



# Tauroursodeoxycholic Acid Improves Motor Symptoms in a Mouse Model of Parkinson's Disease

Alexandra Isabel Rosa<sup>1</sup> · Sara Duarte-Silva<sup>2,3</sup> · Anabela Silva-Fernandes<sup>2,3</sup> · Maria João Nunes<sup>1</sup> · Andreia Neves Carvalho<sup>1</sup> · Elsa Rodrigues<sup>1,4</sup> · Maria João Gama<sup>1,4</sup> · Cecília Maria Pereira Rodrigues<sup>1,4</sup> · Patrícia Maciel<sup>2,3</sup> · Margarida Castro-Caldas<sup>1,5</sup> 

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## Abstract

Parkinson's disease (PD) is characterized by severe motor symptoms, and currently there is no treatment that retards disease progression or reverses damage prior to the time of clinical diagnosis. Tauroursodeoxycholic acid (TUDCA) is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD; however, its effect in PD motor symptoms has never been addressed. In the present work, an extensive behavior analysis was performed to better characterize the MPTP model of PD and to evaluate the effects of TUDCA in the prevention/improvement of mice phenotype. MPTP induced significant alterations in general motor performance paradigms, including increased latency in the motor swimming, adhesive removal and pole tests, as well as altered gait, foot dragging, and tremors. TUDCA administration, either before or after MPTP, significantly reduced the swimming latency, improved gait quality, and decreased foot dragging. Importantly, TUDCA was also effective in the prevention of typical parkinsonian symptoms such as spontaneous activity, ability to initiate movement and tremors. Accordingly, TUDCA prevented MPTP-induced decrease of dopaminergic fibers and ATP levels, mitochondrial dysfunction and neuroinflammation. Overall, MPTP-injected mice presented motor symptoms that are aggravated throughout time, resembling human parkinsonism, whereas PD motor symptoms were absent or mild in TUDCA-treated animals, and no aggravation was observed in any parameter. The thorough demonstration of improvement of PD symptoms together with the demonstration of the pathways triggered by TUDCA supports a subsequent clinical trial in humans and future validation of the application of this bile acid in PD.

**Keywords** Parkinson's disease · MPTP · TUDCA · Behavioral tests · Neuroinflammation

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Alexandra Isabel Rosa and Sara Duarte-Silva are joint first authors.

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✉ Margarida Castro-Caldas  
mcastrocaldas@ff.ulisboa.pt

- <sup>1</sup> Research Institute for Medicines (iMed.U LISboa), Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal
- <sup>2</sup> Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, 4710-057 Braga, Portugal
- <sup>3</sup> ICVS/3B's PT Government Associate Laboratory, University of Minho, Guimarães, Braga, Portugal
- <sup>4</sup> Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal
- <sup>5</sup> Department of Life Sciences, Faculty of Science and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal

## Introduction

The cellular mechanisms underlying dopaminergic cell death in Parkinson's disease (PD) are still not fully understood, but neuroinflammation, mitochondrial dysfunction and oxidative stress are thought to contribute to dopaminergic cell loss in both familial and sporadic cases [1, 2].

Glia play a key role in the antioxidant defense, as well as in supplying molecules to support and monitor the neuronal micro-environment [3]. When activated by dying neurons, these cells are known to phagocytose cellular debris and to secrete a number of pro-inflammatory mediators [1, 4, 5], further contributing to neuronal degeneration, hence creating a vicious cycle of inflammation and cell death, which exacerbates and propagates the neurodegenerative process [6]. Indeed, glial activation plays an important role in the progression of neuronal dysfunction in PD, as reactive microglia and astrocytes, and increased levels of cytokines have been reported in PD patient's brains [7–13].

*Post-mortem* observations in PD patients have also revealed decreased activity of mitochondrial Complex I in the *Substantia nigra pars compacta* (SNpc), platelets and skeletal muscle [14–16]. The fact that mitochondrial inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that recapitulate PD pathological features [17–19], can induce glial activation and induction of pro-inflammatory cytokines [20], further reinforces the idea that mitochondrial dysfunction is involved in PD pathogenesis. Epidemiological studies revealed that approximately 90% of PD cases have a sporadic origin, and the remaining 10% are familial forms of the disease [21]. One gene responsible for monogenic PD, is *PARK2* that encodes for parkin, an E3 ubiquitin ligase that has been demonstrated to be involved in different aspects of mitochondrial turnover [22]. Mutations in parkin are the most common cause of early onset PD [21, 23]. Interestingly, wild-type parkin dysfunction also plays a pivotal role in sporadic PD, due to nitrosative and oxidative stress-induced post-translational modifications [24, 25]. Together with phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1), parkin regulates mitophagy [22]. On the other hand, parkin regulates mitochondrial biogenesis by modulating the levels of parkin interacting substrate (PARIS), a transcriptional repressor of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha) [26]. Interestingly, parkin also triggers the expression of DJ-1, a multi-functional anti-oxidative protein, via transcriptional repression of p53 [27].

There is currently no treatment that significantly retards disease progression or reverses damage prior to the time of clinical diagnosis in PD. Symptomatic therapeutic approaches currently used to restore dopamine levels and alleviate motor symptoms have associated long-term usage secondary effects [28]. Hence, there is still an unmet need for disease-modifying therapies. The fact that mitochondrial impairment and oxidative stress are early events in the progression of PD makes them important therapeutic targets and renders the pharmacological modulation of oxidative stress and the clearance of dysfunctional mitochondria a promising therapeutic approach in PD.

Tauroursodeoxycholic acid (TUDCA) is the endogenous taurine conjugate of ursodeoxycholic acid (UDCA), a hydrophilic bile acid that is approved by the U.S. Food and Drug Administration (FDA) for the treatment of certain cholestatic liver diseases. It is orally bioavailable, crosses the blood-brain barrier, and is relatively nontoxic. There is a growing body of research on the potential therapeutic effects of TUDCA on a wide variety of non-liver diseases, particularly in neurodegenerative disease models. In fact, TUDCA is neuroprotective in mouse models of Huntington's and Alzheimer's disease [29–32]. Importantly, our previous work demonstrated that TUDCA is neuroprotective in the MPTP mouse model of PD [33–35]. The actual mechanisms underlying TUDCA neuroprotection in experimental models of PD may be multiple,

including activation of the pro-survival Ser/Thr kinase Akt and anti-oxidative mechanisms dependent on nuclear factor E2-related factor 2 (Nrf2) pathway, as well as parkin-mediated mitochondrial turnover [33–35].

Surprisingly, reports on the effects of MPTP treatment on motor dysfunction are scarce. Animal performance in conventional motor tests, such as the rotarod test, are conflicting and different toxin administration protocols as well as different test variants make comparisons between studies difficult [36–38]. In addition, to the best of our knowledge, the impact of TUDCA treatment on the motor performance of MPTP-insulted mice has never been demonstrated.

Herein, an extensive behavior analysis was performed to evaluate the effects of TUDCA in the prevention/improvement of the phenotype of MPTP-treated mice, and to better characterize this model of PD. The cellular mechanisms underlying these effects were also evaluated. Our work shows that a single administration of MPTP induces long-term severe motor coordination deficits in C57BL/6 mice. Importantly, administration of TUDCA, either before or after MPTP, was effective in rescuing the MPTP-induced motor impairments. Although TUDCA dosage used in this *in vivo* experimental work may be further optimized to consistently maintain the protective effects throughout the entire experimental time course, the results suggest that TUDCA is a promising candidate for subsequent clinical studies in PD.

## Materials and Methods

### Ethics Statement

All procedures were conducted in accordance with European regulations (European Union Directive 2010/63/EU). Animal facilities and the people directly involved in animal experiments (A.I.R., S.D.S.) were certified by the Portuguese regulatory entity - Direção Geral de Alimentação e Veterinária.

All protocols were approved by the Animal Ethics Committee of the Life and Health Sciences Research Institute, University of Minho.

### Animals and Drug Administration

Twelve-week-old male C57BL/6 mice were purchased from Charles River and housed in groups of 4 or 5 animals in filter-topped polysulfone cages 267 × 207 × 140 mm (370 cm<sup>2</sup> floor area) (Tecniplast, Buguggiate, Italy), with corn cob bedding (Scobis Due, Mucedola SRL, Settimo Milanese, Italy) in a conventional animal facility. All animals were maintained under standard laboratory conditions: an artificial 12 h light/dark cycle (lights on from 8:00 to 20:00 h), with an ambient temperature of 21 ± 1 °C and a relative humidity of 50–60%; the mice were given a standard diet (4RF21, Mucedola SRL,

Settimo Milanese, Italy) and water ad libitum. Health monitoring was performed according to FELASA guidelines [39, 40] confirming the Specified Pathogens status of sentinel animals maintained in the same animal room. Humane endpoints for experiments were defined (20% reduction of the body weight, inability to reach food and water, presence of wounds in the body, dehydration), but not needed in practice, as animals did not reach these endpoints in the study period.

The acute MPTP (Sigma) paradigm (single dose, 40 mg/Kg, i.p.) was used to recapitulate sporadic PD. This model is highly accepted allowing an accurate time-dependent analysis of nigro-striatal neuroadaptive changes [41–43]. TUDCA (Sigma) dose (50 mg/Kg, i.p., for 3 days) was based on its pharmacokinetics, and on our previous experience [34, 35, 44].

