Cardiac Tyrosine Hydroxylase Activation and MB-COMT in Dyskinetic Monkeys

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Research

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Abstract

Background: The impact of age-associated disorders is increasing as the life expectancy of the population increments. Cardiovascular diseases and neurodegenerative disorders, such as Parkinson's disease, have the highest social and economic burden and increasing evidence show interrelations between them. Particularly, dysfunction of the cardiovascular nervous system is part of the dysautonomic symptoms of Parkinson's disease, although more studies are needed to elucidate the role of cardiac function on it .

Methods: We analyzed the dopaminergic system in the nigrostriatal pathway of Parkinsonian and dyskinetic monkeys and the expression of some key proteins in the metabolism and synthesis of catecholamines in the heart: total and phosphorylated (phospho) tyrosine hydroxylase (TH), and membrane (MB) and soluble (S) isoforms of catechol-O-methyl transferase (COMT).

Results: The number of dopaminergic neurons in the *Substantia Nigra pars compacta* and the optical density of TH+ fibers and dopamine transporter in the striatum were significantly decreased in all MPTP-intoxicated monkeys. MPTP- and MPTP+L-DOPA-treated animals also showed a decrease in total TH expression in both right (RV) and left ventricle (LV). We found a significant increase of phospho-TH in both groups (MPTP and MPTP+L-DOPA) in the LV, while this increase was only observed in MPTP-treated monkeys in the RV. MB-COMT analysis showed a very significant increase of this isoform in the LV of MPTP- and MPTP+L-DOPA-treated animals. However, we found no significant differences in S-COMT levels. These data suggest that MB-COMT is the main isoform implicated in the cardiac noradrenergic changes observed after MPTP treatment, suggesting an increase in NA metabolism. Moreover, the increase of TH activity indicates that cardiac noradrenergic neurons still respond despite MPTP treatment.

Conclusions: These results could help to elucidate the possible role of alterations in the catecholaminergic system that can contribute to noradrenergic deficiency in the hearts of PD patients. Therefore, this information might be relevant to clinical field, contributing to the therapeutic design of the disease.

Introduction

Aging is the leading risk factor for Parkinson's disease (PD), the second most diagnosed neurodegenerative disorder, affecting almost 1% of the population over age 60 (Blauwendraat et al., 2019). Typical neuropathological hallmarks of PD include the selective loss of dopaminergic cell bodies in the Subtantia Nigra pars compacta (SNpc) (Schapira et al. 2014). Non-motors symptoms often precede and/or accompany PD onset and progression, but the underlying pathological alterations in the autonomic pathways are not fully understood. So, the study of dysautonomic symptoms is receiving growing attention in the recent years, in part because its incidence is increasing due to the aging of the population. Degenerative changes in autonomic pathways affect multiple systemic organs (Sanval et al.

2012), which has been described in both, cardiac entities (such as orthostatic transplant patients) (Nygaard et al. 2019) and in neurodegenerative diseases, such as PD (Knudsen and Borghammer 2018). Particularly, dysautonomic manifestations observed in PD and related disorders include, among others, cognitive dysfunction, behaviour disorder, orthostatic hypotension and arrhythmias (Cuenca et al. 2019). In addition, dysfunction of the cardiovascular autonomic nervous system is common in PD undermining quality of life and contributing to a higher mortality (Bouhaddi et al., 2004). Studies from cardiac sympathetic neuroimaging indicate that some of the mechanisms involved in PD dysautonomia could be the loss of post-ganglionic noradrenergic nerves (Cuenca et al. 2019) and the severe myocardial noradrenaline (NA) depletion (Jain and Goldstein 2012). Moreover, noradrenergic sympathetic nervous system alterations are evidenced in the heart (Mitsui et al. 2006; Orimo et al. 2008). Particularly, myocardial tissue obtained from PD patients showed decreased tyrosine hydroxylase positive (TH+) neurons, thus indicating cardiac sympathetic denervation (Orimo et al. 2002). In addition, right ventricular (RV) and left ventricular (LV) myocardium have been reported to differ in their pathophysiological response to several factors (Friehs et al. 2013). Differences in the genetic make-up, morphology, and functional environment suggest that the RV response to abnormal stress conditions, such as treatment with 1-metil-4-fenil,6-tetrahidropiridine (MPTP) and the undesired peripheral effects of L-DOPA, may differ from that of the LV.

Although there is not an experimental model that represents all the manifestation of PD, the intoxication with MPTP is well characterized to provoke nigrostriatal neurotoxicity and cell death, inducing Parkinsonism in humans (Langston et al. 1983) and in monkeys (Langston et al. 1984). When MPTP is administered to non-human primates, they show a bilateral Parkinsonian syndrome with behavioural and neuroanatomical similarities to human condition (Pérez-Ortuño et al. 1992).

However, the mechanisms of MPTP toxicity to peripheral catecholaminergic system are not very well understood. It has been reported that, in mice, MPTP reduces myocardial NA concentrations (Luthman and Sundström 1990) and NA transporter density in their hearts (Fukumitsu et al. 2006). Additionally, there are other cardiac potential abnormalities such as increased vesicular permeability (Plotegher et al. 2017) or the decrease in NA turnover (Almela et al. 2019).

The aim of our study was to identify the functional abnormalities that contribute to myocardial noradrenergic deficiency in PD. For this, we analyzed the expression and activation of TH, as the rate-limiting enzyme in catecholamine biosynthesis, and the expression of membrane (MB) and soluble (S) isoforms of catechol-O-methyl transferase (COMT), an enzyme that metabolizes NA and L-DOPA, in MPTP or MPTP + L-DOPA-treated monkeys.

