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Development and biochemical characterization of a mouse model of Parkinson's disease bearing defective glucocerebrosidase activity

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ABSTRACT

GBA1 gene encodes for the lysosomal membrane protein glucocerebrosidase (GCase). GBA1 heterozygous mutations profoundly impair GCase activity and are currently recognized as an important risk factor for the development of Parkinson's disease (PD). Deficits in lysosomal degradation pathways may contribute to pathological α-synuclein accumulation, thereby favoring dopaminergic neuron degeneration and associated microglial activation. However, the precise mechanisms by which GCase deficiency may influence PD onset and progression remain unclear. In this work we used conduritol-β-epoxide (CBE), a potent inhibitor of GCase, to induce a partial, systemic defect of GCase activity comparable to that associated with heterozygous GBA1 mutations, in mice. Chronic (28 days) administration of CBE (50 mg/kg, i.p.) was combined with administration of a classic PD-like inducing neurotoxin, such as MPTP (30 mg/kg, i.p. for 5 days). The aim was to investigate whether a pre-existing GCase defect may influence the effects of MPTP in terms of nigrostriatal damage, microglia activation and α-synuclein accumulation. Pre-treatment with CBE had tendency to enhance MPTP-induced neurodegeneration in striatum and caused significant increase of total α-synuclein expression in substantia nigra. Microglia was remarkably activated by CBE alone, without further increases when combined with MPTP. Overall, we propose this model as an additional tool to study pathophysiological processes of PD in the presence of GCase defects.

1. Introduction

Glucocerebrosidase 1 (GBA1) gene encodes for the lysosomal membrane protein glucocerebrosidase (GCase), which cleaves the beta-glucosidic link of glucosylceramide, an intermediate in glycolipid metabolism (Beutler, 1992). Homozygous mutations of the GBA1 gene cause Gaucher disease (GD), a lysosomal storage disorder characterized by accumulation of glycosylceramide in cells of the macrophage-monocyte system and a complex clinical picture that, in the neuronopathic forms of the disease, also includes neurological symptoms. In GD, GBA1 mutations cause GCase misfolding and a dramatic reduction of enzymatic activity, with patients typically showing only 10–20% of GCase residual activity, compared to normal individuals (Migdalska-Richards and Schapira, 2016; Sardi et al., 2011, 2015, 2017).

Heterozygous mutations in the GBA1 gene have a lesser impact on GCase activity and do not cause GD, but they have been recently pointed out as a major contributing player in the development of Parkinson's disease (PD) (Migdalska-Richards and Schapira, 2016; Cilia et al., 2016). Indeed, approximately 10% of patients with sporadic PD carrying GBA1 heterozygous mutations (Sidransky and Lopez, 2012; Migdalska-Richards and Schapira, 2016). They show a phenotypic profile similar to that of PD patients without GBA1 mutations, except for a slightly younger age of onset and, above all, more severe signs of cognitive decline (Cilia et al., 2016). Considering that the presence of a GBA1 mutation increases the risk of developing PD by 20 to 30 times, this condition is currently considered the most relevant risk factor for PD, after age (Migdalska-Richards and Schapira, 2016).

Clarifying the link that apparently binds pathological α-synuclein (α-syn) accumulation to defective GCase activity is a crucial step in the understanding of PD pathogenesis (Migdalska-Richards et al., 2017). The discovery of mutant GCase in Lewy bodies of patients with synucleinopathies suggests that the fate of these two proteins is most likely connected (Goker-Alpan et al., 2010). Recently, one of the pivot roles in PD progression was given to macroautophagy dysregulation (Dehay et al., 2010), mediating accumulation of cytoplasmic proteins (including α-syn), organelles and glucosylceramides (Zunke et al., 2017). Moreover, it is widely recognized that neuroinflammation is a crucial player in the pathogenesis of PD (Blandini, 2013) and that

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accumulation of pathological α-syn is a powerful trigger of microglial activation, which ultimately concurs to the degeneration of dopaminergic neurons (Ambrosi et al., 2017; Sznejder-Pachołek et al., 2017).

GCase activity can be inhibited by conduritol-β-epoxide (CBE), a compound that binds covalently to the catalytic site of the enzyme, causing accumulation of GCase substrate glucosylceramide. CBE has long been used to abolish GCase activity in an attempt to replicate GD-like features in rodents (Kanfer et al., 1975); more recently CBE-induced complete inhibition of GCase activity was associated with increased α-syn accumulation and microglia activation, without signs of nigrostriatal degeneration (Sardi et al., 2015; Rocha et al., 2015).

The aim of our study was to further investigate the link between GCase deficiency and PD pathogenesis by exploring whether a partial defect of GCase activity, comparable to that associated with heterozygous GBA1 mutations (not exceeding 50%), may enhance the effects of a classic PD-like inducing neurotoxin, such as 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP). To this end, we developed a mixed pharmacological model of PD with partial deficiency of GCase activity, caused by chronic treatment with a low doses of CBE and combined with sub-chronic MPTP treatment in mice. Using this protocol, we quantified the brain levels of GCase activity, nigrostriatal neurodegeneration, α-syn expression, and neuroinflammation. Our data might improve knowledge on neurodegeneration in animals with partial GCase deficiency and promote further investigation of the connection between deficiency in GCase activity and the intrinsic mechanisms underlying PD.

2. Materials and methods

2.1. Animals

Male C57BL/6N mice (Charles River, Italy), 8 week-old, were used in this study. All experimental procedures on animals were carried out in accordance with the European Communities Council Directives (2010/63/EEC; D.L., 27.01.1992, number 116) and the guidelines for animal experimentation approved by the Animal Care Committees of the University of Pavia. Maximum efforts were made to limit the number of animals used in this study.

