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**Glucocerebrosidase deficiency and mitochondrial impairment  
in experimental Parkinson disease**

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**Running title :** Glucerebrosidase and PD

**ABSTRACT**

Gaucher disease is an autosomal recessive disease, caused by a lack or functional deficiency of the lysosomal enzyme, glucocerebrosidase (GCCase). Recently, mutations in the glucocerebrosidase gene (GBA) have been associated with Parkinson disease (PD) and GBA mutations are now considered the most important genetic vulnerability factor for PD. In this study, we have investigated (i) *in vivo* whether inhibition of the enzyme glucosylceramide synthase by miglustat may protect C57Bl/6 mice against subchronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication and (ii) *in vitro* whether a decrease of GCCase activity may render dopaminergic neurons susceptible to MPP<sup>+</sup> (1-methyl-4-phenylpyridinium) or alpha-synuclein ( $\alpha$ -Syn) toxicity and amenable to miglustat treatment. We could demonstrate that reduction of glucocerebrosidase by inhibition of glucosylceramide synthase partially protects mice against MPTP-induced toxicity. Conversely, we could show that inhibition of GCCase activity with conduritol-B-epoxide (CBE) enhances both  $\alpha$ -Syn and MPP<sup>+</sup> induced toxicity *in vitro*. However, only CBE-induced enhancement of MPP<sup>+</sup> toxicity could be reversed by miglustat. Moreover, we were unable to reveal any alterations of complex I activity or cell respiration upon treatment with either CBE or miglustat. Our findings suggest that the reduction of GCCase activity rather than an accumulation of glucocerebrosidase increases  $\alpha$ -Syn toxicity.

**Key words** : Glucocerebrosidase, Gaucher disease, Parkinson disease, dopamine cell death, MPTP,  $\alpha$ -Syn, mitochondria.

**Abbreviations:**

$\alpha$ -Syn, alpha-Synuclein; CBE, conduritol-B-epoxide; CCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; CS, citrate synthase; Cx I, Mitochondrial respiratory chain complex I; DA, dopaminergic; DIV, day in vitro; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; ECAR, extracellular acidification rate; GBA, glucocerebrosidase gene; GCCase : glucocerebrosidase; GDNF, Glial cell-derived neurotrophic factor; HPLC, high-performance liquid chromatography; HVA, homovanillic acid; LB, Lewy bodies; LDH, lactate dehydrogenase; LUHMES, Lund human mesencephalic cell line; MPP<sup>+</sup>, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NeuN, neuronal nuclei; OCR, oxygen consumption rate; PBS, phosphate buffered saline; PD, Parkinson disease; PFA, paraformaldehyde; SH-SY5Y, human neuroblastoma cell line; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

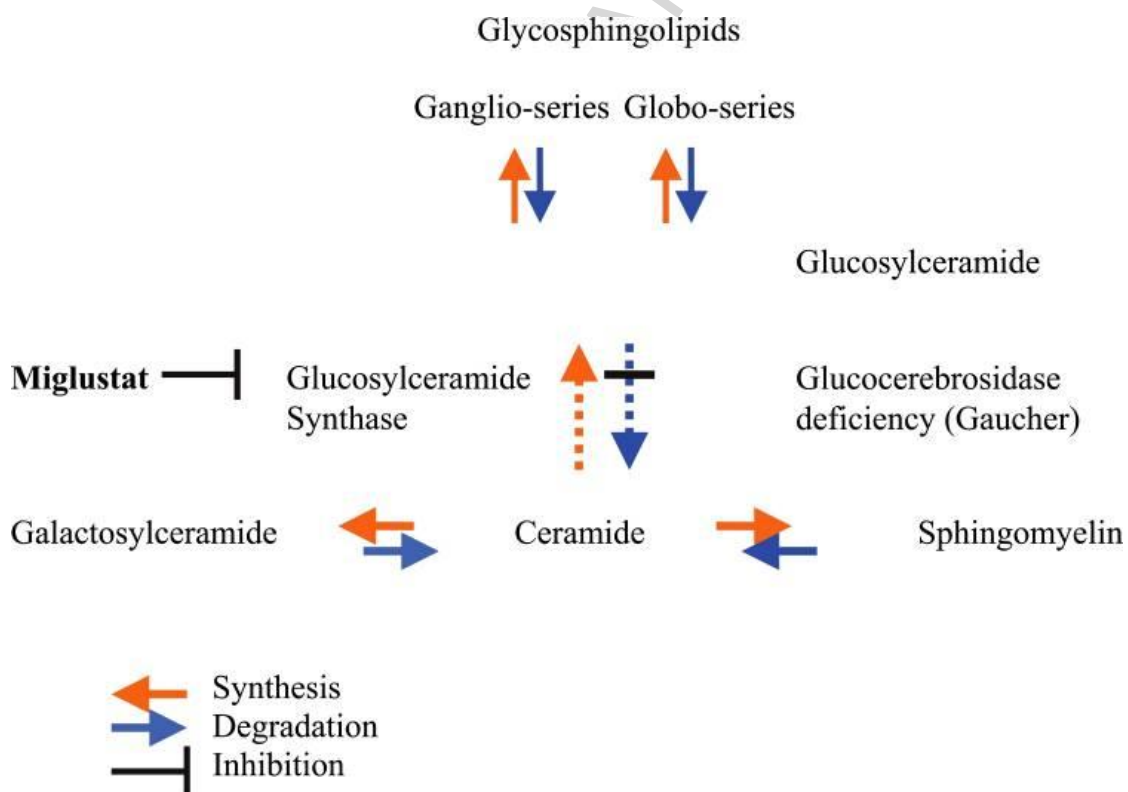
## 1. INTRODUCTION

Gaucher disease is an autosomal recessive disease, caused by a lack or functional deficiency of the lysosomal enzyme, glucocerebrosidase (GCase), which catalyses the breakdown of glucocerebroside to glucose and ceramide [1]. One therapeutic approach is the treatment with N-butyldeoxynojirimycin, known as miglustat, an imino sugar that inhibits glucosylceramide synthase, a glucosyl transferase enzyme responsible for the first step in the synthesis of most glycosphingolipids, including glucosylceramide (**Fig. 1**). Thereby, miglustat reduces intracellular storage of glucosylceramide. Recently, mutations in the glucocerebrosidase gene (GBA) have been associated with Parkinson disease (PD), a common neurodegenerative disorder characterized clinically by akinesia, rigidity and rest tremor. The cardinal neuroanatomical feature of PD is a massive and preferential loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), resulting in a drastic decline in striatal dopamine concentrations. Additionally, aggregates of fibrillated alpha-synuclein ( $\alpha$ -Syn) are the major component of so-called Lewy bodies (LB) which are the pathologic hallmarks of PD [2, 3].

In a large multicenter study, 3% of patients with PD, so far classified as 'idiopathic', displayed the frequent N370S or L444P GBA mutations [4]. When taking into consideration rarer mutations, this figure has been estimated to rise to 7% [5]. Moreover, this association appears not to be restricted to Ashkenazi Jews, as initially suggested [6], but is present worldwide in a multitude of ethnicities; thus, GBA mutations can be considered the most important genetic vulnerability factor for PD identified to date [7-9]. Interestingly, in the substantia nigra of sporadic PD patients without GBA mutations, GCase deficiency could be demonstrated very recently [10], lending support to the notion that GCase function may be altered in synucleinopathies [11, 12]. Conversely, dysfunction of GCase has been shown to increase the levels of intracellular  $\alpha$ -Syn [13-15]. However, the mechanism underlying these findings remains unclear to date. Different hypotheses have been raised including a gain-of-novel-function model contributing to the enhanced aggregation of  $\alpha$ -Syn either by a direct or indirect interaction between GCase and  $\alpha$ -Syn.

