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Cannabidiol Exposure During Neuronal Differentiation Sensitizes Cells Against Redox-Active Neurotoxins

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Abstract Cannabidiol (CBD), one of the most abundant *Cannabis sativa*-derived compounds, has been implicated with neuroprotective effect in several human pathologies. Until now, no undesired side effects have been associated with CBD. In this study, we evaluated CBD's neuroprotective effect in terminal differentiation (mature) and during neuronal differentiation (neuronal developmental toxicity model) of the human neuroblastoma SH-SY5Y cell line. A dose-response curve was performed to establish a sublethal dose of CBD with antioxidant activity (2.5 μM). In terminally differentiated SH-SY5Y cells, incubation with 2.5 μM CBD was unable to protect cells against the neurotoxic effect of glycolaldehyde, methylglyoxal, 6-hydroxydopamine, and hydrogen peroxide (H_2O_2). Moreover, no difference in antioxidant potential and neurite density was observed. When SH-SY5Y cells undergoing neuronal differentiation were exposed to CBD, no differences in antioxidant potential and neurite density were observed. However, CBD potentiated the neurotoxicity induced by all redox-active drugs tested. Our data indicate that 2.5 μM of CBD, the higher dose tolerated by differentiated SH-SY5Y neuronal cells, does not provide neuroprotection for terminally differentiated cells and shows, for the first time, that exposure of CBD during neuronal differentiation could sensitize immature cells to future challenges with neurotoxins.

Keywords Cannabidiol · Neuroprotection · Neurodevelopmental toxicity model · SH-SY5Y cells · Neurotoxicity · Side effects

Introduction

Cannabis sativa has been used for medicinal/recreational purposes for thousands of years [1]. The two major components are Δ^9-tetrahydrocannabinol (Δ^9-THC, the main psychoactive ingredient) and cannabidiol (CBD, which is devoid of

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psychoactive effects) [2–4]. These phytocannabinoids, together with the endocannabinoids *N*-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), mainly target the cannabinoid receptor type 1 (CB1), widely expressed in the nervous system, and the cannabinoid receptor type 2 (CB2), primarily expressed in immune cells [5–9]. Along the neuronal development, CB1 and CB2 regulate protein kinase cascades involved in cell proliferation and survival, with major consequences on progenitor cell fate decisions [10]. While CB1 expression increases during neuronal differentiation, CB2 decreases [11–13].

Most reports describing the adverse effects of cannabis are attributed to Δ^9-THC [14]. In contrast, CBD is associated with anti-inflammatory/antioxidant potential [15, 16], has a protective effect on neurons and astrocytes, and improves neurobehavioral performance in hypoxic/ischemic newborn animals [17–19]. CBD is antipsychotic, anxiolytic, antidepressant [20], and antiepileptic [21]. There are some studies evaluating the neuroprotective role of CBD in vivo and in vitro against redox-active neurotoxins such as 6-hydroxydopamine (6-OHDA) [22], amyloid-beta (Aβ) peptide, and hydrogen peroxide (H_2O_2) among others [23, 24]. In a recent study, CBD was able to reverse iron-induced reductions in synaptophysin levels and increases in caspase-3 levels [25], and it has either improved memory impairments associated to iron toxicity in a rat model [26]. CBD administration after hypoxia-ischemia in newborn rats reduces brain injury and restores neurobehavioral function [19].

As CBD is not often associated with relevant described side effects [27, 28], it has been predicted as innocuous (or harmless) from adult to newborn animal models [18, 19, 22]. Actually, a medicine containing CBD combined with THC (Sativex®) has been licensed for the symptomatic treatment of spasticity and pain associated with multiple sclerosis [22, 28], and parents are already using CBD for treatment-resistant epilepsy children, although data of cannabidiol use among children are inconclusive about its safety and tolerability [29]. Despite the intense preclinical research into numerous neurodegenerative disorders [30–32], CBD's molecular mechanisms of action are yet to be completely identified [15]. Moreover, few studies to date evaluated the effect of CBD over terminally differentiated human neuronal cells.

Most studies with CBD used in vivo models, primary cultures derived from rodents, or tumor-derived human cell lines [23]. In this context, in vivo neurotoxicity testing evaluating the effects of compounds on neurobehavioral and neuropathological processes is expensive, time-consuming, and unsuitable for screening a large number of chemical and, as other animal models, is not sensitive enough to predict human neurotoxicity [33]. Moreover, tumoral cells do not have the molecular and morphological characteristics of human neurons [34]. For this purpose, the human neuroblastoma SH-SY5Y cell line has been widely used for neurotoxicological evaluations [34], presenting several advantages for neuroscience studies such as its human origin, the facility to grow and maintain, and regardless of its tumoral origin, the neuronal morphology/physiology that can be accessed using retinoic acid (RA) [35]. A recent study has characterized the molecular phenotype of RA-differentiated SH-SY5Y cells and concluded that these cells have a neuronal dopaminergic phenotype and provide a good cellular screening tool to find compounds that affect neurologic processes [36].

Thus, the RA-differentiated SH-SY5Y cells are considered as a more suitable in vitro model to evaluate neuroprotection/neurotoxicity of compounds [35, 37] and can also be used as a neuronal cell model to screen the effect of drugs during neuronal development when these drugs are administered during the differentiation process [33, 34]. Herein, we evaluate CBD's effects in terminally differentiated (mature) as well as differentiated (neuronal developmental toxicity model) neurons using the RA-differentiated human neuroblastoma SH-SY5Y cell line.