Animals were housed two per cage at the Centralized Animal Facility of the University of Pavia, under controlled conditions of light (12-h light/dark cycle) and temperature (20 ± 2 °C), with free access to food and water. Upon arrival, mice were left at the animal facilities for at least 1 week before the beginning of any experimental procedures.

2.2. Drugs

CBE (Calbiochem—EMD Millipore, Billerica, MA) was dissolved in 10% DMSO and administered intraperitoneally (*i.p.)* at a dose of 50 mg/kg for 28 consequent days. MPTP (Sigma–Aldrich, Co., St. Louis, MO) was dissolved in sterile saline and administered *i.p*. at a dose of 30 mg/kg/day for 5 consequent days. All solutions were made fresh daily.

2.3. Pharmacological inhibition of GCase activity combined with dopaminergic deficiency

A pilot study was conducted to identify the concentration of CBE capable of inducing a 50% reduction of GCase activity in the brain tissue. During this preliminary study animals were treated for 14 days with different doses of CBE (1, 50 and 100 mg/kg/day, i.p.); at sacrifice, brain tissue (cerebral cortex) was collected for evaluation of GCase activity. The 50 mg/kg/day dose was the one granting the desired reduction (see Results) and it was then used for the 28-day chronic treatment. In addition, animals were co-treated with MPTP or vehicle for 5 consequent days, starting from the day 10 of CBE treatment. All experimental groups were balanced with appropriate control groups (CBE + MPTP, CBE + Vehicle, MPTP + Vehicle, Vehicle).

2.4. Fluorometric assay of GCase activity

GCase activity was determined as previously described with slight modifications (Sardiello et al., 2009); mouse brain tissues (~5 mg) were homogenized in 300 µl of ice-cold lysis buffer (CelLytic, Sigma) containing diluted phosphatase (1:10, Roche) and protease inhibitors (1:25, Roche) Samples were diluted in a 2 mg/ml bovine serum albumin (BSA), citric acid sodium phosphate buffer (pH 5), 40 µg of sample lysate was added to 200 µl of reaction mix (0.1 M sodium citrate phosphate, pH 5.6; 0.1% Triton X-100; 0.25% sodium taurocholate and 2.5 mM 4-Methylumbelliferyl-b-D-glucuronide, 4-MUG). After incubation with the substrate for 60 min at 37 °C, the reaction was terminated using 500 µl of stop solution (0.1 M Glycine, pH 10). Plates were read (Ex 360/Em 460) in SpectraMax Gemini-XS Spectrofluorometer (Molecular devices LLC) plate reader using Softmax Pro software. Enzymatic activity was assessed from a 4-methylumbelliferyl (4-MUG, Sigma) standard curve and normalized to protein content in each sample as determined using a bicinchoninic acid (BCA) assay (Thermo Scientific Pierce).

2.5. Immunohistochemistry

At sacrifice, animals were deeply anesthetized (sodium-thiopental 150 mg/kg i.p.) and perfused with 4% paraformaldehyde (Merck VWR, Stockholm, Sweden). Brains were removed, post-fixed in sucrose solutions (30%) and stored at −80 °C. Coronal brain sections containing striatum and SNc were cut at 40 µm on a freezing sliding microtome (SM 200R, Leica, Milan; Italy) and stored at −20 °C in a solution containing 30% ethylene glycol, 20% glycerol and 0.05 mol/l sodium phosphate buffer until use.

Tyrosine hydroxylase (TH): for evaluation of dopaminergic neurodegeneration, striatal and SNc sections were processed with rabbit anti-TH primary antibody (Millipore AB152, 1:2000), biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, 1:500) and revealed using a commercial kit based on the avidin-biotin technique (Vectastain ABC Elite kit, Vector Laboratories). Reaction products were developed using nickel-intensified 3′-3′-diaminobenzedine tetra-hydrochloride (DAB Substrate Kit for Peroxidase, Vector Laboratories).

Microglia activation: assessment of neuroinflammation in the SN and striatum was performed by microglia and dopaminergic cells double immunofluorescent labeling. The reaction was processed with rabbit anti-TH primary antibody (Chemicon AB152, 1:500) and mouse anti-CD11b antibody (Santa Cruz Biotechnology, 1:300); sections were covered with Prolong mounting medium containing DAPI to label cell nuclei.

α-syn: assessment of total α-syn in the SNc and striatum was performed by α-syn and dopaminergic cells double immunofluorescent labeling. The reaction was processed with rabbit anti-TH primary antibody (Chemicon AB152, 1:500) and mouse anti-α-syn antibody (BD 610786, 1:500); sections were covered with Prolong mounting medium containing DAPI to label cell nuclei.

2.6. Immunostaining of insoluble α-synuclein aggregates

To analyze insoluble α-syn aggregates, tissue sections were premounted on polylisinate slides and incubated with proteinase K solution (20 µg/ml; Promega) for 25 min at 37C°. Endogenous peroxidases were quenched in 3% hydrogen peroxide for 7 min and placed in a blocking solution (Vectashield MOM kit) for 1 h at room temperature. Tissue sections were incubated with mouse anti-α-syn (BD 610786, 1:500); and rabbit anti-TH (Chemicon AB152, 1:500) antibodies 48 h at 4C° using primary antibody diluent (Vectashield MOM kit). Further sections were incubated with appropriate secondary antibodies and

covered with fixation solution containing DAPI (Prolong mounting medium).

