ROTENONE TOXICITY IN STRIATAL AND CORTICAL ORGANOTYPIC SLICE CULTURES

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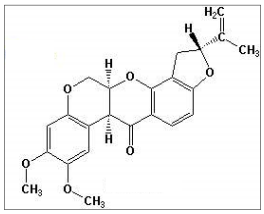
Rotenone



1,2,12,12-alpha-Tetrahydro-2-alpha-isopropenyl-8,9-dimethoxy-(1)benzopyrano(3,4-b)furo(2,3-h)(1)benzopyran-6(6-alpha-alpha-H)-on

Introduction

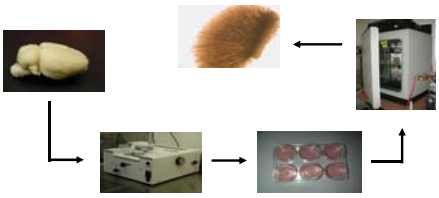
Complex 1 inhibitors of the electron transport chain represent model drugs for investigations on neurodegenerative diseases accompanied by mitochondrial dysfunctions, e.g. Parkinson's disease (Betarbet et al., 2000).

The pesticide rotenone, a mitochondrial poison, is a highly lipophilic substance that easily crosses cell membranes and accumulates in cellular organelles. Several studies indicate a selective degeneration of dopaminergic neurons after rotenone exposure (Sherer et al., 2003; Alam u. Schmidt, 2002).

In the present study cortical and striatal organotypic slices were studied for their sensitivity to rotenone.

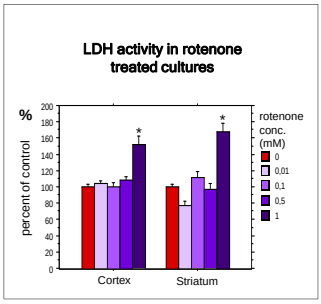
Conclusion

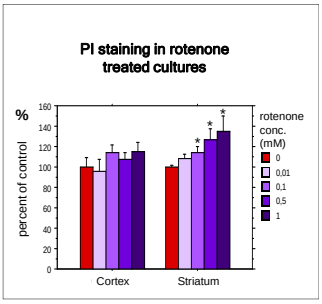
The differential effects of rotenone in the cortex and the striatum indicate a specific sensitivity of dopaminergic cells to this substance due to a higher vulnerability for damages induced by reactive oxygen and nitrogen species. Rotenone might exert its neurotoxicity in organotypic cultures via formation of NO.

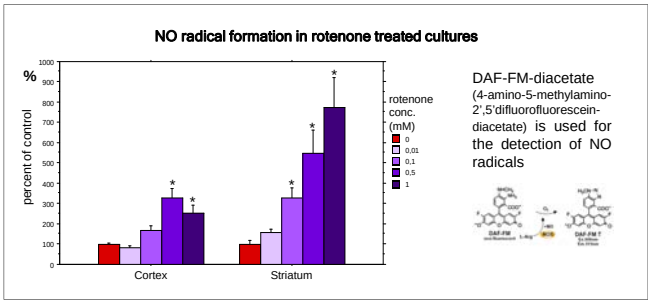


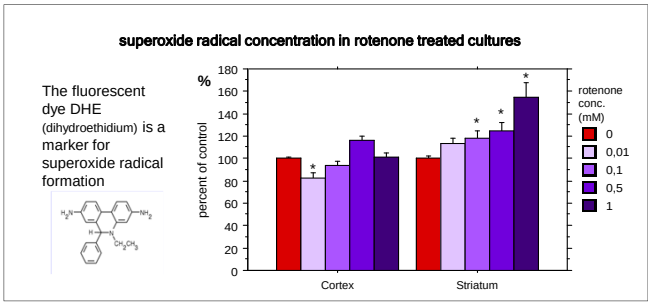
preparation of organotypic slice cultures

striatum and cortex are isolated from the mouse brain and cut into 400µm slices with a Mc Illwain Tissue Chopper. Organotypic slices are placed on Millipore-CM-filters and cultivated in a 5% CO_2 atmosphere at 37° C according to the method of Stoppini et al. (1991).









Method

Organotypic striatal cultures of adult C57/Bl6 mice were prepared using the method described by Stoppini et al. (1991). Slices were cultivated in control or rotenone-containing medium (0.01, 0.1, 0.5, 1 mM) for 48 hours.

Measurement of LDH activity and propidium iodide (PI) staining were used as general markers for cellular damage and were performed according to common protocols. Fluorescent dyes for the detection of NO (DAF-FM) and superoxide radical (dihydroethidium) formation were used and evaluated by fluorescence microscopy.

Statistical significant differences were performed using the Chi2-test after Kruscal Wallis (H)-test.

Data are expressed as mean ± S.E.M., $p<0.05$ considered significant.

Results

The neurotoxic effects of rotenone after 48h show an increase in the LDH activity at 1mM concentration (up to 52% in the cortex, 67% in the striatum) and a significantly higher PI uptake in the striatum. These data correspond with results obtained from the fluorescent dyes DAF-FM and DHE. An excessive rise of superoxide radical (up to 195% compared to control cultures) and nitric oxide radical (up to the 7 fold) were seen in striatal cultures even at lower concentrations of rotenone. In the cortex the formation of NO was increased up to 327%, while superoxide levels did not significantly raise.

This study was supported by the Bank Austria Creditanstalt

References

Betarbet R. et al. (2000) Nat. Neurosci. 3, 1301- 1306; Stoppini L., Buchs P.A., Muller D. (1991) J Neurosci Meth 37: 173- 182; Sherer T.B. et al. (2003) Exp. Neurol. 179: 9- 16; Alam M., Schmidt W. J. (2002) 136: 317- 324

THYMOQUINONE SHOWS RESCUEING EFFECTS ON MURINE MESENCEPHALIC CULTURE NEURONS AFFECTED BY COMPLEX I INHIBITION

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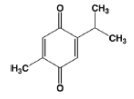
Introduction

Thymoquinone is a main constituent of black seed (*Nigella sativa*). In the Middle East, black seed oil is widely used as a medicine since some thousand years (GALI-MUTHASIB et al, 2006). Thymoquinone in a dose- and time-dependent manner reduces nitrite production, a parameter for NO synthesis, and decreases both gene expression and protein synthesis levels of iNOS without affecting the cell viability. Stimulation of polymorphonuclear leukocytes with TQ showed protective action against superoxide anion radical indicating to its potent superoxide radical scavenger abilities (SALEM, 2005). In addition, this quinone derivative is discussed a potent neuroprotective drug whose beneficial action might be based on its antioxidant properties (ALI and BLUNDEN, 2003). Since in Parkinson´s disease (PD) mitochondrial impairment entails oxidative stress, thymoquinone may provide an additive to conventional applied therapies. In this study, we used 1-methyl-4-pyridinium (MPP^+) and the pesticide rotenone to induce damages particularly on dopaminergic neurons. MPP^+ and rotenone are specific complex I inhibitors, and represents model substances for PD. Aim of our study was to investigate whether thymoquinone is neuroprotective, and whether putative neuroprotective effects of this compound are based on radical scavenging capabilities.

