Repositioning Doxycycline for treating Parkinson’s Disease: evidence from a pre-clinical mouse model

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Research

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Abstract

Background

Parkinson's disease remains orphan of valuable therapies capable to interfere with the disease pathogenesis despite the large number of symptomatic approaches adopted in clinical practice to manage this disease. Treatments simultaneously affecting \( \alpha \)-synuclein (\( \alpha \)-syn) oligomerization and neuroinflammation may counteract Parkinson's disease. Recent data demonstrated that Doxycycline an antibiotic of the tetracycline class, can inhibit \( \alpha \)-syn aggregation and exert anti-inflammatory activity. We herein investigate, for the first time, the potential therapeutic properties of Doxy in a human \( \alpha \)-syn A53T transgenic Parkinson's disease mouse model by the evaluation of behavioural, biochemical and histopathological parameters.

Methods

human \( \alpha \)-syn A53T transgenic mice were treated with Doxycycline (10 mg/Kg daily ip) for 30 days, the effect of treatment on motor and cognitive behaviour impairment and daily live activity of mice were examined, successively immunocytochemical, electrophysiological and biochemical analysis of cerebral tissue was performed.

Results

Doxy treatment abolished cognitive and daily life activity deficiencies in A53T mice. The effect on cognitive functions was associated with neuroprotection, inhibition of \( \alpha \)-syn oligomerization and gliosis both in the cortex and hippocampus. Doxy treatment restored hippocampal long-term potentiation in association with inhibition of pro-inflammatory cytokines expression. Moreover, Doxy ameliorated motor impairment and reduced striatal glial activation in A53T mice.

Conclusions

Our findings promote Doxy as a valuable multi-target therapeutic approach counteracting both symptoms and neuropathology in the complex scenario of \( \alpha \)-synucleinopathies

Background

Parkinson's Disease (PD) and related disorders are devastating neurodegenerative diseases orphan of valuable therapeutic strategies either for halting or modifying the disease course. Although motor symptoms are core PD clinical manifestations, cognitive deficits and dementia are acquiring an increasing interest being recognized as first causes of patient institutionalization [1]. Neuroinflammation and intraneuronal inclusions, mainly composed by alpha-synuclein (\( \alpha \)-syn), are both culprits and
neuropathological hallmarks of PD and its related disorders. From an etiological standpoint, α-syn oligomeric assemblies (α-synO) are recognized as the main neurotoxic moieties capable of causing cognitive deficits, long term potentiation (LTP) impairment, inflammation, synaptic damage and neuronal loss [2–11], although other conformers might exerted similar neurotoxic activity [12–15]. We hypothesized that a therapeutic approach based on a single molecule affecting at the same time α-syn aggregation and neuroinflammation may offer a promising tool to alleviate PD progression. In such a perspective, doxycycline (Doxy), a tetracycline antibiotic, represents a promising alternative, endowed with anti-amyloidogenic activity against several proteins including α-syn [16, 17] and anti-inflammatory action reported in various contexts [18–21]. In addition, Doxy has appealing pharmacological properties such as a safe toxicological profile, a favorable blood-brain-barrier permeability [22], and is already widely used in the clinic. Based on this evidence, we herein investigated in A53T PD transgenic (Tg) mice, carrying the human missense mutation A53T, the effectiveness of a treatment with Doxy. Our results demonstrate that the treatment with Doxy at sub-antimicrobial dose leads to beneficial effects in A53T mice. In fact, Doxy ameliorates motor functions, restores cognitive functions and increases neuronal survival while decreasing α-synOs and neuroinflammation. Notably, the treatment with Doxy is not associated with adverse effects in non-transgenic mice, thus supporting Doxy as a valuable compound for long-lasting treatments as already proved in clinical studies.

**Materials And Methods**

**Mice**

Eight-month-old hemizygous B6.Cg2310039L15RikTg(Prnp-SNCA*A53T)23Mkle/J (referred to as A53T mice) and their non-transgenic matched littermates (NTG) (The Jackson Laboratory, USA) were exploited. A53T mice express the A53T missense mutant form of human α-syn under the control of the murine prion promoter. Transgenic mice from line G2-3(A53T) express the A53T mutant α-syn protein at approximately six times the level of endogenous mouse α-syn. Mice spontaneously develop adult-onset neurodegeneration affecting several brain regions and a progressive motor and cognitive dysfunction [23, 24].

All experimental procedures were conducted in conformity with institutional guidelines, that are in compliance with national (D.L. n.26, G.U. 4 March 2014) and international guidelines and laws (EEC Council Directive 86/609, OJ L 358, 1, 12 December 1987, Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996), and were reviewed and approved by the intramural ethical committee.

