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# The neuroprotection of cannabidiol against $MPP^+$-induced toxicity in PC12 cells involves trkA receptors, upregulation of axonal and synaptic proteins, neuritogenesis, and might be relevant to Parkinson's disease

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ABSTRACT

Cannabidiol (CBD) is a non-psychoactive constituent of *Cannabis sativa* with potential to treat neurodegenerative diseases. Its neuroprotection has been mainly associated with anti-inflammatory and antioxidant events; however, other mechanisms might be involved. We investigated the involvement of neuritogenesis, NGF receptors (trkA), NGF, and neuronal proteins in the mechanism of neuroprotection of CBD against $MPP^+$ toxicity in PC12 cells. CBD increased cell viability, differentiation, and the expression of axonal (GAP-43) and synaptic (synaptophysin and synapsin I) proteins. Its neuritogenic effect was not dependent or additive to NGF, but it was inhibited by K252a (trkA inhibitor). CBD did not increase the expression of NGF, but protected against its decrease induced by $MPP^+$, probably by an indirect mechanism. We also evaluated the neuritogenesis in SH-SY5Y cells, which do not express trkA receptors. CBD did not induce neuritogenesis in this cellular model, which supports the involvement of trkA receptors. This is the first study to report the involvement of neuronal proteins and trkA in the neuroprotection of CBD. Our findings suggest that CBD has a neurorestorative potential independent of NGF that might contribute to its neuroprotection against $MPP^+$, a neurotoxin relevant to Parkinson's disease.


## 1. Introduction

Cannabidiol (CBD) is a non-psychoactive compound of *Cannabis sativa* with anti-inflammatory and antioxidant properties and consequently, a potential for neuroprotection (Zuardi et al., 2006; Fernandez-Ruiz et al., 2013). Several studies have suggested the potential of CBD in the treatment of neurodegenerative diseases (ND) such as Parkinson's disease (PD) (Chagas et al., 2014), Alzheimer's disease (AD) (Fernandes-Ruiz et al., 2005) and Huntington's disease (HD) (Sagredo et al., 2007). Despite that, the precise molecular mechanisms underlying the therapeutic potential of CBD remain unclear. Many targets of CBD such as receptors, ion channels, enzymes and transporters have been identified (Fernandez-Ruiz et al., 2013); however, the role of neuritogenesis in the neuroprotection of CBD has not been clarified yet.

Neurite loss is an early event in neurodegenerative diseases and the regeneration of the network of neurites constitutes a strategy for the treatment of such disorders. Neurotrophic factors play a critical role in neuronal regeneration, but their clinical use is limited by their inability to cross the blood brain barrier (Gottlieb et al., 2010). It is noteworthy that, CBD is not psychoactive, but it can readily cross the blood brain barrier and exert its beneficial effects on the brain (Deiana et al., 2012). The trophic signaling of nerve growth factor (NGF) has been associated with the prevention of neuronal apoptosis mediated by activation of the anti-apoptotic protein $Bcl_2$. The lack of stimulation by NGF and the resulting inactivation of $Bcl_2$ trigger the intrinsic pathway (mitochondrial) of apoptosis through mitochondrial permeability transition (MPT), release of cytochrome c and activation of the caspase cascade (Wolozin and Behl, 2000).

Loss and dysfunction of trophic factors seem to be involved in the pathogenesis of AD and PD (Connor and Dragunow, 1998; Siegel and Chauhan, 2000). Decrease of neurotrophic factors such as BDNF and NGF has been reported in the brain and cerebrospinal fluid of PD patients (Mogi et al., 1999; Nagatsu et al., 2000). Additionally, a study showed significant reduction of serum levels of NGF in parkinsonian rats and in patients with PD. Interestingly, NGF levels in early stages of

*Abbreviations:* AD, Alzheimer's disease; BDNF, brain derived neurotrophic factor; CBD, cannabidiol; FBS, fetal bovine serum; GAP-43, growth associated protein; GDNF, glial-derived neurotrophic factor; HD, Huntington's disease; $MPP^+$, 1-methyl-4-phenylpyridinium iodide; MPT, mitochondrial permeability transition; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NGF, nerve growth factor; PD, Parkinson's disease; trk receptors, tyrosine kinase receptors.

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the disease (Grades I–II) were lower in comparison to those found in advanced stages (Grades III–IV) (Lorigados Pedre et al., 2002).

Based on these premises, the present study investigated the neurorestorative potential of CBD and the pathways that mediate it by using PC12 cells treated with the neurotoxin $MPP^+$, a metabolite of MPTP (1-methyl-4-phenyl-1,2,5,6tetrahydropyridine), known to induce Parkinsonism in vivo. The involvement of the NGF-pathway was evaluated by using a specific inhibitor of trk receptors and two cell lines (PC12 and SH-SY5Y) with distinct phenotypes for these receptors. The role of NGF in the protective mechanism of CBD was also investigated.

## 2. Materials and methods

### 2.1. Chemicals

Only high purity reagents were used (analytical grade minimum). Reagents were obtained from Sigma-Aldrich®, unless specified differently. Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). Reagents for Western blot were purchased from Bio-Rad®. Type I water (ultra-pure) obtained in the purification system by reverse osmosis (Rios DI-3), followed by purification in a Milli-Q Gradient system (Millipore, Bedford, USA) was used in the preparation of solutions. CBD was obtained from THC-Pharm, Frankfurt, Germany. CBD solutions were prepared in DMSO, and therefore controls were treated with the same volume of DMSO.

### 2.2. Cellular cultures

PC12 and SH-SY5Y cells were obtained from the American Type Culture Collection, ATCC (Rockville, MD).

PC12 cells were grown in DMEM high glucose with 10% horse serum, 5% fetal bovine serum and 1% PNS (5 mg/ml penicillin, 10 mg/ml neomycin and 5 mg/ml streptomycin, GIBCO®). PC12 cells were cultured in 75 $cm^2$ tissue-culture flasks at 37 °C under a humidified atmospheric condition of 5% $CO_2$ and 95% air. Medium was replaced every 2 days. Confluent cultures were washed with pre-warmed phosphate buffered saline (PBS), detached with trypsin/EDTA solution (GIBCO®), inactivated with growth medium, centrifuged, and subcultured (1:2; every 7–9 days). Third-passage cells with 80% confluence were used in the experiments.

SH-SY5Y cells were grown in F12 nutrient mixture (F12 HAM; Sigma Cell Culture, St. Louis, MO) supplemented with 15% fetal bovine serum (GIBCO) and 1% PNS. Cells were cultured in 75 $cm_2$ tissue-culture flasks at 37 °C under a humidified atmospheric condition of 5% $CO_2$ and 95% air. Medium was replaced every day. Confluent cultures were detached with trypsin/EDTA solution (GIBCO®), inactivated with growth medium, centrifuged, and subcultured (1:2; every 2–3 days). Third-passage cells with 80% confluence were used in the experiments.

### 2.3. $MPP^+$/PC12 model

The rat pheochromocytoma cell line PC12 is a widely used cell model to study neuronal differentiation. The toxicity was induced with 1 mM $MPP^+$ (IC50) for cell-viability-related assays (MTT and caspase-3) or 100 μM $MPP^+$ (sub-lethal concentration) for neurite-related assays. These concentrations were based on our previous findings showing that the impairment of neurite outgrowth is an early event that precedes cell death and occurs at sub-lethal concentrations of $MPP^+$ (dos Santos et al., 2014). Cells were plated in 12-well (neurites assay) or 96-well (cell viability) plates at the appropriate density for each assay and incubated for 24 h prior to the following additions: 1 mM/100 μM $MPP^+$; CBD (1, 5, 10, 25 and 50 μM); and 1 mM/100 μM $MPP^+$ + CBD (1, 5 and 10 μM).