Methods

Animals

12 adult male monkeys (*Macaca fascicularis*, 4–6 kg, 4–5 years old, R.C. Hartelust BV, Netherlands) were divided into three groups: 1) control (n = 4); 2) MPTP (n = 4); and, 3) MPTP + L-DOPA (n = 4). Control group

animals remained untreated and intoxication, and treatment of groups 2 and 3 are detailed below. All the animals lived individually in cages with dimensions following primates housing regulations. They were placed under controlled conditions of: humidity (50% ± 5%), temperature (24 ± 1ºC), light (12:12 light:dark cycle) and food (Masuri primate diet; Scientific Dietary Services, UK). Water and fresh fruit were available *ad libitum*. Qualified personnel in animal health care was in charge of monitoring the monkeys' welfare throughout the study.

All the studies were done in accordance with the European Convention for the protection of Vertebrate Animals used for Experimental and the Council of Europe (nº 123, June 15th, 2006) and with The Code of Ethics of the EU Directive 2010/63/EU. All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Murcia.

Induction of Parkinsonism

Animals from MPTP and MPTP + L-DOPA groups were intoxicated with MPTP (Sigma-Aldrich, St. Louis, USA). MPTP was dissolved in saline and was administered intravenously, 1 injection (0.3 mg/kg) every 2 weeks for 7 months. At the end of the experiment, animals received a cumulative dose of 7.0 ± 0.2 mg/kg, which has been described as a reproducible intoxication regimen that leads to the first appearance of Parkinsonian clinical signs (Barcia et al. 2004). We assessed the level of Parkinsonism using a motor scale for MPTP Parkinsonian primates that evaluates different parameters (Kastner et al. 1994).

DOPA treatment

When the monkeys included in the MPTP + L-DOPA group reached a stable Parkinsonism, they were treated with Madopar® (Roche, 100 mg/kg) until dyskinesias were developed (Almela et al. 2019). The administration regimen consisted in 4 months of daily oral administration of Madopar ® + Benserazide (25 mg/kg). Then, the intensity of the dyskinesias was evaluated, every 30 minutes of a total of 180 minutes, for each segment of the body (face, neck, trunk, arms and legs), using a dyskinesia disability scale.

Tissue preparation for post-mortem analyses

The animals were sacrificed 4 h after the last L-DOPA administration by an overdose of sodium pentobarbital (150 mg/kg, intravenous). Brains were fixated by immersion in a 4% paraformaldehyde solution (in 0.01M phosphate buffered saline, PBS) for 5 days and subsequently placed in a 30% sucrose solution in PBS. Brains were cut coronally along the rostral axis (40 µm-thick) with a freezing microtome (Leica, Germany), collected in 0.125M PBS + 0.05% sodium azide, and stored at − 20 °C for further free-floating immunohistochemistry. Each heart was dissected and cut longitudinally to obtain LV and RV, which were immediately frozen with dry ice and stored at -80º C for further analyses.

Immunohistochemical analyses and quantification

Brain slices that contained the anterior striatum were used for TH and dopamine transporter (DAT) immunohistochemistry, while ventral mesencephalon sections (III cranial pair exit) were immunostained for TH. Sections were incubated with 0.02% hydrogen peroxide (in PBS) for endogenous peroxidase blocking and then incubated for 30 minutes with normal goat serum (5% in 0.2% Triton X-100, Sigma-

Aldrich). After, the sections were incubated in the same solution overnight at 4ºC with the primary antibodies anti-TH (mouse monoclonal, 1:1000; Chemicon, CA, USA) and anti-DAT (rat monoclonal, 1:1000; Chemicon). Then, sections were washed with PBS and incubated with the secondary antibodies (30 minutes): biotinylated goat anti-mouse (1:200 in PBS; Agilent, CA, USA) and biotinylated rabbit anti-rat (1:200 in PBS; Chemicon). They were incubated with the Vector avidin-biotin complex (1:200, Vectastain Elite ABC kit, Vector Laboratories, USA) and DAB substrate kit (Vector Laboratories) was used to stain the sections. Additionally, mesencephalon slices were selected and stained for thionine. All the slices were washed and mounted on gelatin-coated slides and coverslipped (DPX, Sigma-Aldrich).

TH and DAT optical density was performed in the striatum of all animals at the rostral level (anterior commissure + 2 mm) including the head of the caudate and putamen. The relative optical densities of TH + and DAT + fibers were quantified using computer-assisted image analysis (ImageJ 1.41, National Institutes of Health, USA) in four different areas: dorsolateral (DL), dorsomedial (DM), ventrolateral (VL) and ventromedial (VM). Seven sections from rostro-caudal levels, equally spaced (intervals of 2.4 mm), were examined for each monkey. Three sections were more rostral and 4 sections more caudal to the level where the anterior commissure crosses the midline.

The number of TH + neurons was quantified in the ventral mesencephalon by the optical fractionator in the SNpc and Ventral Tegmental Area (VTA) (West 1999).

All images were taken in black and white 8-bit monochrome using with a digital camera (AxioCam HRc, Zeiss, Germany) coupled to an interactive computer system consisting of a Zeiss Axioskop optical microscope (Oberkochen, Germany).

