Vitamin D Treatment Attenuates Neuroinflammation and Dopaminergic Neurodegeneration in an Animal Model of Parkinson's Disease, Shifting M1 to M2 Microglia Responses

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Abstract
Microglia-mediated neuroinflammation has been described as a common hallmark of Parkinson's disease (PD) and is believed to further exacerbate the progressive degeneration of dopaminergic neurons. Current therapies are unable to prevent the disease progression. A significant association has been demonstrated between PD and low levels of vitamin D in patients serum, and vitamin D supplement appears to have a beneficial clinical effect. Herein, we investigated whether vitamin D administered orally in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced preclinical animal model of PD protects against glia-mediated inflammation and nigrostriatal neurodegeneration. Vitamin D significantly attenuated the MPTP-induced loss of tyrosine hydrlase (TH)-positive neuronal cells, microglial cell activation (Iba1-immunoreactive), inducible nitric oxide synthase (iNOS) and TLR-4 expression, typical hallmarks of the pro-inflammatory (M1) activation of microglia. Additionally, Vitamin D was able to decrease pro-inflammatory cytokines mRNA expression in distinct brain areas of the MPTP mouse. Importantly, we also assessed the anti-inflammatory property of vitamin D in the MPTP mouse, in which it upregulated the anti-inflammatory cytokines (IL-10, IL-4 and TGF-β) mRNA expression as well as increasing the expression of CD163, CD206 and CD204, typical hallmarks of alternative activation of microglia for anti-inflammatory signalling (M2). Collectively, these results demonstrate that vitamin D exhibits substantial neuroprotective effects in this PD animal model, by attenuating pro-inflammatory and up-regulating anti-inflammatory processes.

Introduction
Neuroinflammation is a common mechanism that plays a crucial role in the pathogenesis of various brain diseases. Initiation of a neuroinflammatory response involves a complex interplay of glia and neurons. Activated glial cells, mainly microglia and astrocytes, are histopathological hallmarks of neurologic diseases (González et al. 2014).

Inflammatory mediators [e.g., nitric oxide (NO), reactive oxygen species (ROS), proinflammatory cytokines, and chemokines] released by activated glia are neurotoxic and can cause neuronal damage (Wang et al. 2006). In the last decades, neuroinflammation has been considered to be have an important role during the

onset and progression of Parkinson's disease (PD) (Whitton 2007). PD is the second most prevalent human neurodegenerative disease, characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and decreased dopamine levels in the striatum of the basal ganglia (Lees et al. 2009). Overactivated or dysregulated microglia are constantly involved in the pathogenesis of PD, thus contributing to neuronal damage caused by pathological stimuli and toxins, which in turn, induces more widespread damage to the neighboring neurons (Block and Hong 2005; Teismann et al. 2003).

Microglia can be polarized to different functional phenotypes. Recently, it has been suggested that activated microglia may polarize in the same way as peripheral macrophages in the central nervous system (CNS), assuming a pro-inflammatory M1 phenotype or alternatively an anti-inflammatory M2 phenotype according to cytokine production (Varnum and Ikezu 2012).

Vitamin D is a steroid hormone that is important in the regulation of plasma calcium concentrations. Ultraviolet exposure of 7-dehydrocholesterol present in the skin results in the photochemical production of vitamin D, which is biologically inactive. The liver enzyme 25-hydroxylase then converts vitamin D into 25-hydroxyvitamin D3 [25(OH)D3, cholecalciferol], the biologically active form of vitamin D. In the kidney proximal tubule, 1-α-hydroxylase converts 25(OH)D3 into 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], the hormonally active vitamin D metabolite (Norman 2012).

Clinical studies suggest that a vitamin D insufficiency is associated with an increased risk of developing brain diseases, such as Alzheimer's disease (AD) (Yu et al. 2011), PD (Sanchez et al. 2009), and ischemic brain injury (Wang et al. 2000). Vitamin D has potent immunomodulatory activities in both innate and adaptive immunity (Mora et al. 2008) but the mechanisms underlying the role of vitamin D in neuroinflammation remain unclear.

In this work the effect of 25(OH)D3 on an in vivo PD animal model, being an MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced preclinical mouse model of Parkinson's disease, has been investigated. In particular, we tested the hypothesis that vitamin D is able to reduce dopaminergic neurons injury by shifting microglia polarization from detrimental M1 to beneficial M2 phenotypes.

Materials and Methods
Animals and Treatment
Forty-eight adult male C57BL/6 N mice (22–24 g body weight, 8–10 weeks of age) purchased from Harlan, Italy, were kept under environmentally controlled conditions (ambient temperature: 20 ± 2 °C; humidity: $50 \pm 5\%$) on a 12 h light/dark cycle with food and water ad libitum. Experimental procedures involving animals were carried out in strict accordance with the European Council Directive 86/609/EEC and the Italian animal welfare legislation (art. 4 and 5 of D.L. 116/92).

One group of 24 animals received 1 μg/kg vitamin D (Sigma-Aldrich, Milan, Italy) daily by intragastric gavage between 8.00 and 9.00 am every day for 10 days, while the other group of 24 mice was given only the vehicle (olive oil). On day three, 12 mice from each group were given four intraperitoneal injections of the neurotoxin MPTP (20 mg/kg) for a total of four doses over an 8 h period and sacrificed 7 days later (Jackson-Lewis and Przedborski 2007). Control mice were treated with sterile saline solution.

Immunohistochemistry
Six mice from each group were sacrificed by CO_2 inhalation and transcardially perfused with tris-buffered saline (pH 7.6) followed by 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4 at 4°C. Brains were subsequently post-fixed in the same fixative, paraffin embedded and 10um slices were obtained with a rotating microtome (Leica, Milan, Italy). Immunohistochemistry was performed following a standard avidin-

biotin complex procedure. Briefly, specimens were incubated with mouse primary monoclonal antibody (MoAb) anti-tyrosine hydroxylase (TH) at a ratio of 1:1500 (BioLegend, San Diego, CA, USA), a mouse MoAb anti-glial fibrillary acidic protein (GFAP) at a ratio of 1:1000 (Merck Millipore, Vimodrone MI, Italy) or a rabbit policlonal (p)Ab anti-Iba1 at a ratio of 1:1000 (Wako, Neuss, Germany) overnight and then with an anti-mouse biotinylated secondary Ab (Dako, Milan, Italy) at a 1:1000 dilution for 1 hour. To visualize the formation of the antigen-Ab complex, sections were incubated for 1 hour with extravidin peroxidase (Sigma-Aldrich) diluted 1:1500 and colour development was obtained with 3,3′-diaminobenzidine.

For co-localisation experiments, sections were incubated with a solution containing a mouse MoAb anti-Iba1 at a ratio of 1:500 (Merck Millipore) and either a rabbit pAb anti-CD204, or rabbit pAb anti-CD163, or rabbit pAb anti-CD206, at a ratio of 1:200 (Santa Cruz Biotechnology, Inc., Milan, Italy) overnight. Next day, sections were incubated with a solution containing a goat anti-rabbit IgG secondary antibody Alexa fluor 546 and a Goat anti-mouse IgG secondary antibody Alexa fluor 488 (Thermo Fisher Scientific Inc., Monza, Italy). Immunocomplexes were visualized with a Zeiss LSM 800 confocal microscope (Carl Zeiss S.p.A., Milan, Italy).