In the present, proof-of-concept study, we have investigated *in vivo* whether inhibition of the enzyme glucosylceramide synthase by miglustat may protect mice against intoxication with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin selectively targeting dopaminergic neurons. Based on our *in vivo* results, we have further attempted to clarify *in vitro* whether a decrease of GCCase activity may render dopaminergic neurons more susceptible to MPP<sup>+</sup> (1-methyl-4-phenylpyridinium), the active metabolite of MPTP, or  $\alpha$ -Syn-mediated toxicity, and if inhibition of glucosylceramide synthase also protects dopaminergic neurons under these conditions.

**Figure 1:**



**Inhibition of glucosylceramide synthesis with miglustat by Can Ficicioglu [16]**

## 2. MATERIALS AND METHODS

### 2.1. Animals

Animals were housed, handled, and cared for in accordance with the Guide for the Care and Use of Laboratory Animals [NCR (National Research Council) 1996] and the European Union Council Directive 86/609/EEC, and the experimental protocols were carried out in compliance with institutional ethical committee guidelines for animal research. All efforts were made to minimize the number of animals used and their suffering. For all studies, mice were maintained on a 12:12 h light/dark cycle with lights on at 6.30 a.m. The room temperature was kept at 23°C, with free access to standard diet and tap water.

### 2.2. Subchronic MPTP intoxication of mice and miglustat treatment

Nine week old male C57/Bl6 mice (Janvier Breeding Center, France) were used (n=8-10 per group). The mice were injected i.p. with either 30 mg/kg MPTP-HCl once daily for a total of 5 days or a corresponding volume of NaCl 0.9% [17]. For miglustat treatment, the mice were fed 3 times daily by gavage (840 mg/kg body weight) or a corresponding volume of saline, respectively resulting in the following treatment groups of mice: saline treated control animals fed with either miglustat or saline (NaCl 0.9%) gavage (NaCl 0.9%/ miglustat or NaCl 0.9%/NaCl 0.9%) or mice treated intoxicated with MPTP receiving miglustat or saline (NaCl 0.9%) gavage (MPTP/miglustat, MPTP/NaCl 0.9%).

Miglustat administration was started one day after the MPTP intoxications and continued until sacrifice 7 days after the end of intoxications. Subsequently, the animals were killed by cervical dislocation and their brains were processed for further analysis.

All systemic injections were administered through the intraperitoneal route and the neurotoxins were dissolved in saline. All procedures involving MPTP injections in mice were performed according to standard procedures [18].

### 2.3. Neurochemical analysis

The striatum of the animals was used for high-performance liquid chromatography (HPLC) measurement of DA and homovanillic acid (HVA). Dopamine was analysed by reversed phase chromatography combined with a two-channel electrochemical detector under isocratic conditions as previously described [19]. Briefly, detector potential was set at +750 mV using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase (0.14 g octanesulfonic acid sodium salt, 0.1 g Na<sub>2</sub>EDTA, 6 ml triethylamine, adjusted to pH 2.8 with H<sub>3</sub>PO<sub>4</sub> in 1 l millipore Q water containing 35 ml acetonitrile) was delivered at a rate of 0.5 ml/min at 22°C onto the reversed phase column (125x3 mm with pre-column 5x3 mm; Nucleosil 120–3 C18; Knauer, Berlin, Germany). Twenty-microliter aliquots were injected by an autosampler with the cooling module set at 4°C.

#### **2.4. Immunohistochemistry**

The posterior part of the brains was postfixed with 4% paraformaldehyde (PFA) and cryoprotected. Immunohistochemistry was performed as described previously [20] on free-floating cryomicrotome-cut sections (20 µm) encompassing the entire midbrain. Sections were incubated with a rabbit polyclonal antibody against tyrosine hydroxylase (TH; 1:1000; Pel-Freez Biologicals, Rogers, AR). Sections were then treated with secondary antibodies (Vectastain, Vector Laboratories, Burlingame, CA) and subsequently incubated with avidin-biotinylated horseradish peroxidase complex. Peroxidase was revealed by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride containing 0.008% hydrogen peroxide. Sections were Nissl counter-stained (for details see [21]).

#### **2.5. Cell counting *in vivo***

Quantification was performed as described previously [21]. Tyrosine hydroxylase-positive neurons were visualized in bright field (Nikon Optiphot-2) and quantified stereologically on regularly spaced sections covering the mesencephalon from the rostral pole of the SNpc to the locus coeruleus (Mercator T4.18 stereology software). The SNpc was identified

according to established anatomical landmarks [22]. Cell loss was verified by Nissl counterstaining.

## 2.6. Primary mesencephalic cell cultures

For mouse mesencephalic cultures, E 13.5 Swiss mouse embryos were used (Janvier Breeding Center). The dissected tissue pieces were processed according to previously described protocols [23, 24]. Briefly, after mechanical dissociation in modified L15 medium with no enzymatic treatment, the cells were plated at a density of  $1.5\text{--}2.0 \times 10^5$  cells/cm<sup>2</sup>. The cells were then allowed to mature and differentiate in N5 culture medium [24], supplemented with 5% horse serum and 0.5% fetal calf serum except for the first 3 days *in vitro* (DIV), when the concentration of FCS was raised to 2.5%. Ara-C (2  $\mu\text{m}$ ) was added to the medium at DIV 1-2 after plating to inhibit proliferation of non-neuronal cells (astrocytes, microglia), as well in the presence of 1  $\mu\text{M}$  MK-801 to prevent secondary excitotoxic stress [25]. Mesencephalic cultures were treated either with 1  $\mu\text{M}$  MPP<sup>+</sup> (Sigma-Aldrich) at DIV 4 and DIV 5 as previously described [26] combined with 50, 100, 200 or 400  $\mu\text{M}$  conduritol-B-epoxide (CBE; Sigma-Aldrich) at DIV 1-10 or in combination of both. The cultures were left to recover until DIV 10 in the presence of control medium

## 2.7. $\alpha$ -synuclein overexpression in LUHMES cells

Experiments on  $\alpha$ -Syn overexpression were done in LUHMES cells, transduced with adenoviral vectors to overexpress human wild-type  $\alpha$ -Syn and untransduced cells for control conditions respectively [27]. LUHMES cells were cultured in Nunclon  $\Delta$  Surface (Nunc, Roskilde, Denmark) plastic cell culture flasks and multi-well plates pre-coated with 0.1 mg/mL poly-L-lysine and 5  $\mu\text{g}/\text{mL}$  fibronectin (Sigma-Aldrich). Proliferation medium consisted of Dulbecco's modified Eagle's medium/F12 (Sigma-Aldrich), 1% N-2 supplement (Life Technologies, Carlsbad, CA, USA), and 40 ng/mL recombinant basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA). LUHMES cells were grown at 37 °C in a humidified 95% air, 5% CO<sub>2</sub> atmosphere. Proliferating cells were enzymatically dissociated with trypsin



(138 mM NaCl, 5.4 mM KCl, 6.9 mM NaHCO<sub>3</sub>, 5.6 mM D-Glucose, 0.54 mM EDTA, 0.5 g/L trypsin from bovine pancreas type-II-S; Sigma-Aldrich) and passaged 1:10 when they reached 80% confluence. For differentiation the cells were transferred to multi-well plates at a density of 55,000/cm<sup>2</sup> in growth medium and 24 hours after plating the medium was exchanged to differentiation medium, consisting of Dulbecco's modified Eagle's medium/F12 (Sigma-Aldrich), 1% N-2 supplement (Life Technologies), 1 µg/ml tetracycline (Sigma-Aldrich), 0.49 mg/ml dibutyryl cyclic AMP (Sigma-Aldrich), and 2 ng/ml GDNF (R&D Systems). Virus transduction was performed 48 hours into the differentiation process at an multiplicity of infection of 5 as previously described [27].

