Neuroprotection by $\Delta^9$-Tetrahydrocannabinol, the Main Active Compound in Marijuana, against Ouabain-induced In Vivo Excitotoxicity

M. van der Stelt1*, W.B. Veldhuis2,3*, P.R. Bar3, G.A. Veldink1, J.F.G. Vliegenthart1 & K. Nicolay2

Based on: Van der Stelt et al., Journal of Neuroscience, 2001, 21, 6475-6479

*These authors contributed equally to the work.
1Dept. of Bio-organic Chemistry, Bijvoet Center for Biomolecular Research, Padualaan 8, 3584 CH, Utrecht University, Utrecht, The Netherlands
2Dept. of Experimental in vivo NMR, Image Sciences Institute, Bolognalaan 50, 3584 CJ, Utrecht, University Medical Center Utrecht, The Netherlands
3Dept. of Experimental Neurology, University Medical Center Utrecht, Heidelbergaan 100, 3584 CX, Utrecht, The Netherlands
Abstract

Excitotoxicity is a paradigm used to explain the biochemical events in both acute neuronal damage and in slowly progressive, neurodegenerative diseases. Here, we show in a longitudinal Magnetic Resonance Imaging (MRI)-study that ∆9-tetrahydrocannabinol (∆9-THC), the main active compound in marijuana, reduces neuronal injury in neonatal rats injected intracerebrally with the Na+/K+-ATPase inhibitor ouabain to elicit excitotoxicity. In the acute phase ∆9-THC reduced the volume of cytotoxic edema by 22%. After 7 days, 36% less neuronal damage was observed in treated rats compared with control animals. Coadministration of the CB1 cannabinoid receptor antagonist SR141716 prevented the neuroprotective actions of ∆9-THC, indicating that ∆9-THC afforded protection to neurons via the CB1-receptor. In ∆9-THC-treated rats the volume of astrogliotic tissue was 36% smaller. The CB1 receptor antagonist did not block this effect. These results provide evidence that the cannabinoid system can serve to protect the brain against neurodegeneration.

Introduction

The endogenous cannabinoid system comprises two cannabinoid receptors, designated CB1 and CB2, which have been cloned and characterized1. Two main endogenous ligands based on fatty acids, i.e. anandamide and 2-arachidonoylglycerol (2-AG) have been identified1. The CB1 receptor is mainly found in the central nervous system, whereas the CB2 receptor is almost exclusively expressed by cells of the immune system 2. The discovery of the endogenous cannabinoid system initiated intense research into the therapeutic potential of cannabinoids in a variety of neurological and neurodegenerative disorders, such as gliomas, cerebral ischemia, and multiple sclerosis3-6. (Endo)cannabinoids have also been tested in models of excitotoxicity, which is a concept of neuronal cell death caused by overactivation of excitatory amino acid receptors. The excitotoxicity hypothesis is used to explain the common biochemical basis behind many acute and chronic neurodegenerative disorders such as stroke, traumatic brain injury, amyotrophic lateral sclerosis, Parkinson’s, Huntington’s, and Alzheimer’s diseases7,8. N-acyl ethanolamines, including anandamide, and their precursors and 2-AG accumulate, when tissues and cells are subjected to excitotoxic stress3-6,9,12. Whether this increase in endocannabinoids is neuroprotective and if so via which mechanism is still under debate13-19.
The therapeutic effects of cannabinoids in \textit{in vivo} models of cerebral ischemia are also not consistent. Chronic $\Delta^9$-THC administration has been shown to reduce the impact of an ischemic insult evoked by a reduced blood pressure and 12 min bilateral carotid artery occlusion. The involvement of the CB$_1$ receptor was not studied\textsuperscript{5}. However, no protective effect could be found for WIN55.212, a synthetic CB receptor agonist, in rats when the middle cerebral artery was occluded for 2 h. Surprisingly, the CB$_1$ receptor antagonist SR141716 was protective (C.J. Hillard, personal communication). Remarkably, WIN55.212-2 afforded protection to hippocampal and cortical neurons in CB$_1$-dependent manner in rats with a permanent middle cerebral artery occlusion or global ischemia\textsuperscript{5}. The reason for this discrepancy is not known at the moment, but (endo)cannabinoid-induced vasorelaxation\textsuperscript{20} may have a different impact on the pathway of neuronal demise in each of these stroke models.