2.7. Western blotting

Animals were sacrificed by decapitation and brain areas including striatum and cortex were rapidly removed and frozen on dry ice, and stored at −80 °C. Protein lysates were obtained by re-suspending the different brain areas in ice-cold lysis buffer (CelLytic, Sigma) containing diluted phosphatase (1:10, Roche) and protease inhibitors (1:25, Roche). After centrifugation, the supernatant was collected and protein concentration was measured using a BCA Assay (Sigma). Protein lysates were run on 10% gels, transferred onto nitrocellulose membranes (Biorad) and western blot was performed. Membranes were blocked (Odyssey blocking buffer, LiCor) and incubated overnight with the following primary antibodies: goat anti-actin (Santa Cruz Biotechnology 1:4000) as housekeeping protein, rabbit anti-TH (Chemicon AB152 1:4000); mouse anti-α-syn (BD 1:1000). As secondary antibodies, IRDye® 700 donkey anti rabbit (1:10000), IRDye® 800 donkey anti-mouse (1:5000) (LiCor, Biosciences) were used. Image analysis of western blots was performed using the fluorescent near-infrared Odyssey® scanner and software (LiCor, Biosciences) and fluorescence signal was normalized with the corresponding actin signal.

2.8. Sandwich ELISA for α-syn

Commercially available kit for α-syn detection in mouse tissue was used according to attached protocol (ELISA kit for SNCA mouse, SEB222Mu, Cloud-Clone Corp., USA). Samples of striatal extracts were dissolved 1:40 in PBS and loaded in volume 100 ul to the microplate pre-coated with biotin-conjugated antibody specific to α-syn. Plates were read (Em 450) in SpectraMax Gemini-XS Spectrofluorometer plate reader (Molecular devices LLC) using Softmax Pro software. A-syn concentration was assessed from a 4-parameters standard curve and normalized to protein content in each sample determined using a BCA assay (Thermo Scientific Pierce).

2.9. Image analysis

Image analysis was performed using an AxioSkop2 microscope connected to a computerized image analysis system (AxioCam MR5) equipped with a dedicated software (AxioVision Rel 4.2).

Nigrostriatal Degeneration: the number of TH+ neurons in the SNc was counted bilaterally on every fifth section throughout the entire nucleus using the unbiased stereological optical fractionator method (Stereo Investigator System, Microbrightfield Inc).

Neuroinflammation: activation of microglia within the striatum and SNc was determined by a quantitative analysis, performed by analyzing three different SNc sections, chosen according to their rostro-caudal coordinates. Cell density was assessed by counting CD11b + cells from the picture (0.04 mm^2 frame, 40× magnification) taken from the same areas of the same striatal or SNc section.

α-syn: the number of neurons co-expressing α-syn and TH+ in the SNc was counted bilaterally on every fifth section throughout the entire nucleus using the unbiased stereological optical fractionator method (Stereo Investigator System, Microbrightfield Inc) and expressed as percent to total amount of neurons expressing TH+.

2.10. Statistical analysis

All values are expressed as mean ± SEM. Statistical evaluation of data was performed using a dedicated software (Prism 3 Software, GraphPad Software). One-way ANOVA followed by Bonferroni post-hoc test were used to analyzed the differences between experimental groups. Statistical significance was set at $p < 0.05$.

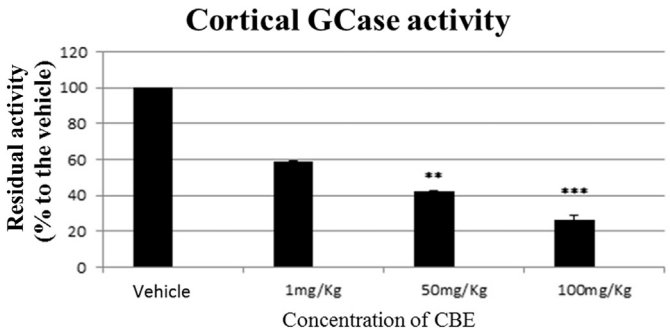

Fig. 1. Effect of CBE treatment on residual GCase activity.
Residual activity of the enzyme glucocerebrosidase in mice cerebral cortex after treatment with different concentrations of CBE, pilot study (flourometric assay). Data are expressed as mean ± SEM. **p < 0.01 *vs.* vehicle; ***p < 0.001 *vs.* vehicle (n = 6 per group). One-way ANOVA with Bonferroni post-hoc test.

3. Results

3.1. GCase activity

Preliminary study showed that systemic treatment with 1, 50 or 100 mg/kg CBE caused dose-dependent reduction in GCase activity in the cerebral cortex of treated mice (Fig. 1). The intermediate dose (50 mg/kg) induced a degree of GCase reduction similar to that observed in PD patients carrying heterozygous GCase mutations, with approximately 50% of residual GCase activity. This dose was then used for the experiments.

Animals treated chronically with CBE for 28 days (CBE + Vehicle and CBE + MPTP groups) showed significant reduction of GCase activity in cortical samples compared to animals that did not receive CBE (Vehicle and MPTP + Vehicle groups) ($p < 0.001$, one-way ANOVA, N = 10). Also in these groups, the dose of CBE caused approximately a 50% reduction of GCase activity. MPTP, either alone or when co-administered with CBE, did not affect GCase activity (Fig. 2).