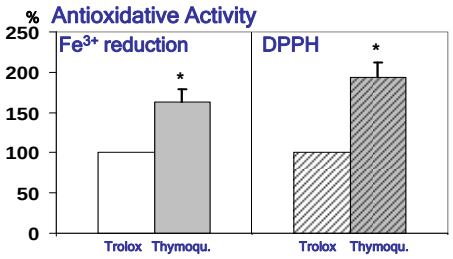


(2-isopropyl-5-methyl-1,4-benzoquinone)

Thymoquinone

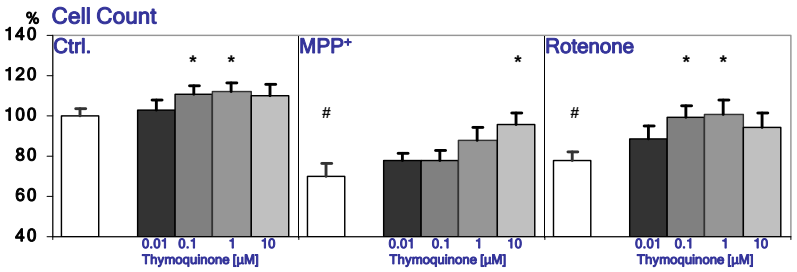
Method

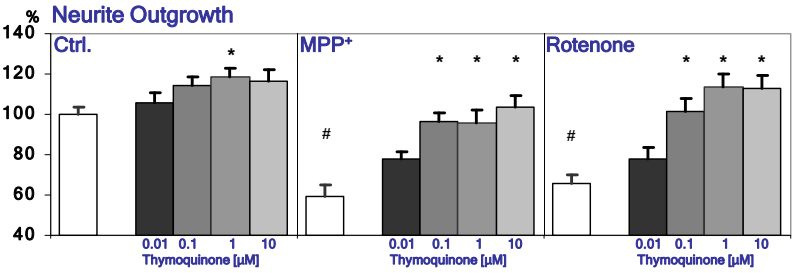
Primary dopaminergic cell cultures were prepared from mouse mesencephala according to RADAD et al. (2004). After 8 DIV, cultures were treated with thymoquinone (0.01, 0.1, 1, 10 µM), thymoquinone/MPP^+ (10 µM), or thymoquinone/rotenone (20 nM) for 48 hrs. The tyrosine hydroxylase (TH) immunostaining followed a common protocol. The fluorescent dyes DAF-FM (marker for NO production) and dihydroethidium (DHE, marker for superoxide radical formation) were used to measure the cell protection by fluorescence microscopy. Antioxidative activity of thymoquinone was photometrically determined by measurement of Fe^{2+} -TPTZ complex (at 593nm) or the reduction of stable radical DPPH (at 515nm). Results are referred to vitamin E derivative TROLOX (set as 100). Data (n=6) are and expressed as mean ±S.E.M. Statistical differences are done with Kruskal-Wallis or Mann-Whitney test, $p<0.05$ considered significant.

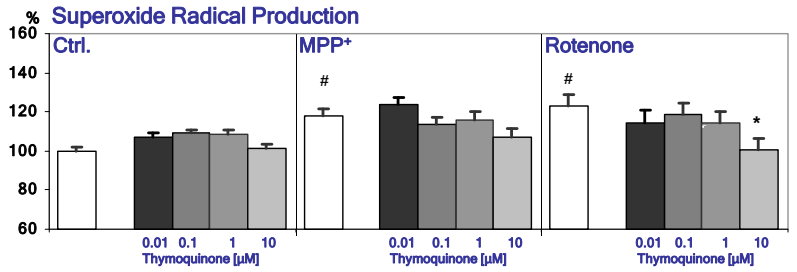


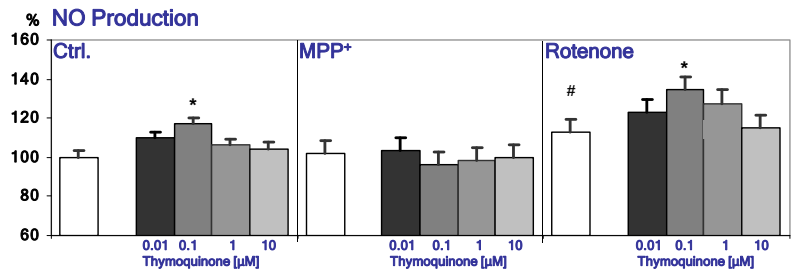
Results

Administration of thymoquinone (0.01 to 10 µM) for 48hrs dose-dependently increased the number of dopaminergic neurons up to 20%. Rotenone (20 nM) and MPP^+ (10 µM) led to a loss of about 30% or 50% of the dopaminergic neurons, respectively. Co-treated with rotenone or MPP+, thymoquinone rescued dose-dependently up to 90% of the cells compared to controls. Both toxic conditions next to cell death also caused significant deteriorations in the remaining cells as rugged appearance of cell morphology, lost and shrunk neurites and nuclear changes. This effect could be counteracted by thymoquinone to a large extent. Additionally, rotenone- or MPP^+-induced increase of superoxide radical formation can be countervailed only by high doses (10µM) of thymoquinone, whereas thymoquinone was not able to drop increased NO radical levels after complex I inhibition. At a concentration of 0.1 µM of thymoquinone, the NO formation was significantly increased. The anitoxidative activity of thymoquinone was 1.62 or 1.93 times higher than Trolox as determined by photometrical assays.







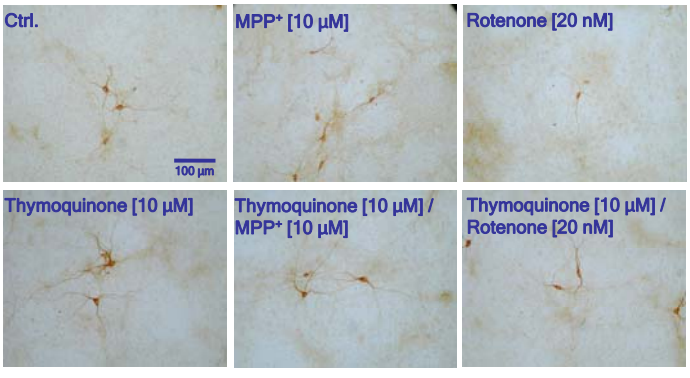


Conclusions

The definite mode of action of thymoquinone is still not understood. Since other antioxidants are beneficial to counteract rotenone- or MPP^+-induced damages on dopaminergic neurons, thymoquinone likewise may act through direct radical scavanging or indirect antioxidant effects. The protective mechanism of thymoquinone in complex I inhibition remains unclear. Nonetheless, in this study we could show that this quinone can prevent neuronal degeneration in our model of mitochondrial impairment in PD shown by an increase of numbers and an improvement of cell morphology of dopaminergic neurons. After 48 hours of co-administration, thymoquinone did not result in a reduction of NO radical concentrations that were increased by complex I inhibitors. Also a very high concentration of this quinone was required to reduce the formation of superoxide radicals. The antioxidant abilities of thymoquinone seem to play a minor role. Thus another unknown protective mode of action has to be assumed.