### 2.4. Cell viability — MTT assay

The colorimetric assay MTT is the most widely used indicator of cell viability. It evaluates the cellular mitochondrial function based on the enzymatic reduction of the tetrazolium salt by mitochondrial dehydrogenases of viable cells (Mosmann, 1983). A previously described procedure (Hansen et al., 1989) with minor modifications was used. Treated cells ($2.0 \times 10^4$ cells/well) were incubated in 96-well plates for 24 h. The medium was removed and 20 μl of MTT (5 mg/ml in phosphate buffered saline) was added to each well (final concentration 0.5 mg/ml). Then the plate was incubated for 3 h at 37 °C. A negative control (cells + MTT solution) and a positive control (cells + Triton X-100 + 0.2% MTT solution) were assayed. After the incubation period, the supernatant was removed and 200 μl DMSO was added to promote cell lysis. The plate was mixed (37 °C, 5 min) and absorbance was determined at 570 nm, in a microplate reader (Multiskan FC, Thermo Scientific).

### 2.5. Caspase-3 activity

An aliquot of 1 ml of cell suspension ($10^7$ cells/ml) was incubated with 1 mM $MPP^+$ (positive control) or 1 mM $MPP^+$ plus 1 μM CBD in 24-well plates (at 37 °C, for 24 h). Controls were incubated without additions. After that, the medium was removed and cells were assayed for caspase-3 activity by using the Colorimetric Caspase 3 Assay Kit (Sigma, MO, USA) according to the manufacturer's instructions. The activity of caspase-3 was assessed by monitoring the absorbance at 405 nm.

### 2.6. Neurite outgrowth: quantitative assay in PC12 cells

PC-12 cells were incubated in 24-well plates ($1 \times 10^5$ cells/well) for 24 h for adhesion. Then, the medium was replaced by Ham's F-12K (Kaighn's) Medium supplemented with 1% horse serum and 1% antibiotic mixture (penicillin/streptomycin/neomycin, PSN GIBCO®). Cells were then incubated (37 °C, 72 h) with one of the following additions: 1 μM CBD, NGF ("nerve growth factor from *Vipera lebetina* venom", 100 ng/ml), 100 μM $MPP^+$ or 100 μM $MPP^+$ + 1 μM CBD. Untreated cells were used as controls. The neurite outgrowth was assessed by inverted phase contrast microscopy (Carl Zeiss Axio Observer A1 inverted microscope, 400× magnification). Phase-contrast photomicrographs of 4 fields per well were taken after 72 h-incubation. The percentage of cells with neurites was determined in digitalized images by using the Image J open source software (Rasband, 1997-2014). Only those cells with at least one neurite with a length equal to or greater than the diameter of the cell body were considered differentiated (Das et al., 2004).

### 2.7. Neurite outgrowth: quantitative assay in SH-SY5Y neuroblastoma cells

Cells were incubated in 24-well plates ($3 \times 10^4$ cells/well) in F12 HAM supplemented with 15% fetal bovine serum (FBS) and 1% antibiotic mixture (penicillin/streptomycin/neomycin, PSN GIBCO®) for 24 h for adhesion. After this period, the medium was replaced (F12 HAM supplemented with 1% FBS, 1% PSN and retinoic acid 10 μM) and cells were incubated for 7 days, during which the medium was renewed every 2 days. Then, cells were treated with 1 μM CBD and incubated at 37 °C for 72 h. Untreated cells were used as controls. The neurite outgrowth was assessed by inverted phase contrast microscopy (Carl Zeiss Axio Observer A1 inverted microscope, 400× magnification). The percentage of cells with neurites was determined in digitalized images by using the Image J open source software (Rasband, 1997-2014). Only those cells with at least one neurite with a length equal to or greater than the diameter of the cell body were considered differentiated (Das et al., 2004).

### 2.8. Trk receptors inhibition assay

K-252a (Sigma-Aldrich®, St. Louis, MO, USA) is a specific inhibitor of trk (tyrosine kinase) receptors and thus, selectively blocks the effect of nerve growth factor (NGF) and agonists of trkA in PC12 cells (Tapley et al., 1992). PC-12 cells were incubated in 24-well plates ($2 \times 10^5$ cells) for 24 h for adhesion. Then, the medium was replaced by Ham's F-12K (Kaighn's) Medium supplemented with 1% horse serum and 1% PSN antibiotic (penicillin/streptomycin/neomycin GIBCO®). Cells were then incubated (37 °C, 168 h), with one of the following additions: CBD (1 μM), NGF (100 ng/ml), K252a (100 nM) + CBD (1 μM) and K252a (100 nM) + NGF (100 ng/ml). Untreated cells were used as controls. The neurite outgrowth was assessed by inverted phase contrast microscopy (Carl Zeiss Axio Observer A1 inverted microscope, 400× magnification). Phase-contrast photomicrographs of 4 fields per well were taken after 72 h, 96 h, 120 h, 144 h and 168 h of incubation. The percentage of cells with neurites was determined in digitalized images by using the Image J open source software (Rasband, 1997-2014). Only those cells with at least one neurite with a length equal to or greater than the diameter of the cell body were considered differentiated (Das et al., 2004).

### 2.9. Determination of NGF levels

Cells (2.0 × 105 cells/well) were plated in 24-well plates and incubated at 37 °C for 24 h prior to the following additions: 1 μM CBD, NGF 100 ng/ml (positive control), 100 μM $MPP^+$ or 100 μM $MPP^+$ + 1 μM CBD (untreated cells were used as controls). Then cells were incubated at 37 °C for 72 h. After that, an aliquot of 100 μl of the supernatant was analyzed by using the Enzyme-Linked Immunosorbent Assay kit RAB0381 (Sigma-Aldrich®, St. Louis, MO, USA) as recommended by the manufacturer. The absorbance at 450 nm was determined in a microplate reader (Multiskan FC, Thermo Scientific).

### 2.10. Western blot analysis — synaptophysin, synapsin and GAP-43

After additions and incubation (72 h, 37 °C), cells ($2 \times 10^5$ cells/well) were rinsed with cold PBS and mixed with 200 μl Tris–Triton lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, Triton X-100 1%, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1:200 protease inhibitor cocktail, and 1% phosphatase inhibitor). The lysis procedure was performed with tubes placed on ice to reduce the activity of proteases. The cells were then harvested and transferred to centrifuge microtubes and incubated (300 rpm, 30 min, 4 °C). After centrifugation (12,000 rpm for 20 min at 4 °C), the precipitates were discarded, and cell lysates (supernatants) were stored in a freezer (−80 °C) until assayed. A 10 μl aliquot of each cell lysate was assayed for protein determination by the Bradford method described below.