Immunoblotting Assay
The remaining mice were sacrificed by cervical dislocation, each brain was rapidly removed and the striatum and SNpc were dissected out with the aid of a stereomicroscope according to Jackson-Lewis and Przedborski. Subsequently, SNpc and striatum were minced in ice-cold PBS and then homogenized in a buffer containing lysis buffer [50 mM Tris pH 8, 0.02 g/mL NaCl, 0.2% SDS, 1% Triton-X, 4 U/ml Aprotinin, 2 μM Leupeptin, 100 μM phenylmethanesulfonylfluoride (PMSF)]. Lysates were centrifuged at $13000 \times g$ for 20 min at 4 °C. The protein concentration in the lysates was determined by Bradford's method (Bradford 1976) and 25 μg of protein from each sample were size-fractionated on a denaturing, reducing 10% polyacrylamide minigel and electrophoretically transferred onto a nitrocellulose membrane. Specific proteins were detected using optimal concentrations of the following specific Abs, mouse MoAb anti-TH, mouse MoAb anti-GFAP (Merck Millipore), mouse MoAb Iba1 (Merck Millipore), rabbit polyclonal antibody (pAb) anti-TLR-4, goat pAb anti-iNOS; rabbit pAb anti-CD204; rabbit pAb anti-CD163; rabbit pAb anti-CD206, (Santa Cruz Biotechnology). The β-actin protein level was used as protein loading control in western blotting. The binding of antibodies was detected with horseradish peroxidase (HRP)-conjugated secondary Abs (Santa Cruz Biotechnology) and visualized by the chemiluminescence method (BioRad, Milan, Italy).

Densitometric Analysis
The visualized bands obtained after immunoblotting experiments were submitted to densitometric analysis using 1D Image Analysis software (Kodak Digital Science). β-actin was used for normalisation of immunoblotting products. Results (means ±SD) were expressed as relative optical measured density.

qRT-PCR
Total tissue RNA, extracted from treated and untreated animals with Trizol isolation reagent (Invitrogen, Milan, Italy), according to the manufacturer's instructions, was reverse-transcribed to cDNA, using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Invitrogen, Milan, Italy). The obtained cDNA was amplified in a 7300 Real-Time PCR System (Life Technologies), where a target cDNA (IL-1β, TNF-α, IL-10, IL-4, TGF-β) and a reference cDNA (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) were amplified simultaneously using an oligonucleotide probe with a 5′ fluorescent reporter dye (6-FAM) and a 3′ quencher dye (NFQ). Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems, CA). Data were analyzed using the comparative threshold cycle (Ct) method and results were expressed as fold difference.

Statistical Analysis

Statistical analysis of experimental data was performed using Statgraphics Centurion (Statpoint Technologies Inc., Warrenton, Virginia, USA); the multiple comparison to determine which means were significantly different from which others was evaluated by ANOVA and Tukey's post hoc test. Values of $p < 0.05$ were considered significant.

Results

Effects of Vitamin D on Dopaminergic Neurons Damage

Results of TH staining (Fig. 1a) showed a marked TH-immunoreactivity decrease in striatum and SNpc following MPTP administration in comparison to controls. Conversely, TH immunostaining in MPTP-treated mice that received vitamin D by intragastric gavage was more pronounced as compared to the immunostaining in those that received MPTP alone, suggesting that vitamin D protects dopaminergic neurons against MPTP induced cell death. Animals treated with vitamin D alone exhibited a very similar TH immunoreactivity pattern to that observed in controls (Fig. 1a).

Results of the immunoblotting densitometric analysis of TH protein expression, reported in Fig. 1b, confirmed that TH loss was significantly reduced in the brain of animals treated with MPTP + vitamin D in comparison with those treated with MPTP alone.

Effects of Vitamin D on Astroglial Activation

Astroglial activation was characterized by GFAP expression analysis (Fig. 2). MPTP treatment determined an increase of immunoreactive cell bodies (Fig. 2a), suggesting astrocyte activation, while the administration of vitamin D in MPTP-treated animals reduced the presence of GFAP immunoreactive cells in both striatum and SNpc. These data were confirmed by the results of densitometric analysis of GFAP protein expression (Fig. 2b), revealing a significant increase of the GFAP protein band following MPTP treatment, that resulted significantly reduced by vitamin D administration.

Effects of Vitamin D on Microglia Activation in the Brain Regions

To determine whether vitamin D neuroprotection was associated with an inhibition of MPTP-induced microglial activation, we assessed the extent of Iba1-expressing microglial cells within the striatum and SNpc of mice after MPTP-intoxication. In controls, ramified resting microglia were observed. After neurotoxin injection, numerous Iba1-positive activated microglia with stouter cell processes and a round shape were observed both in striatum and SNpc (Fig. 3a). Conversely, in mice treated with MPTP and vitamin D, few activated microglia were detected (Fig. 3a).

Results related to western blotting analysis confirmed that Iba1 expression was significantly increased in the MPTP-treated group in comparison to control mice (Fig. 3b). By contrast, in vitamin D-treated mice we detected comparable Iba1 levels to those in controls.

Interestingly, in MPTP mice receiving vitamin D, Iba1 levels resulted significantly reduced in comparison to animals given MPTP alone.

Effects of Vitamin D on the pro-Inflammatory Responses

To assess the effects of vitamin D on the pro-inflammatory responses in MPTP treated mice, the IL-1β and TNF-α mRNA transcripts were determined by qRT-PCR analysis.

Real-time PCR results showed that the levels of IL-1β and TNF-α mRNA were significantly increased both in the striatum (Fig. 4a) and in SNpc of MPTP-treated mice compared with the SNpc of control mice (Fig. 4b). Following vitamin D treatment, the mRNA levels of IL-1β and TNF-α in the MPTP + vitamin D groups were all significantly decreased compared with the MPTP group. The concentrations of IL-1β and TNF-α in the mice treated with vitamin D alone did not exhibit significant differences compared to the control group.

Toll-like receptors, such as TLR-4, have been found to be involved in progressive neurodegenerative diseases (Letiembre et al. 2007; Panaro et al. 2008), and TLR-4 is a well known M1 responses marker (Jeong et al. 2015). Results of analysis of TLR-4 protein expression are reported in Fig. 4c, d. In striatum of MPTP treated mice, TLR-4 protein levels were much higher than in controls (see Fig. 4c). Values of densitometric analysis of TLR-4 protein levels demonstrated significantly reduced amounts of this receptor in MPTP treated mice which received vitamin D. No significant differences were found in the TLR-4 protein levels of mice treated only with vitamin D as compared with controls (Fig. 4c). The same results were obtained in the SNpc (Fig. 4d).

Microglia activation leads to the release of proinflammatory enzymes, including iNOS, in PD (Kim and Joh 2006; Mosley et al. 2006). Thus, we also evaluated iNOS expression in the different brain regions. In this respect, we observed that iNOS levels, up-regulated in MPTP animals, resulted significantly diminished in MPTP mice receiving vitamin D, as reported in Fig. 4e, f. No effect on iNOS expression was observed in mice treated only with vitamin D.