Nigella sativa

TH immunocytochemistry



References: RADAD et al. (2004): J Neural Transm 111; 37-45 - GALI-MUTHASIB et al. (2006): Int Jv Biochem Cell Biol 38; 1249-53 - SALEM (2005): Int Immunopharmacol 5; 1749-70 - ALI and BLUNDEN (2003): Phytother Res 17; 299-305

Effects of resveratrol in a dopaminergic cell culture model for excitotoxicity

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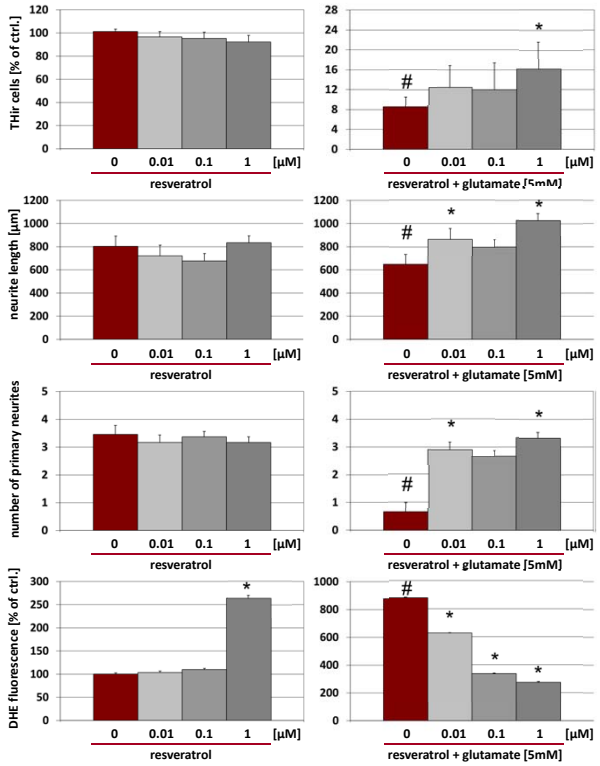
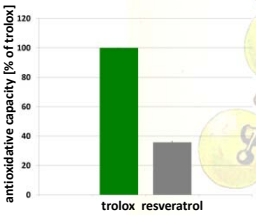
Introduction

The stilbene Resveratrol (trans-3,5,4'-trihydroxystilbene) is an antimicrobial phytoalexin from several plants, including Vitis species. The antioxidative capacity of Resveratrol was the main part for the postulation of the French paradox, the hypothesis that red wine consumption is responsible to the comparably low levels of cardiovascular diseases in France. Resveratrol interacts with the complex III of the respiratory chain and is therefore not just a radical scavenger, but also a substance suppressing radical formation in the mitochondria. This influence on radical formation led to several studies about its putative relevance in the therapy of Pakinson s diseases (PD). Oxidative stress is considered an important factor for the loss of dopaminergic neurons in PD. Excitotoxicity describes the lack of inhibitory or an overflow of excitatory neurotransmitter in the brain resulting in overactivity of neurons followed by oxidative and calcium-mediated damages and cell death. In cell cultures, this overactivity can be modeled by administration of high concentrations of glutamate, a wide-spread excitatory transmitter.

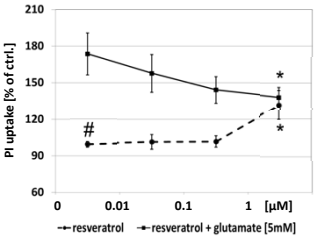
Mesencephalic Cultures

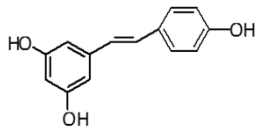




Conclusions

In this study, partial beneficial action of resveratol in a mesencephalic culture system after glutamate affection could be shown. The mode of action might be multifaceted. Glutamate significantly damaged mesencephalic cultures but the number of THir neurons were unaltered when cultures were exposed to resveratrol alone. Resveratrol provided protection against glutamate toxicity in culture by scavenging superoxide radicals. Nonetheless, next to direct antioxidative mechanisms, resveratrol may exert dopaminergic neuroprotective activity by microglial inhibition or antiapoptotic mechanism what may be interesting for the potential use of resveratrol in treatment of PD.

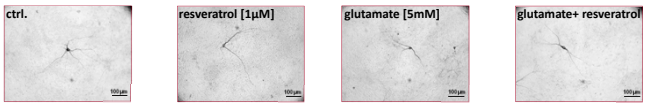




Resveratrol

trans-3,5,4'-trihydroxystilbene

TH immunocytochemistry



Results

Cell count was unaffected in resveratrol treated cultures. Glutamate treatment reduced the number of dopaminergic neuron to 8.54% compared to controls. There was a trend to a preservation of these neurons after resveratrol administration which was significant at 1µM. The number of surviving cells was nearly doubled related to the glutamate control. Neurite outgrowth measurement is a tool to determine the morphologic integrity of neurons. Resveratrol treatment neither influenced neurite length nor number. The averaged length of primary neurites was 803µm. Glutamate led to a shorter averaged length by 155µM. This was counteracted significantly by resveratrol by 216µm at a concentration of 0.01µM and 397µm at 1µM, respectively. DHE visualize the superoxide radical levels. Resveratrol (1µM) increased the level to 163.88% compared to controls, glutamate nearly multiply it nine-fold. A dose dependend decrease could be observed in resveratrol-treated cultures.

Methods

Cultures:
Primary mesencephalic cell cultures were prepared from brains of embryonic OF1/SPF mice. On gestation day 14, mice were sacrificed, their uteri dissected and the mesencephala isolated and dissociated. Dissociated cells were resuspended and plated into 48-well multiplates. Cultures were grown at 37 C in an atmosphere of 5% CO_2. On the 6th DIV, culture medium was changed to a serum-free medium. To investigate the effect of resveratrol on THir neurons in primary mesencephalic culture, resveratrol (0, 0.01, 0.1, 1µM) was added on the 10th DIV for 48h. Glutamate (5mM) was added to the cultures for 15min before medium was replaced by control medium or medium with resveratrol.

Identification of tyrosine hydroxylase immunoreactive (THir) neurons:
To determine the number of THir neurons in cultures, cells were stained followed a common protocol with an anti-TH primary antibody. Cells were counted afterwards with a Nikon inverted microscope at 100x magnification. On the 14th DIV, the average number of THir cells in the various experiments was between 1000 and 1500 cells/well.

Determination of neurite outgrowth in THir neurons:
After anti-TH staining, neurite lengths and numbers of dopaminergic cells were measured. For this reason, photographs of six cells per condition and well were taken. The pictures were edited with Adobe Photoshop and the neurite lengths calculated by pixel evaluation.

Fluorescence staining:
Propidium iodide (PI) and dihydroethidium (DHE) staining was performed on the 14th DIV. The fluorochromes were incubated at 37 C. After incubation with each fluorescence dye, cultured cells were washed and 10 photos were taken (at 100x magnification). Colour intensities for each photo were analyzed by Adobe Photoshop with the histogram modus.
Data are expressed as mean S.E.M. Statistical differences are identified with Kruskal-Wallis followed by Mann-Whitney test. $p<0.05$ was considered significant.

Effects of Phytocannabinoids CBD, CBDV, CBG, CBN and THC on murine neural cell cultures

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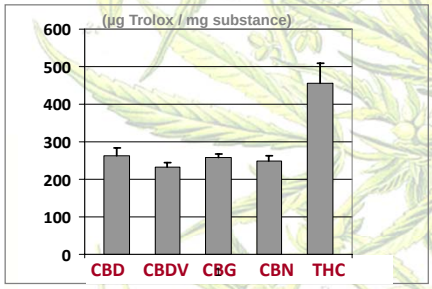
Introduction

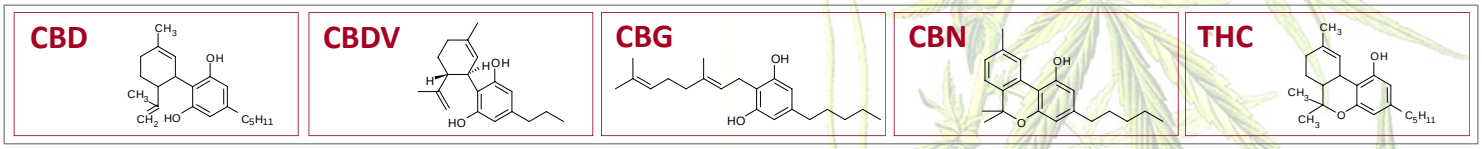
Phytocannabinoids (PCs) are terpenphenoles deriving from *Cannabis* species. They affect cells by receptor binding and non-receptor mediated mechanisms and exhibit antioxidative action. Therefore, PCs are discussed as putative drugs in neurodegenerative diseases. Nonetheless, little is known about putative toxic or protective effects on cultured neurons. In our study, we investigated the effects of cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), cannabidivarine (CBDV) and tetrahydro-cannabinol (THC) on murine dissociated mesencephalic cultures and neuroblastoma cells N18TG2, a cell line expressing CB1 cannabinoid receptors.