Mice were sacrificed at different time-points post-MPTP injection (p.i.). For the long-term evaluation scheme, animals were divided in 4 groups:

- i) Control: mice that received vehicle (saline)
- ii) MPTP: mice treated with a single injection of MPTP
- iii) TUDCA+MPTP: mice that received daily injections of TUDCA for 3 consecutive days. At day 3, 6 h after TUDCA administration mice received MPTP injection.
- iv) MPTP+TUDCA: mice treated with MPTP followed by daily injections of TUDCA for 3 consecutive days. The first TUDCA injection (at day 1) was performed 3 h after MPTP. At this time-point MPP<sup>+</sup> levels peak in the striatum following a 40 mg/Kg single MPTP injection [43], and it is the time that we and others have demonstrated to be the onset of the active phase of MPTP-induced dopaminergic neurodegeneration [33, 43, 45].

All mice from these 4 groups were sacrificed 52 days p.i. together with controls.

Additionally, we used a short-term animal evaluation scheme also composed by 4 groups as follows:

- i) Control: mice that received vehicle (saline)
- ii) MPTP: mice treated with MPTP, and sacrificed 3 h after MPTP administration
- iii) TUDCA+MPTP: mice that received daily injections of TUDCA for 3 consecutive days, followed by MPTP administration at day 3, 6 h after the last TUDCA injection. These animals were sacrificed 3 h after MPTP administration
- iv) MPTP+TUDCA: mice administered with MPTP followed by daily injections of TUDCA for 3 days, starting at day 1, 3 h after MPTP injection. These mice were sacrificed 6 h after the last TUDCA injection.

TUDCA on its own was shown to have no impact in motor performance in the open arena and in the rotarod paradigms in rats and mice, when administered i.p. or s.c., respectively [29,

46]. Unpublished work from our labs has also shown that TUDCA given orally to mice for several weeks does not affect performance in a set of motor behavior paradigms (Duarte-Silva, unpublished). Therefore, to minimize the number of animals used, according to the principles of the 3R's, an animal group treated with TUDCA only was not included in our experiments.

After behavioral assessment, animals were deeply anesthetized with a mixture of ketamine hydrochloride (150 mg/Kg) plus medetomidine (0.3 mg/Kg) and sacrificed by exsanguination perfusion with saline. Brains were collected and separated into hemispheres: one hemisphere was destined to immunohistochemistry and was immediately embedded in OCT and frozen at  $-80^{\circ}\text{C}$  for further processing in the cryostat. The other hemisphere was dissected as previously described [45] to remove the entire midbrain region, containing the SNpc, and the whole striatum, for protein extraction. All behavioral and biochemical studies were performed by investigators blind to the treatment conditions.

## Behavioral Analysis

To register the acute effects of MPTP in C57BL/6 mice, the animals were videotaped for 5 min, 90 min after the MPTP injection. Two independent experimenters analyzed all the videos. Analyzed parameters included palpebral closure, salivation, fur erection, tail erection, resting time, spontaneous activity and vertical exploratory activity.

Mouse behavior assessment was performed at 7, 30 and 45 days p.i.. Body weight was registered in all time-points analyzed. Since the rotarod protocol may cause fatigue to the animals, this test and other phenotypic assessments were performed in alternate weeks.

## Motor Swimming Test

To analyze voluntary locomotion, mice were trained for 2 consecutive days (3 trials each animal) to cross a clear perspex water tank to a safe platform at the end, as previously described [47, 48].

The perspex tank was 100 cm long and the platform at the end was made from black perspex. The latency to cross the water tank was measured from a distance of 60 cm (the tank was labeled with a blue line to mark the initiation). The water temperature was monitored to  $23^{\circ}\text{C}$  using a thermostat. Mice were tested for 3 consecutive days (2 trials per animal) and the latency to traverse the tank was registered by the experimenter.

## Adhesive Removal

A round adhesive (8 mm diameter) was placed on the mouse snout and the time the animal took to remove it was registered.

Time values were measured in 3 consecutive days starting at day 7, 30 and 45 p.i.

### Pole Test

Mice were placed facing up on top of a pole (37.5 cm long) located in their cages. The time the animals took to turn down and to reach the cage was registered. If the animal fell or did not turn down after 5 min, this was considered one failed trial. Time values were registered in 3 consecutive days starting at day 7, 30 and 45 p.i.

### Beam Walk Balance Test

This test was performed to assess balance and fine motor coordination, as previously described [47, 48]. The animals were trained during 3 days in the square beam (12 mm). In the fourth day, they were tested in the training beam and also in one round beam (17 mm). If the animal fell or turned around in the beam, this was considered one failed trial. Each animal had the opportunity to fail twice in each beam. The time the animal took to cross the beam was counted, and time was discounted if the animal stopped in the beam.

### Rotarod

Mice were tested in a rotarod apparatus specific for mice (TSE systems, Bad Homburg, Germany) to evaluate their motor performance. The animals were subjected to 4 trials in 4 consecutive days on an acceleration rod (4–40 rpm, for a maximum of 5 min) with a 20-min-long interval between each trial and the latency to fall was registered automatically by the software.

### SHIRPA

The protocol for phenotypic assessment used in this study was based on the primary screen of the SHIRPA protocol, which mimics the diagnostic process of general neurological and psychiatric examination in humans [49].

### Footprint Analysis

The footprint pattern was obtained to assess gait as described previously [47, 48]. To evaluate the severity of foot dragging, the footprinting pattern was classified at each time-point considering six consecutive steps ( $0 = \text{absent/mild}$ , dragging evident in up to three steps;  $1 = \text{mild}$ , dragging in more than three steps out of six;  $2 = \text{severe}$ , dragging in all six steps). The stride length was measured manually as the distance between two paw prints. Three measurements were obtained in consecutive steps.

## Immunohistochemistry

Immunohistochemistry was performed using standard protocols. Briefly, cryostat coronal slices at the level of the midstriatum (Bregma 1.00) and SNpc (Bregma  $-3.20$ ) were fixed in 4% PFA for 30 min and rinsed in PBS twice for 5 min.

Tyrosine-Hydroxylase (TH) detection was performed using the protocol described previously [50]. TH-positive fiber density was quantified in the striatum ( $n = 3$  for each condition, 2–4 slides per animal). Digital slides were obtained using the high-resolution scanner NanoZoomer S60 (Hamamatsu, Japan), allowing whole slide imaging (objective lens 20 $\times$ ). After digital slide acquisition, TH-positive fiber densitometry was determined using ImageJ software (integrated density) (National Institutes of Health, USA) and normalized for total area.

For detection of glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba-1), after fixation, slices were processed as previously described [33]. Fluorescence images of 10–15 random microscopic fields were acquired per sample, under 400 $\times$  magnification, using a fluorescence microscope (model AxioScope.A1) with integrated camera AxioCam HR (Carl Zeiss, Inc). The results were expressed as the percentage of total area, using the ImageJ software analysis (National Institutes of Health).

Control experiments for non-specific binding were performed in parallel by omission of the primary antibody.

Information regarding the antibodies used is provided in Supplementary Table I.

### Western Blot Analysis

Protein extracts were prepared from striatum and midbrain fragments as described previously [35]. Briefly, brain fragments were homogenized in PBS supplemented with Complete Mini Protease Inhibitor Cocktail (Roche) using a tissue grinder and centrifuged at 3000 r.p.m. for 10 min, at 4 °C. The supernatant was discarded, and the pellet suspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin) supplemented with Complete Mini Protease Inhibitor Cocktail, 200 mM Na<sub>3</sub>VO<sub>4</sub> and 1 M NaF, and further incubated 30 min on ice. After 6 times sonication for 5 s, samples were centrifuged at 13000 r.p.m. for 15 min, at 4 °C, and the supernatants collected and stored at  $-80$  °C. Protein concentration was determined using the Bradford method according to the manufacturer instructions. Brain tissue extracts were added (5:1) to denaturing buffer (0.25 mM Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 0.2% bromophenol blue, 1%  $\beta$ -mercaptoethanol), and boiled for 5 min. Brain tissue extracts were subjected to SDS-PAGE gels and electrotransferred to Immobilon P membranes (Millipore, Bedford, MA, USA). Immunoblot was performed using

specific primary antibodies indicated in Supplementary Table 1. The immunocomplexes were visualized by chemiluminescent detection with ECL Western blotting detection reagent or SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) in a ChemiDoc™ MP imaging system from Bio-Rad Laboratories (Hercules, CA, USA).  $\beta$ -actin or GAPDH were used as a loading control. The relative intensities of protein bands were analyzed using the Image Lab™ analysis software from Bio-Rad Laboratories.

### Measurement of ATP Levels

ATP levels were assessed with the ATP-Glo™ Bioluminometric Cell Viability Assay (Biotium, Hayward, CA, USA), according to the manufacturer's instructions. Briefly, midbrain protein extracts were assayed for luciferase activity in a luminometer (Berthold Systems), and results were normalized to total protein content as previously described [35]. Each condition was assayed in triplicate.

### Statistical Analysis

The experimental unit used in this study was a single animal. Continuous variables with normal distributions (Kolmogorov-Smirnov (K-S) test  $p > 0.05$ ) were analyzed with the two-way ANOVA repeated measures (Factors: time-point and treatment). Behavioral data were subjected to the nonparametric Mann-Whitney  $U$  test or pair wise Kruskal-Wallis when variables were non-continuous or when a continuous variable did not present a normal distribution (K-S test  $p < 0.05$ ). Categorical variables in the SHIRPA protocol were analyzed by contingency tables (Fisher's exact test). All statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL).

IHC, WB analysis and ATP measurement data are expressed as mean  $\pm$  SEM values. Data were analyzed by one-way ANOVA and differences between groups were determined by post hoc Bonferroni's test, using GraphPad Prism 5.0 (San Diego, CA, USA).

A critical value for significance of  $p < 0.05$  was used throughout the study.

