Cannabinoid CB₁ receptor stimulation affords neuroprotection in MPTP-induced neurotoxicity by attenuating S100B up-regulation in vitro

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Abstract In this study, we investigated the mechanism of S100B neurotoxicity and the effect of cannabinoids, in C6 cells treated with 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) and co-cultured with differentiated PC12 cells. MPTP concentration- and time-dependently increased S100B density in C6 cells. This effect was followed by increased C6 cell proliferation and decreased cell viability of co-cultured PC12 cells. An antibody against S100B, given to PC12 cells before co-culture, led to their survival. Treatment with arachidonyl-2-chloroethylamide, a CB₁ agonist, significantly inhibited MPTP-induced S100B density in C6 cells and protected co-cultured PC12 cells from cell death. Because MPTP selectively increased the levels of anandamide in C6 cells, the involvement of the endocannabinoid system was investigated by using selective inhibitors of endocannabinoid inactivation (cellular re-uptake or enzymatic hydrolysis) and selective cannabinoid CB₁ and CB₂

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receptor antagonists and by silencing the CB₁ receptor. Our data suggest that selective activation of CB₁ receptors by either exogenous or endogenous cannabinoids might afford neuroprotection in MPTP-induced neurotoxicity also by controlling S100B up-regulation in activated glial cells.

Keywords Cannabinoid · MPTP · Neurotoxicity

Introduction

The neurotoxin 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) induces in primates and in rodents neurological and pathological changes comparable to those observed in Parkinson's disease (PD) [1]. MPTP neurotoxicity has been demonstrated to involve energy depletion and free radicals production [2]. Its detrimental action is initiated by 1-methyl -4 phenylpyridinium (MPP⁺), a noxious metabolite generated by the monoamino-oxidase B, an enzyme located in the brain mostly in astrocytes. MPP⁺, taken up into dopaminergic neurons [3], is then accumulated into the mitochondria, where it disrupts oxidative phosphorylation, thereby leading to adenosine triphosphate production impairment, intracellular calcium enhancement and overt increase in free radical production.

Reactive gliosis represents a common response of the brain to a neuronal injury [4]. It is characterized by activation of either microglia or astroglia, both able to respond to insults and to participate to the pathological process. The glial reaction observed in PD experimental models has been believed to contribute significantly to neuronal degeneration [5, 6] because activated astrocytes and microglia express a broad array of neurotoxic molecules, including pro-inflammatory cytokines and chemokines, in addition to reactive oxygen and nitrogen species. In the reactive gliosis secondary to MPTP exposure, it has been reported that astrocyte activation parallels the time course of neuronal cell death and lasts long after most neurons have died as a consequence of MPTP toxicity. Although evidence suggests that astroglial activation may be crucial for MPTP-induced neuronal loss [7], the precise role of reactive astrogliosis in the cascade of deleterious events ultimately leading to neuronal death remains still not clearly elucidated.

S100B is a calcium/zinc-binding protein produced and secreted by astrocytes; it is expressed throughout the brain and is observed primarily in the cytoplasm of astrocytes [8]. The role of S100B is still under debate. In fact, on the one hand, it was believed that S100B is neuroprotective because it may decrease neuronal injury and/or contribute to repair after traumatic brain injury [9]; on the other hand, early investigations have shown that exaggerated expression of S100B can result in neuronal damage [10]. Consistent with the latter observations, evidence has been provided suggesting that S100B up-regulation behaves as a worsening factor in some neurodegenerative pathologies, such as Down's syndrome and Alzheimer's disease [11]. It is interesting to note that more recent studies have demonstrated that S100B-positive cells may be involved in MPTP-induced neuronal death [12], although the information regarding the precise relationship between S100B over-expression and MPTP-caused neurodegeneration is still

very limited. Therefore, further investigations are needed to better understand the exact mechanism of the role of astrocyte S100B over-production in MPTP neurotoxic effects.

Activation of the cannabinoid system has been proposed to act 'on demand' to limit neuronal damage during toxic events and to be neuroprotective in different experimental models [13, 14]. Cannabinoids can modulate inflammatory response [15], and it has been reported that they can affect astroglial functions [16]. Astrocytes have been observed to possess a functional endocannabinoid system (ECS) because (a) they express cannabinoid receptors, termed CB₁ and CB₂, (b) they produce the endogenous ligands of these receptors, anandamide (AEA) [17] and 2-arachidonoyl glycerol (2-AG) [15] and (c) they also contain the molecular machinery to inactivate these compounds by cellular uptake and degradation [18] either by fatty acid amide hydrolase [19] or monoacylglycerol lipase [20]. This close relationship between the ECS and glial cells has attracted much attention in the attempt to identify new molecular targets aimed to attenuating or even halting reactive gliosis. Therefore, based on the above background, the major purpose of the present study was to elucidate (1) the involvement of S100B in MPTP-induced neurotoxicity, (2) the molecular mechanism of S100B neurotoxicity and (3) the role of CBs and the ECS in the control of S100B toxicity, in MPTP-exposed C6 rat glioma cells co-cultured with rat pheochromocytoma PC12 cells differentiated into neurons.

Materials and methods

All materials for cell culture were purchased from Bio-Whittaker (Caravaggio, BG, Italy). Foetal calf serum and horse serum (HS) were from HyClone (Logan, UT). MPTP, nerve growth factor, FURA-2 and all the material unless otherwise stated were from Sigma-Aldrich (St.Louis, MO). Anti-S100B antibody was from AbCam (Cambridge, UK), anti-CB₁ antibody was from Affinity BioReagent (Golden, CO) and anti-CB₂ antibody was from Cayman (Ann Arbor, MI); AEA, arachidonyl-2-chloroethylamide (ACEA), JWH-015 were from Tocris (Cookson, Bristol, UK), SR141716A and SR144528 were a gift from Dr. Madaleine Mosse (SANOFI-Reserche, Montpellier, France) and VDM-11 and arachidonoylserotonin (AA-5-HT) were synthesized in the laboratory of prof. Vincenzo Di Marzo (CNR-Pozzuoli, Italy).