In light of the ambiguous results from both \textit{in vitro} models of excitotoxicity and \textit{in vivo} models of cerebral ischemia, we investigated the neuroprotective properties of $\Delta^9$-THC in an \textit{in vivo} model of secondary excitotoxicity. Neurodegeneration was elicited by inhibition of the Na$^+$/$K^+$-ATPase. Diffusion-weighted magnetic resonance imaging (MRI), T$_2$-weighted MRI, and histology were used to study the effects of $\Delta^9$-THC in both the acute and late phases after the induction of excitotoxicity.

**Animals, Materials and Methods**

**Animal model**

Neonatal Wistar rats (U:Wu/Cpb; 7- to 8-day-old) were anesthetized with ether and immobilized in a stereotactic frame. A small burr hole was drilled in the cranium over the left hemisphere, 2.5 mm lateral of bregma. A 1 $\mu$l syringe was lowered into the left striatum to a depth of 4.0 mm \textsuperscript{21}, Ouabain (0.5 $\mu$l 1mM; n=30, Zwijndrecht, The Netherlands) or vehicle (0.5 $\mu$l 40 mM Tris-HCl buffer, pH 7.4; n=2) was injected at a rate of 0.125 $\mu$l/min with a microdrive. After injection the needle was left in situ for 2 min to avoid leakage of injection fluid from the needle tract. Animals were then positioned in the magnet and anesthesia was continued with a mixture of halothane (0.4-1%) in N$_2$O/O$_2$. Body temperature was maintained at 37°C using a water-filled heating pad and an infrared heating lamp. Animals were treated with $\Delta^9$-THC (Sigma Aldrich, n=12), THC + SR141716 (Sanofi Recherche, n=5, Montpellier, France), SR141716 (n=6) (all drugs at 1 mg/kg in 1 ml/kg bodyweight 18:1:1 v/v Phosphate Buffered Saline (PBS)/Tween80/Ethanol) 30 min prior to toxin injection. There was no difference in body weight and growth rate between any of the groups. The vehicle injection \textit{i.p.} did not affect lesion size. The University’s Animal Experimental Committee approved all protocols.
MRI-experiments

MRI was performed on a 4.7T Varian horizontal bore spectrometer. Excitation and signal detection were accomplished by means of a Helmholtz volume coil (9 cm $\varnothing$) and an inductively coupled surface coil (2 cm $\varnothing$), respectively. A single-scan diffusion-trace MRI-sequence (four $b$-values: 100-1300 s/mm$^2$, repetition time (TR) 3s, time of echo (TE) 100 ms) was used to generate quantified images of tissue water trace apparent diffusion coefficient (ADC). Diffusion trace- and $T_2$-weighted-imaging (TE=18, 40, 62 and 84 ms; TR=2s, nt=2) were performed in all animals, starting at $t=15$ min after injection on day 0 and were repeated one week later.

Both the $T_2$-weighted and the diffusion-weighted datasets consisted of seven consecutive, 1.5 mm thick slices, with 0 mm slice gap To minimize interference at the slice boundaries, slices were acquired in alternating order (1,3,5,7,2,4,6), thus maximizing the time between excitation of two neighbouring slices. For the diffusion-weighted imaging we used a double spin-echo pulse sequence with four pairs of bipolar gradients with specific predetermined signs in each of the three orthogonal directions. The combination of gradient directions leads to cancellation of all off-diagonal tensor elements, effectively measuring the trace of the diffusion tensor. This provides unambiguous and rotationally invariant ADC values in one experiment, circumventing the need for three separate experiments. For each $b$-value, two scans were averaged. The total scan time for acquisition of seven slices, with four $b$-values and two averages, was 17 minutes.