## **2.8. Immunocytochemical procedures *in vitro***

Primary mesencephalic cell cultures were fixed for 15 min at room temperature with 4% PFA and washed three times with PBS. For primary mesencephalic cell cultures, survival of DA neurons was determined by TH immunocytochemistry. The cultures were first incubated for 24h at 4°C with a rabbit-polyclonal anti-TH antibody (Pel-Freez Biologicals) diluted 1:1000 in PBS containing 0.2% Triton X-100, then incubated for 1 h at room temperature with an anti-rabbit IgG Alexa488 conjugate (1:500; Sigma/RBI). Mesencephalic cultures contained between 1 and 2% TH+ cells at the time of plating [23]. All neuronal cells, regardless of their neurotransmitter phenotype, were identified by staining of neuronal nuclei with a monoclonal biotin conjugated antibody anti-neuronal nuclei (NeuN) (MAB-377B, Chemicon, Temecula, CA, USA) diluted 1:100 and a secondary antibody (biotin conjugated, Invitrogen, 1:500 in PBS and 5 % horse serum).

## **2.9. Quantification of *in vitro* neuron survival**

Survival of mesencephalic DA neurons was quantified by counting the number of tyrosine hydroxylase–positive neurons in primary cultures. Survival of LUHMES cells was quantified by the measurement of LDH released into the cell culture medium, using the CytotoxOne Membrane Integrity Assay (Promega, Fitchburg, WI, USA) according to the manufacturer's

instructions as previously described. Data were normalized to LDH levels measured in  $\alpha$ -Syn overexpressing cells. Positive controls were cells lysed with 10% Triton X in water as lysis buffer.

### **2.10. Measurement of cell respiration**

Respiration was analyzed using Seahorse Biosciences technology as described elsewhere [28]. To this end, SH-SY5Y (p3-p7) cells [29] (human neuroblastoma cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, France) supplemented with 15% fetal calf serum (FCS), 1 mM L-glutamine, 100U of penicillin/ml, and 100  $\mu$ g of streptomycin sulfate/ml and equilibrated with 5% CO<sub>2</sub>-95% air at 37°C. In brief, a Seahorse 24-well plate was seeded with approximately 300,000 SH-SY5Y cells/per well. At the day of plating, a fourth of the wells was treated with 800  $\mu$ mol CBE, 100  $\mu$ mol miglustat, the same volume of vehicle (PBS), and 100  $\mu$ mol miglustat followed one hour later by 800  $\mu$ mol CBE, respectively for 24 hours. After 24 hours, wells were processed to measure oxygen consumption rate (OCR) to quantify mitochondrial respiration and the extracellular acidification rate (ECAR), an indicator of glycolysis. After determining the basal respiration in the cells, oligomycin (2  $\mu$ M), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (CCCP, 0.5  $\mu$ M) and potassium cyanide (2 mM) were sequentially added to the media and the OCR and ECAR was quantified for each well in parallel. Data were expressed as % of baseline. The results from three independent plates with each 5-6 wells per condition were grouped.

### **2.11. Measurement of Complex I activity**

Mitochondrial respiratory chain complex I (Cx I) and citrate synthase (CS) activity were measured as previously described [30, 31]. In brief, SH-SY5Y were cultured as described above. Cells were treated with 800  $\mu$ mol CBE, 100  $\mu$ mol miglustat, the same volume of vehicle (PBS), and 100  $\mu$ mol miglustat followed one hour later by 800  $\mu$ mol CBE, respectively for 48 hours. After 48 hours, cells were homogenized in homogenization buffer (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA), nuclei and cell debris were removed by

centrifugation at 1,500 g, and mitochondria were isolated by centrifugation at 11,800 g. The mitochondrial pellet was then resuspended in homogenization buffer. Cx I and CS activities were measured by spectrophotometric methods in these mitochondrial preparations as reported [30, 31]. Data were expressed as ratios of Cx I/CS activity in % of control. Analysis was performed in four independent runs.

### **2.12. Statistical analysis**

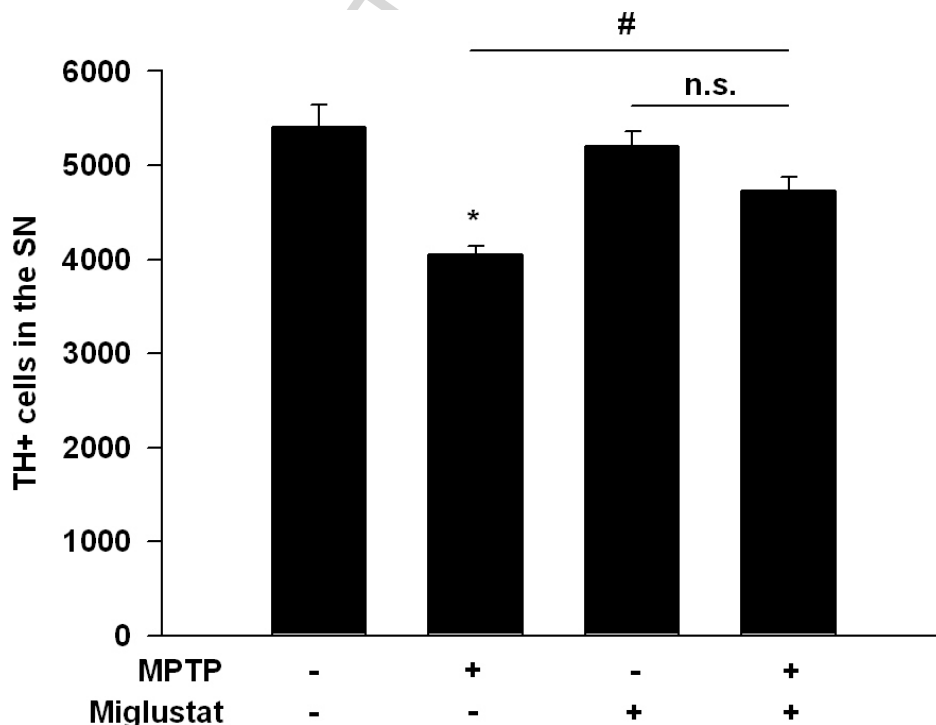
Data are expressed as the percent of corresponding control values. Each data point represents mean  $\pm$  S.E.M. Multiple comparisons against a single reference group were performed by one-way ANOVA followed by a post-hoc Dunnett's test. When pairwise comparisons were carried out, one-way ANOVA was followed by a post-hoc Student-Newman-Keuls test. The null hypothesis was rejected at an  $\alpha$  risk of 5%. All *in vitro* experiments were performed in triplicates at least, with a minimum of three wells per experimental condition.

### 3. RESULTS

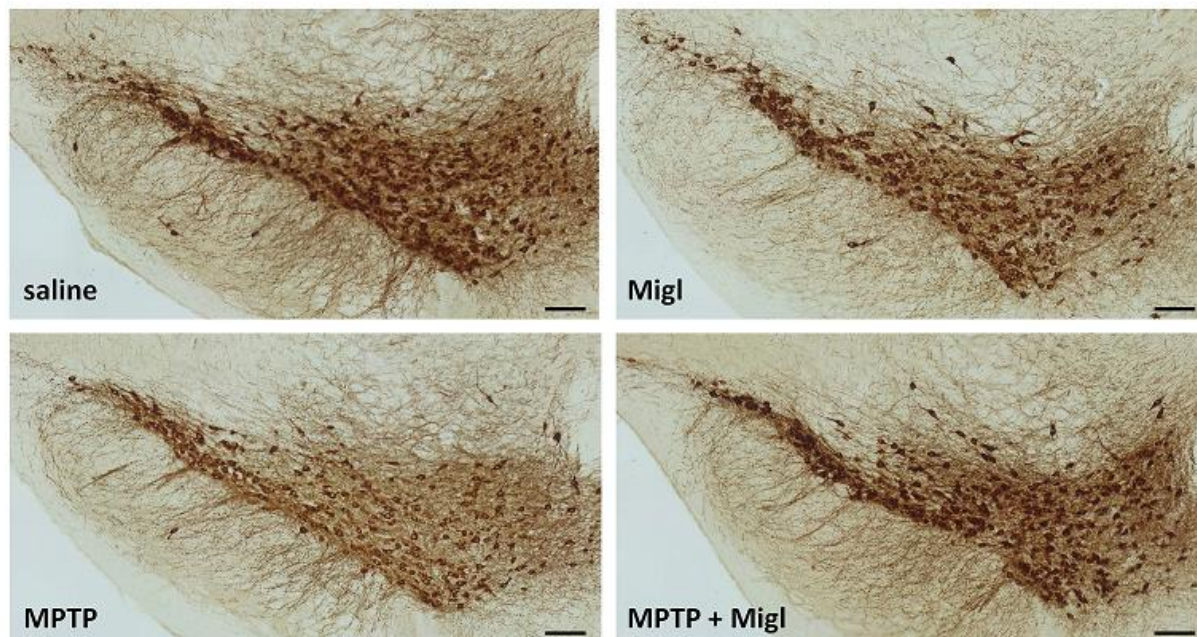
#### 3.1. Miglustat protects nigral DA cell bodies in MPTP-intoxicated mice