#### 2.10.1. Determination of protein in cell lysate (Bradford)

The reactive color Protein Assay Dye Reagent Concentrate (Bio-Rad) was used according to the protocol suggested by the manufacturer. The lysates were diluted with water (1:5) and a calibration curve of BSA (40, 100, 200 and 400 mg/ml) was prepared. The color reagent was diluted with water (1:5). The color reaction was performed in 96-well plates by adding 10 μl of diluted sample or standard and 200 μl of diluted dye reagent. The absorbance (595 nm) was determined in a microplate reader after 5 minute-agitation. Due to the known interference of some components of the lysis buffer in the reaction of Bradford, controls containing 10 μl of diluted lysis buffer were carried out and the average absorbance of them was subtracted from the absorbance values of samples. Concentrations of protein in samples were calculated based on the calibration curve response and multiplied by the dilution factor (×5).

#### 2.10.2. SDS-PAGE

Samples were diluted in equal volume of Laemli sample buffer (2×) and heated for 10 min at 95 °C. Aliquots of 35 μl containing 10 μg total protein were applied to 10% polyacrylamide gel (10 wells) and separated by electrophoresis (1 h, 160 V) in an electrophoresis tank containing 1 l of Tris/glycine buffer (Bio-Rad).

#### 2.10.3. Transfer

Proteins were transferred to nitrocellulose membranes (1 h, 0.37 A) in an electrophoresis tank (Bio-Rad) containing 1 l of Tris/glycine/SDS buffer (Bio-Rad).

#### 2.10.4. Immune reaction

The membranes were blocked (30 min, RT, 300 rpm) with 5% BSA in Tween 20/TBS buffer (TTBS), and incubated (overnight, 4 °C, 300 rpm) with primary antibody: anti-synaptophysin (1:400), or anti-GAP-43 (1:1250), or anti-synapsin I, (1:1000); washed with TTBS and incubated (1 h, RT, 300 rpm) with a secondary antibody conjugated to horseradish peroxidase (anti-mouse or anti-rabbit IgG-HRP, 1:20,000). The membranes were washed with TTBS and TBS and treated with 3 ml of chemiluminescence enhancer detection reagents (1:1). Bands were detected by chemiluminescence and images were captured by using a ChemiDoc system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantitation of the digitalized images was performed based on the optical densitometry (OD) of the bands by using the open source software Image J (Rasband, 1997-2014). Subsequently membranes were stripped (2% SDS, 62.5 mM Tris pH 6.8 and 100 mM mercaptoethanol) and reprobed for β-actin protein (loading control). Immunoreaction was carried out by using a monoclonal anti-β-actin antibody (1:3000) and anti- mouse IgG-HRP (1:20,000). The procedures for chemiluminescent detection were repeated. After quantification of β- actin bands, OD values of synaptophysin, synapsin and GAP-43 were divided by the OD values of β-actin for normalization of the results (L'Episcopo et al., 2011). All antibodies were purchased from Sigma-Aldrich® (St Louis, MO, USA) and the dilutions used were within the range recommended by the manufacturer.

### 2.11. Statistical analysis

Data shown are the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicate (n = 9). Statistical comparisons were performed using One-way ANOVA with Tukey's multiple comparisons test and the software GraphPad Prism version 6:00 for Windows (La Jolla California USA). $p < 0.05$ was used as the level of significance.

## 3. Results

### 3.1. Cell death induced by $MPP^+$. Dose–response curve

The values of the absorbance obtained from the MTT assay were normalized (0–100% range) and the concentrations of $MPP^+$ were transformed into the corresponding logarithm. The IC50 was calculated using the equation: Y = 100/(1 + 10 ^ (LogIC50 − X) * hillslope) and it was 0.9836 to 1.025 mM (95% confidence interval). Therefore, the concentration used in the viability assays was 1 mM. The non-linear regression curve is presented in Fig. 1.

### 3.2. CBD increased cell viability in $MPP^+$-treated PC12 cells

When assayed alone, CBD (10 μM) did not alter the viability of PC12 cells (94.39 ± 2.97) as compared to control (99.6% ± 1.05%). $MPP^+$ (1 mM) significantly reduced cell viability (41.10% ± 0.39) as compared to control (99.6 ± 1.05%). CBD significantly increased cell viability (1 μM, 62.56 ± 1.481%; 5 μM, 52.93 ± 1.27%; and 10 μM, 51.18 ± 1.33%) as compared to $MPP^+$ alone (41.10% ± 0.39). Controls were

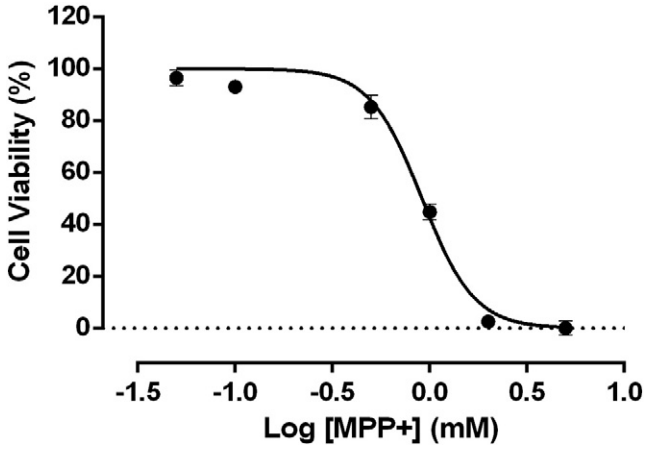


**Fig. 1.** Effect of $MPP^+$ on viability of PC12 cells. Dose–response curve. Cells were incubated (24 h/37 °C) with different concentrations of $MPP^+$ (0 to 5 mM). IC50 = 1 mM. The trace is representative of three independent experiments; each experiment was performed in triplicate.

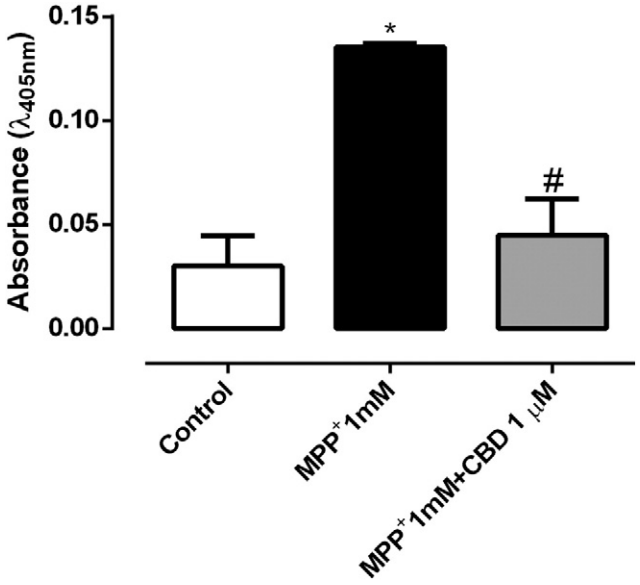


**Fig. 3.** Effect of CBD on caspase-3 activation. Data are representative of three independent experiments; each experiment was performed in triplicate. *Significantly different from control group, #Significantly different from $MPP^+$, ($p < 0.05$).

arbitrarily set as 100% and all the other values were normalized based on controls (Fig. 2).

### 3.3. CBD decreased the activity of caspase-3 in $MPP^+$-treated PC12 cells

$MPP^+$ significantly increased the activity of caspase-3 (0.1354 ± 0.00163) as compared to controls (0.0301 ± 0.01443); whereas CBD decreased the activity of caspase-3 (0.04485 ± 0.01764) as compared to the $MPP^+$ group (Fig. 3).