As expected, at the early time point no changes in $T_2$-weighted MRI were detected. Animals not scanned at day 0, were kept under halothane anesthesia for equal durations as the scanned animals to prevent anesthesia-induced bias.

Data analysis

Mono-exponential fitting using the Interactive Data Language software package generated ADC and $T_2$ maps. Parametric images were analyzed in anatomic regions of interest using Image Browser (Varian). Pixels in the ipsilateral hemisphere were considered pathological when their ADC- or $T_2$-value differed more than twice the SD from the mean value in the contralateral hemisphere. The ventricles were segmented out in the average ADC and $T_2$ measurements. The lesion volume per slice was calculated by multiplying the lesion area ($=\text{number of pathological pixels} \times \text{field-of-view in cm}^2 / \text{number of points acquired per image}$) by the slice thickness. The total lesion volume was obtained by summation of the lesion volumes for all slices. The absence of a slice gap makes interpolation of lesion areas between slices unnecessary, reducing systematic errors to within-slice ‘averaging’ of signal intensity.

Statistical analysis was performed with SPSS 9.0. Differences between groups were analyzed using Student’s t-test; reported $p$-values correspond to two-tailed significance.

Histology

After the last MRI-measurements animals, needed for histology were transcardially perfused with 4% paraformaldehyde in 0.1 M PBS. Dissected brains were post fixed overnight by immersion in the same fixative, cryoprotected in 10% sucrose in PBS for 24 hours, followed by 25% sucrose in
PBS for 72 hours and quickly frozen in liquid nitrogen-cooled isopentane. We cut 10 µm coronal sections and stained for glial fibrillary acidic protein (GFAP), Nissl-substance or hematoxylin/eosin with standard procedures. Position of the histological slices was matched to the position of MRI-images by known position relative to bregma, after which a gross correlation was done.

Results

Loss of cellular ion homeostasis was initiated by unilateral intrastriatal injection of 0.5 µl of the Na+/K+-ATPase inhibitor ouabain (1 mM) into 7- to 8-day-old Wistar rats \(^{23-26}\). Twelve animals received an additional injection \(i.p\). with \(\Delta^9\)-THC (1 mg/kg) and five animals received both \(\Delta^9\)-THC and the CB \(_1\) antagonist SR141716 (1 mg/kg) 30 min prior to ouabain-injection.

ADC maps of brain tissue water, calculated from diffusion-weighted MR images acquired 15 min after ouabain injection, showed hypo intense regions with reduced ADC values (~0.67 \(\times\) \(10^{-3}\) mm\(^2\) s\(^{-1}\)) in the ipsilateral hemisphere in all animals (Fig. 1). Normal ADC values (~1.11 \(\times\) \(10^{-3}\) mm\(^2\) s\(^{-1}\)) were measured in the contralateral hemisphere of the ouabain-injected rats (Fig. 1) and in the brain of the control animals, which received only vehicle (0.5 µl Tris-HCl; 40 mM, pH 7.4). The reduction in ADC values in the ipsilateral hemispheres after ouabain-injection is considered to reflect neuronal swelling, i.e. cytotoxic edema, because of a relocation of part of the extracellular water into depolarized cells \(^{21,27}\). In this acute phase, the volume of brain tissue with cytotoxic edema was 22% smaller in the \(\Delta^9\)-THC-group (\(p<0.05\)) (Figs. 1 and 2). Coinjection of the CB \(_1\) receptor antagonist SR141716 completely abolished the \(\Delta^9\)-THC-induced effect (Figs. 1 and 2). The same brain regions, including the caudate putamen, cortex and hippocampus, were affected in all animals (Fig. 1).