First, we tested whether reduction of glucocerebroside by inhibition of glucosylceramide synthase upon miglustat treatment may protect mice against MPTP in a subchronic intoxication protocol. MPTP-induced TH+ cell loss in the SN was  $25 \pm 1.7\%$ . In mice treated orally thrice daily with miglustat, TH+ cell loss was reduced to  $12.4 \pm 2.7\%$  ( $p < 0.05$ ) (**Fig. 2A/B**). Since the number of Nissl-positive/TH-negative neurons did not increase in MPTP-treated animals, the decrease in TH-positive neurons could be attributed to cell loss rather than loss of TH protein expression (percentage of TH-negative and Nissl-positive neurons in SNpc: miglustat-treated mice ( $96.3\% \pm 7.2\%$ ), saline-treated mice ( $94.9\% \pm 8.6\%$ ). However, striatal dopamine concentrations and HVA/DA ratios remained unaffected by miglustat treatment (**Fig. 3 A/B**), suggesting that protection did not extend to synaptic terminals.

**Figure 2A/B: Miglustat protects nigral DA cell bodies in MPTP-intoxicated mice**

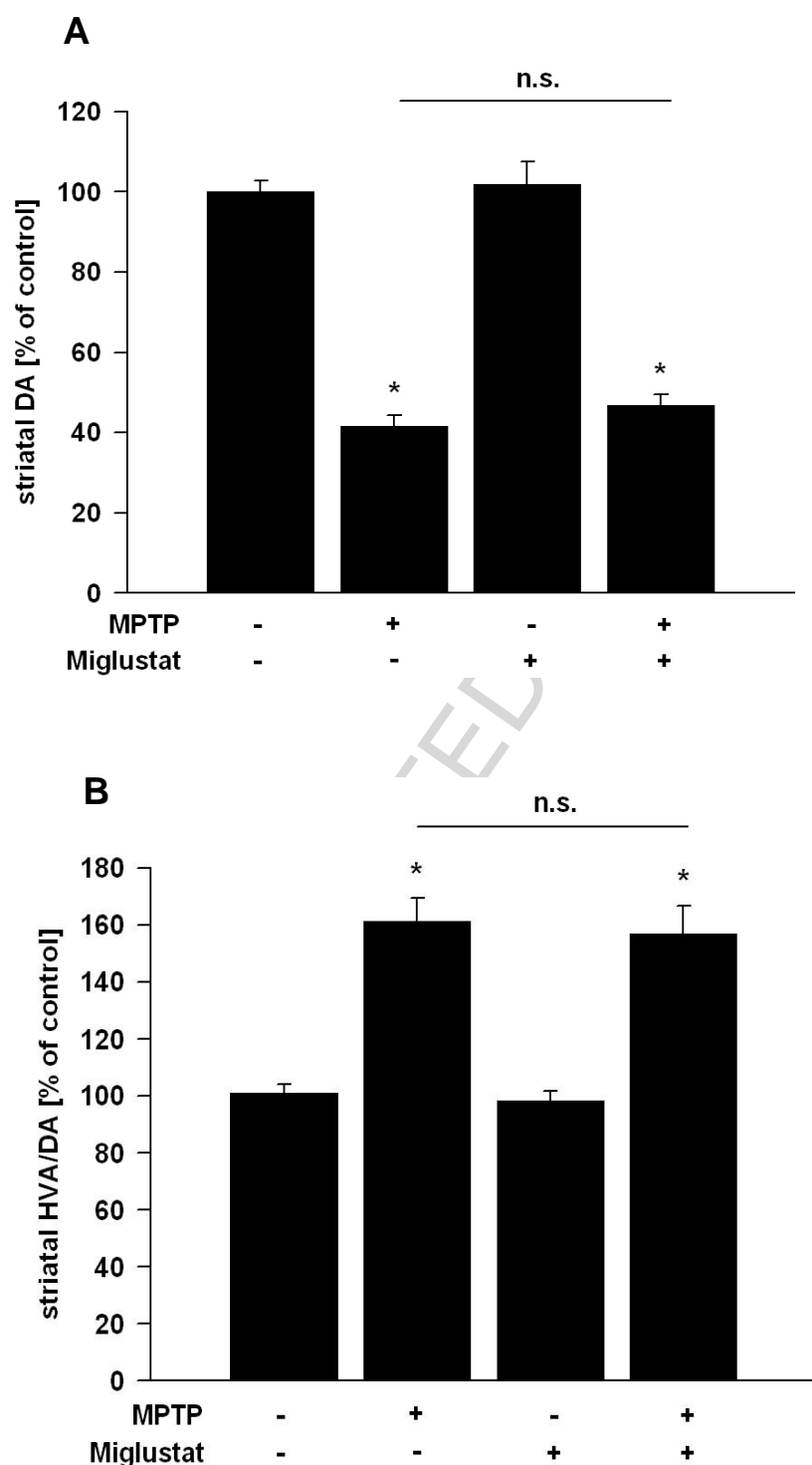


**2A: Effects of miglustat treatment on subchronic MPTP induced nigral dopaminergic cell death.** Data are expressed as percent of corresponding control values and represent mean  $\pm$  S.E.M. \* $p < 0.05$  compared to control values, # $p < 0.05$  for pairwise comparison.



**2B: Representative photographs of the SNpc (TH staining) of each treatment group.** Note the reduced number of TH+ cells in the SNpc after MPTP intoxication compared to control; miglustat attenuated MPTP-induced cell loss. Magnification: 20x; Scale bar: 50  $\mu$ m.

Figure 3: Striatal dopamine concentrations and HVA/DA ratios are unaffected by miglustat treatment



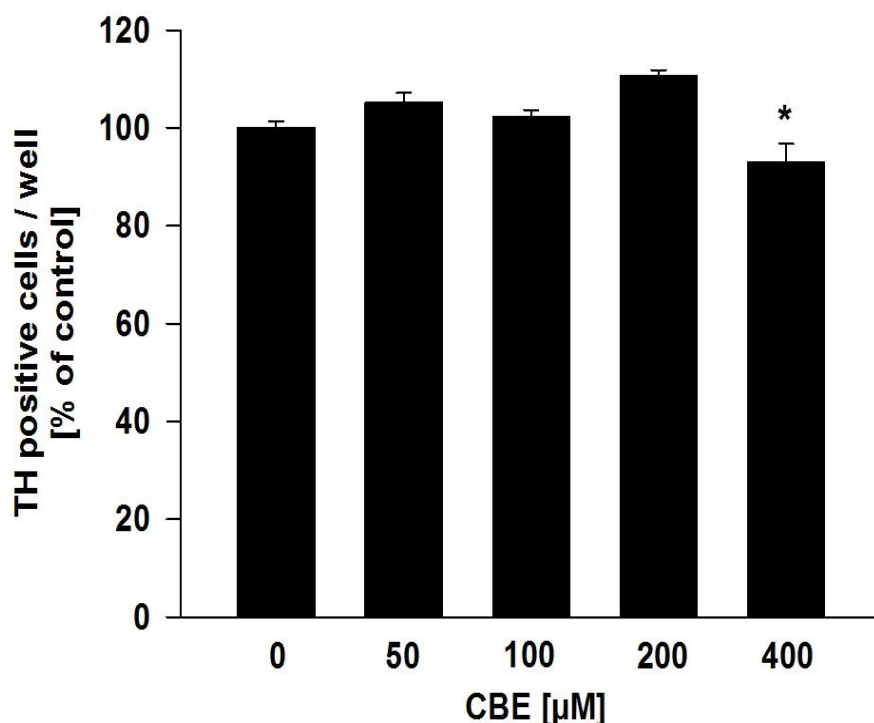
Effects of miglustat treatment on striatal neurotransmitter levels after MPTP intoxication (A: DA; B: HVA/DA ratio). Data are expressed as the percent of corresponding control values. Each data point represents mean  $\pm$  S.E.M. (\* $p$ <0.05, compared to control values).