Rat C6 glioma cell culture and treatments

C6 rat glioma cells (American Type Tissue Collection CCL-107) were cultured in 10-cm Petri dishes in Dulbecco's

Modified Eagle's Medium (DMEM) supplemented with 5% foetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO₂/95% air. Confluent cells were washed three times with phosphate-buffered saline (PBS), detached with trypsin/ethylenediamine tetraacetic acid, plated and left to adhere for 24 h at 37°C. Thereafter, confluent C6 cells were treated with different concentration of MPTP (ranging up from 1 to 100 µM) for different time points according to the experimental protocol and in presence or absence of the following substances: a selective CB₁ agonist, ACEA (10⁻⁸–10⁻⁶ M), a selective CB₂ agonist, JWH015 (10⁻⁸–10⁻⁶ M), the endocannabinoid AEA (10⁻⁸–10⁻⁶ M), the inhibitor of AEA enzymatic hydrolysis AA-5-HT (10⁻⁸–10⁻⁶ M) [21], added to C6 cells 15 min before MPTP in the presence or in the absence of selective CB₁ and CB₂ receptor antagonists, SR141716A (10⁻⁸–10⁻⁶ M) and SR144528 (10⁻⁶ M). In the control experiments, we also tested the effect of SR141716-A and ACEA (10⁻⁶ M) on C6 cell in absence of MPTP (100 µM). In some experiments, VDM-11 (10⁻⁷–10⁻⁶ M), a synthetic inhibitor of AEA transport across the cell membrane [22], was added 30 min before or 12 h after MPTP (100 µM) stimulation to C6 cells. In other experiments, an anti-S100B-neutralizing antibody (dilution 1:1,000–1:10,000 v/v) or an unrelated IgG (anti-tubulin) was added to cells 15 min before MPTP (100 µM).

Rat PC12 pheochromocytoma cell culture

In our study, we used differentiated rat pheochromocytoma PC12 cells that are currently considered a useful model for in vitro study of neuronal toxicity, as in cited references [23]. Moreover, PC12 cells can be considered as dopaminergic-like neurons because they synthesize and release dopamine [24]. PC12 cells (American Tissue Type Collection CRL-1721) were cultured in 10-cm Petri dishes in DMEM supplemented with 5% FBS, 15% HS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO₂/95% air and differentiated with retinoic acid as described by Koh et al. [25]. Depending on the experiments, PC12 cells were treated with a MPTP-conditioned C6 cell medium, with S100B protein (Sigma Aldrich; 0.005–5 µM) or co-cultured with C6 cells. In some experiments, an anti-S100B-neutralizing antibody (dilution 1:1,000 v/v) or an anti-tubulin (dilution 1:1,000 v/v) antibody were added directly to PC12 cells in the experiments with co-cultures or 'conditioned medium.'

Experiments with co-cultures of C6/PC12 cells

In 'co-culture' experiments, confluent PC12 cells were seeded in sterile Falcon inserts at the density of 2.5 × 10⁵ cells/well. In parallel, C6 cells (1 × 10⁶ cells/well) were treated for 24 h with

Scheme 1 General procedure for the 'co-culture' experiments (see experimental procedures)

MPTP (100 µM) in a six-multiwell plate with or without ACEA (10⁻⁸–10⁻⁶ M), JWH015 (10⁻⁸–10⁻⁶ M), AEA (10⁻⁷–10⁻⁶ M) and AA-5HT (10⁻⁷–10⁻⁶ M) in the presence or absence of SR141716A (10⁻⁶ M) given 15 min before MPTP. In other experiments, SR141716A (10⁻⁸–10⁻⁶ M) alone was given 15 min before MPTP. Then, the C6 cell supernatant was removed, and cells were extensively washed to remove any residual MPTP or MPTP metabolites or tested drugs. C6 cells were then supplemented with fresh medium, and PC12 cells were inserted in the six-multiwell plate. PC12 and C6 cells were then co-cultured for a further 24 h at 37°C (Scheme 1). After this time, the inserts were removed, and PC12 cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT)–formazan conversion assay (see below).

Experiments with PC12 cells and the conditioned medium from MPTP-treated C6 cells

PC12 cells were incubated for different time periods with the 'conditioned medium,' deriving from C6 cells previously treated with MPTP (100 µM) for 24 h, to measure [Ca²⁺]_i levels and caspase-3 activity. In some experiments, an S100B-neutralizing antibody (1:1,000 v/v dilution) was added to the PC12 cell medium 15 min before their exposure to the C6 cell 'conditioned medium.'

Preparation of cytosolic fractions from C6 and PC12 cells and Western blot analysis for S100B