Conclusion

None of chosen PCs affected the survival of dopaminergic neurons in a complex primary culture system of mice which is relevant to studies on Parkinson´s disease and related disorders to a large extend. Nonetheless, CBD, might have toxic effects that should be ascertained in further studies. CBDV and CBN impair N18TG2 cells. Whether this is a cell line specific or CB1 receptor mediated effect should be clarified by using neuroblastoma cell lines without those receptors.

Antioxidative Activity (DPPH assay)

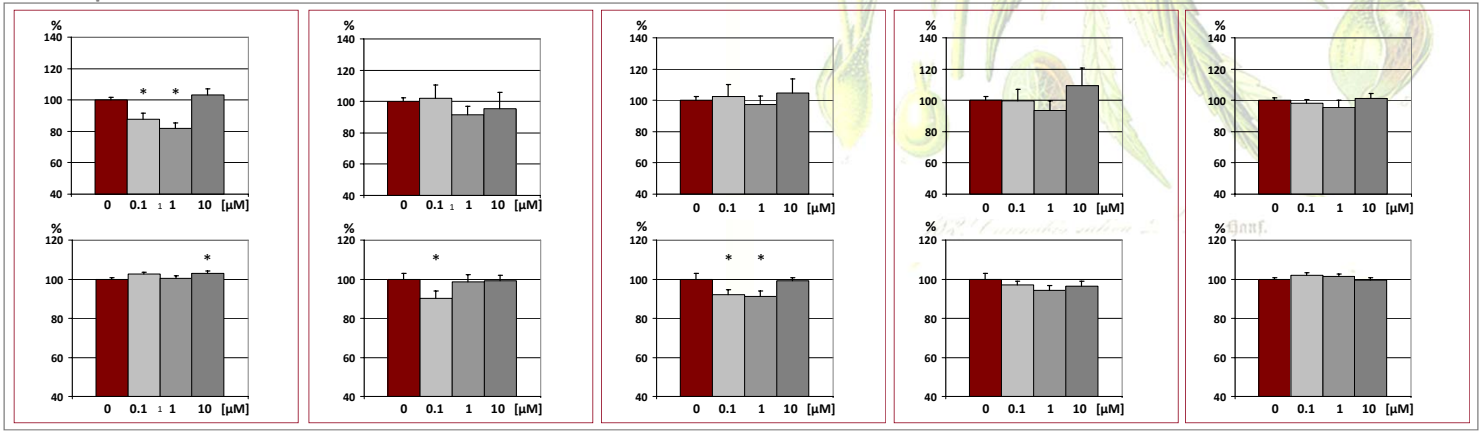




Mesencephalic Cultures

Dopamin. Neurons

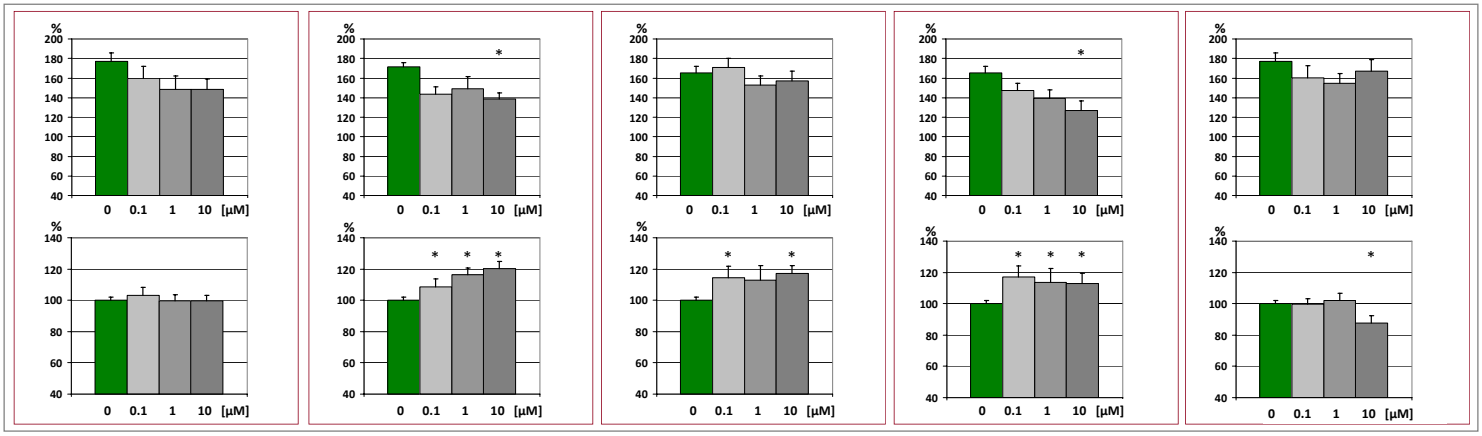
Glutathione Levels



Neuroblastoma Cells

Formazan formation

PI Uptake



Methods

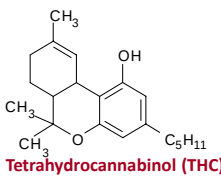
Primary dopaminergic cell cultures were prepared from mouse mesencephala according to RADAD et al. (2004). On the 12th day *in vitro*, cannabinoids (0.1 to 10µM) were administered for 48h. Using tyrosine hydroxylase immunocytochemistry, dopaminergic neurons were detected for counting. Additionally, glutathione levels were determined with a commercially available kit (Cayman Chemical). N18TG2 were treated with same concentrations (0.1 to 10µM) for 48h and detected by measurements on formazan formation (alamarBlue® assay) and propidium iodide uptake. Antioxidative actions of Phytocannabinoids were measured by DPPH assay. Statistical significance was determined using non-parametric Kruskal-Wallis (H)-test followed by the Chi2-test. Differences with $p<0.05$ were regarded as statistically significant (*).

Results

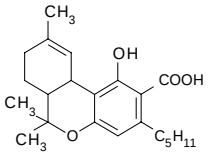
Most PCs did not alter the number of dopaminergic neurons, but CBD administration led to a decrease of cell number of 12% and 18% by 0.1µM and 1µM. Glutathione levels were unchanged by CBN and THC, wheras CBDV led to a significant decrease by 10% at 0.1µM. CBG treatment resulted in a significant decrease by 8% and 9% at 0.1µM and 1µM. Instead of this, CBD showed a significant increase of 3% at 10µM. N18TG2 cells showed some vulnerability to most PCs. Propidium iodide uptake was changed by all cannabinoids except CBD. CBDV and CBN had significant influence at all concentrations with highest increase by 20% at 10µM (CBDV) and by 17% at 0.1µM (CBN). CBG treatment led to a significant increase of PI uptake. In contrast to the other cannabinoids, THC treatment resulted in a significant decrease by 13% at 10µM. Formazan formation was unchanged by CBD and CBG. CBN and CBDV showed a significant decrease at 10µM by 39% (2h, CBN) and 33% (2h, CBDV) at 10µM. All PCs have similar radical scavenging characteristic (250µg Trolox/mg substance) expect THC which showed nearly doubled values (280 µg Trolox/mg substance).