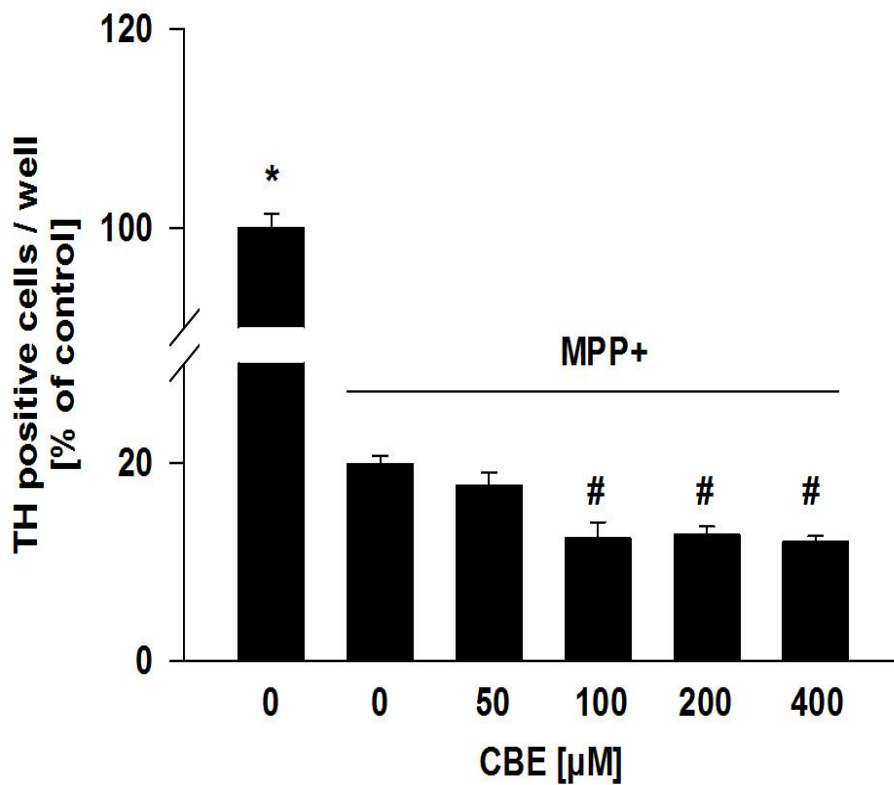
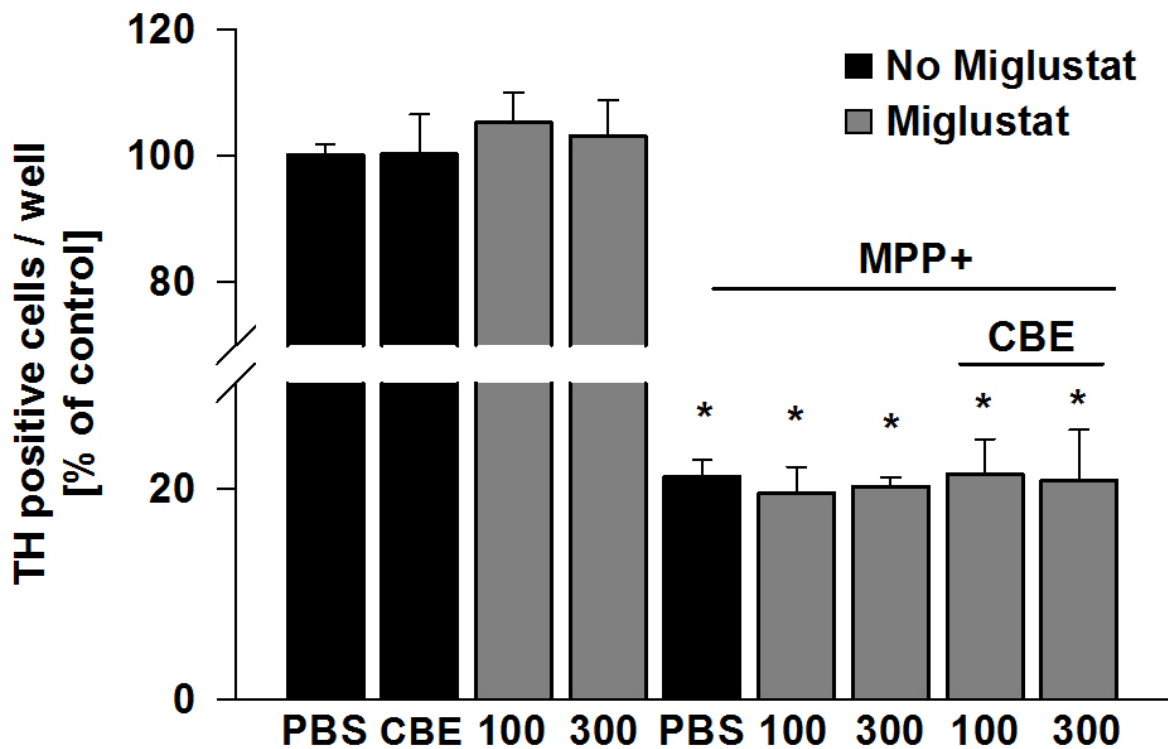
### 3.2. GCase inhibition enhances MPP<sup>+</sup> toxicity *in vitro*

Next, we tested the effect of GCase inhibition on MPP<sup>+</sup>-induced toxicity *in vitro*. Primary mesencephalic cell cultures were treated with different concentrations of CBE (50 - 400  $\mu$ M): this treatment did not alter TH<sup>+</sup> cell number at concentration of 50-200 $\mu$ M (**Fig. 4A**). Cultures were then treated with MPP<sup>+</sup> (1  $\mu$ M): TH<sup>+</sup> cell loss under these conditions was  $80.2 \pm 0.8$  %. When CBE was added, TH<sup>+</sup> cell number further decreased by  $8.1 \pm 0.8$  % (**Fig. 4B**). These results show that CBE is devoid of intrinsic toxic properties on TH<sup>+</sup> neurons in primary mesencephalic cell cultures, but is able to potentiate the toxic effects of MPP<sup>+</sup>. Miglustat did not protect against MPP<sup>+</sup> toxicity alone; however, miglustat was able to counteract CBE-enhanced toxicity of MPP<sup>+</sup> (**Fig. 4C**).

Figure 4:

#### A CBE (50 - 200 $\mu$ M) does not alter the number of TH<sup>+</sup> cells



**B** CBE causes increased MPP<sup>+</sup> toxicity**C** Miglustat is able to counteract CBE-enhanced toxicity of MPP<sup>+</sup>



### Effects of CBE, a GCase inhibitor, on the survival of TH-positive cells *in vitro* in the absence (A) or presence (B) of MPP<sup>+</sup> and the effect of co-treatment with miglustat (C).