Extracts of C6 and PC12 cells treated as described above were prepared as previously reported [26]. Briefly, harvested

cells (1×10^6) were washed two times with ice-cold PBS and centrifuged at $180 \times g$ for 10 min at 4°C . The cell pellet was resuspended in 100 μL of ice-cold hypotonic lysis buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM phenylmethylsulfonylfluoride, 1.5 $\mu\text{g}/\text{mL}$ soybean trypsin inhibitor, 7 $\mu\text{g}/\text{mL}$ pepstatin A, 5 $\mu\text{g}/\text{mL}$ leupeptin, 0.1 mM benzamide, 0.5 mM dithiothreitol) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle for five to six times, and the cytoplasmic fraction was then obtained by centrifugation for 1 min at $13,000 \times g$. Cytosolic fraction proteins were mixed with non-reducing gel-loading buffer (50 mM Tris/10% sodium dodecyl sulphate [SDS]/10% glycerol/10%/2 mg of bromophenol per mL) in a ratio of 1:1, boiled for 3 min and centrifuged at $10,000 \times g$ for 10 min. Cytosolic fraction proteins were mixed with gel-loading buffer (50 mM Tris/10% SDS/10% glycerol/10%/2-mercaptoethanol /2 mg of bromophenol per mL) in a ratio of 1:1, boiled for 3 min and centrifuged at $10,000 \times g$ for 10 min. Protein concentration was determined, and equivalent amounts (100 μg) of each sample were electrophoresed in a 15% discontinuous polyacrylamide mini-gel. The proteins were transferred onto nitro-cellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4°C overnight with 10% non-fat dry milk in PBS and then incubated with 1:1,000 v/v anti-mouse S100B antibody, 1:250 v/v anti-rabbit CB₁ (Affinity SpioBio), 1:250 v/v anti-rabbit CB₂ (Cayman) or 1:1,000 v/v anti-mouse tubulin antibody. The membranes were washed three times with 1% Triton 100-X in PBS and then incubated with anti-mouse or anti-rabbit immunoglobulins coupled to peroxidase (1:2000 v/v). The immunocomplexes were visualised by the ECL chemiluminescence method (Amersham, Buckinghamshire, UK). Subsequently, the relative density of S100B protein in the cytosolic fraction was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad, Hercules, CA) and a computer programme (Molecular Analyst, IBM).

MTT–formazan assay

Cell viability was measured both on C6 and on PC12 cells using the MTT assay, as previously described [26]. MTT (5 mg/mL in DMEM) was added to the cells treated as above described, and cells were incubated for additional 3 h at 37°C . After this time interval, cells were lysed, and dark blue crystals were solubilized with a solution containing 50% (v/v) *N,N'*-dimethyl formamide, 20% (w/v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620-nm

filter. The cell viability was thus calculated as percent of cell viability = $(\text{OD treated}/\text{OD control}) \times 100$.

Determination of $[\text{Ca}^{+2}]_i$ levels in C6 cells and in PC12 cells

Intracellular calcium $[\text{Ca}^{+2}]_i$ levels were measured, at different time points, both in C6 and in PC12 cells by using the fluorescent probe FURA-2AM as described by Grynkiewicz et al. [27]. Both cell types, after the specific treatment described, were incubated at the density of 2.5×10^5 cells in Hanks Balanced Salt Solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 0.49 mM MgCl_2 , 0.44 mM KH_2PO_4 , 0.64 mM Na_2HPO_4 , 3 mM NaHCO_3 , 5.5 mM glucose, 1.26 mM CaCl_2 and 20 mM HEPES, pH 7.4) buffer containing 4 μL (5 μM) FURA-2 probe at 37°C for 1 h. Afterwards, cells were centrifuged at 1,200 rpm for 5 min and washed with fresh HBSS buffer, and a volume of 200 μL of cells (5×10^3) were subjected to 340/380 nm dual wavelength measurement using a spectrofluorometer with an LS55 luminescence spectrometer (Perkin Elmer). Calcium influx was so expressed as percent of increase in basal.

ELISA assay

Nunc Maxisorp 96-well microtiter plates (Gibco, Paisley, UK) were coated overnight at 4°C with 1 $\mu\text{g}/\text{well}$ of monoclonal anti-S100B antibody, diluted in 0.5 M Na_2CO_3 . The wells were washed three times and then blocked with PBS containing 0.05% (v/v) Tween 20 and 0.3% FBS (enzyme-linked immunosorbent assay [ELISA] buffer) for 30 min at room temperature. The standards and samples were added in a 100- μL volume and incubated at 37°C for 1.5 h. The standard curve was generated using 0.1 ng to 1 $\mu\text{g}/\text{well}$ of purified S100B, diluted in ELISA buffer. Supernatant from C6 cells, treated with MPTP (100 μM) in the presence or absence of ACEA (10^{-8} – 10^{-6}) or JWH-015, was diluted (1:100) in ELISA buffer, and 100 μL was assayed in triplicate. After four washes in ELISA buffer, monoclonal anti-S100B antibodies were diluted 1:500 and added to the wells for 1 h. The wells were washed four more times and then incubated with the secondary antibody: anti-mouse Ig, biotinylated species-specific F(ab8)2 fragment from the donkey (Amersham), diluted 1:1,000, for 1 h. After a further four washes, the wells were incubated with 100 μL of streptavidinbiotinylated horseradish peroxidase complex (Amersham) diluted 1:1,000 in ELISA buffer for 1 h. After a final six washes in ELISA buffer, 200 μL of a 0.4 mg/mL solution of *o*-phenylenediamine dihydrochloride (Sigma) in a 0.05-M phosphate citrate buffer was added to each well, and colour was allowed to develop for up to 10 min. The colour reaction was stopped by the addition of HCl, and ODs at 490 nm were

measured using a microplate reader. The absorbance values were corrected by subtracting background readings of wells in which the antigen was omitted. The sensitivity of the assay was 0.1 ng/mL purified S100B. All data were read from the linear portion of the standard curve (1–100 ng/mL).

RNA Interference

Transfection of 25-nucleotide small interfering RNA (siRNA) duplexes (Santa Cruz Biotechnology) for targeting endogenous genes was carried out using OligofectAMINE (Invitrogen) and 70 pmol siRNA for each transfection according to manufacturing instructions (Santa Cruz Biotechnology). Transfected C6 cells were serum starved and assayed 3 days after transfection.

