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EARLY PARKINSON'S DISEASE-LIKE PATHOLOGY IN A TRANSGENIC MOUSE MODEL INVOLVES A DECREASED CST3 MRNA EXPRESSION BUT NOT NEUROINFLAMMATORY RESPONSE IN THE BRAIN

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Abstract

Pathological aggregation and accumulation of α-synuclein in neurons play a core role in Parkinson's disease (PD) while its overexpression is a common PD model. Autophagy-lysosomal pathways are general intraneural mechanisms of protein clearance. Earlier a suppressed autophagy in the brain of young transgenic mice overexpressing the A53T-mutant human α-synuclein (mut(PD)) was revealed. Previous studies have recognized that Cystatin C displays protective activity against neurodegeneration. This cysteine protease inhibitor attracts particular attention as a potential target for PD treatment related to autophagy modulation. Here we evaluated the mRNA levels of *Cst3* encoding Cystatin C in different brain structures of 5 m.o. mut(PD) mice at standard conditions and after the chronic treatment with a neuroprotective agent, ceftriaxone (100 mg/kg, 36 days). The inflammatory markers, namely, microglial activation by IBA1 expression and mRNA levels of two chitinases genes *(Chit1, Chia1)*, were also assessed but no significant difference was found between control and transgenic mice. *Cst3* mRNA levels were significantly reduced in the striatum and amygdala in the transgenic PD model. Furthermore, this was associated with autophagy decline and might be added to early signs of synucleinopathy development. We first demonstrated the modulation of mRNA levels of *Cst3* and autophagy marker *Becn1* in the brain by ceftriaxone treatment. Taken together, the results support the potential of autophagy modulation through Cystatin C at early stages of PD-like pathology.

Keywords

Introduction

Pathological aggregation and accumulation of α-synuclein appear to play a core role in the pathogenesis of Parkinson's disease (PD) [1] and overexpression of α-synuclein is a common PD model [2]. Autophagy-lysosomal pathways, degradation and recycling of proteins by the ubiquitin/proteasome system are the main components of the cellular protein control system and general intraneural mechanisms of protein clearance [3] that are disturbed in PD [4]. A deeper understanding of the mechanisms that may lead to autophagy defects in PD is required to develop the new therapeutic interventions. Cystatin C (Cst3) attracts particular attention as a potential therapeutic target for treatment of neurodegenerative disorders.

Cst3 is a cysteine protease inhibitor that possesses a broad spectrum of biological roles [5, 6]. Human or mouse brain tissue and cerebrospinal fluid contain the highest Cst3 concentration compared to other organs and tissues [7, 8]. Cst3 is implicated in neuroprotection and repair in the nervous system in response to diverse neurotoxic conditions

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and neurodegeneration [9, 10]. Changes in the expression and secretion of Cst3 in the brain have been observed in the models of Alzheimer's disease, amyotrophic lateral sclerosis, as well as in the clinical bio-samples of patients with those neurodegenerative disorders [11]. However, few studies have explored the role of Cst3 in PD. In humans, elevated serum Cst3 was associated with cognitive disturbances and progression of PD [12], while in experimental studies, Cst3 prevented degeneration of dopaminergic neurons [13, 14]. Cst3 was suggested to play a protective role in neuronal challenge by inducing autophagy [15]. Hence, here we evaluated the mRNA levels of Cst3 encoding Cst3 and the levels of markers related to autophagy induction and regulation in different brain structures of young adult transgenic mice with overexpression of A53T-mutant α-synuclein at standard conditions and after the chronic treatment with a neuroprotective agent, ceftriaxone (CEF). The emerging evidence supports the potential effect of CEF to alleviate the symptoms of different experimentally induced neurological disorders including PD [16, 17]. Recently we revealed its inhibitory effect on the augmented autophagy level in the brain of Aβ-induced mouse model of Alzheimer's disease [8], while its effects in PD models with the decreased basal levels of autophagy in the brain and possible involvement of Cst3 were not clear.

Microglial activation is generally considered as a consequence of neurodegeneration. However, some recent reports indicate that an inflammatory reaction because of the over-activation of microglia is an important factor of the neurodegeneration progression in PD [1, 18] and suggest a set of inflammatory biomarkers for PD diagnostics [19]. Here we also evaluated the contribution of this mechanism by the expression levels of inflammatory markers in the brain.

Materials and Methods

Animals and procedures.

Five-month-old male mice of B6.Cg-Tg(Prnp-SNCA*A53T)23Mkle/J) (further – mut(PD)) and control WT strain were purchased from the SPF-vivarium of the Institute of Cytology and Genetics SB RAS (Novosibirsk, Russia). Mut(PD) hemizygous mice were produced by the insertion of human A53T missense mutant form of alpha-synuclein cDNA in the mouse genome downstream of a mouse prion Prnp promoter (https://www.jax.org/strain/006823). All mice were housed in groups of 5-6 per cage (40 x 25 x 15 cm) under standard conditions (temperature: 18-22oC, relative humidity: 50-60%, 14/10 h light/dark cycle (lights off at 15-00)) with food and sterile water *ad libitum.*

In the experiment with the CEF treatment, mice were subdivided into four groups (5–6 animals each): 1) WT mice were treated with the intraperitoneal (i.p.) injections of saline (0.9% NaCl solution, 100 μl/10 g) for 36 days (WT+Saline); 2) WT mice were treated with the i.p. injections of CEF (100 mg/kg/day for 36 days) (WT+CEF); 3) mut(PD) mice were treated with the i.p. injections of saline for 36 days (Mut(PD)+Saline); 4) mut(PD) mice were treated with the i.p. injections of CEF for 36 days (Mut(PD)+CEF). In the present study we applied the dose of 100 mg/kg/day and treatment course of 36 daily drug injections of CEF that appeared to be effective for correction of both behavioral and neuronal deficits as well as for the modulation of the expression of genes in the brain of a genetic model of Alzheimer's disease (rats of OXYS strain) [20, 21].