### 3.4. CBD alone induced cellular differentiation on PC12 cells

CBD alone (27.19 ± 0.047%) and NGF (17.38 ± 0.93%) induced differentiation in PC12 cells as compared to controls (10.14 ± 0.65%) (Fig. 4A). Phase-contrast photomicrographs of controls, NGF and CBD are presented in Fig. 4B,C and D, respectively.

### 3.5. K252a reduced the cellular differentiation induced by CBD on PC12 cells

No significant difference was observed between cells treated with K252a alone (72 h, 6.29 ± 0.15; 96 h, 14.42 ± 0.21; 120 h, 13.77 ± 1.27; 144 h, 12.82 ± 0.12 and 168 h, 6.32 ± 0.95) and controls (untreated, non-NGF stimulated cells) during the whole period of incubation. NGF (72 h, 14.08 ± 0.74; 96 h, 22.26 ± 1.37; 120 h, 23.77 ± 1.99; 144 h, 21.19 ± 1.92 and 168 h, 14.90 ± 1.12%) and CBD (72 h, 12.99 ± 0.82; 96 h, 22.69 ± 1.18; 120 h, 21.67 ± 0.59; 144 h, 21.10 ± 1.48 and 168 h, 14.21 ± 0.95%) significantly increased the differentiation as compared to controls (72 h, 6.21 ± 0.22; 96 h, 14.50 ± 0.12; 120 h, 13.22 ± 0.87; 144 h, 12.44 ± 1.11 and 168 h, 6.39 ± 0.26). The percentage of differentiated cells in the NGF + K252a group was decreased (72 h, 7.30 ± 0.81; 96 h, 12.85 ± 0.77; 120 h, 12.97 ± 0.54; 144 h, 8.98 ± 0.86; and 168 h, 6.66 ± 0.94%) as compared to NGF alone (72 h, 14.08 ± 0.74; 96 h, 22.26 ± 1.37; 120 h, 23.77 ± 1.99; 144 h, 21.19 ± 1.92 and 168 h, 14.90 ± 1.12%). Differently from NGF effect, the inhibition of CBD effect by K252a was only significant after 120 h of incubation (72 h, 12.68 ± 0.72; 96 h, 20.61 ± 0.88; 120 h, 15.65 ± 1.27; 144 h, 10.37 ± 0.63 and 168 h, 8.57 ± 0.67%) as compared to CBD alone (72 h, 12.99 ± 0.82; 96 h, 22.69 ± 1.18; 120 h, 21.67 ± 0.59; 144 h, 21.10 ± 1.48 and 168 h, 14.21 ± 0.95%) (Fig. 5A–E).

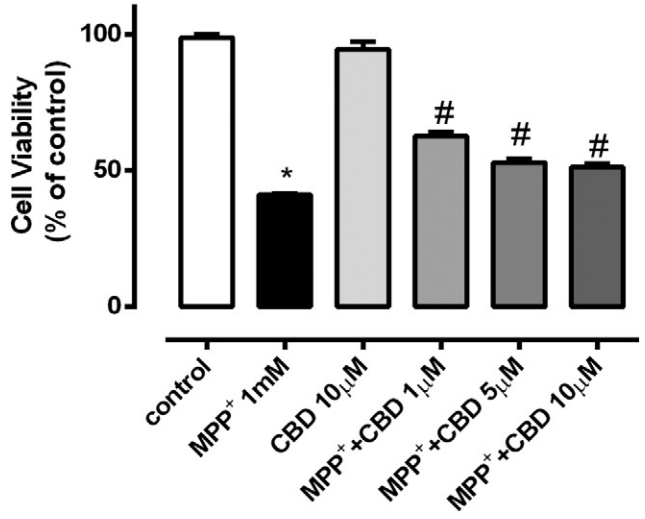


**Fig. 2.** Effect of CBD on $MPP^+$-induced cell death. Data are representative of three independent experiments; each experiment was performed in triplicate. *Significantly different from control group ($p < 0.05$).

### 3.6. CBD protected against the inhibition of cellular differentiation induced by $MPP^+$

CBD significantly increased the cellular differentiation (4.90 ± 0.59) in cells treated with $MPP^+$ (1.02 + 0.60) as presented in Fig. 6A. Phase-contrast photomicrographs of the $MPP^+$ group and the $MPP^+$ + CBD group are presented in Fig. 6B and C, respectively.

### 3.7. The effect of CBD on neuritogenesis is not additive to the effect of NGF

NGF (9.8 ± 0.41%), CBD (12.39 ± 0.70%) and CBD + NGF (12.77 ± 0.34%) increased the percentage of differentiated cells in relation to control (2.69 ± 0.25%). No significant difference was observed between the effects of CBD alone and CBD + NGF (Fig. 7).

### 3.8. CBD did not increase the levels of NGF in PC12 cells

$MPP^+$ decreased (0.0053 ± 0.00067) and CBD did not significantly affect (0.021 ± 0.0018) the levels of NGF as compared to control

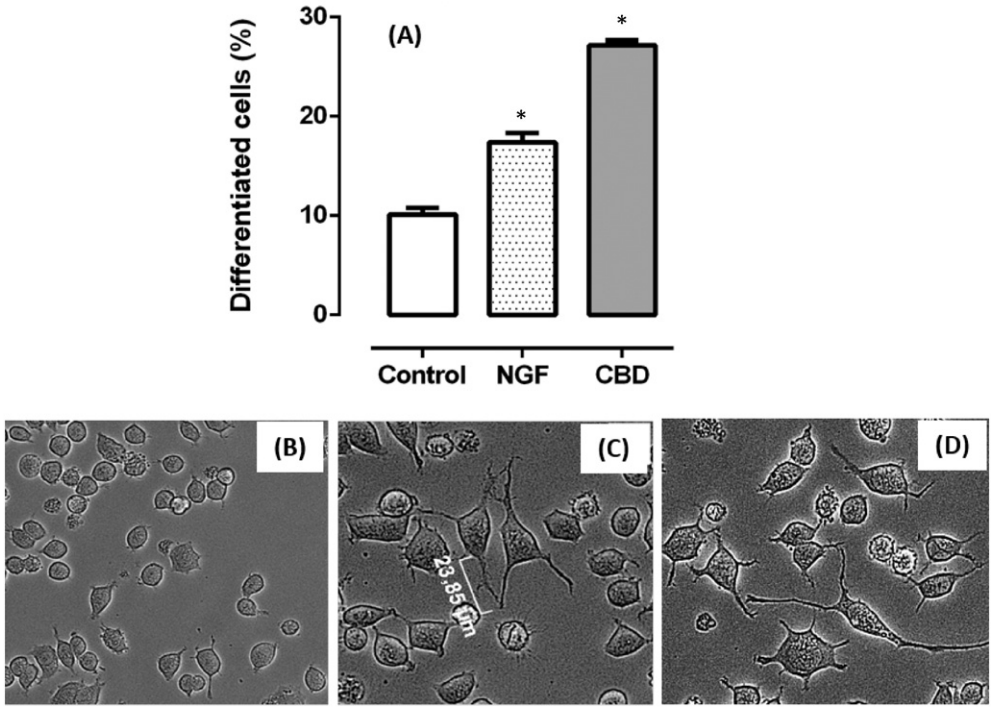


**Fig. 4.** Effect of CBD on differentiation of PC12 cells. Cells were incubated for 72 h with/without NGF 100 ng/ml or CBD 1 μM. Cells with at least one neurite with a length equal to or greater than the cell body were counted and expressed as a percentage of total cells in the field. (A) Bar graph representing the mean ± SEM of 3 different experiments performed in triplicate (n = 9). *Significantly different from controls, $p < 0.05$. (B–D) Phase-contrast photomicrographs of (B) Control (no-treatment), (C) NGF 100 ng/ml and (D) CBD 1 μM.