After seven days, sharply delineated hyperintense regions were observed in the ADC maps (data not shown), indicative of the formation of vasogenic edema as well as tissue loss and ventricle dilatation. The volume of infarcted tissue as calculated from ADC maps, was 36% smaller in \(\Delta^9\)-THC-treated rats (\(p<0.01\)) (Fig. 2). SR141716 abolished the protective effect (\(p<0.005\)) (Fig. 2). A 10% increase in infarct volume was observed in the CB \(_1\)-antagonist-treated rats compared to non-treated rats (Fig. 2). This trend did not reach statistical significance. Neuroprotection was observed in brain regions known to express CB \(_1\) receptors, such as the hippocampus, caudate putamen, and cortex \(^{28}\). Western blot analysis confirmed the presence of CB \(_1\)-like receptors, but as expected, not of CB \(_2\)-like receptors, in 7- and 14-day-old rat brains (data not shown). The effects of \(\Delta^9\)-THC treatment on neuronal damage after seven days was also assessed using T\(_2\)-weighted imaging and verified with a histological procedure. T\(_2\) maps demonstrated both hyper- and hypointensities (Fig. 3). Both types of T\(_2\) abnormalities indicate pathological changes. Hyperintense areas correspond to vasogenic edema, tissue loss, and ventricle dilatation.
whereas hypointensities correlate to astrogliosis, i.e. phenotypic changes (hypertrophy) and proliferation of astroglial cells in response to neuronal injury (Fig. 4)\textsuperscript{27,29}. Lesion volumes, based on the combination of hyper- and hypointense abnormalities on T\textsubscript{2} maps, were reduced by 36\% (\(p<0.005\)) in \(\Delta^9\)-THC-treated rats compared with the control group (Figs. 2 and 3). Infarct size based on T\textsubscript{2}-hyperintense abnormalities was reduced by 35\% in the \(\Delta^9\)-THC-treated group (\(p<0.05\)) compared to the control animals (Figs. 2 and 3). This effect could be blocked by the CB\textsubscript{1} antagonist (\(p<0.05\)) (Figs. 2 and 3). Conventional histology (Nissl- and hematoxylin/eosin staining) showed the same lesion pattern on brain sections and confirmed the assessment made by ADC and T\textsubscript{2} map analysis (data not shown).

Figure 1  Three adjacent coronal ADC maps of neonatal rat brain 15 min after ouabain injection: effect of THC-pretreatment

a) no treatment, b) THC-treatment, c) THC + SR-141716 treatment. Hypointensities correlate to cytotoxic edema.
The hypointense regions on the T2 maps corresponded to regions exhibiting increased staining for GFAP staining, which is typical of astrogliosis, on brain sections of ouabain-treated rats (Fig. 4). No indications were found for hemorrhage. Astrogliotic tissue constituted 40% of the lesion on T2-maps and usually surrounded the edematous tissue and the dilated ventricles (Fig. 3). The volume of astrogliotic tissue in $\Delta^9$-THC-treated rats was reduced by 37% compared with nontreated rats ($p<0.05$). Importantly, this effect was not blocked by the CB1 receptor antagonist (Fig. 2).

Discussion

In the brain at least 40% of the energy produced by mitochondrial respiration is required by the Na$^+$/K$^+$-ATPase to maintain ion gradients across the cell membranes. Energy levels in the brain can be compromised by a lack of glucose and oxygen or by defects in the respiratory chain such as occurring in stroke and Parkinson’s disease, respectively. Na$^+$/K$^+$-ATPase function is inhibited during energy failure. This may lead to a prolonged depolarization of the neuron, excessive release, and reversal of the uptake of excitatory amino acids, i.e. the induction of excitotoxicity$^{7,8}$. Ouabain inhibits Na$^+$/K$^+$-ATPases and is a very potent neurotoxin that leads to pancellular necrosis and infarction$^{23}$. It is used to study the involvement of Na$^+$/K$^+$-ATPase in central nervous system
ouabain rapidly perturbs ion homeostasis, induces cell swelling and glutamate dependent damage of cells, which can be prevented, at least in part, by blockade of the NMDA receptor\textsuperscript{23-26,30}.

