, 4.1 mM and 0.08 min^{-1}, respectively (Fig. 6, *E* and *F*). Overall, the efficiency of inactivation (k_{inact}/K_I) of GBA by CBE was 300-fold greater than that observed for GBA2.

Measurement of GBA2 Activity—The results presented above are relevant for the measurement of the GBA2 β-gluco-

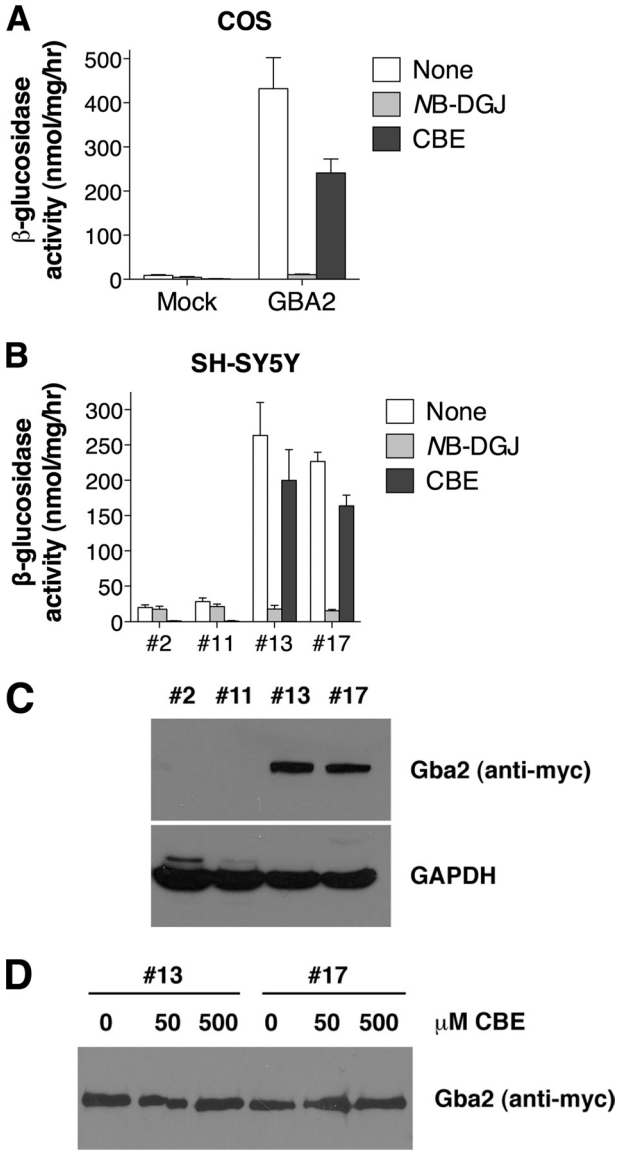

FIGURE 4. **Impact of *N*B-DGJ and CBE on the β-glucosidase activity in cells overexpressing GBA2.** *A,* COS cells transiently transfected with a Neo^R/GBA2 cDNA expression vector; *B,* four SH-SY5Y-derived cell lines stably transfected with this vector were assayed for β-glucosidase activity either without inhibitors, in the presence of *N*B-DGJ, or after preincubation with CBE. In GBA2-cDNA-transfected COS cells and SH-SY5Y-derived cell lines 13 and 17, total β-glucosidase activity as well as CBE-resistant β-glucosidase activity were much higher than in mock-transfected COS cells and SH-SY5Y-derived cell lines 2 and 11, respectively. The CBE-resistant β-glucosidase activity was 56, 72, and 76% of total activity in GBA2-transfected COS cells and cell lines 13 and 17, respectively. Furthermore, *N*B-DGJ reduced the β-glucosidase activity in GBA2-transfected COS cells and cell lines 13 and 17 to similar levels as in mock-transfected COS cells and SH-SY5Y-derived cell lines 2 and 11, respectively ($n = 2$). *C,* SH-SY5Y-derived cell lines 2, 11, 13, and 17 were assayed for expression of Myc-tagged GBA2 by Western blotting using anti-Myc antibodies; GAPDH was used as loading control. *D,* GBA2-overexpressing cell lines 13 and 17 were cultured in the presence of CBE, processed for Western blotting, and probed with an anti-Myc antibody. The CBE treatment did not affect the level of GBA2.