**A:** First, CBE was used in different concentrations (50-400  $\mu\text{M}$ ) on primary mesencephalic cultures. Only at a concentration of 400  $\mu\text{M}$  a significant decrease of TH<sup>+</sup> neurons were detectable (\* $p < 0.05$  compared to control values).

**B:** TH<sup>+</sup> cell loss after MPP<sup>+</sup> treatment (1  $\mu\text{M}$ ) was  $80.2 \pm 0.8\%$ . Co-treatment with CBE (50-400 $\mu\text{M}$ ) causes an increased MPP<sup>+</sup> toxicity (\* $p < 0.05$  compared to control values, # $p < 0.05$  for pairwise comparison compared to MPP<sup>+</sup> without CBE).

**C:** TH<sup>+</sup> cell loss after MPP<sup>+</sup> treatment (1  $\mu\text{M}$ ) was  $80.2 \pm 0.8\%$  (\* $p < 0.05$  compared to PBS control). Treatment of miglustat at a concentration of 100 and 300  $\mu\text{M}$ , respectively did not protect against MPP<sup>+</sup> toxicity. However, miglustat was able to counteract CBE-enhanced-toxicity (100 $\mu\text{M}$ ) of MPP<sup>+</sup>.

Data are expressed as the percent of corresponding control values (TH<sup>+</sup> cells per well in % of control, control= 100%= 257 cells/well). Each data point represents mean  $\pm$  S.E.M (\* $p < 0.05$  compared to PBS control values).

### 3.3 $\alpha$ -Syn enhances toxicity of GCase inhibition *in vitro*

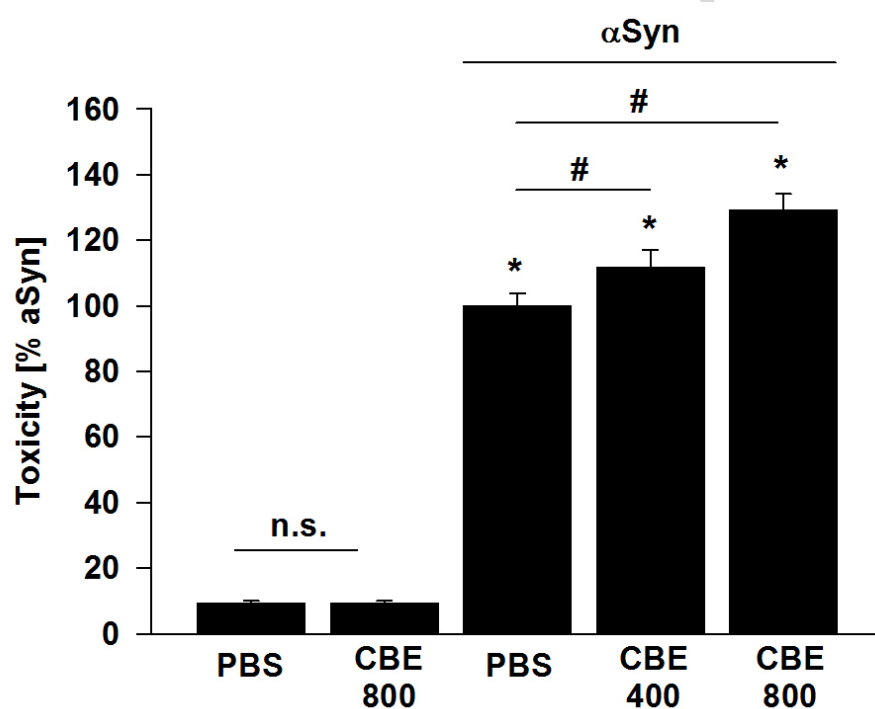
It is known that a reduction in GCase activity leads to an accumulation of  $\alpha$ -Syn in neuroblastoma cells [13-15]. Therefore, we wanted to test whether GCase inhibition by conduritol-B-epoxide (CBE) would increase toxicity in  $\alpha$ -Syn-overexpressing LUHMES cells, a human dopaminergic neuronal cell line. Overexpression of  $\alpha$ -Syn led to significant cell death (set as  $100.0\% \pm 3.9$ ,  $\alpha$ -Syn) compared to occasional occurring cell death in untreated controls ( $9.3\% \pm 0.8$ , PBS). Whereas CBE alone at 800  $\mu\text{M}$  did not lead to altered cell survival ( $9.3\% \pm 0.7$ ),  $\alpha$ -Syn induced toxicity was further enhanced by co-treatment with CBE in a dose dependent manner ( $111.7\% \pm 5.4$  with 400  $\mu\text{M}$  and  $129.2\% \pm 4.9$  with 800  $\mu\text{M}$ , **Fig. 5A**).

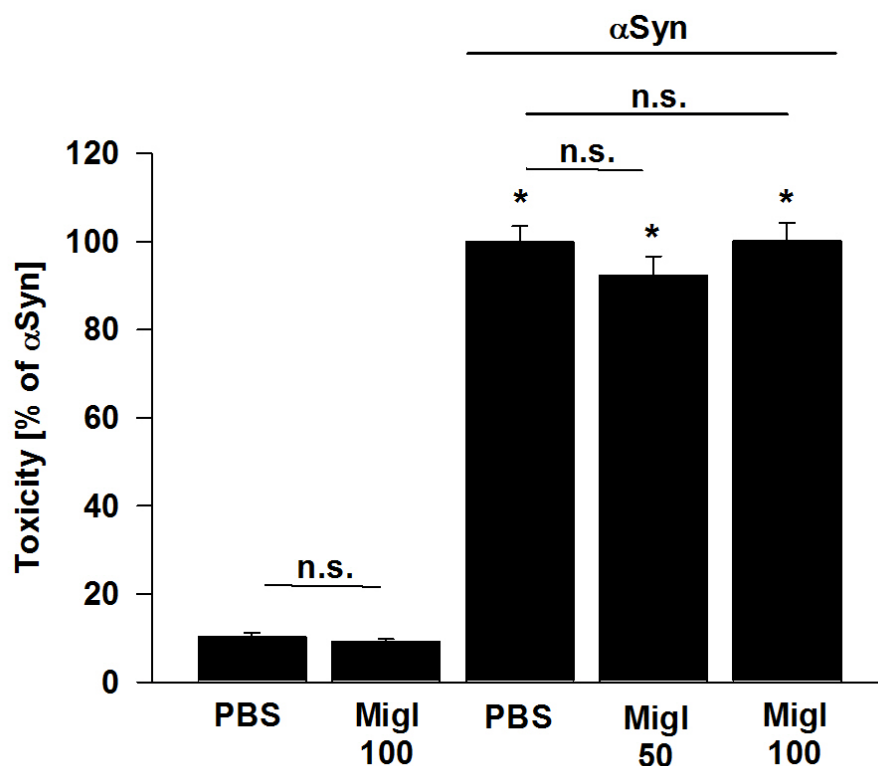
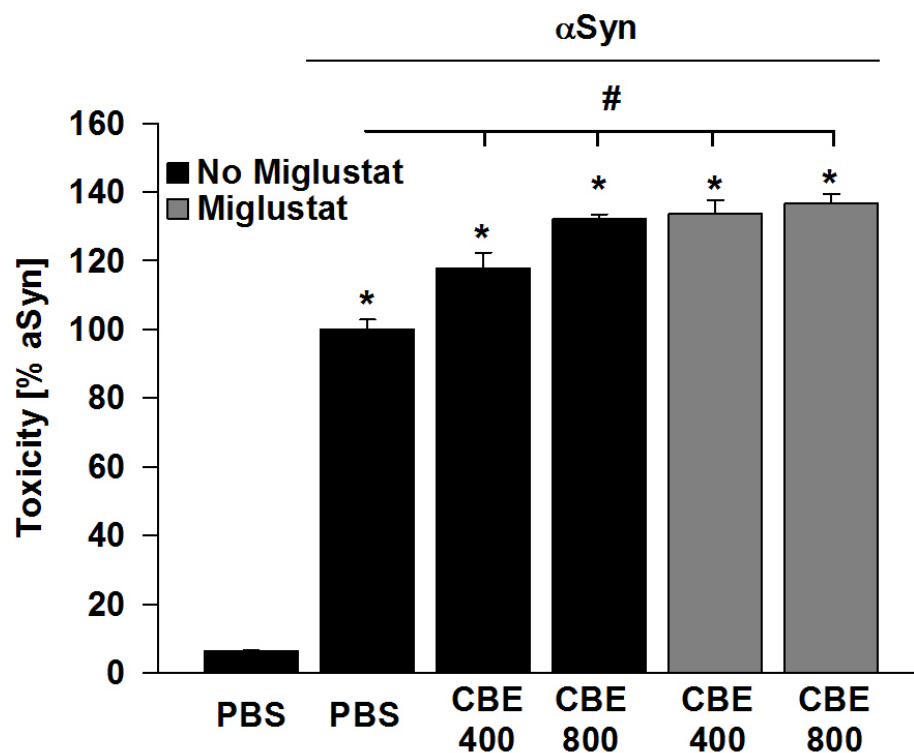
Next, we tested whether inhibition of glucosylceramide synthase by miglustat might protect against  $\alpha$ -Syn toxicity. Overexpression of  $\alpha$ -Syn in LUHMES cells led to significant cell death (set as  $100\% \pm 5.0$ ,  $\alpha$ -Syn) compared to occasional occurring cell death in untreated controls ( $10.4\% \pm 1.0$ , PBS; and  $9.1 \pm 0.7$  in miglustat-treated controls, respectively). Co-treatment with miglustat could not reduce  $\alpha$ -Syn-induced toxicity ( $99.0\% \pm 4.3$  (miglustat 50  $\mu\text{M}$ ) and  $98.8\% \pm 5.1$  (miglustat 100  $\mu\text{M}$ , **Fig. 5B**). Similarly, when  $\alpha$ -Syn-overexpressing LUHMES cells were treated by CBE, no protective effect of miglustat was