## Results

### Mouse Welfare Assessment During the Acute Phase of MPTP Toxicity

Mice treated with saline (vehicle), MPTP or TUDCA before MPTP injection were videotaped for 5 min, 90 min after MPTP injection (during the acute phase of MPTP toxicity), and were evaluated for different welfare parameters.

We observed no differences in palpebral closure or salivation between the groups (Fig. 1a, b). However, around 30% of the MPTP-injected animals exhibited an elevated tail, i.e. a "Straub tail" phenomenon ( $p = 0.051$ , Fig. 1c). Fur erection (Fig. 1d) was found in all animals treated with MPTP in the presence or absence of TUDCA ( $p < 0.001$ ). During this initial assessment, MPTP-injected animals were markedly less active than vehicle-treated animals spending more time immobile ( $p < 0.001$ , Fig. 1e), less time moving spontaneously ( $p < 0.001$ , Fig. 1f) and less time exploring (given by the rearing natural behavior,  $p < 0.001$ , Fig. 1g). Pre-treatment with TUDCA did not prevent these acute effects of MPTP, except for the Straub tail. Interestingly, animals treated with TUDCA prior to MPTP administration showed absence of this phenotype, and were similar to controls (Fig. 1c).

### Motor Coordination and Gait Characterization

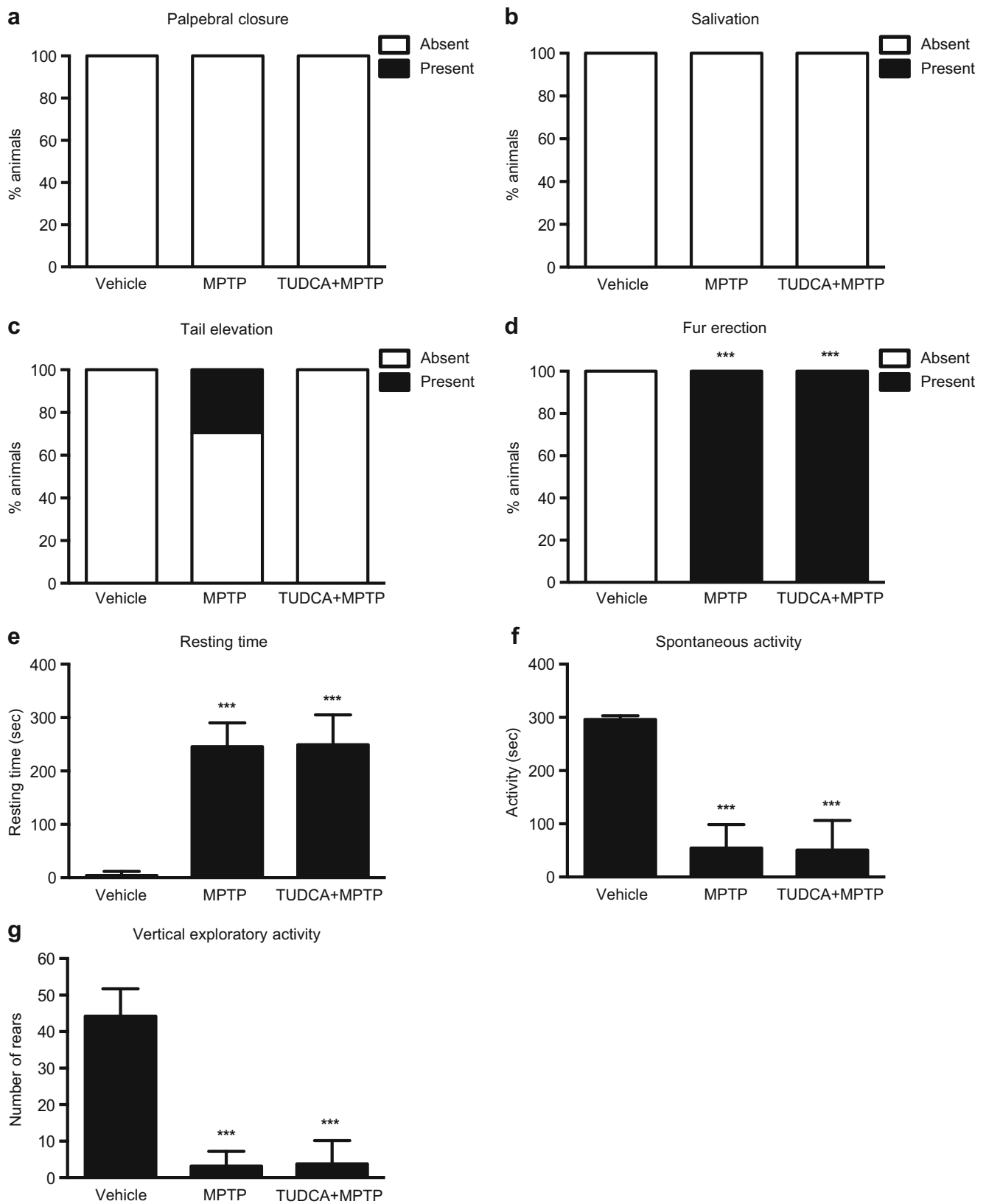
As expected, mice gradually increased their body weight with age and there were no differences in weight variation between groups (Supplementary Fig. 1a).

Motor coordination was assessed using the motor swimming test. Administration of a single dose of MPTP significantly increased swimming latency of the animals throughout the time course of the experiment ( $p < 0.001$ , Fig. 2a). The swimming pattern of these animals was also clearly altered: while vehicle-treated mice presented a straight-forward pattern, the MPTP-treated mice presented a more angled pattern (zigzag swimming), touched the pool wall more frequently and used their tails to aid the swimming task (Supplementary movies). Notably, when animals were treated with TUDCA before or after MPTP, the latency to cross the pool and the swimming pattern were similar to control and significantly different from MPTP-treated mice ( $p < 0.001$  vs MPTP, Fig. 2a).

MPTP-injected animals showed abnormal gait quality (qualitative assessment) 30 days p.i. ( $p < 0.05$ , Fig. 2b), an effect that was prevented by TUDCA treatment prior to or after MPTP ( $p < 0.05$  vs MPTP, Fig. 2b). However, the effect of MPTP in gait quality seems to be transient since the observed gait abnormalities were not maintained 45 days p.i. (Fig. 2b).

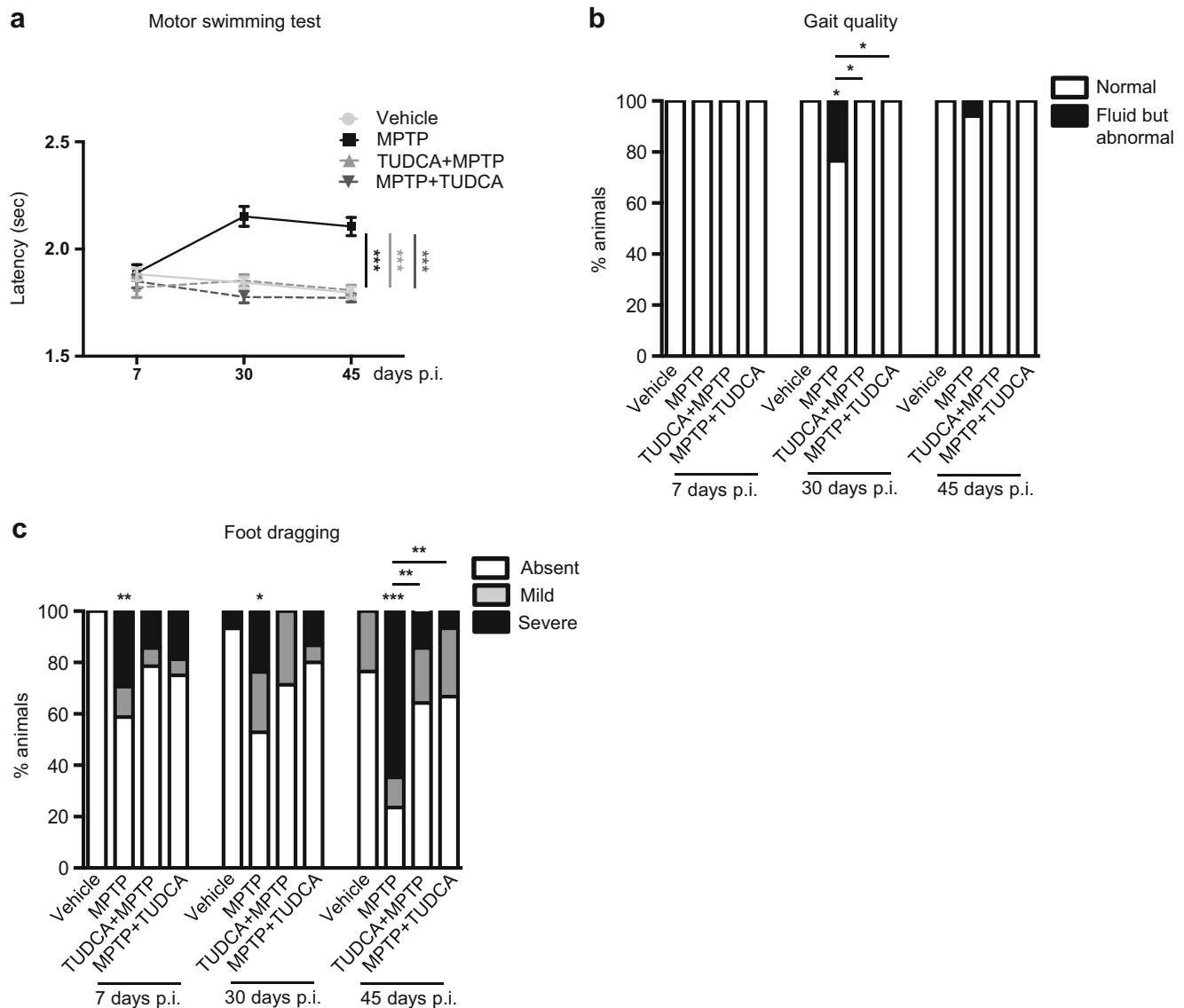
Foot-dragging was detected in mice treated with MPTP at days 7, 30 and 45 after MPTP injection, ( $p < 0.01$ , 0.05 and 0.001, respectively, Fig. 2c). Interestingly, this phenotype was prevented in animals treated with TUDCA before or after MPTP, particularly at 45 days p.i. ( $p < 0.01$  vs MPTP, Fig. 2c). Importantly, while animals exposed to MPTP showed significant symptom aggravation, TUDCA treatment was able to improve this phenotype over time.