sidase activity. Originally, the nonlysosomal β-glucosidase activity of GBA2 was specified as the membrane-associated, detergent-independent β-glucosidase activity that is CBE-resistant (8, 34, 62). This definition needs to be revised in light of our finding that CBE inhibited not only GBA but also GBA2. By contrast, *N*B-DGJ differed from CBE in that it was selective in its inhibition of membrane-associated β-glucosidases; *N*B-DGJ inhibited GBA2 but not GBA (see above). Accordingly, GBA2 activity can be measured as the β-glucosidase activity that is sensitive to inhibition by *N*B-DGJ. We compared the CBE-resistant and *N*B-DGJ-sensitive β-glucosidase activities in various mouse tissues and cell lines. In mouse tissues (except spleen), the *N*B-DGJ-sensitive β-glucosidase activity was 3–9-fold higher than the CBE-resistant β-glucosidase activity (Fig. 5, *left panel*), and in monocyte/macrophage and neuroblastoma cell lines, the *N*B-DGJ-sensitive β-glucosidase activity was 1.5–6-fold higher (10-fold for neuronally differentiated SH-SY5Y cells; Fig. 5, *right panel*). The GBA2 activity varied significantly among mouse tissues, with testis being the most active, followed by brain and liver (Fig. 5, *left panel*).

Also, as a percentage of the total detergent-independent β-glucosidase activity of mouse tissue membranes, the *N*B-DGJ-sensitive activity was higher and more variable (average 59%, range 24–89%) than the CBE-resistant activity (average 13%, range 6–8%, except brain, 32%). The difference between these parameters was especially broad for testis, in which 8% of the β-glucosidase activity was CBE-resistant, and 74% was *N*B-DGJ-sensitive. Furthermore, compared with the relatively uniform levels of CBE-resistant activity among mouse tissues (6–8%), the *N*B-DGJ-sensitive β-glucosidase activity was significantly different between all tissues except testis-brain and testis-liver. We made similar observations in the monocyte/macrophage and neuroblastoma cell lines; as a percentage of the total detergent-independent β-glucosidase activity, the *N*B-DGJ-sensitive activity was higher (average 33%, range 14–43%) than the CBE-resistant activity (average 9%, range 3–9%, except RAW cells 23%). Taken together, in mouse tissues and in cultured cells, the GBA2 (*N*B-DGJ-sensitive β-glucosidase) activity was responsible for a considerably larger proportion of the detergent-independent β-glucosidase activity and was more variable, compared with the CBE-resistant activity.

GBA2 Is Sensitive to Inhibition by CBE in Live Cells—CBE is often applied to cultured cells with the aim of generating cell culture models of Gaucher disease (82, 83). Having established that CBE inhibits GBA2 *in vitro*, we sought to establish whether CBE also inhibits GBA2 in live monocyte/macrophage and neuroblastoma cells. We found that, at 500 μM CBE, cellular GBA2 activity was reduced on average to 32% of control cells (range 44–5%), depending on the cell line (Table 3). The decrease in *N*B-DGJ-sensitive β-glucosidase activity in SH-SY5Y cell lines 13 and 17 was not due to a decrease in the protein level of GBA2, as shown by Western blotting (Fig. 4*D*). These data demonstrate that culturing cells in the presence of CBE can reduce their GBA2 activity.

Sensitivity of GBA2 to Inhibition by Alkylated Imino Sugars—Previously, the sensitivity of GBA2 to inhibition by imino sugars was determined *after* incubating membrane preparations in 1.0–2.5 mM CBE (8, 9, 34). Following this approach, the IC_{50} value of *N*B-DNJ toward GBA2 was determined at 140–310 nM (9, 34). Furthermore, in contrast to *N*B-DGJ, *N*B-DNJ inhibits GBA *in vitro*, with an IC_{50} of 424–520 μM (34, 36). Having established that GBA2 is itself affected by CBE, we sought to reassess the sensitivity of this enzyme to inhibition by alkylated imino sugars. Therefore, we prepared membranes from GBA2-