Effects of Vitamin D on the M2 Anti-Inflammatory Responses
Then, we tested the effects of vitamin D on the M2 phenotype, evaluating both anti-inflammatory cytokines and M2 markers in the brains of animal tested by qRT-PCR (Fig. 5). Brain levels of the anti-inflammatory cytokines, IL-10, TFG-β and IL-4, were significantly increased in vitamin D-treated mice in comparison to controls, thus demonstrating that vitamin D is able to up-regulate anti-inflammatory mediators both in striatum (Panel A) and in SNpc (Panel B), as reported in Fig. 5a, b. Interestingly, MPTP mice receiving vitamin D exhibited significantly increased levels of these cytokines in comparison to animals treated with MPTP alone, that demonstrated very low levels, comparable to those in control mice, as reported in Fig. 5. To evaluate the alternative M2 phenotype of microglia in MPTP-treated mice and the therapeutic effects of vitamin D, we analyzed CD206, CD163 and CD204, typical markers of the M2 phenotype. Results of immunostaining showed an immunoreactivity increase of these markers in striatal microglia in MPTP animals treated with vitamin D following MPTP administration in comparison to those treated with MPTP alone. Conversely, in mice treated with MPTP alone, the levels of CD163, CD206 and CD204 resulted very low, virtually comparable to those observed in controls (Figs. 6, 7 and 8a). Results concerning western blotting analysis showed an up-regulation of these markers both in striatum and in SNpc of vitamin D-treated mice in comparison to controls (Fig. 6, 7 and 8b). In addition, an increased expression of all these M2 markers was detected in MPTP animals treated with vitamin D as compared to those treated with MPTP alone. Finally, in mice treated with MPTP alone, the levels of CD163, CD206 and CD204 resulted very low, virtually comparable to those observed in controls (Figs. 6, 7 and 8b).
Discussion
Increasing evidence has demonstrated that microglial activation is involved in the pathogenesis of PD (Hirsch and Hunot 2009). In this work the neuroprotective action of vitamin D on striatum and SNpc dopaminergic neurons was assessed by mapping TH, the rate-limiting enzyme for dopamine biosynthesis (Fig. 1). Interestingly, we observed that in vivo vitamin D treatment produced a significant neuroprotection of dopaminergic neurons against MPTP-induced neurotoxicity. The causes of the dopaminergic neuronal cell death in PD are not yet fully known and pharmacological treatments currently employed for PD are primarily dopamine replacement therapies, which can only alleviate certain symptoms and are often associated with serious side effects (Olanow and Schapira 2013). In the last decades, neuroinflammation and innate immunity have been considered to have an important role during the onset and progression of PD (Tansey and Goldberg 2010; Chen et al. 2008). M1 microglia are mainly considered to cause inflammation and neurotoxicity, whereas M2 microglia have shown roles in anti-inflammation and tissue repair (Blandini 2013). The unbalanced M1/2 microglia phenotype might contribute to accelerate neuron death.

In this study, we used a PD model based on C57BL/6 J mice treated with MPTP, a neurotoxin that causes the loss of dopaminergic neurons in the SNpc (Meredith and Rademacher 2011) generating a neuroinflammatory picture (Lofrumento et al. 2011, 2014), including an accumulation of Iba1-positive activated microglia that indicate local inflammation (Ito et al. 2001; Pikhovych et al. 2016) Based on a large volume of literature in the relevant field of macrophage polarization, we selected the markers of microglia M1 polarization, such as iNOS, IL-1β and TNF-α, as well as the markers of M2 polarization, such as CD206, CD163, TGF-β, IL-4 and IL-10 (Mstriatumherson et al. 2014; Benoit et al. 2008; Kou and Babensee 2011; Bosurgi et al. 2011; Genin et al. 2015).

A high expression of inflammatory markers was used as an index of activated M1 microglia cells. In our model, not only did vitamin D treatment in PD mice attenuate MPTP-induced iNOS and pro-inflammatory cytokines expression, but it was also able to upregulate the expression of the anti-inflammatory markers. Dopaminergic neurons death induced by MPTP administration triggers a primary inflammation response, which is suppressed by vitamin D administration.

An increase in neurotoxic microglia and inflammatory cytokines has been widely reported in PD, associated to dopaminergic neurons degeneration (De Lella Ezcurra et al. 2010; Pott Godoy et al. 2008; McCoy and Tansey 2008; Leal et al. 2013; Walsh et al. 2011). Activated microglia and pro-inflammatory cytokines have been described in different brain areas of parkinsonian subjects and in PD animal models (Imamura et al. 2003; Barcia et al. 2011). An elevated expression of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, as well as their receptors, has been described after MPTP intoxication in mice, and a reduction of their expression is neuroprotective in PD models (Sriram et al. 2006; Lofrumento et al. 2011).

In the current study, we found that the expression of several pro-inflammatory factors such as iNOS, TNF-α, and IL-1β was strongly suppressed by vitamin D treatment in the MPTP mouse model. These results are in agreement with a previous study showing that the exogenous administration of vitamin D significantly decreased 6-OHDA- and MPTP-induced dopaminergic neuronal loss in the SNpc by preventing the activation of microglia and inhibiting pro-inflammatory cytokines expression (Kim et al. 2006). Interestingly, we observed that the expression of several anti-inflammatory markers, including IL-10, TFG-β, IL-4, as well as CD163, CD204 and CD206, was also significantly increased by vitamin D treatment, thus demonstrating, for the first time, changes in anti-inflammatory cytokine production in the MPTP model of PD mice receiving vitamin D treatment. In this respect, our results suggest that Vitamin D in MPTP animals attenuates MPTP-initiated neuroinflammation via shifting M1 pro-inflammatory responses to M2 anti-inflammatory responses. In this context, it was reported that vitamin D can affect the innate and inflammatory responses by modulating the expression of specific functional markers (Kim et al. 2013; Verma and Kim 2016; Yin et al. 2015), thus supporting the idea that vitamin D may exerts immunoregulating properties.

TGF-β has been reported to have a protective activity on the TH-positive neurons against the neurotoxicity of MPTP and its metabolite–MPP+ (Krieglstein et al. 1995; Unsicker et al. 1996). Although there are also reports suggesting that TGF-β enhances the degeneration caused by MPTP, TGF-β has been described to influence the glial cells activity through limiting inflammatory cytokine expression and consequently decreasing the cytotoxic properties of activated microglia (de Sampaio e Spohr et al. 2002).

IL-10 is a potent anti-inflammatory cytokine secreted by innate immune cells, including dendritic cells, macrophages, mast cells, natural killer cells, eosinophils and neutrophils, and by adaptive immune cells, including TH1, TH2, TH17 and regulatory T cells (Tregs), as well as B cell subsets (Moore et al. 2001; Saraiva and O'Garra 2010). In the CNS, IL-10 is expressed by microglia, astrocytes and neurons (Gutierrez et al. 2014; Tarazi et al. 2014; Yan et al. 2014). IL-10 is implicated in neuroimmune diseases of varying

etiologies, including PD-associated neurodegeneration, where an insufficiency in IL-10 signaling/bioavailability and ongoing inflammation are described. Activation of IL-10 receptors leads to the inhibition of TNF-α, IL-1β, IL-6, IL-8, IL-12 and IL-23 release and an enhanced release of anti-inflammatory mediators such as IL-1 receptor antagonists and soluble TNF receptors (sTNFRs) from innate immune cells (Sabat et al. 2010).

Interestingly, IL-10 itself can also promote the M1 to M2 transition (Deng et al. 2012). Recently, an in vitro study has reported that vitamin D facilitates M2 polarization and upregulates TLR10 expression on human microglial cells, exerting an anti-inflammatory action (Verma and Kim 2016). Moreover, Boontanrart et al. reported that in the presence of vitamin D, activated microglia were able to reduce the expression of pro-inflammatory cytokines and increase the expression of IL-10 and IFN-β and that the reduction of pro-inflammatory responses was dependent on IL-10 induction of SOCS3 (Boontanrart et al. 2016). Although the precise mechanism of the different expression profiles of M1 and M2 after vitamin D treatment has not yet been clarified, this effect seems to be a promising aspect requiring further investigation.

To the best of our knowledge, the polarizing effect on microglia responses of vitamin D that we have observed in a MPTP preclinical mouse model of PD has never previously been reported, so our results provide novel support of the neuroprotective role of vitamin D in slowing down the progression of PD and, potentially, other degenerative diseases.