Experimental Procedures

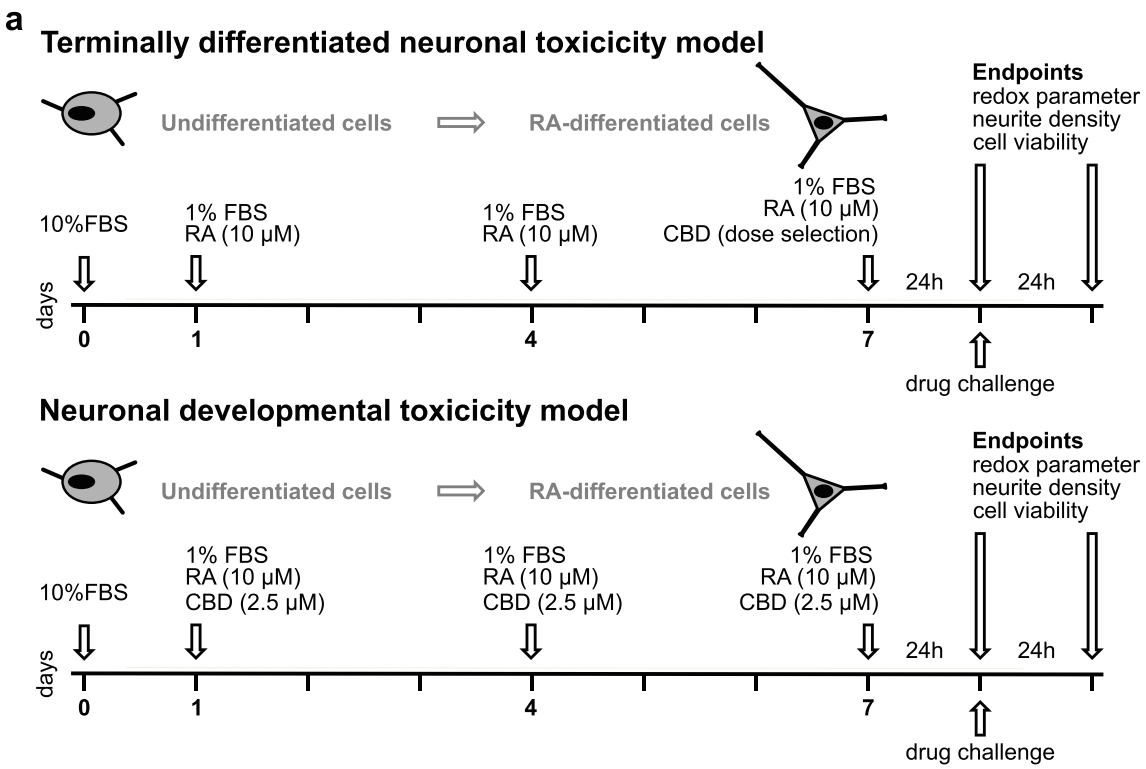
Chemicals

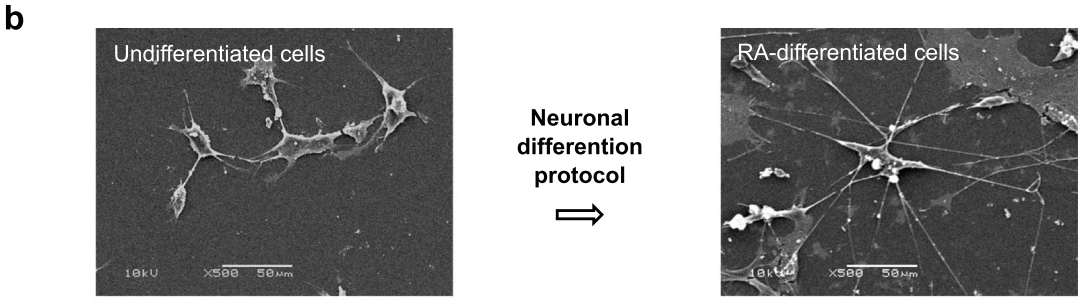
Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cannabidiol (99.9 %) is from THC Pharm (Frankfurt, Germany). Protein contents were measured by the Bradford assay [38].

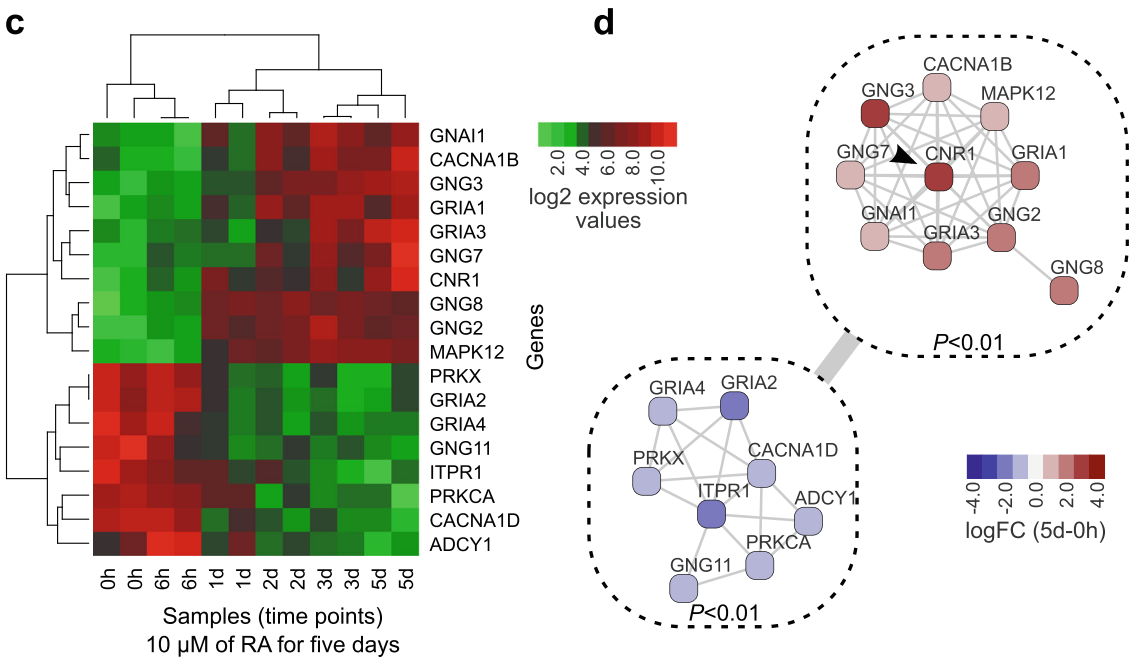
Cell Culture, Differentiation, and Treatments