(0.026 ± 0.0008). $MPP^+$ + CBD significantly increased the levels of NGF (0.0150 ± 0.0018) as compared to $MPP^+$ alone. NGF was added as positive control and its absorbance was significantly higher (0.034 ± 0.0025) than control (0.026 ± 0.0008) (Fig. 8).

### 3.9. CBD did not induce cellular differentiation in SH-SY5Y neuronal model

Retinoic acid (RA, 10 μM) induced cell differentiation (35.10 ± 3.72) as compared to control (5.36 ± 1.51). CBD (1 μM) did not induce cell

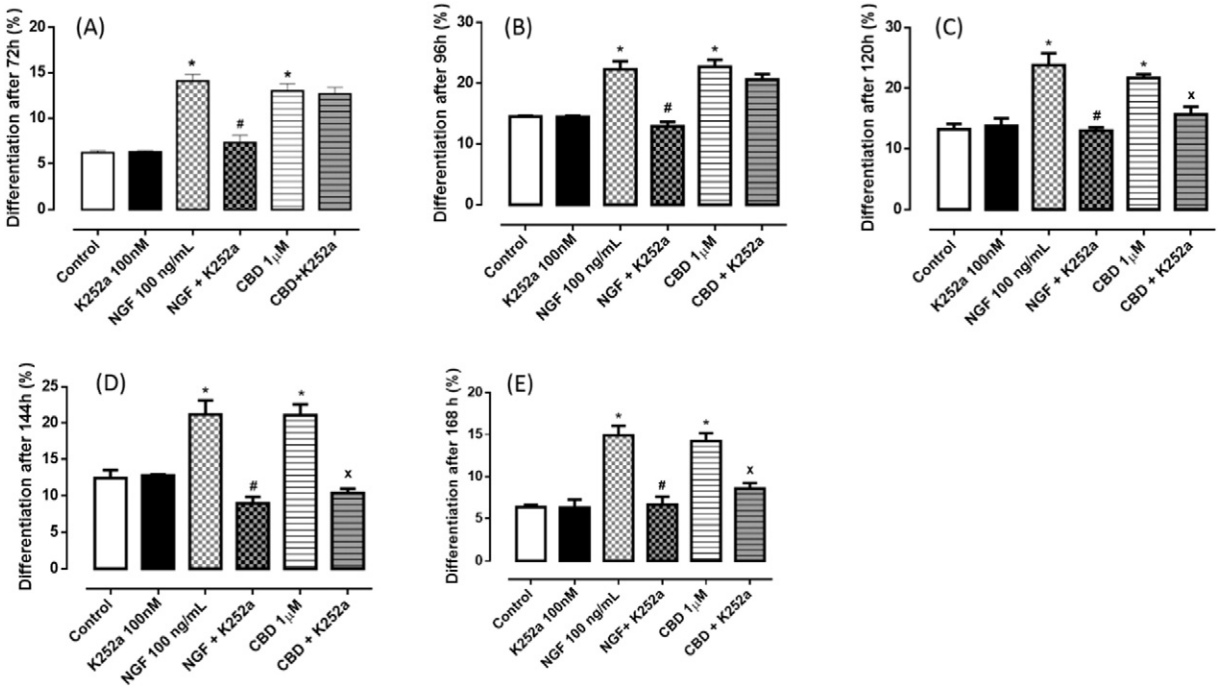


**Fig. 5.** Effect of CBD on the differentiation of PC12 cells treated with K252a (trk inhibitor). Cells were incubated with NGF 100 ng/ml or CBD 1 μM in the presence/absence of 100 nM K252a and analyzed after (A) 72 h, (B), 96 h, (C), 120 h, (D) 144 h and (E) 168 h. Cells with at least one neurite with a length equal to or greater than the cell body were counted and expressed as a percentage of total cells in the field. The results are expressed as mean ± SEM of 3 different experiments performed in triplicate (n = 9). *Significantly different from controls; #Significantly different from NGF alone, ˣSignificantly different from CBD alone, $p < 0.05$.

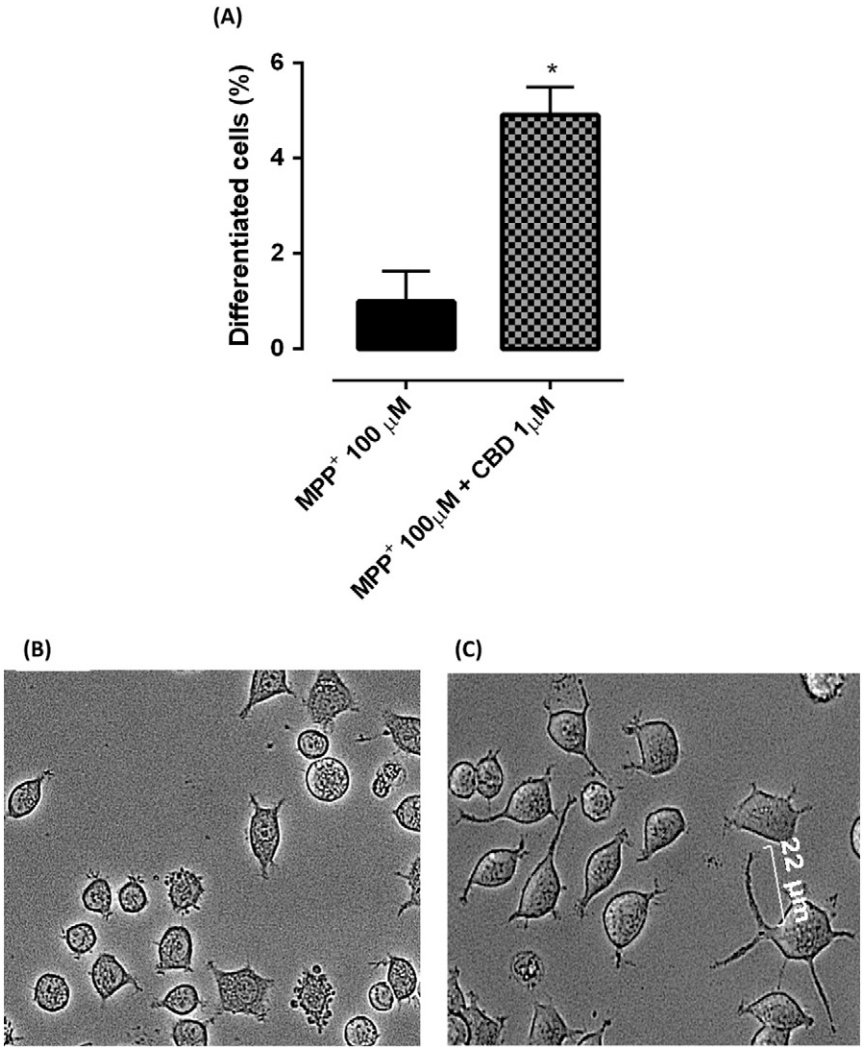


**Fig. 6.** Effect of CBD on the differentiation of PC12 cells treated with $MPP^+$. (A) Bar graph; (B) and (C) photomicrographs of $MPP^+$ group and $MPP^+$ + CBD group, respectively. Cells with at least one neurite with a length equal to or greater than the cell body were counted and expressed as a percentage of the total cells in the field. The results expressed are mean ± SEM of 3 different experiments performed in triplicates (n = 9). NGF 100 ng/ml; MPP + 100 μM and CBD 1 μM. *Significantly different from control; **Significantly different from NGF; #Significantly different from MPP+; $p < 0.05$.

differentiation (5.11 ± 0.91) in relation to control (5.36 ± 1.51) and reduced (23.60 ± 3.30) the differentiation induced by RA alone (35.10 ± 3.72) as presented in Fig. 9. Phase-contrast photomicrographs of RA-induced cells and RA plus CBD treated cells are presented in Fig. 9B and C, respectively.