Mice were sacrificed by decapitation. Immediately after sacrifice, brain structures (frontal cortex, hippocampus, amygdala, hypothalamus, and striatum) were rapidly dissected on ice, put in RNAlater solution (Invitrogen, USA) with following storage of the samples at -20°C until the total RNA extraction. For immunohistochemical analysis (IHC), separate cohorts of transgenic or WT mice (5–6 animals each) were used. 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.) [22]. On the day of euthanasia, mice were anesthetized with CO2. 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. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and approved by the Local Ethical Committee of the Institute. All efforts were made to minimize the number of animals used and their suffering.

Quantitative real-time PCR (qPCR).

The relative amount of target mRNA was measured by qPCR according to previously published protocol with minor modifications [20]. Extraction of total RNA from the brain samples was performed using the Purelink RNA Mini Kit (Ambion, USA) according to the manufacturer's instructions for fresh-frozen tissue. Synthesis of cDNA was performed in the volume of 25 μl from initial 1 μg RNA using the Reverse Transcription System kit (Promega, USA) according to the manufacturer's instructions. Sequences of specific primers (Biosynthesis Ltd., Russia) for the reference genes (*Actb, Gapdh, Rpl13a, Ppia*) and target genes (*Cst3, Becn1, Chit1, Chia1*) were designed using the Primer 3 (NCBI) (Table 1); exon spanning primer sets including large introns were used to eliminate the detection of residual genomic DNA. qPCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) and LightCycler 480 II (Roche, Switzerland) using HS-qPCR Mix SYBR Green (2x) (Biolabmix, Russia). All samples were analyzed in triplicate. PCR efficiency,

slope, correlation coefficient (R2) were calculated using CFX Manager™ 3.0 Software. Fold-change values were determined using the ΔΔCt relative quantification method. For *Becn1*, the quantitative analysis was performed according to Ruijter et al. (2009) [23] to calculate the starting concentration (N0) of each cDNA template. Levels of the target genes were normalized to the geometric average of the reference gene mRNA levels.

IHC analysis.

The IHC analysis was performed on 30-μm-thick cryosections according to a protocol described in detail previously [24]. Coronal slices along the frontal cortex (AP: 2.93 – 2.45 mm), striatum (AP: 0.49–0.37 mm), or hippocampus / amygdala / hypothalamus (AP: –2.03 to –2.15 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 goat polyclonal antibody (NB100-1028, 1:200 dilution, Novus Biologicals, USA) as the primary antibody to detect microglial marker AIF-1/IBA1. A fluorescently labeled (Alexa Fluor 488–conjugated) goat anti-rabbit IgG antibody (ab150077, 1:600 dilution, Abcam, UK) or Alexa Fluor 488–conjugated donkey anti-goat IgG antibody (ab150129, 1:200 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 then analyzed in Image Pro Plus Software 6.0 (Media Cybernetics, CA, USA). Fluorescence intensity (IBA1 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. The area of interest was 18 192 μm2 in the striatum, amygdala, or the 3rd layer of the frontal cortex; 103 893 μm2 in the hypothalamus; 26 077 μm2 in the hippocampal CA1 or CA3 areas.

ELISA.

Cst3 levels in serum and urine of mice were measured by specific sandwich Cystatin C Mouse ELISA kits (BioVendor, Czech Republic) using STAT FAX 2100 reader (Awareness Technology, USA) according to the manufacturer's instructions.

Statistics.

All results were expressed as the mean ± SEM. Statistical processing of data was performed using Student's t-test and two-way ANOVA followed by post-hoc Fisher LSD test, or nonparametric Mann-Whitney U-test and Kruskal–Wallis ANOVA followed by multiple comparisons of mean ranks for all groups (in case of the lack of normal distribution of the data in the studied groups). The independent variables for two-way ANOVA were Genotype (WT or mut(PD) strain) and Treatment (Saline or CEF). STATISTICA 10.0 software was used to perform all the statistical analyses.

Results

Analysis of mRNA levels of the target genes (Becn1, Cst3, Chit1, and Chia1) in the brain of a transgenic mouse PD model.

In transgenic mice, *Becn1* expression was substantially decreased in the frontal cortex (Figure 1). Cst3 gene expression analysis in the brain of mice with transgenic PD model revealed a statistically significant decrease in the striatum and amygdala vs. control WT mice (Figure 2A). Both *Chit1* and *Chia1* were expressed at low but detectable levels in the mouse brain areas studied (Figure 2B, C). There was no difference in the expression of both chitinases genes studied vs. control WT mice.

IHC analysis of LC3-II and IBA1 levels in the brain of a transgenic mouse PD model.

In transgenic mice, the expression of an autophagy marker LC3-II was significantly decreased in the striatum, amygdala, and hypothalamus (Figure 3). The levels of LC3-II in the frontal cortex or hippocampus were not changed in mut(PD) mice. Transgenic mice had a reduced expression of microglial marker IBA1 in the striatum and frontal cortex but this parameter was markedly augmented in the hypothalamus of transgenic mice compared to WT (Figure 4). The groups did not vary significantly in the levels of IBA1 in the amygdala or hippocampus. No morphological signs of microglia activation were observed in the brain structures of transgenic mice.

Effects of CEF on the mRNA levels of the target genes (Cst3, Becn1) in the brain of a transgenic mouse PD model.

Abundance of mRNA species in the amygdala or frontal cortex is summarized in Table 2. *Cst3*. According to two-way ANOVA, there were a significant influence of the "Genotype" factor ($F_{1,15} = 32.1$, $p < 0.001$), "Treatment" factor ($F_{1,15} = 28.7$, $p < 0.001$) and a significant effect of the interaction between the factors ($F_{1,15} = 13.3$, $p < 0.01$) on the mRNA levels of Cst3 in the amygdala. LSD *post-hoc* test revealed that the parameter was significantly higher in the mice of "WT+saline" group vs. that in the mice of "WT+CEF" group ($p < 0.001$), "Mut(PD)+saline" ($p < 0.001$) or "Mut(PD)+CEF" ($p < 0.001$) group and there was a tendency to decrease in the "Mut(PD)+CEF" group vs. "Mut(PD)+saline" group ($p = 0.209$). No significant differences between the groups were found in the mouse hypothalamus ($H(3, N=18) = 3.8$, $p > 0.05$; data not shown). *Becn1*. According

to two-way ANOVA, there was a significant influence of the interaction between the factors of “Genotype” and “Treatment” ($F_{1,12} = 10.2$, $p < 0.01$) on the mRNA levels of *Becn1* in the mouse frontal cortex while the effects of the “Genotype” factor ($F_{1,12} < 1$) or of the “Treatment” factor ($F_{1,12} < 1$) were insignificant. LSD post-hoc test revealed that the parameter was significantly higher in mice of the “WT+saline” group vs. that in mice of the “Mut(PD)+saline” ($p < 0.05$) or “WT+CEF” ($p < 0.05$) group. There was a tendency to increase in the parameter in mice of the “Mut(PD)+CEF” vs. “Mut(PD)+saline” group ($p = 0.055$). No significant differences between the groups were found in the rest brain structures studied (data not shown).