No differences were found between animal groups in the square (12 mm) and in the round (11 mm) beam test (balance evaluation), nor in the rotarod test or in the stride length determination (Supplementary Fig. 1b-e).



**Fig. 1** Initial mouse welfare assessment. Ninety min after MPTP injection, mice from vehicle, MPTP and TUDCA plus MPTP groups were videotaped for 5 min and evaluated for several parameters as follows: **a** Palpebral closure, **b** salivation, **c** tail elevation, **d** fur erection,

**e** resting time, **f** spontaneous activity, and **g** vertical exploratory activity.  $n = 14\text{--}17$  for each group. Data are presented as mean  $\pm$  SD of the different groups.  $***p < 0.001$  using the Mann-Whitney test



**Fig. 2** TUDCA improves the motor performance of MPTP-treated mice. Evaluation of mice motor performance using **a** the motor swimming test, **b** gait quality (qualitative assessment), and **c** the foot dragging phenotype.  $n = 14\text{--}17$  for each group used. Data are presented as mean  $\pm$  SD of the

different groups. **a**  $***p < 0.001$  using repeated measures ANOVA considering time-point and treatment factors. **b, c** \*, \*\*, and  $*** p < 0.05$ , 0.01, and 0.001, respectively, using the Mann-Whitney test. p.i. - days post-MPTP injection

### Evaluation of Spontaneous Activity

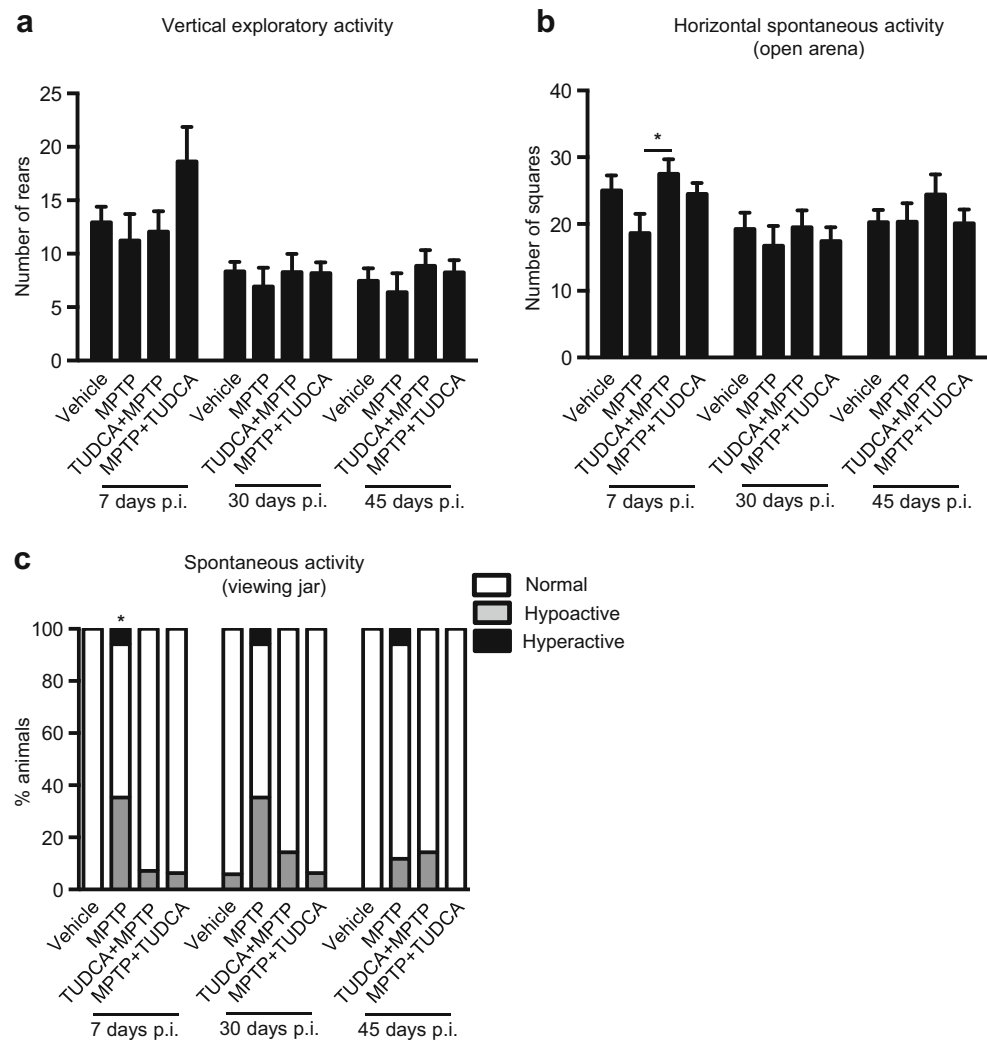
Spontaneous activity was characterized in terms of vertical movements counted while animals explored a cylinder viewing jar (rears), in terms of horizontal movement in an arena (number of squares crossed) and qualitatively (scored by the experimenter as normal, hypoactive and hyperactive). Results presented in Fig. 3a indicate that rearing behavior did not differ between animal groups during the duration of this trial. Accordingly, horizontal activity was also not significantly altered by MPTP administration. However, 7 days p.i. horizontal activity tended to be reduced in MPTP-treated mice as compared to vehicle-treated, and animals treated with TUDCA before MPTP exhibited an improved phenotype

compared to MPTP-treated mice ( $p < 0.05$  vs MPTP, Fig. 3b). Regarding qualitative assessment of spontaneous activity (Fig. 3c), mice injected with MPTP showed a significant effect 7 days p.i., and a mild effect in the subsequent time-points evaluated ( $p < 0.05$ ). Importantly, MPTP-induced hyperactivity was prevented by TUDCA administration either before or after MPTP.

### Evaluation of the Ability to Initiate Movement, Tremors, Tail Position and Hindlimb Clasping

To evaluate bradykinesia in mice we determined the ability of animals to initiate movement by using the adhesive removal test. As expected, MPTP-injected animals were significantly

**Fig. 3** Evaluation of mice exploratory activity. **a** Vertical exploratory activity was determined by the number of vertical movements (rears). **b** Horizontal spontaneous activity was determined by the number of squares travelled in an open arena for 1 min. **c** Spontaneous activity was scored by the experimenter as normal, hypoactive and hyperactive.  $n = 14\text{--}17$  for each group. Data are presented as mean  $\pm$  SD of the different groups.  $*p < 0.05$  using the Mann-Whitney test. p.i. - days post-MPTP injection