### *3.10. CBD increased the expression of synaptophysin, synapsin I and GAP-43 in $MPP^+$-treated PC12 cells*

CBD significantly increased the expression of synaptophysin (3.04 ± 0.12); GAP-43 (3.94 ± 0.12) and synapsin I (4.59 ± 0.59) as compared to $MPP^+$ alone (1.54 ± 0.16; 1.40 ± 0.44 and 2.55 ± 0.39, respectively (Fig. 10A–D).

## 4. Discussion

The neurodegenerative diseases share common mechanisms of toxicity, namely: neuroinflammation, excitotoxicity, mitochondrial dysfunction and reduced trophic support (Fagan and Campbell, 2014). The neuroprotection of CBD has been observed in different models of neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's diseases, and it has been mainly associated with antioxidant and anti-inflammatory mechanisms; however, these properties alone cannot explain all the biological effects found in preclinical and clinical investigations with CBD (Zuardi, 2008; Iuvone et al., 2009). The present study evaluated the neurotrophic potential of CBD as a mechanism of neuroprotection as well as the involvement of the NGF-pathway. For this purpose, cell viability, neuritogenesis, expression of NGF and neuronal proteins, and the involvement of NGF receptors (trkA) were investigated in three cellular systems: (i) PC12 cells treated with $MPP^+$; (ii) NGF-stimulated PC12 cells and (iii) retinoic acid (RA)-stimulated SH-SY5Y cells. PC12 cells were used as a model system for neuronal differentiation because they express membrane-bound receptors and cytosolic macromolecules similar to those expressed in neurons. Besides that, PC12 are electrically excitable cells and secrete neurotransmitters such as dopamine, norepinephrine and acetylcholine (Shafer and Atchison, 1991; Satpute et al., 2010). The neurotoxin $MPP^+$ is the active metabolite of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine), which induces PD symptoms in exposed humans. The mechanism of toxicity involves the uptake of $MPP^+$ by the dopaminergic neurons of the brain, inhibition of complex 1 of the mitochondrial electron transport chain, mitochondrial dysfunction and

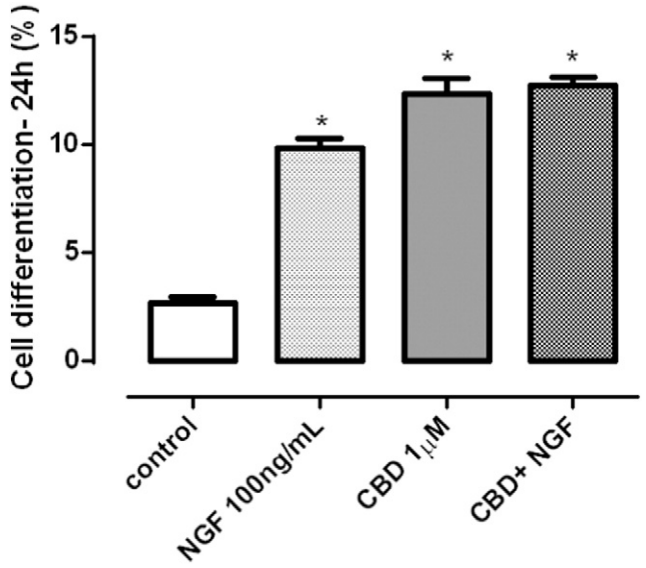


**Fig. 7.** Effect of CBD on the neuritogenesis of PC12 cells treated with NGF. No significant difference was observed between the effects of CBD alone and CBD + NGF. Data were expressed as mean ± SEM of three experiments performed in triplicate. *Significant as compared to control ($p < 0.05$).

dopaminergic cell death (Korecka et al., 2013). In PC12 cells, $MPP^+$ depletes the dopamine content and induces cell death (Xu et al., 2005). Therefore, $MPP^+$ is a relevant neurotoxin to Parkinson's disease.

In order to establish the neurotoxicity model and calculate the IC50, a dose–response curve (cell viability assay) of the neurotoxin $MPP^+$ was performed in PC12 cells. The IC50 of $MPP^+$ (1 mM) was used to evaluate the neuroprotection of CBD against the cell death induced by $MPP^+$. We also investigated the possible neurotoxic effect of CBD on PC12 cells by treating them with a high concentration (10 μM) of CBD alone. This concentration of CBD (10 μM) did not alter the viability of PC12 cells as compared to control. Then, cells were treated with 1 mM $MPP^+$ (IC50) and CBD (1-10 μM) to investigate the protection against $MPP^+$-induced cell death. All concentrations of CBD increased cell viability of $MPP^+$-treated PC12 cells; however, the lowest concentration of CBD

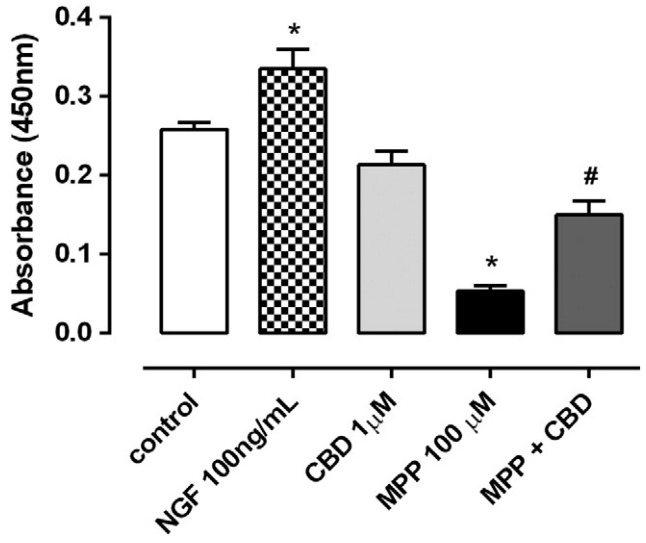


**Fig. 8.** Effect of CBD on the levels of NGF of PC12 cells. No significant difference was observed between the effects of CBD and control. Data were expressed as mean ± SEM of three experiments performed in triplicate. *Significantly different from control; #Significantly different from $MPP^+$; $p < 0.05$.