References: Giuffrida et al. (1999): Nat Neurosci 2: 358-63 - Marinelli et al. (2007): Neuropsychopharmacology 32: 298-308 - RADAD et al. (2004): J Neural Transm 111:37-45

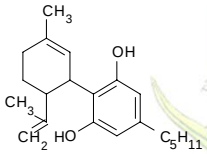
EFFECTS OF THC, THC ACID AND CBD ON MPP+ OR GLUTAMATE AFFECTED DISSOCIATED MESENCEPHALIC CULTURES OF MICE



Tetrahydrocannabinol (THC)



THC acid



Cannabidiol

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Introduction

Cannabinoids are terpenphenoles deriving from *Cannabis* species. In the brain, their activity is mainly mediated by specific receptors, and they exhibit antioxidative action. Therefore, they might be candidates for therapy of oxidative stress in neurodegenerative diseases. Mesencephalic dopaminergic neurons that are degenerating in Parkinson´s disease (PD), express cannabinoid receptor 1 (CB1) (Matsuda et al., 1993). Two events leading to cell degeneration in PD are oxidative stress and excitotoxicity. In cell culture systems, these events can be induced by the use of either the complex I inhibitor MPP+ or high concentration of the excitatoric neurotransmitter glutamate. In our study, we investigated the effects of tetrahydrocannabinol (THC), THC acid (THCA) and cannabidiol (CBD) on MPP+ or glutamate affected dissociated mesencephalic cultures of mice.

Method

Pregnant animals were housed and handled in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. Primary dopaminergic cell cultures were prepared from mouse mesencephala according to RADAD et al. (2004). On the 8th day *in vitro*, cannabinoids (0.001 to 10µM) were administered alone or concomitantly with MPP+ (10µM) or glutamate (30µM) for 48h. Using tyrosine hydroxylase immunocytochemistry, dopaminergic neurons were stained and counted. Additionally, the outgrowth and number of primary neurites were measured.

Cell Morphology

Number fo Neurites in MPP+ treated Cultures						
	ctrl.		1µM		10µM	
	%	s.e.m.	%	s.e.m.	%	s.e.m.
CBD	72,82	6,09	75,84	6,05	63,72	5,57
THC	73,36	4,94	66,32	6,08	71,82	5,34
THCacid	62,29	5,60	68,68	6,07	68,244	5,05

Neurite Length in MPP+ treated Cultures						
	ctrl.		1µM		10µM	
	%	s.e.m.	%	s.e.m.	%	s.e.m.
CBD	83,43	5,63	86,23	7,68	86,36	9,04
THC	81,99	6,03	84,79	8,98	88,95	4,92
THCacid	79,63	7,79	73,94	5,98	78,30	4,70

Untreated Control Cultures were set as 100%

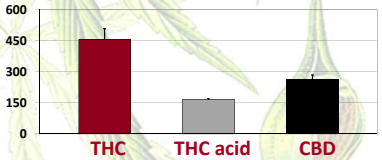
Results

The investigated cannabinoids differ concerning their protective action. While 10µM of CBD decreases the dopaminergic cell number, THCA has no effect and THC increases the number of surviving neurons at a concentration of 1 and 10µM. MPP+ treatment results in a degeneration of about a half of the dopaminergic cells. Against MPP+ induced damages, THCA and CBD display neuroprotective effect at 10µM, THC at 1 and 10µM.

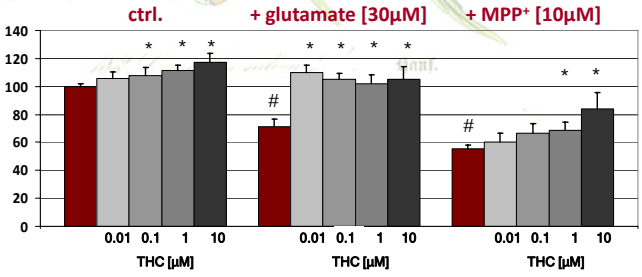
The most prominent effect of cannabinoids can be observed in glutamate induced excitotoxicity. Administration of glutamate for 48h leads to a reduction of dopaminergic cell count by about 30%. THCA and THC can abolish this degeneration in all chosen concentrations. The cell number equals the control levels in co-treated cultures. CBD can support the cell survival in glutamate treated cultures as well. This effect starts at a concentration of 10nM, but at 10µM, the damaging effects of CBD itself counteract the prevention against glutamate. Interestingly, the number and length of primary neurites was not increased in the surviving MPP+ affected dopaminergic neurons after administration of any chosen cannabinoid.

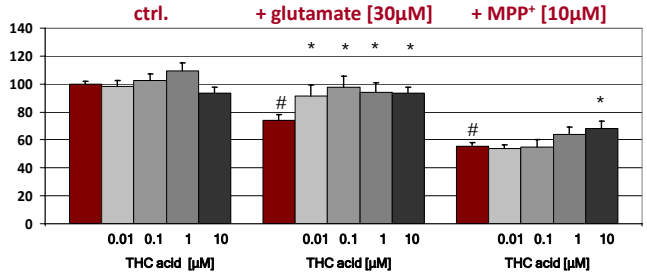
Antioxidative Activity (DPPH assay)

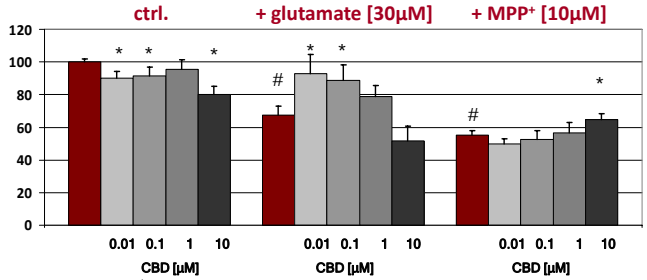
(µg Trolox / mg substance)



Number of dopaminergic Neurons [% of ctrls.]



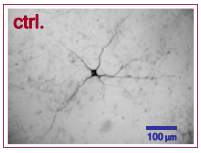


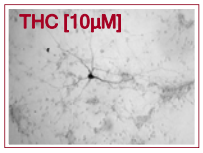


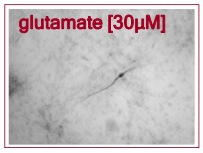
Conclusion

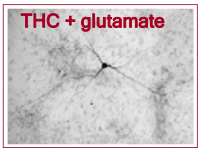
Data show protective effects of cannabinoids on dopaminergic neurons when treated concommitantly with either MPP+ or glutamate. It could be shown by Giuffrida et al. (1999). that the cannabinoid antagonist SR141716A enhances the stimulation of the motoric system by quinpirole, an agonist of the dopamine receptor D2/D3. In another study, the strong interaction between these systems is shown by Marinelli et al. (2007). Since the outgrowth of primary neurites is not improved by cannabinoids in MPP+ damaged cells, the cannabinoids might bias the surviving of the cells on a critical point rather than acting as an ameliorator of cell function. Whether the observed effects of cannabinoids against glutamate damages are related solely via the CB1 receptor remains to be investigated, but cannabinoids might be candidates for neuroprotective agents in disorders in which excitotoxicity and oxidative stress occur.