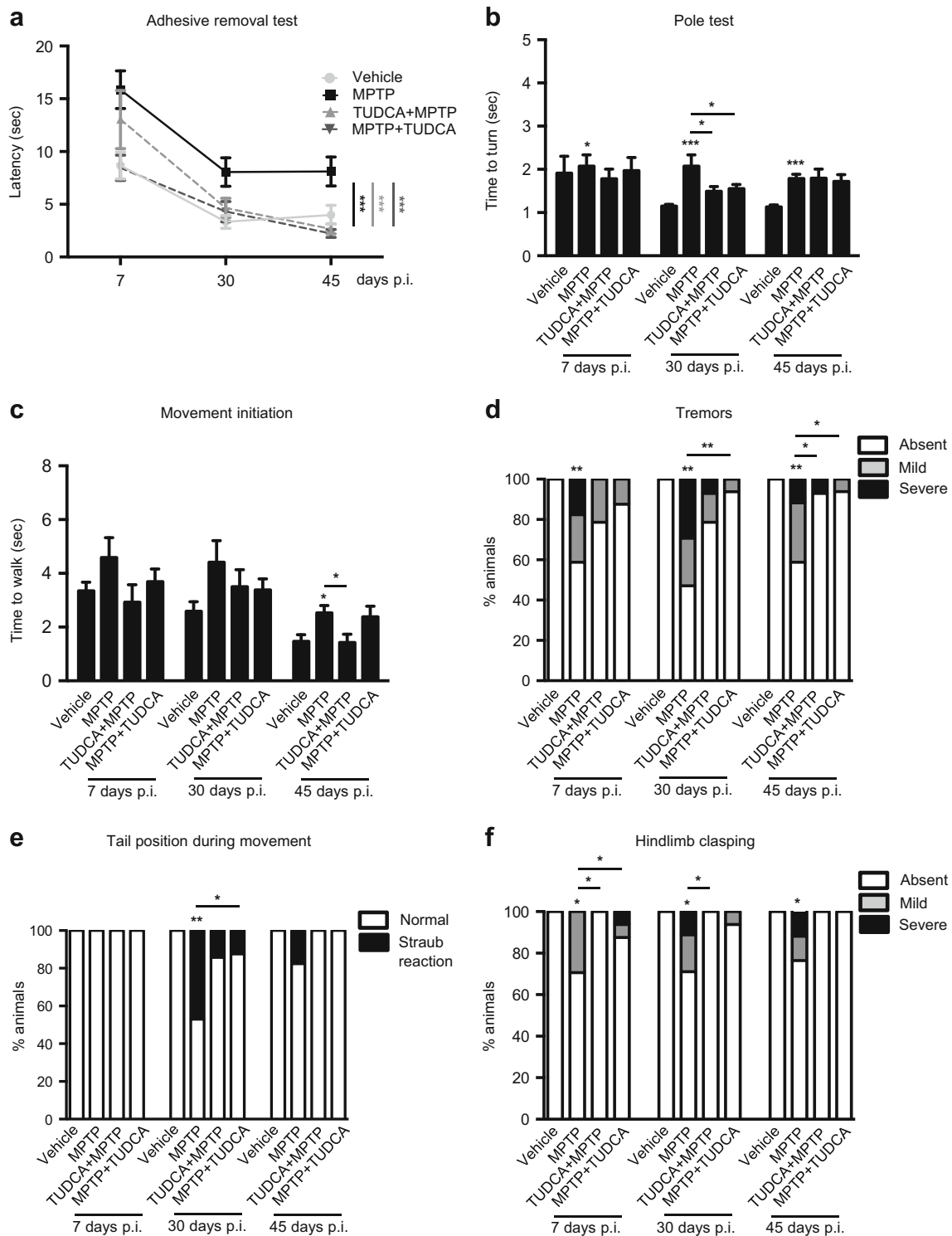
slower to remove the adhesive that was placed on their snouts than vehicle-treated mice at 45 days p.i. ( $p < 0.001$ , Fig. 4a). Relevantly, at that time-point TUDCA treatment ameliorated mice ability to remove the adhesive, when administered before or after MPTP ( $p < 0.001$  vs MPTP). The ability to initiate movement was also evaluated by the pole test. MPTP administration increased mice latency to turn downward at all evaluated time-points (7 days,  $p < 0.05$ ; 30 and 45 days,  $p < 0.001$ , Fig. 4b). In contrast, TUDCA administration, prior to or after MPTP, improved this phenotype, particularly at 30 days p.i. ( $p < 0.05$  vs MPTP, Fig. 4b). However, the time the animals took to reach the cage in the pole test was similar in all groups (Supplementary Fig. 2). Forty-five days p.i., the latency to initiate movement in the arena was significantly increased in MPTP-injected animals as compared to the vehicle-injected group. Importantly, TUDCA treatment prior to MPTP administration was able to significantly improve this phenotype ( $p < 0.05$ , Fig. 4c). Together, our data indicate that MPTP-treated mice have an aberrant phenotype regarding

movement initiation that was corrected by TUDCA administration either before or after exposure to the neurotoxin.

Besides bradykinesia, tremors are also a key feature of PD [51]. Therefore, we have evaluated this symptom in MPTP-treated mice. MPTP-injected animals presented tremors since 7 days p.i., that were sustained and increasingly more severe throughout the course of the experiments ( $p < 0.01$ , Fig. 4d). Importantly, TUDCA was able to ameliorate this symptom at 30 days when administered after MPTP ( $p < 0.01$  vs MPTP, Fig. 4d), and at 45 days when administered either before or after MPTP ( $p < 0.05$  vs MPTP, Fig. 4d). Interestingly, mice treated with TUDCA after MPTP administration only showed mild tremors that ameliorated over time.

In addition, 30 days after injection with MPTP, a Straub tail reaction was found in 50% of the MPTP-injected mice ( $p < 0.01$ , Fig. 4e). This phenotype was significantly less frequent in animals that were treated with TUDCA after MPTP ( $p < 0.05$  vs MPTP, Fig. 4e). Importantly, although animals from the 3 experimental groups showed a transient symptom





**Fig. 4** TUDCA increases the ability of MPTP-treated animals to initiate movement and ameliorates tremors, “Straub tail” reaction and hindlimb claspings. Capability of movement initiation was evaluated using the **a** adhesive removal test, in which the period of time each animal took to remove an adhesive placed on its snout was measured; **b** the Pole test, which consists in the evaluation of the latency to turn downwards; and **c**

the latency of each animal to initialize movement in the arena. Evaluation of **d** tremors, **e** Straub tail reaction, and **f** hindlimb claspings.  $n = 14-17$  for each group. Data are presented as mean  $\pm$  SD of the different groups. **a**  $***p < 0.001$  using repeated measures ANOVA considering age and treatment factors. **b-f** \*, \*\*, and  $***p < 0.05, 0.01, \text{ and } 0.001$ , respectively, using the Mann-Whitney test. p.i. - days post-MPTP injection

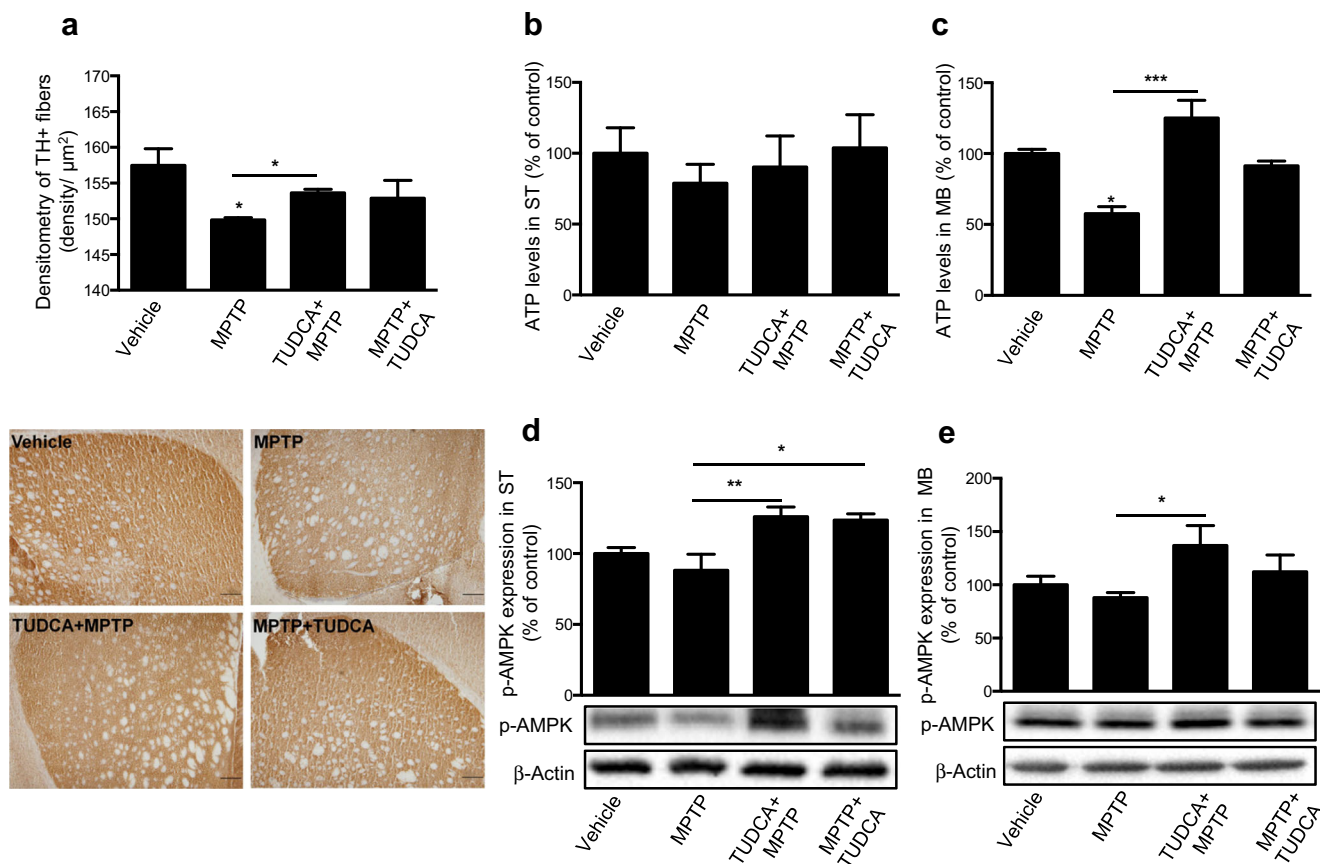
aggravation 30 days p.i., at 45 days a Straub tail reaction was only exhibited in animals that were injected with MPTP.

Hindlimb claspings was also evaluated as a measure of basal ganglia dysfunction [52]. MPTP-treated mice exhibit significant hindlimb claspings from 7 days p.i. onwards, with symptom aggravation from 7 to 30 days p.i. ( $p < 0.05$ , Fig. 4f). On the other hand, although mice treated with TUDCA after MPTP administration showed mild and severe limb claspings 7 days p.i., this symptom improved over time and was no longer detected at 45 days post the neurotoxic insult. Importantly, hindlimb claspings was never observed when animals were pre-treated with TUDCA, at any time-points evaluated. In fact, TUDCA improved this phenotype 7 days p.i. when administered before or after the neurotoxin ( $p < 0.05$  vs MPTP, Fig. 4f), and 30 days p.i. when administered before MPTP ( $p < 0.05$  vs MPTP, Fig. 4f). Other parameters included in the SHIRPA protocol were evaluated and no differences were found between experimental groups (listed in Supplementary Table II).