**Experimental design**

Mice were daily treated with doxycycline (10 mg/Kg, Sigma) or saline (Veh) for a total of 34 days. Starting from day 27, mice were tested in several behavioral tasks (1 task/day) Between day 27 and 29 mice were tested in the novel object recognition test. Between day 30 and 32 the nesting behavior was
assessed. On day 33 mice were evaluated in the beam walk test and, on day 34 in the paw grip. Mice were sacrificed immediately after the end of the last behavioral test.

**Novel object recognition test**

Novel object recognition test was performed to assess long term recognition memory as previously described [9]. Memory was expressed as percentage of time spent in the exploration of the familiar and novel object, the time spent by mice to explore the objects was measured by a blinded operator.

**Nesting test**

Activity of daily living was assessed through the Nesting test. The task is a sensitive test based on natural mouse behaviour which allow to detect early cognitive and behavioural deficiencies [25].

The quality of the nests was assessed by two blind operators at 1, 24 and 48 hours after cotton bulk placement through a five-point scale [26].

**Beam walk test**

The beam walk test measures the foot slips and latency of a mouse walking twice along an elevated wooden beam (8 mm wide and 100 cm long). Before the test, mice are trained in three habituation trials (intertrial interval: 30 sec). Foot slips and latency were assessed 60 sec after the last training trial. Results are expressed as mean ± SEM of the two trials (intertrial interval: 60 sec) in the test phase. Mice with gait instability had more foot slips and a longer latency than not impaired animals.

**Paw grip test**

The paw grip test has been performed as previously described [27]. Briefly, mice were placed on a horizontal grid at about 30 cm from the table and the tail is gently pulled until they grasp the grid with their fore and hind paws. The lid is then gently turned upside down and the latency time of the mouse to fall on the table is recorded for a maximum of 300 s. Each mouse is given up to three attempts and the longest latency is recorded.

**Immunohistochemistry**

Mice were anesthetized and transcardially perfused with 50 mM phosphate buffer saline (PBS) pH 7.4. Serial coronal sections (30 μm) were collected in 100 mM PBS for immunohistochemical analyses of the neuronal marker (NeuN), the human a-syn, the astroglial marker (GFAP) and the microglial marker (IBA1). Briefly, slices were blocked for 1 hour at room temperature (RT) with an appropriate blocking solution (Neun and GFAP: 3% NGS, Triton 0.4% in PBS 100 mM, human a-syn and IBA: 10% NGS, Triton 0.3% in PBS 100 mM). Then slices were incubated with mouse anti-Neun (1:1000, MAB37, Millipore), mouse anti-human a-syn (1:2000, Syn505, 35-8300, Invitrogen), mouse anti-GFAP antibody (1:3500, MAB3402, Millipore), or rabbit anti-IBA1 antibody (1:1000, 019-19741, Wako) at 4°C overnight. After incubation with the anti-mouse or anti-rabbit biotinylated secondary antibody (1:200,
Vector Laboratories, 1 hour RT), immunostaining was developed using the avidin-biotin kit (Vector Laboratories) and diaminobenzidine (Sigma, Italy).

**Image analysis**

The quantitative analyses of the Neun+ cell density was measured in the pre-frontal cortex (PFC), NeoCTX and CA3 hippocampal subfield. Data were expressed as percentage of NTG+Veh treated animals. Human a-syn optic density was measured in the NeoCTX and hippocampus of A53T+Veh or +Doxy treated mice. Data was expressed as percentage of A53T+Veh treated animals. The marked area for each specific marker was quantified applying dedicated home-made macros through Fiji software\(^{26}\). Data are normalized on the area of the brain region considered and are presented as percentage (mean±sem) of NTG+Veh treated mice or A53T+Veh treated mice as specified in each result sections.

Quantitative analyses were performed by a blind operator. Image acquisition was done with the Olympus VS120-S6-FL-078. Neun-, human a-syn-, GFAP- and IBA1-immunoreactivity were analyzed with homemade macros and Fiji software \[^{28}\].

**Western blot analyses**

Cortices and hippocampi from 30 days NTG and A53T treated animals were dissected and snap frozen on dry ice. Samples were homogenized in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100, protease inhibitor cocktail tablets (Roche) and phosphatase inhibitors (Pierce). Lysates were incubated for 30 min on ice, centrifuge at 150000 g for 60 min at 4°C. The pellet (Triton X-100 insoluble) and supernatant (Triton X-100 soluble) were collected. The Triton X-100-insoluble pellets were redissolved in the previously described lysis buffer containing 2% SDS \[^{29}\].

**Acute brain slices preparation and extracellular field recordings**

Acute brain slices were obtained from 9 months old NTG and A53T mice, treated with Veh or Doxy for 30 days. Coronal cortico-hippocampal slices (350 mm thick) were cut in ice-cold modified artificial cerebrospinal fluid (aCSF) as previously described \[^{8}\].