Exponentially growing human neuroblastoma SH-SY5Y cell line, obtained from ATCC (Manassas, VA, USA), was maintained at 37 °C in a humidified atmosphere of 5 % CO_2. Cells were grown in a mixture of 1:1 of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of fetal bovine serum (FBS), 2 mM of glutamine, 1,000 U/mL penicillin, 1,000 μg/mL streptomycin, and 2.5 μg/mL of amphotericin B. Neuronal differentiation was triggered by

Fig. 1 Protocol design, morphological changes, and gene expression analysis of endocannabinoid signaling pathway in RA-differentiated SH-SY5Y cells. **a** RA differentiation protocol, CBD treatments, and endpoints (*arrows*) in terminally differentiated (*top*) or during neuronal differentiation (*bottom*) of SH-SY5Y cells. **b** Representative images with increased neurite outgrowth in RA-differentiated SH-SY5Y cells. **c** Heat map showing log2 expression values of endocannabinoid signaling pathway genes responding differently over time (for $P<0.01$) in RA-differentiated SH-SY5Y cells. **d** Co-expression associations. Significant associations among genes listed in the heat map were obtained by permutation analysis ($P<0.01$). Modules are defined by genes sharing positive associations (Pearson's coefficient $R>0$), while negative associations are assigned between modules (Pearson's coefficient $R<0$). *Node coloring* depicts differential expression as log2 fold change (*logFC*) of the max peak observed in the time series


a
Terminally differentiated neuronal toxicicity model
Undifferentiated cells
RA-differentiated cells
Endpoints
redox parameter
neurite density
cell viability
10%FBS
1% FBS
RA (10 μM)
1% FBS
RA (10 μM)
1% FBS
RA (10 μM)
CBD (dose selection)
24h
24h
days
0
1
4
7
drug challenge
Neuronal developmental toxicicity model
Undifferentiated cells
RA-differentiated cells
Endpoints
redox parameter
neurite density
cell viability
10%FBS
1% FBS
RA (10 μM)
CBD (2.5 μM)
1% FBS
RA (10 μM)
CBD (2.5 μM)
1% FBS
RA (10 μM)
CBD (2.5 μM)
24h
24h
days
0
1
4
7
drug challenge


b
Undjfferentiated cells
Neuronal
differention
protocol
RA-differentiated cells


c
GNAI1
CACNA1B
GNG3
GRIA1
GRIA3
GNG7
CNR1
GNG8
GNG2
MAPK12
PRKX
GRIA2
GRIA4
GNG11
ITPR1
PRKCA
CACNA1D
ADCY1
Genes
2.0
4.0
6.0
8.0
10.0
log2 expression
values
0h
0h
6h
6h
1d
1d
2d
2d
3d
3d
5d
5d
Samples (time points)
10 μM of RA for five days
d
CACNA1B
GNG3
MAPK12
GNG7
CNR1
GRIA1
GNAI1
GRIA3
GNG2
GNG8
P<0.01
GRIA4
GRIA2
PRKX
CACNA1D
ITPR1
ADCY1
PRKCA
GNG11
P<0.01
-4.0
-2.0
0.0
2.0
4.0
logFC (5d-0h)

lowering the FBS to 1 % with the addition of 10 μM RA during 7 days [35]. In the seventh day of RA-induced neuronal differentiation, SH-SY5Y cells were treated with CBD for 24 h. For evaluation of CBD's effects over neuronal development, CBD was co-administered with RA during neuronal differentiation (protocol design in Fig. 1a, bottom). In the seventh day, CBD and RA were replaced and experiments were performed 24 h later. For cell viability and reactive species (RS) generation assays, cells were seeded in 96-well plates at a density of 2×10^4 cells/well.

Neurite Density

The neuronal (stellate) morphology and neurite density were analyzed by scanning electron microscopy (SEM) and immunofluorescence, respectively. Cellular treatments were performed in 12-well plates at a density of 10^5 cells/well. Cells were washed with phosphate-buffered saline (PBS), fixed with methanol/acetone solution (1:1) for 20 min, and permeabilized with PBS/Tween 0.2 %. After washing with PBS, blocking was performed with 1 % BSA solution for 1 h. Then, cells were incubated overnight with anti-βIII tubulin antibody (Alexa 488-conjugated), and after which, the nuclear dye Hoechst 33342 was added. Five microscopic fields were randomly selected and photographed using an Olympus IX70 inverted microscope and were analyzed with NIS-Elements software. Neurite density was assessed using the AutoQuant Neurite software (implemented in *R*) and was expressed as arbitrary units (AU). For SEM, the cells were grown on 13-mm round glass tissue culture coverslips, fixed in 0.1 M phosphate-buffered 1.25 % glutaraldehyde (pH 7.4) at 4 °C, dehydrated, dried in a critical point drier, and coated with gold using a sputter coater. The specimens were examined with a JEOL JSM-5800 Scanning Electron Microscope.

