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Therapeutic activation of autophagy by combined treatment with rapamycin and trehalose in a mouse MPTP-induced model of Parkinson's disease

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ABSTRACT

The neuroprotective effect of autophagy activation by rapamycin and trehalose was studied in a mouse model of Parkinson's disease (PD) induced by neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Both rapamycin (10 mg/kg/day, 7 days) and trehalose (2% in drinking water, 7 days) increased the expression of LC3-II (a marker of autophagy activation) in the frontal cortex and striatum of normal C57Bl/6J mice, with signs of an additive effect. Autophagy stimulation in the striatum was confirmed by a lysosomal osmotic test. In the model of MPTP-induced PD, the two drugs were applied starting from the 2nd day after subchronic daily MPTP administration (20 mg/kg/day, 4 days). A marked increase in LC3-II expression in the striatum was detected under the action of trehalose and in the *S. nigra* after combined treatment with rapamycin and trehalose. The drugs had a positive effect for recovery of dopaminergic neurons and neuroprotection after MPTP-induced PD-like injury. The therapeutic effect was proven by active restoration of tyrosine hydroxylase (TH) content in the striatum and *S. nigra* and by improved cognition measured by the passive avoidance learning task. The results revealed the additive effect of the combined treatment with rapamycin and trehalose on dopaminergic deficits (according to the levels of TH expression in the nigrostriatal system) but not on the behavioral performance in the mouse PD model. Thus, the autophagy activation through different pathways by the combination of rapamycin and trehalose reverses both neuronal dopaminergic and behavioral deficits in vivo and seems to be a promising therapy for PD-like pathology.

1. Introduction

Induction of autophagy promotes cell survival, which is especially important for neurons lacking a proliferative resource. Cell models of neurodegenerative processes are suggestive of a therapeutic effect of autophagy activation (Sarkar et al., 2007; Martinez-Vicente, 2015). Similarly, positive effects were obtained in animal models of Alzheimer's (AD) disease and Parkinson's disease (PD) and other illnesses (Menzies et al., 2017). A strong inducer of autophagy is rapamycin, which binds to its target protein mTOR (mammalian target of rapamycin), which is a part of protein complexes mTORC1 and mTORC2 (Switon et al., 2017). Induction of autophagy in the brain by rapamycin has been successfully performed in vivo (Dehay et al., 2010; Liu et al., 2013). The use of rapamycin in animal models of neurodegeneration often yields a positive therapeutic result (Caccamo et al., 2010; Spilman et al., 2010; Ehrnhoefer et al., 2011), accompanied by a decrease in neuronal death and improvement in motor activity (Malagelada et al., 2010; Liu et al., 2013; Vidal et al., 2014; Moors et al., 2017).

Prospects of therapeutic induction of autophagy are associated with the possibility of combined activation of various pathways and mechanisms underlying protein quality control mediated by autophagy and the proteasome system. Hence, the combination of rapamycin with other agents activating autophagy through the additional mTOR-independent pathways seems to be a promising therapy for neurodegenerative disorders. Among such autophagy inducers are lithium salts (Sarkar et al., 2008; Motoi et al., 2014), carbamazepine (Li et al., 2013), trehalose (Sarkar et al., 2007; Schaeffer et al., 2012), and some other compounds.

Disaccharide trehalose attracts special attention because it induces autophagy and has a therapeutic effect on cellular and in vivo models of

Abbreviations: AD, Alzheimer's disease; DA, dopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OD, optical density; PD, Parkinson's disease; TH, tyrosine hydroxylase; TFEB, transcriptional factor EB

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neurodegeneration (Chen and Haddad, 2004; He et al., 2016; Hosseinpour-Moghaddam et al., 2018). Trehalose reduces the accumulation of protein aggregates in models of neurodegenerative diseases (Aguib et al., 2009; Zhang et al., 2014). It simultaneously has the properties of a chaperone, an inducer of chaperone-mediated autophagy, and an mTOR-independent inducer of macroautophagy (Sarkar et al., 2007; Rodríguez-Navarro et al., 2010). Another useful cellular effect of trehalose is activation of a factor called TFEB regulating the biogenesis of lysosomes (Palmieri et al., 2017). During its action on the brain, trehalose significantly attenuates degeneration of substantia nigra dopamine (DA)ergic neurons (Wu et al., 2015; He et al., 2016) and reduces the motor deficits caused by α-synuclein (He et al., 2016).

In vitro models have revealed an additive effect of autophagy induction by rapamycin and trehalose (Sarkar et al., 2007). Combined use of rapamycin and trehalose also exerts a neuroprotective effect on an in vitro model of accumulation of α-synuclein aggregates (Underwood et al., 2010). Meanwhile, effects of such a combined therapy have barely been studied on in vivo models of PD.

Modeling of PD is based on targeted lesioning of DAergic neurons located in the *S. nigra* and of their neurites in the striatum. Apparently, a genetic model based on overexpression of α-synuclein (Hatami and Chesselet, 2015) seems to be the most appropriate for this purpose, because in this case, the process of degeneration of DAergic neurons and their death proceed slowly (Meredith and Rademacher, 2011). Standard intoxication with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces massive DAergic neuron damage and simultaneously signs of autophagy activation (Wang et al., 2016). Similarly, acute or subchronic exposure of DAergic neurons to, for example, 6-hydroxydopamine or rotenone is accompanied by toxic damage to neurons, induction of an inflammatory response, and concomitant autophagy (Bazzu et al., 2010; Nopparat et al., 2014; Li et al., 2015; Liu et al., 2015). The induction of autophagy in response to MPTP apparently proceeds via the nonclassical pathway because autophagy inhibition by wortmannin or 3-methyladenine does not block the neurotoxin-induced autophagy and mitophagy (Zhu et al., 2007).

Activation of autophagy during pharmacologically induced DAergic neuron damage promotes removal of the forming cytotoxic proteins and structures. In comparison with the genetic model of PD, after the application of MPTP, there is progressive weakening of cytotoxicity rather than its enhancement inherent in PD. Nevertheless, the model of MPTP-induced PD is widespread and generally accepted. In our experiments here, we studied the therapeutic potential of rapamycin and trehalose in the treatment of subchronic MPTP-induced damage to DAergic neurons in mice. A 7-day treatment was administered after 4-day neurotoxin application.

2. Materials and methods

2.1. Reagents

The following reagents were used: rapamycin (MedChem Express, USA), D-(+)-trehalose dihydrate (Tokyo Chemical Industry, Japan), MPTP (hydrochloride; MedChem Express, USA), chloroquine diphosphate (Sigma, UK), Triton X-100 (Sigma, USA), 4-methylumbelliferyl-β,D-galactopyranoside (Melford Laboratories Ltd., England), rabbit polyclonal antibodies to autophagosome marker LC3B (i.e., MAP1LC3B; cat. # NB100-2220, 1:400 dilution, Novus Biologicals, USA), a mouse monoclonal anti–tyrosine hydroxylase antibody (MAB318, 1:300 dilution, Millipore), an Alexa Fluor 488–conjugated goat anti-rabbit IgG antibody (ab150077, 1:600 dilution, Abcam, UK), Tween 80 (Oleon, Germany), and polyethylene glycol 400 (PEG-400) (Serva, Germany).