**Gene expression analysis.**

Total RNA was extracted from hippocampi of NTG+Veh, A53T+Veh and A53T+Doxy animals after 30 days of treatment by PureLink RNA Mini Kit (Invitrogen) following the manufacturer's instructions. As previously described \[^{30}\], samples were treated with DNase I (Invitrogen) and reverse-transcribed with random hexamer primers using cDNA Reverse Transcription Kit (Applied Biosystem). The analysis was performed using Power syber green pcr master mix (Applied Biosystem) and 7300 Real-Time PCR System (Applied Biosystems). mRNA expression of target genes was evaluated using primers listed in Table 1. \(\beta\)-actin was used as housekeeping gene to normalize mRNA levels. Relative gene expression was determined by \(\Delta\Delta\text{Ct}\) method. Data are expressed as fold difference from the NTG+Veh group.
Table 1 List of primers used for real-time reverse transcription polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI refSequence</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>NM_007393</td>
<td>CGCGAGCACAGCTTTTCTTT</td>
<td>GCAGCGATATCGTCATCCCAT</td>
</tr>
<tr>
<td>IL-1b</td>
<td>NM_008361.3.3</td>
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<tr>
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<td>NM_013693.2</td>
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<td>TTGCTACGACGTGGGCTACA</td>
</tr>
<tr>
<td>BDNF</td>
<td>NM_007540.4</td>
<td>AGGCACTGGAACTCGCAATG</td>
<td>AAGGGCCCGAACATACGATT</td>
</tr>
</tbody>
</table>

Results

Doxy ameliorates motor performance

Motor deficits are core manifestations in PD. Gait instability and muscle weakness significantly affect the daily living of patients who constantly experience a disabling fear of falling [31].

To evaluate the effect of Doxy treatment on gait instability and muscle weakness, NTG and A53T mice, receiving either Veh or Doxy, were tested in the beam walk and paw grip test. As shown in Fig. 1A and B, A53T+Veh-treated mice were significantly impaired in their gait stability experiencing a higher number of footslips and spending a longer time in performing the task than Veh- and Doxy-NTG animals. Notably, Doxy significantly improved the gait stability in A53T mice which performed significantly better than A53T+Veh mice. A53T+Doxy-treated mice, indeed, displayed a significant reduction of both the number of footslips and the time spent for performing the task compared to Veh-treated Tg animals. Besides an effect on mouse gait stability, Doxy treatment also significantly ameliorated muscle strength in A53T mice. In fact, A53T+Doxy-treated animals showed a longer latency in the paw grip test compared to the Veh-treated Tg group (Fig. 1C).

Doxy restores cognitive functions and daily life activity in A53T mice

PD patients also develop deficits in memory and lose their ability to execute daily life activities. To assess the effects of the Doxy treatment at cognitive level, IP treated A53T and NTG mice were tested in both NORT and nesting test starting from treatment day 27. We found that, while A53T+Veh-treated mice showed a significant memory impairment, spending an equal time investigating the novel and familiar object, Doxy completely restored memory in A53T mice which spent a longer time on the novel object rather than on the familiar one, showing both a higher % of investigation time on the novel object and a discrimination index (DI) comparable with the control condition (Fig. 2A, B). Doxy also significantly ameliorated the daily life activity measured through the nesting test in A53T mice. As shown in Figure 2C
and D, Doxy rescued the nesting behavior of A53T mice 24 and 48 hours after the nest presentation. At 48 hours the nesting score of Doxy-treated Tg mice was comparable to both NTG experimental groups.

**Doxy prevents cortical and hippocampal neuronal death in A53T mice**

Since Doxy treatment restored cognitive functions in A53T mice, we sought whether such a positive outcome was associated with a Doxy-induced neuronal protection in several brain areas involved in cognitive functions, such as the pre-frontal cortex (PFC), neo-cortex (neoCTX) and hippocampus (HP). NeuN staining of both the PFC and the neoCTX demonstrated that Doxy counteracts neuronal loss in A53T mice. Indeed, through the quantification of NeuN⁺ cell density, a slight but significant reduction was observed in the PFC and neoCTX of A53T+Veh mice, whereas a neuronal density comparable to NTG+Veh- and NTG+Doxy-treated mice was found in the same brain regions in A53T mice receiving Doxy (Fig. 3A-C).

Aside from a neuroprotective effect of Doxy in cortical regions, we found that it also promoted neuronal survival in the HP. As shown in Figure 3D, we found a significant reduction of NeuN⁺ cell density in the hippocampal CA3 subfield of A53+Veh-treated mice when compared to both Veh- and Doxy-treated NTG mice. In contrast, neuronal loss did not occur in A53T+Doxy treated animals as confirmed by the quantitative analysis of NeuN⁺ cell density (Fig. 3E).