Neurotoxicity/Neuroprotection Assays

Neurotoxicity of CBD was evaluated by the quantification of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Neuroprotection evaluations of CBD were performed, and after treatment or differentiation with selected dose of CBD, cells were washed with PBS and challenged with the following toxins: 6-OHDA (median lethal dose (LD_{50})=15 μM; sublethal dose=6.25 μM), methylglyoxal (MG, LD_{50}=1,350 μM; sublethal dose=625 μM), glycolaldehyde (GA, LD_{50}=115 μM; sublethal dose=25 μM), and H_2O_2 (LD_{50}=750 μM; sublethal dose=300 μM). At the end of the treatment, cells were incubated with 0.5 mg/mL of MTT during 1 h at 37 °C, and after which, medium was discarded and DMSO was added to solubilize the formazan crystals. The absorbance was measured at 560 and 630 nm using a SoftMax Pro Microplate Reader (Molecular Devices, USA). The results were expressed as a percentage of untreated cells (mean±SD value) of at least four independent experiments performed in triplicates (n=4).

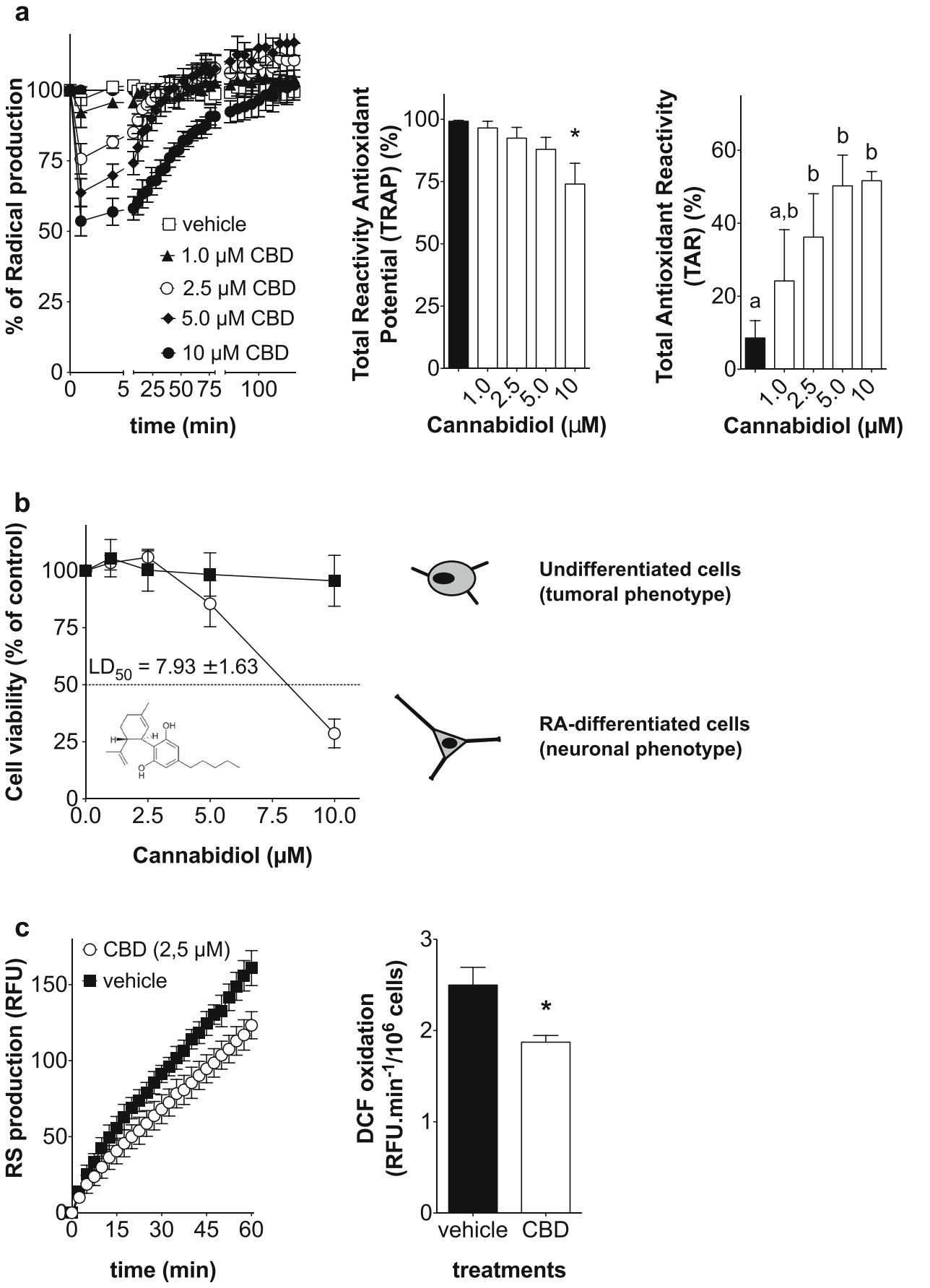
Reactive Species Generation, Total Radical-Trapping Antioxidant Potential, Total Antioxidant Reactivity, and Reduced Thiol (−SH) Levels

To evaluate the generation of reactive species (RS) in CBD-treated cells (RS), we used the probe 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) [39, 40]. After CBD treatment, the medium was removed and 10 μM DCF-DA was added. After 1-h incubation, medium was changed and the fluorescence was measured in a SoftMax Pro Microplate Reader (Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm. The nonenzymatic antioxidant capacity of CBD was assessed through the total radical-trapping antioxidant potential (TRAP) assay [41, 42]. The luminescence was monitored using a Wallace 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer (Perkin Elmer). The total antioxidant reactivity (TAR) assay [41] was performed. −SH levels were determined by measuring absorbance of DTNB at 412 nm and expressed in nanomoles of −SH per milligram of protein [43]. The results are a mean±SD value of at least four independent experiments performed in triplicates (n=4).