Western blot analysis

Western blot was performed for total TH, phosphorylated (phospho) TH, and MB- and S-COMT in the RV and LV. Samples were placed in homogenization buffer [PBS, 2% sodium dodecylsulfate (SDS) plus protease inhibitors (Roche, Germany) and phosphatase inhibitors Cocktail Set (Calbiochem, Germany)], homogenized (50 s) and centrifuged (6000 g, 20 min, 4 °C). Total protein concentrations were determined using the bicinchoninic acid method. 50 µg of protein/lane from each sample were loaded on a 10% SDS–polyacrylamide gel (SDS–PAGE), electrophoresed, and transferred onto a PVDF membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories, CA, USA). Membranes were blocked with 1% bovine serum albumin (BSA) in tris buffer saline tween (TBST: 10 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, and 0.05% Tween 20). Blots were incubated at 4 °C with primary antibodies: polyclonal anti-TH; 1:1000; AB152, Chemicon), polyclonal anti-P-TH Ser-40 (1:500; AB5935, Chemicon), monoclonal anti-COMT (1:5000; AB5873, Chemicon), in TBST with BSA. After TBST washes, the membranes were incubated (1 h, RT) with the peroxidase-labelled secondary antibodies: anti-rabbit sc-2004 for total and phosphorylated TH (1:2500), and anti-mouse sc-2005 for COMT (1:5000). Blots were washed and immunoreactivity was detected with a chemiluminescent/chemifluorescent detection system (ECL Plus, GE Healthcare, LittleChalfont, Buckinghamshire, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). Anti β-actin (Cell Signaling, 45 kDa) was used as loading control.

The ratio total TH/β-actin, phospho-TH/β-actin, MB-COMT/β-actin and S-COMT/β-actin was plotted and analysed.

Quantification of immunoreactivity corresponding to total TH (60 kDa), P-TH Ser-40 (60 kDa), MB-COMT and S-COMT (30 and 25 kDa, respectively) bands was carried out by densitometry (AlphaImager, Nucliber, Madrid).

Statistical analysis

Data was collected and analyzed blindly. Differences in TH + neurons were analysed using one-way repeated measures analyses of variance (ANOVA) followed by the Bonferroni *post-hoc* test. Comparison of TH + and DAT + stainings among striatal subregions in MPTP-treated monkeys as well as total TH and phospho-TH, S-COMT and MB-COMT analysis was performed using one-way ANOVA followed by Newman-Keuls *post-hoc* test. Two-sided *p* values of less than 0.05 were considered significant. Statistical analyses were done with GraphPad Prism software (GraphPad Prism version 5.0 software for Windows; California, USA).

Results

Brain tissue

We performed TH and DAT immunostaining in two relevant brain areas in PD (striatum and ventral mesencephalon) in order to confirm that MPTP induced the histopathological model of the disease.

Dopaminergic neuronal loss in the ventral mesencephalon

The stereological count of TH + neurons in the SNpc revealed a very significant reduction ($P < 0.001$) in comparison to control animals in the groups of MPTP and MPTP + L-DOPA monkeys, in which, approximately, only 26 and 29% of the TH + neurons survive, respectively (Fig. 1b). In the VTA, we also observed a very significant reduction in the number of TH + cells in both groups ($P < 0.001$; Fig. 1c).

Assessment of terminal densities in the striatum

A reduction in the level of immunoreactivity for both dopaminergic markers (TH and DAT) was observed in the anterior striatum of all MPTP and MPTP + L-DOPA-treated monkeys (Fig. 2). TH immunostaining was very significantly decreased in all MPTP and MPTP + L-DOPA groups, both in the putamen (dorsomedial: $P < 0.001$; dorsolateral: $P < 0.001$; ventromedial: $P < 0.001$; $P < 0.001$) and in the caudate (dorsomedial: $P < 0.001$; dorsolateral: $P < 0.001$; ventromedial: $P < 0.001$; ventrolateral: $P < 0.001$) (Fig. 2a). Similar results were obtained in the DAT + of the putamen (dorsomedial: $P < 0.001$; dorsolateral: $P < 0.001$; ventromedial: $P < 0.001$; $P < 0.001$) and in the caudate nucleus (dorsomedial: $P < 0.001$; dorsolateral: $P < 0.001$; ventromedial: $P < 0.001$; ventrolateral: $P < 0.001$) (Fig. 2b).

Heart tissue

Effects of Parkinsonism and L-DOPA treatment on total TH and phospho-TH at Ser-40 expression

We studied total TH expression and TH phosphorylation at Ser-40 in the RV and LV to determine the severity of the Parkinsonism in the groups treated either with MPTP or with MPTP + L-DOPA. As expected, MPTP-treated animals showed a decrease in total TH expression in both RV and LV versus control group. However, this relationship became significant only in the LV. Similar results were obtained in L-DOPA-treated animals and this drug also induced a significant reduction of total TH expression in LV versus MPTP-treated animals (Fig. 3a,b). Moreover, there was a significant correlation between the number of TH^+ neurons in the SNpc and total TH levels in the heart tissue ($r^2 = 0.85$, $P = 0.0004$, Fig. 3c).

In the RV, a significant increase of phospho-TH was observed in MPTP-treated monkeys with respect to the other groups ($P < 0.01$). However, in the LV, we found an important increase of phospho-TH in both groups (MPTP or MPTP + L-DOPA treatment) compared to control animals ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 3d,e), suggesting that L-DOPA treatment could activate a phosphorylation mechanism in TH protein and exert a compensative effect in the LV myocardium. Moreover, we observed that there was an inverse significant correlation between TH^+ neurons in the SNpc and phospho-TH levels in the heart tissue (r^2=-0.77, $P = 0.0019$, Fig. 4f), reinforcing the idea of a close relationship between central and peripheral catecholaminergic changes in MPTP-treated monkeys.