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Figures

Fig.1
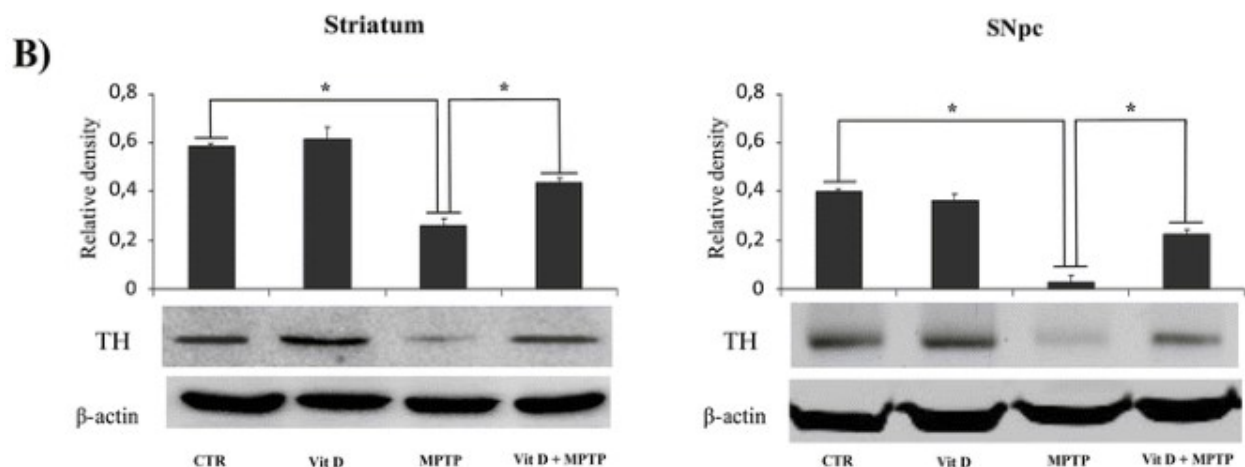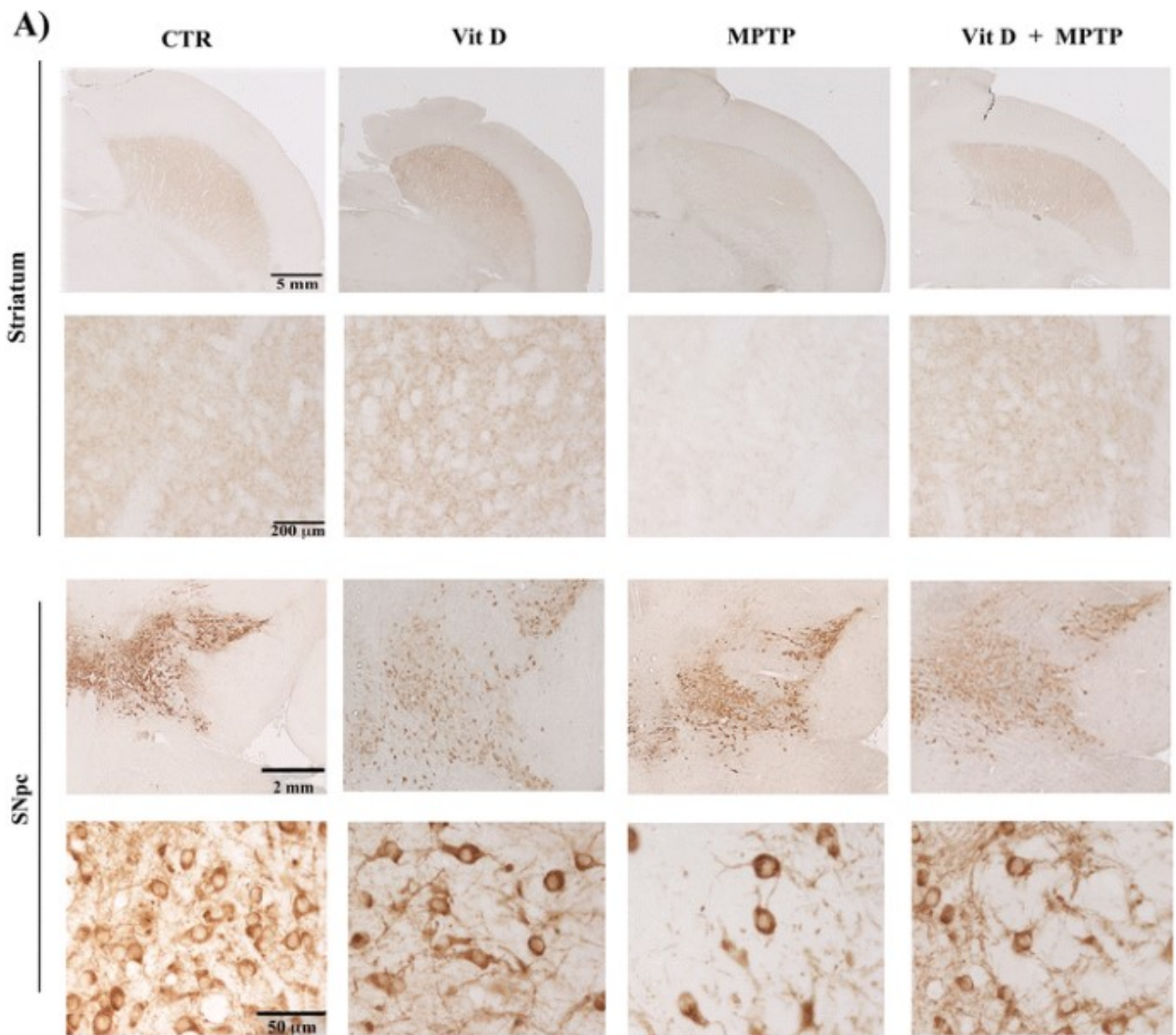
Tyrosine hydroxylase (TH) analysis. (a) TH immunoreactivity in the striatum (upper) and SNpc (lower) in controls (CTR), mice treated with vitamin D (Vit D), MPTP, vitamin D and MPTP (Vit D + MPTP). Scale bar: (Striatum) upper 5 mm, lower 200 μm; (SNpc) upper 2 mm, lower 50 μm. (b) Densitometric analysis of western blot bands showing TH expression levels in striatum (left) and SNpc (right). For TH protein expression analysis, values represent the relative optic density after normalization against β-actin expression. All values are expressed as means ±SD (n = 5 per group, 5 replicates). *$p < 0.05$ significantly different