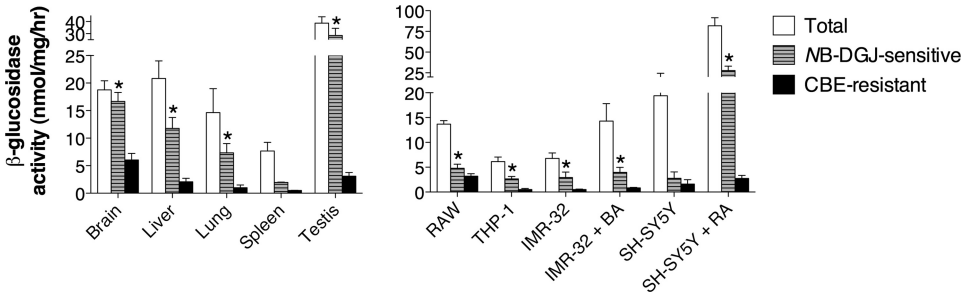

FIGURE 5. **Assessment of β-glucosidase activity in various mouse tissues and cell lines: comparison of different ways to measure GBA2 activity.** *Left panel,* membranes were prepared from various mouse tissues. *Right panel,* homogenates from various cell lines were assayed for β-glucosidase activity either without inhibitors, in the presence of *N*B-DGJ, or after preincubation with CBE. Shown are total β-glucosidase activity, *NB-DGJ-sensitive* β-glucosidase activity, and *CBE-resistant* β-glucosidase activity. For most tissues and cell lines, except spleen and SH-SY5Y cells, the *N*B-DGJ-sensitive activity was significantly higher than the CBE-resistant β-glucosidase activity. *IMR-32 + BA,* IMR-32 cells differentiated with butyric acid into a neuronal phenotype; *SH-SY5Y + RA,* SH-SY5Y cells differentiated into a neuronal phenotype with all-*trans*-retinoic acid. * indicates where *N*B-DGJ-sensitive β-glucosidase activity was significantly higher than CBE-resistant β-glucosidase activity ($p < 0.01$); $n = 5$ (*left panel*) or $n = 3–4$ (*right panel*).

TABLE 2

Percentage of total β-glucosidase activity that is sensitive to inhibition by CBE as well as by *N*B-DGJ, determined in various mouse tissues and cell lines

Data in this table were derived from those presented in Fig. 5. All tissues were from adult WT mice, except when indicated otherwise. WT, wild-type; GBA(−), GBA-deficient; BA, butyric acid; RA, all-*trans*-retinoic acid; TX, transfected.

Brain, 12-day, WT (30 min CBE)	28.3
Brain, 12-day, GBA(−) (30 min CBE)	43.6
Brain, 12-day, WT (60 min CBE)	37.8
Brain, 12-day, GBA(−) (60 min CBE)	79.5
Brain	56.7
Liver	46.7
Lung	43.1
Spleen	17.9
Testis	65.7
RAW264.7	11.7
THP-1	34.7
IMR-32	35.7
IMR-32 + BA	21.8
SH-SY5Y	6.0
SH-SY5Y + RA	34.7
Mock-TX COS cells	37.2
GBA2-TX COS cells	41.7
SH-SY5Y-GBA2 line 13	17.2
SH-SY5Y-GBA2 line 17	20.9

overexpressing SH-SY5Y cells and wild-type mouse testis and brain, and we compared the impact of a wide range of *N*B-DNJ and *N*B-DGJ concentrations (3.3 pM to 3.3 mM) on the β-glucosidase activity of control and CBE-treated membranes.

NB-DNJ v NB-DGJ in Control Membranes—Considering that *N*B-DNJ inhibits both GBA and GBA2, we expected that the highest concentration of this compound would suppress all β-glucosidase activity, which is indeed what we found (Fig. 7, *A–C*). The *N*B-DNJ titration curve was complex and fitted best with a two-site model, which assumes two different IC_{50} values, IC_{50}Hi and IC_{50}Lo (Fig. 7, *A–C*). IC_{50}Hi values of *N*B-DNJ were ~6 nM, whereas IC_{50}Lo values were at least 3 orders of magnitude higher, 105–189 μM for SH-SY5Y cells and testis (Table 4), which is in the same range as the *N*B-DNJ IC_{50} determined previously for GBA (Table 1). Accordingly, we attributed the IC_{50}Hi and IC_{50}Lo values of *N*B-DNJ to GBA2 and GBA, respectively.