2.2. Experimental procedures involving animals

Male C57Bl/6J mice (3 months old, 22–28 g) were kept on a standard laboratory diet and under standard conditions (20–22 °C, 14 h light). All the experimental procedures were carried out in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the SRIPhBM. Every effort was made to minimize the number of animals used and their suffering.

In the experiment on autophagy activation with rapamycin and trehalose in normal mice, there were the following groups (5–7 animals each): {1} Vehicle control (solvent of rapamycin, 5% Tween 80 + 5% PEG-400), {2} Rapamycin (1 mg/kg), {3} Rapamycin (5 mg/kg), {4} Rapamycin (10 mg/kg), {5} Trehalose (2% in drinking water), {6} Rapamycin (10 mg/kg) + Trehalose. Increasing concentrations of rapamycin were evaluated because its effective dose was not clear.

In the experiment on therapeutic autophagy activation in the model of MPTP-induced PD, the following groups were employed: {1} Control (mice injected with saline instead of MPTP), {2} MPTP (in saline, 20 mg/kg per day, 4 days), {3} MPTP + solvent of rapamycin, {4} MPTP + rapamycin (10 mg/kg), {5} MPTP + trehalose, and {6} MPTP + rapamycin + trehalose.

Rapamycin was dissolved in ethanol and diluted 20-fold or more with a 5% Tween 80 solution containing 5% PEG-400. The drug was administered in the indicated doses intraperitoneally (i.p.) daily for a week. Trehalose was added to drinking water (2%), which was freely available, also for 1 week. MPTP was administered at the dose of 20 mg/kg (i.p., daily) for 4 days. The treatment was started 2 days after the last MPTP injection. The mice were euthanized 1 day after the end of all exposures for assessment by a lysosomal osmotic test, and 5 days later for immunohistochemical (IHC) analysis (the interval was scheduled for behavioral tests).

2.3. IHC analysis

IHC analysis of the expression of the autophagy marker LC3-II protein was preceded by the administration of chloroquine (16 h prior to euthanasia, 30 mg/kg, i.p.) (Iwai-Kanai et al., 2008). On the day of euthanasia, mice were anesthetized with CO_2. The animals were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS, then the brains were rapidly excised and postfixed in PBS containing 30% sucrose at 4 °C until further neuromorphological analysis.

The IHC analysis was performed on 30-mm-thick cryosections according to a protocol described in detail previously (Weng et al., 2016; Pupyshev et al., 2018). Coronal slices along the frontal cortex (AP: 2.93–2.45 mm), striatum (AP: 1.21–0.73 mm), or *S. nigra* (AP: −2.91 to −3.15 mm) of each mouse brain were made. We applied a rabbit polyclonal antibody (NB100-2220, 1:400 dilution, Novus Biologicals, USA) as a primary antibody to detect autophagosome marker MAP1LC3B or a mouse monoclonal anti–tyrosine hydroxylase antibody (MAB318, 1:300 dilution, Millipore) as the primary antibody to detect DAergic neurons and projections. A fluorescently labeled (Alexa Fluor 488–conjugated) goat anti-rabbit IgG antibody (ab150077, 1:600 dilution, Abcam, UK) or Alexa Fluor 568–conjugated goat anti-mouse IgG antibody (ab175473, 1:400 dilution, Abcam, UK) served as the secondary antibodies, respectively. Fluorescent images were finally obtained by means of an Axioplan 2 (Carl Zeiss) imaging microscope and confocal laser scanning microscope LSM 510 META (Carl Zeiss) and then analyzed in Image Pro Plus Software 6.0 (Media Cybernetics, CA, USA). Fluorescence intensity (TH expression) was measured as background-corrected optical density (OD with subtraction of staining signals of the non-immunoreactive regions) in the images converted to grayscale. Fluorescence intensity of punctate LC3 immunostaining was measured with subtraction of low diffuse fluorescence of some areas (punctate staining vs. background staining of the non-punctate regions) in the images converted to gray-scale (Kabeya et al., 2000; Klionsky et al., 2008). The area of interest was 5712 μm^2 in the striatum and the 3rd layer of the frontal cortex, or 99,744 μm^2 in the *S. nigra*.