The sequence of the siRNA used for rat CB₁ receptor was: ACGTGGGCTCGAATGACATTCAGTA accession number U40395. Specific silencing of targeted genes was confirmed by Western blot analysis for CB₁ receptor. Transfected C6 cells were then treated for 24 h with MPTP (1–100 μ M).

Quantification of endocannabinoids in MPTP-treated rat C6 glioma cells

After stimulation, media plus cells were treated with 2 vol of cold methanol and transferred to 50-mL Falcon tubes. At this point, 100 pmol of d⁸-AEA, d⁴-palmitoylethanolamide and d⁵-2-AG were added as internal standards. The mixture was then extracted three times with 4 vol of chloroform by 30-s sonication. The three organic phases were then pooled, lyophilised and pre-purified by open-bed chromatography on silica gel, eluted with chloroform/methanol (9:1 v/v). The eluate was then lyophilised and analysed by isotope dilution–liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (MS) using conditions described in Marsicano et al. [28] and allowing the separation of 2-AG and AEA. MS detection was carried out in the selected ion monitoring mode using *m/z* values of 356.0 and 348.0 (molecular ions +1 for deuterated and undeuterated AEA), 384.35 and 379.35 (molecular ions +1 for deuterated and undeuterated 2-AG) and 304.0 and 300.0 (molecular ions +1 for deuterated and undeuterated palmitoylethanolamide). The area ratios between signals of deuterated and undeuterated AEA varied linearly with varying amounts of undeuterated AEA (30 fmol–100 pmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100 pmol–20 nmol interval. AEA and 2-AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. The amounts of endocannabinoids are expressed as pmoles per milligram of lipid extract.

Results

Effect of MPTP on S100B protein density

The density of S100B protein in C6 cells treated with MPTP was evaluated, in two different sets of experiments, by Western blot analysis and the band corresponding to protein molecular weight was quantified by densitometry. In the first set of experiments, incubation with MPTP (1–100 μ M) for 24 h resulted in a significant and concentration-dependent increase in S100B protein density in C6 cells with respect to basal levels (unstimulated cells). In a second set of experiments, C6 cells were treated for 24 h with MPTP (100 μ M) and extensively washed, and S100B protein density was evaluated after further 24 h. Under these conditions, S100B protein density in C6 cells was still significantly increased with respect to basal levels (Fig. 1).

Effect of MPTP on $[Ca^{+2}]_i$ levels in C6 cells

Because S100B is a calcium-binding protein, we tested the hypothesis that an increase in intracellular calcium ($[Ca^{+2}]_i$) levels after MPTP administration may be a trigger signal for S100B up-regulation. Therefore, we measured $[Ca^{+2}]_i$ levels in MPTP-treated C6 cells after different intervals of time.

Fig. 1 Effect of MPTP (1–100 μ M) on S100B protein density in C6 cells. **a** Western blot analysis of S100B protein at 24 and 48 h. **b** Relative densitometric analysis. Each bar shows the mean $\pm$ SEM of *n*=4 experiments. **P*<0.05, ****P*<0.001 vs control

When MPTP was added to C6 cells, $[Ca^{+2}]_i$ levels were significantly increased starting from 20 up to 120 min, reaching a peak at 60 min, in comparison to basal levels (unstimulated cells; no Ca^{+2} detection by FURA-2 was revealed). At 60 min, moreover, MPTP (1–100 μ M) concentration-dependently increased $[Ca^{+2}]_i$, in comparison to basal levels (Fig. 2).

Effect of MPTP on C6 cell proliferation

Incubation of C6 cells with MPTP (1–100 μ M) concentration-dependently increased cell proliferation at 48 h. The effect of MPTP on C6 cell proliferation was significantly and concentration-dependently inhibited by the anti-S100B antibody given to the cells 15 min before MPTP challenge, while the unrelated anti-tubulin IgG was ineffective (Fig. 3).

Effect of MPTP-conditioned C6 cell medium on PC12 $[Ca^{+2}]_i$ levels

Because it has been described that S100B protein directly increases Ca^{+2} influx in neuronal cell lines and this is accompanied by a pro-apoptotic effect [29], we evaluated the increase in $[Ca^{+2}]_i$ levels in PC12 cells treated for different time intervals (5–15–30–60–120 min) with the ‘conditioned medium’ from MPTP (100 μ M)-treated C6 cells.

$[Ca^{+2}]_i$ levels increased in PC12 cells starting from 5 to 120 min with respect to basal levels (unstimulated cells), reaching a peak at 30 min and progressively decreasing at 120 min (Fig. 4a). When PC12 cells were treated with anti-S100B antibody (dilution 1:1,000 v/v), given 15 min before the MPTP-‘conditioned medium,’ this $[Ca^{+2}]_i$ increase was significantly reduced at all time points (Fig. 4a). The

Fig. 2 Effect of MPTP (1–100 μ M) on C6 $[Ca^{+2}]_i$ levels. $[Ca^{+2}]_i$ levels were measured at 60 min, i.e. at their peak and expressed as Δ increase in comparison to basal level. Each bar shows the mean $\pm$ SEM of $n=6$ experiments. ** $P<0.05$ vs control; *** $P<0.001$ vs control

Fig. 3 Effect of MPTP (1–100 μ M) on C6 cell proliferation at 48 h. Each bar shows the mean $\pm$ SEM of $n=5$ experiments. The effect of S100B-neutralizing antibody (1:10,000–1:1,000 dilution v/v) on MPTP (100 μ M)-induced C6 cell proliferation at 48 h is also shown. Each bar shows the mean $\pm$ SEM of $n=5$ experiments. * $P<0.05$, *** $P<0.001$ vs control. °° $P<0.01$, °°° $P<0.001$ vs MPTP (100 μ M)

S100B-neutralizing antibody given alone to PC12 cells did not modify $[Ca^{+2}]_i$ (Fig. 4a).