## Neuroprotective Effect of TUDCA Against MPTP-Induced Neurodegeneration

To evaluate dopaminergic neuron degeneration, mouse coronal brain slices at the level of the midstriatum were subjected to immunohistochemistry against TH (Fig. 5a). In accordance with the behavioral data, densitometry analysis of TH-positive fibers showed a significant reduction in MPTP-treated mice striatum ( $p < 0.05$ ), which was prevented by TUDCA when administered prior to MPTP ( $p < 0.05$ , Fig. 5a). These results also show that in animals treated with TUDCA after MPTP, and in spite of some beneficial effects at the behavior level, TH-positive fiber density was not significantly different from MPTP-injected mice.

To better characterize the degenerative effects triggered by MPTP in this work, we determined ATP levels in mouse brain extracts. In the striatum, despite the fact that there was a clear tendency for ATP levels to be decreased in MPTP-treated



**Fig. 5** Neuroprotective effect of TUDCA against MPTP-induced neurodegeneration. **a** Microscopy images of TH-positive fibers in the striatum from vehicle, MPTP, TUDCA prior to MPTP or TUDCA after MPTP-treated mice, sacrificed 52 days p.i. Scale bar = 200  $\mu\text{m}$ . TH-positive fibers densitometry was determined with the ImageJ software and normalized for total area. \* $p < 0.05$  using the Mann-Whitney test. ATP levels were quantified in mouse striatum (**b**) and midbrain (**c**) extracts. Levels of p-AMPK were determined in the striatum (**d**) and midbrain (**e**) by

Western blot analysis. Representative p-AMPK immunoblots and relative band intensity are shown.  $\beta$ -Actin was used as loading control. Relative band intensity was quantified using the Image Lab™ software and expressed as percentage of control. Data represent mean values  $\pm$  SEM of at least three independent experiments, indicated as percentage of control. \*, \*\*, and \*\*\*  $p < 0.05$ , 0.01, and 0.001, respectively, using one-way ANOVA with Bonferroni's post hoc test

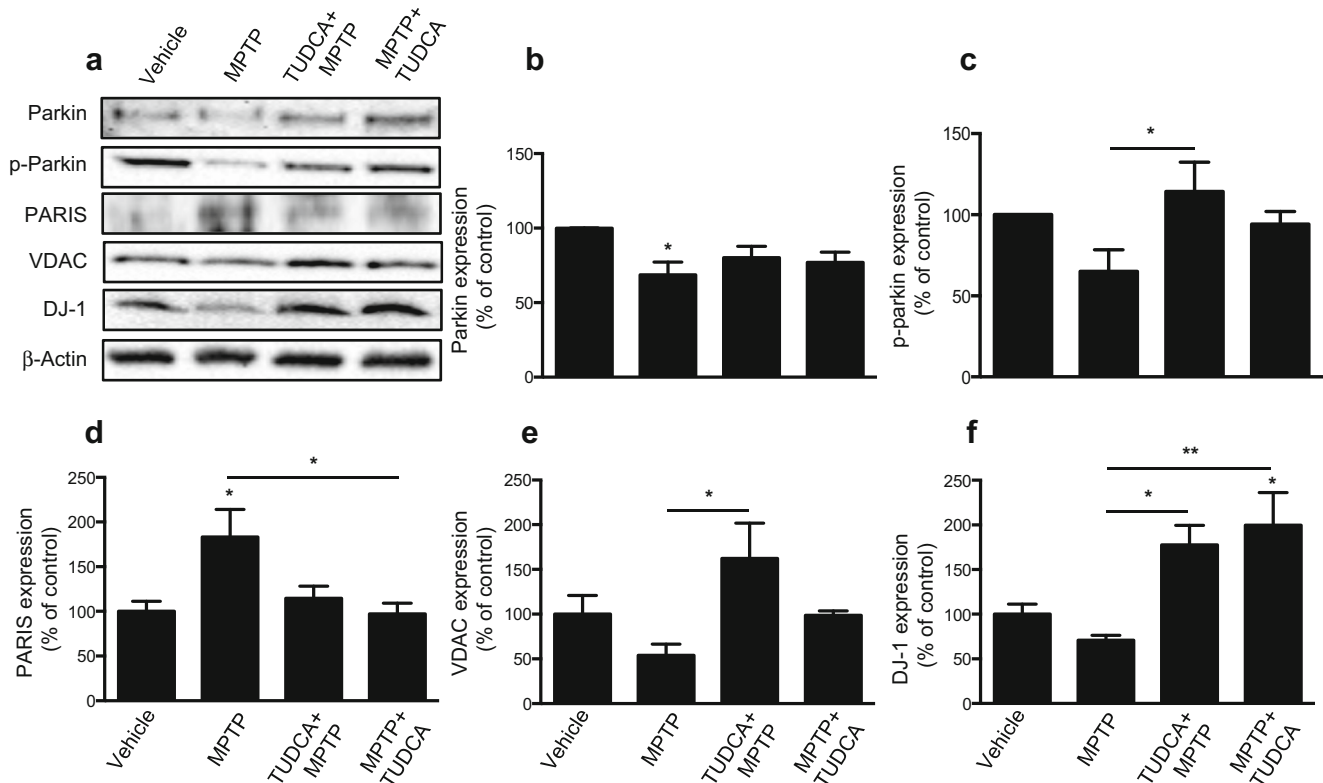
mice, no differences were found between groups (Fig. 5b). However, in the midbrain, MPTP-treated mice showed a significant decrease of ATP levels as compared to vehicle-treated animals ( $p < 0.05$ , Fig. 5c). Importantly, pre-treatment with TUDCA prevented the decrease of ATP found in MPTP-treated animals ( $p < 0.05$ , Fig. 5c). Although mice that were treated with TUDCA after MPTP showed ATP levels similar to the ones found in controls (Fig. 5c), no significant differences were detected between MPTP and MPTP+TUDCA groups.

To further evaluate the protective role of TUDCA on ATP depletion induced by MPTP, we evaluated the phosphorylated levels of the energy sensor AMPK. Strikingly, results show that in the striatum of TUDCA-treated animals the levels of activated AMPK (p-AMPK) were significantly higher than in MPTP-treated mice (TUDCA prior to MPTP:  $p < 0.01$ ; TUDCA after MPTP:  $p < 0.05$  Fig. 5d). Accordingly, in the midbrain extracts from animals treated with TUDCA before MPTP the levels of p-AMPK were also significantly higher than in MPTP-treated mice ( $p < 0.05$ , Fig. 5e).

Altogether these results suggest that TUDCA prevents MPTP-induced dopaminergic neurodegeneration.

## Effect of TUDCA on Mitochondrial Homeostasis-Associated Proteins in MPTP-Treated Mice

We have previously shown that at early time-points after MPTP injection (3 and 6 h), pre-treatment with TUDCA promotes mitophagy via the E3 protein-ubiquitin ligase parkin [35]. Thus, to evaluate the effect of TUDCA on mitochondrial homeostasis in the striatum of animals subjected to long-term MPTP treatment, the levels of parkin were determined by Western blot. Results presented in Fig. 6 show that total parkin levels significantly decrease in the striatum of MPTP-treated animals ( $p < 0.05$ , Fig. 6a and b). Phosphorylated parkin (Ser65) levels tend to decrease in MPTP-treated animals, and are partially reverted in TUDCA-treated animals, despite the fact that this increase is only significant in animals treated with TUDCA before MPTP ( $p < 0.05$  vs MPTP, Fig. 6a and c). As expected, the levels of PARIS, a parkin target that inhibits mitochondrial biogenesis [26], were increased in the striatum from MPTP-injected mice ( $p < 0.05$ , Fig. 6a and d). Importantly, TUDCA treatment reverted the increase of PARIS expression levels triggered by MPTP injection ( $p < 0.05$  vs MPTP, Fig. 6a and d). We next evaluated voltage-



**Fig. 6** TUDCA modulates the expression of mitochondrial homeostasis proteins in mouse striatum. Representative immunoblots (a) and respective quantification of parkin (b), p-parkin (c), PARIS (d), VDAC (e), and DJ-1 (f) are shown for striatum extracts. Mice were treated with vehicle, MPTP, TUDCA prior to MPTP or TUDCA after MPTP, and were sacrificed 52 days p.i.  $\beta$ -Actin was used as loading control.

Relative band intensity was quantified using the Image Lab™ software and expressed as percentage of control. Data represent mean values  $\pm$  SEM of at least three independent experiments, indicated as percentage of control. \* and \*\*  $p < 0.05$  and  $0.01$ , respectively, using one-way ANOVA with Bonferroni's post hoc test

dependent anion channel (VDAC) expression as a readout of the mitochondrial pool. Results presented in Fig. 6a and e show that in animals treated with TUDCA before MPTP administration, the expression levels of VDAC were significantly higher as compared with MPTP-injected animals ( $p < 0.05$  vs MPTP, Fig. 6a and e). In accordance, DJ-1 levels were significantly increased in the striatum of animals treated with TUDCA after MPTP administration, comparing to vehicle-treated mice ( $p < 0.05$ , Fig. 6a and f). Furthermore, the levels of this protein were also significantly increased in the striatum of animals treated with TUDCA before or after MPTP administration, comparing to MPTP-treated mice ( $p < 0.05$  and  $p < 0.01$ ).

Altogether these results indicate that TUDCA treatment affects mitochondrial homeostasis proteins in the striatum in long-term MPTP-intoxication, suggesting that this is one of the mechanisms underlying the neuroprotective role of this bile acid in PD.