**Doxy reduces α-syn oligomers in A53T mice**

As aforementioned α-syn deposits are typical neuropathological hallmarks of PD. Thus, through an immunohistochemical approach we investigated whether functional effects of Doxy were associated with changes in deposited α-syn. As shown in Fig 4A, we found that Doxy did not affect α-syn deposition both in the CTX and HP of A53T mice, which was confirmed through a quantitative analysis (Fig 4B and C).

Among α-syn assemblies, oligomers have been pointed out as the higher neurotoxic moieties capable of affecting neuronal survival, as well as cognitive functions and synaptic plasticity [3-6, 8, 9, 32]. To investigate whether Doxy treatment affected α-synO production in A53T mice, we performed western blotting analyses for α-syn in the Triton X-100 soluble and insoluble fraction of both CTX and HP from A53T+Veh- and A53T+Doxy-treated mice. While not significant differences were detected in the amount of α-syn monomer and oligomers in the cortical and hippocampal soluble fraction (data not shown), we found differences in the insoluble one. In particular, while both in the CTX (Fig. 4D and E) and HP (Fig 3G and H) of A53T mice Doxy did not affect the α-syn monomer protein levels, the levels of oligomeric assemblies were significantly reduced in both brain areas.

**Glial cell activation is reduced by Doxy treatment in A53T mice**

Neuroinflammation is a key factor fostering PD as well as cognitive deficits [6, 9, 10, 33-37]. α-synOs have been previously reported to trigger glial activation [32], and it is well-known that Doxy exerts pleiotropic
activities such as inhibition of α-syn aggregation and gliosis [20, 38]. To assess whether the recovery of A53T cognitive functions was linked to a Doxy-mediated reduction in neuroinflammation, we addressed the extent of glial activation in the neoCTX and HP of A53T mice. As shown in Fig. 5A and Fig. 5D, the extent of either microglial (IBA1) or astroglial (GFAP) cell activation was decreased in both area upon Doxy treatment. Indeed, in both areas A53T+Veh-treated mice displayed microglia and astrocytes with an enlarged soma, whereas in A53T mice receiving Doxy these cells showed a resting morphology with a smaller soma and a lower number of branches. To further define the extent of gliosis, we quantified the % of IBA1- and GFAP-marked area in neocortical and hippocampal area among experimental groups. In the neoCTX, Doxy completely abolished microglial cell activation, indeed the % of IBA1-marked area was significantly reduced in Doxy-treated A53T mice compared to A53T receiving Veh, and similar to either Veh- or Doxy-treated NTG mice (Fig. 5B). Similar to microglial activation, the quantitative analysis of the % of GFAP-marked area was significantly reduced in Doxy treated A53T mice compared to Veh-treated Tg animals. Moreover, Veh-treated A53T mice had a larger neocortical area occupied by GFAP+ cells compared to Veh and Doxy treated NTG mice (Fig. 5C).

We also examined the degree of gliosis in HP, a crucial brain region involved both in long term memory and daily life activity [39, 40]. As shown in Fig. 4E Doxy treatment significantly reduced the % of IBA1-marked area in A53T+Doxy mice compared to A53T+Veh ones, which had a significantly higher % of IBA1-marked area than Veh- or Doxy-treated NTG mice (Fig. 5E).

Our data also highlight a inhibitory effect of Doxy on astroglial cells in the HP of A53T mice (Fig. 4F). In fact, while the hippocampal area occupied by GFAP+ cells was significantly larger in A53T+Veh-treated mice, compared to either untreated or treated NTG mice, it was significantly smaller in Doxy-treated A53T mice.

Striatal inflammation commonly occurs in PD [41] and it has been also associated with motivation and motor impairment [42]. Thus, we assessed gliosis in the striatum of either Veh or Doxy-treated NTG and A53T mice. While microglial activation was significantly hampered in A53T+Doxy-treated mice compared to Veh-treated A53T animals (Fig 5A and B), astroglial cell activation was not affected (Fig. 6A and C).

**Doxy restores hippocampal LTP in association with inhibition of proinflammatory mediators, and an increased expression of BDNF**

Synaptic plasticity, functionally assessed through the LTP, is a key neuronal mechanism underling learning and new memory formation.