Cst3 levels in serum and urine.

There were no changes in Cst3 level in serum of transgenic mice vs. control (388.0 ± 10.3 vs. 389.0 ± 16.2 ng/ml, respectively, $p > 0.05$). Cst3 concentration in urine was higher in the transgenic mice (360.0 ± 5.7 vs 140.0 ± 1.70 ng/ml in control, $p < 0.01$) indicating some kidney problems in the model.

Discussion

Neurodegeneration is tightly connected to neuroinflammation. Although microglial activation is enhanced in animal models of PD where lesions or toxins are used to induce death of neurons acutely, microglial involvement in PD remains a controversial issue. Particularly, many studies in humans have shown a lack of microglial activation in the vicinity of Lewy bodies [25]. Macrophage-derived chitinases (chitotriosidase and acid AMKase) as well as chitin-binding proteins lacking the enzymatic activity are regarded as markers of microglial activation and inflammation in the CNS [26-29]. *In vitro* studies showed that mutant α-synuclein could activate microglia more powerfully than WT α-synuclein [30]. *In vivo*, 12-month-old mice expressing the human A53T variant of α-synuclein had an increased number of IBA1-positive microglial cells in the striatum [31]. However, another study revealed the significant changes in the levels of inflammatory markers in the striatum or s.nigra of 12-month-old transgenic mice overexpressing human mutant α-synuclein only after a provocative injection of the inflammogen lipopolysaccharide at the age of seven-month-old [32]. Transgenic mice overexpressing the mutant α-synuclein of younger age (seven-month-old) also differed from the WT mice in the microglia activation only at additive provocative procedure, a chronic mild stress model [33]. Our data supports those findings since we have not observed a significant difference in the mRNA levels of both chitinases genes studied in the brain between WT and transgenic mice. Those observations were further confirmed by IHC analysis with IBA1 marker. We found a significant reduction in IBA1 fluorescence in the striatum and frontal cortex of transgenic mice and a relative increase in the parameter in the hypothalamus of mut(PD) mice compared to WT mice. It should be noted that no morphological signs of microglia activation were observed in the brain structures of transgenic mice. Relative decrease and increase of microglia abundance in certain brain structures seem to be a specific feature of microglia distribution in transgenic mice. Thus, neuroinflammation seemingly is not an early event in the PD-like pathology associated with the α-synuclein overexpression and certain provocative impacts and / or aging factor are necessary to trigger its progression.

In our previous study, we have shown a suppressed autophagy in the dopaminergic structures of five-month-old transgenic mice overexpressing human A53T α-synuclein compared to control WT mice as an early event at synucleinopathy progression [34]. Here we confirmed those findings on a reduced autophagy by LC3-II expression in the striatum of mut(PD) mice. We also found a decreased autophagic activity in the amygdala and hypothalamus but not in the hippocampus or frontal cortex in the transgenic mice. Interestingly, mRNA levels of *Becn1* encoding a regulatory protein of autophagy Beclin 1 were significantly reduced in the frontal cortex but not in the striatum in the transgenic mouse PD model. Autophagy marker LC3-II is a membrane-bound protein of autophagosomes and it is eliminated rapidly from the internal side of their membrane after their fusion with lysosomes. In general, its expression clearly reflects autophagy activity while the levels of Beclin1 are used as an additional parameter of autophagy regulation [35]. The results suggest the disturbances in autophagy activation in the striatum, amygdala, and hypothalamus in the transgenic mice and some alterations in the autophagy regulation in the frontal cortex of the mice. Thus, at early stage of PD-like pathology alterations in autophagy are not limited to the nigrostriatal system.

Cst3 induces fully functional active autophagy via the mTOR pathway; moreover, neuroprotective effects of Cst3 were prevented by autophagy inhibition with *Becn1* siRNA or 3-methyladenine [15]. Another regulatory pathway implies Cst3 direct inhibitory effect on neutral cysteine proteinases calpains that are involved in autophagy inhibition by means of mTOR-independent channels and apoptosis [36, 37]. In a recent study, neuroprotective effects of the elevated Cst3 expression at stroke were associated with autophagy induction in the affected brain regions. Moreover, exogenous Cst3 reduced the neurological deficits and infarct volume after brain ischemic injury, while 3-methyladenine partially reversed this neuroprotection [38]. Upregulation of Cst3 expression can represent a neuroprotective mechanism and may have therapeutic implications for treatment of neurodegenerative disorders [13, 39]. Indeed, the injections of Cst3 into s. nigra of transgenic mice expressing the human A53T variant of α-synuclein had neuroprotective effects

by upregulating VEGF and autophagy and downregulating α-synuclein and apoptosis [14]. Noteworthy, neuroprotection was associated with an increase in brain levels of both Cst3 and *Cst3* mRNA [40].

Our findings on the decreased expression of Cst3 mRNA in the striatum and amygdala and concurrent autophagy reduction in these brain regions of the transgenic mouse PD model are in a good agreement with the previous findings and support the notion about an important regulatory and neuroprotective role of Cst3 at neurodegeneration. We also examined the peripheral levels of Cst3 in the serum and urine of WT and transgenic mice. However, we did not find changes in the peripheral indices that would correspond to the decreased brain levels of *Cst3* expression. There were no significant differences in the serum Cst3 levels between the mice of WT and transgenic PD model while the urine levels in transgenic mice were even higher than that in WT mice. Clinical data indicated higher serum levels of Cst3 in PD patients compared to that in the healthy persons [41, 42] and a gradual increase in the parameter with the disease progression [41]. Hence, although serum Cst3 level might be regarded as a peripheral biomarker of PD progression, its relation to the brain Cst3 levels does not seem unambiguous, at least, for the early stages of the disease.