The diffusion-weighted MRI data acquired 15 min after the injection of ouabain showed that activation of the CB\textsubscript{1} receptor by ∆\textsubscript{9}-THC attenuates \textit{in vivo} cell swelling in an early phase after the induction of excitotoxicity. Activation of the CB\textsubscript{1} receptor on presynaptic-neuron terminals can lead to inhibition of the Ca\textsuperscript{2+} influx via N-, and P/Q-type voltage-sensitive calcium channels, thereby preventing the release of glutamate and subsequent depolarization of other neurons\textsuperscript{17,31}. Furthermore, cannabinoids can induce hyperpolarization via the CB\textsubscript{1}-mediated activation of inward rectifying and A-type K\textsuperscript{+}-channels\textsuperscript{32}. Hyperpolarization raises the threshold to depolarization, which therefore may lead to cell protection.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Three adjacent coronal T\textsubscript{2}-maps of neonatal rat brain 7 days after ouabain injection: effect of THC-pretreatment.}
\label{fig:figure3}
\end{figure}

\textbf{Table 3} Three adjacent coronal T\textsubscript{2}-maps of neonatal rat brain 7 days after ouabain injection: effect of THC-pretreatment.

\begin{tabular}{|c|c|c|}
\hline
\textbf{a)} no treatment & \textbf{b)} THC treatment & \textbf{c)} THC + SR141716 treatment \\
\hline
Hyperintensities correlate to ventricle dilatation, vasogenic edema and tissue loss, whereas hypo-intensities correlate to astrogliosis.
\hline
\end{tabular}
contribute to the observed reduction in the development of cytotoxic edema.

ADC and T2 data acquired after seven days, demonstrated that Δ⁹-THC or its CB₁-active metabolite 11-HO-Δ⁹-THC reduced neuronal damage by 36%. Various mechanisms could underline the observed effects:

(i) Δ⁹-THC-induced hypothermia. In our experimental setup, the body temperature of the rats was externally controlled by an infrared lamp and a water-heated pad, making the contribution of cannabinoid-induced hypothermia to the protective effects unlikely.

(ii) Anti-oxidative properties of Δ⁹-THC. Anti-oxidant activity most likely does not play a major role in our model, because a) the neuroprotective effects were blocked by the CB₁ antagonist and b) the dose of Δ⁹-THC (1 mg/kg) is low compared to the high dose of anti-oxidant (50-100 mg/kg) required for effective protection in other studies.

(iii) Down-regulation of brain-resident mast cells by activation of a CB₂-like receptor. This process is also unlikely to be effective in our model, because a) the neuroprotective effects were blocked by SR141716, b) Δ⁹-THC has been shown to have a low efficacy on the stimulation of the CB₂ receptor and c) a CB₂ like receptor could not be detected.

(iv) Closing of N- and P/Q-type calcium channels via a CB₁-receptor-mediated mechanism. Reduced influx of calcium decreases directly the activation of destructive pathways, e.g. it prevents the activation of neuronal NO-synthase and it reduces glutamatergic transmission, i.e. induction of excitotoxicity. This CB₁-mediated mechanism is likely to dominate the observed neuroprotective effects in the late phase in our model.
Neuroprotection by $\Delta^9$-THC was observed in the hippocampus, striatum and cortex. Western blots verified the presence of CB$_1$ receptors in neonatal rat brain. Previously, radioligand binding studies have demonstrated that CB receptors were expressed in the cerebral cortex, striatum, hippocampus, cerebellum and brain stem at postnatal day 5$^{28,38}$. The presence of mRNA transcripts for the CB$_1$ receptor was also observed in some forebrain areas, such as the subventricular zone of the striatum, nucleus accumbens and neocortex. The abundance of the mRNA transcripts was high at gestational day 21 but tended to wane to postnatal day 5 and disappeared at day 30$^{38}$. Thus, these data support a CB$_1$-mediated neuroprotection.