Differential Gene Expression Analysis

Microarray data were obtained from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/), GSE9169 dataset [44], which comprises time-course gene expression data from the human SH-SY5Y neuroblastoma cells treated under RA-inducible conditions for 5 days. Raw data (CEL files) was preprocessed and normalized using Bioconductor RMA implementation [45]. Time-course differential expression analysis was performed for six time points (0 h, 6 h, 1 day, 2 days, 3 days, and 5 days) using the R/Bioconductor package Limma [46]. Differential expression

Fig. 2 Dose selection of cannabidiol. **a** In vitro total radical-trapping antioxidant potential (TRAP) and total antioxidant reactivity (TAR) of CBD. The *left figure* presents representative TRAP traces of the effect of CBD. The *central figure* represents the *AUC* values and is expressed as a percentage of radical produced compared to vehicle (*black bar*) (*P<0.05) (one-way analysis of variance). The *right figure* represents TAR profile of CBD, expressed as a percentage of radical scavenging in comparison to vehicle (*black bar*). Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences (P<0.05) (one-way analysis of variance). **b** Cytotoxicity curve of CBD in RA-differentiated SH-SY5Y cells. Cells were treated with CBD during 24 h, and cell viability was evaluated by MTT assay (as described in Fig. 1a, *top*). Results are expressed as a percentage of vehicle. **c** RS production of cells treated with 2.5 μM CBD for 24 h was evaluated by DCF assay and expressed as relative fluorescence units (*RFU*). Data are presented as mean±SD of four independent experiments carried out in triplicates (n=4). *P<0.05 (Student's t test)


a
% of Radical production
time (min)
vehicle
1.0 μM CBD
2.5 μM CBD
5.0 μM CBD
10 μM CBD
Total Reactivity Antioxidant Potential (TRAP) (%)
Cannabidiol (μM)
Total Antioxidant Reactivity (TAR) (%)
Cannabidiol (μM)
b
Cell viability (% of control)
LD50 = 7.93 ±1.63
Cannabidiol (μM)
Undifferentiated cells (tumoral phenotype)
RA-differentiated cells (neuronal phenotype)
c
CBD (2,5 μM)
vehicle
RS production (RFU)
time (min)
DCF oxidation (RFU.min-1/10^6 cells)
vehicle
CBD
treatments

calls and contrasts have been set to interrogate whether there are any significant differences between time points related to time 0 h ($P<0.01$, false discovery rate (FDR)-adjusted for multiple testing).

Co-expression of Gene Network Analysis

The co-expression analysis was performed in the R/Bioconductor package RedeR [47] by computing Pearson's correlation values for a set of genes in a pairwise adjacency matrix. A null distribution is derived via permutation analysis and used to remove the nonsignificant associations in the adjacency matrix ($P<0.01$, FDR-adjusted for multiple testing). Additional detains are available in the R package documentation.

Statistical Analysis

Data are expressed as a percentage of untreated cells (control) (mean±SD) from at least four independent experiments ($n=4$). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant at $P<0.05$.