We also studied the potential effects of a multipotent antibiotic drug CEF, which has neuroprotective activity, on the modulation of mRNA levels of *Cst3* and *Becn1* genes. CEF revised cognitive and neuronal deficits in the animal models of PD and Alzheimer's disease [21, 43, 44]. Earlier we found the inhibitory effect of CEF treatment on the augmented autophagy level in the brain of a pharmacological Aβ-induced model of Alzheimer's disease in mice [8]. However, its effects in PD models with the decreased basal levels of autophagy in the brain were not clear. In WT mice, the effects of CEF treatment were similar to those observed in the model of Alzheimer's disease [8] or traumatic brain injury [45]: there was a significant decrease in autophagy marker (*Becn1* mRNA cortical level) in mice chronically treated with CEF. In the transgenic mice treated with CEF, *Becn1* mRNA levels augmented up to the level of control WT mice. CEF reduced significantly *Cst3* mRNA levels in WT mice but did not produce further significant attenuation of this index in transgenic mice. It points to different mechanisms of CEF action in WT and transgenic mice. *Cst3* gene expression was found to be strongly positively regulated by TGF-β via AP-1 transcription factor [46]. On the other hand, TGF-β was markedly down-expressed following treatment with CEF in rat renal tissues [47]. Hence, the involvement of TGF-β and AP-1 in the modulation of Cst3 expression by CEF in the brain of WT mice might be suggested. In the transgenic mice, the contribution of Cst3 in the regulation of autophagy at CEF treatment does not seem significant due to the reduced initial levels of autophagy and Cst3 expression. Monomeric and fibrillated α-synuclein stimulates glutamate release [48], while glutamate-dependent promotion of mTOR phosphorylation leading to autophagy reduction was revealed in glial cells [49]. The up-regulation of glutamate transporter EAAT2 in glial cells is responsible for CEF-mediated neuroprotection via its ability to reduce extracellular glutamate levels and subsequent excitotoxicity [17]. Thus, one may suggest that the influence of CEF on autophagy in the transgenic mice overexpressing human A53T α-synuclein is mediated mainly via the glutamatergic regulation.

Conclusion

Apparently, a significant decrease in mRNA levels of *Cst3* in the brain revealed here and associated with the autophagy decline might be added to the early signs of synucleinopathy formation. Noteworthy, the alterations in autophagy were not limited to the nigrostriatal system of the transgenic mice but occurred in the amygdala, hypothalamus, and frontal cortex as well. The study first demonstrated the modulation of *Cst3* mRNA levels in the brain by CEF treatment. The effect was observed in WT mice but not in a transgenic mouse PD model. We did not reveal significant changes in the markers associated with inflammation in the brain of young transgenic mice overexpressing human A53T α-synuclein. The results point to the potential of autophagy modulation at early stages of PD-like pathology and suggest Cst3 as a promising therapeutic tool.

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Conflicts of Interest Statement

All authors claim that there are no conflicts of interest.