3.2. Nigrostriatal degeneration

MPTP administration caused significant reduction of the TH signal in the striatum (−52 ± 4%, $p < 0.01$, one way ANOVA, n = 5 per group) and loss of TH+ cell bodies (−31 ± 4%, $p < 0.05$, one way ANOVA, n = 10 per group) in the SNc of the MPTP + Vehicle group, compared to Vehicle-treated animals. Reduction of the TH signal was even more pronounced in the striatum of animals treated with CBE + MPTP, compared to vehicle-treated animals (−77 ± 6%,

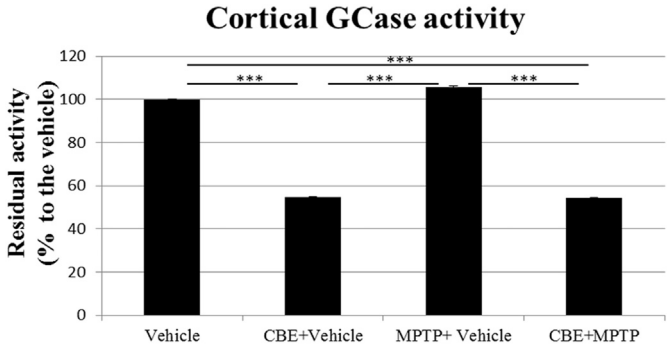

Fig. 2. Glucocerebrosidase activity in the cerebral cortex.
Evaluation of cortical residual glucocerebrosidase activity by flourometric assay. The results expressed as a percentage in respect to vehicle. Data are shown as mean ± SEM. ***p < 0.001, n = 10 per group. One-way ANOVA with Bonferroni post-hoc test.

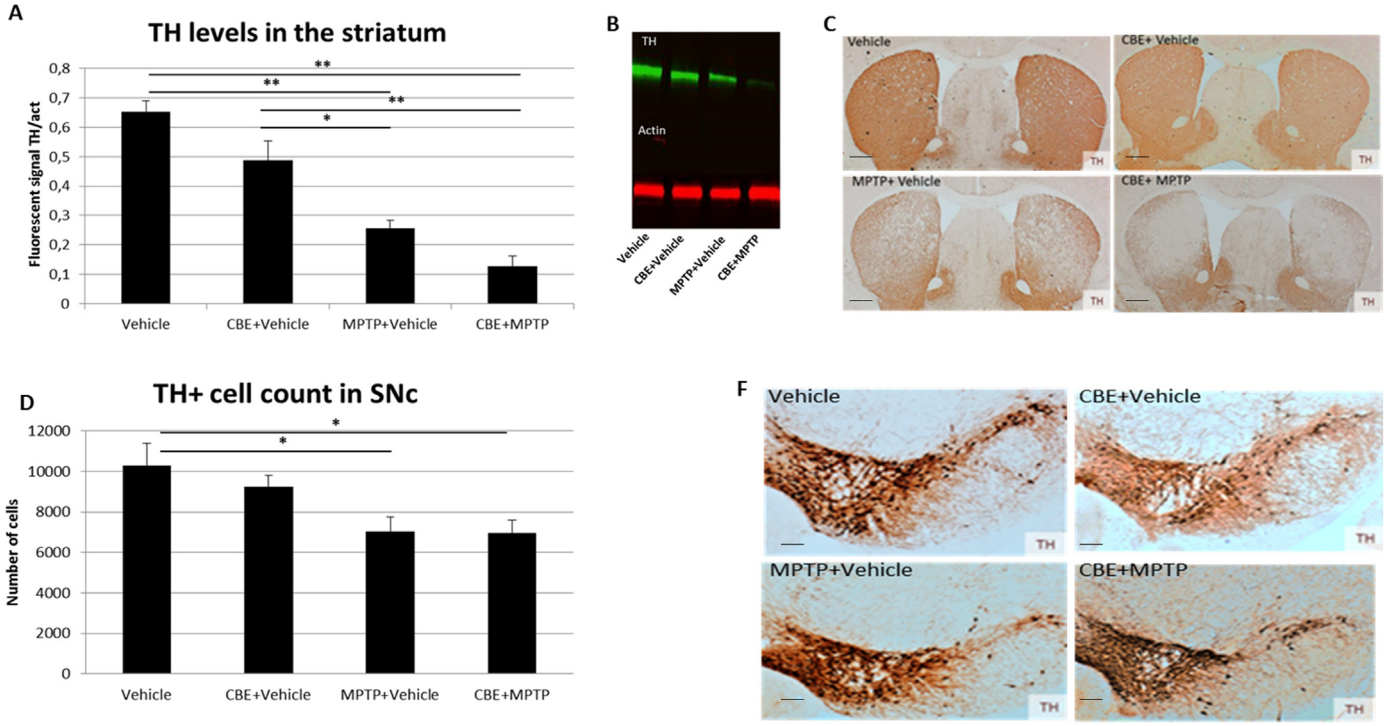

Fig. 3. Nigrostriatal neurodegeneration profile.
Loss of dopaminergic, TH+ terminals in the striatum of animals treated with CBE and/or MPTP was evaluated by western blot (A, B; n = 5 per group) and confirmed by immunofluorescence assay for TH in bright field (C; 1×, scale bar 1 mm). Stereological count of surviving TH+ cell bodies in SNc (D; n = 10 per group) and representative images (F; 10×, scale bar 100 μm) of dopaminergic neurons survival in SNc of animals subjected to the different pharmacological treatments (immunofluorescence assay for TH in a bright field). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, one-way ANOVA with Bonferroni post-hoc test.

p < 0.01, one way ANOVA, n = 5 per group), but in comparison with MPTP-treated group this tendency did not reach statistical significance (Fig. 3A-C). Within the SNc (Fig. 3D-F) this treatment regime did not further enhance the degenerative process. Slight, nonsignificant reductions in striatal TH expression and TH+ neuronal cell bodies were observed in the CBE + Vehicle group, indicating that treatment with CBE alone was not potent enough to cause degeneration of dopaminergic neurons.

3.3. Microglia activation

Investigation of microglia activation within the SNc showed a significant increase in the number of CD11b + cells in CBE-treated compared to vehicle-treated mice (+56 ± 9%, p < 0.05, one way ANOVA, n = 7 per group) (Fig. 4A, B-SNc). A similar increase in CD11b + cell count was observed in animals treated with MPTP alone (+45 ± 9%; p < 0.05, one way ANOVA, n = 7 per group). Co-administration of CBE and MPTP did not promote further microglia changes; in fact, animals of the CBE + MPTP group showed a less pronounced increase in CD11b + cell count over control values (+37 ± 14%), which did not reach statistical significance.