COMT activation after chronic MPTP and L-DOPA treatment

Considering the fact that little or nothing is known about the differences of the physiological roles of S-COMT and MB-COMT in the heart, we found interesting to evaluate in this study both isoforms of this protein. We obtained two bands at approximately 30 and 25 kDa. Based on the sizes and relevant abundance, the 30 kDa bands are presumed to be the membrane subunit MB-COMT in the heart, and the 25 kDa ones correspond to S-COMT. Quantitative analysis showed non-significant changes in S-COMT expression in any of the groups studied (Fig. 4d,e). In contrast, we observed a significant increase in MB-COMT expression in the RV of MPTP- and MPTP + L-DOPA-treated animals compared to the control group ($P < 0.05$). Similar results were observed in the LV, an increase in this protein after MPTP or MPTP + L-DOPA treatment with respect to control animals ($P < 0.01$ and $P < 0.001$, respectively, Fig. 4a,b).

Consistently with our previous findings demonstrating a close relationship between catecholaminergic central and peripheral changes, we also observed an inverse significant correlation between TH^+ neurons in the SNpc and MB-COMT in the heart tissue (r^2=-0.71, $P = 0.046$, Fig. 4c). In contrast, we did not observe a significant correlation between TH^+ neurons in the SNpc and S-COMT in the heart tissue (r^2=-0.39, P = 0.07, Fig. 4f).

Discussion

Several studies have reported that PD entails profound alterations in cardiac noradrenergic pathways that affect, not only to neuronal loss, but also to other neuronal functions such as NA biosynthesis via TH or NA turnover (Almela et al. 2019; Goldstein et al. 2019). Importantly, in the present study, we report a strong correlation between neuronal cell death in the SNpc and cardiac sympathetic pathways. This result is consistent with a previous study in monkeys demonstrating that MPTP exposure can induce

nigral dopaminergic cell loss in parallel with the degeneration of cardiac TH-ir (Carmona-Abellan et al. 2019). In addition, we evaluate MPTP-induced changes in NA synthesis and metabolism, including L-DOPA treatment. The different responses observed in LV and RV to MPTP toxicity suggest that the pattern of cardiac sympathetic dysfunction in PD is not homogeneous in both ventricles.

The effect of MPTP on the central nervous system has been largely reported by others and us in the literature (Barcia et al. 2004; Fukumitsu et al. 2006; Almela et al. 2019). In the present study, a nigrostriatal degeneration with a marked decrease in the number of striatal TH + fibers and dopaminergic cells in the SNpc and in the VTA was observed. Additionally, we found that the striatum of Parkinsonian monkeys chronically treated with L-DOPA exhibited a small number of TH + fibers, similar to the MPTP-treated group, when compared to control animals (Herrero et al. 1996a). Similar to these results, DAT optical density was dramatically decreased in the striatum of MPTP and MPTP + L-DOPA groups compared to the control one, both in the putamen and in the caudate nucleus (Herrero et al. 1996b).

On the other hand, clinical and pathological studies have provided strong evidence of the involvement of cardiac sympathetic nerves in PD patients (Dickson et al., 2008; Fujishiro et al., 2008). In our study, we determined that sympathetic dysfunction was present in Parkinsonian monkeys without L-DOPA treatment. According with this result, different studies have suggested the denervation of Parkinsonian heart with a decrease in TH-ir axons in the epicardial layer of LV anterior wall (Taki et al. 2000; Jain and Goldstein 2012). However, studies reporting functional evidence of cardiac denervation are uncommon. Present results demonstrate different ventricular responses to MPTP toxicity and posterior L-DOPA treatment, being the LV the most sensible. The amount and distribution of sympathetic loss in PD seems to vary among individuals and it has been reported as either diffuse, at times extensively, or following no uniform pattern with maximal loss in the apex and the inferior and lateral walls of the LV (for revision, see Weihe et al. 2005). Consistently, we observed a decrease in TH protein expression in monkey cardiac tissue in both ventricles, being significant only in the LV, concomitantly with an enhancement in TH phosphorylation at Ser-40. The decrease in TH protein level observed in our study can be explained as a compensatory mechanism of catalytic activity by increased phosphorylation (Nakashima et al. 2013). TH phosphorylated at Ser-40 increased levels in both ventricles accelerated TH activity, thereby stimulating production of neurotransmitter in catecholaminergic terminals (Dunkley et al. 2004). The increase of sympathetic activity observed in this study could be implicated in the increase of heart rate described in MPTP-treated mice (Liu et al. 2020). Our results demonstrating an increased TH activity in parkinsonian monkeys are in agree with the idea that loss of sympathetic noardrenergic innervation induce abnormalities in residual nerves that are dysfunctional but extant (Goldstein et al. 2019). Importantly, present results, together with a previous study (Almela et al. 2019) which demonstrated an increased in the NA turnover after MPTP + L-DOPA treatment, support this idea.

Together with the increased TH activity, we found an enhancement of COMT expression in both ventricles in Parkinsonian monkeys before and after L-DOPA treatment without any changes in S-COMT. Moreover, we found a strong correlation between the number of TH + SNpc neurons and MB-COMT, suggesting that membrane isoform of COMT has an important role in NA metabolism. In this line, our results support a

recent study where it was suggested that MB-COMT has a higher catalytic activity than S-COMT (Magarkar et al. 2018). Our study also concordats with the use of COMT inhibitors which is currently chosen as a complementary therapy to L-DOPA such as opicapone, being a safe and successful clinical choice (Fabbri et al. 2018; Reichmann et al. 2020), even if Zeng and collaborators found no significant changes in brain and liver COMT levels (Zeng et al. 2010).