Fig.2
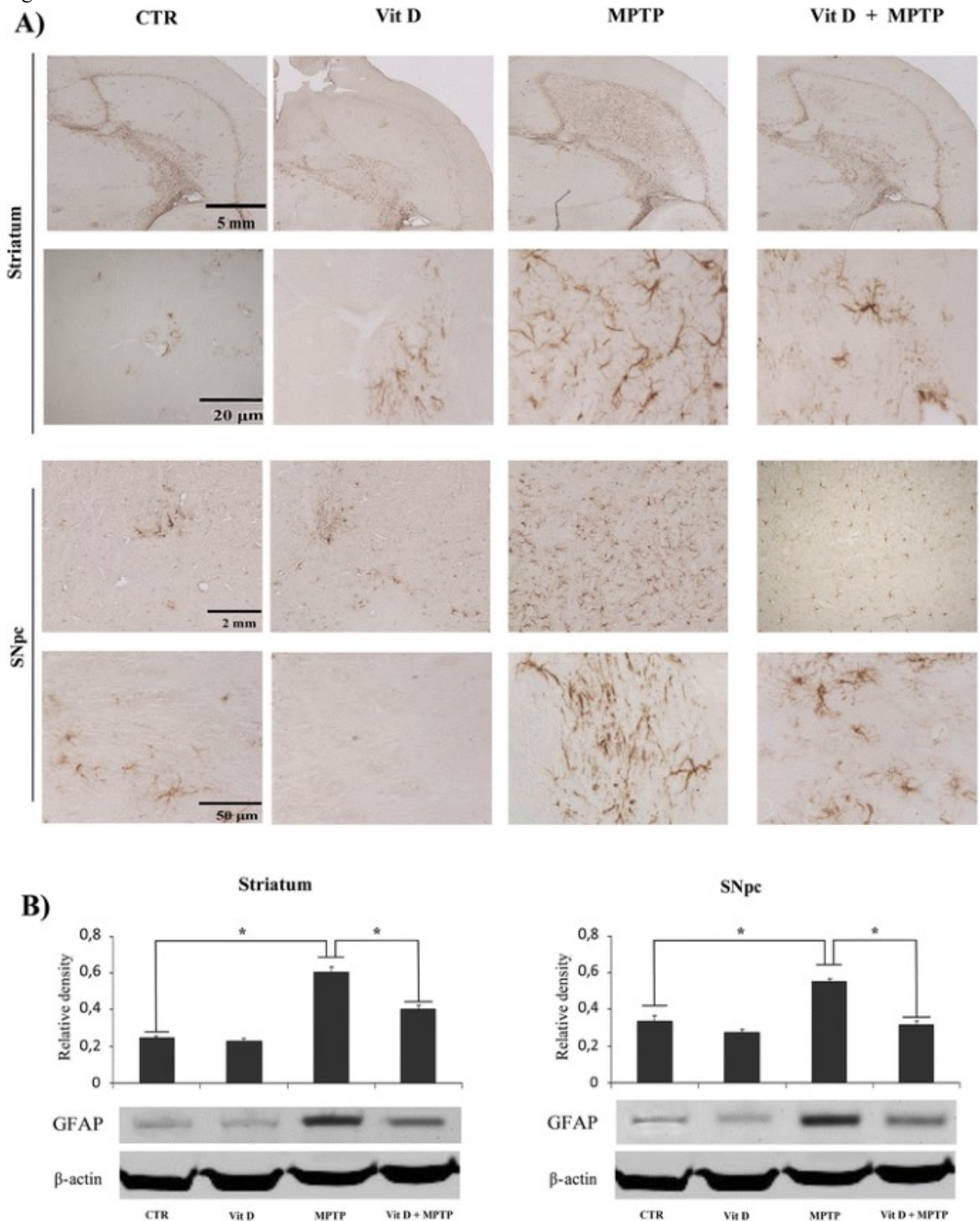
GFAP analysis. (a) GFAP immunoreactivity in the striatum (upper) and SNpc (lower) of controls (CTR), mice treated with vitamin D (Vit D), MPTP, vitamin D and MPTP (Vit D + MPTP). Scale bar: (Striatum) upper 5 mm, lower 20 μm; (SNpc) upper 2 mm, lower 50 μm. (b) Densitometric analysis of western blot bands showing GFAP expression levels in striatum (left) and SNpc (right). For GFAP protein expression
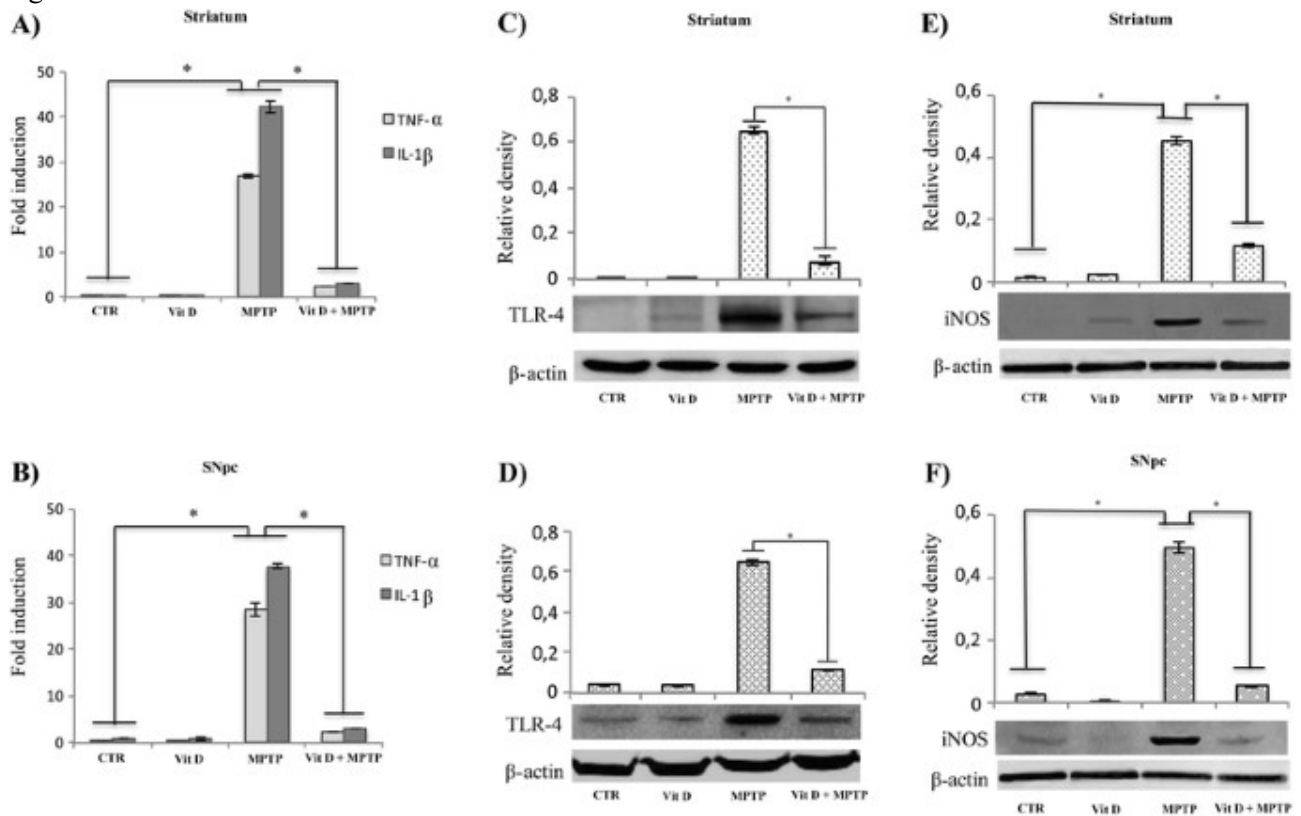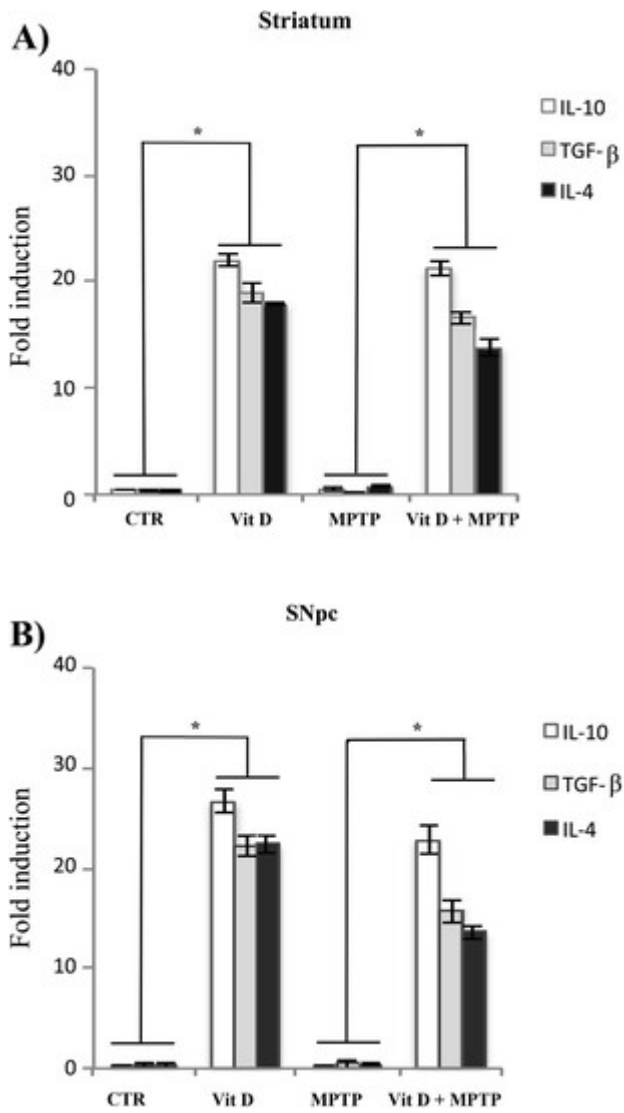
analysis, values represent the relative optic density after normalization against β-actin expression. All values are expressed as means ±SD (n = 5 per group, 5 replicates). *p < 0.05 significantly different