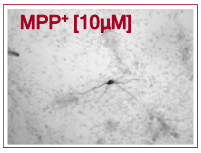
TH immunocytochemistry

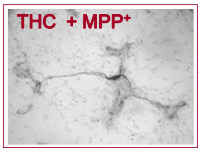






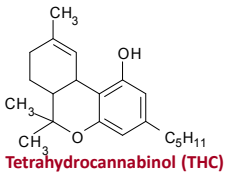






References: Giuffrida et al. (1999): Nat Neurosci 2: 358-63 - Marinelli et al. (2007): Neuropsychopharmacology 32: 298-308 - MATSUDA et al. (1993): J Comp Neurol 327: 535-50 - RADAD et al. (2004): J Neural Transm 111:37-45

THC exerts neuroprotective effects in glutamate affected murine primary mesencephalic cultures and neuroblastoma N18TG2 cells



Tetrahydrocannabinol (THC)

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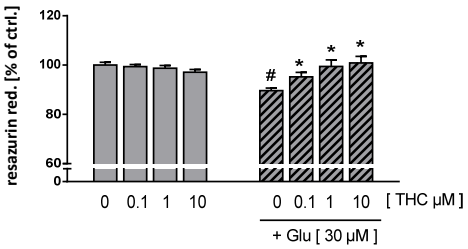
Introduction

Cannabinoids are terpenphenoles deriving from *Cannabis* species. In the brain, their activity is mainly mediated by specific receptors, and they exhibit antioxidative action. Therefore, they might be candidates for therapy of oxidative stress in neurodegenerative diseases. Mesencephalic dopaminergic neurons that are degenerating in Parkinson´s disease (PD), express cannabinoid receptor 1 (CB1) (Matsuda et al., 1993). Two events leading to cell degeneration in PD are oxidative stress and excitotoxicity. In cell culture systems, these events can be induced by the use of either the complex I inhibitor MPP^+ or high concentration of the excitatory neurotransmitter glutamate. In our study, we investigated the effects of tetrahydrocannabinol (THC) on glutamate affected dissociated mesencephalic cultures of mice and N18TG2 cells.

1a

THC restores metabolic activity in glutamate affected primary mesencephalic cultures

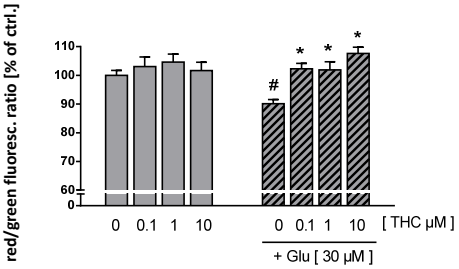
Method: resazurin reduction assay



1b

Additionally, mitochondrial potential ($\Delta\Psi m$) is stabilized by THC

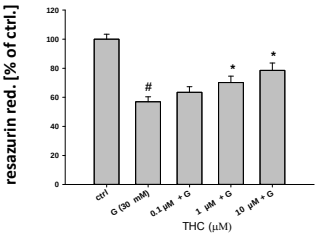
Method: JC-1 fluorescence microscopy



3a

Also in N18TG2 cells THC restores metabolic activity in glutamate affected cultures

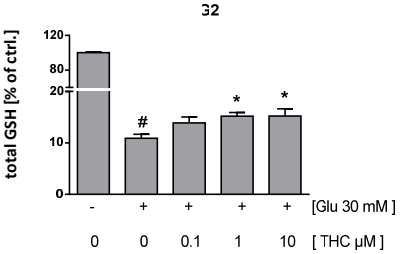
Method: resazurin reduction assay



3b

THC counteracts glutamate damages by increasing GSH levels in N18TG2 cells

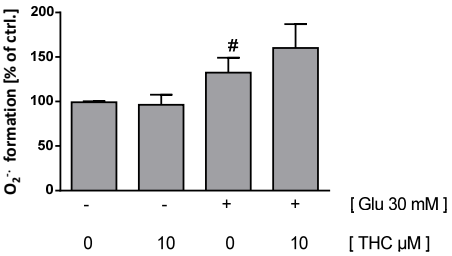
Method: kit for measuring total glutathione



3c

Glutamate increased formation of superoxide radicals cannot be counteracted by THC in N18TG2 cells

Method: electron spin resonance



Conclusion

THC counteracts glutamate induced damages in different ways:

-by antioxidative action by increasing GSH levels (3b), not scavenging superoxide radicals (3c),

-by restoring mitochondrial function (1b) and promotion of overall metabolic activity (1a, 3a),

-and most prominently by antiapoptotic activity (4).

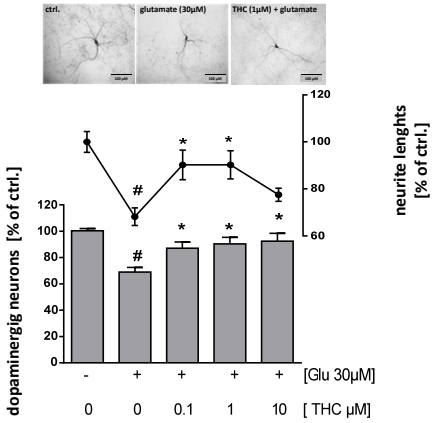
Since it is still discussed which effects of THS are receptor-dependent ones, it is remarkable that in our glutamate models the protective effect is CB1 receptor-mediated (2b).

Method for the preparation of mesencepahlic cultures: Pregnant animals were housed and handled in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. Primary dopaminergic cell cultures were prepared from mouse mesencephala according to RADAD et al. (2004). On the 8th day *in vitro*, cannabinoids (0.001 to 10µM) were administered alone or concomitantly with MPP^+ (10µM) or glutamate (30µM) for 48h.

2a

THC increases survival rate of dopaminergic neurons in mesencephalic cultures

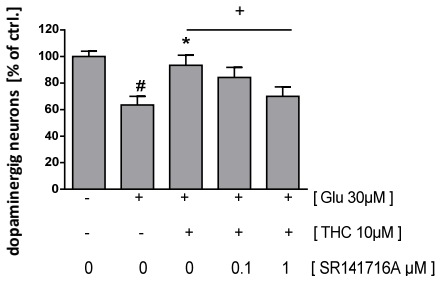
Method: tyrosine hydroxylase ICC



2b

This effect is CB1-receptor Dependent

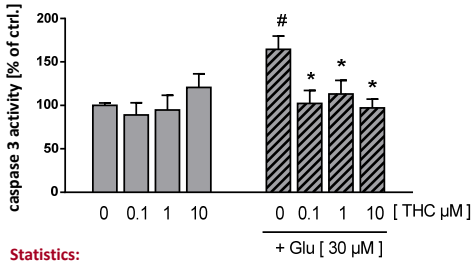
Method: tyrosine hydroxylase ICC



4

The main effect of THC in mesencephalic cultures is an antiapoptotic one

Method: caspase-3 assay



Statistics:

Data used for statistical analysis were obtained from 4 to 6 independent experiments. Data were presented as mean ± SEM, and analysis with non-parametric Kruskal-Wallis (H)-test followed by the Chi square (χ^2) test. For evaluation of glutamate effects vs. control, the non-parametric Mann–Whitney U-test was used. $P < 0.05$ was considered as statistical significant.

Reference:

MATSUDA et al. (1993): J Comp Neurol 327: 535-50 RADAD et al. (2004): J Neural Transm 111:37-45

Phytocannabinoids tetrahydrocannabinol and cannabidiol act against rotenone induced damages in murine cell cultures

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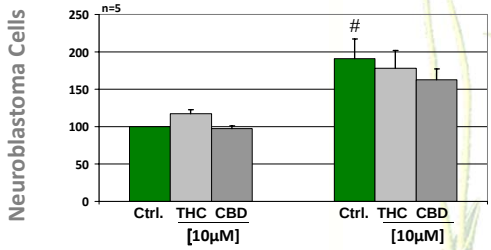
Introduction

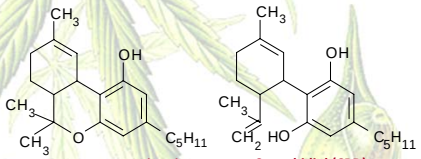
Phytocannabinoids are terpenphenoles deriving from *Cannabis* species. In the brain, their activity is mediated by specific receptors such as the cannabinoid receptor 1 type (CB1). This receptor is expressed in mesencephalic dopaminergic neurons which are degenerating in Parkinson´s disease (PD). It could be shown by Giuffrida et al. (1999) that the cannabinoid antagonist SR141716A enhances the stimulation of the motoric system by quinpirole, an agonist of the dopamine receptor D2/D3. In another study, the strong interaction between these systems is shown by Marinelli et al. (2007). Phytocannabinoids may display antioxidative capacities. Since one major event in PD is oxidative stress, cannabinoids are potential neuroprotective drugs. Experimentally, oxidative stress can be induced by the complex I inhibitor rotenone. We investigated the effects of tetrahydrocannabinol (THC) and cannabidiol (CBD) on rotenone affected dissociated mesencephalic cultures and neuroblastoma (N18TG2) cells of mice.