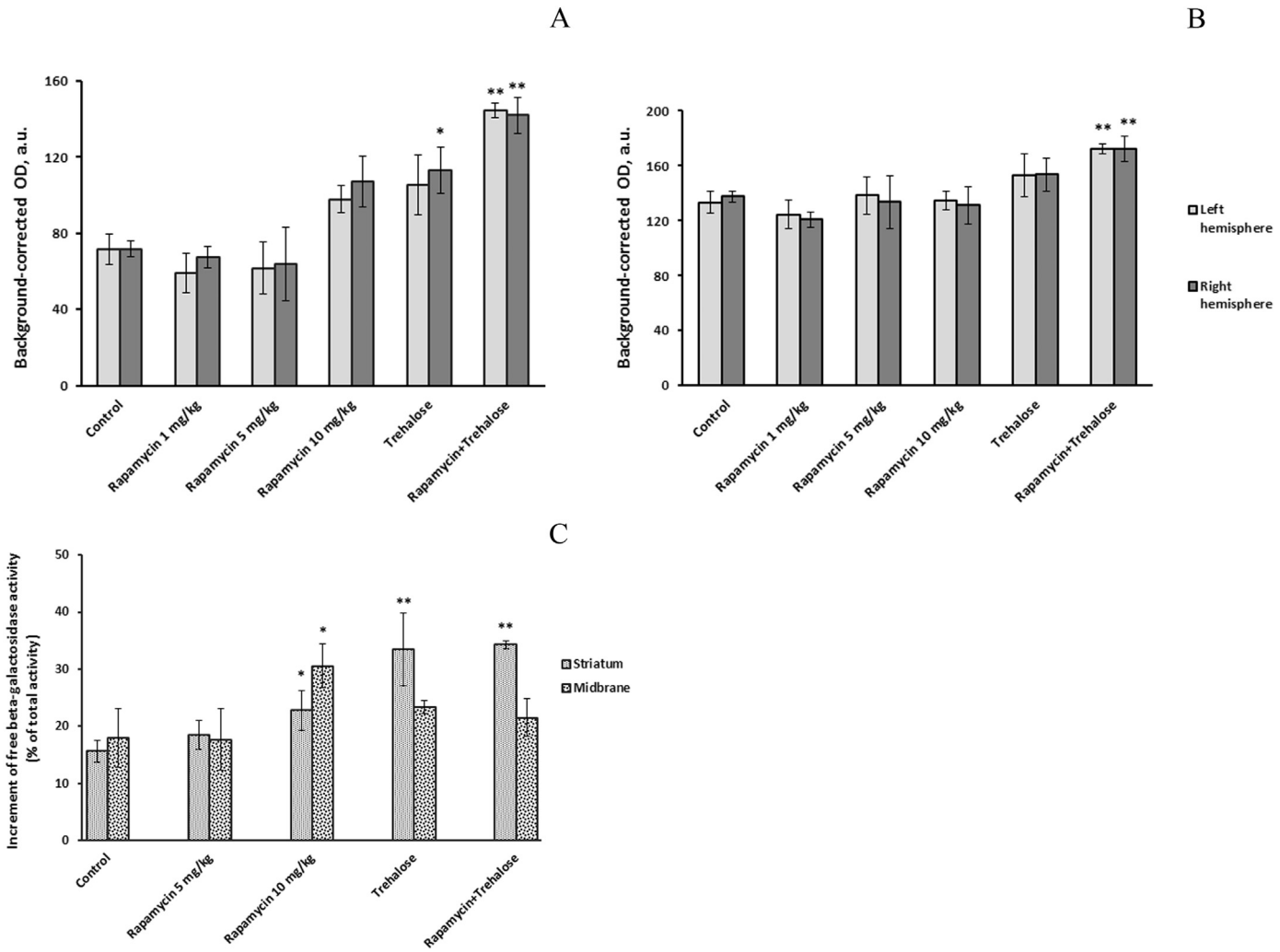

Fig. 1. Effect of treatment with rapamycin (1, 5, or 10 mg/kg/day, 7 days), trehalose (2% in drinking water, 7 days), or their combination on autophagy activity measured by LC3-II expression in the frontal cortex (A) and striatum (B) or by osmotic susceptibility of lysosomes in the striatum and midbrain (C) in C57Bl/6 J mice. The data are expressed as the mean ± SEM. *p < 0.05, **p < 0.01 compared to the Control group given vehicle (solvent of rapamycin, 5% Tween 80 + 5% PEG-400).

2.4. Lysosomal osmotic test on autophagy

We performed an in vitro test of autophagy; this assay is based on high osmotic susceptibility of the autophagolysosomal membrane in comparison with primary lysosomes or lysosomes in the cell steady state (Deter and De Duve, 1967). The method was developed for detection of an increased population of autophagolysosomes in parenchymal organs and was applied here to the brain for the first time.

A 2% brain homogenate was prepared in an isotonic solution of 0.25 M sucrose, 1 mM Na-EDTA, pH 7.4, at 0–2 °C. Hypotonic treatment consisted of addition of a cold distillate (1:1 by volume) to the samples and incubation for 30 min at 0–2 °C, after which activities of free and total lysosomal β-galactosidase were determined. Free β-galactosidase activity is associated with the maintenance of maximal integrity of lysosomes (in an isotonic sucrose solution), and the total activity was estimated during complete labilization of lysosomal membranes by means of 0.1% Triton X-100. The activity of acid β-galactosidase was determined with the substrate 4-methyl-umbelliferil-β,D-galactopyranoside (Pupyshev et al., 2005). The fluorescent product was registered on a Shimadzu RF 5301-PC spectrofluorimeter (Japan). The results were expressed as the ratio of free to total enzymatic activity (%), and its increment caused by the hypoosmotic treatment was a metric of the osmotic sensitivity of lysosomes, increasing with the autophagy induction (Deter and de Duve, 1967).

2.5. Behavioral tests

The passive avoidance test evaluates learning and memory function. Training on the passive avoidance reaction was performed by a standard single-session method in an experimental chamber with dark and light compartments and an automated Gemini Avoidance System apparatus (San Diego Instruments, CA, USA). Mice were habituated to the apparatus the day before passive avoidance acquisition. On the training day, mice were placed in the light compartment with the tail towards the open door. After transfer to the dark compartment, the door was closed and the mouse received a painful electric shock (0.5 mA, 2 s). After 10–20 s, the animal was transferred to the home cage. 24 h later the mouse was again placed in the light compartment with the door opened for 180 s. The Gemini program was used for automatic recording the latency of the transfer to the dark compartment and the data of testing served as a measure of acquisition of the conditioned passive avoidance reaction.

An open field test. This test was carried out in an apparatus with a square arena (40 × 40 cm) and plastic walls 37.5 cm high brightly lit from above (1000 lx). Performance on the test was monitored via a video camera (Sony, China) positioned above the apparatus and

operated with original EthoVision XT software (Noldus, Netherlands). A mouse was placed near the wall, and its movements were recorded for 10 min. The following parameters were determined: general locomotion (the distance travelled in cm); vertical locomotor and exploratory activity (rearing number); anxiety (time spent in the central part of the arena); and emotionality (defecation number).

2.6. Statistics

All the results are expressed as the mean ± SEM. Statistical analysis of data was performed by one- and two-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Induction of autophagy in the normal mice

First of all, we evaluated the possibility of autophagy activation by rapamycin and trehalose in the brain regions of normal mice (7-day treatment). According to two-way ANOVA, there was a significant influence of the group (treatment) factor ($F_{5,12} = 8.0$, $p < 0.01$) and no significant effect of the hemisphere ($F_{1,12} < 1$) or of the interaction between these factors ($F_{5,12} < 1$) on IHC signals of autophagy marker LC3-II in the frontal cerebral cortex. This parameter increased approximately 1.5-fold under the action of rapamycin (10 mg/kg) and trehalose (Fig. 1A). The greatest effect (approximately a twofold increase) was caused by joint application of rapamycin (10 mg/kg) and trehalose; this finding is suggestive of additive stimulation of autophagy by these compounds. Low doses of rapamycin were ineffective.

A similar but less pronounced modulation of autophagy was observed in terms of the expression of LC3-II in the striatum (Fig. 1B). Two-way ANOVA uncovered a significant influence of the group (treatment) factor ($F_{5,12} = 7.89$, $p < 0.01$) and no significant effect of the hemisphere ($F_{1,12} < 1$) or of the interaction between these factors ($F_{5,12} < 1$) on the above parameter. Trehalose increased the expression of LC3-II by approximately 15%, but significant growth was obtained only with the combined use of rapamycin and trehalose.

To evaluate the activation of autophagy in certain brain regions, an osmotic test was conducted. Osmotic susceptibility of lysosomal membranes (related to the autophagolysosomal activity) was estimated next (Fig. 1C). This indicator was significantly influenced by the group (treatment) factor ($F_{4,16} = 10.46$, $p < 0.001$) in the striatum. Rapamycin at a dose of 10 mg/kg caused a slight but significant increase in this indicator. A significant twofold increase was obtained with trehalose, and an even higher increase for the joint application of rapamycin and trehalose. The osmotic sensitivity of lysosomes in the striatum changed generally in accordance with the expression of LC3-II in the frontal cortex and striatum. Thus, according to the main alterations, the greatest and most reliable activation of autophagy in the striatum was caused by trehalose and its combination with rapamycin. In the midbrain, which includes *S. nigra* as its small subregion, a significant influence of the group (treatment) factor ($F_{4,13} = 4.16$, $p < 0.05$) on the osmotic susceptibility of the lysosomal membranes was also seen. Nonetheless, we did not observe clear autophagic stimulation in response to the joint action of rapamycin and trehalose.

3.2. Induction of autophagy in the MPTP-induced mouse model of PD

Taking into account the above results, we studied the therapeutic activation of autophagy in the model of PD caused by quadruple administration of MPTP. Here, we used rapamycin in its effective dose of 10 mg/kg and standard application of trehalose. In our experiments, the toxin caused a significant increase in the expression of LC3-II in the striatum (Fig. 2A): the periphery of DAergic neurons, where autophagosomes are formed. At the same time, an opposite reaction was obtained in the *S. nigra* (Fig. 2B), where the bodies of DAergic neurons and glial cells are located.