In contrast to *N*B-DNJ, the highest *N*B-DGJ concentration did not suppress all β-glucosidase activity but left a residual activity (Fig. 7, *D–F*), which is in line with the selectivity of *N*B-DGJ toward GBA2. *N*B-DGJ inhibited the β-glucosidase activity following a sigmoidal curve (Fig. 7, *D–F*), with IC_{50}

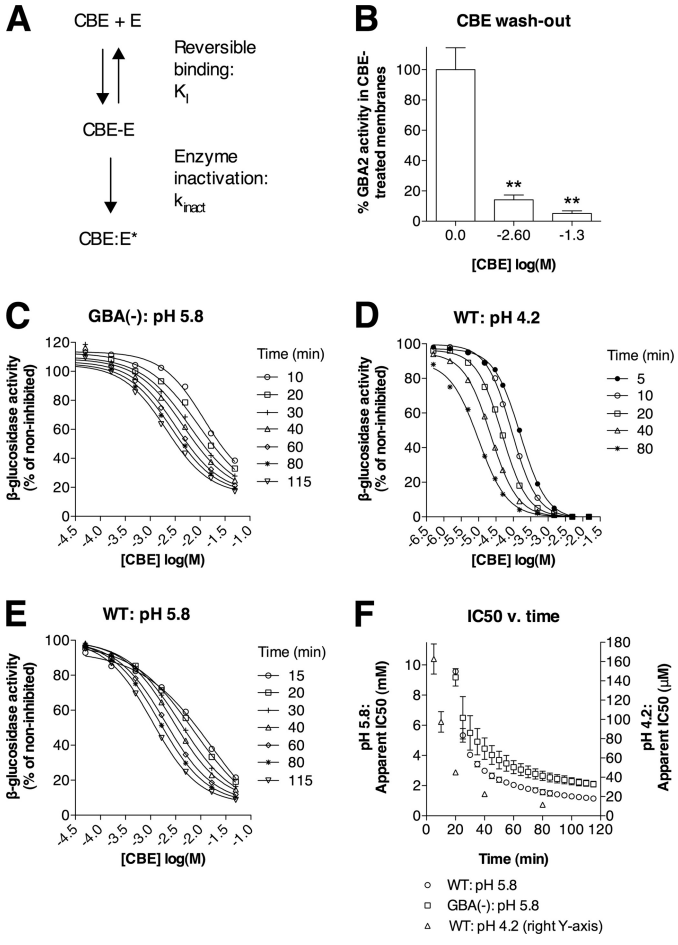

FIGURE 6. **CBE inhibits GBA2 in an irreversible time- and dose-dependent fashion.** *A,* model of the interaction of CBE with an enzyme (*E*), distinguishing reversible binding (*CBE-E*) and inactivating the enzyme by covalent binding (*CBE:E**), and the kinetic parameters that apply to these interactions, K_I and k_{inact}, respectively. *B,* wild-type brain membranes were incubated with or without CBE for 60 min, repeatedly diluted and sedimented by centrifugation, and assayed for their remaining *N*B-DGJ-sensitive β-glucosidase activity. Transient exposure to CBE was sufficient to reduce the membrane-associated GBA2 activity ($n = 3$). *C–E,* to determine time-dependent IC_{50} values of CBE toward GBA2 and GBA, different enzyme preparations were incubated for increasing lengths of time with substrate and CBE (without preincubation); pH 5.8 GBA-deficient brain membranes (containing only GBA2) (*C*), pH 4.2 detergent extract (exhibiting only GBA activity) (*D*), and pH 5.8 wild-type brain membranes (*E*). Typical results are shown. *F,* decrease of CBE IC_{50} values over time, derived from data in *C–E*. Data are from 2 or 3 independent determinations. **, $p < 0.001$.

TABLE 3

Effect of CBE on the GBA2 activity in cultured cells

Various cell lines were cultured in the presence of CBE for 2 days. Cells were harvested and assayed for GBA2 activity *in vitro*. Expressed is the level of *N*B-DGJ-sensitive β-glucosidase activity in the CBE-treated cells as percentage of control values. Average values are from 3 to 4 independent experiments ± S.D. Abbreviations are as in Table 2.

Cell line	% of GBA2 activity relative to control cells, CBE in culture medium	
	50	500 μM
IMR-32	73.4 ± 17	32.7 ± 5.8
IMR-32 + BA	90.4 ± 11	43.5 ± 7.9
SH-SY5Y + RA	16.5 ± 1.7	4.7 ± 0.5
RAW	83.1 ± 7.8	34.5 ± 8.3
THP-1	73.5 ± 8.4	38.0 ± 5.2
SH-SY5Y-GBA2 line 13	91.6 ± 17	40.2 ± 7.8
SH-SY5Y-GBA2 line 17	96.4 ± 6.1	40.7 ± 2.9

values ranging from 1 to 6 μM (Table 4). *N*B-DNJ and *N*B-DGJ thus affected the β-glucosidase activity of control membranes differently, diverging in curve fit and in their IC_{50} values toward GBA2.