We also observed increased systematic microglial response in substantia nigra pars reticulata (Fig. 4B-SNr) of animals from all experimental groups that had undergone pharmacological treatment with CBE, MPTP or both.

In the striatum, we detected increased CD11b + immunoreactivity only in the group treated with CBE (Fig. 4A, C), but without reaching a statistically significant difference with respect to control animals.

The results show that CBE *per se* is able to activate a neuroinflammatory response within the nigrostriatal pathway and that such phenomenon is not affected by the combined treatment with MPTP.

3.4. Total and insoluble α-synuclein in the nigrostriatal pathway

The effects of single or combined treatment with CBE and/or MPTP on α-syn levels were evaluated both in striatum (Fig. 5A, B) and in SNc (Fig. 5C, D). In the midbrain, CBE and MPTP - when given separately - induced small to moderate increases in amount of TH+ cell bodies expressing α-syn, compared to vehicle-treated animals, which did not reach statistical significance. At the same time we found a considerable augmentation in the number of α-*syn*-positive dopaminergic cells in the CBE + MPTP group compared to controls (+318 ± 62%, p < 0.05, one-way ANOVA) (Fig. 5C).

In opposite, in the striatum we were not able to reveal the difference between experimental groups in the levels of total α-syn neither by western blot nor by ELISA (5A, B).

In our samples, we also observed an initial stage of accumulation of insoluble α-syn aggregates (proteinase-K resistant inclusions) in the neurons of SNc of animals treated with CBE+ MPTP, but in animals treated with MPTP or CBE alone amount of DA cells containing aggregates was negligible. The total amount of neurons expressing α-syn proteinase-K resistant signal, however, was not sufficient to apply a reliable statistical analysis (Fig. 5E). At the same time, as positive control, we checked samples of the experimental group from the pilot study, receiving CBE at dose 100 mg/kg for 14 days, and detected the presence of insoluble α-syn aggregates within TH+ neurons of SNc.

4. Discussion

The presence of heterozygous mutations in the GBA1 gene and the resulting GCase enzyme deficiency is currently considered the most prominent risk factor for PD, after age (Sidransky and Lopez, 2012). The mechanisms linking the GCase deficit to the disease, however, are poorly understood and have been prompting considerable interest in the scientific community in recent years, making this subject one of the

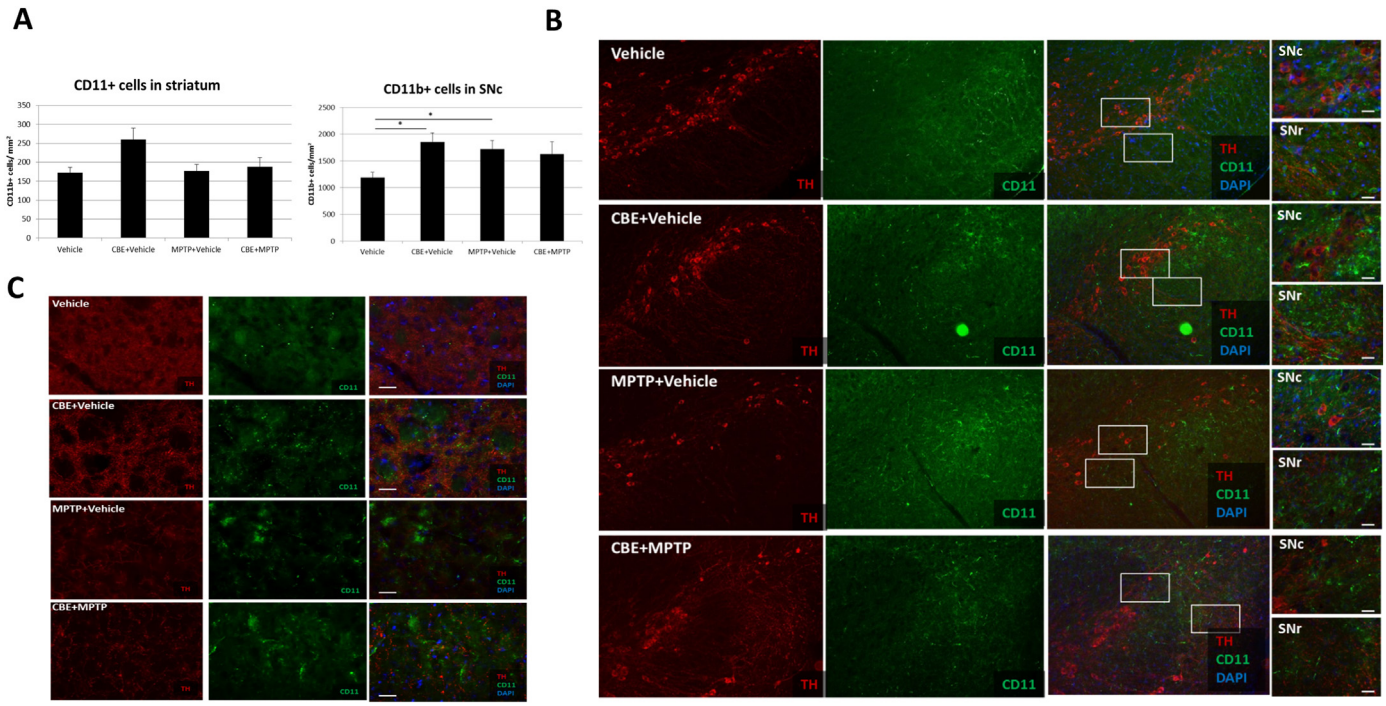

Fig. 4. Microglial activation profile.
Quantitative (A; n = 7 per group, frame cell count) and qualitative representation of the data obtained with double immunofluorescence assay with markers for microglial activation (CD11b+) and TH+ on coronal slices of SN (B; 10×/40×) and striatum (C; 40×). Results are shown as mean ± SEM. *p < 0.05, one-way ANOVA with Bonferroni post-hoc test. Scale bar 20 μm.

hottest topics in PD research.