Two-way ANOVA showed a significant influence of the group (treatment) ($F_{5,12} = 7.1$, $p < 0.01$) and no significant effect of the hemisphere ($F_{1,12} < 1$) or of the interaction between these factors ($F_{5,12} < 1$) on the above parameter in the striatum. The expression of LC3-II did not differ between the groups treated with rapamycin (or rapamycin together with trehalose) and the respective control (MPTP + solvent of rapamycin; Fig. 2A). By contrast, an almost twofold increase in the expression of LC3-II was observed in the trehalose-treated group vs. MPTP-treated group. Activation of autophagy with trehalose in the striatum was significant, whereas coadministration of trehalose with rapamycin seemed to suppress the effect. Two-way ANOVA also showed a significant influence of the group (treatment) ($F_{5,11} = 43.25$, $p < 0.001$) and no significant effect of the hemisphere ($F_{1,11} = 4.03$, $p > 0.05$) or of the interaction between these factors ($F_{5,11} < 1$) on the above parameter in *S. nigra*. This brain structure responded to MPTP intoxication with apparent inhibition of autophagy, whereas trehalose produced substantial autophagy activation. Although rapamycin treatment did not affect LC3-II expression in *S. nigra*, the combined treatment with rapamycin and trehalose yielded well-pronounced autophagy activation (Fig. 2B). IHC images of LC3-II expression in the *S. nigra* (Fig. 2C) suggested that the strong fluorescence might be attributed to glial or microglial cells as well and possibly interfered with signals presumably attributed to DAergic neurons. Nevertheless, IHC staining for microglial marker IbaI did not reveal substantial differences among the experimental groups (data not shown).

In total, the changes in the immunofluorescence of LC3-II in the *S. nigra* after separate application of rapamycin and trehalose were weak, although trehalose statistically significantly stimulated expression of the marker. Its combination with rapamycin, it increased the expression of LC3-II more than threefold. Similarly, a noticeable activating effect of rapamycin in combination with trehalose was detected vs. the effect of trehalose alone. In the striatum, only stimulation of autophagy with rapamycin was noted, with a weak effect of the combination of the drugs.

3.3. Therapeutic efficacy of rapamycin and trehalose

3.3.1. Recovery of TH expression

The quantitative IHC index of TH expression was calculated as a marker of neuronal density or damage and recovery. Two-way ANOVA uncovered a significant influence of the group (treatment) ($F_{5,12} = 14.79$, $p < 0.001$) and no significant effect of the hemisphere ($F_{1,12} < 1$) or of the interaction between these factors ($F_{5,12} = 1.59$, $p > 0.05$) on the expression of TH in the s. nigra (Fig. 3). MPTP caused a marked decrease in TH expression in the s. nigra (by ~20%). In comparison with this level, the amount of TH was strongly restored under the influence of rapamycin or trehalose, with the maximal effect seen after the joint application of these drugs.

According to two-way ANOVA, there was a significant influence of the group (treatment) ($F_{5,12} = 10.37$, $p < 0.001$) and no significant effect of the hemisphere ($F_{1,12} = 1.37$, $p > 0.05$) or of the interaction between these factors ($F_{5,12} = 1.96$, $p > 0.05$) on the expression of TH in the striatum. IHC evaluation of the TH expression indicated that MPTP strongly reduced the amount of this marker in the striatum (Fig. 4). Trehalose and its combination with rapamycin effectively restored the level of TH in the striatum up to the normal level. Unexpectedly, no effect was observed with rapamycin alone; however, there was a positive effect of its combination with trehalose.

3.3.2. Behavioral tests

The efficacy of rapamycin and trehalose in eliminating the symptoms of neurodegeneration and in recovering cognitive and locomotor activity was estimated via behavioral evaluation: the test of passive

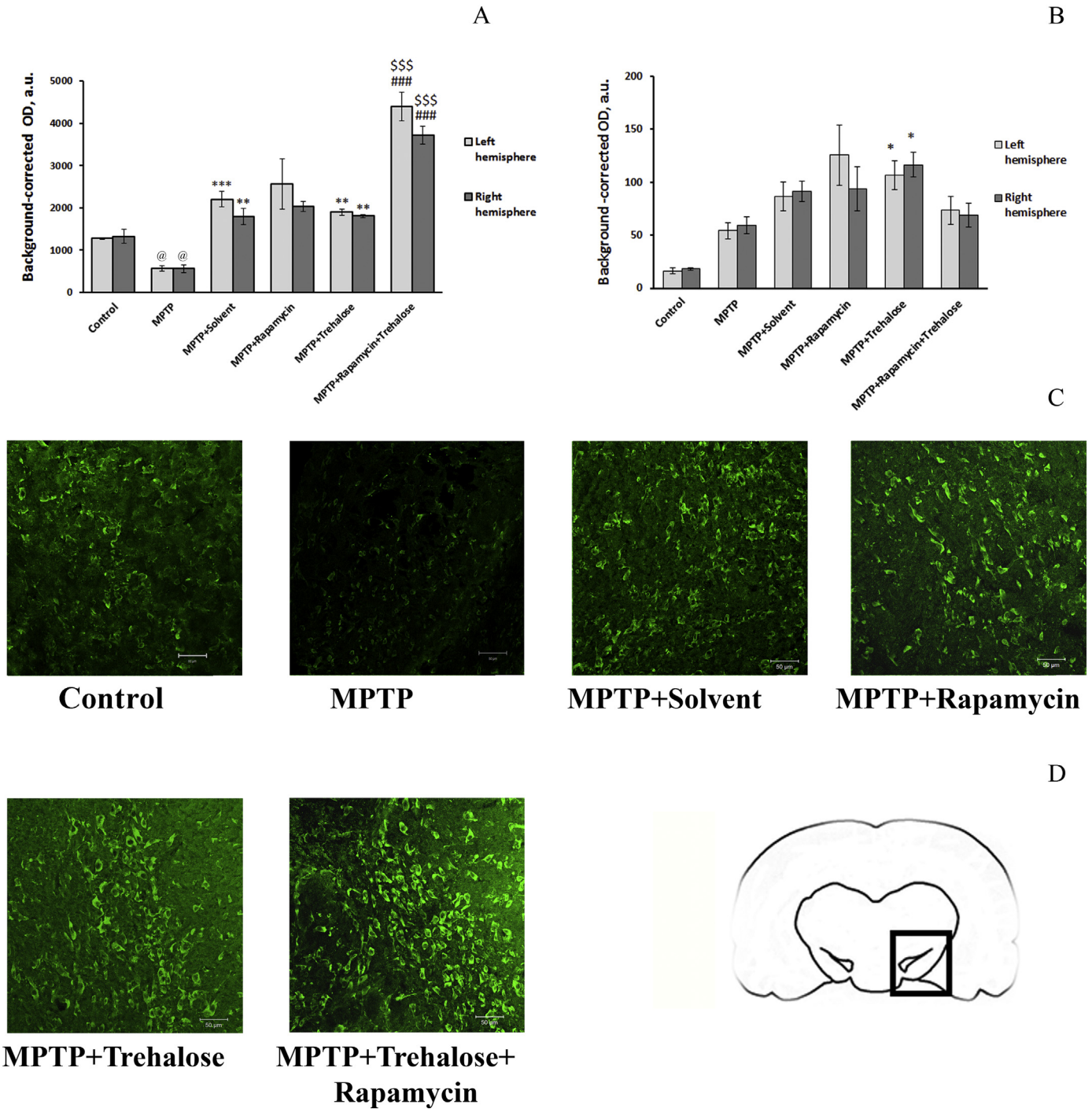

Fig. 2. Effect of treatment with rapamycin (10 mg/kg/day, 7 days), trehalose (2% in drinking water, 7 days), or their combination on autophagy activity measured by quantified immunoreactivity of LC3-II in the *S. nigra* (A) or striatum (B) in MPTP-induced mouse model of Parkinson's disease (PD). To induce PD-like pathology, MPTP was administered at the dose of 20 mg/kg (i.p., daily) for 4 days, the Control group received saline injections. The treatment was started 2 days after the last MPTP injection. MPTP + solvent group was given solvent of rapamycin (5% Tween 80 + 5% PEG-400; i.p.) for 7 days. The data are expressed as the mean ± SEM. C: LC3-II immunoreactivity in the *S. nigra*. Magnification, 200×; bar, 50 μm. The rectangle in (D) indicates the area shown in (C). $^{@}p < 0.05$ vs. the Control group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. the MPTP-treated group; $^{\#\#\#}p < 0.001$ vs. the MPTP + solvent group; $^{\$\$\$}p < 0.001$ vs. the MPTP + trehalose group.