(1 μM) was the most effective. Accordingly, a previous study showed the protection of CBD against $MPP^+$ in primary dopaminergic cell culture (Moldzio et al., 2012). The neuroprotective effect of CBD against another dopaminergic neurotoxin, 6-OHDA, was shown in primary mixed glial cultures from C57BL/6 mice and in male Sprague–Dawley rats (Lastres-Becker et al., 2005). The cannabinoid receptors CB1 and CB2 seem to play an important role in neuroprotection; however, CBD has very low affinity for these receptors. The activation of CB2 is associated with decrease of microglial activation, and the activation of CB1 receptors is associated with the neuroprotection of the psychotropic cannabinoid delta-9-tetrahydrocannabinol ($\Delta^9$THC) (Ashton and Glass, 2007; Carroll et al., 2012). The mechanism of neuroprotection of CBD remains unknown (Chagas et al., 2014; Szaflarski and Bebin, 2014) and it might occur via different pathways; mechanisms like normalization of glutamate homeostasis (Fernandez-Ruiz et al., 2013) and reduction of microglial activation (Martin-Moreno et al., 2011) have been proposed. The present study suggests the involvement of neuritogenesis in the neuroprotective mechanism of CBD. We observed that CBD alone induced neuritogenesis on PC12 cells cultivated in reduced-serum medium not supplemented with NGF and therefore, almost deprived of growth factors. It is known that deprivation of growth factors leads to apoptosis (Mesner et al., 1995). The treatment with CBD might have restored the trophic signaling, therefore decreasing apoptosis (as demonstrated by caspase-3 activity), and therefore, increasing the viability of PC12 cells. Besides inducing the neuritogenesis of PC12 cells, CBD also protected against the inhibition of neuritogenesis induced by the neurotoxin $MPP^+$, which suggests the contribution of neuritogenesis in the mechanism of neuroprotection of CBD. It is known that, PC12 cells express trkA receptors, the main target of NGF. In the presence of NGF, PC12 cells cease proliferation, differentiate, form neurites and increase excitability, like neurons (Slotkin and Seidler, 2010), therefore, PC12 cells constitute a useful system for studying trkA function (Tsoulfas et al., 1996). In the present study, we evaluated trkA function by using K252a, a selective inhibitor of trk neurotrophin receptors (Morotti et al., 2002) and we observed that it reduced the neuritogenesis induced by CBD alone and by NGF alone. The effect of NGF, was inhibited after 72 h-incubation, whereas the inhibition of CBD by K252a was only significant after 120 h-incubation. This difference might be related to different interactions with the trkA receptor. Different ligands might have distinct affinities or even induce phosphorylation at different sites of trk receptors (Huang and Reichardt, 2003; Longo and Massa, 2013). We also observed that the neuritogenic effect of CBD is not additive to the effect of NGF nor dependent on the presence of NGF. Moreover, CBD did not increase the levels of NGF. However, CBD attenuated the decreased level of NGF induced by $MPP^+$. Since CBD seems not to increase the expression of NGF, the higher levels of NGF observed in $MPP^+$ + CBD as compared to $MPP^+$ alone might be due to an indirect mechanism. Altogether, these findings strongly suggest that CBD induces neuritogenesis by activation of trkA receptors by a mechanism independent of NGF. In neurons, the main pathway regulating apoptosis seems to be mediated by growth factors and trk receptors. By this pathway, the anti-apoptotic protein Bcl2 inhibits the mitochondrial release of cytochrome c and the subsequent activation of the caspase cascade that culminates with the activation of caspase-3, the final executor of apoptosis (Wolozin and Behl, 2000). Therefore, the activation of trkA receptors by CBD might have contributed to the decreased activity of caspase-3 induced by CBD in cells treated with the neurotoxin $MPP^+$.

CBD did not induce neuritogenesis on SH-SY5Y neuroblastoma cells, which do not express trkA receptors (Encinas et al., 1999; Edsjo et al., 2001). In line with our findings, a study showed that CBD did not protect differentiated (retinoic acid-treated) SH-SY5Y neuroblastoma cells against $MPP^+$ toxicity (Carroll et al., 2012). Another study reported that CBD did not increase neurite density or the viability of retinoic acid-treated SH-SY5Y neuroblastoma cells exposed to 6-OHDA, a neurotoxin also relevant for Parkinson's disease (Schonhofen et al., 2015). The distinct phenotypes of PC12 and SH-SY5Y neuroblastoma cells might

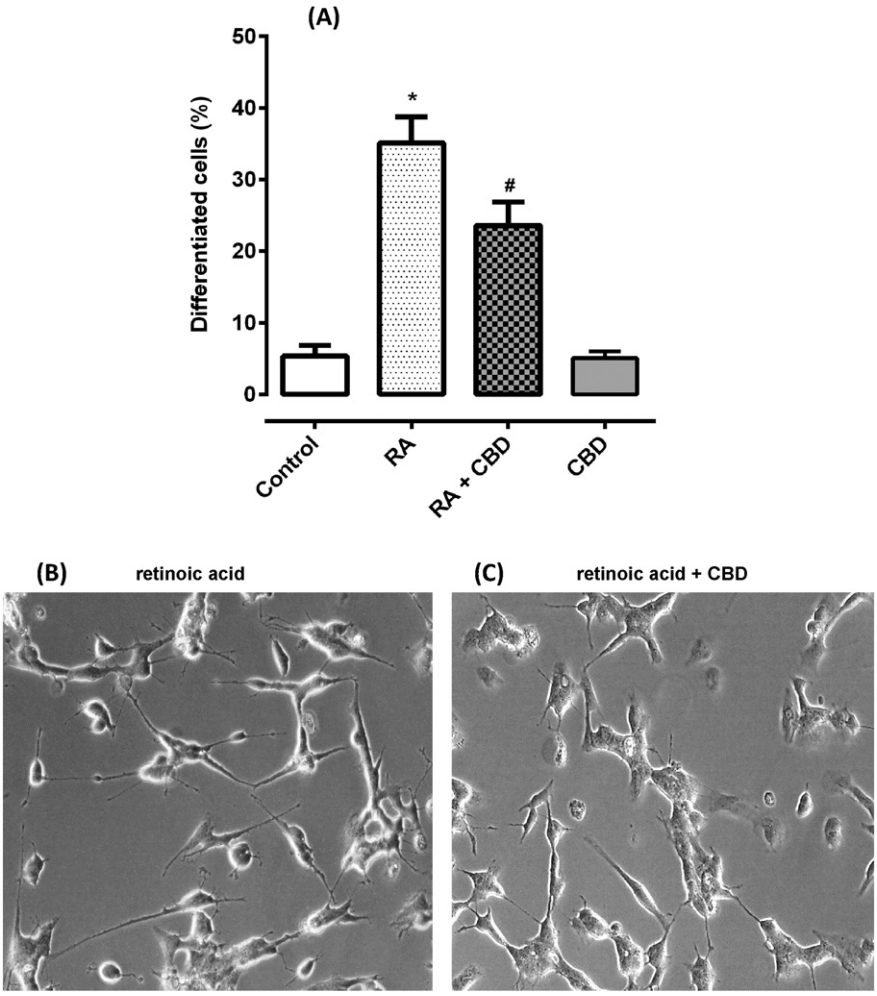


**Fig. 9.** Effect of CBD on the differentiation of SH-SY5Y cells (A) Bar graph; (B) and (C) photomicrographs of SH-SY5Y cells treated with retinoic acid and retinoic acid plus CBD, respectively. The results expressed are mean ± SEM of 3 different experiments performed in triplicate (n = 9). RA, retinoic acid 10 μM and CBD 1 μM. *Significantly different from control, $p < 0.05$. #Significantly different from RA, $p < 0.05$.