NB-DNJ Versus NB-DGJ in CBE-treated Membranes—In these membranes, *N*B-DNJ reduced the β-glucosidase activity in standard sigmoidal fashion (Fig. 7, *A–C*), with apparent IC_{50} values that were 10 times higher than the IC_{50}Hi values measured in control membranes (Table 4). The IC_{50}Lo side of the *N*B-DNJ titration curves seen in control membranes was absent in CBE-treated membranes. *N*B-DGJ IC_{50} values were similar to those found for control membranes (Fig. 7, *D–F*; Table 4). However, for a part of the concentration range, *N*B-DGJ *increased* the β-glucosidase activity of CBE-treated SH-SY5Y and brain membranes (Fig. 7, *D* and *F*).

Preincubating membranes with CBE thus resulted in apparent *N*B-DNJ IC_{50} values that were higher than those measured in control membranes and reversed the effect of some *N*B-DGJ concentrations on the β-glucosidase activity, from inhibitory to activating. It is currently not clear how the interaction of *N*B-DGJ and CBE has this effect on the β-glucosidase activity.

Finally, we evaluated the impact of AMP-DNJ on β-glucosidase activity in brain membranes. AMP-DNJ (Fig. 1*E*) decreased the enzyme activity with similar efficiencies in control and CBE-treated membranes, fitting a standard sigmoidal curve with an IC_{50} of 0.8 nM (Fig. 7*G*; Table 4). The highest AMP-DNJ concentration extinguished all enzyme activity, irrespective of CBE pretreatment (Fig. 7*G*). Overall, the imino sugar titration curves of CBE-treated membranes appear to reflect both the irreversible inhibition of GBA and GBA2 by CBE and the reversible inhibition of GBA2 (and GBA in the case of *N*B-DNJ and AMP-DNJ).

DISCUSSION

To fully appreciate the physiological and pathological roles of GBA2, as well as its pharmacology, it is fundamental to characterize the β-glucosidase activity exerted by this enzyme and to distinguish its activity from that of GBA. To delineate GBA2 activity, we have assayed β-glucosidase activities at different pH values and utilized the distinct small molecular β-glucosidase inhibitors, *N*B-DGJ and CBE.

We first established that GBA in its native membrane-bound state and accompanied by the required co-factor is not sensitive to inhibition by *N*B-DGJ, irrespective of the type of substrate used. Our results are in agreement with a previous study using GBA purified from human placenta, which was impervious to *N*B-DGJ (36). The lack of an effect of *N*B-DGJ on GBA thus appears consistent. Accordingly, the finding of Wennekes *et al.* (84) that *N*B-DGJ inhibited GBA (Table 1) may need to be re-examined.

Next, by comparing wild-type and GBA-deficient brain membranes, we determined that GBA2 had a mildly acidic pH optimum (5.5–6.0), which overlapped with that of GBA (3.5–5.5). Accordingly, pH 4.2 was chosen to selectively assay GBA activity in samples prepared from wild-type tissues, in agreement with earlier studies (11, 77).

We further examined the sensitivity of GBA2 for inhibition by CBE, which is a small molecular compound that is well established as an irreversible inhibitor of GBA (63–67). GBA2 activity has been operationally defined as the membrane-associated β-glucosidase activity that is not inhibited by CBE (34, 62). However, Matern *et al.* (35) reported that the activity of GBA2 toward glucosylated bile acids is sensitive to inhibition by CBE, albeit with different sensitivities depending on whether the enzyme is membrane-associated or not. We addressed this inconsistency by specifically assessing the β-glucosidase activity of GBA2 for its sensitivity to inhibition by CBE, using membrane preparations that are devoid of GBA. We found that GBA2 is inactivated by CBE, in a time-dependent fashion, which is typical for mechanism-based enzyme inhibitors (80, 81). Measurement of K_I and k_{inact} values showed that CBE bound GBA2 with less affinity and that CBE inactivated GBA2 at a lower rate, as compared with the interaction of CBE and GBA. The K_I value we obtained for GBA, 140 μM, is very close to the 166 μM by Grabowski *et al.* (67). However, our value for k_{inact} (0.59 min^{-1}) was 10-fold higher than that found in the earlier study (0.051 min^{-1}) (67). This discrepancy is possibly due to differences in the assay and analytical methods used to obtain these kinetic parameters.