References

1. Rocha EM, De Miranda B, Sanders LH. Alpha-synuclein: Pathology, mitochondrial dysfunction and neuroinflammation in Parkinson's disease. Neurobiol Dis. 2018;109(Pt B):249–57. https://doi.org/10.1016/j.nbd.2017.04.004
2. Jagmag SA, Tripathi N, Shukla SD, Maiti S, Khurana S. Evaluation of Models of Parkinson's Disease. Front Neurosci. 2016:503. https://doi.org/10.3389/fnins.2015.00503
3. Deleidi M, Maetzler W. Protein clearance mechanisms of alpha-synuclein and amyloid-Beta in lewy body disorders. Int J Alzheimers Dis. 2012;2012:391438. https://doi.org/10.1155/2012/391438
4. Cerri S, Blandini F. Role of Autophagy in Parkinson's Disease. Curr Med Chem. 2019;26(20):3702–18. https://doi.org/10.2174/0929867325666180226094351
5. Seronie-Vivien S, Delanaye P, Pieroni L, Mariat C, Froissart M, Cristol JP, et al. Cystatin C: current position and future prospects. Clin Chem Lab Med. 2008;46(12):1664–86. https://doi.org/10.1515/CCLM.2008.336
6. Amin F, Khan MS, Bano B. Mammalian cystatin and protagonists in brain diseases. J Biomol Struct Dyn. 2020;38(7):2171–96. https://doi.org/10.1080/07391102.2019.1620636
7. Keppler D. Towards novel anti-cancer strategies based on cystatin function. Cancer Lett. 2006;235(2):159–76. https://doi.org/10.1016/j.canlet.2005.04.001
8. Korolenko TA, Shintyapina AB, Pupyshev AB, Akopyan AA, Russkikh GS, Dikovskaya MA, et al. The regulatory role of cystatin C in autophagy and neurodegeneration. Vavilov Journal of Genetics and Breeding. 2019;23(4):390–7. https://doi.org/10.18699/vj19.507
9. Pérez-González R, Sahoo S, Gauthier SA, Kim Y, Li M, Kumar A, et al. Neuroprotection mediated by cystatin C-loaded extracellular vesicles. Sci Rep. 2019;9(1):11104. https://doi.org/10.1038/s41598-019-47524-7
10. Kaur G, Levy E. Cystatin C in Alzheimer's disease. Front Mol Neurosci. 2012;5:79. https://doi.org/10.3389/fnmol.2012.00079
11. Gauthier S, Kaur G, Mi W, Tizon B, Levy E. Protective mechanisms by cystatin C in neurodegenerative diseases. Front Biosci (Schol Ed). 2011;3:541–54.
12. Hu WD, Chen J, Mao CJ, Feng P, Yang YP, Luo WF, et al. Elevated Cystatin C Levels Are Associated with Cognitive Impairment and Progression of Parkinson Disease. Cogn Behav Neurol. 2016;29(3):144–9. http://dx.doi.org/10.1097/wnn.0000000000000100
13. Xu L, Sheng J, Tang Z, Wu X, Yu Y, Guo H, et al. Cystatin C prevents degeneration of rat nigral dopaminergic neurons: in vitro and in vivo studies. Neurobiol Dis. 2005;18(1):152–65. https://doi.org/10.1016/j.nbd.2004.08.012
14. Zou J, Chen Z, Wei X, Chen Z, Fu Y, Yang X, et al. Cystatin C as a potential therapeutic mediator against Parkinson's disease via VEGF-induced angiogenesis and enhanced neuronal autophagy in neurovascular units. Cell Death Dis. 2017;8(6):e2854. https://doi.org/10.1038/cddis.2017.240
15. Tizon B, Sahoo S, Yu H, Gauthier S, Kumar AR, Mohan P, et al. Induction of autophagy by cystatin C: a mechanism that protects murine primary cortical neurons and neuronal cell lines. PLoS One. 2010;5(3):e9819. https://doi.org/10.1371/journal.pone.0009819
16. Yimer EM, Hishe HZ, Tuem KB. Repurposing of the β-Lactam Antibiotic, Ceftriaxone for Neurological Disorders: A Review. Front Neurosci. 2019;13:236. https://doi.org/10.3389/fnins.2019.00236
17. Tai CH, Bellesi M, Chen AC, Lin CL, Li HH, Lin PJ, et al. A new avenue for treating neuronal diseases: Ceftriaxone, an old antibiotic demonstrating behavioral neuronal effects. Behav Brain Res. 2019;364:149–56. https://doi.org/10.1016/j.bbr.2019.02.020
18. Gelders G, Baekelandt V, Van der Perren A. Linking Neuroinflammation and Neurodegeneration in Parkinson's Disease. J Immunol Res. 2018;2018:4784268. https://doi.org/10.1155/2018/4784268
19. Li T, Le W. Biomarkers for Parkinson's Disease: How Good Are They? Neurosci Bull. 2020;36(2):183–94. https://doi.org/10.1007/s12264-019-00433-1
20. Tikhonova MA, Amstislavskaya TG, Belichenko VM, Fedoseeva LA, Kovalenko SP, Pisareva EE, et al. Modulation of the expression of genes related to the system of amyloid-beta metabolism in the brain as a novel mechanism of ceftriaxone neuroprotective properties. BMC Neurosci. 2018;19(Suppl 1):13. https://doi.org/10.1186/s12868-018-0412-5
21. Tikhonova MA, Ho SC, Akopyan AA, Kolosova NG, Weng JC, Meng WY, et al. Neuroprotective effects of ceftriaxone treatment on cognitive and neuronal deficits in a rat model of accelerated senescence. Behav Brain Res. 2017;330:8–16. https://doi.org/10.1016/j.bbr.2017.05.002
22. Iwai-Kanai E, Yuan H, Huang C, Sayen MR, Perry-Garza CN, Kim L, et al. A method to measure cardiac autophagic flux in vivo. Autophagy. 2008;4(3):322–9. https://doi.org/10.4161/auto.5603
23. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 2009;37(6):e45. https://doi.org/10.1093/nar/gkp045
24. Pupyshev AB, Tikhonova MA, Akopyan AA, Tenditnik MV, Dubrovina NI, Korolenko TA. Therapeutic activation of autophagy by combined treatment with rapamycin and trehalose in a mouse MPTP-induced model of Parkinson's disease. Pharmacol Biochem Behav. 2019;177:1–11. https://doi.org/10.1016/j.pbb.2018.12.005