To investigate whether Doxy treatment was capable of restoring synaptic plasticity in A53T mice, hippocampal LTP was measured in NTG and A53T mice after 30 days of treatment with Veh or 10 mg/Kg of Doxy. As shown in Figure 5D-F, a significant impairment of LTP in A53T mice receiving Veh was completely rescued by Doxy treatment. In A53T+Doxy-treated mice LTP amplitude was comparable to NTG receiving either Veh or Doxy (Fig 7A). Notably, the Doxy treatment did not affect LTP in NTG mice (Fig. 7A-C), further confirming the absence of potential adverse effects.
Proinflammatory cytokines and neurotrophic factors can influence neuronal functions and LTP. In particular, increased levels of IL-1β, IL-6 and TNF-α, as well as the reduced expression of the neurotrophic factor BDNF, can inhibit LTP [43-49]. Since we have found that A53T mice showed an impaired LTP and Doxy was able to restore it, we have investigated in A53T mice whether Doxy could affect the level of IL-1β, IL-6, TNF-α and BDNF in the hippocampus of Tg compared to NTG mice. Our analysis revealed that IL-1β expression (Fig. 7C) was significantly higher in A53T+Veh-treated mice compared to NTG animals receiving Veh, and that Doxy treatment significantly reduced its expression. Consistent with the results obtained for IL-1β expression in the hippocampus, we found a significantly higher expression of IL-6 and TNF-α in Veh-treated A53T mice compared to the NTG ones, and Doxy significantly dampened also their expression (Fig 7D, F).

It has been recently demonstrated that Doxy favors the expression of the neurotrophic factor BDNF [50] which is involved in memory processing as well as LTP. Thus, we assessed BDNF expression in A53T mice. As shown in Fig. 7G, while the expression of BDNF was significantly lower in A53T+Veh compared to NTG+Veh mice, in A53T mice receiving Doxy, it was comparable to the level of NTG+Veh mice.

Discussion

Neurodegenerative disorders, such as PD and Alzheimer's disease (AD), are protein-misfolding related disorders sharing common pathological mechanisms and the urgent need for efficacious therapies [6, 51]. The role played by alpha synuclein in PD has been investigated at experimental level using different form of aggregates, oligomers, protofibrils and fibrils, our investigation was focussed on soluble form of aggregates, α-synOs, that more consistently explain PD pathogenesis [6]. Alpha-synOs or amyloid-β oligomers (AβOs), represent crucial players in PD and AD pathogenesis respectively as they lead to cognitive impairment, synaptic failure, neuroinflammation and neuronal death [6, 51–53]. Moreover, recent data highlight neuroinflammation not merely as a secondary event, but as a mediator of oligomeric-induced deleterious effects, as well as a linking event between genetic predisposition and environmental factors fostering neurodegenerative disorder onset and progression [8, 29, 54] Thus, a therapeutic strategy counteracting at the same time oligomer formation and/or their detrimental actions as well as neuroinflammation, may represent a useful approach for halting and modifying PD progression as well as related disorders known as α-synucleinopathies. Since the development of new molecules and the evaluation of their therapeutic effectiveness require years, the repurposing of an already available drug in a new context may significantly accelerate its transition from the bench to the bed side. Indeed, as previously mentioned, the main advantage of Doxy is that it has a safe pharmacological profile and easily crosses the blood brain barrier as determined through pharmacokinetic studies at preclinical level [22]. In addition, its administration at sub-antimicrobial doses does not trigger the acquisition of pharmacological resistances in bacteria [15].

As reported by Lucchetti et al (2019) [22] the cerebral levels of doxycycline after a treatment with 10-100 mg/kg daily in mice are comparable with the cerebral concentration of doxycycline observed in humans after a chronically treatment of 100 mg/daily [55]. This evidence is important in terms of translational
value of our results in A53T mice since doxycycline can exerts anti-inflammatory and anti-aggregation activity without substantial side effects in humans [56].

In the present study, we have assessed the therapeutic potential of Doxy in A53T mice. The motor behavior is impaired in A53T mice and the treatment with Doxy improved gait stability and muscle strength in these mice. The positive effect was associated with an inhibitory action on microglial cell activation in the striatum as well as in the midbrain, brainstem and deep cerebellar nuclei (data not shown). The striatal astrogliosis is modestly affected by Doxy, the quantification did not show significant difference between A53T mice treated with vehicle or Doxy. Moreover, astroglial cells (GFAP+ and S100β+) seems to not been activate in the midbrain, brain stem and deep cerebellar nuclei of A53T+Veh treated mice compared to NTG+Veh treated animals (data not shown). This different sensitivity to drug treatment of microglial and astroglial activation is reminiscent of different reactivity of these glial cells to the combination of local exposure to α-syn and peripheral inflammation. In fact, while microglial cells potentiate their activation acquiring a pro-inflammatory phenotype, astroglial cells do not and acquire an atrophic-like morphology [10]. Thus suggesting that the two glial population can differentially react to a same stimulus.

Together with motor behavior improvement the Doxy treatment almost completely abolished the cognitive and daily life activity impairment in nine-month-old A53T mice; both their recognition memory performance and their ability to build a well-defined nest were significantly improved by the treatment (41, 42]. Of note, the nesting test provides an index of cognitive and daily living dysfunction in mice (57]. Our results demonstrate the beneficial effects of Doxy at functional level, and particularly on non-motor functions which impairment has been described as first cause of patient institutionalization (1].