Effect of MPTP on co-cultured PC12 cell viability

In co-cultured PC12 cells, we observed a reduction in cell viability in comparison to control cells (PC12 cells co-cultured with unstimulated C6 cells=100% viability). The anti-S100B antibody (1:1,000 v/v dilution) reversed PC12 cell death, while the unrelated anti-tubulin IgG was ineffective, suggesting that the effect was mediated, at least in part, by S100B release from MPTP-treated C6 cells (Fig. 4b).

Effect of exogenous S100B on PC12 cell-viability

Treatment with S100B protein (0.005–5 μ M) significantly and concentration dependently reduced PC12 cells viability (Fig. 4c).

Effect of the conditioned medium from 24-h MPTP-treated C6 cells on caspase-3 activation in PC12 cells

To study whether PC12 cell death occurred because of apoptosis, we evaluated the appearance of caspase-3 from the inactive precursor, pro-caspase, in cellular extracts of PC12 cells co-cultured for 3 or 6 h with the ‘conditioned medium’ derived from MPTP (100 μ M)-treated C6 cells, in the presence or absence of an anti-S100 β antibody.

Analysis of the pro-caspase/total caspase-3 ratio showed that PC12 cells started to undergo apoptosis 3 h after co-culture with the ‘conditioned medium,’ although a marked apoptosis was evident only 6 h after the treatment. Apoptosis was counteracted by the anti-S100 β antibody (1:1,000 v/v dilution), added to PC12 cells 15 min before the ‘conditioned medium’ from MPTP-treated C6 cells (Fig. 4d,e).

Effect of ACEA and JWH015 on MPTP-induced S100B protein density in C6 rat glioma cells

The selective CB₁ receptor agonist, ACEA (10^{-8} – 10^{-6} M) added to MPTP (100 μ M)-treated C6 cells, concentration-

dependently reduced S100B protein density at 24 h. By contrast, the selective CB₂ receptor agonist, JWH015 (10^{-6} M), did not reverse the effect of MPTP (Fig. 5).

Effect of ACEA and JWH-015 on MPTP-induced S100B protein release in C6 rat glioma cells

When ACEA (10^{-8} – 10^{-6} M) was added to MPTP (100 μ M)-treated C6 cells, it significantly and concentration-dependently reduced S100B protein release in the cultured medium 24 h after treatment. By contrast, the selective CB₂ receptor agonist, JWH015 (10^{-6} M), did not reverse the effect of MPTP (Fig. 6).

Fig. 4 **a** Time-dependent effect of MPTP-conditioned C6 cell medium on PC12 $[Ca^{2+}]_i$ levels in the presence or absence of S100B-neutralizing antibody (1:1,000 dilution v/v). Each point shows the mean $\pm$ SEM of $n=5$ experiments. *** $P<0.001$ vs MPTP-conditioned C6 cell medium. **b** Effect of MPTP (1–100 μ M)-conditioned C6 cell medium in the presence of S100B-neutralizing antibody (1:1,000 dilution v/v) given to PC12 cells on co-cultured PC12 cell viability. Each bar in panel **b** shows the mean $\pm$ SEM of three experiments. * $P<0.01$ vs control; *** $P<0.001$ vs control. **c** Concentration dependent effect of S100B protein on PC12 cells viability. **d**

Western blot analysis showing the effect of the conditioned medium from 24-h MPTP-treated C6 cells on caspase-3 activation in PC12 cells, evaluated at 3 and 6 h, in the presence and absence of S100B-neutralizing antibody (1:1,000 dilution v/v). **e** Densitometric analysis of Pro-caspase-3/caspase-3 ratio of corresponding bands (optical density). **d** is representative of $n=3$ separated experiments. Each bar in **e** shows the mean $\pm$ SEM of $n=3$ experiments. ** $P<0.05$ vs control *** $P<0.001$ vs control. °°° $P<0.001$ vs MPTP-conditioned C6 cell deriving medium

Fig. 5 Effect of ACEA, JWH015 and SR 141716-A on MPTP-induced S100B protein expression in C6 rat glioma cells. **a** Western blot analysis of S100B protein expression at 24 h in MPTP (100 μ M)-treated C6 cells in the presence of ACEA (10^{-8} – 10^{-6} M), JWH015 (10^{-6} M) and SR141716A (10^{-6} M). **b** Corresponding densitometric analysis. Each bar shows the mean $\pm$ SEM of $n=4$ experiments. *** $P<0.001$ vs control. °°° $P<0.001$, °° $P<0.05$ vs MPTP. ### $P<0.001$ vs ACEA

Effect of ACEA and JWH015 on MPTP-induced $[Ca^{+2}]_i$ levels in C6 rat glioma cells

ACEA (10^{-8} – 10^{-6} M) added to MPTP (100 μ M)-treated C6 cells significantly and concentration-dependently reduced $[Ca^{+2}]_i$ levels in the cells evaluated 60 min after MPTP addition (Fig. 7), whereas under the same experimental conditions, JWH015 (10^{-6} M) did not show any effect (data not shown).

Fig. 6 Effect of ACEA (10^{-8} – 10^{-6}) and JWH-015 (10^{-6}) on MPTP-induced S100B levels in C6 rat glioma cells medium. ELISA analysis of S100B protein levels at 24 h in MPTP (100 μ M)-treated C6 cells in the presence of ACEA (10^{-8} – 10^{-6} M) and JWH015 (10^{-6} M). Each bar shows the mean $\pm$ SEM of $n=4$ experiments. *** $P<0.001$ vs control. °°° $P<0.001$, °° $P<0.05$ vs MPTP

Effect of ACEA and JWH015 on MPTP-challenged C6 cells and corresponding effect on PC12 cells co-cultured with C6 cells

When PC12 cells were co-cultured for 24 h with MPTP (100 μ M)-treated C6 cells, a significant and concentration-dependent reduction in cell viability was observed. Under the same experimental conditions, ACEA (10^{-8} – 10^{-6} M) but not JWH015 (10^{-8} – 10^{-6} M), given to C6 cells 15 min before MPTP, resulted in a significant and concentration-dependent inhibition of co-cultured PC12 cell death (Fig. 8).