Fig.3

Iba1 analysis. (a) Representative microphotographs of Iba1 expression in striatum (upper) and SNpc (lower) on slices immunostainings of controls (CTR), mice treated with vitamin D (Vit D), MPTP, vitamin D and MPTP (Vit D + MPTP). Scale bar: (Striatum) upper 1 mm, lower 50 μm; (SNpc) upper 1 mm, lower 50 μm.
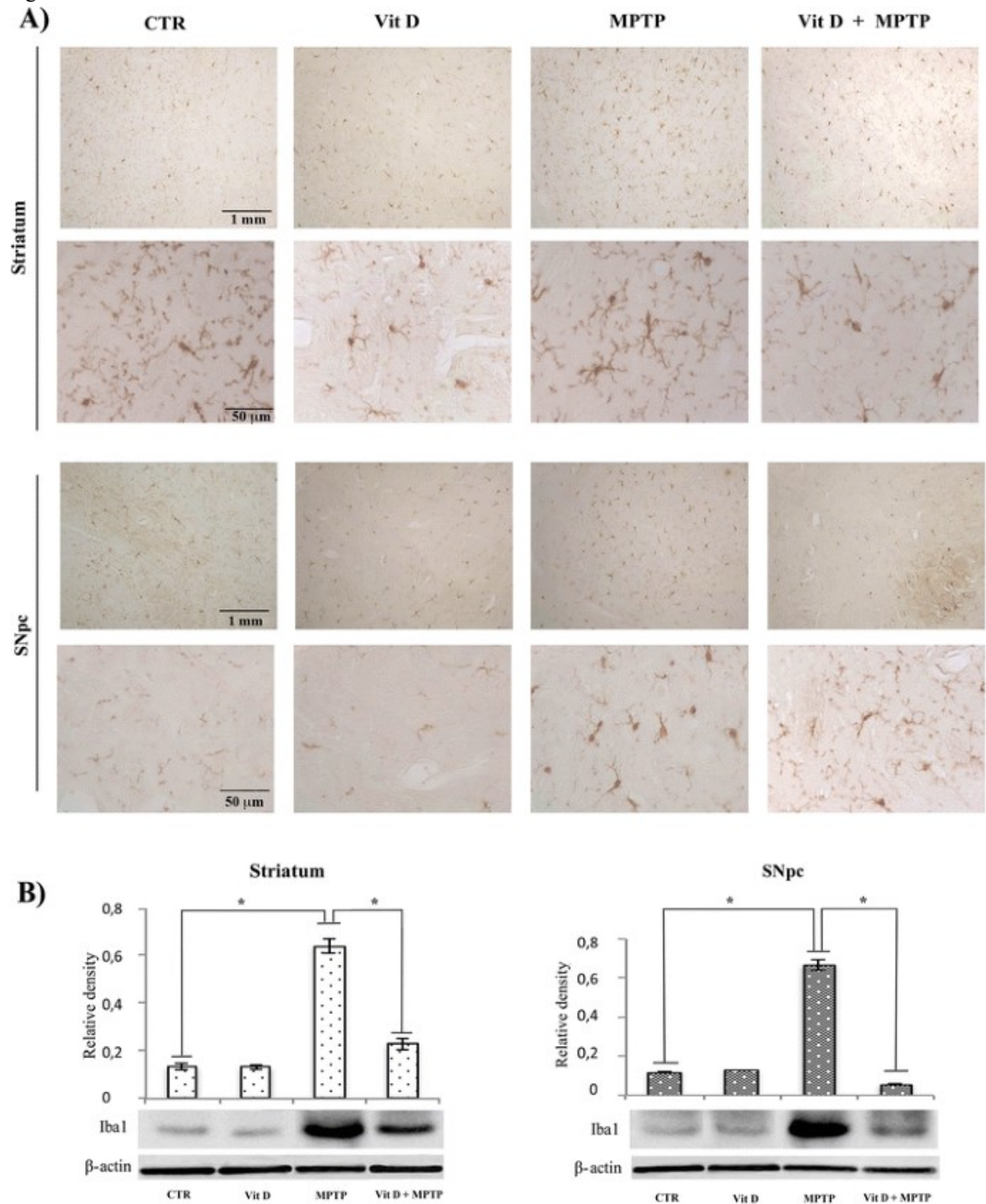
(b) Densitometric analysis of western blot bands showing Iba1 expression levels in striatum (left) and SNpc (right). For Iba1 protein expression analysis, values represent the relative optic density after normalization against β-actin expression. All values are expressed as means ±SD (n = 5 per group, 5 replicates). *p < 0.05 significantly different

Fig.4

Analysis of pro-inflammatory responses. Real-time PCR analysis of TNF-α and IL-1β mRNA expression levels in striatum (a) and SNpc (b) of controls (CTR), mice treated with vitamin D (Vit D), MPTP, vitamin D and MPTP (Vit D + MPTP). For mRNA real-time PCR analysis, values represent the mRNA fold changes relative to GAPDH used as resident control. All values are expressed as means ±SD (n = 5 per group, 5 replicates). *p < 0.05 significantly different. Densitometric analysis of western blot bands showing TLR-4 expression levels in striatum (c) and SNpc (d), and iNOS expression levels in striatum (e) and SNpc (f). For protein expression analysis, values represent the relative optic density after normalization against β-actin expression. All values are expressed as means ±SD (n = 5 per group, 5 replicates). *p < 0.05 significantly different