To get more insights on neuropathological aspects underlying the positive functional outcome mediated by Doxy, we have assessed neuronal survival and α-syn pathology in A53T mice. Particularly, we focused our analyses on hippocampus and cortex since these two brain regions are strongly involved in nesting behavior as well as in recognition memory (26, 58-61]. We found that Doxy treatment leads to neuronal protection in the PFC, neoCTX and CA3 hippocampal sub-field in A53T mice. Neuroprotection was not associated with any significant reduction in α-syn deposits upon Doxy treatment.

Despite α-syn inclusions are considered as the histopathological hallmarks of PD, their role in neuronal cell death remains a matter of intense debate (62]. In latest years, researchers have focused their attention on oligomeric α-syn moieties. In fact, α-synOs are now considered as the main neurotoxic species able to induce cognitive and synaptic dysfunction, activation of the inflammatory response and neuronal death (3, 7, 10, 63, 64].

Since Doxy has been shown to lower α-syn oligomerization, we have investigated whether Doxy was reducing α-synO production in our Tg mice. Consistent with previous data (16], we confirmed the ability of Doxy to significantly decrease α-synO production both in the CTX and HP. To be noticed that in contrast to data obtained by Dominguez-Meijide and colleagues (17], we have not found an effect of Doxy on α-syn
deposits. Such a difference may be ascribable to several elements including the different animal model used and the amount of Doxy that effectively reaches the brain. A possible explanation for the lack of effect on α-syn aggregates is the limited period of Doxy exposure; similar evidence was reported also in AD mice where the reduction of Aβ plaques was observable only after a three-month treatment with Doxy (17).

To further address possible Doxy mechanisms of action, we focused our investigation on neuroinflammation. Astroglial and microglial cells are immunocompetent cells within the central nervous system. These cells have key functions in tuning and orchestrating the neuronal activities (65). When chronically over-activated as in PD, glial cells release several pro-inflammatory mediators which in turn affect neuronal functions and survival. Since α-synOs do activate glial cells, and inflammation mediates α-synO detrimental effects (10), we evaluated the extent of glial cell activation in the CTX and HP of NTG and A53T either treated or untreated mice. Our results corroborate the anti-inflammatory potential of Doxy. In fact, Doxy lowered astroglial and microglial activation in both areas of Tg mice. Moreover, further analysis of Doxy effects at functional level in NTG and A53T mice through the LTP measurement revealed that Doxy completely restored LTP which was inhibited in A53T mice receiving Veh.

Besides LTP restoration, we demonstrated that Doxy treatment in A53T mice normalizes the expression of several factors involved in the control of synaptic plasticity (43-49). Indeed, while A53T+Veh treated mice showed an increased expression of pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α, in the HP, their expression was reduced in A53T mice receiving Doxy. In association with memory and LTP recovery, we also found that Doxy significantly restored the down-regulated neurotrophic factor BDNF in the HP.

Conclusions

We demonstrated that Doxy represents a valuable therapeutic approach for treating cognitive and motor impairments and for counteracting crucial neuropathological events in A53T mice. Our results are consistent with recent data published in the field of α-synucleinopathies (16, 17) and further highlight the potential multi-target effects of Doxy that strongly encourage the repurposing of Doxy for the treatment of α-synucleinopathies

Abbreviations

PD: Parkinson’s disease
α-syn, alpha-synuclein
α-synO : alpha-synuclein oligomers
Doxy: doxycycline
**A53T mice**: transgenic mice express the A53T mutant a-syn protein

**NTG**: non transgenic

**aCSF**: artificial CSF

**neoCTX**: neo-cortex

**PFC**: prefrontal cortex

**GFAP**: glial fibrillar acidic protein

**IBA1**: ionized calcium-binding adapter molecule 1 (microglia marker)

**NeuN**: neuronal marker

**PBS**: Phosphate-buffered saline

**BDNF**: brain-derived neutrophic factor

**Declarations**

Ethics approval and consent to participate: N/A

Consent for publication: All the authors approved the publication of the paper in this form

Availability of data and materials: the datasets used and/or analysed in the current study are available from the corresponding authors on reasonable request.

Competing interests: all authors declare no competing interests

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Authors' contributions:

**PL** Experimental execution, data elaboration, conceptualization, writing original draft, **LM** Experimental execution, data elaboration, **MC** Experimental execution, data elaboration writing review & editing, **CP** Experimental execution, data elaboration, **LD** Experimental execution, data elaboration, **SL** Experimental execution, data elaboration **CB** conceptualization writing review & editing, **GF** conceptualization writing final version of the paper, supervision.