avoidance learning (Fig. 5A) and the open field test (Fig. 5B). There was a significant influence of the group (treatment) ($F_{5,30} = 4.1$, $p < 0.01$), learning (repeated measures) ($F_{1,30} = 106.6$, $p < 0.001$), and of the interaction between these factors ($F_{5,30} = 4.4$, $p < 0.01$) on the step-through latency. Latency to enter a dark compartment during training (before the foot shock) did not differ significantly among the experimental groups (Fig. 5A). As evidence of learning on testing day, 24 h after receiving the foot shock, control (untreated) mice showed increased step-through latencies, often ~10-fold greater than latencies on training day. In contrast to control group, the step-through latencies of MPTP-treated or MPTP + solvent of rapamycin–treated mice were sharply reduced, approximately threefold, indicating memory impairment. Inducers of autophagy largely restored this behavioral response. All three types of treatment (rapamycin or trehalose alone or their combination) prominently improved learning deficit as evidenced by a significantly longer retention latencies, twofold or more, as compared

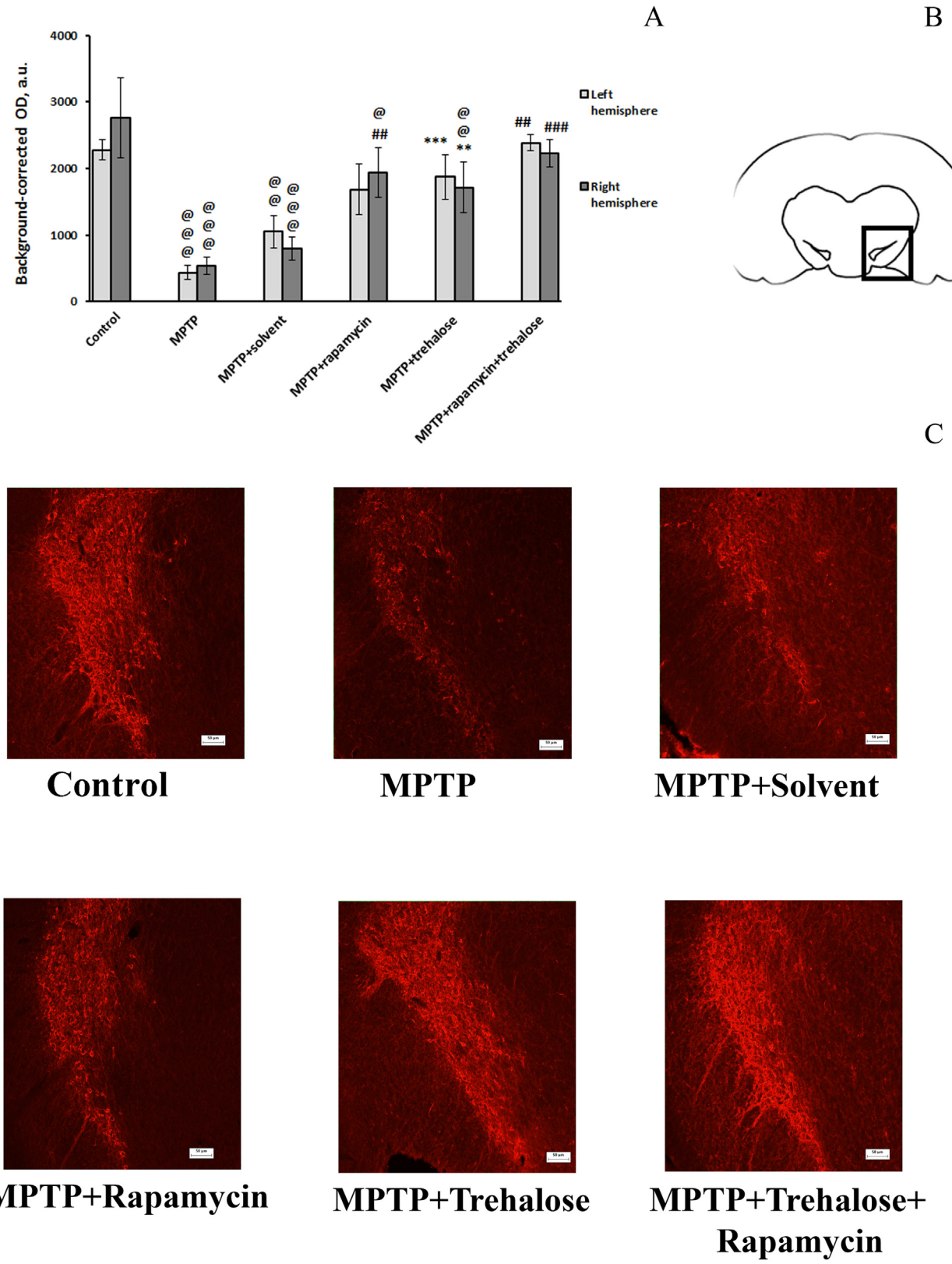

Fig. 3. Effect of treatment with rapamycin, trehalose, or their combination on tyrosine hydroxylase (TH) expression in the *S. nigra* in MPTP-induced mouse model of Parkinson's disease. Animals were treated as in the Fig. 2. A: Quantitative results. The data are expressed as the mean ± SEM. C: TH immunoreactivity in the *S. nigra*. Magnification, 100×; bar, 50 μm. The rectangle in (B) indicates the area shown in (C). $^{@}p < 0.05$, $^{@@}p < 0.01$, $^{@@@}p < 0.05$ vs. the Control group; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. the MPTP-treated group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the MPTP + solvent group.