### Role of TUDCA on MPTP-Induced Glial Activation and Neuroinflammation

Glial activation in striatum and SNpc was evaluated by determination of GFAP or Iba-1 expression levels, markers for astro- and microgliosis, respectively. In the striatum and midbrain from MPTP-treated mice, GFAP expression levels were significantly increased ( $p < 0.05$  (striatum) and  $p < 0.001$  (midbrain), Fig. 7a). Importantly, astrocyte activation was completely prevented in animals treated with TUDCA prior to MPTP in the striatum ( $p < 0.01$  vs MPTP), and in animals treated with TUDCA prior to or after MPTP in the midbrain ( $p < 0.05$  vs MPTP). Accordingly, results from Fig. 7b show that Iba-1 expression levels were significantly higher in striatum and midbrain from MPTP-treated mice ( $p < 0.001$ ). Once again, pre- and post-treatment with TUDCA completely prevented MPTP-induced Iba-1 expression ( $p < 0.001$  vs MPTP).

To better assess the role of MPTP and TUDCA on neuroinflammation, the levels of the cytokine interleukin-1 $\beta$  (IL-1 $\beta$ ) and of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) were also determined in the striatum. Previous studies have described that the expression of inflammatory mediators occurs short after MPTP administration [53–55]. Therefore, here we used striatal samples from animals treated with MPTP for 3 h in the presence or absence of TUDCA (short-term evaluation scheme). As expected, our results show that MPTP treatment leads to a rapid and significant increase of both IL-1 $\beta$  and COX-2 protein levels. Importantly, administration of TUDCA before and after MPTP prevented the up-regulation of these pro-inflammatory agents ( $p < 0.01$  vs MPTP, Fig. 8a–c). On the other hand, the expression levels of Annexin-A1 (ANXA1), an anti-inflammatory protein [56], were significantly decreased in MPTP-treated animals ( $p < 0.01$ , Fig. 8d). Importantly, mice that received TUDCA

prior to or after MPTP injection exhibited striatal ANXA1 expression that did not differ from vehicle-treated mice (Fig. 8d), indicating that TUDCA prevents the MPTP-induced decrease of ANXA1 levels.

Together these results show that MPTP induces a rapid pro-inflammatory state, which is maintained throughout time, in both striatum and midbrain, and is efficiently prevented when animals are treated with TUDCA either before or after the neurotoxin.

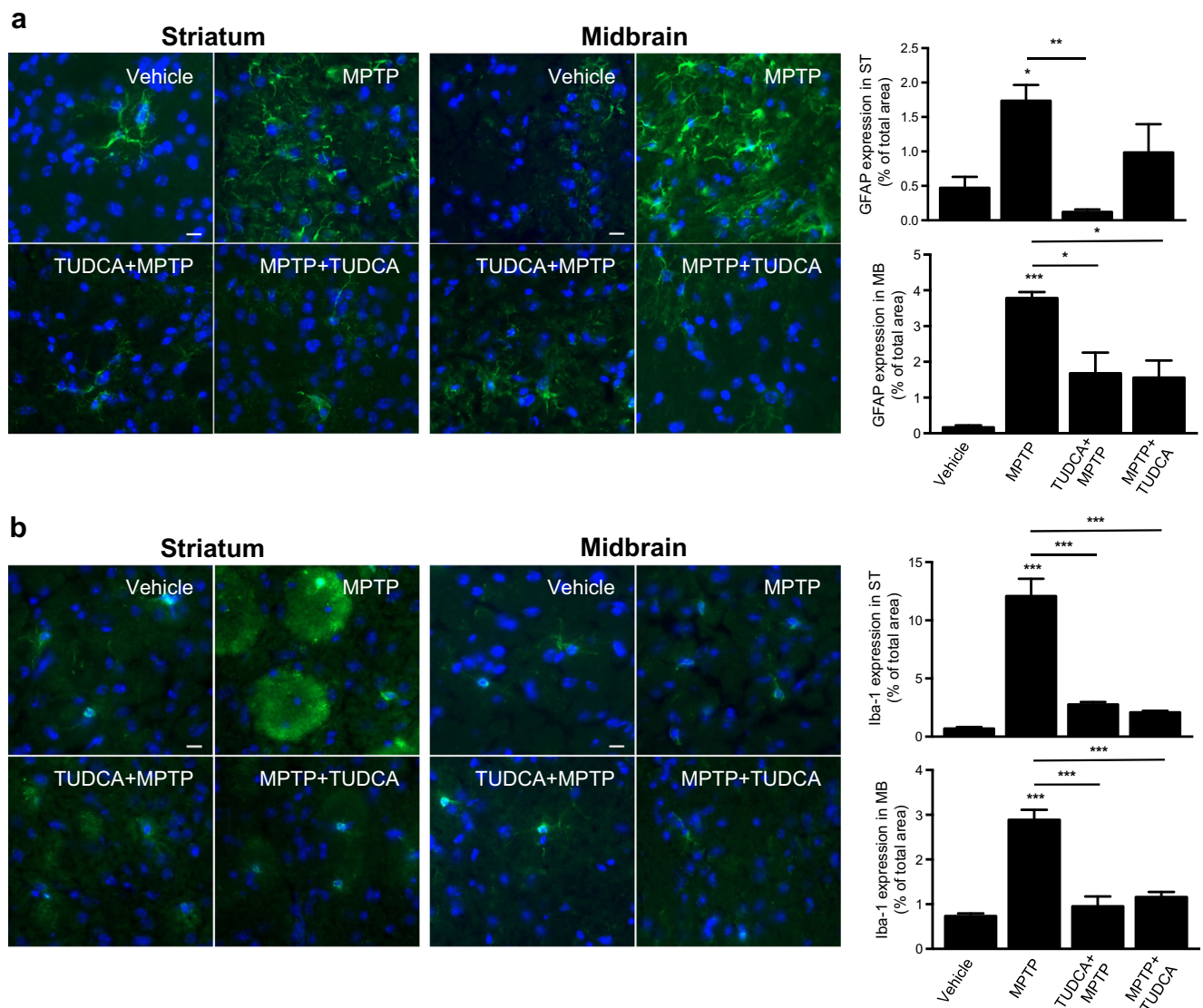
## Discussion

Using the in vivo MPTP mouse model of PD, we evaluated whether treatment with the bile acid TUDCA was able to alleviate motor symptoms, and investigated the possible mechanisms underlying its neuroprotective effects.

The first objective of this study was to extensively characterize motor behavior impairment in MPTP-injected mice. Here we show that even an acute MPTP administration induced significant long-term alterations in general motor performance paradigms, namely increased latency in the motor swimming test, as well as altered gait and foot dragging. It is controversial whether MPTP administration has an impact in motor performance in the classical rotarod test [37, 38]. In our experiments, we could not detect any effect of MPTP in the rotarod test performance nor in the balance beam test, suggesting that the balance of these animals was preserved after MPTP administration. Regarding tests aiming to assess specific PD symptoms, as expected, spontaneous activity was decreased by MPTP injection, as well as the capacity to initiate movement, assessed by the adhesive removal test, the pole test and the time to initiate movement in an open arena. Interestingly, in the pole test, no differences were found in the time that the animals took to reach the cage. However, in this test, we observed an increased time to initiate movement, which is in accordance with the core symptomatology of PD. Importantly, MPTP-treated animals also exhibited tremors. This supports the face validity of this animal model of PD, reinforcing its relevance for pre-clinical studies.

Strikingly, TUDCA administration, prior to or after MPTP, significantly prevented the MPTP-mediated increase in swimming latency, improve gait quality and decrease foot dragging. TUDCA treatment also prevented the decrease of spontaneous activity and ability to initiate movement, as well as the tremors induced by MPTP.

Regarding gait quality and spontaneous activity, the deleterious effects of MPTP were progressively reduced throughout time. This functional recovery effect is reported in the literature and may be caused by increased dopamine turnover in the remaining dopaminergic neurons [57] or by increased dopaminergic receptors in the post-synaptic cells [58], but additional studies are needed to better understand the



**Fig. 7** TUDCA modulates glial activation in the striatum and midbrain of MPTP-treated mice. Fluorescence microscopy images showing GFAP (**a**) and Iba-1 (**b**) staining in the striatum (ST) and midbrain (MB) of vehicle, MPTP, TUDCA prior to MPTP or TUDCA before MPTP-treated mice, sacrificed 52 days p.i. GFAP and Iba-1 were stained in green and nuclei were counterstained with the Hoechst dye 33258. Scale bar = 10  $\mu$ m. The

percentage of area occupied by GFAP- and Iba-1-positive cells was measured using the ImageJ software. Data shown are mean values  $\pm$  SEM of at least three independent experiments. \*, \*\*, and \*\*\*  $p < 0.05$ , 0.01, and 0.001, respectively, using one-way ANOVA with Bonferroni's post hoc test