25. Streit WJ, Xue QS, Tischer J, Bechmann I. Microglial pathology. Acta Neuropathol Commun. 2014;2:142. https://doi.org/10.1186/s40478-014-0142-6
26. Correale J, Fiol M. Chitinase effects on immune cell response in neuromyelitis optica and multiple sclerosis. Mult Scler. 2011;17(5):521–31. https://doi.org/10.1177/1352458510392619
27. Hall S, Surova Y, Öhrfelt A; Swedish BioFINDER Study, Blennow K, Zetterberg H, Hansson O. Longitudinal Measurements of Cerebrospinal Fluid Biomarkers in Parkinson's Disease. Mov Disord. 2016;31(6):898–905. https://doi.org/10.1002/mds.26578
28. Xiao Q, Yu W, Tian Q, Fu X, Wang X, Gu M, et al. Chitinase1 contributed to a potential protection via microglia polarization and Aβ oligomer reduction in D-galactose and aluminum-induced rat model with cognitive impairments. Neuroscience. 2017;355:61–70. https://doi.org/10.1016/j.neuroscience.2017.04.050
29. Steinacker P, Verde F, Fang L, Feneberg E, Oeckl P, Roeber S, et al. Chitotriosidase (CHIT1) is increased in microglia and macrophages in spinal cord of amyotrophic lateral sclerosis and cerebrospinal fluid levels correlate with disease severity and progression. J Neurol Neurosurg Psychiatry. 2018;89(3):239–47. http://dx.doi.org/10.1136/jnnp-2017-317138
30. Zhang W, Dallas S, Zhang D, Guo JP, Pang H, Wilson B, et al. Microglial PHOX and Mac-1 are essential to the enhanced dopaminergic neurodegeneration elicited by A30P and A53T mutant alpha-synuclein. Glia. 2007;55(11):1178–88. https://doi.org/10.1002/glia.20532
31. Sirabella R, Sisalli MJ, Costa G, Omura K, Ianniello G, Pinna A, et al. NCX1 and NCX3 as potential factors contributing to neurodegeneration and neuroinflammation in the A53T transgenic mouse model of Parkinson's Disease. Cell Death Dis. 2018;9(7):725. https://doi.org/10.1038/s41419-018-0775-7
32. Gao HM, Zhang F, Zhou H, Kam W, Wilson B, Hong JS. Neuroinflammation and alpha-synuclein dysfunction potentiate each other, driving chronic progression of neurodegeneration in a mouse model of Parkinson's disease. Environ Health Perspect. 2011;119(6):807–14. https://doi.org/10.1289/ehp.1003013
33. Wu Q, Yang X, Zhang Y, Zhang L, Feng L. Chronic mild stress accelerates the progression of Parkinson's disease in A53T α-synuclein transgenic mice. Exp Neurol. 2016;285(Pt A):61–71. https://doi.org/10.1016/j.expneurol.2016.09.004
34. Pupyshev AB, Korolenko TA, Akopyan AA, Amstislavskaya TG, Tikhonova MA. Suppression of autophagy in the brain of transgenic mice with overexpression of A53T-mutant α-synuclein as an early event at synucleinopathy progression. Neurosci Lett. 2018;672:140–4. https://doi.org/10.1016/j.neulet.2017.12.001
35. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2012;8(4):445–544. http://dx.doi.org/10.4161/auto.19496
36. Ahumada-Castro U, Silva-Pavez E, Lovy A, Pardo E, Molgó J, Cárdenas C. MTOR-independent autophagy induced by interrupted endoplasmic reticulum-mitochondrial Ca2+ communication: a dead end in cancer cells. Autophagy. 2019;15(2):358–61. https://doi.org/10.1080/15548627.2018.1537769
37. Williams A, Sarkar S, Cuddon P, Ttofi EK, Saiki S, Siddiqi FH, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol. 2008;4(5):295–305. https://doi.org/10.1038/nchembio.79
38. Fang Z, Feng Y, Li Y, Deng J, Nie H, Yang Q, et al. Neuroprotective Autophagic Flux Induced by Hyperbaric Oxygen Preconditioning is Mediated by Cystatin C. Neurosci Bull. 2019;35(2):336–46. https://doi.org/10.1007/s12264-018-0313-8
39. Wang R, Chen Z, Fu Y, Wei X, Liao J, Liu X, et al. Plasma Cystatin C and High-Density Lipoprotein Are Important Biomarkers of Alzheimer's Disease and Vascular Dementia: A Cross-Sectional Study. Front Aging Neurosci. 2017;9:26. https://doi.org/10.3389/fnagi.2017.00026
40. Kaur G, Mohan P, Pawlik M, DeRosa S, Fajiculay J, Che S, et al. Cystatin C rescues degenerating neurons in a cystatin B-knockout mouse model of progressive myoclonus epilepsy. Am J Pathol. 2010;177(5):2256–67. https://doi.org/10.2353/ajpath.2010.100461
41. Chen WW, Cheng X, Zhang X, Zhang QS, Sun HQ, Huang WJ, et al. The expression features of serum Cystatin C and homocysteine of Parkinson's disease with mild cognitive dysfunction. Eur Rev Med Pharmacol Sci. 2015;19(16):2957–63.
42. Xiong Y, Mahmood A, Chopp M. Current understanding of neuroinflammation after traumatic brain injury and cell-based therapeutic opportunities. Chin J Traumatol. 2018;21(3):137–51. https://doi.org/10.1016/j.cjtee.2018.02.003
43. Ho SC, Hsu CC, Pawlak CR, Tikhonova MA, Lai TJ, Amstislavskaya TG, et al. Effects of ceftriaxone on the behavioral and neuronal changes in an MPTP-induced Parkinson's disease rat model. Behav Brain Res. 2014;268:177–84. https://doi.org/10.1016/j.bbr.2014.04.022
44. Weng JC, Tikhonova MA, Chen JH, Shen MS, Meng WY, Chang YT, et al. Ceftriaxone prevents the neurodegeneration and decreased neurogenesis seen in a Parkinson's disease rat model: An immunohistochemical and MRI study. Behav Brain Res. 2016;305:126–39. https://doi.org/10.1016/j.bbr.2016.02.034
45. Cui C, Cui Y, Gao J, Sun L, Wang Y, Wang K, et al. Neuroprotective effect of ceftriaxone in a rat model of traumatic brain injury. Neurol Sci. 2014;35(5):695–700. https://doi.org/10.1007/s10072-013-1585-4
46. Huh C, Nagle JW, Kozak CA, Abrahamson M, Karlsson S. Structural organization, expression and chromosomal mapping of the mouse cystatin-C-encoding gene (Cst3). Gene. 1995;152(2):221–6. https://doi.org/10.1016/0378-1119(94)00728-B
47. Abdel-Daim MM, El-Sayed YS, Eldaim MA, Ibrahim A. Nephroprotective efficacy of ceftriaxone against cisplatin-induced subchronic renal fibrosis in rats. Naunyn Schmiedebergs Arch Pharmacol. 2017;390:301–9. https://doi.org/10.1007/s00210-016-1332-5

48. Sarafian TA, Littlejohn K, Yuan S, Fernandez C, Cilluffo M, Koo BK, et al. Stimulation of synaptoneurosome glutamate release by monomeric and fibrillated α synuclein. J Neurosci Res. 2017;95(9):1871–87. https://doi.org/10.1002/jnr.24024

49. Lopez-Colome MA, Martinez-Lozada Z, Guillem AM, Lopez E, Ortega A. Glutamate transporter-dependent mTOR phosphorylation in Muller glia cells. ASN Neuro. 2012:4(5):e00095. http://dx.doi.org/10.1042/AN20120022

Tables

Table 1. Reference and target genes PCR primer sequences (5' - 3'), amplicon size.

Symbol	Sequence (5' - 3')		Amplicon size (bp)
	Forward Primer	Reverse Primer	
	Reference genes		
Gapdh	GCTCCTCCCTGTTCCAGAGAC	CCAATACGGCCAAATCCGTTCA	103
Actb	TTCTACAATGAGCTGCGTGTG	GGGGTGTTGAAGGTCTCAAA	102
Rpl13a	CATGAGGTCGGGTGGAAGTA	TTCCGTAACCTCAAGATCTGC	110
Ppia	AAAGTTCCAAAGACAGCAGAAAA	GCCAGGACCTGTATGCTTTAG	207
	Target genes		
Cst3	AGGAGGCAGATGCCAATGAG	GGGCTGGTCATGGAAAGGA	227
Chit1	CGGCAGGAACTAAATCTTCCAT	TGGGCGTGGCTCAGGTAT	70
Chia1	TTTTGGCAGTGCATCAATGG	GCAGCAATTACAGCTGGTATCAA	80
Becn1	GAACTCACAGCTCCATTACTTA	ATCTTCGAGAGACACCATCC	121

Table 2. Relative mRNA levels of *Cst3* and *Becn1* in the brain structures. Data are presented as the Mean±S.E.M. of the values obtained in an independent group of animals (n=5-6 per group). Statistically significant differences: *p < 0.05, ***p < 0.001 vs. "WT+Saline" group.