Fig.5

Analysis of anti-inflammatory responses. Real-time PCR analysis of IL-10, TGF-β and IL-4 mRNA expression levels in striatum (a) and SNpc (b) of controls (CTR), mice treated with vitamin D (Vit D), MPTP, vitamin D and MPTP (Vit D + MPTP). For mRNA real-time PCR analysis, values represent the mRNA fold changes relative to GAPDH used as resident control. All values are expressed as means ±SD (n = 5 per group, 5 replicates). *$p < 0.05$ significantly different
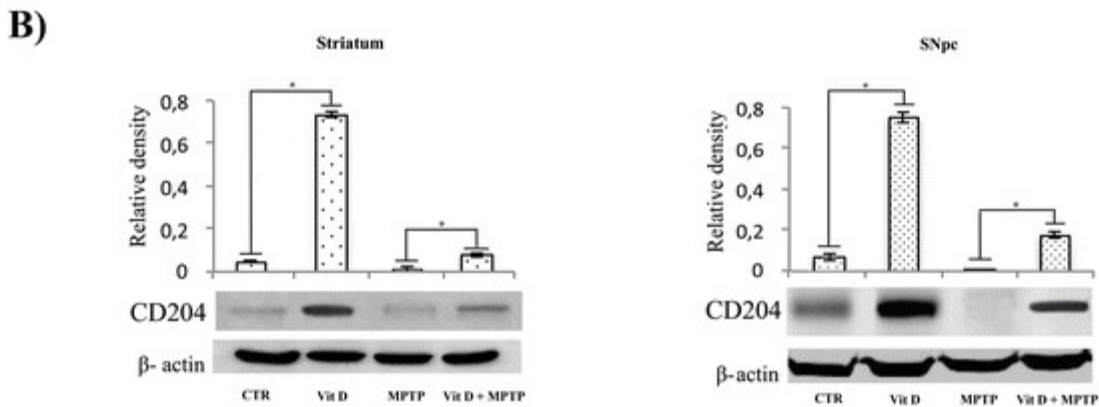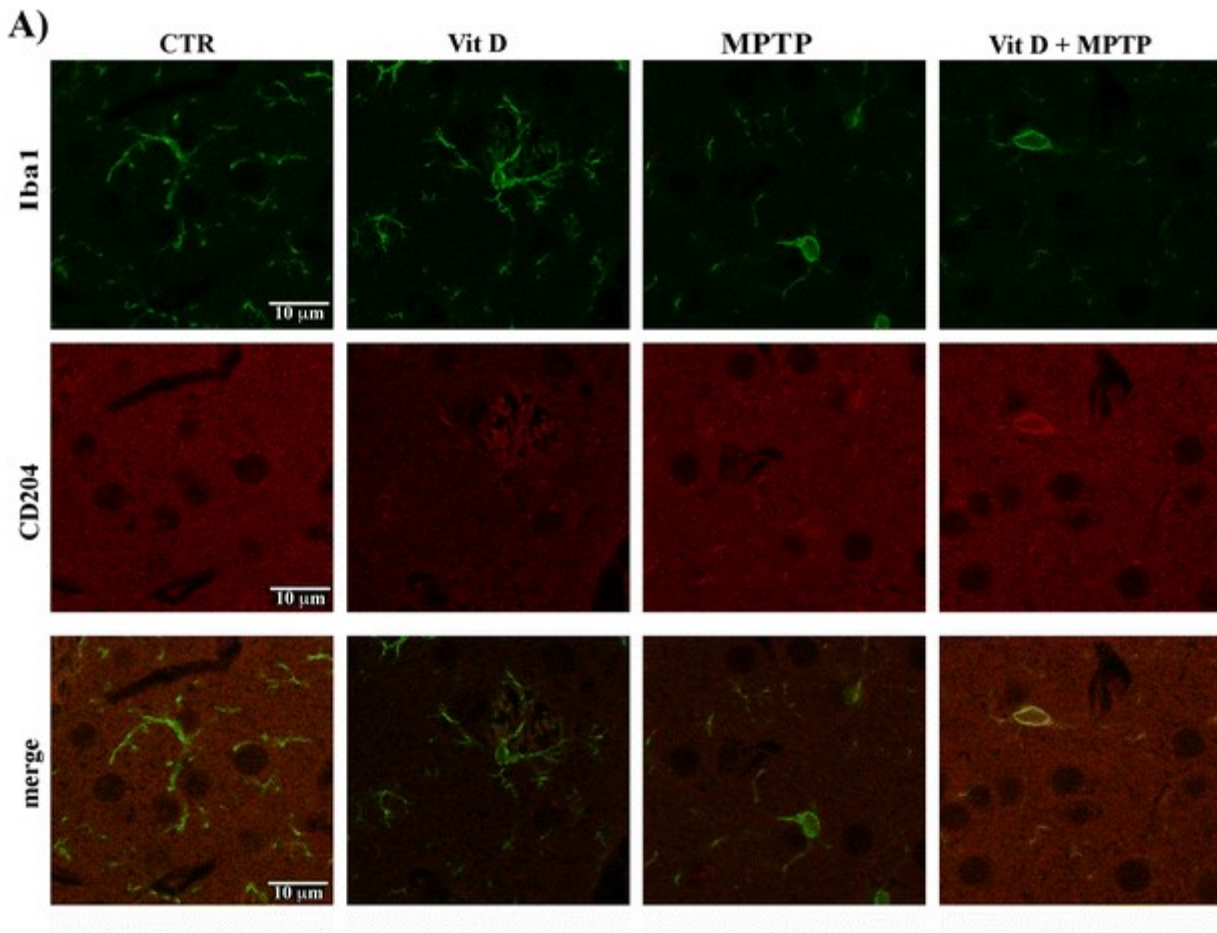
Fig. 6
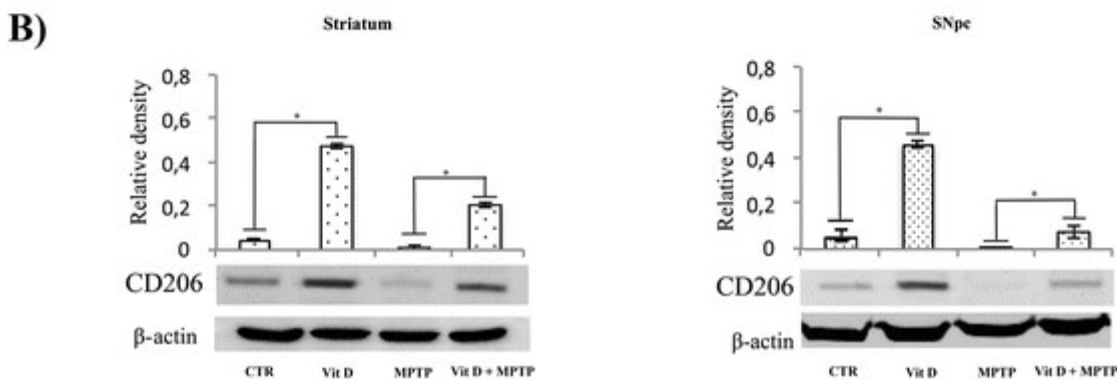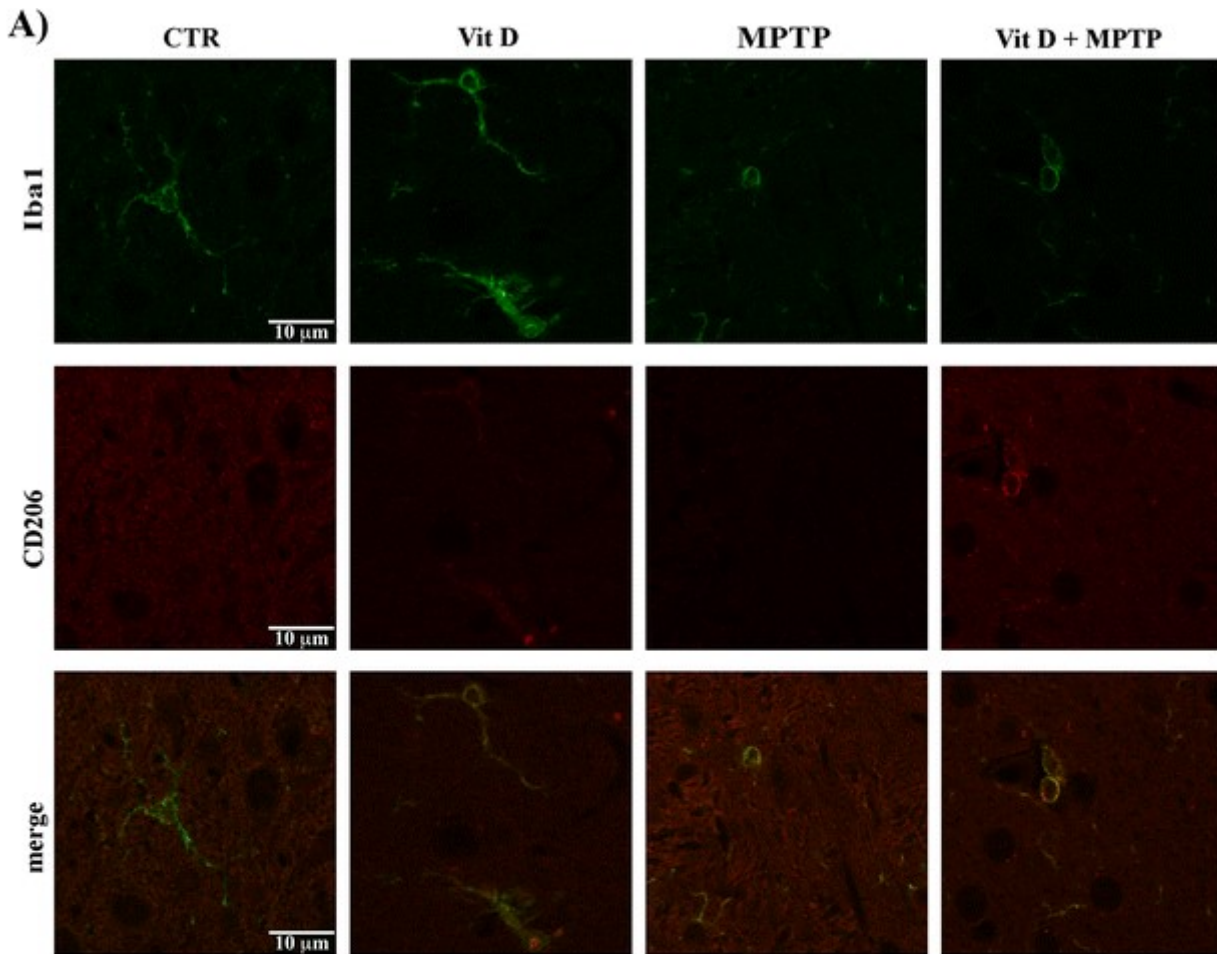
Analysis of CD206 expression. (a) Representative microphotographs of CD206 expression in Iba1-immunoreactive cells in striatum of controls (CTR), mice treated with vitamin D (Vit D), MPTP, vitamin D and MPTP (Vit D + MPTP). Scale bar: 10 μm. (b) Densitometric analysis of western blot bands showing CD206 expression levels in striatum (left) and SNpc (right); For protein expression analysis, values represent the relative optic density after normalization against β-actin expression. All values are expressed as means ±SD, (n = 5 per group, 5 replicates). *p < 0.05 significantly different