The enhancement in MB-COMT could be responsible for the increase of normetanephrine content and NA turnover showed after MPTP + L-DOPA treatment in a previous study (Almela et al. 2019). However, whether L-DOPA is neurotoxic or neuroprotective is still an issue of debate and no studies have directly evaluated the toxic or protective cardiac effects of this drug (Noack et al. 2014). Some studies showed that L-DOPA could improve the abnormality in the variability of heart rate and did not exacerbate orthostatic hypotension (Noack et al. 2014). Although, other studies demonstrated that L-DOPA decreased blood pressure and increased orthostatic hypotension (Haapaniemi et al. 2000). Our results could also be interesting for other cardiovascular areas. For example, dysautonomic symptoms and cardiac denervation has been detected in patients after orthotopic heart transplantation, giving rise to arrythmia and altered orthostatic hypotension (Rivinius et al. 2018; Nygoard et al. 2019).

Conclusions

In conclusion, our results indicate impairments of NA synthesis and metabolism in Parkinsonism, with enhancement in the expression of MB-COMT. These results support the hypothesis that the residual cardiac innervation is able to respond to different events, so the noradrenergic neurons are altered, but still exist. In addition, knowing that aging is a common risk factor for PD and cardiac alterations, these data can contribute to our knowledge about the mechanisms implicated in PD, which may be useful for treatment's design and, therefore, to retard its clinical progression and impact.

List Of Abbreviations

COMT, catechol-O-methyltransferase; DA, dopamine; DAT, dopamine transporter; L-DOPA, levodopa; LIDs, L-DOPA-induced dyskinesias; LV, left ventricle; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NA, noradrenaline; NMN, normetanephrine; PD, Parkinson's disease; RV, right ventricle; SNpc, *Substantia Nigra pars compacta*; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

Declarations

Ethics approval and consent to participate

All the studies were done in accordance with the European Convention for the protection of Vertebrate Animals used for Experimental and the Council of Europe (nº 123, June 15th, 2006) and with The Code of Ethics of the EU Directive 2010/63/EU. All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Murcia.

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors designed the study. Lorena Cuenca-Bermejo, Pilar Almela, Pablo Gallo-Soljancic, José E. Yuste, Vicente de Pablos, Víctor Bautista-Hernández and Emiliano Fernández-Villalba1conducted the literature survey and data analysis; E. Yuste, Vicente de Plablos and Emiliano Fernández-Villalba performed de experiments; Pilar Almela and Victor Bautista-Hernández drafted the manuscript; María-Luisa Laorden and María-Trinidad Herrero provided writing ideas and supervised the preparation of the final version of the paper.