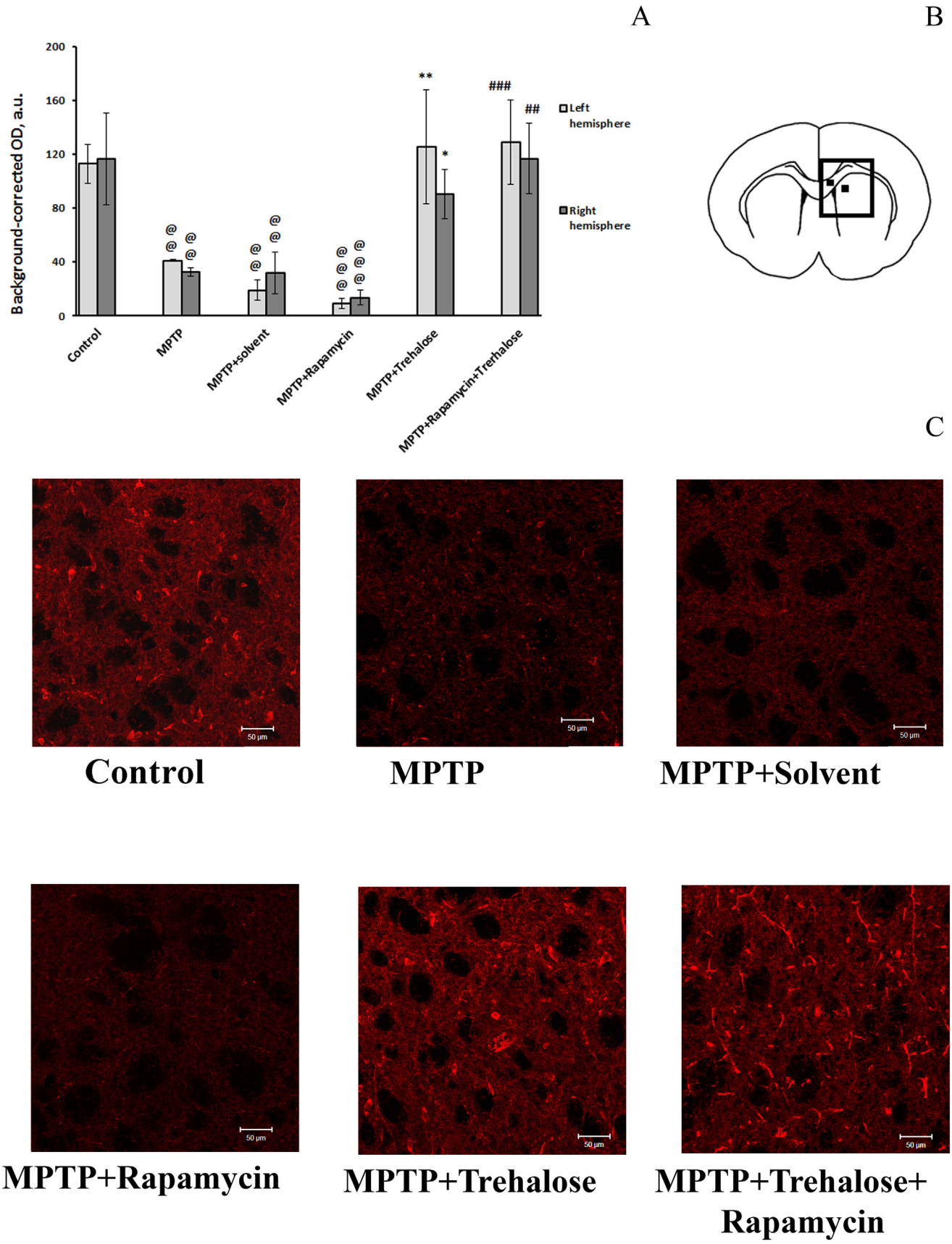

Fig. 4. Effect of treatment with rapamycin, trehalose, or their combination on tyrosine hydroxylase (TH) expression in the striatum in MPTP-induced mouse model of Parkinson's disease. Animals were treated as in the Fig. 2. A: Quantitative results. The data are expressed as the mean ± SEM. C: TH immunoreactivity in the striatum. Magnification, 200×; bar, 50 μm. The rectangle in (B) indicates the area shown in (C), and the two small black squares inside the rectangle indicate the areas used for measuring the optical density (OD) in the striatum. $^{@@}p < 0.01$, $^{@@@}p < 0.05$ vs. the Control group; $^{*}p < 0.05$; $^{**}p < 0.01$ vs. the MPTP-treated group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the MPTP + solvent group.

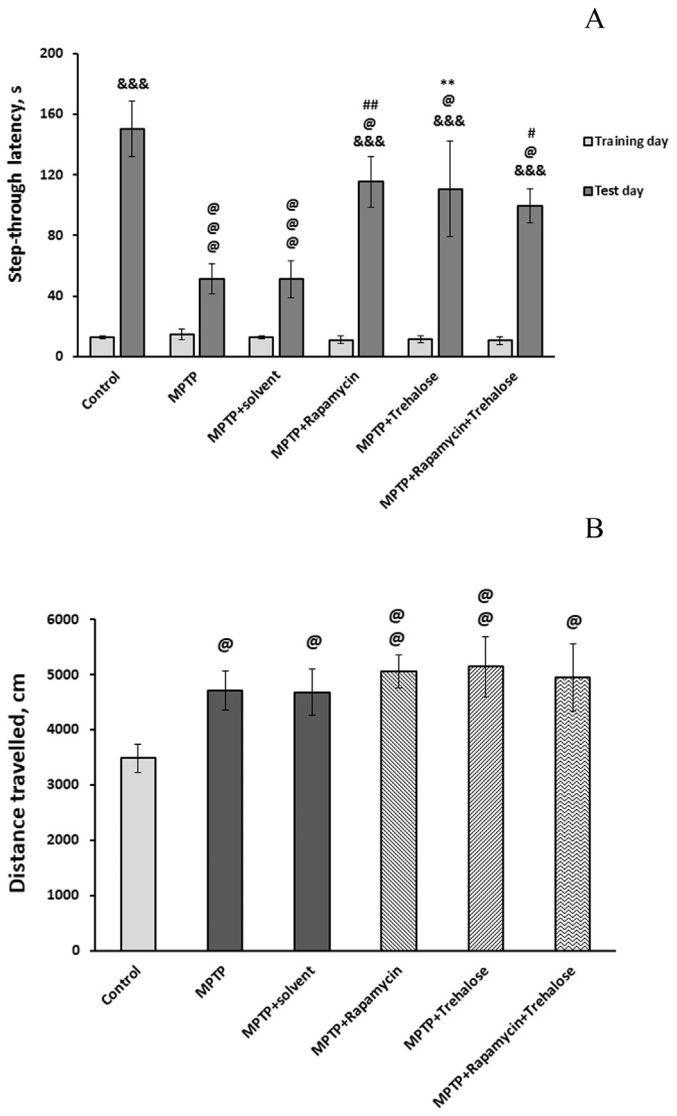

Fig. 5. Effect of treatment with rapamycin, trehalose, or their combination on the passive avoidance learning (A) or locomotion (distance travelled) in the open field test (B) in MPTP-induced mouse model of Parkinson's disease. Animals were treated as in the Fig. 2. The data are expressed as the mean ± SEM. $^{\&\&\&}p < 0.001$ compared to values on the training day; $^{@}p < 0.05$, $^{@@}p < 0.01$, $^{@@@}p < 0.05$ vs. the Control group; $^{**}p < 0.01$ vs. the MPTP-treated group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the MPTP + solvent group.

to the respective controls, MPTP-treated or MPTP + solvent of rapamycin–treated groups. Nonetheless, no additive therapeutic effect was seen for the combination of rapamycin and trehalose.

Evaluation of motor function was carried out by an open field test (Meredith and Kang, 2006). General locomotion (the distance travelled) and some other parameters (not shown here) were registered. Locomotor activity was significantly influenced by the group (treatment) ($F_{4,33} = 3.86$, $p < 0.05$). Modeling of DAergic damage by MPTP intoxication exerted a paradoxical effect of stimulation of locomotor activity (Fig. 5B). MPTP-treated mice that received rapamycin and trehalose were characterized by the increased locomotion as compared to one control group, but locomotor activity in those groups treated with autophagy inducers did not differ from that in the MPTP-treated group.