have contributed to the different effects of CBD on both cells. Neuroblastoma derived cells have no or low expression of neurotrophin receptor genes and treatment with retinoic acid induces the expression of functional trkB and trkC, but not trkA (Encinas et al., 1999; Edsjo et al., 2001). Additionally, a study reported that if SH-SY5Y cells are transfected with trkA, they respond to NGF by increasing the expression of the neuronal protein synaptophysin (Edsjo et al., 2001). Accordingly, in our assays, CBD did not induce differentiation on SH-SY5Y cells, and, surprisingly, reduced the neuritogenesis induced by retinoic acid. Additionally, a clinical study suggested that the beneficial effects of CBD in PD patients were not associated with alterations in the plasma levels of BDNF, a ligand of trkB receptors (Encinas et al., 1999; Chagas et al., 2014). Moreover, studies in animal models have shown that CBD does not increase or might even decrease the expression of BDNF and trkB receptors in the brain of mice and rats, suggesting that the neuroprotective effect of CBD is not mediated by trkB receptors (Magen et al., 2009; Zanelati et al., 2010; ElBatsh et al., 2012), which supports our findings. Altogether, these data suggest that CBD activates trkA receptors, but does not activate trkB or trkC receptors. This is the first study to show the involvement of trkA receptors in the mechanism of neuroprotection of CBD. To further explore this mechanism, we evaluated the effect of CBD on the expression of neuronal proteins related to synaptogenesis (synaptophysin and synapsin I) and axonal growth (GAP-43) that are knowingly induced by NGF (Das et al., 2004). GAP-43 is the most abundant neuron-specific protein in the growth cones, and it is used as a marker of neuronal differentiation (Encinas et al., 1999). Synaptophysin is an abundant integral membrane protein on synaptic vesicles (Cheung et al., 2009), whereas synapsin I is associated with the cytoplasmic side of synaptic vesicles. Synaptophysin and synapsin I represent useful markers of the synaptic vesicle membrane of nerve terminals (Valtorta et al., 1989). CBD significantly increased the expression of synaptophysin, GAP-43 and synapsin I as compared to $MPP^+$ alone. These data suggest that CBD induces neurite formation and elongation via up-regulation of GAP-43 and induces the formation of synaptic vesicles via up-regulation of synaptophysin and synapsin I. This is the first study to show that CBD protects against the down-regulation of these neuronal proteins induced by $MPP^+$. The induction of neuritogenesis and the restoration of the neuronal network have been suggested as possible strategies for the treatment of neurodegenerative diseases (Sofroniew et al., 2001; Lad et al., 2003; Gottlieb et al., 2010). However, the use of neurotrophic factors such as glial-derived neurotrophic factor (GDNF), BDNF and NGF in neurorestorative therapies is limited by the blood brain barrier (BBB), poor plasma stability and pleiotropic effects mediated by the interaction with multiple receptors (Allen et al., 2013; Longo and Massa, 2013). The identification of agents able to cross the BBB and to modulate specific systems of neurotrophin/receptors would contribute to the treatment of neurological disorders. The activation of trkA, for example, has been suggested as a therapeutic strategy for Alzheimer's disease (Longo and Massa, 2013). CBD is able to cross the blood brain barrier (Deiana et al., 2012) and to

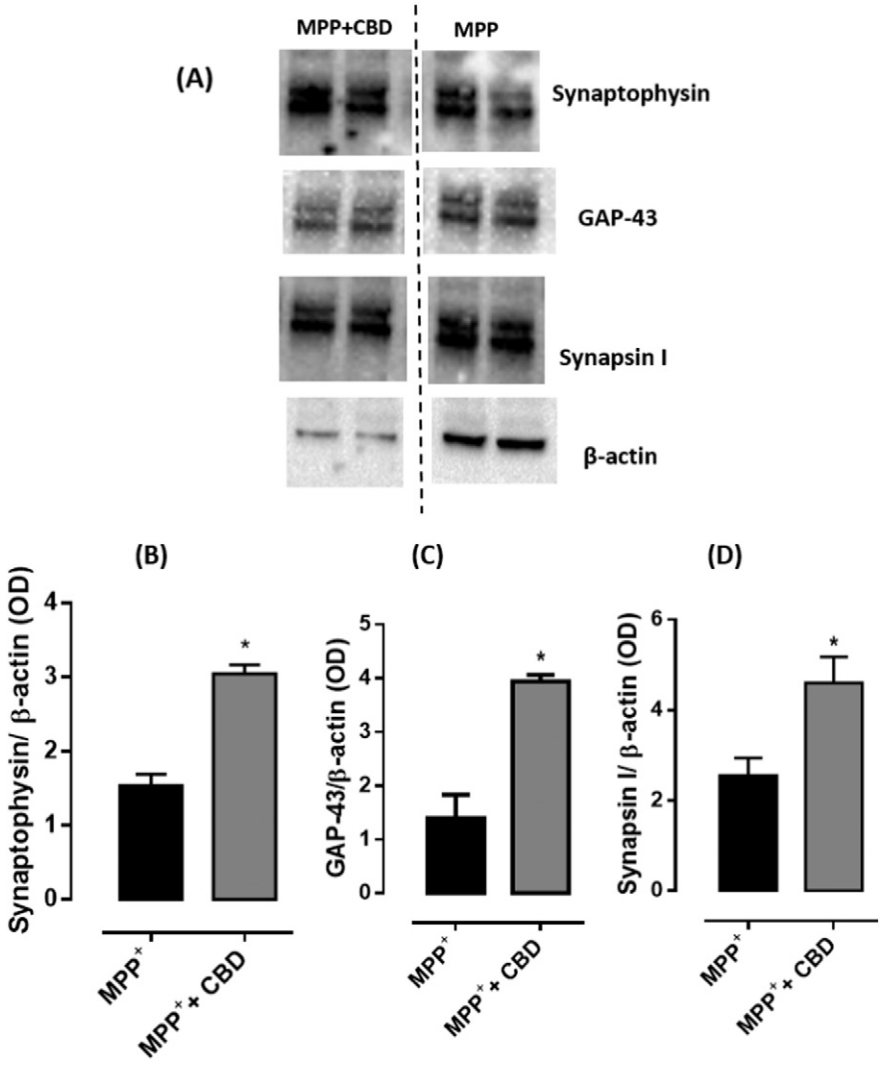


**Fig. 10.** Effect of CBD on the expression of neuronal proteins in PC12 cells treated with $MPP^+$. (A) Western blot membranes and bar graphs of (B) synaptophysin, (C) GAP-43 and (D) synapsin I. The results expressed are mean ± SEM of 3 different experiments performed in triplicate (n = 9). $MPP^+$ 100 μM and CBD 1 μM. *Significantly different from $MPP^+$, $p < 0.05$.

produce some neuritogenic effects similar to those induced by NGF, namely, activation of trkA receptors and increased expression of neuronal proteins related to axonal growth and synaptogenesis. These effects might be beneficial in neurodegenerative processes such as Parkinson's disease and should be further investigated.

## 5. Conclusions

CBD protects against the cell death and neurite loss induced by the neurotoxin $MPP^+$ on PC12 cells. The neuroprotection is not dependent or additive to NGF and might involve the activation of trkA receptors and increased expression of axonal and synaptogenic proteins. CBD seems not to activate trkB (main receptor of BDNF) or trkC (main receptor of GDNF) in SH-SY5Y cells and diminishes the neuritogenesis induced by retinoic acid on these cells. Therefore, SH-SY5Y cells might not be a suitable model to study the neuroprotective effects of CBD. The NGF-like effects of CBD contribute to its neuroprotective activity against the toxicity induced by $MPP^+$, a neurotoxin relevant to Parkinson's disease.

## Conflicts of interest

The authors declare no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tiv.2015.11.004.

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