Results and Discussion

Experimental Design and Dose Selection

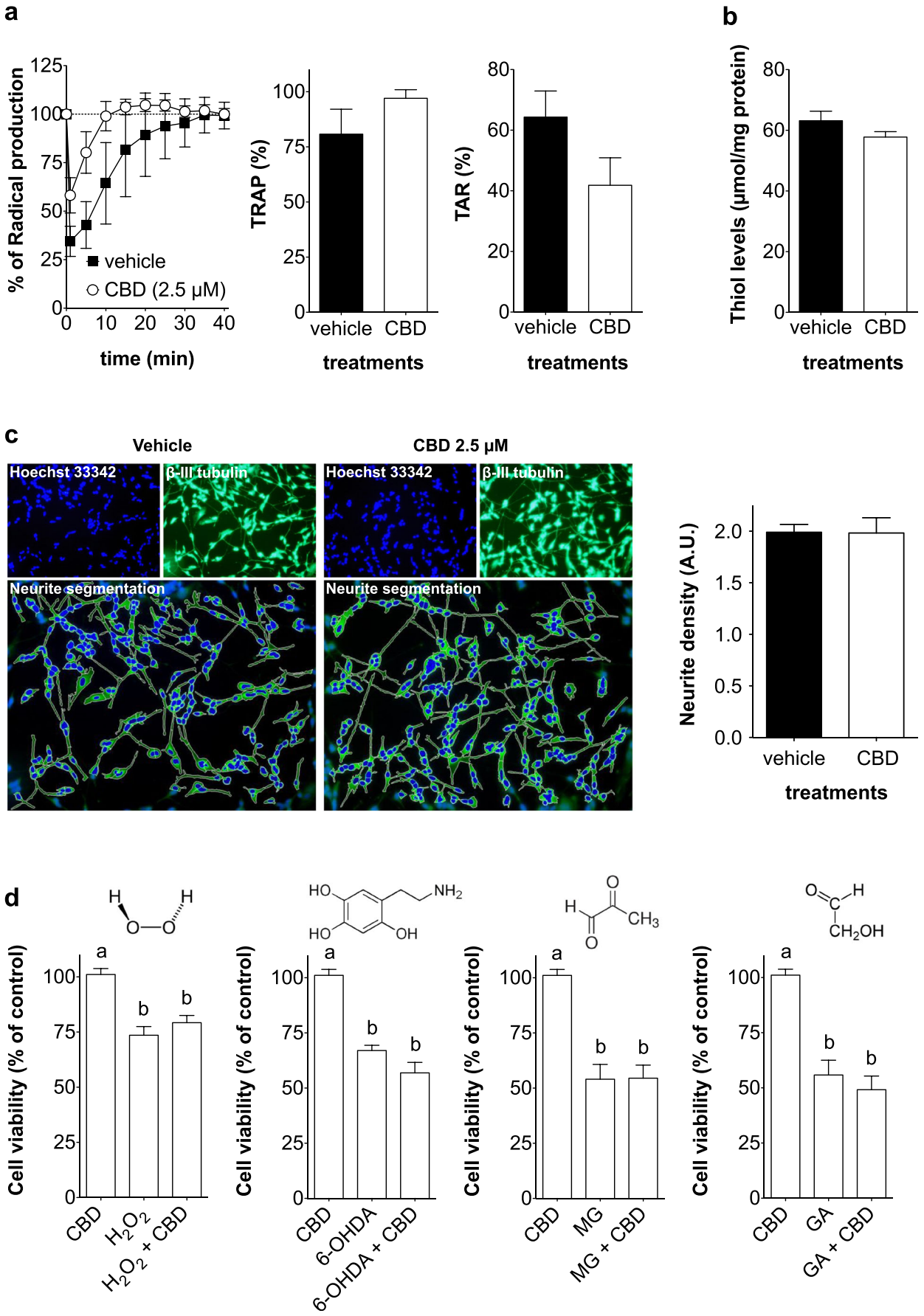
The experimental design of our study is presented in Fig. 1a. The RA-differentiated SH-SY5Y human neuroblastoma cell is an interesting in vitro model to access CBD's effects because many of the neurodevelopmental processes that occur in vivo, including cell differentiation and neurite outgrowth (as shown in Fig. 1b), can be accessed [33, 34]. Regarding the differential gene expression of the endocannabinoid signaling pathway, emphasis should be made on the increase in CB1 receptor gene expression (CNR1 gene) in RA-differentiated cells (Fig. 1d, arrow), which is in accordance to previous studies reporting an upregulation of CB1 along neuronal development (a complete list of genes is presented in Supplementary Table 1) [11–13].

As CBD is known as a potent antioxidant molecule, and since oxidative stress is related to pathophysiologic mechanisms of many neurodegenerative diseases [48], a CBD dose-response curve was designed to find the CBD dose that presents high in vitro antioxidant potential with concomitant low neurotoxicity to undifferentiated (tumoral phenotype) and RA-differentiated (neuronal phenotype) SH-SY5Y cells, which was based on previous studies [15]. Although by TRAP assay CBD was able to scavenge peroxyl radical only in the higher concentration (10 μM), TAR assay shows a significant antioxidant reactivity of CBD as low as 2.5 μM (Fig. 2a). Data on CBD-treated RA-differentiated SH-SY5Y cells show that CBD does not affect cell viability until 2.5 μM (Fig. 2b). As this dose has also presented lower rate in the reactive species production in SH-SY5Y cells (Fig. 2c), it was selected for all further experiments. The same CBD dose-response curve was tested in undifferentiated SH-SY5Y cells (tumoral phenotype) without significant changes in cell viability (Fig. 2b), which is in agreement with previous reports [23].

Effects of Cannabidiol in Terminally Differentiated SH-SY5Y Cells

In order to evaluate the effects of CBD in mature neurons, we used RA-differentiated SH-SY5Y cells treated with 2.5 μM of CBD for 24 h (protocol design in Fig. 1a). No differences were found in redox parameters (Fig. 3a, b) and in neurite densities derived from CBD-treated cells (Fig. 3c). Neuroprotective effect of CBD was evaluated by challenging treated cells with the LD_{50} of the redox-active neurotoxins 6-OHDA, MG, GA, and H_2O_2. Glycotoxins, such as MG and GA, are used in neurodegenerative models of diabetic neuropathy [49]. 6-OHDA, one of the most used toxins in experimental models of Parkinson's disease [35, 37, 50], accumulates inside the neurons causing oxidative damage [51]. H_2O_2 is an oxidant used in neurotoxicity models and associated with several neuropathologies [23, 52–55]. Although no morphological changes (such as change in neurite densities) and cellular viability were observed in terminally differentiated CBD-treated SH-SY5Y cells, CBD was not able to protect RA-differentiated SH-SY5Y cells over the significant loss of cell viability induced by the redox-active toxins tested (Fig. 3d). These data corroborate with another study in which

Fig. 3 The effect of sublethal dose of CBD over terminally differentiated human neuroblastoma SH-SY5Y cells. Cells were treated for 24 h with CBD at a concentration of 2.5 μM. **a** The *left figure* represents TRAP traces of the effect of CBD or vehicle on cells. The *central figure* represents the *AUC* values and is expressed as a percentage of radical produced compared to vehicle (*black bar*). The *right figure* represents TAR profile of treated cells, expressed as a percentage of radical scavenging in comparison to vehicle (*black bar*). **b** Elmann's reduced thiol levels. **c** Representative phase contrast and fluorescent images of nuclear dye Hoechst 33342 and cytoskeleton labeled with anti-βIII tubulin of cells treated for 24 h with vehicle (*first column*) or CBD (*second column*). The *right figure* represents the quantification of the neuritis density per cell body. **c** Evaluation of neuroprotection of sublethal dose of CBD against redox-active toxins. Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences ($P<0.05$) (one-way analysis of variance). Data are presented as mean± SD of four independent experiments carried out in triplicates ($n=4$)