4. Discussion

The induction of autophagy in the brain with rapamycin and trehalose was registered here by IHC evaluation of the expression of autophagy marker LC3-II and the osmotic test of lysosomes (Fig. 1). The effect corresponded to the frontal cortex and striatum, which contain neurites of DAergic neurons and are affected in PD. Nonetheless, in the midbrain, which contains the somas of DAergic neurons in the *S. nigra*, the combined treatment with these drugs did not cause significant activation of autophagy. To interpret these results, specific features of autophagosome translocation from the periphery (striatum) to the perinuclear zone of DAergic neurons (*S. nigra*) should be taken into account. The latter is the destination of the LC3-II protein, which is mostly digested after the fusion of autophagosomes with lysosomes. Thus, it is difficult to register autophagosomes in the *S. nigra* (or midbrain) material. In addition, there is a strong contribution of the glial and/or microglial cells (surrounding the neurons of the *S. nigra* that also carry the LC3-II marker) to the total LC3-II fluorescence. Probably, we should better rely on the results of the LC3-II expression in the striatum than in the midbrain or *S. nigra* because the striatum is more related to the autophagosomes than the *S. nigra* is. Another reason is that the *S. nigra* is not a big part of the midbrain and its specific features might get mixed with and get lost in the properties of other midbrain regions.

While studying the possibility of activation of autophagy in the brain of normal mice, we found that the combined use of rapamycin and trehalose yields the maximal effect and is suggestive of an additive therapeutic effect of these drugs in vivo, which is in a good agreement with their additive positive effect in vitro (Sarkar et al., 2007). This additive effect piqued our interest and prompted our subsequent experiments with a mouse PD model.

The experiments were carried out on a popular PD model based on subchronic damage to DAergic neurons by MPTP (four daily doses of 20 mg/kg). Under similar conditions, signs of early activation of autophagy in DA neurons were reported, followed by neuronal death accompanied by an inflammatory response and compensatory neurochemical and motor activation (Bazzu et al., 2010; Meredith and Rademacher, 2011). The popular acute or subchronic form of MPTP intoxication does not reproduce the progressive nature of accumulation of abnormal protein and inclusions inherent in PD (Petroske et al., 2001; Meredith et al., 2008). The resulting acute damage to DAergic neurons inevitably involves an inflammatory process associated for example with massive neuronal death, activation of microglia, and involvement of cytokines. Note also that unlike the damage to neurons by MPTP, neurodegeneration in PD starts from the periphery of neurons: from dendrites and/or neurites (Bezard et al., 1997, 2013). To overcome this contradiction, researchers employ mild chronic neurotoxin administration, where progressive death of DAergic neurons is implemented (Miyara et al., 2016). Nevertheless, acute or subchronic damage to DAergic neurons remains a common model of PD (Meredith and Rademacher, 2011). These features of the model must be taken into account during interpretation of the results.

Neurons in vitro have revealed varied effects of MPTP on autophagy, apparently depending on experimental conditions (Zhu et al., 2007; Dehay et al., 2010). For acute or subacute intoxication, accumulation of the marker of autophagy LC3-II was noted, and this effect seems to be associated with inhibition of the marker removal, rather than an increase in the biosynthesis of autophagosomes (Miyara et al., 2016). To discriminate these situations, it was proposed to differentiate autophagic and lysosomal fluxes by hindering the fusion of autophagosomes with lysosomes (Klionsky et al., 2016; Lumkwana et al., 2017).

In experiments on mice, it has been shown elsewhere that acute intoxication with MPTP causes an early (already after 1 day) accumulation of LC3-II in DAergic neurons and a subsequent decrease to an almost normal level in 1 week (Dehay et al., 2010; Li et al., 2015; Lamine-Ajili et al., 2016). It is believed that this phenomenon reflects changes in the activity of autophagy. Sometimes autophagy is suppressed under the influence of MPTP in vivo, and this effect was attributed to a decrease in DAergic neuron survival (Liu et al., 2013). Under these conditions, stimulation of autophagy with rapamycin for 7 days after intoxication (i.e., after the end of the acute inflammatory

reaction) reduced the levels of α-synuclein and increased the survival of neurons (Liu et al., 2013). The effects of repeated MPTP administration might result in the ultimate inhibition of autophagy by stimulation of potent production of reactive oxygen species. In such cases, the activation of autophagy with rapamycin or TFEB may promote the survival of neurons, especially at the late stages of intoxication both in vitro and in vivo (Dehay et al., 2010; Dagda et al., 2013).

In our experiments, MPTP significantly increased the expression of autophagy marker LC3-II in the striatum (Fig. 2A), where autophagosomes are formed, but reduced this indicator in the *S. nigra* (Fig. 2B), where the LC3-II antigen is cleaved in autophagolysomes. We regard this increase in LC3-II content of the striatum as an indication of autophagy activation because the autophagic inducers under study clearly elevated this indicator. Apparently, we are talking about activation of autophagy in the striatum, the periphery of DAergic neurons, and a decrease in the number of autophagosomes and/or autophagolysomes in the perinuclear region of the neurons, where the fusion of autophagosomes with lysosomes occurs predominantly. It is worth noting that our IHC LC3-II data were obtained under conditions of the inhibition of LC3-II cleavage in autophagolysosomes by pretreatment with chloroquine, which blocks the fusion of autophagic and lysosomal fluxes. Thus, the results seem to indicate an MPTP-induced increase in the rate of autophagosome formation in the striatum and its decrease in the *S. nigra* rather than accumulation of autophagosomes in the striatum owing to the reduction in their removal (Klionsky et al., 2016; Lumkwana et al., 2017). Moreover, damage to DAergic neurons during MPTP intoxication is usually accompanied by neuroinflammation (Miyara et al., 2016) associated with the death of neurons and activation of glia and/or microglia. The fluorescent signals of LC3-II in glial and microglial cells interact with those attributed to neurons and require cautious interpretation of the results.

In the model of MPTP-induced PD, the result of activation of autophagy with rapamycin and trehalose largely depended on the initial effect of the neurotoxin on autophagy marker LC3-II. In the striatum, there was a pronounced increase in LC3-II expression under the influence of the neurotoxin, and a strong activating effect was noticed only for trehalose (not rapamycin; Fig. 2A). Regarding the joint action of rapamycin and trehalose, an explicit response of autophagy was not obtained; this finding is hardly consistent with their effectiveness when acting separately. It is likely that MPTP induces an imbalance in the cellular response to the compounds under study. In the *S. nigra*, MPTP, on the contrary, reduces the expression of the LC3-II autophagy marker (Fig. 2B). As compared to this level, trehalose activated autophagy, as did its combination with rapamycin (vs. appropriate controls). As a consequence, stimulation of autophagy with rapamycin and trehalose in the mouse model of MPTP-induced PD was more significant in the *S. nigra* than in the striatum. In neurons in vitro, rapamycin suppressed inhibition of autophagy and cell death caused by either low (10 μM) or high (200 μM) concentration of toxin MMP^+, that is, rapamycin protects neurons by activating autophagy during various forms of MPTP-like intoxication (Miyara et al., 2016).