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Figures

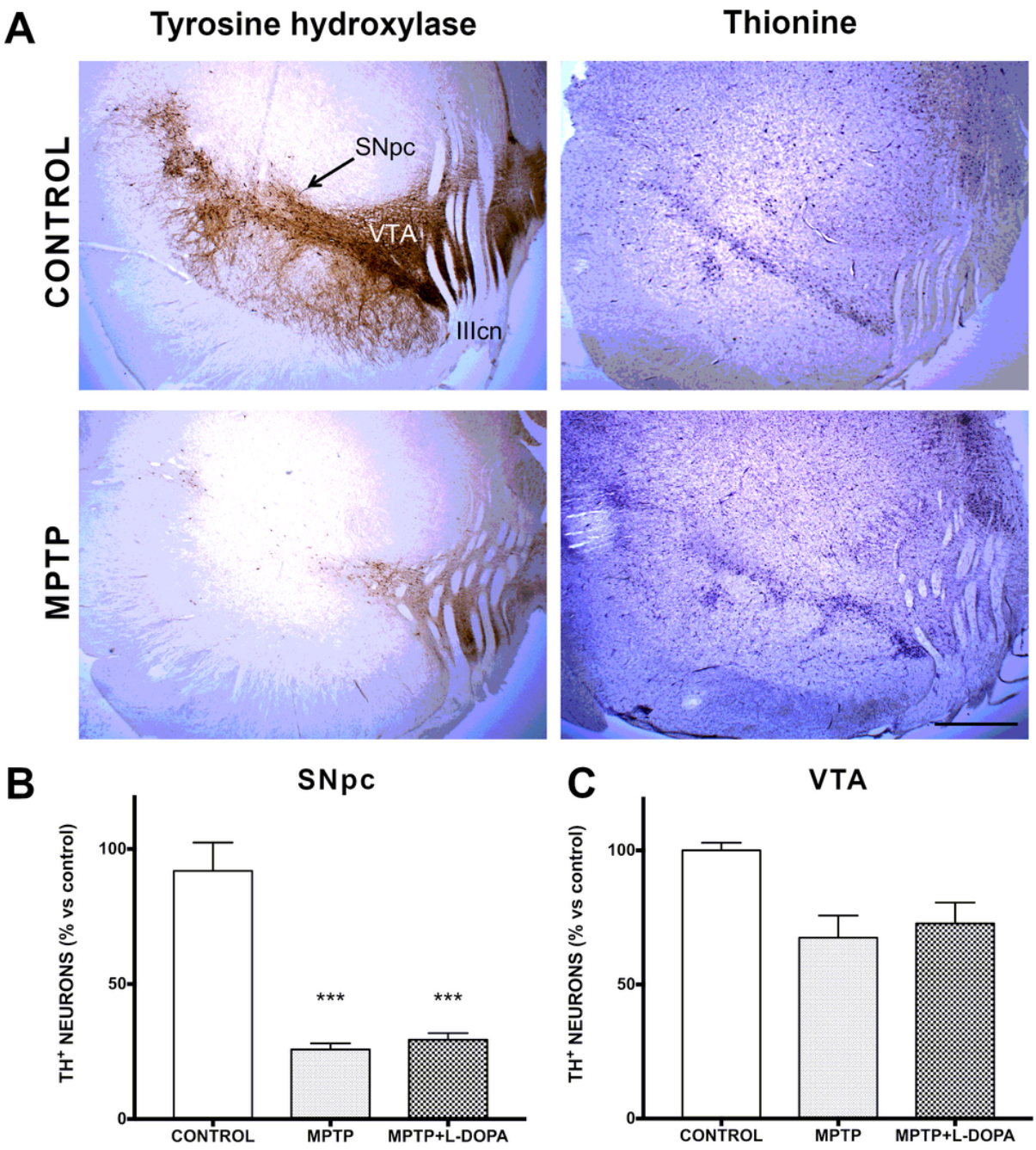

Figure 1

Evaluation of dopaminergic neuronal loss in the ventral mesencephalon. A) Representative micrographs of TH immunolabeling and thionine staining (scale bar = 1 mm; SNpc: Substantia Nigra pars compacta; VTA: ventral tegmental area; IIIcn: III cranial nerve). B and C) Scatter plots representing the number of TH+ir cells (% versus control, Mean ± SD) in the SNpc (B) and in the VTA (C). Data were compared by one-way ANOVA followed by the Bonferroni post-hoc test. Values are represented as mean ± standard deviation. Two-sided p values < 0.05 were considered significant; *** Indicate significant differences with P< 0.001 compared to the control group (n=4 biological replicates and 7 technical replicates/animal).

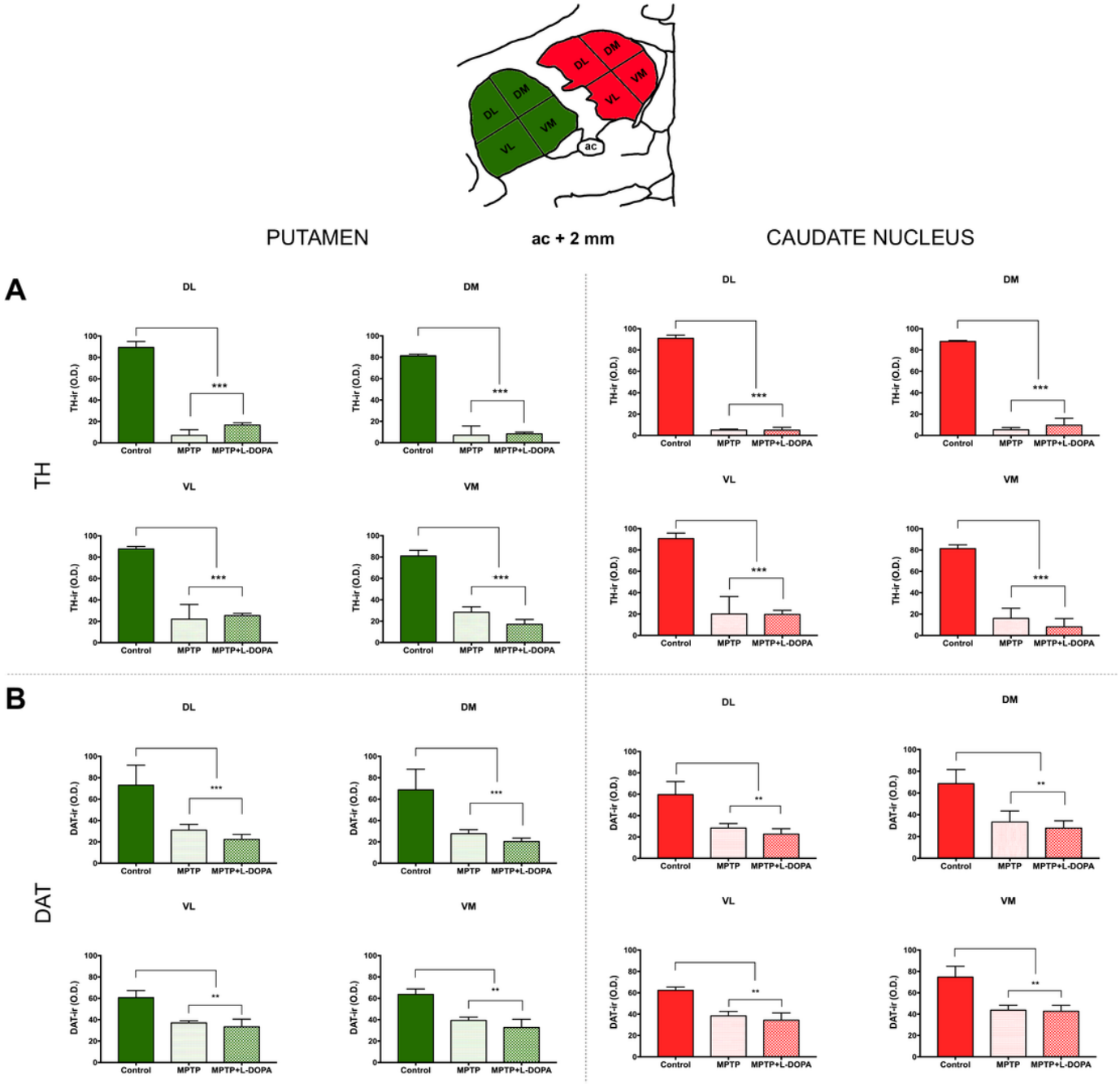

Figure 2

Striatal TH+ir fibers distribution and DAT expression in the anterior striatum (anterior commissure = +2 mm). A) Quantification of TH optical density in the putamen (left, green) and caudate nucleus (right, red). B) Quantification of DAT optical density in the putamen (left, green) and caudate nucleus (right, red). Data were compared by one-way ANOVA followed by Newman-Keuls post-hoc test. Values are represented as mean ± standard deviation. Two-sided p values < 0.05 were considered significant Symbols indicate significant differences compared with the control group: ** P<0.01 and *** P<0.001.