a
% of Radical production
125
100
75
50
25
0
0 10 20 30 40
time (min)
vehicle
CBD (2.5 μM)
TRAP (%)
100
75
50
25
0
vehicle CBD
treatments
TAR (%)
80
60
40
20
0
vehicle CBD
treatments
b
Thiol levels (μmol/mg protein)
80
60
40
20
0
vehicle CBD
treatments
c
Vehicle
CBD 2.5 μM
Hoechst 33342
β-III tubulin
Hoechst 33342
β-III tubulin
Neurite segmentation
Neurite segmentation
Neurite density (A.U.)
2.0
1.5
1.0
0.5
0.0
vehicle CBD
treatments
d
Cell viability (% of control)
100
75
50
25
0
a
b
b
CBD
H2O2
H2O2 + CBD
HO
HO
NH2
OH
CBD
6-OHDA
6-OHDA + CBD
O
H
CH3
O
CBD
MG
MG + CBD
O
C
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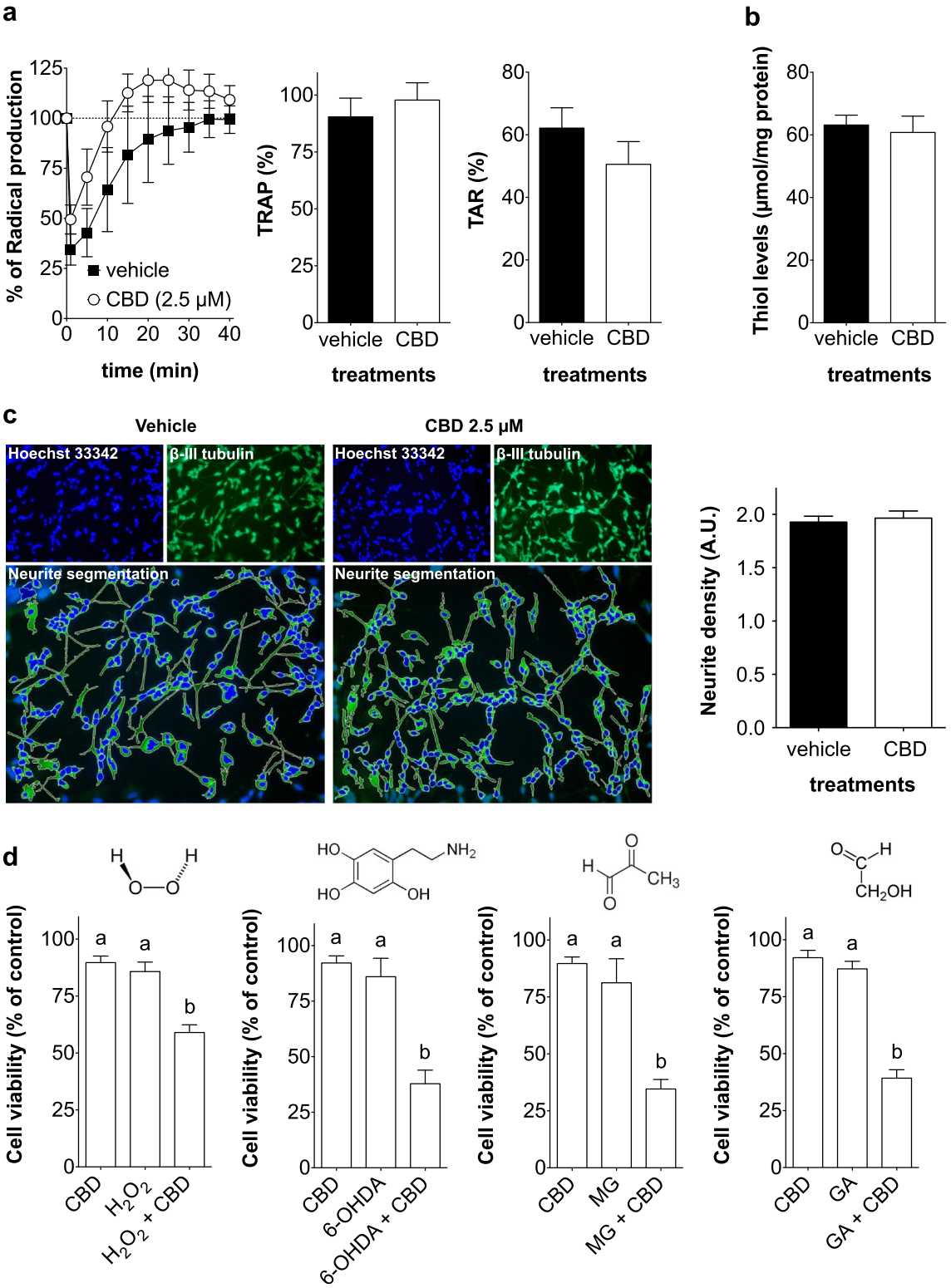

CBD at 0.01, 0.1, and 1.0 μM had no protective effect against 7 mM of 1-methyl-4-phenylpyridinium (MPP+) toxicity in RA-differentiated SH-SY5Y [24]. In contrast, cannabidiol improved cell viability in response to *tert*-butyl hydroperoxide in PC12 rat pheochromocytoma cells and in undifferentiated SH-SY5Y cells, while it was not able to inhibit amyloid-beta and H_2O_2 toxicity at 1.0 and 10.0 μM [23].