The therapeutic effect of activation of autophagy in mice with DAergic neuron damage was assessed by expression analysis of TH and by behavioral tests. In our study, TH content [related to DA formation and DAergic neuron population (Kirik et al., 2002; Feve, 2012)] was considerably restored in the *S. nigra* by the autophagy inducers. This restoration was stimulated with rapamycin, trehalose, and their combination, and the latter had the strongest effect (Fig. 3). It is noteworthy that with the combined use of rapamycin and trehalose, TH expression approximated to the normal levels registered in untreated mice: this change can be considered a striking therapeutic effect. In the striatum (Fig. 4), TH expression was also restored up to normal levels of untreated mice by the combined treatment with rapamycin and trehalose. The efficacy of the combination was slightly higher than that of trehalose alone, whereas rapamycin alone had no significant effect on TH expression. Our results are in a good agreement with the positive effect of trehalose therapy on the mouse model of MPTP-induced PD: namely, this effect partially restored the disturbed DAergic function of the nigrostriatal system according to the levels of TH and DA transporter DAT in the striatum and *S. nigra* as well as the striatal levels of DA, HIAA, and HVA (Sarkar et al., 2014; Ferguson et al., 2015). According to some reports, rapamycin treatment fully restores TH counts (the number of TH-positive neurons in the *S. nigra*) in a mouse model of acute MPTP-induced PD and partially reduces the number of degenerated neurons in the *S. nigra* during subchronic MPTP intoxication (Malagelada et al., 2010). Another study showed that rapamycin treatment partially restores TH counts in the *S. nigra* and moderately increases the expression of TH as well as DA and DOPAC levels in the striatum under the subchronic regimen of MPTP intoxication in mice (Liu et al., 2013). Given that both drugs did not restore DAergic function completely in the PD model produced by subchronic MPTP intoxication in mice, a potential additive effect of the combined treatment is of interest. The present study shows an additive effect of the combined treatment with rapamycin and trehalose on DAergic deficits according to the levels of TH expression in the nigrostriatal system.

It is known that rapamycin influences behavior and cognitive function. In an animal model of synaptic plasticity impairment (hindlimb unloaded mice), rapamycin at a low dose (0.5 mg/kg per day for 14 days) produces a decrease in anxiety, restoration of the exploratory activity, and an LTP response in the hippocampus as well as a reduction of the oxidative stress in the hippocampus (Zhai et al., 2018). A rapamycin analog (everolimus; 5 mg/kg per day for 2 or 3 weeks) does not worsen the performance of normal C57BL/6J Rj mice in the Morris water maze test of learning and memory function (Dubois et al., 2014), whereas in a model of betamethasone 21-phosphate disodium (BTM)-induced depression and cognitive decline in DBA/2 mice, it improves the parameters of learning and memory in control mice and reverses the impairment of these functions in the BTM-treated group along with promoting hippocampal neurogenesis and synaptogenesis (Russo et al., 2016). In models of Alzheimer's disease, long-term rapamycin treatment (2.24 mg/kg, 13 weeks) or even subchronic rapamycin administration (3.5 mg/kg, 14 days) contributes to the recovery of cognitive function in the Morris water maze test, triggers autophagy, and decreases amyloid-β accumulation (Spilman et al., 2010; Zhu et al., 2014). It is interesting that the therapeutic effect of rapamycin disappears during inhibition of autophagy by means of 3-methyladenine (Zhu et al., 2014). In a mouse model of 6-OHDA-induced PD, acute rapamycin administration counteracts the impairment of novel object recognition (Masini et al., 2018).

Trehalose is also reported to correct behavioral deficits. This compound improves traumatic brain injury–induced cognitive impairment including the performance in the Morris water maze and Y-maze (Portbury et al., 2017a). It restores cognition in a transgenic Tg2576 mouse AD model thus significantly improving the performance on the Morris water maze test, which evaluates learning and memory function (Portbury et al., 2017b). In a rat model of PD induced by the injection of viral vectors expressing human A53T mutant α-synuclein into the *S. nigra*, trehalose treatment significantly attenuates DAergic neuron degeneration including impaired DAergic neuron survival and DA turnover as well as α-synuclein accumulation and aggregation in the nigrostriatal system and α-synuclein–mediated motor deficits (He et al., 2016). By contrast, it fails to activate autophagy in the spinal cord and accordingly has no effect on the motor impairment in human-mutant-P301S-tau transgenic mice (Schaeffer et al., 2012). On the other hand, the effects of combined treatment with rapamycin and trehalose on behavioral deficits have not been studied to date.

Patients with PD show not only motor impairment but also cognitive deficits, including memory, recognitive, and emotional deficits: a condition referred to as PD dementia (PDD) (Riedel et al., 2010). Almost all patients with PD will experience a cognitive decline over the years although many already have subtle deficits up to several years before the onset of motor symptoms (Brown and Tanner, 2017; Jellinger, 2018).

Some studies revealed that at 10 days after MPTP lesioning, the motor deficits disappear, but there are cognitive dysfunctions including working memory and recognition deficits (Wang et al., 2009, 2010; Hsieh et al., 2012). Both acute (Essawy et al., 2017) and subchronic (Zhao et al., 2017) MPTP intoxication causes disturbances in cognitive function of mice according to the passive avoidance task. Our results are in a good agreement with those findings because MPTP administration produced a significant decrease in the latency time in the passive avoidance test. Therapeutically important results were obtained here for all three versions of treatment (rapamycin, trehalose, and their combination): a significant increase in the latency duration in the drug-treated groups compared to respective MPTP damage groups. Nonetheless, no additive therapeutic effect was observed for the combination of rapamycin and trehalose. Of note, all types of treatment did not affect general locomotion in the open field test in comparison with MPTP damage groups, and thus the improvement in passive avoidance learning was specific. Moreover, other studies did not detect any effect of rapamycin (Masini et al., 2018) or trehalose (Kara et al., 2013; Ferguson et al., 2015) on locomotion either. An increased locomotor activity in the MPTP damage group was also observed elsewhere despite damage to motor DAergic neurons (Ferguson et al., 2015). There is a phenomenon known as compensatory acceleration of DA metabolism and synaptic conductivity in these neurons (Bazzu et al., 2010), apparently with the effect of hypercompensation.

Accordingly, in normal C57Bl/6J mice, rapamycin and trehalose exert a stimulating effect on autophagy in the striatum and to a lesser extent in the DAergic neurons of the midbrain. The use of these compounds for stimulation of autophagy for restoration of DAergic neurons and neuroprotection after the damage by neurotoxin MPTP had a positive influence. The therapeutic effect was demonstrated by active restoration of TH content of the *S. nigra* and striatum and improved cognition as determined by the passive avoidance learning task. This study offers evidence of an additive effect of the combined treatment with rapamycin and trehalose on DAergic deficits (according to the levels of TH expression in the nigrostriatal system) but not on the behavioral performance in the mouse model of PD induced by subchronic MPTP intoxication. Further studies on other PD models are needed to confirm the additive effect of the combined treatment with rapamycin and trehalose on PD-like pathology. In particular, research on transgenic models of PD that are associated with progressive accumulation of α-synuclein toxic material in neurons and corresponding gradual neuronal death are of particular interest because they better correspond to the pathogenesis of PD in humans.

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Disclosure

The authors of this manuscript have no conflicts of interests.

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