(n=4 biological replicates and 7 technical replicates/animal). ac: anterior commissure, DAT: dopamine transporter, DL: dorsolateral, DM: dorsomedial, VL: ventrolateral, VM: ventromedial, TH: tyrosine hydroxylase.

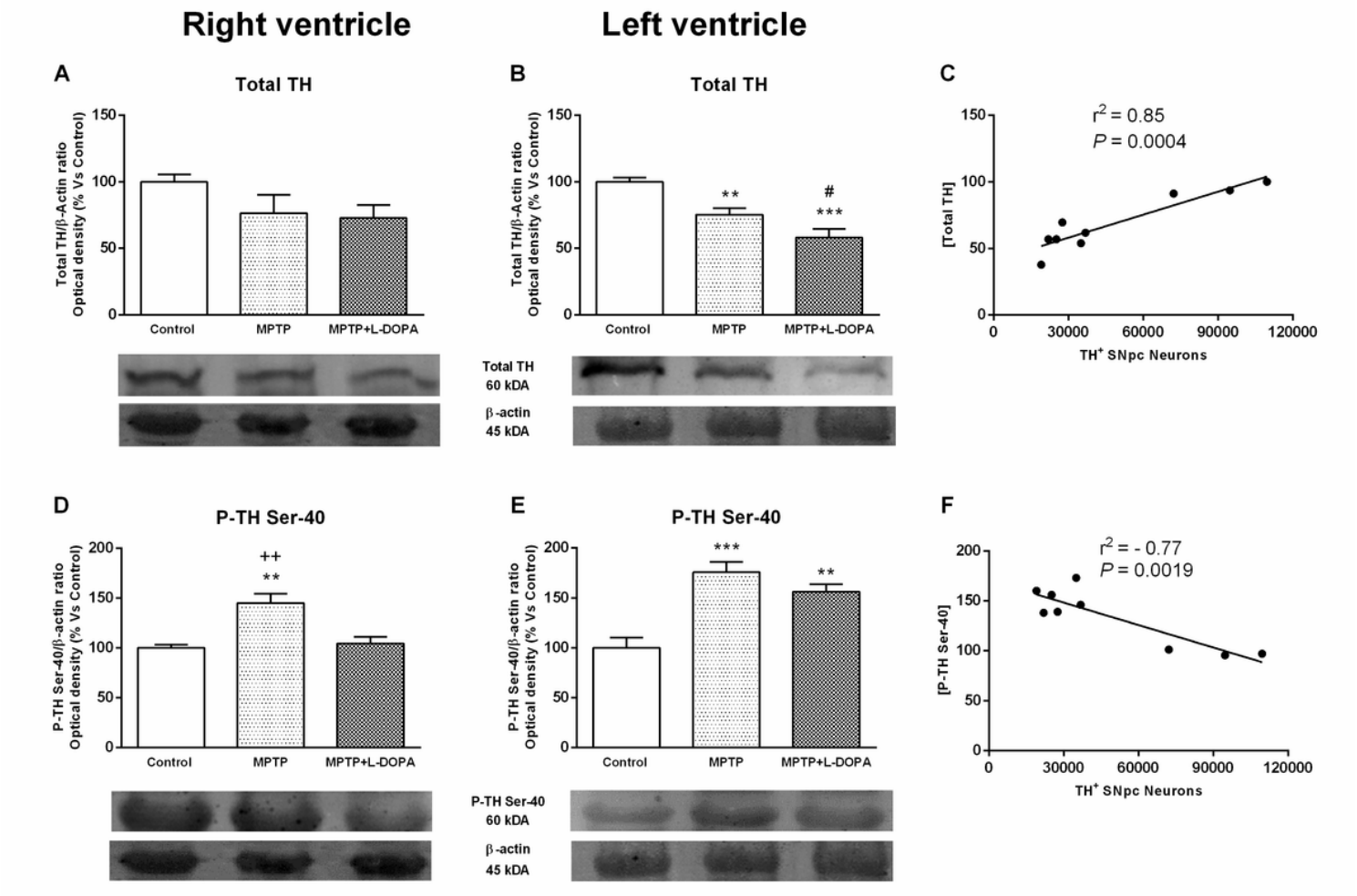

Figure 3

Effects of MPTP and L-DOPA treatment on total TH and phospho-TH at Ser-40 expression in the monkey heart. A and B) Total TH/β-actin ratio (optical density, % vs control) in controls, MPTP-treated monkeys and in the MPTP+L-DOPA group. Total TH expression was significantly decreased in the LV in MPTP-treated monkeys and in the MPTP+L-DOPA group versus control group and in the MPTP+L-DOPA group versus MPTP-treated animals. C) Strong correlation between the number of TH+ neurons in the SNpc and total TH levels in the heart tissue (r2=0.85, P=0.0004). D and E) P-TH at Ser-40/ β-actin ratio (optical density, % vs control) in controls, MPTP-treated monkeys and in the MPTP+L-DOPA group. TH phosphorylation at Ser-40 was significantly increased in the RV with respect to the other two groups. Besides, in the LV, we found an important increase of phospho-TH at Ser-40 in both groups (MPTP or MPTP+L-DOPA treatment) with respect to controls. F) Strong correlation between phospho-TH and TH+ neurons in the SNpc (r2=0.77, P=0.0019). Data were compared by one-way ANOVA followed by Newman-Keuls post-hoc test. Values are represented as mean ± standard deviation. Two-sided p values < 0.05 were considered significant; (**P<0.01, ***P<0.001 versus control group; #P<0.05 versus MPTP-treated

animals; ++P<0.01 versus MPTP+L-DOPA). TH: tyrosine hydroxylase, MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, L-DOPA: levodopa, P: phospho. (n=4 animals/group and 2 technical replicates/animal).