Conclusions

Data show protective effects of cannabinoids on dopaminergic neurons when treated concomitantly with rotenone. Radical scavenging does not appear to be the main mechanism of the phytocannabinoid action. The increase of glutathione levels is eminent, so that an upregulation of endogenous antioxidative mechanisms might play a crucial role in phytocannbinoid induced neuroprotection. Whether the observed effects of cannabinoids against rotenone damages are related solely via the CB1 receptor remains to be investigated, but cannabinoids might be candidates for neuroprotective agents in disorders in which oxidative stress is involved.

Superoxide Radical Formation [% of ctrl.]

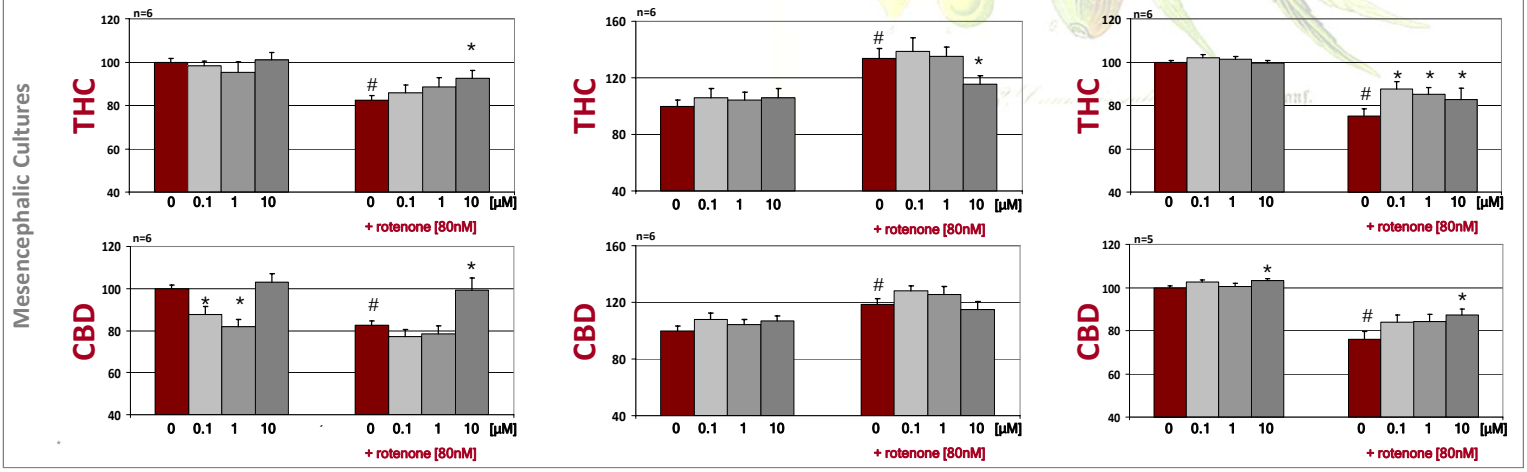




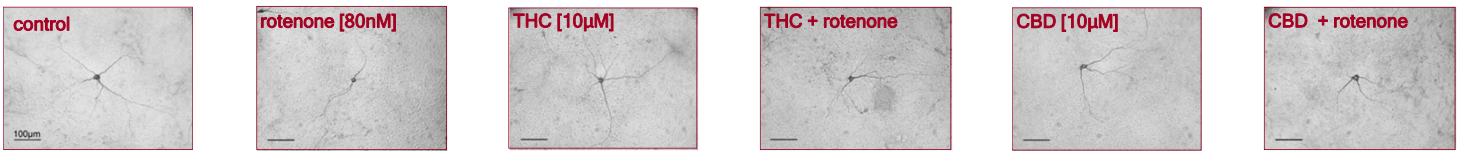
Dopaminergic Neurons [% of ctrls.]

Propidium Iodide Uptake [% of ctrls.]

Glutathione Levels [% of ctrls.]



TH immunocytochemistry



Methods

Pregnant animals were housed and handled in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. Primary dopaminergic cell cultures were prepared from mouse mesencephala according to RADAD et al. (2004). On the 8th day *in vitro*, cannabinoids (0.1 to 10µM) were administered alone or concomitantly with rotenone (80nM) for 48 h. Tyrosine hydroxylase ICC was performed to detect dopaminergic neurons for counting and evaluation of cell morphology. Glutathione levels were determined with a commercially available kit (Cayman Chemical). Superoxide radical formation in N18TG2 neuroblastoma cells was determined by electron spin resonance (ESR) spectroscopy which is based on absorption of microwave radiation stimulated by an electromagnetic field in free radicals and unpaired electrons. $O_2^{\cdot-}$ radicals react with CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) to form stable nitroxyl radicals, which can be quantified by ESR. Statistical significance was determined using non-parametric Kruskal-Wallis (H)-test followed by the Chi2-test. Differences with $p<0.05$ were regarded as statistically significant (*). Non-parametric Mann–Whitney U-test was used to determine statistical significance between two independent sample groups followed by the Chi2-test. Differences with $p<0.05$ were regarded as statistically significant (#).

Results

THC alone exhibited no significant effect on quantity of dopaminergic neurons. In contrast, treatment with CBD showed a significant decrease in cell number at 0.1µM and 1µM of 12% and 18%, respectively. Interestingly, a higher concentration of 10µM CBD had no such effect. Treatment with 80nM rotenone led to a decline in number of dopaminergic neurons by 18%. This toxic effect is significantly counteracted by both THC and CBD at 10µM. Mesencephalic cells treated with rotenone exhibited a significant increase in propidium iodide stained cells of 33% compared to untreated cells. Cultures treated with rotenone and THC simultaneously showed a significant decrease of non-viable cells by nearly the half. In contrast, CBD had no significant effect. In Neuroblastoma cells, no rescue could be observed (data not shown). Under control conditions, production of superoxide radicals ranged between 0.132 to 0.766 pmol/min. Rotenone led to an increase of superoxide radicals of 91%. Neither THC nor CBD were capable of reducing superoxide radical formation in neuroblastoma cells significantly. Interestingly, CBD (10µM) alone also enhanced production of superoxide radicals significantly, whereas THC (10µM) had no such effect. THC alone did not alter glutathione levels in mescencephalic cells. Rotenone depleted glutathione levels by 25%. This effect was counteracted by treatment with THC in all tested concentrations (0.1µM, 1µM and 10µM). Treatment with CBD alone led to a significant increase of glutathione levels at a concentration of 10µM. CBD was also capable of reducing rotenone-induced depletion of glutathione significantly at 10µM and exhibited a tendency to do so at a concentration of 0.1µM and 1µM.