Effect of MPTP on endocannabinoid levels in C6 rat glioma cells

Incubation with MPTP (100 μ M) resulted in a significant and almost fourfold increase in AEA levels but not of the levels of 2-AG or of other cannabimimetic fatty acid amides, such as palmitoylethanolamide (PEA), in C6 rat glioma cells (Fig. 9).

Effect of AEA, AA-5-HT and SR141716A on MPTP-induced S100B protein density in C6 cells

AEA and AA-5-HT (10^{-6} M), a selective inhibitor of AEA degradation via fatty acid amide hydrolase added to MPTP (100 μ M)-treated C6 cells, reduced S100B protein density at 24 h. This effect was attenuated by the concomitant treatment of MPTP-stimulated C6 cells with AEA plus SR141716A (10^{-6} M) or AA-5-HT plus SR141716A (10^{-6} M). Under the same experimental conditions, SR141716A (10^{-6} M) alone slightly, although significantly, increased MPTP (100 μ M)-induced S100B protein density.

Fig. 7 Effect of ACEA and SR141716-A on MPTP induced $[Ca^{+2}]_i$ levels in C6 rat glioma cells. $[Ca^{+2}]_i$ levels were measured at 60 min, i.e. at their peak and expressed as $\Delta\%$ increase in comparison to basal level. ACEA (10^{-8} – 10^{-6} M) and SR141716A (10^{-6} M) were given 15 min before MPTP (100 μ M). Each bar shows the mean $\pm$ SEM of $n=6$ experiments. *** $P<0.001$ vs control; °°° $P<0.001$ vs MPTP. ### $P<0.001$ vs ACEA

Fig. 8 Concentration dependent effect of ACEA and JWH-015 on PC12 cell viability co-cultured with C6 cells. ACEA and JWH-015 (10^{-8} – 10^{-6} M) were given to C6 cells 15 min before MPTP (100 μ M), thereafter PC12 were co-cultured with C6 cells. Each bar shows the mean $\pm$ SEM of $n=4$ experiments. *** $P<0.001$ vs control; °°° $P<0.001$ vs MPTP

However, the partial reversal of AEA and AA-5-HT effects by SR141716A appeared to be higher than the effect exerted by SR141716A per se (Fig. 10a).

Effect of CB₁ siRNA on MPTP-induced S100B density

As shown in Fig. 10d, both CB₁ and CB₂ receptors are expressed in resting C6 cells. Western blot analysis showed that in CB₁ siRNA-treated C6 cells, the levels of MPTP (100 μ M)-induced S100B protein were increased to a higher extent than in non-treated C6 cells (Fig. 10d).

Effect of ACEA, AEA, AA-5-HT, SR141716A and SR144528 on MPTP-induced C6 cell proliferation

MPTP (100 μ M) increased C6 cell proliferation at 48 h. ACEA (10^{-8} – 10^{-6} M), AEA (10^{-8} – 10^{-6} M) and AA-5-HT (10^{-8} – 10^{-6} M) concentration-dependently reduced C6 cell

proliferation. SR141716A (10^{-7} – 10^{-6} M) co-administered with ACEA, AEA and AA-5-HT to C6 cells 15 min before MPTP reversed their effects (Fig. 11a). Treatment with SR141716A (10^{-6} M) or ACEA (10^{-6} M) had no effect on C6 cell proliferation in the absence of MPTP (Fig. 11a).

Under the same experimental conditions, SR141716A (10^{-8} – 10^{-6} M) significantly and concentration-dependently increased MPTP-induced C6 cell proliferation, whereas SR144528 (10^{-6} M) had no effect (Fig. 11b).

Effect of VDM-11 on MPTP-induced C6 cell proliferation

Experiments with an inhibitor of AEA transport across cell membrane, VDM-11 (10^{-7} – 10^{-6} M), given to cells 30 min before MPTP (100 μ M), significantly and concentration-dependently increased MPTP-induced C6 cell proliferation. Conversely, when VDM-11 (10^{-7} – 10^{-6} M) was given to the cells 12 h after MPTP (100 μ M), a significant reduction in MPTP-induced C6 cell proliferation was observed, and this effect was reversed by SR141716A (10^{-7} – 10^{-6} M; Fig. 11c). VDM-11 had no effect on C6 cell proliferation in the absence of MPTP (data not shown).

Effect of ACEA, AEA, AA-5-HT and SR141716A given to MPTP-treated C6 cell on co-cultured PC12 cells

When MPTP (100 μ M)-treated C6 cells were extensively washed and placed in contact with PC12 cells for further 24 h, this resulted in a significant and concentration-dependent reduction in PC12 cell viability. ACEA (10^{-7} – 10^{-6} M), AEA (10^{-7} – 10^{-6} M) and AA-5-HT (10^{-7} – 10^{-6} M), given to C6 cells 15 min before MPTP, increased co-cultured PC12 cell viability. SR141716A (10^{-6} M) co-administered with ACEA, AEA and AA-5-HT to C6 cells 15 min before MPTP abolished their effects (Fig. 12a). On the contrary, SR141716A (10^{-8} – 10^{-6} M) given alone significantly and concentration-dependently decreased PC12 co-cultured cell viability (Fig. 12b), whereas SR144528 (10^{-6} M) had no effect (data not shown).