The gliotic response to neuronal injury after ouabain-injection has been reported in adult rats$^{25}$. We also observed astrogliosis surrounding vasogenic edema in our model. $\Delta^9$-THC-treatment reduced the volume of brain tissue with astrogliosis. Although astrocytes express CB$_1$-like receptors sensitive to SR141716$^{28}$, administration of the SR141716 did not block the reduction in astrogliotic tissue. Therefore, this process does not seem to be mediated by a CB$_1$-like receptor. Noteworthy, dexamabinol, a non-psychotropic cannabinoid, inhibits tumor necrosis factor-$\alpha$ (TNF-$\alpha$) release from astrocytes$^{39}$. It is thought that TNF-$\alpha$ sets the stage for inflammatory reactions including glial cell activation and proliferation$^{29}$. $\Delta^9$-THC is known to inhibit the release of TNF-$\alpha$ from immune cells$^{40}$. Thus, $\Delta^9$-THC or one of its metabolites might also inhibit the release of TNF-$\alpha$ from astrocytes (or immune cells) and reduce astrogliosis. Furthermore, non-psychotropic cannabinoid metabolites inhibit prostaglandin synthesis$^{41}$. Cyclooxygenase-2 activation has been shown to induce astrogliosis$^{42}$. Thus, it is also possible that non-psychotropic metabolites of $\Delta^9$-THC reduce astrogliosis via this mechanism. Further research is required to investigate the mechanism of $\Delta^9$-THC-induced reduction of astrogliosis.

Our data may suggest that endogenous cannabinoids could be released upon neuronal injury and protect neurons in the periphery of the infarct: on the ADC-maps we observed a trend towards a larger infarct (+10%) in antagonist-treated rats compared with non-treated rats (Figs. 2 and 3). It should be noted that tissue was considered to be pathological only in case ADC or T$_2$ values differed more than twice the SD of the mean value in the contralateral hemisphere. The periphery of the infarct with smaller changes in ADC or T$_2$ is not incorporated in this way, but may nevertheless have benefited from endogenous release of cannabinoids. Interestingly, the cortex was not severely damaged in the non-treated animals, whereas in the SR141716-injected animals this area was infarcted (Figs. 3a and 3c). It has been shown that glutamate-induced neurotoxicity leads to the formation of anandamide and its precursor N-acylphosphatidylethanolamine$^{9,10}$. However, SR141716 is an inverse agonist. It is possible that SR141716 blocks constitutively active CB$_1$-receptors$^2$. Yet, Mechoulam and co-workers have found that the endogenous cannabinoid 2-AG is upregulated in the first hours after closed head injury in mice, and
that administration of 2-AG reduces edema formation via the CB1-receptor, which strongly corroborates our findings (R. Mechoulam, personal communication).

In summary, we have shown that in an in vivo model of neurodegeneration ∆9-THC reduces neuronal damage via a CB1 receptor-mediated mechanism. This holds in both the acute and late phase after induction of excitotoxicity. ∆9-THC inhibits astrogliosis via a non-CB1-receptor-controlled mechanism. The results strengthen the concept that the endogenous cannabinoid system may serve to establish a defense system for the brain. This system may be functional in several neurodegenerative diseases in which excitotoxicity is thought to play a role, such as amyotrophic lateral sclerosis, Huntington’s, and Parkinson’s diseases, and also in acute neuronal damage as found in stroke and traumatic brain injury. It is conceivable that the endogenous cannabinoid system can be exploited for therapeutic interventions in these types of primarily incurable diseases.

Acknowledgements:

We are indebted to H. Veldman and G. van Haaften for expert technical assistance. Sanofi Recherche is gratefully acknowledged for the gift of SR141716. We thank dr. R. Dijkhuizen for fruitful discussions and dr. R. van Sluis for the development of the data analysis programme. WBV is financially supported by the Netherlands Organisation for Scientific Research, Medical Sciences council.
References


36 Huang, C. C.; Lo, S.W.; Hsu, K. S. Presynaptic mechanisms underlying cannabinoid inhibition of excitatory synaptic transmission in rat striatal neurons *J Physiol* 2001, 532, 731-748.