References: Giuffrida et al. (1999): Nat Neurosci 2: 358-63 - Marinelli et al. (2007): Neuropsychopharmacology 32: 298-308 - RADAD et al. (2004): J Neural Transm 111:37-45

CBD and THC decrease mitochondrial respiration in N18TG2 neuroblastoma cells without influencing cell survival

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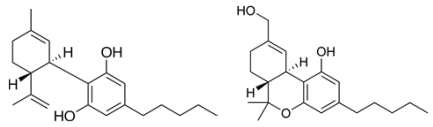
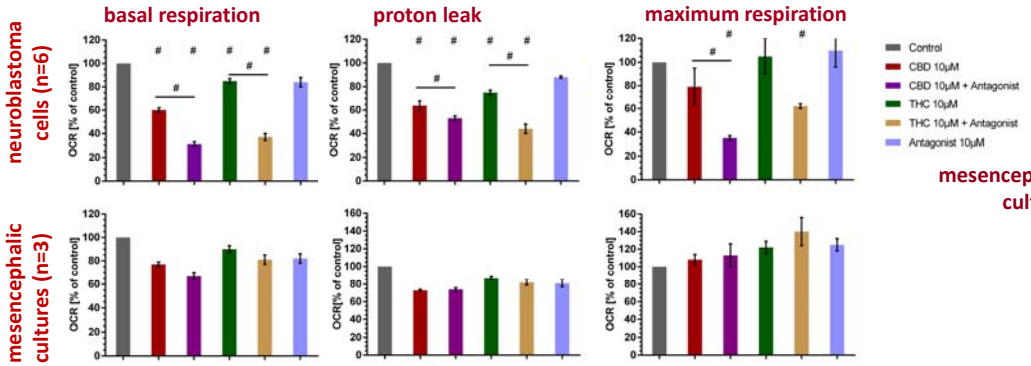


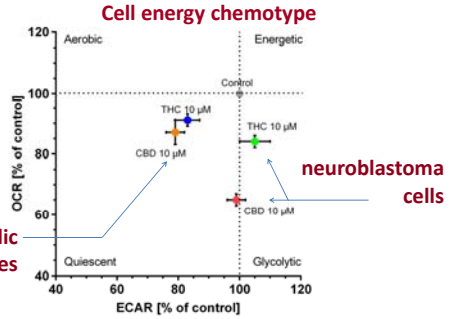
Objectives

Phytocannabinoids are discussed to be beneficial for Parkinson´s Disease (PD) patients and display neuroprotective effects in cellular PD models. Nonetheless it is known that these compounds can inhibit mitochondrial function. This is interesting since mitochondrial impairment in dopaminergic neurons is a main characteristic of PD. In our study, we wanted to know whether this inhibition has an influence on cell survival. Therefore, we investigated mitochondrial oxygen consumption and cell viability in neuroblastoma cells treated with THC or CBD. Additionally, we repeated the experiments in primary mesencephalic cell cultures, in which both substances are neuroprotective against MPP^+, a complex I inhibitor and a model substance for PD (Moldzio et al., 2012).

Effects of THC and CBD on metabolic activity of neuroblastoma cells and primary mesencephalic cultures



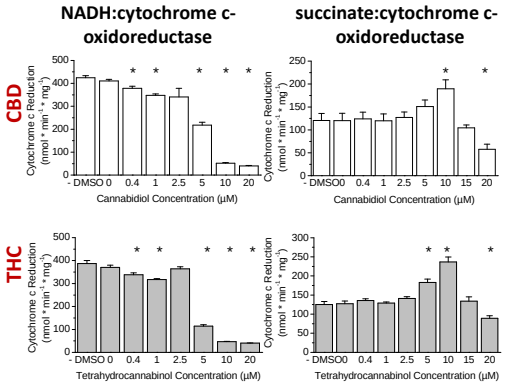
Cell energy chemotype



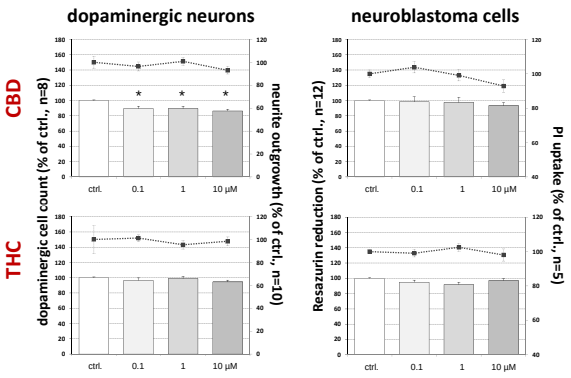
Results

Neither CBD nor THC reduces cell viability in N18TG2 cells whereas in mesencephalic primary cultures, CBD but not THC, reduces the number of dopaminergic neurons. While the effects of the cannabinoids are alike in submitochondrial particles in which they inhibit enzymes of the electron transport chain, these phytocannabinoids affect mitochondrial respiration differently in our chosen cell cultures. Basal oxygen consumption and ATP production were less reduced after administration of THC (by 16 and 13%) than after treatment with CBD (by 39 and 42%). These decreases are not accompanied by a shift to glycolysis and cannot be counteracted by co-treatment with SR141716A. Additionally, neither cannabinoid seems to induce mitochondrial uncoupling.

THC and CBD inhibit mitochondrial activity in submitochondrial particles (n=6)



Effects of THC and CBD on cell survival parameters in N18TG2 cells and dopaminergic neurons



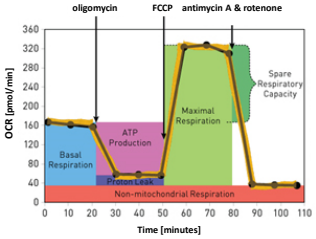
Conclusions

In N18TG2 cells and murine primary mesencephalic cultures, THC and CBD inhibit mitochondrial respiration. CBD reduces mitochondrial activity to a larger extend, which may be responsible for the significant cell toxicity to dopaminergic neurons. The moderate reduction of the overall cell metabolism by THC in either culture may be one of the ways how this phytocannabinoid exhibits neuroprotective effects in neurodegenerative diseases such as PD.

Method

N18TG2 cells were treated for 48 hrs with CBD or THC (0.1 to 10 µM). Afterwards, a resazurin reduction assay for measuring the overall metabolic activity and propidium iodide (PI) staining for evaluating cell viability were performed. Murine mesencephalic cultures were prepared from E14 mice and treated on the 12th DIV in the same way as the N18TG2 cells. Dopaminergic neurons were counted after visualization with tyrosine hydroxylase activity. Neurite outgrowth was examined by measuring neurite lengths. Functional mitochondrial metabolic analysis was investigated in both cell culture types using a fluorometric system that allows real-time measurement of the two main metabolic energy pathways within cells – oxidative phosphorylation and glycolysis (Seahorse XF Analysis, Agilent Technologies). The oxidative phosphorylation couples the electron transfer via mitochondrial complexes I to IV, and finally oxygen consumption, to ATP synthesis. The efficiency of oxidative phosphorylation is judged from the measurement of oxygen consumption rates (OCR) via oxygen-sensitive fluorophores under different metabolic conditions. Simultaneously, the extracellular acidification rate (ECAR) is quantified via pH-sensitive fluorophores. The ECAR is an indicator for the cellular capability of performing glycolysis and therefore the formation of pyruvic or lactic acid (Divakaruni et al., 2014). Experiments were performed in cell cultures treated with either cannabinoid (+/- CB1 receptor antagonist SR141716A) for 4 hrs. Additionally, in submitochondrial particles, activities of NADH:cytochrome c-oxidoreductase and the succinate:cytochrome c-oxidoreductase were measured spectrophotometrically at 550 nm (increase of absorption due to reduction of cytochrome c).

Mitochondrial respiration



References:

Divakaruni AS, Paradyse A, Ferrick DA, Murphy AN, Jastroch M. Methods Enzymol. 2014;547:309-54

Moldzio R, Pacher T, Krewenka C, Kranner B, Novak J, Duvigneau JC, Rausch WD. Phytomedicine. 2012;19:819-24.