In membranes prepared from tissues of wild-type mice, containing both GBA and GBA2, the inhibitory effects of CBE and *N*B-DGJ were not mutually exclusive. Instead, a proportion of the membrane-associated β-glucosidase activity was sensitive to CBE as well as *N*B-DGJ. Having established that *N*B-DGJ does not affect GBA, these results indicate that the β-glucosidase activity that is sensitive to CBE as well as *N*B-DGJ needs to be attributed to GBA2. We conclude that, under our experimental conditions, CBE irreversibly inhibits all GBA activity as well as a proportion of the GBA2 activity in membranes prepared from mouse tissues and in homogenates of human and murine cell lines.

Korschen *et al.* (11) assessed the impact of up to 100 μM CBE on GBA2 activity in post-nuclear supernatants prepared from various mouse tissues and GBA2-overexpressing HEK293 cells without preincubation, and continuously measured the release of the cleavage product 4-methylumbelliferone (over an unspecified time period). When assayed in this fashion, CBE only caused a minor decrease in the GBA2 activity in mouse tissues and GBA2-overexpressing HEK293 cells (11). The lack of an effect of CBE on GBA2 in this study is likely due to the

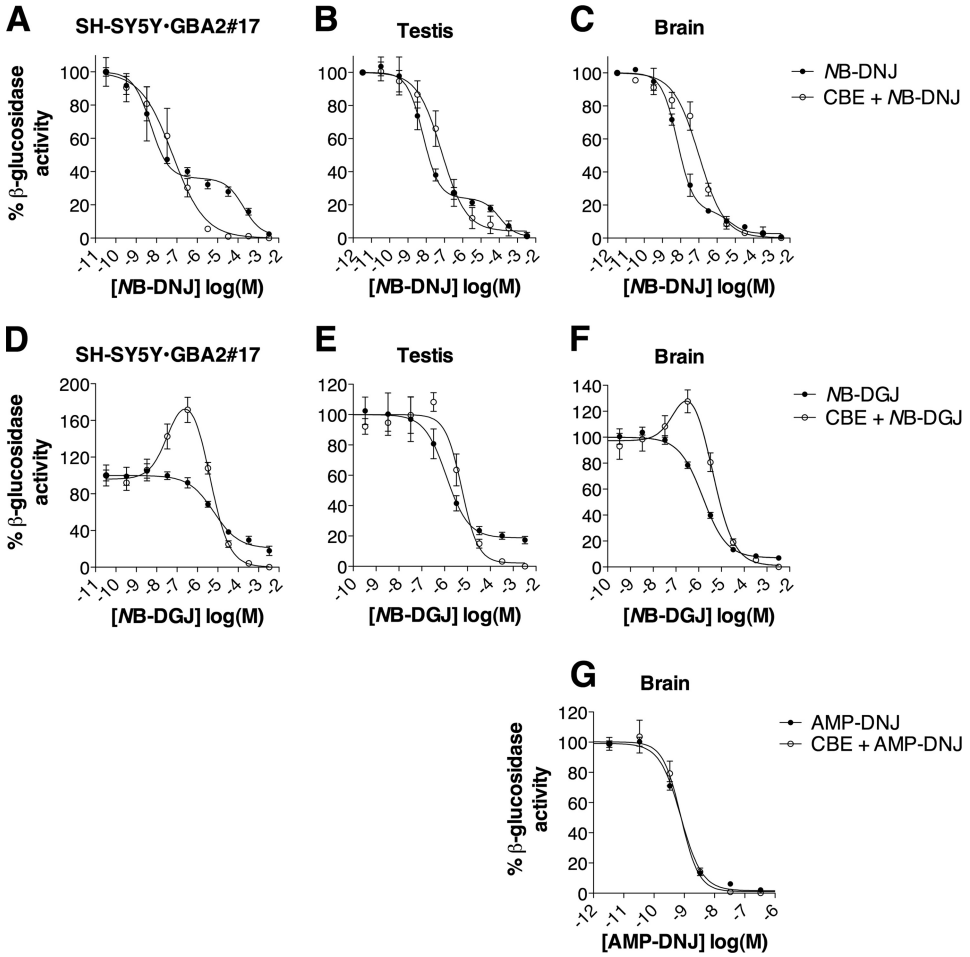

FIGURE 7. **CBE alters the effects of *N*B-DNJ and *N*B-DGJ on the membrane-associated β-glucosidase activity.** Membranes prepared from GBA2-overexpressing SH-SY5Y cell line 17 (*A* and *D*), adult mouse testis (*B* and *E*), and adult mouse brain (*C, F,* and *G*) were preincubated with or without 2.5 mM CBE prior to titration of the β-glucosidase activity for its sensitivity to inhibition by *N*B-DNJ (*A, B,* and *C*), *N*B-DGJ (*C, D,* and *E*), and AMP-DNJ (*G*). *A, B,* and *C,* without CBE treatment, the membrane-associated β-glucosidase activity had two components, one highly sensitive to inhibition by *N*B-DNJ, and one less sensitive to *N*B-DNJ (see Table 4 for IC_{50} values). In CBE-treated membranes, *N*B-DNJ inhibited the β-glucosidase activity following a standard sigmoidal curve, without a low *N*B-DNJ-sensitive component. *D, E,* and *F,* in the absence of CBE, *N*B-DGJ reduced the β-glucosidase activity in a sigmoidal fashion, with 20 to 10% of the β-glucosidase activity being insensitive to *N*B-DGJ. In contrast, in CBE-treated membranes, *N*B-DGJ affected the β-glucosidase activity following a bell-shaped biphasic curve, increasing the activity at certain concentrations. *A–F,* in CBE-treated membranes, the β-glucosidase activity was fully inhibited at the highest *N*B-DNJ and *N*B-DGJ concentrations. *G,* CBE did not affect the inhibition of GBA2 by AMP-DNJ, which suppressed all β-glucosidase activity at the highest levels. Data are from three independent assays.

TABLE 4

IC_{50} values of alkylated iminosugars toward the β-glucosidase activity measured in the absence or presence of CBE, derived from data presented in Fig. 7

β-Glucosidase activity was measured in membranes prepared from GBA2-overexpressing SH-SY5Y cells, mouse testis, and mouse brain. Best curve fit for enzyme activity data was dependent on the presence of CBE, and on the type of imino sugar.

	*N*B-DNJ			*N*B-DGJ		AMP-DNJ	
	−CBE[a]		+CBE[b]	−CBE[b]	+CBE	−CBE[b]	+CBE[b]
	nM	μM	nM	μM	μM	nM	nM
SH-SY5Y-GBA2 line 17	5.2	189	62.5	6.4	3.9[c]		
Testis	6.8	105	70.0	1.2	5.6[b]		
Brain	6.7	5.2	102	1.5	4.7[c]	0.7	0.9

[a] Two-sites curve fit.
[b] Sigmoidal curve fit.
[c] Bell-shaped curve fit.

relatively low CBE concentrations, considering our finding of apparent CBE IC_{50} values in the range of 2–9 mM.

AMP-DNJ has been applied as a tool to assess GBA2 activity, as a specific GBA2 inhibitor (85). However, the *in vitro* IC_{50} values of AMP-DNJ toward GBA2 (0.8 nM (this study) or 1–2 nM (post-CBE (34, 84)) are rather close to its IC_{50} value toward membrane-bound GBA (48 nM) (Table 1) (34). Consequently, it is hard to choose any concentration of AMP-DNJ that fully inhibits GBA2 and at the same time does not affect GBA at all. This is evident in our titrations of β-glucosidase activity in brain membranes, where increasing concentrations of AMP-DNJ are paralleled by a continuously decreasing enzyme activity, until 100% inhibition is reached. Clearly, AMP-DNJ inhibited both the GBA2 and GBA activities. In contrast, the *N*B-DNJ IC_{50} values toward GBA2 and GBA are very far apart, 6–20 nM and 100–500 μM, respectively (Table 1). When care is taken not to inhibit GBA, *N*B-DNJ is therefore a potential alternative to selectively inhibit GBA2 activity and thus to measure GBA2 activity. Indeed, GBA2 activity has been measured as the *N*B-DNJ-sensitive β-glucosidase (11, 86, 87).