Acknowledgements N/A

**References**


34: 1412–1429.


Figures
Figure 1

Sub chronic Doxy treatment improves motor deficits in A53T mice. (A) Scatter plots and bars are the footslips (mean±SEM) of NTG+Veh (n=7), NTG+Doxy (n=6), A53T+Veh (n=6) and A53T+Doxy (n=7) treated animals in the beam walk test, (p<0.001 Kruskal-Wallis test followed by post hoc Dunn’s test, *p<0.05, ***p<0.001 and ****p<0.0001). (B) Scatter plots and bars are the time (mean±SEM) spend by NTG+Veh (n=7), NTG+Doxy (n=6), A53T+Veh (n=6) and A53T+Doxy (n=7) treated animals in the beam...
walk test (p<0.001, (p <0.001,Kruskal-Wallis test followed by Dunn's post hoc test, ***p<0.001
****p<0.0001). (C) Scatter plots and bars are the latency (mean±SEM) of NTG+Veh (n=7), NTG+Doxy
(n=6), A53T+Veh (n=6) and A53T+Doxy (n=7) treated animals in the paw-grip test (***p<0.001 Kruskal-
Wallis test followed followed by Dunn's post hoc test, *p<0.05, **p<0.01 ***p<0.001 ****p<0.0001).
**Figure 2**

**Sub chronic Doxy treatment abrogates memory and daily life activity deficits in A53T mice.** (A) Scatter plots and bars are the percentage of time (mean±SEM) spent by NTG+Veh (n=7), NTG+Doxy (n=6), A53T+Veh (n=6) and A53T+Doxy (n=7) treated animals in the exploration of the familiar object and the novel one during the NORT. (B) Scatter plots and bars of the DI (mean ± SEM) of NTG and A53T mice injected with either Veh or Doxy (****p<0.0001, Two-way ANOVA followed by Tukey's post hoc test). (C) Representative images of the nest building up by NTG and A53T mice receiving either Veh or Doxy at 1, 24 and 48 hours after cotton bulk placement. (D) Scatter plots and bars are the nesting score (mean±SEM) assessed at different time point after cotton bulk presentation in each experimental group (p<0.0001, Kruskal-Wallis test followed by Dunn's post hoc test, *p<0.05, ***p<0.001, ****p<0.0001).
Figure 3

**Figure 3**

**Doxy promotes neuronal survival in A53T mice.** (A) Representative images at 20X and 40X magnification (insets) of NeuN staining in the PFC and neoCTX of NTG and A53T treated with Veh or Doxy. (B) Plots and bars show the quantitative analysis of the percentage of NeuN\(^+\) cell density (% of NTG+Veh mean±SEM) in the PFC of each experimental group (n=6/group, *p<0.05, **p<0.01 and ****p<0.0001, Two-way ANOVA followed by Tukey’s post hoc test). (C) Plots and bars show the quantitative analysis of...
the percentage of NeuN+ cell density (% of NTG+Veh mean±SEM) in the neoCTX of each experimental group (n=6/group, ***p<0.001 and ****p<0.0001, Two-way ANOVA followed by Tukey’s post hoc test). (D) Representative images at 20X and 40X magnification (insets) of NeuN staining in the HP and CA3 subfield hippocampal region of NTG and A53T treated with Veh or Doxy. (E) Plots and bars show the quantitative analysis of the percentage of NeuN+ cell density (% of NTG+Veh mean±SEM) in the CA3 hippocampal subfield of each experimental group (n=6/group, *p<0.05 and **p<0.01, Two-way ANOVA followed by Tukey’s post hoc test).
Figure 4

Effect of Doxy treatment on α-syn deposits in A53T mice. (A) Representative images of α-syn staining in the CTX and HP of NTG and A53T mice treated either with Veh or Doxy. (B) Plots and bars show the quantification of α-syn optic density (% of NTG+Veh mean±SEM) in the CTX of each experimental group (n=6). The Two-way ANOVA finds a significant effect of genotype (****P<0.00001). (C) Plots and bars show the quantification of α-syn optic density (% of NTG+Veh mean±SEM) in the HP of each experimental group (n=6). The Two-way ANOVA finds a significant effect of genotype (****P<0.00001).
experimental group (n=6). The Two-way ANOVA finds a significant effect of genotype (**p<0.00001).