detectable, whereas  $\alpha$ -Syn overexpression led to cell toxicity in the same range as in prior experiments ( $100.0\% \pm 2.9$  compared to  $6.3\% \pm 0.3$  in control conditions). Addition of CBE increased toxicity to  $117.8\% \pm 4.6$  (400 nM) and  $132.3\% \pm 9.4$  (800 nM). Co-treatment with miglustat did not alter this toxicity ( $133.7\% \pm 4.0$  and  $136.7 \pm 2.9$ , respectively) (**Fig. 5C**).

Figure 5 :

**A** GCCase inhibition by CBE causes increased toxicity of  $\alpha$ -Syn



**B Miglustat does not reduce  $\alpha$ -Syn induced toxicity****C Co-treatment of miglustat does not alter the effect of CBE on  $\alpha$ -Syn toxicity**

**Effects of conduritol-B-epoxide (CBE 400 and 800  $\mu$ M) (A) and miglustat (Migl 50 and 100  $\mu$ M) (B) on  $\alpha$ -Syn induced toxicity in LUHMES cells overexpressing human WT  $\alpha$ -Syn ( $\alpha$ Syn) or untransduced cells.**

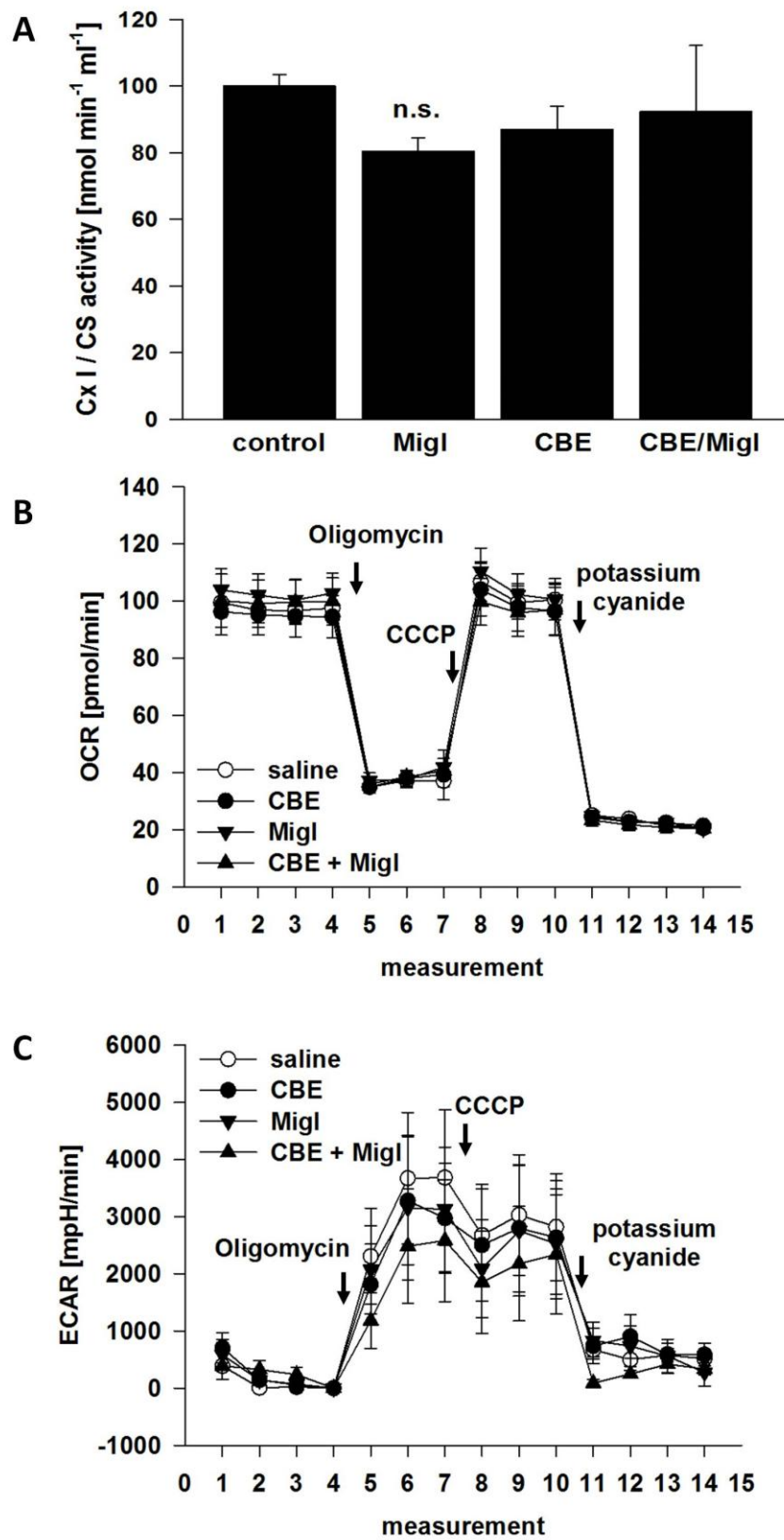
Data are expressed as percent of corresponding values of  $\alpha$ -Syn overexpressing cells and represent mean  $\pm$  S.E.M. \*p < 0.05 compared to control values, #p < 0.05 for pairwise comparison.

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### 3.4 No alterations of complex I activity or cell respiration upon treatment with either CBE or miglustat *in vitro*

We tested the cell energy metabolism by measuring the oxygen consumption rate (OCR) for the aerobic and the extracellular proton production (extracellular acidification rate, ECAR) of the glycolytic pathway. Since primary mesencephalic cultures contain only about 1-3% of dopaminergic neurons (TH<sup>+</sup>) [32], we carried out the tests on a dopaminergic cell line, SH-SY5Y cells. Neither the inhibition of GCCase by CBE (800 $\mu$ M) nor the inhibition of glucosylceramide synthase by miglustat (100  $\mu$ M) altered complex I activity in these cells compared to controls (**Fig 6A**). Whereas the oxygen consumption was lowered by oligomycin and increased by CCCP, neither the inhibition of GCCase by CBE (800 $\mu$ M) nor the inhibition of glucosylceramide synthase by miglustat (100 $\mu$ M) altered the respiratory chain under basal conditions, stress and complete inhibition (**Fig 6B**) supporting the findings on complex I activity. Also, the glycolytic pathway, measured by the extracellular pH indicated by the ECAR, was comparable among all treatment conditions (**Fig 6C**) suggesting that neither the inhibition of GCCase nor of glucosylceramide synthase have an impact on cellular energy metabolism in this experimental set up.