Our conclusion that GBA2 is sensitive to inhibition by CBE warrants a reassessment of the method used to measure GBA2 activity. Clearly, as CBE will inhibit a proportion of the GBA2

activity in samples of interest, the CBE-resistant β-glucosidase will be considerably lower than the total GBA2 activity. The application of CBE in assays to measure GBA2 activity therefore needs to be reconsidered. In contrast, we found that submillimolar concentrations of *N*B-DGJ do not affect GBA at all, and at the same time, these concentrations of *N*B-DGJ inhibit over 99% of the β-glucosidase activity in GBA-deficient membranes, *i.e.* GBA2 activity. *N*B-DGJ thus distinguishes GBA from GBA2, it exclusively inhibits GBA2 and can therefore be utilized to delineate GBA2 activity. We found that the *N*B-DGJ-sensitive β-glucosidase activity was 3–9- and 1.5–10-fold higher than the CBE-resistant activity in mouse tissue membranes and cell homogenates, respectively. Our data also show that instead of representing less than 10% of the total β-glucosidase activity, the GBA2 activity was responsible for the majority of the detergent-independent β-glucosidase activity of most tissues and cell lines.

We further observed that the sensitivity of the membrane-associated β-glucosidase activity to inhibition by *N*B-DNJ and *N*B-DGJ was altered by the inclusion of CBE. Following a preincubation with CBE, the β-glucosidase activity was 10-fold less sensitive to *N*B-DNJ compared with the untreated enzyme. Without exposure to CBE, the *N*B-DNJ IC_{50} toward GBA2 was around 6 nM, in membranes prepared from mouse brain and testis as well as that from SH-SY5Y cells overexpressing human GBA2. This *N*B-DNJ IC_{50} value was close to those found by Korschen *et al.* (12–20 nM) (11) but considerably lower than the IC_{50} values reported in earlier studies (Table 1). Furthermore, CBE dramatically altered the response of GBA2 to certain concentrations of *N*B-DGJ, from inhibitory to activating. Our findings suggest that the inhibitory potency of alkylated imino sugars and other monosaccharides toward GBA2 may be reliably assessed when the enzyme is exposed to such molecules in the absence of CBE, eliminating the confounding impact of CBE on GBA2 activity.

We also observed that exposing live cells to 500 μM CBE for 2 days lowered the GBA2 activity by ~60% (measured as *N*B-DGJ-sensitive β-glucosidase activity), irrespective of the absolute level of GBA2 activity. This finding is relevant in light of the application of CBE to establish chemically induced models of Gaucher disease in cultured cells (200–500 μM (88–90)) and *in vivo* (100 mg/kg/day (91, 92)). In Gaucher disease, however, only GBA is affected, whereas it is now evident that CBE impacts both GBA and GBA2. Considering that these two enzymes both act on GlcCer, it will be difficult to distinguish which of the consequences of CBE treatment are due to inhibition of GBA and of GBA2. The combined inhibition of GBA and GBA2 could therefore be a confounding factor in the interpretation of observations made in CBE-treated cells, especially in light of the recent finding of mutations in the *GBA2* gene in neurological syndromes (13, 14).

Our data and those of Korschen *et al.* (11) indicate that GBA2 is more sensitive to inhibition by *N*B-DNJ than previously estimated. It is therefore likely that, in Gaucher and Niemann-Pick type C patients who are prescribed *N*B-DNJ (miglustat, Zavesca®), GBA2 activity may be more reduced than assumed thus far. This is also suggested by our earlier finding of highly elevated GlcCer levels in the brain of normal mice that had been treated with relatively low doses of *N*B-DNJ (9). The potential clinical relevance of GBA2 inhibition by *N*B-DNJ remains to be established.

In summary, we have further characterized the enzymatic activity of GBA2 and propose a robust approach to discriminate GBA2 activity from that of GBA. Our findings will permit a more thorough assessment of the potential role of GBA2 in Gaucher disease, especially because, in Gaucher cells, the accumulation of GlcCer appears not to be limited to the endolysosomal compartment (93). Aureli *et al.* (85) and Burke *et al.* (86) recently reported that the GBA2 activity is increased in Gaucher fibroblasts and leukocytes and in GBA-deficient mouse brains, and Körschen *et al.* (11) found that the GBA2 activity is reduced in murine GBA-deficient fibroblasts and in type 2 Gaucher fibroblasts. In addition, the ability to assess GBA2 activity in full will be an asset in establishing the contribution of GBA2 to sphingolipid metabolism, to determine the pharmacological properties of newly developed monosaccharide mimetics, and to evaluate the roles of GBA2 in physiological and pathological processes, including the recently discovered involvement of GBA2 in hereditary motor neuron disorders (13, 14), malignant melanoma (15), and may thus be helpful in clinical practice.

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