Fig.7
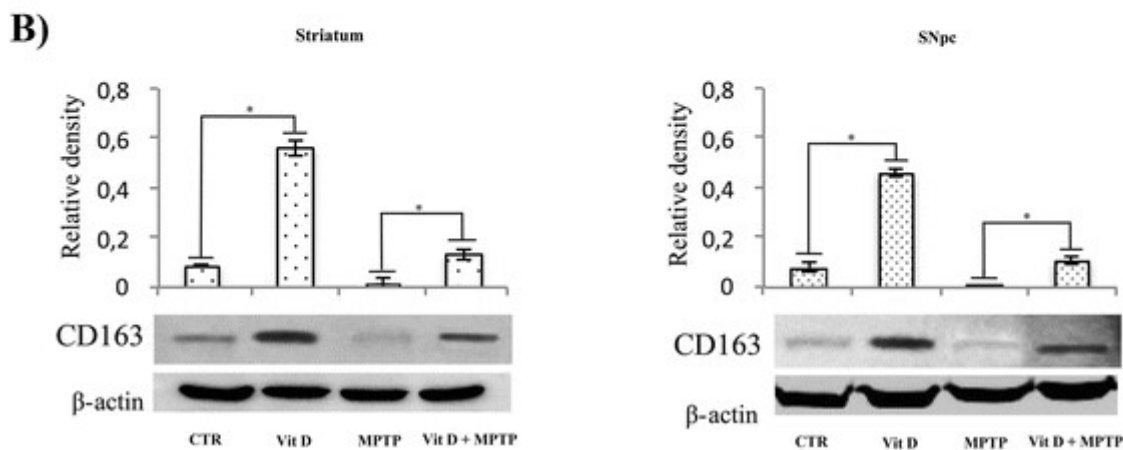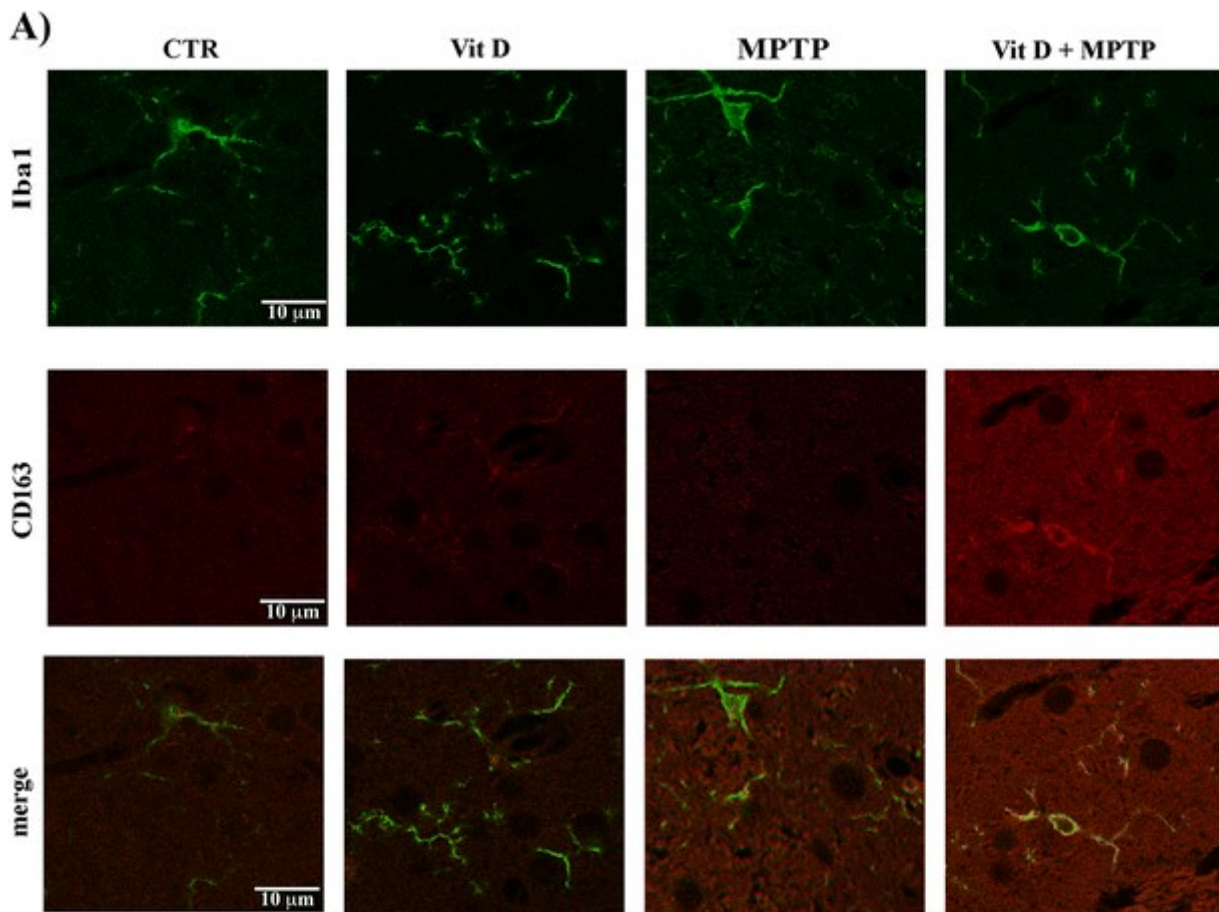
Analysis of CD163 expression. (a) Representative microphotographs of CD163 expression in Iba1-immunoreactive cells in striatum of controls (CTR), mice treated with vitamin D (Vit D), MPTP, vitamin D and MPTP (Vit D + MPTP). Scale bar: 10 μm. (b) Densitometric analysis of western blot bands showing CD206 expression levels in striatum (left) and SNpc (right); For protein expression analysis, values represent the relative optic density after normalization against β-actin expression. All values are expressed as means ±SD, (n = 5 per group, 5 replicates). *p < 0.05 significantly different

Fig.8

Analysis of CD204 expression. (a) Representative microphotographs of CD204 expression in Iba1-immunoreactive cells in striatum of controls (CTR), mice treated with vitamin D (Vit D), MPTP, vitamin D and MPTP (Vit D + MPTP). Scale bar: 10 μm. (b) Densitometric analysis of western blot bands showing CD206 expression levels in striatum (left) and SNpc (right); For protein expression analysis, values represent the relative optic density after normalization against β-actin expression. All values are expressed as means ±SD, (n = 5 per group, 5 replicates). *p < 0.05 significantly different