(D) Western blotting analyses of α-syn in the CTX of A53T mice receiving Veh or Doxy. (E) Plots and bars show the quantitative analyses of the optic density (% of Veh) of cortical monomeric α-syn normalized on the corresponding β-actin in A53T+Veh or +Doxy treated animals (n=5/group). (F) Plots and bars show the quantitative analyses of the optic density (% of Veh) of cortical aggregated α-syn normalized on the corresponding β-actin in A53T+Veh or +Doxy treated animals (n=5/group, *p<0.05, Student’s T-test). (G) Western blotting analyses of α-syn in the HP of A53T mice receiving Veh or Doxy. (H) Plots and bars show the quantitative analyses of the optic density (% of Veh) of hippocampal monomeric α-syn normalized on the corresponding β-actin in A53T+Veh or +Doxy treated animals. (n=5/group). (I) Plots and bars show the quantitative analyses of the optic density (% of Veh) of hippocampal aggregated α-syn normalized on the corresponding β-actin in A53T+Veh or +Doxy treated animals (n=5/group, *p<0.05, Student’s T-test).
Figure 5

Doxy treatment significantly dampened cortical and hippocampal glial activation in A53T mice. (A) Representative images (40X magnification) showing microglial (IBA1) and astroglial (GFAP) cells in the CTX of NTG and A53T mice after 1 month of either Veh or Doxy administration. (B) Scatter plots and bars show the quantitative analysis of the cortical percentage IBA1-marked area (% of NTG+Veh mean±SEM) of the different experimental groups (n=6/group, ****p<0.0001, Two-way ANOVA followed by Tukey’s post
hoc test). (C) Scatter plots and bars show the quantitative analysis of the cortical percentage GFAP-marked area (% of NTG+Veh mean±SEM) of the different experimental groups (n=6/group, **p<0.01, ***p<0.001 and ****p<0.0001, Two-way ANOVA followed by Tukey's post hoc test). (D) Representative images (10X, 20X and 40X magnification) showing microglial (IBA1) and astroglial (GFAP) cells in the HP of NTG and A53T mice after 1 month of either Veh or Doxy administration. (E) Scatter plots and bars show the quantitative analysis of the cortical percentage IBA1-marked area (% of NTG+Veh mean±SEM) of the different experimental groups (n=6/group, ***p<0.001 and ****p<0.0001, Two-way ANOVA followed by Tukey’s post hoc test). (F) Scatter plots and bars show the quantitative analysis of the cortical percentage GFAP-marked area (% of NTG+Veh mean±SEM) of the different experimental groups (n=6/group, *p<0.05, ***p<0.001 and ****p<0.0001, Two-way ANOVA followed by Tukey’s post hoc test).
**Figure 6**

**Effect of sub chronic Doxy treatment on striatal gliosis in A53T mice.** Representative images (40X magnification) showing microglial (IBA1) and astroglial (GFAP) cells in the striatum of NTG and A53T mice after 1 month of either Veh or Doxy administration. (B) Scatter plots and bars show the quantitative analysis of the striatal percentage IBA1-marked area (% of NTG+Veh mean±SEM) of the different experimental groups (n=6/group, **p<0.01 and ***p<0.001, Two-way ANOVA followed by Tukey's post
hoc test). (C) Scatter plots and bars show the quantitative analysis of the striatal percentage GFAP-marked area (% of NTG+Veh mean±SEM) of the different experimental groups (n=6/group, Two-way ANOVA found a significant effect of genotype ****P<0.0001).

Figure 7
Doxy treatment restores hippocampal LTP and the expression of inflammatory trophic factors in A53T mice. (A) LTP time course in the hippocampal CA1 subfield from Veh or Doxy treated NTG or A53T mice. Data are presented as mean±SEM of fEPSP slopes (% of baseline). The arrow indicates the time of theta-burst stimulation (TBS) delivery. (B) Representative fEPSC traces from LTP experiments pre- and post-TBS stimulation. (C) fEPSP slope 40–50 min post-TBS relative to baseline established prior TBS (nmice/slice NTG+Veh 4/5, NTG+Doxy 4/5, A53T+Veh 5/6, A53T+Doxy 5/6, ***p<0.001 and ****p<0.0001, Two-way ANOVA followed by Tukey’s post hoc test). (D) Scatter plots and bars (mean±SEM) show the hippocampal IL-1b mRNA expression normalized in NTG+Veh treated mice in each experimental group (n=6/group, **p<0.01 and p<0.001, One-way ANOVA followed by Tukey’s post hoc test). (E) Scatter plots and bars (mean±SEM) show the hippocampal IL-6 mRNA expression normalized in NTG+Veh treated mice in each experimental group (n=6/group, *p<0.05, One-way ANOVA followed by Tukey’s post hoc test). (F) Scatter plots and bars (mean±SEM) show the hippocampal TNF-a mRNA expression normalized in NTG+Veh treated mice in each experimental group (n=6/group, ****p<0.0001, One-way ANOVA followed by Tukey’s post hoc test). (G) Scatter plots and bars (mean±SEM) show the hippocampal BDNF mRNA expression normalized in NTG+Veh treated mice in each experimental group (n=6/group, p<0.05 and **p<0.01, One-way ANOVA followed by Tukey’s post hoc test).