Gene, brain structure	*Group*			
	WT+Saline	**WT+CEF**	**Mut (PD)+Saline**	**Mut (PD)+CEF**
Cst3, amygdala	1.058 ± 0.1791	0.271 ± 0.0866***	0.244 ± 0.0342***	0.095 ± 0.0308***
Becn1, frontal cortex	8.65 ± 3.333	0.69 ± 0.346*	0.75 ± 0.140*	6.99 ± 2.185

Figures

Figure 1. Effect of the overexpression of A53T-mutant α-synuclein on mRNA levels of *Becn1* in the frontal cortex, hippocampus, and striatum in 5 m.o. mice. The data are expressed as the means ± SEMs of the values obtained in an independent group of animals (*n*=5-6 per group). Statistically significant differences: * p<0.05 vs. WT mice.

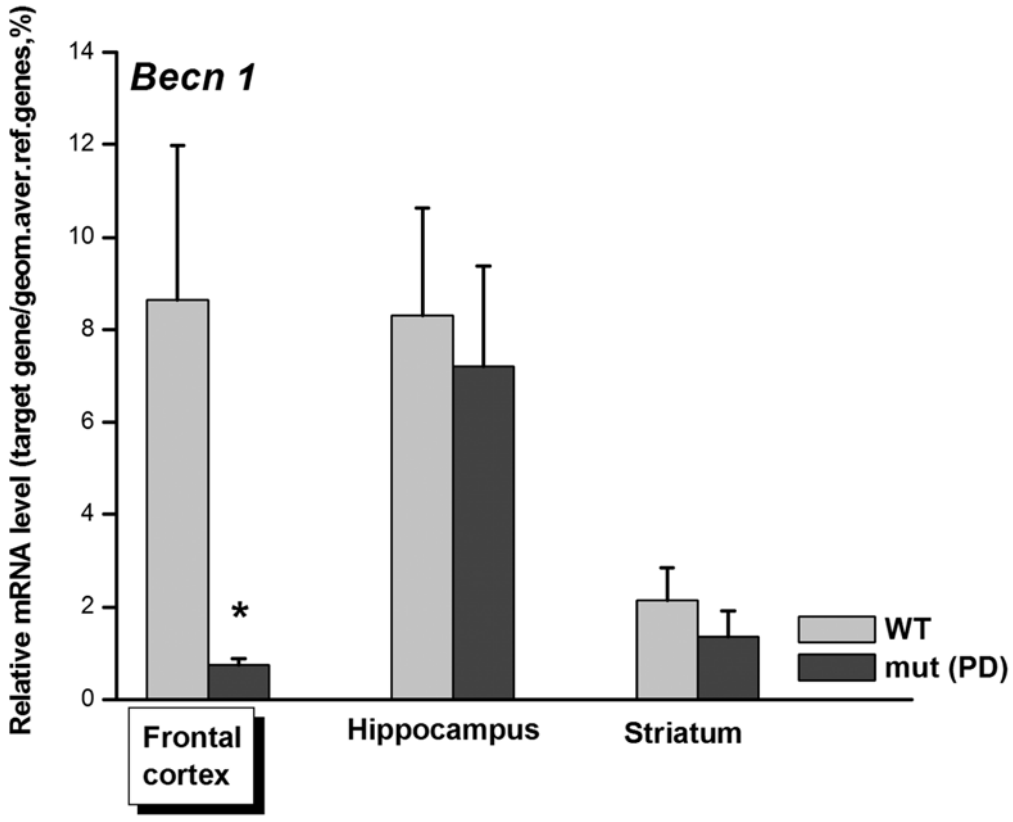

Figure 2. Effect of the overexpression of A53T-mutant α-synuclein on mRNA levels of *Cst3* (A), *Chit1* (B), and *Chia1* (C) in the striatum, amygdala, hippocampus, and hypothalamus in 5 m.o. mice. The data are expressed as the means ± SEMs of the values obtained in an independent group of animals (*n*=5-6 per group). Statistically significant differences: * p<0.05, ** p<0.01 vs. WT mice.

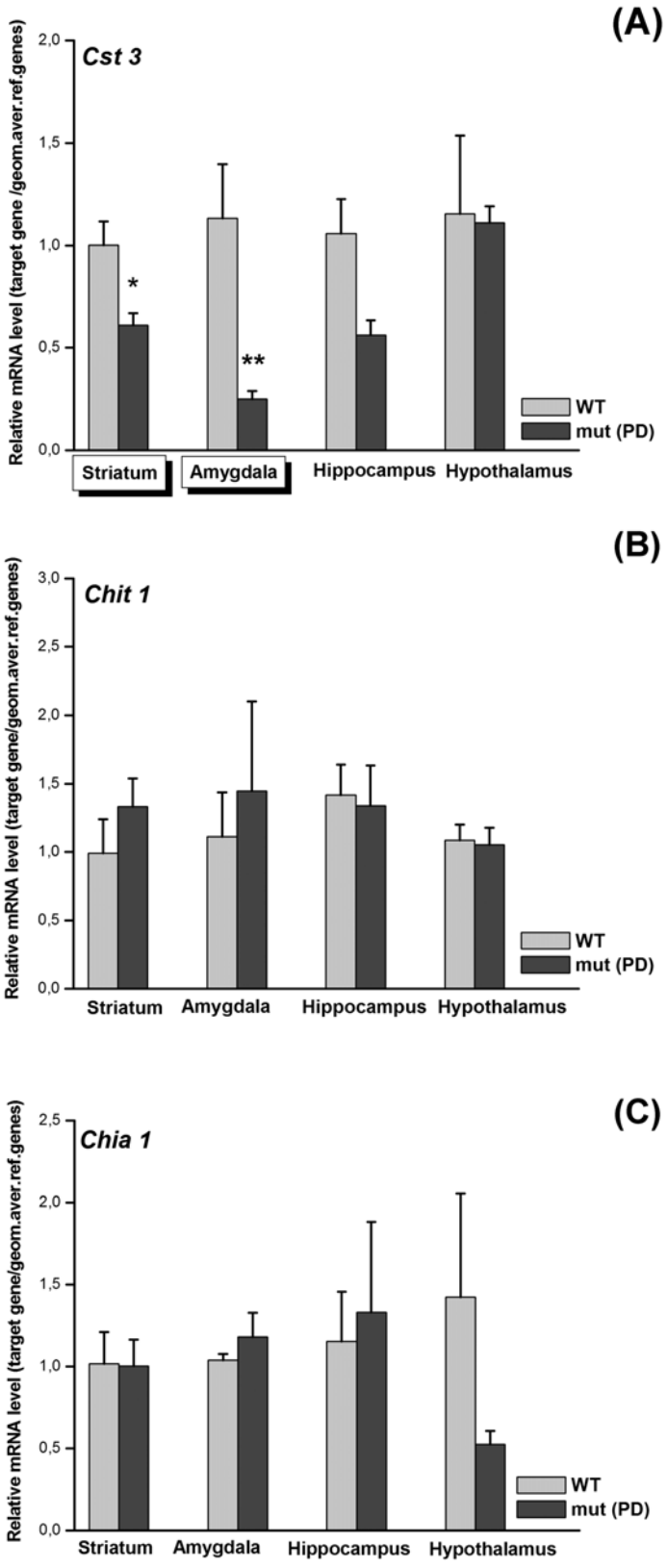

Figure 3. Effect of the overexpression of A53T-mutant α-synuclein on autophagy activity measured by quantified immunoreactivity of LC3-II in the striatum, amygdala, hippocampal CA1 and CA3 areas, hypothalamus, and frontal cortex in 5 m.o. mice. A: Quantitative results. The data are expressed as the Mean±S.E.M. of the values obtained in an independent group of animals (*n*=3-6 per group). Statistically significant differences: * p<0.05, ** p<0.01 vs. WT mice. B: LC3-II immunoreactivity in the striatum, amygdala, and hypothalamus. Magnification, 200×; bar, 50 μm.

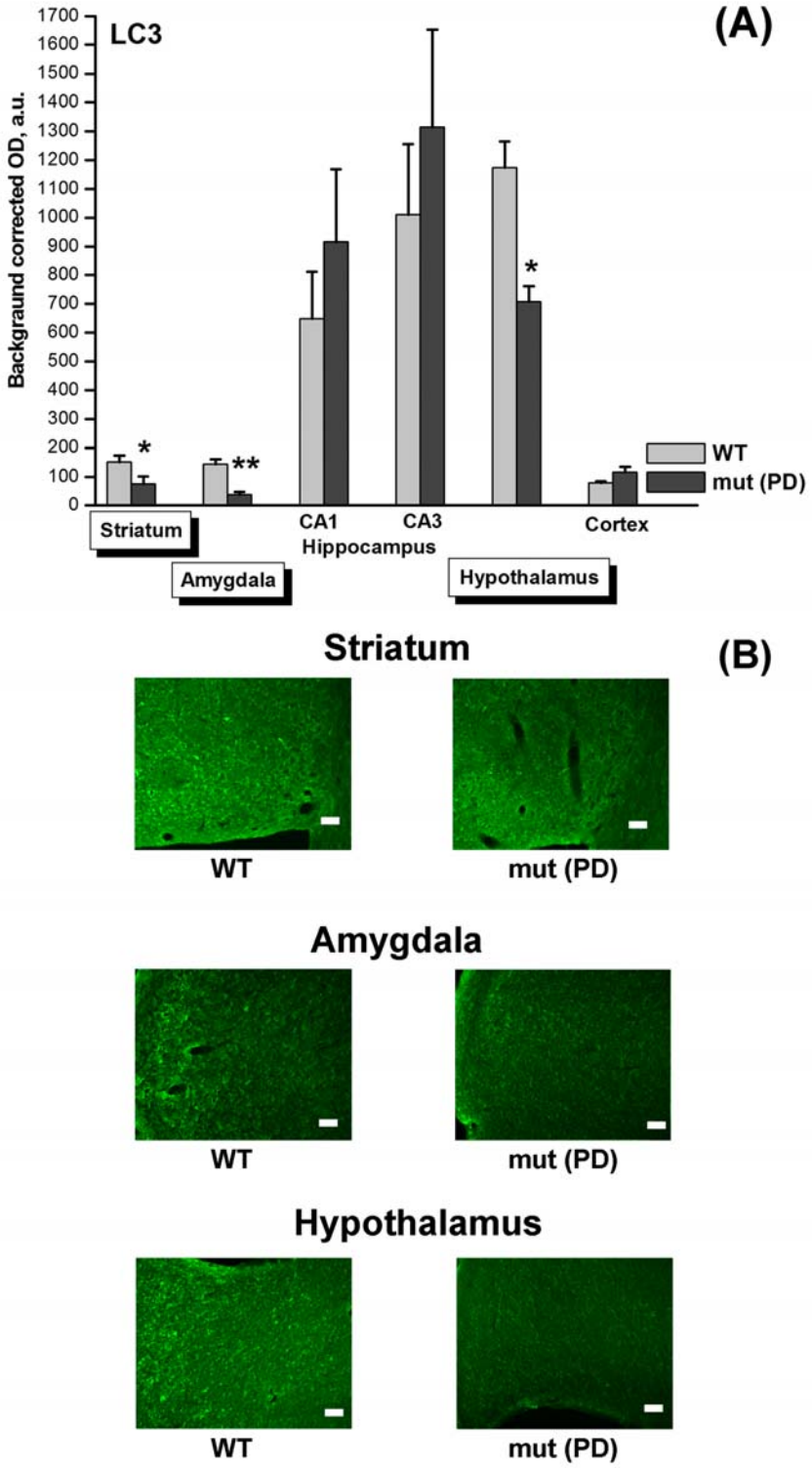

Figure 4. Effect of the overexpression of A53T-mutant α-synuclein on the expression of microglial marker IBA1 in the striatum, amygdala, hippocampal CA1 and CA3 areas, hypothalamus, and frontal cortex in 5 m.o. mice. A: Quantitative results. The data are expressed as the Mean±S.E.M. of the values obtained in an independent group of animals (*n*=3-6 per group). Statistically significant differences: * p<0.05, *** p<0.001 vs. WT mice. B: IBA1 immunoreactivity in the striatum, frontal cortex, and hypothalamus. Magnification, 200×; bar, 50 μm.