Figure 6 :



**Effects of CBE (800  $\mu$ M) and/or miglustat (100 $\mu$ M) on complex I activity or cell respiration.** Mitochondrial respiratory chain complex I (Cx I) and citrate synthase (CS) activity were measured **(A)**. Data were expressed as ratios of Cx I/CS activity in % of control. Cell respiration and glycolytic pathway indicated oxygen consumption rate (OCR) **(B)** and the extracellular acidification rate (ECAR) **(C)**, after sequential treatment with oligomycin (ATPase inhibitor), CCCP (carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone) an uncoupler to show maximal respiration, and potassium cyanide to show nonmitochondrial oxygen consumption. Data are expressed as % of baseline.

#### 4. DISCUSSION

Our results support a link between GCase activity and sporadic PD. We demonstrate that MPTP toxicity in mice can be diminished by decreasing glucocerebroside, the substrate accumulating when GCase activity is reduced. In addition, we show that GCase inhibition potentiates the toxic effects of MPP<sup>+</sup>-induced toxicity on primary mesencephalic cell cultures. This GCase-related toxicity is blocked by treatment with miglustat. Moreover, we show that GCase inhibition by CBE causes an increased toxicity in  $\alpha$ -Syn-overexpressing LUHMES cells, a human dopaminergic neuronal cell line. In contrast to MPP<sup>+</sup>-induced toxicity, miglustat does not protect against  $\alpha$ -Syn toxicity alone or enhanced by CBE. As to a possible mechanism by which CBE or miglustat alter MPP<sup>+</sup>- and synuclein-induced toxicity, we were unable to reveal any alterations of complex I activity or cell respiration upon treatment with these agents.

To date, available cell culture or mouse models of Gaucher disease have not demonstrated any toxicity on dopaminergic neurons. *In vivo*, Sardi et al. [33] could show that a mouse model of Gaucher disease (Gba1D409V/D409V) exhibits characteristics of synucleinopathies, including progressive accumulation of proteinase K-resistant  $\alpha$ -synuclein/ubiquitin aggregates in hippocampal neurons and a coincident memory deficit. However, no cell loss, especially in the SNpc, was reported. *In vitro*, a recent study has shown that survival of differentiated SH-SY5Y cells treated with CBE (50-200 mM) is not compromised [14]. However, increased expression of  $\alpha$ -Syn was observed in cell extracts. *In vivo*, these authors also treated C57Bl/6 mice with a single i.p. dose of 200 mg/kg CBE or DMSO (as controls). No data were provided on DA cell numbers in the SNpc but as *in vitro*,  $\alpha$ -Syn concentrations were increased in extracts from ventral mesencephalon (48h survival time). Furthermore, increased  $\alpha$ -Syn staining could be observed in nigral astrocytes of mice treated with CBE compared to DMSO. Thus, these results indicate that inhibition of GCase activity by CBE increases  $\alpha$ -Syn levels *in vitro* and *in vivo* [14]. More recently, Manning-Bog et al. [34] demonstrated, also in SH-SY5Y cells, that GCase inhibition by CBE (50  $\mu$ M) causes mitochondrial respiratory chain dysfunction, ATP reduction and free radical



production without, however, causing actual cell death. Also, Manning-Bog et al. and Cleeter et al. [14, 34] show that GCase inhibition increases  $\alpha$ -Syn protein levels in cell extracts. Both *in vitro* studies thereby indicate that GCase inhibition by CBE is insufficient to cause cell death in SH-SY5Y cells – a result that we reproduce in primary dopaminergic cultures. Moreover, our data suggest that an additional environmental factor, such as direct complex I inhibition of the mitochondrial respiratory chain by MPP<sup>+</sup> in conjunction with reduced GCase activity may be necessary to elicit actual cell death.

Mazzuli and colleagues [11] provide a potential mechanistic link between Gaucher disease and PD. They showed that loss of GCase activity in primary neuronal cultures and in human neurons derived from induced pluripotent stem cells from a Gaucher disease patient causes preferential accumulation of  $\alpha$ -Syn by interfering with its degradation in lysosomes. What is special about these results is that they reveal a bidirectional positive feedback loop in which  $\alpha$ -Syn inhibits GCase activity and reduction in GCase activity boosts  $\alpha$ -Syn accumulation [35]. Such homeostatic processes that keep the feedback loops in check may fail with time, supporting age as the major risk factor in neurodegenerative diseases. With regard to sporadic PD, this means that even in the absence of GBA mutations,  $\alpha$ -Syn accumulation can affect GCase function. It is well established that  $\alpha$ -Syn is involved in mediating the toxic effects of MPTP as mice deleted for  $\alpha$ -Syn are less sensitive to MPTP [19, 36, 37]. A plausible mechanism for the toxic effects of  $\alpha$ -Syn figures an increase MPTP-induced oxidative stress [38, 39]. This in turn could provide an explanation why miglustat is effective against MPTP toxicity *in vivo* despite the absence of GBA mutations. It is also the most likely explanation for the differing *in vivo* and *in vitro* results regarding the protective effects of miglustat when administered with MPTP or MPP<sup>+</sup> alone. Indeed, the kinetics of intoxication are very different in both conditions: subchronic *in vivo* versus acute *in vitro*. We postulate that in the latter, there might not be sufficient time for build-up of  $\alpha$ -Syn and GCase inhibition, for which reason miglustat cannot exert any protective effect.

It has been reported that GCase dysfunction results in an accumulation of dysfunctional mitochondria due to defects in both protein and organellar degradation machinery [34, 40]. Thus, and in light of the mitochondrial actions of MPTP/MPP<sup>+</sup>, we wondered whether a direct inhibition of GCase or a reduction of glucosylceramide by miglustat would lead to alterations in complex I activity or cell respiration and energy metabolism. However, we were unable to reveal any alterations of complex I activity or cell respiration following treatment with either CBE or miglustat. This difference to previous studies could be explained by an acute inhibition of the enzyme compared to an accumulated deficit in GBA-mutated or -deleted cells, as previously discussed [34, 40]. Thus, we conclude that the previously reported effects on cell respiration and complex I activity is an indirect consequence of GCase dysfunction, resulting in impaired mitochondrial degradation and increase of oxidative stress rather than a direct effect of GCase dysfunction or glucosylceramide accumulation.

To summarize, we find that deleterious effects of mitochondrial dysfunction mediated by MPTP or MPP<sup>+</sup> can be partially rescued by reduction of glucocerebroside and is aggravated by GCase inhibition. In contrast, reduction of glucocerebroside is without effect on  $\alpha$ -Syn induced toxicity, but this toxicity is aggravated by GCase inhibition. This suggests that enhanced  $\alpha$ -Syn toxicity is favored by reduced GCase activity rather than glucocerebroside accumulation.

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**Highlights “Glucocerebrosidase deficiency and mitochondrial impairment in experimental Parkinson disease”:**

- Inhibition of glucosylceramide synthase partially protects mice against subchronic MPTP intoxication
- *in vitro* a decrease of GCase activity may render dopaminergic neurons susceptible to MPP<sup>+</sup>
- *in vitro* a decrease of GCase activity may render dopaminergic neurons susceptible to  $\alpha$ -Syn toxicity
- Results suggest an interference of glucocerebrosidase activity in nigral dopaminergic cell death

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