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Glucocerebrosidase deficiency and mitochondrial impairment in experimental Parkinson disease

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ABSTRACT

Gaucher disease is an autosomal recessive disease, caused by a lack or functional deficiency of the lysosomal enzyme, glucocerebrosidase (GCase). Recently, mutations in the glucocerebrosidase gene (GBA) have been associated with Parkinson's 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 GCase activity may render dopaminergic neurons susceptible to MPP^+ (1-methyl-4-phenylpyridinium) or alpha-synuclein (α-Syn) toxicity and amenable to miglustat treatment. We could demonstrate that reduction of glucocerebroside by inhibition of glucosylceramide synthase partially protects mice against MPTP-induced toxicity. Conversely, we could show that inhibition of GCase activity with conduritol-B-epoxide (CBE) enhances both α-Syn and MPP^+ induced toxicity *in vitro*. However, only CBE-induced enhancement of MPP^+ 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 GCase activity rather than an accumulation of glucocerebroside increases aSyn toxicity.

1. introduction

Gaucher disease is an autosomal recessive disease, caused by a lack or functional deficiency of the lysosomal enzyme, glucocerebrosidase (GCase), which catalyzes 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's 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

Abbreviations: α-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; GCase, 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+, 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.

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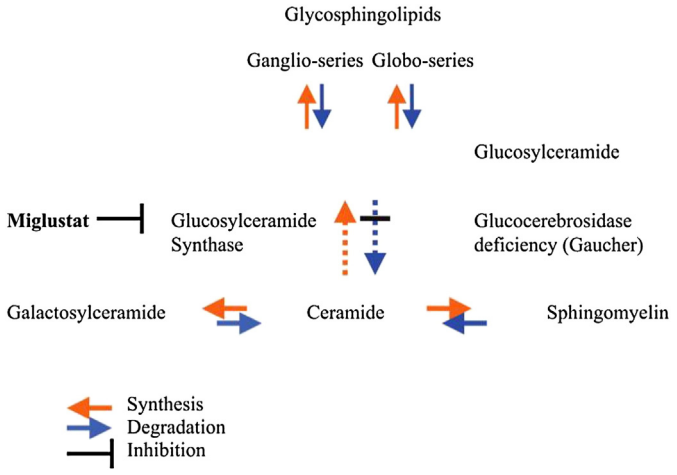

Fig. 1. Inhibition of glucosylceramide synthesis with miglustat by Can Ficicioglu [16].

(DA) neurons in the substantia nigra pars compacta (SNpc), resulting in a drastic decline in striatal dopamine concentrations. Additionally, aggregates of fibrillated alpha-synuclein (α-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 α-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 α-Syn either by a direct or indirect interaction between GCase and α-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 GCase activity may render dopaminergic neurons more susceptible to MPP^+ (1-methyl-4-phenylpyridinium), the active metabolite of MPTP, or α-Syn-mediated toxicity, and if inhibition of glucosylceramide synthase also protects dopaminergic neurons under these conditions.

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 analyzed 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_2EDTA, 6 ml triethylamine, adjusted to pH 2.8 with H_3PO_4 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 (125 × 3 mm with pre-column 5 × 3 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

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