Fig. 9 Effect of MPTP on endocannabinoid levels in C6 cells. Cells were treated with MPTP (100 μ M) for 24 h and endocannabinoid (AEA, 2-AG) and palmitoylethanolamide (PEA) levels, were analysed

by LC-APCI-MS. Each bar shows the mean $\pm$ SEM of $n=4$ separate experiments. * $P<0.001$ vs control

Fig. 10 Effect of AEA and AA-5-HT in the presence or absence of SR141716A and SR141716A alone on MPTP-induced S100B protein expression in C6 cells. **a** Western blot analysis of S100B protein at 24 h. **b** Relative densitometric analysis. **c** Effect of silencing mRNA (*siRNA*) for CB₁ receptor in C6 cells and **d** CB₂ receptor expression

in C6 cells and Jurkat cells as control. **e** Effect of CB₁ siRNA on MPTP-induced S100B density. **f** Relative densitometric analysis. Each bar shows the mean $\pm$ SEM of $n=4$ experiments. *** $P<0.001$ vs control; °°° $P<0.001$, °° $P<0.01$ and ° $P<0.05$ vs MPTP

Discussion

The results of the present study demonstrate that the neurotoxin MPTP is able to directly promote the density and the release of the glial neurotrophin S100B in C6 rat glioma cells. This up-regulation was thereafter responsible for two opposite effects: the increased glial cell proliferation and the extensive death of co-cultured PC12 neuronal cells. It is interesting to note that the neuronal injury because of MPTP exposure appeared closely related to the over-density and the increased release of S100B protein. Although the biochemical apparatus accounting for such effect remains to be fully clarified, it is reasonable to assume that the over-expression and release of S100B by reactive C6 cells plays a key role in the induction of PC12 cell loss. The observation that a significant increase in intracellular Ca^{+2} levels in C6 cells was caused by exposure to MPTP allows us to suggest that this is one of the mechanisms contributing to the increased density and release of S100B occurring after MPTP treatment. A similar increase in intracellular Ca^{+2} levels, with consequent activation of caspase-3 and reduction in PC12 cell viability, was also observed in PC12 cells exposed to the ‘conditioned medium’ from C6 cells, suggesting that released S100B promotes a pro-apoptotic process. Indeed, the specific S100B-blocking antibody was able to reverse Ca^{+2} level increase, caspase-3 activation and neuronal cell

death, thus confirming the crucial role played by extracellular S100B under the present experimental conditions.

All the above reported findings suggest that S100B, in agreement with its dual nature, operates also under our experimental conditions in two opposite ways: as an autocrine, pro-proliferative agent in C6 cells and as a paracrine, toxic agent for co-cultured PC12 cells.

We also found that a selective CB₁ receptor agonist, ACEA, but not a selective CB₂ receptor agonist, JWH-015, significantly inhibited intracellular Ca^{+2} levels in MPTP-treated C6 cells. This inhibition was accompanied by the reduction in both S100B protein density and the subsequent glial cell proliferation. As a consequence, stimulation of CB₁ receptors in C6 cells by ACEA also protected PC12 cells from S100B-induced intracellular Ca^{+2} elevation, and the subsequent caspase-3 release and apoptosis, which were observed when PC12 cells, were placed in contact with the ‘conditioned medium’ from C6 cells.

Because it is well known that AEA synthesis is triggered by increased intracellular Ca^{+2} levels, the role of this endocannabinoid in a possible tonic regulation of S100B density in C6 cells was also investigated. In these experiments, MPTP elevated AEA levels in parallel to the increase in intracellular Ca^{+2} in C6 cells. It is interesting to note that MPTP-induced endocannabinoid synthesis was selective for AEA and did not involve a cannabinoid receptor-inactive AEA congener as PEA nor the other

major endocannabinoid, 2-AG. This is in agreement with the recent observation that increases in intracellular Ca^{2+} might not be sufficient alone and must be accompanied by activation of phospholipase C β by metabotropic receptors, to cause 2-AG biosynthesis [30]. More importantly, elevation of AEA levels, either by administration of exogenous AEA or by inhibition of AEA enzymatic hydrolysis with AA-5-HT, caused here a CB $_1$ receptor-mediated inhibition of MPTP-induced C6 cell proliferation. This effect was due again to inhibition of S100B density. Furthermore, experiments with VDM-11, a selective inhibitor of AEA transport across the cell membrane, allowed to better understand the possible role played by endogenous AEA under the present experimental conditions. In fact, VDM-11 exerted opposite effects on MPTP-treated C6 cells, depending on whether it was administered: simulta-

neously or after MPTP. In the latter case, it inhibited MPTP-induced glial cell proliferation; however, when VDM-11 was given concomitantly to MPTP, this resulted in a significant increase in glial cell proliferation. Therefore, because VDM-11 is known to inhibit both AEA release and re-uptake from cells [31], it seems likely that when this compound was given 12 h after MPTP (when AEA had already been synthesized and released from the cells), it inhibited AEA re-uptake and, subsequently, increased extracellular AEA concentrations and CB $_1$ activation. In this case, VDM-11 thus causes a very potent and significant inhibition of glial cell proliferation, similar to what was seen with exogenous ACEA, AEA and AA-5-HT. Conversely, when VDM-11 was given concomitantly to MPTP, it might have interfered with the release of newly synthesized AEA from the cells, thereby reducing extracel-