In this study, we combined the well-known toxic effects of MPTP on dopaminergic neurons with a pharmacologically-induced, functional deficiency of GCase caused by chronic treatment with covalent inhibitor CBE. The purpose was to establish the impact of a pre-existing condition of GCase defect on the neurodegenerative and neuroinflammatory processes triggered by MPTP, as well as on the accumulation of α-syn in surviving nigrostriatal neurons. In a very recent paper, Yun et al. (2018) have reported that GCase deficiency associated with L444P GBA heterozygous mutation favors α-syn accumulation and renders dopaminergic neurons more susceptible to MPTP intoxication in mice. Our results confirm the concept that GCase dysfunction renders nigrostriatal neurons more susceptible to neurodegeneration, although we choose a pharmacological tool to induce of a chronic GCase defect. Indeed, in comparison to existing transgenic mouse models of heterozygous GBA1 mutation (Sardi et al., 2011; Migdalska-Richards et al., 2017), our pharmacological model allowed us to manipulate exclusively and precisely the level of GCase inhibition and might be used as a flexible tool to determine the extent to which a decrease in GCase activity may enhance vulnerability to neuropathological events typical of PD.

It has been previously described that chronic CBE treatment at 100 mg/kg completely inhibits GCase activity, mimicking the pathophysiological conditions causing GD (Ginns et al., 2014; Rocha et al., 2015; Rockenstein et al., 2016). Our purpose, however, was to reproduce a defect of GCase activity comparable to that observed in subjects carrying a heterozygous mutation of the GBA1 gene (Neumann et al., 2009; Asselta et al., 2014). Additionally, studies conducted in heterozygouse GBA1-mutant mice showed that GCase deficiency alone caused prominent increase in α-syn accumulation, but did not lead to the development of other behavioral and biochemical PD-like features during the lifespan of the mouse (Migdalska-Richards et al., 2017). To negotiate with these limitations, we combined chronic treatment with CBE 50 mg/kg/day (causing a 50% reduction of GCase activity in the brain of treated animals) with sub-chronic administration of MPTP (Li et al., 2016). In general, the presence of CBE in the administration protocol potentiated MPTP effects, but with notable exceptions, as discussed below.

4.1. Nigrostriatal degeneration

Our MPTP administration protocol induced degeneration of nigrostriatal dopaminergic neurons, with an efficiency similar to that previously reported (Vila et al., 2000; Li et al., 2016). The MPTP-induced loss of striatal dopaminergic terminals was further enhanced in the animals that were receiving CBE, but without reaching statistical significance. On the other hand, the degree of MPTP-induced neurodegeneration observed at the SNc was apparently lower than the loss of TH signal in the striatum and was not aggravated by combination with CBE. One potential explanation for this apparent discrepancy may lie in the concept that the degree of terminal loss in the striatum appears to be more pronounced than the magnitude of SNc dopaminergic neuron loss, suggesting that striatal dopaminergic nerve terminals are the primary target of the degenerative process and that neuronal death in PD may result from a “dying back” process (Dauer and Przedborski, 2003).

In our experimental conditions, the group receiving CBE alone showed just negligible neuronal loss either in striatum or in SNc. These results are in line with data obtained in animal models of PD, where chronic administration of a higher doses of CBE (100 mg/kg) did not cause significant degeneration of nigrostriatal neurons (Ginns et al., 2014; Rocha et al., 2015), while causing a complete deficit of GCase activity. In past decade, one of the pivot roles in PD progression was given to macroautophagy dysregulation (Levine and Kroemer, 2008; Vogiatzi et al., 2008). Autophagy refers to the global process by which intracellular components are degraded by lysosomes (Luzio et al., 2007). Compromised autophagic lysosomal reformation was observed in cells lacking functional GCase (Magalhaes et al., 2016). In addition, Rocha and colleagues (Rocha et al., 2015) demonstrated elevated level of LC3-II protein in striatum and SN of animals chronically treated with CBE 100 mg/kg/day; indicating the contribution of GCase inhibition in