◀ **Fig. 4** The effect of sublethal dose of CBD administered during the RA differentiation of SH-SY5Y cells. **a** TRAP of treated cells. The *left figure* represents TRAP traces, representing the effect of RA differentiation with CBD or vehicle on cells. The *central figure* represents the *AUC* values and is expressed as a percentage of radical produced compared to vehicle (*black bar*). The *right figure* represents TAR profile of treated cells, expressed as a percentage of radical scavenging in comparison to vehicle (*black bar*). **b** Elmann's reduced thiol levels. **c** Representative phase contrast and fluorescent images of nuclear dye Hoechst 33342 and cytoskeleton labeled with anti-βIII tubulin of cells RA-differentiated treated with vehicle (*first column*) or differentiated with CBD (*second column*). The *right figure* represents the quantification of the neuritis density per cell body. **c** Evaluation of neuroprotection of sublethal dose of CBD against redox-active toxins. Significant differences are expressed by *letters*, where *equal letters* represent no significant differences and *different letters* represent significant differences ($P<0.05$) (one-way analysis of variance). Data are presented as mean±SD of four independent experiments carried out in triplicates ($n=4$)

Effects of Cannabidiol Exposure During the Neuronal Differentiation of SH-SY5Y Cells

Although in mature neuronal cells 2.5 μM of CBD, the highest dose tolerated by RA-differentiated SH-SY5Y cells, seems to not have neuroprotective effects (Fig. 3d), previous studies reported CBD interactions with CB1 receptors [56], which expression is induced during the neuronal development (Fig. 1d). Indeed, stimulation of the endocannabinoid system exerts a regulatory role on neural progenitor cell proliferation, differentiation, and migration in the developing nervous system and the restricted neurogenic areas that persist in the adult brain [57–62], and CBD can also promote adult hippocampal neurogenesis by activating CB1 [56]. In several studies, both phytocannabinoids and endocannabinoids detained the development of early embryos through CB1 regulation [57–60]. However, CB1, CB2, and endocannabinoids expression are induced during the formation of embryonic stem cells, and pharmacological blockade of these receptors are lethal, suggesting a role of endocannabinoid system in the survival of embryonic stem cells [63, 64].

In order to elucidate CBD's actions over the human neuronal development, SH-SY5Y cells under the RA-differentiation process was used as a model for screening of a neurotoxic/neuroprotective profile of CBD (protocol design in Fig. 1a). No statistically significant results were found in redox parameters (Fig. 4a, b) and in neurite densities derived from CBD-treated cells (Fig. 4c). However, once CBD-treated cells were challenged with sublethal doses of 6-OHDA, MG, GA, and H_2O_2, we found a significantly decrease in cellular viability in all drugs tested (Fig. 4d), arguing that the exposure of CBD during the neuronal differentiation sensitizes the cells to further challenges with redox-active toxins. These effects of CBD in developing neurons might be due to disturbance in CB1 receptor signaling [15], since CB1-mediated neuroprotection occurs through a decrease of intracellular calcium during a neurotoxic event [65]. In the nanomolar range, CBD can antagonize the pharmacological effects of CB1 agonists [9, 66], but in the micromolar range, CBD has low affinity with CB1. Moreover, some studies found that the protective effect of CBD is unlikely to be mediated by CB1 [22, 24]. Yet, at higher doses, although CBD shows a higher antioxidant potential, it is strongly cytotoxic towards terminally differentiated human SH-SY5Y cells (Fig. 2c).

There are some reports about adverse effects of *Cannabis* consumption in human. For instance, fetal development is affected by prenatal maternal *Cannabis* use, while in infancy there is negative impact in cognitive or behavioral outcomes [67]. Moreover, a search on electronic databases for preliminary clinical trials found that high-dose oral CBD, although exerts a therapeutic effect for social anxiety disorder, insomnia, and epilepsy, may cause mental sedation [68]. Cannabinoids also impair all stages of memory including encoding, consolidation, and retrieval [69]. On the other hand, most of previous studies have shown positive results for CBD. For instance, administration of CBD to newborn piglets shortly after hypoxia-ischemia has a protective effect on neurons and astrocytes, preserves brain activity, prevents seizures, and improves neurobehavioral performance [17, 18]. In an in vitro model of hypoxia-ischemia damage to newborn brains, CBD also mediated prevention of necrotic and apoptotic cell death [19]. These previous results indicate that CBD would be a useful partner for therapeutic strategies, such as hypothermia in newborn brains [19]. However, our data have shown that CBD administered during the development sensitizes neurons against future challenges with redox-active neurotoxins.

Conclusions

We present here results about the potential deleterious effects of cannabidiol (CBD). Besides not being neuroprotective for mature neuronal cells, CBD presented hazardous unwanted effects in a neuronal developmental toxicity in vitro model. Exposure of CBD during neuronal differentiation sensitized immature neuronal cells to future challenges with redox-active neurotoxins. Our data show that the potential harmful effects of CBD are actually hidden and become evident only when cells are exposed to CBD during neuronal development and further challenged with redox-active toxins. Since until now no relevant undesired side effects have been associated with CBD, our data reinforce that clinical trials and carefully risks evaluation criteria might be still necessary before CBD could be recommended to infants and adults. In this context, there are already many synthetic cannabinoids under test, designed in order to enhance protective properties, which might be a better target for new treatment strategies with these compounds [70–72].

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Author Contributions

P.S., L.M.M., I.J.B., and F.M.L. performed experiments. M.A.A.C., M.A.B..., and F.K. analyzed and interpreted the data. F.K., J.A.S.C., and F.K. conceived and designed the experiments. P.S., M.A.A.C., R.B.P., and F.K. wrote the manuscript.