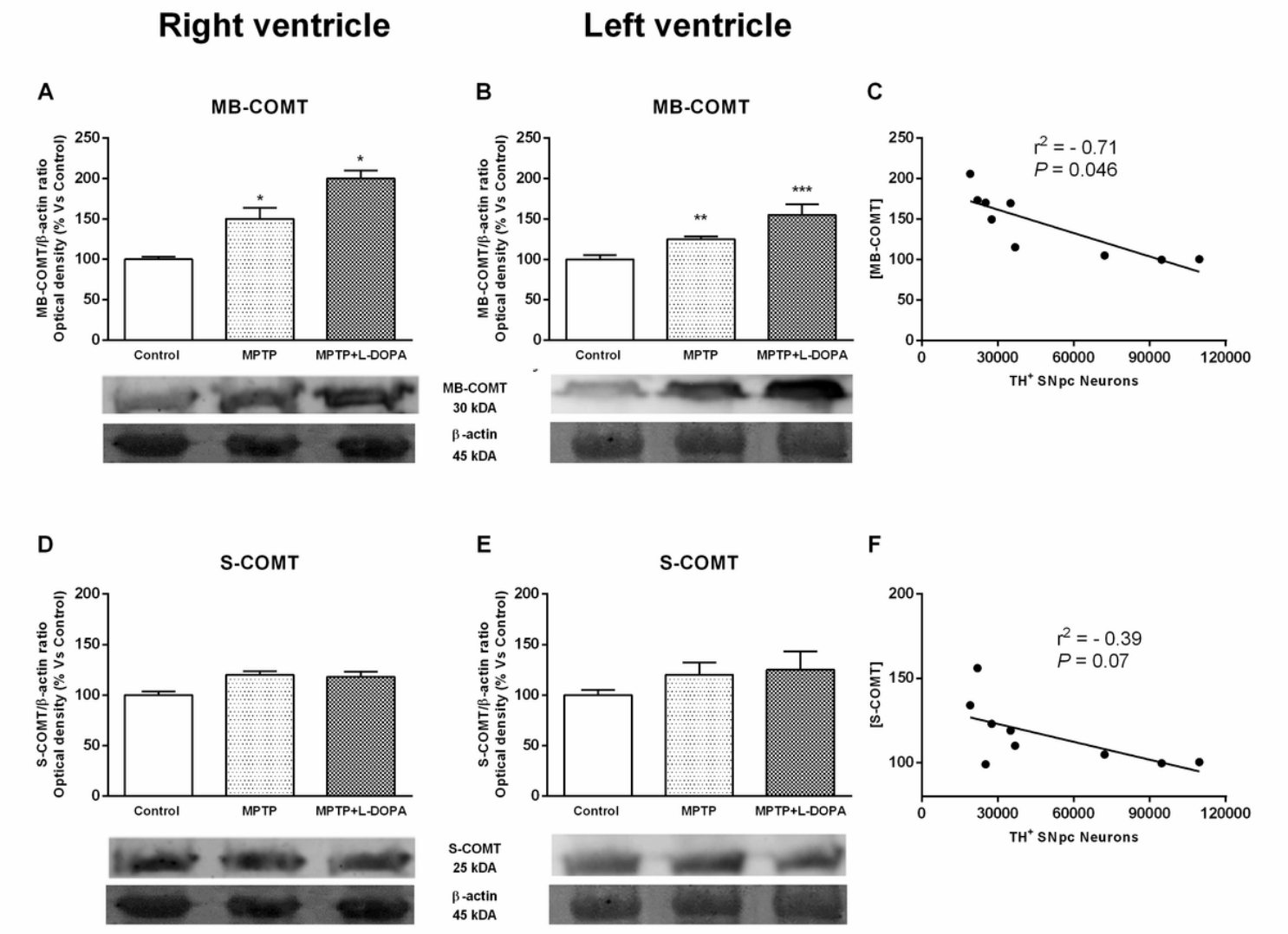

Figure 4

Effects of MPTP and L-DOPA treatment on COMT in the monkey heart. A and B) MB-COMT/β-actin ratio (optical density, % vs control) in controls, MPTP-treated monkeys and in the MPTP+L-DOPA group. MB-COMT expression was increased in the RV of MPTP- and MPTP+L-DOPA-treated animals compared to control group. C) Significant correlation between MB-COMT and TH+ neurons in the SNpc (r2=0.71, P=0.0046). D and E) S-COMT/β-actin ratio (optical density, % vs control) in controls, MPTP-treated monkeys and in the MPTP+L-DOPA group. The quantitative analysis showed no differences between groups. F) No correlation between S-COMT and TH+ neurons in the SNpc was observed. Data were compared by one-way ANOVA followed by Newman-Keuls post-hoc test. Values are represented as mean ± standard deviation. Two-sided p values < 0.05 were considered significant; (*P<0.05, **P<0.01, ***P<0.001 versus control group). MB: membrane, S: soluble, COMT: catechol-O-methyl transferase,

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, L-DOPA: levodopa. (n=4) animals/group and 2 technical replicates/animal).