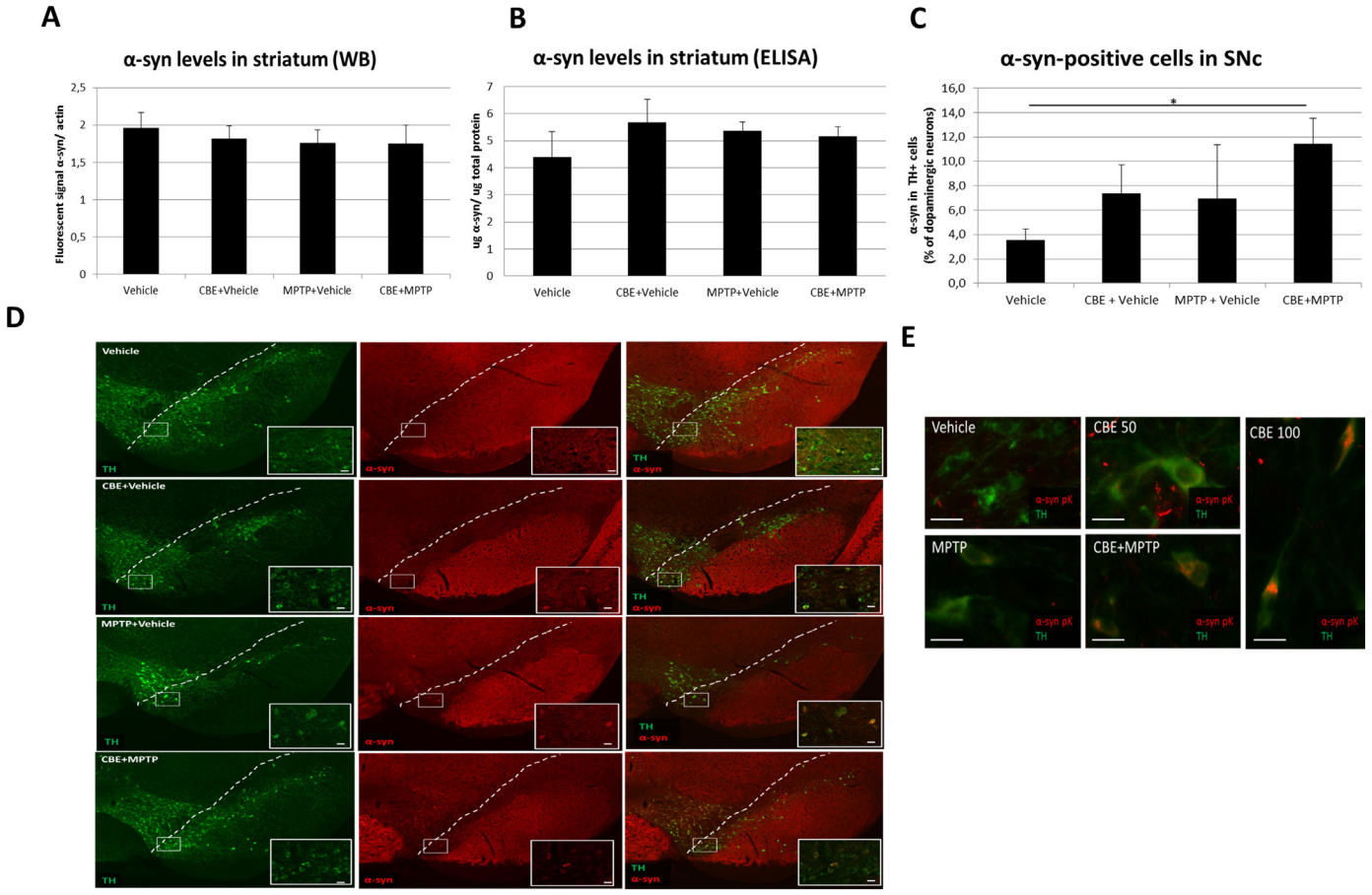

Fig. 5. Total and proteinase K-resistant alpha-synuclein expression profiles.
No changes in levels of total α-syn in striatum was detected between groups neither by western blot (A; n = 7 per group) nor by ELISA assay (B; n = 7 per group). Quantitative cell count (C; n = 7 per group) and qualitative representation (D; 10×/40×) of the data on total α-syn IF signal in survived dopaminergic neurons in SNc obtained with double immunofluorescence assay (α-syn and TH) on coronal brain slices. (E) Representative images of insoluble α-syn accumulation observed in neurons of SNc after applying the protocol of immunostaining for proteinase K-resistant synuclein. Brain sections obtained from animals treated with CBE 100 mg/kg/day for 14 days (pilot study) were used as positive control. Results are shown as mean ± SEM. *$p < 0.05$, one-way ANOVA with Bonferroni post-hoc test. Scale bar 20 μm.

disruption of macroautophagy pathway. Further, Dehay and colleagues (Dehay et al., 2010) showed in MPTP mouse model of PD that neurodegeneration process were preceded by a significant decrease in the amount of lysosomes within dopaminergic neurons. Taken together, these data might help us to justify more profound striatal neurodegeneration caused by combined treatment with CBE and MPTP, without significant changes in the levels of neuroinflammation and α-syn accumulation in this nuclei. Strong lysosomal deficit elicited by MPTP *per se* might be further exacerbated by disturbances in macroautophagy caused by CBE, thereby blocking the "cleaning machinery" of neurons and bringing them to death. Interestingly, more pronounced reduction of presynaptic nigrostriatal dopaminergic terminals was observed also in carriers of severe GBA1 mutations in comparison to non-carriers (Cilia et al., 2016).

4.2. Microglia activation

We analyzed microglia activation in the striatum and SNc, as a measure of the neuroinflammatory response to treatments, as it is known that neuroinflammation accompanies nigrostriatal degeneration in the brain of PD patients and animal models (Zhang et al., 2005; Blandini, 2013; Ambrosi et al., 2017; Schwenkgrub et al., 2017). Within the SNc both CBE and MPTP, when given separately, caused remarkable increases in CD11b + cell count. This increase was maintained also in the group of animals that received combined treatment with CBE and MPTP, but without further augmentation in the number of CD11b + cells. The lack of additive effect of the combined treatment could be explained by the fact that all available microglia cells might have been activated by each drug separately. In this conditions, additional stimuli could not cause further neuroinflammation.

The tendency was similar in the striatum, with the difference that neuroinflammation was not observed for the group treated with MPTP. This lack of pro-inflammatory properties of MPTP in striatum was previously shown by Hurley and colleagues in studies on primates, where they observed reduced glial immunoreactivity independently to the degree of neurotoxin-induced striatal neurodegeneration (Hurley et al., 2003).