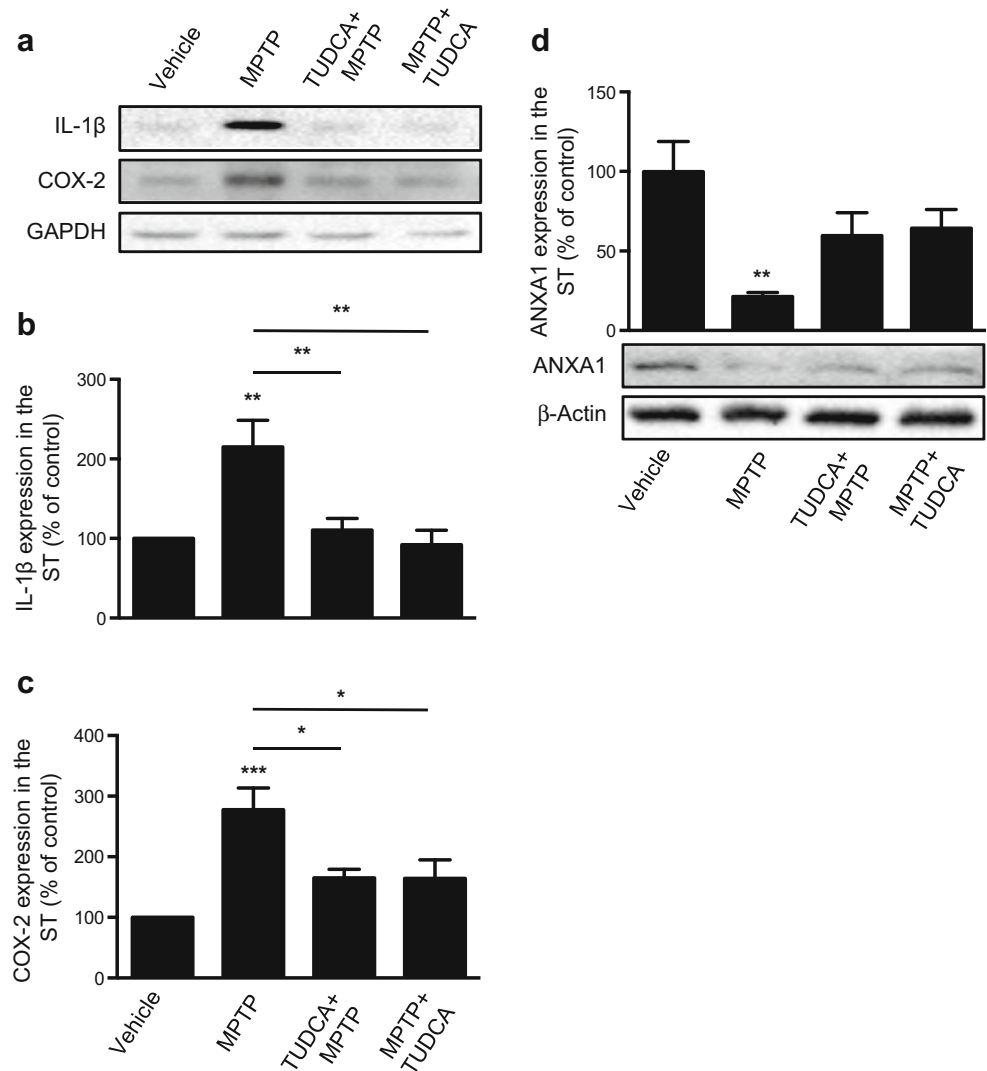
mechanisms employed here. Nonetheless, long-lasting MPTP effects were observed in the motor swimming test, in the foot dragging phenotype, in the adhesive removal test, in the pole test, in the time to initiate movement, in tremors and in hindlimb claspings at day 45 after MPTP administration. Interestingly, the paw-claspings phenotype is observed in several mouse models with damaged basal ganglia, including those pharmacologically induced by MPTP [52, 59], and here we show that it was ameliorated by TUDCA treatment.

Overall, results show that MPTP-injected mice present motor symptoms that resemble human parkinsonism. Moreover, as in humans, motor symptoms were aggravated throughout time in parkinsonian mice, proving that the experimental

paradigm is adequate. Strikingly, in TUDCA-treated animals PD motor symptoms were absent or mild, and no aggravation was observed in any evaluated parameter. In a medical perspective, in the context of a chronic progressive neurodegenerative disease like PD, these results are significant. TUDCA may prevent neurodegeneration of still healthy neurons and glial activation, keeping a larger pool of dopaminergic neurons, which may account for the delay of disease and symptoms progression.

Behavioral deficits were accompanied by a loss of TH-positive fibers in the striatum, analyzed 52 days after acute MPTP administration. Importantly, TUDCA treatment prevented the loss of TH-positive fibers in the striatum

**Fig. 8** TUDCA modulates neuroinflammation in mouse striatum of MPTP-treated mice. Representative immunoblots (a) and respective quantification of IL-1 $\beta$  (b) and COX-2 (c) are shown for striatal samples from mice treated with vehicle, MPTP, TUDCA prior to MPTP or TUDCA after MPTP, and sacrificed 3 h p.i.; and d ANXA1 in striatal samples from mice treated with vehicle, MPTP, TUDCA prior to MPTP or TUDCA after MPTP, and sacrificed 52 days p.i. GAPDH and  $\beta$ -Actin were used as loading controls. Relative band intensity was quantified using the Image Lab™ software and expressed as a percentage of control. Data shown are mean values  $\pm$  SEM of at least three independent experiments. \*, \*\*, and \*\*\*  $p < 0.05$ , 0.01, and 0.001, respectively, using one-way ANOVA with Bonferroni's post hoc test



confirming our previous observations [33, 35]. In a recent study, we have also shown that TUDCA protects neurons against MPP<sup>+</sup>-induced cell death [35], and this bile acid was also neuroprotective in other experimental models of neurodegenerative diseases [29–32], further strengthening our current results. The neuroprotection of dopaminergic cells by TUDCA may explain the maintenance of ATP levels when animals were treated with the bile acid.

Loss-of-function mutations of parkin are the major cause of recessively inherited early-onset PD and wild-type parkin may also be inactivated by post-translational modifications in sporadic PD [24, 60], which leads to impaired mitochondrial turnover. Herein we observed that MPTP-injected mice showed significantly decreased expression of parkin, whereas treatment with TUDCA maintained parkin levels and most notably of phosphorylated parkin in the striatum. PARIS, a parkin target that accumulates upon parkin inactivation, was concomitantly increased in the striatum of MPTP-injected mice, but not when animals

were treated with TUDCA. These results are in accordance with our previous observations, showing that TUDCA triggers parkin expression and phosphorylation in the striatum of MPTP-injected mice [35]. We have previously demonstrated that parkin activation is an early event occurring *in vivo* after MPTP administration [35]. Moreover, we have also shown using *in vitro* systems that parkin mediates some of the neuroprotective effects of TUDCA in neuronal cells upon MPP<sup>+</sup> exposure [35]. Interestingly, here we show that modulation of parkin by TUDCA in the presence of MPTP is maintained throughout longer time periods, confirming that this activation is an important mechanism underlying TUDCA neuroprotective role in experimental PD. The fact that parkin is involved in mitochondrial turnover makes it a perfect candidate to be a TUDCA mediator. The pharmacological upregulation of parkin by TUDCA is crucial in the context of PD and other neurodegenerative disorders where mitochondria also play a key role, and deserves further investigation.

Additionally, parkin and DJ-1 form a complex to promote degradation of misfolded proteins and their PD-pathogenic mutations impair E3 ligase activity of the complex [61]. Interestingly, DJ-1 protein expression was increased in the striatum of animals treated with TUDCA. This is also in accordance with our previous observations, in the striatum and midbrain from mice treated with MPTP for 3–6 h, showing that DJ-1 expression was upregulated when animals received TUDCA before MPTP [34]. These results suggest that DJ-1 is also an important target protein mediating TUDCA neuroprotective effects *in vivo*.

Taking in consideration our previous and present work, data strongly indicate that TUDCA promotes neuroprotection through the maintenance of a healthy mitochondrial pool in MPTP-intoxicated mice.

Besides mitochondrial dysfunction, neuroinflammation and glial activation are also hallmarks of PD [7, 9, 11–13]. Here we have measured the expression levels of IL-1 $\beta$  and COX-2 as surrogate markers of inflammation in the striatum. Accordingly, MPTP-treated mice exhibited an early overexpression of these inflammatory mediators, and subsequent astro- and microglyosis both in the striatum and the midbrain. The early protein changes in inflammatory mediators have already been described after MPTP insult [53–55] and may be the consequence for long-term sustained glial activation. In fact, sustained glial activation in the surrounding environment of dopaminergic injured cells is in accordance with previous reports [20, 62]. Accordingly, IL-1 $\beta$  levels have already been shown to be increased in PD brains [7] and the deletion of the pro-inflammatory enzyme COX-2 in mice protects from MPTP-induced dopaminergic cell loss [63]. Besides its ability to impair oxidative stress, TUDCA has recently been shown to have an anti-inflammatory role in glial cultures and in an experimental mouse model of neuroinflammation [64, 65]. However, to the best of our knowledge, there is no available data concerning the effect of TUDCA on neuroinflammation and glial activation in models of PD. Interestingly, TUDCA prevented the induction of IL-1 $\beta$  and COX-2 expression and glial activation when administered both before and after MPTP. Furthermore, we have shown that ANXA1, an anti-inflammatory protein, which is able to decrease ROS and neuroinflammation by reducing the expression levels of pro-inflammatory cytokines [66], is decreased in the striatum of MPTP-injected mice but not when animals were treated with TUDCA. Altogether, our results suggest that one of the mechanisms underlying TUDCA neuroprotective effect towards MPTP toxicity may be the modulation of glial activation and neuroinflammation. The changes in these inflammatory molecules precede or are coincident with the manifestation of MPTP-induced damage in the striatum. Therefore, they may contribute to nerve damage and cell loss and/or be a response to those processes. The fact that TUDCA

efficiently prevented the inflammatory reaction further contributes to the validation of its neuroprotective role.

Although we found that TUDCA was not protective towards MPTP acute toxicity, the results presented here are promising and indicate that TUDCA improves motor performance in MPTP-intoxicated mice, although longer/higher TUDCA dosage, as well as different time-points of administration following MPTP injection, should be optimized to extend the neuroprotection. The demonstration of the prevention and/or the alleviation of symptoms together with the demonstration of the pathways triggered by TUDCA should contribute to a subsequent clinical trial in humans and future validation of the therapeutic application of this bile acid in PD.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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