Fig. 11 C6 cell proliferation at 48 h. **a** Concentration-dependent effect of ACEA, AEA and AA-5-HT (10^{-8} – 10^{-6} M) in the presence or absence of SR141716A (10^{-7} – 10^{-6} M) on MPTP (100 μM)-induced cell proliferation. Effect of ACEA and SR141716A (10^{-6} M) given alone on C6 cell proliferation. Maximal concentration of ACEA, AEA and AA-5-HT (10^{-6} M) was used in the presence of CB $_1$ antagonist. **b** Effects of SR141716A and SR144528 (10^{-6} M) on MPTP (100 μM)-induced cell proliferation. **c** Effect of VDM-11 (10^{-7} – 10^{-6} M) given 30 min before MPTP or 12 h after MPTP. Maximal concentration of VDM-11 (10^{-6} M) was used in the presence of CB $_1$ antagonist (10^{-7} – 10^{-6} M). Each bar shows the mean $\pm$ SEM of $n=4$ experiments. *** $P<0.001$ vs control; ° $P<0.05$, °° $P<0.01$, °°° $P<0.001$ vs MPTP; § $P<0.05$, §§ $P<0.01$, §§§ $P<0.001$ vs MPTP; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs MPTP. ## $P<0.05$ and ### $P<0.001$ vs ACEA @ $P<0.05$ and @@@ $P<0.001$ vs AEA; §§ $P<0.05$ and §§§ $P<0.001$ vs AA-5-HT

lular AEA concentrations and CB₁ receptor activation and failing to control glial cell proliferation. Overall, these data suggest that after MPTP exposure, C6 cells release not only S100B with an autocrine, pro-proliferative, action and a paracrine, pro-apoptotic, effect on neurons but also AEA, which counterbalances both S100B effects, via CB₁ receptors. Therefore, AEA acts as an ‘on demand’ neuroprotective response to S100B over-density, similar to what was previously shown for kainate-induced neuronal excitotoxicity [32]. To strengthen this hypothesis, blockade of CB₁ receptors with SR141716A induced a pro-proliferative effect on MPTP-treated C6 cells and decreased viability of PC12 cells co-cultured with MPTP-treated C6 cells. This latter finding was unlikely due to the inverse agonist properties of SR141716A because the compound exerted no effects on C6 cells when they were not treated with

MPTP. Therefore, these data, together with our observation of increased AEA levels after MPTP exposure, strongly suggest the existence of an AEA tonic inhibitory action on MPTP-induced S100B over-expression. This result was further supported by our findings with silenced CB₁ receptors in C6 cells because under these conditions, we found an increase in MPTP-induced S100B significantly higher than in non-transfected C6 cells.

The results of the present study allows to suggest also that CB₁ activation, by suppressing S100B density in glial cells, might afford a dual ‘rebound’ neuroprotection through the inhibition of both glial cell proliferation and S100B-induced neurotoxicity. On the basis of these results, it is possible to assume that the tonic, negative control played by AEA and CB₁ receptor activation on S100B protein density ultimately down-regulates also reactive gliosis. Intriguingly,

Fig. 12 Effect of ACEA, AEA, AA-5-HT and SR141716A given to MPTP-treated C6 cell on co-cultured PC12 cell viability. **a** Concentration-dependent effects of ACEA, AEA and AA-5-HT in the presence or absence of SR141716A. Maximal concentrations of ACEA, AEA and AA-5-HT (10^{-6} M) were used in the presence of the CB₁ antagonist. **b** Concentration-dependent effect of SR141716A. Each bar shows the mean $\pm$ SEM of $n=3$ experiments. $^{\circ\circ\circ}P<0.001$ vs control; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs MPTP

Scheme 2 Anandamide and CB₁ receptors as an endogenous 'on demand' neuroprotective response against S100B-induced neuronal toxicity and gliosis. Possible scenario for the neuroprotection induced by endogenous anandamide (AEA) against the neurotoxic effects of glial cell-derived S100B. Stimulation of glial cells with MPTP causes elevation of intracellular Ca²⁺, which in turns triggers S100B density and AEA formation. S100B is then released by glial cells and stimulates their proliferation while inducing elevation of intracellular Ca²⁺, capsase-3 activation and apoptosis in neighbouring neurons. AEA acts on CB₁ receptors in glial cells to suppress S100B density.

S100B protein density and CB₁ receptor activation seem to regulate cell activity in an opposite ways. Whilst S100B leads to G_s protein activation, intracellular cyclic adenosine monophosphate (cAMP) elevation and increased Ca²⁺ levels in glial and neuronal cells [29], CB₁ receptor activation is coupled to G_i protein activation, decreased intracellular cAMP and inhibition of Ca²⁺ entry into the cells via voltage-activated Ca²⁺ channels in cultured neurons [33]. Therefore, it is possible that CB₁ activation might also counteract S100B neurotoxic signalling in neurons and not only its production by glial cells.

In conclusion, the above reported results point to the autocrine (pro-gliotic) and paracrine (neurotoxic) effects of S100B as a new possible mechanism contributing to MPTP-induced neurotoxicity (Scheme 2). In this context, CB₁ receptor activation by both exogenous and endogenous cannabinoids may be regarded as a putative candidate for inhibition of S100B over-production and hence as an endogenous control system over MPTP-dependent gliosis and neuronal loss. Non-psychotropic inhibitors of endocannabinoid metabolism, such as VDM11 and AA-5-HT, may represent, by this way, a new tool to attenuate MPTP-

Inhibitors of the intracellular hydrolysis of AEA to arachidonic acid (AA) and ethanolamine (EtNH₂) by fatty acid amide hydrolase (FAAH), such as arachidonoylserotonin (AA-5-HT), potentiate the CB₁-mediated neuroprotective action of AEA. Inhibitors of AEA release/re-uptake, such as VDM-11, exert opposing actions on AEA stimulation of CB₁ receptors and subsequent neuroprotective effects, depending on whether they are administered to glial cells concomitantly or after MPTP. Finally, blockage of CB₁ receptors by elective antagonists enhances the pro-proliferative (on glial cells) and toxic (on neurons) effects of S100B

induced neurotoxicity and other neuropathological conditions where S100B plays a major role.

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