If it is well known that subchronic treatment with MPTP causes microglia activation in the SNc (Blandini and Armentero, 2014; Pisanu et al., 2014; Li et al., 2016); the fact that CBE *per se* was able to trigger a similar phenomenon represents an intriguing observation. Indeed, microglia activation was the only phenomenon - among those analyzed in our study - CBE was able to elicit by itself. It was previously reported that chronic administration of CBE 100 mg/kg, a dose that completely inhibits GCase activity, induces significant microglia activation and increases levels of inflammatory mediators (Ginns et al., 2014; Rocha et al., 2015). However, this is the first time that the same phenomenon is observed with this dose of CBE (50 mg/kg), which only abates GCase activity by 50%. In various studies on genetic and pharmacological animal models of GD, activated microglia and astrocytes were detected

prior to cell loss in brain regions that are affected by the disease (Farfel-Becker et al., 2011; Vitner et al., 2012). This process is triggered by glucosylceramide accumulation under conditions of GCase deficiency, which in turn may enhance vulnerability to injury, cellular dysfunction and cell loss (Ginns et al., 2014). It is widely known that once activated, microglia can be polarized and - depending on the modulatory effects of the microenvironment and the duration of the insult - driven towards a cytoprotective or a cytotoxic phenotype (Blandini, 2013). It is, therefore, the dynamic balance between these phenotypes that affect the different level neuroinflammation and intensity of the associated neurodegeneration (Ambrosi et al., 2017).

4.3. Changes in α-syn levels

Animals treated with MPTP alone showed slight, nonsignificant increases in the levels of native α-syn in the striatum and SNc, while combined treatment with CBE and MPTP significantly increased amount of DA cells expressing α-syn in SNc, also prompting deposition of spare insoluble aggregates in that nuclei. On one hand, this finding is in keeping with the observation that sub-chronic treatment with MPTP does not lead to accumulation of total and pathological α-syn within the nigrostriatal pathway (Meredith and Rademacher, 2011); on the other hand, our data show that the CBE-induced reduction of GCase activity creates favorable conditions for accumulation of both soluble and insoluble forms of α-syn in midbrain, in the presence of a specific trigger of dopaminegic degeneration, such as MPTP. These findings are consistent with published data in other models of PD, where it was shown that reduced GCase activity results in increased plasmatic α-syn levels in SN (Manning-Boğ et al., 2009; Cullen et al., 2011; Mazzulli et al., 2011; Xu et al., 2011; Cleeter et al., 2013; Fishbein et al., 2014). The lack of this tendency for total α-syn levels in striatum goes along with data obtained by group of Papadopoulos (Papadopoulos et al., 2018), where they showed no difference in striatal levels of monomeric α-syn in mice treated chronically with CBE for 8 weeks (100 mg/kg/day, 3 days per week), while indicating significant increase of this synuclein fraction in SN. Importantly, even small changes in α-syn homeostasis can trigger the formation of pathological amyloid fibrils, suggesting that aberrant metabolism of lipids due to lysosomal malfunctioning and α-syn accumulation are downstream effects of PD (Rockenstein et al., 2016). Functional deficits in lysosomal degradation of α-syn caused by GCase deficiency contribute to neurodegeneration process, also by promoting accumulation of oligomeric α-syn (McCormack et al., 2012). The tight association between α-syn aggregation and PD neurodegenerative phenotypes in human patients and animal models strongly highlights the importance of abnormal aggregation of this protein in the disease pathogenesis. Existing experimental models based on inhibition of the GCase activity demonstrate an increase in glycolipid levels and consequent pathological α-syn accumulation (Mazzulli et al., 2011; Rocha et al., 2015; Sardi et al., 2017). In our experiments, we were able to detect the initial stage of the formation of insoluble α-syn (proteinase-K resistant) only in SNc of animals treated with combination of CBE and MPTP, but we did not observe sufficient amount of neurons with these aggregates in nigrostriatal pathways to be able to apply statistical analysis for a reliable comparison between experimental groups. This issue may be connected to our experimental time course, which may have not been long enough to allow a robust deposition of these aggregates. Indeed, Rocha et al. (2015) reported formation of α-syn aggregates in nigrostriatal pathways after 28 days of treatment, but with twice as much dose we used (100 mg/kg instead of 50 mg/kg). It goes along with our data on animals from preliminary set (treated with CBE 100 mg/kg for 14 days) where we observed formation of α-syn aggregates in nigral neurons. Interestingly, in studies conducted in heterozygous GBA1-mutant mice with residual GCase activity around 50%, the proteinase K-resistant forms of α-syn were not detected, which suggests that α-syn accumulation in the brain of these animals was not reaching the threshold required for α-syn aggregation (Migdalska-Richards et al., 2017). In fact, we were able to see α-syn aggregates formation after 28 days only in the animals where CBE treatment was combined with MPTP, which possibly acted as an additional trigger of insoluble α-syn accumulation, pumping up the effect of CBE.

In conclusion, the described protocol of partial GCase inhibition by chronic treatment with low doses of CBE combined with MPTP administration was able to cause significant increase in the number of DA neurons expressing α-syn in SN, leading to the initiation of the process of its primary aggregation. In striatum administration of CBE tended to enhance MPTP-induced neurodegeneration without significant changes in the levels of neuroinflammation and α-syn accumulation. In both nuclei CBE *per se* promoted marked microglia activation, but this process was not further aggravated by administration of neurotoxin. Overall, we believe this model may be used as an additional tool to study the biochemical processes underlying pathophysiology characterizing PD in the presence of GCase defects.

Author contributions

Study concept and design: F.S., S.C., F.B.; Acquisition and analysis of the data: L.M, F.S., S.C., Cr.G., C.G., Drafting the manuscript and/or figures: L.M., F.S., S.C., F.B.

Potential conflicts of interest

Nothing to report.

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